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CHARACTERIZATION AND MPLECLAR DETECTION OF PURIFIED PROTEUS MIRABILIS PMBS41 ALPHA-HEMOLYSIN

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Key words: Molecular detection, *Proteus mirabilis,* Alpha-hemolysin..

ABSTRACT

The Alpha- hemolysin, produced by *Proteus mirabilis* PMBS41 grown in a chemical defined medium was purified 9.77 fold with a yield (14.90 %) . Alpha-hemolysin was estimate the molecular weight which was shown to be 88,750KD by using gel filtration chromatography using Sepharose -6B and 109.64 KD by using SDS-electrophoresis and exhibited an optimum temperature of 35 and 40°C, and pH optimas at 8.0. Whereas hemolysin reserve a full of its activity at a pH 8 and a temperature at 25° - 30°C.Molecular dectection was done by using specific primer to each *HpmA* and *HpmB* genes that encode for Hemolysin as a virulence factor of *proteus mirabilis* by using MPCR and electrophoresis technique.

The PCR assay results identified (53) isolate possessed *hpmA* and *hpmB* genes of the *proteus mirabilis* bacteria diagnosed , This explains the blood analysis of all pathogenic bacterial isolates but with different ratio and the importance of PCR in detection of virulence of *proteus mirabilis* in clinical urine samples of urinary tract infection (UTI) .

INTODUCTION

Hemolysin is one of the major virulence factors in pathogenic bacteria, and a variety of haemolysins were identified in a wide range of bacterial species. It is a toxin that inserted to the membranes of target eukaryotic cell forming a pore causing the efflux of ions and subsequent cell damage [1].

P.mirabilis hemolysin is a calcium independent hemolysin system consisting of two proteins HpmA and HpmB. hemolysin (HpmA) is the protein identified as a cytotoxin in *P. mirabilis*. This pore-forming secreted cytotoxin is produced by *P. mirabilis* during the mid-exponential or late exponential phase of growth [2]. Hemolysin, which is induced during swarmer cell differentiation of *P. mirabilis* and during infection [3], lyses nucleated cells and erythrocytes [4]. *P. mirabilis* produces hemolysin HpmA, that used to damage the kidney tissues. This hemolysin is associated to the cell, calcium-independent, former of pores, Encodes by two genes, *hpmA* and *hpmB*, that codify the HpmA (166 kDa) and HpmB (63 kDa) proteins, respectively [5]. HpmA hemolysin is responsible for tissue damage and is activated when its N-terminal peptide is cleaved, resulting in active HpmA (140 kDa), and HpmB is responsible for HpmA activation and transport [6,5].The HpmA haemolysin toxin of *Proteus mirabilis* production is upregulated co-ordinately with the synthesis and assembly of flagella during differentiation into hyperflagellated swarm cells [3]. The levels of hemolysin in *P. mirabilis* correlate with its ability to invade cultured kidney cells, and an isogenic *P. mirabilis hpmA* mutant is minimally invasive in cultured cells [7].

The aim of the study is to study the biochemical characterization of purify of hemolysin and detection of the genes encodes of Hemolysin including: DNA extraction from *P.mirabilis* isolates and detection of hemolysin genes by using PCR and electrophoresis.

MATERIALS AND METHODS

Alpha-hemolysin was extracted from the selected local producer isolate of *P. mirabils* PMBS41 by centrifugation, applied on an 24 hrs cultured at 37°C of brain heart infusion broth, at 10000 xg for 20 min at 4°C for the removal of cells from the bacterial culture medium. The cell-free supernatant fluid was decanted and used as crude extract .

The hemolytic activity assay was determined using Senior and Hughes [8] methods with some modification as follow. 0.1 ml of an overnight nutrient-broth culture of each strain was added to 10 ml of BHI broth and incubated with shaking at 37°C, small amount of samples (150µl) were removed into micro-centrifuge tubes containing 1 ml of a washed suspension of red blood cells 2% v/v in saline with and without 150 mM $CaCl_2$. The tubes were incubated with gentle agitation in a water bath at 37°C for 1hr., after that the tubes were then centrifuged at 13000 rpm for 1 min. to

pellet the red blood cells and bacteria. The amount of free haemoglobin in the supernate (measured by its absorbance at 540 nm) indicated the degree of hemolytic activity. Protein concentration was carried out using the Bradford [9].

