

Selective Isolation and Identification of Arginine Degrading Bacteria; the Optimized Arginine Deaminase Production by *Enterobacter sp. sgn1* as a New Source of This Potentially Anti-Tumor Enzyme

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ABSTRACT

Since certain tumor cells are auxotrophic for arginine, depletion of the extracellular arginine by means of arginine degrading enzymes might be exploited to target such tumors. Among the arginine degrading enzymes, arginine deaminase has attracted more attention as a potential anti-cancer agent for arginine-auxotrophic tumors. In order to find some new sources for arginine degrading enzymes, this study was designed to screen bacterial species, applying a simple and convenient procedure, using a selective medium and Nessler's reagent test. The characterization of the isolated samples was carried out using 16S ribosomal DNA gene sequence analysis, which were further identified as *Enterobacter sp.*, *Bacillus sp.*, *Erwinia sp.*, *Oceanomonas sp.*, *Acinetobacter sp.*, *Cronobacter sp.* *Enterobacter sp. sgn1* was further studied as the arginine deiminase producing bacteria, by investigating the effect of some factors on enzyme production (using Minitab software and measuring the amount of citrulline as the product of ADI enzyme). According to our results; the arginine deiminase production and activity of *Enterobacter sp. sgn1* were affected by: temperature, the concentration of glucose and Mg⁺ ion. Moreover, the phylogenetic tree was constructed using Mega 6 software and it was observed that among the isolated species, *Bacillus* species are very close to *Mycoplasma arginini*.

INTRODUCTION

Some tumors require the extra cellular sources of some amino acids, which are considered as non-essential in normal cells, due to metabolic deficiencies (Stone *et al.*, 2010; Kuo *et al.*, 2010). Thus, enzymatic degradation of these amino acids can be an effective strategy in the suppression of such tumors (Shen *et al.*, 2006). Asparaginase is the example of amino acid depriving enzymes, which has been applied in the treatment of

acute lymphoblastic leukemia for many years (Piatkowska-Jakubas *et al.*, 2008). A similar approach might be exploited for arginine auxotrophic tumors (Feun *et al.*, 2008). Arginine degrading enzymes consist of three main types of enzymes: arginine deiminase, arginase and arginine decarboxylase which exist in archaea, bacteria, and eukaria (Zúñiga *et al.*, 2002; Knodler *et al.*, 1998). To discover new sources for arginine degrading enzymes, one possible way is screening, isolation and characterization of the microorganisms. Among the arginine degrading enzymes, arginine deiminase (ADI) has been considered as an anti-tumor enzyme to treat hepatocellular carcinomas (HCCs) (Izzo *et al.*, 2004, Ensor *et al.*, 2002) and melanomas (Kuo *et al.*, 2010, Feun and Savaraj, 2006) which are auxotrophic for arginine.

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The catabolism of arginine to citrulline and ammonia is catalyzed by this enzyme (Ni *et al.*, 2011) in the arginine deiminase pathway, which is utilized by a number of microorganisms such as *Pseudomonas*, *Mycoplasma* (Ni *et al.*, 2009), *Halobacterium* (Monstadt and Holldorf, 1991), *Lactobacillus* (Arena *et al.*, 1999), *Lactococcus* (Kim *et al.*, 2007), and *Streptococcus* (Niven Jr *et al.*, 1942) species) as an energy source (Ni *et al.*, 2008, Mercenier *et al.*, 1980). The aim of the present study was to isolate bacterial species, which are able to produce arginine degrading enzymes. For this purpose, a number of microorganisms were screened from various samples to obtain the species which can produce arginine degrading enzymes; using a convenient and sensitive screening procedure on phenol red indicator plates.

Furthermore, the enzyme activity was measured by Nessler's reagent, and the isolated species were characterized using 16S rRNA gene sequence analysis. Among the isolated species, *Enterobacter sp. SgnI*, which has not been previously studied as a source of ADI, was selected for further research. Since the optimization of the enzyme production can result in achieving higher amounts of enzyme, the effect of temperature, pH, the concentration of glucose and Mg^{+} ion on the enzyme production was evaluated.

Minitab software was applied to design the experiments and citrulline was measured as the product of ADI activity. The ADI sequences of the isolated species were compared to the sequence of *Mycoplasma arginini* (the source of ADI enzyme, which is under investigation in clinical trials) to find the species with the similar sequences.

MATERIALS AND METHODS

Materials and microorganisms

L-Citrulline, diacetylmonoxime and thiosemicarbasiade were obtained from the Sigma Aldrich Company. Glucose, L-arginine, NaCl, KH_2PO_4 , $MgCl_2$, $MnCl_2$, $FeSO_4$, $CaCO_3$, agar, sulfuric acid (95-98%), phosphoric acid (85%) and $FeCl_3$ were purchased from the Merck Company. Phenol red was obtained from the Lobachemie Company, and the bacterial strains were isolated from various regions of Fars and Boushehr province.

