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Extraction, Purification and Characterization (A) of Arginine Deiminase Enzyme from a Local Higher Productive Isolation of Enterococcus faecium M1

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Abstract
Arginine deiminase (ADI) enzyme was extracted from *Enterococcus faecium* M1 isolate and lysozyme was efficient in bacterial cell lyses more than Triton-X100 and Glass beads.ADI was more active in the range of (50-80%) ammonium sulfate saturations. After purification by anion exchange and gel filtration chromatography the specific activity of ADI reached to 59.2 U/mg protein with 11.23 folds and 42.93% yield. The purity of enzyme was estimated by Native-PAGE electrophoresis under non denatured conditions. The whole average molecular weight of ADI was 186 KDa, included two non- identical bands on SDS- PAGE, one of them had 52 KDa and the other had40 KDa. By concluding, ADI may contain four polypeptide subunits (tetrarmeric enzyme). Optimum pH for enzyme activity was ranged between (6.5 to 7.5) with maximum activity at pH 7.0 and still active over a wide range of pH values (4.0-10), ADI was more stable at pH level (6.0-7.5) with full remaining activity. By concluding, the maximum activity and stability of ADI in neutral pH encouraged us to use it as anticancer treatment agent and for other applications in human body.
Keywords: Arginine deiminase, *E. faecium* M1, Extraction, Purification, Characterization (A).

Introduction

Arginine deiminase regarded as arginine-depleting enzyme and being a potential anticancer agent because tumour cells have a high requirement for arginine by enhances tumor growth (Wheatley, *et al*. 2000; Kim, *et al*. 2009 and Syed, *et al.* 2013). ADI enzyme could be used in other biological applications (Kubo, *et al*. 2006; Izzo, *et al*. 2007; Gallego, *et al.* 2007; Kameya and Asano, 2014). A number of microorganisms generate ATP fermentatively from arginine (as intracellular enzyme) in which is deiminated by arginine deiminase and promoting higher survival in stationary phase by ADI enzyme (Kim, *et al*. 2009 and Verges, *etal.*1999), Although *E. faecalis* is the paradigm for biochemical studies on the arginine deiminase pathway of fermentative arginine catabolism (Barcelona, *et al*.2002). Lysozyme is an active enzyme in lyses of Gram positive cell wall peptidoglycan (Mitani, *et al*. 2005), Metcalf and Deibel (1973) reported that in a low ionic environment the majority of enterococcal cell wall was hydrolyzed, but cellular integrity was preserved and almost all cellular protein under these conditions. ADI of *E. faecalis* was successfully purified by using ammonium sulfate precipitation, Q-Sepharose fast flow anion exchange chromatography and Sephadex-G75 gel filtration chromatography(Cheng-Fu, *et al*. 2008) and the authors stated that the purified ADI of *E. faecalis* was consists of four identical subunits, but they found that the enzyme consisted of one band with 46 KDa and the whole enzyme MW was 190 KDa. Most studies reported that ADI enzyme was active and stable at a wide range of pH values (Cheng-Fu, *et al*. 2008 and Weickmann *et al*. 1978). Lindcvist, (2005) Mentioned that the stability of *Mycoplasma arthritidis* ADI is due to the unique feature of the composition especially in the presence of 36 half-cystine residues, the state of oxidation of the half-cystines appears to be well established as 16 disulfide and 4 sulfhydryl groups. The aim of this study is to find the most suitable method for extraction of ADI from a local higher productive isolate *E. faecium* M1 and purify it in order to retain the best specific activity for this important enzyme as a potent anticancer agent and for other biological applications.

