Biotransformation of Diphenylamine by *Stenotrophomonas DL18* by UV-Vis spectroscopy and HPLC

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Abstract: Most of the times industrial effluent is observed alkaline. The waste is heavily loaded with aromatic amines like Diphenyl amine (DPA), nitroamines, aniline, anisidine, benzidine etc. It is necessary to make the water free from these contaminants presents in it for healthy ecosystem. Physicochemical methods are harsh and costly. To overcome this problem biodegradation in alkaline condition can be a good remedy. Hence an attempt was made to study the way of bio-transformation using an alkaliphilic Strain *Stenotrophomonas* DL18. It was noted that 99 % of 20mg/100ml DPA was degraded within 24 hrs by the experimental strain; while 100% of DPA was degraded for other experimental concentration of DPA within the same time. Aniline, Catechol, Muconic acid was detected by HPLC as catabolites. CYP450, Superoxide dismutase, Cat 1, 2 dioxygenase, Cat 2, 3 dioxygenase, Acetanilide hydroxylase enzymes were found induced within 24 hr experimental time. The Strain *Stenotrophomonas* DL18 was actively involved in biodegradation.

Key Words: Biotransformation, Stenotrophomonas, Diphenyl amine (DPA) .Xenobotics, HPLC.

Abbreviatioons: SOD – Superoxide dismutase, CYP450- Cytochrome P450, Cat 1,2 dioxxygenase – Catechol 1,2 dioxygenase.

1. Introduction: Several antimicrobial and antifungal compounds were synthesized from Diphenylamine [1]. Diphenylamine showed inhibitory effects on the photosynthesis of phototrophic bacteria. It was also reported that DPA is inhibitor of carotenoid synthesis of a few photosynthetic bacteria [2]. It is carcinogenic and also affecting kidney functions in mammals. Contact to eyes is corrosive and lead corneal opacity, blackening of urine, decrease in erythrocyte count, significant increase in alkaline phosphatase, and relative increase in weight of liver, spleen kidney, gonads if ingested. Hematopiosis and cystitis also observed at higher concentration or over exposure. Although it has various eco-toxicological effects, few of the microorganisms can catabolise the contaminants aerobically and or anaerobically in environment. They use it as a carbon and energy source [3]. As the Diphenyl amine is toxic and used for controlling fruit pest; its residual content was determined [4]. Many amines are mutagenic and carcinogenic [5]. Diphenylamine (DPA) is a primary pollutant widely used as a stabilizer in explosives and as precursor for pesticides, pharmaceutical products and dyes [6].

DPA analysis from gun propellant was carried by Jelisavac, L. and M. Filipovic [7]. Diphenylamine is a common contaminant at polluted sites as well as at aniline manufacturing sites. DPA also reacts with nitric oxides to form nitrated derivatives of DPA [8]. Most of the aniline and diphenylamine manufacturing sites are contaminated with aniline resulting in contamination of ground water as well as surface water resources. Aniline can seriously affect on human health [9]. Biodegradation is the process in which persistent organic substances are transformed by living organisms into less toxic or non toxic substances in presence of oxygen or in absence of oxygen. Little is known about the biodegradation of Diphenylamine in alkaline condition. An attempt was made to investigate the biotransformation of Diphenyl amine using *Stenotrophomoas maltophila* DL18. The NCBI access number of the experimental isolate from Salt Lake Lonar, Buldana, (MS) was noted as JN995612.

2. Material and methods:

Chemicals: Inorganic chemicals were purchased from Sigma and SRL Mumbai. Culture media for bacteria were taken from Hi media Mumbai. DPA was obtained from Department of Biochemistry, Pune University.

2.1 Bacterial isolation and media preparation:

The Strain *S. maltophila* DL18 was isolated by Serial dilution and Streak plate technics on agar plate and cultured on composition containing yeast extract, peptone, NaCl - 5g/L respectively and the micronutrients' in mg /L were KH_2PO_4 -170, Na_2HPO_4 - 290, $(NH_4)_2SO_4$ -100, $MgSO_4$, MgO-0.1, $FeSO_4$ -0.05, $CaCO_3$ 0.20, $ZnSO_4$ - 0.08, $CuSO_4$ - 0.016, $CaSO_4$. 0.016, Boric acid 0.06, and pH 9. The pH was adjusted by sodium bicarbonate (0.1 M or 1M) solution. The media was sterilized by autoclaving at 121 ^{0}Cfor 15 pounds at 20 minutes. The solid media was prepared in same way by adding 2% agar. The same broth media of pH 9 was used for biotransformation study except the pH parameter changed. The Diphenyl amine was added aseptically during experimental start up.

