Multienzyme Mevalonate Pathway Bioreactor

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Abstract: The five-carbon metabolic intermediate isopentenyl diphosphate constitutes the basic building block for the biosynthesis of all isoprenoids in all forms of life. Two distinct pathways lead from amphibolic intermediates to isopentenyl diphosphate. The Gram-positive cocci and certain other pathogenic bacteria employ exclusively the mevalonate pathway, a set of six enzyme-catalyzed reactions that convert 3 mol of acetyl-CoA to 1 mol each of carbon dioxide and isopentenyl diphosphate. The survival of the Gram-positive cocci requires a fully functional set of mevalonate pathway enzymes. These enzymes therefore represent potential targets of inhibitors that might be employed as antibiotics directed against multidrug-resistant strains of certain bacterial pathogens. A rapid throughput, bioreactor-based assay to assess the effects of potential inhibitors on several enzymes simultaneously should prove useful for the survey of candidate inhibitors. To approach this goal, and as a proof of concept, we employed enzymes from the Gram-positive pathogen *Enterococcus faecalis*. Purified recombinant enzymes that catalyze the first three reactions of the mevalonate pathway were immobilized in two kinds of continuous flow enzyme bioreactors: a classical hollow fiber bioreactor and an immobilized plug flow bioreactor that exploited a novel method of enzyme immobilization. Both bioreactor types employed recombinant acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase from *E. faecalis* to convert acetyl-CoA to mevalonate, the central intermediate of the mevalonate pathway. Reactor performance was monitored continuously by spectrophotometric measurement of the concentration of NADPH in the reactor effluent. Additional potential applications of an Ni^{++} affinity support bioreactor include using recombinant enzymes from extremophiles for biosynthetic applications. Finally, linking a Ni^{++} affinity support bioreactor to an HPLC-mass spectrometer would provide an experimental and pedagogical tool for study of metabolite flux and pool sizes of intermediates to model regulation in intact cells.

Keywords: bioreactor; mevalonate; HMG-CoA; acetoacetyl-CoA thiolase; HMG-CoA synthase; HMG-CoA reductase; hollow-fiber reactor

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INTRODUCTION

Reactors that employ enzymes as catalysts offer the advantages of chiral specificity, selective catalysis of a single reaction, and the consequent absence of undesired by-products. While these advantages are partially offset by the sensitivity of enzymes to extremes of temperature, pH, and heavy metals, and the expense of some enzymes and cofactors, enzyme-based bioreactors find wide applications in industrial processes such as the hydrolysis of lactose, resolution of racemic amino acids, and waste treatment (Drioli and Giorno, 1999; Giorno and Drioli, 2000; Horvath and Engasser, 1974). Recombinant DNA techniques have dramatically expanded the spectrum of possible applications of enzyme-based bioreactors. Costs have been significantly lowered and the purification of enzymes previously isolated from living organisms by tedious, low-yield protocols have been greatly simplified.

The continuous removal of products that characterizes plug-flow reactors offers significant advantages for the catalysis of reactions that are thermodynamically unfavored or are product-inhibited. Enzymes may be segregated in a module, immobilized in or on a membrane, or attached to a support by physical absorption, ionic binding, or covalent attachment (Giorno and Drioli, 2000). Plug-flow bioreactors employ enzymes immobilized to ion-exchange or hydrophobic supports or covalently linked to a support. These reactors provide stability and productivity but have the potential disadvantage, particularly for covalent attachment, of possible denaturation of enzymes during the binding step (Giorno and Drioli, 2000; Arcos et al., 1999). Hollow fiber bioreactors provide a large surface area for substrate and product flux, easy replacement of spent catalyst, and an attractive alternative for enzymes sensitive to covalent attachment techniques (Fink and Rodwell, 1975). Hollow fiber bioreactors also are suited to therapeutic applications such as a bioartificial pancreas or liver, or blood detoxification (Michaels, 1991).

