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Biodegradation of *p*-Chloroaniline by bacteria isolated from Contaminated Sites

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Abstract

Enrichment of water from a contaminated site in a textile industry in Ikeja resulted in the isolation of two bacteria belonging to the genera *Alcaligenes* and *Cellulomonas*. These bacteria were able to mineralize para-chloroaniline (*p*-chloroaniline). Time course degradation of *p*-chloroaniline using pure cultures of these organisms showed that *p*-chloroaniline supported the growth of these isolates. An initial increase in cell densities of 7.50-9.46 cfu/mL was recorded from day 0-9th day for *Cellulomonas* sp. while for *Alcaligenes denitrificans* it was 7.20-9.40 cfu/mL. After day 9, a decrease in population occurred, indicating non-availability of nutrients or toxicity of the medium. Simultaneously, a decrease in the pH, indicative of increased acidity of the medium, was also observed from the first day. The result of the GC analysis of the pure isolates on *p*-chloroaniline shows that 86.5% of the *p*-chloroaniline was degraded by the *Cellulomonas* sp. while 81.2% was degraded by the *A. denitrificans* in 30 days. These bacterial isolates utilized other hydrocarbons such as pyrene, anthracene, crude oil and chlorobenzoates as sole source of carbon and energy but not phenanthrene, naphthalene and biphenyl. The two isolates tolerated NaCl concentration of up to 5%.

Keywords: Biodegradation; *p*-chloroaniline; sole nitrogen; *A. denitrificans, cellulomonas* sp.

INTRODUCTION

Environmental pollution caused by the release of a wide range of compounds as a consequence of industrial progress has now assumed serious proportions. Thousands of hazardous wastes sites have been generated worldwide resulting from accumulation of xenobiotics in soil and water over the years (Rakesh et al., 2005).

Anilines are extensively used in many industrial processes as solvents, as precursors for amino aromatic derivatives and in synthesis of dyes, plasticizers, pharmaceuticals, pesticides, polymers including polyurethane and rubber additives, photographic chemicals and varnishes (Gheewala and Annachhatre, 1997; Yen et al., 2008). They are also formed during microbial transformation of nitroaromatic compound found in explosives, dinitroaniline, urea herbicides and nitropyrenes (Hallas and Alexander, 1983; Kinouchi and Ohniski, 1983).

For many years anilines and chloroanilines have been one of the most important industrially produced amines. In recent years, they have attracted increasing attention owing to their widespread use, ubiquity in the environment and endocrine disrupting activity (Lyons et al., 1984; Yen et al., 2008). As a consequence of this widespread use, they are detected in many industrial waste waters (Sarasa et al., 1998; Lacorte et al., 1999; Rai et al., 2005). Aniline is particularly toxic to aquatic organisms, which is due to their impediment on the oxygen carrying capacity of the blood supply; thus effectively suffocating the aquatic organisms (Liu et al., 2002). The primary toxic effect resulting from acute exposure to aniline, by inhalation or by oral route, is methemoglobinemia, usually accompanied with anoxia, erythrocyte damage and spleen effects (USEPA, 1985; Bombard and Herbold, 2005).

The environmental fate of anilines is affected by a number of physicochemical processes, including evaporation, photo-oxidation and chemical binding

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(Bollag et al., 1978). However, biodegradation is the main mechanism of aniline removal in aquatic environment (Lyons et al., 1984). In soils, mineralization of anilines may occur slowly (Suss et al., 1978) or they may react to form recalcitrant molecules such as azobenzenes and triazenes (Minnard et al., 1977; Parris, 1980).

Aniline is rapidly metabolized in contrast to chloroaniline, which is more persistent in the environment (Suss et al., 1978; Horrowitz et al., 1983). Therefore, many efforts have been undertaken to isolate bacteria capable of degrading anilines and its derivatives. *Moraxella* sp. strain G (Zeyer et al., 1982) was the first strain isolated that could use 4-chloroaniline as sole source of carbon and energy. Other bacterial species such as *Pseudomonas* (Hinteregger et al., 1992), *Frauteria* (Murakami et al., *2003*), *Delftia* sp. AN3 (Liu et al., 2002) and so many others have also been implicated. In this paper we report the isolation and characterization of parachloroaniline (*p*-chlororoaniline) degrading bacteria from textile effluents.

