



HAL
open science

d -Serine as a Key Building Block: Enzymatic Process Development and Smart Applications within the Cascade Enzymatic Concept

Nazim Ocal, Mélanie L'enfant, Franck Charmantray, Loredano Pollegioni, Juliette Martin, Pascal Auffray, Jérôme Collin, Laurence Hecquet

► **To cite this version:**

Nazim Ocal, Mélanie L'enfant, Franck Charmantray, Loredano Pollegioni, Juliette Martin, et al.. d -Serine as a Key Building Block: Enzymatic Process Development and Smart Applications within the Cascade Enzymatic Concept. *Organic Process Research and Development*, 2020, 24 (5), pp.769-775. 10.1021/acs.oprd.0c00024 . hal-02995483

HAL Id: hal-02995483

<https://hal.science/hal-02995483>

Submitted on 9 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

D-Serine as a Key Building-Block : Enzymatic Process Development & Smart Applications within Cascade Enzymatic Concept

Nazim Ocal, † Mélanie L'enfant, † Franck Charmantray, † Loredano Pollegioni, §

Juliette Martin, ‡, Pascal Auffray, ‡, Jérôme Collin, ‡* Laurence Hecquet, †**

† Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut de Chimie de Clermont-Ferrand (ICCF) F-63000 Clermont-Ferrand (France)

‡ Protéus by Seqens, 70 Allée Graham Bell F-30035 Nîmes (France)

§ Department of Biotechnology and Life Sciences, Università degli Studi dell'Insubria, Varese (Italy)

KEYWORDS. D-Serine, D-Threonine Aldolase, D-Amino Acid Oxidase, Transketolase, Ketoses

ABSTRACT. An efficient enzymatic way catalyzed by an enzyme from D-Threonine Aldolase family was developed for D-serine production at industrial scale. This process was applied to the synthesis of two valuable ketoses, L-erythrulose and D-fructose, within cascade enzymatic concept involving both other enzymes. Indeed, D-serine was used as a substrate of D-amino acid oxidase

1
2
3 for the *in situ* generation of the corresponding ketoacid, hydroxypyruvate, a key donor substrate
4 of Transketolase. This enzyme catalyzed the irreversible transfer of the ketol group from
5 hydroxypyruvate to an aldehyde acceptor to form a (3*S*)-ketose by stereoselective carbon-carbon
6 bond formation. The compatibility of all enzymes and substrates allowed to develop a three step
7 enzymatic process avoiding the purification of intermediates. This strategy was validated with two
8 TK aldehyde substrates to finally obtain the corresponding (3*S*)-ketoses with a high control of the
9 stereoselectivity and excellent aldehyde conversion rates.
10
11
12
13
14
15
16
17
18
19
20

21 INTRODUCTION

22
23
24
25

26 Small molecule drug candidates in the Pharmaceutical pipeline today are becoming more complex.
27 The increasing complexity requires ever more sophisticated and selective processes, especially in
28 the realm of asymmetric synthesis. The mild neutral conditions coupled with the high specificity
29 and low relative cost inherent with the use of enzymes has led to an emergence of the use of
30 biocatalysts in the synthesis of bulk chemicals and drug compounds.
31
32
33
34
35
36
37

38 Like chemocatalysts, biocatalysts are designed to perform highly selective and specific chemistry,
39 but can be comprised of engineered enzymes. They have the advantage of being heavy-metal free,
40 and thus do not require the removal of metal impurities after completion of a reaction. Consistent
41 with the homochirality of biomolecules, the reactions catalyzed by enzymes generally exhibit high
42 stereo- and regioselectivities, generating products highly enriched for single enantiomers.
43 Additionally, biocatalysts are highly efficient, allowing catalysis to be performed under mild
44 reaction conditions, with reduced energy consumption and greenhouse gas emissions in
45 comparison to chemical catalysis.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 In addition to being used to replace chemocatalysts in straightforward asymmetric reactions,
4 biocatalysis can sometimes be used to complete chiral transformations with significantly fewer
5 steps. One such example with molecules harboring more than one of the same type of functional
6 group: while chemocatalysis is often not selective for one specific of those group (thus requiring
7 protection/deprotection steps), enzymes have the capability to selectively react with a single
8 functional groups in a molecule, reducing the overall process steps, time and cost. There are also
9 cases where enzymes are able to achieve transformations that cannot be performed effectively or
10 at all using classical chemical methods or chemocatalysis. In many cases, biocatalysis can mediate
11 complex transformations that generate complex intermediates or APIs with multiple chiral centers.
12 Here, biocatalysis affords access to novel structures and can potentially have significant impacts
13 on manufacturing time and cost.
14
15
16
17
18
19
20
21
22
23
24
25
26
27

28 Biocatalysts are extensively used in the industrial production of bulk chemicals and
29 pharmaceuticals, and over 300 processes have already been implemented¹. Moreover, in the past
30 10 years, the application of biocatalysis in API synthesis has become a method of choice in many
31 industrial processes². Initially the focus was on the application of hydrolytic enzymes (e.g., lipases,
32 proteases, esterases) and biocatalysts for asymmetric reduction of ketones (e.g., alcohol
33 dehydrogenases, ketoreductases). Recently the scope of biocatalysis has broadened significantly
34 with new reaction platforms coming on stream³, including transaminases, oxidases, nitrilases,
35 aldolases, and ammonia lyases. Increasingly biocatalysts are available commercially “off-the-
36 shelf” in forms that are readily adaptable to synthetic organic chemistry operations, which enable
37 chemists to rapidly identify applications of enzymes using specific target intermediates of interest.
38 To aid synthetic chemists in identifying where biocatalysts might be usefully applied, a recent
39 publication highlighted the need for rules and guidelines⁴ for “biocatalytic retrosynthesis”. The
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

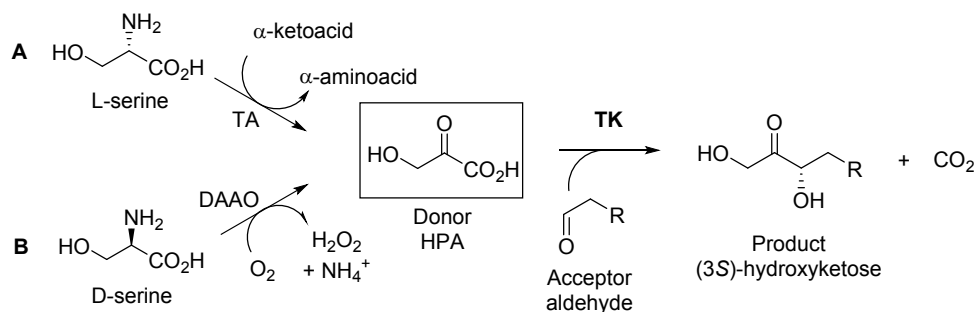
1
2
3 main objective of “biocatalytic retrosynthesis” is to encourage synthetic organic chemists to
4 include consideration of biocatalytic step(s) when planning a synthetic route to a target molecule.
5

