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REVIEW

Progress in biocatalysis with immobilized viable whole cells: systems development, reaction engineering and applications

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Abstract Viable microbial cells are important biocatalysts in the production of fine chemicals and biofuels, in environmental applications and also in emerging applications such as biosensors or medicine. Their increasing significance is driven mainly by the intensive development of high performance recombinant strains supplying multienzyme cascade reaction pathways, and by advances in preservation of the native state and stability of whole-cell biocatalysts throughout their application. In many cases, the stability and performance of whole-cell biocatalysts can be highly improved by controlled immobilization techniques. This review summarizes the current progress in the development of immobilized whole-cell biocatalysts, the immobilization methods as well as in the bioreaction engineering aspects and economical aspects of their biocatalytic applications.

Keywords Biocatalysis · Immobilization methods · Immobilized whole-cell biocatalyst · Multienzyme cascade reactions · Process economics · Reaction engineering

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Introduction

Whole-cell biocatalysis has been an important route to the industrial production of some bulk and fine chemicals, and pharmaceuticals (Ladkau et al. 2014; De Carvalho 2016). Heterologous enzymes and synthetic pathways have been increasingly employed in whole-cell biocatalysis due to the rapid development of DNA-recombinant techniques (Carballeira et al. 2009; Milner and Maguire 2012). In spite of industrial applications of whole-cell biocatalysts, the potential of fundamental biocatalytic research has not been effectively exploited yet. One limitation is that the process development often starts only after a biocatalyst is designed. Lima-Ramos et al. (2014) made a thorough analysis of process engineering aspects and solutions that should be considered in early stages of the biocatalytic process

development to achieve industrial implementation. It means that bioreactor and downstream processing design and solutions problems optimization should be envisaged throughout the investigation of biocatalyst properties and performance.

An important technique to consider in the context of applications of whole cells is immobilization (Table 1). Immobilization of whole-cell biocatalysts can enhance their performance mainly by extending their lifetime (Kisukuri and Andrade 2015). Further two important benefits of immobilized whole-cell biocatalysts utilization are the increase of specific biocatalyst loading, and simplification of biocatalyst recycling and downstream processing (Liese and Hilterhaus 2013). Industrial use of immobilized whole-cell biocatalysts requires the knowledge of various physical and chemical properties of specific biocatalytic systems. Guidelines for their characterization by standardized procedures have been proposed (Buchholz et al. 2012a) and standardization was also applied for the comparison of the performance of different immobilized preparations (Prüsse et al. 2008; de Vos et al. 2009; Schenkmayerová et al. 2014).

The objective of this review is to map current trends in the development of immobilized whole-cell biocatalysts and to analyze the key issues of their successful industrial implementation. An overview of novel trends in the immobilization methods and applications of whole-cell biocatalysts is provided. Furthermore, the review presents the state-of-the-art in the bioreactor engineering employing these biocatalysts and analyzes economical aspects of the whole-cell biocatalytic production processes. The second part of the series deals with the characterization techniques mainly employing modern methods for the determination of physiology of immobilized cells and morphology of cell-containing particles.

Whole-cell biocatalysis with viable free and immobilized cells

Methods to provide technically useful whole-cell catalysts include "classical" methods such as screening a wide range of strains for desired activities (Carballeira Rodriguez et al. 2004) or genetic engineering. Genetic engineering can provide whole-cell catalysts with a missing activity, or multiple activities creating a pathway. Strategies and tools to develop complex industrial host organisms through systems biotechnology (Kuhn et al. 2010) and directed evolution (Cobb et al. 2013) have been reviewed recently.

Advances in molecular biology enable rational engineering of whole-cell biocatalysts for multiple fields of application including industrial biotechnology, biosensors, biofuels and remediation. Available reviews summarize the advances in the application of genetic engineering in new drug development (Stryjewska et al. 2013), production of fine chemicals (Liese and Villela Filho 1999), stereoselective redox reactions (Carballeira et al. 2009), and engineering of whole-cell biocatalysts for neurotoxic organophosphates detoxification (Kim et al. 2014a). Perspectives of biodiesel production by biotechnology have also been reviewed (Uthoff et al. 2009).

Many processes utilizing genetically engineered catalysts have the potential to be further engineered or improved by immobilization techniques. The most notable recent applications of immobilized genetically engineered whole-cell biocatalysts in various fields are summarized in Table 2.

Production of fine chemicals is currently the fastest growing area of genetically-modified whole-cell biocatalysts applications. Natural compounds and their derivatives are the main class of chemicals produced by biotransformations including processes with whole-cell biocatalysts (Straathof 2006). *Escherichia coli* is the most frequently used host organism for expressing an enzymatic activity not dependent on a coenzyme. *E. coli, Saccharomyces cerevisiae* and *Corynebacterium glutamicum* as synthetic platforms have been reviewed by Becker and Wittmann (2015). Environmental applications focus on the decontamination of organophosphates. In addition to immobilization in various materials as it is being reviewed here also direct adsorption to surface is an accepted approach in environmental application and its use in bioremediation was reviewed recently (Bayat et al. 2015). Another aspect of use of recombinant microorganisms in environmental applications is the legal approval by EEA, EPA and national agencies. Currently, the legislative processes in environmental applications are to certain extent in the shadow of public discussion on the use of genetically-modified organisms (GMO) in agriculture and food production. The potential of genetically-modified biocatatalysts for biofuel production, including bioethanol, biobutanol and biodiesel, is clearly recognized as well (Sakuragi et al. 2011). Biodiesel production requires a lipase as a single

