# Microbial Transformation of Citral by *Aspergillus niger*-PTCC 5011 and Study of the Pathways Involved

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## Abstract

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Citronellol and hydroxy citronellal are fragrance ingredients used in decorative cosmetics and fine fragrances. They have many applications in flavourings, extracts, foods, and drugs manufacturing. Their use worldwide is in the range exceeding 1000 metric tons per annum. Previous studies investigated the biotransformation of citral by the free cell method and immobilised cell method with *Saccharomyces cerevisae.* In this research, the microbial transformation of citral by the sporulated surface cultures method with *Aspergillus niger*-PTCC 5011 was studied. In experiments, *A. niger* was inoculated on solid media in conical flasks. The transformation took place for the periods of time of 6 days and 15 days. The results were analysed by the theoretical study, Fourier transform infrared spectroscopy, gas chromatography, and gas chromatography-mass spectroscopy. The major compounds produced in 6 days were acetone (26.2%) and hydroxy citronellal (37.0%), while the major compounds after the 15-day period were acetone (15.0%) and citronellol (36.0%). As citronellol has numerous commercial applications, the 15-day biotransformation period was found to be preferable.

**Keywords**: biotransformation; bioconversion; *Aspergillus niger*; fungi; citronellol

Monoterpenes are widely distributed in nature and find extensive applications in the flavour and fragrance industry. Their simple structures make them ideal targets for microbial biotransformations to yield several commercially important products (Werf *et al.* 1997). The use of microorganisms for the transformation of complex chemicals resulting in single and specific transformations has been effectively exploited in recent decades. They are useful to the biological and food industries for the production of organic and food compounds either in view of improving the yield of already known substances or novel substances production. Since the first such patent in 1937, bacteria and fungi have been widely employed in steroid biotransformation studies (Smith 1984). Among the various kinds of microorganisms, microalgae had been less investigated in the conversion of steroid com-

pounds. Earlier studies have already demonstrated the potential of microalgae for steroid modifications. Abul-Hajj and Qian (1986) showed the ability of different strains of microalgae such as *Anabaena cylindrical, Scenedesmus quadricauda,* and *Coelastrum proboscideum* in the conversion of androstendione to testosterone.

Other researches proved that compared to conventional chemical catalysts, some biocatalysts function under mild conditions to perform reactions that are regio-, stereo-, and enantiospecific. Chiral building blocks, pharmaceutical and agrochemical compounds, and food additives are commercially considered as pure enantiomers only when one enantiomer is present in excess of ca. 98%. Although the majority of flavour and pharmaceutical compounds are racemic, usually only one of the enantiomers has the desired activ-

ity. The other enantiomer may be inactive, or it may have an unwanted activity. The United States Food and Drug Administration has declared that if a drug is chiral, the biological effects of both enantiomers must be studied because the non-therapeutic enantiomer may have unwanted side effects (Jirage & Martin 1999).

Citronellol is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps, and other toiletries as well as in noncosmetic products such as household cleaners and detergents. As citral has a lessen use in fragrances, shampoos, and other production, its conversion to citronellol is of commercial interest (Larroche & Gros 1989; Larroche et al. 1989). Recently, we reported on the bioconversion and study of the pathways involved during the biotransformation of citral to citronellol by the free cell method and immobilised cell method using *Saccharomyces cerevisae.*

In the course of the research related to bioconversion of monoterpene aldehyde by fungi, the microbial transformation of citral by *Aspergillus niger*-PTCC 5011 was investigated. The biotransformation of (l)-citronellal to (l)-citronellol by free and immobilised *Rhodotorula minuta* has been reported (Velankar & Heble 2003). Bioconversion of geranyl and neryl acetate by *Aspergillus niger* has been described (Madyastha & Krishna Murthy 1988a,b). Some investigations have shown that the main reaction to liquefied *A. niger* was hydrolysis of terpene to 8-hydroxy derivatives (Goto 1967). In 1969, wood used the sporulated surface cultures method (SSCM) to convert geraniol to linalool and partially oxidised it to citral. Demyttenaere et al. (2000) compared microbial transformation of geraniol and nerol by five *A. niger* strains and three *Penicillium* strains using SSCM and the submerged liquid method. Several investigators used different fungi to bioconvert citral (Massada 1976; Larroche & Gros 1989; Larroche et al. 1989; Demyttenaer & De Pooter 1998; Ramaswami et al. 1998; Adams 2001).

