COMPARISON OF ORAL MICROFLORA ISOLATED FROM RURAL MEDICAL CAMPS AND URBAN MEDICAL CLINICS USING ENZYME ESSAY, GC-MS AND 16s RIBOSOMAL RNA SEQUENCE ANALYSIS

M.Sc. Thesis

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Tribhuvan University

Kirtipur, Kathmandu, Nepal

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Mukesh Thapa

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Mukesh Thapa

Supervisors

Principal supervisor
Prof.Dr. Rajani Malla
TU

Research supervisor
Dr. Hari Datta Bhattarai
RIBB

Registration No.: 5-2-37-923-2008

Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Date……

Recommendation

This is to certify that Mr. Mukesh Thapa has successfully completed her dissertation work entitled "**Comparison of oral microflora isolated from rural medical camps and urban medical clinics using enzyme essay, GC-Ms and 16s ribosomal RNA sequence analysis.**" under my supervision. This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 110. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

……

Prof. DR.Rajani Malla

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Nepal

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date……

Certificate of evaluation

This is to certify that this thesis entitled "**Comparison of oral microflora isolated from rural medical camps and urban medical clinics using enzyme essay, GC-Ms and 16s ribosomal RNA sequence analysis.**" presented to evaluation committee by Mr. Mukesh Thapa is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Head of Department

Prof. Dr.Rajani Malla

……

External examiner

Prof. Dr.Megha Raj Banjara

……

Internal examiner

Mrs.Smita Shrestha

……

Principal Supervisor

Prof. Dr.Rajani Malla

……

Research supervisor

Dr. Hari Datta Bhattarai

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Mukesh Thapa

List of Abbreviations

RNA	Ribonucleotides
DNA	Deoxyribonucleotide
rRNA	Ribosoma RNA
3D	Three Dimensional
EPS	Extracellular polymeric substance
PIA	polysaccharide intercellular adhesion
DMFT	Decaye- missing- filled teeth
VAP	Ventilator-associated pneumonia
CV-I	Crystal violet- iodine
RDP	Ribosomal Database Project
IDNS	Internationalized domain name services
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
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
eV	Electron volt
NIST	National Institute of Standards and Technology
CMC	Carboxymethyl cellulose
ATCC	American Type Culture Collection
CDBT	Central Department of Biotechnology

MS	Mass Spectrometry
GC-MS	Gas chromatography-Mass spectroscopy
UV	Ultra Violet
WHO	World Health Organization

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Abstract

A biofilm is an assemblage of surface-associated multiple or single microbial species cells enclosed in an extracellular polymeric substance matrix (Bersan *et al*., 2014). Gathering the knowledge of microbial diversity and biochemical mode of action can enlighten nature, resistance mechanism and pathogenic property of oral biofilms (Geethashri *et al.*, 2014). This study explores the possibility of obtaining knowledge of microbial population and their biochemical mode of action among two sets of oral samples, urban people (17) and rural communities (12) by using various molecular methods. Sequencing of 16S ribosomal RNA was employed after proper selection of colonies for identification of sample obtained isolates. Biochemical mode of action was signified using understanding of Enzyme assay and identification of possible metabolites using Gas Chromatography-Mass spectroscopy analysis. In enzyme assay except 35% colonies remaining had shown some kind of enzyme activity with maximum for protease then for cellulase and amylase but none for pectinase. GC-MS of bacterial extract provided the possible evidence of 255 compounds where only 107 compounds were significantly different. Majority of compounds identified provides idea of bacterial virulence and adaptation inside oral cavity by using alkyl compounds, organic acids, amines, amides, ester acid ester. Antibacterial biocompounds like abietic acid, O-toluic acid ester, Phthalice acid ester, Phenol acetate, ergostol acetate were also identified. Thirty similar compounds were obtained between 91 compounds identified from urban clinic and 50 of rural camps. By using 16s rRNA sequencing 17 different genuses with 33 species was isolated from oral samples. *Enterococcus, Enterobacter, Klebsiella, Bacillus, Staphylococcus, Serratia, Citrobacter, Pseudomonas* with more than one strain types and *Proteus mirabilis, Escherichia marmotae, Ochrobactrum anthropi, Stenotrophomonas chelatiphaga, Achromobacter pulmonis, Novosphingobium capsulatum, Chryseobacterium vietnamense, Obesumbacterium proteus, Flavobacterium oceanosedimentum, Lyinibacillus macroides* were identified with both sets of samples again showing similar pattern of commensal and pathogenic bacteria and *Escherichia marmotae* which was never isolated before in oral sample gives idea of anonymous nature inside oral cavity. Conclusively, comparative study between urban and rural samples with respect with their bacterial community and their biochemical mode of action shows similarity among prevalence, pathogenicity and virulence.

Keywords: BIofilm, Enzyme assay, Sequencing, GC-MS.

CHAPTER 1. INTRODUCTION

1.1 Oral microflora and their roles

Oral health relates all the parts present in the oral cavity but the major problem occurs in the dental portion of oral cavity. This problem seems to be increasing because of presence of both hard and solid surface for the colonization of bacteria which further develops dental biofilms and create dental carries (Kirby *et al*, 2014).The problem of oral health is prevalence worldwide in all age group and societies. In Nepal also the oral health is major concern but research relating these aspects are rarely done so proper data are not available for these subjects, but because of difference in geographic region and cultural habits there should be diversity in oral microorganism in Nepal. Biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix in a surface of some substance (Donlan, 2002). Microbial biofilms are communities formed when multiple number of single cell microorganisms become firmly adhered to a solid surface covered by an extracellular polysaccharide matrix; extracellular polymeric substances such as polysaccharides, proteins and nucleic acids (Narayan, 2013). It is found that biofilm can be formed from multiple or single microbial species (Bersan *et al*., 2014). Oral cavity is the entrance of digestive tract so it is considered as the initial source of most digestive tract microorganisms. The oral cavity is one of the most densely microbial populated areas of digestive tract. The oral cavity has two main types of surfaces for microbial colonization: non-shedding surfaces (teeth) and shedding surfaces (mucosa). Saliva is another content of oral cavity and it contains a large number of bacteria and enzymes liberation from glands (Kort *et al*., 2014). The heterogeneity of tissue types in the oral cavity, such as gingival crevices, tongue, hard palate, soft palate, cheeks, and lips, teeth means that a variety of sites with different features are available for colonization to oral microorganisms. A number of studies have shown that each of these type of surfaces provide a range of habitats with a characteristic microbiota. These microorganisms colonize oral surfaces where they form a microbial consortium referred to as dental plaque or oral biofilm (Takahashi, 2005).The dental biofilm is a microbial community that forms at high density on tooth and tissue surfaces of oral cavity including tongue, mucosa and other variant surfaces. This is one of the most complex and pathogenic biofilm that exist in humans. 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 (Narayana, 2013). Dental biofilm causes major health hazards not only relating to mouth but also other systemic diseases. Dental caries and periodontal diseases are among the most important global oral health problems (Bhardwaj , 2006).

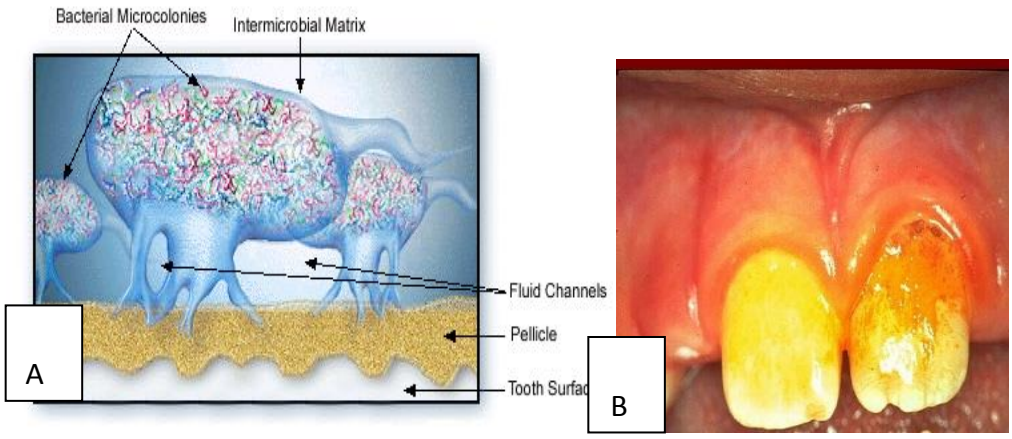

Figure 1.1 Biofilm formations on tooth surface (A) 3D structure (B) superficial visible structure

1.2 Microbial flora of dental biofilm

Oral biofilms distinctly contain more than 500-700 different bacterial taxa. These micro-organisms seem to have considerable inter- individual variation among them self. Heterogeneity of oral biofilm is reported not only in various parts of same oral cavity but also on the same teeth of an individual (Stoodleyl, 2005). These organisms seems to have developed variety of genetic and physiological abilities to sustain oral environment and to be part of dental biofilm (Geethashri *et al.*, 2014). Various procedures are used to detect oral bacteria, such as microbial culture, immunological assays, enzymatic methods and molecular biology, polymerase chain reaction (PCR)-based diagnostic technique, 16s RNA sequencing. These procedures have helped scientists to identify 500 different bacterial taxa in oral cavity. Much of these community constitute of commensal, free living, nonpathogenic, pathogenic bacteria in abundance but most of the oral problems are caused by group of 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). Although most of the peoples don't take oral problem quite serious, it as one of the major health concern in present scenario. Dental caries, gingivitis and periodontitis are the major oral health hazards and bacterial dental plaque is considered to be the primary etiological factor in the development of these problems (Garhwal, 2011). Along with these normal oral diseases it is also strongly associated with other diseases such as cardiac disease and cancers (pancreatic, gastrointestinal, oral and pharyngeal cancers like oral squamous cell carcinomas) (Chakraborty *et al*., 2014). WHO record shows significant results giving rising concerns relating oral health hazards among all age group, communities. 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. Globally, about 30% of people aged 65–74 have no natural teeth (Oral health, 2012).

1.3 Statement of problem

Oral health and dental problems has been the concern of every society since earlier period to recent day. An increasing number of oral problems and diseases in spite of recent development in this sector have raised the serious concern in our understanding of oral microbial environment. Many significant efforts are done by modern researchers to understand the microbial environment and relative disease caused in and around oral cavity including tooth. Mouths being the ambiguous and enormous source of both culturable and non culturable microorganisms, insignificant knowledge are available till now. Again looking at the current scenario of our country, Nepal, not any effort is done to understand the microbial diversity of oral cavity and dental biofilms. For this type of research, the sequencing based screening of microorganism isolated from different oral samples and GC-MS based bio-compound with authenticity can be first step in our country.

1.4 Hypothesis

Large source of diverse microbial community are found in oral cavity. Sequence based screening may identify variable aerobic or anaerobic, pathogenic or commensal group of microorganisms where as these microorganisms may produce different virulent and adaption based secondary metabolites. Rural samples should acquire vast majority of bacterial community with strict pathogens different from urban samples.

1.5 Rationale

Oral cavities have pool of different structural and sites for microbial attachment and different self-maintained environment for growth. Bacteria compatibility with these conditions is abundant in counting and identification of these species can give light to the oral problem itself. Pathogenic and commensal but opportunist pathogens are found to cause most of oral health hazards and associated systemic diseases where these bacteria are supposed to adopt and change by producing variable virulent secondary metabolites to cause disease condition. No significant researches have been done in this area. Hence there is a need for proper identification and isolation of microbial community along with bio- compounds produced by them which may be significant as marker for oral health. Along with this comparable study between population deprived of proper oral medical facilities who only attends medical camps, urban population and people of cities having ability and knowledge of oral health also need to be done.

1.6 Scope

This study creates an interest among the researcher for exploration of microbial biodiversity and if found to be noble and correlative opens a new arena for further research to understand association of these micro floras with diseased conditions and finally can go for medical advantages. This research will give a scientific validation on the

bioactive compounds produced by the common oral microorganism which can be further analyzed for marker use. Apart from this study would be a preliminary step towards identification of bacterial community and bio-active compounds produced by them in Nepalese population. The most important thing is that it will create awareness among the oral product associated companies. The information may pave the better way for exploiting finding of this work.

1.7 Objectives

1.7.1 General objective:

To identify and characterize oral microflora by using molecular techniques

1.7.2 Specific objectives:

1. To identify the oral microbial species from different medical camps and clinical populations using 16S rRNA sequence analysis.
2. To characterize the microflora enzymatically by using protease, cellulase, amylase, pectinase activities of each isolates.
3. To identify the secondary metabolites secreted by microorganism producing biofilm.

CHAPTER 2. LITERATURE REVIEW

2.1 Biofilm establishment

In past scientific research Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces as biofilm, so he can be credited with the discovery of microbial biofilms. A detailed examination of biofilms was done further with the help of introduction of the electron microscope, which allowed high-resolution and much higher magnifications. In a wastewater treatment plant after invention of scanning and transmission electron microscopy research showed them to be composed of a variety of organisms (Donlan, 2002). Biofilm establishment is regular and spontaneous process, if attachment sites are available. Attachment is the first step to obtain a sustainable biofilm. Initially pellicle is formed on the surface to create sites of attachment. Following pellicle formation, there is passive transport of oral bacteria on to the tooth surface by using weak, long-range physicochemical interactions among the pellicle coated tooth surface and the microbial cell. But short-range strong interactions between specific molecules on the bacterial cells and the complementary receptor proteins on the pellicle surface mainly contribute on establishment of proper attachment (Marsh, 2004). 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. To obtain clear picture of attachment one should considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and various properties of the cell surface (Characklis *et al.*, 1990). The co-adhesion of the later incoming bacterial colonizer to the already present biofilm continues to thicken the layer. These later co-adhesion involve many specific interactions between bacterial receptors and adhesions and build up the biofilm to create a more diverse environment including the development of unusual morphological structures. These interactions between diverse bacterial species now begin to create a number of synergistic and antagonistic biochemical interactions (Eg; bacterial food chains) among themselves either interspecies or intra-species. Both obligate anaerobes and aerobes are involved in co-adhesion and these biochemical interactions ensure the favorable environment to anaerobic bacteria's survival in the oxygen-rich oral cavity inside layer of biofilm (Marsh, 2006). 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. As the biofilm thickens and becomes more mature, matrix contributes in structural maturation and strengthening and in these 3D structre anaerobic bacteria continues to live deeper within biofilm after thick and matured multilayer plaue is established which will further protect them from the oxygen-rich environment within the oral cavity (Sbordone, 2003). It appears that early colonizers of dental plaque play an important role in progressive biofilm formation and the

characterization of mature dental plaque. After initial colonization they further maintain cell signaling and increase constant unification of cell layers to form thicker and thicker layer (Narayana, 2013). On both the tooth surface and periodontal tissues of the human oral cavity Dental plaque, a multispecies biofilm is organized (Kolenbrander, 2000).

