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Purification of *Azoreductase* for Industrial Applications: A Review

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Abstract

Bacterial *azoreductases* were exploited for research purposes to assess their potential to degrade azo dyes. Azo dyes are toxic and needs to be removed, degraded converted into less toxic forms. Various bacterial strains isolated from the azo-dye contaminated soils, industrial effluent discharge, waste water etc. were the potential source for their isolation, recombinant DNA technology has enabled to increase the expression of azo-dye degrading genes in different expression systems to be used in industries. The demand to purification varies upon the potential use of the *azoreductase* in industries keeping in view their effects on human health. This review article is concerned with the purification strategies which are involved in extraction (in case of intracellular and membrane bound enzyme) and purification of bacterial *azoreductases* along with their activity and toxicity assays.

Keywords: Xenobiotics; Flavoenzymes; Ion-exchange chromatography; Hydrophobic interaction chromatography; Azo dyes

INTRODUCTION

Now-a-days, bioremediation is the latest clean up approach that utilizes microbes to clear toxic wastes from the environment with the help of biotransformation enzymes. These microbes can convert highly toxic compounds to harmless form as well [1]. So, it's important to find a dye degradation strategy which consists of different techniques. In the recent era, use of mixed culture of microbes result in best dye degradation strategy and it is more economically balance as well. Use of microbial enzyme technology is also important for the removal of xenobiotics from the environment. Azo dyes are classified by the presence of azo groups in their chemical structure. It is found that synthetic dyes mostly contained azo group. Because of their vast usage in food, textile and pharmaceutical industry, azo dyes are being produced in greater amount.

These dyes show much persistence in the environment just because of their stability to light and aerobic conditions. In the recent research, its toxicity and mutagenic behavior have been noted and that's why they are considered highly toxic. Derivative of 3,3 dichlorobenzidine, derivative of 2-amino-4-nitrotoluene, phthalocyanin is mostly in the tattoo colorant of synthetic azo dyes. It increases a number of industrial effluents because of its toxic metabolic products when released improperly in the environment. The treatment process for waste water having dye generally involves biological methods using plant extracts and microbes. Azo dye is mostly degraded in aerobic conditions [2].

Several microbes isolated from soil have shown a great impact on the degradation of dye. The phenomenon involves in degradation is reducing the azo bonds present in azo dyes by microbes and results in amine formation. *Azoreductase* enzyme present in bacteria has the ability for the reduction of azo bond by NADPH [3]. NADPH is considered as an electron donor in lab conditions. *Azoreductases* are the pivotal enzyme produced by a number of azo dye degrading bacteria. Few proteins have been discovered so far depicted the *azoreductase* activity. Some of the bacteria including Enterococcus faecalis, *Xenophilus azovorans*, KF46F, Bacillus sp, Staphylococcus aureus and Escherichia coli. Due to enzymatic efficacy of bacteria, its use for industrial purpose is highly considered. It is also under consider that bacteria usage is also rearing for a long period of time whereas its enzyme can nullify this crucial step and results in cost

effective strategy *Chromobacterium violaceum* group of bacteria are found in water and soil of subtropical areas of the world [4].

It is evident that *C. violaceumis* not similar to the rest of its bacterial group in case of nucleotide sequence. It has a greater amount of potency to degrade azo dyes in oxygenic conditions [3]. It is estimated that over 10 million tons of dyes are produced annually [5,6] and 50% are azo dyes [4]. Azo dyes are mostly used in textile industry [7]. *Azoreductases* are considered as flavin dependent or flavin independent [8] according to the cofactor requirements. They are further sub divided into subgroups based on their need to NADHPH or NADH used as an electron donor [9].

The need of coenzyme namely NADH or NADPH is considered as a limiting factor in the applications of waste water treatment [3]. Moreover, these days an *in-situ* coenzyme regeneration technique has been in use to incorporate NADH or NADPH. Recently, two most common enzymes are used as coenzyme regeneration known as *Formate dehydrogenases* (FDHs) and *Glucose dehydrogenases* (GDHs). One of the successful experiments was conducted for dye removal by using integrated enzyme system having glucose 1-dehrogenase and *azoreductase*. Another experiment was done by using FDHs as the coenzyme for the reduction of NADH in *E. coli* [10] and it showed an account of 30 % decolorization in twelve hours [11]. The use of GDHs has been increased in the industrial applications because of high enzymatic stability as compared to FDHs which limit the activity coenzyme system [9].

