Environmental Engineering and Management Journal

August 2016, Vol.15, No. 8, 1849-1854
http://omicron.ch.tuiasi.ro/EEMJ/

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ECO-FRIENDLY PRODUCTION OF CHEMICALS 1. IMPROVEMENT OF ENZYMATIC PRODUCTION OF ACETOPHENONE BY DIRECT EXTRACTION

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Abstract

Acetophenone can be enzymatically produced by conversion of methylbenzylamine using transaminase. The enzymatic process is strongly affected by the product inhibition, thus requiring the acetophenone removal from the media during its synthesis. In this purpose, the individual and selective extraction of acetophenone and methylbenzylamine with the biocompatible solvent n-heptane containing 1-octanol, D2EHPA or TOA has been analyzed at three values of pH (5, 7, and 9). Regardless of the solvent used and pH-value, the highest efficiency has been reached for extraction of acetophenone, the difference between the extraction yields of acetophenone and methylbenzylamine being amplified during the separation of these compounds from their mixture. On the basis of the experimental selectivity factors and taking into consideration both the possible loss of substrate from the media and the pH required for enzymatic reaction, pH = 7, it has been concluded that the optimum solvent combination is the mixture between n-heptane and 1-octanol. This solvent mixture allowed reaching high selectivity factor of 315, corresponding to the extraction yield of acetophenone of 94.5 % and of methylbenzylamine of only 0.3 %.

Key words: acetophenone, extraction, methylbenzylamine, 1-octanol, selectivity factor

Received: February, 2016; Revised final: August, 2016; Accepted: August, 2016

1. Introduction

Acetophenone is an aromatic ketone having important applications in chemical industry (as raw material for production of resins used in adhesives, inks, and coating manufacture), food and perfumery industries (as precursor of cherry, jasmine, almond or strawberry fragrances), pharmaceuticals production, and as reagent for various synthesis at laboratory scale (Burdock, 2005; Clark and McQuarrie, 2002; Gadamasetti and Braish, 2007; Siegel et al., 2002). This compound can be found in vegetables as apple, apricot, banana, and cauliflower, but the extraction from natural sources cannot respond to the increased demand for acetophenone (Garcia-Salas et al., 2010; Lasekan et al., 2013).

At industrial level, acetophenone is obtained by oxidation of cumene or ethylbenzene, chemical synthesis from benzene and acetylchloride or acetic anhydride, catalytically from acetic and benzoic acids, or as by-product in the Hock phenol synthesis (Bryant et al., 2004; Clark and McQuarrie, 2002; Ogata, 2012; Siegel et al., 2002). Although the chemical synthesis methods allow obtaining the desired quantity of acetophenone, they require important materials consumption and, implicitly,

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high costs for materials and energy, and produce high amount of pollutants.

For these reasons, the microbial or enzymatic conversion of various substrates to acetophenone represents an important challenge for the traditional methods of this compound production, causing an increased interest from the researchers. Therefore, acetophenone can be obtained by fermentation of bacteria (*Arthrobacter sp*., *Comamonas sp.*, *Nocardia sp.*, *Pseudomonas sp*., etc.) or fungi (*Cunninghamella sp*., *Helminthosporium sp*., *Mortierella sp*.) grown on different substrates (hexadecane, ethylbenzene, toluene, cinnamic acid, amines) (Cripps et al., 1978; Cox and Parker, 1979; Farbood et al., 2002; Hilton and Cain, 1990; Holland et al., 1987; Jobst et al., 2012; Lee and Gibson, 1996). Recently, microbial technologies have been improved, from the point of view of production yield, purity, and environmental impact, by developing the enzymatic synthesis of acetophenone. In this purpose, aminotransferase or transaminase from *Escherichia coli* has been used in free or immobilized form (Halim et al., 2013; Kaulmann et al., 2013; Martin et al., 2007; Shin and Kim, 1997; Yun et al., 2003). One of the most attractive enzymatic synthesis of acetophenone is by methylbenzylamine conversion under transaminase action, according to the reaction mechanism shown in Fig. 1 (Halim et al., 2013).