In this article, microbial transformation of the pure terpene aldehyde citral is carried out by SSCM. Biotransformation of citral over a period of 15 days produced citronellol, primarily. The product of the biotransformation was extracted with $Et_2O$ three consecutive times and directly analysed by the theoretical study (TS), Fourier transform infrared spectroscopy (FT-IR), gas chromatography (GC), and gas chromatography/mass spectroscopy (GC-MS).

## MATERIAL AND METHODS

***Microorganisms***. Our laboratories isolated a strain of *Aspergillus niger* from soil taken in Tehran prefecture, which was identified according to its physiological and morphological characteristics. The strain was identified as *A. niger* (PTCC 5011) according to the Persian Type Culture Collection, Iranian Research Organization for Science and Technology, Tehran, Iran. A spore suspension of *A. niger* was prepared in a nutrient broth solution for the inoculation of fungi in Petri dishes.

***Growth medium and conditions***. The solid medium Sabouraud dextrose agar (SDA), containing glucose (4.0%), and agar (1.5%), and mycological peptone (1.0%), was used for the isolation, growth, and conservation of the fungi in Petri dishes.

The solid agar medium was inoculated with spores of *A. niger*. First germination of the spores and mycelial growth took place, with the growth medium stored at room temperature. After one week, the surfaces of the Petri dishes were covered with spores, and the biotransformation reaction started.

***Experiments with spore suspension***. The spores were recovered from 1-week-old surface cultures of *A. niger* and *Penicillium* sp. grown in Petri dishes on SDA. This was done by adding 10 ml of a sterile Tween 80 solution (0.2%) in distilled water to each culture, bringing the spores into suspension. A total spore suspension of 50 ml was obtained, which was shaken in a 250-ml conical flask.

To this spore suspension 1 ml of the solution of 5% menthol in ethanol was added, and the suspension was placed on a shaker at 180 rpm. After 6days and 15 days, the product of the biotransformation was extracted with $Et_2O$ three consecutive times, and the products were directly analysed by TS, FT-IR, GC, and GC-MS.

***Biotransformation***. The methodology for biotransformation described by Leuenberger (1984) was employed. After 48 h of growth the activated spores were added to 100 ml SDA containing 4.47 g/l citral. 0.1 g/l methanol was used as a solubilising agent. The transformation efficiency was determined using the following formula:

X grams of *Aspergillus* transforms X grams of citral to citronellol per day = Number spore suspension adjust $1 \times 10^8$ ml per spore (0.5 g/l for a spore) × $(4.47 \times 10^{-3}$ g citral /l) $= 2.2 \times 10^5$ g/l

***Optimum conditions for biotransformation***. 500-ml Erlenmeyer flasks were used for *A. niger*

growth in 250 ml media for studying optimum conditions necessary for biotransformation. The suitable growth phase after which the cells could be harvested was determined by using different growth periods (6 days and 15 days). The optimum value of pH was estimated in the range of pH 5.5 and optimum temperature at 27°C. Agitation speed of 150 rpm was employed.

***Extraction and analysis***. On completion, the contents of the flasks were extracted with 3× 50 ml $Et_2O$, demoisturised by the addition of 1 g sodium sulfate/l, and distilled in a water bath at 60°C. The products were directly analysed by TS, FT-IR, GC, and GC-MS.

The method described by SPEELMANS *et al.* (2008) was with some modifications standardised for the analysis of the products on a gas chromatograph. The products were further identified by GC and GC-MS analyses. The composition in relative percentages was computed by the normalisation method from the GC peak areas and the percentage conversion was used as the performance criterion.

***Analysis of the samples with IR, GC, and GC-MS***. GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a capillary column that used DB-5 (50 m × 0.2 mm, film thickness 0.32 μm).

