

TREASURE WRAPPED IN AN ENIGMA: CHEMISTRY AND INDUSTRIAL RELEVANCE OF ENZYMES FROM RARE ACTINOMYCETES

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ABSTRACT

Microbial enzymes are known for their versatile catalytic property. With the advent of enzyme engineering, stringent environmental rules restraining the use of toxic chemicals, and need for the sustainable resource, there is a mounting demand for the utilization of these enzymes. Classified under Gram-positive filamentous bacteria, actinomycetes are ubiquitous and are one of the major sources of enzymes, antibiotics, and various such bioactive molecules. Rare actinomycetes are a less explored genera of actinomycetes. However, they are also a potential source of a diverse spectrum of enzymes that are principal of commercial importance. Enzymes produced by rare actinomycetes have a wide array of applications ranging from bioremediation techniques to the estimation of serum cholesterol levels. This untapped resource is industrially as well as biotechnologically valuable. Oxidative enzymes and esterases are two very important classes of enzymes produced by rare actinomycetes. The fundamental principles of catalysis applied by the organic catalysts are also relevant to the enzymes. This review highlights how this unexploited resource could be effectively exploited for various commercial applications and gives an overview of the industrial and biochemical applications of oxidative enzymes and esterases produced by rare actinomycetes. Protein engineering and modern biotechnology have been capable of manipulating the enzyme design making it a more stable and efficient asset to the industries.

Keywords: Rare Actinomycetes, Green Enzymes, Oxidation, Chemical Moiety, Esterification, Biotransformation, Therapeutic Applications.

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INTRODUCTION

The use of microbial enzymes is long-established but the first stated commercial use dates back to 6000 BC. Over the years, enzymes of microbial origin have been recognized worldwide for their role in different sectors of commercialization. The global market for enzymes was assessed to be around USD 9.9 billion in 2019 and the largest contribution to this growth has been from the microbial sector. This is projected to advance at a compound annual growth rate of approximately 7.1 % from 2020 to 2027 reaching almost 14.9 billion. Microbial enzymes play a key role in various fields like pharmaceutical, food, textile, and polymer industries. They are also extensively used in the clinical sector for diagnostic and therapeutic purposes. They are used as fibrinolytic, antitumor agents, antioxidants, antibiotics, and in the estimation of urea, glucose, cholesterol, and triglycerides. The industrial and clinical domains are now looking forward to using novel microbial enzymes due to various promising benefits they offer in terms of higher catalytic potential,

economic viability, ease of optimization, and superior stability compared to plant and animal counterparts. Actinomycetes are aerobic Gram-positive filamentous bacteria from the Actinobacteria class and order Actinomycetales.¹ They can be largely found in soil and aquatic systems and may exist as pathogens and commensals.² They produce enzymes in abundance, antibiotics, and other bioactive molecules, the majority of which have been isolated from the *Streptomyces* species.³ The most important antibiotics produced by *Streptomyces* include aminoglycosides, tetracyclines, macrolides, cycloserine, and amphenicols.⁴ Actinomycetes have constantly been explored for the production of enzymes with commercial importance; some of which include xylanase, cellulase, chitinase, amylase, pectinase, and protease. The only difference seen is the reaction conditions in which they act. These large biomolecules are basically proteinaceous in nature and are known for their specificity to both the substrate and the reaction. Being a protein, enzymes are made up of amino acid residues. A couple of them can possess another non-protein component called as a coenzyme which acts as the enhancer of the enzymatic activity but by itself is devoid of any activity. Biocatalysts are able to catalyze numerous organic reactions which makes them an outstanding resource for a wide range of applications. Rare actinomycetes are actinomycetes whose isolation using conventional methods is more difficult compared to *Streptomyces* species. *Nocardia*, *Micromonospora*, *Mycobacterium*, *Salinispora*, and *Corynebacterium* are some of the well-known rare actinomycetes.⁵ They are known to produce antibiotics like glycopeptides, aminoglycosides, anthracyclines⁶, and other substances having anti-inflammatory, cytotoxic, and antibacterial properties. Enzymes produced by rare actinomycetes have a wide array of applications ranging from bioremediation techniques to the estimation of serum cholesterol levels. Inability to isolate using traditional laboratory techniques, the need for pre-treatment with chemicals to inhibit the growth of *Streptomyces* and other undesirable organisms⁷ make rare actinomycetes under-explored genera. Thus, broadening the horizons of classical analytical techniques can help us solve this enigma. Exploring enzymes from unconventional sources can help us bridge the research gap in biodiscovery.