2.2 Biotransformation Study:

Six 250 ml conical flasks with 100 ml alkaline broth media were used for bio-degradation study.1% inoculum of 24 hr. grown culture possessing 0.6 OD at 600 nm was added and allowed to grow for 24 hr in shaking incubator at 37 ^{0}C, 120 rpm. At the end of 24 hr experimental concentration of Diphenyl amine was added in each flask keeping one as an abiotic control among them separately. The experimental flasks were monitored after six hr. intervals. All experiments were carried in dark.

2.2.1 Determination of Spectral changes and residual concentration:

The spectral changes in DPA were noted at 250 to 320 nm in the supernatant after cold centrifugation at 10000g.

The residual remains were analyzed by using Spectrophotometer Jasco Varian 630; monitoring the aliquots absorbance at 285 nm with proper dilution of the supernatant.

2.2.2 HPLC Analysis:

As per the 0 to 6 hr. interval the working flasks were used for solvent extraction with Dichloromethane. The residual metabolites were identified by HPLC running standards and comparing the directory of Software LC solutions. The HPLC instrument used was Shimadzu UFLC Prominance, column used was C18 Phemonex Luna, Colum length and diameter 250 x 6 mm diameter, 5micron particle size, 20 micro liter Rheodyne manual sample injector loop, Column at Room temperature, Detector UV- vis, flow rate 1ml/Min, methanol: water 1:1 as a Solvent

2.2.3 Enzyme study:

Cell mass was harvested after 24 hours induction with Diphenylamine by Du-Pont Sorvall RC-5B centrifuge by spinning at 10000 x g for 15 min at 4^0C. The cell mass was washed with phosphate buffer pH 8.0 twice and physiological saline. Cell disruption was carried by Sonicator Ultra O Sonic (Mumbai) in Tris buffer pH 7.50. The resulting homogenate was centrifuged in cold condition at 15000 x g for 20 min. Protein determination and enzyme activities were carried by Standard methods.

3 Results and discussion:

3.1 UV-Vis Spectrophotometric Analysis:

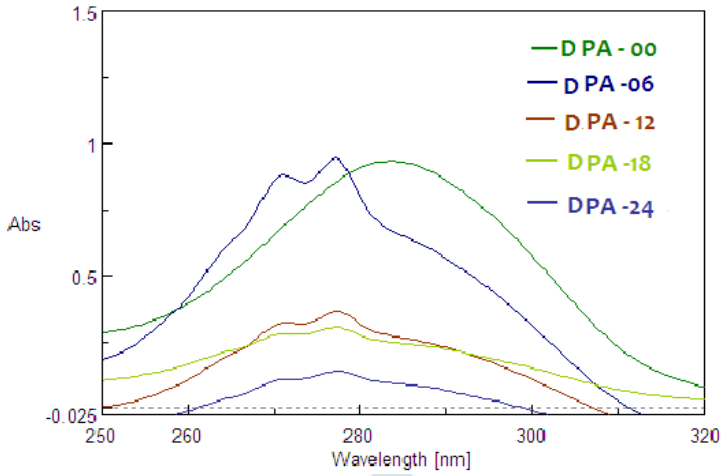

Fig.1 Biotransformation of Diphenyl amine by *Stenotrophomonas maltophila* DL18

The **(Fig.1)** clearly indicates that the DPA spectrum observed at 285 nm at 0 hr. Shifted to left from 285 nm indicating the biotransformation in Diphenylamine. On the basis of reduced OD at 285 nm of properly diluted aliquots the residual concentration was monitored after 6 hr interval and interpreted (**Fig. 2**) in the form of residual percentage.