Table 1 Overview of industrial processes employing immobilized whole cell biocatalysts published in Liese et al. (2006)

Production strain	Immobilization method	Product	Capacity (tonnes/year)	Company
Bacillus coagulans				Novo-Nordisk (Denmark)
Actinplanes missouriensis	Not specified	High fructose corn syrup	$>7 \times 10^6$	DSM (The Netherlands)
Streptomyces rubiginosus				Nagase & Co., Ltd. (Japan)
Protaminobacter rubrum	Not specified	Palatinose (isomaltulose)	>4000	Südzucker AG (Germany) Mitsui Seito Co., Ltd. (Japan)
Rhodococcus rhodochrous J1	Not specified	Nicotinamide (vitamin B3)	6000	Lonza AG (Switzerland)
Escherichia coli, Kluyveromyces citrophila, Bacillus megaterium	Not specified	Beta-lactam antibiotics: cefaclor, cephalexin, cefadroxil, ampicillin, amoxicillin	2000	DSM (The Netherlands)
Pseudomonas pseudoalcaligenes	Not specified	D-Maleic acid	Not specified	DSM (The Netherlands)
Recombinant *Escherichia coli*	Not specified	4-Cyanopentanonic acid	Not specified	Du Pont de Nemours & Co. (USA)
Pseudomonas chloraphis B23	Entrapment in calcium alginate	5-Cyano-valeramide	Several	Du Pont de Nemours & Co. (USA)
Alcaligenes sp., *Pseudomonas* sp.	Entrapment in calcium alginate	R-3-Chloropropane-1,2-diol	Not specified	Daiso Co. Ltd. (Japan)
Fusarium oxysporum or *Brevibacterium protophormia*	Entrapment in calcium alginate	Pantoic acid; enantiopure D-pantolactones and L-lactones, respectively	Not specified	Fuji Chemical Industries Co., Ltd. (Japan)
Bacillus brevis	Not specified	Amino acid derivatives: D-*N*-carbamoyl D-hydroxyphenyl glycine; 5-(4-hydroxy-phenyl)-imidazolidine-2,4-dione	300–700	Kanegafuchi Chemical Industries Co., Ltd. (Japan)
Rhodococcus rhodochrous J1	Entrapment in polyacrylamide	Acrylamide and related products	>30,000	Nitto Chemical Industry Co., Ltd. (Japan)
Pseudomonas dacunhae and *Escherichia coli*	Not specified	L-Alanine and D-Aspartic acid	61 114	Tanabe Seiyaku Co., Ltd. (Japan)
Brevibacterium flavum	Entrapment in κ-carrageenan	*S*-Maleic acid	468	Tanabe Seiyaku Co., Ltd. (Japan)
Escherichia coli B ATCC 11303	Entrapment in polyacrylamide or κ-carrageenan	L-Aspartic acid	700	Tanabe Seiyaku Co., Ltd. (Japan)

enzymatic activity catalyzing transesterification of oils from various sources. Overexpression of lipase has the potential to further improve the effectiveness and economic feasibility of biodiesel production. Additionally, one-step whole-cell biotransformations of non-natural substrates for the preparation of chiral intermediates for the synthesis of chemical specialties have been introduced in the last decades (Bornscheuer et al. 2012). This includes intermediates for the synthesis of drug candidates such as antivirally acting C-nucleosides using recombinant whole-cell biocatalysts (Bianchi et al. 2013).

Table 2 Recent examples of the application of immobilized genetically-modified whole-cell biocatalysts

Area of application	Genetic modification and host organism	Immobilization material	References
Fine chemicals	4-Oxalocrotonate tautomerase	Alginate	Djokic et al. (2015)
	L-Arabinitol dehydrogenase	Alginate	Gao et al. (2015)
	L-Amino acid deaminase in *E. coli*	Alginate	Song et al. (2015)
	L-Arabinitol dehydrogenase (coupled with NAD + regeneration)	Alginate	Gao et al. (2015)
	L-Amino acid deaminase from *Proteus mirabilis*	Alginate	Hossain et al. (2014)
	Monoamine oxidase in *E. coli*	Polyvinylalcohol	Zajkoska et al. (2015)
	Cyclopentatnon monooxygenase	Polyvinylalcohol	Rebroš et al. (2014)
	L-Amino acid deaminase	Alginate	Hossain et al. (2014)
	Arabinose isomerase in *E. coli*	Alginate	Kim et al. (2014b)
	Ammonia lyase and ammonia transferase	Polyvinylalcohol	Casablancas et al. (2013)
	Cyclopentanone monooxygenase in *E. coli*	PEC capsules	Schenkmayerová et al. (2012)
	Omega transaminase in *E. coli*	Polyvinyalcohol	Cardenas-Fernandez et al. (2012)
	Nitrilase in *E. coli*	PVA/SA copolymer	Liu et al. (2012)
	PNPase expressed in *E. coli*	Agar	Luo et al. (2011)
	Cyclopentanone monooxygenase in *E. coli*	PEC capsules	Bučko et al. (2011)
	L-Arabinose isomerase in *E. coli*	Alginate	Zhang et al. (2010)
	Cyclopentanone monooxygenase in *E. coli*	PEC capsules	Hucík et al. (2010)
	Alcohol dehydrogenase in *E. coli*	Alginate	Ng and Jaenicke (2009)
	Toluene ortho-monooxygenase in *E. coli*	Alginate	Garikipati et al. (2009)
	Nitrile hydratase in *E. coli*	Alginate	Mersinger et al. (2005)
Environmental	Organophosphorus hydrolase	Surface display	Tang et al. (2014)
	Nitrilase	Surface display	Detzel et al. (2013)
	Organophosphorus hydrolase	Surface display, glass beads	Mansee et al. (2000)
	Organophosphate hydrolase	Propylene fabric	Mulchandani et al. (1999)
Biofuels	Lipase expressed in *P. pastoris*,	Not immobilized	Yan et al. (2014a, b)
	Genetically modified *R. oryzae*	Not immobilized	Chen et al. (2008)
	Lipase from *R. oryzae* expressed in yeast	Surface-displayed	Matsumoto et al. (2002)
	Substrate-induced lipase	Biomass support particles	Ban et al. (2001)
Other	Resistance to ethanol	Surface display	Perpina et al. (2015)
	Chitinolytic activity on yeast	Surface display	Li et al. (2014)
	Lipase expressed in *E. coli*	Suface display	Kranen et al. (2014)
	Lipase in *P. pastoris*	Surface display	Pan et al. (2012)

