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The Power of Simplicity: Efficient and Selective Carboligation with Whole-Cell Biocatalysts in Pickering Emulsion

Robert Röllig, Christoph Plikat, and Marion B. Ansorge-Schumacher*

Abstract: Pickering emulsions are particle-stabilized multiphase systems with promising features for synthetic application. We here describe a novel, simplified set-up employing catalytic active whole-cells for simultaneous emulsion stabilization and synthetic reaction. In the stereoselective carboligation of benzaldehyde to *(R)*-benzoin catalyzed by a benzaldehyde lyase in *E. coli*, the set-up yielded maximum substrate conversion within very short time, while economizing material demand and waste. Formation and activity of freshly produced PE were enhanced when the catalytic whole-cells were covered with hydrophobic silicone prior to PE formation. Benchmarked against other easy-to-handle whole-cell biocatalyses in pure organic solvent, neat substrate, an aqueous emulsion in substrate, and a micro-aquatic system, respectively, the cell-stabilized PE outperformed all other systems by far. Given the observed combination of synthetic performance and system's simplicity we expect it to hold great potential for becoming a future platform technology for biocatalyzed multiphase synthesis.

Particle stabilized, so-called Pickering emulsions (PE) have in recent years been recognized as valuable tools for multiphase catalytic processes [1], mainly because they offer beneficial solubility effects at a high resistance to breakdown processes without employing surfactants. Thus, surfactant induced adverse effects such as deactivation of catalysts or product contamination do not occur [2]. In 2011, our group successfully introduced the concept to biocatalysis demonstrating promising activity and stability of isolated enzymes in a water-in-oil (w/o) Pickering emulsion [3]. In that set-up, and the research to follow [1], the enzymes were located in the discontinuous aqueous phase emulsified in an organic solvent by non-reactive microparticles (mostly hydrophobized silicate). Qu, Ren and co-workers employed whole cells as interfacial catalysts by encapsulating single cells in a shell of calcium phosphate doped with Fe_3O_4 nanoparticles and coated with sodium monododecyl, using this construct for PE stabilization [4]. The catalytic performance of the whole-cells improved considerably compared to cells encapsulated in macroscopic alginate gels, demonstrating PE as promising reaction systems for whole-cell biocatalysis involving non-aqueous media. However, material preparation was rather complex, and in addition provided only oil-in-water (o/w) emulsions, which are of very limited utility in biocatalysis.

Here, we report an innovative and broadly applicable strategy for implementing PE in whole-cell biocatalysis, yielding a highly efficient and competitive system with native or slightly modified microbial cells. Due to simultaneous emulsion stabilization and catalysis by the cells, addition of extra particles to the emulsion is not required. At the same time, the use of whole cells instead of enzymes avoids material and cost intensive catalyst purification. The system provides the formation of w/o emulsions, in which a hydrophobic organic solvent forms the continuous phase for both substrate supply and product extraction. The hydrophilic (water) phase acts as a flexible support for the biocatalysts helping to ensure favorable pH and water activity. Thus, the system can address the vast number of syntheses where reaction partners have low water solubility.

Scheme 1. (*R*)-benzoin condensation catalyzed with benzaldehyde lyase (BAL).

The investigation involved a standard lab strain of *Escherichia coli* (SG13009) as a microbe with high relevance and broad applicability in whole-cell biocatalysis. It contained a recombinant benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* Biovar I catalyzing the stereoselective carboligation of benzaldehyde to *(R)*-benzoin (Scheme 1). Thus, it allowed direct comparison of catalytic active PE with alternative reaction systems for non- and semi-aqueous biocatalysis [5]. To inverstigate influences of surface hydrophobicity on eligibility for emulsion stabilization cells coated with hydrophobic and more hydrophilic silicone, respectively, were tested in comparison to unmodified cells. Detailed information on cell production, biocatalyst purification and coating are provided in Supporting Information. As organic phase, methoxycyclopentane (CPME), 2-methyltetrahydrofuran (2-MeTHF) and (*1Z,5Z*)-1,5-cyclo-octadiene (1,5-COD) were selected according to their ecologic sustainability and, in case of CPME and MeTHF, broad applicability in biocatalysis [6] including distinct aptitude for benzoin transformation.

