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Marl clay bacteria in Mosul City, Iraq

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Marl is a mineral clay that residents of Mosul City use as a geophagic, food, medicine, and cosmetic ingredient. The interaction of clay minerals with microorganisms is common worldwide and holds significant environmental and economic importance. This study aims to investigate and evaluate microbial diversity present in marl clays to determine how far this marl clay may serve as a potential source of bacterial isolates with valuable applications in medicine, industry and bioremediation, also to gather essential information on the safety of consuming these clays as food, medicine and cosmetics, and to address the lack of research in this area. Thirty marl samples were collected and analyzed using X-ray diffraction (XRD), showing that the marl clay was characterized by a unique mineral composition, mainly containing clay minerals: kaolinite, montmorillonite, palygorskite, quartz, and high content of calcite, while some marl samples contained dolomite and chlorite. The results revealed that the pH was neutral. The study ultimately resulted in the isolation and identification of bacteria from marl clay in Mosul City through molecular methods, specifically 16S rRNA sequencing. Numerous bacterial isolates were identified including Actinobacteria, Betaproteobacteria, Firmicutes and Gammaproteobacteria. Neither pathogenic bacteria not and Enterobacteriaceae were detected. The evolutionary relationships were determined using advanced software tools MEGA11 and iTOL v6.

Keywords: geophagic clay; marl; phylogenetic analysis; the pour-plate.

Introduction

The term marl or marl soil is used with somewhat different meanings in different fields. In agriculture, marl is defined as a limnic soil (Mineralogy clay Classes: Limnic subgroups cannot have terric or hydric layers (Saurette & Deragon, 2022)) which is moist, has a color value 5 or more, and reacts with dilute HCL to produce CO_2.. Marl contains very little organic matter. Marl contains primarily calcium carbonate ($CaCO_3$) so called calcareous shale or shaley limestone (Luino et al., 2024), and clays of different percentages. Marl clay consist from 30–70% calcite, and around 20–50% clay, with occasional traces of organic matter, silt, or sand (Arifuzzaman et al., 2017; Jiang & Mokhtari, 2019; Sadeghi et al., 2020).

For millennia, clay minerals have been widely used with numerous applications. Their medicinal properties have long been exploited in the pharmaceutical and biomedical industries, including in pelotherapy (mud therapy).Their role in drug delivery and tissue engineering enhances their potential (Nomicisio et al., 2023). Some types of clay possess antibacterial characteristics and can effectively treat wounds, soothe irritated skin, and stop bleeding (Davies & Behroozian, 2020) because interactions of clay minerals with microorganisms (biogeochemical cycling of elements and formation of ore deposits) are ubiquitous and have great environmental and economic significance. The presence of bacteria in clay affects the chemical, electrical, and mechanical behaviors of clay. This phenomenon has attracted increasing interest over recent decades (Fomina & Skorochod, 2020; Dong et al., 2022; Joo et al., 2023).

Marl is a geophagic clay or kaolin clay type. The ingestion of clay or soil is a prevalent practice among animals and humans, being especially more common in children and among young pregnant women. Marl is a potential source of heavy metals (Co, Cr, Ni, Cu, Zn, Ca, Fe, K, Na, and Cu) (Kortei et al., 2019; Ali & Naser, 2021; Bonglaisin et al., 2022).

The microbiology of geophagic clays is an area where there is relatively scarce information (Bisi-Johnson et al., 2013). There has long been a close interaction of microbes with clay but the complexities of this jointly evolving relationship have only been addressed by scientists in a fragmentary way. Clearly, this relationship requires systematic research by clay scientists and microbiologists (Fomina & Skorochod, 2020). Bacteria can interact with clay minerals and alter them: dissolution, refinement and transformation, reduction of trace elements incorporated in the clay minerals and uptake of trace elements from these minerals, e.g., by the production of siderophores and chelators and enhancement or reduction of absorbance of trace elements on clay minerals. In addition, bacteria can influence layer charge, cation exchange capacity (CEC), and exchangeable cations (Mueller, 2015; Clark & Sharma, 2025).

Microorganisms are ubiquitous on the Earth's surface and near subsurface (Chan et al., 2019).They are by far the most abundant living beings, and have been on the planet much longer than other life forms. They, in great proportion, live in mineral-rich substrate playing key active roles in the biosphere, particularly in the areas of element biotransformations and biogeochemical cycling, metal and mineral transformations, decomposition, weathering, soil and sediment formation (Gadd, 2010; Cuadros, 2017).