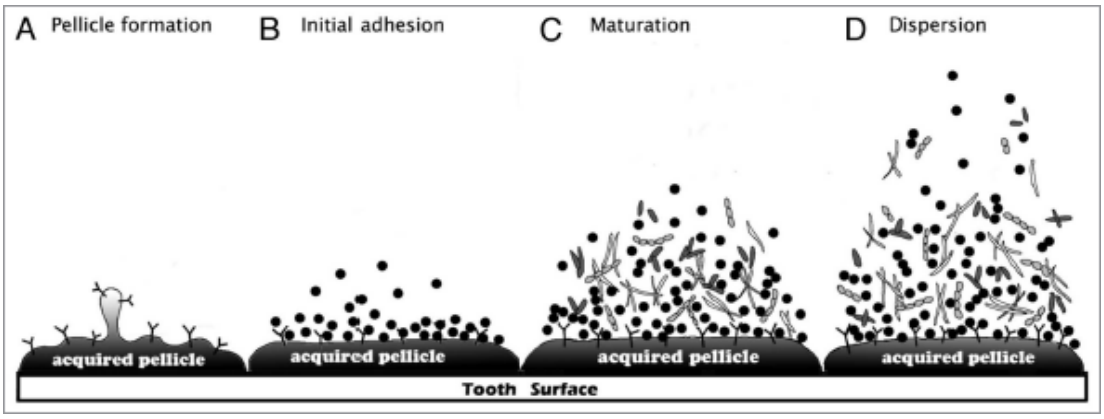

Figure 2.1 Steps during establishment of dental biofilm (Rashid *et al*., 2014).

2.2 Microorganism's variable nature inside biofilm

The water system biofilm is highly complex corrosion products, clay material, fresh water diatoms, and filamentous bacteria; on the other hand biofilm on the medical device, appears to be composed of a single, coccoid organism and the associated extracellular polymeric substance (EPS) matrix. Dentalbiofilms 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 inter connected system is maintained rather than free floating (Socransky *et al.*, 2002). Due to the complexity of biodiversity of Biofilm cells, they are capable of persisting in the presence of antimicrobials at concentrations that are up to 1000-fold higher than those necessary to eradicate a planktonic population. 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. Coaggregation partner are mostly associated for attachment. 1,000 oral bacterial strains that have been examined have at least one highly specific coaggregation partner, for example between the oral bacteria *Capnocytophaga gingivalis* and *Actinomyces israelii* or between *Prevotella loescheii* and *Streptococcussanguinis*(Kolenbrander *et al* ., 2010). To permit passage 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. The process called

quorum sensing within the biofilm enables bacterial 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 *Streptococcous* and *Porphyromonas gingivalis* can be utilized by *Veillonellae*. Similarly, to stimulate the growth of *T. denticola* isobutyrate secreted by *P. ginivalis* is used (Kolenbrander PE *et al*., 2002).

Adhesin-ligand mediated physical interactions are also seen in organisms like *Streptococcusgordonii* and *P. gingivalis* (Kuramitsu *et al*., 2007). Thick exopolymer matrix in dental biofilms prevents the penetration of antimicrobials and enables the microbial cells to be drug resistant, decreased growth rate. It is also considered possibility of resistant genes expression makes antimicrobials ineffective to biofilm microbial cells (Lewis, 2001). Thus organized structure and mechanisms of biofilm with variable but co existing microbial community are responsible for the emergence of drug resistant bacteria (Geethashri *et al*., 2014).

2.3 Oral health

The efforts to maintain good oral hygiene are found in various historical texts considering first known dentist Hesi Re used to promote rinsing with Bicarbonate of Soda to clean the mouth around 3000 B.C. Hippocrates(460-377 BC) attributed dental disease to “a combination of natural predisposition and the corroding action of accumulated filth” so he also recommended cleaning tooth. Chewing sticks were used to clean the teeth prior to the development of toothbrushes as we know them now. To clean the teeth Mohammed (570-632) encouraged the use of Miswak (a twig from the Salvadora Persica tree) before prayer. With the passage of time development of scientific equipment and techniques enhances the study of disease and evolve them from simple knowledge to the application of scientific method and logic in their investigation about its aetiology and usage of modern techniques for prevention. Scientists such as GV Black (1836-1915) were able to study dental pathology by the 19th century, and WD Miller (1853-1907) also developed his study of bacteriology in same period (Hyatt, 1921). In the 19th century major advances were made when dentistry evolved from a trade to a profession. In the UK the Dentist Act was passed in 1878 following the British Dental Association formed in 1879. Journal of Dental Research (JDR) was founded in 1919 by William J. Gies in order to bring scientific advances and to bear on the study of oral diseases. The founding in 1920 of the International Association for Dental Research (IADR) follows JDR.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). Dental caries is defined as an infectious, microbial disease that is characterized by demineralization of the inorganic portion and the destruction of the organic substances of the teeth (Shafer *et al*., 1993) Periodontal diseases are chronic inflammatory conditions characterized by loss of connective tissue, alveolar bone desorption, and formation of periodontal pockets as a result of the complex interaction between pathogenic bacteria and the host's immune response. Periodontitis starts with inflammatory lesions of the gingival, which, if left untreated,

may progress and eventually involve and compromise the entire periodontal apparatus of the affected teeth. Dental plaque is the primary etiologic factor in periodontal disease (Page & Kornman, 1997).The antiplaque agents can be delivered in the form of mouthwashes, dentifrices, chewing gums, gels, and chips. Chlorhexidine, triclosan, cetyl pyridinium chloride, essential oils, and fluoride-based solution are some of the antimicrobial agents tested against oral microbes. Chlorhexidine is an established antimicrobial agent; because of its anticariogenic and remineralization properties, it is extensively used in prevention of dental caries. Whereas high amount of fluoride ingestion may lead to acute poisoning and its low repeated ingestion causes fluorosis, especially in children. So, sodium fluoride mouth rinse is not recommended for children younger than 6 years as they may swallow it (Chatterjee *et al*., 2012). Green tea originated secondary metabolite, Catechin, was found to have antiplaque and antibacterial properties and contributed in caries prevention and gingival enhancement. Rasheed and Haider described the antibacterial effect of green tea catechins against *Streptococcus mutans* bacteria and stated that catechins are of great value in the reduction of *S. mutans* and caries prevalence (Rasheed & Haider , 1998).

2.4 Variability of microorganism in oral cavity

The digestive tract is anatomically continuous and harbors approximately 1×10^{14} microorganisms, which is more than the approximately 6×10^{13} cells that constitute the entire human body. Oral health is integral to general well-being and relates to the quality of life that extends beyond the functions of the craniofacial complex .In excess of 750 microorganism species have been isolated from the oral cavity using recently developed molecular biological methods (Kort *et al*., 2014). 50% of oral microflora is yet to be identified and it is considered that number of these is implicated in oral diseases. 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). The microrganisms adopts into different attachment oral environment promoting genetic and physiological changes on them which are also the main concern of oral hazards. In each stage of its development oral biofilm like other biofilms seems to acquire new microbial species, including *Lactobacillus casei, Streptococcus. mutant, S. sanguis, S. sobrinus and S. mitis* which due their pathogenicity could damage the enamel and gum tissue and create variety of health hazards (Bersan *et al*., 2014).

2.4.1 Microbial population in diseased individuals

Different problems associated to oral health hazard are mostly initiated with the establishment of bacterial film in different locations inside oral cavity. 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). Commensal

partners inside different sites of Humans body have evolved together. While most commensal organism's benefits human health, some can also serve as pathogens if possible advantages are available in exact time. Following the ingestion of dietary carbohydrate,most of these 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 *et al.*, 2009). After biofilm formation different nonpathogenic bacteria forms changes into pathogenic forms and causes medical problems in respect to their locations like supragingival dental plaque initiates dental caries, subgingival plaque causes periodontitis, carious dentine causes infected pulp or chronic caries and tongue surface debris facilates oral malodor (Li, 2014). 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).

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's includes *Porphyromonas gingivalis, Actinobacillus actinomyc etemcomitans, Treponema denticola, Prevotella intermedia* (Junaid *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 &Hart, 2013). An increasing body of evidence suggests that laboratory cultivated bacteria share few characteristics with infectious biofilms.

2.5 Variable morphological characters and enzymes of bacteria

Only shape is never enough but morphology is a significant and important selectable trait which can be used as experimental approach like any other subject. Motility imposes a heavy selective pressure on cell shape as fast cells are better off as rods with a certain length-to-width rather than being coccous (Pernthaler, 2005). Bacterial morphology is understood to regulate cell shape and at the same time morphological changes affect survival in different conditions (Young, 2007). Some species can modify

their colony morphology in response to environmental cues and in certain cases during the course of pathogenesis (Justice *et al.,* 2004). Morphological studies together with gram staining are used in clinical detection of Ventilator-associated pneumonia in many species associated VAP conditions (Raghavendran *et al.,* 2007).

2.5.1 Enzymes of oral bacteria

Bacterial population produces major group of exoenzymes as adoption. In the cases of stress, virulence, the produces major of enzymes using their adoptive genes (Pinto-Tomás, 2007). 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 (Josephine *et al*., 2012).

To catalyses the hydrolysis of starch into sugars for initiation of digestion in human oral cavity Amylase is present in the saliva is used where with the help of amylase starch are hydrolysed into small disaccharides and tri-saccharides (Silverman, 2002). All amylases enzymes are are glycosidehydrolases that 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 usedto degrade extracellular starches (Rejzek *et al*., 2011).

Pectin is the jelly-like matrix used to cement plant cells together and in which other cell wall components, such as cellulose fibrils, are embedded. Pectinase breaks down pectin which is a polysaccharide found in mostly cell walls. It includes major three groups of enzymes pectolyase, pectozyme, and polygalacturonase. Pectinase enzymes are commonly used in degradation**.** Major pectinase producing microorganisms are fungi like *Aspergillus niger*, *Penicillium jenseni* and *Penicillium citrinum* rather than bacterial species (Nwodo-Chinedu, *et al*., 2005).

Any enzymes or mixture of enzymes that catalyze cellulolysis, the decomposition of cellulose and cellulose related polysaccharides (hemicellulose, lichenin) by hydrolysis of the 1,4-beta-D-glycosidic linkages are cellulase (Worthington Biochemical Corporation , 2014). After cellulolysis, cellulases break down the cellulose molecule into monosaccharides or oligosaccharides. Cellulose makes a major constituent of plants so cellulases are considerable important for plant consumers (Lo *et al.*, 2011). Cellulases are produced by a few termite animals and in herbivorous ruminant animals such as like cattle and sheep cellulases are produced by symbiotic bacteria living on their gut wall (Watanabe, 1998).

Any enzyme (trypsin, chymotrypsin, plasmin, elastase ,thrombin) that initiates protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain to perform proteolysis are protease or peptidase or proteinase. Proteases are widespread in nature; all organisms, from prokaryotes to eukaryo vtes to viruses. Microbes serve as a preferred source of these enzymes. Bacillus produces a wide variety of extra-cellular proteases. Several

Bacillus species involved in protease production are e.g. *B. cereus, B. sterothermophilus, B. mojavensis, B. megaterium and B. Subtilis* (Sookkheo, 2000). A bacterium uses proteases to hydrolyses and break the proteins down into their constituent monomers (amino acids) (Sims, *2006).* 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).

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). On the basis of their substrate spectra, Lipases and esterases can be distinguished, as true lipases have marked preference for 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). Triglyceride substrate forms an emulsion to activate the lipase activity in industrial uses (Thota *et al.*, 2012).They can catalyze esterification, interesterification, and transesterification (Jaeger *et al.*, 1994). Some important lipase producing bacterial genera include *Bacillus, Pseudomonas and Burkholderia* etc. Lipase/esterase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs and variable oral samples (Vakhlu *et al.*, 2006).

2.6 Modern and real time technique for bacterial analysis and identification

Although traditional techniques are tedious and take hours to days to yield a result, still present using traditional culture-based techniques large numbers of microbiological samples are analyzed annually. These techniques are above all very unsuitable for non-culturable microorganisms. 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 placemodern and real time technique is quiet handy in gathering usable data's relating the development and changes of organism (Veal *et al*., 2000).

2.6.1 Fluorescence staining and flow cytometry

Dependency on microbial culture is released and rather modern flow cytometry offers the prospect of real-time microbial analysis of individual microorganisms. However, part some analogue technology first developed 20-30 years ago with little improvement and mainly due to the need for trained flow cytometrists, the high cost, complexity of instrumentation and the lack of assay kits with appropriate biological reagents flow cytometry has been rarely used as a tool for routine microbial analysis in place of other modern instruments which are relatively simple to operate without a specialist operator. For simple use, low cost and robust microbial analyses, the incorporation of modern, a solid state opto-electronics combined with digital signal processing technology and micro-fabrication on addition to traditional cytometry is required.

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).