3.2 Residual concentration of DPA after degradation by *Stenotrophomonas maltophila* DL18:

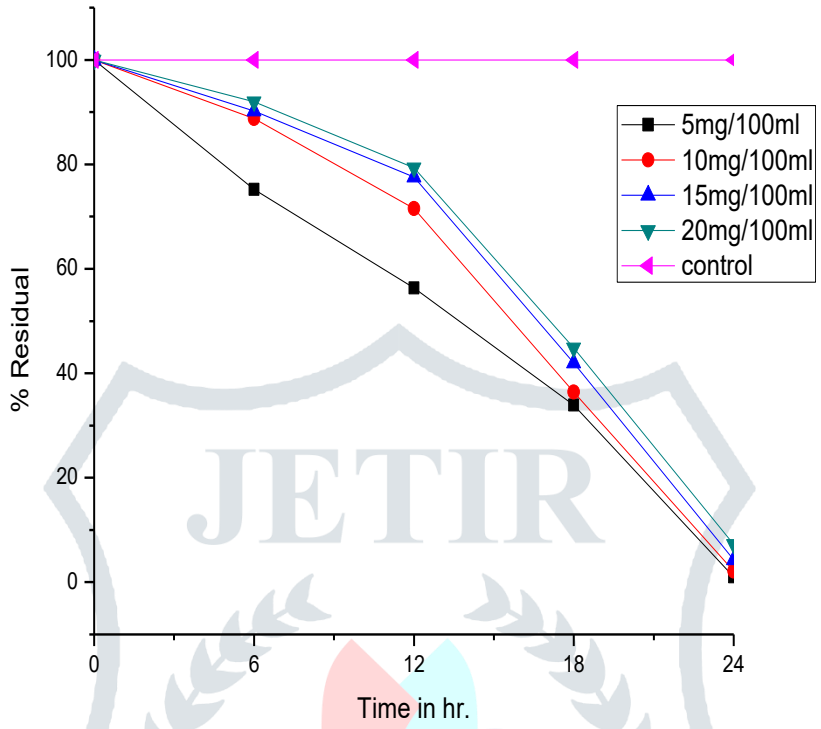

Fig .2 Residual concentration of DPA in percentage after degradation at various times.

The (**Fig.2**) showed that the higher concentration of DPA has vanished to zero level within 24 hrs. It suggested that the strain *Stenotrophomonas* DL18 have high degradation capacity for DPA. This might be due to resistance factors on its main genome or plasmids. As the variation in degradation concentration was marginal. As it was adapted for DPA for three to four months it made habitual for it. Although it is an indigenous isolate of Salt Lake Lonar, Buldana (MS.) as an extremophile it survived and tolerated higher concentration in alkaline state. The (**Fig.2**) illustrates almost 100% degradation by the experimental strain within 24 hrs.

