



## Purification and Characterization of L-Arginine deiminase from *Vibrio alginolyticus* 1374

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### ABSTRACT:

**Aim:** The aim of our present study was to purify and characterize L-arginine deiminase isolated from marine *Vibrio alginolyticus* 1374.

**Methodology:** Arginine deiminase (AD), an arginine-degrading enzyme, has been used in the treatment of tumours, sensitive to arginine deprivation, such as malignant melanoma and hepatocellular carcinoma. Proteins of microbial origin are always associated with mild to severe hypersensitivities. Moreover, proteins from terrestrial sources were found to exhibit stability problems at physiological environment. Hence considering the fact that the characteristics of the enzyme depends upon sources from which it has been isolated, we have used marine organism for the present study expecting to yield therapeutically and physiologically stable enzyme. ADI production was carried out under optimal conditions by shake flask method. The enzyme thus obtained was purified by ammonium sulphate fractionation, ion exchange and gel permeation chromatography. The purity of the enzyme was further confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and it was characterized to know its properties.

**Results:** It was active at physiological pH, showed high substrate specificity towards L-arginine. It also exhibited high salt and temperature tolerance indicating good scope for its industrial and therapeutic applications.

**Conclusions:** ADI obtained from *Vibrio alginolyticus* 1374 can be a good candidate for the treatment of human cancers.

**Keywords:** L-Arginine deiminase (ADI), *Vibrio alginolyticus* 1374; GU726873, ion exchange chromatography, gel permeation chromatography.

Received on : 28-01-2016

Revised on : 15-03-2016

Accepted on : 21-03-2016

### INTRODUCTION:

*Vibrio alginolyticus* is a halophilic microorganism generally found in marine waters<sup>1,2</sup>. It is recognized as an important human pathogen causing mild superficial wound and ear infections<sup>3</sup>. *Vibrio alginolyticus* and various other microorganisms use ADS for energy

production and for survival under extreme acidic conditions<sup>4,5</sup>. Arginine metabolism via ADS (Arginine Deaminase System) has been shown to generate sufficient energy to maintain growth of several bacteria such as *Enterococcus faecalis*<sup>6-10</sup>. Next to the natural role of energy production, arginine deiminases were found to be effective in treatment of arginine succinate synthetase (ASS) negative tumors<sup>11,12</sup>. These tumors are auxotrophic in nature and they face nutrient starvation in the presence of arginine degrading enzymes such as ADI or L-arginase<sup>13-18</sup>. Hence these enzymes can be used for arresting the growth of several cancer cells. Beneficial effects of application of ADI have been also shown in treatment of certain cancers<sup>19</sup>. Even though the

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presence of ADI is found in many micro-organisms, the yield obtained from them is very less. Hence in our preliminary studies we have isolated a potential ADI producer from marine sources and optimized various nutritional as well as physical parameters for optimal production of the enzyme<sup>20</sup>. More over to use ADI as an effective candidate in the treatment of human cancers, it should be stable at biological pH and temperature. Hence in the present study we have purified and characterized the enzyme to know its properties. Immunogenicity and short plasma t ½ time associated with bacterial proteins such as ADI in the human body can be prevented by linking with polymers such as polyethylene glycol, dextran etc without loss of enzymatic activity.

**MATERIALS AND METHODS:**

**Chemicals:**L-Arginine,Nutrient agar used in the present study was procured from Hi-Media Laboratories, Bombay. Ammoniumsulphate, DEAE Sephacel, Sephacryl S-200 column, Sodium dodecyl sulphate polyacrylamide gel and remaining chemicals were purchased from Sigma Aldrich, Bengaluru, India.

**Inoculum preparation :** Marine *Vibrio alginolyticus* 1374 isolated from coastal areas of Chirala beach was maintained in fresh cultures of nutrient agar slants. 5ml of sterile distilled water was added to the nutrient agar slants, mixed well and inoculated into sterile nutrient broth medium and incubated at 37 ° C. The 2% of the resulting suspension was used as an inoculum.

**Production:** Production media containing maltose – 2g, soya bean meal -2g and l-arginine – 2g in sea water based mineral arginine medium of pH 8 was found optimal for the production of ADI in our previous studies. A 2% of the inoculum was added into the production medium and incubated at 37°C for 120 h<sup>21</sup>.

