4. Result and Discussion

4.1 Isolation of bacteria

Dal flour from six different pulse samples was naturally fermented and diluted serially with microbial population ranging between 3-5 log cfu/ml upon 24-48 h of propagation (Table 11), at 37℃ with 4.5 pH. The fermentation set presented in Table 21 for all the chosen dal flour batters. Growth was not observed during the start of fermentation. As time succeeded, LAB population started increasing while declination in batter pH was noted. A hike in the population of yeast was observed with an incline in batter volume. Increase in batter volume supported linear link between yeast growth and leavening process [65], which is similar in case of *idli* batter, incorporation of LAB, hence adding acidification and gas production or entrapment of air in the medium [66]; a representative image of batter is shown in Figure 8. A complex synergy between microbiota is usually observed during natural fermentation, which is generally achieved by yeast and LAB [67].

Conversion of pulse-derived sugars or starch into ethanol, CO_2 and low amounts of energy by aerobic fermentation via glycolysis pathway to form pyruvic acid is the main role of yeast cells while carrying out fermentation [68]; maximum number of acids are produced as end-products by dominant LAB through pyruvic acid under the actions of several enzymes [69,70]. In addition to this, several secondary metabolites influence the texture and aroma, but LAB are particularly involved in producing flavor compounds, and peptides that prevent the growth of undesirable organisms during spontaneous fermentation [67]. Yeast species dominate at the end of 48 h and LAB start to appear after 24 h of fermentation [67]; participation of both allows the enzymatic breakdown of carbohydrates through fermentation inside the pulses [69]. During natural or desired fermentation, these organisms keep changing their dominant microbiota and act typically in sequential or parallel manner [69].

A similar succession of microbiota takes place during natural fermentation of cereals, which is majorly dominated by LAB, when cleaned cereals are kept for some days soaking in water. The most prevalent species of LAB obtained during natural fermentation along with yeast are *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, and lactobacilli such as *L. fermentum*, *Lev. brevis*, *L. paracasei*, *Lpb. plantarum*, *etc* [71]. There are

certain other popular fermented foods across the world such as *kenkey*, *ogi*, *koko*, and *busaa*, a cereal and maize based product where LAB, especially lactobacilli are predominantly involved in the fermentation process directly or indirectly [72].

4.2 Preliminary identification of bacteria from fermented dal flour

The preliminary identification of lactobacilli involves morphological, physiological and biochemical characteristics.

4.2.1 Morphological characteristics

Suitable dilutions were plated on specific bacteriological media and incubated at 37°C for 48

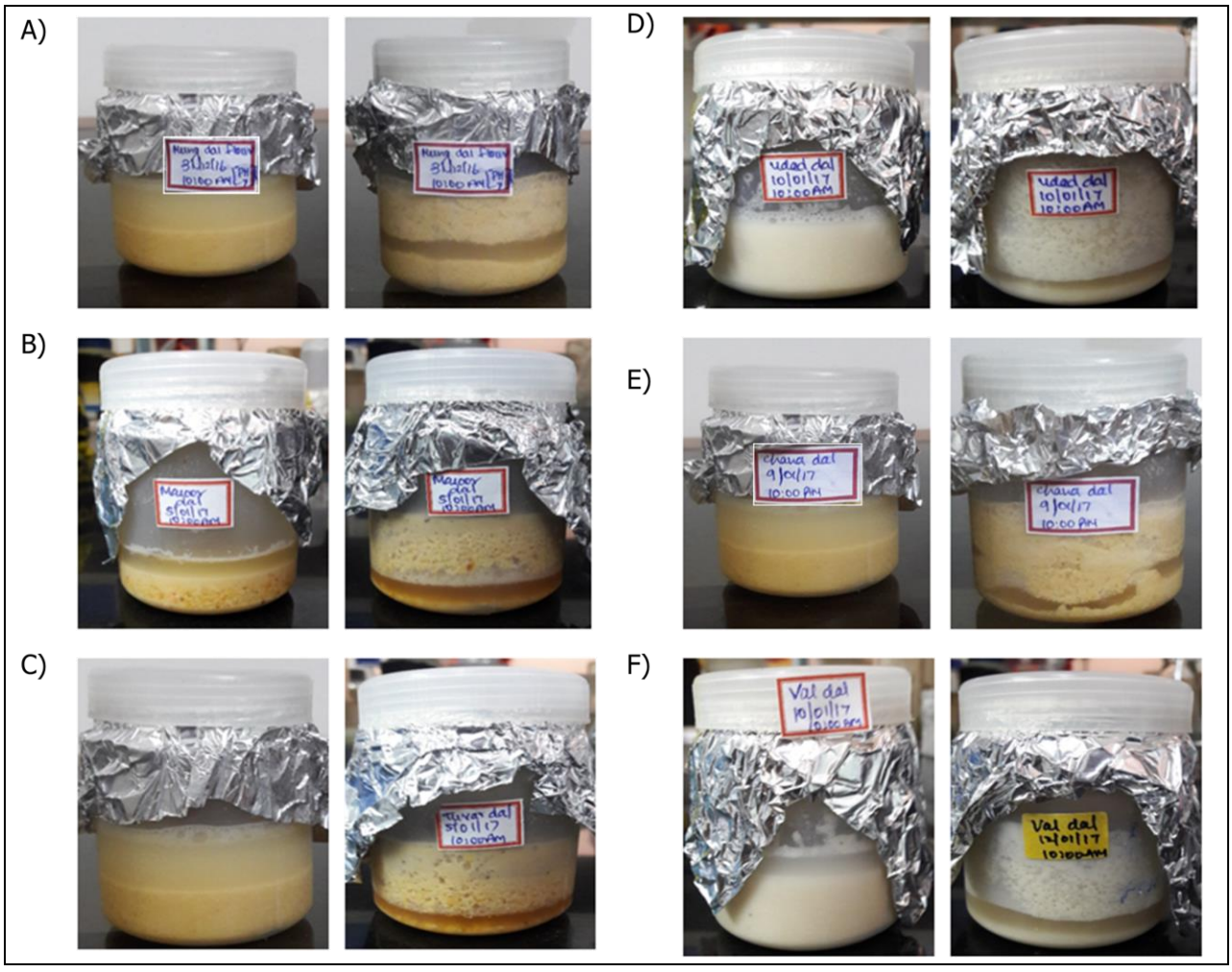

Figure 8: Flours made from different pulse dal (left). (A) mung (B) masoor (C) tuvar (D) urad (E) chana (F) val flour, allowed to ferment naturally and change in property (right), visualized after 48 h of fermentation.

Table 11: Summary of physicochemical parameter of naturally fermented dal flour batter

Sample	Sample code	Vol. of batter (ml) before fermentation at 0 h	Vol. of batter (ml) after fermentation after 24 h	pH of batter after 24 h	LAB count (log cfu/ml) after 24 h	No. of Isolates
Masoor dal	M1	50	90	4.5	5	1
Udad dal	U1	50	200	4.5	5.07	1
Tuvar dal	T1, TIP1, WT1	50	80	4.5	4.30	3
Val dal	V1, VIP1	50	85	4.5	5.04	2
Mung dal	Mu1	50	90	4.5	3.14	1
Chana dal	C1, WC1	50	80	4.5	5	2

hours. A total of eleven well isolated colonies were picked up. Based on colony characters, maximum colonies were selected for further studies. These isolates were mainly catalase negative, non-motile, non-sporulating colonies differentiated into long rods, short rods and cocci in shape. Most of the microbiota was Gram positive after confirmation with Gram's staining (Figure 9). The appearance of colonies was small, usually pin pointed to medium, off-white to creamy in colour and shiny colonies that smelled like yogurt (Figure 10; Table 12). The cultures were surface plated on selected bacterial mediums and stored in duplicates.

4.2.2 Biochemical characteristics

All the bacterial isolates from MRS medium that were Gram positive, rod shaped and catalase negative (Figure 11) were considered as presumptive *Lactobacillus*. The biochemical tests are based on change of color by an acid-base indicator. The tests including citrate reduction, H_2S production, MR, VP, and urease test were performed for all the nine isolates. However, these isolates were only positive for MR test; no change in color was observed for the rest of cultures (Table 13).

Table 12: Colony characteristics of isolates

Colony characteristics	**Mu 1**	**U1**	**V1**	**C1**	**T1**	**M1**	**I8**	**VIP 1**	**TIP1**
Size	Medium to large	Small to medium	Medium	Medium to large	Medium	Small to medium	Small to medium	Small to medium	Small to medium
Shape	circular	circular	circular	circular	Circular	Circular	circular	circular	Circular
Edge	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex	Capitate	Convex	Capitate	Convex
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Moist	Butyrous	Moist	Butyrous	Moist
Optical character	Cretaceous	Opaque	Cretaceous	Cretaceous	Opaque	Opaque	Opaque	Opaque	Opaque
Color	Creamy white	Creamy white	Off-white	Creamy white	Off-white	Creamy white	Off-white	Creamy white	Off-white
Gram staining	+ve, small rod	+ve, long rod	+ve, small rod	+ve, small rod	+ve, small rod	+ve, long rod	+ve, small rod	+ve, long rod	+ve, small rod

4.2.3 Carbohydrate profile

All isolates utilized sugars, which are the main fermenters. More or less, similar lactobacilli were identified phenotypically from some naturally fermented products such as *idli*, *dhokla*, *khaman* batter and *kinema* (fermented soybean) [73]. Most organisms obtain their energy to function through an enzymatic reaction taking place within them, leading to a biological oxidation of a substrate, frequently sugars. Thus, different carbohydrates were used to determine fermentation

profile and further characterization of isolates were accomplished [74]. For this, the isolates were subjected to fermentation reactions using various carbohydrate-containing mediums for knowing their fermentation pattern (Table 14). Bacteria produce certain enzymes that can ferment carbohydrates and form acidic products, indicating yellow color in the end. These carbohydrate tests help to detect pH change through fermenting carbohydrates.

4.3 Physiological characteristics

4.3.1 Growth at different temperatures

The isolates were exposed to temperatures like 4℃, 20℃, 37℃, 45℃, 50℃ *etc* (Table 15). All isolates grew well at 37℃ and 45℃; however, as the temperature was increased to 50 ℃ none of them survived at this elevated temperature. Similarly, all isolates were able to survive at minimum temperature i.e. 20 ℃, with moderate growth. However, C1, Mu1, T1, V1, and I8 isolates showed minimal growth at 20 ℃ and no growth at 45 ℃.

4.3.2 Growth at different pH

The isolates exhibited good growth at pH 2, 3, 4, 7, and 9; maximum alkalinity tolerated by the isolates was pH 9 and minimum acidity isolates can retain is 4. Though, TIP 1, C1, Mu1, T1, V1, I8 isolates showed no growth, moderate growth was observed in VIP1, U1, and M1 isolates at pH 3.5 (Table 15).

4.3.3 Growth at various concentrations of NaCl

The other physiological criterion for growth of the cell is to analyse sodium chloride concentration as salt solution prevents cells from osmotic shock [74]. Thus, growth of isolates at various NaCl concentrations was determined. All isolates could grow well at both 0.5 and 1.5 %; when the concentration was increased to 2.5 % and above, a sudden shock to the cell was observed and no growth occurred. However, growth was moderate even when salinity was 1.5% for isolates in the culture medium (Table 15). The isolates exhibited resistance to temperature, acidity, and saline stress. The presumed organism had the capacity to tolerate high temperature which in a way helps to keep the medium free from contamination. The results for pH were in line as reported by Ayo-Omogie and Okorie, (2016), for an acid-fermented beverage, *kunu*, reported to show low pH during fermentation due to organic acid production by lactobacilli species [75]. The stress tolerance capacity was also not more than 1.5 % as the uptake of high

salt could help in selecting species for such food, which helps the product in maintaining quality and longer shelf-life.

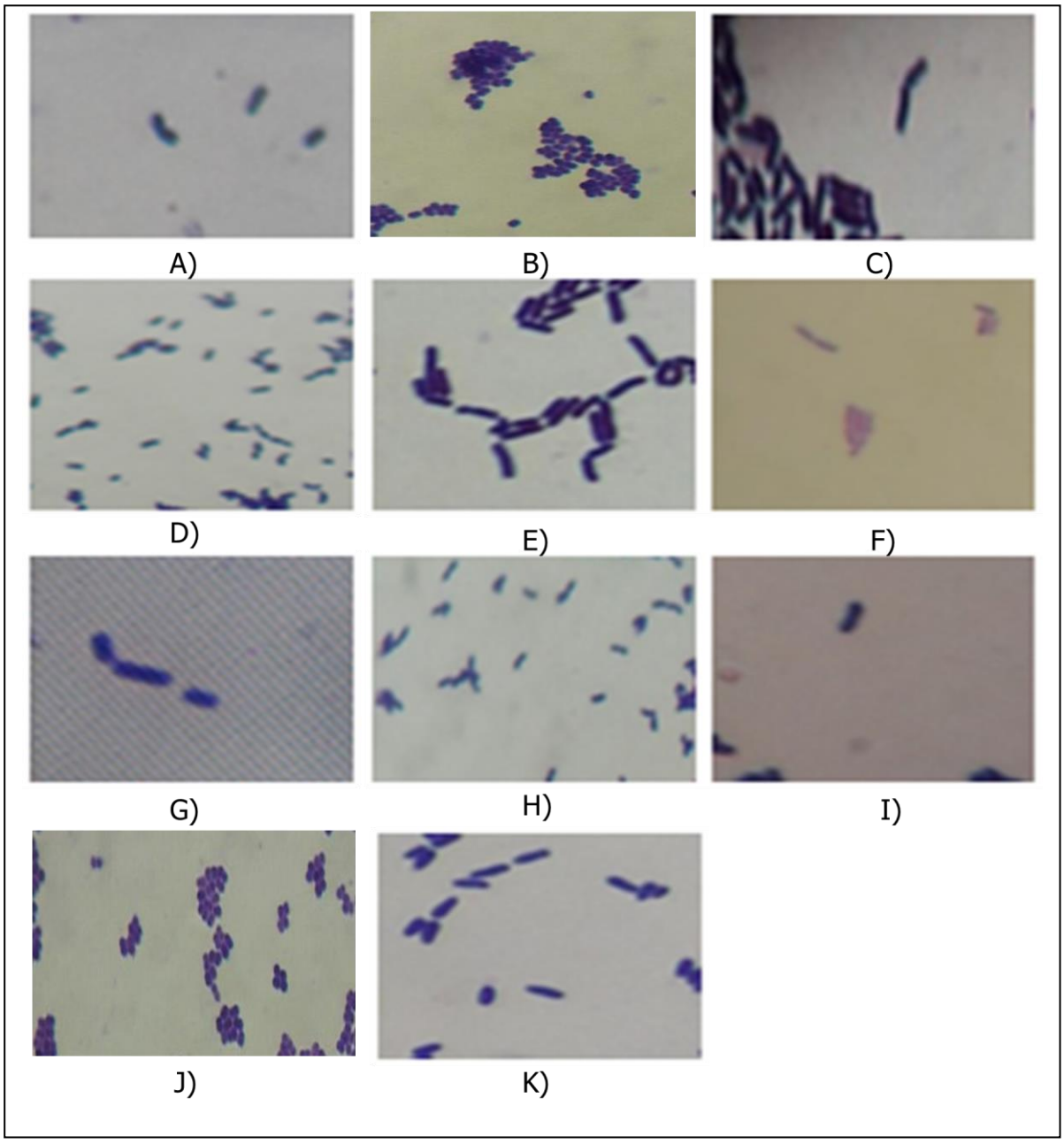

Figure 9: Microscopic images of isolated microbial colonies from fermented batters. Chana-C1, WC1 (A & B), vaal-VIP1, V1 (C & D), urad-U1 (E), masoor- M1 (F), idli batter-I8 (G), tuvar-T1, TIP1, WT1 (H, I & J), mung-Mu1(K).

