

Bioproduction of 2-Phenylethanol in a Biphasic Ionic Liquid Aqueous System

MOR SENDOVSKI, NETTA NIR, AND AYELET FISHMAN*

Department of Biotechnology and Food Engineering, and Institute of Catalysis, Science and Technology,
Technion—Israel Institute of Technology, Haifa 32000, Israel

2-Phenylethanol (PEA) is a commercial flavor and fragrance compound, with a rose-like odor, used in the cosmetics and food industries. *Saccharomyces cerevisiae* strains produce PEA in a growth-associated manner but are prone to product inhibition, resulting in low production yields. The aim of this study was to use immiscible ionic liquids (ILs) in a biphasic system to enhance the PEA concentration by means of *in situ* product removal (ISPR). Nine ILs were tested for their influence on growing yeast cells, and five of them were found to be biocompatible. A correlation between the IL structure and the effect on yeast growth was investigated. $[\text{Tf}_2\text{N}]$ anions were found to be the most biocompatible in comparison to $[\text{PF}_6]$ and $[\text{BF}_4]$, and the pyridinium and ammonium cations were slightly preferable than the imidazolium cation. Furthermore, the longer the alkyl side chain on the imidazolium ring, the less it is biocompatible, with major significance above six carbons. The five biocompatible ILs were tested for PEA recovery capability by determining their distribution coefficients (K_D), with the highest value of 17.6 obtained for BMIM $[\text{Tf}_2\text{N}]$. Finally, ILs were tested for their efficiency as ISPR solvents under stress conditions of a high product concentration. A 3–5-fold increase in the total PEA concentration produced by the cells was obtained with MPPyr $[\text{Tf}_2\text{N}]$, OMA $[\text{Tf}_2\text{N}]$, and BMIM $[\text{Tf}_2\text{N}]$, demonstrating the potential of ILs for enhancing productivity in bioprocesses using growing cells.

KEYWORDS: Ionic liquid; 2-phenylethanol; *Saccharomyces cerevisiae*; *in situ* product removal; extraction

INTRODUCTION

2-Phenylethanol (PEA) is an important flavor and fragrance compound, with a rose-like odor (1). The fragrance of roses is highly popular and desired, making PEA one of the most commercially used fragrance chemicals in perfume and cosmetics (2). It is also used in flavor compositions of food products, such as soft drinks, cookies, chewing gum, pudding, and more. PEA occurs naturally in the essential oils of many flowers and plants, such as roses, jasmine, narcissi, and lilies, but in most cases, concentrations are too low to justify extraction. Most commercial PEA is produced using chemical methods, via a Friedel–Craft reaction of ethylene oxide with benzene in the presence of molar quantities of aluminum chloride or by catalytic reduction of styrene oxide with Raney nickel as a catalyst (1). Both methods involve toxic reagents and harsh conditions, thereby creating a byproduct that reduces the quality of the final PEA. Removal of the undesired contaminants is necessary before marketing the product (1). Consequently, a biotechnological route may provide natural PEA at high purity by an environmentally friendly process.

It has been demonstrated previously that various yeast strains produce PEA from L-phenylalanine by the action of three enzymes via the Ehrlich pathway (3, 4). In this metabolic pathway,

L-phenylalanine (L-Phe) is transaminated to phenylpyruvate by a transaminase, decarboxylated to phenylacetaldehyde by a decarboxylase, and subsequently, reduced to PEA by a dehydrogenase. Synthesis of PEA in the cell depends upon the nitrogen source in the medium. Only if amino acids represent the sole nitrogen source does the Ehrlich pathway predominate (1). PEA production by yeast is growth-associated (2) and yeast-type-dependent (4, 5). The production of PEA from L-Phe by yeast is limited by the inhibitory effects of PEA on the cell (6). Damage to cell membranes (7), reduced uptake of glucose and amino acids (8), and reduction in respiratory capacity (9) are some mechanisms involved in this inhibition phenomena.

We have recently described a stress-tolerant *Saccharomyces cerevisiae* strain (Ye9-612) capable of producing 4.5 g/L PEA in a fed-batch fermentation (10). To further improve productivity, *in situ* product removal (ISPR) is necessary (11). Biphasic systems employing oleyl alcohol or oleic acid have been used previously to increase the PEA productivity of various yeast strain cultures by 3–4-fold (6, 12). Because the yeast cells need to be in viable and growing status to produce PEA, the ISPR solvent should have both good extracting capacity and biocompatibility with the yeast. Ionic liquids (ILs) have been studied extensively as a medium for enzyme catalysis in view of the fact that enzyme activity in a non-aqueous environment is well-documented (13, 14). In contrast, there is limited information on the use of ILs with whole-cell biotransformations, and reports are mostly restricted

*To whom correspondence should be addressed. Telephone: 972-4-829-5898. Fax: 972-4-829-3399. E-mail: afishman@tx.technion.ac.il.

