Chapter 4

Application of Biotechnology to the Production of Natural Flavor and Fragrance Chemicals

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Most natural flavor/fragrance chemicals are heavily dependent on plant and animal origins. However, the quality and the supply of traditional natural flavor/fragrance chemicals are somewhat limited. Viable alternative and innovative ways to synthesize flavor and fragrance chemicals include biotechnological routes, i.e., microbial fermentation and plant tissue culture. Microorganisms are being used not only in the brewing and food industries to produce fermentation products, but also to produce aroma chemicals. The ability to produce aroma chemicals by microbial fermentation may supplement and enhance the quality of plant-based flavor/fragrance chemicals. Recent developments of commercialized processes to produce and/or to biotransform natural precursors into valuable flavor/fragrance chemicals via microbial metabolic pathways include the following; 1) Production of Tuberose lactone (a new GRAS chemical) via hydroxylation of unsaturated fatty acids and limited β-oxidation of the hydroxylated fatty acids; 2) Production of chirally active (R)-styrallyl acetate by regioselective reduction of acetophenone to styrallyl alcohol and subsequent esterification; and 3) de novo synthesis of chirally pure (+)-jasmonic acid and subsequent esterification to methyl jasmonate. Microbial biotransformation and biosynthesis of flavor and fragrance chemicals offer the potential benefits of producing optically active isomers which often have marked differences in flavor and fragrance quality and sensory intensity.

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Introduction

Biotechnology can be applied in the flavor and fragrance industry in terms of the production of flavor and fragrance chemicals, of the production flavor and fragrance modulators and of the analysis of chemosensory systems. Probably, the production of natural flavor and fragrance chemicals is the most feasible way to apply biotechnology in the flavor and fragrance industry. Most natural flavor and fragrance chemicals are heavily dependent on plant and animal origins. However, the quality and the supply of traditional natural flavor and fragrance chemicals are somewhat limited. The supply of natural chemicals from plants is dependent on uncontrollable weather and sociopolitical instability of major supplying areas. Viable alternative and innovative ways to synthesize natural chemicals include biotechnological routes, i.e., microbial fermentation and plant tissue culture. Natural flavor chemicals especially, can be produced by fermentation, enzymology and natural reaction process under the regulation established by FDA (US Code of Federal Regulation, 21 CFR101.22.a3, 1990) (*19*).

Microorganisms are traditionally used not only to produce fermentation products, but also to produce aroma chemicals to enhance product quality in the brewing and food industries. The ability to produce aroma chemicals by microbial fermentation may supplement and enhance the quality of plant-based flavor and fragrance chemicals. The application of diverse microbial metabolic abilities is a major approach for the bio-production of flavor and fragrance chemicals in terms of feasibility and economics. A recent application of biotechnology in the flavor and fragrance industry is the production of natural chemicals using combinatorial approaches to develop environmentally benign bio-organic preparations of natural chemicals. The bio-organic processes, including whole cell fermentation, used in the commercial production of flavor and fragrance chemicals are summarized in Table I. Unlike chemical synthesis, bio-organic synthesis can have added benefit of producing optically active chemo-, regio- and stereo-selective chemicals in addition to the production of new natural chemicals. Such selectivity has marked effects on flavor and fragrance quality and intensity (*16*). The optically active and stereo-selective flavor and fragrance chemicals developed using biotechnology can be produced by; 1) de novo biosynthesis of pure isomers, 2) biotransformation of optically inactive precursors to optically active isomers, and 3) resolution of racemic mixtures to select optically active isomers.