Determination of Molecular Weight of Hemolysin by Gel- Filtration Chromatography:

Determination of the Void Volume of the Column:-

The column void volume (Vo) was determined, by the estimation of the total volume of fractions as characterized with start point movement of the blue dextran to that of climax of absorbency of the blue dextran.

Determination of Hemolysin Elution Volume (Ve)

Sepharose-6B column (80× 1.5 cm) was prepared, packed and Equilibrated for a second time. 5 ml of purified Hemolysin sample was passed through the column carefully, and equilibrated with 0.02 M Tris-base buffer pH 8.0, with a flow rate of 50 ml/hour. Fractions of 5 ml were collected. The elution volume(Ve) was estimated for the separated fractions of purified hemolysin, by following the absorbance at 280 nm.

Measurement of Standard Protein Elution Volumn (Ve)

Different standard proteins (Casein , 31KD; Pepsin 34KD; Alkaline phosphatase 80KD ; DNA Agyrase 199KD and Urease 409KD) were applied through sepharose-4B column, and then eluted with 0.02 M Tris-HCl buffer pH 8.0, with a flow rate of 50 ml/hour,

The elution volume was estimated for each standard protein by following the absorbance for the separated fractions at wave length 280 nm. The (Ve/Vo) ratio was calculated for each standard protein and for the separated fractions of purified Hemolysin . Then standardization was done by plotting the elution volume (Ve) of each standard proteins to the void volume (Vo) of the blue dextran 2000 (Ve/Vo) versus the log value of molecular weight [10]. The hemolysin molecular weight was accordingly calculated.

SDS - Polyacrylamide gel electrophoresis.

Another enzyme purification procedure were applied by utilizing the technique of SDS-polyacrylamide gel electrophoresis (SDS-PGE) according to [11] .

Extraction of the *Proteus mirabilis* DNA

The extraction of the DNA was carried out. *P. mirabilis* Isolates were grown in Brain Heart broth for 24 hrs at 37°C.From these strain cultures, DNA was extracted from bacterial cells using Genomic DNA Mini kit supplemented by the manufacturing company (Geneaid, Korea). The classical method of using Genomic DNA mini kit, (Geneaid, Korea) has a protocol for gram negitive bacteria

RESULTS AND DISCUSSION

The molecular weight was estimated by gel filtration depending on the size of the separated molecules with their charge. It was possible that the different methods of estimation may be used (12).Sepharose 6B for gel filtration was used for estimation the molecular weight of α–hemolysin that purified from *P.mirabilis* PMSB41 in the presence of five standard protein, urease (409KDaltons), DNA gyrase (199 KDaltons), Alkaine phosphatase (80 KDaltons), Pepsin (34KDaltons), Casein (31KDaltons). Blue dextran 2000 was applied first to column to detection the void volume (Vo).α -hemolysin and each of standard proteins were applied to column and eluted separately. The elution volume (Ve)of each of standard protein was recorded, then Ve/Vo was estimated for each one. The molecular weight of α -hemolysin was determined by plotting the relationship between Ve/Vo and Log of MW of standard proteins as shown in figure (1). By the aid of the standard curve, the molecular weight of the –hemolysin is estimated and it is nearly (102 KDaltons).

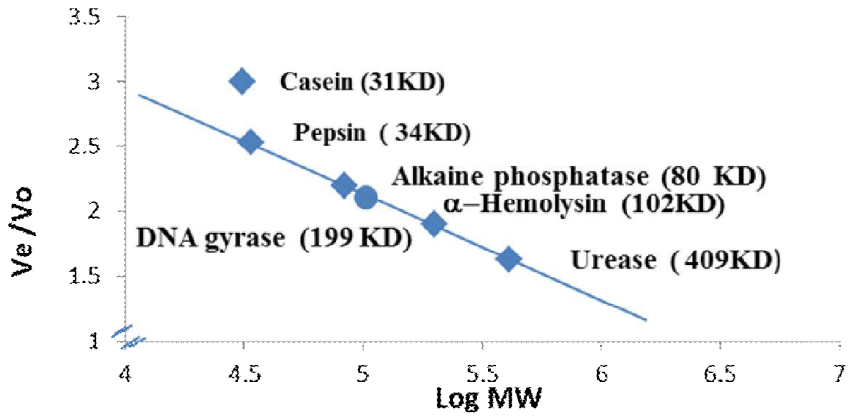


Figure (1) : Determination of the molecular weight of *Proteus mibrils* PMBS41 α -hemolysin using standard proteins of different molecular

