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Isolation and characterization of phthalate degrading bacteria

Pradeep S[1]., Anupama Jayaprakash[1], Sarath Josh M.K[1]., Sudhadevi R[2]., Balachandran S[3]. and Sailas Benjamin[1]
[1]Enzyme Technology Laboratory, Biotechnology Division, Department of Botany, University of Calicut, Kerala - 673635.
[2]Department of Chemistry, Mannam Memorial NSS College, Kottiyam, Kollam, Kerala - 691571.
[3]Department of Chemistry, NSS College Cherthala, Alappuzha, Kerala - 688556.

Abstract: *Phthalates are esters of phthalic acid (PAE) and are mainly used as plasticizers, i.e., substances added to plastics to increase their flexibility, transparency, durability and longevity. There exists many phthalates of varying chain lengths. PAE with longer alkyl chains i.e., di-(2-ethyl hexyl) phthalate (DEHP) and di-octyl phthalate (DOP) are poorly degraded under aerobic and anaerobic conditions. PAE are synthesized in massive amounts to produce various plastics and became a menace to the environment, following their release as a result of extensive usage and production. PAE are known to cause hepatotoxic, teratogenic and carcinogenic effects in humans, apart from inducing sterility and disrupting endocrine system. These environmental and health concerns demand intervention of biotechnology to develop a suitable strategy for reduction and remediation of these toxic chemicals. With this purpose, employing biochemical techniques we have isolated 9 bacteria in pure form from the sludge and soil samples, which utilize DEHP or DOP as sole carbon source. Of them, six isolates were capable of degrading DOP and three degrading DEHP.*

INTRODUCTION

Phthalates (phthalic acid esters- PAEs) are used in a large variety of products, from enteric coatings of pharmaceutical pills and nutritional supplements to viscosity control agents, gelling agents, stabilizers, dispersants, lubricants, emulsifying agents and suspending agents. End applications include: adhesives and glues, building materials, personal care products, medical devices, detergents and surfactants, packagings, children's toys, modeling clay, waxes, paints, printing inks and coatings, pharmaceuticals, food products and textiles. The most widely-used phthalates are the di-2-ethyl hexyl phthalate (DEHP), the diisodecyl phthalate (DIDP) and the diisononphthalate (DINP). DEHP is the dominant plasticizer used in polyvinyl chloride (PVC), due to its low cost (Ling *et al*., 2008).

When used as plasticizers, phthalates are not chemically bonded to the plastic polymer and can therefore eventually migrate from the plastics into the environment. When the environment (air, oil water, blood, *etc*.) has a very high affinity for the plasticizer, the migration rate is dependent upon the ability of the plasticizer to diffuse through the resin to the attracting media (Graham, 1973).

Release of phthalates into the ecosystem or wastewater effluent occurs during the production phase and *via* leaching and volatilization from plastic products during their usage and after disposal (Chen *et al*., 2007). Potential adverse health effects of diisononyl phthalate (DINP) are due its migration from children's toys during mouthing activities, and concern has focused on their potential chronic effects on the kidney and liver (Matsumoto *et al*., 2008**).** Phthalate exposure can be through direct use or indirectly through leaching and general environmental contamination. Diet is believed to be the main source of DEHP and other phthalates in the general population. Fatty foods such as milk, butter, and meats are a major source. Low molecular weight phthalates such as di-2-ethyl phthalate (DEP), dibutyl phthalate (DBP), *etc*., may dermally be absorbed.

A number of studies on biodegradation of phthalates in natural water, wastewater and soil have been known to date. Many bacteria have been isolated from rivers, soil, and even marine regions for their ability to degrade phthalate both aerobically and anaerobically. To date it is well known that PAE with shorter alkyl chains (*i.e*., DEP, DBP) are very easily degraded, while PAE with longer alkyl chains (*i.e*., di-octyl phthalate (DOP), DEHP) are poorly degraded under aerobic and anaerobic conditions; and it is also confirmed that a correlation exists between increasing length of the ester side chain and decreasing biodegradability (Chen *et al*., 2007). Phthalates and their metabolites have been found to be potentially harmful for human and environment due to their hepatotoxic, teratogenic and carcinogenic characteristics (Liang *et al*., 2008).

Numerous studies have demonstrated that microorganisms play major roles in phthalate degradation in the environment. It was reported that complete degradation of phthalates can be achieved at a concentration as high as 2.6 g/l in 48h using *Comamonas acidovorans* strain FY-1 isolated from activated sludge .Two consortia using DEP as a substrate showed capability of degrading 500 mg/l dimethyl phthalate (DMP) to monomethyl phthalate (MMP) and phthalate as the intermediate before cleavage of the aromatic structure (Wang *et al*., 2003). The degradation pathways of phthalates will help to understand their materialization process and the toxicological behaviour of their metabolites. Generally, the phthalate biodegradation pathways consist of two processes:

primary biodegradation from phthalate diesters to phthalate monoesters and then to phthalic acid and ultimate biodegradation from phthalic acid to CO_2 or CH_4 (Staples *et al*., 1997). Until the problem of endocrine disruption was proposed, the studies of phthalate esters degrading microorganisms were mainly focused on wastewater treatment and some microorganisms have been isolated for use in cleansing the phthalate ester contaminated wastewater. In the light of the new status of phthalate ester as endocrine disruptors, there is a current need for a technology for the reduction and remediation of these chemicals. For this purpose, isolation and characterization of microorganisms that utilize DEHP and DOP as their sole carbon source have been focused in this study (Nakemiya *et al*., 2005).

