RESEARCH ARTICLE

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Isolation and identification of *Staphylococcus aureus* and studying effect of physical and chemical mutagenesis on Hyaluronidase production from Iraqi patients

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ABSTRACT: Isolation and identification of *S.aureus* bacteria from Iraqi patients using microbiological and molecular identification by 16s rRNA system using PCR amplification. Secondary metabolite production from wild strains is very low for economical purpose therefor certain strain improvement strategies are required to achieve hundred times greater yield of metabolites. The present study was conducted for enhanced production of hyaluronidase from *Staphylococcus aureus* S9 by mutagenesis using ultraviolet (UV) radiation, and Mitomycin C (MitC) as a mutagens. Results showed that a hyaluronidase over producer mutant symbol *S.aureus* S9-63 was obtained after mutagenesis with UV irradiation with higher enzyme specific activity (209.0 U/mg protein) in comparison to the wild type (118.02 U/mg protein). On the other hand, another overproducer mutant symbol *S.aureus* S9- 121 was obtained after chemical mutagenesis with MitC characterized with high hyaluronidase production. The enzyme specific activity in its crude filtrate was 188.4 U/mg protein in comparison with wild type. It could be concluded that physical mutagenesis using UV irradiation was more efficient than the chemical mutagenesis by MitC to enhance the ability of *S.aureus* A9 in hyaluronidase production.

Keywords: *Staphylococcus aureus*, Mutant, UV, Mitomycin C

INTRODUCTION

Staphylococcus aureus stays one of the most injurious species of staphylococci faced, it reasons of pneumonia, bacteremia, myocarditis, acute endocarditis, pericarditis, encephalitis, meningitis, chorioamnionitis, osteomyelitis, and scalded skin syndrome mastitis (Klevens et al., 2007). Human sickness and transience in hospital sets are largely initiated by staphylococcal bacteremia (Becker et al., 2004). The pathogenic capability of *Staphylococcus aureus* stays visibly dependent on its manufacture of exoproteins and toxins. The species is identified on the basis of a variety of conservative biochemical and molecular characters. The key characters for *S. aureus* exist colony pigment, free coagulase, clumping factor, protein A, heat-stable nuclease, lipase, and acid production from mannitol (Bisaws et al., 2015). Also *Stapylococcus aureus* identified by PCR methods using 16S rRNA gene which is the most valuable and widely scrutinized taxonomic marker molecules (Lancette and Tatini, 1992). The dominance of *S. aureus* was because of production of virulence factors such as hyaluronidase, lipase, protease and possibly staphylokinase, facilitated dissemination by their enzymatic breakdown of connective tissues ground substance (Monday and Bohach, 1999). Hyaluronidases are a family of enzymes that catalyse the degradation of hyaluronic acid (HA). Karl Meyer classified these enzymes in 1971 into three distinct groups, a scheme based on the enzyme reaction products (Harris et al., 2002). The three main types of hyaluronidases are two classes of eukaryotic endoglycosidase hydrolases and a prokaryotic lyase-type of glycosidase. Some bacteria, such as *S. aureus*, *Streptococcus pyogenes* (Shulman and Nahmias, 2003) and *Clostridium perfringens* (Murray et al., 2003) produce hyaluronidase as a means of using hyaluronan as a carbon source. It is often speculated that Streptococcus and Staphylococcus pathogens use hyaluronidase as a virulence factor to destroy the polysaccharide that holds animal cells together, making it easier for the pathogen to spread through the tissues of the host organism, but no valid experimental data are available to support this hypothesis (Murray et al., 2003). By catalyzing the hydrolysis of hyaluronan, a constituent of the extracellular matrix (ECM), hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability. It is, therefore, used in medicine in conjunction with other drugs to speed their dispersion and delivery. Common applications are ophthalmic surgery, in combination with local anesthetics. It also increases the absorption rate of parenteral fluids given by hypodermoclysis, and is an adjunct in subcutaneous urography for improving resorption of radiopaque agents. Hyaluronidase is also used for extravasation of hyperosmolar solutions. Hyalurodinase is used by plastic surgeons and dermatologists to reverse the effects of hyaluronic acid injections used as dermal fillers whenever the patient receiving the injections is unhappy with the results (Korman, 1963; Becker et al., 2004). The role of hyaluronidases in cancer has been historically controversial due to contradictory observations (Meyer, 1971), namely that levels of hyaluronidase (HYAL1/2) are increased in some cancers (bladder, prostate, breast and brain), whereas low expression of HYAL1 is correlated with a decrease in survival of pancreatic adenocarcinoma patients (Stern et al., 2007).

