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THE SPATIAL VARIABILITY AND DIVERSITY OF HETEROTROPHIC BACTERIA AND EVALUATION OF TWO CULTURE MEDIA FOR ISOLATION OF BACTERIA FROM THE SEDIMENT OF LAKE ZENGENA

By

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JULY, 2020

BAHIR DAR, ETHIOPIA

The spatial variability and diversity of heterotrophic bacteria and evaluation of two culture media for isolation of bacteria from the sediment of Lake Zengena

MSc Thesis Submitted to the Department of Biology in Partial Fulfilment of the Requirements for the Award of Master of Science Degree in Biology (Applied Microbiology)

By

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July, 2020

Bahir Dar, Ethiopia

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I hereby certify that I have supervised, read and evaluated this thesis/dissertation titled "The spatial variability and diversity of heterotrophic bacteria and evaluation of two culture media for isolation of bacteria from the sediment of Lake Zengena" by Belaynew Kelkay prepared under my guidance. I recommend the thesis/dissertation be submitted for oral defense.

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Approval of Dissertation/thesis for defense result

As members of the the board of examiners, we examined this dissertation/thesis entitled "The spatial variability and diversity of heterotrophic bacteria and evaluation of two culture media for isolation of bacteria from the sediment of Lake Zengena" by Belaynew Kelkay. We hereby certify that the thesis/dissertation accepted for fulfilling the requirements for the award of the degree of Master of Science Degree in Biology (Applied Microbiology)

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DECLARATION

I Belaynew Kelkay, student of the applied microbiology of Bahir Dar University, aware of my responsibility of the penal law, declare and certify with my signature that my thesis entitled: the spatial variability and diversity of heterotrophic bacteria and evaluation of two culture media for isolation of bacteria from the sediment of Lake Zengena is my own work. I have faithfully and accurately cited all my sources, including books, journals, handouts and unpublished manuscripts, as well as any other media, such as the Internet, letters or significant personal communication.

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ACKNOWLEDGEMENTS

I am greatly indebted to my major advisor, Dr. Baye Sitotaw for his valuable advice, excellent guidance; right direction from the proposal development, supervision in my laboratory work, constructive comments and for the support of major components of R2A media by him alone that made this works successful.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BCC	Bacterial Community Composition
DO	Dissolved Oxygen
HPC	Heterotrophic Plate Count
MR - VP	Methyl Red and Voges Proskauer
NTU	Nephelometric Turbidity Units
OTU	Operational Taxonomic Units
R2A	Reasoner and Geldreich agar
SIM	Sulphide Indole Motility
SPSS	Soft Were Package for Social Science
TDS	Total Dissolved Solids
TSA	Tryptone Soy Agar

ABSTRACT

Sediment is a special habitat in freshwater ecosystem and harbors a variety of heterotrophic bacterial communities. Studying bacterial diversity has an important role for a better understanding of the functioning of aquatic systems however, studies on biodiversity have mainly focused on macroorganisms and little attention have been directed toward microorganisms. The objective of this study was to investigate the diversity and spatial variability of heterotrophic bacteria and evaluate efficacy of two culture media on isolation of bacteria from the sediment of Lake Zengena. Sediment samples were collected from two sampling sites of Lake Zengena via corer sampling device aseptically. Sediment samples were plated in triplicate aerobically on TSA media for three days and R2A media for five days at 25^oC following a serious of serial dilution technique. A total of 200 isolates were picked, purified and grouped into 73 Operational Taxonomic Units (OTUs) according to Bergey's manual of determinative bacteriology from the basis of the result of gram stain, colony morphology and biochemical tests. Diversity indices were used to measure the diversity between sites and media type. Analysis of variance was used to test the mean difference of Operational Taxonomic Units (OTUs) abundance between sites and media. Seventeen of the 73 OTUs were common for both sites and had 61(30%) isolates and fourteen OTUs were common for both media types and represented by 53 (26.5%) isolates. There were 29 and 27 unique OTUs in site one and two respectively. Moreover, R2A media had 29 unique OTUs while TSA media had 22 unique OTUs and variation in both sites and growth media indicate different bacterial abundance. Members of the cultured bacterial community in this study were identified into 9 genera belonged to 4 phyla: Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. There was a statistically significant difference in OTUs abundance between sites at ($p=0.014$) and media type at ($p=0.02$). OTUs diversity was higher in R2A media site one ($H=3.269$) than TSA media site one ($H=3.082$). Furthermore, OTUs diversity was higher in R2A media site two ($H=3.167$) than TSA media site two ($H=3.054$) due to varied composition of organic compounds in the growth media. The result of this study revealed that sediment bacterial communities in Zengena Lake were diverse and mainly composed of phyla that are typical to freshwater sediment. Future work needs to identify each OTUs at the species level to understand the ecological role of the bacterial community.

Key words: Bacteria, Culture-based method, Diversity, Lake Zengena, Sediment

1. INTRODUCTION

1.1. Background of the study

Freshwater lake ecosystems are among the most valuable and widely used natural systems worldwide and they give significant ecosystem services to many millions of people (Zhang *et al*., 2018). Freshwater environments provide excellent habitat for microorganism and they differ from other marine environments in many ways including salinity, average temperature, depth and nutrient content. They are highly variable in the resource and condition available for microbial growth. A large number of microorganisms in a body of freshwater lake generally indicate a high nutrient level in the water (Aryal *et al*., 2015).

Sediment is a special habitat among the aquatic ecosystem and the numbers of microorganisms are much divers than the corresponding water part. Sediment sustains a matrix of complex nutrients and solid surface for microbial growth by receiving deposition of microbes and organic matter from the upper water layer (Zinger *et al*., 2011). Most freshwater sediment is quite diverse ecosystem, which give rise to different environmental niches even on a millimeter scale. They, therefore, entertain highly complex microbial communities concerning species composition and metabolic activity (Ren *et al*., 2019).

The microbial community composition and the process mediated by microbes in aquatic sediments are subjected to several controlling factors. A study showed that bacterial activity in lake sediment is greatly affected by the amount of organic matter and nutrient elements in addition to other factors such as pH and redox potential (Jiang *et al*., 2006). Organic matter that accumulates at the bottom of the lake can be turned into mineral and gas by microorganisms thereby releasing nutrients into the water body and the atmosphere. The physicochemical and biological processes in lake profile maintain the diversity of microorganisms by providing suitable habitat that enhances their metabolic activities. Bacterial communities from nutrient rich sediment have found to display a high range of catabolic response to allochtonous carbon sources because of their ability to use different types of substrates, but nutrient poor lake sediment showed reduced efficiency. Therefore, depending on the nutritional status of inland waters (oligotrophic, mesotrophic and eutrophic), the sediment may not have the same organic matter content and consequently may have a different microbial community (Torres *et al*., 2011).

Microbial biomass and activity are fundamental variables in determining the importance of microorganisms in a particular environment. In the freshwater ecosystem, microbial communities harbored in the sediment play a key role in biogeochemical cycling due to their involvement in the transformation of elements, organic matter demineralization and biochemical degradation (Holmer and Storkholm, 2001). Bacterial activity and biomass are generally high near the surface of sediment but decrease with depth. Regarding the high abundance of bacteria in sediment, the bacterial activity in that respect is often low. This implies that the cell specific activity of sediment bacteria is low or that only a small fraction of the bacterial community is metabolically active (Fischer *et al*., 2002). In addition to the ecological role in sediment ecosystems, bacteria are nowadays gaining attention for their byproducts and are being extract for the production of useful chemicals (Gurung *et al*., 2013).

In their natural habitat, microorganisms coexist in mixed communities, the complexity of which is specific to a particular environment. Identification of these bacterial population into the different group have fundamental importance to microbial systematics and scientists involved in many other areas of applied research and industry. Accurate identification requires a system of ordering organisms into groups as well as an unambiguous nomenclature for naming them (Truper and Schleifer, 2006). Culture based studies and molecular techniques are the common methods for identification and characterization of ecologically significant prokaryotes. Although efforts have been made to reveal the microbial ecosystems in freshwater sediment based on traditional cultivation methods, about 0.001 to 0.25% in the sediment of the total cell count in the environmental sample can be cultured (Tamaki *et al*., 2005). Hence, the traditional cultivation method cannot be directly applied to the whole microbial diversity analyses. However, cultivation based study remains important, because the ecological role of prokaryotes in a natural environment can assessed only when they are successfully cultured and characterized.

Culturing of an organism depends on essential macronutrients and growth factors in isolation media (Bollman *et al*., 2007) however, the majority of culture media used to date have been nutrient-rich. These conditions may favor the growth of faster-growing bacteria at the expense of slow-growing species, some of which thrive in nutrient poor environments (Connon & Giovannoni, 2002). Thus, efficient cultivation of heterotrophic bacteria from different biotopes of oligotrophic habitats requires growth media that contain not only low nutrient concentrations but also mineral and vitamin components.

The important features of bacterial community in particular niche are characterized by the number of species present and their numerical compositions, which is known as bacterial diversity. Studying bacterial diversity have an important role in understanding the functioning of aquatic systems, provides potentially descriptive information about the degree of contamination and trophic status (Sigee, 2005). To compare the bacterial diversity from samples of microorganisms, varieties of approaches have used. Shannon-Weaver and Simpson diversity indices are widely used in bacterial diversity measurement by means of operational taxonomic unit (OTU). Bacterial diversity index depends on both specie richness and the evenness (Kim *et al*., 2017).

The microbial diversity in freshwater sediments ware studied in different parts of the world as well as in Africa. For instance, in Lake Kasumigaura in Japan (Tamaki *et al*., 2005); Poyang Lake in China (Kou *et al*., 2016); in Lake Pamvotis Greece (Touka *et al*., 2018), plateau freshwater lakes (Zhang *et al*., 2015) and in sediment of different Kenyan Lakes (Baringo and Victoria) (Dadheech *et al*., 2009). These study shows that the freshwater lake sediment contains a variety of bacteria such as Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Nitrospirales, Acidobacteria, and Planctomycetes. Ethiopia possesses different freshwater Lake including Lake Zengena but to my knowledge, information about bacterial diversity in the sediment ecosystem is limited. Thus, this study conducted to identify diversity and abundance of heterotrophic bacteria from sediment samples of Lake Zengena by culture based technique.