2.6.2 Molecular based methods

Without using traditional culture techniques different recent 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 mucosa, tongue, saliva, tooth surfaces, and periodontal pockets using these tools. The results have been compiled in the NIH-funded "Human Oral Microbiome Database" (Dewhirst *et al.*, 2010).

2.6.2.1 PCR based methods

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). At its 5' end oligonucleotide probe, with a reporter fluorescent dye attach and a quencher dye attached to its 3' end creating hybridization to the target gene. The quencher dye is break in to fragments by the 5' nuclease activity of Taq polymerase resulting in the accumulation of reporter fluorescence during PCR amplification which is released during amplification allows for rapid detection and quantification of DNA (Heid *et al.* 1996). Compared with other methods like flow cytometry and DNA-DNA hybridization for quantifying oral pathogens TaqMan real-time PCR assay has advantages in terms of sensitivity and rapidity (Socransky *et al.*, 1994).

2.6.2.2 Sequencing

Among the various molecular assays available, 16S rRNA sequencing stands out as a useful technique for detecting uncultivable bacteria. The introduction of molecular diagnostics significantly enhanced the ability to diagnose culture-negative infections. 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 a Carl Woese three decade ago (Woese *et al.*, 1997). Sequencing of various bacterial genomes and comparison between genome alongside 16S rRNA gene phylogeny has continued to solidified the status of 16s rRNA sequencing to confirmed the representativeness of the 16S rRNA gene in bacterial phylogeny (Snel *et al.*, 1999). 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). 16S rRNA sequencing provides solutions to problems, (like yielding unambiguous data), even for unusual and slow-growing isolates, often within 48 h, which are reproducible among laboratories and represents a universal technology (Weinstein *et al.*, 1997). This method also 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). Because of availability of this technique it has become possible to identify thermotolerant Campylobacter fetus rather quicker and to prescribe antibiotic treatment to these bacteria is much easier and specific (Woo *et al.*, 2001). For so called 'unidentifiable' bacteria 16S rRNA sequencing results in a higher percentage of species identification than conventional or commercial methods. For example in provided anaerobic Gram-positive rods, even for genus identification conventional methods are simply not reliable; 16S rRNA sequencing identifies many previously ignored anaerobic species in medical cases of bacteraemia (Bosshard *et al.*, 2002). Difficulties' in identification by 16S rRNA sequencing are known to present in certain groups of bacteria, so these bacteria should be excluded, and other housekeeping gene targets, e.g. rpoB, if available, are required(Janda, 2007). The databases of RDP-II and SmartGene IDNS contain sequences downloaded from GenBank, whereas all sequences in the databases of RIDOM and MicroSeq were obtained by sequencing the 16S rRNA (Woo *et al*., 2008). Various researchers used a combination of phenotypic and genotypic methods to identify the isolates in their cases which show more reliability then single approach (becker *et al.*, 2004).Microarray analysis system (HOMIM) for assessment of oral micro biota Based on 16S rRNA gene sequencing is recently invented which detects about 300 of the most prevalent oral bacterial species, including those that cannot yet be grown in vitro. Similarly the strategy of combining PCR-RFLPs with sequencing of the 16SrRNA gene have also used in recent studies(Chakraborty *et al.*, 2014).

2.7 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Characterization of indigenous bacteria like actinomycetes reflects their adaptations, resulting into secondary metabolite synthesis for virulence, defence, use of available nutrients. Scientists are trying to understand this phenomenon and trying to understand bacterial biocombound basis along with possibility of using them as biomarkers orpharmaceutically (Mangamuri *et al*., 2015). Gas chromatography coupled with mass spectroscopy is being used as one of the main method to identify possible compounds in any bacterial extracts.

In GC/MS, Gas chromatography is used for direct analysis of components existing in plants and bacterial extract (Sermakkani & Thangapandian, 2012). For direct qualitative and quantitative analysis of compounds present at molecular levels with very high precision, Gas chromatography coupled with mass spectroscopy (GC-MS) is normally used where GC first separates the individual chemical components and then MS ionizes and identifies them by their structure and molecular weight as main advantage of GC-MS even at very low concentrations. GC-MS has been widely utilized in many cases of quality control, forensic science, pollution studies, trace element analysis, etc. On recent times, GC-MS have been increasingly used for the analysis of volatile substances, non-polar components, alcohols, alkaloids, phenols long chain an Nature branched chain hydrocarbons, acids, esters, and other bioactive components (Sivakumar *et al*., 2015). Differential distribution coefficients is the main basis of Separation where a carrier gas (an inert gas such as helium or an un reactive gas such as nitrogen) is mobile phase and microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column as stationary phase. In 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)

2.8 Oral health scenarios of Nepal

The World Health Organization (WHO) defines oral health as “a state of being free from mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual’s capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing”(Theilade, 1986).

Orthodontic problems are a public health concern which requires to be treated in a cumulative manner. The present day scenario and future development of orthodontic is on the basis of its history and past tremendous efforts. Since 2008 the malocclusion data on Nepalese population sample has been sought almost every year. According to a study, the occurrence of discrepancies are: increased over jet in 43.8%, increased overbite in 20.7%, open bite in 8.2%, cross bite in 23.3%, and displacement in 65.7%. The need of orthodontic treatment of the Nepalese orthodontic patients are No/Little treatment need for 16%, Borderline treatment need 20%, Great/Severe treatment need for 64% according to Dental Health Component scale; and No/Little treatment need for 26%, Borderline treatment need for 32%, Great/Severe treatment need for 42% (Shrestha,2013). The proportion of male and female orthodontic patients according to hospital samples are 35.6% and 64.4% respectively. Mean duration of fixed orthodontic treatment in adolescent patients is 30.4 months and in adults are 28.8 months. Mean duration of orthodontic treatment in non-extraction case are 25.5 months (Bhattarai,

2013). The proportion of non-extraction and extraction cases 54.2% and 45.8% respectively (Bhattarai, 2010). The periodontal health status of Nepalese orthodontic patient shows 36% having gingival bleeding and calculus deposit and 60% having varying depths of periodontal pocket. The prevalence of dental caries is 79.2% with the mean DMFT value 2.87 among Nepalese orthodontic patients (Shrestha, 2013). However, the study did not consider the malocclusion status of the Nepalese people. An epidemiological data on malocclusion with the study on commonly prevailing occlusal problems in Nepalese people are necessary. The situational analysis should be done on status of orthodontic problem, human resource and availability of service in Nepal. The evaluation of malocclusion data and treatment need is required for planning and prioritizing the orthodontic service in the country.

2.9 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).

H2S exerts antimicrobial effects on certain oral *Streptococcous*, 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). SIimilarly, *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 (Srinivasprasad *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*, *Actinobacillu, Actinomyc 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 METHODOLOGY

3.1 Materials

3.1.1 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 total research work was conducted at the Central Department of Biotechnology, TU (Tribhuvan University) and RIBB (Research Institute for Bioscience and Biotechnology).

3.1.2 Instruments

The instruments that were used during this thesis were Centrifuge, Autoclave, Microwave oven, Incubator, UV spectrophotometer, laminar hood, microscope, sequencer, GC-MS, pcr machine. All of these devices were in good working condition**.**

3.2 Sampling

3.2.1 Patients:

A cross-sectional study was carried out in nearly 29 patients with oral health problems. 12 general medical camp samples from camps setup in rural area were collected in camps established in Baitadi (2), Nuwakot (3) and Sinduplanchok (7). Similarly, 17 dental clinic samples from different clinics of urban area like Pokhara (2), Kathmandu (4), Jhapa (5), Butwal (7) were collected.

3.2.2 Sample collection:

Oral cavity biofilm samples including oral samples like extracted teeth (most), dental plaque (1), and dental calculus (6) were collected from the people mentioned on above stated locations.

3.3 Methodology

3.3.1 Plate culture

All cultivable micro-organisms including was isolated using standard microbial techniques. Several agar plates including 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 and blood agar (BA) and brain heart infusion agar (BHA) was used to isolate colonies of pathogenic bacteria. And finally for possible fungal strains potato dextrose agar (PDA) was used for strain isolation. The grown colonies was carefully observed and purified by using repeated streaking method until we obtained a pure colony.

3.3.2 Identification of micro-flora by colony morphology

Morphological study of isolated micro-organism was carried out using both basic and microscope observation and general characteristic of a pure isolated colony considering characteristic: shape, size, color (pigmentation), margin, opacity, texture, surface, elevation. This information's were used as basis for deducting colony count of the micro-organisms as similar characteristic colonies were collected as same bacteria.

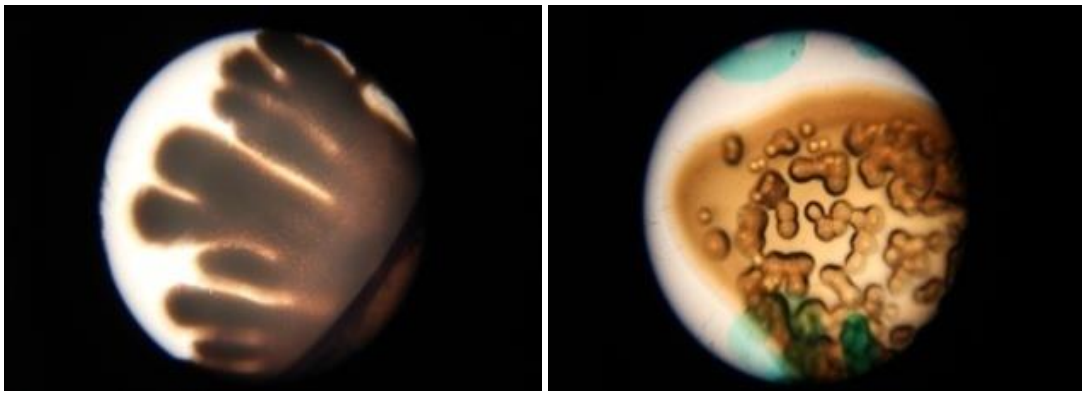

Figure 3.1 Colony morphology on plates

3.3.3 Enzymatic essay

Several extracellular enzyme secretion activities of the isolated bacteria were investigated using spot on lawn assay. In this assay the bacterial colony were grown in an agar plates containing target enzyme activating material and hallow zones were signified as positive enzymatic activity. The protease producing microbial colonies were screened by growing them in Skim milk agar plates. The clear zone around the grown colony was indicated as positive protease activity. Similarly, cellulase enzyme activity was screened by growing the colonies in CMC media and clear halozone after pouring 1% congo red indicator and 1N NaCl wash was indicated as positive cellulase activity. Likewise for amylase enzyme screening was performed on starch plates after pouring 0.1% iodine solution. Same 0.1% iodine was used for screening of pectinase enzymes on pectin based plates.

3.3.4 Gram staining

Four basic and significant steps included in gram staining process were performed. Primary staining of crystal violet binding with grams iodine to trap the CV-I complex inside cell follows with alcohol decolorization and finally counter-strain of safranin. Considering, after decolorization, the gram-negative cell loses its purple color so they retained the color of counter stain (safranin), which was pink. On the other side the gram-positive cell doesn't lose CV-I complex and remained purple in spite of later process. Gram-variable bacteria didn’t function any of above mechanism so they gave a mix of pink and purple cells (Davies *et al.*, 1983).

3.3.5 Molecular identification

3.3.5.1 16s ribosomal RNA sequencing

After comparative study of most of morphological characteristic, enzymatic assay, and gram staining most repetitive and unique 39 (23 urban dental clinic and 16 camps samples) isolated bacterial colonies were selected and their 16S rRNA sequencing was performed.

For 16S rRNA gene sequencing, 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 by 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.

3.3.5.2 Identification of 16s rRNA templates

The sequence of the 16S rRNA gene was compared with varioustypes of strains already available in the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) (Kim *et al*. 2012) to find closely related species. After comparision of identified sequence with strains available in database most probable strains of our sequence were identified.

			(%)	accession
Taxon	DB-251	Enterococcus faecalis ATCC 19433(T)	100.00	ASDA01000001
Taxon	DB-247	Bacillus licheniformis ATCC 14580(T)	99.92	AE017333
Taxon	DB-239	Enterobacter xiangfangensis 10-17(T)	99.54	HF679035
Taxon	DB-236	Lysinibacillus macroides LMG 18474(T)	99.92	AJ628749
Taxon	DB-235	Enterococcus faecium CGMCC 1.2136(T)	99.93	AJKH01000109
Taxon	DB-211	Klebsiella pneumoniae subsp. pneumoniae DSM 30104(T)	99.85	AJJI01000018
Taxon	DB-188	Staphylococcus sciuri subsp. sciuri DSM 20345(T)	100.00	AJ421446
Taxon	DB-138	Enterococcus faecalis ATCC 19433(T)	100.00	ASDA01000001
Taxon	DB-131	Proteus mirabilis ATCC 29906(T)	99.85	ACLE01000013
Taxon	DB-117	Enterococcus faecium CGMCC 1.2136(T)	100.00	AJKH01000109
Taxon	DB-116	Enterococcus faecium CGMCC 1.2136(T)	100.00	AJKH01000109
Taxon	DB-112	Bacillus aerophilus 28K(T)	100.00	AJ831844
Taxon	DB-105	Enterococcus faecium CGMCC 1.2136(T)	100.00	AJKH01000109
Taxon	DB-104	Bacillus amyloliquefaciens subsp. plantarum FZB42(T)	99.93	CP000560
Taxon	DB-99	Bacillus cereus ATCC 14579(T)	100.00	AE016877

Figure 3.2 Pictures extracted from Eztaxon showing some bacterial sequence with its most similar organisms.

3.3.6 Construction of phylogenetic tree and analysis of identified bacterial species

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).

3.3.7 GC-MS analysis

3.3.7.1 Bacterial 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. For ethyl acetate extraction, the bacterial broth and equal volume of Ethyl acetate was kept in continuous 250 rpm movement for another day and only extracted ethyl acetate was separated using pipette. This collected ethyl acetate was subjected to rota-vaporization in vaporization instrument. 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.