A split/splitless injector and flame ionisation detector were heated at 250°C. $N_2$ was used as the carrier gas (1 ml/min). The oven temperature was kept at 60°C for 3 min and then heated to 220°C with a 5°C/min rate and kept constant at 220°C for 5 minutes. Relative percentage amounts were calculated from the peak area using a Shimadzu C-R4A Chromatopac Integrator without correction.

GC-MS analysis was performed using a Hewlett-Packard 5973 with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm). The oven temperature was kept at 60°C for 3 min and programmed to 220°C at a rate of 5°C/min and kept constant for 5 min at 220°C. The flow rate of helium as a carrier gas was 1 ml/min in an electronic impact mode of 70 eV. The identification of the oil constituents was made by comparison of their mass spectra and retention indices with those given in the literature and of authentic samples (FRAN *et al.* 1977; CHEN *et al.* 1982). FT-IR was recorded in $CHCl_3$ as follows for the main compounds citronellol, acetone, and hydroxy citronellal:

Citronellol: 156[$M^+$]: 41(100), 69(82), 55(55), 82(30), 67(37), 81(32), 57(), 95(20). FT-IR (KBr) $v_{max}$ $cm^{-1}$: 3460, 1648, 1344, 1034

Acetone: 58[$M^+$]: 41(100), 58(75), 15(24), 42(80), 27(50), 39(40), 26(6), 59(30). FT-IR (KBr) $v_{max}$ $cm^{-1}$: 1668, 1344, 1034

Hydroxy citronellal: 172[$M^+$]: 59(100), 43(70), 81(45), 71(40), 95(30), 137 (22), 111(20), 121(20). FT-IR (KBr) $v_{max}$ $cm^{-1}$: 3460 (C-OH), 1638 (C=C)

## RESULTS AND DISCUSSION

In this experiment, microbial transformation of citral by SSCM of *A. niger* (PTCC 5011) grown in medium culture flasks was monitored for two weeks. The cultures were grown in Petri dishes on solid medium SDA containing menthol. After incubation, the SDA culture was extracted. It was observed that after six days, the cultures with citral were fully grown and sporulation had occurred. The cultures with 0.1% citral covered only part of the surface. The suspension was extracted with $Et_2O$ three consecutive times and directly analysed by TS, FT-IR, GC, and GC-MS.

In these analyses, various chemicals were determined. The main products obtained in the bioconversion of *A. niger* of citral over a period of six days were citronellol, ethanol, ethyl acetate, acetic acid, 4-isopropyl, 4-hydroxyl-penta-2-one (less than 5%), acetone (26.2%), and hydroxy citronellal (37.0%). When the biotransformation was allowed to continue for a period of 15 days, the major compounds obtained were acetone (15.0%), ethyl acetate (2.8%), hydroxy citronellal (4.7%), and citronellol (36.0%). Scheme 1 shows the possible pathway of the bioconversion of citral to its products over the contrasting periods of 6 and 15 days.

The energy of citral is compared with other products, especially citronellol and hydroxy citronellal, was investigated at the HF levels of theory, using the density functional theory with the Becke3LYP functional and the 6-311$^{++}$G basis sets. It showed citronellol to possess less energy (HF = –468.314 Hartee); others have high energy, but they are unstable. FT-IR $v_{max}$ $cm^{-1}$: 3112, 1560, 1347, 1023.

Scheme 2 shows the role of oxidation in the bioconversion of citral to hydroxy citronellal and citronellol. In the other, more direct method, citral was oxidised to produce hydroxy citronellal. This conversion offered twice the reduction