6
7 This new approach will require a greater understanding by synthetic chemists regarding which
8 possible transformations and suitable intermediates might be considered using biocatalysis, and
9 importantly how they might impact the overall efficiency, sustainability, and safety of the final
10 manufacturing process.
11

12
13 Approaches in synthetic route design, leading to some key building blocks within competitive
14 process synthesis is essential for efficient use in mainly complex molecules synthesis. Moreover,
15 enzymatic strategies have emerged as powerful approaches to the eco-friendly, highly selective
16 synthesis of valuable chiral compounds. To compete with the productivity of traditional methods,
17 the use of two or even more enzymes in cascade can considerably improve the efficiency of a
18 multistage synthesis by obviating the isolation of intermediates, thus saving time, resources,
19 reagents and energy, while reducing waste⁶⁻⁸. The best option is when cascade reactions can be
20 performed using a one-pot strategy: to avoid limitations, such as substrate/product/reagent
21 inhibition or incompatibility of reaction conditions (pH, temperature), a telescoped, sequential
22 one-pot procedure can be used.⁶
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 In the area of chiral polyols, particularly monosaccharides and analogs, largely used in API,
42 enzymatic reactions are particularly useful to generate asymmetric centers with high
43 stereoselectivity, avoiding protection and deprotection steps required by the chemical ways. Beside
44 enzymatic aldose-ketose isomerization,⁹ enzymes catalyzing a stereospecific C-C bond formation
45 such as aldolases¹⁹ and transketolases are also popular.¹¹ Transketolase, TK (EC 2.2.1.1),
46 belonging to the thiamine diphosphate-dependent enzyme family, catalyzes a two-carbon chain
47 elongation by transferring an α -hydroxy carbonyl (ketol) group from a donor to an aldehyde
48
49
50
51
52
53
54
55
56
57
58
59
60

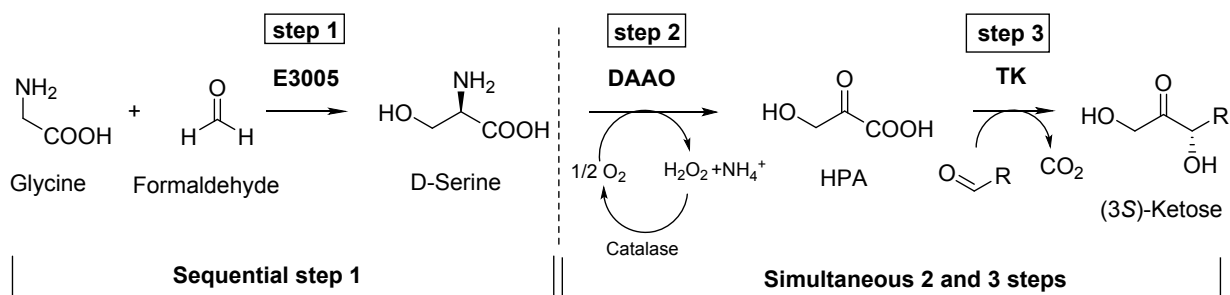
1
2
3 acceptor, thereby producing the C_{n+2} (3*S*)-ketose by the stereoselective formation of the C2-C3
4 bond. (Scheme 1) In nature, TK is involved in the pentose phosphate metabolic pathway where
5 this enzyme transfers a ketol group to reversibly equilibrate ketoses and aldoses carrying a terminal
6 phosphate ester group. Previous *in vitro* studies showed that non-phosphorylated substrates can
7 also be used as TK substrates leading to the corresponding free ketoses in one step. Particularly,
8 release of carbon dioxide from hydroxypyruvate (HPA) as donor confers a major advantage on the
9 TK-catalyzed reaction by rendering the reaction irreversible, thus making TK a powerful tool for
10 the asymmetric synthesis of ketoses and related acyloin compounds (Scheme 1).¹¹
11
12
13
14
15
16
17
18
19
20
21



22
23
24
25
26
27
28
29
30
31
32
Scheme 1: *In situ* generation of HPA from TA or DAAO-catalyzed reaction coupled with TK-catalyzed reaction.

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
The main problem of the TK-catalyzed reaction for synthetic purposes conducted at industrial-scale, is the HPA synthesis, which is commonly obtained by hydrolysis of toxic bromopyruvic acid in the presence lithium hydroxide, leading to a moderate yield.¹² This compound is also commercially available but highly expensive. In addition, HPA is unstable in water at temperature > 25°C.¹³ To circumvent all these limitations, enzymatic *in situ* generation of HPA has been previously described, in particular from L-serine using transaminases (TA)¹⁴ and more recently, from D-serine using D-amino acid oxidase (Scheme 1).¹⁵ This last way offers major advantages

compared to TA strategy, such as an irreversible reaction and no requirement of an additional substrate.



Scheme 2: One pot three-steps enzymatic cascade catalyzed by a crude extract of *E. coli* strain E3005 and purified D-amino acid oxidase from *Rhodotorula gracilis* (DAAO_{Rg}) and transketolase from *Geobacillus stearothermophilus* (TK_{gst}) with three different TK aldehyde substrates glycolaldehyde (R = -CH₂OH), D-glyceraldehyde (R = -CHOH(R)-CH₂OH) and D-erythrose (R = -CHOH(R)-CHOH(R)-CH₂OH).

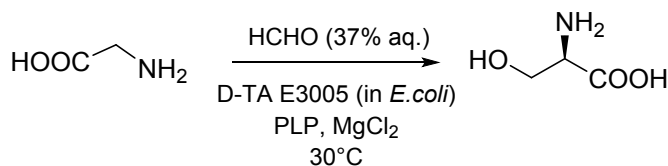
In this work, we first carried out a detailed investigation of D-serine production at industrial scale catalyzed by an enzyme from the D-Threonine Aldolases family (D-TA) overexpressed in *Escherichia coli* strain. This process was then applied in a sequential cascade enzymatic concept for the *in situ* generation of HPA from D-serine, allowing the efficient synthesis of two valuable (3S)-ketoses (Scheme 2). In a first enzymatic step, D-serine, was produced from achiral compounds glycine and formaldehyde according a process developed at industrial scale by Sequens Company. Then, two simultaneous enzymatic steps were developed at laboratory scale. DAAO from *Rhodotorula gracilis* (DAAO_{Rg}) transformed D-serine into the corresponding ketoacid, hydroxypyruvate¹⁶ used by TK from *Geobacillus stearothermophilus* (TK_{gst})¹³ in the presence of an aldehyde as acceptor for obtaining the ketose product. The one pot three steps process was validated using three TK aldehyde acceptors, glycolaldehyde, D-glyceraldehyde and