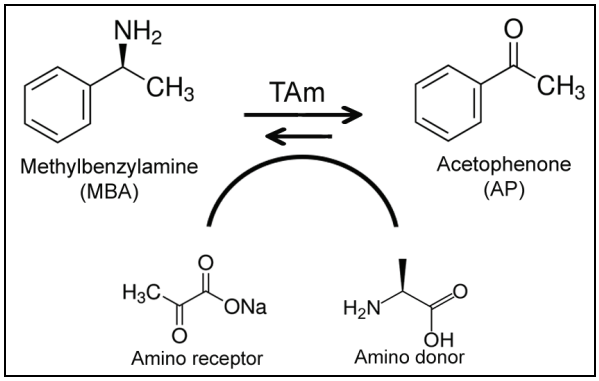

Fig. 1. Enzymatic conversion of methylbenzylamine to acetophenone using transaminase (TAm) (Halim et al., 2013)

The main problem of acetophenone biosynthesis is its severe inhibitory effect, which can affect the efficiency of microbial or enzymatic process (Shin and Kim, 1997). For removing acetophenone from the fermentation or enzymatic medium, several methods have been tested: vacuum distillation, physical extraction, separation through membranes or hollow-fiber membranes, selective inclusion in ß-cyclodextrin, and capillary electrophoresis (Chai and Ji, 2014; Shin and Kim, 1997; Sekar et al., 2013; Shin et al., 200; Yun et al., 2003). The extraction method seems to be the most efficient one, the extraction capacity and the biocompatibility being the main criteria required for solvent selection. However, the highest extraction yields of acetophenone were reached for solvents exhibiting a toxic effect against the microorganism or enzymes (benzene, toluene, cyclohexanone, dioctylphtalate) (Shin and Kim, 1997). The other mentioned separation methods do not allow reaching satisfactory efficiency for applying them at larger scale.

In this context, this work is dedicated to the study on the possibility to remove efficiently acetophenone from the enzymatic medium by extraction with a biocompatible solvent, namely n-heptane. For increasing the extraction efficiency, different extractants and a phase modifier have been added in the solvent phase, their effects being quantified in terms of extraction yield and selectivity. In the experiments, the enzymatic synthesis of acetophenone from methylbenzylamine has been considered.

2. Material and methods

The extraction experiments have been carried out using an extraction column with vibratory mixing, which offers high interfacial area and the possibility to reach rapidly the equilibrium state. The laboratory equipment has been described in detail in previous paper (Cașcaval et al., 2011). The phase mixing has been made by means of a perforated disk with 45 mm diameter and 20 % free section. The vibrations had a frequency of 50 s^{-1} and 5 mm amplitude. The perforated disk position has been maintained at the initial contact interface between the aqueous and organic phases. The extraction time has been 1 minute at a constant temperature of 25 ^{o}C. The resulted emulsion has been broken in a centrifugal separator at 5000 rpm.

The initial concentrations of methylbenzylamine (MBA) or / and of acetophenone in the aqueous solution were 3 g/l for each compound. These compounds have been extracted individually or selectively from their mixture.

The extraction has been carried out using four organic phases: n-heptane, mixture between n-heptane and 10 % vol. 1-octanol (phase modifier), mixture between n-heptane and 20 g/l di-(2-ethylhexyl) phosphoric acid (D2EHPA), and mixture between n-heptane and 20 g/l tri-n-octylamine (TOA) (extractants). The volumetric ratio of aqueous and organic phases has been of 1 (20 ml of each phase).

In all cases, identical extraction conditions have been used.

For respecting the enzymatic reaction pH (Halim et al., 2013), the pH-value of initial aqueous solutions has been 5, 7, and 9, respectively. The pH adjustment has been made with a solution of 3 % sulfuric acid or 3 % sodium hydroxide, depending on the prescribed pH-value. The pH-values were determined using a digital pH-meter of Consort C836 type and have been recorded throughout each experiment. Any pH change has been recorded during the extraction experiments.

The extraction process has been analyzed by means of the extraction yield and selectivity factor.

