

A study on biodegradation of propoxur by bacteria isolated from municipal solid waste

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Abstract- Five different types of bacteria were isolated from Municipal Solid Waste and identified as *Neisseria subflava*, *Staphylococcus aureus*, *Corynebacterium kutscheri*, *Bacillus pasteurii* and *Aeromonas* species. Of these five different types, *Neisseria subflava* and *Staphylococcus aureus* were capable of growing on propoxur containing media. These two bacteria were grown on synthetic broth containing 100 and 200 ppm of propoxur respectively for 12 days. Residual phenol produced as a metabolite was estimated every 24 hours by colorimetry using 4 – aminoantipyrine method. Degradation pattern showed that, *Neisseria subflava* and *Staphylococcus aureus* degraded propoxur into residual phenol and basic compounds. *Neisseria subflava* showed constant increase in degradation of propoxur over the time after 144hrs of exposure to propoxur at 100 and 200 ppm. Nevertheless, degradation of 200 ppm propoxur was comparatively less than that of 100 ppm propoxur by *Neisseria subflava*. *Staphylococcus aureus* showed zigzag pattern of degradation indicating that for every 24 or 48 hrs of degradation of propoxur, there was decrease in growth rate indicating that the metabolite of propoxur was inhibiting the growth and then there was recovery once again leading to increased growth rate for another 24 hrs.

Keywords- Municipal solid waste (MSW), Bacteria, Carbamate, Propoxur, Phenol derivative, Metabolism, Hydrolysis, biodegradation

Introduction

Municipal Solid Waste is a waste generated by households and commercial establishments, and collected usually by local municipal corporation and dump it in the yard. Microorganisms that dwell in these wastes are grouped under Solid Waste Microflora (SWM). The most common organisms that are found in solid waste are bacteria and fungi. Aerobic fungi, *Streptomyces*, thermophilic bacteria like *Brevibacillus borstelensis*, *Bacillus galactosidilyticus*, *Bacillus licheniformis* RH101 and RH104, sulfidogenic bacteria, acetogenic bacteria and photosynthetic bacteria have been reported [1, 2]. Synthetic carbamates have been developed as an alternative to the recalcitrant organochlorine pesticides. However, many of these carbamates are highly toxic and inhibit acetylcholinesterase [3], an enzyme vital to the functioning of the nervous system. Propoxur, commonly called as Baygon is one of the Carbamate insecticides that have the molecular formula $C_{11}H_{15}NO_3$. It exists in different chemical forms, commonly, 2 – (1 – methylethoxy) phenyl N - methylcarbamate and 2 - isopropoxy phenyl N - methylcarbamate [4, 5]. Propoxur is a broad spectrum insecticide; used in hospitals, factories, houses and stables at a concentration of above 0.5% active matter for control of flies, ants, aphids, mosquitoes, cockroaches and millipedes [6]. Propoxur is readily degraded by soil microorganisms in most soils. Environmental conditions that favor the growth and activity of microorganisms also favor degradation. Hydrolysis is a major degradation pathway in soil. Propoxur is reported to be biodegraded quite rapidly in water, particularly when the bacterial activity and temperature is

high. 2-Isopropoxyphenol is a product of propoxur biodegradation [4, 6, 7, 20]. Bacteria capable of degrading carbamate pesticides have been isolated from the soil [4, 6-10, 20]. Studies have implicated the involvement of *Pseudomonas* species in the degradation of propoxur [11]. Many studies have indicated that the first step in the microbial degradation of carbamate compounds is the hydrolysis of the carbamate linkage [7, 10,12, 13]. In this study, we describe the isolation of bacteria from Municipal Solid Waste i. e. *Neisseria subflava*, *Staphylococcus aureus*, *Aeromonas* species, *Bacillus pasteurii* and *Corynebacterium kutscheri* as well as biodegradation of propoxur by *Neisseria subflava* and *Staphylococcus aureus*. The residual phenol produced by degradation of propoxur was analyzed by colorimetric 4 – aminoantipyrine method. Concentration of residual phenol was found from plot of standard phenol, which was then plotted against the time to get the degradation pattern. The propoxur - degrading bacteria may be usable for the decontamination of certain toxic components of industrial waste.

