Optimum Conditions for Mutacin Production From *Streptococcus mutans* S2

Aya R. Salih, Hameed M. Jasim and Mohsan H. Risan
College of Science, Al-Nahrain University, Baghdad-Iraq.
E-mail: aya.raad@yahoo.com.

Abstract

Mutacin production by *Streptococcus mutans* S2 was detected by inoculating production medium (Brain Heart Infusion broth supplemented with yeast extract 2% (w/v) and $CaCO_3$ 1% (w/v)) with fresh culture of the bacterial isolate. The inhibitory effect of mutacin produced by *S. mutans* S2 was studied by detection the inhibitory effect of mutacin against the test microorganism (*Streptococcus pyogene*s) grown on BHI agar plates. Optimum conditions for mutacin production from *S. mutans* S2 includes optimum carbon, nitrogen and phosphate sources and their concentrations, optimum temperature and pH. were studied. Results showed that these conditions include the supplementation of the production medium (Brain-Heart Infusion broth containing 1%$CaCO_3$) with sucrose as a sole source for carbon and energy at a concentration of 3%, and yeast extract as a nitrogen source at a concentration of 2%, and KH_2PO_4 as a phosphate source at a concentration of 0.5%, with initial medium pH of 7, then inoculating with fresh culture (OD 0.8), and incubation at 37°C for 24 hours in candle jar. Under these conditions the maximum mutacin production *S. mutans* S2, causing maximum inhibition effect against test microorganism (*S. pyogene*s) reached 35 mm in diameter.

Introduction

The increase of microbial resistance to antibiotics has led to a continuing search for newer and more effective drugs (1). 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 antimicrobial activity. It is well known that bacteria, induced by stress, produce bacteriocins which is promising antimicrobial substances that may cure infectious diseases (2). Bacteriocins 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 (3). Mutacin as bacteriocins are proteinaceous antibacterial substances produced by *Streptococcus mutans* (4).

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 adhesions 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* 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 (5). Bacteriocins produced by the oral bacterium *Streptococcus 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 depend upon the collaborative activity of two polypeptides to exert their full antimicrobial activity(6). The lantibiotics are small peptides containing lantionina, β-methyl-lantionina dehidratados residues and are synthesized by ribosomes and are modified after translation (7). This study was achieved to produce mutacin from *S. mutans* under optimum conditions.

Materials and Methods

Bacterial isolate

Bacterial isolate identified as *S. mutans* S2 was obtained from dental caries sample (8). This isolate was maintained on Brain Heart Infusion agar medium.

Mutacin Production

Mutacin production by *S. mutans* S2 was detected by inoculating production medium (Brain Heart Infusion broth supplemented with yeast extract 2% (w/v) and $CaCO_3$ 1% (w/v)) with fresh culture of the bacterial isolate, then cultures were incubated at 37°C for 24 hours (O.D 0.8) under anaerobic conditions. After incubation, cultures were placed in water bath at 70 °C for 10 minutes to kill bacterial cells and inhibit protease activity according to (9), then centrifuged at 6000 rpm for 10 minutes. Supernatant was regarded as a crude mutacin.

Detection of the inhibitory effect of mutacin

The inhibitory effect of mutacin produced by *S. Mutans* S2 was studied by detection the inhibitory effect of mutacin against the test microorganism (*S. pyogene*s) grown on BHI agar plates. The antagonistic effect against the test microorganism was achieved according to the well diffusion assay method described by (9).

Optimum conditions for mutacin production

Different nutritional and growth factors were studied to determine the optimum conditions for bacteriocin production by *S.mutans* S2. These conditions include optimum carbon, nitrogen and phosphate sources and their concentrations, optimum temperature and pH.

Effect of carbon source

To determine the optimum carbon source for mutacin production, five carbon sources (maltose, sucrose, galactose, fructose, glucose) were added to the production medium at a concentration of 3% (w/v).

Effect of carbon source concentration

To determine the optimum concentration of carbon source for mutacin production, eight concentrations (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.

Effect of nitrogen source

Six nitrogen sources were used to determine the optimum for mutacin production. These sources are yeast extract, pepton, malt extract, NaNo3, ammonium chloride and ammonium nitate and were added to the production medium in a concentration of 2% (w/v).

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 used to supplement the production medium to determine the optimum for mutacin production.

