PRODUCTION OF NATURAL FLAVOUR COMPOUNDS

Bioconversion of monoterpenes by spores of
Penicillium digitatum

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Ain't got no inspiration
There's nothing left to tell
Ain't got no inspiration
Everything's already said
I can't write no song no more
Writing is just such a bore
An empty paper in front of me
and an pencil in my hand

(De Boegies, Kwait nait)

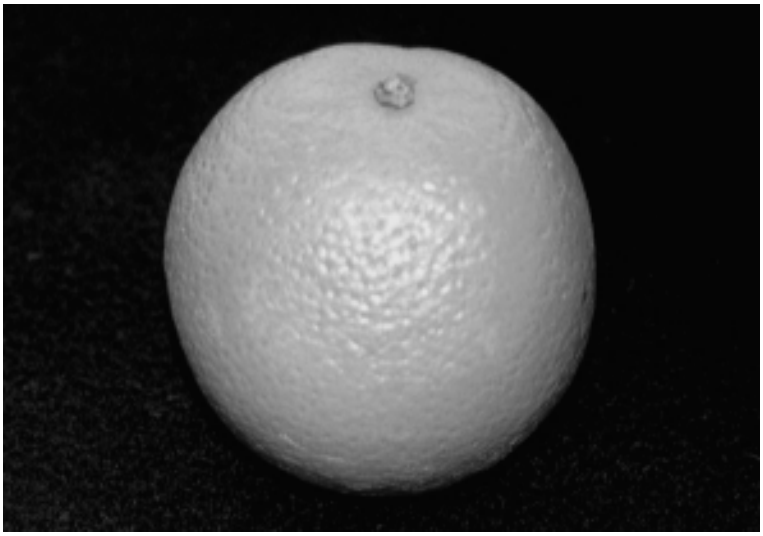
Abstract

Consumers prefer natural over artificial additives. Consequently natural flavour-compounds fetch higher prices than the corresponding artificial compound. Various natural means of flavour production are discussed in this dissertation. The major part is focused on the biotransformation of geraniol into the flavour-compound methylheptenone by spores of the fungus *Penicillium digitatum*. The biotransformation pathway was elucidated and a unique enzyme in this pathway, citral lyase, was purified from the spores. This cofactor-independent enzyme, combining the actions of a hydratase with that of an aldolase, converts citral, an intermediate of the geraniol conversion, in addition to other α,β-unsaturated aldehydes. Furthermore, spores were found to be on average 2.5 times more resistant to the toxic effects of the substrates, intermediates and products of the geraniol conversion. Finally, this dissertation describes the use of amino acids as catalysts in the production of natural flavour compounds. They can for instance be used to catalyse the conversion of cinnamaldehyde into the second most applied flavour compound, benzaldehyde.

Contents

Chapter 1

General introduction



NATURAL FLAVOUR PRODUCTION

Flavours play a very important role in the quality perception of food and beverages. Due practices such as premature harvesting, extended storage and physical treatment, aromas may be lost and the addition of flavour supplements to foodstuffs is often required. Consumers have a strong preference for natural food additives over chemically synthesised compounds. As a result, the 'natural' label, allocated by the European and US food legislation, represents a strong marketing advantage (Krings and Berger, 1998).

In the US, the terms 'natural flavour' and 'natural flavouring' are defined as 'the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavouring rather than nutritional' (Code of Federal Regulations, title 21, section 101, part 22).

Based on this definition it can be concluded that products produced by (micro)organisms, enzymes and even by mild chemical reactions (by the flavour industry referred to as 'kitchen chemistry') can be considered natural as long as natural raw materials are used. The difference in price of a natural compound and its chemically synthesised counterpart can be considerable. For example synthetic vanillin costs ± US$ 12 kg^{-1} while vanillin extracted from vanilla pods costs ± US$ 4000 kg^{-1} (Krings and Berger, 1998). Although, it was estimated that approximately 100 aroma compounds could be produced microbiologically, only a few are actually being produced by microorganisms (Feron *et al.*, 1996). That microbial aroma production can be successful was demonstrated for the aroma-compound γ-decalactone, the impact flavour compound of peach. Its price decreased from ± US$ 20000 kg^{-1} in the early 1980s down to ± US$ 1200 kg^{-1} in 1995 because of a move to microbial production (Feron *et al.*, 1996).

With over 400 different naturally occurring structures, monoterpenes represent one of the largest classes of flavour compounds. Monoterpenes are 10-carbon compounds derived from two isoprene units, with strong sensory qualities. They are widely found in nature, being the major constituent of many plant-derived essential oils. They are valuable compounds for the flavour and fragrance industries. Monoterpenes are produced algae, fungi and higher plants like grapes and hops, which produce monoterpenes important for the flavour of wine and beer (King and Dickinson, 2000; van der Werf *et al.*, 1997). These terpenes form the basis of the sensory perception of the wine bouquet, which is typical of its variety. All five monoterpenes studied in this

thesis (geraniol, nerol, neral, geranial and geranic acid) are among the top 25 of most important wine flavours (Mateo and Jimenez, 2000).

Bioconversion of terpenes has been reported as early as 1915 when yeast was found to convert citronellal into citronellol. The importance of this conversion to aroma chemists was immediately recognised as the initial citrus like aroma was converted into a more rose like aroma (Mayer and Neuberg, 1915). Not withstanding problems with respect to chemical instability, low solubility, volatility and toxicity, much effort has been putt into monoterpene biotransformation reactions in the past decades (van der Werf *et al.*, 1997). Although many of these studies are of pure academic interest, some of them do have industrial potential. For instance, a process has been patented for the production of the isomers of 2-methyl-5-isopropylhexa-2,5-dienal (an aroma compound with a odour described as spicy and citrus woody) from α-pinene oxide using a *Pseudomonas* strain (Harries *et al.*, 1988).

CONVERSION OF MONOTERPENES BY *PENICILLIUM DIGITATUM*

The central microorganism in this thesis is *P. digitatum*, a spore forming fungus that is best known as a spoilage organism of citrus fruits. It is one of the most economically important post-harvest decay organisms of citrus (Palou *et al.*, 2002) and the strain used in this dissertation (*P. digitatum* ATCC 201167) has actually been isolated from a spoiled tangerine by Demyttenaere and De Pooter (1996). They described the conversion of the monoterpene geraniol by sporulated surface cultures of this fungus. These findings formed the starting point of the EC-financed project 'production of bioflavours by fungal spores' (FAIR CT 98-3559). The main objective of this project was to produce natural flavour ingredients by use of microbial spores, so called bioflavours. Central to the sub-project reported in this dissertation was studying the feasibility of using spores for terpene bioconversion rather the vegetative cells.

OUTLINE OF THE THESIS

Chapter 2 provides an overview of the use of microbial spores. Advantages and disadvantages of using spores are discussed and their application in areas like biowarfare, biocontrol, biocatalysis and biosensing are discussed.

The elucidation of the geraniol biotransformation pathway in spores of *P. digitatum* is described in **chapter 3**. The conversion of geraniol and homologous substrates into methylheptenone was studied using spore suspensions. The pathway was deduced from the substrate degradation and product accumulation studies in both spores

and spore extracts and by measuring the enzyme activities of the enzymes involved in spore extracts.

The key enzyme of the pathway described in chapter 3, citral lyase, was studied in more detail in **chapter 4**. The induction of this enzyme in spores of *P. digitatum* by the substrate citral was studied and the enzyme was subsequently purified. The pH and temperature optimum of citral lyase as well as its substrate specificity were determined.

In **chapter 5** the difference in toxicity of terpenes towards spores and mycelium was studied. The toxicity of the substrates, intermediates and products of the geraniol biotransformation described in chapter 3, were tested. The toxic effects on survival as well as bioconversion activity were determined for both spores and mycelium of *P. digitatum*.

During the study of the enzymatic conversion of citral into geranic acid (chapter 3) an interesting chemical conversion was observed as a side-reaction. This amino acid-catalysed conversion of citral is the chemical equivalent to the action of citral lyase and is discussed in **chapter 6**. The conversion of citral into methylheptenone and acetaldehyde as well as the isomerisation of the citral isomers geranial and neral is described. A mechanism for this reaction is postulated in accordance with the observed effects of pH and amino acids.

In **chapter 7** the amino acid-catalysed retroaldol condensation of citral, described in chapter 6, is extended to other α,β-unsaturated aldehydes. The glycine-catalysed conversion of cinnamaldehyde into benzaldehyde was optimised with respect to temperature, pH and cinnamaldehyde and glycine-concentration.

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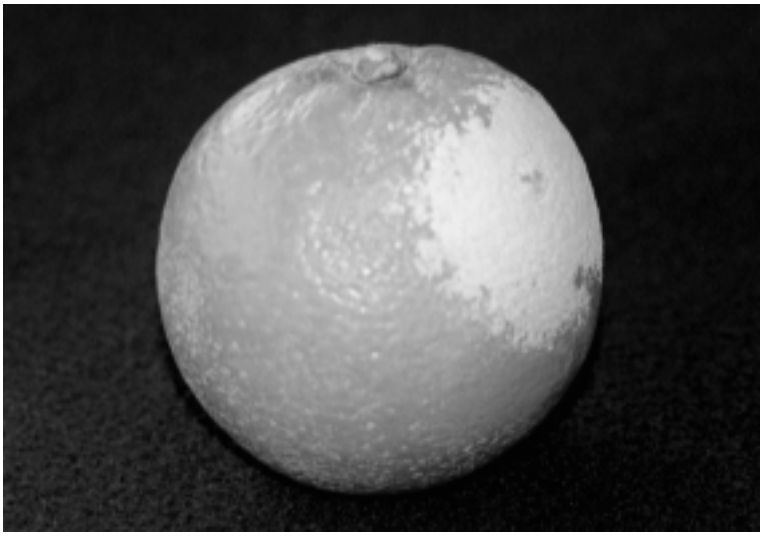
Chapter 2

What can spores do for us?



This chapter has been submitted for publication as: **Wout AM Wolken, Johannes Tramper, Mariët J van der Werf** What can spores do for us?

ABSTRACT

A range of organisms have the capability to form spores, a remarkable and resistant phase in their life cycle. The variety that exists within the microbial spores is considerable. Sizes vary from 0.25 to 340 μm and shapes from round to extremely elongated (a length to with ratio of 250). The surface of a spore can be completely smooth, but also elaborately ornamented. Compared to vegetative cells, spores have several advantages (resistance to toxic compounds, temperature, desiccation and radiation, the resulting easy and long storage, specific activities of spores, etc.) making them better suited for different uses. The application of spores that, unfortunately, comes to most people's minds first is that of biowarfare, more specifically, the use of spores of *Bacillus anthracis*. A related but more positive field in which spores are used is biological control. Since the first spore-based biological control agent was registered in the 1950's, a large range of (commercial) biocontrol applications have been developed. Spores have been proven successful in the biocontrol of insects, fungal diseases and weed infestations. Although often considered to be metabolically inert, spores can carry out a variety of biotransformation processes. After it was discovered that spores were essential in the production of bleu-cheese aroma the use of spores as biocatalyst really became subject of extensive study. Other uses of spores can be found in the fields of probiotics, tumour detection and treatment, biosensing and in the 'war against drugs'.

INTRODUCTION

Spores are a remarkable and resistant phase in the life cycle of a range of organisms (ferns, worts, algae, fungi, bacteria and protozoa). It is impossible to give an unambiguous definition of a spore, as spores are very diverse in origin and morphology (Box 1). Although essentially microscopic, spores vary greatly in size and shape (Figure 1). The smallest spores are the bacterial endospores, but none of the endospores reported is less than 0.25 μm in diameter (Holt *et al.*, 1984-1989). The largest microbial spores are the fungal ascospores, some of which are nearly visible with the unaided eye (e.g. the two-celled ascospore of *Varicellaria microsticta* may be as large as 340 by 115 μm) (Ingold, 1971). Generally microbial spores have a diameter in the range of 1 to 50 μm.

Most spores are initially smooth-walled, spherical or ovoid cells (Figure 1 D and E). Some spores assume during their development more complex shapes (kidney- or needle-shaped, etc. (Hawker and Madelin, 1974). Another external feature of some spores is ornamentation. They vary in size from appendages (like flagella to smaller ornamentations (like spines and ribs) (Figure 1 B, C, E, and G). Even spores that appear smooth under the light microscope may reveal ornamentation when examined under an

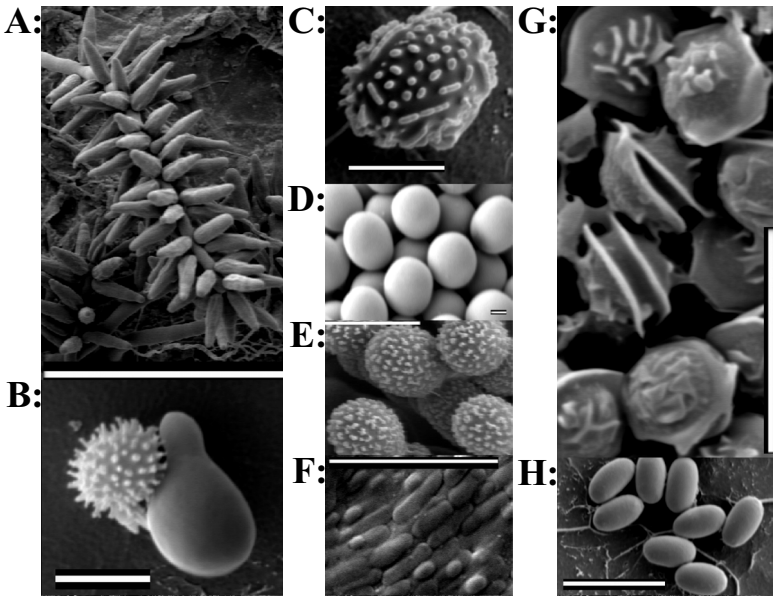


Figure 1. Selection of different spores. A, *Helminthosporium solani* forms large multi-celled conidia on a large spore-bearing structure (Bar: 100 µm); B, Germinated ascospore of *Talaromyces macrosporus*, the outer cell wall of the spore is shed and still visible, attached to the germling (Bar: 5 µm); C, Ascospore of *Talaromyces galapagensis*. Note the characteristic ornamentation on the outer cell wall (Bar: 5 µm); D, Ordered configuration of smooth sporangiospores of the *Zygomycete Mycotypha* spp on a sporangiophore (Bar: 1µm); E, Finely ornamented conidia of *Aspergillus oryzae* on a conidiophore (Bar: 5 µm); F, Formation of bacterial endopores within a colony of *Bacillus cereus*. The sporeforming rodlike cells are visible in the colony and a number of them bear oval spores within them (Bar: 10 µm); G, Ascospores of *Neosartorya pseudofisheri* with ornamentation and lateral ridge (Bar: 10 µm) and F, smooth ascospores of *Talaromyces muroii* (Bar: 10 µm). (With courtesy of J. Dijksterhuis and K.G.A. van Driel, CBS, Utrecht, The Netherlands).

electron microscope (Ingold, 1971). Furthermore, spores may differ in colour due to pigmentation, which may be situated in the spore wall or in the interior. The most common type of pigmentation is the black pigment melanin, but many spores are brightly coloured, e.g. blue-green (*Penicillia*), pink (*Fusaria*), orange (*Uredinales*) or yellow-brown (*Agrarics*) (Hawker and Madelin, 1974).

Based on their biological function, spores can be divided into two groups. First the resting-spores: spores that remain in their place of origin, helping the species to get through an unfavourable period, such as a drought or famine (e.g. bacterial endospores). The second group of spores are the dispersal-spores: spores that are dispersed and initiate vegetative growth in more or less distant places and so secure geographical spread (Madelin, 1966). A good example of this group is the formation of

explosive asci by *Ascomycetes*. A build up of hydrostatic pressure leads to a bursting of the ascus and a violent discharge of ascospores. This spore discharge typically has a reach of 0.5 - 2.0 cm, but reaches of up to 40 cm have been reported (Ingold, 1971).

Spores are produced by a variety of organisms (Box 1), but only spores of microorganisms are discussed in this chapter. Considering the diversity found within the spores one can imagine the extent of possible applications. Nevertheless, spores are too neglected in the search for a suitable microorganism and even scientists working with spores are seldom aware of the full extent of their applications. In the next sections we will give an overview of their different application as well as advantages and disadvantages of spores. This chapter does not aim to be comprehensive, but wants to point to the unique features of spores and to the potential of this all too often 'overlooked' physiological state.

Box 1: General definition of a spore (Luck, 1999)
Spore: Small reproductive body that detaches from the parent organism to produce new offspring without having to fuse with another reproductive cell. Mostly microscopic, spores may consist of one or several cells (but do not contain an embryo) and are produced in large numbers. Some germinate rapidly, others 'rest,' surviving unfavourable environmental conditions. Spores are formed by ferns, horsetails, mosses, fungi, bacteria and some protozoa. Spores are formed during the sporophyte stage in the lifecycle of such organisms.

ADVANTAGES OF USING SPORES OVER VEGETATIVE CELLS

Microbial spores may have several advantages when compared to vegetative cells. The most obvious advantage of using spores instead of vegetative cells is the higher resistance towards several external factors. One of the external factors to which spores are generally more resistant is mechanical force. An extreme example of mechanical stress is the use of explosives to disperse a biological warfare agent. Another problem associated with the dispersal of biowarfare as well as biocontrol agents is desiccation, as the agents are generally distributed in very small droplets, which dry out quickly. So the higher resistance of spores to these effects is a big advantage over vegetative cells (Jackson, 1999). Resistance to loss of activity due to desiccation is of course also an important factor during production of dry spores (e.g. for the use as inoculum). Radiation is another important external factor. The resistance of spores towards solar radiation is essential when applying spores for biowarfare and biocontrol purposes.

Spores are also much more resistant to high temperature, e.g. some yeast spores can survive heat treatment up to 350 times longer as compared to the vegetative cells (Dijksterhuis and Samson, 2002). The high temperature resistance of *Bacillus* spores is the reason why they are used as bioindicators for sterilisation problems. But this high resistance also adds to the survival rate of biowarfare agents dispersed by explosives and spore immobilisation at high temperatures.

The presence of toxic compounds is another important external factor. For instance, in biocatalysis the substrates and products of the conversions are often toxic. Spores are less susceptible to the toxicity of these compounds as was shown for the conversion of the mono terpene geraniol. The spores of *Penicillium digitatum* are more resistant to the toxic effects of the substrate and products of the conversion (Wolken *et al.*, 2002). This resistance towards toxic compound is also advantageous in biocontrol when the agent is combined with conventional (chemical) control agents. For instance in the biological control agent System3 (Table 1) spores of *Bacillus subtilis* are combined with the chemical pesticides metalaxyl and quintozene. When using spores as probiotics resistance towards the toxicity of conjugated bile salts is essential (Spinosa *et al.*, 2000). In the process of immobilisation also often toxic compounds are used as well as other harsh conditions (like temperature). Thus the use of spores in stead of vegetative cells during the immobilisation might be advantageous (Ohlson *et al.*, 1980)

The high shelf life that spore products can have is a direct consequence of the high resistance towards these external factors is. For instance, spores of *Aspergillus ochraceus* were stored without any detectable loss of hydroxylating activity for 1 year at -20°C or 3 months at 4°C (Singh *et al.*, 1968) and the commercial biocontrol agent Green Muscle (Table 1) has a shelf life of over 3 years at 4°C or 1 year at 26 to 32°C. An extreme example of the longevity of spores is given by a spore (related to *Bacillus sphaericus*) that was revived and cultured after having been dormant for over 25 million years within a piece of amber (Cano and Borucki, 1995).

The ability to germinate selectively in response to external triggers (e.g. host surface wax, plant hormones, extreme heat or pressure, etc.) (Dijksterhuis and Samson, 2002; Minton *et al.*, 1995; Rotman, 2001) is a property, which gives spores an advantage in applications like biological control, biosensing and tumour control.

In spores bioconversion activities have been reported that are not, or to a lesser extend, present in the vegetative cells of the same organism. For instance, a large selection of enzyme activities was tested in both spores and vegetative cells of *Saccharomyces cerevisiae*. Of the 64 enzyme activities tested, over 70% were more active in the spores (Murata, 1993). Similar results were obtained when spores and vegetative cells of the bacterium *Bacillus subtilis* were compared (Murata, 1993). However the lack of a certain activity in spores also might be advantageous in for

instance biocatalysis. Whereas spores of *Aspergillus* species produce only the desired product 11α-hydroxyprogesterone from progesterone its mycelium produces six side-products due to undesirable activities (Zedan *et al.*, 1976). Similarly, the lack of activity of most enzymes is what makes non-germinating *Bacillus* spores suitable for their biosensing application (Rotman, 2001).

The mycelial forms of fungi and *Streptomycetes* result in a viscous medium, which is hard to aerate and mix and furthermore makes product recovery difficult. When using the relative small, oval or round, spores instead of the long and fibrous vegetative forms of the same microorganisms these problems are overcome (Larroche and Gros, 1997).

LIMITATIONS TO THE USE OF SPORES

One of the largest restrictions to the use of spores is that spore-production is obviously limited to the spore-forming microorganisms. So a large number of microorganisms are excluded from use. When spores are produced the production is accompanied (or proceeded) by the production of vegetative cells/mycelia. This leads to a lower yield of biomass per gram of substrate. However, this is often compensated by a higher specific (bioconversion-, biocontrol-, etc) activity. Another problem associated with the spore production is that not all organisms are easily cultivated. In biocontrol there are examples of the target organism being used as the 'substrate' for the spore production. In several cases however, the spore production has been successfully optimised. For instance, the biocontrol fungus *Conothyrium minitans* was cultivated on a simple and cheap substrate (oats) from which it produced $5 * 10^{12}$ spores per kg (Oostra *et al.*, 2000).

The same features that give spores their high resistance towards external influences might also limit their application. In biocatalysis the diffusion of the substrate into (and product out of) the spores might be severely hindered by the spore-wall, limiting the overall conversion rate.

BIOLOGICAL WARFARE

It is unfortunate that of all possible applications bio-warfare is the one that seems to be the first in most people's minds when thinking of spores. More specifically, current public attention on bio-warfare seems to focus mainly on the use of spores of *Bacillus anthracis*. For a large part this is due to the anthrax letters send after the 11th September 2001 attacks. Anthrax, the disease of cattle and sheep caused by this organism, can also kill humans. Whereas the production of *B. anthracis* spores is relatively easy, the

processing of the crude bacterial slurry into a form suitable for dispersal is much more difficult. In order to retain the spores inside the victim's lungs, causing the pneumonic form, spore-containing particles of the exact right size (1 to 5 μm) have to be prepared. However, when this is actually achieved, a plane equipped with a crop sprayer carrying 100 kg of anthrax spores, could deliver a fatal dose to up to three million people (Taylor, 1996).

In the list of biological weapons given by the United Nations office for drug control and crime protection several other spore forming microorganisms are named. For instance, several *Clostridia* species like *Clostridium botulinum*, which can lead to foodborn botulism, colonisation of the GI tract of infants and wound botulism (*Clostridium perifringens* causes similar problems) and spores of *Clostridium tetani*, which cause the severe infection disease tetanus with a mortality rate of 50%. Other microorganisms of which spores can be used for biowarfare are *Aspergillus flavus*, *Coccidioides immitis*, *Coxiella burnetii* and *Histoplasma capsulatum* (see http://www.undcp.org). Although probably much research has been performed to adapt these organisms for use as biowarfare agent, (luckily) little has been published, for obvious reasons.

A large-scale biological attack on people needs a carefully ‘weaponised’ germ to ensure that pathogens normally spread by close physical contact can be transmitted through the air and still be infectious. However, a biological attack aimed at foodcrops would be a far less ambitious undertaking (MacKenzie, 1999).

'WAR ON DRUGS'

Not only could biowarfare, strongly resembling the biocontrol of weeds, be aimed at foodcrops but also at different plants used in the production of drugs. This type of biological warfare is developed by the United States government (who prefers to call it biological control) as part of their 'war on drugs'. One of the agents that is being developed by the US (in collaboration with the United Kingdom and the United Nations drug control program) is based on spores of *Pleospora papaveracea*, which like *Dendryphion penicillatum* destroys opium poppies (*Papaver somniferum*) (O' Neill *et al.*, 2000; Belvadi, 2001). Similar agents are developed for the control of marijuana (*Cannabis sativa*) and coca (*Erythroxylon coca*) production (Kleiner, 1999).

BIOCONTROL

Chemical pesticides in particular have been used extensively to control pests (insects, fungi and weeds) for many years. An awareness of recent problems associated with the use of chemical pesticides such as adverse effects on man and the environment has led to a focus on biological control alternatives to chemical pesticides (Jackson, 1999).

Biocontrol of insects

The spore-forming bacterium *Bacillus popilliae* has been registered for control of the Japanese beetle in the USA since about 1950; the first registration of any insect pathogen as a microbial control agent. The bacterial spores are produced commercially in collected larvae, which are injected with bacterial cells, incubated until they develop a milky appearance and then crushed and dried to give a spore powder. The spore powder is applied to grass and is aimed solely against the larvae of the beetle that eat the spores. The use of *B. popilliae* has proved remarkably successful. Between 1939 and 1953 over 100 tons of spore powder were applied to grass in over 160,000 sites in the USA as part of a Government programme. *B. popilliae* products are marketed by several companies (Table 1) (see http://helios.bto.ed.ac.uk/bto/microbes/control). Another insecticidal bacterium that has been successfully used as biocontrol agent is *Bacillus thuringiensis*. Its spores have been marketed worldwide for control of many important plant pests (e.g. mainly caterpillars of the *Lepidoptera* (butterflies and moths), mosquito larvae and simuliid blackflies that vector river blindness in Africa) (see http://helios.bto.ed.ac.uk/bto/microbes/bt).

Biocontrol of insects is far from limited to bacterial spores (Table 1) as is demonstrated by the example of locusts and grasshoppers. Over 500 species of locusts and grasshoppers have been listed as mayor pest of crops and pastures (Peveling *et al.*, 1999). Especially locust plagues capture the imaginations as they can grow very big (in Kenya in 1954 a swarm covering 1000 km^2 was reported), however, their close relatives, the grasshoppers, probably do much more crop damage on average, year after year (Douthwaite *et al.*, 2001). Several products containing spores of the protozoan *Nosema locstae* are commercially available for the biocontrol of locusts and grasshoppers, as well as products containing spores of the fungus *Metarhizium anisopliae* (Table 1).

Part of the effectiveness of spore-forming organisms in their role as biocontrol organisms is the formation of new spores using the target organism as substrate. For instance, after the conidia of *Entomophthora muscae* have infected their target (the cabbage-root fly) they form new conidia that can in turn infect more of the target species (Figure 2, step 5). Furthermore, also thick-walled resting spores are formed which

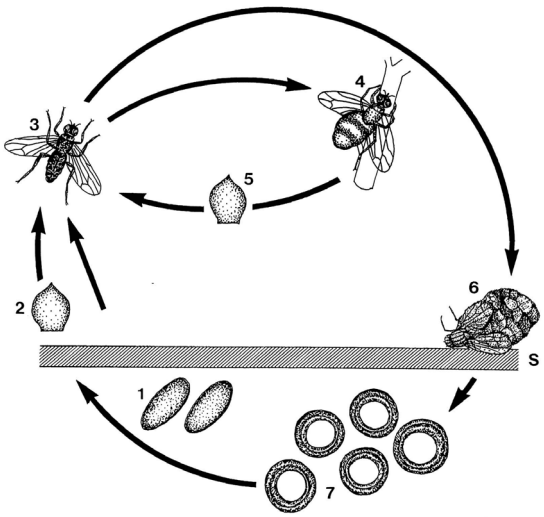


Figure 2. Life cycle of the biocontrol fungus *Entomophthora muscae* in the host, *Delia radicum* (cabbage root fly), not drawn to scale. 1, Overwintering pupae of *D. radicum*l; 2, Infective conidia of *E. muscae* are produced from the resting spores; 3, Adult *D. radicum* becomes infected; 4, Conidiophores emerge from the dead fly. 5, Conidia are discharged and infect other adult *D. radicum* (several cycles can take place during one season); 6, After midsummer resting spores instead of conidia develop. Dead flies, their abdomens filled with resting spores (azygospores), drop to the soil surface (S); 7, Thick walled resting spores surviving the winter to germinate in the next spring. (With courtesy of J. Eilenberg, Department of Ecology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark; with permission of CABI Publishing)

survive the winter (Figure 2, step 1, 6 and 7) and can in the subsequent year start a new cycle of biocontrol (Figure 2, step 2 and 3) (Pell *et al.*, 2001). In this way the total effective dose of biocontrol agent is much higher and also the effective time period is extended

Biocontrol of fungi

When using spores in the fight against fungi which affect different corps (e.g. peanut, cucumber, conifers, etc.) leading to great losses (Table 1) there are basically three different ways of achieving control. The first is the use of a mycoparasite like the fungus *Coniothyrium minitans.* The fungus *Sclerotinia sclerotiorum* (a widespread pathogen that reduces the yield of many crops) can be controlled by applying spores of *C. minitans* to the crops. After germinating the hyphal tips of *C. minitans* penetrate the cell

Table 1. The use of spores for biological control.