Table 1. ILs Used in the Research To Create Biphasic Systems with an Aqueous Medium

IL	Structure
1-Butyl-3-methylimidazolium PF ₆ (BMIM[PF ₆])	
1-Hexyl-3-methylimidazolium PF ₆ (HMIM[PF ₆])	
1-Methyl-3-Octylimidazolium PF ₆ (OMIM[PF ₆])	
1-Decyl-3-methylimidazolium PF ₆ (DMIM[PF ₆])	
1-Benzyl-3-methylimidazolium BF ₄ (Benzyl-MIM[BF ₄])	
1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (BMIM[Tf ₂ N])	
Methyltrioctylammonium bis(trifluoromethylsulfonyl)imide (OMA[Tf ₂ N])	
1-Methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide (MPPyr[Tf ₂ N])	
Trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate (CYPHOS IL-104)	

to nongrowing cultures (resting cells) (15–17). For example, a biphasic system for the asymmetric reduction of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol was studied with *S. cerevisiae* using BMIM[PF₆] (18) or nine different ILs with recombinant *Escherichia coli* cells (19). Pfruender et al. examined the viability of *E. coli* or *S. cerevisiae* after exposure to various ILs but not the ability to grow and produce metabolites in their presence (18). Two recent works used ILs in two-phase systems comprising growing whole cells. Baumann et al. tested the biocompatibility of phosphonium ILs on three xenobiotic-degrading bacteria during growth (20). CYPHOS IL 109 [trihexyl(tetradecyl)phosphonium (Tf₂N)] was found to be biocompatible and was subsequently used in a two-phase partitioning bioreactor for phenol degradation. The phenol degradation was at rates very similar to previously shown aqueous–organic solvent systems (20). Stephens' group recently showed that water-immiscible ILs, [NMeOct₃][Tf₂N] and [P_{6,6,6,14}][Tf₂N], are biocompatible with *E. coli*, and the latter IL was used successfully on a large scale to improve productivity and yield in the toluene dioxygenase-catalyzed conversion of toluene to toluene *cis*-glycol using a recombinant *E. coli* strain (21).

Roosen et al. pointed out in their recent review on ILs in biotechnology that “especially the combination of reaction and

separation will be of interest in the future as here the potential of ILs can be exploited to full extent” (15). Conforming to this view, the aim of the present work was to expand the use of ILs as ISPR solvents for the growth-associated bioproduction of PEA. Furthermore, the effect of nine ILs on the growth of *S. cerevisiae* was examined, and a correlation between IL structure and its biocompatibility was sought.

MATERIALS AND METHODS

Chemicals. Oleic acid, oleyl alcohol, L-Phe, and PEA were purchased from Sigma-Aldrich Chemical Co. (Sigma-Aldrich, Rehovot, Israel). All materials used were of the highest purity available and were used without further purification.

Nine ILs were used in this research (Table 1), seven of which were purchased from Iolitec, Denzlingen, Germany. 1-Benzyl-3-methylimidazolium BF₄ was purchased from Chemada, Israel, and methyltrioctylammonium bis(trifluoromethylsulfonyl)imide was purchased from Sigma-Aldrich Chemical Co. (Sigma-Aldrich, Rehovot, Israel).

Growth Media. Yeast peptone dextrose (YPD) medium contained: 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, and 20 g/L agar, for solid plates. Nitrogen essential optimal (NEO) medium (for PEA production from L-Phe, 10) contained: 20 g/L glucose, 4 g/L L-Phe, 4 g/L KH₂PO₄, 0.4 g/L MgSO₄·7H₂O, and 1 g/L yeast extract.

Yeast Growth in the Presence of Selected ILs. The yeast strain used in this research was *S. cerevisiae* Ye9-612 (10). Yeast cells were initially streaked from glycerol stocks ($-80\text{ }^{\circ}\text{C}$) on YPD plates and incubated at $30\text{ }^{\circ}\text{C}$ overnight. A starter was prepared by inoculation to 10 mL of liquid YPD medium for overnight growth at $30\text{ }^{\circ}\text{C}$ with shaking at 250 rpm (Orbital Shaker Incubator, MRC, Holon, Israel). Cells were inoculated to 27 or 24 mL of liquid YPD medium to obtain an initial OD_{600} of 0.1. The flasks were shaken at 250 rpm at $30\text{ }^{\circ}\text{C}$, and the absorbance at 600 nm was measured every 90 min in the aqueous phase. A total of 3 or 6 mL of IL were added to flasks at either the beginning (with the yeast inoculation) or at $\text{OD}_{600} = 1$. Growth was carried out for 72 h. A standard curve was created in which optical density (at 600 nm) measurements were correlated to dry cell weight concentrations (gram of dry weight per liter, g of dw/L). Dry weight of biomass was determined in duplicate samples of 10 mL each by centrifuging for 10 min at 13400g (Sigma, 4K15 centrifuge with 12172-H rotor, Osteroid, Germany). The pellets were washed twice with distilled water by resuspension and centrifuged again at the same conditions. The wet pellets were dried for 24 h in an oven at $90\text{ }^{\circ}\text{C}$ (MRC Ltd., Holon, Israel) after which they were weighed. Subsequently, all OD_{600} measurements were converted to dry cell weight (DCW) concentrations using the determined correlation factor of $0.41\text{ g of dw L}^{-1}\text{ OD}^{-1}$ (10). Experiments were conducted in duplicates ensuring a standard deviation lower than 10%. Live cell counts during yeast growth were performed by diluting a sample from the aqueous phase and spreading $100\text{ }\mu\text{L}$ on YPD plates using a Drigalski spatula. Plates were incubated at $30\text{ }^{\circ}\text{C}$, and colonies were counted after 24 h.