Screening of the arginine degrading bacteria using the plate method assay

The test samples were collected from various regions of Fars and Boushehr province. Adding the sterile water to the soil samples, 1 mL of the diluted sediment samples was transferred to the selective screening medium (Ghasemi *et al.*, 2008) and incubated for 24 hours at 37°C.

The screening medium (pH=7) consisted of: 0.01% glucose, 1.5% L-arginine, 0.002% NaCl, 0.075% KH_2PO_4 , 0.05% $MgCl_2$, 0.01% $MnCl_2$, 0.0005% $FeSO_4$, 0.1 M $CaCO_3$, 1.7% agar and 0.005% phenol red, as the pH indicator. Colonies with the purple zone were considered as the arginine degrading species and

were subsequently sub cultured for several times to get the pure colonies (colonies A-K).

Activity determination of the arginine degrading enzymes

The obtained pure colonies were subjected to further activity analysis of arginine degrading enzymes using Nessler's reagent to measure the ammonia concentration (Singh *et al.*, 2013) as the product of arginine degrading enzymes. Arginine was used as the sole nitrogen source in the culture media. Cultured in 20 mL of the defined medium, the bacteria were incubated for 24 hours at 37°C, after which 2 mL of each growth medium was collected and centrifuged to assess the concentration of ammonia in the supernatant. Then, 50 μ L of Nessler's reagent was added to 1 mL of the obtained supernatants, the concentration of the produced ammonia was quantified by measuring the absorbance at 480 nm (UV/Vis spectrometer, PG Instruments) (according to the standard curve of ammonium sulfate). The calculated values were correlated to the activity of arginine degrading enzymes within 24 hours of incubation (number of experiments=3).

Identification of the isolated bacteria by sequence analysis of 16S rRNA gene

Along with the morphological and biochemical characterization, molecular identification of the arginine degrading bacteria was carried out by 16S rRNA sequence analysis, using the forward and reverse primers with the sequences of (5'-CAGCCGCGGTAATAC-3') and (5'-ACGGGCGGTGTGTAC-3'), respectively. The 16S rRNA gene was amplified using PCR amplification, under the following conditions: initial denaturation at 99°C for 5 min; followed by 30 cycles of denaturation (94°C for 30 s), annealing (53-56°C for 30 s) and extension (72°C for 2:30 s); the final extension was initiated at 72°C for 10 min. The size of the resulted amplicon was confirmed by agarose gel electrophoresis and following gel purification (QIAquick gel extraction kit, Qiagen USA); furthermore, they were subjected to further sequence analysis (CinnaGen Co., Iran). The obtained sequences were compared with sequences deposited in GenBank database of National Center for Biotechnology Information (NCBI) by Basic Local Alignment Search Tool (BLAST).

Optimization of the nutritional conditions for ADI enzyme production

Among the isolated species, *Enterobacter sp. sgnI*, which was not previously studied for the ADI production, was selected for further studies. To optimize the ADI enzyme production, some culture parameters were evaluated via response surface methodology (RSM) (Kazemi *et al.*, 2014) and Box-Behnken design (using Minitab software, version 16). These parameters were selected based on the reported parameters about other microorganisms: glucose concentration (Arena *et al.*, 1999, Simon *et al.*, 1982), Mg^{+} concentration (Baur *et al.*, 1989, Park *et al.*, 1984), temperature (Kakimoto *et al.*, 1971, Liu *et al.*, 2008) and pH (Kakimoto *et al.*, 1971, Park *et al.*, 1984).

Table 1: The Box-Behnken design for the four independent variables and the observed results for the designed experiments.