The major flavor and fragrance companies are currently focused on biotechnological research efforts in discovery, identification and development of new flavor and fragrance ingredients to provide advantages in creating unique, globally natural, kosher, consumer preferred, high performance flavors and fragrances. The guiding principles drawn on by flavor and fragrance industries for the production of natural chemicals are the following: 1) source materials must occur in nature, 2) the resulting materials from processes must be found in

Table I. Commercial Bio-organic Processes used for the Production of Natural Flavor and Fragrance Chemicals

Chemical Reactions	Products
Oxidation of primary alcohols	Aldehydes and Acids
β-oxidation (aborted)	Methyl ketones
Hydroxylation (Oxidation) of aromatic acids	Hydroxyl aromatic acids
Hydroxylation of fatty acids	Saturated lactones
Hydroxylation & limited β-oxidation of fatty acids	Unsaturated lactones
Baeyer-Villiger oxidation	Alcohols
Reduction of Ketones	Secondary alcohols
Reduction of acids	Aldehydes and Alcohols
Reduction of double bonds	Saturated aromatic chemicals
Amino acid metabolism	Unsaturated & branched acids
De novo synthesis	Acids and alcohols

nature or in traditional foods, and 3) processing conditions must meet the criteria of traditional or in-home preparation techniques (*20*). In this paper, three newly commercialized bioprocesses for the production of, and or biotransformation of, natural precursors into new valuable flavor and fragrance chemicals via microbial metabolic pathways are discussed.

Tuberose Lactone

Lactones are among the major ingredients used for the creation of flavor formulae in many categories, especially in fruit and dairy flavor formulae. Lactones are intramolecular esters that can be formed in plants and microorganisms. Over the years, a variety of saturated and unsaturated γ- and δ-lactones are produced by both chemical synthesis and microbial fermentation. The microbial processes for lactone production can be divided into two different processes; 1) the lactones can be produced by direct hydroxylation of fatty acids to produce d/l (+/-) 4- or 5-hydroxy fatty acids followed by a lactonization reaction, and 2) the lactones can be produced by two step processes of fatty acids, hydroxylation of unsaturated fatty acids and limited β-oxidation of hydroxy fatty acids to produce 4- or 5-hydroxy fatty acids followed by a lactonization reaction. Most commercial developed bioprocesses utilize microbial abilities to produce 4-hydroxy fatty acids for the production of γ-lactones. Various molds including *Aspergillus sp.* are known to directly hydroxylate at the C4-position of short chain fatty acids (*8*). Unlike the direct hydroxylation by molds, unsaturated fatty acids can be hydrated at different double bond positions, especially at the C10-position by various microorganisms (*12*). The resulting 10-hydroxylated fatty acids are converted to lactone intermediates, 4-hydroxy fatty acids, via limited β-oxidation of various yeasts for the production of saturated and or unsaturated γ-lactones. Although certain commercial fermentation processes have been well developed, the metabolic pathways for the accumulation of lactones in microorganisms are poorly understood (*22*). It has been speculated that the lactone intermediates, 4-hydroxy fatty acids, may cause structural hindrance for β-oxidation related enzymes and allow transient accumulation of intermediate 4-hydroxy fatty acids during β-oxidation processes of certain yeast cell cultures (*8,22*). The intermediates can be further oxidized to 3,4- and 3,5-dihydroxy fatty acids and eventually degraded to CO_2 and ATP as final products of β-oxidation beta during prolonged fermentation.

Natural and kosher tuberose lactone is one of the lactones that was produced using a two-step fermentation and the a schematic process of tuberose lactone is described in Figure 1. Tuberose lactone, a mixture of saturated and unsaturated C12 lactones, was produced from hydrolyzed flaxseed oil. Flaxseed oil contains high concentration of C-18 unsaturated fatty acids (>90%),

