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Research Article

Oral Microbial Diversity Among Nepalese Individuals Across Various Geographical Regions

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Abstract

A comprehensive study encompassed the collection of 153 samples derived from oral patients across 17 diverse locations throughout Nepal. The assortment of samples included extracted teeth, dental plaque, and dental calculus, procured from dental clinics, dental hospitals, and dental camps. Employing six distinct culture media, namely nutrient agar (NA), Muller Hilton agar (MHA), mannitol salt agar (MSA), blood agar (BA), brain heart infusion agar (BHA), and potato dextrose agar (PDA) for potential fungal strains, plates were meticulously incubated at 37°C for 5-7 days. The ensuing bacterial colonies were judiciously isolated, and their morphological and biochemical traits were scrutinized. The microscopic structures of the bacterial cells were examined, considering shape, size, colour, opacity, and texture. Gram-staining was employed, and each colony's biochemical attributes were assessed for protease, pectinase, cellulase, and lipase enzymes. From the 1200 colonies isolated from dental samples, 300 diverse colonies, distinguished by morphological and biochemical characteristics, were chosen for further taxonomic identification. Subsequent sequencing revealed the identification of 60 distinct species within 21 genera of bacterial isolates, including *Achromobacter*, *Bacillus*, *Chryseobacterium*, *Citrobacter*, *Curtobacterium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Kocuria*, *Lyinibacillus*, *Novosphingobium*, *Ochrobactrum*, *Proteus*, *Pseudomonas*, *Sporosarcina*, *Staphylococcus*, *Stenotrophomonas*, *Serratia* and *Streptococcus*. The research underscored the presence of various pathogenic bacterial species in oral samples.

Keywords: 16S rRNA, Oral bacteria, Oral disease, Oral hygiene

Introduction

The human body serves as a diverse habitat for a multitude of microorganisms, with certain microbes playing pivotal roles in influencing human health and contributing to various diseases. The oral cavity, in particular, provides a conducive environment for the thriving of numerous bacteria and fungi. These microorganisms establish colonies on oral surfaces, giving rise to dental plaque or oral biofilm (Takahashi, 2005). This biofilm becomes a breeding ground for common oral infections such as dental

caries, gingivitis, periodontitis, and peri-implantitis, posing significant health risks not only locally in the mouth but also contributing to systemic diseases.

The dental biofilm forms a microbial community on tooth and tissue surfaces within the oral cavity, encompassing the tongue, mucosa, and other variant surfaces. The implications of such biofilms extend beyond oral health, impacting global health concerns. Dental caries and periodontal diseases are recognized as critical global oral health issues (Bhardwaj & Bhardwaj, 2012). Furthermore, the link between poor oral health, the invasive potential of oral microbes in the body, and their impact on cardiac health and cognitive function has been explored (Noble et al., 2013). The far-reaching consequences of oral health also extend to an association with various cancers, including pancreatic, gastrointestinal, and oral/pharyngeal cancers like oral squamous cell carcinomas (Chakraborty et al., 2014). Despite being a pervasive concern affecting individuals of all ages and societies worldwide, oral health problems seem to be escalating due to the presence of hard surfaces facilitating bacterial colonization, leading to the development of dental biofilms and the onset of dental caries (Kirby et al., 2014).

In developing countries, particularly in rural areas, maintaining oral hygiene poses a significant challenge due to limited awareness and practice. While oral health is a major concern in Nepal, research in this field is scarce. The geographical and cultural diversity in Nepal suggests potential variations in oral microorganisms, emphasizing the need for more extensive research in this underexplored domain.

Materials and Methods

Sampling

A total of 153 samples were gathered from oral patients residing in diverse locations across the country, spanning 17 different places within 16 districts (Table 1). The selection for sampling was conducted randomly, contingent upon the availability of oral samples. The collected samples comprised extracted teeth, dental plaque, and dental calculus, sourced from a variety of settings including dental clinics, dental hospitals, and dental camps.

Table 1: The list of geographical locations for oral sample collection.