Isopentenyl diphosphate constitutes the fundamental building block for synthesis of isoprenoids, ubiquitous compounds that serve multiple metabolic roles in all forms of

life (Cane, 1999). The best-characterized of the two metabolic pathways that form isopentenyl diphosphate is the six enzyme mevalonate pathway that converts acetyl-CoA to isopentenyl diphosphate (Fig. 1). The Gram-positive cocci such as *Enterococcus faecalis* use exclusively the mevalonate pathway to form isopentenyl diphosphate. Their survival thus requires a fully functional set of mevalonate pathway enzymes (Wilding et al., 2000). These enzymes therefore represent attractive potential targets for novel inhibitors for use as antibiotics to combat multidrug-resistant

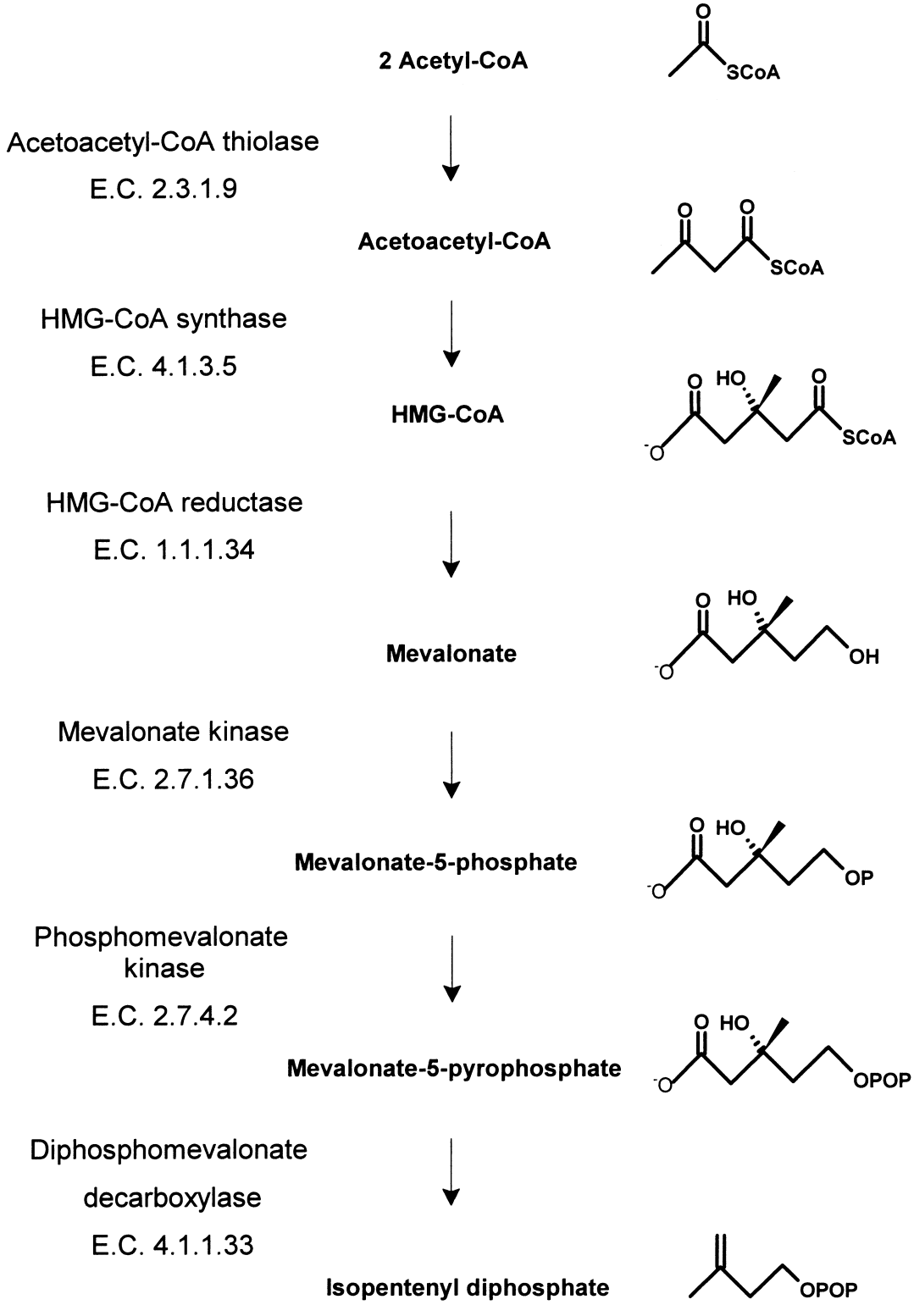

Figure 1. Intermediates and enzymes of the mevalonate pathway of isopentenyl pyrophosphate biosynthesis.

strains of bacterial pathogens. To facilitate the antibiotic discovery process, we developed a bioreactor to assess the effects of potential inhibitors on several bacterial enzymes simultaneously. Two types of bioreactor were employed: a hollow fiber bioreactor and a plug flow bioreactor that exploited a novel method of enzyme immobilization. Both bioreactors used purified recombinant *E. faecalis* acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase to catalyze the first three reactions of the mevalonate pathway. Additional potential applications include using recombinant enzymes from extremophiles for biosynthetic applications. Finally, linking a bioreactor to an HPLC-mass spectrometer would provide an experimental and pedagogical tool for study of metabolite flux and pool sizes of intermediates to model regulation in intact cells.

