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Enzymatic Synthesis of Isoamyl Acetate by Nanoconventional Techniques

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Abstract: *Chemical methods for the synthesis of esters require the use of high temperatures in the presence of chemical catalysts resulting in undesirable by-products. To avoid these problems the use of biocatalysis can lead to greener products. The aim of this papar is to study the synergism of enzyme catalysis with unconventional techniques suc as microwave or ultrasound assited processes. The effect of different reaction parameters, such as temperature, enzyme loading, ultrasonic amplitude and duty cycle, on the enzymatic synthesis of isoamyl acetate has been evaluated. To highlight the efficiency of unconventional techniques, experiments using conventional methods were also performed. The concetrations of isoamyl acetate obtained by unconventional methods were higher than those achieved under conventional heating, as 478 $mg_{ester}/g_{mixture}$ was obtained with ultrasounds in 1 h using 25g/L of enzyme loading, at 50 ^oC as compared to 387 $mg_{ester}/g_{mixture}$ by conventional methods. Mild process conditions and using green techniques (microwaves and ultrasounds) make the biocatalytic procedure a useful way to synthetise esters with application on food, pharmaceutical and cosmetic industries.*

Keywords*: enzymatic esterification, ultrasounds, microwave, isoamyl acetate*

1.Introduction

The current trend in catalysis is moving to "green" chemistry due to environmental impact [1]. Employing enzyme as biocatalyst is safe for the environment keeping high performance and avoiding the formation of undesirable products for food, pharmaceutical and cosmetic industries (a major disadvantage of conventional chemical synthesis). Therefore, biocatalyzed chemical synthesis has become attractive due to its advantages, especially high chemo-, regio- and stereo-selectivity of enzymes [2]. A commonly process using enzymes as catalysts is the lipase-catalysed esterification synthesis of valuable flavour and fragrance compounds such as isoamyl acetate (banana flavor) [3, 4] and isoamyl butyrate (pear flavor) [5]. Worldwide demand for flavour and fragrances is expected to increase at a CAGR of 5.0% from 2019 to 2025. Aroma chemicals is projected to hold the largest share of flavors and fragrances market with more than 70.0% in terms of revenue by 2025 [6].

Isoamyl acetate, a banana flavor compound, is one of the most industrially exerted flavor ester in the food industry, especially in making fruit jam, fruit punches, honey, butterscotch, artificial coffee and beverages [7, 8]. Isoamyl acetate is usually obtained by chemical reaction of an alcohol with an organic acid in the presence of a catalyst, generally an acid; or by extraction from natural sources, a very expensive method [9]. Biotechnological production of this ester by employing lipases is becoming more and more attractive due to greener concept compared to chemical synthesis, and economic aspect compared to extraction from natural sources [3, 4, 10]. Lipases are used to catalyze the chemical reactions of esterification, transesterification [11-13] and hydrolysis [14]. Immobilized enzymes are preferred due to their functional efficiency, prolonged availability and their reusability. Enzyme immobilization consists of enzyme enclosure on a matrix/support. The usage of immobilized enzymes is still low because of their costs, deactivation and storage problems [15, 16] A competitive alternative to traditional enzymatic synthesis is the employment of microwave and/or ultrasounds in order to improve mass transfer and enzyme activity.

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Microwaves, a form of electromagnetic radiation with frequencies between 300 MHz (1 m) and 300 GHz (1 mm), combine thermal and non-thermal effects [17]. Most common microwave wavelength used for enzymatic reactions is 12.24 cm for a frequency of 2.45 GHz [18] Microwave energy together with polar molecules in the reaction generate vibrations leading to ionic conduction and localized superheating [19, 20]. A major advantage of microwaves is the short irradiation time needed in order to achieve similar or better results compared to conventional heating, where the reaction can take place for several hours. Microwaves impact molecules with high dipole moments. Enzymes have significant dipole moments and under microwave irradiation modify their configuration, by conformational flipping of active sites [1, 21]. The microwave field disturbs the polar hydrogen bonding in the protein possibly increasing the enzymatic activity and assisting the substrate to interact efficiently with the active site of enzymes, as well as faster product release [22-25].