Materials and methods

***E.faecium* M1 isolate:** this isolate was obtained from (Mahdi, 2013).

Assay of Arginine deiminase activity: ADI activity was estimated after drawing the standard curve of citrulline (enzyme product) concentrations in the reaction mixture (1ml) containing 0.4ml of 50mM phosphate buffer (pH 7.0), 0.4ml L-arginine (50 mM and 0.2ml cell suspension and incubated at 37ºC for 15min., the reaction was stopped by the addition of 10% trichloroacetic acid, and centrifuged at 6000 rpm for 30 min. The citrulline formed was measured by the modified Archibald method (Crow and Thomas, (1982), 1.4ml of acid mixture (18 M H_2SO_4-14 M H_3P0_4; 1:3,vol/vol) was added to 1 ml of the supernatent after centrifugation of trichloroacetic acid-treated sample, and 0.5 ml of 3% diacetylmonoxime were added, mixed together, and boiled in the dark for 15min then cooled in the dark for 10 min, the absorbance was measured at 490 nm and the enzyme activity was

estimated according to the standard curve of citrulline.
Enzyme activity (unit) is defined as the amount of enzyme that liberates 1 micromole of citrulline in one minute at the assay conditions.

Assay of protein concentration
Protein was assayed by Lowry method (Lowry *et al.* 1951) after drawing the standard curve of bovine serum albumin (protein) concentrations.
Extraction of ADI enzyme from bacterial cells: For disintegration of *E. faecium* M1 cell wall, bacterial cells collected from the production medium after centrifugation at (6000r/min for 30 min in cooling centrifuge), the wet weight was calculated and resuspended in extraction buffer with 0.2mg/ml,1mM of mercaptoethanol was added. More than one method was used to disintegrate the cell wall of bacterial cells:
1- Lysozyme with 200 µg/ml were added to suspension of bacterial cells, incubated for 20 min at 37ºC, the suspension was centrifuged at 8000 r/min for 15 min in cooling centrifuge.
2- Bacterial cells were suspended in the extraction buffer was mixed with sterilized glass beads, vortexes for 20 min in cold condition, the suspension was removed by Pasteur pipette, centrifuged(8000 r/min for 15 min in cooling centrifuge.
3- K2HPO4 (12.5%) and 2% of Triton x-100was added to suspension of bacterial cells, incubated at 25ºC for 30 minute then centrifuged at 8000 r/min for 30 minute in cooling centrifuge(Al-Naima, 2007). The pellet was discarded and the supernatant was collected for calculation of enzyme activity, protein concentration and specific activity for three mentioned methods.

Purification of arginine deiminase
1- **Ammonium Sulfate precipitation**: Certain weights of ammonium Sulfate were added step wise to enzyme solution to obtain a graduated (%) saturation (20-90%) then centrifuged at 6000 rpm for 30 minute, the precipitate was dissolved in minimum volume of phosphate buffer and the enzyme solution was dialyzed after that enzyme activity, protein concentration and specific activity of enzyme solution after each step of ammonium precipitation were measured.
2- **Ion exchange chromatography**: The enzyme was passed through DEAE-Sephadex anion column (2.7 x7 cm), then eluted with 20mM potassium phosphate buffer at pH 7.0 containing stepwise NaCl (0.1– 0.5 M). The volume of fraction was 5ml at flow rate 30ml / hr. Optical density for each fraction was measured at 280 nm, enzyme activity was measured in the peaks, active fractions were collected, volume protein and enzyme activity were measured and after that the purified enzyme was concentrated by dialysis.
3- **Gel filtration chromatography**: The concentrated enzyme was applied to the Sephacryl S-300 column (1.6x33.5cm) that was pre equilibrated with 20mM potassium phosphate buffer at pH 7.0 .The enzyme was eluted with the same buffer conditions then 3mL fractions were collected in each tube with flow rate of 30 ml/hr. The optical density for each fraction was measured at 280 nm, then the enzyme activity was measured in the peaks and the active fractions were collected, protein volume and enzyme activity were measured, followed by concentrating the purified enzyme by dialysis.

