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BIODEGRADATION OF RECALCITRANT POLLUTANTS BY BACTERIAL ISOLATES FROM MUNICIPAL WASTE WATER

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ABSTRACT: Biodegradation of recalcitrant pollutants are highly challenging because of their heterocyclic aromatic constituents. Four bacterial strains have capacity to utilize phenol as sole source of carbon were isolated from municipal wastewater. On the basis of biochemical characterization, these isolates identified KKS1 to KKS4 as Serratia sp., Alcaligenesfaecalis subsp. faecalis strain, Bacillus sp., Klebsiellapneumoniae strain respectively. These bacterial strains can sustain the initial phenol concentration of 1200 mg/l and also showed 40 to 55% degradation within 24h. Salinity studies revealed them to be moderately halophilic. The presence up to 50% concentration of heavy metals observed no inhibition on the growth of these strains. The unique quality of these strains was utilizing tendency of recalcitrant pollutants as sole source of carbon and energy, without any growth enhancer. Biodegradation of these compounds was examined by the production of secondary metabolites of the corresponding compounds. The utilization of recalcitrant pollutants as a growth substrate by a pure bacterial culture at micro-aerophilic condition is reported.

Keywords: Recalcitrant pollutants, Heterocyclic aromatic compounds. Micro-aerophilic. Phenol.

I. INTRODUCTION

The leather industry plays a significant role in today's global economy, by transforming animal hides/skins into valuable leather goods by subjecting them to chemical and mechanical sequential processes (10). Effluent generated from leather processing contains a high level of organic, inorganic, aromatic chemicals (15) and heavy metals. Phenol and its derivatives with different functional groups attached to the benzenoid ring structures such as, hydroxyl, phenyl, methyl, sulphonic or amide are the basic chemicals used in leather processing. Hence, the leather industry poses serious environmental impact with pollution load resulting in high oxygen demand with other heterocyclic aromatic chemicals. About 30 40 m3 of wastewater is generated per ton of raw material (hide/skin) during leather processing. The wastewater characteristic varies in accordance with variations in raw material, process, chemicals and water consumption. Biodegradation of toxic organic and aromatic constituents is challenging due to their recalcitrance (13) and hence pose difficulties in meeting the mandatory discharge limits set by the pollution control boards.The recalcitrant fraction of Chemical Oxygen Demand (COD) in wastewater contributes 10 to 20 % and it remains in conventional wastewater treatment plants as residual COD. At present, regulatory authorities have enforced zero liquid discharge (ZLD). The major limitation of the aerobic process is its high operational costs due to aeration (8). For cost reduction in aerobic treatment, several researches tried different Dissolved Oxygen (DO) levels with reduced oxygen supply of 0.3 to 0.9 mg /l of DO, in treating domestic wastewater (16, 18 and 19). At this DO concentration, there is a reduction in air supply of about 60 to 80 %, resulting in lower operational cost when compared with conventional aerobic treatment where 2 to 2.5 mg/l DO is required. One more disadvantage in aerobic treatment is the bulking of sludge due to proliferation by filamentous bacterial species if DO levels fall below 1 mg/l (17). The microorganisms present in microaerophilic conditions provide versatile metabolic activity and involves numerous physiological processes (18). The availability of O2 decides a primary environmental signal for switching between growth modes, allowing the organism to utilize C and N sources by different metabolic strategies (9). Microaerobic condition where dissolved oxygen is very low, finds increasing application in the environmental biotechnology, especially in the biodegradation of recalcitrant chemicals. Therefore, the present study focus bioremediation potential of bacterial strains isolated from municipal wastewater to degrade heterocyclic aromatic compounds that are present as recalcitrant pollutants in leather industrial wastewater. These studies were also done in detail on the isolated strains to find out their efficiency to be employed in the treatment of wastewater.

II. MATERIALS AND METHODS

A. Isolation and Identification of Bacterial Strains

Total fifty four bacterial strains were isolated from municipal wastewater. It was operated with pH constant at 7.0 and temperature at 30°C so as to maintain the conditions similar to conventional leather industrial treatment plants in tropical countries, in the presence of 1g/l phenol as the sole carbon source.The screened phenol degrading microaerophilic bacterial strains were examined on heterocyclic aromatic compounds specifically on the recalcitrant pollutants generated from leather industrial wastewater, as sole carbon and energy source.The Mineral Salt Medium (MSM) with phenol (2) was used as enrichment medium for isolation of microorganisms in micro-aerophilic condition. For degradation studies of heterocyclic aromatic chemicals, the MSM was modified by substituting nitrate and sulphate of ammonium salts with corresponding chloride salts in order to utilise the aromatic chemicals as sole carbon and energy

