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Effect of Cultivation Conditions on Hemolysin Production from Clinical Isolates of *Serratia marcescens*

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ABSTRACT

Sixty-four isolates collected from clinical sources in Baghdad and Mosul city. The identification of *S. marcescens* confirmed by using API 20E and VITEK-2 systems. Twelve isolates of *S. marcescens* produced hemolysin, which detected by two ways, qualitative hemolytic assay and quantitative hemolytic assay. The optical density of hemolysin producing isolates at standard condition (37ºC, pH=7, 24h) showed that *Serratia marcescens* (SmU9) isolate gave the highest absorbance of hemolysin production (0.7) but at (25°C, pH=7,24h) the absorbance of hemolysin production turn into (3.0), compared with the absorbance at 571nm for Control without hemolysin (0.060) and completely hemolysis by Triton x-100 (6.0). However, the optimum optical density of the best hemolysin producing isolate (SmU9) at the optimum cultivation conditions (25°C, pH=9, 24h, 150 rpm, 1% inoculum size in Nutrient broth) was (6.0).

KEYWORDS: *Serratia marcescens* , hemolysin , cultivation condition

الخلاصة

اربعة وستون عزلة جمعت من مصادر سريرية في مدينة بغداد والموصل . تشخيص بكتريا Serratia marcescens تم التأكد منها باستخدام انظمة API 20E و VITEK-2. اثني عشر عزلة من S.marcescens انتجت هيمولايسين التي كشفت عن طريقتين،فحص التحلل النوعي وفحص التحلل الكمي . الكثافة الضوئية للعزلات المنتجة للهيمولايسين في الظروف القياسية (37مئوية ، 7pH ،24h) اظهرت ان عزلة (SmU9 (S.marcescens اعطت اعلى كثافة ضوئية لانتاجية الهيمولايسين (0.7) ولكن عند (25 مئوية، 7 pH، 24h) الامتصاصية لانتاجية الهيمولايسين اصبحت (3.0)، مقارنة مع الامتصاصية عند 571nm للكنترول بدون هيمولايسين (0.060) والتحلل الكلي بواسطة Triton x-100 (6.0) لكن الكثافة الضوئية المثلى لافضل عزلة منتجة للهيمولايسين (S.mU9) عند الظروف الزرعية المثلى(25 ،1% ،150 rpm ،24 h ، PH=9 ، حجم oC اللقاح البكتيري في وسط Nutrient broth) كانت (6.0).

INTRODUCTION

Serratia marcescens microorganisms are related to the Enterobacteriaceae rod-shaped family, Gram-negative bacteria. They are motile, catalase-positive and oxidase-negative microorganisms. This bacterium has the capacity to generate prodigiosin pigment, which binds to the cell membrane of bacteria [1]. Because *S. marcescens* has a high adaptation and survival capacity, aswell as the ability to utilize a wide range of nutrients, it can live and thrive in a variety of harsh environments, such as disinfectant solutions, antiseptics, and double-distilled water [2]. *Serratia marcescens* has strong cell surface hydrophobicity, thus the bacteria rapidly colonize surfaces and are plentiful near the air-water interface [3]. Furthermore, *S. marcescens* has been demonstrated to be able to use surface-bound nutrients such as long chain fatty acids [4]. *Serratia* hemolysin A (*ShlA* haemolysin) is one key virulence factors of *S. marcescens* secreted via a two-partner secretion system of type Vb, which consists of the *Serratia* hemolysin B (*ShlB*) translocater and *ShlA* [5]. Unless phosphatidylethanolamine is introduced to the activation, highly pure *ShlB* cannot convert inactive *ShlA* to hemolytic *ShlA* [6]. *ShlA* lyses red blood cells, releasing heme and hemoglobin, which is then picked up by the extracellular

protein HasA and transported into the bacterialcell [7]. This study aims to selection of best production of hemolysin isolates and studying the effect of cultivation condition (temperature, incubation period, pH, inoculum size, culture media and aeration) on hemolysin production from clinical isolates of *Serratia marcescens*.

MATERIALS AND METHODS

Bacterial suspension

The selected isolate of *S. marcescens* was activated by picked 4-5 colonies from original culture and suspended in 5ml of Brain heart infusion broth in test tube. The spectrophotometer measurement of turbidity at 625nm was set to 0.5 McFarland standard suspensions.

