

Comparison of Different Chemoenzymatic Process Routes to Enantiomerically Pure Amino Acids

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Abstract: Five common biocatalytic process platforms for the production of enantiomerically pure amino acids are compared along four different dimensions of merit: i) enantioselectivity, ii) overall yield, iii) biocatalyst operating stability, and iv) reactor space-time yield. All processes practiced on industrial scale utilize biocatalysts of very high enantioselectivity. Short efficient process routes are a necessity, giving an inherent advantage to routes based on lyase reactions or reduction of prochiral keto acids. For processes based on the splitting of a racemate, recycling the unwanted enantiomer is crucial. The pKa value of abstracting the α -proton serves as a first indication of the difficulty of achieving overall yields far in excess of 50% through dynamic resolution. High solubility of substrates and products was found to favor high reactor productivity (space-time-yield s.t.y. > 1 kg/(l·d)) and high biocatalyst productivity (enzyme consumption number e.c.n. < 500–1000 U/kg of product).

Keywords: Amino acid · Enantioselectivity · Operating stability · pKa value · Volumetric productivity

1. Process Performance Parameters Pertinent to Enantiomerically Pure Compounds

There are five common biocatalytic methods for the production of enantiomerically pure amino acids; in rough historical order, these are: i) the acylase process, ii) the amidase process, iii) the lyase processes to L-aspartic acid *via* aspartase, to L-alanine with the help of L-aspartate- β -decarboxylase (Asp-DC), and to L-phenylalanine with phenylalanine ammonia lyase (PAL), iv) the hydantoinase/carbamoylase process, most notably to D-amino acids, and v) the reductive amination or transamination starting from prochiral α -amino acids.

There are four dimensions of merit that pertain to any process route, whether biologically or chemically catalyzed, which are regarded as most important:

- selectivity or rather, in the case of manufacture of enantiomerically pure products, enantioselectivity,
- overall yield,
- (bio)catalyst operating stability, and
- reactor space-time yield.

Enantioselectivity. The enantiomeric excess of any optically active product slated for use in the pharmaceutical or food industry is a key product attribute. As the FDA requires separate toxicological data for each impurity (including the undesired enantiomer) of greater than 1% of total composition, an enantiomeric excess of > 98% ee is necessary, usually even > 99% ee. Use in other industries such as agriculture often does not necessitate such high enantioselectivity; the herbicide (*S*)-Metolachlor, one of the most successful examples of the application of homogeneous catalysis, is sufficiently pure for application with 80% ee because even the (*R*)-enantiomer has herbicidal activity but although weaker than the (*S*)-enantiomer [1][2].

Overall Yield. While the lyase processes (iii) and the reductive amination or transamination processes (v) start from

prochiral substrates and thus, in principle, allow up to 100% conversion, the main three processes in operation (acylase, amidase, and hydantoinase/carbamoylase) work on the basis of the separation of a racemate. Yields at or even below 50% are increasingly unsatisfactory, however, because they are uneconomical as well as environmentally unacceptable. Various methods that are discussed in Section 3 have been developed to circumvent the limitation of 50% overall yield.

Biocatalyst Operating Stability. Sufficient (bio)catalyst operating or process stability is a crucial parameter for the success of any process. With such a parameter, stability of the (bio)catalyst is scaled against product formation. The result is a total turnover number (TTN) (Eqn. (1)), a term which is most often used in the homogeneous catalysis field. In enzyme technology, the enzyme consumption number (e.c.n.) is more commonly used (Eqn. (2)):

$$\text{TTN} = \frac{\text{moles of product generated}}{\text{mole of spent catalyst}} \quad (1)$$

$$\text{e.c.n.} = \frac{\text{mass of enzyme preparation spent (in g)}}{\text{kg product}} \quad (2)$$

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For the operating stability of the (bio)catalyst to be considered sufficient, 10 000 is a useful threshold number for TTN; TTNs below that number can only be sustained in case of a very inexpensively produced (bio)catalyst or a high value-added product. Provided the molar masses of enzyme and product are known, TTN and e.c.n. can be mutually interconverted:

$$\text{e.c.n.} = \text{MW}_{\text{enzyme}} / (\text{MW}_{\text{product}} \cdot \text{TTN}) \quad (3)$$

Whereas the parameter TTN does allow comparison between stabilities of homogenous and biological catalysts, the molecular mass especially of an enzyme catalyst is not a suitable reference for the complexity or cost of manufacture. In contrast, both contributions of e.c.n. in Eqn. (3) can be expressed in monetary terms so that the relevance of the biocatalyst cost *vis-à-vis* other costs can be assessed. Assuming $\text{MW}_{\text{product}}$ of 200 and $\text{MW}_{\text{enzyme}}$ of 50 000, a threshold of 10 000 for TTN translates into an e.c.n. of 25 g enzyme preparation per kg of product.

The relevant parameter to measure for the calculation of the e.c.n. and thus for the assessment of operating stabilities of enzymes is the product of active enzyme concentration $[E]_{\text{active}}$ and residence time τ , $[E]_{\text{active}} \cdot \tau$. In a continuous stirred tank reactor (CSTR) the quantities $[E]_{\text{active}}$ and τ are linked by the following equation:

$$([E]_{\text{active}} \cdot \tau) / [S_0] = x / r(x) \quad (4)$$

where $[S_0]$ signifies initial substrate concentration, x the degree of conversion and $r(x)$ the conversion-dependent reaction rate [3][4].

Often, biocatalyst stability is reported as the half-life $\tau_{1/2}$ of an enzyme catalyst. Under the following two conditions, half-lives $\tau_{1/2}$ are equivalent to enzyme consumption numbers: i) there are no diffusion limitations (the effectiveness factor $\eta \approx 1$), and ii) the substrate fully saturates the enzyme ($[S] \gg K_M$, the reaction is zeroth order). Then, both in a CSTR and a plug-flow reactor (PFR), with both constant flow as well as constant conversion policy, a true half-life is calculated if either the degree of conversion (constant flow policy) or flowrate or enzyme concentration (constant conversion policy) are at half the initial values [5].

Reactor Space-Time Yield. Independent of the catalyst, space-time yield is a universal criterion for volumetric reactor productivity:

$$\text{s.t.y.} = \text{amount of product generated} / (\text{reactor volume} \cdot \text{time}) \quad (5)$$

If reactor volume is taken as equivalent to the liquid volume of the reacting solution, an increase of s.t.y. necessarily corresponds to an increase in reaction rate. The rate, expressed in dimensions of $[\text{mole}/(\text{l} \cdot \text{s})]$, in turn tends to be high either at high substrate concentration $[S]$, *i.e.* at high substrate solubility, or in case of a short reaction timescale. Such a short timescale is facilitated in the case of a fast catalyst, otherwise, a higher catalyst load is required. As in the case of enantioselectivity (ee) and (bio)catalyst operating stability (TTN), a threshold number can be provided for the specific activity of the (bio)catalyst, the turnover frequency (tof): a tof of 1 s^{-1} is considered the minimum number for a successful catalyst except in cases of extremely high stability.