**Purification:**Cells from the medium were harvested by centrifugation at 10,000 rpm for 30 min. The supernatant thus obtained was subjected to purification by ammonium sulphate fractionation followed by ion-exchange column (1.5X30 cm of DEAE-Sephacel) and gelfiltration chromatography (SephacrylS-200 column (2.5x55cm). The purity of the enzyme was further confirmed by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) as described by Laemmli<sup>22</sup>.

**Analytical techniques:** Citrulline concentration was used to measure enzyme activity colorimetrically using a modified version of the method described by Oginsky<sup>23</sup>. Enzyme protein: Protein content in the sample was determined by Lowry’s method and the values were expressed in mg<sup>24</sup>.

**Enzyme characteristics:** The purified ADI was characterized for its various properties. The characters analyzed included effect of pH (4 -10) and temperature (30 -80°C) on enzyme activity and stability, effect of NaCl (0-20%) on enzyme activity, effect of substrate

concentration (L - arginine; 0.01 - 1.0 M) on enzyme activity, effect of L-arginine (0.01M) and NaCl (10%) on temperature stability of ADI and the substrate specificity the enzyme.

**RESULTS:**

**Enzyme purification:**

ADI was purified to homogeneity after successive steps of purification. The crude extract contained 360 mg of protein and showed a total ADI activity of 192U units with a specific activity of 0.53 IU/mg protein. After final step of purification process the extract showed the specific activity of 280.6 IU/mg with yield of 43.5% (Table 1).

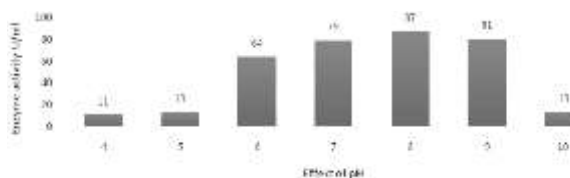
**Determination of molecular weight**

SDS-polyacrylamide gel electrophoresis of the purified l-arginine deiminase together with several proteins with known molecular weight indicated that the enzyme had a molecular mass of approximately 48 kDa (not shown).

**Table 1:** Purification of L-arginine deiminase

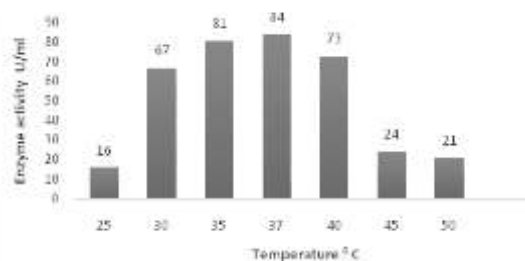
Step	TotalEnzyme Activity(IU)	TotalProtein (mg)	Specific Activity (IU/mg)	%Recovery	Purification Fold
Crudeextract	192	360	0.53	100	1
(NH4)2SO4 fractionation	169	265	0.637	88	1.20
Ion-exchange chromatography	112	0.87	128.7	58.3	242.8
Gelfiltration chromatography	87	0.31	280.6	45.3	529.43

**Enzyme characteristics:**The enzyme was active over a range of pH 4-10 with optimum at pH 8.0 (Fig.1). The activity considerably decreased at both low pH (5.0) and high pH (10.0).

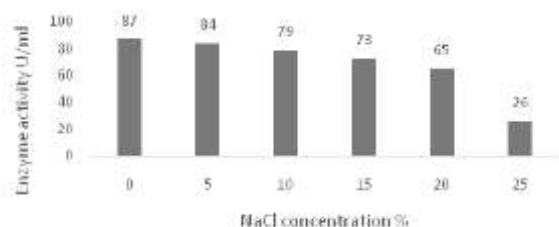


**Fig 1:** Effect of pH on the enzyme activity

The enzyme was active over wide temperature range of 25 and 50°C (Fig. 2). The enzyme had highest activity at 37 °C.