Screening of *S. marcescens* isolates for hemolysin production

Plate Method (Qualitative hemolytic assay)

Bacterial suspensions in sterile saline matching to (1.5 x10^8 CFU/ml) were done from 18h cultures of *S. marcescens* strains. 10µl of each suspension was dropped on the surface of the blood agar media and was incubated at 37°C for 16h. After 16h, the hemolysis was examined [8].

Spectrophotometric Method (Quantitative hemolytic assay)

The hemolysin production was detected in liquid medium by spectrophotometric method described by Di Venanzio *et al*., 2014 [9] with some modification, *S. marcescens* were grown over-night (37ºC) in Nutrient broth. 20µl of *S. marcescens* compared to 0.5 McFarland wasadded to 1980µl of Nutrient broth and incubated at 37 ºC for 24h . Red blood cells suspension was prepared by washing 2ml of blood with 8ml PBS buffer in centrifugation at 3000 rpm for 5 min(3times), the sediment suspended in PBS buffer (0.8ml RBC were added to 9.2ml PBS buffer),1ml of bacterial culture was added to 1ml of RBC suspension and incubated at 37ºC for 1 h , on the other hand, the supernatant of *S. marcescens* culture was tested for hemolysin presence, centrifugation were done after incubation at 12,000 rpm for 10min to collect the supernatants, and the hemolysin production was measured by spectrophotometer at 571nm. The percentage of hemolysis was the relative optical density for sample compared to (OD) for Complete hemolysis of RBC, (Complete hemolysis of RBC was carried out by adding 1% Triton X-100) [10].

Calculation of hemolysis percent

hemolysis%=A571(sample with hemolysin)–A571(control without hemolysin)/A571(total lysis caused by Triton x-100)–A571 (control without hemolysin)*100

Determination of optimum cultivation conditions for hemolysin production from clinical isolates of S. marcescens

1. Temperature Nutrient broth tubes were inoculated with bacterial suspension of *S. marcescens* (SmU9) and incubated at different temperature (25, 30, 37 and 40) ºC for (24, 48, 72) hrs. After incubation, the percentage of hemolysin production were estimated for each temperature.

2. IncubationTime Nutrient broth were inoculated with bacterial suspension (SmU9) and incubated at best temperature (section-1) for (24, 48 and 72) h, then the percentage of hemolysin production were estimated for each incubation time.

3. PH Nutrient broth were prepared with different pH value (5,6,7,8 and 9), pH were adjusted by using electronic pH-meter and 1N of NaOH solution and 1N of HCl solution, then media were prepared, autoclaved and inoculated with bacterial suspension (SmU9) and incubated at best temperature (section-1) for best incubation time (section-2). After that the percentage of hemolysin production were estimated for each pH value.

4. Inoculum Size To determine the effect of inoculum size on hemolysin production, the culture medium (Nutrient broth) was adjusted to the best pH (section-3), then inoculated with various inoculum size (1, 2 , 4 , 6 , 8 , 10)% of inoculum(1.5 x 10^8)CFU/ml for (SmU9) isolate and incubated at the best temperature (section-1) for the best incubation time (section-2). The percentage of hemolysin production was estimated for each inoculum size.

5. Culture Media Three types of media were used, Nutrient broth, Brain heart infusion brothand Tryptic soy broth. Each of the broth was adjusted to the best pH (section-3), then inoculated with best inoculum size (section-4) of bacterial suspension (SmU9), then incubated for the best incubation time (section-2) at best temperature (section-1) and the percentage of

hemolysin production were estimated for each culture media.

6. Aeration For studying the influence of aeration on hemolysin production, the best culture medium (Nutrient broth) (section-5) was adjusted to best pH (section-3), inoculated with best inoculum sizeof (SmU9) (section-4**)** and incubated at best temperature (section-1) with shaker (150rpm) or without shaking (static) for best incubation time (section-2), then the percentage of hemolysin production were estimated for each status.

RESULTS AND DISCUSSION

Screening of *S. marcescens* isolates for hemolysin production

Plate Method (Qualitative hemolytic assay)

All *S. marcescens* isolates was tested for production of hemolysin using agar plates. The results demonstrated the appearance of clear zones of hemolysis after the end of incubation period around the growing colonies with different diameters as in (Table 1) and revealed that 18.75% *S. marcescens* isolates had the ability for producing this enzyme and 81.25% did not produce hemolysin.

