Australian Journal of Basic and Applied Sciences
2018 December; 12(12): pages 51-54
DOI: 10.22587/ajbas.2018.12.12.9
Original paper
Journal home page: www.ajbasweb.com
AENSI Publications

Carrot Roots as Clean and Sustainable Biocatalyst for Obtaining Natural Menthol

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Received date: 15 November 2018, **Accepted date:** 20 December 2018, **Online date:** 31 December 2018

Abstract

Biotransformation using whole plant cells is an area of green chemistry that has the purpose of minimizing impacts to the environment, coupled with the characteristics of high selectivity, chemo, regio and stereoselective reactions. The objective of the present work is the sustainable synthesis of menthol by biocatalysis through the bioreduction reaction of prochiral ketones present in the EOMP (Essential oil of *Mentha piperita* L.) mediated by reductase enzymes contained in carrot cells (*Daucus carota*). The substrates and the carrot biomass were taken to the Orbital Shaker at 35 Cº, 150 rpm, where they remained for 72 hours. At each 24-hour, 2 ml aliquots of the erlenmeyers were collected for reaction follow-up, the aliquots were transferred into test tubes and mixed with 1 mL of ethyl acetate (EtOAc). The organic fraction was analyzed by thin layer chromatography (TLC) and gas chromatography coupled to mass spectrometry (GC/MS). After the *D. carota* bioreduction reaction, the menthol contained in the essential oil of *Mentha piperita* L. raised from 46 to 82% by bioreduction of the carbonyl monoterpenes naturally present in the essential oil of *Mentha piperita* L., demonstrating the capacity of sustainable and efficient biotransformation of *D. carota* even in a complex matrix such as EOMP.

Key words: Bioreduction; *Daucus carota*; essential oil; green chemistry; *Mentha piperita L.*

INTRODUCTION

Since the end of the 1950s, there has been a great increase in environmental legislation, making it more severe to developed countries in terms of pollution of air, water and hazardous waste (Kang, 2010). This new scenario has brought about major changes in the industrial sector, especially in the chemical sector. All these rigorous implications and regulations have boosted the emergence of a new area of chemistry in order to minimize impacts to the environment, this area is known as Green Chemistry (Anastas and Warner, 1998; Farias and Fávaro, 2011).

Within the Green Chemistry methodologies, it's possible to emphasize the biocatalysis that can be defined in general as the use of enzymes, whole cells of microorganisms or plants (Vandenberghe et al., 2013). These enzymes sources are biocatalysts in organic synthesis, a very desirable use due to the attractive characteristics of biocatalysis, such as its high selectivity, which can be chemo, regio and stereoselective providing the reduction or elimination of the use of protective groups and simplifying separation processes that can be performed under mild conditions of temperature and pressure, with consequent reduction of costs and waste (Carvalho, 2011; Clouthier and Pelletier, 2012)

Among the various biocatalytic reactions we can highlight the bioreductions of prochiral carbonyl groups, these reactions generate chiral alcohols, which are start materials widely used in the synthesis of drugs, flavorings, essences and agricultural products (Ni and Xu, 2012; Rowan, 2013). The chiral alcohol menthol is the major compound followed by its carbonyl derivatives of the essential oil of the plant *Mentha piperita* L., popularly known as mint. The economic importance of menthol is linked to several industrial sectors due to its cooling effect and refreshing flavor, being an important input in the production of medicines, beverages flavoring, food, oral hygiene products and tobacco (Saharkhiz et al., 2012).

Obtaining natural menthol to meet industrial demand requires the cultivation of hundreds of thousands of acres of mint and its leaves also need to undergo expensive processes of steam distillation and filtration. It is also possible to obtain menthol by synthetic methods, however, in most cases there is the formation of its undesirable isomers and this will greatly increasing the cost of the process due to separation methods (Brady et al., 2012; Toogood et al., 2015). A more practical, economic and environmental alternative in obtaining menthol would be biocatalytic bioreduction with pieces of carrot (*Daucus carota*), without the need

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for aseptic handling conditions or buffered media, the cost of carrots is negligible and are widely available, these characteristics added to the efficiency and selectivity of thes reaction make it feasible and attractive (Kazici, Bayraktar and Mehmetoglu, 2016; Omori et al., 2016).
In this context, the present work proposes a sustainable alternative for the synthesis of menthol using *Daucus carota* in the bioreduction of carbonyl monoterpenoids present in the essential oil of *Mentha piperita* L. (EOMP).