Effect of phosphate source

To determine the effect of phosphate source on mutacin production by the selected isolate of *S. mutans*, two phosphate sources (KH_2PO_4 and K_2HPO_4) were added to the production medium at a concentration of 0.1%.

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, 1, and 1.5 %) were used to supplement the production medium to determine the optimum in mutacin production.

Effect of temperature

In order to determine the optimum incubation temperature for mutacin production by the selected isolate, culture of *S. mutans* were incubated at different temperatures (20, 25, 30, 37, 40 and 45°C) to determine the optimum for mutacin production.

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).

Results and Discussion

Mutacin production

Ability of *S. mutans* S2 for mutacin production was examined first by detection its inhibitory effect against *S. pyogene*s. This was achieved by propagating the bacterial isolate in

mutacin production medium for OD (0.8), then culture was centrifuged and the crude filtrate was regarded as crude mutacin. Results showed that mutacin inhibited the growth of *S.pyogenes* in a diameter of 20 mm according to well diffusion assay method.

Optimum conditions for mutacin production

Optimum carbon source

In order to examine the optimum carbon source on the ability of locally isolated *S.mutans* S2 in mutacin production, the production medium (BHI broth) was supplemented with five different carbone sources includes sucrose, glucose, maltose, fructose and galactose. These carbon sources were added to the production medium in a concentration of 3%. Results indicated in Fig.(1) that maximum production of mutacin was achieved when sucrose was used to supplement the production medium as a sole source for carbon and energy to induce mutacin production by *S.mutans* S2. Inhibition zone of mutacin against *S.pyogen* was increased to 22 mm in comparison with the other carbon sources.

S. mutans was found 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*. (10). On the other hand, this result was in agreement with Nicolas *et al*. (4) who found that the best production of mutacin was obtained by using sucrose as a sole source for carbon and energy.

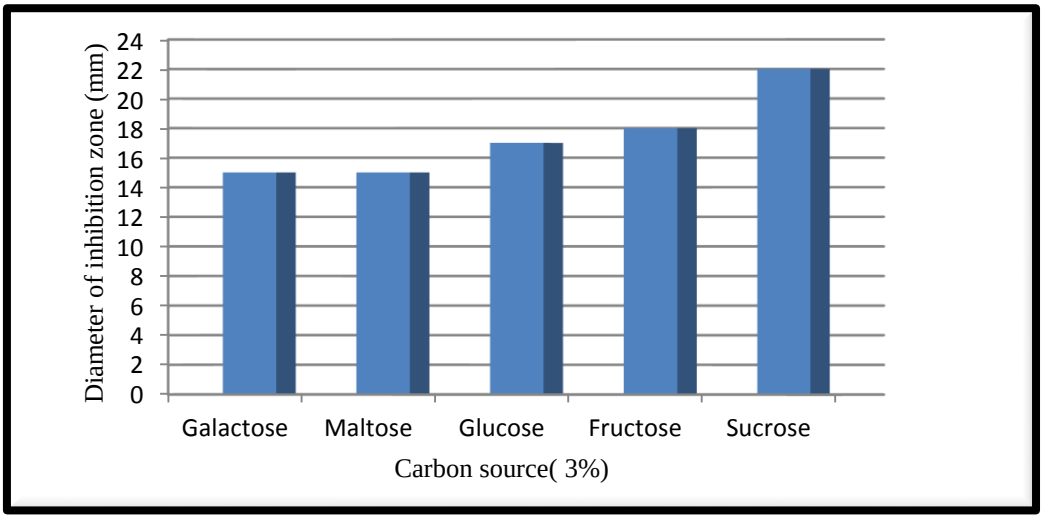

Fig.(1): Effect of different carbon sources on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

Optimum sucrose concentration

In order to determine the optimum sucrose concentration for mutacin production by locally isolated *S. mutans*, different concentration of sucrose (1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5%) were added to the production medium. Results shown in figure (2) indicate that maximum mutacin production was obtained when sucrose was added to the production medium at a concentration of 3%.

