CHAPTER II

LITERATURE REVIEW

Historical Background

Evidence of dental caries can be traced back to the remains of one million–year-old Rhodesian man, however, relatively little caries existed even as late as 1200 A.D. Many hypotheses and theories on the etiology of dental caries were proposed, from the idea of tooth worms that bore holes in the teeth, to the year 1890 when W.D. Miller proposed that initial decalcification of the tooth was caused by bacteria, which through its fermentation of carbohydrates, produced acids, including lactic acids that are capable of destroying the enamel [18]. Substantial clinical and laboratory evidence has supported Miller's hypothesis.

Streptococcus mutans was first described by a microbiologist, J. Kilian Clarke in 1924. Clarke studied the microbiology of dental caries disease. He found small, coccoid chained bacteria in deep dentin caries lesions. He suggested that they resembled mutant streptococci and called them *Streptococcus mutans* [19]. Since that time there have been extensive studies confirming the association of *S. mutans* with carious lesions [20,21]. More evidence in the literature using animal models has proven that caries is a communicable, transmissible, infectious bacterial infection [22,23].

Taxonomy

The genus *Streptococcus* includes the lactic, pyogenic, anaerobic and oral groups of streptococci. These Gram-positive, facultative anaerobic bacteria are spherical or ovoid, 0.5-2.0 μm in diameter, occurring in pairs or chains when grown in liquid media. They are sometimes elongated in the axis of the chain to a lanceolate shape. Streptococci require nutritionally rich media for growth and sometimes 5% CO_2. Growth is usually restricted to 25-45°C (optimum temperature is 37°C). The metabolism is fermentative, producing mainly lactate but no gas. The streptococci are catalase-negative, and they commonly attack red blood cells, with either greenish discoloration

(α-hemolysis) or complete clearing (β-hemolysis). Streptococci mainly inhabit the oral cavity and respiratory tract [24]. Streptococci constitute a major population in the oral cavity, with several different species colonizing the various ecological niches of the mouth.

