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Hydrophobic microspheres for *in situ* removal of 2-phenylethanol from yeast fermentation

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Abstract
The commercial production of the fragrance compound 2-phenylethanol (2-PE) from phenylalanine by yeast is limited by the accumulation of the toxic product, and therefore, *in situ* product removal techniques are required. We describe the use of hydrophobic polymethylmethacrylate (PMMA) microspheres of narrow size distribution of $1.53 \pm 0.10\,\mu m$ diameter for continuous removal of 2-PE from the fermentation medium by a mechanism of swelling. In shake flask experiments with conditions simulating 2-PE stress, a 10-fold increase in productivity was measured for systems containing >10% (w/v) microspheres. A 1 L fed-batch fermentation with 8% (w/v) of PMMA microspheres resulted in a total 2-PE concentration of 7.05 g/L, from which 5.40 g/L was incorporated inside the resin, implying 76% encapsulation. This ratio of 0.07 g/g of product per resin is among the highest reported to date. Scanning electron microscopy revealed a concomitant increase in sphere diameter confirming that swelling occurred.

Keywords: 2-phenylethanol, *Saccharomyces cerevisiae*, *in situ* product removal, polymethylmethacrylate microspheres, single-step swelling

Introduction

Large volume commodity and speciality chemicals produced by biotechnological methods are in high demand in recent years (Wohlgemuth, 2010). The main reason is to avoid the use of hazardous chemicals that are prevalent in conventional chemical production and that can cause environmental and health problems. Furthermore, biocatalysis may provide basic chemicals from sustainable sources (Demain, 2007). Biocatalytic methods have been employed in the food industry to facilitate the production, and therefore the reduction in market cost of natural flavours and fragrances. Such compounds are gaining preference by consumers who believe that they are free from harmful manufacturing traces and by products (Gounaris, 2010).

It is known that 2-phenylethanol (2-PE) is an aromatic alcohol with a rose-like fragrance. It is abundant in flowers, fruits and fermented foods such as cocoa and wine (Garavaglia et al., 2007) and is in high demand in the cosmetics industry (Etschmann et al., 2002). In the food industry, 2-PE is added to ice cream, candy, non-alcoholic beverages, gelatin puddings and chewing gum (Fabre et al., 1998; Garavaglia et al., 2007). Industrial production of 2-PE is carried out mainly by the Friedel–Craft reaction of ethylene oxide with benzene in the presence of molar quantities of aluminium chloride, or by catalytic reduction of styrene oxide using Raney nickel as a catalyst (Etschmann et al., 2002). These chemical synthetic methods involve toxic reagents that are extremely hazardous to the environment and may contaminate the final product. Alternatively, 2-PE can be produced from L-phenylalanine (L-phe) in a natural manner by several plants and microorganisms such as yeast (Fabre et al., 1998; Yang et al., 2009). Among the yeast strains reported are *Kluyveromyces marxianus*

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and *Sacchromyces cerevisiae* (Wittmann et al., 2002; Eshkol et al., 2009).

The main drawback of industrial production by yeast is the toxic effect of 2-PE on the cells, which causes product inhibition and low final concentrations (Etschmann et al., 2002). Overcoming this problem by means of *in situ* product removal (ISPR) has been suggested. ISPR involves quick removal of the formed product from the aqueous phase in which it was produced, and it is a well-established concept in bio-processing (Bechtold and Panke, 2009). Among the various approaches described in the literature for 2-PE removal are the liquid–liquid extraction and solid–liquid extraction methods. Bi-phasic systems include polypropylene glycol 1200 (Etschmann and Schrader, 2006), oleic acid (Stark et al., 2002), oleyl alcohol (Etschmann et al., 2003) and ionic liquids (Sendovski et al., 2010). Serp et al. (2003) advanced this approach using a polymeric matrix for entrapment of the solvent dibutyl sebacate to limit the solvent toxicity to the cells. A similar method comprising of microcapsules containing an organic solvent core surrounded by a hydrogel enabled an increase in 2-PE concentration from 3.8 to 5.6 g/L (Stark et al., 2003). These hybrid resin-solvent matrices are difficult to prepare for large-scale use, and solvents in general are considered as pollutants.

To circumvent the need for organic solvents and the possible formation of emulsions, ISPR of 2-PE using solid extraction was employed. The non-polar macroporous resin D101 showed high adsorption capacity for 2-PE. When 2 g of hydrated resin was added to 30 mL of media, a total concentration of 6.17 g/L was obtained, accompanied by a 0.045 g/g adsorption ratio (Mei et al., 2009). Gao and Daugulis (2009) screened six commercial polymers for adsorption capability and obtained optimal results with Hytrel 8206®, a block copolymer of polybutylene ester and polyether. Usage of 500 g of polymer in a 3 L bioreactor in batch mode resulted in a concentration of 13.7 g/L 2-PE and an adsorption ratio of 0.025 g/g.

This article describes an alternative solid–liquid extraction system for 2-PE removal based on encapsulation within polymethylmethacrylate (PMMA) microspheres via a swelling process. Recently, Margel and co-workers have developed a new and simple encapsulation technique of hydrophobic ligands (e.g. dyes, drugs and iron carbonyl complexes) within polystyrene (PS) microspheres (Bamnolker and Margel, 1996; Ziv et al., 2008; Goldshtein and Margel, 2009a, b). This process was based on the entrapment of these ligands within uniform PS template microspheres, by swelling these template particles with methylene chloride emulsion droplets containing the ligands, followed by evaporation of the methylene chloride from the swollen PS microspheres. The aim of this study was to evaluate the feasibility of PS and PMMA microspheres for *in situ* removal of 2-PE produced by growing yeast. The microspheres were first evaluated for their encapsulation ability under non-biological conditions and later tested for biocompatibility and performance in a bioreactor.

Methods

Chemicals

L-phe, 2-PE, D-(+)-glucose and sodium dodecanoate (lauric acid) were purchased from Sigma–Aldrich Chemical Co. (Rehovot, Israel). Lecithin was purchased from Alfa Aesar Chemical Co. (Ward Hill, MA), sodium dodecyl sulphate (SDS) from BDH Laboratory (Poole, England), Tween 80 from Fisher Scientific Co. (Fair Lawn, NJ) and Tween 20 from Merck Co. (Munchen, Germany). Bacto™ pepton and yeast extract were purchased from Becton, Dickinson and Co. (Le Ponte de Claix, France), KH_2PO_4 from Carlo Erba Reagents Co. (Val De Reuil, France), $MgSO_4 \cdot 7H_2O$ from Frutarom Ltd. (Haifa, Israel) and Agar from Acumedia® (Lansing, MI).

All materials used were of the highest purity available and used without further purification.

For the synthesis of PS and PMMA microspheres, the following analytical grade chemicals were purchased from Aldrich and used without further purification: Methyl methacrylate (MMA was washed with a 10% NaOH aqueous solution and passed through an activated aluminium oxide column to remove the inhibitor), styrene (styrene was passed through activated alumina to remove inhibitors before use), 2,2′-azobisisobutyronitrile (AIBN), ethanol (high-performance liquid chromatography, HPLC grade), methanol (HPLC grade), 2-methoxyethanol (HPLC grade), benzoyl peroxide (BP; 98%) and polyvinylpyrrolidone (PVP, molecular weight 360 000). Water was purified by the passage of deionized water through an Elgastat Spectrum reverse osmosis system (Elga Ltd., High Wycombe, UK).