MATERIALS AND METHOD

Sampling

Three different types of effluents (treated, untreated and contaminated water) were collected from textile factory in Ikeja, Lagos, Nigeria. The samples were collected using sterile bottles and transported to the laboratory for immediate analysis.

Physicochemical analysis

Physicochemical properties of the samples were determined by standard methods (Nelson and Sommers, 1982; AOAC, 1990; Eaton et al., 1995). Parameters assayed were dissolved solids, alkalinity, total hardness, chlorides, biological oxygen demand (BOD), and concentrations of nitrite, nitrate, phosphate, sulphate and heavy metals.

Enrichment and isolation of microorganisms

Continuous enrichment technique was used to isolate bacteria able to degrade *p*-chloroaniline. The minimal salt medium (MSM) described by Kastner et al. (1994) in which the ammonium chloride was excluded was used. The medium contained per litre 2.13 g Na_2HPO4, 1.3 g KH_2PO4, 0.5 g $MgS0_4.7H_20$ and 0.055 g yeast extract (Difco). Trace element (1 mL) described by Bauchop and Eldsen (1960) was sterilized separately and added to the medium. 1.5mM *p*-chloroaniline was added to 250 mL Erlenmeyer flask in acetone and vented off in a fume chamber overnight before the addition of 45 mL

of MSM.

Inocula for enrichments were prepared by adding 5 mL of the textile effluents into 45 mL of MSM. Cultures were carried out with 50 mL medium in 250 mL Erlenmeyer flasks under aerobic condition incubated at 25°C in the dark to exclude the effect of photodegradation for a period of 14-21 days on a rotary shaker (200 rpm). Growth was measured in terms of turbidity. All enrichments were transferred three times into homologous medium. Aniline degraders were isolated by plating out dilutions of the final flask on *p*-chloroaniline supplemented MSM solidified with agar at 1.5%, by spread plate method. Upon growth, the colonies were further purified by subculturing on the same medium. The isolates were inoculated into *p*-chloroaniline supplemented MSM flasks, incubated under aerobic condition for 14 days at 25°C on a rotary shaker. These isolates were re-inoculated on Luria-Berthani and nutrient agar for identification. Pure isolates were preserved in glycerol: Luria-Berthani broth (50:50) at -20°C

Identification and characterization of isolates

Bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics following the scheme of (Barrow and Feltham, (1995). The multiple-test system API 20 NE and API CORYNE (Biomerieux –Vitek, Tokyo, Japan) was also employed.

Salt tolerance of isolates was determined by inoculation of isolates from 24-h culture into LB broth (6 mL) containing NaCl ranging from 1-10% (w/v). Incubation was carried out at room temperature (29±2°C) for 14 days, with intermittent shaking of the test tubes and observation for growth as indicated by turbidity.

Substrate specificity

The ability of the isolates to utilize pure aromatic compounds as sole sources of carbon and energy was tested by growing them in liquid media supplied with the respective hydrocarbons at 50 ppm. Incubation was carried out under similar conditions as described above. Degradation was monitored by visual observation for turbidity. The hydrocarbons tested were crude oil, phenanthrene, biphenyl, naphthalene, anthracene, pyrene, 2, 5-dichlorobenzoate, 3-chlorobenzoate and 4-chlorobenzoate.

Evaluation of aniline biodegradation

p-chloroaniline degradation was monitored by inoculating replicate flasks containing 50 mL of MSM supplemented with *p*-chloroaniline at 50 ppm. The flasks were inocul-

Table 1. Physicochemical composition of effluents

Parameter	Treated	Gutter	Raw sample	FEPA/National environment regulations standard
Cl^- mg/L	88.0	20.0	340.0	500 mg/L
NO_3 mg/L	0.15	0.08	0.12	2.0 mg/L
NO_2 mg/L	0.46	0.05	0.13	20 mg/L
SO_4^{2-} mg/L	0.78	0.19	0.84	500 mg/L
Ca mg/L	16.0	4.0	160.0	NA
Cr mg/L	0.25	0.21	0.40	100 mg/L
Ni mg/L	0.01	ND	ND	0.1 mg/L
Fe mg/L	1.01	0.72	1.03	10 mg/L
Na mg/L	11.01	7.86	133.68	NA
K mg/L	3.20	1.50	5.20	NA
Zn mg/L	0.11	0.18	0.15	5 mg/L
Alkalinity mg/L	196.0	144	10,000	30-500 mg/L
Total hardness	144.0	12.0	300.0	100
DO mg/L	1.0	2.0	0.0	NA
BOD mg/L	6.5	7.0	6.0	80 mg/L