MATERIAL AND METHODS

Chemical reagents

All the solvents used were purchased from Synth®, the substrate acetophenone was obtained from Vetec®, the essential oil of *Mentha piperita* L. was obtained from Química Moderna®, and sodium borohydride ($NaBH_4$) was obtained from Nuclear®.

Chemical reduction of acetophenone

To monitor the bioreduction reaction of acetophenone it was necessary to obtain the product 1-phenylethanol by chemical reduction. For this reaction 0.1 mL of acetophenone was added to a 15 mL flask containing magnetic bar, then 5.0 mL of methanol was added and kept under stirring at 0 ºC. Thereafter, a small amount of sodium borohydride was added ($NaBH_4$) gradually to the total of 0,1 g in the reaction medium, stirring for 60 minutes. After this time, 20 mL of saturated ammonium chloride solution (NH_4Cl) was added. The extraction was performed through a separatory funnel using two 20 mL portions of dichloromethane (CH_2Cl_2) and adding anhydrous sodium sulfate (Na_2SO_4), followed by filtration of the organic phase. The solvent was evaporated under vacuum on a rotary evaporator. In this way, the racemic 1-phenylethanol standard was obtained, whose formation was characterized by Thin Layer Chromatography (TLC) and gas chromatography coupled to mass spectrometry (GC/MS) (Carey and Sundberg, 2008).

Procedure for bioreduction

Fresh carrots (*Daucus carota*) were obtained in a local market, washed in running water, cut into slices around 0.5 cm and immersed in 1% sodium hypochlorite solution. Subsequently, they were weighed into a 125 ml Erlenmeyer flask on an analytical scale until the required amounts of the vegetable were obtained, followed by the addition of 40 ml of sterile water and the respective substrates (Xu, 2010; Omori, Portas, Oliveira, 2012).

Soon after the erlenmeyers containing the substrates and the carrot biomass were taken to the Orbital Shaker at 35 Cº, 150 rpm, where they remained for 72 hours. At each 24-hour incubation, 2 ml aliquots of the erlenmeyers were collected for reaction follow-up, the aliquots were transferred into test tubes and mixed with 1 mL of ethyl acetate (EtOAc). All handling was done aseptically. The organic fraction was analyzed by thin layer chromatography (TLC).

After the completion of the reaction, the liquid phase was transferred to a 125 mL separatory funnel and extracted ethyl acetate (2 x 10 mL). The organic phase was dried by adding anhydrous sodium sulfate (Na_2SO_4), filtered and the solvent was evaporated under vacuum on a rotary evaporator.

Chromatographic Analyzes

Monitoring of the biocatalysis reactions and monitoring the purification of the product were performed by analytical TLC using silica gel coated silica gel chromate plates with UV_{254} nm fluorescence indicator (Macherey-Nagel) under elution with ethyl acetate and hexane in the ratio 1: 4 as mobile phase for acetophenone, ethyl acetate and toluene 1: 9 mobile phase for EOMP. The development of TLC compounds was performed by immersing the plates in a solution of *p*-anisaldehyde (*p*-anisaldehyde, H_2SO_4, acetic acid and ethanol in the ratio of 1: 2: 1: 100 v/v/v/v) and subsequent heating at 300 °C with a heater gun (Armarego and Chai, 2009).

GC/MS analyzes were performed using an Agilent 7890B chromatograph coupled to the Agilent 5977A MSD mass spectrometer, operating with an electron source with ionization energy of 70 eV. An HP-5MS IU capillary column (30m x 0.25mm x 0.250mm) filled with stationary phase composed of 5% phenyl and 95% dimethylpolysiloxane. The injected volume of the appropriately diluted samples was 2 µL and the programmed conditions were: oven temperature at 100 ^oC and increased 3 $^oC\ min^{-1}$ to 180 ^oC, after 180 ^oC the temperature was increased by 60 $^oC\ min^{-1}$ to 280 ^oC remaining for 2 minutes, totaling the analysis time of 30.3 minutes. The mode of injection of the samples was split at the 1:20 ratio with a constant flow of 1.0 mL min^{-1} of Helium as the entrainment gas. The injector temperature and the transfer line were 230 ^oC and 250 ^oC, respectively. In the mass detector, the temperature of the ionization chamber was 230 ^oC and the quadrupole temperature was 150 ^oC. In the mass spectrometer the MS detection system was used in the scan mode operating in the mass/ charge ratio *m/z* 35 - 250, with solvent delay of 3 min. Compounds were identified by comparing their mass spectra with mass spectra of NIST 11.0 library and concentrations were obtained by relative area analysis. (Gören et al., 2002; Adams, 2007; Jesus, Nogueira and Fonseca, 2013).

