The effect of chemical and physical factors on biofilm production in *Staphylococcus epidermidis*

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Abstract

The study was aimed to evalute the effect of chemical factors (sugars, salts, and amino acids) and physical factors (media, temperature, PH, incubation periods, and airation) on production of biofilm by *Staphylococcus epidermidis*. The study was carried out on 20 bacterial isolates of *S. epidermidis*, cultured in three different media, which are: Luria-Bertani broth (LB), Tryptic soya broth (TSB) and Brain heart infusion (BHI). Three types of sugar were used in this experiment (Glucose, Lactose, Maltose), prepared in three concentrations (1 , 5 and 10)mg\ml, three types of amino acids (Glycine, arginine, and valine) were prepared in four concentrations (5 , 10 , 20 and 40)mg\L, and six types of salts ($Fecl_3$.6H2O, Mgcl, Zncl, Kcl, Nacl, Cacl) were used in four concentrations (1 , 3 , 5 and 10)mg\ml.

Results of this study showed that all bacterial isolates of *S. epidermidis* formed biofilms. The highest percent of biofilm formation was recorded for glucose at 10 mg\L , arginin at 20 mg\L and 40 mg\L concentration, and calcium at 3 mg/ml concentration. The optimal conditions for biofilm formation by *S. epidermidis* were 25 Cº temperature, 100 rpm\min airation, a 48-hour incubation period, and alkaline PH at 9 degree. From this study, we can conclude that, chemical and physical factors could affect biofilm formation by *S. epidermidis.*

Key word: *S. epidermidis*, biofilm formation**,** chemical and physical factors, sugars, salts, and aminoacid.

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Introduction

Staphylococcus epidermidis, a member of the coagulase-negative staphylococci group, is found in every human's commensal skin flora (1). *S. epidermidis* is the most common staphylococcal species known in humans. It is a commensal bacteria that colonizes the skin and mucous membranes of mammals. Healthy adults carry between 10 and 24 distinct strains of *S. epidermidis* at any given time, according to epidemiological studies (2). The species is critical for the maintenance of local homoeostasis in its native habitat (3) and the prevention of more pathogenic bacteria, such as *S. aureus*, from attaching (4,5). The capacity of *S. epidermidis* to build biofilm and colonize biomaterials is its most well-known pathogenicity factor. Biofilm is a complex interaction of unicellular organisms that is often coated in a polysaccharide, protein, and nucleic acid extracellular matrix (6).

Biofilm formation is traditionally broken down into at least three phases. Bacteria adhere to the surface to be colonized during the primary attachment phase, while bacteria initiate the formation of a three-dimensional, multi-cellular, and multi-layered architecture during the accumulative phase, in which, intriguingly, most bacteria do not have direct contact with the surface (7,8). *S. epidermidis* can then dismantle the biofilm structure once more, and the freed cells are thought to allow *S. epidermidis* to colonize other body areas. Attachment, maturation, and dissemination are all part of the bacterial biofilm development process (9).

The physic-chemical properties of the cell membrane, substratum surfaces, and incubation conditions have all been shown to influence adhesion (10). Biofilm production can be influenced by a variety of environmental elements in the food industry, including food contact surfaces, nutrient availability, temperature, and maturation time (11). For example, under the same culture conditions, bacteria isolated from the interior surfaces of facilities in a milk powder processing factory formed more biofilms on stainless steel than on polystyrene surfaces (12). The isolated strains were capable of growing and producing biofilm in nutrient broth and tryptic soy broth at both mesophilic and thermophilic temperatures (30–55 °C) (12).

The study was aimed at evaluating the effect of chemical (sugars, salts, and amino acids) and physical (temperature, PH, incubation periods, and ventilation) factors on the biofilm production by *Staphylococcus epidermidis*.

Materials and methods

1-Preparation of bacterial inoculation

According to CLSI (2021), a single colony was selected from the nutrient agar plate by a cotton swab. The swab was dropped into the liquid medium and was swirled. The culture medium was loosely covered with a sterile cap that is not airtight. . Bacterial culture was incubated at 37°C for 12-18hr. The incubated bacterial tube was standardized with McFarland (1.5 $\times 10^8$ CFU/ml).

