Chapter-1

Review of Literature
1.1 Human dentition and tooth

The teeth are the most distinctive and long-lasting component of the human mouth. They are the first in the chain of energy production in the body as they help in chewing and grinding the food. Mammals are diphyodont meaning that they grow two sets of teeth. The first or deciduous teeth start appearing at six month of age and are 20 in number. The second or permanent teeth appear from seven years and are 32 in number. The permanent teeth are four in types-incisors, canines, molars and premolars. The tooth has four major parts- Enamel (the hardest part in the body), dentin, cementum and pulp (Figure 1.1). Paleontologists use teeth for their studies as they are preserved for years, yet there are ailments that can destroy it very early and one such cause is dental caries.

1.2 Dental caries and its epidemiology

Dental caries is a localized and progressive decay of the teeth. Not only does it cause many people to experience a great deal of pain, it leads to continuous discomfort through inconvenient treatment. WHO’s report on the Global Problem of Oral Diseases, notes that oral diseases such as dental caries (tooth decay), periodontitis (gum disease) and oral and pharyngeal cancers are global health problem in both the industrialized and the developing countries, especially among poorer communities (Petersen, 2003). Dental caries is a major oral affliction.
Figure 1.1 Anatomy of a human tooth
(Source:http://en.wikipedia.org/wiki/Tooth_development)
in developing countries, affecting 60-90% of the school children and the vast majority of adults. An estimated five billion people worldwide have experienced dental caries (Petersen, 2003). Figure 1.2 highlights the dental caries experience among 12-year-old children in the six WHO regions in the year 2000, based on the DMFT (Decayed, Missing and Filled Teeth) Index, which measures the lifetime experience of dental caries in permanent dentition. In developing countries like India, the rate of dental caries is rising and since more than 80% of the world’s children live in these countries, dental caries is considered to be a major public health problem (Cirino and Scantlebury, 1998). Table 1.1 shows the caries assessment in a study conducted in Indian school children where the percentage of affected children found is alarming.

1.3 Causes of dental caries

Dental caries is a chronic infectious disease in which the active agent or agents are members of the indigenous oral flora (Shaw, 1987). Oral cavity harbors a rich and diverse microbial flora because of its ideal humidity and temperature, the frequent passage through it of most nutrients needed by many microbial species and presence of several ecological niche. The presence of a myriad of microorganisms is a natural part of proper oral health. Oral microbes can adhere to surfaces throughout the oral cavity (Loesche, 1986). These include the tongue, epithelial cells lining roof of the mouth and the cheeks, and enamel of the teeth.
Figure 1.2 Dental caries experience (DMFT) of 12-year-old children according to WHO regional offices (Source: WHO Global Oral Health Data Bank and WHO Oral Health Country/Area Profile Programme, 2000).
Table 1.1 Dental caries assessment in 5 and 12 years old caries-active subjects in Chennai City, Tamil Nadu, India (Kumar et al., 2005)

<table>
<thead>
<tr>
<th>Age Group</th>
<th>% affected</th>
<th>DMFT</th>
<th>D</th>
<th>M</th>
<th>F</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 years*</td>
<td>83</td>
<td>3.5</td>
<td>3.4</td>
<td>0.07</td>
<td>0.0</td>
<td>2003</td>
</tr>
<tr>
<td>12 years*</td>
<td>80</td>
<td>3.94</td>
<td>3.90</td>
<td>0.03</td>
<td>0.0</td>
<td>2003</td>
</tr>
</tbody>
</table>

*600 children each 5- and 12-year-old age groups
Hundreds of species of bacteria have been identified to form adherent oral communities, known as biofilms (Burne, 1998). Development of the adherent populations of microorganisms in the oral cavity begins with the association and irreversible adhesion of certain bacteria to the tooth surface. Components of the host oral cavity, such as proteins and glycoproteins from the saliva, also adhere. This early coating is referred to as the acquired enamel pellicle (AEP). The AEP alters the chemistry of the tooth surface, encouraging the adhesion of other microbial species (Jefferson, 2004). Over time, as the biofilm thickens, gradients develop within the biofilm. Such environmental alterations promote the development of different types of bacteria in different regions of the biofilm (Marsh and Martin, 1999). This changing pattern represents what is termed bacterial succession. Examples of some bacteria that are typically present as primary colonizers include *Streptococcus, Actinomyces, Neisseria,* and *Veillonella.* Examples of secondary colonizers include *Fusobacterium nucleatum,* *Prevotella intermedia,* and *Capnocytophaga* species. With further time, another group of bacteria can become associated with the adherent community. Examples of these bacteria include *Campylobacter rectus, Eikenella corrodens,* *Actinobacillus actinomycetemcomitans,* and the oral spirochetes of the genus *Treponema* (Liljemark and Bloomquist, 1996).

Under normal circumstances, the microbial flora in the oral cavity reaches equilibrium, where the chemical by-products of growth of some microbes are utilized by other microbes for their growth (Burne, 1998). Significant parameters
regulating homeostasis in the mouth include the integrity of host defenses (including saliva flow) and the composition of the diet (Marsh, 1999). In any ecosystem, microbial homeostasis can break down on occasion due to a substantial change in parameter that is critical to maintaining ecological stability at a site, resulting in the outgrowth of previously minor components of the community. An example, when the diet is high in sugars that can be readily used by bacteria, the pH in the adherent community is lowered, which selects for the predominance of acid-loving bacteria, principally *Streptococcus mutans* and *Lactobacillus* species. These species can itself produce acidic products that damage the tooth resulting in dental caries.

