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Reviews

Continuous Biocatalytic Processes

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Abstract:

In this review article, recently developed continuous biotransformation processes are discussed. The processes are used to carry out resolution, redox reactions, hydrolysis/esterification, C−C bond formation, and other reactions. Examples of continuous downstream processing are also included.

Introduction

Biocatalytic processes continue to remain an area of intensive research, with a desire to develop alternative routes to the organic synthesis of fine chemicals. For example, the annual reports for 2006 and 2007 on biotransformations related to synthetic organic chemistry cite 98 and 87 references, respectively.[1,2] Continuous processes have several advantages over batch processes, including very low down-times for the reactors, higher space-time yields, optimized energy consumption, reactions under steady-state conditions, and many more. On the other hand, continuous processes necessitate the use of more expensive equipment, the use of catalysts with a long life, automation to a large degree in instrumentation and process control, and the availability of downstream processing technologies to separate the product from the unreacted reactants, intermediates, and byproducts.

Compared to continuous processes in the chemical industry, continuous biocatalytic processes, namely processes which employ biocatalysts to catalyze biotransformations, are unique in several ways. Biotransformations generally occur in aqueous media and commonly follow Michaelis−Menten kinetics.[3] The temperature range in which biocatalysts remain active is limited. The pH of the biotransformation reaction is also generally neither highly acidic nor highly basic. Many approaches to continuous biocatalytic processes use immobilized biocatalysts, which are embedded in a structured, flow-through reactor. In former times, the production costs of purified enzymes were often too high to allow batch-wise biocatalytic processing. Here continuous processes with their intrinsic biocatalyst recycling helped to increase biocatalyst utilization (i.e., costs) and were thus instrumental in paving the way for biocatalysis strategies in the chemical industry.

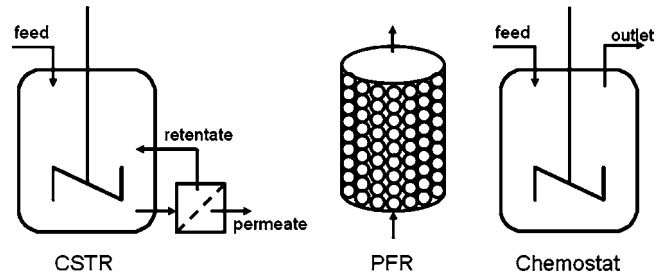

Figure 1. **Schemes of CSTR, PFR, and chemostat.**

In the case of continuous biocatalytic processes with microorganisms, the composition of the microbial population may keep changing. In the case of enzymes as the biocatalysts, immobilization techniques have largely led to improvements in enzyme stability and performance.

The scope to develop newer continuous biotransformation processes and to improve existing ones remains an area of intensive research and development. This review summarizes the recent developments in this field.

Continuous Process Reactors in Biotechnology. The basic reactor configurations and methods of biocatalyst retention will be briefly introduced here. More details on reactor configurations, membrane retention, immobilization methods, and reaction engineering can be found elsewhere.[4]

The Continuous Stirred Tank Reactor (CSTR). In a continuously operated stirred tank reactor (CSTR, Figure 1), the concentration is the same in every volume element as a function of time, assuming ideal mixing. The CSTR works under product outflow conditions, with the concentration at the outlet of the reactor being the same as in every volume element of the CSTR. At steady state, the conversion is controlled by the catalyst concentration and the residence time. When substrate surplus inhibition occurs during a biotransformation, CSTRs are preferred to batch reactors.

Often, instead of one CSTR, several CSTRs are used in a cascade, with the product concentration increasing from one

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Table 1. **Continuous biotransformation processes with biocatalyst retention using immobilization techniques**

substrate	product	immobilized enzyme	STY [$g \cdot L^{-1} \cdot d^{-1}$]
urea	ammonia	urease from *Lactobacillus fermentum*	n.a.
N-acetyl-D,L-3-(4-thiazolyl)alanine	3-(4-thiazolyl)alanine	aminoacylase from *Aspergillus niger*	n.a.
fumaric acid	L-aspartate	aspartase from *Escherichia coli*	3000
glucose	fructose	xylose isomerase from *Bacillus coagulans/Streptomyces rubiginosus*	n.a.

substrate	product	immobilized whole cells	STY [$g \cdot L^{-1} \cdot d^{-1}$]
pantolactone	pantoic acid	*Fusarium oxysporum* (lactonase)	360−380
L-aspartic acid	L-alanine	*Pseudomonas dacunhae* (aspartate β-decarboxylase)	170
fumaric acid	malic acid	*Brevibacterium flavum* (fumarase)	n.a.

Table 2. **Continuous biotransformation processes with biocatalyst retention using membrane techniques**

substrate	product	solubilized enzyme	STY [$g \cdot L^{-1} \cdot d^{-1}$]
2-oxo-4-phenyl-butyric acid	2-hydroxy-4-phenyl-butyric acid	D-lactate dehydrogenase from *Staphylococcus epidermidis*	410
(*R*)-3-(4-fluorophenyl)-2-keto propionate	3-(4-fluorophenyl)-2-hydroxypropionic acid	D-lactate dehydrogenase from *Leuconostoc mesenteroides*	560
trimethylpyruvic acid	*tert*-leucine	leucine dehydrogenase from *Bacillus sphaericus*	638
D,L-phenylalanine-isopropylester	L-phenylalanine	Subtilisin Carlsberg from *Bacillus licheniformis*	14
N-acetyl-D,L-methionine	L-methionine	L-aminoacylase from *Aspergillus oryzae*	592

reactor to the next one. The cascade of CSTRs approximates the plug flow reactor.[4]

The Chemostat. Instead of obtaining batch cultures for fermentation, a continuously operated chemostat (Figure 1) enables cells to grow under controlled conditions, which provide an environment of stable biomass and constant substrate and product concentrations. A controlled, steady metabolic state under optimum conditions can be achieved for the cells, leading to optimization of the biotransformation reaction. This can lead to higher productivity, better process control, and less fluctuation in product quality.

The Plug Flow/Packed Bed Reactor. Plug flow reactors (PFRs) are particularly suited when product inhibition occurs, since the concentration of the product increases only gradually along the reactor, leading to a higher average reaction rate than the CSTR. Packed bed reactors (PBRs) may be considered as plug flow reactors containing solid catalysts. The PBR is one of the most frequently used biotransformation systems for continuous production, for example, in the case of lipase-catalyzed resolutions. The enzyme/substrate ratio is maintained high, and the reactor volume is low. The voidage in a PBR is much lower than in a stirred tank reactor and is often less than half of that of the latter.

Methods of Biocatalyst Retention. In the case of continuous biotransformation processes, it is essential to have an efficient method of retaining the biocatalyst in the bioreactor and, in addition, its biocatalytic activity. Different methods have been developed to this end and are briefly described below.

