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Biosynthesis of new volatile and flavor compounds by using *Bjerkandera adusta* and *Wolfiporia cocos* as destroying agents of secondary metabolites and assuming of chemical bio reactions

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Abstract

Biosynthesis of new volatile and flavor compounds by using *Bjerkanderaadusta* (BAD) and *Wolfiporiacocos* (WCOC) is described together with the assumption of chemical changes likely to occur during fermentation. In total, we used 14 basidiomycetes (BAD, PCH, WCOC, PSA, BCI, SHIBO, MSC, HFA, MPS, PFLA, ICO, LED, TSU and LYP), which were cultivated in submerged media by using roots of *Hypericum perforatum*(L.) as the only source of carbon and nitrogen. We observed production of an ester, aldehydes, chlorinated organic compounds and two unidentified compounds during the interaction of BAD with the secondary metabolites present in roots of the genetically modified *Hypericum perforatum*(L.), while during the interaction of WCOC with the secondary metabolites present in roots of the genetically modified *Hypericum perforatum*(L.) we observed biosynthesis of mostly alcohols, some esters, terpenes and an unidentified compound. Beside the formation of new volatile and flavor compounds, we propose using of WCOC as dealkylation agent and BAD as dealkylation and reduction agent in organic synthesis, due to the changes we observed daily in our biotransformed product. The identification of compounds was performed by comparison of Kovats indices (KI) and mass spectra to those of authentic reference compounds on a polar VF-WAXms column, using headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS).

Keywords: New volatile and flavor compounds, basidiomycetes, *Hypericim perforatum* (L.).

1. Introduction

The use of bacteria and fungi in various fermentation processes is applied to many food products, such as lactic acid bacteria in milk, ferment lactose into lactic acid or production of cheese from milk by using of the enzyme chymosin, sometimes known as renin. Some types of cheese are produced in same way, for example the method used by Brie and Camembert involves the application of the mycelium of fungus *Penicilliumcamemberti* [1]. Fungi of the *Penicillium* family are applied in different food products and the process is generally about the benefit of carbon dioxide, which comes from the bacteria of the milk and as a result, the cheese has irregular cavities. The reason why basidiomycetes are able to cause a lot of changes is due to their extracellular enzymes. By using fungi, we are working in "green chemistry", where the toxicity that comes during classical synthesis is eliminated [2,3]. For fungal growth, carbohydrates are mostly needed. The carbohydrate active enzymes (CAZymes) are responsible for degradation of plant cell, and mostly, six classes are known: glycosyl hydrolases (GHs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), carbohydrate-binding modules (CBMs), glycosyltransferases (GTs), and auxiliary activities (AAs) [4,5].

Among the enzymatic reactions mentioned are those of oxidation of the C-H bond, which convert relatively simple and inexpensive molecules into applicable and high-cost products [6]. By allelic oxidation, alkenes can be converted into α, ß-unsaturated carbonyl compounds, based on the method of Muzard, Muller and Khoi, and Dauben et al. [7-9].. These protocols were later implemented using different metals, such as catalytic agents [10-14]. The use of basidiomycetes is also very effective in production of flavor compounds, which are popular because of their use in

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food industry. In this context we can mention that by using of *Pleurotussapidus (PSA)* (+)-valencen, a nonaromatic compound can be convert into (+)-nootaton, which is a flavor one [14,15]. This transformation was reported also by using solid culture of ascomycete *Chatomiumglobusum* [16,17]. The effect of basidiomycetes in biotechnology is to destroy large molecules by involving certain enzymes. These enzymes are activated depending on the material, for example degradation of cellulose and hemicellulose is carried out in the presence of active enzymes, such as endocellulose (EC 3.2.1.4), exocellulose (CBH cellobiohydrolase, EC 3.2.1.91), glucano-hydroloase (EC 3.1.74) and beta-glucosidase (EC 3.2.1.21) [47]. The importance of basidiomycete enzymes is confirmed by the Association of Manufactures and Formulators of Enzyme Product (AMFFEP 2009), according to which, from a total of 260 commercialized enzymes, approximately 156 are generated by fungi, like hydrolasis 85%, oxidoreductasis 8%, lyases 4%, transferases 2% and isomerases 1% [18]. In this context, using micro-organism or certain enzymes, currently is the best method for production of wanted pure compound, an enantiomer for example, which can be difficult to produce via other synthetic routs [19-22]. Using fungi for generation of flavor compounds is well explained by several authors [23,24]. To activate the enzymes of basidiomycetes, it is necessary to have a material which is rich in carbon and nitrogen.