MATERIALS AND METHODS

Reagents

(*R,S*)-3-Hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA), acetyl-CoA, β-NADPH, and Tris were purchased from Sigma (St. Louis, MO). Ni-NTA-Agarose was from Qiagen (Chatsworth, CA).

Expression Plasmids

Plasmid pET28efTR (formerly MvaEef-pET28-6blunt) contains a hexahistidine-tagged construct of the *E. faecalis mvaE* gene that encodes a dual-function polypeptide that catalyzes both the acetoacetyl coenzyme A thiolase and HMG-CoA reductase reactions (Hedl et al., 2002). pET28efS2 (formerly MvaS-pET28-6H) encodes a hexahistidine-tagged construct of *E. faecalis* HMG-CoA synthase (Sutherlin et al., 2002). Homogeneous unfused *E. faecalis* HMG-CoA reductase (residues 400–823 of the fusion protein) was also expressed from pET28-efR as a hexahistidine construct (Hedl, 2003).

Enzymes

Plasmids that encoded recombinant enzymes were transformed into *Escherichia coli* BL21(DE3) cells, which were then grown on Luria Broth (Sambrook et al., 1989)

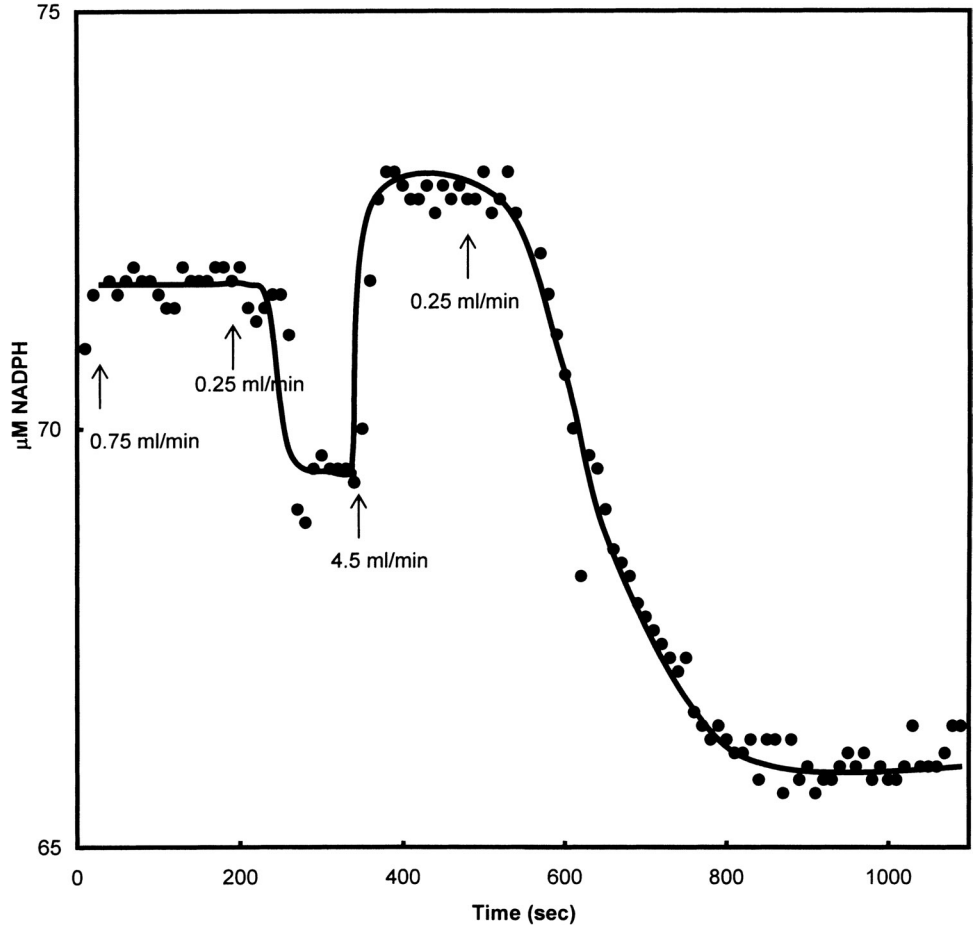

Figure 2. Hollow fiber bioreactor. Effect of flow rate on the conversion of acetyl-CoA to mevalonate. The initial flow-rate, 0.75 ml/min, was varied as indicated.