MATERIALS AND METHOD

Sample collection

Soil samples were collected from 10cm depth in $1m^2$ chosen areas. All the areas from where the samples collected were heavily contaminated with plastics. Samples were collected aseptically with the aid of sterile spatula, scalpels, gloves and plastic bottles and were fully labeled with description and date. Samples are representatives of a site. A total of five samples were collected during the months of March and April, 2009 which included samples from the following areas, near Canoly Canal (AKP), Calicut; Terumopenpol Limited, Puliyarakonam (ATP, BTP), Trivandrum (manufacturer of blood bank equipment and blood bags); and Njelianparambu (ANP,BNP) Calicut. Collected samples were used for the isolation and characterization of phthalate-degrading bacteria.

Screening for phthalate-degrading bacteria

Samples were examined immediately after they were brought to the laboratory. The samples were plated after serial dilution (up to 10^{-6} times). Pure cultures were obtained after repeated streaking and subculturing. By using the pure cultures, various staining and biochemical tests were conducted.

Serial dilution

Approximately 1g soil sample was suspended in 9 ml of sterile water with shaking. Serial dilution was made using standard protocol.

Spread plate technique

After the serial dilution of the soil samples collected from Canoly Canal and Trivandrum, 0.1ml of each sample was added to petri plates containing autoclaved solidified basal medium (1.0 g K_2HPO_4, 1.0 g NaCl, 0.5 g NH_4Cl, 0.4 g $MgSO_4$/l distilled water). DEHP and DOP were used as the carbon source. The concentration of DEHP and DOP added to the plates were 50µl. The pH was adjusted to 7. 2 (Chen *et al*., 2007). The plates were incubated at 37 ^oC in incubator.

Streak plate technique

Standard procedure was employed for the streak plate technique. Each Petri plate was observed for the growth of bacteria into colonies. Discrete colonies farthest away from the streak were selected for further studies.

Table 1. Descriptions of the isolates obtained by the culturing of the five samples collected.

Sl. No.	Isolates	Colony characters observed	Days taken for the appearance of colonies
1	AKP	Pinpoint, white, spherical colonies	3
2	ATP	Small cream coloured, slightly raised colonies	3
3	ANP	Cream coloured colonies	2
4	BTP1	Pinpoint, white colonies	3
5	BTP2	Spherical cream-white coloured colonies	4
6	BTP3	white coloured, spherical colonies	4
7	BTP4	White, spherical, raised colonies	4
8	BNP1	Cream coloured, spherical colonies.	2
9	BNP2	Cream coloured spherical colonies	2

Sub culturing

Pure colonies obtained were preserved for further studies by periodic sub culturing (bi-weekly) and maintained on agar slants. Specimens from these samples were taken for further studies.

Study of biochemical activities of microorganisms

The various biochemical tests performed for the characterisation of selected pure cultures were Gram-staining, spore-staining, starch hydrolysis, nitrate reduction test, urease test, carbohydrate fermentation, indole production test, methyl red test, Voges-Proskauer test, citrate utilisation test, hydrogen sulphide production test.

RESULTS AND DISCUSSION

The prime objective of this study was to isolate phthalate degrading bacteria. PAE with longer alkyl chains (*i.e*., DOP and DEHP) are poorly degraded under aerobic and anaerobic conditions (Chen *et al*., 2007). In this study, DEHP and DOP were used as the sole source of carbon and successfully isolated nine bacteria. Microorganisms are the only known organisms capable of completely degrading phthalate compounds. Most often, these microorganisms belong to the group of *Pseudomonas, Aspergillus, Micrococcus*, and *Bacillus* sp., and are found to be associated with the degradation of these products (Karegoudar *et al*., 1984). Six isolates capable of degrading DOP and three isolates capable of degrading DEHP were isolated from the soil. Biochemical tests were conducted with the nine isolates. Phthalate biodegradation in soil has been widely studied in the last decade (Liang, 2008). Previous studies suggest that the soil or the sludge samples used for the isolation were collected from regions heavily contaminated with plastic pollutants, *i.e*., mostly rivers and near factories (Feng *et al*., 2002, Ahn *et al.*, 2004, Chen *et al.*, 2007). Soil samples collected for the present study were also from highly plastic contaminated regions.

All the five samples collected were subjected to serial dilution, repeated spread and streak plate (Fig. 1) culture techniques. Pure cultures obtained by these techniques were subjected to Gram-staining (Fig. 2), spore staining and biochemical tests.

