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Production of *R*-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium

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Abstract

The use of two agro-residues (liquid cassava waste and orange essential oil) in the biotransformation of *R*-(+)-limonene was investigated. The main components of orange essential oil were determined by GC–MS and *R*-(+)-limonene was shown to be the predominant constituent, accounting for more than 94% of the total content. Cassava wastewater proved to be a suitable substrate for mycelial growth, leading to good, rapid growth with all the fungal strains tested, reaching 29.4 g/l (dry weight) after 3 days of growth (*Penicillium* sp. 2025). The best *R*-(+)-α-terpineol yields were achieved when the strains were grown in cassava media and the mycelia then transferred to a new flask containing mineral medium and orange essential oil as the sole C- and energy source. One of the strains tested, *Fusarium oxysporum* 152B, converted *R*-(+)-limonene to *R*-(+)-α-terpineol, yielding nearly 450 mg/l after 3 days of transformation. Growth in the presence of a solution of 1% orange essential oil in decane did not increase the transformation yields.

Keywords: Biotransformation; Industrial residues; *R*-(+)-limonene; *R*-(+)-α-terpineol; *Fusarium oxysporum*

1. Introduction

In recent years, there has been an increasing trend towards efficient utilization and value addition of agro-industrial residues such as coffee pulp and husk, cassava bagasse, cassava flour wastewater (manipueira), cassava sugar cane bagasse, sugar beet pulp and apple pomace, as well as many essential oils. On one hand, the application of agro-industrial residues in bioprocesses provides alternative substrates, and on the other hand it helps to solve pollution problems. With the advent of biotechnological innovations, many new possibilities are emerging (Pandey et al., 2000).

Liquid cassava waste (manipueira), arises from the pressing of cassava roots (generated at the rate of 250 l/ton of cassava) (Damasceno, Cereda, & Pastore, 1999) and from one point of view can be considered as a "harmful" (Vieites, 1998) pollutant waste, due to its high organic content and the presence of cyanide. From the other point of view, it can be considered as a residue rich in nutrients, and can therefore be used for other applications. Amongst recent studies, the production of biosurfactant by a wild-type *Bacillus* sp. (Nitschke & Pastore, 2003), production of fruit flavour by *Geotrichum fragrans* in manipueira (Damasceno et al., 1999) and the production of citric acid by *Aspergillus niger* (Leonel & Cereda, 1995), can be mentioned.

Essential oils are dominated by monoterpene hydrocarbons, which are regarded as process waste, mainly because of their low sensory activity, low water solubility and tendency to autoxidise and polymerise (Berger, Krings, & Zorn, 2002), which turns terpene hydrocarbons, such as limonenes, pinenes and terpinenes, into ideal starting materials for microbial transformations. Furthermore, *R*-(+)-limonene is a popular starting product for bioconversions because of its widespread and cheap availablity (Adams,

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Demyttenaere, & De Kimpe, 2003). "Natural" flavours can be produced via biotransformation or bioconversion, adhering to consumer demand for natural products (Berger, 1995; Carvalho & Fonseca, 2002).

An interesting end product resulting from the bioconversion of limonene is the monoterpene alcohol α-terpineol. Bioconversion of limonene to *R*-(+)-α-terpineol has been described, using a wide range of microorganisms as catalysts: a *Cladosporium* strain (Kraidman, Mukherjee, & Hill, 1969), *Penicillium digitatum* DSM 62840 (Abraham, Stumpf, & Kieslich, 1986), *Pseudomonas gladioli* (Cadwallader & Braddock, 1992); *Aspergillus cellulosae* M-77 (Noma, Yamasaki, & Asakawa, 1992), *Bacillus stearothermophillus* BR388 (Chang, Gage, & Oriel, 1994), *Pseudomonas aeruginosa* (Acosta, Mazas, Mejias, & Pino, 1996), *Escherichia coli* (expressing a limonene degradation pathway from *Bacillus stearothermophillus* BR388) (Chang, Gage, & Oriel, 1995), *Penicillium digitatum* (Adams et al., 2003; Tan & Day, 1998a, 1998b; Tan, Day, & Cadwallader, 1998).