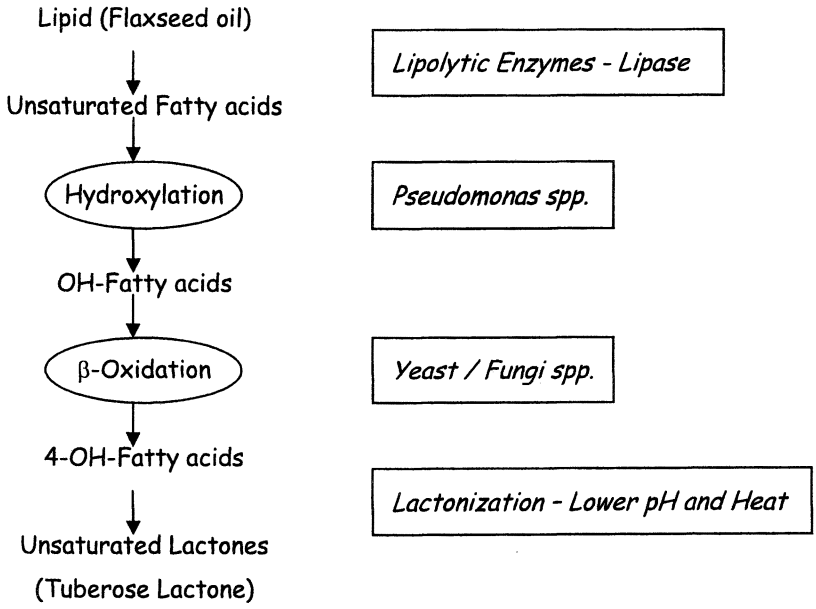

Figure 1. Schematic production flow diagram for the production of tuberose lactone – Two-step fermentation process

including >50% linolenic acid, >20% linoleic acid and >20% oleic acid. Tuberose lactone consists of three different lactones, (Z,Z)-6,9-dodecadien-4-olide (45-50%), (Z)-6-dodecen-4-olide (20-25%) and d-dodecan-4-olide (γ-dodecalactone, 20-25%), reflecting the composition of flaxseed oil (Figure 2).

The production requires 2 fermentations. In the first fermentation, lipase enzymes liberate the unsaturated fatty acids of flaxseed oil. During the first fermentation, the hydrolyzed fatty acids, linolenic acid, linoleic acid and oleic acid, are converted to (respectively) 10-hydroxy-12(Z),15(Z)-octadecadienoic acid, 10-hydroxy-12(Z)-octadecenoic acid and 10-hydroxydecanoic acid by *Pseudomonas sp.* NRRL-2994. *Pseudomonas sp.* produced stereochemically pure d (R)-isomers of each of the hydroxy fatty acids (>95.8%) (*23*) at a concentration of >12 g/L in the fermentation broth. The resulting hydroxy fatty acids were recovered by phase separation technique, and used for the second fermentation.

The second fermentation was developed using yeast strains, such as *Yarrowia lipolytica* ATCC 34088, that has limited β-oxidation abilities. The recovered hydroxy fatty acids were fed into a new fermenter and sterilized with other ingredients before inoculation with *Y. lipolytica* culture. *Y. lipolytica* converted C-18 10-hydroxy fatty acids to the corresponding lactone intermediates, 4-hydroxy C12 fatty acids via a limited β-oxidation. The fermentation was usually terminated at the point of a maximum accumulation of lactone intermediates, at the concentration of ~5 g/L in the fermentation broth. After the fermentation process was complete, the lactone intermediates were lactonized at a pH in the range of 3-5 and at a temperature of >100°C. The resulting lactones were recovered and purified from the fermentation broth by solvent extraction followed by fractional distillation.

Natural and kosher tuberose lactone was recently listed in the GRAS 20 list of flavor chemicals. It is mainly used for the preparation of natural fruit and dairy flavors. They can also can be used in various flavor and fragrance formulae as an enhancer or as a modulator due to their prolonged and tenacious sensory properties.

Styrallyl Acetate

Esters are among the major aroma chemicals in flavors and fragrances of all categories and can be synthesized by microorganisms and microorganism derived enzymes. However, microorganisms and enzymes exhibit substrate specificities that limit their application to the production of various esters. It is especially difficult to produce secondary alcohol esters using commercially available esterification catalyzing enzymes. The commercially developed process for secondary alcohol esters includes two separate processes: 1) the

