To accomplish the objectives of the present investigation, the work was divided into following sections:

3.1 Isolation of a bacterial strain capable of producing an Arginine Deiminase from various food products.

3.2 Biochemical and molecular characterization of the bacterial isolate.

3.3 Characterization of Arginine Deiminase gene.

3.4 Optimization of upstream procedures for production of Arginine Deiminase at flask level.

3.5 Purification and downstream processing of Arginine Deiminase.

3.6 To assess *in vitro* cytotoxicity of purified Arginine Deiminase against human Hepatocellular Carcinoma Cell line by studying apoptosis.

3.1 Isolation of a bacterial strain capable of producing an Arginine Deiminase from various food products.

3.1.1 Procurement of chemicals:

All the chemicals and reagents were of analytical grade and procured from Glaxo India Limited, Hi-Media, Sigma, Sisco Research Laboratories, Pharmacia, Novagen, S.D. Fine Chemicals Limited, Merck India Ltd., Qualigens Fine Chemicals, Merck Specialities Private Limited- Genei and Bioserve.

3.1.2 Preparation of culture media:

3.1.2.1 Modified MRS medium:

Medium is based on the formulations of de Man, Rogosa and Sharpe (MRS) (**table 3.1**). Modified MRS broth is prepared by addition of 5% (*w/v*) yeast extract and 28 mM maltose in

MRS media (De Angelis *et al*., 2002). This enriched medium was used for revival of lactic acid bacteria from dairy and milk products.

Table 3.1: Composition of MRS medium

Ingredients	Quantity(g/l)
Dextrose	20
Beef extract	10
Peptone	10
Yeast extract	5
Sodium acetate	5
Di-potassium phosphate	2
Tri-ammonium citrate	2
Magnesium sulphate	0.1
Manganese sulphate	0.05
Tween80	1ml
pH	5.6±0.2

3.1.2.2 MAM medium:

This enriched medium was used for the isolation of arginine hydrolyzing micro flora (De Angelis *et al*., 2002). Composition of MAM medium is given in **table 3.2**.

Table 3.2: Composition of MAM medium

Ingredients	Quantity(g/l)
Tryptone	10
Glucose	5
Yeast extract	5
Arginine	3
Di-hydrogen potassium phosphate	0.5
Magnesium sulphate	0.2
Manganese sulphate	0.05
Tween80	1ml
pH	5.6±0.2

3.1.2.3 TGYE medium:

This enriched medium was usually used for propagation of *Enterococcus* species (Shekh and Roy, 2012). Composition of TGYE medium is given in **table 3.3**.

Table 3.3: Composition of TGYE medium

Ingredients	Quantity(g/l)
Tryptone	5
Glucose	1
Yeast extract	3
pH	7.0±0.2

3.1.2.4 *Enterococcus* Confirmatory Broth:

This enriched medium *Enterococcus* Confirmatory Broth was recommended for confirming the presence of *Enterococci* in water supplies and other sources (Edwards and Baker, 1990). Composition of *Enterococcus* Confirmatory Broth is given in **table 3.4**.

Table 3.4: Composition of *Enterococcus* Confirmatory Broth

Ingredients	Quantity(g/l)
Casein enzymic hydrolysate	5
Dextrose	5
Yeast extract	5
Sodium azide	0.40
Methylene blue	0.01
pH	8.0±0.2

3.1.2.5 M9 Minimal Salt Media:

The components M9 Minimal Salt media supply the required nutrients for proper bacterial growth (Davis *et al*., 1986). Composition of M9 Minimal Salt media is given in **table 3.5**.

Table 3.5: Composition of M9 Minimal Salt Media

Ingredients	Quantity(g/l)
Disodium hydrogen phosphate	33.90
Dextrose	20
Potassium dihydrogen phosphate	15
Sodium chloride	2.5
Ammonium chloride	5.0
Magnesium sulphate	0.4
Calcium chloride	0.01
pH	7.0±0.2

3.1.3 Preparation of Standard curves:

3.1.3.1 Standard curve of ammonia:

0.5mg/ml stock solution of ammonium chloride was prepared. Different concentrations of ammonium chloride ranging from 10-70 µg of were taken and final volume was made 1ml. Optical density was determined at 450nm using UV-VIS spectrophotometer (Imada *et al*., 1973).

3.1.3.2 Standard curve of citrulline:

1mg/ml stock solution of citrulline was prepared in 50 mM acetate buffer (pH 5.5). Different concentrations of citrulline ranging from 10-100 µg were taken and final volume was made1ml using acetate buffer. 1.5ml of acid mix and 250 µl of diacetyl monoxime were added to the above solution and boiled in dark for 30 min. Solution was cooled for 10min and optical density was determined at 460nm using spectrophotometer (De Angelis *et al*., 2002).

3.1.3.3 Standard curve of protein:

A standard curve for protein estimation was made by Lowry's method (Lowry *et al*., 1951).

Principle: The intense blue color that develops is due to the coordination of peptide bonds with alkaline copper (Biuret reaction) and the reduction of the Folin-Ciocalteau reagent (phosphomolybdate-phosphotungustate) by tyrosine and tryptophan residues in the protein. The protein was estimated by Folin-Lowry method. The detailed procedure is given as under:

Reagent A: Copper sulphate (0.5g) and Sodium potassium tartarate (1.0g) were dissolved in 100 ml distilled water.

Reagent B: Sodium carbonate (2%) was prepared in 0.1 N NaOH.

Reagent C: The reagent B and A were mixed in the ratio of 50:1.

Reagent D: Folin-Ciocalteau reagent was prepared by mixing water with Folin-Ciocalteau in ratio 1:1 with water.

Procedure: To the different (10-100 µg) dilutions of protein solution was added 5 ml of reagent C for 10 minutes. Then 0.5 ml of reagent D was added and after 30 minutes the absorbance of the blue color was detected at 660 nm wavelength.

3.1.4 Screening and isolation of arginine catabolizing LAB strains:

For screening and isolation of arginine-catabolizing LAB strains, various dairy products were collected from Patiala (Punjab). Samples were mixed well with saline (0.85 % NaCl) and processed immediately for isolation of lactic acid bacteria. LAB strains were multiplied in enrichment media, i.e., modified MRS medium as described by De Angelis *et al*., (2002). The pure and healthy colonies of bacterial strains were further isolated by streaking on enrichment medium, i.e., MAM agar plates (De Angelis *et al*., 2002). The optical densities of the liquid cultures in MAM broth were adjusted to 1.0. Inoculum size of 1 % (*v/v*) was used in various assays. Cultures were incubated at 37 °C for 24 h and subcultured thrice in MAM broth, before being subjected to the ADI assay.

3.1.4.1 Arginine deiminase (ADI) activity assay:

To determine enzyme activity, cultures grown for 24 h were centrifuged at 8,000 rpm for 10 min. Cell-free supernatant was assayed for extracellular protein and enzyme activity. $O.D_{600}$ of culture broth was adjusted to 1.0 before assaying enzyme activity. For assaying intracellular ADI activity, cell pellets were resuspended in lysis buffer (BugBuster Protein Extraction Reagent, Novagen). BugBuster Protein Extraction Reagent consists of benzonase in 50% glycerol and 50 mM Tris-HCl pH-8.0, 20 mM NaCl, and 2 mM $MgCl_2$. 1 ml culture pellet was suspended in 300 µl lysis buffer. Total protein was estimated by Lowry's method and quantified using a standard curve of bovine serum albumin.

