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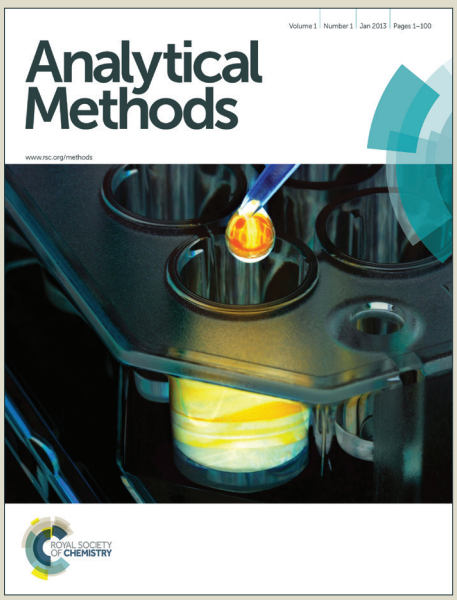

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Optimization of headspace solid-phase microextraction conditions to determine fruity aroma compound produced by *Neurospora sitophila*.

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Abstract

The biotechnological production of aroma compounds is an important alternative to chemical synthesis for flavor ingredients to food industry. On that account, an accurate and precise analytical technique for measurement of biotechnological production of aroma compounds is of important. This paper performed an optimization of the extraction conditions for headspace solid-phase microextraction coupled to GC-FID and GC-MS for determination of ethyl hexanoate produced by *Neurospora sitophila* in malt extract medium. Three SPME fibers were tested: polydimethylsiloxane (PDMS), carboxen-polydimethylsiloxane (CAR/PDMS) and polydimethylsiloxane-divinylbenzene (PDMS/DVB). The PDMS/DVB was the most efficient fiber to trap the volatile aroma compound. The optimized SPME extraction conditions were 30 °C for the extraction temperature and 10 min for the extraction time. The method showed good

linearity and the limits of detection (LOD) and quantification (LOQ) of the targeted aroma compound were 0.6 and 1.9 mg L^{-1}, respectively. The HS-SPME methodology proved to be a simple and rapid approach to quantify ethyl hexanoate accurately and precisely in the biotechnological production process.

Keywords: ethyl hexanoate, fruity aroma, solid phase micro-extraction, gas chromatography-mass spectrometry, optimization.

Introduction

Flavors and aroma compounds are key substances in the food and fragrance industry, with a world market estimated in US$ 18.6 billion in 2008. [1, 2] The food flavoring compounds are mainly produced by chemical synthesis or by extraction from natural materials. [3] However, these last mentioned procedures have some disadvantages. In chemical synthesis, the products are labeled as “artificial” or “nature identical”, decreasing their economic interest. Since market surveys have shown that consumers prefer foodstuff that can be labelled as “natural” and there are interference of climatic features, possible ecological problems (involved with the extraction) and low yields in extraction from natural materials. [3, 4] Thus, an alternative process such as biotechnological generation of flavor molecules would be preferable. This procedure has advantages: the process occurs at mild conditions, it presents high regio- and enantio- selectivity - and the products obtained are recognized as “natural” which represents an important market strategy. [3, 5] The biotechnological production of aroma compounds may be performed using two different strategies: *de novo* synthesis or biotransformation. The *de novo* synthesis produces complex substances from simple

molecules through complex metabolic pathways. On the other hand, the process of biotransformation involves single reactions catalyzed enzymatically using specific precursors. [3, 4, 5]

Production of volatile fruity aromas occurs during the growth of several microbial species in different culture media. [7] Esters are the most important group involved in the fruity flavor and they are employed in fruit-flavored products and dairy products. [3] There is evidence in the literature indicating the generation of aromatic esters using different microorganism through *de novo* synthesis. For example, the moulds *Saccharomyces* sp., *Hansenula* sp. and *Candida utilis* could produce ethyl acetate in a medium containing ethanol, and *Neurospora* sp. has the potential for produce ethyl hexanoate. [7, 8, 9, 10, 11] *Neurospora sitophila* is filamentous fungi very versatile for industrial applications and it is considered a model of model microbes for biochemical genetics and molecular biology has also been seen as a valuable organism for biotechnological applications. The literature describes the use of *N. sitophila* for the production of biomass, proteins, cellulases, lipases and aroma compounds with application in the food industry. [29, 30, 31, 32]

