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# Fruity aroma production by *Neurospora sitophila*: Influence of precursors

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## ABSTRACT

The biotechnological production of aroma compounds is an emerging field and it was stimulated by the increasing preference of consumers for products bearing the label "natural". The genus *Neurospora* belongs to a group of filamentous fungus and it is reported as a producer of ethyl hexanoate, which is characterized by a strong fruity aroma with great industrial interest. This compound has a fruity, banana, pineapple, apple, strawberry and peach aroma with a threshold of 1 $\mu g.L^{-1}$. It is a typical fragrance compound of alcoholic beverages (such as Japanese sake and Chinese liquor), ice cream, baked goods, jams and jellies. Malt extract broth was shown to be a suitable substrate for the production of fruity aroma by *Neurospora sitophila*. Among the precursors tested in this study (soybean oil, glucose, hexanoic acid, ethanol and glycerol with some combinations among them), the soybean oil, glucose, ethanol and glycerol demonstrated to be more suitable for higher production of ethyl hexanoate. This fact could be explained probably by the pathway used by this microorganism to produce this ester, which may occur by alcoholysis of acyl-CoA compounds or by esterification of an acid with an alcohol. Ethyl hexanoate gave a strong pineapple aroma and the maximum production was after 24 hours of fermentation. Besides ethyl hexanoate, some compounds were identified by GC/MS analysis, among them: acetaldehyde, 1-octen-3-ol, isoamyl alcohol, phenylethyl alcohol, ethyl acetate, ethyl butyrate, ethyl heptanoate and ethyl octanoate. The following stage of this research will be the optimization of the production of ethyl hexanoate using experimental design.

**KEYWORDS:** *de novo* synthesis, bioflavors, ethyl hexanoate

## 1. INTRODUCTION

Recent market surveys have shown that consumers prefer foodstuff that can be labelled as "natural" [1]. Biotechnological processes represents 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 labeled as "natural" [2, 3]. In this sense, biotechnology is an interesting approach for the production of bioflavor and encouraged companies to direct their attention towards flavor compounds of biological origin [4]. Different groups of fungi have been reported as producers of volatile fruity aromas during growth in culture medium [5]. Among them, the genus *Neurospora* have received extensive attention due to their powerful production of a pleasant and fruity odor, subsequently identified as ethyl hexanoate by some authors [5, 6, 7, 8]. This compound has a fruity, banana,

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pineapple, apple, strawberry, peach aroma with a threshold of 1 µg.L$^{-1}$. It is much used in alcoholic beverages, such as Japanese sake, Chinese liquor, ice cream, baked goods, jams and jellies [5, 8, 9, 10, 11, 12, 13, 14]. However, there is little information in the literature regarding to production of ethyl hexanoate. In this work, the ability of *Neurospora sitophila* to produce ethyl hexanoate in malt extract with the addition of precursors was explored.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism and chemical

The filamentous fungus used in this study was isolated from Beiju (Brazil's traditional cake made of cassava) and it was identified as *Neurospora sitophila*. The culture was maintained on potato dextrose agar (PDA) at 4°C and sub-cultured every week. Ethyl hexanoate and 2-heptanol were purchased from Acros Organics and Sigma Aldrich, respectively, and diethyl ether from Merck. All the chemicals and solvents used were of analytical grade.

### 2.2. Culture mediums

Nine different combinations of precursors with malt extract broth were used for aroma production: (1) malt extract broth (50 g.L$^{-1}$ of malt extract); (2) malt extract broth with soybean oil (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of soybean oil); (3) malt extract broth with soybean oil and glucose (50 g.L$^{-1}$ of malt extract, 5 g.L$^{-1}$ of soybean oil and 5 g.L$^{-1}$ of glucose); (4) malt extract broth with glucose (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of glucose); (5) malt extract broth with glycerol (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of glycerol); (6) malt extract broth with ethanol (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of ethanol); (7) malt extract broth with hexanoic acid (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of hexanoic acid); (8) malt extract broth with hexanoic acid and ethanol (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of hexanoic acid and 5 g.L$^{-1}$ of ethanol); (9) malt extract broth with hexanoic acid and glycerol (50 g.L$^{-1}$ of malt extract and 5 g.L$^{-1}$ of hexanoic acid and 5 g.L$^{-1}$ of glycerol).

### 2.3. Preparation of the pre-inoculum and fermentation

*Neurospora sitophila* was inoculated into a slant tube of PDA at 30°C for 72 h. After fungi growth, a spore suspension was prepared by adding 10 mL of sterile water to the tube and scraping the mycelia into the water. All volume was transferred into a 250 mL Erlenmeyer flasks filled with 50 mL of Yeast and Malt broth (YM: bacteriological peptone 0.5%, glucose 1.0%, malt extract 0.3% and yeast extract 0.3%). The flasks were incubated in an orbital shaker operating at 30°C and 200 rpm for 24 h. Following this incubation, the culture broth was filtered through an acetate membrane (0.45 µm pore size, Sartorius®, Germany) and the mycelia was washed with sterile water before being used as inoculum at the fermentation experiments.

