An Overview on Biocatalysts and It's Applications

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Abstract **- Biocatalysis underpins some of the oldest chemical transformations known to humans. Biocatalysis is the use of natural catalysts, such as protein enzymes to perform chemical transformations on organic compounds. Both enzymes that have been more or less isolated and enzymes still residing inside living cells are employed for this task. More than one hundred years ago, bio catalysis was employed to do chemical transformations on non-natural man-made organic compounds and the last 30 years have seen a substantial increase in the application of bio catalysis to produce fine chemicals, especially for the pharmaceutical industry. Here the author emphasizes on the various application of biocatalysts useful in the pharmaceutical industry.**

Index Terms **- Biocatalysis, Enzyme, Enzyme synthesis.**

1.INTRODUCTION

Biocatalysis is the use of natural catalysts, such as protein enzymes, to perform chemical transformations on organic compounds. Both, enzymes that have been more or less isolated or enzymes still residing inside living cells are employed for this task. Biocatalysis is the term used to describe the application of any type of biocatalyst (enzymes, as isolated preparations of wild-type or genetically modified variants, or whole cells, either as native cells or as recombinant expressed proteins inside host cells) in a given synthetic schedule[1]. One type of applied biocatalysis, also called a biotransformation[2], takes advantage of the excellent enzymatic precision inherent to its use, in terms of chemoselectivity, regioselectivity or stereoselectivity. The use of bio transformations has increased considerably in recent decades, complementing classical chemical synthesis in multiple industries, mainly for the preparation of pharmaceuticals [3-12], fine chemicals [13-15] or food products [16-18]. Additionally, and based on the principles and metrics of green chemistry [19-20] and sustainable chemistry [21-24], biocatalysis fits perfectly into this framework; in fact, biocatalyzed procedures are highly efficient, economical, and generate less waste than conventional organic syntheses [25-27]. As such, the interest in the application of biocatalysis within the pharma industry is not surprising, as this industry is by far the biggest waste producer. Furthermore, as biotransformations are generally conducted under approximately the same temperature and pressure conditions, the possibility of carrying out coupled cascade processes is enabled, providing additional economic and environmental advantages [28-30].

Finally, most biotransformations can be easily developed in standard multipurpose batch reactors without requiring costly specific devices, such as high-pressure equipment, allowing the costless implementation of continuous processes [31-33]. In recent decades, the increase of the impact of biocatalysis within the pharma industry has not been linear. In fact, it was relatively minor until the last two decades, when a clear increase the third wave of biocatalysis was caused by the popularization of the genetic manipulation of biocatalysts by directed evolution, a method involving fast generation of enzyme mutants using new molecular biology techniques combined with selective pressure via screening conditions. This technique, recognized by the Nobel Prize in Chemistry being awarded to pioneer Frances Arnold in 2018 [34, 35], has allowed the redesign of enzymes to fulfil industrial requirements in terms of specificity, activity, and robustness, while keeping or even increasing its outstanding precision. Thus, with this potent tool in our hands, we are facing what has been called the 4th wave of biocatalysis, which will be fully implemented within the pharma industry when the speed of the overall process needed to create an improved biocatalyst (rational directed evolution in a design make test cycle, combining multiple disciplines in a continuous industrialized workflow is improved by at least 200%–1000%.

Biocatalysis has been extensively reviewed else-where, in particular the more established reaction classes, and we are not attempting to provide comprehensive coverage of the field, rather some insights and observations from an industrial perspective. Biocatalysis has established itself over the past few years as an integral part of the technology suite to develop greener, more sustainable and cheaper chemical manufacturing in many industries including pharmaceutical manufacturing. Compared to other chemical technologies, such as chemo catalysis, biocatalysts have several advantages and distinct challenges. Being genetically encoded provides easy access to much greater diversity either directly from nature (e.g., genomic and metagenomic databases) or via genetic engineering approaches to produce novel proteins. Advances in sequencing also mean that it is straightforward to know at least the primary structure of all the biocatalysts available or created. The path to scale up of production of a biocatalyst is also straightforward and predictable avoiding uncertainties that may be associated with other technologies (e.g., geopolitical uncertainties and price fluctuations for precious metal catalysts). The main advantage of biocatalysts is as a result of their chemo-, regio- and stereo selectivity which often allows for the shortening of synthetic routes by avoiding protecting group manipulations, resolutions, by-product formation etc. However, this selectivity is also their Achilles heel as individual biocatalysts tend to have limited substrate scope requiring a capability to source and screen multiple enzymes to find one with suitable properties. Biocatalysts also tend to be relatively fragile under industrial processing conditions and often require engineering to increase solvent tolerance, functional operating life and reduce substrate and/or product inhibition to allow process intensification to enable an economically viable manufacturing.