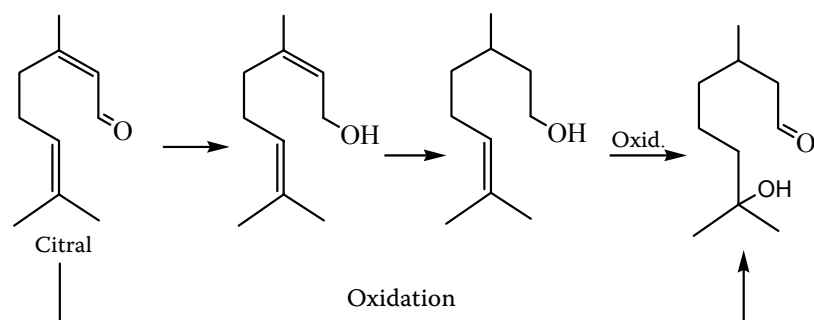Scheme 1. Possible pathway of bioconversion of citral by *A. niger*
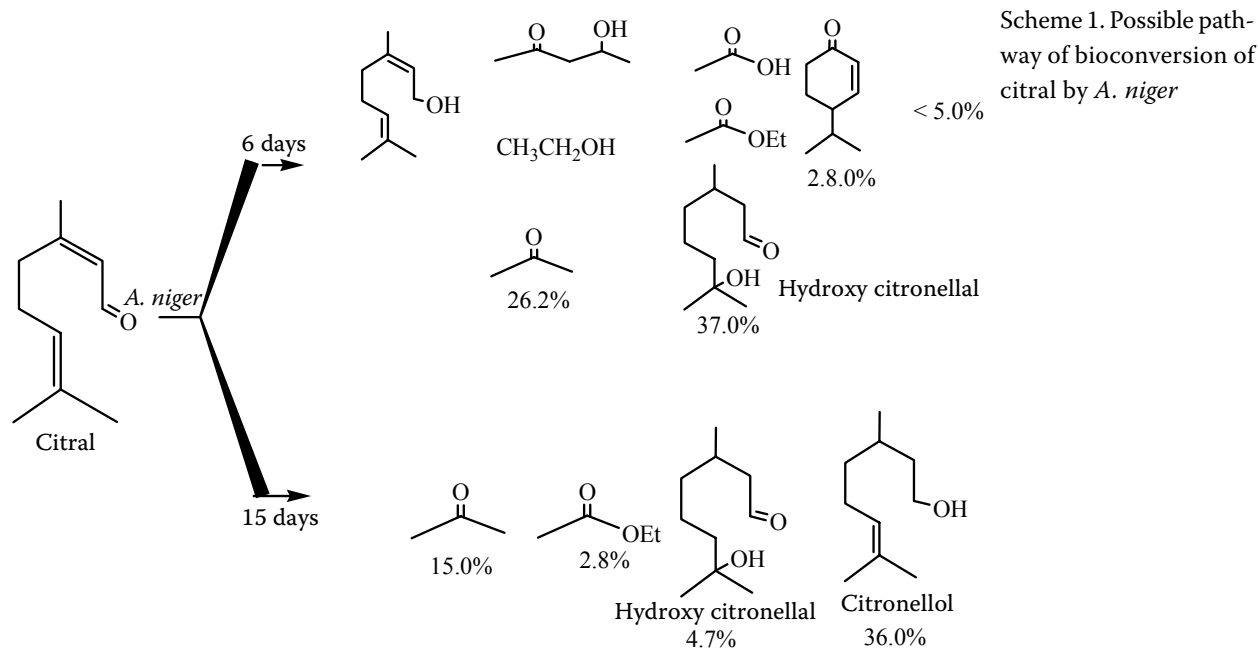
and produced citronellol and then, by oxidation, hydroxy citronellal. In this case citral C=C was reduced and the -OH group bioconverted to hydroxy citronellal.

The results suggested that SSCM over a period of 15 days is suitable for small samples. Because of the form, structure, and stability of citronellol, citral mostly converted to citronellol during biosynthesis.

