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Original Article

Characterization (B) of Arginine deiminase enzyme isolated from a local higher productive isolation of *Enterococcus faecium* M1

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ABSTRACT

Arginine deiminase (ADI) is a very active and stable enzyme especially when it used as a cancer treatment agent. In this study purified ADI from *Enterococcus faecium* M1was characterized, the maximum activity of ADI was 9.2 U/ml at 45ºC and still active for a wide range of temperatures. The activation energy of this enzyme was 6501.3 calorie/mol, ADI enzyme was very stable and had a maximum remaining activity at 20-37ºC. Km and V max were 0.36 mM and 0.01298 M/min, respectively. When ADI incubated with EDTA and cysteine a slight inhibitory effect occurred on enzyme remaining activity (R.A) but it increased with 2-mercaptoethanol, which means that this enzyme may considered a thiol enzyme. The (R.A) of ADI increased in the presence of some metal ions like MnCl2, MgCl2, FeCl2, and HgCl2, but it little decreased with CuCl2, ZnSO4. The incubating results of ADI with some drugs and antibiotics were enhanced ADI activity, The enzyme remained active (90%) when it was stored in a freeze degree (-18Cº) for more than one year. By concluding, the present study was designed to characterize the important ADI enzyme in order to use it in many industrial applications in the future.

Keywords: Characterization (B), Arginine deiminase, higher productive isolation, *Enterococcus faecium* M1

INTRODUCTION

Arginine deiminase (ADI, EC 3.5.3.6) is a potential antitumor drug for the treatment of arginine-auxotrophic tumors and antiangiogenic activity [1].The metabolism of arginine towards ATP synthesis has been considered a routinely test for identification of *Enterococcus faecium* and a major source of energy for microorganisms in anaerobic conditions [2,3]. ADI enzyme purified from *E. faecium* M1 was stable at different pH values[2]. The maximum temperature for ADI activity of *E. faecalis* was 50ºC (maximum enzymatic reaction rate) but when the temperature exceeds 50C the enzymatic reaction rate decreased rapidly and they found that under the conditions of 37℃ after 24h ADI stability is very high and the enzyme loss only 6% of its activity [4], many researchers noted that the stability of this enzyme due to a marked tendency for disulfide bonds to be buried in hydrophobic regions and the most remarkable feature of the enzyme is its extraordinarily high content of disulfide bonds [5].

The Michael is constant of ADI isolated from *M arginini* for L-arginine was 0.3 mM, which is about 30 times lower than that of bovine liver arginase [6]. Km of ADI from *E. faecalis* was 3.2686mMol/L and the Vmax was 2.44 micro Molar/min[4]. The Km and Vmax values for arginine deiminase isolated from *Lactococcus lactis* ssp. were 8.67+/-0.045 mM and 344.83+/-1.79 micromole/min/mg, respectively [7]. When the effect of metal ions onADI activity estimated which was dependent on them at their adequate concentration, Mn2+, Mg2+ and Co2+ were the effective promoter, while superfluous Zn2+and Co2+ inhibited ADI activity(4). ADI enzyme isolated from *Halobacterium salinarium* was inhibited quantitatively by 1 mM-Nethylmaleimide, HgCl2 and AgNO3, whereasCuSO4 and AlCl3 had no effect on ADI and the addition of guanidine as a possible substrate had no effect on the activity of the halophilic enzyme[8]. ADI from *Pseudomonas putida* is inhibited almost quantitatively by Hg2+,Al3+, Cu21 and Ag+ ions [9].

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This paper estimated to study the kinetics and effect of chemical compounds, metal ions on purified arginine deiminase isolated froma local higher productive isolation of *E. faecium* M1, and to study the effect of some drugs and antibiotics on this enzyme, in order to use it as a cancer treatment agent and for other applications in the future.

MATERIALS AND METHODS

Purified arginine deiminase enzyme: this enzyme was obtained from [2].

Determination the effect of temperature on arginine deiminase activity: Arginine deiminase activity was determined after incubation of the purified enzyme with the substrate (for 30 minute) at different temperatures (10-60ºC). The activation energy was estimated according to Arrhenius equation [10].

