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MOLECULAR AND CULTURE ANALYSIS OF BACTERIA FROM EARTHWORM MIDDEN OF DIFFERENT TROPICAL FOREST SOILS OF RANCHI

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KEYWORDS

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Midden

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ABSTRACT

Important physicochemical characters and bacterial biomass in earthworm midden from three different forest (Eucalyptus, Acacia and mixed) soil sample have been studied over on incubation period of 42nd day. Earthworm midden from acacia forest indicated enhanced pH, organic C, N, P and K with respect to eucalyptus and mixed forest midden. In acacia, bacterial population (number/g midden) was gradually decreased from $55.5 \pm 0.88X10^9$ to $19.60 \pm 0.818X10^9$ on 0 to 42nd day. Initial bacterial population was $19.4 \pm 0.503X10^9$ and $39.5 \pm 0.850X10^9$ in eucalyptus and mixed forest respectively, which was gradually increased up to 28th and 21st day ($38.7 \pm 0.873X10^9$ and $55.2 \pm 1.123X10^9$) and thereafter that population were declined $21.1 \pm 0.65X10^9$ and $22.8 \pm 0.702X10^9$ on 42nd of observation in midden of eucalyptus and mixed forest respectively. Punctiform and irregular shape colonies were dominant colonies found in the earthworm midden. One more colony *i.e.* circular in shape and pink in color which was found only in the midden of eucalyptus forest. Molecular based analysis identified the bacteria *Aeromonas punctata* strain JM10, *Bacillus cerus* probio-32 and *Kocuria* sp. HO-9042, based on 16SrDNA probe. The paper deals in detail molecular analysis of bacteria found in midden of different forest soils.

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INTRODUCTION

Soil microbial component is considered to be one of the major determinants for nutrient cycling and organic matter stabilization in soil (Ladd *et al.,* 1993). Microbial biomass, a labileGe fraction of soil organic matter (Jenkinson and Ladd, 1981) acts as both source and sink of plant available nutrients (Singh *et al.,* 1989).

Earthworms have been described as being one of the main groups of soil engineers in tropical and temperate ecosystems because they change the structural properties of soil and thus influence soil microorganisms and plant growth (Kimmins, 1987; Jongman *et al.,* 1995; Judas, 2002; Muratake, 2005 and Sautter *et al.,* 2006). Earthworms midden are hot spots of microbial activity and nutrient dynamics and represent a suitable model for studying earthworm mediated influence on soil microbial communities by alteration of the patch structure of the microbial environment (Aira *et al.,* 2009; Kumari *et al.,* 2009). They affect nutrient cycling by modifying soil porosity (Ammer *et al.,* 2005) and aggregates structure (Sheehan *et al.,* 2006).

No two soils are same. The physical properties allow a unique microbial community to develop in each soil environment. Molecular techniques including DNA sequencing have been vital in determining the composition of the vast diversity of soil and in understanding the interactions between organisms and their environment.

The objective of this study was to analyze the bacterial population and bacteria from earthworm midden by 16S rDNA techniques, a basis on which the phylogeny of the species has been traced.

MATERIALS AND METHODS

Soil sample collection

The soil samples from the three different tropical forests of Ranchi, Jharkhand were collected and study was carried out in laboratory by culturing the earthworms in plastic container under oxygenated and moist condition. The middens were collected from the plastic container and used for microbial study.

Bacterial culture and isolation

Dilution plate method (Parkinson *et al.,* 1971) was used for estimating the bacterial population in middens, 1 mL inoculums of the primary suspension were taken and Cazapek dox agar media was used for culture. After plating petridishes were kept inverted at 37°C for 48h in incubator. After that colony count and identification were continued at every interval of 7 days till 42nd day.

Physico chemical estimation of soil

Organic matter (Walkley and Black, 1934) and total N content of soil was estimated according to Kjeldahl and Jackson method (1973). Potassium and phosphorus content of soil was measured according to method described by Misra (1973)

and pH was measured by BDH pH paper.

