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Active Solutions for Health


# Enzyme production for biocatalysis application: Recombinant Transaminase Case Study

## From miniaturized scale to pilot plant (10m$^3$): the Brindisi development approach

**Biotechnology Platform** - R&D Brindisi - January 2024

# ABSTRACT

This white paper describes the case study of recombinant transaminase production process development, from enzyme screening through miniaturized system to final scale-up in pilot plant (10m$^3$), performed by **EUROAPI Brindisi R&D division**.

The whole transaminase case study developed by R&D Brindisi department starts with miniaturized screening of *E. coli* strains expressing different recombinant transaminases. This step, aimed to select the enzyme with highest bioconversion activities of native and industrial substrate, was performed in microtiter plate (working volume of **0.5-1 mL**), supported by automated system for screening.

Then, enzyme production process was developed at **1L scale,** using a parallel bioreactor system, testing different process parameters to define optimal enzyme expression conditions, and then transferred at **20L** lab fermenter scale. Process was successfully scaled-up and the transaminase was produced at **10m$^3$** scale pilot plant.

**Downstream** process for active enzyme recovery was performed with lab-scale equipment from **10m$^3$** fermentation broth.

# 1 BACKGROUND

Brindisi site, part of EUROAPI Group, is historically engaged in manufacturing of active ingredients from fermentation processes, particularly antibiotics and glucocorticoids (Fig. 1). The site has recently expanded its range of services in the areas of research, development, and manufacturing, in line with the Contract and Development Manufacturing Organization (CDMO) Group footprint and strategy.


**Fig. 1 Brindisi Plant (Italy), EUROAPI Group**

New market demands and the vision from the entire EUROAPI Group have driven the Brindisi site and its R&D department (Fig. 2) to expand the range of services and products of interest over the past years. Leveraging on the site historical expertise, new technologies have been implemented to translate the consolidated experience in the field of fermentation for antibiotic production and new fermentation processes, into the area of precision fermentation to obtain new classes of products of industrial and pharmaceutical interest, including Active Pharmaceutical Ingredients (APIs), intermediates, recombinant products and other molecules, opening the doors to biotechnology products of newer generation compared to the Group's historical activities. Indeed, R&D activities are currently focused not only on the more classic production of antibiotics, but also on the set-up, improvement and scale-up of production processes, from fermentation to purification, related to biological products, APIs, or other pharmaceutical intermediates.

**Fig. 2 R&D Department, Brindisi (Italy), EUROAPI Group**

The transaminase case-study presented in this white paper, describing the development approach of **recombinant enzyme for conversion of chemical compounds**, is perfectly in line with the strategy just described.

# 2 RECOMBINANT ENZYMES FOR INDUSTRIAL AND PHARMACEUTICAL APPLICATIONS

Enzymes are defined as specific proteins that act as biological catalyst by accelerating chemical reactions. In a typical enzymatic reaction (Fig. 3), substrates are converted in different products through enzyme catalytic activity. Due to their high selectivity, enzymes are widely used for industrial and pharmaceutical biocatalysis applications, catalysing varieties of reactions such as hydrolysis, oxidation–reduction, group transfers, isomerization, bond cleavage, ligation and bond formation. The increased interest in the production of chiral intermediates and green synthetic processes in pharmaceutical environment are currently rising interest in the application of enzymatic biocatalysis in these fields. As described in the "Global Industrial Enzymes Market by Product (Carbohydrases, Lipases, Nucleic Acid Enzymes), Source (Animals, Micro-Organisms, Plants), Application - Forecast 2023-2030" report, the global industrial enzyme market was estimated at a substantial USD 6.94 billion in 2022 and is expected to expand to USD 11.34 billion by 2030.

