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Isolation of agarose digesting *Achromobacter* sp. and *Brevundimonas diminuta* using customized earthworm gut as an enrichment system

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Abstract

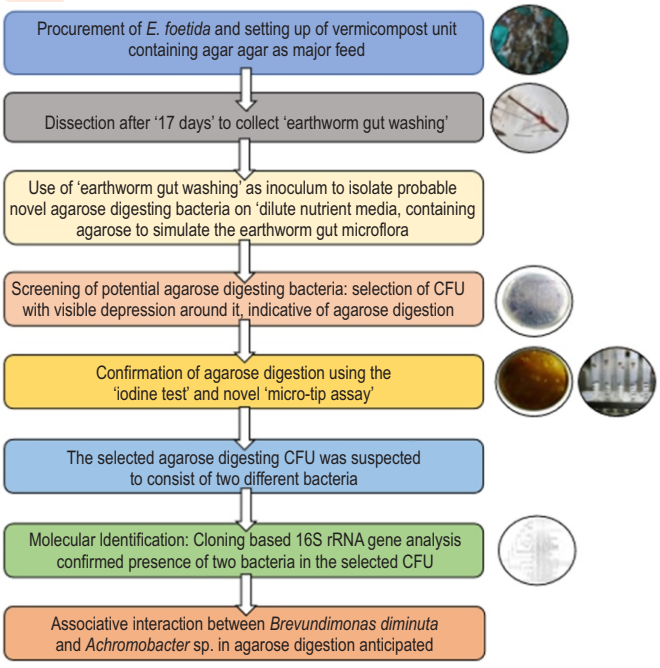
Aim: To isolate potential agarose digesting bacteria from gut of *Eisenia fetida* fed with agar agar as a major feed.

Methodology: *E. fetida* was fed on agar agar for 17 days in a comprehensive vermicompost unit, and gut-washing was collected. A specially designed dilute nutrient medium containing agarose as a solidifying agent was used to grow and isolate agarose digesting bacteria. Agarose digestion was confirmed by iodine test and novel 'micro-tip' assay.

Results: A selected CFU comprising two bacterial species showed depressions on the media, indicative of agarose digestion. Subsequently, cloning based 16S rRNA gene identification revealed the two isolates to be *Brevundimonas diminuta* and *Achromobacter* sp. with 99% similarity. These two could not be separated as pure cultures, indicating a likely associative interaction.

Interpretation: The present study is the first of its sort to demonstrate a probable associative interaction between *Brevundimonas diminuta* and *Achromobacter* sp. in agarose digestion, while using "Customized Earthworm Gut Enrichment System."

Key words: Agarose digesters, Earthworm, Enrichment system, Gut microflora



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Introduction

Agarase, the enzyme that catalyzes the hydrolysis of agarose, has wide applications in the medical, cosmetic, and food industries. It is used as a tool in various biological, physiological, and cytological studies and has essential applications in the isolation of protoplasts from seaweeds, recovery of DNA from agarose gel, and characterization of cell wall of seaweed (Fu and Kim, 2010). Considering these extensive applications, numerous agarases have been isolated from marine and terrestrial bacteria, as well as engineered microorganisms (Fu and Kim 2010). A novel β-agarase has also been isolated from an agar-degrading bacterium, *Microbulbifer* sp. Q7 from sea cucumber gut (Su *et al.,* 2017). We used the approach of isolating agarose digesting bacteria from the gut of *Eisenia fetida* by modulating their feed to majorly comprise agar agar. The gut microbiome of earthworm; *Eisenia fetida*, is known to play a vital role in its capacity to compost a diverse range of organic matter; feed being a major modulator of dynamic gut flora (Horn *et al.,* 2003; Khyade, 2008; Medina-Sauza *et al.,* 2019).