Determination of ADI purity and whole molecular weight by Native- PAGE under non denaturized conditions(Blackshear, 1984)**.**
Electrophoresis steps were conducted with initial current intensity 2mA/tube for 30 minit was appliedand then increased to 3mA/tube for 4 hours. The distances of protein bands migrated to anode were measured and the relative mobility (Rm) for each protein was calculated to plot the relationship between Rm and log molecular weight for each protein, as follows: Rm is the distance migrated by the protein / distance migrated by the dye. The relationship between Rm of standard proteins and their molecular weights was plotted; the M.W of enzyme was estimated depending on this plot. Electrophpresis was achieved with the presence of standard proteins (γ-globuline, Ova – transferrin, Bovine serum albumin, Pepsin, Trypsin) with molecular weight (150, 80, 67, 34 and 20 KDa) respectively.
Determination of ADI molecular weight by (SDS- PAGE)(Blackshear, 1984)
SDS- PAGE used for determination the M.W of ADI subunits in the presence of denaturated conditions, the same standard proteins used in the Native PAGE and the M.W estimated by measuring the Rm of these proteins. Initial current intensity used with 2mA/tube for 30 minute, then increased to 5mA/tube for 4hours.
Determination the optimum pH for purified ADI activity
Enzyme solution was added to 20mM of substrate solutions prepared at different pHs (5-9) of phosphate and carbonate-bicarbonate buffer solutions and inoculated at 37Cº for 30 minute then the reaction was stopped and the enzyme activity was assayed.
2-2-8-3 Determination the optimum pH for purified ADI stability
The purified enzyme was mixed with buffer solutions at different pH values ranging between 4.0 and 10.0 and

inoculated at 37C° for one hour then directly cooled in ice bath, the enzyme activity was estimated and the remaining activity was measured.

Results

Extraction of ADI from bacterial cells: The results indicated that there was a little variation in specific activity of enzyme among the three methods (table1). The specific activity of ADI was 5.4 U/mg when lysozyme used for disintegration of *E. faecium* M1cell wall. Triton-X100 a non-charged surfactant gave a specific activity of 5U/mg and glass beads gave a specific activity of 4.7 U/mg protein, but the S.A of ADI without treatment (intact cells) was 4.3 U/mg protein.

Table (1): Methods used for extraction of ADI from *E. faecium* M1 cells

Method	Exposure time	Specific activity(U/mg) protein
Lysozyme	20 minute at 37 ºC	5.4
Glass beads	20 minute in cold condition	4.7
2% Triton-X 100 with 12.5% K2HP04	30 minute at 25 ºC	5
Cells without treatment	-	4.3

Purification of arginine deiminase:

Ammonium sulfate precipitation: It was noticed that ADI activity was at the most concentrations of ammonium sulfate (20-90%) but it was more in 50, 60, 70 and 80% saturations, the specific activities in these concentrations were nearly equal, ranged(6.5, 6.7, 6.7 and 6.6U/mg). It may be worthy to mention that there were no or little precipitates in the samples after centrifugation but a floating layer was noticed above the liquid (supernatant), the activity of ADI was higher in this layer, no or low activity was found in the liquid solution.

Ion exchange chromatography: The results described (figure1) that there was one protein peak appeared in the washing step, while three main protein peaks appeared in the elution by a gradient concentrations of sodium chloride. Protein peaks were assayed to detect ADI activity. The first peak appeared in a washing step contained arginine deiminase activity 6.5U/ml. The second and third peaks hadn't ADI activity thus they were neglected. The forth protein peak had ADI activity reached to 7.0U/ml.The peak of protein in the washing step was passed through cat ion of CM-cellulose (batch wise), the washing fraction contained most of the enzyme activity while there was no activity in the eluted fraction with NaCl solution**.**