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MATERIALS AND METHOD

Bacterial Isolates and Culture Methods

From January 2019 to July 2020 from different hospital in Bagdad in Iraq, a total of 60 samples were collected by swab, which included 30 samples of burn skin and 30 samples of wound sector. Culturing on nutrient broth over night at 37◦C, then culturing on mannitol salt agar (MSA) media, incubated overnight at 37◦C (Biswas et al., 2015).

Microbiological diagnosis

Phenotypic Identification of *Staphylococcus aureus* as each isolated from MSA media was initially estimated by gram stain and culturing on blood agar (5%), incubation periods overnight at 37◦ C, then tested for catalase and coagulase test (Lancette Gand Tatini, 1992).

Molecular diagnosis

Identification by PCR amplification of 16S rRNA Gene. The bacterial isolates which were establish to be *Staphylococcus aureus* using specific phenotypic features that tested by PCR consuming specific primer for 16S rRNA in figure 1 (Whattcott et al., 2011). The primer amplify region with 228 bp of 16S rRNA gene segment of *Staphylococcus aureus* was very conserved at species level. PCR programming as in figure 2.

primer		Sequence	Length (bp)
16S rRNA gene Staphylococcus aureus	F	GTA GGT GGC AAG CGT TAT CC	228
	R	CGCACATCAGCGTCAG	

Figure 1. *Specific primer of 16S rRNA gene of Staphylococcus aureus*

Amplified genes	Initial denaturation	No. of cycles	Denaturation	Annealing	Elongation	Final extension
16S rRNA gene Staphylococcus aureus	94°C/2min	35	94°C/ 30sec	59 °C/ 30sec	72 °C/ 1min	72 °C/ 4 min

Figure 2. *PCR programming for 16S rRNA gene Staphylococcus aureus*

S. aureus isolates were cultured and maintained on Blood agar medium at 37ºC for 24h under aerobic conditions.

Hyaluronidase production and assay

- A volume of 0.5 m Becker et al., 2004; of HA stock solution (0.2 mg / mL) was placed in a series of clean tubes and incubated for 5 minutes at 37ºC.
- Bacterial culture was centrifuged at 8000 rpm for 20 min at 4°C, pellets were discarded and 0.5 mL of crude extract of the enzyme was added to HA. Tubes were incubated for 30 minutes at 37ºC and then cooled directly by an ice bath (to stop the interaction between the enzyme and HA).
- Albumin reagent (9mL) was added to each tube and left for 10 minutes at room temperature.
- The absorbance for each tube was read at 540 nanometers.
- Amount of the enzyme digested HA was calculated by the following equation:-mg HA digested = 0.2mg-mg HA remained,
- Amount of the remaining HA was calculated by measuring the absorbance at 540 nm according to the standard curve (Figure 2).
- Enzyme activity in TRU\mL was defined as the amount of enzyme required for the reduction of turbidity formed by HA in 30 minutes at 37ºC.

An equation calculating the effectiveness of the enzyme

$$\text{TRU\mL} = \frac{\text{HA digested} \times 30 \text{ min}}{\text{enzyme in the reaction (mL)}}$$ (Bouga et al., 2010).

Protein concentration

Protein concentration was determined according to the method described by Bradford, (Starr and Engleberg, 2006).

Physical mutagenesis

Mutagenesis by UV irradiation was achieved according to Khattab and Mohamed (Zukaite and Biziulevicius, 2000). *S. aureus* S9 was exposed to UV rays at distance of 10 cm from the UV lamp for 5,10,20,30,40,50,60,70,80 and 90 seconds. All these exposures were performed in dark room to avoid any photoreaction in the production of mutants. Aliquot of 0.1 mL of irradiated cell suspension was then spread on nutrient agar plates, and were incubated at 37ºC for 24 hours.

Chemical mutagenesis

Chemical mutagenesis by Mitomycin C was achieved according to Borwring and Morris (Koraki et al., 2007), by incubating 1 mL fresh culture of *S. aureus* S9 with 1 mL of 0.1 mg/mL Mitomycin C at 37ºC for 120 minutes, then aliquots of 0.1 mL of cell suspension was taken every 20 minutes of incubation, streaked on nutrient agar plates and incubated for 48h at 37ºC.