Key: ND = Not detected
NA - Not available

inoculated with MSM washed 18-h LB agar grown cells to achieve an initial optical density (OD) of 0.01-0.03, and incubated with shaking at 200 rpm for 21 days at room temperature. At an interval of 3 days, flasks were removed for total viable count (TVC) by spread plate method. Optical density (OD_{600}) was measured using a biomedical colorimeter (USA) while pH was determined using a digital pH meter (Jenway 3051).

Analytical methods / determination of aniline in culture

Residual aniline in culture was extracted by liquid-liquid extraction as described by Adebusoye et al. (2007). Sample (20mL) was introduced into a separate funnel. Hexane (5 mL) was introduced into the funnel and both shaken vigorously until a clear separation of the organic and aqueous phase was seen. The aqueous phase was then run-off and the organic phase was poured into a vial for Gas chromatographic (GC) analysis. The residual concentration of the p-chloroaniline left in the culture was analyzed by GC (Hewlett Packard) fitted with flame ionization detector (FID). The column was OV-101, and SE 30 with length of 60 m. The injector and detector temperatures were 200 and 350^oC, respectively. The initial temperature of 70^oC and final temperature 320^oC were used for the analysis, programmed to rise at the rate 10^oC per min. Nitrogen (N) was used

as the carrier gas with flow rate of hydrogen at 45 mL/min, N at 22 mL/min and air flow rate at 450 mL/min.

RESULTS

Physicochemical properties of the effluents

The physicochemical properties of the textile effluents used in this study are presented in Table 1.The results obtained showed BOD value of 6.5 mg/L in the treated effluents, chromium one of the heavy metals was also detected at a conc of 0.21-0.25mg/L as well as higher alkalinity level of 144-196 mg/L after treatment.

Identification of bacterial strains

The continuous enrichment of the effluents with MSM supplemented with p-chloroaniline led to the isolation of several p-chloroaniline degraders. Two bacterial strains with the best degradative abilities on p-chloroaniline based on visual observation for turbidity were used for this study. In the culture flask of these two isolates slight colour changes were observed. Further incubation for two weeks produced intense yellow colouration in the two flasks.

The first isolate, strain PA1, was a yellow pigmented Gram-positive, non-motile rod, strictly aerobic and negative for oxidase, urease, indole and citrate utilization.
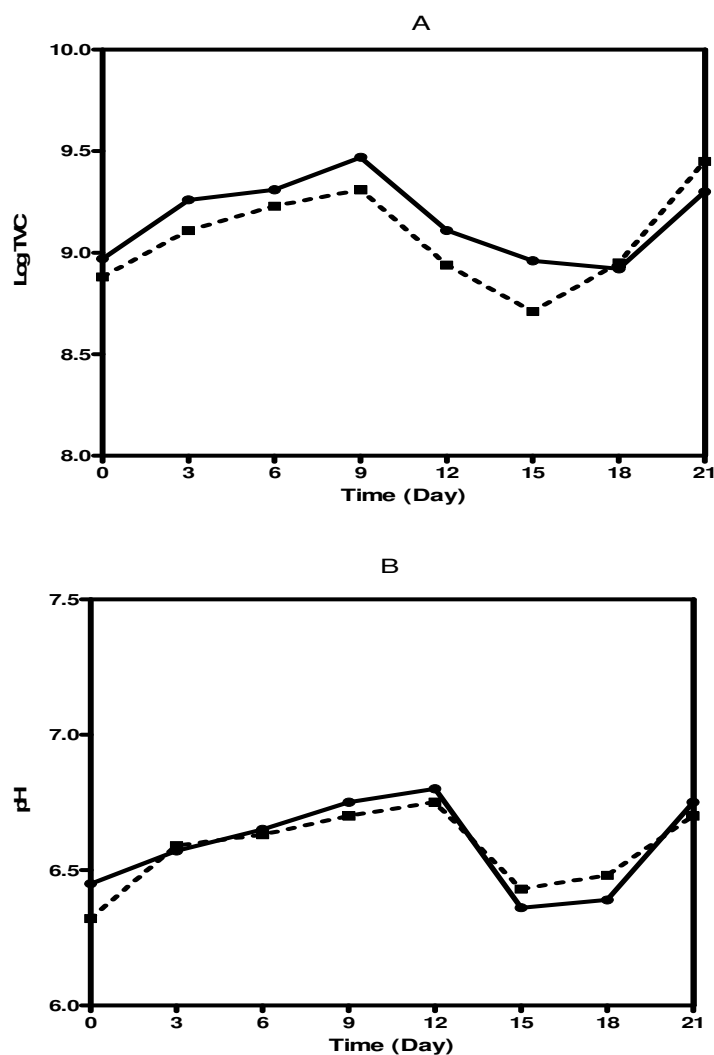
A

