

Biodegradation of aniline by alkaliphilic strain *Bacillus badius* D1

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Abstract: Aromatic amines are well known for their properties as potential mutagens as well as carcinogens. Aniline is an aromatic amine and it is used as an intermediate of many synthetic organic compounds. Several azo dyes and nitro amine compounds, on degradation produce aniline which causes environmental pollution. In the present study, attempts were made to investigate biodegradation of aniline by using an alkaliphile, *Bacillus badius* D1 strain with various concentrations ranging from 0.28 to 1.55g/L at 37°C under shaking condition at pH 9. The alkaliphile, *Bacillus badius* D1 strain was found to have promising degradation potential towards aniline. The effect of various parameters such as, temperature, pH, salinity and additional carbon and nitrogen sources showed significant increase in degradation. The degradation process of aniline by bacteria was monitored by spectroscopic analysis at various time intervals. The tentative degradation pathway was established from the structures of intermediates of aniline by ¹HNMR, FTIR and GCMS.

Key words: Biodegradation, *Bacillus badius*, Aniline

Abbreviations: , mM : milli moles , µl: micro liter, SOD- superoxide dismutase

I. Introduction:

Alkaliphiles are the organisms thriving in harsh alkaline conditions. Although alkaliphiles are being employed for environmental cleanup [1-6], the literature regarding the study of aromatic amines or aniline degradation is less reported as compared to the other degradation studies. The present study is planned to examine the degradation potential of alkaliphile, *Bacillus badius* D1 strain towards aniline. Most of the industrial waste is alkaline in nature which is generally not degraded by neutrophiles and imparts potential threat to the environment. Organic compounds like azo dyes, Nitro amines lead to form aniline as one of the catabolic intermediate. Monocyclic aromatic amines are environmental pollutants and are genotoxic. These impose hazards to human health and aniline is one of them. It is being released into the soil and water bodies due to its expanded use in an industry [7]. Aniline is a synthetic organic compound used in dyes, herbicides and medicines. It is the most important intermediate used in polyurethane, rubber and agricultural products. Aniline cancer had been detected in dye workers particularly as a bladder cancer. Aniline is weak aromatic base; it remains stable in alkaline conditions and adversely affects the aquatic system. Azo dyes on biotransformation produce aromatic amines like p-phenylene diamine, benzedene or aniline. Aniline induces intra chromosomal recombination between repeated sequences in *Saccharomyces cerevisiae*, resulting in deletion (DEL) of intervening sequences. It has reported that the generation of oxidative free radical species by aniline and/or its metabolites may be responsible for its recombinogenic activity in the yeast. Aniline cytotoxicity was many-fold increased in the strains of *S. cerevisiae* lacking the antioxidant enzyme superoxide dismutase [8]. As aniline is highly toxic, it damages the balance of the ecosystem. Alkaliphiles grow well in alkaline conditions and the organic pollutants could be utilized by these bacteria as carbon and nitrogen source.

II. Materials and methods:

2.1 Chemicals and reagents: Aniline 98% pure, ethyl acetate and chloroform methanol (SRL, Mumbai, India) Peptone, Yeast extract, Agar (Hi-media, Mumbai, India)

2.2 Media preparation:

The broth media used for biodegradation study consist of yeast extract, peptone, NaCl -5 g/L respectively. The micronutrients' in mg /L were KH₂PO₄ -170, Na₂HPO₄ .290, (NH₄)₂SO₄ -100, MgSO₄ .MgO-0.1, FeSO₄ -0.05, CaCO₃ 0.20, ZNSO₄ 0.08, CuSO₄ 0.016, CaSO₄ , Boric Acid 0.06, pH-9. The media were sterilized by autoclaving at 121 °C at 15 psi for 20 min. The solid media were prepared in the same way by adding 2% agar.

2.3 Experimental setup:

2.3.1 Bacterial strain adaptation: Alkaliphilic *Bacillus badius* D1 strain isolated by standard method like serial dilution. It was screened for aniline degradation and further adapted [9] for enhancing more potential of degradation by adding more aniline aseptically in broth media around 3 months.