Wolfiporia cocos(Schwein.)Ryvarden*et*Gilb. (Basidiomycota, Polyporaceae) is an edible mushroom used also as e herbal medicine. As a well-known fungus it parasitizes the roots of different species of *Pinus.* It is prevalent in a different part of the World, including East Asia, China, Japan, Korea etc [25-27]. It contains a different pharmaceutical and phytochemical compounds, which are responsible for their use in treatment of a wide range of illness as anti-tumor, anti-viral, anti-rejection, anti-hyperglycemic, antibacterial, anti-inflammatory, free-radical-scavenging, etc. [26-30]. Production of flavor compounds by using microorganisms, especially fungi are preferred nowadays because of their natural origin [31,32]. From all potential aroma producers, basidiomycetes are generally most known microorganisms and according to their application in production of flavor compounds, basidiomycetes have been shown great opportunity in production of variety flavor compounds such as vanillin, benzaldehyde and cinnamaldehyde [33-35]. In general, basidiomycetes are rich with extra cellular enzymes, like lignin peroxidases (LiP), manganese peroxidases (MnP) and most known enzymes are aryl alcohol oxidases (AAO) which are able to oxidize aryl alcohols into their corresponding aldehydes [36,37].

Bjerkandera adusta (BAD) is a ligninolyticbasidomycete used since early time in production of halogenated and flavor compounds [38]. The studies have shown that AAO activity increase in immobilized cells of BAD, what corresponds with benzaldehyde wields [32].

In this manuscript except following formation and identification of new volatile and flavor compounds, we have also assumed application of WCOC and BAD in organic synthesis, due to their ability to change chemical structure of new compounds daily for eight days.

2. Material and methods

2.1 Substrates

Roots of the genetically modified plant of *Hypericum perforatum* (L.) were produced from the Department of Plant Physiology at Ss.Cyril and Methodius University of North Macedonia. The material was stored in -20^{0}C until used for biotransformation while they were homogenized prior to biotransformation.

2.2 Microorganisms

For this research, we have used in total 14 basidiomycetes, which were obtained from the **Central Office for Fungal Cultures** (CBS, Utrecht, Netherlands), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the Department of Molecular Wood Biotechnology and Technical Mycology (WBTM, Göttingen, Germany).

2.3 Chemicals

All volatile compounds suggested as new compounds were purchased from Sigma-Aldrich, Fisher Scientific, Alfa Aesar, TCI Deutschland, Th. Geyer and VWR. When we take in consideration BAD and WCOC, we have in total 18 new volatile compounds, from which 14 were identified by injection of analytical standards on VF-WAXms column of GC/MS-MS/O (formic acid heptyl ester, (E)-2-octenal, 2,4-decadienal, 1,3-dichloro-2-methoxybenzene, 3-chloro-4-methoxybenzaldehyde, octanal, 2-nonanol, 3-octanol, 2-decanol, 2-undecanol, ethyl hexanoate, ethyl nonanoate, D-limonene, linalool). NIST 2011 database also was used for comparison of Kovats indices and mass spectra of the compounds. From all 18 volatile compounds, 4 seem are unable to be identified.

2.4 Surface screening

Based on previous research, hairy roots of genetically modified *Hypericum perforatum* (L.) were autoclaved at 121^{0}C for 20 minutes in a small Erlenmeyer flask covered with aluminum foil. Malt extract agar (MAE) solution 2% (2g agar-agar/100mL H_2O) was autoclaved separately in the same condition as hairy roots [39]. After autoclaving, the suspension was poured into small Petry dishes where and after they have been cooled at room temperature, all 14 fungus mentioned above were inoculated, while their growth was evaluated visually daily. When Petri dishes were evaluated approximately 70% by the mycelium, the surface cultures were sniffed by the trained panel in triplicity.