2. ADVANTAGES OF BIOCATALYSIS

The key word for organic synthesis is selectivity which is necessary to obtain a high yield of a specific product. There are a large range of selective organic reactions available for most synthetic needs. However, there is still one area where organic chemists are struggling, and that is when chirality is involved, although considerable progress in chiral synthesis has been achieved in recent years.

3. ENZYMES DISPLAY THREE MAJOR TYPES OF SELECTIVITY'S

- Chemo selectivity: Since the purpose of an enzyme is to act on a single type of functional group, other sensitive functionalities, which would normally react to a certain extent under chemical catalysis, survive. As a result, biocatalytic reactions tend to be "cleaner" and laborious purification of product(s) from impurities emerging through side-reactions can largely be omitted.
- Regioselectivity and Diastereoselectivity: Due to their complex three-dimensional structure, enzymes may distinguish between functional groups which are chemically situated in different regions of the substrate molecule. Enantioselectivity: Since almost all enzymes are made from L-amino acids, enzymes are chiral catalysts. As a consequence, any type of chirality present in the substrate molecule is "recognized" upon the formation of the enzyme-substrate complex. Thus, a prochiral substrate may be transformed into an optically active product and both enantiomers of a racemic substrate may react at different rates.

These reasons and especially the latter are the major reasons why synthetic chemists have become interested in biocatalysis. This interest in turn is mainly due to the need to synthesize enantiopure compounds as chiral building blocks for drugs and agrochemicals. Another important advantage of biocatalysts are that they are environmentally acceptable, being completely degraded in the environment. Furthermore, the enzymes act under mild conditions, which minimize problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.

4. APPLICATIONS

1) Natural methods of protein stabilization: Thermostable biocatalysts: [36]

In this the authors used the approach to study i.e., to learn how nature has managed to stabilize these proteins using a detailed knowledge of their biochemical properties and

three-dimensional structures. This is illustrated with several different classes of enzyme that have been studied at Exeter. They used alcohol dehydrogenase, aminoacylase, pyroglutamyl carboxypeptidase, -lactamase, dehalogenase and lysophospholipase. Enzymes that are naturally found in thermophilic and hyperthermophilic organisms are being used as robust biocatalysts in the fine chemical and pharmaceutical industries. They have important use in these industries due to their increased stability which is often required during commercial reaction conditions.

2) Biocatalyst activity in non-aqueous environments correlates with centisecond-range protein motions: [37]
In these recent studies explore the relationship between enzymatic catalysis and protein dynamics in the aqueous phase have yielded evidence that dynamics and enzyme activity are strongly correlated. Given that protein dynamics are significantly attenuated in organic solvents and that proteins exhibit a wide range of motions depending on the specific solvent environment, the non-aqueous milieu provides a unique opportunity to examine the role of protein dynamics in enzyme activity. Thus Variable-temperature kinetic measurements, X-band electron spin resonance spectroscopy, 1H NMR relaxation, and 19FNMR spectroscopy experiments were performed on subtilisin Carlsberg colyophilized with several inorganic salts and suspended in organic solvents. The results indicate that salt activation induces a greater degree of transition-state flexibility, reflected by a more positive S†, for the more active biocatalyst preparations in organic solvents. In contrast, H† was negligible regardless of salt type or salt content. As a result, 19F chemical shift measurements and hyperfine tensor measurements of biocatalyst formulations inhibited with 4-fluorobenzenesulfonyl fluoride and 4-ethoxyfluorophosphinyl-oxy-TEMPO, respectively, suggest that enzyme activation was only weakly affected by changes in active-site polarity.