R-(+)-α-terpineol has a floral, typically lilac odour, while (*S*)-(−)-α-terpineol has a coniferous odour characteristic. α-Terpineol one of the most commonly used fragrance compounds. It is mostly produced chemically and is thus commercially available at a relatively low price. A great advantage of enzymatic processes as compared to chemical synthesis is enantiospecificity. However, terpene transformations in general suffer from the volatility of the substrate and from the toxicity of terpenes towards microorganisms (Onken & Berger, 1999).

This study reports the use of two relevant brasilian industrial wastes (orange essential oil and cassava waste water) in a biotechnological process. The biotransformation of limonene (present in great amounts in the essential oil) as the sole C-source by a *Fusarium oysporum* strain developed in cassava waste water, was also investigated. The use of inexpensive raw materials represent higher possibilities to applicate such processes in larger scales.

2. Materials and methods

2.1. Agro-industrial residues

The industrial residues, orange essential oil and manipueira (cassava water flour) were obtained, respectively, from an orange juice industry and from a cassava flour plant, both situated in the State of Sao Paulo, Brazil.

2.2. Chemicals

R-(+)-α-terpineol (Fluka, ∼99%), *S*-(−)-α -terpineol (Fluka, ∼99%), *R*-(+)-limonene (Aldrich, ∼99%), *S*-(−)-limonene (Aldrich, ∼96%) were purchased from the Aldrich Chemical Company. All the chemicals and solvents were of the best available commercial grade.

2.3. Microorganisms and cultivation

The strains *Penicillium* sp. 2025, *Aspergillus* sp. 2038, and *Fusarium oxysporum* 152B from the Bioflavours Laboratory Culture collection, were cultivated and conserved by periodic replications (once a week) on Yeast-Malt agar (YM: bacteriological peptone 0.5%, glucose 1.0%, malt extract 0.3%, yeast extract 0.3%, and agar 2.0%, pH 6.0).

2.4. Cassava medium preparation

Cassava effluent obtained from the manufacture of cassava flour was collected and stored at −18 °C until needed. The medium was prepared by heating the waste to boiling to remove solids. After cooling, the substrate was centrifuged at 10,000*g* for 20 min. The supernatant was sterilized (121 °C for 20 min). The resulting pH prior to autoclaving was 5.3 and was not adjusted.

2.5. Total carbohydrates analyses in cassava medium

Total carbohydrates were determined by a colorimetric method based on Phenol reaction with glucose (Daniels, Hanson, & Phyllips, 1994).

2.6. Determination of reducing sugars contents in cassava medium

Total reducing sugars were quantified according to Somogy (1945).

2.7. Determination of nitrogen contents in cassava medium

Protein content was calculated from nitrogen determination by the Kjeldahl procedure using 6.25 as conversion factor (AOAC, 1995).

2.8. Determination of minerals contents in cassava medium

One millilitre of cassava medium was "incinerated" for 3 h at 500 °C. 5.0 ml of HCl 6 M was added and evaporated. The solid was transferred to a volumetric balloon of 50 ml and filtered. The minerals were determined by a Atomic Emission Spectrophotometer (Jobin Yvon, model JY 50P). The spectral lines for each element were (in nm): P, 178.2; K, 766.5, Ca, 317.9; Mg, 279.9; Mn, 257.6; Fe, 259.9; Cu, 324.7; Zn, 213.8; and S, 180.6.

2.9. Analysis of chemical oxygen demand (COD) of cassava waste water and total solids

Chemical oxygen demand (COD) and total Solids analysis of cassava waste water were done according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

2.10. Mineral medium composition

The composition was 0.5 g of $MgSO_4$; 3.0 g of $NaNO_3$; 1.0 g of K_2HPO_4; 0.5 g KCl; 0.01 g of Fe_2SO_4 in 1.0 l of distilled water (Brunerie, Benda, Bock, & Schreier, 1987). The medium was sterilized at 121 °C for 20 min. The pH was 7.0 and was not adjusted.

2.11. Biotransformation experiments

Biotransformation experiments with all the strains and media were run for 7 days (all in duplicate). The fungi were cultivated in 250 ml conical flasks containing 50 ml of liquid medium.