Trial number	Temperature (X ₁)	pH (X ₂)	Glucose (%) (X ₃)	Mg ²⁺ (M) (X ₄)	Observed amount of citrulline (µg/mL)	Predicted amount of citrulline by RSM (µg/mL)
1	44 (1)	7 (0)	4 (1)	25 (0)	0	2
2	30 (-1)	7 (0)	0.1 (-1)	25 (0)	5	3
3	30 (-1)	7 (0)	2.05 (0)	50 (1)	8	4
4	37 (0)	7 (0)	2.05 (0)	25 (0)	18	16
5	37 (0)	9 (1)	4 (1)	25 (0)	11	14
6	37 (0)	7 (0)	4 (1)	0 (-1)	30	24
7	30 (-1)	7 (0)	4 (1)	25 (0)	9	11
8	44 (1)	7 (0)	2.05 (0)	50 (1)	0	0
9	37 (0)	5 (-1)	2.05 (0)	0 (-1)	24	24
10	37 (0)	5 (-1)	0.1 (-1)	25 (0)	10	8
11	44 (1)	7 (0)	2.05 (0)	0 (-1)	0	5
12	44 (1)	5 (-1)	2.05 (0)	25 (0)	0	1
13	37 (0)	7 (0)	4 (1)	50 (1)	16	17
14	37 (0)	5 (-1)	2.05 (0)	50 (1)	10	12
15	30 (-1)	7 (0)	2.05 (0)	0 (-1)	5	10
16	37 (0)	7 (0)	0.1 (-1)	0 (-1)	21	19
17	44 (1)	9 (1)	2.05 (0)	25 (0)	0	0
18	30 (-1)	5 (-1)	2.05 (0)	25 (0)	5	6
19	30 (-1)	9 (1)	2.05 (0)	25 (0)	9	8
20	37 (0)	5 (-1)	4 (1)	25 (0)	30	28
21	37 (0)	9 (1)	0.1 (-1)	25 (0)	20	22
22	37 (0)	9 (1)	2.05 (0)	50 (1)	17	17
23	44 (1)	7 (0)	0.1 (-1)	25 (0)	0	0
24	37 (0)	7 (0)	2.05 (0)	25 (0)	15	16
25	37 (0)	7 (0)	2.05 (0)	25 (0)	14	16
26	37 (0)	9 (1)	2.05 (0)	0 (-1)	22	20
27	37 (0)	7 (0)	0.1 (-1)	50 (1)	6	11

Conducting 27 experiments, four variables were screened (Table 1) in which the factors of the highest assurance levels were defined into separate levels: temperature (X₁), pH (X₂), glucose concentration (X₃), MgCl₂ concentration (X₄); testing at three levels: a high level denoted by (+1), medium level denoted by (0), a low level denoted by (-1) (listed in Table 1). Other culture conditions and medium constituents were in consistent with the first culture medium, applied in the activity assay (L-arginine 1.5%, NaCl 0.002%, KH₂PO₄ 0.075%, MnCl₂ 0.01%, FeSO₄ 0.0005%, CaCO₃ 0.1 M).

The equation of the effectors is expressed as follows:

$$\beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 = y$$

In this equation, "y" is the predicted response, " β_0 " is the model constant; " x_1 ", " x_2 ", " x_3 " and " x_4 " are the independent variables; " β_1 ", " β_2 ", " β_3 " and " β_4 " are the linear coefficients; " β_{12} ", " β_{13} ", " β_{14} ", " β_{23} ", " β_{24} " and " β_{34} " are the cross-product coefficients; and " β_{11} ", " β_{22} ", " β_{33} " and " β_{44} " are the quadratic coefficients.

ADI enzyme activity assay

The ADI enzyme activity was analyzed based on the photometric measurement of the citrulline concentration (which is formed following the L-arginine hydrolysis by ADI enzyme), according to the modified diacetylmonoxime thiosemicarbazide (DMT) method (Ni *et al.*, 2011, Boyde and Rahmatullah, 1980). The applied reagents were prepared as follows: 1) acid-ferric solution: 250 mL of the concentrated sulfuric acid (95-98%) was added to 550 mL of distilled water, following by the addition of

200 mL of concentrated phosphoric acid (85%). Cooling to the room temperature, FeCl₃ (2.50 mg/L) was dissolved in the aforementioned solution. 2) diacetylmonoxime solution: 500 mg of diacetylmonoxime (DAMO, 2, 3 butanedione) was added to 100 mL of distilled water. 3) chromogenic-reagent: 5 mg of thiosemicarbazide was added to 50 mL of the "reagent 2", following by the addition of the "reagent 1" (100 mL). The solution was used in 1 hour of the preparation.

The selected colony (*Enterobacter sp.sgn1*) was cultured in 20 mL of the designed culture media. Incubating for 24 hours, 2 mL of each growth medium was collected and centrifuged to measure the amount of liberated citrulline in the supernatants. For this purpose, 3 mL of the chromogenic solution (reagent 3) was added to 1 mL of the supernatant and boiled for 5 min at 100°C banmurry. Cooling to the room temperature, the absorbance was measured at 530 nm. The amount of the citrulline was calculated based on the standard curve of the L-citrulline (measured by the same method) and was correlated to the activity of ADI enzyme (which converts L-arginine to L-citrulline).

Sequence and phylogenetic analyses

Twenty-four of the arginine deiminase related sequences were retrieved in FASTA format from NCBI database. The sequences were selected based on the isolated species in this research and also those species which were studied by other researchers (Table 2). The selected sequences were aligned using T-Coffee (<http://www.ebi.ac.uk/Tools/msa/tcoffee/>) to compare the amino acid sequences among different species (Nezafat *et al.*, 2015). Mega 6 software and Neighborhood Joining method were applied to construct the phylogenetic trees.

