biologics like proteins face di erent opportunities and challenges and are not the focus of this review

Problem Metabolites

Reactive metabolites in a cell-free system may inactivate enzymes. For example, high concentrations of input-reducing sugars that may glycylate proteins or aldehydes that are generated can react with nucleophiles on proteins. ese problems may be mitigated by engineering of enzymes to remove vulnerable sites, or using process or system optimization to keep sugar and aldehyde concentrations low. Cells o en compartmentalize toxic intermediates and it is possible that a similar bioinspired strategy could be used **in vitro**, perhaps by channeling toxic substrates to the next enzyme , thereby keeping its concentration low. Reactive oxygen species that are generated must be removed expeditiously; for example, by including catalases to eliminate peroxide. Like cells, high titters of product compounds may destabilize and inactivate enzymes. ese problems can o en be reduced by improving enzyme stability (see above) or through continuous product removal strategies such as an organic overlay. e advantage of cell-free systems is that problematic enzymes can generally be readily identi ed and the full panoply of engineering tools can be used to x them.

Enzymes can make errors and some metabolites are prone to spontaneous chemical alteration. us, unwanted, dead-end side products may build up, leading to a decrease in yield. Perhaps more thorny, the side products may inactivate enzymes in the system. In cells, there can be metabolite repair mechanisms to deal with these unwanted metabolites. In a cell-free system it may be possible to adjust conditions to minimize side reactions or it is necessary to introduce repair systems, thereby adding to complexity. For example, Opgenorth and colleagues introduced a two-enzyme metabolite salvage pathway in a cell-free system to deal with the undesired products of a promiscuous enzyme.

Cofactors

Another important issue in cell-free systems is cofactor costs [ATP, NAD(P)H, CoA, etc.]. For low-cost production, it is essential that cofactors are regenerated **in situ** and used many times. A crude back of the envelope calculation illustrates the challenge in the example of converting isoprenol into limonene. e pathway requires 4 ATP per limonene made. At a bulk ATP price of $1000/kg, the ATP cost for