2-Bacteria identification

The study was conducted on 20 isolates of *S. epidermidis* for a period extending from December 2020 to July 2021 in the al-Ramadi and Al-Faluja cities in Al-Anbar governorate of Iraq. The isolates were collected from clinical specimens (231) of skin,urin,wounds,eye, blood,catheter, teeth are plate onto blood agar ,Manitol salt agar at 37C for 24-48hr and gram staining to guarantee the purity and to study their morphologic structure based on the gram staining results , biochemical tests and identification was performed by VITEK 2 system according to the instructions of the manufacturers.(22)

3- Biofilm formation

Most *S.epidermidis* isolates were giving strong biofilm and some moderate biofilm and measured by modified crystal violet assay done according to (21). Discard the bacterial containing from each well and washing with distilled water lightly, drying the plate and left it 10 minutes ,staining with crystal violet stain for 15min , washing with distilled water and drying, dding glacial acetic acid for 3 min then reading optical density with eliza reader..The average was acconting from taking 6 readings for each type of media then observed that BHI broth media was giving highest average for bacteria growing . Then the plates were washed three times with distilled water following incubation, then oven-dried at 60 °C for 45 min, The wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove un adsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125 µl of ethanol to destain the wells. One hundred microliters (100 µl) of the destaining solution was then transferred to a new plate and the absorbance determined (OD) at 630 nm using a micro plate reader. The mean absorbance (OD 630 nm) was used for evaluating the percentage inhibition of biomass formation for each concentration of the test materials based on the following equation:

Percentage inhibition = 100 - [{OD630 nm experimental well with test matrial / OD 630 nm control well without test matrial}x 100].

4- Effect of chemical factors

S. epidermidis isolates exposure different chemical factors were used in this experiment .

1- Effect of sugars

Three types of sugar were used in this experiment (Glucose,Lactose, Maltose) ,prepared in four concentrations (1,5,10)mg\ml in tubes , then transferd to the wells of microtiter plate that containing 200microliter of BHI(brain heart infusion broth) media with 3replicate of each concentration of suger,then adding 10 microliter of inoculm to every well and incubated at 37c for 24hr ,then done microtiter plate method (crystal violet assay).

2-Effect of Amino acids

Three types of amino acids were used in this experiment (Glycin , arginine, Valin) ,prepared in four concentrations (5,10,20,40)gm\L in tubes , then transferd to wells of microtiter plate that containing 200 microliter of BHI(brain heart infusion broth) media with 3replicate of each concentration of suger,then adding the inoculm to every well and incubated at 37c for 24hr ,then done microtiter plate method (crystal violet assay).

-3-Effect of ionic strength

Six types of salts(Fecl3.6H2O, Nacl, Zncl, Cacl2, Mgcl, Kcl) were used , prepared in four concentrations (3, 1, 10,5)mg\ml in tubes ,then transfered to the wells of microtiter plate that containing 200 microliter of BHI(Brain heart infusion broth) media with three replicate of each concentration of salts then adding the inoculm to every well and incubated at 37C for tow days ,then done the microtiter plate method(crystal violet assay).

5- The effect of physical factors

1- Effect of type of culture media .

Staphylococus epidermidis isolates were cultured on three types of media are 1-Luria-Bertani broth (LB) , 2-Tryptic soya broth(TSB) ,3-Brain heart infusion broth (BHI), on microtiter plate ,then incubate at 37C for one day ,after 24hr apply the crystal violet assay .

2-Effect of PH

Microtiter plate that contain media and bacterial inoculation exposure to three degree of PH factor (Alkaline 5 , Nutrilized 7, Acidity 9) ,Hcl used for obtain acidity degree while NaOH used to obtain alkalinity degree.

3-Effect of Incubation period

After inoculate the media (brain heart infusion broth) with bacterial isolates and tranfered to the wells of microtiter plate , incubate the plate in different incubation degrees (12,24,48,72) hr, after 24hr read the optical density on elisa reader

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4-Effect of Temperature

After inoculate the media (brain heart infusion broth) with bacterial isolates and tranfered to the wells of microtiter plate , incubate the plate in different temperature degrees (15, 25, 30, 35, 37, 40) Centigrate, after 24hr read the optical density on elisa reader .