3.3.7.2 GC-MS

Gas chromatography mass spectrometry(GC-MS) analysis of crude extract of dental bacteria was performed in Clarus 500 gas chromatograph (Perkin Elemer) 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 (Perkin Elemer) 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.

3.3.7.3 Identification of possible compounds

The mass fragmentation pattern of GC separated compounds was analyzed and compared with NIST and WILEY libraries. The obtained graphs are compared to the database of library and then chemically similar organic compound with proper structural picture, compound atomic number were identified.

CHAPTER 4. RESULTS

4.1 Colony characters

Most characters; shape, size, texture, opacity, margin, surface, elevation and there significant numbers were observed. High numbers of colonies were moist (186) but significant amount were dry and mucoid. Similarly, most of colonies were irregular (213) and only 6 shown filamentous characters. Most colonies were thick, opaque or translucent (almost 90%).112 colonies were smooth and 121 were rough only few were wrinkles and shiny. Almost 50% were undulated and remaining was entirely marginal or lobulated but 5 were filiforms. None were concave but about 50% were raised.

4.2 Enzyme activity

Detailed enzymatic activity of these isolated bacteria on respective agar plates had shown clear evidences of major three enzymes. All of the bacterial isolates hasn't shown complete set of enzymatic activity in each regular enzymatic essay plates but more than 63% (173) organisms had shown at least one enzyme activities.118 isolated colonies were lacking any enzyme activities, 27 colonies only amylase, 81 only protease, 18 only cellulose, 21 both cellulose and protease, 19 both protease and cellulose, 4 all cellulose, amylase, and protease, no pectinase and cellulose, but none were equipped with all enzyme activity. Among all enzymatic activities, 56 % of colonies were protease positive in skimmed milk plates. Similarly amylase (24%) and cellulose (19%) activity were also observed but pectinase enzyme was inactive for all the strains. After sequence analysis, the species of colonies were identified. Table no 4.1 gave the information about group of organisms who give positive activity on enzyme (Amylase, Cellulase, Protease) plates along with those organisms who didn't attend any enzyme activity. After sequence analysis our culture strains were identified and results were obtained. Strains of *klebsiella pneumoniae* and *Achromobacter pulmonis* gave evidence of enzyme production for three different enzymes, whereas strains of *Enterobacter xiangfangensis, Bacillus cereus, Bacillus aerophilus, Bacillus amyloliquefaciens, and 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.

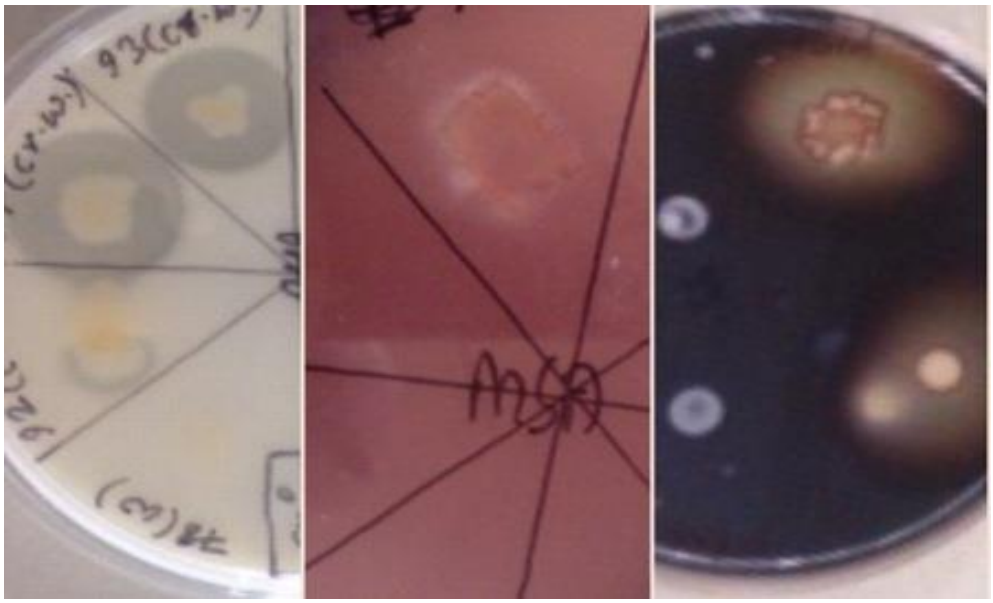

Figure 4.1 Bacterial isolates showing both positive and negative enzyme activities (A) colonies in skimmed milk agar showing clear hallow zone. (b) Colonies in CMC plates showing clear zone after pouring 1% congo red indicator and 1N NaCl wash(C) colonies in starch plates showing hallow zone after pouring 0.1% iodine solution.

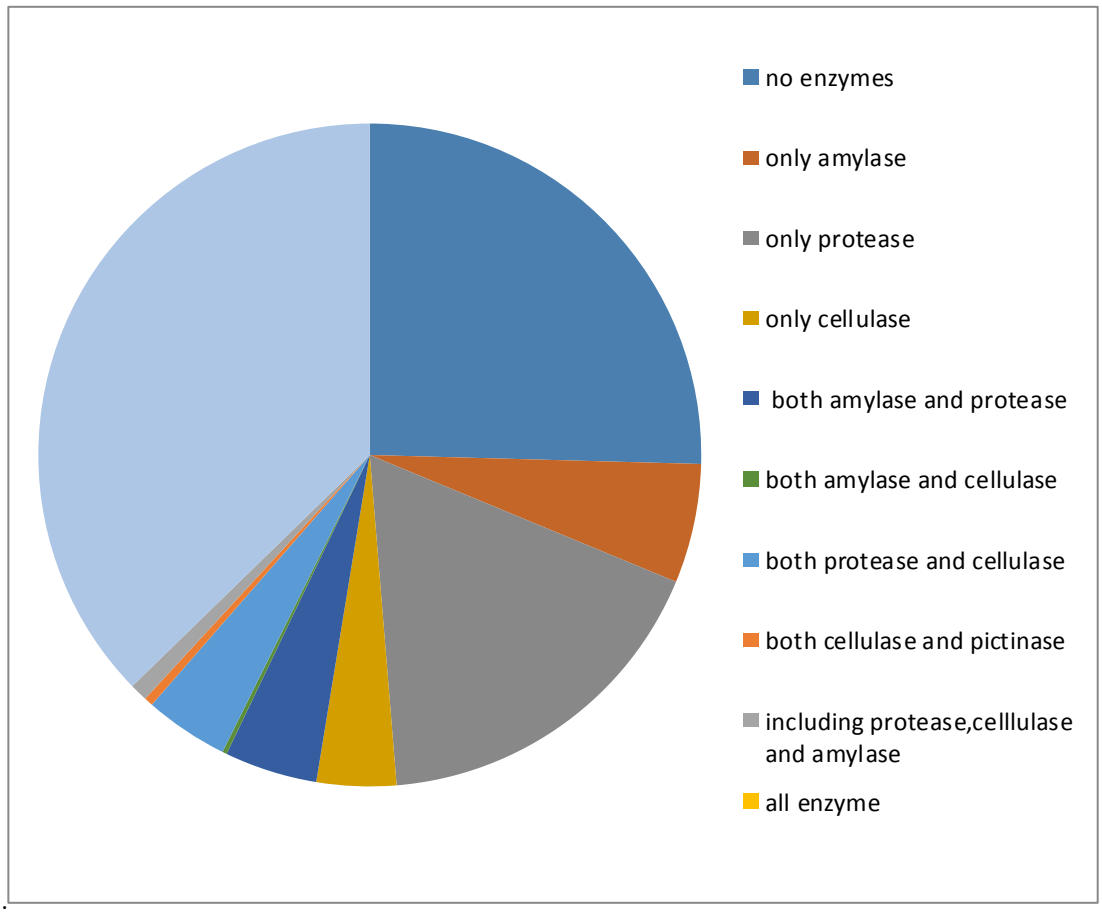

Figure 4.2 Pie chart representations of number of strains with respective enzyme essay results

4.1 list of organisms with enzyme activities

Enzyme	Species identified after sequence analysis
Amylase	*Enterobacter xiangfangensis*
	Enterococcus faecium
	Bacillus aerophilus
	Bacillus amyloliquefaciens subsp. Plantarum
	klebsiella pneumoniae subsp. Pneumoniae
	Kocuria rosea
	Achromobacter pulmonis
	Novosphingobium capsulatum
Cellulase	*Bacillus cereus*
	Klebsiella pneumoniae subsp. Pneumoniae
	Pseudomonas taiwanensis
	Citrobacter freundii
	Achromobacter pulmonis
	Stenotrophomonas chelatiphaga
	Obesumbacterium proteus
Protease	*Enterobacter xiangfangensis*
	Enterococcus faecalis
	Bacillus licheniformis
	Bacillus cereus
	Bacillus circulan
	Bacillus amyloliquefaciens subsp. Plantarum
	Bacillus aerophilu
	Klebsiella pneumoniae subsp. Pneumoniae
	Pseudomonas taiwanensis
	Achromobacter pulmonis
	Escherichia marmotae
	Ochrobactrum anthropi
pectinase	*none*
None	*Staphylococcus epidermidis*
	Citrobacter koseri
	Serratia grimesii
	Chryseobacterium vi etnamense
	Sporosarcina contaminans

4.3 Gram staining

Gram staining of most bacterial colonies had provided enough evidence to convince the presence of major number of both gram positive and gram negative bacteria. Possibility of presence of gram Negative bacteria belonging to *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 significant.

4.4 Identification of bacterial species

After comparative study of most of morphological characteristic, enzyme assay and gram staining most repetitive and unique 39(23 urban medical clinic and 16 rural camps samples) isolated bacterial colonies were selected and their 16s rRNA sequencing was performed. Sequence of 16s rRNA were specified after sequencing procedure following comparison of identified sequence with already registered sequences in Ez TAXON of EZBIOCLOUD program. Table no.4.1 shows the detailed list of variety of bacterial isolates from sequencing. From the table it was clearly stated that wide variety of bacteria were identified in the sample. From 39 sequencing (23 clinical isolated colonies and 16 camp isolated colonies) results 33 different organisms were identified. These 33 different species were of wide variety. 17 different Genus of bacterial were isolated. *Enterococcus, Enterobacter, klebsiella, Bacillus, Staphylococcus, Serratia, Citrobacter, Pseudomonas* with more than single species were identified. Similarly, single species of other bacterial were also identified belonging to; *Proteus mirabilis, Escherichia marmotae, Ochrobactrum anthropi, Stenotrophomonas chelatiphaga, Achromobacter pulmonis, Novosphingobium capsulatum, Chryseobacterium vietnamense, Obesumbacterium proteus, Flavobacterium oceanosedimentum, Lyinibacillus macroides.* When separate analysis was made in camps (rural) samples and clinic (urban) samples, some subtypes of Bacillus and klebsiella were identified in both sections. In rural samples *Enterococcus faecalis, Staphylococcus saprophyticus, Stenotrophomonas chelatiphaga, Pseudomonas geniculata, Proteus mirabilis, kocuria rosea, Obesumbacterium proteus, Ochrobactrum anthropi, Chryseobacterium vietnamense* were isolated along with *bacillus and klebsella.* Similarly in urban samples 3 subtypes of *Citrobacter, 2 subtypes of Enterococcus, Enterobacter, Serratia* and *Staphylococcus epidermidis, Pseudomonas taiwanensis, Achromobacter pulmonis, Escherichia marmotae, Novosphingobium capsulatum, and Lyinibacillus macroides were* isolated. Isolate includes commensal, pathogenic, opportunistic pathogen bacteria, N2 fixing bacteria and a novel species *Escherichia marmotae.* It was obvious from the tree that wide varieties of microorganisms were isolated from the samples. Some of the species had shown close relationship among each other and common ancestor origin. The relationship of these bacteria with already identified oral pathogenic bacteria was also understood.