B

Figure 1A. Growth profile of isolates in 1.5 mM p-chloroaniline showing *Cellulomonas* sp.strain PA1(■)and *A.denitrificans* strain PA2 (●)

1B. Changes in pH of broth culture during growth In 1.5 mM p-chloroaniline showing *Cellulomonas* sp. strain PA1 (■) and *A.denitrificans* strain PA2 (●)

It did not ferment mannitol and mannose but fermented glucose and fructose with production of acid from glucose as carbon source. No production of H_2S was observed. It was catalase positive and also exhibited positive reaction to methyl red and Voges Proskaeur. It was thus identified as *Cellulomonas* sp. strain PA2 was a yellow pigmented Gram-negative, non-motile rod, positive for citrate, oxidase and catalase. It failed to ferment most sugars tested and was also positive for methyl red and Voges Proskaeur reaction. It produced a yellow pigmentation and was thus identified as *Alcaligenes denitrifican*s.

Growth kinetics of isolates on *p*-chloroaniline

Following a 21-days incubation of the two isolates on *p*-chloroaniline as a sole source of carbon, nitrogen and energy source. Significant increase in cell densities with a corresponding sharp decline in pH in the cultures of both organisms were observed within the first week as shown in Figure 1A and 1B.

The *Cellulomonas* sp. strain PA1 grew with an initial OD of 0.03 to final OD of 0.18 at day 21, while the *A. denitrificans* strain PA2 grew from an initial of 0.01 to final

Table 2. Substrate ranges of isolates

Substrate	*Cellulomonas* sp strain PA1	*A.denitrificans* strain PA2
Crude oil	+	+
Phenanthrene	-	-
Biphenyl	-	-
Napthalene	-	-
Anthracene	+	+
Pyrene	+	+
2,5-chlorobenzoate	+	+
2,6-chlorobenzoate	++	++
3-chlorobenzoate	++	++
4-chlorobenzoate	++	++

Note: ++ = Good growth, + = Moderate growth, -= No growth

Table 3. Kinetics of the degradation of p- chloroaniline by isolates

Isolate	% degradation Day 15	% degradation Day 30	Overall degradation rate mg/L/day
Cellulomonas sp	55.7	86.6	2.89
*A. d*enitrificans	66.2	81.2	2.70

p-chloroaniline was supplied at a concentration of 1.5 mM

of 0.17. From 7.50×10^7 cfu/mL strain PA1, Cell densities grew to 9.46×10^7 cfu/mL on day 9 while strain PA2 ranged from 7.20–9.40 cfu/mL (Figure 1A). The pH of culture ranged from 6.36 to 6.80 for PA1 while for PA2 it ranged from 6.32 to 6.75 (Figure 1B). The result of the GC analysis of the pure isolates on *p*-chloroaniline showed that 55.7%–86.6% of the *p*-chloroaniline was degraded by the strain PA2, while 66.2–81.2% was degraded by the *s*train PA1 as shown in (Table 3, Figure 2 and 3).