3.3 HPLC Analysis:

3.3.1 The DPA HPLC peak at RT 4.7 min monitored at UV detector

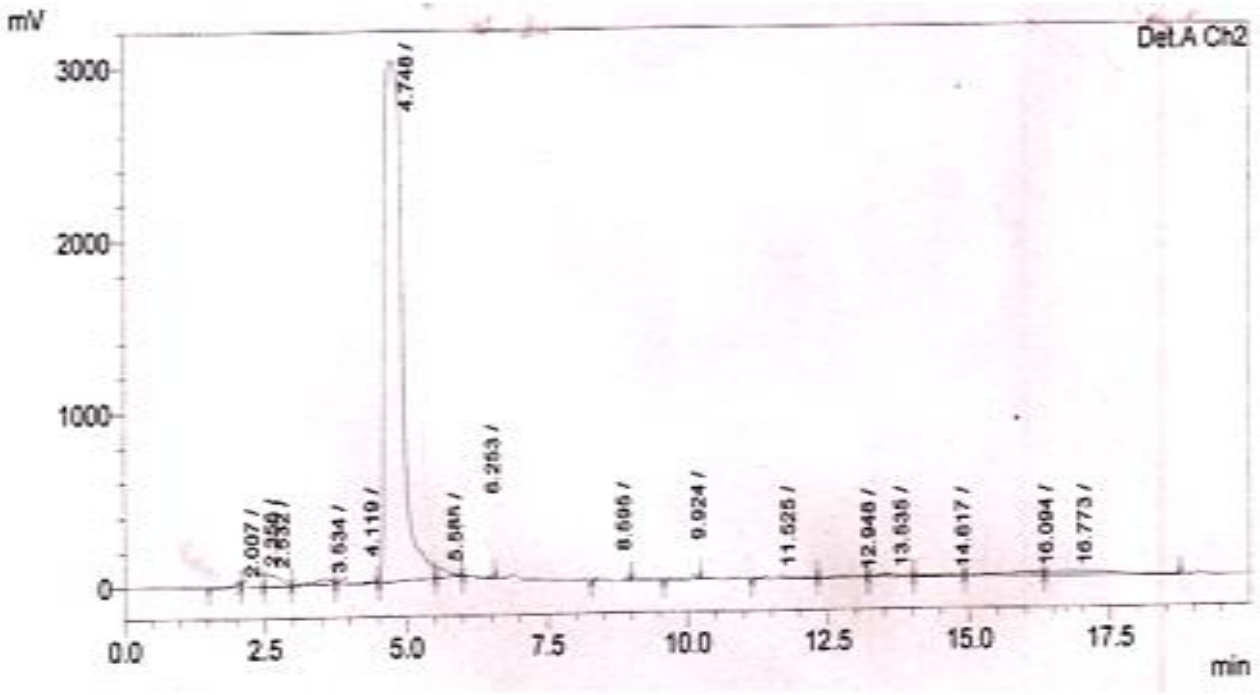

Fig.3a. Diphenylamine at 0 hr. by HPLC with UV-Vis. Detector

The HPLC data (**Fig. 3 a, Fig.3b, Fig.3c, Fig.3d, Fig.3e**) are showing the peaks at different time intervals with different retention time. Newly emerging peaks indicates the new metabolites generating from DPA. At the same time earlier few peaks observed were found disappeared. Particularly from (**Fig. 3a**) it was noticed that all DPA (**RT - 4.72**) has been converted to central metabolite (**RT-11.4 min**) as catechol (**Fig.3d**). The (**Fig.3e**) showed one another metabolite peak identified as cis-cis muconic acid at (**RT- 15.3 min**).

3.3.2 HPLC Data at 6hr:

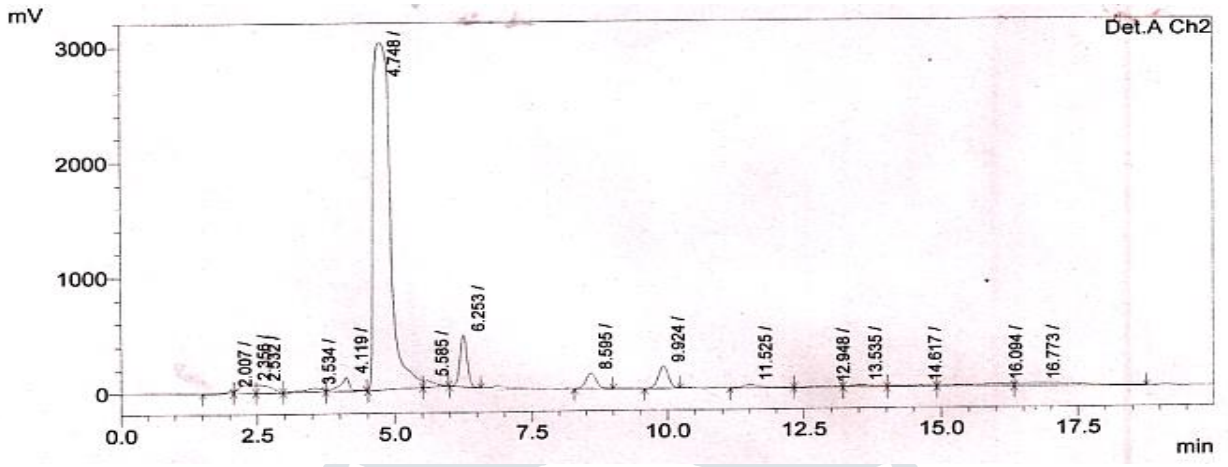

Fig.3b. Diphenylamine metabolites emerging at 6 hr. by HPLC with UV-Vis detector

The difference in (**Fig.3a to Fig.3b**) clearly indicates that several new peaks were emerged indicating the transformation of DPA. However no change in abiotic control flask was observed.

In case of (**Fig. 3b**) the peak appearing prior to DPA (**RT- 3.7min**) might be aniline between (**RT - 3.55 min** and **4.11min**). The peak emerging at (**RT- 6.2**) min was confirmed as acetanilide. The peak appeared in (**Fig.3b at RT- 8.5**) o-aminophenol. The metabolite observed **in (Fig. 3b at RT - 9.9**) might be anthranilic acid. The peak became enlarge in (**Fig.3c)** confirmed as p-aminobenzoic acid at (**RT 8.9**)

