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# Green Routes to Green Notes

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The typical fresh and pleasant top notes of crushed fruits and many green parts of plants, often referred as "green" note, arise from volatile C6-compounds, such as (E2)-hexanal (leaf aldehyde), and (Z3)-hexenol (leaf alcohol).
These compounds can be extracted from the plant tissues but, as they are required in industrial products in concentrations comparable to those in the source material, this utilizes high amounts of materials and is generally not economically realistic. Chemical synthesis of these compounds can meet the amounts required in industry but results in "synthetic compound" labelling that is not well perceived by consumers whose demand, especially in Western Europe, is in favour of "natural product".
Biotechnology represents a very attractive alternative for the production of flavors and fragrances that can be considered as natural. In addition, compared to chemical synthesis, the biotechnological approach offers the resolution of enantiomeric mixtures as they occur in nature, thus eliminating subsequent separation steps and improving the perception of scent that could be affected by chirality.
Here we report on a novel way for the biosynthesis of (E2)-hexanal and (Z3)-hexenol by biotechnological means; Green note compounds were produced by growing engineered bacteria in a specifically tailored medium supplemented with 10% PUFA-rich oil. Bacteria transformed with a novel gene NX6125 degraded the Poly Unsaturated Fatty Acids, such as linoleic- and linolenic acid, into leaf aldehydes and alcohols. In addition, a brand new method for downstream processing i.e. isolation, concentration, and purification of the desired products, is introduced. This innovative development constitutes an elegant protocol for the industrial-scale production of natural green notes.

## Introduction

Volatile aldehydes and alcohols are key compounds in the fresh and green sensorial notes of vegetables and fruits (Rabetafika et al, 2008). They are produced by plants in response to various stresses and therefore play a major role in plant defense mechanisms (Blee, 2000). These compounds are formed via lipoxygenases, which degrade plant PUFA's (linolenic acid) and form hydroperoxides. These in turn are cleaved by

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hydroperoxide lyases to form the volatile aliphatic 6- to 9 carbon including cis-3-hexenol (6C) (leaf alcohol) and trans-2-hexanal (6C) (leaf aldehyde).

Due to their aromatic properties, green alcohols and aldehydes have a wide application in the food, feed, cosmetic, chemical and pharmaceutical sectors. Many of them are still produced via chemical synthesis or via extraction from plant sources; however, a rapid switch towards the bio-production and use of flavor compounds of (micro) biological origin - bioflavors – is observed. Reasons are among others, the fact that chemical synthesis results often in an environmentally unfriendly production processes and in undesirable racemic mixture compounds. Furthermore, the consumer has developed a "chemophobic"-attitude towards chemical or synthetic (even nature-identical) compounds, especially when related to food and home-care products (Cheetham, 1997).

Presently, certain plant sources remain an important source of green notes, but these compounds are present in minor quantities, making extraction, isolation and formulation very expensive. The other bio-route for the synthesis of these important compounds is based on *de novo* microbial fermentation or on bioconversions of natural precursors with microbial cells or enzymes. Biotechnological processes usually require less damaging process conditions to the environment and yield the desirable enantiomeric flavor compounds.

The enzymes involved in the catalysis of these compounds have not been detected so far in bacteria or fungi; they are now obtained as by-products of the essential oil industry (Hatanaka, 1993). Alternatively, cloning of the plant genes, coding for these genes in microorganisms, can allow the formation of the green note flavor by fermentation directly from added linolenic acid (Gallo et al., 2001).

In this work we employed a bacterial system that expresses a newly isolated gene NX6125. We hypothesized that this gene may cleave PUFAs to yield valuable aromatic compounds such as cis-3 hexenol and hexanal**.** Cis-3-hexenol, also known as (Z)-3-hexenol and leaf alcohol, a colorless oily liquid with an intense grassy-green odor of freshly cut green grass and leaves (Blee, 2000). Leaf alcohol is a very important aroma compound that is used in fruit and vegetable flavors and in perfumes (Goff and Klee, 2006; Dudareva and Pichersky, 2008). Hexanal, or hexanaldehyde, is an alkyl aldehyde used in the flavor industry to produce fruity flavors including apple, apricot, banana, blueberry, grape, passion fruit, and many more.