Cell surface-displayed enzymes

Display of proteins on the surface of whole-cell biocatalysts has become an increasingly popular strategy to prepare genetically-modified biocatalysts. The displayed protein is fused to the anchoring motif through *N*-terminal, *C*-terminal or sandwich fusion as schematically presented in Fig. 1.

Using a spacer can further customize the surface display for its intended use for biocatalysis, biosensors, vaccines or other. Surface display is a well-developed and understood technology. This has been

extensively reviewed with focus on the display of proteins in microorganisms (Lee et al. 2003), bacteria (Samuelson et al. 2002), Gram-positive bacteria (Hansson et al. 2001) and yeasts (Schreuder et al. 1996). The use of synthetic scaffolds for pathway enhancement was also recently reviewed (Siu et al. 2015). Currently, the most frequent use of the surface display provides whole-cell catalysts with missing enzymatic activities which is not co-factor dependent, e.g. lipase, hydrolase, oxidase etc. (listed in Table 2). Modification of the cell surface with non-catalytic molecules aimed at the increase of solvent resistance has also been reported (Perpina et al. 2015).

Surface display has immense potential in the preparation of whole-cell biocatalysts. It can be considered an immobilization technique employing recombinant technology which is also used to effectively solve engineering problems such as reduction of diffusion limits and the need for chemical immobilization. Further development in this field towards multiple surface-displays can be expected; maybe even a combination of intracellular and surface-displayed activities creating a multi-step compartmentalized designer biocatalyst.

Creating multi-step and cascade pathways

Designing multi-enzyme cascade reactions in vivo presents a challenge beyond just expressing the enzymatic activities using molecular biology tools. Multi-enzyme systems need to be efficiently assembled within intracellular environments to interact well with the intracellular background, e.g. the supply of cofactors. In addition to molecular biology, also other tools need to be implemented to design custom whole-cell biocatalysts such as systems biology tools (Kuhn et al. 2010), combinatorial biocatalysis (Rich et al. 2002) as well as protein engineering and directed evolution methods (Turner and O'Reilly 2013). Recent reviews summarize advances in the use of multi-enzyme cascade reactors (Ricca et al. 2011), multi-enzyme reactions carried out in a single reactor (so called "in-pot" process) (Santacoloma et al. 2011) and the use of multi-enzyme systems to produce fine chemicals (Kohler and Turner 2015; Liese and Villela Filho 1999). Primary field of practical applications of cascade systems is the synthesis of complex products requiring multiple enzyme activities such as chiral compounds (Ricca et al. 2011), complex saccharides (Härle and Panke 2014) and amino acids (Hibi et al. 2015).

The potential of multi-step synthesis using whole-cell organisms is clearly understood. Application of retrosynthesis allows syntheses by transforming a target molecule into simpler precursors using the concept of "one-pot" cascade reactions (Oberleitner et al. 2013). Cascade reactions allow shifting the reaction equilibrium in favor of enantiomerically pure products from prochiral or racemic substrates, eliminate product inhibition problems and avoid degradation and dilution of substrates (Oroz-Guinea and Garcia-Junceda 2013). Cascade reactions have become a useful tool for the synthesis of extremely important C–C bond formation (Ricca et al. 2011) and C–O functional group chemistry (Schrewe et al. 2013). However, only a few practical examples of using multi-enzyme whole-cell biocatalysts have been reported. Single cell biocatalysts designed de-novo with a two-enzyme pathway, containing transketolase and transaminase, expressed in *E. coli*, was reported to produce amino-alcohols (Ingram et al. 2007). A whole-cell biocatalyst containing an even more complex "mini pathway" designed de-novo was reported to produce functionalized chiral compounds in high yields (Oberleitner et al. 2013). This

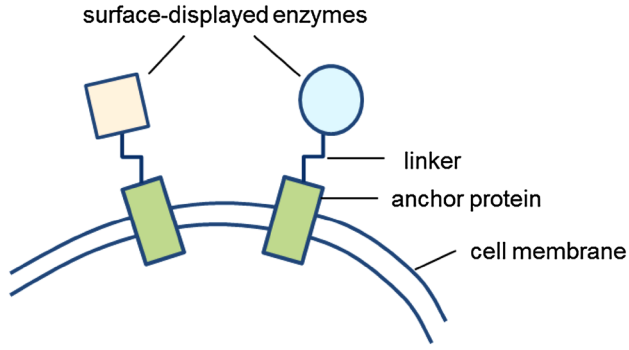

Fig. 1 Principle of surface-displayed enzymes in cells

"mini pathway" consisting of alcohol dehydrogenase, enoate reductase, and Baeyer–Villiger monooxygenase was co-expressed in *E. coli* (Oberleitner et al. 2013, 2014). Cascade catalysis, strategies and challenges en route to preparative synthetic biology have been reviewed by the same team (Muschiol et al. 2015). An even longer pathway, consisting of four enzymes, was used for the enantioselective synthesis of various D-amino acids (Nakajima et al. 1988) though only in an ex vivo system. Engineered metabolic pathways often suffer from flux imbalance since they do not have any regulatory mechanisms. An approach to solve this using synthetic protein scaffolds that spatially recruit metabolic enzymes in a designable manner was reported (Dueber et al. 2009). Overall, the recent advances in surface displaying, designing metabolic pathways, including scaffolding and compartmentalization, seems to be significantly enhancing the potential for application of recombinant microorganisms.

