Full Length Research Paper

Biofilm formation potential of oral streptococci in related to some carbohydrate substrates

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In this study, the effect of sugars type (glucose, sucrose, fructose and the mixture of glucose and fructose) and best substrate concentration are investigated on biofilm accumulation. Then, the role of this substrate examined on gtfB expression level by real time RT PCR. A total of 40 Streptococci strains isolated from dental caries and plaque of patients. The ability of them to form biofilm measured in the presence of some sugars on micro titer plates. The mean of resulted optical density of formed biofilm in the presence of sucrose was higher than other carbohydrates significantly ($p < 0.05$). In the next step biofilm formation of best *Streptococcus mutans* isolates monitored at time intervals and revealed that the number of attached bacteria increased with the increase of sucrose concentration. The results corresponded to a non linear increase of attached bacteria. The line of best fit for the calibration of the attached bacteria versus sucrose concentration required a 3rd -order polynomial ($R^2=1$). The comparison between the mRNA level of gtfB in planktonic, biofilm and unattached cells of *S. mutans* by real time RT PCR showed that, the level of gtfB gene expression in the biofilm condition was significantly higher than the planktonic condition.

Key words: Biofilm formation, carbohydrate, gene expression, oral streptococci.

INTRODUCTION

Dental plaque as a microbial biofilm is defined as the diverse community of micro-organisms found on the tooth surfaces, embedded in an extra cellular matrix of host and microbial polymers. The streptococci are the pioneer strains in plaque formation and *mutans* streptococci are the main etiological agent of dental plaque and caries.

These oral diseases affect the majority of the world's population (Marsh, 2003; Donlan and Costerton, 2002). In general, biofilm formation is a step-wise process, which begins by adhesion of planktonic cells to the surfaces. The fermentable dietary carbohydrates are the key environmental factors in concerned with biofilm initiation and development. Evidences show that expression of required genes, such as gtfB, gtfC and ftf,

is well- regulated after initial adhesion and results in forming dental plaque, caries and other periodontal disease (Zero, 2004).

Although, fluoride and other preventive efforts have led to a dramatic decline in dental caries, the ability to control the actual infection has been limited and dental disease remains a "silent epidemic" in the world. So, as a primary bacterial agent of dental caries, the mechanisms by which Streptococcus mutans adheres to tooth surfaces are important potential targets for anti-cariogenic intervention (Caglar et al., 2005).

The role of sugars in caries etiology is quite understood. In the oral cavity, Streptococci depend on sugars as an energy source; the main energy supply is carbohydrates. The cariogenicity of sugar-containing foods can be modified by many factors including the amount and type of carbohydrates, protective components (proteins, fats, calcium, phosphate, fluoride) and physical and chemical properties (liquid vs. solid,

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retentiveness, solubility, pH, buffering capacity). The accepted sugars are important etiologic factor in dental caries that has been with us since the appearance of civilized man, but the discussion around this subject is a more recent phenomenon (Zero, 2004). The fact that sugars are readily metabolized by oral bacteria, leading to the production of organic acids in sufficient concentration to lower the pH of dental plaque and extra cellular polysaccharides such as glucan and fructan was shown repeatedly in clinical studies (Zero, 2004; Touger et al., 2003).

So, the effect of sugars type (glucose, sucrose, fructose and the mixture of glucose and fructose) and best substrate concentration in relationship to biofilm accumulation is investigated and then, the role of this substrate on gtfB expression level by real time RT PCR was examined, in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 40 streptococci strains were isolated from dental caries (caries lesion class I) or plaque (normal teeth, without any periodontal disease) of patients referring to diagnostic center of dental school of Islamic Azad University- Khorasgan Branch (Isfahan- Iran). These patients were of both sexes with the mean age of 22 years. The samples were prepared by means of sterile dentistry curette. The standard strain was *S. mutans* ATCC 35668. All strains were cultured on the media such as blood agar and mitis salivarius agar (Merck co) in a CO_2 enriched atmosphere, and the biochemical tests were done for their identification (Noel and Holt, 1998). All the experiments were carried out in 3 replicates.