Ultrasound, a type of no radiant energy, cover frequencies between 20 kHz and 5 MHz. Acoustic waves propagate into liquid due to alternate compression and rarefaction cycles. Consequently the cavitation phenomenon takes place. Cavitation leads to a large quantity of energy being dissipated locally, caused by the generation of micro bubbles or cavities, growing and collapsing violently [26]. The effect of ultrasounds on enzymes involve three separately or combined main mechanisms, resulting in either improving enzyme activity or in the denaturation of the biocatalyst [27]. First mechanism is thermal caused by the existence of hot spots achieved during cavitation. The second mechanism involves the generation of free radicals by ultrasonolysis in water or polar liquids. The last mechanism emerge from the mechanical shear forces created by microstreaming and shock [28]. Using ultrasounds in optimum conditions can intensify mass transfer between enzymes and reagents, diminishing the barrier of transportation and reducing reaction time [27-31]. Application of ultrasound also prevents enzyme agglomeration [32].

The goal of our paper is to intensify the enzymatic esterification by nonconventional methods by applying ultrasounds and microwaves in order to synthesize isoamyl acetate, an important ester used as aroma and flavor in the food and cosmetic industry. The microwave or ultrasound assisted reactions have been studied using lipase Lypozyme 435 from *Candida antarctica* as biocatalyst. The reaction parameters, including temperature, enzyme concentration, ultrasound amplitude and duty cycle were ana

lyzed to establish the relationship among them and the ester concentration. Finally, it was evaluated the best method to be used, by comparing these two unconventional methods microwave and ultrasound assisted processes.

2.Materials and methods

2.1. Materials

All chemicals used for the esterification reaction: isoamyl alcohol (Merck), acetic acid (Merck), isoamyl acetate - standard for GC analysis (Aldrich) were analytical grade. The biocatalyst, lipase Lipozyme 435 from *Candida antarctica* was provided by curtesy of Novozymes A/S (Denmark). The enzyme support, Duolite A 568 (Duolite International SA, Paris), is a porous granular, weak base anion exchange resin based on a cross-linked phenol-formaldehyde polycondensate with a hydrophilic structure and controlled pore size distribution. Water was removed using molecular sieves (Type 3 Grace Davison SYLOBEAD MS 564 C).

2.2. Methods

2.2.1. Esterification of acetic acid with isoamyl alcohol

The esterification was carried out in solvent free system, using lipase Lypozyme 435 from *Candida antarctica* as biocatalyst. For each nonconvetional method the influence of the main parameteres has been studied. Acetic acid (100 mmol), isoamyl alcohol (200 mmol), molecular sieves and corresponding amount of enzyme, Lipozyme 435 were mixed in a batch reactor (20 mL) with continuous stirring and heated to the reaction temperature. The esterification progress was monitored by collecting samples after each hour to be analysed by gas-chromatographic analysis (GC analysis). The microwave assited

experiments were carried out in a microwave generator (Biotage Initiator). Experiments in the presence of ultrasound were performed with an ultrasonic horn (Vibracell VCX 750) equipped with an amplitude and duty cycle variation system. The considered parameters were: enzyme concentration, temperature and ultrasonic parameters (amplitude and duty cycle). In order to establish the best esterification conditions, the experiments were carried out by changing one parameter and maintain all the others constant.

2.2.2. GC analysis

Quantitative analysis of the esters was performed usingan HP 6890 gas chromatograph equipped with flameionization detector (FID) [29]. The oven is set to heat the columnfrom 50 to 250°C with a gradient of 10°C/min. Helium isused as column carrier gas (flow rate 1 mL/min). n-Butanol is used as internal standard to determine the ester concentrations. The volume of the injected mixture was 1µL. Individual standards of isoamyl alcohol and isoamyl acetate were analysed and their retention time was recorded and the ester samples were compared under similar conditions. The ester concentration was determined using a calibration curve of pure isoamyl acetate with concentration ranging from 5 to 60 mg ester/g. Samples were diluted 1:10 with a standard solution of 5% n-butanol in methanol before analysis. The samples were analysed in duplicates.

