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Cloning and expression of recombinant hyaluronidase enzyme from *Staphylococcus aureu*s using *Escherichia coli*

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Hyaluronidase (HysA) is an important microbial enzyme that has medical importance. Hyaluronidase enzyme is produced by *Staphylococcus aureus* and is responsible for the spread of the organism during infection. A total of 85 clinical isolates of *S. aureus* were examined for hyaluronidase production. Strain of *S. aureus* S10 was associated with the highest productivity of hysA enzyme among the investigated isolates. The *hysA* gene was amplified from isolate S10 and *S. aureus* Newman by PCR method and cloned into the expression vector pRSET-B. The protein expression of the cloned *hysA* of *S. aureus* Newman (HysA-New protein) and *S. aureus* S10 (HysA-S10 protein) was performed in *Escherichia coli* BL21 and the optimum time for its expression was found to be 6 h after isopropyl-β-D-thiogalactoside (IPTG) induction. Successful expression was confirmed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE) and western blot analysis which showed a protein with a molecular mass of ~94 kDa. The expressed protein was concentrated by size exclusion column with 30 MWCO and showed activity of 435 IU/ml and 401 IU/ml for hysA-S10 and hysA-*S. aureus* Newman recombinant proteins, respectively. The activity of recombinant protein in the spent media of the transformed *E. coli* BL21 was further analyzed under the effect of different pH, temperature and some chemical compounds.

Keywords: Cloning, hyaluronidase gene expression, recombinant, western blot

Introduction

Hyaluronidase enzyme is capable of degrading hyaluronic acid (HA) which is an essential component of the extracellular matrix of human tissues[1]. Moreover, hyaluronic acid is a modulator to cellular immunity by elimination of inflammation and delaying allergic occurrences[2]. Hyaluronidases are produced by various organisms and have been found in both Gram-positive and Gram-negative bacteria. They have been implicated in a disease process as a way to invade and penetrate tissues[3]. Bacteria produce hyaluronidase to allow its spreading to deeper tissues so the infection becomes more virulent *via* hydrolysis of hyaluronic acid[4].

Staplylocuccus aureus is a main human pathogen that causes a variety of clinical infections[5] and is also considered the major pathogen in nosocomial infections[6]. It produces numerous exoenzymes including DNAse, hyaluronidase, protease, lipase and collagenase. These enzymes have the ability to break host tissue hence bacterial propagation is stimulated and the potential of invasive diseases is increased[7]. Hyaluronidase is one of the most important factors in the virulence of *S. aureus*, whereas hyaluronidase breaks hyaluronic acid during infection and also it may represent a diagnostically significant characteristic for differentiating *S. aureus* from other members of this genus[8]. Also a previous study showed that mutation in hyaluronidase gene in *S. aureus* resulted in reduced virulence in a mouse abscess model[9].

Hyaluronidase enzyme was used therapeutically to enhance the diffusion of injected fluids especially local anaesthetics in ophthalmology[10] also it was used with the chemotherapeutics. Additionally, hyaluronidase shows great role in treatment of joint diseases[11]. Moreover, in case of transplanted organ rejection hyaluronidases reduce oedema, and they can treat vitreous haemorrhage[12]. And also it has ability to enhance the penetration of cancer medications so chemotherapy-resistant tumors become more susceptible to treatment[13]. Therefore, production of hyaluronidase by recombinant protein expression would be of a great value due to its medical importance[14]. Recombinant hyaluronidase from *Streptococcus pyogenes* was successfully expressed in *E. coli*[13]. Also purification of this enzyme from

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recombinant *E. coli* has been reported from *Streptococcus pneumonia*[15]. More recently, Abbasian *et al*[16] produced the recombinant antigenic regions of hyaluronidase enzyme from *Streptococcus pyogenes* in *E. coli.* Therefore, the purpose of this study was to produce the recombinant hyaluronidase enzyme of *S. aureus* in *E. coli* as well as characterization of its activity at different physical conditions and in the presence of different compounds was investigated.

Materials and Methods

Microorganisms and Plasmids Used in this Study

Eighty five isolates of *S. aureus* were collected from Mansoura University Hospitals, Dakhlia, Egypt. The isolates were obtained from different clinical sources; 9 were isolated from sore throat, 20 were isolated from burn, 31 were isolated from wound infection, 11 were isolated from diabetic foot and 14 were isolated from nasal discharge. The isolates were purified and identified biochemically as *S. aureus* according to Laboratory Biochemical Standards[17]. *S. aureus* Newman was used as a reference strain (NCTC 8178). All bacterial strains were maintained at -80^{o}C in brain heart infusion (BHI) broth medium containing 20% glycerol until further analyzes were performed. *E. coli* Top 10 (Thermofisher Scientific) was used as the primary host for plasmid clones, while *E coli* BL-21 (DE3) (Invitrogen, USA) was used for protein expression. For recombinant protein production, the expression vector pRSET-B (Cat. No. V351-20, Invitrogen, USA) was used. Luria-Bertani (LB) medium, with or without 1.5% agar, was used for bacterial cultivation[18].

