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Research Paper

Purification and characterization of the enzymes of arginine deiminase (ADI) pathway from Pediocin-sensitive and resistant strain of *Enterococcus faecalis*

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Abstract
Arginine is an essential amino acid for bacterial system and known to be catabolized by arginine deiminase pathway. Therefore in this study, three core enzymes; arginine deiminase (ADI), ornithine transcarbamoylase (OTC) and carbamate kinase (CK) of ADI pathway were purified from pediocin-sensitive and resistant strain of Enterococcus faecalis to investigate a linear correlation between arginine utilization and resistance progression under glucose limitation. Lysozyme was used for efficient bacterial cell lyses and enzymes were found highly active upon precipitation by ammonium sulfate (80% saturation). Further purification was performed with DEAE-Sepharose fast flow anion-exchanger column and gel filtration chromatography with Sephadex G-150 column. Total protein content, enzyme activity and specific activity along with purification fold were calculated at every step of purification. ADI, OTC, and CK enzyme had apparent molecular masse of 46, 39, and 37 kDa, respectively. The optimum pH (7.0) and temperature (37°C) were estimated to determine the maximum enzyme activity from a range of pH (3-10) and temperature (0-100°C). Hence, this work important with respect to the introduced methodology to isolate and/or purify intact enzyme from any bacterial strain.
Keywords: *Enterococcus faecalis, ADI pathway enzymes, ADI, OTC, CK, Purification,*

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I. Introduction

Bacterial system composes the most prosperous form of life to survive in the changing environmental habitats. The basic key of this survival success is their ability to sense, rapidly respond for fluctuating environmental stimuli and admirable genotypic and phenotypic plasticity (Lambert and Kussell, 2014). Bacteria has established a wide array of mechanisms to promote its survival in various harsh conditions including; innate and adaptive immune response of the host (Belkaid and Hand, 2014), exposure of the antimicrobial peptides and antibiotic (Batoni et al. 2015; Munita and Arias, 2016) and nutrients limitation (Chou et al. 2001). During specific response of energy starvation, bacteria turned into the state of viable but non-culturable (VBNC) rather than die and survive for longer time under nutrients limitation. The VBNC physiology has been observed in many bacteria such as *Vibrio, Pseudomonas, Enterococcus* and *Escherichia coli* (Oliver 1993). Previously, it was reported that under carbohydrate limitation, arginine plays a crucial role for bacterial growth and acts an alternative energy source (Deibel RH, 1964; Stuart et al. 1999). Arginine degradation via ADI pathway has reported in several studies (Townsend et al. 1996; Morita et al. 1997; Zuniga et al. 1998; Stuart et al. 1999; Noens and Lolkema, 2017). Typically, ADI operon contains genes; *arcR* encodes transcriptional regulator; *arcA* encodes ADI (EC 3.5.3.6); *arcB* encodes OTC (EC 2.1.3.3) and *arcC* encodes CK (EC 2.7.2.2) (Gallego et al. 2012). ADI pathway plays a vital role in supplying intermediates (citrulline, ornithine) for amino acids biosynthesis (Townsend et al. 1996), producing NH_4^+ for bacterial protection from acid stress (Casiano-Colon and Marquis, 1988; Tonon T. and Lonvaud-Funel A. 2000; Lindgren et al. 2014), producing ATP for growth when carbohydrate is limited (Verges et al. 1999; Kim et al. 2009), producing carbamoyl phosphate for substrate-level phosphorylation (Colon et al. 1988) and pyrimidines as nucleic acids precursor molecules

(Morita et al. 1997). The regulation of ADI pathway is highly depends on transcriptional regulator of *arcR* family and carbon catabolite protein A (ccpA) and factors including; arginine and glucose concentration, pH, temperature, level of intracellular ATP, NH_4^+, NADH and CO_2 also play major role (Lindgren et al. 2014). *Enterococcus* represents the paradigm for biochemical studies of enzymes isolation of ADI deiminase pathway (Angelis et al. 2002; Cheng-Fu et al. 2008). ADI, OTC, and CK enzyme successfully purified using ammonium sulfate precipitation, Q-Sepharose fast flows anion exchange chromatography and Sephadex-G75 gel filtration chromatography with molecular masses 46, 39 and 37 kDa respectively and their specific activity, purification fold and %yield were calculated for *Lactobacillus sanfranciscensis* CB1 (Angelis et al. 2002) and *Lactococcus lactis* (Mahdy et al. 2015). Many studies reported the active and stable form of these enzymes on a wide range of pH, temperature and substrate concentration (Angelis et al. 2002; Mahdy et al. 2015; Remaux et al. 2012). Therefore, the specific objective of this study was to isolate and/or purify all three enzymes of ADI pathway to perform further enzymatic investigations. Along with, this work will be very useful and can be consider as a reference to purify intact enzyme from any bacterial strains.

II. Materials And Methods

2.1 Bacterial strain and culture media

Pediocin-sensitive and resistant *E. faecalis* isolated from raw buffalo milk (Kumar et al. 2019) were selected as test strains to examine the behavior of arginine metabolic pathway during resistance acquisition against pediocin. CDM (pH 7.0) was used throughout the study and prepared using several components; 1 gram (g) K2HPO4, 5 g KH2PO4, 0.6 g ammonium citrate, 1 g sodium acetate, 2.5 g NaHCO3, 0.25 g tyrosine, 0.24 g alanine, 0.42 g aspartic acid, 0.180 g cysteine, 0.5 g glutamic acid, 0.15 g histidine, 0.21 g isoleucine, 0.475 g leucine, 0.44 g lysine, 0.275 g phenylalanine, 0.675 g proline, 0.34 g serine, 0.225 g threonine, 0.05 g tryptophan, 0.325 g valine, 0.175 g glycine, 0.125 g methionine, 0.1 g asparagine, 0.2 g glutamine, 0.5 g L-ascorbic acid, 38.5 milligram (mg) adenine sulfate, 27.5 mg guanine, 22 mg uracil, 10 mg xanthine, 2.5 mg D-biotin, 1 mg vitamin B12, 1 mg riboflavin, 5 mg pyridoxamine-HCl, 10 microgram (µg) *p*-aminobenzoic acid, 1 mg pantothenate, 5 mg inosine, 1 mg nicotinic acid, 5 mg orotic acid, 2 mg pyridoxine, 1 mg thiamine, 2.5 mg lipoic acid, 5 mg thymidine, 200 mg MgCl2, 50 mg CaCl2, 16 mg MnCl2, 3 mg FeCl3, 5 mg FeC12, 5 mg ZnSO4, 2.5 mg CoSO4, 2.5 mg CuSO4, and 2.5 mg (NH4)6 Mo7O2 dissolved in one litter of distilled water (Veith et al. 2015). CDM supplemented with 63.14 mM arginine was used to grow sensitive and resistant *E. faecalis*.