Traditionally, a multi-enzyme system would be contained within a cell or a capsule serving as an "artificial cell". A few non-conventional and innovative approaches to assemble multi-step systems have been published recently. For example, a multi-enzyme system consisting of 3α-hydroxysteroid dehydrogenase, diaphorase and NADH was confined in a novel hollow nanofiber-based artificial cell that performs multi-step reactions involving efficient coenzyme regeneration (Ji et al. 2014).

Immobilization methods

Utilization of recombinant cells with non-natural cascades of enzymes as biocatalysts for complex reaction sequences is currently one of the main trends in biocatalysis. Since these reactions can only be performed by viable cells, their immobilization should keep the cells in a viable state allowing stabilization of their catalytic efficiency and enable their repeated use. Several methods for whole cells immobilization are available and thoroughly reviewed (Carballeira et al. 2009). Additionally, transformations mediated by novel Baeyer–Villiger monooxygenases (BVMOs) including cascade reactions as well as whole-cell BVMOs immobilization strategies have been reviewed (Bučko et al. 2016). A newly-discovered cyclohexanone monooxygenase from the family of BVMOs was tested also in the form of viable whole cells as a promising industrial biocatalyst due to broad spectrum of substrates as well as high regio-, enantio- and chemo-selectivity (Romero et al. 2016). Regardless of the utilized immobilization technique, higher enzyme stability of immobilized cells compared to free cells is considered as the original motivation for the immobilization of living microorganisms explained by more efficient (re)synthesis of enzymes and cofactors, and their regeneration (Buchholz et al. 2012b).

Cell immobilization, in particular by their inclusion in the structures of semi-permeable polymer matrices, shows many advantages over the use of free cells. The main advantages include higher cell density, increased specific productivity, easier separation of products and biocatalysts, the possibility of continuous bioreactors arrangement without cell wash-out (Obradovic et al. 2004) as well as the biocatalyst reuse and reduction of cost (Rao et al. 2006). The main additional benefits of immobilization, notably encapsulation in capsules with semi-permeable membrane are the highly defined encapsulation process and biocompatible microenvironment for viable cells (Hucík et al. 2010).

The most used technique of whole-cell biocatalysts immobilization is based on the formation of stable porous gels based on ionotropic gelation of water-soluble polyelectrolytes, usually polysaccharides containing charged functional groups (alginate, pectate, carrageenan, chitosan) with oppositely charged ions (usually Ca^{2+}, K^{+}, polyphosphates) (Buchholz et al. 2012b). Among the hydrogels, calcium alginate, κ-carrageenan and polyacrylamide were used in industrial bioprocesses (Table 1). Thermoreversible gelation of poly(vinylalcohol) in form of LentiKats (Jekel et al. 1998) also found applications in industry due to improved properties such as higher catalytic activity, mechanical stability, lower mass transfer resistance and lower cost (Rebroš et al. 2009; Trögl et al. 2012). Other types of biomaterials used for cell immobilization include natural polymers such as collagen, hyaluronic acid and agarose as well as synthetic polymers such as poly(glycolide), poly(lactide), polyanhydrides, poly(ethylene oxide) and poly(-ethylene glycol) (Riddle and Mooney 2004). Efforts to obtain alternative organic–inorganic composite materials with alginate as organic and silica hydrogels as inorganic components for viable cells immobilization were also described (Spedalieri et al. 2015).

Biocatalytic efficiency of viable recombinant cells *E. coli* with overexpressed Baeyer–Villiger monooxygenase (BVMO) was improved by their encapsulation

in biocompatible polyelectrolyte complex (PEC) capsules of controlled size, membrane thickness, permeability and mechanical strength (Bučko et al. 2012). For this purpose, a unique multiloop reactor (Anilkumar et al. 2001) was used for continuous encapsulation based on polyelectrolyte complexation of sodium alginate and cellulose sulfate as polyanions, poly (methylene-co-guanidine) as polycation, $CaCl_2$ as gelling and NaCl as antigelling agents (Lacík 2006). Additionally, a rare comparative study of the key physical and bioengineering parameters of PEC capsules and LentiKats showed, that highly defined PEC capsules are suitable for laboratory investigation of encapsulated cells (Schenkmayerová et al. 2014). On the other side, the use of LentiKats for cell immobilization is also attractive in terms of potential industrial applications including viable whole-cell BVMOs biocatalysts (Rebroš et al. 2014).