2.11.1. Biotransformation experiments in cassava medium (CM) and mineral medium (MM)

Fifty millilitres of experimental culture were inoculated with 2.5 ml of an aqueous spore ($1–7 \times 10^7$ spore ml^{-1}) suspension and incubated in a rotary shaker at 160 rpm and 30 °C. After 3 days, biotransformation was initiated with the addition of 50 μl of orange essential oil (under sterile conditions). Two subsequent additions of 50 μl every 24 h, were performed. Incubation was continued for two more days after the last addition.

2.11.2. Biotransformation experiments in two steps

Fifty millilitres of CM culture were inoculated with 2.5 ml of aqueous spore ($1–7 \times 10^7$ spore ml^{-1}) suspension and incubated in a rotary shaker at 160 rpm and 30 °C. After 3 days, the culture was filtered through Whatman filters under sterile conditions. The mycelia were washed three times with sterile (121 °C for 20 min) distilled water and transferred to another flask containing 50 ml of mineral medium. Biotransformation was started by the addition of 50 μl of orange essential oil (under sterile conditions). Two subsequent additions of 50 μl were made every 24 h. The experiments were continued for two more days after the last addition.

In the same way, chemical blanks at the same final pH values (the pH adjustments were done with HCl prior to autoclaving) as the biotransformation experiments, were performed, but without mycelium, to ensure the absence of chemical transformation reactions. Twenty-four, 48, 72 and 96 h after the addition of orange essential oil, 5-ml samples were taken and extracted with 5 ml of diethyl ether. The samples were analysed directly by gas chromatography/flame ionisation detection (GC/FID) (decane was the internal standard).

2.12. Fusarium oxyzporum grown in the presence of orange essential oil in decane

A 200 μl portion of an aqueous cell suspension was spread on a Yeast-Malt agar slant. After cultivation for 2 days, 10 ml of a 0.1% solution of orange essential oil in decane was added to the tubes and incubated at 30 °C for 5 days without stirring (Oda, Sugai, & Ohta, 1999).

A portion of the suspension (2.5 ml) was then inoculated in a conical flask containing 50 ml cassava medium. The biotransformation experiments were conducted in the same way as described in Section 2.11.2. Camphor was the internal standard.

2.13. Analysis of the samples by GC and gas chromatography/mass spectrometry (GC–MS)

GC analyses of the biotransformation products were performed with a Chrompack CP9001 gas chromatograph, equipped with a split/splitless injector, an FID and a fused silica capillary column. The stationary phase was CP-Sil CB (60 m length × 0.25 mm i.d.; coating thickness of 0.25 μm). The working conditions were: injector 220 °C, detector 250 °C (carrier gas He at 1 ml/min). The oven temperature was programmed from 40 to 210 at 5 °C/min, with an initial holding time of 1 min and a final holding time of 5 min. Quantification was performed by calibration according to the internal standard decane or camphor. Chiral GC analyses were performed with the same GC, equipped with a Beta Dex 120 (SUPELCO) chiral column (60 m × 0.25 mm i.d., coating thickness 0.25 μm). The working conditions were: injector 220 °C, detector 250 °C (carrier gas He at 1 ml/min). The oven temperature was programmed from 110 to 170 °C at 1 °C/min, with an initial holding time of 10 min and a final holding time of 5 min.

GC–MS analyses were performed using a Varian Saturn gas chromatograph equipped with an EM-IT mass selective detector, a CP-Sil 8CB Low bleed/MS capillary column (30 m × 0.25 mm × 0.25μm) and split/splitless CP1177 injector. The working conditions were: injector 280 °C, transfer line to MSD 260 °C. The oven temperature was programmed from 40 °C (1 min) to 160 °C (3 min) at 5 °C/min and from 160 to 250 °C with a final holding time of 5 min, with a scan range of m/z 40–500, carrier gas (He) at 1 ml/min; split ratio 1/50; ionisation electron impact (EI) at 70 eV; scan rate, 1 s^{-1}.

Positive identifications were made by matching sample retention indices (RI) and mass spectra of the samples with those of the standards, analysed under identical conditions.