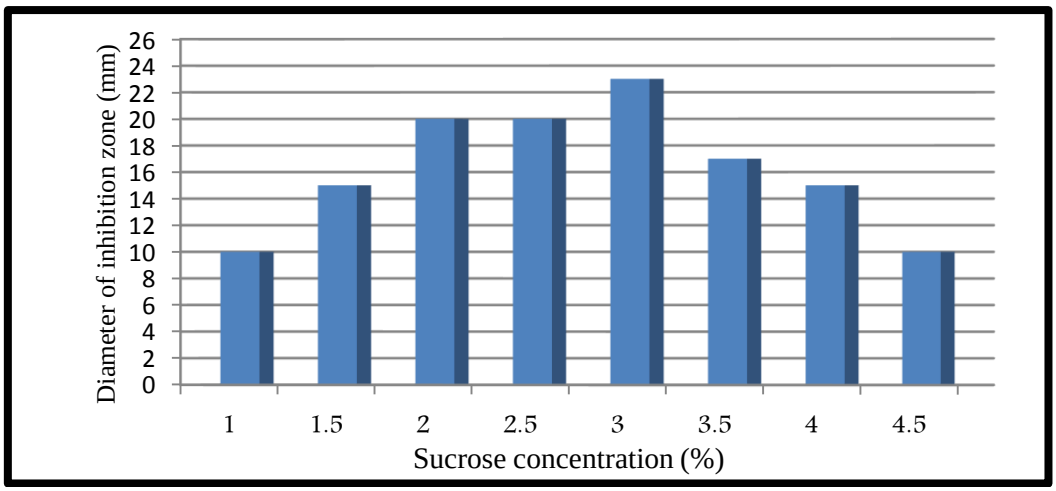

Fig.(2): Effect of sucrose concentration on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S.pyogenes.

At this concentration, anatagonistic effect of mutacin in culture filtrate of S. mutans S2 against S. pyogenes reached the maximum because the inhibition zone was increased to 23 mm in comparison with the other concentrations of sucrose that gave less production of mutacin and less inhibitory effect against S.pyogenes. This result was in agreement with Nicolas et al. (4) who mentioned that mutacin production by S. mutans was obtained when production medium was supplemented with sucrose at concentration lower than 5 %.

Optimum nitrogen source

Different nitrogen sources were added to the production medium in order to determine the optimum for mutacin production by S.mutans S2. Results shown in Fig.(3) showed that the maximum mutacin production was achieved when yeast extract was used as nitrogen source. Crude mutacin in culture filtrate of S. mutans S2 caused the higher inhibitory effect against S. pyogenes by increasing the inhibition zone to 27 mm in diameter.

It has been suggested that yeast extract contains peptides that are essential for biosynthesis of mutacin or may act as inducers of mutacin production. The positive effect of yeast extract was related to the high content of mineral, vitamins and amino acid such as serine, cysteine and threonine. Furthermore, yeast extract was also shown to contain fermentable sugars which could contribute to the increase mutacin production (Nicolas et al. (4)).

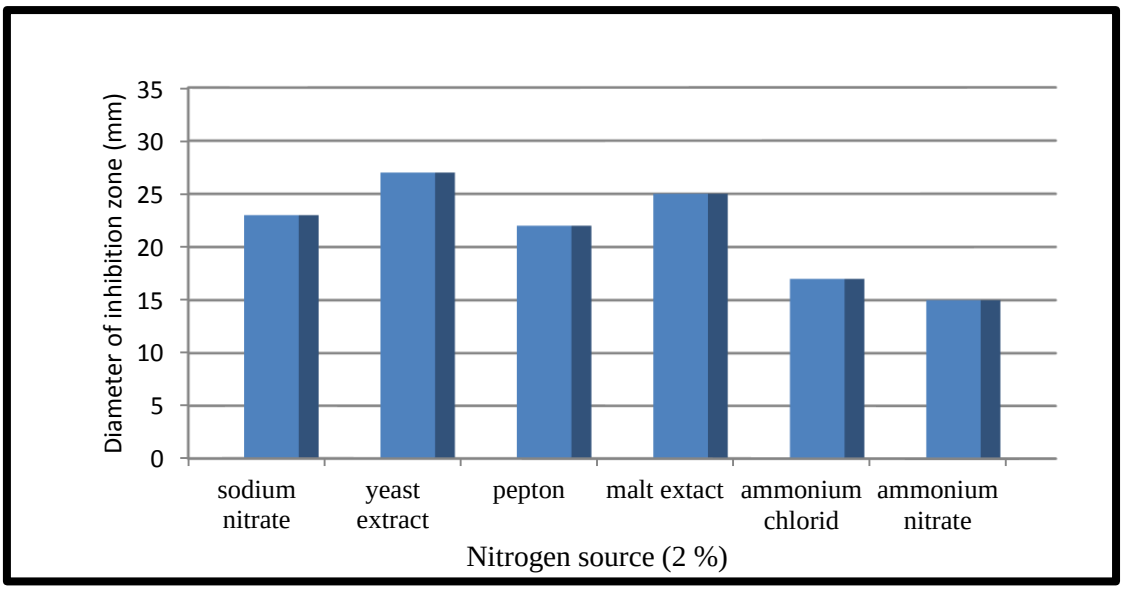

Fig.(3): Effect of different nitrogen sources on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