Overview of the Status of Development of Catalytic Processes

To assess different processes and biocatalysts in the following sections, it is useful to compare results for the dimensions of merit with pertinent benchmark cases. Table 1 provides numbers that have been achieved for mostly enantioselective processes, both biologically and homogeneously catalyzed.

From Table 1 it can be discerned that only few processes meet all of the threshold criteria mentioned above. In the case of (*S*)-metolachlor, 80% ee is sufficient for use as a herbicide as the unwanted enantiomer is not toxic and even somewhat active; however, both tof and TTN are impressive. The key performance pa-

rameter in the process to (–)-menthol based on the homogeneous catalytic isomerization is the TTN in excess of 100 000. The LeuDH-catalyzed reductive amination of trimethylpyruvate to *L*-tert-leucine (see below) scores high on enantioselectivity and on stability. The different operating stability numbers for chloroperoxidase-(CPD-) catalyzed reactions demonstrate the possible dependence of TTN on substrate and product instead of just on the catalyst.

2. Processes for the Manufacture of Enantiomerically Pure Amino Acids

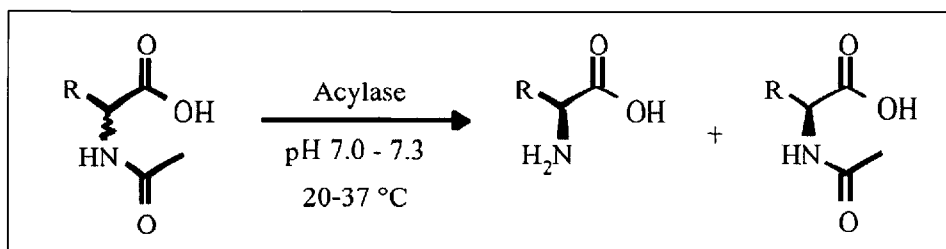
2.1. The Acylase Process

N-Acetyl-*L,D*-amino acids can be transformed to *L*-amino acids and *N*-acetyl-*D*-amino acids *via* *L*-aminoacylase (Acylase I, Scheme 1). With *L*-aminoacylase, Tanabe Seiyaku in 1969 introduced an immobilized enzyme into industrial scale [9–13]. In 1982, Degussa began to operate the first enzyme membrane reactor (EMR) for the manufacture of pharmaceutical intermediates, again with *L*-aminoacylase [3][4][14][15]. Currently, the EMR process is operated for several hundreds of tons annually of *L*-amino acids, especially *L*-methionine and *L*-valine (Scheme 2).

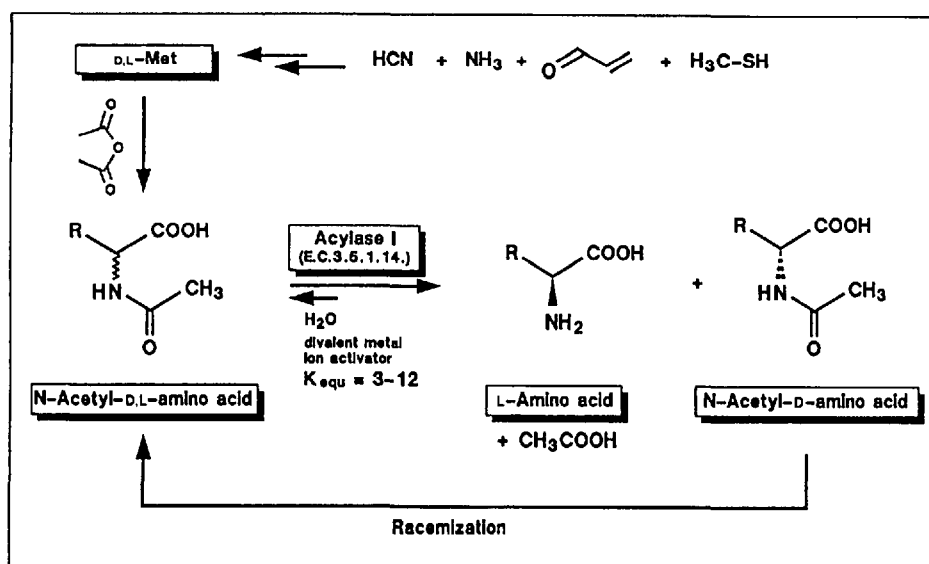
The EMR is operated as a continuous stirred tank reactor (CSTR) with a recycle ratio (feed flow rate into reactor/rate of recycled flow) of up to 200. On pilot and production scale the ultrafiltration membranes are configured as hollow fibers with a molecular weight cut-off around 10 kDa and a retention rate of considerably larger than 99.9%.