D-erythrose for the synthesis of corresponding valuable (3*S*)-ketoses, L-erythrulose, a tanning agent in the cosmetics industry,¹⁷ and D-fructose, a sweetener with very low glycemic index.¹⁸

RESULTS AND DISCUSSION

D-serine Enzymatic Process Development

In the one pot three steps cascade enzymatic concept, the first step was performed independently for the production of D-serine by the *Escherichia coli* strain E3005 (whole cells) and with glycine and formaldehyde as substrates. This strategy was developed by Seqens Company and applied to the production of D-serine in an enantiomerically pure form (>99% ee) at multiton scale per year. The corresponding biocatalyst, an enzyme (E3005) from the *D-Threonine Aldolases* family (D-TA) was identified. The enzyme activity requires a cofactor generally pyridoxal-5-phosphate (PLP) and magnesium chloride (MgCl₂) to ensure the protein stability (Scheme 3).



Scheme 3: Enzymatic aldolization of glycine with formaldehyde by biocatalyst D-TA expressed in *E. coli* E3005 for selectively producing D-serine.

The reaction is carried out in water with glycine (300g.L⁻¹) and formaldehyde (37% aq.) in presence of D-TA, produced by *E. coli* E3005 (10% w/w). The pH is continuously controlled during the synthetic reaction step by adding KOH. At the end of the reaction, the pH is decreased, the reaction mixture is then concentrated followed by the elimination of the whole cells. The crude D-serine product is treated with charcoal to remove the salts, filtered to remove any cell/protein residues followed by a recrystallization step in a water/alcohol mixture. The enzyme candidate

was selected among our off-the-shelf enzymes portfolio¹⁹ which provided the required level of enantioselectivity (>99% ee). In this work, we describe the biotransformation process reactions conditions that were developed in order to ensure high performance, high productivity and robustness for multi-ton manufacturing. Among the different studied parameters, we have determined the optimal amounts of PLP, temperature, pH and glycine concentrations.

Parameters	Process Values
pH	7 ± 0,5
MgCl ₂	0.003 eq
PLP	0.05% w/w
Base	KOH 35.7%
Temperature	30°C
Conversion rate of both substrates	~90%

Table 1: Conditions for glycine and formaldehyde biotransformation into D-serine by whole cells expressing D-TA

PLP

PLP is the cofactor required for full functioning of the enzyme during the reaction: its amount is determinant for an efficient cost effect. The best results were obtained within 0.05% w/w of PLP (chart 1).

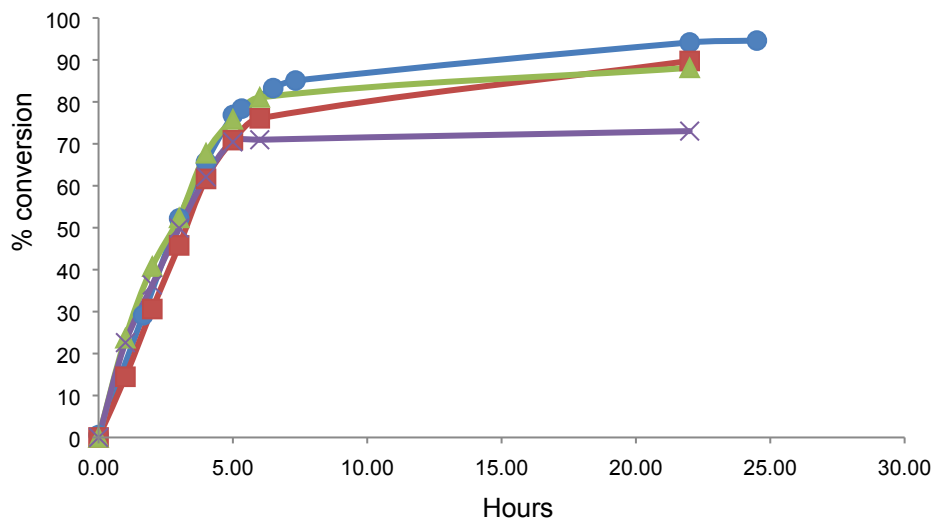


Chart 1. Influence of PLP concentration on conversion rate of glycine ● 0.3% w/w; ■ 0.1% w/w; ▲ 0.05% w/w ; X 0.01% w/w

Temperature

At temperature above 40°C, the conversion rate is rapidly limited by the degradation of formaldehyde yielding inhibition of the enzymatic activity. Furthermore, significant amount of impurities was observed between 40 and 50°C (not shown). We therefore focused the operational temperature range between 25 and 35°C: the best conversion was observed at 30°C (Chart 2).

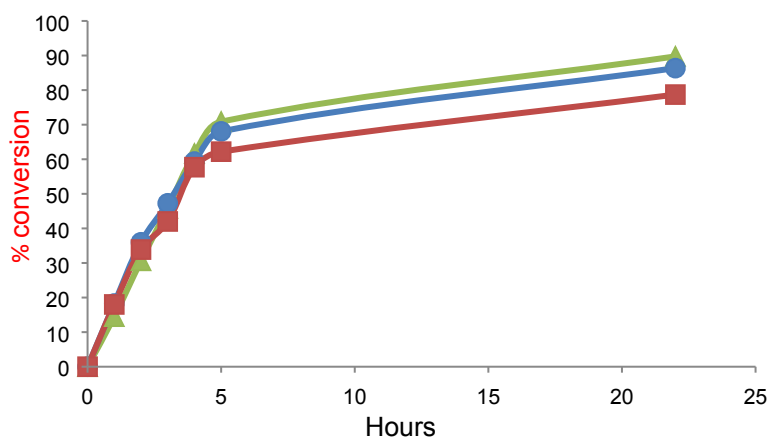


Chart 2: Influence of temperature on conversion rate of glycine ▲ 35°C; ● 30°C ; ■ 25°C.

pH optimum and its control

We have evaluated the effect of pH in the 6-8 range on the enantioselective conversion (Chart 3). The optimal activity was observed at pH 7 - 7.5. The control of this pH range allows to ensure high conversion profile with limited impact on racemization, conducting to the formation of less than 1% formation of L-serine.