Materials and methods

Thirty dry marl clay specimens were collected from Mosul city during the period between October 30 to 1 December 2023. The marl clay (Fig. 1) was analyzed with X-ray diffraction (XRD) and the pH of the marl was analyzed by pH meter. The specimens were crushed and ground directly in the laboratory of the Biology Department, College of Science, Mosul University, the dilutions 10^{-1}–10^{-5} were prepared, the pour-plate technique of cultivation was performed, enumerations were employed on three replicate agar media to each dilution. This media included nutrient agar and Tryptone soy agar (Scharlau made in Spain, European Union). The pour-plate technique (Clark, 1967; Madigan et al., 2021) allows the creation of both aerobic and anaerobic conditions. This method helps to provide a range of environments wherein the surface of the agar supports aerobic bacterial growth, while deeper layers can create anaerobic conditions, allowing the cultivation of a variety of bacterial species under different oxygen conditions. The cultures were incubated for 1–2 weeks.

The pure cultures of bacterial isolates were identified using morphological characteristics and 16S rRNA. Molecular characterization of bacterial isolates of marl clay by genomic DNA was performed using the prestoTM Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan). The 16S rRNA fragment was amplified by using 16S rRNA universal primers for most bacteria (Shen, 2019) Forward 27F AGA

GTTTGATCMTGGCTCAG and Reverse 1522R AAGGAGGTGA TCCARCCGCA (Macrogen, Korea). Forward and reverse DNA sequencing reactions were performed using a Go Taq® G2 Green Master Mix (USA country of origin).

Fig. 1. Marl clay

The normal condition for 16S rRNA amplification number of steps. Temperature and incubation time: 1 – denaturation 94 °C, 2 minutes; 2 – subsequent denaturation 94 °C, 1 minute; 3 – annealing 55 °C, 30 seconds; 4 – extension 72 °C, 30 seconds; 5 – extension 72 °C, 10 minutes (Persing, 2016; Khaleel et al., 2023a). The PCR implication was performed by Sensoquest Labcycler Gradient (PCR Thermocycler) Germany. Agarose Gel Electrophoresis – Consort Power EV243 – Belgium in College of Science, ladder 100 bp Company TransGen Biotech Co., Ltd., China. 2% (tris-borate-EDTA 10x TBE solution Bio Basic Canada INC) TAE. Agarose gel (Scharlau, Spain, European Union), stained with gel stain red safe Europe, Nippon Genetics Co., Ltd. Japan. Loading volume – 5 µL. Sequences performed by the Sanger method Psomagen sequencing company (Maryland, USA). The Nucleotide Basic Local Alignment Search Tool (BLASTn) program was used to search for homology to the input sequence against entire sequences which are available on the sequence NCBI GenBank database (Khaleel et al., 2023; Younis & Faisal, 2024).

The Neighbour Joining method was employed to elucidate the evolutionary history (Saitou & Nei, 1987). The optimal tree is presented. Above the branches of the tree we give the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to construct the phylogenetic tree. We calculated the evolutionary distances using the Maximum Composite Likelihood method (Tamura et al., 2004) and these are in the units of the number of base substitutions per site. This analysis used 51 nucleotide sequences (Fig. 5). All doubtful positions were deleted for each sequence pair (pairwise deletion option). Ther final dataset included 982 positions in total. We carried out the evolutionary analyses in MEGA11 (Tamura et al., 2021) and iTOL v6: "Interactive Tree of Life" – https://itol.embl.de.

Results

The X-Ray diffraction (XRD) analyses model ADX-2700 USA, current 30 mA, voltage 40 kV in Bagdad University, type of X-ray beam: monochromatic, source of X-ray: cupper Kα, filter element nickel, scatter slit 1 degree, divergence slit 1 degree, wave length: λ = 1.541 A (Fig. 2) showed that the untreated marl mainly contained clay minerals: kaolinite, montmorillonite, palygorskite, quartz, and high contents of calcite, while some marl samples contained dolomite and chlorite.