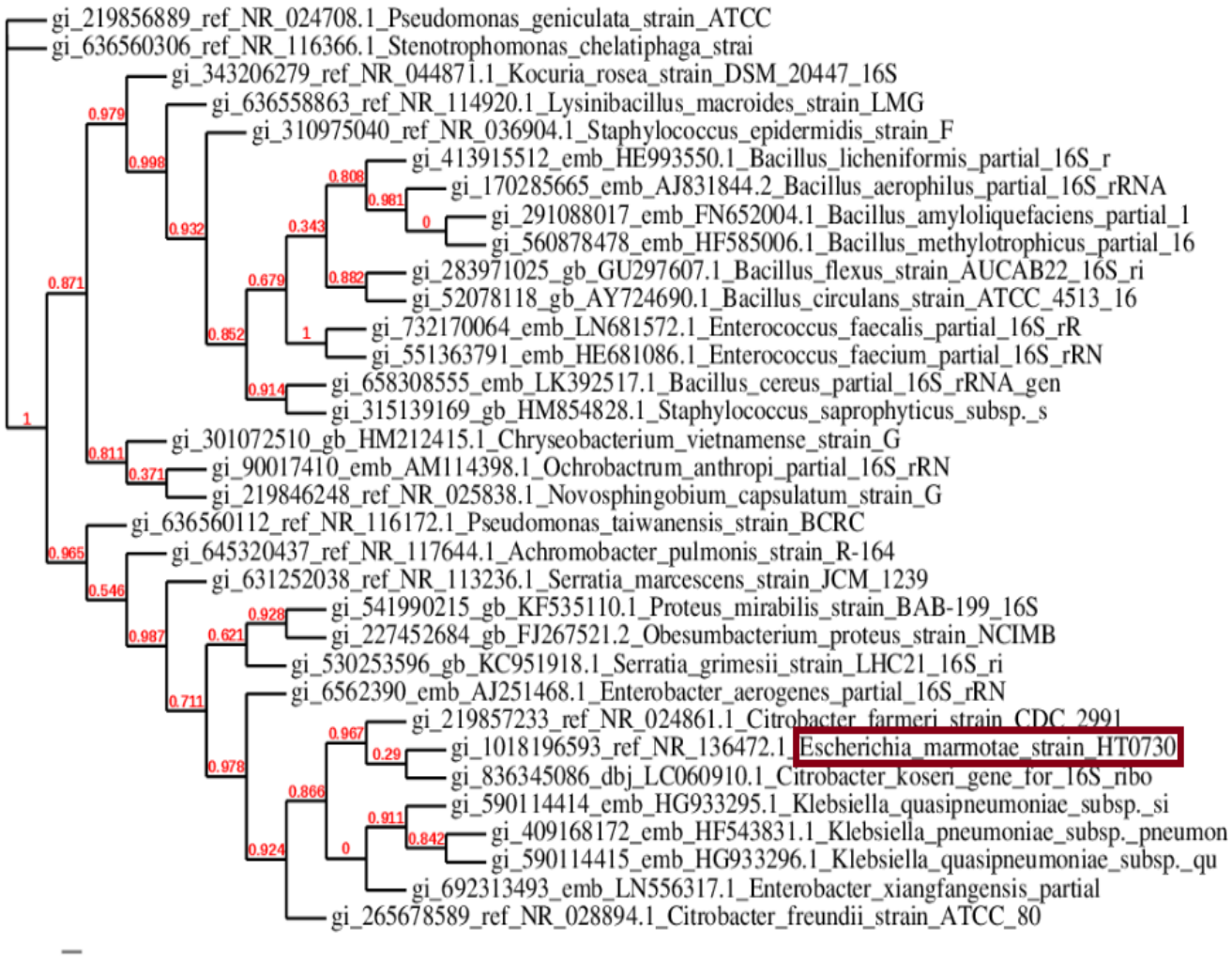

Figure 4.3 Phylogenetic representations of all isolated bacterial strains

Table 4.2 list of bacterial isolates identified by 16s rRNA sequencing

S.N	GENEUS SPECIES	ACCESS NO
	Bacterial strains isolated from rural camp samples	
1	*Enterococcus faecalis*	ASDA01000001
2	*Bacillus licheniformis*	AE017333
3	*Bacillus cereus*	AE016877
4	*Bacillus flexus*	AB021185
5	*Bacillus circulans*	AY724690
6	*Bacillus amyloliquefaciens subsp. Plantarum*	CP000560
7	*klebsiella pneumoniae subsp. Pneumoniae*	AJJIO1000018
8	*Klebsiella quasipnaumoniae subsp. Similipneumoniae*	CBZR01000004(

9	*Staphylococcus saprophyticus subsp. Saprophyticus*	AP008934
10	*Stenotrophomonas chelatiphaga*	EU573216
11	*Pseudomonas geniculata*	AB021404
12	*Proteus mirabilis*	ACLE01000013
13	*Kocuria rosea*	X87756
14	*Obesumbacterium proteus*	AJ233422
15	*Ochrobactrum anthropi*	CP000758
16	Chryseobacterium vietnamense	HM212415
	Bacterial species isolated from clinical samples	
1	*Enterococcus faecalis*	ASDA01000001
2	*Enterococcus faecium*	AJKH01000109
3	*Enterobacter aerogenes*	CP002824
4	*Enterobacter xiangfangensis*	HF679035
5	*Bacillus licheniformis*	AE017333
6	*Bacillus aerophilus*	AJ831844
7	*Bacillus amyloliquefaciens subsp. Plantarum*	CP000560
8	*Bacillus methylotrophicus*	JTKJ01000077
9	*Bacillus cereus*	AE016877
10	*Klebsiella quasipneumoniae subsp. Quasipneumoniae*	HG933296
11	*Klebsiella pneumoniae subsp. Pneumoniae*	AJJIO1000018
12	*citrobacter koseri*	AF025372
13	*Citrobacter farmeri*	AF025371
14	*Citrobacter freundii*	ANAV01000046
15	*Staphylococcus sciuri subsp. Sciuri*	AJ421446
16	*Staphylococcus epidermidis*	L37605
17	*Serratia marcescens subsp. Marcescens*	JMPQ0100005
18	*Serratia grimesii*	AJ233430
19	*Pseudomonas taiwanensis*	EU103629
20	*Achromobacter pulmonis*	HE798552
21	*Escherichia marmotae*	JANBP01000188
22	*Novosphingobium capsulatum*	D16147
23	*Lyinibacillus macroides*	AJ628749

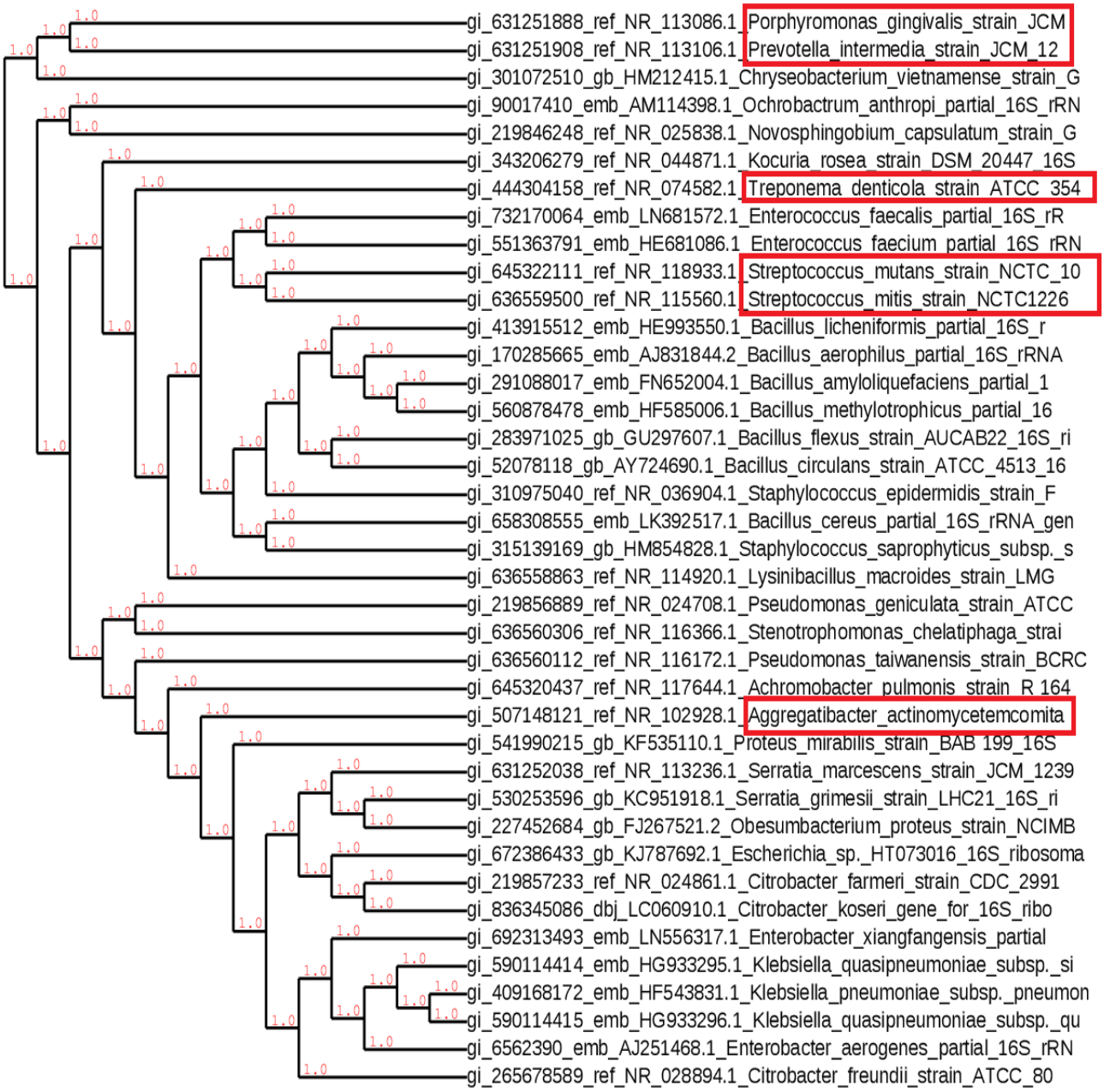

Figure 4.4 Similarities between common oral pathogenic bacteria strains with isolated strains represented in phylogenetic tree

4.5 Identification of possible compounds in bacterial extract using GC-MS technique

GC-MS process of bacterial extract for same 39 isolated sent for identification using 16S rRNA sequencing had provided the possible evidence of 255 compounds but among them only these 107 possible compounds were significantly different. These were the some repetitive compounds but most of the compounds were produced by few colonies only. Similarly, in this list all type of organic compounds were significantly present, 10 were alkyl based compounds (only C & H), 40 oxygen containing organic compounds, 8

nitrogen containing organic compounds, 2 sulfur containing compounds, 16 organic compounds contains both nitrogen and oxygen, 15 sulfur containing compounds along with oxygen or nitrogen or sometimes both, 9 chlorine and 3 bromine containing compounds were identified. Similarly other compounds containing silicon, cobalt, phosphorus and fluorine, in organic form were far and few.

When separate analysis was performed considering the samples from dental camps and medical clinics separately, 91 different possible compounds were identified from GC-Ms of medical clinics samples and 50 different possible compounds from medical camps. Both set of samples had have voluble numbers of alkyl based compounds (only C & H), oxygen containing organic compounds, nitrogen containing organic compounds, organic compounds containing both nitrogen and oxygen, sulfur containing compounds along with oxygen or nitrogen or sometimes both, and few compounds containing bromine, chlorine, silicon, cobalt, phosphorus and fluorine. Both set produced 31 similar compounds and among them few compounds were highly repetitive.

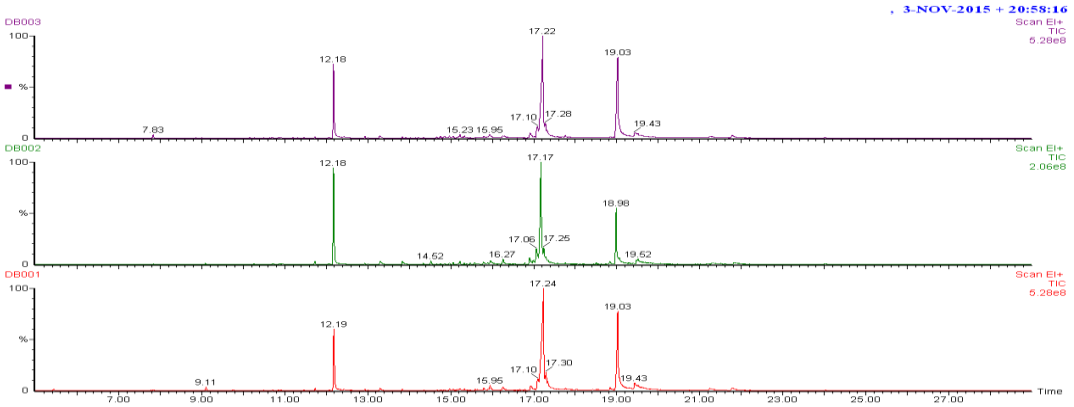

Figure 4.6 Compound peaked graph obtained after completion of GC-MS of samples(*Bacillus cereus*).

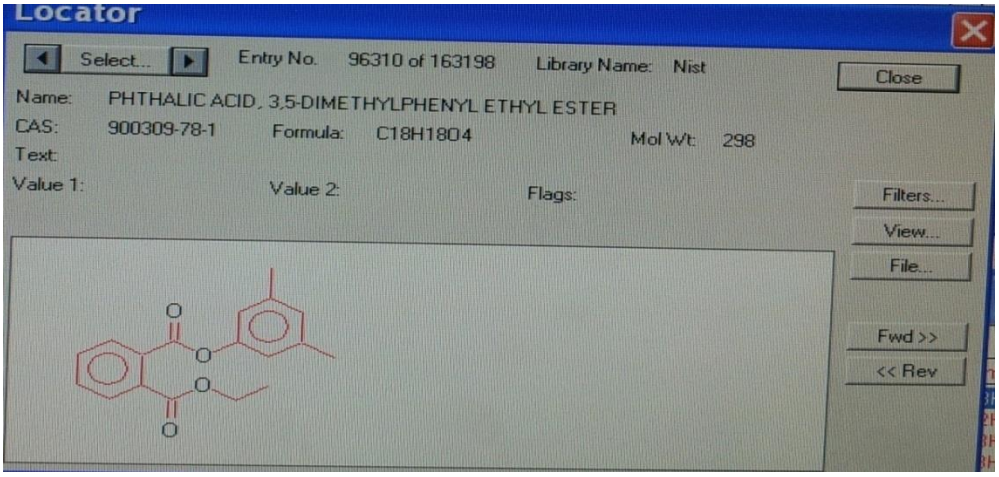

Figure 4.7 Possible compound (PHTHALIC ACID, 3,5-DIMETHYLPHENYL ETHYL ESTER) identified in single peak of GC-MS obtained graph