Immobilization Techniques. Immobilization techniques for biocatalysis are broadly classified into physical and chemical methods. Physical methods of immobilization include the entrapment of the biocatalyst in natural or synthetic gel matrices. Commonly used materials include alginates, κ-carrageenan, and polyacrylamide gel. Chemical methods of immobilization include cross-linking (including the formation of cross-linked enzyme crystals) and covalent bond formation, usually onto inert carrier materials. Table 1 gives an overview of continuous biotransformation processes with biocatalyst retention using immobilization techniques.

Biocatalyst Retention. Biocatalyst retention by physical membrane processes is another frequently employed strategy for biotransformations as well as in downstream processing. To this end, microfiltration, nanofiltration, and ultrafiltration are commonly used.

Rios et al. have reviewed the phenomena which influence the performance of enzyme membrane reactors (EMRs), the development of new catalytic membrane devices, and the trends in new integrated processes.[5] In addition to being a reactor with continuous substrate feeding for continuous processes, the EMR has also been employed as a membrane contactor. Table 2 gives an overview of continuous biotransformation processes with biocatalyst retention using membrane techniques.

Continuous Biotransformation Processes. *Continuous Resolution Processes.* In the case of biotransformation reactions, where one enantiomer is selectively obtained from a racemic mixture, the other enantiomer has to be racemized again, and the biotransformation to the desired enantiomer has to be repeated. Continuous dynamic kinetic resolution (DKR) processes assume significance wherever enzymatic kinetic resolution is carried out to obtain a pure single enantiomer. The latter process has a maximum chemical yield of 50%. When the "undesired" enantiomer is racemized in situ by using a so-called dynamic kinetic resolution, this problem can be overcome.

The (*R*)- and (*S*)-enantiomers of 1,3-butanediol are useful building blocks for active pharmaceutical ingredients. N. Itoh et al. immobilized *E. coli* cells overexpressing mutated phenylacetaldehyde reductase (PAR) or *Leifsonia* alcohol dehydrogenase (LSADH) and applied this system for the continuous

(5) Rios, G. M.; Belleville, M. P.; Paolucci, D.; Sanchez, J. *J. Membr. Sci.* **2004**, *242*, 189–196.

production of the chiral alcohol in a PBR.[6] Immobilization by cell aggregation was carried out using polyethyleneimine (PEI) P-70 or 1,6-diaminohexane (DAH) and glutaraldehyde. The immobilized cells in the packed bed reactor continuously produced (*R*)-1,3-butanediol with a yield of 99% from a 5% (w/v) 4-hydroxy-2-butanoate solution over a period of 500 h. The cosubstrate 2-propanol was oxidized to acetone. There was a decrease in biocatalytic activity after 500 h, although the ee of 99% could be maintained for (*R*)-1,3-butanediol. The concentration of 2-propanol was kept lower than 20%. The authors found that using 2-propanol solution is more effective for immobilizing whole cell systems than for intact whole cell systems. The biocatalyst could be stored as a dry powder before reuse.

Another example for a DKR is the racemization of sec-alcohols via a hydrogen-transfer process using transition metal complexes and acid supported on solid matrix. P. Lozano et al. have designed an enzymatic process in an ionic liquid (IL)/supercritical carbon dioxide ($scCO_2$) biphasic system.[7] They used *Candida antarctica* lipase B (CALB)-catalyzed kinetic resolution of *rac*-1-phenylethanol in the water-immiscible [btma][NPfc], in which phase the enzyme was present. The substrates/products are transported by the $scCO_2$ phase. The enzyme was found to be both active and stable in the IL, exhibiting an increase in half-life time up to 1600 times with respect to that in hexane. The continuous enzyme DKR process was carried out in $scCO_2$ at 50 °C and 100 Mpa, using both enzymatic and chemical catalyst coated with the IL. The CALB was immobilized in silica gel and was used simultaneously with silica modified with benzenesulfonic acid groups (SCX) catalyst. A weight ratio of 25:1 of immobilized enzyme/SCX was used for the DKR experiments of *rac*-phenylethanol in $scCO_2$. The simultaneous presence of both immobilized CALB and SCX particles in the reactor resulted in a clear improvement of the DKR process. The enzymatic and acidic catalysts were physically separated by glass wool in the reactor. The enantioselective synthesis of (*R*)-1-phenylethyl propionate reached 78% (92% ee) in the continuous chemoenzymatic DKR.

The combination of the racemization of the slower-reacting stereoisomer with kinetic resolution in a one-pot reaction has been investigated by Teo et al.[8] They have studied the dynamic resolution of secondary alcohols through enzymatic stereoselective transesterification and heterogeneously catalyzed racemization of the alcohol over several zirconia-containing catalysts. A fluorous phase-switching technique coupled with fluorous extraction was used to facilitate product separation. Continuous extraction in a membrane contactor allowed for easy recovery of the fluorous tagged species in a scaleable operation. More than 90% conversion could be achieved.

The resolution of racemic 1-phenylethanol was investigated using 2′2′2′-trifluoroethanol 1*H*,1*H*,2*H*,2*H*-perfluoro-undecanoate as the acyl donor. For the extraction, the membrane contactor consisted of a hollow-fiber module containing a bundle of microporous polypropylene fibers. The fluorous extraction solvent was circulated through the lumen side of the contactor, and the phase cotaining the material to be extracted was circulated through the shell side. Both streams were recycled and passed through the contactor in concurrent mode. The immobilized lipase Novozym 435 (CALB immobilized on a macroporous acrylic resin) showed a very high activity. For the racemization of the undesired stereoisomer, acetophenone assisted the reversible reaction with phenylethanol. The aluminum-free Zr-zeolite beta (Zr-100, Si/Zr = 100) showed a very high activity and was only slightly poisoned by the alkyl donor.

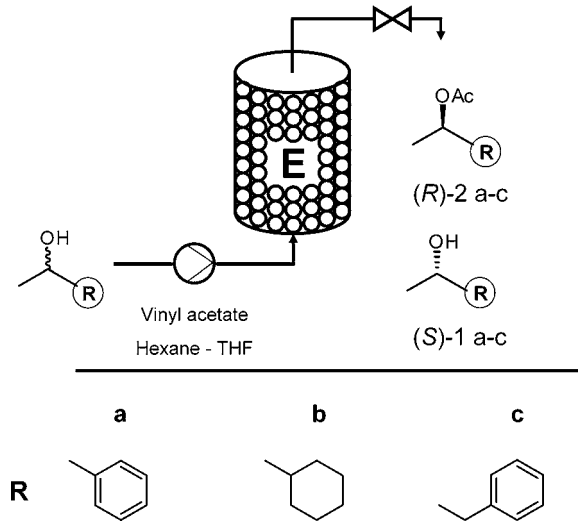

Figure 2. **Kinetic resolution of racemic secondary alcohols *rac*-1a−c in continuous-flow enzyme reactors.**

In a membrane-assisted separation process, after 6 h of continuous operation, when the extract had been exposed to 75 volumes of fluorous solvent, more than 90% of the fluorous ester was recovered from the evaporator. No phenylethanol passed into the fluorous phase. The solubility properties allowed for the facile separation of the fluorous ester from the unreacted (*S*)-alcohol by extraction with a fluorous solvent. The Zr-zeolite beta, however, also catalyzed certain side reactions, and the ee at 95% conversion was about 70%.