Table 1. Benchmark numbers for dimensions of merit for catalytic processes

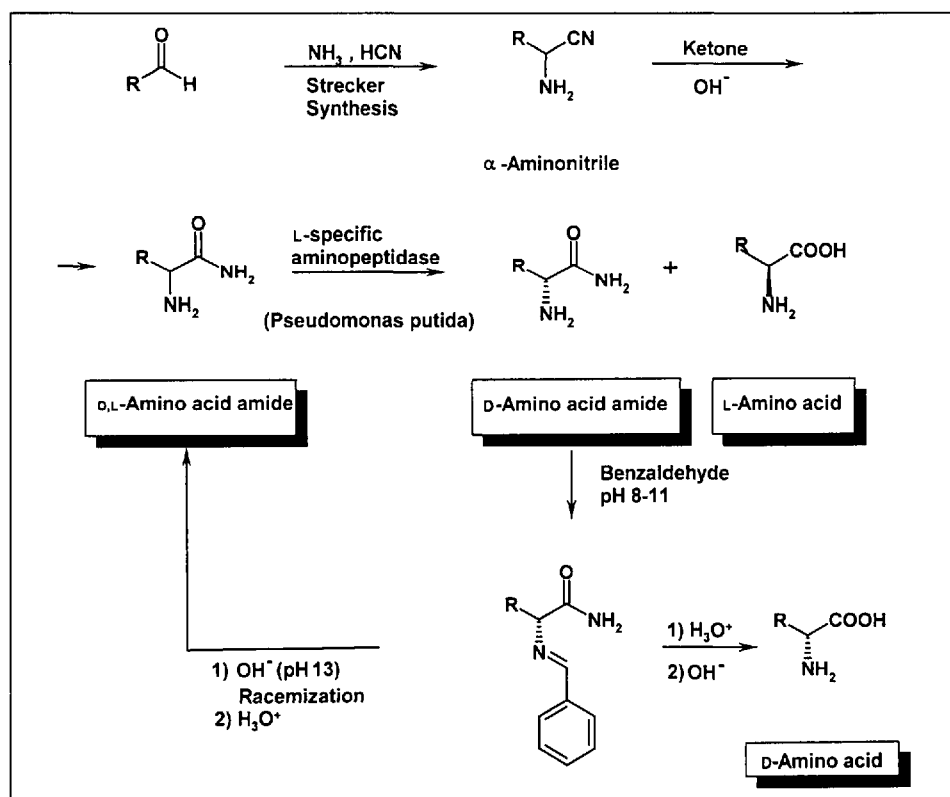
Product	Process	Catalyst	Use, company	tof [s^{-1}]	ee [%]	TTN [-]	Ref.
(<i>S</i>)-metolachlor	catal. hydrogenation	Ir, ferrocene	herbicide, Novartis	≤ 100	80	10^6	[1][2][6]
(–)-menthol	catal. isomerization	Rh ⁺ -(<i>S</i>)-BINAP	food, Takasago		> 99	$3 \cdot 10^5$	[7]
<i>L</i> -tert-leucine	reductive amination	LeuDH	pharma, degussa.	50	> 99.9	10^7	[7]
butylene oxide	epoxidation	CPD	pharma intermediate	$\ll 1$	> 98	$4 \cdot 10^3$	[8]
oxindol	epoxidation	CPD	Crixivan intermediate	$\ll 1$	n.a.	$8.4 \cdot 10^5$	[8]



Scheme 1. L-Aminoacylase reaction.



Scheme 2. L-Acylase process of degussa.



Scheme 3. Amidase process (DSM).

Of the numerous investigations on acylase I the reader is referred specifically to the literature on substrate specificity [16], reaction engineering [17], operational stability [18], and breadth of applicability [19].

2.2. The Amidase Process

Amino acid amides, generated from nitriles through chemical or enzymatic hydrolysis, in turn can be hydrolyzed to L-amino acids with the help of L-amino acid amidase (Scheme 3). The catalyst is used as crude permeabilized whole cells in the pH range of 8–10 and 20% substrate by weight [20].

The substrate specificity of L-amidase is wide [21], similar to L-acylase. The enzyme can be isolated from a wide variety of microorganisms; DSM has developed furthest those from *Pseudomonas putida* and *Ochrobactrum anthropi*. A comprehensive and fairly recent review on the amidase process has been published in [22].

2.3. The Lyase Processes

While several more processes have been developed with lyase enzymes such as a process to hydroxy and amino acids with oxynitrilase (hydroxynitrile lyase, HNL) [23–26], only three are discussed here; the processes to L-aspartate from fumaric acid with L-aspartase, the reaction from L-aspartate on to L-alanine catalyzed by L-aspartate- β -decarboxylase, and the synthesis of L-phenylalanine from cinnamic acid via phenylalanine ammonia lyase, PAL [27].

L-aspartate is generated with the help of aspartase from inexpensive fumaric acid, preferentially used as ammonium fumarate, via addition of ammonia (Scheme 4). While use of living cells was effective, higher productivities and stabilities were achieved by using immobilized cells. In 1974, the first commercial process based on immobilized cells was introduced by Tanabe Seiyaku. Since then, a large number of immobilization protocols have been developed [28] resulting in one of the most efficient enzyme processes known today (see below). Today, the aspartase reaction is virtually the only process by which L-aspartate, a precursor of Aspartame[®], is manufactured in quantities of > 5000 tons annually by several producers around the world.

The L-aspartate- β -decarboxylase route is based on the thermodynamically favorable β -decarboxylation of L-aspartate via L-aspartate- β -decarboxylase (L-Asp- β -DC, E.C. 4.1.1.12.) (Scheme 5).

This reaction has some features ideal for large-scale processing:

- The irreversible decarboxylase reaction allows complete conversion of L-aspartate to L-alanine and carbon dioxide in up to 100% isolated yield, in contrast to separation of racemates.
- Since the chiral α -C-atom is not involved in key reaction steps, the L-aspartate- β -decarboxylase reaction proceeds enantioconservatively and the enantiomeric purity of the L-alanine product is not compromised. The gene coding for L-aspartate- β -decarboxylase has been cloned and overexpressed [29] so that the alanine racemase activity commonly occurring in the wildtype enzyme cannot be detected anymore.
- The CO_2 generated acts as buffer at the pH optimum of the enzyme, pH 6, so that only L-alanine is obtained besides volatile co-products when ammonium aspartate is used as substrate.

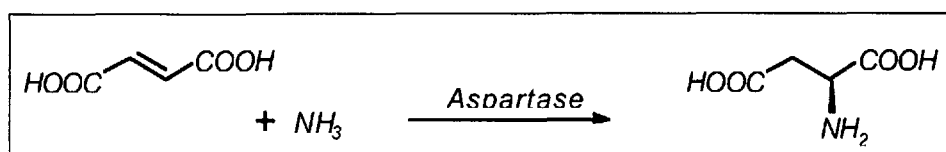
Different pH optima of L-aspartase (pH 8.5) and L-aspartate- β -decarboxylase (pH 6.0) preclude use of a single reactor to convert fumaric acid directly to L-alanine [30].

L-phenylalanine can be obtained by simple addition of ammonia to *trans*-cinnamic acid catalyzed by phenylalanine ammonia lyase, PAL [27] (Scheme 6). The process had been scaled up for the production of L-phenylalanine for Aspartame by Genex but ultimately had to be abandoned because the costs to L-phenylalanine *via* fermentation from glucose were lower than those for the enzymatic process.

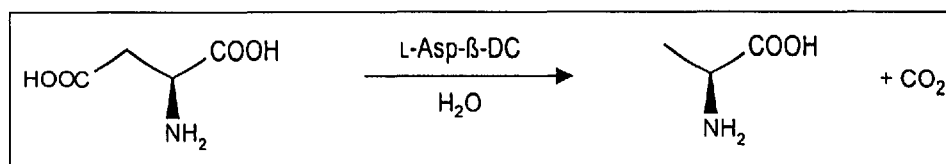
2.4. The Hydantoinase/Carbamoylase Process

Hydantoin, which can be viewed as cyclically protected amino acids, can be converted to D- or L-carbamoyl amino acids through the action of D- or L-hydantoinases (Scheme 7). The carbamoyl amino acids can be further converted to the D- or L-amino acids with the respective carbamoylases. Only innocuous by-products such as ammonia and carbon dioxide are formed. The hydantoinase/carbamoylase process is widely used to produce D-phenylglycine and D-*p*-hydroxyphenyl-glycine, sidechains of the antibiotics ampicillin and amoxicillin, respectively, with annual production volumes exceeding 5000 tons annually worldwide.