In this context, the analytical task of qualitative and quantitative analysis of aroma compounds is not simple and different analytical methods have been developed and employed for this issue. The most typically methods for extraction and pre-concentration aroma compounds are: headspace techniques, purge-and-trap, liquid–liquid extraction, and simultaneous distillation and extraction. However, these methods are laborious, lengthy and error-prone. The formation de artifacts can be induced during the sample preparation by the use of different solvents and by the elimination of solvents to concentrate the volatiles. [12] Considering the limitations of the traditional sample preparation method, headspace solid-phase microextraction (HS-SPME), liquid-

phase microextraction (LPME) and liquid–liquid microextraction (DLLME) are attractive alternatives. [13, 14, 15, 16, 17, 18] In this context, SPME is a solvent-free sample preparation technique that integrates sampling, isolation/extraction and concentration into a single uninterrupted process, resulting in high sample throughput. [19, 20] For all these reasons, SPME has been employed for the extraction of volatile compounds in biotechnological processes, such as is the case for limonene, citronellol and linalool were used as substrate in biotransformation assays.[6, 21, 22] In addition, a large number of scientific papers used SPME to identify and quantify ethyl hexanoate in various matrices as cider, wine, beer, liquor, distilled drinks and fruit.[23, 24, 25, 26, 27, 28] HS-SPME is an equilibrium technique that requires proper optimization of key parameters affecting the extraction efficiency. These parameters include fiber sorbent material phase, extraction temperature and time, to name a few. .

The aim of this study was to develop a simple, robust, reliable and solvent-free technique, based on HS-SPME combined with gas chromatography mass spectrometry (GC-MS) and gas chromatography flame ionization detector (GC-FID), in order to allow a qualitative and quantitative screening of aroma compounds produced by *Neurospora sitophila* in malt extract medium.

Experimental

Chemicals and SPME fibers

Ethyl hexanoate (ethyl caproate) 99.9% was purchased from Acrós Organics (USA). The SPME fibers 100 µm polydimethylsiloxane (PDMS); 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) and 65 µm divinylbenzene/polydimethylsiloxane (DVB/PDMS) were purchased from Supelco (Bellefonte, PA, USA). The fibers were conditioned prior to use according to the

instruction from the supplier (into the gas chromatograph injector at 250 °C for 1 hour). The standard solution of n-alkanes used for the determination of the linear temperature-programmed retention indices (LTPRI) was purchase from PolyScience (Illinois, EUA).

Sample preparation and SPME procedures

For SPME analysis, 20 mL of the culture broth was transferred into a 100 mL serum vial septa containing a microstirring bar. The three fibers were tested with concentration of 100 mg L^{-1} to select the one presenting the best capacity to extract the ethyl hexanoate. All the fibers were exposed to the sample headspace under the following conditions: equilibrium time of 10 min, extraction time of 30 min, extraction temperature of 30 ºC (conditions arbitrarily established by the authors in the choice-of-fiber step); after extraction, the fibers were introduced into the gas chromatograph injector for the analyte desorption at a temperature of 250 ºC in the splitless mode for a period of 1.0 min. After the extraction and desorption procedures, each of the fibers was reconditioned at 250 ºC for 10 min. The fiber reconditioning procedure was carried out to guarantee the absence of peaks in the blanks run and the good quality of SPME extraction and chromatographic procedures. With the aim at defining the best condition for the SPME procedure, three different fibers, five equilibrium times (0, 3, 5, 7, 10 min) and four extraction times (10, 20, 30, 40 min) were tested for headspace analysis of ethyl hexanoate from malt extract brots. All the fibers were tested in triplicate and the results presented represent the mean values.

Preparation of stock standard solution

Sterile distilled water and ethyl hexanoate were used for the preparation of the standard solution. The stock solution (100 mg L^{-1}) was prepared daily and kept cool until the end of the day. Appropriate dilutions of the stock solution were prepared for the construction of the calibration curve.

