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Immobilized Whole Cells as Effective Catalysts for Chiral Alcohol Production

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Recombinant *Escherichia coli* overexpressing the gene *Lb*ADH, which encodes for an alcohol dehydrogenase from *Lactobacillus brevis*, was successfully transformed and cultured. The cells are able to catalyze the reduction of pro-chiral ketones, e.g. ethyl acetoacetate into *R*-(−)ethyl hydroxybutyrate (EHB) with high conversion and enantiomeric excess >99%. Immobilizing the whole cells in alginate beads leads to a catalyst with improved stability and ease of handling while maintaining the high activity of the free cells. The whole-cell catalyst was tested in a stirred batch reactor (CSTR) and in a continuously operated packed-bed reactor. An Mg^{2+} concentration of 2 mM was crucial for maintaining the activity of the biocatalyst. After a partial optimization of the process conditions, a productivity of 1.4 $g_{EHB}\ g_{wcw}^{-1}\ h^{-1}$ could be maintained in a continuous flow reactor over a prolonged period of time.

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Introduction

Most biologically active compounds including many pharmaceuticals have chiral molecular structures with one or more stereogenic centres. Because of safety, therapeutic, and regulatory concerns, it is necessary to evaluate and test every drug in their enantiomerically pure forms.[1] The ready access to chiral synthons is therefore very important. Optically active secondary alcohols are widely used by the pharmaceutical industry for the introduction of chirality.[2] Routes to single enantiomers of small molecules can be classified into four groups: chiral pool synthesis, resolution, biological, and chemical asymmetric techniques.[3] Asymmetric reduction of the corresponding pro-chiral ketones proved to be one of the most effective and promising routes to chiral alcohols.[4] The environmentally friendly biocatalytic approach using oxidoreductases is attractive compared with chemical methods involving transition metal catalysts and organic solvents. The biocatalyst, used either as the isolated enzyme or as the whole cell, catalyzes the reduction of pro-chiral ketones with remarkable chemo-, regio-, and stereoselectivity.[5] Functional group protection/deprotection strategies are usually not necessary in reactions catalyzed by enzymes, in contrast to chemical catalysts. Moreover, biocatalytic reductions can be carried out at ambient temperature and atmospheric pressure.[6] Thus, biotransformations can simplify manufacturing processes, making them economically more attractive and at the same time more environmentally acceptable.[7]

Alcohol dehydrogenases (ADHs) catalyze the reversible reduction of functional groups such as ketones and ketoesters to chiral alcohols, and provide access to either the (*R*) or (*S*)-form, depending on the enzyme used. The most commonly used ADHs are those available from yeast, horse liver, and *Thermoanaerobium brockii*. All these enzymes reduce the ketone by transferring the pro-*R* hydrogen to the re-face of substrate forming (*S*)-alcohols in a process characterized by Prelog's rule.[8] However, enzymes isolated from the *Lactobacillus* strains *L. kefir* or *L. brevis* catalyze the formation of (*R*)-alcohols from a wide range of substrates in good yields and high enantiomeric excess (ee) (94–99%).[9,10]

Free enzymes have disadvantages that make them less suitable for technical applications, notably insufficient long-term stability, and limited substrate acceptance.[11] When isolated enzymes are used for oxidation or reduction reactions, the necessity to provide the required coenzymes constitutes a major drawback. The cost for stoichiometric amounts of (reduced) nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) is prohibitive, but even when the cofactors are regenerated, the required processes are complex, proving a significant economic obstacle against using ADH for commercial syntheses.[12]

Cofactor regeneration can be achieved in different ways, such as coupled enzyme,[13,14] coupled substrate,[15] as well as by electro-enzymatic, photochemical, and chemical approaches.[16] Whole cell fermentation offers an alternative to the use of isolated enzymes. The whole cell biocatalyst acts as a mini-reactor with all the necessary cofactors and sequences of enzymes available within one cell. The same enzyme, ADH, catalyzes the reduction of the substrate and the oxidation of the cosubstrate isopropanol to regenerate the NAD(P)H as shown in Fig. 1.[17] In addition, isopropanol acts as the cosolvent and increases the solubility of the organic substrate in the aqueous phase in which the biocatalytic reaction is carried out.