1.2. Statement of the problem

Studies on biodiversity and its relations to ecosystem structure and functions have mainly focused on macroorganisms and little attention have directed toward microorganisms (Shen *et al*., 2014). However, studying microbial diversity has become an important issue due to their role in energy and matter transformation. Knowledge about bacterial community structure and diversity is vital to understand the relationship between environmental factors and ecosystem functions. Such knowledge used to evaluate the effect on ecosystems of environmental stress and perturbations like pollution, agricultural exploitation and global changes. Likewise, in Lake Zengena some researcher conducted their studies in terms of physicochemical characteristics of the lake water (Goraw Goshu, 2007), community based ecotourism development (Zemenu Bires, 2017) and floristic diversity (Desalegn Tadele *et al*., 2014). In contrast, lake bacterial diversity remains untouched. Thus, this study intended to investigate the diversity of heterotrophic bacteria from the sediment of Lake Zengena using culture dependent technique.

1.3. Objectives of the study

1.3.1. General objective

The general objective of this study was to investigate the diversity and spatial variability of heterotrophic bacteria and evaluate efficacy of two culture media on isolation of bacteria from the sediment of Lake Zengena.

1.3.2. Specific objectives

- To investigate the diversity of heterotrophic bacteria from the sediment of Lake Zengena using culture based technique
- To determine special variability on the bacterial diversity of Lake Zengena sediment
- To evaluate the effect of media type on the diversity of bacteria isolated from the sediment of Lake Zengena

1.4. Significance of the study

Microbial diversity is the key to human survival and economic wellbeing and provides a huge reservoir of resources that we can utilize for our benefit. Microbes are able to recycle nutrients, produce and consume gases that affect the global climate, destroy pollutants, treat our wastes and they can used for biological control of plant and animal pests. Since sediment contains diverse microbial communities, studying them also indirectly indicates their important role in ecosystem functioning and processes such as biogeochemical cycles and nutrient transformation. Thus, this study has an important indication for the optimization of integrated ecosystem assessment of freshwater lake sediment and provides interesting information for the conservation of bacterial diversity in the lake ecosystem.

1.5. Scope of the study

This study covers mainly the heterotrophic bacterial diversity and abundance using culture based techniques. Geographically, this study was covered Lake Zengena sediment, which is located in Banja Woreda, Awi Nationality Administration. Particularly the research was focused to identify heterotrophic bacterial diversity in two sediment sites.

1.6. Limitations of the study

Studying heterotrophic bacterial diversity in the deepest part of the lake sediment was the main limitation of this work. Additionally, the study was limited to anaerobic cultures, the physiological characteristics of the isolates in terms of pH, temperature and salt, molecular characterization and identification to species level by using more biochemical tests.

2. LITERATURE REVIEW

Microbial diversity that we see today is the result of nearly billions of years of evolutionary change. This diversity can viewed in several ways such as cell size and morphology, physiology, motility, pathogenicity, adaptation to the different environmental conditions and mechanism of cell division (Madigan *et al*., 2012). Microbial diversity at the species level consists of two components. The first component is the total number of species present, which is species richness, or it refers to the variation among species. The second component is the occurrence of individuals among these species referred to as evenness. One major difficulty is that evenness is unknown in bacterial systems because individual cells very rarely identified to the species level (Harpole, 2010).

The diversity of the Operational Taxonomic Unit (OTU) or even communities may give us a better estimation of the functioning of an ecosystem. Species diversity is a community parameter that pertains to the degree of stability of that community. Any diversity index must measure the diversity of information kept within the community. Well-structured communities that cover a particular level of diversity are stable (Yannarell and Triplett, 2005). If some kind of stress introduced to this community, the stability may fail and the diversity will change.

2.1. The diversity heterotrophic bacteria in freshwater lake sediments

Freshwater bacteria are a very different group of prokaryote organisms, varying in their morphology, physiology and ecological preferences. Bacteria are able categorized into various natural assemblages based on characteristics such as cell shape, spore-forming abilities, oxygen requirement for growth and other cellular characteristics. Most bacteria in freshwater environments are Gram-negative, with few Gram-positive representatives (Sigee, 2005).

The majority of freshwater microorganisms are heterotrophic which obtain their energy by using complex organic compounds as a source of carbon. Bacteria are widespread throughout the freshwater environment, forming extensive pelagic and benthic populations in lakes. Even if some bacteria, such as *Escherichia coli*, are present as accidental contaminants, most freshwater bacteria have close physiological adaptations to their environment thus; strict anaerobes are confined to anoxic sediments. In some cases, particular organisms (e.g. *Bacillus pituitans)* have a very restricted habitat range, while others such as *Pseudomonas aeruginosa* are very widespread being routinely found in freshwater, soil and aerial samples (Sigee, 2005).

Bacterial diversity is the key to human survival and economic security as it provides a variety and reservoir of resources that can be utilized by humans for their benefits (Berdy, 2005). Diverse microbes mainly bacteria play an important role in biological products such as antibiotics, drugs, enzymes, herbicides and growth promoters useful to humans. The study of bacterial diversity is also important to solve new and emerging challenges like diseases and to give a boost to biotechnology. Exploration, evaluation, and exploitation of bacterial diversity are essential for scientific, industrial and social development (Bhat, 2013).

Previous studies based on both culture-dependent and culture-independent methods have been able to identify a broad range of bacteria phylum in freshwater lake sediments. These include Proteobacteria (β-, γ-, δ- and α-Proteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospirales, Planctomycetes and Verrucomicrobia (Kou *et al*., 2016; Tamaki *et al*., 2005 and Touka *et al*., 2018, plateau freshwater lakes (Dadheech *et al*., 2009; Zhang *et al*., 2015).

2.1.1. Phylum Proteobacteria

The phylum Proteobacteria is a group of Gram-negative bacteria encompassing the majority of recognized agriculturally, industrially and medically relevant organisms and therefore is the most studied group of the bacterial phyla. The phylum was formally established using phylogenetic analysis of 16S rRNA gene sequences by Garrity *et al*. (2005) with five constituent classes. This phylum contains all known Gram-negative bacteria like Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria (McAllister *et al*., 2011). This group of bacteria, considered as purple bacteria that encompass a very complex assemblage of phenotypic and physiological attributes including many phototrophs, heterotrophs and chemolithotrophs. The proteobacterial group is of great biological significance as it includes a large number of known human, animal and plant pathogens (Gupta, 2000).

2.1.2. Phylum Actinobacteria

This phylum comprised Gram-positive organisms with a high G+C content constitutes one of the largest phyla within the bacteria (Stackebrandt and Schumann, 2006). The different genera in this phylum show vast diversity in terms of their morphology, physiology and metabolic abilities (Stach and Bull, 2005). The morphologies of Actinobacteria species vary from coccoid (e.g. Micrococcus) or rod-coccoid (e.g. Arthrobacter) to fragmenting hyphal forms (e.g.

Nocardia) or differentiated branched mycelia (e.g. Streptomyces). Spore formation is not ubiquitous among Actinobacteria even if it is common. The species of this group also exhibit plentiful physiological diversity, as evidenced by their production of a variety of extracellular enzymes and metabolic products like antibiotics (Chater *et al.*, 2010). Molecular studies revealed that members of the Actinobacteria are common and often numerically important components in a variety of freshwater habitats (Zwart *et al.*, 2002).

The freshwater Actinobacteria are present in the bottom water (hypolimnion) and sediment of Lake Ecosystem (Boucher *et al.*, 2006), but their abundance often decreases with decreasing oxygen concentrations (Allgaier and Grossart, 2006). This high level of abundance in the epilimnion has proven to be consistent across lake types, as the Actinobacteria are common among oligotrophic (Humbert, 2009), mesotrophic (Zeder, 2009) and dystrophic (Newton *et al.*, 2006) lakes.

2.1.3. Phylum Bacteroidetes

The phylum Bacteroidetes are found in various environments including freshwater sediment, marine habitats and soda lakes (Humayoun *et al.*, 2003), indicating their divers in nature. Bacteroidetes are commonly known to dominate particle-associated bacterial communities of freshwaters and found at depth in lakes, where they may degrade recalcitrant macromolecules (Yannarell and Kent, 2009). They also known to be abundant and play an important role as a major degrader of organic compounds in saline and freshwater environments (Krieg *et al.*, 2010).

This phylum consists of a Gram-negative rod that exhibits enormous phenotypic and metabolic diversity (Newton *et al.*, 2011). Within the Bacteroidetes there are three distinct classes including Bacteroidetes, Flavobacteriales and Sphingobacteriales. In Lake Epilimnia, biopolymers degradation can occur mainly due to these bacteria. The Lake Bacteroidetes abundance increases following cyanobacteria blooms and can sometimes account for more than 40% of the total bacterial biomass in a lake (Zwart *et al.*, 2002).

2.1.4. Phylum Firmicutes

This phylum mostly contains a Gram-positive cell wall structure. The Firmicutes phylum includes all Gram-positive bacteria, but scientists recently defined them to be one of a central group of related forms called the low-G+C group. Firmicutes play an important role in the fermentation processes like beer, wine and cider spoilage. The group typically divided into

the *Clostridia*, which are anaerobic, the *Bacilli*, which are obligate or facultative aerobes (Wolf *et al*., 2004). Spore forming Firmicutes contain both autotrophs and heterotrophs, many of which used as classical organisms for different studies. Chemolithoautotrophs include a variety of hydrogen oxidizing bacteria that grow by reducing sulfur, sulfate or nitrate (Chivian *et al*., 2008) and others grow by oxidizing minerals, including ferrous iron (Galperin, 2013).

2.2. Spatial diversity of bacteria in sediments

Understanding the mechanisms that generate and sustain biodiversity is vital to predicting ecosystem responses to future environmental changes. The difference in community composition with geographic distance is a universal biogeographic pattern observed in communities from all domains of life (Green *et al*., 2004). The spatial distributions of bacterial communities in sediment may be driven by many environmental factors. Thus, understanding the interactions between bacterial distribution and environmental factors is vital for understanding sediment stability and the functioning of freshwater lake ecosystem.

Several studies reveal that bacterial distributions may be spatially predictable rather than random (Ettema and Wardle, 2002). Differences in environmental factors along sediment horizontal gradients largely govern bacterial community composition and diversity like water content (Badin *et al*., 2011), C and N availability (Lin *et al*., 2012), temperature (Redmond and Valentine, 2012) and pH (Lindström *et al*., 2005).