The scale-up of hydrogel particles production from laboratory quantities to the operational scale is important for the application of immobilized whole-cell biocatalysts. Development of instrumentation techniques for biocatalysts immobilization by entrapment within hydrogel particles allowed acceleration of research in this field in the 1980s (Bučko et al. 2012; Prüsse et al. 2008). Current commercially available high-performance devices operating on different physical principles enable to produce hydrogel particles in amounts of up to tens of kg per hour (Büchi Labortechnik AG, geniaLab BioTechnologie—Produkte und Dienstleistungen GmbH, LentiKat AS, Nisco Engineering AG). Utilization of immobilized viable cells for larger scale biocatalytic purposes is a promising application due to the adaptability of the most frequent entrapment and encapsulation materials to the mentioned high performance devices and the significant progress in the characterization techniques.

Reaction engineering and process economics

A SciFinder database search showed that the total number of scientific papers on cell immobilization has increased exponentially during the last four decades; from about 2000 journal articles published in 1976–1985 to 15,000 articles in 2006–2015. It is, however, interesting that the total share of the papers dedicated to reactor issues decreased significantly from 6% to only 2.8%.

The boom of bioreaction engineering research of immobilized biocatalysts occurred from the mid of 1970s to the mid of 1990s. Most books and reviews dealing with immobilized cell reactors were written in this period and they are still the key reference sources of general information (Kasche 1979; Moo-Young 1988; Webb et al. 1986; Webb and Dervakos 1996; Willaert and Baron 1996; Willaert et al. 1996).

The last two reviews with a broad scope surveying the results of intensive activities were published in the last century (Freeman and Lilly 1998; Riley et al. 1999). The paper of (Riley et al. 1999) was essentially an epilogue to the vivid research on the quantitative characterization of diffusion properties of immobilized cell systems. Relatively few works dealt with the determination of effective diffusivities of substrates and products of biocatalytic processes in the following period (Fidaleo et al. 2006; Polakovič et al. 2001; Schenkmayerová et al. 2014; Xiao et al. 2008). Freeman and Lilly (1998) analyzed in their review the ways ensuring a stable continuous operation of aerobic fermentations and cultivations in immobilized cell reactors. However, these processes found only a few applications on industrial scale. On the other hand, some of these research achievements are pertinent to the systems with immobilized non-growing cells and should be considered in a larger extent in the current research activities in the area of biocatalytic oxidations using air oxygen. Recent literature contains several review papers dedicated to various applications of immobilized cell reactors mainly for the production of alcoholic beverages and wastewater treatment (Table 3).

Biocatalyst productivity, stability and process costs

Dicosimo et al. (2013) showed an evident disparity between the thriving immobilization science and the very modest industrial application of immobilized biocatalysts in the last 20 years. It is evident that a successful implementation of immobilization technology can be achieved only by mastering the material science for efficient immobilization, process engineering for high bioreactor productivity and yield and process economics for feasible production. Tufvesson et al. (2011) have published recommendations for early-stage economic assessment of biocatalyst production: the cost of immobilized cells can be 5–10

Table 3 Recent review articles on the applications of immobilized cell reactors

Application	References
Continuous beer fermentation	Brányik et al. (2005)
Continuous beer and beverages fermentation	Verbelen et al. (2006)
Batch and continuous wine production	Genisheva et al. (2014)
Biofilm wastewater treatment	Gullicks et al. (2011), Lazarova and Manem (2000), Yu et al. (2005)
Lactic acid production	Dagher et al. (2010)
Alcohols, organic acids, antibiotics, and enzymes	Demirci et al. (2007)
Biodiesel production	Guldhe et al. (2015), Parawira (2009)

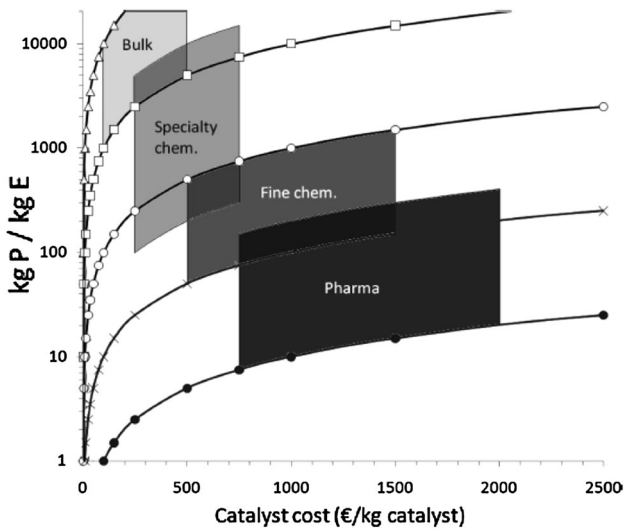

Fig. 2 Effect of biocatalyst cost and allowable cost contribution on the requirements for biocatalyst productivity in terms of kilogram of product per kilogram of biocatalyst used for production of bulk, fuel, or specialty chemicals employing immobilized enzymes. Allowable cost contribution of 0.01 €/kg V *triangle*, 0.1 €/kg *rectangle*, 1 €/kg *open circle*, 10 €/kg ×, 100 €/kg *filled circle* Reproduced with permission from Tufvesson et al. (2011). Copyright by the American Chemical Society

times higher than that of free cells which can reduce or eliminate the advantages brought by the bioreactor applications of immobilized cells.

The cost of industrial biocatalysts used on large scale is typically in the range of 50–500 US$ per kg protein contained and it often forms only a small fraction of product costs, e.g. 1 cent per liter of starch-derived bioethanol (Dicosimo et al. 2013). The mentioned cost is however achieved only when the biocatalyst yield from the fermentation is in units of g/l. The biocatalyst yield for specialty chemicals and pharmaceutical applications can be only in hundreds of mg/l and the biocatalyst production cost will thus increase in inverse proportion to the concentration. A feasible biocatalyst cost depends very much on the biocatalyst application. Figure 2 shows that for pharmaceutical applications it can reach up to €1000–2000 per kg of biocatalyst for the specific productivity of the process of only 10 kg product per kg of immobilized biocatalyst. On the contrary, biotechnological production of bulk chemicals requires the specific productivity as high as 10,000 kg/kg.