3.1.4.2 Nesslerization method (Imada *et al*., 1973):

Preliminary screening of arginine catabolizing LAB strains was carried out by quantitative analysis of ammonia production by Nesslerization method (Imada *et al*., 1973). The standard assay mixture consisted of 200µl of 0.04M arginine, 100µl of 0.05M tris-HCl buffer (pH 7.2), and 100µl distilled water. 100µl crude enzymes were obtained by centrifugation of culture at 8,000 rpm for 10 min. Controls without substrate and without enzyme were included. After incubation at 37^{o}C for 1h, reaction was stopped by adding 100µl of 1.5M trichloroacetic acid (TCA). 100µl of above mixture was mixed with 750µl distilled water and 500µl of Nessler's reagent and incubated at 30^{o}C for 20 min. Ammonia production was estimated by measuring absorbance at 450nm. One unit of ADI is the amount of enzyme which catalyzed the formation of 1 µmol of ammonia per min at 37^{o}C (Imada *et al*., 1973).

3.1.4.3 Citrulline assay (De Angelis *et al*., 2002):

The confirmatory assay of ADI activity was performed according to the method described by De Angelis et al. (2002). Under standard conditions, the reaction mixture consisted of 150 µl of 50mM arginine, 2.3 ml of 50mM acetate buffer (pH 5.5), 50 µl of cell wall or cytoplasm preparation, and 3.6 µl of sodium azide (final concentration, 0.05 % *w/v*). Controls without substrate and without enzyme were included. After incubation at 37 °C for 1 h, reaction was stopped by adding 0.5 ml of a solution of 2N HCl, and precipitated protein was removed by centrifugation. The citrulline content of the supernatant was determined by the method described by Archibald (1944). One milliliter of 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 butanedione monoxime) in 10 % (*v/v*) methanol, mixed and then boiled in the dark for 30 min. After cooling for 10 min, the absorbance was measured at 460 nm. One enzyme unit was defined

as the amount of enzyme required to catalyze formation of 1 μmol citrulline per min. Finally, specific ADI activity was calculated as International Enzyme Units present per mg (IU/mg) of protein (De Angelis *et al*., 2002).

3.1.4.4 Citrulline estimation by HPLC (Bai *et al*., 2007):

Formation of citrulline was estimated qualitatively and quantitatively by High-Performance Liquid Chromatography (HPLC) (Shimadzu, UV detector, column C-18, length- 25 cm, and ID-4.6 mm), according to the method given by Bai *et al*., (2007). The mobile phase consisted of acetonitrile: 0.03 M potassium phosphate (20:80), pH 3.2 at the flow rate of 0.5 ml/min with 30 °C column temperature and 20 μl sample injection volume. Citrulline was used as standard.

3.2 Biochemical and molecular characterization of the bacterial isolate.

3.2.1 Identification of microorganism at Generic level:

Preliminary identification of the isolates was carried out according to Bergey's Manual of Determinative Bacteriology, Ninth Edition (Holt *et al*., 1994). Different morphological and physiochemical properties studied include:

1. Gram-Stain reaction: Gram-staining is a quick procedure used to characterize bacteria as Gram-positive or Gram-negative, based on the chemical and physical properties of their cell walls.

Reagents:

Primary Stain	-	Crystal Violet
Mordant	-	Grams Iodine
Decolourizer	-	Ethyl Alcohol
Secondary Stain	-	Safranin

Procedure:

With a sterile cooled loop, a loopful of the broth culture was placed on the slide and spreaded by means of circular motion of the inoculating loop to about one centimeter in diameter. The smear was air-dried by passing the entire slide through the flame of a Bunsen burner two to three times with the smear-side up. Smear was gently flooded with crystal violet and allowed to stand for 1 minute. The slide was tilted and gently rinsed with tap water using a wash bottle. Smear was gently flooded with Gram's iodine and allowed to stand for 1 minute. The slide was tilted and gently rinsed with tap water using a wash bottle. The smear appeared as a purple circle on the slide and it was decolorized using 95% ethyl alcohol. Tilted the slide slightly and applied the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear and immediately rinsed with water. Gently flooded with safranin to counter-stain and allowed to stand for 45 seconds. The slide was tilted slightly and gently rinsed with distilled water using a wash bottle and blot dried the slide with bibulous paper. The smear was viewed using a light-microscope under oil-immersion.

2. Morphological Examination: Gram stained slide of the isolates were viewed at 100X magnification under oil immersion and the most common appearance of the cells and their predominant arrangements was observed using binocular microscope.

3. Motility: A drop of culture was poured on a sterile and clean glass slide. Cover slip was placed on the culture drop and viewed under the microscope at 100X magnification and observed for wriggling movements of cells.

4. Growth at Different Temperatures: Growth pattern of isolate at different temperatures were studied. MAM broth was inoculated at 1 % (*v/v*) inoculum and initial pH of the media

was set 6.0. Cultures were incubated at different temperatures viz. 4, 15, 25, 30, 37, 42, 55°C for 24h. $O.D_{600}$ was measured using a UV-VIS spectrophotometer after 24h of culturing and diluting the pelleted cells with 1 ml normal saline.

5. Growth at different pH: MAM broths were adjusted to pH 5, 7.5 and 12, autoclaved and inoculated at 1 % (*v/v*) inoculum of the LAB isolate. After 24h of the incubation at 37°C, growth was measured by taking OD of 1ml pelleted cells (resuspended in equal volume of saline) at 600nm using a UV-VIS spectrophotometer.

6. Growth in the presence of 6.5% (w/v) NaCl: MAM broth (pH 6.5) was prepared and NaCl was added so as to get a final concentration of 6.5%. It was inoculated with 1% (*v/v*) inoculums of the LAB strain and incubated at 37°C for 24h. After incubation was over, growth was compared with controls lacking inoculums, by measuring OD_{600}.

7. Methyl Red test: Methyl Red test was carried out to study the ability of strain to perform mixed-acid fermentation. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. After incubation, the pH indicator methyl red is added to the broth. Methyl red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and would be considered a negative result (Smibert and Krieg, 1994).

8. Voges-Proskaeur test: Voges-Proskaeur test was used to detect the production of acetoin (acetyl methyl carbinol). It was performed by inoculating in MRVP (Methyl-Red-Voges-Proskauer) broth with bacterial culture and incubated at 37 °C for 48 h. When 1 ml of potassium hydroxide and 3 ml of 5 % solution of α-napthol in absolute alcohol was added, a

development of pink color in 2-5 min and crimson in 30 min indicate positive reaction (Smibert and Krieg, 1994).