Unique characteristics of bacterial biology like small size and environmental hardiness may prevent bacteria from exhibiting spatial variability. Furthermore, if they are flexible in habitat requirements and physiological abilities, or if they can easily obtain traits through horizontal gene transfer that are necessary for survival in a given habitat, then taxa–area relationship may not expected (Horner-Devine *et al*., 2004).

2.3. Media for cultivation of bacteria from sediments

The majority of culture media commonly used to date have been complex in nutrient contents. It is now thought that these conditions may favor the growth of faster-growing bacteria at the expense of slow-growing species, some of which thrive in nutrient poor environment (Connon & Giovannoni, 2002).

Many bacteria, found in oligotrophic environment, are very slow growing. Growth media with low nutrient contents and long incubation times are essential for the cultivation of such bacteria, with the added advantage that faster-growing members within the mixed populations progressively die off over time, decreasing the bacterial competition. The culture of biofilm bacteria via R2A media for five up to 7 days has revealed increasing colony counts and an increased recovery of isolates with time (Sukhanova *et al*., 2019). Similarly, long-term incubation for up to 28 days has been successful for the isolation of heterotrophic bacteria (Segawa *et al*., 2011).

Some bacteria are frankly resistant to culture in isolation on conventional media. Certain bacteria have fastidious growth requirements including specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere. Kopke et al. (2005) investigated the effect of different substrates and culture conditions on the growth of bacteria from different samples of coastal sediments and found that the several cultivation approaches resulted in the isolation of different groups of bacteria specific to each method.

2.4. Factor affecting bacterial communities in Lake

Understanding the factors determining the bacterial community composition in freshwater ecosystems can potentially help in the assessment of the physical condition of these systems because microbes respond fast to the fluctuation of environmental conditions by particular changes that are detectable physiologically and metabolically. Recent studies in microbiology show the extent to which variation in microbial communities shaped by deterministic processes and how these associates to variation in local environmental parameters such as physicochemical environment, climate, overlying plant community and disturbance regime or evolutionary events (Bandh *et al*., 2019).

The physical properties of water exert important and wide-ranging influence on the biology of freshwater microorganisms through their effects on the nearby aquatic environment. The physicochemical properties of the Zengena Lake show no significant seasonal changes in conductivity, turbidity, total dissolved solids and pH observed (Goraw Goshu, 2007). This may indicate that mixing of the water column due to either internal waves or wind action is absent. The previous study shows the surface water temperature of the lake ranged from 19 to 26 °C, dissolved oxygen from 4 to 10 mg 1^{-1}, turbidity from 5 to 30 NTU and pH from 7.7 to 8.7. The conductivity of water ranged from 55 to 185 µS cm^{-1}, total dissolved solids from 33 to 150 mg 1^{-1} and nitrate from 2.6 to 4.5 mg 1^{-1} No_3 –N.

The abundance of bacteria in the aquatic environment can also depend on numerous factors including the concentration of pathogens, the size of the watershed, human activities, land use management practices and the extent of aquatic plants and sediment (Liang *et al*., 2013). Water bodies partly or fully covered with pastures are more contaminated than those located within forests and cultivated areas (Johnson *et al*., 2010). Zengena Lake is covered with different plant species. A previous study on floristic diversity shows that 50 woody plant species belonging to 31 families found in the lake surrounding (Desalegn Tadele *et al.*, 2014) and these involve in the composition of bacterial diversity. The direct excretions of animals to water bodies are also a source of bacteria (Islam *et al*., 2018). Lake Zengena is rich with a range of wildlife that constitutes birds, reptiles, fish and mammals.

2.5. Methods of studying bacterial diversity

Cultivating bacteria means to put them in conditions fortunate enough to allow their development. These conditions include the definition of physicochemical and metabolic parameters (temperature, pH, salinity and oxygen) which allow the cell for access to an energy source and nutrient. During the culturing of a sample, microorganisms placed in a new environment that carried out on different growth media types (Maier *et al*., 2009).

Approaches for assessing microbial diversity can categorize into two group mainly culture-dependent and culture-independent approaches. Approaches to investigating microbial ecosystems in freshwater sediment based on conventional culture methods are important since, via this approach, the ecological role of the cultivated and characterized prokaryotes can be estimate. However, using this method, only minute fractions of the bacteria get cultivated, leaving a vast majority uncultivated. Over the years, significant advancements have made to minimize some of the drawbacks of culture-based methods (Mwirichia *et al*., 2010). However, it is realized that the majority of microbes could not still be enumerated by using cultivation approaches, mainly due to the lack of information on microbial ecology, biology and hence cultivation techniques.

Culture-independent methods based on small subunit rRNA has also used for studies of microbial diversity in freshwater sediment (Altmann *et al*., 2003; Purdy *et al*., 2003). A combination of two or more approaches is likely to provide a more comprehensive picture of bacterial diversity in an environmental sample since there is a likelihood that the drawback of one method could overcome by another method.

2.5.1. Dilution plating and plate counts

Successful isolation and cultivation of bacteria critically depend on the selection of suitable growth media and incubation conditions. The most traditional cultivation method for assessment of microbial diversity is selective and differential plating and following viable counts. Counts of viable (metabolically active) heterotrophic bacteria can readily carried out by plating bacteria on agar plates and counting the number of colonies that develop an isolated colony. Bacterial counts expressed as colony-forming units (CFU), records those organisms that can grow and multiply on the nutrient media (Mwirichia *et al.*, 2010).

This method is fast, cheap and offers information about the active and culturable heterotrophic microbial population. Factors that limit the use of these methods include the difficulties in selecting suitable growth media, provision of specific growth conditions, inability to culture a large number of bacterial species through techniques available at present and the potential for inhibition or spreading of colonies other than our interest. The culturable portion of the microbial community is an important ecological parameter and it is important to assess bacterial activity (Tabacchioni *et al.*, 2000).

2.5.2. Isolation and identification of bacteria

The identification of individual bacterial species involves laboratory culture, isolation and characterization. Identification of bacteria into a different level of classification like families, genera and species is based on a variety of phenotypic characteristics and biochemical attributes (Konneke *et al.*, 2005). The first step in the identification procedure is to accumulate information that pertains to the organism's morphological, cultural and biochemical characteristics.

The phenotypic identification provides direct functional information that reveals what metabolic activities are taking place to aid the survival and growth of the bacteria. Phenotypic methods are accurate and reliable even if having its limitations. However, this technique is solely applicable for cultivable organisms, time consuming and variability of culture due to different environmental conditions may lead to ambiguous results and subjective interpretation (Bosshard *et al.*, 2004).

2.5.3. Bacterial diversity measures

There are many ways to measure diversity and methods vary in the particular aspect of diversity that they measure, their sensitivity to different abundance and their failings. The numbers of existent species and their numerical compositions characterizes the significant features of bacterial communities in certain niche. Different diversity indices used for bacterial diversity measurement using operational taxonomic units (OTUs) (Lozupone and Knight, 2008). OTUs inferred to exist based on sequence data and can defined at different levels of resolution (phylum, class, order, family, genus, and species).

For the study of microbial community diversity, there is no overall covenant on which diversity index is appropriate to use (Hughes and Bohannan, 2004). However, the uses of Shannon-Weaver and Simpson diversity indices have been suggested to measure microbial diversity (Haegeman *et al*., 2013). Shannon-Weaver and Simpson's diversity indices give more inference about the community structure than simple species richness or evenness. A diversity index depends on both species richness and the evenness concerning relative abundances. However, both diversity indices have specific biases. The Shannon-Weaver index seats a greater weight on species richness, while the Simpson index reflects species evenness more than species richness in its measurement (Schloss *et al*., 2009).

Besides, the Shannon-Weaver index measures the average degree of uncertainty in predicting where individual species chosen at random will belong. The value increases as the number of species increases and as the distribution of individuals among the species become even (Lemos *et al*., 2011). On the other hand, the Simpson index indicates the species dominance that considers the probability of two individuals that belong to the same species being randomly chosen and ranges from zero to one.

2.6. Role of sediment bacteria

Lake sediment is important grounds for series of biogeochemical cycling of essential nutrients (carbon, nitrogen and phosphorus) and contaminants (Bouskill *et al*., 2010). Sediment microorganisms especially bacteria play a great role in these critical processes. Bacterial mediated transformations in sediment lead to an active exchange of energy and materials with the water column and intimately unite sedimentary processes with other different aquatic ecosystem function (Ranjard *et al*., 2000; Urakawa *et al*., 2000). Bacterial community composition (BCC) in freshwater lakes has extensively studied partly because of their abilities

in predicting biogeochemical functions. Early studies have shown that lake sediment BCC may affected by physicochemical factors such as pH, temperature, streamflow and nutrient concentrations (Sauvain *et al*., 2014).

Bacteria play a key role in the processes of decomposition of organic matter accumulated in freshwater beaches. Most of the organic matter in aquatic ecosystems consists of compounds of a high molecular weight and polymeric structure mainly proteins, starch, lipids, pectin, cellulose, chitin, nucleic acids or lignin. For heterotrophic bacteria, those high molecular weight biopolymers contain an important source of carbon, nitrogen and energy used for biosynthesis or respiration (Patel *et al.*, 2000). As polymeric molecules are too large to be directly incorporate into bacterial cells, they have to be decomposed by extracellular enzymes into smaller and simpler compounds that can easily diffuse into the periplasmic space (Hopp *et al*., 2002). Many heterotrophic bacteria have genetic and metabolic abilities to produce and control extracellular enzymes which can breakdown and change a large variety of natural polymers in aquatic environments (Mudryk and Skorczewski, 2004).

Besides their ecological role, sediment microorganisms are nowadays also gaining attention for their byproducts. Most aquatic sediment bacteria are a rich source of hydrolytic enzymes such as amylases, lipases, proteases, phospholipase and catalases, which have industrial importance (Keller and Zengler, 2004). Microorganisms have become important as producers of industrial enzymes due to their biochemical diversity and ease with which enzyme concentration may increase by genetic and environmental manipulation (Pandey *et al*., 2000).

3. MATERIALS AND METHODS

3.1. Description of the study area

Lake Zengena is a crater lake found in Banja Woreda of Awi zone at an elevation of 2523m above sea level. The absolute geographical position of Lake Zengena is 10° 54' 50" N, 36° 58' 00"E and the diameter of the lake reaches 930 meters from North to South and 970 meters from East to West. Lake Zengena covers a surface area of about 20 - 25 hectares and a maximum depth of 166 meters in the middle of the lake. The main rainy season ranges from mid-June to mid-October with maximum rainfall occurring between July and August and mean annual rainfall ranging between 1,300 and 1,800 mm. The mean annual temperature ranges between 16 and 20°C (Banja Woreda stastical office, 2020).