Dental caries is the second most common of all maladies in humans, next only to the common cold (Slavkin, 1998). Dental caries typically proceeds in stages (Figure 1.3). Discoloration and loosening of the hard enamel covering of the tooth precedes the formation of a microscopic hole in the enamel. The hole subsequently widens and damage to the interior of the tooth usually results. If damage occurs to the pulp and the roots anchoring the tooth to the jaw, the tooth is usually beyond saving (Shaw, 1987). Removal of the tooth is necessary to prevent accumulation of bacterial products that could pose further adverse health effects. Caries is thus a complex disease caused by imbalance in physiological equilibrium and environmental factors play a critical role in the initiation and progression of the disease (Arends and christoferren, 1986).
Figure 1.3 Developmental stages of dental caries. (Source: http://greenfield.fortunecity.com/rattler/46/upali3.htm) (1) Acids present in the oral cavity destroy the enamel of the tooth. (2) The dentine is then attacked by acids and bacteria invade the cavity. (3) Inflammation of the pulp. (4) Necrosis of the pulp tissue. (5) Periapical abcess formed at the apex of the root.
Of 200-300 species that are predominant in the human dental plaque, only a finite number may be considered as dental pathogens and dental caries can be regarded as a specific treatable disease.

1.4 Is dental caries infectious?

The infections and transmittable nature of dental caries was brought in focus by studies of Keyes on gnotobiotic rodents in 1960. He found that germ free hamsters developed caries when they were caged together with caries-active hamsters. Further proof emerged when certain streptococci caused rampant decay in previously caries-active animals (Fitzgerald and Keyes, 1960). These bacteria later identified as *Streptococcus mutans*, gave rise to the concept of caries being due to a specific infection with mutans streptococci.

1.5 Mutans Streptococci

Streptococcal species contribute approximately 1/3 of the total viable organisms of plaque (Newbrun, 1989). The microorganisms co-habiting within the plaque ecosystem are *Streptococcus mutans*, *Lactobacilli acidophilus* and *Actinomyces israelii* (van Houte *et al.*, 1994; Schupbach *et al.*, 1996). The mutans group of streptococci consists of seven species: *Streptococcus cricetus*, *S. ferus*, *S. macacae*, *S. downei*, *S. rattus*, *S. sobrinus* and *S. mutans* (Whiley and Beighton, 1998). *S. mutans* and *S. sobrinus* are most commonly encountered in human dental plaque and are associated with dental caries (Loesche, 1986). Of these, *S.*
mutans are more prevalent in active-caries than S. sobrinus. Also, subjects harboring both S. mutans and S. sobrinus have a significantly higher prevalence of dental caries than those with S. mutans or S. sobrinus alone (Okada et al., 2002; Wu et al., 2003).

1.6 Streptococcus mutans

Kingdom: Bacteria
Phylum: Firmicutes
Class: Cocci
Order: Lactobacillales
Family: Streptococcaceae
Genus: Streptococcus
Species: mutans

Streptococcus mutans is a Gram-positive, facultatively anaerobic bacteria commonly found in the human oral cavity. The natural habitat of S. mutans is the human mouth. The organism can be isolated frequently from faeces in human (Finegold et al., 1975). It was first observed by Clarke who found a small, chained coccobacillus which was more oval than spherical in shape. He suggested that these microorganisms were mutant streptococci and called them Streptococcus mutans (Clarke, 1924). The cells are spherical or ovoid, 0.5-2.0 μm
in diameter, occurring in pairs or chains when grown in liquid media, and stain
Gram-positive (Figure 1.4). The optimum temperature for growth is 37°C, and
growth is usually restricted to 25-45°C.

1.7 Role of Streptococcus mutans in dental caries

Numerous studies have shown that mutans streptococci can bring about caries
in pits and fissures as well as on smooth and root surfaces of the teeth of both
gnotobiotic and conventional animals (Michalek et al., 1976). Moreover, the
caries induced by mutans streptococci is more severe than caused by other
streptococci. Mutans streptococci involvement in the etiology of caries has
come from immunization studies. In one such study, the oral administration of
streptococci cells to gnotobiotic rats induced the production of secretory IgA in
the saliva and this correlated with the reduction in caries incidence in these
animals (Michalek et al., 1976). Intravenous administration of Streptococcus
mutans cells to monkeys led to a serum body response and an associated
decrease in caries incidence (Bowen, 1969b). The mode of action of these
antibodies is inhibition of the adherence and possibly metabolic activities of S.
mutans (Russell and Hajishengallis, 1999). Some antisera against whole cells
of S. mutans significantly inhibit the enzymatic activity of extracellular
GTFases and hence the subsequent adherence to smooth surfaces (Evans, 1973;
Hamada and Mizuno, 1979).
Figure 1.4  Arrangement and shape of *Streptococcus mutans* (A) microscopic view of *S. mutans* (40X). The bacteria stain positive with gram stain (B) The shape of *S. mutans* (Source of figure B- http://media-2.web.britannica.com/eb-media/95/58695-004-601B37BC.jpg)
In a longitudinal study, it was found that levels of mutans streptococci in plaque increased, 6-24 months before the clinical appearance (Loesche et al., 1984). Increase in the proportion of mutans streptococci was observed to occur before root caries lesion development or when these lesions become active. In general high titers of salivary and serum antibodies to mutans streptococci antigens have been found in low caries population and the prevalence of caries is high in immuno-compromised patients (Taubman, 1992).

1.8 Virulence factors of Streptococcus mutans

The term virulence describe the ability of a parasite (microorganism) to cause infection in its host, the organism it lives on or in. The property is quantitative and expresses the degree of pathogenicity. Virulence factors in S. mutans help protect the bacterium against possible host defenses and maintain its ecological niche in oral cavity while contributing to its ability to cause host damage.

1.8.1 Adhesins

Streptococcus mutans antigen (Ag) I/II, a cell surface fibrillar protein also known as protein B, P1, PAc, MSL-1, and SR, is implicated in the adherence of S. mutans to salivary pellicle-coated dental enamel (Russell et al., 1994). Both S. mutans (Gibbons et al., 1984) and Agl/II (Russell and Mansson-Rahemtulla, 1989) bind selectively to human saliva-coated hydroxyapatite (SHA), which simulates pellicle-coated enamel, but isogenic Agl/II-deficient
mutants of *S. mutans* lack the protein fuzzy coat on the cell surface and bind poorly to experimental salivary pellicles (Lee *et al.*, 1989). Furthermore, Agl/II competitively inhibits *S. mutans* adherence (Bleiweis *et al.*, 1992), and it is a target of *S. mutans* adherence-inhibiting human secretory immunoglobulin A (S-IgA) antibodies (Hajishengallis *et al.*, 1992). This streptococcal protein also mediates saliva-induced aggregation of *S. mutans*, as shown by aggregation studies comparing wild-type and isogenic Agl/II-deficient strains (Lee *et al.*, 1989), and by the findings that *S. mutans* aggregation is inhibited by free Agl/II (Demuth *et al.*, 1990b) or by anti-Agl/II monoclonal antibodies (Brady *et al.*, 1992).