2.2 Purification of the ADI pathway enzymes

Enzymes of the ADI pathway were purified using four purification steps; (i) cell lysate preparation, (ii) ammonium sulfate precipitation, (iii) ion exchange chromatography and (iv) gel filtration chromatography. Enzyme activity, specific activity and protein concentration were measured along with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis at every purification step.

2.3 Preparation of cell lysate

Pediocin-sensitive and resistant strain was cultured in CDM for 12 h and cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C. Cell pellets were washed thrice with potassium phosphate buffer (50 mM, pH 7.0), resuspended in Tris-EDTA extraction buffer (20 mM, pH 8.0) containing lysozyme (5 mg ml^{-1}) and incubated at 37°C for 1.5 h. After incubation, sonication was done with 30 sec off and on cycles 4 times to get cell suspension and cell lysate was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C. Total protein concentration of cell lysate was estimated using Bovine Serum Albumin (BSA) as standard (1mg ml^{-1}) (Lowry et al. 1951) along with enzyme activity and specific activity measurement. Further, cell lysate was used for SDS-PAGE analysis and next purification step.

2.4 Ammonium sulfate precipitation

Cell lysate solution was precipitated by adding certain weight of ammonium sulphate step by step at magnetic stirrer to obtain 0-80% saturation. Precipitates were isolated from precipitated solution by centrifugation at 10,000 rpm for 30 minutes at 4°C and dissolved in potassium phosphate buffer (50mM, pH 7.0). Further, protein concentration, enzyme activity, and specific activity were measured of ADI pathway enzymes of pediocin-sensitive and resistant strain. Solution was concentrated using 3 kDa cut-off membrane ultrafilters (Amicon) and used for SDS-PAGE analysis and next purification steps.

2.5 Ion-exchange chromatography

Concentration solution was passed through DEAE-Sephadex fast flow anion exchanger column and eluted with potassium phosphate buffer (50 mM, pH 7.0) containing NaCl (0.1-0.5 M). Fractions (2 ml each) were collected with flow rate 30 ml h^{-1}. Collected fractions were used to measure the absorbance at 280 nm and activity was calculated by performing enzyme assay to get the active fractions. Then, active fractions with higher enzyme activity were concentrated using 3 kDa cut-off membrane ultrafilters (Amicon) and used for SDS-PAGE analysis. Enzyme activity, specific activity and protein concentration were measured using active fractions and used for next purification step.

2.6 **Gel filtration chromatography**

Activated concentrated fractions were applied to Sephadex G-150 gel filtration column and eluted with potassium phosphate buffer (50mM, pH 7.0). Fractions (3 ml) were collected with flow rate 30 ml h^{-1}. Collected fractions were used to measure the absorbance at 280nm and activity was calculated by performing enzyme assay to get the active fractions. Active fractions with higher enzyme activity were concentrated using 3 kDa cut-off membrane ultrafilters (Amicon) and used for SDS-PAGE analysis. Enzyme activity, specific activity and protein concentration were measured using concentrated fractions and used for further study.

2.7 SDS-PAGE profiling

Characterization of ADI, OTC and CK of pediocin-sensitive and resistant *E. faecalis* was done during purification steps based on their molecular weight analysis by SDS-PAGE (Laemmli UK, 1970) with the reference protein ladder (Bio-Rad, #161-0374). Electrophoresis was performed using Bio-Rad Mini apparatus with 30 µg of protein loaded in the well of 12% acrylamide gel (thickness, 1 mm; separation distance, 10 cm) under denatured conditions. A constant voltage supply (70V) was set until tracking dye reached to the bottom of the gel. To characterize a single subunit of each enzyme, the distance of protein bands migrated toward anode was measured with their relative mobility (*Rm*).

2.8 Enzyme assay

Enzymes assays were performed to purify ADI, OTC, and CK enzyme from pediocin-sensitive and resistant *E. faecalis* and to calculate their activity. Enzyme assay for ADI was estimated using standardized method of Mahdy et al. (2015) with slight modifications and it activity was estimated by drawing standard curve of citrulline (enzyme product). Briefly, the reaction mixture containing 100 µl potassium phosphate buffer (50 mM, pH 7.0), 100 µl L-arginine (100 mM) and 100 µl purified enzyme, mixed well and incubated at 37ºC for 15 min. After incubation, the reaction mixture was centrifuged at 6,000 rpm for 10 min at 4°C and supernatant was collected. Then, 600 µl COLDER solution was added to supernatant and incubated at 95ºC in dark water bath for 15 min as suggested by Crow and Thomas (1982). Simultaneously, tubes of control without enzyme and blank without substrate and enzyme were also prepared. Enzyme activity was calculated by estimating citrulline production using recorded absorbance at 540 nm. Unit (U) of enzyme activity is defined as the amount of enzyme which catalyzes the conversion of 1 µmole of substrate per minute. The specific activity gives a measurement of enzyme purity from mixture and defined as the moles of product formed by an enzyme in a given time at standard conditions with per mg of total protein. Enzyme activity OTC was estimated by a standardized method (Ruepp et al. 1995). Briefly, the reaction mixture containing 100 µl potassium phosphate buffer (50 mM, pH 7.0), 100 µl L-ornithine (50 mM), 100µl carbamoyl phosphate (10 mM), 100 µl purified enzyme, mixed well and incubated at 37ºC for 10 min. After incubation, the reaction mixture was centrifuged at 6,000 rpm for 10 min at 4°C and 600 µl COLDER solution was added to the collected supernatant and incubated at 95ºC in dark water bath for 15 min. Enzyme activity was calculated by citrulline estimation using recorded absorbance at 540 nm. Simultaneously, tubes of control without enzyme and blank without substrate and enzyme were also prepared. Enzyme activity CK was estimated using the method of Liu (1995). The reaction mixture containing 650 µl acetate buffer (50 mM, pH 7.0), 100 µl ADP (50 mM) and 100 µl $MgCl_2$ (100 mM), mixed well and kept at room temperature for 10 min. Then, 100 µl carbamoyl phosphate (10 mM) was added and incubated 37ºC for 15 min. A reaction was initiated by addition 100 µl purified enzyme and liberated ammonia was measured using ammonia assay kit (Cat. no. AA0100-1KT) to estimate enzyme activity. Simultaneously, tubes of control without enzyme and blank without substrate and enzyme were also prepared.

2.9 Optimum substrate concentration for maximum enzyme activity

Maximum activity of the purified enzymes ADI, OTC and CK from pediocin-sensitive and resistant *E. faecalis* was measured using a range of substrates concentration (0-500 mM for arginine and 0-100 mM for carbamoyl phosphate). The enzyme assay was performed with increasing substrate concentration and reaction absorbance was recorded to determine the enzyme activity and products (citrulline and NH_4^+) formation. Enzyme assays were performed in triplicate to calculate the standard error.