2.3.2 Biodegradation studies on Aniline: Thirteen 500 ml Conical flasks containing Sterilized 250 ml alkaline broth pH-9 were inoculated by 1 % *Bacillus badius* culture possessing 1.6 OD at 600 nm aseptically and grown for 24 hrs at 37 °C with shaking on Orbital shaker at 110 rpm. The 24 hrs grown culture flasks were induced by adding appropriate concentration of aniline. These flasks were removed sequentially from 0 to 72 hours from a 6 hour interval. The removed flasks were used for OD at 600 nm to check the growth and then spun to DuPont Sorvall Cold centrifuge at 10000 x g. An aliquot of centrifuged supernatant used for quantitative analysis by diazo coupling with N-1- Naphthyl ethylenediamine. Remaining part was used for solvent extraction. The residue obtained after Rota evaporation used for UV- visible spectroscopic studies, further it was purified by preparative TLC or column chromatography using neutral alumina. The purified residue was further subjected to FTIR, ¹HNMR and GC-MS for structural determination. Similarly another flask with same media was kept as abiotic control by adding the appropriate concentration of aniline aseptically without bacterial culture and aliquots were used for quantitative analysis with respect to the time intervals. All experiments were carried in the dark.

2.4 Enzyme Activities:

2.4.1 Preparation of Cell extract: Cell mass was harvested after 24 hours induction with aniline by Du-Pont Sorvall RC-5B centrifuge by spinning at 10000 x g for 15 min at 4 °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 [10] using BSA as standard.

2.4.2 Cytosolic content of Cytochrome P-450: The content of cytosolic cytochrome P 450 was measured by the procedure of Oura and Sato [11, 12]. The content of CYP -450 calculated by using 91 cm⁻¹mM⁻¹ as an extinction coefficient. All operations were carried at room temperature. CYP 450 content was reported as n mole of CYP 450 mg⁻¹ of cytosolic protein.

2.4.3 Super Oxide Dismutase [SOD]: SOD was measured spectrophotometrically according to the procedure of Mishra and Fridovich [13] on Jasco V-630 double beam spectrophotometer at room temperature. The inhibition of auto-oxidation of epinephrine at pH 10.2 by SOD was monitored by measuring the formation of androchrome at 480 nm.

2.4.4 Acetanilide hydroxylase assay: Acetanilide hydroxylase was assayed by the protocol of Shenkman et al, [14]. The p-hydroxy acetanilide formed during the hydroxylation of acetanilide was measured according to the method of Weiseberg and Goodall [15]. The activity of the enzyme was expressed as n moles of p- hydroxyl acetanilide formed / min / mg of cytosolic protein.

2.4.5 Catechol 1, 2 Dioxygenase: catechol 1, 2 dioxygenase activity was studied by Guzik Urszula, Gren Izabela et al [16]. The enzyme activity was expressed as μM / min / mg of protein.

2.4.6 Catechol 2, 3 Dioxygenase: catechol 2, 3 dioxygenase activity was studied by J.M Sala Trepas and W.C. Evans [17, 18]. The enzyme activity noted as μM/ min/ mg of protein.

2.4.7 Quantification of aniline: Remaining residual aniline during the microbial degradation was determined by the procedure of George Norvit [19].