Packed bed reactors have been employed to achieve higher productivities in the continuous lipase-catalyzed enantiomer-selective acylation of racemic alcohols. C. Csajagi et al. have employed various lipases in their studies.[9] With Lipase (B lipase) from *C. antarctica* (CALB) immobilized on acrylic resin, preparative-scale kinetic resolutions of the racemic alcohols 1-phenylethanol (a), 1-cyclohexylethanol (b), and 1-phenylpropan-2-ol (c) could be achieved by using vinyl acetate for the acetylation reaction (Figure 2). The pressure in the reactor had no significant effect on the specific reaction rate or on the enantiomer selectivity.

Continuous Redox Processes. Cross-linked enzyme crystals (CLECs) of laccase have been investigated by Roy and Abraham for the continuous biotransformation of pyrogallol to purpurogallin.[10] Purpurogallin is reported to have antioxidant and biological activity and is an inhibitor of epidermal growth

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factor receptor protein tyrosine kinase activity and of xanthane oxidase. The continuous biotransformation was carried out in a packed bed reactor at 28 ± 2 °C under exclusion of light. The product was separated from unreacted pyrogallol using a silica gel column. The CLECs were prepared by crystallizing laccase enzyme using ammonium sulfate (75% saturation), followed by cross-linking with 1.5% glutaraldehyde. Retention of activity to the extent of 64% was observed. The crystals were further lyophilized with 1 mM·L^{-1} of β-cyclodextrin, which functioned as a surfactant, to get the CLECs in powder form. Maximum conversion to the extent of 76.28% was obtained with 3 mM·L^{-1} of pyrogallol and a residence time of 7.1 s. The laccase CLEC had a high stability.

Carrying out biotransformations in multiphase reactors has been the subject of investigation in several laboratories.[11] One strategy in the case where product inhibition occurs is to bind the product onto a solid phase and thereby maintain only nontoxic concentrations of the product in the medium. Prpich and Daugulis have investigated the bioproduction of 3-methylcatechol, starting from toluene, by *Pseudomonas putida* MC2 cells in a solid−liquid two-phase partitioning bioreactor.[12] As the solid phase, a commercially available thermoplastic polymer, HYTREL was used at a 10% (w/w) solid beads to liquid phase ratio. Compared to the biotransformation in a single-phase bioreactor, the product yield incresaed 2.7-fold to 350 mg·L^{-1}·h^{-1}. When sheets made out of the polymer beads were placed around the interior circumference of the bioreactor, a similar increase was observed. The biotransformation could be carried out continuously by circulating the culture medium upwards through an external extraction column containing HYTREL beads. Replacement of the extraction column as the 3-methylcatechol concentration increased to about 500 mg·L^{-1} in the reactor by a fresh column enabled the process to be carried out continuously. The 3-methylcatechol could be desorbed from the beads by elution with methanol. *P. putida* MC2 is a recombinant strain designed specifically to overproduce 3-methylcatechcol. HYTREL is a poly(ether-ester) block copolymer. A total of three extrusion columns were used. Although the continuous experiments were performed over a period of 12.5 h, the results indicate that with a single start-up period and prolonged operation in continuous mode, enhanced productivity can be achieved.

With the increasing number of problems associated with the disposal of electronic waste and the challenges associated with the recovery of precious metals from them, the use of microorganisms for the recovery of precious metals is being studied by various groups. For example, the microorganism *Desulfovibrio desulfuricans* reduces Pd(II) to Pd(0) at the expense of hydrogen. The latter can be generated electrochemically in a so-called electrochemical bioreactor (Figure 3). By separating the primary electrolysis chamber from the chamber in which the biocatalysis takes place, different solutions and pH conditions can be used in each chamber. Besides, a low voltage can be used for the generation of hydrogen. The continuous removal of Pd, Pt, and Rh to the extent of 88%,

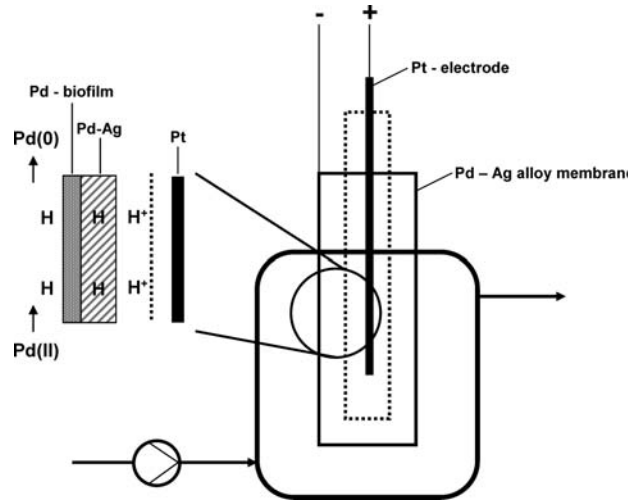

Figure 3. **Electrobioreactor for the bioreductive recovery of platinum group metals.**

99%, and 75%, respectively, at a flow residence time of 10−20 min and input pH of 2.5 could be achieved when the total metal concentration was about 5 mM. In addition to the Pd−Ag electrode and the Pt counter electrode in the electrobioreactor, the cells were incorporated as self-immobilized Biofilm on the front side of the Pd−Ag alloy membrane electrode facing the perfusing solution. The system, with a chamber volume of 20 mL, was run under continuous flow conditions at 1−2 mL·min^{-1} at 22 °C under a constant current of 20 mA, and the contents were mixed with the help of a magnetic stirrer.[13]

Sirisansaneeyakul et al. have studied the production of lactic acid from glucose by immobilized cells of *Lactococcus lactis* IO-1.[14] Microencapsulation was found to be the more effective method of immobilization. The packed bed reactor with immobilized cells was operated in batch and continuous mode. For the continuous mode of operation, the reactor system was first started in the batch mode and switched to the continuous mode after all glucose had been consumed. The dilution rate in the stirred tank was 0.5 h^{-1}. A constant recycle ratio was maintained through the packed bed, and a steady state was eventually achieved. Compared to a volumetric productivity of 1.8 g·L^{-1}·h^{-1} in shake flasks, the productivity of the packed bed of encapsulated cells with recycle of the broth through the bed was increased to 4.5 g·L^{-1}·h^{-1}, partly as a result of reduced product inhibition. The PBR could be run repeatedly to produce lactic acid.