**Fig 2:** Effect of temperature on the enzyme activity

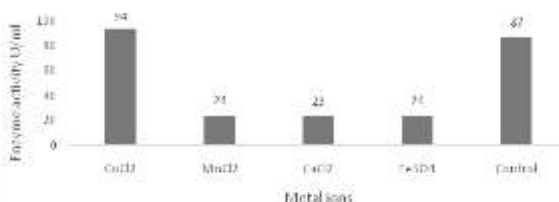


**Fig.3:** Effect of NaCl concentration on enzyme activity.

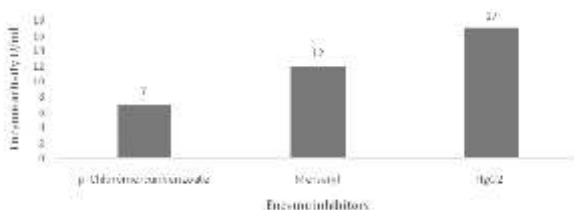
It retained its maximum activity between 0 to 15% NaCl concentration which indicated its salt tolerant nature of ADI from *Vibrio alginolyticus* (fig 3).

Effects of metal ions and some compounds: As shown in fig. 4, cobalt ions were important for the activity of the enzyme. Other metal ions such as manganese, calcium and iron tested hardly affected the enzyme activity.

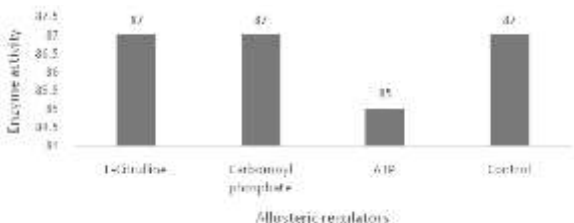
Arginine deiminase was inhibited by sulfhydryl inhibitors like p-chloromercuribenzoate,  $Hg^{2+}$  and mersaryl (fig.5). As shown in fig. 6 ADI is not allosterically regulated by the intermediates and metabolic products of arginine degradation. No significant changes in ADI activity were observed in the presence of ATP, citrulline, and carbamoyl phosphate, respectively. The enzyme shows high substrate specificity towards L-arginine (fig.7).



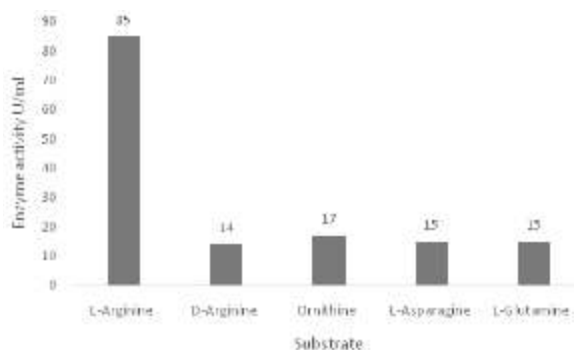
**Fig.4:** Effect of metal ions on enzyme activity.



**Fig.5:** Effect of enzyme inhibitors on enzyme activity.



**Fig.6:** Effect of allosteric regulators on enzyme activity.



**Fig. 7:** Effect of substrates on enzyme activity.

### DISCUSSIONS :

The impurities present in crude extract were removed by step by step process of purification. The specific activity of crude extract before purification was 0.53 IU/mg. Upon precipitation with ammonium sulphate, the specific activity was found to be increased by 1.2 fold and total protein content was found to be decreased by 1.35 fold. This indicates that the fractionation range ammonium sulphate used for precipitation was effective enough in removing proteins which are contaminants, with subsequent loss of total activity. With ion exchange chromatography, the specific activity was found to increase by 242.8 fold with decrease in protein concentration to about 413.7 times which indicates an efficient removal of contaminants. GFC was performed as final step of purification. The specific activity after GFC was found to be increased by 529.43 fold, with total protein content decreasing by 1161.2 fold. Three bands were observed in SDS-PAGE after IEC. After GFC, only one band was observed. From the bands observed, it can be claimed that the enzyme was purified successfully and its molecular mass was found to be approximately 48 kDa. Whereas in case of *Mycoplasma arthritidis* and *Mycoplasma arginini* it was found to be 49 and 48 kDa respectively on SDS PAGE<sup>25,26</sup>. The specific activity of the purified enzyme from *Vibrio alginolyticus* 1376 was found to be 280.6 IU/mg which is more when compared to *Enterococcus* (5.1IU/mg), *Pseudomonas putida* (76 ± 0.03 IU/mg) and recombinant *E. coli* (30-34 U/mg)<sup>27-29</sup>.