It is demonstrated that GIT of human contains four hundred to five hundred bacterial species and some of them are characterized having great amount of azo nitro reductase activity [12]. The use of azo drugs is considered in the treatment of bowel diseases in humans by the activation of NADPH (quinone oxidoreductase). Actually, quinone oxidoreductase is the ortholog of *azoreductase* [13]. Infact, its precise function in drug metabolism is still not known [14]. In the recent study, it is indicated that colon specify drug delivery can be made possible by *azoreductase* sensitivity [15].

Its structure indicated that it is homodimeric or monomeric in nature as shown in Figure 1. When isolated from *S. aureus*, it is observed that it has tetrameric form of NADPH-dependent as well. The structure of flavin free *azoreductases* are monomeric mostly [16]. When the structure *Enterrococuus feacalis* is studied, it is found that FMN dependent Azo A exists in homotetramer.

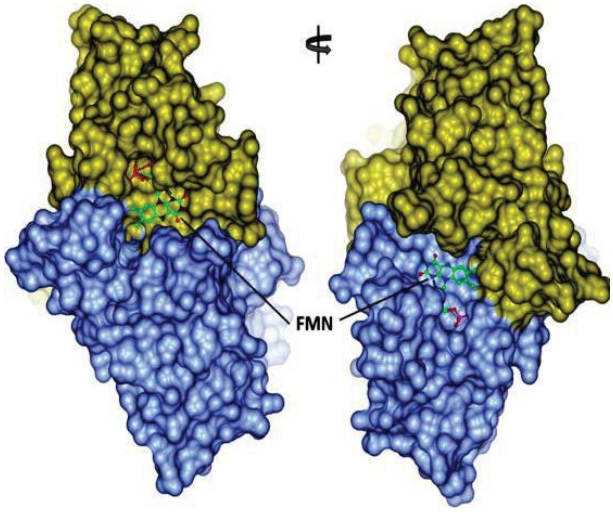


Figure 1: Crystal structure of Flavin dependent azoreductase [19].

One monomer binds with substrate methyl non covalently and other binds as a cofactor with FMN. It is also stated that Azr A from *Baccilus* and FMN-dependent Azo R from *E.coli* also come in the form of homodimers [17]. Size exclusion column chromatography depicts that *azoreductases* from neutrophilic and bacterial strains have the monomeric appearance. Some investigations related to the structure also depicted the sensitivity to oxygen by anaerobic and aerobic bacterial strains. A number of enzyme activities having reduction mechanism are also prescribed for the conditions. Several scientific reports indicated that the *azoreduction* is done by mostly flavoprotein present in the microbial electron transport chain as shown in Figure 1 [14].

Azo reductase is mainly considered for its ability in breaking the azo linkage while the dye metabolism is in progress under anaerobic and aerobic conditions. In this case, aromatic amines are produced by reduction that degraded by aerobic conditions by enzymes namely dioxygenases and hydrolases. One of the most important activity is nitro reduction process which utilizes toxic nitro aromatic compounds [18]. Due to lack of strategies and economic pressure, only a few anaerobic *azoreductases* are being isolated and characterized only. Morrison [19] had done characterization of AzoC from the bacterial sp. Clostridium perfringens and it shown the best enzyme activity at basic pH at 25°C. *Azoreductases* follow ping-pong-bi-bi mechanism in falvin *azoreductases* revelaed by kinetic studies supported by plots of concentration of substrate vs initial velocity [20]. Another bi-reactant mechanism was suggested by scientists for the flavin-free *azoreductases*. Some kinetic constants were also

described for carboxy-orange II reductase and found that enzyme activity is greater for NADH.