Given this fact, various microscopic, cultivation-based, as well as molecular approaches are being applied for studying the biodiversity and role of microorganisms in the earthworm gut (Brito-vega and Espinosa-victoria, 2009). While cultivation-independent methods are essential in revealing the vast microbial diversity, cultivation-based methods are nevertheless crucial for understanding the detailed metabolism and functions of these organisms (Aslam *et al.,* 2010). In the present study, we focused on isolating agarose digesting bacteria from the gut of *Eisenia fetida*, an epigeic earthworm, mainly feeding on decaying organic matter and commonly used in vermicomposting facilities worldwide. These worms have high metabolic and reproductive rates that allow them to adapt to the changing environmental conditions of the soil surface. They also display high consumption rates, digestion, assimilation of organic matter and play a key role as litter transformers. Given these facts, we hypothesized that feeding *Eisenia fetida* a particular substrate/ feed (*i.e.* agar agar) would allow us to enrich their guts with the ideal microbiota (*i.e.* agarose digesters). Agar agar was therefore used as the primary source of feed for the earthworms with an aim to identify potentially new agarose-digesting bacteria from their gut (*i.e.* as part of their intestinal flora). Additionally, the earthworms' natural intestinal micro-environment (*i.e.* in-vivo environment) was simulated in the laboratory by addition of earthworm gut washing in the cultivation media and by use of low-nutrient cultivation media. According to literature, both these factors play a crucial role in elevating the chances of isolating novel agarose digesting bacteria (Watve, 2000; Pham and Kim, 2012; Stewart, 2012).

Materials and Methods

Procurement of earthworms and setting up of the vermicompost unit: The study was performed from 2017 to 2018. Twenty-five earthworms (*E. fetida*), were procured from an active vermicompost unit and immediately used in bin setup. The vermicompost unit was set up at the VPM's B.N. Bandodkar College of Science, Thane, Maharashtra, India. The vermicompost unit (Fig. 1a) was a fabricated plastic container (2.5 ft x 1.5 ft x 1 ft) which was perforated at the bottom and covered with a shade net for draining out water. Round pebbles and small stones comprised the bottom-most layer (1"- 1.5" height). Brick pieces made the second layer, and above this, a thin layer of coarse sand was set. Sugarcane bagasse was collected, soaked in water for 30- 45 min, washed to remove all sugar content, and dried completely (1- 2 days) to remove all residual moisture. This dried bagasse formed the third layer (2 "- 3"), over which a layer of dried cow dung (pellets) was added (1"- 2"). This was layered with vegetable waste (3"- 4") and sprinkled with water. The setup was kept in shade such that the unit was protected from ants and other crawling insects. The worms were fed with sterile 3% agar agar (HiMedia) as primary feed throughout the experimentation. The bin was regularly watered and monitored for agar digestion. Accordingly, fresh agar was added as and when required.

Dissection of earthworms and collection of gut-washing: In our earlier study, increased agar agar digestion was noted after 17 days, for earthworms fed with microbial agar waste as feed (Mulye *et al.,* 2015). Hence, in this study, the worms were dissected and washing was collected on the 17th day after setting-up the bin. Worms were collected on 17th day after setting-up of the bin. They were washed thrice with sterile double-distilled water, sedated with 40% ethanol and pinned down vertically on a sterilized dissecting board with the dorsal part downward. The ventral part was cut open longitudinally along the length using sterilized dissecting instruments. The gut was removed, and gut-washing was collected in sterile containers by squeezing intact worms from the anterior to the posterior end. The gut-washing was preserved at 4°C until further use. It was used as an inoculum as well as a component of cultivation medium during the study.

Designing and preparation of cultivation medium for isolation of agarose digesters: The cultivation medium comprised 1:100 diluted nutrient broth (0.013 g NB, HiMedia in 100 ml distilled water), 1% earthworm gut-washing diluted with saline, and 2% agarose as solidifying agent (pH 6.5). The medium was sterilized by autoclaving.

Isolation of agarose digesting bacteria: The cultivation media plates were streaked with earthworm gut-washing as inoculum and incubated at room temperature. The plates were regularly observed for isolates manifesting visible depressions.