Furthermore, the enzymes are more stable within the cell where they are in their natural environment.[18] The wild forms of microorganisms frequently contain several enzymes that can act on the same substrate, usually with different stereoselectivity. This compromises the ee. In a study by Ribeiro et al. on

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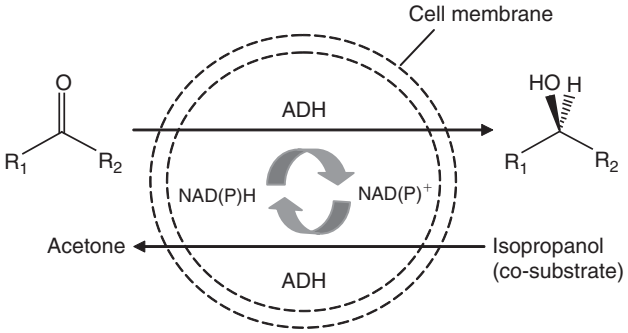


Fig. 1. Whole-cell reduction: substrate and cosubstrate enter the cell where the enzymatic reduction takes place under regeneration of the cofactor NAD(P)H.

the reduction of ethylacetoacetate by different wild type microorganisms, the highest ee was 81% with a *Hansenula* sp.[19] This situation improved dramatically with the development of 'designer bugs' in which only those enzymes specific for the desired biotransformation are overexpressed.[20]

Continuously operated processes are favoured on an industrial scale because they offer higher volumetric productivity and avoid the setup times associated with batch reactions. However, less than a quarter of all biotechnological processes currently conducted on an industrial scale are operated in a continuous mode.[21] Schroer et al. demonstrated the first continuously operated biotransformation process catalyzed by recombinant *Escherichia coli* using a membrane reactor.[17] By applying the coupled substrate approach, these authors successfully produced (*R*)-methyl-3-hydroxybutyrate at a maximum space time yield of 700 g L^{-1} per day. They used 50 g_{wcw} L^{-1} biomass in a 0.05 L reactor and retained the cells by an ultrafiltration membrane with a molecular weight cutoff of 10 kDa. However, problems such as membrane failure are common for membrane reactors, and the higher cost of the ultrafiltration membrane adds to the cost of production. Immobilization of the biocatalyst in a porous carrier allows for an effective continuous production of chiral alcohols at a much lower biomass concentration. Immobilization of the cells on a support is promising for cell reuse, because it eliminates the costly processes of cell recovery by centrifugation or cross-flow filtration, and moreover, offers stability advantages over free cells.[22,23] The principal methods for whole cell immobilization are adsorption, covalent binding, encapsulation, entrapment, crosslinking, or combinations thereof.[24–26] The successful immobilization of a variety of cells in different matrices has been reported.[27–29] The present study describes the optimization of the immobilization of recombinant *E. coli* in calcium alginate beads and the reaction conditions for the chiral reduction of various β-ketoesters to the corresponding alcohol, in particular the reduction of ethylacetoacetate (EAA) to (*R*)-ethyl-3-hydroxybutyrate (EHB).