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was used for detection the degree of α- hemolysin purity, also used as another technique for determination of molecular weight of purified a-hemolysin. In addition to the five standard proteins (Aldolase ,154000 Dalton; Alkaline phosphatase ,80000 Dalton; BSA , 67000 Dalton; Ovalbumin, 43000 Dalton; Trypsin, 23000 Dalton) that were migrated in the same gel.

Figure (2) showed that the analysis of protein profile of purified α– hemolysin for *Proteus mibrils* PMBS41 reveal appearance of one protein band showed high purity of hemolysin with molecular weight (109640 Dalton).

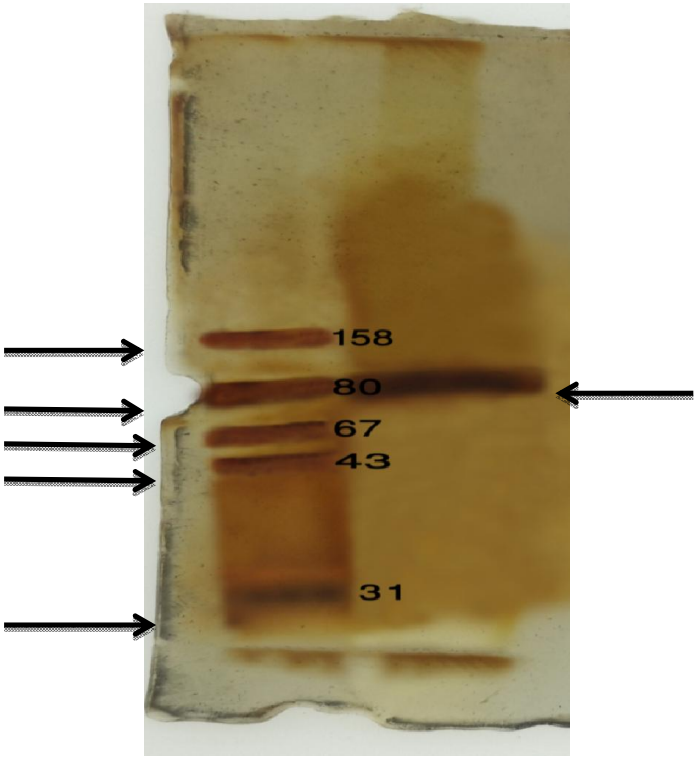


Figure (2): Sodium dodecyl sulfate-polyacrylamide gel analysis of pure α-hemolysine from *Proteus mirabilis* PMBS41.