9. Production of ammonia from arginine:

Production of ammonium from arginine was assessed in arginine broth. Incubation of test samples of the culture with Nessler's reagent (0.09 mol/l of potassium tetraiodomercurate in 2.5 mol/l of potassium hydroxide), a yellow or orange color indicates positive test (Niven *et al*., 1942).

10. Catalase reaction: The reaction was carried out using standard methodology of Smibert and Krieg, (1994). To 0.5 ml culture broth of each LAB strain, an equal volume of 3% (*v/v*) H_2O_2 was added and observed for bubbling immediately after addition of H_2O_2 and after 5 min of H_2O_2 addition. Bubbling in the reaction indicates a positive catalase activity.

11. Oxidase test: The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to purple color product (indophenols) and in the absence of an enzyme; the reagent is reduced and become colorless. Oxidase test was performed by adding 1% (*v/v*) tetramethyl-p-phenylene diamine dihydrochloride to a test tube containing 24h old culture (Niven et al 1942).

12. Citrate utilization test: Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as only carbon source and inorganic ($NH_4H_2PO_4$) as sole fixed nitrogen source. Simmons citrate agar (0.2% sodium citrate, ammonium dihydrogen phosphate and bromophenol indicator) is inoculated and incubated at 37 °C for 24 h and change from green to blue in the medium indicates a positive test for growth using citrate (Tang *et al*., 1998).

13. Indole production test: Some bacteria have the ability to breakdown tryptophan using enzyme tryptophanase. When tryptophan was broken down, the presence of indole test can be detected by colorimetric reaction with a Kovac's reagent (p-dimethyl-aminobenzaldehyde). Indole test was performed using tryptone broth. Peptone water (10 g/l-peptone and 5 g/l-sodium chloride) was inoculated and incubated overnight. After 24h Kovac's reagent (2-3 drops) was added and formation of red color ring in alcohol layer indicates positive test whereas, yellow colors ring (color of Kovac's reagent) indicates negative test (Smibert and Krieg, 1994).

14. Sugar fermentation profile: The ability of an organism to ferment carbohydrates in a medium results in production of acid or acid and gas, used to characterize a specific species of bacteria. Fermentation reactions are detected by the color change of a pH indicator when acid products are formed and Durham tubes are used to detect gas production. The sugar fermentation profile of isolate was checked for glucose (1%), lactose (5%), mannitol (1%), sucrose (1%), dulcitol (1%), rhamnose (1%), sorbitoland (1%), xylose (1%) and trehalose (1%) by adding 1 ml of Andrades indicator in MRS broth containing inverted Durham's tube (Smibert and Krieg, 1994). Appearance of pink to yellow color (pH 5.0-8.0) indicates sugar fermentation and bubbles in Durham tubes depicts gas formation.

3.2.2 Molecular characterization of LAB isolate:

The 16SrRNA gene sequencing of the selected GR7 isolate was carried out at MTCC, IMTECH, Chandigarh (India). The sequence was aligned with a non-reductant DNA database (blast.ncbi.nlm.nih.gov) and sequence homologies were studied using the CLUSTALW program (srs.ebi.ac.uk/ClustalW). Pairwise evolutionary distances of the homologs were calculated using Kimura's two-parameter model (Kimura, 1980). A

phylogenetic tree was constructed from distance matrices by the Neighbor-Joining method, using MEGA 5.10 software (Tamura *et al*., 2011).

3.3 Characterization of Arginine Deiminase gene.

3.3.1 Isolation of genomic DNA:

Genomic DNA was isolated from *E. faecium* GR7 using the GeNei ™ Bacterial DNA purification kit. 1 ml culture broth was taken in an eppendrof tube under proper sterile conditions. Tubes were centrifuged for 10 minutes at 6,000 g, 4°C to form a pellet. Supernatant was discarded and pellet was collected. Pellet was washed twice in 1X TE buffer (containing Tris base-0.12g/l, EDTA-0.037g/l; pH-8). Pellet was resuspended in 1ml of 2 mg/ml lysozyme solution. Sample was incubated for 2h at room temperature. Sample was gently flipped after every 10 minutes. 100µl of 10% SDS and 150µl of 10 mg/ml Proteinase K was added and the suspension was incubated at 37 °C for 30 min. Supernatant was collected by centrifugation at 10,000 rpm for 5 minute at 4°C and transferred into a sterile tube. It was mixed with an equal volume of phenol:chloroform: isoamyl alcohol mixture (25:24:1 *v/v*). The tube was gently flipped several times. Sample was centrifuged at 14,000 rpm for 10 minutes at 4°C to precipitate the genomic DNA. Supernatant was decanted and 500µl of 95% ethanol was added to the sample, which was then centrifuged at 12,000 rpm for 5 minutes. Supernatant was discarded and 500µl of 70% ethanol was added to the sample. Centrifugation was done at 12,000 rpm for 5 minute. Supernatant was discarded and pellet was dried at 37°C for 1- 2 hours. The pellet was suspended in 50µl of 1X TE buffer and stored at -20°C. Extracted genomic DNA was analyzed by running 0.8% (*w/v*) agarose gel electrophoresis.

