3. MATERIALS AND METHODS

3.1 Chemicals

Media and general constituents were acquired from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Raffinose, Thiobarbituric acid, 5-Diazo uracil, Tannic acid, Phytic acid, Saponins, 5-Sulfosalicylic acid dihydrate, and Vanillin were procured from Sisco Research Laboratories Pvt. Ltd. Mumbai. ρNPG and TLC Silica gel 60 F_{254} were brought from Sigma-Aldrich Inc, USA. Ferrous chloride was collected from S.D Fine-Chem Ltd. Mumbai. All the glass wares and plastic wares used in the present study were purchased from Borosil, India; Qualigens, India; Bangalore Genei, India; Tarsons, India.

3.2 Standard strain

Standard strain *Lactiplantilactobacillus plantarum* 1407^T (previously known as Lactobacillus plantarum 1407^T) was procured from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh. This type strain was isolated from fermented cabbage and was preserved at 4°C on MRS slants.

3.3 Isolation of lactobacilli

3.3.1 Sample collection

Pulses dal such as vaal, tuvar, masoor, udad, chana and mung were collected from pulse market in Vadodara. This dal was milled using mortar and pestle to fine size flour. These flours were stored in an airtight container at room temperature (RT).

3.3.2 Fermentation setup for isolating bacterial strains

A fermentation setup was established for the isolation of lactobacilli from pulses dal flour. The flours were mixed in a 1:2 (w/v) ratio with sterile deionized water in a sterile container and level of flour batters were noted before fermentation. Similarly, batters were mixed thoroughly and fermentation was allowed for rest of flours. Flour batters were kept for 24 h at 37°C to obtain lactobacilli. Sample collection was done at an interval of 12 h for analysis.

The ready to use *idli* batter sample was collected from a local market near Valsad. The sample was kept at 37°C for incubation till the pH decreases and samples were procured every 24 h for isolation of bacteria.

3.3.3 Preparation of media

The de-Man Rogosa sharpe (MRS) agar medium was used in the isolation of lactobacilli. MRS is an enriched selective medium consisting of media (g/l) components such as Peptone 10g; Yeast extract 5g; Beef extract 10g; Glucose 20g; Tween 80 1ml; K_2HPO_4 2g; Sodium acetate 5g; $MnSO_4.4H_2O$ 0.2g; Triammonium citrate 2g; $MgSO_4.7H_20$ 0.2g; Agar-Agar 15g. The inhibiting agents like ammonium citrate and sodium acetate help grow lactobacilli by suppressing moulds and other bacteria in the medium. Additionally, medium was supplemented with antifungal antibiotic, 0.01 % cycloheximide (HiMedia, Mumbai) to suppress the growth of fungi. The pH was adjusted 6.5 [41].

3.3.4 Isolation method

Isolation of lactobacilli from flour batters was done using serial dilution technique. Ten grams of each sample were weighed aseptically and suspended in saline water. The sample was homogenized for 30 seconds with Genie vortex mixer, India, diluted to a ten-fold serial dilution with the same. 100 µl of aliquot suitable as per ten-fold dilution were plated on MRS. Plates were incubated at 37°C, 2-3 days. A randomly selected colony from plates of higher dilution was picked up for each sample. A repetitive sub-culturing of obtained isolates was done to achieve pure colonies. The pure colonies were maintained on MRS agar slants.

3.4 Identification of isolates

Identification of lactobacilli was performed according to Bergey's Manual of Determinative Bacteriology [42]. Strains were selected for species level of identification which includes the following methods:

3.4.1 Phenotypic methods

3.4.1.1 Colony Morphology

The colony characteristics like forms, elevation, margins, color were examined under light microscope with 100x magnification.

3.4.1.2 Gram's staining

Overnight grown bacterial cells were used for gram staining. One ml of bacterial cells was transferred aseptically into sterile eppendorf tubes. After centrifugation, supernatant was discarded and cells were stained using the procedure given by Gram, 1884. Cells of the isolate were heat fixed on a clean glass slide. Cells were flooded with filtered crystal violet and allowed to stand for 30 sec. The slides were then washed for 5 sec with water and Gram's iodine was poured. Thereafter, slides were rinsed again for 5 sec with water. The decolorization of cells was achieved with 95% ethanol till the crystal violet washes off and slides were rinsed for 5 sec with water. Cells were counterstained using safranin for 1 min and slides were washed to remove the excessive staining. The slides were kept for drying and samples were examined under oil immersion microscope at different magnification [43].