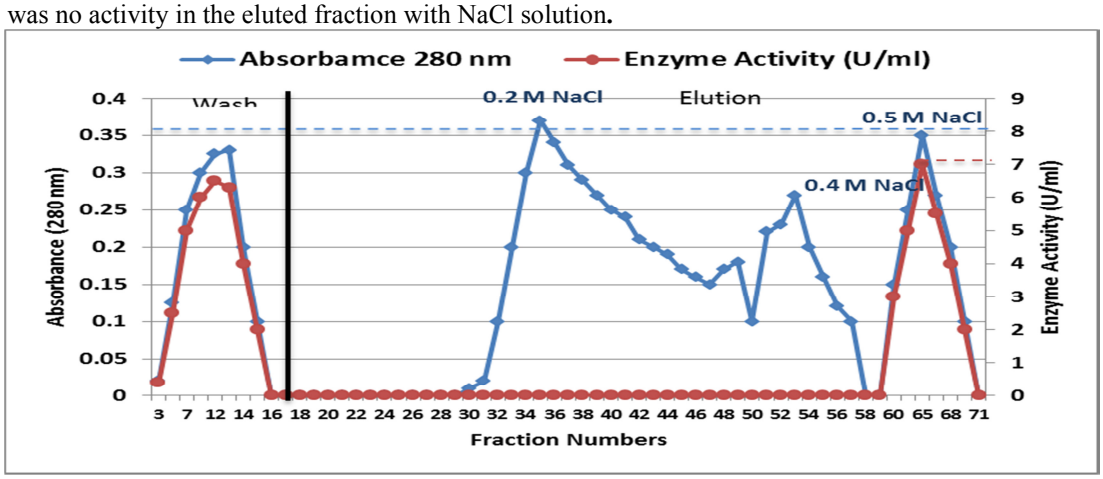

Figure (1): Ion exchange chromatography of ADI from *E. faecium* (M1) using DEAE-Sephadex (2.7x7cm) column, fraction volume was 5ml at a flow rate of 30ml/hr.

Gel filtration chromatography: Results in figure (2) showed three peaks appeared in the eluted fractions but only the first peak had the enzyme activity that was 8.3 U/ml, the specific activity reached 59.2 U/mg with 11.71 purification folds and 42.93 % yield. ADI purification steps of *E. faecium* M1 are summarized in table 2.

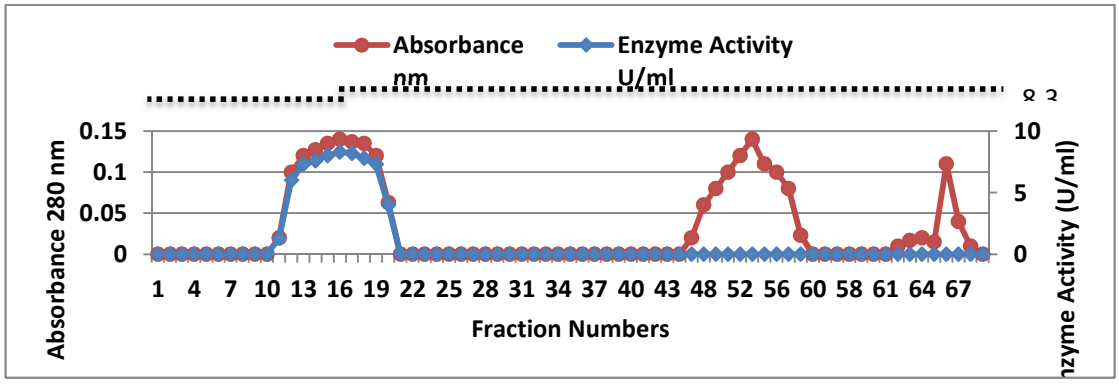

Figure (2): Gel filtration chromatography for *E. faecium* M1 ADI purification using Sephacryl S-300 column (1.6 × 33.5 cm), fraction volume was 5ml at flow rate 30 ml/hr.