Figure 1.5 shows the map and different regions of Agl/II. It consists of a protease-sensitive N-terminal region, AgI, and protease-resistant AgII, which represents the C-terminal and membrane-proximal one-third of the molecule (Russell *et al.*, 1980a; Kelly *et al.*, 1990). Immune responses to the Agl component, but not to AgII, have been associated with caries protection in the monkey model (Russell *et al.*, 1980b). Another study using four overlapping recombinant polypeptides covering the whole adhesin, localized the adhesion domain of Agl/II in the region specified by residues 816-1213 which includes the proline rich repeat region (P region), whereas the polypeptide containing the A region did not inhibit *S. mutans* adherence to SHA (Munro *et al.*, 1993).
Agl/II: 1565 a.a./ Mr 170,615

<table>
<thead>
<tr>
<th>Signal</th>
<th>A-region</th>
<th>V-region</th>
<th>P-region</th>
<th>Anchor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>(a.a.219-464)</td>
<td>(a.a. 465-825)</td>
<td>(a.a. 851-967)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.5  Schematic representation of different regions of Agl/II of \textit{S. mutans}
While it is not known whether the P region-including segment is also involved in *S. mutans* aggregation, a fusion protein containing the P region appears to bind directly to the whole Agl/II molecule (Nakai *et al*., 1993).

Several salivary molecules have been found to promote *S. mutans* adherence, including SAG (Lee *et al*., 1989), acidic proline-rich proteins (Gibbons and Hay, 1989), and adhesion-promoting proteins in the mucin fraction of submandibular-sublingual saliva (Kishimoto *et al*., 1989), suggesting that Agl/II may have multiple receptors and consequently multiple binding domains. Alternatively, it is possible that these salivary proteins display some common binding sites, eg. SAG and adhesion-promoting proteins may share oligosaccharide chains. The possibility of carbohydrate involvement is supported by the findings that *S. mutans* Agl/II recognizes fucose and lactose, whereas the homologous SSP-5 protein of *S. sanguis* M5 recognizes sialic acid (Demuth *et al*., 1990b). Studies with site-directed mutagenesis showed that adhesion to salivary film under conditions of flow was reduced in the *S. mutans* isogenic mutants. The disruption of the protein also results in alteration of bacterial surface charge (Petersen *et al*., 2002).

Coaggregation between oral bacteria is considered to be an important mechanism driving the development of biofilm communities. Specific interactions between bacteria provide for nutritional synergy (Palmer Jr *et al*., 2001), facilitate binding of secondary colonizers (Lamont *et al*., 2002).
and promote invasion of oral tissues (Love, 2000). The Agl/II family polypeptides have been implicated in mediating coaggregation between streptococci and several different genera of oral microorganisms (Lamont and Jenkinson, 2000). The specificity for coaggregation appears to reside within the N-terminal region of the Agl/II protein (Jakubovics et al., 2005). Multiple discrete regions within the primary sequences of the Agl/II polypeptides are selectively utilized in adhesion to oral cavity receptors. The N-terminal portion, comprising the A and V (variable) region, appears to determine specificity of Agl/II polypeptide recognition of *A. naeslundii* strains. These sequences would therefore potentially operate to drive the formation of Streptococcus-mixed species communities. Sequences within the C terminal portion of the polypeptides downstream of the P (proline rich) aa repeat blocks and within the C-terminal 400 aa residues, are not necessary for coaggregation with *A. naeslundii*, but are functionally obligate in recognition of gp340. The evidence that specificity of receptor recognition by these proteins results from multiple interactive domains suggests that binding to different receptors might be selectively targeted. Because Agl/II polypeptides are intimately involved in initial adhesion of streptococci to surfaces and in the subsequent establishment of oral microbial communities, modulation of their activities may be effective in controlling the development of polymicrobial biofilms that are ultimately associated with a range of human disease process.
1.8.2 Glucosyltransferases and fructosyltransferase

*Streptococcus mutans* metabolizes sucrose to synthesize water soluble and/or insoluble glucans. The reactions are catalyzed by three isozymes of glucosyltransferases (*Gtfs*) i.e. (*GtfB*, *GtfC* and *GtfD*). These enzymes catalyze the transfer and addition of a glucosyl moiety to the terminal site of a primer or elongating glucan (*Monchois et al.*, 1999).

\[ n.\text{sucrose} \rightarrow (\text{glucose})_n + n.\text{fructose} \]

The equilibrium of this reaction is almost irreversibly to the right. Practically, sucrose is the sole substrate for *Gtfs*. The products of *gtfB* and *gtfC* genes respectively primarily catalyze the synthesis of water-insoluble glucans, whereas *Gtf-S*, the product of *gtfD* genes catalyze the synthesis of water soluble glucans. In fact, water-soluble glucan from *S. mutans* has been reported to consist of an \( \alpha-(1-6) \)-linked linear glucose polymer with \( \alpha-(1-3) \) glucosidic branch linkages. The water-insoluble glucan possess more \( \alpha-(1-3) \) glucosidic linkages than water-soluble glucan and is hence resistant to the enzymatic action of \( \alpha-(1-6) \) glucanase, i.e., dextransase (*Hamada and Slade*, 1980). The sticky nature of glucan facilitates the adherence of bacteria to the tooth and resists its detachment by normal mechanical force such as mastication, swallowing and chewing. The nucleotide sequence of *gtf* genes from different oral streptococci comply with the same basic pattern and *Gtfs* are
approximately 1500 amino acids long (Russell, 1994). Streptococcal Gtfs have two common functional domains. The amino terminal portion, the glucan binding protein is responsible for glucan binding. In addition to glucan synthesis bacteria uses one fructosyltransferase to synthesize fructans with β-(2-1)-D-fructo-furanosidic linkages with an average repeating unit of 8 and 27 sugar residues (Birkhed et al., 1979).