Biocontrol organism (type[a])	Trade name (company)	Target organism (common name)	Protected crop	References
The use of spores against insects and nematodes:				
Bacillus popilliae (B)	Doom (Fairfax Biological Laboratory, USA), Milky Spore (ARBICO, USA) and Grub Attack (Ringer, USA)	*Popillia japonica* (japanese beetle)	Various	H
Bacillus sphaericus (B)		*Culex quinquefasciatus* (mosquito)		(Ampofo, 1995)
Bacillus thuringiensis (B)	Thuricide (Bonide Products, USA)	*Lepidoptera* spp. (butterflies and moths)	Various	H
Beauveria bassiana (F)	Ostrinin (NPP, France)	*Diatraea saccharalis* (sugarcane borer)	Maize	(Arcas *et al.*, 1999)
Beauveria bronginiartii (F)	Melocont (Kwizda, Austria)	*Melolontha melolontha* (cockchafer)	Cereals and grasses	(Keller *et al.*, 1997)
		Psacothea hilaris (yellow spotted longicorn beetle)	Mulberry and figs	(Higuchi *et al.*, 1997)
Entomophthora muscae		*Delia radicum* (the cabbage-root fly)	cabbage	(Pell *et al.*, 2001)
Metarhizium anisopliae (F)	Green Muscle (Biological Control Products, South Africa and CABI BioScience, UK)	*Acrididae* spp. (locusts and grasshoppers)	Various	(Douthwaite *et al.* Peveling *et al.*
		Blattela germanica (German cockroach)		(Pachamuthu *et al.*, 1999)
Nosema locstae (P)	Nolo bait (M.R. Durango, U.S.A.)and Semaspore (Bozeman, U.S.A.).	*Acrididae* spp. (locusts and grasshoppers)	Various	(Lomer *et al.*, 1999)
Paecilomyces Fumosoroseus (F)	Memisin (Probioargo, Venezuela)	*Bemisia tabci* (sweet potato whitefly)	Various	(Jackson, 1999)
Pasteuria penetrans (B)		*Meloidogyne* spp. (root-knot nematodes)	Tomato	(Fould *et al.*, 2001)
Pseudomonocystis spp. (P)		*Leucopholis coneophora* (coconut root grub)	Coconut	(Dangar and Abraham, 1997)
Syngliocladium tetanopsis (F)		*Tetanops myopaeformis* (sugarbeet root maggot)	Sugar beet	(Wozniak, 1999)
Vairimorpha spp. (P)		*Plutella xylostella* (diamondback moth)	Cruciferous crops	(Haque *et al.*, 1999)
Verticillium lecanii (F)	Mycotal (Koppert, the Netherlands)	(whitefly)	Vegetable crops	(Florido *et al.*, 2002)
The use of spores against fungi:				
Actinomycetes spp. (B)		*Pythium ultimum* (damping-off)	Lettuce	(Crawford *et al.*
Aspergillus flavus and *Aspergillus parasiticus* (nontoxyogenic strains) (F)		*A. flavus* and *A. parasiticus* (aflatoxin production)	Peanut	(Horn *et al.*, 2001)

Bacillus subtilis (B)	Kodiak (Gustafson, USA) and System3 (Helena Chemical Company, USA)	*Rhizoctonia solani* (root cankers)	Peanut	(Emmert and Handelsman, 1999)
Coniothyrycum minitans (F)	Contans WG (Prophyta, Germany)	*Sclerotinia sclerotiorum* (white mold)	Several	(de Vrije *et al.*, 2001)
Penicillium oxalicum (F)		*Fusarium oxysporum* (*Fusarium* wilt)	Tomato	(de Cal *et al.*, 2000)
Phlebiopsis gigantea (F)	Rotstop (Kemira Agro Oy, Finland)	*Hetrobasidion annosum* (root and butt rot)	Conifers	H
Pseudozyma flocculosa (Y)		*Sphaerotheca fuliginea* (cucumber powdery mildew)	Cucumber	(Avis and Belanger, 2002)
Rhodotorula spp. (Y)		*Monilinia fructicola* (post harvest brown rot)	Stone fruits	(Hong *et al.*, 1998)
Talaromyces flavus (F)		*Verticillium dahliae* (*Verticillium* wilt)	Eggplant	(Engelkes *et al.*
Trichoderma harzianum (F)	T-22 HC (Bioworks, USA)	*Botrytis cinerea* (cucumber grey mould)	Cucumber	(Munoz *et al.*, 1995)
Trichoderma virens (F)		*Rhizoctonia solani* (cotton seedling desease)	Cotton	(Howell *et al.*, 2000)
		Basidiomycete decay fungi (soft rot decay)	Wood	(Brown and Bruce, 1999)
The use of spores against weeds:				
Alternaria alternaria (F)		*Lantana camara* (lantana)	Various crops, trees and livestock	(Saxena and Pandey, 2002)
Ascochyta caulina (F)		*Chenopodium album* (common lambsquarters)	Corn and sugar beet	(Kempenaar *et al.*
Colletotrichum gloeosporioide (F)	Collego (Encore Technologies, USA)	*Aeschynomene virginica* (nothern jointvetch)	Rice and soybean	(Daniel *et al.*, 1973)
Colletotrichum malvarum (F)		*Sida spinosa* (prickly sida or teaweed)		(Templeton, 1974)
Colletotrichum orbicular (F)		*Xanthium spinosum* (spiny cocklebur)		(Auld and Say, 1999)
Colletotrichum truncatum (F)		*Sesbania exaltata* (hemp sesbania)	Soybean	(Silman *et al.*, 1993)
F. oxysporum (F)		*Orobanche cumana* (sunflower broomrape)	Sunflower	(Thomas *et al.*, 1998)
Phytophthora palmivora (F)	DeVine (Abbott laboratories, USA)	*Morrenia odorata* (strangervine)	Orchard crops	(Evans, 2002)
Puccinia canaliculata (F)	Dr. Biosedge (Tifton Innovation, USA)	*Cyperus esculentus* (yellow nutsedge)		(Evans, 2002)

[a]Abbreviations: B, bacterial; F, fungal; P, protozoan; Y, yeast; H, see http://helios.bto.ed.ac.uk/bto/microbes.

walls of *S. sclerotiorum* resulting in disintegration of cells (de Vrije *et al.*, 2001). Spores of this fungus are commercially produced and sold as biocontrol agent (Table 1). The second way of controlling fungi is by introducing a competitor, which out-competes the target fungus for space and food. For instance, butt rot of coniferous trees (caused by *Heterobasidion annosum*) can be stopped by applying spores of another fungus (*Phlebiopsis gigantea*) onto fresh stump surfaces where it colonises the stump, giving it protection against butt rot (Pratt, 1989). Similarly, spores of *Aspergillus flavus* can be uses to stop the normal, *afla*-toxin producing *A. flavus* strains from developing on peanut pods (Horn *et al.*, 2001). The third mode of action used in preventing damage by fungal infections is the use of spores to induce resistance in the crop to be protected. In this manner tomato plants develop a resistance to *Fusarium* wilt when exposed to spores of *Penicillium oxalicum* (de Cal *et al.*, 2000) and cotton seedlings develop a resistance towards *Rhizoctonia solani* when exposed to spores of *Trichoderma virens* (Howell *et al.*, 2000).

Biocontrol of weeds

A final target group for biocontrol is weed. During the 1970s it was demonstrated that mycoherbicides are a save and practical alternative to agrochemicals in controlling weeds. In 1981 this lead to the first registered product DeVine (Table 1). This mycoherbicide is based on a wet spore formulation of *Phytophthora palmivora* and is used for the control of stranglervine (Evans, 2002). Several other fungal species were found to be useful against a range of weeds. Especially spores of the genus *Colletotrichum* are widely used as mycoherbicides. The durable spores of *Colletotrichum gloeosporioides* are produces abundantly in artificial culture, resulting in a commercial mycoherbicide introduced in 1982 which is used against the weed northern jointvetch (Table 1) (Evans, 2002; Daniel *et al.*, 1973). Other *Colletotrichum* species; *Colletotrichum malvarum*, *Colletotrichum orbiculare* and *Colletotrichum truncatum*, are effective against teaweed, spiny cocklebur and hemp, respectively (Templeton, 1974; Auld and Say, 1999; Silman *et al.*, 1993)

BIOCONVERSION

Flavour production

Although many scientists still consider spores to be metabolically inert, it is already a considerable time ago when the first report on spore-catalysed conversions appeared; in 1919 Kopeloff and Kopeloff described the inversion of sucrose by spores of 4 different

fungi. However, scientific interest in the application of spores for biocatalysis really started as a result of the work of Gehrig and Knight published in 1958. They discovered that the flavour formation during the maturation of blue-veined cheeses was the result of spores, rather then the mycelium of *Penicillium roqueforti*. The major flavour compound, methylketone, could also be produced by spores of *P. roqueforti* from fatty acids in a simple (cheese-free) system giving a economical alternative to the use of blue-cheese as flavouring agent (Figure 3). This alternative production of blue-cheese flavour is even up to now subject of investigation by several groups. Different process variations (immobilisation, two-phase systems, additions of enzymes, etc.), substrates and spores were tested to optimise the process (Table 2). Also other spore-specific flavour productions have been described, such as the conversion of the terpene geraniol into the aroma compound methylheptenone by sporulated surface cultures (Demyttenaere and De Pooter, 1996) and spore suspensions (Wolken and van der Werf, 2001) of *P. digitatum* (Table 2).

Steroid and antibiotic biotransformation

After describing the fatty-acid conversion by spores of *P. roqueforti*, Gerigh and Knight ended their 1958 publication on spore biotransformation with the following statement: 'Immediately one wonders if other mould spores, like these, might not have physiological possibilities that are now unknown or are ascribed to the vegetative cells.' With this in mind attention was focussed on the field of steroid biotransformation. A variety of steroids (like the hormones oestrogen, progesterone and testosterone) are widely used as anti-inflammatory agents, contraceptives, anti-cancer agents and other applications. In the steroid industry the 11α-, 11β- and 16α-hydroxylations are exclusively achieved by microbial transformations (Mahato and Majumdar, 1993). These hydroxylations were also among the first to be identified as catalysed by microbial spores. 11α-Hydroxylation catalysed by spores of *A. ocraceus*, was the first to be described (Figure 3) (Schleg and Knight, 1962) and to be used on a limited scale in industry (Vezina, 1987). It was soon followed by 11β-hydroxylation by spores of *Stachylidium theobromae* (Vezina *et al.*, 1968) and 16α-hydroxylation by spores of the fungus-like aerobic bacteria S*treptomyces* (Vezina *et al.*, 1963). These initial results lead to a whole range of spore-catalysed steroid transformation. In Table 2 a summary of all reactions described so far is given. In addition, other pharmaceutically relevant conversions were described as for instance the conversions of different antibiotics (El-Kersh and Plourde, 1976; Singh and Rakhit, 1971; Singh *et al.*, 1969) and the conversion of the inactive drug lucanthone into the active compound hycanthone (Zedan *et al.*, 1983) (Table 2).

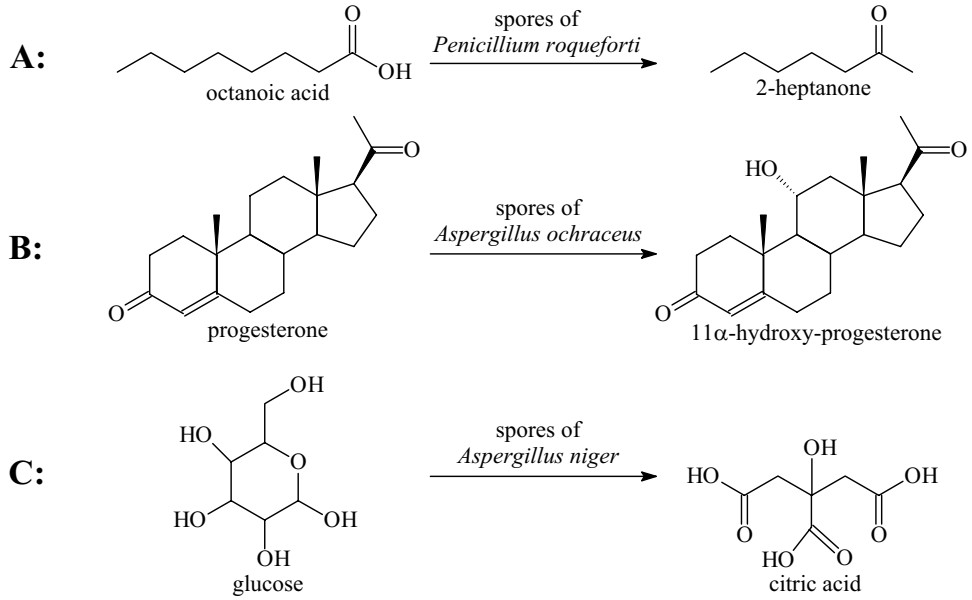


Figure 3. Selected spore-catalysed reactions. The production of, the bleu-cheese flavour compound 2-heptanone (A), the anti-androgen 11-α-hydroxyprogesterone (B) and the food additive citric acid (C) by spores of *P. roqueforti*, *A. ochraceus* and *A. niger*, respectively.

Carbohydrate and ketoester biotransformation

The use of fungal spores for carbohydrate conversion, first described in 1919 by Kopeloff and Kopeloff, was forgotten and reinvented 50 years later (Johnson and Ciegler, 1969). They immobilised the spores on different ion-exchange resins and used them for the inversion of sucrose, giving equal molar amounts of glucose and fructose (Table 2). The formation of polysaccharide, then thought to be levan, by spores of *Aspegillus sydowi*, described by Kopeloff and Kopeloff (1920), was also subject of research years later. The product formed from sucrose by the spores was now identified as a polyfructan (Kawai *et al.*, 1973). Whereas spores of *A. sydowi* catalyse the formation of polysaccharides, the opposite is true for spores of *Aspergillus wentii*, which convert soluble starch to glucose (Johnson *et al.*, 1968). Glucose in turn can be converted into mannitol or gluconate by actions of other spores (Table 2).

Citric acid is one of the most commonly used acids in the food industry and is mainly produced biotechnologically (Tsay and To, 1987). Besides mycelium, also spores of *Aspergillus niger* convert several sugars into citric acid (Figure 3). To explore the possibilities of spores, several studies were undertaken using different substrates and production methods (Table 2). For instance, the efficiency of the citric acid production

was doubled by adding a second phase of silicone oil, which increases oxygen availability (Ates *et al.*, 2002).

A last example of the use of spores as biocatalyst is the reduction of β-ketoesters by spores of the fungus *Mucor rouxii.* In aqueous solution isopropyl acetoacetate is efficiently reduced by spores resulting in a product having an enantiomeric excess of the (*S*) form of 70%. When the same reaction is carried out in a water/hexane two-phase system, the enantiomeric excess even reaches 95%. Enantiopure products, obtained from prochiral substrates, are used as building blocks of fine chemicals like pharmaceuticals, flavours and fragrances (Mangone *et al.*, 2002).

The potential of spore bioconversions is far from fully explored considering the amount of spore-forming microorganisms currently in use as biocatalyst. In the past several different screening methods have been tested, with some success, to find new spore-catalysed conversions (Vezina *et al.*, 1969; Zedan and Plourde, 1971; Demyttenaere *et al.*, 2001). If with the current knowledge of biotransformations and screening techniques attention would be focused on spore catalysts, a large range of interesting processes could be developed. Especially when focusing on the processes where spores will be particularly advantageous e.g. when using toxic compounds (substrates, products, solvents, etc.) or other harsh conditions (temperature, pH, agitation, etc.).

THE SPORE AS BIOSENSOR

Dormant spores of *Bacillus* species carry out no detectable macromolecular synthesis or oxidative metabolism, but acquire, within minutes, normal cell functions in response to specific 'germinants' in the environment. Because of this feature these spores can be used as nanodetectors for instance to detect and identify microorganisms (based on the presence and absence of certain enzyme activities). The unknown microorganism is for instance tested for β-galactosidase activity. If the tested organism exhibits this activity it will produce adenosine from adenosine-β-D-galactopyranoside. The adenosine will trigger the germination of the spores and the germinating spores in turn convert diacetyl fluorescien into fluorescent products, which are easily detectable (Rotman, 2001). The non-germinating spores can also be used in the rapid detection of biotoxic contaminants. For instance, a sample (containing the biotoxic contamination) is mixed with a germinant (L-alanine), spores of *Bacillus licheniformis* and an indicator for germination. After a short incubation the effects of the biotoxic contaminant are revealed by the intensity of the indicator (a low intensity is correlated to a high concentration of biotoxin) (Citri, 1997).

Table 2. The use of spores as biocatalyst.

Reaction	Process[ab]	Microorganism (type[a])	References
Blue cheese flavour production:			
Fatty acids ➔ 2-ketones		*Penicillium roqueforti* (F)	(Gehrig and Knight, 1958; Larroche *et al.*, 1994)
	I	*P. roqueforti* (F)	(Larroche *et al.*, 1989)
	I, T	*P. roqueforti* (F)	(Creuly *et al.*, 1992)
		Aspergillus spp. (F), *Penicillium* spp. (F), *Paecilomyces varioti* (F) and *Scaputariopsis brevicqulis* (F)	(Gehrig and Knight, 1961)
	I, T	*Penicillium* spp. (F), *Aspergillus niger* (F) and *Trichoderma koningii* (F)	(Creuly *et al.*, 1990)
Triglycerides ➔ 2-ketones		*P. roqueforti* (F)	(Lawrence, 1967)
Food fat ➔ 2-ketones	L	*P. roqueforti* (F)	(Knight, 1963; Kosikowski and Jolly, 1979)
Coconut oil ➔ 2-ketones	L	*P. roqueforti* (F)	(Jolly and Kosikowski, 1975; Pratt, 1989; Chalier and Crouzet, 1998)
Alkyl esters of fatty acids ➔ 2-alkanones	I, T	*P. roqueforti* (F)	(Park *et al.*, 2000)
Dispersed blue cheese ➔ blue cheese flavorant	L, P	*P. roqueforti* (F)	(Groesbeck *et al.*, 1995)
Terpenes:			
Geraniol ➔ methylheptenone	S	*Penicillium digitatum* (F)	(Demyttenaere and De Pooter, 1996)
		P. digitatum (F)	(Wolken and van der Werf, 2001)
Citronellal ➔ citronellol	S	*A. niger* (F)	(Demyttenaere, 1998)
Linalool ➔ α-terpineol		*A. niger* (F)	(Demyttenaere and Willemen, 1998)
Limonene ➔ α-terpineol	S	*P. digitatum* (F)	(Demyttenaere *et al.*, 2001)
Steroids:			
Progesterone ➔ 11 α-hydroxyprogesterone		*Aspergillus ochraceus* (F)	(Schleg and Knight, 1962)
	I	*A. ochraceus* (F)	(Bihari *et al.*, 1984; Dutta and Samanta, 1997)
Progesterone ➔ 16 α-hydroxyprogesterone		*Streptomyces argenteolus* (B)	(Vezina *et al.*, 1963)
Progesterone ➔ androstadienedione 1-dehydrotestololactone		*Fusarium solani* (F) and *Cylindrocarpon radicicola* (F)	(Zedan *et al.*, 1976)
Compound S ➔ 11 β-compound S		*Stachylidium theobromae* (F)	(Vezina *et al.*, 1968)
17 α-Hydroxyprogesterone ➔17 α-hydroxypregna-1,4-diene-3,20-dione		*F. solani* (F)	(Plourde *et al.*, 1972)

Cortexolone ➔11-epicortisol + cortisool		*Cunninghamella elegans* (F)	(Jaworski *et al.*, 1982)
conversion of several steroids		*Aspergillus* spp. (F), *Cylindrocarpon* spp. (F), *Didymella* spp. (F), *Fusarium* spp. (F), *Gliocladium* spp. (F), *Mucor* spp. (F), *Penicillium* spp. (F), *Pestalotia* spp. (F), *Septomyxa* spp. (F), *Sporotrichum* spp. (F), *Stachylidium* spp. (F) and *Streptomyces* spp. (B)	(Vezina *et al.*, 1968; Vezina and Singh, 1975)
Antibiotics:			
Phenoxymethyl penicillin ➔ 6-amino penicillanic acid		*Fusarium moniliforme* (F)	(Singh *et al.*, 1969)
Antimycin A ➔ inactive acids		*A. ochraceus* (F)	(Singh and Rakhit, 1971)
Chloramphenicol degradation		*Streptomyces* spp. (B)	(El-Kersh and Plourde, 1976)
Carbohydrates:			
Sucrose ➔ glucose + fructose		*A. niger* (F), *Aspergillus sydowi* (F), *Aspergillus flavus* (F) *and Penicillium expansum* (F)	(Kopeloff and Kopeloff, 1919)
	I	*Aspergillus oryzae* (F), *Aspergillus wentii* (F) and *P. roqueforti* (F)	(Johnson and Ciegler, 1969)
Sucrose ➔ levan		*A. sydowi* (F)	(Kopeloff *et al.*, 1920)
Sucrose ➔ polyfructan + oligofructans		*A. sydowi* (F)	(Kawai *et al.*, 1973)
Starch ➔ glucose		*A. wentii* (F)	(Johnson *et al.*, 1968)
Glucose ➔ mannitol production		*Aspergillus candidus* (F)	(Nelson *et al.*, 1971)
Several sugars ➔ citric acid	I	*A. niger* (F)	(Tsay and To, 1987)
Cane molasses ➔ citric acid	I	*A. niger* (F)	(Kahlon *et al.*, 1991)
Sucrose ➔ citric acid	I	*A. niger* (F)	(Bayraktar and Mehmetoglu, 2000)
	I, T	*A. niger* (F)	(Ates *et al.*, 2002)
Glucose ➔ gluconate		*A. niger* (F)	(Moksia *et al.*, 1996)
Other:			
L-alanine ➔ D-alanine		*Bacillus spp.* (B)	(Stewart and Halvorson, 1952)
Lucanthone ➔ hycanthone		*Aspergillus* spp.(F)	(Zedan *et al.*, 1983)
Soybean oil ➔ lactones	L	*P. roqueforti* (F)	(Chalier and Crouzet, 1992)
Naringin ➔ sweetning agent		*Penicillium charlesii* (F)	(Ciegler *et al.*, 1971)
Reduction of isopropyl acetoacetate	T	*Mucor rouxii* (F)	(Mangone *et al.*, 2002)
Methylglyoxal glutathione ➔*S*-lactoylglutathione	I	*Saccharomyces cerevisiae* (Y)	(Murata, 1993)

[a]Abbreviations: B, bacterial; F, fungal; I, immobilised; L, lipase added; P, protease added; S, sporulated surface cultures; T, two phase system; Y, yeast. [b]Unless stated otherwise free spores are used in an aqueous system.

Spores of *Bacillus* are also used as bioindicators in the validation of thermal sterilisation processes (Spicher *et al.*, 2002). For instance an ampoule that contains nutrient broth, sugar, a pH indicator and spores of *Bacillus stearothermophilus* is placed into the autoclave along with the batch to be sterilised. After autoclaving, the success of the sterilisation process is checked by incubation of the ampoules. No growth of *B. stearothermophilus* indicates adequate sterilisation, whereas growth shows inadequate sterilisation (see http://www.emscience.com).

TUMOUR CONTROL

Germination also plays a central role in the use of spores in tumour control. Some bacterial spores have the ability to germinate selectively in tumour cells. Intravenously injected Clostridial spores exhibit a remarkable specificity for tumour cells and consequently germinate exclusively in tumours (Minton *et al.*, 1995). This is explained by the fact that rodent and most human solid tumours have hypoxic/necrotic regions. The hypoxic condition (deficiency of oxygen in the tissues), normally absent in healthy tissues, allow the Clostridial spore to germinate (Theys *et al.*, 2001). Already in 1970 Möse used this fact to devise a serological method for tumour detection. After injection of spores into mice only the tumour bearing mice produced antibodies against *Clostridia*. Thus formation of antibodies against *Clostridia* could be used to detect tumours (Mose, 1970).

Remarkably, it was 25 years later before this unique feature of Clostridial spores was successfully used for tumour control. By genetic engineering, a gene encoding for nitroreductase was introduced into *Clostridium beijerinckii*. The spores accumulate in the tumour cells, germinate and produce the nitroreductase. This enzyme in turn converts a non-toxic pro-drug (which is systematically introduced into the patient), into a toxic metabolite, thus specifically targeting the tumour cells (Minton *et al.*, 1995). This system has been successfully tested in mouse tumour models, it lacks toxic effects and the growth of *C. beijerinckii* spores is highly selective (Hawkins *et al.*, 2002). These successes and the current need for new, selective, cancer treatments suggest that clinical trials should start as soon as possible.

SPORES AS PROBIOTICS

A serious problem of effective pro-biotic utilisation is the survival of the microorganisms in the stomach and intestines after the administration (Spinosa *et al.*, 2000). If spores are used as probiotics instead of vegetative cells the survival and subsequent inoculation levels might be much higher. A good example of spores used as

probiotics was given for poultry. Newly hatched chicks (free of the test-pathogen) were dosed with spores of *B. subtilis*. When the chicks were subsequently challenged with a virulent *Escherichia coli* strain it was found that infections numbers were dramatically decreased (La Ragione *et al.*, 2001). In the agricultural industry spores are receiving increasing attention as potential alternatives to antibiotics as growth promoters even though the mode of action is still unknown (Casula and Cutting, 2002). *Bacillus* spores are also used for the treatment of diarrhoea in humans. Although probiotic spore-suspensions are commercially available there is still an absence of controlled trials to prove their efficiency (Mombelli and Gismondo, 2000).

CONCLUDING REMARKS

The use of spores rather than vegetative cells can have a number of advantages. The high resistance to external factors like toxic compounds, temperature, mechanical force, radiation, etc. is the most obvious advantage. This and other advantages makes that spores have a vast potential, which is all too often overlooked. In the field of biocontrol, where the use of spores is now generally accepted, much research has been done and as a consequence a large number of spore-based products are now commercially available (Table 1). Unfortunately, in other promising fields where the use of spores is less well known and accepted, like biocatalyses and tumour control, research and consequently their application is still limited. The answer to our title question 'what can spores do for us?' is as follows: 'More than you think!'

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Chapter 3

Geraniol biotransformation-pathway in spores of *Penicillium digitatum*

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ABSTRACT

Spores of *Penicillium digitatum* ATCC 201167 transform geraniol, nerol, citral, and geranic acid into methylheptenone. Spore extracts of *P. digitatum* convert geraniol and nerol NAD^+-dependently into citral. Spore extract also converts citral NAD^+-dependently into geranic acid. Furthermore, a novel enzymatic activity, citral lyase, which cofactor-independently converts citral into methylheptenone and acetaldehyde, was detected. These results show that spores of *P. digitatum* convert geraniol via a novel biotransformation pathway. This is the first time a biotransformation pathway in fungal spores has been substantiated by biochemical studies. Geraniol as well as nerol are converted into citral by citrol dehydrogenase activity. The citral formed is subsequently converted by citral lyase activity, forming methylheptenone and acetaldehyde. Moreover, citral is converted reversibly into geranic acid by citral dehydrogenase activity.

INTRODUCTION

Spores, although often considered to be metabolically inert, catalyse a large variety of biotransformation reactions. Gehrig and Knight (1958) were among the first to report on fungal spores transforming organic compounds. They described the conversion of octanoic acid to 2-heptanone by conidia of *Penicillium roquefortii*, which led to a commercial process for blue-cheese flavor production (Watts and Nelson, 1963). Since the discovery of spore biotransformations the conversion of many more organic compounds has been reported (e.g. steroids, triglycerides, fatty acids, antibiotics, flavanoids and carbohydrates) (Larroche and Gros, 1997). The use of spores instead of mycelium for bioconversion reactions has several advantages. When using spores instead of mycelium the medium is less viscous, resulting in easier aeration and product recovery (Moskowitz, 1979). Moreover, spores are generally easier to store, and the lack of pellet formation results in greater homogeneity of the biocatalyst (Larroche and Gros, 1997). In some cases biotransformation reactions are carried out exclusively by spores, or vegetative cells cause the biotransformation less efficiently and for a considerably shorter period of time (Knight, 1966). Although spore biotransformations have been reported for some time, to the best of our knowledge, so far no pathway in fungal spores has been substantiated by biochemical studies.

The linear monoterpene geraniol occurs in many terpene-containing essential oils. Palmarosa oil contains 70-85% geraniol, and also geranium and rose oils contain large quantities of geraniol (Bauer and Garbe, 1985). Geraniol is a colourless liquid, with a flowery-roselike odour that is a frequently used terpenoid fragrance material

(Bauer and Garbe, 1985). The bioconversion and biodegradation of geraniol has been well studied (Bock *et al.*, 1988; Brunerie *et al.*, 1988; Cantwell *et al.*, 1978; Demyttenaere and De Pooter, 1995; Demyttenaere and De Pooter, 1996; Hylemon and Harder, 1998; King and Dickinson, 2000; Rama Devi and Bhattacharyya, 1977; Rama Devi and Bhattacharyya, 1978; Seubert and Fass, 1964). In many instances, methylheptenone was a major biotransformation product (Bock *et al.*, 1988; Brunerie *et al.*, 1988; Demyttenaere and De Pooter, 1995; Demyttenaere and De Pooter, 1996; Rama Devi and Bhattacharyya, 1977; Rama Devi and Bhattacharyya, 1978). Methylheptenone has a strong, fatty green, citrus-like odour and a bittersweet taste reminiscent of pear (Burdock, 1995). Geraniol is converted to methylheptenone by strains of the fungus *Botrytis cinerea* (Figure 1A) through the oxidation of the terminal alcohol followed by a carboxylation. *B. cinerea* strain 5882/1 formed up to 83% methylheptenone from geraniol (Bock *et al.*, 1988). Geraniol is also converted to methylheptenone by *Pseudomonas incognita* (Figure 1B) in a reaction initiated by an epoxydation. However, in this microorganism the methylheptenone formed was further metabolised (Rama Devi and Bhattacharyya, 1977; Rama Devi and Bhattacharyya, 1978). Geraniol also is transformed into methylheptenone by spores of different fungi (Demyttenaere and De Pooter, 1995). Especially *Penicillium digitatum* ATCC 201167 was shown to be an efficient biocatalyst (Demyttenaere and De Pooter, 1996). In addition, nerol and citral were also transformed into methylheptenone by spores of this strain (Demyttenaere and De Pooter, 1998). A pathway for the biotransformation of geraniol into methylheptenone was proposed (Demyttenaere and De Pooter, 1996), based on the work of Rama Devi and Bhattacharyya (1977, 1978).

