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ORIGINAL ARTICLE

Biochemical interaction of *Bacilllus badius* with alpha Naphthol

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ABSTRACT

The Study regarding biotransformation of alpha naphthol was carried using Bacillus badius in alkaline condition. 24 hr grown adapted culture was used for seeding the experiment. 30mg/L concentration of alpha Naphthol added in working flask containing bacteria with alkaline medium 200 ml. The experimental concentration of alpha naphthol was used in each Erlenmeyer flask including abiotic standard and in working flasks for growth and bio-transformation. The experiment was carried for 24 hr and the flasks were removed by 6 hr interval for observing residual concentration as well as searching for the metabolites generated. The metabolites were extracted by solvent extraction method using DCM or Ethhyl acetate. The residual metabolites were characterized by UV-Vis spectrophotometry, IR, NMR and GCMS. 3, 4 dihydroxy benzoic acid acid and catechol, 1 carboxy muconic acid semialdehyde, were well characterized in this process.
***Key Words**: alpha Naphthol, Bio-transformation, Bacillus badius*

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INTRODUCTION

2-Naphthol (β-Naphthol) has a water solubility of 0.6 - 0.8 g/L while 1 Naphthol (α –Naphthol) is little more soluble than that 0.85 g/L. It is a toxic hydroxylated metabolite of the (PAH) naphthalene. Alpha (α) Naphthol is slightly soluble in water, soluble in alkali and in organic solvents. It has acute oral toxicity, irritation in mucous membrane, toxic to lungs. Alpha naphthol 5% is being used for VP test(Voges-Proskauer A) reagent in quantitative analysis of gram negative bacteria in fermentation of glucose to acetoin (acetyl methyl carbenol) and 40 % KOH added further. An alpha naphthol is being used as coloring agent in hair dyeing. Sometimes it is used in cosmetic products. Repeated or prolonged exposure to human being can produce target organs damage and may produce general deterioration of health. It is toxic, infectious or corrosive. 1-naphthol (α -Naphthol) is a high-volume industrial product widely used in the synthetic dyes, perfumes, and pesticides like Carbaryl. It is weakly acidic (pKa9.34). Its stability in aqueous systems is thought to be affected by irradiation, oxygenation, and microbial decomposition [1].
Although there are several reports on biodegradation of phenolic waste; particularly alpha naphthol degradation in alkaline condition are very rare. Biodegradation of alpha-Naphthol in industrial waste water was studied by using *Aspergillus niger* isolated from the soil of industrial waste water load [2].
It is also released by microbes into the environment as a metabolic intermediate of various PAH. Naphthol is often liberated into the environment due to biological oxidation of naphthalene by various fungi and bacteria. Phenol and phenolic compounds are common water pollutants affecting on hormones negatively. They are affecting skin to form blisters and also found genotoxic in fishes in phenolic polluted water bodies.
Therefore an attempt was made to observe alpha Naphthol degradation under alkaline condition.

MATERIAL AND METHODS

Chemicals: All Chemicals were purchased from SRL Mumbai, Bacteriological media yeast extract, peptone Purchased from Hi-Media Mumbai.
Growth media: The broth media used for biotransformation study consist of yeast extract, peptone, NaCl -5 g/L respectively. 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, CaCO3 0.20, $ZnSO_4$ 0.08, $CuSO_4$ 0.016, $CaSO_4$, Boric acid 0.06, pH-9. The media were sterilized by autoclaving at 121 ^{0}C at15 psi for 20 min. The solid media were prepared in the same way by adding 2% agar.

Bio-transformstion and residual concentration: Five 500 ml conical flasks containing sterilized 200 ml alkaline broth of pH-9.00 were inoculated by 1 % *Bacillus badius* culture possessing 1.6 OD at 600 nm aseptically. These culture flasks were incubated for 24 hrs at 37 ^{0}C with shaking on Orbital shaker at 110 rpm. The 24 hrs grown culture flasks were induced by adding concentration 30 mg/L of alpha Naphthol. These flasks were removed sequentially from 0 to 6 hr. interval. The removed flasks were used for OD at 600 nm to check the growth and then spun to DuPont Sorvoll Cold centrifuge at 10000 x g. Similarly one another flask was kept as abiotic control by adding experimental concentration of alpha Naphthol. The residual concentration was investigated using Spectrophotometric method [3] by Jasco Varian-630. These experiments were repeated thrice. The residual sample was characterized by IR, NMR, and GCMS analysis after solvent extraction.