Oxidative Enzymes and Esterase

Oxidative enzymes are recognized to catalyze a range of chemical reactions using NADP or NAD⁺ as co-factors.⁸ They comprise the following classes of enzymes: peroxidases, oxidases, reductases, hydroxylases, dehydrogenases, and oxygenases. With the advent of enzyme engineering⁹, stringent environmental rules restraining the use of toxic chemicals, and need for the sustainable resource, there is a growing demand for the utilization of this class of enzyme. They can be used as better substitutes for chemo-catalysts due to minimal hazardous by-product formation and their ability to operate under mild reaction conditions. Esterase, which falls under the category of hydrolase, promotes cleaving followed by ester bond formation.¹⁰ Trans esterification, esterification, and inter esterification reactions are also known to be catalyzed by them¹¹ and for their stereoselectivity, stability, and ability to act without co-factors. They have been frequently used for bioremediation and biodegradation and in food, detergent, paper, and pulp industries.¹²

Oxidative Enzymes

Laccases

These belong to the category of multicopper oxidase. They are involved in catalyzing the oxidation of several phenolic as well as non-phenolic compounds concomitantly producing water as a byproduct, because of which they are also referred to as 'green enzymes'. Laccases fall under the class of ligninolytic enzymes. The complex structure of lignin makes the decomposition of lignocellulosic biomass difficult which can be overcome by laccases. These enzymes are known to improve fermentability, surface hydrophobicity, and porosity of lignocellulosic microfiber.¹³ It is therefore used in biofuel production where the various physicochemical methods currently employed are uneconomical and not environment-friendly. *Thermobifida fusca*-derived laccase has excellent thermostability and is alkaline stable. It has the ability to oxidize 2,6-dimethylphenylalanine and p-aminophenol which are dye intermediates that can be utilized to produce new colors¹⁴ making it useful in hair coloring. Copper present in laccases may be responsible for the biodegradation of polyethylene. This was demonstrated in the actinomycete *Rhodococcus ruber* strain in a study.¹⁵ Bioinformatic search performed by Ausec *et al.* revealed that laccase-like genes were observed in *Arthrobacter*, *Mycobacterium*, and *Nocardia* species.¹⁶

Cholesterol Oxidase

Cholesterol oxidase (CHOX), belonging to the family of flavin-specific oxidoreductases, is associated with catalyzing the dehydrogenation of cholesterol to cholestenone.¹⁷ It was first isolated from the bacterium, *Rhodococcus erythropolis*.¹⁸ They are present in actinomycetes strains such as *Mycobacterium*, *Corynebacterium*, *Brevibacterium*, *Rhodococcus*, *Arthrobacter*, and *Streptomyces* species. CHOX is used for degrading cholesterol, a source of carbon in non-pathogenic bacteria and the enzyme aids in the pathogenicity of infective bacteria (*Mycobacterium*, *Rhodococcus equi*) due to its membrane damaging effect. Hence this can be considered a potential target for treating infectious diseases caused by such organisms. The ability of CHOX to degrade cholesterol can offer a promising solution for the therapeutic management of cardiovascular diseases. These enzymes are commonly used in the detection of cholesterol levels in the blood. A multiparameter fiber optic biosensor making use of this immobilized form of the enzyme was developed which could determine the levels of cholesterol.¹⁹ Furthermore, biosensors can be used to determine the cholesterol content in the food.²⁰ Some of the biosensors underuse include CHOX/Carbon Nanotube-Adorned Platinum (CHOX/CNT-Pt) biosensor, Fourier Transformation Continuous Cycle Voltmeter (FFTCCV), Poly-Pyrrole-Polyvinylsulfonate (PPy-PVS), N[3-(trimethoxysilyl) Propyl] and Multiwall Carbon Nanotubes/Glassy Carbon Electrode (MWCNTs/GCE). The enzyme has a significant role in the transformation of steroids which finds its utilization in the production of steroid hormones. CHOX from *Rhodococcus erythropolis* also have been used for optical resolution of an allylic alcohol.²¹