Tests for confirmation of agarose digesting activity: Agarase activity of the isolates giving visible depressions on agarose-containing media was confirmed using Lugol's iodine [1% potassium iodide and 0.5% iodine (Loba Cheme) in distilled water] which stained the undigested polysaccharide of agarose dark brown while leaving the digested agarose unstained (Saraswathi *et al.,* 2011). This was observed as a zone of clearance around the agarose digesting CFU. To support this finding, a novel micro tip assay was developed. Micropipette tips

(1000 µl capacity, Tarsons) were marked with 100 µl calibrations and placed in an autoclaved test tube stand. They were sealed at the bottom end using 50 µl sterile 1% molten agarose. After effective sealing, sterile 1% molten agarose was filled in the tips and allowed to solidify. For the test, 50 µl test culture was overlaid as inoculum. The negative control (control) consisted 50 µl of sterile distilled water instead of the test culture. To rule out the possibility of a reduction in volume of agarose due to desiccation (false positives), a distilled water control was set (i.e. without the enriched culture). All the tips were sealed at the top end using parafilm (HiMedia) and incubated at room temperature. The level of agarose in the tips was regularly monitored for up to five days.

Molecular identification and phylogenetic analysis of cultures: The culture pellet of the CFU responsible for agarose digestion was outsourced for molecular identification to Chromous Biotech Pvt. Ltd., Bengaluru, Karnataka, India. For molecular identification, genomic DNA from the CFU was extracted, followed by 16S rRNA gene amplification by Polymerase Chain Reaction using consensus primers. The amplification product (1.3kb) was then sequenced bi-directionally by Sangers method (Sanger *et al.,* 1977). The 16S rRNA gene amplification product was further subjected to cloning, Sanger's sequencing and molecular identification.

Amplification of bacterial 16S rRNA genes: The reactions mixture PCR contained of the following: DNA; dNTPs 2.5 mM each; 10X Taq DNA polymerase assay buffer; Taq DNA polymerase; 16S rRNA gene primers forward [5'–AGHGTBTGHTCMTGNCTCAS–3'] and reverse [5'–TRCG GYTMCCTTGTWHCGACTH–3']); PCR grade distilled water to make up the volume 100 µl (Chromous Biotech Pvt. Ltd.). Amplification conditions were as follows: initial denaturation at 95°C for 5 min; denaturation at 94°C for 30 sec (35 cycles); annealing at 50°C for 30 sec; extension at 72°C for 1.30 min; final extension at 72°C for 7 min. The PCR product of 1.3 kb was separated by agarose gel electrophoresis along with molecular weight marker.

Amplicon sequencing: Sanger's sequencing was carried out in ABI 3500 Genetic Analyzer according to the BigDye Terminator v.3.1 Cycle Sequencing Kit protocol (Applied Biosystems, United States). The reaction mixture contained the following: ready reaction premix; primer; template; milli Q water. Reaction conditions were as follows: 25 cycles (96°C, 5 mins; 96°C, 30 sec; 50 °C for 30 sec; 60°C, 1.30 min).

Phylogenetic analysis: The 16S rRNA gene sequences were analyzed by nucleotide BLAST and seqmatch tool of the Ribosomal Database Project. The sequences have been deposited in the ENA (European Nucleotide Archive) sequence repository (accession numbers LS992279 and LS992286). Phylogenetic analyses were conducted using the MEGA7 software package. Sequences were aligned with closest BLASTn matches using the Clustal W algorithm. Molecular phylogenetic analyses were inferred using the Maximum Likelihood method. Genetic distance between homologous sequences was calculated using the Tamura 3-parameter nucleotide substitution model. Both the trees were rooted with the outgroup *Deinococcus radiodurans* strain DSM 20539 (NR 026401.1) and *Clostridium perfringens* strain ATCC 13124 (NR 121607.2).