Analytical Methods. Conversion of L-Phe to PEA was determined by high-performance liquid chromatography (HPLC) with an Agilent 1100 series instrument (Agilent Technologies, Santa Clara, CA) using an Eclipse XDB C18, $5\text{ }\mu\text{m}$, $4.6 \times 150\text{ mm}$ column (Agilent Technologies, Santa Clara, CA). An isocratic method comprising 70:30 water/acetonitrile was used. A diode array detector was used at a fixed wavelength of 215 nm. A total of $1\text{ }\mu\text{L}$ of filtered samples was injected to the column, and under these conditions, L-Phe eluted at 1.3 min and PEA eluted at 4.2 min.

For quantification of PEA in four ILs, an isocratic method of 70:30 water/ACN was used and PEA eluted at 4.2 min. For quantification of PEA in BMIM[Tf_2N], 95:5 water/ACN was used and PEA eluted at 45.4 min.

PEA Distribution Coefficients in ILs. A 10 mL mixture of 20% organic media (IL or other) and 80% water was prepared in 16 mL glass vials. To the mixture, $30\text{ }\mu\text{L}$ (0.25 mmol) of PEA were added. The vials were shaken at $25\text{ }^{\circ}\text{C}$ and 1000 rpm for 1 h (Vibramax 100, Heidolph, Germany), and phase separation was facilitated by a short centrifugation step ($13000g$ for 10 min) (Minispin, Eppendorph, Hamburg, Germany). The IL phase was diluted by 2-fold in ACN, and the PEA concentration (mM) in each phase was analyzed by HPLC.

PEA Production in the Presence of ILs. Yeast cells were initially streaked from glycerol stocks ($-80\text{ }^{\circ}\text{C}$) on YPD plates and incubated at $30\text{ }^{\circ}\text{C}$ overnight. A starter was prepared by inoculation to 10 mL of liquid YPD medium for overnight growth at $30\text{ }^{\circ}\text{C}$ with shaking at 250 rpm. Cells were inoculated to 20 mL of liquid NEO media to obtain an initial OD_{600} of 0.1, in a 250 mL flask to allow for maximal oxygen transfer. The flasks were shaken at 250 rpm and $30\text{ }^{\circ}\text{C}$, and the absorbance at 600 nm was measured at 0, 7, and 72 h. A total of 5 mL (20%, v/v) of IL or another solvent was added to flasks at $\text{OD}_{600} = 1$. After 72 h, the broth was centrifuged and samples from each phase were analyzed by HPLC for PEA concentration.

PEA Production under Stress Conditions with ISPR. Yeast cells were initially streaked from glycerol stocks ($-80\text{ }^{\circ}\text{C}$) on YPD plates and incubated at $30\text{ }^{\circ}\text{C}$ overnight. A starter was prepared by inoculation to 10 mL of liquid YPD medium for overnight growth at $30\text{ }^{\circ}\text{C}$ with shaking at 250 rpm. Cells were inoculated to 24 mL of liquid NEO media at an initial OD_{600} of 0.1 in a 100 mL flask. To these experimental flasks, PEA was added at an initial concentration of 2.5 g/L in the aqueous phase. The flasks were shaken at 250 rpm at $30\text{ }^{\circ}\text{C}$, and the absorbance at 600 nm was measured at 0, 5, and 72 h in the aqueous phase. A total of 6 mL of IL (20%, v/v) was added to flasks after 5 h. Growth was carried out for 72 h, after which the broth was centrifuged and samples from each phase were analyzed by HPLC for PEA concentration.

RESULTS AND DISCUSSION

Although the solvent in an ISPR system is present as a second phase, molecular and phase toxicity to the biocatalyst are likely.

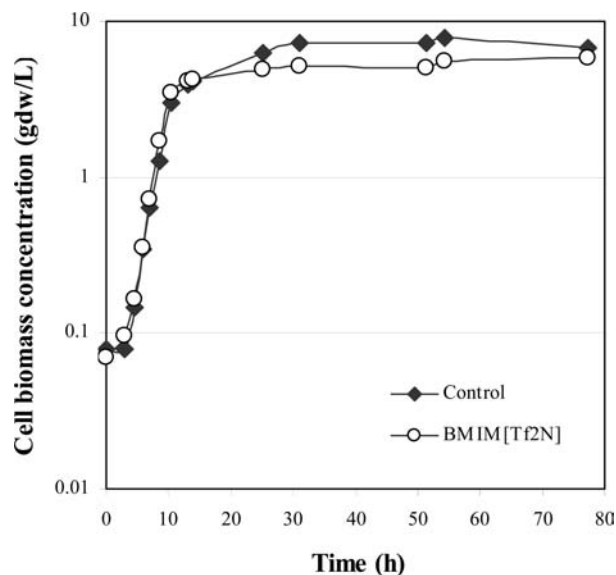


Figure 1. Typical growth curves of strain Ye9-612 in a biphasic system comprising 20% (v/v) IL in the aqueous medium (○) in comparison to a control (◆) without IL. Cells were grown on YPD medium with an initial DCW = 0.05 g of dw/L. The IL was added at DCW = 0.5 g of dw/L.