III. Results:

3.1 Degradation of aniline by *Bacillus badius* D1 at concentration of 1.55 g/L with different time intervals.

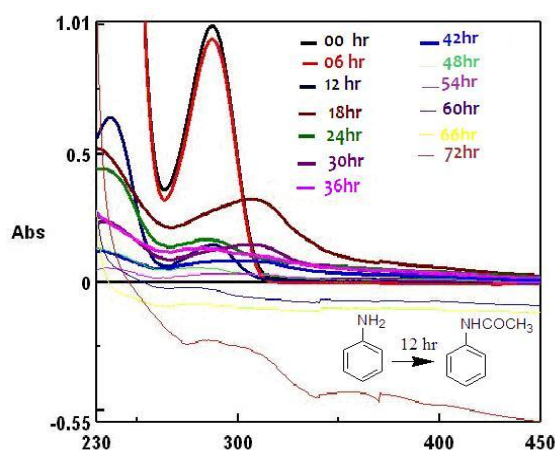


Fig. 1 UV- visible pattern of aniline degradation by *Bacillus badius* D1

Fig.1 indicates that the degradation pattern of aniline by *Bacillus badius* D1, aniline at a concentration of 1.55g/L was incubated with bacterial strain at pH 9.0 up to 72 hours. The pattern of degradation was monitored by spectrophotometer at each 6 hour of interval. The peak at 288 nm was found to be decreased by 6hr incubation and new peak was detected at 235 nm by the end of 12 hour incubation. Another peak at 275 nm was observed at the end of 24 hour incubation, indicating the possibility of catechol. Further incubation up to 72 hours resulted in maximum utilization of aniline by the experimental bacteria.

3.2. Effect of concentrations on degradation of aniline.

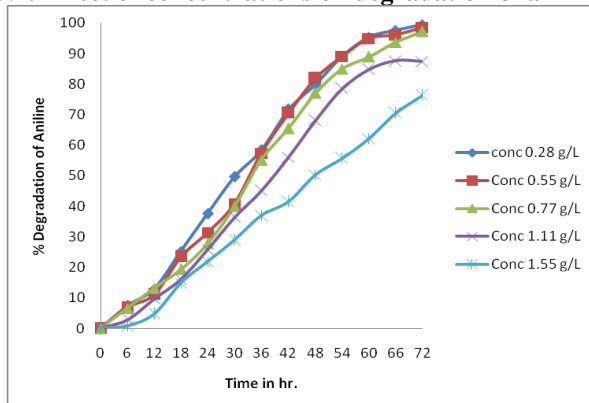


Fig. 2 Effect of concentrations on aniline degradation

Fig. 2 depicts the effect of various concentrations of aniline on the degradation. Aniline at various concentrations (0.28 to 1.55 g/L) was incubated with bacteria for 72 hours. At concentrations of 0.28 and 0.55 g/L maximum degradation of aniline 99% was observed within 72 hours. With an increase in concentration of aniline the degradation rate was found to be lowered 78% up to 72 hours.

3.3. Effect of pH on aniline degradation:

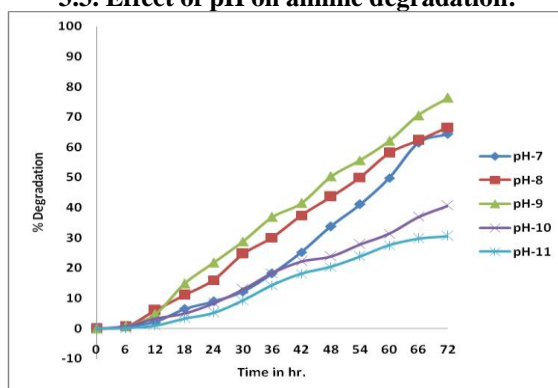


Fig. 3 Effect of pH on aniline degradation

Fig. 3 shows the effect of pH on the degradation of aniline by *Bacillus badius* D1. In order to examine the effect of pH on the degradation of aniline by this bacterial strain, *Bacillus badius* D1 was incubated with aniline at a concentration of 1.55 g/L for 72 hours. At 37 °C with pH range 7.0 -11. Approximately 78% of aniline was degraded at pH 9.0. A further increase in the pH values the rate of aniline degradation was found to be reduced marginally.

