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Bio-Catalytic Action of Pseudomonas DL17 on Environmental Contaminant Sunset Yellow FCF

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Abstract: Sunset yellow is one of the dyes used in food and other allied industries having yellow to orange red color existing as an environmental contaminant. It could impair hepatic function, and show acute toxicity to leucocytes, growth retardation etc. Prolonged oral administration increases the liver glutathione content as well as induces of hepatotoxicity, genotoxicity, anorexia and decrease in body weights, hair loss, ulcerated skin rashes etc. An effort was done to biomineralization using an adapted alkaliphile Pseudomoas DL7 isolated from Lonar Lake(MS) Buldana .600mg/LSunset Yellow FCF was found completely degraded within 48 hrs. 4-aminonaphthalene-5, 6 diol, 1-sulfonic acid, 4-aminonaphthalene-1-sulfonic acid, catechol were confirmed as a catabolites by GCMS. pH 9.00 was found optimum for biomineralization. Enzyme induction study showed the experimental bacteria actively plays important role in biomineralization.
Keywords: Sunset Yellow FCF, Pseudomonas DL7, biomineralization, contaminat.

I. INTRODUCTION

Sunset yellow shows higher toxicity like chromosomal aberrations, such as deletions/and or fragments, of ring chromosomes. It affects on meristematic cells of *Brassica campestris.* Sunset yellow is one of the dyes used in food and other allied industries having yellow to orange red color existing as an environmental contaminant. Several tons of dye being produced (nearby 900000 metric tons) per year worldwide. Out of which 70% is observed as an azo dye. Thus colored wastewater produced by dyeing practices, are heavily polluted with several chemicals from textile auxiliaries and dyes directly dumped in various water bodies harms the environment. The properties of textile wastewater depend on the production technology and chemicals used. This not only affects the aquatic life but also exerts toxic and carcinogenic effects to human being as well as other aquatic organisms being targeted [1]. Generally the colored fraction adsorbs on the surface particles; if oily reflects sunlight entering the water and interferes in the photosynthetic process of aquatic species affecting growth. It was observed that decolonization of azo dye is an anaerobic reduction process involving four electrons transfer to break one azo bond. Aerobic /anaerobic sequential treatment process was said satisfactory for the decolonization and dye removal[2]. Bacteria from different area can degrade the dye till complete mineralization at its optimal condition[3]. The electron withdrawing nature of the azo bond makes the compound less susceptible to oxidative degradation. Due to high solubility and absorption through in skin it lead to allergic responses. Metabolites generated through azo dye degradation are generally carcinogenic aromatic amines. These could get oxidized metabolically to reductive electrophilic species and can bind to DNA by irreversibly causing mutation and finally affecting the enzyme functions. These xenobiotics increase BOD, COD leading to several diseases and sporadic disorders. Chronic effect of these dyes mainly associated with kidney and liver damage. These dyes may alter the biological composition of soil and also the properties of enzymes and microbial species. Physicochemical methods including adsorption, coagulation, flocculation, ozonization, use of activated charcoal, membrane filtration, electrokinetic coagulation etc. are expensive and inefficient. Oxidation and electro dialysis methods are found very much efficient in color removal but these methods are quite expensive and have operational problems. The sludge formation, and rejuvenation cost of it makes adsorption as an unattractive method for decolorization. So biodegradation becomes an important and attractive decolorizing and detoxification method. Most of the biodegradation reports observed in case of neutrophiles and or mesophiles. Few of the experiments are carried in alkaline condition to detoxify the textile azo dyes by using alkaliphiles[4].

An interesting experiment of reductive degradation of azo dye was carried by using microbial nanotechnology in which the degradation of azo dyes were achieved by hydrogenation. The molecular hydrogen, was concomitantly generated by *C. pasteurianum* used in the reduction reaction[5]. Thus bio-nanoparticles of metals having catalytic properties also being exploited for bioremediation.

Halophilic and halo tolerant bacteria can bethe good representatives mineralization of recalcitrants at high salt concentrations and pH. In recent years it seems the researchers have been focused on halophiles and their abilities of decolorization the azo dyes or various other recalcitrants[6]. Thus an effort was made to mineralization of such contaminant using an alkaliphile.