After biomass recovery, 1 g of the biomass was added to flasks containing 50 mL of each culture medium and precursors described in the paragraph above and homogenized under sterile conditions using an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA). The flasks were incubated on a rotary shaker at 30°C and 200 rpm, for 27 h. Samples were extracted using solvent and analyzed by gas chromatography (GC-FID) at 21, 24 and 27 hours.

### 2.4. Liquid-liquid extraction

For the analysis of the volatile compound, 5 mL of cultured broth was collected into a tube containing 0.1 g NaCl. Samples were vortexed (10 s) and then extracted using 1 mL of diethyl ether containing 0.003% of 2-heptanol as an internal standard. The mixture was then vortexed for 30 seconds, and 1 µL of the organic extracts were directly analyzed using GC-FID. The experiments were made in triplicate.

### 2.5. GC-MS conditions

GC-MS analyses were carried out using a GC-MS system (Agilent 5975C, inert MSD) using the following instrumental conditions: HP-5 MS fused silica capillary column (Agilent Technologies i.d. = 0.320 mm, length = 30 m, film thickness = 0.25 µm, USA); injector mode and temperature, splitless, 250°C; purge time, 1 min; purge flow, 20 mL.min$^{-1}$; helium flow rate, 1.0 mL.min$^{-1}$; oven, 50°C for 1 min and then 10°C.min$^{-1}$ up to 150°C followed by 20°C.min$^{-1}$ up to 200°C and hold for 3 min; transfer line temperature, 250°C; energy of impact, +70 eV, 35-350 m/z. A mixture of aliphatic hydrocarbons ($C_5$-$C_{20}$) was injected using the above temperature program to calculate the retention index (I) of ethyl hexanoate.

This component was identified by mass spectra and it agreed with standard. Further, spectra were compared with Adams [15] and NIST 2005 mass spectral database libraries. Similarity was higher than 90% and was supported by retention index (I) data. For the others compounds, they were identified by mass spectra compared with NIST 2005 mass spectral database libraries with similarity higher than 90%.

**2.6. Statistical analyses**

Data were analyzed using ANOVA followed by Tukey post hoc tests ($p<0.05$). The statistical package used was Statistica™ 8.0 data analysis software by Statsoft Inc., USA.

## 3. RESULTS AND DISCUSSION

### 3.1. Quantification of the target compound in different culture mediums

The intervals of analysis were defined based on previous studies that demonstrated that the maximum production in malt extract was carried out in 24 hours. Because of that, the analyses were carried out in 21, 24 and 27 hours.

Table 1 shows the maximum production of ethyl hexanoate in different culture mediums, described in the literature with an aroma like pineapple, apple, peaches and pears [7, 8, 9, 10, 13, 16].

Ethyl hexanoate was detected in all samples, however, when hexanoic acid was used as precursor, the concentration of ethyl hexanoate was under the limit of quantification on the analytical method used. The concentrations of 0.38-10.54 $mg.L^{-1}$ of ethyl hexanoate were detected and the highest concentration (10.54 $mg.L^{-1}$) was produced at 24 hours of fermentation, with addition of soybean oil and glucose, as shown in Table 1, which differed statistically, at 95% confiance level, from the concentrations of others.

When soybean oil was added to the malt extract medium, a large increase in the production of ethyl hexanoate was observed, increasing from 4.72 $mg.L^{-1}$ to 7.61 $mg.L^{-1}$. Further additions of glucose and soybean oil kept the production increasing, what could be explained probably by the pathway used by this microorganism to produce this ester, which may occur by alcoholysis of acyl-CoA compounds and alcohols to their corresponding esters by alcohol acyltransferase. Thus, the ethyl hexanoate might be mainly synthesized from hexanoyl-CoA and ethanol *via* catalysis by this enzyme. Other possibility could be by the esterification of an acid with an alcohol, achieving this interesting aromatic ester [6, 17].

*Neurospora* sp. ATCC46892 proved to be a stronger producer of ethyl hexanoate, expressed an AATase activity, which is highly active on various acyl-CoAs longer than *n*-hexanoyl-CoA. This enzyme is present in the cytoplasm and is highly active on hexanoyl-CoA [6, 17].

