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Research Article

# Utilization of Bulk Drugs by a Highly Antibiotic Resistant Microorganism Isolated from Waste Water of a Bulk Drug Industry

Basu S[1,*], Dastidar SG[2], Mukhopadhyay S[3], Gangopadhyay A[3]

[1]*Environmental Engineering Division, Civil Engineering Department, Jadavpur University, Kolkata-700032,*
[2]*Department of Microbiology, Herbicure Health Bio-Herbal, Research Foundation, Pailan, Kolkata-700104*
[3]*Civil Engineering Department, Jadavpur University, Kolkata-700032*
***Address for correspondence:** subrata.basu@ymail.com; +919433170814*

## Abstract

An inoculum is collected from the activated sludge of the biological treatment plant of a basic drug manufacturing company in Kolkata producing Citric Acid. This inoculum is cultured and a microorganism is isolated and identified as *Pseudomonas aeruginosa* named as *Pseudomonas aeruginosa* SSP1. It is understood that the organism *Pseudomonas aeruginosa* SSP1 grows by utilizing Citric Acid as a carbon source. This bacterium is found to be highly resistant to a large number of antibiotics and non-antibiotics, thereby proving the danger that may be present in the pharmaceutical wastewater in one side and on the other confirming the potentiality of the bacteria to degrade the compounds and bio-remediate the pharmaceutical effluents.

***Keywords*:** *Pseudomonas aeruginosa*, drugs, wastewater, antibiotics, non-antibiotics

## Introduction

Water consumption and wastewater discharge of pharmaceutical industries are quite large. The wastewater of pharmaceutical formulation as well as bulk drug industries contains many organic and inorganic matters. Steps adopted for treatments of effluent from these industries are screening, equalization, sedimentation and aerobic stabilization. The specific bacterial species acclimatized in presence of the compounds are to be used for aerobic stabilization. Several researchers have isolated and identified bacterial species from the sludge of the biological treatment plant for their further utilization in degradation of many other similar compounds. Present study is an attempt in the same direction.

Many researchers have worked on the identification of species isolated from the sludge of biological treatment plants in pharmaceutical industries and have used those bacteria for utilization of other pharmaceutical ingredients. Following are some of the research findings. Mansour HB et al. (2012) found *Pseudomonas putida* mt-2 to be very efficient in reducing chemical oxygen demand (COD), total dissolved solids (TDS) and turbidity of the pharmaceutical effluents by 85.5%, 89.1% and 81.5%.[1] Das et al (2012) used a *Pseudomonas* species for the bioremediation of pharmaceutical effluent to reduce sulphate, COD, TSS and TDS.[2]

Ali Elkarmi (2008) isolated a bacterial strain capable of utilizing 2, 4-dichlorophenol as the sole carbon source and the strain was identified as *Pseudomonas alcaligenes.*[3] Jayasuriya (1955) identified and characterized an oxalate decomposing bacterial strain as *Pseudomonas.*[4] The organisms when isolated from patients suffering from various infections in hospitals in Kolkata are found to be highly resistant to a large number of antibiotics. Hence the treatment of such patients is found to be really difficult.[5]

The objective of the present paper is to identify an organism from activated sludge of a biological wastewater treatment plant of a basic drug industry and to study on the utilization characteristics of this microorganism with different active pharmaceutical ingredients to establish whether this organism may be used to degrade these drugs in wastewater and bio-remediate the same.

## Materials and methods

### Materials

All media used was obtained from Oxoids, Hampshire, United Kingdom. The strain *Pseudomonas aerugenosa* SSP1 was isolated in our laboratory. *Pseudomonas aerugenosa* ATCC 27853 was obtained from American Type Culture Collection (ATCC), 10801, University Boulevard, Manassas, VA 20110, USA. Antibiotics and

non-antibiotics were obtained from Sigma Chemicals, USA.

## Collection of sludge sample

A sample of activated sludge from the reactor of biological treatment plant of a bulk drug industry has been collected and subjected to isolation and identification of the specific bacteria responsible for biodegradation of the ingredients present in the effluent of the bulk drug industry.

## Preservation of cultures

All the strains of the microorganisms have been preserved in freeze dried ampoules and also in stab agar media. Regular sub-culturing of the bacteria on bromothymol blue lactose agar has been done. To ensure purity of cultures, they are always identified before following standard protocol.