Table 1. Hemolysin production by the clinical isolates of *S. marcescens* grown on blood agar after 24 h.

***Serratia marcescens* isolates No**.	**Diameters of hemolytic zones (mm)**	**Sources**
SmB1	15	Burn
SmB2	15	Burn
SmU1	7	UTI
SmU2	8	UTI
SmU3	9	UTI
SmU4	10	UTI
SmU5	15	UTI
SmU6	20	UTI
SmU7	20	UTI
SmU8	20	UTI
SmU9	30	UTI
SmW1	10	Wound

S. marcescens isolate (SmU9) isolated from urinary tract infection (UTI) gave the highest zone of hemolysin (30mm) among all isolates. These results agreed with Shimuta *et al.*, 2009 who showed *S. marcescens* have hemolytic activity on blood agar and *ShlA* was expressed at lower temperature [8]. However, at 37°C, its expression was decreased. Moreover, Gulbahar, 2019 who revealed that *S. marcescens* produce hemolysin enzyme on blood agar [11]. In an experimental rat pyelonephritis model, Marre *et al.*, 1989 foundthat bladder colonization of the *Serratia* hemolysin producing strain was significantly higher than renal colonization higher than that of *ShlA* negative recipient strain, indicating that the *Serratia* hemolysin contributes to uropathogenicity and increased leukocyturia and thickening of the bladder walls were shown to be signs of involvement of *ShlA* in inflammatory reactions [12]. The capacity of bacteria to survive in humans is dependent on their ability to quickly adapt to changing environmental factors such as temperature, pH, osmolality, incubation duration, oxygen tension, and nutrient availability and Iron reduction, which significantly increased the virulence factors of hemolysis [13].

Spectrophotometric Method (Quantitative hemolytic assay)

S. marcescens isolates were examined for their ability to produce hemolysin by liquid medium. Results showed the isolates that gave hemolysin production, as illustrated in the (Table 2), compared to the optical density at 571nm for Control without hemolysin (0.060) (1%) and Complete hemolysis (6.0) (100%).

Table 2. Hemolysin production from *S.marcescens* isolates by liquid medium.

***Serratia marcescens* isolates No.**	**Absorbance of hemolysin (OD-571 nm)**	**Hemolysis (%)**
Control without hemolysin	0.060	1
Complete hemolysis by Triton x-100	6.0	100
SmB1	0.5	7.4
SmB2	0.5	7.4
SmU1	0.4	5.7
SmU2	0.3	4.0
SmU3	0.5	7.4
SmU4	0.5	7.4
SmU5	0.4	5.7
SmU6	0.6	9.0

SmU7	0.5	7.4
SmU8	0.6	9.0
SmU9	0.7	10.7
SmW1	0.5	7.4

The result in line with agar medium method and *S. marcescens* isolate (SmU9) showed the highest yield of hemolysis (10.7%), Urine had been the source of it and it was chosen as the best isolate for the production of hemolysin. This result agreed with Maarib *et al.*, 2020 they found highest absorbance of hemolysin production at405nm was (1.09) at (pH=7, 37°C ,24h) [14].This result also agreed with result of Alonso & Baquero, 1994 who found that 19.4% of *S. marcescens* strains were hemolytic, however, only highly hemolytic strains could be identified using standard hospital hemolytic tests [15]. Variation in hemolysin production, on the other hand, might be attributed to *S. marcescens* genetic variation as well as differences in isolation source (blood, wound, burn, urine and sputum) [16].

Effect of cultivation conditions on hemolysin production from clinical isolate of *S. marcescens* (Sm7)

- ***Temperature*** The selected isolate *S. marcescens* (SmU9) was incubated for different temperature (25, 30, 37, 40) °C for determinationthe optimum temperature for hemolysin production. The optimum temperatures of hemolysin production was 25°C by which theabsorbance at 571nm was (3.0) but at 40°C the hemolysin production declined to (0.2) (Figure1).

