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INVESTIGATION OF THE MELANIN IN*STAPHYLOCOCCUS AUREUS* ISOLATED FROM PATIENTS WITH URINARY TRACT INFECTIONS.

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Abstract

The current study included isolating *Staphylococcus aureus* from the urine of men with urinary tract infections, where 17 isolates belonging to this species were obtained, which were diagnosed traditionally and by polymerase chain reaction. Then, the ability of the isolates to produce melanin was tested, as it is a substance that has great medical benefits, as it can be isolated and used as an antibacterial and antifungal, it has many other benefits. All isolates were melanin-producing, but the best of them were isolates 1, 2, 3and 10, which gave melanin amount of 3.1, 2.8, 3, and 2.9 mg/L, respectively, and gave a dry weight of 8.8, 6.5, and 6.1and 3.6 mg/l. Nitrogen sources gave close amounts of melanin, as follows: soybeans produced melanin with an amount of 2.7, yeast extract 2.6, and peptone 2.65 mg/l, while the best carbon sources culture medium was starch2.8 mg/l, then glucose came after that with 2.6 mg/l, then glycerol, maltose, fructose, sucrose, dextrose, and mannitol, with a yield of 2.4, 2.2, 2.2, 1.7, and 1.5 mg/l, respectively. The best temperature for melanin production was 35℃, followed by 30℃, then 25℃, then 40℃, then 20℃, with a melanin production amount of, 2.8, 2.3, 1.9, 1.7 and 1.1mg\l respectively. As for the pH, the best was 7, followed by 6, then 8, followed by 5, then 4 with melanin amount of 2.7,2.2, 1.7,1.7 and 1.3 mg\lrespectively.

Keywords: Melanin, *Staph. aureus*, nitrogen source, carbon source ,temperature and pH .

Introduction

Melanin is a protein that gives tissues a dark color [1]. There are pigments found in human skin, hair, and eyes, as well as in plants, birds, reptiles, foods, insects as well as in microorganisms, with some differences in quantities and chemical composition. There are specialized cells to produce melanin called melanocytes, and their effectiveness increases whenever they are exposed to sunlight[2]. The process of melanin production takes place through the oxidation of the amino acid tyrosine, which is the cornerstone of the production process[3]. Melanin has many functions, it protects tissues from harmful radiation that causes cancer, especially ultraviolet radiation, and prevents the oxidation process in the cell and helps microorganisms resist dehydration, hunger, and toxic substances, and it has an antibacterial role. It also gives harshness and roughness to the organism's body, which helps it to stand firm in harsh environments[4]. One of the distinctive things in which melanin plays a pivotal role is the process of disguise, changing color according to the environment, and evading enemies by camouflaging[5].Melanin pigment spreads in various living organisms, and there are three types of melanin according to size, color, composition, and location [6]. The first type is eumelanin, and it is characterized by a very dark color, as is the case in dark hair, as well as dark-colored colonies of bacteria, fungi, some parasites, and others. It is the most effective in absorbing UV light by 99%. The second type is pheomelanin, which is reddish in color, as is the case in the lips, tongue, and sexual organs in humans. It is also responsible for the pink colors of some plants and mushrooms. The third is neuromelanin, and we find it in cells with unclear features, squirrel color, such as neurons in humans. It is also found in plants, fungi,bacteria, and others[6].
Recently, have been shed light on microorganisms as producers of melanin, especially bacteria and fungi. Studies have shown that bacteria can oxidize tyrosine into an intermediate compound, L-DOPA, and then turn into DOPA quinone, which decomposes into cysteinyl-DOPA and chromium DOPA, which crystallize to give The first is eumelanin and the second is pheomelanin in fungi there are another type of melanin can produce it called allomelanin [7].Scientists have recently paid attention to the melanin found in bacteria and found that the bacteria that produce melanin are characterized by being more virulent than others, as they resist the body's immunity, radiation, hunger, thirst, and oxidation,toxins, adhesion, or hemolysis. Many methods have been devised to investigate the ability of bacteria to produce melanin, as well as to purify it and evaluate its effect on pathogenic fungi and bacteria, which opens new horizons in the field of treating bacterial diseases [8, 9].