2.10 Optimum pH for maximum enzyme activity

Optimum pH for the enzymes ADI, OTC and CK was determined from a pH range (3-10) using reaction mixture prepared with potassium phosphate buffer (50 mM) of different pH. Reaction absorbance was recorded from the essay at different pH to determine optimum pH for maximum enzyme activity. Enzyme assays were performed in triplicate to calculate the standard error.

2.11 Optimum temperature for maximum enzyme activity

Optimum temperature for the enzymes ADI, OTC and CK was determined by assays performed at different temperature (0-100°C). Standardized reaction mixture for the enzymes was set and incubated at different temperature for 15 min. Reaction absorbance was recorded from the essay at different temperature to determine the optimum temperature for maximum enzyme activity. Enzyme assays were performed in triplicate to calculate the standard error.

2.12 Statistical analysis

Enzymatic measurements from three independent experiments were subjected to analysis of variance (ANOVA) for statistical study using GRAPHPAD Prism 5.01 (GRAPHPAD software, San Diego, CA, USA) to determine significant differences ($P<0.0001$).

III. Result

3.1 Purification of ADI, OTC, and CK enzyme

Purification of the enzymes of ADI pathway from pediocin-sensitive and resistant strain was done based on their molecular weight and enzyme activity. At every step of purification, total protein concentration, enzyme activity and specific activity of the enzymes were calculated to draw purification fold tables (1, 2 and 3) of sensitive (a) and resistant strain (b). Variations in protein concentration, enzyme activity and specific activity were noticed at every purification step.

Table 1. Purification tables of the ADI enzyme purified from pediocin-sensitive (a) and resistant (b) strain

(a)

Purification of ADI (Sensitive)	Total Protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	% Yield	Purification fold
Cytoplasmic extract	3.73	1167.22	12.49	100	1
Ammonium Sulfate ppt.	1.32	837.07	42.06	71.71	3.36
Ion exchange chromatography	0.75	714.31	94.98	61.19	7.6
Gel filtration chromatography	0.14	383.2	548.21	32.83	43.89

(b)

Purification of ADI (Resistant)	Total Protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg)	% Yield	Purification fold
Cytoplasmic extract	4.09	1731.54	16.93	100	1
Ammonium Sulfate ppt.	1.35	1201.7	59.34	69.4	3.5
Ion exchange chromatography	0.7	983.45	140.48	56.8	8.2
Gel filtration chromatography	0.16	841.16	1051.87	48.5	60.69

3.2 Ion-exchange chromatography

A wide peak was observed upon absorbance analysis of the eluents collected during ion-exchange chromatography, but maximum absorbance along with highest specific activity was recorded of the fractions eluted with 0.3 and 0.4 M NaCl. Results obtained with maximum absorbance and higher specific activity were shown in figure 1 (for ADI (a and b for sensitive) (c and d for resistant)), figure 2 (for OTC (a and b for sensitive) (c and d for resistant)) and figure 3 (for CK (a and b for sensitive) (c and d for resistant)).

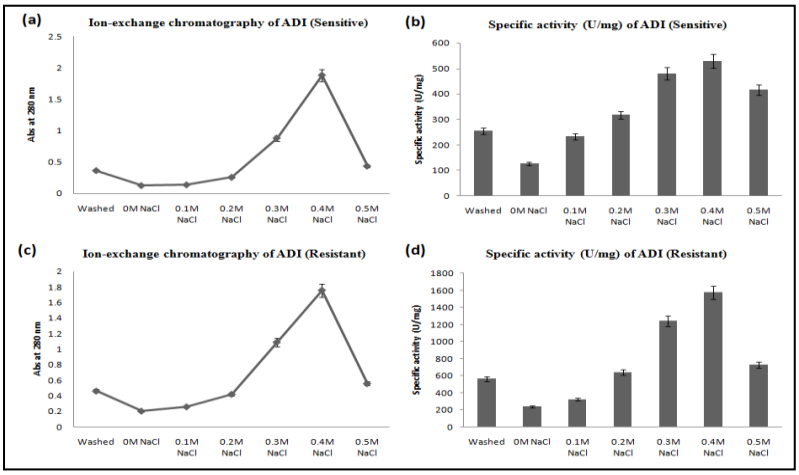

Figure 1. Ion-exchange chromatography of ADI; Ion-exchange chromatography using DEAE-Sephadex fast flow anion exchanger column and specific activity (U/mg) estimation of the ADI enzyme; active fraction collection during ion-exchange chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

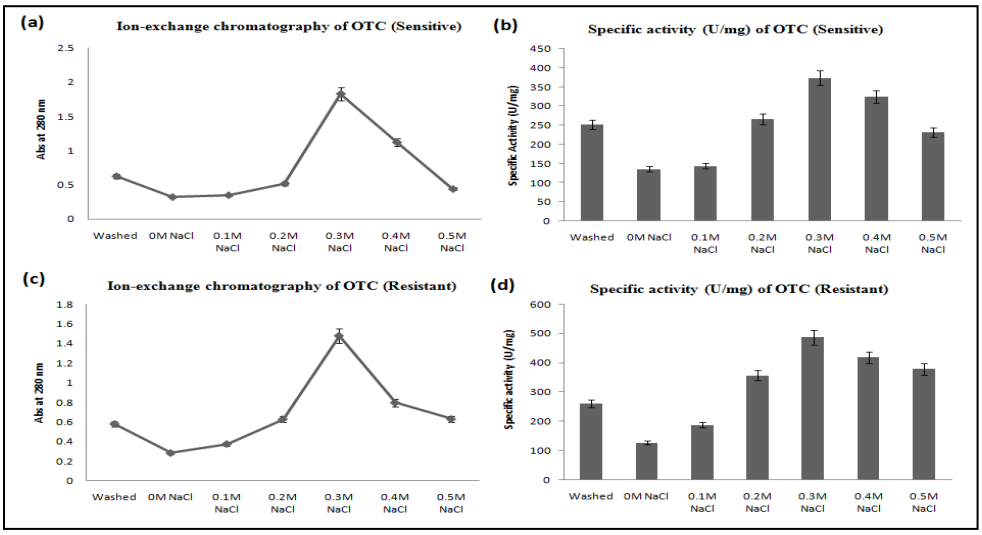

Figure 2. Ion-exchange chromatography of OTC; Ion-exchange chromatography using DEAE-Sephadex fast flow anion exchanger column and specific activity (U/mg) estimation of the OTC enzyme; active fraction collection during ion-exchange chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