Table 2: The arginine deiminase related sequences retrieved in FASTA format from NCBI database for sequence and phylogenetic analyses.

Bacterial species	Accession number	Protein length
<i>Bacillus cereus</i> AH1273	228740297	410
<i>Bacillus cytotoxicus</i> NVH 391-98	152022936	410
<i>Bacillus licheniformis</i>	765364190	411
<i>Bacillus thuringiensis</i> IBL 4222	228860522	410
<i>Citrobacter amalonaticus</i>	923246961	406
<i>Citrobacter freundii</i> GTC 09629	486077046	406
<i>Citrobacter youngae</i> ATCC 29220	291069141	407
<i>Cronobacter sakazakii</i> SP291	449099762	406
<i>Enterobacter asburiae</i> LF7a	345091594	406
<i>Enterobacter cloacae</i> str. Hanford	523458876	406
<i>Enterococcus faecium</i> TX0082	313646064	409
<i>Enterobacter lignolyticus</i> SCF1	308750386	406
<i>Enterococcus faecalis</i> EnGen0310 = MMH594	802262237	408
<i>Erwinia mallochorhiza</i>	594397452	406
<i>Escherichia coli</i> ATCC 25922	674303266	406
<i>Halobacterium salinarum</i>	499206621	486
<i>Lactobacillus fermentum</i>	215271739	407
<i>Lactobacillus sakei</i>	2764611	409
<i>Mycoplasma arginini</i>	761598075	410
<i>Mycoplasma arthritis</i>	5901880	409
<i>Mycoplasma penetrans</i>	768722109	410
<i>Pseudomonas aeruginosa</i>	674743455	418
<i>Pseudomonas plecoglossicida</i>	154183755	417
<i>Pseudomonas putida</i> W619	169761387	417

Statistical analysis

All experiments were conducted in triplicates, presented by means and standard deviation. Statistical analyses were performed by means of one-way analysis of variance (ANOVA), (p -value < 0.05) and also based on the Minitab software.

RESULTS AND DISCUSSION

Isolation of the arginine degrading bacteria

In order to screen the arginine degrading bacteria, a selective culture medium was designed with arginine as the sole nitrogen source; in which only the arginine degrading bacteria were able to utilize the nitrogen source and grow consequently. Moreover, as ammonium is liberated in the arginine degrading pathways, the primary screening was carried out using phenol red as the pH indicator (Liu *et al.*, 2008) and colonies with the purple zone around were selected as arginine degrading bacteria.

Considering the arginine rich sources and the suitable conditions for the growth of arginine degrading bacteria, different environmental samples were collected. Some of these bacteria have been mentioned as halophilic, according to other studies (Monstadt and Holldorf, 1991); in this regard, a sample was collected from the beach of Oman Sea (sample F). Since some of the arginine degrading bacteria can tolerate the acidic conditions (Casiano-Colón and Marquis, 1988), some of the samples were collected from acidic environments such as vinegar (sample K).

Through the aforementioned primary screening, 11 strains (A-K) were isolated from different areas of Fars and Boushehr province in Iran, which all had the purple zone around and were selected for further analysis.

Activity determination of arginine degrading enzymes

These species were further analyzed by Nessler's reagent, in order to quantitatively determine the activity of arginine degrading enzymes, according to the amount of the liberated ammonia. This method has been also applied for the determination of the ammonia concentration as a product of enzymatic degradation by asparaginase (Ghoshoon *et al.*, 2015) and cytosine deaminase (Gholami *et al.*, 2015).

The concentration of the liberated ammonia by test samples is presented in figure 1. One way ANOVA analysis revealed a significant difference between 11 bacterial species (p -value=0.046), considering the amount of the produced ammonia. Conducting Dunnett's test, as the post hoc test, the amounts of the produced ammonia by different samples were compared to the sample "C".

It was observed that the amount of the produced ammonia by the sample "I" is significantly higher than sample "C" (p -value= 0.032). Among the isolated species, sample "I", which was further identified as *Bacillus sp. sgn10*, produced the highest amount of ammonia (0.077 mg/mL) through the degradation of arginine; which can be assigned to the highest amount or activity of arginine degrading enzymes (Fig.1).