Substrate specificity of isolates

The two isolates (PA1 and PA2) showed good growth on 3-chlorobenzoate, 4-chlorobenzoate, and 2, 6-chlorobenzoate. It grew moderately on 2, 5-chlorobenzoate, pyrene, anthracene and crude oil but failed to grow on phenanthrene, biphenyl and naphthalene. The same pattern of growth was also observed on PA2 (Table 2)

Salt tolerance

The two isolates were tested for their ability to withstand low and high salt concentration .It was observed that the *A. denitrificans* strain PA2 grew well at salt concentration of 1%-5% and no growth was recorded at concentration above this. The *Cellulomonas* sp. strain PA1 on the other hand, grew well at concentration of 1–2% with poor growth at 3–5%

DISCUSSION

Potential microbial processes for developing feasible remediation technology to combat environmental pollution due to dye bearing waste waters have been reported by several research groups (Kecharia and Madamwar, 2003). In the last few years, several microorganisms have been found to decolorize, transform and even completely mineralize azo dyes (Stolz, 2001).

The biological oxygen demand (BOD) value obtained in the physicochemical parameters after treatment of the effluent is an indication of polluted water. The results of Odokuma and Okpokwasili (1993) showed that BOD value of 2.5 mg/L indicated fairly polluted water while a value 4.0 or 5 mg/L and above indicated polluted water. The value of chromium one of the heavy metals was detected as well as the alkalinity, was also above the recommended value. The chromium can bioaccumulate through the food chain to toxic levels in man.

The green and blue colour observed in the effluents is as a result of the dyes present. These dyes impart persistent colour to receiving streams and interfere with photosynthesis of the phytoplankton which also has a negative impact on the food chain (Cunningham and Saigo, 2001). Wong and Yu (1990) reported that colour is usually the first contaminant to be recognized in waste

FIG 2

FIG 3

Retention time (min)
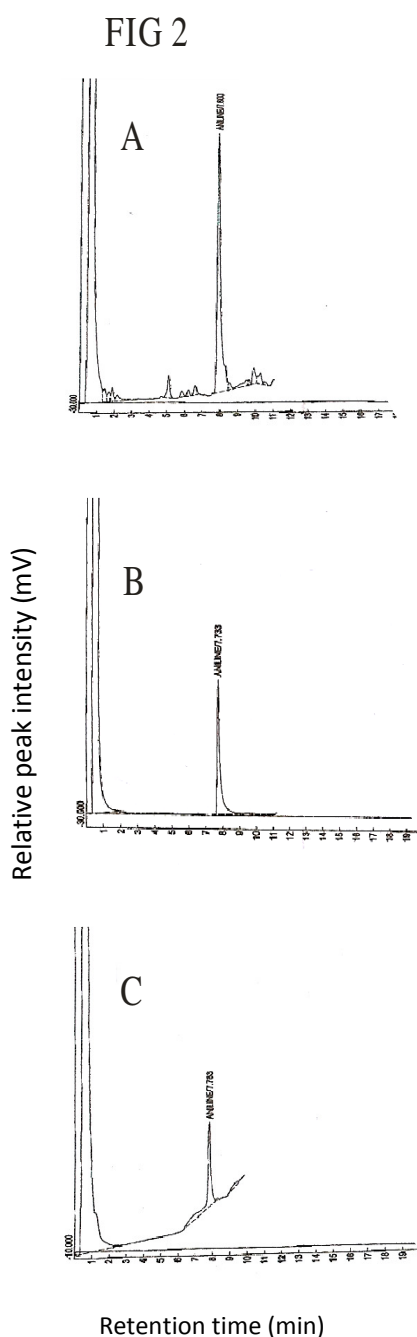
Figure 2. Gas chromatographic fingerprints of p-chloroaniline from flasks inoculated with PA1(A), experimental flasks recovered containing PA1 after incubation for 15days (B) and experimental flasks containing PA1 after incubation for 30days (C).