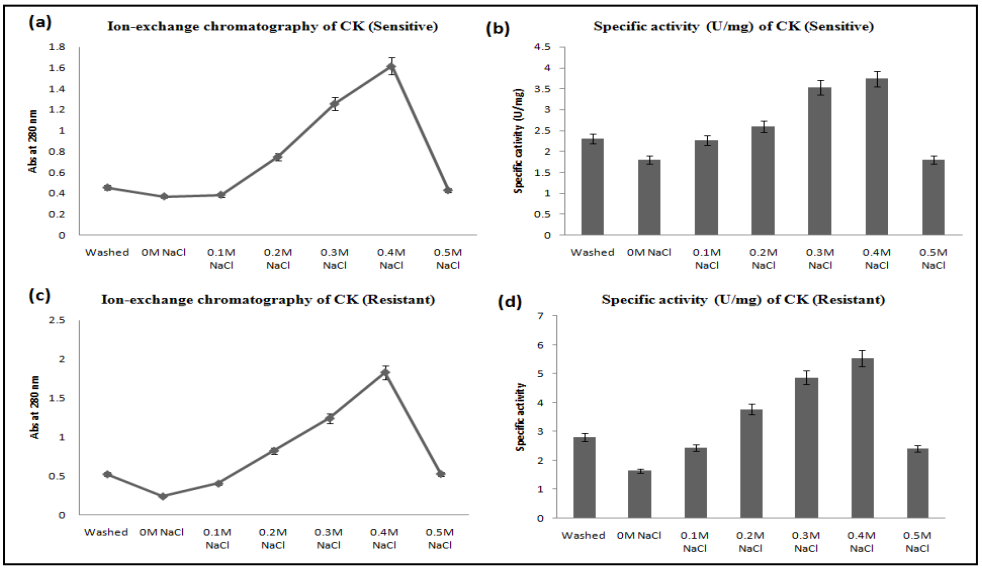

Figure 3. Ion-exchange chromatography of CK; Ion-exchange chromatography using DEAE-Sephadex fast flow anion exchanger column and specific activity (U/mg) estimation of the CK enzyme; active fraction collection during ion-exchange chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

3.3 Gel filtration chromatography

Likely, three peaks were noticed upon absorbance analysis of eluents collected during gel filtration chromatography, but maximum absorbance along with highest specific activity was noticed of the initial fractions. Results obtained with maximum absorbance and higher specific activity were shown in figure 4 (for ADI (a and b for sensitive) (c and d for resistant)), figure 5 (for OTC (a and b for sensitive) (c and d for resistant)) and figure 6 (for CK (a and b for sensitive) (c and d for resistant)).

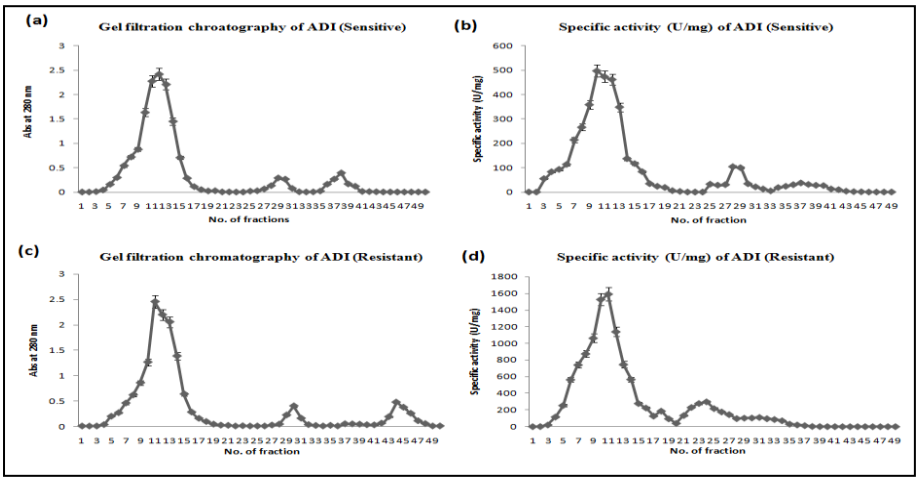

Figure 4. Gel filtration chromatography of ADI; Gel filtration chromatography using Sephadex G-150 gel filtration column and specific activity (U/mg) estimation of the ADI enzyme; active fraction collection during gel filtration chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

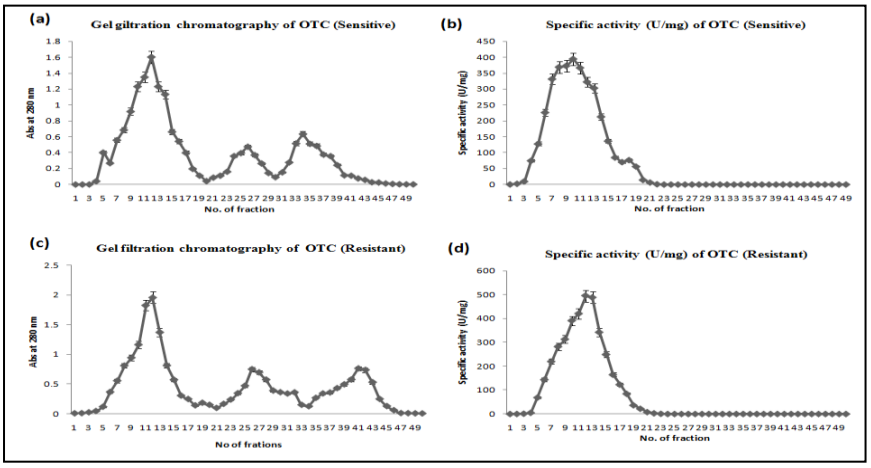

Figure 5. Gel filtration chromatography of OTC; Gel filtration chromatography using Sephadex G-150 gel filtration column and specific activity (U/mg) estimation of the OTC enzyme; active fraction collection during gel filtration chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

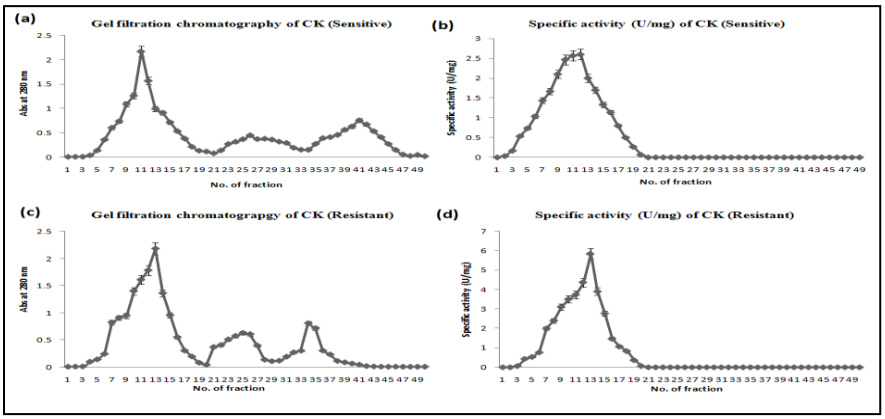

Figure 6. Gel filtration chromatography of CK; Gel filtration chromatography using Sephadex G-150 gel filtration column and specific activity (U/mg) estimation of the CK enzyme; active fraction collection during gel filtration chromatography (a for sensitive and c for resistant) and specific activity estimation (b for sensitive and d for resistant).

