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Full Length Research Paper

Isolation and characterization of a bacterial strain for aniline degradation

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Aniline, a serious environmental threat and health risk to living organisms is being released into the soil and water bodies owing to its expanded use in industry. The objective of the present study was to isolate a strain from rhizospheric soil samples of wheat (*Triticum aestivum* L.) taken from an agricultural site near the industrial area of Faisalabad, with the capability of degrading aniline with its maximum activity. The isolated strain was identified as *Staphylococcus aureus* ST1 a newly reported strain for aniline degradation. The strain ST1 showed tolerance up to 2000 ppm for aniline on mineral salt media plates and its degradative ability was checked through shake flasks experiments using HPLC. The strain was capable of degrading aniline and utilizing it as a sole source of carbon and energy. Maximum reduction of aniline concentration in medium up to 59.65% was observed after 72 h. An enhancement in biodegradation was observed using glucose as an additional growth substrate. The degradative products analyzed by HPLC were catechol, phenol and some other unknown compounds. Plasmid curing showed the involvement of plasmid encoded genes which was later followed by the isolation of plasmid DNA, which was found to be a large one of ~40 kb having restriction sites for enzymes (*Eco*RI, *Bam*HI, *Cla*I, *Stu*I, *Pst*I, and *Hind*III) used.

Key words: Aniline, biodegradation, *Staphylococcus aureus*, HPLC, plasmid curing, restriction sites.

INTRODUCTION

Rapid industrialization and improper discharge of industrial effluents, wastes, accidental spills or deliberate release of certain hazardous chemicals that are mutagenic, carcinogenic and recalcitrant, pose a serious threat to environment including soils, groundwater as well as open water bodies (Tani et al., 1998). These effluents have a variety of unusual chemicals including a range of aromatic hydrocarbons and their derivatives (Van der Meer et al., 1992) which the microbes enzymatically decompose and utilize in cellular metabolism (Phale et al., 2007).

Aniline is a toxic chemical present in the effluent of many industries as it is widely used as a raw material in the manufacturing of a number of products such as dyes, plastics, resins, pharmaceuticals, petro-chemicals, herbicides, pesticides among others. Its other source is through natural microbial transformation of several nitro aromatic compounds (Hallas and Alexander, 1983; Kinouchi and Ohnishi, 1983).

Aniline is a major breakdown product of diphenylamine and p-aminoazobenzene (Zissi et al., 1997). To treat wastewaters and affected soils containing aniline and most of its derivatives, remedies often used are photodecomposition, auto-oxidation, electrolysis, resin adsorption or ozone oxidation but the cost of such treatments limits their application to small scale facilities (Gheewala and Annachhatre, 1997). Many herbicides are transormed to anilinic compounds whose fate in soil and other environments is only partially clarified and when such anilinic compounds are applied to soil, a greater portion of them is apparently bound both by physical and chemical adsorption (Laanio and Blattmann, 1978; Chung and Boyd,

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Table 1. Growth of seven bacterial isolates at different concentrations of aniline in mineral salt media with and without glucose.

Bacterial strains		Concentration of aniline (ppm)														
		100	200	300	400	500	600	700		100	200	300	400	500	600	700
ST1	PNR-aniline	+++	+++	+++	+++	+++	+++	+++	PNR-G-aniline	++	+++	+++	+++	+++	+++	+++
ST2		+++	+++	+++	++	++	++	++		+++	++	++	++	++	++	++
ST3		++	+	+	-	-	-	-		++	+	+	-	-	-	-
ST4		++	+	-	-	-	-	-		++	+	-	-	-	-	-
ST5		++	+	-	-	-	-	-		+	-	-	-	-	-	-
ST6		++	+	-	-	-	-	-		+	+	-	-	-	-	-
ST7		++	+	+	-	-	-	-		++	++	+	-	-	-	-

+++ = Rich growth; ++ = good growth; + = poor growth; - = no growth.

1987). Aniline and its derivatives are considered to be a health risk due to their strong toxicity and mutagenicity (Saint et al., 1990; Holder, 1999).