Biofilm production assay by microtiter plate test

Quantification of biofilm formation was done using the usual titer plate method. To assay biofilm formation potential of the *S. mutans* isolates, an overnight culture of each was grown in Brain Heart Infusion broth (BHI, Merck, Germany) for 18 - 20 h at 35°C. One ml of each overnight culture was transferred to 10 ml of sterile BHI with or without following carbohydrates, 1% glucose, 1% fructose, 1% Sucrose and a mixture of 0.5% glucose and 0.5% fructose, separately for production of biofilm. The suspensions were adjusted with the same BHI medium to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08 - 0.1 at 625 nm) in a spectrophotometer (Schimadzu, model UV-120-01, Japan), corresponding to approximately 108 CFU/ml. Then, from each culture, 250 l volumes were transferred into eight wells of a micro titer plate (Dynatech, Immulon) . Blank wells contained broth, only. Plates were made in duplicate, covered, and incubated for 24 h at 35°C. At the end of 24 h, the planktonic suspension and nutrient solutions were aspirated and each well was washed three times with 300 l of sterile physiological saline. The plates were strongly shaken in order to remove all no adherent bacteria.

The remaining attached bacteria were fixed with 250 l of 96% ethanol per well and, after 15 min, plates were made empty and left to dry. Each well was then stained for 5 min with 200 of 2% crystal violet (CV Gram stain, Merck, Germany). The stain was rinsed off by placing the plates under running tap water. CV stain is suitable for determining the amount of biofilm (Pitts et al., 2003). After drying the stained plates, biofilms were visible as purple rings formed on the sides of each well. The quantitative analysis of

biofilm production was performed by adding 200 l of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Then the optical density (OD) of the stain was measured at 492 nm by an ELIZA reader (TECAN co. Spectra SLT) as described previously (O`toole and Kolter, 1998). Based on the OD of the bacterial films, strains were classified into the following categories, as previously described (Stepanovic et al., 2000): OD ODC = not a biofilm producer; ODC < OD 2ODC = a weak biofilm producer; 2ODC < OD 4ODC = a moderate biofilm producer; 4ODC < OD= a strong biofilm producer. ODC and OD were defined as the mean OD of the blank wells and wells with biofilm, respectively.

Biofilm formation monitoring on glass slides

Alternatively, biofilms were formed on glass slides, in order to biofilm development monitoring of strong biofilm former in the presence of different concentrations of the best substrate. To do this, sucrose was selected and used for assessing the biofilm formation in concentrations such as 0.1, 0.5, 1 and 2% and times interval. A 500- l over night culture of the strains adjusted with 0.5 on the McFarland turbidity standard was added to 50 ml BHI broth supplemented with or without different concentrations of sucrose in a beaker containing two glass slides and the chamber was incubated at 35°C with shaking at 100 rpm for 24 h. At the end of each time interval (4, 16 and 24 h), the slides was washed three times with distilled water. One of the slides of each beaker dried at room temperature for 30 min. Dried biofilms on slides were gram stained and examined under a microscope (Bos et al., 1999). Light microscopic observation was performed using a Nicon eclipse E200 (Japan) equipped with a digital camera (Olympus Co.). The other slide used for enumeration of the number of attached bacteria on glass slides at time intervals by drop plate method (Herigstad et al., 2001).

The assessment of gtfB gene expression level

The assessment of gtfB gene expression level was done in three different conditions:

1. The *S. mutans* cells in planktonic culture in a media without sucrose.
2. Cells of *S. mutans* biofilm formed in presence of sucrose (attached cells).
3. Unattached or detached cells of *S. mutans* in presence of sucrose.