Screening for Hyaluronidase Production and Quantitative Estimation of its Level

Screening of hyaluronidase production among clinical isolates was performed by hyaluronidase plate assay method which was described by Hart *et al*[19] with minor modifications. Overnight cultures of the purified *S. aureus* strains in BHI broth medium were incubated at 37^{o}C, for overnight with shaking at 200 rpm. Afterwards, the cultures were adjusted to the same OD 630 nm (0.01 - 0.05) using fresh BHI broth. The culture (1 ml) was centrifuged at 8000 rpm for 5 min at 4^{o}C, and the supernatants containing the hyaluronidase enzyme (spent media) were filtered before storage at -80^{o}C until needed. Cups (7 mm diameter) were made in plates containing 1% agarose, 1% bovine serum albumin (BSA) and 0.1 mg/ml of HA (Sigma, H-10504) in 0.3M sodium phosphate buffer (pH 5.3) and 100 µl of spent media was applied into each well then the plates were incubated at 37^{o}C for 24 h before flooding with 2M acetic acid. A background of opaque precipitated BSA conjugated to the undigested HA and their diameters were measured in millimeters. Hyaluronidase production was indicated by a clear zone that was visualized around the well due to degradation of HA that is present in the applied medium by hyaluronidase. The diameter of the clear zones (mm) was indication of the hyaluronidase activity. A standard curve was constructed in a separate plate by blotting different concentrations of standard hyaluronidase (Sanofi, 1500 IU) against the diameter of the corresponding clear zones as previously mentioned by Abdelkader *et al*[20]. The hyaluronidase activity in the spent medium of each isolate was measured and expressed as IU/ml by plotting the obtained clear zone on the standard curve of hyaluornidase and calculating the corresponding enzymatic activity. *S. aureus* (isolate S10) showed the highest level of hyaluronidase activity and was selected for production of recombinant hyaluronidase enzyme.

Chromosomal DNA Isolation

Genomic DNA was extracted from cultures of *S. aureus* Newman and *S. aureus* S10 strains according to the method described by El-Moafy *et al*[21]. Cell pellets were collected by centrifugation 5 ml of overnight bacterial culture at 13000 rpm for 5 min, then they were re-suspended in 200 µl breaking buffer [NaCl 100 mM, Tris HCl (pH 7.5, 0.5 M), triton 2%, EDTA (pH 8, 0.5 M) and SDS 1%] in addition to 0.2 gm glass beads (0.25 - 0.5 mm) (Roth, Carlsruhe, Germany) and 200 µl of phenol: chloroform: isoamyl alcohol mix (25:24:1). The suspension was subjected to alternative intervals of vortexing (30 sec) and immersion in ice (30 sec) for a total of 10 min followed by mixing with 100 µl Tris-HCl (10 mM, pH 8). After that the suspension was centrifuged at 8000 rpm for 5 min to remove the glass beads and the supernatant was transferred to clean Eppendorf tube. Genomic DNA was precipitated by addition of 1 ml absolute ethyl alcohol (Sigma, USA) followed by centrifugation. The supernatant was withdrawn and the DNA pellet was dissolved in 100 µl Tris HCl (10 mM, pH 8) and then stored at -20^{o}C until further use. The concentration and purity of the DNA were measured via the Nano Drop spectrophotometer by measuring the absorbance at 260 nm. The purity of the DNA was determined by calculating the ratio of A260/280. Pure DNA has an A260/A280 ratio of 1.7 - 1.9[22].

Gene Amplification

PCR was used to amplify hyaluronidase (*hysA*) gene of both *S. aureus* S10 and Newman using the following pair of primers; the forward primer (5'TCGCAA*GGATCC*GATGACATATAGAATAAAG AAATGGC 3') contained *Bam*H1 site and the Reverse primer (5'GACATT*AAGCTT*TTATTTAGTTAATTCA AAGTGCACGC3') contained recognition site for *Hin*dIII with Phusion high fidelity DNA polymerase as instructed by the manufacturer (NEB; UK). The following conditions were used for amplification: primary denaturation at 98°C for 30 sec followed by 35 cycles of: denaturation at 98°C for 10 sec, annealing at 48°C for 30 sec and extension at 72°C for 3 min and lastly a cycle of final extension at 72°C for 10 min. The PCR product was analyzed by electrophoresis in 1.5% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified from agarose gel by QIA quick PCR purification kit (Cat No. 28104, Qiagen) as described as in the manufacturer's guidelines.

Cloning of Hyaluronidase Gene in Expression Vector

The purified PCR products and pRSET-B plamid were digested with *Bam*HI and *Hin*dIII restriction enzymes. Then the digested PCR products were ligated into digested pRSET-B using T4 DNA ligase enzyme (NEB; UK) at 16°C overnight incubation. Moreover, *E. coli* Top10 and *E. coli* BL21 competent cells were prepared by calcium chloride method and then they were used for transformation of the constructed plasmids. *E. coli* BL21 was transformed with ligation product by heat shock method[23]. Fifty µl of chemically competent cells was transformed with 5 µl ice-cooled plasmid or ligation products and left in ice for 30 min. Then this mixture was exposed to heat shock at 42°C for 1 min followed by immersion in ice for 2 min. LB medium was added to the transformed cells and incubated for 1 h with shaking at 37°C. Then centrifugation was done and the cell pellets were re-suspended in 50 µl LB medium and grown on LB agar plates contained ampicillin (100 µg/ml) which allowed only the positive transformed cells to grow. Plasmids were isolated from the transformed cells using a Qiagen plasmid kit (Qiagen Inc.) and were confirmed for successful cloning by restriction digestion analysis. To confirm successful cloning of the *hysA* insert of S10 isolate in the correct orientation, the plasmid pl-HysA-S10 was sequenced using T7 promoter primer.