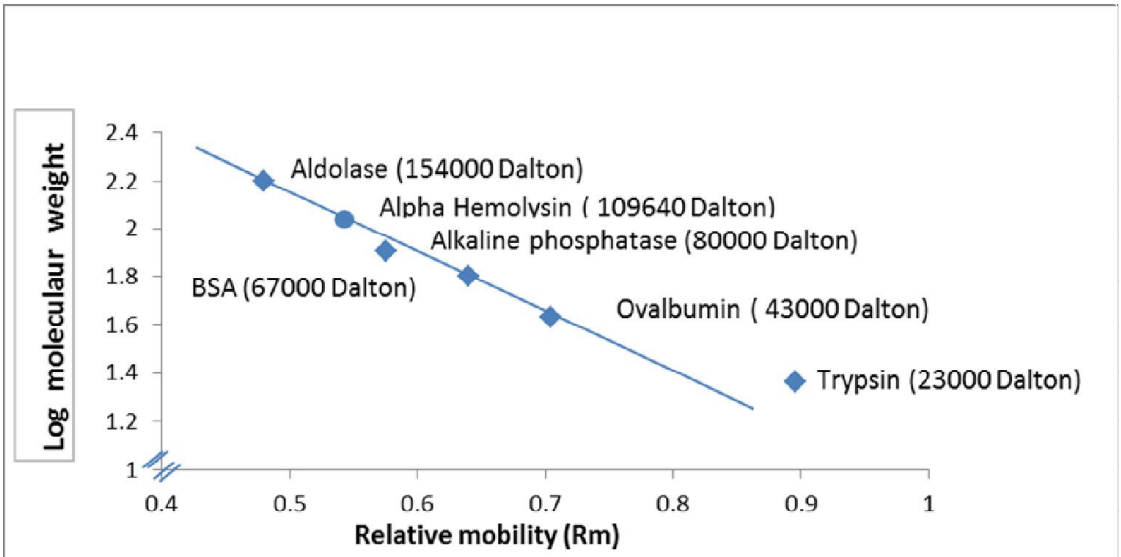
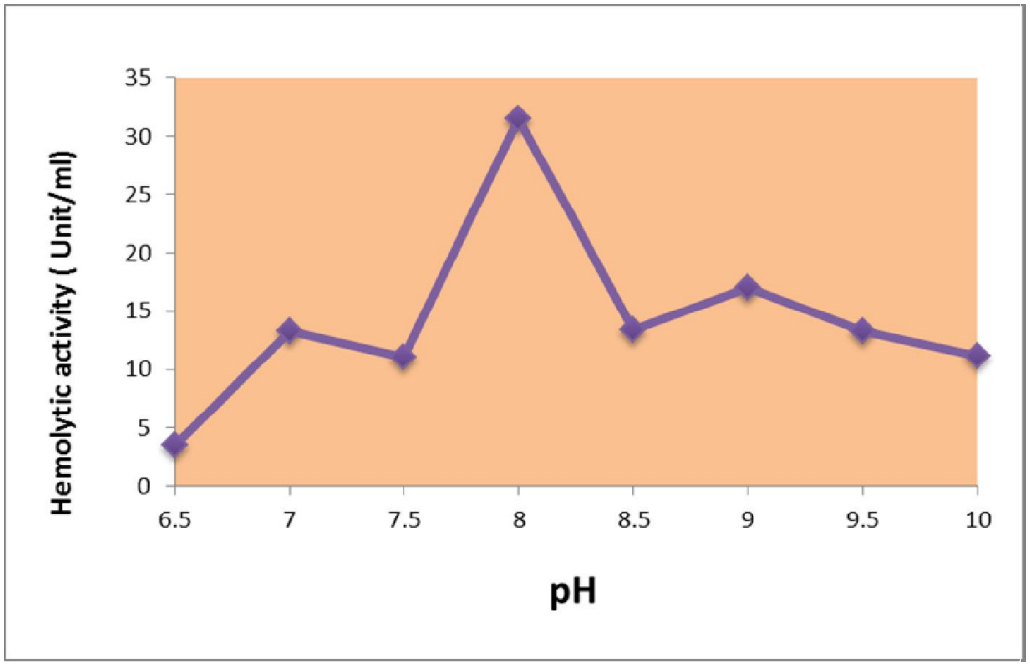


Figure (3) : Standard curve to determine molecular weight of Alpha hemolysin produce by *Proteus mirabilis* PMBS41. Using SDS-

The optimum pH for Hemolysin activity

Different pH values ranging from (6.0-9.5) were used to determine the optimum pH for hemolysin activity because the activity of the hemolysin was affected by differences in the pH buffer. The optimum pH of hemolysin activity purified from *P.mirabilis* was 8.0 (figure 4) .where as the optimal activity of *E.coli* hemolysin was 7.9 that reported by [13]. while the results demonstrated by [14] that the best hemolysin production from *Staphylococcus aureus* was in the pH near neutrality (pH 7.0-7.5).In another study conducted by [15], working with entero invasive *E. coli*, they showed that hemolysin expression at optimum pH appeared to be a strain specific feature and was found maximum inthe pH range 7.5-8.0 coinciding with the results of [16] who reported an optimum pH of 7.5 for hemolysin production by *Aeromonas hydrophila* in TSB ,While another strainspecific study indicated that *B. pertussis* showed maximum activity at 7.5, whereas *B. parapertussis* and *B. bronchiseptica* showed activity at 7.5-8.0. Both studies revealed results that are nearly similar to the result of this study and the slight indifference might be attributed to the involvement of different determinant in host interaction and virulence of various bacterial species and strains within these species [17].