Streptococcus mutans produces 3 types of Gtfs, whose cooperative action is considered to be essential for its cellular adherence to the tooth surface. However, the precise mechanisms for synthesizing adhesive glucans and the specific roles of each Gtf in cellular adherence to smooth surfaces have not been elucidated. In a study by Ooshima et al. (2001) seven types of isogenic mutants of S. mutans MT8148 lacking GtfB, GtfC, and/or GtfD activities were constructed by inactivation of the genes encoding GtfB, GtfC, and/or GtfD. Using these Gtf-deficient mutants and rGtfs, sucrose-dependent adherence of S. mutans resting cells and subsequently the role of each Gtf in vitro was examined. The highest level of sucrose-dependent adherence was found at the ratio of 20 rGtfB:1 rGtfC:4 rGtfD in both the resting cells of Gtf-deficient mutants and insoluble glucan synthesized by rGtfs. Moreover, when rGtfC and rGtfD were both present at concentrations of 1.5 mU and 6 mU, respectively, the insoluble glucan synthesized from sucrose by the rGtfs showed a high level of adhesiveness to smooth surfaces, even without rGtfB. These results suggest that the presence of all three Gtfs at the optimum ratio is
necessary for sucrose-dependent adherence of \textit{S. mutans}, and that GtfC and GtfD may play significant roles in the synthesis of adhesive and insoluble glucan from sucrose.

1.8.3 Other proteins related to sucrose metabolism

In addition to Gtfs, \textit{S. mutans} also produces three Glucan binding proteins (Gbps); GbpA, GbpB and GbpC. GbpA and GbpC are considered to participate in the initial adherence and hence contribute to cariogenicity of \textit{S. mutans} (Matsumura \textit{et al.}, 2003). Studies with GbpB-deficient mutant strain suggest that GbpB may have an important role in cell-wall construction and be involved in cell separation and cell maintenance (Fujita \textit{et al.}, 2007).

A global response regulator (\textit{vicR}) plays important roles in \textit{S. mutans} \textit{ftf} and \textit{gtf} expression in response to a variety of stimuli. Moreover, dietary carbohydrates influence the expression of these genes. This suggests regulatory circuits for exopolysaccharide gene expression in \textit{S. mutans} (Shemesh \textit{et al.}, 2006).

1.8.4 Biofilm

The formal formation of dental plaque leading to dental caries by \textit{S. mutans} is a multistep mechanism (Figure 1.6). Initial attachment of \textit{S. mutans} is followed by its accumulation and proliferation, leading to the formation of a sessile,
Figure 1.6 Stages leading to colonization and infection of \textit{S.mutans} on tooth surface.

A- Normal oral flora. B- Initial adhesin-mediated attachment of \textit{S. mutans}.
C- Synthesis of extracellular polysaccharide by bacteria resulting in aggregation of \textit{S.mutans}. D- The acid released by aggregated cells leads to demineralization and cavitation of tooth. \textit{S. mutans} and other oral microbes - \(\triangle\), \(\Box\), adhesin - \(\bigcirc\) Sucrose moeity - \(\triangleleft\)
expolymer shrouded community known as biofilm. Biofilms can tolerate numerous adverse conditions such as antimicrobial agents, variation in pH, nutrient and oxygen deprivation. The physiology of the organism in such surface associates communities is different from that of planktonic cells (Davey and O’toole, 2000).

LuxS-mediated quorum sensing has been shown to regulate important physiologic functions and virulence in a variety of bacteria. The role of luxS of Streptococcus mutans in the biofilm formation is important. Reporter gene fusions showed that inactivation of luxS resulted in a down-regulation of fructanase, a demonstrated virulence determinant, by more than 50%. The LuxS-deficient strain (TW26) showed increased sensitivity to acid killing but could still undergo acid adaptation. This study demonstrated that luxS-dependent signaling plays critical roles in modulating key virulence properties of S. mutans (Wen et al., 2004). The expression of more than 200 genes was found by microarray analysis to be altered in cells lacking biofilm regulatory protein BrpA (P < 0.01). The loss of brpA can dramatically influence the transcriptome and significantly affect the regulation of acid and oxidative stress tolerance and biofilm formation in S. mutans, which are key virulence attributes of the organism (Wen et al., 2006).
1.8.5 Acidogenicity and acid tolerance

The mutans Streptococci ferment many different sugars, and they may appear to metabolize sugar to lactic acid more rapidly than other oral bacteria. *S. mutans* can grow at low pH, some growing at less than pH 4. This helps in the caries process, i.e. decalcification of dental enamel, taking place at relatively low pH. Acid production in *S. mutans* is thought to be related to the multitude of enzyme systems catalyzing the reactions of transport and metabolism of sucrose expressed by these organisms (Kuramitsu, 1993). These metabolic reactions render the dental plaque acidic in the presence of a fermentable carbon source, and the acid tolerance of the mutans Streptococci enables them to continue metabolisms even at low pH. The strains of mutans Streptococci are more tolerant than of all other bacteria examined, with the exception of lactobacilli (Loesche, 1986). An inducible property exists in *Streptococcus mutans* which permits adaptation to acidic environments (Hamilton and Buckley, 1991; Birkhed et al., 1993). Bender et al. (1986) found that the property of acid tolerance appears to be connected with the membrane-associated H⁺ (Proton)-translocation ATPase of these organisms. This is attained along with expression of stress response proteins that help bacteria in adapting to acidic conditions (Svenaster et al., 2000). In *S. mutans* a pH change from 7.4-5.5 resulted in induction of an acid-tolerance response over a 2 hour period that increased cell survival at pH 3.0 (Svenaster et al., 1997). The pH change resulted in significant alterations in protein synthesis;
extration followed by one dimensional electrophoresis, revealed the up regulation of 36 proteins, with 25 of these being acid responsive proteins appearing within the first 30 minutes of the pH change (Hamilton and Svenaster, 1998). The synthesis of many of the proteins was transient during the 2 hour adaptation period. The identity of the proteins is not known, but the molecular mass comparison suggested that both acid specific proteins (i.e. components of H+/ATPase) and general stress protein (i.e. heat-shock protein) are present in the extract of the acid-induced cell (Svenaster et al., 2000). This overlap is highly significant for the survival of cells in nutritionally limited environments and transient exposures to carbohydrate rich areas. Prolonged acidification of \textit{S. mutans} cells results in the increased specific activity of membrane F1-ATPase involved in proton efflux during pH homeostasis.