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II. METHODOLOGY

A. *Chemicals*

Yeast extract, peptone purchased By Hi Media Mumbai, Other organic and inorganic chemicals were purchased by SRL Mumbai,

B. *Media Composition for bacterial Isolation*

The Strain Pseudomonas DL17 (JN595813.1) was isolated by serial dilution and streak plate technique from sediment of Lonar Salt lake(MS). Yeast extract, Peptone and NaCl were used- 5g/L respectively and the remaining micronutrients' in mg /L were KH_2PO_4 -160, Na_2HPO_4 - 280, $(NH_4)_2SO_4$ -95, $MgSO_4$, MgO-0.25, $FeSO_4$ -0.061, $CaCO_3$ 0.20, $ZnSO_4$ - 0.075, $CuSO_4$ - 0.016, $CaSO_4$ - 0.015, Boric acid 0.06, and pH 9.00. 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 21 minutes. The solid media was prepared by adding 2% agar in it. An adapted 24 hr. fully grown 1 ml bacterial culture ($1x10^6$ cells) were used for seeding the alkaline broth of pH 9.00. This culture was further grown for 24 hr. and induced by the experimental concentration (600 mg/L) of dye Sunset Yellow FCF adding aseptically. The pH for both media was used f 9.00 for biodegradation purpose except the study for pH parameter. The dye was added aseptically in working as well as control flasks. The dye degradation study was carried for 48 hrs. By removing the working flasks by 6 hr interval sequentially from shaking incubator at 33^{0}C. further used for solvent extraction and knowing the remains of residual concentration. The residue was purified by column chromatography and the metabolites were characterized by FTIR, NMR and GCMS.

C. *Isolation of Cytosolic Fraction and Enzymatic Study*

Twenty four hrs.' fully grown cell culture was used for enzyme induction with experimental concentration of dye and harvested by Du-Pont Sorvall RC-5B centrifuge by spinning at 10000 x g for 15 min at 4 ^{0}C. The cell mass was washed thrice with phosphate buffer pH 8.0 twice and physiological saline. Cell disruption was carried by sonicator Ultra O Sonic (Mumbai) in the same buffer. The resulting homogenate was centrifuged in cold condition at 15000 x g for 20 min. The cytosolic protein was measured by Lowry using BSA as standard[7].

The enzyme induction study for azo reductase was performed by Punj and others[8,9]. Cytochrome P450 was carried by using (Omura and Sato) method[10]. Catechol 1, 2 dioxygenase and Catechol 2, 3 activities were studied by Nozaki and others[11,12]. The enzyme activity expressed as µM/ min/ mg of protein containt.

III. RESULTS

A. *FTIR at zero hr*

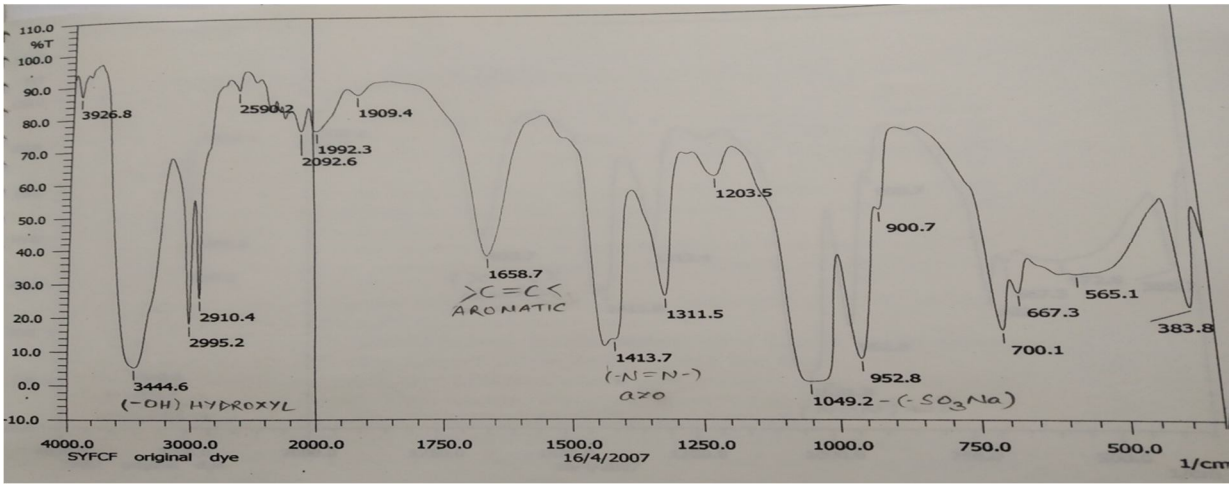

Fig. 3.1 FTIR spectrum of sunset Yellow FCF pure.

The[Fig3.1] clearly indicates the higher absorption of aromatic region at 1658 Cm^{-1}, 1413.7 Cm^{-1}azo bond, 1049.2 $Cm^{-1}SO_3Na$ etc has changed in [Fig3.2] one can find various changes in [Fig3.2] indicates change in functional group absorption. As it is of scientific concern to know the spectrum of R-OH is found broaden indicating the acidic group of azoic dye. Even the '-N=N-' absorption region found narrow than earlier one [Fig3.1]. It suggests the concentration in media lowered during mineralization of the experimental dye.

