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Isolation and characterization of a *Pseudomonas* strain that degrades 4-acetamidophenol and 4-aminophenol

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Abstract

Though many microorganisms that are capable of using phenol as sole source of carbon have been isolated and characterized, only a few organisms degrading substituted phenols have been described to date. In this study, one strain of microorganism that is capable of using phenol (3000 ppm), 4-aminophenol (4000 ppm) and 4-acetamidophenol (4000 ppm) as sole source of carbon and energy was isolated and characterized. This strain was obtained by enrichment culture from a site contaminated with compounds like 4-acetamidophenol, 4-aminophenol and phenol in Pakistan at Bhai Pheru. The contaminated site is able to support large bacterial community as indicated by the viable cell counts (2×10^4–5×10^8) per gram of soil. Detailed taxonomic studies identified the organisms as *Pseudomonas* species designated as strain STI. The isolate also showed growth on other organic compounds like aniline, benzene, benzyl alcohol, benzyl bromide, toluene, ρ-cresol, trichloroethylene and *o*-xylene. Optimum growth temperature and pH were found to be 30 °C and 7, respectively, while growth at 4, 25 and 35 °C and at pH 8 and 9 was also observed. Non growing suspended cells of strain ST1 degraded 68, 96 and 76.8% of 4-aminophenol (1000 ppm), phenol (500 ppm) and 4-acetamidophenol (1000 ppm), respectively, in 72 hrs. The isolation and characterization of *Pseudomonas* species strain ST1, may contribute to efforts on phenolic bioremediation, particularly in an environment with very high levels of 4-acetamidophenol and 4-aminophenol.

Introduction

Phenol and its homologous compounds from coal carbonization, heavy synthetic chemicals, petrochemicals and pharmaceutical industries are among the pollutants most frequently found in rivers, industrial effluent discharges in industrialized countries. 4-Acetamidophenol is one of the most extensively used analgesic/antipyretic worldwide. 4-Acetamidophenol and 4-aminophenol are also used in the manufacture of azodyes and photographic chemicals (Merck index). 4-Acetamidophenol is synthesized from 4-aminophenol (Sandler et al., 1989), ρ-nitrophenol, nitrobenzene and monochlorobenzene (Smilkstein et al., 1988) and also a major metabolite of phenacetin and acetanilide. 4-Aminophenol is synthesized from phenylhydroxylamine (Sone et al., 1981). 4-Aminophenol is formed by microbial transformation of hydroxylaminobenzene (Schenzle et al., 1997), 4-nitrophenol (O'Connor and Young, 1996) and aniline (Cerniglia et al., 1981); it is also produced in the metabolism of aniline in the human erythrocytes and rat and hamster liver (Mieyal et al., 1976; McCarthy et al., 1985; Ulgen et al., 1994).

4-Aminophenol is highly toxic and carcinogenic, irritating to eyes, brain and respiratory systems (Zissi et al., 1997; Wang and Barlaz, 1997). 4-Acetamidophenol is capable of causing methemoglobinemia (Goodman and Gilman, 1971), DNA strand breaks, and inhibition of replication and DNA repair systems (Rannug et al., 1995). Harnagea-Theophilus et al. (1999) mentioned that 4-acetamidophenol induces breast cancer cell proliferation.

The prevalence of these compounds in the environment has stimulated investigations into the biodegradation of hazardous substances in water or contaminated soil. This effort is often limited by the

antimicrobial action of the pollutants. High concentrations of toxic chemicals usually reduce the capabilities of microorganisms to remove these compounds (Dean-Ross and Rahimi, 1995). 4-Aminophenol is extremely toxic to the cyanobacteria *Agmenellum quadruplicatum* strain PR-6 (Batterton et al., 1978; Cerniglia et al., 1981). The degradation process can be accelerated by an adaptation to toxins (Shimps and Pfaender, 1987; Aamand et al., 1989).

Bioremediation is an attractive alternative for cleanup because of cost effectiveness and mineralization of phenols into innocuous products. Among the biggest challenges in biological treatment of phenolics in aqueous phase is finding the microorganisms that can efficiently degrade the target pollutants. It becomes very important to explore the potential of new isolates for the degradation of compounds like 4-acetamidophenol and 4-aminophenol. There is a scarcity of literature describing the aerobic biodegradation of 4-acetamidophenol and 4-aminophenol, so little is known about their degradation. There is no report of a microorganism capable of degrading 4-acetamidophenol. Adams et al. (1997) reported the aerobic biodegradation of 4-aminophenol by unacclimated activated sludge microorganisms but the degradative pathways remained unknown.

