MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF THE ORAL MICROFLORA ISOLATED FROM VARIOUS REGIONS OF NEPAL

M.Sc. Thesis

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Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by

Nirmal Panthi

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Abstract

Formation of biofilm is a survival strategy for bacteria and fungi to adapt to their living environment. Poor oral hygiene is one of the major causes for oral and dental diseases. And to understand the root cause of these oral diseases the identification of bacterial community is must. Along with oral community if biochemical mode of action of these microorganisms can be identified then possible mode of action towards situation of disease can be understood. So in this current work we try to obtain knowledge of microbial population and their biochemical mode of action among some of the Nepalese population using various molecular techniques to understand possible disease causing factor inside these oral cavity. Similarly, microbial enzymatic activity by these bacterial colonies was also explored. After the 16s rRNA sequencing 49 different organisms are identified having 19 different genera of oral microbial diversity in Nepalese population from various altitudes, ethnic groups, and various age groups. The bacterial isolates belonging to genus *Enterococcus, Enterobacter, klebsiella, Bacillus, Pseudomonas, Staphylococcus, Serratia, Citrobacter, Stenotrophomonas, Achromobacter* and their sub types are identified. GC-MS of bacterial extract provided the presence of 141 different compounds. Bacteria's with varying organic compounds secretion are obtained like alkyl, amines, esters phenols, chlorine and sulphur containing where these compounds either help them to establish formidably inside oral biofilm or help them to initiate and establish disease condition like oral malodors(sulphur compounds), carcinogenicity(acid and acid esters, chlorine compounds), caries(acid, acid eser). *Escherichia marmotae* the bacteria which was never isolated before in oral sample shows the anonymous nature in the oral cavity.The overall scientific data has paved the way to describe the distribution, pathogenic potential, physiology and variation of oral micro- organisms in Nepalese communities.

Keywords: Biofilm, Enzyme assay, Sequencing, GC-MS

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List of Abbreviations

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid
EPS	Extracellular polymeric substance
PIA	Polysaccharide intercellular adhesion
VAP	Ventilator-associated pneumonia
CV-I	Crystal violet- iodine
RDP	Ribosomal Database Project
RIDOM	Ribosomal Differentiation of Medical Micro-organisms Database
HOMIM	Human Oral Microbe Identification Microarray
RFLPs	Restriction Fragment Length Polymorphism
RIBB	Research Institute for Bioscience and Biotechnology
TU	Tribhuvan University
F	Female
M	male
NA	Nutrient agar
MHA	Muller Hilton agar

MSA	Mannitol salt agar
BA	Blood agar
BHA	Brain heart infusion agar
PDA	Potato dextrose agar
rpm	Revolutions per minute
mm	millimeter
min	Minutes
eV	Electron volt
CMC	Carboxymethyl cellulose
C	Carbon
H	Hydrogen
ATCC	American Type Culture Collection
CDBT	Central Department of Biotechnology
gm	Gram
GC-MS	Gas chromatography-Mass spectroscopy
mg	Milligram
ml	Milliliter
MS	Mass Spectrometry

WHO	World Health Organization
NIST	National Institute of Standards and Technology
OPD	Out Patient Department
PCR	Polymerase Chain Reaction
http	Hyper Text Transfer Protocol

INTRODUCTION

1.1 Background

Oral diseases are a big public health problem with high prevalence and incidence all around the world. Improving oral health in the rural children is a big challenge in a developing country like Nepal(Bono *et al,* 2006). The dental problems seem to be increasing because of presence of hard surface for the colonization of bacteria which further develops dental biofilms and create dental carries (Kirby *et al*, 2014).An oral disease qualifies as major public health problems owing to their higher prevalence and significant social impact (Humagain, 2011). Nepal is a developing country with little awareness and practice regarding oral health amongst rural Nepalese population (Prasai *et al.,* 2013). The microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or more recently as the oral microbiome. The term microbiome was coined by Joshua Lederberg "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg and McCray,2001).

1.2 Biofilm

A biofilm is a thin layer of microorganisms that adhere to the surface of an organic or inorganic structure together with their secreted polymers. Biofilms are the predominant phenotype of nearly all bacteria in their natural habitat, whether pathogenic or environmental. Biofilm bacteria attach to surfaces and are surrounded by an extracellular matrix called the Extracellular Polymeric Substance (EPS) (Costerton *et al*., 1999). Biofilms are composed primarily of microbial cells and Extracellular Polymeric Substance. Extracellular Polymeric Substance may account for 50% to 90% of the total organic carbon of biofilms and can be considered the primary matrix material of the biofilm (Flemming *et. al*, 2000). EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-

glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pryruvates confers the anionic property (Sutherland *et al.,* 2001). In the case of some gram-positive bacteria, such as the staphylococcus, the chemical composition of EPS may be quite different and may be primarily cationic. Hussain *et al.,* found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins. EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. EPS may be hydrophobic, although most types of EPS are both hydrophilic and hydrophobic (Sutherland *et al*., 2001).

1.3 Oral Micro biota

More than 700 different bacterial species or phytotypes have been detected in the oral cavity over 50 % of which have not been cultivated. The microflora on teeth, the tongue, buccal epithelium, soft and hard palate, and the vestibulum revealed 20-30 different predominant species at each site, and the number of predominant species per individual was in the range of 30-70 (Aas *et al*., 2005). The most common species belonged to the genera *Gemella*, *Granulicatella, Streptococcus,* and *Veillonella.* The digestive tract is anatomically continuous and harbors approximately 11014 microorganisms, which is more than the approximately 61013 cells that constitute the entire human body and oral cavity is the entrance of digestive tract so it is sometimes considered as the initial source of most digestive tract microorganisms. The bacterial flora of the mouth also varies according to age, diet and eating habits, and other factors such as the degree of oral hygiene in an individual (Lenander-Lumikari *et al.*, 2000).Saliva plays an important role in oral health, regulating and maintaining the integrity of the oral hard and soft tissues (Dodds *et al*., 2005; Llena-Puy, 2006). Several functions can be defined for saliva: lubrication and protection, buffering action and clearance, maintenance of tooth integrity, and digestion. Saliva provides a protective surface coating of mucosa and teeth.

1.4 Dental Biofilm

The dental biofilm is a microbial community that forms at high density on tooth and tissue surfaces of oral cavity. Dental biofilm contains a dynamic microbial community which forms high cell density on the tooth and tissue surfaces. This microbial environment attaches dental pellicle which favors the successive bacterial colonization. After initial

colonization they further maintain cell signaling and increase constant unification of cell layers to form thicker and thicker layer. Dental biofilm causes major health hazards not only relating to mouth but also other systemic diseases(Suresh & Narayana., 2013). Dental caries and periodontal diseases are among the most important global oral health problems(Bhardwaj & Bhardwaj., 2006). Plaque is not only present on natural teeth but also on dental materials and implant components as well. Dental caries and periodontal diseases being highly prevalent in developing countries are a major public health problem.

1.5 Formation and maturation of oral biofilm

Biofilm formation is a very ordered and coordinated process, involving sequential formation of acquired pellicle, colonization of primary colonizers and then secondary colonizers. In the absence of communication, these orderly changes would be random (Kolenbrander *et al*, 2002). The acquired pellicle, which is composed of a variety of host-derived molecules, coats the enamel surface within minutes after professional cleaning and is a source of receptors for primary colonizers of the plaque—*Streptococci* spp., *Actinomyces* spp., *Capnocytophaga* spp., *Eikenella* spp., *Haemophilus* spp., and *Veillonella* spp. As soon as the pioneer bacteria attach on the pellicle, they begin to excrete extracellular polymeric substance, which helps the bacteria stay bound together and attach to the pellicle (Huang *et al*., 2011). The pioneer bacteria provide specific binding sites for the subsequent bacterial colonization. Bacteria coaggregate, forming typical corncob forms, bristle brush form, or other forms in mature oral biofilm. Various procedures are used to detect oral bacteria, such as microbial culture, immunological assays, enzymatic methods and molecular biology, a polymerase chain reaction (PCR)-based diagnostic technique, 16sRNA sequencing. These procedures have help scientists to identify those 500 different bacterial taxa in oral cavity. Much of these community constitute of commensal, free living, non-pathogenic, pathogenic bacteria in abundance but most of the oral problems are caused by group or organisms rather than single individual (Suzuki *et al.*, 2005). Most of these commensal bacteria are required to keep equilibrium in the mouth ecosystem and are major constitute of oral ecosystem. only sometimes they seems to play a key role in the development of oral diseases such as dental caries, periodontal disease(Chakraborty *et al*., 2014).

1.6 Oral Microbiome

Molecular analysis of oral microbial communities by cloning and sequencing the bacterial 16SrRNA genes present has indicated that the oral cavity provides a habitat for approximately 700 species of bacteria, of which between 100 and 200 different species are present in the healthy mouth of any individual (Frias-Lopez and Duran-Pinedo, 2012 ; Kolenbrander *et al*., 2010). Heterogeneity of oral biofilm is reported not only in various parts of same oral cavity but also on the same teeth of an individual (Hall- Stoodleyl and Stoodleyl, 2005). These organisms seems to have developed variety of genetic and physiological abilities to sustain in oral environment and to be part of dental biofilm(Geethashri *et al*, 2014). Most of the commensal bacteria are required to keep equilibrium in the mouth ecosystem only sometimes they plays a key role in the development of oral diseases such as dental caries, periodontal disease(Chakraborty *et al*, 2014). Oral biofilm like other biofilms seems to acquires new microbial species in each stage of its development, including *Lactobacillus casei, Streptococcus sanguis, S. mutans, S. mitis and S. sobrinus*, which due their pathogenicity could damage the enamel and gum tissue (Bersan *et al*, 2014). Actinomyces and lactobacilli, *S.gordonii, S. parasanguinis, and S. mitis, S. epidermidis* are major commensal species of oral cavity. *Streptococcus mutans* are found in almost all dental caries and have significant role in initiating carries(Geethashri *et al*., 2014). *E. faecalis* and S. *aureus* are almost present in every root canal of tooth. *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Treponema denticola, Prevotella intermedia* are pathogenic and malodor causing bacteria's present in oral cavity(Ahmed *et al*., 2014) .

1.7 Oral Health Concern

The pathogenicity of oral biofilm is a result of a shift in bacterial composition due to environmental changes, e.g. a pH change of the oral cavity or a dietary change of the host. Adhesion, colonization, and growth of oral bacteria on tooth surfaces lead to a multispecies biofilm, called dental plaque (Rosan and Lamont 2000; Hicks *et al*. 2003; Marsh 2004). It is the cause of common oral infectious diseases such as dental caries, gingivitis, periodontitis, and peri-implantitis. In the oral cavity, a compositional imbalance of oral biofilm can cause gingivitis, periodontitis and caries. Along with these normal oral

diseases it is also strongly associated with other diseases such as cardiac disease and cancers (pancreatic, gastrointestinal, oral and even oral/pharyngeal cancers like oral squamous cell carcinomas) (Chakraborty *et al*., 2014). Bacteria, released from oral biofilm and transmitted by blood or other routes to remote areas of the human body, can cause for example atherosclerosis (Reyes *et al*., 2013), endocarditis (Nagata *et al*., 2005; Wilson *et al*., 2008), bronchopneumonia (Imsand *et al*., 2002), otitis media (Topcuoglu *et al*., 2012), rhinosinusitis, osteomyelitis (Rana and Monnis, 2011) and infections related to biomaterial implants or devices (Mombelli and Decaillet, 2011).Worldwide, 60–90% of school children and nearly 100% of adults have dental cavities, similarly severe periodontal (gum) disease, which may result in tooth loss, is found in 15–20% of middle-aged (35-44 years) adults and now Globally, about 30% of people aged 65–74 have no natural teeth (oral health, 2012) . In the 2004, National Pathfinder survey reported that, dental caries in the deciduous dentition, especially in young children attending urban schools, was still above recommended targets, thus making dental caries one of the most prevalent childhood diseases (67.0%) in Nepal. The study also reported untreated dental caries having an effect on the quality of life leading to pain and loss of teeth.

1.8 Rationale of the study

Human oral cavity makes an easy site for various micro biomes in their self-maintain environment for their growth. Various opportunistic pathogens are found to cause oral health problems and associated diseases where they adapt in the oral environment and prolong the disease conditions. As oral problem in world is in higher number and although the modern dental technologies have increased but the number of dental patients are not decreasing. This may be due to unhealthy diet, tobacco use, harmful alcohol use and poor oral hygiene and social determinants. In Nepal also oral problems are in large scale and this may be due to different nutritional practice, bad sanitary habits and most importantly lack of medical guidance and medical personals in rural areas. So it is very significant to identify and understand the type of microbial community that is causing these problems in different Nepalese population.

1.9 Objectives

1.9.1 General objective:

Identification of the microbial flora of oral cavity (extracted tooth) by molecular and biochemical techniques.

1.9.2 Specific objectives:

- To isolate and characterize the oral microbial species of different people from different places of Nepal using 16SrRNA sequence analysis.
- To explore enzymatic activities (protease, cellulase, amylase, pectinase etc.) of each oral microbial isolate.
- To prepare extract using methanol extraction method.
- To explore secondary metabolites (small molecules) secreted by biofilm micro-organisms in bacterial extracts.

CHAPTER 2. LITERATURE REVIEW

2.1 History of Biofilms establishment

Microbial communities attached to surfaces (biofilms) had been observed long before people had the tools to study them in detail. The discovery of microbial biofilms can be attributed to Antonie van Leeuwenhoek, who first observed microorganisms on the plaque on his own teeth. The phenomenon of adherence of bacteria was investigated by Henrici*,* Windogradsky, Cholodny, and Conn. An important observation made by these scientists was that bacteria that grew attached to the surface express different phenotypic characters from planktonic counterparts (Lappin-Scott, 1999). In 1969, Jones *et al* used for the first time the transmission electron microscope to study biofilm created on filter irrigation plants, while at the same time, using a special ruthenium-red staining, these scientists showed that the matrix in which the bacteria were immersed was of polysaccharide nature (Jones *et al*., 1969).

Costerton *et al* stated a theory of biofilm development. According to their theory, the majority of bacteria grow in matrix enclosed biofilms adhered to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic counterpart (Costerton *et al*., 1978). Biofilm formation is a complex and spontaneous process that is carried out in the availability of attachment sites in several stages.

- ATTACHMENT STAGE
- AGGREGATION STAGE,
- MATURATION STAGE,
- MATURE BIOFILM STAGE, and
- DISPERSION STAGE.

In natural environment, microorganisms do not adhere to the substrate themselves but they rather stick onto this so-called conditioned layer, which is known to be formed on most substrates as the result of chemical surface modification (Percival SL *et al.,* 2011). Attachment is the first step to obtain a sustainable biofilm. Initially pellicle is formed on the tooth surface to create sites of attachment. The solid-liquid interface between a

tooth surface and an aqueous medium (e.g., water, blood) provides bacteria for an ideal environment for the attachment and growth. The process of adhesion onto a certain surface is carried out through two stages: primary adhesion (the stage of reversible binding) and secondary adhesion (the stage of irreversible binding). The first step in biofilm formation occurs when microorganisms encounter the surface, which enables the primary bacteria adhesion. Bacterial cells could be moved passively (due to the effects of hydro-dynamics, Brown's motion, or sedimentation) (Ginn TR *et al.,* 2002) or actively (due to locomotors organs, or taxis). When bacteria come close enough to the surface, the adherence will depend on the net sum of the attraction and repulsion forces created between two surfaces.

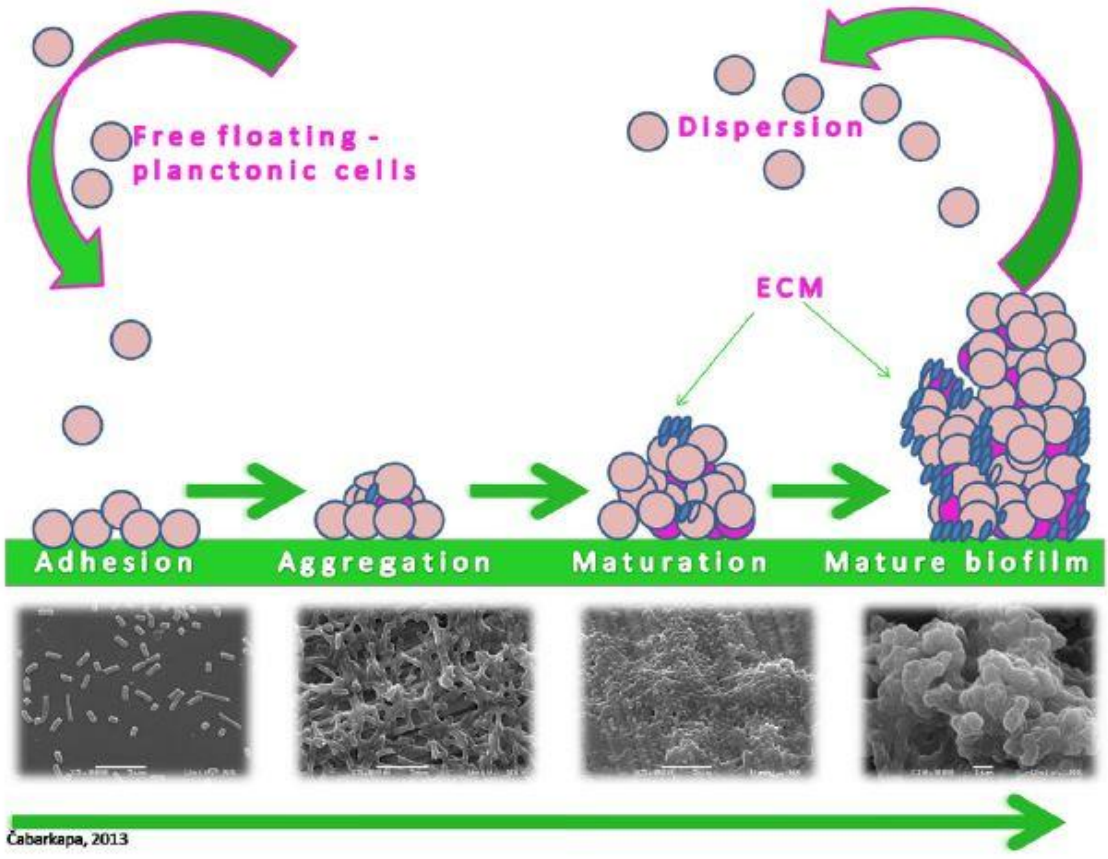

Figure 2.1 Stages of biofilm development

Diverse surface structures, such as fimbriae, flagella and pili, which play the key role in the adhesion, are found on the bacterial cell surface. Fimbriae are amyloid cell-surface proteins, involved in adhesion to surfaces, cell aggregation, environmental persistence and biofilm development. Flagella participate in the initial binding of bacterial cells with the surface by possessing numerous ion groups that are covalently bonded with surface

molecules around the cell wall leading to a strong electrostatic interaction of bacteria with the surface, while pili enable twitching motion generated by pili extension and retraction (Zottola and Sasahara, 1994) In the temporal function, the bond between bacteria and substrate is stronger, and it turns binding into irreversible process. Strengthening of the bond between bacteria and substrate is the result of production of extracellular polymeric substance (EPS), or biofilm matrix. Aggregation is the second stage of biofilm development and it is the result of simultaneous accumulation, growth, and multiplication of microorganisms. The cell division initiates the "quorum sensing" (QS) molecules and further EPS production. In addition to enabling aggregation of bacterial cells by forming micro colonies, biofilm matrix contributes to further adhesion of biofilm to the surface on which it is formed (Kaplan JB, 2010). These first colonists facilitate the arrival of others by providing adhesion sites and the matrix that holds the biofilm together. *Streptococcus mutans*, *Lactobacilli* spp. and *Candida albicans* are the predominant microorganisms found in dental plaque associated with a caries lesion (Signoretto *et al.,* 2009). *Streptococcus mutans*, *Lactobacilli* spp. are considered crucial for the initiation and progression of dental caries as they have shown by their more acidogenic and acidophilic properties than those of other oral bacteria (Shu *et al.,* 2000). They can convert dietary carbohydrates into acid, which lowers the pH of the environment, and solubilizes the calcium phosphate of the enamel to produce a caries lesion. The development of biofilm is because of adherence of new planktonic cells in combination with continuous growth of already bonded cells and EPS production. Within the formed micro colonies, bacteria are bonded with intercellular bonds, surrounded with EPS that binds them together. The bacterial cells already colonized and incoming continue to divide until a three-dimensional mixed-culture biofilm is formed. The extracellular matrix Polymer production causes the development of further complexity, consisting of soluble and insoluble glucans, fructans, and heteropolymers that contributes in structural and functionally organized(Marsh, 2004). This matrix is one of the key structural aspects of the plaque biofilm. The growth potential of one bacterial biofilm is limited with availability of nutrients, capacity of their perfusion to the cells and elimination of metabolic products. Other factors that affecting biofilm maturation are pH, oxygen perfusion, sources of carbon and osmolarity (Milanov, 2008). Biofilm maturation changes the conditions within the micro-environment that surrounds the bacteria in terms of cell population density, pH, presence of nutrients and

oxygen, while the difference in metabolic and reproductive cell functionality arises as the consequence of heterogeneity of the environment (Kaplan, 2010). The biofilm development process is quite slow, and biofilm can be evaluated as mature after several days (Stoodley, 2002). Fully mature biofilm consists of bacterial cells, EPS, and interstitial water channels that enable the exchange of nutrients and elimination of waste metabolic products (Fux, 2004). Dispersion is the last stage of biofilm development characterized by deadhesion of microorganisms from the biofilm structure as the response to the changed environmental conditions, irrespective if they are caused by the lack of nutrients or by other unfavorable impacts. The dispersion mechanism can be active (implies the mechanisms initiated by the bacterial cells themselves) or passive (refers to dispersion of bacteria mediated by shear forces, abrasion, human factors, predation over bacteria in biofilm) (Kaplan,2010). Deadhesion of bacteria from the biofilm can be carried out via erosion, sloughing, seeding, and abrasion (Kaplan *et al* ,2010; Sheffield *et al.*,2012). Biofilm growth and deadhesion are mutually dependent processes, and deadhesion rate increases with biofilm maturation.