Determination the optimal temperature for arginine deiminase stability: The Purified enzyme was incubated at different temperatures (10-60 ºC) for one hour, then immediately placed in to an ice bath. Activity was assayed for each treatment. The remaining activity (%) for ADI was calculated and plotted against the temperature (ºC).

Estimation of ADI enzyme kinetics: 1- Different concentrations (0.1-10Mm) of L-arginine were prepared in phosphate buffer solution (0.02) at pH 7.0. 2- The enzyme activity was measured by the modified Archibald method [11]. 3- The relationship between substrate concentrations was plotted against ADI activity in order to determine Michael is constant (Km) and maximum velocity value (V- max) of enzyme by using Line weaver-Burk plot method [10]. The remaining activity was calculated.

***Effect of reducing and chelating agents on ADI activity*.** The effect of some reducing and chelating agents on purified ADI activity was studied .This was achieved by incubating the purified enzyme with 2-mercaptoethanol, EDTA, cysteine and sodium azide at two concentrations (5 and 10 mM) for one hour. Enzyme activity was then assayed after each treatment and the enzyme solution was incubated without treating with chemical solutions treatment then the enzyme activity was also estimated for control reaction.

***Effect of some metal ions on arginine deiminase activity*.** The effect of metal ions on purified ADI was studied, these metals include: (HgCl2, FeCl2, CuCl2, MgCl2, NaI, KI, CoCl2, KCl, CaCl2, NaCl, ZnSO4, MnCl2). The enzyme was incubated with equal volume of different metal ions at a concentration of 5 and 10 mM at 37 ºCfor 60 minutes, the enzyme activity was assayed for each treatment. The control was the enzyme solution without any of these compounds. The remaining activity was assayed for each treatment.

Effect of some drugs and antibiotics on ADI activity

The purified enzyme was treated with different drugs and antibiotics (Analgesic drug ampule, Gentamycin ampule, Sefotaxime ampule, Meropenem ampule, Cloxaplus (Ampicillin–Cloxacilline) ampule, Doxycycline capsule and Ceftriaxone ampule at different concentrations (5, 10, 50, 100mg/ml), 0.2ml of the enzyme solution was added to 0.4ml of these solutions and 0.4ml of reaction solution (Potassium phosphate buffer 50mM, at pH 7.0 containing 50mM L- arginine) incubated at 37Cº for 60 minute and the enzyme activity was assayed , The control was the enzyme solution without treatment with drug.

RESULTS AND DISCUSSION

Effect of incubation temperature on arginine deiminase activity: Temperature is an important factor which affects enzyme activity. The favorable temperature for ADI activity may differ with different bacterial types. In order to determine the optimum temperature for ADI activity, enzyme activity was assayed at different temperatures ranged between (20 to 70ºC). The activity of *E. faecium*M1 ADI increased with temperature increasing and reached to 9.2 U/ml at 45ºC which was the maximum ADI activity and still active for a wide range of temperatures ranging from 35 ºC until 60 ºC (figure 1). At physiological temperature 37ºC the enzyme activity was 8.5 U/ml. This proves that this enzyme can stay active at a wide range of temperatures.

Generally at low temperatures the enzyme activity decreased and when the temperature slightly increased the velocity of reaction increased too and the rate of product production will become stable, but if the temperature increased more than this, it leads to protein denaturation and changing the active site and decreasing the activity of enzyme. The maximum activity for ADI enzyme of *E. faecalis* was at 50ºC [4].

Activation energy of arginine deiminase: Activation energy is the minimum energy required for the molecules to be converted to product. One of the important characters of enzyme is acceleration of chemical reactions and lowering the activation energy which lead to increase the speed of enzymatic reactions more than non- enzymatic chemical reactions[12].

The activation energy for conversion of arginine to citrulline by ADI enzyme was 6501.3 calories/mol (figure 2).

In general activation energy of the chemical reactions ranged between (6000-15000) calories /Mol, whenever activation energy is low, the enzyme is more efficient in converting the substrate to product, that indicated ADI enzyme isolated from *E. faecium M1* isolate is very efficient in converting L-arginine to citrulline because it require low activation energy to convert the substrate to product.