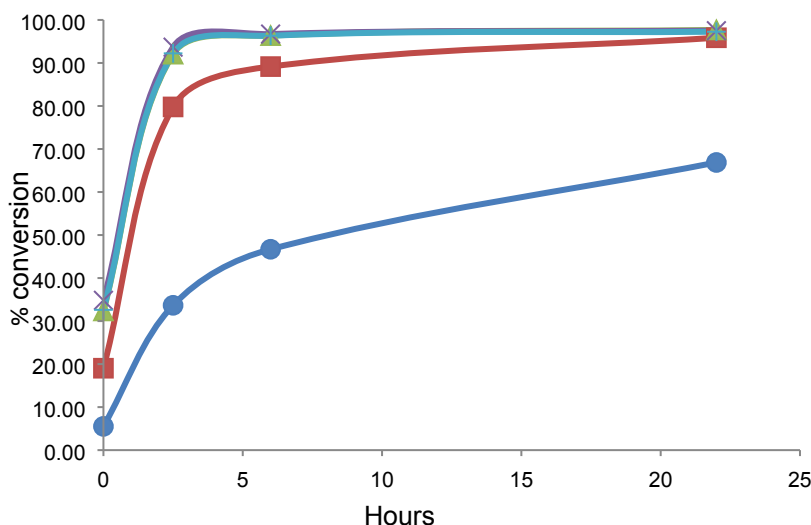


Chart 3: Influence of pH on conversion rate of glycine. ● 1B pH 6 ; ■ 2B pH 6.5 ; ▲ 3B pH 7 ; X 4B pH 7.5 ; + 5B pH 8.

Nature of the base for pH control

During the reaction, the medium tends to acidify due to HCHO addition. Among the different studied bases, NH_4OH and KOH were the best compounds to achieve full conversion into D-serine and avoiding potential racemization (Chart 4). The conversion in the presence of KOH (test F16514-05B) is significantly better, while the use of NH_4OH allows a simple distillation step at the end of reaction.

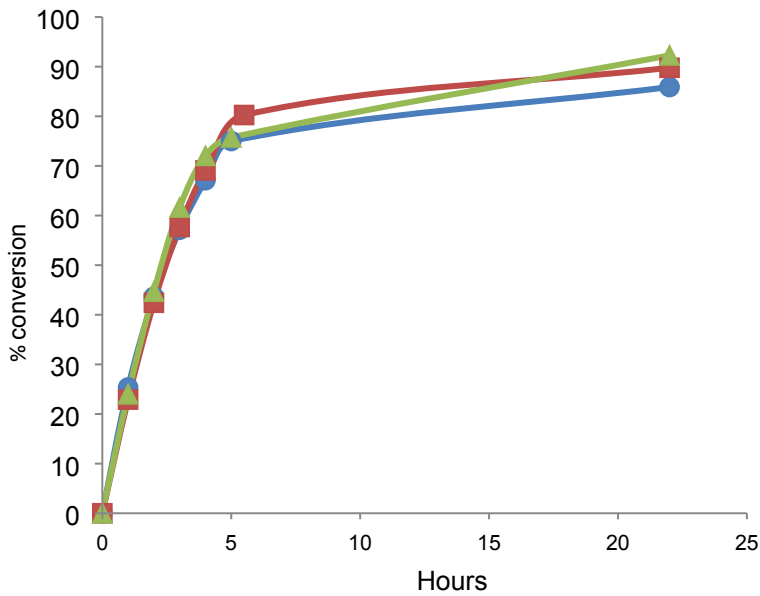


Chart 4: Influence of the nature of the base used to control pH on conversion rate of glycine. ● NH₄OH-F16514-056 ■ KOH 35.7%-F16514-58 ; ▲ KHCO₃-F16514-072.

Scale-up studies

At first, we developed the fermentation process of the D-TA enzyme overexpressed in *Escherichia coli*. To define the fermentation process, a Statistical Experimental Design has been carried out in order to determine the critical and important parameters, such as oxygen concentration, glucose feed and its optimal concentration, and other medium components impact. This approach allowed us to determine a robust domain of operations for producing the overexpressed enzyme. The Fermentation at different scales, from lab scale (2L) to several cubic meters volume fermenter size (up to 15 m³), have been implemented successively before ensuring production in large fermenters (Figure 4).

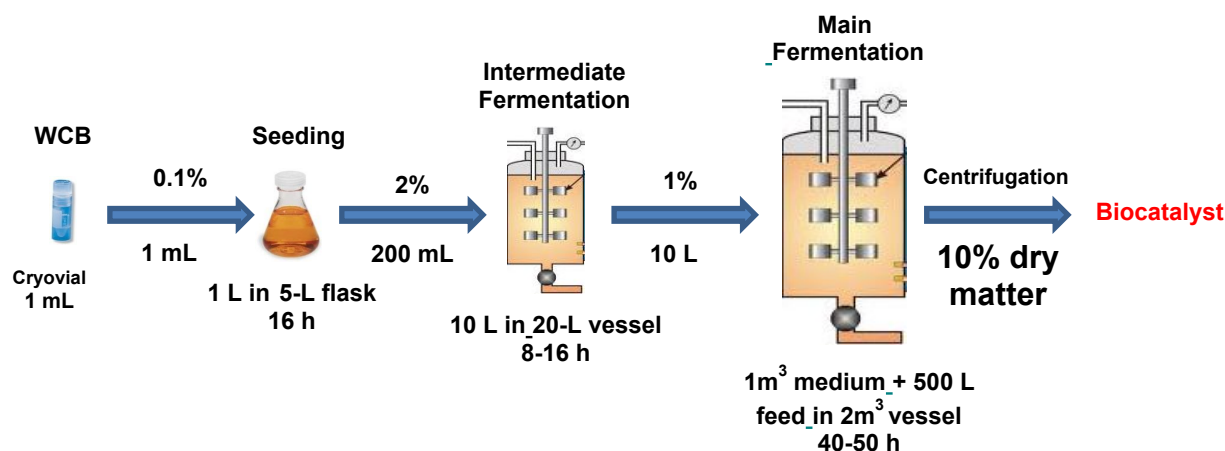


Figure 4: Different steps of fermentation process for biocatalyst production (D-TA enzyme overexpressed in *Escherichia coli*).

The obtained biocatalyst is represented by the *E. coli* E3005 wet biomass that is introduced into the biotransformation step. The corresponding heterogeneous mixture can lead to foaming issues. Indeed, control of foaming in bioprocesses at larger scale remains a dilemma, since a highly surface active heterogeneous system has to be adequately aerated and agitated while the bioreaction is proceeding without interruption. Special emphasis has been made by testing chemical antifoaming agents and to the determination of their efficiencies. The foam suppression or collapse is a result of a fine balance between the effects of various surface active agents. We have therefore investigated the efficiency at industrial scale correlating agitation turbulence and speed for ensuring full control on the conversion rate while maintaining high enantioselectivity.

Once the reaction is stopped, the mixture is directly filtrated onto a filter press combined with a filtration adjuvant, such as diatomaceous earth, in order to avoid clogging issues.

