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Full Length Research Paper

Fruity aroma production by *Neurospora sitophila* using green coffee beans as an alternative culture media

Daniele Souza de Carvalho[1*], Gustavo Molina[1], Mário Roberto Maróstica Junior[2], Gláucia Maria Pastore[1]

[1]Laboratory of Bioflavors, Department of Food Science, Faculty of Food Engineering (UNICAMP), P.O. Box 6121, 13083-862, Campinas-SP, Brazil
[2]Laboratory of Nutrition and Metabolism, Department of Food Nutrition, Faculty of Food Engineering (UNICAMP), P.O. Box 6121, 13083-862, Campinas-SP, Brazil

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Biotechnological processes appear as an attractive alternative for the production of flavours. The use of *de novo* synthesis allows the production of chemically different volatile flavors, such as short-chain alcohols, esters, aldehydes, ketones, methylketones and acids, as well as pyrazines and lactones that could be formed concurrently. This paper evaluated the use of green coffee beans as an alternative culture media for the development of a fruity aroma by the different strains of *Neurospora* sp. Moreover, the study also described the validation method for the aroma production and the average recovery of volatile compound using SPME technique was 30.5%. The highest production of ethyl hexanoate in green coffee beans was 25.43 mg.L^{-1} after 72 hours of fermentation by the strain LB26DSC. As far as the authors known, this is the first report dealing with the production of ethyl hexanoate by *Neuropora* sp. using green coffee beans as culture medium, which represents a promissory alternative to produce this aroma compound.

Keywords: Flavors, aroma production, green coffee and *Neuropora* sp.

INTRODUCTION

The growing market for flavored and fragranced products requires novel strategies for aroma chemical production and a rapid switch towards the bio-production and use of flavor compounds from biotechnological origin (bioflavors) has been observed (Krings and Berger, 1998).

Biotechnological processes appear as an attractive alternative for the production of flavors, since it occurs at mild conditions, presents high regio- and enantio-selectivity, does not generate toxic wastes and the products obtained may be labelled as "natural" (Bicas et al., 2010).

The use of *de novo* synthesis allows the production of chemically different volatile flavors, such as short-chain alcohols, esters, aldehydes, ketones, methylketones and acids, as well as pyrazines and lactones that could be formed concurrently (Krings and Berger, 1998).

*Corresponding Author E-mail: danisc31@gmail.com

Different groups of fungi have been reported as producers of volatile fruity aromas during its growth in the culture medium (Pastore et al., 1994). Among them, the fungi from the genus *Neurospora* have received extensive attention due to their powerful production of a pleasant and fruity odour (Yamauchi et al., 1991, Yoshizawa et al., 1988; Pastore et al., 1994).

Currently, several researches evaluate the utilization of low-cost sources of nutrients and the application of non-conventional media for the production of new added-value compounds (Bicas et al., 2010), such as the production of fruity banana and pineapple flavor by *Ceratocystis fimbriata* grown on steam-treated coffee husk (Soares et al., 2000) or sugar cane bagasse (Christen et al., 1997).

Thus, this paper evaluated the use of green coffee beans as an alternative culture media for the development of a fruity aroma by the different strains of *Neurospora* sp. Moreover, the study also described the validation method for the aroma production, the average recovery of volatile compound and the product

quantification.

METHODS

Preparation and characterization of the green coffee

Green coffee was collected from ITAL (Campinas, SP/ Brazil) and transported to the Laboratory of Bioflavors (Unicamp) at room temperature. The substrate was characterized regarding the content of total nitrogen (AOAC, 2005), the mineral fraction - P, K, Ca, Mg, S, Al, B, Cu, Fe, Mn, Zn, Cd, Cr, Ni and Pb - (Maróstica and Pastore, 2007), ammonia, nitrate (Bremer and Keeney, 1965) and pH.

Preparation of pre-inoculum and fermentation

Five strains of *Neurospora* sp. (LB8DSC, LB11DSC, LB12DSC, LB23DSC and LB26DSC) were inoculated into slant tube of Potato Dextrose agar (PDA) at 30°C for 72 h. After fungal growth, a spore suspension was prepared by adding 10 mL of sterile water into the tube and scraping the mycelia. The whole spore suspension volume was transferred to 250 mL Erlenmeyer flasks containing 50 mL of Yeast Malt Broth (YM: 10g.L^{-1} glucose, 5g.L^{-1} peptone, 3g.L^{-1} yeast extract, 3g.L^{-1} malt extract). This flask was subsequently transferred to an orbital shaker operating at 30°C and 200 rpm for 24 h for fungal growth. After this period, the culture broth was filtered through a membrane acetate filter (pore size: 0.45µm - Sartorius) and the mycelia washed with sterile water before being used as inoculum in the experiment of fruity aroma production. The inoculum consisted of 0.2 grams of biomass added in a flask containing 20 mL of processed green coffee. The flasks were incubated on a rotary shaker (New Brunswick Scientific) at 30°C for 72 h under agitation (200 rpm). Each 24 h, samples were collected and analyzed, using HS-SPME gas chromatography.