5-Effect of airation

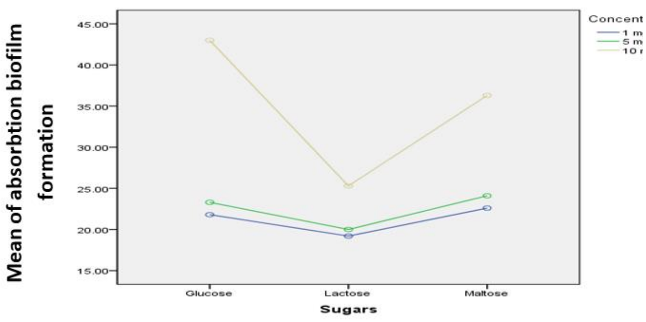
On different degrees of airation (50, 100, 150, 250, 350, 650)rpm\min , microtiter plate was read and apply crystal violet assay.

Results and discussion

Three concentrations of each sugar was used (1,5,10)mg\ml. Results of this study showed that all bacterial isolates of *S. epidermidis* was forming biofilms. The highest percent of biofilm formation was recorded for glucose in 10 mg/ml concentration at 43% (strongly produced), followed by maltose in 10 mg/ml concentration at 36.3% (strongly produced) this mean that high concentration of sugars in the media result in more bacterial activity and multiplication and therefore more biofilm formations and vice versa. While, maltose in different concentrations was moderately biofilm forming. Table (1) and Fig. (1).

Table 1. Effect of different concentrations of sugars on biofilm formation by *S. epidermidis*.

Sugars Conc	Concentration					
	1 mg/ml		5 mg/ml		10 mg/ml	
Glucose	21.8	Moderate	23.3	Moderate	43	Strong
Lactose	19.2	Moderate	20	Moderate	25.3	Moderate
Maltose	22.6	Moderate	24.1	Moderate	36.3	Strong



Sugars concentration

Figure 1. Effect of different concentrations of sugars on biofilm formation by *S. epidermidis*.

Four concentrations of each amino acid was used (5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L).

Results of current study showed that all bacterial isolates of *S. epidermidis* were forming biofilms in the presence of different amino acid in different percent. The highest percent of biofilm formation was recorded for arginine at 20 mg/L and 40 mg/L concentration (strongly produced), while other amino acids (arginine and valine) in different concentrations were moderately biofilm forming, this is due to the microorganism *S. epidermidis* is prefer arginine as a nutrient source that encouraging production of biofilm by this bacteria. Table (2) and Fig. (2).

Table 2. Effect of different concentrations of amino acids on biofilm formation by *S. epidermidis*.

Amino acid Conc	Concentration							
	5 mg/L		**10** mg/L		**20** mg/L		**40** mg/L	
Glycine	25.1	Moderate	27.7	Moderate	21.4	Moderate	21.6	Moderate
Arginine	19.8	Moderate	28	Moderate	34.1	Strong	34	Strong
Valine	27	Moderate	28.5	Moderate	29	Moderate	29.8	Moderate

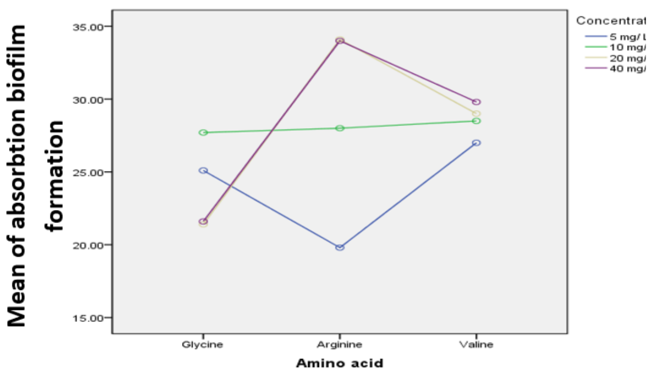


Figure 2. Effect of different concentrations of amino acids on biofilm formation by *S. epidermidis*.

Four concentrations of each salt was used (1 Molarity, 3 Molarity, 5 Molarity and 10 Molarity). The results of recurrent investigation revealed that the presence of calcium in the media in a 3 Molarity concentration (strongly produced) recorded the highest percent of biofilm formation, this finding suggest that calcium in a 3 mg/ml encouraging bacterial growth and thereby biofilm formation in high amount. While potassium (K) 10.1 Molarity and ferrum (Fe) 12 Molarity recorded the lowest percent of biofilm formation and this mean that those tow salts inhibit *S. epidermidis* growth and decrease biofilm production. Table (3) and Fig. (A).

Table 3. Effect of different concentrations of salts on biofilm formation by *S. epidermidis*.