3. Results and discussion

3.1. Orange essential oil composition

The orange essential oil used in this study was analysed by GC–MS. The compounds identified and their percentages are shown in Table 1. Ten compounds, representing 99% of the GC profile, were identified, but other minor compounds could not be identified. Compounds with concentrations below 0.1% were not considered. The major constituents of the oil were limonene, myrcene, carvone and carveol. Some oxidation compounds, such as carvone,

Table 1
Composition of orange essential oil

Compound	%
α-Pinene	0.53
Myrcene	1.21
Terpinene	0.20
Limonene	94.29
Dihydromyrcene	0.55
Unknown	0.26
trans-p-2,8-Menthadien-1-ol	0.10
Carveol	0.72
Carvone	1.12
Limonene oxide	0.15
(*Z*)-citral	0.17
Unknown	0.10

carveol and limonene oxide, were identified. These were taken into account in the biotransformation analyses and were recovered mainly in the chemical blank (medium with orange essential oil, but without the culture). The chromatographic profiles obtained from the biotransformation experiments were compared to those from the diluted orange essential oil (to confirm that the α-terpineol was really a biotransformation product and did not come from the oil) and to those from the chemical blanks (to ensure that the α-terpineol was not chemically transformed).

3.2. Composition of cassava wastewater (manipueira)

Manipueira is a residue generated in great amounts during the manufacture of cassava flour, a very common ingredient in Brazilian cookery. The major nutrients present in cassava waste are sugars (sucrose, glucose, fructose and maltose), nitrogen and mineral salts. Although disposal of this residue is a problem due to its high organic load, it is also a very attractive substrate for biotechnological processes. Economic aspects are very important for an industrial process once the raw material represents 30% of the total costs of biotechnological processes (Cameotra & Makkar, 1998). The composition of the manipueira used in this study is shown in Table 2.

3.3. Manipueira as a bioconversion medium

The cassava medium has a high content of organic material and minerals. On the other hand, manipueira also contains high contents of substances that could limit the growth or inhibit the capacity of biotransformation, such as cyanide. The cassava medium (CM) was tested in the bioconversion of *R*-(+)-limonene by the strains *Penicillium* sp. 2025, *Aspergillus* sp. 2038, *Fusarium oxysporum* 152B. The appearance of the shaken-flask cultures was evaluated. The cassava medium was shown to be an excellent medium for the growth of all the fungal strains. The dry matter obtained is shown in Table 3. It was concluded that manipueira was an excellent medium for biomass production and was an appropriate biotransformation medium for *Fusarium oxysporum*.

Table 2
Composition of the Manipueira used in this work compared with Nitschke and Pastore (2003) and Damasceno et al. (1999)

Components	Concentration		
	This study	Nitschke (2003)	Damasceno (1999)
Total solids (g/l)	60.0	62.00	62.0
COD (g/l)[a]	53.4	55.8	60.0
Total sugars (g/l)	39.5	41.45	58.18
Non-reducing sugars (g/l)	20.1	23.3	20.2
Reducing sugars (g/l)	19.4	18.3	38.0
Total Nitrogen (g/l)	1.72	2.08	1.60
Phosphorous (mg/l)	369	245	83.3
Potassium (mg/l)	3640	3472	895
Calcium (mg/l)	236	293	184
Magnesium (mg/l)	438	519	173
Sulphur (mg/l)	61.4	154	38.0
Iron (mg/l)	2.72	7.80	8.00
Zinc (mg/l)	3.01	2.80	4.50
Manganese (mg/l)	3.46	1.70	1.50
Copper (mg/l)	1.11	1.00	0.75
pH (mg/l)	5.3	5.8	5.5

[a] Chemical oxygen demand.