## Tests for identification of the bacteria

*Morphology*

Smear and heat fixed a drop of liquid culture on a glass slide, crystal violet is covered for 30 sec, wash with water, covered with Lugol's iodine for 30 sec water wash, then cover with iodine-acetone solution for 10 sec, wash with water. Smear is covered with dilute carbol-fuchsin for 1 min.,wash with water. The smear is seen under oil immersion lens (100x) of a compound microscope.[6]

*Motility*

A drop of live culture is placed on a centre of a cover slip and inverted over a grooved slide, so the drop hangs inside. Then a drop of cedar wood oil is placed over the cover slip and is seen through a oil immersion lens (100x) of a compound microscope.[7]

## Biological characterization tests

These include the following tests:

*Fermentation of carbohydrates*

Fermentation of different carbohydrates like glucose, lactose, sucrose, mannitol, dulcitol, salicin adonitol, inositol, sorbitol, arabinose, raffinose, rhamnose, maltose, xylose and mannose have been tested and the results have been recorded daily up to 96h and again on the 7th day of incubation.

*Indole Test*

The production of indole may be detected by the use of solution of paradimethyl-amino-benzaldehyde in alcoholic hydrochloric acid. A pink colour indicates the production of indole.[8]

*Oxidation-Fermentation Test*

Stab are prepared with Hugh and Leifson media containing tryptone, yeast ext., glucose, agar, bromocresol purple, two stabs are inoculated with organism, one tube is covered with 1ml liq. Paraffin, incubate for 5 days at 37 °C. A colour change from green to yellow is positive test.[9]

*Methyl-Red*

Organism is inoculated in glucose phosphate peptone water; incubate at 37 °C for 48 h. 5 drops of methyl red are added, bright red is positive, yellow is negative.[10]

*Acetyl-Methyl Carbinol Production Test*

For Voges–Proskaur reaction, in same inoculated and incubated media for methyl red, 1ml 40% KOH and 3ml 5% alpha-naphthol in alcohol are added. Positive reaction indicated by pink colour in 2-5 min, crimson in 30 min.[11]

*Catalase test*

A small amount of of bacterial culture from nutrient slant was smeared on a slide; a drop of hydrogen peroxide was added to it and looked for immediate effervescence of gas bubbles from the culture, for positive reaction.

*Oxidase test*

A small filter paper is soaked with 1% kovacs oxidase reagent and dried. A loop full of fresh culture is rubbed on treated filter paper. Color changes to dark purple within 5 to 10 sec or 60 to 90 sec proves positive.[12]

*Urease test*

The presence of urease, an enzyme which can split urea to ammonia, may be detected by growing the organism on Christensen's medium which consists of a simple medium of urea and phenol red indicator. No colour change in negative.[13]

## Utilization activity of the identified microorganism on other pharmaceutical ingredients

The ingredient present in the bulk drug industrial effluent under present study is citric acid. As the organism grows by utilizing Citric Acid as a carbon source, so it is taken for further study of its utilization activity on other Active Pharmaceutical Ingredients. Sixteen Antibiotics and three non-antibiotics are used for this study. Those are: antibiotics like Penicillin (Pc), Streptomycin (Sm), Tetracycline (Tc), Gentamicin (Gm), Ciprofloxacin (Cf), Norfloxacin (Nf) Chloramphenicol (Cm), Carbenicillin (Cn), Piperacillin (Pp), Amikacin (Ak), Ampicillin (Ap) ,Azithromycin (Az), Meropenem (Mp), Ceftazidime (Cd), Cefoperazone (Cz) ,Cefuroxime (Cr) and non – antibiotics like Thioridazine (Tz),Chlorpromazine (Cpz), Methylglyoxal (Mg).

## Determination of minimum inhibitory concentration (MIC) values of different antibiotics and non-antibiotics

The Minimum Inhibitory Concentration (MIC) is defined as the

lowest concentration of an antibiotic and non-antibiotic that completely inhibits the growth of the organism. The isolated and identified organism is allowed to grow in presence of different antibiotics and non-antibiotics to find MIC values of the ingredients. Simultaneously, the MIC values of a standard strain of *Pseudomonas aeruginosa* ATCC 27853 are also determined. Both organisms are grown overnight on nutrient agar/ Muller Hilton agar plates containing increasing amounts of antibiotics at 37˚C and observed during the stationary phase after 24 hand incubated further up to 72h, if necessary. The MIC values of the antibiotics are determined by agar dilution method by adding the antibiotics individually at concentrations of 0 (control), 25, 50, 100, 200, 500, 1000 and 5000 $\mu$g/ml. MIC values of non-antibiotics are determined similarly.