The optimum temperature for stability of arginine deiminase: To determine the optimum temperature for ADI stability, the enzyme was incubated at different temperatures for one hour. Figure (3) showed that ADI is stable over a wide ranges of temperatures ranged between 20-45ºC.

The enzyme had a maximum remaining activity(100%) at 20-37ºC and at 40ºC it retained about 98%, this result agree with most studies about the stability of ADI at different temperatures (13). The remaining activity decreased slightly at 45ºC to 95% of them it become 80% at 50ºC and the enzyme lost most of its activity after incubation at 60ºC which reached to 27% then to 0% at 70ºC that prove the ADI enzyme of *E. faecium* M1 denatured after one hour of incubation at this temperature. The stability of this enzyme was explained by the presence of disulfide bonds buried in hydrophobic regions and the most remarkable feature of the enzyme is its extraordinarily high content of disulfide bonds [5]. ADI enzyme isolated from *E. faecalis* lost its activity after incubation for 50 minute at 60ºC.

Increasing of temperature may lead to denaturation of enzyme by destructing the three dimensional structure of protein and formation of random polypeptide chains, these causes a change in the active site which inadequate binding of enzyme and substrate? There were also heat stable enzymes (from thermophiles organisms) which may not be affected by high temperature due to their hydrophobic and disulfide bridges which strengthen their structure [14].The large numbers of cystine residues contribute significantly in their stability of the enzyme, as well as its resistance to unfolding, probably this is linked to its exceptionally high cystine content [15]. Its worthy to say that enzyme ADI produced from *E. faecium* M1was stable during storage for more than one year with 90% remaining activity at (-18ºC).

The kinetic constants of arginine deiminase: Michaelis –Menten constant (Km) and maximum velocity (Vmax) of *E. faecium* M1 (ADI reaction) were calculated according to Lineweaver- burk plot. Results showed that the average of Km and Vmax of ADI were0.36 mM and 0.01298M/min, respectively [4].

The small value of Km means that there is a high affinity between ADI enzyme isolated from *E. faecium* M1 and its substrate in the cell.

The Km and Vmax of ADI enzyme isolated from *E. faecalis* were 3.2686mM and 2.44µM/min [4]. The Km of ADI from *Mycoplasma arginini* was 0.2mM and the Vmax was 50U/mg (13). The Km and Vmax values for arginine deiminase of *Lactococcus lactis* were 8.67 mM and 344.83µmol/min, respectively [16].

The purpose of use Michaelis–menten constant is to estimate the approximate value of substrate levels in the cells for using it in a comparative study between enzymes isolated from different microorganisms when Km is low or small, the affinity of enzymes to substrate will be high[10].

Effect of reducing and chelating agents on ADI activity: Some chelating and reducing agents (2-mercaptoethanol, EDTA, Cystein and sodium azide) were added to the purified enzyme at two concentrations (5 and 10 Mm) in order to determine their effect on ADI activity which give some information about this enzyme and characterize it. ADI of *E. faecium* M1 activity increased to 110% when treated with 5mM of 2-mercaptoethanol and maximum remaining activity (130%) was achieved with 10mM mercaptoethanol (table 4). This explains that this reducing agent may positively interact with the active site of ADI enzyme to support the enzyme activity. This conclude that this enzyme may be a thiol enzyme which has thiol groups involved in the active site of the enzyme [17]. EDTA had little effect on enzyme activity, the remaining activity was 80% at 5mM and 83% at 10mM. The results presented in table (1) showed that the ADI activity is not affected when 5mM of cysteine was added but it decreased to 72% when10Mm of cystein was used.

The activity of the halophilic enzyme isolated from *Halobacterium salinarium* depends on free thiol groups [18]. Sodium azide had noeffect on the enzyme activityat5 and 10mM.

Effect of metal ions on arginine deiminase activity: In this study the effect of some metal ions **(**$HgCl_2$, $FeCl_2$, $CuCl_2$, $MgCl_2$, NaI, KI, $CoCl_2$, KCl, $CaCl_2$, NaCl, $ZnSO_4$ and $MnCl_2$) on purified ADI was determined. Results in table (2) explained that ADI activity increased when the enzyme was incubated with $MnCl_2$, $FeCl_2$, $MgCl_2$ and $HgCl_2$

with5 and 10 mM as ADI activators**,** the second concentration gave a higher activity than 5mM. $MnCl_2$ was the best metal ion for increasing the activity of ADI enzyme, maximum remaining activity (127%) was achieved when the enzyme was incubated with 10mM of MnCl2 which may indicate that this enzyme may exhibit as ametaloenzyme which activated in the presence of these metal ions.