For calculating these parameters, the concentrations of methylbenzylamine and acetophenone in the initial aqueous solution and in the raffinate have been measured, the mass balance being applied for the entire extraction system. These compounds concentrations have been determined by high performance liquid chromatography technique (HPLC), according to the previous method described in literature (Halim et al., 2013).

Each experiment has been repeated for two or three times under identical conditions, the average value of the considered parameters being used. The maximum experimental error was 5.46 %.

3. Results and discussion

Depending on the extraction system characteristics, the separation by extraction occurs by means of the physical processes of diffusion and solubilization (physical extraction), or by means of the interfacial interactions of hydrogen, ionic exchange, and solvation types between the solute and the extractant (reactive extraction). The selection of physical or reactive extraction method for separating a given compound is conditioned by the solute physical or chemical properties (solubility, acidity index, chemical reactivity and stability, etc.), as well as by solvent or extractants characteristics (capacity of solubilization, dielectric constant, rate of interfacial reaction with solute, rate of solute release into the stripping phase, stability and solubility into the aqueous phase of the interfacial compound formed between solute and extractant, etc.) (Caşcaval et al., 2013; Poştaru et al., 2014). If the extraction is used for separating directly various compounds from microbial or enzymatic media, the solvent phase has to exhibit no toxicity against the microbial cells or enzymes.

In the case of enzymatic conversion of methylbenzylamine into acetophenone, for establishing the optimum conditions required for an efficient and selective removal of product during the enzymatic process, the individual extraction of the two compounds has been initially studied.

The results of individual extraction of MBA, for the tested solvents, are plotted in Fig. 2.

The yield of physical extraction of methylbenzylamine with n-heptane varies between 41 and 53 %, being slightly enhanced by increasing the pH-value of aqueous phase. Because n-heptane is a low-polar solvent (dielectric constant 1.90 at 25 ^{o}C (Weast, 1974)) which can solubilize especially the nondissociated molecules, the positive influence of the pH increase can be related to the reduction of amine protonation degree at pH-domain close to neutral one (pKa = 4.25 (Weast, 1974)).

The addition of the phase modifier 1-octanol increases the polarity of organic phase (dielectric constant of 1-octanol is 10.3 at 25 ^{o}C (Weast, 1974)), due to the superior ability of solvents with higher polarity to solubilize the dissociated molecules. Therefore, the physical extraction of methylbenzylamine is improved, its yield increasing with 7 to 15 % by varying the pH-value from 5 to 9.

The most important improvement of methylbenzylamine extraction has been recorded by using the reactive extraction with D2EHPA (Fig. 2). In this case, the extraction yield is enhanced with up to 32 % compared to the physical extraction with n-heptane, and with maximum 25 % compared to the physical extraction with the mixture between n-heptane and 1-octanol.

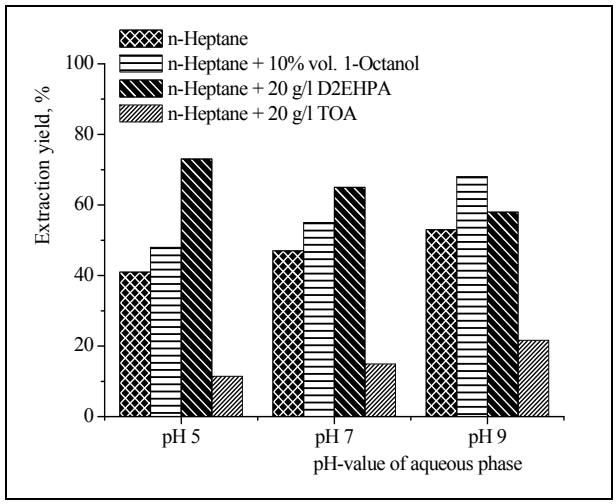

Fig. 2. Influence of pH-value on yields of methylbenzylamine individual extraction

Contrary to the above discussed systems of physical extraction, by varying the pH-value from 5 to 9, the efficiency of reactive extraction with D2EHPA is reduced. This influence is due to the mechanism of amine separation, which is based on the interfacial reaction of ionic exchange type (HP symbolizes the organophosphoric extractant):

$$C_6H_5\text{-}CH(NH_3^+)\text{-}CH_{3(aq)} + HP_{(o)} \rightleftharpoons C_6H_5\text{-}CH(NH_3{+}P^-)\text{-}CH_{3(o)} + H^+_{(aq)}$$