Figure(4): Effect of different pH on activity of purified hemolysin of production from *Proteus mirabilis* PMBS41.

The optimum pH for Hemolysin stability

Essential to assay of an eproteins or preparation at regulare intervals under standared conditions is to determine that it remains stable; or if an protein was unstable, It steadily loses activity with time [18]. In this study used deferent buffer pH (6.0-9.5) to detect the optimal pH for the hemolysin stability. The results show that the optimum pH for the hemolysin purified form *P.mirabilis* stability was 8.0 as well as the hemolysin keeps 100% of its activity. This means that the protein stays at its optimum state in this pH compared with other. Figure (5) shown the pH stability decrease gradually from pH (8.5 and 9.0) because of the effect of the alkaline on the nature of protein, while at pH 6.0 stability was very few, This result refers to a decrease in the protein stability at a hydrogen number which is far of pH 8.0. This decline is due to the pH effect on the enzymatic protein structure or irreversible denaturation may occur in a high acidic or basic solution which leads to change in the active site of the enzyme making it lose. its activity [19].

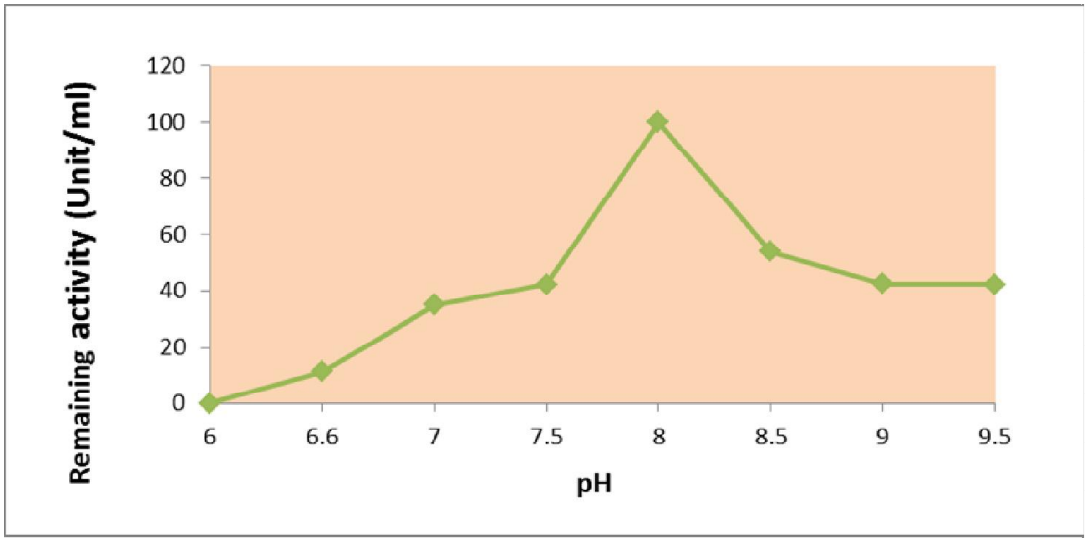


Figure (5): Effect of pH on purified hemolysin stability from *Proteus mirabilis* PMBS41 . The purified enzyme incubated at different pH values 6-10 for 1 hours at 37^{o} C.

The optimum temperature for Hemolysin activity

Different incubation temperature was used to study the effect of temperature on Hemolysin activity because each proteins and enzyme works within a range of temperature specific to the kind organism. The effect of temperature on the purified hemolysin activity was studied; the results show that the activity of purified hemolysin reached its maximum value at (25-30)^{0}C see figure (6), the hemolysin activity then decreased with the continuous increase in temperature and the activity was lost. These results were nearly agree with those of Li *et al*.,(2011) who mentioned that the optimal temperature for enzyme and proteins was most active at 35 ^{o}C an increase in the enzyme activity was observed when the temperature was increased until 35^{0}C and then begin to decrease see Figure (6). An increase in temperature generally leads to an increase in reaction rates, a given increase because higher temperatures lead to a sharp decrease in reaction rates [18].