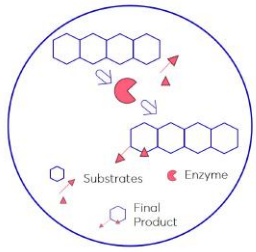


**Fig. 3 Graphic representation of enzymatic reaction**

Several examples of **biocatalysis** applications are currently available in different industrial fields, from food, agrochemicals and feed to biofuel, biochemical and biopolymer production. **Biocatalysis approach in pharmaceutical processes can find application in different steps of API manufacturing** such as group protection and deprotection, selective acylation and deacylation, resolution of racemic mixtures, esterification, transesterification, and other reactions.

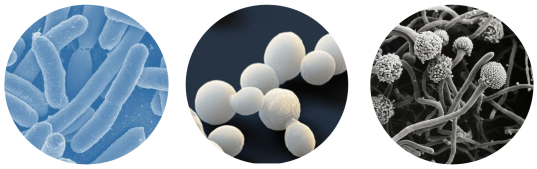

**Fig. 4 Microscopic observation of different microorganisms** ***E. coli, P.pastoris, A. fumigatus***

Industrial enzymes, including those used in pharmaceutical industries, are produced through fermentation of selected microbial strains, mainly **bacteria and fungi (both yeast and filamentous fungi) (Fig. 4)**. These enzymes are secreted by the producing microbial cells in the fermentation medium or accumulated in the intracellular environment and then recovered through the downstream process as crude extract or pure enzyme according to final application and purity grade requirements. Industrial enzymes may be used as isolated free forms or immobilized forms (as whole cells or isolated enzyme immobilized on a suitable support).

Microbial strains commonly used for fermentative enzyme production (Fig. 4) can be divided in microorganisms that naturally produce certain class of enzymes, such as cellulase production from filamentous fungi (e.g., *Trichoderma sp., Aspergillus sp.*), and recombinant microorganisms, including all the strains (e.g., *Escherichia coli, Pichia pastoris*) that are genetically modified with the technology of recombinant DNA to produce, ideally with high productivity, selected enzymes specifically encoded by external DNA sequences (cloned in the host strain as insertion in the genomic DNA or as extra-genomic plasmid).

# 3 RECOMBINANT TRANSAMINASE: STATE OF ART AND INDUSTRIAL APPLICATIONS

Transaminases (TAms) are class of enzymes used for production of chiral amines, valuable as building blocks, for the pharmaceutical and fine chemical industries. Compared to conventional chemical synthesis of chiral amines, TAms show different advantages including excellent stereoselectivity, possibility to perform reactions under mild conditions, replacement of often toxic transition metal catalysts as alternative technology, and reduction of the use of volatile organic solvents in chemical manufacturing, offering a potential "green alternative"

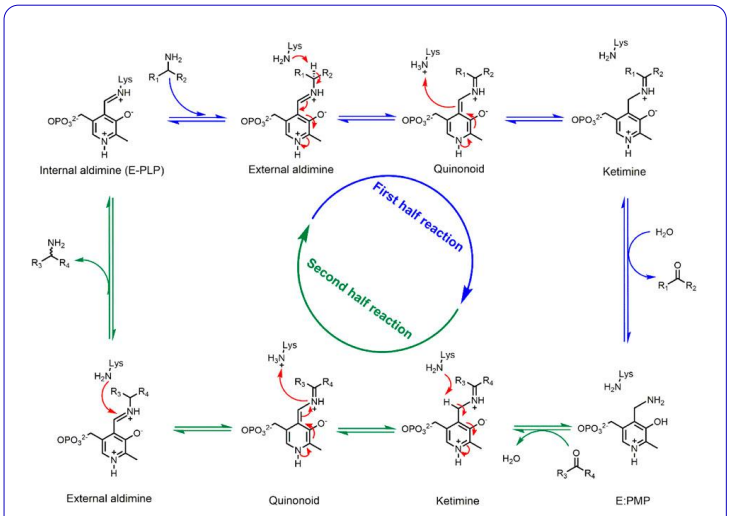


**Fig. 5 Schematic representation of TAm mechanism of action**

to overcome the drawbacks associated with conventional chemical methods. TAms are pyridoxal-5-phosphate (PLP) dependent enzymes where the cofactor PLP is fully regenerated at the end of the reaction.