Growth media composition

Yeast peptone dextrose medium (YPD) contained (g/L): glucose – 20, peptone – 20, yeast extract – 10 and agar – 20, for solid plates. Nitrogen Essential Optimal medium (NEO) (for 2-PE production from L-phe) contained (g/L): glucose – 20, L-phe – 4, KH_2PO_4 – 4, $MgSO_4 \cdot 7H_2O$ – 0.4, and yeast extract – 1.

Analytical methods

Conversion of L-phe to 2-PE was determined by HPLC with an Agilent 1100-series instrument (Agilent Technologies, Santa Clara, CA) using an Eclipse XDB C18, 5 μm, 4.6 × 150 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. Then, 1 μL of filtered samples was injected to the column and under these conditions, L-phe was eluted at 1.3 min and 2-PE at 4.2 min. Production of ethanol (EtOH) was determined by gas chromatography–mass spectrometry (GC/MS). Samples for GC/MS were analysed using a GC 6890 N (Agilent Technologies, Santa

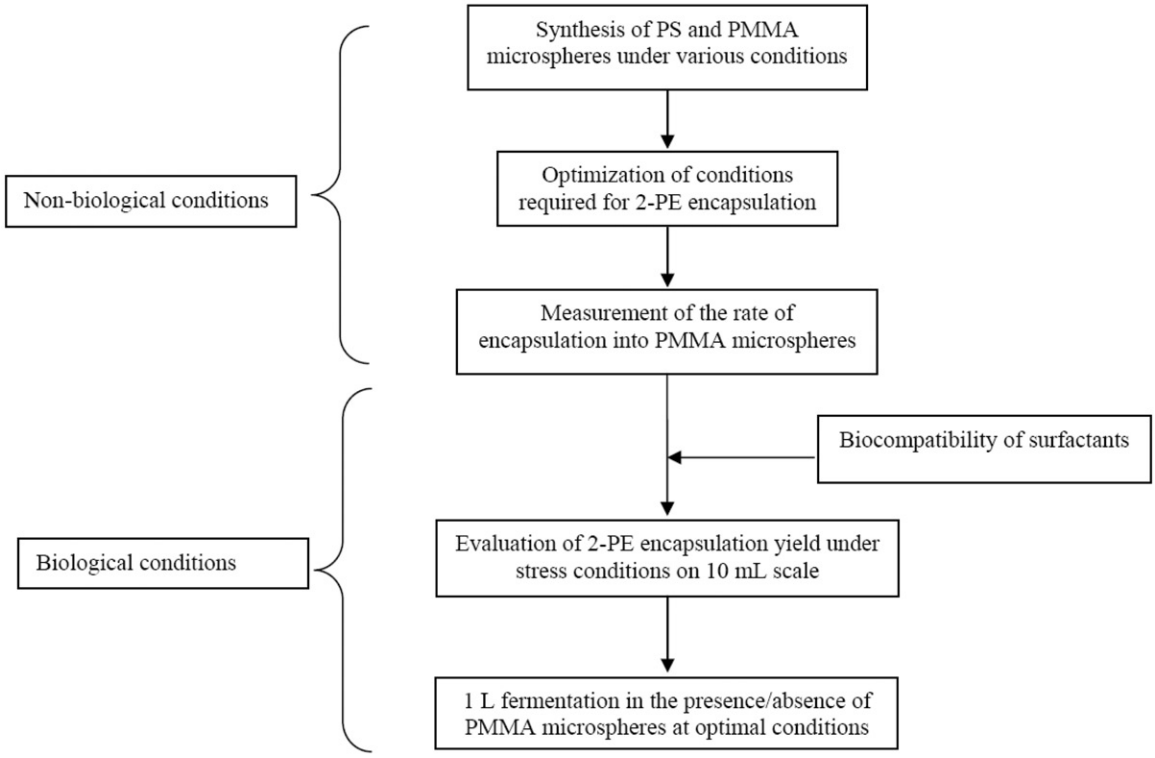


Figure 1. A flowchart of the work methodology undertaken for creating an ISPR system for 2-PE removal based on encapsulation via swelling of hydrophobic microspheres.

Clara, CA) instrument equipped with a capillary HP-5 column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies) and an HP-5975 mass spectra detector (Agilent Technologies, Santa Clara, CA). The temperatures profile was programmed as follows: $T_1 = 50^\circ$C (5 min), $T_2 = 50^\circ$C (dT/dt = 30°C/min), $T_3 = 200^\circ$C (2 min), with a split ratio of 1:3. Under these conditions, the retention time of EtOH was 1.55 min. GC/MS sample were prepared by the extraction of samples with ethyl acetate (1:1 v/v).

Data analysis

All the results obtained in the experiments with PMMA microspheres (with or without yeast) are an average of at least two independent measurements from which standard deviations (SDs) were calculated.

The following experimental sections describe the procedures taken to develop the ISPR process according to the flowchart in Figure 1.

Synthesis of PS microspheres

PS template microspheres of narrow size distribution were prepared according to a procedure described in the literature (Bamnolker and Margel, 1996). Briefly, these microspheres were synthesized in a three-neck round-bottom flask with a condenser in a constant temperature silicone oil bath at 73°C. In a typical experiment, PS microspheres of 2.75 ± 0.09 μm diameter were formed by addition of PVP (3.75 g, 1.5% w/v of total solution) dissolved in a mixture of ethanol (150 mL) and 2-methoxy ethanol (62.5 mL) into the reaction flask (1 L). Nitrogen was bubbled for 15 min to exclude air and then nitrogen atmosphere maintained over the solution during the polymerization period. Subsequently, a de-aerated solution containing the initiator BP (1.5 g, 0.6% w/v of total solution) and styrene (37.5 mL, 16% w/v of total solution) was added to the reaction flask. The polymerization reaction continued for 24 h with mechanical stirring (200 rpm), and was then stopped by cooling to room temperature. The microspheres were washed by extensive centrifugation cycles with ethanol and then with water. The particles were dried by lyophilization. Polymerization yield: 70–80%.

Synthesis of PMMA microspheres

PMMA template microspheres of narrow size distribution were prepared according to a procedure similar to that described in the literature (Kim et al., 2006; Jiang et al., 2007; Lee and Lee, 2008). Briefly, these microspheres were synthesized in a 250 mL flask in a constant temperature water bath at a 73°C. In a typical experiment, PMMA microspheres with an average diameter of 1.53 ± 0.10 μm were formed by introducing into the reaction flask a solution containing PVP (3 g) dissolved in a mixture of methanol (105 mL) and water (45 mL). Nitrogen was bubbled through the solution for 10 min to exclude air. A de-aerated solution containing the initiator AIBN (0.15 g) and MMA (15 mL) was then added to the reaction flask.

The polymerization reaction continued for 18 h and was then stopped by cooling to room temperature. The microspheres formed were washed by extensive centrifugation cycles with ethanol and then with water. The particles were then dried by lyophilization. Polymerization yield: $86 \pm 8\%$. PMMA microspheres of various diameters were prepared similarly, by changing polymerization parameters, e.g. monomer concentration, stabilizer concentration, M_w, etc.

Characterization of microspheres

The diameter and size distribution of the various microspheres dispersed in aqueous phase were determined using a micron particle analyzer, model LS100, Coulter Electronics (Fullerton, CA). The reported values are an average of at least five replications of each measurement. Fourier transform infrared (FT-IR) analysis was performed with a Bomem FT-IR model MB100 spectrophotometer (ABB Bomem Inc., Quebec, Canada). The analysis was performed with 13 mm KBr pellets that contained 2 mg of the detected material and 198 mg of KBr. The pellets were scanned over 100 scans at a $4\,cm^{-1}$ resolution.