Expression of Hyaluronidase from Transformed *E. coli* BL-21

A colony of *E. coli* BL-21 which was transformed with pRSET-B harboring either the *hysA* ORF of *S. aureus* S10 (pl-hysA - S10 plasmid) or *hysA* of *S. aureus* Newman (pl-hysA-New plasmid) was cultured in 5 ml LB medium supplemented with ampicillin (100 µg/ml) at 37°C with agitation for 24 h. One ml of the overnight cultures of *E. coli* BL-21 which was transformed with either pl-hysA-new orpl-hysA- S10 plasmids was used for inoculation of 50 ml LB broth containing ampicillin (100 µg/ml**)** and 1 mM IPTG followed by incubation at 37°C with 200 rpm shaking. Samples (1 ml) from each culture of *E. coli* BL-21 were collected in a period of 3-7 h intervals and were investigated for hyaluronidase production by the plate assay method with parallel measuring of OD 630 nm. Subculture of *E. coli* BL-21 which was transformed with empty vector was similarly prepared (negative control). For detection of *HysA* by SDS-PAGE, the 50 ml subcultures were harvested after 6 h of isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The spent media which contained the soluble recombinant protein were collected by centrifugation at 8000 rpm for 30 min at 4°C, then it was concentrated using size exclusion column with 30 MWCO (Microsep Pall) at a speed of 4000 rpm till the final volume of the spent media reached to 150 µl. The concentrated proteins were analyzed by SDS-PAGE (12%)

Western Blot Analysis

The expressed protein was analyzed by western blot analysis according to the method described by Mellick and Rodgers[25] using diluted (1:1000) 6-histidine epitope tag antibody (Sigma Aldrich) then visualization of the membrane by exposing it to tetramethylbenzidine (TMB; Sigma Aldrich) substrate solution at room temperature for few minutes to detect the target blotted HysA-S10 protein.

Effect of the Physical and Chemical Factors on the Relative Activity of the Expressed HysA from *E. Coli* BL-21

The effect of pH on the activity of the expressed HysA proteins (HysA-New and HysA-S10) in the spent media was measured in the range of pH (3-10). This was performed using citrate-sodium phosphate buffer (pH 3-7), phosphate buffer (pH 6-8) and NaOH-glycine buffer (pH 9-10)[26]. The spent media which were collected from sub-culturing of the transformed *E. coli* BL- 21 in LB medium contained ampicillin (100 µg/ml) and 1 mM IPTG

for 6 h at 37°C with shaking were diluted with 10X of the investigated buffers then the activity of the enzyme was determined by plate assay method. On other hand the spent medium which was diluted (1:10) by sterile distilled water was used as a control and its HysA activity was considered as 100% activity. The activity of HysA at different pH values was calculated as a percent relative to such control. The activity of the expressed HysA proteins (HysA-New and HysA-S10) was also measured at different temperature. The spent media were diluted by 10X phosphate buffer (pH 6) and incubated at 4°C (control), 37°C, 40°C, 45°C, 50°C, 55°C and 60°C for 1 h. The incubated spent media were analyzed for HysA activity by plate assay method. The HysA activity of the control that was incubated at 4°C was considered as 100% activity. The residual activity of HysA at different temperatures was calculated as a percent relative to such control.

The effect of Ca^{2+}, Mg^{2+}, Mn^{2+}, EDTA and urea on the activity of the expressed HysA proteins (HysA-New and HysA-S10) was investigated. Each of these chemicals was included in the spent medium at a final concentration of 10, 50 and 100 mM followed by incubation at room temperature for 30 min. Afterwards, spent media were analyzed for HysA activity by plate assay method. The HysA activity of the control was considered a 100% activity. The activity of HysA enzyme in present of metal ion or EDTA or urea was determined as a percent relative to this control.

Results

Hyaluronidase Activity Among the Clinical *S. aureus* Isolates by Plate Assay

Hyaluronidase production was detected in 82.3% (70 of 85) of examined *S. aureus* strains where as, they produced a zone of clearance (Fig. 1), while 15 isolates showed no hyaluronidase activity. Quantitative estimation of the hyaluronidase production was performed by hyaluronidase plate assay method. The hyaluronidase concentration was calculated using a standard curve, where the diameter of clearance zone was directly proportional to the logarithmic concentration of hysA enzyme. *S. aureus* isolate no. S10 (from burn) showed the highest level of hyaluronidase production (2511.89 IU/ml). *S. aureus* isolate (S10) was selected as a template for amplification of hyaluronidase gene and engineering of the recombinant vector. By investigation of the correlation between HysA production and the source of isolates, it was found that 7 out of 9 isolates from sore throat, 16 out of 20 isolates from burns, 26 out of 31 isolates from wounds, 9 out of 11 isolates from diabetic foot and 12 out of 14 isolates from nasal mucosa were positive HysA producers.

PCR and Cloning of Hyaluronidase Enzyme Gene

The amplified gene of hyaluronidase enzyme from the genomic DNA of *S. aureus* Newman strain and S10 strain had the expected size of 2424 bp (Fig. 2A). PCR products were purified and cloned into expression vector pRSET-B after digestion of each of them with *Bam*H1 and *Hin*dIII and then transformation occurred in *E. coli* Top 10. The positive clones were selected into LB/ampicillin agar. PCR reaction and digestion of recombinant plasmid from the selected clones by *Bam*H1 and *Hin*dIII confirmed successful cloning of recombinant gene into *E. coli* Top 10. Also to confirm the transformation of pRSET-B-HysA in *E. coli* BL21, enzymatic digestion with *Bam*HI and *Hin*dIII produced two bands at 2424 and 2900 bp representing the insert ORF of hyaluronidase, pRSET-B vector respectively (Fig. 2B). Moreover, sequencing showed that *hysA* insert of S10 isolate was inserted in the correct orientation in the plasmid pl-HysA-S10 (Fig. 3).