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B. FTIR at 24 hr

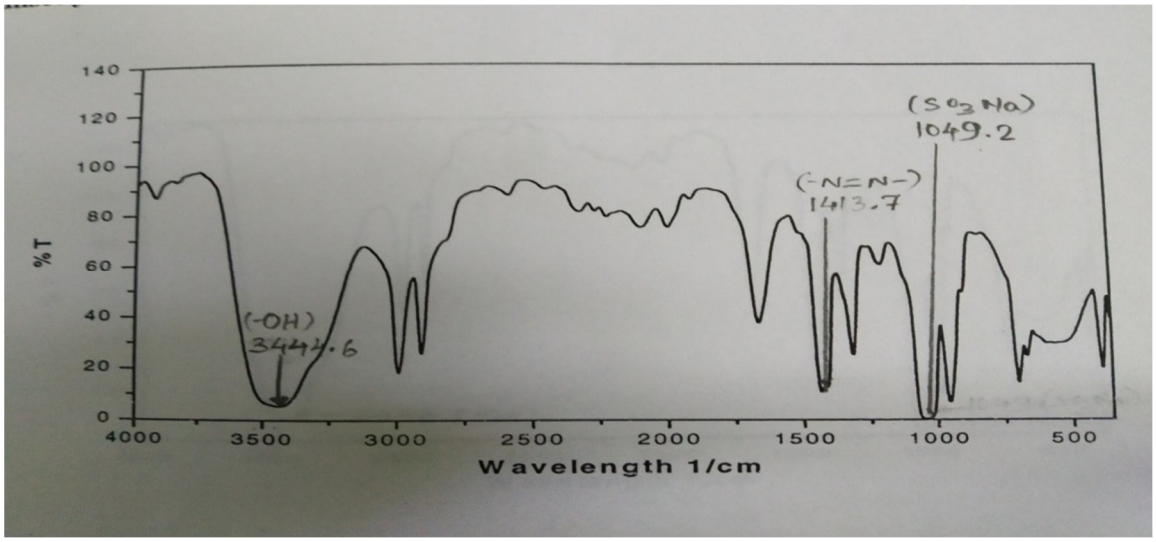

Fig 3.2 Change in FTIR of Sunset Yellow FCF after 24hr.

The spectrum observed at 3442.6 seemes broadens indicating more hydroxylation of the metabolite. Absorption at 1600 Cm^{-1} has almost vanished indicates remaining of traces of aromatic compound or new metabolites generated. Comparatively the azoic absorption also found reduced indicates as far as the dye came across the bacterial cells might have converted into respective amines or amino phenols by breaking the azo bond and the color of the dye also found decreasing slowly indicating transformation in original molecule.

C. FTIR at 36 hr

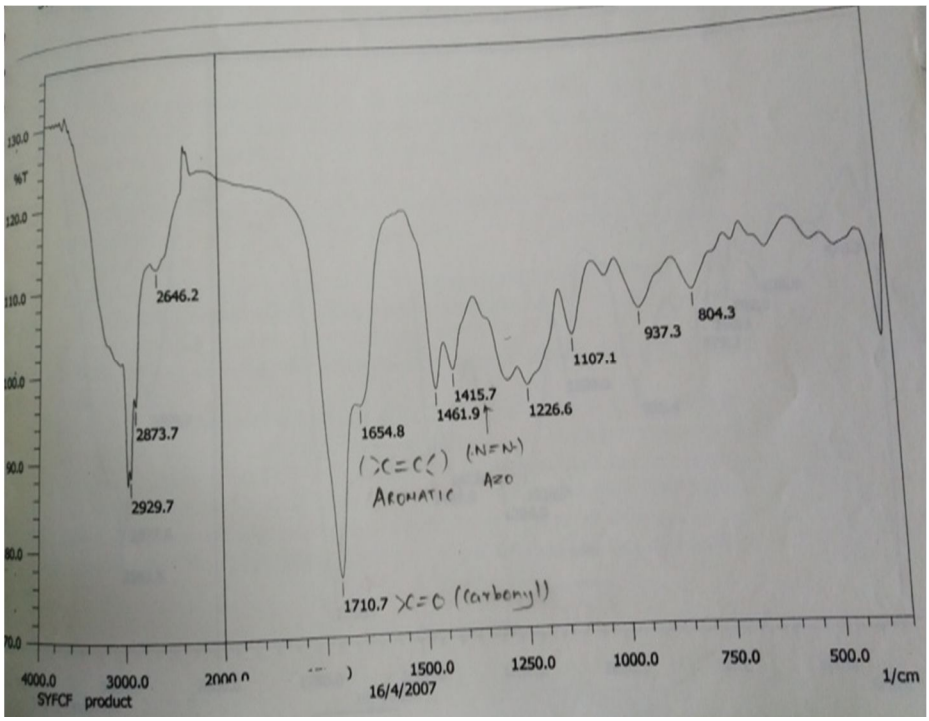

Fig 3.3. Change in FTIR of Sunset Yellow FCF after 36hr.

The [Fig3.3] clearly indicates that azoic bond absorption is very least. It means the dye has been transformed in to respected metabolites. Similarly in [Fig3.4] showed almost all dye degraded by the experimental organism.

