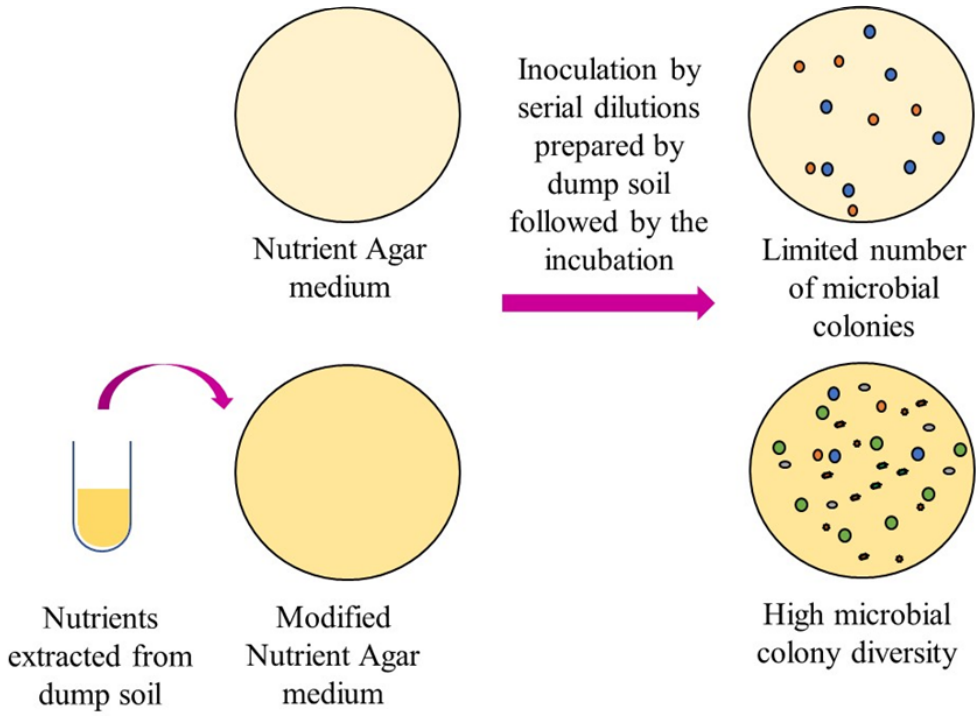
RESEARCH ARTICLE

Modification of nutrient agar medium to culture yet-unculturable bacteria living in unsanitary landfills

C. Devika, I.D. Singhalage* and G. Seneviratne



Highlights

- Addition of soil extract improved bacteria isolation potential of Nutrient Agar.
- Un-culturable bacterial diversity of landfill soil is considerably high.

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RESEARCH ARTICLE

Modification of nutrient agar medium to culture yet-unculturable bacteria living in unsanitary landfills

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Abstract: The microbial diversity in waste dumps is considered to be high. Generally, *ca*. 99% of bacterial species remain unculturable in standard media. This research was focused to modify Nutrient Agar (NA) mimicking the natural substrate where bacteria actually live. NA medium was modified by adding 40% (M1), 32% (M2), 24% (M3), 16% (M4), and 8% (M5) (v/v) soil extracts obtained from the dump site to isolate its bacteria. Conventional NA was the control. The isolates were characterized by FTIR spectroscopy. Seventy-eight and 18 bacterial strains were isolated from the modified media (M1-M5) and the control, respectively. Absorbance of the FTIR spectra constituted six clusters. According to the cluster analysis, the 100% similarity was observed in three bacterial couples, which were considered as three strains. Thus, the modified media of the present study facilitated the culturing of 75 unculturable bacterial strains over the conventional NA and percent improvement of isolation was 417% over NA. Results revealed the potential of extracted soil biochemicals and nutrients to modify the NA for culturing yet-unculturable bacteria living in unsanitary landfills.