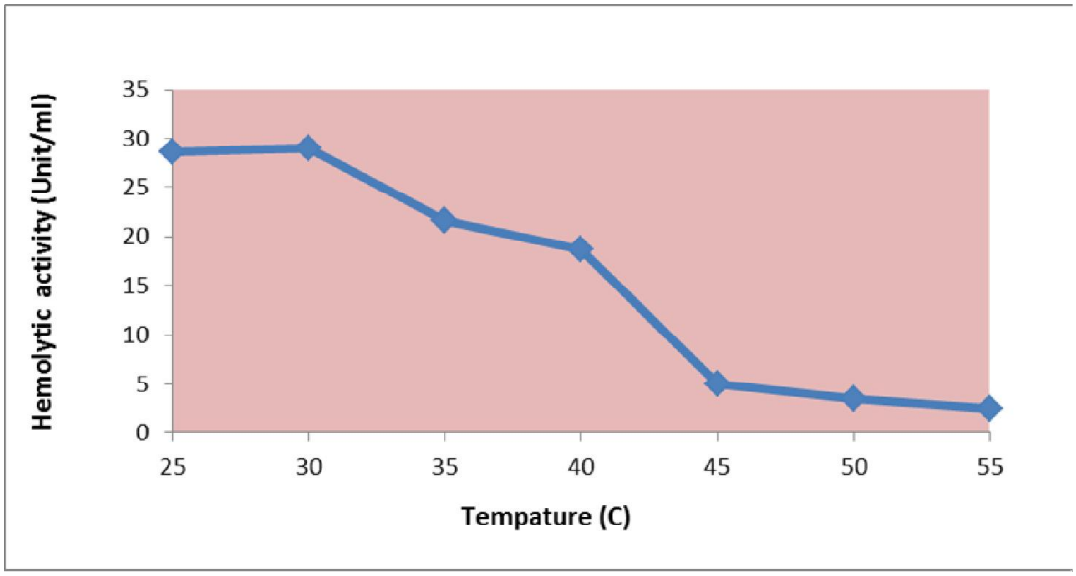


Figure (6): Effect of different temperatures on activity of purified hemolysin of production from *Proteus mirabilis* PM BS41.

The Optimum Temperature for Hemolysin Stability

The purified hemolysin was affect by an increase in the temperature because of their proteinaceous nature. The hemolysin production by *P.mirabilis* PMBS41 was stable at temperature range of (35-40) °C and then the activity began to decrease with the increase of temperature Figure (7). This was due to the denaturing of protein structures resulting from the breakdown of the weak ionic and hydrogen bonding that stabilized three dimensional structure of the enzyme [18] . Most proteins were more stable at low temperature, and in order to maintain the activity of the proteins is preferably saved it in low temperatures [21].The results showed that the hemolysin stability began to decrease gradually with the increase in the temperature that caused distortion in the active site of protein due to loss of activity to the breakdown of substrates. There may be several explanations including the facts that (i) a heat-sensitive step occurs early in the reaction sequence and takes place before heat denaturation occurs, or (ii) the red cell - hemolysin complex is more stable than is the hemolysin alone.The studies indicate that the hemolysin-erythrocyte interaction is complex.