Table 2. The results obtained with Gram staining of isolates from 5 samples collected from different places summarized.

Sl. No.	Sample	Gram (+ ve / -ve)	Bacterial shape	Length (µm)	Width (µm)	Endo-spore (+ / -)	Spore shape	Spore position	Motility
1	AKP	- ve	Rod	0.1±1.5	0.1±0.5	-	-	-	motile
2	ATP	-ve	Rod	0.1±0.5	0.1±0.5	-	-	-	motile
3	ANP	+ve	Rod	0.2±0.5	0.2±0.5	+	E	C	non-motile
4	BTP1	+ve	Rod	1.5±1.5	1±0.5	+	E	C	motile
5	BTP2	-ve	Rod	0.5±1	1±1	+	E	C	motile
6	BTP3	-ve	Rod	0.1±2	0.1±1.5	-	-	-	motile
7	BTP4	-ve	Rod	1±0.5	1±0.5	-	-	-	motile
8	BNP1	-ve	Rod	1±1	0.5±0.5	+	E	C	non-motile
9	BNP2	+ve	Rod	0.1±0.5	0.1±0.2	+	E	C	motile

+ present, - absent, E- ellipsoidal, C-central.

All the isolates were subjected to spore staining (Sherman and Cuppuccino, 1996). Three isolates were Gram positive (Table 2). Isolates showed the presence of endospores, which were ellipsoidal and centrally positioned (Table 2, and Figure 3).

All the isolates were subjected to 8 biochemical tests such as starch hydrolysis, carbohydrate fermentation test (glucose, lactose and sucrose), nitrate reduction test, urease test, indole production test, Voges-Proskauer test and citrate utilization test (Table 3) (Aneja, 1993).Test for citrate utilization was conducted to determine the ability of the bacteria to utilize citrate. The isolates that utilize citrate changed the indicator from green to blue. Five isolates showed positive results and the other 4 isolates showed negative result (Table 3, Fig. 4). Nitrate reduction test showed clear purple colour of the starch iodine paper, when immersed in culture broth (Table 3). Six isolates showed positive results and the other 3 isolates showed negative results. Test for starch hydrolysis was performed to determine the production of the exoenzyme amylase, by observing the clear zone around and beneath the bacterial growth on the agar plate flooded with Gram's iodine (Table 3; Fig. 5). Four isolates showed positive results and all the others showed negative results. Carbohydrate fermentation test was conducted and all the isolates showed negative results for lactose and sucrose fermentation. Five isolates showed positive results for glucose fermentation and the other 4 showed negative results (Table 3; Fig. 6). Urease test was conducted to determine the ability of the bacteria to produce the enzyme urease. Four isolates showed

positive results and the other five isolates showed negative results (Table 3). All the isolates showed negative result for indole production, Voges-Proskauer and methyl red tests (Table 3).

Table 3: Summary of the results of eight biochemical tests performed with 9 isolates; citrate utilization test (CUT), nitrate reduction test (NRT), glucose fermentation (GF), lactose fermentation (LF), sucrose fermentation (SF), carbohydrate fermentation test (CFT), starch hydrolysis test (SHT), urease test (UT), indole production test (IPT), Voges-Proskauer test (V-PT), methyl red test (MRT).

S.No	Isolates	CUT	NRT	GF	LF	SF	SHT	UT	IPT	V-PT	MRT
1	AKP	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	ATP	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
3	ANP	-ve.	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
4	BTP1	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
5	BTP2	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
6	BTP3	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7	BTP4	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	BNP1	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
9	BNP2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve

A gram positive *Microbacterium* sp. strain CQ0110Y with round morphology, smooth and glossy surface and yellowish colouring was isolated by Chen *et al.* (2007). Similar results were shown by the isolates ANP, BNP1 and BNP2 (Table 2). The morphological tests conducted in the nine isolates revealed that four isolates (AKP, ATP, BTP2, BNP2) are gram positive rods, and all of them produced endospore that are ellipsoidal in shape and centrally positioned.

Another gram positive, motile and rod *Bacillus* strain T-36 was isolated from sea mud in the Tagonoura harbor, Japan (Sakagami, 1982). The spores were ellipsoidal and central or paracentral. The growth on agar slants was abundant, rough, opaque, dull, waxy, spreading and off white. The growth in broth produced uniform turbidity. It produced acid but no gas from glucose, the pH in cultured glucose broth was 7.6 after 7 days incubation. Citrate was utilised and nitrite was produced from nitrate, but gas was not produced from nitrate broth under anaerobic conditions (Sakagami, 1982). Similarly various biochemical tests conducted in the nine samples revealed that, seven isolates (AKP, ATP, ANP, BTP1, BTP2, BTP4 and BNP1) reduced nitrates to nitrites (Fig. 7). Four isolates (AKP, BTP1, BTP2 and BTP3) utilized citrate (Fig. 6). Starch was hydrolyzed by four isolates (ATP, ANP, BTP1 and BTP2) (Fig. 5). Five isolates (BTP1, BTP3, BTP4, BNP1 and ANP) produced acid from glucose but no gas (Fig. 6). Five isolates (ATP, ANP, AKP, BTP1 and BTP2) are endospore forming. Endospores produced are ellipsoidal, centrally positioned (Fig. 3). Seven isolates (AKP, ATP, BTP1, BTP2, BTP3, BTP4 and BNP2) are motile. In summary, out of nine isolates, three were DEHP degrading and the remaining six were DOP degrading bacteria. This preliminary information need to be confirmed by rDNA PCR.