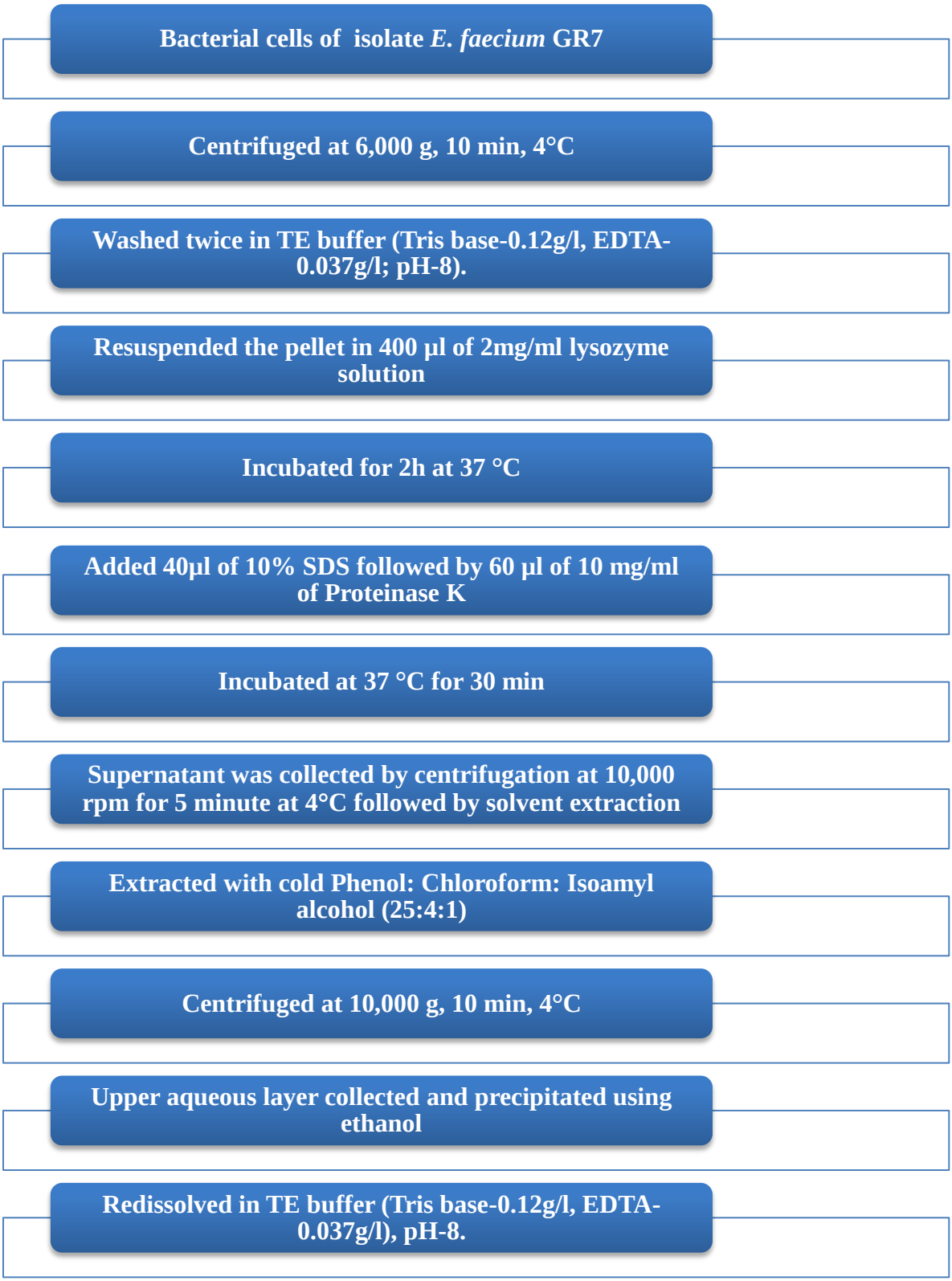

Fig. 3.1: Flow chart of genomic DNA isolation technique

3.3.2 Designing PCR primers for amplification of *arcA*, *arcB* and *arcC* genes from genomic DNA of LAB isolate *E. faecium* GR7:

E. faecium Aus 0004, *arcA*, *arcB* and *arcC* gene sequences (Genbank no. CP003351.1) were retrieved from NCBI. For the amplification of 3906 kb sequence, 2 pairs of primers were designed, one set was designed for the amplification of 1,260 bp region containing *arcA* gene and the second set was designed to amplify 2,750 bp region containing *arcB* and *arcC* genes using Gene Runner 3.05 (Hastings Software Inc.), PCR Primer Stat and PCR Product Tools of Sequence Manipulation Suite (SMS). Various factors like length of primers, primer melting temperature, primer annealing temperature, percent GC content, GC Clamp, primer secondary structures (hairpins, self-dimer, bulge loops, internal loops), repeats, runs and 3' end stability were kept in mind for primer designing.

3.3.3 PCR amplification of partial *arc* operon of *E. faecium* GR7 bearing *arcA*, *arcB* and *arcC* genes:

PCR amplification was carried out using designed primer set including forward primer 1 (5'-gactgccgtcaggaaacaaattacttcaag-3') and reverse primer 1 (5'- gcaacacaacatggataaacctattcacg-3') to amplify 1260 bp containing *arcA* (**table 3.6**); forward primer 2 (5'-cgttgtatcttcttcattcggcttcc-3') and reverse primer 2 (5'- caatcgctccaggtgttgtagtcact-3') to amplify 2750 bp containing *arcB* and *arcC* genes (**table 3.7**). DNA sequences bearing partial arc operon were amplified using Taq DNA polymerase and a Techne programmed thermal cycler according to parameters depicted in (**table 3.8 and 3.9)**. PCR products were analyzed in 1.2% agarose and after ethidium bromide staining, visualized on UV transilluminator. Amplicons of 1.2 kb and 2.5 kb were further sequenced by GenScript, New Jersey, USA.

Table 3.6 Sequence attributes of Primer set 1 used for the amplification of *arcA* gene

PARAMETERS	FORWARD PRIMER	REVERSE PRIMER
Sequence	5'-gactgccgtcaggaaacaaattacttcaag-3'	5'- gcaacacaacatggataaacctattcacg-3'
Length	30	29
Tm(°C)	60	59
% GC	43.33	41.38
Hairpin	0	0

Table 3.7 Sequence attributes of Primer set 2 used for the amplification of *arcBC* gene

PARAMETERS	FORWARD PRIMER	REVERSE PRIMER
Sequence	5'- cgttgtatcttcttcattcggcttcc-3'	5'- caatcgctccaggtgttgtagtcact-3'
Length	26	26
Tm(°C)	58	60
% GC	46.15	50
Hairpin	0	0

Table 3.8: Composition of the PCR reaction mixture

COMPONENTS	AMOUNTS (µl)
Template	1
Forward primer	1
Reverse primer	1
***Taq* DNA polymerase (3U/µl)**	0.5
dNTPs	2
$MgCl_2$	2
10X T*aq* DNA polymerase buffer	3
Double distilled water	2
Total volume	12.5

Table 3.9: Thermal parameters used for amplification of *arcA* and *arcBC* genes

CYCLE	TEMPERATURE (°C)	TIME (min)	Number of Cycles
Denaturation	94	5	1
Denaturation	94	0.5	32
Annealing	58	0.5	
Extension	72	3	
Final Extension	72	10	1
Holding	4	5	1

3.3.4 *In silico* analysis of sequenced partial *arc* operon:

The homology analysis of the nucleotide sequences was carried out using the National Center for Biotechnology Information (NCBI)'s BLASTn program. Sequenced ADI operon fragments were aligned using the BLASTn tool (blast.ncbi.nlm.nih.gov). The ClustalW tool (srs.ebi.ac.uk/ClustalW) was used for multiple sequence alignment analysis among homologs. Various control regions of sequenced ADI genes, i.e., promoter and ribosomal binding site (RBS), encoded polypeptides or open reading frame (ORF) and terminators were identified based upon their homology with *E. faecium* Aus 0004 as a reference sequence using various bioinformatics tools such as BLASTp, the reverse translate and translation tool of SMS and the ORF finder. Stem-loops were localized with program REPEATS and their ΔG values were calculated with the program Mfold (RNA mfold, version 2.3, server (http://bioinfo.math.rpi.edu). Fingerprints of the amino acid sequence were analysed by the P-val FingerPRINTScan tool provided with PRINTS, and used to align the protein sequence to profiles derived from the PRINTS database. Multiple sequence alignment of protein was carried out using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

3.4 Optimization of upstream procedures for production of Arginine Deiminase at flask level.

The present study was aimed to screen and optimize media components that increase ADI activity of *E. faecium* GR7. RSM, formerly known as Box-Wilson methodology, is the most widely used statistical technique to evaluate relationship between a set of controllable experimental factors and observed results (Kennedy and Krouse, 1999; Lee and Chen, 1997; Liu *et al*., 2003). RSM is capable to find optimum set of experimental factors that produce maximum or minimum value of response and represent the direct and interactive effect of process variables through two-dimensional and three-dimensional graphs. Keeping in view, the great commercial application of ADI as a therapeutic agent; an attempt was made to optimize the composition of the ADI production media for *E. faecium* GR7 which was carried out in two steps. Independent experiments for the selection of most influential process parameters including fermentation media and environmental conditions were conducted followed by application of RSM involving Central Composite Design (CCD) for final optimization of media components to enhance ADI activity and biomass production using influential process variables.

