Chapter III Biodegradation of Aniline by alkaliphilic Bacillus badius D1

3.1. Introduction: Monocyclic aromatic amines MAAs are environmental pollutants. Many of them are genotoxic and impose hazards to human health. The mutagenicity of more than 80 of these amines was reviewed with primary emphasis on evaluation by the Ames Salmonella microsome testing system (233). Many amines are mutagenic in salmonella tester strains TA98 and TA100. Aniline is a serious environmental threat and health risk to living organisms. It is being released into the soil and water bodies owing to its expanded use in industry (234). Aromatic amines have been recognized as carcinogen since 1895. Aniline is synthetic organic compound used in dyes, herbicides and medicine. It is most important intermediate product of various chemical industries. It is a metabolite of diphenyl urea degradation pathway (235). It is used in polyurethane, rubber and agricultural products as well as in some medicines. Aniline cancer had been detected in dye workers particularly as bladder cancer. It is an organic aromatic weak base partially soluble (36 g/l) in water and increase toxicity in aquatic system. Aniline was reported to generate hydrazobenzene, 4-amino diphenyl amine, 2-amino diphenyl amine, benzidine and azobenzene by sunlight (236). It has endocrine disrupting nature (237) and erythrocyte (238) spleen toxicity239). It causes methamoglobinemia leading to less oxygen carrying capacity of hemoglobin (240). Hemangiosarcoma is a rare, rapidly growing, highly invasive variety of cancer. It is a sarcoma arising from the lining of blood vessels; that is blood-filled channels and spaces are commonly observed microscopically. A frequent cause of death is the rupturing of this tumor, leading the patient to rapidly bleed and death (241) is one another severe effect of aniline and other aromatic amines. In 1981 Spanish toxic Oil Syndrome was reported due to denatured repressed fraudulently processed oil containing aniline derivatives and marketed as edible oil (242). There are several reports stating that the azo dye on biotransformation leads to produce aromatic amines (243), like p-phenylene diamine, benzedene or aniline also
It has reported that benzidine is one of the metabolite of chemical and enzymatic reaction of Direct black 38 and Direct brown 1 which are carcinogenic amine (245). Aniline has long been known to be capable of producing methemoglobinemia and hemolytic anemia (246). Aniline produced chemically or enzymatically induced intrachromosomal recombination between repeated sequences in *Saccharomyces cerevisiae*, resulting in deletion of intervening sequences. It is not clear that, whether the free radicals generated during aniline biotransformation or its metabolites are responsible for its recombinagenic activity in yeast. The toxicity and recombinagenicity of aniline in yeast was 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 (247). Microbial and plant metabolism of acetanilide, phenylurea, phenyl carbamate and nitro aniline herbicides produce free and bound aniline residues. It was studied that transformations in aniline or acetanilide may alter the toxicity and bioavailability of the aniline residues in forage(248). Although some biodegradation work has carried with various organic compounds in alkaline and neutral conditions, there is no data available on the degradation of aniline by alkaliphilic bacteria and the reports are scare. Therefore attempt has been made to use alkaliphilic microbes ie. *Bacillus badius* for biodegradation of this compound.

3.2. Material and Methodology:

3.2.1 Chemicals: Aniline and other chemicals like sodium nitrite, sulfamic acid, N-naphthylethylene- diamine dihydrochloride were purchased from SRL Mumbai and material for media was purchased from Hi-media as described in (Chapter II).

3.2.2 Media for biodegradation study:

The broth media used for biodegradation study was similar to the media used in (chapter no. II) unless otherwise the physicochemical parameters like pH, concentration, salinity, carbon source, nitrogen source changed. The pure resistant *Bacillus badius D1* culture maintained in laboratory on slant agar amending little concentration of aniline.
3.2.3. **Adaptation:** This wild strain further adapted for enhancing more potential of degradation to aniline, other aromatic amines and phenols amending the broth with respected pollutant around 3 months.

3.2.4. **Methodology for biodegradation study:** Thirteen 500 ml Conical flask 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 from 0 to 72 Hrs by 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. Aliquot of centrifuged supernatant used for quantitative analysis by diazo coupling with N-(1-Naphthyl ethylenediamine) method (249). Remaining part used for solvent extraction. The residue obtained after Rota evaporation used for UV-vis spectroscopic study, further it was purified by preparative TLC or column chromatography using alumina neutral. The purified residue was further subjected to FTIR, ¹HNMR and GC-MS for structural determination. Similarly one another flask with same media kept as abiotic control by adding the appropriate concentration of aniline aseptically without bacterial culture and aliquots were used for quantitative analysis with respected time intervals. All experiments carried in dark.

