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Detection the role of physiological factors to produce carotenoid pigment in *Staphylococcus aureus*

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ABSTRACT

Three *Staphylococcus aureus* isolates were used in the present study for extraction and quantitation of carotenoid pigment production. Different culture conditions were used to determine optimum pigmentation such as type of culture media, different pH values, temperature, and finally daily bacterial sub-culturing for more than 2 weeks. Nutrient agar was found to be the best medium with the highest production (1.09). Optimum pH value was 6.5 and gave (1.8) carotenoid. Also results showed that pigment was produced at 37^{o} C more than at 42^{o}C. Daily repeated sub culturing had a negative impact on pigment production. Colonies gradualy lost pigmentation and became white in color. Also, sub-culturing was shown to affect the volume of the colonies as they became smaller on solid media.

Introduction

Carotenoids are natural pigments distributed vastly in the nature with a wide diversity of functions and chemical composition. The major cause for using microbes to produce such composites that can otherwise be extracted by animals and vegetation or chemically synthesized is the easiness of augmenting production by ecological and genetic manipulation [1] .

Carotenoids are hydrophobic pigments that are embedded inveterately in the photosynthetic membrane. They are typically yellow, brown, red, brown, or green and absorb light in the blue region of the spectrum [2].

Carotenoids are produced by *Staphylococcus aureus* (*S. aureus*), and gram-positive cocci relatively resistant to reduced water potential and it is tolerating desiccation and high salt (NaCl) concentration. This bacterium is aerobic desiccation with a typical respiratory metabolism (catalase-positive). They are yellow-pigmented species [3].

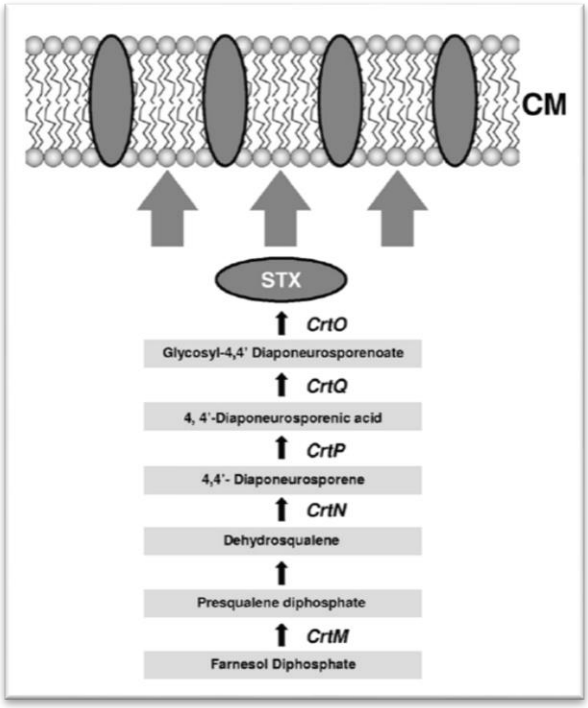
One study [4] estimated that almost all *S. aureus* bacteria isolated from human diseases are pigmented Staphyloxanthin (STX) producing is an triterpenoid membrane-bound carotenoid. The pathway of STX biosynthetic for is illustrated in Figure (1),which includes those *crt* operon genes involved at the different STX synthesis steps [5] .

Epithet appellation of *S. aureus* bacterium reflects the color of the colonies (aureus means golden). This pigment has a role in pathogenicity, when it has been analyzed: identifying as triterpenoid carotenoids possessing a C_{30} chain, with biosynthesis genes are regulated in an operon, *crtOPQMN*, controlled by a σ^{B}–dependent promoter upstream of *crtO* and termination area downstream of *crtN* [6, 7].

Carotenoids are among the bulk plentiful natural pigments available in environment. These pigments received big interest because of their biotechnological applications, also interestingly, due to their useful uses in human healthcare, pharmaceuticals, cosmetics and food processing, the modern standards for a healthy lifestyle and ecofriendly practices give escalate to searche for natural biocompounds as alters to synthetic ones. So, currently, biomass of microorganisms is being used to obtain naturally-available carotenoids with high antioxidant capacity. This is an alternative to the in vitro synthesis of carotenoids, which is costly and propagate a large number of residues, and the compounds synthesized are sometimes inactive biologically [8].