***Keywords*:** Unculturable bacteria; soil extracts; nutrient agar; FTIR.

INTRODUCTION

The deposition of untreated municipal solid waste in landfills demonstrates a strong impact on the environment (Ferronato and Torretta, 2019). The decomposition of municipal solid waste is primarily mediated by microorganisms. Generally, microbial diversity of unsanitary landfills is high (Gautam *et al*., 2012). According to Gautam *et al*. (2012), 49 out of 250 microbes isolated from solid waste produced cellulase enzyme and among them *Trichoderma viride* was the best. Methanogen populations such as Methanomicrobiales, Methanosarcinaceae, and Methanobacteriales were common in waste dumps (Bareither *et al*., 2013), where Firmicutes, Proteobacteria, and Bacteroidetes were the dominant bacterial phyla (Wang *et al*., 2017). Bacteria, fungi, algae, virus, and protozoa living in waste dumps are highly beneficial in techniques like composting, activated sludge, trickling filters, and oxidation ponds (Adebayo and Obiekezie, 2018). Thus, it is essential to isolate and culture the participating microorganisms in order to accelerate beneficial activity. However, the current laboratory culturing techniques are unable to grow many microbial species from their natural sources (Stewart, 2012). Therefore, more than 99% of bacterial species living in natural habitats are remained unculturable (Pham and Kim, 2012).

Artificial media are often incapable of mimicking the endogenous abiotic and biotic factors required for microbial growth (Pham and Kim, 2012). However, scientists have paid their attention to change and modify the culture media to improve the cultivability of microorganisms. Liebeke (2009) showed the possibility of culturing unculturable bacteria in solubilized organic and inorganic matter extracted from oak forest soil. Buddhika and Seneviratne (2019) used microbial biofilm exudates to culture unculturable soil bacteria. Tagliavia *et al*. (2019) modified and adjusted the thiosulfate-citrate-bile salts-sucrose agar medium for culturing marine vibrios, which greatly recovered the vibrios species. Separate autoclaving of ingredients of the medium has greatly improved the cultivability of microorganisms by mitigating oxidative stress over the medium where ingredients have autoclaved together (Kato *et al*., 2018). Soil properties of unsanitary landfills are very complex and extremely different in comparison to that of natural lands (Parveen *et al*., 2012). Thus, microbes living in landfill soil could have more adaptations and heterogeneity in accordance with the habitat characteristics (Bleuven and Landry, 2016). To culture the uncultivable bacteria living in landfill soil, the composition of standard culture media can be mimicked using soil extracts which are obtained from the unsanitary landfill itself where the relevant bacteria actually lives in. Therefore, this study focused on the modification of NA medium by using different concentrations of the soil extracts to improve the cultivability of bacterial species living in unsanitary landfills.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from an unsanitary landfill in Uva province of Sri Lanka situated at 60˚ 59′ 5″ North

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and 81˚ 03′ 23″ East. Six soil samples (100.0 g each) were collected randomly from 10 cm below the surface, into sterile bags and brought to the laboratory of Uva Wellassa University, Badulla. Soil samples were air-dried, mixed together and passed through a 2 mm sieve.

Extraction of soil biomolecules and nutrient, and preparation of modified NA media

Thirty grams of soil (< 2 mm) was added to 100 mL of distilled water, stirred (20 min) and filtered to get soil biomolecules and nutrients extract. NA medium was modified in five ways by adding 40% (M1), 32% (M2), 24% (M3), 16% (M4), and 8% (M5) (v/v) of the soil extract. NA medium without any modifications was used as the control. All modified media and the control were adjusted to pH 7.0 and sterilized.

Culturing soil bacteria

One gram of soil was dissolved in 100 mL of sterile distilled water and dilution series was prepared. Soil bacteria were cultured in modified media and the control by spreading 100 µL of inoculum taken from 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions and incubated (36 ℃, 48 h). Bacterial colonies were differentiated according to their colony morphologies and sub cultured by continuous streaking on each media compositions to get pure cultures. Bacterial strains isolated from media M1, M2, M3, M4, M5, and the control were coded as m11-m1n, m21-m2n, m31-m3n, m41-m4n, m51-m5n, and c1-cn, respectively. At the end of the isolation, the number of pure colonies in each modified media and the control was counted by colony counter. The pure cultures were used during the FTIR analysis mentioned bellow.