3.4. Effect of Temperature on aniline degradation:

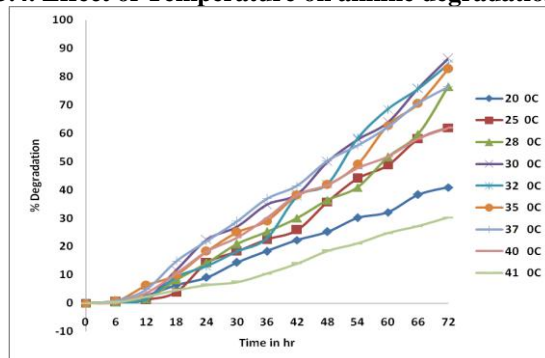


Fig. 4 Effect of Temperature on aniline degradation

Fig. 4 shows the effect of temperature on the degradation of aniline by *Bacillus badius* D1 to observe the effect of temperature on aniline degradation, alkaliphilic strain *Bacillus badius* D1 was incubated with aniline at a concentration of 1.55 g/L for 72 hours at various temperatures i.e. 20 - 40 °C. The maximum degradation of aniline was observed at a 30 °C. Further increase in the temperature resulted in the slight decrease of aniline degradation.

3.5. Effect of Salinity:

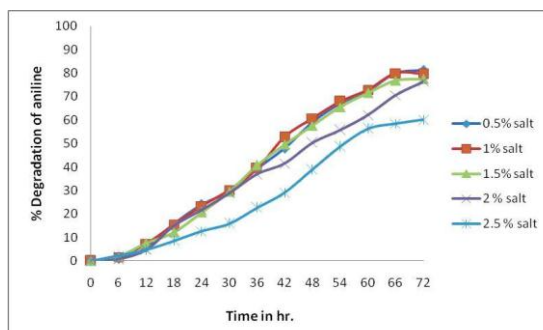


Fig. 5 Effect of Salinity

Fig. 5 indicates the effect of salinity on aniline degradation. The saline tolerant bacterial strain *Bacillus badius* D1 was incubated with aniline at 1.55 g/L of growth medium, pH 9.0 at 37 °C for 72 hours with NaCl concentration ranging from 0.5% to 2.5%. The maximum degradation of aniline (90 %) was observed between 0.5 – 1% of NaCl with an increase in NaCl concentration up to 2.5% in media decreases the rate of degradation of aniline marginally.

3.6. Effect of additional carbon sources on aniline degradation:

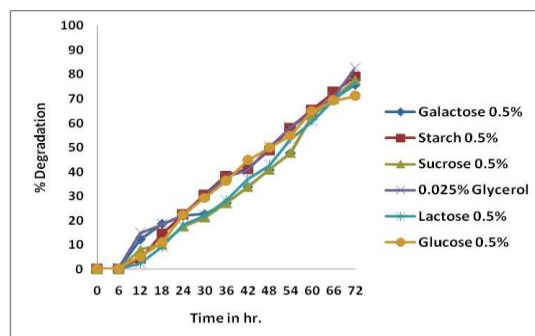


Fig. 6: Effect of additional carbon sources on aniline degradation

The effect of additional carbon sources on aniline degradation is shown in Fig. 6. On incubation of bacterial strain *Bacillus badius* D1 in broth medium having aniline concentration 1.55 g/L with different carbon sources like galactose, starch, sucrose, lactose, glucose etc at 0.5% and glycerol 0.025% at temperature 37 °C, pH 9.0 for 72 hr. It was observed that in the presence of all these carbon sources maximum degradation (90%) of aniline was observed.

3.7. Effect of additional Nitrogen source:

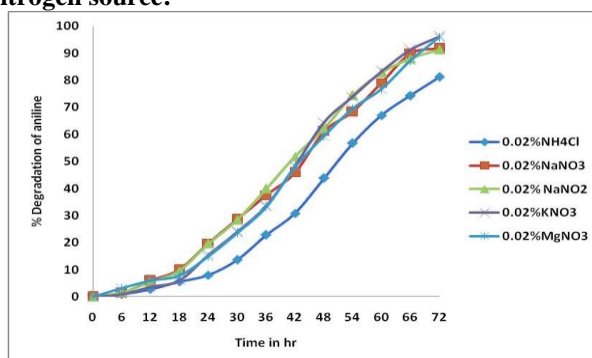


Fig. 7 Effect of additional Nitrogen source

The effect of additional Nitrogen source is indicated in Fig. 7. To examine the effect of various nitrogen sources on the degradation of aniline by *Bacillus badius* D1, the bacterial strain was incubated with 1.55 g/L aniline in growth medium, pH 9.0 at 37 °C for 72 hours with several nitrogen sources such as NH₄Cl, NaNO₃, NaNO₂, KNO₃, and MgNO₃ at 0.02%. All the added nitrogen sources have supported the degradation of aniline.