Interesting industrial applications of TAms are related to biocatalytic asymmetric synthesis of chiral amines in API production, such as the TAm catalysed synthesis of the antidiabetic drug *Sitagliptin* that replace a rhodium-catalysed hydrogenation and the TAm catalysed synthesis of the chiral scaffold of *Sacubitril*, an antihypertensive drug. Typical mechanism of actions is illustrated in Fig. 5. Transaminases have been grouped into six classes according to the catalytic properties:

- L-aspartate transaminases (class I)
- L-alanine transaminases (class II)
- ω-transaminases (class III)
- D-amino acid transaminases and branched chain transaminases (class IV)
- L-serine transaminases (class V)
- Sugar transaminases (class VI)

# 4 EUROAPI APPROACH: FROM MINIATURIZED SCALE TO PILOT PLANT PRODUCTION OF RECOMBINANT TRANSAMINASE

An example of EUROAPI's approach for recombinant enzyme production for biocatalysis application is the development case-study of a recombinant transaminase identified for the bioconversion of an intermediate within the synthetic pathway of an active pharmaceutical ingredient owned by EUROAPI Group. The goal was to identify a strategy to convert a chemical synthesis step into a bioconversion step catalysed by a biotechnologically produced enzyme. This process, from enzyme variant selection to production and purification, was developed starting from a miniaturized and automated scale, to ensure efficient screening of different variants, then scaled up to laboratory scale (1L-20L) and finally transferred to pilot plant (10m$^3$). Main steps of the project are summarised in Fig. 6 and described in the paragraphs below.

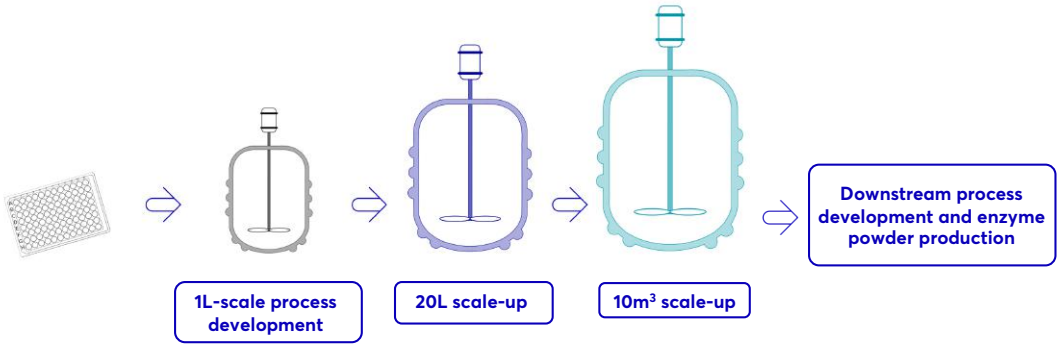


**Fig. 6 Brindisi development workflow for recombinant transaminase production process development**

## First step: Miniaturized transaminase screening

This project started from a library of recombinant *E. coli* BL21 strains expressing ten different heterologous transaminases, previously selected *in silico* for bioconversion of an industrial intermediate. Strains were screened into a miniaturized system for transaminase production and biocatalytic activity, both for conversion of native and industrial substrate. Miniaturized strain screening was performed using 96-well plate (microtiter plate, MTP) system supported by automated liquid handling platform for the seeding step. This system, available in R&D Brindisi, is composed of four different automated stations for liquid handling suitable for **high-throughput screening application** and allows to screen several strains and production conditions per time. Miniaturized **enzyme production** has been performed in batch mode with IPTG induction for gene expression.