Aims of study

The current study is the first of its kind in Iraq that sheds light on the melanin produced by the bacterium *Staph. aureus* to know its ability to produce and purify it ,this melanin can be used as anti-inflammatory agent or in other fields[10].

Material and Methods

Isolation of *Staph. aureus*

Staph. aureus was isolated from urinary tract adult male patients attending the Teaching Hospital in Al-Diwaniyah with the help of laboratory staffs. The period of study includes three months from Abril to August 2022.The first urine drops were discarded while Med-stream was adopted[11]. Isolation was done using agar neutrino medium, which was cast in plastic Petri dishes with a diameter of 9 cm, where the isolation was done by the method of dilution as we worked. [12]Several dilutions by sterile distilled water, and the dilution was stabilized at 1×10^3[12]. All plates were then incubated at 37 °C until bacterial colonies appeared, where they were purified and prepared for subsequent experiments.The isolates were diagnosed based on their farm behavior, which includes the shape, color, smell, and texture of the culture. They were also examined under a microscope to see their cluster shape. Biochemical tests were also performed to confirm the diagnosis[13].

Molecular study

The molecular diagnosis of the 10 suspected isolates was carried out by checking for the presence of the 16S rRNA gene, as the instructions attached with the genetic diagnostic kit prepared by Pioneer were followed in terms of extraction, amplification, and electrophoresis, according to the[14-16] .The primer used in this test is 5' GTA GGT GGC AAG CGT TAT CCC GCA CAT CAG CGT CAG 3'implication size with 228 bp [17].

Melanin production test

Melanin phenotype examination in *Staphylococcus aureus* was performed according to the method used in[18], bacterial isolates were grown on neutrino broth medium at a rate of 10 ml in a test tube and inoculated with lubrication of loop only once and then left. To grow at 37 m until stagnation after one week and then take 1 ml of the bacterial suspension and mix with 0.4% of the substrate which is L-tyrosine. The reaction mixture was mixed well, then incubated at 30 m for half an hour, where the color of the solution became red, then examined by a spectrophotometer at a wavelength of 480 nm[19].

Optimization of isolates in melanin production

Many factors were tested for the purpose of knowing which one's support melanin production and which ones inhibit its production, and this is directly related to the pathogenesis of *Staph aureus*, where the effect of carbon source, nitrogen source, temperature and pH was tested by manipulating the culture medium and incubation method, according to what was stated in[18].Also biomass were evaluated via centrifuging the liquid culture and the sediments were takenand drayed then the dray weight were taken.

Extensive melanin production

The shake flask method was adopted by[20].1000 ml of production medium consisting of nitrogen source- 0.2%, soyabean meal and 1.0% of sugarcane waste concentration, for 7 days incubation at 35°C, pH 7 and salinity 15ppt.Then, melanin pigments were extracted by centrifuging the liquid culture at 3000 rpm for half an hour, after which an equal volume of chloroform, ethyl acetate and methanol was taken. Add with cell-free supernatant and mix well. These steps were repeated 2-3 times. Then the solvent was evaporated. The powder was collected while the pigment residue was added[21].

Results and Discussion

Isolation of *Staph. aureus*

Figure 1 shows the number and percentage of bacteria isolated from patients with urinary tract infection under study. The study showed that there are many bacterial genera present in the infected persons and may be responsible for the infection alone if in cooperation with other genera. *E. coli* bacteria were in the foreground with several 35 isolates accounted for 41%, followed by Staphylococcus aureus with 17 isolates and a rate of 20%.

Then came Klebsiella spp, Pseudomonas aeruginosa, Enterobacter spp and *Proteus mirabilis*, with rates of 13, 11, 9 and 5%, respectively.