Dried particle size and size distribution were measured with a scanning electron microscope (SEM). SEM pictures were obtained with a FEI SEM model Inspect S (Eindhoven, Holland). For this purpose, a drop of dilute microsphere dispersion in the continuous phase was spread on a glass surface, and then dried at room temperature. The dried sample was coated with gold under vacuum before viewing it with SEM. SEM images of PMMA microspheres before and after 2-PE encapsulation were obtained with a same FEI SEM instrument, but in low vacuum conditions without gold coating. The average particles size and distribution were determined by the measurement of the diameters of more than 100 particles with image analysis software (Analysis Auto, Soft Imaging System GmbH, Germany).

The molecular weight of PS and PMMA microspheres was measured by gel permeation chromatography consisting of an Applied Bioscience RI-71 detector (Waltham, MA). The samples were eluted with tetrahydrofuran through a linear Phenogel 5 μm column at a flow rate of 1 mL/min. The molecular weight was determined with respect to PS standards with a Clarity computer program.

The polymerization yield was calculated by the following expression:

$$\text{Polymerization yield (weight \%)} = \frac{W_{PMMA}}{W_{MMA}} \times 100 \qquad (1)$$

where W_{PMMA} is the weight of the beads (PS or PMMA microspheres) and W_{MMA} the initial weight of the monomers (styrene or MMA).

Rate of 2-PE encapsulation within PMMA microspheres

The encapsulation rate of 2-PE within PMMA beads was tested in a system comprising 1 g PMMA microspheres placed in 10 mL vials containing 10 g/L 2-PE and L-phe with 0.3% (v/v) Tween 80 in a shaker at 30°C and 250 rpm. The quantity of 2-PE and L-phe in the aqueous phase was analysed by HPLC over 72 h. In a similar experiment, 1%, 5% and 10% (w/v) PMMA microspheres were employed to evaluate the effect of PMMA concentration on the encapsulation.

Yeast cultivation

The yeast strain used in this research was *S. cerevisiae* Ye9-612 (Eshkol et al., 2009). Yeast cells were initially streaked from glycerol stocks (−80°C) on YPD plates and incubated at 30°C overnight. A starter culture was prepared by inoculation to 10 mL of liquid YPD medium for overnight growth at 30°C with shaking at 250 rpm (Orbital Shaker Incubator, MRC, Holon, Israel).

Yeast growth in the presence of selected surfactants

Ye9-612 was cultivated as described above and 0.25 mL of it was inoculated to 25 mL of liquid YPD media to obtain an initial OD_{600} of 0.1 and the following surfactant concentrations were added: SDS (0.1%, 0.5% and 1% v/v), Tween 20 (1% v/v), Tween 80 (0.5% and 1% v/v), lecithin (0.5%, 1%, and 2% w/w) and sodium dodecanoate (1% and 3% w/w). The flasks were shaken at 250 rpm at 30°C, and the absorbance at 600 nm was measured every 90 min in the aqueous phase, except for lecithin that was measured with a counting cell under the microscope. Growth was carried out for 24 h for SDS and Tween 20, and 48 h for Tween 80, lecithin and sodium dodecanoate. Experiments were conducted in duplicates, ensuring a SD lower than 10%. Live cell counts were performed by diluting a sample from the aqueous phase and spreading 100 μL on YPD plates using a Drigalski spatula. Plates were incubated at 30°C and colonies counted after 24 h.

Biocompatibility of the microspheres with yeast

Ye9-612 was cultivated as described; cells were inoculated into 50 mL of liquid NEO media to obtain an initial OD_{600} of 0.1, in a 500 mL flask in order to allow for maximal oxygen transfer. Tween 80 (5% v/v) and PMMA microspheres (0.5 g, 1% w/v) were added with the inoculation of the cells. The flasks were shaken at 250 rpm and 30°C, and the absorbance at OD_{600} was measured. The 2-PE concentration in the media was analysed by HPLC.

2-PE production under stress conditions with ISPR

Ye9-612 was cultivated as described and 100 μL from the starter culture was inoculated into 10 mL of liquid NEO media in a 100 mL flask. To these experimental flasks, 2-PE was added at an initial concentration of 2.0 g/L in

the aqueous phase. The flasks were shaken at 250 rpm at 30°C, and PMMA microspheres were added at different concentrations 10%, 20% and 30% (w/v). Samples for HPLC analysis were taken prior to microsphere addition and 10 min after the addition. Growth was carried out for 72 h, after which a 1 mL sample was centrifuged and analysed by HPLC for 2-PE concentration. Ethyl acetate (1 mL) was used to extract the 2-PE from the washed PMMA microspheres followed by HPLC analysis.

2-PE production in a 3 L bioreactor with ISPR

Ye9-612 was cultivated as described above and 24 mL from the starter culture was inoculated into 1 L medium in a Bioflow 3000 reactor (New Brunswick Scientific, Edison, NJ) to obtain an initial OD_{600} of 2.0. The fermentation medium contained 10 g L-phe, 20 g glucose, 4 g KH_2PO_2, 0.4 g $MgSO_4 \cdot H_2O$ and 1 g yeast extract. The fermentation lasted 7 days with agitation at 400 rpm and 30°C. Aeration was maintained at 1.3 vvm and dissolved oxygen tension was measured using a pO_2 electrode to ensure that the yeast are not under oxygen starvation. The pH was maintained at 5.0 with 2 M NaOH and 2 M H_2SO_4. Glucose levels were tested using a glucose assay kit (Sigma GAGO20) throughout the fermentation and glucose was periodically added to maintain a concentration of 15 g/L. Sampling was designed to overcome volume changes due to glucose dilution. After 24 h, PMMA microspheres (9 g, 0.9% w/v or 80 g, 8% w/v) were added with Tween 80 (3 mL, 0.3% (v/v); for minimizing foaming while maintaining the surfactant's ability to facilitate encapsulation). A standard curve was created in which optical density (600 nm) measurements were correlated to dry cell weight concentrations (gram dry weight per litre – gdw/L). Dry weight of biomass was determined in duplicate samples of 10 mL each by centrifugation for 10 min at 13 400 × g (Sigma, 4K15 centrifuge with 12166 rotor, Osteroid, Germany). The pellets were washed twice with distilled water, and centrifuged again under the same conditions. The wet pellets were dried for 24 h in an oven at 90°C (MRC Ltd., Holon, Israel) and then weighed.

2-PE extraction from PMMA microspheres

The beads and cell biomass were recovered from the fermentation broth by centrifugation at 8000 rpm (Sigma, 4K15 centrifuge with 12166 rotor, Osteroid, Germany) and the solids washed with phosphate buffer and centrifuged again at 8000 rpm. The PMMA microspheres and the biomass were extracted with 50 mL ethyl acetate using shaking at 250 rpm, room temperature for 48 h. The concentration of 2-PE was measured by HPLC. The total amount of 2-PE was measured following ethyl acetate evaporation. In some experiments, only 1 g sample was removed from the broth and treated as described using 10 mL ethyl acetate for extraction. The amount of 2-PE encapsulated was calculated by the following expression:

$$C_F \cdot V_{EX} \cdot \frac{W_{TP}}{W_{WP}} = W_{EN} \quad (2)$$

where C_F is the extracted 2-PE concentration (g/L), V_{EX} the volume of the extractant (L), W_{TP} the total weight of PMMA microspheres (g), W_{WP} the weight of the PMMA sample that was used for the extraction and W_{EN} the total weight of 2-PE that was encapsulated within the PMMA microspheres during the fermentation process.