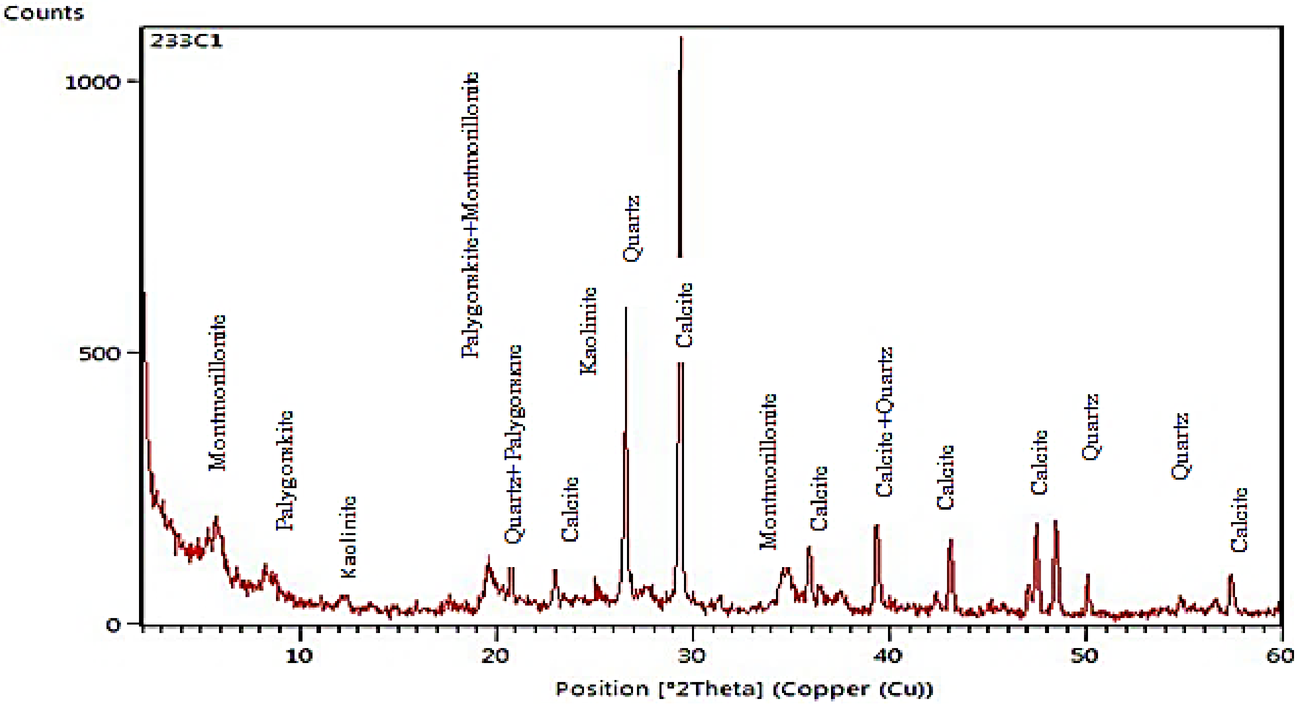

Fig. 2. X-ray diffraction (XRD) analysis of marl clay

The marl clay samples had bacterial counts of between 86.3 × 10^2 CFU/g, while dilution 10^{-1} showed overgrowth (Fig. 3) and 10^{-3}, 10^{-4}, 10^{-5} showed very low numbers of bacterial colonies.

Identification of isolated cultures was carried out using 16S rRNA. The genomic DNA was isolated from the 62 selected marl bacterial isolates and their 16S rRNA genes amplified resulting in amplicon of size 1495 bp for *Streptomyces* as shown in Figure 4.

Polymerase Chain Reaction (PCR) amplification of 16S rRNA gene sequences of the marl bacterial isolates was subjected to the BLAST similarity search by 16S rRNA gene sequence analysis. Following NCBI blasting identified at 90–99% identity for most isolates. The bacterial isolates were identified as Actinobacteria including *Streptomyces*, *Arthrobacter*, *Micrococcus*, *Janibacter*, *Kocuria*, *Saccharopolyspora*, *Amycolatopsis*, Betaproteobacteria including (*Vario-*

vorax), Firmicutes including *Paenibacillus* and Gammaproteobacteria including *Pseudomonas*, Table 1.