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D. FTIR 48 hr

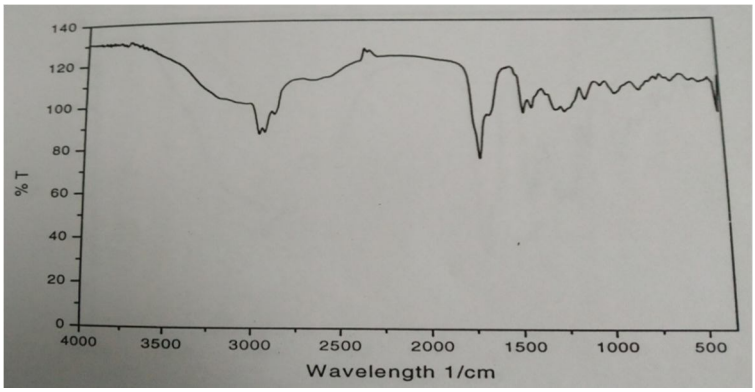

Fig 3. 4 Change in FTIR of Sunset Yellow FCF after 48hr.

The [Fig 3. 4] indicates that most of the infra red light absorbing functional groups are lost; indicating that the contaminant might have undergone several bio-transformations losing its toxicity profile.

E. Effect of pH on Biodegradation

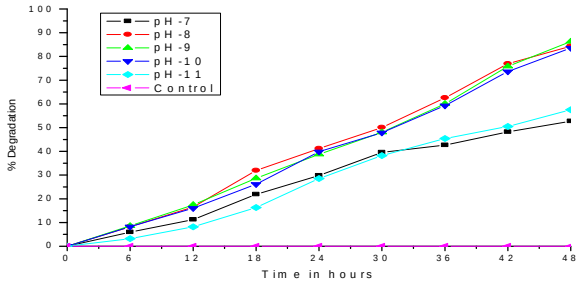

Fig. 3.5 Percent degradation of Sunset Yellow FCF after at various pH

Sunset yellow FCF degradation at various pH was studied and observed that **[Fig 3. 5]** pH 7.00 and pH 11.00 are not so favorable for degradation while pH 9.00 found optimum for degradation. Influence of salts and pH on growth of alkaliphile tolerating high salinity was observed in case of *Thioalkalibacter halophilus* isolated from South-Western Siberian soda lakes[13] supports the more degradation at its optimum P^H. 97.00% degradation of azo dye was reported at p^H 9.00.

F. Effect of Additional Carbon Sources on Biodegradation

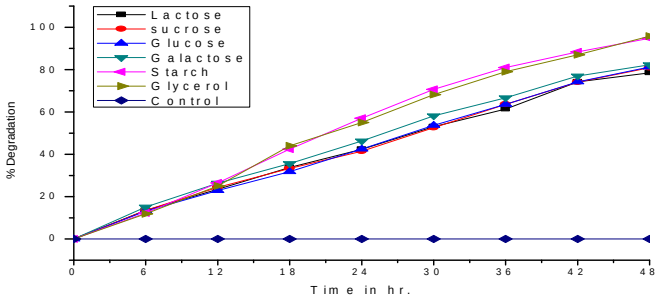

Fig 3. 6 % degradation of Sunset Yellow FCF after bio-augmentation of Carbon 0.2% sources

Influence glucose on biodegradation of 4-aminophenol was reported earlier[14]. Similarly [**Fig 3.6**] showed bio-augmentation of starch and glycerol positively affected on biomineralization of the experimental dye. 100% dye degradation was recorded in case of starch and glycerol augmentation