Results and Discussion

Concentration of Sodium Alginate

Different concentrations (1%, 2%, 3%, and 4%) of sodium alginate were used to prepare the immobilized biocatalyst. The conversion of EAA to EHB was independent of the concentration of sodium alginate. With all of the immobilized biocatalysts, ~98% conversion of 100 mM EAA could be achieved within 3 h reaction time. Because EAA is a rather small molecule, diffusion through the alginate network does not noticeably affect

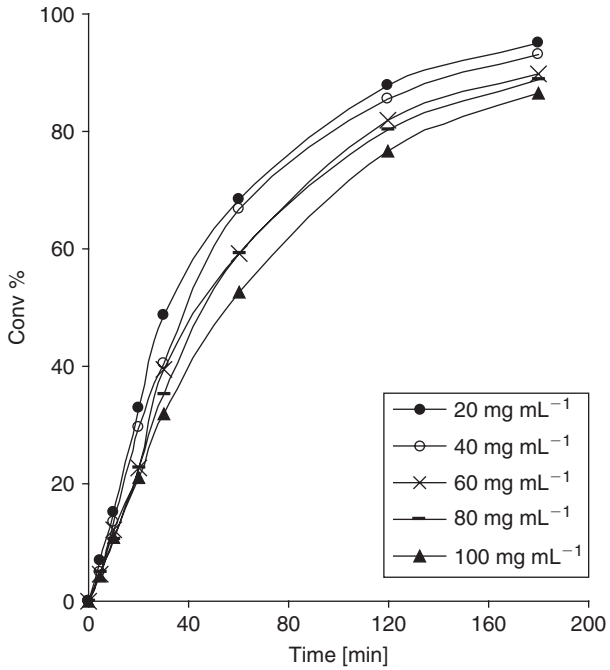


Fig. 2. Effect of cell loading on the bioreduction of ethyl acetoacetate.

the reaction rate. However, 3% and 4% sodium alginate solutions are more viscous, making the extrusion of the solution into the hardening bath more difficult. 2% Sodium alginate solution was preferred over 1% for further studies because of the higher mechanical strength which increases the potential for reusability.

Cell Concentration

The bioreduction of EAA was investigated as a function of the cell loading within the 2% sodium alginate beads. The result is shown in Fig. 2. A higher concentration of cells resulted in a lower conversion of EAA. Under these conditions, the cells agglomerate, and this reduces the surface area available for the bioreduction. Only cells near the surface take part in the reaction, whereas cells deeper inside the beads cannot be reached by the substrate. Smaller pores might also lead to additional diffusion limitations.

Influence of the Cations for the Hardening Bath

It is known that the affinity of alginates for alkali metals increases in the order $Mg^{2+} << Ca^{2+} < Sr^{2+} < Ba^{2+}$.[30] Calcium and barium ions were chosen as the divalent ions in the hardening bath to study the effect on the mechanical strength of the beads. The result is shown in Fig. 3. Both the calcium alginate and the barium alginate immobilized biocatalyst gave similar results when tested over 15 cycles at 37°C. Calcium alginate dissolves in sodium citrate solution, with the citrate acting as a chelating agent for Ca^{2+} ions. However, barium alginate is tough enough to remain in bead shape after stirring in citrate buffer for 24 h. However, the increased robustness of the barium alginate beads did not lead to a better performance during the recycling, and in both cases, the biocatalysts lost ~25% of their initial activity after 15 cycles of re-use. It might be that cell leaching only occurred within the beads' outer surfaces, or the decrease of activity is simply due to the deactivation of the biocatalyst.

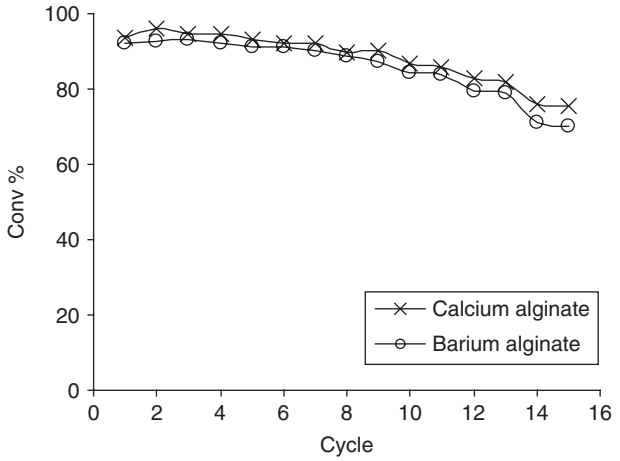


Fig. 3. Effect of divalent ions used in the hardening bath.