Preparation of Cell extract and Biocatalysis: Cell mass was harvested after 24 hours induction with alpha Naphthol 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 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 [4].

The bio-catalytic study for cytochrome P450 was carried by using (Omura and Sato) standard methods [5]. Catechol 2, 3 dioxygenase activity by[6] Nozaki and others[7] and Catalase Paroxidase, SOD by standard experimental methods[8,9],. The enzyme activity expressed as µM/ min/ mg of protein.

RESULTS

UV- Vis Spectrophotometic study:

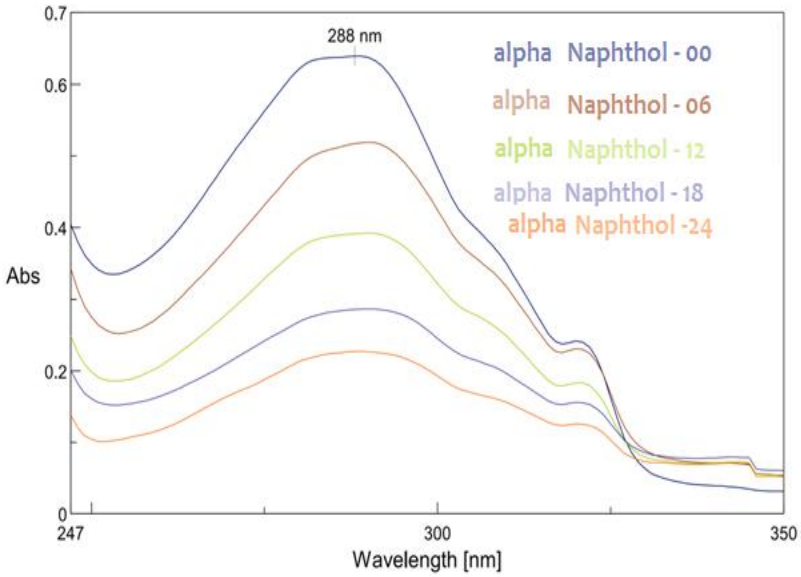


Fig.1. Biodegradation pattern of alpha Naphthol by *Bacillus badius*

The decreasing absorption of alpha Naphthol at certain time interval clearly indicates the use of alpha Naphthol as food source or its transformation into another product.

Percent degradation and bio-transformation study:

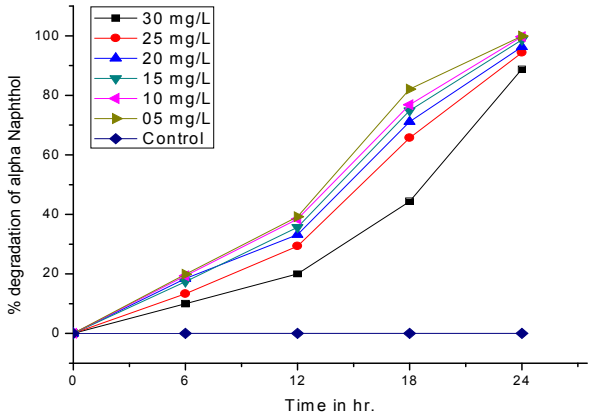
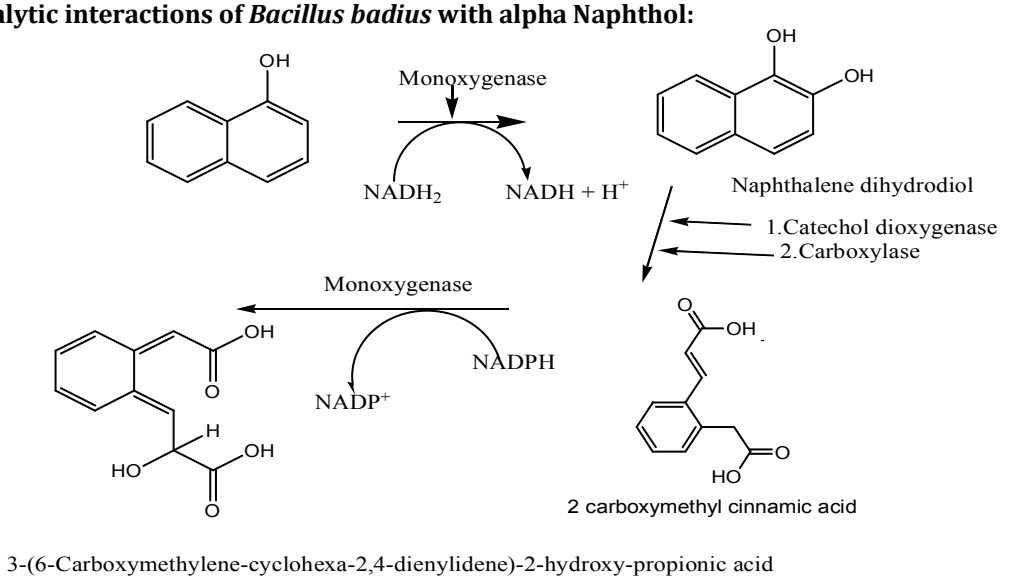