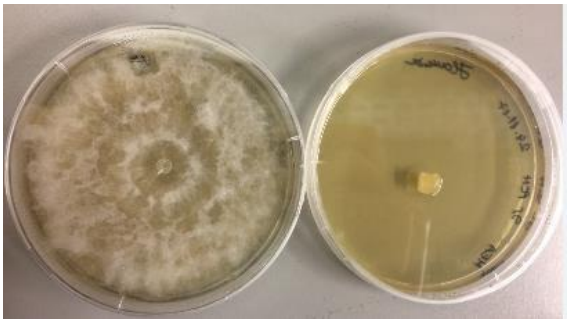


Figure 1.Formation of fresh Petry dishes

2.5 Submerged cultures

Submerged cultivation seems to be very effective in identification of new volatile and flavor compounds formed during biotransformation processes, because of its ability to analyze the product via GC/MS-MS/O methods. For submerged cultivation, precultures have to be formed first, and the procedure for all 14 basidiomycetes is based in protocols used in the previous papers [16,39]. Basidiomycetes like *Bjerkanderaadusta* (BAD), *Wolfiporiacocos* (WCOC), *Phenerochaetechrysosporium* (PCH), *Pleurotussapidus* (PSA), *Botryciscineraea* (BCI), *Stereumhisutum* (SHI-BD), *Mycetinisscorodonius* (MSC), Hypholomafasciculare (HFA), *Mycenapseudocorticola* (MPS), *Pleurotusflabellatus* (PFLA), *Irpexconsoros* (ICO C), *Lentilulaedodes* (LED), *Trametessuaveolens* (TSU) and *Lycoperdonpyriforme* (LYP) were inoculated into Standard Nutrition Solution (SNS) which contains 30 g/L glucose monohydrate, 4.5 g/L asparagine monohydrate x H2O, 1.5 g/L mono potassium phosphate (KH2PO4), 0.5 g/L magnesium sulfate nH2O (MgSO4 × H2O), 3 g/L yeast extract, 1 ml/L trace element solution (CuSO4 × 5 H2O 5 mg/L; FeCl3 × 6 H2O 80 mg/L; MnSO4 × H2O 30 mg/L; ZnSO4 × 7 H2O 90 mg/L; EDTA 0.4 g/L) [33]. In all cases, the pH value of the solution must be 6 by adding sodium hydroxide solution (1M) or hydrogen chloride (1M) prior to sterilization. Medium was autoclaved at 121^0 of Celsius for a period of 20 minutes. After inoculation, erlenmeyer flasks with the solution (250mL / 100mL) were incubated on a rotary shaker in the dark (24^0C, 150rpm, 25mm shaking diameter). When precultures were ready, 10mL of the material was precipitated by centrifugation with 4.000 rpm (2.150xg) for 10 min at 24^0C. For being sure that all SNS is removed, the mycelium was washed three times with sterile water and in the end the pellets were re-suspended again in 10mL of sterile water. This suspension was added into Erlenmeyer flask (250mL) containing 100 mL of hairy roots medium (10g/L). In parallel three blank samples were prepared, 1) plant with water; 2) fungus with minimal medium (MM); 3) fungus with sterile water. The MM solution contains 6.24 g/L mono sodium-L-aspartate × 1 H2O, 3 g/L glucose, 2.4 g/L ammonium nitrate (NH4NO3), 1.5 g/L potassium hydrogen phosphate (KH2PO4), and 1 ml/L trace element solution (CuSO4 × 5 H2O 5 mg/L; FeCl3 × 6 H2O 80 mg/L; MnSO4 × H2O 30 mg/L; ZnSO4 × 7 H2O 90 mg/L; EDTA 0.4 g/L). The fermented product was taken daily for 8 days, in parallel samples were prepared for GC/MS-MS/O analysis and sensory evaluation. Everything was done in triplicity.