Classification of *Azoreductases*

Some of the bacterial enzymes are known that can reduce azo dyes and their gene sequence has been determined so far. Some of the enzymes act on substrates as compare to azo dyes. It is classified on the basis of catalysis which shows if it is affected by molecular oxygen or not [14]. It is described hat if the reaction is degassed with nitrogen then oxygen sensitive *azoreductases* increase their activities. This process is observed in *Clostridum perfringes* as shown in Table 1 and Bacillus sp. It is regarded as the best process because sensitivity is attributable to the formation of hydrogen peroxide from $FMNH_2$. Moreover, presence of oxygen shows no impact on oxygen sensitive *azoreductases* enzymes obtained from *Klebsiella oxytoca and Bacillus velezensis* [21]. It is also explained in that some are oxygen sensitive and some are not. On the basis of co-factors, it is divided into two categories independent and free enzymes [22].

Potential Applications of *Azoreductases*

In some cases, gene deletion has been examined for azoR so far as these are not essential in cell growth. To invoke thiol oxidative stress, electrophilic quinones are used. For this purpose, *azoreductase* activity may be utilized for stress responses. Azo cleavage is important in degradation and decoloration, it is involved mostly in clean up systems to remove pollutants from the environment. As *azoreductases* use unstable donors and utilized electron donor for this purpose. Some of the *azoreductase* sytems are achieved to get integrated process in labs made up of glucose dehydrogenase. For the treatment of inflammatory bowel disease, 5-ASA is used. Hypoxia, which is induced by cell apoptosis is triggered by the mitochondrial damage which evoke cytochrome c in the cytoplasm that's why cytochrome c acts as a biomarker. Some scientists reported a nano sensor also for the detection of cytochrome c which was activated when *azoreductases* cleaved it. These are dominant in mammal cells mostly. Some of the *ampiphilic* copolymers also described having azo linkage. The copolymer combined into micellar structure with the help of hydrophobic interactions but disassembled when the azo linkages are cleaved [22].

Table 1: Brief description of known azoreductases from different bacteria.

Organism	Flavin/NADPH dependent	Thermal stability in °C	References
Shewanella oneidensis MR-1	NADH	25	[46]
Clostridium perfringens	NADH	NA	[23]
Lysinibacillus sphaericus	NADH	70	[18]
Bacillus badius	NADH	60	[17]
Halomonas elongate	NADH	NA	[11]
Aquiflexum sp.	NADH	80	[20]
Pseudomonas Putida	NADH	NA	[12]

Sources of *Azoreductases*

There are many sources and sites that have been explored to and find beneficial for harvesting the bacteria that produce *azoreductase* enzyme. Following are some predominant sources of *azoreductase* producing bacteria that are discussed briefly:

Due to the anaerobic conditions prevailing in the human gut system, most of the bacteria isolated from the human gut capable of producing *azoreductase* were anaerobes. In an experiment consisting of plaque assay, the bacteria were grown on media having azo dyes, the reduction of azo dyes indicated the production of the desired enzyme [23]. The bacteria were isolated from the fecal sample. The results indicated that *Clostridium clostridioforme, Bacteriodes sp., Butyrivibrio sp., Clostridium nexile, Eubacterium sp. and Clostridium paraputrificum* were clearly the producers of *azoreductase* enzyme. In another study the bacteria such as *Escherichia coli, E avium, B cereus and E faecalis* also showed the activity of reducing the dye [24]. As the effluents from the industries, possessing waste dyes have azo dyes a major component, this effluent flows through different lands. When bacteria from halophilic conditions where effluent passed were cultured in laboratory, it expressed the ability to reduce the azo dyes. This is Halomonas elongata. This bacterium was then further used in isolating *azoreductase* enzyme gene and was cloned in *E. coli* for further studies and experiments [25].

As methods have been devised for the treatment of the dyes that are the waste that is exposed as hazardous waste for the environment, meantime certain bacteria also dwell in such environments by the mercy of nature that are reducing the azo dyes in the effluent from the industries. They are working without any human intervention to save the environment and the ecosystem [1]. The bacteria that were isolated from the effluent of textile industries mainly comprised of *Bacillus thuringiensis, Nocardiopsis alba, Bacillus subtilis, Alcaligenes sp. and Bacillus odyssey and Rhodococcus sp*. [5]. Soil also harvested a wide range of bacterial strains. A