Fig.2 percent degradation of alpha Naphthol by *Bacillus badius*

The **[Fig.2]** clearly shows 100 % degradation of alpha Naphthol within 24hr. with marginal difference; while no change in abiotic control was observed. As the metabolites are being part of TCA cycle after catechol pathway the xenobiotics are turning into CO_2 and Water in this metabolic process.

Bio-catalytic interactions of *Bacillus badius* with alpha Naphthol:



3-(6-Carboxymethylene-cyclohexa-2,4-dienylidene)-2-hydroxy-propionic acid

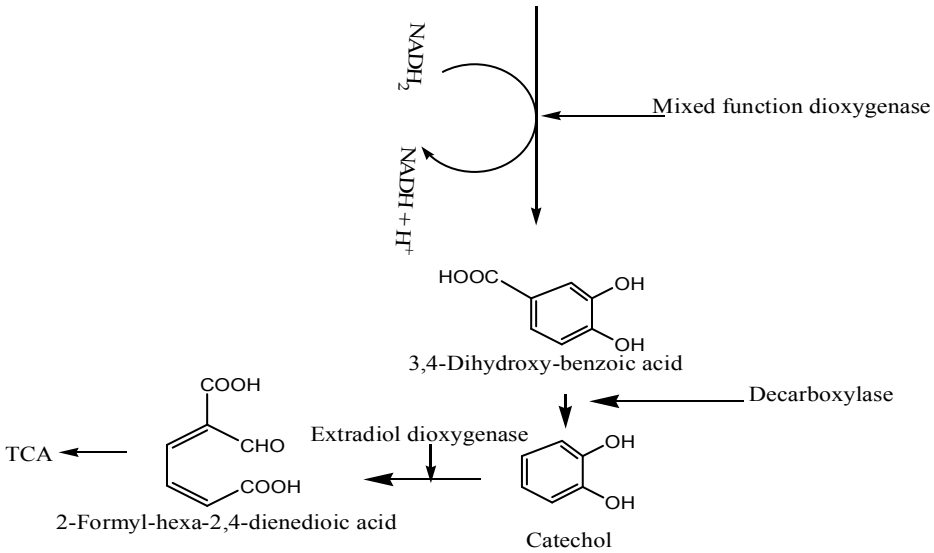


Fig. 3. Mechanism of alpha Naphthol bio-transformation by *Bacillus badius*.

Bio-catalysis study:

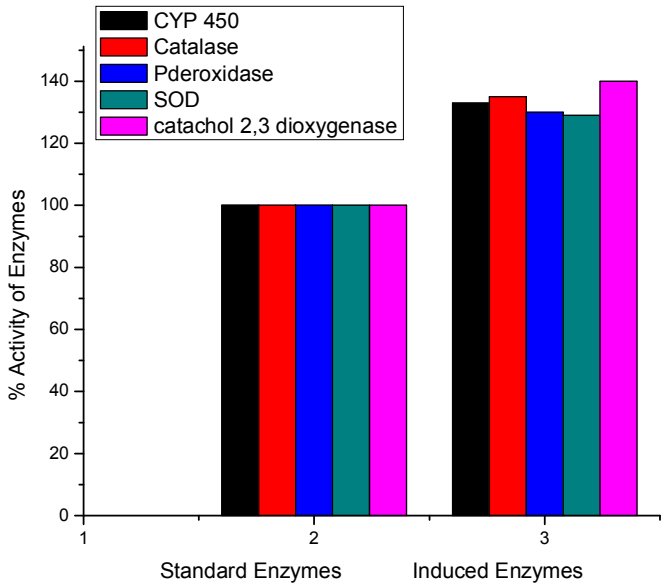


Fig.4. The enzymes involved in biotransformation

Although; many of the enzymes are involved in biotransformation process. Few of the enzymes activity experiments were performed invitro. [**Fig.4].** It shows the CYP450, Catalase, Peroxidase, SOD, Catachol dioxygenase are found more induced than normal due to the alpha Naphthol in medium