This paper describes the isolation and characterization of a new 4-acetamidophenol and 4-aminophenol degrading microorganism that was isolated from wastewater contaminated site of a pharmaceutical plant at Bhai Pheru in Pakistan. The wastewater was generated by the plant containing high concentrations of phenolics (phenol, 4-aminophenol, 4-nitrosophenol, 4-acetamidophenol etc.) through the production of 4-acetamidophenol and aspirin using phenol as the basic raw material. The strain isolated will be further used for the effective treatment of their wastewater.

Materials and methods

Growth media, isolation and cultivation conditions

The liquid growth media used were nutrient broth and a defined mineral salts medium (DeFrank and Ribbons, 1976) containing 4-acetamidophenol, 4-aminophenol or phenol as carbon source at final concentration of 250–5000 ppm. Agar 2% w/v (Difco Laboratories, Detroit) was used in solid media and glucose was added in 5 mM concentration. Phenolics were added from concentrated stock solution (1% v/v in distilled water). Soil samples from contaminated soil (1 g) were suspended in sterile distilled water, serially diluted in 10-fold increments, and plated on nutrient agar plates and incubated at 28 °C for 24 hrs. A number of colonies were counted and corrected for the dilution factor and viable cell counts were enumerated. Colonies showing good growth were transferred to mineral salt agar containing 4-aminophenol, phenol and 4-acetamidophenol (250 ppm each). Plates were incubated for several days at 30 °C and colonies showing growth were purified on nutrient agar and after several transfers on phenolics containing media, they were checked again on nutrient agar to be pure. Purity was also tested by microscopy of 48 hrs growth. Pure colonies isolated were tested for growth on higher concentrations of 4-acetamidophenol, 4-aminophenol and phenol (500–5000 ppm) in mineral salt agar with and without glucose supplementation. The isolated strain showing best growth on the compounds under study was selected and characterized further.

Bacterial identification

The isolate was characterized by taxonomic studies based on Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham, 1993) and identified according to the criteria of Bergey's manual of determinative bacteriology (Holt et al., 1994). Colony morphology of pure cultures was studied on nutrient agar plates. Cell morphology was determined by microscopy, gram and spore staining were performed according to the standard protocols and motility was assessed by direct microscopic observation during growth which was confirmed by growing the organism on MIU medium. For preliminary identification at species level, the multiple test system API 20E (bioMeneux-Vitek) was also employed. Triple sugar iron agar slants were used to determine fermentation of glucose, lactose, H_2S and gas production. Other tests were, citrate utilization, urease, oxidase and catalase activity, indole production, nitrate reduction, methyl red test, Vogues Proskauer test, starch hydrolysis, casein hydrolysis, tween 20 hydrolysis, tween 80 hydrolysis, brown pigment from tyrosine and fermentation of many carbohydrates.

Physiological characterization

Utilization of different organic compounds as carbon and energy sources was tested in agar plates of mineral salt medium supplemented with 250 ppm of these

compounds or volatile compounds supplied in vapors. The pH range and optimum pH for growth of the strain were determined by monitoring the growth after 24 and 48 hrs of cultures incubated into nutrient broth having different initial pH values (3–9). Range and optimum growth temperature were also determined by observing growth in nutrient broth at different temperatures, i.e. 4, 25, 30, 35, 40 °C. A 5% v/v inocolum was used in these experiments.

Analytical methods

Concentrations of 4-acetamidophenol, 4-aminophenol and phenol were measured by reverse phase high performance liquid chromatography (HPLC, Shimadzu, Japan), fitted with a detector (Shimadzu) operating at an absorbance wavelength of λ270 nm. Mobile phase contained, acetonitrile: water (35:65 v/v). The flow rate was 1.5 ml/min, and column was operated at room temperature. Authentic samples were cochromatographed to aid the concentration determination of the substrate. Bacterial growth was measured spectrophotometrically at λ600 nm with a Shimadzu model UV 240 spectrophotometer.