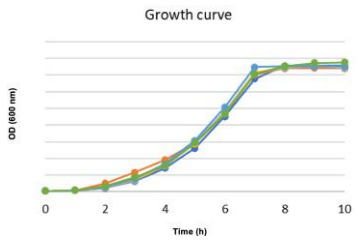


**Fig. 7 Strains growth curve in 96-well plate**

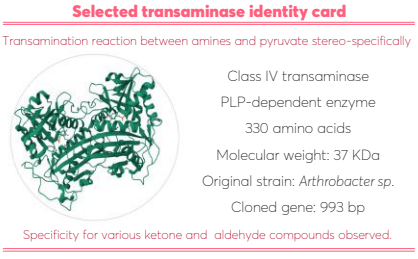


**Fig. 8 Selected transaminase key information**

After a first phase of MTP protocol optimization, ten strains were tested for enzyme expression (different strains growth curve in MTP, monitored as optical density (OD) at 600nm, is reported in Fig. 7) and produced enzymes were tested for bioconversion of pyruvate, as natural substrate, and an industrial ketone as industrial substrate. **Among the ten different transaminases**, two showed the highest activity for bioconversion of substrates in the corresponding amine form. **Only one** of these last two enzymes, **(Fig. 8) has been chosen for the next steps due to its highest bioconversion rate of both substrates**.

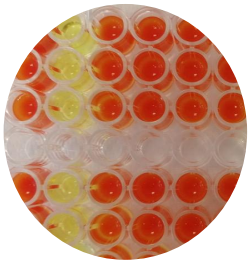

Fig. 9 Example of colorimetric bioconversion assay

Enzymatic bioconversion activity has been evaluated starting from efficient colorimetric method developed by Baud et al. for high-throughput screening of transaminases. The assay is based on the reaction of transaminase cell lysate in presence of 2-4-nitrophenyl-ethan-1-amine hydrochloride (NPE) as amine donor, an aldehyde or a ketone as amine acceptor, PLP as co-factor and potassium phosphate buffer pH 6.0 to pH 9.0. In these conditions, reaction between pyruvate, or industrials substrate and NPE, produce red-coloured product, as observed in Fig. 9. Red-coloured product formation is monitored evaluating increasing of absorbance at specific wavelength during the enzymatic reaction.

## Second step: Enzyme production process at 1L and 20L scale and downstream development

Fig. 10 Upstream development Lab equipment

The selected *E. coli* strain, expressing the transaminase identified for bioconversion of industrial substrate of interest, was used for process development at **laboratory fermenter scale**. The Upstream (USP) Lab of Brindisi R&D unit is equipped with a **1L-scale parallel bioreactor system (4 parallel vessels), 20L-scale fermenters (13), 30L-scale (2), 40L scale fermenters (2), cell-tainer, pure oxygen line supply and off-gas analyser systems** for process development and optimization (Fig. 10). Historically dedicated to fermentation processes of filamentous bacteria (e.g., *Streptomyces sp.*) for antibiotics production, USP equipments are currently also dedicated to different production processes, such as high-cell density fermentation for recombinant protein production.

Process development for selected transaminase production was performed starting from 1L parallel bioreactor system and then scaled-up at 20L fermenter scale. The optimized production process is a fed-batch high-cell density fermentation with recombinant *E. coli* strain based on process steps described below (Fig. 11).

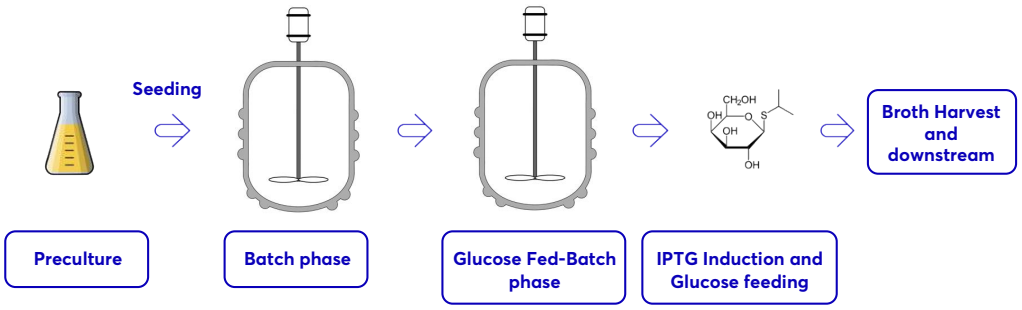


Fig. 11 Transaminase production process

Process development was performed in order to test different parameters to increase fermentation performances including:

- Fermentation medium and feeding strategy
- Process temperature
- IPTG concentration
- Harvest time

Fermentation process was monitored for cell growth profile (optical density, $OD_{600nm}$), pH trend, dissolved oxygen (DO) concentration, oxygen transfer rate (OTR) and oxygen uptake rate (OUR), glucose consumption and $CO_2$ production.
Enzyme production was evaluated at different fermentation time as total amount of protein produced per $OD_{600nm}$ unit and enzymatic activity using pyruvate as bioconversion substrate.

In parallel, a downstream process (DSP) at laboratory scale for enzyme recovery was set-up. Starting from 20L-scale fermentation harvest broth, different technologies were evaluated to define an efficient protocol for transaminase purification, keeping the same enzymatic activity. Several technologies and different process parameters were tested, including:

- Cell lysis and homogenization
- Microfiltration
- Depth filtration
- Cellular debris flocculation with different flocculation agents and conditions
- pH effect on protein precipitation
- Spray dryer vs Freeze dryer

A complete workflow for intracellular enzyme purification from *E. coli* broth was set-up as graphically described in Fig. 12.

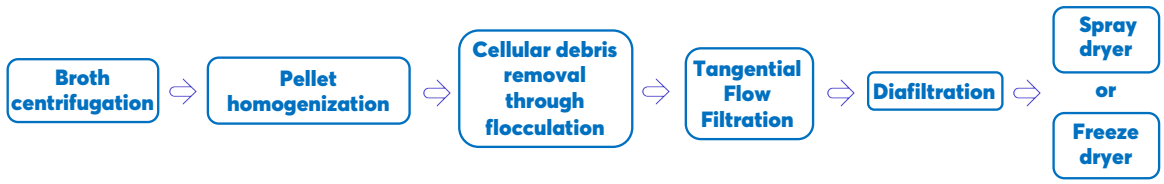


**Fig. 12 Downstream process developed for transaminase recovery**

R&D downstream laboratory is fully equipped to develop and optimize purification processes of products deriving from 20L-scale fermentation, including lab scale **ultracentrifuge, homogenizer, microfiltration and ultrafiltration system (TFF cassette), chromatographic system for protein purification (fast protein liquid chromatography, FPLC), spray dryer and freeze dryer.**

**Fig. 13 Downstream development Lab equipment**

In addition to the strong historical experience related to downstream processes for several classes of antibiotics, different technologies have been implemented, in the last few years, to build new competencies in purification of recombinant protein, enzymes and other products (e.g., oligosaccharides). Currently, the Brindisi R&D department counts not only lab-scale DSP equipment (for gram-batch size), but also pilot scale train for recombinant protein and enzyme purification, including **disc stack centrifuge, homogenizer, TFF multi-purpose system, FPLC system and freeze dryer** for pilot scale fermentation broth produced at 600L-10m$^3$ and product (Kg-batch size).

## Third step: Enzyme production process scale-up at 10m$^3$ scale

The transaminase case-study was completed with successful production process scale-up at 10m$^3$ scale. The fermentation process developed at 20L scale was transferred to pilot scale, performing fed-batch high-cell density fermentation at 10m$^3$ scale (working volume 7m$^3$). The pilot plant, equipped with 600L and 10m$^3$ non-GMP bioreactors, connected with DSP pilot equipment, is suitable for different fermentation processes, such as antibiotic production, but also recombinant protein and enzyme, as demonstrated by this case study.
In this example, fermentation process was monitored, as well as 20L scale process, for $OD_{600nm}$, DO concentration, OTR and OUR, glucose consumption and $CO_2$ production (example of some process parameters are reported in Fig. 14).