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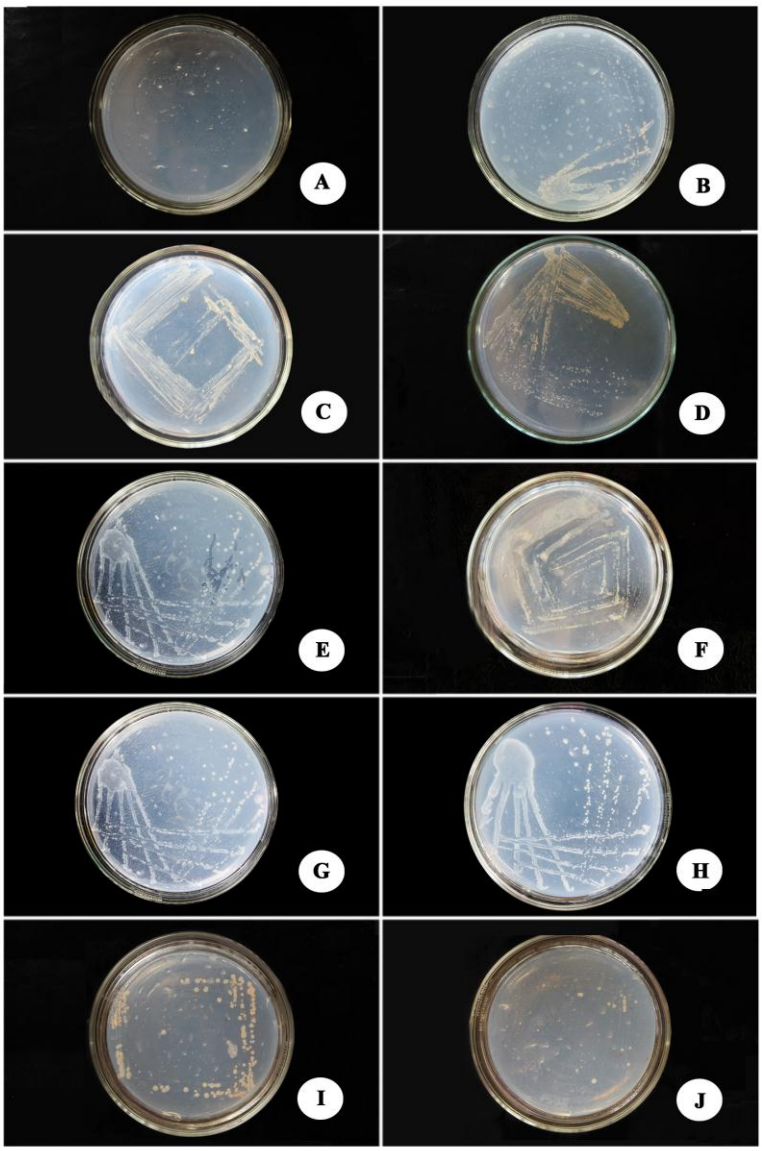

Fig. 1: Streak plate method of samples collected from different places, viz.; A.Control;B.Kanoli soil(AKP);C.TVM(ATP);D.Njeliyanparambu soil(ANP);E. TVM (BTP 1); F. TVM (BTP 2); G. TVM (BTP 3); F. TVM (BTP 4); I. Njeliyan-parambu (BNP 1); J. Njeliyanparambu (BNP 2).

