Immobilised Enzymes for Sesquiterpene Synthesis in Batch and Flow Systems

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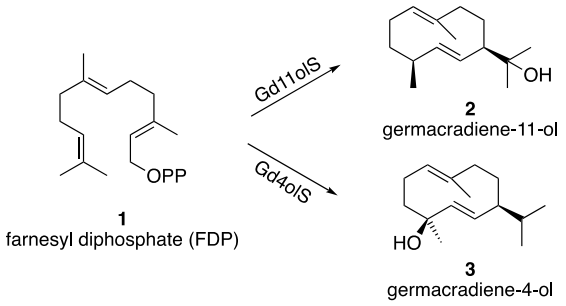
Abstract: Sesquiterpene synthases catalyse the bioconversion of farnesyl diphosphate into sesquiterpenes. The immobilisation of sesquiterpene synthases on controlled porosity glass through metal-ion affinity binding is reported. The immobilised sesquiterpene synthases were able to maintain 50% catalytic activity for at least 50 cycles under continuous flow conditions.

The application of enzymes for the synthesis of chemical compounds that are not easily obtainable via chemical synthetic routes has been significantly expanded. In general, reducing protection/deprotection steps in biocatalytic routes decreases the requirement of solvents, reagents, time and energy.[1] Synthesis of sesquiterpenes as a large group of natural products is not an exception. Regardless of their structural diversity, all sesquiterpenes are synthesised from farnesyl diphosphate (**1**, FDP) or its analogues in high purity with recombinant terpene synthases (Scheme 1).[2] The overall rate of these enzymatic reactions is typically limited by the release of the hydrophobic product into the aqueous solvent. We have reported that the use of organic-aqueous biphasic systems for rapid extraction can enhance sesquiterpene synthesis.[3] However, in addition to the necessity of enzyme expression and purification, low turnover numbers and low productivity of sesquiterpene synthases hinder their use in medium and large scale syntheses. To overcome these limitations, enzyme reusability is a fundamental requirement which can be obtained using various immobilisation techniques.[4] Sesquiterpene synthase immobilisation on a solid support should provide reusability and easier downstream processing and could be used to develop continuous processes.



Scheme 1. Germacradienols **2** and **3** generated from FDP **1** by germacradien-11-ol synthase (Gd11olS) and germacradien-4-ol synthase (Gd4olS).

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Affinity immobilisation is a well-known method where the selective attachment of the enzyme with an affinity tag to a complementary solid support is exploited. This method minimizes conformational changes of an immobilised enzyme by controlling the enzyme orientation. Metal-ion affinity binding is a known method for tethering the enzymes onto a support where a metal ion localised on the matrix interacts with a fused terminal histidine tag (His_6-tag).[5] Recently, a hybrid-controlled porous glass (EziG), coated with an organic polymer and chelated Fe^{3+} has been used for enzyme immobilisation.[6] This carrier has a highly porous network providing a maximum binding capacity of ~30% (w/w, enzyme/carrier). It has already been applied to the (co)immobilisation of a broad range of enzymes including lipases, transaminases, monooxygenases, reductases and dehydrogenases, which showed highly efficient biocatalytic performance in batch and in flow systems.[6-7]

Herein we present the application of EziG with a hydrophilic derivatised silica surface for the immobilisation of germacradien-4-ol synthase (Gd4olS) and germacradien-11-ol synthase (Gd11olS) and analyse their performance in organic-aqueous biphasic batch and flow systems. The N-terminal His-tag fusion proteins of Gd11olS and Gd4olS (referred to henceforth as His-Gd11olS and His-Gd4olS, respectively) were produced in *E. coli* and purified to homogeneity (see SI, Figure S1). The particle pore sizes of EziG are 50 ± 5 nm diameter and enable relatively unrestricted access of His-Gd11olS (enzyme diameter ~5 nm) and His-Gd4olS (enzyme diameter ~4.7 nm) to the internal pore surface area. Characteristic enzyme diameters were obtained with the CalcTool where the diameter is estimated from an empirical relationship calculated from its molecular weight.[8] The immobilization process was evaluated in terms of incubation time and binding capacity of the carrier. If not mentioned otherwise, purified enzymes were used for all experiments. Given low turnover numbers for most sesquiterpene synthases, highest level of enzyme loading on the carrier were targeted. Figure 1A shows that the maximum binding capacity of the carrier is 260 $mg_{Gd11olS}/g_{carrier}$ (± 22, N = 10) corresponding to ~20% (w/w, enzyme/carrier). Thompson et al. reported the maximum binding capacity of 15 – 32% for different classes of enzymes.[7c] The process of saturating the carrier was also shown by SDS-PAGE (see SI, Figure S2). The progress of immobilisation showed that about 80% of the enzyme was bound to the carrier after 10 min and the immobilisation process was essentially complete after 30 min. A facile and applicable immobilisation of sesquiterpene synthases was demonstrated that comprised approx. 5.8 $\mu mol_{Gd11olS}/g_{carrier}$.