In a previous study on bioconversion, citral and nerol were transformed by the spores of *Penicillium digitatum* into 6-methylhept-5-en-2-one by SSCM (Madyastha & Krishna Murthy 1988a). Microbial transformation of geraniol, nerol, and citral by *Aspergillus niger* produced linalool and α-terpineol. Bioconversion of nerol with *Penicillium chrysogenum* yielded mainly α-terpineol and some unidentified compounds. With *Penicillium rugulosum,* the major bioconversion product derived from nerol and citral was linalool (Madyastha & Krishna Murthy 1988b). In a study

of microbial transformation of the monoterpene cis-*p*-menthan-7-ol using *Aspergillus niger* and *Penicillium* sp., it was found that the main products obtained by SSCM were limonene, *p*-cymene, and γ-terpinene (Esmaeili *et al.* 2009c). The main bioconversion products of (-)-menthol obtained with *Mucor ramannianus* using SSCM were *trans*-*p*-menthan-8-ol, *trans*-menth-2-en-1-ol, sabinene, *p*-menthane-3,8-diol, isomenthol, and 1,8-cineole. Most of the main products of microbial transformation of citral in this study resemble those obtained in a former work (Esmaeili *et al.* 2009b). The main biotransformation products obtained from menthol by *Penicillium* sp. were α-pinene (18.0%), *trans*-*p*-menthan-1-ol (10.6%), *p*-menth-1-ene (5.8%), sabinene (3.9%), 1,8-cineole (6.4%), and limonene (3.2%) (Esmaeili *et al.* 2009a). The experimental work suggests that microbial transformation with *Penicillium* and *Aspergillus* caused an oxidation reaction and transformation of monoterpenes to a more stable product. Bioconversion using SSCM



Scheme 2. Possible pathway of bioconversion of citral to citronellol and hydroxy citronellal by *A. niger*

with *A. niger* could selectively provide one product in a high percentage.

The biotransformation products were identified using TS, FT-IR, GC, and GC-MS. The C=C and C-OH groups in FT-IR spectra for 6 and 15 days were more clear, in addition to citronellol, hydroxy citronellal, and acetone products. With this method, citral was transformed to citronellol, hydroxyl citronellal, and acetone of high percentages Acetone could be the product of ozonisation of C=C citral. Citronellol is used in the manufacture of cosmetics, fine fragrances, shampoos, toilet soaps, and other toiletries. Its use worldwide is in the range exceeding 1000 metric tons per annum. Hydroxy citronellal is used for supplying berry and cherry flavours and other exotic applications. This research indicates that citral, which is unsuitable for many such applications, can be changed to citronellol, which is used to a greater extent in flavour, extract, food, and drug manufacture.

## CONCLUSIONS

(1) The purpose of this research was to study the pathways involved during microbial transformation of citral by SSCM using *A. niger*.

(2) The major components of the microbial transformation of citral derived over a period of six days were citronellol, ethanol, ethyl acetate, acetic acid, 4-isopropyl, 4-hydroxyl-penta-2-one (< 5%), acetone (26.2%), and hydroxy citronellal (37.0%). Acetone (15.0%), ethyl acetate (2.8%), hydroxy citronellal (4.7%), and citronellol (36.0%) were the major compounds derived over the longer period of 15 days.

(3) Limiting the transformation period to 6 days by *A. niger* with SSCM was not effective for small samples, while it can maintain the activity over prolonged periods, for example in the production of Ca-alginate or continuous production of selective compounds, a system well known for the bioconversion of fatty acids to methyl ketones.

(4) Many different terpenoid compounds can be tested for the bioconversion reactions with the same batch of fungus. The present work confirms the findings of two earlier studies.

(5) Citronellol, hydroxy citronellal, and acetone are major products of SSCM. Citronellol finds many applications in flavour, extract, food, drug, household cleaner, and detergent manufacturing, as well as, being a fragrance ingredient, in decora-

tive cosmetics, fine fragrances, shampoos, toilet soap, and other toiletries. Its use worldwide is in the range exceeding 1000 metric tons per annum. Hydroxy citronellal is used in berry and cherry flavours and other exotic applications.

(6) TS of citral is compared to other products, especially citronellol, hydroxy citronellal, and acetone with HF levels of theory, using the density functional theory with the Becke3LYP functional and the $6\text{-}311^{++}G$ basis set, showed citronellol, hydroxy citronellal, and acetone to have generally less energy and compound stability.

(7) TS, FT-IR, GC and GC-MS were employed for the determination of the primary biotransformation products.

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