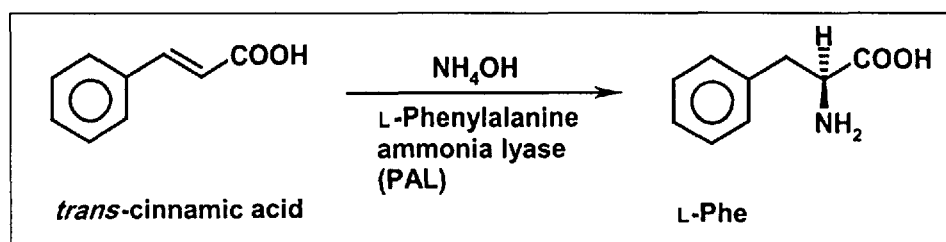
The D-hydantoinase/D-carbamoylase process has been described in detail [31]. The commercially available D-hydantoinase has a wide substrate specificity [32] with already a high enantioselectivity.



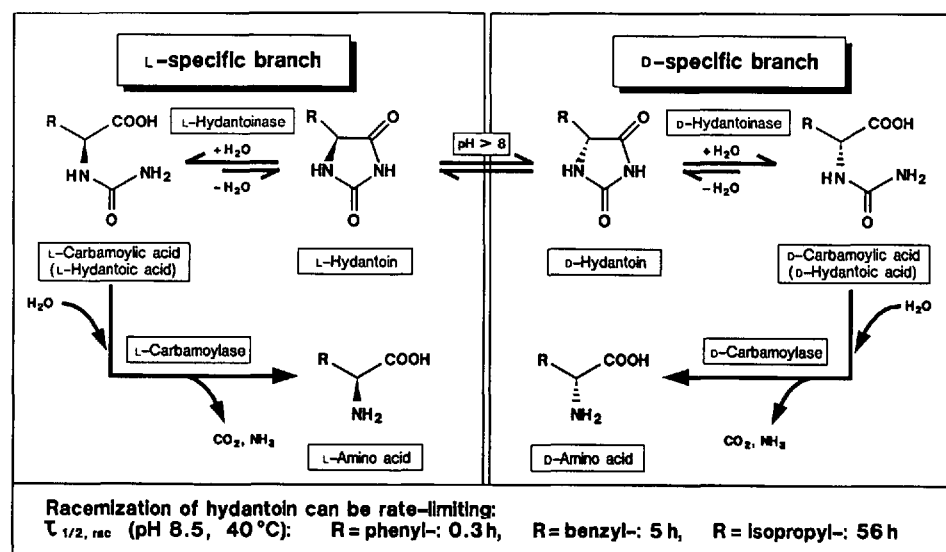
Scheme 4. Aspartase reaction.



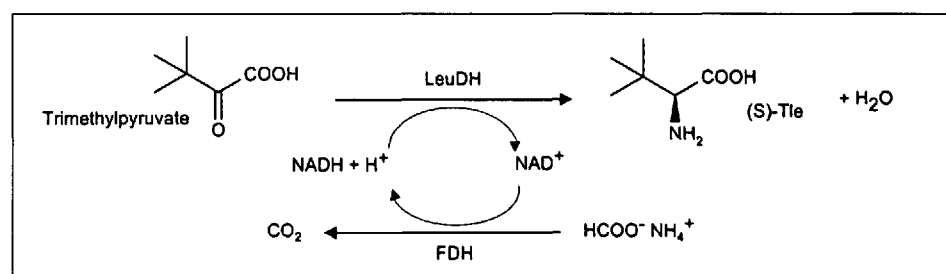
Scheme 5. Aspartate- β -decarboxylase reaction.



Scheme 6. Process to L-phenylalanine from cinnamic acid *via* PAL.



Scheme 7. Hydantoinase/carbamoylase process to D- or L-amino acids.



Scheme 8. Reductive amination process with cofactor regeneration.

The D-carbamoylase is very enantiospecific, thus enhancing the action of the D-hydantoinase, but is rather unstable so it has not been used in isolated form. The L-branch of the system is much less well known [33]. L-hydantoinase is found to possess a low enantiospecificity which often even favors the D-side with a particular hydantoin substrate. Only recently the L-hydantoinase was evolved towards either higher L- or D-enantioselectivity [34]. For a review and comparison of both D- and L-hydantoinase/carbamoylase systems the reader is referred to reference [35].

2.5. Reductive Amination or Transamination Starting from Prochiral α -Amino Acids

α -Keto acids can be reduced to α -amino acids with the help of amino acid dehydrogenases. Leucine dehydrogenase (LeuDH) and phenylalanine dehydrogenase (PheDH) are of particular interest for synthesis. The area has been reviewed by Hummel and Kula (1989) [36], Ohshima and Soda (1989) [37] and, most recently, by Brunhuber and Blanchard (1994) [38].

Enzymatic reductive amination with NADH as cofactor can only be operated on large scale if the cofactor is regenerated. Wandrey and Kula have developed a regeneration scheme using formate as reductant of NAD⁺ generated upon reductive amination. The formate is oxidized irreversibly to CO₂ by formate dehydrogenase (FDH, E.C. 1.2.1.2.) (Scheme 8) [39].

For soluble reactants and products, enzymes are preferentially immobilized in an enzyme-membrane reactor (EMR). To keep the cofactor from penetrating through the membrane, it can be enlarged with polyethyleneglycol (PEG) [40]. With the advantage of quantitative use of keto acid substrate and with a suitable process of cofactor regeneration, enzymatic cofactor-dependent reductive amination of trimethylpyruvate is the route of choice for large-scale synthesis of enantiomerically pure (*S*)-Tle. Degussa operates an industrial process producing high-quality (*S*)-Tle on tons scale *via* this route [41].