3. Results and discussions

3.1. Intensification of esterification process by nonconventional methods

The influence of the type of esterification process on the ester concentration in the enzymatic reaction was studied. Three different techniques were used: conventional esterification, microwaves (MW) and ultrasounds (US) assisted processes. For the conventional and microwave methods the constant reaction conditions were: acid to alcohol molar ratio 1 : 2, temperature 50°C, stirring rate 900 rpm and reaction time 60 min. For the ultrasound assisted experiments additional conditions were set: 20% amplitude and ultrasonication in pulses with 3 s on/6 s off (duty cycle 34%). The results are graphically represented in Figure 1.

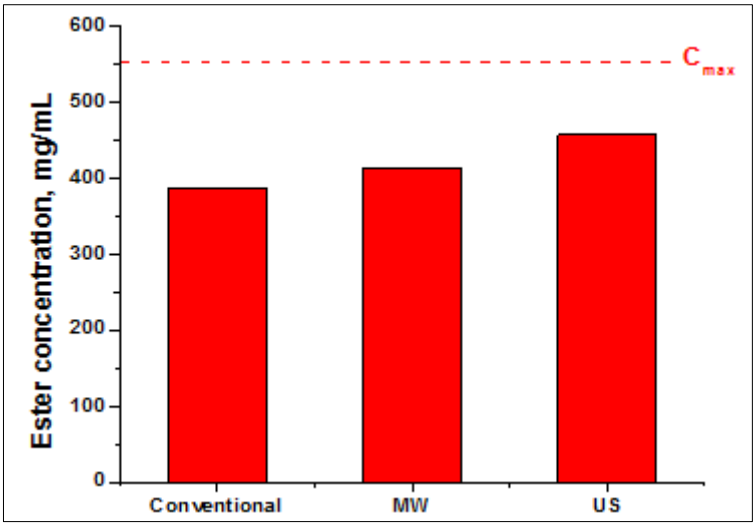

Figure 1. The influence of the esterification technique on the ester concentration

As is shown in Figure 1 the concentration of the ester obtained by conventional heating is lower than that obtained using microwaves and of the ester obtained by ultrasound assay. Thus, microwave irradiation and ultrasounds act synergistically with enzymatic catalysis. In order to establish the best conditions for esterification process, all further studies were carried out under microwave amd ultrasound irradiation as considerable increase in total ester concentration was observed.

3.2. Influence of the enzyme concentration in the microwave assited enzymatic esterification

The influence of the enzyme (Lipozyme 435) amount under microwave irradiation s on the ester concentration was studied in the range of 15 - 35 g/L. The constant experimental conditions were: acid to alcohol molar ratio 1 : 2, temperature 50°C, stirring rate 900 rpm and reaction time 60 min. Figure 2 shows the dependence of the concentration of isoamyl acetate obtained under microwave irradiation depending on the enzyme concentration.