2.6 Sensory evaluation of cultures

All 14 basidiomycetes (BAD, PCH, WCOC, PSA, BCI, SHIBD, MSC, HFA, MSP, PFLA ICO C LED TSU and LYP) were screened in submerged cultivation by taking sample for sensory evaluation and for GC/MS-MS/O analysis daily for eight days. Sensory analysis was performed by heating an aliaquot of 5mL culture at 40^0C for a few minutes in a sterile cup. The same analysis was done for three blank samples. Odor intensity was rated from 1 to 4 (1: low intensity of flavor – 4: strong intensity of flavor).

2.7 Headspace solid-phase microextraction (HS-SPME)

HS-SPME seems to be very effective method for determination of volatile and flavor compound. Everything needed when using this method is choosing adequate fiber/fibers. We have focused in two most used fibers DVB/CAR/PDMS and PDMS/DVB and respective chromatograms were compared. Because PDMS/DVB was able to adsorb more compounds, we decided to work with it further. The PDMS/DVB fiber had this performance: film thickness 75 μm, fiber length 1 cm; Supelco, Steinheim, Germany. It works by combining with a GERSTEL MPS 2XL multi-purpose sampler (GERSTEL, Mülheim and der Ruhr, Germany).

After optimization of the method, ten mL sample taken from the submerged cultivation was transformed daily into a sterilized headpace vial (20mL) and capped. Agitation was done for 10 minutes (250rpm) at 40^0C followed by headspace extraction for the next 30 minutes at the same temperature. The analytes adsorbed on the fiber were directly desorbed in the split/splitless inlet (250^0C; SPME liner, 0.75 mm i.d; Supleco) on the GC/MS-MS/O system for 1 min. After, the fiber was moved automatically to the SPME fiber conditioning station and prepared for the next measurement by heated at 250^0C for 20 min.

2.8 Gas chromatography/mass spectrometry-mass spectrometry/olfactometry (GC/MS-MS/O)

Gas chromatography apparatus used for all measurements was from Agilent Technologies, (Waldbronn, Germany) 7890A equipped with an Agilent 7000B triple quadrupole mass spectrometry (MS/MS) detector (Agilent Technologies). A polar Agilent VF-WAXms column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent Technologies) was used for analysis. Helium was used as carrier gas at a constant flow rate of 1.56 ml/min. At the end of the

column, the carrier gas was split 1:1 into the triple quadrupole mass spectrometer and into an olfactory detector port (ODP 3, GERSTEL, Mülheiman der Ruhr, Germany). Temperature of the program 40°C (3 min)/5°C/min to 240°C (5 min); injection temperature 250°C; septum purge flow rate, 3 ml/min; MS modes, scan mode in Q1; scan range *m/z* 33–330; electron ionization energy, 70 eV; source temperature, 230°C; quadrupoles temperature, 150°C; MS/MS transfer line temperature 250°C; He quench gas, 2.25 ml/min; N2 collision gas, 1.5 ml/min; ODP 3 transfer line temperature, 250°C; ODP 3 mixing chamber, 150°C; ODP 3 make up gas, N2. GC-MS/MS analyses were performed using an Agilent 7,890 gas chromatograph from Agilent Technology, equipped with a model 5975C mass spectrometry detector, also from Agilent Technology (carrier gas, helium; constant flow rate, 1.2 ml/min; inlet temperature, 250°C; split ratio, 10:1; septum purge flow rate, 3 ml/min; 30 m x 0.25 mm i.d., 0.25 μm Agilent JandW DB-5MS column; scan range, *m/z 33–330*; electron ionization energy, 70 eV; source temperature, 230°C; quadrupole temperature, 150°C; transfer line temperature, 250°C). The condition used in this research was in accordance with a scientific work conducted by Tang et al.[30].

2.9 Identification of volatile and flavor compounds

Identification of volatile and flavor compounds was performed by calculation of Kovats indices (KI) and their comparison of analytical standards injected at the same apparatus in which all measurements were done , also authentic references of NIST 2011 database were consulted included KI and mass spectra.

3. Results and discussion

As a material into this research, we have used genetically modified roots of *Hypericum perforatum (L.)* [40]. Mostly, the transformations by using specific conditions are preferred to avoid the influence of external factors (temperature, climate changes etc.) on the quality and quantity of secondary metabolites [41].