SN	Location	Number of samples
1	Baglung	2
2	Baitadi	5
3	Bhairahawa	8
4	Butwal	11
5	Dang	3
6	Dharan	7
7	Ilam	5
8	Janakpur	9
9	Jhapa	9
10	Kathmandu	8
11	Biratnagar	12
12	Myagdi	3
13	Nuwakot	14
14	Palpa	5
15	Pokhara	6
16	Sindhupalchowk	46
17	Udaypur	1

Microbial organism culture

Standard microbial techniques were employed to isolate all cultivable microorganisms. The isolation process utilized six different culture media, namely nutrient agar (NA), Muller Hilton agar (MHA), mannitol salt agar (MSA), blood agar (BA), brain heart infusion agar (BHA), and, for potential fungal strains, potato dextrose agar (PDA). The grown colonies underwent careful observation and purification through repeated streaking until a pure colony was achieved. Incubation took place at 37°C for 5-6 days, with most samples exhibiting growth in the nutrient agar medium.

The morphological characteristics of the microorganisms were scrutinized, encompassing considerations of their shape, size, colour, margin, opacity, and texture. This comprehensive approach facilitated the thorough examination and categorization of isolated microorganisms, enhancing the understanding of their diverse traits and behaviours.

Identification of bacteria

Gram staining: The gram staining procedure involves the execution of four fundamental and crucial steps. Initial staining employed crystal violet, binding with grams of iodine to entrap the CV-I complex within the cell. This was succeeded by alcohol decolourization and, ultimately, a counter-stain application of safranin. Following

decolourization, gram-negative cells lost their purple colour, retaining the safranin counter-stain, resulting in a pink hue. In contrast, gram-positive cells retained the CV-I complex, maintaining their purple colour throughout the subsequent stages. Gram-variable bacteria exhibited a distinct behaviour, deviating from the aforementioned mechanisms, leading to a mixture of pink and purple cells in the staining process (Davies et al., 1983).

16S ribosomal RNA sequencing: Genomic DNA extraction was carried out using the Labo Pass Mini Tissue Genomic DNA Isolation Kit from Cosmogentech Inc., Korea. Subsequently, the 16S rRNA genes were PCR-amplified utilizing universal primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The resulting PCR products underwent purification with the Labo Pass PCR Purification Kit from Cosmogenetech, Seoul, Korea, and were subsequently sequenced using the same primers employed for amplification. To identify closely related species, the obtained 16S rRNA gene sequences were compared with those of type strains accessible in the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) as outlined by Kim et al. (2012). This comparative analysis facilitated the determination of the genetic similarities and relationships between the sequenced 16S rRNA genes and the reference strains in the EzTaxon-e database.

Phylogenetic analysis: Two online tools, ClustalW with the Clustal Omega multiple alignment tool, and a robust phylogenetic analysis tool on Phylogeny.fr, were employed in this study. Both tools share a common underlying principle: the extracted sequences were initially aligned using the Clustal Omega multiple alignment tool. Subsequent steps included curation, phylogenetic analysis, and tree rendering to ultimately generate a comprehensive phylogenetic tree. The construction of a rooted phylogenetic tree was facilitated by utilizing ribosomal RNA partial sequences extracted from NCBI for all identified bacteria. Additional sequences, particularly those associated with pathogenically significant oral bacteria, were also incorporated into the analysis. These tools, as described by Dereeper et al. (2010), played a crucial role in the alignment, curation, and subsequent generation of a phylogenetic tree, offering insights into the evolutionary relationships among the identified bacterial species.

Enzymatic assay of the colonies

Isolated colonies were cultivated on agar plates containing compounds that activate target enzymes. To screen for protease-producing colonies, they were grown on skim milk agar plates. The presence of a clear zone around the grown colony served as an indicator of protease activity. Similarly, cellulase enzyme activity was assessed by cultivating colonies in Carboxymethylcellulose (CMC) media. Positive cellulase activity was confirmed by the appearance of a clear halo zone after pouring a 1% congo red indicator and subsequent washing with 1N NaCl. For amylase enzyme screening, starch plates were employed, and 0.1% iodine solution was applied. Additionally, the same 0.1% iodine solution was utilized to screen pectinase enzymes on plates containing pectin. These screening methods allowed for the identification of colonies exhibiting amylase and pectinase enzyme activities, respectively, based on the visual cues provided by the iodine solution on the respective agar plates.