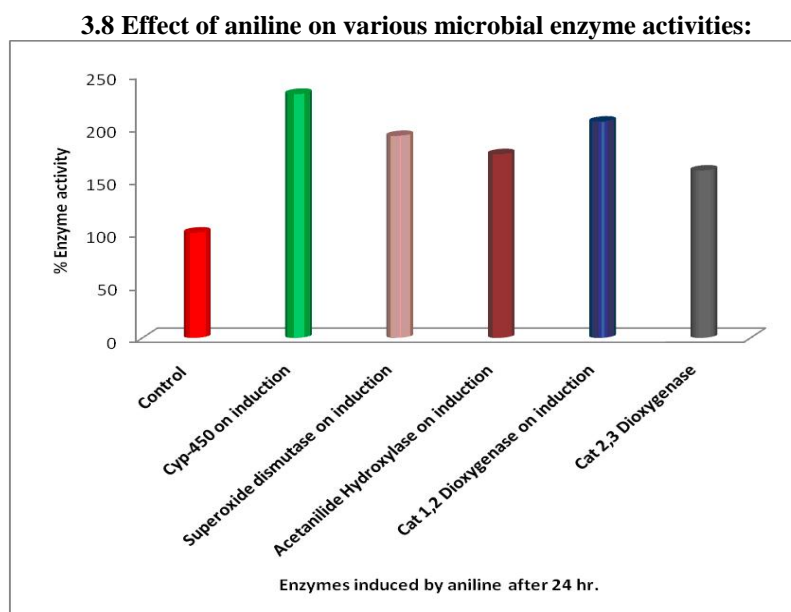
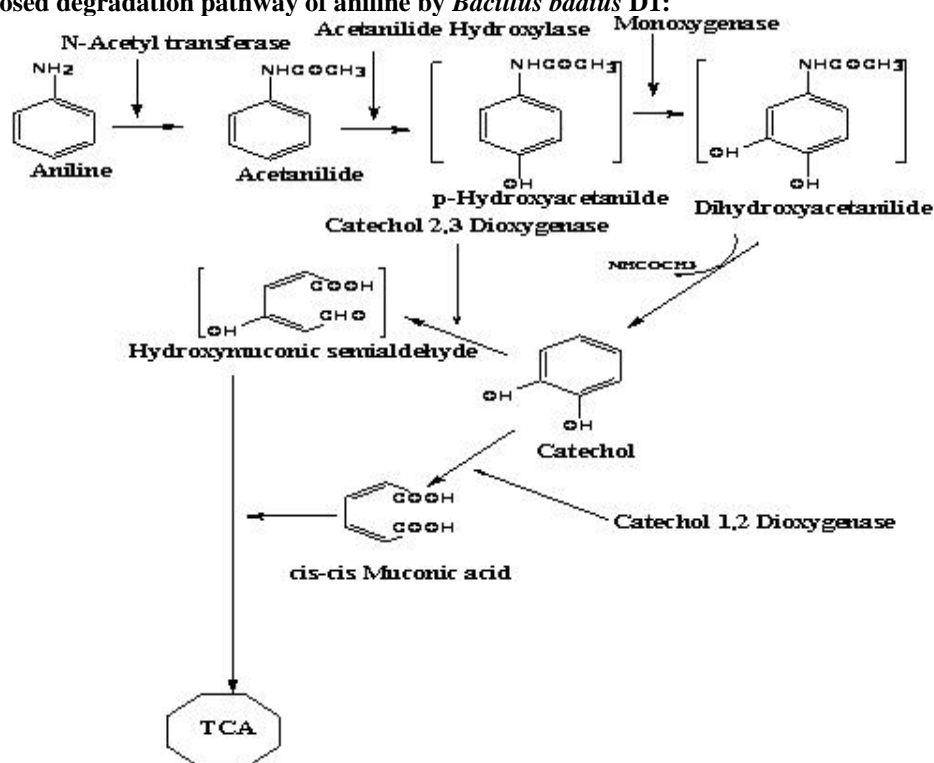