The main reason why we have been focused on roots of the genetically modified *Hypericum perforatum* (L.) was based on the previous studies [39]. Roots are mostly rich with secondary metabolites like lignin and ability of basidiomycetes in destruction of large molecules can lead in production of small molecules which are potentially flavors.

All volatiles emitted from hairy roots of genetically modified *Hypericum perforatum* (L.) were analyzed by GC/MS-MS and GC/MS-MS/O at 40^0C (3min)/5^0C/min to 240^0C (5min) and after three measurements we were able to find five tentatively identified compounds[42] with very low peak intensity.

We consider that from an initial material (roots of genetically modified *Hypericum perforatum* (L.)) with only five tentatively identified volatile compounds, we have managed biosynthesis and identification of 18 new volatile and flavor compounds by involving WCOC and BAD, together with the assumption of application of fungi mentioned above in organic synthesis, is an achievement in itself.

3.1 Results from surface screening, sensory evaluation and submerged cultivation

The easiest way for selection of effective basidiomycetes is using surface screening by using Petry dishes. The protocol for formation of Petry dishes and their sensory evaluation was described in 2.5 and 2.6. From all 14 basidiomycetes (BAD, PCH, WCOC, PSA, BCI, SHIBD, MSC, HFA, MPS, PFLA ,ICO C, LED, TSU and LYP) here we will be focused on BAD and WCOC.

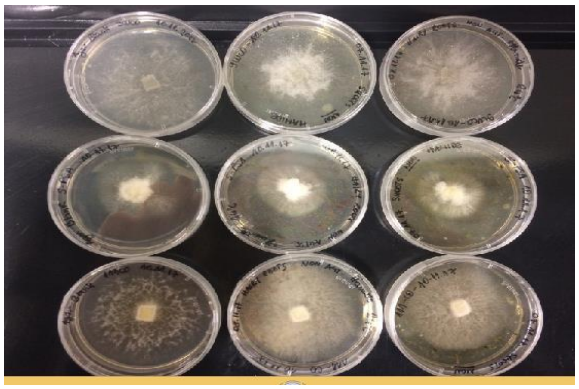


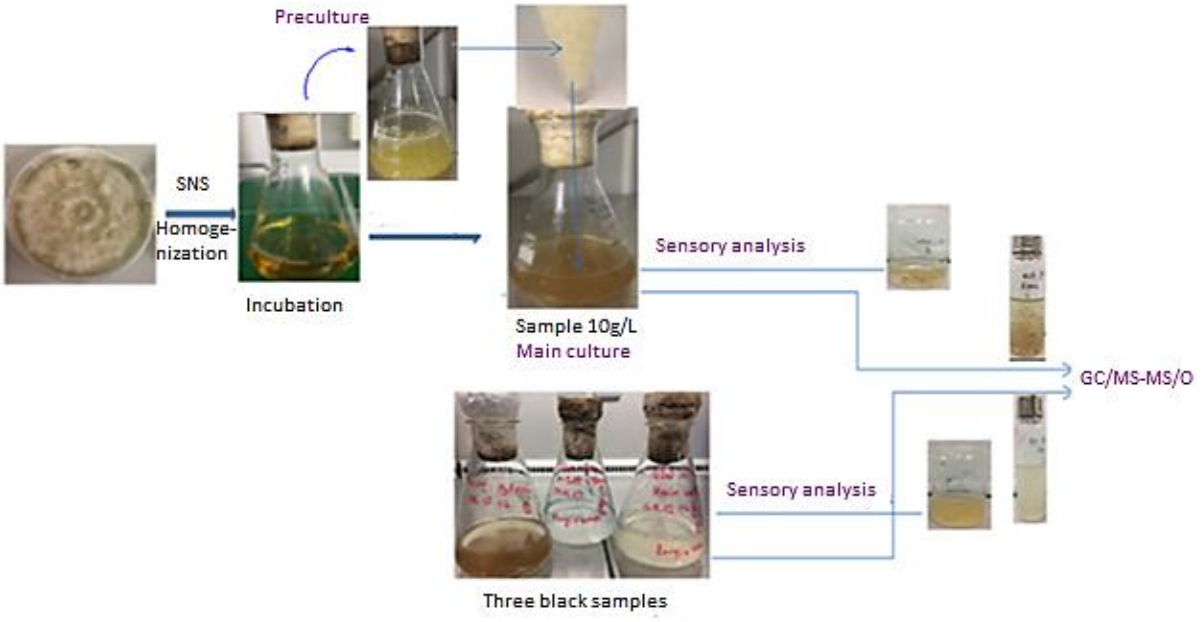
Figure 2.Formation of Petry dishes between roots of genetically modified Hypericum perforatum (L.) and basidiomycetes in the agar-agar 2% solution