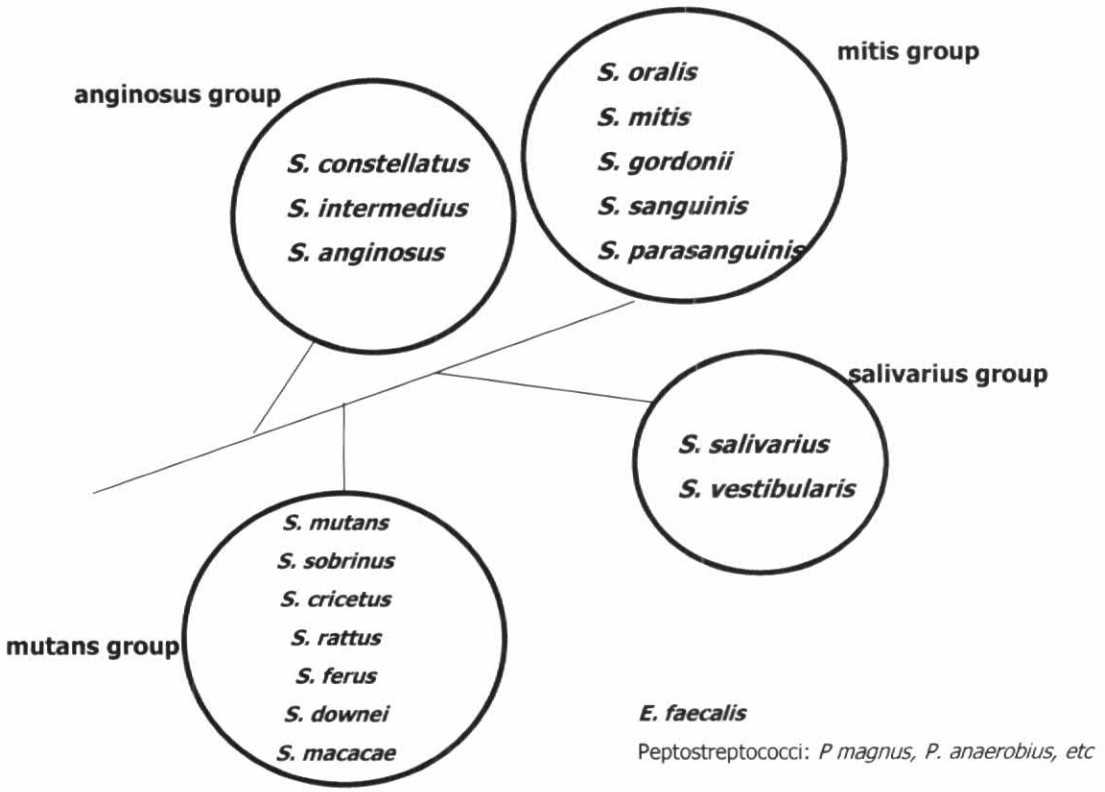

Figure 2.1 Phylogenetic relationship among oral streptococci groups [25].

The oral group is often referred to as viridans streptococci, referring to the greenish color that results from partial hemolysis on blood agar. However, some species classified as viridans streptococci are not detected in the oral cavity, and not all of oral streptococci are α-hemolytic. The current classification of the oral streptococci places the bacteria into four species groups; the anginosus, mitis, salivarius and mutans groups (Fig. 2.1). The classification is based on chemotaxonomic and genotypic data, especially DNA-DNA base pairing and 16S rRNA gene sequence analysis [26]. Historically, the bacteria previously referred to as *S. mutans* are actually seven distinct species, which are collectively called the mutans streptococci. Mutans

streptococci may be rod shaped on primary isolation from oral cavity. On blood agar, colonies are white or gray, circular or irregular, 0.5-1.0 mm in diameter, sometimes very hard and coherent and tending to adhere to the surface of the agar. They usually are α- or γ-hemolytic, but occasional β-hemolytic strains are found. On sucrose-containing agar, most strains produce rough, heaped colonies irregular in margin and adherent, about 1 mm in diameter, often with beads of frosted glass-like droplets or puddles of liquid on or around the colonies. Some may form smooth or mucoid colonies. Most strains will grow to some extent in air but growth is enhanced under anaerobic conditions [27]. The mutans group includes *S. mutans, S. sobrinus, S. cricetus, S. rattus, S. downeii* and *S. macacae.* Although the phylogenetic position of *S. ferus* has yet to be determined by 16S rRNA sequencing, other data indicate that *S. ferus* also belongs to the mutans group [26]. The differentiation within the mutans streptococci is based on differences in biochemical reactions (Table 2.1), on physicochemical surface properties [28], and other distinctions detected by molecular techniques [26]. The mutans streptococci represent eight serotypes [26,29], classified by the difference in the polysaccharides located in or on the cell wall (Fig 2.2). These are composed primarily of a combination of sugars, such as glucose, galactose and rhamnose (Table 2.2). The most recent taxonomy of *S. mutans* therefore is confined to serotypes c, e, f only, whilst *S. mutans* serotype c is the most common oral isolate. [30-32]. *S. mutans* cells are about 0.5-0.75 μm in diameter. *S. mutans* occurs in pairs or in short- or medium-length chains. Under acid conditions in broth and on some solid media, these cocci may form short rods 1.5-3.0 μm in length. *S. sobrinus*, comprising serotypes d and g are about 0.5 μm in diameter. *S. sobrinus* occurs in pairs and in chains. The word sobrinus means male cousin on mother's side and refers to the "distant relationship" between this species and *S. mutans. S. rattus, S. ferus* and *S. cricetus* are about 0.5 μm in diameter, occurring in pairs or chains [27].