Therefore, the biocompatibility of the solvent to be used is the most important factor in a whole-cell biocatalytic process. In growth-associated biotransformations, such as in L-Phe conversion to PEA, it is crucial that growth is unaffected for the biotransformation to occur and to reach high product yields. It was discovered previously that PEA production becomes significant at mid-log growth phase (10), and therefore, to limit the exposure of cells to the IL, the latter was added to the medium at a cell biomass concentration of 0.5 g of dw/L. The cell biomass concentration was measured in the aqueous phase of the biphasic systems comprising 10 or 20% IL (v/v). **Figure 1** presents a typical growth curve of yeast in the presence of 20% BMIM[Tf_2N], in comparison to the control without a second phase. Similar curves were obtained for MPPyr[Tf_2N] and OMA[Tf_2N] (results not shown). **Figure 2** presents the growth curves of yeast in the presence of 20% ILs containing the [PF_6] anion. Both 10 and 20% IL resulted in similar growth curves for all ILs tested (results not shown), and therefore, 20% IL was used in future experiments to ensure better extraction of PEA from the aqueous phase. It is evident from **Figures 1** and **2** that the growth rate is mostly unaffected by the presence of the IL but the final cell concentration is in some cases lower than the control. The effect of 20% (v/v) ILs on yeast growth was examined for nine different ILs: three containing the [Tf_2N] anion with different cations, four comprising the same anion [PF_6] and with an imidazolium cation of increasing alkyl chains, and two other ILs (**Figure 3**). The ILs containing the [Tf_2N] anion showed good biocompatibility accompanied by a mild decrease in the final cell concentration, correlating well with the results of Pfruender et al. for resting yeast cells in the presence of OMA[Tf_2N] and BMIM[Tf_2N] (18). In contrast, BMIM[Tf_2N] was reported previously to be toxic to growing *E. coli* cells (21). Apparently, the robust yeast cell wall protects it from the inhibitive effects of the ILs in comparison to the more vulnerable *E. coli* cell membrane.

Benzyl-MIM[BF_4], DMIM[PF_6], and CYPHOS IL-104 inhibited growth of *S. cerevisiae* completely following their addition to the growth medium at mid-log phase. The last IL was previously shown to inhibit growth of *Pseudomonas putida* S12TPL (20). The cell viability in the presence of DMIM[PF_6] and benzyl-MIM[BF_4] was measured after a 25 h exposure. The cell count

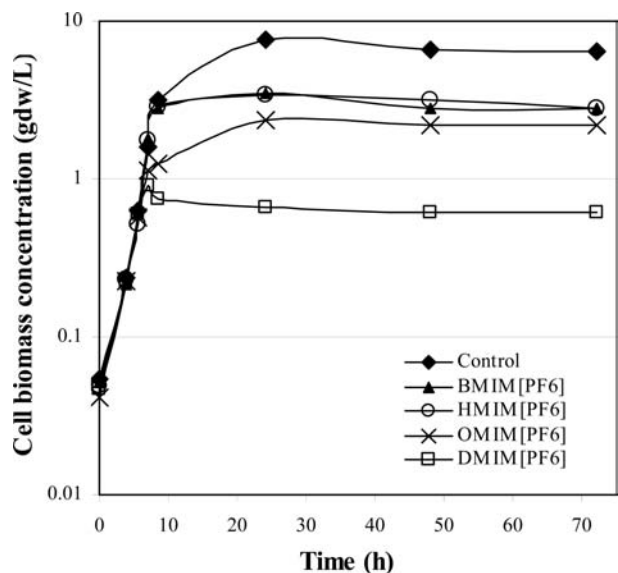


Figure 2. Growth curves of strain Ye9-612 in a biphasic system comprising 20% (v/v) ILs with a [PF₆] anion and with an imidazolium cation of increasing alkyl chains [4 (▲), 6 (○), 8 (×), or 10 (□) carbons] in comparison to a control (◆) without IL. Cells were grown on YPD medium with an initial DCW = 0.05 g of dw/L. The IL was added at DCW = 0.5 g of dw/L.

measured as colony forming unit (CFU)/mL decreased from 4.36×10^7 to 4.35×10^3 in benzyl-MIM[BF₄] and from 2.71×10^7 to < 10 in DMIM[PF₆]. In the case of DMIM[PF₆], there were no live cells, indicating that the IL was lethal to them. In the presence of benzyl-MIM[BF₄], some yeast cells still survived but the live count was 4-fold less than the control, suggesting the IL is toxic to the cells but to a lesser extent than DMIM[PF₆]. These results explain the lack of growth presented in **Figure 3**.

