



Isolation of Ethidium Bromide Degrading Bacteria from Jharkhand

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Abstract

Ethidium bromide (EtBr) is a carcinogenic and mutagenic agent which is widely used in research laboratories to probe nucleic acids by gel electrophoresis. It is generally buried underground (for solid waste) or disposed of pouring it down the sink (in case of liquid waste). Soil or drain microbial community may be able to take care of such substance else it will lead to contamination of our underground resources or others through defined and undefined routes. In view of the above assumption and literature reports the present study was undertaken to isolate and evaluate bacteria for removal, by bioaccumulation and /or biotransformation, of EtBr from contaminated sources and wastes, before their disposal to the environment. Two distinct bacteria both motile BR3 and BR4 could be identified from agarose-gel-waste containing 0.5-1.0 µg/ml ethidium bromide. Both bacteria were found to grow on EtBr-NA plate (Nutrient-Agar supplemented with EtBr at a concentration of 30 µg/ml) however only BR3 isolate showed large non-fluorescent-halo zone (characteristic to degradation of EtBr) when exposed to trans-UV light. Other isolate BR4 could accumulate EtBr within the colony biomass but did not show clear (non-fluorescent) hallow zone around it. However the bacterium was not able to utilize the EtBr as a sole carbon source.

Keyword: EtBr; Bioremediation; Biodegradation; Non-fluorescent-halo.

Introduction

Ethidium bromide (EtBr) is a red color powder and water soluble fluorescent dye. It absorbs light in UV range (UV absorbance maxima at 210 and 285 nm) and gives bright orange fluorescence in visible range with wavelength 605 nm (Sabnis 2010). Earlier it was being used as trypanocide medicine to treat trypanosomiasis in cattle (Stevenson *et al.*, 1995). It is, now, being widely used in research to detect nucleic acids in gel under trans-UV light (Diaz *et al.*, 2002; Olmsted III and Kearns 1977), as a model of mouse ventral spinal cord demyelination to characterize behavioral function, inflammation, myelin status and axonal viability

(Kuypers *et al.*, 2013), and nuclear translocation of estradiol receptors (Andre *et al.*, 1997). Due to its characteristic structure it intercalates between stacked nucleotide bases of nucleic acids and manifests carcinogenic and mutagenic effects via affecting biological processes of DNA replication, transcription, structural and functional alteration of the cell (Andre *et al.*, 1997; Kuypers *et al.*, 2013; Röding *et al.*, 1986; Waring 1965). There are other dyes available as an alternative to EtBr such as methylene blue, thiazole orange homodimer and dihydroethidium and others which are reported to be non-mutagenic, less toxic or converted to mutagenic form within the cells only (Budd *et al.*, 1997; Saiki *et al.*, 1986; Singer *et al.*, 1999; Wilke *et al.*,

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1994). Despite availability of alternatives to EtBr, application of this carcinogenic and mutagenic agent is widespread and inevitable in research.

Being a threat as mutagenic and carcinogenic agent EtBr contaminated wastes are recommended to be decontaminated or treated prior to its disposal. Treatment with bleach, incineration, processing of solution through Rohm and Haas Amberlite XAD-16 resin, and other methods and products have been recorded and suggested for decontamination of EtBr contaminated wastes (Armour 2003; Joshua 1986; Lunn and Sansone 1987 and 1991; Sigma-Aldrich-MSDS, 2017; Zoher *et al.*, 1988). Other product such as EtBr Destroyer (Favorgen Biotech Corp), a chemical based product, claims to degrade and destroy EtBr as quickly as five minutes leading to non-fluorescent and non-mutagenic by-products. Removal of EtBr using activated charcoal and ion-exchange-resin and incineration are the recommended methods of ethidium bromide decontamination (Sabnis 2010; Saeidnia and Abdollahi, 2013; Sigma-Aldrich-MSDS).

Currently bioremediation are considered to be the choice of methods of waste treatment and management of any kind of hazardous material, if possible. EtBr, though inhibits microbial growth at higher concentration, can be degraded by microbes residing in nature either under normal condition or EtBr contaminated sites (Singer *et al.*, 1999). Different research groups have isolated EtBr degrading microorganisms namely *Bacillus*, *Bacillus thuringiensis*, *Neisseria canis*, *Neisseria subflava*, *Pseudomonas chlororaphis*, *Neisseria macacae*, *Pseudomonas putida* and *Aeromonas hydrophila* from uncontaminated-soil (Lone *et al.*, 2015; Patil and Berde 2015; Sukhumungoon *et al.*, 2013). Based on literature cited above and necessity to utilize indigenous microbes to decontaminate EtBr at source. The current study aims to isolate bacteria from EtBr-containing waste in lab and to employ them efficiently in treatment or management of EtBr-contaminated-wastes.