3.4.2 Physiological method

3.4.2.1 Gas production

For hetero-fermentative isolates, a glucose test for CO_2 production was performed. For this purpose, inverted Durham's tubes in MRS broth were used. In 4 ml of test media, 50 µl of active grown culture were transferred and kept for 24-48 h, 37°C. An accumulation of gas in Durham's tube was observed as evidence for the production of CO_2 from glucose [44].

3.4.2.2 Effect of temperature

MRS broth was prepared for bacterial isolates to test their ability to grow at temperatures like 4, 20, 37, 45, and 50°C. For this, 50 µl of bacterial cultures were inoculated in 4 ml of MRS broth medium. The development of turbidity by isolates in MRS tubes was marked as the ability to grow at various temperatures. The turbidity was measured using spectrophotometer (*Shimadzu,* Japan) [43].

3.4.2.3 Effect of pH

Culture was inoculated in broth containing varying pH such as 2, 3, 4, 7 and 9, at 37°C, 48 h. Growth was observed on a spectrophotometer [45].

3.4.2.4 Effect of NaCl concentration

50 µl of active culture were transferred into MRS broth tubes containing 4 ml of each 0.5, 2.5, 4.5, 6.5 % (w/v) concentration of NaCl medium. These tubes were incubated at 37°C, 48 h. Growth was measured on spectrophotometer at 600 nm OD [45].

3.4.3 Biochemical test

All the obtained isolates were tested for biochemical characterization. The media was prepared containing different biochemical tests such as urease, H2S production, citrate, Methyl red (MR), Voges-Proskauer (VP), and catalase. Each test medium contained 50 µl of bacterial cells incubated 24 h, 37°C. The result of change in color was recorded using control. A control was an uninoculated sample to compare color change as positive and negative.

3.4.3.1 Catalase test

Some organisms have the capacity to produce catalase enzymes that can break down a toxic hydrogen peroxidase (H_2O_2) into water (H_2O) and molecular oxygen (O_2). The occurrence of catalase enzymes is thus indicated by generation of gas bubbles. Hence, to differentiate genus from other organisms, this test was performed. For this purpose, bacterial inoculum from pure culture stocks was streaked on MRS plates and incubated. Culture was located onto a clean slide and presence of enzyme was confirmed by dropping hydrogen peroxide (3%). A culture was observed as positive or negative. However, a rapid formation of O_2 is evidence of a bubble indicating a positive result. While no bubble indicates the test as negative [46-48].

3.4.3.2 Methyl Red and Voges-Proskauer test

VP and MR test was carried to detect the end product of fermentable glucose. An organism either metabolizes glucose to compounds like acetoin, 2,3-butylene glycol *etc* using dimethylene glycol pathway or converted to LA, AA and ethanol as an end product using multiple pathway of acids. MR test, identify mixed-acid fermentation pathways whereas VP test, butylene glycol

fermentation pathway. The colonies of isolates were inoculated in test medium, after for 24 h incubation few drops of MR, was added as a pH indicator and tubes were labelled. In another tube, Barritt's Reagent A and B were appended for VP test. The red color in MR tubes signify positive signs while no change of color signify negative signs. Moreover, VP test medium starts to develop its color within 10-15 min after addition of reagent and indicates positive if appeared red while copper color is indicated as negative [46-48].

3.4.3.3 Citrate Utilization test

Some organisms have the capacity to utilize citrate, nitrogen that leads to production of an alkaline environment due to ammonia. In this test, the enzyme citrase breaks down citrate to acetate and oxaloacetate. In a medium also Bromothymol blue is added as an indicator to detect alkaline pH, color change to yellow indicates acidic pH to 6.0 as positive and blue color indicates pH above 7.6 as negative [46-48].

3.4.3.4 Urease test

Urease tests are performed to detect alkaline fermentation of urea with the end product ammonia by organism. Hydrolysis of urea results in production of NH_3 and CO_2. The NH_3 combines with CO_2 and H_2O to form $(NH_4)_2CO_3$ and turns alkaline medium in pink color, giving an indication of positive test. A negative control was an uninoculated slant [49].