The Lake is circular and it is a closed basin probably formed through volcanic eruption. Like the other crater lakes, it is circular and surrounded by cliff covered with vegetation. There are no permanent rivers which tribute to the lake and no outlet. Lake Zengena is surrounded by forest with different woody plant species (Desalegn Tadele, 2014). The Lake has both exotic and native fish species (Yared Tigabu, 2010) and serves tourism resources (Zemenu Bires, 2017). The map of study and specific location of area is shown below figure 1.

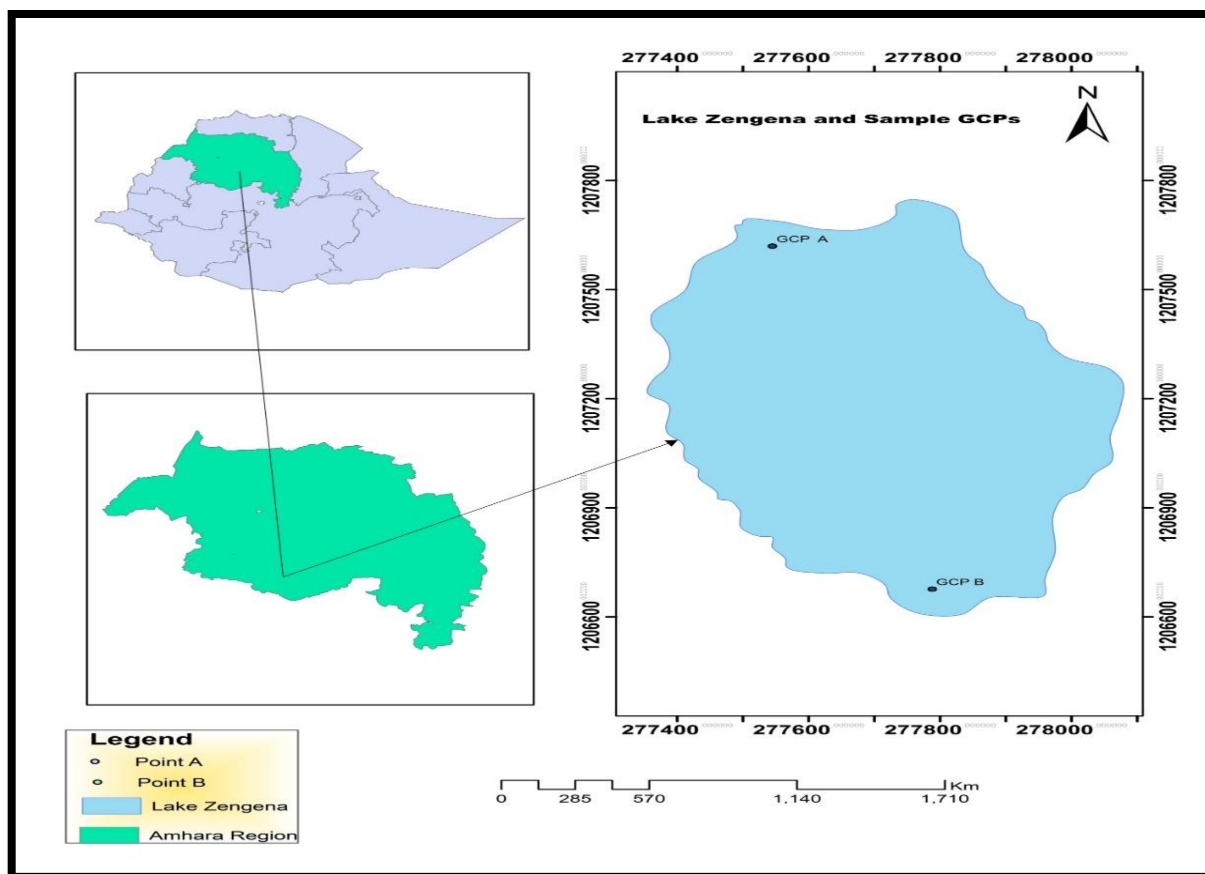


Figure 1: Map of Ethiopia showing the study site

3.2. Study design and period

The experimental study design was conducted to study heterotrophic bacterial diversity and abundance from the sediment of Lake Zengena from January 2020 to June 2020. Experimental procedures were also used for characterization and identification of heterotrophic bacteria. The study was conducted on two sites and two culture media type based on the following design (Table 1).

Table 1: The different culture types indicating both media type and sites

No	Code	Culture type description
1.	S1R2A	Site one and R2A media
2.	S1TSA	Site one and TSA media
3.	S2R2A	Site two and R2A media
4.	S2TSA	Site two and TSA media

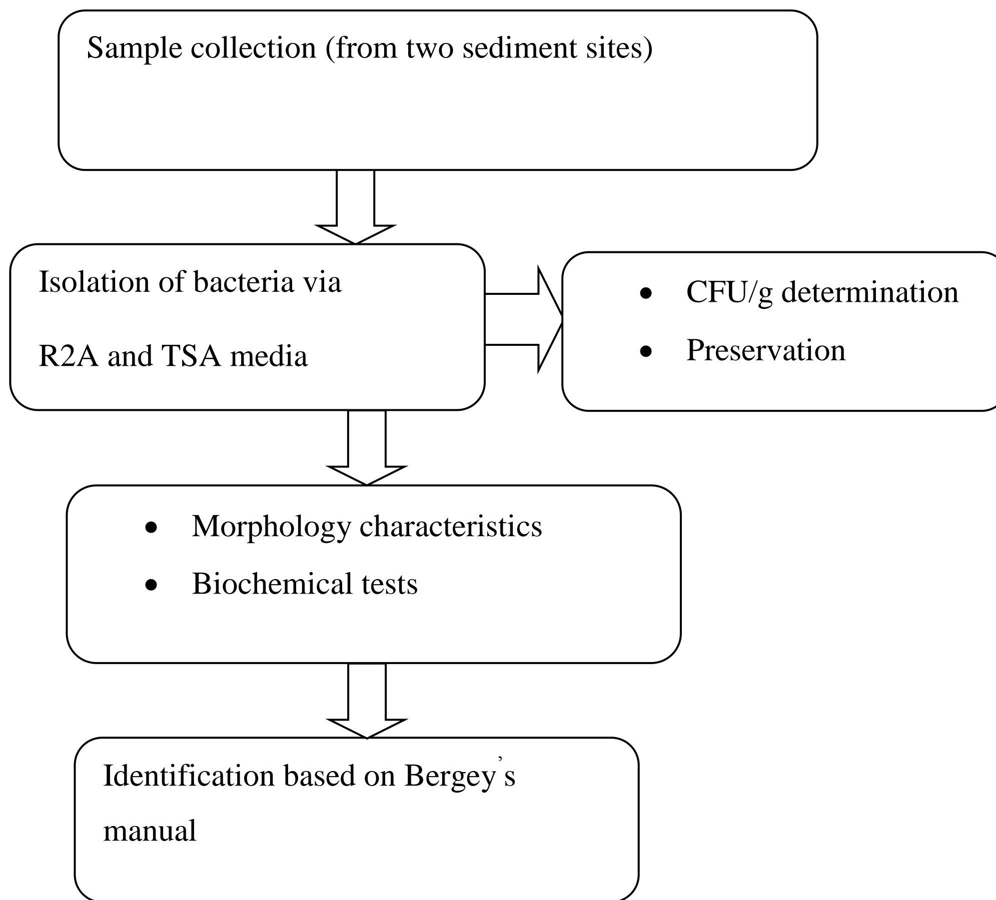


Figure 2: Workflow of sample collection to identification of bacterial genera

3.3. Sampling sites and sample collection

To study sediment microbial community, sediment samples were collected from two sampling sites. These sites were chosen to cover lake with human activities and free from human activates as the nature of the lake has only one entrance site. Site one is located at 36.96° 33' 60" N, 10.91° 59' 01" E (near the entrance of the Lake) and site two is located at 36.96° 44' 66" N, 10.91° 15' 36"E (far from the entrance of the Lake) (figure 1). The two sediment samples were collected using sterilized corer sampler on January 4/2020 and transferred to sterilized 1L glass bottle. Sediment samples were collected aseptically kept in the icebox, transported to Bahir Dar University, department of Biology microbiology laboratory, and stored at $4^{o}C$ until laboratory analysis was performed.

3.4. Isolation of bacteria

Isolation of heterotrophic bacteria was done by the spread plate method as described by Hayakawa (2008) with a few modifications. A stock solution was prepared by diluting 1g of sediment in 9 ml of sterile physiological saline water and shake well by using a vortex mixer. From the stock solution, 1 ml was used to prepare serial dilution up to 10^{-6} and from five consecutive dilutions (10^{-2}- 10^{-6}) 0.1 mL was spread on Tryptone soy agar (TSA) (MERCK, Germany) and R2A agar media (appendix I) in triplicate aseptically. The plates were incubate aerobically at 25°C for 3 days and 25°C for 5 days respectively. After successful growth, the pure cultures of bacteria were subcultured in nutrient broth (HIMEDIA, India) and store in 40 % glycerol for subsequent studies (figure 2).

3.5. Isolation and characterization of bacterial isolates

For identification of isolates, the bacterial colonies were selected by considering their color, size, shape, elevation, margin, surface texture and consistency. Bacterial isolate were characterized and identified based on their colony morphological features, gram reactions and biochemical characteristics. After incubation, 50 morphologically distinct colonies from each culture were selected randomly from a plate containing 30-300 colonies. Although colonies were selected at random, an effort was made to ensure that representatives of all distinct colony types present on the plates were included in the colonies transferred. They further characterized based on selected tests for each group as per Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994).

3.5.1. Morphological characterization

Morphological characterization of isolate was based on conventional macroscopic techniques of shape, size, margin, color, opacity, elevation, surface and texture of pure colonies. These colony characteristics of isolates were described using standard microbiological criteria to give suggestive information as to the identification of an organism (Sousa *et al.*, 2013).

The shape of the cell (rod, cocci and spiral) was noted down from the freshly grown culture of bacteria microscopically. Classification of the isolates as Gram positive or Gram negative was done by following standard method of gram stain reaction and for confirmation, a 3% KOH sensitivity test was also performed as the test used to quickly identify Gram negative and Gram positive bacteria. Spore staining abilities of isolates and motility were tested by following the

Schaeffer-Fulton method and agar stabbing method respectively (Cappuccino and Sherman, 2002).