L-Amino Oxidase

They are flavoprotein-containing enzymes that catalyze the oxidative deamination of L-amino acids to finally produce α keto acids.²² This reaction occurs via the formation of unstable imino acid intermediate along with the release of hydrogen peroxide and ammonia. They have been isolated in various micro-organisms. Among the actinomycetes, *Rhodococcus opacus*, *Cellulomonas cellulans*, *Streptomyces* sp., and *Corynebacterium* are some of the important sources.²³ These enzymes demonstrate antimicrobial properties which are believed to be mainly due to the release of H₂O₂. The LAAO (L-amino oxidase) from *Corynebacterium*²⁴ and *Rhodococcus* have broad substrate specificity as they act on both D and L amino acids. The enzymes can be intracellular like in *Rhodococcus*²⁵ or extracellular as observed in *Cellulomonas cellulans*.²⁶ LAAO from *Rhodococcus* sp AIU Z-35-1 has been effective in producing a wide range of D-amino acids from DL-amino acids through kinetic resolution. The enzyme used in Levodopa biotransformation to α -keto acid and in the production of amino adipic derivatives that are β -lactam antibiotics precursors is also demonstrated. The use of this enzyme for performing the assay of L-aminoacylase was exhibited to be more effective than the traditional ninhydrin assay.²⁷

Putrescine Oxidase

Putrescine is one of the common polyamines found in actinomycetes. This polycationic compound is abundantly found in eubacteria.²⁸ Putrescine can be isolated from *Rhodococcus erythropolis* and *Kocuria rosea* (*Micrococcus rubens*) as well as many others.^{29,30} It is among the important biogenic amines found in food and is found in meat, fish, cheese, etc.³¹ Polyamines are crucial for regulating the synthesis of macromolecules in many organisms. Polyamines are also vital for the growth of most organisms. Putrescine oxidases are known to catalyze the process of oxidative deamination of putrescine to 4-aminobutanol, hydrogen peroxide, and ammonia. Putrescine can be utilized as a marker to detect spoilage of food due to Enterobacteriaceae and *Clostridium* spp.³² Biogenic amines can be detected using ultra-performance liquid chromatography, gas chromatography, and thin layer chromatography.^{33,34} Use of biosensors can also be a suitable method as it helps in the rapid detection of putrescine compared to high-performance liquid chromatography.

Sarcosine Oxidase

Sarcosine oxidase is an enzyme involved in the catabolism of creatinine. Creatinine gets converted to creatine by the enzyme, creatininase. Sarcosine is then produced from the hydrolysis of creatine by creatinase. Sarcosine oxidase hydrolyses sarcosine to produce glycine, formaldehyde, and hydrogen peroxide. H₂O₂ or formaldehyde produced during this reaction can be used to estimate the levels of

creatinine and /or creatine.³⁵ Atsushi Sogabe et.al invented a method for determining levels of creatine and creatinine in a sample using creatine amidinohydrolase and sarcosine oxidase. Sarcosine oxidase to be used for this method can be obtained from genera *Arthrobacter*, *Micrococcus*, and *Corynebacterium*. Sarcosine oxidase of *Corynebacterium* is the most extensively studied sarcosine oxidase in relation to kinetics as well as structure. Cloning and sequencing of the gene which encodes for sarcosine oxidase in *Arthrobacter* spp have also been.³⁶ Genomic studies have revealed the presence of sarcosine oxidase genes in several actinobacterial species.^{37,38} Nishiya *et.al* reported a method to determine the concentration of chloride ions in serum as well as other samples using mutant sarcosine oxidase. The method involves the reactivation of enzymes in the presence of chloride ions, after which, it converts sarcosine to H₂O₂. Then, the amount of H₂O₂ produced is measured. The rate of this reaction enhanced with the increase in the concentration of chloride ion.³⁹

Catechol 1,2-dioxygenase (C1,2O) and Catechol 2,3-dioxygenase (C2,3O)