3.4.3.5 Hydrogen sulfide production test

Hydrogen sulfide (H_2S) is performed to check the production of H_2S gas induced by organisms. These tests show whether an organism has the capacity to reduce the sulfide compound in the test medium through the metabolism process. The color change to black indicates positive result and no organism was added hence, no color change was observed in control after 24 h [49].

3.4.3.6 Carbohydrate fermentation pattern of isolates

Most organisms obtain their energy by fermenting sugars through a series of an orderly and integrated chemical reaction leading to bio-oxidation of substrate. Thus, different carbohydrates were used to determine the fermentation profile of obtained isolates. The fermentable sugars such as glucose, galactose, xylose, lactose, fructose, maltose, sucrose, raffinose, mannitol and

starch were used. Cells were activated in MRS broth for 12-18 h or until the absorbance reached 0.5 OD. In each test medium, bacterial cells were inoculated and tubes were incubated for 24 h. The development of yellow color from pink is indicated as positive; conversion to orange was noted as weak positive while no change was considered as negative as in control [48].

3.5 Characterization and molecular phylogenetic analysis of isolates

For genotyping characterization of isolates, pure cultures on MRS slants were sent to Labreq Bioscientific, Ahmedabad. The culture was followed by isolation of genomic DNA from bacteria and 16S rRNA sequencing method was carried out. This sequencing was done using BigDye^{TM} Terminator Cycle Sequencing Kit on a 3730xl DNA Analyzer (Applied Biosystems, CA, USA). This 16S rRNA tool was applied for identification of bacteria. The sequences in the database also help in determining the position in the evolutionary tree and recognize the most likely species of organism in the sample using the BLAST program. These BLAST programs assist in finding the similarity between biological sequences and calculate the statistical significance of isolates. The sequence identified was then aligned using MEGA X software [50]. The evolutionary distance was measured for selected sequence as per method of Maximum Composite Likelihood method [51]. The Phylogenetic tree was derived using the Neighbor-Joining method [52]. The bootstrap analysis was done using Laser gene sequence analysis software inferred from 1000 replicates [53].

3.6 Utilization of carbohydrates by isolates

In 250 ml of Erlenmeyer flask, modified MRS (mMRS) was prepared. The different disaccharide, trisaccharide and polysaccharide sugars like sucrose, starch, raffinose were selected. These sugars were added singly and in combination in mMRS medium. The combinations made were as follows: sucrose, soluble starch + sucrose, raffinose, and raffinose + soluble starch. The concentration of these sugars were sucrose 1%, starch 0.3% + sucrose 0.5%, raffinose 1%, raffinose 1% + starch 0.3% were added separately in different flasks. A colony of fresh culture from the MRS plate was added in respective MRS broth media containing carbon source and allowed to grow at 37°C till the OD reaches 0.5. Each 1 ml of culture was added in the flask with carbon source and kept for incubation. Sample was withdrawn every 4 h and centrifuged. Pallet was removed; supernatant was collected and kept in refrigeration until further

use. Samples were used in monitoring bacterial counts, decrease in sugars, pH drop, *etc*. No culture was inoculated in the control flask and so was considered as blank.

3.7 Chemical analysis

3.7.1 Growth curve via colony forming unit

A 24 h old culture was inoculated into the respective growth at 37°C till OD reaches 0.5. The culture was procured at an interval of 4 h. Microorganisms growth was also determined by assessing microbial load at OD 600 nm [54]. The microbial load in a liquid sample was analyzed using:

$$\text{cfu/ml} = \frac{\text{(no. of colonies x dilution factor)}}{\text{Volume of culture plate}}$$

3.7.2 Lactic acid determination

A simple, systematic and inexpensive spectrophotometric method was used to find out concentration of LA in a liquid culture sample. Lactic acid with a known concentration (1.2g/ml; 89%) in volumetric flask (10 ml) and distilled water. A concentration range between 0.3-10 g/l of LA was built. A solution of iron (III) chloride (0.2 %) was prepared in a 250 ml of volumetric flask containing distilled water (100 ml). A 50 µl solution of lactic acid was added to the corresponding concentration of 0.2% of 2 ml of $FeCl_3$ solution and mixed properly. The reaction of lactic acid with $FeCl_3$ in aqueous solution results in a color formation of yellowish-green iron (III) lactate in a solution. The absorbance of the lactate ions with $FeCl_3$ solutions was studied at 390 nm. The color of mixture is stable for 15 min. For test culture, the liquid sample was separated from bacterial cells by centrifugation and sample was analyzed. Sample was kept in the refrigerator until further use [55].