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G. Effect of Inorganic Salt

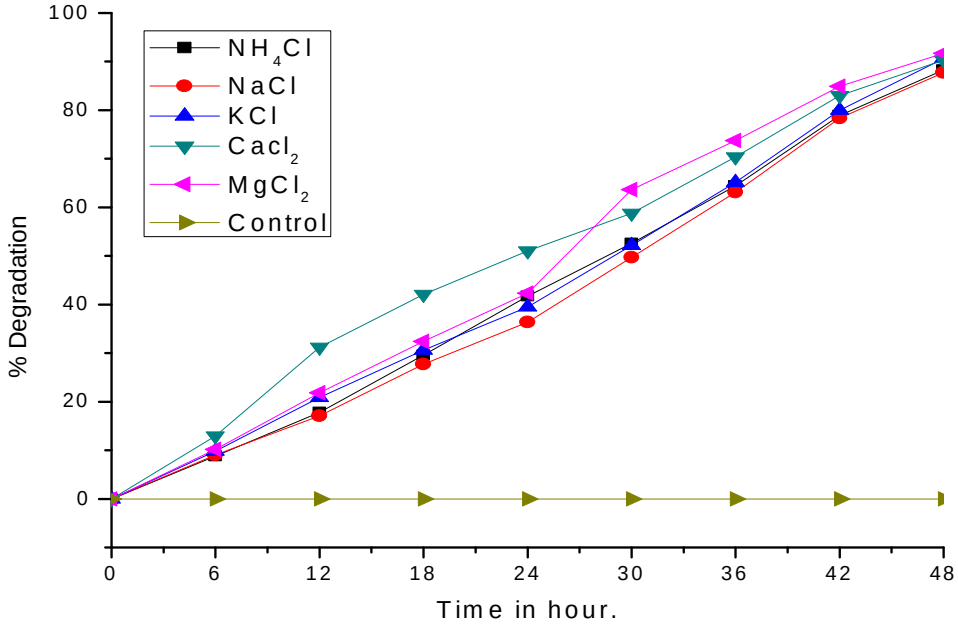

Fig 3. 7 % degradation of Sunset Yellow FCF after addition of Inorganic Salts 0.3 mg/L excess

The trace minerals also play very important for growth and additional concentration of the inorganic salts might favor the degradation rate. The [Fig 3. 7] showed marginal effect on degradation of Sunset Yellow FCF found similarly to that of phenol removal[15].

H. Enzymatic Study

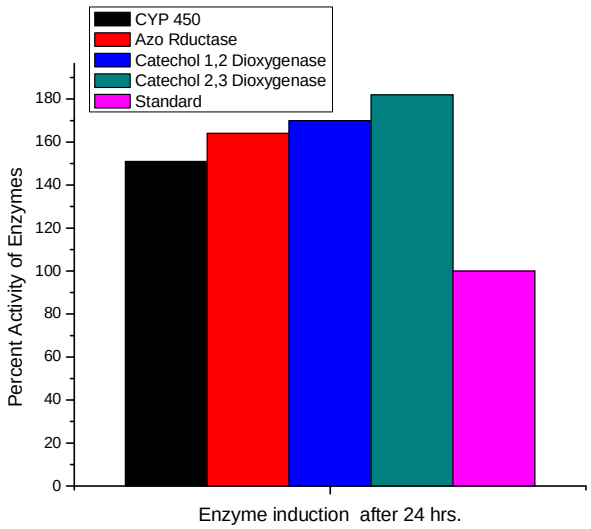

Fig. 3. 8 Enzyme activity after 24 hr Experimental Dye induction

The [Fig 3. 8] Showed CYP450, azo reductase, Catechol 1,2 dioxygenase, Catechol 2,3 dioxygenase hiked ther percent activity vlue. This indicated that the experimental strain actively participated in biomineralization of the dye.

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I. GCMS study and Probable mechanism of Biocatalysis

4-aminonaphthalene-5, 6 diol,1-sulfonic acid, 4-aminonaphthalene-1-sulfonic acid, catechol were confirmed as a catabolites by GCMS study with molecular spectrum of masses 255, 223, 110 respectively.