Biological treatment facilities are better suited to deal with affected soils and for large volumes of wastewaters where the traditional activated sludge process is not effective in treating aniline (Gheewala et al., 2004). Activated sludge results in the production of derivatives of aniline which are difficult to degrade and inhibit the biodegradation of other chemicals (Wang et al., 2006). O' Neill et al. (2000) isolated a consortium of bacteria capable of degrading aniline found in wastewaters produced by oil-fields, marine mud, acid peat bog water and soils. The majority of the bacteria which metabolize aniline belong to the genera *Rhodcocci, Alkaligenes*, *Pseudomonas* and *Bacilli.* Under anaerobic conditions, the biodegradation of aniline and substituted anilines by *Paracoccus* sp occurs (Wang et al., 2006), while under aerobic conditions, biodegradation of pentylaniline and aniline by *Pseudomonas* sp was reported by Bollag and Russel (Bollag and Russel, 1976).

Anilines are metabolized to the corresponding catechols via one or more oxidative reactions catalyzed by aniline dioxygenases, liberating ammonia and subsequently undergoing metabolic transformations (Liodl et al., 1990; Fuchs et al., 1991). Bacterial degradation of aromatic compounds has frequently been shown to be plasmid encoded (Chakrabarty, 1976). The first degradative phenotypes attributed to catabolic plasmids were found in *Pseudomonas* and were for camphor, octane and salicylate (Chakrabarty, 1972). Plasmids encoding the ability to utilize aniline have been recognized such as pCITI in *Pseudomonas* sp. strain CITI, pTDN1 in *Pseudomonas putida* UCC22 and pYA1 in *Acinetobacter* sp. strain YAA (Saint et al., 1990; Fujji et al., 1997).

The objectives of the present study were the isolation and selection of strain that harbour maximum concentrations of aniline and to check the biodegradative ability of the strain for aniline and ultimately its fate into its different degradative products (catechol, phenol among others).

MATERIALS AND METHODS

Soil Samples

Rhizospheric soil samples of wheat (*Triticum aestivum* L.) were taken from an agricultural site near Faisalabad (31.36 °N 72.99 °E) where industrial effluent was thrown. The soil samples were further processed immediately after it reached the laboratory.

Selection, adaptatiion and screening of aniline degrading bacteria

Seven different strains of bacteria (ST1, ST2, ST3, ST4, ST5, ST6 and ST7) were isolated from the soil samples by streaking on nutrient agar plates. The bacterial colonies of these seven strains were streaked on sterilized mineral salt (PNR) media with 100 ppm of aniline concentration and the strains showing growth at this concentration were then restreaked on higher concentrations of aniline up to 700 ppm (Table 1) of aniline with and without glucose for the screening of the most tolerant strains. Maximum growth showing strains were further restreaked on PNR media at aniline concentrations up to 2000 ppm with and without glucose (Table 2) to make them adopted for higher concentrations of aniline and selection of the final one for further experiment.

Mineral salt media of the composition, 13.6% KH_2PO_4, 2.4% $(NH_4)_2SO_4$ and 2.5% NaOH for PN salt (20×) and 8.0% $MgSO_4$, 0.2% Fe_2SO_4 and 4% HCl for R salt, was used to check the growth with 4-aminophenol as sole source of carbon.

Characterization of colonies

Individual colonies were characterized on the basis of colony morphology (shape, size, texture and colour), gram staining and conventional biochemical tests. Streak plate method was used to obtained single and pure colony. The isolated colony was streaked on nutrient agar slants and incubated at 37°C for 24 h to obtain optimum growth. The preliminary characterization was based on colony morphology on nutrient agar plates after 2 - 3 days at 37°C of incubation. Each isolate was subjected to gram staining (Baker, 1962) and examined for cellular morphology.

Bacterial suspensions for the API-50-CHB/E test were prepared using isolates pregrown on nutrient agar. Colonies were picked up and suspended in sterile CHB medium to get turbidity equivalent to 2 McFarland. The suspension was then added to the tubes according to the manufacturer's instruction. Reading of reaction within each test was taken after 24 and 48 h incubation at 37°C. The

Table 2. Tolerance of ST1 and ST2 on different concentrations of aniline with (PNR-G) and without glucose (PNR).

