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Research Article

Investigation on the production of an anticancer enzyme from *Vibrio alginolyticus* strain under solid state fermentation using various natural substrates

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ABSTRACT

Vibrio alginolyticus 1374; GU726873 is a gram negative rod shaped marine bacteria isolated from chirala beach of Andhra Pradesh. It was screened and confirmed for the enzyme production and used for the present study. Solid state fermentation was carried out for the production of L-arginine deiminase by *Vibrio alginolyticus* 1374; GU726873 using different natural substrates including wheat bran, rice bran, maize bran, ragi powder, black gram and red gram as solid substrates. L-arginine deiminase has received significant attention in recent years owing to its potential applications in medicine as an anticancer agent and as a biosensor. Maximum production was noticed with wheat as a solid substrate at pH 8, 37°C, with 2% inoculums, 2% L-arginine concentration, 2% maltose, 2% soya bean meal and 120 h of incubation period. Both physico-chemical and nutritional parameters played a significant role in the production of the enzyme.

Keywords: ADI- L-Arginine deiminase; *Vibrio alginolyticus* 1374; GU726873; MAM- minimal arginine media; SSF- solid state fermentation.

INTRODUCTION

L-Arginine deiminase (EC 3.5.3.6) is a therapeutic enzyme that catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia (Simon *et al.*, 1982). It is widely expressed in bacteria including *Mycoplasma arginini*, *Pseudomonas aeruginosa* and some species of *Enterococcus*. Interest on amidohydrolases started with the discovery of their anti-cancer properties and since then, a lot of efforts have gone into extensive studies on microbial L-arginine deiminase with the intention of developing them as anticancer agents.

Cancer is a global problem and in spite of sincere efforts paid in the past, search for efficient drugs to solve this problem is being continued worldwide. Interest in amidohydrolases such as L-arginine deiminase started with the discovery of their anticancer properties (Szlosarek PW *et al.*, 2006, Yoon CY *et al.*, 2007, Kim RH *et al.*, 2009, Noh EJ *et al.*, 2004, Kobayashi E 2010, *et al.*). Cancer cells are exact in their nutritional requirement. They require specific nutrients for their growth. Some of the cancer cells cannot synthesize these nutrients (amino acids) and depends upon their external

source for their supply, arginine is one among them. It is an efficient source of nitrogen, which is used for nucleic acid and protein synthesis by the cancer cells. ADI acts by depleting the levels of arginine thereby inhibiting both nucleic acid and protein synthesis (M. Ikeda *et al.*, 2009, T. Utagawa *et al.*, 2004)

Even though L- Arginine deiminase activity was reported in numerous micro-organisms, production of L- Arginine deiminase from microbial sources is very scanty. Hence an effort was made to isolate a novel potent marine producer and the production was carried out to get an optimal yield. A comparative study for the ADI production was done using different fermentation techniques.

While commercial production of various therapeutic enzymes is carried out using submerged fermentation (SmF) technique, using natural substrates or agricultural by products/residues has also gained much attention due to its various advantages over SmF. In recent years, solid state fermentation (SSF) has emerged as a promising technology for the development of several bioprocesses which include the production of industrial enzymes on a large scale.

In the present study we report the production of ADI from a novel *Vibrio alginolyticus* 1374; GU726873 strain by solid state fermentation technique using natural substrates. The effect of both physico-chemical and nutritional parameters was also seen.

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MATERIALS AND METHODS

Microorganism and inoculum

Soil and water samples collected from the chirala beach of Andhra Pradesh were serially diluted and inoculated on sterile enrichment medium. Upon incubation at 37°C for 24-48 h, total 35 colonies were formed, out of which 20 were positive. Screening of enzyme production was done by dye based method using minimal arginine medium. Further the ADI production was confirmed by estimating the citrulline levels calorimetrically. The potential isolate was selected and was identified as *Vibrio alginolyticus* 1374.

Preparation of solid substrate: Commercially available wheat bran of <0.24 cm particle size was used in the solid state fermentation (SSF) studies for the production of ADI. 5g of wheat bran taken in conical flask was thoroughly mixed with 5ml of MAM in the flask was autoclaved for 15 min and allowed to cool down to room temperature (28 °C).

Inoculation and incubation: The flasks were inoculated with 2ml of inoculum. The contents were mixed thoroughly and the flasks were incubated in a slanting position at 35°C in an incubator with 65-70% relative humidity for 24-28 hours.

