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Optimization and Genetic study on Locally Isolated *Streptococcus mutans* for Mutacin Production

A Thesis

Submitted to the Council of College of Science Al-Nahrain University in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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Summary

In order to isolate *Streptococcus mutans*, 80 swab samples were collected from dental caries of patients attended Al-Dora Health Center and Al-Zewiya Health Center in Baghdad city. From these samples, 98 bacterial isolates were obtained. Results of identification depending on morphological, cultural and biochemical tests showed that 10 of these isolates were belonged to *Streptococcus mutans*. The results of identification were confirmed by using VITEK-П system. All isolates were screened to examine their ability to mutacin production. Results showed that all isolates were mutacin producers with variable degrees depending on mutacin inhibitory effect produced in the culture filtrates of these isolates against the test microorganism (*Streptococcus pyogene*s).

S. mutans S2 which gave a highest inhibition zone (20mm) against *S. pyogenes* was selected for further studies. The optimum conditions for mutacin production was supplemented the production medium (brain-heart infusion broth) with 3% sucrose (as a sole source for carbon and energy) , 2% yeast extract (as a nitrogen source) and 0.5% KH_2PO_4 (as a phosphate source), with an intial medium pH of 6, in addition to incubation at 37°C for 24 hours under anaerobic conditions. Under these conditions the maximum mutacin production by *S. mutans* S2 against *S. pyogenes* was reached 35mm in diameter.

When antibiotic susceptibility of *S. mutans* S2 was examined against different antibiotics, results showed that this isolate was resist to bacitracin and erythromycin, while sensitive to amoxicillin, ampicillin chloramphenicol, vancomycin, clindamycin, impenem, gentamicin and cephalothin.

Plasmid content of *S. mutans* S2 was studied to investigate its role in mutacin production by extraction of plasmid DNA, then electrophoresis on agarose gel. Results showed this isolate had one plasmid DNA band,

but not responsible for antibiotic resistance and for mutacin production according to the obtained results of curing by using interchalating ethidium bromide dye in a concentration of 800 μg/ml. adversely, cured cells were still having ability of mutacin production which indicats that this trait may be is a chromosomally encoded.

1. Introduction and Literatures review

1.1 Introduction

The increase of microbial resistance to antibiotics has led to a continuing search for newer and more effective drugs. Antimicrobial peptides are generally found in animals, plants, and microorganisms and are of great interest to medicine, pharmacology, and the food industry. These peptides are capable of inhibiting pathogenic microorganisms(Al-Aridhi, 2008). They can attack parasites, while causing little or no harm to the host cells. A substance to be deemed a useful chemotherapeutic agent must have toxic selectivity for the parasite; this means that, at an effective concentration in the tissues, the substance must have low toxicity to host cells and high toxicity to the infective agent; it also should not alter the natural defense mechanisms of the host, such as phagocytosis and synthesis of antibodies (Coutinho *et al*., 2008).

Antimicrobial peptides present new possibility for combating infectious diseases. They inhibit the growth of pathogenic microorganisms, without affecting the host or the animals and plants that produce them, and have a broad spectrum of antimicrobial activity. It is well known that bacteria, induced by stress, produce bacteriocins which is promising antimicrobial substances that may cure infectious diseases (Bianchi and Baneyx, 1999). These peptides may be found in all living organisms; they can be modified in the laboratory or they can be obtained from organisms that produce them for their own defense (Lamberty *et al*., 2001).

Bacteriocin are found in almost every bacterial species examined to date, and within a species tens or even hundreds of different kinds of bacteriocins are present, and more recently, have been described as universally produced by some members of the archaea (Riley and Chavan, 2007).

Mutacins as bacteriocins are proteinaceous antibacterial substances produced by *Streptococcus mutans*. Mutacins used as possible anticaries agents and the role they play in the colonisation of the oral cavity have raised much interest . They have also been studied as a bacterial fingerprinting tool and to assess the distribution of mutacin-producing strains . New application of mutacin are developed as potential food preservatives and as new effective antibiotics (Nicolas *et al*., 2007).

S. mutans, the chief etiologic agent of dental cavities in humans , colonizes the oral cavity shortly after tooth eruption and is found in approximately 98% of the population worldwide (Spatafora *et al*., 1999).

Because of the limited studies and importance of both(*S. mutans* and mutacin as " designer drugs" alternatives to traditional antibiotics that target specific bacterial pathogens) in genetic engineering, agricultural, biological control, and medicine, the present work was aimed to:

1. Isolation of *S. mutans* from dental caries.
2. Identification of bacterial isolates according to their morphological and cultural characteristics and biochemical tests.
3. Detection the ability of bacterial isolates in mutacin production.
4. Selection the most virulent isolate according to the ability in mutacin production.
5. Studying plasmid profile for the selected isolate.
6. Detection of the role of plasmid content of the selected isolate in mutacin production.

1.2 Literature review

1.2.1 Genus *Streptococcus*

Genus *Streptococcus* comprises important pathogens that have a severe impact on human health (Richards *et al*., 2014). Genus *Streptococcus* was first recorded in 1683 in Van Leeuwenhoek's drawings of microscope images of the material removed from his teeth. *Streptococcus* comes from the Greek strepto (twisted) and coccus (spherical) **(**Nobbs *et al*, 2009). The earliest attempt at differentiating the streptococci was probably made in 1903 by Shottmuller , who used blood agar to differentiate strains that were beta-hemolytic from those that were not. Before 1933, fermentation and tolerance tests were the only tests used for differentiating many of the streptococci. In 1933 Lancefield reported the technique of demonstrating specific carbohydrate "group" antigens associated with the beta-hemolytic strains . In 1937, Sherman proposed a scheme for placing the streptococci into four categories. These categories were organized by hemolytic reaction, group carbohydrate antigens, and phenotypic tests (primarily fermentation and tolerance tests) (Facklam, 2002).

According to the classification in Bergey's Manual of Determinative Bacteriology, 9th ed. (Holt *et al.*, 1994), the genus *Streptococcus* includes the pyogenic, oral and anaerobic groups of streptococci, as well as a group of other streptococci. The cells are spherical or ovoid, 0.5- 2.0 μm in diameter, occurring in pairs or chains, and stain Gram-positive. Streptococci require nutritionally rich media for growth. 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).

Optimum temperature for growth is 37°C, and growth is usually restricted to 25-45°C (Gronroos, 2000).

The family *Streptococcaceae* includes the species *Enterococcus*, generally associated with faeces, *Lactococcus*, associated with plants and dairy products, and *Streptococcus***.** (Makarova and Koonin, 2006). The members of the genus *Streptococcus* are clinically important species and these organisms are often isolated from human clinical specimens (Tong *et al*., 2003). They are subdivided into six major clusters or species groups: the *mitis*, *anginosus*, *salivarius*, *mutans*, *bovis*, and pyogenic groups (Makarova and Koonin, 2006).

1.2.2 Microorganisms Present in the Mouth

The oral microorganism functions as a part of the host defense by acting as a barrier, by competition for essential nutrients and creation of unfavorable conditions to exogenous organisms that may be pathogenic to the host. Over 700 bacterial taxa have been found in the oral cavity, however they are not all present in the same mouth (Aas *et al*., 2005). The composition varies in different sites in the oral cavity, with a large and more diverse bacterial load on the dorsum of the tongue. Most of these microbes are harmless, but under certain conditions some can cause oral infections like caries or periodontal disease (Sakamoto *et al*., 2005). Oral streptococci, like *Streptococcus mutans*, are associated with pyogenic and other infections in various sites including mouth, heart, joints, skin, muscle, and central nervous system (Holt *et al*., 1994).

1.2.3 Oral *Streptococcus*

Oral streptococci are in general referred to as viridans streptococci, but this is not an exclusive classification because the viridans streptococci contain as well members not isolated from the oral cavity. Oral

streptococci are divided into five different groups: (1) Mutans group (prominent members are *Streptococcus mutans* and *Streptococcus sobrinus*), (2) Salivarius group (*Streptococcus salivarius*), (3) Anginosus group (*Streptococcus anginosus* and *Streptococcus intermedius*), (4) Sanguinis group (*Streptococcus sanguinis* and *Streptococcus gordonii*), and (5) Mitis group (*Streptococcus mitis* and *Streptococcus oralis*). Some of the oral streptococci are known to cause infective endocarditis when disseminated through the blood stream, like the early colonizer *S. gordonii, S. sanguinis*, and the recently identified *Streptococcus oligofermentan*s (Kreth *et al*., 2009).

Oral cavity microbes are often cited as viridans group streptococci because colonies cause greening of blood agar (Nobbes *et al*., 2009).

The oral or viridians *streptococcus* from an important component of the normal microbial flora of the mouth and the upper respiratory tract of humans. These sites appear to be their normal habitats, although some species can be isolated from other sits and from feces (Hardie and Whiley, 2006).

1.2.4 Dental plaque

Dental plaque is the term commonly used for the biofilm that is formed on the tooth surface and consists of a complex microbial community embedded in a matrix of polymers of bacterial and salivary origin (Spratt and Pratten, 2003). The formation of acid end-products through the metabolism of carbohydrates by acidogenic microorganisms within these biofilms is an important factor in the development of dental caries (Svensater *et al.,* 2003). The essential process involves demineralization of the tooth structure by high concentrations of organic acids.(Zanin *et al*., 2005). *Streptococcus mutans* has been implicated as the primary a etiological agent because of its relatively high numbers in

plaque prior to the appearance of carious lesions, its ability to degrade carbohydrates rapidly with the formation of abundant acid and its ability to induce a tolerance to low pH environments (Svensater *et al*., 2001). The current treatment for plaque-related diseases involves the use of traditional antimicrobials in conjunction with the mechanical removal of the biofilm. In the case of caries, a more attractive proposition would be to kill the causative organisms in situ (Wilson, 2004).

Dental caries is a transmissible infectious disease in which mutans streptococci (MS) play the major role (Napimoga *et al*., 2005). Although the species *S. mutans* was not included in the eighth edition of Bergeys Manual of Systematic Bacteriology , subsequent taxonomic developments established a group that now consists of six species: *S.mutans, S. sobrinus, S. cricetus, S. rattus, S. macacae and S. downei* (Hardie and Whiley, 2006).

1.2.5 *Streptococcus mutans*

S. mutans is a Gram-positive bacterium, which plays a key role in the formation of the dental plaque biofilm as an early coloniser (it produces adhesins which attach the organism to the acquired pellicle of the teeth) and is the most important bacterium in the formation of dental caries**,** *S. mutans* do not have flagella, but do have pili**.** On agar. *S. mutans* are Gram-positive ovoid cocci, that typically occur in pairs or chains, are aciduric (grow well in acid medium) and acidogenic (produce acid) and are non-motile facultative anaerobes that grow optimally at 37°C (Koning, 2010).

S. mutans have a thick cell wall, the cell wall composed of peptidoglycan (murein) and teichonic acid that prevent osmotic lysis of cell protoplast and confer rigidity and shape on cell. *S. mutans* have

capsule that is composed of polysaccharide and it is structural unit is dextran glucose (Balakrishnan *et al.*, 2000).

S.mutans classified into four serotype (c , e, f, k) based on chemical composition of its cell surface serotype specific Rhamnose-glucose polymers (RGPs), which form backbone of rhamnose polymer with side chain of glucose polymers (Nakano *et al*., 2007).

S. mutans play a central role in the etiology of dental caries , because these can adhere to the enamel salivary pellicle and to other plaque bacteria . Mutans streptococci and lactobacilli are strong acid producers and hence cause an acidic environment creating the risk for cavities .Usually, the appearance of *S. mutans* in the tooth cavities is followed by caries after 6-24 months. (Forssten *et al*., 2010).

The primary habitats for *S. mutans* are mouth, pharynx, and intestine (Forssten *et. al*., 2010). *S. mutans* metabolize carbohydrates, such as glucose and sucrose, to produce acid and enhance biofilm formation with the early colonizing bacteria to induce dental caries. important contributor to the formation of the cariogenic biofilm since *S. mutans* utilizes dietary sucrose to synthesize large amounts of glucan and is highly acidogenic (Lee *et. al*., 2012)**.** *S. mutans* was first isolated from carious lesions by Clarke *et. al*. as early as 1924, *S. mutans* was the name given to all oral streptococci that were isolated from carious lesions that could ferment mannitol and sorbitol, that produced extracellular glucans and were cariogenic in rodent models of caries. They were called “mutans” due to their appearance on Gram stains where they resembled mutant versions of streptococci, possessing a smaller and more oval appearance(Lynch, 2010)**.**

1.2.6 Virulance factors

Virulence factors in *S. mutans* help protect the bacterium against

possible host defenses and maintain its ecological niche in the oral cavity, while contributing to its ability to cause host damage(Ajdic´ *et al.*, 2002).

1.2.6.1 Mutacin

The increase in bacterial resistance to antibiotics impels the development of new anti-bacterial substances. Mutacins (bacteriocins) are small antibacterial peptides produced by *S.mutans* showing activity against bacterial pathogens.(Nicolas *et al*., 2011).

Bacteriocins are synthesized by ribosomes as precursor peptides with a signal peptide at the N-terminus, typical of secreted proteins ,which is cleaved concomitantly with the export of the mature peptide across the membrane . The production of bacteriocins by Gram-positive bacteria is generally associated with the transition from log phase to stationary phase of bacterial growth or the cell density in the culture medium (Nes and Holo, 2000).

Bacteriocins produced by the oral bacterium *S. mutans* are divided into two groups: (i) the lanthionine-containing (lantibiotic) mutacins and (ii) the unmodified mutacins. While most bacteriocin activities characterized to date consist of a single active polypeptide, several two-component lantibiotic and nonlantibiotic bacteriocins have also been described, and these are dependent upon the collaborative activity of two polypeptides to exert their full antimicrobial activity(Hale *et al*, 2005c). The lantibiotics are small peptides containing lantionina, β-methyl-lantionina dehidratados residues and are synthesized by ribosomes and are modified after translation(Sahl *et al*., 1995).

Kamiya *et al*., 2011 divided the bacteriocins from *S. mutans* into two groups according to their molecular weight and sensitivity to heat. The

first group was inactivated with heating at 80C° for 20 minute and possessed a molecular mass less then 10,000 Da, the second group was composed of small molecules (more then 10,000 Da) and heat stable. But the spectrum of activity, sensitivity to either, chloroform and the trypsin varied considerably within the same group. Bacteriocins produced by Gram-positive bacteria are peptide antibiotics classified as class I (lantibiotics) or class II (non-lantibiotics), based on their post-translational modifications, in *S. mutans*, both classes of bacteriocins are produced. The lantibiotic mutacins show in figure (1-1) have a wide spectrum of activity against Gram-positive bacteria, including multiple-drug-resistant pathogens , while the non-lantibiotic mutacins are more specific to closely related streptococcal species such as the mitis group streptococci and group A streptococci (Tsang *et al*., 2005).

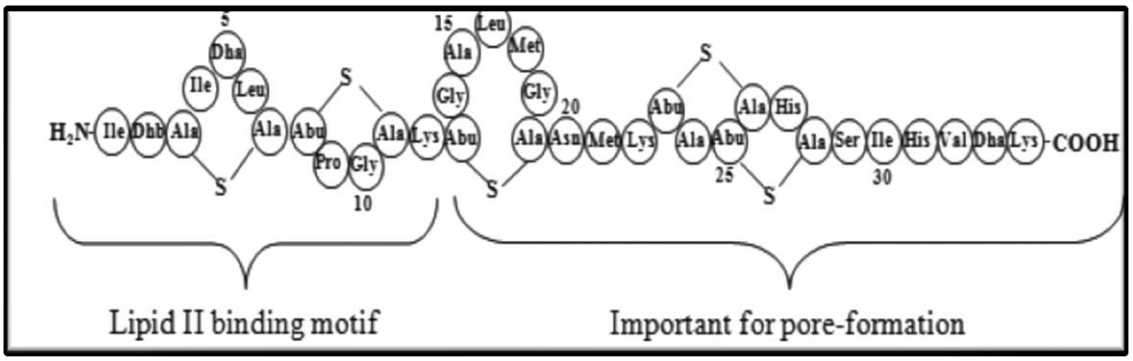


Figure (1-1): Chemical structure of lantibiotic mutacin (Paiva *et. al*., 2011)

Mutacins could play an important biological role in the regulation and composition of dental biofilm due to their synergistic or antagonistic activity, suggesting that wide spectrum mutacins may be more important in the colonization and stabilization of this cariogenic species, mainly in

the stable niche of highly complex microbial activity (Napimoga *et al*, 2005).