3.7.3 Starch determination

A rapid, simple, accurate method developed using a colorimeter to determine the content of starch which is based on the iodine complex formation with amylose and amylopectin. This technique helps in determining the amount of starch present in the sample. For this, sample (1 ml) was mixed with distilled water (5 ml). The test tube was covered with aluminum foil and the solution was kept on heating for 15 min at 85°C, cool diluted using distilled water (50 ml). To

this, iodine solution (5 ml) was added, sample was mixed thoroughly. Sample was incubated at RT, 15 min. Starch-iodine mixture in the sample was observed as blue color, absorbance was recorded at 590 nm [56]. Standard starch sample was run using the concentration range between 0.1 to 10mg. A standard curve was plotted using OD and concentration of starch in mg/ml. The distilled water was used in a blank test tube with iodine reagent and OD was measured.

3.7.4 Extraction of free sugars

In this method, whole bean seeds (10 g) were soaked in distilled water overnight. The next day beans were washed again with distilled water and appended in 80% ethanol. The suspension was kept for 1 h at shaking condition and obtained residue was filtered. Residue was mixed with of distilled water (100 ml) and allowed to stir for 30 min; filtrate was obtained again. The final filtrate was combined with distilled water (100 ml). Same procedure was performed using culture supernatant incubated at 37°C. A sample was procured at different time intervals of 12, 18, 24, 36 and 48 h.

3.7.5 Qualitative determination of oligosaccharides

A rapid thin layer chromatography method was developed by Tanaka et al. (1976) to analyze monosaccharides, disaccharides and oligosaccharides in whole seed. 10 µl of control and culture treated samples were loaded on a silica gel 60 F_{254} TLC plate along with standards (sucrose, raffinose). System was (n-propanol: ethyl acetate: water (6:1:3, v/v)) allowed to settle for 30 min. The modified α- naphthol reagent prepared in 70% ethanol and orthophosphoric acid were used as a spraying agent. The plates were kept for drying in oven, 15 min. The sugar spots obtained were identified by retention factor (R_f) [57].

3.7.6 Quantitative analysis of oligosaccharides

This analytical procedure for sugars helps in determining the amount of sucrose and raffinose in legume beans by thiobarbituric acid method. To 1 ml of standard sugar sample, 0.02M of 1 ml thiobarbituric acid and conc. hydrochloric acid 1 ml was added and heated, 6 min in boiling water bath and later cooled in running water. The yellow color obtained was read on a spectrophotometer at 432.5 nm. The corresponding concentration of sugar was calculated from a standard curve, ranging from 10-100 mg [57].

3.7.7 Glucose estimation using DNSA method

3,5-dinitrosalicylic acid (DNSA) is a sensitive and cheaper method used for quantitative measurement of reducing sugars. In this method 3,5-dinitrosalicylic acid on heating with reducing sugar is turned down to 3-amino-5-nitro salicylic acid. In simple terms, the aldehyde group is oxidized to the carboxyl group under alkaline conditions. The color tests were made with 3 ml of sugar solution and DNSA reagent. Mixture was kept in boiling water, 5 min and later cooled. Color of the solution changes to reddish-brown from yellow. The solution was placed in a cuvette and color intensity was read at 540 nm. The control consisted of deionized water considered as blank, while the concentration range of standard glucose solution was in between 10-100 µl. The unknown sample was plotted using a standard curve against OD [58].