Results and discussion

The steps undertaken in order to develop an ISPR process based on swelling of hydrophobic microspheres are presented in Figure 1.

Characterization of the PS and PMMA microspheres

Previous publications by Margel and co-workers described the encapsulation of various substances within uniform PS microspheres, e.g. radiopaque iodinated compounds, magnetic precursors such as iron pentacarbonyl, UV absorbers and others (Shpaisman and Margel, 2005; Galperin et al., 2006; Goldshtein and Margel, 2011). Here, the objective was to use PS and PMMA microspheres for continuous removal of 2-PE formed during the fermentation of *S. cerevisiae* strain Ye9-612 on L-phe as the nitrogen source. SEM images showed a spherical shape and smooth surface morphology of both PS and PMMA microspheres (results not shown). The measured hydrodynamic and dry diameters of these microspheres were 2.90 ± 0.12 and 2.75 ± 0.09 μm for PS particles and 1.8 ± 0.11 and 1.53 ± 0.10 μm for PMMA beads, respectively. The larger hydrodynamic diameter includes the adsorbed solvents (methanol and water) while the SEM image shows the dry diameter only (Horak et al., 2000). Molecular weight distribution of PS and PMMA microspheres were 10 000 and 67 813 g/mol, and average molecular weight per number (M_w/M_n) values 1.1 and 3.2, respectively. The FT-IR spectrum of PS and PMMA particles were similar to that of the monomer, except for disappearance of the absorption peak at about 1640 and 1630 cm^{-1}, respectively, corresponding to the C = C double bond stretching band, indicating the lack of a residual monomer within the particles.

Effect of co-solvent concentration

Water was a co-solvent in the dispersion protocol for MMA polymerization. This co-solvent was used for decreasing the solubility of the monomer thus allowing smaller particle diameter of the PMMA microspheres. This was accomplished by keeping the total solvent (MeOH, methanol and H_2O) volume constant while altering the volume ratio between the two solvents. The effect of the H_2O

concentration on the hydrodynamic diameter and size distribution of the formed PMMA microspheres was measured. It was shown that increasing the water concentration from 1% to 3% and 3% and 10% led to a decrease in particle diameter from 3.40 ± 1.10 to $2.00 \pm 0.20\,\mu m$ and $0.40 \pm 0.05\,\mu m$, respectively. A similar effect of water concentration on the PMMA diameter and size distribution was reported previously (Yu et al., 2005; Kim et al., 2006). Low water concentration resulted in bigger particle diameter and size distribution, suggesting that the particle size was dependent on the solubility parameter of the co-solvent that was used in dispersion polymerization. Thus, a solvent which has a closer solubility parameter to the monomer would give a larger particle diameter because a smaller number of nuclei are produced in the nucleation stage. In addition, increasing the amount of water increases the polarity of the reaction medium, resulting in a decrease in the diameter of the microspheres.

Effects of the PVP concentration and molecular weight on the microspheres

Figure 2 demonstrates the effects of the PVP concentration and its molecular weight (40 000 and 360 000) on the dry diameter size distribution of the formed PMMA microspheres. Increasing the PVP concentration from 0.5% to 2.0%, 10.0% and 20.0% led to a decrease in diameter and

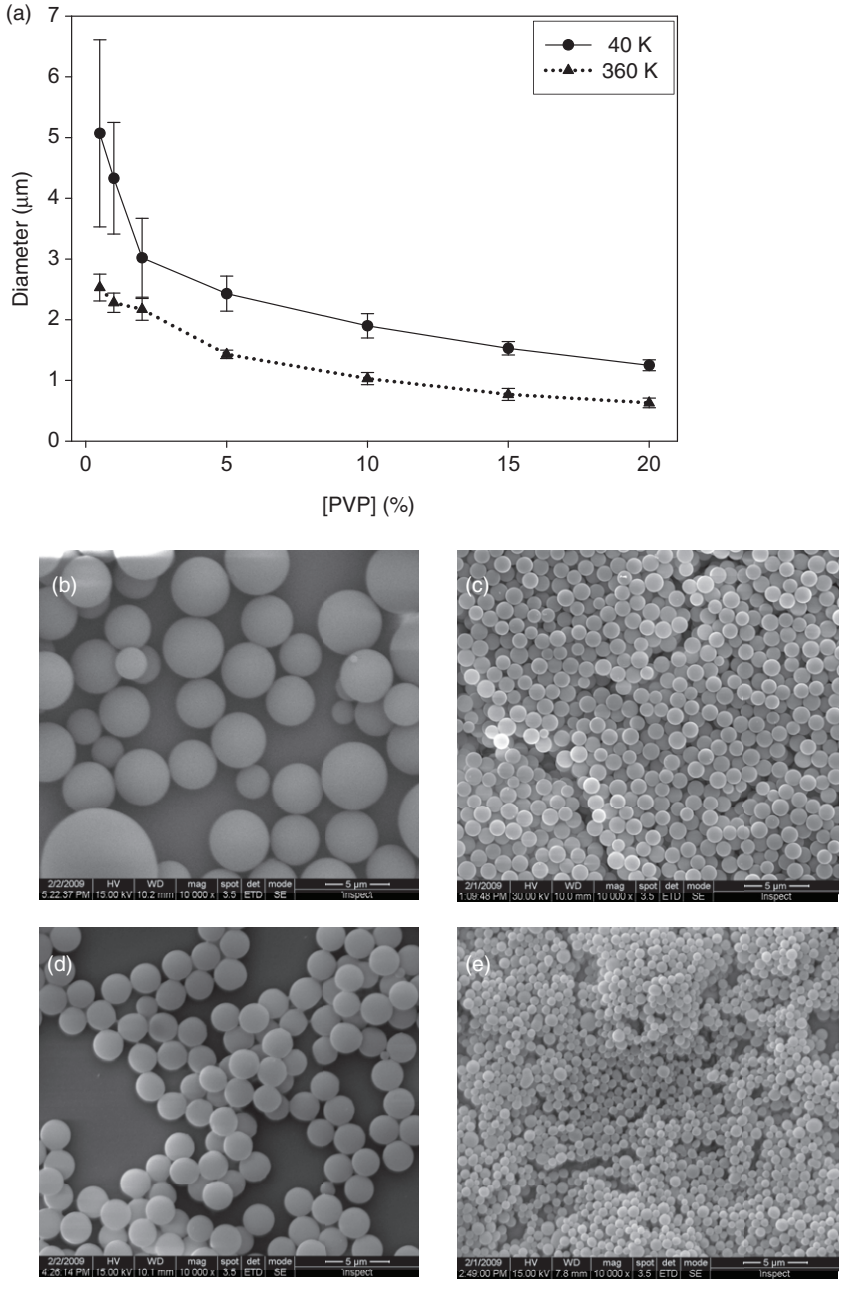


Figure 2. Influence of the stabilizer concentration and molecular weight on the size of the PMMA microspheres (a). SEM images of PMMA microspheres prepared in the presence of 0.5% and 20% PVP with molecular weights of 40 000 (b, d) and 360 000 (c, e). The PMMA microspheres were synthesized in the presence of 10% MMA and different concentrations of PVP of two molecular weights, according to the experimental section.