Polycyclic aromatic hydrocarbons are toxic as well as carcinogenic in nature.⁴⁰ Most of the aromatic compounds get degraded to catechol or protocatechuate based on their chemical structure. The catechol formed is then cleaved by either C1, 2O via ortho-pathway or by C2, 3O via meta-pathway.^{41,42} Active-site iron present in C1,2O or C2,3O is important for the activity of the enzymes. These two enzymes may also find use in polycyclic aromatic hydrocarbon biodegradation and bioremediation techniques related to wastewater treatment. A study on C1, 2O, and C2, 3O of *Gordonia polyisoprenivorans* carried out by Silva *et al*, found that the activity of the latter was more than that of the former under most of the environmental conditions tested.⁴³ Gene catA, which encodes for C1,2O, can be utilized as a marker to check the catabolic ability of the bacteria in the case of bioremediation techniques.⁴⁴ Phenols, one of the common constituents in industrial effluents, can be degraded by C1,2O. A study carried out by Nadaf *et.al* found through Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis that the molecular weight of C1,2O produced by *Rhodococcus* sp. NCIM 2891 is 30kDa and the activity of this enzyme was fully inhibited by ions of certain metals like Cu⁺⁺, Hg⁺⁺, and Fe⁺⁺⁺.⁴⁵ C1,2O produced by *Rhodococcus rhodochrous* N75 has broad substrate specificity. Aniline and its derivatives present in agricultural soil ⁴⁶, chlorinated benzenes, dibenzothiophene, biodiesel, and diesel can also be degraded by C1, 2O, and C2,3O.⁴⁷

Peroxidase

Peroxidases are a class of oxidoreductases that bring about oxidation using H₂O₂ as an electron acceptor.⁴⁸ Present in both eukaryotic and prokaryotic organisms, these are involved in various activities ranging from biosynthesis to degradation and immunological mechanisms. They are broadly categorized into plant and animal peroxidases where the former is subdivided into 3 classes.⁴⁹ Dyp (dye decolorizing peroxidase) are heme-containing novel peroxidases. First identified in fungi, it has now been recognized in bacteria.⁵⁰ They have been widely studied for their ability to degrade synthetic dyes making them useful in the bioremediation of dye-contaminated wastewater. A study carried out by Van Bloois demonstrated that TfuDyP identified from *Thermobifida fusca* was found to be active on anthraquinone dyes, azo dyes, and aromatic sulfides. Unlike other Dyp-type peroxidases, TfuDyp could carry out enantioselective sulfoxidation. Alkali tolerant Dyp-type peroxidase (*SviDyP*) produced from *Saccharomonospora Viridis* was investigated for its bio-bleaching properties. It could degrade dyes such as Anthraquinonnic and triarylmethane dyes. The properties it possesses make it suitable for use in dye wastewater disposal and paper-pulp industries. Dyp-type peroxidases are also receiving attention due to their lignin degradation ability.

This was observed in actinomycetes such as *Thermomonospora* ⁵¹, *Thermobifida*, *Rhodococcus*, and *Cellulomonas*. Peroxidase identified in non-filamentous actinomycetes *Nonomuraea gerezanensis* had properties comparable to fungal peroxidases in lignin degradation along with higher thermostability. The enzyme also demonstrated dye decolorization properties.⁵² Among actinomycetes, the Streptomycetaceae family has been recognized as possessing these enzymes. It was demonstrated that peroxidase from *Streptomyces* was capable of degrading plastic polyethylene more successfully than the fungal strains. Overall actinobacterial peroxidases have been largely studied for their ligninolytic ability, degradation of xenobiotic and dyes, soil humification, and biopulping.⁵³

Esterase

Esterases that catalyze cleavage and formation of ester bonds fall under the category of hydrolases. Feruloyl esterase (FAE) and acetyl xylan esterase are two important lignocellulolytic enzymes that hydrolyze the ester bands in triacylglycerides to form fatty acid and glycerol. FAE comes under the category of carboxylic ester hydrolases. They have the capability of breaking the ferulate ester in hemicellulose and lignin cross-linking thus hydrolyzing the plant cell wall. They have been increasingly used in the production of biofuel.⁵⁴ Figure-1 gives an overview of steps in biofuel production from lignocellulosic biomass.