3) Cytochrome *c* as a biocatalyst: [38]
The present study shown that Type c cytochromes, which are involved in the electron transport system, are also able to catalyze peroxidase-like reactions in the presence of an electron acceptor, such as hydrogen peroxide or an organic hydroperoxide. This work reviews the catalytic activity of cytochrome c, and the potential design by site-directed mutagenesis and chemical modification of new biocatalysts for environmental purposes.

4) Site-directed mutagenesis improves the biocatalytic activity of iso-1-cytochrome c in polycyclic hydrocarbon oxidation: [39]
In this iso-1-Cytochrome c from Saccharomyces cerevisiae is able to oxidize polycyclic aromatic hydrocarbons (PAH) in the presence of hydrogen peroxide. Anthracene and pyrene are oxidized by yeast cytochrome c to form anthraquinone and 1,8-pyrenedione, respectively. Iso-1- cytochrome c from S. cerevisiae was modified by site- directed mutagenesis of Phe82 and Cys102. The Phe82 substitution significantly altered the kinetic behavior of the protein; Cys102 modification affected neither the kinetic nor the stability constant. The Gly82; Thr102 variant was 10 times more active and showed a catalytic efficiency 10- fold greater than the wild-type iso-1-cytochrome c. However, Phe82 variants showed lower stability against inactivation by hydrogen peroxide than the wild-type protein. These site-directed mutations did not significantly alter the stability and activity of the hemo protein increasing concentrations of tetrahydro furan.

5) De novo design of biocatalysts: [40]
The challenging field of de novo enzyme design has begun to yield proteins with impressive catalytic efficiency. However, the current methods are not sufficient to design efficient enzymes for many reactions.

6) Intermediates of thiamine catalysis immobilized on silica surface as active biocatalysts for ketoacid decarboxylation: [41]
The authors used two ‘active aldehyde’ intermediates of thiamine catalysis immobilized on a silica surface by a convenient method via their phosphate groups. These bio- composite materials have been evaluated as catalysts for pyruvate and benzoyl-formate decarboxylation in either the presence or not of an aldehyde additive. They are stable and very effective catalysts for the production of 2-hydroxy- ketones, acetoine and benzoin.

7) Solubilization of cytochrome *c* in organic media with fluoroalkyl end-capped *N*-(1,1-dimethyl-3-

oxobutyl) acrylamide oligomer: a new approach to fluorinated biocatalyst in organic media: [42]
The authors suggested self-assembled molecular aggregates of fluoroalkyl end-capped N-(1,1-dimethyl-3- oxobutyl)acrylamide oligomer can solubilize cytochrome c in organic media such as methanol, although the corresponding non-fluorinated polymer cannot solubilize cytochrome c in organic media. Interestingly, the resulting fluorinated oligomer–cytochrome c aggregate was found to act effectively as a new fluorinated biocatalyst for the oxidation of pinacyanol chloride with hydrogen peroxide in the non-aqueous methanol.

8) Hydantoinases and related enzymes as biocatalysts for the synthesis of unnatural chiral amino acids: [43]
The authors introduced the hydantoinase process as an economically attractive method for the production of many unnatural chiral amino acids, which are components of potential pharmaceuticals.

9) Lipases as practical biocatalysts: [44]
The authors were states that novel developments in the study of lipases, ubiquitous enzymes used in numerous industrial processes, offer exciting opportunities leading to further practical applications of lipases in synthetic organic chemistry.