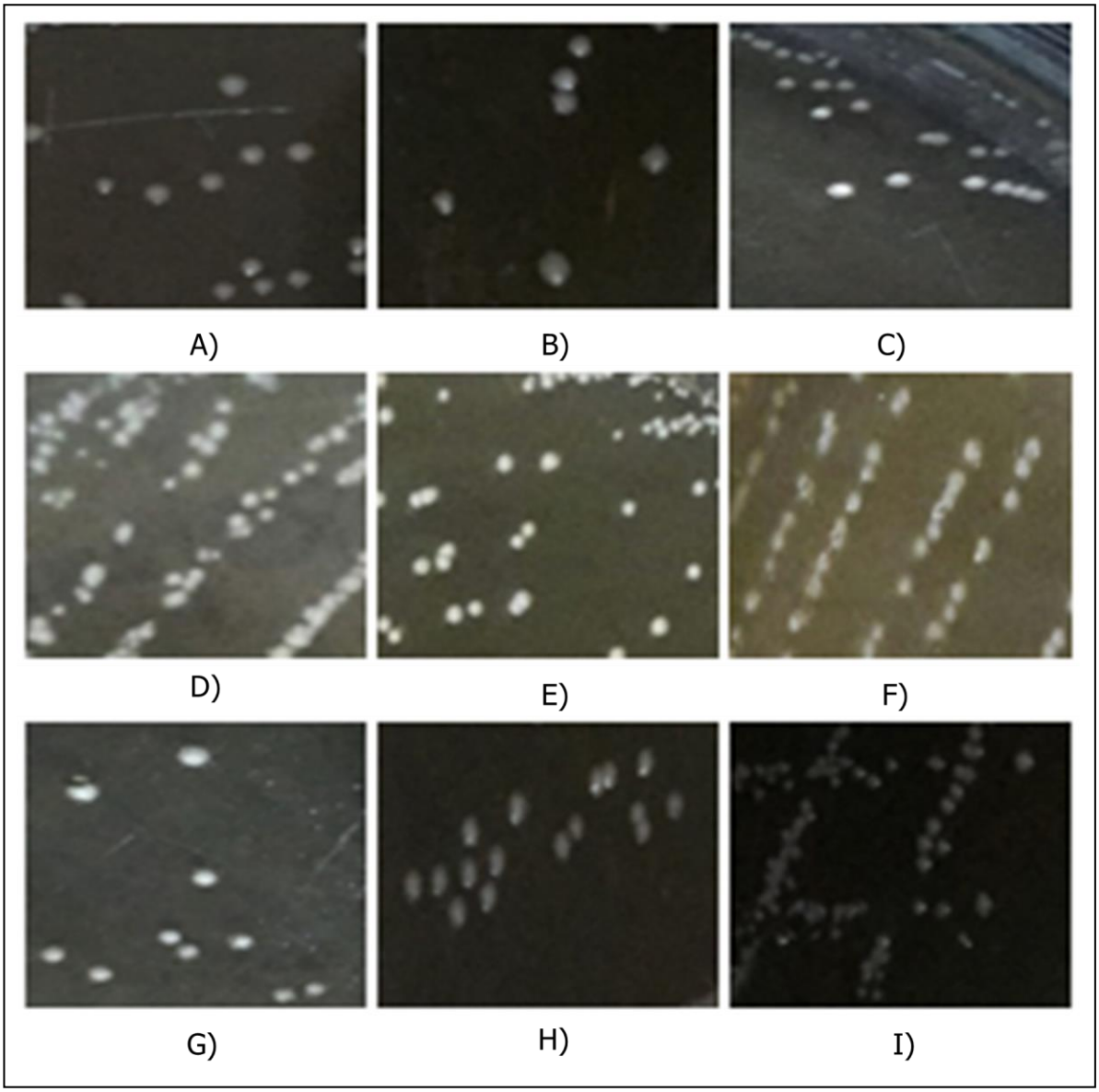

Figure 10: Colony morphology of isolated bacteria from fermented batters. C1 (A), V1 & VIP1 (B & C), U1 (D), M1 (E), I8 (F), Mu1(G), T1 & TIP1 (H & I).

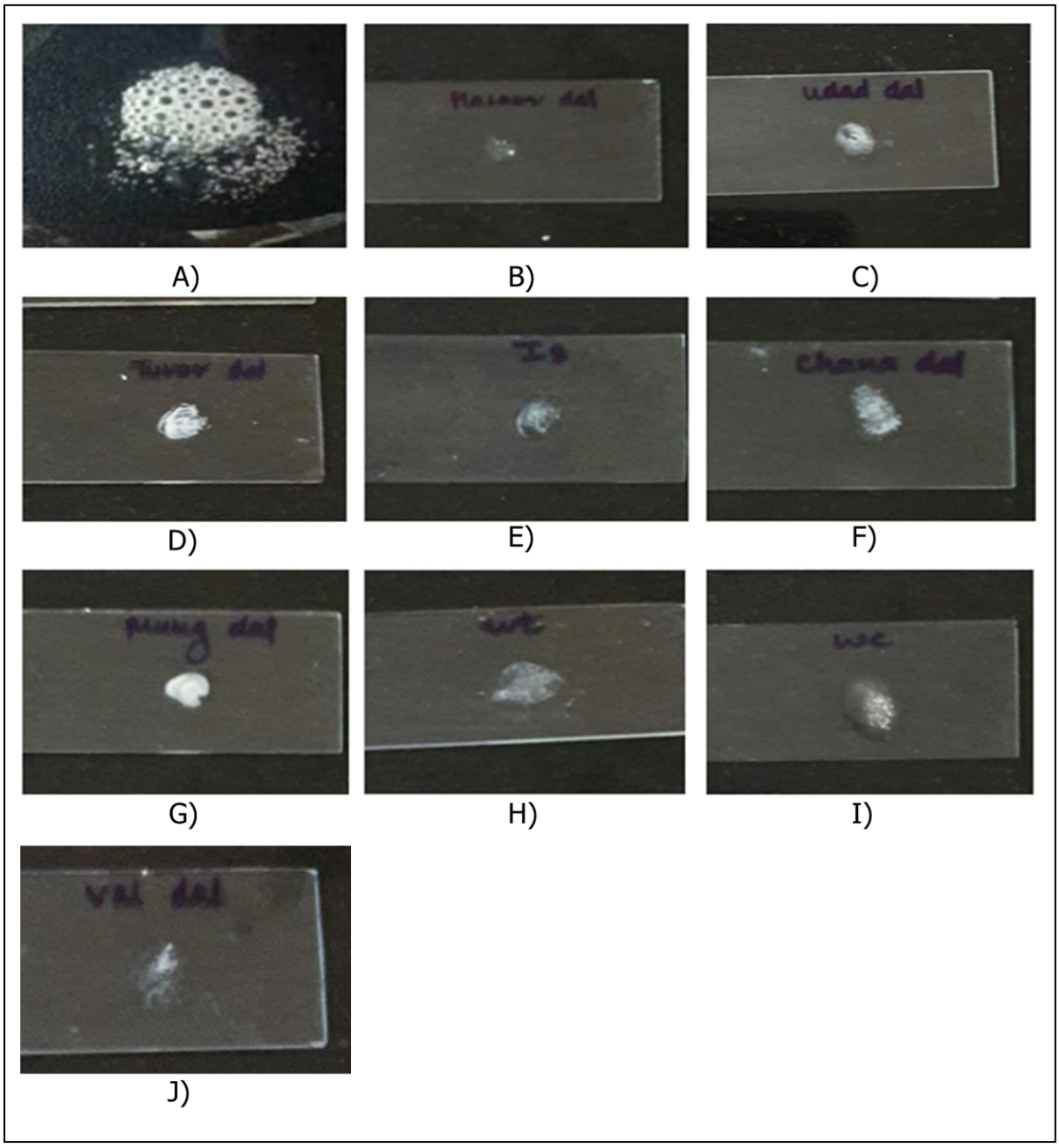

Figure 11: Catalase test of isolates. (A) Positive control (*Bacillus megaterium*) (B) M1 (C) U1 (D) T1 (E) I8 (F) C1 (G) Mu1 (H) WT1 (I) WC1 (J) V1.

Table 13: Biochemical test of isolates

Characteristics	M1	U1	VIP1	TIP1	V1	C1	I8	Mu1	T1
Catalase	-	-	-	-	-	-	-	-	-
Gas production from glucose	+	+	+	+	+	+	+	+	+
Fermentative type	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
H_2S production	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-	-	-	-
Methyl red test	+	+	+	+	+	+	+	+	+
Urease test	-	-	-	-	-	-	-	-	-
Citrate reduction	-	-	-	-	-	-	-	-	-

(+) growth; (-) no growth; He- Hetero-fermentative; ND- not determined

Table 14: Carbohydrate fermentation profile of isolates

Carbohydrates	M1	U1	VIP1	TIP1	T1	V1	C1	I8	Mu1
Glucose	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	-	+	+	+	-	+
Mannitol	+	+	+	-	-	+	+	-	+
Lactose	+	+	+	-	+	+	+	-	+
Maltose	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	-	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+

(+) growth; (-) no growth

Table 15: Phenotypic characteristics of isolates from fermented batter

Isolates	Growth at Temperature (°C)					Growth at pH					Growth on NaCl (%)			
	4	20	37	45	50	2	3	4	7	9	0.5	2.5	4	6
M1	-	+	+++	+	-	-	+	+++	+++	++	+++	-	-	-
U1	-	+	+++	-	-	-	-	+++	+++	++	+++	-	-	-
VIP1	-	+	+++	+	-	-	+	+++	+++	++	+++	-	-	-
TIP1	-	+	+++	+	-	-	-	+++	+++	++	+++	-	-	-
I8	-	+	+++	-	-	-	-	+++	+++	++	+++	-	-	-

(+) growth; (-) no growth

4.3.4 Gas production from glucose

The bacteria use sugar in different patterns depending on enzymes present. Since, substrates during fermentation of carbohydrates undergo aerobic and anaerobic dissimilation and produce acids, sometimes accompanied by the gas production at the end of the fermentation [74]. Therefore, all isolates were subjected to utilization of carbohydrates in order to check the production of gas by them. It was observed that tubes containing glucose as a sole carbon source produce gas seen in an inverted Durham's tube and, therefore, resulted as positive, were considered as hetero-fermentative. Thus, growth in the isolates were marked that showed fermentation of sugars in the medium (Table 13).

4.4 Identification of species by 16S rRNA sequencing and phylogenetic tree analysis

All eleven strains isolated from fermented dal flour batter were identified by sequencing their genes by 16S Sanger sequencing method. To obtain the partial rRNA sequence, genomic DNA from different bacterial isolates was extracted using phenol: chloroform method. Using this genomic DNA, genes were amplified using forward and reverse universal primers i.e. 27F and 1107R. These isolates were identified using the V3 region of 16S rRNA genes. The V3 region is a highly variable region that helps to differentiate between closely related microbial communities

at species level [76]. Sequencing was done based on the modification done by the sanger principle. The electrophoregram was generated as an output forms from the sequencer (Figure 12), to check the quality and reliability for single species DNA. The final sequence (Figure 13) obtained in FASTA format was opened with BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the maximum likelihood and identity of isolated bacterial species (Table 16). All eleven isolates from different fermented dal flour batters were identified using their sequence for 16S partial rRNA gene obtained from National Center for Biotechnology Information (NCBI) and confirmed upto species level. The partial 16S rRNA sequence with the accession numbers given in Table 16 were preserved at NCBI. All isolates revealed more than 98 % identity for particular species.

Based on morphological, physiological, biochemical, and molecular characterization, the species identified belonged to the *Lactobacillaceae* family. The isolates M1, U1, VIP1, TIP1, V1, C1, T1, Mu1, and I8 were identified. The population of LAB in pulse dal flour batter constitute of an extremely large variety of LAB species: *Lpb. plantarum, Lpb. pentosus, Lpb. plantarum, Lev. brevis, Weissella paramesenteroides, W. confusa, W. hellenica, W. jogaejeotgali,* and *Lev. brevis.* When compared to *idli* batter [77], these large varieties of LAB species and strains are common, and previously identified in wheat sourdoughs [78]. LAB constitutes about 85 % of the population throughout fermentation. *L. plantarum* is ubiquitous in nature and found everywhere in the food ecosystem [79]. *W. paramesenteroides* were also isolated from fermented kidney beans flours [80] and chickpea sourdough [73] from north western argentina. Moreover, studies reported the involvement of *Weissella* strains in the leavening process of pizza dough, which occurs by mechanical process of transforming flour proteins to polypeptide chains where starch granules are embedded [81]. The heterofermentative lactobacilli and *Weissella* has been reported in *idli* batter fermentation, and detected in various cereal based fermented products [82].

Phylogenetic tree prepared from the gene sequence of all cultures isolated from different sources is shown in Figure 14. The isolates from pulse dal batter belong to the *Lactobacillales* family and phylum *Firmicutes* from the phylogenetic analysis. The reference isolates obtained from *idli* batter [82] and sourdough [83] present in each clade of tree are closely related with the isolates of flour samples used in the study. Comparison of 16S rRNA sequence of four different

lactobacilli indicated less similarity between and more similarity within groups. The evolutionary analysis was prepared in MEGA X. The closest similarity of 100 % was obtained among species of *Lev. brevis* TIP1 and *Lpb. plantarum* VIP1 followed by that of *Lpb. plantarum* M1 and *Lpb. pentosus* U1 whereby 64 % of their genetic material was found similar. The species having closest relations were isolated from different sources.