Fourier Transform Infrared (FTIR) spectral analysis of bacterial cultures

Bacterial colonies were scraped out from culture plates by a clean, sterilized spatula and transferred into a clean, sterilized glass slides separately, and allowed to air-dry under aseptic conditions. One slide per colony was used to avoid the contaminations. Then, dried colonies were powdered (each dried colony was around 20 mg in weight) and used for spectral analysis. Attenuated Total Reflectance (ATR) infrared spectroscopy was used to record the spectra. Spectra were obtained by scanning powdered bacterial samples in the 500 - 4000 cm^{-1} region using FTIR Spectrometer (Bruker, USA). The 120 scans were taken for each interferogram at 4 cm^{-1} resolution. The spectra were recorded by FTIR software, Bruker OPUS (version 7.5). Spectrography (version 1.2.12) software was used for spectral data processing. Average absorbance of each spectrum was calculated for statistical analysis.

Statistical analysis

Spectral data were analyzed by hierarchical cluster analysis. Average absorbance of each FTIR spectrum was used as variables of the distance matrix. Average linkage and Pearson distance measurement were used in the cluster analysis. Statistical analysis was performed by Minitab (version 17).

RESULTS AND DISCUSSION

Total of 96 bacterial strains were isolated from all media compositions. The modified media M1, M2, M3, M4, M5, and the control isolated 19, 13, 15, 14, 17, and 18 bacterial strains, respectively. The spectral records of each bacterial strain on each medium composition are given in Figure 1. According to the FTIR spectral characterization of bacterial strains, all strains showed the characteristic Amide I and Amide II bands at ~1635 cm^{-1}, and ~1545 cm^{-1}, respectively (Beech *et al*., 1999; Garip, 2005; Mistry, 2009; Dean *et al*., 2010) indicating that all cultures are bacterial strains.

The dendrograms given in the Figure 2 show the similarities and the differences of the bacterial strains isolated from the unsanitary landfill soil on different modified NA media and the control. According to the Figure 2, the closest relatives of the media M1, M2, M3, M4, M5, and control are given respectively as m15 and m16 (96% similarity), m212 and m213 (92% similarity), m311 and m312 (94% similarity), m42 and m43 (93% similarity), m511 and m512 (93% similarity), and c6 and c7 (95% similarity). The highest similarity given by m15 and m16 in M1 medium couldn't be considered as same species because according to the literature *Streptococcus faecalis*, *Streptococcus faecium*, and *Streptococcus pyogenes* showed the similarity ratios of 98.7%, 98.5%, and 98.3% during the cluster analysis of FTIR data. Such S*treptococcus* species are having more than 98% similarity, but still they have considered as different species (Helm *et al*., 1991). It has been well-documented that the FTIR spectroscopy is a user-friendly tool to identify and classify bacteria based on the whole-cell biochemical profile (Helm *et al*., 1991; Schmitt and Flemming, 1998; Beekes *et al*., 2007; Mapelli, 2008). Scientists have also examined bacterial peptidoglycan (Naumann *et al*., 1982; Nichols *et al*., 1985) and lipid extracts (Hedrick *et al*., 1991) during the classification of bacteria into different groups using FTIR. FTIR has also been used to characterize the cell components of prokaryotic and eukaryotic cells (Beech *et al*., 1999). Further FTIR provide the specific spectral fingerprints which are highly typical for different microorganisms (Preisner *et al*., 2007). Therefore, present study too used FTIR spectral characteristics to distinguish one bacterial isolate from another, because the similarities and the differences between bacterial isolates can easily be detected due to high sensitivity of the spectroscopy in detecting the changes in the functional groups belonging to different components of bacteria. The FTIR patterns of the bacterial isolates have been used to type the bacterial strains (Helm *et al*., 1991; Kirschner *et al*., 2001). The previous studies on the modification of NA medium also suggested that the addition of different elements can widen the culturable bacterial diversity (Leadbetter *et al*., 1999; Uphoff *et al*., 2001; Stevenson *et al*., 2004).