3.3.3 HPLC data at 12 hr.

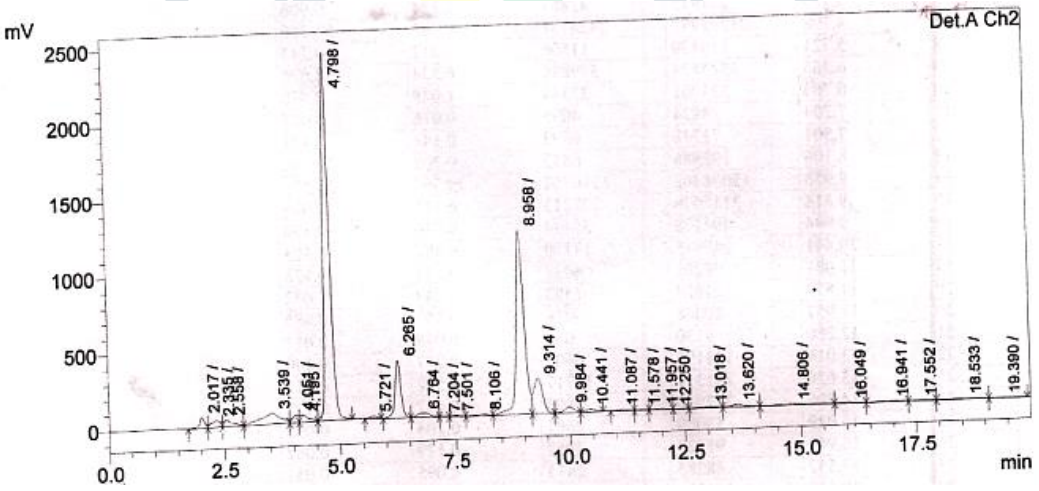

Fig.3c. Diphenylamine metabolites emerging at 12 hr. by HPLC with UV-Vis detector

The Fig. No. 3c showed a new peak at (RT- 9.3) might be acetaminophen.

3.34 Metabolite appeared at 18 hr.

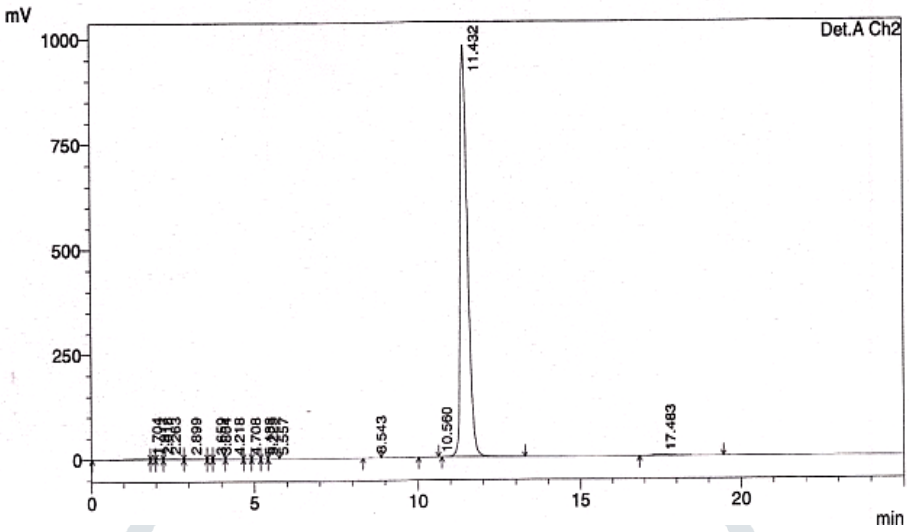

Fig.3d. Diphenylamine metabolites emerging at 18 hr. by HPLC with UV-Vis detector

The (Fig. 3d) Showed a single peak identified as Catechol at RT 11.4. Generally the toxicants goes for ortho or meta cleavage of catechol or sometimes follow both ways. Catechol also exerts toxicity by formation of benzoquinone therefore these types of pathways might be followed. As the benzoqunone forms it affects the DNA level.

3.3.5 Metabolites at 24 hr. (Fig.3e)

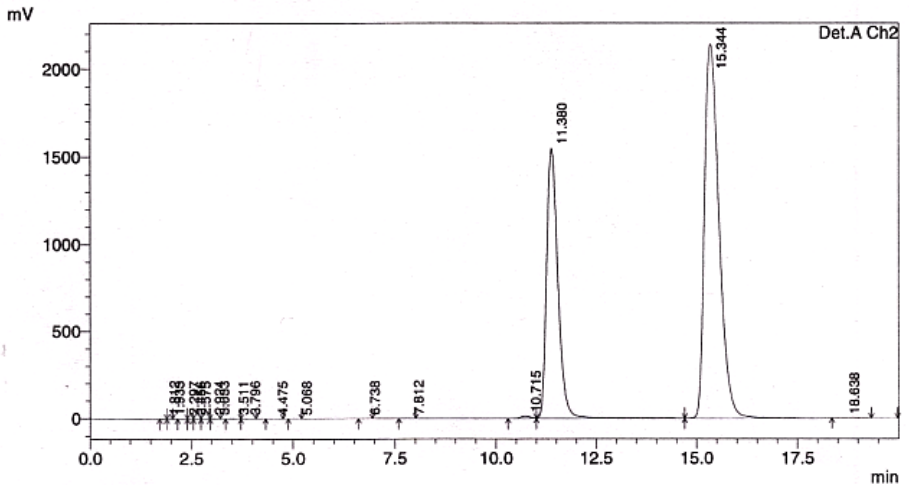

Fig.3e. Diphenylamine metabolites emerging at 24 hr. by HPLC with UV-Vis detector

The (**Fig.3e**) showed two peaks at RT-11.3 as a catechol and RT-15.3 the cis- cis muconic acid. It is clearly indicating that the ortho cleavage occurred in catechol.