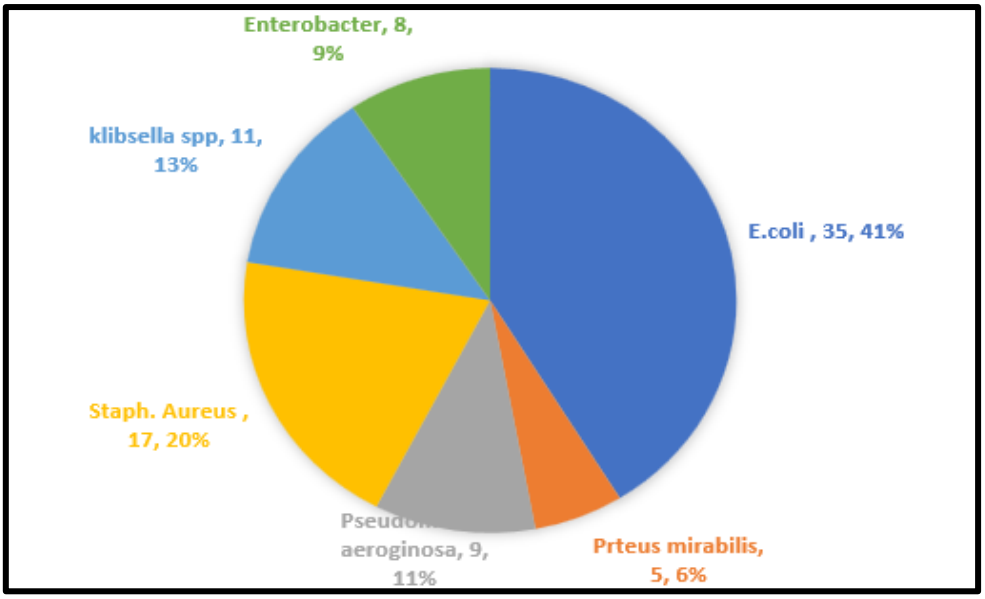

Figure (1) number and percentage of bacteria isolated from UIT inflammation patients.

The urinary tract is a very suitable place for the growth of many microorganisms, in terms of humidity, temperature, appropriate pH, as well as the availability of nutrients[13]. Some of these microorganisms are harmless and are part of the natural flora, especially *Staph. aureus* bacteria, which may turn into a pathogen in the event of a weakening of the immune system because of drug use[17]. Some immunosuppressive drugs or infection with some chronic diseases and other pathological symptoms such as AIDS, asthma, and allergies, where then an overgrowth of these bacteria occurs and attacks the human body and causes congestion and ulceration of the tissue lining the urinary system[22].*Staphylococcus aureus* is characterized by its ability to adapt, withstand difficult conditions, resist drugs, and produce toxins, which increases its risk, especially in people with low immunity who were mentioned above.[23]mentioned that the staphylococcus aureus bacteria lives in a throwing form in many parts of the body, but it may turn into a pathogen in some people due to its possession of some factors such as hydrolytic enzymes and endotoxins, and the risk increases when the environment is suitable for the bacteria and is not suitable for the host.

Identification

Regarding the diagnosis of bacterial isolates, it was done initially by relying on the clinical diagnosis made by the doctors, then by relying on the direct examination of the urine sample, then by the method of culture and biochemical tests, where 17 isolates belonging to *Staph.aureus* were obtained. There was some doubt about ten isolates of them, so an examination was conducted Its PCR showed a complete match of 100%, which confirms that these isolates are Staphylococcus aureus. Figure 2 below shows the results of the PCR examination in the agarose gel, where we note the appearance of the bands of the ten isolates under test at the base pair 228 when compared with the referenced isolate. It has the letter M.