Extraction of total RNA

The above bacterial cells were disrupted by means of Hybaid ribolyser instrument and kit according to the manufacturer's protocol in which RNA-containing supernatant from ribolyser tube was transferred to new RNase free microtube, centrifuged and treated with 300 l of chloroform – isoamyl alcohol, vortexed and centrifuged. Then, total RNA was recovered by precipitation with isopropanol and dried under appropriate sterile conditions. The RNA concentration was determined spectrophoto-metrically with the aid of a biophotometer (Eppendorff, Rs 232-C, Germany). The integrity of the RNA was assessed by agarose-gel electrophoresis.

Reverse transcription

A reverse transcription (RT) reaction mixture (20 L) containing 50 ng of random hexamers primers, 2 g of total RNA sample and up to 12 l RNase free water was incubated at 70°C for 5 min to

Primer	Sequence (5′ - 3′)	Fragment location	Accession number
gtfB – F	AGCAATGCAGCCAATCTACAAAT	1150 - 1172	M17361
GtfB – R	ACGAACTTTGCCGTTATTGTCA	1224 - 1245	M17361

remove any secondary structure and was placed on ice. Then, 5X RT buffer (4 l), 20 U/ l Ribonuclease Inhibitor (1 l) and 10 mM dNTPs mix (2 l) (cinagen) were added to each reaction mixture, after 5 min incubation in 37°C, 1 l reverse transcriptase was added. Then, the mixture was incubated at 42°C for 60 min. The reaction was terminated by heating the mixture at 70°C for 10 min, and the cDNA samples were stored at -20°C to be used later.

Real-time quantitative PCR

Using the ABI-step I (Applied Biosystems, USA) with SYBR Green PCR Master Mix (Qiagene), amplification, detection and analysis of mRNA were performed. The method and primers of gtfB gene were selected according to Tam et al. (2006) with uniformity in size (~100 bp) and melting temperature of primers. Primers and their sequences are shown in Table 1. The reaction mixture (20 L) contained 1X SYBR Green PCR Master Mix (Qiagene), the appropriate forward, reverse PCR primers (1 M) and 1 L of the cDNA sample. PCR conditions included an initial denaturation at 95°C for 5 min, then, 40 cycle amplification consisting of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Using the two-step protocol described above without the addition of RNA template. All primer pairs were checked for primer–dimer formation. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out without reverse transcriptase in order to identify whether the RNA samples were contaminated by residual genomic DNA. The critical threshold cycle (Ct) is defined as the cycle in which fluorescence becomes detectable above the background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules.

RESULTS

Characteristics of streptococcal isolates

The isolated streptococci from dental plaque and caries lesion were or hemolytic on blood agar plates. Their esculin hydrolysis was positive and all of the *mutans streptococci* were able to ferment sugars like: Lactose, Inulin, Manitol, Sorbitol and Raffinose. The VP test of *mutans* streptococci was positive.

Biofilm formation potential

The ability of the streptococcal isolates to form biofilm on micro titer plates was measured and the isolates were classified into four categories (Figure 1). In the medium without any sugars the potential of biofilm formation was very low and about 94% of tested isolates were non adherent. In the presence of sucrose, the percentage of strongly adherent bacteria was about 33.3% which is not

seen in the presence of other carbohydrates. Figure 2 shows the comparison between the effects of sugars on the adherence of Streptococcal isolates.

According to the results, it is cleared that sucrose is the best substrate for adherence of *S. mutans* isolates to surfaces. The mean of the resulted optical density of formed biofilm in the presence of sucrose was higher than other carbohydrates significantly ($p < 0.05$) and in the absence of any sugars, the significantly low optical density was observed an increase of sucrose concentration (Figure 3a) . Increasing the number of attached bacteria in 16 and 24 h biofilms was up to 1 - 1.5% of sucrose (Figure 3b, c). These results correspond to a non linear increase of attached bacteria. The line of best fit for the calibration of the attached bacteria versus sucrose concentration required a 3rd-order polynomial ($R^2=1$). The biofilm formation on glass slide was monitored at the end of 4, 16 and 24 h. The results showed that the number of attached cells increased over time (Figure 4).