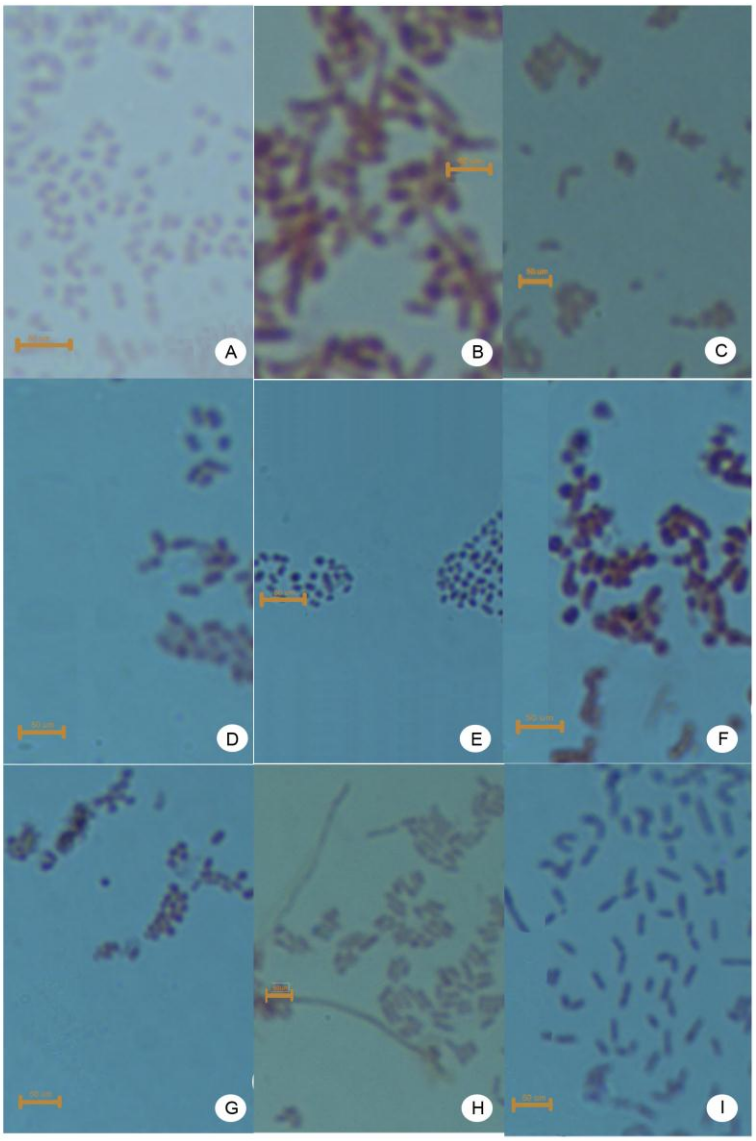

Fig. 2: Gram staining of sample collected from different places. A. Kanoli (AKP); B. TVM (ATP); C. Njeliyanparambu (ANP); D. TVM (BTP1); E. TVM (BTP2); F. TVM (BTP3): G. TVM (BTP4); H. Njeliyanparambu (BNP1); I. Njeliyanparambu (BNP2).

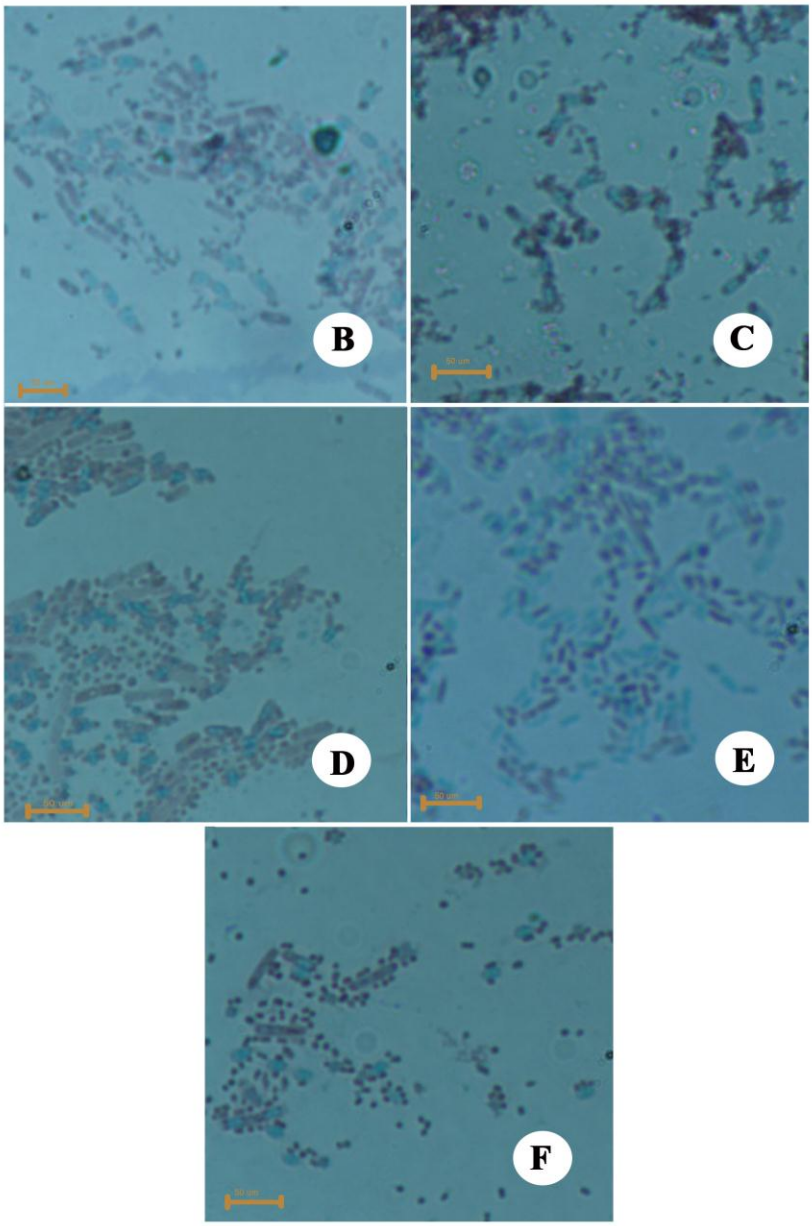

Fig. 3: Spore staining of samples collected from different places, viz.; A.Control;B. Kanoli soil(AKP);C.TVM soil(ATP);D.Njeliyanparambu soil(ANP);E.TVM soil(BTP1);F.TVM soil(BTP2).