For the production of lactic acid, Lin and Wang have studied a multistage integrated continuous fermentation process, with each stage consisting of a mixing tank bioreactor, cell recycle unit and an extractor. Best results, including highest overall productivity and conversion, were obtained with three stages. A biocompatible solvent, such as a tertiary amine and oleyl alcohol, is added into the extractor to extract the lactic acid.[15]

Zhang et al. have demonstrated for the first time the efficient recycling of the cofactor NADPH in a bioreduction with

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(15) Lin, H.-T.; Wang, F.-S. *AIChE J.* **2007**, *53*, 449–459.

permeabilized cells of a single microorganism.[16] Thus, permeabilized cells of *Bacillus pumiles* Phe-C3 containing a NADP-dependent ketoreductase and a glucose-6-phosphate dehydrogenase (G-6-PDH) were used to reduce ethyl-3-oxo-4,4,4-trifluorobutanoate to (*R*)-ethyl-3-hydroxy-4,4,4-trifluorobutanoate in 95% ee.The NADPH was recycled 4220 times from the externally added $NADP^+$. The permeabilized cells were stable and active for long periods, enabling continuous bioreduction by renewed addition of $NADP^+$. High product concentration could be achieved.

The reduction of prochiral ketones with whole cells can also be carried out in continuous processes. Haberland et al. describe the reduction of 2,5-hexanedione to the corresponding (2*R*,5*R*)-hexanediol in excellent optical purity (ee, de > 99.5%) with whole cells of *Lactobacillus kefir*. The CSTR is operated for 6 days, achieving space-time yields of up to 60 $g \cdot L^{-1} \cdot d^{-1}$. This process also includes an online downstream processing consisting of a countercurrent extraction of the aqueous phase with ethyl acetate and solvent recycling.[17]

Tailor-made microorganisms were successfully applied for the asymmetric reduction of the β-ketoester methyl acetoacetate by Schroer et al. in a CSTR process. Here, recombinant *E. coli* cells overexpressing the alcohol dehydrogenase from *L. brevis* (LbADH) are used as biocatalysts. Surprisingly, the best mode of cofactor regeneration is the substrate-coupled approach, where only 2-propanol is used as reductant and the LbADH is used both for the production reaction and the intracellular cofactor recycling. The reactor was operated for 7 weeks with maximal space-time yields of 700 $g \cdot L^{-1} \cdot d^{-1}$ of the (*R*)-methyl hydroxybutanoate (ee > 99.5%).[18]

Hildebrand et al. reported on the immobilization of alcohol dehydrogenase from *L. brevis* by covalent coupling. The enzyme was immobilized to Sepabeads as carrier material with amino-epoxy functionalities. One advantage that could be achieved by enzyme immobilization was an increase of biocatalyst stability. The authors demonstrated how the enzyme stability could be positively affected. To increase the stability, the remaining functional groups of the support were blocked, and the immobilized enzyme was cross-linked by glutardialdehyde. Cross-linking the enzyme leads to factor 60 having a stabilizing effect compared to soluble enzyme. Using both methods to immobilize the enzyme to the support, it was possible to operate a plug-flow reactor continuously for more than 70 days. As a result, a total turnover number of 2,500,000 could be achieved.[19]

Continuous Hydrolyis/Esterification Processes. Oleyl oleate is a high-molecular weight ester resembling jojoba oil and has wide industrial applications. The esterification of oleic acid to oleyl oleate by chemical and enzymatic methods has been studied by Aracil et al.[20] Commercial immobilized lipases were used as the enzymes in four fixed reactors in a continuous biotransformation process. While the reaction in a batch enzymatic process had to be carried out under vacuum in order to remove the byproduct water continuously, the continuous enzymatic biotransformation was carried out at about atmospheric pressure, with the formed water being removed after each reactor by flash evaporation.

In the continuous enzymatic process for oleyl oleate production, the catalyst consumption was found to be 0.386 kg per ton of product, with a catalyst life of 500 h. In the batch enzymatic biotransformation, the catalyst consumption was 0.465 kg per ton of product. In both cases, the reaction occurred at significantly lower temperatures than in esterification processes using conventional catalysts. Although high pressure drops are associated with packed bed reactors, since water was removed by flash evaporation after each reactor, the pressure drop through the enzymatic bed was balanced.

Banerjee et al. have investigated the continuous production of 6-APA by agarose-immobilized penicillin acylase in a packed bed reactor.[21] The enzyme was sourced from *E. coli* ATCC 11105 and was entrapped physically on agarose gel. Unlike synthetic polymers, agarose is cheap and biodegradable. The continuous nature of the process reduced the inhibitory effect of the acidic 6-APA. The experiments were carried out with partially purified enzymes. The half-life of the immobilized enzyme was found to be 5 days. A 2% concentration of the penicillin G substrate was found to give the highest productivity. The enzyme activity was also found to be maximal at this substrate concentration.

Hexyl esters are popular emollient materials in several cosmetic industrial applications. While chemical esterification reactions use strong catalysts, enzymatic esterification offers a milder and "greener" alternative. Using a lipase from *Rhizomucor miehei* (Lipozyme IM-77) to catalyze the esterification of lauric acid with 1-hexanol in *n*-hexane solvent, Chang et al. had shown previously that the reaction could be successfully carried out on a batch scale. The authors have now reported on the continuous synthesis of hexyl laurate in a packed bed reactor. The optimum conditions for the biotransformation were found to be 45 °C reaction temperature, substrate molar ratio 1:2, and reaction flow rate 4.5 $mL \cdot min^{-1}$. The actual production rate was about 437 $\mu mol \cdot min^{-1}$. Byproduct formation was very low, and the system stability in the PBR was found to be high.[22]

With increasing demand for γ-aminobutyric acid (GABA), a non-protein amino acid which acts as a major inhibitory neurotransmitter in the central nervous system, biosynthetic methods are being investigated as alternatives to chemical methods. Huang et al. have immobilized *L. brevis* CGMCC 1306 cells into calcium alginate beads and carried out the biotransformation of monosodium glutamate to GABA[23] (Scheme 1). Continuous production of GABA was achieved with a high yield by incorporating cell recultivation using the packed bed reactor. The strain used by the authors was isolated from fresh milk without pasteurization and then mutagenized with UV

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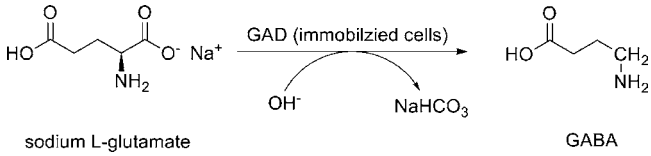
Scheme 1. **Biotransformation of sodium L-glutamate to γ-aminobutyric acid (GABA) by single-step α-decarboxylation with glutamate decarboxylase**



treatment and ^{60}Co radiation. At higher substrate concentration, the conversion in the packed bed reactor was much higher than in the batch reactor. In the continuous process, as the GAD catalytic activity declined, the immobilized cells could be recultivated to a certain extent and the re-encapsulated beads added to the fermentation medium.