Based on reports stating that the natural surface hydrophobicity of microbial cells can be sufficient to stabilize PE [7], we attempted dry (lyophilized) unmodified *E. coli* cells for PE formation. This was successful with all three investigated solvents

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at a phase ratio of 1:5 (w/o). The obtained emulsions mostly were of multi-phasic character (Figures 1A and S1), and appeared considerably more viscous than the phase forming fluids alone. 1,5-COD was only partly integrated in the emulsion and formed an additional separate phase (Figure S1). In all PEs, *E. coli* cells containing recombinant BAL also showed catalytic activity (Figure S1). It was highest in CPME yielding a specific activity of 6.2 ± 0.2 kU $mg^{-1}_{protein}$ after 15 min (1 U referring to a substrate conversion of 1 µmol·min^{-1}), and a product yield of 558,6 mg ± 33.5 mg (*R)*-benzoin from a 14-mL batch (see Supporting Information for details on analysis).

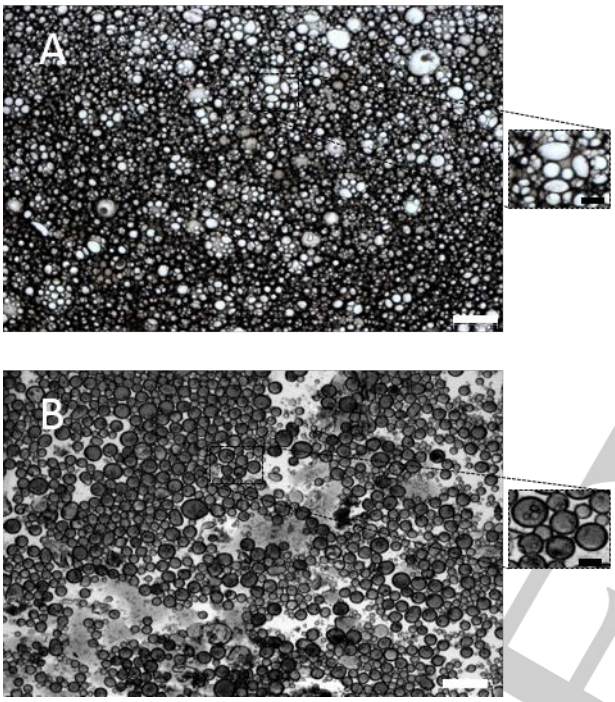

Figure 1. Light microscopic picture immediately after formation of w/CPME (1:5 (v/v)) PE stabilized by unmodified (**A**) and surface-modified (**B**), lyophilized *E. coli* SG13009 cells containing recombinant BAL. (3 % (w/v_{water})). o/w/o-Type multi-emulsion are designated by white droplets in grey droplets (zoom A), while emulsions of w/o-type build entirely grey droplets (zoom B). The white scale bars refer to 200 µm, the black bars in the zoom pictures to 50 µm.

In the formation and stabilization of PE, surface wettability of the employed particles plays a key role [8]. The occurrence of multi-emulsions in the cell-stabilized PE indicates that the cell surface properties of *E. coli* enable both adoption of contact angles less than 90° as well as more than 90° [9] connected to unclear wettability. We expected phase complexity of the cell-stabilized PE to reduce upon modification of hydrophobicity, and thus wettability, of the cell surface. According to a method previously developed in our working group [10], we deposited a hydrophobic silicone (derived *via* Pt-catalyzed hydrosilylation from commercial siloxanes DVS-50 and SiH 50/10; for details see Supporting Information) on the surfaces of lyophilized *E. coli* SG 13009 cells containing recombinant BAL (Figure S2). With these surface-modified cells, considerably less multi-emulsions were observed directly after formation, and after stirring at 220 rpm for up to 24 hours (Figures 1B and S3, respectively). Associated was an obviously lower viscosity of the emulsion, while it was equally stable as the emulsion obtained with unmodified cells (Figure 1A) over time.

PEs with defined phases were also achieved when we used a more hydrophilic silicone (obtained from C11-PEG400 and SiH-50/10 siloxanes according to [11]; Table S1) for cell coating. Emulsions stabilized by these cells initially exhibited a chimeric character, displaying small aqueous droplets comparable to those in PEs stabilized by cells modified with hydrophobic silicone (~10 µm in diameter) and considerably bigger aqueous droplets (~100-200 µm in diameter) with enclosed solvent droplets (resembling o/w/o-type emulsions). Over time, however, this morphologic difference to PEs stabilized by cells modified with hydrophobic silicone faded, and converged after 24 hours (Figure S3).