Table 2.1 Differential characteristics of the mutans streptococci group [a]

	Species						
	S. mutans	*S.rattus*	*S. sobrinus*	*S. cricetus*	*S. downei*	*S. macacae*	*S. ferus*
Characteristic							
Growth in air	Δ	Δ	Δ	Δ	+	w	Δ
Growth at							
10°C	-	-	-	-	ND	ND	-
45°C	Δ	Δ	Δ	Δ	-	-	-
Hemolysis	γ	ND	γ or α	γ	ND	α	ND
Fermentation of							
mannitol	+	+	+	+	+	+	+
sorbitol	+	+	Δ	+	-	+	+
raffinose	+	+	Δ	+	-	+	-
inulin	+	+	Δ	Δ	+	-	+
melibiose	Δ	+	-	ND	ND	ND	ND
salicin	+	+	-	+	+	ND	+
trehalose	+	+	Δ	+	+	+	ND
Hydrolysis of							
arginine	-	+	-	-	-	-	-
esculin	+	+	Δ	Δ	-	+	+

	S. mutans	*S.rattus*	*S. sobrinus*	*S. cricetus*	*S. downei*	*S. macacae*	*S. ferus*
Production of							
Hydrogen peroxide	-	-	+	-	-	-	-
α-galactosidase	Δ[b]	ND	-	ND	ND	ND	ND
β-glucosidase	+	ND	-	ND	ND	ND	ND
Voges-Proskauer test	+	+	+	+	+	ND	Δ
Bacitracin resistance	+	+	+	-	-	-	-

Δ, 11-89% of strains are positive

+, 90% or more of strains are positive

-, 90% or more of strains are negative

w, weak growth

ND, not determined

α, alpha-hemolysis, greenish discoloration on blood agar

γ, gamma-hemolysis, no clearing

[a], Information from Beighton et al., 1991 [33]; Maiden et al., 1992 [29]; Whiley and Beighton, 1998 [26].

[b], According to Beighton et al, 1991 [33]. *S mutans* strains that do not ferment melibiose neither produce α-galactosidase

Figure 2.2 Diagram showed the cell wall components of *S. mutans*.

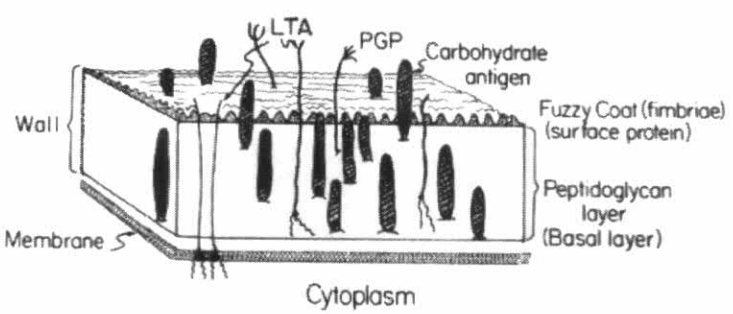

Table 2.2 Classification of the mutans streptococci group [a]

Species	Serotype[‡]	Major cell wall serotype polysaccharide constituents[b]	Primary host
S. mutans	c, e, f	Glc, Rha	human
S. rattus	b	Gal, Rha	human/ rodent
S. sobrinus	d, g	Glc, Gal, Rha	human
S. cricetus	a	Glc, Gal, Rha	human
S. downei	h	ND	monkey
S. macacae	c	ND	monkey
S. ferus	c	Glc, Rha[c]	monkey

[a] Information from Maiden et al., 1992 [29], Whiley and Beighton, 1998 [26].

[‡] Brathall, 1970 [30], Coykendall et al, 1976 [32].

[b] Abbreviations: Rha, rhamnose; Glc, glucose; Gal, galactose; ND, not determined [34].