3.5.2. Biochemical characterization

The identification of isolated bacteria was carried out by subjecting the bacterial colonies to different biochemical tests. The common biochemical tests performed in this research were catalase test, triple sugar iron test, mannitol fermentation, indole production, methyl red, Voges Proskauer test and citrate utilization tests (Cappuccino and Sherman, 2002).

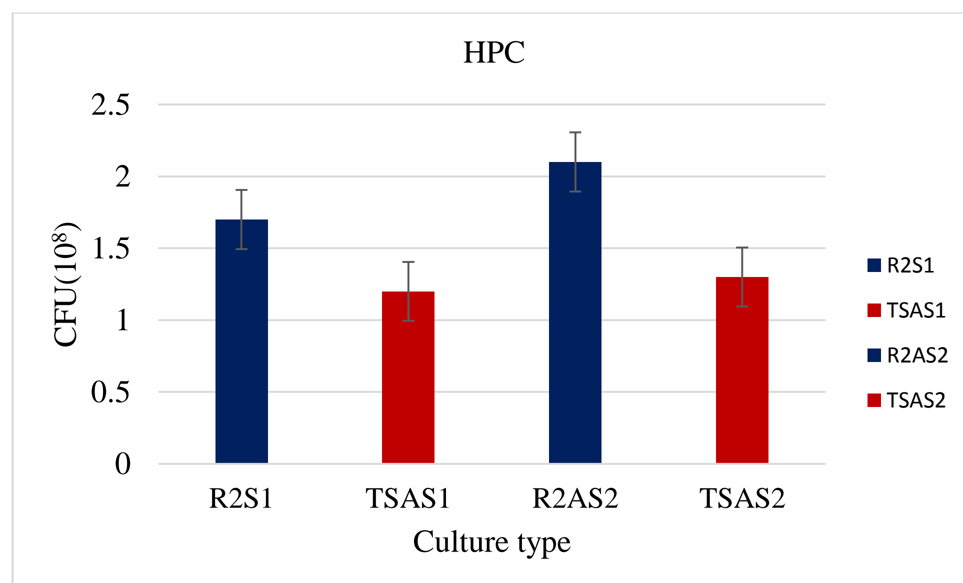
3.6. Data Analysis

Rarefaction curve was calculated to determine the sample sufficiency. The computer based Statistical Package for the Social Science (SPSS version 21 for windows) was used for data analysis. One-way ANOVA was applied to test for significant differences in bacterial diversity between sites and media type ($P \leq 0.05$, at $\alpha = 0.05$). The evenness and richness of each culture in terms of the operational taxonomic unit were analyzed using Simpson’s index and Shannon Wiener index of diversity.

4. RESULTS AND DISCUSSION

4.1. Heterotrophic bacterial count for Lake Zengena sediment

The HPC was based on the enumeration of the growth of heterotrophic culturable microorganisms on non-selective media (R2A and TSA) under defined cultivation conditions. The mean heterotrophic plate counts (HPC) of bacteria range from 1.2×10^8 CFU/g in site one TSA media to 2.1×10^8 CFU/g of sediment site two R2A media. Bacterial counts in R2A media were greater than TSA media in both sites (Figure 3). This indicates R2A media increases the cultivability of heterotrophic bacteria. This finding is similar to the other report, in which the count of heterotrophic bacteria was higher in R2A than TSA growth media. Sukhanova et al. (2019) reported that that the highest CFU of cultured bacteria from Lake Baikal epilithic biofilms were observed on R2A than other media. The report by Segawa et al. (2011) showed a high CFU of cultured bacteria by R2A medium than Luria Broth and the cultivability of bacteria increased as the growth medium diluted.



Where: R2A: Reasoner and Geldreich agar, TSA: Tryptone Soy Agar and S: Site

Figure 3: Bacterial counts of sediment samples in the different culture types

4.2. Heterotrophic bacterial diversity

The sample rarefaction curve indicates that 200 isolates were enough to explain the diversity at the given methods. As more individuals are sampled, the total number of OTUs recorded in the sample increases and species accumulation curve was generated (Figure 4). The curves shows somewhat reached a horizontal asymptote, so it is reasonable to infer that rarefaction analysis plots (R) has converged on a optimum estimate of the correct value. The rarefaction curve shows site one R2A media was more diverse and diversity decreased in site two TSA media (Figure 4).

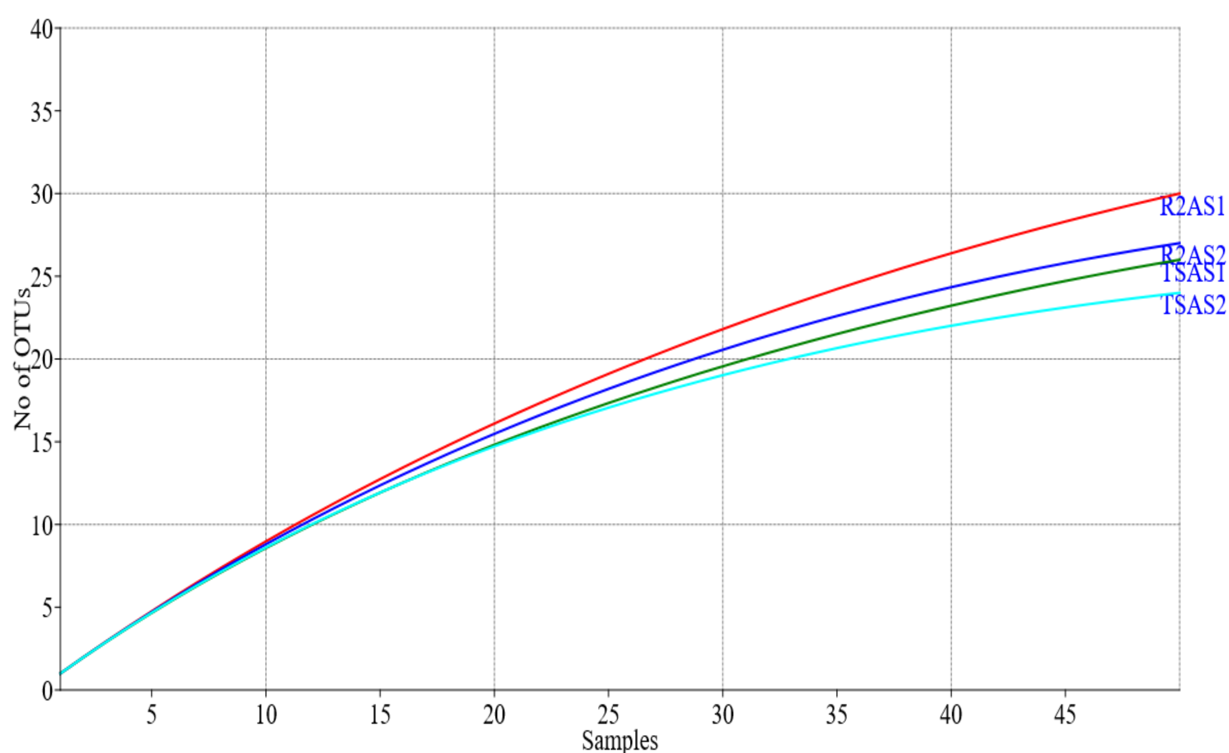


Figure 4: Rarefaction curve of individual culture type

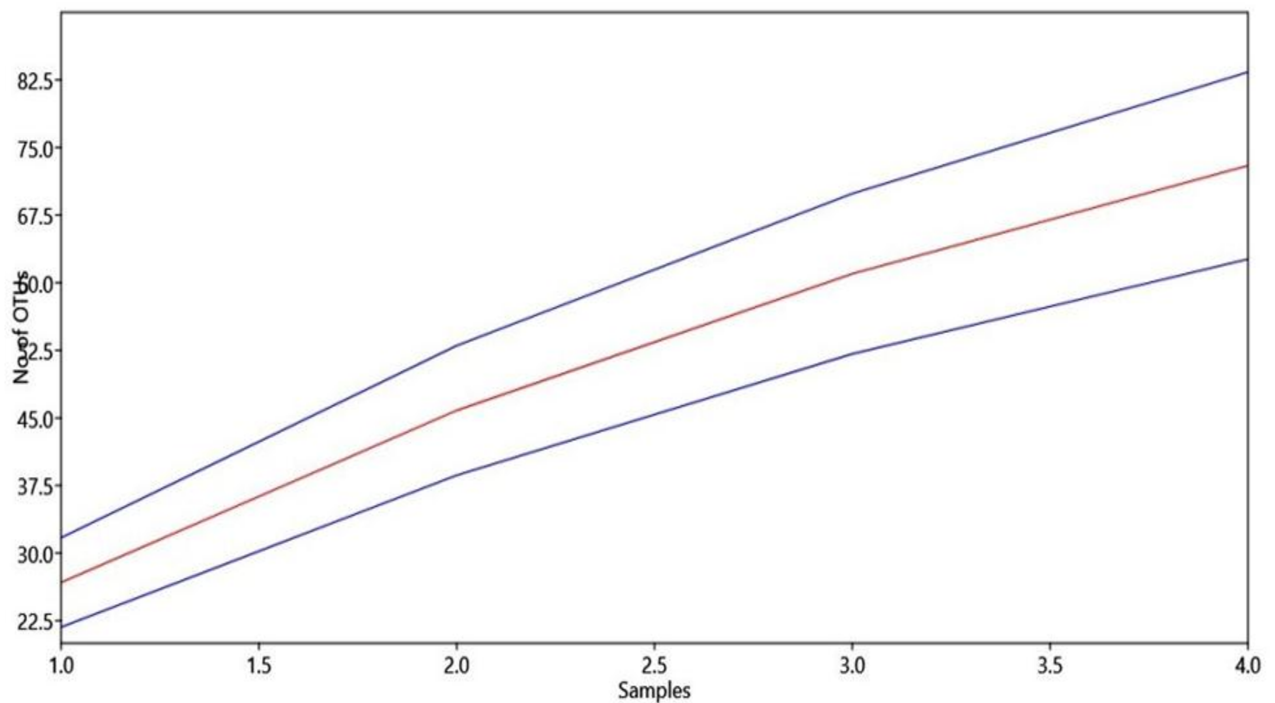


Figure 5: sample rarefaction curve

Bacterial diversity was assessed in terms of unique operational taxonomic units (OTUs). There were 73 OTUs, which demonstrate unique morphological and biochemical features and can be a proxy for at least species. From all the 73 OTUs, three OTUs (OUT 29, OUT 47 and OUT 53) were found in all conditions (culture types). OTU 33 and 70 were present in all conditions except site two R2A media while OUT 58 were present in all conditions except site one R2A medium. These 6 OTUs were abundant and constitute about 16% of the total number of isolates. OUT 54 had 8 isolates and OUT 37, 42 and 46 each had 6 isolates (Appendix II). This indicates that these OTUs thrive in all culture conditions are probably stable and permanent microbial communities.

