ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Molecular identification and characterization of an arginine catabolizing *Entercoccus faecium* NM216 via arginine deiminase enzyme (ADI) pathway and evaluation its effect *in vitro* on tumor cell lines.

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ABSTRACT

The microbial Arginine deiminase (ADI) enzyme encoded by arcA gene has been shown as a potential anti-cancer agent against different types of tumors. Enterococcus sp. are members in Lactic acid bacteria (LAB) and known as normal commensal bacteria in the human gut microbiota. Here, the human isolate NM216 was selected as high ADI producer and comprises the arcABC operon. The isolate was identified by the molecular method using the sequencing analysis of the 16S rRNA gene which by blast search at the database revealed 95% homology to Enterococcus faecium strains subsequently it was submitted to the Genebank under the accession no. KX155648. Optimization of the production media of the ADI enzyme by the NM216 strain was investigated. The investigation of the suggested virulence enterococcal genes and the hemolytic activity confirmed its safety. In addition the strain was evaluated in vitro against four human tumor cell lines: HePG2 (hepatocellular carcinoma); MCF7 (Caucasian breast adenocarcinoma); A549 (Lung carcinoma); HCT116 (Colon). The results showed the sensitivity of HePG2, MCF7, and HCT116 to cell free supernatant of Ent. faecium NM216 suggesting it as potential therapeutic probiotic agent against cancer and as a promising cell factory for ADI production for therapeutic functions.

Keywords: ADI enzyme, antitumor, arcA gene, Enterococcus Spp

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ISSN: 0975-8585

INTRODUCTION

The amino acid arginine has a great ability to boost tumor progression contrariwise; the catabolism of arginine prevents the development of metastatic tumors [1, 2]. Hence, many research studies suggested the usage of L-arginine catabolizing enzymes as a prospective anticancer mediator [3]. Many studies suggested the ability of arginine deiminase (ADI) for inhibiting the cell proliferation in hepatocellular, melanoma, leukaemia and prostate cancer cell lines [4-7] Lactic acid bacteria (LAB) have been known for their role in maintaining the human gut health and protection from diseases and different types of cancer [8-10] but the exact mechanisms for their action are not clear. The energy metabolism of Lactic acid bacteria (LAB) is rely on their proteolytic activity, sugar fermentation, arginine deamination, in addition to acid and amino acid decarboxylation [11]. In LAB, arginine catabolism occurs via the ADI pathway, which involves three enzymatic reactions. The first drives via ADI enzyme which hydrolyses L-Arginine into L-citrulline and ammonia, second by the action of ornithine transcarbamylase enzyme (OTC), L-citrulline is degraded into ornithine and carbamoyl phosphate. Followed by the third step where the carbamoyl phosphate is further degraded into ammonia and CO_2 by carbamate kinase (CK). This hydrolysis process converts one mole of arginine to two moles of ammonia and one mole each of ornithine, ATP and carbon dioxide, which provides energy and shield against a low external pH conditions. However, this property as energy foundation or as a defensive structure from acidic surroundings diverges not only amongst LAB but also among the strains which considered as strain specific [12] and the gene organization multiplicity of the ADI operons has been shown [13]. The key enzymes of the ADI pathway i.e. ADI, OTC, CK are coded by *arc*A, *arc*B, *arc*C genes respectively, are conserved among LAB. In the present work we aimed to isolation and characterization new candidate according to its ability to degrade arginine via ADI and its effect against human tumor cell lines. Thus would have great impact in both food and pharmaceutical industries.

MATERIALS AND METHODS

Human fecal sampling

Fecal samples were collected from human subjects aged from 3 months up to 40 years. Two gm of each sample was homogenized into 10 ml sterilized 0.9% sterile saline solution and handled at once for bacterial isolation.

Chemicals

Chemicals used in this study were purchased from Sigma Aldrich (Sigma–Aldrich, St. Louis, MO, USA). The media and media supplementation used were purchased from Oxoid (Oxoid Basingstoke, UK).

Isolation of Lactic Acid Bacteria (LAB) and growth conditions

Fecal samples were inoculated in de Man, Rogosa and Sharpe (MRS) broth overnight at 37 °C anaerobically in jar with AnaeroGen. Then the cultures were plated on MRS agar plates anaerobically at 37 °C for 48 h and single bacterial colonies were picked, purified, and preserved as stock in 40% glycerol at – 40 °C. The isolates were examined by catalase test, gram stain and hemolysis test.