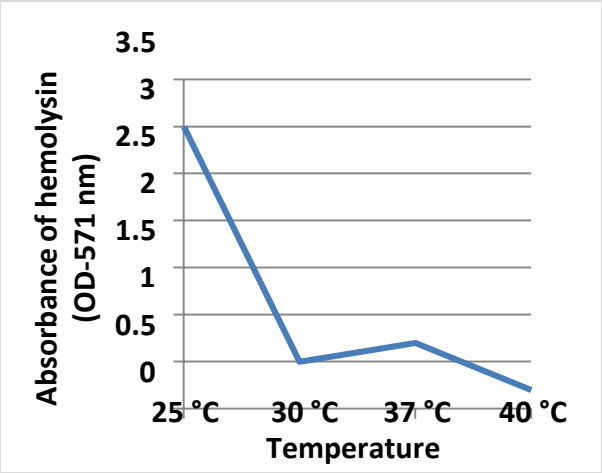

Figure 1. Effect of temperature on hemolysin production from clinical isolate of *S. marcescens* (SmU9).

These results agreed with those reported by Poole & Braun, 1988 which observed that bacterial cells cultured at 37°C gave 10-fold less lysis compared with those grown at 30°C because of altering in LPS structure and decreasing the levels of the outer membrane protein *ShlB* at 37°C, also these results indicated a significantly increased in expression of *shlA* gene in 30°C [17,18]. Research found that there is a connection between enzyme production and energy metabolism in bacteria, which is influenced by temperature and oxygen acquisition, temperature had been shown to affect the release of extracellular enzymes through modifying the physical characteristics of the cell membrane [19].

- ***Incubation Time*** This study investigated optimal incubation periods that were used to estimate hemolysin production by *S. marscecens* and the results of the survey after using varied incubation period (24,48,72) h showed that thebest incubation period was 24h, at this time the absorbance at 571nm for hemolysin productionwas (3.0), (Figure 2).

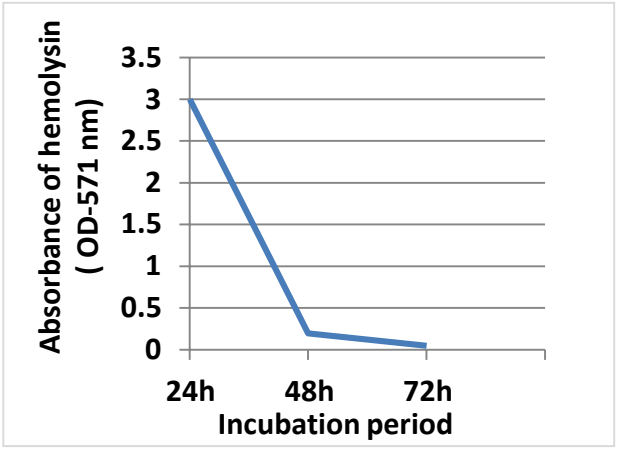

Figure 2. Effect of incubation time on hemolysin production from clinical isolate of S. marcescens (SmU9).

These results disagreed with Maarib *et al*., 2020. They showed that optimum incubation time was 48hr [14], but agreed with Carbonell *et al*., 1996. They found that bacterial growth looked to be a significant factor in the generation of cytotoxin by *S. marcescens* [20].

During the mid-to-late logarithmic phase of growth *S. marscecens* hemolysin was formed [21]. The activity of *S. marcescens* hemolysin is controlled by the growth of culture state. In the late logarithmic growth phase, expression of *shlA* and *shlB* is optimum [22]. Previous research had

revealed that specific nucleoid based environmental conditions influence the expression of several virulence genes [23, 24].

- ***PH*** Variety of media pH values (5, 6, 7, 8, and 9) was used to determine the best pH forproducing hemolysin. It appeared that the best hemolysin yield was at pH 9.0 because the absorbance at 571nm was 6.0 (Figure 3).

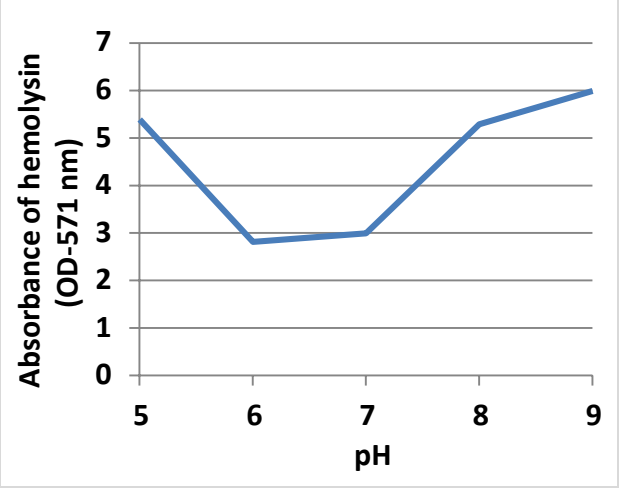

Figure 3. Effect of pH on hemolysin production from clinical isolate of *S. marcescens* (SmU9).