Gas chromatography conditions

Ethyl hexanoate was analyzed using a Shimadzu GC-17A/QP-5000 (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (for quantitative analysis) and high performance quadrupole mass spectrometer (for qualitative analysis). The column used consisted of a nonpolar HP-5 (5% phenyl 95% dimethylpolysiloxane) (length 30 m, 0.25 mm i.d., film thickness 0.25 μm), and helium at 1 mL $min^{-1}$was the carrier gas. The transfer line and the ion source operated respectively at 240 ºC and 200 °C, and energy of impact: +70 eV, (35-350m/z). Desorption of SPME fiber was done in gas chromatograph injection port for 1 min at 250 ºC in a splitless mode. Oven temperature was programmed at 50 °C for 1 min, then increased 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. The positive identification of the ethyl hexanoate in samples was done by mass spectrum and retention index agreed with standard and comparing mass spectra with Adams (2007) and NIST 2008, with similarities higher than 90%.

Method validation

Analytical method validation was performed including the following parameters: linearity, precision, limits of detection (LOD) and quantification (LOQ) and relative recovery at different levels of fortification for ethyl hexanoate in different fermentation

times (0, 24, 48 and 72 hours). The performance was determined, using the optimized HS-SPME extraction conditions, with a standard solution that was added to the matrix (matrix was defined as the culture medium and *Neurospora* sp. autoclaved after 24 hours). Linearity was determined by constructing of calibration curve with standard solutions, in matrix containing ethyl hexanoate in the range of 2–25 mg L^{-1}. Three injections were made at each of the five concentration levels. Reproducibility was evaluated using five concentrations and calculating the relative standard deviation (RSD) of three replicates of each concentration over three different days (inter day) and three replicates of three concentrations over the same day (intra day). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the calibration curves constructed for ethyl hexanoate. The LOD was considered as three times the RSD of the analytical blank values and LOQ was ten times the RSD of the analytical blank values. Accuracy and precision data were obtained with recovery studies carried out by spiking samples with ethyl hexanoate at levels of 5, 10, 15, 20 and 25 mg L^{-1}. The spiked samples as well as the unspiked controls were analyzed in three replicates. Repeatability of the method was evaluated through the relative standard deviation (RSD) associated to measurements of the ethyl hexanoate performed during recovery analyses.

Quantitative analysis

Quantification was performed using the external calibration curve, with the following concentrations of ethyl hexanoate: 5, 10, 15, 20 and 25 mg L^{-1}. The ethyl hexanoate standard was directly diluted in the artificial matrix, which containing 0.4 g of the inoculums autoclaved and malt extract broth to provide a matrix effect. All the experiments were carried out in triplicate.. The recovery was carried out during the time

of fermentation and the above concentrations of ethyl hexanoate were added into the fermentation broth vial.

Preparation of pre-inoculum and fermentation

Neurospora sitophila was inoculated into a 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 distilled water (sterilized at 121 °C for 20 min) and scraping the mycelia. The spore suspension was transferred for a 250 mL erlenmeyer flasks containing 50 mL of Yeast Malt Broth (YM: 10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract and 3 g L^{-1} malt extract). The flask was placed in an orbital shaker at 30 °C and 200 rpm for 24 h for fungal growth. After this period, the culture broth was filtered throughout a acetate filter membrane (pore size: 0.45 μm) and the mycelia were washed with distilled sterilized water (121 °C for 20 min) before being used as inoculum in the fermentation experiments. The inoculum consisted of 0.2 g of biomass added in a flask containing 20 mL of malt extract medium. The flasks were incubated on a rotator shaker (New Brunswick Scientific) at 30 °C for until 72 h under agitation (200 rpm). Samples were collected, each 24 h and extracted by HS-SPME.

Statistical analysis

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

Results and discussion

SPME extraction efficiency

To compare the efficiency of SPME fibers to trap the volatile compound, each fiber was exposed to the headspace under the same conditions of equilibrium time, extraction time and temperature, and although the extraction conditions were the same, the differences in the areas obtained revealed the behavior of each type of coating used for each fiber tested. The results for total area for the target analyte obtained for SPME fibers tested (Fig.1) were significantly different (Tukey $p<0.05$). The PDMS/DVB fiber has mainly mesoporous, which are ideal for trapping low molecular weight (C6-C15) analytes, like ethyl hexanoate, probably due to their low vapor pressure. [19, 20] Based in these results, the fiber PDMS/DVB was chosen for the following experiments.