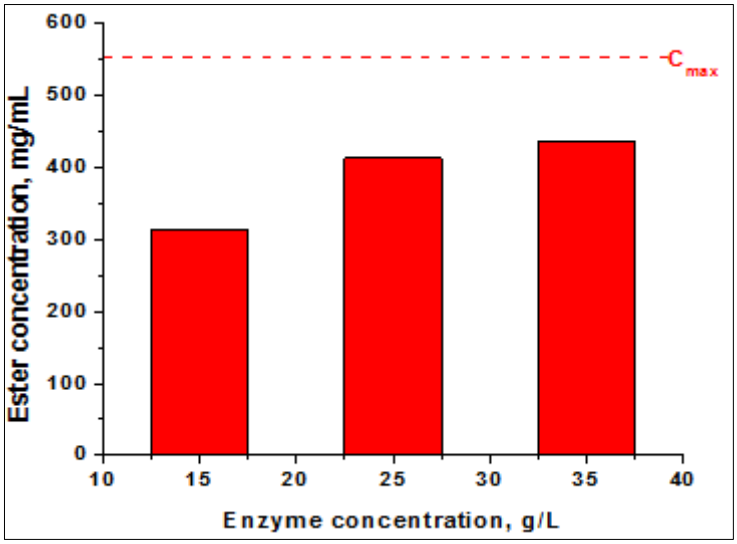

Figure 2. Influence of enzyme loading in microwave assisted esterification

The enzyme loading significantly influences the concentration of the ester obtained, by the microwave assisted esterification reaction, the higher the enzyme concentration, the higher the concentration of the isoamyl acetate was obtained. Increase of catalyst concentration from 15 to 25g/L increased the ester concentration by around 25%. But, further increase of catalyst concentration to 35g/L increased the ester amount only slightly (Figure 2). This behavior may suggest that all accessible active sites of the enzyme are occupied by reactant molecules. Additional increase in enzyme concentration will not have a significant effect as a little amount of reactant molecules is available and thus the active sites of enzyme would be unoccupied [30]. Moreover, due to the cost of enzyme, the lower the quantity of lipase, the more cost effective is the esterification process, and thus, 25g/L of Lipozyme 435 was used in the further experiments as a optimum catalyst concentration.

3.3. Influence of temperature on microwave assisted esterification

The reaction temperature is a critical parameter in enzymatic catalysis due to the risk of denaturation of enzymes at higher temperatures. The influence of temperature on the esterification reaction was investigated by comparing ultrasound and microwave assisted processes. Activity of the lipase Lipozyme 435 was studied over a temperature range from 30 to 60°C. The same amounts of reactants, molecular sieves and enzyme were used as in previous experiments. The constant experimental conditions were: acid to alcohol molar ratio 1 : 2, temperature 50°C, stirring rate 900 rpm and reaction time 60 min. Figure 3 shows the dependence of the concentration of isoamyl acetate obtained under microwave irradiation (A) and using ultrasounds (B) on the temperature.

As shown in Figure 3(A) the temperature influences the concentration of isoamyl acetate obtained by microwave assisted esterification. A slightly increase in isoamyl acetate concentration from 40°C to 50°C is observed. However, a further increase of temperature to 60°C led to a considerable decrease in ester concentration, that could be attributed to the deactivation of enzyme at high temperatures. Another explanation for the deactivation of the enzyme could be due to the high microwave power provided to

the system at the beginning of the reaction to reach the reaction temperature in a short time, leading to local overheating.

Regarding the ultrasound assisted esterification, the biocatalyst is sensitive to higher ultrasound powers because under harsh conditions, intramolecular hydrogen bonds breakdown inside the enzyme leading to the modification of the conformational structure, and subsequently to deactivation or reduction of catalytic activity of lipase [28, 29]. In addition, at high temperatures, the vapours entering inside the cavitation bubbles cushion the cavitation effect acting as shock absorbers [26]. For all these reasons the reaction in the presence of ultrasounds were carried out at temperatures ranging between 30 - 50°C. For ultrasound experiments the constant conditios were amplitude 20%, ultrasonication in pulses 3 s on and 9 s off (duty cycle 25%).