3.4.1 Microorganism and culture conditions:

E. faecium GR7 was revived in MAM medium at 37 °C for 24h. Optical density of the inoculum was adjusted to 1.0 and 1% (*v/v*) culture was used in each experiment.

3.4.2 Arginine deiminase (ADI) activity assay:

To determine enzyme activity, 24 h-old cultures were centrifuged at 8,000 rpm for 10min. Cell free supernatant (CFS) was assessed for extracellular protein and enzyme activity. $O.D_{600}$ of culture broth was adjusted to 1.0 before pursuing with each activity assay. For

assaying intracellular ADI activity, cell pellet was resuspended in lysis buffer (Bug Buster Protein Extraction Reagent, Novagen). Total protein was estimated by following Lowry's method (Lowry *et al*., 1951). ADI activity was assayed using standard method of De Angelis *et al*., (2002). Citrulline content after enzyme assay was determined by Archibald's method (1984).

3.4.3 Selection of Basal Medium for ADI Production:

Six culture media including MAM (De Angelis *et al*., 2002); MRS (de Man *et al*., 1990); TGYE (Shekh and Roy, 2012); (modified) MRS broth (De Angelis *et al*., 2002); *Enterococcus* confirmatory broth (Edwards and Baker, 1990); and M9Minimal salt media (Davis *et al*., 1986) each supplemented with 15mM arginine were selected to investigate production of ADI in *E. faecium* GR7. Inoculums were subcultured twice in MAM broth at 37°C for 24h and used at 1% (*v/v*) for each experiment. ADI specific activity was determined following standard procedures of De Angelis *et al*., (2002) and Archibald (1994). Total protein was estimated by following Lowry's method (Lowry *et al*., 1951).

3.4.4 Growth profile of *E. faecium* GR7 and ADI production in TGYE basal medium:

To study the growth profile of *E. faecium* GR7 and to determine the duration of lag, log and stationary phase, TGYE was seeded with 1% (*v/v*) inoculum and incubated at 37°C for 24h. The optical density of the culture in TGYE basal medium was taken at regular intervals each at 600 nm for 24 hours. ADI specific activity of the culture in TGYE basal medium was determined by following standard procedures of De Angelis *et al*., (2002) and Archibald (1994) and total protein was estimated by following Lowry's method (Lowry *et al*., 1951).

3.4.5 Optimization of Process Parameters for Enhancing ADI Activity:

E. faecium GR7 was grown in the selected medium (TGYE) and the effect of various parameters, that is, initial pH (5.0 to 7.0), incubation temperature (25°C to 50°C), culture incubation conditions and that is, aerobic vs. anaerobic, shaking (200 rpm) vs. stationary, inoculum size (1 to 5% *v/v*) and subculturing period (1 to 4 days) on ADI production was studied individually, by varying one factor at a time. At each step, the selected factor was included in the basal medium (selected from the previous experiment) for getting a set of conditions that enhanced ADI activity in *E. faecium* GR7. Rest of the conditions and enzyme assays were as described previously.

3.4.6 Effect of Various Nutrients on Enzyme Production:

The effect of various carbon and nitrogen sources on enzyme production in *E. faecium* GR7 was investigated. In the production medium, carbon of the basal media was replaced with glucose, galactose, sucrose, maltose, lactose, and fructose, and nitrogen with yeast extract, peptone, tryptone, and beef extract which were tested at levels ranging from 5 to 20 g/L (*w/v*).

3.4.7 Effect of Inducer Concentration:

Effect of inducer concentration, that is, arginine on ADI production was studied by supplementing basal medium with different concentrations of arginine ranging from 5 to 25mM (*w/v*).

3.4.8 Effect of Salts and Surfactants on Enzyme Production:

Basal medium was supplemented with various salts (*w/v*) such as NaCl (0.5–5 g/l), K_2HPO_4 (0.5–5 g/l), $MnSO_4$ (0.2–2mM), $CuSO_4$ (0.025–0.075mM), $ZnSO_4$ (2–6mM), and surfactants

including CTAB, SDS, Tween 80, and Triton X-100 (1%) to investigate their role in improving enzyme production and secretion by *E. faecium* GR7.

3.4.9 Experimental Validation of EAPM media:

Important process variables, identified based on independent experiments, were validated under optimized conditions in triplicates. *Enterococcus* ADI production media (EAPM), composed of lactose 20 g/L; tryptone 20 g/L; arginine 15 mM; $MnSO_4$-0.6mM; NaCl 1.0 g/L; K_2HPO_4 3 g/L; Tween-80 1%; pH-6.0. Media was inoculated with (2% *v/v*) inoculum after 2nd subculturing and cultures were incubated at 30°C for 24 h. ADI specific activity of the culture in EAPM media was determined by following standard procedures of De Angelis *et al*., (2002) and Archibald (1994) and total protein was estimated by following Lowry's method (Lowry *et al*., 1951).

3.4.10 Optimization of media components using CCD design:

Important process variables, identified based on independent experiments, were finally used to optimize composition of the ADI production medium for *E. faecium* GR7. RSM is a successive and exploratory tool for establishing the influence and interaction among variables on biological activities (Dandavate *et al*., 2009). Experimental design central composite design (CCD) of RSM using Design Expert Software trial version 8.0.2 statistical software (State-Ease Inc., Minneaopolis, MN, USA) was applied for improving enzyme activity and cell densities in the LAB isolate. A quadratic model obtained by a multiple regression technique for three factors, that is, tryptone, lactose, and arginine was studied at five different levels along with four constant variables (**table 3.10**), so that, interactions among these variables at different levels could be studied for two responses, that is, ADI activity and biomass production. During CCD experiments, concentrations of K_2HPO_4 (3

g/L), NaCl (1 g/L), $MnSO_4$ (0.6mM), and Tween-80 (1% *w/v*), pH (6.0), and temperature (30 °C) and 24 h old cultures under aerobic conditions were kept as constant factors. In all the CCD experimental runs, biomass and enzyme activities were assayed using standard protocols. A total of 20 experiments (**table 3.11**) were employed in CCD to estimate curvature and interactions among selected variables, and finally, significance of the obtained model was checked by F-test and Goodness of Fit by multiple correlation R as well as determination $R2$ coefficients. The calculated regression equation for the optimization of media constituents assessed the specific activity (Y) and growth (G) as a function of these variables. Multiple regression analysis of the experimental data was carried out. All design matrices were generated and analyzed using Design-Expert 8.0.2 to illustrate the relationships between experimental and predicted values, and the results were depicted as 2-dimensional contour plots.