sample taken from the soil near waste treatment plant for industrial effluents was analyzed, the bacteria that had found to be effective in reducing azo dyes was *Bacillus sp*. OY12 [26]. The genes from these bacteria were isolated and cloned in other bacteria to express recombinant proteins to enhance the activity of reducing the azo dyes. The *azoreductase* enzyme was then able to express itself in aerobic conditions that other than is not possible in normal conditions. Bacillus badius is a bacterial strain that is usually found in alkaline habitats. This has that unique ability to produce *azoreductase* to reduce the azo dyes. These special bacteria were found in India, Maharashtra state from the pristine alkaline Crater lake of Lonar. The strain can degrade the azo dyes in aerobic conditions. It has the potential to act at the pH 7.4 and the enzyme remains stable at the temperatures as high as 85-degree celsius [20]. A brief tabular representation of the isolation sites of the *azoreductase* producing bacteria with their important strains is given below in Table 2.

Recombinant *Azoreductases*

Due to the promising benefits of the *azoreductase* enzyme, a lot of research is dedicated for the exploration of its potentials. With the emergence of genetic engineering, it is aimed to modify the enzyme to enhance its reducing abilities with a wider spectrum of dyes to act. The gene is modified to obtain as many benefits as we can. Since the discovery of this enzyme, different models for genetic engineering are being set up. Some of them are discussed here. This bacterium is specially known for its ability to decolorize the dyes by breaking the azo bonds. The strain was isolated from rocks present in China. The gene for the *azoreductase* was expressed to a minimal level among the other known strains producing this enzyme. Plasmid vectors pET28a, pET32a and pET20bl were used and *E. coli* was used for the expression system [27]. *Shewanella xiamenensis* was grown on LB broth at 30-degree celsius at 150 rpm. *E. coli* was grown at LB media at 37-degree celsius at 200 rpm. AzoR was integrated into the vector plasmid by generating sticky ends. The expression system over expresses the protein of interest and protein is analyzed and purified in the later steps [28]. The bacteria Klebsiella pneumoniae is among one of the most primitive *azoreductase* producers. It was isolated from the sludge near the effluent industrial water. The gene of interest was cloned and expressed in E. coli. The gene was already able to encode an *azoreductase* that had the ability to reduce mono, di and tri azo dyes, after recombination and expression in *E. coli* BL 21 it was able to reduce a model azo dye called methyl orange [29]. The vector plasmid for cloning used was pGEM-T. For the overexpression of the protein of interest by the recombination of pET-28b-azoK in *E. coli* is done. The results of the electrophoresis showed that the molecular mass of the protein was 28kDa. The recombinant enzyme was successfully able to reduce the test dye. The presence of a redox mediator was seen to enhance the reducing ability of the enzyme [30].

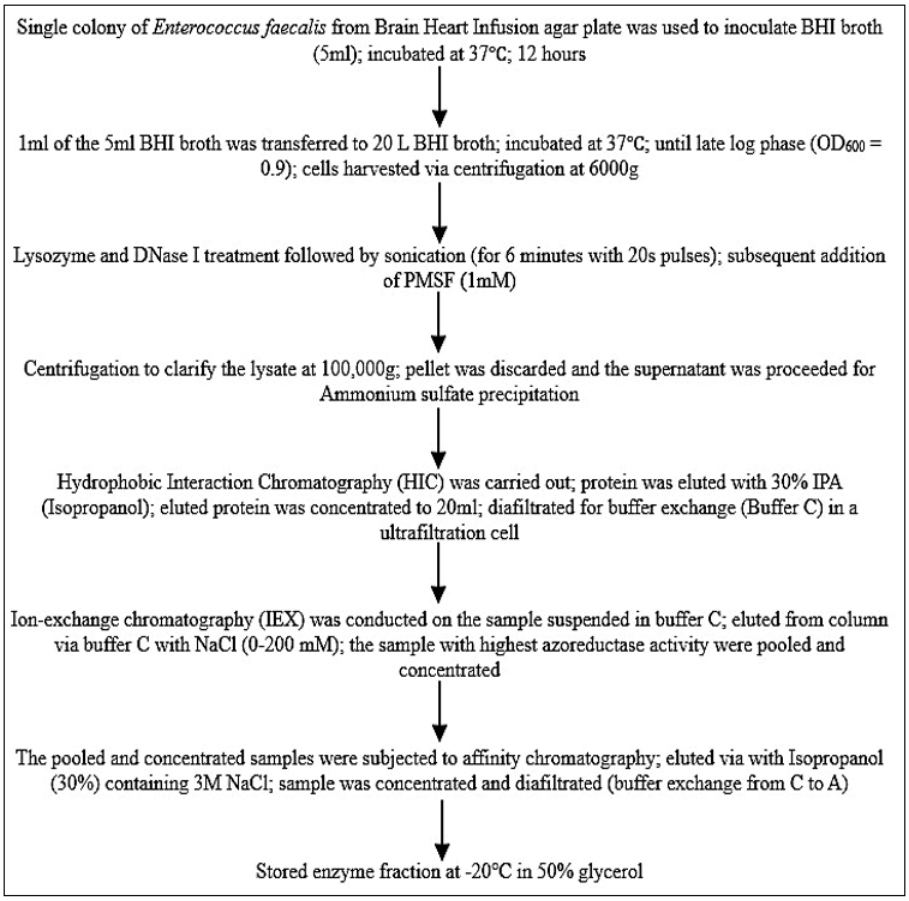
Table 2: Sites for the Isolation of bacterial species producing azoreductase.