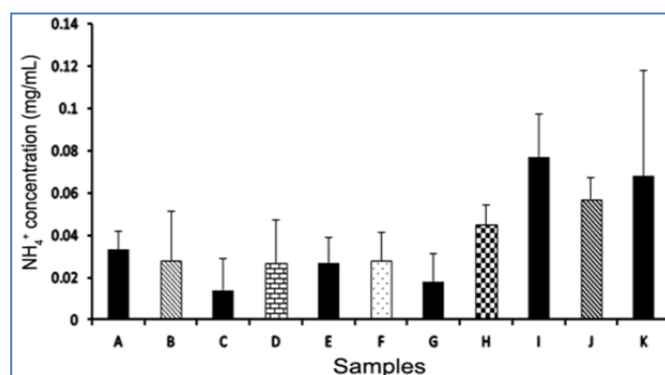


Fig. 1: NH₄⁺ concentration (mg/mL) produced by the isolated bacteria: A. *Enterobacter sp. sgn1*, B. *Cronobacter sp. sgn2*, C. *Cronobacter sp. sgn3*, D. *Erwinia sp. sgn4*, E. *Acinetobacter sp. sgn6*, F. *Oceanimonas sp. sgn7*, G. *Enterobacter sp. sgn8*, H. *Erwinia sp. sgn9*, I. *Bacillus sp. sgn10*, J. *Bacillus sp. sgn11*, K. *Erwinia sp. sgn12*. Data are expressed as mean ± SD of three independent samples. The statistically significant differences in ammonia concentration, compared to the sample "C", are represented by asterisks (*).

Identification of the isolated bacteria by sequence analysis of 16S rRNA gene

Comparing the sequences of 16S rRNA genes with complete GenBank database (using BLASTN), the isolated samples, represented 99-100% homology with *Enterobacter sp.* (samples A and G), *Cronobacter sp.* (samples B and C), *Erwinia sp.* (samples D, H and K), *Acinetobacter sp.* (sample E), *Oceanimonas sp.* (sample F) and *Bacillus sp.* (samples I and J).

The isolated species were supposed to produce arginine degrading enzymes such as arginine deiminase, arginase and arginine decarboxylase. The sequences of 16S rRNA gene were deposited in GenBank under the specific accession numbers (Table 3).

Table 3: Accession numbers of the 11 environmentally isolated bacteria published in NCBI.

Samples	Accession numbers	Bacteria
A	JX456240.1	<i>Enterobacter sp. sgn1</i>
B	JX456241.1	<i>Cronobacter sp. sgn2</i>
C	JX456242.1	<i>Cronobacter sp. sgn3</i>
D	JX456243.1	<i>Erwinia sp. sgn4</i>
E	JX456244.1	<i>Acinetobacter sp. sgn6</i>
F	JX456245.1	<i>Oceanimonas sp. sgn7</i>
G	JX456246.1	<i>Enterobacter sp. sgn8</i>
H	JX456247.1	<i>Erwinia sp. sgn9</i>
I	JX456248.1	<i>Bacillus sp. sgn10</i>
J	JX456249.1	<i>Bacillus sp. sgn11</i>
K	JX456250.1	<i>Erwinia sp. sgn12</i>

For further study, ADI enzyme was considered (among the arginine degrading enzymes), regarding its advantages over other arginine degrading enzymes like more affinity for arginine compared to arginase, and fewer side effects compared to arginine decarboxylase (Savaraj *et al.*, 2010).

Therefore, among the isolated species, *Enterobacter sp. sgn1* (sample A) was selected for further study, as to the best of our knowledge; this species was not studied as a source of ADI, previously.

Optimization of ADI enzyme production and activity assay

Using RSM (Box-Behnken design), the effect of four factors (temperature, pH, glucose and $MgCl_2$ concentration) on ADI enzyme activity produced by *Enterobacter sp. sgn1* was determined. Other researches have reported the effect of various factors, which affected the ADI enzyme in different microorganisms such as: *Pseudomonas plecoglossicida* (Liu *et al.*, 2008), *Euglena gracilis* (Park *et al.*, 1984), *Halobacterium salinarum* (Monstadt and Holldorf, 1991), *Pseudomonas putida*

(Kakimoto *et al.*, 1971), *Lactobacillus plantarum* (Arena *et al.*, 1999), *Streptococcus faecalis* (Simon *et al.*, 1982), *Pseudomonas aeruginosa* (Baur *et al.*, 1989). Using this method, we could examine the influence of 4 factors at a time with 27 designed experiments. Such a designing also helps us to determine the function of the designed factors' interaction on enzyme production and activity.