Optimum nitrogen source concentration

Five concentrations of yeast extract were used to determine the optimum for mutacin production by *S.mutans* S2. Results shown in Fig.(4) indicate that maximum mutacin production was obtained when yeast extract was added to the production medium in a concentration of 2% (w/v).

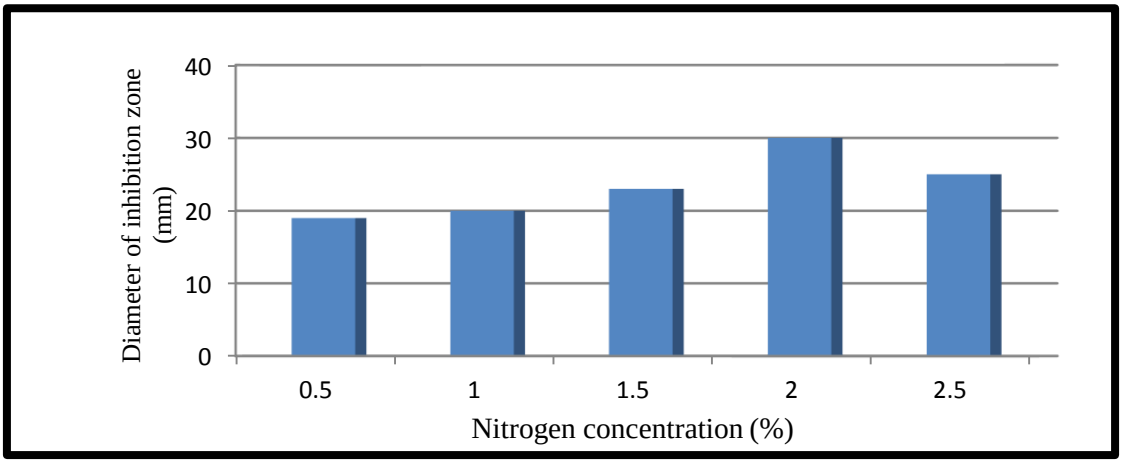

Fig.(4) : Effect of different concentration of yeast extract on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

At this concentration the antagonistic effect of the produced mutacin against *S.pyogenes* reached the maximum and increase the inhibition zones to 30 mm in comparison with the other concentrations that cause less production of mutacin and then less effect of antagonism against *S.pyogene*s.

This result confirmed that obtained by Nicolas *et al*.(4) who found that mutacin production improved when yeast extract was added to the production medium in a concentration of 2%, while Li *et al*.(11) found that mutacin yield increased with increasing the amount of yeast extract up to a concentration of 1%.

Optimum medium pH

Results shown in Fig.(5) indicate that the maximum mutacin production was obtained when the pH value of mutacin production medium was adjusted to 6. A reduction in mutacin production was occurred when the initial pH values of the production medium were higher or less than pH 6 which might refer to their effect on the microorganism metabolism, ionization, stability and solubility of the biomolecules in culture medium as it was mentioned by Hammami *et al*. (12).