Table (2) purification steps for ADI produced by *E. faecium* M1

Purification step	Volume (ml)	Enzyme activity u/ml	Protein concentration mg/ml	Specific activity (U/mg)	Total activity U	Purification folds	Yield (%)
Crude enzyme	100	5.8	1.1	5.27	580	1	100
Ammonium sulfate precipitation	58	6.6	1.2	5.5	382.8	1.04	66
DEAE - Sephadex	42	7	0.34	20.58	294	3.9	50.6
Sephacryl S-300	30	8.3	0.14	59.2	249	11.23	42.93

Determination of arginine deiminase purity: ADI purity was detected by electrophoresis using polyacrylamide gel under non denatured condition as another step of purification and for determining the efficiency of purification steps. The protein profile of the *E. faecium* M1purified arginine deiminase to homogenity which gave one protein band located in the upper part of the gel (figure 3).

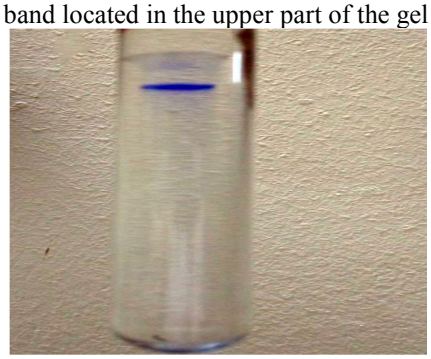

Figure 3: Native- PAGE for the purified ADI from *E. faecium* M1

Characterization of purified arginine deiminase

Whole molecular weight of arginine deiminase enzyme on Native- PAGE

One protein band located in the upper part of the gel was detected, that indicate ADI had a large molecular weight. The method used for determining ADI M.W by measuring the relative mobility (Rm) of ADI as described in figure (4) depending on the (Rm) of standard proteins. Result showed that the approximate molecular weight of ADI was (186000 Daltons)

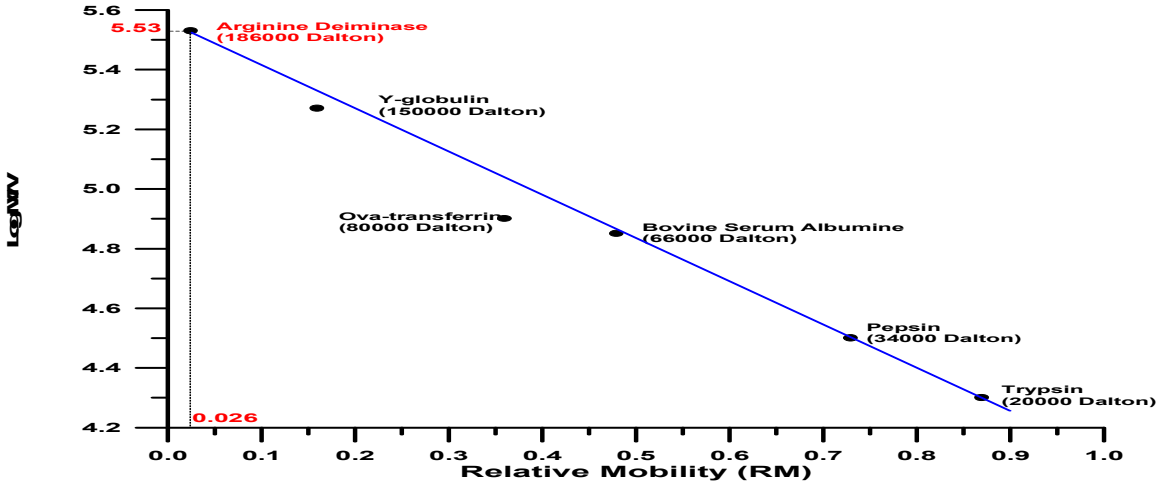

Figure (4): Standard curve for determination *E. faecium* M1 ADI molecular weight using Native PAGE

Molecular weight of ADI subunits by SDS-PAGE gel electrophoresis under denatured conditions

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was also used to determine the M.W of ADI subunits (Figure 5 and 6). Two protein bands appeared for ADI subunits, the upper band had approximately MW 52000 Daltons and the lower band had 40000 Daltons. The overall MW of the 2 non identical subunits equal 92000 Daltons.