Additional production costs include the costs for immobilization materials, labor, and equipment. Moreover, the development of an immobilized preparation requires additional costs and additional time. Especially, the extra time needed for introducing a product to the market can disqualify the application of

immobilized biocatalysts in the production of high added-value products. The comparison of cost advantages of immobilized and free biocatalysts is thus often unequivocal. The final decision can be affected also by other factors such as better control and production stability of continuously operated immobilized cell reactors.

In each case, the immobilized biocatalyst must provide good activity and operational stability. The biocatalyst activity values can be found in almost every paper dealing with the investigation of immobilized whole cells. Hundreds of papers demonstrated a good stability of developed biocatalysts, which was typically achieved by carrying out several repeated batch runs. Much less frequent, but more valuable, are the works considering longer-term operation; e.g. Samin et al. (2014) designed a packed-bed bioreactor with a concurrent liquid and air flows for aerobic degradation of 1,2,3-trichloropropane from wastewater using immobilized genetically engineered *Pseudomonas putida* cells that was operated in a stable steady state for two months.

A rigorous approach to the characterization of biocatalyst stability is based on the investigation of inactivation kinetics. The kinetics of inactivation of immobilized whole-cell biocatalysts was evaluated for the production of ethyl esters of fatty acids by *Mucor circinelloides* (Andrade et al. 2012), reduction of ketones by *Monascus kaoliang* (Quezada et al. 2009), decolorization of an azo dye by *Pseudomonas luteola* (Chen and Lin 2007), and orthohydroxylation of bisphenol A by *Agaricus bisporus* containing a tyrosinase (Kampmann et al. 2015). These authors fitted the inactivation data at individual temperatures with first-order kinetics and subsequently the temperature dependence of the rate constant was fitted with the Arrhenius equation. Kaul et al. (2006) used an integral form of series-inactivation kinetics to describe the loss of activity of *Alcaligenes faecalis* nitrilase at a single temperature. They enhanced their investigation by a rigorous thermodynamic analysis of the temperature dependence of the kinetics of stereoselective hydrolysis of mandelonitrile.

The combined effect of long-term stability and activity of biocatalysts is conveniently characterized by the total turnover number (*ttn*), the amount of product obtained per amount of biocatalyst spent during its lifetime. The comparison of *ttn*-values of free and immobilized biocatalysts can provide principal information about the suitability of immobilization. Unfortunately, such data are scarce for whole cells although they are quite common for enzymes. We were not able to find any published *ttn*-values for immobilized whole cells. Pennec et al. (2014) evaluated *ttn* of free whole cells and crude extracts for 12 different cytochrome P450 monoxygenase mutants catalyzing the hydroxylation of cycloalkanes and linear alkanes. They found that *ttn* of the whole cells of most active mutants was about 3000 mol/mol for cycloalkanes and 5000 mol/mol for linear alkanes. Since the typical enzyme concentration was about 1 μmol/g of dry cell mass, the dry cell mass-based *ttn* were about 200–300 g/g.

It is often more convenient to express *ttn* of whole-cell biocatalysts as the ratio of mass of product formed per mass of biocatalyst preparation. Kratzer et al. (2011) investigated the conversion of *o*-chloroacetophenone into chiral 1-(*o*-chlorophenyl)-ethanols catalyzed by recombinant reductase whole-cell biocatalysts from *E. coli and S. cerevisiae* and determined the *ttn* value of only about 0.2 g/g. Such a low value demonstrates very low biocatalyst stability caused by the toxic effect of the substrate. Bertóková et al. (2015) compared the activity and stability of free and immobilized whole-cell *Gluconobacter oxydans* in the oxidation of 2-phenylethanol to phenylacetic acid in a bubble column reactor. About thrice larger amount of phenylacetic acid per dry cell mass was produced using the immobilized biocatalyst in repetitive batch cycles compared to the free one. Moreover, the immobilized biocatalyst had still about 50% of the initial activity after twelve cycles whereas the free biocatalyst was completely inactivated after seven cycles. The *ttn* value of the immobilized biocatalyst can thus be estimated to be about one order of magnitude higher.

Difference in *ttn* of free and immobilized biocatalyst is low if substrates or products are toxic. In such a case, immobilization is not economically feasible. Also, high values of *ttn* of free biocatalysts and/or high-added value products make immobilization only little beneficial because the biocatalyst cost forms a very small fraction of the overall product price. Immobilization is thus recommended when *ttn* of free biocatalysts is neither low nor high (Liese and Hilterhaus 2013). It is difficult to specify the limits of low and high values of *ttn* for whole-cell biocatalysts. Kragl and Dwars (2001) did this for enzyme

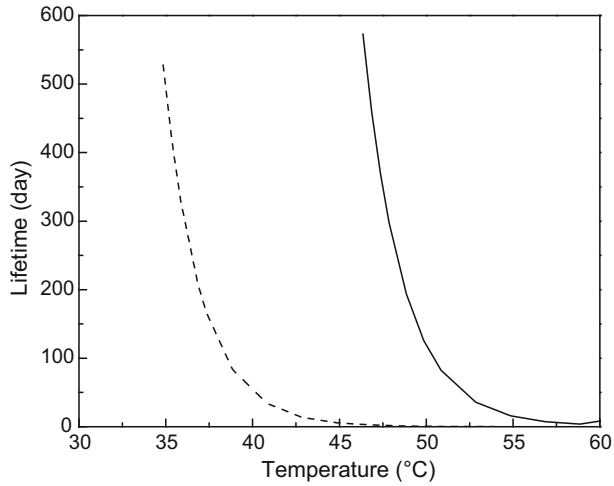

Fig. 3 Lifetime of *Aureobasidium pullulans* fructosyltransferase versus temperature in the reaction mixture containing about 55 mass% of saccharides (solid line) and a phosphate-citrate buffer solution (*dashed line*) Reproduced from Onderková et al. (2001)

catalysts and suggested that the minimal value should be around 1000 for small-scale, expensive products and 50,000 for large-scale, less expensive products.