3.2.5. **Enzyme activities:**

3.2.5.1. **Preparation of cell extract:**

Cell mass was harvested after 24 hr 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 (Mumbai) in the same buffer. The resulting homogenate was centrifuged in cold condition at 15000 x g for 20 min. Cytosolic protein was measured by Lowry (250) using BSA as standard.
3.2.5.2. Cytosolic content of cytochrome P-450:

The content of cytosolic cytochrome P 450 was measured by the procedure of Omura and Sato(251) The reaction mixture 6 ml containing 1.2 ml cytosole in 50 mM Tris HCl buffer pH 7.4 was divided equally into sample and reference cuvettes. Few grains of sodium dithionite were added to both sample and reference cuvette, a base line of equal absorbance was recorded by scanning on Jasco V-630 double beam recording spectrometer from 500 to 350 nm, CO was paseed through a KOH trap was bubbled through the suspension in the sample cuvette for 30 sec. The difference of absorbance was recorded at 450 nm. The content of CYP -450 calculated using 91 cm \(^{-1}\)mM\(^{-1}\).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.

3.2.5.3. Super Oxide Dismutase [SOD]: SOD was measured spectrometrically according to the procedure of Mishra and Fridovich (252) 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. Total assay mixture 1 ml contain 960 µl 0.05 M carbonate buffer pH 10.2 containing 0.1 mM EDTA and 40 µl of 20 mM epinephrine. The respective blank was prepared by taking 1000 µl of bicarbonate buffer pH 10.2. The androchrome formation was recorded at 480 nm at interval of 30 sec. for 5 min. The inhibition of autoxidation of epinephrine was measured by taking the same ingredient assay mixture plus 20 µl of enzyme preparation and again formation of androchrome was measured at 480 nm.

\[ \text{SOD Activity} = \frac{\text{Control OD} - \text{Experimental OD}}{\text{Control OD} \times \text{Protein concentration}} \times 2 \]

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3.2.5.4. Acetanilide Hydroxylase:

Acetanilide Hydroxylase was assayed by the protocol of Shenkman et al (253) The reaction mixture containing 50 mM Tris HCl buffer pH 7.4, 5 mM MgCl₂, 8 mM acetanilide, 0.2 mg/ml cytosolic protein, total volume 5 ml The reaction was initiated by addition of 1mM NADPH and incubated for 10 min at 37 °C in water bath with continuous shaking. The reaction was terminated by addition of 1 ml of ice cold 10 % TCA. The precipitate was removed by 3000 x g for 15 min and protein free supernatant was used to estimate the product formed.

The p-hydroxy acetanilide formed during the hydroxylation of acetanilide was measured according to the method of Weiseberg and Goodall (254). 1 ml of protein free supernatant was made to 2.0 ml with distilled water. To this 1 ml 0.03 M sodium nitrite was added and kept at 0 °C for 10 min. and then 1 ml 0.03 M R salt [2 naphthol 3,6 disulphonate] in 0.5 N NH₄ OH added. After attaining room temperature resultant redish orange colour was measured at 484 nm. The amount of p- hydroxyl acetanilide was calculated after standardization with standard p-hydroxyacetanilide. The activity of enzyme was expressed as n moles of p- hydroxyl acetanilide formed/min /mg of cytosolic protein.

3.2.5.5. Catechol 1, 2 Dioxygenase: catechol 1, 2 dioxygenase activity was studied by Guzik Urszula, Gren Izabela et al (214). The reaction mixture contain 2880 µl phosphate buffer of pH 8.0, 60 µl 10 mM catechol. The reaction was started by addition of 60 µl enzyme. The formation of cis-muconic acid was monitored at 260 nm for 2 to 3 min and the activity was calculated by using molar extinction coefficient of the product 16.8. The enzyme activity was expressed as µM/ min/ mg of protein.