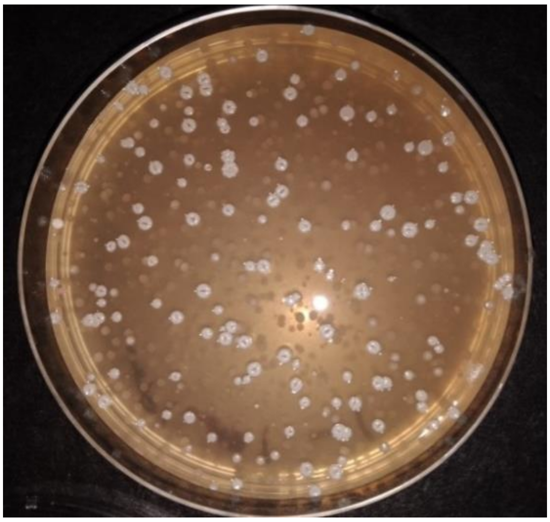

Fig. 3. The microbial colonies in dilution 10^{-1}

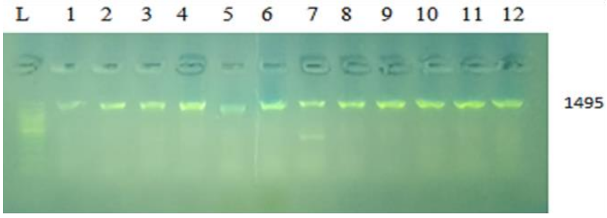

Fig. 4. Agarose gel electrophoresis of 16S rRNA gene bands (1495 bp) for some marl clay bacteria represent *Streptomyces* sp.: lane L, ladder (100-bp DNA ladder); lanes 1–12 samples positive results at Volte 50 and 35–40 minutes

The 16S rRNA sequences for all bacterial isolates identified in this study were submitted to Gene Bank, with the following accession numbers: PP384391.1, PP384319.1, PP380193.1, PP380192.1, PP380191.1, PP380177.1, PP380176.1, PP380170.1, PP380164.1, PP380160.1, PP380158.1, PP380156.1, PP380151.1, PP380150.1, PP380148.1, PP380147.1, PP380146.1, PP380143.1, PP380142.1, PP380141.1, PP380139.1, PP380138.1, PP380137.1, PP380134.1, PP380128.1, PP380126.1, PP380096.1, PP380091.1, PP129647.1, PP126125.1, PP125910.1, PP125841.1, PP125836.1, PP125835.1, PP125832.1, PP125828.1, PP125825.1, PP125823.1, PP125805.1, PP125803.1, PP125802.1, PP124605.1, PP124588.1, PP124585.1, PP117928.1, PP117079.1, PP112177.1 and PP112105.1, PV707140.1, PV701143.1. All these have been deposited in Gene Bank www.ncbi.nlm.nih.gov/genbank.

The 16S rRNA sequence of the bacterial marl isolates was aligned with programs MEGA11 and iTOL v6: Interactive Tree of Life, https://itol.embl.de.

The 16S rRNA gene sequences of marl bacteria strains were determined and compared. A phylogenetic tree (Fig. 5) was constructed using the 16S rRNA sequences of marl bacteria strains. The phylogenetic tree root contains 51 leaves.

Discussion

The results our study of marl compositions applied with (XRD) (Arifuzzaman et al., 2017) were interpreted by a geology expert at the University of Mosul, Iraq. The marl pH is 7.5–8.0.

The most abundant phyla of marl clay were Actinobacteria, Betaproteobacteria, Firmicutes and Gammaproteobacteria, without any findings of pathogenic bacteria and Enterobacteriaceae. According to Saadouli et al. (2022) the most abundant genera in extreme environments are *Streptomyces*, *Kocuria* and *Arthrobacter*.

Actinobacteria are widespread in nature and can be isolated from various natural habitats (Yadav et al., 2018). Also the most abundant actinobacteria were *Streptomyces*, according to Clark et al. (2019), Law et al. (2019), Pacios-Michelena et al. (2021) and Omar et al. (2022). This genus of bacteria represents the major antibiotic producing bacteria, with biotechnological potential, producing anticancer effects and biocontrol. Nocardioides belong to *Nocardia* and also represent an important source for natural products such as polyketide, nonribosomal peptide, and terpenoids (Barka et al., 2015; Männle et al., 2020). They were identified in marl clay (Li et al., 2019).There have been interactions between actinobacteria and mineral clay, which include formation, transformation of clay mineral and elements cycles.