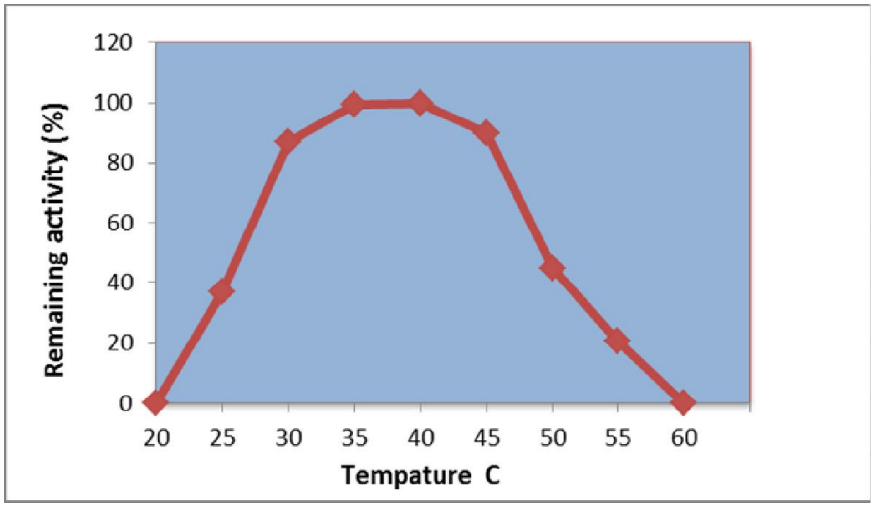


Figure (7): Effect of temperatues on purified hemolysin stability from *Proteus mirabilis* PMBS41 . The purified enzyme incubated at values 6-10 for 1 hours at 37° C.

Bacterial DNA Extraction

DNA was extracted from colonies identified as *P.mirabilis* by using genome DNA purification kit (Geneaid/ korea) by concentration 50-80 ng/µl. The results were detected by electrophoresis on 1% agarose and exposed to U.V light in which the DNA appears as compact bands, see figure (8).

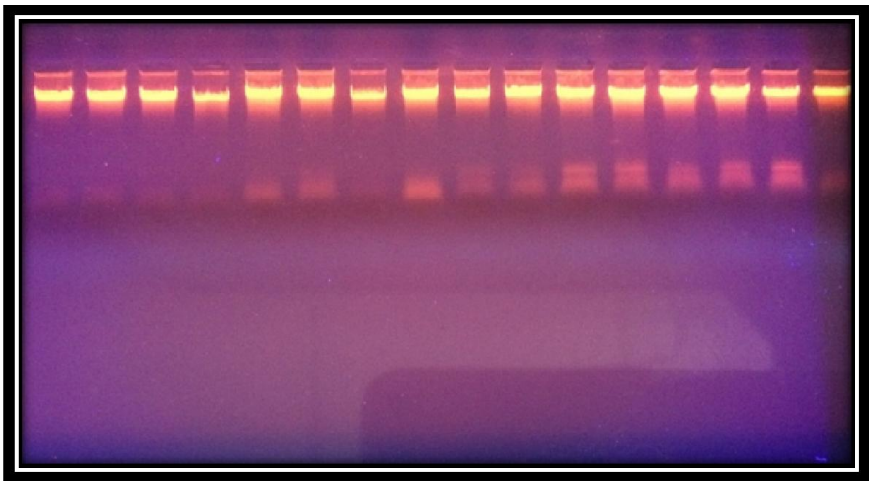


Figure (8): Genomic DNA extraction electrophoresis on 1% agarose at 70 volt/cm for 30min.

Detection of Hemolysin Genes Using Multiplex PCR (MPCR)

P.mirabilis hemolysin differ from other *Proteus* spp. that codified by two genes, *hpmA* and *hpmB,* that encodes for the HpmA and HpmB proteins respectively [5, 6].

Amplification and melting conditions were optimized for the MPCR assay, using specific primers sequences for *HpmA* and *HpmB* genes. in this study all P.mirabilis isolates presented the hpmA and hpmB genes by MPCR see figure (9) ,these result agree with the result reported by [22].The presence of the *hpmB* gene in the isolates that had the *hpmA* gene was in line with the description [6)]and [23] who showed the need to cleave the N-terminal peptide of the HpmA by HpmB to activate and transport the hemolytic HpmA protein to outside the cell, This suggests that HpmA is a factor in the pathogenesis of *P. mirabilis* samples isolated from human urine.