So far, all geraniol biotransformation pathways, forming methylheptenone, proposed were based on the bioconversion products formed. None of these pathways have been substantiated by biochemical studies. In this report the biotransformation pathway of geraniol into methylheptenone by spores of *P. digitatum* is elucidated.

MATERIALS AND METHODS

Organism

Penicillium digitatum ATCC 201167 (*P. digitatum* CLE) was isolated from a spoiled tangerine (Demyttenaere and De Pooter, 1998). The culture was maintained as a spore suspension stored at –80°C

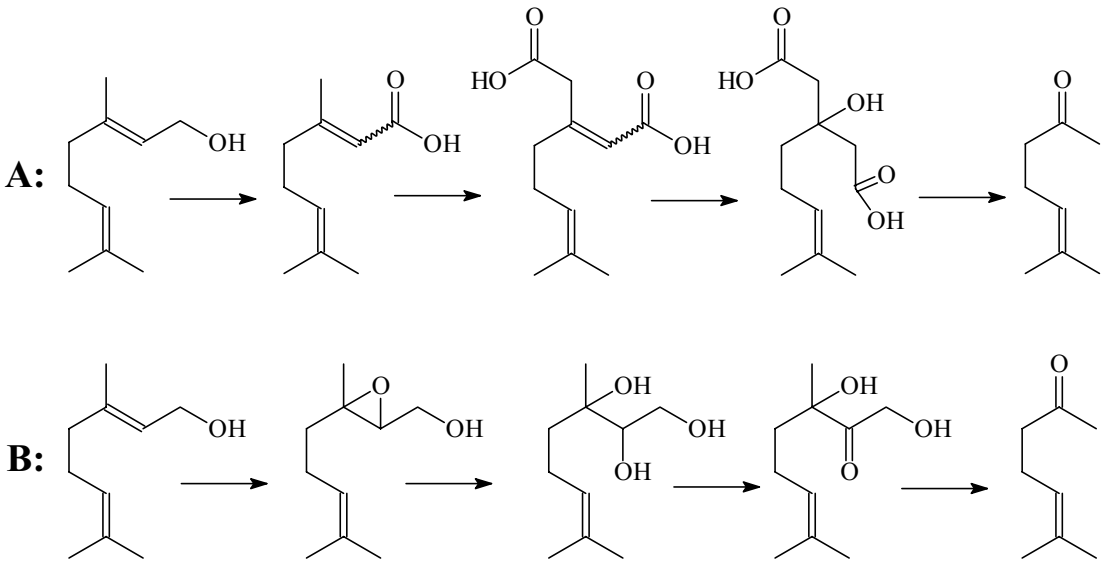

Figure 1. Pathways for the biotransformation of geraniol into methylheptenone as proposed in literature. A, *Botrytis cinerea* (Brunerie *et al.*, 1988); B, *Pseudomonas incognita* (Rama Devi and Bhattacharyya, 1978).

Production of spores

Spores were obtained by growing *P. digitatum* on a modified defined mineral salts medium (Hartmans *et al.*, 1989) containing (per liter of demineralised water) 15 g of agar, 20 g of glucose·H_2O, 2 g of aspargine·H_2O, 10 mg of EDTA, 2 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $MnCl_2 \cdot 4H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$ and 50 mM phosphate buffer (pH 7.0). Medium was autoclaved at 121°C for 20 minutes. After solidification of the agar the surface (500 ml in a 5-liter Erlenmeyer flasks) was inoculated with 5 ml spore suspension (see below). After growth of the mycelium at 21°C for 8 days, spores were harvested by washing the surface of the culture twice with 50 mM phosphate buffer (pH 7.0), containing 0.1% Tween 80. The spore suspension obtained was concentrated by centrifugation (25000 g, 15 min), dissolved in the same buffer (10 ml), and stored at -20°C until used.

Spore bioconversions

Experiments were carried out in 15 ml vials fitted with Teflon Mininert valves (Supelco, Zwijndrecht, The Netherlands). To a spore suspension substrate (1 mM final concentration) was added, in a total volume of 1 ml 50 mM phosphate buffer (pH 7.0). After the addition of substrate the vials were vigorously shaken for 30 seconds, to obtain a homogenous mixture, and then placed in a shaking water bath (250 rpm, 25°C). For every point in time a vial was taken from the water bath and the contents was extracted with 1 ml of ethyl acetate. The vials were shaken vigorously to quantitatively extract the terpenes. The ethyl acetate phase was separated from the aqueous phase by centrifuging the mixture in a 2 ml microcentrifuge tube (1 min, 13000 g). Subsequently 5 µl of the ethyl acetate phase was analysed by gas chromatography (GC).

Enzyme assays

Spore extract was prepared by thawing aliquots (10 ml) of a frozen spore suspension and adding an equal volume of glass beads (∅ = 0.5 to 0.75 mm). Subsequently the spores were broken with a Retsch (Haan, Germany) model MM 2000 bead mill (15 min, 1580 rpm, 4°C). Cell debris was removed by centrifugation at 13000 g for 10 min at 4°C. The supernatant was used as the spore extract. Dialysed spore extracts were obtained by dialysing the spore extract overnight against 100 parts buffer (4ºC, molecular cutoff 5 kDa).

Citrol (geraniol, nerol or a mixture of both) dehydrogenase activity was determined in a reaction mixture containing spore extract, 10 mM NAD^+, 100 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.5), and 1 mM of substrate (nerol or geraniol). The reaction mixture was placed in a shaking water bath (250 rpm, 25°C). In time 0.5-ml samples were taken from the incubation and extracted with 0.5 ml ethyl acetate. Subsequently 5 µl of the ethyl acetate phase was analysed by GC. Alternatively, citrol dehydrogenase activity was determined spectrophotometrically by monitoring the formation of NADH (ε_{340} = 6.22 mM^{-1} cm^{-1}). Activity was measured at 25°C in an incubation (1 ml) containing 100 µl spore extract, 1 mM NAD^+, and 100 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.5). The reaction was started by the addition of 1 mM substrate. Specific activities were calculated from the linear part of the reaction and were corrected for endogenous activity.

Citral dehydrogenase activity was determined in a reaction mixture containing spore extract, 100 mM glycine/NaOH buffer (pH 8.5), 2.8 mM citral, and various concentrations of NAD^+. The reaction mixture was placed in a shaking water bath (250

rpm, 25°C) and samples (0.5 ml) were taken in time. The reaction was terminated by the addition of 0.5 ml ethyl acetate, and subsequently 50 µl of H_2SO_4 was added to facilitate the extraction of geranic acid. Five µl of the ethyl acetate phase was analysed by GC.

Citral lyase was determined using spore extract obtained from spores that were incubated for 3 hours with 0.56 mM citral to induce citral lyase activity. To 1 ml of spore extract (containing 50 mM phosphate buffer (pH 7.0)) in a 15 ml vial, 1 mM citral was added. The vials were placed in a shaking water bath (250 rpm, 25°C). During the biotransformation, headspace samples were taken and analysed by GC for citral consumption, and methylheptenone and acetaldehyde formation.

Partial purification of citral lyase

Spore extract (30 ml, 30 mg of protein) was applied to a DEAE-Sepharose CL-6B (Pharmacia) column (2.5 by 25 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) at 4ºC. The column was washed with 63 ml of the same buffer (0.7 ml min^{-1}; collected fraction volume, 7 ml) and subsequently the proteins where eluted with a NaCl gradient in the same buffer (total volume, 602 ml).

Analytical Methods

All terpenes were analysed by GC using a fused silica cyclodextrin capillary column (type α-DEX 120; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm; Supelco, Zwijndrecht, The Netherlands). GC was performed with a Hewlett Packard 6890 GC, equipped with a flame ionisation detector with N_2 as the carrier gas. The detector and injector temperatures were 250 and 200°C, respectively, and the split ratio was 1:50. The samples were analysed isocratically at an oven temperature of 140°C. Acetaldehyde was determined in the headspace of the samples using the same GC and column. Samples of 100 µl were injected and the split ratio was 1:10. The oven temperature was held at 80°C for 2 min and subsequently raised to 140°C (120°C min^{-1}) were it was held for 9 min.

GC-mass spectrometry (GC-MS) analyses were carried out on a Hewlett Packard (Wilmington, DE) 5970 MSD GC equipped with a fused silica capillary column (type HP-5MS; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm). The flow rate of the carrier gas (He) was 1.0 ml min^{-1}. The injector temperature was 220°C, and the oven temperature was increased from 70 to 175°C at 7°C min^{-1}. The injection volume was 1 µl, and the split ratio was 1:50. Electron impact MS data were obtained at 70 eV.

Geraniol, nerol, geranial, neral, geranic acid and methylheptenone were identified by comparing GC retention times and MS spectra with those of authentic samples. All the MS spectra were identical to those in the NIST spectral database (NIST Mass Spectrometry Data Center, S. E. Stein, director). Acetaldehyde was identified by comparing the GC retention time with that of authentic acetaldehyde and enzymatically by using an acetaldehyde test kit (Boehringer, Mannheim, Germany).

The protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. Before analysis samples and standards were boiled for 15 min with 1 ml of 1 M NaOH per ml of sample.

Chemicals

Geraniol (2-*trans*-3,7-dimethyl-2,6-octadien-1-ol), nerol (2-*cis*-3,7-dimethyl-2,6-octadien-1-ol), citral (mixture of *cis*- and *trans*-3,7-dimethyl-2,6-octadien-1-al), geranic acid (2-*trans*-3,7-dimethyl-2,6-octadienoic acid), and methylheptenone (6-methyl-5-hepten-2-one) were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade (purity ≥ 99%).

RESULTS

Conversion of geraniol, nerol, citral and geranic acid by spores of *P. digitatum*

The bioconversion of geraniol into methylheptenone by spores of *P. digitatum* is shown in Figure 2A. After 5 hours geraniol is completely converted and an equimolar amount of methylheptenone was formed. Small amounts of geranic acid accumulated transiently during the conversion of geraniol. Nerol, the *cis-trans* isomer of geraniol, was transformed into methylheptenone at a similar rate (Figure 2B). Only after 3 hours maximal geraniol and nerol conversion rates were observed.

Commercial citral, a mixture of 60% geranial and 40% neral, also was converted to methylheptenone by spores of *P. digitatum* (Figure 2C). Within one hour citral was completely converted, forming a mixture of methylheptenone (40%), geraniol (25%), nerol (25%), and geranic acid (10%). Initially, methylheptenone is formed at half the rate of citral conversion, but when all citral is converted, methylheptenone formation stops for approximately two hours. After five hours the geraniol, nerol, and geranic acid formed were completely converted, resulting in a quantitative increase in the methylheptenone concentration (Figure 2C). The maximal methylheptenone formation rate from citral is three times higher than the maximal methylheptenone formation rate with geraniol or nerol as the substrate.

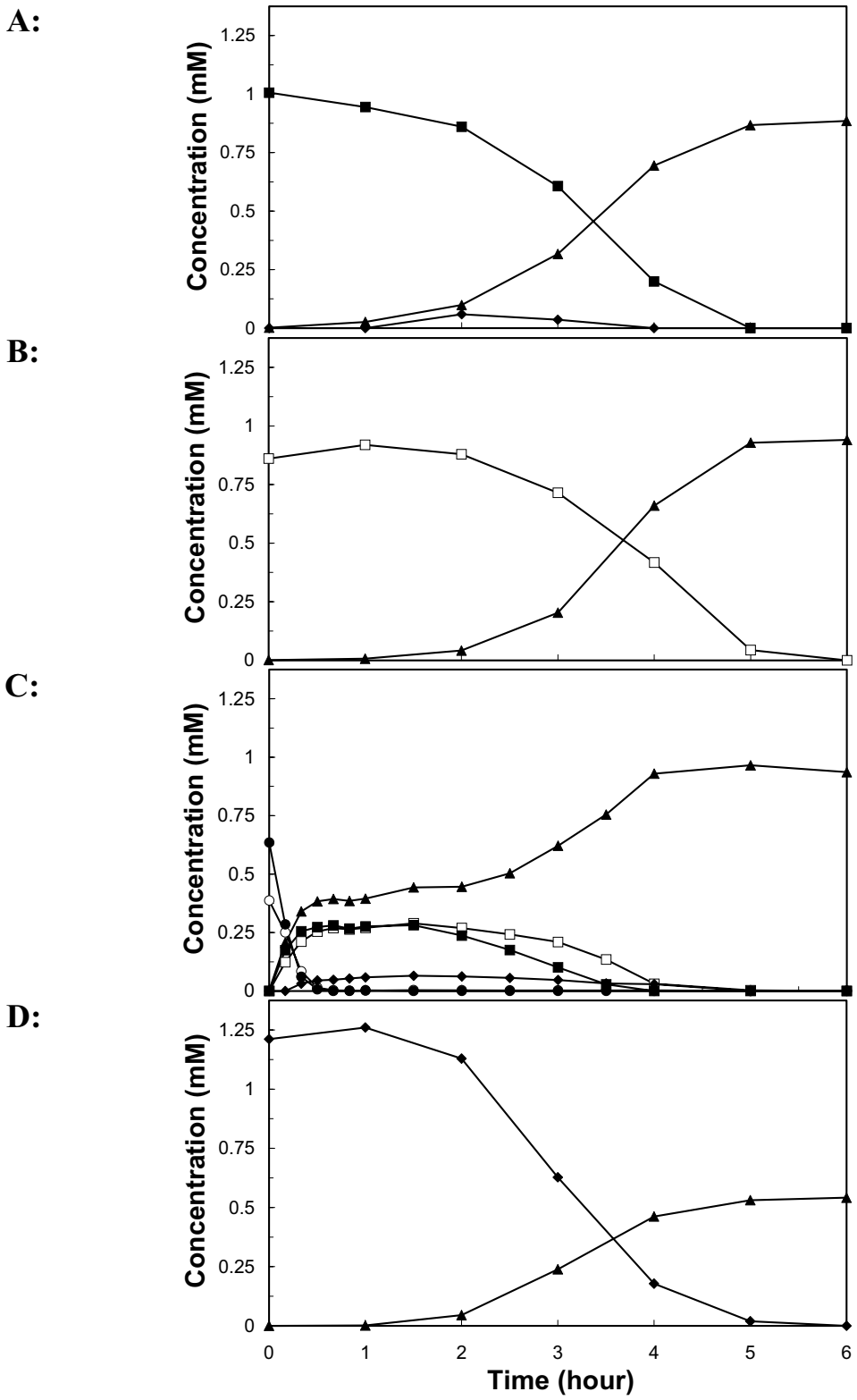

Figure 2. Biotransformation of geraniol (A), nerol (B), citral (C) and geranic acid (D) into methylheptenone by spores of *P. digitatum*. Reaction mixtures (25°C) contained *P. digitatum* spores (4.3 mg protein ml^{-1}), 50 mM potassium phosphate buffer (pH 7.0), and 1 mM substrate. Symbols: ■, geraniol; □, nerol, ●, geranial; ○, neral; ◆, geranic acid and ▲, methylheptenone.

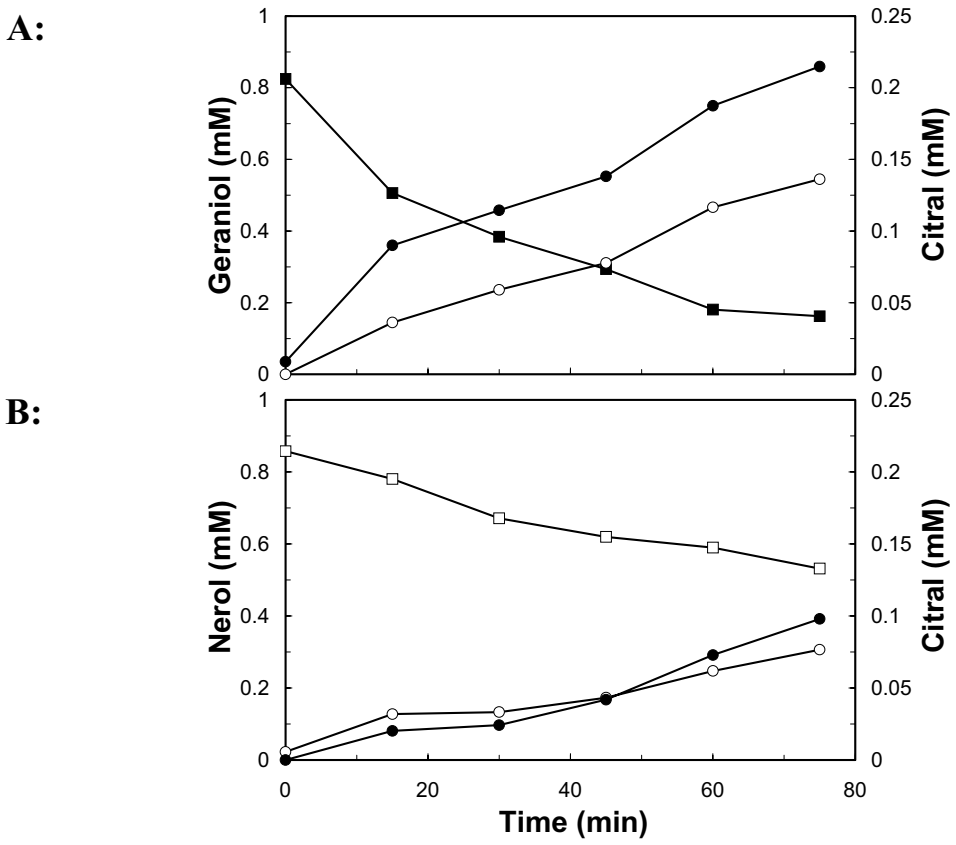

Figure 3. Biotransformation of geraniol (A) and nerol (B) by spore extracts of *P. digitatum*. Reaction mixtures (25°C) contained spore extract (1.7 mg protein ml^{-1}), 10 mM NAD^+, 100 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.5), and 1 mM substrate. Symbols: ■, Geraniol; □, Nerol; ●, geranial and ○, neral.

Geranic acid, which transiently accumulated during the conversion of geraniol and citral (Figure 2A and C), also is converted to methylheptenone (Figure 2D) by spores of *P. digitatum*. Geranic acid is converted at a similar rate as geraniol and nerol. With geranic acid less than half the molar amount of the supplied substrate was found as methylheptenone. No other products were detected with the methods used.

Citrol dehydrogenase

NAD^+-dependent citrol dehydrogenase activity was present in spore extracts of *P. digitatum*, with both geraniol and nerol as the substrates (Figure 3). There was no activity when using $NADP^+$ as a cofactor. Citrol dehydogenase activity was relatively unstable; storage of spore extracts for one hour on ice resulted in a 20% loss of enzymatic activity.

Geraniol as well as nerol were converted into citral (Figure 3). Geraniol was converted faster than nerol, as was confirmed by the spectrophotometric citrol-dehydrogenase assay (see also Table 1). The citral produced from geraniol contains more geranial than neral and at the end of the conversion geranial accounts for 60 and neral for 40% of the formed citral (Figure 3A), which is in accordance with the chemical equilibrium (Wolken *et al.*, 2000). The citral produced from nerol, however, contains more neral than geranial during the first half-hour of the conversion towards the end of the conversion the percentage of geranial increases but never reaching the 60% value of the chemical equilibrium (Figure 3B). This suggests that geraniol is transformed into geranial, while nerol is transformed into neral. After formation of either isomer isomerisation occurs resulting in a mixture of both isomers. This *cis-trans* isomerisation of neral into geranial and *vice versa* is probably catalysed by proteins and amino acids present in the reaction mixture (Wolken *et al.*, 2000).

Citral dehydrogenase

The transformation of citral into geranic acid by spore extracts of *P. digitatum* is dependent on NAD^+; using $NADP^+$ as the cofactor no transformation of citral into geranic acid was observed. The citral dehydrogenase activity was highest at an NAD^+ concentration of 1 mM. The specific activity is much lower than that of citral lyase and citrol dehydrogenase (Table 1). No accumulation of neric acid was observed during the transformation of citral

Table 1. Biotransformation of terpenes by spore extracts of *P. digitatum.*

Enzyme	Substrate	Specific activity (nmol min^{-1} mg^{-1})	
		GC[a]	Spec.[b]
Citrol dehydrogenase	Geraniol	3.0	20.3
	Neral	1.3	7.4
Citral dehydrogenase	Citral	1.2	ND[c]
Citral lyase	Citral	18.1	NA[d]

[a]**GC**, based on product formation as determined by GC; [b]**Spec.**, based on NADH formation determined spectrophotometrically; [c]**ND**, not determined, [d]**NA**, not applicable.

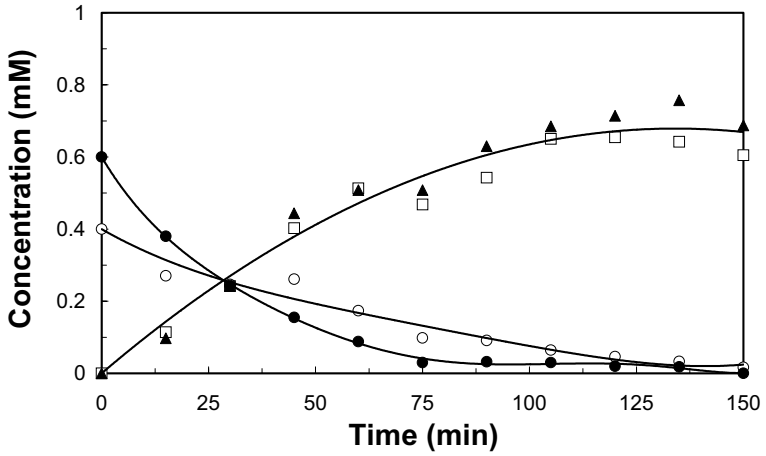

Figure 4. The transformation of citral into methylheptenone and acetaldehyde by spore extracts of *P. digitatum*. Reaction mixtures (25°C) contained spore extract (0.56 mg protein ml^{-1}), 50 mM phosphate buffer (pH 7.0), and 1 mM citral. Symbols: ●, geranial; ○, neral; □, acetaldehyde and ▲, methylheptenone.

Citral lyase

Spore extracts of *P. digitatum* converted citral into methylheptenone even in the absence of added $NAD(P)^+$. Equimolar amounts of methylheptenone and acetaldehyde were formed as the products (Figure 4). Dialysing the spore extract did not affect the methylheptenone formation rate (data not shown). Boiling the spore extract for 5 min resulted in total loss of activity.

To determine if one or more enzymes are responsible for the conversion of citral into methylheptenone citral lyase activity was partially purified using anion-exchange chromatography (Figure 5). Most of the activity was found in one peak, suggesting that one enzyme is responsible for the citral lyase activity. During the conversion of citral by the partially purified enzyme, geranial was transformed three times faster than neral (not shown). A similar phenomenon was observed during the transformation of citral with spores (Figure 2C) and spore extract of *P. digitatum* (Figure 4).

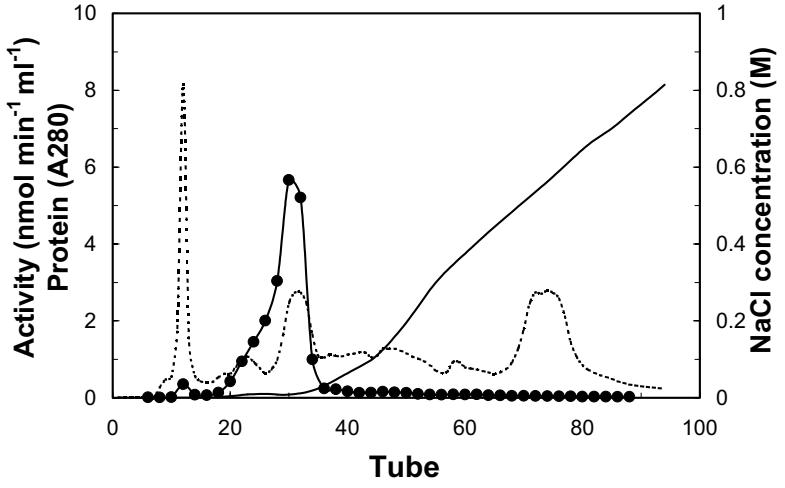

Figure 5. Partial purification of citral lyase from spore extracts of *P. digitatum* by anion exchange chromatography. Symbols: ●, citral lyase activity (based on methylheptenone formation); (.....), protein concentration and (———), NaCl concentration.

DISCUSSION

This chapter describes the biotransformation pathway for geraniol into methylheptenone by spores of *P. digitatum* (Figure 6). This is the first time that a geraniol biotransformation pathway has been substantiated by biochemical studies; it was based on enzymatic (Table 1), and substrate degradation and product formation studies (Figure 2).

Geraniol is converted into geranial by NAD^+-dependent citrol dehydrogenase activity. The geranial formed is the subject of a-biotic *cis-trans* isomerisation, under the influence of proteins and amino acids (Wolken *et al.*, 2000), resulting in a mixture of 60% geranial and 40% neral. Likewise, nerol is transformed NAD^+-dependently into neral, which also is isomerised a-biotically into a citral mixture. The citral formed is subsequently converted by citral lyase activity, forming methylheptenone and acetaldehyde. The geranial formed from geraniol also is partially transformed into geranic acid by NAD^+-dependent citral dehydrogenase activity. Whole spores also catalysed the reverse reaction, i.e. the conversion of geranic acid into citral. All tested substrates (geraniol, nerol, citral and geranic acid) were, in time, transformed into methylheptenone by spores of *P. digitatum*. The biotransformation pathway presented here differs greatly from the previously postulated geraniol to methylheptenone biotransformation pathways (Bock *et al.*, 1988; Brunerie *et al.*, 1988; Demyttenaere and

De Pooter, 1995; Demyttenaere and De Pooter, 1996; Rama Devi and Bhattacharyya, 1977; Rama Devi and Bhattacharyya, 1978). These pathways are either initiated by an epoxidation, or by the oxidation of the alcohol followed by a carboxylation (Figure 1). All pathways proposed in literature involve at least four enzymatic steps whereas the geraniol biotransformation pathway of *P. digitatum*, presented here, involves only two enzymatic activities. However, the previously described results do not rule out the possibility that these microorganisms also use the pathway as present in *P. digitatum*. The pathways proposed in literature are based on bioconversion products found during the conversion of geraniol. These products might very well be the result of side reactions or a-biotic conversion of these relatively unstable terpenes.

During the biotransformation of the terpene alcohols geraniol and nerol, we observed a lag phase of several hours (Figure 2A and B). This lag is most likely caused by lack of NAD^+, required for the transformation of citrol into citral in the spores. Before the start of the biotransformation of either geraniol or nerol, cofactor regeneration has to be achieved. We do not think that the lag is caused by a lack of citrol dehydrogenase activity, since the enzyme responsible for the reverse reaction, converting citral into citrol, which is probably the same enzyme, is already active in spores at the start of the conversion (Figure 2C). Furthermore, spore extracts exhibit citrol dehydrogenase activity (Table 1).

The enzymes of *P. digitatum* have a preference for the *trans* isomer of the tested terpenes; geranial is transformed faster than neral to the corresponding alcohol by whole spores (Figure 2C), and geranial also is transformed faster into methylheptenone than neral by spore extracts (Figure 4). Furthermore, spore extracts convert geraniol three times faster into citral than nerol (Figure 3), and remarkably, geranic acid is formed by spores (Figure 2A and C) as well as by spore extracts (Table 1), whereas neric acid is not.