source. MSM containing the chemicals used in leather processes of 25 mg/l each of Biocide, (TCMTB); Surfactant, (Luwet- 40); Vegetable tannin, (Wattle powder); Phenolic Syntan, (Phenol formaldehyde); Synthetic tannin, Naphthalene sulfonic acid, Melamine resin; Acrylic polymer, (Relugan); Vat dye (Direct black 38), Synthetic Fatliquor - (FB-II) and Solvent (Benzene) were added independently. It was inoculated with 10 % of isolated bacterial cultures and incubated in a screw capped flask until dense growth was obtained. About four bacterial strains utilized all the ten recalcitrant chemicals tested. They were designated as KKS1, KKS2, KKS3 andKKS4 Selected isolates were identified through biochemical analysis (6).The isolated bacterial strains were maintained on Luria agar and stored at 4°C until further use.

B. Measurement of Phenol Degradation

At the optimised condition of pH 7.0, temperature 30 ºC and dissolved oxygen of 1.0 mg/l, the phenol degradation potential of all the four bacterial strains, KKS 1 to KKS4 were evaluated. The effect of initial concentration of 1000 to 2000 mg/l of phenol was evaluated in MSM. An initial cell density of 0.034 was inoculated individually into the culture medium in 50 ml screw capped Erlenmeyer flasks and incubated for 24 h. Growth was measured by turbidity at 600 nm and after removing the cells by centrifugation at 8,000 rpm for 10 min, the supernatant was immediately measured for the concentration of phenol by 4-aminoantipyrine method at 510 nm (2)using a UV-Vis spectrophotometer (Shimadzu UV2450), at different time intervals until complete degradation.

C. Salinity Effect

The composite wastewater discharged in common effluent treatment plants (CETPs) is a mixture of different processes from different tanneries. The salinity of composite wastewater varies between 1 to 1.5%. Salinity hinders the activity of microorganism therefore, substrate degradation is inhibited. So effect of each KKS strains on salinity was evaluated in 50 ml screw capped Erlenmeyer flasks by inoculating individually into the culture medium containing 0.5% to 1.5% sodium chloride. After 24 h of incubation, growth of KKSs was measured at 600 nm.

D. Tolerance of heavy metals

Chromium is the major heavy metal used in chrome tanning of leather processes. Zirconium (Zr) aluminium (Al) are used in tanning and retanning as a substitute for chromium salts, and certain metal salts are used in dyeing processes (3). Effect of heavy metal resistance with initial concentration of 25 mg/l of metal salts such as chromium, aluminium, zirconium, zinc, barium and Magnesium were tested. The growth of each strain was determined individually on all KKS strains.

E. Sensitivity to Antibiotics

At present due to the widespread usage of antibiotics, microbial species have developed numerous mechanisms that render them resistant to them. For an antibiotic to be effective against bacteria the existence of a susceptible antibiotic target must be present in the cell, quantity of the antibiotics to the target should be sufficient, and the antibiotic should be active. Susceptibility for all KKSs to different antibiotics namely erythromycin, neomycin, penicillin, ampicillin, polymixin-B, cephaloridine, tetracycline, Ciprofloxacin, Co-trimazole, Gentamycin, Kanamycin and Streptomycin was determined by disc diffusion method (4).The antibiotic impregnated discs (Oxoid) were placed on freshly prepared lawn of bacterial isolate on Mueller Hinton agar plates, and incubated at 30 ± 1°C for 24 h. The bacterial isolate was classified as resistant or susceptible by examining the zone of inhibition on the lawn of bacterial culture, according to the criteria recommended by the national committee for clinical Laboratory Standards, 2001.

F. Metabolic versatility

The experiments on metabolic versatility of four KKS strains as consortium were carried out with MSM supplemented with different synthetic chemicals used in leather processing as sole carbon and energy at a final concentration of 25 mg/l. Recalcitrant chemicals used in leather processing, specifically pesticide, acid and basic dyes, synthetic chemicals used in finishing units and its probable secondary metabolites, secondary amines, were also evaluated to confirm the degradation of recalcitrant chemicals.