Carotenoids are present in a wide diversity of bacteria, algae, fungi, and plants having the ability to absorb light energy, food colorants, oxygen transporters [9], antioxidants, and consequently, they could possess antitumor activity; also, certain

carotenoids exhibit pro-vitamin A activity; and lastly, they may exhibit a protective effect against the development of degenerative diseases, such as cancer and heart diseases, or they may be able to prevent metabolic diseases, such as type 2 diabetes [10, 11].



(Mishra *et al*., 2011)[5]
Fig.(1): biosynthesis pathway to produc Carotenoid; CM: Cell Membrane; STX: Staphyloxanthin; CrtO,Q,P,N,M: proteins of *crt* operon genes.

Due to the significant of carotenoid pigment extracted from microorganisms, so, the current study aimed to optimize pigment production with respect to type of culture medium, pH, incubation temperature, and repeated subculture of *S. aureus* on production of this pigment, and to utilize a typical conditions to produce it for applied benefit.

Materials and methods

▪ **Bacterial isolates:** Three isolates of *S. aureus* were obtained from Department of Biology / college of science / University of Mosul. The sources of isolation were from clinical cases.

Assertion of identification was achieved before the beginning of the study according to morphological, microscopic characteristics and biochemical tests according to Procop and coworkers [12] , these isolates were named *S. aureus* 1, *S. aureus* 2, and *S. aureus* 3.

▪ **Extraction and Quantitation of Carotenoid pigment:**

Extraction was done according to Dufosse and coworkers [13], by taking each of three bacterial isolates from heavy growth of 24 hours from agar medium by rinseing each plate with 1.5 ml of Double distilled water (ddw), then bacterial cells are centrifuged(Labnet. USA) at 6000 g for 15 minute.

The pigment was extracted from bacterial cells pellets as follows:

- Pellets were mixed with 8 ml of 99.9% methanol in a sterilized tube, and wrapped using aluminum foil to preclude exposure to light.
- The tube was placed in agitator at (50 rpm) till cells are bleached (2 hours).
- Methanol portion was dissociated from biomass after centrifuging (Remi, India) at (6000 g) for 15 minute.
- Purification was performed by another centrifugation at (10000 g) for 15 minutes.
- Content of pigment was measured by the absorption at 450 nm using UV- spectrophotometer.

▪ **Effect of some physiological factors on carotenoid production:**

Production of carotenoid was measured in *S. aureus* bacteria after modification in physiological conditions mentioned below for detection the effect of each factor dependently in production positively or negatively as follow:

- Culture media: Three culture media were used to determine its effect on pigmentation, they were: Tryptic soy agar (TSA), Manitol salt agar (MSA) and Nutreint agar (NA).
- pH: the pH of medium was altered at 6.3, 6.5, 6.8 before bacterial incubation.
- Temperature: inoculated plates were incubated at 37 and 42^{o} C.
- Repeated subculture: daily subculture was achieved for two weeks, (16days), four measurements were done for carotenoid production. The first day was as control.

Results and discussion

Following confirmed identification of the three *S. aureus* isolates depending on their characteristics and tests according to Procop and coworkers[12], pigment production was studied under various physiological factors namely; culture media types, pH value, incubation temperature and repeated subculture.

Results showed-through measuring content of carotenoid extracted by spectrophotometer of 450 nm-that NA was the best among three **media** TSA, MSA, and NA, in carotenoid pigment production by *S. aureus* as shown in table (1) where the value and pigment production was 0.5 and 0.3 for isolate (1) and (3) respectively, while the isolate (3) gave the highest production of pigment equal to 1.04.

MSA medium was the least efficient for isolate where it gave 0.3, 0.24, and 0.29 respectively. On TSA medium, the value of pigment production were 0.38, 0.32 and 0.4 respectively.