Results and Discussion

From 153 oral samples collected across 17 different locations in Nepal, a total of 1200 colonies were isolated. Six diverse culture media were employed to isolate bacterial colonies. Among these, a meticulous selection process was undertaken, choosing only 300 colonies based on criteria such as colony morphology, colour, cellular morphology, biochemical characteristics, and gram-staining properties for further taxonomic identification.

A considerable portion of the isolated colonies, numbering 186, exhibited a moist texture, while a substantial amount displayed dry and mucoid characteristics. The majority of the colonies, specifically 213, demonstrated irregular shapes, with only 6 showcasing filamentous attributes. Nearly 90% of the colonies appeared opaque, contrasting with the few that were transparent. Smooth colonies accounted for 112 instances, while 121 were categorized as rough, and a small number exhibited wrinkled and shiny features.

Approximately half of the colonies displayed undulated patterns, while the remainder exhibited entirely marginal or lobulated structures, with 5 colonies adopting filiform shapes. None of the colonies displayed concave attributes, but

approximately 60% were raised. This detailed analysis provides a comprehensive overview of the varied morphological characteristics observed among the isolated colonies, adding depth to the understanding of their diverse traits.

Within the isolated bacterial genera, those identified as gram-negative included *Chryseobacterium*, *Flavobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Proteus*, *Escherichia*, *Ochrobactrum*, *Serratia*, *Novosphingobium*, *Achromobacter*, *Klebsiella*, *Enterobacter* and *Citrobacter*. Conversely, the gram-positive genera encompassed *Bacillus*, *Enterococcus*, *Staphylococcus*, *Lysinibacillus*, *Kocuria*, *Streptococcus* and *Curtobacterium*. This categorization sheds light on the diverse bacterial composition within the isolated samples, offering insights into the distribution of both gram-negative and gram-positive genera in the examined oral microbiome.

Subsequent to the sequencing of the 16S rRNA of 300 colonies, a noteworthy outcome emerged: the identification of 60 different species (Table 2) spanning 22 distinct genera. The breakdown of these genera is as follows: *Achromobacter* (3 species), *Bacillus* (14 species), *Chryseobacterium* (1 species), *Citrobacter* (3 species), *Curtobacterium* (1 species), *Enterobacter* (4 species), *Enterococcus* (2 species), *Escherichia* (1 species), *Flavobacterium* (1 species), *Klebsiella* (6 species), *Kocuria* (1 species), *Lyinibacillus* (1 species), *Novosphingobium* (1 species), *Proteus* (1 species), *Obesumbacterum* (1 species), *Serratia* (4 species), *Ochrobactrum* (1 species), *Pseudomonas* (5 species), *Sporosarcina* (1 species), *Streptococcus* (1 species), *Stenotrophomonas* (2 species) and *Staphylococcus* (5 species). This comprehensive analysis (Figure 1) provides valuable insights into the diversity and distribution of bacterial species within the oral microbiome in the context of Nepalese populations.

Table 2: Identified bacterial species by using 16S ribosomal RNA sequencing.