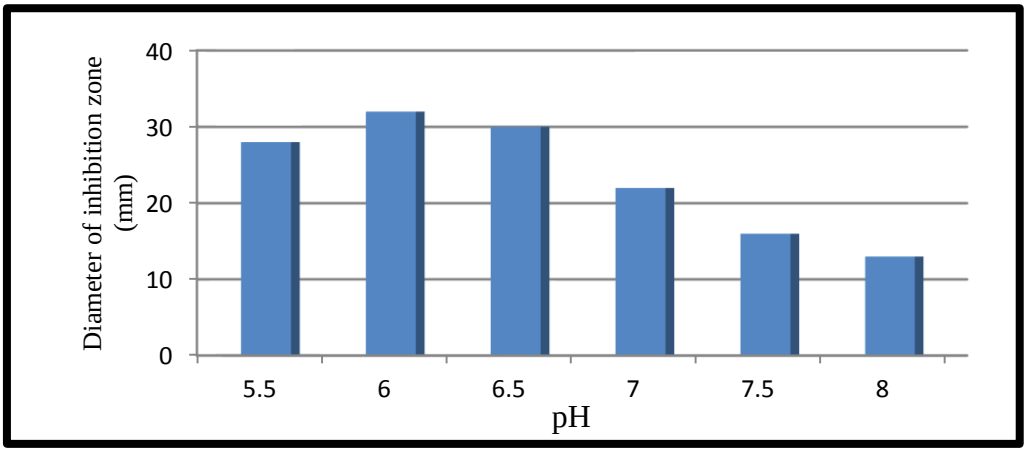

Fig.(5) : Effect of pH on mutacin production byS. mutans S2 after incubation at 37°C for 24 hrs. under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

Optimum Temperature

Different incubation temperatures were used to determine the optimum temperature for mutacin production by*S.mutans* S2. Results shown in Fig.(6) 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* was increased causing higher antagonistic effect against *S.pyogene*s with an inhibition zone of 33 mm.

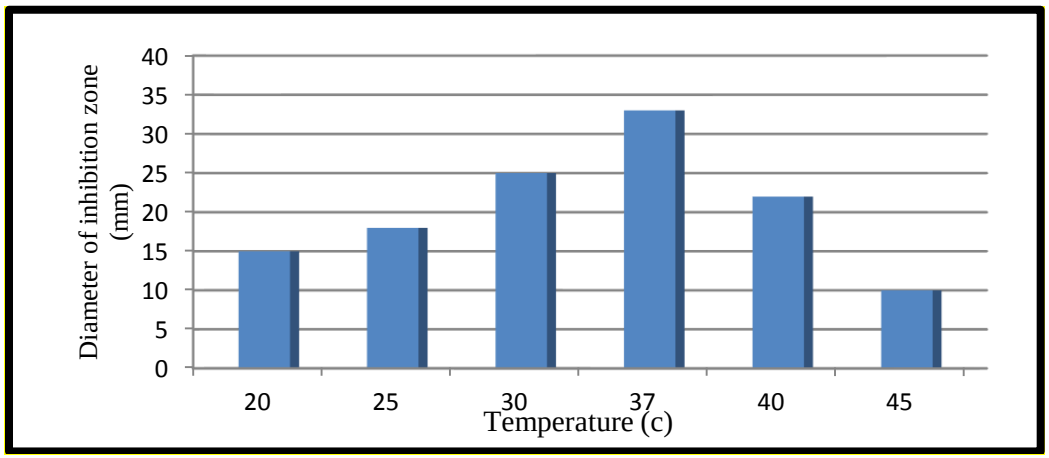

Fig.(6): Effect of different incubation temperature on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs. under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

This result was agreed with that recorded by Ma and Marquis (13), 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 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 and on kinetic energy of molecules, and these affects mutacin production (14).

Optimum phosphate source

Two types of phosphate sources (KH_2PO_4 and K_2HPO_4) were used in concentration of 0.1 % (w/v) to determine the optimum for mutacin production by *S.mutans* S2. Results shown in Fig.(7) indicate that the maximum production of mutacin was obtained when the production medium contain KH_2PO_4. By using this phosphate source the antagonistic effect of the mutacin against *S.pyogene*s reached its maximum with an inhibition zone of 35 mm**.** The presence of phosphate in the culture medium works as a buffering capacity when the medium become acidic because of bacterial growth (15).