3.2.5.6. Catechol 2, 3 dioxygenase: catechol 2, 3 dioxygenase activity was studied by J.M Sala Trepat and W.C. Evans (255). The reaction mixture contain 2880 µl phosphate buffer of pH 8.0, 60 µl 10 mM catechol. The reaction was started by addition of 60 µl enzyme. The optical density noted at 375 nm. The enzyme activity noted as µM/ min/ mg of protein.
3.2.6. Reagents of N Napthyl ethylene diamine (NNa) method:

Sodium nitrite solution (1%) and sulfamic acid solution (3%), N-Na reagent (0.75%). 0.375 g of N-naphthylethylene-diamine dihydrochloride added to about 45 ml of water while stirring and diluted to 50 ml.

3.2.7. Analytical method for quantification of aniline: To determine the unutilized aniline during the microbial degradation experiments, aniline was estimated by the procedure of George Norvit (256). Standard concentration of aniline was used 0.010 mg of aniline/ml. 0.00, 2.00, 4.00, 5.00, and 6.00 ml of standard aniline solutions were taken to plot the standard graph. These standard solutions were transferred to 50-mL volumetric flasks and diluted to about 35 ml with water. Further 2.0 ml of 1 N hydrochloric or sulfuric acid added in it. Then 1.0 ml of sodium nitrite solution (1%), added with swirling and allowed to stand 5 min. Further 1.0 ml of sulfamic acid solution (3%) added with swirling and washed down the sides of the flask allowing to stand for 10 min. 2.5 ml of N-Na reagent (0.75%) added to the same with swirling, and diluted to the mark. It was mixed properly and removed the stopper to escape of nitrogen gas and allowed to stand for 75 min or more. The absorbance was noted at 555 nm against the blank and the amount of unutilized aniline was determined from the standard graph.
3.3. Results:

3.3.1 UV-visible pattern of aniline degradation by *Bacillus badius D1* at concentration of 1.55 g/L. with different time intervals.

![UV-visible pattern of aniline degradation by Bacillus badius D1 at concentration of 1.55 g/L. with different time intervals.](image)

Fig 3.3.1 UV-visible pattern of aniline degradation by *Bacillus badius D1* at concentration of 1.55 g/L. with different time intervals.

Fig 3.5.1 indicate that the degradation pattern of aniline by *Bacillus badius D1* at 0 hr. of incubation aniline have shown peak at 288 nm and the trough at 270 nm. With further incubation has resulted in gradual decrease in the peak at 288 nm and forming the new peaks corresponding to acetanilide at 235nm, catechol 275 nm 24 hr. Further incubation up to 72 hr. resulted in complete utilization of aniline by the bacteria.
3.3.2 Effect of various concentrations of aniline on bacterial growth:

Fig 3.3.2 Effect of various concentrations of aniline on *Bacillus badius* D1

Fig 3.5.2 shows the effect of various concentrations of aniline on bacterial growth. To see the effects of various concentrations of aniline on bacterial growth, the strain *Bacillus badius* was incubated with aniline at concentration ranging from 0.28 – 1.55 g/L. The maximum growth of bacteria was observed at 0.28 g/L aniline and the reduction of growth was observed with increase in aniline concentration.
3.3.3. Effect of concentrations on degradation of aniline.

![Graph depicting the effect of concentrations on aniline degradation.](image1)

Fig 3.3.3. Effect of concentrations on aniline degradation

Fig 3.3.3 depicts the effect of various concentrations of aniline on the degradation. At concentrations of 0.28 and 0.55 g/L maximum degradation of aniline was observed within 72 hr. 99%. With increase in concentration of aniline the degradation rate was found to be low [78%] up to 72 hr.

3.3.4 Effect of various pH on bacterial growth

![Graph depicting the effect of various pH on bacterial growth.](image2)

Fig 3.3.4 Effect of various pH on bacterial growth
Fig 3.3.4. indicates the growth of bacteria at various pH 7.0 – 11.0 in presence of aniline at concentration of 1.55 g/L. Maximum growth of bacteria was observed at pH 9.0. With further increase in pH, gradual decrease in bacterial growth was observed with 1.55 g/L aniline.

### 3.3.5 Effect of pH on aniline degradation:

![Graph showing the effect of pH on aniline degradation](image)

Fig 3.3.5 Effect of pH on aniline degradation

Fig 3.3.5 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 concentration of 1.55 g/L for 72 hr. at 37 °C with pH range 7-11. Approximately 78% of aniline was degraded at pH 9.0. Further increase in the pH values the rate of aniline degradation was found to be reduced marginally.
3.3.6. Effect of temperature on aniline degradation

![Effect of temperature on aniline degradation graph]

**Fig 3.3.6** shows the effect of temperature on degradation of aniline by *Bacillus badius* D1 to observe the effect of temperature on aniline degradation, alkalophilic strain *Bacillus badius* D1 was incubated with aniline at a concentration of 1.55 g/L for 72 hr. at various temperatures 20-40 °C. The maximum degradation of aniline was observed at 30 °C. Further increase in the temperature resulted in the slight decrease of aniline degradation.