No.	Name of the identified species	No.	Name of the identified species
1.	*Achromobacter marplatensis* B2(T)	31.	*Klebsiella michiganensis* W14
2.	*Achromobacter pulmonis* LMG 26696	32.	*Klebsiella pneumoniae* subsp. *ozaenae*ATCC 1196
3.	*Achromobacter spiritinus* LMG 26692	33.	*Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T)
4.	*Bacillus aerophilus 28K*(T	34.	*Klebsiella quasipneumoniae* subsp.*quasipneumoniae* 01A030
5.	*Bacillus altitudinis* 41KF2b(T)	35.	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044
6.	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42(T)	36.	*Klebsiella variicola* DSM 15968
7.	*Bacillus aryabhattai* B8W22	37.	*Kocuriarosea* DSM 20447
8.	*Bacillus cereus* ATCC 14579(T)	38.	*Lysinibacillus macroides* LMG 18474(T)
9.	*Bacillus circulans* ATCC 4513	39.	*Novosphingobium capsulatum*GIFU11526
10.	*Bacillus flexus* IFO 15715	40.	*Obesumbacterium proteus* DSM 2777
11.	*Bacillus licheniformis* ATCC 14580(T)	41.	*Ochrobactrum anthropi* ATCC 49188
12.	*Bacillus methylotrophicus* KACC 1310	42.	*Proteus mirabilis* ATCC 29906(T)
13.	*Bacillus paralicheniformis* KJ-16(T)	43.	*Pseudomonas aeruginosa* JCM 5962
14.	*Bacillus safensis* FO-36	44.	*Pseudomonas geniculata* ATCC 19374
15.	*Bacillus siamensis* KCTC 13613(T)	45.	*Pseudomonas hibiscicola* ATCC 19867
16.	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429	46.	*Pseudomonas japonica* NBRC 103040
17.	*Bacillus tequilensis*KCTC 13622(T)	47.	*Pseudomonas taiwanensis* BCRC 17751
18.	*Chryseobacter iumvietnamense* GIMN1 .005	48.	*Serratia glossinae* C1(T)
19.	*Citrobacter farmeri* CDC 2991-81	49.	*Serratia grimesii* DSM 30063(T)
20.	*Citrobacter freundii* ATCC 8090	50.	*Serratia liquefaciena* ATCC 27592(T)
21.	*Citrobacter koseri* CDC3613-63	51.	*Serratia marcescens* subsp.*marcescen* ATCC 13880
22.	*Curtobacter iumoceanosedimentum* ATCC31317(T)	52.	*Sporosarcina contaminans* CCUG 53915
23.	*Enterobacter aerogenes* KCTC 2190	53.	*Staphylococcus epidermidis*ATCC 14990
24.	*Enterobacter ludwigii* EN-119	54.	*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305
25.	*Enterobacter tabaci* YIM Hb-3(T)	55.	*Staphylococcus sciuri* DSM 20345(T)
26.	*Enterobacter xiangfangensis*10-17(T)	56.	*Staphylococcus sciuri* subsp. *sciuri* DSM 20345(T)
27.	*Enterococcus faecalis* ATCC 19433(T)	57.	*Staphylococcus xylosus* ATCC 29971(T)
28.	*Enterococcus faecium* CGMCC 1.2136(T)	58.	*Stenotrophomonas chelatiphaga* LPM-5
29.	*Escherichia marmotae* HT07301	59.	*Stenotrophomonas maltophilia* MTCC 434
30.	*Flavobacter iumoceanosedimentum* ATCC 31317	60.	*Streptococcus mutans* NCTC 10449