Isolation of DNA and genomic analysis

DNA was isolated from the pure culture of bacterial colony. Its quality was evaluated an 1.2% Agarose gel, a single band of high molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing was carried out by using BDT v 3.1 cycle sequencing kit on ABI 3730 X 1 genetic analyzer and consensus sequence was generated by Aligner software. The 16S rDNA gene sequence was used to carry out BLAST with nr database of NCBI gene bank database (Marchler Bauer *et al.,* 2000; Pruitt *et al.,* 2005) Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program clustal W Distance matrix and the phylogenetic tree was constructed by Neighbor joining method (Saitou and Nei, 1987) with MEGA 4 program.

RESULTS AND DISCUSSION

Soil physicochemical properties of three different forest sites are presented in Table 1. As evident from the table, pH of the midden of Eucalyptus forest was higher than other. The levels of soil organic C showed the maximum value in Acacia forest on 7th day on incubation and minimum was observed in mixed forest on 42nd day of observation. The level of N was consistently higher in Acacia forest with respect to other. The levels of phosphorus and K were at maximum in Acacia forest and

Table 1: Physico-chemical properties of experimental soil

Days → Parameters↓	0	7	14	21	28	35	42
Eucalyptus forest							
pH	5.96±0.32	6.02±0.34	6.11±0.22	6.92±0.29	7.02±0.12	6.15±0.29	5.82±0.01
Org. C(mg C/g)	6.82±0.13	6.96±0.21	7.01±0.28	7.2±0.17	7.07±1.32	6.86±1.12	6.57±0.32
Nitrogen (mg N/g)	0.56±0.08	0.59±0.12	0.58±0.13	0.62±0.05	0.61±0.15	0.57±0.18	0.52±0.11
Phosphorus (kg P/hec.)	28.370±1.32	28.68±1.2	29.71±2.01	30.57±3.04	28.07±2.03	27.53±1.38	27.42±2.31
Potassium (kg K/hec.)	136.21±5.02	138.31±4.18	139.02±6.08	142.04±5.32	137.32±4.06	135.12±3.2	134.02±4.52
Mixed forest							
pH	5.78±0.29	5.81±0.27	6.42±0.24	6.33±0.33	6.17±0.31	5.96±0.30	5.92±0.28
Org. C(mg C/g)	6.96±0.96	7.18±1.2	7.32±0.89	7.45±1.45	7.28±1.2	7.02±0.92	6.72±1.31
Nitrogen (mg N/g)	0.57±0.23	0.58±0.32	0.62±0.21	0.59±0.10	0.58±0.19	0.61±0.16	0.53±0.24
Phosphorus (kg P/hec.)	21.82±1.65	21.96±2.01	22.04±1.89	22.67±2.18	22.51±1.95	21.72±2.21	21.68±2.32
Potassium (kg K/hec.)	143.08±5.02	146.72±4.56	150.21±6.5	149.1±7.01	147.20±5.62	137.04±6.32	138.32±5.95
Acacia forest							
pH	6.53±0.25	6.51±0.32	6.42±0.27	6.21±0.19	6.08±0.20	5.82±0.18	5.71±0.22
Org. C(mg C/g)	8.12±1.89	8.21±1.56	7.96±0.95	7.87±1.65	7.82±1.74	7.76±1.21	7.80±1.32
Nitrogen (mg N/g)	0.62±0.11	0.64±0.21	0.59±0.15	0.61±0.12	0.57±0.11	0.54±0.13	0.51±0.12
Phosphorus (kg P/hec.)	33.02±1.45	33.01±2.11	32.02±1.56	31.12±2.31	32.18±1.75	30.16±1.81	28.10±2.12
Potassium (kg K/hec.)	159.3±7.32	160.2±6.52	163.4±5.23	157.6±6.52	155.1±6.48	152.5±7.32	151.2±5.96

Table 2: Morphological details of bacterial colonies in midden of different forest