NaCl and CoCl2 at 5mM enhanced the activity of ADI more than 10mM, 5mM KI increased the remaining activity to 107% but it was 96% at 10 Mm (table 2). In the other hand CuCl2, ZnSO4 and NaI ions slightly decreased the activity of ADI enzyme. It can be concluded that ADI of *E. faecium* is not inhibited by most of metal ions and it can be activated by many of them. Cheng-Fu et al. (2008) found that the superfluous Zn+2 and Co+2 inhibited ADI activity. ADI from *Pseudomonas putida* inhibited almost quantitatively by Hg2+, Al3+, Cu+2 and Ag+ ions [19].

Effect of some drugs and antibiotics on ADI activity: ADI is considered as a potent cancer treatment agent and the patients of this fatal disease often exposed to secondary microbial infections, thus they are forced to use antibiotics and pain remover drugs, in order to know if some drugs can interact with the activity of ADI enzyme, thus six different broad-spectrum antibiotics with one kind of pain killer drug were examined to determine their effects on *E. faecium* M1 ADI activity.

The effect of some drugs and antibiotics on purified ADI activity at four concentrations (5,10,50 and 100 mg/ml) was studied. In general there was no inhibitory effect for all the drugs and antibiotics which mean they had synergistic effects for ADI activity except for Cloxaplus(Ampicillin-Cloxacilline) at 5mg and somehow at 10mg with partially antagonistic effects as mentioned in(table 3) but when 50 and 100mg used the remaining activity increased to117% and 103% respectively. Gentamycin enhanced the activity of enzyme at three concentrations(5, 10 and 50 mg/ml) but at 100mg/ml the remaining activity was 88%(table 3).These results show that ADI of *E. faecium* M1 may be allosteric enzyme that have more than one site (regulatory site) in addition to active site to bind with chemical compounds because this enzyme have more than one subunit(quaternary structured enzyme), thus if it bind to inducer effector (at some concentrations of drugs), it will be change the conformation of enzyme to be more suitable to bind with substrate in the active site of enzyme that lead to increase enzyme activity, on the contrary if the enzyme bind to inhibitor (concentrations), that will be decrease the enzyme activity [20].

Table(1): Effect of reducing and chelating agent on activity of ADI enzyme purified from *E. faecium* M1

Materials	Concentration (Mm)	Remaining activity (%)
2-mercaptoethanol	5	110
	10	130
EDTA	5	80
	10	83
Cystein	5	100
	10	72
Sodium azide	5	100
	10	100

Table(2) Effect of some metal ions on *E.faecium* M1 ADI enzyme activity

Materials	Concentration (Mm)	Remaining activity (%)
$MnCl_2$	5	108
	10	127
$MgCl_2$	5	106
	10	113
$CuCl_2$	5	95
	10	97
$FeCl_2$	5	110
	10	113
KCl	5	106
	10	112
NaCl	5	113
	10	105
$CoCl_2$	5	108
	10	105
$HgCl_2$	5	103
	10	112
KI	5	107
	10	96
$ZnSo_4$	5	82
	10	78
NaI	5	81
	10	77

Table (3).The effect of some drugs on *E. faecium* M1 ADI activity

Drug name	Remaining activity (RA)			
	5mg/ml	10mg/ml	50mg/ml	100mg/ml
Analgesic	101	108	111	118
Gentamycin	119	112	101	88
Meropenem	101	107	116	122
Cloxaplus(Ampicillin –Cloxacilline)	48	71	117	103
Doxycilline	80	86	115	109
Ceftriaxone	102	115	119	121
Tetracycline	100	112	118	124