Increasing of enzyme production was monitored at different fermentation stages as total amount of protein produced per $OD_{600nm}$ unit and enzymatic activity was tested using pyruvate and industrial ketone as bioconversion substrate using the colorimetric method previously described (Baud et al.). Increasing concentration of red-coloured product is monitored as Absorbance increasing at a fixed wavelength (Fig. 15).
Pilot scale harvested broth was processed for enzyme recovery with final DSP protocol applied at lab-scale, according to the workflow previously described in Fig. 12.

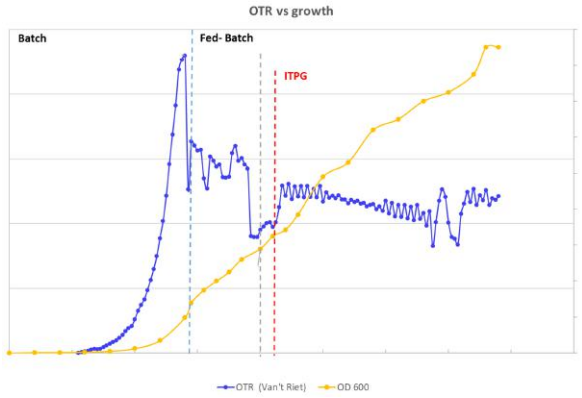


**Fig. 14 OTR vs growth trend in 10m$^3$ fermentation**

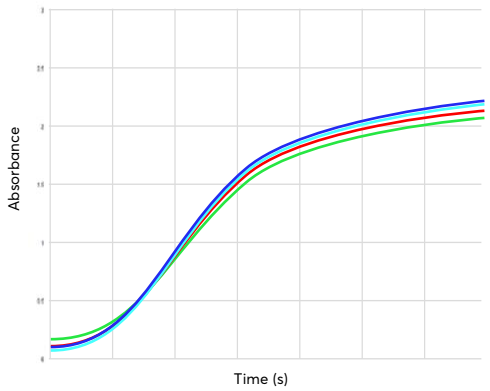


**Fig. 15 20L vs 10m$^3$ scale transaminase activity**

Finally, 10m$^3$ scaled-up process resulted comparable to 20L scale standardized process, in terms of *E. coli* growth curve profile (with final $OD_{600nm}$ reached of about 150), oxygen demand (no oxygen limitation observed), glucose consumption, enzyme expression profile (monitored as total amount of protein produced and SDS page profile) and enzymatic bioconversion rate, as shown in Fig. 15.

The defined downstream process allowed to obtain final enzyme powder with high-purity level and significant retained enzymatic activity using both spray drying and freeze drying technology.

The same process, fully scaled-up at 10m$^3$, can easily be transferred also at 600L scale.

# 5 CONCLUSION

The results described in this white paper prove the end-to-end development approach (Fig. 16) applied by EUROAPI R&D team to recombinant enzyme production. The case study presented starts with strain screening for different enzyme variants evaluation, supported by miniaturized high-throughput systems, and moves to fermentation process set-up (1L-20L-40L scale), with parallel development of lab-scale downstream process, up to scale-up studies and kilo-lab batch manufacturing at 10m$^3$ pilot plant.

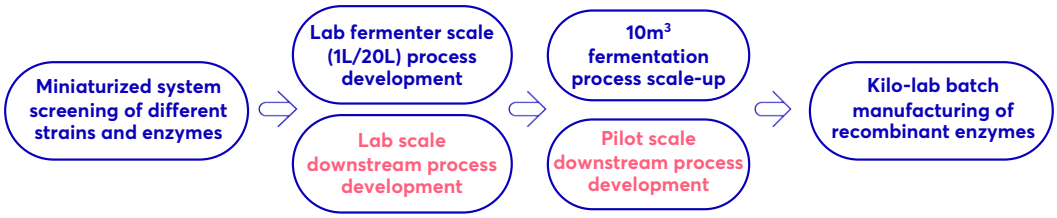


**Fig. 16 Development approach from strain selection to kilo-batch manufacturing**

The transaminase case-study demonstrates the suitability of Brindisi equipment, both at laboratory and pilot scale, for development and kilo-lab batch production of recombinant enzyme for biocatalysis application. These results allow to offer full process development train, from lab to pilot scale with non-GMP batch manufacturing up to 10m$^3$ scale, in the field of CDMO business.