D-Serine isolation

The crude product is then recrystallized in a mixture of water and alcohol. Based on serine solubility profiles,²⁰ we were able to determine the most appropriate isolation process in respect with the zwitterionic specie, within high yield recovery (>70%). This process offers many advantages compared to the other microbial procedures²¹ such as asymmetric synthesis for full conversion of achiral, cheap and affordable substrates, no requirement of enzyme purification, high stereoselectivity of the enzyme-catalyzed reaction.

Smart Application to ketose production within Cascade Enzymatic Concept

The production of D-serine catalyzed by D-TA-E3005 cell free extract (CFE)^o was first studied in terms of substrate concentrations (Table 1).

Table 1. Influence of substrate concentrations on D-serine production.

D-TA cell free extract (mg of proteins)	Substrates(mM)		Reaction time (h) ^o	Glycine conversion rate (%) ^a
	Glycine	Formaldehyde		
3.5	50	50	24h	94
3.5	100	100	24h	82.5

^a determined by *in situ* ¹HNMR

The best results were obtained with glycine and formaldehyde at 50 mM allowing an almost complete conversion of both substrates after 24h. The D-serine quantification determined by *in situ* ¹HNMR revealed a concentration of 50mM. The reaction mixture was treated with methanol


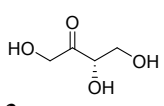
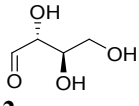
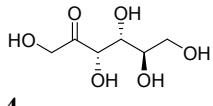
1
2
3 to precipitate CFE proteins to avoid by-products formation from substrates and reagents used in
4
5 steps 2 and 3. After filtration and methanol evaporation, D-serine was used in the second part of
6
7 the process in which both DAAO_{Rg} and TK_{gst} enzymes were coupled for the synthesis of the
8
9 ketoses.
10

11
12 DAAO_{Rg}, overexpressed in *E. coli* and purified by Ni²⁺ chelating affinity column chromatography
13
14 as reported earlier (SI),¹⁶ was used to convert D-serine into HPA. The choice of this enzyme can
15
16 be explained by its broad amino acid spectrum including D-serine while the best substrates for L-
17
18 AAOs are hydrophobic amino acids.²² In addition, DAAO_{Rg} showed much higher turnover
19
20 number, better stability under a wide range of reaction conditions, and a larger active site to
21
22 accommodate various substrates than the other source of DAAOs.^{24,23} DAAOs (EC 1.4.3.3) are
23
24 flavin adenine dinucleotide (FAD)-containing flavoenzymes that catalyze the deamination of D-
25
26 amino acids to their imino acid counterparts with concomitant reduction of FAD which is
27
28 subsequently re-oxidized by molecular oxygen with generation of hydrogen peroxide. The released
29
30 imino acid spontaneously hydrolyses to the corresponding α -keto acid and ammonia (Scheme 2).
31
32
33 The thermostable TK_{gst} was selected for its properties compatible with industrial applications such
34
35 as high stability and robustness against unusual conditions, reusability after immobilisation²⁴ and
36
37 evolvability.²⁵ TK_{gst} was overexpressed in *E. coli* and purified by Ni²⁺ chelating affinity column
38
39 chromatography as reported earlier (SI).¹³
40
41
42
43
44

45 The two-step cascade reaction catalyzed by DAAO_{Rg} and TK_{gst} was performed using D-serine
46
47 obtained with D-TA-3005 and an appropriated aldehyde as TK acceptor substrate. To validate the
48
49 strategy, two aldehydes having an increased carbon chain length were chosen, such as
50
51 glycolaldehyde C₂ (**1**), and D-erythrose C₄ (**2**) leading to the corresponding valuable C_{n+2} ketoses,
52
53 L-erythrulose C₄ (**3**), and D-fructose C₆ (**4**) respectively (Table 2).
54
55
56
57
58
59
60

The influence of different concentrations of both enzymes and TK aldehyde substrates were first studied and the best conditions are indicated in table 2.

Table 2. Conditions and results of one-pot cascade reactions for ketoses **3** and **4** synthesis from aldehydes **1** and **2** and D-serine, obtained by coupling DAAO_{Rg} and TK_{gst}

TK acceptors Aldehydes ^a	TK products Ketoses	DAAO _{Rg} /TK _{gst} (mg) ^a	Aldehyde conversion rate (%) ^b	Reaction Time (h) ^c	Isolated product yield (%)	<i>e.e.</i> or <i>d.e.</i> (%) ^d
		2/6	82	8	60%	>95%
1	3					
		2/6	>95	8	57%	>95%
2	4					

^a Reactions were carried out with DAAO_{Rg} (2 mg) and TK_{gst} (6 mg), ThDP (0.1 mM), MgCl₂ (1 mM), aldose (50 mM), D-serine (50 mM) at pH 7 and 25°C. ^b Aldehyde acceptor conversion determined by *in situ* ¹H NMR analysis. ^c ¹H NMR of reaction mixture (SI). ^d Enantiomeric excess (*e.e.*), diastereoisomeric excess (*d.e.*) determined by ¹H NMR.

For all reactions, the same concentration (50 mM) of both substrates D-serine and aldehyde **1** or **2** was used. The ratio DAAO_{Rg}/TK_{gst} was adjusted to obtain a total conversion of D-serine considering that a minimum amount of both enzymes was used and to avoid DAAO_{Rg} inhibition by HPA. Hence, the bi-enzymatic cascade allowed an almost total (82% and 95% for **1** and **2** respectively) and rapid (8h) conversion of both substrates as observed by *in situ* ¹H NMR (Table 2). Both ketoses were purified and characterized as the only products, with high enantio- and diastereoselectivity (> 95%) and obtained with good isolated yields (57-60%).

CONCLUSIONS

1
2
3
4
5 An efficient enzymatic way catalyzed by D-TA overexpressed in *Escherichia coli strain* E3005
6 was developed in whole cells for D-serine production at industrial scale. A Smart Application of
7 this D-TA catalyzed process was applied to ketose production within cascade enzymatic concept
8 involving both other enzymes, DAAO and TK. The three enzymatic steps were performed with
9 cell free extracts for the synthesis of two valuable (3*S*)-ketoses, L-erythrulose **3** and D-Fructose **4**
10 with good yields and excellent stereoselectivities. This process allowed to resolve the major
11 drawback of TK-catalyzed reaction for potential industrial applications. Indeed, HPA the key TK
12 donor substrate rendering the reaction irreversible was *in situ* generated by D-TA and then DAAO
13 from achiral glycine and formaldehyde.. This environmentally friendly procedure from affordable
14 and cheap precursors represents an interesting alternative to the conventional chemical synthesis
15 of HPA from toxic bromopyruvate and to the *in situ* HPA generation by reversible enzymatic
16 Transaminase-catalyzed reaction from L-serine, requiring an additional α -ketoacid as amino
17 acceptor and an excess of one substrate to shift the equilibrium. Since the three enzymes are active
18 under similar conditions, the multistep cascade synthesis obviated the isolation of intermediates,
19 thus allowing to save time, resources, reagents and energy, while reducing waste.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39