[c] *S. ferus* included in the mutans group by DNA-DNA hybridization, but not by multilocus enzyme electrophoresis

For primary isolation of mutans streptococci, the most frequently used medium is mitis salivarius bacitracin (MSB) agar [35], which is composed of mitis salivarius agar with sucrose, bacitracin and potassium tellurite. MSB agar is selective for *S. mutans, S. sobrinus* and *S. rattus*. On MS agar, *S. mutans* colonies are small, raised, irregularly margined and adherent, while *S. sobrinus* colonies are surrounded by a zooglea with a gelatinous consistency [21]. TYCSB agar contains TYC agar (trypticase, yeast extract and cystine) with sucrose and bacitracin [36]. GSTB agar contains a basal GS agar (trypticase and yeast extract) with glucose, sucrose, bacitracin and potassium tellurite [37]. TSY20B agar contains trypticase soy agar, yeast extract, sucrose and bacitracin [38]. MSKB medium is composed of mitis salivarius agar, sorbitol, kanamycin sulfate,

bacitracin and potassium tellurite [39]. Differences in culture result when different media are used [38,40-42]. Various studies have claimed higher recovery rate of mutans streptococci among different type of media, yet, MSB agar has been found to give the most consistent recovery [38,40,42].

Epidemiology

Epidemiological studies show that in human populations world-wide virtually all adults carry mutans streptococci [20,43]. Of the serotypes, c, e, f and d / g have been detected in humans with high frequency. Some reports of finding serotypes *a (S. cricetus)* and *b (S. rattus)* have also been published [20,28]. Serotype *c* is predominant in plaque and saliva samples from humans [20]. In samples from Finnish children, 75-90% of *S. mutans* isolates are serotype *c*, 10-20% serotype *e*, and only a few percent represent serotype *f* [44,45]. *S. sobrinus* is not as prevalent as *S. mutans* and is usually detected together with *S. mutans* [20]. The prevalence of *S. sobrinus* has been reported to range between a very low frequency and 30% in different populations. In selected subject groups, a higher frequency of *S. sobrinus* has been reported [46]. Only a few studies report finding *S. sobrinus* as the sole mutans species, and subjects who harbor both *S. mutans* and *S. sobrinus* tend to have higher salivary mutans streptococcal counts than subjects harboring *S. mutans* alone [47].

Cariogenesis

Current concepts of the etiology of caries have been derived from laboratory animal models, clinical studies, and epidemiological studies in humans. The process includes a breakdown of enamel, and a subsequent breakdown of the underlying dentin, which may eventually expose pulp and compromise the tooth vitality. The disease is multifactorial in that multiple factors are required for caries to occur [48,49]. The original still-prevailing theory explaining the disease process implicates carbohydrates, oral microorganisms, and acids that demineralize the tooth surface as the main factors in the caries process. This acidogenic theory states that dental decay

is a chemico-parasitic process consisting of two stages, the decalcification of enamel, which results in the decalcification of dentin, followed by dissolution of the softened residue. The acid, which affects this primary decalcification, is derived from the fermentation of starches and sugar lodged in the retaining centers of the teeth. Today, *S. mutans* is considered to be the main etiological species in caries disease as they posses the properties that fulfill the requirements damaging the tooth structure.

Virulence of *Streptococcus mutans*

Virulence consists of bacterial properties required in the interaction between host and parasite. As regards *S. mutans*, properties that affect its ability to cause dental caries are factors that promote its colonization and survival in the oral biofilm, the dental plaque, which covers the tooth surfaces. Recognized virulence factors also include certain cell surface proteins, acid production, acid tolerance, and production of glucosyltransferases, bacteriocin and intracellular polysaccharides [17]. In addition to the recognized virulence factors, its proteolytic activity could contribute to further damage of the organic matrix of tooth structure [20,50-52]. The mechanisms by which these organisms initially colonize tooth surfaces are not fully understood; however, adherence to the salivary pellicle or to existing plaque is considered important (9, 13, 25). A number of salivary components, such as mucins, proline-rich proteins, histatins, lysozyme, fibronectin, and secretory immunoglobulin A (sIgA), have been found to interact with *S. mutans* [53-57]. Salivary agglutinins can also bind to hydroxyapatite, the main component in tooth enamel and mediate adherence of *S. mutans* to this solid support [58].