Bacterial Strain	Concentration of aniline (ppm)											
	100	300	500	700	900	1000	1300	1500	1600	1800	1900	2000
PNR-G-aniline												
ST-1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
ST-2	+++	+++	++	++	+	+	-	-	-	-	-	-
PNR-aniline												
ST-1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
ST-2	+++	+++	++	++	+	+	-	-	-	-	-	-

+++ = Rich growth; ++ = good growth; + = poor growth; - = no growth.

results were then interpreted for bacterial identification according to the manufacturer's instruction and also using the results of conventional biochemical tests (Holt et al., 1994).

Quantification by high performance liquid chromatography (HPLC)

Staphylococcus sp. ST1 was cultured overnight in nutrient broth and was used as inoculum (10% v/v) for 100 ml media with and without glucose having different concentrations of aniline (100, 200 and 300 ppm) and was incubated at 37°C in shaking incubator at 150 rpm. Samples taken at different time intervals (0/start time and after 72 h) of incubation were spun at 10,000 rpm for 10 min and supernatant was filtered by 0.2 µm filter and analyzed by HPLC. HPLC analysis was carried out with a Waters instrument and isocratic separations were done on a C-18 reverse phase column (thermo Hypersil 150 x 4.6 mm 5µ Hypersil) with the mobile phase composition (ACN: H_2O = 78:22 v/v), at a flow rate of 0.8 ml/min at room temperature. The aniline eluted out was detected with a UV detector at 254 nm. Qualitative and quantitative data were obtained by comparing the peaks area of the chromatograms obtained.

Plasmid curing

Before isolation of plasmid having susceptible degradation gene, plasmid curing suggested by Hardy (1993) was performed to ensure that the gene degrading aniline is plasmid encoded or chromosomal encoded. For this, the strain was cultured in 4 ml nutrient broth overnight. Then 0.2 ml of culture was added in 4 ml nutrient broth and placed in shaking incubator at 29°C for 2 - 4 h. The log phase 200 µL culture was then added in each tube with 2 ml broth containing different concentrations of ethidium bromide (20 µg/ml to 10 mg/ml). Positive control containing only cells (without the curing agent) while negative control containing only ethidium bromide were also run and all tubes were incubated (in dark) at 29°C overnight. Tubes with highest concentrations of ethidium bromide (in which growth was manifested) and bacterial growths were selected. These were then serially diluted with sterilized distilled water. Following the spreading technique, equal volumes of inoculum from different dilutions were spreaded on nutrient agar plates and also on PNR media containing aniline of 1400 ppm (in which rich growth and clear zones of hydrolysis of culture were manifested). Plates were then incubated at 37°C for 24 h and then colonies were counted. Colonies were then replica plated on nutrient agar and transferred to selective media containing aniline.

Plasmid isolation

A single colony from fresh plate was inoculated in nutrient broth (10 ml) and was incubated at 37°C. The next day, the inoculum was spun at 14,000 rpm for 1 min and pallet of bacterial cells was obtained. Finally, the pallet of bacterial cells was resuspended in 100 µl of solution 1 and 200 µl of solution 2 and placed for 5 minutes after each addition at room temperature. 150 µl of solution 3 was then added, placed for 20 - 30 min at room temperature and spinned at 14,000 rpm for 5 min. Subsequently, the supernatant was extracted three times with an equal volume of chloroform: Isoamyl alcohol (24:1) and by centrifuging at 14,000 rpm for 5 min. The supernatant was then taken and mixed with twice the volume of ethanol and allowed to stand at -70°C for 30 min following spinning at 14,000 rpm for 10 min. The DNA pallet obtained was air dried, resuspended in 20 µl TE buffer and stored at 4°C (Mini prep DNA isolation procedure). The plasmid isolation was also done using Gene Jet Miniprep Kit, Fermentas according to the instructions given by manufacturer.