Early studies of \textit{S. mutans} characterized it as a homolactic fermentor (Drucker and Melville, 1968). LDH was shown to be produced constitutively, but its activity was found to be entirely dependent on the presence of fructose 1,6-diphosphate (Brown and Wittenberger, 1972). Under growth conditions where intracellular fructose 1,6-diphosphate concentrations are low, such as with limiting glucose, fermentation end products other than lactate have been observed. Anaerobically the activity of pyruvate formate-lyase accounts for the observed production of formate, acetate, and ethanol (Abbe et al., 1982). In an aerobic environment, pyruvate formate-lyase is inactivated by oxygen, and instead, pyruvate dehydrogenase activity can account for the observed
production of acetate and acetoin (Hillman et al., 1987). It was initially presumed that these alternate pathways for pyruvate dissimilation would enable an LDH deficient mutant of *S. mutans* to thrive under both aerobic and anaerobic cultivation conditions. The finding that LDH deficiency was lethal in *S. mutans* suggests that these pathways are less active, resulting in growth inhibition from NAD-NADH imbalance and/or the accumulation of glycolytic intermediates. It was also demonstrated that LDH lethality in *S. mutans* can be overcome by limiting the supply of glucose.

### 1.8.6 Mutacin Production

Bacteriocins are proteinceous antibacterial substances produced by some bacteria to inhibit or interfere with the growth of other bacteria. These bacteriocins are ribosomally synthesized and usually require extensive post-translational modification for activity (Madigan et al., 1997a & b). The genes involved in the synthesis and modification of bacteriocins are often carried by a plasmid or a transposon. Bacteriocins are frequently named according to the bacterial species producing them; bacteriocin produced by streptococci is called mutacin. Mutacin production in usually not plasmid encoded (Caufield et al., 1990). It is active against other streptococcal species and non-streptococcal gram positive bacteria (Hamada and Ooshima, 1975). The production of the bacteriocin helps in the efficient establishment and hence colonization of this bacterium inside the oral cavity (Rogres, 1976). Hillman et
al. (1987) found that bacterial strains with increased mutacin production could colonize the oral flora of an adult even after a single application.

1.8.7 Production of intracellular polysaccharides

Many plaque bacteria can synthesize intracellular iodine staining polysaccharide (IPS) from high concentration of various sugars. Most *S. mutans* strains produce a storage IPS which may contribute to the pathogenicity of *S. mutans* (Berman et al., 1967). These IPS are similar to those of the oral streptococci and are glucose homopolymers with α-(1-4) and α-(1-6) linkages (van Houte and Jansen, 1970). The synthesis of IPS is linearly proportional to the intracellular carbohydrate concentration (glucose or sucrose) (Freedman and Coykendall, 1975). The metabolism of IPS may foster the development of caries by prolonging the exposure time to organic acids when bacterium is devoid of an external food source. Numerous reports confirm IPS as an important contributor to the cariogenicity of *S. mutans* (Harris and Michalek, 1992).

1.9 Remedies for dental caries

1.9.1 Habits and hygiene

The lifestyle of a person and behavioural factors that manipulate the oral hygiene undoubtedly, influence the susceptibility to dental caries (Selwitz et al., 2007).
Continuous brushing and flossing of teeth removes the bacteria and fermentable substances as well. The continuous flow of saliva reduces the cariogenic flora on the tooth. Saliva also acts as a buffering agent during the continuous acid production in the oral cavity (Kleinberg, 2002). Individuals with dry mouth syndrome are hence more prone to dental caries. This has been observed with people following radiation treatment of head and neck cancer, using narcotics and patients with Sjogren's syndrome (Dreizen and Brown, 1976; Lowenthal, 1967).

Dental caries is correlated with sugar uptake in the diet. Increase in urbanization has led to the replacement of crude sugar by refined sugar that worsened the situation (Peterson, 2003). Numerous studies indicate the linear correlation of sugar consumption with dental caries in population worldwide (Marthaler, 1990; Burt, 1993). However, starchy foods and fresh fruits are reported to be less cariogenic. Foods that involve extensive mastication stimulate production of saliva and hence have low cariogenic potential. Intake of fibrillar and firm fruits like apple and carrots act as natural toothbrush, as they clean the tooth surface during chewing. Substitution of fermentable carbohydrates by xylitol, saccharin and aspartame has been efficiently used for reducing caries (Naylor, 1986). Fluids like juices and milk seems to be less cariogenic as they are lesser retained in oral cavity. Consumption of carbonated drinks however pose a higher risk to dental caries (Sowers et al., 2006).
1.9.2 Fluoride-uptake

Our teeth (enamel and dentin) are comprised of minerals mainly carbonated hydroxyapatite and can be approximately represented by the formula

$$\text{Ca}_{10-x} \text{(Na)}_x \text{(PO}_4\text{)}_{6-y} \text{(CO}_3\text{)}_y \text{(OH)}_{2-a} \text{(F)}_a$$ (Featherstone, 2000)