Selection of arginine deiminase (ADI) producer strains

The bacterial isolates were screened for arginine deiminase (ADI) enzyme production according to Kaur and Kaur (2013) [14] with some modification in minimal arginine medium (MAM) containing (g/l): tryptone, 2.5; glucose, 1; arginine, 5; KH_2PO_4, 1. Cultures were incubated at 37°C for 48 h.

DNA extraction and PCR

Microbial genomic DNA was extracted using AxyPrep miniprep kit (Axygen Biosciences, Union City, CA, USA) according to the producer's guidelines and quantitative by electrophoresis on 1% agarose gel. PCR reactions for amplification the 16s rRNA, detection of the *arc* operon and the enterococcal virulence genes were carried on the PCR machine Mj Mini (Bio Rad, Hercules, CA, USA) using Thermo Scientific PCR master mix. Primers used in this study were ordered from Sigma Scientific Services, Cairo, Egypt and the primers

sequences [15-17] are listed in Table (1). Amplified PCR fragments were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the producer's guidelines.

Table (1): List of Primers (Forward (F), and Reverse (R)) used in this study.

Primer name/ target	Primer sequence 5' -3'	References
16s rRNA		[15]
FDI	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	
RDI	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	
arcA operon		[16]
(*arc*A) ADI F	CAYGCNATGATGCAYYTNGAYACNGT	
ADI R	GTRTTNSWNCCRTCRTTCCAYTGYTCOTC	
(*arc*B) OTC F	ATGCAYTGYYTNCCNGCNTTYCAYGA	
OTC R	CCNARNGTNGCNGCCATDATNGCYTT	
(*arc*C) CK F1	CAYGGNAAYGGNCCNCARGTNGGNAA	
CK R1	CKNCKNYANCCNCKNCCNGCRTCYTC	
Enterococcal virulence genes		[17]
(*agg*): F	AAG AAA AAG AAG TAG ACC AAC	
R	AAA CGG CAA GAC AAG TAA ATA	
(*cyl*A): F	TGG ATG ATA GTG ATA GGA AGT	
R	TCT ACA GTA AAT CTT TCG TCA	
(*efa*Afs): F	GAC AGA CCC TCA CGA ATA	
R	AGT TCA TCA TGC TGT AGT A	
(*efa*Afm): F	AAC AGA TCC GCA TGA ATA	
R	CAT TTC ATC ATC TGA TAG TA	
(*cob*): F	AAC ATT CAG CAA ACA AAG C	
R	TTG TCA TAA AGA GTG GTC AT	
(*ccf*): F	GGG AAT TGA GTA GTG 0AAG	
R	AGC CGC TAA AAT CGG TAA AAT	

Sequence analysis

The amplified pure PCR products were sequenced by Labtechnology, Cairo, Egypt. The homology analysis of the nucleotide sequences was carried out using nucleotide BLAST program at the National Center for Biotechnology Information (NCBI).

Preparation of culture supernatants and cell-free extracts.

The isolates were inoculated in MRS medium at 37 °C for 2 days. The culture supernatant was obtained by centrifuging at 10,000 g for 20 min at 4 °C and filtered using 0.22 µm pore size cellulose acetate filter. The bacterial pellet was resuspended in 3 ml of the PBS buffer followed by sonication 4 times at 15 s intervals and 1 min on ice and the cell-free extracts were obtained by centrifuging at 10,000 g for 20 min at 4 °C.

Enzyme assays

Cell free supernatant was assessed for extracellular arginine deiminase activity (ADI) according to De Angelis *et al*. (2002) [12]. Assay mixture containing 150µl of 50 mM arginine, 2.3 ml 50 mM acetate buffer pH 5.5, 50 µl culture filtrate and 3.6 µl 0.05% sodium azide. After incubation for 1 h at 37°C, the reaction was stopped by adding 0.5 ml of 2N HCl, and precipitated protein was removed by centrifugation. Citrulline content was determined according to Archibald (1944) [18]. One milliliter of the supernatant was added to 1.5 ml of an acid mixture of H_3PO_4-H_2SO_4 (3/1 V/V) and 250 µl of diacetyl monoxime (1.5% 2, 3 butnedione monoxime) in 10% methanol, mixed and boiled for 30 min. After cooling for 10 min, absorbance was measured at 460 nm and one arginine deiminase unit was calculated as the amount of required to produce 1 µmol citrulline per min. Finally, specific activity was calculated as international enzyme units present per gm (IU/ mg) of protein. Protein concentrations were determined by Bradford method the Bradford method [19] using bovine serum albumin (BSA) as standard.