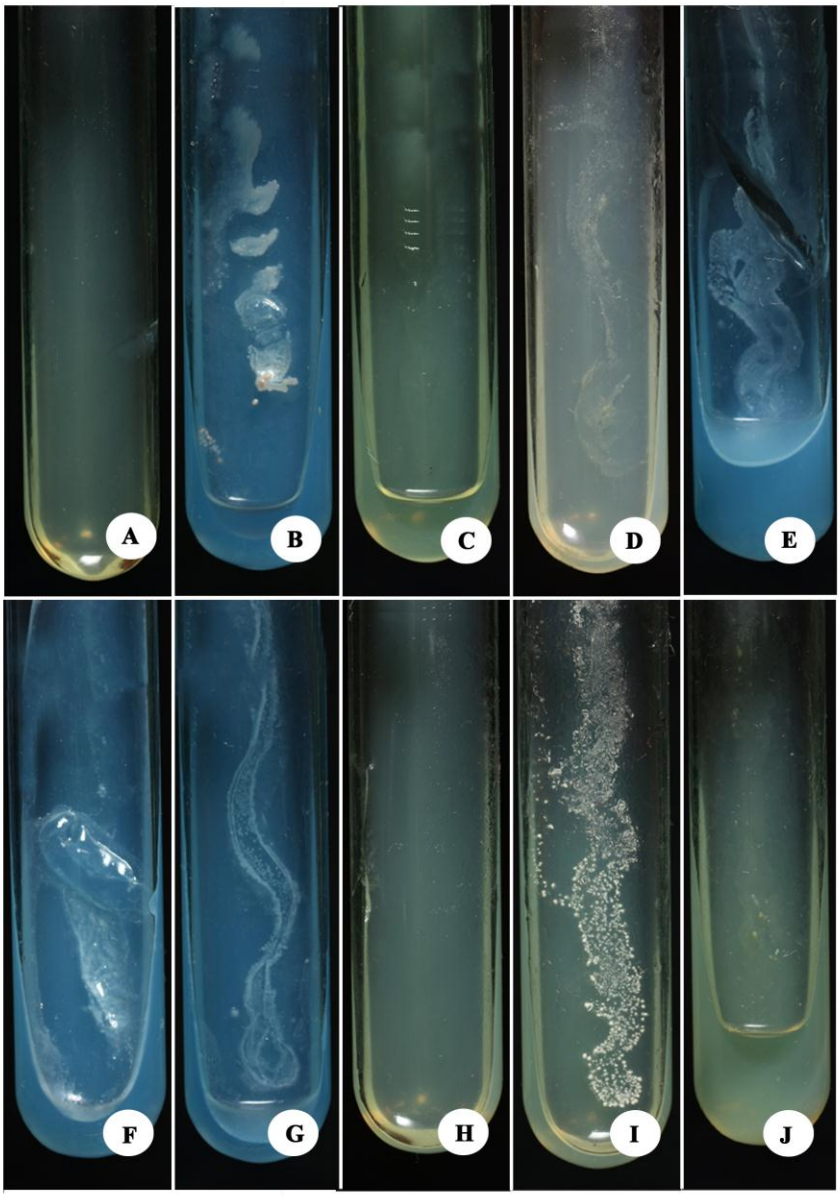

Fig. 4: Citrate utilization test of samples collected from different places viz., **A.** Control; **B.** Kanolisoil(AKP); C. TVM(ATP); D. Njeliyanparambu soil(ANP);E.TVM(BTP1);F.TVM(BTP2);G.TVM(BTP3);H.(BTP4);I.Njeliyanparambu soil(BNP1);J.Njeliyanparambu soil(BNP2).