In the presence of BMIM[PF₆] or HMIM[PF₆], there was no difference in the growth rate but a marked decrease in the final cell concentration to 40% of the control was measured. This observation of unchanged growth rate accompanied by a reduction in microbial yield was previously reported by our group upon the application of thermal stress on *S. cerevisiae* yeast (22). The effects of BMIM[BF₄] and BMIM[PF₆] on the growth of three commonly used microorganisms in biotransformations, *E. coli*, *Pichia pastoris*, and *Bacillus cereus*, have recently been examined by Ganske and Bornscheuer (23). BMIM[BF₄], a miscible IL, was toxic and hence delayed growth in all three organisms at a mere 1% concentration. BMIM[PF₆], an immiscible IL, was toxic to *E. coli* growth, in contrast to earlier reports that showed *E. coli* cell viability was maintained in this IL (18). This suggests that the IL may retard growth but will maintain viability of microorganisms. A concentration of 10% BMIM[PF₆] did not affect the growth of *P. pastoris*, similar to the results presented here for *S. cerevisiae* Ye9-612, but was not biocompatible with the two prokaryotes (23). In addition, while testing the effect of BMIM[PF₆] on *P. pastoris*, Ganske and Bornscheuer obtained a similar trend to the one reported here, of unchanged growth rate accompanied by a decrease in the final cell OD for IL concentrations of 0.1 and 1.0%. Lenourry et al. reported that BMIM[PF₆] inhibited caffeate reduction by *Sporomusa termitida* and suggested that the inhibitory effects of [PF₆] are due to its release of toxic HF through hydrolysis (24).

For the first time, a correlation between the immiscible IL structure and its effect on yeast growth was sought. Four imidazolium ILs with an alkyl chain length of 4, 6, 8, or 10 carbons on the cation were tested (BMIM[PF₆], HMIM[PF₆], OMIM[PF₆], and DMIM[PF₆], respectively) (**Figure 2**). At 4 and

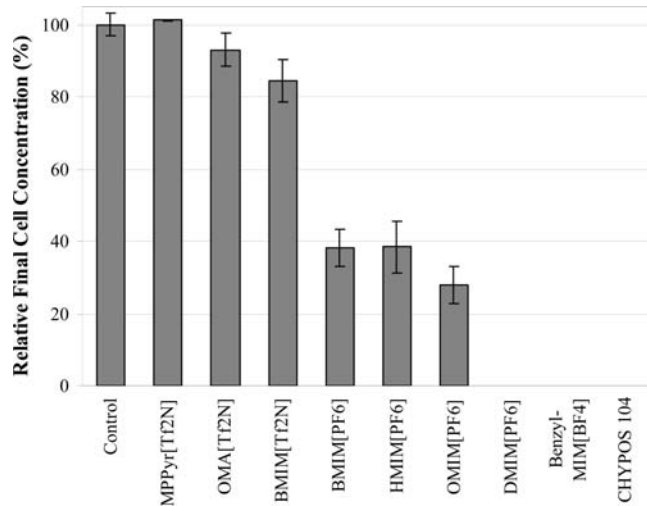


Figure 3. Relative final cell concentration in the presence of various ILs at 20% (v/v). Cells were grown on YPD media with an initial DCW = 0.05 g of dw/L. IL was added at DCW = 0.5 g of dw/L. Experiments lasted 72 h. Results represent an average of duplicates. The cell concentration value representing 100% was 11.3 g of dw/L.

6 carbons on the side chain, a similar effect was observed (60% lower final cell biomass concentration or no change in the growth rate), but at 8 carbons, a significant effect on the growth rate was observed (approximately a 75% decrease), as well as a decrease of 70% in the final cell biomass. At 10 carbons, the IL was completely toxic to the yeast. It is hypothesized that the effect of the IL in the case of BMIM[PF₆] and HMIM[PF₆] is pronounced in later stages of the growth, causing them to enter the stationary phase earlier, resulting in a lower final cell concentration. A live count performed after exposure of the culture for 24 h to the ILs also supported the OD measurements in all four solvents (results not shown). Because DMIM[PF₆] completely inhibited cell viability, it is speculated that the long hydrophobic chain penetrates the cell membrane and kills the yeast cell in a manner similar to cationic detergents (25). Docherty and Kulpa showed a similar tendency for imidazolium ILs, with a Br⁻ anion on *Vibrio fischeri* resting cells at 5–15 min exposure (25). Although the ILs tested in that study were miscible with water (because of the Br⁻ anion), the trend observed was an increase in toxicity with the increase in hydrophobicity, corresponding to the increase in alkyl chain length, as presented here. A comparison of two different anions to an imidazolium cation (BMIM[PF₆] versus BMIM[TF₂N]) revealed that the anion [TF₂N] significantly contributed to the biocompatibility with yeast growth (**Figure 3**). This again substantiates the hazardous nature of the anion [PF₆], as was reported earlier (24). The three ILs comprising the [TF₂N] anion with different cations had a similar influence on yeast growth (less than 15% decrease in the final cell biomass concentration). Pyridinium or ammonium cations were slightly preferable than the imidazolium cation; however, all three ILs were highly biocompatible and were therefore employed in further experiments studying PEA production.

To further investigate the effect of ILs on the growth of yeasts, selected ILs (OMA[TF₂N] and BMIM[TF₂N]) were added with cell inoculation. The growth rate was still unaffected by the presence of IL (results not shown), and the final cell concentrations were as shown before.