Results and Discussion

The ecosystem engineers- earthworms, are well known to play crucial roles in nutrient cycling and plant growth through direct as well as indirect effects. While their gut is known to lack sufficient enzymes to digest the complex soil organic matter, the

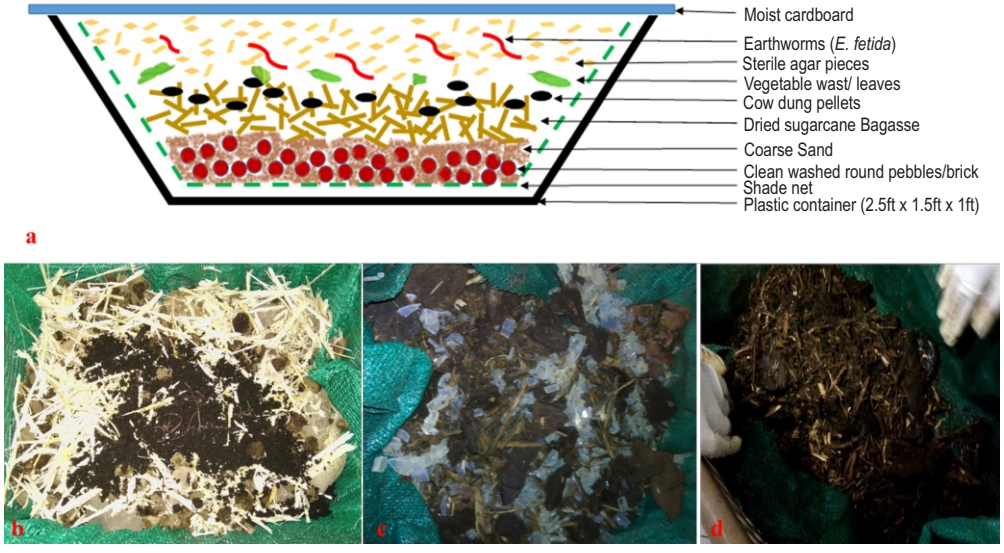

Fig. 1: Vermicomposting Unit: (a) Diagrammatic representation of the vermicompost unit set used in the present study; (b) Unit on Day 1 of set up; (c) Unit on Day 2 of set up with freshly added sterile agar pieces (indicated by the red arrow) and (d) Unit on day 9, after complete digestion of agar.