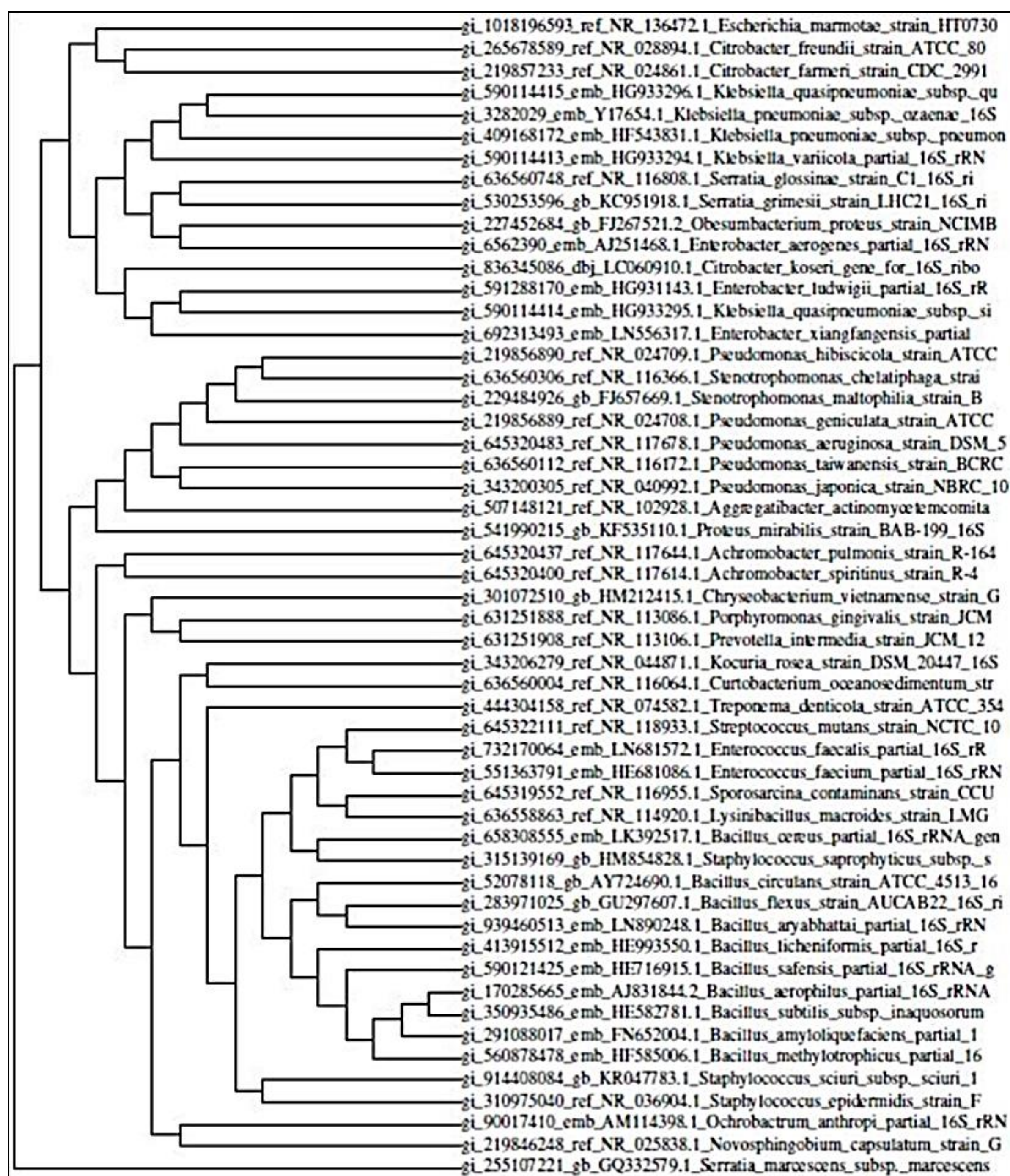

Figure 1: Phylogenetic tree of the isolated bacterial strains.

Figure 2 illustrates that over 50% of the organisms (173) demonstrated at least one enzyme activity, while 127 colonies exhibited no enzyme activities. Specifically, 27 colonies exhibited amylase activity, 81 colonies showcased protease activity, and 18 isolates displayed cellulase activity. Furthermore, 21 colonies demonstrated both cellulase and protease activities, 19 colonies exhibited both protease and cellulase activities, and 4 colonies displayed all three enzyme activities (cellulase, amylase, and protease). This detailed enzymatic analysis on respective agar plates provided clear evidence of the prevalence of three major enzymes.

Notably, strains of *Klebsiella pneumonia*e and *Achromobacter pulmonis* were observed to produce all three enzymes (amylase, cellulase, and protease). Additionally, strains of *Enterobacter xiangfangensis*, *Bacillus cereus*, *Bacillus aerophilus*, *Bacillus amyloliquefaciens*, and *Pseudomonas taiwanensis* exhibited positive results for two enzymes, namely cellulase and protease.