The effects of equilibrium time and extraction time were also investigated. The results revealed that equilibrium time is not a significant parameter to the recovery of the specific analyte, probably because the PDMS/DVB fiber has a high affinity of ethyl hexanoate and because in the temperature of the assays (30 °C) the equilibrium of the analyte with the gas phase had fast reached (Fig. 2). The best results were obtained at 30 °C and 10 min (Fig. 3). Hence, the extraction time for subsequent analyses was fixed at 10 min.

Method validation

The external standard calibration showed linearity in the range of 2-25 mg L^{-1}, with correlation coefficients (r) higher of 0.995. Relative standard deviations (RSD) of the three replicate injections ranged from 5% to 8% (intraday) and 3% to 15% (inter day) showing good repeatability. [6] The recovery data for the ethyl hexanoate were performed in five different spiking levels. The mean value of recovery was 73 %. The

LOD was 0.6 mg L^{-1} and the LOQ was 1.9 mg L^{-1}. The results of the method validation indicate that the HS-SPME sample preparation coupled with the GC-FID analysis is suitable for the determination of ethyl hexanoate. [6]

Analysis of fermentation samples

The validate method was employed to determine the concentrations of ethyl hexanoate produced in malt extract broth by *Neurospora sitophila*. A decrease occurred in the amount of the ethyl hexanoate during fermentation based on figure 4, the concentration of ethyl hexanoate increases to a maximum of 8 mg L^{-1} at 24 hours with a drop to less than 2 mg L^{-1} after 70 hours. After 24 h, the ethyl hexanoate were detected in all samples, at concentrations of 2.0 – 8.0 mg L^{-1} and the highest concentration (8.0 mg L^{-1}) was detected in the first 24 h of the experiment. The production of ethyl hexanaote by *Neurospora* was investigated by different research groups. [7, 8, 10, 20] However, at the moment, the production obtained for all research groups are not sufficient for an industrial application. Several researches have pointed that simple modification on processes conditions, like the change of culture medium, temperature, pressure, agitation or pH could significantly improve productivity. Some experiments about the use of Response Surface Methodology are now being carried out by us and the results will be released soon.[4, 6, 29]

Conclusions

A HS-SPME method enabling a simple and rapid determination of ethyl hexanoate in malt fermentation broths was optimized and validated. The method had a LOD and LOQ of 0.6 and 1.9 mg L^{-1} and a mean recovery, accuracy and precision of

73%, respectively. The method was used to follow the generation of ethyl hexanoate for a period of over 70 hours in a malt-based fermentation broth of *Neurospora sitophila*.

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Figure captions:

Fig. 1. Efficiency of HS-SPME fiber coatings in the extraction of ethyl hexanoate. The results are the means of triplicates of the total areas obtained on GC-MS chromatograms.

Fig. 2. The effect of HS-SPME equilibrium time on the extraction efficiency of ethyl hexanoate. The results are the means of triplicates of the total areas obtained on GC-MS chromatograms.

Fig. 3. The effect of HS-SPME extraction time on the extraction efficiency of ethyl hexanoate. The results are the means of triplicates of the total areas obtained on GC-MS chromatograms.

Fig. 4. Kinetic of ethyl hexanoate production by *Neurospora sitophila* in malt extract broth at 30°C and 200 rpm.

Table 1. Parameters for the regression equation (y=ax+b), where (y) is the concentration (mg/L) and (x) is the peak area

312 Table 2. Fortification experiments at different levels (recovery), limit of detection
313 (LOD) and limit of quantification (LOQ).