α -Keto acids can also be reduced to α -L-amino acids *via* transamination (Scheme 9) [42]. In this process, L-aspartate serves as the ultimate amino donor; however, only the broad-range transferase can accept L-aspartate directly. The branched-chain transferase (BCAT, branched-chain aminotransferase) only accepts L-glutamate, so an additional re-

dox cycle between L-aspartate and L-glutamate catalyzed by aspartate aminotransferase (AAT) is necessary. This latter cycle is driven by the spontaneous decarboxylation of the oxaloacetate generated from aspartate into pyruvate and CO₂. Again, pyruvate has to be removed because it can serve as substrate for BCAT, so it is condensed with itself with the help of acetolactate synthase (ALS) which in turn spontaneously decarboxylates again, to volatile acetoin which can be removed by distillation. As in the cases of aminoacylase and amidase, substrate specificity is not very tight, allowing for conversion of a variety of keto acid substrates. This feature of transaminases and others has been reviewed in recent years [43][44].

3. Assessment of Quality of the Different Processes

3.1. Enantioselectivity

A comparison of results regarding enantiomeric excess of products for the different enzyme systems discussed in this article reveals that all of them offer enantioselectivities above 99% ee, at least in the last crucial step yielding the amino acid product (L-amidase: [45], L-acylase: [46], L-carbamoylase [33], and transaminase [42]). In the hydantoinase/carbamoylase system, this function rests on the carbamoylase which has been found to be highly enantioselective. However, the enantioselectivity of hydantoinase, the enzyme catalyzing the penultimate step from hydantoin to carbamoylic acid, varies with the substrate and is always imperfect [34].

Routes that do not offer the required level of enantiomeric purity have to be

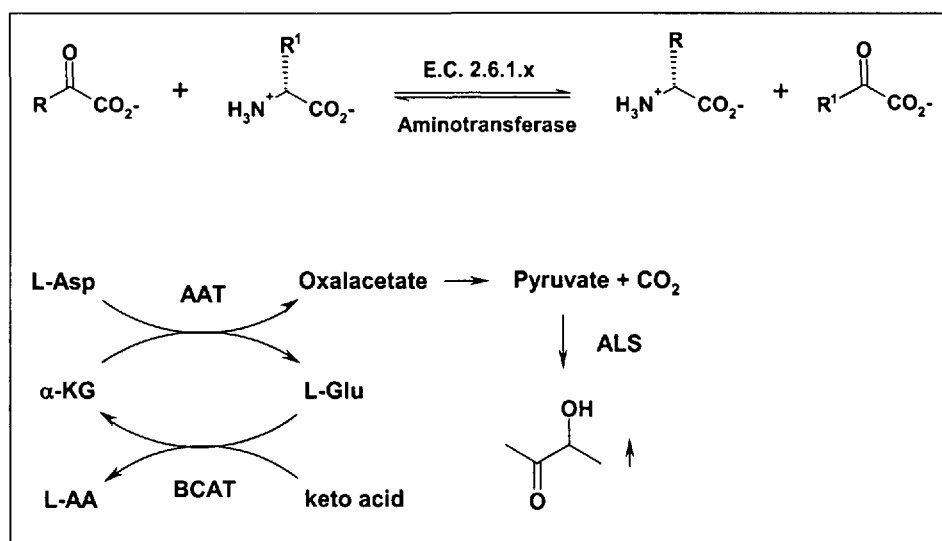
extended with additional purification and polishing steps which often compromise efficiency, overall chemical yield, or space-time yield. As an example, the lipase-catalyzed ring opening of azlactones in toluene yielded the substituted *L-tert*-leucine ester which upon direct hydrolysis in 6N HCl yielded *L*-Tle with 73% ee (c.y. 80%), however, an additional hydrolysis step with alcalase followed by treatment with HCl resulted in an enantiomeric purity of 97% ee [47].

It deserves to be noted that, in contrast to processes based on homogeneous catalysis, any process to enantiomerically pure compounds that was developed to large scale, uses an enzyme with very high to near perfect enantioselectivity, at least in the last step. As the example of hydantoinases demonstrates, enzyme catalysts in any penultimate step do not have to be completely enantioselective to be considered for a large-scale process. However, as issues of overall yield and space-time-yield arise, efforts are made to improve even enzymes of penultimate processing steps [34].

3.2. Overall Yield or Substrate Utilization: Thermodynamic Yield Limitations, Prochiral Selectivity or Ease of Racemization

The chemoenzymatic amino acid processes fall into two different categories:

- The acylase, amidase, and hydantoinase/carbamoylase processes utilize one enantiomer of a racemic mixture. Per pass (*i.e.* without recycle), the maximum yield is limited to 50%.
- The processes based on addition (aspartate), elimination (*L*-asp- β -decarboxylase), or reductive amination of a keto group (amino acid dehydro-



Scheme 9. Transamination process to α -amino acids (NSC).

genases and transaminases) can utilize up to 100% of the substrate.

Before investigating any other yield-limiting factor, possible thermodynamic limitations should be ascertained. Among the processes based on a separation of racemates, only the amidase process is not thermodynamically limited. The hydrolysis reaction of N-acetyl amino acids is equilibrium-limited, however, the equilibrium is far on the side of the hydrolysis so that at low substrate concentrations conversion is nearly quantitative. For the case of N-acetyl methionine, the equilibrium constant K ($K = [\text{acetate}][\text{L-Met}]/[\text{N-Ac-L-Met}]$) was determined to equal 2.75 at 37 °C and pH 7 [17]; equilibrium conversion x_e (based on N-Ac-L-Met) at $[S_0] = 100$ mM is 96%, and decreases to 91% at $[S_0] = 300$ mM and 86% $[S_0] = 500$ mM. In the hydantoinase/carbamoylase system, there is an equilibrium between the hydantoin and the carbamoylic amino acid ($K \approx 10$ favoring the carbamoylic acid), however, as the carbamoylase reaction is irreversible (Scheme 7), the combined enzymatic system produces yields up to 100%.

Lyase-catalyzed reactions discussed in this article (aspartase, L-aspartate- β -decarboxylase and phenylalanine ammonia lyase) are not thermodynamically limited to any significant degree. The two amination processes, however, offer a stark contrast on this point: whereas the reductive amination process with amino acid dehydrogenases is not limited significantly (for leucine/ ketoleucine at pH 11.0, $K_{eq} = 9 \cdot 10^{12}$ [48]), the equilibrium constant of the transamination process is in the order of 1 ($K = 1.86$ for L-Ala/ α -ketoglutarate and pyruvate/L-Glu [49]; for additional equilibrium constants, see [50]).

For the processes based on the separation of racemate, the yield can be increased beyond 50% if the unconverted enantiomer is racemized and recycled. The pKa value of the labile α -hydrogen serves as an indicator of the ease of racemization.