ISSN: 0975-8585

Optimization of medium components for enhancing ADI activity through two steps statically factorial design:

Experimental design using Design Expert Software trial version 7.0.0 statistical software was used for improving ADI activity. **First step**, the Plackett-Burman (PB) experimental design was used to select significant factors that influence ADI activity which included eleven factors as shown in Table (2). Total number of experiments was 12 runs based on the rule n+1, where n represents the number of factors under investigation. In the experimental design, each row represented an experiment, and each column represented an independent variable (Table 3). The signs '+' and'_' represent the higher and lower levels of the independent variable under investigation. The statistical significance was determined by F-value, and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R2.

Table (2): The Plackett–Burman design for nutrient screening in ADI production.

Variables		Levels	
Factor code	**Factor**	**Low (-1)**	**High (+1)**
A	Glucose (g/l)	0.5	5
B	Lactose (g/l)	0.5	5
C	Tryptone (g/l)	5	7
D	CuSO4 (g/l)	0	0.5
E	NaCl (g/l)	0	
F	Tween 80 (ml/l)	0	0.1
G	CaCl (g/l)	0	0.25
H	MnSO4 (g/l)	0	0.05
J	K2HPO4 (g/l)	0.5	2
K	ZnCl (g/l)	0	0.25
L	Arginine (g/l)	10	20

Table (3): The Plackett–Burman design variables with ADI specific activity as response.

Run	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	**K**	**L**	**ADI specific activity (IU/mg)**
1	0.50	0.50	7.00	0.00	0.50	0.10	0.00	0.05	2.00	2.50	10.00	8.65
2	5.00	5.00	7.00	0.00	0.00	0.00	0.25	0.00	2.00	2.50	10.00	6.3
3	5.00	0.50	5.00	0.00	0.50	0.00	0.25	0.05	0.50	2.50	20.00	3.3
4	0.50	0.50	5.00	0.50	0.00	0.10	0.25	0.00	2.00	2.50	20.00	8
5	0.50	5.00	7.00	0.50	0.00	0.00	0.00	0.05	0.50	2.50	20.00	1.25
6	0.50	0.50	5.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	10.00	4.25
7	5.00	0.50	7.00	0.50	0.00	0.10	0.25	0.05	0.50	0.00	10.00	0
8	5.00	5.00	5.00	0.00	0.00	0.10	0.00	0.05	2.00	0.00	20.00	2.5
9	5.00	0.50	7.00	0.50	0.50	0.00	0.00	0.00	2.00	0.00	20.00	10.15
10	5.00	5.00	5.00	0.50	0.50	0.10	0.00	0.00	0.50	2.50	10.00	2
11	0.50	5.00	5.00	0.50	0.50	0.00	0.25	0.05	2.00	0.00	10.00	0
12	0.50	5.00	7.00	0.00	0.50	0.10	0.25	0.00	0.50	0.00	20.00	12.85

Second step, the optimum level of selected variables tryptone, arginine, NaCl and K_2HPO_4 that were screened from the PB design was determined by Taguchi L9 (3^4) orthogonal array design. These variables were tested at three levels (Table 4).

Table (4): Factors and their levels for optimization of ADI production by the orthogonal array of L9 (3ˆ4) design.