size distribution of PMMA microspheres from 5.1 ± 1.5 to $3.0 \pm 0.6\,\mu m$, $1.9 \pm 0.2\,\mu m$ and $1.3 \pm 0.1\,\mu m$ in the presence of PVP with a molecular weight of 40 000, and particle diameter and size distribution decreased from 2.5 ± 0.2 to $2.2 \pm 0.2\,\mu m$, $1.0 \pm 0.1\,\mu m$ and $0.6 \pm 0.1\,\mu m$ in the presence of PVP with a molecular weight of 360 000. Inverse proportionality between the stabilizer concentration and the average size of the particles formed by dispersion polymerization of monomers such as 2-hydroxyethyl methacrylate (HEMA), pentabromobenzyl acrylate, 2-(2′-hydroxy-5′-methacryloxyethylphenyl)-2H-benzotriazole and styrene was also reported previously (Bamnolker and Margel, 1996; Horak, 1999; Goldshtein and Margel, 2009a, b). A higher stabilizer concentration resulted in increasing amounts of stabilizer adsorbed onto the nuclei and, therefore, in better protection against the growing process. In addition, the figure illustrates the effect of the molecular weight of the PVP on the PMMA microsphere diameter. Increasing the PVP molecular weight results in PMMA particles of smaller size and size distribution. For example, in the presence of 2% PVP with molecular weights of 40 000 and 360 000 g/mol, the size and size distribution of the PMMA microspheres was decreased from 3.0 ± 0.6 to $2.2 \pm 0.2\,\mu m$, respectively. A similar effect of the stabilizer molecular weight on the average size and size distribution of PS, pentabromobenzyl acrylate and MAOETIB microspheres prepared by dispersion polymerization in the presence of different molecular weights of the stabilizer PVP was reported previously (Bamnolker and Margel, 1996; Galperin et al., 2006; Goldshtein and Margel, 2009b). The explanation for this behaviour is that PVP of a higher molecular weight was adsorbed more strongly to the nucleus surfaces and therefore stabilized the particles more effectively.

Based on the results obtained above, the optimal conditions for preparing PMMA microspheres were 30% (w/w) of the co-solvent (H_2O) and 2% (w/w) of the PVP stabilizer with molecular weight 360 000 g/mol.

Yeast growth in the presence of selected surfactants

In this research, a new ISPR system which is based on encapsulation by swelling hydrophobic microspheres was employed. The hypothesis was that swelling would be facilitated by chemical hydrophobic interactions, and a suitable surfactant would be needed to mediate between the 2-PE and the microspheres. Indeed it was discovered that in non-biological systems employing microspheres, 2-PE and surfactants, with mixing via sonication, there was an advantage for the usage of surfactants in the system. The influence of surfactants on the encapsulation was tested with concentrations of range 0.1–3.0%, which are akin to living organisms and are tolerated by technical operational conditions (higher concentrations maybe toxic to cells and may cause excess foaming in a bioreactor; results not shown). The next step was to test the biocompatibility of various surfactants on *S. cerevisiae* Ye9-612, a strain that has shown both good 2-PE production and robustness (Eshkol et al., 2009). The surfactants were chosen based on their desirable properties such as food grade or common use in the industry and the concentrations were similar to the non-biological systems.

Yeast strain Ye9-612 was grown in the presence of these surfactants and the live cell count indicated the toxicity (Table 1). SDS did not have a toxic effect at a low concentration (0.1%), but showed a toxic effect at a higher concentration (0.5%). Lauric acid showed high toxicity at a low concentration (0.3%). Tween 80, Tween 20 and lecithin showed either no toxic effect or only a mild one on the yeast growth and were suitable for use in the ISPR system. Tween 80 was chosen for further work because it did not form turbidity in the medium and therefore yeast growth could be monitored by optical density measurements.

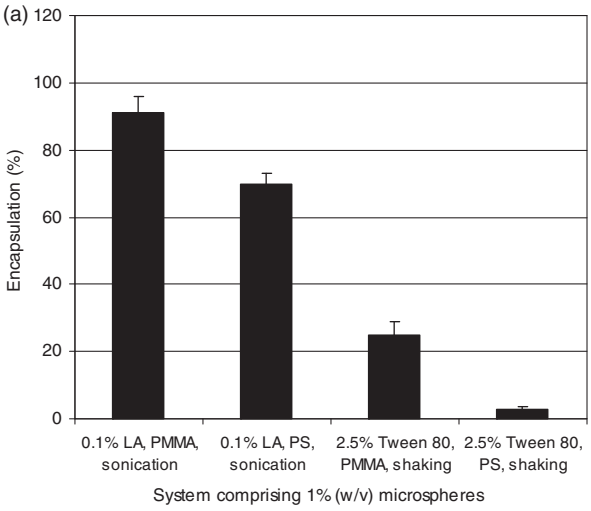
Optimization of the 2-PE encapsulation system

PS and PMMA microspheres were tested for their capability to encapsulate 2-PE in two systems: (1) using sonication (a method used in non-biological systems) and (2) rapid mixing in a shaker with small and large amounts of 2-PE. The results showed that there was a high encapsulation level (~70–90%) in a non-biological system with sonication (Figure 3a) and there was a moderate level of encapsulation (~25%) under rapid mixing conditions which are akin to biological growth conditions (Figure 3a). In addition to the encapsulation experiments, pictures that were taken under a light-microscope showed enlargement of the microcapsules (PMMA or PS) in the presence of Tween 80 and 2-PE, indicating that encapsulation of the product did occur. PMMA microcapsules showed better encapsulation ability under biological conditions (Figure 3a) and were therefore chosen for further work. It is believed that the less hydrophobic nature of PMMA

Table 1. Biocompatibility of surfactants with *S. cerevisiae* strain Ye9-612.

Surfactant	Concentration (% v/v)	Yeast [CFU] (24 h)	Yeast [CFU] (48 h)
None (control)	–	5.6×10^7	2×10^8
Tween 20	0.1	4.7×10^6	ND
Tween 80	0.5	ND	2.5×10^7
Tween 80	1	ND	2.9×10^7
Lecithin	0.5	9.4×10^7	1.2×10^8
Lecithin	1	1.1×10^7	1.1×10^8
Lecithin	2	9.7×10^7	1.3×10^8
SDS	0.1	2.4×10^7	ND
SDS	0.5	1.3×10^4	ND
SDS	1	2.8×10^3	ND
Lauric acid	0.3	$< 1.1 \times 10^2$	ND

Notes: CFU, colony forming units; ND, not determined.
Experimental conditions: Yeast cells were grown in YPD medium in the presence of various concentrations of surfactants. The flasks were shaken at 250 rpm and 30°C for 24–48 h. Live cell counts were performed by diluting a sample from the aqueous phase and spreading 100 μL on YPD plates using a Drigalski spatula. Plates were incubated at 30°C and colonies were counted after 24 h.



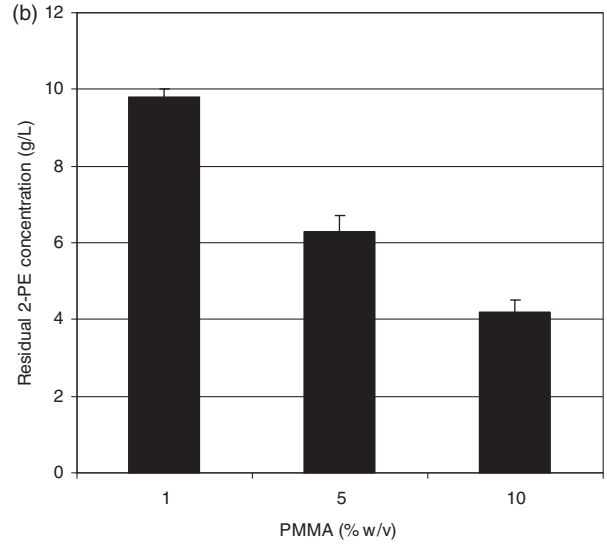


Figure 3. Optimizing system parameters in the absence of yeast. (a) Percentage of 2-PE encapsulated as determined from the residual concentration in the liquid phase using HPLC analyses in different systems. The reaction mixture contained 1% (w/v) microspheres (PS or PMMA), 1% 2-PE, surfactant (Lauric acid or Tween 80) and sonication or shaking at 200 rpm, and room temperature. (b) The residual concentration of 2-PE in the liquid phase in the presence of different concentrations of PMMA microspheres. The initial 2-PE concentration was 10 g/L. Reactions were performed in 10 mL of distilled water with shaking at 200 rpm and room temperature. The results are an average of three independent measurements.

microspheres enables better encapsulation of 2-PE than the very hydrophobic PS microspheres.