Salts Con	Concentration							
	1 mg/ ml		3 mg/ ml		5 mg/ ml		10 mg/ ml	
Fe	33.8	Strong	34.7	Strong	12	Moderate	30.3	Moderate
Mgcl	34.9	Strong	32.9	Strong	33.9	Strong	31.4	Moderate
Zncl	29.3	Moderate	31.8	Moderate	49.3	Strong	37	Strong
Kcl	10.1	Moderate	40.7	Strong	24.8	Moderate	26	Moderate
Nacl	37.2	Strong	34.8	Strong	51	Strong	22.8	Moderate
Cacl	37.7	Strong	67.3	Strong	12.3	Moderate	18.6	Moderate

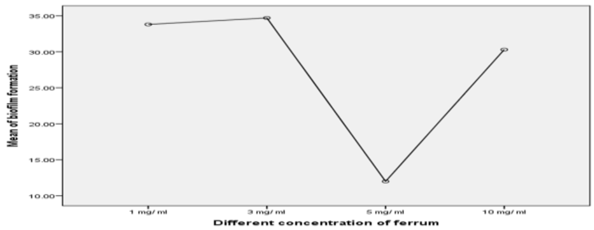


Figure A. Effect of different concentrations of ferrum (Fe) on biofilm formation by *S. epidermidis*.

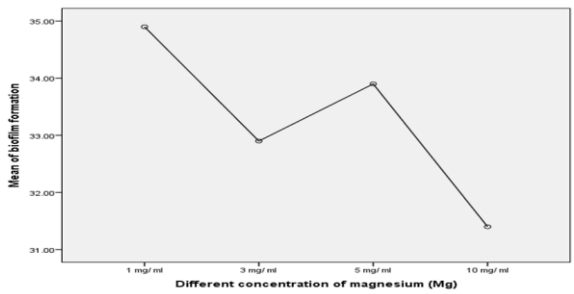


Figure B. Effect of different concentrations of magnesium (Mg) on biofilm formation by *S. epidermidis*.

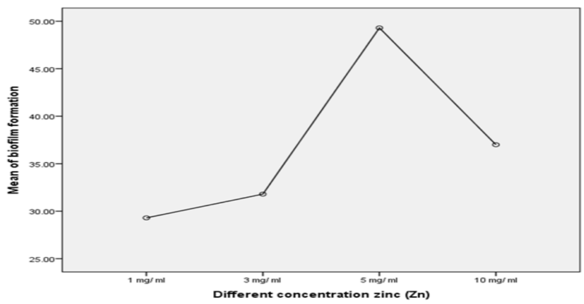


Figure C. Effect of different concentrations of zinc (Zn) on biofilm formation by *S. epidermidis*

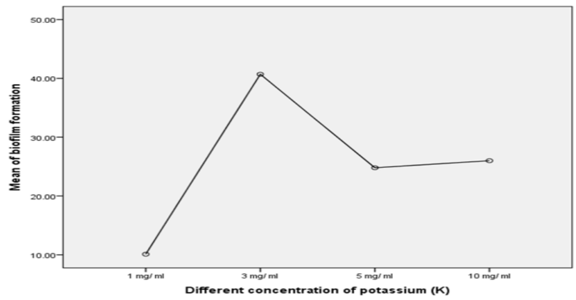


Figure D. Effect of different concentrations of potassium (K) on biofilm formation by *S. epidermidis*.

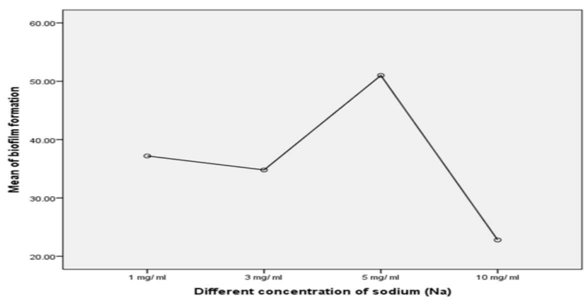


Figure E. Effect of different concentrations of sodium (Na) on biofilm formation by *S. epidermidis*

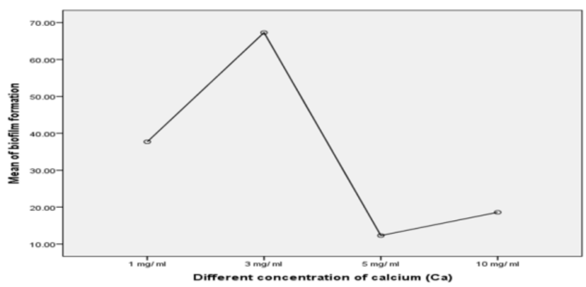


Figure F. Effect of different concentrations of calcium (Ca) on biofilm formation by *S. epidermidis*.