S.No.	Shape	Margin	Elevation	Color	Gram stain
Eucalyptus forest	60%Punctiform	Entire	75%Flat 25%Raised	75%White 25% Yellow	-ve cocci
	30% Irregular	Undulate	80%Convex 20%Raised	80% Cream 20% White	+ve bacilli
	8% Circular	Erose	100% Convex	100% Pink	+ve cocci
	2% Circular	Entire	70% Convex 30%Umbonate	70% Yellow 30% White	+ve cocci
Mixed forest	70%Punctiform	Entire	60%Flat 40%Raised	60%White 40% Yellow	-ve cocci
	22% Irregular	Undulate	70%Convex 30%Umbonate	70% White 30% Yellow	+ve bacilli
	5% Circular	Entire	80% Raised 20% Convex	80% Cream 20% White	+ve cocci
	3% Filamentous	Filamentous	100% Raised	50% Green 50% White	+ve bacilli
Acacia forest	78%Punctiform	Entire	80%Flat 20%Raised	80%White 20% Yellow	-ve cocci
	15% Irregular	Undulate	75%Convex 25%Umbonate	75% White 25% Yellow	+ve bacilli
	5% Circular	Entire	70% Raised 30% Pulvinate	70% Cream 30% White	+ve cocci
	2% Filamentous	Filamentous	100% Raised	50% Green 50% White	+ve bacilli

Table 3: Sequence producing significant alignments of the *Aeromonas punctata* strain JM10

Accession	Description	Max. score	Total score	Query coverage	E value	Max ident
EU862311.1	Uncultured bacterium clone Niu10	2617	2617	99%	0.0	100%
GQ259885.2	*Aeromonas punctata* strain 159	2614	2614	100%	0.0	99%
GU205197.1	*Aeromonas punctata* strain JM10	2614	2614	100%	0.0	99%
GU205195.1	*Aeromonas punctata* strain JW04	2614	2614	100%	0.0	99%
FJ494901.1	*Aeromonas* sp. B27	2614	2614	100%	0.0	99%
FJ168776.1	*Aeromonas punctata* strain 219c	2614	2614	100%	0.0	99%
FJ168775.1	*Aeromonas punctata* strain 176c	2614	2614	100%	0.0	99%
FJ168774.1	*Aeromonas punctata* strain 360c	2614	2614	100%	0.0	99%
DQ979324.1	*Aeromonas punctata* strain MPT4	2614	2614	100%	0.0	99%
AY987761.1	*Aeromonas punctata* strain RK 65541	2614	2614	100%	0.0	99%

Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

Table 4: Sequence producing significant alignments of the *Bacillus cereus* strain Probio-*32*

Accession	Description	Max. score	Total score	Query coverage	E value	Max ident
GU812900.1	*Bacillus cereus* strain JBS10	2636	2636	100%	0.0	100%
GU826154.1	*Bacillus cereus* strain Q34	2636	2636	100%	0.0	100%
GU566345.1	*Bacillus* sp R5(2010)	2636	2636	100%	0.0	100%
GU471752.1	*Bacillus cereus* strain Probio-32	2636	2636	100%	0.0	100%
AB542372.1	*Bacillus* sp. TSA4w	2636	2636	100%	0.0	100%
GU125426.1	*Bacillus cereus* strain IMAU80004	2636	2636	100%	0.0	100%
GU125425.1	*Bacillus cereus* strain IMAU80003	2636	2636	100%	0.0	100%
GQ383905.1	*Bacillus* sp. 4CCS8	2636	2636	100%	0.0	100%
FJ188297.1	*Bacillus cereus* strain BU040901-022	2636	2636	100%	0.0	100%
FJ803926.1	*Bacillus cereus* strain 0-9	2636	2636	100%	0.0	100%

Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

Table 5: Sequence producing significant alignments of the *Kocuria* sp. HO 9042

Accession	Description	Max. score	Total score	Query coverage	E value	Max ident
DQ531634.2	*Kocuria* sp. HO-9042	2588	2588	100%	0.0	100%
EU660350.1	*Kocuria rosea* strain CT22	2555	2555	100%	0.0	99%
AY345428.1	*Bacterium* K225	2553	2553	100%	0.0	99%
DQ448711.1	*Kocuria* sp. CNJ770PL04	2510	2510	100%	0.0	99%
DF675625.1	*Kocuria* sp. RM1	2497	2497	100%	0.0	98%
AB302331.1	*Actinobacterium* C18 gene	2481	2481	99%	0.0	98%
GU217694.1	*Kocuria* sp. ljh-7	2475	2475	100%	0.0	98%
AB330815.1	*Actinobacterium* C20	2471	2471	99%	0.0	98%
DQ059617.1	*Kocuria aegyptia* strain YIM7003	2459	2459	99%	0.0	98%
EU372971.1	*Kocuria* sp. E7	2453	2453	100%	0.0	98%