Extraction procedure

For SPME analysis, 20 mL consisting of 5% green coffee, diluted in distilled water, were transferred into a 100 mL serum vial containing a micro stirring bar. The vial was sealed with a Teflon-lined septa and screw cap, and then immersed in a water bath at 30°C. The SPME fiber coated with DVB/PDMS was manually exposed to the sample headspace for 10 min. The sample was continuously agitated with a magnetic stirring bar during the extraction process to allow faster equilibrium condition. Finally, the fiber was withdrawn into the needle holder, immediately introduced into the GC injection port and held for 10 min to completely desorb the volatile compounds.

GC-FID conditions

The volatile compounds were analyzed using a gas chromatograph (GC 7890A, Agilent Technologies) equipped with a flame ionization detector (FID) and a HP-5 column (Agilent Technologies, length = 30 m, i.d. = 0.320 mm, film thickness = 0.25 µm, USA). Desorption proceeded in the injection port of the gas chromatograph during 0.75 min at 250ºC using split less mode. Oven temperature was programmed at 50°C for 1 min, then ramped to 150°C at 10°C.min^{-1} and held for 1 min and finally raised to 200°C at 20°C.min^{-1} and held for 3 min at the final temperature. Nitrog en was used as the carrier gas with a flow-rate of 1 mL.min^{-1}. Detector temperature was 250°C. After desorption, the fiber remained 10 min further in the injector port to eliminate the possibility of any carry-over of analyte.

GC-MS conditions

For GC-MS analyses, an Agilent GC 7890A-5975C equipped with a HP-5 (5% phenyl 95% dimethylpolysiloxane) fused silica capillary column (Agilent Tecnhologies i.d. = 0.320 mm, length = 30 m, film thickness = 0.25µm, USA) was used under the following conditions: injection mode was split less, injector temperature was 250°C; purge time was 1 min., purge flow was 20 mL.min^{-1}; helium flow rate of 1.0 mL.min^{-1}, oven was initially kept at 50°C (1 min.) and temperature was risen at 10° C.min^{-1} up to 150°C and at 20° C.min^{-1} up to 200°C for 3 min; transfer line temperature was 240°C, energy of impact: +70 eV, 35-350m/z). A mixture of aliphatic hydrocarbons (C_5–C_{20}) (PolyScience, Illinois, EUA) was loaded into the vial and submitted at HD-SPME extraction and injected under the above temperature program to calculate the retention index (I) of ethyl hexanoate.

Method validation

The method was validated under the optimized conditions by determining the limits of detection (LOD) and quantification (LOQ), the inter- and intra-day precisions (RSD), the linearity and the relative recovery at different levels of fortification.

Quantification

Quantification was performed using the external calibration curve with the following concentrations of ethyl hexanoate: 1, 5, 10, 15, 20, 25 e 30 mg.L^{-1}.

Table 1. Physicochemical composition of green coffee.

Components	Green coffee
Humidity (%)	8.0
Total nitrogen ($g.Kg^{-1}$)	23.1
Phosphorous ($mg.L^{-1}$)	1.4
Potassium ($mg.L^{-1}$)	12.8
Calcium ($mg.L^{-1}$)	3.4
Magnesium ($mg.L^{-1}$)	2.0
Sulfur ($mg.L^{-1}$)	1.7
Iron ($mg.L^{-1}$)	61.5
Boron ($mg.L^{-1}$)	12.3
Copper ($mg.L^{-1}$)	20.2
Manganese ($mg.L^{-1}$)	49.5
Zinc ($mg.L^{-1}$)	7.5
Ammonia [NH_4^+] ($mg.L^{-1}$)	43.4
Nitrate [NO_3^-] ($mg.L^{-1}$)	115.8
pH	5.8
Organic Carbon (g/Kg)	552.4
Carbon/ Nitrogen Ratio	23.9

Table 2. The concentration range, regression equations, R^2, recovery, LOD, LOQ and RSD for the ethyl hexanoate analysis.

Matrix	Concentration range ($mg.L^{-1}$)	Regression equation	R^2	Recovery (%)	RDS range %	LOD ($mg.L^{-1}$)	LOQ ($mg.L^{-1}$)
Green coffee	1-30	y = 546.71x + 3885.7	0.9951	30.45	1.89- 8.78[a] 2.89-14.65[b]	0.46	1.41

Statistical analysis

The data obtained was analyzed using ANOVA ($p<0.05$). The statistical package used was Statistica ™ 7.0 data analysis software by Statsoft, Inc.,USA.

RESULTS AND DISCUSSION

Green coffee composition

The composition of the green coffee is shown in Table 1. The green coffee is rich in nitrogen and mineral salts, and the presence of C-source turns this substrate into a suitable medium for the cultivation of microorganisms, contributing to make this a feasible industrial process (Cameotra and Makkar, 1998; Maróstica and Pastore, 2007). The presence of high levels of several important micronutrients for microbial development makes it an important and promising substrate, since it does not require any nutrient supplementation (Barros et al., 2008).

Identification

The identification of ethyl hexanoate was made by mass spectrum and retention index agreed with standards and comparing spectra with Adams (2007) and NIST 98 mass spectral database libraries, with similarities higher than 90% and supported by retention index data.