Fig. 8 Effect of aniline on various microbial enzyme activities

Fig. 8 shows the effect of aniline on the activities of microbial biotransformation enzymes. To evaluate the effect of aniline on these biotransformation enzymes, bacterial strain *Bacillus badius* D1 was incubated with aniline at a concentration of 1.55g/L, for 24 hours at 37 °C, in growth media pH 9.0. Incubation with aniline for 24 hours was resulted in an increase in the content of cytosolic cytochrome P450 and in the activities of acetanilide hydroxylase, super oxide dismutase, Catechol 1,2 dioxygenase and cortical 2,3 dioxygenase as compared to their respective controls. However, the magnitude of increase in the content of cytochrome P450 and the activities of superoxide dismutase, catechol 1, 2 dioxygenase were found to be higher than acetanilide hydroxylase and catechol 2, 3 dioxygenase.

3.9 Spectroscopic analysis of intermediate metabolites during aniline degradation by *Bacillus badius* D1:

The structural analysis of isolated metabolites/intermediates of aniline at each time interval of 6 hours is determined by, NMR, FTIR, and GCMS. The formation of acetanilide was confirmed by delta values from NMR δ - 2.151,(S-3H), δ -7.5,(m-2H), FTIR stretches at 1662,1498-1535,3294, the mass spectrographic data. The molecular ion peak appeared at 135 and fragmentation peaks at 93, 77, 66, 43, and 40. The metabolite acetanilide was isolated at the end of 12 hour incubation. Acetanilide was further transformed to catechol by the bacterial strain *Bacillus badius* at the end of 24 hours incubation. The confirmation was made by NMR data δ - 5.33 (S-2H), δ -6.8 (m-4H), FTIR Ar-OH-3229, Ar-1517-159 and mass spectrographic data showing molecular ion peak at 110 and its fragmentation observed at 92, 80, 64, 53, 40. Catechol was further converted into cis -cis muconic acid at the end of 48 hours. The metabolite structure was confirmed by NMR delta values δ -12.9 (S-2H), δ - 6.21(2-H), δ -7.59(2H), the FTIR stretches observed at Ar-COOH – b-3043-2575,Ar-1635-1674, while mass spectrographic data indicated the molecular ion peak at 142 and the fragmentation pattern appeared at 142,123,114,96,83,71,56,43,38.

3.10. Proposed degradation pathway of aniline by *Bacillus badius* D1:Fig. 9: Proposed degradation pathway of aniline by *Bacillus badius* D1

The parent molecule aniline was transformed to acetanilide, p-hydroxy and dihydroxy acetanilide, catechol and cis-cis muconic acid finally muconic acid enters into TCA cycle. The biotransformation of aniline could be attributed to the involvement of microbial enzymes like N- acetyl transferase, Acetanilide hydroxylase, monooxygenase, catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase.