Table 4.3list of compounds identified by GC-MS

compounds Alkane based	Chemical 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
$C_{12}H_{14}$	BENZENE, 1,3.HEXADIENYL-	158
$C_{16}H_{16}$	CYCLOBUTANE, 1,3-DIPHENYL-, TRANS	208
$C_{14}H_{10}$	DIPHENYLETHYNE	178
$C_{18}H_{18}$	BENZENE, 1,1'-(1 ,5-HEXADIENE-1 ,6-DIYL)BIS-	234
$C_{10}H_{12}$	BENZENE, 4-ETHENYL-1,2-DIMETHYL-	132
$C_{18}H_{26}$	PHENANTHRENE, 9-BUTYL-1,2,3,4,5,6,7,8-OCTAHYDRO-	242
$C_{19}H_{28}$	1H-INDENE,2-BUTYL-3-HEXYL-	256
$C_{15}H_{18}$	NAPHTHALENE, 1,6-DIMETHYL-4-(1-METHYLETHYL)-	198
C_9H_{10}	INDANE	118
O_2containing	chemical identified	molecular weight
$C_{16}H_{12}O_5$	5,10-DIHYDROXY-2-NETHOXY-7-METHYL-1,4-ANTHRACENEDIONE	284
$C_{16}H_{16}O_2$	O-TOLUIC ACID , 2-PHENYLETHYL ESTER	240
$C_{17}H_{24}O_4$	PHTHALICE ACID,BUTYL 2-PENTYL ESTER	292
$C_{13}H_{14}O_6$	2-BENAOYLOXYSUCCINIC ACID , DIMETHYL ESTER	266
$C_{16}H_{24}O$	2,5-CYLCOHEXADIEN-1-ONE, 2,6-BIS(1 ,1-DIMETHYLETHYL)-4-ETHYLIDENE-	232
$C_{18}H_{18}O_4$	PHTHALIC ACID, 3,5-DIMETHYLPHENYL ETHYL ESTER	298
$C_{17}H_{16}O_4$	PHTHALIC ACID , METHYL 2-PHENYLETHYL ESTER	284
$C_{23}H_{18}O_3$	FLUOREN-9-OL, 3,6-DIMETHOXY-9-(2-PHENYLETHYNYL)-	342
$C_{13}H_{16}O_4$	PHTHALIC ACID , ETHYL ISOPORPYL ESTER	236
$C_8H_{10}O_3$	PHENOL, 3,5-DIMETHOXY	154
$C_{15}H_{20}O_3$	3,4-DIMETHYL-2-(3-METHYL-BUTYRYL)-BENZOIC ACID, METHYL ESTER	248
$C_{15}H_{24}O$	PHENOL, 2,4,6-TRIS(1-METHYLETHYL)-	220
$C_{11}H_{18}O_5$	DIETHYL 4-OXO PIMELATE	230
$C_{10}H_{12}O_4$	PHENOL, 3,5-DIMETHOXY-,ACETATE	196
$C_{24}H_{34}O_4$	PHTHALIC ACID,PROPYL TRIDEC-2-YN-1-YL ESTER	386
$C_{14}H_{22}O$	PHENOL, 2,5-BIS(1 , 1-DIMETHYLETHYL)-	206
$C_{22}H_{26}O_4$	PHTHALIC ACID, HEXYL 2-PHENYLETHYL ESTER	354
$C_{22}H_{34}O_4$	PHTHALIC ACID, 6-ETHYL-3-OCTYL BUTYL ESETR	362
$C_{11}H_{14}O_2$	BENZOIC ACID, 2-METHYL-,(2-METHYLPHENYL)METHYL ESTER	178
$C_{12}H_{14}O_4$	DIETHYL PHTHALETE	222
$C_{16}H_{16}O_3$	2H-PYRAN-2-ONE, 6-[2-E-(3-ETHOLPHENYL)ETHENYL]-4-METHOXY-	256
$C_{14}H_{14}O$	PHENOL, 2-(1-PHENYLETHYL)-	198

$C_{14}H_{12}O_3$	SPIRO[NAPHTHALENE-2(1H),2'-OXIRAN]-1-ONE, 3'-ACETYL-3'-METHYL-, CIS-(+	228
$C_{22}H_{22}O$	PHENOL, 2,4-BIS(1-PHENYLETHYL)-	302
$C_{18}H_{18}O_6$	1-,2,3,4-BUTANETETROL, 1,4-DIBENZOATE,(R*,S*)-	330
$C_9H_{12}O_3$	3-ETHOXY-4-METHOXYPHENOL	168
$C_{10}H_{16}O_5$	DIME THYL 4-OXOOCTANE-1,8-DIOATE	216
$C_{17}H_{24}O_3$	7,9-DI-TERT-BUTYL-1-OXASPIRO(4,5)DECA-6,9-DIENE-2,8-DIO	276
$C_{14}H_{20}O_2$	2,5-DI-TERT-BURYL-1,4-BENZOQUINONE	220
$C_{32}H_{53}O_2$	ERGOST-5-EN-3-OL, 22,23-DIMETHYL-, ACETATE, [3.BETA.]-	470
$C_{21}H_{24}O_4$	PHTHALICACID,BUTYL 4-ISOPROPYLPHENYL ESTER	340
$C_{12}H_{14}O_2$	2H-1-BENZOPYRAN, 7-METHOXY-2,2-DIMETHYL-	190
$C_{32}H_{54}O_2$	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
$C_{24}H_{26}O_2$	4,4'-((P-PHENYLENE)DIISOPROPYLIDENE)DIPHENOL	346
$C_{20}H_{30}O_2$	ABIETIC ACID	302
$C_{15}H_{14}O_4$	1,3-BENZENEDIOL, 4-(3,4-DIHYDRO-7-HYDROXY-2H-1-BENZOPYRAN-3-YL)-	258
$C_{11}H_{16}O_2$	2(4H)-BENAOFURANONE, 5,6,7,7A-TETRAHYDRO-4,4,7AA-TRIMETHYL-,-	180
N containing	chemical identified	molecular weight
$C_{10}H_{20}N_4$	3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE	196
$C_{12}H_{11}N$	1,2-DIHYDRO-5-ACENAPHTHYLENAMINE	169
$C_{18}H_{24}N_2$	1,4-BENZENEDIAMINE, N-(1-,3-DIMETHYLBUTYL)-N'-PHENYL-	268
$C_{15}H_{15}N$	ACRIDINE, 9,10-DIHYDRO-9,9-DIMETHYL	209
$C_{12}H_{13}N$	2-NAPHTHALENAMINE,N-ETHYL-	171
$C_{12}H_{12}N_2$	4-TERT-BUTYLPHTHALONITRILE	184
$C_{10}H_6N_2$	6-CYANOQUINOLINE	154
$C_{13}H_{15}N$	1-CYANO-4-CYCLOHEXYLBENZENE	185
O and N containing	chemical identified	molecular weight
$C_{14}H_{16}O_2N_2$	PYRROLO[1,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-	244
$C_{10}N_{12}O_4$	PHENOL, 3,5-DIMETHOXY-,ACETATE	196
$C_{12}H_{18}ON_2$	4'-DIETHYLAMINOACETANILIDE	206
$C_{13}H_{12}O_2N_4$	LUMIFLAVINE	256
$C_{20}H_{23}O_2N_3$	1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1,1'-BIPHENYL[-4-YLOXY)-.ALPHA. -(1.	337
$C_{19}H_{23}ON$	N-BENAYL-N-ETHYL-P-ISOPROPYLBENZAMIDE	281
$C_{23}H_{41}O_4N$	L-PROLINE, N-ALLYLOXYCARBONYL-, TETRADECYL ESTER	395
$C_{17}H_{21}O_3N$	1,3-DIOXOLANE-2-HEPTANENITRILE, .ALHA.-METHYL-.DELTA.-OXO-2-PHEN	287
$C_{16}H_{31}ON$	7-METHYL-2-(7-OXONONYL)-PERHYDROAZEPINE	253

$C_{14}H_{23}O_3N$	CYCLOPENTANECARBOXYLIC ACID, 1,2,2-TRIMETHYL-3-[PYRROLIDINE-1-CARBONYL]-	253
$C_{14}H_{13}ON$	MURRAYAFOLINE	211
$C_9H_{11}O_2N$	4-PYRIDINEMETHANOL, 3-METHYL-,ACETATE (ESTER)	165
$C_{18}H_{24}O_3N_2$	OXAZOLIDIN-2-ONE, 4-HYDROXY-4,5,5-TRIMETHYL-3-[2-[1,2-DIMETHYL-3-INDOLYL]ETHYL]-	316
$C_{18}H_{16}O_7N_2$	PYRROLO[1,2-A]QUINOXALINE-1,2,3-TRICARBOXYLIC ACID,4,5 DIHYDRO-5-M	372
$C_{15}H_{13}O_3N_3$	BEZOIC ACID , 2-[(BENZOYLAMINO)CARBONYL]HYDRAZIDE	283
$C_{22}H_{39}ON$	1-OCTYL-2-[6-(4,4-DIMETHYL-2-OXAZOLIN-2-YL)HEXYL]-CYCLOPROPENE	333
$C_7H_8ON_2$	BENZALDEHYDE, 3-AMINO-,OXINE	136
S containing	chemical identified	molecular weight
$C_{20}H_{36}S$	2,3-DIMETHYL-5(2,6,10-TRIMETHYLUNDECYL)THIOPHENE)	308
$C_{10}H_{16}S$	THIOPHENE, 2-BUTYL-5-ETHYL	168
O,S containing	chemical identified	molecular weight
$C_{10}H_{16}O_2S$	THIOPHENE, 3,4-BIS(ETHOXYNETHYN)-	200
$C_9H_{10}O_2S$	2-THIOPHENECARABOXYLICACID,CYCLOBUTYL ESTER	182
$C_7H_6O_2S$	1 ,2-PROPANEDIONE, 1-(2-THIENYL)-	154
$C_8H_{10}OS$	1-BUTANONE, 1-(2-THIANYL)-	154
$C_7H_8O_2S$	THIOPHENE-2-CARBOXYLIC ACID ETHYL ESTER	156
$C_5H_4O_2S$	2-THIOPHENECARBOXYLIC ACID	128
S, N containing	chemical identified	molecular weight
$C_6H_{10}N_4S$	2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE	170
$C_9H_{10}ON_4S$	2-IMINO-6-MERCAPTO-4,4-DIMETHYL-1,2,3,4-TETRAHYDRO-PYRIDINE-3,5-DIC	206
$C_{14}H_{20}N_4S_2$	1,4-BIS-(2-METHYL-THIAZOL-4-YLMETHYL)-PIPERAZINE	308
$C_8H_{11}NS$	6-METHYLCYCLOHEXATHIAAOLE	153
S,O, N containing	chemical identified	molecular weight
$C_{14}H_{21}O_3NS$	L-ALANINE, N-(2-THIENYLCARBOLYL-,HEXYL ESTER)	283
$C_{12}H_{17}O_3NS$	L-ALANINE , N-(2-THIENYLCARBONYLA)-.BUTYL ESTER	255
$C_{18}H_{19}O_2NS$	QUINOLINE, 1,2,3,4-TETRAHYDRO-1-((2-PHENYLCYCLOPROPYL)SULFONYL)-,	313
$C_{17}H_{16}ON_2S$	N-(4,DIMETHYL-THIAZOL-2-YL)-2-NAPHTALEN-1-YL-ACETAMDI	296
$C_{11}H_{13}O_3N_3S$	TRANS-3-AZIDO-1 ,2,3,4-TETRAHYDRO-2-NAPHTHYL METHANESULFONATE	267
Cl containing	chemical identified	molecular weight
$C_{14}H_{14}O_2NClS$	THIOPHENE-2-CARBOXAMIDE, N-[2-(4-CHLORO-2-METHYLPHENOXY)ETHYL}-	295
$C_{18}H_{30}NCl$	4-CHLOROBENZYLAMINE, N-DECYL-N-METHYL-	295
$C_{10}H_9O_2Cl_3$	ACETIC ACID, TRICHLORO-, 2-PHENYLETHYL ESTER	266

$C_9H_{14}NCl$	PHENYLTRIMETHYLEMMONIUN CHOLORIDE	171
$C_{11}H_7O_2ClS$	2-THIOPHENECARBOXYLIC ACID, 4-CHLOROPHENYL ESTER	238
$C_{15}H_{15}NCl_2$	PHENETHYLAMINE , P-CHLORO-N-(P-CHLOROBENZYL)-	279
$C_{19}H_{19}O_4Cl$	PHTHALIC ACID, BUTYL, 4-CHLOROBENZYL ESTER	346
$C_{17}H_{12}O_3N_3Cl$	2'CHLORO-4-(4-METHOXYPHENYL)-6-(4-NITROPHENYL)PYRIMIDINE	341
$C_{12}H_{14}O_2N_5Cl$	1 ,2,5-OXADIAZOLE-3-CARBOXAMIDE, 4-AMINO-N-[2-[[(CHLOROPHENYL)MET	295
Br containing	chemical identified	molecular weight
$C_{17}H_{12}NBrS$	2-(P-BROMOPHENYL)-8-METHYL-8H-THIENO(2,3-B)INDOLE	341
$C_{13}H_9ON_3BrF_3S$	N-(4-BROMO-2-TRIFLUOROMETHYL-PHENYL)-2-(PYRIMIDIN-2-YLSULFANYL)-A	391
$C_{14}H_{19}O_2Br$	6-BROMOHEXANOIC ACID, 2-PHENYLETHYL ESTER	298
remaining others	chemical identified	molecular weight
$C_{14}H_{23}O_3P$	BUTYLPHOSPHONIC ACID , ETHYL 2-PHENYLTHYL ESTER	270
$C_{16}H_{34}OSi$	1-METHYL-1-[6-ETHYL-3-OCTYLOXY]-1-SILACYCLOHEXANE	270
$C_{11}H_{14}O_2NF$	CARBAMIC ACID, 4-FLUOROPHENYL-,BUTYL ESTER	211
$C_{22}H_{18}Co$	COBALT , .ETA.-5-CYCLOPENTADIENYL-.ETA.-5-1,2-DIPHENYLCYCLOPENTADI	341

Table 4.4 Highly repetitive compounds identified by GC-MS.