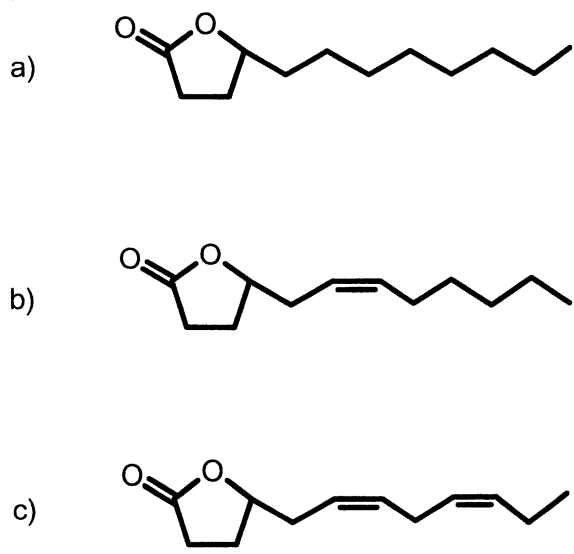

Figure 2. Tuberose lactone as a mixture of three different lactones: a) d-Dodecan-4-olide (20-25%), b) d-(z)-6-Dodecen-4-olide (20-25%), and c) d-(z,z)-6,9-Dodecadien-4-olide (45-50%)

microbial reduction of ketones to secondary alcohols, and 2) the esterification reaction of secondary alcohols with acetic acid under natural reaction conditions. The microbial reduction of ketones for the production of chiral secondary alcohols has been well known and used in the production of particular stereoisomers of specific secondary alcohols by the flavor and fragrance industry (Figure 3). The hydrogenation of ketones to form chiral secondary alcohols using *Clostridia* such as *Clostridium kluyveri* has been reported, and specially used in the production of styrallyl alcohol stereoisomers (*17*). Asymmetric reduction of acetophenone to styrallyl alcohol with enzymes of acetic acid bacteria was also reported by Adlercreutz (*2*).

Novel mixtures of optical isomers of natural and kosher styrallyl alcohol (α-phenylethyl alcohol), and their corresponding acetate esters of styrallyl alcohol (α-phenylethyl acetate) were prepared by multiple fermentation processes and an azeotropic esterification reaction. In the first step, natural acetophenone was produced by bioconversion of cinnamic acid by *Pseudomonas sp.* (*9*), *Comanonas sp.* and *Arthrobacter sp.*(*6*). In the first microbial oxidation process, the side chain of cinnamic acid was oxidized to the ketone to form acetophenone that was transiently accumulated in the fermentation broth (*9*). The current commercial fermentation process yielded >5g/L of acetophenone in the fermentation broth following 2 days of incubation using *Arthrobacter sp.* The resulting acetophenone was recovered and purified from the fermentation broth by solvent extraction followed by fractional distillation. Acetophenone itself can be used in creating flavor formulations and in enhancement of aroma and taste or both.

In the second step, a microbial process of reducing acetophenone to produce styrallyl alcohol stereoisomers was developed using a culture of *Kluyveromyces polysporus* ATCC 22028. *K. polysporus* quantitatively converted acetophenone to styrallyl alcohol at a concentration of >10 g/L in the fermentation broth following 2 days of incubation. The resulting styrallyl alcohol isomers (87% R- and 13% S-isomers) were recovered and purified by solvent extraction followed by fractional distillation. The crude styrallyl alcohol recovered from a short path distillation was used in the esterification reaction. Styrallyl alcohol itself also can be used in creating flavor and fragrance formulations.

Finally, the esterification reaction with styrallyl alcohol and acetic acid was carried out under natural reaction conditions using esterification catalysts such as citric acid or by means of bioprocess reactions using commercial ester-forming enzymes (*5*). The catalytic conversion of styrallyl alcohol to styrallyl acetate did not change the stereoisomer ratio. The resulting stereoisomeric mixture of styrallyl acetate was recovered and purified by solvent extraction followed by fractional distillation.

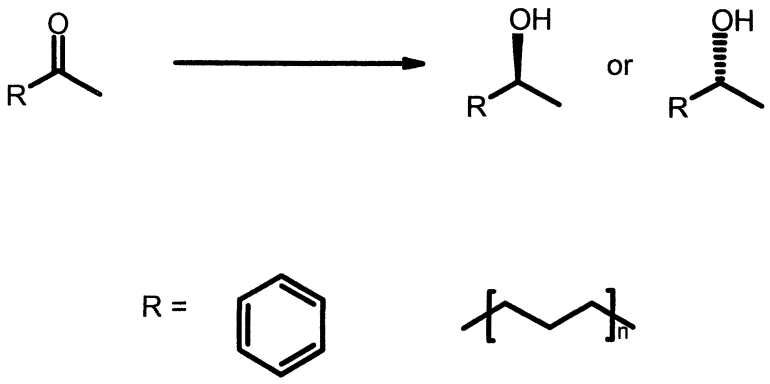

Figure 3. Ketone reduction by microorganisms. Ketones are selectively reduced to either S- or R-isomers

The overall bioprocesses produced a stereoisomeric mixture of natural styrallyl acetate with enantiomeric excess of 87% of the R-isomer. Styrallyl acetate mainly can be used for natural savory, fruit and dairy flavors, and fresh fruity and floral perfume compositions.