Monitoring of biofilm formation at time intervals

The results of drop plate method for biofilm formation monitoring at time intervals in different sucrose concentration are presented in Figure 3.

Expression of gtfB in different condition

The gtfB expression level in planktonic, attached and unattached cells of *S. mutans* in biofilm environment was compared by real time RT PCR. The results showed that, the mRNA level of gtfB was up regulated in the biofilm condition. The level of gtfB gene expression in the biofilm condition was significantly higher than in the planktonic condition (Figure 5).

DISCUSSION

The ability of biofilm formation assay in presence and absence of some dietary carbohydrates showed that the more biofilm formation is related to presence of the sugars. Numerous studies have established the role of sugars in caries etiology and the importance of sugars as the principal dietary substrate that drives the caries process (Caglar et al., 2005; Touger et al., 2003; Loo et al., 2003). Brown et al. (2005) have mentioned that adding carbohydrates to growth media of oral streptococci
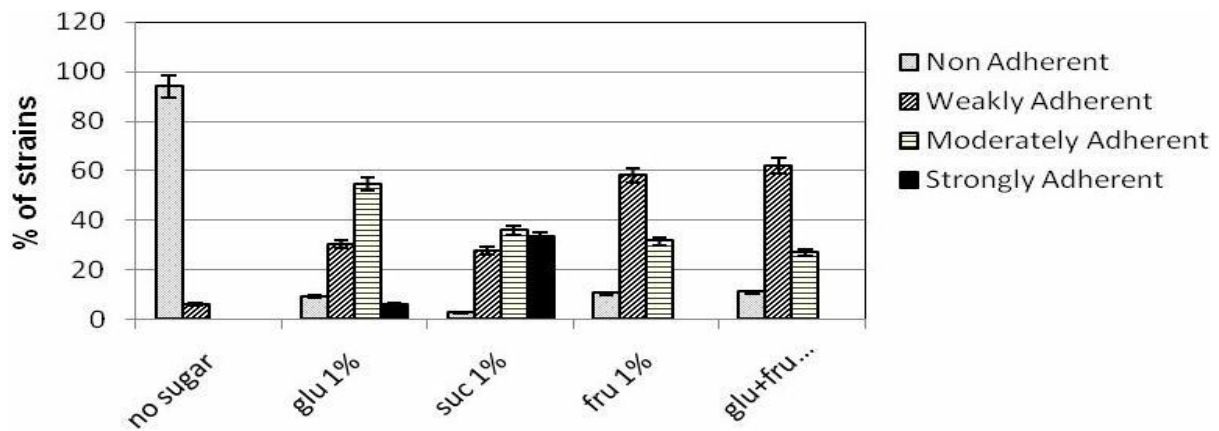
Figure 1. The effect of different substrates in the adherence potential of streptococcal isolates. All isolates were classified into four groups. Data are expressed as means and standard deviations of triplicate experiments.
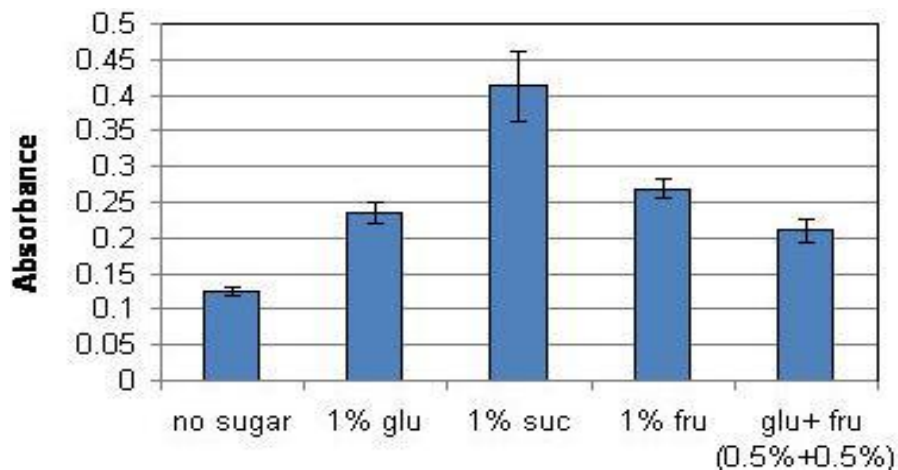