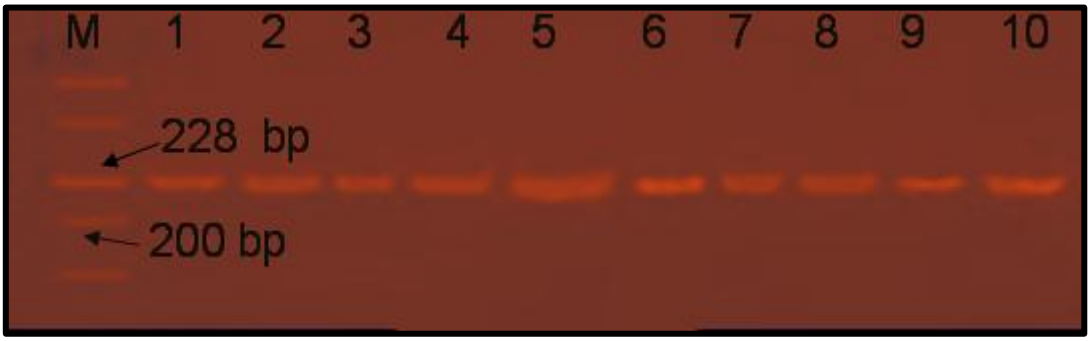

Figure (2) The PCR products of amplifying of 16s rRNA gene in 1.5 % agarose gel ,the numbers indicate the Staph. aureus isolates and M the lane.

Genetic diagnosis based on polymerase chain reaction is one of the crucial methods of diagnosis, which has become routinely performed in various laboratories around the world, and this, of course, does not mean the abolition of the traditional initial diagnosis, which is also indispensable in the sense that both methods complement each other, and many newer and more accurate methods have appeared recently by using more stable primers, which gives results that cannot be challenged.Scientists are now targeting specific genes to ensure their presence and spread among bacterial strains, as well as evolutionary relationships and the extent of their gene expression, such as drug resistance and virulence genes[24].

Melanin Production test

Table No. 1 shows the ability of the isolates from Staphylococcus aureus to produce melanin in the medium for this test and the relationship of production to the biomass of bacteria in terms of dry weight, where the ability of the isolates to produce melanin ranged between high production, such as isolates No. 1, 2, 3, and 10, which produced melanin of 3.1, 2.8, 3, and 2.9 mg/l, respectively, and gave a dry weight of 8.8, 6.5, and 6.1and 3.6 mg/l, respectively.

The difference in the ability to produce melanin by *Staph. aureus* isolates can be attributed to two main reasons, namely the genetic aspect, as some isolates contain the genes necessary for melanin production, such as the *mel*A gene[7], and not only this, but the gene must be able to express itself clearly and efficiently[25]. The other side is the external environment includes heat, nutrients, salts, pH, and other competing microorganisms. The carbon and nitrogen source also plays a major role in this, as food rich in tyrosine facilitates the process of melanin production[26].

All that we mentioned previously applies to the biomass, which the more it increases, the more it supports the production of melanin, as the cell becomes full of nutrients that support the interaction and increase its duration and increase productivity [27].

Table (2) ability of *Staph. aureus* for melanin production

Isolate no.	Melanin gm/l	Biomass gm/l
1	3.1	8.8
2	2.8	6.5
3	3	6.1
4	2.2	5.1
5	1.9	3.5
6	2.7	3.5
7	1.9	3
8	2.2	2.7
9	1.1	3
10	2.9	3.6
11	0.4	1.4
12	0.9	1.1
13	0.2	1.3
14	0.3	0.5
15	0.8	1.6
16	0.4	1
17	0.3	0.7

Optimization of melanin production

Carbon source

Regarding the carbon source, the results in Figure 3 showed that starch was the best among the sources used, as the amount of melanin produced in the culture medium was 2.8 mg/L, then glucose came after that with 2.6 mg/l, then glycerol, maltose, fructose, sucrose, dextrose, and mannitol, with a yield of 2.4, 2.2, 2.2, 1.7, and 1.5 mg/l, respectively.

The differences in the amount of production that appeared when changing the carbon source were mainly due to the metabolic reactions of the bacterial cell and the extent of the chemical suitability between the carbohydrate substance and the metabolism of melanin production, and this depends on the chemical composition of the carbohydrates, the number and type of bonds and the effective free aggregates[28].