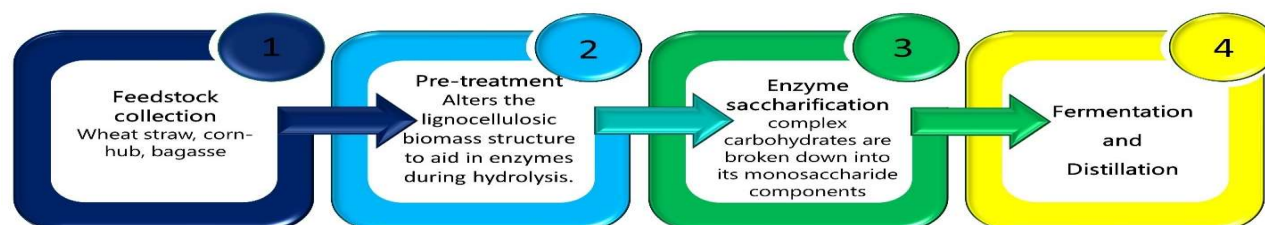


Fig.-1: Biofuel Production from Lignocellulosic Biomass

FAE along with other enzymes like xylanase and cellulase remove the lignin from the surface and enhance the saccharification of wheat straw.⁵⁵ They have been mainly isolated from the fungi but also have been identified in the bacteria. Actinomycetes strain *Nonomuraea* was identified to produce this enzyme. Thermophilic actinomycetes were also capable of generating FAE which increased the production of ferulic acid from destarched wheat bran. The ferulic acid thus obtained is having potent antioxidant properties. Acetyl xylan esterases are another group of enzymes that play a vital role in the deacetylation of Xylooligosaccharides, and acetyl xylan, which are important degradation products of xylan.⁵⁶ *Thermobifida fusca* NTU22, a thermophilic actinomycete could produce acetyl xylan esterase enzyme both intracellularly and extracellularly which along with xylanase caused the degradation of xylan from oat spelled liberating acetic acid.⁵⁷ Heroin esterase enzyme has been identified only in *Rhodococcus* sp strain H1 which can thrive on heroin using it as a sole carbon source and energy. The enzyme deacetylates it to morphine which along with morphine dehydrogenase obtained from *Pseudomonas* species can be widely used as a biorecognition system for developing a sensor for the detection of heroin.⁵⁸ Another biosensor was developed for detecting cocaine, an illicit drug. It made use of cocaine esterase produced from *Rhodococcus* sp strain MB1 which depends on cocaine as a source of nitrogen and carbon. Ecgonine methyl ester and benzoate, the enzyme-catalyzed degradation products of cocaine were further metabolized by the microorganism.⁵⁹ Apart from this, the enzyme has been observed to be of use as therapy for cocaine intoxication and addiction. In rodents and Rhesus monkeys, the enzyme could improve cocaine-induced CV disturbances.⁶⁰ A thermostable esterase from *Actinomadura* stable at 50°C and 60°C for 120 min and having 90% activity was isolated. It was tolerant to ionic and non-ionic solvents and mild detergents making it useful in industries.⁶¹ Esterases from *Rhodococcus* sp CR-53 could perform kinetic resolution of tertiary alcohols which is of benefit in pharmaceutical production.⁶²

Polyester Degrading Esterases

The degradation of plastics has been a universal concern because of the severe environmental problems they create.⁶³ Use of bioplastics has been one such solution to this. PLA (Polylactic acid) is an aliphatic polyester used as a component in bioplastic. Biodegradation of plastics using enzymes has been extensively studied. Polyester degrading enzymes mainly belong to the family of carboxylic ester hydrolases which includes lipases, cutinases, and esterases. Actinomycetes are one of the largest sources of these enzymes. Short-chain water soluble acyl glycerol and short and medium acyl chain of P-nitrophenol esters are acted upon by carboxylesterases. Thermophilic actinomycetes were able to produce these types of esterases which could hydrolyze synthetic cyclic PET (Polyethylene Terephthalate) trimers.⁶⁴ Degradation of the aliphatic-aromatic co-polyester film was demonstrated by an esterase from *Thermobifida alba* strain AHK119. Cutinase is a serine hydrolase type also coming under the category of carboxylic ester hydrolase.⁶⁵ They have been widely studied for their ability to degrade both aliphatic and aromatic type polyesters.⁶⁶ *Thermobifida alba* AHK119 has been found to produce cutinases capable of degrading plastic.

[illegible]

Fig.-2: Applications of Oxidative Enzymes and Esterases Produced From Rare Actinomycetes

Rare actinomycetes are one of the harbingers of sustainable technology. They play a significant role in discovering commercially important bioactive molecules. They are one of the mainstream suppliers of antibiotics, enzymes, and other unique chemical moieties. The biologically active enzymes of rare actinomycetes are also a potential budding resource for a broad range of industrial, pharmaceutical, and biochemical relevance like biofuel production, bioremediation, optical resolution of allylic alcohol, L-aminoacylase assay, and estimation of creatinine, cocaine, and chloride ion concentration in samples. Robust stability and a multitude of applications of enzymes from rare actinomycetes make it a propitious source for commercial use. Future research and enzyme engineering should be directed towards efficiently exploiting the chemical properties of these enzymes for industrial applications. Exploring a novel ecological environment can help us address the research gap in biodiscovery and hence contribute to the bioeconomy.

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