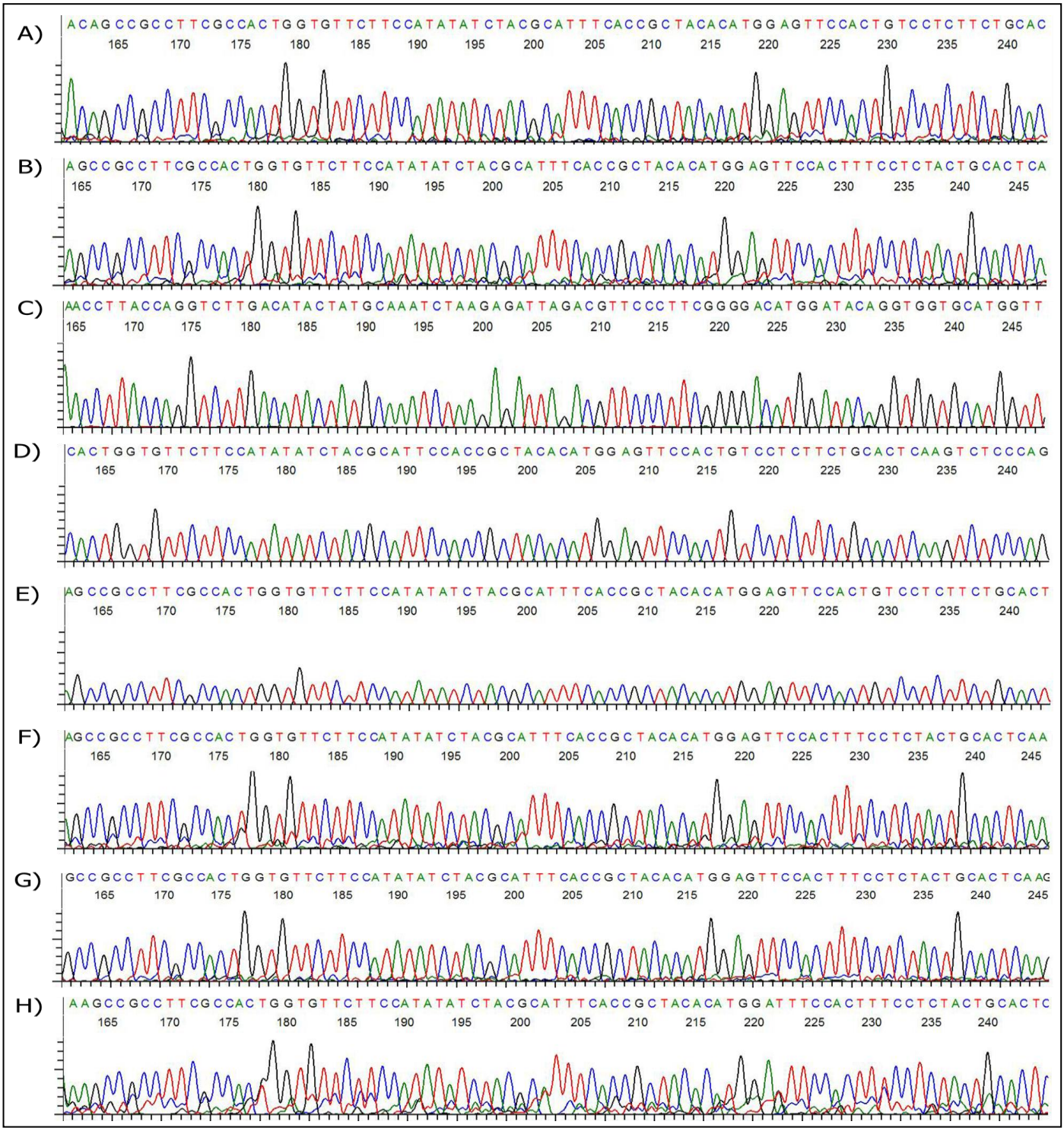

Figure 12: An electropherogram of pulse dal flour isolates. (A) U1, (B) V1, (C) M1, (D) TIP1, (E) VIP1, (F) C1, (G) Mu1, (H) T1.

Figure 13: A representative sequence of pulse dal flour isolates.

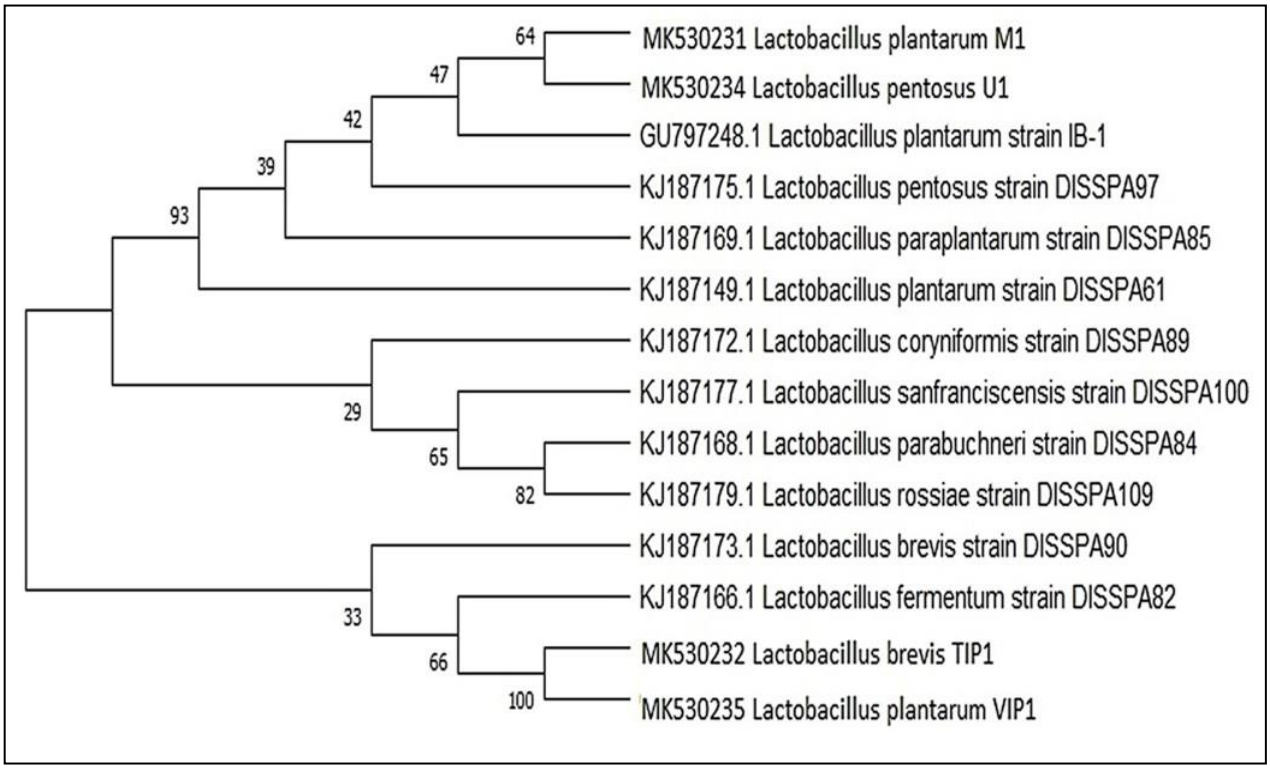

Figure 14: Phylogenetic tree based on 16S rRNA gene sequence. Inferred using Neighbor-Joining method showing the position of isolates and related strains. Each isolate has been designated with accession number in the starting of the isolate obtained from NCBI database.

Table 16: Results of 16S identification by BLAST analysis

Sample	Sequencing result	Accession number	Total score	Query coverage	Max Identity
M1	*Lpb. plantarum*	MK530231	1288	94%	99%
U1	*Lpb. pentosus*	MK530234	1655	90%	99%
VIP1	*Lpb. plantarum*	MK530235	1657	99%	99%
TIP1	*Lev. brevis*	MK530232	8189	99%	100%
Mu1	*Weissella jogaejeotgali*	-	12351	91%	98%
T1	*Weissella hellenica*	MK530233	1424	88%	96%
C1	*Weissella paramesenteroides*	MK530237	1616	94%	99%
V1	*Weissella confuse*	MK530236	1537	89%	98%

4.5 Growth kinetics of isolates using glucose as carbon source

A mathematical model was used to study behavior of bacterial growth and other growth parameters using glucose as a carbon source. Lactobacilli and *Weissella* have reported to generate maximum LA from different sugars [84]. The assessment of such metabolites like LA, functions as conservative that could be helpful during pulses bio-processing [85]. Also, the type of sugar used may affect the way of LA production [84]. Hence, the ability of microorganism isolated from fermented batters was analyzed to induce maximum production of LA. For this, MRS medium containing glucose was considered based on the composition of sugars in pulse seeds. Five lactobacilli and four *Weissella* species were studied for bacterial growth evaluation after incubation for 48 h. Glucose as carbon source was used in submerged batch culture medium.

Figure 15 represents cell growth, pH, LA production and TTA profile for all LAB isolates. Microbial densities of 9.91, 9.99, 8.92, 9.81, 9.64, 9.23, 9.62 and 9.32 log cfu/g were obtained for M1, U1, VIP, TIP1, Mu1, T1, C1, V1. When glucose was used for LA production, the highest titer reached was 15.40 g/l in U1 isolate; while M1, Mu1, VIP1, C1, TIP1, T1, V1 managed to produce 14.80, 14.00, 13.91, 13.90, 11.70, 11.20, 10 g/l of LA after 48 h of incubation. Among *Weissella* spp., C1 isolate showed maximum LA with 14.5 g/l at 36 h, this declined at 48 h to 13.90 g/l. Previous studies stated that growth terminates once concentration in LA increases that could be due to lack of nutrients other than sugars [86]. This process is called energy uncoupling which happens between cell and LA wherein the energy obtained by conversion for making lactate is not utilized for growth rather used in maintainence of pH [87].

LA concentration is directly proportional to microbial growth, but inversely in proportion to decrease in biomass and increase in LA production [87]. Also in some strains, decrease in microbial growth was noted that could be due to an over accumulation of lactate in medium resulting in growth inhibition [88]. Mostly, this inhibition is observed due to accumulation of anions or heavy acidification in cytosol [89]. Fermentation of medium having glucose decrease pH from 6.5 to 4.5 in lactobacilli and *Weissella* species at the end of 48 h incubated at 37˚C. TTA was significantly increased in medium during incubation and ranged from 12.61 to 16.01

g/l for all isolates, except T1 after 48 h. An increase in acidity was observed during fermentation which decreases dominance of contaminated microorganisms in the medium [90].

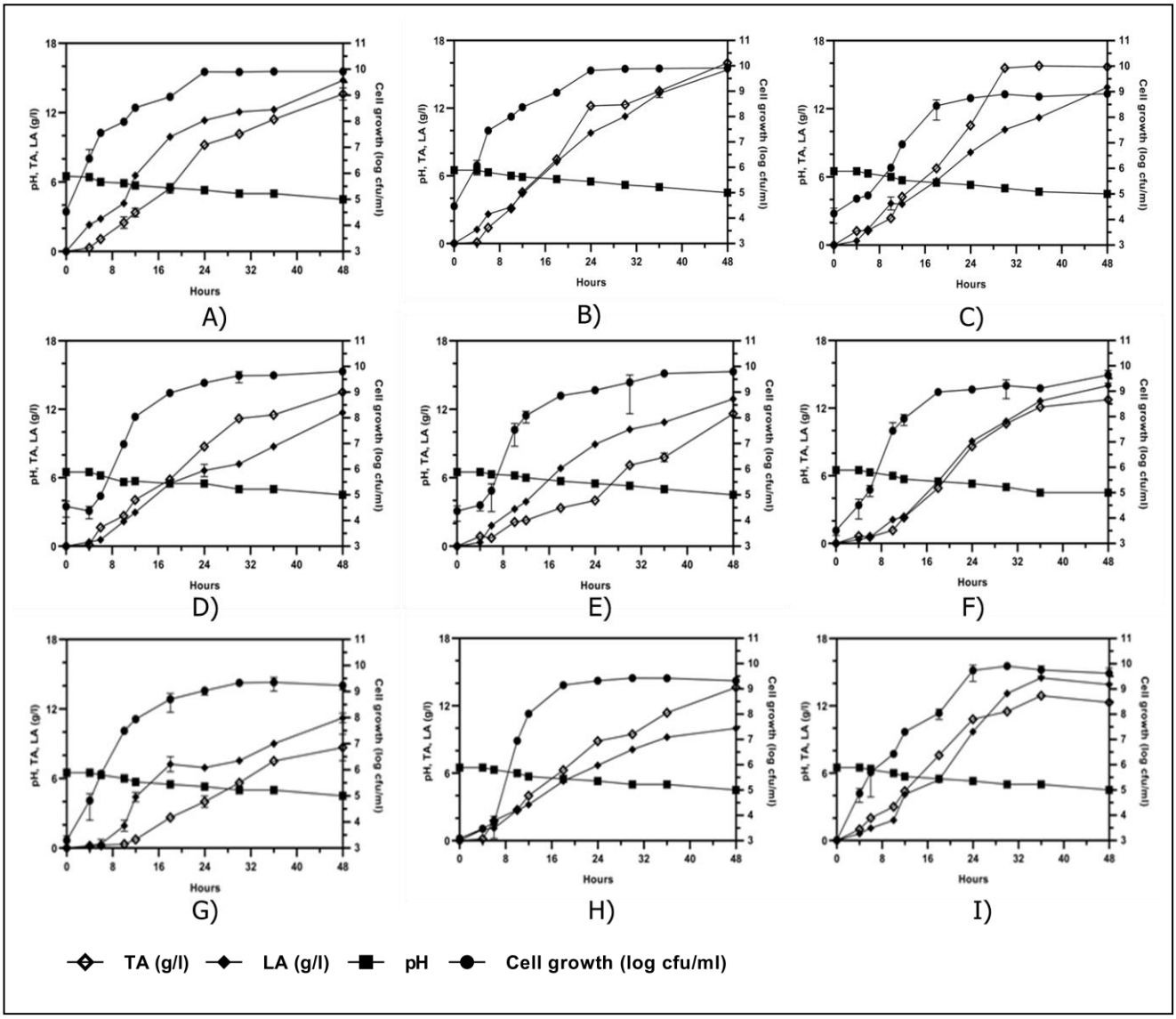

Figure 15: Profile of growth, pH, lactic acid production and titratable acidity during fermentation. (A) M1, (B) U1, (C) VIP1, (D) TIP1, (E) I8, (F) Mu1, (G) T1, (H) V1, (I) C1 isolates using dextrose concentration of 20 g/l, at 37˚C static condition for 48 h.

Further, evaluation of maximum LA production and maximum LA productivity from medium with 20 g/l of glucose was investigated. Lactobacilli and *Weissella* spp. exhibited similar lag phase of 4 h with maximum biomass of 1.50 and 1.58 (OD_{600} nm) in U1 and C1 isolate. Strains displayed longest stationary phases from 24 to 40 h and highest LA production was

accomplished. LA productivities in lactobacilli ranged from 0.24 to 0.32 g/l/h and maximal LA productivities obtained between 1.80 to 3.70 g/l/h. While, for *Weissella* spp. LA productivities ranged from 0.21 to 0.29 g/l/h and 1.8 to 4.0 g/l/h maximum LA productivities were achieved. U1 and T1 culture exhibited the maximal LA productivities from 20 g/l of glucose concentration (Figure 15; Table 17).

The kinetics of acidification was obtained using Gompertz equation in MRS-glu medium and indicated slightly low latency phase for all isolates. Factors such as microbial growth rate, fermentation hours, utilization of substrate and acid formation significantly influence the process [91]. Kinetic parameters like V_{max} and ΔpH depends on microbial concentration in logarithmic phase, where V_{max} value measured was 0.1 dpH/h, highest ΔpH obtained was 0.6 and lag phase lasted for 6 h (λ) for both lactobacilli and *Weissella* spp. pH value was found similar among all isolates.

Table 17: Kinetic parameters of lactic acid production using MRS-glu medium

Isolates	**Conc. of glucose (g/l)**	**OD $_{600}$ nm [a]**	**LA conc. (g/l)[b]**	**P_{LA} (g/l/h)[c]**	**Max P_{LA} (g/l/h)[d]**
M1	20	1.49 ± 0.01	14.8 ± 2.17	0.30	3.73 (6h)
U1	20	1.50 ± 0.01	15.40 ± 0.0	0.32	1.8 (6h)
VIP1	20	1.29 ± 0.09	13.91 ± 0.0	0.28	2.2 (6h)
TIP1	20	1.48 ± 0.20	11.70 ± 0.26	0.24	2.4 (6h)
I8	20	1.49 ± 0.05	12.9 ± 0.07	0.27	2.2 (6h)
Mu 1	20	1.36 ± 0.13	14.0 ± 0.0	0.29	1.8 (6h)
T1	20	1.48 ± 0.20	11.23 ± 0.40	0.23	4.0 (6h)
V1	20	1.37 ± 0.03	10.12 ± 0.10	0.21	2.1 (6h)
C1	20	1.58 ± 0.03	13.90 ± 0.0	0.28	3.0 (6h)

[a]OD, highest optical density; [b]Maximum lactic acid production after 48 h. [c]Maximum productivity of lactic acid at the end of 48 h using glucose concentration of 20 g/l; [d]Highest lactic acid productivity at desired time.