Similar to starch hydrolysates, protein hydrolysates are gaining significance as useful starting materials for their bioconversion to useful bioactive products. The enzyme membrane reactor could offer a suitable system for such biotransformations on a continuous scale, particularly in order to avoid inhibition caused by end products and also to make the enzymes reusable. Kapel et al. used a continuously operated enzyme membrane reactor to hydrolyze an industrial alfalfa white protein concentrate (AWPC) using the commercially available enzyme Delvolase (DSM). One of the hydrolysis products was an ACE inhibitory peptide. The EMR employed twin tubular ultrafiltration modules made of zirconium oxide mineral membranes with 10 kDa molecular weight cutoff. The EMR was operated at 40 °C and pH 9.5 and had a working volume of 32 L. In downstream processing, the polyphenolic compounds were removed by an anion-exchange polystyrene resin. Desalting was carried out by electrodialysis. The AWPC hydrolysate production was carried out for 20 h.[24]

In contrast to naturally occurring lipids, structured lipids are restructured fats or oils in which the composition and positional distribution of fatty acids are modified chemically or enzymatically. The use of immobilized lipase in continuous packed bed reactors has been investigated by Kim and Akoh for the preparation of structured lipids from roasted sesame oil and caprylic acid (CA) by *R. miehei* lipase-catalyzed acidolysis. The total incorporation of CA in the SL was 42.5 mol % with an acyl migration of 3.1 mol %. The lipase exhibited a half-life of 19.2 days. Commercially available, immobilized Lipozyme RM IM (Novozyme) was used. The reactor temperature was kept at 45 °C, and the sesame oil/caprylic acid ratio was 1:6 in the feed stream. The substrate mixture was fed from below into the PBR.[25]

The hydrolysis of the cheaply available and renewable raw material starch leads finally to various types of sugars. Chemical and enzymatic processes are used to this end. The hydrolysis of maltodextrin in a continuous mode has been reported by Rebros et al.[26] They immobilized the glucoamylase from *A. niger* into poly(vinylalcohol) hydrogel lens-shaped capsules, LentiKats. Immobilization led to high long-term operational stability during the hydrolysis reaction. The pH during the

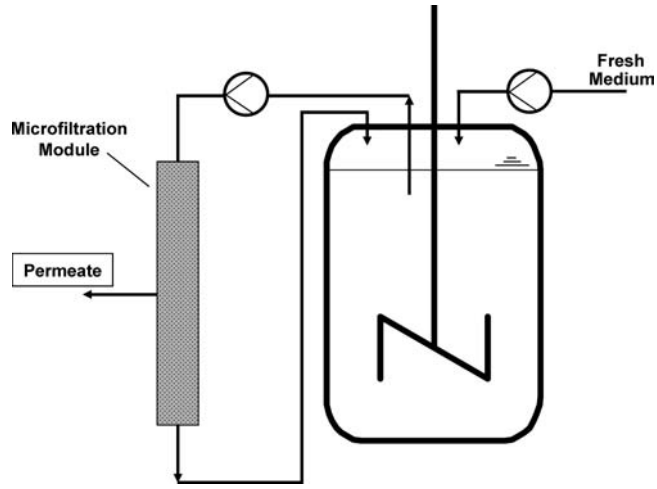


Figure 4. **Continuous chitinase production.**

continuous hydrolysis in a stirred bioreactor was maintained at 4.5, with a different operational temperature and conversion degree. After 500 h of hydrolysis, contamination by *A. niger* was observed. This could be overcome by continuous culture at high substrate conversion and at higher temperature. As a result, no contamination was observed even after 3 days. After continuous hydrolysis at 45 °C, the immobilized glucoamylase enzyme particles were stored in storage solution for four months at 4 °C without any decrease in enzyme activity or contamination.

The continuous hydrolysis of cellulose to glucose in a special tubular membrane reactor in which a porous stainless steel filter was covered by a nonwoven technical textile layer has been studied by Bélafi-Bako et al. This textile layer provided a fine, hairy surface for simultaneous adsorption of the cellulose particles and the biocatalyst, which was the enzyme preparation Celluclast 1.5 L (Novozyme A/s). As substrates, Maxicell pellets and Solka Floc BW 200 powder were investigated. The product glucose permeated easily across the membrane. In comparison to biotransformation in a sheet membrane bioreactor, the conversion of Solka Floc substrate in the hairy tubular reactor was 10% higher.[27]

Kao et al. have employed a bioreactor (Figure 4) with a membrane outer recycling loop in order to evaluate the effect of membrane pore size on cell retention efficiency, permeate flow rate, fouling, and chitin recovery in the permeate during the continuous chitinase production by *Paenibacillus* sp. CHE-N1. A ceramic microfiltration M9 column with a nominal pore size of 300 kDa exhibited the best microfiltration characteristics. In comparison to the batch mode, the membrane mode of operation resulted in a 78% increase in the total chitinase activity over a period of 132 h. Continuous chitinase production with the chitinase activity ranging from 13 to 15 mU·mL^{-1} could be achieved when chitin was fed every 3−4 days.[28] By reversing the flow direction with supplemented air sparging for 10 min·h^{-1}, the average permeate flow rate could be maintained.

The enzymatic synthesis of galacto-oligosaccharide starting from lactose using a commercially available β-galactosidase enzyme from *Kluveromyces lactis* has been studied by Chock-

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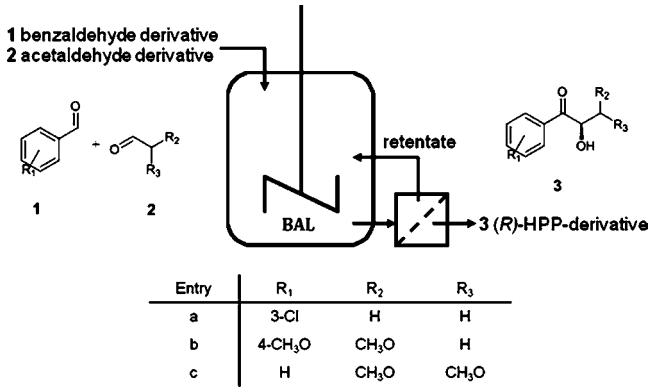


Entry	R_1	R_2	R_3
a	3-Cl	H	H
b	4-CH_3O	CH_3O	H
c	H	CH_3O	CH_3O

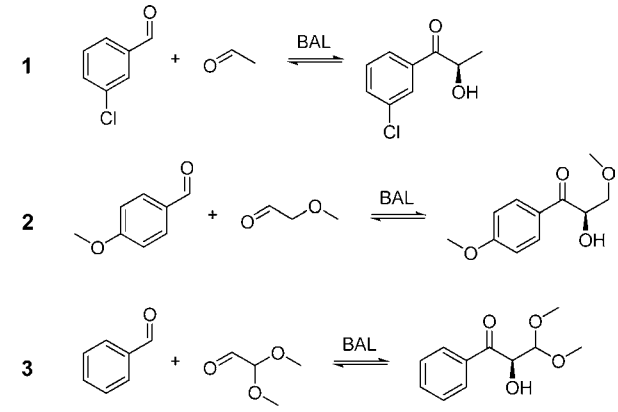
Figure 5. **Reactor concept for the continuous production of synthetically important 2-hydroxyketones with benzaldehyde lyase from *P. fluorescens* Biovar I in an enzyme membrane reactor (EMR).**

chaisawadee et al.[29] The product formation was dependent on lactose concentration and not on the enzyme concentration. Galactose was found to be a competitive inhibitor, and glucose, a noncompetitive inhibitor. The continuous process was carried out in a cross-flow mode in a UF membrane reactor with the cellulose membrane having a NMWCO of 10 kDa. The production performance in the continuous mode was superior to that of the batch mode of operation. The product spectra for both batch and continuous synthesis using high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) were found to be identical, but they differed from that of a commercial product.