Materials and Methods

Sampling and Isolation of Bacteria

Teaching lab of Centre for Life Science, Central University of Jharkhand, Brambe, Ranchi-835205, Jharkhand, India was chosen as the site of sample collection. Sample was collected from the container in which all the EtBr containing agrose-gel and liquids were discarded and stored for safe disposal of the waste. Water, at the bottom of EtBr-waste-container, was collected into sterile micro-centrifuge tube with the help of sterile pipette. 4 µl sample was streak plated on EtBr-NA (Nutrient Agar supplemented with filter sterilized EtBr at the concentration of 30 µg/ml). Plate was incubated overnight (16 hours) in dark at 37 °C to allow growth of bacteria. Based on colony morphology different forms of colonies were marked. Colonies in the plate were screened under UV-Trans-illuminator at 302 nm to observe

non-fluorescent-halo or fluorescent dye accumulation by colonies. Both types of colonies were picked-up and purified by subsequent repeated sub-culture by streak plate method. The pure cultures were maintained on separate NA as well as EtBr-NA plated at 4 °C.

Study of EtBr Degradation and Bioaccumulation by Bacteria

Bacteria namely isolate-BR3, isolate-BR4, standard reference organism *E. coli*.DH5α (Gifted by Dr. PN Jha, BITS, Pilani, Rajasthan) and *Lactobacillus* (Lactic acid bacillus from Nutrolin-B, CIPLA LTD.) were grown overnight in nutrient broth (NB). Cultures were centrifuged at room temperature at 10,000 rpm to get pellet. Pellets were washed and suspended in 10 ml saline (0.85% NaCl). OD at λ₆₀₀ nm of all the bacterial cell suspension was adjusted to ~1.0. 5.0 µl drop of each cultures were placed on both NA and EtBr-NA bed in Petri plates. Plates were incubated in dark at 37 °C and/or at room temperature to grow bacteria. Plates were scanned over Trans-UV (302 nm) to monitor EtBr degradation/accumulation. Change in fluorescence intensity or change in the diameter of non-fluorescent-halo zone was considered as a measure of EtBr biodegradation or bioaccumulation. To test the growth inhibitory effect, around the concentration of EtBr used under this study, and hence degradation the isolates were grown at different EtBr concentration from 0 to 100 µg/ml at 37 °C in dark for 24 hours. Biomass growth and size of non-fluorescent zone was observed to test growth inhibition and EtBr degradation respectively. Photography was done in white light/under Trans-UV by normal camera and Gel-documentation system (AlphaImager-Cell Biosciences Inc., USA) as required.

Characterization of the Isolates

Bacteria were grown on solid LB-agar plate and morphological characteristics of the bacterial colonies were observed and recorded as per the guidelines of Willey *et al.*, (2008). Shape and cell wall characteristics of the bacteria were observed following gram staining by standard protocol using gram staining kit (HiMedia, India). Slide preparation was observed under microscope at 100X in oil immersion. Isolates were further subjected to catalase test. Loop-full cells from culture plates were transferred to clean-sterile glass slide and overlaid with 3% H₂O₂. Formation of air bubbles gives catalase positive test. Motility test was performed in semisolid LB medium in tubes. Bacteria were grown in LB broth overnight and bacteria were stab-inoculated, with inoculation needle, into semisolid (0.4 % agar) LB-Agar in tubes. Tubes were incubated un-agitated at 37 °C overnight. Growth patterns of the stab cultures were recorded to assess motility of bacteria.

Test of EtBr Utilization as a Sole Carbon Source

Bacterial cultures BR3 and BR4 were grown overnight on LB broth medium at 37 °C. Cultures were harvested by

centrifugation at 8000 rpm for 5 minutes at 25 °C. Pellets were washed 10 times with 1.0 ml M9 medium. Finally, the pellets were suspended into 1.0 ml M9 medium. The cell density (OD₆₀₀) was adjusted to 1.0 and 100µl was added in different tubes as per table 1. Tubes were incubated at 37°C for 20 hours and observations were recorded. To check viability of culture in M9, with/without EtBr, over 20 hours the serial dilutions of S No 3 and 4 were drop (5 µl) plated on LB agar plate and incubated overnight at 37 °C. Increase in turbidity was marked as utilization of EtBr as a sole carbon source (if no growth is observed by 20 hours LB broth, final strength 1/5 of original LB, will be added to S No 3 and 5 containing EtBr, M9 and culture to ensure growth and biodegradation of EtBr. For this, the negative control will be set without LB).