1.2.6.2 Adhesins

Streptococcus mutans is capable of forming biofilms through a number of mechanisms: one of these mechanism is ability to express the surface protein called adhesin P1(Li *et al*., 2002; Sato *et al*., 2004; Biswas and Biswas , 2005).

Biofilm development occurs in two distinct phases: during the first, bacterial surface proteins interact with host or bacterial products adsorbed on the tooth surface. In the second phase, biofilm forms as bacteria accumulate by aggregation with the same or other species and produce an extracellular polysaccharide matrix(Napimoga *et al*., 2005).

The adhesion P1 participate in sucrose-independent colonization of tooth surfaces. P1 possesses multiple sites contributing to it is interaction with the high-molecular-weight salivary glycoprotein(Fontana *et al*.,2000 ;Crowley *et al*., 2008). *S.mutans* cells can attach initially to saliva coated surfaces through sucrose-independent mechanisms mediated primarily by interaction between specific pellicle protein and bacterial adhesion(Zhu *et al*., 2006; koo *et al*., 2009). So that this surface protein consider as virulence factor for *S. mutans* (Nakano *et al*., 2009).

The SloC protein, an adhesin belonging to the lipoprotein receptor antigen I (LraI) family, was recently characterized . Members of the LraI family were found in a range of bacteria, and have a dual role: transport and adhesion.

S. mutans can adhere to salivary agglutinin, other plaque bacteria, extracellular matrix, and epithelial cell-surface receptors(Ajdic´ *et al*., 2002).

1.2.6.3 Exoenzymes

Streptococcus mutans has the genes to produce many exoenzymes involved in extracellular sucrose metabolism, including well known glucosyltransferases I, SI, and S, fructanase, fructosyltransferase, and dextranase. The *gbpB* (also known as *sagA*) gene that encodes a putative peptidoglycan hydrolase important for cell wall integrity**(**Ajdic´ *et al.*, 2002).

glucosyltransferases from *S. mutans* (Gtfs) play critical roles in the development of virulent dental plaque. Gtfs adsorb to enamel synthesizing glucans in situ, providing sites for colonization by microorganisms and an insoluble matrix for plaque. Gtfs also adsorb to surfaces of other oral microorganisms converting them to glucan producers. *S. mutans* expresses three genetically distinct Gtfs; each appears to play a different but overlapping role in the formation of virulent plaque. GtfC is adsorbed to enamel within pellicle whereas GtfB binds avidly to bacteria promoting tight cell clustering, and enhancing cohesion of plaque. GtfD forms a soluble, readily metabolizable polysaccharide and acts as a primer for GtfB (Bowen and koo, 2011).

The *gtfB* and *gtfC* genes are in an operon-like arrangement and encode enzymes that produce mainly water-insoluble glucans, whereas the *gtfD* gene, which is not linked to the *gtfBC* locus, encodes an enzyme that catalyses the formation of a water-soluble glucan. It is the water-insoluble glucans made by GtfBC that play important roles in adhesion and accumulation of the organisms on the tooth surfaces, and in establishing the extracellular polysaccharide matrix that is responsible for the structural integrity of dental biolms (Li and Burne, 2001).

1.2.6.4 Acidiogencity and acid tolerance

The mutans streptococci ferment many different sugars, and they

appear to metabolize sucrose to lactic acid more rapidly than other oral bacteria. This is thought to be related to the multitude of enzyme systems catalysing the reactions of transport and metabolism of sucrose expressed by these organisms .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 (Gronroos, 2000).

the plaque environment is continuously experiencing changes in pH from above pH 7.0 to as low as pH 3.0 due to ingestion of dietary carbohydrates (Lemos *et. al*., 2005). The extreme acidic environments pose a considerable stress on organisms living within the dental plaque. *S. mutans* along with several other oral streptococci posses several acid adaptive strategies, some of which are distributed among all the species and some that are unique to *S. mutans* . It has been shown that prior exposure of *S. mutans* to a sub-lethal pH of 5.5 results in enhanced survival at the extremely low pH of 3.0-3.5(Hamilton and Svensater, 1998; Svensater *et al*., 1997; and Welin-Neilands and Svensater, 2007).

This adaptive response, termed as acid tolerance response (ATR), involves changes in the physiology, including alteration in gene and protein expression, increased glycolytic activity, and increased proton-extrusion via the F1/F0-ATPases, which lead to enhanced survival(Banas, 2004; Lemos *et al*., 2005; and Welin-Neilands and Svensater, 2007). Glycolytic enzymes in *S. mutans* are maximally active around pH 6.5 for cells growing in acidic conditions, while for other oral bacteria maximum glycolysis is obtained around pH 7.5 (Hamilton and Buckley, 1991).

1.2.6.5 Glucan binding proteins(GBP)

Streptococcus mutans is a principal etiologic agent in the development of dental caries due to its exceptional aciduric and acidogenic properties, and its ability to adhere and accumulate in large numbers on tooth surfaces in the presence of sucrose. Sucrose-dependent adherence is mediated by glucans, polymers of glucose synthesized from sucrose by glucosyltransferase (Gtf) enzymes. *S. mutans* makes several proteins that have the property of binding glucans. glucan-binding proteins (Gbps), Gbps A, B, C and D, contribute to the cariogenicity of *S. mutans*(Lynch *et al*., 2013).

GbpC most closely fits the definition of a cell receptor for glucan since it is a cell wall-anchored protein. Sequence analysis revealed that the GbpC shares some homology with the major streptococcal surface protein P1. Most recently, a fourth Gbp, GbpD, was discovered and isolated based on sequence analysis of the complete, annotated sequence of *S. mutans* UA159 strain . GbpD possesses amino acid repeats similar to those in the glucan-binding domains of GbpA and the Gtfs (Shah and Russell, 2004).

GbpA of *S.mutans* has been shown to contribute to the architecture of glucan-dependent biofilms formed by this species and influence virulence in rat model (Banas *et al.,* 2007).

GbpB has been shown to induce protective immunity to dental caries in experimental models (Smith *et al*, 2003).

1.2.6.6 Intracellular polysaccharide(IPS)

Intracellular polysaccharide (IPS) is accumulated by *S. mutans* when the bacteria are grown in excess sugar and can contribute toward the cariogenicity of *S. mutans*. IPS is important for the persistence of *S. mutans* grown in batch culture with excess glucose and then starved of

glucose.IPS metabolism distinct from providing nutrients is important for persistence. IPS was not needed for persistence when sucrose was the carbon source.(Busuioc *et al*., 2009).

Intracellular polysaccharides (IPS) are glycogen-like storage polymers which contribute significantly to *Streptococcus mutans*-induced cariogenesis. IPS can serve as metabolic substrates for acid production during periods of carbohydrate limitation in the oral cavity . The utilization of IPS by *S. mutans* can therefore exacerbate tooth decay by prolonging the period of exposure of host tissues to organic acids, such as during the periods between meals(Spatafora *et al*., 1999).

1.2.7 Types of mutacin

Mutacins that have been well characterized to date are: I, II , III , IV, B-Ny266, 1140 , N, M46, M19, H7 ,H-29B . Six of these mutacins (I, II, III, B-Ny266, 1140, and H- 29B) are lantibiotics, which are lanthionine-containing antibacterial peptides with dehydrated amino acid residues and thioether bridges resulting from posttranslational modifications(Nicolas *et al*, 2004).

1.2.7.1 Mutacin I

Mutacin I is produced by *S. mutans* CH43 and UA140. The mature peptide is composed of 24 amino acids with a molecular mass of 2364 Da. Mutacin I belongs to the lantibiotic type AI and epidermin group. The biosynthetic operon was reported to encompass 14 ORFs, Upstream of the biosynthetic genes is the alanyl t-RNA synthetase *ats* (SMU.650) gene (Nicolas *et al*., 2007).

1.2.7.2 Class II bacteriocins and Mutacin H-29B :

Class II bacteriocins are small (<10kDa) non-lanthionine containing bacteriocins, heat-stable peptides without any post-translational modification(Cotter *et. al.,* 2005). Most of the class II bacteriocins cause disruption of proton-motive force by permeabilizing the cell membrane and act in nano molar range . Class II can be further divided into several subclasses . Class IIa consists of listeria-active pediocin like peptides, class IIb consists of two-peptide bacteriocins, and other multi-component peptides are included into class IIc (Nissen-Meyer *et al*., 2009). that the amino acid sequence of mutacin H-29B is identical to the already known mutacin II (J-T8) over the first 24 residues(Nicolas *et al*., 2006).

1.2.7.3 Mutacin III

Mutacin III has a complete sequence of the peptide was deduced from the sequence of the gene and the molecular mass was calculated to be 2266 Da. Biosynthesis of mutacin III is controlled by an operon composed of eight ORFs, namely *mutRAA'BCDPT* (Nicolas *et al*., 2007). Mutacin III thus belongs to the type AI lantibiotics (Chatterjee *et al*., 2005).

1.2.7.4 Mutacin IV (cyclic bacteriocins)

This class of bacteriocins has covalently linked N- and C- terminal structure. They are cationic, hydrophobic and their size varies from 3.4 kDa to 7.2 kDa. All of them permeabilize the target cell membrane to small molecules that cause the disruption of membrane potential, which ultimately results in cell lysis (Nissen-Mayer *et al*., 2009). Enterocin is the best characterized cyclic bacteriocin which contains 70 residues and produced by several species of enterococcus(Martinez-Bueno *et al*., 1998 and Gonzalez *et al.*, 2000). Cyclic structure might protect the bacteriocins

from proteolysis and are active against both gram-positive and gram-negative bacteria (Maqueda *et al.*, 2004).

1.2.7.5 Mutacin B-Ny266

This type consist of 22 amino acids long and possesses a calculated mass of 2270 Da. Mutacin B-Ny266 was the first mutacin lantibiotic to be completely sequenced(Nicolas *et al*., 2007). Mutacin B-Ny266 shown in figure (1-2) belongs to type AI lantibiotics (Chatterjee *et al*., 2005).

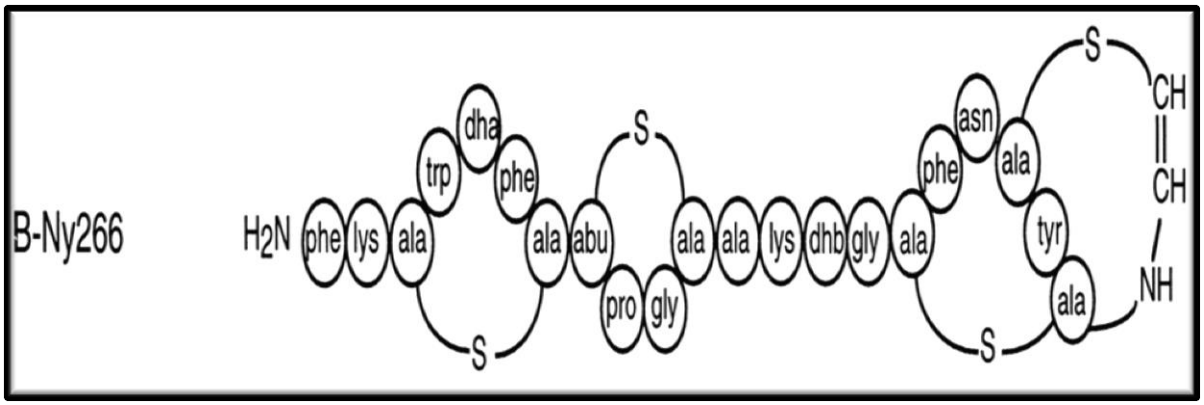


Figure (1-2): Chemical structure of mutacin B-Ny266((Paiva *et al.*, 2011).

1.2.7.6 Mutacin 1140

Mutacin 1140 is composed of 22 amino acids with a molecular mass of 2263 Da, and the reported sequence makes it very similar to the lantibiotics belonging to the epidermin group, differing from mutacin B-Ny266 by only two amino acids (Smith *et al*., 2003).

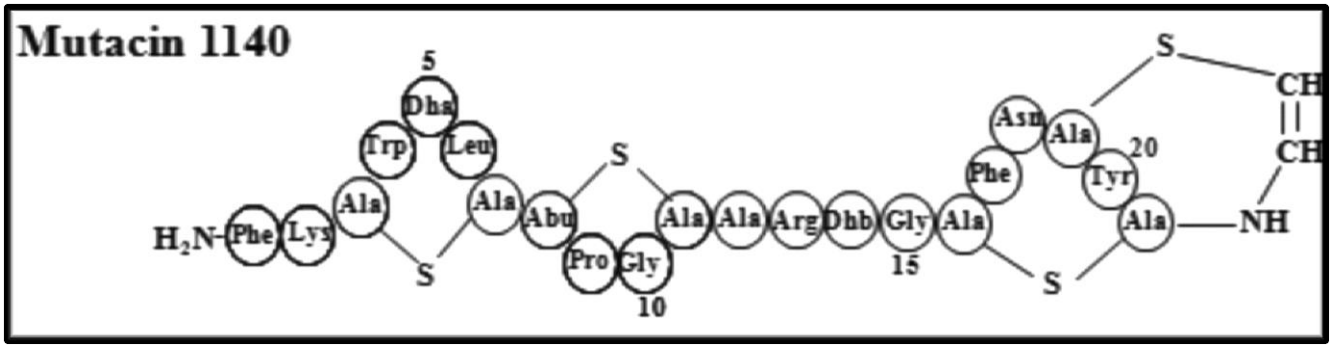


Figure (1-3): Chemical structure of mutacin 1140 ((Paiva *et al.*, 2011)

1.2.7.7 Mutacin N

Mutacin N produced by *S. mutans* N is a non-lantibiotic mutacin composed of 49 amino acids and has a molecular mass of 4806 Da (Nicolas *et al*., 2007).

1.2.8 Disease cause by *Streptococcus mutans*

1.2.8.1 infective endocarditis

S. mutans, considered to be a pathogen for infective endocarditis (Nomura *et al*., 2007).

About 20% of the endocarditis cases attributed to viridians Streptococci are due to *S. mutans* (Banas, 2004). IE is a life-threatening bacterial infection of the endocardium, a smooth layer of tissue that covers the inside the heart to protect the heart muscles (Beynon *et al*., 2006 and Moreillon and Que, 2004). A serotype specific putative adhesin, derived from rhamnose-glucose polysaccharide (RGP), has been identified in *S. mutans* that is thought to be required for attachment to human monocytes, fibroblasts, and platelets (Chia *et al*., 2004 and Engels-Deutsch *et. al*., 2003). Furthermore, the *S. mutans* adhesin, Antigen I/II, may play a role in IE since this adhesin binds to the extracellular matrix components such as type 1 collagen, fibrinogen, fibronectin, and laminin . Although the exact role of *S. mutans* in the pathogenesis of IE is not well understood, the availability of complete genome sequence may suggest various surface adhesins in the pathogenesis(Beg *et al*., 2002).

Oral streptococci can often cause bacteremia following various dental procedure including oral surgery, flossing, or brushing, allowing these organisms to gain and adhere to damaged heart valve, causing IE (Nakano *et al*, 2007).

S. mutans is able to maintain it is presence by forming biofilms in the oral cavity, similar conditions are also encountered in the biofilm formrd

on the heart valves where the inhibitory effects of immune response elements in the blood must be overcome. *S.mutans* along with the other oral bacteria can enter the blood stream after dental extraction and cause transient bacterimia and infective endocarditis (Biswas *et al.*, 2008).

1.2.8.2 Dental caries

The oral cavity is a unique environment consisting of both soft tissue and dental hard tissue, which are readily exposed to the external environment. These structures are constantly bathed with saliva and also serve as biological niches for a large assortment of oral microorganisms (Leone and Oppenheim, 2001).