The four KKS strains were mixed in equal proportion with an initial cell density of 0.034 and inoculated as consortium into the above chemicals. The culture medium was incubated at 30 °C on an orbital shaker in 50 ml screw capped Erlenmeyer flasks at 50 rpm for a period of 1 to 2 days until dense growth was observed. The negative and abiotic controls were conducted during every set of experiments. The reported values are the average of three replicate measurements.

III. RESULTS AND DISCUSSION

A. Isolation and Identification

About four bacterial strains that can degrade all the ten chemicals tested were selected for identification. They were labelled as KKSs. Biochemical characterisation showed that all the four bacterial strains are closer to Alcaligenes, Bacillus and Enter obacteriaceae families. The KKSs are flocculent in nature with high settle ability, resulting in a compact sludge. They were adopted in such a way that either they were capsulated or sporulated or highly motile to face the stressed conditions. The morphological and biochemical identities of the bacterial isolates are given in Table 1. The KKSs were easily cultivable in Luria broth reaching exponential phase (OD 1.267 at 600 nm) within 6 h of incubation. On the basis of biochemical characterization, these isolates identified KKS1 to KKS4 as Serratia sp., Alcaligenes faecalis subsp. faecalis strain, Bacillus sp., Klebsiellapneumoniae strain respectively.

B. Phenol degradation

The potential of bacterial strains KKS 1 to KKS4 was evaluated at 24h for phenol degradation in MSM, with different initial concentrations of phenol at optimum conditions (Fig 1). Phenol degradation was evaluated at micro-aerophilic condition by growing the four bacterial strains independently in 600, 900, 1200 and 1500 mg/l of phenol as sole carbon. 98 to 99 % degradation of phenol was observed by KKS 1 to KKS4 for the initial concentration of 1000 mg/l. At concentration of 900 mg/l phenol, KKS 1 to KKS4 degraded 75%, 72%, 80% and 74% respectively within 24 h. KKSs degraded between 40 to 55 % within 24h when the concentration of phenol was 1200 mg/l. However, inhibition began from the concentration of 1500 mg/l where KKS 1 to KKS4 degraded 34%, 30%, 41% and 30% of phenol respectively.

C. Salinity Effect

The inhibitory effect of salinity from 0.1 to 1.5% was evaluated by the growth of the four bacterial strain KKSs (Fig 4). By the results obtained, they were observed to be moderately halophilic. Each one of the isolates was able to tolerate well up to 0.8 % NaCl with the absorbance value of 0.5 at 600 nm. 0.5% of NaCl was observed to be optimum with maximum growth at 0.1 % NaCl. However, the phenol degradation was inhibited at 1.5 %.

D. Tolerance of heavy metals

The growth potential of KKS strains with heavy metals that are predominantly present in leather industrial wastewater was evaluated as presented in Fig.5. No significant inhibition on growth was observed on all tested metals at 25 mg/l concentration. Maximum growth was observed in the presence of Mg. Next in order were chromium, barium and zinc. Alumium scored better growth when compared with zirconium. Microorganisms that are effective in sequestering heavy metals, (14) are useful to remove metals from polluted wastewater. The observed results proved that the bacterial strains KKSs may be exploited for industrial wastewater treatment.

Table.1 Morphological and Biochemical characteristics of KKS1 to KKS 4

Test	KKS1	KKS2	.KKS3	KKS4
Colony morpholog	circular	Irregular	Circular	Mucoid
Gram Reaction	G-	G-	G+	G-
Shape	Rod	Rod	Rod	Rod
Motility	+	+	+	-
Endospores	-	+	+	+
Habitat	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative aerobe
Gelatin	+	+	+	+
Starch	+	+	+	+
Citrate Utilization	+	+	+	+
Glucose	+	+	+	+
Sucrose	+	+	+	+
Arabinose	+	+	+	-
Lactose	-	-	+	+
Mannitol	+	+	+	-
Sorbitol	+	-	-	+
Rhamnose	+	-	-	-
Adonitol	-	-	-	-
Hydrolysis of				
Casein	+	+	+	+

Voges Proskauer	+	-	+	+
Indole Formation	-	+	-	-
Methyl Red	-	+	-	-
Nitrate to Nitrite	+	-	+	+
Phenyl Alanine	-	-	-	-
Ornithine decorboxyla	+	+	+	-
Lysine Utilization	+	+	+	+
Urease Detection	-	-	+	+
Oxidase Reaction	+	+	-	-
Catalase Reaction	+	-	+	+
H_2S Production	-	-	-	+
Name of the Isolate	Serratia sp.	*Alcaligene s faecalis*	*Bacillus* sp.	*Klebsiella pneumoni*