Table 1: Composition of experimental set-up for utilization of EtBr as a sole carbon source

S. N.	Media composition	Culture
1.	M9 media + 90 µl water	Negative control- 100 µl M9
2.	M9 media + EtBr (30µg/ml)	Negative control- 100 µl M9
3.	M9 media + EtBr (30µg/ml)	100 µl BR3 at OD ₆₀₀ 1.0
4.	M9 media + 90 µl water	100 µl BR3 at OD ₆₀₀ 1.0
5.	M9 media + EtBr (30µg/ml)	100 µl BR4 at OD ₆₀₀ 1.0
6.	M9 media + 90 µl water	100 µl BR4 at OD ₆₀₀ 1.0

Model Designing

Based on the application of EtBr in Lab and need of decontamination of waste before its disposal the design of the “Generalized Laboratory Model of EtBr Degradation Plant” has been proposed by us to operate at lab scale (Fig. 1). The model can be refined based on working volume, type of organism used and level of containment needed for the risk group of organism used. Basically it is comprised of five units: Crude Waste Reservoir, EtBr-Water Reservoir, Inoculum Unit, Degradation Unit and Containment Unit. Number of degradation unit are independent multiple units to manage for duration of degradation and rate of inflow of crude waste and EtBr-Water.

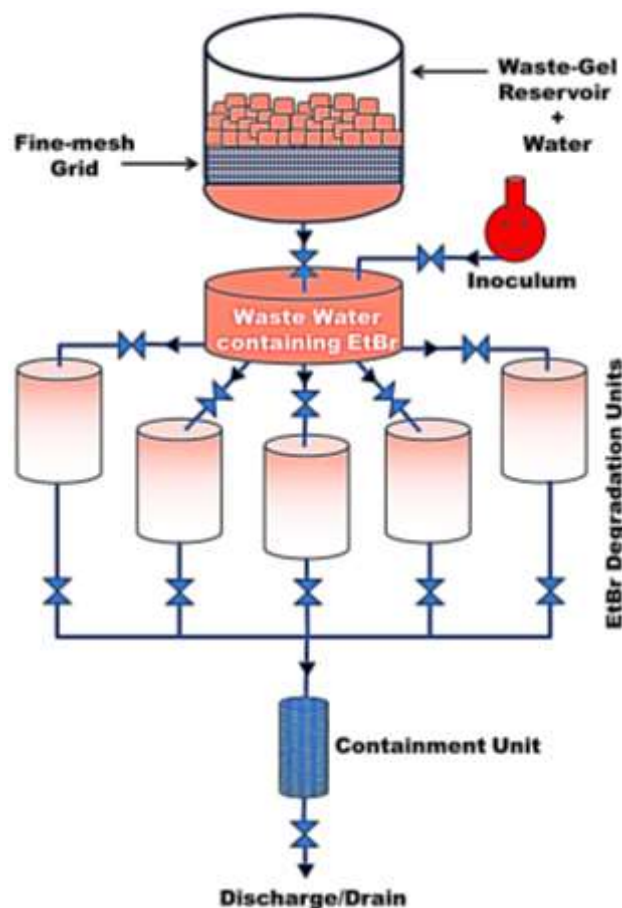


Fig. 1: Generalized Laboratory Model of EtBr Degradation Plant. Direction of flow is indicated by Black arrow. The shape flowchart-collate indicate the points to control the flow of material across the line.

Results and Discussion

Isolation and Characterization of Bacteria

During initial isolation and culture purification from EtBr waste two distinct type of bacterial colony could be observed on streak plate (Fig. 2A). One of the isolate was growing faster and spreading/diffusing rapidly forming ~ 4 different concentric rings/zones (Fig. 2A-1-3). Subsequent separate subculture from these distinct zones 1-3) again resulted in the similar growth pattern in all (Fig. 2B- plate 1, 2, 3). Ultimately we selected the bacteria growing in zone 3/plate 3 of figure 2A/Figure 2B respectively and named it BR3. Other isolate was growing confined into a distinct colony (Fig. 2A-colony 4/ Fig. 2B-plate 4) was named BR4.

Both of the isolates were found to be off-white-cream color colony, gram-negative short rods, catalase-test-positive (Fig. 3) and motile bacteria (Fig. 4). Use of Motile bacteria in biodegradation plant of toxic material may be beneficial as the agitation system may not be required to mix the content rather bacteria itself can reach to the toxic source to act on.