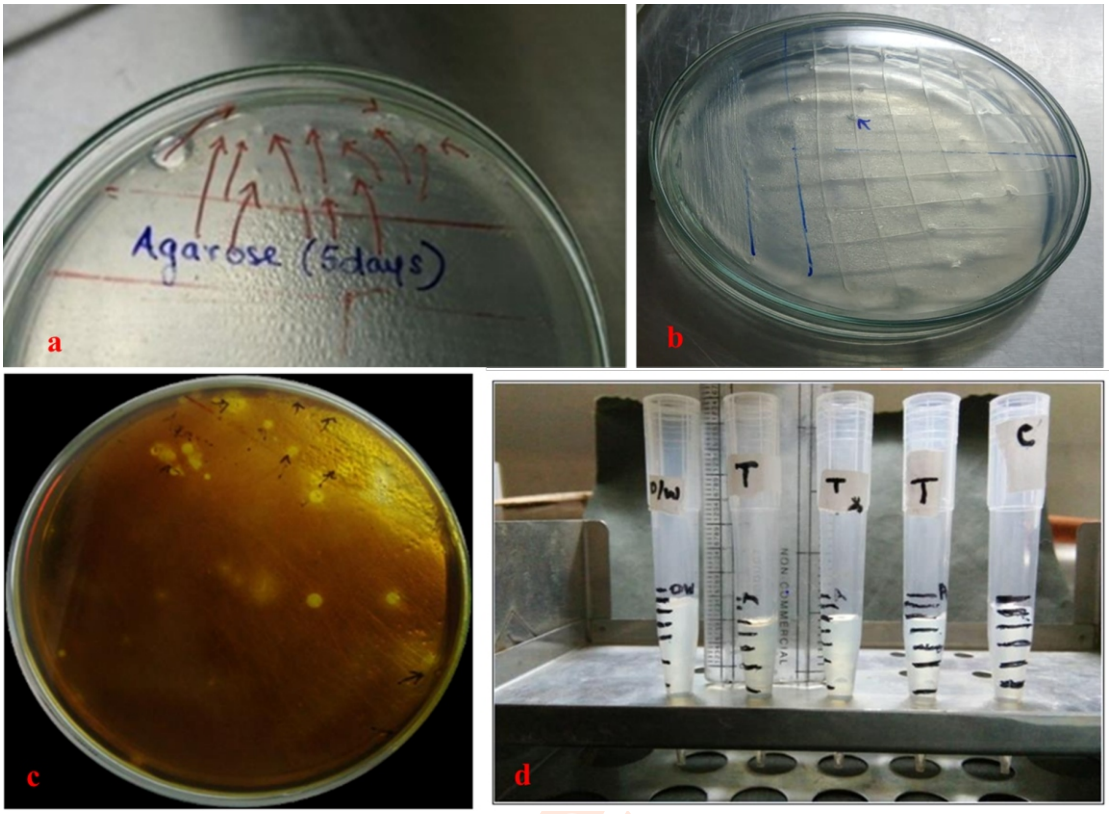

Fig. 2: Identification of agarose digester and confirmation of their activity; A and B: Dilute nutrient medium plates (0.013% nutrient broth, 2% agarose, and 1% gut washing) after 5-day incubation at room temperature showing the selected CFU; C: Iodine test for confirmation of agarose digestion using Lugol's Iodine (potassium iodide, 2 g, and iodine, 1 g in 200 ml of water) poured onto the 5-day old media plate (having the CFUs showing depression) showing the zone of clearance around the selected colony; D: Novel micro tip-assay system (set up in 1000µL tips marked at every 100 µL) showing significant reduction in the agarose level of the test micro tips (marked T/T*) (inoculated with the potential agarose degrading culture) as compared to the distilled water control (marked D/W) and un-innoculated controls (marked C) confirming the agarase activity of the isolate.

worms harbor diverse dynamic gut flora comprising a mixture of transient and resident bacterial species that help the worms in this task (Medina-Sauza *et al.,* 2019). Although it is not yet very clear, if earthworms have their own intestinal microbiome or it comes from the soil, most of the current information indicates that it comes from the soil. Signaling molecules, produced either by the earthworms or most likely by the gut microorganisms, have been speculated to highly influence the earthworm-induced changes in soil functioning and on plant growth (Khyade, 2018; Medina-Sauza *et al.,* 2019).

The resident bacteria also reap many advantages, in return. While dwelling in this specialized ecosystem-the earthworm gut, is known to provide moist, high-osmolarity, substrate-rich conditions and stable temperature, as compared to relatively dry and substrate-poor conditions in the soil. Furthermore, the earthworm gut has been rightly cited as a 'novel colloidal mill' or a 'miniature composting tube' (Horn *et al.,* 2003; Govindarajan and Prabaharan 2014; Kiyasudeen *et al.,* 2014). Interestingly, in our previous study "Efficacy analysis of vermicomposting for microbiological waste treatment" (Mulye *et al.,* 2015), we observed that, as the worms were fed with microbial waste containing agar agar, the rate of agar digestion by the worms kept on increasing gradually. It was thus evident that the earthworm gut was getting enriched with bacteria that could digest the major feed- agar agar, more rapidly with time. Therefore, we hypothesized that the worm gut could be used as 'customized enrichment system' to isolate yet uncultured, novel bacteria involved in the digestion of complex organic compound-agarose.In present study, *E. fetida*, in the vermicompost set-up, was continuously provided with agar agar as major feed.

Fig. 1 (1b-1d) depicts the vermicompost unit used in the present study after addition of agar agar; as well as after its utilization as feed by earthworms. As per our expectation, the agar digestion rate by the worms gradually increased, which might have been due to "enrichment of its gut with agarose digesting bacteria". At this stage, *i.e.,* seventeen days after the vermicompost unit was set up, the bacterial flora of the worm gut extract was analyzed for agarose digestion. Current study focused on isolating "yet-uncultured; novel bacteria" involved in