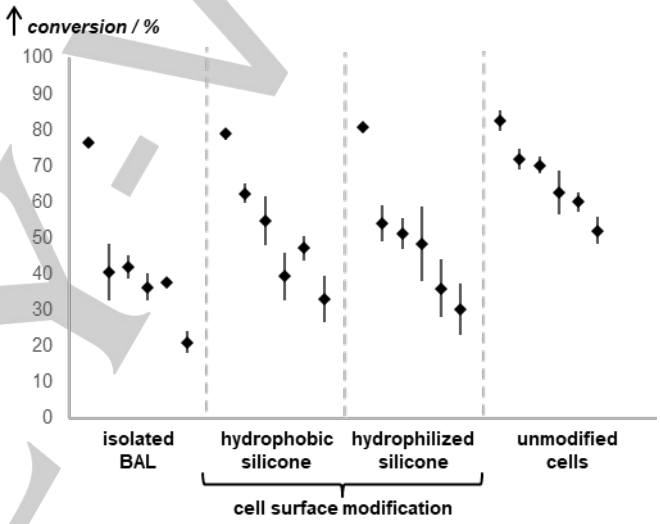

Figure 2. Conversion of benzaldehyde within one hour in PE with free (isolated) BAL and different catalytic active whole-cell formulations in repeated reaction cycles. PE consisted of TEA buffer/CPME at a ratio of 1:5 and 3 % (w/v) of cells. Each data point refers to substrate conversion within one hour. The initial substrate concentration in the organic phase (CPME) was 400 mmol·L^{-1}. Error bars represent standard deviation of results from three independent batches.

For evaluation of the biocatalytic performance of PE stabilized with surface modified catalytic active cells, we benchmarked their activity in benzoin condensation with PE stabilized by unmodified, catalytic active cells and with PE containing free enzyme (BAL not contained in cells) in the aqueous phase. For proper comparability of the systems, the enzyme amount within the cells was estimated from a SDS PAGE after cell disruption (Figure S4), and the free-enzyme system was doped with roughly the same enzyme amount. Additionally, the free-enzyme PE was stabilized with *E.coli* SG13009 cells, which were modified with hydrophobic silicone, but were not catalytically active themselves. Within a reaction time of one hour, all investigated systems (free enzyme in PE, enzyme in unmodified and either hydrophobic or more hydrophilic surface-modified cells stabilizing PE) achieved a substrate conversion of ~76-83 %

(Figure 2), which is in the same range as for BAL-catalyzed benzoin condensation (free enzyme) reported in literature [5]. Assuming that the estimation of enzyme amounts within the catalytic active cells was correct the equal conversion indicates a very small diffusional barrier in all reaction systems. It is noteworthy to mention, that the enantiomeric excess (*ee*) of the reaction product *(R)*-benzoin was >99 % in all batches.

The advantages of using catalytic active whole cells in the PE instead of isolated enzyme became evident upon repetitive use. For repetition, we removed a defined amount of organic solvent from the PE after a reaction time of one hour and replaced it with the same amount of fresh solvent (see Supporting Information for details). Again, the enantiomeric excess of (*R*)-benzoin was >99 % in all cycles. However, the catalytic performance of the isolated enzyme dropped dramatically yielding a conversion of only ~41 % after the first and ~21 % after the fifth recycling. Conversion also dropped when PE with catalytic active whole-cells were used repeatedly, but considerably less than with the free enzyme (Figure 3). The activity loss is probably due to the repeated exposition to the organic solvent, which in the whole cells was cushioned by the protective environment of the cell envelope.

Astonishingly, the PE stabilized with unmodified catalytic active cells performed significantly better upon recycling than PE stabilized with modified cells. A conversion drop of only ~13 % occurred after the first recycling, after the fifth recycling conversion was still at 52.0±3.7 %. This was more than 1.5-fold better than in the PE stabilized with modified cells, regardless of the type of silicone used for modification. Presently, we can only speculate about the reasons. It might be that activity in the coated systems dropped as a delayed effect of the coating procedure, which involved solvent exposure for several hours and might have weakened the cell envelope. Alternatively, the higher viscosity of the PE stabilized with unmodified whole cells may have a protective effect on the cells during the process.

In contrast, PE stabilized with modified cells, in particular cells modified with hydrophobic silicone, showed the highest reaction rate of all systems. It achieved maximum conversion (77.5±5.7 %) within only 15 minutes (Figure S5 and S6, respectively), while PE stabilized with unmodified cells reached only ~86 % of maximum conversion in the same time. PE stabilized with cells modified with hydrophilized silicone achieved ~92 % of the maximum conversion. The observation might be explained by a facilitation of the access of hydrophobic molecules to the cell due to their accumulation in the surficial silicone layer.