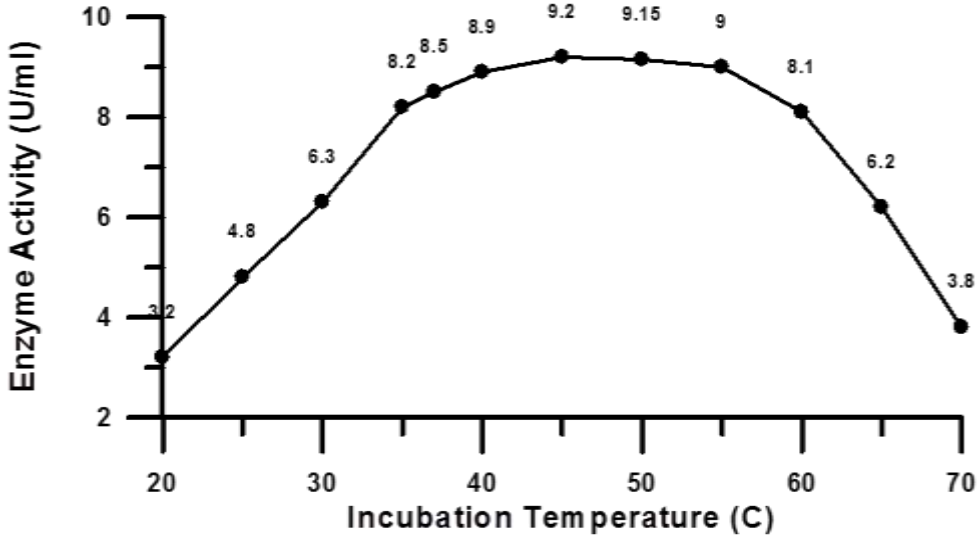
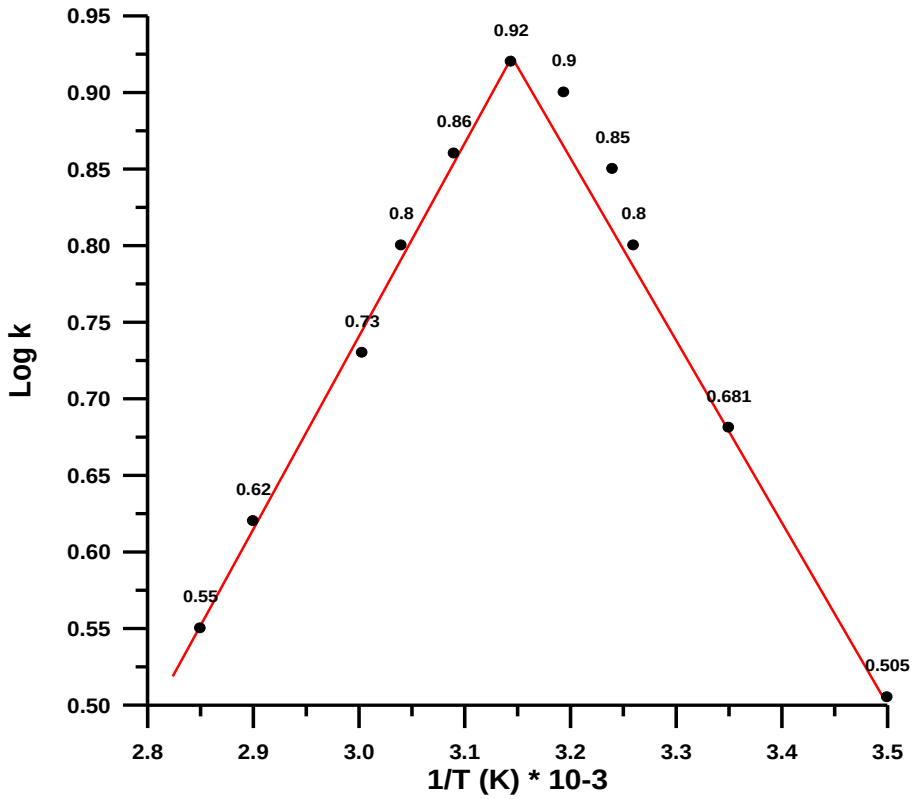


Figure (1): Effect of different temperatures on purified ADI activity of *E. faecium* (M1) at pH 7.0 and 18h incubation



Figure(2) Arrhenius plot for determination the activation energy of ADI produced by *E. faecium* M1 strain