Retention time (min)
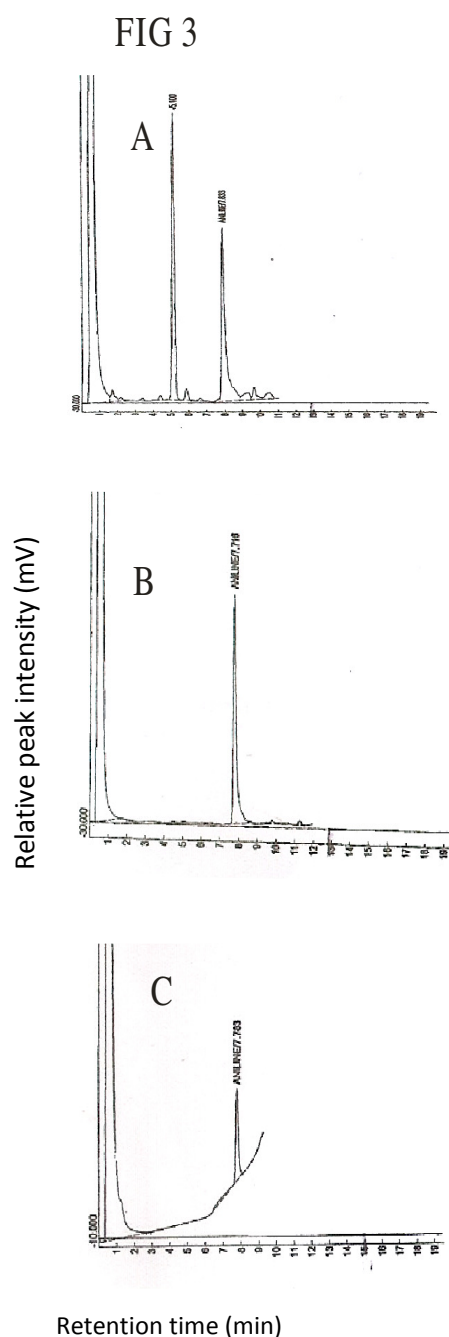
Figure 3. Gas chromatographic finger prints of p-chloroaniline from flasks inoculated with PA2(A), experimental flasks recovered containing PA2 after incubation for 15 days (B) and experimental flasks containing PA1 after incubation for 30 days (C).

waters, and this affects the aesthetics, water transparency and gas solubility.

The biodegradative abilities of two bacterial strains isolated from textiles effluents contaminated sites was examined on *p*-chloroaniline. The two isolates were identified as *A.denitrificans* and C*ellulomonas* sp. Many bacteria capable of degrading xenobiotic chemicals have been reported to belong to *Alcaligenes* and *Pseudomonas*

(Konopka et al., 1989; Galli et al., 1992; Hashizume et al., 1994; Nishihara et al., 1997). The presence of these degraders in these ecosystems can be attributed to the pollution of the ecosystem with elevated amounts of *p*-chloroaniline, which is widely used in the production of dyes used in textile industries.

The continue exposure of microorganisms to xenobiotic compounds facilitates their adaptation through the synthesis of the enzymes for their catabolism. This generally requires the presence of an inducer, which is either the aromatic substrate itself (Worsey et al., 1978) or an intermediate in the degradative pathway (Ornston, 1971). The initial increase in cell densities with corresponding decrease in pH could be due to production of different metabolites in the growth medium. A later decline in population could be as a result of non-availability of nutrients and continued production of accumulated toxic metabolites that inhibits growth.

The yellow coloration observed in the two culture flasks has been reported to indicate the production of chlorohydroxymuconic semialdehyde, an intermediate of chlorinated catechol via meta-cleavage (Masaaki et al., 2004). The GC finger prints of the two isolates at 1.5 mM concentration of *p*-chloroaniline within 15-30 days showed 55.7–86.5% degradation rate by the *Cellulomonas* specie and 66.15%-81.3% for the *A. dentrificans* (Figure 2 - 3).

The rising turbidity of the growth medium as shown by the OD and TVC is in accordance with the high substrate utilization observed in the GC fingerprint. From the result obtained in the salt tolerance of the isolates it was obvious that they cannot thrive well in an environment with high salt concentration. The substrate spectrum analysis of the two isolates on other hydrocarbon substrates used in this study showed different utilization patterns. The inability to utilize the three substrates may be as a result of lack of the enzymes and degradative genes needed for the catabolism of these substrates.

In conclusion, indiscriminate disposal of industrial effluents into water bodies poses a major threat to the environment. Consequently, removal of the pollutant before discharge of effluents is a challenge for environmental management. Microbial degradation of these pollutants is the major natural process that leads to detoxification and elimination from the environment.

This study shows the degradative abilities of *A. denitrificans* and *Cellulomonas* sp. in biodegradation of chlorinated aniline. Effective cleanup of environmental pollutants in effluents can be achieved by these isolates. This will reduce the environmental risks and hazards which aniline and its derivatives are known to cause.

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