Expression of Recombinant Hyaluronidase Enzyme

The expression of hyaluronidase (pRSET-B-hysA-New and pRSET-B-S10) in *E. coli* BL21 was induced

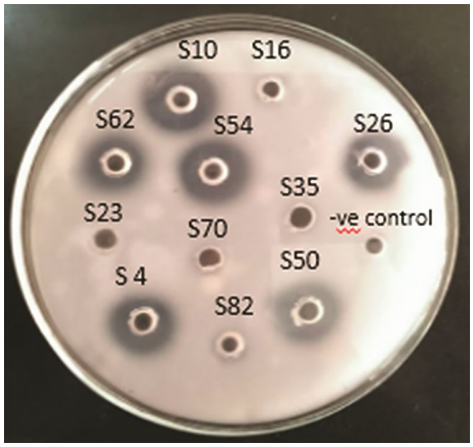

Fig. 1 — Screening of hyaluronidase activity of *S. aureus* representative isolates from various clinical sources using plate assay method; S4 (burn), S10 (burn), S16 (burn), S23 (wound), S26 (wound), S35 (wound), S50 (wound), S54 (nasal discharge), S62 (nasal discharge), S70 (diabetic foot), S82 (sore throat).

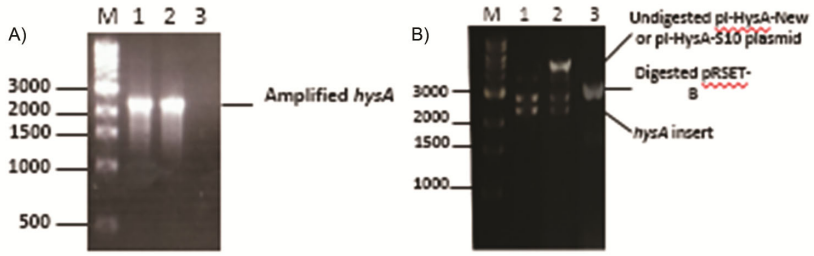

Fig. 2 — Amplification of hyaluronidase gene and confirmation of the recombinant clones. (A) Agarose gel electrophoresis of the amplified hyaluronidase gene. Lane M:1 kbp DNA ladder. Lane 1: Amplified *hysA* from the *S. aureus* Newman genomic DNA. Lane 2: Amplified *hysA* from the *S. aureus* S10 genomic DNA, and lane 3: Negative control. (B) Agarose gel electrophoresis of the digestion of the plasmids isolated from recombinant clone. M: 1 kbp ladder. Lanes 1, 2 and 3: *Bam*HI/*Hin*dIII-digested pl-HysA-New, pl-HysA-S10 and pRSET-B empty vector respectively