Methyl Jasmonate

Jasmonic acid (3-oxo-2-(2'-pentenyl)-cyclopentaneacetic acid), a plant fatty acid metabolite, is an endogenous plant growth regulator and is widely distributed in the plant kingdom (*3*). Jasmonic acid has various physiological functions including fruit ripening, production of pollen, root growth, tendril coiling, wounding, abiotic stress, and plant defense systems from microbial infection at lower concentrations in plants (*3*). Jasmonic acid is produced from the unsaturated fatty acid, linolenic acid, by plant lipoxygenase related metabolic pathways (Figure 4) and is probably a part of lipoxygenase related plant growth regulation (*18,21*). Its production starts with linolenic acid being converted to the 13-hydroperoxide of linolenic acid by lipoxygenase and further converted to the allene oxide by allene oxide synthetase. The allene oxide is metabolized to jasmonic acid via cyclization, double-bond reduction and side chain β-oxidation (*21*). It is also reported that several species of plant pathogenic fungi including *Diplodia sp.* produce jasmonic acid which show a phytotoxic effect at the relatively higher concentration (*11*). Although the biosynthetic pathway of microorganisms is probably similar to that of plants, the function of jasmonic acid in microorganisms is not clearly known. Jasmonic acid has two chiral centers at the C3 and C9 positions, and is expected to have potentially 4 stereoisomers (Figure 5) However, the absolute configuration of jasmonic acid in nature is not clearly known due to the difficulty of isolating pure jasmonic acid stereoisomers from both plants and fungi. It was reported that fungal cultures including *Diplodia sp.* produced mainly (+)-epijasmonic acid and a small amount of (-) jasmonic acid (*12*).

The methyl ester of jasmonic acid (methyl 3-oxo-2-(2'-pentenyl)-cyclopentaneacetate) is also known as both an active plant hormone and an important fragrance and flavor component with sweet-floral and jasmine-like aroma notes (*13*). Methyl jasmonate induces plant systems to provide defense from microbial infection, and is a secondary synthesis product of plants. Methyl jasmonate was also isolated from the Oriental fruit moth, *Grapholitha molesta* as a part of the male sex hormone (*1,15*). The authors demonstrated that the volatile form of jasmonic acid, methyl jasmonate, is a major signal molecule for inter- and intra species communications. Methyl jasmonate is expected to have