In this study, the growth kinetic parameter of lactobacilli and *Weissella* spp. was evaluated. For this, modified equation was fitted to describe bacterial growth behavior incubated at 37˚C [86].

Considering the fact that microorganisms grow faster during log phase, the graph of $y = \ln(N/N_0)$ is often plotted as logarithmic population size against time as narrated by Zwietering et al., (1990) [92]. Parameters like μ_{max} , λ, and A were calculated using the equation. TIP1 and V1 isolate showed highest growth rate of 1.18 and 1.86 per hour, where bacteria utilize carbon source and proliferates rapidly to archive maximum growth (Figure 16; Table 18). The longest doubling time of 7.96 h was observed in VIP1 isolate; while the shortest 1.81 h in Mu1 culture was obtained. An increase in the time of lag phase (3 h) was observed for all the isolate after which bacteria starts to multiply rapidly.

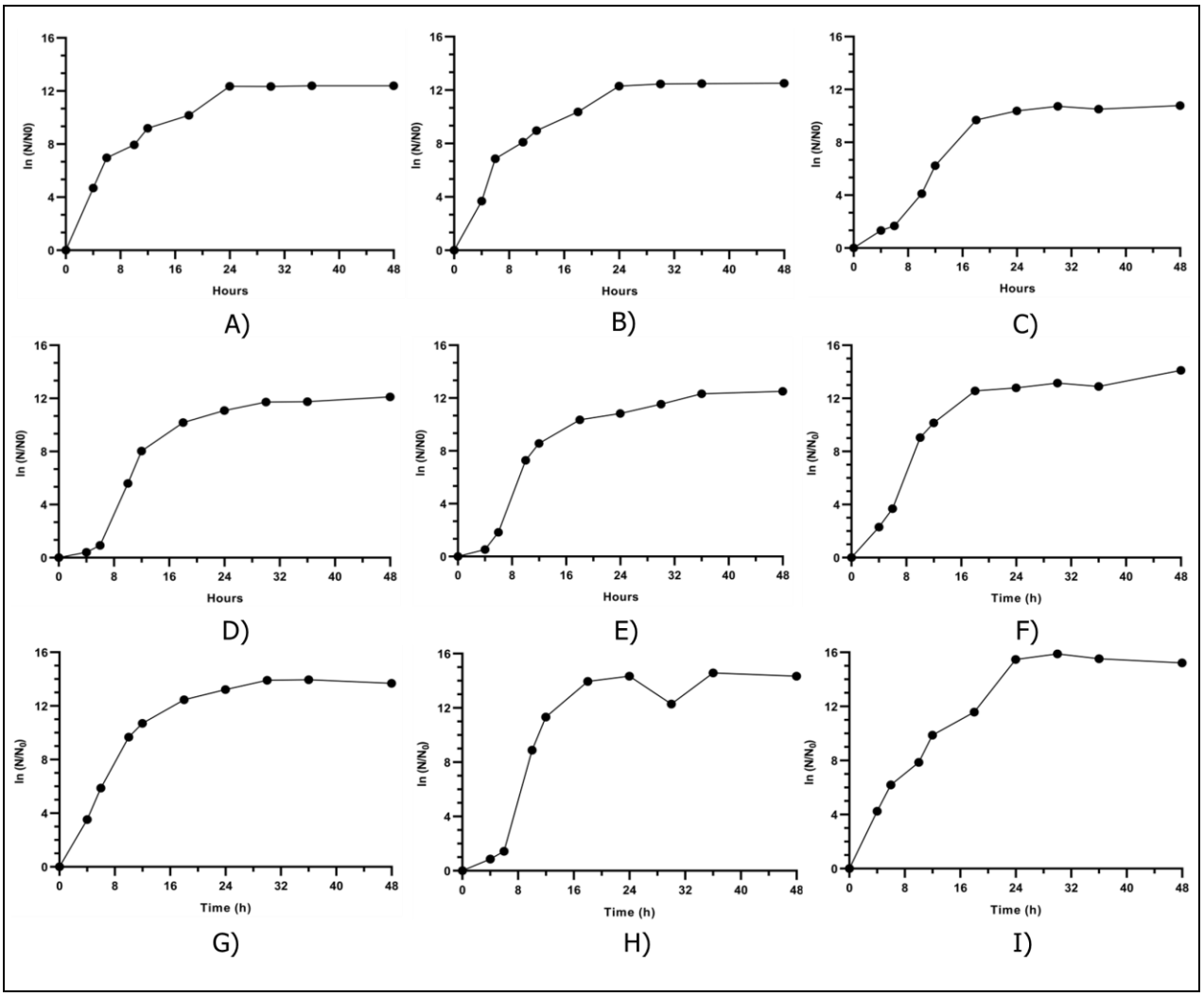

Figure 16: Growth kinetics fitted with modified Gompertz equation, during fermentation. (A) M1 (B) U1 (C) VIP1 (D) TIP1 (E) I8 (F) Mu1 (G) T1 (H) V1 (I) C1 in MRS-glu medium.

This could be due to low inoculum volume that causes clear increase in lag phase [93]. Also, the factor hampering microbial growth is the change in physiological environment of fermentation medium [93]. Obtained experimental data indicated suitability of model by following the exponential law; however, population of bacteria is directly proportional to growth rate that decays with time due to death in organism [94]. Therefore, this bacterial model is the most common for growth kinetics and frequently used among several organisms.

Table 18: Kinetic parameter of bacterial growth curve in MRS-glu medium

Parameters	**M1**	**U1**	**VIP1**	**TIP1**	**I8**	**Mu1**	**T1**	**V1**	**C1**
μ_{max} (h^{-1})	0.37	0.28	0.76	1.18	0.49	1.075	0.95	1.86	0.70
λ (h)	12	18	12	12	12	10	10	10	12
ε (h)	24.83	37.03	8.19	6.81	17.63	9.44	10.17	4.77	14.1
A	9.19	10.37	6.23	8.04	8.57	10.15	9.67	8.88	9.87
Doubling time (h)	7.45	7.14	7.96	3.12	6.93	1.81	3.85	6.86	2.82

Values were obtained using modified Gompertz model for experimental data done in triplicates. D_0, density at $t = 0$; D_t, optical density (OD_{600nm}) at time t; t, growth at particular time (h); A, the asymptotic value (D_0 and OD_{600nm}); μ_{max}, maximum growth rate per hour; λ, duration of lag phase (h); ε, exponential growth time, calculated as $\varepsilon = A/\mu_{max}$.

4.6 Carbohydrate consumption pattern

Pulses, a source of good proteins and carbohydrates (25-65 %) [1]. It constitutes significant portion of starch as storage carbohydrates along with disaccharide such as sucrose [1]. It is distinctly present among species in pulses about 1-3% or slightly higher in amount followed by RFO that accounts to 70% of total carbohydrate [95]. Ganzle et al., (2012) reported that on lactobacilli possess mechanism to metabolize starch, while sucrose is the most extremely preferred substrate [16].

Moreover, raffinose is degraded by sucrose-hydrolyzing enzymes using lactobacilli strains [16]. Therefore, an approach was undertaken using lactobacilli isolates in metabolizing

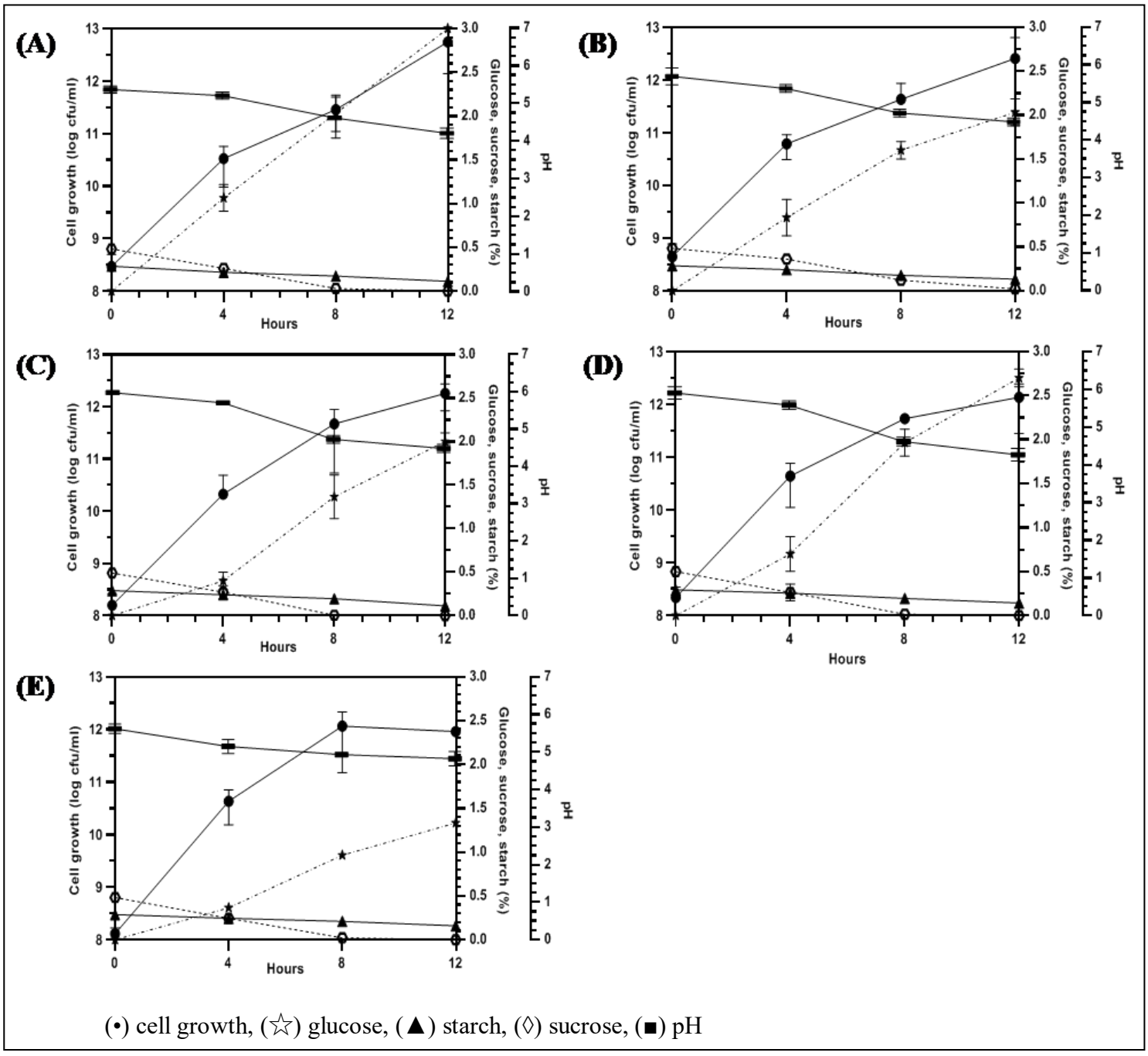

Figure 17: Cell growth, pH as well as starch + sucrose consumption profile. Submerged fermentation using reference strain and (A) M1 (B) U1 (C) VIP1 and (D) TIP1 isolates in mMRS medium at 37˚C for 12 h.

carbohydrate by fermentation. Considering this, a synthetic medium components using raffinose, sucrose, starch as carbohydrate nutrients were taken. Final concentration in medium was established on the basis of quantity present in pulses. However, growth in mMRS was observed

when tested by adding YE while eliminating nitrogen sources. Lactobacilli and standard strain grew efficiently in mMRS medium. Figure 17 (A-E) showed excellent growth pattern in mMRS medium enriched using 0.3 % starch + 0.3 % sucrose, when plotted against time. The logarithmic phase was noted till 8 h followed by stationary phase for some cultures while death phase started in isolates in fermentation medium. The microbial count increased from 8.00-11.50 log cfu/ml reaching maximum to 12.90 log cfu/ml. The microbes tend to grow faster due to proper availability of nutrients in medium. However, bacterial count during fermentation declined to 12.69 log cfu/ml (8 h) and 11.77 log cfu/ml (12 h). Decrease in number of organism during fermentation was perhaps due to huge accumulation of metabolites like AA, LA, ethanol and carbon dioxide [96]. This incline in lactobacilli count could be associated with the capacity of consuming carbohydrate source [96].

Several reporters claimed the decrease in pH by fermentation, leading to acidity and maximize LA accumulation by organism [97,98]. In the present study, similar results were observed, increase in bacterial counts leads to decrease in pH by 4.0 which indicates association between growth and pH [99,100]. Thus, it could be assumed that LA lowers the pH of medium as growth enhances. Literature confirms that lactobacilli utilize starch as direct conversions for generating LA are hence tagged as amylolytic [101,102]. Maximum starch consumption was obtained in reference strain and U1 isolate up to 62 % followed by M1, VIP1 and TIP1 isolate of 50 % after 12 h. Results indicated degradation of starch by starch hydrolyzing enzymes that is usually activated during fermentation and breaks starch to small molecular weight forms like mono- di-saccharides [103-105]. Also, the hike in glucose during fermentation was observed, while reduction in starch content could be due to fermenting microorganism present medium.