3.4 Enzyme characterization by SDS- PAGE profiling

During SDS-PAGE analysis of the purified enzymes of pediocin-sensitive and resistant strain, a sharp band of single subunit of enzyme with expected molecular weight was appeared on the gel. Molecular weight 46, 39, and 37 kDa was found for ADI (1), OTC (2) and CK (3) respectively during purification (cell lysate (a); ammonium sulfate precipitation (b); ion-exchange chromatography (c) and gel filtration chromatography (d)) as shown in figure 7. SDS-PAGE profile represented the enzyme purification at every step (Fig. 8 a) and a separate band of single subunit of the purified ADI, OTC and CK was appeared after 1/10th concentrated to gel filtration fractions (Fig. 8 b) of sensitive and resistant *E. faecalis.*

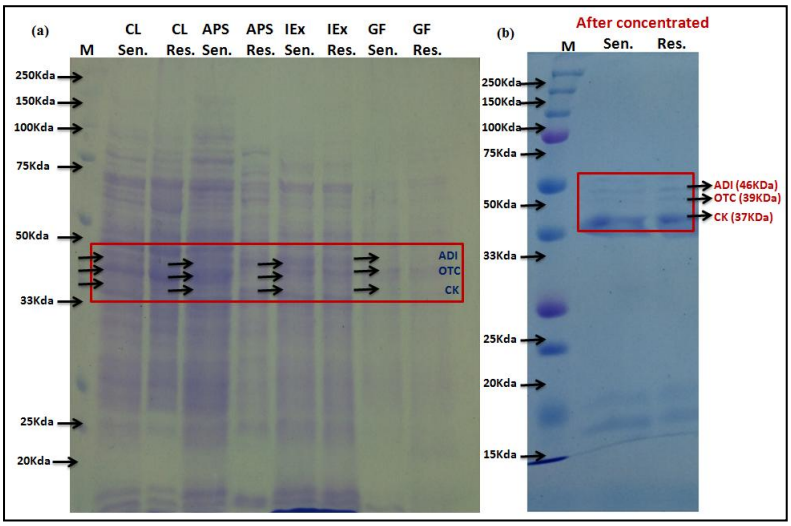

Figure 10. Purification profiling of the ADI pathway enzymes; SDS-PAGE profiling of the ADI pathway enzymes at every purification step (a) and 1/10th concentrated gel filtration fraction of each purified enzymes (b) from pediocin-sensitive and resistant strain of *E. faecalis*.

3.5 Characterization by enzymatic assay

Characterization of the purified enzymes of ADI pathway was also done by enzymatic assay and significantly ($P<0.0001$) higher activities of the enzymes of resistant strain was recorded as compared to sensitive. Enzyme activity of ADI (Fig. 9 a), OTC (Fig. 9 b) and CK (Fig. 9 c) was obtained from the assay performed in triplicate with standard error.

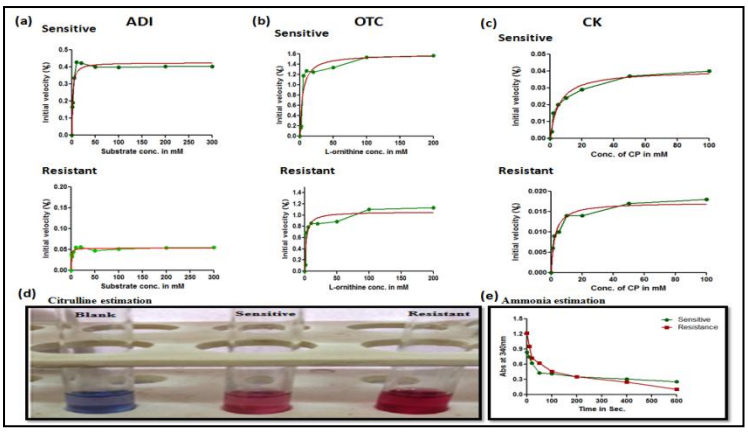

Figure 11. Enzymatic assays of the ADI pathway enzymes; Characterization of the enzymes of ADI pathway by enzymatic assay. Alteration in the enzyme activity of ADI (a), OTC (b) and CK (c) enzyme of pediocin-sensitive and resistant strain with the standard curve. The values are represented as mean ±SD of three independent experiments with the significant difference at $p< 0.001$.

3.6 Impact of substrate concentration on enzyme activity

Activity of the purified enzymes of ADI pathway was found to be affected with changing substrate concentration. Initially, increasing substrate concentration gradually enhanced the enzyme activity but continue increasing substrate concentration, resulted the enzyme saturation. This saturation level indicated the optimum

substrate concentration for maximum activity of ADI (Fig. 10 a), OTC (Fig. 10 b) and CK (Fig. 10 c) enzyme of pediocin-sensitive and resistant *E. faecalis*.

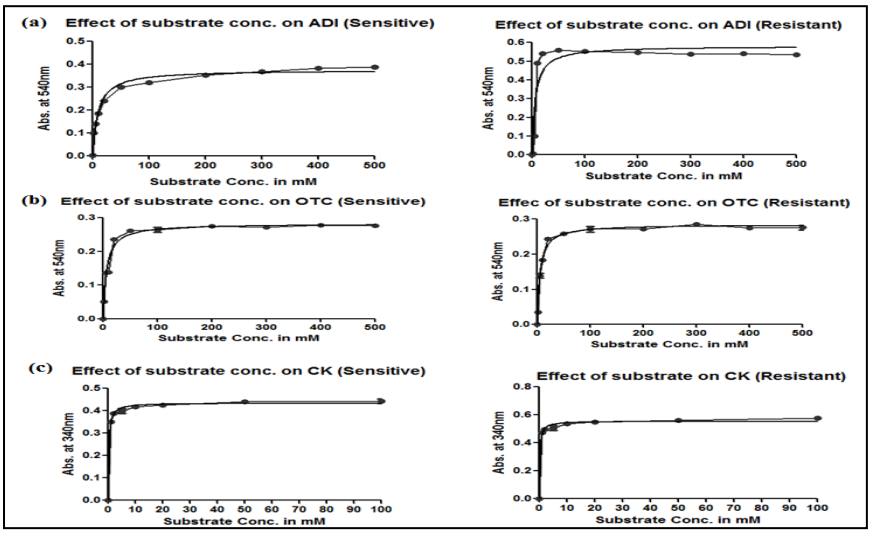

Figure 12. Effect of substrate concentration; Effect of substrate concentration on ADI (a), OTC (b) and CK (c) enzyme of ADI pathway isolated from pediocin-sensitive and resistant *E. faecalis*. The values are represented as mean ±SD of three independent experiments with a significant difference at $p< 0.001$.