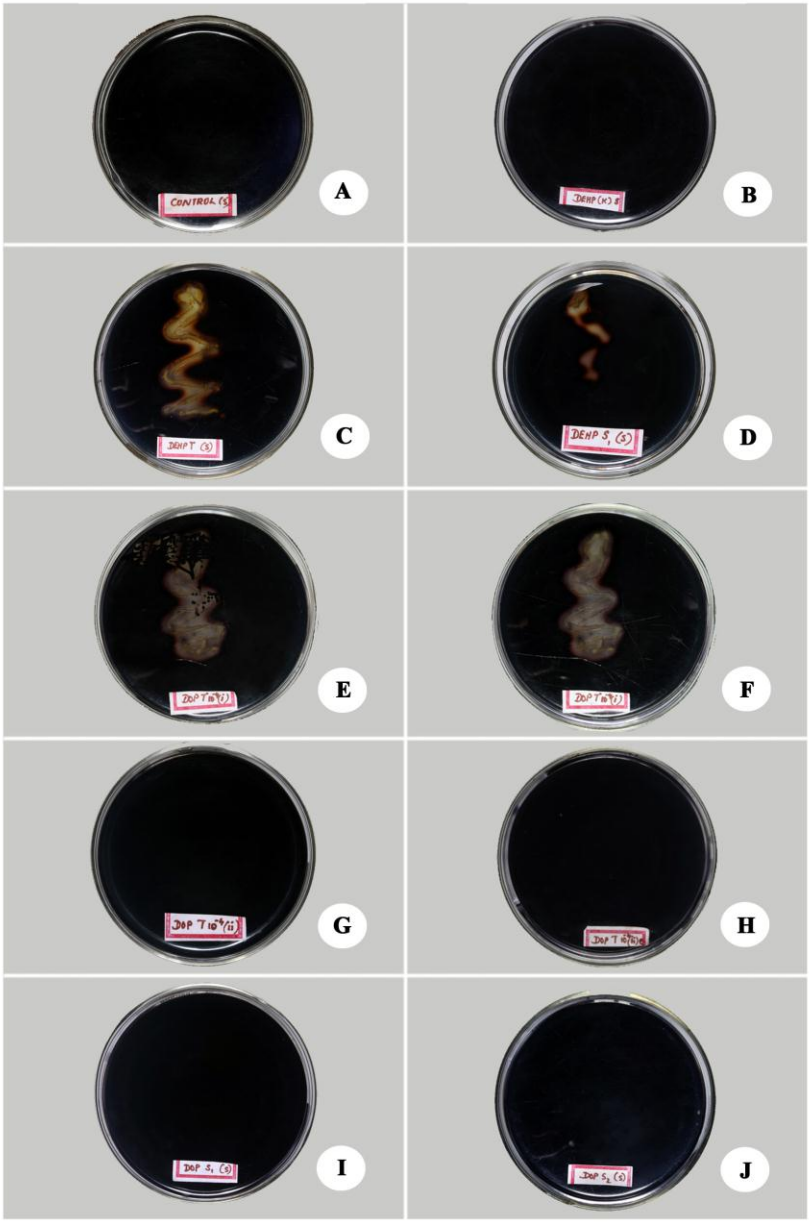

Fig. 5: Starch hydrolysis test of samples collected from different places, viz.; A.Control;B.Kanoil soil(AKP);C.TVM(ATP);D.Njeliyanparambu soil(ANP);E. TVM (BTP 1); F. TVM (BTP 2); G. TVM (BTP 3); F. TVM (BTP 4); I. Njeliyan-parambu (BNP 1); J. Njeliyanparambu (BNP 2).