Table 1
The number of bacterial isolates and their percentage of isolation

Bacteria isolates	Numbers	%
Amycolatopsis roodepoortensis	1	2
Arthrobacter agilis	1	2
Janibacter terrae	1	2
Kocuria rhizophila	1	2
Kocuria sp.	1	2
Micrococcus antarcticus	2	3
Nocardioides albus	2	3
Paenibacillus lautus	1	2
Pseudomonas oryzihabitans	1	2
Saccharopolyspora endophytica	1	2
Streptomyces aquilus	1	2
Streptomyces bellus	1	2
Streptomyces buecherae	1	2
Streptomyces caeruleatus	12	19
Streptomyces canus	1	2
Streptomyces cinereus	1	2
Streptomyces durmitorensis	1	2
Streptomyces galilaeus	3	5
Streptomyces griseocarneus	2	3
Streptomyces griseus	2	3
Streptomyces kanamyceticus	2	3
Streptomyces lincolnensis	1	2
Streptomyces monticola	1	2
Streptomyces muensis	1	2
Streptomyces ossamyceticus	1	2
Streptomyces plumbiresistens	1	2
Streptomyces praecox	1	2
Streptomyces pseudovenezuelae	1	2
Streptomyces sp	11	18
Streptomyces staurosporininus	3	5
Streptomyces thermospinosisporus	1	2
Variovorax soli	1	2

Microbes in such environments were found to be involved in the formation of minerals including dolomite (Alibrahim et al., 2019). *Paenibacillus* and *Streptomyces* play important roles in microbial carbonization by induced calcium carbonate precipitation (Yu et al., 2022).

The kaolinite presence enhanced the processes of acetate assimilation while decreasing catabolic activity in the bacterial metabolism (Fomina & Skorochod, 2020). Also kaolinite limits bacterial growth probably due to its high aluminum concentration, which damages bacterial membranes (McMahon et al., 2016). Bacteria-induced calcium carbonate precipitation takes place (Anbu et al., 2016). According to García et al. (2016), Actinobacteria, Firmicutes, and Proteobacteria, the most abundant phyla in the environment contain calcium carbonate. A nearly neutral pH of 7-8 in marl is optimal for bacterial enzymatic activity. The chemical composition of marl is the source of bacterial macronutrients and micronutrients.

The marl clay studied was without pathogenic bacteria and Enterobacteriaceae because the marl clay contained montmorillonite in its chemical structure. According to Luo et al. (2011), Hossain et al. (2023), Mokhtar et al. (2023), montmorillonite and chlorite are highly active towards pathogenic gram-positive and gram-negative bacteria and can significantly inhibit and eradicate pathogens and harmful biofilms. Dolomite has antibacterial activity against pathogenic gram-positive and gram-negative bacteria.

The consistent detection of 16S rRNA (Fig 3) bands at the expected size confirms the reliability of this method for bacterial identification. This approach provides a robust foundation for downstream sequencing and phylogenetic analysis.