RESULTS

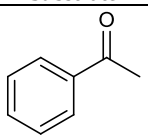
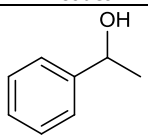
Bioreduction of acetophenone

To determine the conditions the biocatalysis samples were divided as A,B,C being A (20 µL of substrate and 10 g of carrot biomass), B (30 µL and 10 g of biomass) and C (20 µL and 15 g of biomass).

Table 1 shows the percentage ratio of acetophenone bioreduction product, 1-phenylethanol, mediated by *Daucus carota* pieces in a period of 72 h. The results of the conversions were confirmed by the calculation of the relative area of the chromatograms.

The tests with biomass variation and substrate concentration showed that in test A the percentage yield was 98% while tests B and C were above 96% (Table 1).

Table 1: Acetophenone bioreduction by *Daucus carota*

Identification	Substrate	Product	Final Product Yeld %		
Acetophenone			A 98,0%	B 96,4%	C 93,0%

Analysis method: GC/MS

Bioreduction of Essential Oil of *Mentha piperita* L. (EOMP)

Initially the EOMP was analyzed by GC/MS to determine the menthol content present in the sample and to characterize the monoterpenes that could be reduced to menthol. In the crude sample of EOMP the ketone monoterpenoids were found: menthone at retention time of 5.1 minutes, isomenthone in 5.3 minutes, pulegone in 6.8 minutes, piperithone in 7.2 minutes and menthol in 5.5 minutes, with a content of 46,47% (Figure 1).

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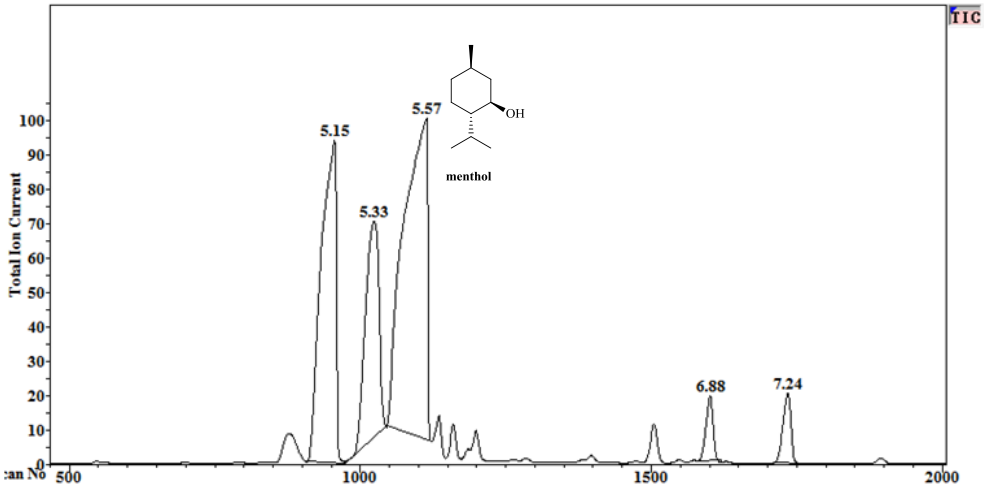


Figure 1: Chromatogram obtained by GC/MS from the crude sample of EOMP.

After 72 h period of incubation of the EOMP substrate with *Daucus carota* biomass, the sample was extracted and analyzed by GC/MS. The results showed a percentage drop of ketone monoterpenoid compounds, more evident in the menthone and isomenthone compounds with an increase of percentage of the menthol compound to 82% (Figure 2 and Table 2).