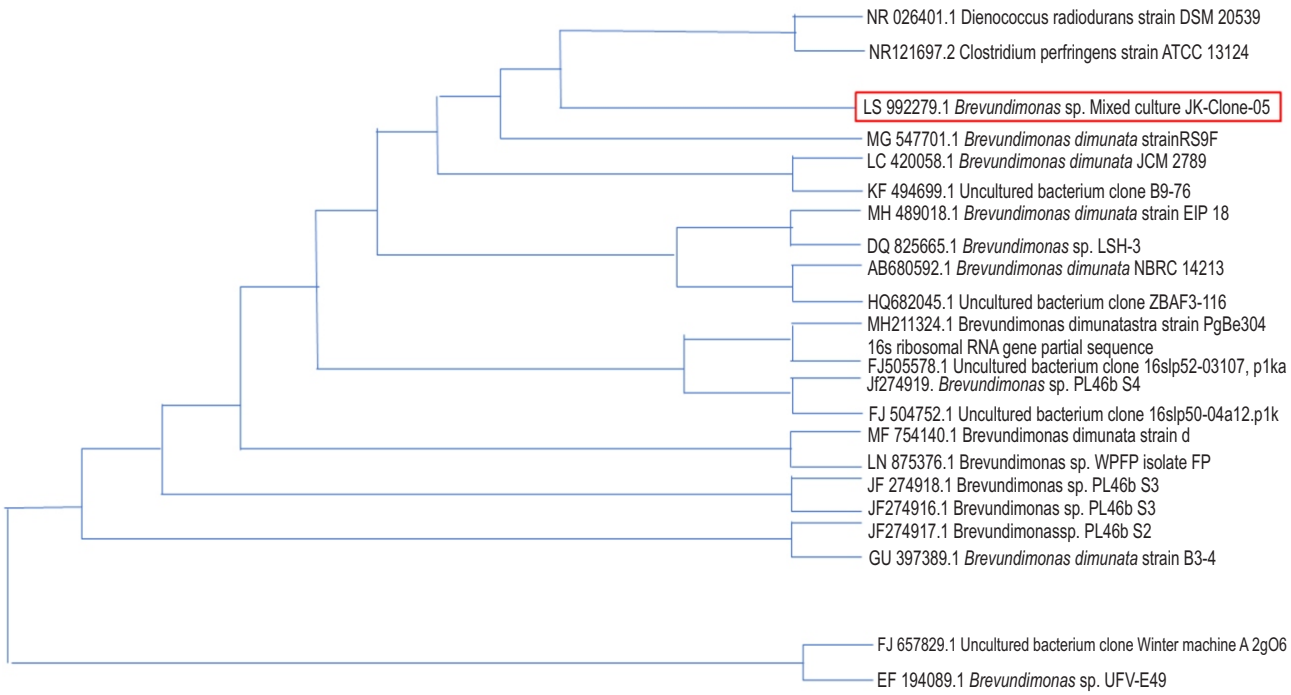

Fig. 3: Maximum-likelihood tree for JK-Clone-5; MEGA software showing phylogenetic relation among JK-Clone-5 and its closest BLASTn matches based on 16S rRNA gene sequences. *Deinococcus radiodurans* strain DSM 20539 and *Clostridium perfringens* strain ATCC 13124 were used as outgroup. Numbers at the node indicate the level of bootstrap support (%) based on neighbor-joining analysis of 100 resampled dataset.

agarose digestion. Therefore, we strategically used low-nutrient cultivation media for this purpose, as it is well known that most of the unculturable bacteria are oligotrophic and are unable to grow in the conventional high-nutrient complex media. Another important advantage of using dilute media was that at low substrate concentrations, r-strategists get outcompeted by k-strategists which are adapted to nutrition depletion, giving opportunity for the k-strategists to flourish predominantly. In this way, we could slowly but very selectively capture only the uncultured k-strategists bacteria with agar digesting capacity while limiting the growth of fast growing r-strategist that would otherwise conveniently outgrow and mask the prior. This was also evident through several earlier studies that used minimal media, more closely mimicking the oligotrophic conditions in nature instead of rich, complex cultivation media to particularly promote growth of hitherto uncultured bacteria (Watve *et al.,* 2000; Vartoukian, *et al.,* 2010; Eevers *et al.,* 2015).