Materials and Methods

Chemicals- Propoxur (2 - isopropoxy phenyl N – methylcarbamate) technical grade (99.1%) was obtained from Bayers India Ltd., Mumbai, 4 – aminoantipyrine from HiMedia Laboratories Pvt. Ltd., Mumbai, Potassium Ferricyanide and Ethyl Alcohol from E. Merck.

Staining reagents- Carbol fuchsin, Crystal violet, Malachite Green, Methylene blue, Safranin stain from E. Merck.

Biochemical test reagents- Nitrate Reduction Discs, Oxidase Discs, Kovac's Reagent, Methyl

red Reagent, α - naphthol 5%, Potassium hydroxide 40% from HiMedia Laboratories Pvt. Ltd., Mumbai.

Culture media- Nutrient Agar, Simmons Citrate agar, Mannitol Motility media, SIM Agar, Skim Milk Agar, Tryptone Broth, Urease Broth from HiMedia Laboratories Pvt. Ltd., Mumbai.

Sample collection and isolation of bacteria- Sample was collected from the Kariyakal Dumpyard of Karkala during rainy season at 27-280 C. Different types of wastes like tree twig, paper, vegetable waste etc. were collected in autoclavable polythene bags. Isolation of bacteria was done by Spread plate method. Pure cultures were obtained by streak plate method [14].

Bacterial Identification- Before being tested, the strains were subcultured overnight in nutrient agar at 30°C. The following phenotypical tests were conducted: Acid – fast staining; endospore staining by malachite green; Oxygen requirement test; production of gas and acid from glucose, fructose, maltose, mannitol, lactose and sucrose under anaerobic conditions (tested with Durham tubes); catalase test; SIM agar was employed to detect H₂S production; presence of caseinase checked by growing bacteria on 10% skim milk agar and growth on starch agar to check the presence of amylase were performed [14]. Grams' staining was conducted [14]; oxidase-reaction was done by transferring a loopfull of solid growth onto an oxidase disc and was checked for deep purple colour [14]. IMViC tests were performed [14]. Organisms were incubated in urea broth to check presence of urease enzyme [14]. Nitrate reduction discs were used to check the ability of organisms to reduce nitrate [14]. Motility test and cellulase test were also performed [14].

In addition to this colony morphology was studied.

Isolation of Propoxur degrading bacteria- Bacteria isolated from MSW were grown in 3 flasks each, having nutrient broth for 24 hrs [15]. Two flasks were treated with 100 and 200 ppm of propoxur. The third flask was maintained as turbidity control. The flasks were incubated at 370C in an incubator shaker, for 12 days.

Analysis of Propoxur biodegradation- Propoxur metabolism results in residual phenol derivatives, which can be detected by colorimetric 4- aminoantipyrine method. This method is based on formation of complex by antipyrine dye with phenolic compounds in the presence of an alkaline oxidizing agent like potassium ferricyanide at pH 10.2. The colour developed is read at 540 nm [16, 17].

Five ml of aliquot was taken at a regular interval of 24 hrs and heated at 900C for 5 minutes and cooled. Bacterial samples were centrifuged at 15000 rpm for 10 minutes. Supernatant was taken in different test tubes, to which 1 ml of 2.4 % potassium ferricyanide and 1 ml of 0.6 % 4 –

aminoantipyrine were added. Allowed to stand for 10 minutes and absorbance was read at 540 nm. The absorbance of the turbidity control was subtracted from the absorbance of the supernatant from the organisms subjected to propoxur. The concentration of residual phenol released by propoxur metabolism was estimated from the phenol standard graph.

Results

Identification of Bacteria-Based on colony morphology, results of staining tests and biochemical tests (Table 1), using Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology isolates were identified as *Neisseria subflava*, *Staphylococcus aureus*, *Bacillus pasteurii*, *Aeromonas* species and *Corynebacterium kutscheri* [14].