Continuous C−C Bond-Formation Processes. Hildebrand et al. reported on a reactor concept (Figure 5) for the continuous production of synthetically important 2-hydroxy ketones with benzaldehyde lyase (BAL; E.C. 4.1.2.38) from *P. fluorescens* Biovar I (chemoselectivity >98% and enantioselectivity >99.5%) in an enzyme membrane reactor.[30] BAL, which is known to catalyze asymmetric C−C bond formation and cleavage, yielding synthetically important 2-hydroxyketones, is used to catalyze the reversible formation of benzoins from aromatic aldehydes and the coupling of aromatic with aliphatic aldehydes, yielding derivatives of (*R*)-2-hydroxy-1-phenyl-propan-1-one ((*R*)-HPP).[31] The continuous enzymatic production of three different products ((*R*)-1-(3-chlorophenyl)-2-hydroxypropan-1-one, (*R*)-2-hydroxy-3-methoxy-1-(4-methoxy-phenyl)propan-1-one, and (*R*)-2-hydroxy-3,3-dimethoxy-1-phenylpropan-1-one, Scheme 2) in gram quantities with space-time yields of up to 330 $g \cdot L^{-1} \cdot d^{-1}$ by utilizing moderate protein amounts (18−56 mg) was reported. Total turnover numbers (*ttn*) of up to 43,000 could be achieved. At optimized residence times the intermediately formed benzoins could be converted quantitatively into HPP derivatives. The application of an EMR for enzyme retention allowed operation periods of up to 60 h without additional enzyme dosage.

The work of Stillger et al. also focuses on the aforementioned reaction of BAL from *P. fluorescens* which catalyzes the

Scheme 2. **Enzyme-catalyzed formation of HPP derivatives with benzaldehyde lyase: 1 [(*R*)-1-(3-chlorophenyl)-2-hydroxypropan-1-one], 2 [(*R*)-2-hydroxy-3-methoxy-1-(4-methoxy-phenyl)propan-1-one], and 3 [(*R*)-2-hydroxy-3,3-dimethoxy-1-phenylpropan-1-one)]**



enantioselective carboligation of benzaldehyde and acetaldehyde, yielding (*R*)-HPP. They used an enzyme membrane reactor for the continuous production of (*R*)-HPP with simultaneous discrimination of the undesired benzoin formation and reached a STY of 1120 $g \cdot L^{-1} \cdot d^{-1}$ (ee > 99%) and total turnover numbers of 188,000. Compared to that of a batch reactor, the STY was increased by more than 30-fold.[32]

The broad applicability of this reactor concept was demonstrated by the production of another (*R*)-HPP derivative (*R*)-(3-chlorophenyl)-2-hydroxy-1-propanone[33] with a STY of 1214 $g \cdot L^{-1} \cdot d^{-1}$ (ee = 99%). These chiral hydroxyketones are known to be versatile building blocks in organic chemistry and common subunits in biologically active molecules.[34]

(*R*)-Phenylacetylcarbinol (PAC) is an important intermediate in the synthesis of L-ephedrine and was one of the earliest chiral intermediates to be obtained by biotransformation.[35] PAC has been produced over 70 years by a fed-batch fermentation of yeast.[36] Goetz et al. reported the use of isolated enzymes to overcome the limitations of the biotransformation, such as the formation of byproduct and the sensitivity of yeast against acetaldehyde. The production of PAC was carried out with a cascade of enzyme membrane reactors while using an isolated pyruvate decarboxylase catalyzing the decarboxylation as the main reaction and the carboligation of two aldehydes as a side reaction. It was shown that the pyruvate decarboxylases of *Saccharomyces cerevisiae* and *Zymomonas mobilis* differ in their potential of use for the carboligation, the latter one being more suited with respect to activity and stability. So the production of PAC was carried out with a cascade of two EMR and an equimolar concentration (50 mM) of both substrates. The low concentration of substrate in the efflux of the first reactor was completed by adding pure aldehydes, yielding a

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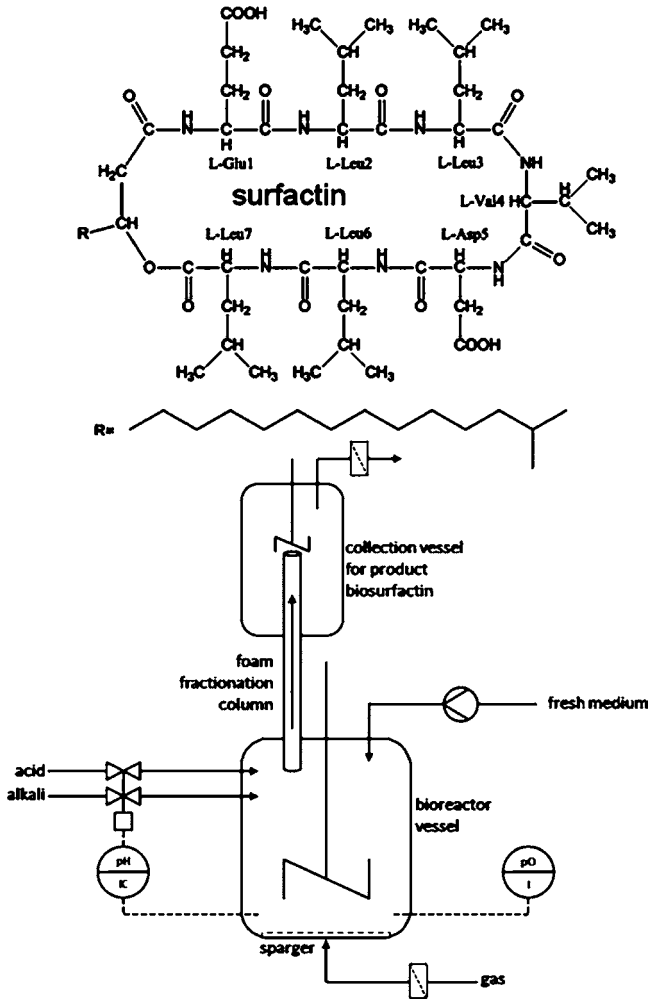

Figure 6. **Molecular structure of surfactin and reactor concept for continuous production of biosurfactant with foam fractionation.**

product concentration of 45 mM in the second reactor and a space-time yield of 81 $g \cdot L^{-1} \cdot d^{-1}$. Using isolated pyruvate decarboxylase in combination with a cascade of two reactors, it was possible to obtain product concentrations that compare to those of conventional fed-batch fermentations.[37]

Other Continuous Biotransformation Processes. Chen et al. have investigated the continuous production of the biosurfactant, surfactin, by the continuous cultivation of *B. subtilis* BBK 006 in a chemostat.[38] The continuous culture was carried out in a 1.5 L integrated bioreactor with foam fractionation using a working volume of 1 L. The system consisted of a stirred tank reactor, a foam fractionation column, and a collection vessel (Figure 6). The surfactin production rate was found to be a function of the dilution rate and the initial concentration of glucose in the feed. The yield of surfactin was highest at low specific growth rates and decreased with increasing specific growth rates. The surfactin concentration was maintained at 18 $mg \cdot L^{-1}$ in the reactor, so that a high surfactin enrichment of about 50 was obtained by foam fractionation. The production of surfactin is known to be related to the microorganism's age, and a simple model has been constructed by the authors to describe how age-related production can be quantified. At steady state, the distribution of physiological states is premature bacteria, bacteria producing surfactin, and older bacteria that have lost the ability to produce surfactin. Submerged microbial fermentation was found to be appropriate for the production of the biosurfactant as it gave economic efficiency. Glucose was used as a carbon source.