containing 50 μg per mL kanamycin. Expression of each encoded enzyme was induced by addition of 0.5 mM isopropyl thiogalactoside. The expressed enzymes were then purified to apparent homogeneity by affinity chromatography on Ni^{++}-agarose as previously described (Hedl et al., 2002; Sutherlin et al., 2002).

Hollow Fiber Bioreactor

The hollow fiber reactor employed a 2-ml process volume module from MicroKros (Spectrum Labs, http://www.spectrapor.com) with 0.5 mm diameter polyethersulfone fibers, surface area 7 cm^2, molecular weight cutoff 10 kD. *Enterococcus faecalis* acetoacetyl-CoA thiolase/HMG-CoA reductase fusion protein (3.75–5.9 nmol), HMG-CoA synthase (3.75–75 nmol), and unfused HMG-CoA reductase (9.0–90 nmol) were present in the compartment that surrounded the fibers. A peristaltic pump was used to apply the input stream (100 μM acetyl-CoA, 0.4 μM NADPH, 200 mM KCl, 4 mM $MgCl_2$ in 50 mM Tris, pH 8.5) to the hollow fibers. Emerging products passed through a spectrophotometer flow cuvet, then to a reservoir for potential product recovery.

Nickel Affinity Bioreactor

The nickel affinity bioreactor contained 22.5 nmol acetoacetyl-CoA thiolase/HMG-CoA reductase fusion protein, 65 nmol HMG-CoA synthase, and 90 nmol unfused HMG-CoA reductase from *E. faecalis* or 200 nmol unfused *E. faecalis* HMG-CoA reductase alone adsorbed on 1 mL of NiNTA affinity resin in a 3-ml Bio-Rad (Hercules, CA) glass column and associated flexible tubing. Either gravity flow or a peristaltic pump was used to apply the input stream (100 μM acetyl-CoA, 0.4 μM NADPH, 200 mM KCl, 4 mM $MgCl_2$ in 50 mM Tris, pH 8.5) to the nickel reactor.

Monitoring Bioreactor Performance

For both types of reactor, detection of the coupled conversion of acetyl-CoA to mevalonate employed a Hewlett-Packard model 8452 diode array spectrophotometer

