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New Catalytic Applications

One of the recent developments in the CLEC-catalyzed reactions was a discovery that CLEC catalysts can be extremely active in neat organic solvents. The potential synthetic benefits of purified lipases in organic solvents, such as higher enantioselectivity, activity, and ease of downstream processing, have not been realized to date. Not only the cost of purified lipases is higher, but their stability and activity in organic solvents is lower than that of the crude counterparts. We have recently developed a novel drying procedure for CLECs of lipases and subtilisin (that, by definition, are pure enzymes) by washing them with solutions of different surfactants in organic solvents [3]. The high specific activity, purity, and stability of CLECs result in the high catalyst productivity in these media which compares favorably with the best asymmetric catalysts.

In addition to their stability against various denaturants [4], CLEC catalysts are mechanically stable for long periods of time under standard fine-chemical production conditions (agitation, pumping, and filtration) [5]. This is extremely important for industrial applications, since as a process is scaled up from the bench through pilot scale to production, there is an increase in mechanical shear and pressure.

So far, the major applications of crystalline enzyme catalysts have been in the production of high fructose corn syrup [6], the resolution of chiral compounds [7], synthesis of peptides [8] and peptidomimetics [9], and C–C bond forming reactions [10]. Synthetic applications of CLEC catalysts will remain the major focus of research activities. In the near future, we will see many more enzymes in CLEC form, including cofactor-dependent dehydrogenases. However, since CLEC technology is broadly applicable to a wide variety of proteins, crystalline materials will undoubtedly find use in other areas.

Materials for Chromatography

One of these areas is separation of molecules *via* chromatography or simulating moving bed (SMB) technology. Unlike crystals of small molecules, protein crystals are macroporous materials and can be thought of as bioorganic zeolites. The uniform solvent-filled channels traverse the body of a crystal and, thus, facilitate the transport of ligands in and out of the crystal. The diameter of the channels depends on the nature of the protein and its crystal form, and ranges from 20 to 100 Å. Unlike the majority of current stationary phases, such as silica, zeolites, and synthetic polymers, protein crystals are asymmetric molecules made of L-amino acids and can, in principle, provide stereoselective adsorption of chiral ligands. Protein crystals made of enzymes, receptors, or antibodies may provide yet another level of selectivity, namely specific affinity binding of small molecules to the binding sites of the stationary phases. Since proteins are weak ion-exchangers with isoelectric points from 2 to 12, one can easily manipulate binding of small molecules by changing pH and buffer content of the eluent. We will present data that demonstrate the unique properties of protein crystals in separation of mixtures of small molecules *via* several different mechanisms.

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Regeneration of Redox Enzymes for Continuous Preparative Processes

Wim A.C. Somers*, **Edwin C.A. Stigter**, **Wim van Hartingsveldt**, and **Jan Pieter van der Lugt**

Introduction

Oxidoreductases are valuable biocatalysts for selective conversions in industrial processes. They are used in several areas such as the pharmaceutical industry, agricultural industry, and the food industry, *e.g.* for the enantioselective synthesis of intermediates or the production of oxidized carbohydrates. Reactions catalyzed by oxidoreductases are characterized by the need of enzyme regeneration, more specifically by the need for cofactor and/or coenzyme regeneration [1][2]. For industrial purposes, this means that application of these enzymes on technological scale is determined by the availability of a cost-efficient process for regeneration of the enzyme.

Enzyme Electrodes for Enzyme Regeneration

Enzyme electrodes are an appropriate tool for the regeneration of redox enzymes. In these devices, oxidoreductases are immobilized together with a conducting in-

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termediate on an electrode surface (see *Fig. 1*).

The mediating polymers are chosen on basis of their redox potential and on their properties with regard to the interaction with the enzymes under study. For preparative purposes, it is necessary that high conversion efficiencies (expressed as current density per unit of electrode surface area) are obtained. In addition, the stability of the electrode has to be high. At TNO, enzyme electrodes were developed for several conversions (see *Table 1*). PQQ-dependent enzymes, having pyrrolo-quinoline quinone as the cofactor, normally do not use oxygen as the electron acceptor and, therefore, hydrogen peroxide is not an intermediate in the reaction cycle [3]. This property makes this type of enzymes attractive to immobilize at electrode surfaces because various compounds may serve as electron acceptor, allowing regeneration of the enzymes [4].