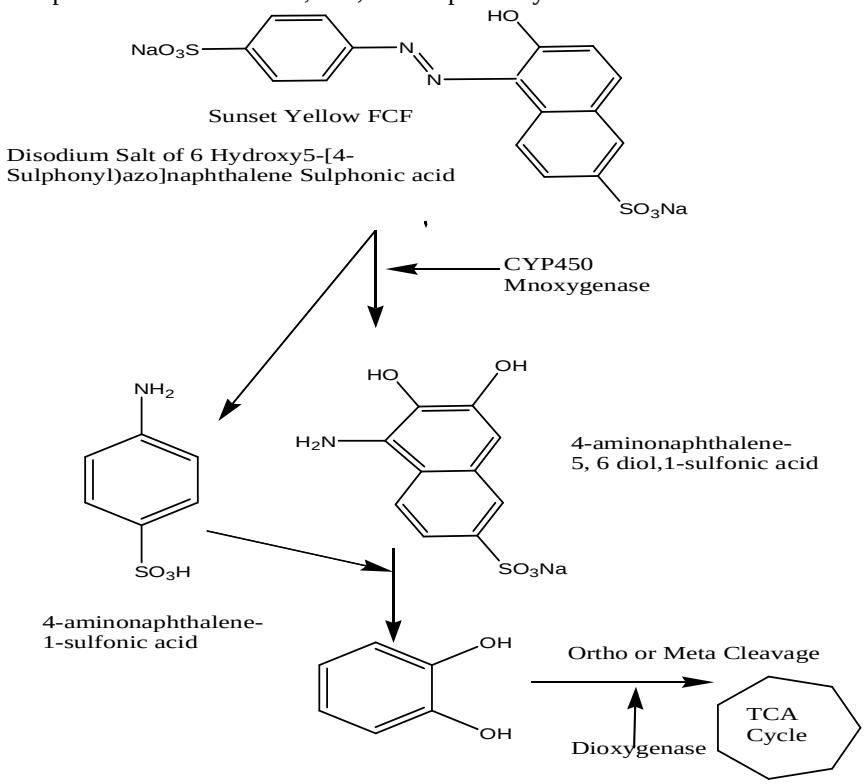

Fig 3.9 Bio-catalytic action of Pseudomonas DL7

IV. DISCUSSION

Bio-remediation is nothing but making the environment free of recalcitrant compounds or reducing the toxicity of it by biological source using algae, fungi or bacteria of different origins. Many of the microbes are having ability to detoxify the chemicals being dumped in environment. They may involve acidophiles, alkaliphiles, neutrophiles etc. Phyto-remediation by using algae's or degradation of sewage by various saprophytic fungi had been implemented for this purpose till today. Bio-sorption, bio-accumulation, bio-augmentation, oxidation ponds, use of bioreactors etc. became a need today to protect the environment from various types of pollution. Certain vital textile dyes of azo group were widely used in textile industry and known for producing aromatic amines which are mutagenic and carcinogenic in nature [16]. *Bacillus lentus* an alkaliphile strain BI377has found useful in color removal of a contaminant sulfonated azo dye named Reactive Red. It was observed that the di-azo dye first followed chromophore cleavage by azo reductase whereas, in case of mono-azo dye; cleavage took place by peroxidase via successive electron transfers to oxide surface forming asymmetric cleavage of the azo bond which leads to mineralization [17]. Thus hazardous aromatic compounds can be treated by extremophiles such as alkaliphiles, which exhibit an enormous potential of to degrade these aromatic compounds and helps in clearing the environment [18]. Bioremediation, especially through bacteria is becoming an emerging and important sector in effluent treatment [19].The bio-sorption is one of the technique in which living and dead microorganisms are implemented for bioremediation. Hetro-polysacharides and lipid components of the cell wall are supposed to be involved in this process. The enzyme azo reductase catalyzes the reaction only in presence of reducing equivalents like FADH and NADH. Both of the coenzymes NADH and NADPH could serve as electron donors for azo reductase but the latter was most preferred. Externally added FMN also shown good reducing power by the azo reductase via ping-pong mechanism but in such cases FMN could act as competitive inhibitor of NADPH, when substrates were methyl red, and nitro-furazone. This new azo reductase found shown broad substrate specificity might be member of a new nitro/FMN reductase family deducing higher potential in bioremediation [20]. Zoo-toxicity as well as phyto-toxicity was studied after decolorization and detoxification of Evans Blue, Triphenylmethane, Brilliant green, Malachite green etc in case of Pseudomonas [21]. It has removed 95 % of Brilliant green and Malachite green in 500 µmol/L concentrations after 12 hr. This can help in cleaning the environment.

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A report regarding monoxygenase was suggesting that heat is being evolved during microbial oxidation. An interesting part of observation showed that it is less for growth substrate catabolites than for their isomers [22]. As an NADH-dependent azo-reductase in *Bacillus sp. strain SF* found responsible for the decolorization of azo dyes. This purified enzyme showed molecular mass about 61.6 KD and an isoelectric pH 5.3. The pH optimum for the azo-reductase observed within the range of pH 8 to 9; while the temperature maximum was reached at 80°C. Thus the alkaliphiles and their enzymes became a need of for environmental detoxification, bio-augmentation, aeration or keeping anaerobic condition [23].Azo dyes can cause allergies diarrhea, hyperactivity and can affect children's intellectual development. If its higher intake exceeds the liver load increases the burden of self regulation and cause some damage to the kidneys and liver [24]. The decolorization of azo dyes was studied at various concentrations (100–300 mg/L) using bacterial consortium, with 5 days experiment using alkaliphiles[25]Recently The percent decolorization and detoxification of the dye Sunset Yellow FCF was shown spectro-photometrically (λmax-485nm).The efficiency of decolorization detoxification was increased with augmentation of carbon and nitrogen sources with 100% efficacy ast 2000 µgm/ml concentration[26]. The bio-degradation and decolorization of azo dyes came in limelight recently due to its eco-friendly and inexpensive nature. As they could follow both aerobic as well as anaerobic metabolic processes and different types of enzymes like Laccase, Peroxidase Catalase, Tyrosinase, Azo- reductases, Oxidoreductase from various sources like algae, fungi, bacteria, actinomycetis and several other. Thus the bio-remediation may involve aerobic or aerobic treatment. Sometimes combine use of aerobic-anaerobic techniques are found more effective. This might be due to formation of aromatic amines which are highly carcinogenic. In the presence of oxygen, aromatic amines can be degraded by oxygen insensitive azo reductases [27]. Mostly it makes difficult to isolate and purify the intracellular enzymes and the questions regarding its stability was somewhere found solved by the extracellular enzyme from *Alishewanlla sp*. CBL-2.It showed 78% secretion of azo reductase in liquid media used[28]. The decolorization of azo dyes by biological processes may take place either by bio-sorption or bio-degradation. Thus variety of bacteria and their enzymes of CYP450 family are involved in biotransformation of such xenobiotics by reductive or oxidative mechanism [29]. The cleaving enzyme makes more smaller fragments and could lead the mineralization process of such bulky aromatic recalcitrant's[30]. Fungi and their enzymes like Laccase, Lignin peroxidase or Mn peroxidase also have major contribution in mineralization by lignin modification and simultaneously attacking azo dyes [31]. The decolorization of azo dyes has been found to be more effective under anaerobic condition but the anaerobic degradation yields aromatic amines which are mutagenic and toxic to humans and cannot be metabolized further sometimes [32]. The bacterial dye decolorization is dependent on edaphic factors which affects the bacterial growth. In an another study it has showed that decolorization was significantly influenced by augmentation of starch followed by beef extract and pH. It must be known that which substrates favors' bacterial growth and decolorization [33]. As azo dyes are highly persistent and ubiquitously distributed in the environment it has created a threat to environment [34].