Run No.	Factors								Response enzyme activity (IU/mg)
	Coded levels				Actual levels				Experimental
	Tryptone	arginine	NaCl	K_2HPO_4	Tryptone	arginine	NaCl	K_2HPO_4	
1	1	3	1	2	2	25	0.75	2	23.45
2	3	2	2	2	15	15	1.5	2	12.5
3	1	1	2	1	2	2	1.5	1	19.45
4	3	3	3	1	15	25	2	1	10.25
5	2	1	3	2	10	2	2	2	14.68
6	3	1	1	3	15	2	0.75	3	8.5
7	1	2	3	3	2	15	2	3	15.45
8	2	2	1	1	10	15	0.75	1	0
9	2	3	2	3	10	25	1.5	3	0

***In vitro* activity of selected strain towards tumor cells**

In vitro bioassay of supernatant and the cell-free extract of the tested bacteria on human tumor cell lines was conducted and determined by the Bioassay-Cell Culture Laboratory, National Research Centre, Egypt. Four different human cancer cell lines were used: HePG2 (Human hepatocellular carcinoma cell line); MCF7 (Human Caucasian breast adenocarcinoma); A549 (Lung carcinoma cell line); HCT116 (Colon cell line). The assay was performed as described previously by Hansen *et al*. (1989) [20]. A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent who gives 100 % lethality under the same conditions [21]. The absorbance was measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm.

Statistical analysis of the results

One-way ANOVA analysis was carried out, and results are presented as mean ± standard deviation of three triplicate experiments and a probability value (p) less than 0.05 was considered as significant statistically.

RESULTS AND DISCUSSION

Isolation of LAB from Human fecal samples

Eighteen isolates were selected from culturing the fecal samples on MRS agar plates as catalase negative, hemolysis negative, and gram positive for further screening of ADI producer strains. The microscopic examination and initial phenotype characteristics revealed that all the eighteen isolates are belonging to *Enterococcus* sp.

Screening of ADI producer LAB strains

The initial degradation of arginine by ADI leads to ammonia and citrulline production. The first screening of the eighteen isolates in the MAM medium revealed only two isolates were able to produce ADI isolate no. 7 and no. 14 (1.8 and 2.5 IU/mg, respectively) and named as strain NM116 and NM216 respectively.

Detection of the *arc*ABC genes by PCR

Three sets of degenerate primers were used to detect the genes *arcA*, *arcB*, and *arcC* in the eighteen LAB isolates by PCR. The amplified products were examined by gel electrophoresis (data not shown). The detection results of *arc*ABC genes is in agreement with their preliminary screening for ADI producers where only the two isolates no. 7 (named NM116) and no. 14 (named NM216) showed positive by giving amplified bands in the predicted size 266, 181, 343 base pair for the *arc*A, *arc*B, *arc*C respectively.

Determination of the enterococcal virulence genes

The two isolates 7 (NM116) and 14 (NM216) were screened by PCR for the presence of six virulence gene; *cyl*A, *cob*, *agg*, *efafs*, *efafm*, *ccf* the predicted size for each gene are; 517, 1,400, 1,500, 705, 735, and 543

bp. The amplified products for the two isolates 7 and 14 were examined on 1% agarose gel as shown in Fig. (1). The results revealed the absences of three virulent factors *cyl*A, *cob*, and *efafs* within isolate 14 while isolate 7 showed absence of the virulent factors *cyl*A and *cob* only.

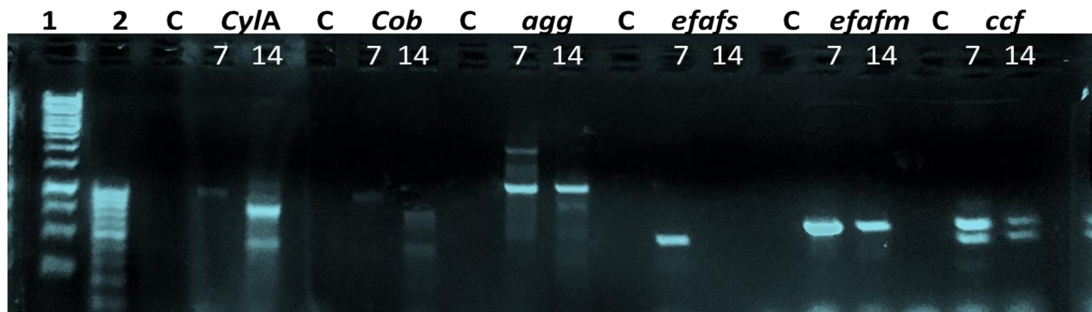

Fig. (1): The Determination of the enterococcal virulence gene within the genomic DNA of the isolates 7 and 14 by PCR. The amplified products were checked on 1 % agarose gel. Lane 1, 2; 1kb and 100 bp DNA ladder, C; negative control for each reaction set.