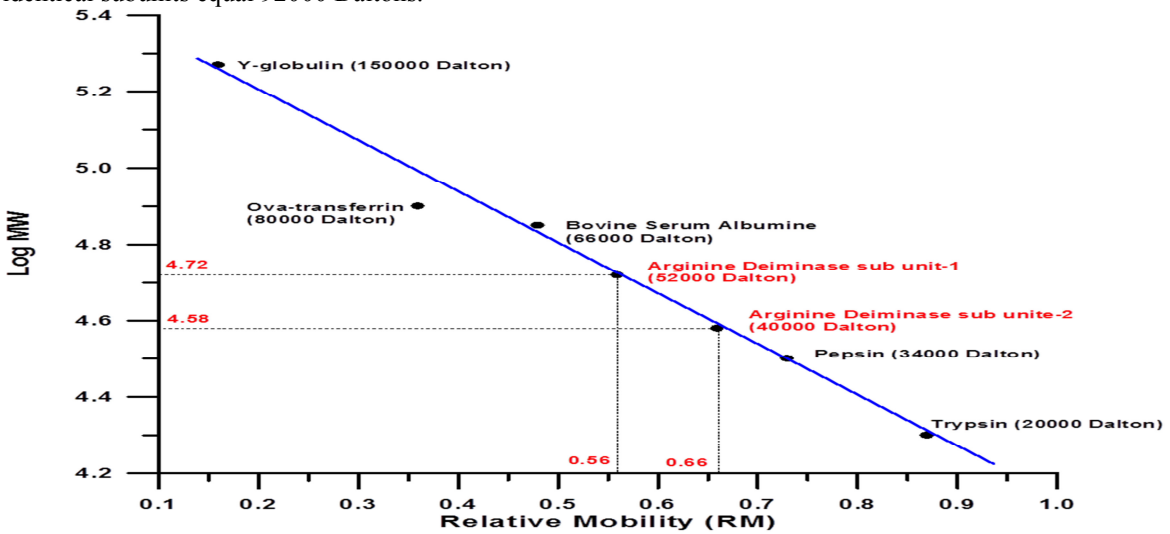

Figure (5): Standard curve for determination *E. faecium* M1 ADI subunits molecular weight using SDS-PAGE

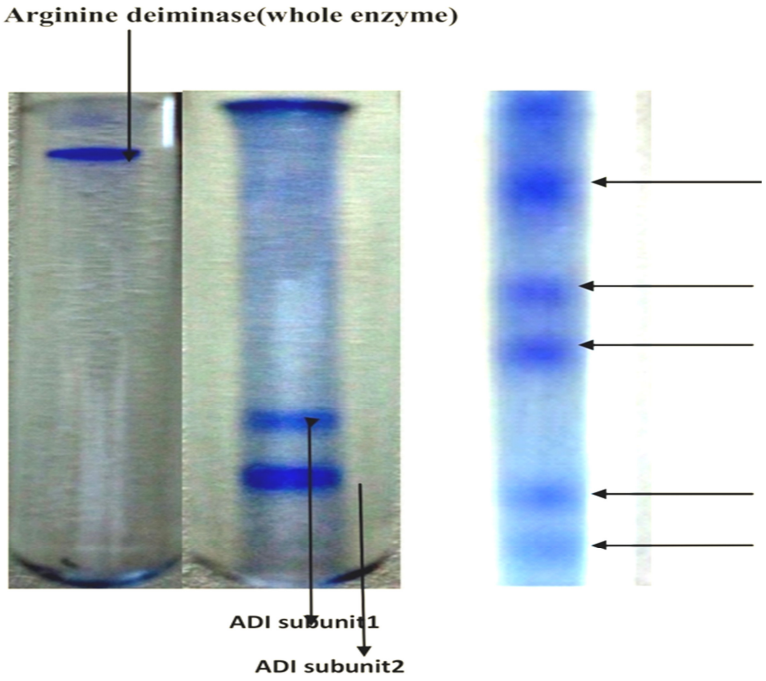

Figure (6): Polyacrylamide gel electrophoresis of purified *E. faecium* M1ADI. A: whole Enzyme (Native-PAGE), B: Enzyme subunits (SDS PAGE), C:standered proteins.