2.1.1 Extracellular polymeric substances (EPS)

The production of extracellular polymeric substance or biofilm matrix is the main precondition for biofilm formation (Branda SS *et al* 2005; Flemming HC *et al.*, 2007). Biofilm matrix is highly hydrated substance, which consists of water, exopolymer, microorganisms, and products of their activities. Biofilm matrix exopolymers are usually composed of polysaccharides (40-95%), proteins (1-60%), nucleic acids (1-10%), and lipids (1-40%). The composition of EPS depends on the present microorganisms, temperature, and availability of nutrients (Flemming *et al.*, 2002).EPS is the basis of three-dimensional biofilm matrix structure and it is responsible for adhesion of biofilm to surfaces as well as for its consolidation (cohesion).

2.2 Quorum sensing

The intercellular communication between bacteria , carried out through production of the signalling molecules marked as autoinducers (AI) (Giaouris *et al.,* 2012). In low population density, the production of signaling molecules is at the minimal level; as the population density rises the production of signaling molecules grows. Since signaling molecules pass

through a membrane of bacterial cells, their concentration in the environment is almost equal to the concentration in the cell. Upon reaching the critical concentration, the signaling molecules return into the cell via diffusion or binding for specific receptors, which initiates a range of cascade reactions enabling coordinated expression or repression of appropriate sets of genes. As this phenomenon is conditioned with the population density, namely it occurs once the appropriate population density (quorum) is reached, it was called quorum sensing (Antunes *et al.*,2010). Quorum sensing is considered the key process in regulation of expression of genes responsible for different physiological activities such as competition, symbiosis, motility, sporulation, bioluminescence, production of antimicrobial peptides, virulence regulation, as well as some of the genes that are responsible for biofilm formation regulated by "quorum sensing" system (Giaouris *et al.,* 2012; Annous *et al.,* 2009). Since "quorum sensing" depends on population density it does not occur in the initial but rather in later stages of biofilm formation.

2.3 Microorganism's variable nature inside biofilm

Biofilms are complex structures consisting of pure or mixed microcolonies surrounded by a glycocalyx and it is often associated with oral diseases such as dental caries, marginal and apical periodontitis, peri-implantitis, and mucositis. Bacteria in biofilms behave differently from those planktonic microorganisms, as in biofilm an organized and interconnected system is maintained rather free-floating (Socransky *et al.*, 2002). Microorganisms enclosed into the biofilm matrix show resistance to antimicrobial agents compared to planktonic cultures of the same microorganism. The researchers in this field show that resistance of microorganisms enclosed into the biofilm formation may be up to 1,000 times higher. Biofilm-associated bacteria found in different body sites are difficult to eradicate, and in cases of medical implant devices removal of the infected device is often required for an efficient treatment of biofilm infection (Cerca *et al.*, 2005). Salivary flow transported out oral microorganisms that cannot adhere to a surface out of the mouth and down the digestive tract. It is therefore all oral bacteria possess mechanisms of adherence to solid surfaces coated with salivary pellicles such as teeth. The extracellular biofilm matrix acts also as a dilution gradient as it slows down the penetration of antimicrobial substances, which provides additional time for cells to

transfer new genes for resistance before they are caught by antimicrobial agent. To allow transfer of nutrients and waste to the overlaying bathing fluid there are water channels inside the biofilm. To enable changes in the biofilm bacteria initiates cell-to-cell communication and start transfer of genetic information (quorum sensing) within the biofilm which enables bacteria to communicate with each other and to other species inside different layers. Bacteria sense one another and regulate variety of physiological activities like symbiosis, virulence, motility, antibiotic production, and biofilm formation using Quorum sensing (Costerton *et al.*, 2003). In the oral flora Cross-feeding or metabolic cooperation is well-documented among certain bacterial species. The lactic acid produced by *streptococci* and *Porphyromonas gingivalis* can be utilized by *Veillonellae*. Similarly, to stimulate the growth of *T. denticola* isobutyrate secreted by *P. gingiivalis* is used (Kolenbrander *et al*., 2002).

2.4 History and development of dental health

Since prehistoric times till 19th century scientific discovery continues to increase in the 20th century and modern scientist and dentist are introducing new technique and methods in dental science (Slavkin, 2014).Molecular-based, culture-independent techniques, such as the use of 16S rRNA profiling, have provided important new insights into the diversity of the microbiome within the oral cavity (Crielaard *et al*., 2011).Molecular-based, culture-independent techniques, such as the use of 16S rRNA profiling, have provided important new insights into the diversity of the microbiome within the oral cavity (Crielaard *et al*., 2011).Molecular-based, culture-independent techniques, such as the use of 16S rRNA profiling, have provided important new insights into the diversity of the microbiome within the oral cavity (Crielaard *et al*., 2011).The human body is home to many indigenous microorganisms, with distinct communities at different anatomical sites (Dethlefsen *et al.*, 2007). The oral cavity is also home to microbial communities with important implications for human health and disease. Chronic periodontitis is one of the most common inflammatory conditions worldwide, and is associated with bacterial community structures that are distinct from those of health.

Efforts to characterize microbial diversity increasingly rely on cultivation-independent, molecular techniques (Hugenholtz, 2002; Schloss and Handelsman, 2004), since the vast

majority of bacteria are yet to be cultivated. Most of these molecular studies are based on the (16S) ribosomal RNA (rRNA) gene because of its universal presence in cellular organisms, the presence of conserved regions, and its reliability for phylogenetic analysis (Woese and Fox, 1977). Recent molecular surveys of the human distal gut microbiota have shown that each individual gut is home to 500 – 3000 bacterial species, with a large degree of interindividual variation (Dethlefsen *et al.*, 2007; Dethlefsen *et al.*, 2008). Using rRNA gene-based techniques, it is estimated that the human oral cavity harbors 500–700 different bacterial species (Dewhirst, 2008). The link between microbial species that form part of the microbiota of the oral cavity with oral diseases is well established (Jenkinson *et al.*, 2005). Genetic and phenotypic diversity completes hallmark of biofilms which enhances the robustness of the bacterial community (Ehrlich *et al.*, 2005).

In each stage of development oral biofilm like other biofilms seems to acquire new microbial species, including *Lactobacillus casei, Streptococcus. mutans, S. sanguis, , S. sobrinus and, S. mitis* which due to their pathogenicity could damage the enamel and gum tissue and create variety of health hazards (Bersan *et al*., 2014).

2.5 Oral health condition in Nepal

Oral health knowledge is considered to be an essential prerequisite for health related behavior (Al-Ansari, 2003). The World Health Organization (WHO) has defined oral health as “A standard of the oral and related issues which enables an individual to eat, speak and socialize without active diseases, discomfort or embarrassment and which contributes to general well-being”(Department of Health Services, Annual Report 2009/10). Oral hygiene is the practice of keeping the mouth and teeth clean to prevent oral problems and oral malodor. In 1990 advent of the first qualified orthodontic practitioner, beginning of orthodontic practice in 1998, establishment of Orthodontic & Dent facial Orthopedic Association of Nepal (ODOAN) in 2007, subsequently ODOAN’s membership to World Federation of Orthodontists (WFO) and Asia Pacific Orthodontic Society (APOS) in 2008, organization of first orthodontic scientific conference in 2008, commencement of orthodontic post-graduate program (MDS) in 2008, and publication of first orthodontic journal (OJN) in 2011. The orthodontic specialists were only two in number till the late 90’s, the present number of qualified orthodontists is about thirty-five. According to the

Nepal national pathfinder survey 2004, it was found that the prevalence of dental decay is low in adolescents who are studying in school (i.e., 25.6% for 12- to 13-year-olds and 25.6% for 15- to 16-year-olds). The probable reason for this is the use of fluoridated toothpaste. However, periodontal problems (especially gingivitis and calculus) in adolescence is high (i.e., 62.8% for 12 to 13 years and 61% for 15 to 16 years).

According to the Annual Report (2009/10), 3,92,831 have dental caries/toothache, 73,309 have periodontal diseases (gum), 62,747 have other disorders of teeth and 1,13,819 have oral ulcer, mucosa and other related diseases. In the total OPD visit, 3.08%is related to oral health (The World Oral Health Report 2003). It shows that Nepal has a high incidence of problems in the area of dental health. There are different divisions working under the Department of Health Services, but not the Oral Health Division. The second long term health plan (1999-2017) has put oral health into essential health care services and oral health for the first time into a primary health care approach. Although the oral health policies and strategies are established, the limited fund resources and manpower are a constraint to implementation.

Poor oral health and untreated oral diseases and conditions can have a significant impact on quality of life. In developing countries like Nepal, oral diseases are the most common problems due to lack of education and poverty. The most common dental problem of the Nepalese is dental decay and periodontal diseases.

2.6 Microbes and oral infections

Older studies suggested a direct, proportional relationship between plaque amount formed at the specific location with level of inflammation and tissue destruction in the site (Lövdal *et al.*, 1958). Recent studies have suggested that several oral commensal bacteria are opportunistic pathogens rather than straight pathogens. they are mostly involved in systemic disease, such as bacterial endocarditis, aspiration pneumonia, pre-term low birth weight and cardiovascular disease (Chakraborty *et al* ., 2014). Following the ingestion of dietary carbohydrate, most of the commensal community shows a shift to an acidogenic, aciduric flora that fosters dental caries. Further, the administration of broad-spectrum antibiotics selective to antibiotic resistant bacteria can result in imbalances in the commensal flora creating opportunistic infections. Amylase-

streptococcus interaction evolution is another fine example (Avila M *et al.*, 2009). After biofilm formation different bacteria changes into pathogenic forms and causes medical problems in respect to their locations. Biofilm disease has been viewed as various diseases that affect a variety of tissues and structures where bacterial biofilms can be established, including ear, nose, throat, mouth, eye, lung, heart, kidney, gall bladder, pancreas, nervous system, skin, bone, and virtually every implanted medical device (Lewis, 2007). More specifically systemic diseases, endocrinopathies (e.g., diabetes mellitus), immunosuppression (e.g., acquired immunodeficiency syndrome), hematologic disorders (e.g., neutropenia) and genetic disorders (e.g., Down's syndrome, leukocyte adhesion deficiency syndrome) have been linked to the destruction of the periodontium caused by oral problems (Kinane, 1999).

Some of the predominant groups present in the mouth include *Streptococcus*, *Neisseria*, *Veillonella*, *Actinomyces* and other obligate anaerobes (Avila, 2009) These organisms maintain a mutualistic relationship with the host by preventing pathogenic species from adhering to the mucosal surface (Liljemark and Bloomquist, 1996). Oral microflorae can cause dental plaques and are also a common cause dental caries and periodontal disease (Kigure *et al* 1995; Sbordone and Bortolaia, 2003).

Streptococcus mutans are found in almost all dental caries and have significant role in initiating carries alongside other common commensal species *Actinomyces and lactobacilli, S. gordonii, S. parasanguinis, and S. mitis, S. epidermidis* of oral cavity (Geethashri *et al* ., 2014). *E. faecalis and S. aureus* are almost present in every root canal of tooth whereas Pathogenic and malodor causing bacteria includes *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Treponema denticola, Prevotella intermedia* (Ahmed *et al* ., 2014). After taking into account factors like smoking, alcohol consumption, socioeconomic status we can tend to persist the relationship of oral bacteria and development of systemic diseases (Chakraborty *et al* ., 2014). Various genetic characteristics relating the oral cavity (e.g., type and quality of bone surrounding the tooth root) and epigenetic modifications are all considerable factors that determine host response to possible insults and confer susceptibility to periodontal health. It has been reported that as compared to non-smokers heavy smokers are facing a two- to eight-fold

increased risk for periodontal attachment loss and/or bone loss (Chien HH & Hart, 2013). Cigarette smoke extract increases biofilm formation in the oral pathogen, *P. gingivalis*, (Bagaitkar *et al*, 2010).Smoking is an important cause of severe gum disease in the United States (Eke *et al*. 2010). It has become clear that smoking promotes bacterial adhesion and biofilm formation in several other key pathogens, including *S. mutans*, *S. aureus*, *P. aeruginosa* and *S. pneumoniae*. Alcohol slows the production of saliva, which helps neutralize the acids produced by plaque. An accumulation of these acids can lead to the early stages of periodontal disease. (Eugênio *et al*, 2015)

The World Health Organization and International Agency for Research on Cancer classified betel nut as a group 1 human carcinogens (Pankaj, 2010). The betel nut may be cytotoxic to periodontal fibroblasts and may exacerbate preexisting periodontal disease as well as impair periodontal reattachment. Some researchers have shown that loss of periodontal attachment and calculus formation is greater in betel chewers. (Chang *et al*, 1998; Anerud *et al*, 1991; Parmar *et al*,2008).

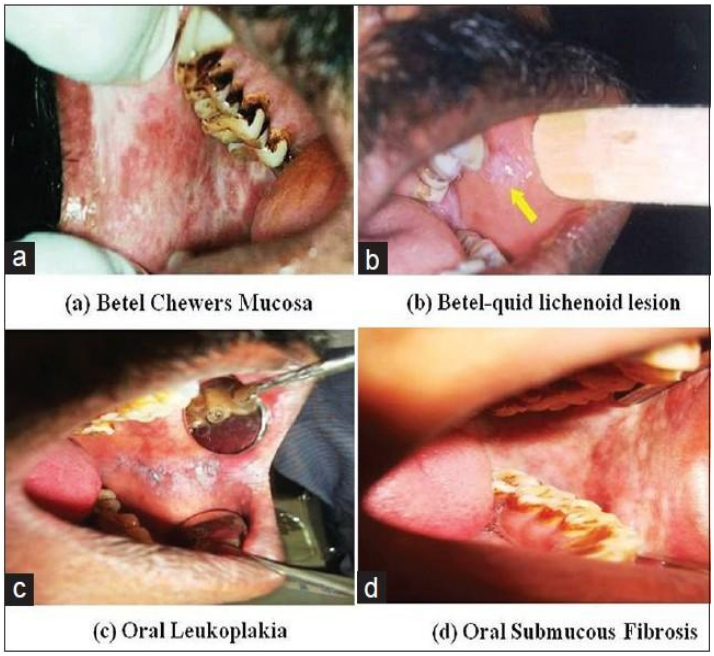

Figure 2.2 Oral health hazard (Anand *et al*, 2014)

2.7 Bacterial enzymes

Bacteria produce exoenzymes in cases of stress, virulence factors using their adoptive genes. Bacteria and fungi produces exoenzymes to digest nutrients in their environment, and these organisms can be used to conduct laboratory assays to identify the presence

and function of such exoenzymes (Roberts, 2013). Some pathogenic species use exoenzymes as virulence factors to assist in the spread of these disease causing microorganisms(Duben, 2010).

Bacteria grown under aerobic conditions when tested for an array of enzymes, including gelatinases, caseinases, lipases, esterases, cellulases, xylanases, amylases and chitinases shows no activity for all enzymatic tests but a pattern of enzymatic activity can be observed (Pinto-Tomás, 2007).

Amylases are glycoside hydrolases that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans where it begins the chemical process of digestion. They can cleave α-1,4-glycosidic bonds by acting on random locations(α-amylase) or specific sites along the starch chain to break down long-chain carbohydrates into maltotriose and maltose from amylose, or maltose, glucose and limit dextrin" from amylopectin (Udani, 2004). Most amylases forms is also produced by plants, some group of fungi (ascomycetes and basidiomycetes) and bacteria (most *Bacilli*) where they are used to degrade extracellular starches (Rejzek *et al.*, 2011).

Cellulases break down the cellulose molecule into monosaccharides ("simple sugars") such as beta-glucose, or shorter polysaccharides and oligosaccharides. Cellulase hydrolyses the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin, and cereal beta-D-glucans. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides such as starch(David *et al*., 2008) Cellulose makes a major constituent of plants so cellulases are considerable important for plant consumers (Lo *et al.*, 2011). Few bacteria synthesize the complete enzyme system that can result in extensive hydrolysis of the crystalline material found in nature. These few bacteria should be called “true cellulolytic “bacteria and those bacteria that produce some endoglucanasesand ß-glucosidases, but not the complete system, are called “pseudocellulolytic” (Coughlan, Mayer, 2013). In many ruminants like cattle and sheep and hindgut fermenters like horses, cellulases are produced by symbiotic bacteria. (*Bignell*, 2011) and some termites (Watanabe, 1998).To catalyze the hydrolysis of triacylglycerols into diacylglycerols, monoacylglycerols, free fatty acids and glycerol enzymes Esterase and Lipases both are functional (Kempka *et al.*, 2008). Lipase helps in digestion, transport and processing of dietary lipids

(e.g. triglycerides, fats, oils) in living organisms. Lipases have marked preference for catalysing the bonds of long chain fatty acids (>10 carbon atoms) while as substrates esterases catalyze the hydrolysis of carboxylic ester bonds of short chain fatty acids (<10 carbon atoms)(Gilham, 2005). Lipases act at a specific position on the glycerol backbone of a lipid substrate (small intestine)human pancreatic lipase (HPL) (Winkler, 1990) which converts triglyceride substrates found in ingested oils to monoglycerides and two fatty acids. Some lipase producing bacteria includes *Bacillus, Pseudomonas and Burkholderia*. Lipase/esterase-producing bacteria have been found in diverse habitats such as oil spilled soil, dairy and industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs and variable oral samples (Vakhlu *et al.*, 2006).

Pectinase constitutes a complex enzymatic system responsible for the degradation of pectic substances (Ahamed *et al*., 2003). Pectinase breaks down pectin which is a polysaccharide found in mostly plant cell walls. Pectic substances are glycosidic macromolecules with high molecular weight. They form the major components of the middle lamella and primary plant cell wall. It includes major three groups of enzymes pectolyase, pectozyme, and polygalacturonase. The main chain of pectin is partially methyl esterified 1, 4 – D-glacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid. This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages.Major Pectinase producers are plants and microorganisms (fungi) like *Aspergillus niger*, *Penicillium jenseni* and *Penicillium citrinum* rather than bacterial species (Nwodo-Chinedu *et al.*, 2005).

Any enzyme that performs proteolysis, by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. A bacterium uses proteases to hydrolyse and break the proteins down into their constituent monomers (amino acids) (Sims, *2006*). *Bacillus* species are major sources of industrial and commercial valuable enzymes; however, some of thermophilic *Bacillus* species have been reported to produce useful enzymes (Rao *et al.*, 1998, Rahman *et al.*, 1994). Few thermophilic strains such as *Bacilluss tearothermophilus* (Sookkheo *et al.*, 2000), *Bacillus licheniformis* (Ferrero *et al.*, 1996), and *Bacillus pumilus* (Kumar, 2002) were explored for protease production. In natural sources impact can be observed at the overall microbial community level as protease breaks proteins ultimately in to carbon, nitrogen, or sulfur limitation. Bacterial protease may also

act as an exotoxin (exfoliative toxin), virulence factor in bacterial pathogenesis and destroy extracellular structures (Sims, 2002). Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006). Several species of strains including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis*) are reported to produce proteases (Ellaiah *et al.*, 2002).

2.8 Bacterial identification and characterization

The traditional techniques are slow and lengthy to obtain a result, but also traditional culture-based techniques are used to analyze the large numbers of microbiological samples till date. In the industrial manufacture of many microbial products, quick and reliable medical examination culture-based techniques cannot provide real-time information on the physiological status of the organism in situ where in place modern and real time technique is quiet handy in gathering usable data's relating the development and changes of organism (Veal *et al*., 2000). Fluorescent nucleic acid binding dyes, which displays a fluorescence, suggests that the dye hexidium iodide (HI) selectively stains gram-positive bacteria florescenses red and SYTO 13 is a group of cell-permeating nucleic acid stains and fluoresces green (Haughland, 1996). These dyes have been found to stain DNA and RNA in live or dead eukaryotic cells (Poot, 1997). The combination of these two dyes applied to mixed bacterial populations would result in all bacteria being labeled, with differential labeling of gram-positive bacteria (HI and SYTO 13) and gram-negative bacteria (SYTO 13 only). The different fluorescence emission wavelengths of the two dyes would ensure differentiation of gram-positive from gram-negative bacteria by either epifluorescence microscopy or flow cytometry when equipped with the appropriate excitation and emission filters.

Flow cytometry is a useful tool for simultaneously measuring multiple physical properties of individual particles (such as cells). Cells pass single-file through a laser beam. As each

cell passes through the laser beam, the cytometer records how the cell or particle scatters incident laser light and emits fluorescence. Using this flow cytometric analysis protocol, one can perform a simultaneous analysis of surface molecules at the single-cell level. Combination of flow cytometry alongside fluorescent staining fulfills modern requirements of real time instrumentation. For example, fluorescent tagging using fluorescent antibodies, fluorescent in situ hybridization and fluorogenic enzymatic substrates can be used to label microorganism's expression of particular antigens, phylogeny and specific enzyme activities. Similarly, viruses based Reagents are also available to enable their direct detection in environments such as sea water by sufficiently bright stain (Veal *et al.*, 2000). Different molecular techniques like polymerase chain reaction and pyrosequencing-based profiling have enabled the isolation of bacterial 16S rRNA gene sequences and classification of bacteria from bio-samples (Choi *et al.*, 1994). More than 1100 taxa were identified from oral cavities. The results have been compiled in the NIH-funded "Human Oral Microbiome Database" (Dewhirst *et al.*, 2010).