Max. score = Maximum score; E value = Expected value; Max. ident. = Maximum identification

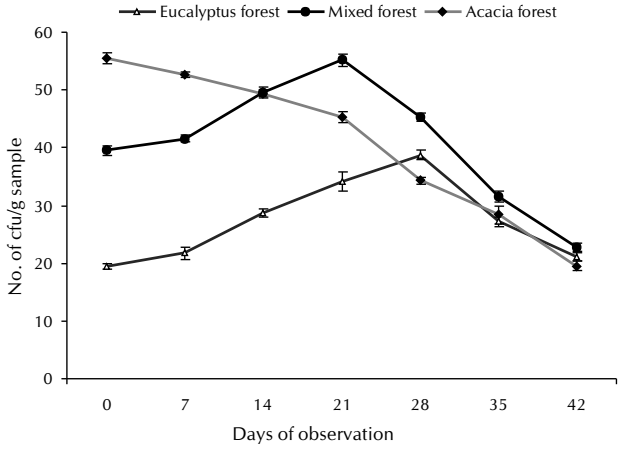

Figure 1: Bacterial population in midden of different forest in time interval of 7 days

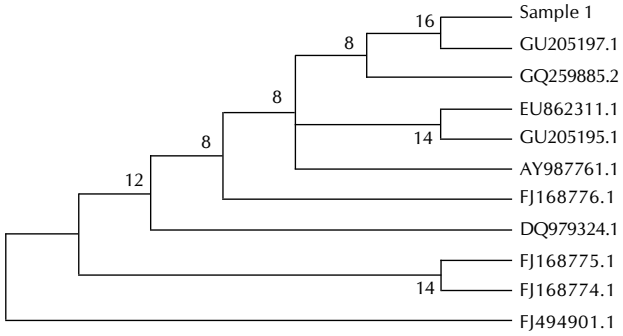

Figure 2: Phylogenetic tree showing position of sample 1 (*Aeromonas punctata* strain JM10)

minimum in Mixed forest. Earthworm midden contains elevated amount of inorganic N relative to surrounding soil. As a consequence of N and can simulate other N transformation such as denitrification (Iman and Rahmani, 2005). By increasing bypass flow of infiltrating water earthworm midden can increase the amount of N and other nutrient leaching from the soil profile (Neirynk *et al.*, 2000). Alternatively earthworms can reduce the amount of nutrients lost in surface runoff by increasing rates of water infiltration into the soil

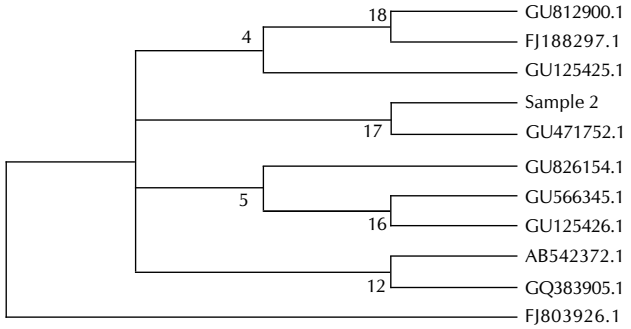

Figure 3: Phylogenetic tree showing position of sample 2 (*B. cereus strain probio- 32*)

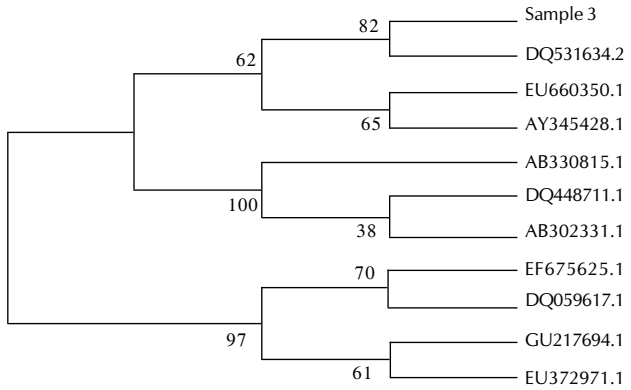

Figure 4: Phylogenetic tree showing position of sample 3 (*Kocuria* sp. HO-9042)