Restriction analysis of plasmid DNA

The dilutions of DNA were made (0.2 – 1 µg of DNA in 20 µl of ddH_2O). 2.0 µl of dilution was taken in PCR tubes and then 2 µl of digestion buffer (10X), 1U of restriction enzyme making a total volume of 20 µl by adding deionized water with mixing by tapping tube was added. Incubation was at 37°C for 1 - 2 h and the reaction was stopped by adding 0.5 M EDTA (pH 7.5). The quality and quantity of the genomic DNA was also inspected by running a small aliquot of plasmid DNA on 1% agarose gel (Sambrook and Russell, 2001). A total of 10 µl (6 µl sample + 4 µl loading dye) was loaded in gel pockets and run for 1.5 h at 80 V. Finally, the gel was analyzed in gel documentation system (Bio Rad Instruments, Italy).

RESULTS

In the present study, the effect of different concentrations of aniline with and without glucose was studied on seven bacterial strains (ST1, ST2, ST3, ST4, ST5, ST6 and ST7) and it was found that these all were capable of growing on aniline (100 ppm). When the bacterial colonies of these strains were streaked on mineral salt media (PNR) with 700 ppm of aniline with and without

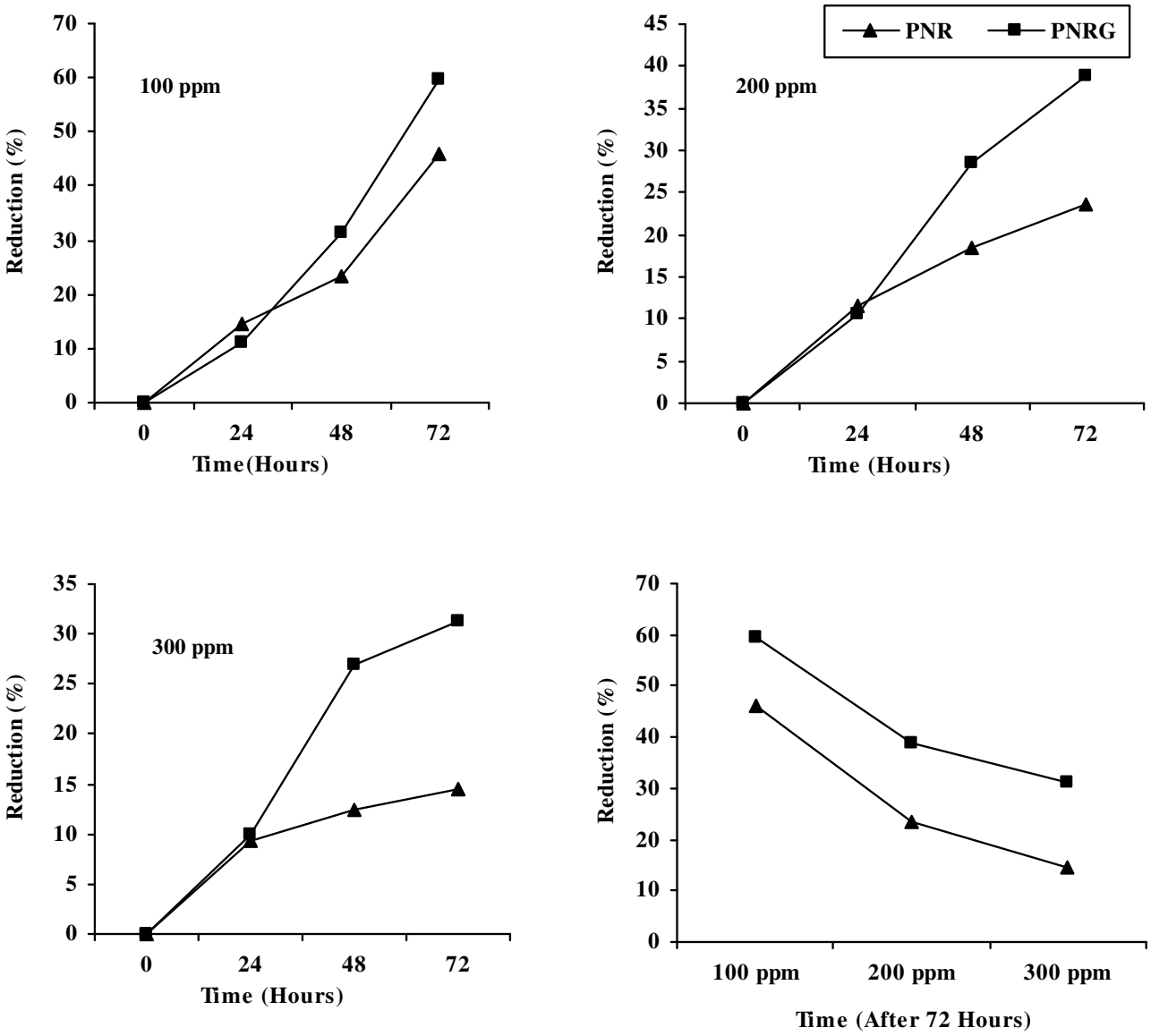

Figure 1. Percentage reduction of aniline at different aniline concentration after 72 h by *Staphylococcus* sp. ST1 determined by HPLC.