Partial purification of the citral lyase activity suggested that one enzyme converts citral into methylheptenone and acetaldehyde. Citral lyase activity, combining hydratase and aldolase activity, has not been described before. However, recently several other hydratase-aldolases, catalysing comparable reactions have been described; i.e. *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (Eaton, 2000), *trans*-2'-carboxybenzalpyruvate hydratase-aldolase (Iwabuchi and Harayama, 1998), and enoyl-CoA hydratase-aldolase (Overhage *et al.*, 1999). The reaction catalysed by citral lyase resembles most that of 6-hydroxy-2-keto-5-methyl-3,5-heptadienoic acid hydratase-aldolase (Laurie and Lloyd Jones, 1999). These enzymes both catalyse the cleavage at a double bond resulting in the formation of a ketone and an aldehyde. The likelihood that only one enzyme is responsible for the conversion of citral into methylheptenone and

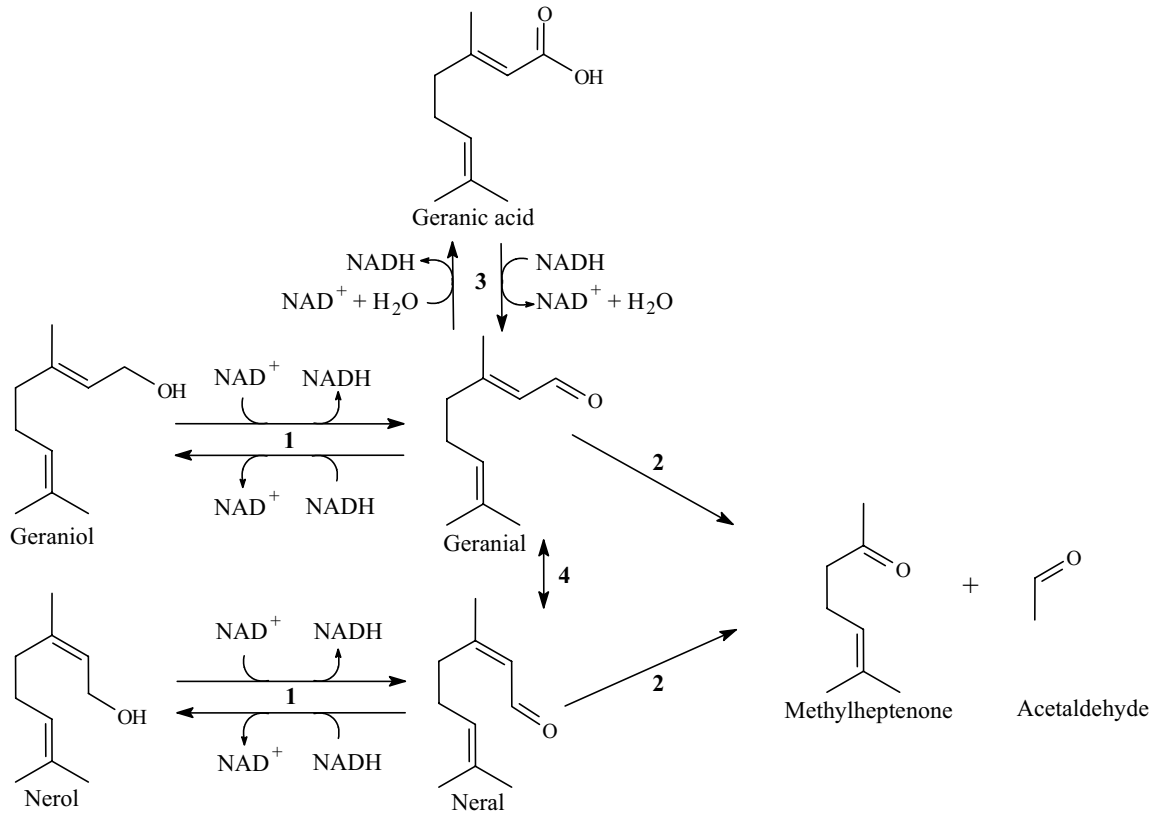

Figure 6. Bioconversion pathway of geraniol and nerol into methylheptenone and acetaldehyde by spores of *P. digitatum* ATCC 201167. 1, citrol dehydogenase; 2, citral lyase; 3, citral dehydrogenase and 4, a-biotic isomerisation (Wolken *et al.*, 2000).

acetaldehyde is supported by the relative ease with which citral is chemically converted (Wolken *et al.*, 2000).

In conclusion, spores of *P. digitatum* ATCC 201167 convert geraniol into methylheptenone via a novel pathway that involves two enzymatic activities; citrol dehydrogenase and citral lyase. This last enzyme catalyses a novel enzymatic activity; the retroaldol condensation of an α,β-unsaturated aldehyde.

ACKNOWLEDGEMENT

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Chapter 4

A novel, inducible, citral lyase purified from spores of *Penicillium digitatum*

This chapter has been published as: **Wout AM Wolken, Willem JV Van Loo, Johannes Tramper, Mariët J van der Werf** A novel, inducible, citral lyase purified from spores of *Penicillium digitatum*. *European Journal of Biochemistry* **2002** 269:5903-5910

ABSTRACT

A novel lyase, combining hydratase and aldolase activity, that converts citral into methylheptenone and acetaldehyde, was purified from spores of *Penicillium digitatum*. Remarkably, citral lyase activity was induced 118-fold by incubating non-germinating spores with the substrate, citral. This cofactor-independent hydratase/aldolase, was purified and found to be a monomeric enzyme of 31 kDa. Citral lyase has a K_m of 0.058 mM and a V_{max} of 52.6 U mg^{-1}. Enzyme activity was optimal at 20°C and pH 7.6. The enzyme has a strong preference for the *trans* isomer of citral (geranial). Citral lyase also converts other α,β-unsaturated aldehydes (farnesal, methyl-crotonaldehyde, decenal and cinnamaldehyde).

INTRODUCTION

The linear monoterpene citral was originally reported to occur in lemongrass, accounting for up to 75% of the oil. Citral was then also found in several other plant oils, e.g. in lemon and lime oil. Commercial citral is obtained by isolating it from citral-containing essential oils or by chemical synthesis from β-pinene or isoprene (Burdock, 2002). Citral is a mixture of the *cis*- and *trans*-isomers of 3,7-dimethyl-2,6-octadiene-1-al, referred to as neral and geranial, respectively. Commercial citral typically contains 60% geranial and 40% neral. Citral is widely used in the flavour and fragrance industry, its application ranges from meat products to hard candy. The amounts used in the products differ from 0.20 ppm in cheese to 429.8 ppm in chewing gum. Citral has a strong, lemon-like odour and a characteristic bittersweet taste (Burdock, 2002). With an annual world consumption of 1200 tons (in 1996) it is one of the most applied flavour compounds (Somogyi, 1996). Moreover, citral has antimicrobial (Onawunmi, 1989) and pheromone activity (Kuwahara *et al.*, 1983; Robacker and Hendry, 1977), and is used in the production of vitamin A and ionones (Shadab *et al.*, 1992).

The biotransformation of citral by several organisms has been described, as for instance bacteria (Joglekar and Dhavlikar, 1969), yeasts (Chatterjee *et al.*, 1999), fungi (Demyttenaere *et al.*, 2000), plants (Dudai *et al.*, 2000) and mammals (Ishida *et al.*, 1989). A pathway for the transformation of citral into methylheptenone by *Botrytis cinerea* was postulated by Brunerie *et al* (Brunerie *et al.*, 1988). In this pathway citral is first converted into the alcohol then into the acid, which, after carboxylation is converted into methylheptenone. Recently we described the biotransformation of citral in spores of *P. digitatum* (Wolken and van der Werf, 2001). Citral is converted into methylheptenone and acetaldehyde by the action of a single enzyme, citral lyase (Figure 1A). We now report on the induction, purification and properties of this novel enzyme.

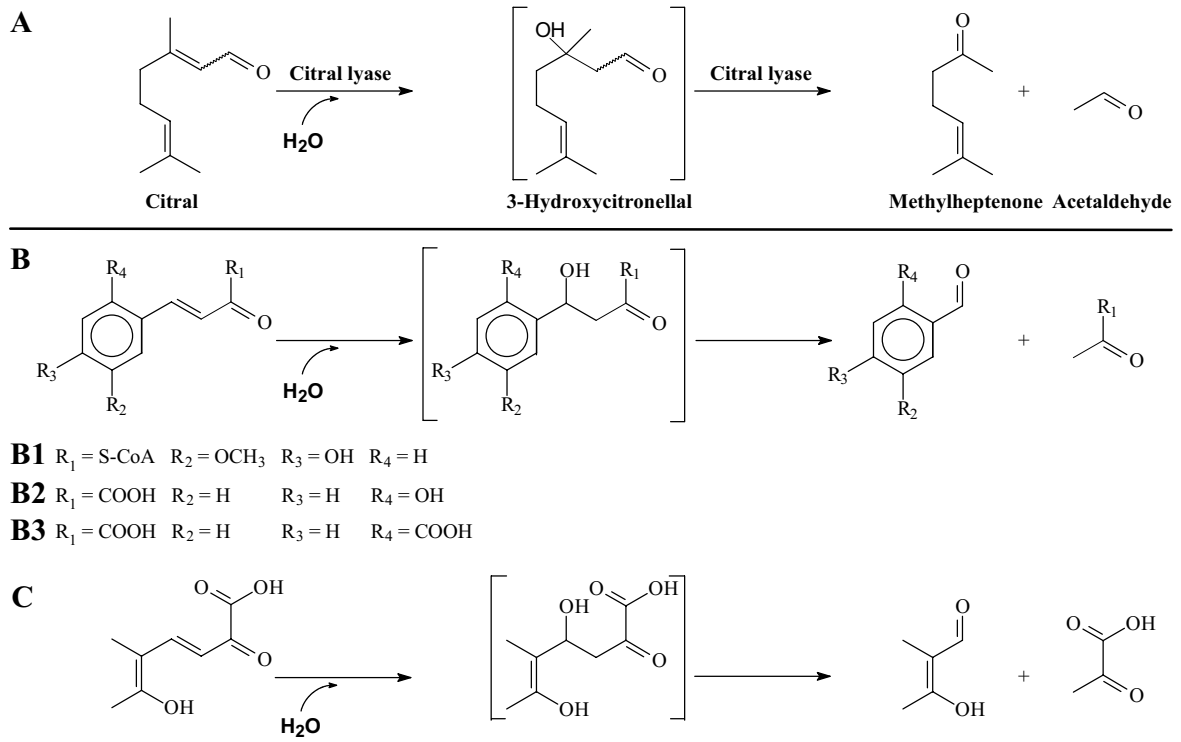


Figure 1. A, Reaction catalysed by citral lyase, combining hydratase and aldolase activity, from *P. digitatum*; B and C, Other hydratase/aldolase enzymes described in literature; B1, enoyl-CoA hydratase/aldolase (Overhage *et al.*, 1999); B2, *trans-o*-hydroxybenzylidenepyruvate hydratase/aldolase (Gasson *et al.*, 1998); B3, *trans*-2-carboxybenzalpyruvate hydratase/aldolase (Iwabuchi and Harayama, 1998) and C, 6-hydroxy-2-keto-5-methyl-3,5-heptadienoic acid hydratase/aldolase (Laurie and Lloyd Jones, 1999).

MATERIALS AND METHODS

Materials

Acetaldehyde (ethanal), hexadienal (2,4-hexadien-1-al), hexenal (*trans*-2-hexenal) and geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) were purchased from Aldrich (Steinheim, Germany). Benzaldehyde was purchased from Merck (Darmstadt, Germany). Cinnamaldehyde (*trans*-cinnamaldehyde), crotonaldehyde, decenal (*trans*-2-decenal) and decadienal (*trans*,*trans*-2,4-decadienal) were purchased from Acros (Geel, Belgium). Citral (mixture of *cis*- and *trans*-3,7-dimethyl-2,6-octadien-1-al), methylcrotonaldehyde (3-methylcrotonaldehyde), methylheptenone (6-methyl-5-hepten-2-one) and octanal (caprylic aldehyde) were purchased from Fluka (Buchs, Switzerland). Farnesal (3,7,11-trimethyl-2,6,10-dodecatrienal) was purchased from

Frinton Laboratories (Vineland, New Jersey, USA). All other chemicals used were of analytical grade (purity ≥ 99%).

Penicillium digitatum and production of spores

Penicillium digitatum ATCC 201167 (*P. digitatum* CLE) was isolated from a spoiled tangerine (Demyttenaere and De Pooter, 1998). The culture was maintained as a spore suspension stored at –80ºC. Spores were obtained by growing *P. digitatum* for 8 days (25°C) on a defined mineral salts agar (pH 7.0) with aspargine as N-source and glucose as C-source (Wolken and van der Werf, 2001). Spores were harvested by washing the surface of the agar with buffer, and, after concentration, spores were stored at -20ºC until use (Wolken and van der Werf, 2001).

Induction of citral lyase activity in spores

Optimisation of induction

To a series of 1 ml spore suspension (7.70 mg ml^{-1} spores in 50 mM phosphate buffer, pH 7.0 containing 0.1% $^{v}/_{v}$ Tween 80) in a 15 ml vial fitted with a Teflon closure, different concentrations of citral (1.11 mM intervals) were added. The vials were placed in a shaking waterbath (2.5 Hz, amplitude 2 cm, 25°C) for different periods of time (4 hour intervals). Subsequently, spores were washed by removal of the supernatant after centrifugation (2 min, 13000 g, 4°C) and activity was determined after resuspending the spores 4 times in fresh buffer (see activity measurements).

Standard induction

Routinely, citral lyase was induced by incubating spores with 2.23 mM citral for 16 hours in 50 mM phosphate buffer (pH 7.0) containing 0.1% $^{v}/_{v}$ Tween 80 (2.5 Hz, 2 cm amplitude, 25°C).

Enzyme purification

All purification steps were carried out, unless stated otherwise, at 4ºC using buffer containing 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and 20% $^{v}/_{v}$ glycerol.

Preparation of crude spore extract

Spore-free extract was prepared by adding an equal volume of glass beads (∅ = 0.5 to 0.75 mm) to 1 ml aliquots of thawed spore suspension and subsequent breaking of the spores with a Retsch (Haan, Germany) model MM 2000 bead mill (6 min, 1580 rpm, 4°C). Cell debris was removed by centrifugation at 13000 g for 10 min. The supernatant was used as the crude spore extract.

Hydroxyapatite and anionexchange chromatography

Crude spore extract was diluted to a concentration of 10 mM phosphate buffer and applied to a HA (hydroxyapatite, Bio-Rad) column (5 × 6 cm) equilibrated with the same buffer, at a rate of 0.3 ml min^{-1}. The column was subsequently washed with 100 ml of the same buffer. Unbound fractions (containing the citral lyase activity) were directly applied to a DEAE-Sepharose CL-6B (Pharmacia) column (2.5 × 25 cm), equilibrated with the same buffer. Protein was eluted by a phosphate buffer gradient: 10 mM (180 ml), 10 to 94 mM (495 ml, linear), 94 to 250 mM (90 ml, linear) and 250 mM (90 ml), at a rate of 0.9 ml min^{-1} (collected fraction volume, 9 ml). Fractions containing citral lyase activity (31 to 46 mM phosphate buffer) were pooled. HA and DEAE were both operated with a Gradifac system (Pharmacia Biotech, Roosendaal, The Netherlands).

Concentration and gelfiltration chromatography

After HA/DEAE active fractions were concentrated (on ice) in an Amicon ultrafiltration unit using a YM-10 membrane at 5 bar of pressure. The concentrated fractions were loaded onto an analytical G75 gelfiltration column (Superdex FPLC, Pharmacia) equilibrated with buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA and 20% $^{v}/_{v}$ glycerol). The enzyme was eluted at 1 ml min^{-1} using a FPLC system (Pharmacia Biotech, Roosendaal, The Netherlands) at room temperature.

Activity measurements

Citral lyase activity was typically determined by incubating the sample (1 ml total volume in a 15 ml vial fitted with Teflon Mininert valves (Supelco, Zwijndrecht, The Netherlands)) with citral, in a shaking water bath (oscillating at 2.5 Hz with an amplitude of 2 cm). Unless stated differently the incubations were carried out for 15 min at 25°C at a substrate concentration of 0.5 mM after which liquid samples were taken and analysed for methylheptenone (see analytical methods). The standard buffer used

contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA and 20% $^{v}/_{v}$ glycerol. One unit of citral lyase activity was defined as the amount of enzyme that produces 1 μmol of methylheptenone or acetaldehyde per minute.

Activity in spores

Citral lyase activity of spores was determined by diluting the spore suspensions 50 times in buffer (50 mM phosphate buffer, pH 7.0 containing 0.1% $^{v}/_{v}$ Tween 80) and incubated for 30 min with 2.83 mM citral.

Stability of citral lyase

The crude spore extract was diluted (1 to 100 times) and stored (0 to 7 days) at 4°C. To determine activity all samples were diluted 100 times and 1.1 mM citral was added.

Conversion profile of citral

The conversion of citral was followed in time by taking headspace samples from 2 to 60 min at 2-min intervals to determine acetaldehyde production and liquid samples in time to determine methylheptenone formation and geranial and neral degradation.

V_{max} and K_m

The V_{max} and K_m were determined by measuring acetaldehyde during the conversion of different concentrations of citral (0.022 to 0.556 mM). The initial activities of the conversions were plotted in a Lineweaver-Burk plot to obtain the value V_{max} and K_m.

Temperature and pH optimum

The temperature dependence of the conversion was determined by varying the temperature during the incubation from 0.4 to 45°C.
The pH dependence of the conversion was determined by varying the pH form 5.66 to 10.19 (by adding 0.8 ml of 0.1 M buffer to 0.2 ml of purified enzyme). The exact pH during the conversion was determined using a WTW microprocessor pH meter (Weilheim, Germany).

Alternative substrates

The conversion of the different substrates was tested using acetaldehyde production in time as a measure for activity. At the end of the conversion liquid samples were taken and analysed by GC and GC-MS to determine the products formed.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess purity of enzyme preparations and determine the molecular mass of the purified enzyme under denaturing conditions. SDS-PAGE was carried out with a Bio-Rad apparatus (mini protean II) and a homogenous 15% polyacrylamid gel, using Coomassie blue staining for detecting protein bands. Prestained protein markers (Bio-Rad) in the 7100 to 209000 molecular mass range were used to estimate molecular mass. The gel was scanned using a Bio-Rad GS-710 Calibrated Imaging Densitometer and interpreted using the Quantity One software (version 4.2.1).

Analytical Methods

Substrates and products of the conversions were detected by extracting the liquid samples with ethyl acetate and subsequent GC and GC-MS analysis, as described earlier (Wolken and van der Werf, 2001). Acetaldehyde and acetone were determined in the headspace of the samples as described earlier for acetaldehyde, only now isocraticaly at an oventemperature of 60°C (Wolken and van der Werf, 2001). Protein concentrations of spore suspensions and spore extracts were determined according to Lowry (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

RESULTS

Induction of citral lyase

Initially, the reproducibility of the results was hindered by variations in the citral lyase activity of the *P. digitatum* spores. Remarkably lyase activity was found to be induced when spores were incubated with the substrate, citral. Induction of citral lyase activity in spores op *P. digitatum* was dependent on both the concentration of citral and the time of induction (Figure 2). Preincubation of the spores with citral for 12 hours resulted in a substantial increase in lyase activity. Longer incubation times did not result in a further increase of activity. The induction was also strongly dependent on the citral

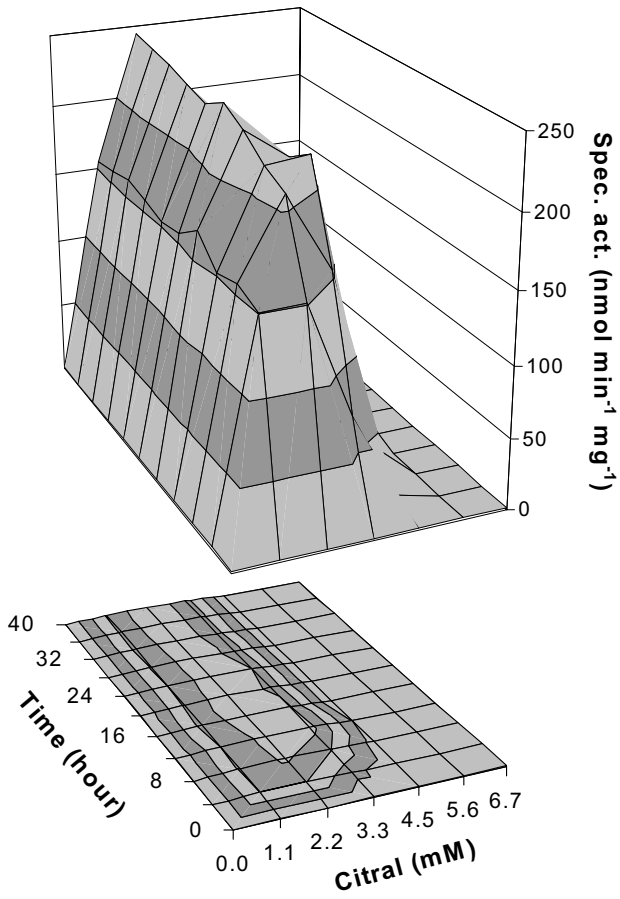


Figure 2. Induction of citral lyase activity in spores of *P. digitatum* (7.70 mg ml^{-1}) using different combinations of citral concentration and incubation time. Specific activity was calculated from the methylheptenone produced in 30 min.

concentration; while there was no induction in the absence of citral, the activity of the induced spores increased strongly with citral concentration reaching a maximum at a concentration of 2.2 mM. Raising the citral concentration to above 3.3 mM lead to a dramatic decrease in activity because of the toxic effects of citral towards spores of *P. digitatum* (Wolken *et al.*, 2002). For optimal induction of citral lyase activity, spores should be incubated for at least 12 hours at a citral concentration between 1.7 and 2.8 mM. Under these conditions the average activity of the induced spores was 204 nmol min^{-1} mg^{-1}, which is a factor 118 higher than the activity of the non-induced spores (1.7 nmol min^{-1} mg^{-1}). Addition of 500 ppm of cyclohexamide, a protein synthesis inhibitor (Santos *et al.*, 1978), inhibited the induction of citral lyase completely. The addition of cyclohexamide after induction did, however, not negatively influence citral lyase activity (not shown). This indicates that citral lyase is induced and not activated. To

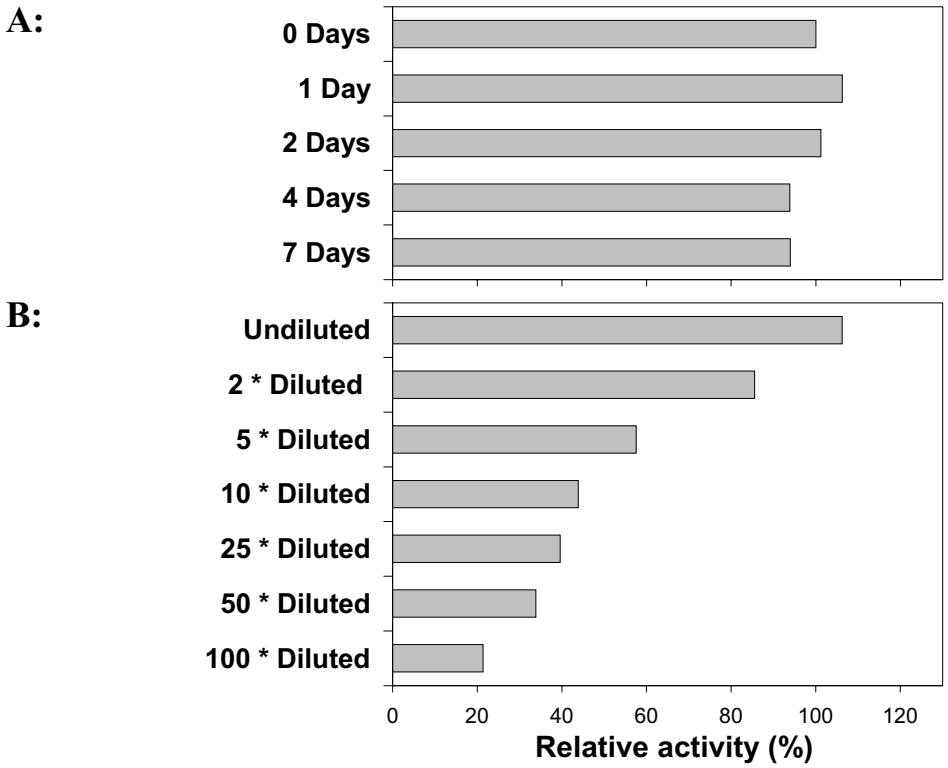


Figure 3. Stability of citral lyase activity in crude spore extract of *P. digitatum*. Activity was determined as methylheptenone formation after a 15 min incubation. The initial activity (storage time 0 days) was set to 100%. A, effect of storage time at 4°C on undiluted crude spore extract (1.8 mg ml^{-1}); B, effect of dilution on activity after one day storage at 4°C.

check for germination, the spores were studied under a light microscope (400 times magnification). There was no appreciable germtube-formation (less than 1 in 1000 spores showed signs of germination) visible during the induction, not even after 40 hours. Furthermore, the total protein content and average spore size did not change during induction.

Stability of citral lyase activity

The activity and stability of citral lyase was dramatically affected by the addition of 20% $^{v}/_{v}$ glycerol and 1 mM EDTA. When glycerol and EDTA were added before disrupting the spores, the activity of the crude spore extract was more than 25-fold higher (not shown). Even when these compounds were added after preparation of the crude spore extract there was a strong positive effect on the activity. Crude spore extract was found to be stable, only minor loss of activity was observed at 4°C over a period of 7 days (Figure 3A). However, dilution of crude spore extract resulted in a reduced stability of

citral lyase (Figure 3B). Upon 100 times dilution of the spore extract, 79% of activity was lost in one day time. Even at 10 times dilution 56% of activity was lost. The stability of citral lyase proved to be a key problem in further purification of citral lyase (see below).

Table 1. Purification of citral lyase from spores of *P. digitatum.*

Fraction	Total Activity (U)	Total Protein (mg)	Specific activity (U mg^{-1})	Purification (-fold)	Recovery (%)
Non-induced spore extract			0.00052		
Induced spore extract	27.7	23.5	1.18	1	100
Combined HA/DEAE	2.03	0.081	25.2	21.4	7.3
Gelfiltration (G75)	0.009	0.008	1.1	0.9	0.03

Enzyme purification

Of several different methods tested, hydroxyapatite (HA) and anionexchange (DEAE) chromatography were the most effective purification steps for citral lyase. Although citral lyase did not bind to HA it was an effective purification step as more than three-quarters of the total protein did bind to the HA column (not shown). To limit the negative effects of dilution, the HA column was directly coupled to the DEAE column. Previously, we showed that citral lyase has a low affinity for DEAE (Wolken and van der Werf, 2001). Simply raising the phosphate buffer concentration was sufficient to elute the enzyme, thus avoiding the use of NaCl or KCl, that have negative effects on stability of the enzyme (results not shown). Using HA and DEAE citral lyase was purified 21-fold with an overall yield of 7% (Table 1). Final purification by gelfitration resulted in a substantial loss of activity. This is in part caused by the need to concentrate the partially purified enzyme before applying it to the column and in part by the fact that gelfiltration was carried out at room temperature. Therefore, inclusion of the gelfiltration step in the overall purification scheme resulted in a reduced purification factor (1-fold) and a very poor yield (Table 1). Because of the large loss of activity in the final purification step the citral lyase characterisation studies were done with the citral lyase preparation after HA and DEAE.

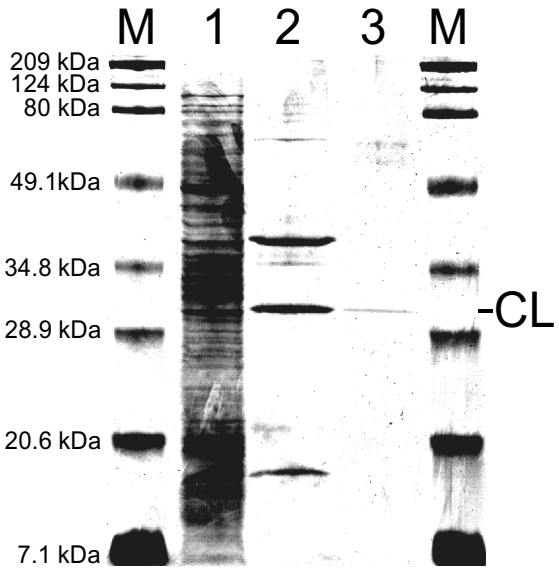


Figure 4. SDS-PAGE of citral lyase from *P. digitatu*m. M, molecular mass markers (6.25 μg); 1, Crude spore extract (10.7 μg); 2, pooled fractions after HA/DEAE (3.9 μg); 3, pooled fractions after gelfiltration (0.3 μg) and CL, citral lyase.

SDS-PAGE of the enzyme after final purification revealed 1 distinct band (Figure 4 lane 3). This band corresponds to 1 of the 3 major bands obtained after HA/DEAE purification visible in lane 2 of the same figure. From the gel it was calculated that the enzyme after HA and DEAE is 11.3% pure.

The native molecular mass of citral lyase was determined to be 25 kDa, based on the elution pattern of citral lyase activity during gelfiltration as compared to molecular mass standards. SDS-PAGE revealed a molecular mass of 30.8 kDa under denaturing conditions (Figure 4). Based on these results it can be concluded that citral lyase is a monomeric enzyme of approximately 30 kDa.

Citral conversion

The conversion of citral by citral lyase was followed in time (Figure 5). Citral lyase has a strong preference for the *trans* isomer of citral (geranial). Whereas geranial was already converted for approximately 45% after 60 minutes no neral (the *cis* isomer of citral) is converted at all. However, once the geranial concentration approaches zero also neral is converted albeit with approximately half the conversion rate as compared to geranial (insert Figure 5). Citral is converted into equimolar amounts of methylheptenone and acetaldehyde.

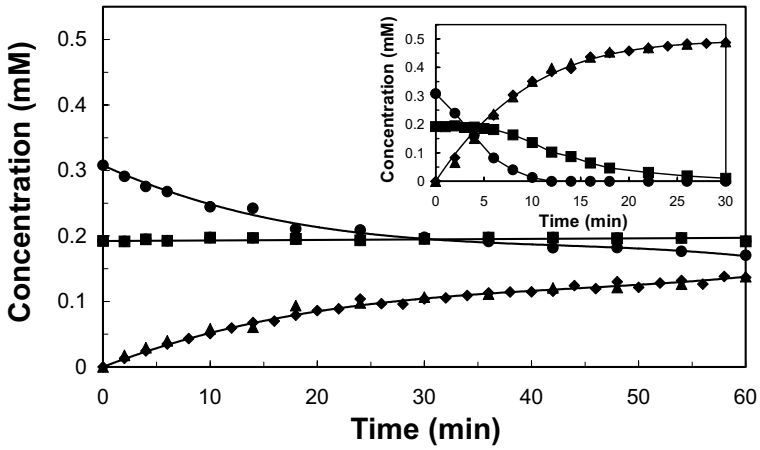


Figure 5. Transformation of citral into methylheptenone and acetaldehyde by purified citral lyase after HA/DEAE (0.221 μg ml^{-1}). Insert, conversion of citral by crude spore extract (36.7 μg ml^{-1}). Symbols: ●, geranial; ■, neral; ◆,acetaldehyde and ▲, methylheptenone.