Ease of Racemization of Hydantoins

The pKa values for the abstraction of a hydrogen atom in position 5 do not differ much (Table 2); they range from 8.5 to 9.5. There is no discernible trend for aromatic and aliphatic, functionalized and unfunctionalized sidechains. Corresponding racemization half-lives at 40 °C and pH 8.5 in the absence of any buffer are listed in Table 3; values for half-lives span more than two orders of magnitude.

Table 2. pKa Values for 5-substituted hydantoins

Sidechain	Amino acid	pKa
Phenyl	Phenylglycine	8.70
3'-Pyridylmethyl	3'-Pyridylalanine	8.78
Methylthioethyl	Methionine	9.00
Imidazolmethyl	Histidine	9.03
Methyl	Alanine	9.10
Benzyl	Phenylalanine	9.11
Isopropyl	Valine	9.14
Carboxyethyl	Glutamic acid	9.26
<i>p</i> -Chlorobenzyl	<i>p</i> -Cl-Phenylalanine	9.28

Table 3. Racemization half-lives

Side chain	Amino acid	τ 1/2 [h]
Hydroxyphenyl	<i>p</i> -OH-Phenylglycine	0.21
Phenyl	Phenylglycine	0.27
Hydroxymethyl	Serine	1.60
Benzyl	Phenylalanine	5.00
Methylthioethyl	Methionine	5.82
Imidazolymethyl	Histidine	16.09
Isobutyl	Leucine	21.42
Methyl	Alanine	33.98
Isopropyl	Valine	55.90
<i>tert</i> -Butyl	<i>tert</i> -Leucine	120

Conditions: 40 °C, pH 8.5, no buffer added
Contents of Table partially excerpted from [35]

Accordingly, hydrogen abstraction already can be afforded by OH⁻ ion at moderate pH values at which hydantoinases and carbamoylases are active and, at least in the D-hydantoinase/D-carbamoylase system, also stable. The pH optimum of hydantoinases has been reported to be at 8.0 to 9.0 [31][33].

Despite pKa values suitable for proton abstraction with common bases such as hydroxide at moderate pH values, racemization proceeds more quickly in the presence of chemical or enzymatic catalysts. When passed over strongly basic ion exchange columns, hydantoins racemize preferentially in the highly basic layer around the beads [51]. In addition, two hydantoin racemases have been found and characterized with respect to sequence and substrate specificity [52–54].

Ease of Racemization of Amino acid Amides

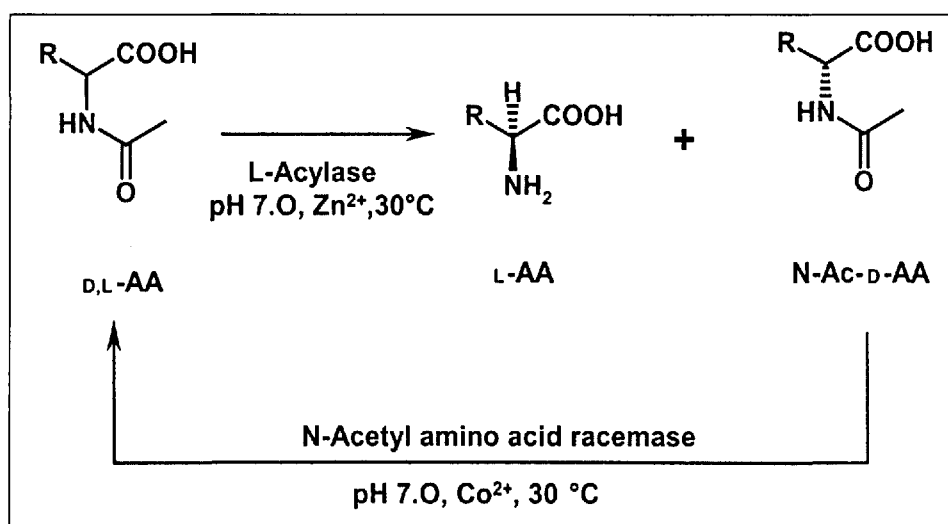
While unsubstituted amino acid amides are not easily racemized ($pK_a \geq 15$) these compounds can be treated with aromatic aldehydes at pH 8–11 resulting in formation of a Schiff base in accordance with the bottom branch in Scheme

3. The latter then can be racemized in a facile manner at pH ≈ 13 and recycled [55].

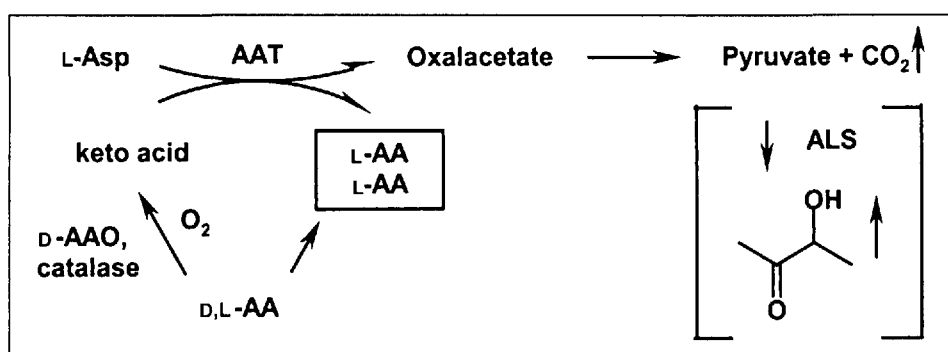
Ease of Racemization of Acetyl Amino Acids

The pKa of abstraction of α -hydrogen atoms from acetyl amino acids is much higher than 8–9, approaching 15. Therefore, either extremes of temperature and pH or extraordinary catalysts are necessary to afford racemization. Nevertheless, N-acetyl amino acid racemases (N-AAARs) have been found, first by Tokuyama *et al.* [56–59] and recently by Verseck [60].

Scheme 10 depicts the desired process scheme for the coupled acylase-acetyl amino acid racemase reaction which increases overall yield from about 45% based on N-Ac-DL-amino acid to nearly 90%. In principle, acetyl amino acid racemase can be coupled with either an L- or a D-acylase. The enzyme requires divalent metal ions to function, the order of activation is $\text{Co}^{2+} > \text{Mg}^{2+} > \text{Mn}^{2+} > \text{Zn}^{2+}$ [60]. Its pH-optimum around pH 8 is compatible with that from acylase at pH 7.



Scheme 10. Yield increase by enzymatic racemization of N-acetyl-D-amino acid.



Scheme 11. Coupling of transaminases with D-amino acid oxidase to yield solely L-amino acid.