glucose, it was observed that of these all only two strains (ST1, ST2) gave rich growth in the 2 to 3 days of incubation (Table 1). These two strains (ST1 and ST2) when restreaked on PNR media containing higher concentrations of aniline with and without glucose for adaptation, strain ST1 showed good and maximum tolerance at all concentrations of aniline while ST2 showed its maximum tolerance at 1000 ppm (Table 2) and as such, strain ST1 was selected for further experiments.

Morphologically, the ST1 gave round to some extent pin point, raised edged, irregular, lobate margin and opaque white to off-white colonies on agar plates while by gram staining, the cells were gram positive cocci forming clusters (bunches) (Holt et al., 1994). The ST1 was also identified on the basis of biochemical tests using API 50 CHB/E kits (Biomerieux, France) and the results were interpreted (Figure 1) and finally the strain was identified as *Staphylococcus* sp. (*Staphylococcus aureus*).

When the effect of aniline alone and aniline with glucose on the degradative efficiency of *S*T1 was observed, aniline reduction in the PNR media containing 100, 200 and 300 ppm of aniline with glucose was more than with no glucose as an additional substrate for each of the concentrations. This is quite obvious from their comparisons individually after different intervals of time (Figure 1). Ultimately with HPLC (Figure 2), overall reduction levels observed after 72 h showed a reduction in aniline of