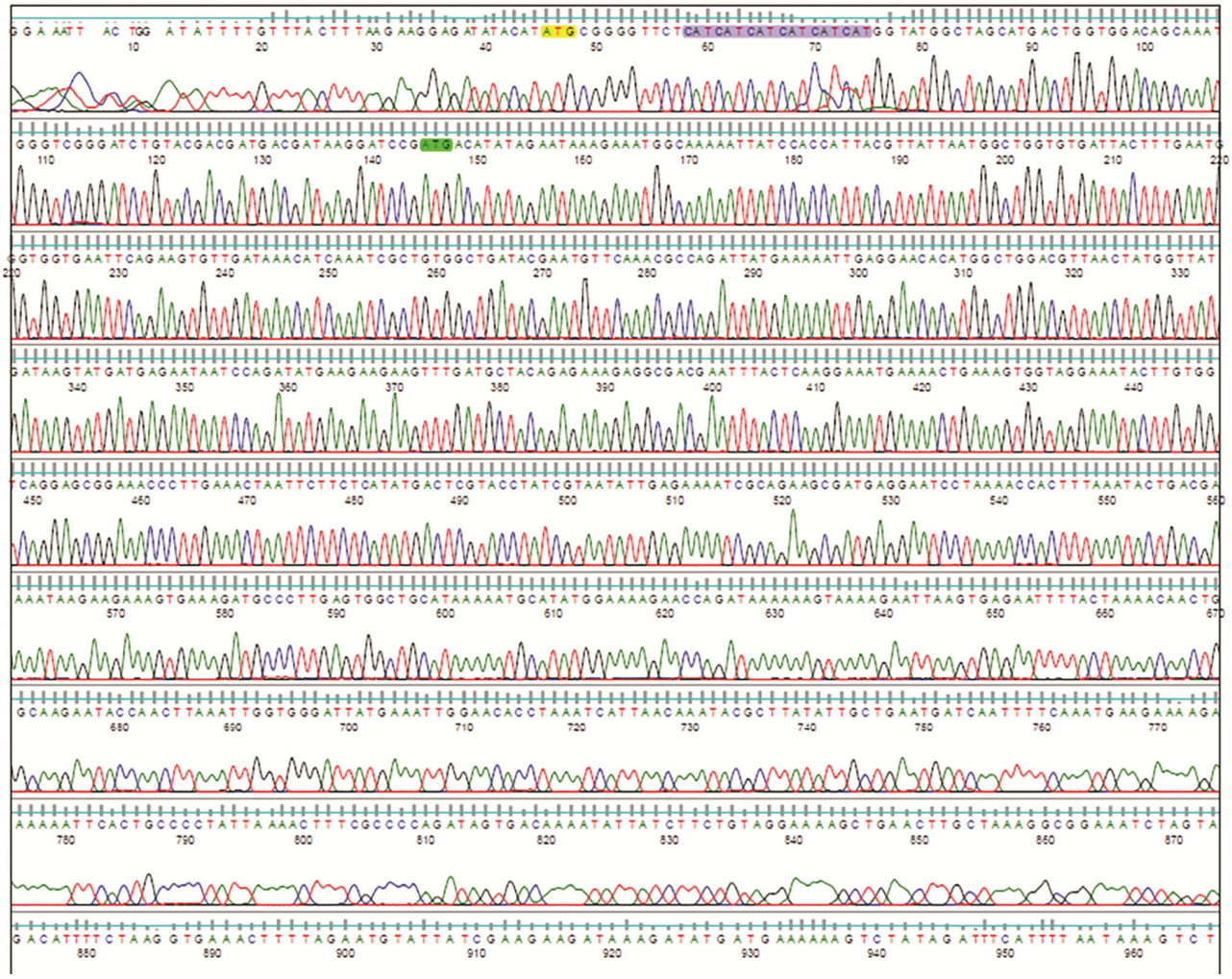

Fig. 3 — DNA sequence of pl-HysA-S10 obtained by T7 promoter primer: First codon of pRSET-B is indicated by yellow shading. The N-terminal His-tag is indicated by purple shading. First codon of *hysA* gene is indicated by green shading.

with 1 mM IPTG. Maximum protein expression was reported 6 h after incubation and the activity of recombinant protein was assayed and calculated as 435 IU/ml for hysA-S10 and 401 IU/ml for hysA-New per 1 ml of initial culture (Figs. 4 & 5; Table 1). SDS-PAGE analysis showed the molecular weight of 94 kDa for recombinant protein (Fig. 6A). Also to confirm the identity of the protein western blot analysis was performed using anti-histidine tag monoclonal antibody (Sigma, Aldrich), where the

recombinant hyaluronidase was detected as a clear band corresponding to its molecular mass (94 kDa) (Fig. 6B).

The Effect of Physical Factors on the Activity of the Expressed HysA Proteins

The optimum pH for the activity of HysA-New protein was found to be 6, where the relative activity increased by 20% in comparison with the 100% activity of the spent medium which diluted by sterile distilled water as demonstrated in Figure 7A. Similarly, the optimum pH for the activity of HysA-S10 protein was at 6 and 7, however the relative activity increased by 50% as shown in Figure 7B**.** The residual activity of the protein in the spent media decreased gradually by increasing the temperature and was totally lost at temperature ≥ 50°C as shown in Figure 8A & B.

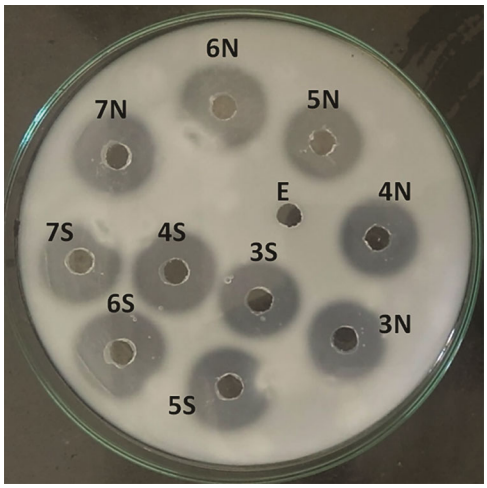

Fig. 4 — Assay of HysA-New and HysA-S10 proteins in the spent media of *E. coli* BL-21 after 3 - 7 h of IPTG induction. 3N, 4N, 5N, 6N and 7N: Spent media of HysA-New after 3 h, 4 h, 5 h, 6 h and 7 h respectively. 3S, 4S, 5S, 6S and 7S: Spent media of HysA-S10 after 3 h, 4 h, 5 h, 6 h and 7 h respectively. E: Spent media of *E. coli* BL-21 transformed with pRSET-B after 6 h of addition of IPTG.

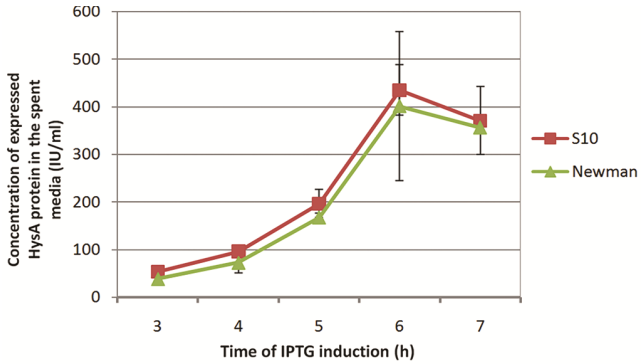

Fig. 5 — Time course screening of expression of HysA-New and HysA-S10 by *E. coli* BL-21 after IPTG induction. Both curves represent the average data from two independent experiments (Mean ± SD).