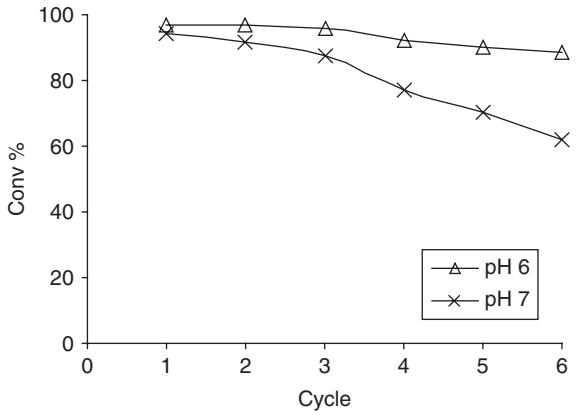


Fig. 4. Effect of pH during the immobilization of the cells in calcium alginate.

Effect of pH in the Preparation of Calcium Alginate Immobilized Cells

The best performance was obtained in solution buffered at near neutral pH. The optimum pH was further fine tuned in the preparation of calcium alginate immobilized cells. Acetate buffer pH 6 and TRIS-HCl buffer pH 7 were used as the buffers in dissolving sodium alginate and preparing the cell slurry. The bioreduction was carried out in buffered solutions with the respective pH at 37°C. Fig. 4 shows the results. Initially, cells immobilized at pH 6 and pH 7 gave similar performance; however, the activity of cells immobilized at pH 7 decreased more rapidly than that of cells immobilized at pH 6 and was lower by ~20% for the 6th cycle of re-use. Slightly acidic conditions were thus identified as optimal for the bioreduction with whole *E. coli* cells.

Reusability

The stability and the reusability of the biocatalyst were studied and the results are shown in Fig. 5. The conversion of EAA to EHB decreased drastically after the free cells had been recycled six times, and less than 5% of the initial activity remained after the 14th run. However, the cells immobilized in calcium alginate demonstrated excellent stability over many cycles, and the conversion of the EAA was more than 70%, even after 15 cycles. Part of the drop in activity of the free cells may be caused by inefficient recovery of the biocatalyst by centrifugation after the reaction. The cell mass was checked at the end of the

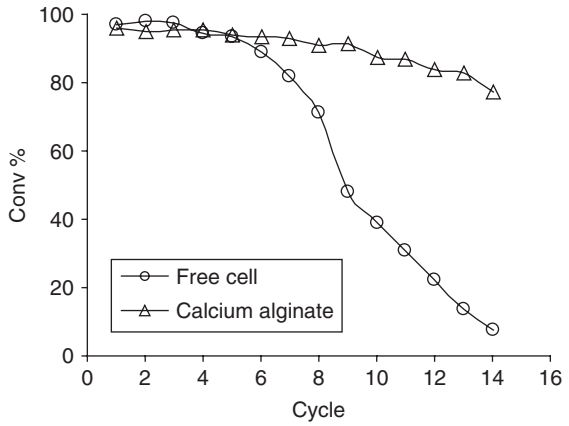


Fig. 5. Process stability for free and immobilized cells during the continuous reduction of ethyl acetoacetate in a batch-wise reaction.

Table 1. Experimental details for the continuous bioreduction of ethyl acetoacetate (EAA)

Tubular reactor	16 mm ID × 480 mm
Reactor volume	0.096 L
Flow rate	0.032 L h^{-1}
Residence time (τ)	3 h
EAA:IPA	1:2
Temperature	37°C
Product monitoring	Optical rotation (Perkin–Elmer 341) and GC (Agilent with capillary column HP-5)

recycling experiment, and it was found that it had decreased significantly, from 0.10 g to 0.018 g. In contrast, ~78% of the cells still remained entrapped in the calcium alginate after 14 cycles. Additionally, the immobilized biocatalyst showed a higher activity as expressed in the productivity of 24.7 $mmol_{EHB}$ g^{-1} cell compared with only 10.6 $mmol_{EHB}$ g^{-1} cell for the free cells.