PYRROLO[1 ,2-A]PYRAZINE-1,4-DIONE, HEXAHYDRO-3-(PHENYLMETHYL)-	$C_{14}H_{16}O_2N_2$
3,6-DIBUTYL-1,2-DIHYDRO-1,2,4,5-TETRAZINE	$C_{10}H_{20}N_4$
4'-DIETHYLAMINOACETANILIDE	$C_{12}H_{18}ON_2$
2-HYDRAZINO-4-METHYL-6-METHYLTHIOPYRIMIDINE	$C_6H_{10}N_4S$
THIOPHENE, 3,4-BIS(ETHOXYNETHYN)-	$C_{10}H_{16}O_2S$
LUMIFLAVINE	$C_{13}H_{12}O_2N_4$
1H-1,2,4-TRIAZOLE-1-ETHANOL, BETA-([1,1' -BIPHENYL[-4-YLOXY)-.ALPHA. -(1.	$C_{20}H_{23}O_2N_3$

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 that wide variety of microorganisms were found in oral cavity. Similarly, due to usage of blood agar and brain heart agar for plating and significant growth of colonies in these plates, it was also clear that many different flora found in these samples were pathogenic and they might cause disease. There was significant similarity in some characteristic and colors considering most of the colonies being white, pale white or yellow colored and show raised elevation, undulated or entire margin, smooth or rough surface, along with opaque, moist and irregular colony character. But again there were also significantly variable and out of order colonies with extra characteristic like pink colored colonies(MSA), transparent opacity(NA), milky white(MSA) colored colonies, and colonies showing haemolysis, filiform margins (Milward,2010). Considering the time of incubation and manner of colony development on plates being non selective, It can be considered that most of the organisms grown are commonly adoptable and easily gradable organisms (Justice *et al*., 2004) supposed to be easily present in human samples and other plate cultivable samples with only few different and dominant in oral samples. Most of the characteristic like mucoid surface, highly opaque colonies supports mucoid exopolysaccharide (Socransky *et al*., 2002) that forms a prominent capsule in the bacterial surface, favoring adhesion later in biofilm formation (Cerca *et al*., 2005), protects the strains from the mucociliary activity, phagocytosis, complement system activity, reduces the antimicrobial action as it prevents the penetration of antimicrobials and enables the microbial cells to be drug resistant (Lewis, 2001).

After checking enzyme activities of these colonies it was found that 63% sample inoculate colonies show at least one enzymatic activity when we check for the enzyme activities in enzyme specific plates 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 adoptation 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 potent strains. Enzymes 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). As adoptive activities are only significant in oral cavity against continuous salivary flow and various innate and adoptive immune response (antibodies, phagocytic cells), whereas in normal condition not most of the organisms show such hard fact (enzyme activities, antibiotic resistance) activities of adaptation like enzyme which may have seen because of stress caused by compound deficiency and anaerobic environment (Ploss *et al*., 2016). Sometimes collaboration with other organisms of biofilms allows horizontal gene transfer (or cell-cell signaling systems) that may initiate enzymatic proteins in some non-enzymatic species. Bacteria mostly alter the transcription of carbohydrate utilization genes and virulence factor production in response to changes in the environmental conditions encountered as only strict nutrient are available in oral cavity. Oral bacteria have developed molecular strategies to directly link the regulation of carbohydrate utilization and virulence factors such as biofilm formation and enzyme production to utilize that carbohydrate (Immanuel *et al*, 2006). None of the strains show all enzyme activities, this is because in biofilm a collaborative environment is established and no single organisms should do all kind of work which influences the byproducts and enzymes they produce. Similarly pectinase enzymatic activity is inactive, no strains showing this enzymatic activity but their 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 food of human are either starch, protein (Hartog *et al*., 2006), and above all these are either amino acids or carbohydrates and rather than pectinase other enzymes amylase and protease along with cellulose are easy usable in adaptation with oral food source. Where amylase is used in breakage of long chain of starch hydrocarbons, protease is used in breakage of milk proteins (Yadav *et al*., 2016). Most of the food rice, wheat, contains starch as the primary source and amylase produced comes handy in usage for oral organisms. Similarly, dairy products like milk are also major source of food consumption in almost every age group (Kromhout *et al*., 2016) so high production of protease (by more than 40%) enzyme were responsible in these strains. Similarly the cell wall lytic activity of these enzymes is eminent in various bactericidal activities of bacteria. *Streptomyces exfoliates* produces protease which exhibited inhibitory activity to various fruit-rotting species of bacteria and fungi (Choudhary *et al*., 2014) so it can be understood that most of bacteria are trying to establish restrict and well adoptive microenvironment.

Because of strict and proper selection mechanism among colonies, small number of 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 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. Most of these bacteria only changes into pathogenic form when they are provided with adoptive and favorable immune-compromised situations. *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 also suggest multiple organisms can be associated with bacterial accumulation and plaque composition and they can be causative in The etiologies of dental caries, periodontal diseases and oral malodor. Most of the isolates are causes of disease in hospital conditions and in immune compromised patients. These species could be used as oral markers for the early detection of oral disease, improving the survival rate considerably in oral cancer as some study have given information of species like *Staphylococcus aureus* and *Enterococcus faecalis* presence in disease and cancer salivary samples (Fábián *et al.,*2008). *Bacillus* species are able to produce copious amounts of enzymes (amylase and protease) and intracellular inclusions of polyhydroxyalkanoates (Baron *et al*., 1996) which may be advantageous for adaptation inside biofilm of oral cavity. Similar is case for other species also which are quite capable of producing extracellular enzymes (Alcaraz *et al*., 2010). Most of these isolates also show hard and strict antibiotic resistance after causing diseases which also give evidence of their ability of adaptation and gene transfer. Biofilm composition varies in significance with different bacterial presence. Similarly there adhesion properties are also altered in these biofilm. Reports claim 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 *K. pneumoniae* are thinner than *Pseudomonas*. *Enterococcus, Staphylococcus, Pseudomonas, Klebsiella* majorly present in most biofilmis mainly present in many medical apparatus used in treatments that contains prominent surface. Recent sources have claimed that they are found in biofilms of venous catheter, Prosthetic heart valve, Urinary catheter. Similar is case for other species of bacterial isolates (Donlan, 2002). *E. 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. E. faecalis is reported to form biofilm successfully in extremely alkaline conditions (Manikandan *et al*., 2013). 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). *E. faecalis* is the main causative organism of failed root canal treatments (Mahmoudpour *et al*., 2007). 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). *S. 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).

S. marcescens produce characteristic red pigment, prodigiosin (tooth surface) (Auwaerter , 2007) and *Chryseobacterium* produces yellow flexirubin type, non-diffusible (Li &Zhu, 2012) slime layer once established, complete eradication is often difficult usually in subgingival biofilm of teeth. *S. marcescens* produces extracellular metallo proteinases which are believed to function in cell-to-extracellular matrix interactions (Auwaerter, 2007). A study demonstrated the extent of horizontal gene transfer among *Staphylococcus* to be much greater than previously expected, and encompasses genes with functions beyond antibiotic resistance and virulence, and beyond genes residing within the mobile genetic elements (Chan *et al*, 2011) which can be well suited in biofilm formation and to bind to the already existing biofilm, creating a multilayer biofilm by producing surface proteins that bind blood and extracellular matrix proteins and by producing an extracellular material, polysaccharide intercellular adhesin (PIA) (Dixie,2002). *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 also isolated in our sample which gives idea that more and more number of oral resident bacteria is still to be identified. Again *Novosphingobium* bacteria 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* secrets 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 *Mutans Streptococcous* 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 aren't 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 comparisons was done with proven oral microbial pathogens of tooth decay(s.mutan and s.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 decay responsible bacteria, S*treptococcus*. Similarly *Serratia, Klebsiella, Proteus, Escherichia, Citrobacter* species are closely linked with each other and all lie in enterobacteraeae family. They are known to cause disease in many system of human. *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 by. Proteins, carbohydrates, are broken down in response to carbon, nitrogen, or sulfur limits with low molecular weights by using enzyme (protease, amylase, cellulase), so biocompound of bacterial extracts contain massive amount of these units (Puente *et al*. 2003) which are subsequently use as metabolic substrates (Takahashi, 2005). This was also supported by our enzyme activity assay, showing more than half bacteria with these enzyme activities. There were large numbers of bio compounds with low molecular weights and only small number were higher molecular weight (around 400), this may be because of enzymatic cleavage. 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 among both sets of samples 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 as much as 30 different samples. So a possible agenda arises where these compounds could be used as biomarker of dental bacterial 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. Various classes of compounds, including extracellular peptides and steroids have been identified as signaling compounds for *Staphylococcus aureus* (Plotkin and Konaklieva, 2007). There was presence of large number of compounds suggesting parts of extracellular peptides and steroids cleavage collected in our compound identification. Coaggregation partner like

bacteria *Capnocytophaga gingivalis* and *Actinomyces israelii* may use these compounds as signal components to strengthen their co dependability (Kolenbrander& Jakubovics, 2010). To enable changes, in the biofilm bacteria, theyinitiate cell-to-cell communication and start transfer of genetic information. The process called quorum sensing within the biofilm enables bacteria to communicate with each other and to other species inside different layers. Bacteria regulate variety of physiological activities like symbiosis, virulence, motility, antibiotic production, and biofilm formation using Quorum sensing (Costerton *et al.*, 2003), these identified compounds may have significant role on these activities. Some clinical evidence suggested that, cariogenic conditions are associated with increased amounts of microorganisms capable of acid production (Takahashi, 2005), and 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 carries. 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. Activity toward cysteine and methionine, alanine produces sulfur compounds, the major components of oral malodor (Takahashi, 2005). Our biocompounds also support this evidence as there are wide varieties of sulfur compounds and also possible cysteine and methionine cleaved compounds to produce malordor causing compounds like are hydrogen sulfide (H2S), methyl mercaptan and dimethyl sulfide (Geethashri *et al*., 2014). 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. 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). Similarly, compounds identified showed anti-bacterial effects towards different groups of species were also identified. 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). Our result also gives us possibility of massive amount of organic acids and acid esters like (Abietic acid, O-toluic acid ester, Phthalice 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. Research conducted by soria MC and Audisio MC provides evidences that other metabolites, different from organic acids, present in the cell-free supernatant also gives bactericidal effect and a large possible compounds to this description are also identified in our GC-MS results$C_{16}H_{34}OSi$ (1-methyl-1-[6-ethyl-3-octyloxy]-1-silacyclohexane) and $C_{13}H_9ON_3BrF_3S$ (n-(4-bromo-2-trifluoromethyl-phenyl)-2-(pyrimidin-2-ylsulfanyl) (Soria & Audisio, 2014) giving our research future prospect to try and identify these compounds. Methyl ester and polyene were also found among the secondary metabolites produced by

Streptomyces exfoliates against fungal infections suggesting our compounds like$C_{13}H_{14}O_6$ (2-Benaoyloxysuccinic acid, dimethyl ester), $C_{14}H_{22}O$ (Phenol, 2,5-bis(1 , 1-dimethylethyl) have possibility of such response too (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). Compounds like C_9H_{10}(indane), Anthracenedione are molecularly similar to indol dione so their anti-bacterial activity is also prospect of further research. A chlorine-containing metabolite,"t-chlorotonilA"was found to exhibit promising anti-malarial activity (Held et al., 2015). Comounds like $C_{14}H_{14}O_2NClS$(thiophene-2-carboxamide, n-[2-(4-chloro-2-methylphenoxy)ethyl}-), $C_{18}H_{30}NCl$(4-chlorobenzylamine, n-decyl-n-methyl-), $C_9H_{14}NCl$(phenyltrimethylemmoniun choloride),$C_{12}H_{14}O_2N_5Cl$(1 ,2,5-oxadiazole-3-carboxamide, 4-amino-n-[2-[[(chlorophenyl)met]]) are chlorine based metabolites identified after GC-MS and these may have similar fate as chloiotonilA against disease parasites. Study had also shown that organic bio compounds like 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) and this could be done to the possible organic compounds we have identified but further detailed study is to be conducted.

After comparative assay of isolated organism from rural and urban samples, subtypes of major common commensal oral microflora; *Bacillus, Enterococcus, Klebsiella, Staphylococcus, Pseudomonas* were isolated from both group and isolates. Remaining isolates identified in both group are mostly opportunistic pathogens and major contributors in pathogenecity and oral problems if favorable environments are available. Similarly, *Escherichia marmotae*, a recently soil isolated microflora was isolated from clinical sample. Likewise, *Chryseobacterium vietnamense*, which is very closely related to oral pathogens like *Porphyromonas* gingivalis was isolated in rural sample which show some availability of medically significant pathogens in rural areas but both groups cant isolate these major pathogenic bacteria *Treponema denticola* , *Porphyromonas gingivalis,* microaerophile *Actinobacillus actinomyctemcomitans, Prevotella. Intermedia* which raise the question on medical problems but this may because of conditions of strict mechanisms to grow these pathogens and evidence of their lacking in our major sample source (extracted tooth). But the fact is understood that both groups of peoples inhabit both commensal and pathogenic bacteria which give a clear understanding that availability of modern knowledge, equipments, and medical workers in urban area somehow haven't been successful in contributing for better oral health in urban area.

Similarly, biocompound analysis also suggest that 31 of the possible identified compounds are similar, which strengthen the evidences supported by results obtained from sequencing. So the basic mechanism of action in oral microenvironment of both set of samples may be similar. This may be because of lacking concern of oral health and sanitation in Nepal where rural population are devoid of proper health knowledgeable and equipped hospitals where as urban population are only concerned of systemic disease and consider oral health problems when they are seriously diseased (Bhattarai, 2010). Along with these evidences, immunity and food habit also play critical role in oral

microflora. Because of proper resourceful environment but lacking proper diet in rural area and resourceful diet but bad polluted environments in urban, these substances could also have little to say. Some evidence have proven that food habit containing probiotic bacteria can sometimes contribute to favor oral health, This may be reason behind still maintained oral health in rural areas. Dairy products have low cariogenic potential and demonstrate anticaries activities which are pure and abundant in rural areas (Sonmez & Aras, 2007) and can help to maintain some stability in oral health comparing with urban areas. Similarly, rural peoples use herbal products much significantly than urban ones and various studies have shown that various herbal plants have significant effect in oral microflora (Marsh *et al*., 2006). So evidence of similar background of microfora in both communities suggests significance of oral health resources, food habit, and herbal use diversity.