40 The entire process could be further optimized, especially by co-immobilization of all enzymes to
41 ensure their reusability and by performing the conversion in a continuous reactor for higher scale
42 production of ketoses. The procedure could also be extended to TK variants or to other HPA-
43 dependent enzymes in place of wild-type TK for obtaining a wide range of (3*S*)-ketoses and
44 analogs.
45
46
47
48
49
50
51
52
53

54 EXPERIMENTAL SECTION

55
56
57
58
59
60

1
2
3
4
5
6 **General.** All chemicals were purchased from Sigma-Aldrich, Alfa-Aesar and CarboSynth.
7
8 Bradford reagent was from Bio-Rad. Ni-NTA resin was obtained from QIAGEN. Proteins and
9
10 enzymes were acquired from Sigma-Aldrich. Lyophilisation was carried out with Triad
11
12 LABCONCO dryer. UV-visible absorbance was measured using a Spark control 10 microplate
13
14 reader from TECAN and an Agilent Technologies, Cary 300 UV-Vis spectrophotometer enabling
15
16 Peltier temperature control. MARCHEREY-NAGEL GmbH & Co KG 60/40-63 mesh silica gel
17
18 for Liquid Flash Chromatography and MARCHEREY-NAGEL GmbH & Co KG 60 F254 silica
19
20 gel TLC plates with anisaldehyde stain for detection were used. Reaction pH for preparative
21
22 synthesis was maintained using a TitroLine®7000 autotitrator. NMR spectra were recorded in D₂O
23
24 or DMSO on a 400MHz Bruker Avance III HD spectrometer. Chemical shifts are referenced to
25
26 the residual solvent peak. The following multiplicity abbreviations are used: (s) singlet, (d)
27
28 doublet, (t) triplet, (m) multiplet.
29
30
31
32

33 ***In situ* ¹H NMR measurements.** Progress of preparative scale enzymatic synthesis were
34
35 monitored by using quantitative *in situ* ¹H NMR relative to 3-trimethylsilyl-2,2,3,3-
36
37 tetradeuteropropionate (TSP-d₄) as internal standard. Aliquots of the reaction mixture were
38
39 removed overtime (450 μL) and mixed with 50 μL of TSP-d₄ (50 mM in D₂O).
40
41
42

43 **Procedure of D-serine synthesis catalyzed by D-TA E3005 (CFE).** To the solution containing
44
45 glycine and formaldehyde (50mM or 100mM) were added PLP (0.05 mM), MgCl₂ (10 mM) and
46
47 cell free extract of D-TA E3005 (133μL of CFE containing 3.5 mg of proteins) in H₂O. The pH
48
49 was adjusted at 7.3 with 0.1M NaOH. After 24 h, methanol (twice the reaction mixture volume)
50
51 was added. After 16 h at 4°C, the solution was filtrated and evaporated under reduced pressure. D-
52
53
54
55
56
57
58
59
60

1
2
3 serine was characterized by HPLC and ^1H NMR (SI) and was obtained as a white solid with 97%
4
5 yield.
6

7
8 **Procedure of HPA synthesis catalyzed by purified DAAO_{Rg}.** To the solution containing D-
9
10 serine (50 mM) previously produced using D-TA were added catalase (1 mg, *i.e.*1998U-4995
11
12 U/2h) and DAAO_{Rg} (2 mg, 86 U) giving a final volume of 20 mL. Oxygen was bubbled into the
13
14 reactor (10 mL · min⁻¹ at atmospheric pressure). The reaction mixture was stirred (100 rpm) at
15
16 25°C, and the pH was automatically maintained at 7 by adding HCl (0.1 M) using a pH stat.
17
18 Oxygen was bubbled into the reactor (10 mL·min⁻¹ at atmospheric pressure). The reaction was
19
20 monitored by measuring HPA appearance using the LDH assay and D-serine consumption using
21
22 the DAAO_{Rg}/LDH assay. The completion of the reaction was evidenced by *in situ* ^1H NMR.
23
24

25
26 **Procedure of simultaneous cascade synthesis catalyzed by purified TK_{gst} and DAAO_{Rg}.** ThDP
27
28 (0.1 mM), MgCl₂ (1 mM), D-serine previously produced using D-TA (50 mM) and acceptor
29
30 aldehyde (50 mM) were dissolved in H₂O and the pH was adjusted to 7 with 0.1 M NaOH. To this
31
32 stirred solution were added catalase (1 mg/2h, 1998-4995 U/2h), TK_{gst} (2mg /2,7h , 4,7 U/2,7h)
33
34 and DAAO_{Rg} (2 mg, 86 U) giving a final volume of 20 mL. Oxygen was bubbled into the reactor
35
36 (10 mL · min⁻¹ at atmospheric pressure). The reaction mixture was stirred (100 rpm) at 25°C and
37
38 the pH was automatically maintained at 7 by adding 0.1 M HCl using a pH stat. Simultaneous
39
40 cascade reactions were monitored by measuring D-serine and aldehydes consumption by *in situ* ^1H
41
42 NMR and TLC. After total conversion of D-serine and/or aldehydes (8-32 hours), silica was added
43
44 to the supernatant and the suspension was concentrated to dryness under reduced pressure before
45
46 dry loading onto a flash silica column. Products **3** and **4** were isolated by silica gel chromatography
47
48 using eluent CH₃-COO(C₂H₅)/CH₃OH (9/1) and CH₂Cl₂/CH₃OH (9:1) respectively.
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **(3S)-1, 3, 4-trihydroxy-butan-2-one 2 (L-erythrulose) 3.** Compound **3** was isolated as a yellow
4
5 oil; yield: 160 mg (44%). $[\alpha]_{\text{D}}^{25} = +6.3$ (c 1.48, H₂O), lit. $[\alpha]_{\text{D}}^{25} = +6.3$ (c 1.1, H₂O),²⁸ $[\alpha]_{\text{D}}^{25} = +6.7$
6
7 (c 1.48, H₂O).²⁹ NMR spectra were identical to those previously described.²⁸ ¹H NMR (400 MHz,
8
9 MeOD): d=4.52 (d, J=19.3 Hz, 1H, 1-HA), 4.45 (d, J=19.3 Hz, 1H, 1-HB), 4.26 (t, J=4.2 Hz, 1H,
10
11 3-H), 3.80 (dd, J=11.6, 4.5 Hz, 1H, 4-H), 3.76 (dd, J=11.6, 4.0 Hz, 1H, 4-H); ¹³C NMR (101 MHz,
12
13 MeOD): d=212.98 (C-2), 77.82 (C-3), 67.75 (C-1), 64.86 (C-4). m/z HRMS found [M+HCOO]⁻
14
15 165.0405, C₅H₉O₆ requires 165.0396.
16
17
18