These results did not agree with the pH demonstrated previously, which were pH 5 and 7 [18, 25] but agreed that the best cytotoxin production was alkaline condition [20] because they showed that pH=8.5 was the optimum pH for cytotoxity activity [26]. Revealed that culture pH has been demonstrated to have a significant impact on numerous enzymatic activities as wellas the transfer of different components across the cell membrane. The physiology of microorganisms, such as the optimum activity of enzymes, can be influenced by PH, which in turn affects the effectiveness of the biological treatment process [27]. Extremely high or low pH levels could cause enzyme activity to be lost completely. The enzymes have good activity at physiological pH levels, which are about 7.0 and the optimal pH value for one enzyme might vary based on a variety parameters, including the source of enzyme [28], existence of materials [29], enzyme immobilization [30], mutations in enzymes [31], environmental motivations such as temperature, pH and nutrient availability [32]. Many pathogenicity of bacteria is related to the expression of particular genes, which required for growth and continued existence under changing ecological conditions [33].

- ***Inoculum size*** the effect of inoculum size on hemolysin production was studied. *S. marcescens* (SmU9) was incubated with various inoculum size (1, 2, 4, 6, 8 and 10) % of (1.5×10^8) CFU /ml. Results showed that the best inoculum size for hemolysin production was 1% (Figure 4).

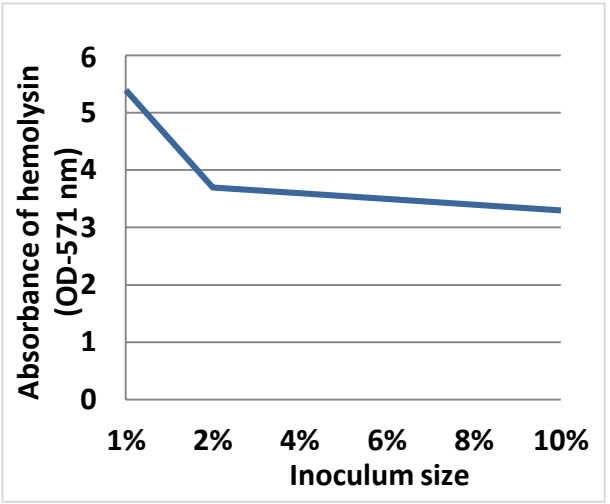

Figure 4. Effect of inoculum size on hemolysin production from clinical isolate of *S. marcescens* (SmU9).

Similar result was also reported; they observed that using a larger inoculum size of 11% (v/v)decreased protease formation more than using a smaller inoculum of 1%(v/v) [34]. As a result, larger inoculum sizes might not always indicate a higher protease production. The greater surface area to volume ratio, which resulted in enhanced protease synthesis, was given as the reason for the rise in protease production utilizing smaller inoculum sizes [35]. If the inoculum size is very small, the number of bacteria existing is inadequate, resulting in a lower amount of released protease [36]. Higher inoculum sizes, on the other hand, may result in or create a shortageof oxygen and nutrient depletion in the culturemedia [35].

There are several hypotheses for the cause of the inoculum effect, including population diversity and quorum sensing, as well as the null hypothesis, which states that the clearly evident inoculum impact and alterations in the growth/no growth border with regard to inoculum size are dependent on the time it requires for a specialinoculum size to achieve growth under the

particular environmental conditions [37]. Thus, only a few studies have looked at these factors in the production of lipases and other enzymes [38,39]. On a two-stage inoculum system, there was also a significant interaction between the primary and secondary inoculums [40]. Additionally, in order to achieve high enzymatic reactions, the inoculum size was a crucial element in the research methodology [41]. Reduced microbial growth and enzyme synthesis at larger inoculum sizes may well be due to fast nutritional depletion. The accumulating of poisonous metabolites might potentially decrease enzyme synthesis [42].

- ***Culture Media*** The effect of culture media on hemolysin production was studied. *Serratia marcescens* (SmU9) was incubated at three different media included Tryptic soy broth (TSB), Brain Heard Infusion broth (BHI) and Nutrient broth (NB). Results showed that the best culture medium for hemolysin production was Nutrient broth in which the absorption of hemolysis at 571nm was 6.0 in optimum conditions, while in BHI and TSB were observed lower hemolysin production 0.4 and 0.3 respectively (Figure 5).