In a different approach, Arora et al. have developed a so-called "separative bioreactor", based on resin wafer electrodeionization, an electrodeionization platform which employs resin wafers fabricated from ion-exchange resins.[39] This separation bioreactor is able to separate the organic acid from the biocatalyst, thereby preventing product inhibition and increasing the reaction rates. Another advantage is the fact that the product is separated as the free acid and not as a salt. The *E. coli* strain C8M1 produced lactic acid on a continuous basis for up to 24 h with a productivity of >200 $mg \cdot L^{-1} \cdot h^{-1}$ and near quantitative product recovery. Using the same approach, gluconic acid was produced continuously for 7 days with a productivity of up to 1000 $mg \cdot L^{-1} \cdot h^{-1}$ at >99% product recovery. The immobilized enzyme glucose fructose oxidoreductase (GFOR) had a reactivity of >30 mg of gluconic acid per milligram of enzyme per hour. The gene for the enzyme was obtained from *Z. mobilis* strain ATCC 29191 and cloned into *E. coli*. In addition to the electrodialysis unit, a bipolar membrane was used in the product stream adjacent to the anodic electrode rinse compartment to prevent ion transport (for example, sodium ions) to the product stream. The resin wafers were prepared by immobilizing ion-exchange resin beads in a porous matrix.

Biotransformations with cheaply available agro residues offer in several cases a viable alternative to the conventional methods of producing fine chemicals. John and co-workers immobilized whole cells of *L. delbrueckii* NCIM 2025 in alginate and carried out the fermentation in a packed column bioreactor, using cassava bagasse starch hydrolysate-based medium. The cassava bagasse was hydrolyzed enzymatically using α-amylase and glucoamylase.[40] The immobilized beads could be reused up to six times without any drop in efficacy. With the PBR, higher productivity could be obtained than that in batch mode. The productivity was between 0.33 and 0.66 $g \cdot L^{-1} \cdot h^{-1}$ over a period of 22 days. The fluctuation was suspected to be due to variation in nutrient levels in the medium.

The aldol reaction of pyruvate with C_3- and C_4- aldoses to give *syn*- and *anti*-3-deoxy-2-acids using immobilized 2-keto-3-deoxygluconate aldolase from the hyperthermophilic *Sulfolobus sulfataricus* has been investigated in a continuous flow packed bed reactor by Lamble et al. 3-Deoxy-2-ulosonic acids are naturally occurring compounds which have important biological functions such as inhibition of certain metabolic pathways. The immobilized biocatalyst was used within a recyclable continuous-flow bioreactor in order to produce gram quantities of C_6- and C_7-3-deoxyhept-2-ulosonic acids.[41]

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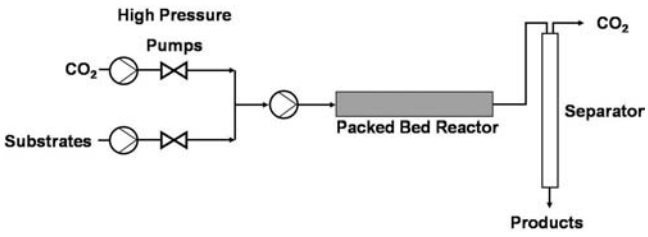

Figure 7. **High pressure plug-flow packed bed reactor.**

Recombinant 2-keto-3-deoxygluconate (KDG)-aldolase was efficiently expressed containing a His-tag at its N-terminus, so that it could be immobilized via metal chelation to Ni^{2+} ions on a His-bind resin.The immobilized biocatalyst was packed into a column to accommodate about 60 mg of the immobilized enzyme. By using a 2-fold molar excess of pyruvate and a 100 mM racemic glyceraldehyde solution, a 50:50 mixture of *rac*-KDG and *rac*-2-keto-3-deoxy-D-galactonate (KDGal) were obtained at a reaction temperature of 50 °C. The enzyme showed no loss of activity over a 60 h continuous run. The products could be separated on a preparative scale by anion-exchange chromatography.

Biocatalysis in unconventional media such as organic solutions may exhibit a wide array of attractive reactions and new substrate availabilities that are not accessible in aqueous media. Pfromm et al. applied subtilisin Carlsberg (immobilized on fumed silica particles) for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester and 1-propanol to *N*-acetyl-L-phenylalanine propyl ester and ethanol in dry hexane. The reaction was carried out in a continuously operated PBR, and a STY of about 6 $g \cdot L^{-1} \cdot d^{-1}$ was achieved.[42]

Supercritical carbon dioxide as solvent was used by Laudani et al. for lipase-catalyzed production of *n*-octyl oleate. The reaction took place in a packed bed reactor (Figure 7) with immobilized lipase from *R. miehei*. With a pressure of 10 Mpa, a yield of about 93% could be attained. The stability of lipozyme was excellent without a decrease over 50 days. It could be shown by Laudani et al. that $scCO_2$ is a potential medium for *n*-octyl oleate synthesis in a continuous mode.[43]

Continuous Down-Stream Processing. For the sequential product formation and separation of organic acids after fermentation, the methods commonly employed include electrodialysis, reverse osmosis, nanofiltration, ion-exchange, ion chromatography, Donnan dialysis, and supercritical fluid extraction, as well as combined reactors. Kumar et al. have developed a process which could be employed for the separation and purification of lactic acid from the fermentation broth.[44] The presence of other organic acids and nonvolatile impurities, as well as the nonvolatile nature of lactic acid, poses certain problems during its separation. By esterifying lactic acid to methyl lactate, separating it from a two-phase CSTR and hydrolyzing it back to lactic acid and methanol, pure lactic acid is obtained from the bottom of the reactive distillation column. In a continuous process, fermentation broth containing a dilute

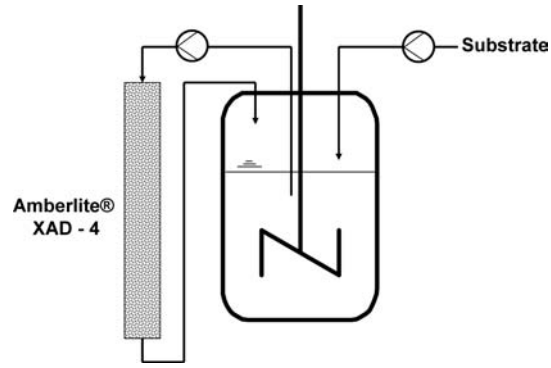

Figure 8. **Biocatalytic synthesis of 3-*tert*-butylcatechol with in situ product recovery.**

solution of lactic acid (16−160 $g \cdot kg^{-1}$) was concentrated in an evaporator to increase the LA concentration to 800 $g \cdot kg^{-1}$. The stream containing concentrated LA and methanol, in the desired mole ratio, is continuously fed to the two-phase CSTR under boiling conditions, where esterification occurs in presence of an ion-exchange resin. The vapour stream of the CSTR, containing methyl lactate, water, and methanol, is fed to the reactive distillation column, in which the LA concentrates at the bottom.