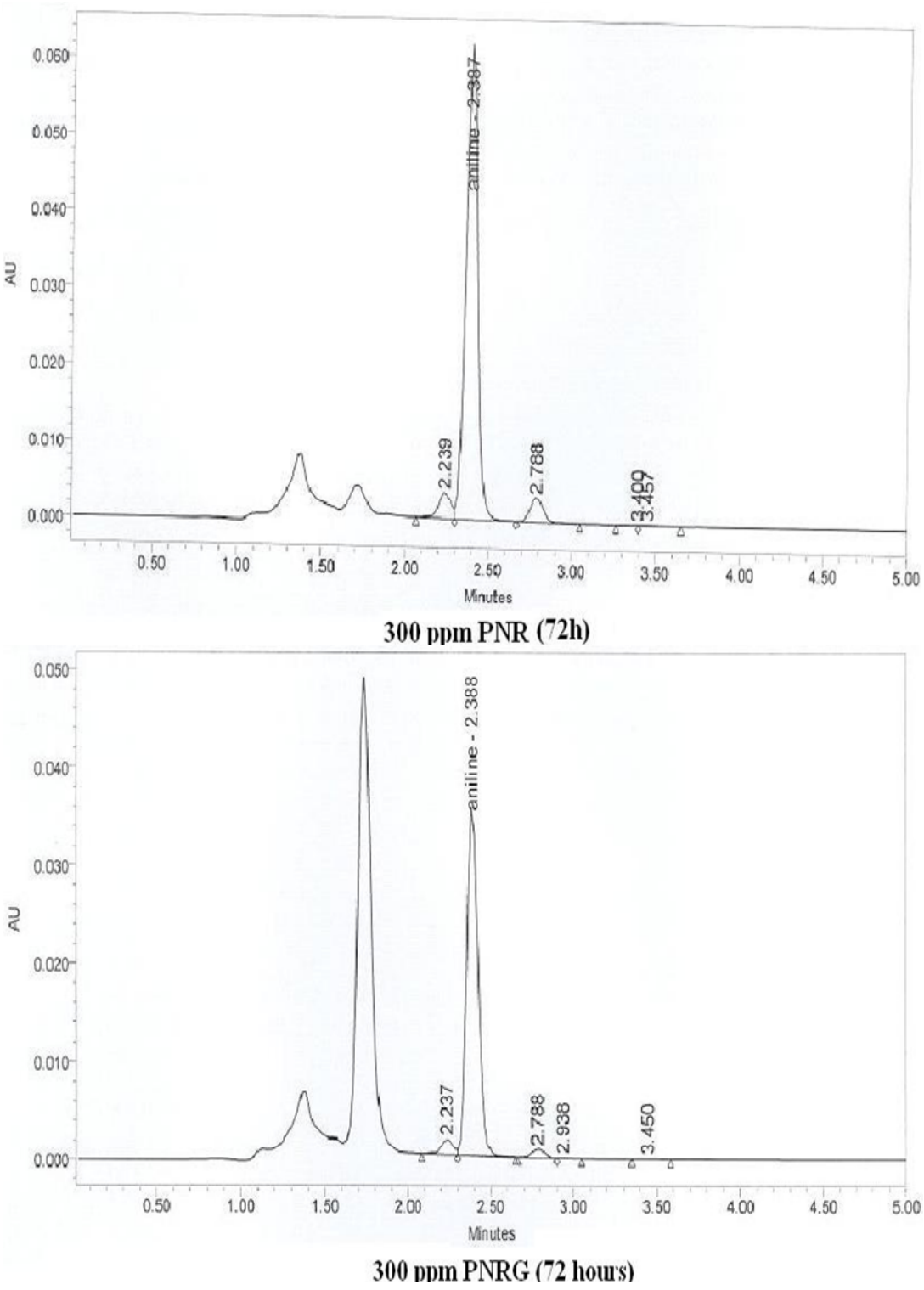

Figure 2. HPLC chromatogram for reduction of aniline at 300 ppm of aniline with and without glucose after 72 h by *Staphylococcus* sp.

45.97% without glucose and 59.65% with glucose at 100 ppm whereas 23.56% reduction without glucose and 38.75% reduction with glucose were observered for 200 ppm. For higher concentration, that is, 300 ppm, 14.5%

reduction without glucose and 31.23% reduction with glucose was seen (Figure 1). The individual and collectively observed levels showed more reduction with glucose as an additional growth substrate.

Plasmid curing, isolation and restriction analysis

In order to determine the involvement of plasmid-encoded genes in aniline degradation, plasmid curing was performed in which ethidium bromide, a mutagenic compound that rendered the plasmid genes inactive was used. It was observed that 200 out of 250 colonies mutated were unable to utilize aniline and thus failed to grow. So it was concluded that the genes involved in aniline degradation were plasmid encoded. The plasmid was isolated and the product obtained was electrophoresed and compared with standard to calculate its molecular weight which was found in the range of ~40 kb. Multiple cloning sites in isolated plasmid DNA of ST1 were also analyzed using restriction endonucleases (*Eco*RI, *Bam*HI, *Cla*I, *Stu*I, *Pst*I and *Hind*III) and two bands for *Eco*RI, *Cla*I and *Stu*I were found showing more than one restriction sites for each but one restriction site for *Bam*HI, *Hind*III and *Pst*I was found as they produce linear or single bands.

DISCUSSION

Wide varieties of aromatic compounds which are being released into the environment through different human activities are metabolized by soil bacteria. This is of great importance in environmental cleanup technologies (Watanabe et al., 1996). Efficient bioremediation process is very important. This is because the bacteria involved perform a complete degradation pathway to eliminate toxic metabolites from soil (Kazunga et al., 2001). In the present study, a bacterial strain *Staphylococcus* sp. (ST1) isolated from rhizospheric soil of wheat crop, was tested for its ability to degrade aniline, a toxic chemical which is present in the effluents of many industries. The strain has been reported in same area in wheat rhizosphere by Arshad et al. (2006) while it is being reported for the first time that the isolated strain has a potential for aniline degradation. The strain grew well in selected media containing aniline with glucose and without glucose showing that it readily decomposed it and utilize it as a sole source of carbon and energy. Similar findings on *Pseudomonas* sp. (PN1001) that actively utilized and degraded pentylaniline and aniline as carbon source with no glucose/starch degradation has being reported by Wang et al. (2006).