Figure 2. The comparison between sugars effect on the adherence of streptococcal isolates to the polystyrene micro titer plates (biofilm formation) by measuring the optical density at 492 nm. Data are expressed as means and standard deviations of triplicate experiments.

affects their biofilm formation on abiotic surfaces. Rosen et al. (2001) also reported that *S. mutans* and sobrinus adherence is related to glucans and the adherence of *Streptococcus gordoni* is related to glucans and fructans. Although, sugars, both naturally occurring and added, and fermentable carbohydrates stimulate bacteria to produce acid and lower the pH, several dietary factors affect the caries risk associated with fermentable carbohydrates. Then, according to SAS statistical analysis, it is revealed that sucrose, fructose and glucose with significant differences are involved in biofim formation of streptococcal isolates, respectively. Therefore, among the various tested carbohydrates in this study and other different researches, sucrose is considered the most cariogenic dietary carbohydrate, because it is fermentable, and also serves as a best substrate for the

synthesis of extracellular and intracellular polysaccharides and dental plaque formation (Brown et al., 2005; Bowen, 2002; Cury et al., 2000; Pecharki et al., 2005; Ribeiro et al., 2005; Leme et al., 2006).

In the next step, the drop plate method was used to determine the number of attached bacteria on glass slide in the presence of different concentrations of sucrose, as the best substrate, and time intervals. The drop plate method has some advantages over the spread plate method. Less time and effort are required to dispense the drops onto an agar plate than to spread an equivalent total sample volume into the agar. By distributing the sample in drops, colony counting can be done faster and perhaps more accurately. However, the relationship between sucrose concentration and attached bacterial population was exponential. During the first 4 h, the
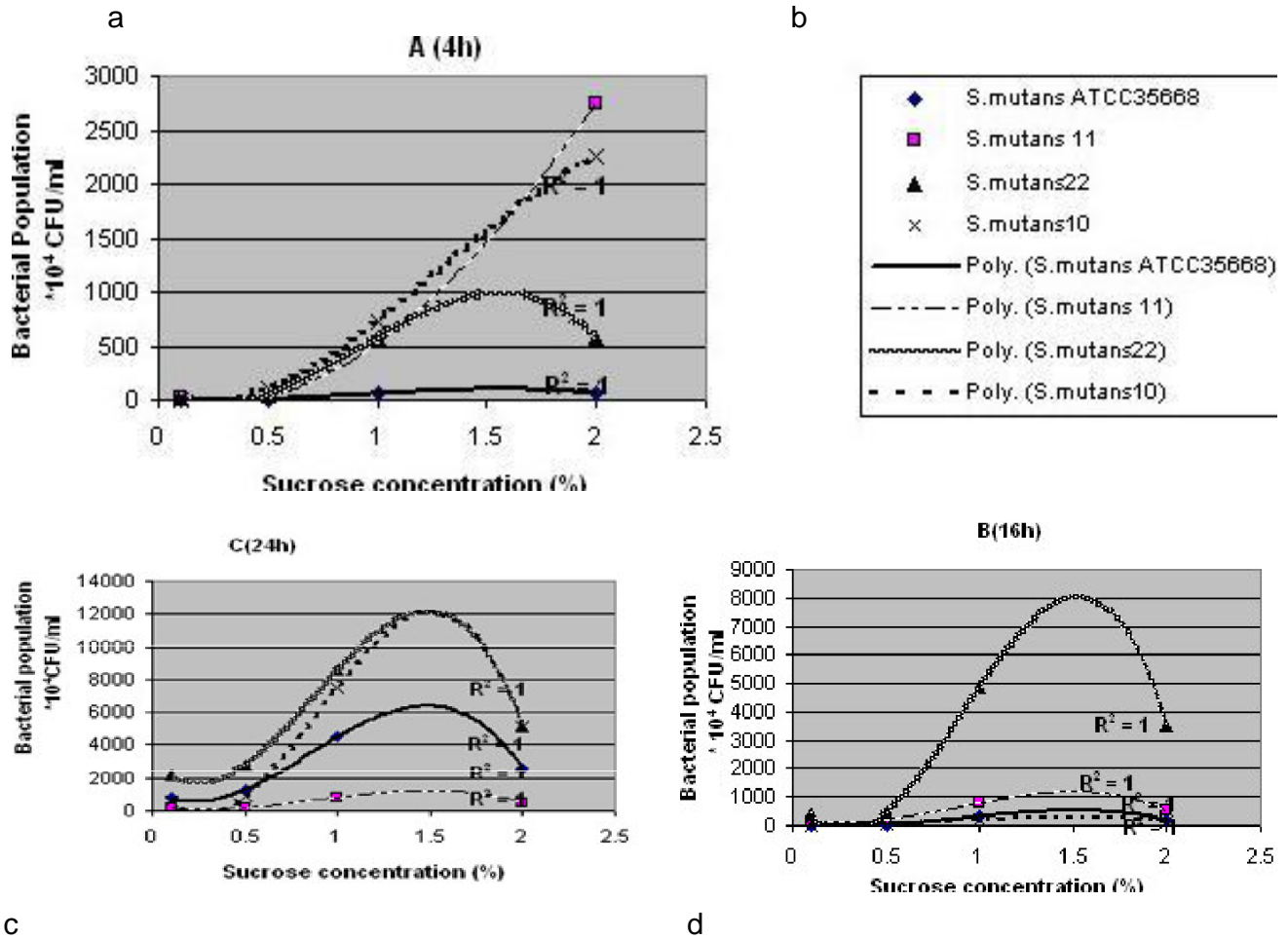
a

A (4h)

Bacterial Population *10⁴ CFU/ml

Sucrose concentration (%)

b

- ♦ S.mutans ATCC35668
- ■ S.mutans 11
- ▲ S.mutans22
- × S.mutans10
- —— Poly. (S.mutans ATCC35668)
- – – – – Poly. (S.mutans 11)
- ∿∿∿ Poly. (S.mutans22)
- - - - - Poly. (S.mutans10)

C(24h)

Bacterial population *10⁴CFU/ml

Sucrose concentration (%)

B(16h)

Bacterial population *10⁴ CFU/ml

Sucrose concentration (%)

c

d

Figure 3. Kinetics of *S. mutans* strains attachment to glass slides in the presence of different concentrations of sucrose. Panel A: at the end of 4 h. Panel B: at the end of 16 h. Panel C: at the end of 24 h. Results are in average of three experiments;
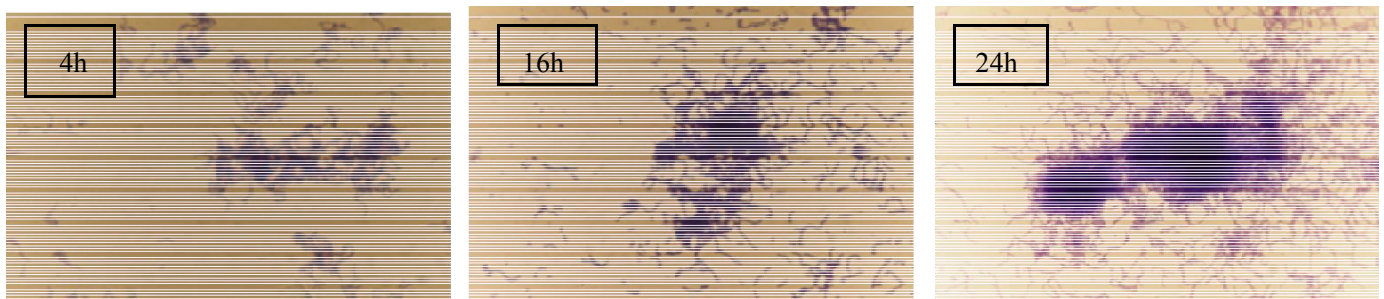