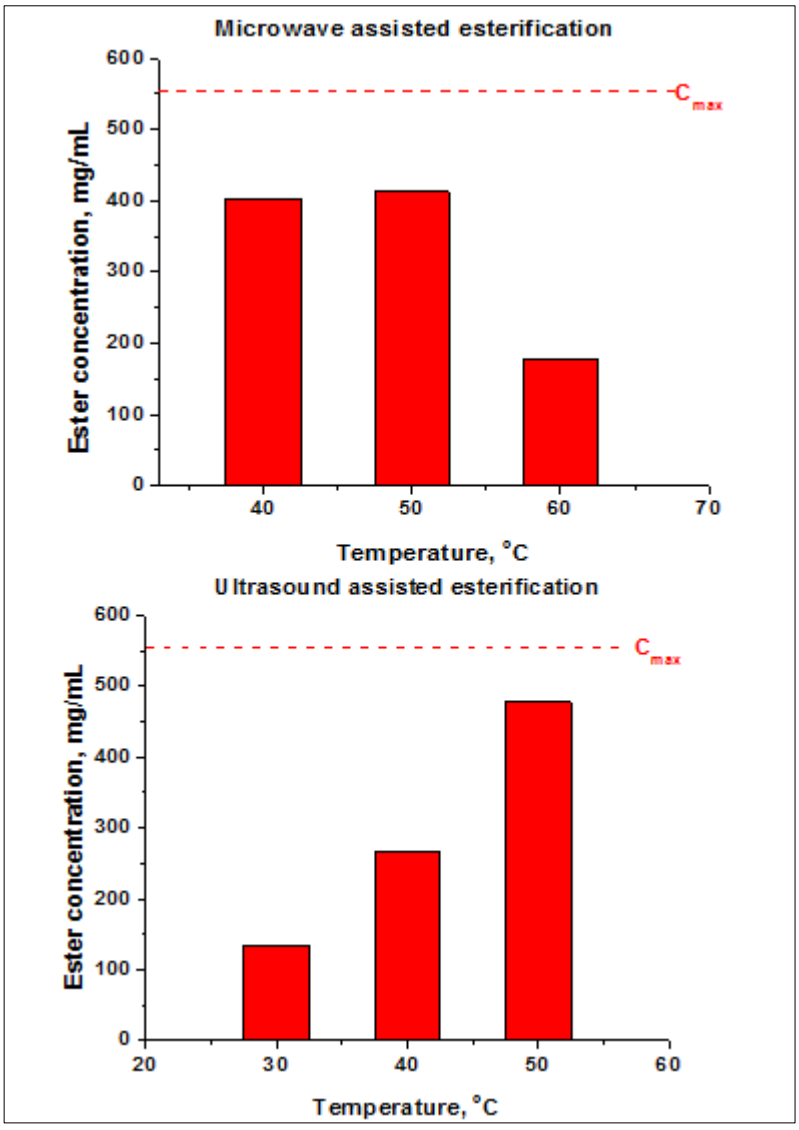

Figure 3. The influence of temperature on the ester concentration (A) microwave assisted esterification and (B) ultrasound assisted esterification

Figure 3(B) shows that the temperature of 50°C was observed as the optimal temperature in terms of increase in ester concentration. The desired ester concentration reached 85% in 60 min at 50°C from the maximum concentration (C_{max}) that can be obtained compared to the low temperature (30°C) that showed only 21% from C_{max}.

3.4. Influence of duty cycle on the ultrasounds assisted enzymatic esterification

Duty cycle is an important factor in ultrasound assisted processes. The effect of duty cycle on esterification was investigated by varying the on-off time of ultrasonic irradiation on the reaction mixture. To determine the influence of the pulse frequency on the isoamyl acetate concentration in the ultrasound-assisted enzymatic esterification reaction, the experiments were performed by varying the sonication time (pulses). The other conditions were kept at the optimum values: acid to alcohol molar ratio 1 : 2, temperature 50°C, stirring rate 900 rpm, ultrasonic aplitude 20%, and reaction time 60 min. Figure 5 presents the effect of ultrasound duty cycle on the ester concentration over time.

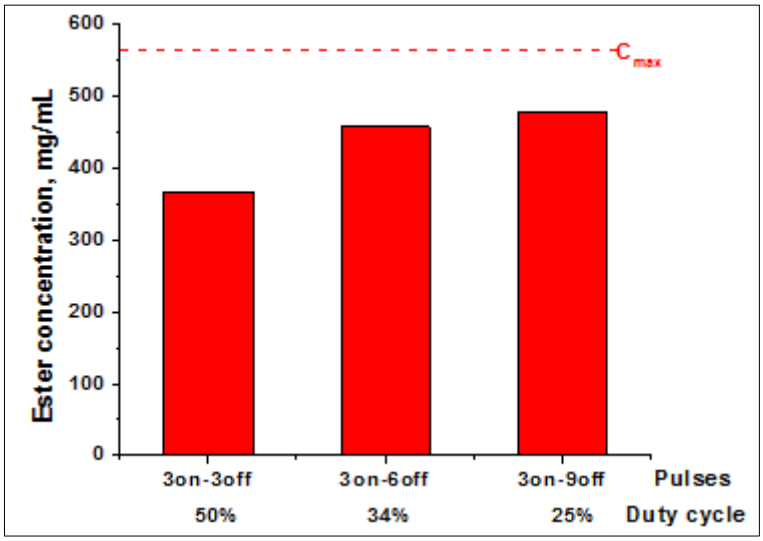

Figure 5. Influence of pulse frequency on ester concentration in ultrasound assisted esterification

The ultrasonic power corresponding to each duty cycle was read on the Vibracell device. The ultrasonic pulses applied for the esterification reaction were 3s on/ 3s off (50%), 3s on/ 6s off (34%), and 3s on/ 9s off (25%). The reported results establish that duty cycle is an important parameter affecting the concentration of isoamyl acetate. Figure 5 illustrates that using pulses of 3 on/9 off increases the ester concentration, after one hour of reaction, to 478 $mg_{ester}/g_{mixture}$. Further increasing the off cycle of the ultrasound irradiation from 3s off to 6, respectively 9s off, leads to an increase in the concentration of isoamyl acetate. Finally, 25% duty cycle was selected as optimum for this reaction.