Surface screening is very fast method, but because there is no analytical way to determine which compounds are responsible for the aroma of the product, although it is still not a completely satisfying method. In order to be sure that this aroma comes from biotransformation processes, including fungus, and to identify these compounds, we did submerged cultivation, described in 2.5, while sensory evaluation is described in 2.6 as the method for aroma identification.

Submerged cultivation, sensory evaluation and preparation of the samples for measuring on GC/MS-MS/O will figuratively be explained with a scheme written below.

TABLE 1.Selected basidiomycetes from the surface screening and their impression

Basidiomycetes	Impression
Bjerkandera adusta (BAD)	Medicine, fresh, fruit, old things
Wolfiporia cocos (WCOC)	Fresh, orange, green



Scheme1. Submerged cultivation and sensory evaluation of the product taken daily for eight days, including three black samples.

Aroma impression from sensory evaluation mostly was similar to those from submerged cultivation, except differences observed in BAD and WCOC which are the reason for the research to go further. The fresh and fruit aroma which are determined during surface screening when biotransformation was done with BAD, was not observed during sensory evaluation, and fresh and fruit and orange impression aroma determined during surface screening when WCOC was used as a fungi, was not perceived during sensory evaluation.

3.2 Flavor analysis and assumption of chemical reaction coming from certain basidiomycetes

BAD in reaction with roots of genetically modified *Hypericum perforatum* (L.) was able to produce an ester (formic acid, heptyl ester), three aldehydes ((E)-2-octenal), 2,4-decadienal, octanal), two chlorinated compounds (1,3-dichloro-2-methoxybenzene, 3-chloro-4-metoxybenzaldehyde) and two no identified compounds.

Table 2. Identification of new compounds produced by BAD.

Compound[a]	Fermentation[b] time/day	Aroma description[c]	Aroma intensity[d]	KI[e](VFWAXms)	Identification[f]
Formic acid, heptyl ester	2	Odorless	1	1316	MS, KI
(E)-2-Octenal	2, 3, 6, 7, 8	Fresh	2	1394	MS, KI, aroma
2,4-Decadienal	3	Odorless	1	1754	MS, KI
n.i	3	Pleasant	3	1297	Aroma
1,3-Dichloro-2-methoksibenzene	2, 3	Fresh/chlorine	3	1694	MS, KI, aroma
3-Chloro-4-methoksibenzaldehide	2, 3	Odorless	1	2316	MS, KI
Octanal	7	Odorless	1	1288	MS, KI
n.i	3, 4, 5, 6,7, 8	Chlorine/Hygienic preparations	3	2055	Aroma

Abbreviations: 1: no aroma; 2: low intensity; 3: middle intensity; 4: high intensity
Compound[a] = compounds formed de novo or during biotransformation
Fermentation[b]=fermentation time expressed in day
Odor description[c]=defined during olfactory measurements
Odor intensity[d]=1-4 (1: no odor; 4-high intensity)
n.i= (not identified compound)