10) Metagenomic, gene discovery and the deal biocatalyst: [45]
In this optimum condition for biotransformation processes can be established without the constraints of the properties of the biocatalyst. These technologies can then be applied to find the 'ideal biocatalyst' for the process. In identifying the ideal biocatalyst, the processes of gene discovery and enzyme evolution play major roles. However, in order to expand the pool genes for in vitro evolution, new technologies, which circumvent the limitations of microbial culturability, must be applied. These technologies, which currently include metagenomic library screening, gene- specific amplification methods and even full metagenomic sequencing, provide access to a volume of 'sequence space' that is not addressed by traditional screening.

11) Biocatalysis- Biological systems for the reduction of chemicals: [46]
Authors described biocatalysis harnesses the catalytic potential of enzymes to produce building blocks and end- products for the pharmaceutical and chemical industry. Located at the interface between fermentation processes and petrol-based chemistry, biotransformation processes broaden the toolbox for bioconversion of organic compounds to functionalized products.

12) Fusion protein of Vitreoscilla hemoglobin with D-amino acid oxidase enhances activity and stability of biocatalyst in the bioconversion process of cephalosporin C: [47]
An artificial flavohemoprotein was constructed by fusing Vitreoscillahemoglobin (VHb) with D-amino acid oxidase (DAO) of Rhodotorulagracilis to determine whether bacterial hemoglobin can be used as an oxygen donor to immobilized flavoenzyme. This chimeric enzyme significantly enhanced DAO activity and stability in the bioconversion process of cephalosporin C. In a 200-mL bioreactor, the catalytic efficiency of immobilized VHb- DAO against cephalosporin C was 12.5-fold higher than that of immobilized DAO, and the operational stability of the immobilized VHb-DAO was approximately threefold better than that of the immobilized DAO. In the scaled-up bioprocess with a 5-L bioreactor, immobilized VHb-DAO (2500 U/L) resulted in 99% bioconversion of 120 mM cephalosporin C within 60 min at an oxygen flow rate of 0.2 (v/v) × min. Ninety percent of the initial activity of immobilized VHb-DAO could be maintained at up to 50 cycles of the enzymatic reaction without exogenous addition of H2O2 and flavin adenine dinucleotide (FAD). The purity of the final product, glutaryl-7aminocephalosporanic acid, was confirmed to be 99.77% by HPLC analysis. Relative specificity of immobilized VHb- DAO on D-aminoadipic acid, a precursor in cephalosporin C biosynthesis, increased twofold, compared with that of immobilized DAO, suggesting that conformational modification of the VHb-DAO fusion protein may be altered in favor of cephalosporin C.

13) The search for the ideal biocatalyst: [48]
While the use of enzymes as biocatalysts to assist in the industrial manufacture of fine chemicals and pharmaceuticals has enormous potential, application is frequently limited by evolution-led catalyst traits. The advent of designer biocatalysts, produced by informed selection and mutation through recombinant DNA technology, enables production of process-compatible

enzymes. However, to fully realize the potential of designer enzymes in industrial applications, it will be necessary to tailor catalyst properties so that they are optimal not only
for a given reaction but also in the context of the industrial process in which the enzyme is applied.

14) Biocatalysts for clean industrial products and processes: [49]
Biocatalysis inherently offers the prospect of clean industrial processing and has become an accepted technology throughout most sectors. The convergence of biology and chemistry has enabled a plethora of industrial opportunities to be targeted, while discoveries in biodiversity and the impact of molecular biology and computational science are extending the range of natural and engineered biocatalysts that can be customized for clean industrial requirements.

15) Protein engineering of oxygenases for biocatalysis: [50]
In this authors explain oxygenase enzymes have limited practical applications because of their complexity, poor stabilities, and often low catalytic rates. However, their ability to perform difficult chemistry with high selectivity and specificity has kept oxygenases at the forefront of engineering efforts. Growing understanding of structurefunction relationships and improved protein engineering methods are paving the way for applications of oxygenases in chemical synthesis and bioremediation.

16) Selection of mutations for increased protein stability: [51]
There are many ways to select mutations that increase the stability of proteins, including rational design, functional screening of randomly generated mutant libraries, and comparison of naturally occurring homologous proteins. The protein engineer's toolbox is expanding and the number of successful examples of engineered protein stability is increasing. Still, the selection of thermostable mutations is not a standard process. Selection is complicated by lack of knowledge of the process that leads to thermal inactivation and by the fact that proteins employ a large variety of structural tricks to achieve stability.