The physiological role of an acetyl amino acid is unclear. There is evidence that the enzyme fortuitously acquired the function of racemizing acetyl amino acids and that the protein's main function is as an O-succinylbenzoate synthase (OSBS) rather than an N-AAAR [61]. Both the k_{cat} as well as k_{cat}/K_M values suggest that the protein is a much better OSBS than N-AAAR.

Currently, however, racemization of acetyl amino acids is effected chemically at low pH values combined with high temperatures and resulting in an overall yield on the order of 80% [15].

Yield Limitations in Amination Processes

The reductive amination and transamination processes from prochiral α -keto acids to the corresponding amino acids do not suffer from yield limitations resulting from an unwanted enantiomer. However, even such process schemes can suffer from other limitations affording yields of less than 100%. At high concentrations, keto acids and ammonia can form dimeric-type by-products (N-acetyl-D,L-amino acid amide) from condensation reactions [62]. In addition to this chemical side reaction, enzymatic trans-

amination reactions face enzymatic side reactions. Even if the aforementioned thermodynamic limitations are alleviated through decarboxylation of oxaloacetate and condensation of pyruvate with acetolactate synthase (ALS), the pyruvate formed can act as a substrate for the branched-chain aminotransferase (BCAT) (Scheme 9), resulting in amino acid mixtures that can be difficult to purify.

An interesting scheme to purify D,L-amino acids enantiomerically is given in Scheme 11 [63]. The D-amino acid is oxidized to the corresponding keto acid with D-amino acid oxidase, available from *Trigonopsis variabilis*; the keto acid is transaminated with L-glutamate to the L-amino acid, and oxaloacetate decarboxylates to pyruvate which in turn is converted to acetoin by condensation and decarboxylation as described earlier.

3.3. Biocatalyst Operating Stability

Data on biocatalyst stability under industrially relevant conditions are notoriously hard to obtain. Following the stability behavior of even a reasonably stable biocatalyst is time-consuming and laborious, especially if the proper proce-

dures is applied, *i.e.* either the constant flow policy with varying degree of conversion or, even less ambiguous, the constant conversion policy with either changing enzyme concentration or changing residence time.

For the amino acid processes discussed here, the numbers are presented in Table 4.

The data in Table 4 demonstrate that half-lives on the order of one month need to be attained for sufficient operating stability. At prevalent high substrate concentrations of often more than 1M, such half-lives correspond with enzyme consumption numbers of between low 100s to low 1000s units per kg of product. While the enzyme consumption number is important, this quantity is modulated by the price to produce one unit of activity. With the advent of efficiently produced recombinant enzymes, even e.c.n.'s of 1000 U/kg can still mean a sufficiently inexpensive biocatalyst for large-scale processing.

The operating stability of acylase I has been particularly well characterized [16][18][66]: In aqueous solution, the resting stability of acylase from *Aspergillus oryzae* was found to depend much more on pH than on concentration: while at room temperature (25 °C) and standard pH (7.0) half-life $\tau_{1/2}$ was around 60 days between concentrations of 30 to 120 g crude enzyme/l, $\tau_{1/2}$ dropped to 45 d at pH 6.5 and to about 30 d at pH 6.0 [66]. Also, in solubilized form, the fungal enzyme is quite stable whereas porcine kidney enzyme is sensitive to auto oxidation and therefore should be kept under nitrogen if stored in solubilized form [16]. Tests for operating stability in repeated-batch mode ([3][16][17][67]) reveal that acylase from *Aspergillus oryzae* again fares much better than the porcine kidney enzyme. Tests for operating stability in a continuous reactor with the acylase from *Aspergillus oryzae* [18] demonstrated

- the superior stability of AA (616 U/kg L-methionine) over PKA (6000 U/kg L-met) both measured with $[\text{Co}^{2+}]$ of $5 \cdot 10^{-4}\text{M}$,
- the tighter binding of Zn^{2+} vs. Co^{2+} at $5 \cdot 10^{-4}\text{M}$ (308 vs. 616 U/kg L-met),
- that loss of metal, commonly Zn^{2+} , is responsible for activity loss and
- the possibility of reconstitution over a timescale of several hours, whereas the time constant of leaching is on the order of 48 h, as well as
- the option of pulsing divalent metal addition resulting in 477 U/kg L-met at $[\text{Zn}^{2+}]$ of $4 \cdot 10^{-4}\text{M}$.

Porcine kidney acylase seems to have a different spectrum of activation [3]: although Co^{2+} activates PKA most strongly, Ca^{2+} is not far behind whereas Zn^{2+} , just like Mg^{2+} or Fe^{2+} , does not seem to exert a strong effect. Both the fungal and the porcine enzyme have moderate thermostability [68] and moderate stability in the presence of organic cosolvents [16].

3.4. Space-Time-Yield: The Influence of Solubility and Dispersion

Conditions for using an EMR Reactor

As far as reactor configurations are concerned, retention can be achieved through immobilization (fixed- or fluidized-bed) or by ultrafiltration, either in successive batch mode, as dead-end filtration or as a recycle reactor [69]. The enzyme membrane reactor has been reviewed several times [15][70].

Employing soluble enzymes retained in an enzyme membrane reactor possesses the advantages of i) absence of mass transfer limitations in connection with nearly perfect retention, ii) ease of replenishment of fresh catalyst which is much easier than in an immobilized enzyme reactor column, and iii) ease of scale-up; fluidity F ($F = V/(\Delta P \cdot t \cdot A)$ [liters/(bar-hour-m²)], V = ultrafiltered volume, ΔP = transmembrane pressure, t = filtration time and A = membrane area) can be assumed to stay constant during scale-up. Degussa-Hüls has wide experience with both continuous and batch membrane reactors on very different scales, ranging from small-scale laboratory models with 10 ml volume via the pilot-plant scale models at 500 cm² to 0.5 m² to the plant-size reactors at several cubic meters [15].

Solubility of Substrates and Products

An EMR reactor can only be used with soluble substrates and products. Table 5 provides an overview of common amino acids including their derivatives.

As enzymes often do not work at all in suspension or are co-precipitated, or as the reaction is performed in an enzyme membrane reactor, solubility of substrates and products is important. From Table 4, however, it is apparent that there is no *a priori* preferred route that features more highly soluble substrates or intermediates among the alternatives discussed in this paper. By combining Eqn. (4) and (5) it can be seen that the maximum substrate concentration attainable sets a limit to reactor productivity. Some such numbers of reactor productivities are listed in Table 6.