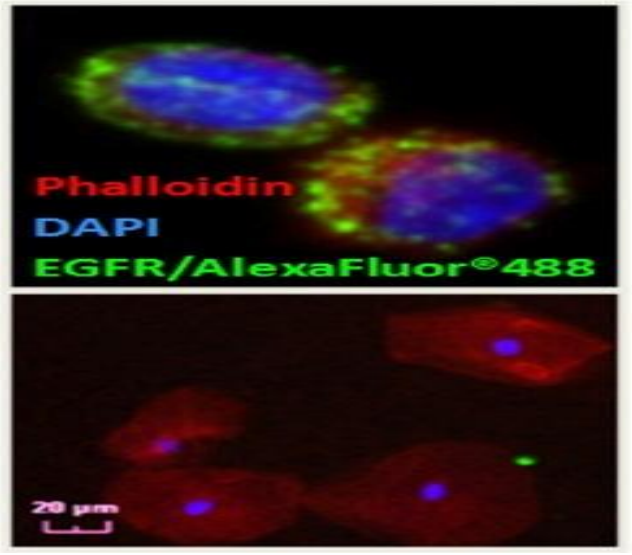

Figure 2.3 Fluorescence-microscopy images of cells stained with a cocktail of fluorescent dyes and antibody-based reagents such that the cytoplasm appears red (Phalloidin), the nucleus blue (4'-6-diamidino-2-phenylindole: DAPI), and EGFR is green (McDevitt *et al*., 2016)

Microarrays consist of an orderly arrangement of probes (Oligonucleotides, DNA fragments, proteins, sugars or lectins) attached to a solid surface. The main advantages of microarray technology are high throughput, parallelism, miniaturization, speed and

automation (Schrenzel,2009). Microarrays combines the potential of simultaneous bacterial identification and speciation. The rapid identification of the bacteria in clinical samples is important for patient management and antimicrobial therapy. DNA microarray-based approach is used for the quick detection and identification of bacteria using species-specific oligonucleotide probes designed for specific regions of various targeted genes. Oligonucleotide microarrays can enable bacterial detection utilizing conserved bacterial genes (Small, 2001), species identification (Wilson *et al*., 2002) as well as genotyping of bacterial pathogens, by using large sets of discriminative epidemiological markers (Chizhikov *et al*., 2001; Call *et al.*, 2003)

2.8.1 PCR Based Bacterial Identification

Using a DNA based assay, the bacterial strains can easily be detected directly from clinical samples or from small amounts of cultured bacterial cells, thus improving the sensitivity and decreasing the time required for bacterial identification. PCR has been particularly useful in this regard, which relies on primer sequences designed to facilitate bacterial identification at any level of specificity: strain, species or genus. A real-time PCR assay using a TaqMan probe (a fluorescent DNA probe based on the 5’ to 3’ exonuclease activity of Taq polymerase) has been developed for quantitative DNA analysis (Holland *et al.,* 1991). In recent years, real-time PCR methods have been developed and described for the rapid detection and identification of several bacterial strains. Real-time PCR is a promising tool for distinguishing specific sequences from a complex mixture of DNA and therefore is useful for determining the presence and quantity of pathogen-specific or other unique sequences within a sample. Real-time PCR facilitates a rapid detection of low amounts of bacterial DNA accelerating therapeutic decisions and enabling an earlier adequate antibiotic treatment. TaqMan real-time PCR assay has advantages in terms of sensitivity and rapidity over other techniques(Socransky *et al.*, 1994).

2.8.2 DNA sequencing

DNA sequencing is a molecular technique which can be used to identify bacteria and fungi (both yeasts and moulds). The analysis uses a conserved region of DNA (ribosomal DNA) found in all bacteria or yeasts which can be compared to a validated reference database to generate a percentage match with known organisms.

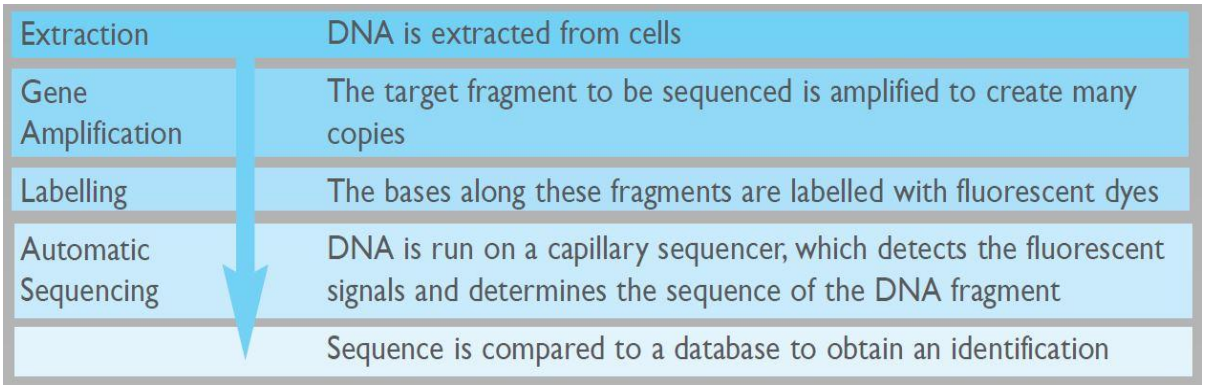

Figure 2.4 steps in sequencing

The analysis and sequencing of the 16S rRNA genes of various bacteria, using DNA sequencing, a state-of-the-art technology for phylogenetic studies was initiated by Carl Woese (Woese *et al.*, 1997). The polymerase chain reaction (PCR) and cloning strategies that target bacterial 16S rRNA genes can be used in combination to determine the bacterial composition of any ecological site. The 16S rRNA gene amplicons are cloned into Escherichia coli after DNA is amplified by PCR using conserved primers for the 16S rRNA genes. Then cloned inserts are sequenced to determine species identity (Paster *et al.*, 2006). This approach has been used in clinical studies to define the association of single or sets of bacterial species with health and disease in the oral cavity. Problems created in previous techniques can be overcome by a single technology Using 16S rRNA sequencing, and considerable discovery of novel genera and species have been made using this technique. Providing genus identification in >90% of cases, and identification of 65–83% of these at the species level considerably shows advantage of this technique (Drancourt *et al.*, 2000). Till date 16S rRNA sequencing is particularly helpful in identifying unusual bacteria that are difficult to identify by conventional methods (Mignard, 2006).

Sequencing contributes to a better understanding of the epidemiology and pathogenic role of identified bacteria, which has not been possible in the past conventional methods. Lie for instance, 16S rRNA sequencing of invasive Streptococcus infections, are now recognized in Asia which had previously been reported in North America only (Sun *et al.*, 2007).16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (Chen *et al.*, 1989)

2.9 Mass spectrometry–based bacterial characterization and identification.

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. Gas chromatography (GC) has proved an extremely valuable tool for identifying Volatile Organic Compounds associated with bacterial infection in dental research (Persson *et al.,* 1990).In most of the cases, Gas chromatography (GC/MS) is used for direct analysis of components existing in plants and bacterial extract (Sermakkani & Thangapandian, 2012).Gas chromatography first separates the individual chemical components and then MS ionizes and identifies them by their structure and molecular weight even at very low concentrations. GC-MS have been increasingly used for the analysis of volatile substances, non-polar components, alcohols, alkaloids, phenols long chain Nature branched chain hydrocarbons, acids, esters, and other bioactive components (Sivakumar *et al*., 2015). The compound to be analyzed by GC/MS it must be sufficiently volatile and thermally stable.

Differential distribution coefficients are the main basis of Separation where a carrier gas (an inert gas such as helium or a unreactive gas such as nitrogen) is a mobile phase and a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column as the stationary phase. In the gas separator, the gaseous compounds interact with the walls of the column in process of being analyzed, coated with different stationary phases. This causes elution of each compound at a different time, the retention time of the compound. Secondly, in an oven the column through which the gas phase passes is located where the temperature of the gas can be controlled, better than column chromatography where no such temperature control is available. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas (Sermakkani & Thangapandian, 2012)

2.1 Some recent and interesting research findings associated with oral microflora.

More than 700 bacterial species or phylotypes, of which over 50% have not been cultivated, have been detected in the oral cavity. Aas *et al* carried out an extensive study

in this regard. It is important to note that micro-organisms in the oral cavity are responsible for various oral diseases, and an existence of an inter-relationship between the two is strongly hinted (Aas *et al.,* 2009). Similarly, Takahashi attempted to study the microbial ecosystem of the oral cavity and its relationship with various oral diseases (Takahashi, 2005). Association was found between oral microflora with other systemic diseases. Gendron suggested that Oral cavity is a reservoir of bacterial pathogens that can provoke focal infections (Gendron *et al.*, 2000). In a study, to understand influence of space maintainers on magnitude of oral microflora strict experiment was carried out and identified that both fixed and removable space maintainers led to an increase in the number of microorganisms in the oral cavity as well as to increases in the periodontal index scores. This gives us proof that space maintainers may serve as a source of infection making it significant that it is more difficult to control the biofilm in metal and other extra-oral surfaces compare to teeth and that special attention must be given to their oral hygiene (Arikan *et al*., 2015). When study was carried out for understanding oral-cariogenic bacteria among cancer treating children, a significant increase in the number of cariogenic bacteria in the saliva during episodes of chemotherapy-induced neutropenia was found suggesting that the activity of dental caries increases in children undergoing antineoplastic treatment (Olszewska & Błaszczak, 2016).

Doxycycline is regarded as an effective treatment for periodontal inflammation. So it has possibility of uses in much of oral medical products (Kim *et al*., 2015). Similar antimicrobial ingredients (such as stannous fluoride, cetylpyridinium chloride (CPC) and triclosan) which are typically effective against a wide range of bacteria are frequently included in dentifrice and rinse formulations to improve their anti-plaque and anti-gingivitis efficacy. For example, stannous fluoride gel has been shown to result in more than a 99% reduction in subgingival microbiota within 30minutes in periodontal pockets. Conversely, microbial sensitivity to antibacterial ingredients can vary significantly: *Enterococcus faecalis* and *Staphylococcus aureus* have been found to be more resistant to stannous fluoride than *Prevotella intermedia* (Huang *et al*., 2016). *Zataria multiflora* extract has antimicrobial properties and can be used for disinfection of elastomeric ligatures. In vivo studies suggests good efficacy of the incorporation of this herbal extract in mouthwashes for orthodontic patients so uses of herbal mouthwashes such as persica and garlic extract has

increased by orthodontic patients in recent days (Hossein *et al*., 2015). Using probiotics has recently been introduced to reduce the incidence of dental caries. It consists of live microbial food supplements that beneficially affect the host, and hence are considered an alternative way to eradicate the infections in gut as well as oral linings. Study had shown that probiotic yogurt could reduce the *Streptococcus mutans* counts of oral cavity significantly. *Sorangium cellulosum* produces chlorine-containing metabolite,"t chlorotonilA" which was found to exhibit promisinganti malarial activity giving noble idea for treatment of parasites other than antibiotic and medicines (Jana *et al*., 2015). Recently, it was demonstrated that volatile organic compounds (VOCs), including hydrocarbons, alcohols, ketones, aldehydes, ethers, esters, terpenes, terpene derivatives, and several heteroaromatic compounds produced by some bacteria can influence the growth of fungi. Similarly, Xenorhabdus and Photorhabdus produce several compounds exhibiting antibacterial and antifungal activity, many of which have been identified from the bacterial cultures. Some of these include compounds such as nematophins (indole derivatives), xenorhabdins (dithiolopyrrlones), xenocoumacins (benzopyran-1-one derivatives) and anthraquinones which act as antibacterial and antifungal metabolites.

Study had suggested that Microbiota affects drug metabolism, directly and indirectly. Since most of medicines are orally administered, so these constituents are inevitably exposed to the oral microbiota and the interplays between medicines constituents with oral microbiota are expected. So administration of drugs for any systemic disease also in synergy affects the oral microflora diversity. And similar is case for gut microflora (Chen *et al*., 2016).

Hydrogen Sulfide exerts antimicrobial effects on certain oral *Streptococcus*, potentially contributing to the decrease in health-associated plaque microflora contributing to oral biofilm formation (Ooi & Tan, 2016). Considering Histatin 5, a salivary antifungal peptide, study was conducted. It was found that *C. albicans* was able to grow in human saliva without addition of glucose, and in the stationary phase could survive for more than 400 hr. *Candida albicans* grown in saliva was more than 10 times less susceptible for salivary histatin 5 than *C. albicans* cultured in Sabouraud medium. This signifies that according to growth microenvironment species can adopt to them (Valentijn-Benz *et al.,* 2015). The antimicrobial compounds , organic acids lactic acid acetic acid phenyl-lactic acid, putative

bacteriocin, hydrogen peroxide, and diacetyl are produced from CFS of lactic acid bacteria were effective in repressing the growth of opportunistic wounding dermal pathogen *Staphylococcus epidermidis* (Liong & Lau, 2014). Similarly, *E. faecium* produced only lactic and acetic acid (24.5 and 12.2 mmol/L, respectively) and was able to inhibit both vegetative cells and spores of the *B. cereus* like bacterias, at a final fermentation pH of 5.0 and at pH 6.5. This would indicate the action of other metabolites, different from organic acids, present in the cell-free supernatant (Soria &Audisio, 2014). *Streptomyces exfoliates* produces protease, fatty acid methyl ester, its organic solvent extracts exhibited inhibitory activity to various fruit-rotting fungi. Antifungal secondary metabolites were found to be polyene in nature and their mode of action seems to vary according to active compounds (Choudhary *et al.*, 2014). Isatin (1H-indole-2,3-dione) scaffold produced in organism of marine areas has shown immense potential as future antibacterial/antifouling candidate (Majik *et al.*, 2014).Variety of organic acids reduced the intensity of the pathogen in disease environment (Maggi *et al*., 2013). Study had shown that 3-methoxy-5-methyl-4-oxo-2, 5-hexadienoic acid produced from *Aspergillus persii* can be used as a lead molecule for development of synthetic bactericides for control of various plant diseases causing pathogens (Nguyen *et al*.,2016). Significant salivary specificity is noted in specific bacterial species, notably *Streptococcus mitis, Capnocytophaga gingivalis, Prevotella melaninogenica*, in oral cancer patients. Thus, these species can be used as salivary markers for the early detection of oral cancers, improving the survival rate considerably. Such a high degree of bacterial specificity in oral cancers has also provoked the designing of new treatment options for cancer prevention by way of vaccine delivery (Srinivas prasad *et al*., 2015). When studied viable bacteria present within oral squamous cell carcinoma tissue variability and unique properties were obtained (Hooper *et al.,* 2006). Research contended to understand alteration in the oral microflora demographics consequently led to local and systemic infections in patients suffering from oral neoplasms, an investigative study on the inhibition of biofilm present on the surfaces of oral squamous cell carcinomas (OSCC's) was warranted. *S. mitis, Staphylococcus aureus* and *Enterococcus faecalis* were isolated from at least twice as many tumor surfaces before the rinsing. Of the aerobic Gram-negative species isolated; *Haemophilus influenzae, Neisseria* spp and *Serratia* spp. were found more frequently before rinsing than after. Furthermore, *Campylobacter*, *Actinobacillus, Actinomycete-*

temcomitans and *Capnocytophaga* were found more frequently and *Porphyromonas* at the same frequency before Meridol rinsing. Of the Gram-positive anaerobes, *Clostridium* was the only species isolated exclusively before rinsing from the tumor surface (Nagy *et al*., 2010).

CHAPTER 3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

All the chemicals and reagents (Appendix A) that were used during the research work were of analytical grade (Merck Co., Mumbai). The research work was conducted at the Central Department of Biotechnology (CDBT), Kirtipur and Research Institute for Bioscience and Biotechnology (RIBB).

The instruments used during this thesis were all in good working conditions**.**

Petri plates,

Reagent bottles,

Autoclave,

Laminar hood,

Sequencer,

Vortex mixer,

Micropipette,

Rotavapor

conical flasks,

Centrifuge,

Incubator,

Microscope,

GC-MS,

UV spectrophotometer,

Refrigerator

3.1.1 Sample collection

One Hundred Forty Eight (148) patients with oral problem were selected from various parts of country (16 districts). The selection for sampling was random as on the basis of oral samples availability.

Samples were collected from dental clinics, dental camps and dental hospitals and each patient had will to participate and cooperated in the process.

148 samples were collected from

- Baitadi 5
- Butwal (Rupandehi) 11
- Dang 1
- Myagdi 1

- Dharan(Sunsari) 7
- Ilam 5
- Janakpur (Dhanusa) 9
- Jhapa 9
- Kathmandu 7
- Baglung 2
- Nuwakot 14
- Palpa 5
- Pokhara (Kaski) 6
- Bhairahawa (Rupandehi) 8
- Udaipur 1
- Sindhupalchok 46

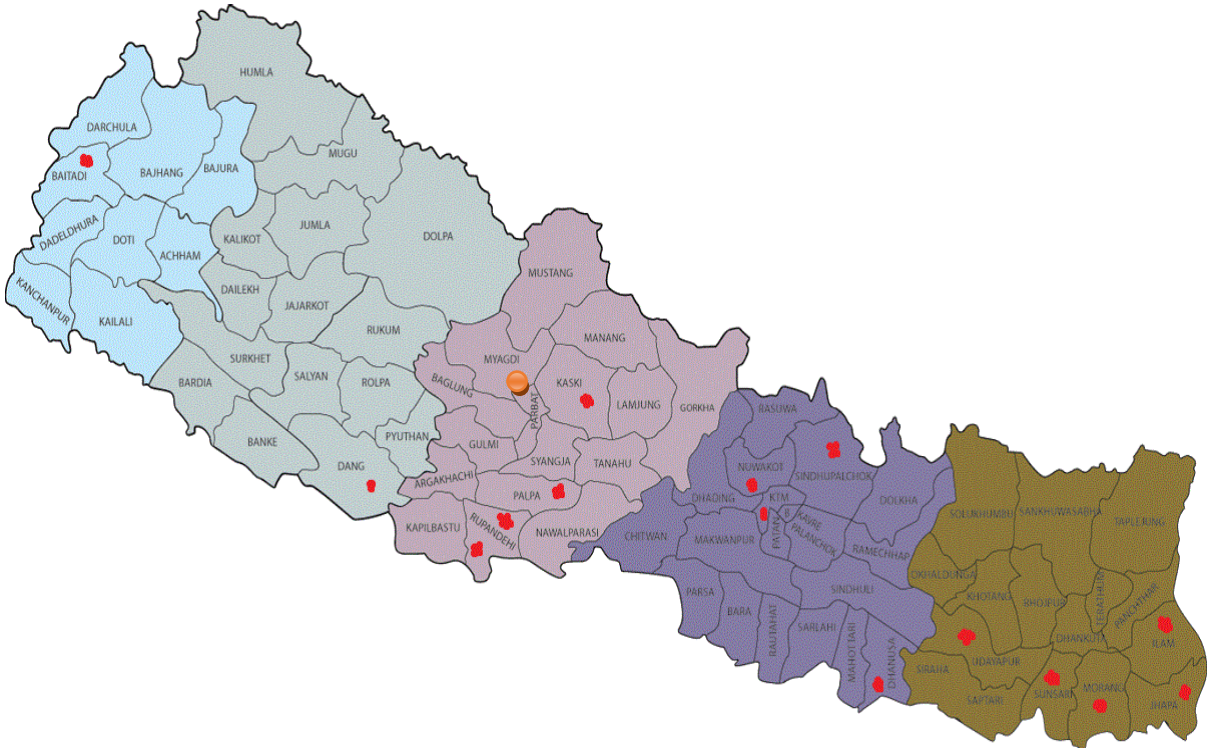

Figure 3.1 Samples collected sites in map of Nepal

Figure 3.2 Sample of collected tooth

3.2 Methodology

All cultivable micro-organisms were isolated using standard microbial techniques. Six (6) different culture media were used for the isolation of different bacteria. Nutrient agar (NA), Muller Hilton agar (MHA) were used to isolate colonies of general cultivable bacteria. Similarly, Mannitol salt agar (MSA) was used to isolate colonies of halophilic bacteria. Blood agar (BA) and Brain Heart infusion agar (BHA) were used to isolate colonies of pathogenic bacteria. For possible fungal strains Potato dextrose agar (PDA) was used(appendix no. 4 to 14). The grown colonies were observed and repeated streaking was done to get a pure colony. For 2/3 days the plates were incubated at 37 degree centigrade, and after that the plates were examined for the growth of bacterial colonies. Most of the sample had positive growth in the nutrient agar plates and the samples that did not showed any growth were discarded. Among the bacterial colonies in the plates with bacterial growth were studied and the morphology of the colony were observed. After the morphological study of the isolated microorganisms by considering their shape, size, color margin, opacity, texture, etc. the microscopic observation was done for reducing the colony numbers having similar appearances and were collected as the same species.

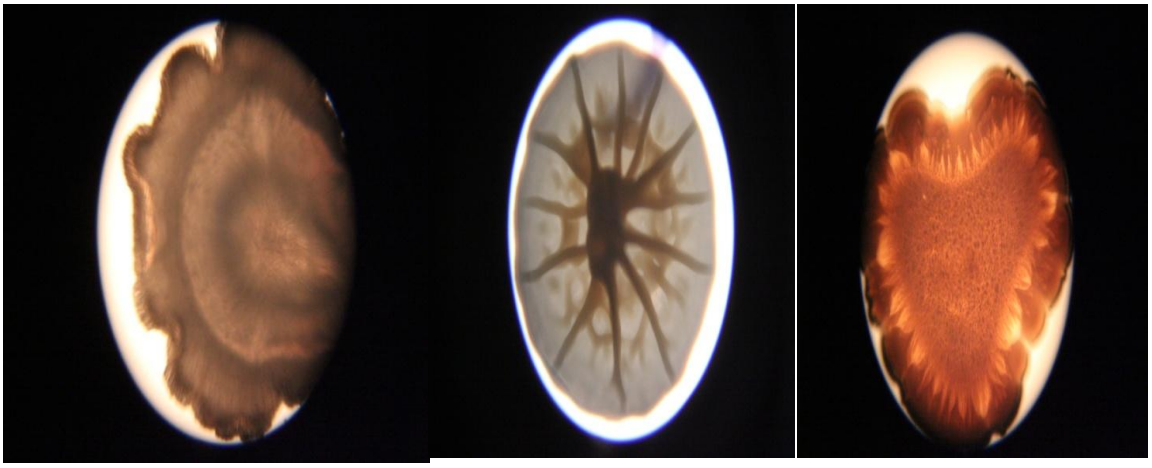

Figure 3.3 Microscope observation of bacterial colony in agar plates

3.3 Enzymatic assay

The isolated colonies were grown in agar plates containing target enzymes substrates. The protease producing colonies were screened by growing in Skim milk agar plates. The clear zone around the grown colony indicates the protease activity. Similarly, cellulase enzyme activity was screened by growing the colonies in Carboxymethylcellulose(CMC) media. The clear halozone observed after pouring 1% congo red indicator and 1N NaCl wash indicates positive cellulase activity. For amylase enzyme screening, 0.1% iodinesolution was poured on the starch plates. For screening of pectinase enzymes, in the pectin plates again 0.1% iodine solution was poured.