Esterases are known to be able to produce ethyl esters. In comparison of ester-forming activity between AATase and esterase in *Neurospora* sp., AATase and esterase produced 48.5 and 1.05 ppm of ethyl hexanoate from 0.6 M ethanol and the same amount of hexanoyl-CoA or hexanoic acid (7.3 mM). This experiment indicated that at physiological concentrations of substrates, AATase

**Table 1.** Maximum production of ethyl hexanoate in different culture medium.

| Culture broth | Ethyl hexanoate ($mg.L^{-1}$) |
|---|---|
| Malt extract | $4.72^{c} \pm 0.04$ |
| Malt extract/ethanol | $2.69^{d} \pm 0.10$ |
| Malt extract/glycerol | $0.38^{f} \pm 0.09$ |
| Malt extract/soybean oil/glucose | $10.54^{a} \pm 0.02$ |
| Malt extract/glucose | $1.83^{e} \pm 0.01$ |
| Malt extract/soybean oil | $7.61^{b} \pm 0.03$ |

Different letters indicate significant differences between treatments by Tukey test ($p<0.05$).

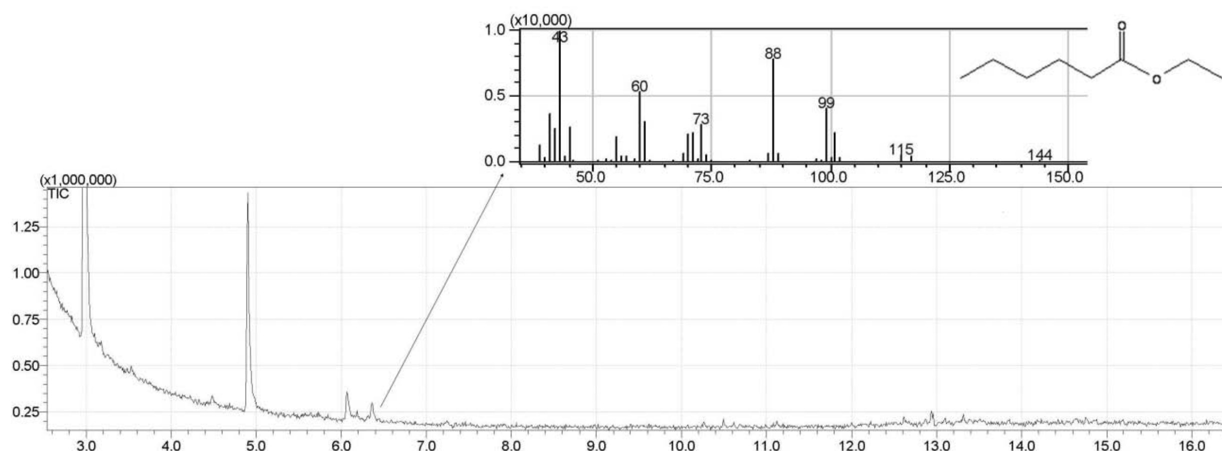


**Figure 1.** GC-MS chromatogram and mass spectra of the ethyl hexanote produced by *Neurospora sitophila*.

is expected to be mainly responsible for ester production in *Neurospora* sp. [17].

The production of ethyl hexanoate by *Neurospora* was investigated by different research groups, especially using malt extract as culture medium [5, 7, 8, 9, 13, 18]. However, at present, the production obtained for all research groups are not sufficient to support an industrial application. Several researches have pointed that simple modification on process conditions, like the changes in the culture medium with addition of precursors, temperature, pressure, agitation or pH could significantly improve the productivity. The use of statistical models, like Response Surface Methodology could help in reducing the number of experiments required in multi-factorial systems [19, 20]. In this way, the further step of this study will aim to evaluate the culture conditions during the fermentation process, to provide an increase in the fermentation yields achieved.

### 3. 2. Identification of ethyl hexanoate and other volatile compounds

The identification of compounds was made by the comparison between the mass spectra retentions index with Adams [15] and NIST 2005 mass spectral database libraries, with similarities higher than 90% and supported by retention index data. The reliability of the identification proposal is indicated by mass spectra and linear retention index agree with standard. For ethyl hexanoate the retention index calculated (I-Cal) and retention index to literature (I-Lit) was I-Cal = 998 and I-Lit 998 [21]. Figure 1 shows the chromatogram and the mass spectra of the ethyl hexanoate produced by *Neurospora sitophila* during growth in malt extract medium.

Besides ethyl hexanoate, some compounds were separated and identified by GC/MS analysis, such as acetaldehyde, 1-octen-3-ol, isoamyl alcohol, phenylethyl alcohol, ethyl acetate, ethyl butyrate, ethyl heptanoate and ethyl octanoate. The major volatile compounds found in the sample were alcohols and esters. Alcohols do not play a predominant role in flavors but are known to contribute to the overall flavor quality and can act as suitable precursors for the production of fruit-like flavoring esters.

## CONCLUSION

*Neurospora sitophila* was capable of producing ethyl hexanoate in 24 hours of fermentation and the most adequate precursors, for the production of this compound, were soybean oil, glucose, ethanol and glycerol. The following stage of this research will be the optimization of ethyl hexanoate production using experimental design.

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