In conclusion of these results, NA medium was the best in pigment production (it has been used in later experiment related to pH, temperature, and repeated subculture) and the isolate *S. aureus* 3 had the highest efficacy among others.

Our results did not agree with researcher Lan and coworkers [14] who mentioned that a strong yellow pigmentation was observed when plated on TSA

suggesting overproduction of pigment. Al-Kazaz and her coworkers [15] mentioned that TSA medium had middle production of STX, while N.A. did not give satisfied result in pigment production, and this differs on our result.

The cause of pigment production from our isolates on N.A medium among other media may be return to contain N.A of beef/ yeast extract which provide *S. aureus* with important nutritional contents such as carbohydrate, vitamins, nitrogen and salts; and this is not present in TSA and MSA. Furthermore MSA as mentioned by Sun and coworkers [16] could not provide suitable of substrate that cause STX production when are consumed .

The **pH** values used demonstrated less variations in pigment production. Generally, pH (6.5) gave the best production, followed by 6.8, while pH equal to 6.3 resulted in low pigment production as show in table (1). Also, results revealed that *S. aureus* (1) gave the highest production at pH= 6.5 where the value of carotenoid production was 1.8.

According to previous data, pH= 6.5 was used in preparation of NA medium to know the effect of temperature and repeated subculture.

Our result agree with Goswami and coworkers [17] who used pH from (1-14) to illustrate optimum pH that gave its effect on pigment production in *S. aureus*, their study revealed that considerable pH was between 6 and 10 with optimum cell growth and production of pigment observed at pH 7. Also they mentioned to a gradual decrease in growth and carotenoid formation with the high pH levels from 7 - 10. and no growth and no pigmentation was noted under pH 6 .

Other comparison about pH is with Al-Kazaz and her coworkers [15] which revealed difference of our results, the best pH value for them was (8) and this is not comports with our study

A study by Mandelli and coworkers [18] revealed in their research, there was a relationship between carotenoid production in particular bacterium with pH, pigment production has increased as pH increase.

Incubation **temperature** was also studied by growing three *S. aureus* isolates on NA medium with pH= 6.5, the better incubation was at 37^{o}C, the results demonstrated as show in table (1) that temperature 42^{o} C inhibited pigment production, reading values seemed low (0.1-0.3) compared with values at 37^{o} C (0.3-0.43). and the isolate *S. aureus* 2 occupied the first rank in pigment production.

Our results agree with Goswami and coworkers [17] who incubated at temperatures ranging from 4^{o} - 45^{o}C. they found that maximum pigment production was at 30^{o}C. There was a gradual declines in growth and carotenoid production with the boosting in degree of temperature, from 30- 40°C. At temperatures above 40 °C and lower than 10°C, minimum pigment content is noted.

As well as, our results agree with study of Al-Kazaz and her coworkers [15] in determination of optimum conditions for STX production, which was pigment production was increased at 37^{o}C, wherase decreased in high temperature more than 37^{o}C, and this agree also with kim and Lee [19]

Because the temperature noteworthy bulk for production of carotenoids, So, the implementation of a two-phase cultivation process, where the production of carotenoids is apart from the cellular growth, would accomplish both, high levels of cellular growth and pigment production [18].

The researcher Tjahjono and coworkers [20] mentioned to *Haematococcus pluvialis* which when grows at elevated temperatures 30 °C could increase the formation of singlet O_2 in cell, that in consequence will stimulate the carotenogenesis.

Table (1): Values of carotenoid pigment content for three *S. aureus* according to physiological factors

Physiological factors	**Culture media**			**pH value**			**Temperature**	
Bacterial isolates	TSA	MSA	NA	6.3	6.5	6.8	37	42
*S. aureus*1	0.38	0.30	0.55	0.43	1.8	0.68	0.3	0.18
*S. aureus*2	0.32	0.24	0.39	0.65	0.6	0.69	0.43	0.28
*S. aureus*3	0.41	0.29	1.04	0.52	0.57	0.42	0.4	0.3

Study of Malodnade and coworkers [21] revealed that the amount of pigments can be varied with modifying some circumstances, like pH, nitrogen and carbon sources, incubated temperature and existence the amount of light with salts values. A good nitrogen source for the production of carotenoids is yeast extract. Also it seems that microorganism growth and this pigment production do not have a direct correlation, A two-stage batch fermentation is shown to be better for the production of this pigment by particular microorganism. In the first stage, the microorganism could grow under high levels of carbon with nitrogen; in the second stage, enhancement of pigment production can be established by placing the it under especial conditions..