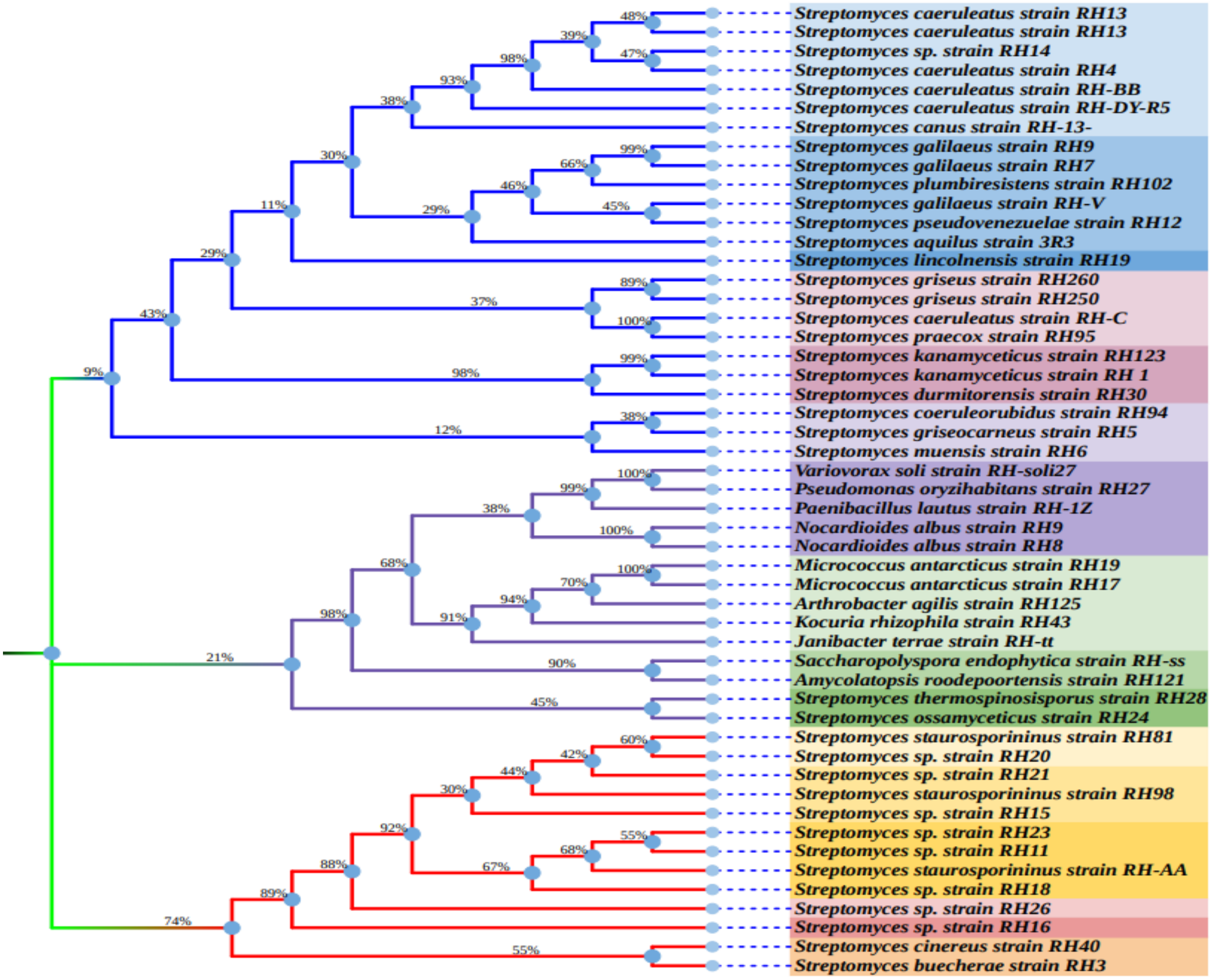

Fig. 5. A phylogenetic tree at scale length 0.05 showing the evolutionary relationships of bacterial taxa isolated from marl clay

The phylogenetic tree of marl bacteria shows the close relation between *Streptomyces* species. The bacteria form a cluster at Maximum similarity value of 100% for the strains *Streptomyces caeruleatus* RH-C and *Streptomyces praecox* RH95, 100% for *Variovorax soli* RH-soli27 and *Pseudomonas oryzihabitans* RH27, 100% for *Micrococcus antarcticus* RH17 and *Micrococcus antarcticus* RH19, 100% for *Nocardioides albus* RH8 and *Nocardioides albus* RH9, 99% for *Streptomyces kanamyceticus* RH1 and *Streptomyces kanamyceticus* RH123, 99%, for *Streptomyces galilaeus* RH7 and *Streptomyces galilaeus* RH9, 90% for *Saccharopolyspora endophytica* RH-ss and *Amycolatopsis roodepoortensis* RH121, 89% for *Streptomyces griseus* RH250 and *Streptomyces griseus* RH260. Other strains showed low similarity; *Streptomyces staurosporininus* RH81 and *Streptomyces* sp. RH20 60%, *Streptomyces* sp. RH23 and *Streptomyces* RH11 55%, *Streptomyces buecherae* RH3 and *Streptomyces cinereus* RH40 55%, *Streptomyces caeruleatus* RH4, *Streptomyces* sp. RH14, *Streptomyces caeruleatus* RH13, 47%, 48%, 39%. Marl strains branched with each other and lined in same clade.

Conclusion

The chemical composition of marl clay may be the stimulus for species richness and diversity of marl bacteria. The marl clay was free of pathogens, which means the marl clay is not a contaminated environment. The marl clay represents a high selectivity environment for growth and diversity of the *Streptomyces* genus. The most abundant marl clay bacteria were *Streptomyces*. This genus has many clinical and biotechnology applications.

The study concluded that it is possible to use this material as food because it is free from enteric bacteria and contains nontoxic components. However, further experiments should be conducted in the future to confirm its potential use as a beneficial food or medical material.

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