Extraction and recovery of enzyme: After the incubation period, the crude enzyme from the fermented substrate was extracted using 0.1M phosphate buffer (pH 8). After mixing the fermented substrate with 40 ml of buffer, the flasks were kept on a rotary shaker at 150 rpm for 30 min. The slurry was centrifuged at 10,000 rpm for about 10 min at 4°C in a cooling centrifuge. Supernatant was collected and used for enzyme assay.

Assay of ADI: Citrulline concentration was used to measure enzyme activity calorimetrically using a modified version of the method described by Oginsky (Oginsky EL 1957). Reaction mixture consisted of 1 part of 80mM di acetyl monoxime (DAMO) and 2mM thiosemicarbazide (TSC) in DI water and 3 parts of 3M H₃PO₄, 6M H₂SO₄ and 2mM NH₄ Fe(SO₄)₂ 12H₂O with DI water was prepared. 200 µl of reaction mixture was added to 60µl of test sample mixture in the tubes. Test sample mixtures consisted 40µl extract and 20µl 10mM arginine solution. Test tubes were then sealed with scotch tape. For enzyme activity measurements, test sample mixture (60µl) in tube was incubated in a 37°C water bath for 30 or 60 minutes, after which the reaction was terminated. Colour development was measured using absorbance in a spectrophotometer at 530nm and the enzyme activity was estimated according to the standard curve of citrulline.

Optimization of various process parameters required for maximum ADI production by *Vibrio alginolyticus* 1374 was performed. The parameters studied for solid state included use of different substrates (wheat bran, rice bran, maize bran, ragi powder, black gram and red

gram) incubation period (24-120 h), initial pH (6-11) of the medium, temperature of incubation (20-45°C), inoculum size (1-5%), NaCl concentration, supplementary carbon sources and nitrogen sources on ADI production. The procedure adopted for optimization of various parameters influencing ADI production was to evaluate the effect of independent parameters keeping others constant and to incorporate it at the optimized level in the next experiment while optimizing another parameter.

Effect of different natural solid substrates on ADI production: A variety of substrates such as wheat bran, rice bran, maize bran, ragi powder, black gram and red gram were procured from the local market and used as the solid substrate to study their effect on the production of ADI.

Effect of incubation period on ADI production: Different incubation periods 24-168 h were employed to study their effect on enzyme production. The optimum incubation period obtained was fixed for the conduct of further experiments.

Effect of initial pH on ADI production: To investigate the influence of initial pH on enzyme production by the organism, the selected production medium was adjusted to various levels of pH (5.0-9.0). Fermentation was conducted and samples were assayed for ADI production. The optimum pH obtained in this study was used in the subsequent studies.

Effect of inoculum size on ADI production: Various inoculum levels of 1%, 2%, 3%, 4%, and 5% were tried to study their impact on enzyme production. The optimum inoculum level achieved by this step was used for subsequent experiments.

Effect of temperature on ADI production: Fermentation was carried out at temperatures of 20°C to 45°C to optimise the temperature for maximum enzyme production under SSF. The experiments followed were conducted at the optimal temperature.

Effect of amount of substrate on ADI production: The influence of the amount of substrate (l -arginine) on ADI production was studied by changing the concentration of substrate to flask volume. Different amounts of substrate were used for fermentation. The minimum quantity of substrate yielding maximum enzyme output was selected for further study.

The effect of sodium chloride: It was evaluated by incorporating various concentrations of 0.1-0.4% in the fermentation medium. The optimum NaCl concentration obtained was fixed for all the subsequent experiments.

Effect of supplementary carbon sources on ADI production

The requirement of additional nutrient supply was studied, by adding different supplementary carbon sources (1% w/w) like glucose, sucrose, maltose, lac