DISCUSSION

Mucosal biotransformation of alpha naphthol had been well reported in the small and large intestine of animals and humans. Even it also reported that gastric mucosa also plays a role in detoxifying ingested material in human gastric epithelial cells which exert an important conjugative effect on the phenolic compound alpha-Naphthol[10]. Isolation and characterization of naphthalene-degrading strains, Pseudomonas sp. CZ2 and CZ5 were reported for following catechol pathway by forming 1, 2 dihydroxy naphthalene[11]. Environmental pollutants are the substances entering into the environment and are toxic to the living forms in a specific chemical form and at specific concentration. They can be solid, liquid and gaseous in nature. When they enter the environment in excess quantities, they lead to toxicity for various life-forms. Thus environmental wastes have to be treated as and when they are introduced into the environment. These wastes can be treated in-situ: at the site of contamination or ex-situ: at a site away from site of contamination [12]. Xenobiotic substances are becoming an increasingly large problem in sewage treatment systems, since they are relatively new substances and are very difficult to categorize. Some xenobiotics are resistant to degradation. e.g., synthetic organochlorides, plastics, pesticides, or naturally occurring organic chemicals such as polyaromatic hydrocarbons and fractions of crude oil, coal etc. However, it is believed that microorganisms are capable of degrading almost all the different complex and resistant xenobiotics found on the earth. Many xenobiotics exert a variety of biological effects, which can be characterized using bioassay [13].

The chemical behavior is governed by its natural physicochemical properties and their metabolic activities. The edaphic factors and biotic factors lead several interactions which contributes the degradation and mineralization of organic compounds. Bulky, nonpolar chemical are lipophilic and/or resistant against biological degradation while polar molecules increases toxicity in water due to high solubility as acute toxicity[14]. Toxicity to human and animals depends on the way of exposure and time. As the physicochemical properties like vapor pressure, solubility, viscosity, adsorption or absorption also affects the rate of biodegradation. In the metabolic pathway of naphthalene or alpha-Naphthol involves the formation of 1, 4-naphthoquinone and 4-hydroxy-1-tetralone. Strong binding of these products to soil constituents may leads formation of non extractable residues.

Sometimes the oxidation of alpha-naphthol in solution leads to form naphthoxy radicals and it could be facilitated by the reaction of O_2 even formation of superoxide radical, hydroxyperoxyl radicals etc; which may subsequently forms HO_2 and O_2^{-2} . These peroxide ions are considered to be the oxidizing species in

aerated water. Naphthoxy radicals are highly reactive and could combine to form alpha- Naphthol dimers, naphthoquinones[15].

Oxygenases are oxidoreductases class of enzymes that have great potential and versatility for catalyzing reactions that are generally not easily possible by chemical routes. These enzymes are important due to high regio, stereo, and enantio selectivity. These enzymes introduce either one or two atoms of molecular oxygen into organic molecules using NADH or NADPH as a cofactor or coenzyme. To eliminate the external addition of a costly cofactor, whole cells expressing oxygenase enzymes are generally used [16]. Oxidoreductases are not stable under certain conditions due to hydrostatic and osmotic pressure, temperature or pH may cause subunit dissociation and it may be another cause of using live cells [17].

In case of Sulphate reducing bacteria the carboxylate group introduced in naphthalene indicating the activation of naphthalene by carboxylation as the initial degradation step. The Cytochrome P450 protein-bound porphyrin complex with the iron-coordinated activate oxygen atom which hydroxylates inert carbon-hydrogen bonds of substrates. This enzyme belongs to oxidoreductase family acting as monoxygenase or dioxygenase introducing one or two molecular oxygen in organic compounds in live cells or in vitro [18]. Algae and cyanobacteria also employed for Phenolic biodegradation by similar mechanism [19].

Alkaline environments not only contain natural soda lakes and soda deserts, but also include the alkaline wastewater derived from anthropogenic activities. In recent years, an increasing number of alkaline waste water with higher pH than 11 have been discharged from cement industry, petroleum refinery, pharmaceuticals, paper and pulp industry [20]. An Alkaliphilic strain thrives in harsh alkaline condition and hence can be used for contaminants removal. Thus employing an Alkaliphiles for biodegradation of toxic compounds in industrial effluents would be of great help [21].