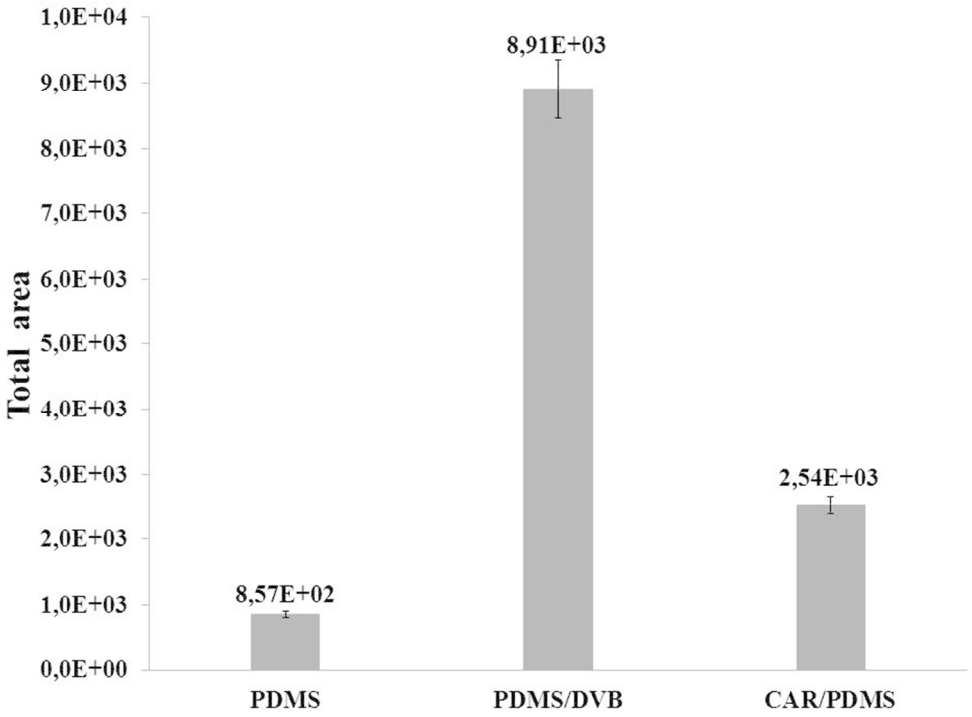
314

Table 1. Parameters for the regression equation (y = ax + b), where (y) is the concentration (mg/L) and (x) is the peak area

	Slope	Intercept	R	Linearity range (mg/L)
Ethyl hexanoate	472.28	2702.5	0.995	2-25

Table 2. Fortification experiments at different levels (recovery), limit of detection (LOD) and limit of quantification (LOQ).

Sample Spiked level (mg/L)	Recovery (%)	LOD (mg/L)	LOQ (mg/L)
2	69	0.6	1.9
5	67		
10	74		
15	82		
20	74		



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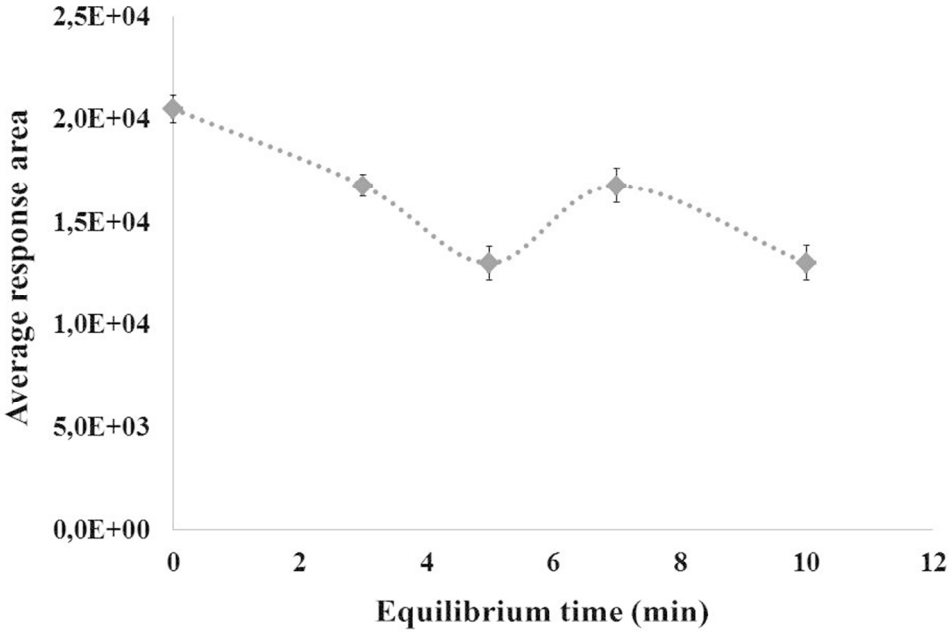