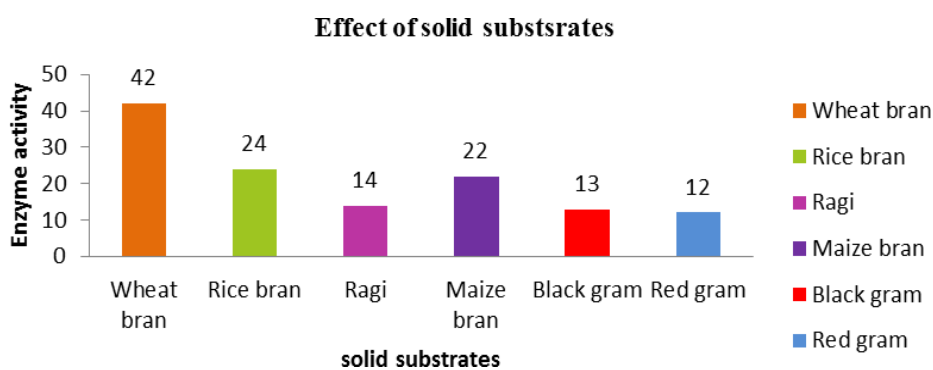


Figure 1: Effect of solid substrate

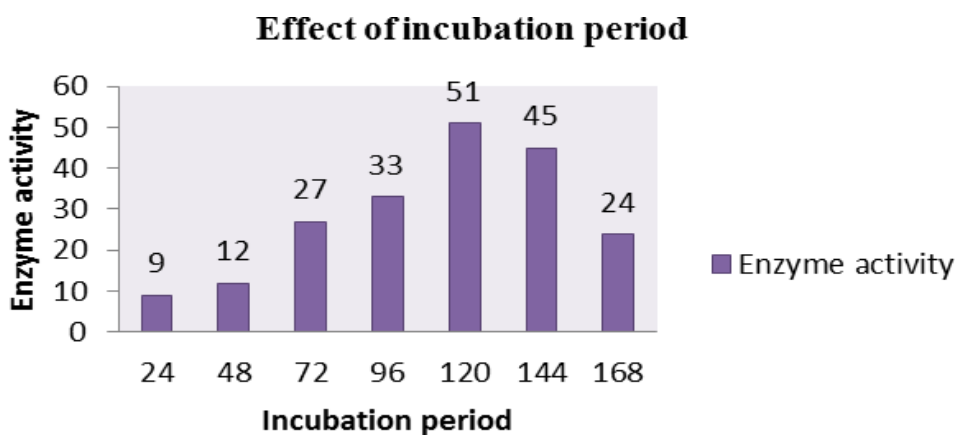


Figure 2: Effect of Incubation period

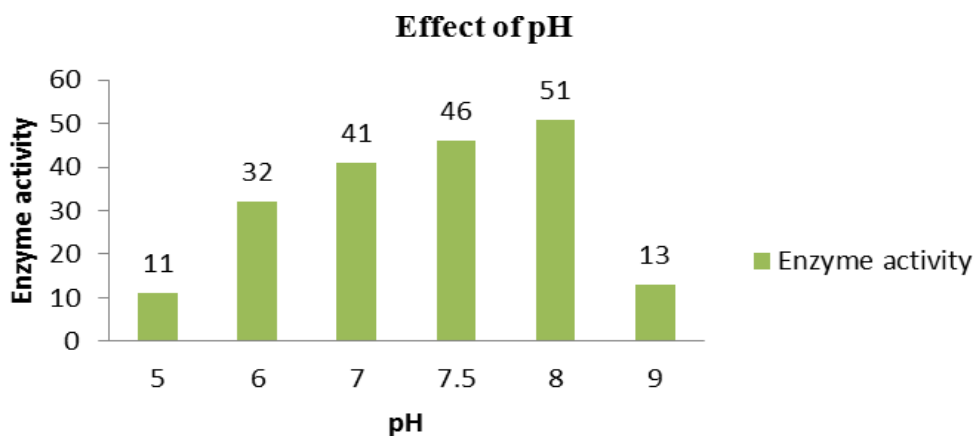


Figure 3: Effect of pH

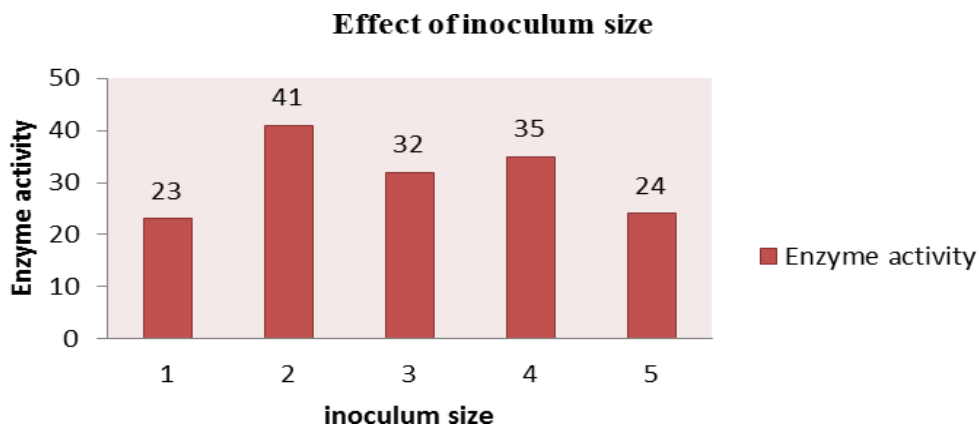


Figure 4: Effect of Inoculum

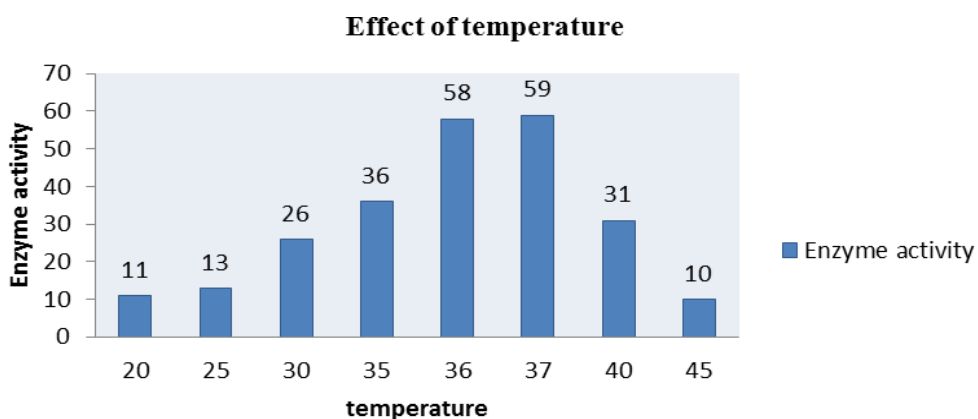


Figure 5: Effect of temperature

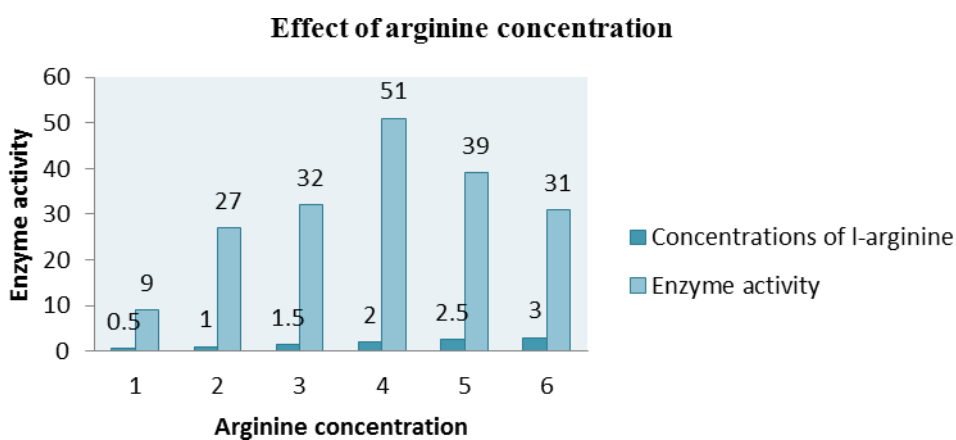


Figure 6: Effect of substrate concentration

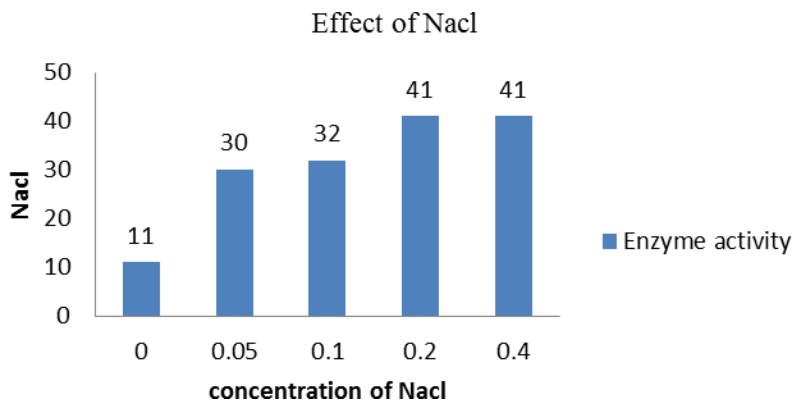


Figure 7: Effect of Nacl concentration

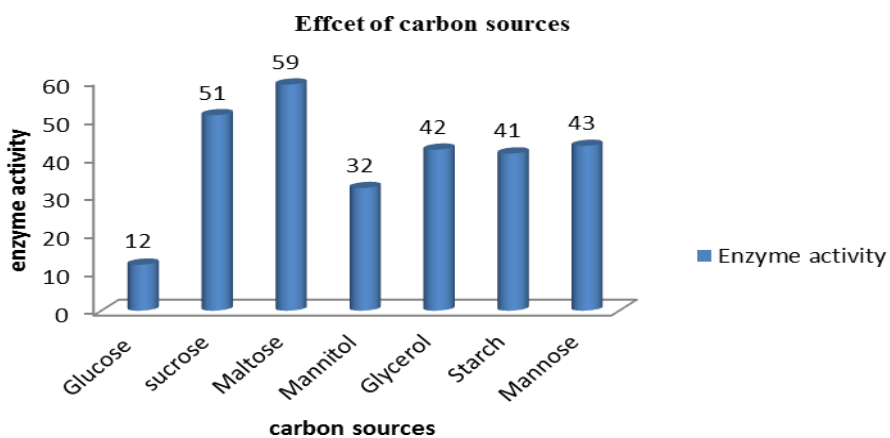


Figure 8: Effect of carbon sources

Effect of nitrogen sources

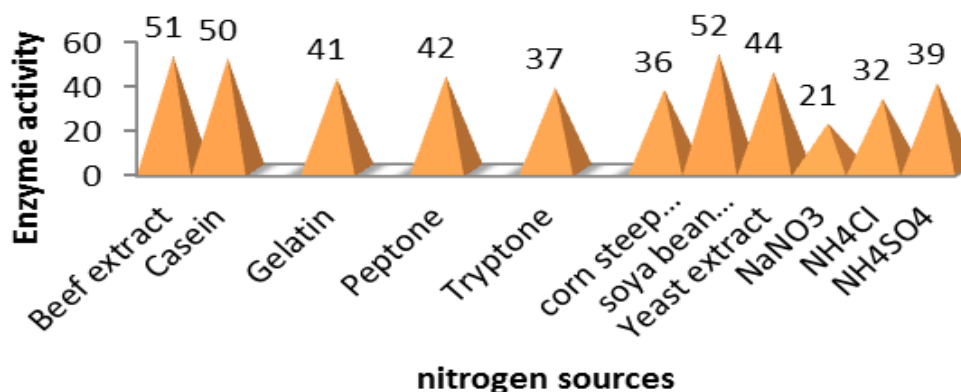


Figure 9: Effect of nitrogen sources

tose, glycerol, starch and mannose to the fermentation medium. The ideal carbon source thus obtained was included in the medium formulation to continue the optimisation studies of the remaining parameters.