Dental caries is essentially a disease of demineralization. Considerable data supports the fluoride-uptake as a remedy to it by acting as an active agent in remineralization. The electrostatic interaction between Ca$^{2+}$ and the F$^-$ is greater than the force between Ca$^{2+}$ and OH$^-$ ions, making the fluoridated apatite lattice more crystalline and more stable (Frazier, 1967). As a consequence it becomes less soluble in acid (Brown et al., 1977). The direct topical administration of fluoride on the tooth after its eruption is better than its intake in the diet (Johnston, 1994). It is hence the most important component in toothpastes and mouthwashes. Fluorides may also inhibit bacterial metabolism. Fluorine in the ionic form (F$^-$) cannot cross the cell wall and membrane of bacteria but can be taken up readily as fluoric acid (HF). When pH in the plaque falls, a portion of F$^-$ combines with hydrogen ions to form HF and diffuses into the cell. After the entry into the cell, HF acidifies the cell and dissociates thereby releasing F$^-$ ions. F$^-$ are toxic to cells as they interfere with the enzyme machinery of the cell [Featherstone, 1999]. Although the first line defense, yet no absolute inverse relationship between fluoride-uptake and caries could be observed universally (De Paola et al., 1975).
1.9.3 Antibiotics

Ever since the establishment of dental caries as an infectious disease, antibiotics are constantly in use for treating it. Chlorhexidine digluconate (CHX) had been considered as a gold standard in this field. It has been used with the aim of elimination or suppression of mutans streptococci in the oral cavity. CHX damages outer cell layers but is insufficient to induce lysis or death (El-Moung et al., 1985). Moreover, it crosses cell wall or outer membrane by passive diffusion and attacks bacterial cytoplasm, followed by leakage of cellular constituents. However, high concentration causes coagulation of intracellular constituents. As a result cytoplasm becomes congealed with a consequent reduction in leakage. Thus there is a biphasic effect on membrane permeabilities: In the presence of CHX, high rate of leakage is observed initially but as CHX concentration soars leakage decreases because of the coagulation of cytosol (Mc Donnell and Russell, 1999). The best results in reducing dental plaque have been observed with chlorhexidine gels and mouthwashes (Emilson, 1994). As disparities in its efficacy against varying subjects have been observed, CHX cannot be regarded as single line defense for the cure of caries (Twetman, 2004).

The pioneering work of McClure and Hewitt indicated the usefulness of penicillin in preventing experimentally induced caries (1946). Many other antibiotics with activity against gram-positive bacteria depress the development of dental caries in experimental animals (Fitzgerald, 1972; 1973). \textit{S. mutans} has been
reported to be highly susceptible to penicillin, methicillin, ampicillin, erythromycin, cephalothin and many other antibiotics (Little et al., 1979). More antibiotics are now screened for their effects against oral biofilms (Nguyen, 2005).

1.9.4 Rediscovering traditional medicines: Herbal cure

Various chewing sticks had been in use since old times for the removal of dental plaque. With less prevalence of caries in people using it, their mode of action is being explored. Extracts from the roots and stems of *Salvadora persica* have been used for treatment of oral infections in animals (Sulaiman et al., 1968). Aqueous and ethanolic extracts were able to remove the smear layer from dentin surfaces (Babay and Almas, 1999). It had been found that aqueous extracts of *S. persica* bark, pulp as well as the whole of it were effective against various bacteria including *Streptococcus mutans* (Almas and Al-Bagieh, 1999).

The chewing sticks used in India are usually from *Azadiracta indica* (Neem). Neem extracts show antimicrobial effects against *Streptococcus mutans* and *S. faecalis* (Almas, 1999). Formulation of mucoadhesive dental gel containing Neem leaves extract (25 mg/g) reduced both, the plaque index and bacterial count (Pai et al., 2004). Acacia is also used as an active constituent in toothpastes in India. The components of its bark and gum chiefly, tannins show antimicrobial and astringent effect (Arias et al., 2004). Plant tannins have the ability to reduce the attachment of *S. mutans* by binding to proline rich protein of the salivary pellicle or with the cell-surface lipoteichoic acid (Wolinsky and Sote, 1984).
Lectins are sugar-binding proteins of non-immune origin that agglutinate cells and/or precipitate glycoconjugate molecules (Naeem et al., 2007). Algal lectins had been shown to inhibit the bacterial attachment to the AEP in-vitro (Teixeira et al., 2007). Plant lectin from Talisia esculenta and Labramia bojeri do not inhibit the growth of Streptococcus mutans but were found to inhibit the bacterial adhesion on AEP (Oliveira et al., 2007).

As most of the commonly used antibiotics are effective against planktonic bacteria hence more studies are now aimed to target biofilm of S. mutans as a whole (Rukayadi and Hwang, 2006). Notably glucan-mediated bacterial attachment is an important feature of biofilm formation, plethora of herbs are evaluated for their effect on this property of bacteria (Table 1.2). Andrographis paniculata, Cassia alata, Chinese black tea, guava and Harrisonia perforale showed decrease in adherence to glass surface as well as saliva-coated hydroxyapatite beads (Limsong et al., 2004). Recently specific compounds from guava have been characterized for their anticariogenic potential (Prabu et al., 2006). Oolong tea extracts show remarkable inhibitory effect on the rate of acid production along with retardation in growth of S. mutans. It is attributed to the polyphenols present in the extracts that also reduce the cell surface hydrophobicity of the bacteria (Matsumoto, 1999).
Table 1.2 List of plants that had been used to study antimicrobial and anti-adhesion effects against *S. mutans*