To evaluate the relationships among bacterial species isolated from different media compositions, a dendrogram was constructed by cluster analysis with all bacterial isolates (Figure 3). All bacterial isolates were

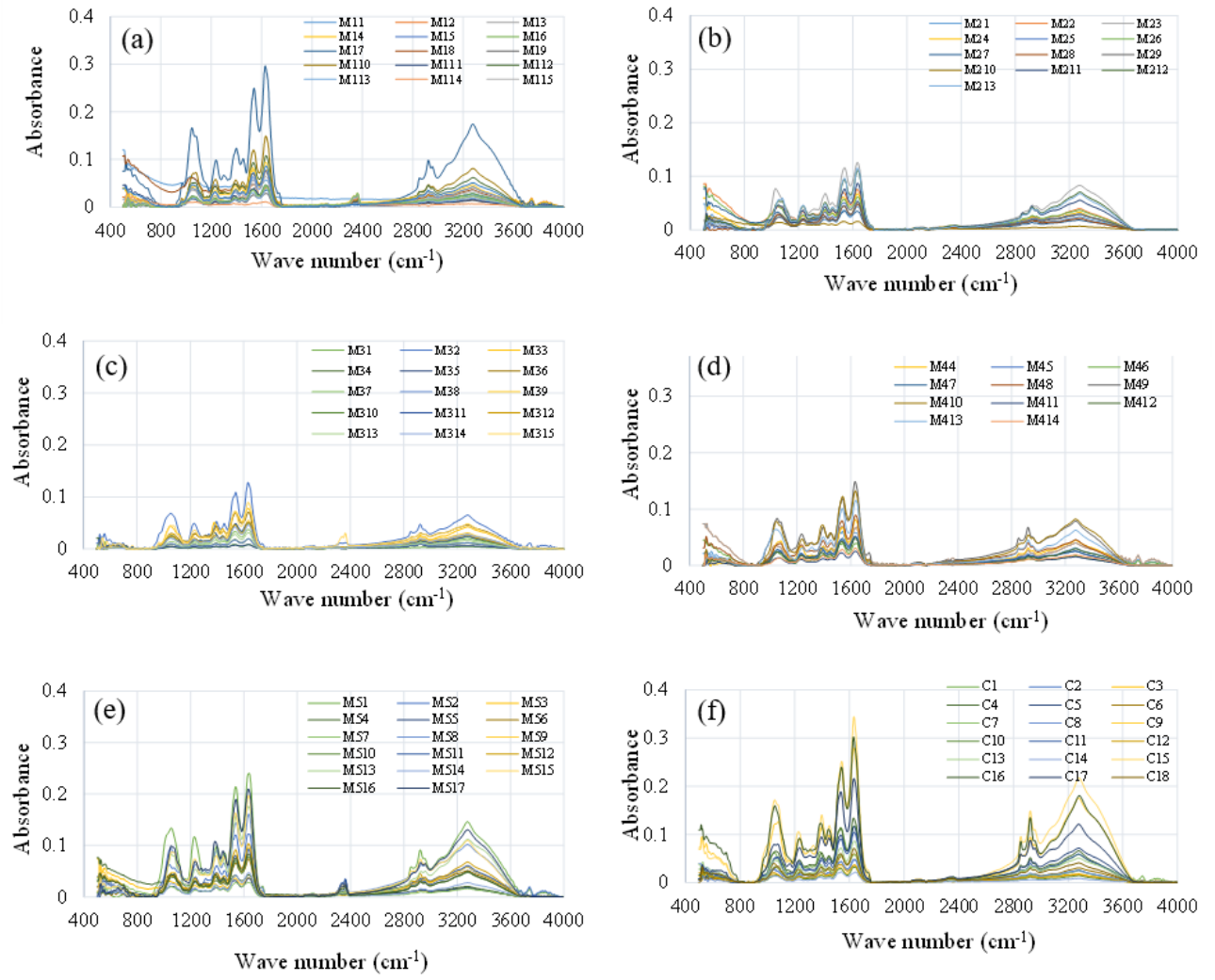

Figure 1: FTIR spectra for bacterial isolates from an unsanitary landfill soil. (a) Conventional NA medium (b) – (f) modified NA medium by adding 40% (M1), 32% (M2), 24% (M3), 16% (M4), 8% (M5) (v/v) of soil extract obtained from the landfill soil.

clustered into six clusters and the members of each cluster are presented in Table 1. The largest clusters were the clusters 1 and 5. The cluster 1 consisted of 39 bacterial species and majority of them were the ones isolated from modified media M1, M2, and conventional NA medium. Although such isolates were clustered together, the isolates from different media did not show 100% similarity to be the same species. Further, all M1, M2, and conventional NA media are derived from natural nutrient sources. Therefore, this is a possibility. The cluster 5 consisted of 26 bacteria isolates which were isolated mainly from modified media M3, M4, and M5. Added soil nutrient concentrations of those media ranged from moderate to least (24%, 16%, 8% v/v). Furthermore, cluster 6 consisted of 5 bacterial species which were isolated from modified media M4 and M5. These media contained least amount of added soil nutrient extract. The nutrient availability could affect the microbial community composition on a medium, and hence by adding soil extracts to the medium, most of the nutrients and other elements required for the microbes might have been added to the medium, thus increasing the microbial diversity. In addition, the microbial cellular structural composition changes in different species and is considered as typical markers for identification (Helm and Naumann, 1995). Since the FTIR enables biochemical scans of whole bacterial cells (Paulina *et al*., 2015) the technique could be used to distinguish microbial isolates of the present study. Further, the isolates of different media did not show 100% similarity and hence were considered as different species.

The left vertical axis of the dendrogram in Figure 3 depicts the increasing heterogeneity. According to the literature, the magnitude of the heterogeneity depends on the number of spectra used in the cluster analysis (Helm, 1991). In Figure 3, 96 FTIR spectra were used and it is always higher than the number of spectra used in Figures 2a, b, c, d, e, and f. Thus, 100% similarities were observed in between some of the isolates (spectra) used in Figure 3. The 100% similarity has been observed in three couples, (m15 and m16), (m311 and m312), and (m42 and m43). Thus, such couples could be considered as three strains and all the other isolates could be considered as different to each other. The present study proved the idea of Zhao *et al*. (2006) that the isolates having 100% 16S rDNA genetic similarities cannot be separated from each other as functionally different species in the phylogenetic analysis,