Dental caries is one of the most common and costly diseases in the world, and although rarely life threatening it is a major problem for health service providers. In order to decrease the prevalence of caries, an improved understanding of the role of the microorganisms in dental diseases is needed(Marsh, 2003).

Dental caries and dental plaque are among the most common diseases worldwide, and are caused by a mixture of microorganisms and food debris. Specific types of acid-producing bacteria, especially *S. mutans*, colonize the dental surface and cause damage to the hard tooth structure in the presence of fermentable carbohydrates e.g., sucrose and fructose (Forssten *et al*., 2010).

Frequent high sucrose meals, combined with factors involving oral hygiene practices, aging, genetic factors, and immune changes, create conditions in the plaque that favor the propagation of the most highly acidogenic and acid-tolerant species such as members of mutans streptococci or lactobacilli**.**

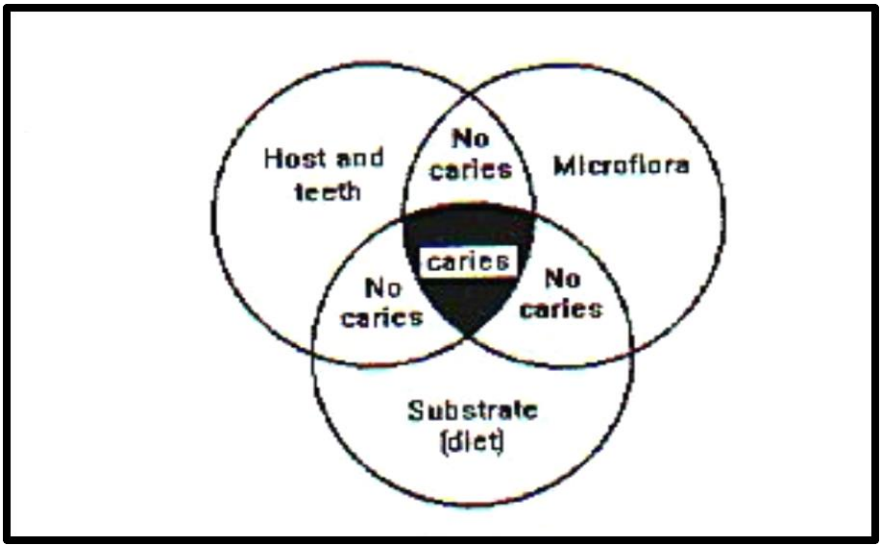


Figure (1-4) Etiological factors of dental caries (El – Shamare, 2007)

In environments with abundant available carbohydrate these acidic bacteria create an unfavorable niche for non acidic commensals leading to greater accumulation of cariogenic species(Mobley, 2003 and Filoche *et al*., 2010).

1.2.9 Bacteriocin immunity

Bacteria that produce bacteriocins always have immunity mechanism which protects them against the cognate bacteriocin. This system is highly specific and usually don't provide protection against other bacteriocins (Stein *et al.,* 2005). even though, some exceptions have been reported (Diep *et al*., 2007 and Franz *et al.,* 2000). For instance, lactococcin G immunity protein provides protection against lactococcin G, but not against enterocin(Nicolas *et al*., 2007).

1.2.10 Mutacin biosynthesis genes

There was no positive correlation between the number of indicator strains inhibited (antimicrobial spectra) and the number of biosynthesis genes expressed . The high diversity of mutacin-producing phenotypes,

associated with high frequency of expression of the biosynthesis genes screened, reveals a broad repertoire of genetic determinants encoding antimicrobial peptides that can act in different combinations.(Kamiya *et al*., 2008)**.**

S. mutans produces the non-lantibiotic mutacin IV, a non-lantibiotic class IIb bacteriocin encoded by the nlmA and nlmB genes (Qi *et al*., 2001), as well as an additional, as-yet unidentified, inhibitory agent (Hale *et al*., 2005b). Furthermore, analysis of the *S. mutans* genome sequence (Ajdic et al., 2002) has revealed ten small open reading frames with high similarity to the leader peptides of NlmA and NlmB that encode class IIb bacteriocins (nonlantibiotics), each possessing a double-glycine-type leader sequence similar to that of NlmA and NlmB (Hale *et. al*., 2005a; Van der Ploeg, 2005). The putative bacteriocins, designated Bsm (bacteriocin *S. mutans*), range in size from 47 to 87 aa, have leader peptides of 22–25 and contain a double glycine motif that can be recognized by the ComAB processing and export system (NlmTE). Some of the genes encoding the putative bacteriocins have been found located in tandem, indicating that they might act cooperatively, as is typical for class IIb bacteriocins; these peptides may represent a large repertoire of antimicrobial substances produced by *S. mutans* (Hale *et al*., 2005a; van der Ploeg, 2005).

The NlmA peptide consists of 44 amino acid residues, while the NlmB peptide consists of 49 amino acid residues. Both peptides contain signal sequences with well conserved GG motifs, the site where peptidase cleavage occurs during export. NlmTE, a transporter complex, cleaves the signal sequence and mediates the export of these peptides (Hale *et al*., 2005d).

S. mutans UA159 also secretes another nonlantibiotic peptide, mutacin V, which is a single peptide encoded by *nlmC*. The NlmC

peptide is 52 residues long, with a signal peptide and three GxxxG motifs. This mutacin is also secreted by the NlmTE export system and appears to have broad antimicrobial activity, ranging from mitis streptococci to lactococci and micrococcus(Hossain and Biswas , 2011).

1.2.11 Bacteriocin function

Bacteriocins are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria and archaea. Although research in this area is still in its infancy, there is intriguing evidence to suggest that bacteriocins may function in a number of ways within the gastrointestinal tract. Bacteriocins may facilitate the introduction of a producer into an established niche, directly inhibit the invasion of competing strains or pathogens, or modulate the composition of the microbiota and influence the host immune system..(Dobson *et al*., 2012).

Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" (Pineiro and Stanton 2007.). They are believed to enhance or maintain the ratio of beneficial to undesirable components in the human gastrointestinal (GI) microbiota (O'Hara and Shanahan, 2007). Several mechanisms of probiotic action have been described, the most common relating to their abilities to strengthen the intestinal barrier, to modulate the host immune system, and to produce antimicrobial substances (Corr *et al*., 2009).

Bacteriocins may function as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche (Riley and Wertz , 2002.). As shown in figure (1-5) alternatively, bacteriocins may act as antimicrobial or killing peptides, directly inhibiting competing strains or pathogens (Majeed *et al.*, 2011).

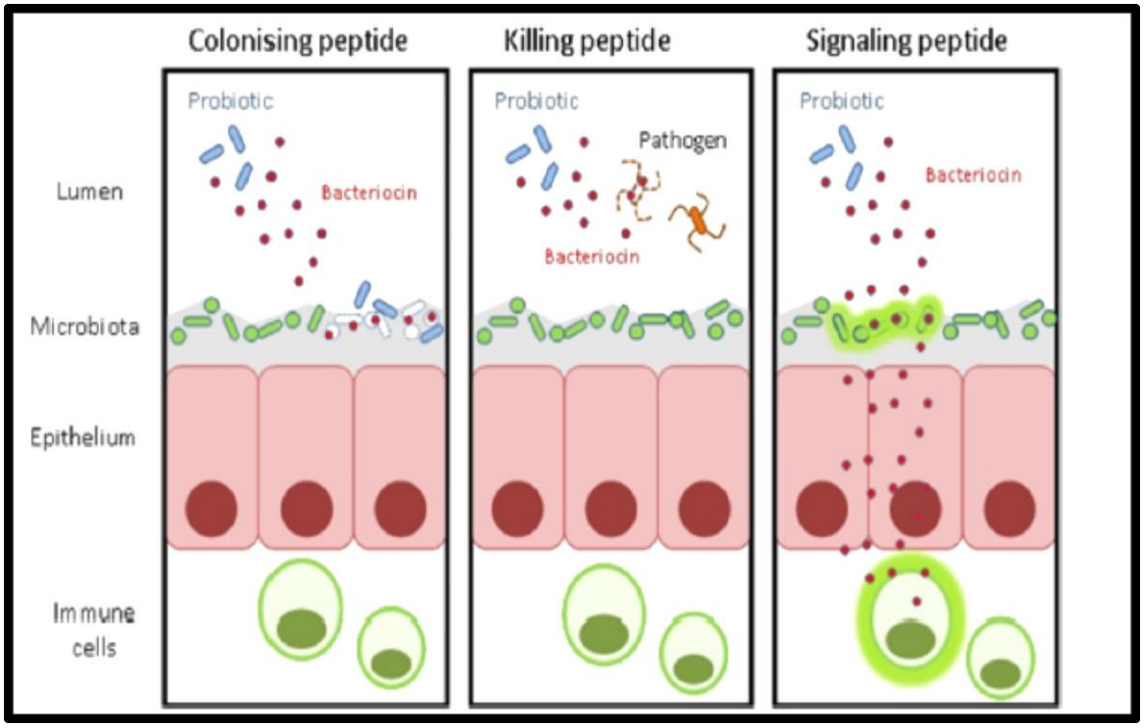


Figure (1-5): Mode of action of bacteriocin (Dobson *et al*., 2012)

1.2.12 In vivo application of bacteriocin

In addition to bacteriocinogenic probiotics, purified or partially purified bacteriocins also hold great promise with respect to the treatment of target pathogenic bacteria and may ultimately be employed as pharmabiotics and/or novel alternatives to existing antibiotics (Gillor and Ghazaryan, 2007). Studies using a mouse model of *Salmonella* Newport infection have shown that treatment with microcin J25 resulted in a 2- to 3-log reduction in viable numbers of the pathogen in both the spleen and the liver compared to those of control mice (Lopez *et al.*, 2007). Mersacidin, produced by *Bacillus* sp. strain HIL Y85, was also active against methicillin resistant *Staphylococcus aureus* (MRSA) in a hydrocortisone treated mouse rhinitis model (Kruszewska, 2004). This bacteriocin was able to completely eradicate MRSA from the nasal epithelium of the mouse, independent of the colonization time and number of inoculations. It has also been shown that a single dose of mutacin B-Ny266, produced by *Streptococcus mutans*, was 100%

protective when administered intraperitoneally to mice previously infected with methicillin-susceptible *S. aureus* (Mota-Meira *et al*., 2005).

Finally, it is noteworthy that both lacticin 3147 and thuricin CD, produced by *Lactococcus lactis* and *Bacillus thuringiensis*, respectively, exhibited inhibitory activity against *C. difficile* in an *ex vivo* model of the colon (Rea, 2010 and Rea, 2011). Thuricin CD is of particular interest, as this two-peptide bacteriocin was shown to be as effective as conventional antibiotics (e.g., metronidazole and vancomycin) in an *ex vivo* model of *C. difficile* infection. However, in contrast to conventional antibiotics, thuricin CD did not result in major alterations of GI populations, a contributing factor in recurrent *C. difficile* infection (Rupnik *et al*., 2009).

1.2.13 *Streptococcus mutans* plasmid

plasmids present in many gram-positive cocci usually encode for a variety of biological activities, including resistance to antibiotics, heavy metals, production of and/or resistance to bacteriocins, metabolic properties, immunity, and factors of pathogenesis. However, a 5.6-kb (corresponding to 3.6 megadalton) plasmid with a G_C content between 31 and 34 mol % in *S. mutans* has unknown functions and is thus still designated as ‘cryptic’. Interestingly, in clinical populations, it shows a relatively constant prevalence ranging from 5 to 13%.(Rheinberg *et. al*., 2013).

Caufield *et al*., (1988) also show that a cryptic plasmid resides in 5% of the isolates of *S. mutans.*

The function of this plasmid remains unknown, although its sequence has been published (Zou *et al*., 2001). Because of its high sequence variability in the hypervariable region (HVR) and its low prevalence, the cryptic plasmid is a useful epidemiological marker for studying transmission (Caufield *et al*., 1982) and, as here, its phylogenic history.

The 5.6-kb plasmid was initially thought to be related to bacteriocin production, because most bacteriocins of gram-positive bacteria are plasmid encoded . Subsequent discovery of the chromosomal locus for mutacins I, II, III, and IV (Qi *et al.,* 1999; Qi *et al*., 2001), coupled with sequencing of the plasmid, showed that mutacins are not plasmid encoded (Zou *et al*., 2001). Nonetheless, virtually all known plasmid-bearing strains of *S. mutans* elaborate either mutacin I or II, and these strains are also naturally competent (Caufield *et al.,* 2007). Moreover, mutacin and competence are coordinately expressed as part of an overall mechanism to acquire DNA (Kreth *et al*., 2005).

1.2.14 Plasmid curing

Deoxyribonucleic acid (DNA) is contained in all cells and is responsible for passing down traits from the parent organism to the offspring. Plasmids are extrachromosomal genetic elements which are wide spread in bacteria, they have their origin of replication, and autonomously replicates with respect to chromosomal DNA and stable inherited (Zelasko, 2005).

Plasmids influence the biology of the host, sometimes dramatically. Plasmids also display great diversity in size, mode of replication and transfer, host range, and the set of genes they carry , making them interesting elements for analysis. As tools, plasmids are indispensable in molecular biology, commonly used for cloning, expression, and mutagenesis(Shibayama, 2011).

In nature plasmid can lost spontaneously from a very few bacterial cells, but the probability of this loss is extremely low. However the majority of plasmids are extremely stable. And require to use of curing agents or other procedures that might increase the plasmid loss and these from the basis of artificial plasmid elimination (Molnar, 1988).

Acridine orange, ethidium bromide, sodium dodecyl sulfate (SDS) and phenothiazines were recognize as effective in vitro antibacterial and antiplasmid agents during the last three decad. Acridine dye and ethidium bromide were excluded from in vitro trails because of their mutagen effect as well as SDS, since i t has detergent action. A series of psychopharmacological agents without mutagenic were found to eliminate plasmid in vitro(Molnar, 1992).

Many studies already known that acridine orange, ethidium bromide and SDS affect plasmid replication. Some physical effects also influence plasmid replication like elevated growth temperature and thymine starvation (Trevors, 1986; Mojgani *et al*., 2006). Some antibiotics like rifampicin, chloramphenicol and mitomycin C also have effect on plasmid replication(Haque, 1979).

Bacterial plasmids have a major impact on metabolic function. Bacterial plasmids can be eliminated from bacterial species grown as pure or mixed bacterial cultures in the presence of sub-inhibitory concentrations of non-mutagenic heterocyclic compounds (Spengler *et al.*, 2006).

The efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the element involved, the bacterial strain, and the mode of action of the curing agent(Trevors, 1986). It is generally assumed that curing activity is related to the ability of these compounds to intercalate into superspiralized DNA and to inhibit its replication (Molnar *et al*., 1992).

2 . Materials and methods

2.1 Materials

2.1.1 Apparatus and Equipments

The following apparatus and equipments were used in this study:

Apparatus or Equipments	Company/Origin
Autoclave	Express/ West-Germany
Automatic pipettes	Witey/Germany
Balance	Ohaus/France
Compound light microscope	Olympus/Japan
Cooling centrifuge	Harrier/U.K.
Distillator	GFL/Germany
Electric oven	Gallenkamp/England
Electrophoresis apparatus	Bio Rad/ Italy
Eppendrof bench centrifuge	Netherler and Hinz/Germany
Incubator	Gallenkamp
Laminar air flow	Memmert/west-Germany
Micro centrifuge	Eppendorf/Germany
Millipore filter paper unit	Miliporecorp/USA
pH meter	Metler-Toledo/ U.K.
Power supply	LKB/ Sweden
Sensitive balance	Delta Range/Switzerland
Shaker incubator	GFL/Germany
Spectrophotometer/visible	Apel-PD303/Japan
Vitek2	Biomerierx/ France
Vortex	Buchi/Switzerland

2.1.2 Chemicals

The following chemicals were used in this study:

Chemicals	Company/Origin
Agar-Agar	Biolife/Italy
Agarose, Ethidium bromide	Sigma/USA
Absolute ethyle alcohol, Ethylenediamine-tetraacetic acid (EDTA), Peptone, Treptone, Ammonium sulphate, HCl, Maltose, K_2HPO_4, KH_2PO_4, NaCl	BDH/England
Glucose, Sucrose, Mannitol, Sorbitol, Raffinose	Sigma/Germany
Ethanol, NaOH	Merck/Germany
Hydrogen peroxide, Fructose	Fluka/ Switzerland

2.1.3 Antibiotics

The following antibiotic discs (supplied by Bioanayse/ Turky) were used during this study:

Antibiotic	Abbreviation	Concentration (µg / disk)
Amoxicilin	AmC	10
Ampicillin	Amp	10
Bacitracin	BA	10
Cephalothin	Cph	10
Chloramphenicol	Chl	30
Clindamycin	Cli	20
Erythromycin	E	15
Gentamycin	Gen	10
Impenem	IpM	10
Vancomycin	Van	30

2.1.4 kit

Kit	Company/ Origin
Plasmid Mini Extraction Kit	Bioneer/ Japan

2.1.5 Test microorganisms

Strain	Source
Streptococcus pyogenes	Department of Biotechnology/College of Science/ AL-Nahrain University
Escherichia coli	Department of Biotechnology/College of Science/ AL-Nahrain University

2.1.6 Media

2.1.6.1 Ready to use Media

The following media were prepared as recommended by their manufacturing companies. pH was adjusted to 7.0 and sterilized by autoclave at 121°C for 15 min.

