Production of 2-phenylethanol (PEA) by yeast with ionic liquids in situ extraction

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Introduction

PEA is a valuable aroma of a subtle rose scent used in food, perfume and household chemicals, as well as a preservative (in medicines) and a disinfectant (e.g. for strawberries). High demand for PEA motivates the ongoing quest for new production methods. An alternative for synthetic PEA is obtaining this fragrance from rose petals. However, the costs of such production are very high – the price of rose oil (containing approx. 66–79% PEA [1]) is approx. 4,600 euro/kg [2]. The paper presents the application of yeasts for the PEA synthesis. The product of such synthesis, under the applicable law, is considered as a natural product [3]. The only problem is the low final concentration of the product. This can be solved using the *in situ* extraction [4]. There are the following methods for the PEA *in situ* extraction available: adsorption [5÷8], microcapsules [9, 10], membrane extraction [11], liquid-liquid extraction using oleic acid [12, 13] or ILs [4, 9, 14÷17]. These methods allow for the increase of the production of PEA to approx. 6 g/L in case of flask cultures and 12 g/L to 26 g/L for fed-batch cultures [18]. The study focuses on the liquid-liquid extraction using ionic liquids due to the possibility to match the ionic liquid for the purposes of research and the simplicity of this solution. The possibility to re-use the ionic liquid after the PEA re-extraction was another motive. The aim of the studies was to find the best ionic liquid for the extraction of PEA *in situ*.

Description

Experimental part

Materials

The studies involved use of YPD broth for yeast growth (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone), while the experiment itself was conducted using production broth (10 g/L glucose, 10 g/L sucrose, 6 g/L *L*–phenylalanine (*L*-Phe), 4 g/L KH_2PO_4, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L yeast extract). The studies involved the strain *Saccharomyces cerevisiae* AM1d from the collection of the Department of Drug Technology and Biotechnology. The following *in situ* extractants were used $[C_8iQuin][NTf_2]$ (the synthesis method and purity were presented in the paper [19]) [BMPyr][NTf_2] (Io-li-tec, >0.99), $[P_{6,6,6,14}][TCM]$(MERCK KGaA, ≥0.98 [20]), [HMPIP][NTf_2] (io-li-tec, >0.99[4]).

Methods

3 yeast colonies were transferred into a 100 mL Erlenmeyer flask containing 10 mL of YPD broth. The culture was left in an incubating shaker (30°C, 220 rpm, Lab Companion SI-600R). On the next day, the over-night culture was used to inoculate production broth in such a manner so the initial optical density of culture is 0.2, when measured at wavelength λ=600 nm (OD 600). The culture was cultivated in the incubating shaker (30°C, 220 rpm). OD_{600} of culture was measured every 1 hour. When OD_{600} reached 1, the culture was poured into 4 flasks (100 mL): 15 mL to each reference flask and 12 mL to each test flask, followed by the addition of 3 mL of the extractant to the test flasks. The cultures were cultivated in two biological replicates. In the extractant-free sample, the yeast growth was measured (OD_{600} measurements for 8 hours). The cultures were centrifuged (25°C, 1000 *g*, 2 min, Eppendorf Centrifuge 5804R) after 24 hours and 48 hours in order to separate the phases and OD_{600} of the aqueous phase mesurment. Then, they were re-centrifuged (25°C, 2000 *g*, 3 min) in order to separate biomass. The phases were collected into test tubes, followed by the addition of an equal volume of acetonitrile (2-fold dilution). The final concentration of 2-fenylethanol in both phases was determined by means of HPLC (Agilent Technologies 1200 series, column C18, 25°C, 1 mL, acetonitrile:water = 50:50) at wavelength λ=220 nm.

Result discussion

Completely new ionic liquids were used as potential PEA *in situ* extractants. The results of cultures presented (Fig. 1) clearly show that the addition of ionic liquids may have various effects on yeast growth. Two ionic liquids [BMPyr][NTf_2] (OD_{600}^{24h}=1.78, OD_{600}^{48h}=1.92), and [HMPIP][NTf_2] (OD_{600}^{24h}=1.78, OD_{600}^{48h}=1.86) intensified the yeast growth, while the other two $[C_8iQuin][NTf_2]$ (OD_{600}^{24h}=1.36, OD_{600}^{48h}=1.50) and $[P_{6,6,6,14}][TCM]$ (OD_{600}^{24h}=1.42, OD_{600}^{48h}=1.43) slowed down the yeast growth in comparison with the reference (OD_{600}^{24h}=1.64, OD_{600}^{48h}=1.65), as it is clearly shown in Figure 1. The slowed growth could have been caused by a need of additional adaption of yeast to these specific ionic liquids. On the other hand, the intensified growth is probably a result of low PEA concentration in the aqueous phase, thus eliminating its inhibiting effect on the yeast growth occurring for the concentrations of approx. 2–3 g/L[21].