Furthermore, only 17 OTUs were common to both sites and represented by 61(30%) isolates and 14 OTUs were common for both media types and represented by 53 (26.5%) isolates. Therefore, variation in terms of location and media type were observed regarding the dominant communities. Concerning unique OTUs, site one had 29 unique OTUs while, sites two had 27 OTUs. Moreover, R2A media had 29 unique OTUs and TSA media had 22 unique OTUs (Table 2). This indicated that bacteria cultivability differ in different sites and culture media as the presence of unique isolates obtained on both site and media.

Table 2: Unique and common OTUs in the different culture types, July 2020

OTUs	Sites		Media types	
	Site I	Site II	R2A	TSA
Total	30	27	26	24
Unique	29	27	29	22
Common	17		14	

OTUs structure in terms of diversity and abundance showed a statistically significant difference between media types ($p<0.05$) and two distinctive clusters were formed due probably to media based factors (Figure 6). Analysis of variance showed a statistically significant difference between site one and site two at ($p=0.014$) (appendix III). The difference in sites in heterotrophic bacterial diversity might be probably due to lack of mixing of the water column due to either internal waves or wind action, as the lake is stable. Furthermore, physicochemical factors in the study sites might be responsible for variation in phylotype diversity and this idea is supported by many studies (Gilbert *et al.*, 2017). There was also a statistically significant difference between R2A and TSA media at ($p=0.02$) (appendix III).

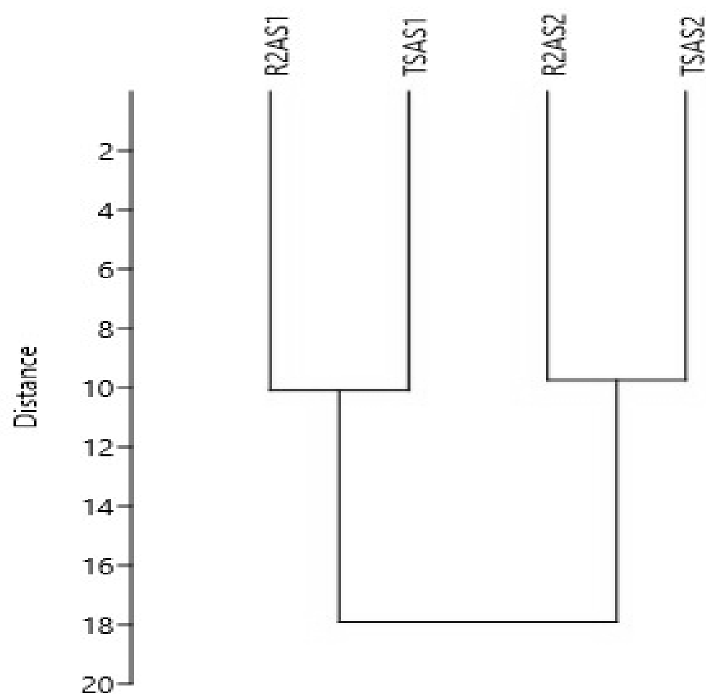


Figure 6: Dendrogram showing bacterial community clustering based on the different culture types

4.3. Bacterial diversity indices

Based on the results of the Shannon-Weiner Index, OTUs diversity was higher in R2A than the TSA medium in both sites. OTUs diversity was higher in R2A medium site one (H=3.269; Hmax= 0.8764 and D=0.044) than TSA medium site one (H=3.082, Hmax=0.8385 and D=0.0552). Likewise, OTUs diversity was higher in R2A medium site two (H=3.167; Hmax= 0.8836 and D=0.0488) than TSA medium site two (H=3.054, Hmax=0.8787 and D=0.0528) (Table 3).

Table 3: OTUs diversity indices, July 2020

OTUs	R2A site one	R2A site two	TSA site one	TSA site two
Taxa_S	30	27	26	24
Individuals	50	50	50	50
Dominance_D	0.044	0.0488	0.0552	0.0528
Simpson_1-D	0.956	0.9512	0.9448	0.9472
Shannon_H	3.269	3.167	3.082	3.054
Evenness_e^H/S	0.8764	0.8787	0.8385	0.8836

This result thus suggested that type of media influence the diversity of OTUs. The reason for the differences in bacterial selectivity might be that these two media have different carbon sources and nutrient concentrations. Tamaki et al. (2005) stated that the type of bacteria that were recovered by plating was influenced by the type of media used and conditions of growth used in the study compared to the bacterial diversity from the natural environment.

4.4. Identified genera from sediment samples of Lake Zengena

Based on the morphological and biochemical characteristics all 200 isolates from the sediment samples of Lake Zengena were identified to nine bacterial genera (appendix IV) that belonged to four phyla: Proteobacteria (56%), Actinobacteria (22%), Firmicutes (11%) and Bacteroidetes (11%). In this study, the phylum Proteobacteria was the dominant phyla represented by two classes and five genera: Gammaproteobacteria (*Aeromonas*, *Pseudomonas*, *Proteus* and *Escherichia*) and Alphaproteobacteria (*Sphingomonas*) (Table 4). The predominance of Proteobacteria revealed that they were actively engaged in the functioning and processes of lake sediment ecosystems (Song *et al*., 2012). Thus, the results of this study revealed that the sediment of Lake Zengena possessed different groups of bacteria

phyla. This finding is more or less similar to that of the bacterial community structure in other Lake mainly Lake Baikal (Sukhanova *et al*., 2019).

Regarding genus, the most dominant genera were *Pseudomonas* 42 (21%) and *Bacillus* 34 (17%) followed by *Flavobacterium* 30 (15%). Other genera ranged from 28 (14%) of *Aeromonas* to six (3%) of *Escherichia* (Figure 7). *Bacillus* and *Pseudomonas* are present ubiquitously and possess a high biological potential and a strong capacity to adapt to various environmental conditions (Sukhanova *et al*., 2019). The dominance of *Pseudomonas* in this freshwater ecosystem might be attributed to their versatile metabolic capacity and broad potential for adaptation to fluctuating environmental conditions (Michaud *et al*., 2012).

The dominance of *Bacillus* might be due to their abilities of spore production, which can resist different environmental conditions. The idea of spore-forming bacteria dominated in the bottom sediments, both in quantitative abundance and in species diversity was supported by Huang and Jiang (2016). This finding indicates that these two genera as the common members of lake sediment.

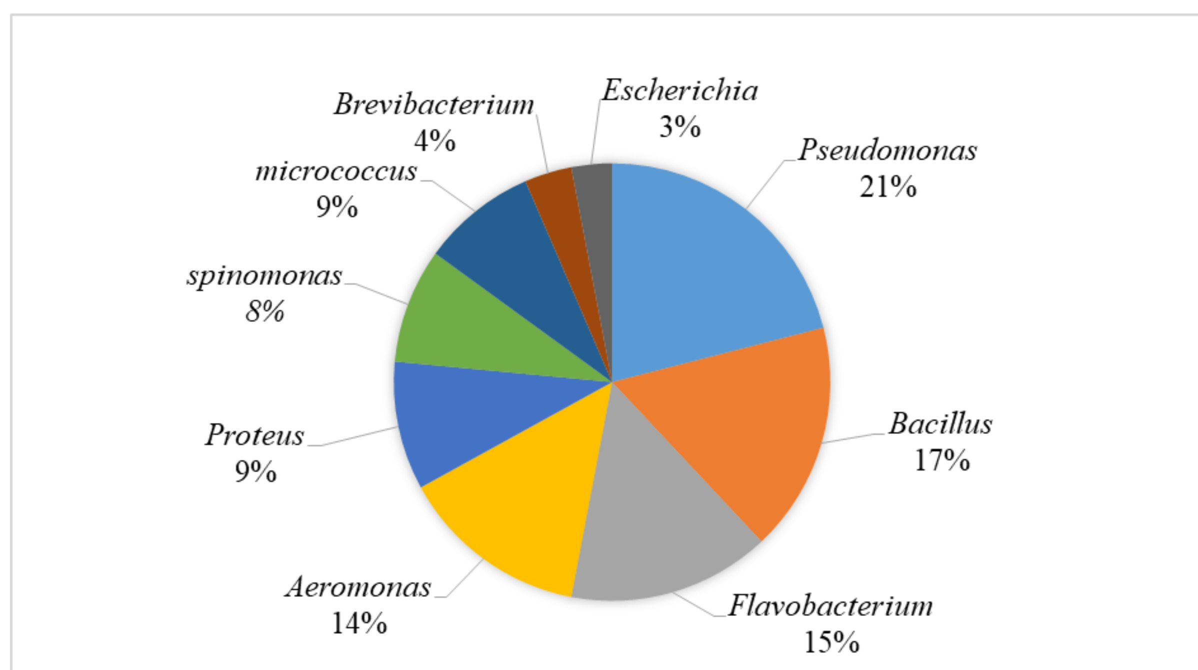


Figure 7: Relative percentage of each genus from sediment samples of Lake Zengena

4.5. Distribution of the identified genera by site and media type

Of all genera, seven genera were found in both sites and media types showing their stable presence in the lake sediment (Figure 8) and most of these common genera were abundant. However, few genera such as *Micrococcus* and *Escherichia* were not detected in site two R2A

media and site two TSA media respectively. *Pseudomonas* was found at the highest percentage at site one (12.5 %) than site two (8.5%). Contrarily, *Bacillus* was found at the highest percentage at site two (13.5 %) than site one (3.5%) as shown below (Figure 9).