As well as its physical nature, such as the composition of its crystals, the speed of its dissolution in the culture medium, and other specifications

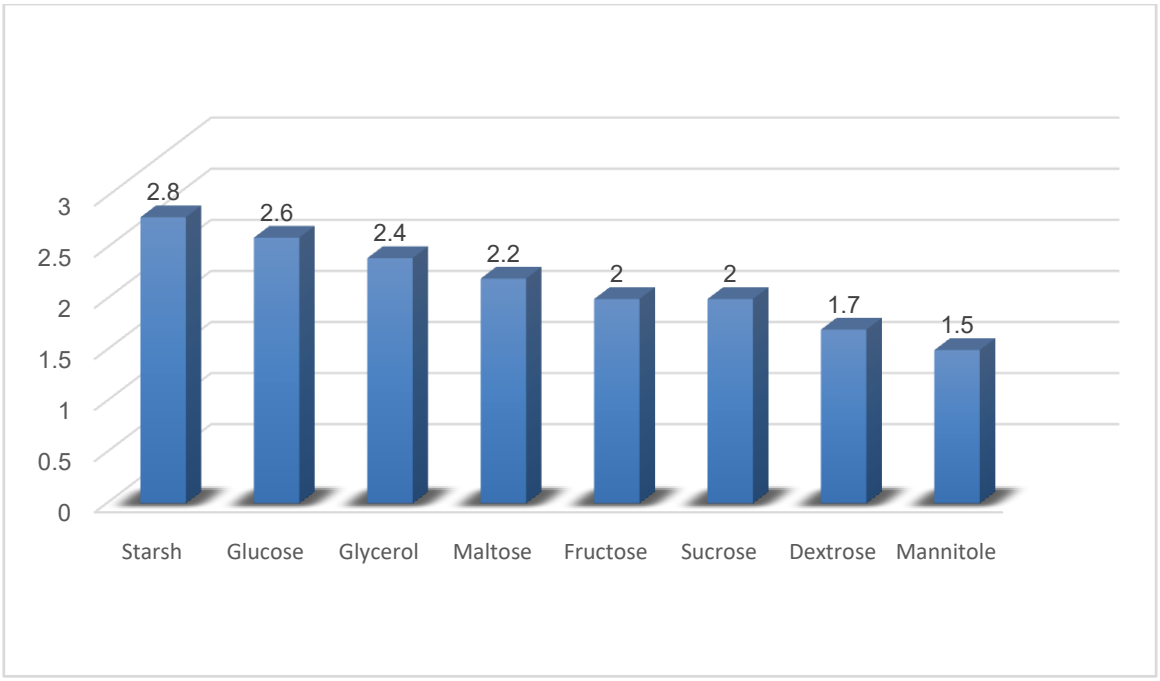

Figure (3) effect of carbon source on melanin production from *Staph. aureus*.

Nitrogen source

The response to changing the nitrogen source was significantly similar, as all nitrogen sources gave close amounts of melanin, as follows: soybeans produced melanin with an amount of 2.7, yeast extract 2.6, and peptone 2.65 mg/l. The nitrogen source is very important, as it strongly supports the production of melanin, especially if we know that melanin metabolism depends on amino acids, and therefore there is an urgent need by the cell for the presence of nitrogen. The results of our study showed that the bacterial cell of Staphylococcus aureus is able, with its enzymes, to exploit nitrogen sources and adapted to produce melanin of two types, eumelanin and pheomelanin[29].