Besides this, media containing environmental portions have also been widely used to culture novel bacteria (Hamaki *et al.,* 2005; Moore *et al.,* 2007). Such media simulates the natural environment and provide unknown nutrients, growth factors etc., that may be absent in conventional cultivation media. George *et al.* (2011) used dilute media containing soil extract to isolate novel bacteria from soil, while Pulschen *et al.* (2017) isolated rare bacteria from Antarctic soil using oligotrophic cultivation media. When earthworm gut washing was inoculated on the agarose containing dilute cultivation media comprising of 1% earthworm gut-washing, pH 6.5; seven distinct CFUs were observed. Out of these, one CFU showed distinctly visible depressions after incubation for five days (Fig. 2a, b). This CFU, presumed to be capable of agarose digestion, was further proceeded for bacterial identification. Gram staining of this selected CFU revealed that it actually comprised of two different bacteria which were seen as Gram-negative rods of two different sizes. To rule out the possibility of pleomorphic culture, the CFU was regularly observed under a microscope (10X magnification) for up to six days. Microscopic observation indicated the presence of two distinct micro-colonies which appeared to be associated with each other. Trials to isolate each of them in pure culture failed even after repeated streaking attempts. Rather it was noticed that they could not grow separately and remained together to show agarose digestion activity seen as depressions on the media.

According to literature, Lugol's iodine has been widely of used as a qualitative test to screen for agarose digestion microorganisms (Fu and Kim, 2010; Saraswathi *et al.,* 2011; Yoon *et al.*, 2022). When the CFUs were re-streaked on different agarose containing media plates, incubated for five days and then flooded with Lugol's iodine, bright clear un-stained zones around them indicated agarose digestion (Fig. 2c). The original preserved culture showing such zone of clearance was selected for further analysis. To support the results of this test, we designed a novel 'micro-tip assay.' This assay could also compare the agar/agarose digesting capacity of different cultures in a semi-quantitative manner. To the best of our knowledge, this is the first