19 **(3S,4R,5R)-1,3,4,5,6-pentahydroxyhexan-2-one (D-fructose) 4.** Compound **4** was isolated as
20
21 colorless oil (118 mg, 65% yield). NMR data were identical to those previously described
22
23 (ratio :73/22/5; lit. Ratio : 75/21/4).^{30,31} ¹H NMR (400 MHz, D₂O) : δ (ppm) β-D-fructo-2,6-
24
25 pyranose 4.09-4.04 (m, 1H, H6a); 4.04-4.02 (m, 1H, H5); 3.93 (dd, J=10.0Hz, J=3.5Hz, 1H, H4);
26
27 3.83 (d, J=10.0Hz, 1H, H3); 3.76-3.72 (m, 2H, H6b, H1a); 3.60 (d, J=11.7Hz, 1H, H1b), β-D-
28
29 fructo-2,5-furanose 4.17-4.13 (m, 2H, H3, H4); 3.84 (m, 1H, H5); 3.83 (m, 1H, H6a); 3.69 (m, 1H,
30
31 H6b); 3.63 (d, J=12.1Hz, 1H, H1a); 3.58 (d, J=12.1Hz, 1H, H1b), α-D-fructo-2,5-furanose 4.17-
32
33 4.13 (m, 2H, H3, H5); 4.05-4.01 (m, 1H, H4); 3.86-3.82 (m, 1H, H6a); 3.74-3.70 (m, 1H, H6b);
34
35 3.72-3.68 (m, 2H, H1a/b). ¹³C NMR (101 MHz, D₂O): δ (ppm) β-D-fructo-2,6-pyranose 99.12
36
37 (C2); 70.72 (C4); 70.25 (C5); 68.61 (C3); 64.94 (C1); 64.42 (C6), β-D-fructo-2,5-furanose 102.54
38
39 (C2); 81.72 (C5); 76.44 (C3); 75.49 (C4); 63.74 (C1); 63.43 (C6), α-D-fructo-2,5-furanose 105.49
40
41 (C2); 83.0 (C3); 82.37 (C5); 77.09 (C4); 63.96 (C1); 62.15 (C6). m/z HRMS found [M+Cl]⁻
42
43 215.0317, C₆H₁₂O₆Cl requires 215.0321.
44
45
46
47
48
49
50
51

52 ASSOCIATED CONTENT
53
54

55 **Supporting Information.**
56
57
58
59
60

1
2
3
4 Production and activity determination of DAAO_{Rg} and TK_{gst}, ¹H NMR, ¹³C NMR spectra of
5
6 ketoses **3** and **4**.
7
8
9

10 AUTHOR INFORMATION

11 **Corresponding Authors**

12
13
14
15 * Laurence.hecquet@uca.fr

16
17 * Juliette.martin@seqens.com

18
19 * Pascal.auffray@seqens.com

20
21 * Jerome.collin@seqens.com
22
23
24

25 ACKNOWLEDGMENTS

26 **Funding Sources**

27
28
29 *This work was funded by the Fonds Régional Innovation Laboratoire (grant DOS00494484/00, to*
30
31 *L.H.).*
32
33
34
35
36

37 REFERENCES

- 38
39
40
41 1. a) M. Adamczak,.; K. H. Sajja, *Food Technol. Biotechnol.* **2004**, *42*, 251–264. b) R. K.
42 Singh, M. K. Tiwari, R. Singh, J.-K. Lee, *Int. J. Mol. Sci.* **2013**, *14*,1232-1277.
43
44 2 R. N.Patel, Ed. *Biocatalysis in the Paharmaceutical and Biotechnology Industries*, CRC
45 Press, 2010.
46
47 3 A. S. Wells,J. W. Wong, P. C. Michels, D. A. Entwistle, K. Fandrick, G. L. Finch, A.
48 Goswami,H. Lee, S. Mix, T. S. Moody, L. Pang, R. K. Sato, N. J. Turner, T. J. Watson,
49 *Org. Process Res. Dev.* 2016, *20*, 594-601.
50
51 4 N. J. Turner E. O'Reilly *Nat. Chem. Biol.*, 2013, *9*, 285-288
52
53 5 R. A. Sheldon, J. M. Woodley *Chem. Rev.*, 2018, *118*, 801–838.
54
55
56
57
58
59
60