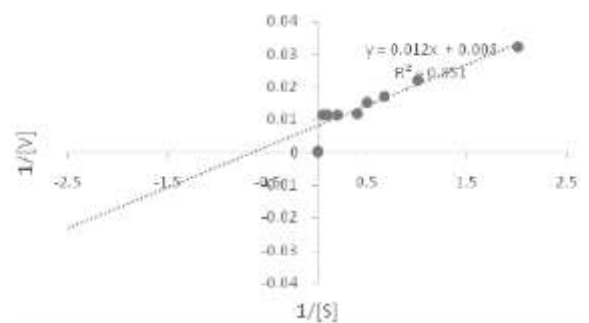
The enzyme was active over a range of pH 4-10 with optimum at pH 8.0. The activity considerably decreased at both low pH (5.0) and high pH (10.0). ADI isolated from various sources were shown to have slightly acidic to neutral pH. ADI isolated from *Streptococcus pyogenes* M4959 was having optimal pH at 6.5 whereas that of *Lactobacillus Buchneri* NCDO110, *Pseudomonas putida*, *Escherichia coli* was at pH 6<sup>30-32</sup>. The enzyme had highest activity at 37 °C. The fact that ADI from *Vibrio alginolyticus* 1374 obviously works best at 37°C and pH 7.4 makes it a suitable candidate for the application in treatment of ASS-negative tumors. Immunogenicity of bacterial proteins such as ADI in the human body can be circumvented by formulation with polyethylene glycol without loss of enzymatic activity.

It retained its maximum activity between 0 to 15% NaCl concentration which indicated its salt tolerant nature of ADI from *Vibrio alginolyticus*. Metal ions acts as co factors for several enzymes. Of various ions tested cobalt ion was important for the activity of the enzyme. Other metal ions hardly affected the enzyme activity. Arginine deiminase was inhibited by sulfhydryl inhibitors like p-chloromercuribenzoate, Hg<sup>2+</sup>, and mersaryl. ADI isolated from *Pseudomonas* has also been reported to be inhibited by sulfhydryl inhibitors<sup>33</sup>.

As shown in fig. 8 ADI of *Vibrio alginolyticus* is not allosterically regulated by the metabolites of arginine metabolism. No significant changes in ADI activity were observed in the presence of ATP, citrulline, and carbamoyl phosphate, respectively. While citrulline and carbamoyl phosphate had no effect on the activity of ADI of other bacteria either, for *L. buchneri* an inhibitory effect of ATP on ADI activity has been shown<sup>34</sup>.

#### The kinetic constants of arginine deiminase:

Michaelis–Menten constant (Km) and maximum velocity (Vmax) of *Vibrio alginolyticus* 1374 (ADI reaction) were calculated according to Line weaver- Burk plot. Results showed that the average of Km and Vmax of L-arginine deiminase were, 1.51 ± 0.23 mM and 120.48 ± 3.24 U/ml/min respectively. The small value of Km indicates high substrate specificity of ADI towards arginine. ADI isolated from *E. faecalis* has Km and Vmax values of 3.2686 mM and 2.44 M/min<sup>35</sup>. The Km of ADI from *Mycoplasma arginini* was 0.2 mM and the Vmax was 50 U/mg<sup>36</sup>. The Km and Vmax values for arginine deiminase of *Lactococcus lactis* were 8.67 mM and 344.83 mol/min, respectively<sup>37</sup>. The purpose of use Michaelis–Menten constant is to estimate the approximate value of L-arginine 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<sup>38</sup>.



**Fig. 8:** The Line weaver –Burk plot to determine the Km and Vmax for ADI

#### CONCLUSIONS:

The ADI produced by the organism has several beneficial properties needed for atherapeutic and industrial enzyme. It has wide range of activity and stability at various temperatures and p H and is highly

salt-tolerant. The substrate specificity towards L-arginine is also very high which means that it could be used in low amounts to achieve the desired effect. Preliminary studies showed that the purified ADI was an effective therapeutic agent in the treatment of cancer (unpublished data). Based on the results obtained in the present study, we conclude that the marine *Vibrio alginolyticus* 1374 has immense potential for large scale production of ADI having several beneficial properties for its use as an anticancer agent and in the food industry.