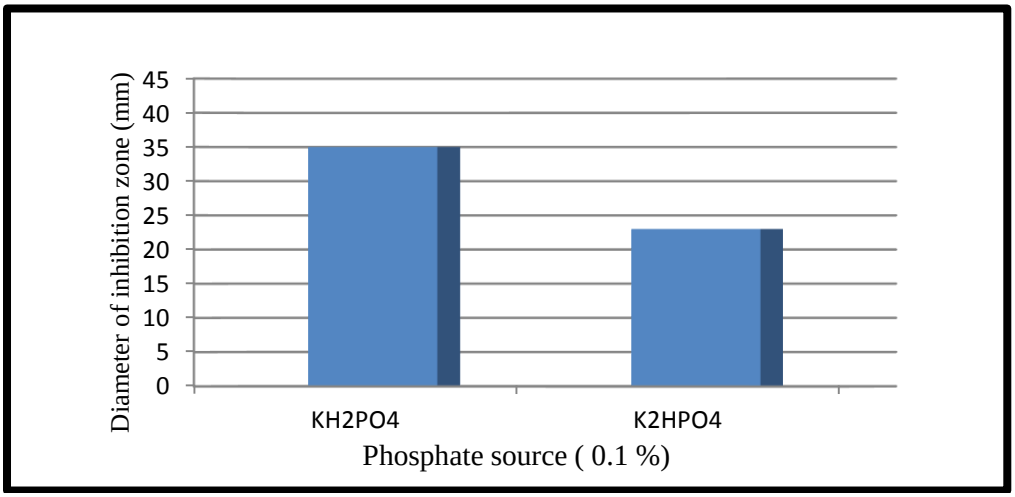

Fig.(7): Effect of different phosphate source on mutacin production by S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

Optimum phosphate source concentrations

Different concentrations of the optimum phosphate source (KH_2PO_4) were used to determine the optimum concentration for mutacin production by *S.mutans* S2. Results shown in Fig.(8) indicate that the maximum mutacin production was obtained when potassium dihydrogen phosphate 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 reacheed 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 filtrate and then less effect of antagonism against *S.pyogene*s. The inhibition zones were, 30 and 24 mm when the production medium contained KH_2PO_4 in a concentration of 0.1 and 1 respectively.

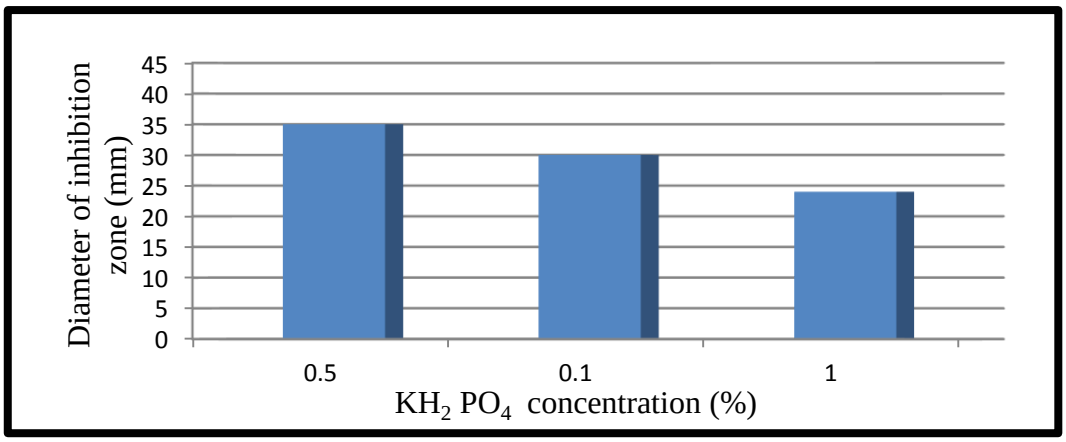

Fig.(8) Effect of different concentration of KH_2PO_4 on mutacin production by locally isolated S. mutans S2 after incubation at 37°C for 24 hrs under anaerobic conditions expressed as its inhibitory effect against S. pyogenes.

Conclusions

1. *S. mutans* was one of the major microflora in dental caries infections.
2. Local isolates of *S. mutans* S2 obtained from dental caries samples were able to produce mutacin.
3. Mutacin production from *S. mutans* S2 can be increased after culturing under optimum conditions of nutritional suppliments and growth factors.

References

[1] Coutinho H. D., Henrique D. M., Lobo K. M., Bezerra D. A. and Lobo I., "Peptides and proteins with antimicrobial activity. J. Pharmacology", 40, 3-9, 2008.

[2] Bianchi A. A. and Baneyx F., "Stress response as a tool to detect and characterize the mode of action of antibacterial agents", Appl. Environ Microbiol, 65, 5023-5027, 1999.

[3] Riley M. A. and Chavan M. A., "Bacteriocin, Ecology and Evolution", Spring Berlin Heidelbrg New York, USA, 2007.

[4] Nicolas G. G., Lavoie M. C. and LaPointe G., "Molecular Genetics, Genomics and Biochemistry of Mutacins", Genes, Genomes and Genomics, 1, 2, 193-208, 2007.