3.4.11 Experimental Validation of Statistical Model:

The response surface model was validated under the predicted conditions in triplicates. On the basis of results obtained in statistical RSM analysis, the optimized medium (g/L) was composed of tryptone-15.0; lactose-10.0; arginine-20.0; $MnSO_4$ - 0.6mM; NaCl-1.0; K_2HPO_4 -3.0; Tween 80-1% (*w/v*); pH-6.0. Media was inoculated with (2% *v/v*) inoculum after 2^{nd} subculturing and incubated at 30°C for 24 h. The samples were collected after regular intervals to estimate specific ADI activity and growth (A_{600}) in the RSM optimized and TGYE basal medium.

3.4.12 Statistical Analysis:

One way Anova analysis was carried out, and the results are presented as mean ± standard deviation of three triplicate experiments. A probability value of *P* value < 0.05 was used as the criterion for statistical significance.

Table 3.10: Variables and their levels for arginine deiminase production by *E. faecium* GR7

Factor (g/L)	Low level star point (-α)	Low level factorial (-1)	Central point (0)	High level factorial (+1)	High level star point (+α)
A-Tryptone	-0.23	0	15	30	40.23
B-Lactose	-6.82	0	10	20	26.87
C-Arginine	-6.82	0	10	20	26.817

Table 3.11: Experimental design of central composite design for ADI specific activity and growth from *E. faecium* GR7

Run	Tryptone (g/L)	Lactose (g/L)	Arginine (g/L)
1	0	0	0
2	0	-α	0
3	0	0	+α
4	-1	-1	-1
5	+1	+1	+1
6	-1	+1	-1
7	-1	-1	+1
8	+1	+1	-1
9	0	0	0
10	+1	-1	-1
11	0	0	0
12	0	0	0
13	0	0	0
14	+α	0	0
15	0	+α	0
16	**+1**	**0**	**+1**
17	+1	-1	+1
18	0	0	0
19	-α	0	0
20	0	0	-α

3.5 Purification and downstream processing of Arginine Deiminase.

3.5.1 Microorganism and culture conditions:

E. faecium GR7 was retrieved in RSM optimized medium (g/L) consisting of tryptone-15.0; lactose-10.0; arginine-20.0; NaCl-1.0; K_2HPO_4 -0.5; $MgSO_4$ -0.2; $MnSO_4$ -0.05 and Tween-80 -1.0 % (*w/v*), pH-6.0 at 30°C for 24 h. Optical density of the inoculum was adjusted to 1.0 and 2 % *v/v* culture after 2^{nd} subculturing was used in each experiment. Cultures were incubated at 30° C for 24 h. The cells were harvested by centrifugation at 4°C. Cell pellet was suspended in lysis buffer (Bug Buster Protein Extraction Reagent, Novagen). This suspension was centrifuged at 10,000g, 4°C for 10 min and cell free supernatant (crude extract) was used to purify native ADI.

3.5.2 Arginine deiminase (ADI) activity assay:

To determine enzyme activity, 24 h-old cultures were centrifuged at 8,000 rpm for 10min. Cell free supernatant (CFS) was assessed for extracellular protein and enzyme activity. $O.D_{600}$ of culture broth was adjusted to 1.0 before pursuing each enzyme activity assay. For assaying intracellular ADI activity, cell pellet was resuspended in lysis buffer (Bug Buster Protein Extraction Reagent, Novagen). Total protein was estimated by following Lowry's method (Lowry *et al*., 1951). ADI activity was assayed using standard method of De Angelis *et al*., (2002). Citrulline content after enzyme assay in CFS was determined by Archibald's method (1984).

3.5.3 Purification of Arginine Deiminase:

3.5.3.1 Ammonium sulphate precipitation (Scopes, 1994):

Principle:

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Since proteins differ markedly in their solubility at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein.

Procedure:

The crude extract was mixed with different amounts of solid ammonium sulphate to get 20% and then successively raised to 100 % saturation. The amount of was calculated for each % saturation according to the below mentioned equation. All steps are carried out at 4°C under mild stirring conditions. After this, the sample was kept for twelve hour (overnight) at 4°C without stirring. When the precipitation occurred, the sample was centrifuged for 20 minutes (10,000 rpm at 4°C) and pellet was dissolved in 20 mM phosphate buffer saline (1M). Enzyme activity (De Angelis *et al*., 2002) and protein content (Lowry et al., 1951) of the dissolved pellet and supernatant was quantified. The fraction with 40-60 % saturation was observed to have maximum specific enzyme activity. Salting out of the entire protein took

place at 60% saturation. The ammonium sulphate added to reach a predetermined concentration was calculated from the following formula:

g = 533 (S2-S1)/100-0.3 S2

Where,

g = gram

S1 = % saturation to take it to S2 % saturation.

3.5.3.2 Ultrafiltration (Li *et al*., 2006):

Principle:

A dialysis membrane is based on principle of selective diffusion of molecules across a semi-permeable membrane (usually a sheet of regenerated cellulose) to separate molecules based on size. Molecules larger than the pores cannot pass through the membrane but small molecules can do so freely. In this manner, dialysis may be used to perform purification or buffer exchange for samples containing macromolecules. In terms of the size of the molecules it retains - it is defined by the Molecular Weight Cut Off (MWCO) of the membrane used. A dialysis membrane with a 20K MWCO will generally elute proteins having a molecular mass of less than 20kDa.

Procedure:

Molecular sieve or dialysis was achieved by using Slide-A-Lyzer G2 Dialysis Cassettes, 20K MWCO (Thermo Scientific, Pierce Biotechnology, USA). The membrane was hydrated by immersing cassette in dialysis buffer for 2 minutes. The sample volume of approximately >1/3 of the cassette's maximum volume was injected into the cassette bearing micro filter. Cassette was floated vertically in the dialysis buffer with mild vortexing. Samples were dialyzed for 2h at room temperature; then dialysis buffer was changed and sample was

dialyzed for another 2h at 4°C. Once again the dialysis buffer was changed and dialysis was carried out overnight at 4°C with decreasing concentration of solutes (salt) to prevent the osmotic pressure from swelling the membrane.

3.5.3.3 Gel permeation chromatography (Li *et al*., 2006):

Principle:

Gel Permeation Chromatography separates molecules on the basis of their size. This differs from other separation techniques which depend upon chemical or physical interactions to separate analytes. The molecules can be separated on the basis of difference in their size by passing them through column packed with gel. The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time. Conversely, larger analytes spend little if any time in the pores and are eluted quickly. The gel consists of an open cross linked three dimensional molecular network cast in bead form for easy column packing and optimum flow characteristics. The pores within the beads are of such size that some are not accessible by large molecules, but smaller molecules can penetrate the process.

Procedure:

Molecular sieve or gel permeation chromatography was achieved by using Sephadex G-100 (Pharmacia, Uppsala, Sweden). A jacketed column of 1.2 X 8.2 cm dimension was filled with Sephadex, at 4°C, at a flow rate of 1 ml/minute. It was equilibrated with 20 mM phosphate buffer saline (1M), pH 7.2 (Li *et al*., 2006). Enzyme solution (2 ml) with specific activity from above step was loaded to the column and elution was carried with same buffer phosphate buffer saline pH 7.2 (0.01M) and fractions of 1 ml each were collected. The

fractions were assayed for protein concentration (Lowry et al., 1951) and enzyme activity (De Angelis *et al*., 2002). Fractions having higher specific activity were pooled.