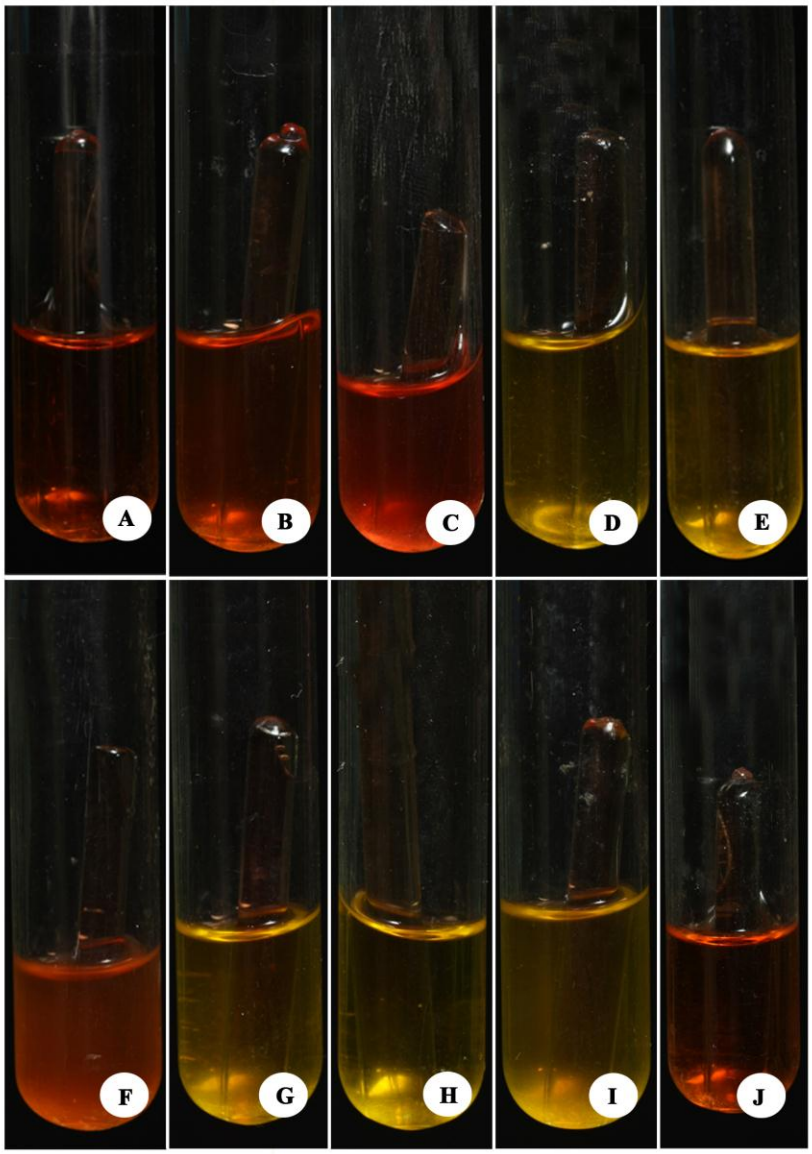

Fig. 6: Glucose fermentation of samples collected from different places viz., A. Control; **B.Kanoli soil(AKP);C.TVM soil(ATP);D.Njeliyanparambu soil(ANP);E.TVM soil(BTP1);F.TVM soil(BTP2);G.TVM soil(BTP3);H.TVM soil(BTP4);I.Njeliyanparambu soil (BNP1);F.Njeliyanparambu soil(BNP2).**

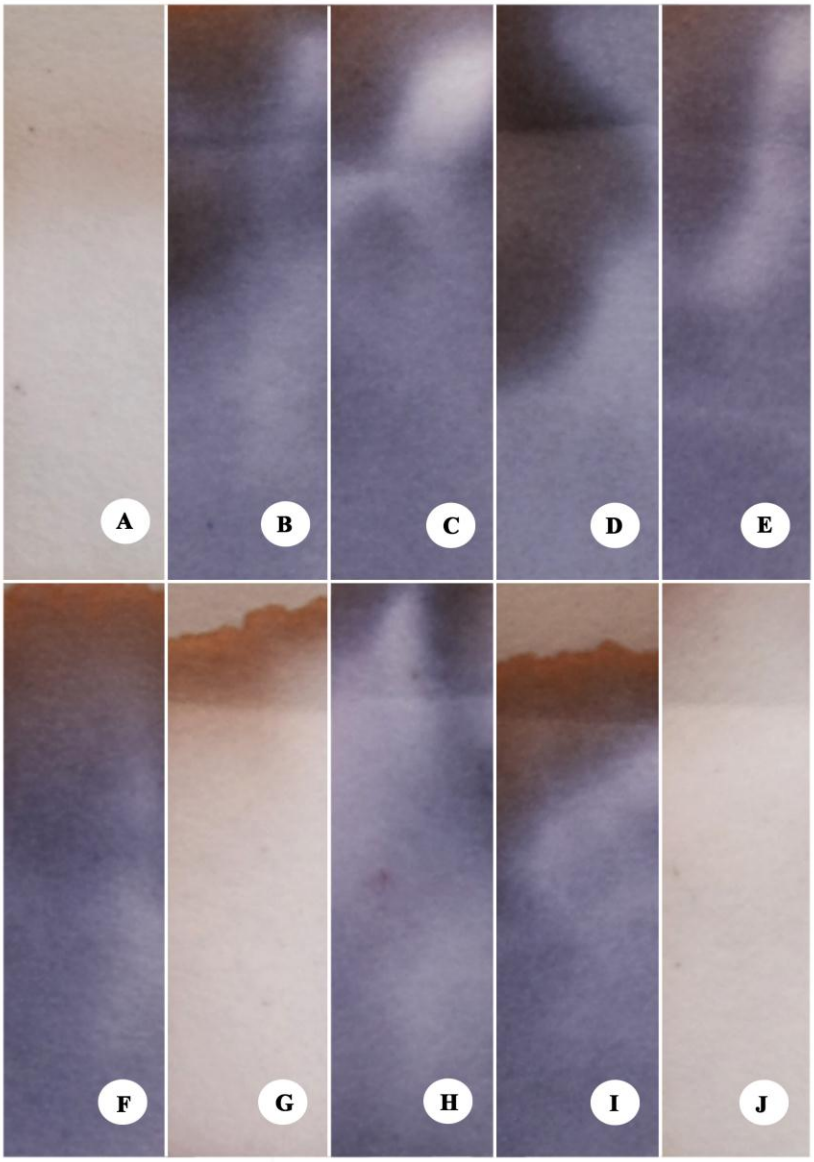

Fig. 7: Nitrate reduction test of samples collected from different places viz., **A**.Control;**B**.Kanolisoil(AKP);C.TVMsoil(ATP);D.Njeliyanparambu soil(ANP);E.TVM(BTP1);F.TVM(BTP2);G.TVM(BTP3);H.(BTP4);I.Njeliyanparambu soil(BNP1);J.Njeliyanparambu soil(BNP2).