The derived regression equation for the optimization of the culture conditions indicated that the ADI activity, which is correlated to the amount of produced citrulline (y), is affected by temperature (x_1), pH (x_2), the concentration of glucose (x_3) and Mg^{+} ion (x_4). The following second order multinomial equation can display the relation between 4 factors, which were analyzed in this study.

$$0.0157 - 0.0034x_1 - 0.0000x_2 + 0.0028x_3 - 0.0037x_4 - 0.0010x_1x_2 - 0.0010x_1x_3 - 0.0007x_1x_4 - 0.0072x_2x_3 + 0.0022x_2x_4 + 0.0002x_3x_4 - 0.0135x_1^2 + 0.0012x_2^2 + 0.0012x_3^2 + 0.0013x_4^2 = y$$

The effects of the variables are shown in table 1 in which the observed responses are compared with the predicted responses which were obtained from Box-Behnken designed experiments. Table 4 represents the interaction between the test variables, *p* values and confidence level (%). According to the calculated *p* values, it is observed that out of the 4 studied variables, three variables (temperature, glucose and Mg^{+} concentration) had a significant effect on the ADI enzyme production and activity (*p* value < 0.05, significant at 5% level). Although the pH value did not significantly affect the ADI enzyme production and activity, the observed values revealed that its interaction with glucose concentration could have significant effect (Fig. 2).

Table 4: The results of Regression Analysis of Box–Behnken Design for ADI Production by *Enterobacter sp. sgn1*.

Variable	Coefficient	SE Coefficient	T statistic	P value	Confidence level (%)
Constant	0.015667	0.002370	6.610	0.000	100
Temperature	-0.003417	0.001185	-2.883	0.014	98.6
pH	-0.000000	0.001185	-0.000	1.000	0
Glucose	0.002833	0.001185	2.391	0.034	96.6
Mg^{+}	-0.003750	0.001185	-3.164	0.008	99.2
Temperature * Temperature	-0.013458	0.001778	-7.571	0.000	100
pH * pH	0.001167	0.001778	0.656	0.524	47.6
Glucose * Glucose	0.001167	0.001778	0.656	0.524	47.6
Mg^{+} * Mg^{+}	0.001292	0.001778	0.727	0.481	51.9
Temperature * pH	-0.001000	0.002053	-0.487	0.635	36.5
Temperature * Glucose	-0.001000	0.002053	-0.487	0.635	36.5
Temperature * Mg^{+}	-0.000750	0.002053	-0.365	0.721	27.9
pH * Glucose	-0.007250	0.002053	-3.532	0.004	99.6
pH * Mg^{+}	0.002250	0.002053	1.096	0.295	70.5
Glucose * Mg^{+}	0.000250	0.002053	0.122	0.905	9.5

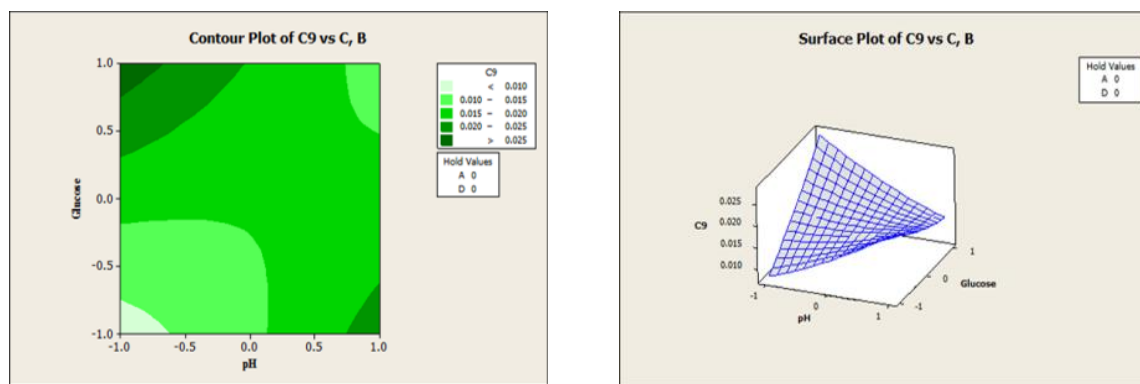


Fig. 2: Counter and surface plots for ADI activity considering glucose and pH level

The amount of the produced citrulline, which is correlated to the ADI enzyme production and activity, varied from 0-30 $\mu\text{g/mL}$ in the lowest and the highest amount in the designed experiments. The highest ADI enzyme production and activity was observed in the designed conditions of experiments 6 and 20. So we can conclude that the best culture condition for ADI production obtains at 37°C with the glucose concentration of 4%, pH of 5-7 and Mg^+ concentration of 0-25 M, while other factors such as L-arginine 1.5%, NaCl 0.002%, KH_2PO_4 0.075%, MnCl_2 0.01%, FeSO_4 0.0005%, CaCO_3 0.1 M are constant in all designed experiments. R^2 value (the coefficient of multiple determinations) which is always between 0 and 1, represents the adequacy of the designed model: the closer the R^2 value to 1, the stronger the predicted response. In this study, the R^2 value was 0.9082 which suggested that the model can explain up to 90.82% of data variation. The observed correlation between the predicted and the experimentally obtained results also proves that the designed model has been appropriate to analyze the obtained responses.