Excessive enzyme loading on carriers might reduce the turnover number of immobilized enzymes. Therefore, the effect of carrier saturation on the performance of loaded enzyme was studied. If not mentioned otherwise, all reactions were done in 200 μL Tris buffer (20 mM, pH = 8) overlaid with pentane containing 43 μM α-humulene as an internal standard and the volume ratio org:aq

(1:1). Figure 1B shows that the conversion has a linear dependence to the amount of immobilised enzyme with 2-12% loading at 10 min reaction time; it levelled off at ~70% conversion, most likely because of mass transfer limitation between the organic and aqueous phases. As the immobilised enzyme performed similarly with no evidence of overloading, saturating of the carrier was not in conflict with the accessibility of immobilised enzyme on the carrier. Other investigations with EziG have shown a similar behaviour.[6] As a consequence, we applied 20% enzyme loading for other experiments. This approach could be expanded to co-immobilise the enzymes of the terpene biosynthetic pathway.[9] Chen *et al.* reported the co-immobilisation of enzymes on Ni-NTA resin in a random manner for amorpha-4,11-diene biosynthesis; however, the full characterisation and optimisation of these complex co-immobilised multienzyme systems remained unclear.[10]

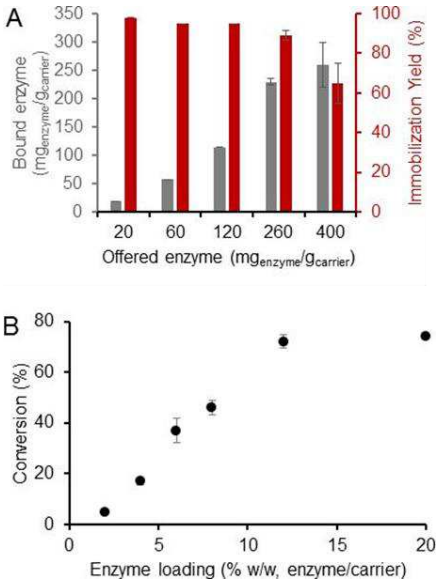


Figure 1. Analysis of EziG binding capacity and characterisation of His-Gd11olS EziG. A) Binding capacity of the carriers in terms of bound enzyme and immobilisation yield. B) Dependency of the conversion on the amount of loaded enzyme used in the reaction. Reaction mixture contained 0.35 mM **1**, 5 mM $MgCl_2$ and 10 mg carrier (containing 4.5-44.7 nmol enzyme). Error bars show standard deviations from two independent experiments.

Typically, the productivity of immobilised enzymes is lower than the productivity of free enzymes due to the attachment to the solid surface and/or mass transfer limitation.[11] The effectiveness factor (η) is defined as the ratio between the activity of immobilised enzyme and the activity of free enzyme indicating the efficiency of immobilisation. The germacradiene-11-ol (**2**) synthesis was followed over time using 4.5 nmol free and immobilised His-Gd11olS (20% loading). Figure 2 shows that the free enzyme is 2.7-fold more active than the immobilised one which gives the η value of 0.36 ± 0.02 (calculated at the initial reaction phase).

The reusability of biocatalysts in batch reactions can compensate the fact that $\eta < 1$ but also overcome the high cost and time of enzyme expression and purification. The reuse of the solid catalyst in several cycles of the reaction is shown in Figure 3. FDP (**1**) was converted to **2** in 30 min with 18 nmol enzyme (20% w/w, enzyme/carrier). About 60% conversion was