Daily repeated subcultures have negatively affected carotenoid production from three *S. aureus* isolates. Fig. (2) demonstrates obvious low in carotenoid content after each production measuring for each isolate. So, for isolate No. 1, pigment production in first day was equal to 0.552 and has decreased gradually to 0.024, the first reading for isolate No. 2 was 0.248 then lowered to 0.016, while the value of carotenoid quantity in isolate No. (3) was 0.44 in primery detection and 0.012 in the last detection. This suggests that repeated subculture weakens *S. aureus* ability in production of carotenoid.

The results of repeated subculture reveale that weakness status in *S. aureus* took place where colonies seemed small and lost its pigment as shown in figure (3), this phenotype is similar to which was characterized by Biswas and coworkers [22], they mentioned *S. aureus* bacterial colonies seemed weak when they were exposured to external factors like pyocyanin produced by *Pseudomonas aeruginosa.* They called this phenotype " small-colony variants" (SCV), they stated that this decline in colour is characteristic of SCV.

Anyway, *S. aureus* bacteria as an adaptable pathogens can avoid these hindrances with the help of choosing of a respiration- defective subpopulation that was characterized with the phenotype (SCV). The clogged of the electron transport path leads *S. aureus* to fermentative pathway, which is followed by a lowered ATP totted and weak growth when lastly results in the formation of tinier colonies. SCVs were known to persist best than their normal counterparts within host cells.

The amount of current scientific knowledge on the genesis of phenotypic variants and the mechanisms of switches between different phenotypes is much smaller than the amount of data on genomic variations. This holds particularly true for the phenomenon of the small colony variant (SCV) phenotype. A deep understanding of the importance of the SCV phenotype considers a general strategy for bacterial survival and growth.

The lifestyle of SCV change is accompanied to a conversion of the bacterium's metabolism, and affects the host-bacteria interplay, the SCV lifestyle fits into a broader concept of persistence for long-term survival of bacteria within their environment [23].

SCVs were designated as a specific kind of staphylococcal "dissociants" or as "dwarf" colonies. *S. aureus* SCVs are characterized by their small colony size, slow growth, and downregulated virulence genes, while genes important for biofilm formation and adhesion are mostly upregulated. Regarding their nature and medical impact, early speculations hypothesized SCVs to have a position in the life cycle of the microorganism or to represent a state of temporarily decreased metabolism; the uncommon physiological, metabolic, and morphological features of SCVs are challenging for the routine diagnostic laboratory [24].

Since SCVs differ from the wild-type phenotype in their generation time, growth, colony morphology, and many metabolic and other physiological characteristics, they are difficult to detect, often overlooked. Otherwise, the normal staphylococcal phenotype is characterized by medium-sized colonies, reaching 1 to 3 mm in diameter within 24 h, SCV colonies have a pinpoint size that is about 1/10 the size of the parental strain. In contrast to isogenic parental strains of a given species, SCVs are nonpigmented or show a strongly reduced pigmentation[23, 24], So, Lan and coworkers [14] demonstrated the *sigB* mutant featuring a colorless phenotype, which indicate that *sigB* is necessary for pigment production. As well as loss SCV for pigment may be due to defect in its biosynthesis which demand acetyl coenzyme A and acetoaetyl-CoA by meralonate pathway [25].