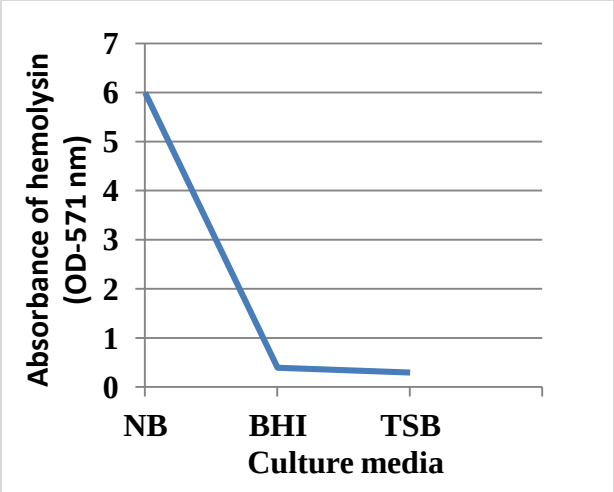

Figure 5. Effect of culture media on hemolysin production from clinical isolate of *S. marcescens* (SmU9).

This result did not agree with Lysyk *etal*., 2002 that showed *S. marcescens* on Nutrient broth did not produce B-hemolysis [43]. Various production of bacteria extracellular toxins and enzymes couldbe influenced by bacterial medium [44]. On the other hand, *S. marcescens* grew poorly on medium containing maltose or inositol and showed very low cytotoxic effect on a monolayer of cells. Bacterial growth was abundant in a minimum medium containing glucose and cytotoxicity was greater than in a medium containing sucrose or galactose [20]. Parameters such as carbon sources, temperature, pH, and incubation period had already a significant impact on enzyme production of microorganisms [45]. However, another study found that not all carbon and nitrogen sourcescould improve simultaneous enzyme synthesis ina single fermentation system in contrast to single- enzyme biosynthesis [46].

- ***Aeration*** When the microorganisms (SmU9) were cultured with shaking at 150rpm. Absorbance of hemolysin at OD-571nm was 5.7 comparing to those grown on static culture. Absorbance of hemolysin at OD-571nm was 5.3no significant variations in hemolysin production were detected, while aeration was considered as the best. This result agreed with Carbonell *et al.*, 1996. They showed that *S. marcescens* growth was poor in static and anaerobic conditions, and that cytotoxin production was minimal [20]. The growth and cytotoxicity of these strains were greatly enhanced by shaking cultures. *Serratia marcescens* is a facultative anaerobic bacterium, which means it might grow aerobically or anaerobically. Besides, it mostly obtains energy from fermentation and contains enzymes superoxide dismutase, catalase, or peroxides that defend it against reactive oxygen [47]. The culture conditions are critical for a successful enzyme synthesis, and optimization parameters like pH and temperature that are essential for the development of this cultivation [48]. Cultivation conditions that affect oxygen passage into culture medium impact metabolism, electron transport, redox balance, and other regulatory processes, resulting in regulatory alterations [49]. The oxygen needs of microorganisms differ. For oxidative processes to produce energy for biological functions, oxygen serves as a terminal electron acceptor. Variations in agitation speed have been shown to affect the extent of mixing in culture flasks, as well as the availability of nutrients [50] and the dissolved oxygen of the culture medium was increased [51]. Decreased thevolume of medium contained in a flask and agitation of the culture were two typical ways for expanding surface area [52].

To summarize, agitation and aeration were major factors for all aerobic processes, and they had a significant impact on the production of most biomaterials. On the other hand, agitation could cause shear forces, which influenced microorganisms by causing changes in

morphology, growth and metabolite formation, and even cell structure injury [53, 54].

CONCLUSION

1. *Serratia marcescens* (SmU9) which was isolated from urinary tract infection (UTI) recorded the highest production of hemolysinin both solid and liquid methods of hemolysin production.
2. The cell-bound hemolysin activity required vitable *Serratia* cells. No hemolysin activitywas found in the culture supernatants.
3. The optimum conditions for hemolysin production were growing *S. marcescens*(SmU9) in NB at 25°C/24h, pH = 9 with 1% inoculum size in aeration (150rpm).

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