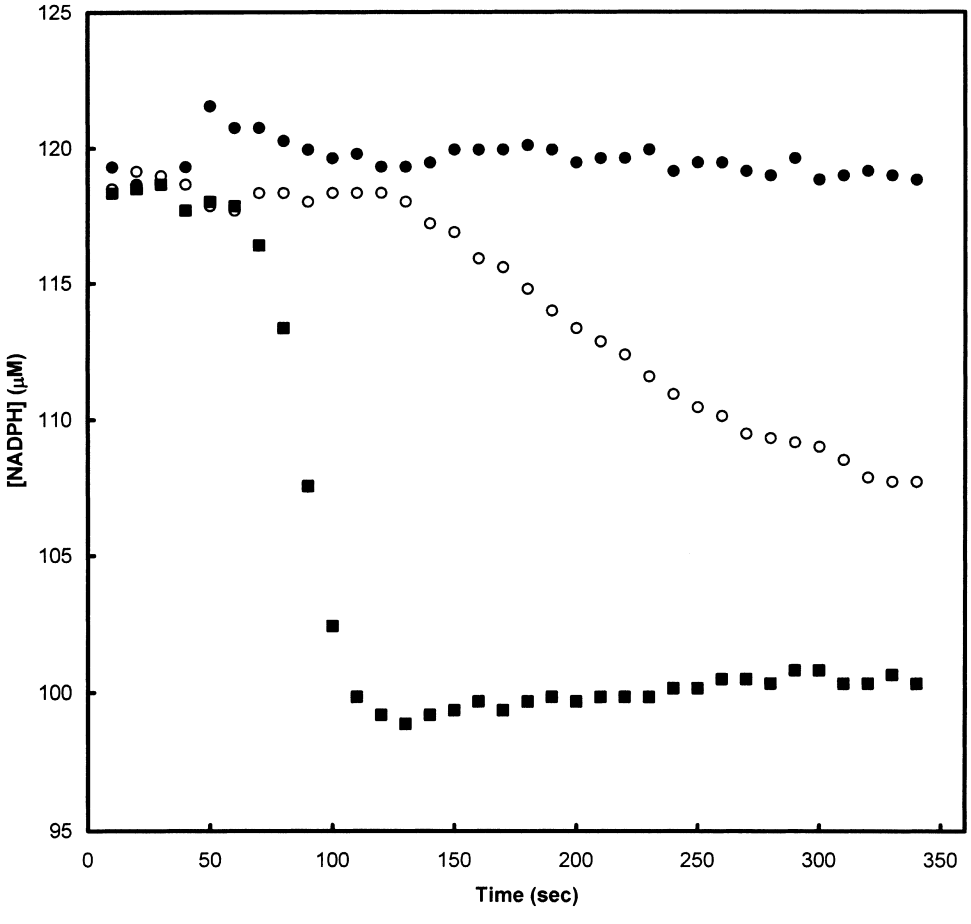

Figure 3. Hollow fiber bioreactor. The HMG-CoA reductase activity of the *E. faecalis* fusion protein is rate-limiting for conversion of acetyl-CoA to mevalonate. Conditions employed equimolar concentrations of the fusion protein in the absence (○) or presence of unfused HMG-CoA reductase (■). The control lacked acetyl-CoA (●).

equipped with a flow-through cell to monitor the decrease in absorbance at 340 nm that accompanies the oxidation of NADPH.

RESULTS

Hollow Fiber Bioreactor

Determination of Optimal Flow-Rate

Bioreactor efficiency was determined by monitoring the fraction (%) of NADPH oxidized by comparing NADPH concentration in the effluent stream to that in a control stream that contained no acetyl-CoA. The effect of flow rate on conversion of acetyl-CoA to mevalonate was studied by the varying flow rate from 0.25–4.5 ml/min. Maximal product formation occurred at 0.25 ml/min, the slowest available setting of the pump employed (Fig. 2).

HMG-CoA Reductase Activity of the Fusion Protein Is Rate-Limiting

In mammalian systems HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway. When the reactor was charged with equimolar (3.75 nmol) concentrations of HMG-CoA synthase and the fusion protein (acetoacetyl-CoA thiolase/HMG-CoA reductase), conversion of NADPH to $NADP^+$ was 10%. Subsequent addition of 9.0 nmol of unfused HMG-CoA reductase increased NADPH conversion to 16% (Fig. 3), illustrating that the fused HMG-CoA reductase was also rate-limiting for the bacterial enzymes under these conditions.

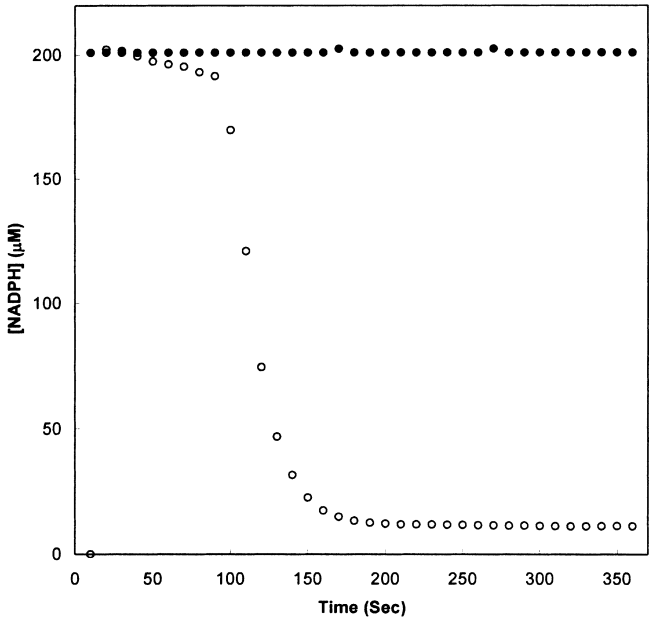

Figure 4. Nickel affinity support bioreactor. Conversion of acetyl-CoA to mevalonate in the absence (●) and presence (○) of HMG-CoA.

Nickel Column Bioreactors

Single Enzyme Bioreactor

The initial Ni^{++} affinity support bioreactor contained 200 nmol of a single enzyme, unfused *E. faecalis* HMG-CoA reductase. The input stream contained 0.5 mM (*R,S*)-HMG-CoA, 0.25 mM NADPH, 20 mM KCl, and 50 mM Tris, pH 7.5. Conversion of NADPH to $NADP^+$ was 95% (Fig. 4).