The citral conversion rate was determined at different citral concentrations. From the Lineweaver-Burk plot of these data, the K_m for citral conversion was determined to be 0.058 (±0.01) mM and the V_{max} of the conversion was 52.6 (±6.7) U mg^{-1}.

Temperature and pH optimum

The temperature dependence of citral lyase is shown Figure 6A. Lyase activity is approximately 50% of maximum at 8°C and rises gradually to a clear optimum at 20°C after which it gradually declines again, reaching 50% activity at 30°C. From the Arrhenius plot (insert Figure 6A) an activation energy of citral conversion of 47.2 kJ mol^{-1} for citral lyase activity was determined. The activation energy for the inactivation of the enzyme was determined to be 103.3 kJ mol^{-1}.

The pH dependence of citral conversion by the purified enzyme is shown in Figure 6B. The activity is approximately 50% of maximum at pH 6.5 and rises gradually to a clear optimum at a pH of 7.6 after which it declines reaching 50% activity at pH 8.2. The buffer used had a significant effect on the citral lyase activity, and the highest activities were found using potassium phosphate buffer. At pH 7.0 five other buffers were tested (mes/NaOH, hepes/NaOH, tris/maleate, imidazole/HCl and mops/KOH), which all resulted in lower (5 to 25 times) activities compared to potassium phosphate buffer (not shown).

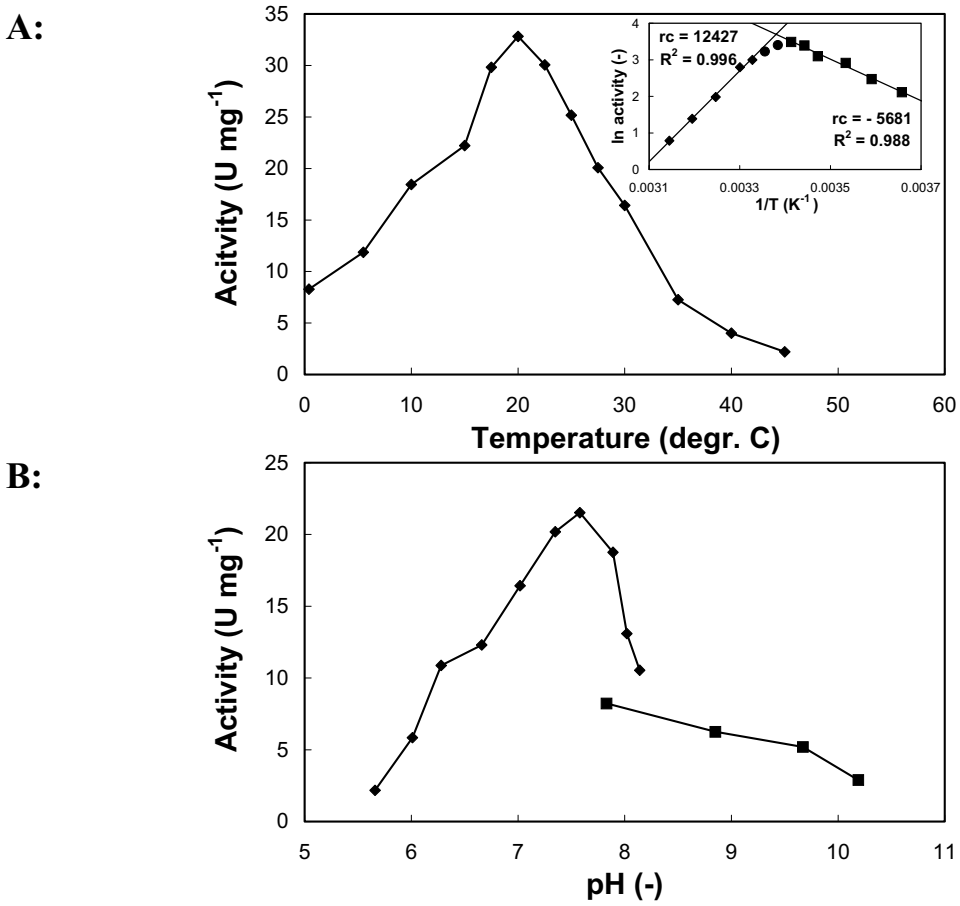
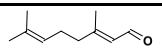
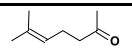
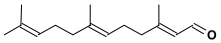
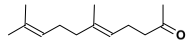
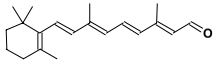
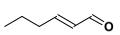
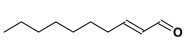
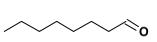
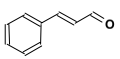
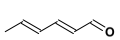
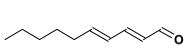


Figure 6. Effect of temperature (A; Insert, Arrhenius plot) and pH (B) on activity of citral lyase. Activity was based on methylheptenone production by purified citral lyase after HA/DEAE (0.221 μg ml^{-1}). A, 50 mM phosphate buffer (pH 7.0); B, 25°C; potassium phosphate buffer (◆) and sodium carbonate/sodium bicarbonate buffer (■).

Substrate specificity

A range of α,β-unsaturated aldehydes were tested as substrates for citral lyase (Table 2). As the total activity of the partially purified citral lyase is relatively low (Table 1) crude spore extract was used to pre screen potential substrates. Farnesal was converted with a rate of 30.6% of that of citral whilst methyl-crotonaldehyde, decenal and cinnamaldehyde were converted to a lesser extent, with 0.6, 0.7 and 0.3%, respectively. Conversion of crotonaldehyde, hexenal, hexadienal and decadienal was not observed. Retinaldehyde was also not converted by the crude spore extract. This was probably because retinaldehyde does not dissolve well in aqueous media and the addition of a co-solvent (10% acetone or ethanol) led to the total loss of enzyme activity. We showed that citral (Wolken *et al.*, 2000) and other α,β-unsaturated aldehydes (chapter 7) are also

Table 2. Conversion of α,β-unsaturated aldehydes by citral lyase from spores of *P. digitatum.*

Substrate		Relative activity (%)				Product		
		Crude spore extract		Cital lyase after HA/DEAE				
Name	Structure	Induced	Non-induced	Induced	Non-induced	Name	Structure	Identification method
Citral		**100**[1]	<0.1	**100**[1]	<1.0	Methylheptenone		GC; GC-MS
Methyl-crotonaldehyde		0.6	<0.1	1.9	<1.0	Acetone		GC
Farnesal		30.6	<0.1	20.6	<1.0	Geranyl acetone		GC-MS
Retinaldehyde		<0.1	<0.1					
Crotonaldehyde		<0.1	<0.1					
Hexenal		<0.1	<0.1					
Decenal		0.7	<0.1	3.6	<1.0	Octanal		GC
Cinnamaldehyde		0.3	<0.1	1.6	<1.0	Benzaldehyde		GC
Hexadienal		<0.1	<0.1					
Decadienal		<0.1	<0.1					

[1]**100,** Set to 100% for crude spore extract and purified enzyme respectively.

converted chemically, albeit at higher pH. As a control for this chemical (and non-specific enzymatic) conversion non-induced crude spore extract (0.05% of citral conversion activity as compared to extracts of induced spores) was used. These controls did not show detectable conversion of the alternative substrates.

After the screening conversion of farnesal, methyl-crotonaldehyde, decenal and cinnamaldehyde by HA/DEAE purified citral lyase was tested, this resulted in similar results. Farnesal, which structurally resembles citral the most of the tested substrates, is converted fastest by citral lyase (20.6% as compared to citral). GC and GC-MS showed that farnesal is converted to form the aroma compound geranyl acetone. The citral lyase also converted methyl-crotonaldehyde and decanal forming acetone and octanal, respectively. Furthermore, cinnamaldehyde was converted into benzaldehyde, one of the most frequently applied flavour compounds (Welsh *et al.*, 1989).

DISCUSSION

In this study, we purified citral lyase from spores of *P. digitatum*. Presently, only a very limited number of reports describing the purification of enzymes from spores have been published. These reports describe enzymes purified from fungal (e.g. *Neurospora crassa* (Say *et al.*, 1996) and *Botrytis cinerea* (Gindo and Pezet, 1999)) as well as bacterial spores (e.g. *Clostridium perfringens* (Miyata *et al.*, 1995) and *Bacillus subtilis* (Suzuki *et al.*, 2000)). There are several reasons to purify an enzyme from spores rather than from vegetative cells or mycelium; The enzyme of interest might be part of the germination machinery of the spores, and thus only present in spores (Miyata *et al.*, 1995). Likewise, some bioconversion activities are only present in the spores, as was demonstrated for *Saccharomyces cerevisiae* and *Bacillus subtilis* (Murata, 1993). Furthermore, there can be differences in the biochemical properties of enzymes expressed in spores as compared to vegetative cells (Say *et al.*, 1996). It has been reported that some enzymes are modified from vegetative type to spore type by a sporulation-specific protease during sporulation, producing differences in molecular and/or catalytic properties (Ujita and Kimura, 1975). Citral lyase, which was first identified in spores of *P. digitatum*, was also expressed in mycelium (not shown). However, due to the higher susceptibility of mycelium towards the toxic effects of citral (Wolken *et al.*, 2002) the enzyme could only be induced by a factor 5 in mycelium (not shown) as compared to the factor 118 induction in spores.

Remarkably, citral lyase could be induced in the non-germinating spores of *P. digitatum*. To the best of our knowledge, the induction of an enzymatic activity in non-germinating spores has so far only been described in spores of *Aspergillus oryzae*; i.e. α-

amylase, invertase and glucose dehydrogenase were induced in spores of *A. oryzae* without the occurrence of germination or swelling (Sinohara, 1970).

The most probable mechanism for the conversion of citral into methylheptenone and acetaldehyde is the addition of water to the α,β-double bond resulting in 3-hydroxycitronellal followed by rearrangement of the hydroxyl group leading to the cleavage of the α,β C-C bond (Figure 1A). This pathway is analogous to that proposed for the amino acid catalysed conversion of citral at high pH (Wolken *et al.*, 2000). For the enzymatic equivalent of this reaction the actions of a hydratase and an aldolase are needed. Citral lyase of *P. digitatum* combines hydratase and aldolase activity in a single enzyme. No other enzyme has been reported to catalyse the conversion of citral into methylheptenone and acetaldehyde, or a similar conversion of other α,β-unsaturated aldehydes. However, there have been reports on enzymes combining the action of a hydratase with that of an aldolase. The best studied is enoyl-CoA hydratase/aldolase (Overhage *et al.*, 1999) (also known as 4-hydroxycinnamoyl-CoA hydratase/aldolase (Mitra *et al.*, 1999)), which is involved in the bioconversion of ferulic acid to vanillin (Figure 1B1). Besides the substrate (feruloyl-CoA), enoyl-CoA hydratase/aldolase also convert the proposed intermediate (4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA) into vanillin (Gasson *et al.*, 1998). An other well known example is *trans-o*-hydroxybenzylidenepyruvate hydratase/aldolase (Eaton, 1994; Ohmoto *et al.*, 2000) (also known as is 2'-hydroxybenzalpyruvate hydratase/aldolase (Kuhm *et al.*, 1993)), which is part of the naphthalene catabolic pathway (Figure 1B2). Furthermore, *trans*-2-carboxybenzalpyruvate hydratase/aldolase (Iwabuchi and Harayama, 1998) (Figure 1B3) and 6-hydroxy-2-keto-5-methyl-3,5-heptadienoic acid hydratase/aldolase (Laurie and Lloyd Jones, 1999) (Figure 1C) have been reported in literature. Four hydratase/aldolases, which are like citral lyase cofactor-independent, have been purified and characterised. One is a homodimer of 63 kDa (Mitra *et al.*, 1999), the other three were all homotrimers of 110 (Ohmoto *et al.*, 2000), 113 (Iwabuchi and Harayama, 1998) and 120 kDa (Kuhm *et al.*, 1993) respectively. Citral lyase is a monomeric enzyme of 30 kDa, which is approximately the monomeric size of these hydratase/aldolase enzymes. All of these enzymes exhibit more then 75% of their maximum activity at pH 7.6, the optimum pH of citral lyase (Mitra *et al.*, 1999; Ohmoto *et al.*, 2000; Kuhm *et al.*, 1993; Iwabuchi and Harayama, 1998). Whereas, many bacterial aldolases require a divalent cation for catalysis, this does not seem to be the case for hydratase/aldolases, which are, like citral lyase, not negatively affected by EDTA (Kuhm *et al.*, 1993).

The citral lyase described in this chapter is the first example of a hydratase/aldolase acting on the α,β-double bond of α,β-unsaturated aldehydes. This novel enzyme was purified from spores of *P. digitatum*, wherein it was found to be

inducible by the substrate citral. Citral lyase seems to have the potential to produce other natural flavour compounds as e.g. benzaldehyde.

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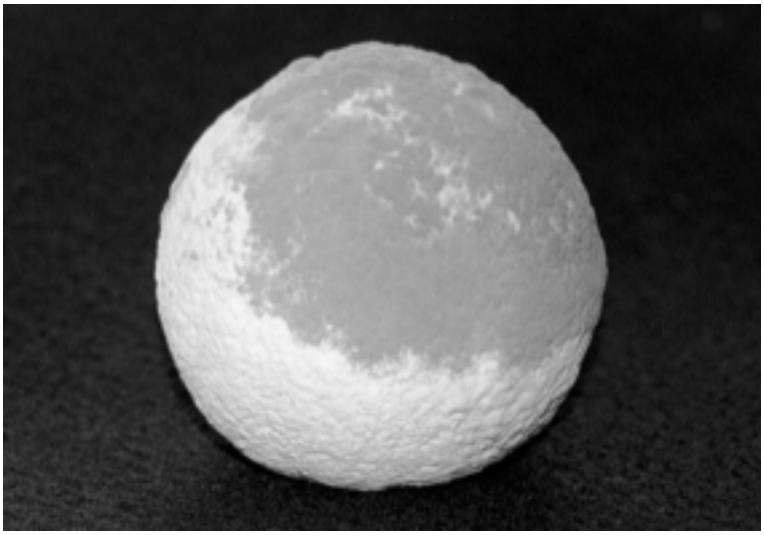
Chapter 5

Toxicity of terpenes to spores and mycelium of *Penicillium digitatum*



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ABSTRACT

Spores, although often considered metabolically inert, catalyse a variety of reactions. The use of spores in stead of mycelium for bioconversions has several advantages. In this chapter, we describe the difference in susceptibility of mycelium and spores against toxic substrates and products. A higher resistance of spores towards the toxic effects of bioconversion substrates and products is advantage that has not been studied in detail until now. This chapter shows that spores of *Penicillium digitatum* ATCC 201167 are on average over 2.5 times more resistant towards the toxicity of substrates, intermediates and products of the geraniol bioconversion-pathway, than mycelium. Furthermore, the higher resistance of spores to citral was shown an advantage in its biotransformation by *P. digitatum*. Using three different approaches the toxicity of the compounds were tested. The order of toxicity toward *P. digitatum* was, starting with the most toxic: Citral > nerol / geraniol > geranic acid > methylheptenone >> acetaldehyde.

INTRODUCTION

Fungal spores are generally considered as a dormant stage in the life cycle of fungi. However, spores have been shown to contain enzymes necessary for a variety of metabolic activities. Since Gehrig and Knight (1958) reported that conidia of *Penicillium roquefortii* catalysed the conversion of octanoic acid to 2-heptanone, several papers have been published on spore bioconversions (Larroche and Gros, 1997; Murata, 1993; Vezina *et al.*, 1968). Several advantages of using spores have been proposed; when using spores instead of mycelium the medium is less viscous, resulting in easier aeration and product recovery (Moskowitz, 1979). Moreover, spores are generally easier to store, and the lack of pellet formation results in greater homogeneity of the biocatalyst (Larroche and Gros, 1997). In some cases biotransformation reactions are carried out exclusively by spores, or vegetative cells catalyse the biotransformation less efficiently (Knight, 1966). However, one obvious advantage of using spores has been suggested in literature (Murata, 1993) but has not been studied in detail, that is the toxicity resistance of spores to substrates and products of the bioconversion. As spores are generally more rigid than mycelium it would be likely that they are less susceptible to toxicity effects.

Microorganisms have been applied in many foods and beverages for their flavour enhancing effects. Microorganisms can either produce flavour chemicals *de novo* or convert precursors into flavour compounds. However, many of these flavours are toxic to microorganisms (Pai, 1990.). Besides products, also the precursors can be toxic, as is the case in vanillin (Krings and Berger, 1998). This is also the case for monoterpenes, which are important flavour and fragrance compounds. The biotransformation of

monoterpenes has been studied quite extensively during the past 30 years. A problem associated with monoterpene biotransformation studies is the toxicity of these compounds to whole cells (van der Werf *et al.*, 1997).

Previously, we described the bioconversion of the linear monoterpene geraniol by spores of *P. digitatum* (Wolken and van der Werf, 2001) and the intermediates and products of this conversion were identified (Figure 1). The compounds that are involved in this conversion, geraniol, nerol, citral, geranic acid, methylheptenone and acetaldehyde are all known to be toxic (Gochnauer *et al.*, 1979; Reichardt, 1981; Scora and Scora, 1998; Yuen *et al.*, 1995). Especially the toxicity effects of the highly toxic citral have been described extensively in the literature (Gochnauer *et al.*, 1979; Knobloch *et al.*, 1988; Kurita *et al.*, 1981; Mahmoud, 1994; Onawunmi, 1989; Pattnaik *et al.*, 1997; Yousef *et al.*, 1978). The known toxicity of citral is an advantage when applying citral as a biocontrol agent, e.g. as grain preservative (Nandi, 1978) and in controlling post harvest decay of lemon fruit (Rodov *et al.*, 1995).

The aim of this study was to determine if spores are more resisted to terpenes than mycelium of the same strain. Moreover, that the use of spores instead of mycelium in bioconversion might be an advantage in biotransformation, more specific in flavour production. In order to prove this geraniol, nerol, citral, geranic acid, methylheptenone and acetaldehyde were tested for their toxicity towards mycelium and spores of *P. digitatum*. Effects on the viability of both spores and mycelium were tested as well as the effects on bioconversion rate.

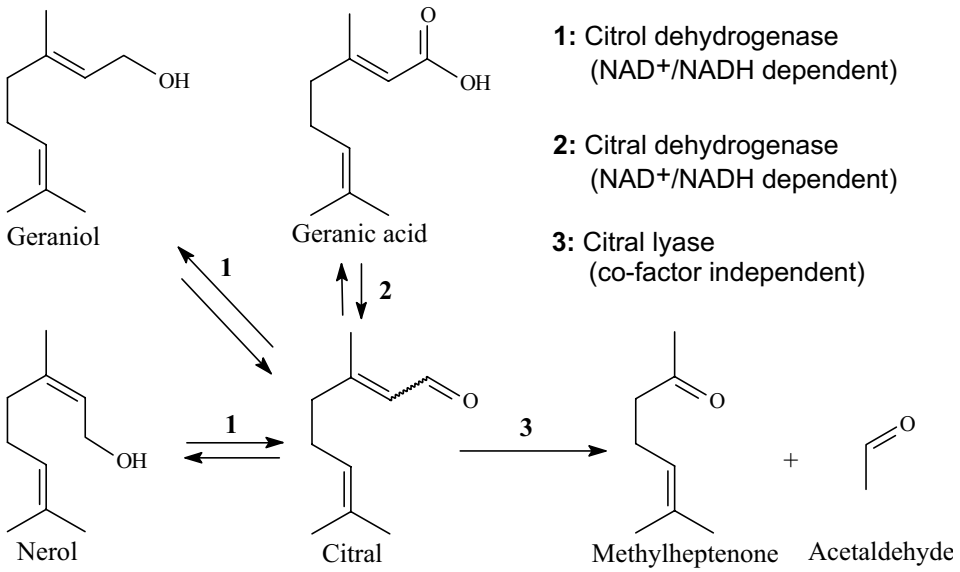

Figure 1. Bioconversion pathway of geraniol and nerol into methylheptenone and acetaldehyde by spores of *P. digitatum* ATCC 201167 (Wolken and van der Werf, 2001).

MATERIALS AND METHODS

Organism

Penicillium digitatum ATCC 201167 (*P. digitatum* CLE) was isolated from a spoiled tangerine (Demyttenaere and De Pooter, 1998). The culture was maintained as a spore suspension in buffer (see below) stored at –80ºC.

Media and buffer

Defined medium (DM) is a modified defined mineral salts medium (pH 7.0) (Hartmans *et al.*, 1989). The N-source is aspargine monohydrate (2 g l^{-1}) and the C-source is glucose monohydrate (20 g l^{-1}). Defined medium agar (DMA) is DM to which 15 g l^{-1} of agar was added. Malt extract agar (MEA) contains 10 g l^{-1} malt extract, 0.5 g l^{-1} pepton, 10 g l^{-1} glucose and 10 g l^{-1} agar. The buffer used throughout the experiments (unless stated otherwise) was a 50 mM phosphate buffer (pH 7.0) containing 0.1% $^{v}/_{v}$ Tween 80.

Production of spores and mycelium

Spores were obtained by growing *P. digitatum* on DMA (0.5 l) autoclaved at 121ºC for 20 minutes in 5-liter Erlenmeyer flasks. After solidification of the agar, the surface was inoculated with 5 ml spore suspension (see below). After 8 days incubation at 21ºC, the spores were harvested by washing the surface of the culture twice with buffer. The spore suspension obtained was concentrated by centrifugation, dissolved in buffer (10 ml) and stored at -20ºC until used.

Mycelium was obtained by inoculating 50-ml portions DM in 300 ml-Erlenmeyer flasks (autoclaved at 121ºC for 20 min) with 0.5 ml of spore suspension. The flasks were incubated at 30ºC on a horizontal shaker oscillating at 1 Hz with an amplitude of 10 cm. After the growth of *P. digitatum* for 2 days the mycelium was harvested by filtration (∅ 40 mm, #0860, Schleicher & Schuell, Dassel, Germany) and subsequent resuspending in buffer.

Induction of citral lyase activity

Spores were induced by adding 0.4 µl citral per ml of spore suspension, and incubating for 11 hours at 25°C whilst shaking (2.5 Hz, 2 cm amplitude). After washing the spores 4 times with buffer, the induced spore suspension was stored at -20 °C for later use.

Induced mycelium was obtained by adding 0.2 µl citral per ml of growing mycelium culture (two days old) and incubating for 15 hours at 30ºC whilst shaking (1 Hz, 10 cm amplitude). The mycelium is used directly after washing it 4 times with buffer.

Chemical properties of the chemicals used

Vapour and liquid phase concentrations (at 25°C) of geraniol, nerol, citral, geranic acid, methylheptenone and acetaldehyde were measured by gas chromatography (GC) to determine water/air partition coefficients ($K_{water/air}$), necessary to calculate the actual concentrations from the added amounts (Table 1). For the compound with the lowest $K_{water/air}$, methylheptenone, this accounts to only 3.3% being in the gaseous phase (in the case of 1 ml aqueous phase in a total volume of 16.5 ml), so evaporation of the compounds plays only a minor role under the test conditions.

Maximum solubility's of geraniol, nerol, citral, geranic acid and methylheptenone were determined under the actual reaction conditions by measuring the concentrations in the aqueous phase after centrifugation (5 min, 13000 g, 25°C) of over-saturated solutions (Table 1). The values in Table 1 are used as the upper limit for the experiments described in this chapter.

Growth in presence of compounds

Experiments were carried out in 250 ml Boston flasks fitted with Teflon Mininert valves (Supelco, Zwijndrecht, The Netherlands) containing 25 ml of sterile DM. Different amounts of test compounds were added and the experiments were started by inoculating with 200 µl of spore suspension (final concentration of spores 2.0 µg protein ml^{-1}). After 3 days of growth at 25°C whilst shaking (2.5 Hz, 2 cm amplitude) the CO_2 production, as a measure for biomass formation, was determined by GC.

Effects of preincubation

To 1 ml buffer (in a 15 ml vial fitted with a Teflon closure) containing mycelium or spores, different amount of the tested compounds were added by micro syringe. The vials were placed in a shaking water bath (4 Hz, 2 cm amplitude, 25ºC) for 30 min after which the activity or viability was tested. For the viability study, non-induced spores and mycelium were used. The same amount of biomass was used for both spores and mycelium (both 23.8 µg of protein) to ascertain that differences in biomass

Table 1. Chemical properties, under the bioconversion conditions, of the tested compounds.

	Chemical name	Max. Solubility (mM)	$K_{water/air}$ (-)
Geraniol	2-*trans*-3,7-dimethyl-2,6-octadien-1-ol	6.0 (0.2)[a]	2984 (171)
Nerol	2-*cis*-3,7-dimethyl-2,6-octadien-1-ol	5.9 (0.2)	2767 (375)
Citral	3,7-dimethyl-2,6-octadien-1-al	6.9 (0.2)	991 (68)
Geranic acid	2-*trans*-3,7-dimethyl-2,6-octadienoic acid	19.8 (0.5)	[b]
Methylheptenone	6-methyl-5-hepten-2-one	22.4 (0.3)	486 (24)
Acetaldehyde	ethanal	[c]	596 (48)

[a], Values in brackets are the standard errors; [b], not detected in gas phase; [c], completely water soluble.

-concentration did not influence the outcome of the study. For the activity study, citral-induced spores and mycelium (both 10.1 μg of protein) were used.

To determine viability, the exposed samples were diluted with sterile buffer, plated on MEA plates and incubated at 20°C. Spores were diluted 500 times, 200 μl was plated and colonies were counted after 3 days. Mycelium was diluted 100 times, 500 μl was plated and colonies were counted after 2 days.

To determine activity, the exposed samples were washed and tested for the conversion of citral into methylheptenone (30 min, 4 Hz, 2 cm amplitude, 25ºC). Spores were washed by removal of the supernatant after centrifugation (2 min, 13000 g, 4°C) and subsequent addition of fresh buffer (3 times); to the final volume of 2.5 ml 1 μl citral was added. Mycelium was washed by filtration (∅ 40 mm, #0860, Schleicher & Schuell, Dassel, Germany) and subsequent addition of fresh buffer (3 times); to the final volume of 5 ml 1 μl citral was added. After 30 min, the mixture was extracted with an equal volume of ethyl acetate. The vials were shaken vigorously to quantitatively extract the terpenes. The ethyl acetate phase was separated and 5 μl of the ethyl acetate phase was analysed by GC.

Biotransformation of citral at different concentrations

The conversion of citral into methylheptenone was determined in a 15 ml vial fitted with a Teflon closure containing 1 ml of spore or mycelium suspension (both at 10.1 μg

protein ml^{-1}) and different amounts of citral. The vials were placed in a shaking water bath (250 rpm, 25ºC) for 5 hours after which the vials were extracted with 1 ml of ethyl acetate. Subsequently 5 µl of the ethyl acetate phase was analysed by GC for methylheptenone formation.

Analytical Methods

All tested compounds were analysed by GC as described previously (Wolken *et al.*, 2000). CO_2 was analysed by injecting 100-µl gas phase samples onto a Hewlett-Packard 6890 gas chromatograph containing a Chrompack Poraplot Q column (Chrompack, Middelburg The Netherlands). The carrier gas was helium at a flow of 3.0 ml min^{-1} and a thermal conductivity detector was used. The column and detector temperatures were 70 and 250°C, respectively. After boiling samples for 10 min in the presence of 1 N NaOH, protein concentrations were determined according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistical analysis

The concentrations at which the tested compounds caused an inhibition of 50% (IC50) was determined using the following general procedure. Each set of experimental values (either CO_2 produced, colony count or methylheptenone produced) was normalised using controls (average of the controls set to 100%). The results of two to six separate experiments were plotted in a single graph; the values in % plotted against the added amount of tested compound. For every IC50 determined 9 to 40 separate samples were used (controls not included). Using the statistical program GENSTAT 5 (release 4.1, Lawes Agricultural Trust, IACR-Rothamsted, UK) a Gompertz curve was fitted through the data (Van der Graaf and Schoemaker, 1999). The 95% reliability interval was determined using the Monte-Carlo method (Manly, 1997).

Chemicals

Geraniol, nerol, citral, geranic acid and methylheptenone were purchased from Fluka (Buchs, Switzerland). Acetaldehyde was purchased from Aldrich (Steinheim, Germany). Agar (no. 1) and malt extract were purchased from Oxoid (Hapshire, England). Peptone (no. 140) was purchased from GibcoBRL (Paisle, Scotland). All other chemicals used were of analytical grade (purity ≥ 99%).

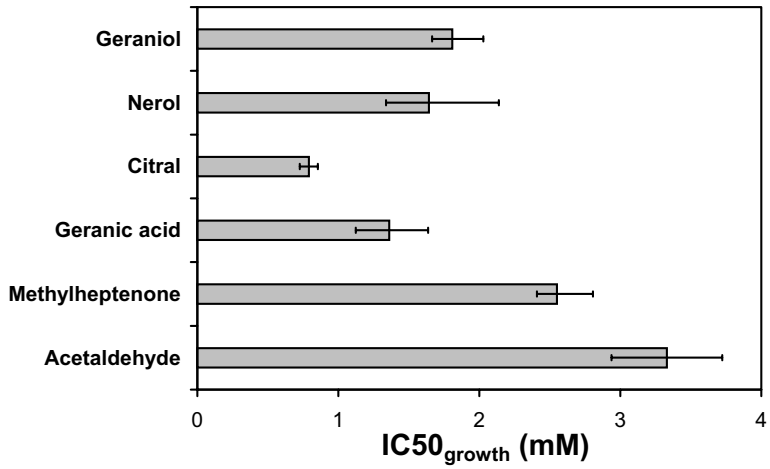

Figure 2. Inhibition of mycelial growth, as determined by measurement of CO_2 production, by the presence of geraniol, nerol, citral, geranic acid, methylheptenone or acetaldehyde during growth of *P. digitatum*. The error bars represent the 95% reliability interval.