The Effect of Chemical Factors (Metal Ions, EDTA and Urea) on the Activity of the Expressed HysA Proteins

The effect of metals at different concentrations on the activity of the expressed HysA proteins was investigated. In case of HysA-New as demonstrated in Figure 9A, the relative activity of the protein was not affected at 10 and 50 mM $CaCl_2$, although it was decreased at higher concentration (100 mM) of this metal. On the other side, the relative activity of the protein increased by 122% and 50% in the presence of $MgSO_4$ (10 mM) and $MnSO_4$ (10 mM), respectively, however the relative activity of HysA-New protein at higher concentrations (50, 100 mM) was decreased in case of $MgSO_4$ or totally abolished in case of $MnSO_4$. Regarding HysA-S10 protein, none of the metals at the investigated concentrations increased the relative activity of the protein. The relative activity of HysA-S10 was maintained at 100% activity in case of $CaCl_2$ (10 mM), $MgSO_4$ (10 and 50 mM). Incorporation of $MnSO_4$ in the assay of the protein decreased its

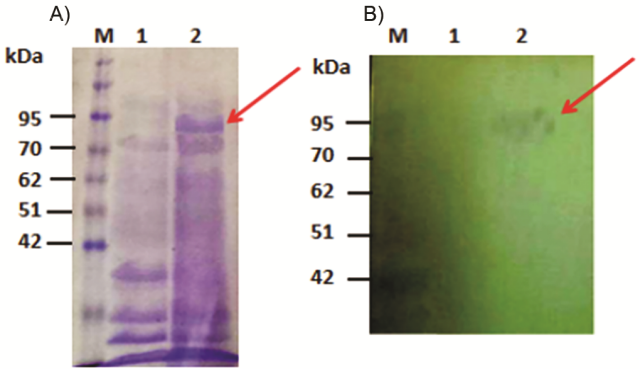

Fig. 6 — Analysis of recombinant hyaluronidase enzyme. A) Coomassie staining SDS-PAGE of the recombinant hyaluronidase protein. B) Western blot detection of recombinant hyaluronidase. Lane M: Chromatein prestained protien ladder. Lane 1: The concentrated spent medium of the *E. coli* BL-21 transformed by pRSET-B (negative control). Lane 2: The concentrated spent medium of the *E. coli* BL-21 transformed by pRSET-B-HysA-S10. The expressed HysA-S10 in the concentrated spent medium of *E. coli* BL-21 is indicated by red arrows.

Table 1 — Activities of crude and recombinant hyaluronidase

Strain	*S.aureus* (S10)	*S.aureus* Newman	*E. coli* BL21/ pl-hysA-S10	*E. coli* BL21/ pl-hysA-new
Hyaluronidase activity (IU/ml)	2511.89	510.5	435	401

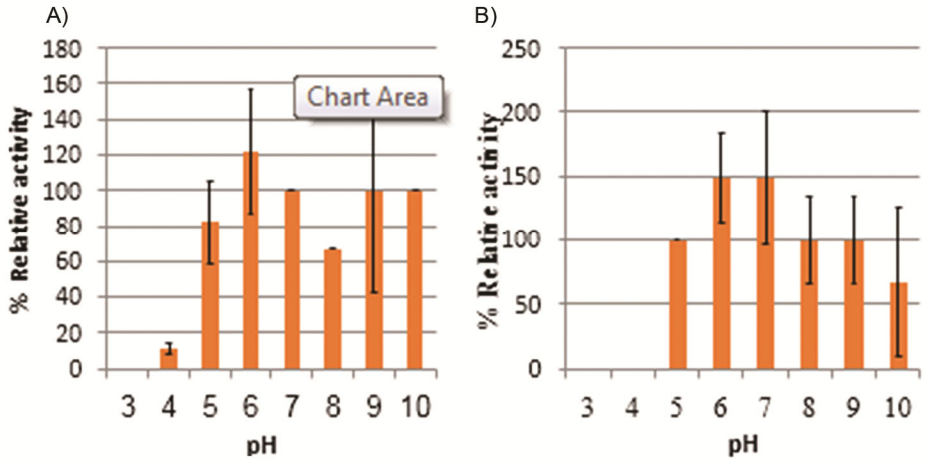

Fig. 7 — The effect of pH on the relative activity of the expressed HysA in *E. coli* BL-21. A) Effect of pH on the activity of HysA-New protein. B) Effect of pH on the activity of HysA-S10 protein. Both charts represent the average data from two independent experiments (Mean ± SD). The activity of HysA in the spent media diluted (1:10) by sterile distilled water was considered 100% activity.

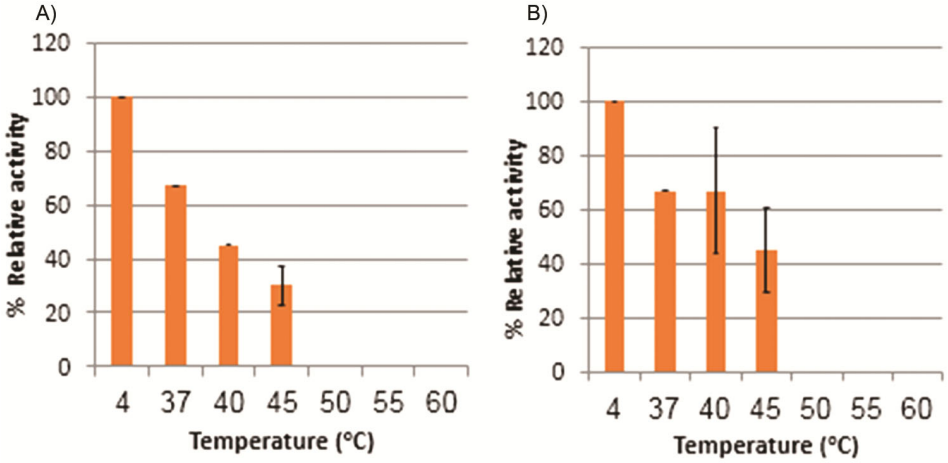

Fig. 8 — The effect of temperature on the residual activity of the expressed HysA in *E. coli* BL-21. A) Influence of temperature on HysA-New activity. B) Effect of temperature on HysA-S10 activity. Both charts represent the average data from two independent experiments (Mean ± SD). The activity of HysA in the spent media diluted (1:10) by 10X phosphate buffer pH 6 at 4 ^{o}C was considered as 100% activity.