In present study, consumption of sucrose was investigated with starting concentration of 1 %. Fermentation enhanced cell population and acidity when incubated at 37°C. Figure 18 (A-E) showed complete utilization of sucrose within 12 h of fermentation with final pH of 4.8. Presence of invertase enzyme breakdown sucrose through catabolism results in glucose and fructose as end product [106]. Similar results were observed by Granito et al., break down of sucrose increases glucose level in natural fermentation of beans [106]. Another report indicated good lactobacilli growth on medium having sucrose [107] including natural sources such

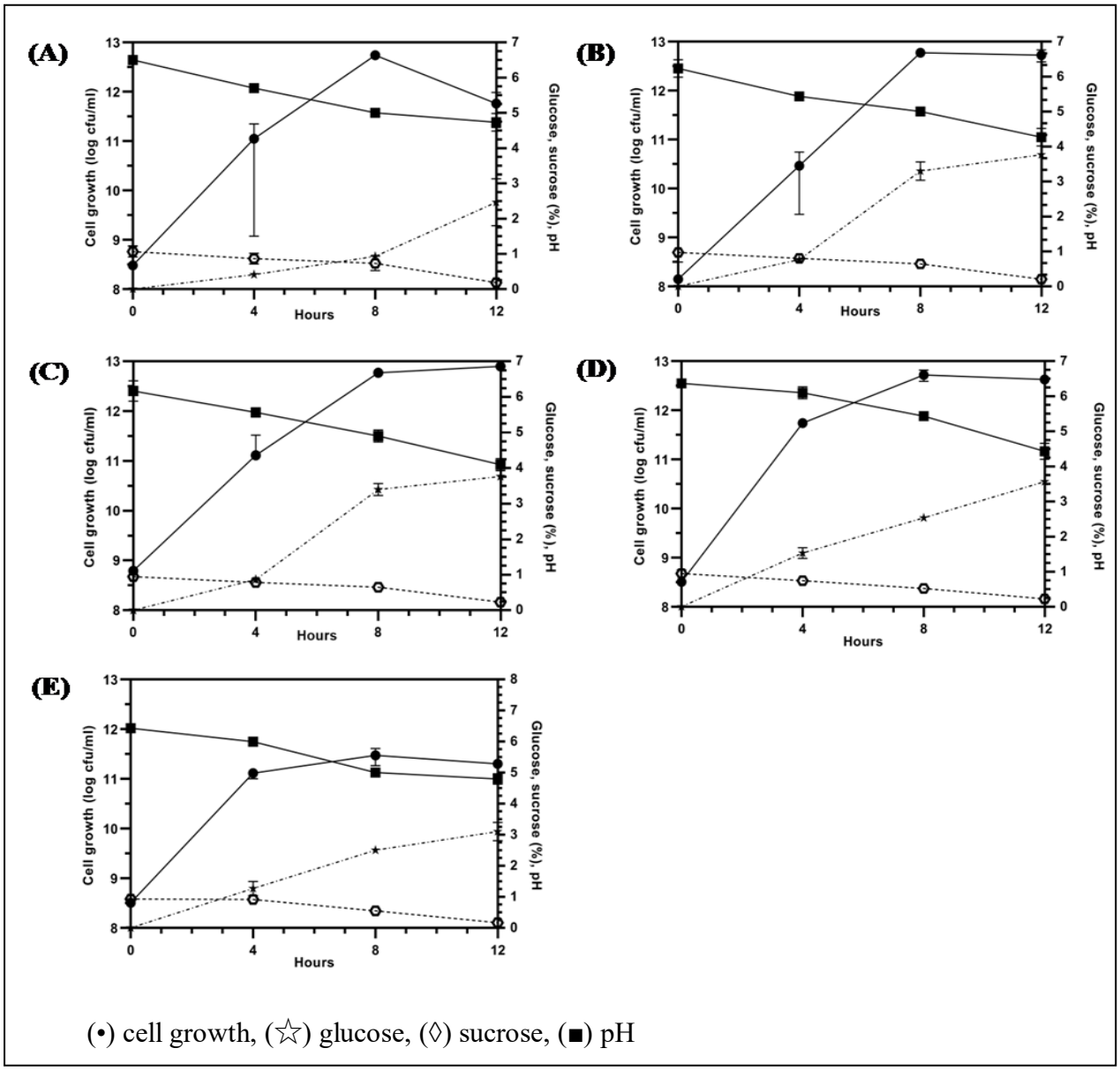

Figure 18: Cell growth, pH as well as sucrose consumption profile. Submerged fermentation using reference strain and (A) M1 (B) U1 (C) VIP1 and (D) TIP1 isolates in mMRS medium at 37˚C for 12 h.

sugarcane juice [108] and cane sugar [109]. Thus, acidic fermentation using low cost sources of sucrose could be a promising process in future.

Further, these lactobacilli was investigated for growth using raffinose as carbohydrate in mMRS medium in Figure 19 (A-E). Isolates showed rapid consumption of raffinose in medium

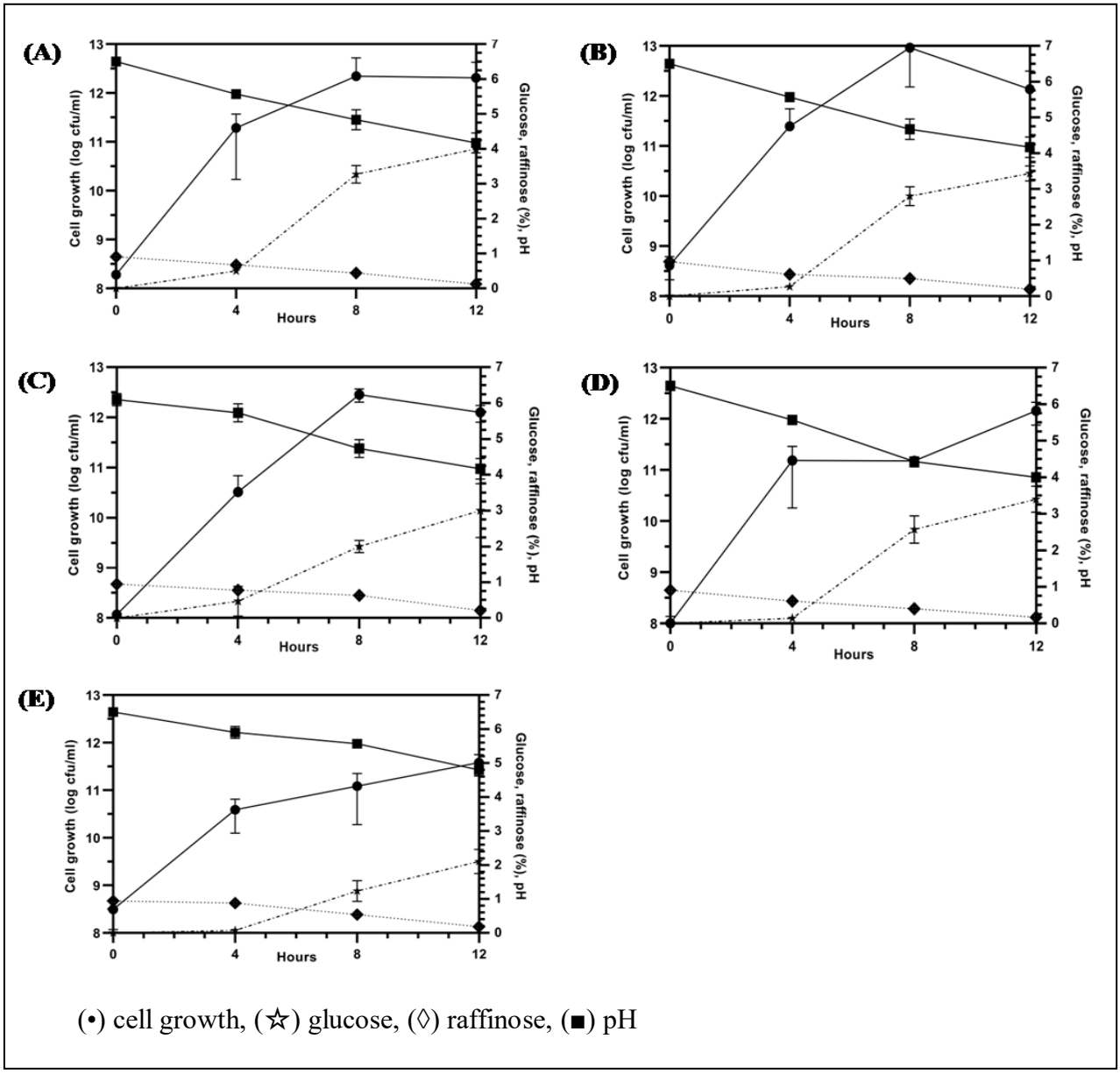

Figure 19: Cell growth, pH as well as raffinose consumption profile. Submerged fermentation using reference strain and (A) M1 (B) U1 (C) VIP1 and (D) TIP1 isolates in mMRS medium at 37˚C for 12 h.

with maximum growth reaching to 12.10 log cfu/ml and acidification capacity lowed to less than 4.5. Similar study reported that *Lpb. plantarum* consumed raffinose substantially and produces glucose from the same [110-112].

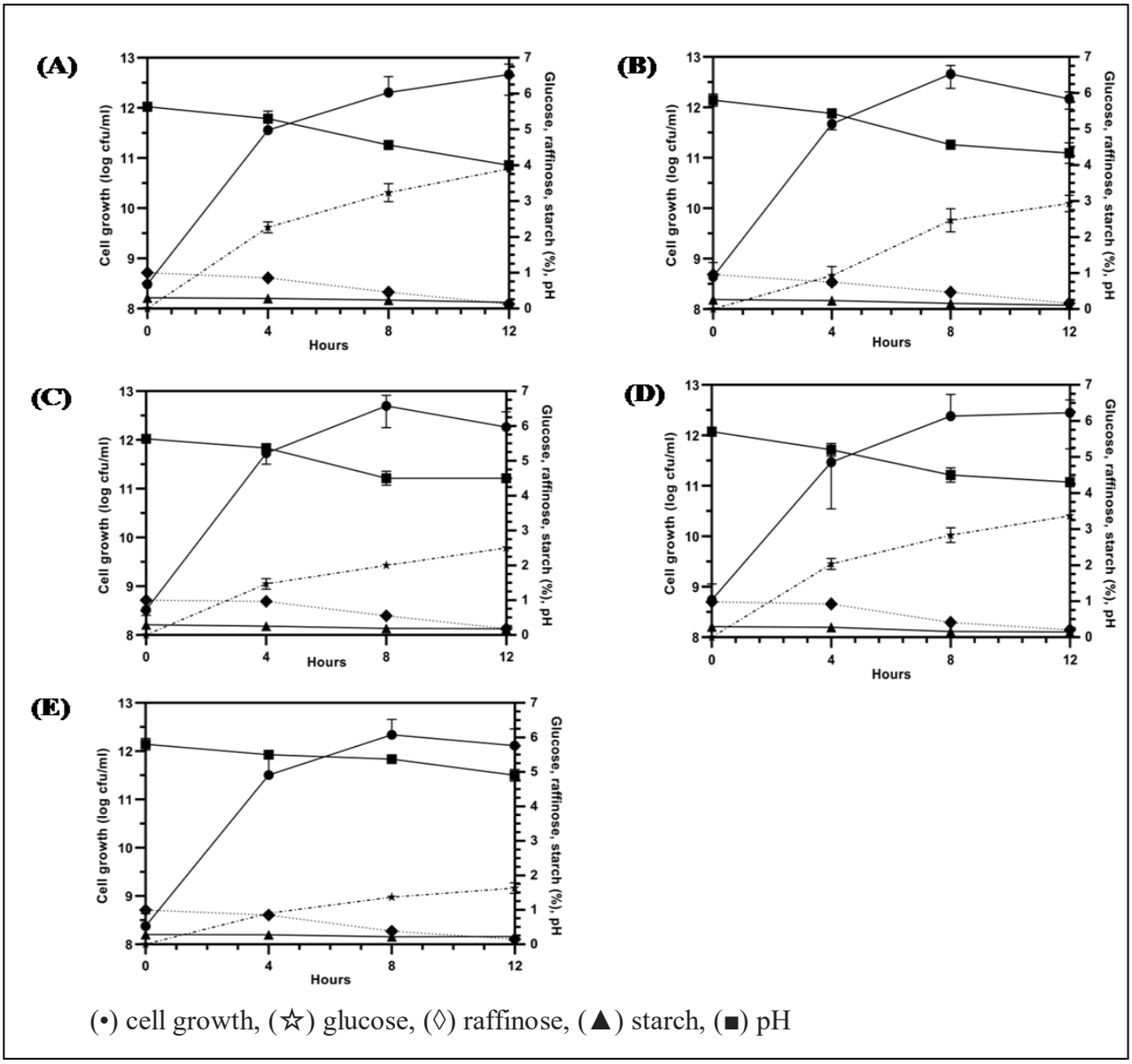

Figure 20: Cell growth, pH as well as raffinose + starch consumption profile. Submerged fermentation using reference strain and (A) M1 (B) U1 (C) VIP1 and (D) TIP1 isolates in mMRS medium at 37˚C for 12 h.

A naturally alorena green table olives was fermented to isolate *Lpb. pentosus*, a probiotic strain showed capacity to utilize raffinose [113]. Another report using *Lev. brevis*, a strain isolated from fermented caper berry indicated no consumption of raffinose [114]. Reference strain including M1, U1, VIP1 and TIP1 were effectively reduced raffinose (80 %) in mMRS medium ($p<0.05$).

Growth profile, pH and carbohydrate utilization of lactobacilli were obtained grown in media containing 1% raffinose + 0.3% starch in Figure 20 (A-E). The inoculum volume was kept constant for all the isolates. Microbial count increase in similar manner and reached to 12.00 log cfu/ml for isolates and reference strain. Raffinose and starch both were present in medium; consumption of sugars was achieved with similar viable cell count in end.

Overall, isolation of lactobacilli from pulse split beans showed outstanding metabolism of sucrose, starch and raffinose in medium. No difference in carbohydrates supplemented in mMRS medium was observed. Also, fermentation hours showed remarkable effect on cell population, carbon consumption and sugar formation at the end of fermentation.

4.7 Screening of isolates for α-galactosidase activity

Total five isolates including reference strain were quantitatively screened for α-galactosidase production. Among five bacterial isolates, only one isolate named, TIP1 showed highest α-gal activity as compared to other isolates. Microbial growth was also calculated which was in between 7.38-7.78 log cfu/ml. Figure 21 shows α-gal activity of TIP1 isolate to be 1.30 ± 0.05 U/ml and specific protein activity 0.81 ± 0.06 U/mg. Extracellular fraction in this study was recorded higher than for intracellular fraction. Results showed that enzymes can be either extracellular or intracellular [115-117]. Literature reported presence of most α-gal as intracellular enzyme; while few strains showing cell bound and extracellular form [40]. However, extracellular enzyme activity was higher in this case. Although, extracellular enzyme provides high yield, good stability and better pH range in comparison to intracellular enzyme [118,119,18].

4.7.1 Effect of temperature

The effect of different temperature ranging from 5 to 60˚C was studied to evaluate α-galactosidase production (Figure 22A). Study of temperature on activity revealed that enzyme production increased at 38 ˚C and started to decrease above 38˚C for TIP1 isolate. This could be probably due to thermal deactivation [4]. Similarly, it has been reported that *L. helveticus* and *L. acidophilus* gives higher activity at 37˚C, *L. fermentum* and *Lpb. plantarum* showed activity at

50˚C [120-124]. The enzyme stability occurred between 20-45˚C and retained its activity at higher temperature (Figure 22B); while 80% of enzyme stability was maintained.

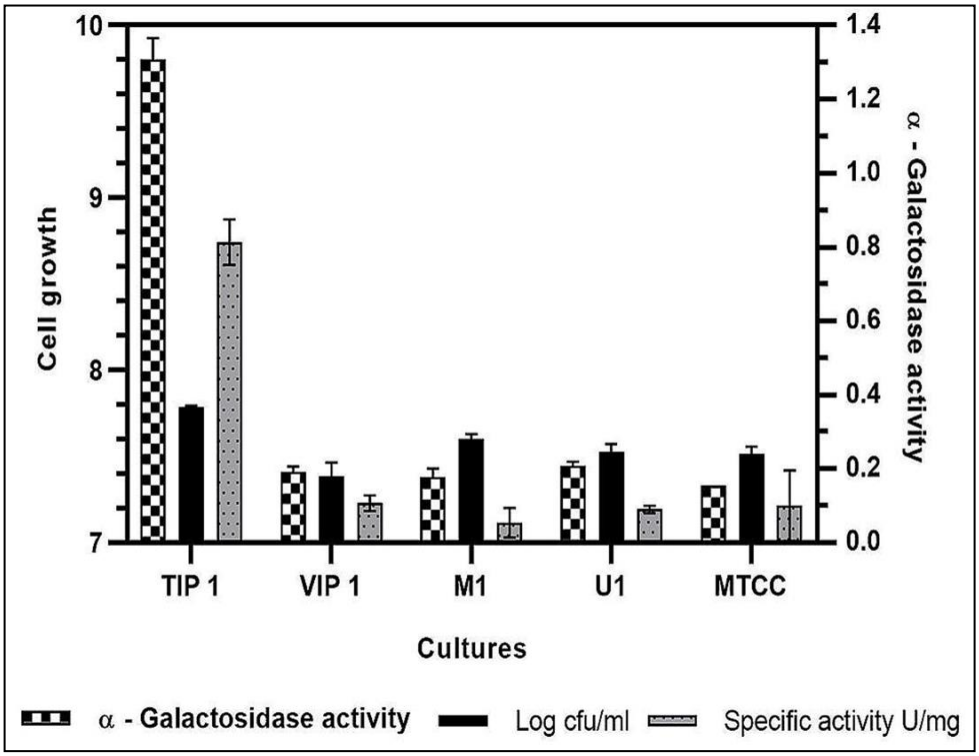

Figure 21: Evaluation of isolates for α-galactosidase activity. Bacterial growth and specific activity in mMRS medium containing raffinose incubated 37˚C, 24 h.