Medium	Company/Origin
Brain- Heart Infusion agar	Difco/ USA
Brain- Heart Infusion broth	Difco
Muller Hinton agar	Oxoid/ England
Luria broth	Oxoid

2.1.6.2 Laboratory Prepared Media

2.1.6.2.1 Mitis salivarius bacitracin agar(MSBA)(Yoo *et al*., 2005)

This medium was consisted of (90 g/L) of mitis salivaris agar and (150 g/L) of sucrose were dissolved in 950 ml distilled water, pH was adjusted to 7.2, then volume was completed to 1000 ml with distilled water, and sterilized by autoclaving. After cooling to 45°C , 1 ml of bacitracin solution (200 I.U/ liter) was added , the medium was poured into sterile plates. This medium was used for isolation and identification of *S. mutans*.

2.1.6.2.2 Carbohydrates fermentation medium (Atlas and Snyder, 2006)

This medium was prepared by dissolving 3.7 g of brain-heart infusion broth and 0.1 g phenol red in 100 ml of distilled water, pH was adjusted to 7.0 and sterilized by autoclaving. After cooling to room temperature, the medium was distributed into sterilized screw-capped tubes with 10 ml increments, then 1 ml of 10% of carbohydrates solution (mannitol, sorbiotl and raffinose) sterilized by filtration was added to each tube under aseptic condition.

2.1.6.2.3 Blood agar (Atlas and Snyder 2006)

This medium was prepared by dissolving 33g of blood base in 950 ml of distilled water, pH was adjusted to 7.0 and sterilized by autoclaving. after cooling to room temperature, 50 ml of human blood was added and mixed thoroughly, then medium was poured into sterile plates. This medium was used to determine the type of hemolysis.

2.1.7 Reagents and Dyes

2.1.7.1 Catalase Reagent (Atlas *et al*., 1995)

This reagent was prepared to be consisting of 3% hydrogen peroxide.

2.1.7.2 Gram stain

Gram stain was prepared according to Atlas *et al*. (1995).

2.1.7.3 Bacitracin solution (200 I.U/ liter)

Bacitracin solution was freshly prepared according to Yoo *et al*. (2005) by dissolving 0.364 g of bacitracin in 100 ml distilled water, mixed thoroughly and sterilized by filtration.

2.1.8 DNA extraction buffers and solutions

Buffers and solutions used for DNA extraction from the bacterial isolate were prepared according to Maniatis *et al*. (1982).

2.1.8.1 SET buffer

This solution consisted of the followings:

NaCl 75 mM

EDTA 25 mM

Tris-HCl 20 mM

pH was adjusted to 8 and sterilized by autoclave at 121°C for 15 min.

2.1.8.2 TE buffer

This solution was prepared from (10 mM) of Tris-HCl and (1mM) of EDTA, then pH was adjusted to 8, and sterilized by autoclave at 121°C for 15 min.

2.1.8.3 Lysozyme solution (3%)

It was freshly prepared by dissolving 30 mg of lysozyme in 1ml of sterile distilled water.

2.1.8.4 5X Tris-Borate-EDTA (TBE) buffer solution

This solution was prepared from the followings:

Tris-OH 54 g

Boric acid 27.5 g

EDTA 20 ml

All components were dissolved in 900 ml of distilled water, pH was adjusted to 8, then completed to 1000 ml and sterilized by autoclave at 121°C for 15 min . After cooling, the solution was kept at 4°C until use.

2.1.8.5 loading buffer 6X

This solution was prepared from (0.25 g) of Bromophenol blue and (40 g) of Sucrose, which dissolved in 100 ml distilled water and sterilized by autoclaving. After cooling, the solution was kept at 4°C until use.

2.1.8.6 Ethidium bromide solution (10 mg/ ml)

It was prepared by dissolving 1 gm of ethidium bromide in 100 ml of distilled water, stirred by magnetic stirrer until ethidium bromide was

completely dissolved, then it was filtered using Whattman filter paper No. 1 and stored in dark bottle at 4°C until use.

2.2 Methods

2.2.1 Sterilization methods

Three methods of sterilization were used in this study as follow:

a- Moist Heat Sterilization (Autoclaving)

Culture media and solutions were sterilized by autoclaving at 121 °C (15 Ib/in^2) for 15 minutes, except for some heat sensitive solutions which were sterilized for 10 minutes under the same condition.

b- Dry Heat Sterilization

Electric oven was used to sterilize glassware and some other tools at 180 °C for 3 h.

c- Membrane Sterilization (Filtration)

Millipore filter unit was used to sterilize the heat sensitive solutions by using Millipore filter (0.45mm).

2.2.2 Collection of samples

Eighty samples were collected by taking swabs from mouth cavity from patient with different dental caries (pit, fissure and dental roots), These samples were put into peptone water and then streaked on semi-selective Mitis Salivaris agar medium (MSA) for the period between November 2013 and January 2014. Those patients were attended Al-Dora Health Center and Al-Zewiya Health Center in Baghdad city.

2.2.3 Isolation of *Streptococcus mutans*

Bacterial isolates obtained from dental caries samples were streaked on selective medium (Mitis Salivaris Bacitracin Agar) prepared in (2-1-6-2-1) for isolation of *S. mutans*, then plates were incubated at 37°C for 48 h under anaerobic conditions in candle jar. This step was repeated until pure culture was obtained (Nolte, 1982).

2.2.4 Identification of *Streptococcus* isolates(Carlsson, 1967; Emilson, 1983)

Bacterial isolates were identified according to their morphological (microscopic) and cultural characteristics, biochemical tests and VITIK-II.

2.2.4.1 Morphological and cultural characteristics

Colony size, shape and color of the bacterial isolates were studied on Mitis Salivaris Bacitracin agar prepared in (2-1-6-2-1) plates after incubation at 37°C for 24 h under anaerobic conditions in candle jar.

Shape , clump and arrangement of cells were studied under microscope.

2.2.4.2 Biochemical tests

2.2.4.2.1 Catalase test (Brown, 2005)

Clump of growth from pure culture of each bacterial isolate was transferred onto a microscopical slide using a wooden stick applicator, then two drops of 3% hydrogen peroxide solution were added on bacterial cells. Presence of gaseous bubbles indicates a positive result (production of catalase).

2.2.4.2.2 Blood hemolysis test (Collee *et al.*, 1996)

This test was used to detect the ability of bacterial isolates to produce hemolysin and determine the type of hemolysis, and was achieved by streaking each bacterial isolate on blood agar medium, then incubated at 37°C for 48 h under anaerobic conditions.

2.2.4.2.3 Carbohydrate fermentation test (Yoo *et al.*, 2005)

This test was used to detect the ability of bacterial isolates to utilize different carbon sources (mannitol, sorbitol and raffinose) as a sole

source of carbon and energy. This was achieved by inoculating test tubes of brain heart infusion broth containing each carbon source (3%) with each bacterial isolate. All tubes were then incubated at 37°C for 24hrs under anaerobic conditions . The change in color of indicator from red to yellow indicates a positive result.

2.2.4.3 Identification by using VITEK-II **identification system**

The innovative VITEK-II microbial identification system includes an expanded identification database, the most automated platform available, rapid results, improved confidence, with minimal training time. Bacterial isolates suspected to be *S. mutans* were completely identification by using VITEK-II identification system.

A sterile swab or wooden stick applicator was used to transfer bacterial colonies from a pure culture and was suspend in 3.0 ml of sterile saline in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted to 0.8 and measured using a turbidity meter called the DensiChek, then identification cards were inoculated with bacterial suspensions using an integrated vacuum apparatus. A test tube containing cells suspension were placed into a special rack (cassette) and the identification card was placed in the neighboring slot, while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed either manually or transported automatically into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the cells suspension was forced through the transfer tube into micro-channels that fill all the test wells.

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. All card types were incubated on-line at 35.5 , then Each card

was removed from the carousel incubator once every 15 minutes, and transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data were collected at 15-minute intervals during the entire incubation period.

2.2.5 maintenance of bacterial isolates

Maintenance of bacterial isolates were achieved according to Maniatis *et al*. (1982).

2.2.5.1 Short-term storage

For routinely working, bacterial isolates were maintained for few weeks on mitis salivaris Bacitracin agar prepared in (2-1-6-2-1) and incubated at 4°C.

2.2.5.2 Medium-term storage

Bacterial isolates were maintained on slants of brain-heart infusion agar medium and incubated for 18 h at 37°C and then stored at 4°C for few months.

2.2.5.3 Long term storage

Bacteria can be stored for many years in medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacterial isolates in screw-capped tubes with final volume 10 ml and stored at -20°C.

2.2.6 Screening bacterial isolates for Mutacin production

Bacterial isolates identified as *S. mutans* were examined to detect their ability in mutacin production.

2.2.6.1 Induction of Mutacin production (Nicolas *et al*., 2004)

Induction of mutacin production by the bacterial isolates were achieved by supplementing brain heart infusion broth (as production medium) with yeast extract 2% (w/v) and $CaCO_3$ 1% (w/v) and inoculating the production medium with activated culture (O.D = 0.8) of *S. mutans*,, then cultures were incubated at 37°C for 24 h under anaerobic conditions. After incubation, the cultures were incubated at 70 °C for 10 minutes to kill the cells and inhibit protease activity, then centrifuged at 6000 rpm for 10 minutes. The supernatant was regarded as a crude mutacin.

2.2.6.2 Detection of inhibitory effect of mutacin (Nicolas *et al*., 2004)

The antibacterial activity of mutacin produced by the local isolates which identified as *S. mutans* were studied by detecting the inhibitory effect of mutacin against the test microorganisms (*S. pyogene*s and *E. coli*) grown on BHI agar plates. The antagonistic effect against the test microorganisms was performed by the well diffusion assay as follows:

- Brain heart infusion broth was inoculated with the fresh culture of test microorganisms (*S. pyogene*s and *E. coli*) and incubated at 37°C for 24 h.
- A portion of 100 µL from the overnight culture of *S.pyogene*s and *E. coli* separately was taken and spread on brain heart infusion broth plates by sterile cotton swabs.

- Immediately wells were made in the agar using sterile cork borer, then each well was filled with 50 µl of crude filtrate of the bacterial isolate (identified as *S. mutans*).
- Plates were then incubated at 37°C for 24 h under anaerobic condition in candle jar. The inhibitory effect of mutacin was detected by measuring the diameter of inhibition zones around *S.pyogene*s and *E. coli* which appeared as clear circular zones surrounding the wells.

2.2.7 Determination of optimal conditions for mutacin production

Different nutritional and growth factors were studied to determine the optimal conditions for mutacin production by the selected isolate of *S. mutans*. These experiments were achieved by inoculated the production medium with activated suspension (O.D = 0.8) of the selected isolate of *S. mutans*, then cultures were incubated at 37°C for 24 h under anaerobic conditions. After incubation, cultures were incubated at 70 °C for 10 minutes to kill the cells and inhibit protease activity, then centrifuged at 6000 rpm for 10 minutes. The supernatant was regarded as the crude mutacin. The inhibitory effect of culture filtrate (crude mutacin) against test microorganism was examined by the well diffusion method.

2.2.7.1 Effect of carbon source

To determine the optimum carbon source for mutacin production, five carbon sources (maltose, sucrose, galactose, fructose, glucose) were added, separately, to the production medium in a concentration of 3% (w/v). The cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7). after determining the optimum carbon source, this source was used in the next experiment of optimization.

2.2.7.2 Effect of carbon source concentration

To determine the optimum concentration of carbon source for mutacin production, eight concentration (1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5% w/v) of the appropriate carbon source were added to production medium, then cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7). Optimum carbon source concentration was used in the next experiment of optimization.

2.2.7.3 Effect of nitrogen source

Six nitrogen sources were used to determine the optimum for mutacin production. These sources including organic source (yeast extract, pepton and malt extract) and inorganic source (sodium nitrate, ammonium chloride and ammonium nitate) were added, separately, to the production medium in a concentration of 2% (w/v). Cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7).Optimum nitrogen source was used in the next experiment of optimization.

2.2.7.4 Effect of nitrogen source concentration

Different concentrations of the best nitrogen source were used to determine the optimum for mutacin production. For this purpose, six concentrations of the appropriate nitrogen source (0.5, 1, 1.5, 2, 2.5 and 3% (w/v)) were added to supplement the production medium . Cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7). Optimum nitrogen source concentration was used in the next experiment of optimization.

2.2.7.5 Effect of pH

In order to determine the optimum pH for mutacin production, pH of mutacin production medium was adjusted to different pH values (5.5, 6, 6.5, 7, 7.5 and 8), cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7). Optimum medium pH was set in the next experiments of optimization to adjust production medium.

2.2.7.6 Effect of temperature

In order to determine the optimum incubation temperature for mutacin production by the selected isolate, production medium were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated at different temperatures (20, 25, 30, 37, 40 and 45°C)and treated as in (2.2.7). Optimum temperature was fixed in the next experiment of optimization for incubation cultures of *S. mutans*.

2.2.7.7 Effect of phosphate source

To determine the effect of phosphate source on mutacin production by the selected isoate of *S. mutans*, two phosphate sources (KH_2PO_4 and K_2HPO_4) were added to the production medium in a concentration of 0.1%. Cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7). Optimum phosphate source was used in the next experiment of optimization.

2.2.7.8 Effect of phosphate source concentration

Different concentrations of the best phosphate source were used to determine the optimum for mutacin production. For this purpose, three concentrations of the appropriate phosphate source (0.5, 0.1, and 1%)

were added to the production medium. Cultures were inoculated with the activated suspension of the selected isolate of *S. mutans* and incubated and treated as in (2.2.7).

2.2.8 Antibiotic susceptibility test (Atlas *et al*., 1995).

The disk diffusion method was used to test the antibiotic susceptibility of the selected isolate toward different antibiotics . A sterile cotton swab was dipped in activated suspension of *S. mutans* (O.D = 0.8) and streaked on the surface of Muller-Hinton agar plates by rotating the plate approximately 60 between streaking to ensure even distribution. The inoculated plates were incubated at room temperature for 10 minutes to allow absorption of excess moisture, then antibiotic disks (bacitracin , erythromycin , amoxicilin, ampicillin, chloramphenicol, vancomycin, clindamycin, impenem, gentamycin and cephalothin) were fixed by sterile forceps on the surface of plates and incubated at 37°C for 18 hrs. in an inverted position.

After incubation, diameters of inhibition zones (clear zone area around each antibiotic disk) were measured in (mm).

2.2.9 plasmid content

plasmid profiles of the selected isolate of *S. mutans* was achieved by using *AccuPrep*® Plasmid Mini Extraction Kit supplied by Bioneer/ Japan as follows:

- A single colony of the selected isolate of *S. mutans* was used to inoculate the 50 ml of brain heart infusion broth in a 50ml and incubated at 37°C for 24 h under anaerobic conditions, then cultures were pelletd by centrifuged at 6000 rpm for 15 min.
- Bacterial pellets were washed twice with 5 ml of SET buffer prepared in (2.1.8.1) , mixed by vortexing, and centrifuged at 6000

rpm for 15 min, then cells were re-suspended in 1.6 ml of SET buffer.