Transformation processes are accelerated by adaptation to aromatic compounds which are basic criterion for microbial selections (Bastos et al., 1995). Shake flask transformation of aniline was studied and selection of ST1 was done by adapting it to higher concentration (2000 ppm) of aniline with and without glucose. It was observed that cell growth was increased with time and aniline concentration was decreased as traced by HPLC.

It was found that the total percent reduction was decreased with increase in aniline concentration. As for the lowest concentration (100 ppm), more reduction (59.65% with glucose) was seen after 72 h than with higher concentrations of 200 and 300 ppm which was 38.75 and 31.23%, respectively with glucose. It showed that at higher concentration of aniline, there could be an inhibi-tory effect on the strain which was clear by the less degradation of aniline by the strain at higher concentrations.

HPLC analysis also showed some other smaller peaks of degradative products of aniline which were also studied and were confirmed as benzene, phenol and catechol by their respective standards. The fate of other carbon ring system remained unclear, because benzene (as a product of reductive cleavage), phenol (as a product of hydrolytic cleavage) were not observed in the experiment with the method employed (Drzyzga and Blotevogel, 1997). The 1,2-dichlorobenzene can be co-metabolized (biodegraded) in the presence of glucose by *Pseudomonas* and *Staphylococcus* species. The catabolic end products can be analyzed in the culture medium (Ziagova and Liakopoulou-Kyriakides, 2006).

Aniline transformation in the presence of glucose showed promising results. Without glucose, less reduction (45.97 %) in aniline concentration was observed after 72 h which is less than aniline with glucose (59.65%) for 100 ppm. The same pattern of aniline reduction was seen for 200 and 300 ppm when glucose was used as an additional supplement. This indicated that the glucose posed a stimulatory effect on the microbes to degrade aniline faster. The induction of aniline oxidizing activity in *Rhodococcus erythropolis* AN-13 was accelerated in the presence of glucose with an increase in cell growth (Aoki et al., 1983). Shukat et al. (1983) reported that *Rhodococcus* sp. AN117 was able to co metabolize 2- and 3-chloroaniline in the presence of glucose and that the addition of supplemental glucose did not have any inhibitory effect on the rate of aniline degradation.

The bacterial transformation of aromatic compounds has frequently been shown to be plasmid encoded as reported for many microbial strains (Chakrabarty, 1972; Dunn and Gunsalus, 1973; Williams and Murray, 1974; Anson and Mackinnon, 1984; Saint et al., 1990). Plasmid curing of ST1 showed that 90% of the colonies failed to grow on PNR-aniline agar plates which was possibly due to fact that aniline catabolic pathway genes were plasmid encoded.

The plasmids of ST1 were of larger size of more than 20 kb approximately in the range of 30 - 40 kb. This was confirmed by comparing with DNA marker of known size and by the slow movement of plasmid DNA on agarose gel. The isolated plasmid DNA was digested with restric-

tion endonucleases (*Eco*RI, *Bam*I, *Cla*I, *Stu*I, *Pst*I *and Hind*III) and the resultant restriction fragments were separated by electrophoresis on an agarose gel against the standard to calculate the molecular weight of different restriction DNA fragments. Two bands for *Eco*RI, *Cla*I *and Stu*I have more than one restriction sites and hence results in digests of smaller size 8 - 10 kb, while linear bands were observed for, *Bam*HI, *Pst*I and *Hind*III. These had one s restriction site for each.

From the present study, it is concluded that *Staphylococcus aureus* ST1 is a newly reported bacterial strain that is capable of utilizing aniline as a growth substrate with a catabolic rate that is up to 59.65%. Glucose on the other hand, proved to be an additional carbon constituent to influence and increase the activity of the strain. The genes responsible for aniline degradation are plasmid encoded with different restriction sites.

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