For the continuous production of bioethanol, recycling of yeast cells has been carried out in order to increase productivity. Arifeen et al. have, in addition, evolved a hybrid system, whereby ethanol was removed from the fermentation broth and concentrated by pervaporation. Ethanol purification was carried out by pressure swing distillation in continuous mode. A nutrient-complete fermentation feedstock was produced based on a continuous process, including fungal fermentation to produce enzymatic and fungal cells integrated with feedstock production by starch hydrolysis and fungal autolysis. The enzyme production was carried out by fungal submerged fermentation.[45]

In the case of continuous biocatalytic processes in which product inhibition can make the process unviable, this limitation can be overcome by implementing in situ product removal techniques. A. Meyer et al. have used the hydrophobic resin Amberlite XAD-4 in order to remove 2-*tert*-butylcatechol continuously from a fermentation bioreactor in which recombinant *E. coli* JM 101 cells were used for the in vivo biocatalytic synthesis[46] (Figure 8).

The binding to the resin also stabilized the catechol, which is known to be labile in aqueous solutions. The substrate, *tert*-butyl phenol, also binds to the resin and is eluted along with the product by using methanol. The product was isolated by being selectively bound over neutral aluminum oxide. A purity of >98% could be achieved for 3-*tert*-butylcatechol. The process had a productivity of 63 $mg \cdot L^{-1} \cdot h^{-1}$.

Newer Strategies and Perspectives. Continuous biotransformation processes are being investigated to improve productivity or purity as well as to increase the stability of the biocatalyst using innovative strategies. A liquid−solid circulating fluidized bed (LSCFB) system has been used for the

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continuous polymerization of phenol using soybean seed hull peroxidase enzymes. The reactor had a 3.8 cm I.D. and 4 m high riser and 12 cm I.D. and 3.5 m high down-comer. The biotransformation occurred in the riser section to which phenol and hydrogen peroxide were introduced at the entrance of the riser. The down-comer was used for the regeneration of the coated immobilized enzyme particles. Thus, biotransformation and enzyme regeneration were carried out simultaneously and independently in the LSCFB system.[47] The biodegradation of phenol at high concentration under continuous operation in an immobilized-cell hollow fiber membrane bioreactor has been successfully carried out. *P. putida* ATCC 49451 was immobilized in asymmetric polysulfone hollow-fiber membranes through entrapment inside the porous regions and through attachment on the membrane surface. With a relatively short startup, steady-state conditions were attained within 160 h. An optimum degradation capacity with respect to phenol loading rate was observed due to the tradeoff in the amount of phenol degraded against a rise in the feed rate. During long-term sustainable continuous operation, no significant biofilm fouling on the membranes was observed.[48]

Microstructured flow reactors are being investigated intensively for applications in biocatalytic processes. Coating of the wall with a biocatalyst layer opens up several new possibilities. Thomson and Nidetzky have functionalized the surface of a stainless steel microreactor with aminopropyl triethoxysiloxane followed by glutardialdehyde activation to bind the thermophilic β-glycosidase CelB from *Pyrococcus furiosus* covalently. About half of the specific activity could be retained. Continuous hydrolysis of a 100 mM solution of lactose to glucose at 80 °C could be carried out with more than 70% conversion, with a space-time-yield of 500 $mg \cdot mL^{-1} \cdot h^{-1}$. Compared to microstructures fabricated from poly(dimethylsiloxane) for biocatalytic applications, no hydrophobic solute-material interactions, which restrict the scope of such microstructures, are seen in this type of stainless steel microreactor. As a consequence, biotransformations such as the conversion of 2-nitrophenyl-β-D-galactoside to 2-nitro-phenol can be carried out with high levels of specific and volumetric activity in the immobilized biocatalyst. The biocatalyst-coated microstructured flow reactor was stable under pressure-driven flow conditions for several days at 80 °C and has the potential for use in preparative synthesis.[49]

Biofilm reactors can be run as CSTR, PBR, TBR and fluidized bed reactor, airlift reactor, and upflow anaerobic sludge blanket (UASB) reactor. As a consequence of biofilm formation, cell densities in the reactor increase, and relatively high cell concentrations can be achieved. Qureshi et al. have reviewed the use of biofilm reactors for the production of various chemicals by fermentation for wastewater treatment and for gas and odour treatment.[50]

On the downstream processing side, simulated moving bed technology for continuous chromatographic separation is being studied with great interest as a preparative HPLC tool in the field of pharmaceuticals, fine chemicals, and biotechnology.[51]

For all the obvious advantages and possibilities which continuous biotransformation processes offer, this field will continue to remain a focus area for research and development.

Glossary

6-APA	6-amino penicillanic acid
ATCC	American Type Culture Collection
AWPC	alfalfa white protein concentrate
BAL	benzaldehyde lyase
CA	caprylic acid
CALB	*Candida antarctica* lipase B
CLEC	cross-linked enzyme crystals
CSTR	continuous stirred tank reactor
DAH	1,6-diaminohexane
DKR	dynamic kinetic resolution
EMR	enzyme membrane reactor
G-6-PDH	glucose-6-phospate dehydrogenase
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
GFOR	glucose fructose oxidoreductase
HPAE-PAD	high performance anion-exchange chromatography with pulsed amperometric detection
IL	ionic liquid
kDa	kilodalton
KDG	2-keto-3-deoxygluconate
KDGal	2-keto-3-deoxy-D-galactonate
LA	lactic acid
LbADH	*Lactobacillus brevis* alcohol dehydrogenase
LSADH	*Leifsonia* alcohol dehydrogenase
LSCFB	liquid−solid circulating fluidized bed
NMWCO	nominal molecular weight cutoff
PAC	(*R*)-phenylacetylcarbinol
PAR	phenylacetaldehyde reductase
PBR	packed bed reactor
PEI	polyethyleneimine
PFR	plug flow reactor
(*R*)-HPP	(*R*)-2-hydroxy-1-phenyl-propane-1-one
$scCO_2$	supercritical CO_2
SCX	benzenesulfonic acid groups
SL	structured lipid
STY	space-time yield
ttn	total turnover number
UF	ultrafiltration
UASB	upflow anaerobic sludge blanket

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