- Aliquot of 100 µl of freshly prepared lysozyme prepared in (2.1.8.3) was added and incubated at 37°C for 1 h.
- Cells were collected by centrifugation at 8000 rpm for 2 minute, then re-suspended in 250 µl of buffer 1 and mixed by vortex.
- Cell suspention was centrifuged at 8000 rpm for 2 minutes, and 250 µl of buffer 2 was added and mixed by inverting the tube gently for 3 to 4 times.
- Aliquot of 350 µl of Buffer 3 and immediately mixed by inverting the tube 3-4 times gently and Tubes were centerifugated at 13000 rpm, at 4℃ for 10 min.
- Clear lysate was transferred into DNA binding column tube and centrifuged at 13000 rpm for 1 min. Pour off the flow-through and re-assemble the DNA binding filter column with the 2.0 ml collection tube.
- Aliquot of 500 µl of Buffer D was added and left to stand for 5 min, then centrifuged at 13,000 rpm for 1 min. Pour off the flow-through and re-assemble the DNA binding filter column with the 2.0 ml collection tube.
- Aliquot of 700 µl of Buffer 4 was added to the DNA binding column, then tubes were centrifuged at 13,000 rpm for 1 min. Pour off the flow-through and re-assemble the DNA binding filter column with the 2.0 ml collection tube.
- DNA binding filter column was transferred to new 1.5 ml microcentrifuge tube, then 50-100 µl of Buffer 5 was added to the DNA binding filter column, and left to stand for at least 1 min. for elution step.

- plasmid DNA was eluted by centrifugation at 13,000 rpm for 1 min.
- The precipitated DNA was dissolved in 100 µl TE buffer and stored at 5 ℃.

2.2.10 Agarose Gel Electrophoresis (Maniatis *et al.*, 1982)

Plasmid DNA samples extracted from the selected isolate of *S. mutans* were loaded into the well of agarose gel (0.7%) after mixing with loading buffer prepared in (2.1.8.5), then gel electrophoresis was run horizontally in Tris-borate EDTA (1X TBE buffer) prepared in (2.1.8.4) for 2-3 h at 5v/cm. After electrophoresis, gel was stained with ethidium bromide in concentration of 0.5 µg/ml for 30-45 min, then DNA bands were visualized under UV ray.

2.2.11 Curing of plasmid DNA

Curing experiment was achived to investigate the role of plasmid of *S. mutans* in mutacin production . Ethidium bromide was used as the curing agent, according to Zaman *et al.* (2010) .

Bacterial cells of the selected isolate was grown in brain heart infusion broth to mid log phase, then 0.1 ml inoculum of the growth culture was used to inoculate each of the series of tubes contaninig 5 ml brain heart infusion broth with different concentrations of ethidium bromide (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml), then all tubes were incubated at 37°C for 24 h.

Growth density of different tubes was observed by nacked eye and compared with the control to determine the effect of ethidium bromide on growth of bacterial cells. The sub-lethal concentration of agent that inhibits the growth of bacterial cells was considered as the minimum inhibitory concentration (MIC). Samples were taken from tubes containing the highest concentration of ethidium bromide that still allow

bacterial growth, and diluted appropriately, then 0.1 ml from the proper dilution was taken and spread on brain heart infusion agar plates and incubated for overnight at 37°C . One hundred of survived colonies were analyzed for the presence or absence of antibiotic resistance traits as a result of plasmid curing. Those colonies were replica plated (using toothpick) on brain heart infusion agar (master plate), and on brain heart agar plates contining antibiotics to which the original isolate was resist.

If colonies were able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it means that cells of this colony are cured cells that lost the resistance of this antibiotic. These cured colonies were tested for their ability to inhibit of *S. pyogene*s growth, in addition to investigate the presence of plasmid in each colony.

3. Results and Discussion

3.1Isolation of *S. mutans*

The mouth cavity samples were put into peptone water and then streaked on semi-selective Mitis Salivaris agar medium (MSA). This medium promote the growth of streptococci and inhibits the growth of other bacterial species because this medium contains crystal violet and potassium tellurite which inhibit most Gram-negative bacilli and Gram-positive bacteria except streptococci(Zimbro *et al*., 1998). Bacterial isolates grown on MSA were then re-streaked on selective Mitis Salivaris Bacitracin agar (MSBA) which composed of MSA , 20% sucrose and 0.2 U/ml of Bacitracin to inhibit growth of most bacteria except *S.mutans* and *S.sorbinus*. The inclusion of sucrose in this medium leads to the formation of glucan and distinctive colony appearance that aids identification of *S. mutans* (Yoo *et al.,* 2007)**.** Bacterial isolates then streaked on brain heart infusion agar plates and incubated at 37°C under anaerobic conditions. Colonies of these isolates appeared as pin points into the culture medium and surrounded by a white opaque halo.

3.2 Identification of bacterial isolates

Bacterial isolates suspected to be *S.mutans* were grown on selective medium (MSBA) and identified according to their morphological and cultural characteristics and biochemical test:

3.2.1 Morphological and cultural characteristics

Bacterial isolates grown on Mitis Salivaris Bacitracin agar medium were the first identification according to their morphological and cultural characteristics. Results showed that these isolates were Gram positive, spherical cells and appear in medium chains . Colonies of these isolates are

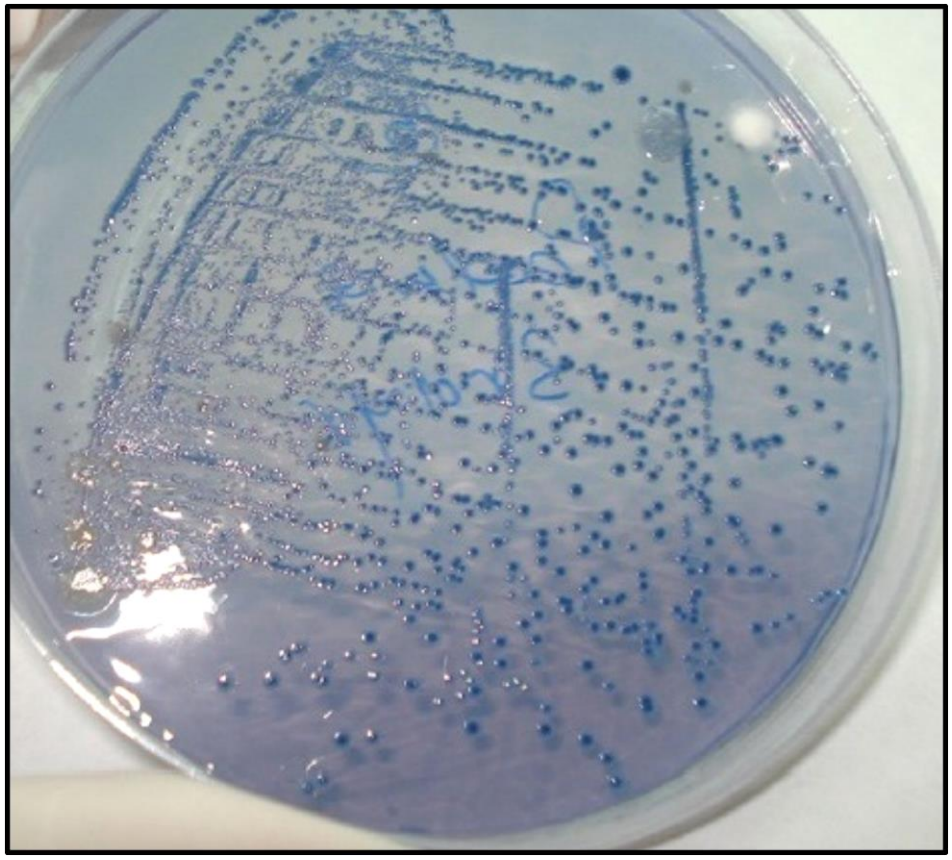


Figure (3.1): *Streptocoocus mutans* on Mitis Salivaris agar after incubation at 37°C for 48 h under anaerobic conditions

highly convex, raised, light – blue, frosted glass appearance with smooth surface as shown in figure (3-1). These colonies were also highly adherent to the agar surface if it picked up by loop, polysaccharide formaters was observed as a glistening drop on top of the colony or as a pool besides the colony. This result was agree with Koneman *et al*, (1998).

3.2.2 Biochemical tests(Yoo *et al*., 2005)

When bacterial isolates suspected to be belong to the genus *Streptococcus* were subjected to examined for their biochemical characteristics, results showed that these isolates were Gram positive, negative for catalase and able to ferment mannitol, sorbitol and raffinose sugars. These isolates were unable to produce hemolysin exhibiting gamma

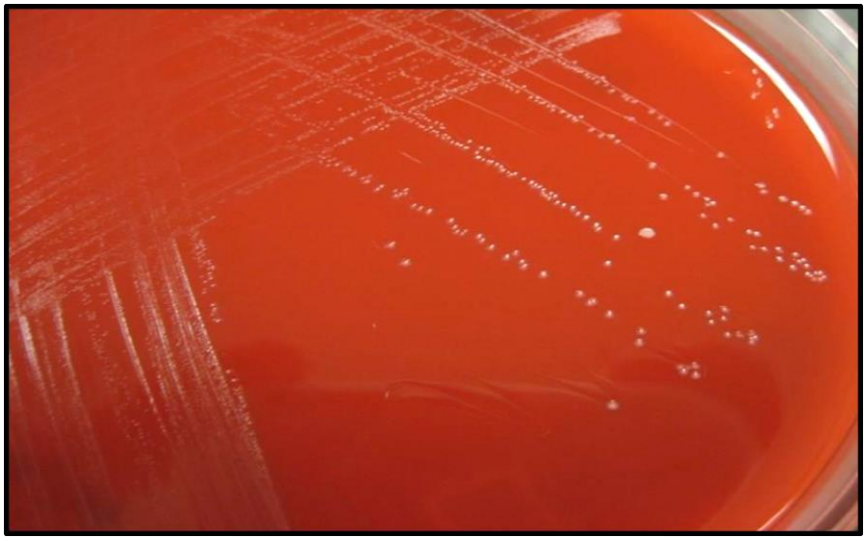


Figure (3.2): Local isolates of *Streptococcus* grown on blood agar medium after incubation at 37°C for 24 h under anaerobic conditions

hemolysis on blood agar medium as shown in figure (3.2). According to the such results, ten isolates were regarded as *S. mutans*.

To confirm the identification of the ten bacterial isolates as *S. mutans*, they were examined also by the VITEK-П system. Result indicated in appendix (1) showed that *S. mutans* were negative to hydrolysis of urease (URE) and arginine dihydrolase1(ADH1) and positive to alpha-glucosidase (AGAL) and fermentation of Lactose (LAC).

3.3 Screening of *S.mutans* isolates in mutacin production

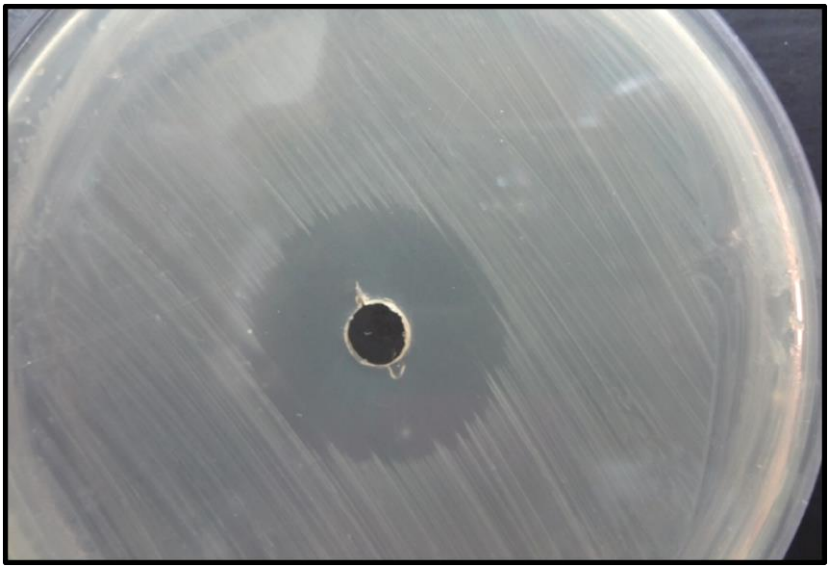
Locally isolated *S.mutans* were screened in order to select the efficient isolate in mutacin production. The ability of these isolates in mutacin production was assayed after culturing at 37°C in brain heart infusion broth and incubation till the optical density was reached 0.8, then crude mutacin in the culture filtrate was used to study the antagonistic effect against the test microorganisms (*S. pyogen*es and *E.coli*) by measuring the inhibition zones according by well diffusion method.

Table (3-1): Ability of local isolates of *Streptococcus mutans* in mutacin production termed by inhibition zones against *Streptococcus pyogene*s.

Isolate symbol	Inhibition zone (mm)
S1	18
S2	20
S3	15
S4	10
S5	7
S6	14
S7	10
S8	15
S9	17
S10	9

Results in table (3-1) showed that all of the 10 isolates of *S.mutans* were mutacin producers according to the inhibition zones produced against *S.pyogen*es . Diameters of inhibition zones were ranged between 7 and 20 mm.

On the other hand, it was found there was no any inhibitory effect of mutacin produced by the local isolates of *S. mutans* on the second test microorganism (*E. coli*). This may be due to the nature of *E. coli* cell wall that haven't specific receptor for mutacin in addition to the selectivity of cell membrane toward different harmful molecules in culture medium as mentioned by Mota-meira *et al*., (2000) which also added that most Gram negative bacteria were resistant to the mutacin.



Figure(3-3): Inhibitory effect of mutacin produced by locally isolated *Streptococcus mutans* S2 against *Streptococcus pyogenes* on brain heart infusion agar after incubation at 37°C for 24 h under anaerobic conditions

Among the ten isolates of *S.mutans*, the *S. mutans* S2 was the best in mutacin production which gave the maximum diameter zone of inhibition (20 mm) against *S.pyogen*es as shown in figure (3-3). According to these results, this isolate was selected to study the optimum conditions for mutacin production.

Mota-meira *et al*. (2005) also showed that mutacin was active against Gram-negative bacteria only after treatments altering the outer membrane thus giving access to the cytoplasmic membrane.

3.4 Optimum conditions for mutacin production by *S. mutans*

Optimum conditions for mutacin production by the locally isolated *S.mutans* S2 were studied using different parameters of growth condition affects mutacin production as follows:

3.4.1 Optimum carbon source

Results indicated in figure (3-4) showed that maximum production of mutacin was achieved when sucrose was used as a sole source for carbon

and energy to induce mutacin production by locally isolate *S.mutans* S2. Inhibition zone of mutacin against *S.pyogene*s was increased to 22 mm in comparison with the other carbon sources (galactose, maltose, glucose and fructose) that gave lower inhibition zone diameters against same bacteria (18, 17, 15, 15 respectively).

As mentioned above, sucrose was the optimal carbon source for mutacin production because it is an important carbohydrate and energy source for the growth and production and can be used easily by microorganism.

S. mutans was able to utilize sucrose as a carbon source for the production of intracellular storage components and for the production of extracellular glucans as mentioned by Kreth *et al*., (2008). In another study, Nicolas *et al*., (2004) found that the best production of mutacin was obtained with the use of sucrose as a sole source for carbon and energy.

Sucrose has been considered the most cariogenic carbohydrate due to its ability to increase porosity of dental plaque matrix formed in its presence.

In addition to a glucan-rich matrix, *in situ* dental plaque formed in the presence of high frequency of sucrose exposure shows a low inorganic concentration of calcium, phosphorous and fluoride , considering that sucrose is also a fermentable carbohydrate, maintaining low pH that may induce an ecological changes in dental plaque, with an increase in mutans streptococci levels, therefore, a high porosity, low inorganic concentration and high levels of mutans streptococci in dental plaque are factors that can explain the high cariogenicity of sucrose(Cury *et al*., 2001).