Phenol Degradation by resting cell cultures

Exponential phase cells grown in phenol, 4-aminophenol or 4-acetamidophenol liquid medium were harvested by centrifugation at 18,000× g for 30 min, washed twice with 50 mM phosphate buffer (pH 7.2) and resuspended in fresh buffer. The transformation of phenolics was checked by adding phenolic compounds to a final concentrations 500, 1000, 2500 and 5000 ppm. Flasks were incubated at 30 °C in a shaking incubator at 150 rpm. At various time intervals (0, 24, 48, and 72 hrs), 1 ml was removed, centrifuged, filtered and analyzed by HPLC.

Results

Isolation and selection of phenolic compounds degrading microbes

Soil samples were taken from the site of a pharmaceutical plant at Bhai Pheru, Pakistan and were subjected to viable counts per gram of soil which were found to be 2×10^4–5×10^8 cells per gram of soil. Pure colonies were isolated by replica plating on nutrient agar on the basis of their morphology. Initially fifty isolates were checked for their growth on phenolics (250 ppm); 4-aminophenol, 4-acetamidophenol, phenol, and 4-nitrosophenol. Fourteen out of these fifty isolates showed different levels of growth with different phenolics. Out of these isolates one best growing strain selected was named ST1 and was used for subsequent studies. The isolated strain ST1 was adapted to grow on higher concentrations of 4-acetamidophenol, 4-aminophenol and phenol (500–5000 ppm) in mineral salt agar with and without added glucose. All the three compounds supported the growth of strain ST1 up to concentration 5000 ppm when supplemented with glucose. 4-Acetamidophenol, 4-aminophenol and phenol served as the sole sources of carbon and energy up to 4000, 4000 and 3000 ppm, respectively (Table 1). The isolated strain ST1 could grow in nutrient broth at a range of pH 5.0–9.0 with optimum pH 7.0. Growth was observed at temperature range 20–40 °C while 30 °C was the optimum temperature (data not shown). The strain was, therefore, cultured at pH 7.0 and 30 °C temperature in subsequent experiments.

Taxonomy of phenolic compounds metabolizing isolate

The isolated bacterial strain designated STI was gram negative, nonspore forming, motile rods. Bacterial colonies on nutrient agar medium were small, circular, and convex with entire margins. Biochemical tests indicated that the strain ST1 was catalase and oxidase positive, utilized citrate, Vogues Proskauer test positive, indole test negative. It used glucose, fructose, ethanol and glycerol with acid production but did not ferment sucrose, dextrose, lactose, mannitol, dulcitol, salicin, raffinose, rhamnose, maltose or inositol. Triple sugar iron agar did not produce gas or H_2S on triple sugar. Starch, casein, tween 20 and tween 80 hydrolysis were negative. It showed growth at 4 and 40 °C and produced brown pigment on L-tyrosine containing nutrient agar (Table 2). These morphological and biochemical properties are characteristic criteria for *Pseudomonas putida* (Barrow and Feltham, 1993; Holt et al., 1994). The API 20E (bioMeneux-Vitek) identification system, which is based on 21 phenetic characters, also classified strain ST1 as *Pseudomonas putida* with 79% probability.

Metabolic versatility

The phenolic compounds degrading isolate was tested for its ability to use different organic compounds as the sole source of carbon and energy. *Pseudomonas* sp. strain ST1 showed broad substrate range as in

Table 1. Growth of bacterial isolate on different concentrations of 4-aminophenol, 4-acetamidophenol and phenol in mineral salt medium with and without glucose

Strain	4-Aminophenol with glucose (ppm)						4-Aminophenol without glucose (ppm)					
	500	1000	2000	3000	4000	5000	500	1000	2000	3000	4000	5000
STI	+++[a]	++[b]	++	++	++	++	+[c]	+	+	+	+	−[d]
Strain	4-Acetamidophenol with glucose (ppm)						4-Acetamidophenol without glucose (ppm)					
	500	1000	2000	3000	4000	5000	500	1000	2000	3000	4000	5000
ST1	+++	+++	++	++	++	+	+	+	+	+	+	−
Strain	Phenol with glucose (ppm)						Phenol without glucose (ppm)					
	500	1000	2000	3000	4000	5000	500	1000	2000	3000	4000	5000
STI	+++	++	+	+	+	+	+	+	+	+	−	−

[a]Rich growth.
[b]Good growth.
[c]Slight growth.
[d]No growth.