Care is, however, needed in applying such rules-of-thumb. Rogers and Bommarius (2010) demonstrated that it is very important to determine the *ttn*-values at real application temperatures and other process conditions so that they correctly represent a combined measure of biocatalyst activity and stability. On the other hand, Gibbs et al. (2005) developed a method of accelerated determination of *ttn* of industrial immobilized biocatalysts to avoid long-term experiments at process conditions. Sensitivity of *ttn* with respect to process parameters should be investigated. For example, biocatalyst lifetime near the optimal temperature is very sensitive to the fluctuations in the reaction mixture composition and process temperature (Fig. 3). A difference of 1 °C can change the biocatalyst lifetime by 100 days (Onderková et al. 2010).

Bioreactor applications

The *ttn* depends on bioreactor configuration and process parameters therefore its representative value cannot be obtained before bioreactor design and optimization are completed. As it has been mentioned above, a small fraction of scientific papers on immobilized cells dealt with reactor operation using the investigated biocatalysts. Many reactor studies are straightforward tests for a single set of operation conditions in batch stirred or packed bed bioreactors. Some more complex experimental reactor studies for certain important applications are worth mentioning (Table 4). Besides a vast number of papers on

Table 4 Packed bed bioreactor studies exploiting immobilized whole-cell biocatalysts

Bioreactor type	Biocatalyst	References
Two-liquid phase packed bed	*R. oryzae* in polyurethane foam	Hama et al. (2007)
Two-liquid phase packed bed	*A. oryzae* in polyurethane foam	Yoshida et al. (2012)
Packed bed	*C. beijerinckii* on ceramic rings and pumice stones	Gungormusler et al. (2011)
Packed bed biofilm	*C. freundii* and *P. agglomerans* in in polyurethane foams	Casali et al. (2012)
Packed bed	*E. coli* on methacrylate beads	Andrade et al. (2014)
Packed bed and bubble column	*A. faecalis* in alginate–chitosan–alginate capsules	Zhang et al. (2014)
Packed bed with anion-exchange column in a recycle loop	*A. faecalis* in Ca alginate gel treated with polyethyleneimine and glutaraldehyde	Xue et al. (2013)

biodiesel production using immobilized lipase, a significant number of works dealing with the bioreactor applications of whole-cell biocatalysts for this process have recently been published. Some of them can be found in the recent review of Guldhe et al. (2015). The group of Akihiko Kondo is the most active one in this area; good illustrative examples are the works of Hama et al. (2007) and Yoshida et al. (2012). The former work compared the performance of packed bed and batch reactors and the effect of the reaction mixture emulsification. The latter one presented a cascade of six packed bed reactors with intermittent glycerol sedimentation separation after each reactor and injection of fresh methanol.

Another important application is the 1,3-propanediol production. Gungormusler et al. (2011) and Casali et al. (2012) investigated the effect of residence time in a packed bed biofilm reactor and compared it with the performance of a batch bioreactor. Andrade et al. (2014) dealt with the immobilization of *E. coli* cells containing overexpressed (*R*)-selective ω-transaminase and the cofactor PLP on methacrylate beads. These cells were used in a packed bed reactor and stable production was achieved up to ten days. Zhang et al. (2014) used bubble column and packed bed bioreactors for the biotransformation of iminodiacetonitrile to iminodiacetic acid with encapsulated *Alcaligens faecalis*. Xue et al. (2013) investigated a packed bed reactor with immobilized whole cells with an in situ product recovery loop for the production of (*R*)-mandelic acid.

The group of Andreas Schmid has presented many investigations in the application of biofilm-membrane immobilized whole cells biocatalytic processes. For example, Gross et al. (2013) studied the technical feasibility of biofilm-based biotransformations of *n*-octanol and (*S*)-styrene oxide by carrying out single membrane tube microreactor experiments. A process scale-up to industrial scale was proposed by multiplication of the number of microreactors that would result in 59 membrane fiber modules (of 0.9 m diam. and 2 m length) consisting of 84,000 polypropylene fibers. Halan et al. (2014) optimized a solid support membrane-aerated biofilm reactor and scaled it up to yield gram amounts of (*S*)-styrene oxide, a toxic and instable high value chemical synthon. A polytetrafluoroethylene membrane was found to be best suited for in situ substrate delivery and product extraction. Lang et al. (2015) dealt with the continuous production of (*S*)-3-hydroxyisobutyric acid. The problem of cyclohexane substrate toxicity was circumvented using an aqueous-air segmented flow biofilm membrane reactor with continuous cyclohexane feed (Karande et al. 2016).