Finally Pathway of DPA biotransformation was constructed on the basis of catebolites observed shown in (**Fig.3F**).

3.3.6 DPA Biotransformation pathway:

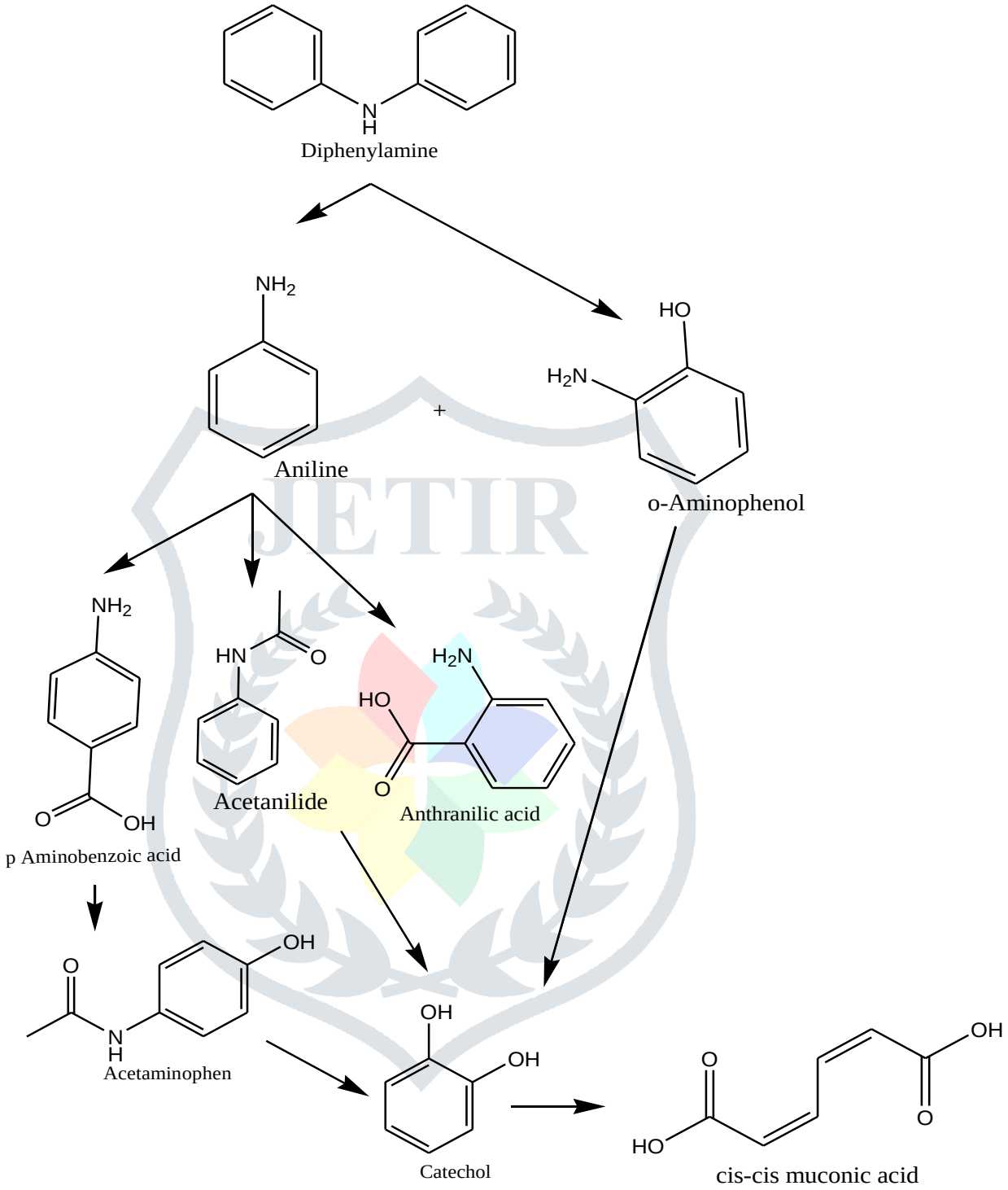

Fig.3F Biotransformation of DPA by *Stenotrophomonas DL18*

3.4. Enzyme Activities:

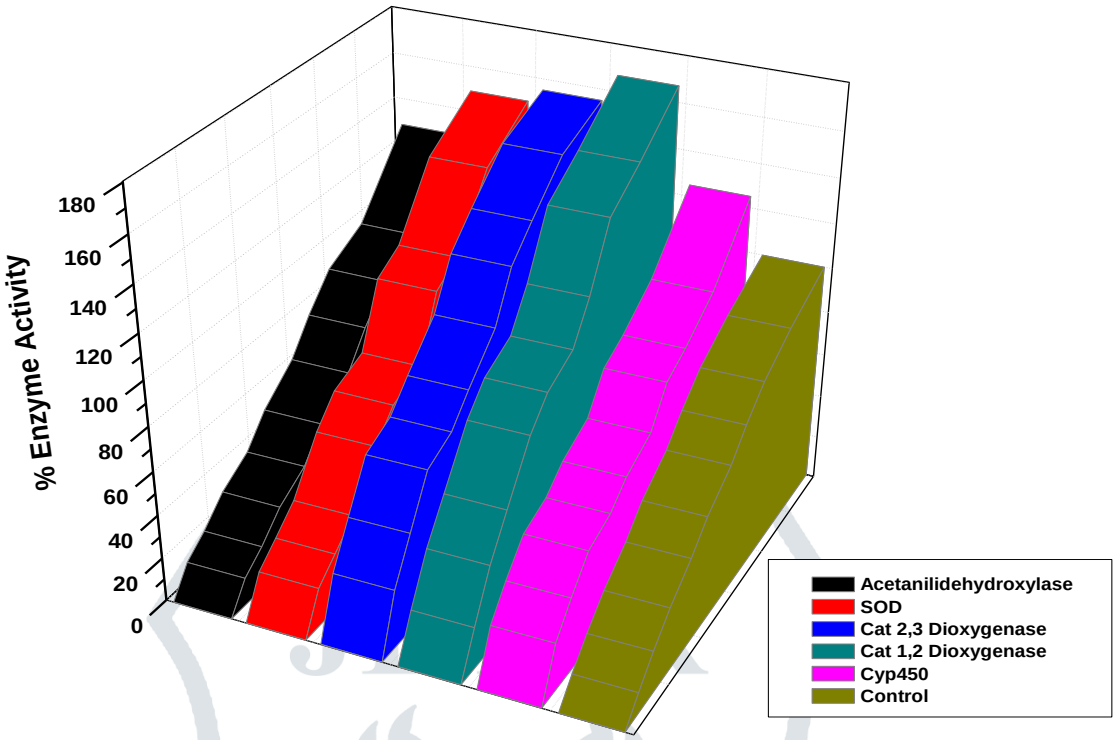

Fig .4. Enzyme inductions at 24 hr.

Biodiversity of microbes is being exploited for various purposes like isolation of enzymes, drugs, and or several other objects. Neutrophiles, acidophiles or alkaliphiles are having different versatile genes on their nuclear material or their plasmids. Many of these organisms have resistant factors on their main genome or plasmids. These show resistance against toxicants. Aromatic amines comprise one of the major groups of carcinogens. At many manufacturing sites the soil and water are extensively contaminated with aniline and DPA [10]. The microbial degradation of recalcitrant molecules takes place rapidly in the environment with acidic or neutral pH but, the hyper saline and extreme halo alkaline conditions of lakes and mangroves limits the microbial hydrolytic activity on complex xenobiotics [11]. Halophiles are being employed for hydrocarbon degradation as well as various metabolites and enzyme isolation [12, 13]. Aromatic amines are generally not metabolized further under anaerobic conditions and hence aerobic post-treatment required for the complete mineralization of azo dyes [14] due to generation of aromatic amines and benzoquinone. Bioremediation of aromatic amines was studied by M. Delnawaz, et al.in 2008 but it mentioned the degradation in terms of COD [15].

Aerobes have an oxygen based metabolism in order to obtain energy. Anaerobic organisms having less growth rate comparative to aerobes and hence they are more important. The aerobic process improves the environmental condition. Extremophilic microorganisms are adapted to grow and thrive under such adverse conditions by which it makes easy the catabolism of xenobiotics. Thus biodegradation is an ideal candidature for the biological treatment of effluents in extreme habitats. Further, those adapted to more than one extreme condition offers a special potential for the biological decontamination.

Halomonas organivorans catabolized several aromatic compounds like benzoic acid, p-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenyl acetic acid, phenyl propionic acid, phenol, p-coumaric acid, ferulic acid ,p-aminosalicylic acid etc [16]. DPA and its derivatives are having potential hazard to ecotoxicological point of view [17]. Halophiles are adapted to extreme salinity and alkalinity; therefore these microorganisms are good candidates for treatment of saline effluents or organic recalcitrant's in alkaline conditions [18].