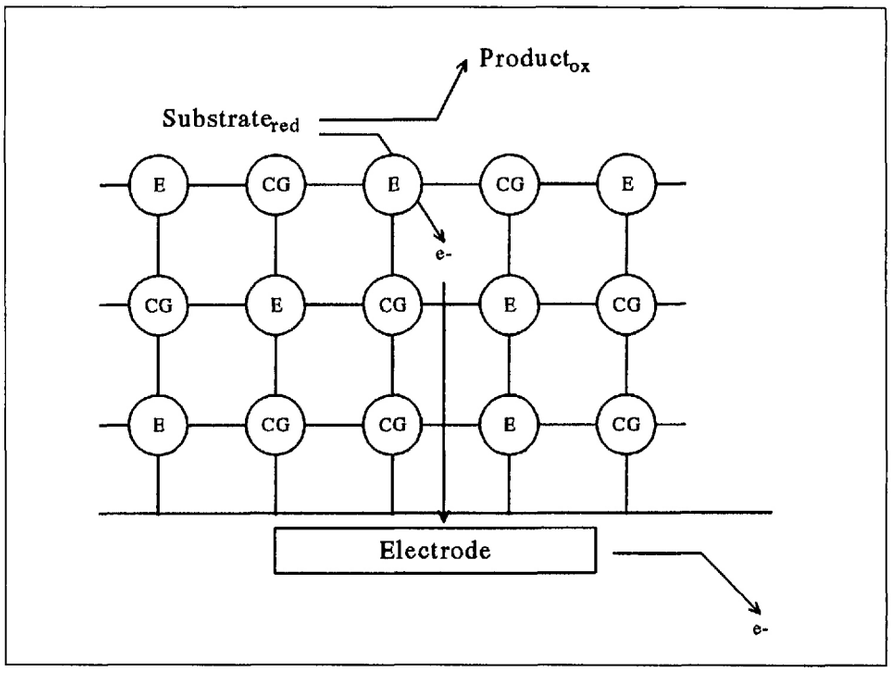

Figure. *Schematic representation of an enzyme electrode.* E = Enyzme, CG = Conducting Group.

Results

PQQ-Dependent Alcohol Dehydrogenase from Comomonas testosteroni*: Enantioselective Oxidation of Secondary Alcohols*

PQQ-dependent alcohol dehydrogenase from *Comomonas testosteroni* (PQQ-EDH) was immobilized in the presence of an osmium-bipyridyl-containing polyvinyl pyridine and a bifunctional crosslinking reagent (di-epoxide) [5]. This enzyme catalyzes the conversion of primary alcohols into their corresponding aldehydes and carboxylic acids and oxidizes secondary alcohols enantioselectively into the corresponding ketones (see *Table 2*). Current densities up to 0.4 A/m^2 were measured for the conversion of a large number of alcohols at PQQ-EDH electrodes. The kinetic parameters of these electrodes are comparable with those of the free enzyme, indicating that mass transfer resistances do not seem to govern the reaction velocity in these electrode systems. The enzyme and the enzyme electrode can be effectively used for the enantioselective resolution of, *e.g.* heptan-2-ol and octan-2-ol (see *Table 2*). Half-lives of 7 d for electrodes containing PQQ-EDH were observed [6].

PQQ-Glucose Dehydrogenase and Its Application in the Production of Aldonic Acids

PQQ-GDH from *Acinetobacter calcoaceticus* is active with a broad range of mono- and oligosaccharides including glucose, lactose, and maltose [3]. Very active enzyme electrodes were prepared with

Table 1. *Oxidoreductases and Their Application in Bio-Electrocatalysis*

Enzyme	Substrate	Product
Oxidations		
Glucose oxidase	Glucose	Gluconate; Gluconic acid
Lactose dehydrogenase	Lactose	Lactobionate; Lactobionic acid
Alcohol oxidase; Alcohol dehydrogenase	Alcohol(s)	Aldehyde(s), Ketone(s)
Reductions		
Nitrate-reducing enzymes	Nitrate	Nitrogen
Alcohol dehydrogenase	Aldehyde(s), Ketone(s)	Alcohol(s)

Table 2. *Oxidation of Alcohols with PQQ-EDH Enzyme Electrodes:* K_m*(app) and* V_{max}*(app) PQQ-EDH* (V_{max} (100%) = 0.4 A/m^2)