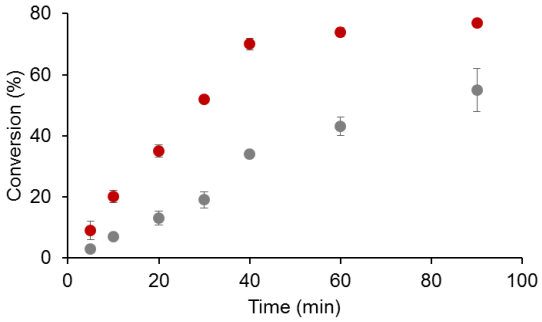
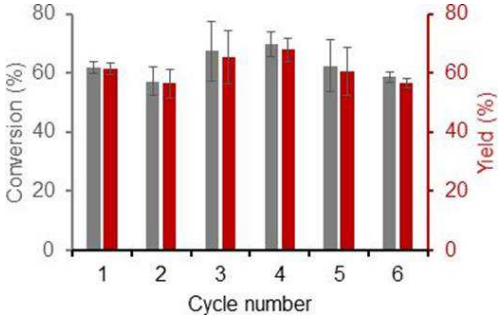


Figure 2. Time course of the synthesis of **2** using free (•) and immobilised (•) His-Gd11olS. Reaction mixture contained 0.35 mM **1,** 5 mM $MgCl_2$ and 4.5 nmol free or immobilised enzyme (0.75 mg carrier). Error bars show standard deviations from two independent experiments.

maintained for six consecutive batches demonstrating the robustness of the biocatalyst under the applied reaction conditions. At 60 % conversion (lower than the highest conversion obtained, 70 %) enzyme inactivation or leakage was not observed. Considering the short reaction times (30 min) used for terpene synthase, a turnover number (μmol product/μmol enzyme) of ~16 was calculated for six cycles. Furthermore, no change in behaviour of the enzyme was observed related to product pattern in each cycle, which demonstrated the stability of immobilised enzyme during the recycling process. Böhmer *et al.* showed the high reusability of transaminase EziG for 16 consecutive cycles (15 min each) in an aqueous phase.[6] The robustness of His-Gd11olS EziG combined with the high loading capacity of EziG therefore indicates a possible application for long-term operation.

Figure 3. Reuse of the immobilized His-Gd11olS. Reaction mixture contained



0.35 mM **1**, 5 mM $MgCl_2$ and 3 mg carrier (containing 18 nmol enzyme). Error bars show standard deviations from two independent experiments.

Continuous flow systems with immobilised enzymes allow precise control over reaction conditions, easy downstream processing and enhances the amount of product over the operation time of the reactor.[12] An efficient use of immobilised enzymes EziG over long periods of time in packed bed reactors (PBR) has been

demonstrated.[6, 7c] To the best of our knowledge, as the first report, we investigated immobilised sesquiterpene synthases on EziG under continuous liquid-liquid segmented flow conditions (Figure 4A).

The concentration of **1** had to be optimised for continuous flow experiments (0.2 mM **1**, 0.5 mM $MgCl_2$) as the batch experiment concentrations (0.35 mM **1**, 5 mM $MgCl_2$) leads to precipitation over time. The dependence of the conversion on the amount of loaded enzyme on carrier under optimised reaction conditions (0.2 mM **1**, 0.5 mM $MgCl_2$) was studied in batch using the same amount of enzyme mentioned in Figure 1B. Similar behaviour was observed in the presence of 0.35 mM FDP/5 mM $MgCl_2$ and 0.2 mM FDP/0.5 mM $MgCl_2$ (see SI, Figure S3). However, the highest conversion of 50% was obtained in the presence of 0.2 mM FDP/0.5 mM $MgCl_2$ which is 1.4-fold lower compared to 0.35 mM FDP/5 mM $MgCl_2$. Limited accessibility of **1** or Mg^{2+} to the loaded enzyme due to reduced concentration in the reaction mixture might cause lower conversion.

The effect of different enzyme loadings between 2% and 20% on the performance was studied under steady state reaction conditions. 200 mg carrier contained 4-48 mg enzyme (0.09-1 μmol) was used for the PBR system. The residence time (τ_{res}) defined as the ratio of reactor volume (void volume = 450 μL) to flow rate was 22.5 min. A conversion of 40% was obtained with 8% enzyme loading, however, the reactor blocked after 9 h operation. The precipitation of **1** inside the pores of the carrier without enzyme might cause a slow clogging of the column. Therefore, 20% enzyme loading where the carrier is fully saturated with the enzyme was used for a continuous flow experiment. Figure 4B shows that the conversion is maintained at 50% for around 19 h of continuous operation which is equal to about 50 cycles of the reactor. The actual reaction time was normalised on τ_{res} to show the number of reactor cycles.