For optimization of this system, different operational conditions were tested (such as different temperatures and shaking conditions); however, no significant difference between the experiments was found (data not shown). All the systems evaluated resulted in a 15–25% encapsulation of 2-PE. It was evident from the above experiments that sonication gave the best encapsulation yield. However, this is not applicable to a biological system comprising growing yeast cells.

A concentration of 1% (w/v) of PMMA microspheres was used in the preliminary experiments; however, this did not give rise to promising encapsulation concentrations.

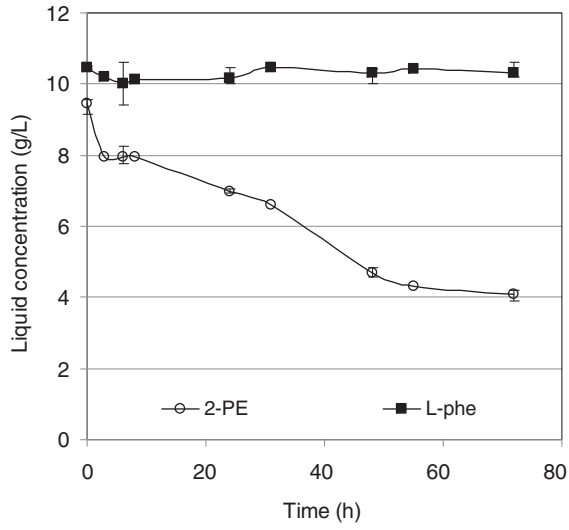


Figure 4. The concentration of 2-PE and L-phe in the liquid phase in the presence of 10% PMMA microspheres (w/v) and 0.3% Tween 80 (v/v). The initial 2-PE and L-phe concentration was 10 g/L. The reaction was performed in 10 mL of distilled water with shaking at 30°C and 250 rpm over 72 h. The results are an average of two to three independent measurements.

Therefore, higher microsphere concentrations were examined (5% and 10% (w/v)) in a system without yeast cells. It was shown that increase in the concentration of the PMMA microspheres gave an increase in 2-PE encapsulation and less 2-PE was present in the liquid phase (Figure 3b). The system comprising 10% PMMA microspheres gave 60-fold higher encapsulation than that obtained with a 1% concentration (6 g/L *vs.* 0.1 g/L, respectively; Figure 3b). A similar trend was shown in other works correlating higher production of 2-PE with and increase in the solid phase concentration. For example, increasing the concentration of D101 resin from 1.6% to 6.6% (w/v) was accompanied by an increase of 1.5 g/L 2-PE in the medium (Mei et al., 2009). In a fermentation containing Hytrel® as the adsorbent, a 5-fold increase in the resin concentration (500 g *vs.* 100 g in a 3 L reactor) enabled a 3.4-fold increase in the total 2-PE productivity (Gao and Daugulis, 2009). Concentrations of 1% and 8% were chosen for the large-scale fermentations.

The selectivity of the PMMA microspheres for the product was next examined using a 10% microsphere concentration to assess that L-phe, the substrate, is not incorporated as well. Results showed that L-phe concentration in the medium was constant, indicating no encapsulation (Figure 4). This result is superior to the commercial resin D-101 that absorbed 84 mg L-phe versus 136 mg 2-PE per gram resin, indicating poor selectivity (Mei et al., 2009), which may affect the product purity. The rate of 2-PE uptake was shown to be time dependent. In the first few hours, the concentration rapidly decreased in the medium at a rate of 0.6 g/L/h, followed by a slower rate of 0.095 in the next 40 h; then, the rate of encapsulation decreased to 0.0085 (Figure 4).

A total of 60% of the initial 2-PE amount was incorporated into the microspheres under conditions simulating yeast growth. This amounts to a maximum of 0.6 g/g under steady-state conditions.

Biocompatibility of the microspheres with yeast

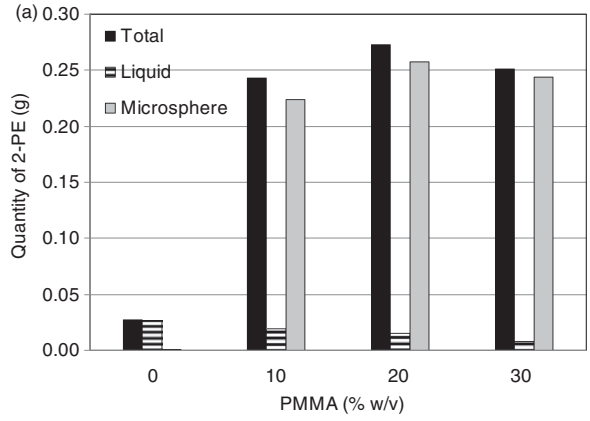
The biocompatibility of PMMA microspheres with yeast strain Ye9-612 was evaluated in shake flasks with a concentration of 1% (w/v). The growth rate was unaffected by the presence of the microspheres (results not shown). However, the 2-PE concentration in the liquid media was slightly lower in the presence of the PMMA microspheres than it was in the system without the microspheres. That result is most likely due to 2-PE encapsulation in the microspheres. Extraction attempts were unsuccessful probably because of the small amount of microspheres in the system (less than 0.5 g).

2-PE production under stress conditions

The final step before carrying out large volume fermentations was mimicking the desirable situation of high 2-PE concentration in the medium in order to establish the usefulness of PMMA microspheres as ISPR agents (Sendovski et al., 2010). The stress system was used to test on small scale: (1) the protective effect that different PMMA microsphere concentrations have on the yeast under a high 2-PE concentration, (2) the encapsulation capability of a high PMMA microsphere concentration ($>10\%$) without the need to synthesize large amounts of microspheres. The results indicated that utilizing a concentration of 20% PMMA (w/v) led to the highest total 2-PE amount (0.27 g; Figure 5a) which is 10-fold better than the system without ISPR; however, the best encapsulation ratio (2-PE/PMMA g/g) was obtained at 10% (w/v) (0.22 g/g). This concentration was therefore later used in the bioreactor. All the systems containing PMMA microspheres showed much higher quantities of 2-PE compared to the control ($\sim$0.25 g *vs.* <0.05 g, respectively; Figure 5a). The concentration of 2-PE in the liquid phase decreased shortly after the addition of the microspheres (Figure 5b). After 72 h of growth, the control system had a concentration of >2.5 g/L 2-PE in the liquid phase, which is inhibitory for yeast cells (Seward et al., 1996). In contrast, the systems containing PMMA microspheres had a much lower concentration (<2 g/L) due to product encapsulation (Figure 5b).