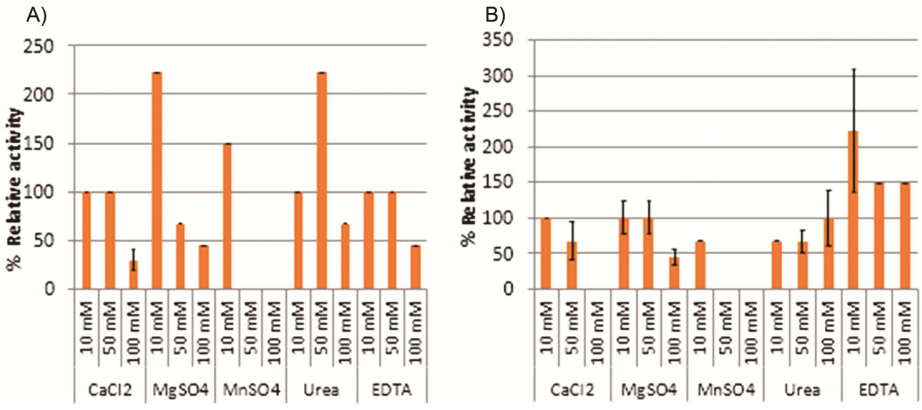

Fig. 9 — Effect of metal ions, EDTA and urea on the relative activity of expressed HysA in *E. coli* BL-21. A) The effect of Ca^{+2}, Mg^{+2}, Mn^{+2}, EDTA and urea on the activity of HysA-New. B) The effect of Ca^{+2}, Mg^{+2}, Mn^{+2}, EDTA and urea on the activity of HysA-S10. Both charts represent the average data from two independent experiments (Mean ± SD). The activity of HysA in the spent media diluted by both 10X phosphate buffer (pH 6) and sterile distilled water was considered 100% activity.

relative activity by 32% in case of 10 mM concentration or totally inhibited the activity at concentrations 50 and 100 mM as shown in Figure 9B. The relative activity of HysA-New protein (Fig. 9A) was increased by 122% in the presence of urea (50 mM) however decreased by 32% by increasing the concentration of urea to 100 mM. In case of HysA-S10 protein (Fig. 9B), we found that the relative activity of the protein was decreased by 32% in the presence of urea (10 and 50 mM) however, it maintained its 100% in higher concentration (100 mM). Concerning EDTA, we observed that the relative activity of HysA-New remained 100% in the presence of EDTA (10 and 50 mM) but decreased by 55% in case of EDTA (100 mM) (Fig. 9A). On the other hand, HysA-S10 relative activity was increased by 122% in the presence of EDTA (10 mM) and by 50% in case of EDTA (50 and 100 mM) (Fig. 9B)

Discussion

The production of recombinant enzymes from bacteria for medical, analytical and industrial applications was taken great interest. Hyaluronidase enzyme has ability to degrade hyaluronic acid which is an essential component of connective tissue. Therefore, hyaluronidase might contribute directly in the invasion of microorganism and plays an important role in the pathogenesis of the bacterium by allowing greater microbial access to host tissue for colonization, also it plays a role in the migration of the organism between tissues[27]. Additionally, hyaluronidase may be an essential biofilm detaching agent owing to its ability for cell dispersal[28-29]. Numerous Gram-positive bacteria produced this enzyme such as *Staphylococcus*, *Propionibacterium*, *Streptococcus* spp., *Clostridium*, *Peptostreptococcus* and *Streptomyces* (Hynes & Waltan, 2000). Hyaluronidase enzyme is considered as a valuable tool for therapeutic purposes. Thus it can be used in some diseases treatment and diagnostic kits production[30]. Also hyaluronidase is considered important anti-tumor strategy[31]. This enzyme is not usually produced by its natural and main sources due to the low amount of product and also the risk of contamination. Therefore, research focused on the production of recombinant hysA enzyme in heterologous organisms. The production of recombinant hyaluronidase enzyme has been reported from various organisms, including *Streptococcus pyogenes*, *Streptococcus pneumonia* and *Clostridium*[32]. Few studies have focused on the production of hyaluronidase from *S. aureus*. Such reports were interested in either screening for hyaluronidase production by *S. aureus* or examination of its importance in the virulence of this bacterium. Also *HysA* gene was identified and sequenced in few studies[33].

In the present study, 85 clinical isolates of *S. aureus* were examined for production of hyaluronidase enzyme, whereas strains which produced a clear zone in the agar plate due to degradation of the present hyaluronic acid were identified as hyaluronidase enzyme producer (Fig. 1). It was found that 82.3% of the isolates produced hyaluronidase enzyme, so it is distributed widely among *S. aureus* isolates and this was consistent with previous studies that showed high percentage of hyaluronidase production among their *S. aureus* isolates[34]. Also this study indicated that the clinical source did not affect on the level of hyaluronidase production in *S. aureus* as the percent of hyaluronidase producing isolates were 77.77%, 80%, 84%, 82% and 86% from sore throat, burns, wounds, diabetic foot and nasal discharge respectively. *S. aureus* isolate S10 from burn had the highest level of hyaluronidase production (2511.89 IU/ml).