CHAPTER 6. CONCLUSIONS

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 variable 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 were studied for identification of possible bacterial community and identification of the 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. The dental samples cover both rural (uneducated, primitive) and urban (educated and modern) to give vision of both type of communities on sanitation and pathogenicity. The samples showed the presence of significant diversity of bacterial with 33 different species of belonging to 17 genera and both sets of samples have basic similarity in microbial community of their oral cavity(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 malordor 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. 30 similar compounds in both sets of samples strengthen the possibility of similarity of mode of action inside biofilm of urban and rural population following the ideas generated because of similar microflora identified after sequence result.

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 byproducts 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 Nutrient broth media

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

5. Composition of Nutrient agar media

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

6. Composition of Mueller Hinton Agar (MHA)

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

7. Composition of Potato Dextrose Agar (PDA)

Components	g/l
Potato	200
Agar	2
Dextrose/Glucose	2

8. Composition of Blood agar

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

9. Composition of Mannitol salt agar

Components	g/l
Proteose peptone	10
Meat extract B#	1
Sodium chloride	75
D-Mannitol	10
Phenol red	0.025
Agar	15
Final pH (at 25°C)	7.4±0.2

10. Composition of brain heart infusion agar

Components	g/l
Magnesium sulphate	0.2
Calcium chloride	0.02
Monopotassium phosphate	1
Dipotassium phosphate	1
Ammonium nitrate	1
Ferric chloride	0.05

Agar	20
Final pH (at 25°C)	7.0±0.2

11. Composition of Starch agar

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

1.5 per cent agar was added at the end

12. Composition of CMC agar

Components	g/l
Carboxymethyl cellulose	2
Sodium nitrate	1
Dipotassium phosphate	1
Potassium chloride	1
Magnesium sulphate	0.5
Magnesium sulphate	0.01
Magnesium sulphate	5
PH	7

1.5 per cent agar was added at the end

13. Composition of pectin agar

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

14. Composition of skimmed milk agar

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

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

Ref no	peaks	chemical 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	
	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	
	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 sequence of samples obtained after 16s RNA sequencing along with closely related species identified after blast analysis

- 13 *Achromobacter pulmonis*

TCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGTGCCCAGTAGCGGGGGATAACTACGC
GAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTGG
AGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTTT
GAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAG
CACTTTTGGCAGGAAAGAAACGTCGTGGGTTAATACCCCGCGAAACTGACGGTACCTGCAGAATAAGCA
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTTTGGAACTGCATTTTT
AACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGC
GGAGGAACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGTCTTCGGAC
CTTGGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCT
ACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGCAGTGCTCGCAAGAGAACCGGAACACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTA
GTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGC
GAGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTC

GGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGT
CACACCATGGGGAGTGGGTTTTACCAGAAGTAGTTAGCCTAACCGCAA

- 58 *citrobacter koseri*

CGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGA
AACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGAT
GTGCCCAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCGAGAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACT
GGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
GGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGC
GTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCT
GGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTT
GCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGT
CAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCT
CGCGAGAGCAAGCGGACCTCATAAAGTATGTCATAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAA
GTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCC

- 70 *Stenotrophomonas chelatiphaga*

CAGCACAGTAAGAGCTTGCTCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACT
TTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA
CCTTCGGGCCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
GGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGA
AGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCTGGTTGGGAT
GACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC
GTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCTGTTGTGAAAGCCCTGGGCT
CAACCTGGGAACTGCAGTGGAAACTGGACAACTAGAGTGTGGTAGAGGGTAGCGGAATTCCCGGTGTA
GCAGTGAAATGCGTAGAGATCGGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACA
CTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGA
ACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATT
CGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTC
GGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTAC
AATGGTGGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCCATCTCAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGA
ATACGTTCCC

- 71 *Obesumbacterium proteus*

TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACGTCTTCGGACCAAAGTGGGGG
ACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTA
GGCGACGATCTCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA
AGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCATTGTGGTTAATAACCGCAGTGATT
GACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGATTAAGTCAGATGTGAAATCCCCGAGC
TTAACTTGGGAACTGCATTTGAAACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCG
ACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTGCTAGAGATAGCTTAGTGCCTTCG
GGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTATCCTTTGTTGCCAGCGCGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAA
ACCGGAGGAAGGTGGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACA
ATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGAT
TGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT

- 89 *klebsiella pneumoniae subsp. Pneumoniae*

TAACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGT
AATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCG
CAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA
GCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTAAG
GTTAATAACCTCAGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCG
GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC
CCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATC
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGA
GATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAG
GAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGG
GCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAG
TATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCA
GAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCA
AAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCAT

- 141 *Staphylococcus epidermidis*

AGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGA
GCTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGAT
GGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC

TTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACT
CTGTTATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC
TGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGG
AGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCC
TTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGA
ATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAA
ATCTTGACATCCTCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGT
TGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGTAGCGAAACC
GCGAGGTCAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTATATGAAGC
TGG

- 145 *Escherichia marmotae*

TAATACATGCAAGTCGAGCGAATCGATGGGAGCTTGCTCCTTGAGATTAGCGGCGGACGGGTGAGTAAC
ACGTGGGCAACCTGCCCTGCAGATGGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCAGTTC
CTCCGCATGGAGGAACTCTGAAAGACGGTTTCGGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCT
TGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGT
CTGATGGAGCAACGCCGCGTGAGCGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTGAGGGAAGAACA
AGTACGGGAGTAACTGCCCGTACCTTGACGGTACCTCATCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCCT
TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTACAGA
AGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACA
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CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCA
CTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGGGCTGCGAACCCGCGA

- 180 *Bacillus amyloliquefaciens subsp. Plantarum*

TAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAAC
ACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTG
AACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG
TGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCT
TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGA
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GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
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AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAA
TCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGACAGAACAAA

- 184 *Citrobacter freundii*

TTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGG
ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
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CGTCACACCATGGGGAGTGGGTTGCAAAA

- 188 *Bacillus licheniformis*

TAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATG
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CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC
CTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
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ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG
TTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAG
CCGCGAGGCTAAGCCAATCCCAC

- 206 *Bacillus circulans*

TAATACATGCAAGTCGAGCGGACTTTAAAAGCTTGCTTTTAAAGTTAGCGGCGGACGGGTGAGTAACAC
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GGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTAC
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AAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGA
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AGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACACTCC
TAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACT
CTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAC
CTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGC

- 208 *Bacillus aerophilus*

TAATACATGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAA
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GAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA
AGTGCAAGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCT
TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGA
AGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAA
CCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGACAGAACAAA

- 219 *Serratia grimesii*

TGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGG
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CTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTGCATTGACGTTACTCGCAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
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GGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
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AGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAACTC
GCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAG
TCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTT
GTGATTCATGACTGGG

- 233 *Chryseobacterium vietnamense*

TAACACATGCAAGCCGAGCGGTATTGTTTCTTCGGAAATGAGAGAGCGGCGTACGGGTGCGGAACACGT
GTGCAACCTGCCTTTATCAGGGGGATAGCCTTTCGAAAGGAAGATTAACACCCCATAATATATTAAGTGG
CATCATTTGATATTGAAAACTCCGGTGGATAGAGATGGGCACGCGCAAGATTAGATAGTTGGTAGGGTA
ACGGCCTACCAAGTCTACGATCTTTAGGGGGCCTGAGAGGGTGATCCCCCACACTGGTACTGAGACACG
GACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGTGCAAGCCTGATCCAGCCATCC
CGCGTGAAGGATGACGGCCCTATGGGTTGTAAACTTCTTTTGTATAGGGATAAACCTTTCCTCGTGAGGA
AAGCTGAAGGTACTATACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCTGATTTGTAAGTCAGTGGTGAAATCTCA
CAGCTTAACTGTGAAACTGCCATTGATACTGCAAGTCTTGAGTGTTGTTGAAGTAGCTGGAATAAGTAGT
GTAGCGGTGAAATGCATAGATATTACTTAGAACACCAATTGCGAAGGCAGGTTACTAAGCAACAACTGAC
GCTGATGGACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCT
AACTCGTTTTTGGTTTTTCGGAATCAGAGACTAAGCGAAAGTGATAAGTTAGCCACCTGGGGAGTACGAA
CGCAAGTTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGTTTAATTCGAT
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ATTTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTAGGTTAAGTCCTGCAACGAGCG
CAACCCCTGTCACTAGTTGCCATCATTAAGTTGGGGACTCTAGGAGACTGCCTACGCAAGTAGAGAGGAA
GGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCCACACACGTAATACAATGGCCGGTAC
AGAGGGCAGCTACACAGCGATGTGATGCAAATCTCGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAA
CTCGACTCTATGAAGCTGGAATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCAAGCCATGGAAGTCTGGGGTACCTGAAGTCGGTGACCGTAACAGGAGCTG
CCTAGGGTAAAACAGGTAACTA

- 247 *Bacillus licheniformis*

AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGA
GCTAATACCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGAT
GGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGA
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GCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCT
GGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGT
CACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCGTC

- 249 *kocuria rosea*

TAACACATGCAAGTCGAACGATGATGCCCAGCTTGCTGGGCGGATTAGTGGCGAACGGGTGAGTAATAC
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CGATGCAACGCGAAGAACCTTACCAAGGCTTGACATTCACCGGACCGCCCCAGAGATGGGGTTTCCCTTC
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ACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGCCGGGGTCA
ACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAA
TGGCCGGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGAA

- 252 *Novosphingobium capsulatum*

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CCTGGTAGTCCACGCCGTAAACGATGATAACTAGCTGTCCGGGCACTTGGTGTTTGGGTGGCGCAGCTAA
CGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCTGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCTTTGACATCCCGCGC
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TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCCAGCATTAAGTTGGGC
ACTCTAAGGAAACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTAC

AGGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAAGCACGCGAGTGTGAGCTAATCT
CCAAAAGCCGTCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGGCGGAATCGCTAGTAATCGC
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GATTCACTCGAAGGCGTTGAGCTAACCCGCAAGGGAGGCAGGCGACCACAGTGGGTTTAGCGAC

- 287 *Ochrobactrum anthropi*

GCAAGGGGAGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGCTACGGAATAACTCAGGGA
AACTTGTGCTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGGCAAAGGATCGGCCCGCGTTGGAT
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TGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
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GTTCGGCTGGACCGGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
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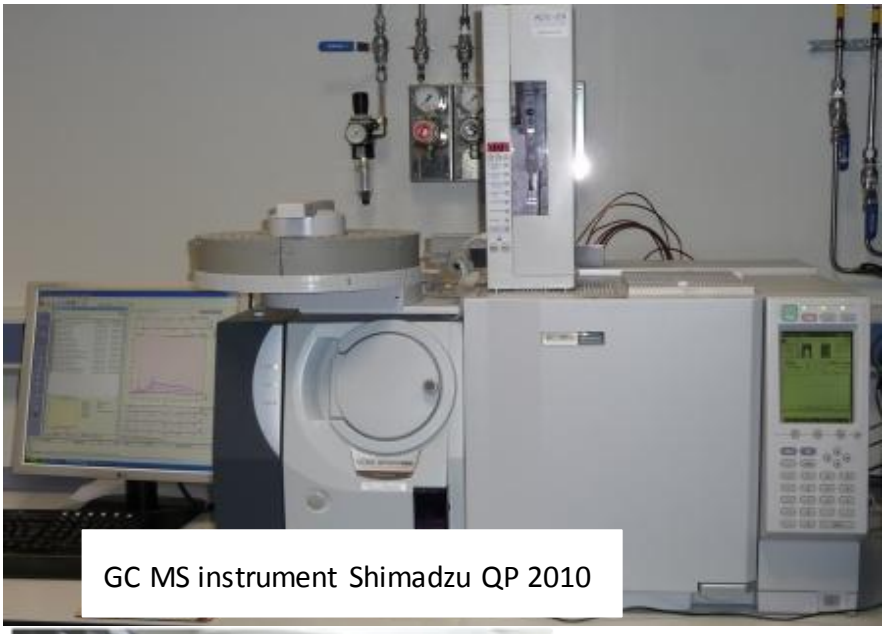
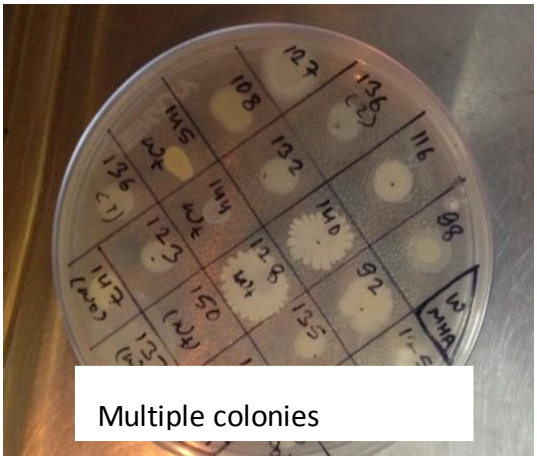
GC Ms library analysis

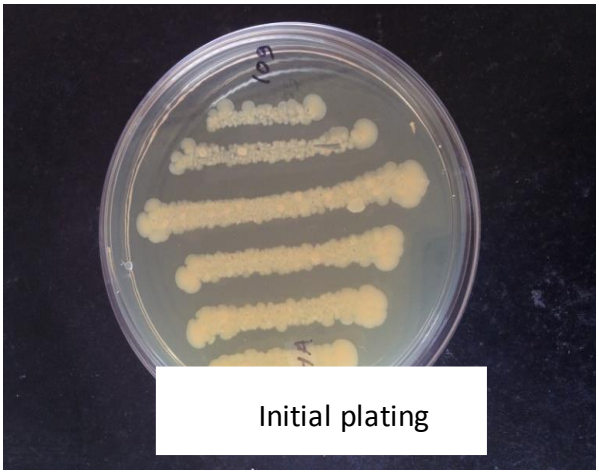


GC MS instrument Shimadzu QP 2010

Apparatus performing GC MS



Multiple colonies



Initial plating