Isolation sites	Bacterial species	References
Human gut microflora	*Clostridium clostridioforme,*	[52]
	Bacteroides sp.,	[35]
	Butyrivibrio sp.,	
	Clostridium nexile, Eubacterium sp.	
	Clostridium paraputrificum	
Halophilic conditions	*Halomonas elongata*	[11]
Textile dye effluent	*Bacillus thuringiensis,*	[37]
	Nocardiopsis alba, Bacillus subtilis, Alcaligenes sp.	[44]
	Bacillus odyssey	
	Rhodococcus sp.	
Soil	*Bacillus sp*. OY1-2	[36]
Alkaline water	*Bacillus badius*	[17]

Purification of *Azoreductases*

Azoreductases are the flavoenzymes which are produced by a range of bacterial and fungal species. Their purification is concerned here regarding their potential applications in various industries. From the literature reviewed the research articles from 2009 to 2020 were studied for the purification strategies used for *azoreductases*. Zimmermann [31] was among the earlier reporters of azoreductase producing bacteria Pseudomonas KF46. The reported *azoreductase* was Orange II azoreductase which was purified (98%) via two successive runs of affinity chromatography varying in chromatographic media with different triazinyl dye as ligands (Blue sepharose CL-6B and matrix Gel Red A). The enzyme was purified 120-fold with 43% yield.

The elution of enzyme from dye gels was carried out via two co-substrates NADPH and NADH (as well as by Orange II). Native and denaturing disc gel-electrophoresis of purified enzyme was carried out to check contaminating proteins. Punj and John [32] reported the production and purification of azoreductase from Enterococcus faecalis, which is a gram-negative opportunistic pathogen in mammalian intestines, heterologously expressed. The purification steps are depicted in the Flowchart 1 below [31]



Flowchart 1: Purification steps of intracellular azoreductase from Enterococcus faecalis (All the steps of purification were carried out at 4°C).

The purification of recombinant azoreductase (AzoB) of Pigmentiphaga kullae K24 was expressed in *Escherichia coli* (cloned via TA cloning) as reported by [33]. The recombinant AzoB was then purified from the IPTG induced *E. coli* supernatant via combination of chromatographies that are HIC and IEX. The enzyme was purified nearly 3.5 folds; 45% of yield with 28.7% of the total proteins in crude cell extract. The specific activity of the purified AzoB was estimated to be 10.1 U/mg protein using Orange I as a substrate in enzyme assay. SDS-PAGE was carried out of the purified protein and the size was estimated to be 22kDa which was further confirmed via Gel Filtration Chromatography.

Yang [34] reported in his research work regarding the extracellular azoreductase production by *Pseudomonas* putida WLY for the decolorization of reactive Brilliant Red X-3B (used as a carbon source). The extracellular enzyme was purified via ion-exchange chromatography and gel-filtration chromatography. The molecular weight was estimated by SDS-PAGE to be 28 KDa.