Continuous Reduction of EAA in a Plug-Flow Reactor

The bioreduction with calcium alginate immobilized recombinant *E. coli* overexpressing *Lb*ADH was carried out under the operational condition shown in Table 1.

To establish the optimum concentration of EAA for highest productivity, EAA concentrations from 0.3 M to 1 M were fed into the reactor. As shown in Table 2, 0.7 M of EAA (91 g L^{-1}) is the optimum concentration at 3 h retention time in the reactor. Higher concentrations may lead to substrate inactivation of the enzyme, or to physiological changes in the cell wall due to the high content of organic solvents in the medium. The maximal productivity was 1.4 g_{EHB} g_{wcw}^{-1} h^{-1} at a conversion of 81%. However, the activity of the catalyst started to decrease gradually after 18 h. Further optimization of the reaction medium was carried out. When 2 mM Mg^{2+} was added to the substrate solution, the reactor could be operated for 30 h before eventually the activity of the catalyst gradually decreased as shown in Fig. 6. Mg^{2+} is present in the active site of *Lb*ADH, and it is known that the binding of Mg^{2+} causes structural changes which activate the enzyme.[31] The effect is element specific, and no positive effect on the activity was observed when Ca^{2+} instead of Mg^{2+} was co-fed with the substrate solution. The performance of the continuous process under optimized condition is shown in Table 3.

Table 2. Optimization of the substrate concentration

Wet cell mass [g]	EAA concentration [mol L^{-1}]	Flow rate [L h^{-1}]	Maximum conversion	Productivity [g_{EHB} g_{wcw}^{-1} h^{-1}]
1.67	0.3	0.034	98%	0.8
1.67	0.5	0.032	97%	1.2
1.67	0.7	0.032	81%	1.4
1.98	1.0	0.026	70%	1.2

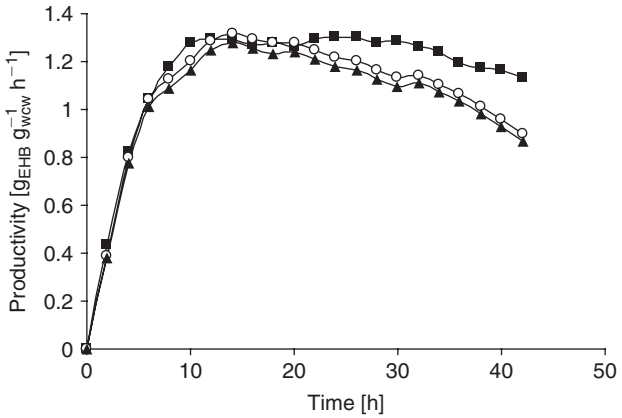


Fig. 6. Stability of the continuous bioreduction of ethyl acetoacetate. (■), 2 mM Mg^{2+}; (▲), 2 mM Ca^{2+}; (○), without divalent ions. EHB, *R*-(−)ethyl hydroxybutyrate.

Table 3. Optimized reaction conditions for continuous production of *R*-(−)ethyl hydroxybutyrate

Ethyl acetoacetate concentration	0.7 M
Conversion	0.81
Biomass concentration	17 g_{wcw} L^{-1}
Space time yield	25 g L^{-1} h^{-1}
Productivity	1.4 g_{EHB} g_{wcw}^{-1} h^{-1}
Enantiomeric excess	>99%