4.7.2 Effect of pH

The effect of different pH between 2 to 11.5 was studied to evaluate α-galactosidase production (Figure 22C). Maximum α-gal activity was achieved at pH 6.5. However, production of enzyme was less when the pH increased i.e alkaline range. Similar to our study, G.Tzortzis et al., (2003) reported maximum α-gal activity in *L. reuteri* during acidic condition [125].

4.7.3 Effect of different carbon sources

To understand the best carbon source, carbon source effect was studied for production of α-gal by isolate TIP1 (Figure 22D). Different carbohydrates such as raffinose, sucrose, starch, sucrose + raffinose and starch + raffinose were supplemented in mMRS medium and α-gal activity was

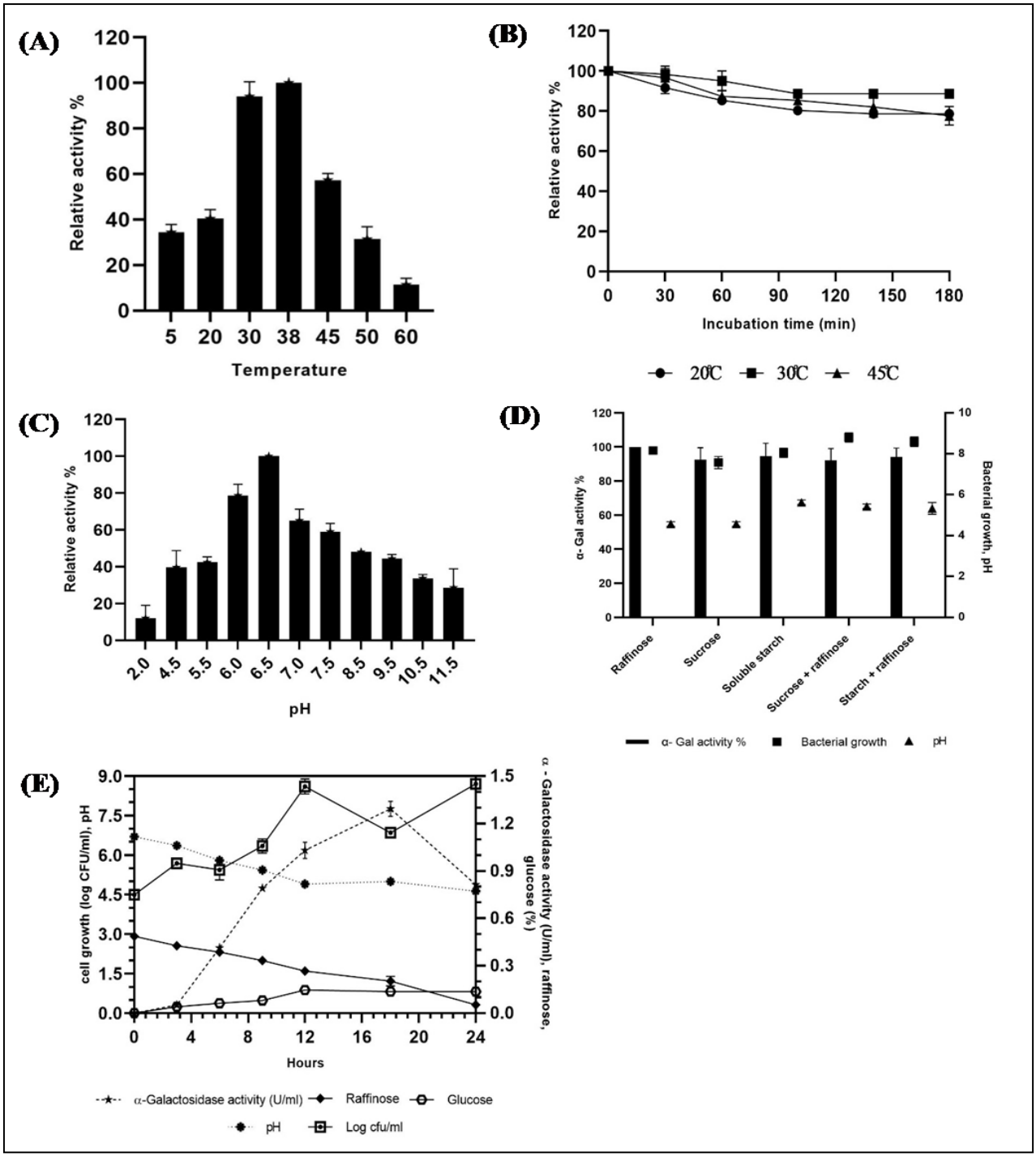

Figure 22: Effect of different parameters on the production of α-galactosidase enzyme. (A) temperatures, (B) temperature stability, (C) pH, (D) carbon source and kinetics of α-galactosidase (E) during fermentation with TIP1 culture in mMRS medium.

measured. Raffinose promoted maximum microbial galactosidase, followed by starch, starch + raffinose, sucrose + raffinose and raffinose.

No noticeable difference in increase of activity from other carbon source was observed, even when used in combination. Similar results were observed in *Lpb. plantarum* where raffinose expressed maximum enzyme production [126]. The studied lactobacilli expresses highest activity at concentration 5.0 g/l, but not increase in activity was noted when concentration raised to 30.0 g/l.

4.7.4 Kinetics profile of α-galactosidase production

Kinetics of TIP1 indicating cell number and product of raffinose determined in mMRS medium at different time is shown in figure 22E. Cell growth was rapid during initial hours and reached to maximum 8.06 log cfu/ml, 12 h. Final pH of medium dropped to 4.60 after 24 h of fermentation. Meanwhile, enzyme activity started to appear after sometime indicating that carbohydrate has been utilized and reached highest activity to 1.29 U/ml after 18 h. related to our study, Garro et al., (2004) reported degradation of raffinose following the growth pattern of bacteria [127]. Utilization of raffinose inclines enzyme activity; however decline in activity was observed during fermentation hours. With course of time, decreased in raffinose went to no detectable point at 24 h.

4.7.5 Production of α-galactosidase enzyme during fermentation

Enzyme activity, bacterial growth and utilization of oligosaccharide or non-digestible oligosaccharides (NDO) were evaluated during bean fermentation. NDO are important part of pigeon pea and causes problem upon consumption [126]. These oligosaccharides are hydrolyzed by microorganisms and produces CO_2, NH_3 and H_2 gas in intestine [126]. TIP showed outstanding growth and highest enzyme activity during initial hours of stationary phase (Figure 23A). TIP showed maximum count till 14 h; cell started to divide and increased to 9.79 log cfu/ml. The rapid growth in medium during the start might be because of active grown culture and better availability of nutrients such as starch, sucrose, *etc* [113]. Moreover, higher content of sugars in medium facilitates organism to long time survival in medium [113].

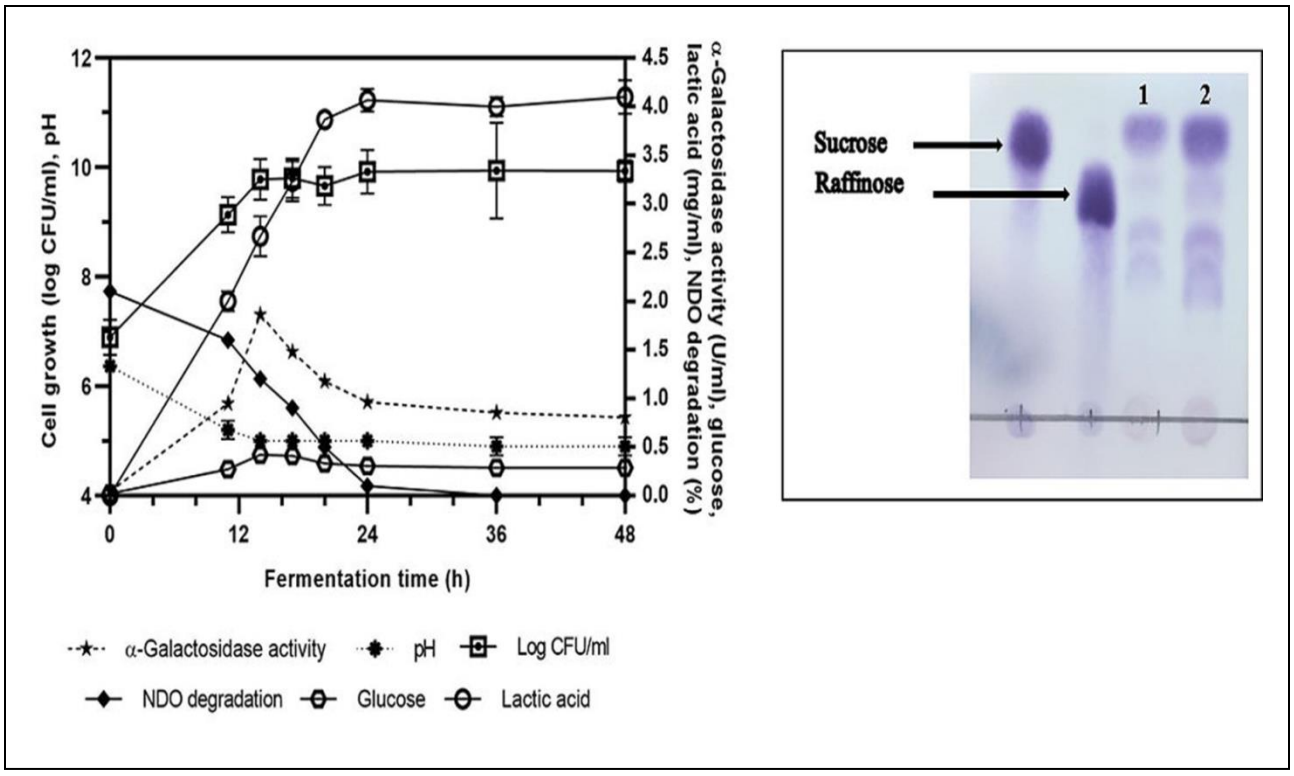

Figure 23: Change in parameters during pigeon pea fermentation with by TIP culture. Residual sugars, bacterial growth, lactic acid, pH reduction, and α-galactosidase activity (A). TLC of NDO in control and test. Lane 1: sample treated for 18-h, Lane 2: untreated sample (B). Experiment was done in triplicates.

At 14th h, growth of organism decreased, mostly because of start of stationary phase which remained constant till 48 h. Also, medium pH dropped to 5.0 at 14 h, due to formation of acidity in medium by TIP1 isolate. TIP1 reduced concentration of NDO significantly ($p < 0.05$) by 0.10 mg/100ml. Gal enzyme breaks galactoside bond existing in raffinose and sucrose, a primary substrate for lactobacilli [16]. Importantly, activity of enzyme was found to be higher in case bean fermentation compared to mMRS medium. Highest activity of enzyme was 1.86 U/ml during log phase that decreased sharply by end. A complete utilization of oligosaccharide was observed in 36 h, an increased activity was noted with time. α- Gal activity is strain dependent and has been recorded in many lactobacilli [119]. Further analysis on reduction of oligosaccharide using TLC is as done by Tanaka et al., (1975).

TIP1 analysis of bean fermentation using enzyme showed hydrolysis of oligosaccharides after 18 h (Figure 23B). TLC indicated a proper decrease in NDO treated bean when culture was added compared to untreated sample i.e. control. Same time another two bands appeared that could be verbascose and stachyose. The concentration of these carbohydrates after sucrose and raffinose are considered higher in pigeon pea [128]. As documented, fermentation with GRAS category organism could effectively decrease galacto-oligosaccharides content in fermented cereals and legumes, due to high enzyme activity [129]. There are other reports confirming removal of raffinose using *Aspergillus niger* in cowpea flours [129] and *Bacillus megaterium* in soymilk [3]. Moreover, advantage of using bacterial enzyme is the cost effectiveness; hence using native culture with maximum benefits could be a promising approach in removing oligosaccharides causing flatulence and also adding probiotic features in product.

4.8 Optimization of medium components using pulses to enhanced lactic acid production

Lactic acid producing lactobacilli that originated from various sources are broadly studied [84]. Lactobacilli are diversely present in fermented foods and represent various functional activities [130]. Lactobacilli are notably high LA producers; it might be applied for establishing valuable fermented products [84]. Pulses are nutritionally rich in carbon sources and its use during fermentation process as raw material does not involve any special treatment to release carbohydrate in fermentable form [131]. Considering the fact, pulses were employed in developing a simple synthetic medium only comprising of nitrogen and mineral element for bacterial growth and LA production. Evaluation of major medium components in proportions affecting the growth and LA production were carried out using these lactobacilli. Further, primary screening of the ingredients was done to investigate the effect of the media components on product formation and few additional components were used for further optimization. Figure 24 shows the flow chart for preparation of fermented beans.

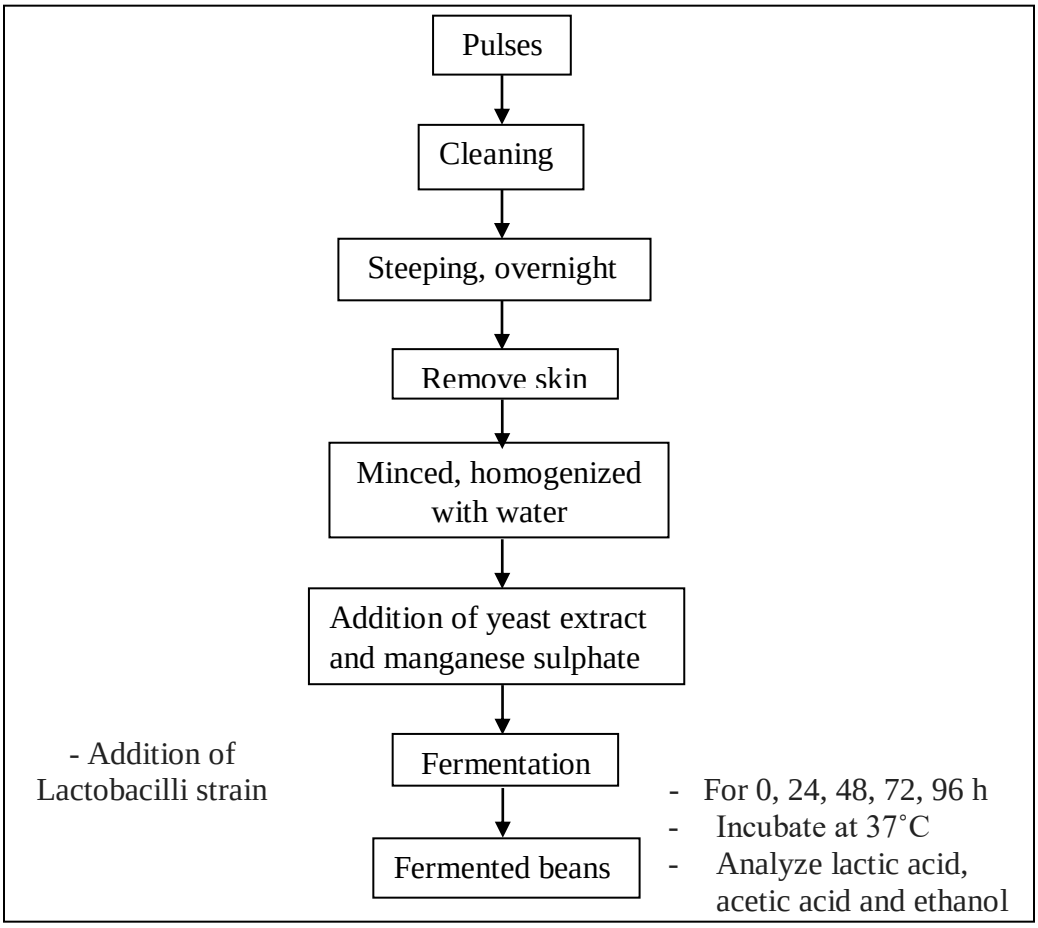

Figure 24: A flow chart for preparation of fermented beans.