Vijaya [35] conducted research on the oxygen insensitive *azoreductase* for the degradation of methyl red, purified from *Pseudomonas aeruginosa*. The enzyme purification was carried out after harvesting the cells from the mineral salt medium (dye decolorized). The cells were disrupted via sonication, centrifuged (12,000g) and the cell debris was removed. Supernatant was further proceeded with ammonium sulfate fractionation, 40% ammonium sulfate saturation to remove impurities and 70% saturation to precipitate *azoreductase*. The precipitated protein was separated via centrifugation (10,000g; 30mins) and the pellet was resuspended in 10 ml phosphate buffer. Desalting was done via overnight dialysis against phosphate buffer. The clear supernatant obtained upon centrifugation of the dialysate was applied on DEAE-cellulose anion exchanger. The protein was eluted from the column via buffer with 0.1-0.3M NaCl. The fraction obtained was then individually dialyzed prior to the *azoreductase* activity assay.

A study conducted by Oturker [36] on the synergistic action of *Bacillus* lentus BI377 (alkaliphilic strain)

azoreductase (flavin containing, NADH dependent) and cytochrome P450 monooxygenase system to carry out the degradation of 98% recalcitrant azoic compounds. The purification of this induced intracellular *azoreductase* was carried out by initial ammonium sulfate precipitation (80%), followed by DEAE-cellulose anion exchange chromatography and finally size exclusion chromatography on Sephadex G-100. Single band of purified protein was visualized on SDS-PAGE.

Purohit and Desai [37] reported one step purification of *azoreductase* (Azor 1 KF803342) produced by *Pluralibacter gergoviae*. The one step purification was by using Hydrophobic Interaction Chromatography (HIC). Shah [38] and Saranaj [37] reported *Nocardia sp. and Alcaligenes sp., Bacillus cereus, Bacillus odyssey, Bacillus subtilis, Bacillus thuringiensis and Nocardiopsis alba* respectively for the production of *azoreductase* and its subsequent purification. The strategy of purification is depicted via Flowchart 2.

The strategy and the combination of purifying *azoreductase* from different bacteria are more or less same as shown in Table 3. However, the table below represents the purification techniques used from 2016 to 2020.

Toxicity Assays

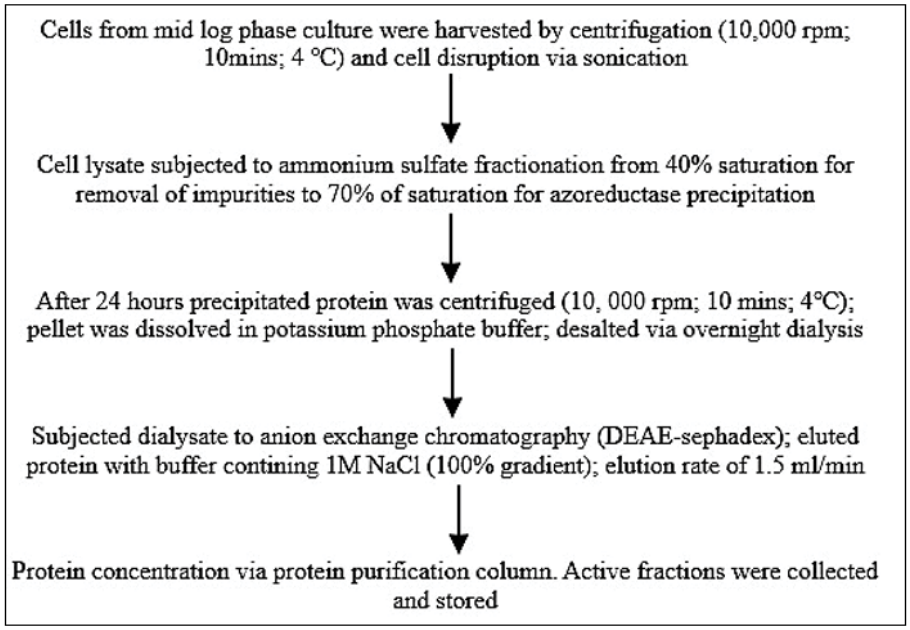
The toxicity of the by-products which are produced as a result of cleavage of azo-dyes by *azoreductases* was analyzed via HPLC and FT-IR, evaluation and the assessment of the metabolites toxicity in cell culture broth as reported. *Pseudomonas luteola* was the microorganism under study. Similarly, the microbial toxicity was analyzed of the decolorized dye via toxicity well assay. This assay was reported for *Alishewanella sp*. CBL-2 [38].