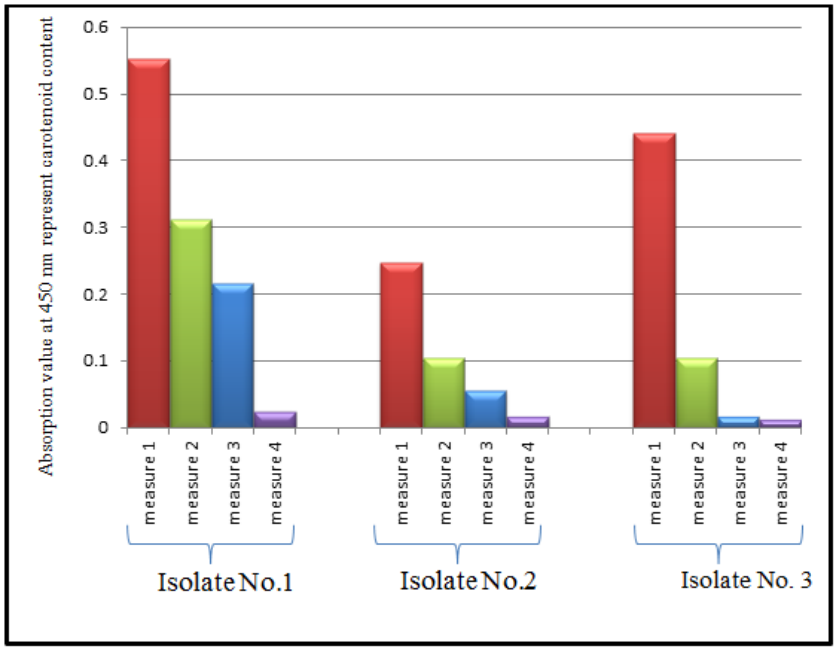
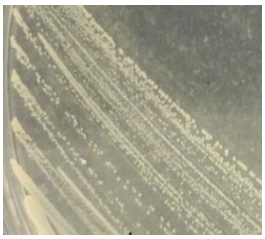
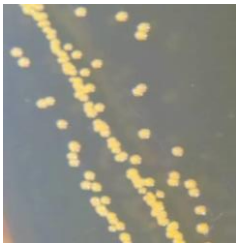


Fig. (2): The effect of repeated subculture on carotenoid production by three isolates of *S. aureus*



Phenotype of *S. aureus* colonies after repeated subculture (small whit colonies)



Wildtype of *S. aureus* colonies (normal-large- golden colonies)

Fig. (3):normal phenotype of *S.aureus* colonies (on the right), SCV of S. aures after repeated subculture (on the left).

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التحري عن دور العوامل الفسلجية لانتاج صبغة الكاروتينويد في جرثومة *Staphylococcus aureus*

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الملخص

استخدمت في هذه الدراسة ثلاث عزلات بكتيرية تعود للنوع *S. aureus* من اجل استخلاص صبغة الكاروتينويد منها وقياس كميتها، والتحري عن انتاجية هذه الصبغة من قبل العزلات بعد اجراء تعديلات في بعض الظروف الفسلجية مثل نوع الوسط الزرعي المستخدم لتنمية البكتريا، وقيمة الدالة الحامضية له فضلا عن الدرجة الحرارية الفضلى لانتاج هذه الصبغة، واخيرا اجراء تكرار للزرع البكتيري بشكل يومي لفترة تجاوزت الاسبوعين. واظهرت النتائج ان افضل وسط زرعي لانتاج صبغة الكاروتينويد كان وسط الاكار المغذي NA اذ كانت اعلى قيمة انتاج للصبغة فيه بقيمة 1.09، وان افضل داله حامضية هي 6.5 التي اعطت اعلى انتاج للصبغة بقيمة 1.8، اما الدرجة الحرارية 37 فقد كانت افضل من درجة 42 ^{o}م. كما بينت النتائج ان عملية اجراء الزرع اليومي المتكرر لبكتريا *S. aureus* قد اثر سلبا على انتاجها للصبغة حيث تناقص محتواها من الكاروتينويد تدريجيا فقد بدت المستعمرات البكتيرية بيضاء فاقدة للصبغة، بالاضافة الى ذلك اثر الزرع المتكرر على حجم المستعمرة البكتيرية مما جعلها تبدو صغيرة الحجم عند نموها داخل الوسط الزرعي.