Exoenzymes and glucan-binding proteins

S. mutans possesses glucosyltransferases (GTFs) and fructosyltransferases (FTFs) that synthesize water-soluble and water-insoluble glucan and fructan polymers from sucrose, respectively [20]. *S. mutans* produces at least one FTF and three GTFs [59,60]. GTF-I and GTF-SI enzymes, products of the *gtfB* and *gtfC* genes, primarily

catalyse the synthesis of water-insoluble glucans, whereas GTF-S, the product of *gtfD*, mainly catalyses the synthesis of water-soluble glucans. The three *gtf* genes from *S. mutans* have been thoroughly assessed by comparative sequence analysis, revealing that differences of *gtfB* and *gtfD* among strains are limited, but *gtfC* exhibits significant variability [61] among strains. The nucleotide sequences of *gtf* genes, however, have a similar basic pattern. The proteins are approximately 1500 amino acids long [59] with two common functional domains. The amino-terminal catalytic domain, is responsible for the cleavage of sucrose, and the carboxyl terminal portion, the glucan binding domain, is responsible for glucan binding [62].

These enzymes also function as anchors for the glucans [63-66] in concert with glucan-binding proteins (GbpA, GbpB, GbpC and GbpD) that allows the tenacious adhesion and accumulation on the tooth surface [20,67-71]. This process is believed to contribute in major ways to adherence and to the formation of biofilm [65,72]. The 58 kDa GbpA, is a secreted protein of *S. mutans* that may contribute to optimal aggregation and plaque biofilm architecture [70,73]. The loss of GbpA changes the architecture of sucrose dependent biofilm deposited on hydroxyapatite coated dishes *in vitro* [73]. The inactivation of the *gbpA* gene in *S. mutans* resulted in the recovery, from infected rats, of mutants that had undergone a recombination of *gtfB* and *gtfC* genes, and resulted in increased virulence in the gnotobiotic rat model [74,75]. Another study had found that a *S. mutans* serotype c strain (MT 8148) defective in *gbpA* was associated with a reduction in cariogenicity [76]. Therefore, GbpA appears to be important but its precise contribution may have to be teased from the peculiarities of the biological systems used to investigate it. *S. mutans* GbpC, a 584 amino acid protein is a cell-wall anchored and known to be involved in dextran-dependent aggregation under defined stressful conditions [68,77]. The most recently discovered GbpD, a 75 kDa protein that functions as a lipase and shares homology in its glucan-binding domain with those in GbpA and GTFs. Loss of this protein results in an extremely fragile biofilm in sucrose containing broth cultures suggesting a role in cell adherence [71]. In addition to the microbial properties, host factors may affect adherence. Salivary components, in particular high molecular weight mucin, agglutinin, and proline-rich

proteins can function as receptors in oral pellicles for microbial adhesion to host surfaces [55,56,78,79].

Surface proteins

S. mutans express a predominant surface protein called SpaP (AgI/II, B, P1, IF, SR or PAc), a multifunctional protein is a major surface antigen of *S. mutans* that has been shown to be important in saliva-mediated aggregation and adherence [56]. It is very well conserved among several species of oral streptococci [80,81]. It is known to recognize a wide range of ligands. The A-region in the polypeptide chain is able to bind to protein motifs on salivary agglutinin [82-85], and on receptors of human epithelial cells [86]. The central domain exhibits the largest degree of variability among SpaP sequences and has been called the variable region (V-region). It has been shown that the V-region of various oral streptococci binds fucosylated and sialylated carbohydrates on monocytic receptors and this recognition promotes the release of TNF-α from stimulated monocytes [87,88]. The C-terminal region of SpaP displays adhesion epitopes involved in adherence of *S. mutans* to saliva-coated microtitre wells [89]. Identification of WapA antigen (Antigen A, Ag III) in *S. mutans* [90] suggested its involvement in specific attachment to tooth surface. WapA, a 29 kDa protein is resistant to most proteases [91]. Whether WapA is involved only in initial attachment or also in sucrose-dependent adhesion is still in dispute [54,92-94]. Inactivation of *wapA* in *S. mutans* was reported to result in a significant decrease in sucrose-dependent adherence to surfaces [93], whereas another study demonstrated that inactivation of *wapA* had an indirect effect on other surface components in *S. mutans* [95]. Whether or not its function depends upon the presence of sucrose , both WapA and SpaP have been considered vaccine candidates for dental caries [96].