4h

16h

24h

Figure 4. The monitoring of biofilm formation at time intervals (4, 16 and 24 h).

number of attached cells was significantly correlated with sucrose concentration (p < 0.05) and bacterial detachment has occurred in mature biofilms (16, 24 h), in the concentration of 2% sucrose. Therefore, the kinetics of cell adhesion was found to be logarithmic, with R2 = 1 (polynomial regression 3rd order).

Shera et al. (2006) reported that increased glucose

concentration resulted in increased biofilm formation of group A streptococcal isolates. Loo et al. (2003) also showed that glucose can enhance the biofilm formation of Streptococci while very rich media of glucose will inhibit it. Tam et al. (2007) reported that increasing sucrose concentration up to 1%, increased *S. mutans* biofilm formation and after that, the separation process will hap-
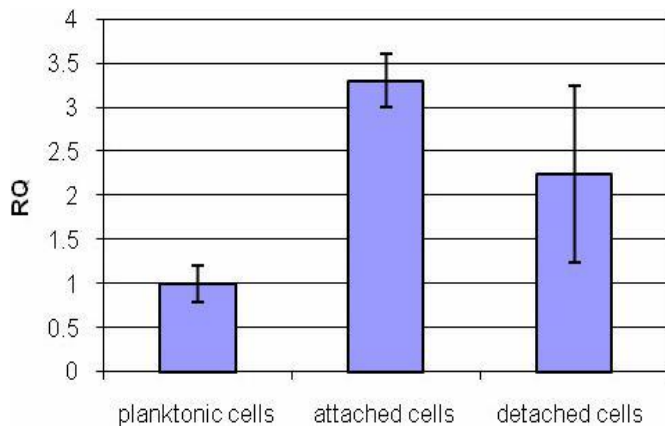
Figure 5. The comparison of gtfB *gene* expression level in different condition (planktonic cells in the absence of sucrose, attached cells in the present of 1% sucrose and unattached or detached cells from biofilm in the presence of 1% sucrose).

pen. They also expressed that the greater availability of sucrose contributed to biofilms with less lag time, lower doubling times and earlier detachment. Then, in the biofilm formation monitoring, actually, visible films form readily within 4 - 24 h under the culture conditions utilized. It is also revealed that in the presence of sucrose, biofilms of these bacterial strains form consistently, as Brune et al. (1997) had mentioned before. The mRNA level of gtfB, the key factor in attach, was influenced significantly by biofilm formation in S. mutans. It was also cleared that, *S. mutans* uses sucrose to synthesize glucan polymers through the actions of three secreted glucosyltrans-ferases, encoded by the gtfB, gtfC, and gtfD genes (Yoshida and Kuaramitsu, 2002). Water-insoluble pro-ducts of the GtfB and GtfC enzymes are major contributors to adhesion to teeth, and are essential for the efficient initiation of dental caries on the smooth surfaces of the teeth (Burne et al., 1997). Huang et al. (2008) reported that, the mRNA level of gtfB, gtfC and ftf in the biofilm condition was significantly higher than in the planktonic condition.

It can be concluded that, despite the fact that, the relationship between sugar consumption and caries is so strong; sugar consumption restriction still has an important role in prevention of caries.

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