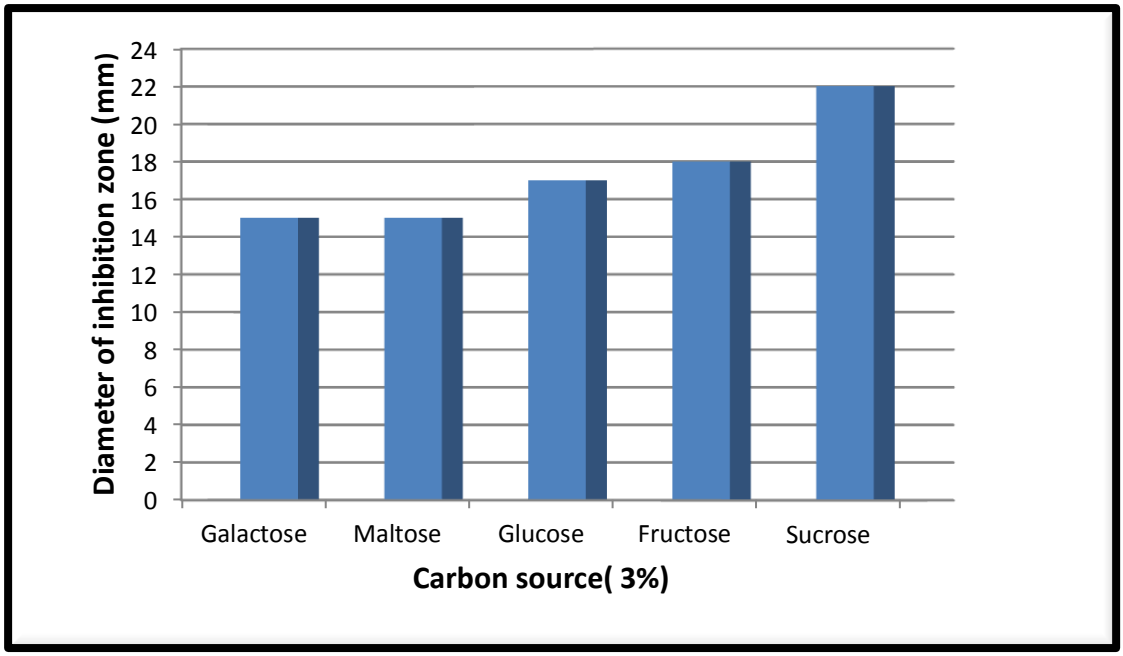


Figure (3-4): Effect of different carbon sources on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

According to this result, the optimum carbon source (sucrose) was used in the next experiment of optimization for mutacin production.

3.4.2 Optimum of sucrose concentration

Results shown in figure (3-5) indicate that maximum mutacin production was obtained when sucrose was added to the production medium in a concentration of 3%. At this concentration, anatagonistic effect of mutacin in culture filtrate of *S. mutans* S2 against *S. pyogenes* reached the maximum when the inhibition zone increased to 23 mm in comparison with the other concentrations of sucrose. Nicolas *et al.*, (2004) showed that mutacin production by S. *mutans* in a liquid medium

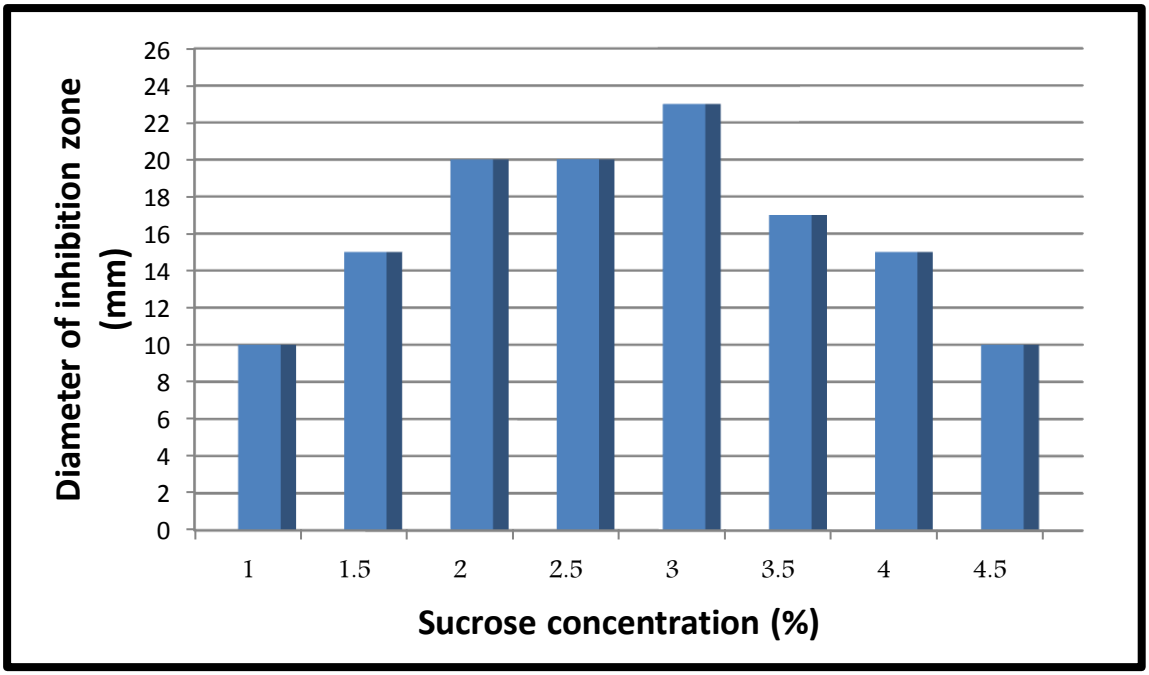


Figure (3-5): Effect of sucrose concentration on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

supplemented was increased by using sucrose at concentration lower then 5 % . According to this result, sucrose in a concentration of 3% was used in the next experiment of optimization for mutacin production.

3.4.3 Optimum Nitrogen source

Results in figure (3-6) showed that the maximum mutacin production was achieved when yeast extract was used as a nitrogen source. Crude mutacin in the culture filtrate of *S. mutans* S2 gave the inhibitory effect on growth of the test microorganism (*S. pyogenes*) when its inhibition zone reached 27 mm in diameter. On other hand, the inhibitory effect of other nitrogen source against *S. pyogenes* was decreased.

In general, organic nitrogen sources (yeast extract, peptone and malt extract) were better than the inorganic sources (sodium nitrate, ammonium

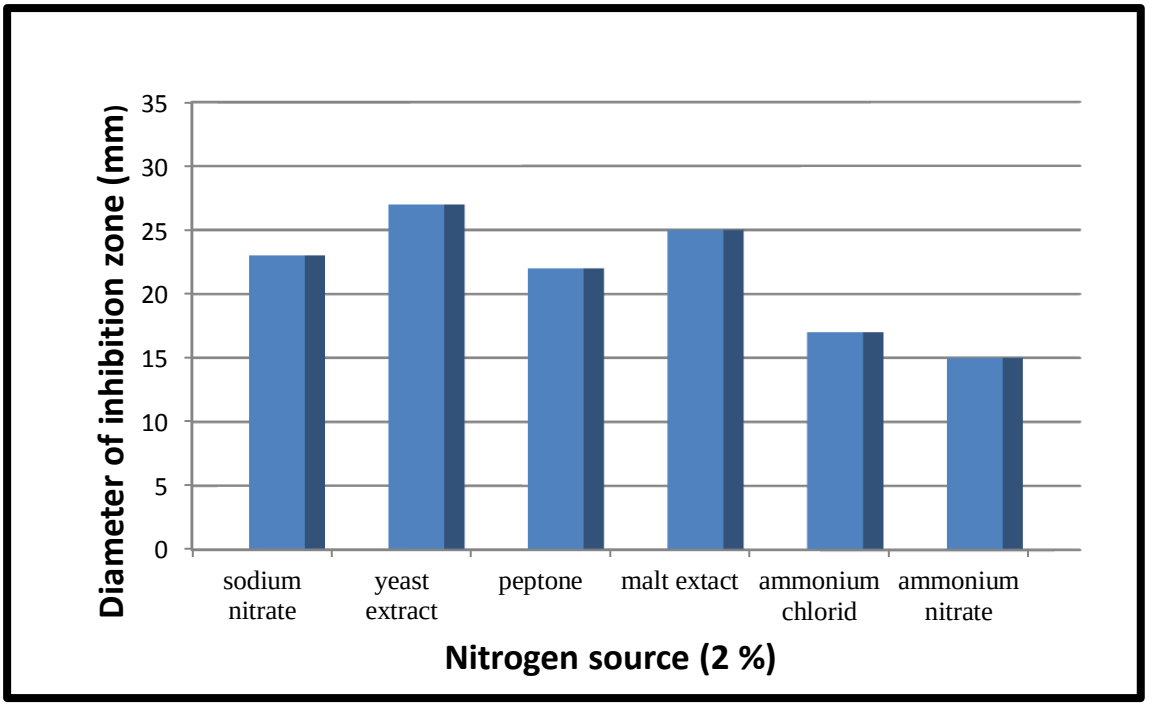


Figure (3-6): Effect of different nitrogen sources on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

chloride and ammonium nitrate) in induction of mutacin production by *S. mutans* S2 as shown in figure (3-6). It has been suggested that the organic nitrogen sources could contain peptides that are essential for the biosynthesis of mutacin or may act as inducers of mutacin production . Their positive effect can also be related to their high content in mineral,vitamins and amino acid such as serine, cysteine and threonine. Amino acids could have a precursor role during mutacin synthesis. Furthermore, yeast extract was also shown to contain fermentable sugars which could contribute to the increase mutacin production (Nicolas *et al*., 2004).

According to these results, yeast extract was used in the next experiments of optimization for mutacin production.

3.4.4 Optimum yeast extract concentration

Different concentrations of the optimum nitrogen source (yeast extract) were used to determine the optimum for mutacin production by locally isolated *S.mutans* S2. Results in figure (3-7) indicate that maximum mutacin production was obtained when yeast extract was added to the production medium in a concentration of 2% (w/v). at this concentration the antagonistic effect of the produced mutacin against *S.pyogenes* reached the maximum according to the result of inhibition zones that reach 28 mm in comparison with the other concentrations that cause less production of mutacin and then less effect of antagonism against *S.pyogen*es. The inhibition zones were 19,20, 23, and 25 when the production medium contain yeast extract in a concentration of 0.5, 1, 1.5 and 2.5, respectively.

This result confirm with Nicolas *et al*., 2004 who found that mutacin production was improved when yeast extract was added to the production medium in a concentration of 2%, while Li *et al*., 2002 found that mutacin yield was increased with increasing the amount of yeast extract up to a concentration of 1%.

According to these results, yeast extract was used in a concentration of 2% in the next experiments of optimization for mutacin production.

3.4.5 Optimum medium pH

To investigate the optimum initial medium pH on mutacin production by *S.mutans* S2, production medium was adjusted to different pH values. Results in figure(3-8) indicate that the maximum mutacin production was obtained when the pH value of mutacin production medium was adjusted to 6. The antagonistic effect of mutacin produced under this pH against *S.pyogenes* reached the maximum with an inhibition zone of 32 mm in

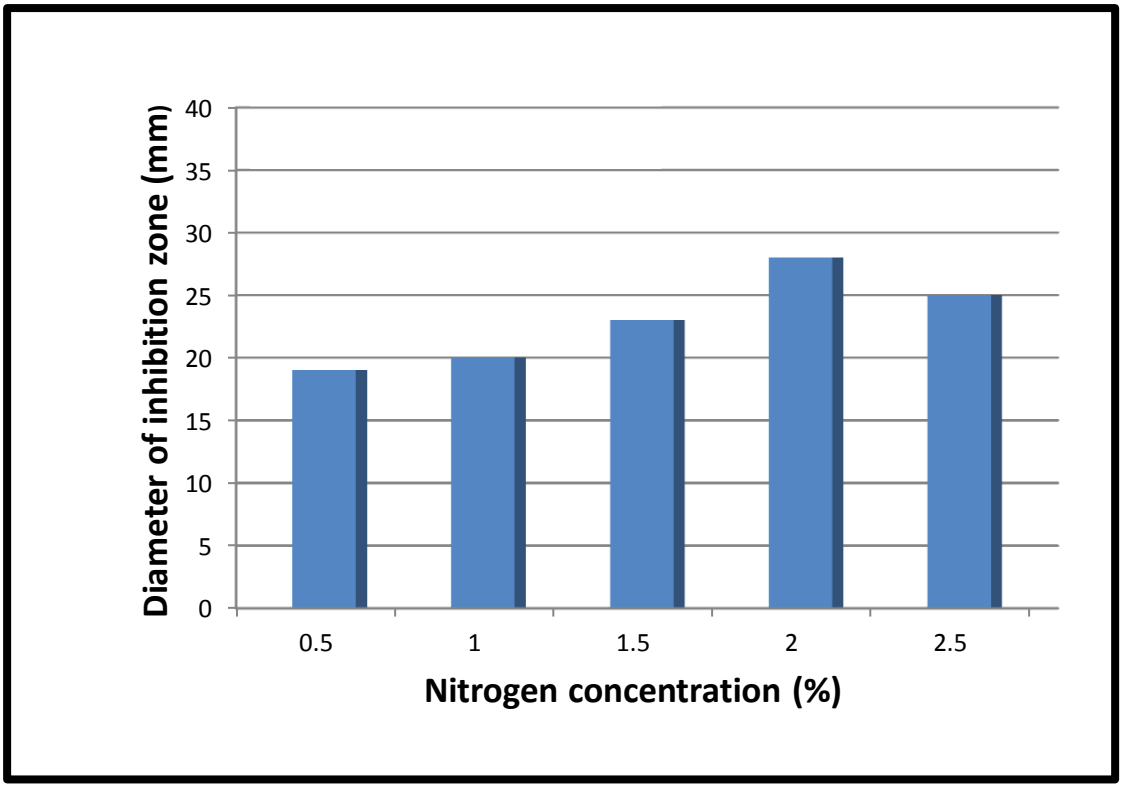


Figure (3-7): Effect of different concentration of yeast extract on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

zones were 28, 30, 31 , 16 and 13mm when the production medium adjusted to pH 5.5, 6.5, 7. 7.5 and 8.0 respectively. In respects of increasing mutacin production in medium with the initial pH 6, The reduction in mutacin production in medium with minimal initial pH values then of pH 6 might be referred to their effect on the microorganism metabolism, ionization, stability and solubility of the bio-molecules in the his may be because of appropriate for growth and production. culture medium (Hammami *et al.*, 2007). Nilsen *et al.*, 2003 recorded that production of bacteriocin by *E. faecalis* was highly depended on the pH of the growth medium and the optimum pH was 6.5 while less production occurred at pHs below 6.