Finally, we evaluated the competitiveness of our bioactive whole-cell PE through benchmarking their catalytic performance against other promising reaction set-ups for BAL-catalyzed benzoin condensation, namely whole-cells (unmodified or modified with hydrophobic silicone) in pure organic solvents, in neat substrates, and in micro-aquatic systems. In addition, we tested a two-phase system consisting of an aqueous solution of (unmodified) whole cells dispersed in pure substrate (substrate emulsion). Like in the previous benchmarking of PE-stabilized systems, we made sure that comparable amounts of enzyme (cells) were present in all set-ups.

As illustrated in Figure 3, within a reaction time of one hour, we observed measurable amounts of (*R*)-benzoin only in PE, substrate emulsion, and the micro-aquatic system. Upon extending the reaction time to 24 hours, small amounts of product were also obtained with unmodified and modified cells in pure CPME (19.6±12.4 mmol·L^{-1} and 79.9±33.1 mmol·L^{-1}, respectively). PE stabilized with modified cells (hydrophobic coating) yielded 148.6±5.7 mmol·L^{-1} (*R*)-benzoin within one hour (designating 100% in Figure 3), exceeding substrate emulsion (2.1±0.3 mmol·L^{-1}) and micro-aqueous system (59.0±19.7 mmol·L^{-1}) by 70- and more than two-fold, respectively. Considering that micro-aqueous systems have to date been among the most efficient reaction systems for whole-cell biocatalysis in non-conventional media [12], this is a formidable performance. It might be explained by a facilitation of diffusion in PE compared to diffusion in the only partially fluid micro-aquatic systems.

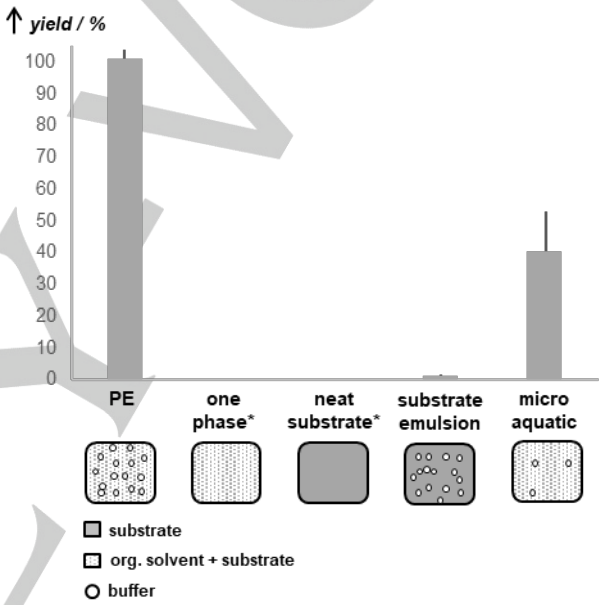

Figure 3. (*R*)-benzoin yields from catalytic active cells after one hour reaction in various set-ups: Pickering emulsion stabilized by cells modified with hydrophobic silicone (PE); pure CPME (one phase); undiluted benzaldehyde (neat substrate); small amounts of buffer in benzaldehyde (substrate emulsion); cell suspension in CPME with addition of a very small amount of buffer (micro-aquatic). Error bars represent standard deviation of results from three independent batches. Benzaldehyde concentration in PE, one phase and mirco aquatic was 400 mmol L^{-1}. Experiments were repeated with an equal amount of unmodified *E. coli* cells* in one phase and neat substrate systems. Water activity of either 0.37, or 0.79 was adjusted for both cells and benzaldehyde in neat substrate systems. PE and substrate emulsions were formed at a water:sovlent ratio of 1:5 (v/v). Water:solvent ratio in the micro aquatic was 1:111 (v/v).

In conclusion, Pickering emulsions using *E. coli* cells for stabilization and simultaneous catalysis provided both efficient and stable systems for biocatalyzed carboligation, overcoming current limits in the use of whole-cell biocatalysts for synthesis. Omission of extra particles and waiver of enzyme purification makes the set-up considerably simpler than biocatalytic PE currently in use. Formation of viscous multi-emulsions can, if desired, easily be overcome through non-covalent cell-surface coating with silicone. As a side effect such a modification can further improve catalytic performance. We expect these findings

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to be fully transferrable to both other reactions and whole-cell biocatalysts implying great potential for becoming a future platform technology in biocatalyzed multiphase synthesis.

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Keywords: Whole-cell biocatalysis • Pickering emulsion • Carboligation • Cell surface modification • Silicone

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