3.4 16s ribosomal RNA sequencing

For 16S rRNA gene sequencing, first genomic DNA was extracted by using a Labo Pass Mini Tissue Genomic DNA Isolation Kit (Cosmogentech Inc., Korea). Then PCR amplification of these 16S rRNA gene was done using universal primers, 27F; 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R; 5'-GGT TAC CTT GTT ACG ACT T-3'. The PCR products were purified using the Labo Pass PCR purification kit (Cosmogenetech, Seoul, Korea) and respectively sequenced with the same primers used for amplification.

The sequence of the 16S rRNA gene was compared with that of type strains available in the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) (Kim *et al*., 2012) to find closely related species.

3.5 Construction of phylogenetic tree and bacterial analysis

Few online tools were available for the identification of phylogenetic relationship among species. For this purpose two online tools (clastalW with Clastal omega multiple alignment tool and robust phylogenetic analysis usingPhylogeny.fr) were used. Both tools use same basic principle where first all the extracted sequences were aligned with multiple alignment tools (clustal omega) which were followed by curation, phylogeny and tree rendering to finally construct a phylogenetic tree. A rooted phylogenetic tree was constructed with the help of NCBI extracted ribosomal RNA partial sequences of all identified bacteria's and another additional along with some pathogenically important oral bacteria using these tools (Dereeper *et al* ., 2010).

3.6 GC-MS analysis

3.6.1 Extract preparation

Each purified colony of oral bacteria was sub-cultured in Nutrient broth (500 ml) at 37°C at 250 rpm for 7 days. There by, after 7 days of incubation, the cells were removed by centrifuging at 13,000 rpm and broth was extracted with Ethyl acetate. The bacterial broth and equal volume of Ethyl acetate was kept in continuous 250 rpm movement for a day and the extracted ethyl acetate was separated with the help of micropipette. The collected ethyl acetate was subjected to rota-vaporization in rotary evaporator. After vaporization of ethyl acetate remaining extracts were again collected in 1ml of ethyl acetate. The extract was dissolved in n-hexane before they were subjected for GC-MS analysis.

3.6.2 Instrumental process of GC-MS

Gas chromatography mass spectrometry(GC-MS) analysis of crude extract of dental bacteria was performed in Clarus 500 gas chromatograph (PerkinElmer) equipped with a 30 m x 0.32 mm PerkinElmer Elite-5MS low bleed capillary column with 0.25 mm film phase where the temperature of injector was 200 °C. Sample analysis was performed following a temperature program, 90 °C for 3min, then 5 °C min^{-1} until 260 °C. The GC was coupled with a Clarus 500 mass spectrometer (PerkinElmer) with a mass limit 1,185. The analysis was performed in EI mode (ionization energy 70 eV, source temperature 180 °C).

After completion of GC coupled with MS in mentioned criteria a mass fragment patterns in multiple peaks representing possible compounds were identified. The mass fragmentation pattern of GC separated compounds was analyzed and compared with NIST and WILEY libraries.

CHAPTER 4. RESULTS

4.1 Colony morphology

The bacteria were grown in simple Nutrient Agar plates. The colony number were reduced to about one fourth of the total colonies that is 300 bacterial colonies from around 1200 isolated colonies. The reduction in colony numbers was done by comparing the characters in the respective plates. The shape, size, texture, opacity, margin, surface, and elevation of the colonies were studied and there significant numbers are depicted in bar-graph representations.(Appendix)

Besides these some colonies also showed some pigmentation, haemolysis, pungent odor and coloration. Most of the colonies were white or yellow in color but some showed pink color in MSA plates. Most of these characteristic were helpful to reduce the number of sample size comparing their similar characteristics. High numbers of colonies were moist (186) but significant amount were dry and mucoid. Most of the colonies were irregular (213) and only 6 showed filamentous character. Most colonies were opaque (almost 90%) and few were transparent. One Hundred Twelve (112) colonies were smooth and 121 were rough only few were wrinkled and shiny. Almost half were undulated and remaining were entirely marginal or lobulated but 5 were filiforms. None were concave but about 60% were raised colonies.

4.2 Enzymatic activity

Bacterial enzymatic activity of colonies in respective Nutrient agar plates shows clear enzymatic activities in different bacterial colonies. Most of the bacterial colonies didn't showed any enzymatic activity in regular enzymatic essay plates but more than 50% (173) organisms showed at least one enzyme activities. One hundred eighteen colonies shows no enzyme activities, twenty seven, eighty one , eighteen isolates screened showed positive activity for amylase, protease, and cellulase respectively. 21 colonies showed both cellulase and protease, 19 colonies showed both protease and cellulase, 4 colonies showed cellulase, amylase, and protease activities, and 2 showed pectinase and cellulase activity, but none showed all enzyme activities.

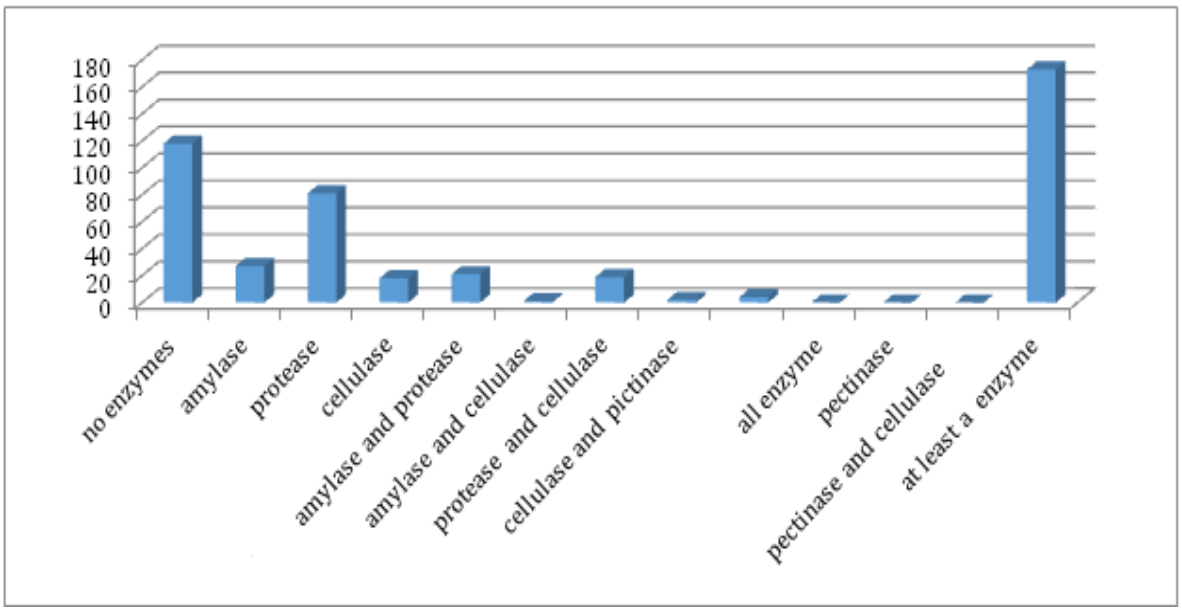

Figure 4.1 Graph showing Enzyme activity of Bacterial colonies

Among all enzymatic colonies, 56 % of colonies (125) showed protease enzyme activity. Similarly amylase (24%) and cellulase (19%) activity are also seen but pectinase enzyme is showed no activity except 2 colonies.

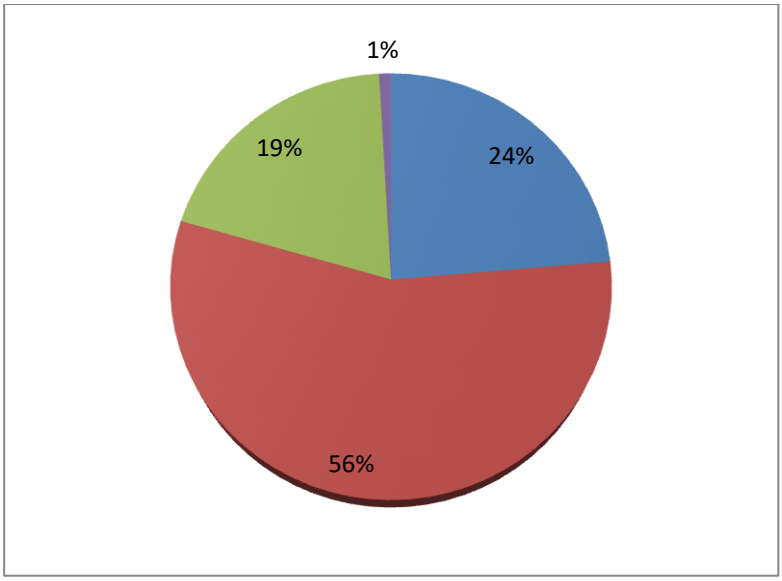

Figure 4.2 Pie chart showing enzyme activity shown by colonies

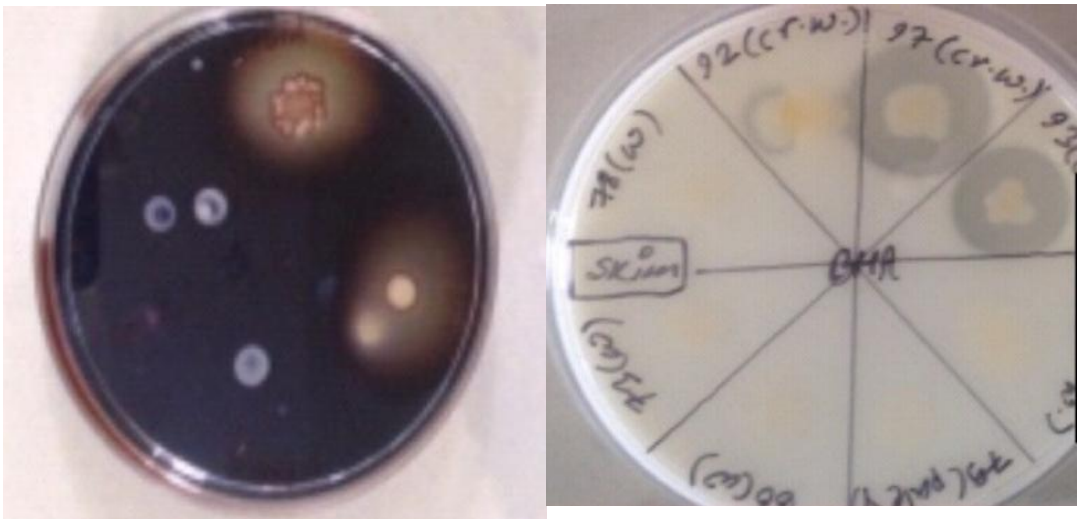

Figure 4.3 Bacterial colonies showing positive and negative enzymatic activities

4.3 Gram staining

For most of the bacterial colonies gram staining was done which gave the evidence of presence of both gram positive and gram negative bacteria. Gram Negative bacteria belonging to subtypes of *Shigella, Salmonella, Klebsella, Proteus, Escherichia*, *Chryseobacterium, Flavobacterium, Pseudomonas, Stenotrophomonas, Ochrobactrum, Novosphingobium, Serratia, Achromobacter, Enterobacter, Citrobacter* and gram positive organisms belonging to *Streptococcus, Bacillus, Staphylococcus* were signified after gram staining.

4.3.1 Bacterial species Identification

The morphology study of the colonies, their enzymatic assay, and gram staining gave most repetitive and unique isolated bacterial colonies. These were selected and their 16S rRNA sequencing was performed. Sequence of 16s rRNA were identified after sequencing procedure. Ez TAXON of EZBIOCLOUD program identifies the most similar species whose 16s rRNA was already stored in the database. The lists of variety of bacterial isolates from colony sequencing are listed. After the sequencing of 100 results 49 different organisms were identified.

These 49 different species are having 19 different genus of bacterial isolates. The bacterial species identified after sequencing belongs to genus *Enterococcus, Enterobacter, Klebsiella, Bacillus, Pseudomonas, Staphylococcus, Serratia, Citrobacter,*

Stenotrophomonas, Achromobacter and their sub types. Some other bacterial species are identified *Proteus, Sporosarcina contaminans, Escherichia, Ochrobactrum, Novosphingobium capsulatum , Kocuria rosea , Chryseobacterium, Lyinibacillus macroides* and others.

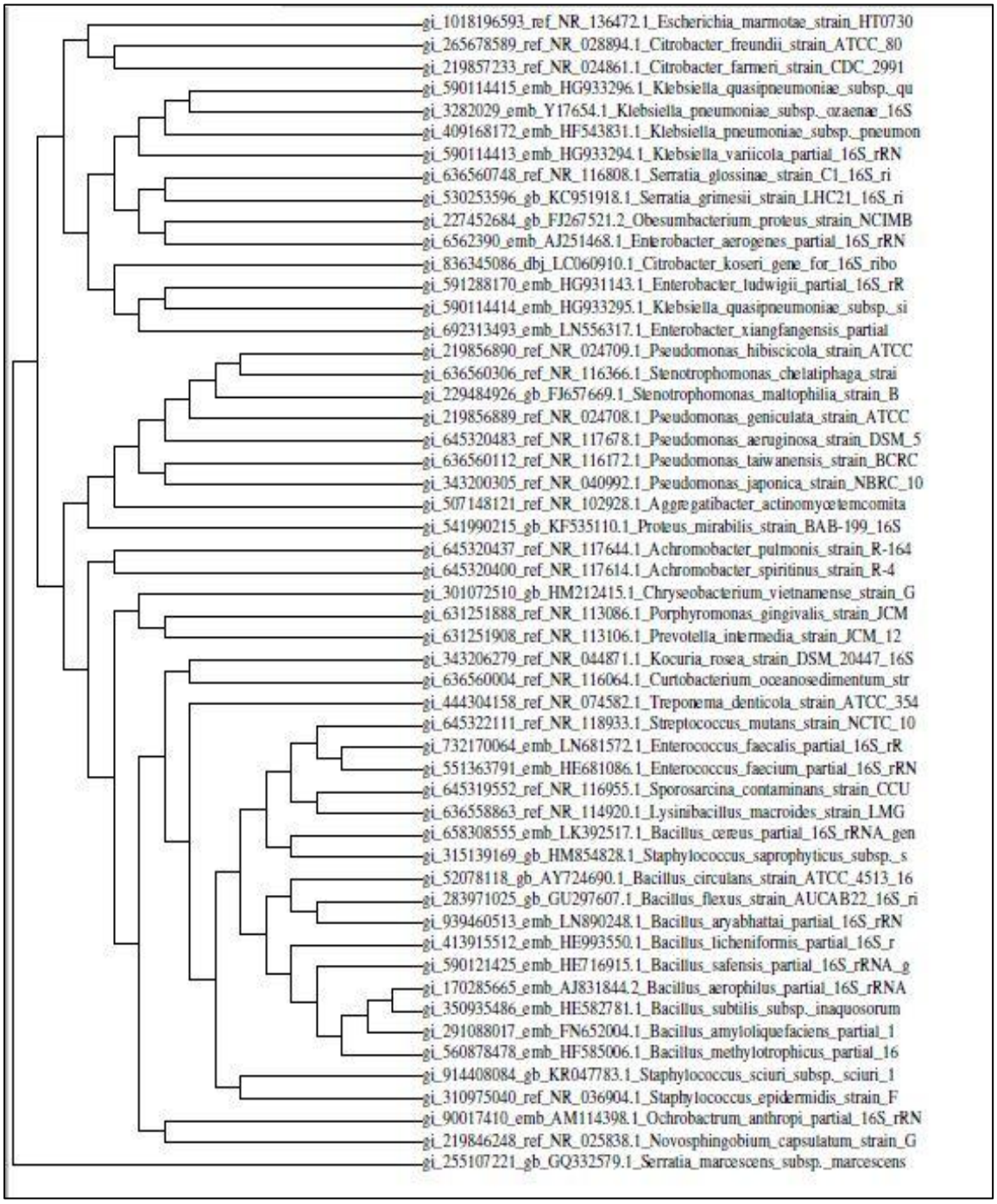

Figure 4.4 Phylogenetic tree of isolated bacterial strains

Table 4.1 The detailed list of variety of bacterial isolates from colony sequencing

S.N	GENUS SPECIES	Similarity (%)	ACCESSION NO.
DB-32	*Enterococcus faecalis ATCC 19433(T)*	*100*	ASDA01000001
DB-247	*Bacillus licheniformis ATCC 14580(T)*	*99.92*	AE017333
DB-134	*Bacillus cereus ATCC 14579(T)*	*100*	AE016877
DB-186	*Bacillus flexus IFO 15715*	*100*	AB021185
DB-206	*Bacillus circulans ATCC 4513*	*100*	AY724690
DB-130	*Bacillus amyloliquefaciens subsp. Plantarum FZB42(T)*	*99.92*	CP000560
DB-111	*Bacillus safensis FO-36b*	*100*	ASJD01000027
DB-224	*Bacillus aryabhattai B8W22*	*100*	EF114313
DB-144	*Bacillus subtilis subsp. Inaquosorum KCTC 13429*	*99.92*	AMXN0100021
DB-89	*klebsiella pneumoniae subsp. Pneumoniae DSM 30104(T)*	*99.72*	AJJIO1000018
DB-160	*Klebsiella quasipnaumoniae subsp. Similipneumoniae 07A044*	*99.77*	CBZR01000004
DB-29	*Klebsiella michiganensis W14*	*99.31*	JQ070300
DB-16	*Klebsiella pneumoniae subsp. Ozaenae ATCC 1196*	*99.70*	Y17654
DB-177	*Klebsiella variicola DSM 15968*	*99.85*	CP010523
DB-247	*Staphylococcus saprophyticus subsp. Saprophyticus ATCC 15305*	99.78	AP008934
DB-70	*Stenotrophomonas chelatiphaga LPM-5*	*98.94*	EU573216
DB-77	*Stenotrophomonas maltophilia MTCC 434*	*99.85*	JALV01000036
DB-232	*Pseudomonas geniculata ATCC 19374*	*99.01*	AB021404
DB-218	*Pseudomonas aeruginosa JCM 5962*	*100*	BAMA01000316

DB-66	*Pseudomonas hibiscicola ATCC 19867*	*99.83*	AP021405
DB-20	*Pseudomonas japonica NBRC 103040*	*98.82*	BBIR01000146
DB-131	*Proteus mirabilis ATCC 29906(T)*	*99.85*	ACLE01000013
DB-249	*Kocuria rosea DSM 20447*	*99.85*	X87756
DB-71	*Obesumbacterium proteus DSM 2777*	*99.45*	AJ233422
DB-68	*Ochrobactrum anthropi ATCC 49188*	*100*	CP000758
DB-233	*Chryseobacterium vietnamense GIMN1 .005*	*97.77*	HM212415
DB-254	*Enterococcus faecium CGMCC 1.2136(T)*	*99.92*	AJKH01000109
DB-249	*Enterobacter aerogenes KCTC 2190*	*99.86*	CP002824
DB-239	*Enterobacter xiangfangensis 10-17(T)*	*99.54*	HF679035
DB-234	*Enterobacter ludwigii EN-119*	*99.45*	ZTLO01000001
DB-208	*Bacillus aerophilus 28K(T)*	*100*	AJ831844
DB-178	*Bacillus methylotrophicus KACC 13105*	*100*	JTKJ01000077
DB-179	*Klebsiella quasipneumoniae subsp. Quasipneumoniae 01A030*	*99.84*	HG933296
DB-58	*Citrobacter koseri CDC3613-63*	*99.38*	AF025372
DB-148	*Citrobacter farmeri CDC 2991-81*	*99.62*	AF025371
DB-34	*Citrobacter freundii ATCC 8090*	*99.64*	ANAV01000046
DB-188	*Staphylococcus sciuri subsp. Sciuri DSM 20345(T)*	*100*	AJ421446
DB-141	*Staphylococcus epidermidis ATCC 14990*	*100*	L37605
DB-240	*Serratia marcescens subsp. Marcescens ATCC 13880*	*99.92*	JMPQ0100005
DB-28	*Serratia grimesii DSM 30063(T)*	*99.85*	AJ233430
DB-79	*Serratia glossinae C1*	*99.85*	FJ790328
DB-44	*Pseudomonas taiwanensis BCRC 17751*	*99.84*	EU103629

DB-13	*Achromobacter pulmonis LMG 26696*	*99.78*	HE798552
DB-193	*Achromobacter spiritinus LMG 26692*	*99.85*	HE613447
DB-113	*Escherichia marmotae HT073016*	*99.14*	JANBP01000188
DB-252	*Novosphingobium capsulatum GIFU11526*	*99.35*	D16147
DB-236	*Lyinibacillus macroides LMG 18474(T)*	*99.92*	AJ628749
DB-256	*Sporosarcina contaminans CCUG 53915*	*99.93*	FN298444
DB-43	*Flavobacterium oceanosedimentum ATCC 31317*	*99.24*	EF592577