Due to pharmaceutical applications of hyaluronidase enzyme, we were interested in heterologous expression of bacterial hyaluronidase in *E. coli* BL-21. So in the present study, the expression of recombinant hyaluronidase enzyme from S10 isolate and from standard *S. aureus* Newman using the expression vector pRSET-B was carried in *E. coli* BL21. *E. coli* is commonly used as a host for production of recombinant proteins in both industry and in research. In the following study BL-21strain of *E. coli* was used as host for expression, where as the absence of cytoplasmic proteases such as Deg P, Lon, HtpR and OmpT leads to increase in expression of hyaluronidase in *E. coli* BL21[35]. Mirjamali *et al* (2014) used pET system in which the hyaluronidase gene in under control of T7 promotor. But in our study expression of the gene of interest *(hysA*) which was present in pRSET-B was controlled by the strong T7 promotor and in *E. coli* BL21 transcription of the gene is performed by T7 RNA polymerase which is included in the genome of *E. coli* BL-21 under the control of this promoter and its expression is induced by IPTG (Sambrook *et al*, 2001). Once sufficient T7 RNA polymerase is produced, it binds to the T7 promotor and transcripts the target gene in the pRSET-B vector. So, in this system cellular factors

which are involved in synthesis of protein did not affect on mRNA and protein production because the gene transcription system in this host cell did not depend on them. Therefore, in this system production is more than the systems in which protein synthesis depends on the host cell polymerases. However, *HysA* was previously expressed using different modified vector (Pnw21) and transformed into *Staphylococcus carnosus*[37]. Interestingly, *hysA* gene from both strains of S10 and *S. aureus* Newman was successfully expressed in *E. coli* BL21 with recombinant hysA enzyme maximum activity in the spent media was detected after 6 h of IPTG addition, whereas (Makris *et al*, 2004) reported that hyaluronidase specific activity was observed to increase rapidly during exponential growth and gradually decrease with time until approximately 24 h when activity was undetectable. The activity of recombinant hysA enzyme from S10 reached 435 IU/ml and from *S. aureus* Newman reached 401 IU/ml which were near to the activity of crude enzyme which was produced by the original *S. aureus* Newman (510.5 IU/ml). The molecular mass of recombinant hyaluronidase was estimated to be 94 kDa by SDS PAGE which agreed with the size of the purified protein of the *S. aureus* 8325-4 hyaluronate lyase in (Farrell *et al*, 1995).

The optimum pH for the activity of HysA was found to be 6. To make sure that the result of the optimum pH for activity of HysA-New and HysA-S10 proteins is related only to the pH and not to the composition of the buffer, the activity of the expressed proteins at pH 6 and 7 was examined into two different buffers (citrate-sodium phosphate and phosphate buffers). Therefore, the investigation of the effect of other factors on the activity of the expressed proteins was performed at pH 6 using phosphate buffer. This was consistent with a previous study that showed that the optimum pH for the activity of HysA by *Bacillus* sp A50 was 6.5. Also it was found in this study there was a gradual loss in the activity by increasing temperature. However, we found that HysA-S10 was more stable than HysA-New, this was indicated by the percent residual activity at 40^{o}C for HysA-New (45%) and HysA-S10 (67%). The activity of the expressed enzyme was totally lost after heating at 50^{o}C for 1 h. However, a previous study showed that relative activity of hyaluronidase obtained from *Bacillus* sp A50 was not affected by heating at 45^{o}C for 1 hr and 90% of the relative activity was totally lost by heating at 50^{o}C for 1 hr.

Additionally, in this study the effect of metal ions (Ca^{+2}, Mg^{+2} and Mn^{+2}), chelator (EDTA) and organic compound (urea) on the activity of both proteins was investigated at three different concentrations (10, 50 and 100 mM). The relative activity of HysA-S10 was found to be not affected at a concentration 10 mM (in case of $CaCl_2$ and $MgSO_4$) or decreased in case of $MnSO_4$. But the relative activity of HysA-New was increased at 10 mM concentration of $MgSO_4$ (122% increase) and $MnSO_4$ (50% increase). However, at higher concentration (50 and 100 mM) of these metals the relative activity was either greatly decreased or totally lost. But the relative activity of hyaluronidase from *Bacillus* sp A50 was increased up to 50% by increasing the concentration of $CaCl_2$ up to 100 mM and it was increased also by 20% in the presence of 100 mM $MgSO_4$ (Guo *et al*, 2014).

Concerning of the urea effect on the activity of the expressed HysA proteins, 50 mM concentration of urea increased the activity of HysA-New by 122%, while no increase in the relative activity of HysA-S10 was observed in the presence of urea. In addition higher concentration of EDTA (100 mM) decreased the relative activity of HysA-New by 55%, while lower concentration of EDTA did not affect the relative activity of the protein. Interestingly, all the investigated concentrations of EDTA increased the relative activity of HysA-S10 protein, however the relative activity of hyaluronidase obtained from *Bacillus* sp A50 was decreased by incorporation of EDTA at concentrations of 10-100 mM (Guo *et al*, 2014). In conclusion, our present data indicates the successful cloning of hyaluronidase gene from *S. aureus* into *E. coli* and production of its recombinant enzyme in soluble and active form.

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