Verification of the model

In order to verify the model which was designed for the optimization of ADI production, an experiment was designed for the analyzed parameters (using Minitab software and according to our results); temperature of about 33°C , glucose concentration of 4%, pH=5 and Mg^+ concentration of 0 M. The amount of the produced citrulline was predicted to be 35 $\mu\text{g/mL}$ in the designed condition which experimentally determined to be 31 $\mu\text{g/mL}$. This result revealed 88.6% accuracy and is an evidence on the validity of the designed model under the investigated circumstances, which indicates the reliability of the model for the maximal ADI production and activity by *Enterobacter sp. sgn1*.

Sequence and phylogenetic analyses of arginine deiminase sequences

Currently, the pegylated form of arginine deaminase (from *Mycoplasma arginini*) is investigating in clinical trials for the treatment of arginine auxotrophic tumors (Ni *et al.*, 2008). In this study, the ADI sequences of the isolated species were

compared to the sequence of *Mycoplasma arginini* to find other ADI producers with similar sequences. Conserved regions were observed in the multiple sequence alignment in 1, 13, 17, 20, 25, 40, 55, 161, 164, 166, 167, 175, 181, 186, 189, 225, 226, 227, 228, 240, 279, 280, 281, 282, 283, 291, 366, 372, 403, 406, 408, 412, 413, 416 and 419 amino acid residues (Fig. 3). Based on the phylogenetic study, three major clusters were observed in the constructed phylogenetic tree (Fig. 4). Among the isolated species in this study, *Bacillus* species (belong to the second cluster) are very close to *Mycoplasma* species (sharing 94% of homology). These species are also close to *Lactobacillus* species and *Enterococcus* species. According to their similarity with *Mycoplasma* species, these species might be considered as a source of ADI, conducting more studies. *Erwinia sp.*, *Cronobacter sp.* and *Enterobacter sp.*, which belong to the first cluster, are very close to each other and share 99% of homology with *Pseudomonas sp.* It was observed that *Halobacterium salinarum* was categorized as a distinct cluster. This species is a member of Archaeobacterial Kingdom and contains the ADI gene which is completely apart from other species.

CONCLUSION

In order to find novel ADI sources, in the present study, a convenient method was established, using a selective medium, to isolate arginine degrading bacteria from local samples. They were further identified as *Enterobacter sp.*, *Cronobacter sp.*, *Erwinia sp.*, *Acinetobacter sp.*, *Oceanimonas sp.* and *Bacillus sp.* Moreover, four factors which could affect the ADI production and activity, produced by *Enterobacter sp. sgn1* as a source of this enzyme, were studied for the first time (using RSM). It was observed that temperature, glucose concentration, Mg^+ concentration and the interaction of pH and glucose concentration had a significant effect on the production and activity of ADI enzyme. Comparing the ADI sequences of the isolated species with *Mycoplasma arginini*, it was observed that *Bacillus* species are very close to *Mycoplasma* species. Conducting more studies about the isolated species, might result in the possible application of them as potential sources for ADI enzyme.