RESULTS

Growth of *P. digitatum* in presence of chemicals

The concentrations at which the mycelial growth of *P. digitatum* was inhibited 50% ($IC50_{growth}$) was determined using CO_2 production as a measure for growth (Figure 2). There was no significant difference in $IC50_{growth}$ between geraniol, nerol and geranic acid (1.4 to 1.8 mM) under these conditions. The $IC50_{growth}$ was lowest for citral, methylheptenone was less toxic than geraniol and nerol and acetaldehyde was found the least toxic of the tested compounds.

Effects of preincubation on viability and citral lyase activity of *P. digitatum*

The viability of mycelium and spores of *P. digitatum* after exposure to the different compounds was tested. Growth on MEA after exposure for 30 min to these compounds was used to determine the viability ($IC50_{viability}$). The results for both spores and mycelium of *P. digitatum* are displayed in Figure 3A. The differences between the tested compounds are much more profound as compared to the $IC50_{growth}$. Citral is again more toxic than geraniol and nerol, which are equally toxic. However, the difference between citral and geraniol or nerol is only significant for the mycelium, not for the spores. Geranic acid and methylheptenone have a much higher $IC50_{viability}$, in both cases 50% inhibition of growth was not reached at the maximal solubility (Table 1) of the tested

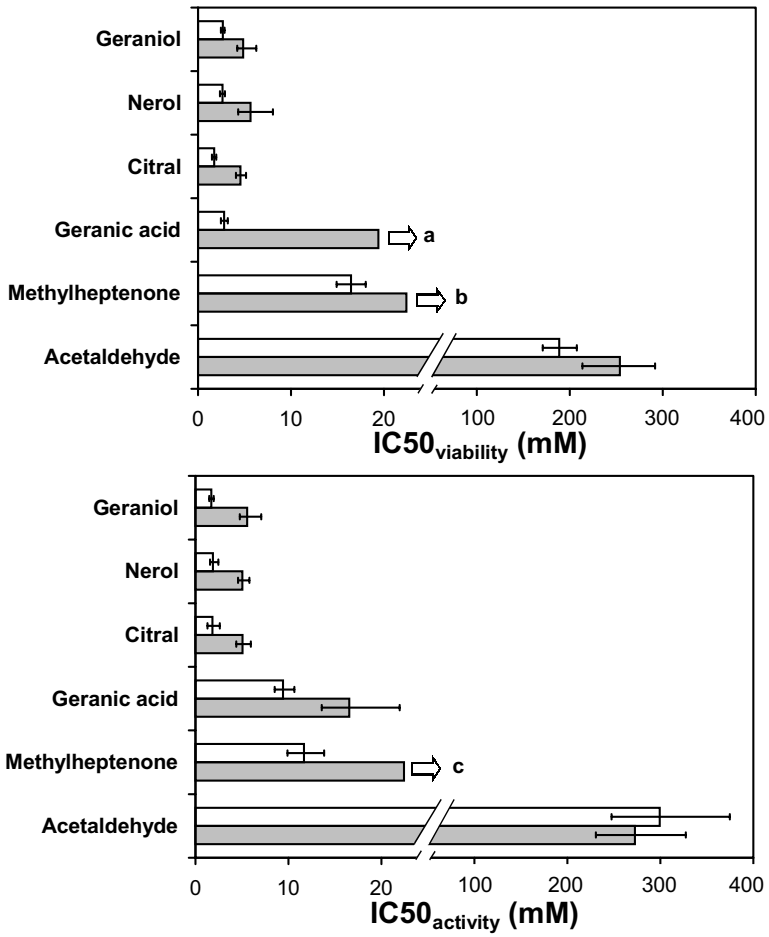

Figure 3. Viability and remaining citral lyase activity of mycelium (open bars) and spores (filled bars) of *P. digitatum* after 30 minutes of incubation with geraniol, nerol, citral, geranic acid, methylheptenone or acetaldehyde. Viability was determined by counting colonies formed on MEA (A) and activity was determined as methylheptenone formation from citral (B). Symbols: ⇨, less than 50% inhibition at saturating concentration; a, no inhibition; b, ± 27% inhibition and c, ± 31% inhibition. The error bars represent the 95% reliability interval.

compound. However, methylheptenone seems to be the more toxic compound of the two, as it showed some loss of viability (± 27%) at maximal solubility, whereas geranic acid showed none. The most remarkable are the findings for acetaldehyde. Where this compound has an only slightly higher $IC50_{growth}$ compared to the other compounds, it has a more than factor 10 times higher $IC50_{viability}$, indicating that the inhibition of growth by acetaldehyde is a largely reversible reaction. For all the compounds tested, the mycelium was more susceptible for the toxic effects than spores, ranging from a factor 1.4 more susceptible for acetaldehyde to a more than a factor 6.9 for geranic acid.

The activity of citral lyase was used as a measurement of the remaining bioconversion activity after exposure of *P. digitatum* to different compounds. The susceptibility of both spores and mycelium was expressed as $IC50_{activity}$, for all the tested compounds (Figure 3B). Geraniol, nerol and citral are equally toxic towards citral lyase activity of *P. digitatum*. Geranic acid is less toxic followed by the even less toxic methylheptenone. Although the difference in $IC50_{activity}$ between geranic acid and methylheptenone is not significant for mycelium, it is evident for spores. Spores exposed for 30 min to methylheptenone at maximum solubility (Table 1) only showed a 31% loss of activity, compared to the controls. Like the $IC50_{viability}$, the $IC50_{activity}$ for acetaldehyde is much higher than for the other compounds tested. Again, except for acetaldehyde, mycelium is much more sensitive to the tested compounds than the spores. The maximum difference between mycelium and spores in this experiment was a factor 3.3 in the susceptibility for geraniol.

Bioconversion activity at different concentrations of citral

Besides the $IC50_{activity}$, also the toxicity effects during the bioconversion were assessed. This measurement was limited to the conversion of citral at different concentrations of citral. The conversion of the other possible substrates (geraniol, nerol and geranic acid) was not studied as these conversions involve more than one enzymatic step and display a 'lag-phase', making them more difficult to assess (Wolken and van der Werf, 2001). In Figure 4 the relationship between citral concentration and activity are depicted for spores and mycelium of *P. digitatum*.

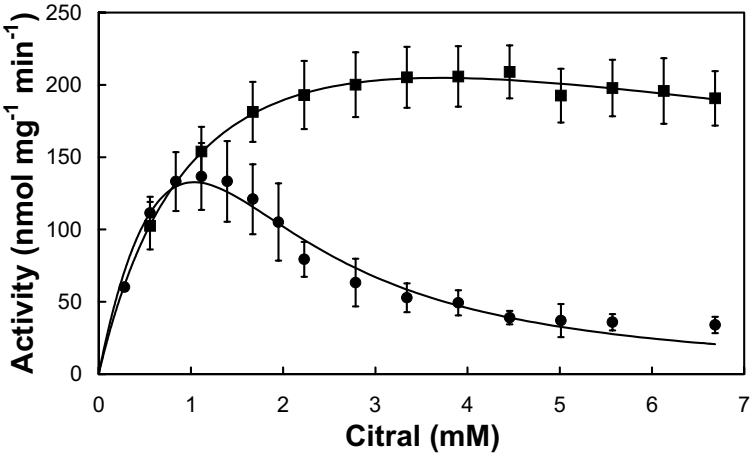

Figure 4. Effect of substrate concentration on the citral bioconversion activity (expressed as methylheptenone formation). Symbols: ●, Mycelium and ■, Spores. The error bars represent the standard error of triplicates.

The conversion of citral by spores and mycelium is comparable at concentrations lower than 1 mM. At higher concentrations, the activity of mycelium decreases considerably compared to spores. This indicates that whilst maximum conversion rate and the affinity constant are comparable there is a considerable difference in inhibitory constant, mycelium being inhibited at a much lower concentration of citral. This is in agreement with the difference in $IC50_{activity}$ and $IC50_{viability}$ found for citral.

DISCUSSION

In this chapter, results are presented which address the toxicity problems associated with monoterpene biotransformation. The order of toxicity of the tested compounds was determined (starting with the most toxic compound); citral > nerol / geraniol > geranic acid > methylheptenone >> acetaldehyde. Considering the order of toxicity, the conversion of geraniol, nerol, geranic acid and citral into methylheptenone might be a detoxification process similar to the conversion of medium chain fatty acids into methyl ketones as described for *P. crustosum* (Hatton and Kinderlerer, 1991). Secondly, with the exception of the $IC50_{activity}$ for acetaldehyde, the mycelium is more susceptible to the toxicity of the tested compounds than spores. On average (for activity and viability), the IC50 is a factor 2.5 lower for mycelium than for spores of *P. digitatum*.

It is difficult to compare the results obtained in this study to literature data as the methods used differ considerably and moreover, the manner in which the results are represented differ to a great extent. Frequently, results are presented as inhibition zone (in mm^2 or mm), days of inhibition (after treatment), % decay (of oranges), or no unit is given at all (tested compounds just put in order of toxicity). However, if more than one compound of interest is tested in the same paper, one can extrapolate some results. There are several reports in which both citral and geraniol are tested for their antifungal and antibacterial properties. In most of the studies citral is more toxic than geraniol (Gochnauer *et al.*, 1979; Knobloch *et al.*, 1988; Pattnaik *et al.*, 1997; Scora and Scora, 1998; Viollon and Chaumont, 1994; Yousef *et al.*, 1978). However, there are also reports indicating no real difference in toxicity (Kurita *et al.*, 1981) or even the opposite (Arora and Pandey, 1977; Mahmoud, 1994). Likewise, geraniol and nerol were tested in the same papers. Several papers claim no real difference in fungi- and bactericidal action of geraniol and nerol (Knobloch *et al.*, 1988; Mahmoud, 1994; Scora and Scora, 1998) and one report showed that geraniol was more toxic (Viollon and Chaumont, 1994). In general, there seems to be no real difference in toxicity between nerol and geraniol. Last, one paper describes that methylheptenone is an, at least five times less efficient, antifungal agent then citral and that acetaldehyde is at least a further factor two less effective (Nandi and Fries, 1976). Extrapolating from the data presented in literature one

comes to the following order of toxicity (starting with the most toxic compound): Citral > geraniol / nerol > methylheptenone > acetaldehyde (geranic acid unknown). This is in agreement with the findings of this report.

The main target for the toxicity of lipophilic compounds, such as terpenes, to eukaryotic cells is the cell membrane (Bard *et al.*, 1988; Sikkema *et al.*, 1995). The terpenes partition preferentially in membranes causing an increase in membrane fluidity, which is leading to a permeabilisation. Furthermore, these lipophilic compounds affect proteins and more specific enzymes. The fact that not only the viability of *P. digitatum*, but also the bioconversion activity is susceptible to the toxicity effects of the terpenes tested, confirms that also for *P. digitatum* the toxicity effect of the terpenes is rather non-specific. If only the mitochondria would have been targeted, as suggested for *S. cerivisiae* (Uribe *et al.*, 1985), only the growth after exposure would have been affected and not the activity of a cofactor-independent enzyme, like citral lyase.

There is a rather big difference, especially for acetaldehyde, when comparing the fungistatic ($IC50_{growth}$) to the fungicidal properties ($IC50_{viablity}$ and $IC50_{activity}$). This indicates that at the concentrations where growth of *P. digitatum* is inhibited the toxic effects are still reversible. The good correlation between the $IC50_{viablity}$ and $IC50_{activity}$ indicates the potential to use an enzymatic activity (in this case citral lyase) as a measure for the viability of a microorganism. Similarly, the enzymatic reduction of MTT is frequently used as a test for cell viability (Bernas and Dobrucki, 1999; Stentelaire *et al.*, 2001).

In conclusion, this chapter shows that spores are more resistant towards toxic substrates, intermediates and products of the geraniol bioconversion-pathway than mycelium of *P. digitatum*.

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Chapter 6

The amino acid-catalysed conversion of citral: *cis-trans* isomerisation and its conversion into 6-methyl-5-hepten-2-one and acetaldehyde

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ABSTRACT

Under alkaline conditions amino acids or proteins catalyse the retroaldol condensation of citral, a major aroma component, resulting in methylheptenone and acetaldehyde formation. 3-Hydroxycitronellal is an intermediate in this reaction. Amino acids also catalyse the *cis-trans* isomerisation of the pure isomers of citral, geranial and neral. Most likely the amino acids are involved in stabilising intermediates of the isomerisation and retroaldol condensation-reaction of citral. Based on our findings some consequences for the application of citral, or its isomers, in food are discussed.

INTRODUCTION

Citral is widely used in the flavour and fragrance industry, its application ranges from meat products to hard candy. The amounts used in the products differ greatly, amounting to as little as 0.20 ppm in cheese and as many as 429.8 ppm in chewing gum. Citral has a strong, lemon-like odour and a characteristic bittersweet taste (Burdock, 1995). It was originally reported in lemongrass, accounting for up to 75% of the oil. Citral is also found in several other plant oils, and it is present in lemon and lime oil. Commercial citral is obtained by isolation from citral containing essential oils or by chemical synthesis from β-pinene or isoprene (Burdock, 1995). With an annual world consumption of 1200 tons (in 1996) it is one of the most applied flavour compounds (Somogyi, 1996). Moreover, citral has antimicrobial (Onawunmi, 1989) and pheromone activity (Kuwahara *et al.*, 1983; Robacker and Hendry, 1977), and is used in the production of vitamin A and ionones (Shadab *et al.*, 1992).

The linear monoterpene citral is a mixture of the *cis*- and *trans*-isomers of 3,7-dimethyl-2,6-octadiene-1-al, referred to as neral and geranial, respectively. Commercial citral typically contains 60% geranial and 40% neral. The *cis-trans* isomers geranial and neral have different characteristics. They differ in odour threshold (neral 8.8 ng l^{-1} air, geranial 12.0 ng l^{-1} air) (Schieberle and Grosch, 1988). Also the aroma profiles of the two citral isomers differ; neral has a somewhat harsh and grassy odour while geranial is milder and more lemon like (Clark and Chamblee, 1992). Furthermore, neral is an alarm pheromone in some types of mites, while geranial is not (Kuwahara *et al.*, 1983).

In this chapter, we report on the amino acid-catalysed retroaldol condensation and isomerisation of geranial and neral, under neutral and alkaline conditions.

MATERIALS AND METHODS

Materials

Citral and methylheptenone were purchased from Fluka (Buchs, Switzerland) Acetaldehyde was purchased from Merck (Darmstadt, Germany). A reference sample of 3-hydroxycitronellal was prepared as described by Fkyerat and Tabacchi (1997). Geranial (containing 95% geranial and 5% neral) and neral (containing 11% geranial and 89% neral) were obtained by separating commercial citral using preparative GC. Of a 10% citral solution in acetone, 30 µl was injected on a packed column (2.0 mm × 4 mm) filled with 8.6 g of Chromsorb 100-120 containing 9.8% Carbowax. GC was performed on a Varian model 3700 GC equipped with a thermo conductor detector. The detector and filament temperatures were 130 and 160°C, respectively. The injector and oven temperatures were 200 and 110°C, respectively, and the flow of the carrier gas (H_2) was 30 ml min^{-1}. The purified isomers were dissolved in hexane and stored at 4°C.

cis-trans Isomerisation

Experiments were carried out in 4 ml vials fitted with Teflon Mininert valves (Supelco, Zwijndrecht, The Netherlands). 200 µl of a geranial or neral solution in hexane (equalling 1 mM final concentration in solution) was added to the vials and subsequently the hexane was evaporated. After the addition of 1 ml reaction mixture the vials were vigorously shaken for 30 seconds, to obtain a homogenous mixture, and placed in a shaking waterbath (150 rpm, 25°C). The reaction mixture was an aqueous solution containing 0.1 M amino acid and buffer (typically a 50 mM phosphate buffer (pH 7.0), or others (0.1 M) when testing the pH dependence). The reaction was followed by analysing samples (volume 100 µl), taken from the headspace, by GC. At the end of each experiment, the reaction mixture was extracted with 1 ml ethyl acetate to determine non-volatile intermediates and products, which could not be detected in the headspace samples. The vials were vigorously shaken to quantitatively extract the terpenes. The ethyl acetate phase was separated from the aqueous phase by centrifuging the mixture in a 2 ml microcentrifuge tube (1 min, 13000 rpm). Subsequently 5 µl of the ethyl acetate phase was analysed by GC.

Conversion of citral into methylheptenone

The glycine-catalysed conversion of citral was determined in 15 ml vials, fitted with Teflon Mininert valves. Reaction mixtures (1 ml) contained 1 mM citral and 0.1 M glycine/NaOH buffer (pH 10.0). At different times, 66.6 µl samples were taken and analysed immediately for acetaldehyde using an acetaldehyde test kit (Boehringer, Mannheim, Germany). The remaining 0.933 ml sample was extracted with a equal amount of ethyl acetate and analysed by GC

The pH-dependence of the transformation of citral into methylheptenone catalysed by different amino acids was monitored in reaction mixtures (1 ml) containing 0.1 M amino acid and 0.25 M buffer. The reaction was started by addition of 1 µl citral (5.66 mM final concentration). After 20 hours in a shaking water bath (300 rpm, 25°C) the terpenes were extracted with 1 ml ethyl acetate and analysed by GC.

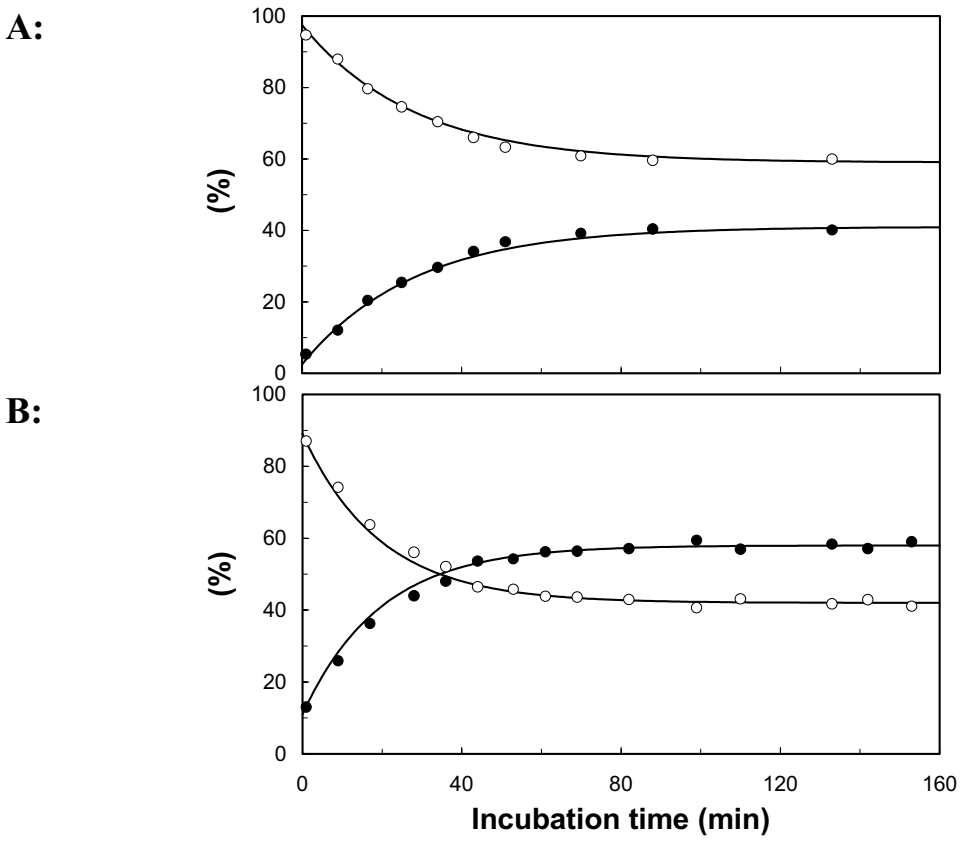

Figure 1. Amino acid-catalysed isomerisation of geranial (A) and neral (B). Each reaction mixture (25°C) contained 0.1 M glycine, 50 mM potassium phosphate buffer (pH 7.0) and 1 mM substrate. Geranial and neral are expressed as a percentage of total citral. Symbols: ●, neral and; ○, geranial.

Identification of products

Geranial, neral, 3-hydroxycitronellal and methylheptenone were identified by comparing 7GC retention times and MS spectra with those of authentic samples. The MS spectra of geranial, neral and methylheptenone were identical to those in the NIST spectral database. The MS spectrum of 3-hydroxycitronellal was: *m/z* (relative intensity) 152 (17), 137 (18), 94 (89), 82 (83), 69 (100), 55 (40), 43 (92). Acetaldehyde was identified by comparing the GC retention time with that of authentic acetaldehyde and enzymatically using an acetaldehyde test kit.

Analytical Methods

All terpenes were analysed by GC using a fused silica cyclodextrin capillary column (type α-DEX 120; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm; Supelco, Zwijndrecht, The Netherlands). GC was performed with a Hewlett Packard 6890 GC, equipped with a flame ionisation detector with N_2 as the carrier gas. The detector and injector temperatures were 250 and 200°C, respectively, and the split ratio was 1:50. The samples were analysed isocratically at 140°C. Acetaldehyde was determined by analysing the headspace (100 µl) using a Hewlett Packard 6890 GC equipped with a CP-sil 19CB wood fused silica column (30 m x 0.32 mm). The oven temperature was held at 30°C for 10 min and subsequently raised to 80°C (10°C min^{-1}). The carrier gas was N_2 (flow 1 ml min^{-1}). GC-MS analyses were carried out on a model Hewlett Packard 5970 MSD GC equipped with a fused silica capillary column (type HP-5MS; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm). The flow rate of the carrier gas, He, was 1.0 ml min^{-1}. The injector temperature was 220°C, and the oven temperature was increased from 70 to 175°C at 7°C min^{-1}. The injection volume was 1 µl, and the split ratio was 1:50. Electron impact MS data were obtained at 70 eV.

RESULTS

Amino acid-catalysed isomerisation of geranial and neral

When studying the bioconversion of the pure isomers of citral, a spontaneous 'chemical' isomerisation was observed in samples containing boiled cell extracts. This spontaneous isomerisation was also observed in samples that only contained glycine/NaOH buffer, whereas phosphate- and tris-buffer did not result in isomerisation. Since the amino acid-catalysed isomerisation of geranial or neral was not reported before, this reaction was studied in more detail.

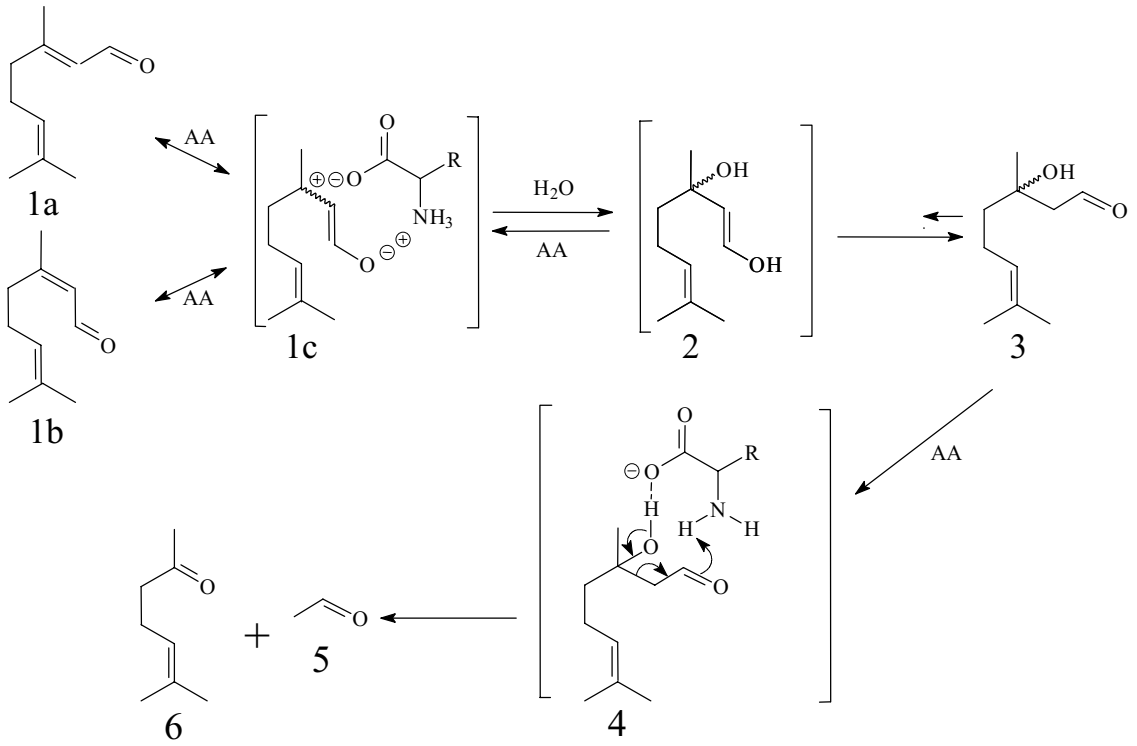

Figure 2. Proposed pathway for the isomerisation of geranial (1a) and neral (1b) and their conversion via 3-hydroxycitronellal (3) into methylheptenone (6) and acetaldehyde (5), catalysed by amino acids (AA), in alkaline aqueous solution.

The glycine-catalysed isomerisation of geranial is a relatively fast process (Figure 1A). After 80 minutes an equilibrium was reached with 60% of the citral in the geranial form and 40% in the neral form (Figure 2). This is the same composition of isomers as present in commercial citral. Starting with neral the same equilibrium was reached (Figure 1B).

Also several other amino acids catalysed the isomerisation of geranial, albeit at different rates (Figure 3). DL-Aspargine was the most efficient catalyst, while DL-aspartic acid was the least efficient at a 3.5 times lower rate. Also the protein bovine serum albumin (10 mg ml^{-1} equalling 0.14 mM of protein or 88 mM of the individual amino acids) catalysed the isomerisation of geranial to neral (Figure 3). In the absence of amino acids, no isomerisation of geranial and neral was observed (not shown). Experiments were performed to determine whether the effect was due to the presence of a positively charged nitrogen (i.e. ammonium) or the carboxyl group (i.e. acetate) as present in amino acids. Ammonium sulphate, acetate and a combination of both did not

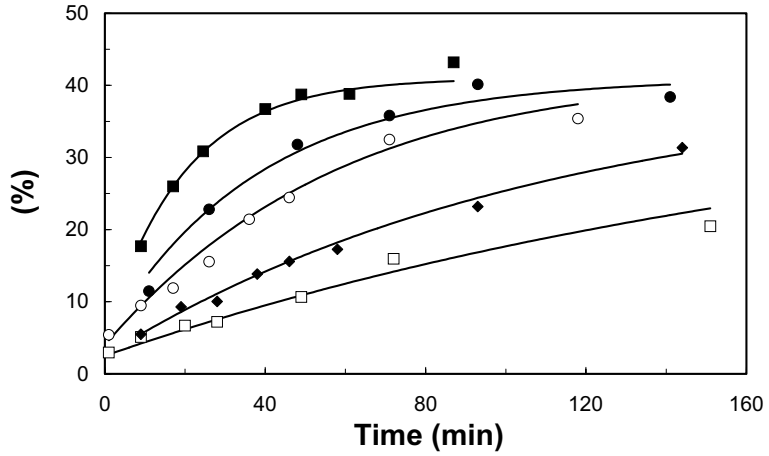

Figure 3. The effect of different amino acids and bovine serum albumin (BSA), on the isomerisation of geranial. Each reaction mixture (25°C) contained 0.1 M amino acid or 10 mg ml^{-1} BSA, 50 mM potassium phosphate buffer (pH 7.0) and 1 mM substrate. The neral formed from geranial is expressed as a percentage of the total amount of neral and geranial. Symbols: ■, DL-aspargine; ●, L-glutamic acid; ○, BSA; ◆, β-alanine and □, DL-asparic acid.

catalyse the isomerisation of geranial. This indicates that the presence of an amino- and carboxyl-group in one molecule is essential for catalysis.

Since most chemical reactions of citral involve oxidative or free radical mechanisms (Clark and Chamblee, 1992; Grein *et al.*, 1994), it was determined whether this was also true for the amino acid-catalysed isomerisation-reaction. The isomerisation catalysed by glycine at neutral pH, was performed in the absence of oxygen (N_2 atmosphere) or in the presence of 25 mM vitamin C, a well-known radical scavenger, respectively. Neither the absence of oxygen nor the presence of vitamin C affected the isomerisation-rate (not shown).

The pH dependence of the isomerisation was tested using glycine as the catalyst (Figure 4). The isomerisation rate was clearly pH dependent, displaying very low activity at pH 4.0 and increasingly higher rates at higher pH.

Amino acid-catalysed conversion of citral into methylheptenone and acetaldehyde

During the glycine-catalysed isomerisation of geranial (1a) and neral (1b) some other reaction products were detected. Of these products the three most dominant were identified as 3-hydroxycitronellal (3), methylheptenone (6) and acetaldehyde (5) (Figure 2).