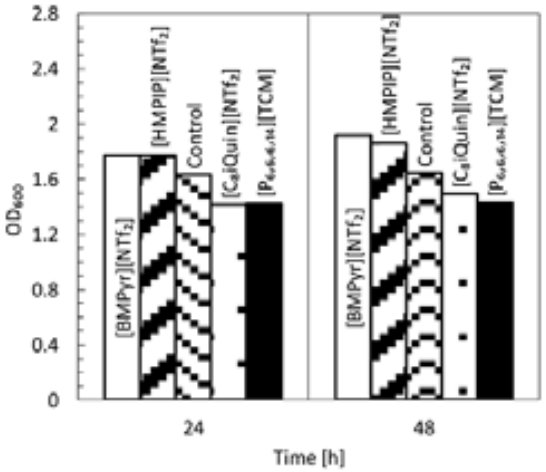

Fig. 1. Culture growth presented by optical density at wavelength λ = 600nm

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For three of the ionic liquids tested after 24h of the cultivation, the PEA concentrations obtained (in grams per litre of culture) were much higher than for the reference sample (no extractant) – 2.10 ± 0.009 g /L. These were in the descending order [BMPyr][NTf_2] (in the IL phase 6.71 ± 0.006 g PEA/L), [HMPIP][NTf_2] (in the IL phase 6.21 ± 0.010 g /L), [C_8iQuin][NTf_2] (in the IL phase IL 3.89 ± 0.004 g/L). For [$P_{6,6,6,14}$][TCM], the PEA concentration in the IL phase after 48-hour culture was only 0.05 ± 0.005 g/L. 2-phenylethanol was still produced, which is indicated by much higher concentrations after 48 hours. The PEA concentration in samples after 48 hours depending on the ionic liquid in the descending order: [BMPyr][NTf_2] (in the IL phase 10.25 ± 0.019 g PEA/L), [HMPIP][NTf_2] (in the IL phase 6.21 ± 0.009 g /L), [C_8iQuin][NTf_2] (in the IL phase 3.89 ± 0.007 g/L), reference (no extractant) 2.10 ± 0.014 g/L, [$P_{6,6,6,14}$][TCM] (in the IL phase 1.92 ± 0.007 g/L). The results are presented in Figure 2.

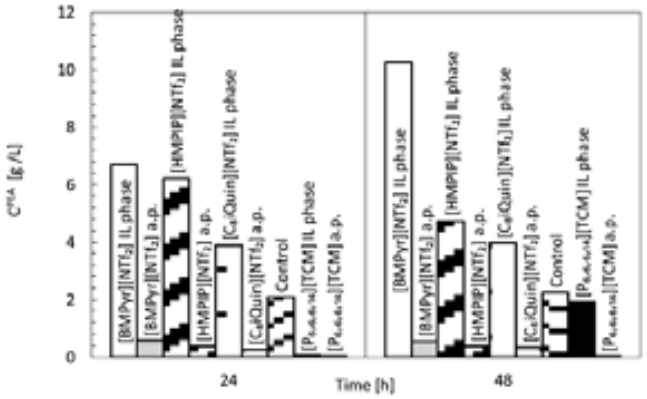

Fig. 2. PEA concentration after 24 hours and 48 hours in grams per litre of culture in the aqueous phase (a.p.) and the ionic liquid phase (IL phase)

The bioconversion results *L*-Phe to PEA (Fig. 3) at 70% for [BMPyr][NTf_2] after 48 hours show that a further conversion was still possible. Therefore, we plan to set new 72-hour cultures. The organic phase/aqueous phase partition coefficients for PEA (Fig. 4) indicate a good PEA extraction by the selected ionic liquids zamiast good PEA extraction by the ionic liquids selected. For [$P_{6,6,6,14}$] [TCM] after 48 hours, the value of the partition coefficient was at its maximum, however, as already mentioned, the total PEA concentrations in the culture were very low, which makes it useless for the *in situ* extraction.