Acidogenicity and acid tolerance

S. mutans ferments many different sugars. It appears to metabolize sucrose to lactic acid rapidly. This is thought to be related to the multitude of enzyme systems catalysing the reactions of transport and metabolism of sucrose [17]. These metabolic reactions render the dental plaque acidic in the presence of a fermentable carbon source. At a certain critical pH of 5.5 or less, with increasing concentration of hydrogen ions in the plaque, more phosphate ion in the form of HPO_4^{2-} and $H_2PO_4^-$ will leave the solid apatite phase. The concentration of calcium and phosphate influences the apatite dissolution rate by the law of mass action. Calcium is the cation associated with phosphates in the apatite crystal structure [97].

$$\text{Apatite} - PO_4 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} HPO_4^{2-} \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} H_2PO_4^-$$

Furthermore, the acid tolerance of *S. mutans* enables it to continue metabolizing even at low pH. It has been demonstrated that *S. mutans* is more acid tolerant than all other bacteria examined, with the exception of lactobacilli [20]. An inducible property permits them to adapt to acidic environments [98,99]. This property of acid tolerance appears to be connected with the membrane-associated H^+ (proton)-translocating ATPase of these organisms [100].

Production of intracellular polysaccharides

Most strains of *S. mutans* produce intracellular polysaccharides (IPS) from sugars, which may contribute to their virulence [17]. Because of this intracellular polysaccharide storage, these cariogenic bacteria have the ability to continue fermentation and acid production in the absence of exogenous food supplies [20].

Trace Metal and Dental Caries

Trace metals have been studied for their roles in cariogenesis [1]. While some elements such as aluminum, selenium and strontium have been associated with a low incidence of dental caries [101,102], high concentrations of copper, manganese and cadmium have been linked to a higher prevalence of dental caries [4,103]. Furthermore, studies of trace metals in drinking water and tooth enamel have suggested a cariogenic potential for manganese [2,3]. Though availability of trace elements rarely limits microbial growth since they are required in such minute amounts, there may be certain conditions under which the concentration of a trace elements diminishes or enhances virulence [104]. An animal model study revealed that manganese added to drinking water resulted in a significant increase in caries levels [4]. Recent research has shown that manganese is required for growth and survival of many living microorganisms [5].

Cellular Function of Manganese

The ionic radius of Mn^{2+} (0·80 A°) in aqueous solutions lies between that of Mg^{2+} (0·65 A°) and Ca^{2+} (0·99 A°), and is close to that of Fe^{2+} (0·76 A°) and several other transition metal ions. It is therefore not surprising that Mn^{2+} and other cations may be interchangeable in the metal-binding sites of many proteins. Most commonly, Mn^{2+} and Mg^{2+} are interchangeable on account of the similarities between chelate structures of these ions. However, manganese is essential for certain metabolic pathways. Recent studies have demonstrated how this trace metal is regulated in living cells in order to respond to cellular demand. Manganese metalloenzymes have many diverse functions within bacterial cells [105,106], for example, oxygenic photosynthesis in cyanobacteria requires a tetra-Mn cluster present in the reaction centre complex of photosystem II [106], and a number of enzymes, including manganese superoxide dismutase, mangani-catalase and arginase, specifically require Mn^{2+} for their activities [105]. In addition, glycolysis cannot proceed fully without 3-phosphoglycerate mutase (PGM) which, in several Gram-positive endospore-forming bacteria, is active only when associated with Mn^{2+} [107]. The pH-dependent dissociation of Mn^{2+} from PGM