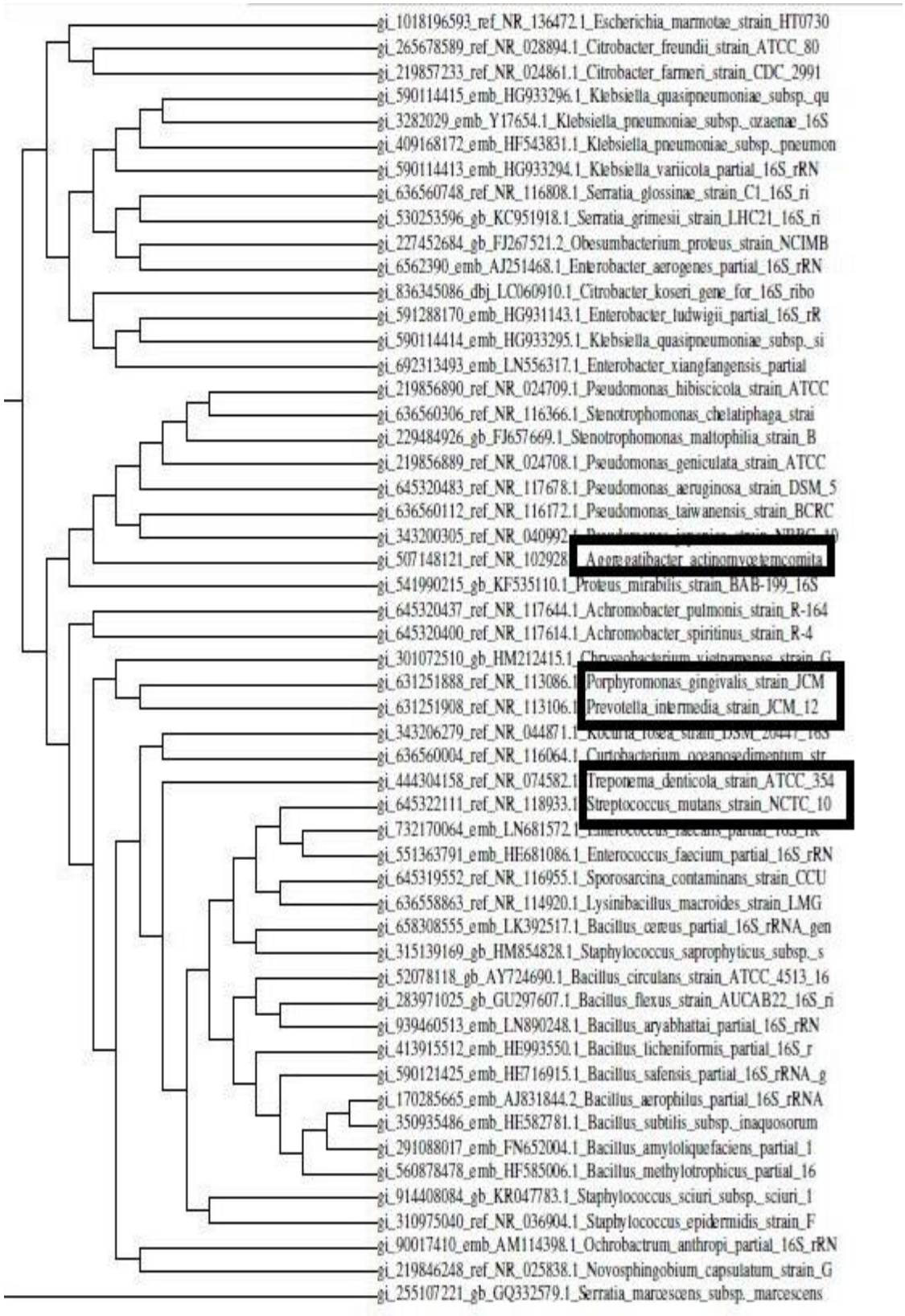

Figure 5. Similarities in the isolated pathogenic bacteria

4.2.1 Compounds identification in bacterial extract using GC-MS

The bacterial extract prepared after growing colonies in nutrient broth were sent for GC-MS process. About 950 compounds were identified but among them only 141 compounds were different. Some compounds are highly repetitively produced by most of the bacteria but most are produced by few colonies only. All type of organic compounds are significantly present, seventeen (17) were alkyl based compounds (only C & H), fifty five (55) oxygen containing organic compounds, twelve(12) only nitrogen containing organic compounds, thirteen (13) organic compounds contains both nitrogen and oxygen, 3 sulfur and nitrogen containing compounds, ten (10) sulfur containing compounds along with oxygen or nitrogen or both, twelve (12) chlorine containing compounds were identified. Similarly, seventeen (17) other compounds contain silicon, cobalt, phosphorus, bromine and fluorine along with nitrogen oxygen and sulphur in organic form.

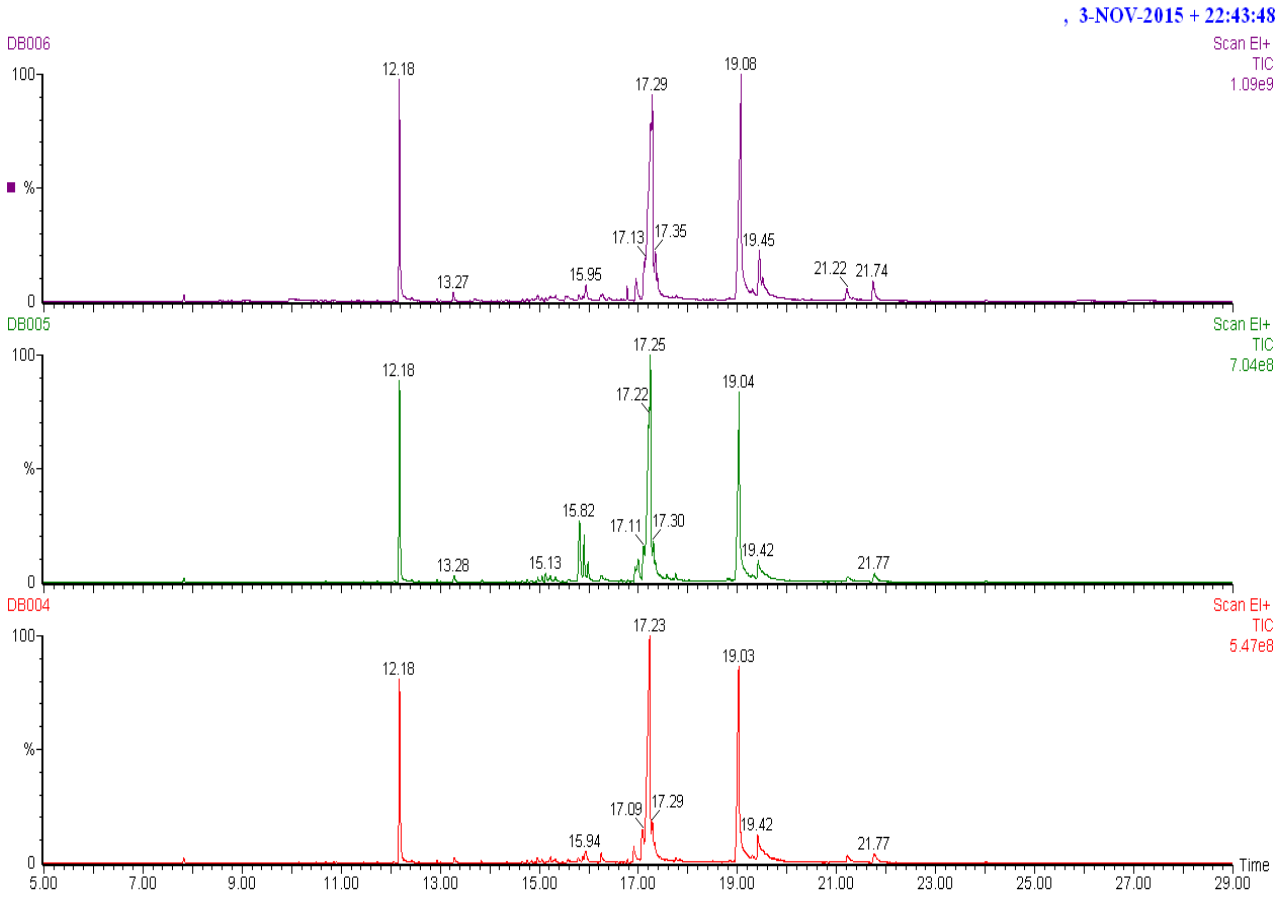

Figure 4.6 GC-MS of three samples peaks compounds

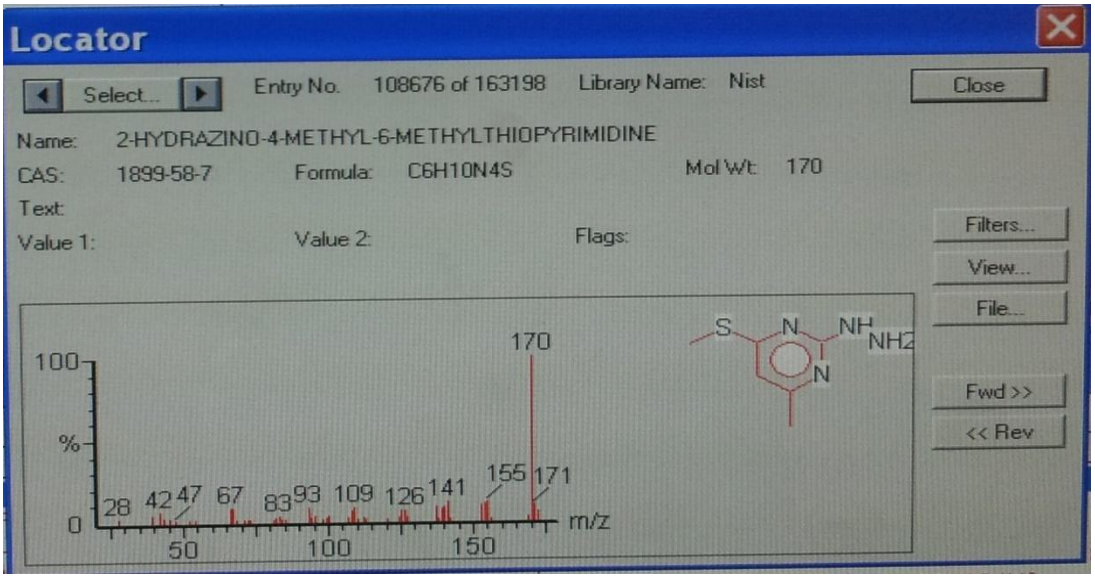

Figure 4.7 Compound identification in GC-MS graph

Table 4.2 List of organic compounds identified after GC-MS

Compounds Alkane	**Compound identified**	**Molecular weight**
$C_{21}H_{32}$	TETRACYCLO[16.1.0.0(2.9).0(10,17)]NONADECA-2(9),10(17)-DIENE, 19,19-DIME	284
$C_{15}H_{18}$	NAPHTHALENE, 1,6-DIMETHYL-4-(1-METHYLETHYL)-	198
C_9H_{10}	INDANE	118
$C_{12}H_{14}$	BENZENE, 1,3.HEXADIENYL-	158
$C_{19}H_{28}$	1H-INDENE,2-BUTYL-3-HEXYL-	256
$C_{10}H_{12}$	BENZENE, 4-ETHENYL-1,2-DIMETHYL-	132
$C_{14}H_{10}$	DIPHENYLETHYNE	178
$C_{18}H_{26}$	PHENANTHRENE,9-BUTYL-1,2,3,4,5,6,7,8-OCTAHYDRO-	242
$C_{16}H_{16}$	CYCLOBUTANE, 1, 3-BIPHENYL-, TRANS-	208
$C_{30}H_{46}$	BENZENE,1,1'-(1,1,10,10-TETRAMETHYL-1,10-DECANEDIYL)BIS[3,4-DIMETHY	406
$C_{12}H_{12}$	NAPHTHALENE, 1,2-DIMETHYL-	156

$C_{10}H_{12}$	BENZENE, 4-ETHENYL-1 ,2-DIMETHYL-	132
$C_{18}H_{18}$	BENZENE, 1,1'-(1 ,5-HEXADIENE-1 ,6-DIYL)BIS-	234
$C_{14}H_{10}$	BNZ[A]AZULENE	178
$C_{16}H_{16}$	BENZENE, 1,1'-(1 ,2-CYCLOBUTANEDIOL)BIS-, CIS-	208
$C_{19}H_{28}$	1H-INDENE, 2-BUTYL-3-HEXYL-	256
$C_{25}H_{42}$	1H-INDENE, 1-HEXADECYL-2.3-DIHYDRO-	342
Oxygen containing	**Compound identified**	**molecular weight**
$C_{11}H_{18}O_5$	DIETHYL 4-OXOPIMELATE	230
$C_{16}H_{12}O_5$	5,10 DIHYDROXY 2-METHOXY 7-METHYL 14 ANTHRACENEDIONE	284
$C_{10}H_{16}O_5$	DIME THYL 4-OXOOCTANE-1 ,8-DIOATE	216
$C_{16}H_{16}O_3$	2H-PYRAN-2-ONE,6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-	256
$C_{10}H_{20}O_4$	PHENOL, 3,5-DIMETHOXY-,ACETATE	196
$C_9H_{12}O_3$	3-ETHOXY-4-METHOXYPHENOL	168
$C_{14}H_{14}O$	PHENOL, 2-(1-PHENYLETHYL)-	198
$C_{17}H_{16}O_4$	PHTHALIC ACID , METHYL 2-PHENYLETHYL ESTER	284
$C_{12}H_{14}O_4$	DIETHYL PHTHALETE	222
$C_{22}H_{22}O$	PHENOL, 2,4-BIS(1-PHENYLETHYL)-	302
$C_{14}H_{22}O_3$	ACETICEACID,2-(2,2,6-TRIMETHYL-7-OXA-BICYCLO[4.1.0]HEPT-1-YL)-PROPEN	238
$C_{11}H_{16}O_2$	2(4H)-BENAOFURANONE,5,6,7,7A-TETRAHYDRO-4,4,7AA-TRIMETHYL-,-	180
$C_{14}H_{12}O_3$	SPIRO[NAPHTHALENE-2(1H),2'-OXIRAN]-1-ONE, 3'-ACETYL-3'-METHYL-, CIS-(+	228

$C_{18}H_{18}O_6$	1-,2,3,4-BUTANETETROL, 1,4-DIBENZOATE,(R* ,S*)-	330
$C_{15}H_{24}O$	PHENOL, 2,4,6-TRIS(1-METHYLETHYL)-	220
$C_{17}H_{24}O_4$	PHTHALIC ACID, BUTYL 2-PENTYLESTER	292
$C_8H_{10}O_3$	PHENOL, 3,5-DIMETHOXY	154
$C_{16}H_{16}O_2$	BENZOIC ACID, 2-METHYL-, (2-METHYLPHENYL) METHYL ESTER	240
$C_{20}H_{22}O_4$	PHTHALIC ACID, BUTYL , 5-DIMETHYLPHENYL ESTER	326
$C_{23}H_{18}O_3$	FLUOREN-9-OL,3,6-DIMETHOXY-9-(2-PHENYLETHYNYL)-	342
$C_{30}H_{38}O_7$	(+)-JABOROL ACETATESPIRO[NAPHTHALENE-2(1H), 2'-OXIRAN]-1-ONE, 3'-ACETYL-3'-METHYL-. CIS-(+)	510
$C_{15}H_{14}O_4$	1,3-BENZENEDIOL,4-(3,4-DIHYDRO-7-HYDROXY-2H-1-BENZOPYRAN-3-YL)-	258
$C_{17}H_{20}O_2$	3-(3,4-DIMETHYL-6-PHENYLCYCLOHEX-3-ENYL) PROP-2-ENOIC ACID	256
$C_{24}H_{34}O_4$	PHTHALIC ACID,PROPYL TRIDEC-2-YN-1-YL ESTER	386
$C_{11}H_{14}O_2$	BENZOIC ACID , 2-METHYLPRORYL ESTER	178
$C_{20}H_{30}O_4$	PHTHALIC ACID , 2-ETHYLHEXYL ISOBUTYL ESTER	334
$C_{33}H_{34}O$	2-BENZYL-1,3-DIPHENYL-2-[2,3,5,6-TETRAMETHYL-BENZYL]-PROPAN-1-ONE	446
$C_{13}H_{14}O_6$	2-BENAOYLOXYSUCCINIC ACID , DIMETHYL ESTER	266
$C_{22}H_{26}O_4$	PHTHALIC ACID, HEXYL 2-PHENYLETHYL ESTER	354
$C_{13}H_{16}O_4$	PHTHALIC ACID, ETHYL ISOPROPYL ESTER	236
$C_{18}H_{24}O_4$	1, 2-BENZENEDICARPOXYLIC ACID, BUTYL CYCLOHEXOL ESTER	304
$C_{32}H_{54}O_2$	ERGOST-5-EN-3-OL, 22, 23-DIMETHYL-,ACETATE, (3,BETA)-	470
$C_{29}H_{46}O_6$	CHOLAN-24-OIC ACID, 3, 12-BIS(ACETYLOXY)-,METHYL ESTR, (3.BETA.,5.ALF)	490
$C_{15}H_{18}O_3$	AMBROSIN	246

$C_{22}H_{34}O_4$	PHTHALIC ACID, 6-ETHYL.3-OCTYL BUTYL ESTER	362
$C_{24}H_{26}O_2$	4,4'-((P-PHENYLENE)DIISOPROPYLIDENE)DIPHENOL	346
$C_{20}H_{30}O_2$	ABIETIC ACID	302
$C_{15}H_{20}O_3$	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER	248
$C_{15}H_{14}O_4$	1-NAPHTHALENEBUTANYC ACID, 2-METHOXY-GAMMA-OXO-	258
$C_{16}H_{24}O$	2,5-CYLCOHEXADIEN-1-ONE, 2, 6-BIS(1 , 1-DIMETHYLETHYL) -4-ETHYLIDENE-	232
$C_{17}H_{24}O_3$	7,9-DI-TERT-BUYTL-1-OXASPIRO[4,5]DECA-6,9-DIENE-2, 8-DIONE	276
$C_{12}H_{14}O_2$	2H-1-BENZOPYRAN, 7-METHOXY-2,2-DIMETHYL-	190
$C_{20}H_{22}O_6$	PHTHALIC ACID ,BUTYL 2,6-DIMETHOXYPHENYL ESTER	358
$C_{21}H_{26}O$	1-HEXENE, 3-METHYL-6-PHENYL-4-(1-PHENYLETHOSY)-	294
$C_{44}H_{58}O_5$	2-PROPYNOIC ACID, 3-[4-[(TETRADECYL) OXY] PHENYL]-,4'-[[(1-METHYLHEPT)]]	666
$C_{10}H_{12}O_4$	PHENOL, 3,5-DIMETHOXY-,ACETATE	196
$C_{23}H_{20}O_5$	PHTHALIC ACID, 4-METHOXYPHENYL 2-PHENYLETHYL ESTER	376
$C_{20}H_{22}O_4$	PHTHALIC ACID, BUTYL 3, 5-DIMETHYLPHENYL ESTER	326
$C_{21}H_{24}O_4$	PHTHALICACID, BUTYL 4-ISOPROPYLPHENYL ESTER	340
$C_{15}H_{20}O_4$	PHTHALIC ACID , BUTYL ISOPORPYL ESTER	264
$C_{18}H_{18}O_4$	PHTHALIC ACID , 3,5-DIMETHYLPHENYL ETHYL ESTER	298
$C_{14}H_{16}O$	5(6H)-BENZOCYCLOOCTENONE, 7, 8-DIHYDRO-8, 8-DIMETHYL-	200
$C_{14}H_{22}O$	PHENOL, 2,5-BIS(1,1-DIMETHYLETHYL)-	206
$C_{17}H_{20}O_4$	PHTHALIC ACID,HEX-2-YN-4-YL PROPYL ESTER	288
$C_{28}H_{30}O_{12}$	TREMULOIDIN, TETRACETATE	558

N containing	**Compound identified**	**Molecular weight**
$C_{12}H_{12}N_2$	4-TERT-BUTYLPHTHALONITRILE	184
$C_{10}H_6N_2$	6-CYANOQUINOLINE	154
$C_{13}H_{15}N$	1-CYANO-4-CYCLOHEXYLBENZENE	185
$C_{12}H_{11}N$	1,2-DIHYDRO-5-ACENAPHTHYLENAMINE	169
$C_{12}H_{13}N$	1-PHENYLCYCLOPENTANENITRILE	171
$C_{15}H_{15}N$	ACRIDINE, 9,10-DIHYDRO-9,9-DIMETHYL	209
$C_{18}H_{24}N_2$	1,4-BENZENEDIAMINE, N-(1-,3-DIMETHYLBUTYL)-N'-PHENYL-	268
$C_{10}H_{20}N_4$	3,6-DIBEUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE	196
$C_{12}H_{11}N$	3-METYL-5-PHENYLPYRIDINE	169
$C_{13}H_{27}N$	2,2-DIPROPYL-N-ETHYLPIPERIDINE	197
$C_{11}H_8N_2$	QUINOLINE-4-CARBONITRILE, 2-METYL-	168
$C_{15}H_{17}N$	BENZENAMINE, 4-(1-METHYLETHYL)-N-PHENYL-	211
O and N containing	**Compound identified**	**Molecular weight**
$C_{13}H_{12}O_2N_4$	LUMIFLAVINE	256
$C_{14}H_{23}O_3N$	CYCLOPENTANECARBOXYLIC ACID, 1,2,2-TRIMETHYL-3-(PYRRROLIDINE-1-CAF	253
$C_7H_{10}O_2N_2$	ETHYL 4-METHYL-5-IMIDAZONECARBOXYLAT	154
$C_{18}H_{24}O_3N_2$	OXAZOLIDIN-2- ONE, 4-HYDROXY-4, 5, 5-TRIMETHYL-3-[2-[1,2-DIMETHYL-3-INDOLYL]ETHYL]-	316
$C_{16}H_{14}O_2N_2$	1 ,2-DIBENZOYL-3-METHYLDIAZIRIDINE	266
$C_{25}H_{29}O_3N$	4,5-2H-OXAZOLE-5-ONE, 4-[3,5-DI-T-BUTYL-4-METHOXYHENYL] METHYLENE	391
$C_8H_{15}O_2N$	1-(3-HYDROXYPROPYL)-2-PIPERIDINONE #	157
$C_9H_{11}O_2N$	4-PYRIDINEMETHANOL, 3-METHYL-, ACETATE (ESTER)	165