Bioreduction of Other Ketoesters and Acetophenone

Recombinant *E. coli* overexpressing an ADH of *L. brevis* is able to convert several ketoesters to the corresponding chiral alcohols with good ee (Table 4). However, the branched molecule, methyl 4-methyl-3-oxopentanoate, was not accepted as substrate (entry 5). This is most likely caused by the more bulky residue attached to the carbonyl group of this ketoester. The activity of the cells for the reduction of simple ketones was also investigated. Acetophenone is reduced at a much lower rate compared with the other ketoesters. This confirms the observation by Weckbecker et al., who reported that the activity of *Lb*ADH towards EAA was 4.36 relative to that of acetophenone which was taken as 1.[11] In all cases, the recombinant cells produce alcohols with the same absolute configuration at the optically active centre. This is generally the (*R*)-form, except for the case of methyl 4-chloro-3-oxobutanoate where the product is (*S*) as a consequence of the Cahn-Ingold-Prelog nomenclature rules. The immobilized biocatalyst performed generally as well as the free cells in terms of conversion and stereoselectivity, and no problems due to diffusion limitations within the beads were observed.

Conclusions

Whole recombinant *E. coli* cells overexpressing *Lb*ADH were successfully immobilized using alginate as immobilization matrix. Cells immobilized by the optimized protocol show a better stability than the free cells, so that the activity of the biocatalyst is maintained over more cycles. The recombinant *E. coli* shows excellent activity for the reduction of several ketoesters to the corresponding chiral alcohols with high conversion and ee. Immobilization does not affect the activity of the cells for the catalytic reduction. The continuous bioreduction of EAA in a plug flow reactor using the immobilized cells is a simple and economic approach, and we demonstrated a productivity of 1.4 g_{EHB} g_{wcw}^{-1} h^{-1} with low biomass concentration.

Experimental

Transformation of E. coli *with Plasmids Bearing the Gene Encoding for* Lb*ADH*

Plasmid pBtac-*Lbadh* (X-zyme, Düsseldorf, Germany) bearing the *adh* gene from *Lactobacillus brevis* as well as an ampicillin resistance gene was employed as expression vector for the *E. coli* BL21 Star (DE3) (Invitrogen, Carlsberg, USA) host cells. Competent *E. coli* cells were transformed by adding 5 μL of the plasmid solution into an Eppendorf tube containing the *E. coli* in a buffer. The cells were incubated for 20 min at 4°C to induce the uptake of plasmids. The cells were then heated in a 42°C water bath for 90 s to inactivate the nucleases, and then cooled to 4°C again for 5 min before 400 μL of Hanahan's SOC medium was added.[32] The cells were then incubated at 37°C for 1 h with light shaking to allow the cells to repair the cell membranes. 25 μL of cell suspension was streaked on a Luria-Bertani (LB)-agar plate containing 100 mg L^{-1} of ampicillin and the plates were incubated overnight at 37°C. The ampicillin present in the LB agar selects for cells that were successfully transformed. A colony of transformed *E. coli* was selected randomly from a LB agar plate and used to inoculate 100 mL LB medium containing 100 mg L^{-1} ampicillin in a 250 mL Erlenmeyer flask. This preculture was incubated overnight at 30°C with stirring at 150 rpm. Aliquots of this preculture were used for subsequent cell cultivation.

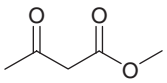
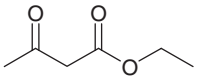
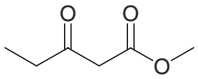
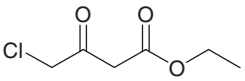
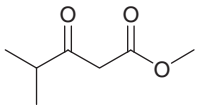
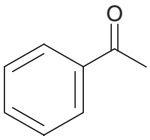
Cell Cultivation

0.5 mL of the preculture was further cultured in 500 mL 'terrific broth' medium containing 200 mg L^{-1} of ampicillin at 37°C and 300 rpm. Antifoam (Anti-foam 204, Sigma) in ppm level was added to reduce foaming in the aerated fermentation. Culturing was continued until the OD_{660} reached to ~0.6–0.8 which took ~6 h. At this point, isopropylthiogalactoside (IPTG) was added to a final concentration of 0.2 mM to induce gene expression. Culturing was continued for a total of 24 h. The cells were harvested by centrifugation (2860*g*, 20 min) and were stored at 4°C as a 50 g L^{-1} cell suspension in 50 mM potassium phosphate buffer pH 6.0.