Table 2. Taxonomic characteristics of bacterial isolate strain ST1

Characteristic	Result	Characteristic	Result	Characteristic	Result
Morphology		Acid production from		Hydrolysis of	
Straight rod	+[a]	Glucose	+	Starch	−
Gram stain	−[b]	Fructose	+	Casein	−
Spore formation	−	Lactose	−	Tween 80	−
Motility	+	Dextrose	−	Tween 20	−
Growth at:		Sucrose	−	Brown pigment	
4 °C	++[c]	Mannitol	−	on L-tyrosine	+++
25 °C	++	Raffinose	−	OPNG	−
30 °C	+++	Rhamnose	−	ADH	−
40 °C	++	Maltose	−	LDC	−
Citrate utilization	+	Inositol	−	ODC	−
Oxidase	+	Dulcitol	−	H_2S	−
Catalase	+	Salicin	−	TDA	−
Gas production	−	Ethanol	+	GEL	−
Urease	−	Glycerol	+	AMY	−
Indol production	−	Melibiose	−	VP	+

[a]Positive result.
[b]Negative result.
[c]Growth.
[d]Rich growth.
Abbreviations: ONPG, o-nitrophenol β-galactosidase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase, TDA, tryptophan deaminase; GEL, gelatin liquefaction; AMY; amygladin; VP, Vogues Proskauer; H_2S, hydrogen sulphide.

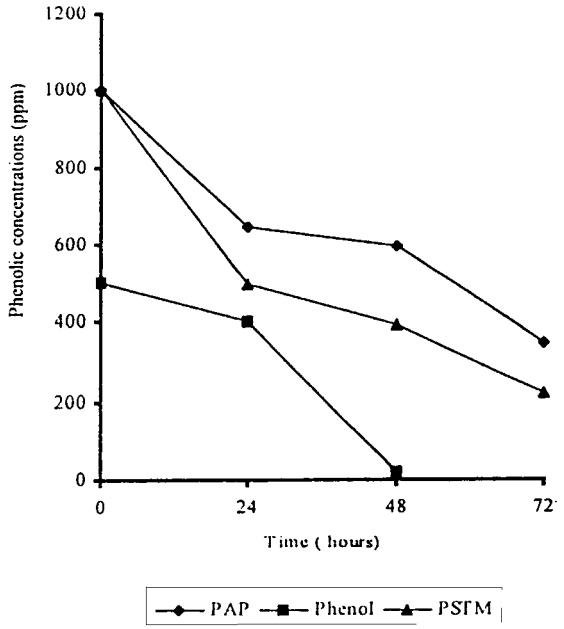

Figure 1. Degradation of phenolics by washed cell suspensions of *Pseudomonas* sp. ST1, lines represents degradation of 4-aminophenol (PAP) by ST1 grown in the presence of PAP, —♦—; phenol by ST1 grown in the presence of PAP, —■—; and 4-Acetamidophenenol (PSTM) by ST1 grown in the presence of PSTM —▲—.

addition to 4-acetamidophenol, 4-aminophenol and phenol growth was also observed on aniline, benzene, benzyl alcohol, benzyl bromide, toluene, *p*-cresol, trichloroethylene and *o*-xylene. Among the compounds checked, only benzoic acid was not utilized by the strain.

Phenolic compounds degradation by non-growing cells

Degradation of higher concentrations of 4-acetamidophenol, 4-aminophenol and phenol non-growing conditions was studied. Cells grown on phenol, 4-aminophenol, and 4-acetamidophenol were harvested in the late exponential phase, washed and resuspended in fresh medium. Under these conditions, 4-aminophenol grown cells of ST1 reduced 4-aminophenol from 1000 to 349 ppm in 72 hrs (Figure 1). Phenol was degraded from 500 to 17.9 ppm after 48 hrs. *Pseudomonas* sp. ST1 cells when induced with 4-acetamidophenol, reduced it from 1000 to 223 ppm within 72 (Figure 1).