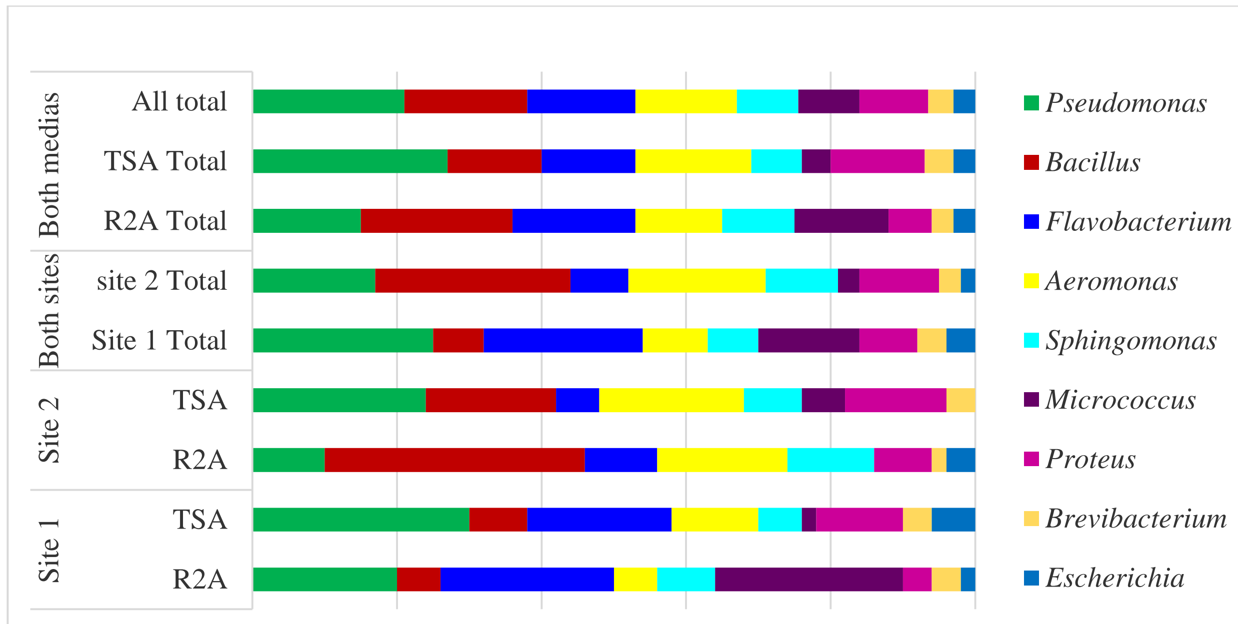


Figure 8: Relative abundance of the identified genera in the sediment sample of Lake Zengena at both media types and sites

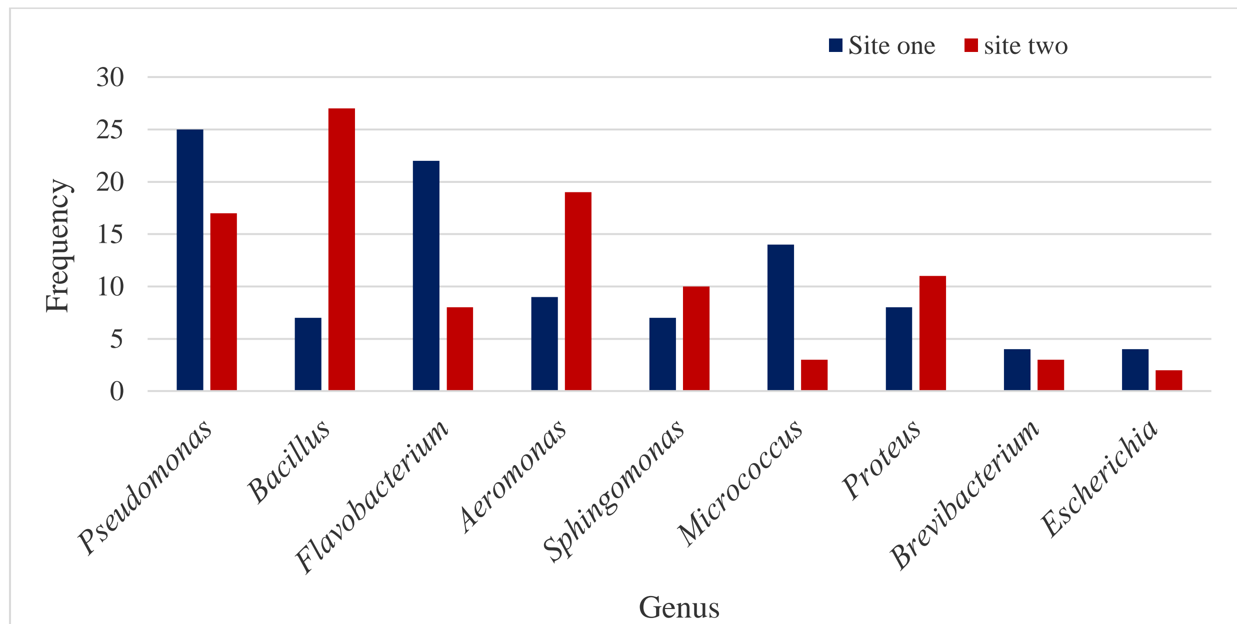


Figure 9: Occurrence of each genus in the site during the study period

The majority of culture media used to isolate microorganisms have been nutrient-rich. It is now thought that these conditions may favor the growth of faster-growing bacteria at the expense of slow-growing one, some of which thrive in nutrient poor environments as culturing of an organism depends on essential macronutrients and growth factors in isolation media (Bollman *et al*., 2007; Connon and Giovannoni, 2002 and Salam *et al*., 2018).

R2A medium is a relatively oligotrophic medium compared with the TSA medium and the medium was able to isolate slow growing and some less common bacteria species. Results of this study showed R2A medium had better selectivity to *Flavobacterium* and *Sphingomonas* than other phyla. The genus *Flavobacterium* and *Sphingomonas* need low nutrient medium relative to other members (Loch and Faisal, 2015; Stewart, 2012).These two genera were more observed on R2A media than TSA (Figure 10). Zhao et al. (2020) reported that the R2A medium had better selectivity for Bacteroidetes. This finding showed that the abundance of cultured microorganisms obtained on growth media with different concentrations of organic compounds was varied. Therefore, to get a higher diversity of cultured bacteria, it is important to employ media varying in the content of organic and mineral compounds. Efficient cultivation of heterotrophic bacteria from different biotopes of oligotrophic habitats needs growth media that contain low organic matter concentrations, mineral and vitamin components, as in R2A. This finding is similar to the other report, in which the R2A media supports the growth of heterotrophic bacteria than TSA and other growth media (Sukhanova *et al*., 2019).

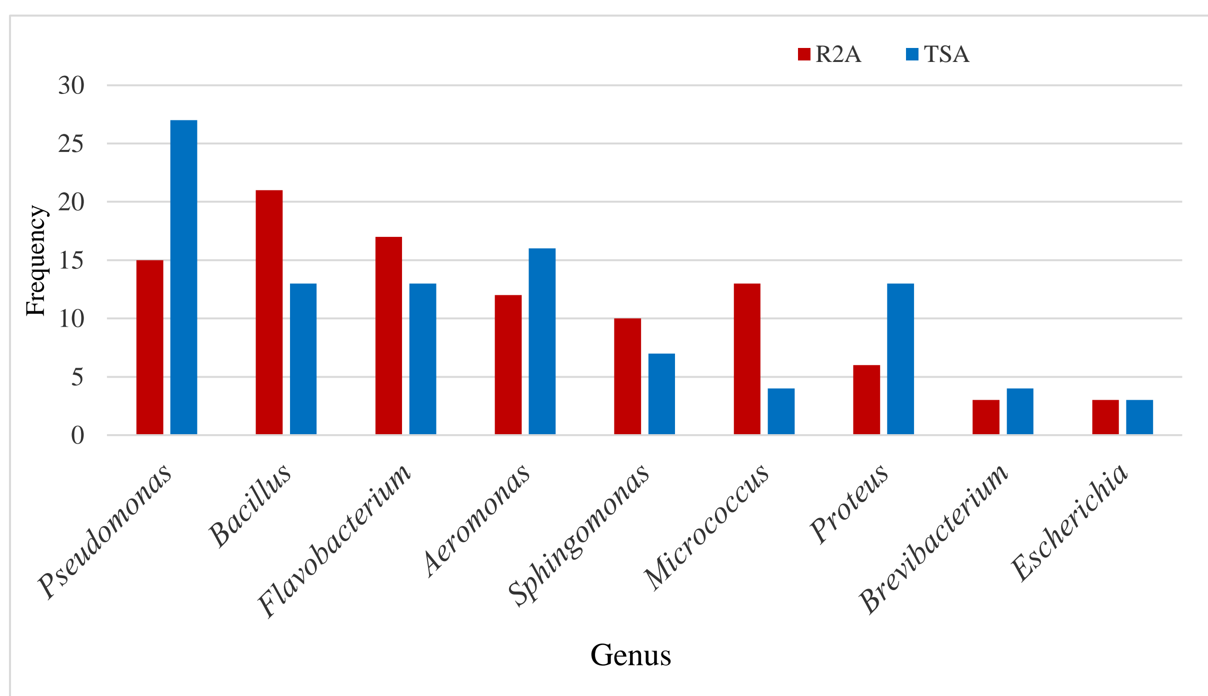


Figure 10: occurrence of each genus in media types during the study period

Overall, Sediment bacterial communities in Zengena Lake were mainly composed of the phylum that is typical to freshwater sediment, including Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Table 4). The microbial diversity was quite diverse and differed between sampling sites and growth media.

Table 4: Taxonomic classification of identified genera from Lake Zengena sediment samples, July 2020.

Genus	Family	Order	Class	Phylum
Pseudomonas	Pseodomonaliscea	Pseodumoales	Gammaproteobacteria	Proteobacteria
Bacillus	Bacilliaceae	Basillales	Bacilli	Firmicutes
Flavobacterium	Flavobacteriaceae	Flavobacteriales	Flavobacteria	Bacteroidetes
Aeromonas	Aeromonadaceae	Aeromonadales	Gammaproteobacteria	Proteobacteria
Sphingomonas	Sphingomonadaceae	Sphingomonadales	Alphaproteobacteria	Proteobacteria
Micrococcus	Micrococcaceae	Micrococcales	Actinobacteria	Actinobacteria
Proteus	Enterobacteriaceae	Enteralesobacteriales	Gammaproteobacteria	Proteobacteria
Brevibacterium	Brevibacteriaceae	Micrococcales	Actinobacteria	Actinobacteria
Escherichia	Enterobacteriaceae	Enteralesobacteriales	Gammaproteobacteria	Proteobacteria

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Determination of bacterial diversity by culture-based method revealed 73 OTUs with unique morphological and biochemical characteristics. The 73 OTUs were identified into nine genera belonging to four phyla and Proteobacteria phylum was the dominant one. A significant difference in terms of OUTs diversity and abundance between media type (p=0.02) was observed. This difference might be due to the different composition of organic compounds and other growth factors in isolation media. OTUs diversity also differed significantly between sites (p=0.014). Different physicochemical factors and lack of mixing (stable environment) might be responsible for variation in OTUs diversity in location. The diversity indices showed differences in growth media in both sites. R2A medium supports more diverse OTUs than TSA in both sites as on nutrient media with different composition of organic compounds. In general, this result revealed that sediment bacterial communities in Zengena Lake were diverse and mainly composed of phyla that are typical to freshwater sediment.