Biodegradation of Propoxur-*Neisseria subflava* and *Staphylococcus aureus* were taken for the study of biodegradation of propoxur at 100 ppm and 200 ppm. The concentration of phenol liberated was found from the phenol standard graph by taking the sample aliquot at regular intervals of 24 hours is shown in Table 2. The degradation pattern was Fig. (1) and Fig. (2).

From the graphs it was observed that at 100 ppm, *Neisseria subflava* started degrading propoxur rapidly for 3 days, a lag was seen for a period of next 3 days, after this the organism degraded the propoxur rapidly and continuously. At 200 ppm, *Neisseria subflava* started degrading propoxur slowly for about six days, then the degradation continued rapidly. At 100 ppm, *Staphylococcus aureus* degraded propoxur for three days, and then there was decline in metabolism for a period of one day and after this the same cycle repeated at an interval of 24 hrs. At 200 ppm, the organism degraded propoxur rapidly for a day, after this a lag was seen for a period of three days. This cycle repeated after every 24 hrs.

Discussion

Identification of Bacteria- *Neisseria subflava* is gram negative cocci usually arranged in the form of pairs. It is microaerophilic, oxidase positive, and catalase positive. It shows twitching motility, and nonspore forming. From these tests it is inferred that the organism is *Neisseria subflava* [14]. *Staphylococcus aureus* is a gram positive cocci arranged in the form of clusters. It is oxidase negative, nonmotile, nonsporeforming facultative anaerobe, fermentation of glucose produces mainly lactic acid ferments mannitol and catalase positive. From these tests it is inferred that the organism is *Staphylococcus aureus* [14]. *Bacillus pasteurii* is a gram positive bacillus. It is facultative anaerobic, non motile, spore forming, ferments only glucose and sucrose, hydrolyses starch and is catalase

positive. From these tests it is inferred that the organism is *Bacillus pasteurii* [14]. *Aeromonas* species is a gram negative bacillus. It is aerobic, non motile, produces acid and gas from D – glucose, and acid from other sugars. The organism is oxidase positive and catalase positive. From these tests it is inferred that this organism is *Aeromonas* species [14]. *Corynebacterium kutscheri* is a gram positive bacillus. It is acid fast negative, aerobic, motile, non spore forming, hydrolyses starch, and catalase positive. From these results it is inferred that the organism is *Corynebacterium kutscheri* [14].

Biodegradation of Propoxur- *Neisseria subflava*, *Staphylococcus aureus*, *Bacillus pasteurii* and *Corynebacterium kutscheri*, when initially subjected to propoxur degradation at very low concentration of 50ppm, only *Neisseria subflava*, *Staphylococcus aureus* showed the ability of degradation of propoxur. Therefore, only *Neisseria subflava*, *Staphylococcus aureus*, were selected for the study of degradation of propoxur provided in the medium at a definite concentration. The results indicate that the two pure isolates of *Neisseria subflava*, *Staphylococcus aureus*, used for degradation of propoxur showed variable potential for degrading propoxur. The lag period for each of the isolate varied, depending on the length of the time for which the cells were grown in synthetic medium. The results indicate that these isolates use propoxur as source of carbon and nitrogen, in addition to the nutrients supplied in the media. The residual phenol levels varied from 1 microgram to 42 microgram, with strain *Neisseria subflava* exhibiting the highest degradation ability, by producing 42 micrograms of residual phenol. *Neisseria subflava* takes almost 6 days to acclimatize to the growth medium provided with 100 and 200 ppm of propoxur, which is represented by an S shaped curve. After this there is an exponential increase in the amount of residual phenol produced. It is also evident from this observation that the organisms require some time to get adapted to the xenobiotic metabolism as well as the metabolites so as to switch on the enzymes required for the metabolism of parent compound as well as its metabolites. On the other hand, the residual phenol released by *Staphylococcus aureus* when grown on synthetic medium containing propoxur at 100 ppm and 200 ppm, the isolate rapidly degrades propoxur into residual phenol within a day. Then a lag phase is observed for the next three days i.e., there is a decrease in the concentration of residual phenol produced. After this the strain recovers from the lag phase and again the degradation of propoxur is observed. The same cycle repeats till the end of the study period. The lag indicates that there is degradation of propoxur to residual phenol and other basic compounds. It can be inferred that