Enzyme Activity Assays

The industrial demand of an enzyme requires retention of specific activity. This enzyme is used for various purposes in industries so, to analyze the specific activity of enzyme after purification and prior to purification is necessary to be assessed. This is done to check the shift in activity after purification. As, mentioned in purification the earlier traditional *azoreductase* assay carried out by Zimmermann [39]. The enzyme assays conducted and the reaction mixtures with specific activity upon purification are tabulated below (Table 4);

Temperature and pH Stability of the Purified Enzyme

Azo Reductases are the enzymes that are proteins. Being proteins after being purified it is very important to save them from denaturation [40]. In order to save the enzyme from denaturation, it must be ensured that the physical parameters remain to an optimum range for a given enzyme. Temperature and pH are among the most important parameters that can act as denaturing agents if not controlled or set to a specific limit will certainly



Flowchart 2: Purification strategy of bacterial azoreductase.

Table 3: Overview of purification steps for bacterial azoreductases.

Azoreductase producer	Purification steps	References
Shewanella xiamenensis BC01	• Recombinant AzrS collected via centrifugation • Sonication • Centrifugation • His-Trap affinity chromatography • Elution with sodium phosphate buffer made with imidazole & NaCl	[50]
Bacillus licheniformis	• Harvested cells were treated with Lysozyme DNase • Lysate clarified by centrifugation • Supernatant was added with ammonium sulfate (4°C) and EDTA (0.5mM) • Ion-exchange chromatography • Affinity chromatography	[39]
Shewanella sp. Strain IFN4	• Membrane bound azoreductase • Crude extract subjected to (NH4)2SO4 precipitation 25% to 70% saturation • Desalt via desalting column PD-10 • Ion Exchange (Q-Sepharose Fast Flow)	[14]
Halomonas elongate	• *E.coli* (BL 21 and Rosetta-gami) transformed with mutated plasmid for improving thermal stability of Azoreductase • Transformed bacteria was harvested via centrifugation • Sonication • Centrifugation and supernatant loaded on nickel column • Elution of protein with 100mM imidazole • Buffer exchanged to remove imidazole via Amicon ultra-15 centrifugal filter unit	[26]
Chromobacterium violaceum	• Culture centrifuged; sonication with lysis buffer • Centrifugation; supernatant loaded on pre-equilibrated Ni-Nitrilotriacetic acid column • Elution with elution buffer (Imidazole in Tris-HCl with NaCl) • Dialyzed purified protein for removal of imidazole • SDS-PAGE (analysis of purified protein & size estimation)	[43]
Bacillus wakoensis A01	• Single step affinity chromatographic purification • Mechanistic and crystallographic studies	[34]

harm the integrity of the protein. Both factors are briefly described below [41,42];

pH

Majority of the known *azoreductases* offer the optimum activity and stability between the pH ranges of 5-9 [43-45]. This range includes some of the *azoreductases* from the alkaliphilic family. The ranges that are deviating from the given optimum range will possibly render the enzyme structure to lose and in return it will be inactivated due to denaturation [46-49].

Temperature

Likewise, pH, temperature is also very important like pH. The stability for the temperature or the thermal stability for the *azoreductase* enzymes lies within the range of 25-85 degree celsius temperature. Out of which the majority of the *azoreductase* enzyme work optimally between the temperature ranges of 35-40 degree celsius. The thermophilic strains that are producing *azoreductase* enzyme may have the temperature stability upto 85 degree [50-52]]. Some of the important examples of organisms that produce *azoreductase* enzyme with their temperature stabilities is given in a Table 5.

FUTURE PERSPECTIVE

Due to the very evident action of *azoreductase* enzymes, it has been explored the vast potential of this enzyme and its variants to reduce the azo bond of the dyes and transform it into a nontoxic alternative. The increase in industrialization and the ever more increase in use of synthetic dyes is going to be very important to treat the toxic effluent from the industries than ever before. To meet this challenge, it would be the need of the hour to

Table 4: Specific azoreductase activity assay for purified enzyme with difference in provided substrates and assay conditions.