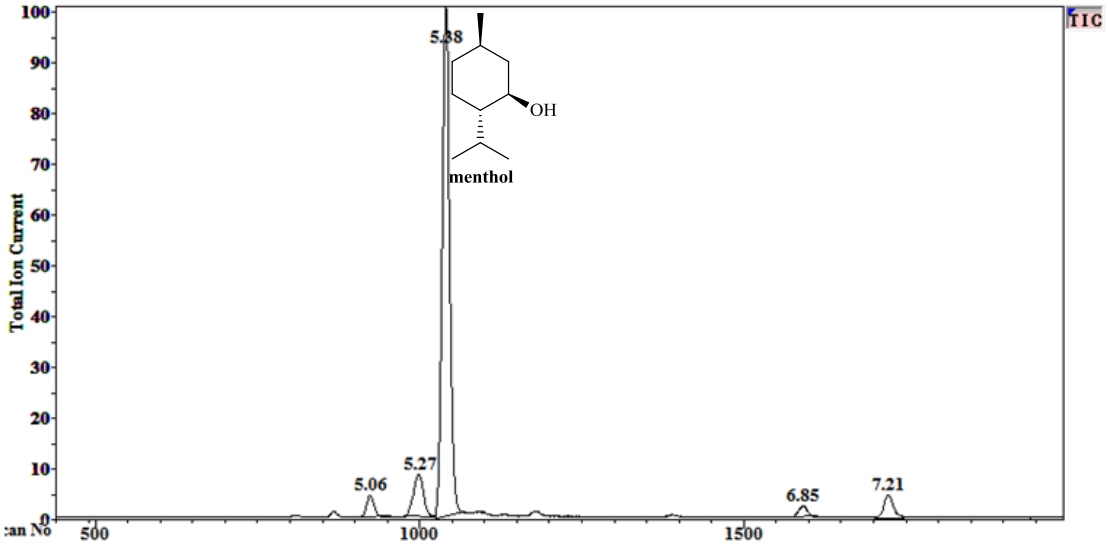
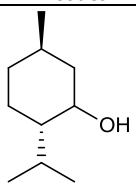


Figure 2: Chromatogram obtained by GC/MS from EOMP bioreduction by *Daucus carota* after 72 h.

Table 2: Bioreduction of menthol by *Daucus carota*

Identification	Substrate	Product	Final Product Yeld %
EOMP	Menthone/Isomenthone/ Pulegone/Piperithone		82,0%

Analysis Method: GC/MS

DISCUSSION

Bioreduction of acetophenone

Initially the ideal reaction conditions were determined based on data from the literature and using as an experimental model the bioreduction of acetophenone (Xu et al., 2010; Liu et al., 2012; Omori, Portas and Oliveira, 2012).

The results showed that *Daucus carota* was efficient in the bioreduction of acetophenone generating as product the chiral alcohol 1-phenylethanol.

The tests with biomass variation and substrate concentration showed that the best reaction condition occurred in test B, with 10 g of carrot biomass and 30 µL of substrate, whose percentage yield was above 96% with a lower amount of biomass and a higher conversion in relation to the amount of substrate.

In relation to essay C, the greater amount of biomass may favor the activity of other enzymes present in the carrot, promoting lateral reactions competing for the same substrate and this resulted in a percentage decrease in the bioreduction of acetophenone (Hudlicky and Reed, 2009).

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Bioreduction of Essential Oil of *Mentha piperita* L. (EOMP)

After the determination of the ideal reaction conditions using the bioreduction of acetophenone as model, the reaction was anew performed following the parameters of test B. The EOMP, however, already presents menthol as the major compound, but with the reduction of the carbonyl monoterpenoids present in this essential oil it is possible to significantly raise the menthol content in the sample (Moghtader, 2013).

Daucus carota used the ketone monoterpenoids as substrates and promoted the bioreduction of these selectively for the menthol compound. The data show that the bioreduction reaction mediated by carrot pieces is also effective in the presence of multiple molecules as in the case of EOMP.

Essential oils represent value-added substances in the industrial sector due to their bioactive and technological potential, they also represent renewable sources to obtain molecules of interest. The molecular modification of these compounds by biocatalytic methods is an economically viable alternative that minimizes processes of purification and extraction. The data obtained in the present work show a further advantage of the use of *Daucus carota* as a source of biocatalyst with the ability to biotransform substrates directly from a matrix and the EOMP without prior purification in the medium of the unbuffered aqueous solution leading to a clean and sustainable of menthol.

CONCLUSIONS

In summary, this work proposes a modification in the traditional biocatalytic methodology, where the EOMP is used as a source of multiple substrates. *Daucus carota* was able to promote the bioreduction of the ketone monoterpenoids present in the EOMP leading to a menthol enrichment in the sample with high selectivity. Thus promoting a cleaner and more sustainable reaction to obtain a compound of great industrial interest.

ACKNOWLEDGEMENTS

The authors are grateful to Unicesumar for having made available both materials and infrastructure to carry out this work and ICETI for the financial contribution.

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