RESULTS

In the this study 60 suspected samples were collected from skin (burned and wound) of infected patients. From 60 sample only 25 samples (42%) were isolated from mannitol salt agar which tolerate high salt concentration and change the color of medium from yellow to pink according to fermented of mannitol in this selective medium and tested as gram positive cocci bacteria. Then these isolates were examined for *Staphylococcus aureus* by different biochemical test and molecular diagnosis . All isolates (25) further identified by tested on blood agar medium, which 50% of the isolates in current study were capable to yield clearing zone rounding the growth on blood agar media representing that they can produce hemolysin. The biochemical tests results which includes in this study showed 84% and 72% positive for catalase and coagulase respectively. Furthermore, samples which is positive for catalase and coagulase undergo molecular diagnosis by using PCR with specific primer for detection 16S rRNA gene. Figure 1 showed that 16S rRNA gene was looked as a band size with 228 bp .

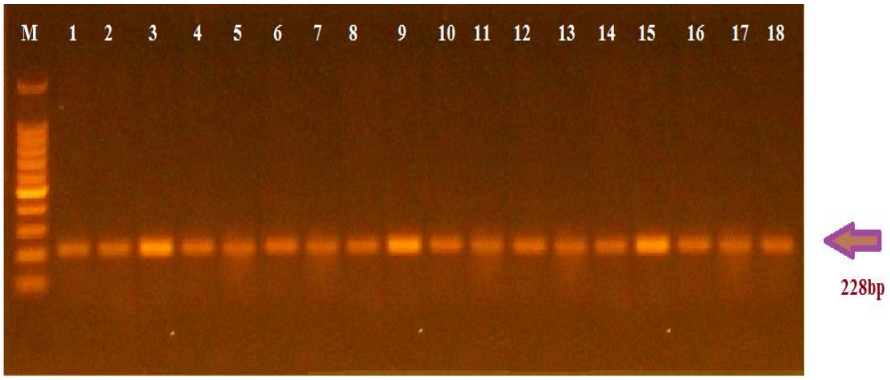

Figure 3. *Showed PCR amplification for 16srRNA gene for Staphylococcus aureus with band of 228bp.*

In order to enhance the ability of *S. aureus* S9 in Hyaluronidase production by random mutagenesis, this isolates were subjected to physical and chemical mutagens. Results of physical mutagenesis illustrated in figure 2 showed that the LD90 was reached after 70 seconds irradiation by UV-ray, and then cultivability of most *S. aureus* S9 was lost after 80 seconds of irradiation. Survivals of irradiated bacterial cells obtained after subjection to LD90 of UV-ray were selected and screened according to their ability to produce Hyaluronidase.

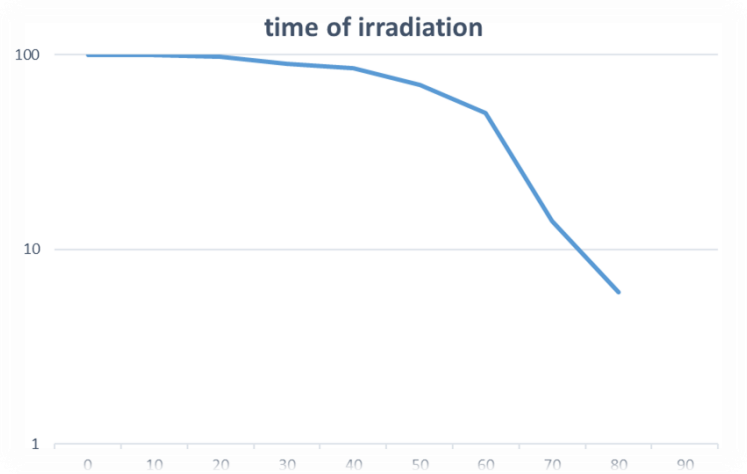

Figure 4. *Survivals of S. aureus S9 after UV irradiation for different periods of times (Seconds)*

Results indicated in table 1 showed that only five mutant out of one hundred were improved their ability in Hyaluronidase production. The most efficient mutant in Hyaluronidase production was S9-63 when its specific activity of Hyaluronidase in culture filtrate was 209.0 U/mg protein compared with 118.02 U/mg protein for the wild type. The strain improvement strategies especially mutagenesis and screening of hyper-producing mutants are very important in the production of secondary metabolites during the fermentation process (Bouga et al., 2010). UV radiation and chemical mutagenesis have been employed to obtain the Hyaluronidase hyper –producing mutant.in the present study, physical and chemical mutagenesis has been employed to mutant *S. aureus* S9 to obtain Hyaluronidase hyper-producing mutants. Physical mutagenesis was done using UV radiation. In a previous study, it was reported that to have powerful mutations and effective screening of mutants, lethality rate should be very high (Kreil, 1995). It is reported that death rate of 70-95% has been optimized for enhanced secondary metabolite production (Krishnapillai et al., 1999).