Similar to the reactive extraction of other compounds containing aminic groups in their molecules, the reactive extraction of methylbenzylamine with D2EHPA is possible only if the aminic group exists in the protonated form into the aqueous phase, respectively at pH-value lower than that corresponding to this amine pKa (Blaga et al., 2008). By increasing pH, the protonation of amine is affected, this compound becoming unable to react with the organophosphoric extractant. For this reason, at pH = 9, the yield of reactive extraction with D2EHPA is higher than that of extraction with n-heptane, but lower than that reached in presence of 1-octanol.

The behavior of extraction system containing TOA differs significantly from those above presented. According to Fig. 2, the efficiency of reactive extraction with this extractant is very low, the extraction yield increasing from 11 %, at pH = 5, to 22 %, at pH = 9. Although in this case the aminic

extractant cannot react with the aminic solute, it has been supposed that the extractant could solvate the solute and, implicitly, enhances its solubility in the organic phase. As it has been reported in literature, the aminic extractants are able to extract different solutes by their solvation without chemical interactions, by forming aminic aggregates especially in non- or low-polar solvents (Caşcaval et al., 2011; Poştaru et al., 2014).

The results plotted in Fig. 2 suggest that the addition of TOA hinders the methylbenzylamine extraction, reducing significantly the extraction yield compared to the other three analyzed systems. This effect can be explained by the presence of TOA in the organic phase which restricts additionally the limited ability of n-heptane to solve ionizable molecules as amines. Depending on the yield of acetophenone extraction, the negative influence of TOA on the methylbenzylamine extraction could represent an important factor enhancing the selectivity of the two compounds separation from their mixture.

Regardless of the used solvent, the yields of individual extraction of acetophenone are considerable higher than those of MBA extraction, being around 90% for all studied extraction systems (Fig. 3).

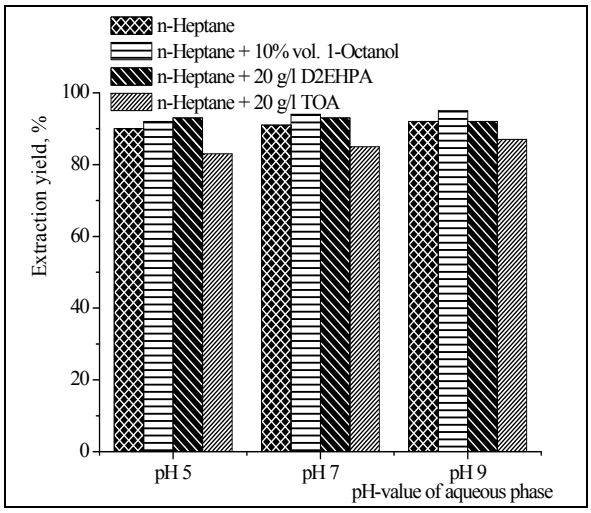

Fig. 3. Influence of pH-value on yields of acetophenone individual extraction

The analysis of acetophenone physical extraction indicates minor differences between the efficiencies of extractions with n-heptane or with n-heptane containing 1-octanol (the addition of 1-octanol leads to the increase of extraction yield with only 3 %). This result is correlated with the less important ionization of acetophenone in the considered pH-domain compared to methylbenzylamine (acetophenone pKa = 6.40 (Chai and Ji, 2014)), its solubility in the organic phase becoming higher than that of amine. For this reason, the addition of phase modifier does not enhance significantly the extraction degree.

The use of D2EHPA allows reaching an extraction yield of acetophenone with about 20 to 35 % greater than that corresponding to the reactive extraction of methylbenzylamine with the same extractant. Moreover, although the variation of pH-value from 5 to 9 induces the reduction of acetophenone extraction efficiency, it amplified the difference between the extraction yields of the two compounds. The more important positive effect of D2EHPA on acetophenone extraction is due to its superior ability to react with D2EHPA cumulated to its superior solubility in the n-heptane phase. Because the efficiency of reactive extraction with D2EHPA is comparable or below that of the physical extraction with the mixture between n-heptane and 1-octanol, it can be concluded that the relative magnitude of the positive influence of enhanced solubility of acetophenone in organic phase exceeds the effect of D2EHPA presence in this phase.