Optimum pH for enzyme activity

The optimum pH for *E. faecium* M1ADI activity was found to be at pH 7.0 that the enzyme activity reached to (8U/ml) and was very active at pH (7.5) with E. a (7.8U/ml) and still active over awide range of pH values as presented in figure (7).

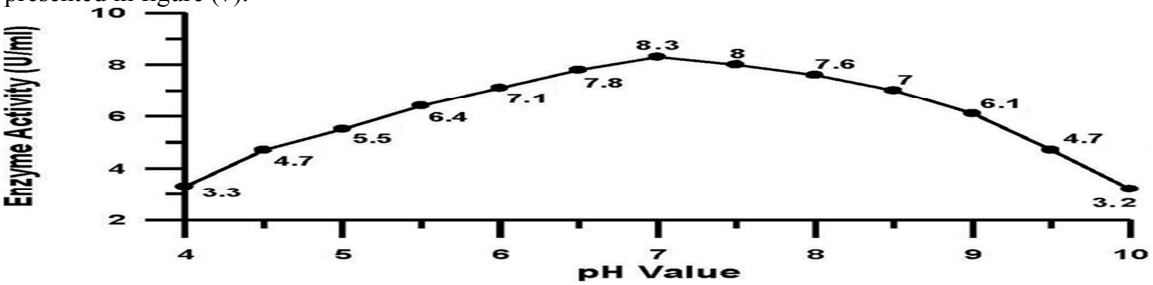

Figure (7): Effect of pH on activity of purified ADI enzyme produced by *E. faecium* M1

Optimum pH for arginine deiminase stability

ADI of *E. faecium* was stable at a wide range of pH (5.5- 9.0) but it was more stable at pH (6.0-7.5) (Figure 8), (12) suggested that the optimal pH for ADI activity in *E. faecalis* with the maximum enzymatic reaction rate was at pH 6.5 but the decline of enzyme activity was more pronounced at pH (4.0) and (9.3).

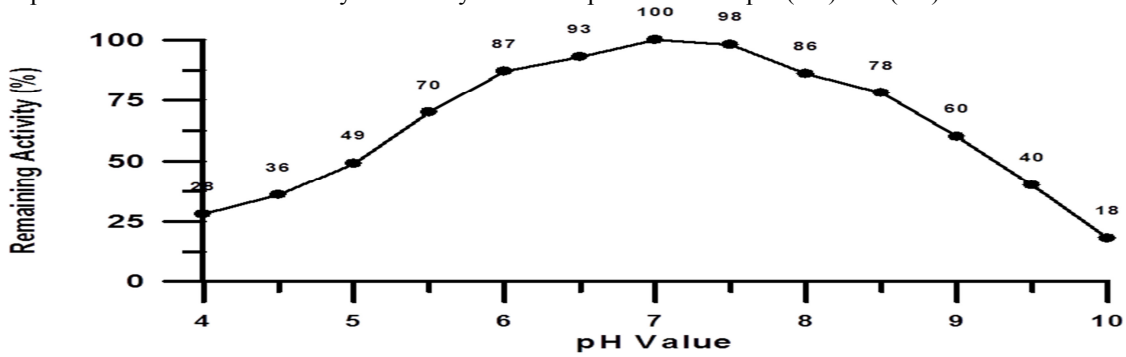

Figure (3-15): Stability of *E. faecium* M1 ADI at different pH values when it was incubated for 60 minute at 37ºC