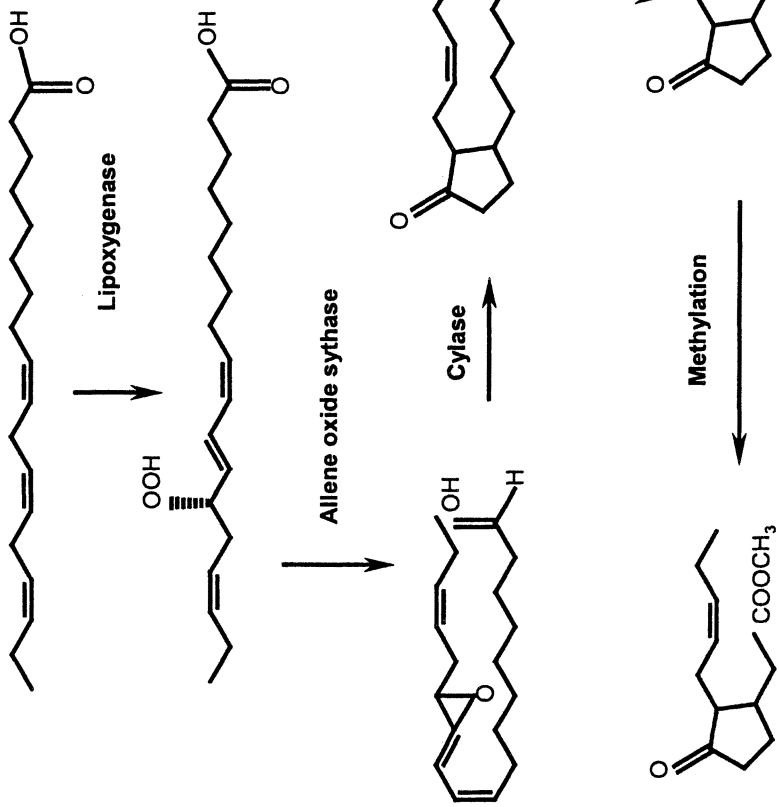

Figure 4. Metabolic pathway for the production of jasmonic acid and methyl jasmonate

potentially 4 optical isomers that may be formed (Figure 5). However, (1R,2S)-(+)-methyl epijasmonate was reported to be the biologically active form that contributed the characteristic odor in nature (*14*). Methyl epijasmonate was not easy to isolate at higher concentration due to the thermodynamically unstable nature of its stereospecific configuration.(*4*)

The schematic bioprocess to produce natural and kosher methyl jasmonate is summarized in Figure 6. The new commercial fermentation process was developed using *Diplodia sp.*, including *D. gossypina* ATCC 10936, that were isolated from citrus plants. *Diplodia sp.* had characteristic hyphal growth rather than pellet growth under submerged conditions (Figure 7). *Diplodia sp.* assimilated various carbon sources and exclusively synthesized (+)-jasmonic acid (>99%) in aerobic conditions as a part of de novo biosynthesis. The commercial fermentation process yielded >1.5 g/L of jasmonic acid in the fermentation broth after 15 days of fermentation using *D. gossypina* ATCC 10936. The resulting jasmonic acid was extracted from the fermentation broth using ethyl acetate. Crude jasmonic acid was recovered by stripping off the solvent at low temperature to prevent epimerization. The crude jasmonic acid was further purified by an anion exchange column method.

The esterification reaction of jasmonic acid with methyl alcohol was carried out in a high-pressure reactor to reduce epimerization. It was known that the cis-form of methyl jasmonate easily epimerized to the trans-form of methyl jasmonate under acid, base and high temperature conditions. The resulting methyl jasmonate mixture was recovered and purified by solvent extraction followed by fractional distillation. To improve the methyl epijasmonate concentration, additional steps of fractionation, such as the use of silica gel, were applied.

The final natural and kosher methyl jasmonate mixture contains various optical isomers, including >5% of biologically active methyl epijasmonate. The methyl jasmonate mixture can be used in creating powerful fresh and sweet impressions in flavor and fragrance formulations, and additionally can be used in secondary and tertiary flavor enhancement.

Summary

Recent consumer preference for "natural" flavors and fragrances prompted the flavor and fragrance industry to re-direct its attention to research in the production of flavor and fragrance ingredients via processes that would be considered "natural". Especially, it is generally recognized in the industry that flavor and fragrance compounds having been prepared by microbial processes can be designated as natural products, and therefore, have an important place in

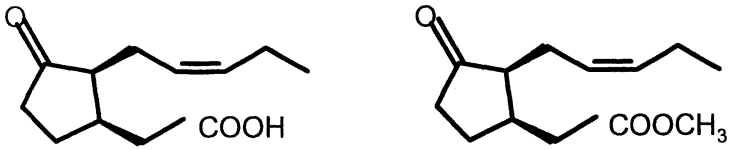

Figure 5. Configuration of biologically active (+) epi-jasmonic acid (3-oxo-2-(2-pentenyl)-cyclopentaneacetic acid) and (+) epi-methyl jasmonate (methyl 3-oxo-2-(2-pentenyl)-cyclopentaneacetate)