<table>
<thead>
<tr>
<th>Plant source</th>
<th>Part used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Piper betle</em></td>
<td>leaf</td>
<td>Sharma et al., 2008</td>
</tr>
<tr>
<td><em>Curcuma xanthorrhiza</em></td>
<td>rhizome</td>
<td>Rukayavadi and Hwang, 2006</td>
</tr>
<tr>
<td><em>Glycyrrhiza uralensis</em></td>
<td>root</td>
<td>Belt, 2008</td>
</tr>
<tr>
<td><em>Cocus nucifera</em></td>
<td>husk fiber</td>
<td>Alviano et al., 2008</td>
</tr>
<tr>
<td><em>Ziziphus joazeiro</em></td>
<td>inner bark</td>
<td>Alviano et al., 2008</td>
</tr>
<tr>
<td><em>Caesalpinia pyramidalis</em></td>
<td>leaves</td>
<td>Alviano et al., 2008</td>
</tr>
<tr>
<td><em>Aristolochia cymbifera</em></td>
<td>rhizome</td>
<td>Alviano et al., 2008</td>
</tr>
<tr>
<td><em>Acronychia baueri</em></td>
<td>bark</td>
<td>Bodet et al., 2008</td>
</tr>
<tr>
<td><em>Kaempheria pandurata</em></td>
<td>rhizome</td>
<td>Hwang et al., 2004</td>
</tr>
<tr>
<td><em>Mikania laevigata</em></td>
<td>leaves</td>
<td>Yatsuda et al., 2005</td>
</tr>
<tr>
<td><em>Allium sativum</em></td>
<td>bulb</td>
<td>Baleri and Douglas, 2005</td>
</tr>
<tr>
<td><em>Coffea arabica</em></td>
<td>coffee beans</td>
<td>Daglia et al., 2002</td>
</tr>
<tr>
<td><em>Magnolia grandiflora</em></td>
<td>bark</td>
<td>Greenberg et al., 2007</td>
</tr>
<tr>
<td><em>Juglandaceae regia</em></td>
<td>stems</td>
<td>Jagtap and Karkera, 2000</td>
</tr>
<tr>
<td><em>Terminalia chebula</em></td>
<td>fruit</td>
<td>Carounanidy et al., 2007</td>
</tr>
<tr>
<td><em>Myrisitica fragrans</em></td>
<td>fruit</td>
<td>Chung et al., 2006</td>
</tr>
<tr>
<td><em>Nidus vespae</em></td>
<td>honeycomb</td>
<td>Xizo et al., 2007</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>fruit</td>
<td>Thimothe et al., 2007</td>
</tr>
<tr>
<td><em>Vaccinium macrocarpon</em></td>
<td>fruit</td>
<td>Gregoire et al., 2007</td>
</tr>
<tr>
<td><em>Psidium cattleianum</em></td>
<td>leaf</td>
<td>Brighenti et al., 2008</td>
</tr>
<tr>
<td><em>Thymus vulgaris</em></td>
<td>leaf</td>
<td>Hammad et al., 2007</td>
</tr>
<tr>
<td><em>Cyperus rotundus</em></td>
<td>tuber</td>
<td>Yu et al., 2007</td>
</tr>
<tr>
<td><em>Polygonum cuspidatum</em></td>
<td>root</td>
<td>Song et al., 2007</td>
</tr>
<tr>
<td><em>Lippia sidoides</em></td>
<td>leaves</td>
<td>Botelho et al., 2007</td>
</tr>
<tr>
<td><em>Cratoxylum formosum</em></td>
<td>gum</td>
<td>Suddasthira et al., 2006</td>
</tr>
<tr>
<td><em>Sagittaria pygmaea</em></td>
<td>whole herb</td>
<td>Liu et al., 2007</td>
</tr>
<tr>
<td><em>Punica granatum</em></td>
<td>fruit</td>
<td>Vasconcelos et al., 2007</td>
</tr>
<tr>
<td><em>Saussurea lappa</em></td>
<td>root</td>
<td>Yu et al., 2007</td>
</tr>
<tr>
<td><em>Asarum sieboldii</em></td>
<td>whole plant</td>
<td>Yu et al., 2006</td>
</tr>
<tr>
<td><em>Eugenia caryophyllata</em></td>
<td>bud</td>
<td>Rahim and Khan, 2006</td>
</tr>
<tr>
<td><em>Psoralea corylifolia</em></td>
<td>Seeds</td>
<td>Katsura et al., 2001</td>
</tr>
</tbody>
</table>
Many essential oils in combination with CHX had been tested for better efficacy in inhibiting growth as well as biofilm of oral flora. Essential oils from cinnamon, *Leptospermum morrisoni*, manuka and tea tree oil exhibited growth inhibitory effect against cariogenic bacteria. They suppress biofilm formation during planktonic growth as well as growth of preformed biofilms. Chlorhexidine in combination with cinnamon, *L. morrisoni* and Manuka oil resulted in better efficiency of Chlorhexidine in inhibiting the growth of *S. mutans* (Filosche, 2005). Compounds found in propolis, effect the growth and glucosyltransferase activity of *S. mutans* (Koo *et al.*, 2000). Topical application of propolis twice a day or its inclusion in drinking water reduced the incidence of dental caries in rats (Ikeno *et al.*, 1991). Of the various components of propolis studied earlier, tt-farnesol was the most effective antibacterial agent while apigenin was found to be most potent inhibitor of glucosyltransferase (Koo *et al.*, 2002).

### 1.9.5 Probiotic therapies

Enormous strides have been made in comprehending the cure for dental caries by the aid of genetic engineering. The sequencing of complete *S. mutans* genome marks the beginning of an era of fashioned therapies (Ajidic *et al.*, 2002). An ideal drug for caries prevention should aim at maintaining the normal homeostasis of the oral cavity and reducing the virulence of *S. mutans* (Figure 1.7).
Figure 1.7  An ideal approach for the control of dental caries. *S. mutans* – 😧, *S. mutans* in oral cavity aggregated on the surface of teeth and ferment sugars to produce acid leading to pathogenicity.