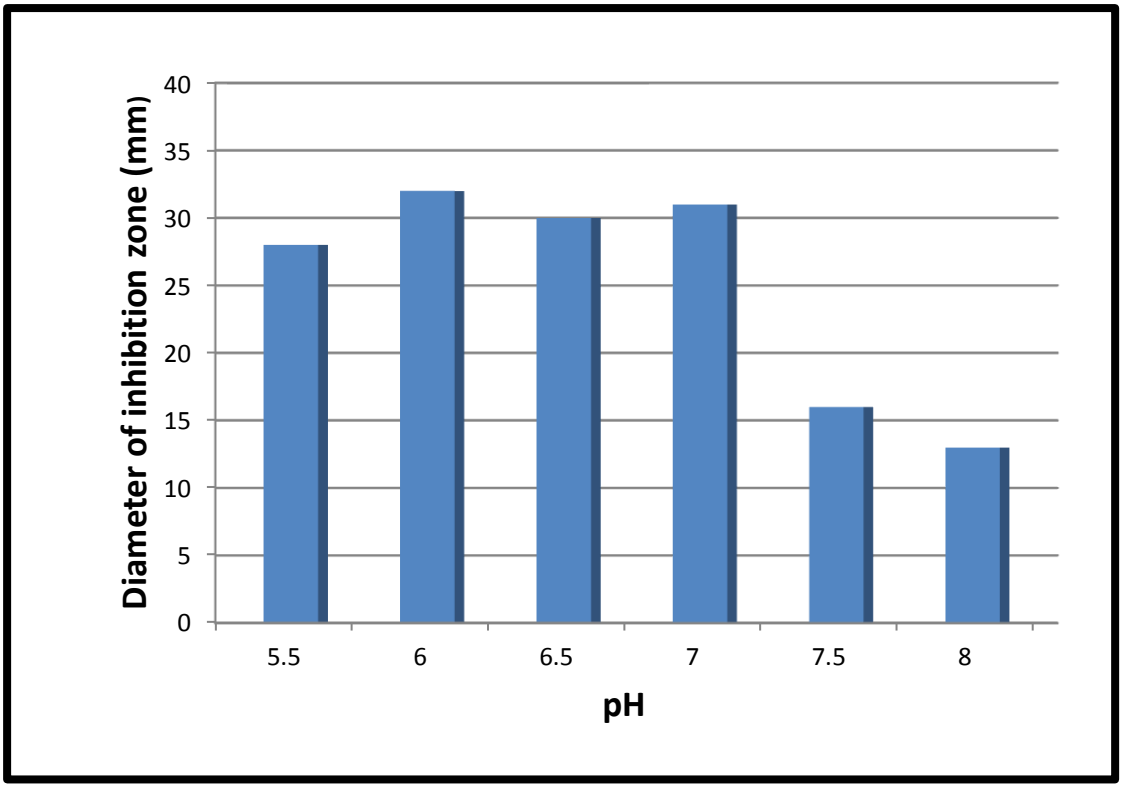


Figure (3-8): Effect of pH on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

3.4.6 Optimum Temperature

Different incubation temperatures were used to determine the optimum for mutacin production by the *S.mutans* S2. Results s in figure (3-9) indicate that the maximum mutacin production was obtained when the culture medium was incubated at 37°C, at this temperature the antagonistic effect of the mutacin against *S.pyogenes* reached it is maximum with an inhibition zone of 33 mm. Other incubation temperatures caused less production of mutacin, then less effect of antagonism against *S.pyogene*s.

This result is similar to that recorded by Ma and Marquis ,(1997), who found that the optimum temperature for growth and mutacin production

by *S. mutans* was 37°C. Reduction in the mutacin production at higher and

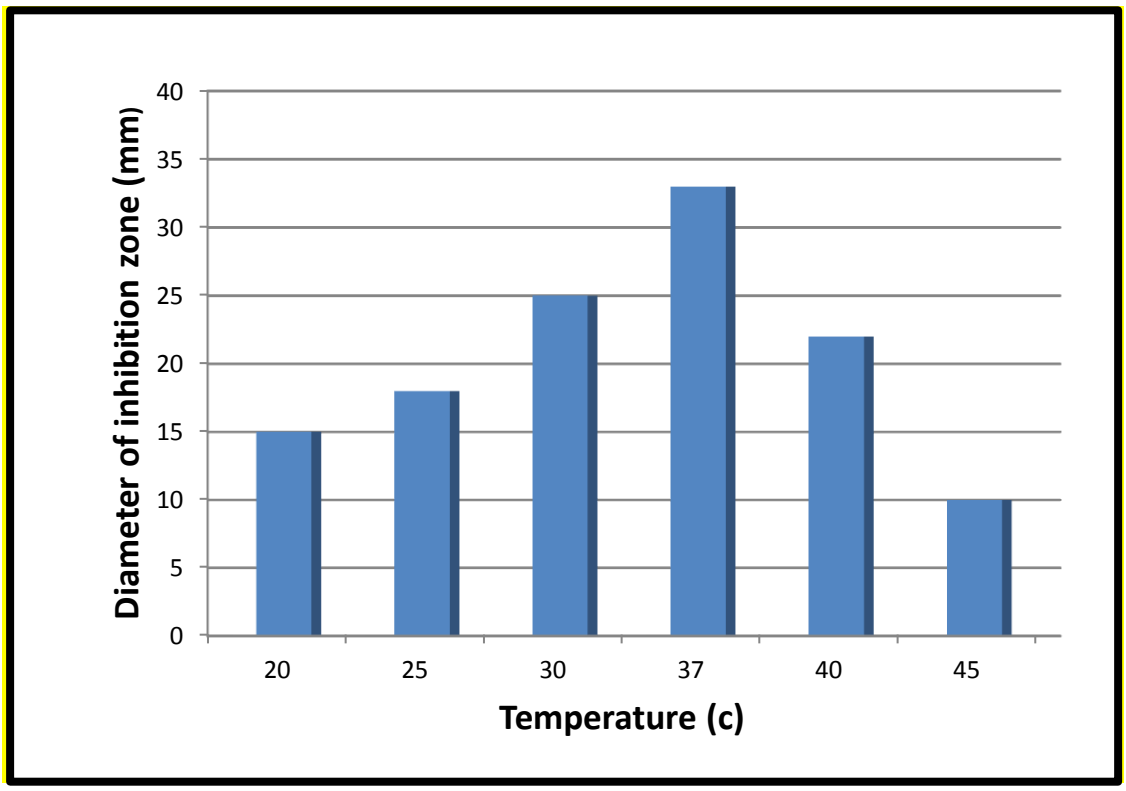


Figure (3-9): Effect of different incubation temperature on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at different temperatures for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

lower temperature may be due to the slow growth that led to retardation of mutacin production. The temperature affects microorganisms through the oxygen solubility in medium, on kinetic energy of molecules, and on reaction velocity in the bacterial cells, and these affects mutacin production (Kandela, 2006).

According to this result, optimum temperature 37°C was used in the next experiments of optimization for mutacin production.

3.4.7 Optimum phosphate source

In order to determine the optimum phosphate source for mutacin production by the locally isolated *S.mutans* S2, two types of phosphate

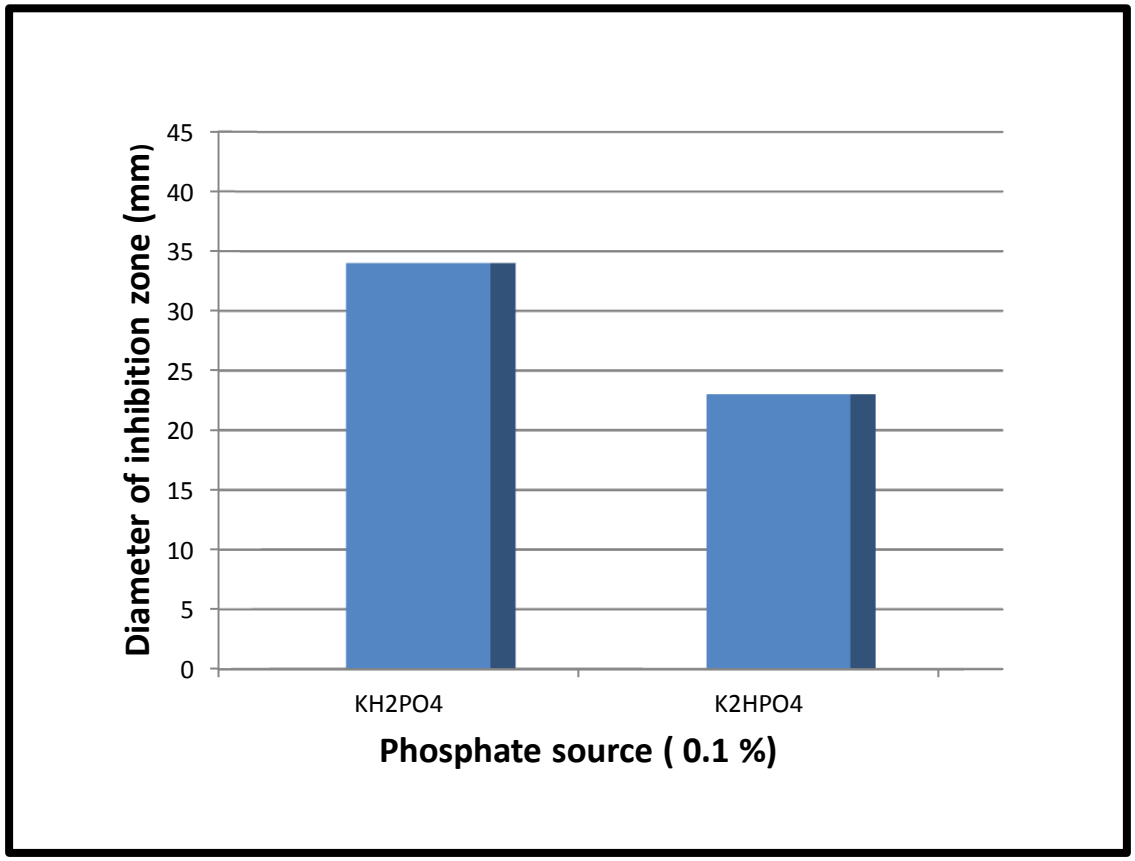


Figure (3-10): Effect of different phosphate source on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

sources (KH_2PO_4 and K_2HPO_4) were used in concentration of 0.1%(w/v).

Results in figure (3-10) indicate that the maximum production of mutacin was obtained when the production medium was consists of KH_2PO_4. By using this phosphate source the antagonistic effect of the mutacin against *S.pyogene*s reaches its maximum with an inhibition zone of 34 mm. other phosphate source caused less production of mutacin, then less effect of antagonism against *S.pyogene*s.

The presence of phosphate in the culture medium works as a buffering capacity when the medium become acidic because of the growth of bacteria. (Brivonese and Sutherland, 1989).

According to this result, optimum phosphate source was used in the next experiments of optimization for mutacin production.

3.4.8 Optimum phosphate source concentrations

Different concentrations of the optimum phosphate source (KH_2PO_4) were used to determine the optimum for mutacin production by the *S.mutans* S2. Results in figure (3-11) indicate that the maximum mutacin production was obtained when KH_2PO_4 was added to the production medium in a concentration of 0.5% (w/v), at this concentration the antagonistic effect of the produced mutacin against *S.pyogene*s reaches the maximum according to the result of inhibition zones that reach 35mm in comparison with the other concentrations that cause less production of mutacin in the culture filterate and then less effect of antagonism against *S.pyogene*s. The inhibition zones were, 32 and 24 mm when the production medium contain KH_2PO_4 in a concentration of 0.1 and 1 respectively.

At this concentration of KH_2PO_4, the buffering capacity may makes the growth condition suitable for mutacin production.

3.5 Antibiotic susceptibility of *S. mutans*

Antibiotic susceptibility pattern of the locally isolated *S. mutans* S2 against different antibiotics was studied. Results indicated in table (3-2) showed that *S. mutan*s S2 was resistant to two antibiotics (bacitracin and erythromycin), while it was sensitive to the other eight antibiotics (amoxicilin, ampicillin, chloramphenicol, vancomycin, clindamycin, impenem, gentamycin and cephalothin). The resistant to these two antibiotics may be encoded by chromosomal DNA and/or plasmid DNA.

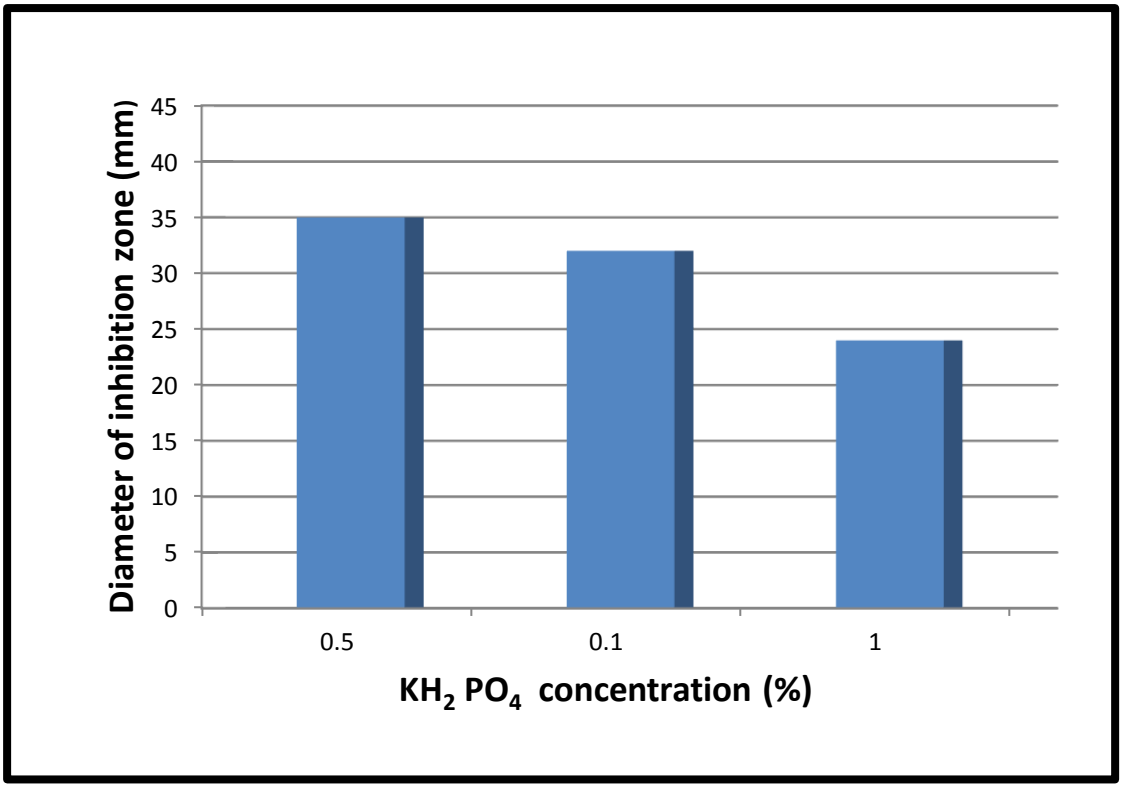


Figure (3-11): Effect of different concentration of KH_2PO_4 on mutacin production by locally isolated *Streptococcus mutans* S2 after incubation at 37°C for 24 h under anaerobic conditions expressed as its inhibitory effect against *Streptococcus pyogenes*

Li *et al*., 2001 referred that *S. mutans* chromosomal DNA harboring a heterologous erythromycin resistance gene(Erythromycin which is an inhibitor of protein synthesis in susceptible organisms) altering the binding site (ribosome) by methylation the 23s rRNA, thereby, blocking binding of the drug (Sator, 2000: King *et al*., 2000).

S. mutans is resistant to bacitracin, which is a peptide antibiotic produced by certain species of *Bacillus*. Tsuda *et al*., 2002 reported that a gene locus (*mbrABCD*), which encodes a putative ABC-transporter (MbrAB) and a two-component regulatory system (MbrCD), is involved in the bacitracin resistance of *S. mutans* and that all *S. mutants* defective in

Table (3-2): Antibiotic susceptibility of locally isolated *Streptococcus mutans* S2

Antibiotics	Concentration (µg / disk)	Symbol	Susceptibility
Bacitracin	10	BA	R
Amoxicillin	10	AmC	S
Erythromycin	10	E	R
Ampicillin	10	Amp	S
Chloramphenicol	30	Ch	S
Vancomycin	20	Van	S
Clindamycin	15	Cli	S
Impenem	10	IpM	S
Gentamycin	10	Gen	S
Cephalothin	30	Cph	S

R: resistance S: sensitive

each *mbr* gene on this locus were about 100 to120 times more sensitive to bacitracin than the wild-type strain.

In 1998 Al-Mizrakichi, demonstrated that 100% of *S. mutans* isolates showed sensitivity to antibiotic Amoxicillin, gentamycin, Erythromycin, and Cephalothin. In (2009) Jain and Pundir demonstrated that *S. mutans* isolates showed sensitivity to Amoxicillin. The result also indicated that *S. mutan*s shown sensitivity to Ampicillin these result similar to the result obtained by Al-Mizrakichi (1998).

3.6 Plasmid content of *S. mutans*

Plasmid profile of *S.mutans* S2 was studied to determine its role in mutacin production, by using *AccuPrep*® Plasmid Mini Extraction Kit for extraction of plasmid DNA. Since most mutacin of Gram +ve are plasmid encoded (Caufield *et al.*, 2007).