Substrate	K_m [mM]	V_{max} [%]
Ethanol	0.8	100
Propan-1-ol	0.06	100
Butan-1-ol	0.006	100
Pentan-1-ol	0.006	100
Propan-2-ol	n.m.	n.m.
(*RS*)-Pentan-2-ol	0.4	43
(*S*)-Hexan-2-ol	0.09	79
(*R*)-Hexan-2-ol	0.6	15
(*S*)-Heptan-2-ol	0.06	75
(*R*)-Heptan-2-ol	0.7	11
(*S*)-Octan-2-ol	0.05	88
(*R*)-Octan-2-ol	0.4	24

n.m. = not measurable

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this enzyme, characterized by current densities of 30 A/m^2 at a cell potential of 600 mV *vs.* Ag/AgCl electrode. This means that an active layer of *ca.* 9000 U of enzyme activity/m^2 is obtained. As a result, glucose and lactose are converted very efficiently at the electrode.

Conclusions

An effective method for the preparation of enzyme electrodes with high current densities is presented. Based on these current densities and on the characteristics of a carbon electrode with a high specific surface area (35000 m^2/m^3), volumetric productivities of 100–940 g product/l·h are calculated for the production of acetaldehyde and gluconate, respectively. On basis of the results, scaling-up of these electrodes is now under study.

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Choice of a Method for *in situ* Recovery of 3-Pyridylacetic Acid Formed by Biotransformation with *Pseudomonas oleovorans*

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Introduction

Biotechnological formation of industrially interesting compounds using microbial systems is often limited due to substrate and/or product inhibition [1–4]. The former may be overcome by suitable substrate-feeding regimes, however, the latter is technically more difficult to avoid or overcome. Ideally, it would be necessary to continuously remove the product at a rate similar to the formation rate using an efficient, non-toxic, regenerable, and cheap process which has a high specificity for the targeted substance, the latter usually present in a complex culture solution. This is the concept of *in situ* Product Recovery (ISPR) [2][4–6], which offers a number of important features: *1*) increased productivity due to low accumulation of the inhibiting compound, *2*) product losses by subsequent reactions or unwanted separations are reduced, and *3*) reduction of downstream processing steps is possible [5]. The choice of the most appropriate ISPR technique is essential to obtain these features, however, this choice will depend upon the nature of the product as well as the physico-chemical environment of the production conditions, including the lability of the biological system employed.

Results and Discussion

3-Pyridylacetic acid (PyAA) formed by biotransformation of 3-ethylpyridine with *Pseudomonas oleovorans* has been chosen as the model system for this work, since PyAA is a toxic metabolite which accumulates to a maximum of 15 g l^{-1}. Moreover, 3-pyridylacetic acid is representative of a large family of industrially interesting compounds: the biologically formed carboxylic acids.

Many separation processes exist, which can be essentially classified in four categories (see also [5]) based on:
1) evaporation of the product,
2) extraction of the product,
3) size-selective permeation of the product,
4) immobilization of the product.

The choice of the best method to apply to the desired product requires a detailed knowledge of the respective advantages and disadvantages of each. This can be combined with physico-chemical properties of the inhibiting compound (molecular weight, hydrophobicity/-philicity, volatility, charge), culture conditions (temperature, pH, presence of living cells, substrates, *etc.*) (see *Table*).

From the *Table*, ion exchange, immobilization by biorecognition, electrodialysis, reactive extraction, and its corollary perstraction are the separation processes that are, *a priori*, the best adapted to the *in situ* recovery of 3-pyridylacetic acid. From these, reactive extraction was chosen for extensive study.

Considering the relatively high hydrophilicity of 3-pyridylacetic acid and the fact that pure organic solvents such as hexane, octane, octan-1-ol, chloroform, and methyl isobutyl ketone are not able to extract it (not shown here), it is clear that carboxylic acids can generally not be separated from water by conventional liquid-liquid extraction (LLE) [7–9]. Thus, it is necessary to add a so-called extractant, to increase the distribution coefficient between organic and aqueous phases of the acid. A complexation reaction is then used (liquid-liquid reactive extraction or LLRE), which adds a certain selectivity to the separation [8].

Two different extractants were tested for PyAA separation. The first, a tertiary amine (*Alamine 336* or trioctyl/decylamine, *Henkel KGaA*, Düsseldorf, Germany), is able to react with the protonated

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