After optimizing the reaction conditions for His-Gd11olS, we performed the same experiment using His-Gd4olS EziG in the flow system. The η of 0.47 ± 0.02 was achieved for His-Gd4olS in batch. 100 mg carrier (contained 13 mg His-Gd4olS, 0.34 μmol) was used for the reactor operation (void volume = 250 μL). As the k_{cat} of Gd4olS (0.079 s^{-1})[13] is 25-fold higher than Gd11olS (k_{cat} = 0.0032 s^{-1})[14], we applied a lower τ_{res} for the operation of the reactor. Figure 4C shows that the conversion of 50% obtained at the τ_{res} of 4.2 min and remained constant for 4.5 h of reactor operation equal to a cycle number of 65 for the reactor. It seems that limited substrate accessibility to the loaded enzyme combined with low product release from the enzyme binding pocket causes incomplete conversion of **1** to sesquiterpenes. After several hours of operation, a grey colour was observed at the inlet of the reactor. This could be related to protein aggregation due to the direct contact with pentane, however, the high enzyme loading turns out to be an advantage of the PBR as the conversion remains constant. The main limitation of the current system is the low concentration limit of **1** fed into the flow reactor due to its precipitation in the presence of Mg^{2+}. *In situ* production of **1** for a simultaneous utilisation with terpene synthases in a flow system may offer a solution to the substrate limitation.

In conclusion, we report a study on the efficient immobilisation of sesquiterpene synthases on a porous carrier. Enzymes with His_6-tags allow facile immobilisation on controlled porosity glass to generate robust and reusable biocatalysts. We demonstrate that the conversion can be maintained at 50% for at least 50 cycles under continuous flow condition for germacradienol synthesis. However, low concentration of **1** (due to its precipitation in the presence of Mg^{2+}) hinders the application of PBR for the production of terpenes at large scale. This system could pave the way for co-immobilisation of enzymes from the terpenoid biosynthetic pathway for the efficient production of valuable sesquiterpenes in biphasic continuous flow systems. Alternative approaches to solubilise FDP (**1**) in high concentrations in the reaction remains an important task for terpene synthesis in packed bed reactors.

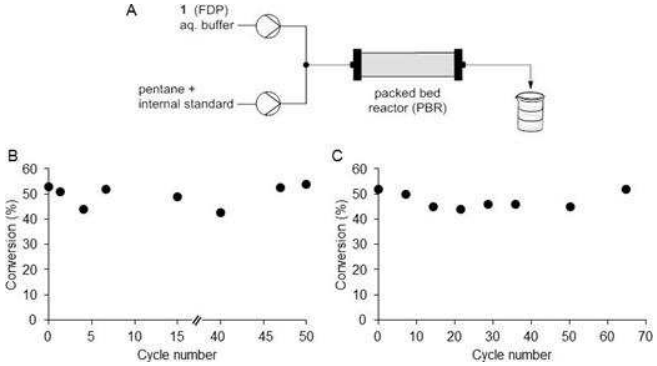


Figure 4. Continuous flow system for the production of sesquiterpenes using immobilised enzymes. A) Liquid-liquid segmented flow system. B) and C) show the long-term operation of the reactor for the production of **2** and **3**, respectively. Aqueous phase contained 0.2 mM **1** and 0.5 mM $MgCl_2$ in Tris buffer (20 mM, pH = 8). Volume ratio org:aq (1:1).

Experimental Section

Experimental Details are explained in SI.

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Keywords: Sesquiterpene • Biocatalysis • Enzyme immobilization • Multiphase reaction • Flow chemistry

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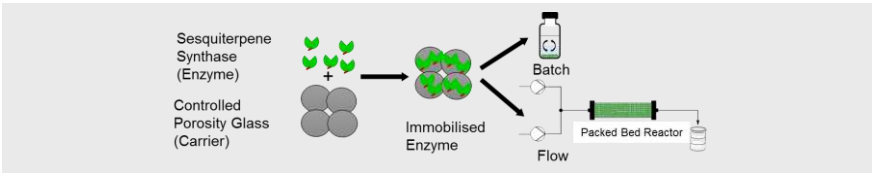

Sesquiterpene synthase immobilisation on controlled porosity glass through metal-ion affinity binding is exploited. This strategy improves enzyme reusability and allows for easier downstream processing and the development of continuous flow processes.

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