Table.2 Utilization of heterocyclic aromatic compounds and its secondary metabolites assole source of carbon by KKS strains as consortium concentration

(a) Pesticide and its Secondary metabolites	Presence of growth
2-thiocyanomethylthiobenzothiazole (TCMTB)	+++
Benzothiazole	+++
3 HydroxyBenzothiazole	+++
2 Methyl Benzothiazole	+++
2 MercaptoBenzothiazole	+++
2 Methyl thioBenzothiazole	+++
2 amino Benzothiazole	+++
Benzothiazolesulfonate	+++

(b) Leather dyes and its Secondary metabolites	Presence of growth
Remazol Red RG	+++
Black DB	+++
Yellow 194	++
Brown 3GV	++
Remazol blue	+++
Black BG	++
Blue RGB	+++
Brown DB	++
Acid green	++
Reactive orange	+++
Black RB	+++
Metanilic acid	+++
Sulfanilic acid	+++

(c) Synthetic Chemicals used in leather processes	Presence of growth
Phenol	+++
Nonyl phenol	+++
Benzene	++
Naphthalene	++
Catechol	+++
Resorcinol	+++
Sodium benzoate	+++
L-Glutamic acid	+++
2 -hydroxybenzoic acid	+++
3-hydroxybenzoic acid	+++
4- hydroxybenzoic acid	++