3.5. Influence of the ultrasound amplitude on enzymatic esterification

Ultrasound intensity in sonochemical processes is an important factor for the efficiency of the reaction. In order to estimate the influence of ultrasound amplitude in isoamyl acetate synthesis, we selected three different ultrasound amplitudes ranging from 20 to 40%. The same amounts of reactants, molecular sieves and enzyme were used as in previous experiments. The constant experimental conditions were: acid to alcohol molar ratio 1 : 2, temperature 50°C, stirring rate 900 rpm, 3s on/ 9s off (25%) and reaction time 60 min. Figure 6 shows the dependence of the concentration of isoamyl acetate on ultrasonic amplitude.

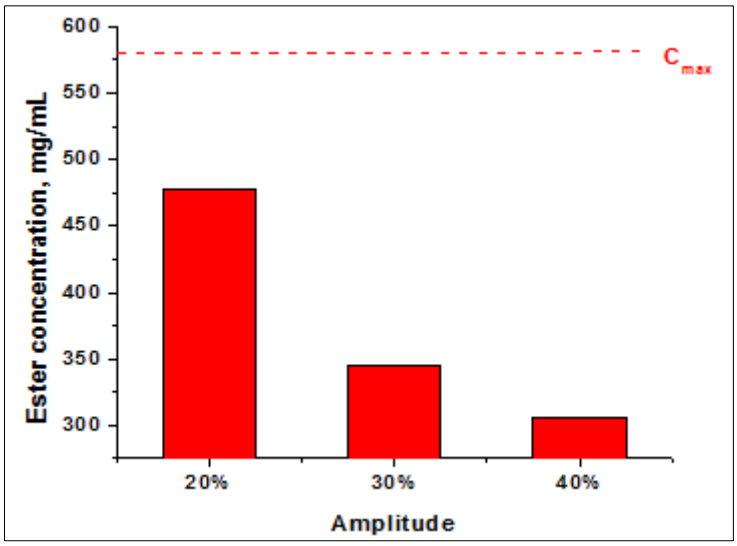

Figure 6. Influence of pulse frequency on ester concentration in ultrasound assisted esterification

As shown in Figure 6 the variation of amplitude significantly influences the concentration of the desired ester. Between amplitudes of 20% and 30% a sudden decrease in concentration is observed, and the decrease continues with increasing amplitude. The power density or applied power is reflected in the amplitude of the ultrasound and should be large enough to have cavitation. This might be explained by the fact that at higher ultrasound amplitudes (high ultrasonic power), cracking and destruction of enzyme can occurs while at low ultrasound power acts as nondestructive and non-invasive.

4. Conclusions

The present research was highlighted on intensifying the esterification process of the lipase catalysed synthesis of isoamyl acetate, an ester with commercial value as aroma (banana) flavour which is commonly used in food, pharmaceutical and cosmetic industries. Microwave and ultrasonication techniques were an added novelty to the conventional method of synthesis of isoamyl acetate from isoamyl alcohol and acetic acid catalysed by Lipozyme 435. The concetrations of isoamyl acetate obtained by unconventional methods were higher than those achieved under conventional heating. Under optimised conditions, maximum concetrations of isoamyl acetate is obtained in 1 h as 478 $mg_{ester}/g_{mixture}$ was obtained with ultrasounds using 25g/L of enzyme loading, at 50ºC as compared to 387 $mg_{ester}/g_{mixture}$ by conventional methods. These results shows a synergism between biocatalysis and unconventional esterification methods and the possible application of microwaves and ultrasounds for enzymatic synthesis of aroma esters.

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