3.8 Screening of isolates for α-galactosidase activity

An overnight grown single bacterial colony was inoculated in modified MRS medium broth containing raffinose (10g/l). pNPG was sterilized by filter sterilization. The mMRS broth containing raffinose was incubated, 37°C, 12-24 h. Change in color indicates production of α-gal enzyme. Cells from broth were harvested by centrifugation. The obtained crude was collected as supernatant to determine extracellular α -gal enzyme. The intensity of color was read at 405 nm. The confirmation of extracellular α –gal was also carried out using cell free supernatant. For that, the cells were allowed to grow in mMRS + raffinose broth and incubated for 48 h, 37°C. The cells were centrifuged and to 45 µl of supernatant, 15 µl of 10 mM pNPG solution was added. Reaction was stopped by 900 µl of 0.25 M Na_2CO_3 solution and absorbance was taken at 405 nm [59]. A unit of enzyme activity (U) is amount of enzyme that is hydrolyzed to produce one µmol of ρ-nitrophenol substrate per minute under the above condition specified.

3.8.1 Determination of protein concentration

A fast, simple method for the quantification of total protein in sample using Coomassie Brilliant Blue G-250 dye-binding assay called as Bradford method. This dye to protein binding assay is based on differential change in color of dye with response to various protein concentrations. The principle of this assay says, binding of dye with protein molecules under acidic conditions results in change of color from brown to blue under above reaction. A standard protein solution was

prepared using bovine serum albumin and concentration range was kept between 10 to 100 µg. A range of protein from 0.1 to 1 ml was pipetted in test tubes and volume of sample was adjusted with deionized water. 1 ml of protein binding dye was added to the test tube including standards and mixed by vortexing. Formation of blue color was measured at 595nm and concentration of protein was plotted against absorbance resulting in a linear graph to determine the unknown protein sample [60].

3.9 Optimization of parameters for extracellular α-galactosidase enzyme

The highest α- gal producing isolates were selected for further studies.

3.9.1 Effect of incubation at different temperatures on α- galactosidase enzyme

To study the optimal temperature for α-gal enzyme, culture including reference strain was incubated at different temperatures ranging between 5°C to 60°C. The strain was incubated with 10 mM pNPG substrate for 18 h at 37°C to find the optimum enzyme activity α-gal activity. To check temperature stability, culture was incubated at various temperatures 20°C, 30°C and 45°C, 18 h and stability of enzyme was determined [61].

3.9.2 Effect of incubation at different pH on α- galactosidase enzyme

To check the optimal pH for α-galactosidase enzyme, different pH values ranging from 2 to 11.5 in mMRS broth including substrates was studied. After incubating it for 18 h culture was studied for the optimal production of enzymes at different pH at 37°C [61].

3.9.3 Effect of incubation in different medium on α- galactosidase enzyme

The culture was evaluated for higher production of α-galactosidase enzymes in various growth mediums containing different carbon sources. The evolution of pH as well as the viability of the strains was determined by colony forming unit after incubating it for 18 to 24 h at 37°C. Substrates mainly raffinose, sucrose, soluble starch, sucrose + raffinose, soluble starch + raffinose were used in the study to find out the optimal enzyme production using appropriate carbon source for α- gal production [62].

3.9.4 Effect of incubation in different carbon concentrations on α- galactosidase enzyme

Various concentrations of raffinose were used to evaluate the effect on enzymes in the mMRS medium. The concentrations used were 0.3%, 0.5%, 1%, 3%, 5% for TIP1 culture. Enzyme assay was performed by adding different carbon concentrations in the production medium to find the potential α-gal activity [62].

3.10 Evaluations of selected isolate to check behavior of pigeon pea pulse during fermentation

The raw material used in this study were pigeon pea beans (Cajanus cajan) commercially called tuvar, red gram, or arhar that were purchased from the local market in Vadodara. Samples were kept in an airtight container at room temperature until use for further analysis. For laboratory scale fermentation samples of pigeon pea beans, 10 g of beans were weighed, washed several times and then soaked overnight in distilled water, in the ratio of gram to beans (10:40, g/ml) at 30±1 ℃ temperature. The next day beans were washed again with distilled water, water was added again. The bacterial culture was grown in 4 ml MRS broth upto the end of late exponential phase or log growth phase at 37℃. From this broth, 2 ml of overnight bacterial culture was pelleted in an eppendorf tube to a final OD_{600nm}= 5. The cells were spun in a microcentrifuge tube for 10 minute at 10,000 rpm at RT. These cells were added to the flask containing beans and allowed to ferment for different times at 37℃. The samples were withdrawn at 0, 10, 14, 18, 20, 24, 36 and 48 hours and results were recorded for several parameters.