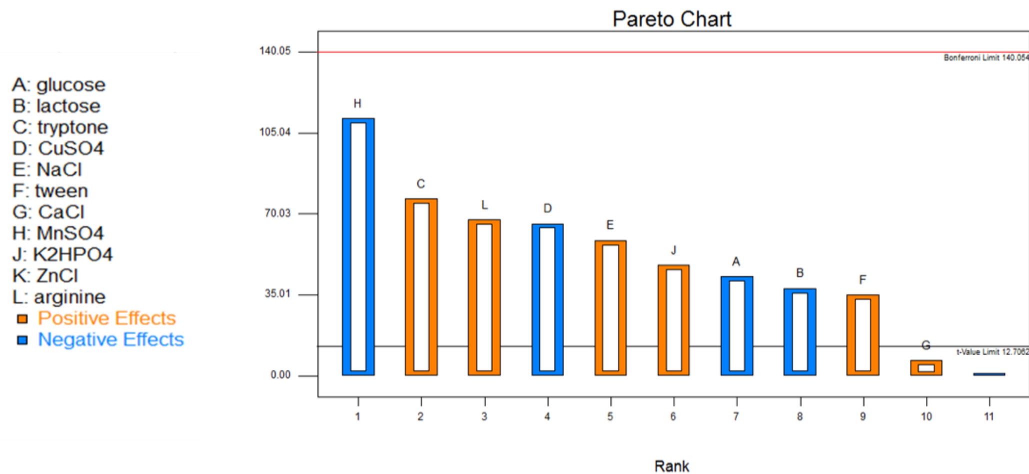

Fig. (2): Pareto chart showed the effect of each variable on the ADI specific activity

Molecular identification of the ADI producer isolate no. 14 (NM216)

According to the higher ADI activity was shown by the isolate 14 (NM216), it was chosen for further work and was identified by amplification and sequencing of the 16s rRNA gene and the sequencing result was analyzed for homology in the NCBI data base. This homology research showed 100% similarity to *Enterococcus faecium.* The strain was designed as *Ent. faecium* NM216 and its partial 16s rRNA gene sequence was submitted to Genbank with Accession no. KX155648.

Enhancement of ADI activity by statically factorial design (PB and Taguchi):

First, PB design was used to investigate the effect of eleven factors as indicated in Table (2) on ADI activity. The yield of ADI expressed as specific activity, determined for each experiment design, was shown in Table (3). The data obtained showed a wide variation in the specific activity of strain NM216 from 0 to 12.85 IU/mg. This indicated the effectiveness of the selected factors. The analysis of variance (ANOVA) for the experiment design The analysis of the regression coefficients of the 11 showed that, G,F,J,E,L, C have positive effect while, B,A,D,H showed negative effect as shown in Fig. (1). According to these results, a medium of the following composition is expected to be near optimum (g/l): glucose, 0.5; lactose, 5; tryptone, 7; NaCl, 0.5; CaCl, 0.25; K_2HPO_4, 0.5; arginine, 20; Tween 80, 0.1 ml/l and pH 6. The ADI specific activity measurement on this medium was 12.85 IU/mg compared to 2.5 IU/mg on screening medium causing 5.14-folds increase in ADI specific activity.

The analysis of variance (ANOVA) for the experiment design showed that, A, B, C, D, E, F, H, J, L are significant model terms. But the factor K is not significant and removed. The first order model equation developed by PB design showed the dependence of ADI specific activity in the medium constituents: Eq. (1).

R1 (ADI specific activity IU/mg) = +4.94-0.90 *A -0.79 *B +1.60 *C -1.37 *D +1.22 *E +0.73 *F +0.14 *G -2.32 *H +1.00 *J +1.40 *L.

There for, the optimum combination of the variables (tryptone, arginine, NaCl, K_2HPO_4) which had the highest significant influence on ADI production was further investigated by a Taguchi method for obtaining the maximum ADI specific activity.

Secondly, Taguchi methodology

Based on the results of PB design, four variables including tryptone, arginine, NaCl, K_2HPO_4 which significantly influenced ADI production, were further investigated for their optimum combination using Taguchi's (L9 3ˆ4) orthogonal array design. The design and results of the experiments together are shown in Table (4) and indicated that the maximum ADI specific activity (23.45 IU/mg) was obtained when tryptone, arginine, NaCl, K_2HPO_4were used with concentrations (g/l): 2, 25, 0.75 and 2, respectively.