Bacteria isolated from human gut were also exploited for azo-reductases activity [35]. In an experiment the enzyme azo reductase having bio-transformation activity was isolated and purified from a Strain *Bacillus badius* from Lonar Lake [36]. Another an alkaliphile *Aquiflexum sp*. DL6 also showed similar potential [37]. Further the researchers added that azo reductase isolated from *Bacillus badius* shown nitro reduction potential also [38]. The azoreductases are observed as a diverse flavozymes or flavin free group of enzymes using NADH or NADPH as a cofactor in many microbes and higher eukaryotic organisms which are mainly responsible for bio-transformation and biodegradation of azo dyes, drugs as well as nitro-aromatic compounds 39]. Textile effluents containing toxic chemicals, alkaline organic contaminants high alkaline pH, heavy load of inorganic salts affecting the aquatic life. Currently enormous interest has been created on the decolorization and detoxification of dyes by alkaliphilic bacterial strains [40]. Although azo dyes are resistant to aerobic degradation some selected aerobic bacterial strains proved their ability to degrade these dyes by oxygen insensitive azo reductases. The exo enzymes of fungi also had shown azo dyes reduction by peroxidase and phenol oxidases at optimum pH. The mechanism of algal dye degradation was similar as that of bacteria. Dye degradation by consortia or fungi also been reported prominently.

It could be said that the reducing activity of the dye does not dependant on the intracellular uptake of the dye as it contain sulphonate like substituents groups, with high molecular weight and unlikely to pass through cell membranes. Even the bacterial membranes are almost impermeable to flavin-containing cofactors and, therefore, restrict the transfer of reduction equivalents such as flavins from the cytoplasm to the sulphonated azo dyes. Hence a mechanism other than reduction by reduced flavins formed by cytoplasmic flavin-dependent azo reductases must be responsible for sulphonated azo dye reduction in intact bacterial cells. Several human intestinal microbes are having azo reductaseactivity which plays an important role in reducing toxicity and mutagenicity. The acpD gene product (AzoEf1) was found responsible for the azo reductase activity in *Enterococcus faecium*. The residues associated with FMN binding, substrate specificity, and specific activity. AzoEf1 utilized both NADH and NADPH for the reduction of azo dyes, and it had leucyl residue at position 104 while; threonyl residue at position 19 shown clear differences from

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AzoA at active site [41]. The huge amount of salts in the wastewater of textile dyeing industry is one of the major limiting factors in the development of an effective bio-treatment method for the removal of dyes from textile effluents [42].Bio-transformation or biodegradation of azo dyes by alkaliphilic bacterial consortium is one of the environmental-friendly methods used for the removal of dyes and detoxification of it from textile effluents[43]. Oblgate alkaliphiles and adapted microbes to higher pH showed higher Cytochrome - C content responsible for thriving in severe alkalinity [44]. Azo reductases activity is found in many pathogenic bacteria as well as in some eukaryotes. In addition to having azo reductase activity these enzymes are also having NAD(P)H quinoneoxidoreductase activity which is having important role in plant pathogenesis[45]. Few of the aerobic bacterial strains found can utilize Azo dyes as growth substrates [46]. These organisms generally have a narrow substrate range. Degradation of aromatic amines depends on their chemical structure and the conditions. Although; some efforts are being made to degrade the dyes such as Tartrazine by Photo-catalysis using Titanium and benzoic acid are not much enough [47]. Hence bioremediation is most important due to it cost effective nature and leading to complete mineralization. The zoo-toxicity and phyto-toxicity also found decreased with reducing the color density of Brilliant green [48]. As some of the bacteria had been reported that enzyme azo-reductase (AZR) particularly from *Rhodobacter sphaeroides* AS1.1737 shown to be a flavodoxin possessing nitroreductase and flavin mononucleotide (FMN) reductase activities[49].It means nitro, amino, azo group containing xenobiotics could be detoxified easily. Thus on the basis of the study elaborated with ***Pseudomonas DL7*** showed good potential for azo dye degradation. The isolated catabolites **Fig [3.9]** 4-aminonaphthalene-5, 6 diol, 1-sulfonic acid, 4-aminonaphthalene-1-sulfonic acid, catechol made easy to construct the biotransformation pathway of the experimental contaminant. **Fig[3.8]** deduce the activation of biocatalyst to transform and detoxify the Sunset yellowFCF. Fig[3.1] to Fig[3.7] are self explanatory suggesting the higher degradation concentration of the experimental dye and change in functional groups leading to loss of toxicity.