**Fig. 17 Spray dryer enzyme powder obtained from 10m$^3$ fermentation broth**

# 6 ABOUT EUROAPI

EUROAPI is focused on reinventing active ingredient solutions to sustainably meet customers' and patients' needs around the world. We are a leading player in active pharmaceutical ingredients with approximately 200 products in our portfolio, offering a large span of technologies, while developing innovative molecules through our Contract Development and Manufacturing Organization (CDMO) activities.

Taking action for health by enabling access to essential therapies inspires our 3,450 people every day. With strong research and development capabilities and six manufacturing sites all located in Europe, EUROAPI ensures API manufacturing of the highest quality to supply customers in more than 80 countries. EUROAPI is listed on Euronext Paris; ISIN: FR0014008VX5; ticker: EAPI). Find out more at www.euroapi.com and follow us on LinkedIn.

# 7 REFERENCES


Puetz, J.; Wurm, F.M. Recombinant Proteins for Industrial versus Pharmaceutical Purposes: A Review of Process and Pricing. Processes 2019, 7, 476. https://doi.org/10.3390/pr7080476

Global Industrial Enzymes Market by Product (Carbohydrases, Lipases, Nucleic Acid Enzymes), Source (Animals, Micro-Organisms, Plants), Application - Forecast 2023-2030 https://www.researchandmarkets.com/reports/5715951/global-industrial-enzymes-market-by-product?gclid=EAIaIQobChMIt7G459CBggMVUZLVCh0yvAQuEAAYASAAEgL8zfD_BwE `

Tandon S et al., Therapeutic enzymes: Discoveries, production and applications; Journal of Drug Delivery Science and Technology Volume 63, June 2021, 102455, https://doi.org/10.1016/j.jddst.2021.102455

Meghwanshi et al. Enzymes for pharmaceutical and therapeutic applications; Biotechnology and Applied Biochemistry, Volume 67, 2020, https://doi.org/10.1002/bab.1919

Pyser J.B. et al., State of the Art Biocatalysis, ACS Central Science, June 2021, https://doi.org/10.1021/acscentsci.1c00273

Kinner, A. et al., Recent Advances in Biocatalysis for Drug Synthesis. Biomedicines 2022, 10, 964. https://doi.org/10.3390/biomedicines10050964

Gautam Kumar Meghwanshi et al., Enzymes for pharmaceutical and therapeutic applications. Biotechnology and Applied Biochemistry 2019 (586-601) https://doi.org/10.1002/bab.1919

Kelly, S.A., Mix, S., Moody, T.S. et al. Transaminases for industrial biocatalysis: novel enzyme discovery. Appl Microbiol Biotechnol 104, 4781–4794 (2020). https://doi.org/10.1007/s00253-020-10585-0

Savile C. K. et al., Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture, Vol 329, Issue 5989, June 2010, DOI: 10.1126/science.1188934

Eliot A. C., and Kirsch J. F., Pyridoxal Phosphate Enzymes: Mechanistic, Structural, and Evolutionary Considerations, Annual Review of Biochemistry, 2004, https://doi.org/10.1146/annurev.biochem.73.011303.074021

Stephen A. Kelly, Stefan Pohle, Scott Wharry, Stefan Mix, Christopher C.R. Allen, Thomas S. Moody, and Brendan F. Gilmore, Application of ω-Transaminases in the Pharmaceutical Industry Chemical Reviews 2018 118 (1), 349-367, 10.1021/acs.chemrev.7b00437

Meng et al., Protein engineering of amine transaminases Front. Catal., 22 November 2022, Sec. Biocatalysis, Volume 2 - 2022 | https://doi.org/10.3389/fctls.2022.1049179

Baud et al., A rapid, sensitive colorimetric assay for the high-throughput screening of transaminases in liquid or solid-phase. Chem. Commun., 2015, 51, 17225 DOI: 10.1039/c5cc06817g