- 1
2
3 6 J. H. Schrittwieser, S. Velikogne, M. Hall W. Kroutil, *Chem. Rev.*, 2018, 118, 270–348.
4
5 7 J. M. Sperl, V. Sieber, *ACS Catal.*, 2018, 8, 2385–2396.
6
7 8 S. Wu, Z. Li, *ChemCatChem*, 2017, 10, 2164–2178.
8
9 9 a) Z. Li, Y. Gao, H. Nakanishi, X. Gao, L. Cai, *J. Org Chem.* 2013, 9, 2434–2445. b) K.
10 Izumori, *J. Biotechnol.*, 2006, 124, 717–722 ; c) L. Wen, K. Huang, M. Wei, J. Meisner,
11 Y. Liu, K. Garner, L. Zang, X. Wang, X. Li, J. Fang, H. Zhang, P.G. Wang, *Angew. Chem.*
12 *Int. Ed. Engl.*, 2015, 54, 12654-12658
13
14 15 a) J. Li, J. Yang, Y. Men, Y. Zeng, Y. Zhu, C. Dong, Y. Sun, Y. Ma, *Appl Microbiol.*
16 *Biotechnol.*, 2015, 99, 7963-7972 ; b) S. M. Dean, W. A. Greenberg, C. H. Wong, *Adv. Synth.*
17 *Catal.*, 2007, 349, 1308-1320 ; c) P. Clapes, W.-D. Fessner, G. Sprenger, A. K. Samland,
18 *Curr. Opin. Chem. Biol.*, 2010, 14, 154-167 ; c) A. Li, L. Cai, Z. Chen, M. Wang, N. Wang,
19 H. Nakanishi, X. D. Gao, Z. Li, *Carbohydr. Res.*, 2017, 452, 108-115.
20
21 22 a) Y. Kobori, D. C. Myles, G. M. Whitesides, *J. Org. Chem.*, 1992, 57, 5899-5907. b) T.
23 Ziegler, A. Straub, F. Effenberger, *Angew. Chem. Int. Ed. Engl.*, 1988, 100, 737–738. c) L.
24 Hecquet, J. Bolte, C. Demuynck, *Tetrahedron*, 1996, 52, 8223–8232. d) F. T. Zimmermann,
25 A. Schneider, U. Schörken, G. A. Sprenger, W.-D. Fessner, 1999, 10, 1643-1646. e) N. J.
26 Turner, *Curr. Opin. Biotechnol.*, 2000, 11, 527–531 f) F. Charmantray, V. Helaine, B.
27 Legeret, L. Hecquet, *J. Mol. Catal. B: Enzym.*, 2009, 57, 6-9.
28
29 30 F. Dickens, D. H. Williamson. *Biochem. J.* 1958, 68, 74-81.
31
32 33 J. Abdoul Zabar, I. Sorel, V. Hélaine, F. Charmantray, T. Devamani, D. Yi V. de Berardinis,
34 D. Louis, P., Marlière, W.D. Fessner, L. Hecquet, *Adv. Synth. Catal.*, 2013, 355, 116-128.
35
36 37 a) L. Hecquet, J. Bolte and C. Demuynck. *Enzymatic. Tetrahedron*, 1996, 52, 8223-823. b)
38 M. Lorillière, M. De Sousa, F. Bruna, E. Heuson, F. Gefflaut, V de Berardinis, T. Saravanan,
39 D. Yi, W.-D. Fessner, F. Charmantray, L. Hecquet, *Green Chem.*, 2017, 19, 425-435. c) F.
40 Subrizi, M. Cárdenas-Fernández, G. J. Lye, J. M. Ward, P. A. Dalby, T. D. Sheppard, H. C.
41 Hailes, *Green Chem.*, 2016, 18, 3158-3165. d) M. Bawn, F. Subrizi, G. J. Lye, T. D.
42 Sheppard, H. C. Hailes, J. M. Ward. *Enzyme Microb. Technol.*, 2018, 116, 16-22.
43
44 45 M. L'enfant, F. Bruna, M. Lorillière, N. Ocal, W.-D. Fessner, L. Pollegioni, F. Charmantray,
46 L. Hecquet. *Adv. Synth. Catal.*, 2019, 361, 2550-2558.
47
48 49 a) L. Pollegioni, A. Falbo, M. S. Pilone, *Biochim. Biophys. Acta*, 1992, 120, 1-16. b) S.
50 Fantinato, L. Pollegioni., S. M. Pilone, *Enz. Microb. Technol.*, 2001, 29, 407–412.
51
52
53
54
55
56
57
58
59
60

- 1
2
3 17 K. Jung, M. Seifert, T. Herrling, J. Fuchs, *Spectrochim. Acta A Mol Biomol. Spectrosc.*,
4 2008, 69, 1423–1428.
5
6 18 H. D. Belitz, W. Grosch, P. Schieberle, in *Food Chemistry*, **2009**, 248-270, "Hiedelber":
7 Springer-Verlag.
8
9 19 Protéus by Seqens has a collection of >300 off-the-shelf enzymes
10
11 20 H. Charmolue, R. W. Rousseau, *AIChE Journal*, 1991, 37, 1121-1128
12
13 21 H. Ikeda, Y. Yonetani, S. I. Hashimoto, Process for producing D-serine, US 7,186,532 B2,
14 Kyowa Hakko Kogyo Co Ltd.
15
16 22 a) L.Pollegioni, G. Molla, S.Sacchi, E. Rosini, R. Verga, M. S. Pilone, *Appl. Microbiol.*
17 *Biotechnol.*, 2008, 78, 1–16 ; b) L. Pollegioni, P. Motta, G. Molla, *Appl. Micr. Biotech.*,
18 2013, 97, 9323-9341.
19
20 23 a) L.Pollegioni, G. Molla, S.Sacchi, E. Rosini, R. Verga, M. S. Pilone, *Appl. Microbiol.*
21 *Biotechnol.* 2008, 78, 1–16 ; b) L. Pollegioni, P. Motta, G. Molla, *Appl. Micr. Biotech.* 2013,
22 97, 9323-9341. b) L. Pollegioni, K. Diederichs, G. Molla, S. Umhau, W. Welte, S. Ghisla,
23 M. S. Pilone, *J. Mol. Biol.* 2002, 324, 535–546. c) L Pollegioni, S Iametti, D Fessas, L
24 Caldinelli, L Piubelli, A Barbiroli, M.S. Pilone, F. Bonomi, *Protein Sci.* 2003, 12, 1018-
25 1029.
26
27 24 G. Ali, T. Moreau, C. Forano, C. Mousty, V. Prevot, F. Charmantray, L. Hecquet,
28 *ChemCatChem*, 2015, 7, 3163–3170
29
30 25 a) J. Abdoul Zabar, M. Lorillière, D. Yi, L. Nauton, F. Charmantray, V. Hélaine, W.-D.
31 Fessner, L. Hecquet, *Adv. Syn. Catal.* 2015, 357, 1715-1720 ;. b) D. Yi, T. Saravanan, T.
32 Devamani, F. Charmantray, L. Hecquet and W.-D. Fessner, *Chem. Commun.* 2015, 51, 480-
33 483; c) M. Lorillière, M. De Sousa, F. Bruna, E. Heuson, T. Gefflaut, V. de Berardinis, T.
34 Saravanan, D. Yi, W.-D. Fessner, F. Charmantray, L. Hecquet, *Green Chem.* 2017, 19, 425
35 – 435 ; d) T. Saravanan, S. Junker, M. Kickstein, J. Hegen, S. Hein, M. K. Link, S. Witt, M.
36 Lorillière, L. Hecquet, W.-D. Fessner, *Angew. Chem. Int. Ed.*, 2017, 56, 5358-5362. e) M.
37 Lorillière, R. Dumoulin, M.L'Enfant, A. Rambourdin, V; They, L. Nauton, W.-D. Fessner,
38 F. Charmantray, L. Hecquet, *ACS Catalysis*, 2019, 9, 4754-4763.
39
40 26 U.K. Laemmli, *Nature*, 1970, 227, 680-685.
41
42 27 L. Hecquet, J. Bolte, and C. Demuynck, *Biosci. Biotech. Biochem.*, 1993, 57 , 2174-2176.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 28 K. Benaissi, V. H elaine, V. Pr evot, C. Forano, L. Hecquet, *Adv. Syn. Catal.*, 2011, 353,
4 1497–1509.
5
6 29 M. E. B. Smith, E. G Hibbert, A. B. Jones, P. A. Dalby, H. C. Hailes, *Adv. Syn. Catal.* 2008,
7 350, 2631–2638.
8
9
10 30 W. Funcke, *Carbohydr. Res.* **1979**, 75, 305–309.
11
12 31 T. Barclay, M. Ginic-Markovic, M. R. Johnston, P. Cooper and N. Petrovsky, *Carbohydr.*
13 *Res.* 2012, 347, 136–141.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60