V. CONCLUSION

600mg/LSunset Yellow was FCF was found completely degraded. 4-aminonaphthalene-5, 6 diol, 1-sulfonic acid, 4-aminonaphthalene-1-sulfonic acid, catechol were confirmed as a catabolites by GCMS. pH 9.00 was found optimum for biomineralization. Enzyme induction study showed the experimental bacteria actively plays important role in biomineralization and could be use for bioremediation of contaminated sites.

VI. ACKNOWLEDGEMENT

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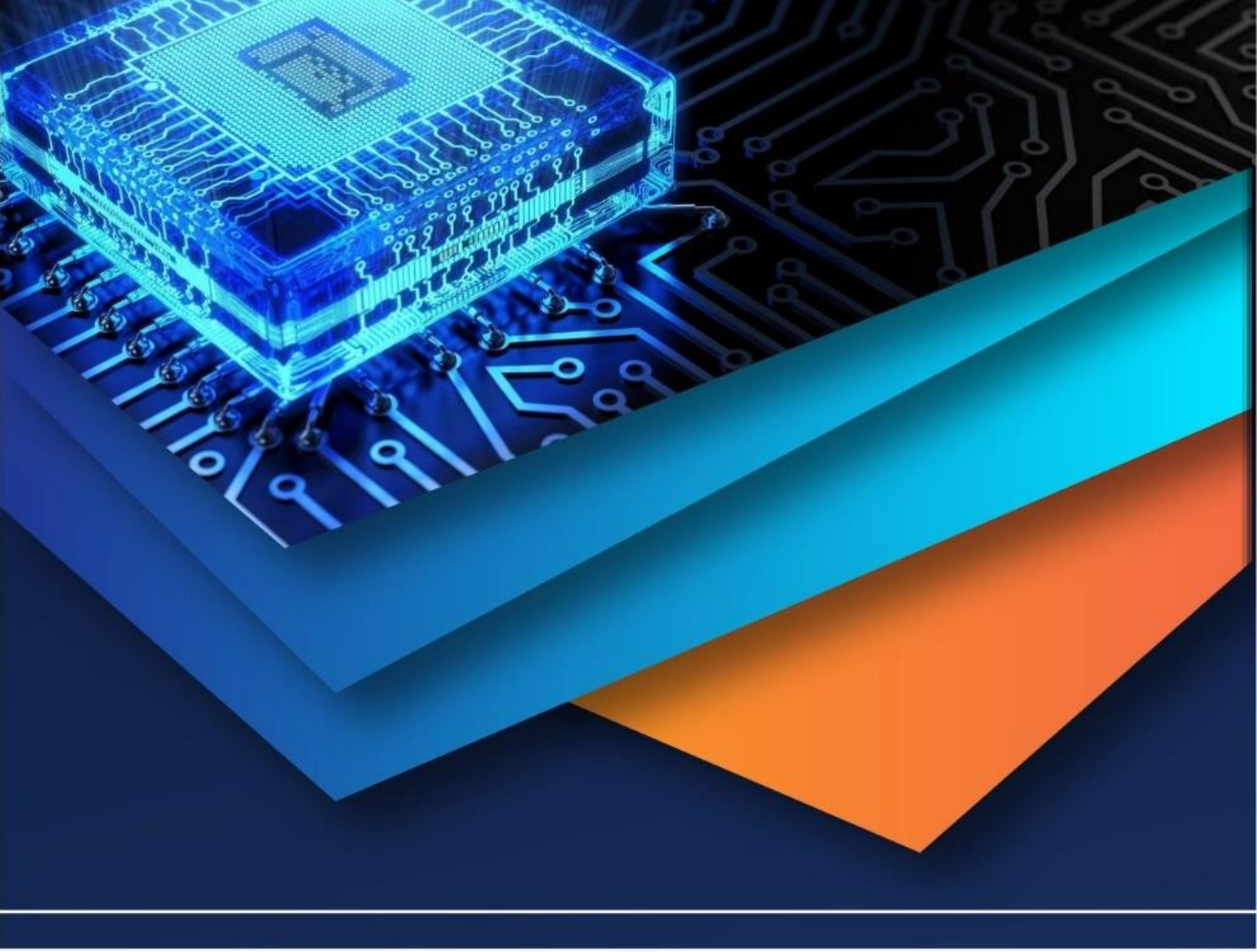

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