the metabolite produced as an end product of degradation of propoxur is probably more toxic than the parent compound. Therefore, there is a lag in the metabolism of the parent compound for the next 24 hrs. It is also possible that the metabolite is further subjected to degradation by another pathway and it is a slow process. Once the metabolite is completely broken down and its inhibition on the biochemical pathway responsible for metabolism of parent compound is no more present, once again the cycle of metabolism of parent compounds starts. Therefore, *Staphylococcus aureus* takes more time to degrade the metabolite than the parent compound. Studies carried out with other organisms like *Pseudomonas* sp. [8, 13, 19], *Blastobacter* sp. [18] have also shown a lag period before the biodegradation of carbaryl.

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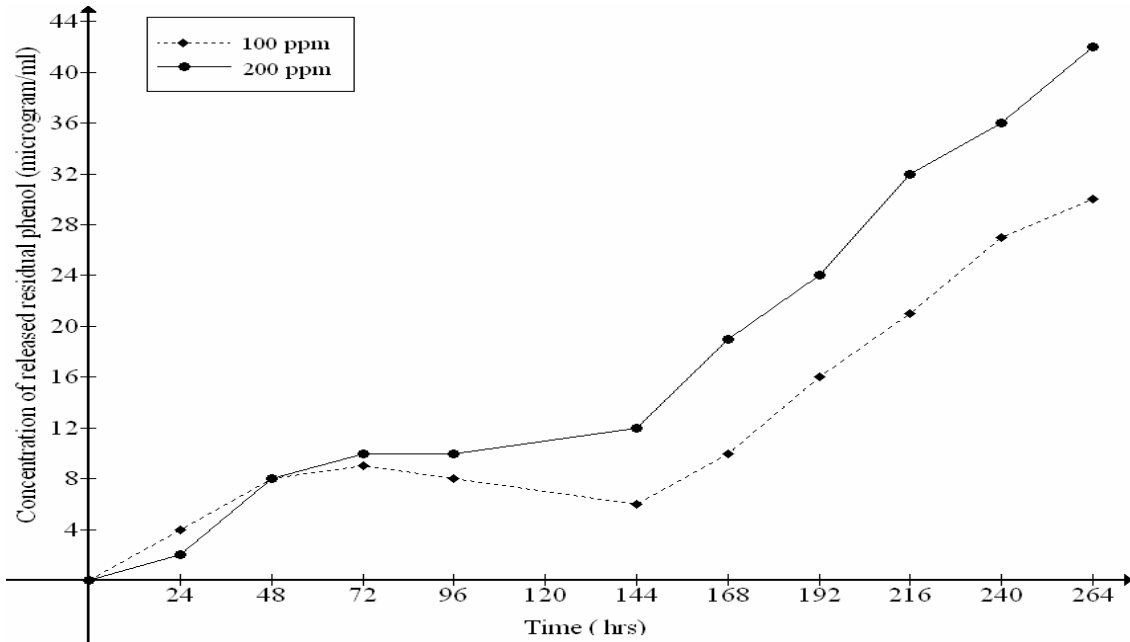


Fig 1- Degradation of 100 and 200 ppm of propoxur provided in the medium by *Neisseria subflava*