3.7 Optimum pH for maximum enzyme activity

Activity of the ADI pathway enzymes purified from pediocin-sensitive and resistant *E. faecalis* was found stable at a range of pH (3-10). The optimum pH 7.0 was noticed for maximum activity of ADI (Fig. 11 a), OTC (Fig. 11 b) and CK (Fig. 11 c) and at pH 7.0, enzymes exhibited the maximum reaction absorbance and products formation.

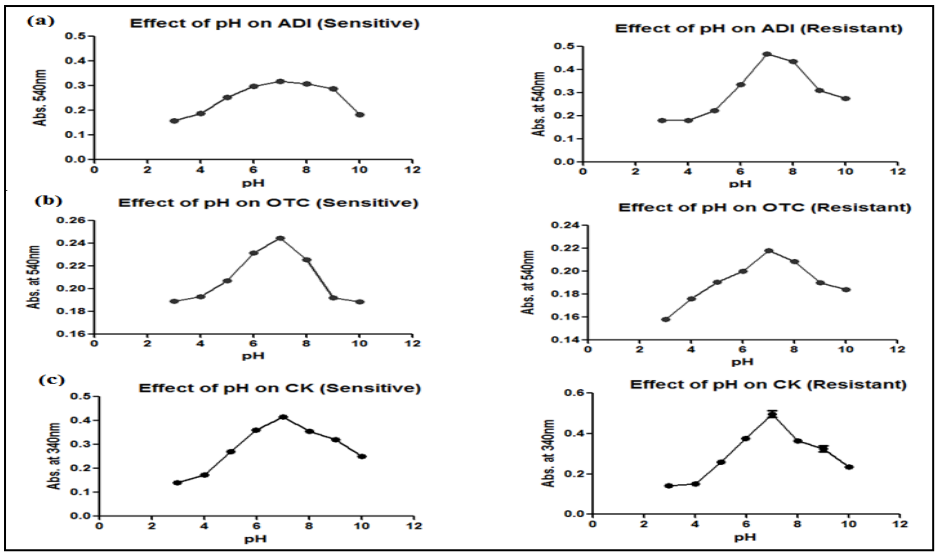

Figure 13. The optimum pH; The optimum pH for the enzymes ADI (a), OTC (b) and CK (c) of ADI pathway to estimate the maximum enzyme activity for pediocin-sensitive and resistant *E. faecalis*. The values are represented as mean ±SD of three independent experiments with a significant difference at $p< 0.001$.

3.8 Optimum temperature for maximum enzyme activity

Activity of the ADI pathway enzymes purified from pediocin-sensitive and resistant strain was observed effected at a range of temperature (10-100°C). The optimum temperature was observed 37°C for maximum activity of ADI (Fig. 12 a), OTC (Fig. 12 b) and CK (Fig. 12 c) and at 37°C temperature, enzymes exhibited the maximum reaction absorbance and products formation.

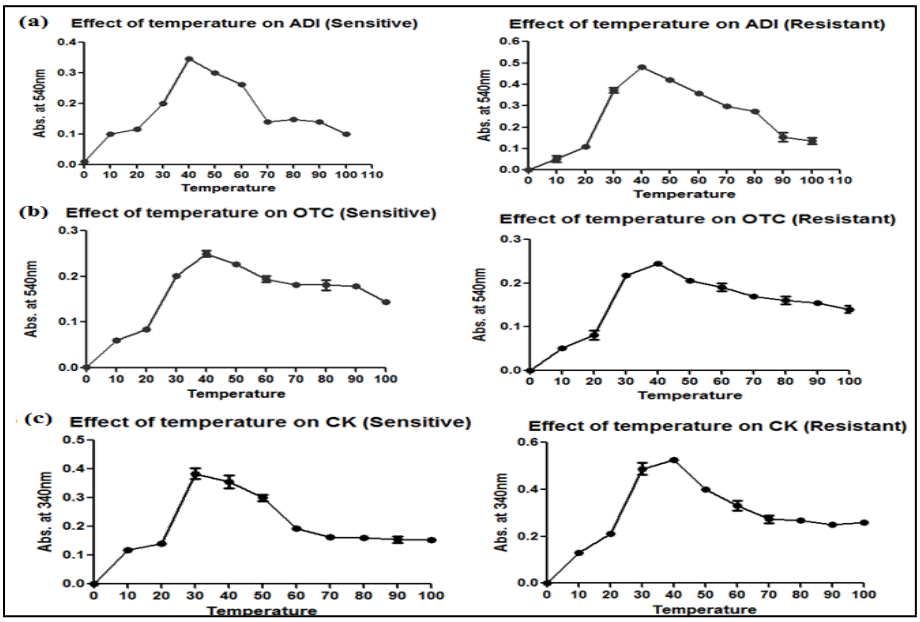

Figure 14. The optimum temperature; The optimum temperature for the enzymes ADI (a), OTC (b) and CK (c) of ADI pathway to estimate the maximum enzyme activity for pediocin-sensitive and resistant *E. faecalis*. The values are represented as mean ±SD of three independent experiments with a significant difference at p< 0.001.

IV. Discussion

ADI pathway has been talked several times to degrade arginine in many bacterial strains including; *Streptococcus faecalis* (Simon et al. 1982), *Streptococcus sanguinis* (Burne et al. 1989), *Lactobacillus sakei* (Champomier-Verges et al. 1999), *Oenococcus oeni* (Liu and Pilone, 1998), *Lactobacillus plantarum* (Arena et al. 1999) and *Lactococcus lactis* (Chou et al. 2001). Especially lactic acid bacteria (LAB) required ADI pathway for their survival in different food environment. Therefore, present study was performed specifically with enzymological aspects to investigate the role of arginine under glucose limitation for persisting resistance of *E. faecalis* against pediocin. Consequently, to purify the enzymes crude cell extract was prepared by treating cell pellets with lysozyme followed by sonication. Further, purification using crude extract was achieved with three steps including; ammonium sulfate precipitation, ion-exchange chromatography using DEAE-Sepharose fast flow anion exchanger column and gel filtration chromatography using Sephadex G-150 column. Simultaneously, total protein content, enzyme activity and specific activity of each enzyme were estimated at every purification step and purity was confirmed by enzyme assay and SDS-PAGE analysis. Previously, characterization of the ADI pathway enzymes of LAB and its effect during sourdough fermentation was majorly focused. Similarly, some heterofermentative sourdough strains such as *L. sanfranciscensis* CB1 (Gobbetti, 1998), *L. sakei* (Zuniga et al. 1998) and *L. lactis* (Chou et al. 2001) showed enzyme activity of ADI pathway. Additionally, to purify the enzymes of ADI pathway, cells treatment with lysozyme emphasized an efficient method of cell lysis than other methods (Glass beads and Triton x-100). Therefore, in this study, lysozyme treatment was used for the efficient cell lysis. Protein content, enzyme activity and specific activity of each enzyme at this initial step of purification were estimated and supported by the purification achieved from *Enterococcus faecium* M1 (Mahdy et al. 2015). The enzymes of ADI pathway were precipitated with maximum activity up to 60-70% saturation (Cheng-Fu et al. 2008) and 80% saturation (Mahdy et al. 2015) using ammonium sulfate. Similarly, in present study, ammonium sulfate precipitation was achieved up to 80% saturation to get maximum precipitation and specific activity of ADI pathway enzymes. Enzyme activity, specific activity and protein content with purification fold of the enzymes were estimated using precipitates and similar purification was achieved as from *L. sanfranciscensis* CB1 and *E. faecium* M1 (Angelis et al. 2002; Mahdy et al. 2015). Further, purification of the enzymes was performed with DEAE-Sepharose fast flow anion exchanger column and gel filtration chromatography with Sephadex G-150 column. During purification, enzymes with maximum specific activity showed similar behavior as purification achieved from *L. sanfranciscensis* CB1 and *E. faecium* M1 (Angelis et al. 2002; Mahdy et al. 2015). Simultaneously, appearance of a sharp band of single subunit of the enzyme on SDS-PAGE gel confirmed purification. The molecular mass of a single subunit of ADI, OTC and CK isolated from *L. sanfranciscensis* CB1was reported 46, 39 and 37 kDa respectively (Angelis et al. 2002). Similarly, molecular mass of ADI and OTC was reported of *L. lactis* (45.98 and 39.45 kDa) (Bolotin et al. 2001), *Staphylococcus aureus* (46.94 and 37.77 kDa) (Kuroda et al. 2001), *L.*