Table 1. *Specific activity of Hyaluronidase produced by mutants of S. aureus*

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
S9-1	116.7	S9-35	103.9	S9-68	114.8
S9-2	117.3	S9-36	102.0	S9-69	100.0
S9-3	109.0	S9-37	100.6	S9-70	109.0
S9-4	100.5	S9-38	116.9	S9-71	133.7
S9-5	107.2	S9-39	116.7	S9-72	118.0
S9-6	111.8	S9-40	118.4	S9-73	158.9
S9-7	116.5	S9-41	111.3	S9-74	113.7
S9-8	94.8	S9-42	97.8	S9-75	109.0
S9-9	109.1	S9-43	117.1	S9-76	111.1
S9-10	100.5	S9-44	89.5	S9-77	98.8
S9-11	101.9	S9-45	110.7	S9-78	111.4
S9-12	97.7	S9-46	118.0	S9-79	108.0
S9-13	80.9	S9-47	99.3	S9-80	108.8
S9-14	100.6	S9-48	98.5	S9-81	115.1
S9-15	99.7	S9-49	90.5	S9-82	97.7
S9-16	117.0	S9-50	118.0	S9-83	118.0

An equation calculating the effectiveness of the enzyme

Table 1. cont.

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
S9-17	112.2	S9-51	110.9	S9-84	101.7
S9-18	110.7	S9-52	116.8	S9-85	89.3
S9-19	99.7	S9-53	87.0	S9-86	111.6
S9-20	67.4	S9-54	112.0	S9-87	107.1
S9-21	100.5	S9-55	119.8	S9-88	116.0
S9-22	112.0	S9-56	113.3	S9-89	112.1
S9-23	98.8	S9-57	118.0	S9-90	117.3
S9-24	101.7	S9-58	99.7	S9-91	115.2
S9-25	107.5	S9-59	100.0	S9-92	98.0
S9-26	115.6	S9-60	114.4	S9-93	106.1
S9-27	90.8	S9-61	116.9	S9-94	114.1
S9-28	118.0	S9-62	117.9	S9-95	103.4
S9-29	117.9	S9-63	209	S9-96	117.6
S9-30	110.6	S9-64	115.5	S9-97	100.0
S9-31	120.1	S9-65	117.8	S9-98	116.9
S9-32	113.3	S9-66	114.2	S9-99	111.4
S9-33	98.9	S9-67	85.8		
S9-34	107.0	Wild Type	118.02		

Results of the present study indicated that the enhanced production of hyaluronidase might be due to some changes in the genetic code of *S. aureus* S9-63 as a result of UV radiation. Over-mutagenesis should therefore be avoided while screening for hyper-producing mutant strain. Optimum dose of mutagen is required in order to get positive mutation. According to the poisson model of mutagenesis, 37% of the survival rate would be unaffected for enhanced metabolic production (Kudo and Anthony, 2001). Chemical mutagenesis was also achieved by using Mitomycin C to induce random mutations in the double stranded DNA helix. MitC was used as mutagenic agent to generate mutants with higher hyaluronidase production. For this purpose, fresh of *S. aureus* S9 was incubated with MitC in a concentration of 0.1mg/mL at 37ºC for different periods of time. Results illustrated in figure 3 showed that survival of bacterial cells was decreased with increasing time of incubation of the mutagen till complete death after 120 minute of incubation with MitC, while the LD90 was reached after 80 min. with the mutagen. Aliquots of cell culture were taken after this time of incubation to evaluate the overproducer hyaluronidase production. It well known that the most efficient mutants were raised after reaching the LD90 (Laemmli, 1970).

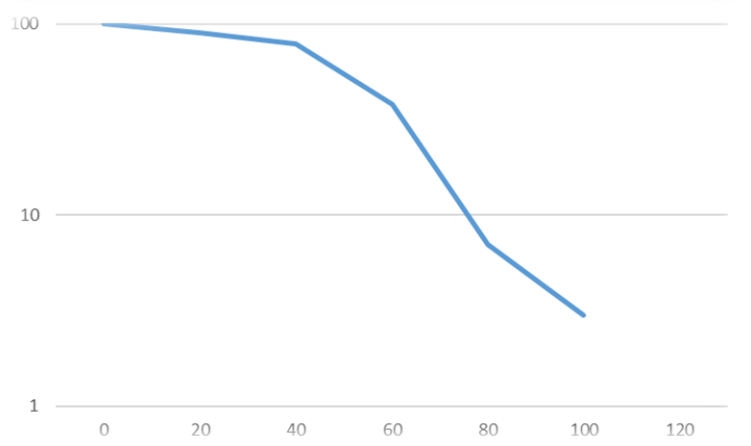

Figure 3. *Survival curve of S. aureus S9 after mutagenesis with 0.1mg/mL Mytomycin C for different periods(Minutes)*