Immobilization of Recombinant E. coli *in Calcium Alginate Gel*

2.0 g alginic acid sodium salt from brown algae (Fluka) was heated in 80 mL of 50 mM acetate buffer pH 6 to give a clear solution. The alginate solution was cooled to room temperature with stirring. 2.0 g of wet cells was washed once with 50 mM acetate buffer pH6 and resuspended in 20 mL of the same buffer and added to the alginate solution to give a final concentration

Table 4. Different substrates for reduction by recombinant *Escherichia coli* cells

Entry	Substrate	Conversion % immobilized	free cells	Enantiomeric excess %
1	 Methyl 3-oxobutanoate	97.5	97.3	>99 (*R*)
2	 Ethyl 3-oxobutanoate	98.3	98.4	>99 (*R*)
3	 Methyl 3-oxopentanoate	93.7	94.1	>99 (*R*)
4	 Ethyl 4-chloro-3-oxobutanoate	100	100	>99 (*S*)
5	 Methyl 4-methyl-3-oxopentanoate	–	–	–
6	 1-Phenylethanone (acetophenone)	54.9	54.9	>99 (*R*)

2% (w/v) sodium alginate solution containing 20 mg mL^{-1} of cells. The homogeneous mixture was extruded drop-wise into 200 mL of 0.1 M calcium chloride solution via a BD spinal syringe needle 20GA 3.5 IN by using a syringe pump at a flow rate of 2.0 mL min^{-1}. The calcium chloride hardening bath was stirred at 100 rpm during the addition. After finishing the addition, the formed beads with immobilized cells were kept stirring for another hour to ensure complete hardening. After that, the beads were filtered off and washed with 100 mL of 0.1 M calcium chloride solution. They were then resuspended in 100 mL of the same solution and kept at 4°C overnight for complete curing. This procedure yields uniform calcium alginate beads with a diameter of ~3 mm.

Effect of Different pH (Preparation of Immobilized Cell)

Two different buffers were used in dissolving the sodium alginate and suspending the cells, namely a 50 mM acetate buffer for pH 6 and a 50 mM Tris buffer for pH 7. The procedure was otherwise the same as for the preparation of calcium alginate immobilized cells described above.

Determination of Cell Loading

The calcium alginate beads were dissolved as described by Xue et al.[33] A known amount of immobilized *E. coli* (10 beads) was weighed and added to a 0.06 M trisodium citrate solution pH 7.8–8.2 in a 5 mL volumetric flask. The beads were stirred at 200 rpm until they were completely dissolved and yielded a cloudy cell suspension. The optical density of the suspension was measured at 660 nm. The cell density corresponding to the OD was determined from a calibration curve.

Batch Bioreduction of EAA

For bioreductions with free cells, 0.1 g of cell pellet was weighed in a conical flask and suspended in 25 mL of 50 mM potassium phosphate buffer pH 6.0. For immobilized cells, the beads were washed once with 50 mM acetate buffer pH 6. Beads containing 0.1 g of cells (based on cell loading) were weighed and suspended in 25 mL of the same buffer. For both cases, ethyl acetoacetate and isopropanol were added to the buffer to a final concentration of 100 mM and 200 mM, respectively. The flask was shaken at 120 rpm at 37°C for 3 h. The cells were separated from the reaction mixture by filtration (immobilized cells) or centrifugation (free cells). The remaining reaction mixture was extracted thrice with an equal volume of diethyl ether. The solvent was evaporated and the ee of the product was determined by GC using a Supelco BetaDex 325 chiral column.

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