3.3.7. Effect of salinity on aniline degradation:

![Effect of salinity on aniline degradation graph]

**Fig 3.3.7. Effect of Salinity**
**Fig. 3.3.7** indicate the effect of salinity on aniline degradation. The 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 hr. 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 increase in NaCl concentration up to 2.5% in media lowered down the rate of degradation of aniline marginally.

### 3.3.8. Effect of additional carbon sources on aniline degradation:

![Graph showing the effect of additional carbon sources on aniline degradation](image)

3.3.8. Effect of additional carbon sources on aniline degradation

Effect of additional carbon sources on aniline degradation is shown in Fig. 3.3.8. 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 presence of all these carbon sources maximum degradation of aniline was observed approximately (90%).
3.3.9. Effect of additional nitrogen source:

Effect of additional Nitrogen source is indicated in Fig.3.5.9. 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 hr. with various nitrogen sources like NH₄Cl, NaNO₃, NaNO₂, KNO₃, MgNO₃ at 0.02%. All the added nitrogen sources has promoted the degradation of aniline.  

![Fig 3.5.9. Effect of additional Nitrogen source](image-url)
3.3.10 Effect of aniline on various microbial enzyme activities:

![Graph showing enzyme activity](image)

Fig 3.3.10 Effect of aniline on various microbial enzyme activities

Fig 3.3.10 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 hr. at 37 °C, in growth media pH 9.0. Incubation with aniline for 24 hr. was resulted in increase in the content of cytosolic cytochrome P450 and in the activities of acetylvaline hydroxylase, super oxide dismutase, Catechol 1,2 dioxygenase and catechol 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 acetylvaline hydroxylase and catechol 2,3 dioxygenase.
3.3.11 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 hr. is determined by, NMR, FTIR, and GCMS. The formation of acetanilide was confirmed by delta values from (Fig. 3.3.11.7) NMR δ- 2.151, (S-3H), δ-7.5, (m-2H), FTIR stretches at 1662, 1498-1535, 3294 (Fig. 3.3.11.8), the mass spectrographic data. The molecular ion peak appeared at 135 and fragmentation peaks at 93, 77, 66, 43, 40 (Fig. 3.3.11.9). The metabolite acetanilide was isolated at the end of 12 hr incubation. Acetanilide was further transformed to catechol by the bacterial strain *Bacillus badius* at the end of 24 hr. incubation. The conformation has done by NMR data δ-5.33 (S-2H), δ-6.8 (m-4H), (Fig. 3.3.11.10), FTIR Ar-OH-3229, Ar-1517-159 (Fig. 3.3.11.11) and mass spectrographic data showing molecular ion peak at 110 and its fragmentation observed at 92, 80, 64, 53, 40. (Fig. 3.3.11.12) Catechol was further converted in to cis-cis muconic acid at the end of 48 hr. The metabolite structure was confirmed by NMR delta values δ -12.9 (S-2H), δ - 6.21 (2-H), δ -7.59 (2H), (Fig. 3.3.11.13) the FTIR stretches observed at Ar-COOH – b-3043-2575, Ar-1635-1674, (Fig. 3.3.11.14) 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 (Fig. 3.3.11.15)
Fig. 3.3.11.1. HNMR of Aniline

Fig. 3.3.11.2. FTIR of Aniline
Fig. 3.3.11.3. Mass Spectrograph of Aniline

Fig. 3.3.11.4. NMR observed during biodegradation of aniline after 12 hr. matched with Acetanilide:
Fig 3.3.11.5 FTIR observed during biodegradation of aniline after 12 hrs with Acetanilide:

Fig 3.3.11.6. Mass Spectrograph of metabolite observed during biodegradation of aniline after 12 hr. matched with Acetanilide:
Fig. 3.3.11.7. NMR observed during biodegradation of aniline after 24 hrs matched with Catechol:

Fig 3.3.11.8. FTIR observed during biodegradation of aniline after 24 hrs matched with catechol:
Fig 3.3.11.9 Mass spectrograph observed during biodegradation of aniline after 24 hrs matched with catechol:

![Mass spectrograph](image)

Fig 3.3.11.10. NMR metabolite cis–cis muconate

![NMR metabolite](image)
Fig 3.3.11.11 FTIR of metabolite cis–cis muconate

\[
\text{cis-cis Muconic acid}
\]

3.3.11.12. Mass spectrograph of metabolite cis–cis muconate:

\[
\text{cis-cis Muconic acid}
\]
3.3.11.13. Detailed Description Table of the metabolites by spectroscopic data:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound</th>
<th>NMR</th>
<th>FTIR</th>
<th>GCMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aniline</td>
<td>δ -3.43(B -S-,2H), δ -6.62(t,2H J-7.6), 6.71-6.76(t,1HJ-7.6), 7.1(t-2HJ-8.2)</td>
<td>NH-3353, Ar-, 1601-1618,</td>
<td>93,78,66,42,40</td>
</tr>
<tr>
<td>2</td>
<td>Acetanilide</td>
<td>δ- 2.151,(S-3H), δ-7.5, (m-2H)</td>
<td>1662,1498-1535,3294</td>
<td>135,93,77,66,43,40</td>
</tr>
<tr>
<td>3</td>
<td>Catechol</td>
<td>δ-5.33(S-2H)  , δ-6.8(m-4H)</td>
<td>Ar-OH-3229,Ar-1517-1599</td>
<td>110,92,80,64,53,40</td>
</tr>
<tr>
<td>4</td>
<td>Muconate</td>
<td>δ -12.9 (S-2H), δ -6.21(2-H), δ -7.59(2H)</td>
<td>Ar-COOH – b-3043-2575, Ar-1635-1674</td>
<td>142,123,114,96,83,71,56,43,38</td>
</tr>
</tbody>
</table>
3.3.11.14. 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, monoxygenase, catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase.

3.4 Discussion:

Environmental pollutants and wide range of toxic organic compounds 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 peoples are at risk from toxic pollution at levels above international levels above international health standards (257-258). Various types of microorganisms like bacteria, fungi, and algae harbours in the soil and water. Most of these microorganisms are eco-friendly and most of them are also pathogenic. 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(259-261) Microorganisms have capacity to get adapted to the changes in the environmental conditions even at extreme environment (262-263). Microorganisms in their environment i.e. in soil and water are continuously exposed with different types of chemicals. These chemicals vary in physicochemical properties. These pollutants are known to interfere with metabolic processes of the organisms. They are known to induce or inhibit many microbial enzymes (264-272). 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 environment is a threat to living organisms. The degradation of aniline by neutrophilic bacteria has been reported by various reporters (273-275). Even the degradation products of aniline have serious toxic effects on biological system (276-277).

Degradation of aniline by alkaliphilic bacterial strain *Bacillus badius* D1 was study to in various experimental conditions like change in concentration, pH,
temperature, salinity, different carbon and nitrogen sources. Effects of initial aniline concentration ranging from 0.2 -1.55 g/L on the degradation studied to determine the maximum aniline concentration tolerated by the alkaliphilic strain *Bacillus badius* D1. The percent of aniline degradation varies with varying initial aniline concentration. For 98% degradation aniline 72 hr required for 0.25 and – g/L. Increasing aniline concentration subsequently reduces degradation rate due to toxic effects of aniline with regard to inadequate cell/aniline ratio or biomass through the inhibition of metabolic activity. Similar results were observed in the degradation of various other organic compounds. The alkaliphilic strain *Bacillus badius* D1 degrade aniline 98 percent up to conc. 0.55 g/L within 72 hr. 59.65% percent of aniline degradation at 300 ppm was reported in case of nutrophilic bacteria by Sarfaraj Ahmed (278). This alkaliphilic bacterial strain tolerates high concentration of aniline indicates the ability of isolate to withstand with higher concentration of aniline in alkaline and aerobic condition.