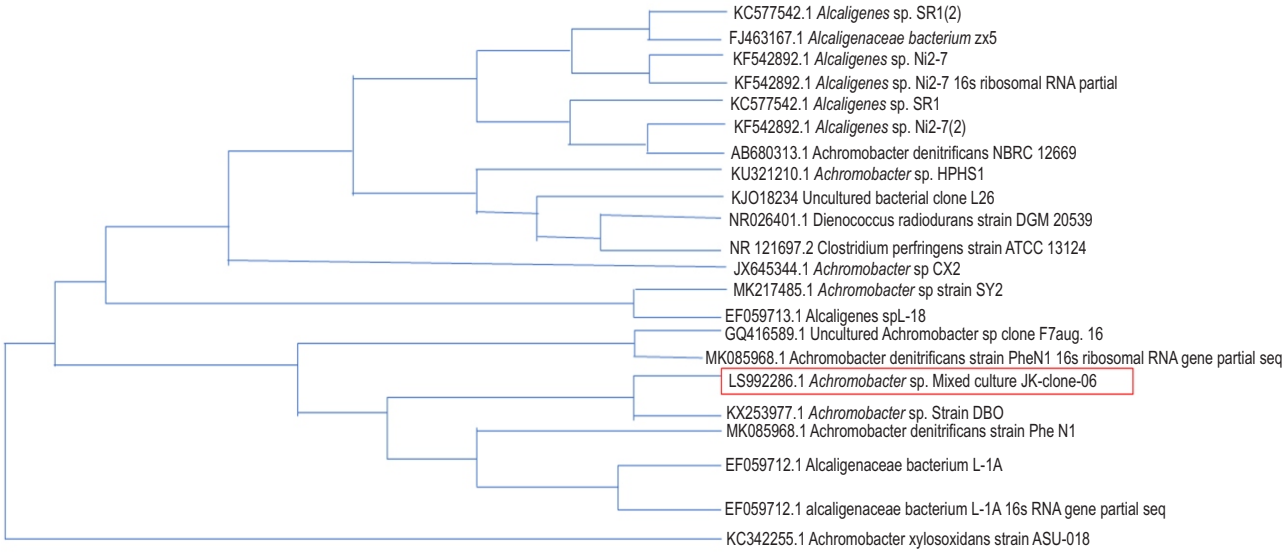

Fig. 4: Maximum-likelihood tree for JK-Clone-6; MEGA software showing phylogenetic relation among JK-Clone-6 and its closest BLASTn matches based on 16S rRNA gene sequences. *Deinococcus radiodurans* strain DSM 20539 and *Clostridium perfringens* strain ATCC 13124 used as out group. Numbers at the node indicate the level of bootstrap support (%) based on neighbor-joining analysis of 100 resampled dataset.

report describing such a simple, cost-effective, semi-quantitative assay to check agar/ agarose digestion. This 'Micro tip assay' showed a decrease in the level of agarose in the tip containing test cultures (T) as compared to control (C and D/W) (Fig. 2d). Thus, agarose digestion by CFU was semi-quantitatively confirmed. To confirm our observation that the two bacterial cultures formed one CFU, and for molecular identification of the cultures, genomic DNA from the CFU was amplified by PCR using 16S rRNA gene primers, followed by sequencing of the amplification product.

The sequence data obtained showed multiple peaks for every data, thus indicating that the culture was not pure. To resolve the issue, the amplification products were subjected to a cloning-based identification technique followed by sequencing, which confirmed that the CFU consisted of two bacteria (JK-Clone-05, JK-Clone-06). The 16S rRNA gene sequence analysis for JK-Clone-05 (LS992279) (nucleotides sequenced: 1281) by BLASTn (similarity score 99.90%) and seqmatch (RDP) (Sab score 0.996) tools revealed that it was similar to *Brevundimonas diminuta* and an uncultured *Brevundimonas*. BLASTn (similarity score 99.7%) and seqmatch (RDP) (Sab score 0.975) tools revealed that the JK-Clone-06 (LS992286) (nucleotides sequenced: 1349) showed similarity to *Achromobacter* sp. and an uncultured *Achromobacter*. However, further phylogenetic analysis confirmed both organisms to be novel and notably distinct from known species of *Brevundimonas diminuta* and *Achromobacter* sp (Fig. 3, 4). Phylogenetic analysis of the sequences, however, indicated that they formed quite distinct lineages when compared to other known members of these genera. Based on this observation, we speculate that they represent novel members of *Brevundimonas* and *Achromobacter* dwelling in the gut of *E. fetida*. Members of the genus *Achromobacter* have been known to be agar digesters for a long time (Goresline, 1993). Strains of the genus *Brevundimonas* are opportunistic pathogens (Berg *et al.,* 2009) and known to be involved in synergistic interactions with *Anabaena* and *Ochrobactrum* in plant growth promotion (Rana *et al.*, 2015). Chen *et al.* (2014) have shown the synergistic association of non-cellulolytic *Achromobacter* sp. CX2 with cellulose-digesting Cytophagales producing β-glucosidase.

In the present study, molecular analysis of CFU; along with the facts that the two micro-colonies were consistently found to be associated with each other, could not be isolated in pure culture despite repeated streaking attempts- all pointed out to the possibility that the members of genera *Brevundimonas* and *Achromobacter* reported in the current work might share some kind of association with each other while dwelling in the gut of *E. fetida*. The exact give-and-take relationship between these two bacteria, however, needs to be explored further. To the best of our knowledge, the present work represents the first report of an associative interaction between *Brevundimonas diminuta* and *Achromobacter* sp. in agarose digestion using 'Customized Earthworm Gut Enrichment System. We wish to explore such systems further to degrade complex biomolecules and confirm the hypothesis.

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Add-on Information

Authors' contribution: J.M. Pawar, K.B. Mulye: Designed study, Analysed data, Contributed new methods, Wrote the

paper; **J.O. Talker, E.O. Talker and A.B. Nikam:** Performed research, wrote paper.

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