In order to circumvent the volatility limitation of these target compounds we developed a novel resin-based *in-situ* product recovery approach using the polymeric Amberlite XAD-4 adsorbent resin. Fig. 1 summarizes the process in which cells were grown on media supplemented with PUFA-rich sunflower oil. Conceivably any oil containing linolenic and linoleic acids may be a suitable source.

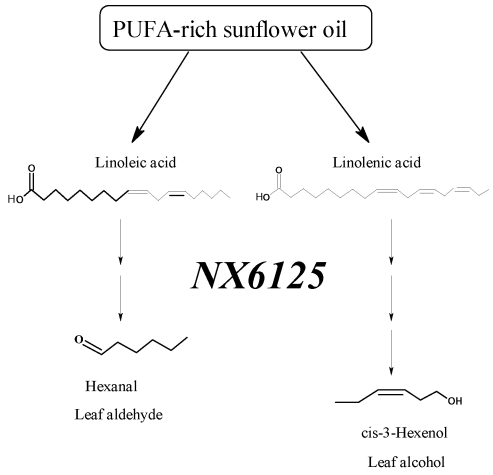


***Fig.1.*** *Proposed pathway leading to green notes biosynthesis. The conversion of PUFAs into green volatiles is canalized by E.coli cells overexpressing NX6125.*

## Results and discussion

We tested the ability of the NX6125 gene product enzyme to cleave a wide array of PUFAs-rich oils as substrate sources. The cleavage was evidenced by appearance of C6 compounds and parallel disappearance of oil, as tested by GC-MS. The highest activity of was observed using sunflower oil. E. coli overexpressing *NX6125* converted linoleic and linolenic acids from the sunflower oil to hexanal, and to *cis*-3-hexenol at a rate of 901.8 (peak area x 1000, and 353.8 (peak area x 1000), respectively. However, NX6125 demonstrated minimal activity on several other PUFAs-rich oils such as canola oil, and *Salvia sclarea* seeds oil. In canola, a rate of 162.2 (peak area x 1000) for hexanal, and 84.8 (peak area x 1000) for *cis*-3-hexenol were obtained, while in *Salvia sclarea* values decreased to 9.0 (peak area x 1000), and for *cis*-3-hexenol to 4.8 (peak area x 1000), respectively. In contrast, NX6125 was found to convert pure linoleic acid to *cis*-3-hexenol at a rate of 4.4 (peak area x 1000), but not to hexanal (Fig. 2). Control cells not expressing NX6125 demonstrated marginal activity indicating these cells harbor similar enzymes as their natural trait but at much lower levels. On the other hand, cells expressing NX6125 demonstrated a 3.6-fold increase over the control levels arose from spontaneous autooxidation of linoleic and linolenic acids (Goers et al., 1987).

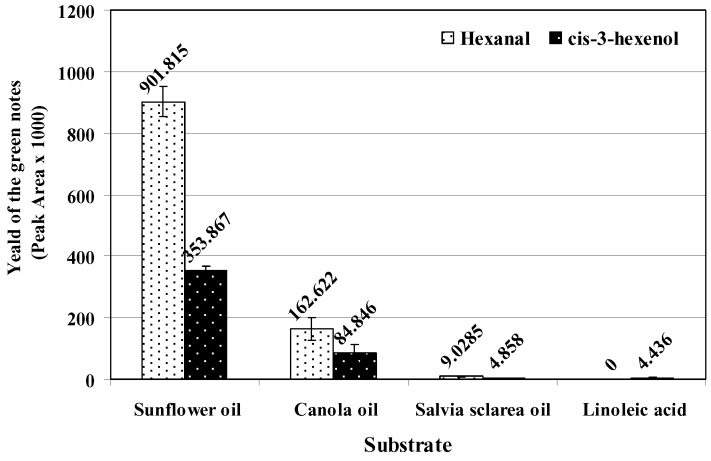


***Fig.2.*** *Detection of cleavage activity using different PUFAs-rich oil as substrate for the production of green notes.*

The recovery of volatile products from a bioreactor soon after it is formed is an important issue in industrial bioprocess development. Here we developed a practical approach to overcome this obstacle, adding the Amberlite XAD-4 polymeric adsorbent to the culture and further eluting with ethanol, resulted in a dramatic increase of up to 10-fold in target compounds (Fig. 3).