Table 4. Effect of physical factors (PH, temperature, media, incubation period, and airation) on biofilm formation by S. epidermidis. *S stronge biofilm *M Moderate biofilm

PH												
	22.3	M	32.7	S	36.6	S						
Temp.	15Cº		25 Cº		30Cº		35 Cº		40Cº			
	27.7	M	48.5	S	22.2	M	19.5	M	17.8	M		
Media	BHI		TSB		LB							
	28.4	M	21.7	M	20.1	M						
Incubation period	12hr		24hr		48hr		72hr					
	24.8	M	27	M	39.4	S	30.8	M				
Airation	50		100		150		250		350		650	
	41	S	48	S	44.4	S	35.2	S	26.2	M	24.2	M

Results of Table (4) illustrated the role of different physical factor (PH, temperature, culture media, incubation periods, and airation) on the biofilm formation by *S. epidermidis*. Three different PH was used (acidic 5, neutralized 7 and alkaline 9), the highest percentage of biofilm formation (strong) was observed at alkaline PH (36.6) and neutralized 7 (32.7) that indicating to the alkaline PH 9 and neutralized PH stimulating biofilm production and acidic PH 5 inhibit biofilm formation by *S. epidermidis* Fig (B). Temperature was used at five different degrees (15 Cº, 25 Cº,30 Cº 35 Cº, and 40 Cº) the highest percentage of biofilm formation (strong) was observed at 25 Cº (48.5) while, the lowest percent was observed at 40 Cº Fig (C). This result meaning that the elevation of environmental temperature result in inhibition of bacterial activity and suppress biofilm formation, as well as the decrease temperature gave the same result while, the temperate heat encouraging bacterial biofilm production.

Three different media was used (BHI, TSB, and LB). There was close proportions of biofilm formation in the three culture medias and in all of them the biofilm formation was moderate. Fig. (D). Four incubation periods were used (12hr, 24hr, 48hr, and 72hr). The highest percentage of biofilm formation (strong) was observed at 48hr incubation period (39.4). Fig. (E). Six levels of airation were used (50, 100, 150, 250, 350). There was strong biofilm formation in the airation level of (50, 100, and 150) while, moderate biofilm formation was observed in airation level of (250, 350,) and illustrating that more level of airation result in decreased biofilm formation and vice versa. Fig. (F).

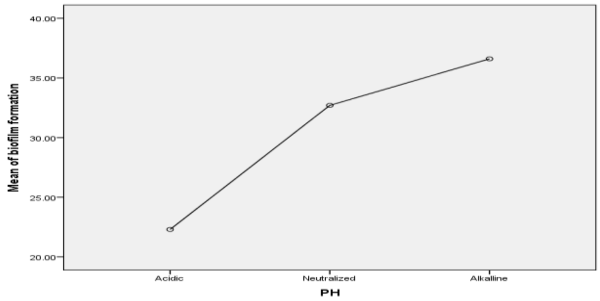


Figure 4. Effect of different PH on biofilm formation by *S. epidermidis*.

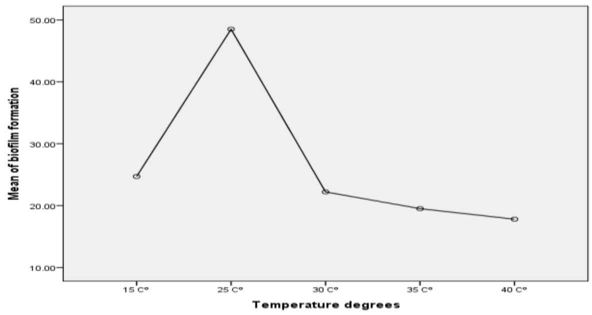


Figure 5. Effect of different degrees of temperature on biofilm formation by *S. epidermidis*.

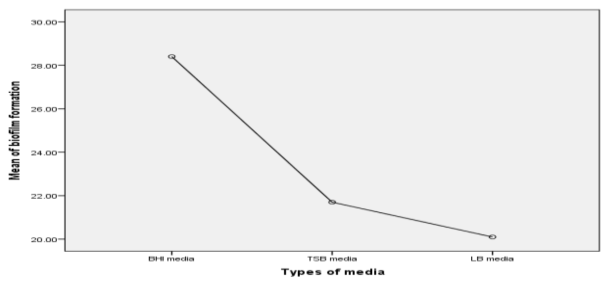


Figure 6. Effect of different culture media on biofilm formation by *S. epidermidis*.