[5] Nakano K., Nemoto H., Nomura R., Homma H., Yoshioka H., Shudo Y., Hata H., Toda K., Taniguchi K., Amano A. and Ooshima T., "Serotype distribution of *streptococcus mutans* a pathogen of dental caries in cardiovascular specimens from Japanese patients", Med. Microbiol., 56, 551-556, 2007.

[6] Hale J. D., Heng N. C., Jack R. W. and Tagg J. R., "Identification of nlmTE, the locus encoding the ABC transport system required for export of nonlantibiotic mutacins in *Streptococcus mutans*", J. Bacteriol., 187, 5036–5039, 2005.

[7] Kamiya R. U., Taiete T. and Gonçalves, R. B., "Mutacins of *Streptococcus mutans*", Braz. J. Microbiol., 42, 4, 1248–1258, 2011.

[8] Aya R., "Genetic study on Locally Isolated *Streptococcus mutans* and it is Ability in Mutacin Production", MSc thesis, College of Science, Al-Nahrain University, 2014.

[9] Nicolas G., Auger I., Beaudoin M., Halle´ F., Morency H., LaPointe G. and Lavoie M. C., "Improved methods for mutacin detection and production", J. Microbiological Methods, 59, 351– 361, 2004.

[10] Kreth J. Zhu L., Merritt J., Shi W. and Qi F., "Role of sucrose in the fitness of *Streptococcus mutans*", Oral Micro biolImmunol, 23, 3, 213-219, 2008.

[11] Li C., Bai J., Cai Z., Ouyang F., "Optimization of a cultural medium for bacteriocin production by *Lactococcuslactis* using response surface methodology", J. Biotechnol., 93, 27– 34, 2002.

[12] Hammami R., Zouhir A., Ban Hamida J. and Fliss I., "Bacteriocin Characterization", BMC Microbiol., 17, 89- 99, 2007.

[13] Ma Y. and Marquis R. E., "Thermophysiology of *Streptococcus*

mutans and related lactic-acid bacteria.Antonie Van Leeuwenhoek", 72, 2, 91-100, 1997.

[14] Kandela N. J., "Production, Purification and Characterization of Enterocin from Enterococcus faecalis Local Isolates from Different Clinical Sources", PhD Thesis College of Science/ Al-Mustansiriya University, 2006.

[15] Brivonese A. and Sutherland W. I., "Polymer production by a mucoid strain of *Azotobacter vinelandii* in batch culture", App. Microbiol. Biotechnol., 30, 97-102, 1989.

الخلاصة

انتاج الميوتاسين بواسطة *Streptococcus. mutans* S2 حددت بواسطة تلقيح وسط الانتاج (مرق نقيع القلب الدماغ) المحتوي على مستخلص الخميرة ٢% و كاربونات الكالسيوم ١% بمزرعة فتية من بكتريا *S. mutans*. التاثير التثبيطي للميوتاسين المنتج بواسطة *S. mutans* S2 درست بواسطة تحديد الثأثير التثبيطي للميوتاسين ضد بكتريا الاختبار *S. pyogenes* النامية على اطباق (مرق نقيع القلب الدماغ). درست الظروف المثلى لانتاج الميوتاسين من بكتريا *S.mutans* S2, وقد اشارت النتائج الى ان هذه الضروف كانت تتضمن تدعيم وسط الانتاج (مرق نتقيع القلب الدماغ المحتوي على ١% كاربونات الكالسيوم) بالسكروز مصدرا وحيدا للكاربون والطاقة بتركيز ٣%, ومستخلص الخميرة مصدرا نايتروجينيا بتركيز ٢%, وفوسفات البوتاسيوم ثنائية الهيدروجين مصدرا فوسفاتيا بتركيز ٠,٥%, وكان الرقم الهيدروجيني الابتدائي للوسط ٧, ثم التلقيح بمزرعة فتية (كثافة ضوئية ٠,٨) من بكتريا *S.mutans* S2 ثم الحضن بدرجة ٣٧ م لمدة ٢٤ ساعة تحتظروف لا هوائية. وقد تم الحصول على اعلى انتاجية من الميوتاسين تحت هذه الظروف معبر عنه بدرجة تثبيط نمو بكتريا الاختبار (*S. pyogenes*) بقطر تثبيط مقداره ٣٥ ملم.