17) Resolution and synthesis of (S)-1-(2- naphthyl) ethanol with immobilized pea protein: as a new biocatalyst: [52]
The authors synthesized (S)-1-(2-Naphthyl) ethanol by immobilized pea (Pisum sativum L.) protein (IPP) from (R, S) 2-naphthyl ethanol (> 99% ee, yield; about 50%), in which the (R)-enantiomer was selectively oxidized to 2- acetonaphthone. IPP could be reused consecutively at least three times without any decrease of yield and optical purity.

5. TYPES OF BIOCATALYST

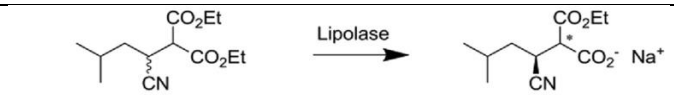
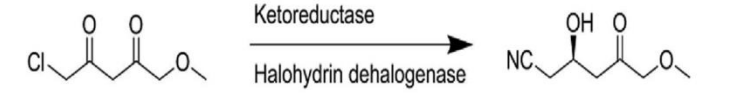
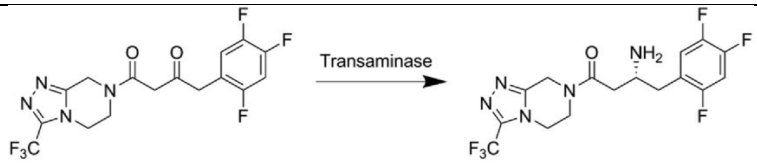
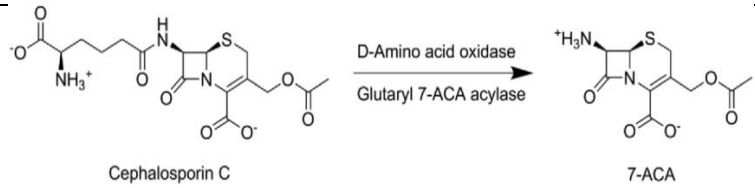
1) Oxidoreductases: Catalyze oxidation /reduction reactions.
   For example, alcohol dehydrogenase converts primary alcohol to aldehydes.
2) Transferases: Transfer a functional group.
   For example, Alanine amino transferase shuffles to alpha amino group between alanine and aspartate.
3) Hydrolases: Catalyze the hydrolysis of various bonds.
   For example, phosphates break the bond of oxygen-phosphorus bond of phosphate esters.
4) Lyases: Formation or removal of double bond with group transfer.
   For example, Dehydratase remove water, as in fumarase (fumarate hydratase)
5) Isomerases: Catalyze isomerization changes within a single molecule. (Rearrangements)
   For example, Triose phosphate isomerase, carry out these rearrangements.
6) Ligases: Removing the elements of water from two functional groups to form a single bond.
7) Kinase: This enzyme in body attaches a phosphate group to a high energy bond. It is very important enzyme required for ATP production and activation of certain enzymes.

6. ROLE OF PHARMACY IN BIOCATALYSIS

Biocatalytic syntheses in pharmaceutical industry:
In the last decades biocatalysis has developed into a key technology for API synthesis in the pharmaceutical industry and is often per se referred to as green technology [53, 54]. Using biocatalysis however imposes reaction conditions also affecting the environment in a certain way, such as the usage of a