Sucrose moiety- 🍴, Sucrose moieties facilitate aggregation of bacterial cells on the tooth surface. Drug candidate-💊, A potent drug combating pathogens to reduce the cariogenicity. The drug should specifically interact with *S. mutans* and inhibit both adhesin and polysaccharide mediated attachment. It should act as a buffering agent and control the acid production by oral microbes (Islam *et al.*, 2007).
1.9.5.1 Vaccines

Immune defense in dental caries is mediated mainly by secretory IgA present in saliva and generated by mucosal immune system. The mode of action of these antibodies is inhibition of adherence and possibly metabolic activities of *S. mutans* (Russell *et al.*, 1999). In view of vaccine development against caries, the virulence factors, specifically the adherence motivating factors are recognized as key antigens. The research focus is mainly on the incorporation of these antigens into mucosal immune systems and delivery with or without adjuvants to mucosal IgA inductive sites (Smith, 2003). Novel strategies using Ag I/II, Gtfs and glucan binding proteins are designed for pursuing vaccine goal as these are main proteins that mediate the attachment of bacteria. Induction of salivary IgA and circulating IgG antibodies have been observed by oral or intranasal immunization with these antigens in many animal models (Jespersgaard, 1999). Similar antigen preparations in human trials have shown successful induction of salivary S-IgA (Childers *et al.*, 2002; 1997). Tailored vaccines possesing immunogenic sites of the virulence determinant traits have showed significant caries-preventive results (Katz *et al.*, 1993). However, chimeric proteins using both Agl/II and Gtfs, has shown most encouraging response (Zhang *et al.*, 2002). DNA vaccines using these proteins are also developed and assayed for their anticariogenic spectrum (Fan *et al.*, 2002). Better efficiency of fusion DNA vaccine coding for both Agl/II and glucosyltransferases in rats has also been reported (Guo *et al.*, 2004).
Numerous approaches for passive immunization are employed to avoid complications that might arise from active immunization (Ferretti et al., 1980). Immunity through antibodies for glucosyltransferases available through cow's milk (Loirmaranta et al., 1998), hen's eggs (Hamada et al., 1991; Hatta et al., 1997) and plantibodies (Ma et al., 1998; 1995) has been reported. Efficient delivery systems are being developed for a continuous and controlled release of vaccines. Both animated (Huang et al., 2001) and non-animated vehicles as liposomes (Michalek et al., 1992) and microparticles (Smith et al., 2000) have been assessed in animal models.

1.9.5.2 Replacement therapy

In the post-genomic era, recombinant DNA technology is being used for finding an answer to dental caries. The recent word in vogue is replacement therapy. Genetic engineering is being used to tailor the effector strain for replacement therapy of dental caries, which acts as a vaccine and should not be pathogenic. Moreover, it colonizes the niche, thereby preventing colonization and outgrowth of wild type strains. Using this approach, a harmless strain is permanently implanted in the host’s oral flora. Once established, the effector strain competes with the wild-type strain and prevents its outgrowth (Hillman, 2002).

An effector strain with all these properties, BCS3-L1 was constructed from a clinical isolate JH1140 with high mutacin activity and genetic stability. The mutagenesis approach has also been used successfully by constructing a
lactate dehydrogenase mutant, which has been reported to reduce cariogenic potential (Johnson et al., 1980). The resultant strain thus produced, was deficient in lactic acid production and had elevated mutacin levels. Colonization studies with the mutant strain BCS3-L1 shows no pathogenic hazards as histopathological examination showed no detectable carious lesions (Hillman et al., 2000).

1.10 Identification of unknown proteins in biofilm formation

*S. mutans* is now regarded as the principal microbiological aetiological agent of dental caries and a target of novel preventive strategies. Beyond initial adherence, it appears that a variety of genes are required for the proper maturation of biofilms formed by *S. mutans* and other oral streptococci. By the use of specific- and random-mutagenesis strategies, many different types of genes that are required for these organisms to transition from adherent microcolonies to complex, three-dimensional biofilms have been identified. These include those for intercellular communication systems and environmental sensing systems, components of the general stress response pathway involved in protein repair and turnover, global regulators of carbohydrate metabolism, and adhesion-promoting genes (Yoshida and Kuramitsu, 2002).

Interestingly, many of the genes identified to affect biofilm formation affect the expression of a large panel of genes, many of which are
either unidentified or have no known function (Shemesh, 2007). Characterization of these genes of unknown function is generally recognized as an essential step toward fully understanding the biology of the host organisms and for establishing potential targets for novel and broadly effective therapeutics against *S. mutans* and other pathogens. There are a large number of hypothetical proteins of as-yet-unknown function present in the annotated genome of *S. mutans* (http://www.stdgen.lanl.gov/oragen). Among these proteins, there could be other unknown surface proteins that play an important role in adhesion and maturation of biofilms. Techniques like 2D protein analysis and microarray are now being used to screen out the expression of all genes involved in the formation of a biofilm (Rathsam *et al.*, 2005; Ajidic and Pham, 2007). An integrated approach to study protein-function relationship was employed to study the variation in *S. mutans* genome (Waterhouse *et al.*, 2007).

The ability to induce mutations has been a major driving force in studying genetics for the past 75 years (Muller and Mott-Smith, 1930). Among the mutagens that have been used to induce mutations, chemical mutagens administered in various ways have become especially popular in studying the forward genetics of bacteria. Alkylating agents, such as ethyl methanesulfonate (EMS), are particularly effective, because they form adducts with nucleotides, causing them to mispair with their complementary bases, thus introducing base changes after replication (Ashburner, 1990). EMS is a potent and efficient
mutagen for generating point mutations. The common effect is to cause G/C-A/T transition, although it does produce some small deletions and other chromosomal rearrangements (Anderson, 1995). Therefore, chemical mutagenesis has become the method of choice for genetic studies, remaining popular even with the advent of sophisticated transgenic technologies that allow for tagging or precise targeting of mutational lesions.

1.1 Objectives

In view of the present background we initiated our study with the following objectives:

a. To evaluate traditionally used Indian herbs for their use in dental caries with special reference to their effect on the growth and biofilm forming ability of S. mutans. The study was also aimed to identify and characterize the active compound from crude herbal extracts.

b. To study the effect of purified lectins on the bacterial growth, adherence to AEP and biofilm formation of S. mutans.

c. To isolate and characterize mutants of S. mutans defective in biofilm formation which may contribute to the understanding of regulatory genes in oral biofilms.