Oil degrading Actinomycetes isolated from Soil of Georgia was reported for bioremidiation[22]. Similalry under saline conditions phenol degradation was also studied [23]. The phenolic compounds are major pollutants of industrial waste waters due to use in many industries like oil refining, coke conversion, pharmaceutical and resin manufacturing plants. The degradation of phenol under saline conditions has been well documented [24]. Characterization of alkalotolerant *Pseudomonas* sp. strain ISTDF1 for degradation of dibenzofuran also reported [25]. Toxicants from Mangrove Ecosystems of Goa, humic acid-reducing bacterium, and certain other xenobiotic degradaers are showing their contribution in alkaline waste remediation [26 - 28]. Among these Pseudomonas sp. Y2 showed a high ability to degrade 1-Naphthol (95.3% of it was degraded within 24 h incubation), which made it very attractive for application in the environmental remediation areas [29].

Similarly *Bacillus badius* also showed high potential for biodegradation and biotransformation by the experimental data [Fig.1]. The decreasing absorption at its wavelength suggests it is being degraded and or bio-transformed into other metabolites. The [Fig.2] is supportive to biodegradation of alpha Naphthol. It indicates 100 % alpha Naphthol has removed from the medium within 24 hr. even at 30 mg/L concentration. The residual sample obtained by Solvent extraction and purification by column chromatography given for IR, NMR, GCMS confirmed the metabolites as Naphthalene dihidrodiol, 3-(6-Carboxymethylene-cyclohexa-2, 4-dienylidene)-2-hydroxy-propionic acid, catechol, 3, 4-Dihydroxy-benzoic acid, 2-Formyl-hexa-2,4-dienedioic acid. This helps to propose a pathway of biotransformation of alpha Naphthol [Fig.3] in alkaline condition**.**

The splitting of Naphthalenee dihidrodiol ring might be as of the thermophile forming caboxy cinnamic acid or carboxy cinnamic ester leading further fragmentation [30]. It might involve catechol and dihydroxy benzoic acid or further broken products into carboxy muconic acid semialdehyde. This made easy to propose probable mechanism of biotransformation and enzyme activity [Fig.3].

Few of these and certain other metabolites such as Gentisic acid, Salicylaldehyde, Salicylic acid, etc. had been identified by other researchers in their biodegradation study[31,32,33] In a previous study 2-naphthol, 1, 2-naphthalene-diol and 1,2-naphthoquinone were detected by HPLC in coupled degradation of beta Naphthol using *Aspergillus niger* and *Bacillus subtilis* was reported[34]. However 2-naphthoic acid, Decahydro-2-naphthoic acid, octahydro-2-naphthoic acid, 5, 6,7,8-tetrahydro-2-naphthoic acid were identified as metabolites in case of anaerobic sulphate reducing culture in which carboxylation was initial step of polarization of naphthalene[35]. Mostly the Naphthalene biotransformation reports were found regarding mesophiles or neutrophiles. Present article is one of the representative of alpha naphthol biotransformation in alkaline condition .

The bio-catalysis was carried by activated oxygen is formed at the site of sixth iron coordination position of the protoporphyrin IX iron complex of CYP450. This catalytic center is surrounded by the enzyme and the substrate located close to the oxygen makes the heme-thiolate proteins catalyze the conversion of a variety of chemically diverse compounds by insertion of an activated oxygen atom as monoxygenase or

sometimes as dioxygenase incorporating two oxygen atoms into an inert C-H bond [36]. In natural ecosystems, many polycyclic aromatic hydrocarbons undergoes to rapid degradation some times by hydroxylated metabolites, which might be higher toxic than the parent compound due to higher solubility in water. As oxygen being incorporated in the molecule, the polarity increases and the dose of ingestion becomes heavy.

Sometimes formation of free radical takes place by the cells to minimize the toxicity or damage created due to the xenobiotic material. This can be done by the enzymes peroxidase or catalase. The **[Fig.4]** gives the idea about the active participation in bio-catalytic interactions of alpha Naphthol, All the enzymes such as CYP450, catalase, peroxidase, SOD, Cat.2, 3 dioxygenase hiked comparative to standard. This shows that the experimental strain is actively involved in biotransformation.

CONCLUSION

Bio-remediation is the best practice to remove the toxicants from nature. One can employ the Alkaliphiles to clean the environment when toxicants are added to the surroundings. Despite of this one can isolate various metabolites if the heavy load of toxicants is known. As *Bacillus badius* is nonpathogenic Strain it can be used on commercial level.

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