5.2. Recommendations

Based on the results of this study, the following points are recommended:

- The identified genera need to be further characterized for their ecological role and identified to species level.
- Anaerobic growth needs to be included to isolate and identified more heterotrophic bacterial diversity.
- The spatial variability of heterotrophic bacteria needs to be analyzed with different environmental parameters and nutrient contents for better understanding of the distribution of bacterial population.

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APPENDICES

Appendix I

Table 5: Composition of Tryptone soy agar

Ingredients	Concentration (g/liter)
Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Final pH at 25^oC	7.3 ± 0. .2

Table 6: Composition of R2A agar

Ingredients	Concentration (g/liter)
Yeast extract	0.5 g
Difco Proteose Peptone no 3	0.5 g
Casamino Acids	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Sodium pyruvate	0.3 g
K2HPO4	0.3 g
$MgSO_4$ * $7H_20$	0.05 g
Agar	15.0g
Final pH at 25^oC	7.2 ± 0.2

Appendix II

Table 7: Individual isolates with their corresponding OTUs based on similar morphological and biochemical characteristic, July 2020

OUT	Isolates
1	ST181, ST182
2	SR127
3	SR106, SR113
4	SR46, SR103
5	SR47
6	ST57, ST89
7	SR48
8	SR142, SR144
9	SR28, ST72
10	SR33, ST61
11	ST190, ST176, ST189
12	SR107
13	SR108, ST163
14	SR149, SR150, ST153, ST160
15	ST70, ST193
16	ST185, ST188
17	SR41, SR43
18	ST97, ST99
19	SR134, SR136
20	ST166, ST168, ST180, ST191

21	ST85, ST86
22	ST88
23	SR128, SR129
24	SR12, ST63, ST73
25	ST94, ST161, ST162, ST167, ST158
26	SR124
27	SR125, SR130
28	ST154, ST156
29	SR30, SR31, SR148, ST55, ST164, ST165
30	ST195, ST183, ST194
31	SR138, SR140
32	SR21, ST170
33	SR1, ST69, ST197
34	ST87, ST90
35	SR126, SR133, ST196, ST200
36	ST173, ST152, ST172
37	SR146, SR147, SR119, SR137, SR139, SR143
38	SR16, ST54
39	ST66, ST67, ST169, ST199
40	SR116, SR117
41	SR20, SR42, SR2, SR6
42	ST62, ST79, ST51, ST56, ST58, ST59
43	ST98, ST53, ST96
44	SR39 ,SR34, SR35

45	SR11,SR25
46	SR40, ST81,ST82, ST52,ST68, ST75
47	SR27, SR32, SR44, SR49, SR50,SR109, ST83, ST157
48	SR4, SR15
49	SR24, SR29
50	ST71,ST74, ST76, ST84
51	ST64, ST65
52	ST77, ST78
53	SR5, SR141, SR120, ST91, ST92, ST159
54	SR112, SR123, SR135, ST171, ST174, ST175, ST186, ST198
55	SR37, SR145
56	ST93, ST95, ST177, ST178
57	SR114, SR110, SR111
58	SR105, SR118, SR104, ST100, ST187
59	SR36
60	SR22, SR102
61	SR115, SR132
62	ST151, ST155
63	SR131, ST80
64	SR38, SR121
65	SR101, SR122
66	SR3, SR45
67	SR14, SR19
68	ST179, ST184

69	SR13
70	SR8, SR18, ST60, ST192
71	SR17, SR23
72	SR9, SR10
73	SR7, SR26

Appendix III

Table 8: ANOVA Table for media type

Descriptive

OTUs	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	Upper Bound	Minimum	Maximum
R2A	100	3.88	2.293	.229	-3.43	4.33	1	9
TSA	100	3.50	2.245	.225	-3.05	3.95	1	9
Total	200	3.69	2.271	.161	-3.37	4.01	1	9

ANOVA

OTUs	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.220	1	7.220	1.402	.02
Within Groups	1019.560	198	5.149		
Total	1026.780	199			

Table 9: ANOVA Table for sites

Descriptive

OTUs	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	Upper Bound	Minimum	Maximum
site one	100	3.89	2.461	.246	-3.40	4.38	1	9
Site two	100	3.49	2.057	.206	-3.08	3.90	1	9
Total	200	3.69	2.271	.161	-3.37	4.01	1	9

ANOVA

OTUs	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	8.000	1	8.000	1.555	.014
Within Groups	1018.780	198	5.145		
Total	1026.780	199			

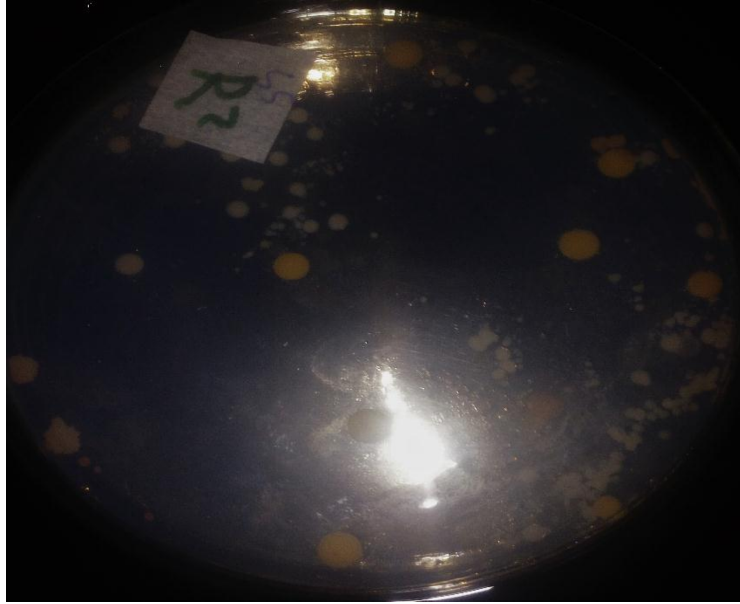
Appendix IV

Table 10: The identified genera based on morphological and biochemical test, July 2020

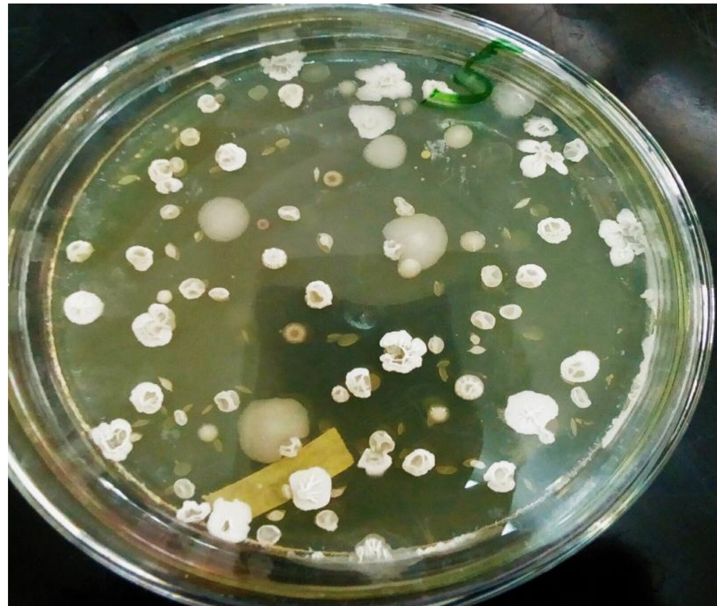
Morphological characteristics									Biochemical characteristics															
Number	Shape	size	Marigion	Color	Opacity	Elevation	surface	texture	Gram stain	Cell shape	Spore stain	motility	catalase	glucose	sucrose	Lactose	gas	H2S	Citrate	indole	M-R	V-P	Mannitol fermentation	Genus
1	Circular	Small	Entire	White/ yellow	Opaque	Flat	smooth	Moist	-	Rod		+	+	+	+/-	-	-	-	+/-	-	-	-		*Pseudomonas*
2	Circular	Large	Entire	White	Opaque/ transparent	Convex	Smooth	Moist	+	Rod	+	+	+	+/-	+	+	-/+	+	+					*Bacillus*
3	Circular	Large	Entire	Yellow	Opaque/	Flat	Smoot	Moist	-	Rod		+	+	-	-	-	-	-	+	-	-	-		*Flavobacterium*
4	Circular	Small	Entire	White / Buffy	Opaque	Raised	Smooth	Moist	-	Rod		+/-	+	+	+	-	+	+/-	+	-	-	+		*Aeromonas*
5	Irregular	Large	Undulate	White	Convex	Flat	Rough	Moist	-	Rod		+	+	+	+	-	+	+	+	+/-	+	+/-		*Proteus*
6	Circular	Small	Entire	Yellow	Opaque	Convex	Smoot	Moist	+	Cocci		-/+	+	+	-	-	-	+					-	*Micrococcus*
7	Circular	Large	Entire	White	transparent	Convex	Smoot	Moist	-	Rod		+	+	-	-	-	-/+	-/+	-	-	-	-		*Sphingomonas*
8	Irregular	Small	Undulat	White	Opaque	Convex	Smoot	Moist	+	Rod	-	-/+	+	+	-	-	-	-	-	-	-	-		*Brevibacterium*
9	Circular	Small	Entire	White	Opaque	Flat	Smoot	Moist	-	Rod		+	+	+	+	+	+	-	-	+/-	+	-		*Escherichia*

Appendix V

Some photo evidences during the study



A



B

Figure 11: Isolated colonies on R2A (A) and TSA (B) media

Appendix VI

A

B

Figure 12: Refresh culture for biochemical tests (A) and Microscopic observation for gram and spore staining (B)