(Aubert *et al*., 2003). Fig. 1 depicts the bacterial population (1 X 10^9 cfu/g soil) in the midden at different time intervals. The highest bacterial count of 55.5 ± 0.88 cfu/g was recorded on the first day of incubation in Acacia forest and lowest in Eucalyptus forest. In Eucalyptus and mixed forest population has been gradually increased up to 21st day and thereafter decreased but in Acacia declining pattern of population was observed (Kumari *et al.,* 2009). The use of microbial activity estimates as ecological markers of soil perturbation or restoration has been suggested by Hart *et al*. (1989). In three forests site lowest bacterial population was observed in Eucalyptus forest. Some studies have indicated that Eucalyptus leaves contain toxic organic compounds (Rice, 1984) which may have a deleterious impact on soil micro-organisms (Dellacassa *et al.,* 1989; Animon *et al.,* 1999). Relatively slower decomposition of Eucalyptus litter due to poor association of decomposing micro flora in comparison with other endemic tree species of Indian subcontinent has also been reported (Sankaran, 1993; Chander *et al.,* 1995; Mahakur and Behera, 1999).

The developed bacterial colonies on nutrient agar plates with respect to their shape and margin were of several types *i.e.* circular entire, circular erose, irregular undulate, punctiform and filamentous has been observed (Table 2). In Eucalyptus forest, 60% of the colonies were punctiform, 30% irregular undulate and 8% of the colonies were circular entire. Filamentous colony was absent in Eucalyptus forest. The elevation of punctiform colonies were either 75% flat or 25% raised with white and yellow color. In Mixed forest, 70% colonies were punctiform, 22% irregular, 5% circular and 3% filamentous. Punctiform colonies was more dominated in Acacia forest at 78% and other colonies were irregular undulate (15%), Circular entire (5%) and filamentous (2%). The bacteria constituting punctiform, irregular undulate colonies were observed to be coccid and bacilli respectively. And one more colony circular entire margin pink in color was coccid and their response to gram's stain was positive.

Genomic analysis of dominated colony, punctiform and irregular undulate in shape and resistant colony circular entire in shape were done. The bacteria was found to be *Aeromonas punctata* strain JM10 (Gen bank accession no. GU205197.1), *Bacillus cerus* strain probio-32(Gen bank accession no. GU471752.1) and *Kocuria* sp.HO-9042 (Gen bank accession no. DQ531634.2) respectively. PCR fragment of 16S rDNA gene from the isolated DNA of bacteria showed amplicon band of 1500bp when resolved in agarose gel.

Aeromonas species are recognized as etiological agents of a wide spectrum of disease in man and animals. In developing countries potentially pathogenic *Aeromonas* sp. is very common in drinking water and in different types of foods. Significant association of *Aeromonas* sp. with diarrhoea in children has been reported from several countries (Ghenghesh *et al.,* 2008). The genus Aeromonas are water borne gram –ve bacteria that are ubiquitous in water, including ground water and chlorinated drinking water (Khun *et al*., 1997; Gavriel *et al*., 1998). *Aeromonas* was found in the isolates from the midden. It appeared that the entire genus found in the midden isolates were also found in the soil isolates except *Aeromonas* (Furlong *et al.,* 2002). *Aeromonas* spp. is typically associated with fish and aquatic habitats. Although they have never been isolated from soil, they have been previously identified as dominant isolate in the midden of the earthworm *E. foetida* (Toyota and Kimura, 2000) and have been associated with the gut of *Pheretima* sp. (Toyota and Kimura, 1994). The midden of *Dichogaster bolavi*, the species of earthworm taken for the present study was dominated by *Aeromonas punctata*. The present study is in conformity with the previous report of Toyota and Kimura (1994 and 2000) even in tropical conditions.

Blast report revealed that the query sequence of the bacterium showed that about more than 200 nucleotides sequences were similar to the 100 blast hits. *Aeromonas punctata* strain JM10 showed 100% similarity with uncultured bacterium clone Niu 10 and 99% similarity with *Aeromonas punctata* strain 159. Other close homologs of *Aeromonas punctata* strain based on blast data are represented in Table 3 and its phylogenitic tree is shown in Fig. 2. The phylogenitic tree shows that *Aeromonas punctata* strain JM10 is closely related to the GU205197.1 and distantly related to FJ494901.1.