2-PE production in a 3 L bioreactor with ISPR using PMMA microspheres and glucose fed-batch mode

The scale up of the ISPR system was performed with a 3 L bioreactor containing a 1 L working volume using periodic addition of glucose to maintain a concentration of 15 g/L (Eshkol et al., 2009). The initial cell concentration was 2 OD_{600} and the microspheres were added after 24 h during the log-phase of growth. In the fermentation systems containing 0%, 1% or 8% (w/v) PMMA microspheres, the final concentration of 2-PE in the media was 3.5 (Figure 6a), 3.1 (data not shown) and 1.65 g/L (Figure 6b), respectively. The encapsulated amounts (W_{EN}) obtained after extraction from the microspheres



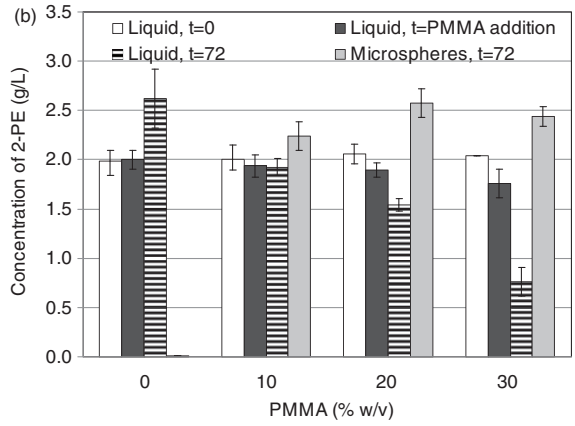


Figure 5. Effect of PMMA microsphere concentration on 2-PE yield under stress conditions. All systems contained an amount of 2 g/L 2-PE. (A) Quantity of 2-PE after 72 h in the liquid medium (stripes), within the microspheres (grey) and the total (black). (B) Concentration of 2-PE in the liquid phase at the beginning of the experiment (white), after the addition of PMMA microspheres (dark grey), and after 72 h (stripes) and the concentration within the PMMA microspheres after 72 h (grey). The experiment was performed in shake flasks at 30°C. The results are an average of two independent measurements.

were 0.47 g/L for the 1% system (data not shown) and –5.4 g/L for the 8% system. Thus, at 1% and 8% (w/v) microsphere concentrations, 15% and 76%, respectively, of the total 2-PE were encapsulated within the microspheres. The total amount of 2-PE produced in the system containing 8% microspheres was 7.05 g/L, which is 2-fold better than the system without ISPR. The encapsulation ratio obtained in the 8% (w/v) system was 0.07 g/g of 2-PE/PMMA, a value which is the highest reported to date, higher than the previously reported 0.025 g/g 2-PE/Hytrel (Gao and Daugulis, 2009) and 0.045 g/g 2-PE/D101 (Mei et al., 2009).

To examine if there was a correlation between encapsulation and microsphere swelling, SEM pictures were taken at different time points. The original average size of the PMMA microspheres was 1.52 ± 0.15 μm, and they swelled to 1.74 ± 0.21 μm shortly after they were added to the broth, which contained at that time more than 3 g/L 2-PE (Figure 7). The final average size of the microspheres was 1.86 ± 0.17 μm at the end of the fermentation, indicating a 22% average swelling (Figure 7). In the fermentation trial with 8% (w/v) PMMA microspheres, ethanol (EtOH)

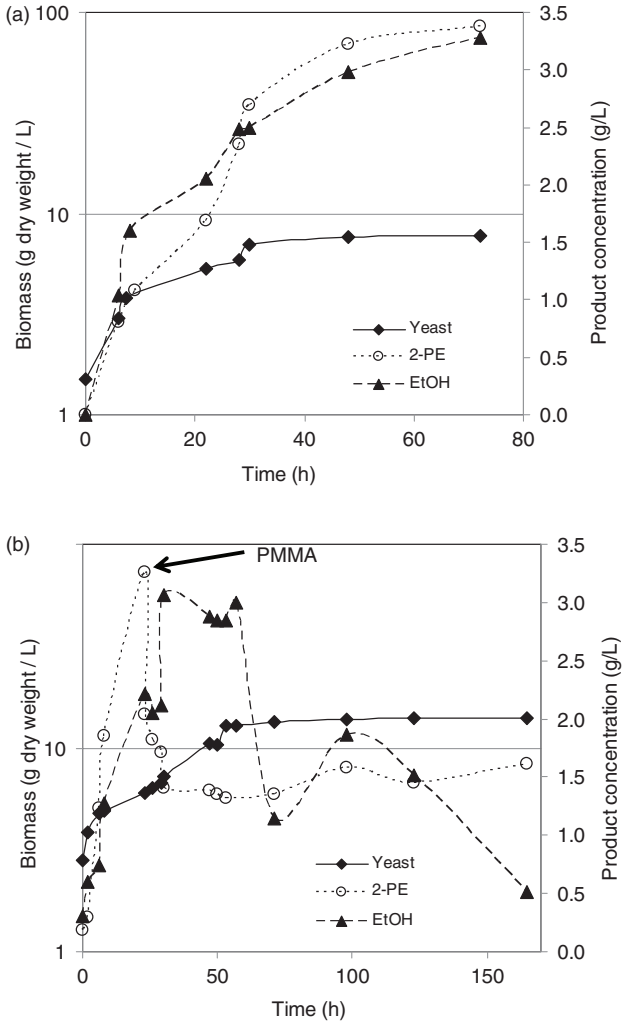


Figure 6. Biomass, 2-PE and ethanol concentrations in a 3 L fed-batch fermentation of *S. cerevisiae* Ye9-612. Glucose concentration was maintained at 15 g/L. (a) Without ISPR (b) With ISPR. PMMA microspheres (8% w/v) and Tween 80 (0.3% v/v) were added after 23 h, indicated with an arrow.

production was also monitored to see if there is a synergistic effect between 2-PE and ethanol. The ethanol concentration did not rise above 0.3% (v/v) (Figure 6b), which is less than the 1% inhibitory level previously reported (Stark et al., 2002), indicating that it was neither limiting nor inhibiting the yeast growth.

Conclusions

We have described a new efficient solid/liquid ISPR system based on swelling of PMMA microspheres. The high surface-to-volume ratio of the microspheres enabled excellent incorporation of 2-PE, resulting in a high uptake ratio per gram of resin. Experiments performed in shake flasks under simulated stress conditions of high 2-PE levels showed a