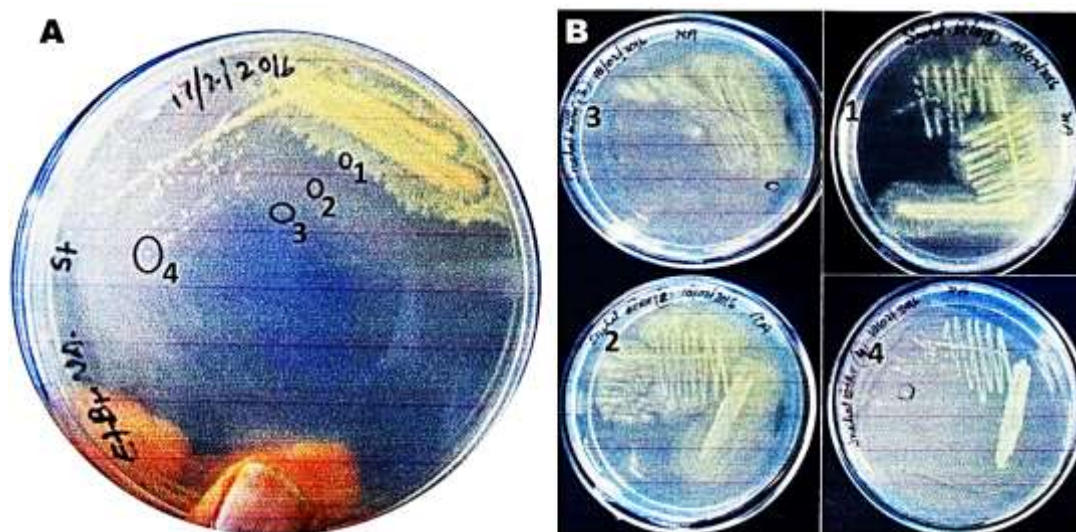


Fig. 2: Isolation and preparation of pure culture by Streak plate Method. Plated show growth characteristics of the both isolates BR3 and BR4. A- Represents primary streak: 1-3 are different growth zone of BR3 and colony 4 is BR4. B- Represents subcultures from zones 1-3 and colony 4 of A. Plates 1-3 show growth characteristics of bacteria from zones 1-3 of A and plate 4 show growth characteristics of bacteria from colony 4 of A.

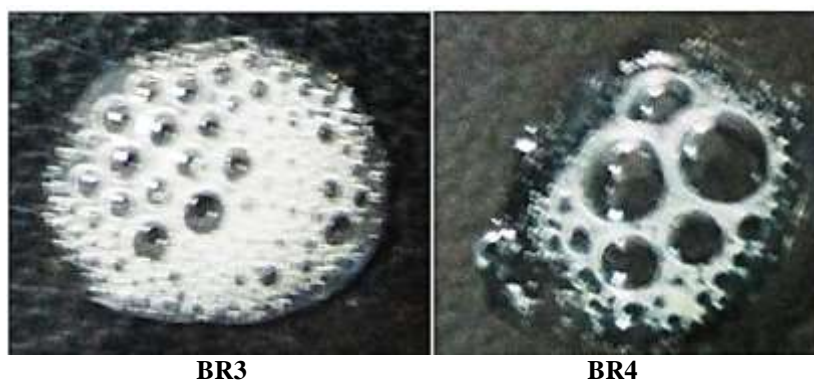


Fig. 3: Catalase Test. Addition of H_2O_2 to cultures of BR3 and BR4 show formation of air bubbles representing conversion of H_2O_2 to release O_2 bubbles.

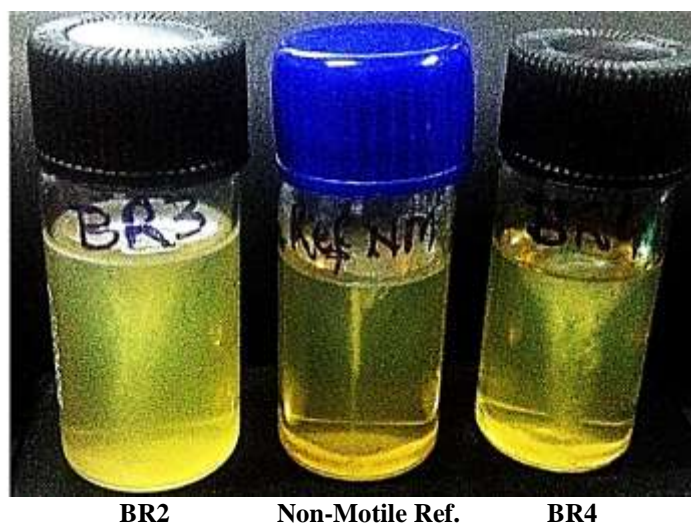


Fig. 4: Motility Test. The tubes show growth characteristics of BR3, a non-motile reference organism and BR4 in semi-solid (0.4 % agar) nutrient agar. Reference non-motile organism is growing in the line of inoculation whereas BR3 and BR4 are growing and diffusing radially away from line of inoculation showing characteristics of motility.