Validation method and aroma production

The method was validated under the optimized conditions by determining the limits of detection (LOD) and quantification (LOQ), the inter- and intra-day precisions (RSD), the linearity and relative recovery at different levels of spiked. These results are presented in Table 2. The external standard calibration curve was constructed with six concentrations in aqueous matrix (water and standard).

The precision of the method was evaluated based on its repeatability, which was ascertained by performing intra-day repeatability from nine sample extractions analyzed on the same day by the same analyst with the same instrument and inter-day repeatability from the variation in results of nine analyses performed on three different days (n=3) by the same analyst using the same instrument. For this purpose, artificial matrix samples (green coffee, microorganisms autoclaved after pre-inoculum) were spiked with analyte at 1, 10, 20 $mg.L^{-1}$. Accuracy was evaluated by recovery tests, analyzing samples spiked with the same concentration levels used in the precision tests. The results were expressed as the

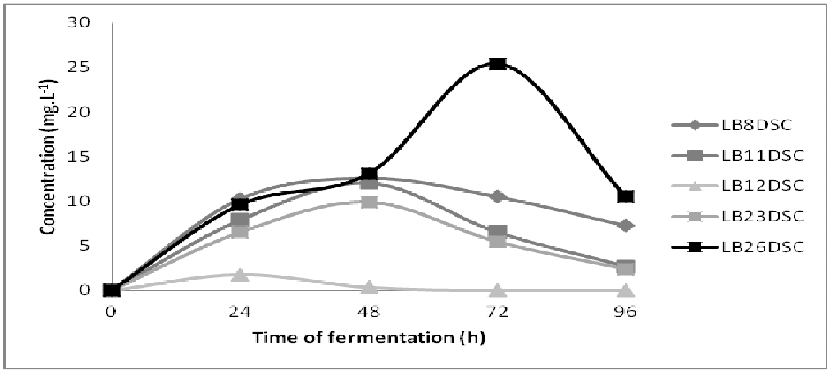

Figure 1. Production of ethyl hexanoate by *Neurospora* sp. using green coffee as alternative culture media under agitation 200 rpm at 30°C

percentage of the expected concentrations (the amounts initially added) of the analytes. The relative standard deviations (RDSs) results are summarized in Table 2. The standard deviations indicated that the dispersion of analysis was independent of the concentration of sample, and the developed method presents a linear range inside the concentrations evaluated. The average RDS % for ethyl hexanoate ranged from 1.89 to 8.78 % for the same day and from 2.89 to 14.65 % for different days. The low average RDSs for this compound indicated that the analytical conditions were found to be acceptable for these matrixes.

As shown in Table 2, the average recovery of volatile compound was 30.5%. This low value could be explained by the fact that SPME is a technique based on an equilibrium extraction and the complexity of matrix, which directly interferes in the analysis.

This method also proved to be linear for ester concentrations between 1 and 30 $mg.L^{-1}$ with R^2 values greater than 0.99. The LOD was calculated from the calibration curves constructed for ethyl hexanoate. LODs (3 x the RDS of the analytical blank values) were calculated from the calibration curve (Table 2). LOQs are almost 3.33 times higher compared to LOD. The results confirmed that the LOD and LOQ were low enough to determinate the ethyl hexanoate in real culture medium with *Neurospora* sp. The LOD and LOQ were 0.46 $mg.L^{-1}$ and 1.41 $mg.L^{-1}$ respectively, Therefore, the results demonstrated that the method was applicable for the analysis of ethyl hexanoate.

Where: a: RDS (relative standard deviation) intra day; b: RDS inter day; LOD: limits of detection; LOQ: limits of quantification.

The identification of ethyl hexanoate was made by the comparison between the mass spectrums and retentions index with Adams (2007) and NIST 2005 mass spectral database libraries, with similarities higher than 90% and supported by retention index data.

Figure 1 shows a decrease in ethyl hexanoate along the fermentation for strain LB26DSC. The results showed that the highest production of ethyl hexanoate in green coffee medium was 25.43 $mg.L^{-1}$ after 72 hours of fermentation by the strain LB26DSC (see Figure 1). The production of ethyl hexanaote by *Neurospora* sp. was explored by different research groups, especially using malt extract as culture medium (Brigido, 2000; Pastore et al., 1995; Pastore et al., 1994; Yamauchi et al., 1991; Yoshizawa et al., 1988). However, as described in this work, the utilization of a new alternative culture medium could support the industrial production of this ester.

CONCLUSION

This paper evaluated the use of green coffee beans as an alternative culture medium, which showed to be a suitable source of nitrogen, mineral salts and carbon source for the microorganism growth and subsequently for the development of the fruity ester. It also studied the validation method for the aroma production and the average recovery of volatile compound using SPME technique was 30.5%. The highest production of ethyl hexanoate in green coffee beans was 25.43 $mg.L^{-1}$ after 72 hours of fermentation by the strain LB26DSC. As far as the authors known, this is the first report dealing with the production of ethyl hexanoate by *Neuropora* sp. using green coffee beans as culture medium, which represents a promissory alternative to produce this aroma compound.

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