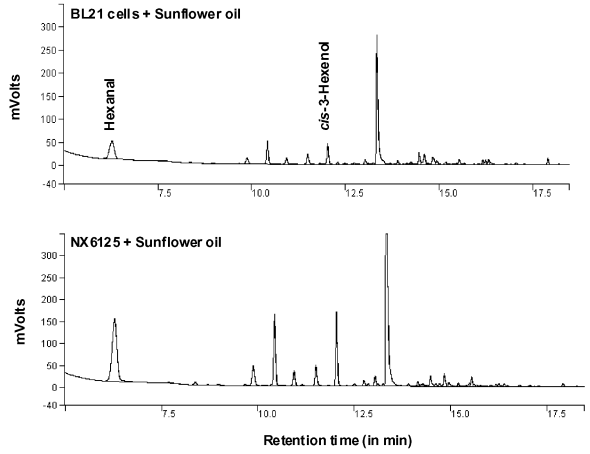


***Fig.3.*** *Representative GC chromatogram of green notes found in E. coli cultures supplemented with sunflower oil. Relative abundance is shown in arbitrary units, based on mVolts. Hexanal and cis-3-hexenol were identified by MS-spectrum and retention time in comparison with authentic standards.*

## Materials and Methods

### *E. coli* cultivation

*E.coli* containing pBK-CMV-NX6125 phagemid vector, were grown minimal salt medium containing 13.3g $KH_2PO_4$, 4g $(NH_4)_2HPO_4$, 1.7 g citric acid , 8.4 mg EDTA, 1.2 g $MgSO_4 \cdot 7H_2O$, 60 ml glycerol (or glucose) 50 % (w/v), 2 ml Trace Elements solution (X100), 4.5 mg thiamine-HCl. Trace Elements solution consisted of 2.5 mg $CoCl_2.6H_2O$, 15 mg $MnCl_2.4H_2O$, 1.5 mg $CuCl_2.2H_2O$, 3 mg $H_3BO_3$, 2.5 mg $Na_2MoO_4.2H_2O$, 13 mg $Zn(CH_3COO)_2.2H_2O$, 100 mg Fe(III)citrate dissolved in 1 L $H_2O$, and shaken at 300 rpm for about 5 h at 37°C. An appropriate volume of cells was added to 50 ml of fresh minimal salt medium, to reach an optical density of 0.6 at 600 nm. Protein expression was induced with 1 mm IPTG at 26°C for 24 h, containing 100 mg / ml Amberlite XAD-4 (Rohm and Haas).

### Volatile extraction

Following 24 h of induction, Amberlite XAD-4 were separated from culture by vacuum filtration, *E. coli* cells were removed and the Amberlite XAD-4 was washed with one volume of $H_2O$. The volatiles were extracted from the resin, by adding 50 ml of 95 % (v/v) ethanol and shaking for 30 min at room temperature. Ethanol layers were dried over $Na_2SO_4$. Samples were concentrated analyzed by GC-MS.

### Product identification by GC-MS Analyses

Samples were analyzed on an HP-GCO apparatus equipped with an HP-5 (30mx0.25mm) fused-silica capillary $1 ml min^{-1}$ column as the gas carrier. Injector temperature was 250°C, set for splitless injection. Oven was set to 50ºC for 1min, and then temperature was increased to 200ºC at a rate of $4 \circ C min^{-1}$. Detector temperature was 280ºC. Mass range was recorded from 45 to 450 m/z, with electron energy of 70eV. Identification of the main components was performed by comparing mass spectra and retention time data with those of authentic standards and supplemented with Wiley GC-MS library.

### Lipase-catalyzed partial hydrolysis of C18 PUFAs

50 mg (700 unit/mg) of lipase powder (Sigma-Aldrich) was added to 4.5 ml of 50 mM Tris-buffer and vigorously shaken for 5 min. lipase solution was supplemented with 4.5 ml PUFAs-rich oil ,100 µl 10% Triton-X100 , and 200 µl 0.5 M $CaCl_2$. The mixture was stirred at 37 °C for 1 h. Free fatty acids were then extracted with diethyl ether. Samples were concentrated and added to the growing cultures.

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