**Table 1. Morphological and biochemical character of *Pseudomonas aeruginosa* SSP1 with respect to standard *Pseudomonas aeruginosa* ATCC 27853**

| Biological tests and staining | Strain of *Pseudomonas aeruginosa* ATCC 27853 | Strain of *Pseudomonas aeruginosa* SSP1 |
|---|---|---|
| Biochemical tests - Indole | - | - |
| Biochemical tests- Methyl Red | - | - |
| Biochemical tests- Citrate | + | + |
| Biochemical tests-Triple Sugar | - | - |
| Biochemical tests - Catalase | + | + |
| Biochemical tests - Oxidase | + | + |
| Biochemical tests - Urease | - | - |
| Morphology- Motility | + | + |
| Morphology and Gram staining | Gram -ve | Gram -ve |

**Table 2. MIC values, utilization of antibiotics and non-antibiotics ($\mu$g/ml) of the isolate and the known strain ATCC 27853**

| Organism | Control | 25 | 50 | 100 | 200 | 500 | 1000 | 2000 | 3000 | 5000 |
|---|---|---|---|---|---|---|---|---|---|---|
| ATCC27853 (Gm) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Gm) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853 (Pc) | + | + | + | + | + | + | + | + | + | + |
| SSP1 (Pc) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853 (Ap) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Ap) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853 (Pp) | + | + | + | - | - | - | - | - | - | - |
| SSP1 (Pp) | + | + | + | - | - | - | - | - | - | - |

| Organism | Control | 25 | 50 | 100 | 200 | 500 | 1000 | 2000 | 3000 | 5000 |
|---|---|---|---|---|---|---|---|---|---|---|
| ATCC27853(Cb) | + | + | + | - | - | - | - | - | - | - |
| SSP1(Cb) | + | + | + | - | - | - | - | - | - | - |
| ATCC27853(Mp) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Mp) | + | + | + | + | + | + | + | + | - | - |
| ATCC27853(Cz) | + | + | + | + | + | - | - | - | - | - |
| SSP1(Cz) | + | + | + | + | + | - | - | - | - | - |
| ATCC27853(Cd) | + | + | + | + | + | - | - | - | - | - |
| SSP1(Cd) | + | + | + | + | + | - | - | - | - | - |
| ATCC27853(Cr) | + | + | + | + | + | - | - | - | - | - |
| SSP1(Cr) | + | + | + | + | + | - | - | - | - | - |
| ATCC27853(Sm) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Sm) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853(Ak) | + | - | - | - | - | - | - | - | - | - |
| SSP1(Ak) | + | - | - | - | - | - | - | - | - | - |
| ATCC27853(Az) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Az) | + | + | + | + | - | - | - | - | - | - |
| ATCC27853(Nf) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Nf) | + | + | + | + | - | - | - | - | - | - |
| ATCC27853(Cf) | + | + | + | - | - | - | - | - | - | - |
| SSP1(Cf) | + | + | + | - | - | - | - | - | - | - |
| ATCC27853(Tc) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853(Tc) | + | + | + | + | + | + | + | + | + | + |
| ATCC27853(Cm) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Cm) | + | + | + | + | + | + | + | + | + | + |

| Organism | Control | 25 | 50 | 100 | 200 | 500 | 1000 | 2000 | 3000 | 5000 |
|---|---|---|---|---|---|---|---|---|---|---|
| ATCC27853(Tz) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Tz) | + | + | + | + | + | + | + | + | - | - |
| ATCC27853(Cpz) | + | + | + | + | + | + | + | + | + | + |
| SSP1(Cpz) | + | + | + | + | + | + | + | + | - | - |
| ATCC27853(Mg) | + | + | + | + | + | - | - | - | - | - |
| SSP1(Mg) | + | + | + | + | + | - | - | - | - | - |

## Results and Discussion

Results of all the biochemical tests (Table 1) have confirmed that the isolated organism is certainly *Pseudomonas aeruginosa*. After the identification of the organism as *Pseudomonas aeruginosa* it is named as *Pseudomonas aeruginosa* SSP1. Results of determination of the MIC values of antibiotics and non-antibiotics of the isolate and the known strain ATCC 27853 (Table 2) have showed that resistance pattern of both the bacteria exist in a very similar manner.