As persistent organic pollutants are bio-accumulative and toxic as well as resistant to degradation. Sometimes they accumulate in the tissues of living organisms, where they can produce undesirable effects on human health and or the environment in certain level. Distinct catabolic genes are either present on mobile genetic elements, such as transposons, plasmids, or the chromosome itself that facilitates horizontal gene transfer and enhances the rapid microbial transformation of toxic xenobiotic compounds[19]. The transformation leads to generate metabolites that can be either less toxic or even more toxic. Mineralization results in complete degradation of an organic chemicals to stable inorganic forms of C, H, N, P, etc.

Aniline cancer had been detected in dye workers specifially as bladder cancer. Aniline however induces intra chromosomal recombination in *Saccharomyces cerevisiae*, resulting in deletion (DEL) of intervening sequences. It has investigated the generation of oxidative free radical species by aniline and/or its metabolites may be lead to recombinagenic activity in yeast. The toxicity and recombinagenicity of aniline in yeast were greatly reduced in the presence of the free radical scavenger IV-acetyl cysteine. Aniline cytotoxicity was many-fold increased in strains of *S.cerevisiae* lacking the antioxidant enzyme superoxide dismutase [20]. There is reductive deamination found in Desulfobacterium anilini through 4-aminobenzoyl Co-A [21]. Catechol is a central intermediate in the degradation pathways of various aromatic compounds [22]. *Delftia tsuruhatensis* AD9 was responsible for the complete metabolism of aniline to TCA-cycle as a sole source of carbon, nitrogen, and energy [23].

Diphenylamine and its derivatives are still produced worldwide by the chemical industries which may be one of the sources of aniline contamination in soil and ground water [24]. However biological treatment methods are additive to physical and harsh chemical methods for remediation. It may be supportive or more beneficial to other methods like adsorption, desorption, filtration or photo catalytic methods employed today.

3.4.1 Enzyme induction Study: The [Fig.4.] describes the induction of biotransforming enzymes. In recent years considerable attention has been dedicated to the mechanisms involved in capacity to metabolize xenobiotics, mainly by cytochrome P- 450 dependent metabolic activities. Studies in human liver microsomes have demonstrated the existence of a number of different cytochrome P-450 isozymes able to carry out drug metabolism.

As alkaliphiles are being employed for various purposes like isolation of erythromycin [25], keratinolytic [26], proteolytic [27] cellulolytic [28] and amylase like enzymes[29]. Interest have been increased in biotransforming enzymes like catechol dioxygenase [30], azo reductase [31], nitroreductase [32] etc. Here an attempt was made to study the induced enzymes during bio-transformation of DPA in *Stenotrophomonas* DL18

Cytosolic protein was estimated by Lawry [33] after cold centrifugation in the supernatant. Superoxide dismutase activity was carried by Mishra and Fredovic [34]. Cytochrome P450 was determined by Omura and Sato [35]. Enzyme activities of Catechol 1, 2 dioxygenase and Catechol 2, 3 dioxygenase were studied by Philip D. Strachan[36]. Acetanilide hydroxylase was performed by Weiseberg and Goodal [37].

There are several reports stating that the azo dye on biotransformation leads to produce aromatic amines like p-phenylene diamine, Benzedene or aniline [38, 39]. Diphenylamine is a common structure of non steroidal anti-inflammatory drugs (NSAIDs) which uncouple mitochondrial oxidative phosphorylation. It decreases hepatocellular ATP content and also creates hepatocytic acute cell injury leading oxidative pseudo energetic mitochondrial swelling and phosphorylation. This induces mitochondrial membrane permeability. Dipheny lamine also bother the safranine binding spectra of mitochondria. This spectral shift indicates the loss of mitochondrial membrane potential. It affects the ATP synthesis and adversely affects the lactose dehydrogenase in eukaryots [40].

Thus the toxicants cause to induce enzymes necessary for the catabolism of xenobiotics when it come across the prokaryotic or eukaryotic cell. In this case Diphenylamine induced the CYP450 class of enzymes. It was known by increased content of active protein. As the activity of catechol 1, 2 dioxygenase and Catechol 2,3 dioxygenase are higher to other enzymes activities investigated. It seems that *Stenotrophomonas* DL18 followed the central catechol pathway by ortho as well as meta cleavage. Catechol is also toxic in higher concentration and hence the bacterial cells might consume catechol with fast rate implementing both the pathways simultaneously. It might happen to be benzoquinone for certain extent from catechol generated by this way or certain other circumstances. The super oxide dismutase induction supports the formation of free radicals generation; as it is involved in protection of the cell from lipid peroxidation or nuclear damage.

4. Conclusion:

Stenotrophomonas DL18 is good candidate for bioremediation of aromatic amines contaminated sites as it degraded higher concentration within 24 hrs. It can be a very good source for several industrially important enzymes.

5. Acknowledgement:

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