The distribution coefficient of PEA in a biphasic IL aqueous system is another important parameter when searching for a suitable ISPR solvent. The distribution coefficient (K_D) of PEA in each IL was examined in comparison to ethyl acetate and other

Table 2. Distribution Coefficients of PEA in Various Biphasic Systems^a

solvent	distribution coefficient (K_D) ($C_{\text{solvent}}/C_{\text{water}}$)
ethyl acetate	20.4
oleic acid	6.5
oleyl alcohol	11.8
OMA[Tf ₂ N]	4.0
BMIM[Tf ₂ N]	17.6
MPPyr[Tf ₂ N]	11.6
BMIM[PF ₆]	9.0
HMIM[PF ₆]	10.5

^a All results are an average of two independent measurements with the standard deviation (STD) lower than 10%.

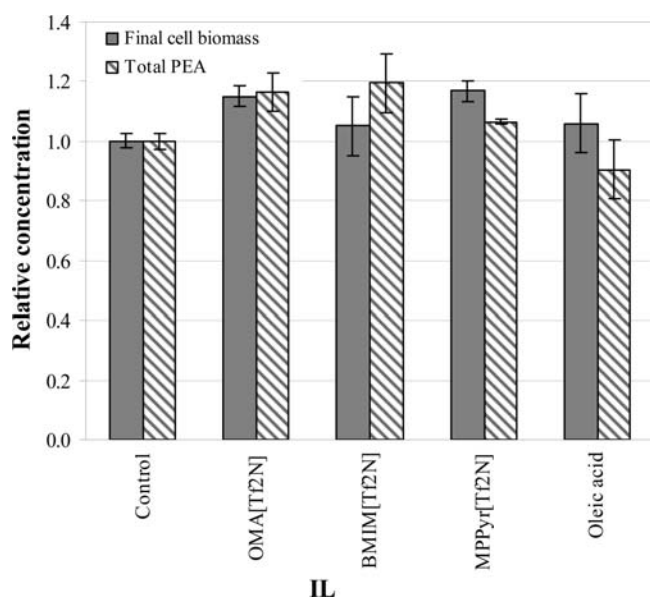


Figure 4. Final cell concentration and final PEA concentration obtained in biphasic systems relative to a control without ISPR. Cells were grown on NEO media with an initial DCW = 0.05 g of dw/L. The aqueous medium volume was 20 mL. ILs (20%, v/v) were added at DCW = 0.5 g of dw/L. PEA concentrations were analyzed by HPLC in each phase and combined to determine the total quantity produced in a system. The relative values are normalized to the control without ISPR designated as 1. The control cell concentration value to which relative values are normalized is 4.3 g of dw/L, and the PEA concentration was 2 g/L.

solvents previously used for extraction of PEA (Table 2). The highest distribution coefficient value was obtained with ethyl acetate (20.4). K_D values obtained with oleic acid and oleyl alcohol (6.5 and 11.8, respectively; Table 2), were similar to the ones reported previously: 6.8 (6) and 13 (12), respectively. The PEA distribution in ILs varied greatly. The lowest K_D value of 4 was obtained in OMA[Tf₂N]. BMIM[Tf₂N], on the other hand, provided the highest K_D among ILs, making it a good potential solvent for extracting PEA. In all other ILs, the K_D values were relatively high and similar to that of oleyl alcohol.

Once the yeast growth was shown to be nearly unaffected by the presence of some ILs, the enhanced production of PEA in a biphasic system was attempted. It was expected that the ILs will extract the PEA during yeast growth and production and facilitate higher PEA yields than the system lacking IL. This concept was recently reported for increasing the yield of toluene *cis*-glycol in the biotransformation of toluene by *E. coli* cells expressing a toluene dioxygenase (21). The ILs examined were OMA[Tf₂N], MPPyr[Tf₂N], and BMIM[Tf₂N] because of their good biocompatibility with strain Ye9-612, and oleic acid was evaluated for comparison. Final cell biomass and PEA concentrations in each

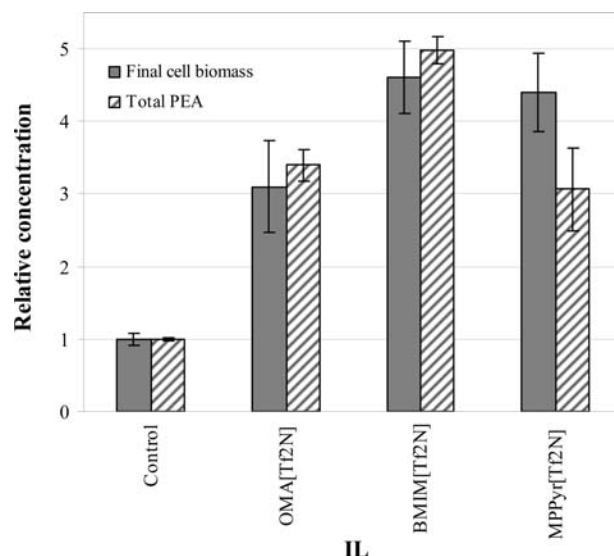


Figure 5. Final cell concentration and final PEA concentration obtained in biphasic systems under PEA stress conditions relative to a control without ISPR. The solvents comprised 20% (v/v) in each system. The aqueous medium volume was 24 mL. Cells were grown on NEO media with an initial DCW = 0.05 g of dw/L and in the presence of 2.5 g/L PEA. ILs were added at DCW = 0.2 g of dw/L. PEA concentrations were analyzed by HPLC in both phases. The relative values are normalized to the control without ISPR designated as 1. The control cell concentration value to which relative values are normalized is 1.1 g of dw/L, and the PEA concentration is 0.6 g/L (in access to the initial amount of 2.5 g/L).