Above experiment shows that the isolated organism *Pseudomonas aeruginosa* SSP1 is resistant to Pc, Sm, Tc, Cm and Ap up to 5000 $\mu$g/ml, Mp, Tz, Cpz up to 2000 $\mu$g/ml, Cz, Cr, Cd, Mg up to 200 $\mu$g/ml, Az, Nf up to 100 $\mu$g/ml, Cf, Cn, Pp up to 50 $\mu$g/ml and sensitive to Ak at 25 $\mu$g/ml. So there is a scope for further study to ascertain the utilization pattern of the isolated organism with other drugs preferably non-antibiotics having antibacterial action.

## Conclusion

*Pseudomonas aeruginosa* is a highly invasive and toxigenic aerobic Gram-negative bacterium. This is non-sporing, non-capsulate and usually motile with the help of one or two flagella. The organism readily grows over a wide range of temperature and media. There are several reasons for the preeminence of this microorganism as a human pathogen, like its adaptability, its innate resistance to many antibiotics and non-antibiotics and its armory of putative virulence factors.[14] MDR Pseudomonas aeruginosa infections are very often life threatening to patients with compromised immunity and its increasingly growing resistance to various antibiotics are creating massive problems. Alternative therapy of treatment with drugs other than antibiotics should be searched for to control and fight with fatal consequences of the infections.

This is one side of the picture. But on the other side, these bacteria will be useful in degrading effluents containing the above mentioned ingredients up to the concentration found as MIC against each.

## References


1. Mansour HB, Mosrati R, Barillier D, Ghedira K, Chekir-Ghedira L. Bioremediation of industrial pharmaceutical drugs, Drug Chem Toxicol 2012; 3: 235-40.

2. Das MP, Bashwant M, Kumar K, Das J. Control of pharmaceutical effluent parameters through bioremediation. Journal of Chemical and Pharmaceutical Research 2012; 4:1061-65.

3. Ali E, Khaled A-E, Muhammad K. Modeling the biodegradation efficiency and growth of *Pseudomonas alcaligenes* utilizing 2, 4-dichlorophenol as a carbon source Pre and Post-exposure to UV radiation. Jordan Journal of Biological Sciences 2008; 1: 7-11.

4. Jayasuriya GCN. The isolation and characteristics of an oxalate-decomposing organism. J Gen Microbiol 1955; 12: 419-28.

5. Mukherjee S, Chaki S, Barman S, Das H, Koley H, Dastidar SG. Effective elimination of drug resistance genes in pathogenic *Pseudomonas aeruginosa* by antipsychotic agent thioridazine. Current Research in Bacteriology 2012; 5(1), p.36-41.

6. Preston NW, Morrell A. Reproducible results with the gram stain. Journal of Pathology & Bacteriology 1962; 84:241.

7. Collee FG, Miles RS, Watt B. In: McCartney's Practical Medical Microbiology, 1996, Churchill Livingstone: New York, p. 131.

8. Cappuccino JG, Sherman N. In: Microbiology: A Laboratory Manual, Addison-Wesley Publishing Company, USA.

9. Huge R, Leifson E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative rods. J Bacteriol 1953; 66:24-26.

10. Edwards PR, Ewing WH. Identification of Enterobacteriaceae, Burgers Publishing Company: Minneapolis, 1972.

11. Barritt MM. The intensification of the Voges-Proskauer by the addition of alpha-napthol, J Pathology & Bacteriology 1936: 42: 441-454.

12. Collee JG, Frasier AG, Marmion BP, Simmons A. In: Mackie & McMartney's Practical Microbiology, 14th edn., Churchill Livingstone:New York, 1996, p.978.

13. Brown R, Collee JG, Poxton IR, Fraser AG. In: Mackie & McMartney's Practical Microbiology, 14th edn., Churchill Livingstone: New York, 1996, p.561.

14. Aendekerk S, Diggle SP, Song Z, Hoiby N, Cornelis P, Williams P, Camara M. The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinilone-dependent cell-to-cell communication. Microbiology 2005; 151: 1113-25.