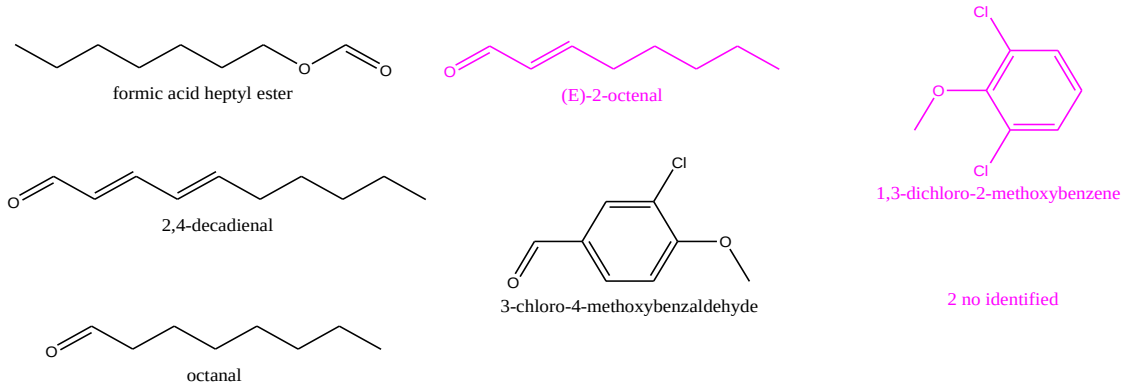


Figure 3.Chemical structure of compounds produced by BAD.

In this research except production of flavor compounds, we were focused also in determination of chemical changes that can be occurred daily between compounds. It was also a reason why biotransformation was followed for eight days. Based on Table 2 and all chromatograms (GC/MS-MS and GC/MS-MS/O) analyzed daily, we observed a correlation between 2,4-decadienal which is produced on the third day of biotransformation and octanal which is produced on the sixth day of biotransformation, when 2,4-decadianal is no longer present. In some previous studies was briefly explained that when white rot fungi are used, alkylation takes place [42,43] and also reduction [19]. Due to our chemical changes and based on the research done in white rot fungi, we propose that when BAD reacts with secondary metabolites present in roots of genetically modified *Hypericum perforatum* (L.), dealkylation and reduction takes place.

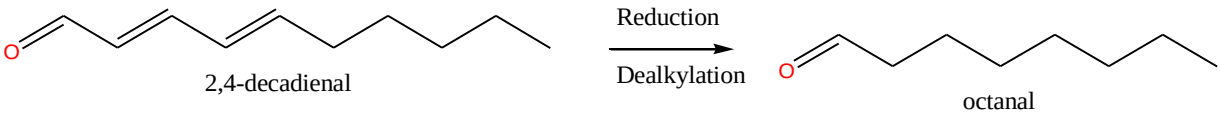


Figure 4.Biotransformation assumed by BAD

WCOC during reaction with roots of genetically modified *Hypericum perforatum* (L.) is able to produce four alcohols (2-nonanol, 3-octanol, 2-decanol and 2-undecanol), two esters (ethyl hexanoate, ethyl octanoate), one monoterpen (D-limonene) and one monoterpenoid (linalool)

Table 3. Identification of new compounds produced from WCOC.

Compound[a]	Fermentation[b] time/day	Aroma description[c]	Aroma intensity[d]	KI[e](VF-WAXms)	Identification[f]
2-Nonanol	4, 5, 6	Odorless	1	1512	MS, KI
3-Octanol	5, 6, 7, 8	Odorless	1	1386	MS, KI
2-Decanol	5, 6, 7, 8	Odorless	1	1613	MS, KI
2-Undecanol	5, 6, 7, 8	Odorless	1	1711	MS, KI
n.i	1	Odorless	1	1634	
Ethyl hexanoate	1, 2	Odorless	1	1230	MS, KI
n.i	1	Undefined	2	1294	Aroma
Ethyl octanoate	1	Fresh-orange	2	1430	MS, KI, aroma
D-Limonen	1, 5	Orange	4	1189	MS, KI, aroma
Linalool	5	Fresh	2	1538	MS, KI, aroma

Abbreviations: 1: no aroma; 2: low intensity; 3: middle intensity; 4: high intensity
Compound[a] = compounds formed de novo or during biotransformation
Fermentation[b]=fermentation time expressed in day
Odor description[c]=defined during olfactory measurements
Odor intensity[d]=1-4 (1: no odor; 4-high intensity), n.i= (not identified compound)