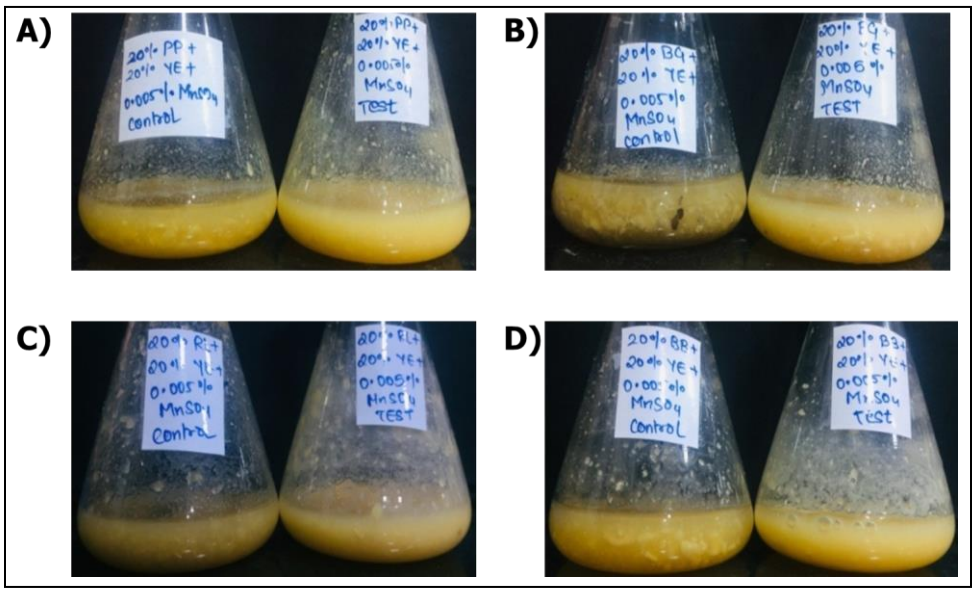

Figure 25: Flask containing different pulses inoculated with respective strains. Medium without inoculation (left) and test flask inoculated (right) with yeast extract, manganese sulphate and strains such as *Lev. brevis* TIP1 in PP (A) *Lpb. pentosus* U1 in BG (B) *Lpb. plantarum* M1 in RL (C) *Lpb. plantarum* VIP1 in BB and kept at 37˚C incubation.

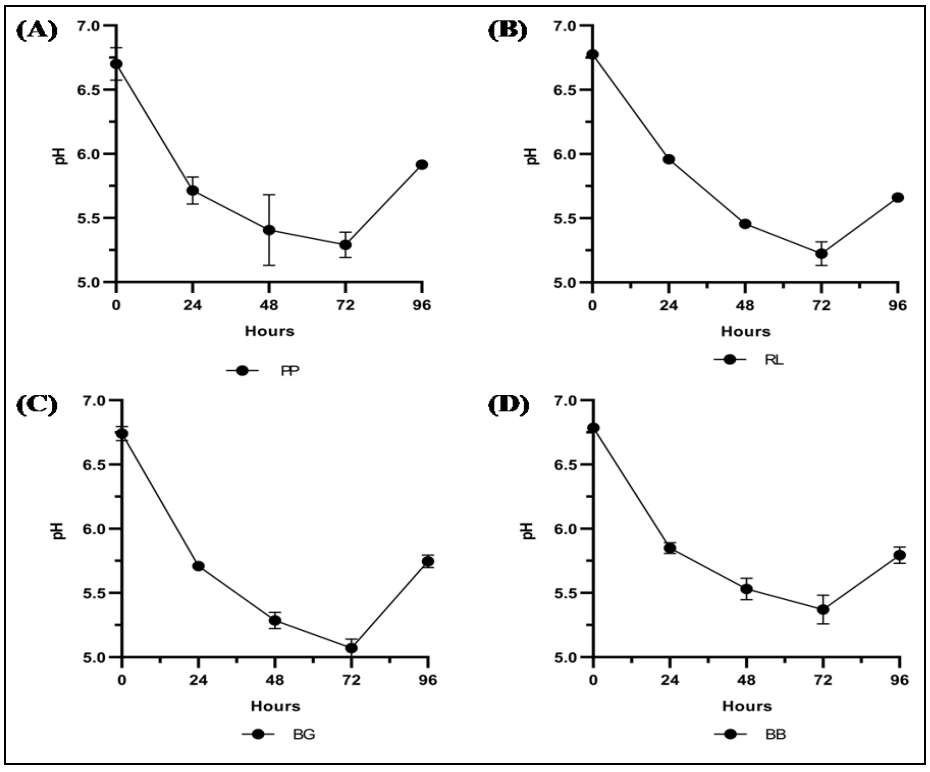

Figure 26: Effect on pH. PP (A), RL (B), BG (C), BB (D) beans using TIP1, M1, U1 and VIP1 cultures in sterile distilled water without the addition of other nutrients, incubated at 37˚C for 96 hrs.

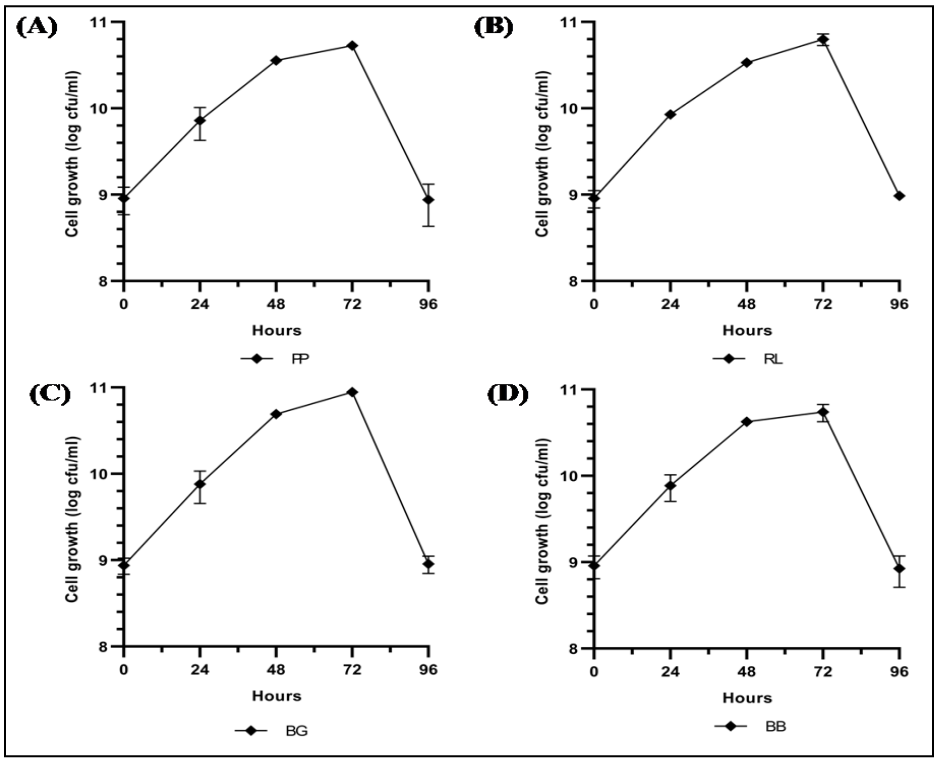

Figure 27: Effect on cell growth. PP (A), RL (B), BG (C), BB (D) beans using TIP1, M1, U1 and VIP1 cultures in sterile distilled water without the addition of other nutrients, incubated at 37℃ for 96 hrs.

pH is considered important for cell growth and LA production (Figure 26). During fermentation, medium with 20% BG maintained relatively more acidic conditions compared to other beans (Figure 27). Similar to this study, Altaf et al., (2005) explained that this might be because of unavailability of certain minerals in the medium, as lactobacilli has a complex nutrient requirement, and the beans were dispensed only in distilled water devoid of nutrients [132]. This explains the necessity for vitamins and specific amino acids for growth of organism, which could be absent in the beans [133].

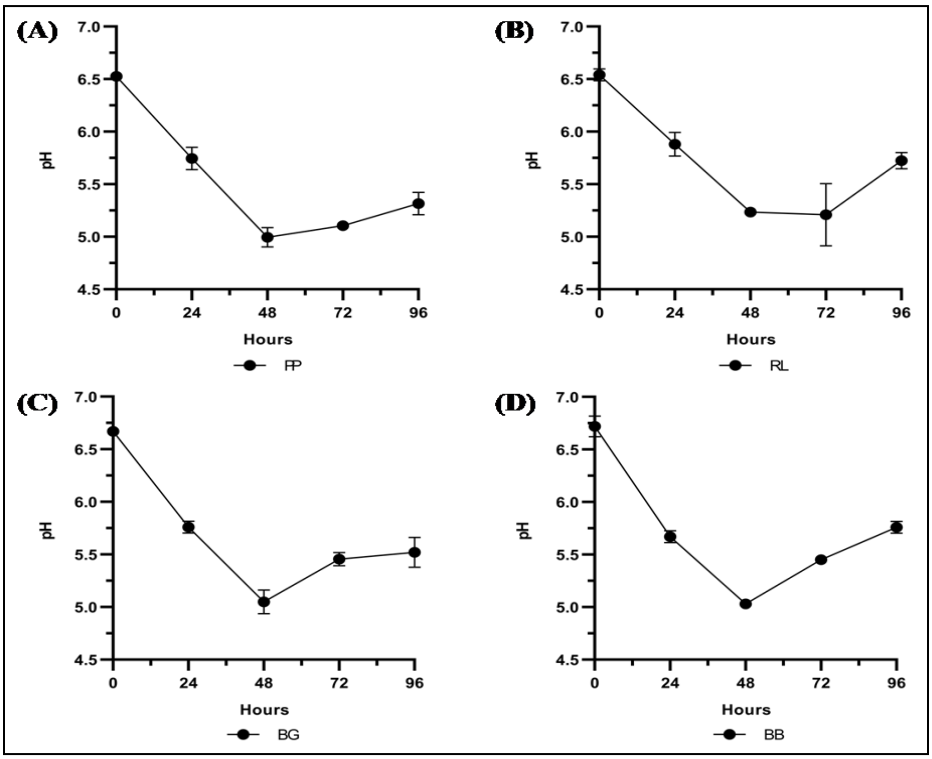

Figure 28: Effect on pH upon addition of yeast extract. TIP1 (A), M1 (B), U1 (C) and VIP1 (D) cultures with yeast extract concentration used was 20 % and the concentration of beans was fixed to 20 %.

Fermentation of beans was performed initially without yeast extract (YE) in order to highlight the performance of isolates. Figure 28 illustrated long fermentation time required to achieve high sugar conversion and pH reduction. Addition of yeast extract in medium ranged from 1 to 20 %, however at 20 % YE, increased in cell growth and reduction in pH time from 72 to 48 h was observed, this is still a long time.

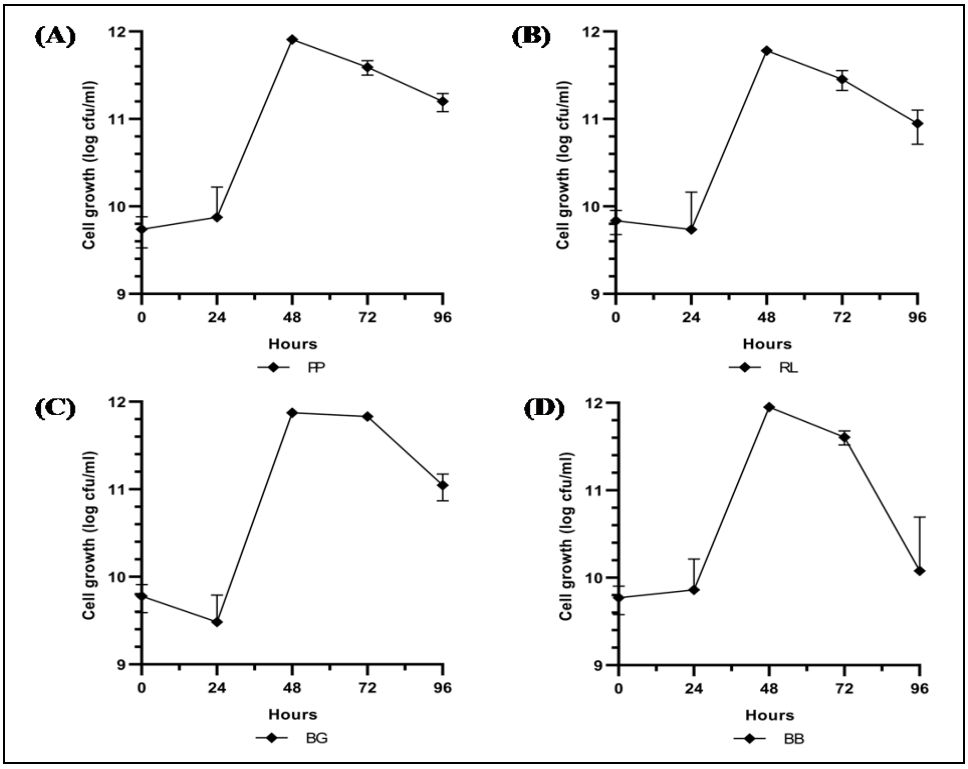

Figure 29: Effect on cell growth upon addition of yeast extract. TIP1 (A), M1 (B), U1 (C) and VIP1 (D) cultures with yeast extract concentration used was 20 % and the concentration of beans was fixed to 20 %.

Several authors reported presence of peptides in YE, contribution of B- vitamin, purine and pyrimidine bases enhances growth of lactobacilli in the medium (Figure 29). The slow cell growth during fermentation could be because of nitrogen deficiency in the medium. Nancib et al., (2005) used date juice and other nitrogen sources with TE and compared them in terms of efficiency for higher acidity. However, none of used sources gave LA as high as that with YE [134,135].