Result shown in figure (3-12) indicates that the *S. mutans* S2 has a plasmid DNA after electrophoresis on agarose gel. This result was agreed with Caufieled *et al*. (2007) who mentioned that a The 5.6-kb plasmid in *S. mutans* was thought to be related to mutacin production, because most bacteriocins of gram-positive bacteria are plasmid encoded. Because of its high sequence variability in the hypervariable region (HVR) and its low prevalence, the cryptic plasmid is a useful epidemiological marker for studying transmission. A cryptic plasmid resides in ~5% of the isolates of *S. mutans* . The function of this plasmid remains unknown, although its sequence has been published (Rheinberg *et al*., 2013).

3.7 Curing of plasmid DNA

An attempt to cure plasmid DNA of the locally isolated *S. mutans* S2 was achived by using ethidium bromide as a curing agent to evaluate the role of this plasmid in mutacin production.

Result indicated in table (3-3) showed that the locally isolated *S. mutans* S2 was still able to grow in brain heart infusion broth in presence of gradual concentration of ethidium bromide till the concentration of 800 µg/ml, (a concentration which was regarded as a sub-lethal concentration). From culture medium containing this concentration of ethidium bromide (800 µg/ml), 100 µl was taken, diluted, and spread on brain heart infusion agar plates, and incubated at 37°C for 24 h. Then 100 colonies were

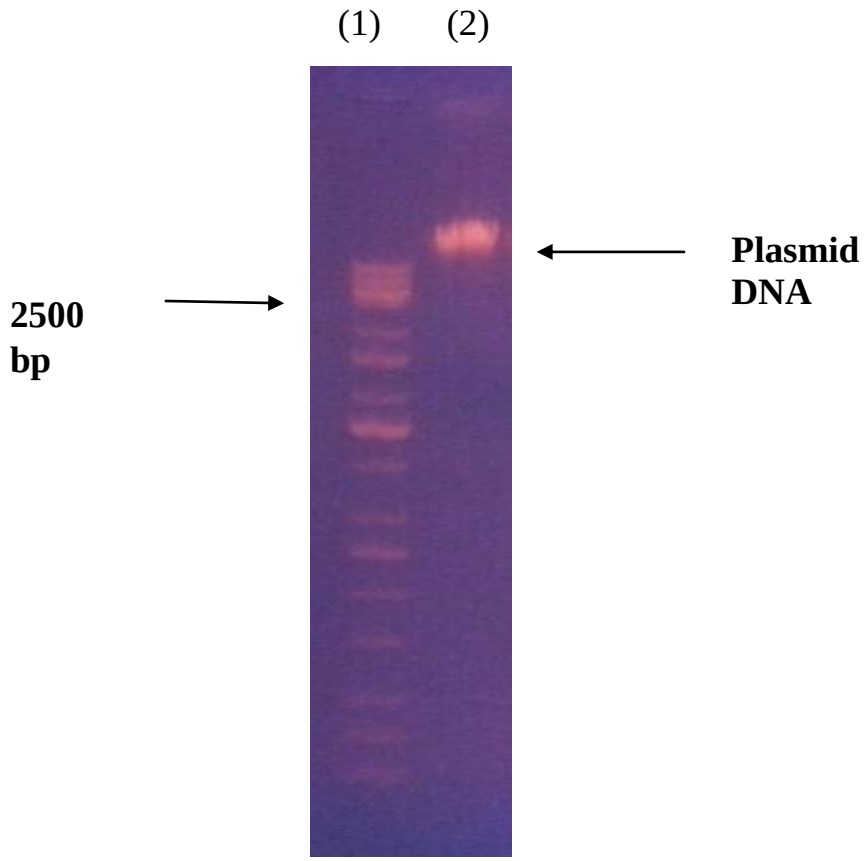


Figure (3-12): Plasmid content of the *Streptococcus mutans* S2 on agarose gel (0.7%) after electrophoresis at 5v/cm for 3 h

Lane (1):DNA ladder marker (2500bp)

Lane (2): *S. mutans*

selected randomly to examine their antibiotic resistance on selective medium contining antibiotic to which wild type is resist (bacitracin and erythromycin) to determine the cured colonies (which can not able to grow on these antibiotic containing media). Result showed that all colonies are still able to grow on medium containing erythromycin and bacitracin. One of these colonies was selected and examined for the presence of its own plasmid by extraction of the plasmidc DNA and electrophoresis on agarose gel. Result shown in figure (3-13) indicates that this colony of *S. mutans* S2 was lost their own plasmid. Cured cells of this colony were examined

Table (3-3): Effect of ethidium bromide on growth of *Streptococcus mutans* S2 after incubation at 37°C for 24 h

Concentration (µg/ml)	Bacterial growth
0	+++
50	+++
100	+++
200	+++
300	++
400	++
500	++
600	++
700	+
800	±
900	–
1000	–

; Very good growth; (++): Good growth; (+): moderate growth(+++): Slight growth; (-): No growth(±):

for the ability to produce mutacin by growing under the optimum conditions for 24 h at 37°C under anaerobic condition. Result showed that these cured cells were still able to produce mutacin in the culture mediumbecause of the inhibitory effect of mutacin produced in crude filtrate against the test microorganism (*S. pyogene*s). These results declared that *S. mutans* S2 plasmid was not responsible for mutacin production, furthermore, this trait is chromosomally located.

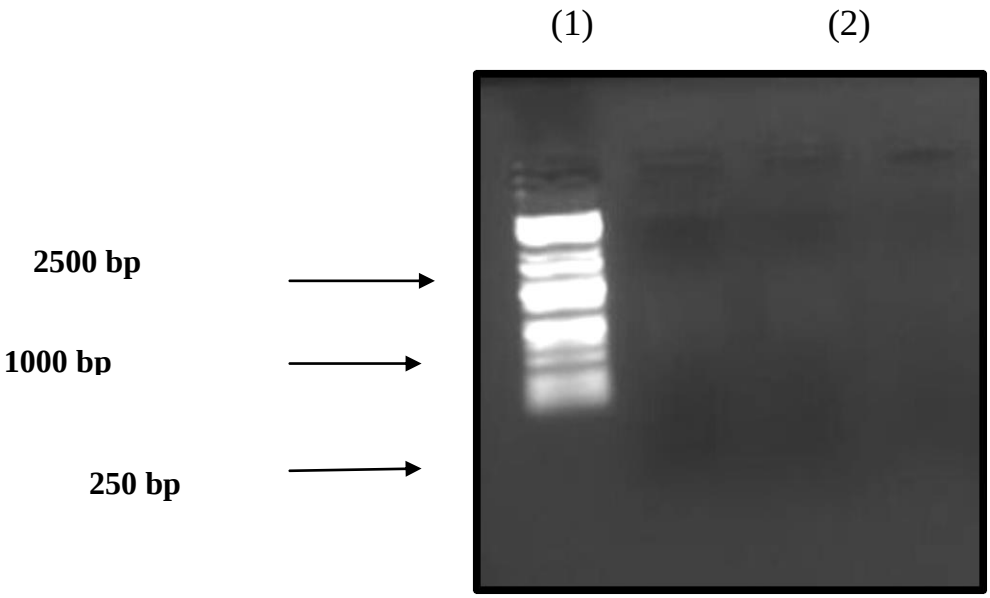


Figure (3-13): Plasmid profile of cured cells of *Streptococcus mutans* S2 on agarose gel (0.7%) after electrophoresis at 5V/cm for 3 h

Lane (1):DNA ladder marker (2500bp)

Lane (2): *S. mutans*

This result was agreed with Kamiya *et al*. (2011) who mentioned that the location of the genes that responsible for mutacin production are chromosomally located in *S. mutans*. Balakrishnan *et al*. (2002) obtained same result when studied on *S. mutans*.

Conclusions

1. *Streptococcus mutans* was one of the major microflora in dental caries infections and was able to produce mutacin in variable degree.
2. Locally isolated *S. mutans* S2 was the most efficient among the other isolates in mutacin production.
3. Mutacin production from *S. mutans* S2 can be increased after culturing under optimum conditions of nutritional suppliments and growth factors.
4. Locally isolated *S. mutans* S2 was sensitive to different antibiotics(amoxicilin, ampicillin, chloramphenicol, vancomycin, clindamycin, impenem, gentamycin and cephalothin), while it was resist only to bacitracin and erythromycin.
5. Locally isolated *S. mutans* S2 was harboring one plasmid DNA.
6. The trait of antibiotic resistance and mutacin production in *S. mutans* S2 was chromosomally located.

Recommendations

- Purification and characterization of mutacin produced by the locally isolated *S. mutans* S2.
- Enhancing the ability of mutacin production by the locally isolated *S. mutans* S2 by physical and chemical mutagenesis.
- Further studies about the possibility of increasing the mutacin production by cloning of mutacin gene into other organisms for commercial .

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Appendix (1) : Result of identification of *S. mutans* according to their biochemical characteristic by using VITEK-Π identification

Biochemical test	Result									
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
D-Amygdalin(D-AMY)	+	+	+	+	+	+	+	+	+	+
Phosphatidylinositol phospholipase C(PIPLC)	-	-	-	-	-	-	-	-	-	-
D-Xylose(Dxyl)	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase1(ADH1)	-	-	-	-	-	-	-	-	-	-
Beta-Galactosidase (BGAL)	-	-	-	-	-	-	-	-	-	-
Alpha-Glucosidase (AGAL)	+	+	+	+	+	+	+	+	+	+
Ala-Phe-Pro Arylamidase (APPA)	-	-	-	-	-	-	-	-	-	-
Cyclodextrin (CDEX)	-	-	-	-	-	-	-	-	-	-
L-Asparate Arylamidase (AspA)	-	-	-	-	-	-	-	-	-	-
Beta-Galactopyranosidase (BGAR)	-	-	-	-	-	-	-	-	-	-
Alpha-Mannosidase (AMAN)	-	-	-	-	-	-	-	-	-	-
Phosphatase(PHOS)	-	-	-	-	-	-	-	-	-	-
Leucine-Arylamidase (LeuA)	+	+	+	+	+	+	+	+	+	+
L-prolin Arylamidase(Pro)	–	–	–	–	–	–	–	–	–	–

Continued

Beta Glucuronidase (BGURr)	−	−	−	−	−	−	−	−	−	−
Alpha-Galactosidase (AGAL)	+	+	+	+	+	+	+	+	+	+
L-Pyrrdidonyl-Arylamidase (PyrA)	−	−	−	−	−	−	−	−	−	−
Beta-Glucuronidase (BGUR)	−	−	−	−	−	−	−	−	−	−
Alanine Arylamidase (AlaA)	+	+	+	+	+	+	+	+	+	+
Tyrosine Arylamidase(TyrA)	−	−	−	−	−	−	−	−	−	−
D-Sorbitol (dsoR)	+	+	+	+	+	+	+	+	+	+
Urease (URE)	−	−	−	−	−	−	−	−	−	−
Polymixn B resistance (Poly B)	+	+	+	+	+	+	+	+	+	+
D-Galactose (dGAL)	+	+	+	+	+	+	+	+	+	+
D-Ribose (dRIB)	−	−	−	−	−	−	−	−	−	−
L-lactate alkalinization(lLATk)	−	−	−	−	−	−	−	−	−	−
Lactose (LAC)	+	+	+	+	+	+	+	+	+	+
D-Maltose (Dmal)	+	+	+	+	+	+	+	+	+	+
Bacitracin resistance (BACI)	+	+	+	+	+	+	+	+	+	+
Novobiocin resistance (NOVO)	+	+	+	+	+	+	+	+	+	+

Continued

D-Mannitol (d MAN)	+	+	+	+	+	+	+	+	+	+
D-Mannose (d MNE)	+	+	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl (NC 6.5)	–	–	–	–	–	–	–	–	–	–
Methyl-B-D-Glucopyranoside (MBdG)	-	–	–	–	–	–	–	–	–	–
Pullulan (PUL)	-	–	–	–	–	–	–	–	–	–
D-Raffinose (d RAF)	+	+	+	+	+	+	+	+	+	+
O/ 129 resistance (Comp vibrio)) (O129R)	-	–	–	–	–	–	–	–	–	–
Salicin (SAL)	+	+	+	+	+	+	+	+	+	+
Saccharose/ Sucrose (SAC)	+	+	+	+	+	+	+	+	+	+
D-Trehalose (d TRE)	+	+	+	+	+	+	+	+	+	+
Arginin dihyrolase2 (ADH2s)	-	–	–	–	–	–	–	–	–	–
Optochin resistance (OPTO)	+	+	+	+	+	+	+	+	+	+

الخلاصة

لعزل بكتريا *Streptococcus mutans* ، فقد جمعت 80 عينة من عينات تسوس الأسنان لمرضى يراجعون المركز الصحي في الدورة والزوية في بغداد. وقد تم الحصول على 98 عزلة بكترية من تلك العينات شخصت 10 عزلات منها على أنها *S. mutans* على أساس خصائصها المظهرية والمزرعية والكيموحيوية. وقد تم تأكيد نتائج التشخيص بأستخدام نظام VITK- Π . أخضعت جميع العزلات المشخصة للغربلة النوعية للتحري عن قابليتها في أنتاج الميوتاسين. وقد أشارت النتائج الى أن جميع العزلات كانت منتجة للميوتاسين وبدرجات متفاوتة على أساس التأثير المثبط للميوتاسين المنتج في رائق المزارع البكتيرية لتلك العزلات ضد بكتريا الأختبار *(Streptococcus pyogenes)* الذي تم دراسته بطريقة تحري الأنتشار البئري (well diffusion method). وقد تم أنتقاء العزلة *S. mutans* S2 لكونها الأكفأ في أنتاج الميوتاسين على أساس قابلية الميوتاسين في رائق مزرعتها في أحداث تثبيط أعلى لنمو بكتريا *S. pyogenes* وبقطر 20 ملم لدراسة الظروف المثلى لأنتاج الميوتاسين منها. وقد أشارت النتائج الى أن الظروف المثلى لأنتاج الميوتاسين كانت تتضمن تدعيم وسط الأنتاج (وسط نقيع الدماغ-القلب السائل) بالسكروز كمصدر وحيد للكاربون والطاقة بتركيز 3%، ومستخلص الخميرة كمصدر نايتروجين بتركيز 2% وفوسفات البوتاسيوم ثناثية الهيدروجين كمصدر فوسفاتي بتركيز 0.5% ، وكان الرقم الهيدروجيني الأولي لوسط الأنتاج هو pH 6 ثم الحضن بدرجة 37 م° لمدة 24 ساعة تحت ظروف لاهوائية. وقد بلغت أنتاجية الميوتاسين من العزلة *S. mutans* S2 أقصاها تحت هذة الظروف ، إذ بلغ التأثير المثبط ضد بكتريا *S. pyogenes* أقصاه و بقطر تثبيط مقداره 35 ملم .

لأنواع مختلفة من مضادات الحياة وقد أشارت *S. mutans* S2 أختبرت حساسية العزلة النتائج الى أن هذه العزلة كانت مقاومة للبستراسين والأرثرومايسين في حين كانت حساسة للأموكسيلين والكلورامفينكول والفانكومايسين والكلندامايسين والجنتامايسين والسيفالوثين.

درس المحتوى البلازميدي للعزلة *S. mutans* S2 للتعرف على دور البلازميد في أنتاج الميوتاسين بأستخلاص الدنا البلازميدي ثم الترحيل الكهربائي على هلام الأكاروز. وقد أشارت النتائج الى أمتلاك العزلة البكتيرية *S. mutans* S2 لحزمة بلازميدية واحدة. وقد وجد بأن هذا البلازميد غير مسؤول عن صفة المقاومة لمضادات الحياة وعن أنتاج الميوتاسين على ضوء نتائج تحييد البلازميد بأستخدام صبغة بروميد الأثيديوم بتركيز 800 مايكروغرام\ مل، إذ حافظت الخلايا المحيدة على قابليتها على أنتاج الميوتاسين مما يدل على أن صفة أنتاج الميوتاسين هي ربما تكون صفة كروموسومية.