high amount of water as solvent and a low substrate load, which have to be considered for process assessment [55]. Calculating the metrics helps to ascertain whether and to which extent the environmental impact improved during process optimizations e.g. the implementation of biocatalytic steps in drug synthesis. Outstanding examples of chemo enzymatic production routes for API synthesis are shown in Table 1. Even though the E factor does not discriminate between the toxicity of waste (such as water and heavy metals e.g. mercury), it gives evidence about the process greenness. In most cases, the E factor was reduced significantly compared to the chemical process, which proves the ability of biocatalysis to establish environmentally benign pharmaceutical production processes. The biocatalysts were selected by screening of natural enzymes or enzymes were modified with protein engineering technologies to achieve adequate catalytic activity for the desired conversion [56-58]. Especially, directed evolution strategies for improving enzyme activities enabled to use biocatalysis more broadly in the pharmaceutical industry. The probably most successful and best known biocatalytic process in the pharmaceutical industry is the synthesis of sitaglitpin with a highly developed transaminase [59]. In order to obtain an enzyme variant with activity on the precursor prositagliptin ketone, an enzyme library was created on the basis of substrate walking, modeling and a mutation approach. The enzyme was then optimized with regard to activity and practical application under process conditions. The developed biocatalytic process has a 13% higher overall yield and a 53% higher productivity compared to the rhodium catalyzed process. Additionally this enzyme has a broad substrate range and can potentially be used for synthesizing other chiral amines from prochiral ketones. However, the attractiveness of biocatalytic processes can sometimes only be assessed by a holistic approach with multiple measures which has been shown for the synthesis of 7-aminocephalosporic acid (7-ACA). The enzymatic synthesis of 7-ACA has a higher E factor than the chemical route, but the chemical process has a larger overall impact considering energy, mass efficiency, hazardous materials and solvents [60].

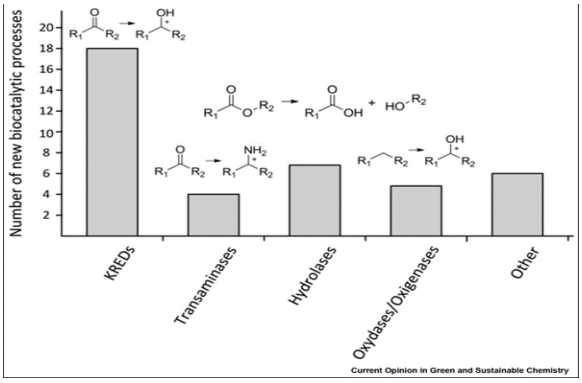
Impact of biocatalysis on the greenness of API syntheses.

Product	Biotransformation	E factor	Ref
Pregabalin	 *Rac*-2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester → 2-Carboxyethyl-3-cyano-5-methylhexanoic acid	12 (86)	[56]
Atorvastatin	 Ethyl 4-chloroacetoacetate → Ethyl (*R*)-4-cyano-3-hydroxybutyrate	6 (n.c.)[b]	[57]
Sitagliptin	 Prositagliptin ketone → Sitagliptin	26 (37)	[59]
7-ACA	 Cephalosporin C → 7-ACA	172 (93)	[60]

a. E factor of the chemoenzymatic process and the chemical process (in brackets).
b. Not calculated (n.c.).
c. 7-Aminocephalosporic acid (7-ACA)

Table No.1: Impact of biocatalysis on the greenness of API syntheses.

The increase of biocatalytic processes for pharmaceuticals in the last decade demonstrates their competitiveness with traditional chemistry (Figure 1). Especially keto reductase (KREDs) catalyzing the synthesis of chiral alcohols have been in great demand due to their broad scope, huge variety of accepted substrates and availability of opposite stereo preference. Biocatalytic processes using KREDs have such high efficiencies and productivities that for some pharmaceutical companies they have become the first choice when designing synthesis routes involving chiral alcohols[61]. The ongoing advances in bioinformatic analysis, screening and protein engineering tools have recently led to new enzymes for synthesizing chiral amines (Table 2) beyond the established transaminase. The recent identification of new imine reductases (IREDs) expands the substrate scope from cyclic imines to exocyclic imines allowing the formation of chiral secondary and tertiary amines [62]. Reductive aminases even extend the substrate scope and enable the formation of a variety of secondary and tertiary amines from amines and ketones. Turner and coworkers discovered a reductive aminase from Aspergillus oryzae which catalyzes the synthesis of secondary and tertiary amines at a preparative scale with total turnover numbers of 32,000 and space time yields up to 3.73 g L 1 d 1 . In general, protein engineering enables the adaptation of existing enzymes to convert many novel compounds and non-natural targets, the creation of entirely new biocatalysts for reactions which cannot be found in nature, as well as the customization of enzyme properties to process specifications.