phase were measured, and the total amounts of PEA obtained in each system were compared to the control (Figure 4). The amount of PEA obtained in the IL biphasic systems was 10–20% higher than in the control. Although these are not significantly higher amounts, they indicate an observable recovery capability of the ILs, especially BMIM[Tf₂N]. Oleic acid was previously shown to successfully increase PEA production by *S. cerevisiae* Giv 2009 in bioreactor experiments using fed-batch or continuous modes of operation by 3.5-fold (6). The fact that in our hands oleic acid did not improve PEA yield at all may be explained by strain dependence and mostly by the use of a batch shake-flask system compared to a fed-batch bioreactor system used by Stark et al. (6). The use of a biphasic system is required to alleviate the inhibitive effect of PEA accumulated in the medium causing cell toxicity. Considering that PEA has to be in high concentrations (above 2.5 g/L) to cause stress (10), the usefulness of an ISPR system can be shown when applying stress during yeast growth. Therefore, the new experimental system contained an initial amount of 2.5 g/L PEA in the medium, posing stress conditions on the growing yeast cells from the very beginning, and the ILs (OMA[Tf₂N], BMIM[Tf₂N], and MPPyr[Tf₂N]) were added at the early log phase. The total quantities of PEA obtained and the final cell biomass concentration in each system were measured (Figure 5). The new experimental setup proved that the ISPR systems managed to significantly improve PEA production. The final cell concentrations as well as the amounts of PEA obtained were significantly higher in the biphasic systems (including oleic acid for which the results are not shown). Among the ILs tested, the highest PEA quantities were obtained in the presence of BMIM[Tf₂N], displaying the highest K_D value (a 5-fold increase in the concentration of PEA produced by the cells in access to the initial amount added). A full correlation was not obtained between the biocompatibility and distribution coefficients (K_D) of the ILs and the PEA yields observed in the stress-containing

systems. This is attributed to the fact that the growth and final cell concentration are affected more by the PEA stress than the IL presence. Furthermore, the total concentration of PEA in the whole system (~5 g/L) is not high enough to reach the maximum extraction capability of the ILs. Thus, the low K_D of OMA[Tf_2N] was sufficient to extract enough PEA from the aqueous phase and alleviate PEA toxicity in a manner similar to MPPyr[Tf_2N], which has a higher K_D . Higher PEA concentrations (10–20 g/L) are needed to evaluate the correlation of K_D with product yields, and this can be reached in fed-batch bioreactors or using higher stress concentrations than the ones tested in the present study.

The PEA can be recovered from the IL phase using low-pressure distillation (15). The combination of fed-batch fermentation with the presence of BMIM[Tf_2N] is currently being studied in our lab to further increase the yields of PEA.

CONCLUSIONS

The present research demonstrates the ability of *S. cerevisiae* strain Ye9-612 to grow and produce PEA in the presence of several immiscible ILs using a biphasic system of 20% (v/v). MPPyr[Tf_2N], OMA[Tf_2N], and BMIM[Tf_2N] enabled a 3–5-fold increase in the PEA concentration. To our knowledge, this is the first report on the use of ILs as ISPR solvents for continuous extraction of a bioproduct from growing and fully functional yeast cells. The ILs extract the inhibitory PEA from the aqueous phase, thereby facilitating higher production yields. The work was performed on a 30 mL scale with direct measurement of PEA in each phase, making the results highly substantial. The experimental setup using growing yeast under stress conditions proved to be useful for the measurement of the ISPR capability of the various solvents. BMIM[Tf_2N] was found to give the best results in terms of extraction capability, biocompatibility, and overall performance in the bioprocess. This work highlights the potential use of ILs for ISPR-coupled fermentation processes. A future decrease in the price of ILs will enable further studies in fed-batch bioreactors that will verify the results presented on a larger scale.

ACKNOWLEDGMENT

We thank Prof. Y. Kashi for providing us with *S. cerevisiae* strain Ye9-612.