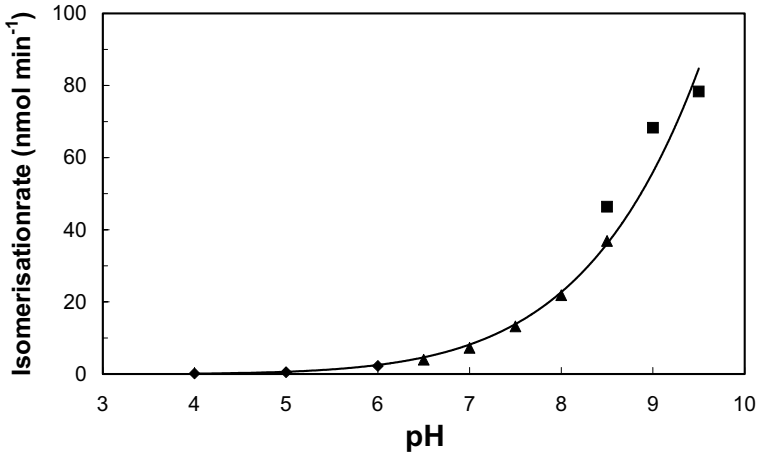

Figure 4. pH-dependence of the glycine-catalysed isomerisation of geranial. Each reaction mixture (1 ml, 25°C) contained 0.1 M glycine, 0.1 M buffer and 1 mM substrate. The isomerisation-rate is expressed as nmol neral formed per min. Symbols: ◆, Acetate buffer; ■, phosphate buffer and ▲, borax buffer.

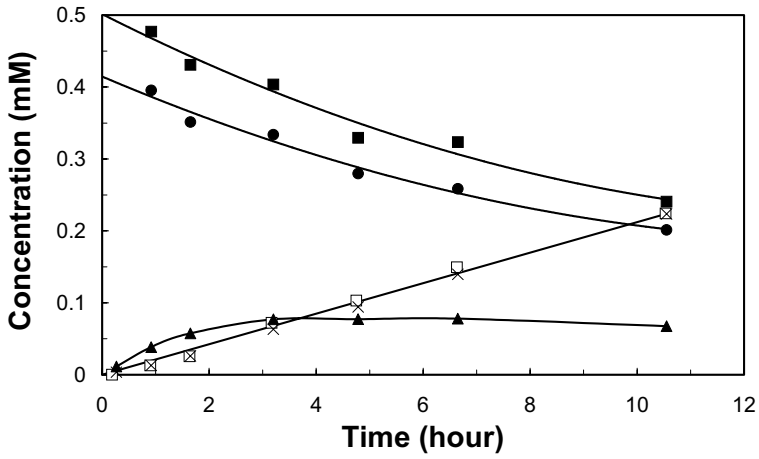

Figure 5. The glycine-catalysed transformation of citral into methylheptenone, acetaldehyde and 3-hydroxycitronellal. Each reaction mixture (25°C) contained 1 mM citral and 0.1 M glycine/NaOH-buffer (pH 10.0). Symbols: ■, Geranial; ■, neral; ×, methylheptenone; □, acetaldehyde and ▲, 3-hydroxycitronellal.

The glycine-catalysed conversion of citral was followed in time (Figure 5). Both geranial and neral were converted at a similar rate. Methylheptenone and acetaldehyde were formed in equimolar amounts. As with the amino acid-catalysed isomerisation, also the formation of these products was catalysed by other amino acids (i.e. L-glutamic

acid, DL-aspartic acid, L-arginine, L-glutamine, L-methionine and DL-aspargine). Of these amino acids L-glutamic acid was the most effective catalyst (0.8 mM methylheptenone formed in 20 hours), while DL-aspargine was the least effective with a 12 times lower methylheptenone formation-rate (not shown). No formation of methylheptenone was found using ammonium sulphate as catalyst or when only buffer was used.

The pH-dependence of the amino acid-catalysed conversion of citral into methylheptenone was studied in more detail (Figure 6A). As for the isomerisation reaction, the retroaldol condensation was fastest at high pH. Especially with glycine, a very clear pH dependence was observed.

Amino acid-catalysed formation and conversion of 3-hydroxycitronellal

During the conversion of citral, also 3-hydroxycitronellal was formed, with an initial rate higher than that of methylheptenone and acetaldehyde formation (Figure 5). The 3-hydroxycitronellal concentration remained constant after the first 3 hours. After 24 hours the substrate was completely converted and also 3-hydroxycitronellal was no longer detected, indicating that it was converted into methylheptenone and acetaldehyde (not shown), suggesting that it is a reaction intermediate.

The formation of 3-hydroxycitronellal from citral was not affected by the addition of 25 mM vitamin C, or flushing with N_2. The 3-hydroxycitronellal formation was shown to be pH dependent, although there was no clear trend (Figure 6B). Besides pH dependent, the 3-hydroxycitronellal formation was also dependent on the buffer used; in samples containing borax buffer the formation rate was much lower than in phosphate buffer (Figure 6B). 3-Hydroxycitronellal formation also depended on the amino acid used as the catalyst; with DL-aspargine a two times higher 3-hydroxycitronellal concentration was observed than with glycine (Figure 6B).

Experiments using 3-hydroxycitronellal as the substrate showed that 3-hydroxycitronellal was converted much faster into citral than into methylheptenone. The rate at which 3-hydroxycitronellal was converted to citral, in the presence of 0.1 M glycine (pH 10.0), was determined to be 2.5 nmol min^{-1} ml^{-1}. This conversion rate is 6 times higher than the rate with which citral is converted into methylheptenone under the same conditions. After 96 hours, 3-hydroxycitronellal was converted completely into methylheptenone.

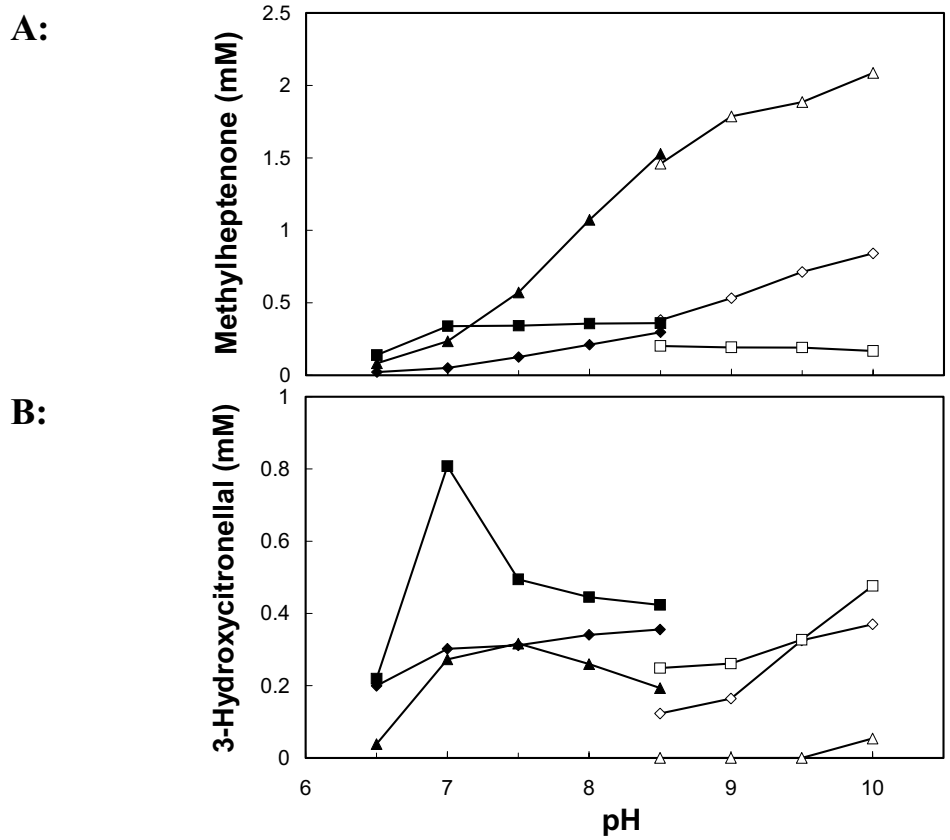

Figure 6. pH-dependence of the amino acid-catalysed transformation of citral into methylheptenone and 3-hydroxycitronellal. Each reaction mixture (25°C) contained 0.1 M amino acid, 5.66 mM citral and 0.25 M phosphate-buffer (solid symbols) or borax-buffer (open symbols). The concentration of methylheptenone (A) and 3-hydroxycitronellal (B) formed were determined after 20 hours. Symbols: ▲, Glycine; ◆, DL-aspartic acid and ■, DL-aspargine.

DISCUSSION

This report describes the amino acid-catalysed conversion of both citral isomers. In the presence of amino acids, and especially under alkaline conditions, the pure isomers undergo *cis-trans* isomerisation and are subsequently (and more slowly) converted via 3-hydroxycitronellal into methylheptenone and acetaldehyde (Figure 2). Citral is converted quantitatively into equimolar amounts methylheptenone and of acetaldehyde. The retroaldol condensation is accompanied by the transient accumulation of 3-hydroxycitronellal, suggesting that this is an intermediate in the reaction.

The proposed mechanism for the isomerisation of geranial and neral and the formation of methylheptenone is shown in Figure 2. Geranial (1a) and neral (1b) are transformed into the resonant state 1c, amino acids, being zwitterions, stabilise 1c. When

addition of water to 1c occurs, intermediate 2 is formed, which is subsequently transformed into the more stable 3-hydroxycitronellal (3) by keto-enol-tautomerisation. Under alkaline conditions amino acids will form a hydrogen bridge with the hydroxyl group of 3-hydroxycitronellal (3) resulting in intermediate 4, which yields methylheptenone (6) and acetaldehyde (5) after rearrangement. When the addition of water to intermediate 1c does not occur, 1c will be converted back into either geranial (1a) or neral (1b), resulting in isomerisation.

The isomerisation of geranial and neral was previously reported by Kuwahara *et al*. (1983). They described the isomerisation of the citral isomers, by bodies of mites, into an equilibrium mixture of 40% neral and 60% geranial. These authors suggest that the reaction was enzymatic or at least protein catalysed, but they did not study the reaction in more detail. Isomerisation of geranial was also described by Kimura *et al*. (1982) in citric acid aqueous solutions. They report the production of cyclic compounds directly from neral rather than from geranial, so isomerisation of geranial into neral is the first step in the cyclisation of geranial.

3-Hydroxycitronellal was also described as an intermediate in the formation of a volatile cyclic compound from citral under oxygen atmosphere in aqueous acidic solution (Grein *et al.*, 1994). They suggest the involvement of oxygen in the formation of 3-hydroxycitronellal from citral. This is in contrast to our results, where we show that the amino acid-dependent 3-hydroxycitronellal formation is oxygen independent.

The conversion of citral into methylheptenone and acetaldehyde has only been described under a more extreme reaction condition. Boiling citral with alkaline (K_2CO_3) results in methylheptenone and acetaldehyde (Karrer, 1963). The retroaldol condensation of several other α,β-unsaturated aldehydes was reported by Grein *et al*. (1993).

Remarkably, aspargine the best catalyst for the isomerisation of geranial, is relatively ineffective in catalysing the conversion to methylheptenone. In addition, the amount of 3-hydroxycitronellal produced is highest when aspargine is used as catalyst. On the other hand, glycine, which is a comparably effective catalyst for the isomerisation reaction, is a much more effective catalyst for the conversion of citral into methylheptenone. This indicates that the function of the amino acid is different in the isomerisation and retroaldol condensation-reaction.

Citral is widely applied as a flavour compound in foods. Most foods have pH values of 7 and lower and the conversion of citral into methylheptenone will not be a major problem, but upon longer storage of foods retroaldol condensation of citral might occur, resulting in an alteration of the flavour of the food. However, the application of citral in protein rich, alkaline foods like West African iru, Japanese natto and Indian kinema (Steinkraus, 1997), obtained by alkaline fermentation using *bacilli*, will be limited. The

conditions of these products (high pH and high concentration of amino acids) will quickly result in the transformation of citral into methylheptenone and acetaldehyde and as a consequence in alteration of the flavour profile. Citral is also used in non-foods like soaps, detergents, creams, lotions and perfumes (Opdyke, 1979). Application of the pure isomers geranial and neral for fragrance differentiation, is only useful in some of these products, dependent on their pH and presence of amino acids or protein.

In conclusion, the retroaldol condensation of citral and consequent alteration of the flavour profile is not expected to be a major problem in many foods, except upon longer storage. The application of pure geranial or neral, however, should be limited to neutral or slightly acidic products or products not containing amino acids or proteins.

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Chapter 7

Amino acid-catalysed retroaldol condensation: the production of natural benzaldehyde and other flavour compounds

This chapter has been submitted for publication as: **Wout AM Wolken, Johannes Tramper, Mariët J van der Werf** Amino acid-catalysed retroaldol condensation: the production of natural of benzaldehyde and other flavour compounds.

ABSTRACT

The amino acid-catalysed retroaldol condensation previously described for citral was extended to other α,β-unsaturated aldehydes. In the presence of glycine and an elevated pH, six other α,β-unsaturated aldehydes also underwent retroaldol condensation. Crotonaldehyde as well as its proposed intermediate, aldol, were converted into acetaldehyde. Hexenal, decenal, methyl-crotonaldehyde, farnesal and cinnamaldehyde were also converted into acetaldehyde plus butanal, octanal, acetone, geranyl acetone and benzaldehyde, respectively. The conversion of cinnamaldehyde into the, for the flavour industry important, compound benzaldehyde was optimised with respect to temperature, pH, the buffer and cinnamaldehyde-concentration. In this way the production rate can be elevated over 23000-fold, resulting in an activity of ± 8.9 mM min^{-1} (= 56.7 g l^{-1} h^{-1}).

INTRODUCTION

Natural flavours and fragrances play an important role in the quality of food and beverages. Due to practices such as premature harvesting, extended storage and physical treatment, aromas may be lost and the addition of flavour supplements to foodstuff is often required. Additionally, consumers are more concerned about food quality and prefer natural food additives to chemically synthesised compounds. As a result, the 'natural' label allocated by the European and US food legislation represents a strong marketing advantage (Krings and Berger, 1998). The difference in price of a natural compound and its chemically synthesised counterpart can be considerable. For example, the price of synthetic vanillin is ± US$ 12 kg^{-1} whereas the price for vanillin extracted from vanilla pods is ± US$ 4000 kg^{-1} (Krings and Berger, 1998) and the price for natural *cis*-2-hexenol is ± US$ 1325 kg^{-1} as compared to ± US$ 67 kg^{-1} for synthetic *cis*-2-hexenol (Manley, 2000).

In the US the term 'natural flavour' or 'natural flavouring' means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavouring rather than nutritional (Code of Federal Regulations, title 21, section 101, part 22). Based on this definition it can be concluded that products, obtained from natural raw materials, by mild chemical reactions (like heating) can be considered natural.

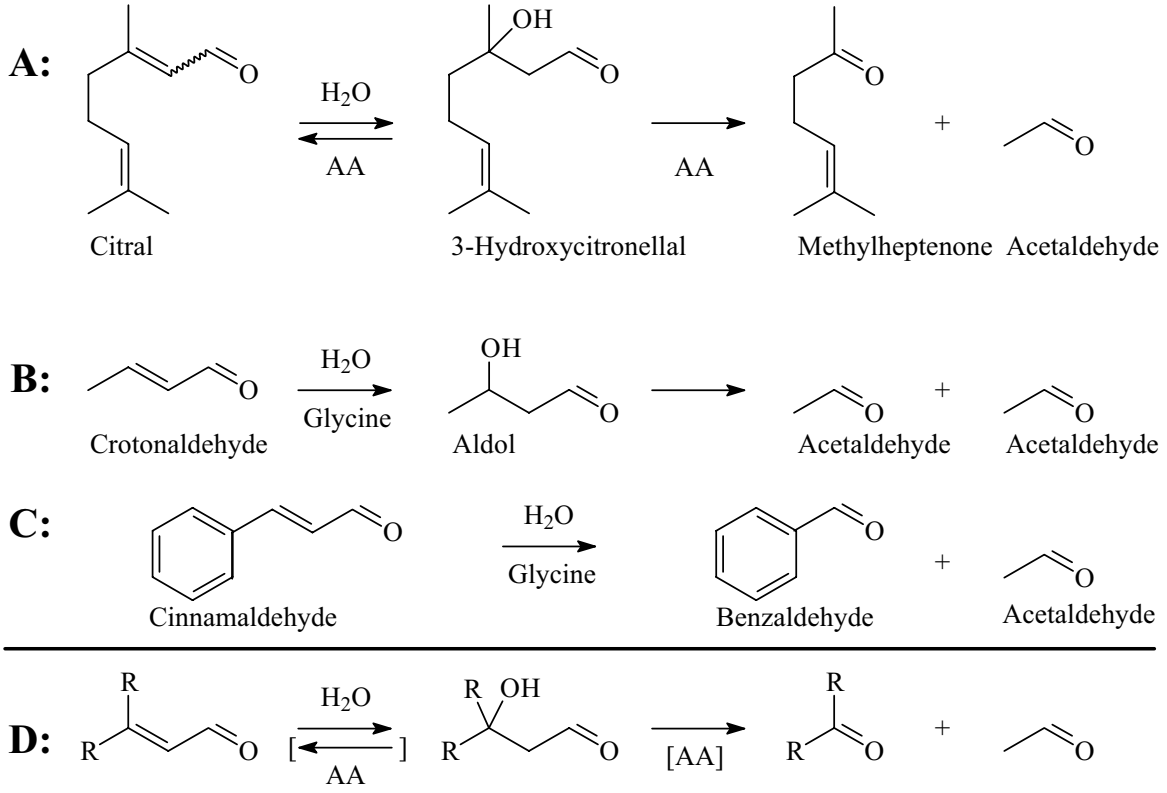


Figure 1. Amino acid (AA)-catalysed conversion of α,β-unsaturated aldehydes: Conversion of citral (A), crotonaldehyde (B) and cinnamaldehyde (C), and general conversion scheme of amino acid-catalysed conversion of α,β-unsaturated aldehydes (D).

Previously we described the retroaldol condensation of citral under mild reaction conditions (Wolken *et al.*, 2000). Citral was efficiently converted into the aroma compounds methylheptenone and acetaldehyde. This pH-dependent reaction was catalysed by several amino acids. Furthermore, ammonium and other buffers did not catalyse the reaction. 3-Hydroxycitronellal was identified as the intermediate of this oxygen-independent reaction (Figure 1A). In this chapter we explore the possibilities to extend this 'kitchen chemistry'-type catalysis to other α,β-unsaturated aldehydes substrates.

MATERIALS AND METHODS

Materials

Acetaldehyde (ethanal), aldol, hexadienal (2,4-hexadien-1-al), hexenal (*trans*-2-hexenal) and geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) were purchased from Aldrich (Steinheim, Germany). Acetone, benzaldehyde and glycine were purchased from Merck (Darmstadt, Germany). Cinnamaldehyde (*trans*-cinnamaldehyde), crotonaldehyde, decenal (*trans*-2-decenal) and decadienal (*trans*,*trans*-2,4-decadienal) were purchased from Acros (Geel, Belgium). Butanal (butyraldehyde), citral (mixture of *cis*- and *trans*-3,7-dimethyl-2,6-octadien-1-al), methyl-crotonaldehyde (3-methylcrotonaldehyde), methylheptenone (6-methyl-5-hepten-2-one) and octanal (caprylic aldehyde) were purchased from Fluka (Buchs, Switzerland). Farnesal (3,7,11-trimethyl-2,6,10-dodecatrienal) was purchased from Frinton Laboratories (Vineland, New Jersey, USA). All other chemicals used were of analytical grade (purity ≥ 99%).

Activity measurements

The retroaldol condensation-activity was typically determined by incubating 1 ml of reaction mixture in a 15 ml vial fitted with a Teflon Mininert valve (Supelco, Zwijndrecht, The Netherlands), in a shaking water bath (oscillating at 2.5 Hz with an amplitude of 2 cm). Unless stated otherwise, the incubations were carried out at 25°C in 0.5 M glycine/NaOH-buffer (pH 10.0) at a substrate concentration of 2.5 mM.

The conversion of the different substrates was tested using acetaldehyde production (as determined in the headspace by GC) in time as a measure for activity. At the end of the conversion the acetaldehyde production was confirmed enzymatically and the remaining reaction mixture was extracted by an equal amount of ethylacetate and subsequently analysed by GC and GC-MS to determine the products formed.

The temperature optimum of the retroaldol condensation of cinnamaldehyde was determined by varying the temperature during the incubation and determining the acetaldehyde concentration in the headspace of the samples by GC. The effects of pH, buffer-concentration and substrate-concentration were studied by incubating reaction mixtures for 30 min at 70°C. After addition of 20% 6 M HCl (to facilitate benzaldehyde extraction) samples were extracted with ethylacetate and subsequently analysed by GC.

Analytical Methods

Benzaldehyde, geranyl acetone, methylheptenone and octanal were detected by extracting the liquid samples with ethyl acetate and subsequent GC and GC-MS analysis, as described earlier (Wolken and van der Werf, 2001) except that the temperature program was 10 min at 40°C followed by a linear gradient of 40 to 160°C (2.0°C min^{-1}) for GC and 4 min at 50°C; linear gradient up to 100°C (1.5°C min^{-1}); linear gradient up to 250°C (10°C min^{-1}); and 20 min at 250°C for GC-MS.

Acetaldehyde, acetone and butanal were determined in 100 µl headspace samples, as described earlier for acetaldehyde (Wolken and van der Werf, 2001), except that an isocratic oventemperature of 40°C was used. Products were identified by comparing the retention times with those of authentic samples and by comparing the MS spectra of the products with those in the NIST spectral database (NIST Mass Spectrometry Data Center, S. E. Stein, director).

For the enzymatic acetaldehyde determination an acetaldehyde test kit (R-Biopharm, Darmstadt, Germany) was used.

RESULTS

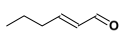
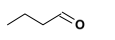
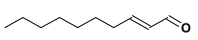
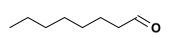
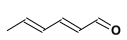
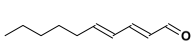
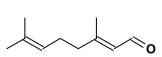
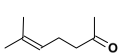
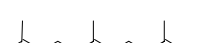
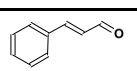
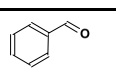
Substrate range

It was demonstrated that under alkaline conditions, amino acids efficiently catalyse the conversion of citral into equimolar amounts of methylheptenone and acetaldehyde (Wolken *et al.*, 2000). Of the amino acids tested, glycine was one of the most efficient catalysts. An additional property of glycine is that it buffers strongly around pH 10, thus omitting the need of an additional buffer or other means of pH control.

Nine different α,β-unsaturated aldehydes were tested for the same reaction and seven were found to be susceptible to glycine-catalysed retroaldol condensation (Table 1). Starting with the most actively converted substrate, methyl-crotonaldehyde, hexenal, crotonaldehyde, citral, decenal, farnesal and cinnamaldehyde were converted. None of the tested compounds were converted (detection limit was 0.2 µM min^{-1}) in the absence of glycine. In all instances the product predicted from the proposed pathway (Figure 1) was formed. Hexadienal and decadienal conversion could not be detected (activity < 0.2 µM min^{-1}).

The proposed intermediate of the retroaldol condensation of crotonaldehyde, aldol (Figure 1B), is commercially available, and was also found to be converted into acetaldehyde. However, in contrast to 3-hydroxycitronellal (the intermediate of the citral

Table 1. Glycine-catalysed conversion of α,β-unsaturated aldehydes.

Substrate		Acetaldehyde formation Activity in μM min^{-1} (Std. Err.)			Product formation		
Name	Structure	Glycine[1]	Control[2]	Identification method	Name	Structure	Identification method
Crotonaldehyde		7.22 (0.44)[3]	<0.2	GC[4]; Enz[5]	Acetaldehyde		GC
Hexenal		5.73 (0.32)	<0.2	GC; Enz	Butanal		GC
Decenal		2.77 (0.18)	<0.2	GC[6]	Octanal		GC; GC-MS[7]
Hexadienal		<0.2	<0.2				
Decadienal		<0.2	<0.2				
Methyl-crotonaldehyde		10.81 (0.79)	<0.2	GC; Enz	Acetone		GC
Citral		2.93 (0.10)	<0.2	GC; Enz	Methylheptenone		GC; GC-MS
Farnesal		0.46 (0.03)	<0.2	GC; Enz	Geranyl acetone		GC; GC-MS
Cinnamaldehyde		0.38 (0.06)	<0.2	GC; Enz	Benzaldehyde		GC; GC-MS

Conversion-rates are determined at 25 °C and a substrate concentration of 2.5 mM. [1]**glycine**, 0.5 M glycine/NaOH-buffer, pH 10.0; [2]**control**, 0.5 M $Na_2CO_3/NaHCO_3$-buffer, pH 10.0; [3], one cortonaldehyde yields two acetaldehyde (crotonaldehyde conversion rate is 3.61 μM min^{-1}); [4]**GC**, retention time as compared to authentic sample; [5]**Enz**, acetaldehyde formation confirmed enzymatically; [6], decenal interferes with the enzymatic acetaldehyde determination; [7]**GC-MS**, MS spectrum as compared to NIST spectral database.

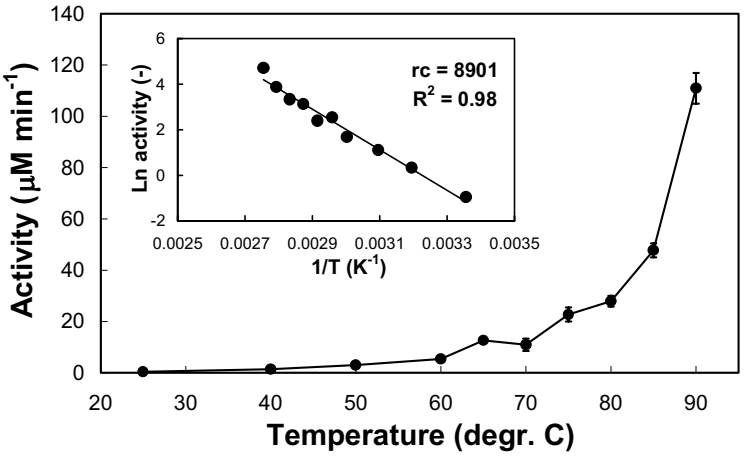


Figure 2. Temperature dependence of the glycine-catalysed conversion of cinnamaldehyde into benzaldehyde and acetaldehyde (25 to 90°C, 0.5 M glycine/NaOH buffer, pH 10.0 and 2.5 mM cinnamaldehyde). Error bars represent standard error of at least triplicates. (Insert: Arrhenius plot).

conversion (Figure 1A, Wolken *et al*., 2000)) aldol is also converted in the absence of glycine. Moreover, in contrast to the analogous reaction with 3-hydroxycitronellal, the reverse reaction of aldol into crotonaldehyde was not observed.

Optimisation of the benzaldehyde production

Of the reactions catalysed (Table 1), the conversion of cinnamaldehyde into benzaldehyde is the most interesting from an industrial viewpoint as benzaldehyde is the second most applied flavour compound worldwide (Welsh *et al.*, 1989). Therefore this reaction was optimised.

The temperature had a pronounced effect on the activity of the conversion, that increased exponentially with the temperature (Figure 2). Raising the temperature from 25 to 90°C resulted in a ± 290 times higher conversion activity. From the Arrhenius plot (insert Figure 2) an activation energy of 74.0 kJ mol^{-1} for the amino acid-catalysed cinnamaldehyde-conversion was calculated.