Final equation in terms of coded factors: Eq. (2).

ADI specific activity= +11.59 -6.69 *A[1] +7.86 *A[2] -1.69 *D[1] +5.29 *D[2]

Analysis of variance (ANOVA) as shown in Table (5) implies the model is significant that F-value of 8.71. Values of "Prob > F" less than 0.0500 indicate model terms are significant, in this case the factors, A-tryptone, D-K_2HPO_4 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant as the factor B-arginine and C-NaCl. The adequate precision value of 8.703 emphasized that an adequate signal and suggests that the model can be used to navigate the design space.

Table (5): Analysis of variance (ANOVA) for the experimental results of the Taguchi method.

Source	Sum of squares	df	Mean square	F value	p-value	Prob>F
Model	455.44	4	113.86	8.71	0.0296	Significant
A-tryptone	324.00	2	162.00	12.40	0.0193	
D-K_2HPO_4	131.44	2	65.72	5.03	0.0810	
Residual	52.27	4	13.07			
Cor Total	507.71	8				

Std. Dev.= 3.61; Mean = 11.59; C.V.% = 31.20; PRESS = 264.62; R-squared = 0.8970; Adj R-squared= 0.7941; Pred R-squared = 0.4788; adeq precision = 8.703.

As a conclusion after the optimization by using Plackett–Burman and Taguchi methods, the optimal medium composition for the ADI production by stain 14 was found to be (g/l): glucose, 0.5; lactose, 5; tryptone, 2; NaCl, 0.75; CaCl, 0.25; K_2HPO_4, 2; arginine, 25; Tween 80, 0.1 ml/l and pH 6. Under the optimized conditions, the maximum yield of ADI achieved was 23.45 IU/mg, which increased about 9.38-folds the initial screening medium.

In vitro determination of *Ent. faecium* NM216 as anti-proliferative effects on different human cancer cell lines

Determination of the cytotoxic effect of the *Ent. Faecium* NM216 culture supernatant and cell-free extract against different human cancer cell line (Hep-G2, MCF-7, HCT-116, and A549) are shown in Table (6). The results revealed that the supernatant compared to its cell-free extract is highly cytotoxic effect to MCF7 and HCT116 cells by expressing half-maximal inhibitory concentration (IC_{50}), 50.0 and 45.2 µg/ml respectively, while has moderate effect on HePG-2 and A549 cells by expressing (IC_{50}) 30.2 and 25.4 µg/ml respectively. These initial investigations have been proved the ability of *Ent. faecium* NM216 as antitumor agent against the breast, colon, liver, and lung cancer cells. This activity was shown in the culture supernatant, the same fraction which demonstrated ADI activity. However the purification of the ADI enzyme and determination its cytotoxic

effect on the human cancer cell line is in progress. Previously Kim *et al*. (2002) [22] have been shown an antiproliferative activity of cytoplasmic fractions of ten different LAB against human stomach and colon cancer cells. Kim *et al*. (2009) [23] showed that the antiproliferative effect of strain *L. lactis* ssp. *lactis* ATCC7962 on stomach cell line is due to ADI enzymes.

Table (6): The anti antiproliferative effect of *Ent. faecium* NM216 Culture supernatant and Cell-free extract

Sample	The half-maximal inhibitory concentration (IC50) value µg/ml			
	Hep-G2	MCF-7	HCT-116	A549
Culture supernatant	30.2	50.0	45.2	25.4
Cell-free extract	8.7	5.4	5.3	6.2

CONCLUSION

The screening of the eighteen LAB isolates for ADI producer either by biochemical or genetic methods delivered *Ent. faecium* NM216 with strong ADI activity and antiproliferative effect against liver, colon, breast, and lung cancer cell lines. Optimization of culture conditions for the ADI production by this strain has been assessed. Thus, *Ent. faecium* NM216 has a potential opportunity as therapeutic agent in pharmaceutical industries. However, further work concerning the isolation and characterization of the *arc*A gene and its pure enzyme ADI from our strain *Ent. faecium* NM216 are in progress.

ACKNOWLEDGEMENTS

This work has been funded by National Research Centre, Egypt under project no. 10010113

Conflict of Interest

The authors declare that they have no conflict of interest.

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