$C_9H_{14}O_3N_2$	PROBARBITAL	198
$C_{16}H_{31}ON$	7-METHYL-2-(7-OXONONYL)-PERHYDROAZEPINE	253
$C_{10}H_{13}O_4N$	2,6-DIETHOXYISONICOTINIC ACID	211
$C_{23}H_{41}O_4N$	L-PROLINE,N-ALLYLOXYCARBONYL,-TETRADECYL ESTER	395
$C_{17}H_{21}O_3N$	1,3-DIOXOLANE-2-HEPTANEITRILE, .ALPHA.-METHYL-.DELTA.-OXO-2-PHEN	287
S and N containing	**Compound identified**	**Molecular Weight**
$C_{14}H_{20}N_4S_2$	1 ,4-BIS- (2-METHYL-THIAZYL-4-YLMETHYL)-PIPERAZINE	308
$C_6H_{10}N_4S$	2HYDYAZINO-4-METHYL-6-METHYL THIOPYRIMIDIMNE	170
$C_9H_{10}N_4S$	2-IMNO-6-MERCAPTO-4,4-DIMETHYL-1,2,3,4-TETRAHYDRO-PYRIDINE-3,5-DIC	206
S, O and N containing	**Compound identified**	**Molecular Weight**
$C_{11}H_{13}O_3N_3S$	TRANS-3-AZIDO-1 ,2,3,4-TETRAHYDRO-2-NAPHTHYL METHANESULFONATE	267
$C_{14}H_{11}O_5NS$	BENZO[D]1,3-DIOXOLANE-5-CARBOXYLIC ACID, 6-(2-THENOYLAMINO)-,METH	305
$C_{12}H_{17}O_3NS$	L-ALANINE, N-(2-THIANYLCARBONYL)-, BUTYL ESTER	255
$C_{21}H_{35}O_3NS$	L-ALANINE,N-(2-THIENYLCARBONYL)-, TRIDECYL ESTER	381
$C_{18}H_{19}O_2NS$	QUINOLINE, 1,2,3,4-TETRAHYDRO-1-((2-PHENYLCYCLOPROPYL)SULFONYL)-,	313
$C_9H_{10}ON_4S$	2-IMINO-6-MERCAPTO-4,4-DIMETHYL-1,2,3,4-TETRAHYDRO-PYRIDINE-3,5-DIC	206
$C_{1O}H_{17}O_3NS$	N-(7 ,7-DIMETHYL-2-OXOBICYCLO[2.2.1]HEPT-1-YL)METHANESULFONAMIDE	231
$C_{14}H_{21}O_3NS$	L-ALANINE, N-(2-THIENYLCARBONYL)-, HEXYL ESTER	283

$C_{17}H_{16}ON_2S$	N-(4,DIMETHYL-THIAZOL-2-YL)-2-NAPHTALEN-1-YL-ACETAMDE	296
$C_{22}H_{17}O_3NS$	4-[3-(2-METHYL-BENZYLOXY)-BENZYLIDENE]-2-THIOPHEN-2-YL-4H-OXAZOL-5	375
Cl containing	**Compound identified**	**Molecular Weight**
$C_{15}H_{15}Cl_2$	PHENETHYLAMINE, P-CHLORO-N-[P-CHLOROBENZYL]-	279
$C_{13}H_{13}OCL$	2-(2-CHLOROBENZYLIDENE)CYCLOHEXANONE	220
$C_{10}H_9O_2Cl_3$	ACETIC ACID, TRICHLORO-, 2-PHENYLETHYL ESTER	266
$C_{18}H_30NCl$	4-CHLOROBENZYLAMINE, N-DECYL-N-METHYL-	295
$C_9H_{14}NCl$	PHENYLTRIMETHYLEMMONIUN CHOLORIDE	171
$C_{15}H_{19}ON_2Cl$	1H-PYRAZOLE, 4-[4-(3-CHLOROPHENOXY)BUTYL]-3,5-DIMETHYL-	278
$C_{11}H_7O_2ClS$	2-THIOPHENECARBOXYLIC ACID, 4-CHLOROPHENYL ESTER	238
$C_{11}H_8ONClS$	THIOPHENE-3-(4'-CHLOROCARBOXANILIDE)	237
$C_{13}H_{11}O_3H_2ClS$	THIOPHENE-2-CARBOXYLIC ACID ,N'-[2-(4-CHLOROPHENOXY)ACETYL]HYDRA	310
$C_{16}H_{16}O_5NCl$	(5-CHLORO-2-METHOXYPHENYL)CARBANIC ACID, 2,6-DIMETHOXYPHENYL E	337
$C_{14}H_{14}O_2NClS$	THIOPHENE-2-CARBOXAMIDE,N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL]-	295
$C_{17}H_{19}O_3N_2CLS$	THIAZOLIDIME-2,4-DIONE, 5-(4-CHLOROBNZYLIDENE)-3-(2,6-DIMETHYLMOR)	366
Remaining others	**Compound identified**	**Molecular Weight**
$C_{14}H_{19}O_2Br$	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER	298
$C_{17}H_{12}NBrS$	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE	341

$C_{15}H_{11}O_3F$	BENZOIC ACID, 3-FLUORO-, 2-OXO-2-PHENYLETHYL ESTER	258
$C_{18}H_{16}O_4BrF$	PHTHALIC ACID, 2-BROMO-4-FLUOROPHENYL BUTYL ESTER	394
$C_{13}H_9ON_3BrF_3S$	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A	391
$C_{10}H_8O_2NBr$	2-BROMOPROPIONIC ACID, 4-CYANOPHENYL ESTER	253
$C_{17}H_{15}O_3NF_2$	L-PHENYLALANINE, N-(2,6-DIFLUOROBENZOYL)-, METHYL ESTER	319
$C_{16}H_{15}ONF_2$	PROPIONAMID, N-(3-FLUORO-2-METHYLPHENYL)-3-(4-FLUOROPHENYL)-	275
$C_{11}H_{14}O_2NF$	CARBAMIC ACID, 4-FLUOROPHENYL-,BUTYL ESTER	211
$C_{14}H_{16}ONF_3$	CYCLOHEXANONE, 2-(3-TRIFLUOROMETHYLPHENYLAMINOMETHYL)-	271
$C_{14}H_{20}ONF$	OCTANAMIDE, N-(4-FLUOROPHENYL)-	237
$C_{22}H_{18}Co$	COBALT , .ETA.-5-CYCLOPENTADIENYL-.ETA.-5-1 ,2-DIPHENYLCYCLOPENTADI	341
$C_{11}H_8ONFS$	THIOPHENE-2-CARBOXAMIDE, N-(2-FUOROPHENYL)-	221
$C_{14}H_{23}O_3P$	BUTYLPHOSPHONICACID, ETHYL 2-PHENYLETHYL ESTER	270
$C_{15}H_{18}OB_2$	DIBOROXIDE, ETHYLMETHYLDIPHENYL-	236
$C_{16}H_{34}OSi$	1-METHYL-1-[6-ETHYL-3-OCTYLOXY]-1-SILACYCLOHEXANE	270
$C_{13}H_{28}OSi$	TRIISOPROPYLSILYLOXYCYCLOBUTANE	228

Table 4.3 Some of the compounds extracted were highly repeated in number by most of the bacteria

PYRROLO [1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
LUMIFLAVINE
5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL]-

4.3 Organisms isolated variation according to age, sex and altitude

From the 100 samples 49 different genus and species were obtained, and the variation in the bacterial count obtained was differentiated according to the age and sex of the patients and the samples from three geographical belts Terai, Hill and Mountain region.Age of the patients was divided as 10-19(teenagers), 20-59(adults) and 60+(older seniors). Seventy (70) percent of microorganisms were found in the cavities of adult population followed by elder people and only 8 adolescents were having bacteria in the samples collected.

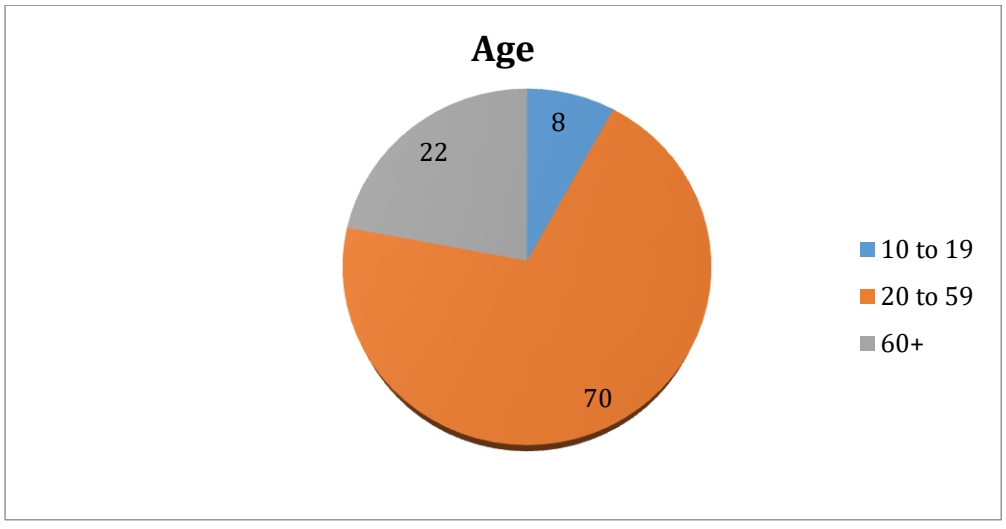

Figure 4.8 Pie chart showing the age of patients and bacteria identified

Among the 100 samples it was found that both male (48) and female (52) are having about similar amount of different bacterial species in the oral cavity.

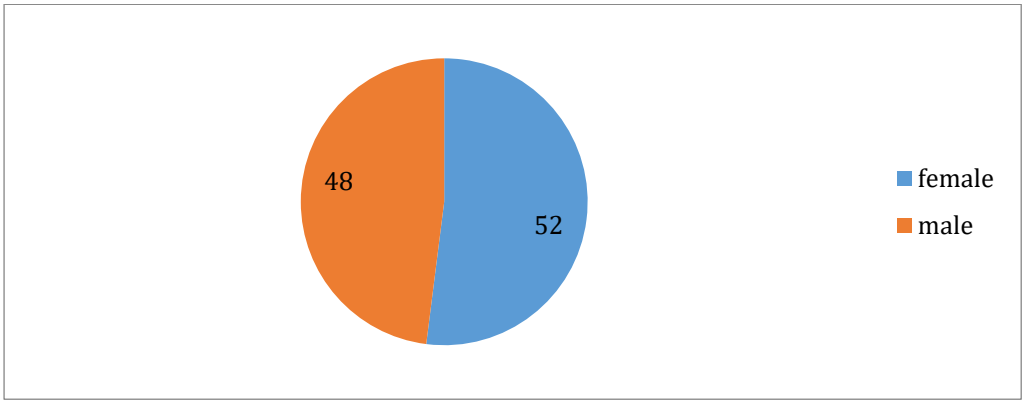

Figure 4.9 Pie chart showing bacterial identification according to sex

Samples were collected from 16 districts of Nepal. Among the 100 samples, about half of the bacterial isolates after sequencing were found out to be from Terai region of Nepal. Similarly 31 bacterial isolates from hilly region and 20 from mountain region were found.

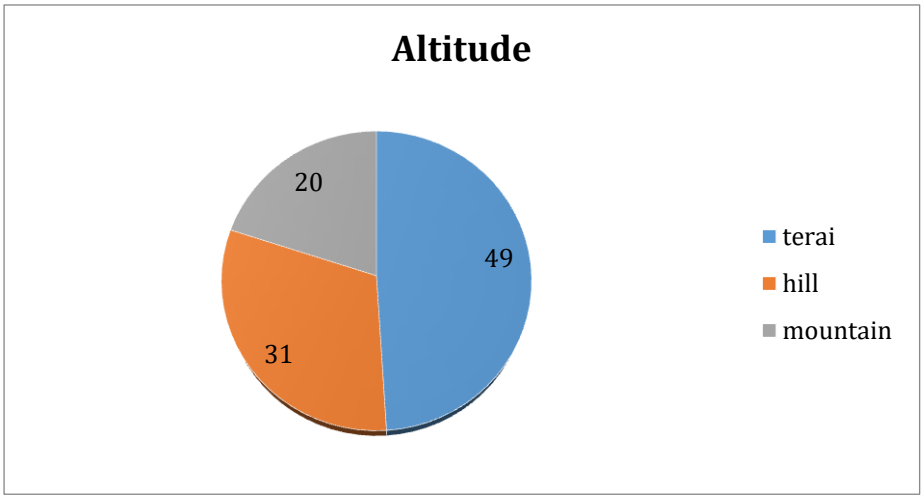

Figure 4.10 Pie chart showing bacterial isolates according to geographical regions

CHAPTER 5. DISCUSSION

Colony characteristic of most of the isolates in different agar plates had significant variation of organism found in oral cavities. Variation in colony characteristic like shape, size , opacity, margin, coloration, surface elevation, clearly stated the wide variety of microorganisms in oral cavity. Considering the time of incubation and manner of colony development on plates all being non selective. It can be considered that most of the organisms grown are commonly adoptable and easily gradable (Justice *et al,* 2004). These organisms are supposed to be easily present in human samples and other plate cultivable samples with only few different and dominant in oral samples. After checking enzyme activities of these colonies it was found that 63% sample inoculate colonies show at least one enzymatic activity,When checked for the enzyme activities in enzyme specific plates. The enzyme activity checked for protease, cellulase, pectinase and amylase activity but remaining colonies didn't show any enzyme activity. Strains of *Klebsiella pneumoniae* and *Achromobacter pulmonis* gave evidence of enzyme production for three different enzymes. So, these strains seems to be most potent strains in biofilm establishment and adaptation whereas, strains of *Enterobacter xiangfangensis, Bacillus cereus, Bacillus aerophilus, Bacillus amyloliquefaciens, Pseudomonas taiwanensis* gave positive results for two enzymes. *Escherichia marmotae*, *Ochrobactrum anthropi*, *Obesumbacterium proteus* gave single enzyme activity. *Staphylococcus epidermidis, Citrobacter koseri, Serratia grimesii, Chryseobacterium vietnamense, Sporosarcina contaminans* didn't show any enzyme activity and this may be because of their correlation and synergic adaptation with other enzyme producing potent strains. Enzymes like protease, cellulase, amylase and pectinase were used for the cleavage of chains of subunits of skimmed milk protein, cellulose, starch and pectin respectively (Yadav *et al*, 2016). Assuming that the production of constitutive enzymes by bacteria is a result of the adaptation of organic matter, microenvironments they live in. The measured enzyme activities can be used as an indication for the diverse and resistant environment (Narantuya *et al.*, 2015). None of the strains show all enzyme activities, this is because in biofilm a collaborative environment is established and no single organism does all sort of work which influences the byproducts and enzymes production. Similarly pectinase showed no activity, but the enzyme activities

relating protease, cellulase, and amylase was significant. This may be because of food habit. Human food habit doesn't really correlates with pectinase enzyme activity. Most of the human food are either starch, protein (Hartog *et al*, 2006), and above all these are either proteins or carbohydrates and rather than pectinase. Other enzymes amylase and protease along with cellulose are easily usable in adaptation with oral food source(Choudhary *et al*, 2014).So, it can be understood that most of bacteria are trying to establish or restrict a well adoptive microenvironment.

Because of strict and proper selection mechanism among colonies, 100 colony sequencing also show large variation within the species. This also verifies that oral cavity is pool of large variation of microorganisms especially bacteria. Similarly, this pool is dominated by both type of organisms (biofilm form and free living together). But most free living organisms can adopt and change into biofilm forms with additional pathogenicity (Takahashi, 2005). All the isolates that are identified after sequencing have diverse features. Among these isolates gram negative-*Chryseobacterium, Flavobacterium , Pseudomonas, Stenotrophomonas, Proteus, Escherichia, Ochrobactrum, Novosphingobium, Serratia , Achromobacter, Klebsiella , Enterobacter, Citrobacter* isolates includes and gram positive includes *Bacillus , Enterococcus, Staphylococcus, Kocuria, Lysinibacillus fusiformis.* Similarly isolates contains bacteria adapted to almost any type of environments. There is presence of aerobic, anaerobic, facultative aerobic bacteria. *Enterococcus, Enterobacter, Achromobacter, Staphylococcus,* are commensal organisms. These commensal microorganisms have the potential to change the environment and their living mode with regular genetic changes through physiological processes such as metabolic activities, subsequently facilitating the introduction of more pathogenic microorganisms *Serratia, Citrobacter, Stenotrophomonas, Pseudomonas, Klebsiella, Bacillus* includes pathogenic (opportunistic) bacteria species. *Proteus, Sporosarcina contaminans, Escherichia, Ochrobactrum, Novosphingobium capsulatum, kocuria rosea, Chryseobacterium, Lyinibacillus macroides* are also found which show wide variety of organisms. Reports claims that 6,768 oral infectious diseases are not caused by a single critical pathogen but rather group of multiple organisms are found to cause single disease. So my result which provides evidence of large variety of microflora inside oral microenvironment also suggest multiple

organisms can be associated with the etiologies of dental caries, periodontal diseases and oral malodor. Most of the isolates are cause of disease in hospital conditions and in immune compromised patients so these species could be used as oral markers for the early detection of oral disease, oral cancer. Some study have given information of species like *Staphylococcus aureus* and *Enterococcus faecalis* as regular microflora around salivary samples of oral cancer patients(Fábián *et al.,* 2008). *Bacillus* species are able to produce copious amounts of enzymes (amylase and protease) and intracellular inclusions of polyhydroxy alkanoates (Baron *et al.*, 1996) which may be advantageous for adaptation inside biofilm of oral cavity. Reports show that in *staphylococci*, the chemical composition of EPS may be quite different and may be primarily thick anion attracting (Sanders & Sanders, 1997). Similarly a biofilm of *Pseudomonas, Klebsiella* are mature biofilm. Pure cultures of *Klebsiella pneumoniae* are thinner than *Pseudomonas*. *Enterococcus, Staphylococcus, Pseudomonas, Klebsiella* majorly present in most biofilm and oral microenvironment. Recent sources have claimed that they are also found in biofilms of venous catheter, Prosthetic heart valve, Urinary catheter. Similar is case for other species of bacterial isolates (Donlan, 2002). *Klebsiella pneumoniae* subsp. *ozaenae* is frequently isolated, which supports the hypothesis of its pathogenicity, even if it is difficult to determine whether it is a pathogen or a colonizer and if in some cases *Pseudomonas aeruginosa* or *Proteus* is simultaneously isolated. *Klebsiella variicola* is known to associate with a number of different plants including banana trees but recent studies associates these strains with different diseases and now presence of them in our oral samples can help to verify if they are associated in oral disease. *Klebsiella michiganensis* was a new Bacterium Isolated from a Tooth Brush Holder so they are also found in our samples. *Pseudomonas japonica* was able to degrade alkyl phenols so they are highly adoptive.

Enterococcus faecalis and different *Staphylococcus* species are the most persistent bacteria in failed root canal treatment (Sundqvist *et al.*, 1998) and cause angular cheilitis, parotitis (Smith *et al.*, 2001) respectively. Colonization of *Staphylococcus* in oral cavity is found to be a possible cause of endocarditis, a life threatening infection of heart (Ohara-Nemoto *et al.*, 2008). This makes clear that although they are commensal microorganisms they are capable to change themselves into more pathogenic form in oral cavity and also

in other parts of human system and cause life threatening diseases (Geethashri *et al.*, 2014). *Staphylococcus epidermidis* along with other *Staphylococcus* species are polyauxotrophic, a mineral medium supplemented can also made it to stay alive in most environments. So they are obviously found in oral cavity where environment favors these bacteria. Similarly, *Pseudomonas aeruginosa* and other *Pseudomonas* species are widely distributed in nature and in hospital environment, and because of its minimal nutritional necessities; it is able to survive in several moist surfaces and humid places like oral samples. These bacterium rarely causes severe infections in healthy individuals, nevertheless, it represents a great threat for hospitalized patients as they are an opportunist bacterium(Coulon *et al.*, 2012). *Serratia marcescens* produce characteristic red pigment, prodigiosin (tooth surface) (Auwaerter, 2007) and *Chryseobacterium* produces yellow flexirubin type, non-diffusible(Li &Zhu, 2012) slime layer. *Proteus mirabilis* shows swarming motility and urease activity which helps to hydrolyzes urea into ammonia produces a very distinct fishy odor because of hydrogen sulfide gas. Because of these feature they contributor to oral malodor rather than pathogenecity (O'hara et *al.,* 2000). *Escherichia marmotae* previously not found in oral habitat but isolated from the soil sample of Qinghai-Tibetan plateau (Liu *et al.*, 2015) were isolated in our sample. This gives idea that more and more number of oral resident bacteria is still to be identified. *Novosphingobium* , significant for nitrogen cycle are also found in our isolates, which further diversify that inside microenvironment of oral cavity, the source and understanding still remains to be understood. A new finding have shown that *Bacillus licheniformis* secretes an enzyme that has proven to be an unexpected tooth decay fighter as it has the ability to cut through plaque or a layer of bacteria(Wilkinson & Tom, 2012). This example verifies that mode of action and defenses of oral bacterial communities can be altered to our benefits but detail study is still needed to be performed.

Most of the dental disease samples comprise of *Streptococcous mutans* and *Porphyromonas gingivalis* but they do not always comprise the major proportion of microflora in initial lesions of oral disease and only increase in number as the disease lesion develops. *Streptococcus, Porphyromonas and Actinomyces* species are predominant

in the supragingival area but our major samples are extracted tooth so these organisms are not identified in our sequencing.