represents a novel signaling mechanism for rapid enzyme inactivation [108]. It has been suggested that similar mechanisms may be responsible for the regulation of other strictly Mn^{2+}-dependent enzymes, such as arginase [108,109]. In addition, it appears that Mn has an important role in bacterial signal transduction. The recently identified *E. coli* proteins PrpA and PrpB belong to a family of Mn^{2+}- containing serine/threonine protein phosphatases that are widely present in eukaryotes where they modulate complex signaling pathways [110]. PrpA and PrpB are linked to the activity of a two-component sensor, CpxAB, and regulation of the periplasmic stress response protease HtrA/DegP [111]. Manganese is also involved in signaling during *Bacillus* sporulation. The biological importance of Mn is not restricted to enzyme-mediated catalysis. For example, Mn^{2+} can detoxify a variety of reactive oxygen species (ROS), protecting cells that lack enzymic defenses. Additionally, non-enzymic Mn^{2+} is crucial for the proper function of a variety of bacterial products, including secreted antibiotics [8], and contributes to the stabilization of bacterial cell walls [11].

Requirements for manganese occur in both Gram-positive and Gram-negative bacteria for growth and metabolic activities [6-10], but too much manganese can also be detrimental [112,113]. Defects in the uptake of manganese has been shown to reduce the virulence of various species of bacteria [6,7]. Manganese was suggested to be required for growth of mutans streptococci [15,16]. Previous studies demonstrated that *S. cricetus* AHT (serotype a) [114,115], *S. mutans* BHT (currently *S. rattus* BHT, serotype b) [16], and *S. sobrinus* OMZ176 (serotype d) [115] exhibited either an absolute requirement for manganese in order to express the GBL or to regulate growth and metabolic activities [9,10].

Many studies in prokaryotes have shown that manganese homeostasis is tightly controlled by multiple transport systems implying an important role for manganese [13,116]. The other metalloenzyme, ABC-type Mn permease was identified by its effect on adhesion of streptococci [117]. A few studies have shown that manganese can interact with transcriptional regulators in many bacteria [12-14]. Two different DNA-binding transcriptional regulators in *Salmonella enterica typhimurium* are known to be co-factored by manganese, Fur and MntR [118-124]. Fur was also found to regulate

some genes in response to acid pH [125]. In *Bacillus subtilis*, MntR, the manganese–dependent repressor, was demonstrated to repress *mntH*, a protein in the natural resistance associated macrophage protein (NRAMP) family. In contrast, the MntR acts as a transcriptional activator of the *mntABCD* operon, encoding an ABC manganese transporter under low manganese conditions [14,126-128]. Upregulation of an ABC-type Mn transporter under Mn^{2+} limiting conditions was also observed in the dental plaque colonizer *Streptococcus gordonii* [13,116]. The *gpmA* gene (glycolytic enzyme, phosphoglycerate mutase) in *Treponema pallidum* is regulated by a Mn-binding transcription factor, TroR [12]. Recently, the *S. mutans* SloR adhesin in the LraI family was proposed as a manganese importer due to homology with TroR and DtxR [116]. Furthermore, manganese has recently been shown to play a role in regulating morphological changes in *Mycobacterium tuberculosis* during cell division through a eukaryotic-type serine/threonine kinase [129,130]. Clearly, manganese can make several contributions to bacterial metabolism beyond an association with superoxide dismutase.

Hypotheses

1. Manganese enhances the growth of *S. mutans* serotype c.
2. Manganese influences the expression of *S. mutans* virulence factors.
3. Manganese promotes cariogenic plaque formation.