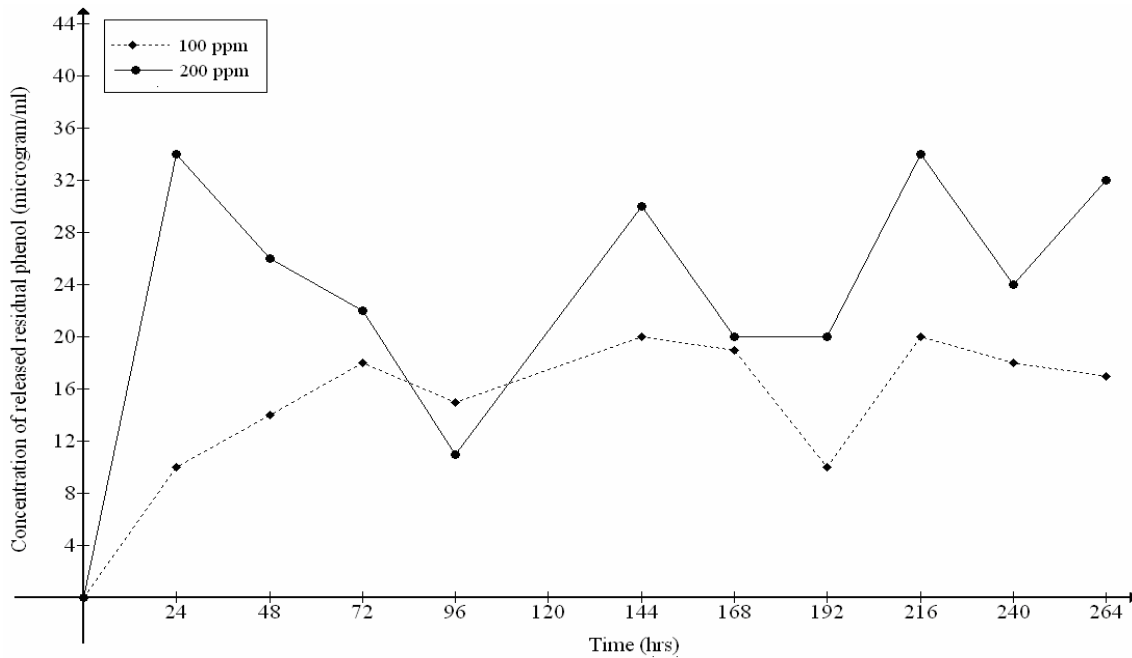


Fig 2- Degradation of 100 and 200 ppm of propoxur provided in the medium by *Staphylococcus aureus*

Table1- Results of tests for identification of bacteria

TESTS	<i>N. subflava</i>	<i>S. aureus</i>	<i>B. pasteurii</i>	<i>Aeromonas</i>	<i>C. kutscheri</i>
				%B ₀	
Cell Shape	cocci	cocci	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	-	+	+	+
NO ₃ Reduction	+	-	+	+	+
O ₂ Requirement	microaero phile	microaero phile	facultative anaerobe	aerobe	aerobe
Indole	-	+	-	+	+
Methyl red	+	+	+	+	+
Voges –Proskauer	+	+	-	-	+
Citrate Utilization	+	+	+	+	+
Endospore Staining	-	-	+	-	+
Glucose fermentation	+	+	+	+	+
Sucrose fermentation	+	+	+	+	+
Fructose fermentation	+	+	-	+	+
Lactose fermentation	-	+	-	+	-
Maltose fermentation	+	+	-	+	+
Mannitol fermentation	+	+	-	+	-
Starch Hydrolysis	+	-	-	+	+
Cellulose Hydrolysis	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+
Urea Hydrolysis	-	-	-	-	+
H ₂ S Production	+	+	-	-	+
Motility	+	-	-	-	+

Table 2- Concentration of released residual phenol estimated at regular intervals of 24 hours produced by the bacteria on treatment with 100 ppm and 200 ppm of propoxur

Sl.No	Time (hrs)	Concentration of released residual phenol			
		<i>N.subflava</i>		<i>S.aureus</i>	
		100 ppm	200 ppm	100 ppm	200 ppm
1	24	4	2	10	34
2	48	8	8	14	26
3	72	9	10	18	22
4	96	8	10	15	11
5	144	6	12	20	30
6	168	10	19	19	20
7	192	16	24	10	20
8	216	21	32	20	34
9	240	27	36	18	24
10	264	30	42	17	32