Table 3
Amounts of α-terpineol, residual non-converted limonene after the biotransformation experiments, final pH and dry matter[e]

Strain	Medium	α-Terpineol (mg/l)	Limonene[a] (mg/l)	pH	Dry matter (g/l)
2025	CM[b]	0.0	4.8	3.8	29.1
2025	MM[c]	7.4	2.3	6.7	–[f]
2025	CM + MM[d]	15.9	1.3	6.5	29.4
2038	CM	15.2	5.2	3.7	28.7
2038	MM	5.5	1.1	6.6	–[f]
2038	CM + MM	23.2	1.4	5.8	27.9
152B	CM	204.5	5.3	6.1	24.5
152B	MM	6.1	1.6	6.3	–[f]
152B	CM + MM	448.7	0.9	6.0	23.5

[a] Concentration of residual limonene in the medium.
[b] CM (Cassava medium).
[c] MM (Mineral medium).
[d] CM + MM (Cassava medium + Mineral medium). The strains were grown in cassava medium and then transferred to the mineral medium – biotransformation started on the addition of orange essential oil.
[e] The values in table 3 are the averages of the final concentrations (duplicate), and were obtained after 7 days of experiments.
[f] The mycelium did not develop, only spores remaining in the medium.

3.4. Comparison of the strains and media

The three strains were compared using the different media, designated as CM, MM and CM + MM for biotransfomation (see Section 2). The concentrations of *R*-(+)-α-terpineol and the residual limonene (from the orange essential oil), and the final broth pH, are shown in Table 3. It is clear that of the strains tested, the strain *Fusarium oxysporum* 152B produced the best yields. The concentration of non-converted limonene was very small in all cases. Losses can be explained on the basis of volatility. Differences with respect to growth and bioconversion were observed. The mineral medium (MM) had no carbon or

nitrogen sources, and did not therefore allow for the development of cellular mass, but a small amount of *R*-(+)-α-terpineol was produced by all the strains, indicating spore activity. The influence of the medium was different for each strain tested and it was not possible to establish a rule, but for all the strains the *R*-(+)-α-terpineol concentration was much higher when the mycelium developed in CM was transferred to MM, and the bioconversion occurred with orange essential oil as the sole carbon source.

The degradation of limonene via the perillyl alcohol route by *Pseudomonas putida* PL has been reported (Dhavalikar & Bhattacharyya, 1966; Duetz, Bouwmeester, van Beilen, & Witholt, 2003), and the authors also reported that limonene was used as the sole carbon and energy source. Thus it can be concluded that the medium has a great influence on bioconversion activity, and the presence of limonene as the sole carbon and energy source in the presence of developed mycelia increases the biotransformation yields.

R-(+)-α-terpineol was not the only product. Small concentrations of perillyl alcohol could be detected. The strain 2025 produced perillyl alcohol in very low concentrations (approximately 1.4 mg/l), after 48 h in the presence of cassava medium (data not shown). The strain 152B also produced perillyl alcohol. The concentrations reached up to 10 mg/l after 96 h of biotransformation, when the fungi developed in the cassava medium were transferred to the mineral medium (Fig. 1).

3.5. Influence on strain growth of the presence of a solution of orange essential oil in decane

The parameter expressing the toxicity of a solvent for microrganisms is the $\log P_{OW}$ (logarithm of the partition coefficient of the substance in the *n*-octanol/water system). Greatest toxicities were observed for solvents with a log-P_{OW} between 1 and 5 (Onken & Berger, 1999). Thus limonene ($\log P_{OW} = 4.8$) is considered toxic for microrganisms. On the other hand, compounds like decane ($\log P_{OW} = 6.0$) are less toxic and can supply oxygen to the cells, due to the high solubility of the gas in the solvent. The effect of co-solvents in biotransformations/bioconversions has already been studied (Adams et al., 2003; Tan & Day, 1998b). In the present study, a 0.1% solution of orange essential oil in decane was used in an attempt to induce the limonene-converting enzymatic system in the 152B strain, which was put in contact with limonene after 2 days of growth in slants. Some authors reported the use of an interface bioreactor as a way of alleviating toxicity (Oda, Inada, Kato, Matsudomi, & Ohta, 1995;Oda & Ohta, 1992; Oda et al., 1999). The toxicity of the limonene at the growth interface (in which the strain was developed) was somewhat alleviated by the presence of the solvent decane. Aliquots of 200 μl were withdrawn aseptically from the slants and analysed by GC, to confirm that *R*-(+)-α-terpineol was not produced in significant amounts during the induction time, only trace amounts being produced.