IV. Discussion

Environmental pollutants and wide range of toxic organic compound accumulates in the environment affects all types of ecosystems, which is the repercussion of anthropogenic activities like efflux of industrial inputs impacted seriously on the pristine nature of our environment. At present, it is estimated that more than 100 million people are at the risk from toxic pollutants above the international levels and above the international health standards [20]. Various types of microorganisms such as bacteria, fungi and algae are present in the soil as well as in the aquatic ecosystem. Most of these microorganisms are eco-friendly and few of them are also known as pathogens. Microbial metabolism potential provides a safer, more efficient and less expensive alternative to physicochemical methods for pollution abatement had been realized and play a crucial role in biogeochemical cycles for sustainable development of biosphere [21, 22]. Microorganisms have the capacity to get adapted to the changes in the environmental conditions even at extreme environment [23, 24]. Microorganisms from soil and aquatic habitats are continuously exposed to different types of chemicals. These chemicals vary in physicochemical properties. These pollutants are known to interfere with metabolic processes of the organisms. They induce or inhibit many microbial enzymes. Among these chemicals various aromatic compounds are reported to interact with biotransformation and antioxidant enzymes in microorganisms. These microorganisms normally involved in degradation of such chemicals. Aniline is an aromatic substance used in various chemical industries. The presence of aniline in the environment is potential threat to living organisms. The degradation of aniline by neutrophilic bacteria have been studied by various reporters [25-27]. The degradation of aniline in alkaline conditions were earlier reported [28, 29], still the degradation of aniline by *Bacillus badius* D1 can't be compared with it due to little different experimental condition.

In the present study the degradation of aniline by *Bacillus badius* D1 was studied in various experimental conditions like concentration, pH, temperature, salinity, additional carbon and nitrogen sources. The effect of initial aniline concentration on the degradation has shown that at lower concentration the rate of degradation is higher. Decrease in the rate of degradation at higher concentration may be due to toxic effects [30] of aniline with regard to inadequate cell/aniline ratio or biomass through the inhibition of metabolic activity. Similar results were observed in various reported microorganisms [31,32]. Our data indicates that the

studied alkaliphile, *Bacillus badius* D1 strain can degrade aniline more efficiently than the earlier reported neutrophilic bacteria [33-34]. The degradation rate of aniline was found to be varied with various pH ranging from 7.0 to 11.0. At pH 9.0 the degradation rate was maximum while the rate was marginally decreased with increase in pH indicating that the alkaliphile, *Bacillus badius* D1 strain degrade aniline in a wide range of pH, while most of the neutrophilic bacteria degrade organic compound at neutral pH. Similarly, the effect of temperature on the degradation of aniline revealed that the bacterial strain could degrade aniline significantly in a wide range of temperature (20-40 °C) indicating no thermal inactivation of bacterial degradation activity at 40 °C. Further increase in temperature resulted in a decreased degradation rate [35]. The optimum degradation temperature [36] observed at 30 °C. The degradation potential of this bacteria has been also studied in the presence of various salinity with additional carbon and nitrogen sources. Significant increase in degradation was observed in the presence of 0.5 to 2.5 % salinity, with all added carbon and nitrogen sources. The ability of *Bacillus badius* D1 strain to degrade aniline even at a higher % of salinity suggest that the strain is halotolerant [37] and can be used to treat the effluent containing high amount of salinity. All additional carbon sources were effective in acceleration of percentage degradation [38,35]. Similar results also observed in case of inorganic nitrogen sources. Additional carbon and nitrogen sources can generate NADH which acts as electron donors for hydroxylation of aromatic compounds by microbial enzymes.

Incubation of bacterial strain with aniline has been resulted in the increase in the activity of acetanilide hydroxylase, superoxide dismutase, catechol 1,2 dioxygenase and catechol 2,3 dioxygenase and cytochrome P450. The induction these enzymes might be due to the effect of parent molecule aniline or its metabolites on genes for coding these enzymes. In order to reveal the mechanism of degradation of aniline by an alkaliphile, *Bacillus badius* D1 strain, the structure of metabolites formed during biotransformation were delineated and elucidated the probable degradation pathway [39-42] of these molecules by various spectroscopic methods.

V. Conclusion:

The alkaliphile, *Bacillus badius* D1 strain was isolated from pristine Crater Lake of Lonar (MS) India, has strong aniline degradation potential. As it tolerates a broad range of temperature, pH, salinity and concentrations, further adaptation of this strain may be useful for the commercial bioremediation purpose at polluted industrial site.

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