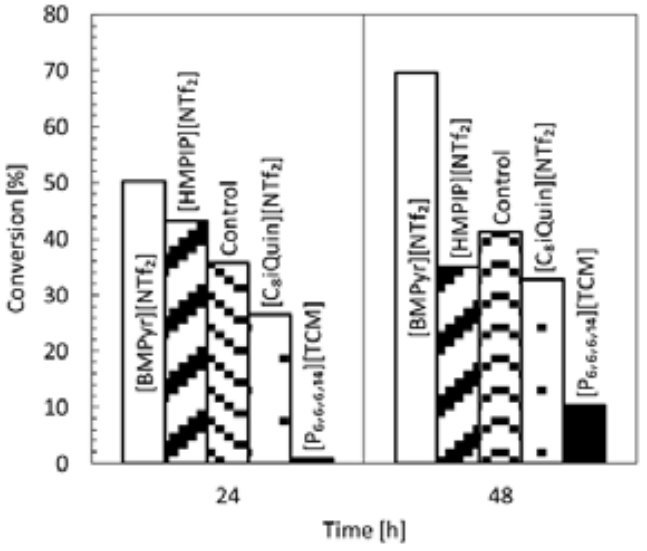

Fig. 3. Bioconversion percentage: *L*–phenylalanine (*L*-Phe) to 2-phenylethanol (PEA)

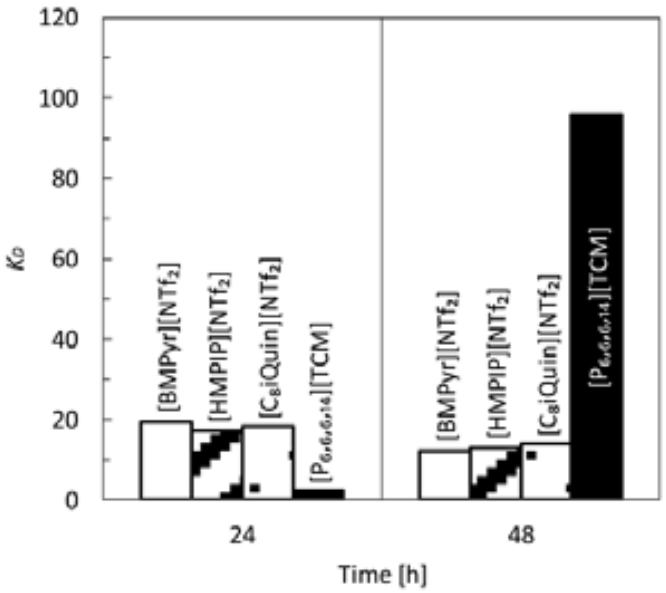

Fig. 4. IL phase/aqueous phase partition coefficient (K_D) for 2-phenylethanol (PEA)

Summary and conclusions

None of the ionic liquids studied proved to be toxic for the yeast *Saccharomyces cerevisiae* AM1d. The results presented clearly show that the best extractant for the *in situ* extraction of PEA is butyl-methyl pyrollidinium bis(trifluoromethylsulfonyl)imide [BMPyr][NTf_2]. This is shown by a high *L*–Phe conversion (50% after 24 hours and 70% after 48 hours), high PEA concentration in the ionic liquid phase (6.71 ± 0.006 g/L after 24 hours and 10.25 ± 0.019 g/L after 48 hours), as well as a high IL phase/aqueous phase partition coefficient for PEA (K_D = 12.22 after 24 hours and K_D = 19.4 after 48 hours). Another promising extractant is [HMPIP] [NTf_2], as indicated by a high *L*–Phe conversion (43% after 24 hours and 35% after 48 hours), close to the reference sample, high PEA concentration in the ionic liquid phase (4.74 ± 0.009 g/L after 24 h and 6.21 ± 0.010 g/L after 48 h), as well as a high IL phase/aqueous phase partition coefficient for PEA (K_D = 17.01 after 24 h and K_D = 12.97 after 48 h). However, the conversion percentage of *L*–Phe (a maximum of 70% after 48 hours) suggests that the culture should be cultivated for longer (e.g. for 72 hours) in order to fully use the substrate. The ionic liquids studied in this work proved to be much better than previously tested ionic liquids containing the same anion and the following cations: 1-benzyl-3-methylimidazolium (~1.2 g PEA/L, K_D = 17.6), 1-methyl-1-propylpiperidinium (~1.2 g PEA/L, K_D = 11.6), methyltrioctylammonium (~1.0 g PEA/L, K_D = 4.0) [15].

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