Phylogenetic analysis of all different species of bacteria shows significant relationship among them but wide diversity of microbes was isolated. When comparison was done with proven oral microbial pathogens of tooth decay (*Streptococcoccus mutans* and *Streptococcus mitis*) and Periodontal Disease (*Treponema denticola* , *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans)* among our isolates, a clear relationship with our isolates was observed (Walter,2006). *Bacillus, Staphylococcus, Enterococcus* are closely related to each other and also with tooth decaying responsible bacteria, S*treptococcus*. Similarly *Serratia, Klebsiella, Proteus, Escherichia, Citrobacter* species are closely linked with each other and all lie in enterobacteraeae family. *Chryseobacterium* species is most distinguished species; this doesn't have much association with the entire pact of microbes but very closely related to oral pathogens *prevotella intemedia* and *Porphyromonas gingivalis*. Another set of organisms close to each other are *Ochrobactrum anthropi, Novosphingobium capsulatum and Stenotrophomonas*. These are also close to *Pseudomonas* and *Lysinibacillus* are close and furthest in the pack. Similarly, *Treponema denticola* and *Actinobacillus actinomycetemcomitans* both are in isolated branches but in the middle of tree which suggest that they are also significant to all the isolated strains.

GC-MS report suggests wide varieties of compounds are produced. Proteins, carbohydrates, are broken down in response to carbon, nitrogen, or sulfur limits with low molecular weights by using enzyme (protease, amylase, cellulase). So, biocompounds of bacterial extracts contain massive amount of these units (Puente *et al,* 2003) which are subsequently used as metabolic substrates (Takahashi, 2005). This was also supported by our enzyme activity assay, showing more than half bacteria with these enzyme activities. The majority of compounds are alkyl (benzene, alkane) based compounds, organic acids, amines, amides, esters, acid esters, suggest wide variety of defense and virulent action to maintain microenvironment in and around bacteria of biofilm. Similarly, a small group of metabolites were highly repetitive including $C_{10}H_{20}N_4$(3,6-dibutyl-1, 2-dihydro-1,2,4,5-tetrazine), $C_{14}H_{16}O_2N_2$(pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-Phenylmethyl) ,$C_{12}H_{18}ON_2$(4'-diethylaminoacetanilide), $C_6H_{10}N_4S$ (2-hydrazino-4-methyl-6-

methylthiopyrimidine), $C_{10}H_{16}O_2S$ (thiophene, 3,4-bis (ethoxynethyn)), $C_{13}H_{12}O_2N_4$ (lumiflavine), $C_{20}H_{23}O_2N_3$(1H-1,2,4-triazole-1-ethanol, beta-(1 ,1' -biphenyl[-4-yloxy]-alpha). These metabolic subunits were repetitive in different samples. So a possible agenda arises where these compounds could be used as biomarker of dental bacteria to determine significance oral biofilms, pathogenicity or associated diseases. Further study still needs to be done but a possibility of metabolite based biomarkers of oral bacterial microflora is open because of these compounds identification.

Some clinical evidence suggested that, cariogenic conditions are associated with increased amounts of microorganisms capable of acid production (Takahashi, 2005).Our isolates identified after sequencing also support this evidence as our major group of possible bio-compounds identified consists of acids and acid esters that helps to maintain the acidic condition to cause dental caries. Acidification results in both demineralization of tooth surface and introduction of more cariogenic microorganisms so, this evidence supports our source of sample, extracted rotten teeth. Our biocompounds also support the evidence that sulfur compounds helps to create oral malodor as there are wide varieties of sulfur compounds and also possible cysteine and methionine cleaved compounds to produce malodor causing compounds like hydrogen sulfide (H2S), methyl mercaptan and dimethyl sulfide (Geethashri *et al*, 2014). Some broth extract provides evidence of production of ammonia based compounds which changes the PH of a compound into neutral or basic, this may be because of environmental adaptation responds of acid intolerant bacteria (*Proteus*) using enzymes to convert proteins into basic compounds to create tolerant environment(Takahashi, 2005).

Most of these identified compounds relate metabolic end products such as short chain fatty acids (propionic, butyric, isobutyric and isovaleric acids), ammonia and sulfur compounds (hydrogen sulfide and methyl mercaptan) which helps to establish bacteria in biofilm microenvironment and subsequently disturb the host defense after impairing host cell functions. Our result also gives us possibility of massive amount of organic acids and acid esters like (Abietic acid, O-toluic acid ester, Phthalic acid ester) and diacetyl based compounds like (Phenol acetate, ergostol acetate) which could be aggressive against opportunist pathogens and gives us idea of some adoptive colonization among oral bacteria.

After sequencing of the collected samples, subtypes of major common commensal oral microflora; *Bacillus, Enterococcus, Klebsiella, Staphylococcus, Pseudomonas* were isolated. Remaining isolates identified are mostly opportunistic pathogens and major contributors in pathogenecity and oral problems. Similarly, *Escherichia marmotae*, a recently soil isolated microflora was isolated . Likewise, *Treponema denticola* , which is very closely related to oral pathogens like *Streptococcus mutans* was isolated. *Porphyromonas gingivalis, and Prevotella Intermedia* shows the close relation among each other.

Among the isolated microorganisms, about each half of the sample showed bacterial isolation from both male and females. This signifies that oral problems are prevalence in equal distribution among both male and females. Teenage sample has a few bacterial isolates but with highest from adults ranging 20 to 59 age groups. Most of the samples in teenage and children are milk tooth degeneration as natural phenomenon so they don't retains most of the pathogenic and problematic microflora of oral cavity that are prevalent in adult people where the extraction of tooth is caused by in most of case because of oral disease. So age groups of 20-59 have wide pool of microorganism. In this age group also the higher group retains most of the species as in old age most of the tooth are degenerated and rotten because of disease environment and bad oral health habit. Half of the sample from terai showed bacterial isolates and less among mountainous region. This shows that terai region with hot climate might be enabling the growth of microorganisms as compared to in the mountain region. Similarly the sanitation habit on terai region is very poor. General and common uses of tobacco, smoking and their products might have contributed in the diminishing of oral health and establishment of disease causing pathogenic bacteria.

CHAPTER 6. CONCLUSION

Knowledge of oral microbial community of country like ours is yet to be understood as no researches have been done relating this aspect. Because of variable and unique food culture and oral health habits compared to develop world it is very hard to correlate our oral statistics with their data's. The study of the microbial community along with possible secondary metabolite produced in the process can be a basis for understanding the cause of oral malfunction, disease, and connection of them with systemic problems. Along with that, if wide distributed sample sources can be used under supervision and comparable research with respect to health practice and food habit is done then, possible health scenario along with their possible reasons can be out lined. Therefore some of the oral samples from possible different parts of Nepal were collected and studied for identification of possible bacterial community and bacterial bioactive compounds produced by them. These identified microbial and their bioactive compounds are the basic for understanding oral bioenvironmental aspects and prospect of oral health hazards caused by them. Forty nine (49) different species belonging to 19 different genera showed the presence of significant diversity of bacteria with different species causing various forms of diseases and oral problems (both commensal and pathogenic). More than half colonies shows some kind of enzyme activity for possible use of substrate(protein and carbohydrate) to produce virulent factors like organic acids, ammonia based compounds and mal odor producing compounds. On the basis of the results obtained after GC-MS of all bacterial extracts we can conclude that most of the bio-compounds secreted were either used as adoptive mechanism to protect the colonization of other species and establish themselves inside protective slime barrier of biofilm or provides them virulent strength to cause disease conditions. Different isolates shows that bacterial organisms are minimal in oral cavities in high mountain regions than low terai. This helps to correlate our oral health with sanitation habit as in terai region use of tobacco and smoking most have elevated the possible bacterial number.

This research is only the preliminary study but it has given powerful insight to the biodiversity of oral cavity in variable Nepalese population. Further research towards isolation, identification and characterization of specific oral bacterial community, their rigorous testing for understanding establishments inside natural biofilm models,

identification of possible and cultural associated biodiversity needs to be done. Now for future references, researchers should further identify these microbial communities and along with this other possible sources of oral infection like virus, fungi should also be identified. Relative anti-microbial activity of present oral products in market and possible formulas which can be used as oral products based on our identified microbial flora and their by products can be done.

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APPENDIX - A (List of Reagents and Culture media)

1. Preparation of 1 N NaCl

To make a 1N aqueous solution of NaCl, 58.5 grams of NaCl was dissolved in some distilled deionized water. Then more water was added to the flask until it totals 1 liter.

2. Preparation of Congo red (1%) -100 ml

To make a 1% aqueous solution of congo red, 1 grams of congo red powder was dissolved in some distilled deionized water. Then more water was added to the flask until it totals 100ml.

3. Composition of Grams Iodine

Components	g/300ml
Iodine	1
Potassium iodide	2
Water	300

4. Composition of media

A. Nutrient broth

Components	g/l
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
PH	7.4 ± 0.2

B. Nutrient agar

Components	g/l
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	15.0
PH	7.4 ± 0.2

C. Mueller Hinton Agar (MHA)

Beef infusion form	300 gm/l
Casein hydrolysate	17.5 gm/l
Starch	1.56 gm/l
Agar	17 gm/l
Final PH	7.3 ± 0.2

D. Potato Dextrose Agar (PDA)

Potato	200 gm/l
Agar	2gm/l
Dextrose/Glucose	2gm/l

E. Blood agar (BA)

Proteose peptone	15 gm/l
Liver extract	2.5 gm/l
Yeast extract	5 gm/l
Sodium chloride	5 gm/l
Agar	15 gm/l
Final pH (at 25°C)	7.4±0.2

F. Mannitol salt agar (MSA)

Proteose peptone	10 gm/l
Meat extract	1 gm/l
Sodium chloride	75 gm/l
D-Mannitol	10 gm/l
Phenol red	0.025 gm/l
Agar	15 gm/l

Final pH (at 25°C)	7.4±0.2

G. Brain heart infusion agar

Calf brain infusion form	200gm/l
Beef heart infusion form	250gm/l
Proteose peptone	10gm/l
Dextrose	2gm/l
Sodium Chloride	5gm/l
Disodium phosphate	2.5gm/l
Agar	15gm/l
Final pH (at 25°C)7.4±0.2	

H. Starch agar

Components	g/l
Peptic digest of animal tissue	5.0 g/l
Beef extract	1.5 g/l
Yeast extract	1.5 g/l
Sodium chloride	5.0 g/l
Starch	2 g/l
PH	7.4 ± 0.2 g/l

1.5 % agar was added at the end

I. CMC agar

Components	g/l
Carboxymethyl cellulose	2 g/l
Sodium nitrate	1 g/l
Dipotassium phosphate	1 g/l
Potassium chloride	1 g/l
Magnesium sulphate	0.5 g/l
Magnesium sulphate	0.01 g/l

Magnesium sulphate	5 g/l
PH	7 g/l

1.5 % agar was added at the end

J. Composition of pectin agar

Components	g/l
Yeast extract	5 g/l
Peptone	5 g/l
Tryptone	10 g/l
Pectin	5 g/l
pH	7.0±0.2 g/l

K. Skimmed milk agar

Components	g/l
skimmed milk	100 g/l
Agar	6 g/l
pH	7.0±0.2 g/l

15. List of bacterial sample number with GC-MS Retention time and library identified compounds

Ref no.	Peaks	Chemicals Identified
251	21.737	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.423	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.039	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.259	LUMIFLAVINE
	16.946	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.251	PHTHALICE ACID,BUTYL 2-PENTYL ESTER

	15.928	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.479	DIPHENYLETHYNE
	14.516	2-BENAOYLOXYSUCCINIC ACID , DIMETHYL ESTER
	13.26	PHTHAIC ACID,
	12.206	4'-DIETHYLAMINOACETANILIDE
	9.11	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE
247	21.732	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.412	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.019	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.254	LUMIFLAVINE
	17.088	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.903	THIOPHENE, 3,4-BIS(ETHOXYNETHYN)-
	14.506	BENZOIC ACID, 2-METHYL-,(2-METHYLPHENYL)METHYL ESTER
	12.211	4'-DIETHYLAMINOACETANILIDE
211	17.041	7-METHYL-2-(7-OXONONYL)-PERHYDROAZEPINE
	16.869	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.234	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	15.921	PHTHALIC ACID, 6-ETHYL-3-OCTYL BUTYL ESETR
	14.59	L-PROLINE, N-ALLYLOXYCARBONYL-, TETRADECYL ESTER
	13.264	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER
	12.033	1 ,3-DIOXOLANE-2-HEPTANENITRILE, .ALHA.-METHYL-.DELTA.-OXO-

		2-PHEN
42	21.739	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	21.194	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.414	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	17.044	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.867	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	16.776	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	15.894	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.223	2-THIOPHENECARABOXYLICACID,CYCLOBUTYL ESTER
	14.573	CYCLOBUTANE, 1,3-DIPHENYL-, TRANS
186	21.732	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.197	2,3-DIMETHYL-5(2,6,10-TRIMETHYLUNDECYL)THIOPHENE)
	19.407	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.003	5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.218	LUMIFLAVINE
	17.062	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.901	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-

		METHYLPHENOXY)ETHYL}-
	15.907	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	13.656	1 ,2-DIHYDRO-5-ACENAPHTHYLENAMINE
	13.254	PHTHALIC ACID, 3,5-DIMETHYLPHENYL ETHYL ESTER
	12.195	4'-DIETHYLAMINOACETANILIDE
206	21.724	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.185	
	19.405	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	17.04	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.873	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.895	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	14.73	PHTHALIC ACID , METHYL 2-PHENYLETHYL ESTER
	14.558	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER
	13.535	BENZOIC ACID , 2-METHYL-, (2-METHYLPHENYL)METHYL ESTER
	13.238	PHTHALIC ACID, HEXYL 2-PHENYLETHYL ESTER
	12.275	O-TOLUIC ACID , 2-PHENYLETHYL ESTER
	12.189	4'-DIETHYLAMINOACETANILIDE
70	21.735	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.195	
	19.405	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.

	17.035	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.864	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	15.901	L-PROLINE, N-ALLYLOXYCARBONYL-, TETRADECYL ESTER
232	21.752	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.432	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	18.979	TETRACYCLO[16. 1 .0.0(2.9).0(10,17)]NONADECA-2(9), 10(17)-DIENE 19,19-DIME
	17.173	LUMIFLAVINE
	16.22	PHENETHYLAMINE , P-CHLORO-N-(P-CHLOROBENZYL)-
	15.903	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.146	BENZENE, 1,3.HEXADIENYL-
	14.571	BENZOIC ACID, 2-METHYL-,(2-METHYLPHENYL)METHYL ESTER
	13.679	1,2-DIHYDRO-5-ACENAPHTHYLENAMINE
	12.201	4'-DEITHYLAMINOACETANILIDE
	9.105	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE
	21.752	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
256	21.742	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	21.132	1,4-BENZENEDIAMINE, N-(1-,3-DIMETHYLBUTYL)-N'-PHENYL-
	19.417	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.034	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE

	17.224	LUMIFLAVINE
	16.246	PHTHALIC ACID,BUTYL ,2-PENTYL ESTER
	15.918	PHENOL, 3,5-DIMETHOXY-,ACETATE
	12.211	4'-DIETHYLAMINOACETANILIDE
	9.76	2-THIOPHENECARBOXYLIC ACID, 4-CHLOROPHENYL ESTER
	9.11	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE
249	13.285	PHTHALIC ACID,PROPYL TRIDEC-2-YN-1-YL ESTER
	12.211	2-IMINO-6-MERCAPTO-4,4-DIMETHYL-1,2,3,4-TETRAHYDRO-PYRIDINE-3,5-DIC
	12.07	PHENOL, 2,5-BIS(1 , 1-DIMETHYLETHYL)-
233	12.206	4'-DIETHYLQMINOACETANILIDE
	11.732	N-BENAYL-N-ETHYL-P-ISOPROPYLBENZAMIDE
	9.105	FLUOREN-9-OL, 3,6-DIMETHOXY-9-(2-PHENYLETHYNYL)-
71	21.742	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.188	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.417	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	16.871	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	15.887	L-PROLINE, N-ALLYLOXYCARBONYL-, TETRADECYL ESTER
	13.265	PHTHALIC ACID , ETHYL ISOPORPYL ESTER

97	21.747	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.213	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.443	H1-1 ,2,4-TRIAZOLE-1-ETHANOL, .BETA.([1 ,1'-BIPHENYL]-4-YLOXY)-ALPH-)1.
	16.785	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.24	PHTHALIC ACID, BUTYL 2-PENTYL ESTER
	15.929	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.529	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	14.511	2-BENZOYLOXYSUCCINIC ACID,DIMETHYL ESTER
160	21.762	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.438	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	18.994	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.032	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.908	PHENOL, 3,5-DIMETHOXY
	12.211	4'-DIETHYLQMINOACETANILIDE
	12.06	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
	11.737	N-BENAYL-N-ETHYL-P-ISOPROPYLBENZAMIDE

287	21.742	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	21.127	1,4-BENZENEDIAMINE, N-(1-,3-DIMETHYLBUTYL)-N'-PHENYL-
	19.448	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.055	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.269	LUMIFLAVINE
	16.785	PHENOL, 2,4,6-TRIS(1-METHYLETHYL)-
	15.943	DIETHYL 4-OXO PIMELATE
	11.737	N-BENAYL-N-ETHYL-P-ISOPROPYLBENZAMIDE
	12.211	4'-DIETHYLAMINOACETANILIDE
	9.105	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE
180	19.407	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	17.037	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.855	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.19	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.887	THI
	15.484	L-ALANINE, N-(2-THIENYLCARBOLYL-,HEXYL ESTER)
	15.221	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	14.974	
	14.863	4-CHLOROBENZYLAMINE, N-DECYL-N-METHYL-
	14.752	PHTHALIC ACID,METHYL 2-PHENYLETHYL ESTER
	14.561	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER
	13.563	ACETIC ACID, TRICHLORO-, 2-PHENYLETHYL ESTER

	13.266	PHTHALIC ACID, 3,5-DIMETHYLPHENYL ETHYL ESTER
	12.827	PHENYLTRIMETHYLEMMONIUN CHOLORIDE
58	15.23	2-THIOPHENECARBOXYLIC ACID, CYCLOBUTYL ESTER
	16.24	PHTHALIC ACID, BUTYL 2-PENTYL ESTER
	18.85	CYCLOPENTANECARBOXYLIC ACID, 1,2,2-TRIMETHYL-3-[PYRROLIDINE-1-CARBONYL]-
	19.41	1H-1,2,4-TRIAZOLE-1-ETHANOL,BETA-{[1,1'-BIPHENYL]-4-YLOXY}-ALPHA-(1,1-DIMETHYLETHYL)-
138	21.218	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.609	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.503	
	19.039	5,10-DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.284	LUMIFLAVINE
	17.108	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.362	2-NAPHTHALENAMINE,N-ETHYL-
	16.241	PHENOL, 3,5-DIMETHOXY-,ACETATE
	15.903	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.499	L-ALANINE, N-(2-THIENYLCARBONYL)-, HEXYL ESTER
	15.232	THIOPHENE-2-CARBOXYLIC ACID ETHYL ESTER
	14.849	THIOPHENE, 2-BUTYL-5-ETHYL
	13,255	DIETHYL PHTHALETE
	12.191	4'-DIETHYLAMINOACETANILIDE

	11.737	N-BENZYL-N-ETHYL-P-ISOPROPYLBENZAMIDE
244	21.742	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.448	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.804	QUINOLINE, 1,2,3,4-TETRAHYDRO-1-((2-PHENYLCYCLOPROPYL)SULFONYL)-,
	17.385	3.6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.79	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.296	PHENOL, 3,5-DIMETHOXY-,ACETATE
	16.246	PHTHALIC ACID, BUTYL 2-PENTYLESTER
	15.958	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.872	BENZENE, 4-ETHENYL-1,2-DIMETHYL-
	15.151	SPIRO[NAPHTHALENE-2(1H),2'-OXIRAN]-1-ONE, 3'-ACETYL-3'-METHYL-, CIS-(+
	13.648	3-METHYL-5-PHENYLPYRIDINE
	13.255	DIETHYL PHTHALETE
	12.216	4'-DIETHYLAMINOACETANILIDE
236	17.148	2H-PYRAN-2-ONE, 6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-
	12.201	4'-DITHYLAMINOACETANILIDE
	9.105	2'CHLORO-4-(4-METHOXYPHENYL)-6-(4-NITROPHENYL)PYRIMIDINE
239	18.3	ERGOST-5-EN-3-OL, 22,23-DIMETHYL-,ACETATE,(3,BETA)-

	18.078	CHOLAN-24-OIC ACID, 3, 12-BIS(ACETYLOXY)-,METHYL ESTR,(3.BETA.,5.ALF)
	17.912	AMBROSIN
	17.281	LUMIFLAVINE
	16.984	ACRIDINE, 9,10-DIHYDRO-9,9-DIMETHYL
	16.5	MURRAYAFOLINE
	16.237	PHTHALIC ACID, 6-ETHYL.3-OCTYL BUTYL ESTER
	16.041	PYRROLO[1,2-A]QUINOXALINE-1,2,3-TRICARBOXYLIC ACID,4,5-DIHYDRO-5-M
	15.834	PHENANTHRENE, 9-BUTYL-1,2,3,4,5,6,7,8-OCTAHYDRO-
	14.871	PHENOL, 2-(1-PHENYLETHYL)-
	14.513	BEZOIC ACID , 2-[(BENZOYLAMINO)CARBONYL]HYDRAZIDE
	13.64	3-METHYL-5-PHENYLPYRIDINE
	12.203	4'-DIETHYLAMINOACETANILIDE
	27.297	4,4'-((P-PHENYLENE)DIISOPROPYLIDENE)DIPHENOL
	23.383	ABIETIC ACID
	22.032	PALUSTRIC ACID
	21.795	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	21.154	1,4-BENZENEDIAMINE, N-(1-,3-DIMETHYLBUTYL)-N'-PHENYL-
	19.485	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.061	5,10-DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
240	19.58	1,3-BENZENEDIOL, 4-(3,4-DIHYDRO-7-HYDROXY-2H-1-BENZOPYRAN-3-YL)-
	17.16	1H-INDENE,2-BUTYL-3-HEXYL-