Azoreductase producer	Reaction mixture	Assay conditions	Specific activity upon purification	References
Pseudomonas KF46 (Purified Orange II azoreducatase)	• Potassium phosphate buffer • NADH • Orange II* • Enzyme solution	Thermostated cuvette (30°C); Air saturated; 482nm	17833 U/g; 43% yield; 119.7 folds	[51]
Pseudomonas aeruginosa (Oxygen Insensitive azoreductase-Methyl Red)	As reported by Zimmermann et al. (1982) with Methyl Red dye in place of Orange II	Room temperature incubation; 430nm	22.5 U/mg; 25.7% yield; 15.73 folds	[45]
Norcadia spp. (Azoreductase- acid red degrader)	• Potassium phosphate buffer • Acid red dye • NADH • Sample	502nm	375 U/mg; 11.9 yield; 41.6 folds	[38]
Pluralibacter gergoviae (Azoreductase-Azor1 KF803342)	• NADPH1 • Enzyme solution	535nm	26388U/mg; 45.2% yield; 25.13 folds	[30]
Shewanella xiamenensis BC01 (Robust recombinant azoreductase- AzrSx_1)	• Phosphate buffer • Methyl red • NADH • Enzyme solution	430nm	97153 U/mg; Vectors were pET32a; pET28a; pET20bl	[50]
Shewanella sp. Strain IFN4 (Membrane bound azoreductase)	Zimmermann et al. (1982) modified method with anaerobic solution of buffer with NADH and Reactive black dye	597nm	139.63U/mg; 42.25 yield; 39.3 folds	[14]
*Orange II can be substituted with other azo-dyes in reaction mixture as 1-(4'sulfophenylazo)-2; 4dihydroxynaphthalene; 1-(4'-sulfophentlazo)-naphthalene. Absorption at 430nm				
[1]Role of co-factors in enzyme activity was analyzed; NADH, NADPH, NADPH+Riboflavin; NADPH+NAD				

Table 5: Important bacterial species with their temperature stability for azoreductase enzyme.

Bacteria	Temperature stability of azoreductase (°C)	References
Pseudomonas aeruginosa	35	[24]
Bacillus badius	60	[17]
Enterococcus faecalis	35	[7]
Halomonas elongata	30	[11]
Shewanella xiamenensis	20	[50]
Escherichia coli	37	[25]
Staphylococcus aureus	55	[8]
Clostridium perfringens	47	[23]
Bacillus lentus	70	[27]
Aquiflexum sp.	80	[20]

treat the effluent enzymatically, and that task would be preferably performed by the *azoreductases*. This would lead in the adaptation of the towards developing new variants of the enzyme through genetic engineering and recombinant gene technologies. This would help in the production of more potent *azoreductase* with higher efficiency for action in different environments. Currently they are being engineered to act on metabolites other than only azo dyes. This might open up for new possibilities in metabolic advances. Still a lot more bacterial strains are being discovered for having the ability to synthesize this enzyme, ongoing studies will help researchers to look up for new domains to be exploited. Attention has been drawn towards the mass production of *azoreductase* enzyme that is definitely going to aid and supplement the ever-increasing demand of *azoreductase* enzyme to bring revolution in industries and also to sustain the environment by reducing the toxic compounds in bulks.

CONCLUSION

This review presents the detailed analysis on the *azoreductase* enzyme. The enzyme reduces the azo dyes by cleaving azo bonds and rendering them nontoxic and non-carcinogenic. This special feature of *azoreductases*

has led the focus of this review towards the various features and their applications, them being sourced by the numerous bacterial strains and producing recombinant strains to get desirable activity and methods that are involved in getting the purified enzyme extracts to be used as per purpose. A lot of work is being done for proper implications of the enzyme. Enzyme assays and toxicity assays are performed to have a better understanding of the potencies and activities of this enzyme. Researchers see a better and safer future that is threatened by the overuse of a variety of toxic dyes, by the mercy of the tremendous ability and activity of the *azoreductase* enzyme.

CONFLICT OF INTEREST

The author declared no conflict of interest.

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