Previously, it was reported that elevating the pH, in the range of 6.5 to 10.0, had a strong positive effect on the glycine-catalysed retroaldol condensation of citral (Wolken *et al.*, 2000). For the cinnamaldehyde conversion the effect of pH of the glycine solution was tested throughout it's buffering range (pH 8.7 to 10.8) and had an optimum at pH 9.2 (Figure 3A). The activity of the amino acid-catalysed conversion was also dependent on the concentration of the glycine/NaOH buffer used. In the absence of glycine buffer no conversion was observed and the activity increased with the amino acid

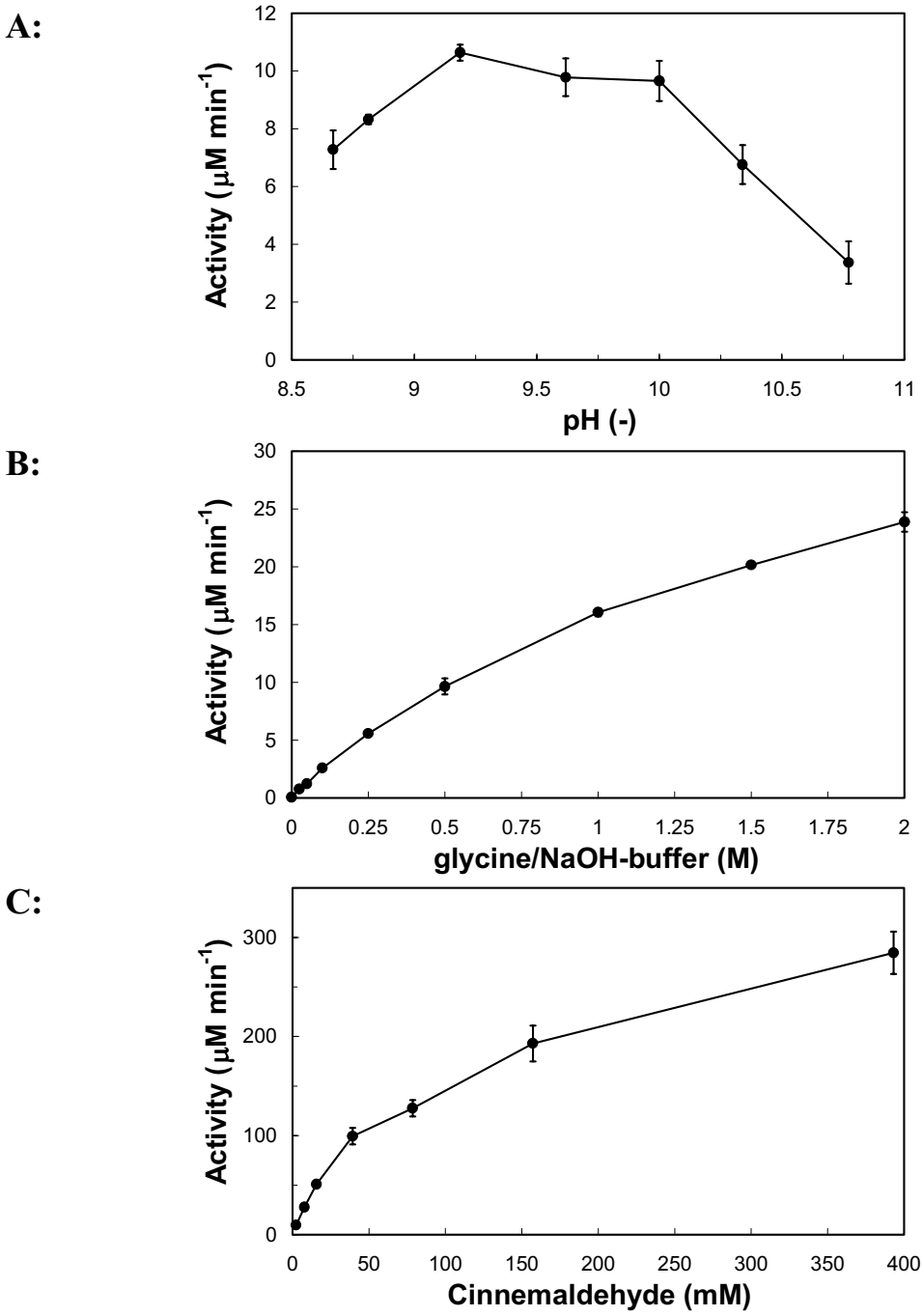


Figure 3. Optimisation of benzaldehyde production-activity at 70°C. A, pH (0.5 M buffer, pH 8.67 to 10.77 and 2.5 mM cinnamaldehyde); B, Buffer-concentration (0 to 2.0 M buffer, pH 10.0 and 2.5 mM cinnemaldehyde); C, Substrate-concentration (0.5 M buffer, pH 10.0 and 2.5 to 393 mM cinnamaldehyde). Error bars represent the standard error of triplicates.

-concentration. At 2 M of glycine the activity was increased ± 2.5-fold as compared to the normal glycine buffer-concentration (0.5 M). Moreover, the effect of cinnamaldehyde concentration on the benzaldehyde formation-activity was tested. The activity of the conversion increased with cinnamaldehyde concentration, but levelled of at concentrations above 40 mM, probably as a result of the low solubility of cinnamaldehyde (± 11 mM at 25°C (Budavari *et al.*, 1996)).

DISCUSSION

In this report, we showed that seven different α,β-unsaturated aldehydes (Table 1) underwent glycine-catalysed retroaldol condensation. Also the proposed intermediate of this reaction, aldol, was converted similar to the conversion of 3-hydroxycitronellal previously shown (Wolken *et al.*, 2000). Based on these findings a general reaction scheme is proposed (Figure 1D); first amino acid-catalysed addition of water to the α,β-unsaturated aldehyde occurs, thus forming a 3-hydroxy intermediate. The 3-hydroxy intermediate is subsequently rearranged resulting in acetaldehyde and the product. For catalyses of this final step amino acids are not in al instances essential, whereas amino acids were essential for catalyses of the first step of the conversion.

All the retroaldol conversion-activities were relatively low, varying between 0.38 and 10.8 μM min^{-1}. This low rate could be improved dramatically as was demonstrated for the conversion of cinnamaldehyde into benzaldehyde. By combining the effects of raising the temperature during the reaction to 90°C (± 290-fold increase), the buffer-concentration to 2 M (± 2.5-fold increase) and the cinnamaldehyde-concentration of 390 mM (± 29-fold increase), and lowering the pH to 9.2 (± 1.1-fold increase) an estimated improvement of over 23000-fold was obtained. In this way natural benzaldehyde can be produced at an activity of ± 8.9 mM min^{-1} (= 56.7 g l^{-1} h^{-1}).

Although natural benzaldehyde is not a very expensive aroma compound (it is the main constituent of bitter almond oil that is sold for ± US$ 400 kg^{-1}, whereas synthetic benzaldehyde is sold for ± US$ 2 kg^{-1}), there is still a great deal of interest in the production of 'natural' benzaldehyde. According to Armstrong et al. (1994) 'natural' benzaldehyde is also commercially produced from cinnamon bark and leave, of which cinnamaldehyde is the major constituent. However a number of firms have protested against the 'natural' status of this benzaldehyde, which is sold for ± US$ 100 kg^{-1}. This is because the use of an inorganic acid or base-catalyst causes concern over the 'natural' status (Armstrong and Brown, 1994; Feron *et al.*, 1996). It is likely that the method patented by Buck et al (1987) was used in this case, a method encompassing a reaction that takes place at pH 12.5 (obtained by addition NaOH) at temperatures of 105°C. This

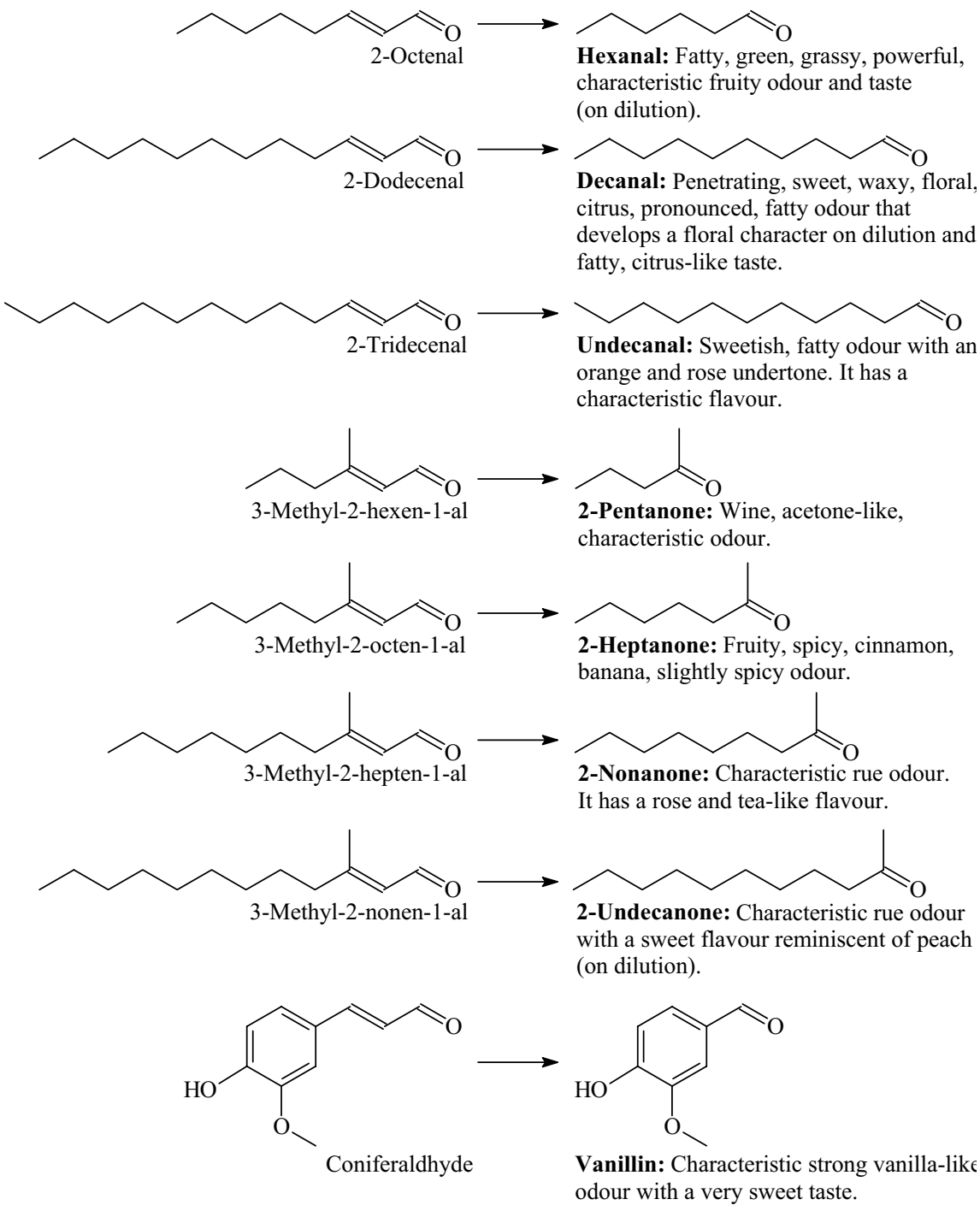


Figure 4. Possible extension of the amino acid-catalysed conversion of α,β-unsaturated aldehydes for the production of flavour compounds. Aroma descriptions are based on Fenaroli's handbook of flavour ingredients (Burdock, 2002).

method of producing benzaldehyde can easily be adapted using the results of this chapter. The resulting production method would take place at a much lower pH (9.2 or even lower if needed) and the fact that glycine is a natural compound and glycine/NaOH is commonly used as a buffer for enzymatic conversion (normally resulting in a natural product), would probably circumvent this reaction being classified as 'catalysed by an organic base'. Although it is not possible to calculate an exact volumetric conversion activity from the data presented in the above-mentioned patent, it can be calculated that the activity is certainly lower than 86 g l^{-1} h^{-1} (at pH 12.5 and 105°C). This indicates that the method presented in this chapter, with an activity of 57 g l^{-1} h^{-1} (at pH 9.2 and 90°C) is certainly commercially feasible.

Besides benzaldehyde also the acetaldehyde, which is produced as 'side-product', is commercially interesting. The flavour compound acetaldehyde has a characteristic pungent, penetrating, ethereal odour. It is, for instance, used as a flavour-enhancer in orange juice to create naturalness, fruitiness and juiciness (Burdock, 2002). If sold as 'natural' compound it has a price of ± US$ 135 kg^{-1} and as a 'synthetic' compound it can still be sold at ± US$ 47 kg^{-1} (Manley, 2000).

The method presented here has the potential to be applied for the production of a range of other natural (aldehyde and ketone) flavour compounds (Figure 4) from α,β-unsaturated aldehydes, similar to the ones successfully tested in this chapter (Table 1). For instance the predominant components of the aroma of Roquefort, as well as Camembert, 2-heptanone and 2-nonanone (Engels *et al.*, 1997), could be produced from the corresponding 3-methyl α,β-unsaturated aldehydes. Finally, the possibility to convert coniferaldehyde (a normal constituent of wood lignin, that is present in sandalwood and conifers (Schoental, 1973)) into vanillin (the most important flavour compound from a commercial point of view (Schwab, 2000)) is postulated.

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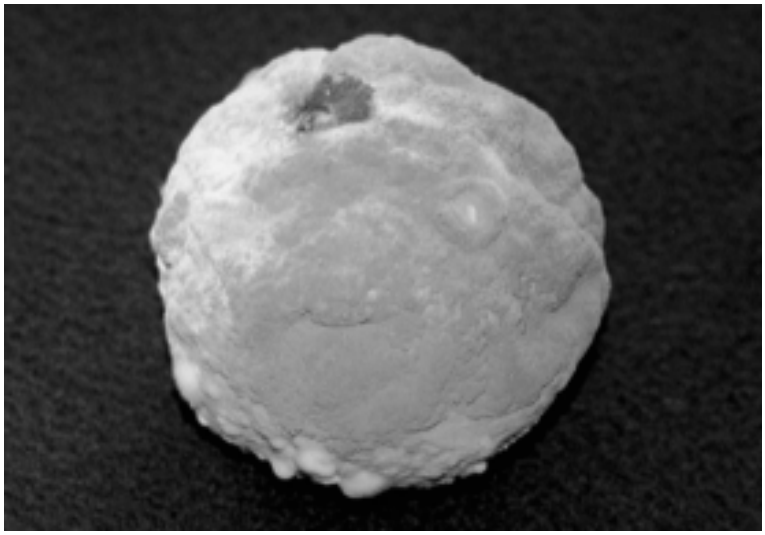
Chapter 8

Summary and concluding remarks



SUMMARY

Natural flavours play an important role in the quality of food and beverages. Consumers nowadays have a strong preference for natural food additives over chemically synthesised compounds. As a result, the 'natural' label allocated to a flavour compound, represents a strong marketing advantage and has resulted in higher prices for compounds of natural origin. According to US law, products obtained by reactions of microorganisms, enzymes or even some mild chemical reactions (like heating) can be considered natural as long as natural raw materials are used. In the research described in this dissertation the natural production of the flavour compound methylheptenone and other aldehydes and ketones is studied in detail.

An important part of this dissertation focuses on the use of spores for the production of methylheptenone. Spores are produced by a range of organisms and represent a remarkable and resistant phase in their life cycle. Microbial spores vary considerably in size (0.25 to 340 μm), shape (round to extremely elongated) and pigmentation, and can be elaborately ornamented. There are several advantages (resistance to external factors like toxic compounds, temperature and radiation, easy and long storage, specific activities of enzymes present in spores, etc.) of using spores rather than vegetative cells. Besides in biotransformation, applications of spores are found in the biocontrol of insects, fungal diseases and weed infestations. Futhermore, spores are used in biowarfare, probiotics, tumour detection and treatment, and biosensing (chapter 2).

In chapters 3 and 4 the bioconversion of geraniol by spores of *Penicillium digitatum* ATCC 201167 was studied. Up to now, this conversion was not studied in detail in this strain and the pathway proposed was based on the geraniol bioconversion pathway of *Pseudomonas incognitas*. Spores of *P. digitatum* transformed geraniol, nerol, citral, and geranic acid into methylheptenone. Extracts of these spores converted geraniol and its *cis*- isomer (nerol) NAD^+-dependently into citral. Also the NAD^+-dependent conversion of citral into geranic acid was demonstrated in spore extract. Furthermore, a novel enzyme, citral lyase, that cofactor-independently converts citral into methylheptenone and acetaldehyde, was detected. These results showed that spores of *P. digitatum* convert geraniol via a novel biotransformation pathway (geraniol is converted by citrol dehydrogenase into citral, which is subsequently converted directly into methylheptenone and acetaldehyde, by citral lyase). This was the first time a biotransformation pathway in fungal spores was substantiated by biochemical studies. Subsequently, the key enzyme in the geraniol bioconversion pathway, citral lyase, was purified from spores of *P. digitatum*. Remarkably, citral lyase activity was induced 118-fold by incubating non-germinating spores with the substrate, citral. This enzyme, that

combines hydratase and aldolase activity, was purified and found to be a monomeric enzyme of 31 kDa and to be highly unstable in the purified form. Citral lyase has a K_M of 0.058 mM and a V_{max} of 52.6 U mg^{-1}, and its activity is optimal at 20°C and pH 7.6. The enzyme has a strong preference for the *trans*-isomer of citral (geranial). Citral lyase also converted other α,β-unsaturated aldehydes like farnesal, methyl-crotonaldehyde, decenal and cinnamaldehyde.

One of the advantages of using spores rather than vegetative cells for biotransformation reactions is the higher resistance of spores towards the toxic effects of substrates, intermediates and products of the reaction. This clear advantage has not been studied in detail in scientific literature, until now (chapter 5). We showed that spores of *P. digitatum* are on average 2.5 times more resistant towards toxic substrates, intermediates and products of the geraniol bioconversion-pathway, than mycelium. The higher resistance of spores to citral was shown an advantage in its biotransformation by *P. digitatum*. The order of toxicity toward *P. digitatum* was, starting with the most toxic compound, citral > nerol / geraniol > geranic acid > methylheptenone >> acetaldehyde.

During the study of the geraniol biotransformation pathway (chapter 3) the chemical conversion of citral was noted. In chapter 6 we studied this conversion in more detail and showed that under alkaline conditions, amino acids as well as proteins catalyse the conversion of citral into methylheptenone and acetaldehyde. 3-Hydroxycitronellal was identified as an intermediate in this amino acid-catalysed reaction. Amino acids also catalyse the *cis-trans* isomerisation of the pure isomers of citral; geranial and neral. Most likely the amino acids are involved in stabilising intermediates of the isomerisation and retroaldol condensation-reactions of citral. Based on our findings some consequences for the application of citral, or its isomers, in food are discussed. The amino acid-catalysed retroaldol condensation described in chapter 6 was extended to other α,β-unsaturated aldehydes in chapter 7. Six other α,β-unsaturated aldehydes (crotonaldehyde. hexenal, decenal, methyl-crotonaldehyde, farnesal and cinnamaldehyde) were also found to be susceptible to retroaldol condensation. In all instances the predicted product (based on the proposed pathway) was formed as well as acetaldehyde. The commercially available intermediate of the crotonaldehyde conversion (aldol) was also converted. The conversion of cinnamaldehyde into benzaldehyde, the second most important compound in the flavour industry, was subsequently optimised with respect to temperature, pH, buffer as well as cinnamaldehyde-concentration. The benzaldehyde production-rate could be increased over then 23000-fold by optimising these parameters.

CONCLUDING REMARKS

In this dissertation we demonstrated that spores have, compared to mycelium, a higher resistant to the toxic effects of substrates, intermediates and products of flavour-bioconversions. The use of these more resistant biocatalysts is a major advantage, allowing the use of a higher substrate and product-concentration, resulting in an improved volumetric productivity and cheaper down-stream processing. Remarkably, the potential of spores as biocatalysts is at the moment far from extensively explored. However, the results described in this dissertation indicate that the possible applications are numerous and that further research on the subject of spore-biocatalyses is more than justified.

Citral lyase is the key enzyme in the geraniol biotransformation-pathway, described in this dissertation. This cofactor-independent enzyme catalyses an uncommon reaction type, retroaldol condensation of α,β-unsaturated aldehydes, that has not frequently been described. This and the fact that the enzyme can be used for the natural production of the major flavour compound, benzaldehyde, makes it a good candidate for further study (from scientific as well commercial as perspective).

An interesting, alternative, approach for the natural production of flavour compounds is found in the amino acid catalyses of retroaldol condensation-reactions. A number of frequently observed flavour compounds can be produced by this relatively mild reaction. This simple but effective method of converting natural α,β-unsaturated aldehydes seems, therefore, of great interest for the industrial production of natural aldehydes and ketones.

Samenvatting

Natuurlijke aromastoffen zijn een belangrijke component in levensmiddelen. Consumenten hebben tegenwoordig een sterke voorkeur voor natuurlijke in plaats van kunstmatige toevoegingen. Hierdoor hebben natuurlijke aromastoffen een aanzienlijk hogere marktwaarde gekregen dan kunstmatige geproduceerde aromastoffen. Volgens de wetgeving in de USA is een product natuurlijk als het geproduceerd is uit een natuurlijke uitgangsstof, gebruik makende van micro-organismen, enzymen of zelfs milde chemische behandelingen. In dit proefschrift wordt de natuurlijke productie van de aromacomponent methylheptenone en andere aldehyden en ketonen beschreven.

Een belangrijk deel van dit proefschrift concentreert zich op het gebruik van sporen voor de productie van methylheptenone. Sporen zijn een opmerkelijke en resistente levensfase van micro-organismen zoals schimmels, bacteriën en protozoa, maar ook van een aantal planten. Microbiële sporen kunnen behoorlijk verschillen in formaat (0,25 tot 340 μm), vorm (rond tot extreem langwerpig) en pigmentatie. Het gebruik van sporen in plaats van vegetatieve cellen heeft diverse voordelen zoals verhoogde resistentie tegen factoren als giftige stoffen, temperatuur en straling. Verder kunnen sporen relatief makkelijk en lang opgeslagen worden en kunnen sporen bepaalde enzym activiteiten bezitten die niet aanwezig zijn in de vegetatieve cel. Behalve bij de biotransformatie van chemicaliën worden sporen ook gebruikt voor biologische bestrijding van insecten, onkruid en schadelijke schimmels. Verder kunnen sporen worden gebruikt in de biologische oorlogsvoering, als probiotica, bij de detectie en bestrijding van tumors, en als biologische detector (hoofdstuk 2).

In de hoofdstukken 3 en 4 wordt de biotransformatie van geraniol door sporen van de schimmel *Penicillium digitatum* ATCC 201167 beschreven. Tot voor kort was de omzetting door deze stam nog niet in detail bestudeerd en was de voorgestelde omzettingsroute gebaseerd op de geraniol omzettingsroute in de bacterie *Pseudomonas incognitas*. Geraniol, nerol, citral en geranic acid worden door sporen van *P. digitatum* omgezet in methylheptenone. In aanwezigheid van NAD^+, werden geraniol en het *cis*-isomeer, nerol, door extracten van deze sporen omgezet in citral. Ook de NAD^+-afhankelijke omzetting van citral naar geranic acid werd aangetoond in extracten van deze sporen. Verder werd een nieuw enzym, citral lyase, ontdekt. Dit enzym katalyseert de cofactor-onafhankelijke omzetting van citral in methylheptenone en acetaldehyde. De resultaten toonden aan dat sporen van *P. digitatum* geraniol omzetten volgens een niet eerder beschreven omzettingsroute: citrol dehydrogenase zet geraniol om in citral, dat vervolgens direct omgezet wordt in methylheptenone en acetaldehyde door citral lyase.

Dit is de eerste keer dat een omzettingsroute in schimmelsporen onderbouwd is door biochemische studies. Vervolgens werd het belangrijkste enzym uit de geraniol omzettingsroute, citral lyase, gezuiverd uit sporen van *P. digitatum*. Citral lyase activiteit werd met een factor 118 geïnduceerd door de sporen met het substraat, citral, te incuberen. Dit enzym, dat de activiteit van een hydratase combineert met die van een aldolase, is een monomeer van 31 kDa. Citral lyase heeft een K_M van 0.058 mM, een V_{max} van 52.6 U mg^{-1}, een optimum pH van 7.6 en een optimum temperatuur van 20°C en bleek zeer instabiel te zijn na zuivering. Dit enzym vertoont een sterke voorkeur voor het *trans*-isomeer van citral (geranial) boven het *cis*-isomeer (neral). Ook andere α,β-onverzadigde aldehyden zoals farnesal, methyl-crotonaldehyde, decenal en kaneelaldehyde worden omgezet door citral lyase.

Een voordeel van het gebruik van sporen in plaats van vegetatieve cellen voor biotransformatiereacties is de relatief hoge tolerantie die sporen hebben tegen de toxische effecten van substraten, intermediairen en producten van de omzetting. Dit voor de hand liggend voordeel was tot nu (hoofdstuk 5) echter nog niet eerder beschreven in de wetenschappelijke literatuur. We hebben aangetoond dat sporen van *P. digitatum* gemiddeld 2.5 keer resistenter zijn tegen de substraten, intermediairen en producten van de geraniol-omzetting, dan het mycelium van deze schimmel. De hogere resistentie van de sporen tegen de toxische effecten van citral bleek een voordeel te zijn in de omzetting hiervan door *P. digitatum* sporen. De volgorde van toxiciteit ten opzichte van *P. digitatum* sporen en mycelium was, startend met de meest toxische verbinding, citral > nerol / geraniol > geranic acid > methylheptenone >> acetaldehyde.

Tijdens het bestuderen van de geraniol-omzettingsroute (hoofdstuk 3) bleek dat citral ook chemisch omgezet kan worden. In hoofdstuk 6 hebben we deze omzetting beter bestudeerd en aangetoond dat onder basische condities zowel aminozuren als eiwitten de omzetting van citral naar methylheptenone en acetaldehyde katalyseren. 3-Hydroxycitronellal werd geïdentificeerd als intermediair in deze reactie. Aminozuren katalyseerden tevens de *cis-trans* isomerisatie van de zuivere isomeren van citral (geranial en neral). Waarschijnlijk stabiliseren de aminozuren de intermediairen van de isomerisatie- en de retroaldol condensatie-reacties van citral. Gebaseerd op onze bevindingen bespreken we de toepassing van citral, of de isomeren daarvan, in levensmiddelen.

In hoofdstuk 7 wordt de aminozuur gekatalyseerde retroaldol condensatie van diverse andere α,β-onverzadigde aldehyden beschreven. Hiervan zijn er zes (crotonaldehyde, hexenal, methyl-crotonaldehyde, farnesal en kaneelaldehyde) eveneens onderhevig aan retroaldol condensatie. In alle gevallen werd het product, zoals verwacht, volgens de voorgestelde omzettingsroute gevormd samen met acetaldehyde. Het commercieel verkrijgbare intermediair van de crotonaldehyde omzetting (aldol)

werd eveneens omgezet. De omzetting van kaneelaldehyde in benzaldehyde, de op een na meest als aromastof toegepaste verbinding, is vervolgens geoptimaliseerd met betrekking tot temperatuur, pH en buffer- en kaneelaldehyde-concentratie. De benzaldehyde-vormingsnelheid kon daardoor met een factor 23000 worden verhoogd.

In dit proefschrift zijn uiteenlopende kanten van de natuurlijke aromastof productie onderzocht. Concluderend kunnen we zeggen dat sporen gebruikt kunnen worden voor de productie van aromastoffen en dat sporen ten opzichte van mycelium resistenter zijn tegen de toxische effecten van substraten, intermediairen en producten van de aromastofomzettingen. Verder is gebleken dat het belangrijkste enzym in de geraniol-omzetting, citral lyase, cofactor-onafhankelijk α,β-onverzadigde aldehyden om kan zetten. Tot slot is er in dit proefschrift een alternatieve 'natuurlijke' manier beschreven om retroaldol-condensatie te katalyseren gebruik makende van aminozuren.

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Wout AM Wolken, Johannes Tramper, Mariët J van der Werf What can spores do for us? *Submitted for publication*

Wout AM Wolken, Johannes Tramper, Mariët J van der Werf Amino acid-catalysed retroaldol condensation: the production of natural of benzaldehyde and other flavour compounds. *Submitted for publication*

Curriculum Vitae

Wout Antonius Maria Wolken werd geboren op 23 juli 1972 in Dinxperlo. Na achtereenvolgens het MAVO en het HAVO diploma te hebben behaald, aan respectievelijk de Pastoor Bluemers MAVO en het Isala College beide te Silvolde, is hij in 1990 gestart met zijn studie levensmiddelentechnologie aan de Hogere Agrarische School (Den Bosch). Tijdens deze opleiding liep hij stages bij Grolsch in Groenlo, Cargill in Bergen op Zoom en Unilever Research in Vlaardingen. In 1994 sloot hij deze opleiding af met een afstudeervak over de toepassing van schimmels in zuivel, in opdracht van MONA (Woerden). In datzelfde jaar startte hij met zijn studie bioprocesstechnologie aan de toenmalige Landbouw Universiteit Wageningen. In 1997 rondde hij deze studie af met een afstudeervak over de productie van α-ketobutyraat door *Zygosaccharomyces rouxii* bij de sectie Proceskunde. In 1998 begon hij aan de toenmalige Landbouw Universiteit Wageningen bij Industriële Microbiologie met het promotie onderzoek dat beschreven staat in dit proefschrift. Vanaf 1 maart 2003 is hij werkzaam als postdoctoraal onderzoeker bij de groep Moleculaire Microbiologie van de Rijksuniversiteit Groningen.

Nawoord

Door de jaren heen heb ik van ongeveer 30 collega's proefschriften mogen ontvangen, maar na 5 jaar is dan nu eindelijk mijn grote ei gelegd; een eigen proefschrift. Ondanks dat ik dit proefschrift trots 'mijn proefschrift' noem heb ik dit natuurlijk niet zonder de hulp van anderen kunnen doen.

Als eerste wil ik daarom mijn promotor, Hans Tramper, en copromotor Mariët van der Werf, bedanken. Hans, bedankt dat je, ondanks dat je niet vanaf het begin bij het onderzoek betrokken bent geweest, toch mijn promotor wilt zijn. Mariët, alhoewel jij na de opheffing van Industriële Microbiologie als een van de eersten een nieuwe baan hebt gevonden, ben je me uitstekend blijven begeleiden. Je was altijd erg snel en uitvoering met het nakijken van de manuscripten en telkens op het moment dat ik het helemaal gehad had met een manuscript vond jij dat we het maar eens moesten opsturen. Bedankt voor al je inspanningen, je snelle reacties en je goede timing!

Ik wil ook graag van deze gelegenheid gebruik maken om al mijn collega's van Industriële Microbiologie, en natuurlijk ook al mijn adoptie collega's van Levensmiddelen Microbiologie, te bedanken voor alle hulp en steun, maar vooral ook voor de gezelligheid, koffiepauzes, publicatie traktaties en het samen genuttigde bier.

Pa en Ma jullie hebben me door al mijn 'studies' heen altijd volledig gesteund, bedankt hiervoor! Corine, zonder jou had dit proefschrift er ongetwijfeld heel anders uitgezien, bedankt voor alles!

There's nothing to tell about the future
nothing about the past
Nanananananana
We've got a song at last
There's nothing to tell about the future
nothing about the past
And maybe it will sound like shit
but it's gonna be a hit!

(De Boegies, Kwait nait)

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Photos on the cover and the first page of each chapter: Different stages of growth of *Penicillium digitatum* ATCC 201167 on its natural substrate, oranges (by Wout Wolken)