Microbioreactors

Microbioreactors are a subject of intensive biochemical engineering research. The most significant applications are directed to the high throughput screening of microorganisms and enzymes with free biocatalysts; however, some works were also performed with immobilized biocatalysts. Wohlgemuth et al. (2015) reviewed the potential of microbioreactors including those with immobilized enzymes and whole cells. The dramatic increase in heat and mass transfer due to the higher surface-to-volume ratio of microreactors and the short diffusion paths are seen as the main advantages of microbioreactors. Most studies were made using immobilized enzymes but only a few examples for immobilized cell microbioreactors can be found. Fidaleo et al. (2006) developed a microbioreactor with a bilayer latex coating to protect non-growing *Gluconobacter oxydans* cells oxidizing D-sorbitol to L-sorbose. Stojkovič and Žnidaršič-Plazl (2012) investigated the production of malic acid using *S. cerevisiae* cells that were first immobilized on inner walls of microchannels and then permeabilized by a detergent. It was demonstrated to be a low-cost and easy-to-use tool for the development of biotransformation processes and fast optimization of reaction conditions. Stojkovič et al. (2014) demonstrated also the construction of microbioreactors with immobilized *E. coli*, *Pseudomonas putida*, and *Bacillus subtilis* cells.

The effect of the microbioreactor diameter with respect to the structure of biofilms formed of the phenol-degrading bacterium *Pseudomonas syringae* was investigated by Akay et al. (2005). They compared the performance of a monolithic microbioreactor with 25 μm pores and a monolayer of immobilized cells with other studies where a packed bed bioreactor was used, revealing the productivity achieved in the microbioreactor being about 20 times higher. Productivity of immobilized cell reactors is often reported in different comparisons; however, without a reference conversion it has little value. A high substrate conversion results in slower reaction kinetics and

therewith in lower productivity. It has also to be considered that the annual production capacity of their bioreactor was only 5 kg. If a large number of parallel microreactors is required to scale-up the production process, the effect of higher volumetric productivity is quickly lost. Capital costs are multiplied by parallelization of microbioreactors whereas the bioreactor scale-up by dimensions increase is governed by the six-tenths rule of the cost–capacity factor exponent. For example, if the mentioned production capacity of 5 kg is increased 1000-fold, the equipment cost increases only about 60-fold. According to Wohlgemuth (2011), the potential drawback of higher microbioreactor costs can be substantiated for aqueous/organic two-phase biocatalytic processes employing toxic reagents that must be efficiently stirred.

Modeling

As it has been mentioned in the initial paragraphs of this section, very few works have dealt with mass transfer and modeling aspects of bioreactors in the recent period. Quezada et al. (2009) carried out a good experimental analysis of mass transfer effects in the reduction of cyclohexanone by *Monascus kaoliang* immobilized in large particles of polyurethane foam (mean size of 1 cm). Despite the very large particle size, the intraparticle mass transfer limitation was negligible. The effectiveness factor was close to one in most cases because the intrinsic reaction rate per particle volume was only 1.7 mmol/l h when the initial cyclohexanone concentration was 10 mM. Such low rates can be expected for many biocatalytic conversions and biotransformations in which toxic substrates are utilized. In the mentioned work, the heterogeneous reaction rates of cyclohexanone reduction were even lower in some cases which was interpreted by NADH regeneration becoming the rate controlling step. The effect of particle size and immobilization procedure on the effectiveness factor of stereoselective nitrile hydrolysis was investigated by Kaul et al. (2006).

The work of Xiao et al. (2008) represents one of the rare cases where rigorous mathematical modeling and its comparison with experimental data was carried out for the reduction kinetics of phenylglyoxylic acid to *R*-(−)-mandelic acid by the immobilized yeast *S. cerevisiae*. The investigated aspects included the influence of cell concentration and particle size on the effective diffusivity of the substrate and effectiveness factor of the heterogeneous reaction. Fidaleo et al. (2006) analyzed the performance of a microbioreactor for D-sorbitol oxidation to L-sorbose using a rigorous mathematical model for which mass transfer and intrinsic kinetic parameters were determined from independent carefully designed experiments. It should be underscored that they also determined the Michaelis constant for oxygen, 0.74 mg/l. This kinetic parameter is generally difficult to determine because the biocatalyst affinity toward O_2 is high. It is thus difficult to achieve an accurate measurement of steady-state dissolved oxygen concentration without avoiding an oxygen-transfer rate controlled regime of the biocatalytic process. Therefore, zero-order kinetics is often assumed for oxygen consumption in immobilized cell particles. Using this assumption and approximate estimates of effective diffusivities, Buque et al. (2002) simulated oxygen profiles for 3-oxo ester reduction by baker's yeast immobilized in 1.2 mm diameter Ca-alginate particles and found that the process became O_2 limited at dry cell concentrations above 30 g/l.

Conclusions

This review analyzes several aspects concerning the preparation and applications of immobilized viable whole-cell biocatalysts. A summary of existing and promising applications of these immobilized biocatalysts demonstrates an enormous influence of protein engineering methods on the progress in this area. In this report, particular attention is given to the description of the preparation of cell surface-displayed enzymes and to the design of multi-enzyme cascades. This review refrains from presenting the myriads of immobilization methods found in scientific or patent literature as the preference was given to the presentation of some of the most useful methods based on the entrapment or encapsulation of cells in polymer matrices. Additionally, key reaction engineering and economic factors determining successful applications of immobilized whole-cell biocatalysts are discussed. These factors must be considered in the very early phase of research when the question is first raised if a whole-cell biocatalyst approach should be immobilized or not.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research involving human participants, animals, plants and microorganisms This article does not contain any studies with human participants or animals performed by any of the authors. Experiments involving plants or microorganisms taken outside the authors'country have been with the correct authorization.

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