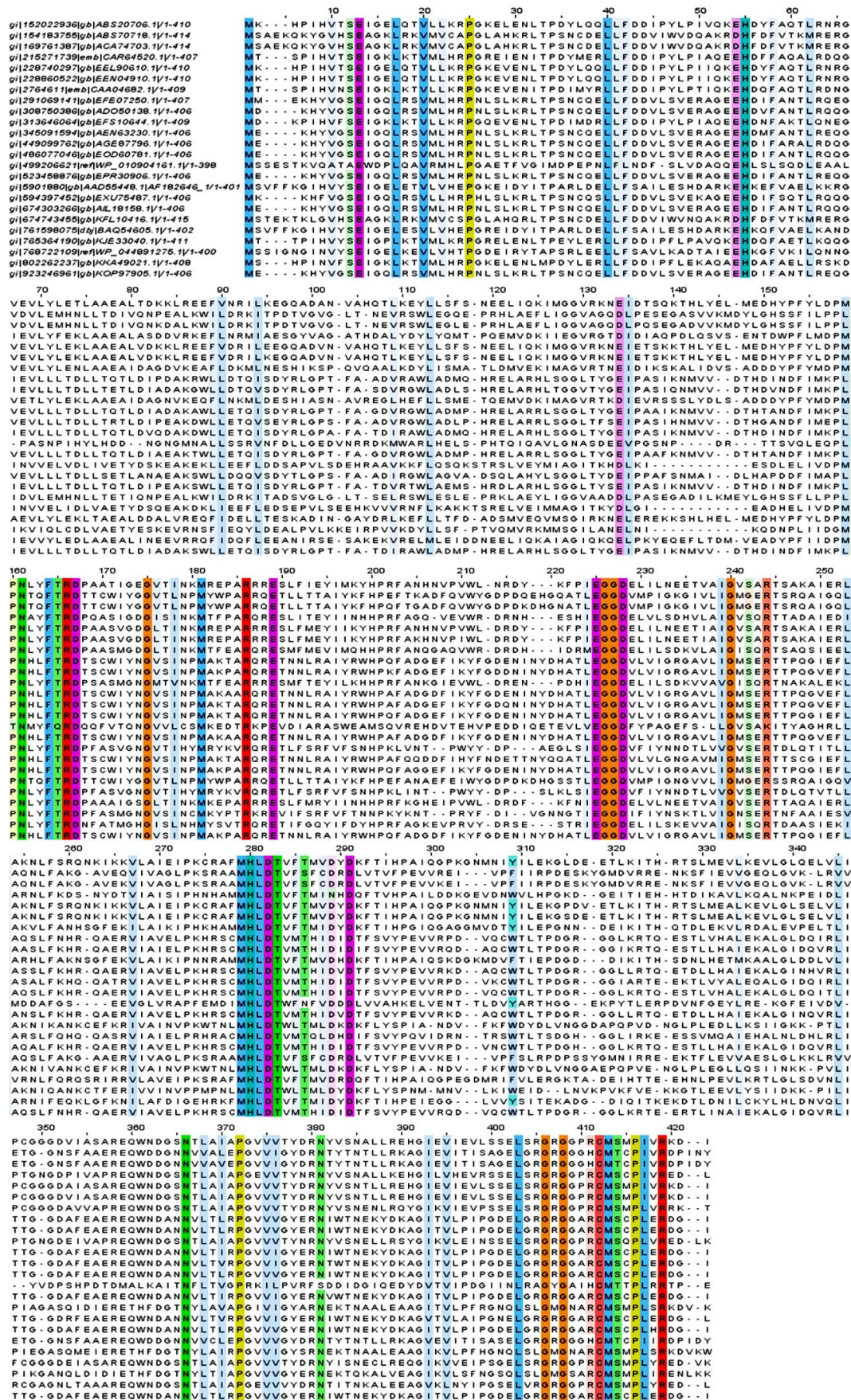


Fig. 3: Multiple sequence alignment of ADI genes (colored based on clustalx matrix and 50% conservation).

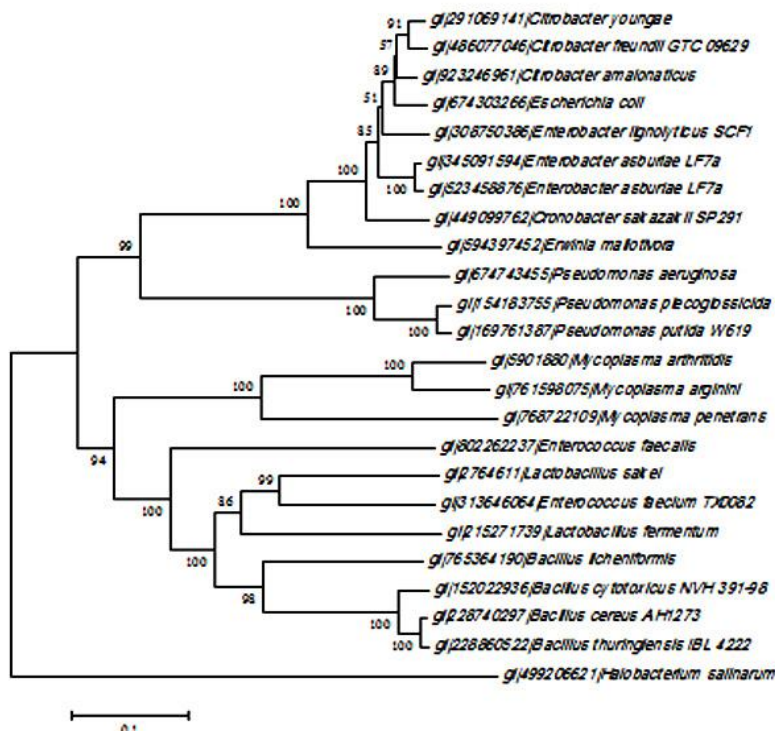


Fig. 4: Phylogenetic trees constructed by Neighbor-Joining method (by means of MEGA 6) based on ADI genes from different species. Bootstrap values and genetic distance are presented.

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