#### LIST OF ABBREVIATIONS

ADS= Arginine deiminase system  
 ADI = L-Arginine deiminase  
 ASS = argininosuccinate synthetase  
 IEC = Ion exchange chromatography,  
 GFC = Gel filtration chromatography,  
 SDS- PAGE = Sodium dodecyl sulphate  
 polyacrylamide gel electrophoresis

ACKNOWLEDGEMENT: Authors are thankful to the authorities of Malla Reddy College of Pharmacy for providing the facilities to carry out this work.

#### REFERENCES:

1. Chowdary MAR, Yamanaka H, Miyoshi S. Ecology and seasonal distribution of *Vibrio parahaemolyticus* in aquatic environments of a temperate region. *FEMS Microbiol Ecol*.1990; 74:1-110.
2. Zanetti S, Deriu A, Volterra L. Virulence factors in *Vibrio alginolyticus* strains isolated from aquatic environments. *Ann Ig*2000;12(6): 487 – 491.
3. Pezzlo M, Valter PJ, Burns MJ. Wound infection associated with *Vibrio alginolyticus*. *Am J Clin Pathol*. 1979;71(4):476-8.
4. A. Casiano-Colon, R.E. Marquis, Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance, *Appl. Environ. Microbiol*. 1988; 54: 1318–1324.
5. B.A. Degnan, M.C. Fontaine, A.H. Doebereiner, J.J. Lee, P. Mastroeni, G. Dougan, J.A. Goodacre, M.A. Kehoe. Characterization of an isogenic mutant of *Streptococcus pyogenes* Manfredo lacking the ability to make streptococcal acid glycoprotein, *Infect. Immun*.2000; 68: 2441–2448.
6. Nada Z. Mahdy<sup>1</sup>, Shatha S. Al-Tahan, Nahi Y. Yaseen .Optimization of ArginineDeiminase production from a local higher productive isolate *Enterococcus faecium* M1.Iraqi *Journal of Cancer and Medical Genetics*, 2014; 7-1.
7. Das K, Butler GH, Kwiatkowski V, Clark AD Jr, Yadav P, Arnold E. Crystal structures of arginine deiminase with covalent reaction intermediates; implications for catalytic mechanism. *Structure*, 2004; 12(4): 657–667.
8. T. Bauchop, The growth of micro-organisms in relation to their energy supply, *J. Gen. Microbiol*. 1960; 23: 457–469.