Study of EtBr Degradation-Bioaccumulation by Bacteria

Comparative study of EtBr degradation by isolates BR3, BR4, standard reference organism *E. coli*.DH5 α and *Lactobacillus* revealed that only BR3 could accumulate and degrade EtBr, BR4 could only accumulate EtBr whereas others did not accumulate or degrade the EtBr (Fig. 5A). Degradation ability is evident from the development of non-fluorescent zone around the colony BR3. We also tested some of the agricultural field isolates and found that some organisms grew and accumulated EtBr while others neither accumulate nor grew on EtBr at 30 μ g/ml (Fig. 5B). From Fig. 5B it is evident that EtBr exhibit some inhibitory effect as some of the bacteria (C-1, C-3, O-3 and T-5) did not grow on EtBr. Toxicity effect of EtBr has been shown in other bacteria at EtBr concentration 10-20 μ g/ml which supports our finding in case of agricultural field isolates but is contradictory to our finding with BR3 and BR4 (Singer et al., 1999). To test the ideal concentration for further study the appearance/size of non-fluorescent zone was tested at different concentration of EtBr. It was found that the diameter of non-fluorescent zone increased up to EtBr 30 μ g/ml and then decreased at EtBr 40 and 100 μ g/ml (Fig.

6). This decrease in diameter of non-fluorescent zone may be attributed to the higher concentration of EtBr and need more time to degrade and attain the comparable size of non-fluorescent zone of EtBr 30 μ g/ml. Decrease in non-fluorescent zone size may not be a inhibitory effect of EtBr because prominent growth is visible at all the concentrations (Fig. 6). From above finding it is clear that isolates from contaminated source proves finding better isolate to resist inhibitory effect of EtBr and some may develop the mechanism to degrade the EtBr. Thus the inhibitory effect of EtBr may be subjective to the organism's ability to degrade/accumulate EtBr and the source of isolation site. EtBr degradation studies have also been conducted by other research groups who used 30 μ g/ml but their non-fluorescent zone diameter appears to be small (Patil and Berde 2015; Sukhumungoon et al., 2013). Furthermore we could see that as early as 6 hours the degradation was efficient and the size of non-fluorescent zone gradually increased with time (Fig. 7). Even within an hour we can see the traces of non-fluorescent zone if we do imaging every hour (Fig. 7- 0 hour).