3.5.4 Polyacrylamide gel electrophoresis:

3.5.4.1 Native and SDS-PAGE (Laemmli, 1970):

Principle:

Electrophoresis is the study of movement of charged molecules in an electric field with help of supporting medium like gel made up of polyacrylamide for proteins. In electrophoresis techniques, the molecules move according to their charge and size. If the biological samples were treated in such a way that they have a uniform charge then the electrophoretic mobility depends primarily on the size.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, separate protein according to electrophoretic mobility of proteins depends primarily on their molecular mass, while in the denatured (unfolded) state. SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. 1.4g of SDS binds with per gram of protein. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density, which is same net negative charge per unit length. The electrophoretic mobilities of these proteins will be a linear function of the logarithms of their molecular weights. Native -PAGE, also known as non-denaturing gels, analyze proteins that are still in their folded state. Thus, the electrophoretic mobility depends not only on the charge-to-mass ratio, but also to the physical shape and size of the protein.

3.5.4.2 Composition of different Reagents of PAGE:

1. Separating (4X) gel buffer

Tris-HCl (18.3 g) was dissolved in 100 ml of deionized water and pH was adjusted to 8.8 with 1 N HCl.

2. Stacking (4X) gel buffer

Tris-HCl (6.055 g) was dissolved in 100 ml of deionized water and pH was adjusted to 6.8 with 1 N HCl.

3. Running buffer (4X):

The electrode buffer was prepared by mixing the components given in **table 3.12**.

Table 3.12 Composition of running buffer

Ingredients	Quantity
Tris HCl	6.05 g
Glycine	28.8 g
SDS	2.0 g
Deionised water	2.0 L
pH	8.3

4. Preparation of Native-PAGE and SDS-PAGE gels:

The separating gel, stacking gel and sample buffer used for SDS-PAGE and Native –PAGE were prepared by mixing the components given in **table 3.13**.

Table 3.13 Composition of SDS-PAGE and Native-PAGE reagents

	SDS-PAGE	Native-PAGE
Separating gel (10%)		
Acrylamide (29.2%) Bis-acrylamide (0.8%)	13.3 ml	13.3 ml
4X Separating gel buffer (pH 8.8)	10 ml	10 ml
SDS (10%)	0.8 ml	-
Glycerol (10%)	1.75 ml	1.75 ml
TEMED	20µl	20µl
Ammonium persulphate (2%)	0.13ml	0.13ml
Deionised water	17.2 ml	18 ml
Stacking gel (4%)		
Acrylamide (29.2%) Bis-acrylamide (0.8%)	1.3 ml	1.3 ml
4X Stacking gel buffer (pH 6.8)	2.5 ml	2.5 ml
SDS (10%)	0.1	-
TEMED	20µl	20µl
Ammonium persulphate (10%)	200 µl	200 µl
Deionised water	5.88 ml	5.98 ml
Sample buffer (2X)		
Tris-HCl buffer (pH 6.8)	62.5 mM	62.5 mM
DTT	50 mM	-
2-Mercaptoethanol	0.4 ml	-
Glycerol	25 %	25 %
Bromophenol blue	1%	1%

Procedure:

This mixture was poured in vertical moulds of plates on the separating gel. Comb was placed in it and gel was allowed to settle for 30 minutes. After the stacking gel was polymerized, the comb was removed. Sample was prepared by mixing the protein sample with sample buffer in equal ratios and for SDS-PAGE it was heated in a boiling water bath for 2-3 minutes but in

case of native- PAGE sample was not heated. The gels were run at 100V. After the run is complete, the gel was taken out and washed with water. Then Coomassie Brilliant Blue R-250 staining was carried out.

3.5.5 Coomassie Brilliant Blue Staining Method (Neuhoff *et al*., 1985):

Principle:

Coomassie Brilliant Blue R-250 is an anionic dye that stoichiometrically binds to proteins, and is therefore preferable to silver staining methods for estimation of relative abundance of proteins useful for differential expression analysis of (2-D) gels. In comparison to Silver staining method, Coomassie Blue staining is a relatively simple and more quantitative method. The protocol involves soaking the gel in a dye solution. Dye that is not bound to protein diffuses out of the gel during destain steps. The proteins are detected as blue spots or bands on a clear background. This method can be used for staining proteins after both 1-DE and 2-DE. The detection limit of this method is between 50-200 ng of protein per spot.

Materials:

Composition of Coomassie Brilliant Blue R-250 staining solutions are given in **table 3.14**.

1. **Gel-fixing solution:** 500ml of USP-grade 95% (*v/v*) ethanol was added to 300 ml of water. 100 ml of acetic acid was added and total volume was adjusted to 1000 ml with water. The final concentrations were 50% (*v/v*) ethanol in water with 10% (*v/v*) acetic acid.
2. **Gel-washing solution:** 500ml of HPLC-grade methanol was added to 300 ml of water. Added 100ml of acetic acid was added and total volume was adjusted to 1000 ml with water. The final concentrations were 50% (*v/v*) methanol in water with 10% (*v/v*) acetic acid.

3. **Stain:** 0.4g of Coomassie Brilliant Blue R-250 stain was dissolved in 200 ml of 40% (*v/v*) methanol in water with stirring. The solution was filtered to remove any insoluble material. 200ml of 20% (*v/v*) acetic acid was added in water. The final concentration was 0.1% (*w/v*) Coomassie Brilliant Blue R-250, 20% (*v/v*) methanol, and 10% (*v/v*) acetic acid.

4. **Destain:** 500ml of HPLC- grade methanol was added to 300 ml of water. 100 ml of acetic acid was added and, after mixing, total volume was adjusted to 1000ml with water. The final concentrations were 50% (*v/v*) methanol in water with 10% (*v/v*) acetic acid.

5. **Storage solution:** 25ml of acetic acid was added to 400ml of water. After mixing, athe final volume was adjusted to 500ml with water. The final concentration of acetic acid was 5% (*v/v*).

Table 3.14 Composition of Coomassie Brilliant Blue R-250 staining solutions

Solution	Components	Amount(g/L)
Coomassie Gel Stain (1L)	Coomassie Brilliant Blue R-250	1g
	Methanol	450 ml
	Glacial acetic acid	100 ml
	Distilled water	450 ml
Coomassie Gel Destain (1L)	Methanol	100 ml
	Glacial acetic acid	100 ml
	Distilled water	800 ml
Fixing Solution (100 ml)	Methanol	25 ml
	Glacial acetic acid	10 ml
	Distilled water	65 ml

Procedure:

After electrophoresis, the apparatus was disassembled and the gel was washed off the glass plates with 500 ml of the gel-fixing solution and soaked in that solution for 1h. The purpose of this step is to gently remove the gel from the plate and begin washing the SDS-containing gel buffers out of the gel. The gel was covered with 500ml of the gel-washing solution, and proteins in the gel were fixed by incubating overnight at room temperature with gentle agitation. The gel should be covered during this process to avoid contamination and to prevent the evaporation of the solution. The gel was covered with 400ml of the Coomassie stain. The gel was stained at room temperature for 3 to 4 h with gentle agitation. The gel was covered with ~250ml of the destain solution and the gel was allowed to destained with gentle agitation. The destain solution should be changed several times. Destaining was continued until the protein bands are seen without background staining of the gel. The gel was equilibrated in the 500ml of storage solution for at least 1 hour and gel should be returned to its original dimensions during this process. The gel was stored in the storage solution.