Effect of supplementary nitrogen sources on ADI production: Beef extract, casein, gelatin, peptone, tryptone, starch, yeast extract, NaNO₃, NH₄Cl and NH₄SO₄.

RESULTS AND DISCUSSION

Out of 20 positive cultures, *Vibrio alginolyticus* 1374 was chosen for ADI production as the organism showed higher enzyme activity in the assay. Screening was done in mineral arginine media by dye based method. Mineral arginine media contains, L-arginine as the only energy source, hence only arginine metabolizing bacteria will grow. Since ADI converts L-arginine to L-citrulline and ammonia. Ammonia production was confirmed by the colour change of the dye. Further the enzyme production was confirmed by estimating the levels of citrulline calorimetrically.

Physicochemical parameters- solid substrates, incubation period, temperature, pH and nutritional parameters- carbon and nitrogen source are the critical factors studied. The physical factors have been the critical components of an industrial or commercial fermentation process, directly affecting the productivity and also the process economics. Optimization of physical parameters will not only support good growth but also enhance product yield. The six different natural solid substrates, wheat emerged as the best substrate for further studies (Figure-1).

There was a gradual increase in the enzyme production between 24 to 120 hours of incubation, above which there was a decrease. The optimal yield obtained at 120 hours of incubation.

There was a steady increase in the enzyme production as the initial pH of the medium was raised from 5 -8, thereafter yield were found to decrease. Maximum activity of 51 IU was observed at pH 8.0. Fig-2.