Discussion

Extraction of ADI from bacterial cells: It was observed that lysozyme is more efficient in cell lysis than other two methods. . In this study the cell suspension was mixed with distilled water in order to produce low ionic environment and treated with lysozyme to disintegrate the cell wall of *E. faecium* M1 and this method was efficient in cell lysis, Metcalf and Deibel, (1973) Found that the action of lysozyme on enterococcal cell differed markedly as a function of the ionic strength of the environment, in high ionic environments, a slow lytic response and decrease in viability were noted but in a low ionic environment the majority of the cell wall was hydrolyzed, but cellular integrity was preserved and almost all cellular protein under these conditions. Triton-X100 a non-charged surfactant gave a good specific activity of ADI,this detergent interacts with phospholipid layer in cell membrane and destroys it (Ramsay *et al*. 1990). Glass beads method had a lesser activity, may be due to its partially harmful effect on ADI during disruption of bacterial cell wall.

Ammonium sulfate (A.S) precipitation

In the first step of purification, the results indicated that ADI is a hydrophobic enzyme because it precipitated with high amount of salt concentrations. This phenomena may interpreted by the presence of a lipid or hydrophobic moiety linked with the enzyme which make it lighter (low density) than the aqueous phase and hence float above it. ADI enzyme from *E. faecalis* was precipitated with 60% and 70% saturations of ammonium sulfate (Cheng-Fu *et al*. 2008).

Ion exchange chromatography

In the second purification stage, there are two peaks had ADI activity presented on DEAE-Sephadex column the first one in washing step, the second one presented anionic ADI peak with highest activity, thus it will be chooses for other steps, because if we collected the fractions of two peaks together, it may be produced heterogeneous ADI with two forms of enzyme. There is more than one probability to explain this result: 1 -The enzyme peak in washing step may be uncharged thus it has no ability to bind with the ion exchangers and hence it was found in washing step. 2-There is more than one form (isoform) of ADI enzyme in the same bacterial strain with different charges. 3-The DEAE - Sephadex columns was saturated with ADI, because this enzyme presented about 8 to 10% of total cell protein in some bacterial species (Brown *et al.* 1998), thus the surplus amount of ADI was dropped in the washing step. 4- It may contain a hydrophobic moiety which reduces its ability to bind with ion exchangers. Weickmann and Fahrney (1977) Reported evidence for two enzyme forms, one form designated arginine deiminase I found only in log phase cultures, the other form, called deiminase II, accounts for 30 to 60% of the enzyme from late log cultures. Lindcvist, (2005) Found that the ADI enzyme extracted from *Streptococcus rattus* was collected during the washing step.

Determination of arginine deiminase purity

The prescence of one band on electrophoresis gel confirmed the purity of ADI enzyme isolated from *E.faecium* that means the purification steps were successful for this enzyme.

Molecular weight of ADI enzyme: The whole M.W of ADI (186 KD) on Native- PAGE indicated that ADI of *E. Faecium* (M1) had a large M.W, but it produced two bands on SDS-PAGE which mean it may consist of four subunits (tetrameric enzyme), each band contains two identical sub units, this demonstrate that ADI is a heterotetrameric enzyme and it composes of 4 subunits, each two are identical**.**

Optimum pH for arginine deiminase activity

From the results we concluded that the maximum activity of*E. faecium* M1ADI enzyme was at pH 7.0 and that will be compatible with pH of tissue culture and human body cells, thus it does not need to genetic engineering to shift its pH like ADI isolated from *Pseudomonas plecoglossicida* which had optimum pH at 6.5 (Zhu *et.al*. 2010). However arginine deiminase of *E. faecalis* M1(Cheng-Fu *et.al*. 2008) still active over a wide range of pH values (4.0-10)

Optimum pH for arginine deiminase stability

The results indicate that ADI of *E. faecium*M1 was very stable and tolerates acidic and alkaline environment, but it favors the neutral pH (6.5-7.5), at this pH the enzyme had maximum remaining activity (98-100%) and thus it will be proper to pH of human body.

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