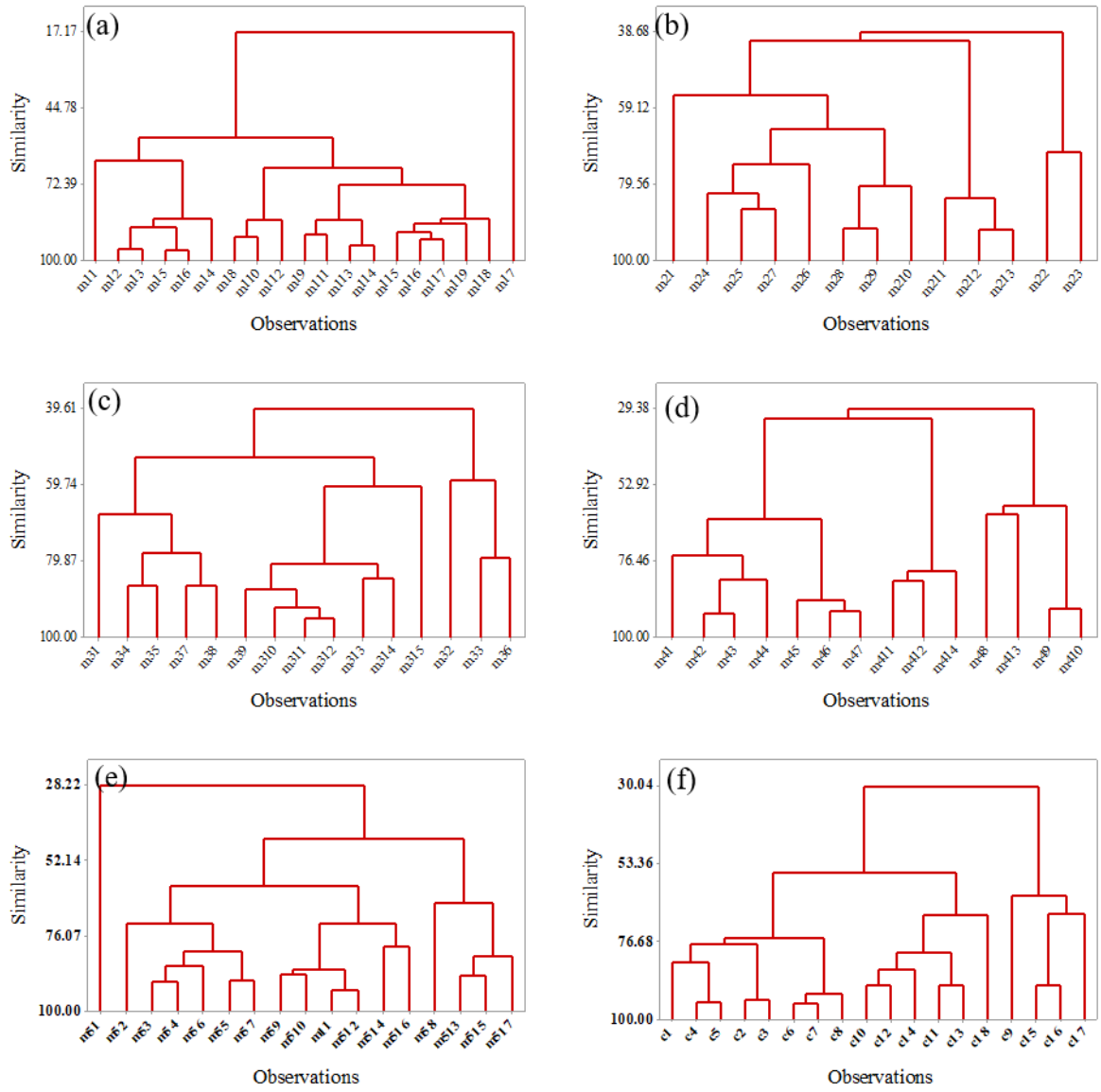

Figure 2: Dendrograms showing similarity between the bacterial strains isolated from the modified NA medium by adding (a) 40% (M1), (b) 32% (M2), (c) 24% (M3), (d) 16% (M4), (e) 8% (M5) (v/v) of soil extract obtained from the landfill soil and (f) the control (unmodified NA).

but using FT-IR, most of the species can be differentiated as functionally different species. The present study mainly focused on the functional heterogeneity. Therefore, FTIR was more beneficial for the present study. The modified media of present study have facilitated the culturing of 75 unculturable bacterial strains over the conventional NA medium. According to the statistical analysis, there were no any overlaps between isolated strains in NA and modified media. Therefore, the 18 strains that were isolated from NA were not included in the 75 strains that were isolated in the modified media. Thus, mimicking the nutrient composition of the NA by adding nutrients from the habitats of bacteria have facilitated a 417% improved bacterial isolation over the NA [Calculation: (Number of bacterial strains isolated in all modified media/number of bacterial strains isolated in un-modified NA) × 100 = (75/18) × 100].