Three Enzyme Bioreactor

A second Ni^{++} affinity support bioreactor contained the first three enzymes of the mevalonate pathway, 22.5 nmol *E. faecalis* HMG-CoA synthase, 65 nmol acetoacetyl-CoA thiolase/HMG-CoA reductase fusion protein, and 90 nmol unfused HMG-CoA reductase. The input stream contained 300 μM acetyl-CoA, 125 μM NADPH, 20 mM KCl, 4 mM $MgCl_2$, and 50 mM Tris, pH 8.5. As for the hollow fiber reactor, maximal conversion of acetyl-CoA to mevalonate occurred at a flow rate of 0.25 ml/min. Maximum conversion of NADPH to $NADP^+$ was 75%, which corresponds to 47% conversion of acetyl-CoA.

Bioreactor Stability

While enzymes from the mesophile *E. faecalis* were not expected to exhibit unusual stability, the NiNTA column retained over 50% of its initial activity after storage in reaction buffer minus acetyl-CoA for 2 weeks at 4°C. However, after subsequent storage at room temperature for an additional 5 days, activity decreased to 4% of its initial value.

DISCUSSION

Of the two reactor types tested, better results were obtained using the Ni^{++} affinity support. This bioreactor is, to our best knowledge, the first to exploit the properties of enzymes with histidine tags for simultaneous immobilization of several enzymes with retention of their catalytic activity. Conversion of NADPH, the limiting reagent, by the three enzyme Ni^{++} affinity support bioreactor was 75% complete, and this reactor retained 50% of its initial activity after storage at 4°C for 2 weeks. Under comparable conditions the hollow fiber bioreactor achieved only 20% conversion, the fibers tended to clog, and osmotic pressure drove fluid from the input stream into the membranes, forcing enzyme back into its reservoir.

A bioreactor that employs recombinant bacterial enzymes of isopentenyl diphosphate biosynthesis presents several attractive options. Foremost among these is the ability to

screen lead compounds for development of new inhibitor-based antibiotics against several enzymes simultaneously in a configuration that models an in vivo situation. Just such antibiotics are urgently needed, as shown by the recent emergence of vancomycin-resistant strains of *E. faecalis* and *Staphylococcus aureus* (Walsh and Howe, 2002; Paulsen et al., 2003), organisms responsible for nosocomial infections, bacteremia, bacterial endocarditis, and urinary tract infections. Since the mevalonate pathway is essential both for Gram-positive bacteria and for humans, a useful inhibitor must selectively inhibit enzymes of the bacterial pathogen and not those of its human or animal host. For at least one enzyme of the mevalonate pathway, this appears feasible. The Class II HMG-CoA reductases of the Gram-positive cocci differ significantly from the human enzyme both structurally (Tabernero et al., 2003) and with respect to regulatory and inhibitory properties (Bischoff and Rodwell, 1996; Hedl et al., 2004). Analogous differences may also be discovered to characterize other enzymes of the pathway. These differences might be exploited for rational drug design; for example, to develop inhibitors of Class II, but not Class I forms of HMG-CoA reductase.

For drug discovery applications it is essential that the bioreactor contain enzymes derived exclusively from the target organism. For this reason, the bioreactors used here contained only enzymes from *E. faecalis*. Recombinant enzymes from extremophiles typically exhibit high activity and stability at elevated temperatures or extremes of pH or salinity, and thus offer potential advantages for biosynthetic applications under conditions unsuitable for enzymes from eubacteria or eukarya. The mevalonate pathway provides illustrative examples. The Class I HMG-CoA reductase of the halophilic archaeon *Haloferax volcanii* is optimally active and optimally stable in 3 M KCl (Bischoff and Rodwell, 1996), and the Class II HMG-CoA reductase of the thermophilic archaeon *Archaeoglobus fulgidus* is maximally active at 85°C (Kim et al., 2000).

Multiple enzyme bioreactors also offer potential beyond immediate applications to drug design or chiral biosynthesis. A continuous flow Ni^{++} affinity support bioreactor containing all 10 enzymes of glycolysis and linked to an HPLC-mass spectrometer would permit study of metabolic regulators on metabolite flux and pool sizes of intermediates. Such a bioreactor would provide a powerful experimental and pedagogical tool to model metabolite flux and metabolic regulation in intact cells.

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