Similar to the extraction of methylbenzylamine, the use of TOA leads to the decrease of the acetophenone extraction yield compared to the other solvents (Fig. 3). But, this reduction is 5 to 10%, being considerably less pronounced than that recorded for methylbenzylamine. This result suggests that the higher solubility of acetophenone in organic phase counteracts the negative effect of TOA on the organic phase capacity to solve this compound, and can be considered an important premise for reaching high selectivity of acetophenone separation from the mixture with methylbenzylamine.

Consequently, by analyzing the individual physical or reactive extractions of methylbenzylamine and acetophenone, it can be concluded that the extraction of acetophenone is more efficient at the pH-value corresponding to the enzymatic reaction, the differences between the extraction yields of the two compounds suggesting the possibility to separate them selectively.

The study on the two compounds separation from their mixture indicates completely changed results for methylbenzylamine extraction, while for acetophenone the extraction efficiency remains rather similar to that corresponding to its individual extraction. Therefore, according to Fig. 4, the yields of methylbenzylamine physical or reactive extractions are strongly decreased compared to the former case, and do not exceed 33 %, value reached only for the reactive extraction with n-heptane and D2EHPA at pH = 9.

Moreover, the amine extraction yield becomes practically 0 (0.2 - 0.3 %) for the physical extraction with n-heptane and 1-octanol at pH = 5 and pH = 7.

These data confirm the lower extractability of methylbenzylamine in the considered solvents and extraction conditions, and suggest the supplementary reduction of the organic phase ability to induce the solvation and, implicitly, solubilization of this compound. This phenomenon could be the result of the change of the intermolecular interactions inside

the organic phase, due to the presence of extracted acetophenone, more soluble in the used solvents.

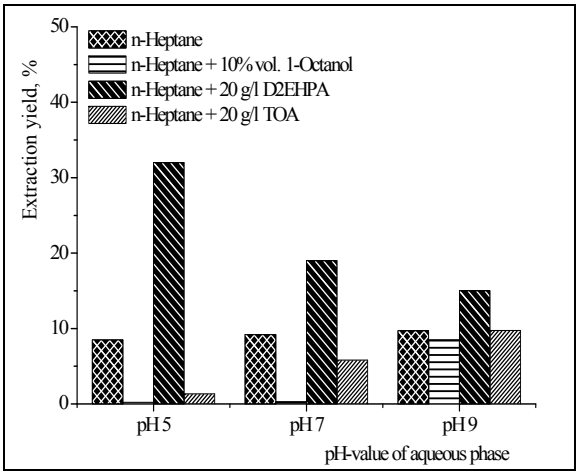

Fig. 4. Influence of pH-value on yields of methylbenzylamine extraction from mixture with acetophenone

As it was mentioned, the extraction of acetophenone occurs with similar high efficiency as in the case of its individual extraction (Fig. 5). For its separation from the mixture with methylbenzylamine, the extraction yield decreases with 4 – 5 % for the extractions with n-heptane and n-heptane containing D2EHPA, increases with about 2 % for the extraction with the mixture between n-heptane and 1-octanol, remaining the same for the extraction with n-heptane and TOA.

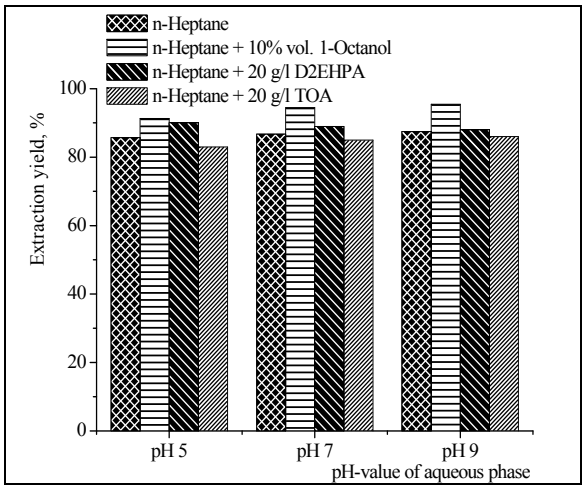

Fig. 5. Influence of pH-value on yields of acetophenone extraction from mixture with methylbenzylamine