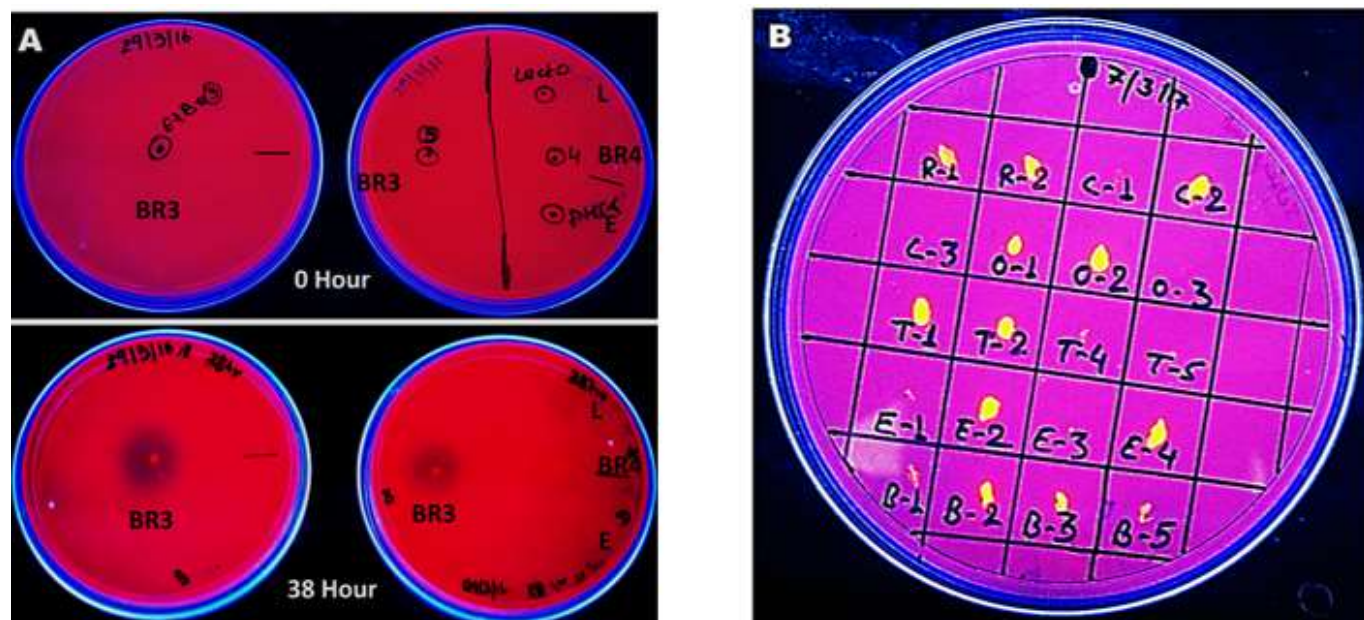


Fig. 5: Growth and degradation of bacteria on nutrient Agar media supplemented with EtBr at 30 μ g/ml. A- Shows EtBr degradation at 38 hour (non-fluorescent zone) by BR3. Other bacteria BR4, *E. coli*.DH5 α and *Lactobacillus* did not show non-fluorescent zone. *E. coli*.DH5 α and *Lactobacillus* did not grow at all. B- shows growth and accumulation of EtBr by bacterial isolates from agricultural fields at 24 hour but they did not degrade EtBr. Isolates C-1, C-3, O-3 and T-5 did not grow on EtBr.

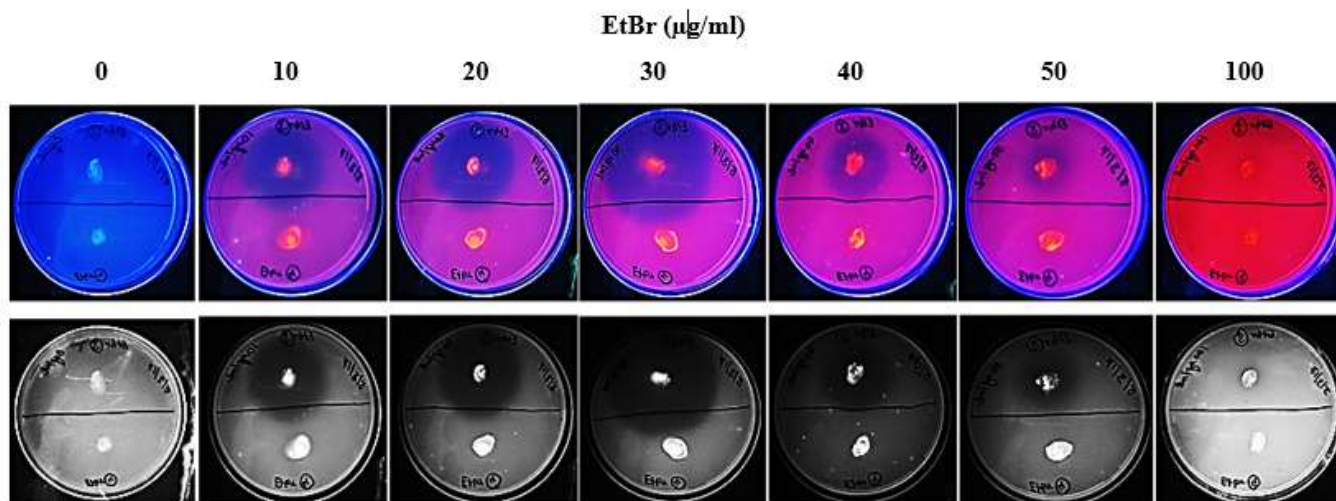


Fig. 6: EtBr degradation and size of non-fluorescent zone at different concentration of EtBr at 24 hour. Top row of figures are taken under trans-UV by normal camera and bottom row are taken under trans-UV by Gel-Doc system (AlphaImager-Cell Biosciences Inc., USA).