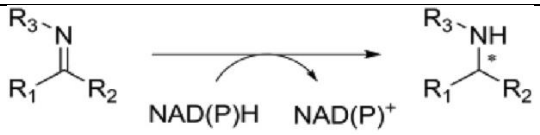
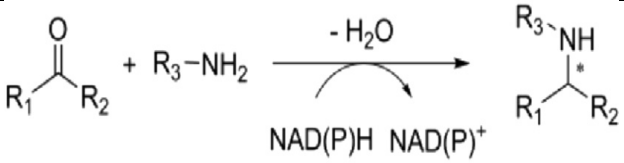
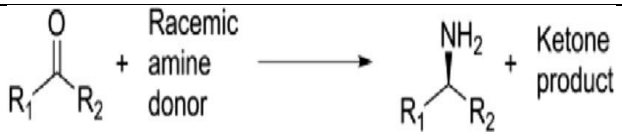
Enzyme	Reaction	Value contribution	Ref.
Imine reductase (IRED)		Novel IREDs for the asymmetric synthesis of amines many of which have a broad substrate scope, good conversion & enantioselectivity	[63,64]
Reductive aminase		Discovery of a reductive aminase with high activity for the reductive amination of ketones and amines	[65]
(S)-Selective transaminase		Creation of a highly stereoselective and active transaminase synthesizing a variety of chiral bulky amines	[66]

Table No.2: Some recent biocatalyst developed for the synthesis of chiral amines. R1, R2 and R3 represent substituent groups.

Lipase B	Candida antarctica	Reboxetine
Carbonyl reductase (YlCR2)	Yarrowia lipolytica	Statins
Oxidase	P.simplicissimum	Pinoresinol
Acyltransferase (LovD)	Whole-cell Escherichia coli strain overexpressing LovD	Simvastatin
Engineered cyclohexanone monooxygenase	-	Armodafinil
(+)-γ-lactamases	Bradyrhizobium japonicum USDA 6	Carbovir, abacavir, melogliptin
Immobilized lipase	Thermomyceslanuginosus	Rasagiline mesylate (active ingredient of AZILECT®)
Expressing tyrosine phenollyase	Erwinia herbicola cells	L-DOPA [57]
E. coli cells expressing cellobiose 2-epimerase	Caldicellulosiruptorsaccharolyticus	Lactulose

Table No. 3: List of biocatalysts and their microbial source employed for the synthesis of pharmaceutical drugs.

7. FUTURE PROSPECTS

Based on the literature available on the role of biocatalysts in the drug/pharmaceutical synthesis, biocatalysts with improved desired characteristics can be achieved by a multifaceted approach. Several tools and techniques represented above will enhance the biocatalytic stability, activity, enzyme-substrate affinity, and thermostability and will lead to higher yield. Also, the incorporation of artificial metabolic pathways, cell factory design, and nanotechnology approaches will further aid towards a suitable biocatalytic process. It will also ensure the quality and productivity of the drugs manufactured by optimizing safe process development. Thus, we envision that biocatalysis will be a more radical approach that is going to feat the arena of pharmaceutical manufacturing as well as other sectors such as bio energy and waste treatment that are far more challenging at present.

8. CONCLUSION

Biocatalysis has made a remarkable journey so far and has been successfully applied for the numerous biotransformation processes in several industries. It has benefitted nearly all sectors, particularly chemical and pharmaceuticals. The flourishing development of economically viable and sustainable chemo enzymatic processes highly depends on the broader availability and applicability of enzymes with robust performance irrespective of extreme conditions. Recent surveys have shown that most of the biocatalysts are being used in the synthesis of pharmaceuticals or drugs or intermediates replacing some of the chemical processes, but their stability, selectivity, and specificity are of prime concern.

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