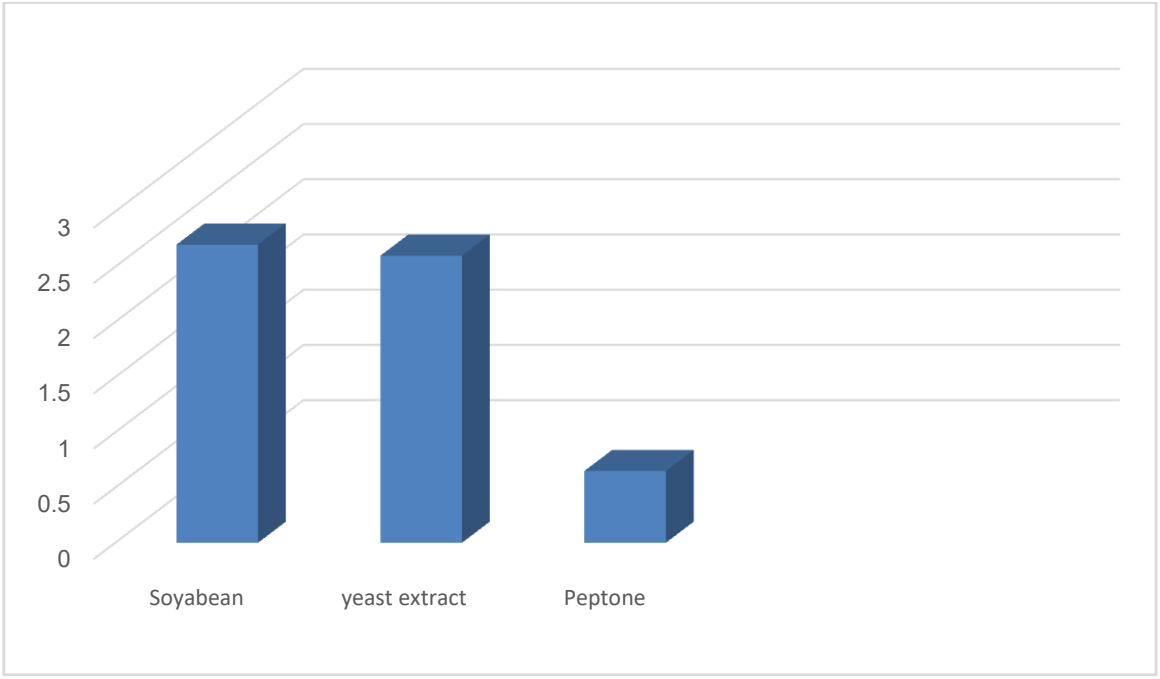

Figure (4) effect of nitrogen source on melanin production from *Staph. aureus*.

Effect of temperature and pH on melanin production

The temperature had a significant impact on the melanin production process, as the amount of melanin production varied according to the temperature, and the best grades were 35, followed by 30, then 25, then 40, then 20, with a melanin production amount of,2.8, 2.3, 1.9, 1.7 and 1.1respectively.As for the pH, the best was 7, followed by 6, then 8, followed by 5, then 4 with melanin amount of 2.7,2.2, 1.7,1.7 and 1.3respectively.

The results also showed that there is a direct relationship between the amount of melanin produced and the biomass of Staphylococcus aureus. The higher the biomass, the greater the amount of melanin produced[30-35].
These results give us the impression that whenever bacteria live in good conditions suitable for their growth and reproduction, they produce a greater amount of melanin. Tyrosine transformations down to the final output. Our words support this also that the largest biomass is the one that gave more melanin due to the abundance of enzymes and the large number of reactions that lead to melanin as a final product[35-40].

Table (2) effect of temperature and pH on melanin produced by *Staph. aureus*.

condition		*Melanin mg/l*	*Biomass mg/l*
Temperature	20	1.1	4.1
	25	1.9	4.2
	30	2.3	6.6
	35	2.8	10.2
	40	1.7	7.1
pH	4	1.3	1.4
	5	1.7	6.6
	6	2.2	10.5
	7	2.7	12.1
	8	2	8.2

Conclusion

- All *Staph. aureus*isolate under study were able to produce melanin but in vary levels.
- All nitrogen sources used in studygave similar effect for melanin production by *Staph. aureus.*
- The starch was the best in support production of melanin by *Staph. aureus*.
- The 35 ℃and 7 pH gave the best results in production of melanin via *Staph. aureus*.
- *Staph. aureus* can be adapted as producer for melanin which can use in many purposes.

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