Further the results of cluster analysis suggested that the grouping of bacterial strains depended on the added nutrient concentration by supporting the idea that the cultivability of bacteria depends on the nutrient composition of the medium. Uphoff *et al.* (2001) also showed that the culture media with complex compounds yielded higher number and more diverse isolates than similar media with only one carbon source. Furthermore, addition of electron transporters (Stevenson *et al.*, 2004), enzymes to cope with reactive oxygen species (Stevenson *et al.*, 2004), inhibitors of undesired organisms (Leadbetter *et al.*, 1999) to the culture media and the combination of an unusual energy source with antibiotics to exclude bacteria (Könneke *et al.*, 2005) have been used to culture unculturable bacteria. Changes in the media formulations, including the use of non-traditional electron donors, electron acceptors and carbon sources have proven efficient in recovery of uncultured species (Köpke *et al.*, 2005). According to previous studies, the absence of one or more essential components in culture media is a major reason for most bacteria to remain unculturable. The dump soil contains Nitrogen and other nutrient sources (Corti *et al.*, 2012) and diverse biochemicals, which could be used to

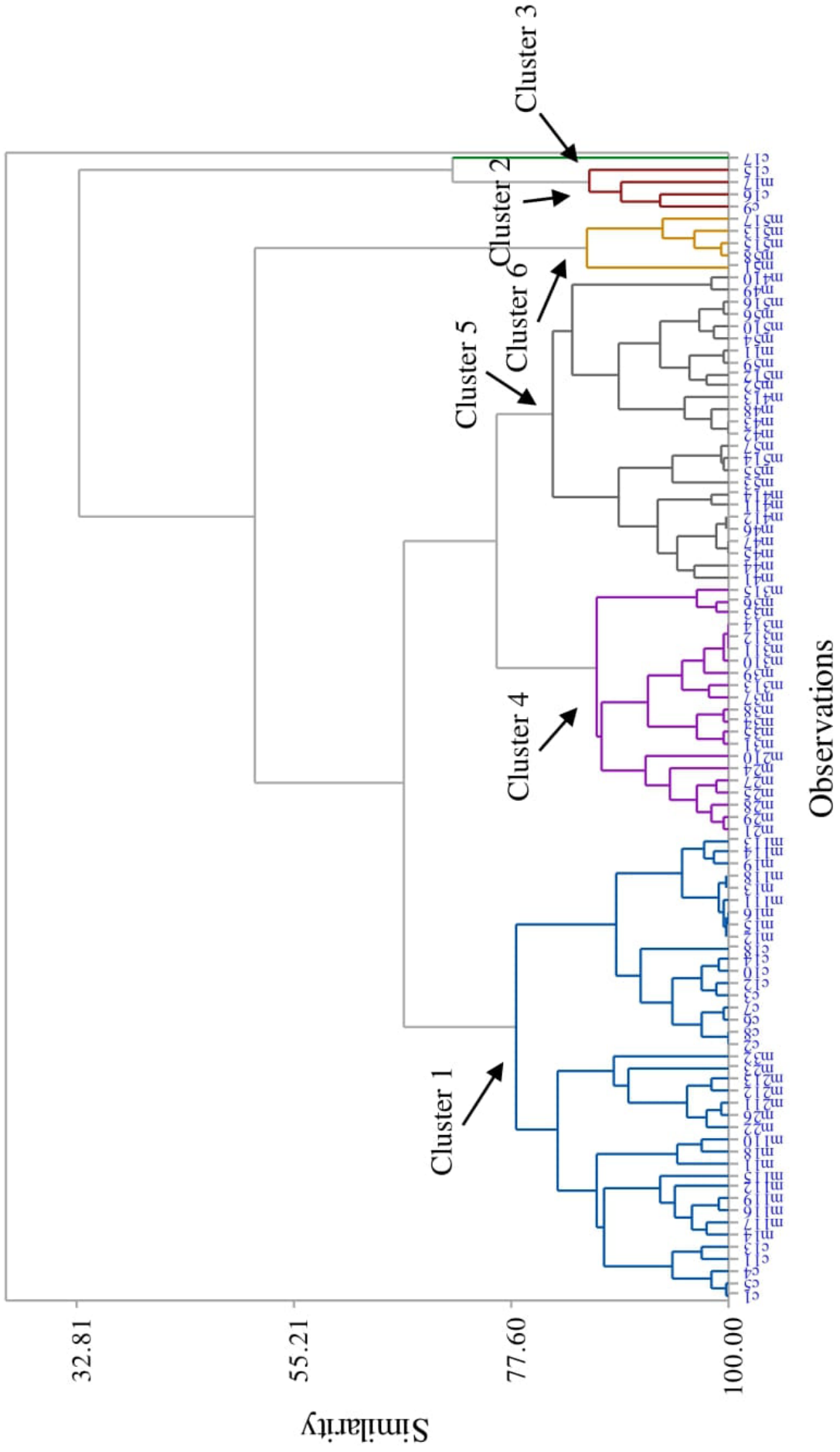

Figure 3: Dendrogram showing the relationship between 96 bacterial strains isolated using differently modified NA and the control.

Table 1: Closest bacterial relatives of the identified clusters of the dendrogram given in figure 3 and their similarity levels.

Cluster	Number of observations	Codes of bacterial isolates	Closest relatives	Similarity (%)
1	39	c1, c5, c4, c11, c13, m14, m117, m116, m119, m112, m115, m11, m18, m110, m22, m26, m211, m212, m213, m23, m32, c2, c8, c6, c7, c3, c12, c10, c14, c18, m12, m15, m16, m111, m13, m118, m19, m114, m113	c1 and c5	99.73
			c11 and c13	97.23
			m14 and m117	97.75
			m116 and m119	98.97
			m18 and m110	97.34
			m26 and m211	97.81