9. Shibatani T, Kakimoto T, Chibata I Crystallization and properties of L-arginine deiminase of *Pseudomonas putida*. *J Biol Chem* 1975; 250(12):4580-4583.
10. H.H. Moustafa, E.B. Collins, Molar growth yields of certain lactic acid bacteria influenced by autolysis, *J. Bacteriol.* 1968; 96 : 117–125.
11. S. Jonsson, E. Clausen, J. Raa, Amino acid degradation by a *Lactobacillus plantarum* strain from fish, *Syst. Appl. Microbiol.* 1983; 4:148–154.
12. V.L. Crow, T.D. Thomas, Arginine metabolism in lactic streptococci, *J. Bacteriol.* 1982; 150: 1024–1032.
13. G.G. Gonzalez, C.V. Byus, Effect of dietary arginine restriction upon ornithine and polyamine metabolism during two-stage epidermal carcinogenesis in the mouse, *Cancer Res.* 1991; 51 : 2932–2939.
14. B.J. Dillon, V.G. Prieto, S.A. Curley, C.M. Ensor, F.W. Holtsberg, J.S. Bomalaski, M.A. Clark, Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation, *Cancer* 2004; 100:826–833.
15. B.J. Dillon, F.W. Holtsberg, C.M. Ensor, J.S. Bomalaski, M.A. Clark, Biochemical Characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production, *Med. Sci. Monitor* 2002; BR248–BR253.
16. K. Miyazaki, H. Takaku, M. Umeda, T. Fujita, W.D. Huang, T. Kimura, J. Yamashita, T. Horio, Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a Mycoplasma infected cell line, *Cancer Res.* 1990; 50 : 4522–4527.
17. R. Philip, E. Campbell, D.N. Wheatley, Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures, *Br. J. Cancer* 2003; 88:613–623.
18. H. Terayama, T. Koji, M. Kontani, T. Okumoto, Arginase as an inhibitory principle in liver plasma membranes arresting the growth of various mammalian cells in vitro, *Biochim. Biophys. Acta* 1982; 720:188–192.
19. P.A. Ascierto, S. Scala, G. Castello, A. Daponte, E. Simeone, A. Ottaiano, G. Beneduce, V. De Rosa, F. Izzo, M.T. Melucci, C.M. Ensor, A.W. Prestayko, F.W. Holtsberg, J.S. Bomalaski, M.A. Clark, N. Savaraj, L.G. Feun, T.F. Logan, Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies, *J. Clin. Oncol.* 2005; 23: 7660–7668.
20. Rahamat Unissa, M. Sudhakar and A. Sunil Kumar Reddy. Screening of marine bacterial cultures for Extracellular production of L-arginine deiminases. *World Journal of Pharmaceutical Research*; 2015: Vol 4, Issue 06.
21. Rahamat Unissa, M. Sudhakar and A. Sunil Kumar Reddy. Condition Optimization and Production of Extracellular L-Arginine Deiminase from *Vibrio alginolyticus* 1374. *Current Biotechnology*; 2015: 4, 254-260.
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
23. Oginsky EL. Isolation and determination of arginine and citrulline. *Meth Enzymol*, 1957; 3: 639-643.
24. Lowry OH, Rosebrough NN, Farr AL, Randall RY. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265-275.
25. Weickmann JL, Fahrney E. Arginine deiminase from *Mycoplasma arthritis*. *J Biol Chem* 1977; 252:2615-2620.
26. Sugimura K, Fukuda S, Wada Y, et al. Identification and purification of arginine deiminase that originated from *Mycoplasma sargine*. *Infect Immun* 1990; 58:2510-2515.
27. Mahdy NZ, Al-Tahan SS, Yaseen NY. Optimization of Arginine deiminase production from a local higher productive isolate *Enterococcus faecium* M1. *Iraqi J Cancer Med Genet* 2014; 1: 7-1.
28. Shibatani T, Kakimoto T, Chibata I. Crystallization and properties of L-arginine deiminase of *Pseudomonas putida*. *J Biol Chem* 1975; 250:4580-4583.
29. Wang Y, Li YZ. Cultivation to improve in vivo solubility of over expressed arginine deiminases in *Escherichia coli* and the enzyme characteristics. *BMC Biotechnol* 2014; 14:53.
30. Silvio Hering et al. Kinetic characterization of arginine deiminase and carbamate kinase from *Streptococcus pyogenes* M49. *Protein Expression and Purification* 2013; 91 :61–68
31. T. Shibatani, T. Kakimoto, I. Chibata, Crystallization and properties of L-arginine deiminase of *Pseudomonas putida*, *J. Biol. Chem.* 1975; 250 : 4580–4583.
32. M.C. Manca de Nadra, A.A. Pesce de Ruiz Holgado, G. Oliver, Isolation and properties of arginine deiminase in *Lactobacillus buchneri* NCDO110, *J. Appl. Biochem.* 1984; 6 : 184–187.
33. T. Shibatani, T. Kakimoto and I. Chibata, *J. Bio. Chern.* 1975; 250 : 4580.
34. M.C. Manca de Nadra, A.A. Pesce de Ruiz Holgado, G. Oliver, Isolation and properties of arginine deiminase in *Lactobacillus buchneri* NCDO110, *J. Appl. Biochem.* 1984; 6 : 184–187.
35. Cheng-Fu T et al. The Research of Enzymology Characterization about Arginine Deiminase from *Enterococcus faecalis* 2008; *Microbiol*, 35, 846-50.

36. Mining Co, Takaku H. A novel arginine deiminase, its manufacturing method and an anti-cancer agent containing this enzyme as an effective ingredient. Ltd./O Japan Energy Corporation. Patent 0414007, 1995.
37. Kim J et al. Arginine deiminase originating from *Lactococcus lactis* ssp. *Lactis* American Type Culture Collection (ATCC) 7962 induces G1-phase cell-cycle arrest and apoptosis in SNU-1 stomach adenocarcinoma cells *Brit J Nutri* 2009; 102: 1469–76.
38. Segal I. *Biochemical Calculation*. John Wiley and Sons: Inc. New York, 1976.