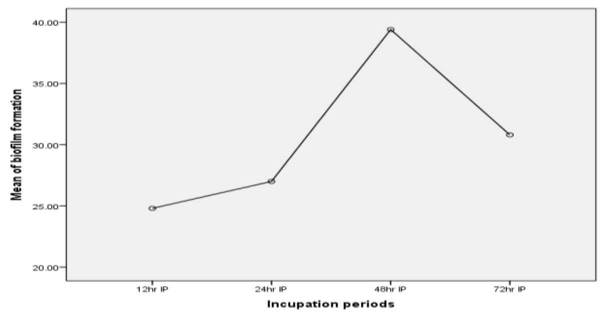


Figure 7. Effect of different incubation periods on biofilm formation by *S.*

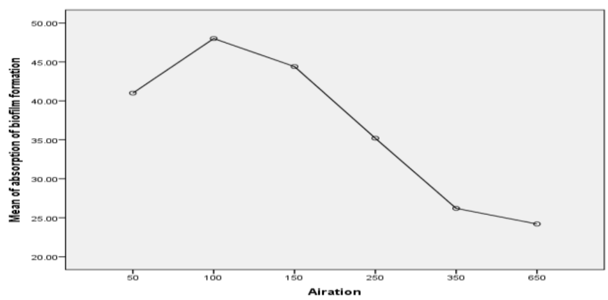


Figure 8. Effect of different levels of airation on biofilm formation by *S. epidermidis*.

Biofilm development is thought to be a major factor in the contamination of biomedical devices by all *staphylococcal* species. Biofilm-producing *staphylococci* isolated from different clinical samples are also of clinical importance since biofilm serves as a reservoir for pathogens and is linked to antibiotic resistance and persistent infections. As a result, an accurate and simple approach to their diagnosis is required (14). Results of the current study showed that all bacterial isolates of *S. epidermidis* formed biofilms. The highest percent of biofilm formation was recorded with glucose at a 10 mg/ml concentration. This result is agreed with by (15), who suggested that *S. epidermidis* developed biofilms under all tested conditions and when glucose was available in the growing environment, the most efficient extracellular polymeric material (biofilm) synthesis occurred. The presence of sodium chloride and glucose in combination aided biofilm development (16).

Results of the present investigation showed that the highest percent of biofilm formation was recorded with arginine in 20 mg/L and 40 mg/L concentrations (strongly produced) (Table 2). This result disagrees with that of (17), who suggested that arginine has an influence on biofilm formation and pathogenesis and reduces polysaccharide intercellular adhesion and accumulation. This may be due to the different media that were used as a bacterial culture. In this study was found that calcium in a 3% concentration (strongly produced) recorded the highest percent of biofilm formation, while potassium (Kcl) 10.1 mg/ml and ferrum (Fe) 12 mg/ml produced the lowest percent of biofilm formation (table3). This finding is agreed with the results of (18), who recorded that K and Fe inhibit bacterial growth and therefore inhibit biofilm formation, while Cacl encourages bacterial growth and biofilm production.

In this study was found that the highest percentage of biofilm formation (strong) was recorded at 25 Cº temperature, and the bacterial ability of biofilm formation decreased as environmental temperature increased(table 4). This is due to the elevated temperature being considered as a stress factor for bacterial growth. This result is nearly similar to that of (16), which suggested that the elevated temperature affected the expression of several biofilm associated genes (e.g. *icaA* and *rbf*). (19) reported that a 16–20-hour incubation period was found to be optimum for biofilm development. (20) reported that at highly acidic (pH 3) and alkaline (pH 12) levels, the biofilm formation was lower, while at pH 7 the adhesion was moderate. So the highly acidic and highly alkaline PH act as a stress factor against biofilm formation. While there were no differences in biofilm formation among the three different media, in all of them the biofilm was moderately formed.

Conclusions

All 20 isolates of *Staphylococcus epidermidis* formed biofilm in different amounts, ranging from moderate to strong, depending on chemical and physical factors. So, chemical and physical factors affect biofilm formation by *S. epidermidis*.

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