The second dominant bacterium was identified as *Bacillus cerus* strain probio-32, which was gram +ve rod shaped bacteria. It is a portably ubiquitous soil bacterium and opportunistic pathogen causing food poisoning manifested by diarrheal or emetic syndromes (Drobniewski, 1993; Helgason *et al.,* 2000; Ivanova *et al.,* 2003). It was closely related to the animal and human pathogen *B. anthracis* and the insect pathogen *B. thuringiensis* (Ivanova *et al.,* 2003). BLAST program was carried carried out to generate significant alignment and the close matches to the query sequence of

Bacillus cereus strain Probio-32. On the query sequence of 1250bp, 161 blast hits matched the alignments scores of > = 200. The 10 different strains of *Bacillus* were also found (Table 4). The Expectation Value (E) of all these *Bacillus* strain was 0.0, which depicts that all the sequence of 10 different strains is homogenous in comparison to *Bacillus cereus* strain Probio-32. Phylogeny is shown in Fig. 3. It was observed that *B. cereus* is closely related to *B*. sp. R5 and distinctly related to *B. cereus* strain 0-9.

In the midden of Eucalyptus forest, about 8% of the colonies were circular entire in shape and pink in color, which was absent in Acacia and Mixed forest, used for genomic analysis. The bacterium was identified as *Kocuria* sp. HO-9042 based on 16S rDNA technique. It was gram +ve aerobic and coccoid cell bacterium. Sequence producing significant alignment by BLAST, the close matches the query sequence of *Kocuria* sp. HO-9042, 10 different strains of *Kocuria* were found (Table 5)**.** The phenotypic features and complete sequence of 16Sr DNA revealed that *Kocuria* sp. HO-9042 strain showed 99% sequence similarity with *Kocuria rosea* strain CT22 (Stackebrandt *et al.,* 1995) and 98% sequence similarity with *Kocuria* sp. RM1 and *Kocuria aegyptia* strain 71M 70003 (Li *et al*., 2008). The type strain *Kocuria rosea* has been reported to cause catheter related bacterium (Altuntas *et al.,* 2004) and the majority of strain are non-pathogenic. Felsenstein (1985) proposed that bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade; alternatives nodes can be rejected if they occur in less than 5% of the bootstrap estimates. Following Felsenstein (1985) description, phylogenitic tree showed that *K*. sp. HO-9042 is in close association with DQ531634.2 which has bootstrap value of 82 at node (Fig. 4). This monophyletic group of *K*. sp. HO-9042 further shows close relation with EU660350.1 (*Kocuria rosea* strain CT22) has bootstrap value 62. *Actinobacterium* C20 shows bootstrap value of 100 with clade of *K*. sp. HO-9042. Harwati *et al.* (2007), first time reported degradation of compounds of Arabian light crude oil by *Kocuria rosea* and *Kocuria aegyptia*. Tamaikina *et al.* (2008) isolated *K. rosea* from the pondweed surface that grew on agar medium with crude oil as carbon source. *K. rosea* CMG2042 grew on all three PAHs (Polycyclic aromatic hydrocarbons). *K. flavia* grow on Naphthalene, phenanthrene (Ahmed *et al.,* 2010). On plates of agar medium with or without yeast extracts colonies of both the strain had accumulated oil around them *K. rosea* had higher growth and oil accumulating in comparision to *K. flava*. It is concluded that *K. flava* and *K. rosea* was able to utilize Naphthalene as sole carbon and energy source (Ahmed *et al.,* 2010).

By the consent of nature, there are micro-organisms ubiquitously distributed in soil and aquatic environment which have hydrocarbons degrading capabilities and considerd to be the major agents for remediation of contimanated sites (Leahy and Colwell, 1990; Boonchan *et al.,* 2000; Widada *et al.,* 2002; Zhong *et al.,* 2007; Lin and Cai, 2008). *Kocuria* sp. was able to degrading hydrocarbon and only found in Eucalyptus forest. Among the three Aeromonas was found to be highly stable in comparisons to the different strains studied.

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