According to the aforementioned reason, the slight change of the solvent phase capacity to solubilize acetophenone is due to the presence of co-extracted methylbenzylamine. These results indicate the possibility to extract selectively acetophenone from the enzymatic media containing its mixture with methylbenzylamine. For selecting the optimum extraction conditions, corresponding both to the highest selectivity of separation and proper efficiency of enzymatic reaction, the influences of the solvent type and pH-value on extraction selectivity have to be quantified. In this purpose, the selectivity factor has been use, being defined as the ratio between the extraction yield of acetophenone and that of methylbenzylamine.

Regardless of the solvent and pH-value, from Fig. 6 it can be seen that the maximum selectivity factor is reached for physical extraction in n-heptane containing the phase modifier, namely 1-octanol. By increasing the pH of aqueous solution from 5 to 9, the selectivity factor related to this solvent is diminished from 397 to 11.5, being 315 at the enzymatic reaction pH = 7 (the extraction yields are 94.5 % for acetophenone and 0.3 % for methylbenzylamine). As it was supposed on the basis of the results obtained for the individual extractions of the two compounds, another solvent which offers high selectivity of extraction is the mixture between n-heptane and TOA, the selectivity factor varying from 62 at pH = 5 to 9 at pH = 9 (at pH = 7, the selectivity factor is 15, being correlated with the extraction yields of 85 % for acetophenone and 5.7 % for methylbenzylamine). However, in this case compared to the use of n-heptane and 1-octanol, the aminic substrate could be partially removed by extraction from the enzymatic media, this affecting the enzymatic process.

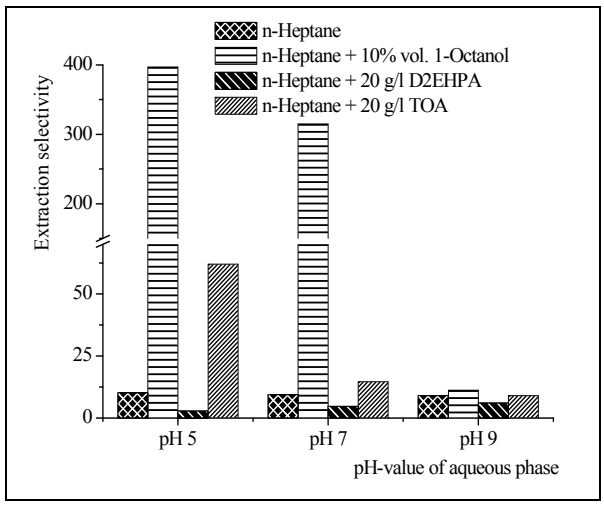

Fig. 6. Influence of pH-value on selectivity factor

4. Conclusions

The studies on acetophenone and methylbenzylamine extraction underlined the possibility to separate them selectively from the enzymatic media. Therefore, by using different solvents consisting of a biocompatible solvent (n-heptane) in which have been dissolved either 1-octanol, as phase modifier, or D2EHPA and TOA, as extractants, it was concluded that the highest extraction efficiencies are related to acetophenone.

The difference between the extraction yields of acetophenone and methylbenzylamine has been amplified during the separation of these compounds for their mixture, this difference allowing to reach high selectivity of extraction. By analyzing the

values of selectivity factor which correspond simultaneously to the avoidance of substrate removal from the media and to the optimum pH for enzymatic reaction, pH = 7, it was concluded that the most adequate solvent is the mixture between n-heptane and 1-octanol. In this case, the extraction yield of acetophenone is 94.5 %, while that of methylbenzylamine is reduced to 0.3 %.

Acknowledgements

This work was supported by the Grant ERA-NET, ERA-IB "MICROTOOLS" authorized by The Romanian Executive Unit for Financing Higher Education, Research, Development and Innovation (UEFISCDI).

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