Table 4. Enzyme consumption numbers and half-lives for amino acid process biocatalysts

Enzyme (organism)	e.c.n. [U/kg]	$\tau_{1/2}$ [d]	Substrate	Ref.
Aspartase (<i>E. coli</i>)	175–690	180–730	NH ₄ fumarate	[64]
L-Acylase (<i>A. oryzae</i>)	306–370		N-Ac-D,L-Met	[4]
Asp- β -decarboxylase	1000–2400	45	NH ₄ aspartate	[64]
LeuDH, FDH	600–800	> 30	Trimethylpyruvate	[65]
Hydantoinase		30	Phenylhydantoin	[64]
Transaminase		30	not known	[64]

Abbreviations: e.c.n. = enzyme consumption number; $\tau_{1/2}$ = half-life.

Table 5. Solubility of amino acids and derivatives

Amino Acid	Xaa	Solub. [M]	Ac-Xaa	Solub. [M]	Hydantoin	Solub. [M]	Carbamoyl-AA	Solub. [M]
Alanine	Ala	2.28	Ac-Ala	1.32				
Aminobutyric acid	Abu	2.25	Ac-Abu	1.05	Ethylhyd.	0.04		
Methionine	Met	1.01	Ac-Met	0.7	MTEH	0.2	Carb-Met	0.52
Leucine	Leu	0.14	Ac-Leu	0.18				
Phenylalanine	Phe	0.25	Ac-Phe	0.45	BH	0.19	Carb-Phe	0.105
Tyrosine	Tyr	0.004	Ac-Tyr	0.29	<i>p</i> -OH-BH	0.28		
Homophenylalanine	Hph	<0.002	Ac-Hph	0.07				

Abbreviations: Xaa = amino acid, MTEH = Methylthioethylhydantoin, BH = Benzylhydantoin, Carb = Carbamoyl.

Conditions: Xaa: water, 40 °C, Ac-Xaa: pH 7.5, 40 °C, hydantoins: pH 8.5, 40 °C, carbamoyl amino acids: pH 8.5, 40 °C.

Table 6. Reactor productivities of biocatalytic processes

Product	enzyme	s.t.y. [g/(l-d)]	[S] ₀ [M]	Ref.
L-Aspartic acid	L-Aspartase	60 000	1.5	[64]
L-Alanine	Asp-DC, fixed-bed, ideal, 4 bar	45 600	1.35	[66]
L-Alanine	Asp-DC, fixed-bed, ideal, 1 bar	31 200	1.3	[29]
L-Alanine	Asp-DC, fixed-bed, dispersion	7 500	1.35	[66]
L-Alanine	Glutamic-pyruvic transaminase	4 800	1.0	[64]
L-tert-Leucine	LeuDH, FDH	638	0.5	[65]
L-Methionine	L-Acylase, soluble	600	0.6	[64]
L-Valine	L-Acylase, immobilized	500	0.4	[11]
L- <i>p</i> -F-phenylalanine	broad-range aminotransferase	480	0.1	[71]
L-Valine	L-Acylase, soluble	350	0.4	[17]

Abbreviations: s.t.y.: space-time-yield, [S]₀ = initial substrate concentration, Asp-DC: L-aspartate decarboxylase, LeuDH: leucine dehydrogenase, FDH: formate dehydrogenase.

Table 6 reveals that experimental data on reactor productivity indeed demonstrate a dependence on substrate concentration as expected: productivities in the range of greater than 1–10 kg/(l-d) cannot be attained with fairly insoluble substrates; however, even productivities of only several hundred grams per liter and day should not be the limiting factor if the product has an attractive price level.

Dependence of Space-Time-Yield on Reactor Configuration: L-Acylase

The L-acylase process has been the pioneering process in enzyme technology for both the immobilized plug-flow column reactor as well the continuous stirred-tank membrane reactor. Sometimes, results for different dimensions of merit for the same process do not point in the same direction: while the half-life of

acylase I immobilized on a nylon membrane as measured by thermal stability of 161 days [72] is superior to the data for immobilized acylase (65 d) [11] or soluble enzyme in an EMR [17], reactor productivity at 0.136 L-valine kg/(l·d) is lower than that for DEAE-Sephadex-immobilized acylase (0.5 kg/(l·d)) [11] or that for a membrane reactor (0.35 kg/(l·d)) [17]. However, as both soluble enzyme and immobilized enzyme processes depend on soluble substrates and products, high solubilities of the reaction components seem to be an almost necessary condition for high space-time-yields.

L-Aspartate- β -Decarboxylase in a Fixed-Bed Reactor: Influence of Dispersion

The dependence of space-time-yield and thus volumetric productivity on factors such as degree of conversion and detailed reactor configuration has been studied in detail for the decarboxylation of L-aspartate to L-alanine with L-aspartate- β -decarboxylase in a fixed-bed reactor [66]. Whereas the s.t.y. at the optimum operating point in a 15 ml-spiral-wound catalytic reactor at an initial substrate concentration $[L-Asp]_0$ of 1.35M at 99% conversion was 45.6 kg L-Ala/(l·d), the corresponding figure in an 83 ml-fixed bed reactor at 90% conversion but otherwise similar conditions was only 7.5 kg L-Ala/(l·d). At longer residence times and thus lower degrees of conversion, higher s.t.y. can be attained; however, these conditions are not relevant for processing.

The cause of the lower s.t.y. was found to be neither the difference in degree of conversion nor internal (pore) diffusion effects (the Wagner-Weisz-Wheeler (WWW) modulus Φ was always $\ll 1$ [73][74]) but non-ideal flow conditions resulting in dispersion effects in the fixed-bed reactor (the Bodenstein numbers Bo were 40–100, the residence-time distribution could be fit best by the tanks-in series model with 20 reactors). The low specific activity of the immobilized enzyme of around 10 U/g silica carrier necessitated a low flowrate in the order of 1 mm/min.

4. Conclusions

The results and discussions of the preceding sections demonstrate that all processes to enantiomerically pure amino acids either practiced on large scale or under consideration, utilize biocatalysts of very high enantioselectivity. Short ef-

ficient process routes are a necessity giving an inherent advantage to routes based on lyase reactions or reduction of prochiral keto acids. Processes based on the separation of a racemate have to emphasize recycle of the unwanted enantiomer. The pKa value of abstracting the α -hydrogen on the remaining unconverted enantiomer serves as a first indication of the difficulty of the prospect for achieving overall yields far higher than 50% through dynamic resolution. Volumetric productivity figures should be critically checked for the conditions employed for their generation: degree of conversion and limiting rate process are especially pertinent parameters towards this end. High solubility of substrates and products, in addition to a short process route, favors high reactor productivity as expressed by space-time yields and high biocatalyst productivity as indicated by the enzyme consumption number for the operating stability.

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