Discussion

The removal of hazardous compounds from industrial aqueous effluent is of great practical significance. Microorganisms capable of using phenolic compounds as the sole source of carbon and energy were readily isolated from the contaminated soil exposed to these compounds. This is not surprising given that phenolic compounds are present in most environments. The results of the present study indicate that the site contaminated with the pharmaceutical effluent containing phenol and its derivatives is able to support a large bacterial community as indicated by the viable cell counts (2×10^4–5×10^8) per gram of soil, consisting of various gram negative rods, coccobacilli and few gram positive bacilli. Nishihara et al. (1997) stated that biodegradability toward certain chemicals could be a result of adaptation of microbial community to chemical contamination in present or past. In 1995, Arguiaqa et al. reported that paint striping wastewater containing high concentration of phenol (1800 ppm) and other organic compounds (2000 ppm) was able to support large bacterial community and gram negatives were dominant. Welikala et al. (1997) stated that *Pseudomonas* sp. and *Acinetobacter* sp. were most abundant among the aniline degraders in river water.

Zilli et al. (1993) found that an increase in degradation efficiency results from step by step adaptation of the microorganisms to the pollutant. The isolated bacterial strain was adapted to grow on higher concentrations of 4-acetamidophenol, 4-aminophenol and phenol (500–5000 ppm). Bastos et al. (1995) developed methods for microbial selection based on adaptation to an aromatic compound, like phenol and isolation of strain utilizing phenol as the sole source of carbon. The lethal dose for phenol was 1300 ppm, best growth was shown at 700 ppm. Aniline concentration higher than 1500 ppm completely inhibited the growth of *Pseudomonas multivorans* An1 (Helm and Reber, 1979).

Our isolate showed a broad range of organic compounds that served as the sole source of carbon and energy. *Pseudomonas* sp. strain ST1 also utilized trichloroethylene as growth substrate along with aniline, benzene, benzyl alcohol, benzyl bromide, toluene, *p*-cresol, and *o*-xylene. Nelson et al., 1987 reported the degradation of trichloroethylene by *Pseudomonas putida* and found that toluene dioxygenase was responsible for this degradation. *Rhodococcus erythropolis* AN-13 isolated by Aoki et al. (1983) had a narrow substrate range as it utilized only acetanilide in

addition to aniline out of 20 aromatic amines including 4-aminophenol tested for the growth of bacterium. Wyndham (1986) isolated strains of *Acinetobacter caloaceticus* that grew only on phenol in addition to aniline.

4-Aminophenol, 4-acetamidophenol and phenol were degraded by *Pseudomonas* sp. ST1, Schenzle et al. (1997) have reported the conversion of phenylhydroxylbenzene into 2- and 4-aminophenols as dead end product metabolites by cell extracts of *Ralstonia eutropha* JMP 134 induced with 3-nitrophenol. O'Connor and Young, 1996 reported the reduction of 4-nitrophenol to 4-aminophenol under methanogenic conditions and then its mineralization under methanogenic and denitrifying conditions by microorganisms. Adams et al. (1997) reported the aerobic biodegradation of 4-aminophenol by unacclimated activated sludge microorganisms. Concentration of phenolics were taken as substrate by the non-growing cells is up to 1000 ppm of 4-aminophenol and 4-acetamidophenol, and 500 ppm of phenol. Higher concentrations tested, 2500 ppm and 5000 ppm for 4-aminophenol and 4-acetamidophenol and 1000 for phenol were not utilized while growth results in agar plates were positive up to 4000 ppm of 4-aminophenol and 4-acetamidophenol (Table 1). This shows that although the strain can tolerate higher concentrations of these compounds but utilize only lower concentrations. Toxic limits for these phenolics is >5000 ppm. Babu et al. (1995) isolated a *Pseudomonas* sp. that used phenol up to 1.4 g/L concentration with optimum growth at pH 7.0 and 30 °C temperature.

In summary, a new microorganism that belongs to genus *Pseudomonas* and is capable of using 4-aminophenol, 4-acetamidophenol and phenol as its sole source of carbon and energy was isolated, identified and characterized. Other researchers have also recently reported *Pseudomonas* sp. that are capable of growth on phenol (Shingler et al., 1989; Watanabe et al., 1995; Zaidi and Imam, 1996). Hence *Pseudomonas* sp. strain ST1 may prove to be important in bioremediation and wastewater treatment. Further studies will focus on 4-aminophenol and 4-acetamidophenol metabolic pathways and on genes and enzymes that mediate this metabolism.

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