Remark: ++ OD < 0.8; +++ OD> 0.8

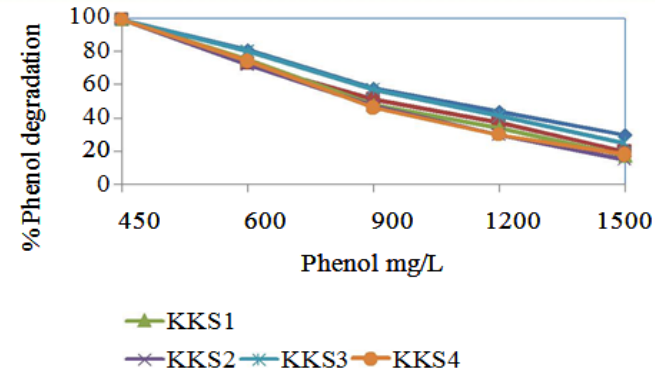

Fig.1 Effect of initial phenol

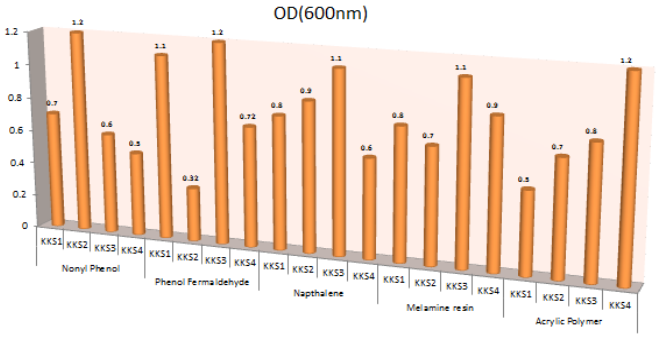

Fig. 2 Growth of KKS strains on heterocyclic aromatic compounds

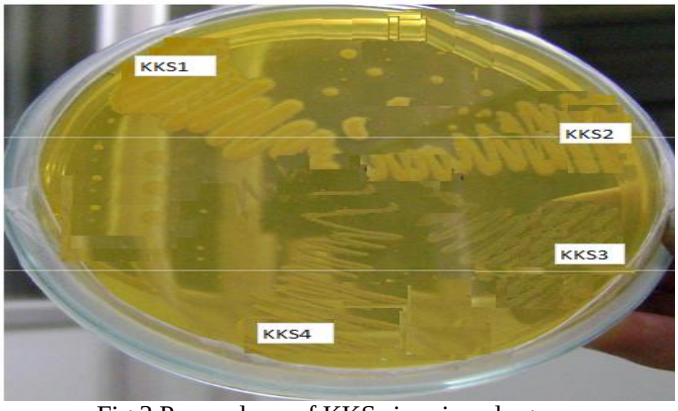

Fig.3 Pure colony of KKSs in mineral agar

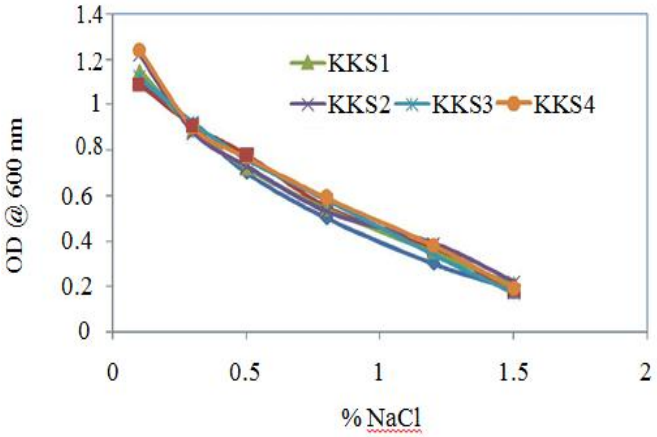

Fig.4 Effect of Salinity tolerance

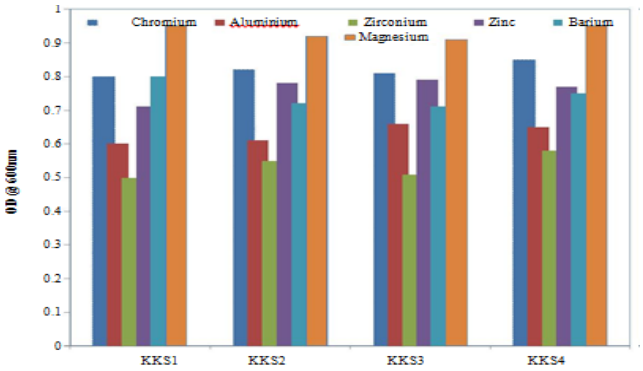

Fig.5 Effect of Heavy metal resistance

E. Sensitivity to Antibiotics

KKS bacterial strains were tested against disks of commonly used antimicrobials to evaluate their sensitivity at microaerophilic conditions. Zone diameter against antimicrobial disks was significantly narrower for erythromycin, neomycin, penicillin, ampicillin and tetracycline (about 1 to 2 mm each) and for chloramphenicol with 3 mm.However, zone was comparatively larger around Streptomycin with 7.5 mm, Kanamycin with 7.5 mm and Gentamycin 8 mm. The zone diameter against polymixin-B, cephaloridine, Ciprofloxacin, Co-trimazole was between 6 to 7 mm. But with vancomycin it was observed to be extreme resistant with nil zone of inhibition. Similar to our results, (5) also reported that micro-aerobic bacteria showed more resistant when compare to aerobic bacteria.

F. Metabolic Versatility

Effect on growth of KKSs as consortium to a variety of structurally different heterocyclic aromatic compounds, used in leather processes was studied. For confirmation of degradation of the tested chemicals, the probable secondary metabolites were tested utilising them as sole carbon and energy. It was confirmed that KKSs grew very well with a wide spectrum of recalcitrant chemicals with an initial concentration of 25 mg/l (Table 2), present in leather industrial wastewater. Growth was observed within 24 h in the range of 0.8 to 1.4 as absorbance (OD 600 nm). It may due to the ortho cleavage pathway of KKSs which releases more energy than meta pathway.

To conclude, recalcitrant chemicals are a common contaminant of industrial wastewaters at present. The biological treatment of these waste streams is strongly inhibited by high salt and heavy metal concentrations (6, 7). Very few studies have been cited in relation to biodegradation of recalcitrant pollutants. It is more energy-efficient than conventional aerobic systems, requiring less energy for aeration and producing minimum sludge (11, 12). This process can be utilised for the removal of recalcitrant aromatic substances under oxygen-limited conditions, which is an important step in wastewater treatment. It is an economically viable process and can be installed easily in an existing treatment plants. The isolated KKS strains are moderately halophilic, highly motile, either capsulated or sporulated to withstand the stress conditions. The unique

feature of the isolates was their capacity to degrade a range of heterocyclic compounds that are used in the leather industry to transform the putrescible hide/skin into valuable leather, by utilising them as sole carbon and energy, even without any growth factors including vitamins, amino acids and peptides. Degradation of these compounds was confirmed by the utilization of secondary metabolites of the corresponding compounds. Resistance to heavy metals is an added feature for KKSs. In the evaluation of antibiotic activity, resistance of KKSs is more pronounced at micro-aerobic condition. Ortho cleavage ring fission was observed by all KKS strains resulting in more energy. This is the first time utilization of recalcitrant pollutants present in leather industrial wastewater as a growth substrate by a pure bacterial culture at micro-aerophilic condition is reported.

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