Escherichia marmotae, *Ochrobactrum anthropi* and *Obesumbacterium proteus* demonstrated single enzyme activity, specifically protease. Conversely, *Staphylococcus epidermidis*, *Citrobacter koseri*, *Serratia grimesii*, *Chryseobacterium vietnamense* and *Sporosarcina contaminans* did not exhibit any enzyme activity. This comprehensive enzymatic analysis provides valuable insights into the diverse enzyme production capabilities of isolated bacteria. Salivary microbiota richness was strongly correlated with poor oral health hygiene, with *Prevotella* and *Veillonella* dominance associated with an increased risk of periodontal disease. Conversely, *Neisseria* dominance was linked to a healthy periodontal condition (Yamashita and Takeshita, 2017). Additionally, *Staphylococcus* colonization in the oral cavity was identified as a potential cause of endocarditis, a severe heart infection (Ohara-Nemoto et al., 2008), while *Escherichia faecalis* was reported as the primary causative organism for root canal treatment failures (Mahmoudpour et al., 2007). This underscores the need for further research to correlate oral microbial specimens with specific oral health symptoms and conditions.

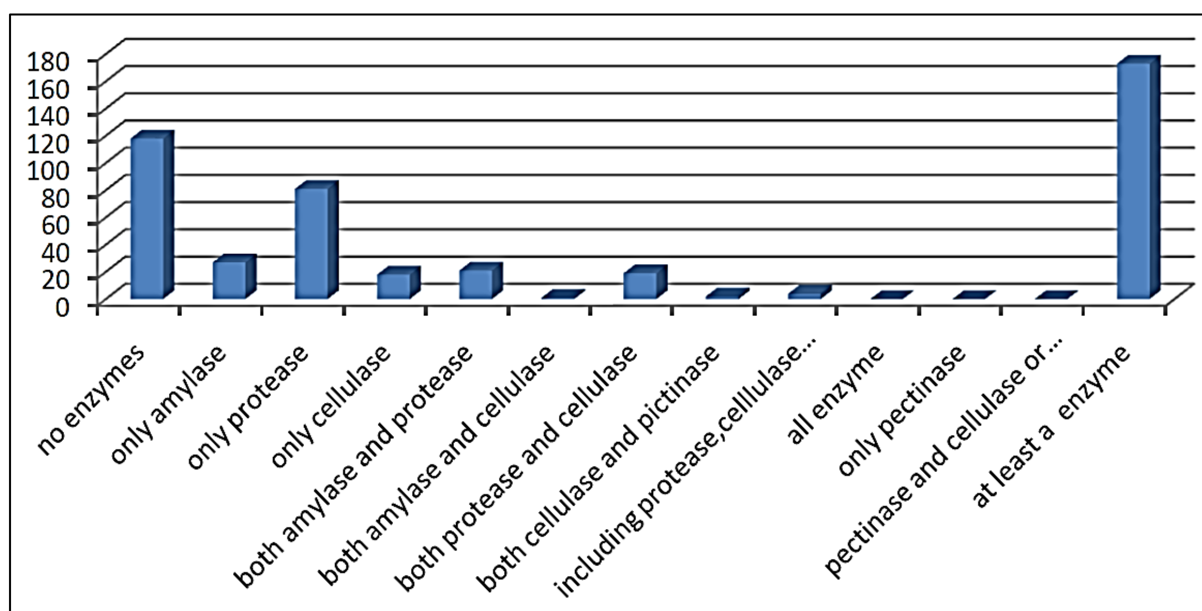

Figure 2: The enzymatic activity of 300 bacterial colonies.

Conclusion

The predominant bacteria associated with dental diseases, *Streptococcus mutans* and *Porphyromonas gingivalis*, were noted, though their prevalence varied in different stages of oral disease lesions. *Streptococcus*, *Porphyromonas* and *Actinomyces* species were identified as predominant in the supragingival area, but as our main samples consisted of extracted teeth, only a subset of these species was identified in our sequencing.

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