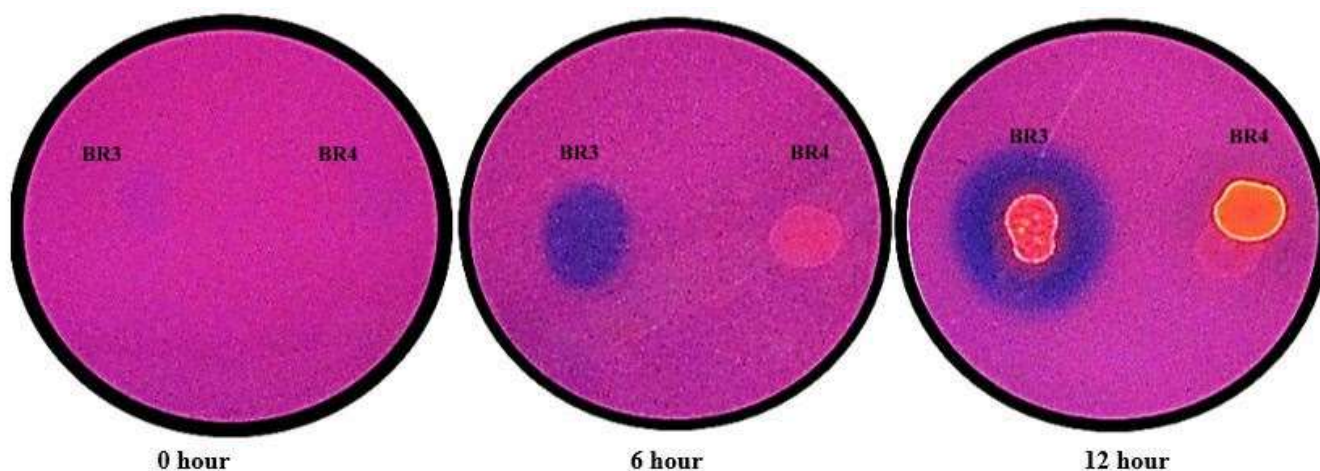


Fig. 7: Time course study of development of non-fluorescent zone over 12 hours. 5 µl drop was inoculated and allowed to absorb (~30 min). 0 hour is equivalent to 30 min.

EtBr degradation ability at 37 °C in dark and unregulated temperature (room temperature in dark) was tested for 434 hours. Growth behavior and degradation ability were different at two experimental conditions (Fig. 8). Bacteria were growing in concentric rings at room temperature while concentric rings did not appear inside incubator at 37 °C. EtBr degradation at room temperature (outside incubator in dark) was found to be faster compared to degradation at regulated temperature at 37 °C. Whole plate became non-fluorescent (except colony biomass) by 40 hours at room temperature however it took 118-142 hours at 37 °C. The disappearance of fluorescence from the bacterial colony

biomass, however, took around 478 hours (Fig. 8). This finding make the study more interesting to employ this organism for decontamination of EtBr containing waste even at room temperature effectively without imposing cost to the additional facility for temperature regulation. Thus isolate BR3 being motile and degrading EtBr effectively at room temperature in short time (6 hours and less) makes it suitable for its application in degradation plant proposed by us (Fig. 1). Further characterization with respect to identity of BR3 and its risk group will help specify the proposed EtBr degradation plant particularly with respect to the containment unit.