Result indicated in table 2 showed that many over-producer mutants were obtained after 80 minutes of incubation with MitC. Among them was mutant designated S9- 121 spesific activity of hyaluronidase in its culture filtrate in comparison with the productivity of the wild type of *S. aureus* (118.02 U/mg protein). Chemical mutagenesis is time and concentration dependent. In the present study, *S. aureus* S9 was treated with 0.1mg/mL of Mitomycin C for time intervals ranging from 20 to 120 minutes. Hyaluronidase hyper-producing mutant was obtained after incubation for 80 min. of mutagen, in the present study, it is also observed that S9-121 higher over producer mutant (188.4U/mg) was obtained.

Table 2. *Specific activity of Hyaluronidase produced by mutants of S. aureus*

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
S9-100	100.7	S9-126	123.9	S9-152	164.8
S9-101	107.0	S9-127	152.0	S9-153	150.0
S9-102	109.0	S9-128	110.6	S9-154	179.0
S9-103	100.5	S9-129	186.9	S9-155	103.9
S9-104	107.2	S9-130	116.0	S9-156	138.0
S9-105	88.7	S9-131	128.4	S9-157	158.0
S9-106	19.5	S9-132	131.0	S9-158	103.7
S9-107	98.4	S9-133	127.8	S9-159	119.0
S9-108	139.1	S9-134	147.1	S9-160	181.1
S9-109	110.1	S9-135	111.5	S9-161	44.8
S9-110	109.8	S9-136	120.7	S9-162	181.4
S9-111	79.7	S9-137	88.0	S9-163	108.0
S9-112	89.9	S9-138	89.3	S9-164	108.9
S9-113	106.6	S9-139	118.5	S9-165	115.1
S9-114	97.7	S9-140	130.5	S9-166	97.7
S9-115	137.0	S9-141	118.0	S9-167	118.0
S9-116	111.2	S9-142	190.9	S9-168	101.7
S9-117	160.7	S9-143	176.8	S9-169	89.3

Table 2. cont.

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
S9-118	100.7	S9-144	87.9	S9-170	111.6
S9-119	69.4	S9-145	172.0	S9-171	107.1
S9-120	107.5	S9-146	159.8	S9-172	116.0
S9-121	188.4	S9-147	123.3	S9-173	112.1
S9-122	99.8	S9-148	108.0	S9-174	117.3
S9-123	121.7	S9-149	129.7	S9-175	115.2
S9-124	117.5	S9-150	140.0	S9-176	118.0
S9-125	165.6	S9-151	184.4	S9-177	116.1
		Wild Type	118.02		

DISCUSSION

Staphylococcus aureus measured a major pathogen that infect and colonizes hospitalized patients with declined immunity in the public. This bacterium is establish obviously on the skin of the human body and can cause infections that is minor and not life-threatening (Lass, 1998). In the this study, 18 isolates (30%) that were established to be *Staphylococcus aureus* from skin in which 10 from burn skin and 8 from wound sector, the skin and mucous membrane are admirable barriers in contradiction of local tissue invasion by *Staphylococcus aureus*. In current study, gram stain, catalase and coagulase considered a significant phenotypic noticing markers of *Staphylococcus aureus* (Murray et al. 2003) . As well as further detecting by PCR using 16S rRNA primers that specific for this species and that in line with Korman finding (Lee et al., 2002). *Staphylococcus aureus* could recognized by PCR methods using 16S rRNA gene which conceder the most valuable and widely investigated molecular marker taxonomy (Li et al., 2001). Furthermore result indicated in table 2 showed that many over-producer mutants were obtained after 80 minutes of incubation with MitC. Among them was mutant designated S9-121 spesific activity of hyaluronidase in its culture filtrate in comparison with the productivity of the wild type of *S. aureus* (118.02 U/mg protein). Chemical mutagenesis is time and concentration dependent. In the present study, *S. aureus* S9 was treated with 0.1mg/mL of Mitomycin C for time intervals ranging from 20 to 120 minutes. Hyaluronidase hyper-producing mutant was obtained after incubation for 80 min. of mutagen, in the present study, it is also observed that S9-121 higher over producer mutant (188.4U/mg) was obtained.

CONCLUSION

The existing effort was strategic to identify and characterize *Staphylococcus aureus* isolates composed from wound and burns of male and female patients hospitalized in the various departments of Bagdad hospital in Iraq. The objectives of the present study were to isolation and identification of *Staphylococcus aureus* by biochemical test and molecular marker (16rRNA), production of Hyaluronidase from mutant isolate of *Staphylococcus aureus*.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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