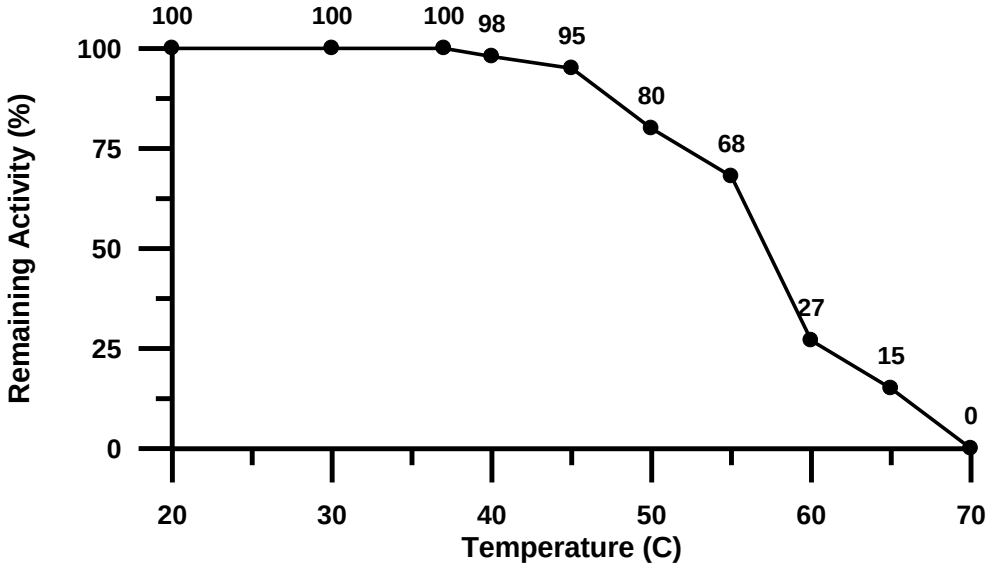


Figure (3): Stability of arginine deiminase for one hour at different temperatures

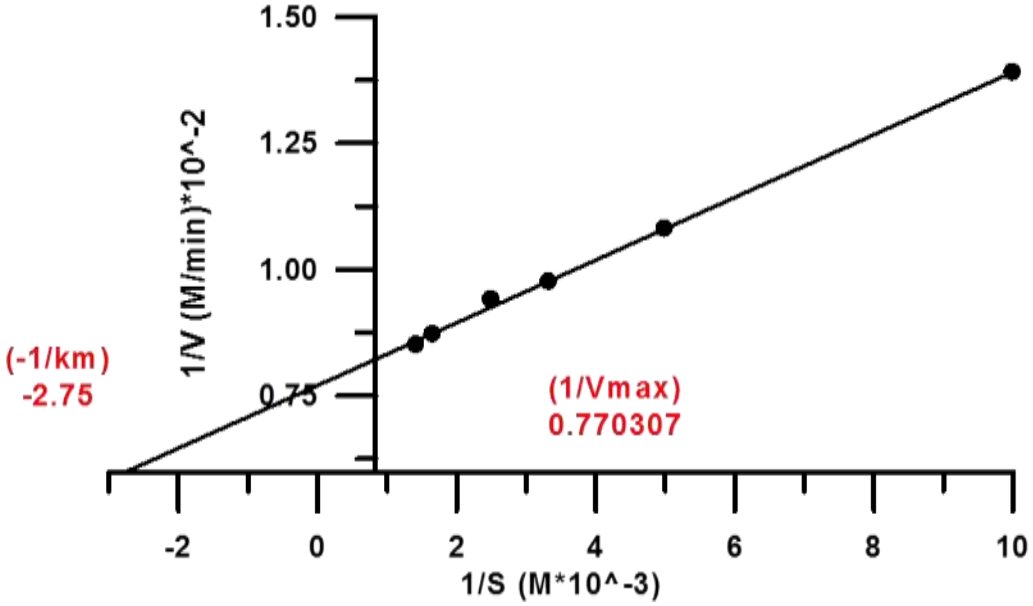


Figure (4): Kinetic constants of ADI purified from *E. faecium*(M1) using L-arginine substrate by Line weaver-Burk plot

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