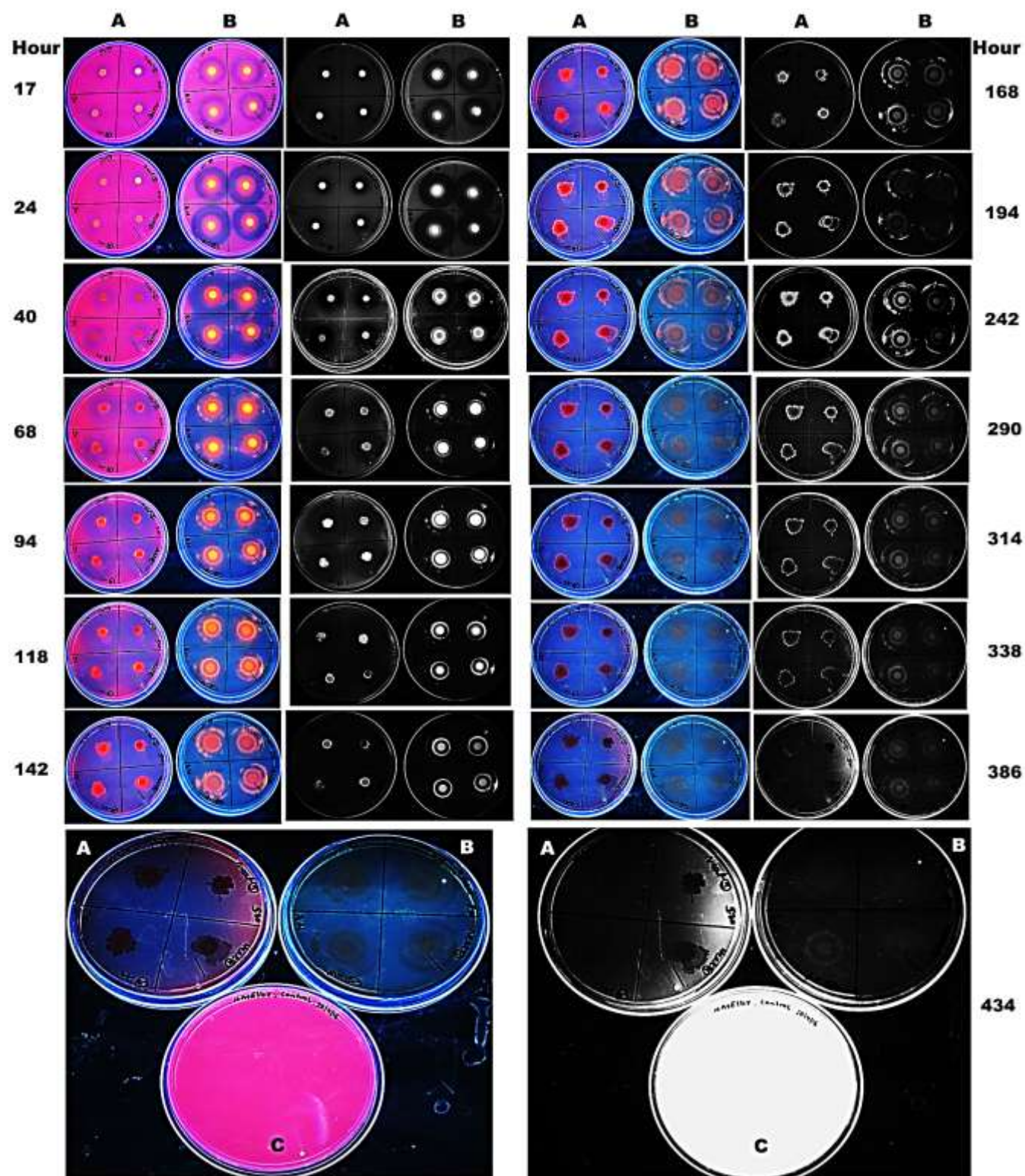


Fig. 8: Time course study of development of non-fluorescent zone over 434 hours. A- Plates were incubated at 37 °C in dark. B- Plates were incubated at unregulated temperature (room temperature in dark). 5 µl drop was inoculated and allowed to absorb (~30 min). 0 hour is equivalent to 30 min. C- control plate containing EtBr 30 µg/ml but inoculum was not added.

Test of EtBr Utilization as a Sole Carbon Source

Test performed in minimal medium M9 supplemented with EtBr did not show growth of the BR3 and BR4 over 20 hours suggesting that they are not able utilize EtBr as a sole

carbon source which is contradictory to finding by others (Patil and Berde, 2015). To check their viability in M9 with EtBr over 20 hours the serial dilution of S No 3 and 4 were drop (5 µl) plated on LB agar plate. Growths were observed in dilutions 10⁻¹ to 10⁻⁶ which suggested that bacterial cells

were viable in experimental set-up but did not grow due to lack of utilizable carbon source. From this study we concluded that bacteria BR3 and BR4 could not utilize EtBr as a sole carbon source. Further addition of LB broth (final strength 1/5 of original LB) to the same EtBr containing M9 and culture lead to resumption of growth of BR3 and BR4 compared to no addition of LB. It also resulted in reduction in fluorescence (BR3) indicating to degradation of EtBr. So while using these bacteria in degradation plant the degradation unit has to be supplemented with other carbon source to augment EtBr degradation. It further requires optimization of the minimum concentration of carbon source to show EtBr degradation capability suitable in the degradation plant.

Conclusion

It is evident, from the current study, that EtBr degradation could be achieved by indigenous microbial population of EtBr contaminated waste reservoir. BR3 evidenced the potential to degrade EtBr but BR4 did not. Though isolates from non-contaminated sites accumulate EtBr but did not degrade it. So probability of finding of EtBr degraders in EtBr contaminated sites is more compared to other sites. However bacteria from other sources do have the capacity to accumulate EtBr and grow too. In our source we could find only two types of culturable bacteria. May be probably the others could not overcome stress/mutation/inhibition by EtBr. Though the bacterium BR3 degrades EtBr but it does not utilize EtBr as a sole carbon source. So it will require the presence of other carbon source to grow and then degrade the EtBr. Thus this study has presented primary investigation of isolation and characterization of efficient EtBr degrading bacteria from our lab in CUJ, Ranchi, Jharkhand. Furthermore the present study has revealed that we can utilize this bacterium to decontaminate our lab-waste containing EtBr as per the model proposed. However this model has to be tested for its performance. This study is still continued to characterize the bacterium to know its identity and the risk group to ascertain its reliability in application.

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Conflict of Interest

The authors have no conflicts of interest

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