LITERATURE CITED

- (1) Etschmann, M. M. W.; Bluemke, W.; Sell, D.; Schrader, J. Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol.* **2002**, *59* (1), 1–8.
- (2) Fabre, C. E.; Blanc, P. J.; Goma, G. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. *Biotechnol. Prog.* **1998**, *14* (2), 270–274.
- (3) Schrader, J.; Etschmann, M. M. W.; Sell, D.; Hilmer, J. M.; Rabenhorst, J. Applied biocatalysis for the synthesis of natural flavour compounds—Current industrial processes and future prospects. *Biotechnol. Lett.* **2004**, *26* (6), 463–472.
- (4) Fabre, C. E.; Blanc, P. J.; Goma, G. Screening of yeasts producing 2-phenylethylalcohol. *Biotechnol. Technol.* **1997**, *11* (7), 523–525.
- (5) Seo, W.-T.; Ahn, K.-C. Production of phenylethanol from L-phenylalanine by *Candida* sp. S-8. *Food Sci. Biotechnol.* **2003**, *12* (6), 644–648.
- (6) Stark, D.; Munch, T.; Sonnleitner, B.; Marison, I. W.; von Stockar, U. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **2002**, *18* (3), 514–523.

- (7) Ingram, L.; Buttke, T. M. Effects of alcohols on microorganisms. *Adv. Microb. Physiol.* **1984**, *25*, 254–300.
- (8) Lester, G. Inhibition of growth, synthesis, and permeability in *Neurospora crassa* by phenethyl alcohol. *J. Bacteriol.* **1965**, *90*, 29–37.
- (9) Stark, D.; Zala, D.; Munch, T.; Sonnleitner, B.; Marison, I. W.; von Stockar, U. Inhibition aspects of the bioconversion of L-phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*. *Enzyme Microb. Technol.* **2003**, *32* (2), 212.
- (10) Eshkol, N.; Sendovski, M.; Bahalul, M.; Katz-Ezov, T.; Kashi, Y.; Fishman, A. Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain. *J. Appl. Microbiol.* **2008**, *106*, 534–542.
- (11) Stark, D.; von Stockar, U. In situ product removal (ISPR) in whole cell biotechnology during the last twenty years. *Adv. Biochem. Eng. Biotechnol.* **2003**, *80*, 149–175.
- (12) Etschmann, M. M. W.; Sell, D.; Schrader, J. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnol. Lett.* **2003**, *25* (7), 531–536.
- (13) van Rantwijk, F.; Sheldon, R. A. Biocatalysis in ionic liquids. *Chem. Rev.* **2007**, *107* (6), 2757–2785.
- (14) Yang, Z.; Pan, W. Ionic liquids: Green solvents for nonaqueous biocatalysis. *Enzyme Microb. Technol.* **2005**, *37* (1), 19–28.
- (15) Roosen, C.; Muller, P.; Greiner, L. Ionic liquids in biotechnology: Applications and perspectives for biotransformations. *Appl. Microbiol. Biotechnol.* **2008**, *81* (4), 607–614.
- (16) Weuster-Botz, D. Process intensification of whole-cell biocatalysis with ionic liquids. *Chem. Rec.* **2007**, *7*, 334–340.
- (17) Pfrüender, H.; Amidjojo, M.; Kragl, U.; Weuster-Botz, D. Efficient whole-cell biotransformation in a biphasic ionic liquid/water system. *Angew. Chem., Int. Ed. Engl.* **2004**, *43* (34), 4529–4531.
- (18) Pfrüender, H.; Jones, R.; Weuster-Botz, D. Water immiscible ionic liquids as solvents for whole cell biocatalysis. *J. Biotechnol.* **2006**, *124* (1), 182–190.
- (19) Brautigam, S.; Bringer-Meyer, S.; Weuster-Botz, D. Asymmetric whole cell biotransformations in biphasic ionic liquid/water-systems by use of recombinant *Escherichia coli* with intracellular cofactor regeneration. *Tetrahedron: Asymmetry* **2007**, *18* (16), 1883–1887.
- (20) Baumann, M. D.; Daugulis, A. J.; Jessop, P. G. Phosphonium ionic liquids for degradation of phenol in a two-phase partitioning bioreactor. *Appl. Microbiol. Biotechnol.* **2005**, *67* (1), 131–137.
- (21) Cornmell, R.; Winder, C. L.; Schuler, S.; Goodacre, R.; Stephens, G. Using a biphasic ionic liquid/water reaction system to improve oxygenase-catalysed biotransformation with whole cells. *Green Chem.* **2008**, *10*, 685–691.
- (22) Nir, N.; Bahalul, M.; Feingersch, R.; Katz-Ezov, T.; Yechezkel, K.; Fishman, A. Improvement of natural isolates of *Saccharomyces cerevisiae* strains for synthesis of a chiral building block using classic genetics. *Appl. Microbiol. Biotechnol.* **2008**, *78* (4), 659–667.
- (23) Ganske, F.; Bornscheuer, U. Growth of *Escherichia coli*, *Pichia pastoris* and *Bacillus cereus* in the presence of the ionic liquids [BMIM][BF₄] and [BMIM][PF₆] and organic solvents. *Biotechnol. Lett.* **2006**, *28* (7), 465–469.
- (24) Lenourry, A.; Gardiner, J. M.; Stephens, G. Hydrogenation of C=C double bonds in an ionic liquid reaction system using the obligate anaerobe, *Sporomusa termitida*. *Biotechnol. Lett.* **2005**, *27* (3), 161–165.
- (25) Docherty, K. M.; Kulpa, C. F. J. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem.* **2005**, *7* (4), 185–189.

Received for review May 4, 2009. Revised manuscript received December 27, 2009. Accepted January 06, 2010.