			m212 and m213	98.28
			c2 and c8	97.29
			c6 and c7	99.54
			c3 and c12	97.19
			c10 and c14	99.00
			m15 and m16	100.00
			m15 and m12	99.79
			m13 and m118	99.81
			m19 and m114	98.50
2	4	c9, c16, m17, c15	c9 and c16	92.89
3	1	c17	-	
4	21	m21, m29, m28, m25, m27, m24, m210, m31, m35, m34, m38, m37, m313, m39, m310, m311, m312, m314, m33, m36, m315	m21 and m29	99.56
			m25 and m27	96.79
			m31 and m35	99.46
			m34 and m38	96.69
			m37 and m313	98.02
			m311 and m312	100.00
			m33 and m36	98.69
5	26	m41, m44, m45, m47, m46, m412, m411, m414, m53, m55, m514, m57, m42, m43, m48, m413, m52, m512, m59, m11, m54, m510, m56, m516, m49, m410	m41 and m44	96.53
			m45 and m47	99.95
			m46 and m412	98.67
			m411 and m414	98.13
			m55 and m514	99.60
			m42 and m43	100.00
			m42 and m48	98.36
			m52 and m512	97.85
			m59 and m11	99.50
			m54 and m510	98.43
			m56 and m516	99.42
			m49 and m410	98.25
6	5	m51, m58, m515, m513, m517	m58 and m515	99.12

improve the composition of conventional NA for microbial growth. Therefore, it can be proved that the addition of essential compounds from natural sources is an effective method to culture yet-unculturables. Under the resource-limited, biotic, and abiotic stress conditions, live microbial cells transform into dormant forms, which are reversible to active cells. Dormant cells in the soil thus contribute to generate a voluminous microbial seed bank, which exists until favorable conditions are met to resuscitate (Buddhika and Seneviratne, 2019). Generally, the dormancy of the seed bank can be broken by diversifying the media composition, as was done in the present study.

CONCLUSION

The addition of soil extracts affected bacterial diversity cultured on NA medium. The results revealed that mimicking natural substrate composition is a potential approach to enhance the culturability of different bacterial strains on NA.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no competing interests.

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