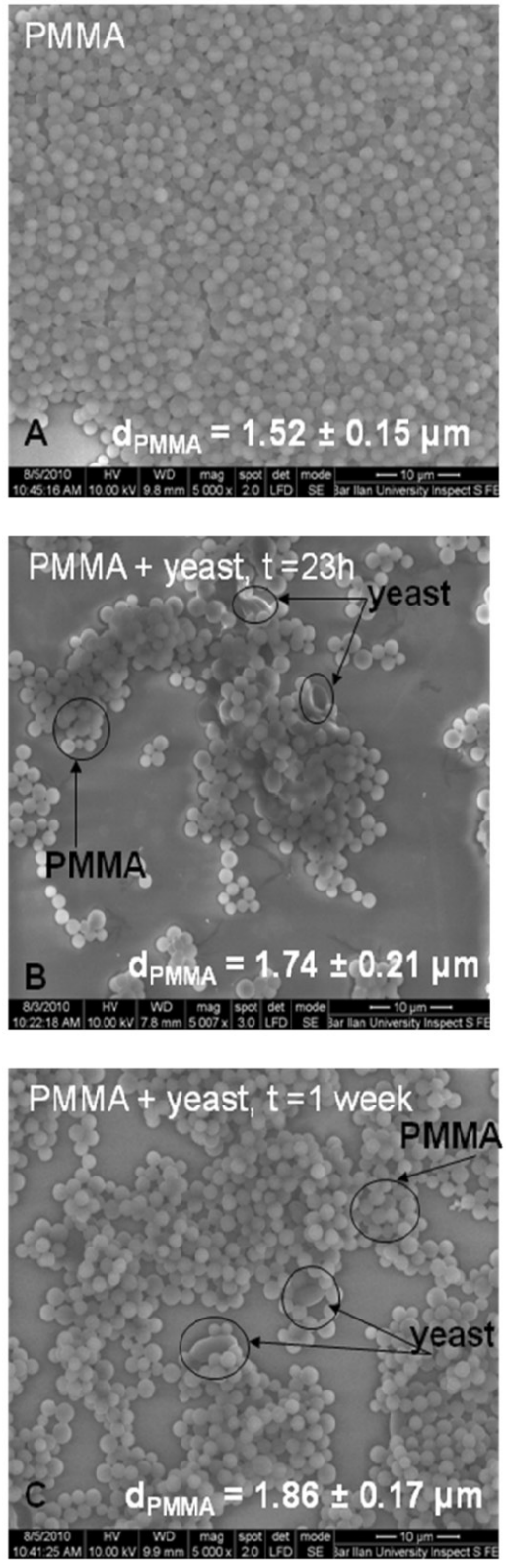


Figure 7. SEM images of PMMA microspheres taken from a 3 L fermentation. (A) Before the beginning of the fermentation. (B) After 1 day of fermentation and 0.5 h after the addition of the microspheres. (C) After 6 days of fermentation. The average diameter is presented revealing an increase of 22% in microsphere size at the end of the fermentation.

10-fold increase in product yield in the presence of 10% PMMA microspheres. In a bioreactor containing 8% (w/v) of microspheres, the increase in product concentration was twofold. The swelling of the microspheres due to 2-PE uptake was also proven by SEM images.

Acknowledgements

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References

Bamnolker H, Margel S. Dispersion polymerization of styrene in polar solvents: Effect of reaction parameters on microsphere surface composition and surface properties, size and size distribution, and molecular weight. J Polym Sci Part A Polym Chem, 1996;34:1857–71.

Bechtold M, Panke S. *In situ* product recovery integrated with biotransformations. Chimia, 2009;63:345–8.

Demain AL. The business of biotechnology. Ind Biotechnol, 2007;3:269–83.

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, 2009;106:534–42.

Etschmann MM, Bluemke W, Sell D, Schrader J. Biotechnological production of 2-phenylethanol. Appl Microbiol Biotechnol, 2002;59:1–8.

Etschmann MM, Schrader J. An aqueous-organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. Appl Microbiol Biotechnol, 2006;71:440–3.

Etschmann MM, Sell D, Schrader J. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. Biotechnol Lett, 2003;25:531–6.

Fabre CE, Blanc PJ, Goma G. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol Progr, 1998;14:270–4.

Galperin A, Margel D, Margel S. Synthesis and characterization of uniform radiopaque polystyrene microspheres for X-ray imaging by a single-step swelling process. J Biomed Mater Res Part A Appl Biomater, 2006;79:544–51.

Gao F, Daugulis AJ. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. Biotechnol Bioeng, 2009;104:332–9.

Garavaglia J, Flôres S, Pizzolato T, Peralba M, Ayub M. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. World J Microbiol Biotechnol, 2007;23:1273–9.

Goldshtein J, Margel S. Synthesis and characterization of new flame-retardant microspheres by dispersion polymerization of pentabromobenzyl acrylate. Eur Polym J, 2009a;45:2987–95.

Goldshtein J, Margel S. Synthesis and characterization of new UV absorbing microspheres of narrow size distribution by dispersion polymerization of 2-(2′-hydroxy-5′-methacryloxyethylphenyl)-2H-benzotriazole. Polym J (Tokyo), 2009b;50:3422–30.

Goldshtein J, Margel S. Synthesis and characterization of polystyrene/2-(5-chloro-2H-benzotriazole-2-yl)-6-(1,1-dimethylethyl)-4-methyl-phenol composite microspheres of narrow size distribution for UV irradiation protection. Coll Polym Sci, 2011;Submitted.

Gounaris Y. Biotechnology for the production of essential oils, flavours and volatile isolates. Flavour Fragr J, 2010;25:367–86.

Horak D. Effect of reaction parameters on the particle size in the dispersion polymerization of 2-hydroxyethyl methacrylate. J Polym Sci Part A Polym Chem, 1999;37:3785–92.

Horak D, Krystufek M, Spevacek J. Effect of reaction parameters on time dispersion polymerization of 1-vinyl-2-pyrrolidone. J Polym Sci Part A Polym Chem, 2000;38:653–63.

Jiang S, Sudol ED, Dimonie VL, El-Aasser MS. Dispersion copolymerization of methyl methacrylate and n-butyl acrylate. J Polymer Sci Part A: Polym Chem, 2007;45:2105–12.

Kim OH, Lee K, Kim K, Lee BH, Choe S. Optimum conditions for preparing micron-sized PMMA beads in the dispersion polymerization using PVA. Colloid Polym Sci, 2006;284:909–15.

Lee KC, Lee SY. Preparation of highly cross-linked, monodisperse poly (methyl methacrylate) microspheres by dispersion polymerization; Part II. Semi-continuous processes. Macromol Res, 2008;16:293–302.

Mei J, Min H, Lu Z. Enhanced biotransformation of l-phenylalanine to 2-phenylethanol using an *in situ* product adsorption technique. Process Biochem, 2009;44:886–90.

Sendovski M, Nir N, Fishman A. Bioproduction of 2-phenylethanol in a biphasic ionic liquid aqueous system. J Agric Food Chem, 2010; 58:2260–5.

Serp D, von Stockar U, Marison IW. Enhancement of 2-phenylethanol productivity by Saccharomyces cerevisiae in two-phase fed-batch fermentations using solvent immobilization. Biotechnol Bioeng, 2003;82:103–10.

Seward R, Willetts JC, Dinsdale MG, Lloyd D. The effects of ethanol, hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status: Synergistic inhibition. J Inst Brew, 1996;106:439–43.

Shpaisman N, Margel S. Synthesis and characterization of air-stable iron nanocrystalline particles based on a single-step swelling process of uniform polystyrene template microspheres. Chem Mater, 2005;18:396–402.

Stark D, Kornmann H, Munch T, Sonnleitner B, Marison IW, von Stockar U. Novel type of *in situ* extraction: Use of solvent containing microcapsules for the bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol Bioeng, 2003;83:376–85.

Stark D, Munch T, Sonnleitner B, Marison IW, von Stockar U. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol Progr, 2002;18:514–23.

Wittmann C, Hans M, Bluemke W. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. Yeast, 2002;19:1351–63.

Wohlgemuth R. Biocatalysis-key to sustainable industrial chemistry. Curr Opin Biotechnol, 2010;21:713–24.

Yang Z, Sakai M, Sayama H, Shimeno T, Yamaguchi K, Watanabe N. Elucidation of the biochemical pathway of 2-phenylethanol from shikimic acid using isolated protoplasts of rose flowers. J Plant Physiol, 2009;166:887–91.

Yu DG, An JH, Bae JY, Ahn SD, Kang SY, Suh KS. Negatively charged ultrafine black particles of P(MMA-co-EGDMA) by dispersion polymerization for electrophoretic displays. Macromolecules, 2005;38:7485–91.

Ziv O, Avtalion RR, Margel S. Immunogenicity of bioactive magnetic nanoparticles: Natural and acquired antibodies. J Biomed Mater Res Part A Appl Biomater, 2008;85A:1011–21.