sakei (45.91 and 37.77 kDa) (Zuniga et al. 1998) and *B. licheniformis* (47.42 and 37.65 kDa) (Maghnouy et al. 1998) and molecular mass of CK was found to be fluctuated in the range of 33.65 to 35.53 kDa (Gobbetti et al. 1996). As per the above outcomes, the molecular mass of ADI, OTC, and CK enzyme of ADI pathway isolated from pediocin-sensitive and resistant *E. faecalis* was observed 46, 39 and 37 kDa respectively in this study. Environmental pH is an important regulator of ADI pathway (Olson, 1993; Vrancken et al. 2009) and highest catabolic activity of ADI pathway was noticed at pH 6.0 to 7.0 (Rimaux et al. 2012). Optimum pH 6.5 was noticed for ADI enzyme isolated from *P. plecoglossicida* from a wide range of pH (4.0-10) (Zhu et al. 2010). Maximum activities of the ADI pathway enzymes were observed at pH 7.0 isolated from *E. faecium* M1 (Mahdy et al. 2015) and pH 7.5 for *P. plecoglossicida* (Zhu et al. 2010). Similarly, optimum pH 7.0 was found for maximum activities of isolated ADI, OTC and CK enzyme in this work. Optimum temperature was determined for the maximum activity from a range of incubated temperatures (25-50°C) and above and below temperatures from 37°C led to decrease in enzyme activity (Ibrahim et al. 2019). Similarly, optimum temperature for maximum activity was observed 37°C for the enzymes of ADI pathway in this work. To determine the maximum activity, substrate (Arginine) concentration also noticed to hamper the activity of ADI pathway and 50 mM arginine concentration was recorded most suitable for maximum (Ibrahim et al. 2019). Likely, in the present study also enzymes of the ADI pathway got affected with increasing substrate concentration from 50 mM and become saturated.

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Conflict of Interest

There is no conflict of interest.

References

[1]. Lambert G., and Kussell E. (2014). Memory and Fitness Optimization of Bacteria under Fluctuating Environments. PLOS genetics, 10(9): e1004556.
[2]. Belkaid Y., and Hand T. (2014). Role of the Microbiota in Immunity and inflammation. Cell, 157:121-141.
[3]. Boeckx J., Hertog M., Geeraerd A., and Nicolai B. (2017). Kinetic modelling: an integrated approach to analyze enzyme activity assays. Plant Method, 13:69.
[4]. Batoni G., Maisetta G., and Esin S. (2016). Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochem Biophy Act –Biomembranes, 1858:1044-1060.
[5]. Munita JM., and Arias CA. (2016). Mechanisms of Antibiotic Resistance. Microbiol Spectr, 2.
[6]. Chou LS., Weimer BC., and Cutler R. (2001). Relationship of arginine and lactose utilization by *Lactococcus lactis* ssp. *lactis* ML3. International Dairy Journal, 11:253-258.
[7]. Oliver JD. (1993). Formation of viable but non cultivable cells. *Starvation in Bacteria, ed.* Kjelleber, 239-271.
[8]. Deibel RH. (1964). Utilization of arginine as an energy source for the growth of *Streptococcus faecalis*. Journal of Bacteriology, 87:988-992.
[9]. Stuart MR., Chou LS., and Weimer BC. (1999). Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. lactis. Applied and Environmental Microbiology, 65:665-673.
[10]. Townsend DE., Kaenjak A., Jayaswal RK., and Wilkinson BJ. (1996). Proline is biosynthesized from arginine in *Staphylococcus aureus*. Microbiology, 142:1491-1497.
[11]. Morita H., Yoshikawa H., Sakata R., and Nagata Y. (1997). Synthesis of nitric oxide from the two equivalent guanidino nitrogens of L-arginine by *Lactobacillus fermentum*. Journal of Bacteriology, 179:7812-7815.
[12]. Zuniga M., Champomier-Verges M., Zagorec M., and Perez-Martinez G. (1998). Structural and functional analysis of the gene cluster encoding the enzymes of the arginine deiminase pathway of *Lactobacillus sake*. Journal of Bacteriology, 180:4154-4159.
[13]. Noens EEE., and Lolkema JS. (2017). Convergent evolution of the arginine deiminase pathway: the ArcD and ArcE arginine/ornithine exchangers. Microbiology open, 6:e00412.
[14]. Gallego P., Planell R., Benach J., Querol E., Perez-Pons JA., and Reverter D. (2012). Structural characterization of the enzymes composing the arginine deiminase pathway in *Mycoplasma penetrans*. PLoS One 7, e47886.
[15]. Casiano-Colon A., and Marquis R. E. (1988). Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Applied and Environmental Microbiology, 54:1318-1324.
[16]. Tonon T., and Lonvaud-Funel A. (2000). Metabolism of arginine and its posi-tive effect on growth and revival of *Oenococcus oeni*. Journal of Applied Microbiology, 89:526-531.
[17]. Lindgren JK., Thomas VC., Olson ME., Chaudhari SS., Nuxoll AS., Schaeffer CR., Lindgren K., Jones EJ., Zimmerman MC., Dunman PM., Bayles KW., and Fey PD. (2014). Arginine Deiminase in *Staphylococcus epidermidis* Functions To Augment Biofilm Maturation through pH Homeostasis. Journal of Bacteriology, 196:2277-2289.
[18]. Champomier-Verges MCC., Zuniga M., Morel-Deville F., Perez-Martinez G., Zagorec M., and Erlich SD. (1999). Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*. FEMS Microbiology Letter, 180:297-304.
[19]. Kim RH., Coates JM., Bowles TL., McNerney GP., Sutcliffe J., Jung JU., Gandour-Edwards R., Chuang FY., Bold RJ., and Kung HJ. (2009). Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Research, 69:700-708.
[20]. Angelis MD., Mariotti L., Rossi J., Servili M., Fox P. F., Rollan G., and Gobbetti M. (2002). Arginine catabolism by sourdough lactic acid bacteria: purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1. Applied and Environmental Microbiology, 68:6193-6201.
[21]. Cheng-Fu T., Kai L., Jia-You L., Qing-Cai J., Qian L., and Tao Y. (2008). The Research of Enzymology Characterization about Arginine Deiminase from *Enterococcus faecalis*. Journal of Bacteriology, 35, 846-850.