	12.206	4'-DIETHYLAMINOACETANILIDE
89	19.508	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	12.025	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
	9.11	COBALT , .ETA.-5-CYCLOPENTADIENYL-.ETA.-5-1 ,2-DIPHENYLCYCLOPENTADI
105	21.757	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.213	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.468	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	16.911	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.785	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.23	PHENOL, 3,5-DIMETHOXY-,ACETATE
	15.908	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.494	L-ALANINE, N-(2-THIENYLCARBONYL)-, HEXYL ESTER
	15.212	N-(4,DIMETHYL-THIAZOL-2-YL)-2-NAPHTALEN-1-YL-ACETAMDE
179	23.352	PHENOL, 2,4-BIS(1-PHENYLETHYL)-
	22.253	PHENOL, 2,4-BIS(1-PHENYLETHYL)-
	22.006	PHENOL, 2,4-BIS(1-PHENYLETHYL)-

	21.728	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.184	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.419	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	18.002	CYCLOPENTANECARBOXYLIC ACID, 1,2,2-TRIMETHYL-3-(PYRRROLIDINE-1-CAF
	17.22	LUMIFLAVINE
	17.049	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.228	2-THIOPHENECARBOXYLIC ACID,CYCLOBUTYL ESTER
	14.981	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.218	2-THIOPHENECARBOXYLIC ACID
	15.137	TRANS-3-AZIDO-1 ,2,3,4-TETRAHYDRO-2-NAPHTHYL METHANESULFONATE
	14.875	PHENOL, 2-(PHENYLETHYL)-
	13.264	PHTHALIC ACID, 3,5-DIMETHYLPHENYL ETHYL ESTER
	9.033	BENZENE, 1,1'-(1 ,5-HEXADIENE-1 ,6-DIYL)BIS-
177	21.744	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.194	
	19.399	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	17.049	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.878	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE

	16.787	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.237	1 ,2,5-OXADIAZOLE-3-CARBOXAMIDE, 4-AMINO-N-[2-[[(CHLOROPHENYL)MET
	15.894	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	13.274	1 ,3-DIOXOLANE-2-HEPTANENITRILE, .ALHA.-METHYL-.DELTA.-OXO-2-PHEN
	12.21	4'-DIETHYLAMINOACETANILIDE
	10.69	2-THIOPHENECARBOXYLIC ACID,5-ETHYL-
	9.069	BENZENE, 1,1'-(1 ,5-HEXADIENE-1 ,6-DIYL)BIS-
188	16.973	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	16.787	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.252	PHTHALIC ACID , BUTYL 2-PENTYL ESTER
	15.914	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	15.511	L-ALANINE, N-(2-THIENYLCARBONYL)- ,HEXYL ESTER
	15.344	2-THIOPHENECARBOXYLIC ACID , CYCLOBUTYL ESTER
	15.228	2-THIOPHENECARBOXYLIC ACD,CYCLO
	15.072	1 ,2-PROPANEDIONE, 1-(2-THIENYL)-
	14.981	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	14.87	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	14.77	1 ,2-PROPANEDIONE, 1-(2-THIENYL)-
	14.674	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	13.257	DIETHYL PHTHALATE

	13.015	1-OCTYL-2-[6-(4,4-DIMETHYL-2-OXAZOLIN-2-YL)HEXYL]-CYCLOPROPENE
	13.015	1-OCTYL-2-[6-(4,4-DIMETHYL-2-OXAZOLIN-2-YL)HEXYL]-CYCLOPROPANE
	12.939	1 2-PROPANEDIONE, 1-(2-THIANYL)-
112	21.732	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
	21.193	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	19.417	
	18.994	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	17.037	5,10DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	16.871	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.887	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	12.196	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
	12.045	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
148	21.756	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.603	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.043	5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
	18.877	2(4H)-BENAOFURANONE, 5,6,7,7A-TETRAHYDRO-4,4,7AA-TRIMETHYL-,-

	15.937	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.15	SPIRO[NAPHTHALENE-2(1H),2'-OXIRAN]-1-ONE, 3'-ACETYL-3'-METHYL-, CIS-(+
	14.853	NAPHTHALENE, 1,6-DIMETHYL-4-(1-METHYLETHYL)-
	14.5	1-,2,3,4-BUTANETETROL, 1,4-DIBENZOATE,(R* ,S*)-
	13.244	DIETHYL PHTHALETE
	12.195	4'-DIETHYLQMINOACETANILIDE
	9.003	INDANE
	8.57	BENZALDEHYDE, 3-AMINO-,OXINE
208	21.732	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.192	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.427	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	19.004	5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.208	LUMIFLAVINE
	17.062	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	14.7222	PHTHALIC ACID , METHYL 2-PHENYLETHYL ESTER
	14.556	BUTYLPHOSPHONIC ACID , ETHYL 2-PHENYLTHYL ESTER
	13.5332	BUTYLPHOSPHONIC ACID , ETHYL 2-PHENYLTHYL ESTER
	13.245	BENZOIC ACID , 2-METHYL-, (2-METHYLPHENYL)METHYL ESTER
	12.271	DIETHYL PHTHALATE
187	19.397	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-

		.ALPHA. -(1.
	17.048	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.882	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-
	16.237	PHTHALICACID,BUTYL 4-ISOPROPYLPHENYL ESTER
	15.905	L-PROLINE, N-ALLYLOXYCARBONYL-, TETRADECYL ESTER
	14.566	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER
	14.51	4-PYRIDINEMETHANOL, 3-METHYL-,ACETATE (ESTER)
	12.196	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
127	21.752	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.208	LUMIFLAVINE
	19.428	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	18.989	5,10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
	17.173	2H-PYRAN-2-ONE , 6-[2-E-(3-ETHYLPHENYL)ETHENYL]-4-METHOXY-
	17.027	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.861	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.205	PHENETHYLAMINE, P-CHLORO-N-(P-CHLOROBENZYL)-
	15.903	PHENOL, 3,5-DIMETHOXY-,ACETATE
44	12.19	3,4-DIMETHYL-2-[3-METHYL-BUTYRYL]-BENZOIC ACID, METHYL ESTER
	13.63	1,2-DIHYDRO-5-ACENAPHTHYLENAMINE
	14.86	PHENOL, 2-[1-PHENYLETHYL]-

	16.03	ERGOST-5-EN-3-OL, 22,23-DIMETHYL-, ACETATE, [3.BETA.]-
	17.03	ACRIDINE,9,10-DIHYDRO-9,9-DIMETHYL-
	19.42	1H-1,2,4-TRIAZOLE-1-ETHANOL,BETA-{[1,1'-BIPHENYL]-4-YLOXY}-ALPHA-(1,1-DIMETHYLETHYL)-
	21.12	1,4-BENZENEDIAMINE,N-[1,3-DIMETHYLBUTYL]-N'PHENYL-
	21.74	PYRROLO[1,2-A] PYRAZINE -1,4-DIONE,HEXAHYDRO-3-[PHENYLMETHYL]
	22.25	PHENOL,2,4-BIS[1-PHENYLETHYL]-
	23.34	PHENOL,2,4-BIS[1-PHENYLETHYL]-
	27.23	OXAZOLIDIN-2-ONE, 4-HYDROXY-4,5,5-TRIMETHYL-3-[2-[1,2-DIMETHYL-3-INDOLYL]ETHYL]-
141	26.861	4-TERT-BUTYLPHTHALONITRILE
	21.742	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.564	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.029	5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
	17.753	2,5-DI-TERT-BURYL-1,4-BENZOQUINONE
	17.254	2H-PYRAN-2-ONE, 6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-
	17.078	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.484	L-ALANINE, N-(2-THIANYLCARBONYL)-, BUTYL ESTER
	12.201	PHENOL, 2,5-BIS(1 , 1-DIMETHYLETHYL)-
219	21.744	DIETHYL PHTHALATE
	21.129	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-

	19.455	1.4-BENZENEDIAMINE, N-(1,3-DIMETHYLBUTYL)-N'-PHENYL-
	19.036	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	18.028	5,10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
	16.979	CYCLOPENTANECARBOXYLIC ACID, 1,2,2-TRIMETHYL-3-(PYRRROLIDINE-1-CAF
	16.787	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.575	7 ,9-DI-TERT-BUTYL-1-OXASPIRO(4,5)DECA-6,9-DIENE-2,8-DIONE
	16.49	1,4-BIS-(2-METHYL-THIAZOL-4-YLMETHYL)-PIPERAZINE
	15.935	2-NEPHTHALENEACETONIRILE , 6-METHOXY-.ALPHA. METHYL-
	13.625	THIOPHENE, 3,4-BIS(ETHOXYMETHYL)-
184	19	5,10-DIHYDROXY-2-METHOXY-7-METHYL-1 ,4-ANTHRACENEDIONE
	17.19	LUMIFLAVINE
	12.196	4'-DIETHYLAMINOACETANILIDE
13	19.42	1H-1,2,4-TRIAZOLE-1-ETHANOL,BETA-{[1,1'-BIPHENYL]-4-YLOXY}-ALPHA-(1.
	18.98	1-METHYL-1-[6-ETHYL-3-OCTYLOXY]-1-SILACYCLOHEXANE
	16.88	THIOPHENE,3,4-BIS[ETHOXYMETHYL]-
	15.91	L-PROLINE,N-ALLYLOXYCARBONYL,-TETRADECYL ESTER
145	15.888	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.464	L-ALANINE, N-(2-THIANYLCARBONYL)-, HEXYLESTER
	14.864	PHENOL, 2-(1-PHENYLETHYL)-

	14.728	PHTHALIC ACID , METHYL 2-PHENYLETHYL ESTER
	14.566	BUTYLPHOSPHONICACID, ETHYL 2-PHENYLETHYL ESTER
	13.527	6 BROMOHEXANOIC ACID , 2-PHENYLETHYL ESTER
	13.245	DIETHYL PHTHALETE
	12.206	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER
	23.351	PHENOL, 2,4-BIS(1-PHENYLETHYL)-
	21.203	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.443	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	18.999	TETRACYCLO[16. 1 .0.0(2.9).0(10,17)]NONADECA-2(9), 10(17)-DIENE 19,19-DIME
	17.234	2H-PYRAN-2-ONE, 6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-
	16.891	6-CYANOQUINOLINE
132	26.881	4-TERT-BUTYLPHTHALONITRILE
	21.757	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.208	LUMIFLAVINE
	19.478	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.044	5, 10-DIHYDROXY-2-METHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.274	LUMIFLAVINE
	16.896	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.785	2,5-CYCLOHEXADIEN-1-ONE, 2,6-BIS(1,1-DIMETHYLETHYL)-4-ETHYLIDENE-
	16.346	2-NAPHTHALENAMINE , N-ETHYL-

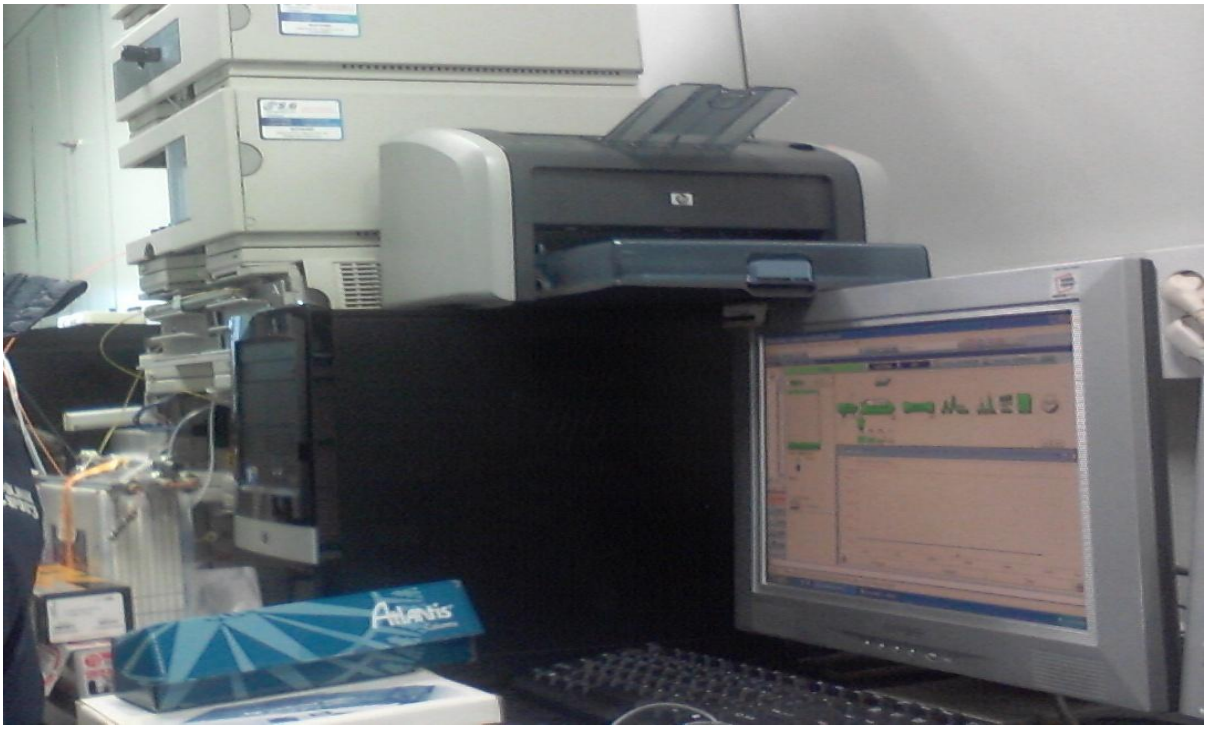
	16.235	PHTHALIC ACID, BUTYL, 4-CHLOROBENZYL ESTER
	15.897	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.484	L-ALANINE , N-(2-THIENYLCARBONYLA)-.BUTYL ESTER
	14.833	3-ETHOXY-4-METHOXYPHENOL
	13.643	1 ,2-DIHYDRO-5-ACENAPHTHYLENAMINE
	13.25	DIETHYL PHTHALATE
	12.196	4'-DIETHYLAMINOACETANILIDE
152	21.751	PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-
	19.456	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE
	19.078	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE
	17.293	LUMIFLAVINE
	17.026	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	16.7	PHENOL, 2,4,6-TRIS(1-METHYLETHYL)-
	16.244	PHTHALIC ACID, BUTYL 2-PENTYLESTER
	15.196	DIETHYL 4-OXO PIMELATE
	15.15	1-CYANO-4-CYCLOHEXYLBENZENE
	13.833	CARBAMIC ACID, 4-FLUOROPHENYL-,BUTYL ESTER
	12.199	4'-DIETHYLQMINOACETANILIDE
	9.108	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE
139	18.979	DIME THYL 4-OXOOCTANE-1 ,8-DIOATE
	17.148	2H-PYRAN-2-ONE, 6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-
	15.333	1-BUTANONE, 1-(2-THIANYL)-

	15.292	1 ,4-BIS-(2-METHYL-THIAZYL-4-YLMETHYL)-PIPERAZINE
	15.227	1 ,2-PROPANEDIONE, 1-(2-THIANYL)-
	15.076	1-BUTANONE, 1-(2-THIANYL)-
	14.975	1 ,2-PROPANEDIONE, 1-(2-THIANYL)-
	14.768	6-METHYLCYCLOHEXATHIAAOLE
	14.672	1-BUTANONE, 1-(2-THIANYL)-
	12.196	PHANOL, 2,5-BIS(1 , 1-DIMETHYLETHYL)-
	12.035	4'-DIETHYLQMINOACETANILIDE
	7.839	2H-1-BENZOPYRAN, 7-METHOXY-2,2-DIMETHYL-
191	21.727	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE,HEXAHYDRO-3-(PHANYLMETHYL)-
	21.193	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A
	19.417	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1 ,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.
	17.229	LUMIFLAVINE
	17.047	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE
	15.887	THIOPHENE , 3,4-BIS(ETHOXYMETHYL)-
	12.196	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER

17. List of accession number of samples obtained after 16s RNA sequencing along with closely related species identified after blast analysis

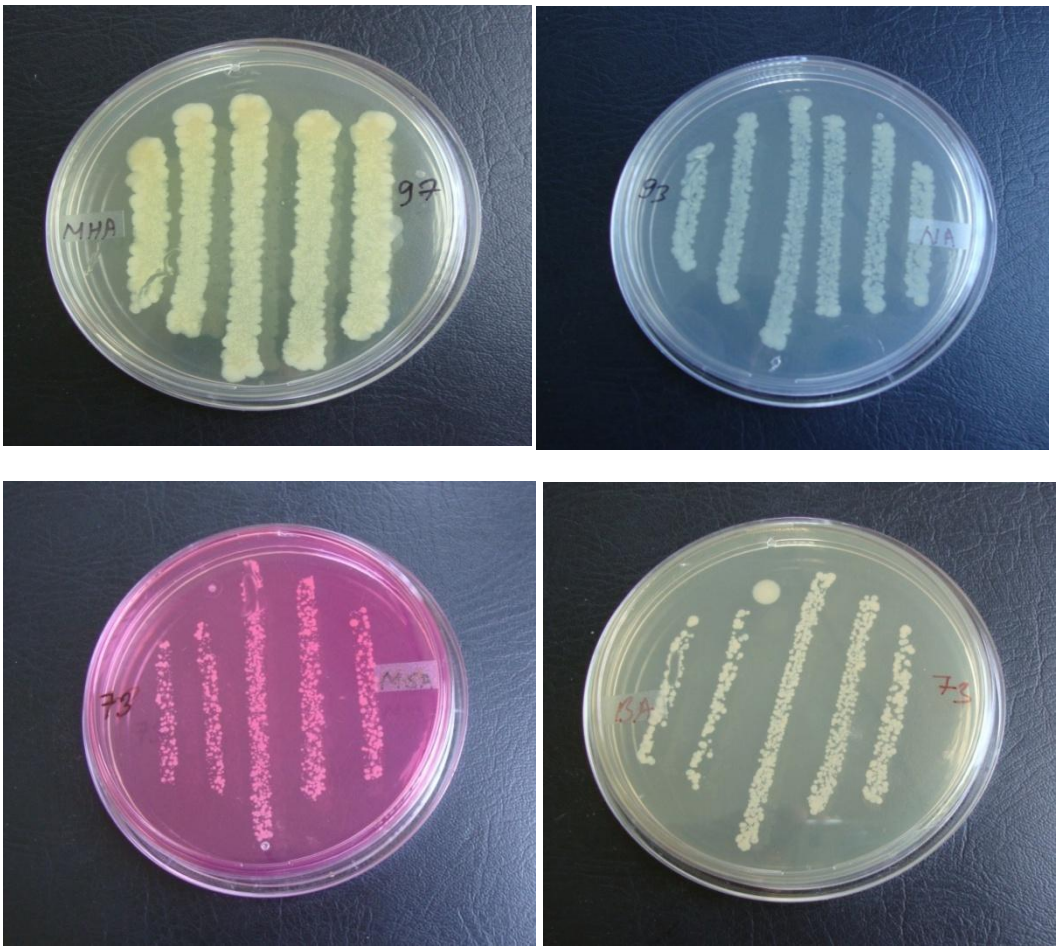
S.N	ACCESS NO.	DB-249	CP002824
DB-32	ASDA01000001	DB-239	HF679035

DB-247	AE017333	DB-234	ZTLO01000001
DB-134	AE016877	DB-208	AJ831844
DB-186	AB021185	DB-178	JTKJ01000077
DB-206	AY724690	DB-179	HG933296
DB-130	CP000560	DB-58	AF025372
DB-111	ASJD01000027	DB-148	AF025371
DB-224	EF114313	DB-34	ANAV01000046
DB-144	AMXN0100021	DB-188	AJ421446
DB-89	AJJIO1000018	DB-141	L37605
DB-160	CBZR01000004	DB-240	JMPQ0100005
DB-29	JQ070300	DB-28	AJ233430
DB-16	Y17654	DB-79	FJ790328
DB-177	CP010523	DB-44	EU103629
DB-247	AP008934	DB-13	HE798552
DB-70	EU573216	DB-193	HE613447
DB-77	JALV01000036	DB-113	JANBP01000188
DB-232	AB021404	DB-252	D16147
DB-218	BAMA01000316	DB-236	AJ628749
DB-66	AP021405	DB-256	FN298444
DB-20	BBIR01000146	DB-43	EF592577
DB-131	ACLE01000013	DB-233	HM212415
DB-249	X87756	DB-254	AJKH01000109
DB-71	AJ233422	DB-68	CP000758

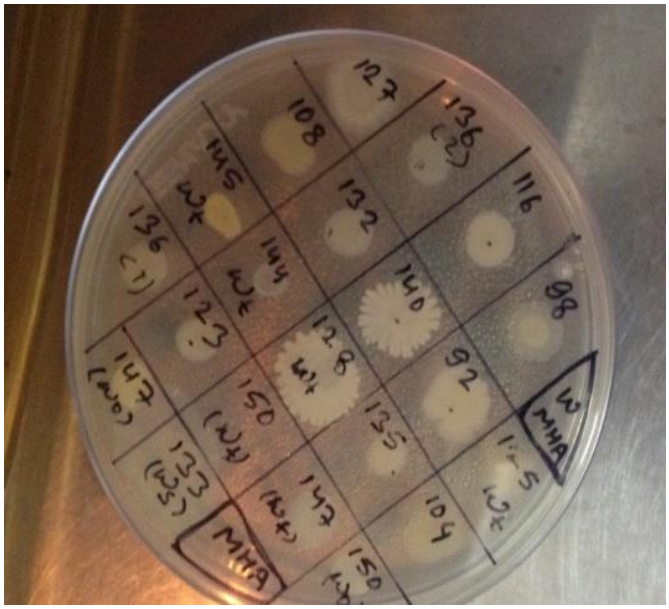


GC Ms library analysis

GC MS instrument Shimadzu QP 2010



Initial plating photos



Multiple colonies

CDBT Lab, TU

RIBB, Sinamangal, Kathmandu