3.11 Screening of pulse beans for optimization of medium

Whole pulse beans such as pigeon pea (*Cajanus Cajan*; tur), red lentil (*Lens culinaris*; masoor), broad beans (*Vicia faba*; vaal), and black gram (*Vigna mungo*; udad) were purchased from local market in Vadodara. These beans were tested as substitutes for peptone and carbon source. Beans were manually sorted and rinsed several times with distilled water and soaked overnight in freshwater. The ratio of beans: water was kept 1:4 (w/v) and allowed to soak for 5 hrs at room temperature. Then the soaked concentration was subjected to crushing using mortar and pestle; skins were removed before mashing the whole pulse. The squashed beans were then weighted with different concentrations such as 1%, 2%, 5%, 10%, 20%, and 50%. From the above obtained concentration of squashed beans, the same steps were applied to different

concentrations of yeast extract. The concentrations such as 1%, 5%, 10% and 20% were considered. The growth and pH were analyzed; based on their observations further experiments using salts components were conducted. The modified medium was exposed to a variety of concentrations of salts such as sodium acetate (0.5%, 1%, 2%, 5%), di-potassium phosphate (0.2%, 1%, 2% 5%) magnesium sulphate (0.01%, 0.1%, 1%, 2%, 5%) manganese sulphate (0.005%, 0.03%, 0.05%, 0.1%), tween 80 (0.1%, 0.5%, 1%, 2%, 5%) *etc*. The mass was then allowed to ferment at 37℃ till the time cfu and pH starts to decline. For seed culture, *Lev. brevis* TIP1 culture was inoculated to MRS medium at 37℃ in 250 ml flask for 24 h. The medium without culture was assessed as control flask for comparison with sample flask. The sampling was opted from each tube at every 0, 24, 48, 72, and 96 h time interval. The collected sample was centrifuged in a micro centrifuge tube and spun, 12,000 rpm, 5 min. Supernatant was monitored for pH profile, cell growth, LA, AA and ethanol production in fermentation media.

3.11.1 Qualitative estimation of lactic acids by HPLC method

Determination of LA is important to monitor pH and microbial growth due to nutritional reason. A successful method was developed to analyze the simultaneous detection of organic acids because of speed, simplicity and stability. High performance liquid chromatography (HPLC) was performed for quantifying organic acid using Atlantis dC18 column (250 mm × 4.6 mm, Waters, Mildford, MA, USA) as described previously [63] with some modifications. A gradient programme two mobile phase: phosphate buffer (Solvent A) (pH 2.2) acetonitrile solvent (Solvent B) were used to separate the compounds. The mobile phase was 20 mM NaH2PO4 aqueous solution. Sample (20µl) was separated on a column set at 30˚C, flow rate 1.25 ml/min. Column was equilibrated for 20 min every time with HPLC water before injecting again the next sample. The absorbance was set at 210 nm monitored by two-channel UV detector, operated using a Breeze software. The determination of linearity was validated using the regression lines. The calibration curve was prepared by plotting five concentration points performed in triplicates. For sample preparation, sample was centrifuged and supernatant was collected. Sample was passed through 0.45 µm poly vinylidene difluoride membrane and diluted with 4.5 mM of H_2SO_4 (extract organic acids); preparation was vortexed for a minute before injecting. The samples were stored at -20˚C [63].

3.11.2 Qualitative estimation of acetic acid and ethanol by GC- FID method

Simple and improved gas chromatography–flame ionization detection (GC–FID) method to measure final product like AA and ethanol in fermentation medium. The sample was performed using GC (Agilent 7890, Santa Clara, CA) equipped with FID. Separation of compound was done using 60 m HP-INNOWAX capillary column, 0.25 mm, coated with polyethylene glycol, using nitrogen as carrier gas, with 2 ml/min flow rate. Obtained chromatogram was recorded using Agilent Chemstation software. For sample, medium was centrifuges to separate sediments. Before injection, clear liquid was filtered to avoid unnecessary block in column. Chromatographic sample were prepared using isobutanol as IS, then loaded into auto sampler [64].