[22]. Mahdy N., Al-Tahan S., Al-Tahan S., and Yaseen N. (2015). Extraction, purification and characterization (A) of arginine deiminase enzyme from local higher productive isolation of *Enterococcus faecium* M1. Extract, 5:10.

[23]. Rimaux T., Riviere A., Illeghems K., Weckx S., De-Vuyst L., and Leroy F. (2012). Expression of the Arginine Deiminase Pathway Genes in *Lactobacillus sakei* is Strain Dependent and Is Affected by the Environmental pH. Applied and Environmental Microbiology, 78:4874-4883.

[24]. Kumar S., Devi S., Sood SK., Kapila S., Narayan KS., and Shandilya S. (2019). Antibiotic resistance and virulence gene s in nisin–resistant *Enterococcus faecalis* isolated from raw buffalo milk modulate the innate functions of rat macrophages. Journal of Applied Microbiology, 127:897-910.

[25]. Veith N., Solheim M., Van-Grinsven KWA., and Olivier BG. (2015). Using a genome-scale metabolic model of *Enterococcus faecalis* V583 to assess amino acid uptake and its impact on central metabolism. Applied and Environmental Microbiology, 81:1622-1633.

[26]. Lowry OH., Rosebrough NJ., Farr AL., and Randall RJ. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193:265-275.

[27]. Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680-685.

[28]. Ruepp A., Muller HN., Lottspeich F., and Soppa J. (1995). Catabolic ornithine transcarbamylase of *Halobacterium halobium* (salinarium): purification, characterization, sequence determination, and evolution. Journal of Bacteriology,177:1129-1136.

[29]. Crow VL., and Thomas TD. (1982). Arginine metabolism in lactic streptococci. Journal of Bacteriology, **150**:1024-1032.

[30]. Fliege R., Tong S., Shibata A., Nickerson KW., and Conway T. (1992) The Entner-Doudoroff Pathway in *escherichia coli* is induced for oxidative glucose metabolism via Pyrroloquinoline Quinone-Dependent Glucose Dehydrogenase. Applied and Environmental Microbiology, 12:3826-3829.

[31]. Liu SQ., Pritchard GC., Hardman MJ., and Pilone GJ. (1995). Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. Applied and Environmental Microbiology, 61:310-316.

[32]. Simon JP., Wargnies B., and Stalon V. (1982). Control of enzyme synthesis in the arginine deiminase pathway of *Streptococcus faecalis*. Journal of Bacteriology, 150:1085-1090.

[33]. Burne RA., Parsons DT., and Marquis RE. (1989). Cloning and expression in *Escherichia coli* of the genes of the arginine deiminase system of *Streptococcus sanguis* NCTC 10904. Infection Immunology, 57:3540-3548.

[34]. Liu SQ., and Pilone GJ. (1998). A review: arginine metabolism in wine lactic acid bacteria and its practical significance. Journal of Applied Microbiology, 84:315-327.

[35]. Arena ME., Saguir F., and Manca de-Nadra MC. (1999). Arginine dihydrolase pathway in *Lactobacillus plantarum* from orange. Internation Journal of Food Microbiology, 47:203-209.

[36]. Gobbetti M. (1998). The sourdough microflora: interactions between lactic acid bacteria and yeast. Trends in Food Science and Technology, 9:267-274.

[37]. Bolotin M., Wincker P., Mauger S., Jaillon O., Malarme K., Weissenbach J., Ehrlich SD., and Sorokin A. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Research, 11:731-753.

[38]. Kuroda M., Ohta T., Uchiyama I., Baba T., Yuzawa H., Kobayashi I., Cui L., Oguchi A., Aoki K. I., Nagai Y., Lian J., Ito T., Kanamori M., Matsumaru H., Maruyama A., Murakami H., Hosoyama A., Mizutani-Ui Y., Takahashi N. K., Sawano T., Inoue R. I., Kaito C., Sekimizu K., Hirakawa H., Kuhara S., Goto S., Yabuzaki J., Kanehisa M., Yamashita A., Oshima K., Furuya K., Yoshino C., Shiba T., Hattori M., Ogasawara N., Hayashi H., and Hiramatsu K. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet, 357:1225-1240.

[39]. Maghnouy A., Sousa de-Cabral TG., Stalon V., and Vander-Wuven C. (1998). The *arcABDC* gene cluster encoding the arginine deiminase pathway of *Bacillus licheniformis* and its activation by the arginine repressor *argR*. Journal of Bacteriology, 180: 6468-6475.

[40]. Gobbetti M., Smacchi E., Fox PF., Stepaniak L., and Corsetti A. (1996). The sourdough microflora, Cellular localization and characterization of proteolytic enzymes in lactic acid bacteria. Lebensmittel-Wissenschaft Technologie , 29:561-569.

[41]. Olson ER. (1993). Influence of pH on bacterial gene expression. Molecular Microbiology, **8**:5-14.

[42]. Vrancken G., Rimaux T., Weckx S., and De-Vuyst L. L. (2009). Environmental pH determines citrulline and ornithine release through the arginine deiminase pathway in *Lactobacillus fermentum* IMDO 130101. International Journal Food Microbiology, 135:216-222.

[43]. Zhu I., Verma R., Roccatano D., Ni Y., Sun ZH., and Schwaneberg U. (2010). A Potential Antitumor Drug (Arginine Deiminase) Reengineered for Efficient Operation under Physiological Conditions. Chembiochemistry, 11(16):2294-2301.

[44]. Ibrahim MM., Alhamami HN., and Briski KP. (2019). Norepinephrine regulation of ventromedial hypothalamic nucleus metabolic transmitter biomarker and astrocyte enzyme and receptor expression: impact of 5′ AMP-activated protein kinase. Brain Research, 17(11):48-57.