Objectives of the Study

1. To determine the effect of manganese on growth in *S. mutans* serotype c.
2. To study the effect of manganese on virulence genes of *S. mutans*.
3. To determine the effect of manganese on the biofilm architecture in the presence and absence of sucrose.

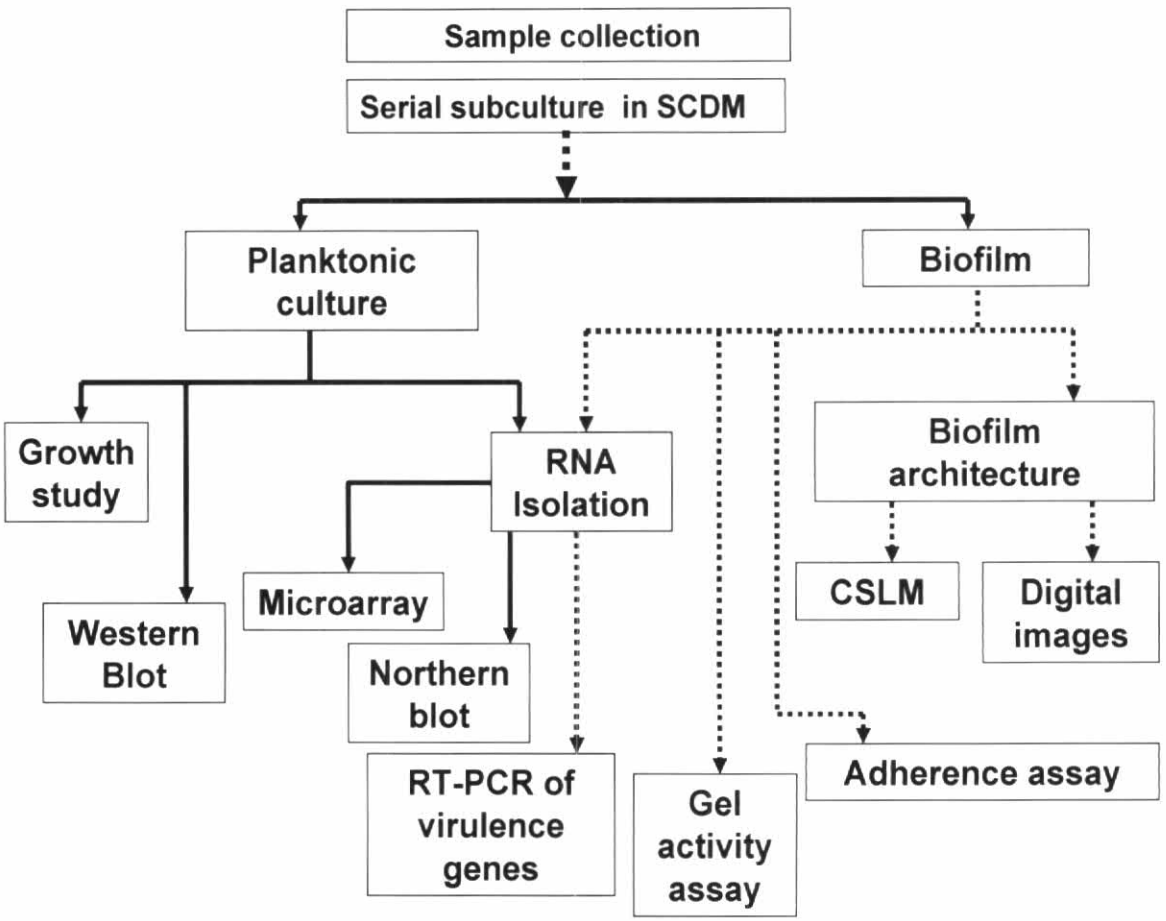

Figure 2.3 **Schematic diagram of research work**