Growth in the presence of a solution of orange essential oil in decane was tested with the *Fusarium oxysporum* strain, and the results for yields of *R*-(+)-α-terpineol and amounts of non-converted limonene are shown in Fig. 2. The inhibition caused by limonene was evidenced by a comparison of the cellular mass obtained in cassava medium without orange essential oil (reaching 32.3 g/l of cellular mass), with 0.1% of the oil (23.5 g/l of cellular mass), and 0.1% of oil in the presence of decane (27.0 g/l). By this data analysis, it can be concluded that decane helps to alleviate the toxicity caused by limonene.

The strain grown in the presence of orange essential oil in decane produced similar yields of *R*-(+)-α-terpineol to that grown in the absence of the solvent. Higher yields were

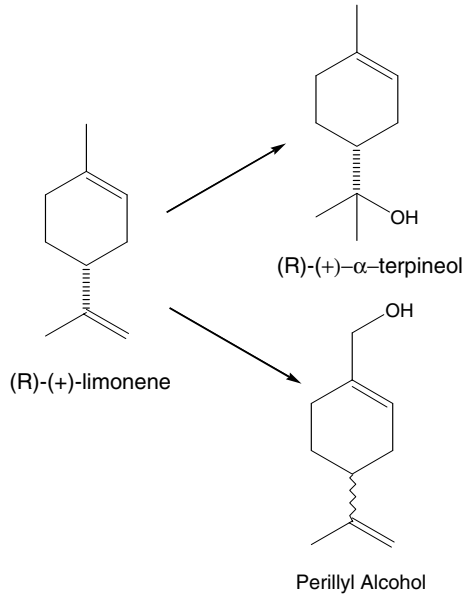

Fig. 1. Biotransformation of (*R*)-(+)-limonene by the *Fusarium oxysporum* strain.

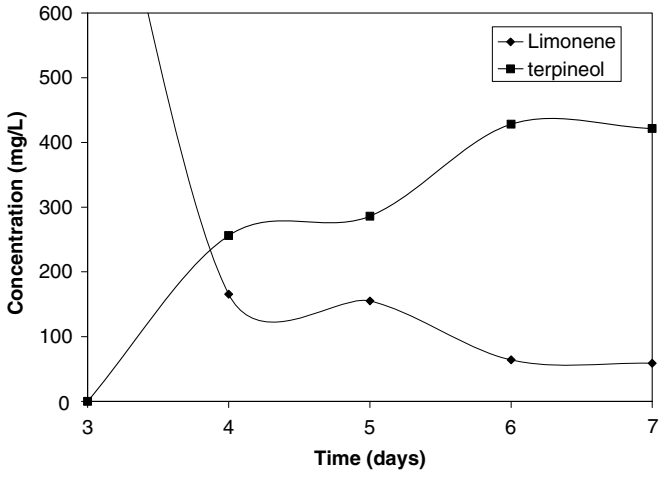

Fig. 2. Biotransformation of limonene by induced *Fusarium oxysporum* strain in the presence of decane, developed in cassava waste water and transferred into mineral medium.

expected due to the induction promoted by the addition of limonene, and the consequent fungal growth in the presence of the substrate, orange essential oil.

The enzyme system converting limonene to α-terpineol was shown to be inducible in *Penicillium digitatum* (Tan et al., 1998). The authors reported that when small amounts of limonene (200 ppm) were spiked into growing cell cultures, a strong corresponding rise in α-terpineol production was observed. Despite having a suitable log P_{OW}, the fungus was inhibited by the high solvent concentration. Unfortunately, in this experiment, the production of *R*-(+)-α-terpineol was not higher in the induction experiments.

4. Conclusions

In this study we showed great growth of the fungi tested in the cassava medium. The biotransformation of orange essential oil was investigated and the production of α-terpineol as the main product was observed. Production of α-terpineol was higher (2-fold for the *Fusarium oxysporum* 152B strain) when orange essential oil was the sole source of carbon and energy, as compared to experiments with a transformation medium including other carbon and nitrogen sources (bioconversion in the presence of cassava medium). Induction with the substrate in solvent did not enhance the yields of α-terpineol. To date, there are no references relating the use of manipueira as a medium for the biotransformation of terpenes.

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