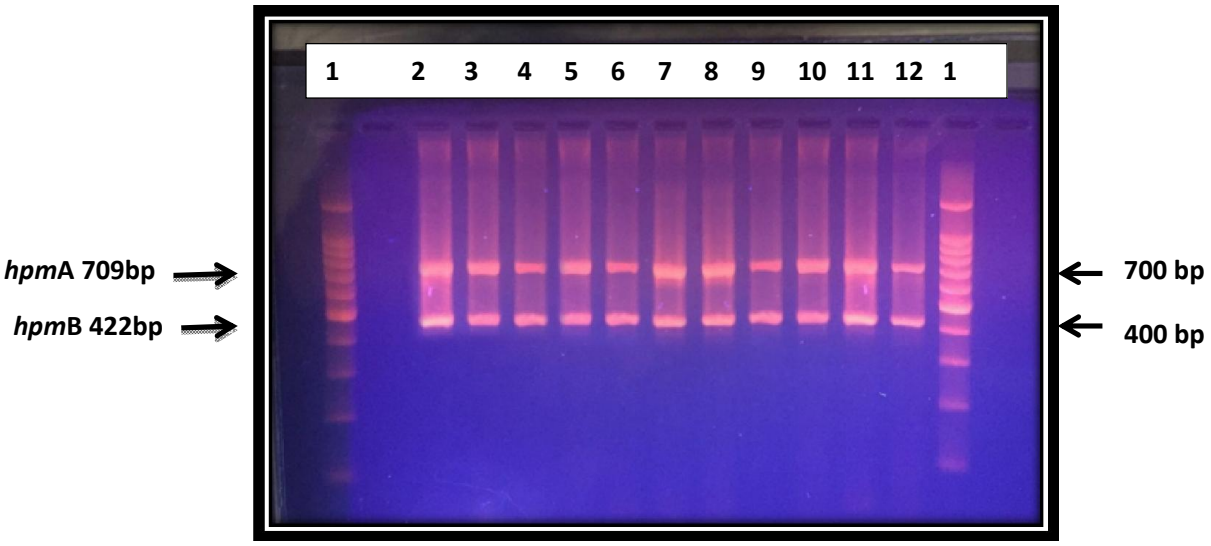


Figure (9): Agarose gel electrophoresis (1.5% agarose, 75 V/cm for 1 hour) of *hpmA* and *hpmB* PCR products (709bp and 422 amplicon) codify for hemolysin of *P.mirabilis* isolates. Lane L (DNA ladder) 100-1100bp molecular marker, lanes 2,3,4,5,6,7,8,9,10,11 isolates are positive results while lane1 and 14 show negative results.

The increase in hemolysin HpmA production is coordinately regulated during the cell differentiation for the swarmer form and during infection, and is correlated with the invasiveness of *P. mirabilis* strains [3].

Study results demonstrate that the detection of *hpmA* and *hpmB* gene by PCR was sensitive enough to be used for the detection of these veriulence factor produced by *P.mirabilis* and The PCR technique were shown to be precise, fast, cheap and more acurate, which were in agreement to many studies that listed before.

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توصيف والكشف الجيني للفا- الهيمولايسين المنقى من بكتريا *Proteus mirabilis* PMBS41

عصام فاضل علوان الجميلي* ساره حسين زغير

*فرع التقنية الاحيائية ، معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا ، جامعة بغداد،بغداد،العراق

الخلاصة

تم انتاج الفا- هيمولايسين بواسطة العزلة *Proteus mirabilis* PMBS41 والتي نميت في الوسط الغذائي المناسب وكانت عدد مرات النقاوة 9.77 والانتاجية بنسبة 14.90% . وقدر الوزن الجزيئي للفا- الهيمولايسين بواسطة كروماتوكرافيا الترشيح الهلامي بأستخدام هلام Sepharose 6B وكان 88,750KD .وكذلك قدر بطريقة الترحيل الكهربائي بوجود SDS فكان 109.64كليو دالتن . أظهرت نتائج توصيف الهيمولايسين ان درجة الحراره المثلى هي 35 و40°C والرقم الهيدروجيني الأمثل هو 8.0، في حين يظهر الهيمولايسين المنقى اعلى مستويات من الفعالية عند درجة حرارة 30°C. - 25° و رقم هيدروجيني 8. تم التشخيص الجزيئي بأستخدام بوادئ متخصصة للجينات المشفرة للهيمولايسين والتي هي *HpmB, HpmA* كعامل ضراوة في بكتريا *Proteus mirabilis* وذلك بأستخدام تقنية PCR .أظهرت النتائج ان جميع العزلات البكتيرية الممرضه Proteus mirabilis المشخصة والمعزولة من عينات الادرار حاوية على جينات عامل الضراوة الهيمولايسين HpmA,HpmB وبذلك فأنها قادرة على تحليل الدم ولكن بنسب متفاوته .

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