Precautions:

The gel must be fixed prior to staining by a non-modifying, precipitation procedure such as the ethanol (or methanol)-acetic acid method. If the protein is not fixed in the gel as a separate step from the staining, the protein will be washed away and results will be compromised. This is especially important for low level digests. Other precautions to be followed are that the handling of the gel should be minimized and whenever handled it should be done with gloves to minimize surface contamination of the gel. Reagents (including the Coomassie stain and destain) should be freshly prepared as freshly prepared

reagents account to a darker, more consistent stain with a lower background along with minimizing the gel contamination.

3.5.6 MALDI-TOF mass spectrometry (Kim *et al*., 2007)

Theory:

MALDI is the abbreviation for "Matrix Assisted Laser Desorption/Ionization." MALDI is a very sensitive technique for determining the mass of proteins, peptides, or polymers. Protein masses are identity of proteins and thus help in proteomics. Thus, MALDI allows protein identification. MALDI sample preparation is relatively fast and easy. It is a first choice when it comes to protein study. Proteins, peptides, and polymers are fragile and tend to fragment when ionized by other ionization techniques.

MALDI is attached to a time of flight (TOF) analyzer which measures time it takes for the molecules to travel a fixed distance. MALDI is a soft ionization technique in which a short laser pulse, instead of continuous laser, of nitrogen gas usually around 237 nm is used to ionize molecules. A protein or peptide sample is placed on a target plate and mixed with an appropriate matrix on the target plate. The mixture of sample and matrix crystallizes due to the vacuum environment and then is irradiated with a short laser pulse. The sample molecules and the matrix now enter gas phase. This leads to release of matrix, samples molecules, and ions from the target plate. The ions then accelerate in TOF analyzer because they are subject to equal electric field. TOF is a field-free flight tube. The ions travel in a strait and linear direction to the detector. The mass to charge ration (m/z) of the sample ions can be calculated using the equation

$T= C1 (m/z)^{0.5} +C2$. Where, C1 and C2 are instrumental constants which can be determined with compounds of known mass.

Procedure:

MALDI-TOF mass spectrometry of purified ADI was carried out using an Ultraflex TOF/TOF instrument with delayed extraction and linear capability (Bruker Daltonics) at NIPER, Mohali (India). A mass spectrum was obtained in the positive ion mode, with an accelerating voltage of 25 KV. 1µl of the undigested and unreduced pure protein was mixed with 1µl of matrix (10mg of sinapinic acid and 0.1% 4-hydroxy-α-cyanocinnamic acid in 1ml of distilled water) and 0.3 µl of this mixture was applied to Teflon-coated plate (Kim *et al*., 2007). BSA was used for calibration.

3.6 To assess *in vitro* cytotoxicity of purified Arginine Deiminase against human Hepatocellular Carcinoma Cell line by studying apoptosis

3.6.1 Procurement and maintenance of cancerous cell lines:

Cell lines were procured from NCIM, Pune and few of them were gifted by Mrs. Bharti Mittu, NIPER, Mohali. Cell lines were revived and maintained in recommended media at growth conditions specified by various culture banks given in **table 3.15**.

Table 3.15 Composition of Growth media used to cultivate cancer cell lines

S.No	Cell line	Growth media	Atmosphere	Temperature
1	HEP G2 CRL-10741	1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium with 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate and 1200 mg/l sodium bicarbonate and supplemented with 0.4 mg/ml G418, 90%; fetal bovine serum, 10%	5% carbon dioxide (CO_2)	37.0 °C
2	MCF7 (HTB-22)	Eagle's minimum Essential medium, 0.01 mg/ml bovine insulin; fetal bovine serum to a final concentration of 10%	5% carbon dioxide (CO_2)	37.0 °C
3	Sp2/0-Ag14) (CRL-1581)	Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum to a final concentration of 10%	5% carbon dioxide (CO_2)	37.0 °C

3.6.2 MTT cell viability assay to assay *in vitro* cytotoxicity of *E. faecium* GR7 (Shashi *et al*., 2006):

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate *in vitro* antiproliferative activity of crude and purified ADI of *E. faecium* GR7 against cancer cell lines according to method given by Mosmann, 1983. Cells (1.0×10^6) were plated on 96 -well plate, and then various concentrations (100-500 µg/mL) of crude ADI and purified ADI were added. Samples were incubated at 37°C with 5% CO_2 for 48 h. Then, 20 µl of MTT solution (0.5 mg/mL) was added to each well and plates were further incubated for 4 h at 37°C to induce reaction. The purple MTT formazan crystals were dissolved by adding 100 µl dimethylnsulfoxide (DMSO). Absorbance at 540 nm was measured by ELISA reader (Thermo Labsystems, Vantaa, Finland). Results were expressed as percentage growth inhibition with respect to untreated cells and calculated using NCL method GTA 2 (Nanotechnology Characterization Laboratory, National Cancer Institute, Frederick).

% Cell viability = [(Sample OD_{540} – Cell free sample blank OD_{540})/ Medium control OD_{540}] × 100.

3.6.3 DNA fragmentation assay (Kim *et al*., 2009):

Fragmentation of the genomic DNA was studied against the most sensitive cell line using modified protocol of Kim *et al*., (2009). Cell (1×10^6) were cultured for 24h, treated with crude and purified ADI of *E. faecium* GR7 for 48h, and then lysed with 250µl lysis buffer containing 100 mM NaCl, 5mM EDTA, 10mM Tris, 5% Triton X-100, 0.25% SDS and 0.2% lithium chloride; pH 8.0. Proteinase K (200µg/ml) and lithium chloride (4 µl) were added and incubated at 50°C for 1 h. After centrifugation at 13,000 rpm for 3 min, supernatant fractions

were extracted using phenol, chloroform and isoamyl alcohol (25:24:1) and again centrifuged at 3,000 rpm for 3 min. DNA was precipitated from the aqueous phase using 3 volumes of chilled ethanol containing 0.3 M sodium acetate at 4°C. Samples were subjected to electrophoresis in 1% w/v agarose gel containing ethidium bromide. The gel was electrophoresed in 1X TBE buffer at 50V and visualized on a UV transilluminator.

3.6.4 Statistics Analysis: Wherever appropriate, data was expressed as mean values ± standard deviations. Data obtained was subjected to one-way analysis of variance in a statistical analysis system. Each set of experiments was performed in triplicates. A probability value of $p<0.05$ was used as the criterion for statistical significance.