Degradation rate of aniline varies with the pH range 7-11. It takes almost 72 hr. for 78.0% degradation of aniline at 1.55 g/L concentration at pH 9.0 and for the concentrations 0.28,0.55,0.77,1.11 g/L, the degradation rate was found to be 99.54,98.49,97.22and 87.32 % respectively. However, at higher pH aniline degradation rate was lower. i.e. 41.68 and 30.62 % degradation was observed at the end of 72 hr. for 1.55 g/L aniline. This alkaliphilic strain *Bacillus badius* D1 degraded aniline in wide range of pH i.e. 7-11 which is a desirable characteristic, in contrast with common degradative bacteria that have a narrow pH range. Most of the neutrophilic bacteria degrade organic compound at neutral pH (279-282). The pH tolerance is quite important because many of organic chemicals including aniline are disposed off from the industry under alkaline conditions. It can be speculated that this alkaliphilic strain efficiently would act on these chemicals (283-284). The temperature effects of aniline degradation were studied at various temperatures ranging from 20 °C to 40 °C. At temperatures 25 °C and 40 °C, the degradation rate was 62% while at temperature 30 °C and 35 °C the degradation rate was 86 % and 82%. The result showed essentially no thermal inactivation of degradation activity under experimental temperature. Hence this strain could
acclimatize to a broad range of temperature. Higher temperature can be attributed to the cell viability. The degradation of aniline was studied in presence of various salt concentrations 0 to 2.5% of NaCl. It was observed that 60.-80 % degradation was observed within 72 hr. for 0.5 to 2.5% salinity for 1.55 g/L aniline, significant degradation of aniline 80% was observed at 0.5% salinity. The ability of alkaliphilic strain Bacillus badius D1 to degrade aniline even at higher concentration suggested that the strain is a halo tolerant culture and can be used to treat the effluents containing high amount of salinity.

Effect of carbon sources on aniline degradation was studied by addition of carbon sources in culture media resulted in marginal increase in the degradation of aniline. Additional carbon sources were ineffective in acceleration of % degradation of aniline which shows quite similarity to observations by (285-287). In contrast additional inorganic nitrogen sources NH\(_4\)Cl, NaNO\(_3\), NaNO\(_2\), KNO\(_3\), and MgNO\(_3\) showed significant increase in percentage degradation of aniline. Addition of nitrogen can regenerate NADH which acts as electron donor for hydroxylation of aromatic compound by microorganisms and thus effective degradation was observed.

For the degradation of organic compound microorganisms employ their enzymes like oxidoreductases, hydroxylases, tyrosinases, catechol dioxygenases, peroxidase, hydrolases etc. Involvement of these enzymes in the degradation of organic material has been reported by many investigators (140,288-291). Incubation of microbial strain with these organic compounds has been resulted in the induction of the activity of these enzymes. In presence studies, incubation of bacterial strain with aniline has been resulted in in the induction of the activities of acetanilide hydroxylase, supper oxide dismutase, and catechol dioxygenases and in the content of cytochrome p-450. The inductive effect of these compounds on the activities of these enzymes could be due to the inductive action of either a parent molecule of aniline or its metabolites on the genes responsible for coding of these enzymes. Similar results were of aromatic amine metabolism by oxygenase system ie. The content of cyp450 and aminopyrine N demethylase has been
induced has been reported in many strain (292-294). Increase in content of cytochrome P450 and activity of marker enzymes as compared to their respective control indicate the involvement of oxidoreductases of bacterial mixed function oxidase system and take part in degradation of xenobiotic compounds (295-296). Our finding showed a similar inductive effect in this microbial enzyme indicative of the important role of these enzymes in achieving the highest level of degradation efficiency. In order to reveal the mechanism of degradation of aniline by alkaliphilic stain *Bacillus badius* D1, delineate the structure of metabolites formed during biotransformation and elucidate the probable degradation pathway of these molecule various spectroscopic methods were used. The UV-vis spectrum of aniline degradation by *Bacillus badius* was taken at different time intervals. Degraded sample showed the disappearance of absorption maxima peak $\lambda_{\text{max}}$ 288 nm in UV range and appearing peak at 275 nm, showing hydroxylation of parent compound finally appeared at 260 nm indicating the aromatic ring fission.

The isolation and characterization of metabolite, its major oxidation and ring fission indicated the multiple routes of enzyme attack. The degradation pathway of aniline by alkaliphilic bacterial strain by *Bacillus badius* D1 was proposed. During the degradation of aniline by this bacteria three metabolites were isolated. In the first reaction isolation of acetanilde was confirmed. This transformation could be attributed to N-acetyl transferase. In next step these bacteria might hydroxylate acetanilide to $p$-hydroxy acetanilide (297) and further to catechol and muconic acid (298 -301). Data from this investigation suggest that N-acetyl transferase, dioxygenase, dehydrogenase and ring cleavage enzymes are present in this bacterial strain.