An inoculum size of 2% was found to be optimum for the production of ADI i.e. 41 IU

Temperature of incubation has shown a considerable impact on ADI production by *Vibrio alginolyticus* 1374. Increasing the incubation temperature from 20°C-37°C lead to an increase in ADI production temperatures higher than 37°C lowered the enzyme yield.

The highest ADI activity of 59 IU was found at 37°C.

Need of amino acids as inducer compound for enhanced enzyme production was evaluated by incorporating different concentrations of the amino acid in the production medium. From the results (Figure 6), it was evident that 2% concentration promoted maximal enzyme production. Further, in general concentration up to 2% were observed to support the enhanced enzyme production.

There was a gradual increase in the enzyme production with the increase in the NaCl concentration from 0 -0.2 further increase in the concentration did not enhanced the enzyme activity.

The process of economization for ADI production with carbon sources supplementation to the medium was carried out with the concentrations of 1.0% level. The different carbon sources studied were glucose, sucrose, maltose, lactose, glycerol, starch and mannose. Among all the carbon sources employed in the present study maltose significantly increased. Maltose (1%) yielded a maximum of 59 IU of ADI at 120 h of fermentation Fig-7.

There was a considerable increase in the enzyme production when the wheat was supplemented with soya bean.

The results revealed that the production of ADI varied according to the type of nutrient sources. Soya bean meal supported ADI production more than other nitrogen sources which reflects the efficiency of this nitrogen source (in the combination with arginine) to provide growth requirements and production of ADI, due to its composition as a protein, supplies essential ami-

no acids for construction of many important compounds in the cells included enzymes. Casein and beef extract followed soya bean meal in supporting ADI production.

CONCLUSION

The strain isolated from marine sediments i.e. *Vibrio alginolyticus* 1374; GU726873 is an efficient strain for the ADI production. The optimization of physical conditions like pH, incubation temperature, inoculum size and process optimization with different nutrient solid substrates, carbon and nitrogen sources has also been studied which will enormously contribute to higher enzyme yield when large scale production is desired. Maximum production was noticed with wheat as a solid substrate at pH 8, 37°C, with 2% inoculums, 2% L-arginine concentration, 2% maltose, 2% soya bean meal and 120 hours of incubation period. Though there are many reports of ADI existence in different microorganisms, bacteria are the most preferred ones. The possibility of obtaining non-allergic source of ADI from the source that we are reporting cannot be denied, moreover the strain seems to be non-pathogenic. Hence the strain can be used for commercial production of the enzyme.

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REFERENCES

- Kim RH, Coates JM, Bowles TL, et al. Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. *Cancer Res.*, 2009; 69(2): 700–708.
- Kobayashi E, Masuda M, Nakayama R, et al. Reduced argininosuccinate synthetase is a predictive biomarker for the development of pulmonary metastasis in patients with osteosarcoma. *Mol Cancer Ther.*, 2010; 9(3): 535–544.
- M. Ikeda, S. Mitsuhashi, K. Tanaka, and M. Hayashi, "Reengineering of a corynebacterium glutamicum L-arginine and L-citrulline producer," *Applied and Environmental Microbiology*, vol. 75, no. 6, pp. 1635–1641, 2009.
- Noh EJ, Kang SW, Shin YJ, et al. Arginine deiminase enhances dexamethasone-induced cytotoxicity in human T-lymphoblastic leukemia CCRF-CEM cells. *Int J Cancer.*, 2004; 112(3): 502–508.
- Oginsky EL. Isolation and determination of arginine and citrulline. *Meth Enzymol*, 1957; 3: 639-643.
- Simon, J. P., B. Wargnies, and V. Stalton. 1982. Control of enzyme synthesis in the arginine deiminase pathway of *Streptococcus faecalis*. *J. Bacteriol.* 150: 1085-1090.

Szlosarek PW, Klabatsa A, Pallaska A, et al. In vivo loss of expression of argininosuccinate synthetase in malignant pleural mesothelioma is a biomarker for susceptibility to arginine depletion. *Clin Cancer Res.*, 2006; 12(23): 7126–7131.

T. Utagawa, "Production of arginine by fermentation," *Journal of Nutrition*, vol. 134, no. 10, pp. 28545–28575, 2004.

Yoon CY, Shim YJ, Kim EH, et al. Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. *Int J Cancer.*, 2007; 120(4): 897–905.