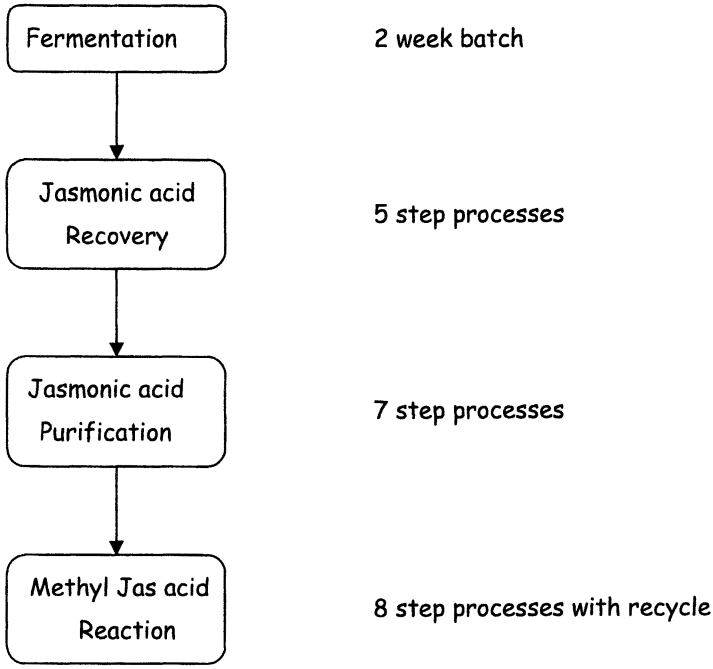

Figure 6. Flow diagram for the production of methyl jasmonate

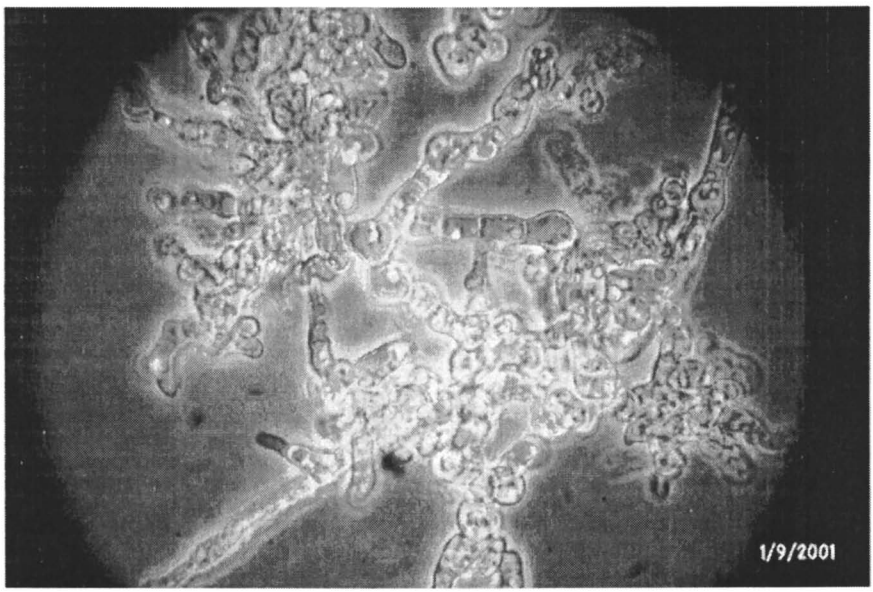

Figure 7. Diplodia gossypina ATCC 10936 with hypal growth that is required for the production of jasmonic acid under submerged conditions

the commercialization of food and other consumer items. As the industry continues to use microbial metabolic properties for the production of natural flavor and fragrance chemicals, the new chemicals will become available for the creation of natural flavors and fragrances, providing important advantages in the market place. It is also noteworthy that another important global trend driving the flavor and fragrance industry is the growing interest in "label-friendly" ingredients reflecting health consciousness, convenience and environmental safety. Environmentally benign bio-organic syntheses should provide alternative ways to produce flavor and fragrance chemicals to meet consumer expectations. It is clear that research in flavors and fragrances should be multidisciplinary to meet the challenges and changes demanded by society and consumers. Recombinant DNA technology will be an important tool in the future, not only for the production of new flavor and fragrance chemicals, but also for improving future understanding of the mechanisms of olfaction and taste

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