The effect of HS-SPME equilibrium time on the extraction efficiency of ethyl hexanoate. The results are the means of triplicates of the total areas obtained on GC-MS chromatograms.
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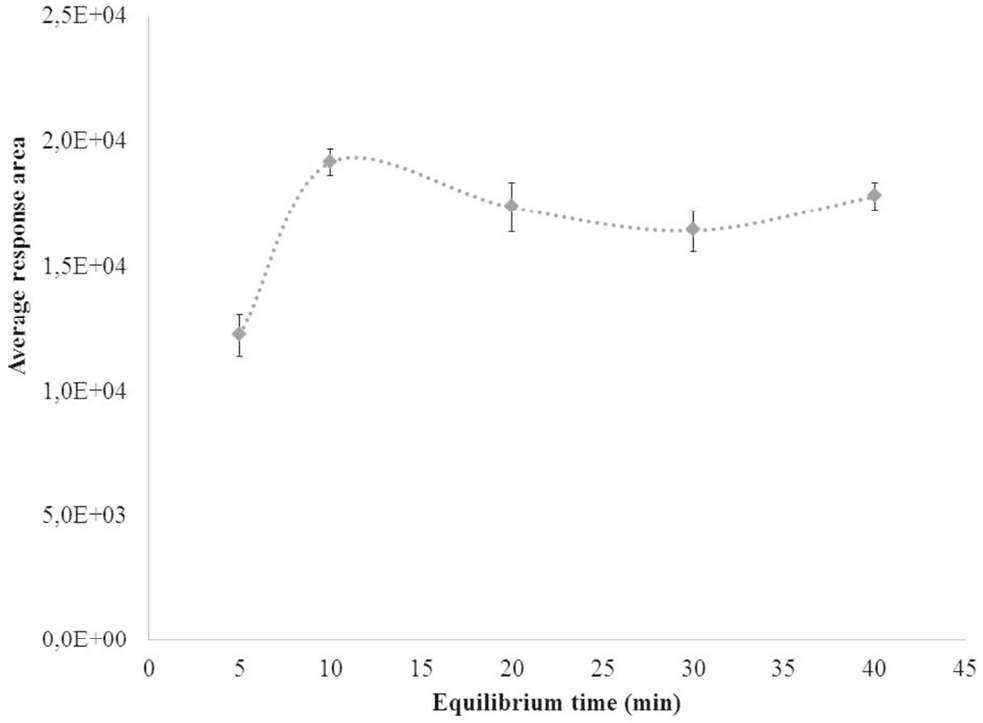

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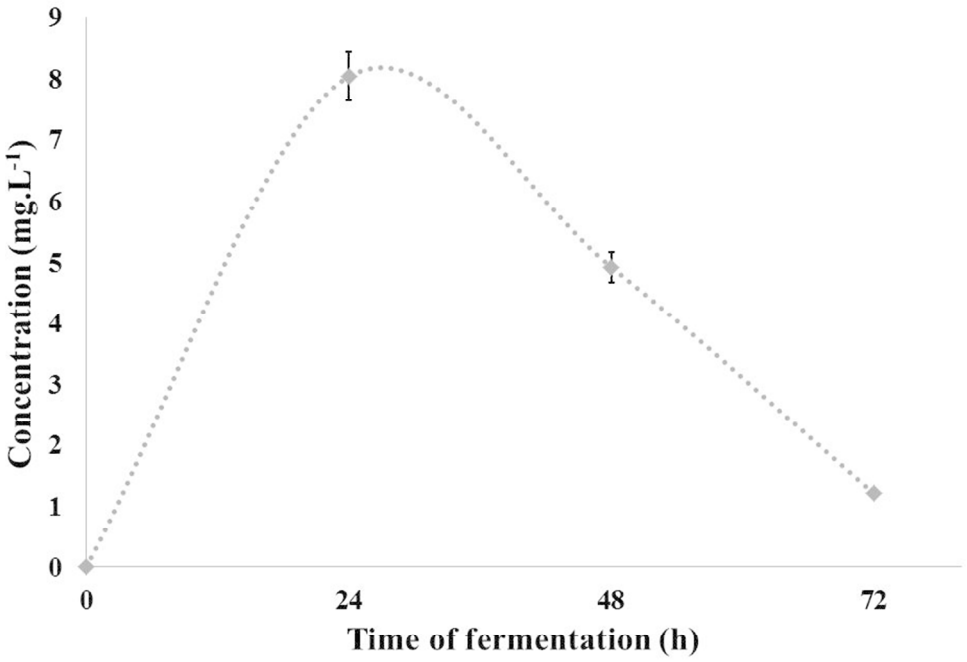

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