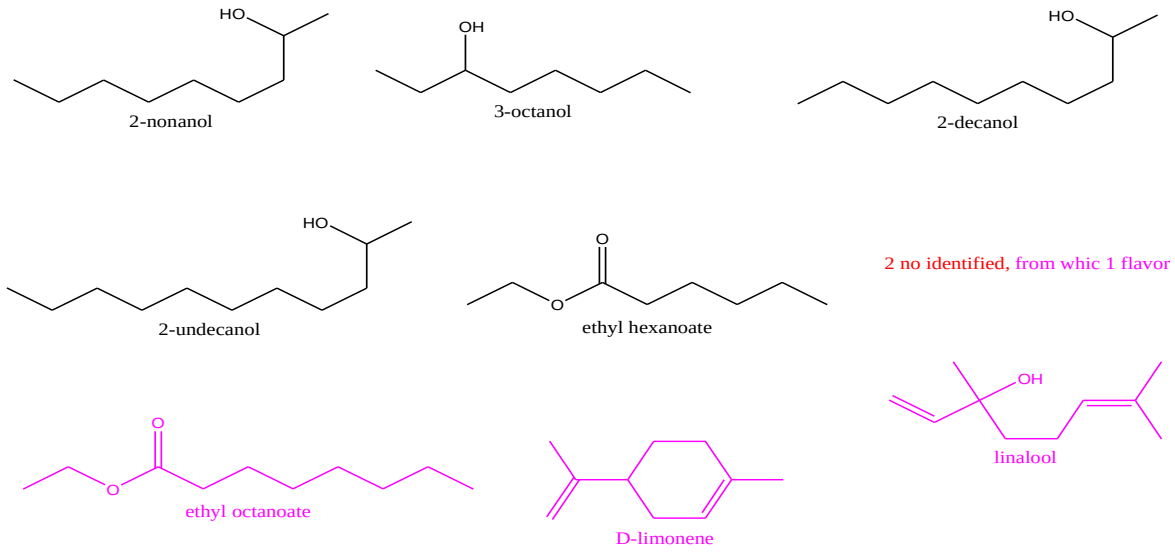


Figure 5.Chemical structure of new compounds produced by WCOC

Based on Table 3 and all chromatograms (GC/MS-MS and GC/MS-MS/O) analyzed daily, we posit that there is a connection between ethyl octanoate which is synthesized on the first day of biotransformation and ethyl hexanoate, for which the maximum of peak intensity is reached on the second day of biotransformation where ethyl octanoate is not still present. Based on some similar research [42,43], we propose that dealcylation takes place here (loosing of ethyl group).

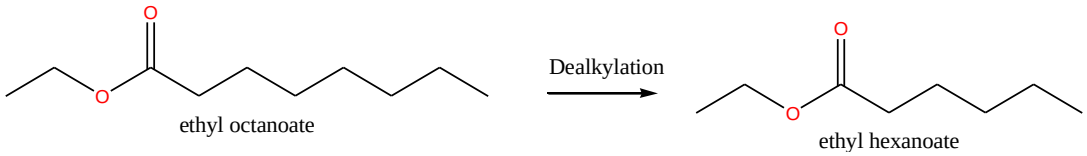


Figure 6.Biotransformation assumed by WCOC

4. Conclusions

When WCOC and BAD react with roots of the genetically modified *Hypericum perforatum* (L.), 18 new volatile compounds are formed, from which 14 were identified by injection of analytical standards and comparing of Kovats Index values together with their mass spectra of the NIST 2011 database (formic acid hepthyl ester, (E)-2-octenal, 2,4-decadienal, 1,3-dichloro-2-methoxybenzene, 3-chloro-4-methoxybenzene, octanal, 2-nonanol, 3-octanol, 2-decanol, 2-undecanol, ethyl hexanoate, ethyl octanoate,D-limonene and linalool, while from 4 no identified compounds, three have shown flavor impression.

Another topic into this research was to see correlation of compounds day by day and proposing of chemical changes happening during biotransformation, which than can be proved by using specific compound and treating them directly with basidiomycetes [44]. To the best of our knowledge there is no paper in which specifically BAD or WCOC are used as dealkylation or reducing agents of organic compounds.

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