Figure 30 represents that Mn^{2+} has significant effects on growth of lactobacilli isolate. Results showed that addition of 0.05 g/l of $MnSO_4$ with 20 % YE substantially increased the cell density, reduced pH level strongly or increased rate of acidity, while, higher concentrations

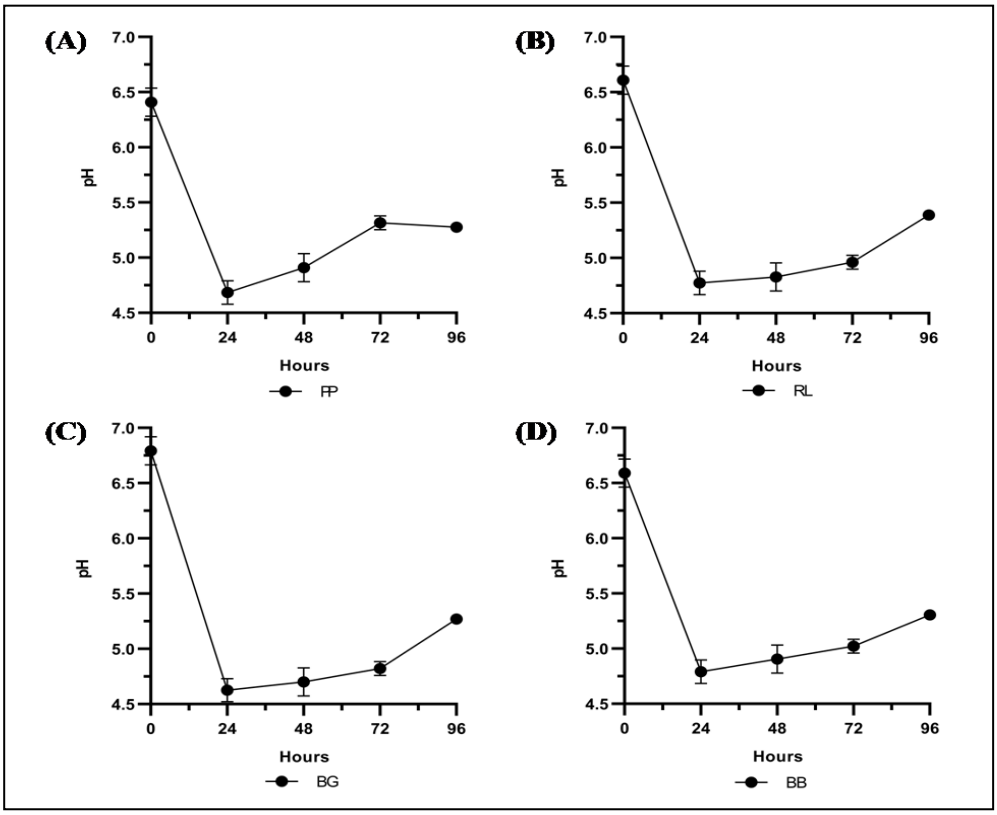

Figure 30: Effect of manganese sulphate, yeast extract and beans on pH. TIP1 (A), M1 (B), U1 (C) and VIP1 (D) culture with $MnSO_4$concentration used was 0.05 g/l, concentration of pigeon pea and yeast extract was fixed to 20 %.

indicated reduced growth rate. At concentrations of 20 % PP + 20 % YE, the maximum number of this microorganism was 11.90 ± 2.19 log cfu/ml at 48 h, respectively, which were notably increased to 12.55 ± 1.42 log cfu/ml, 24 h in medium containing Mn^{2+} (Figure 24). Mn^{2+} showed a productive effect on LA production [136]. Mn^{2+} is a cofactor of certain lactobacilli enzymes such as lactate dehydrogenase (LDH), RNA polymerase, superoxide dismutase and NADH oxidase that helps in improving efficiency and productivity of LA fermentation [137].

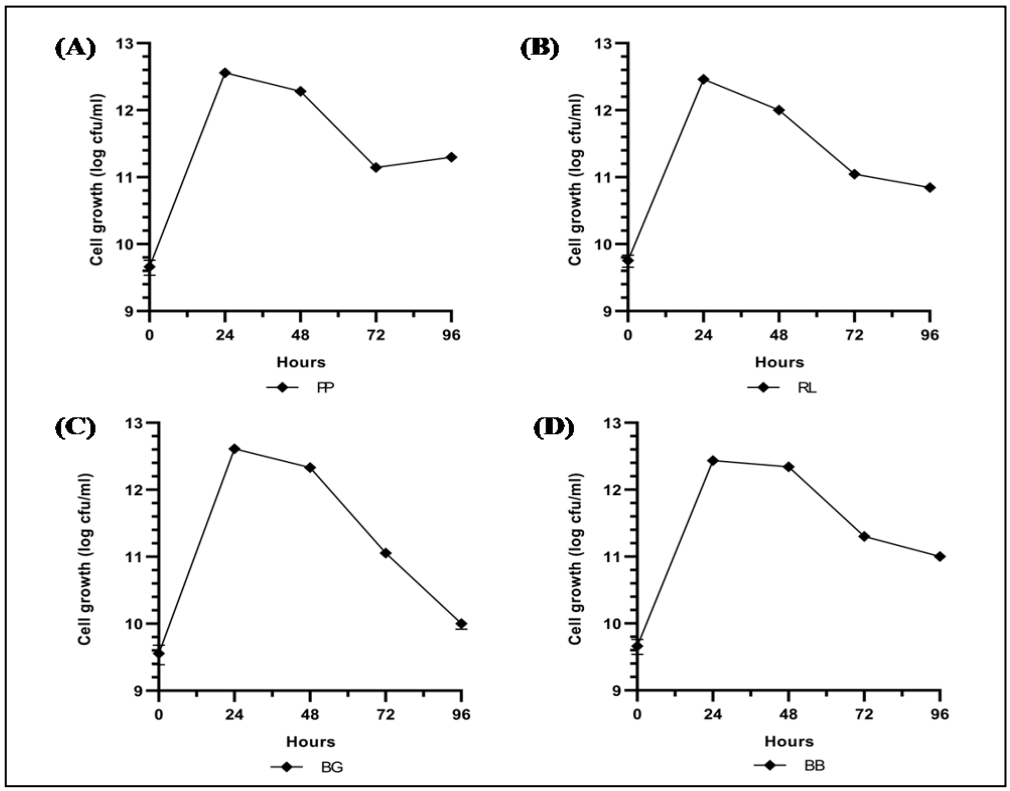

Figure 31: Effect of manganese sulphate, yeast extract and beans on cell growth. TIP1 (A), M1 (B), U1 (C) and VIP1 (D) culture with $MnSO_4$ concentration used was 0.05 g/l, concentration of pigeon pea and yeast extract was fixed to 20 %.

Cheng et al., (2014) demonstrate Mn^{2+} effect on stimulation of LDH by direct conversion of pyruvic acid to LA [138]. The obtained results showed decreases in LA production in absence of $Mn2^{+}$ [139]. Although, *Lpb. plantarum* tolerated high Mn^{2+} concentration, its growth was affected [139]. For this, it becomes necessary to fix concentration of $Mn2^{+}$ to avoid a negative effect on LA production.

4.8.1 Estimation of lactic acid in sample using HPLC

The representative HPLC chromatogram of standard LA is shown in Figure 32. Table 19 shows the results of retention time and concentration for LA analyzed. Similarly, analysis using H_2SO_4 treated samples showed identical retention time near to 4.6 min.

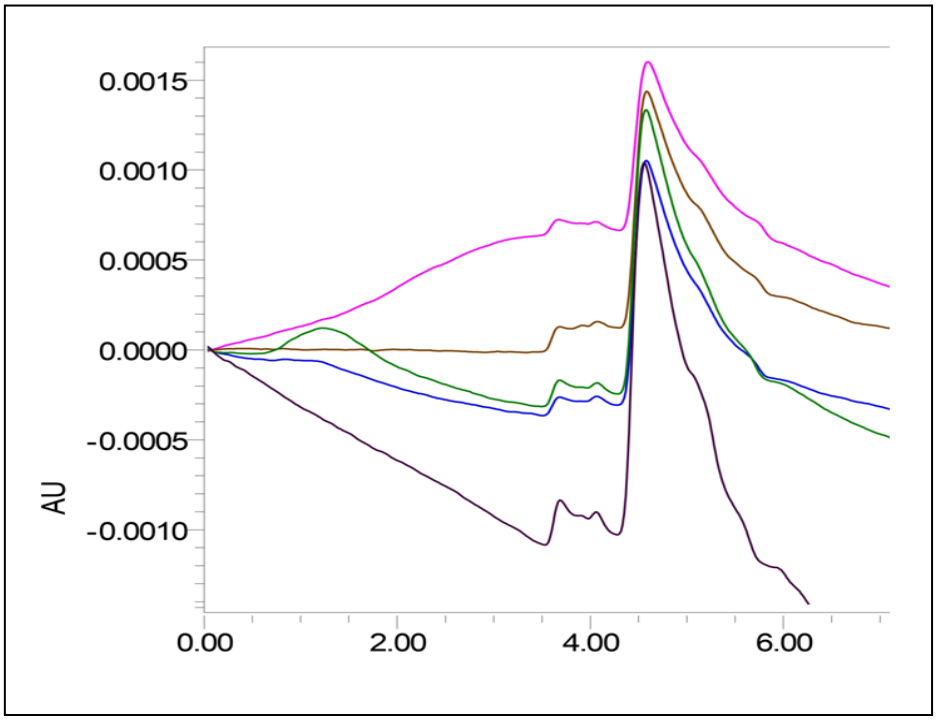

Figure 32: Chromatogram of standard lactic acid.

Table 19: Retention time of standard lactic acid

Standard LA concentration (mM)	Retention time (t_R) (min)	Area (AU)
5	4.561	35037
10	4.579	51407
15	4.583	71430
25	4.588	89752
50	4.598	128728

(I)

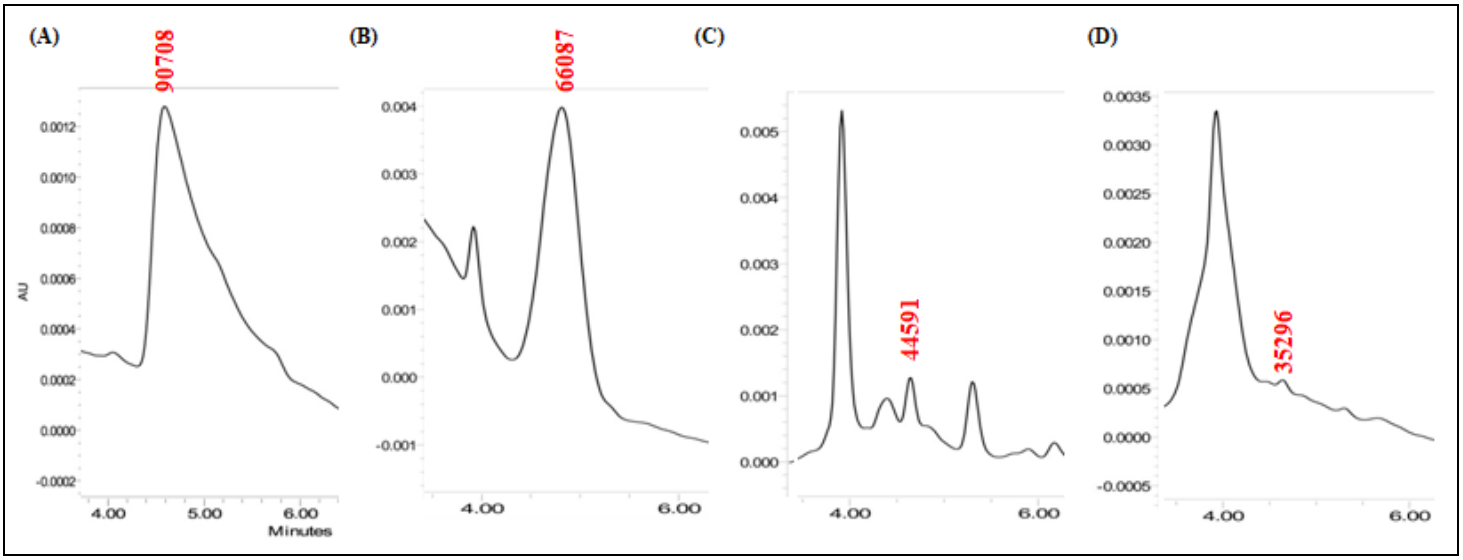

Figure 33: Chromatogram peaks of lactic acid at different fermentation time in PP. (A) 24 (B) 48 (C) 72 and (D) 96 h in 20 % PP + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 20: Retention time (t_R) and area for LA at different hours in PP

Fermentation time (h)	Retention time (t_R) (min)	Area (AU)	LA (g/l)
24	4.592	90708	6.30
48	4.704	66087	5.10
72	4.634	44591	2.34
96	4.855	35296	0.20

(II)

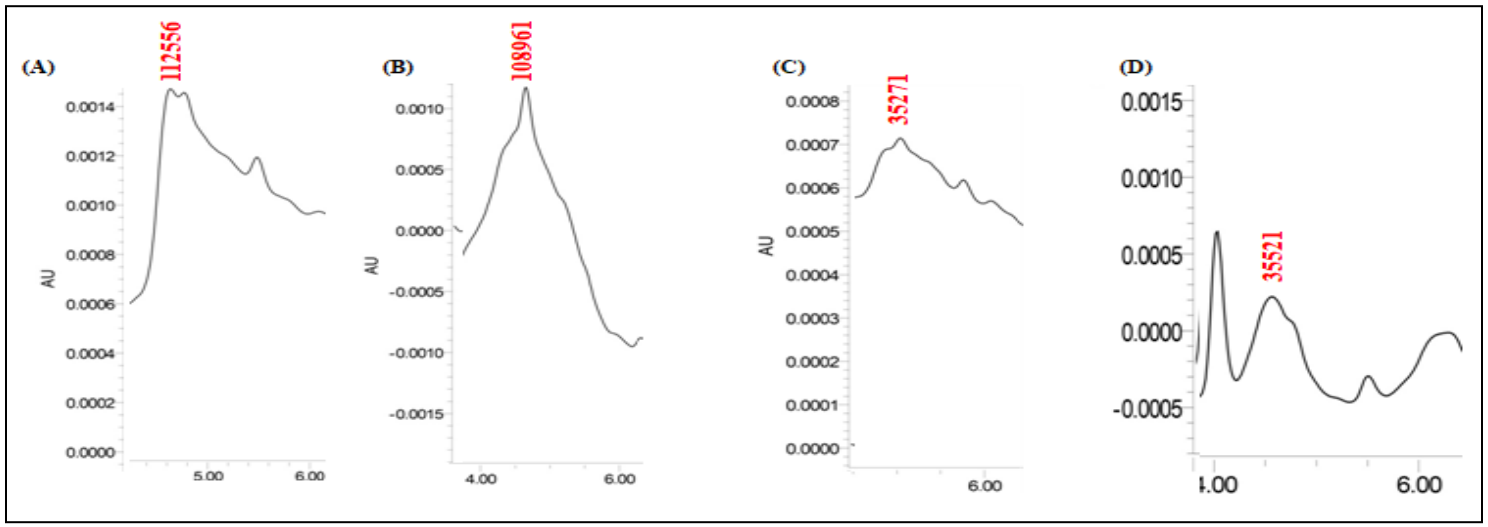

Figure 34: Chromatogram peaks of lactic acid at different fermentation time in RL. (A) 24 (B) 48 (C) 72 and (D) 96 h in 20 % RL + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 21: Retention time (t_R) and area for LA at different hours in RL

Fermentation time (h)	Retention time (t_R) (min)	Area (AU)	LA (g/l)
24	4.693	112556	4.17
48	4.652	108961	3.52
72	4.927	35271	0.41
96	4.799	35521	0.34

(III)

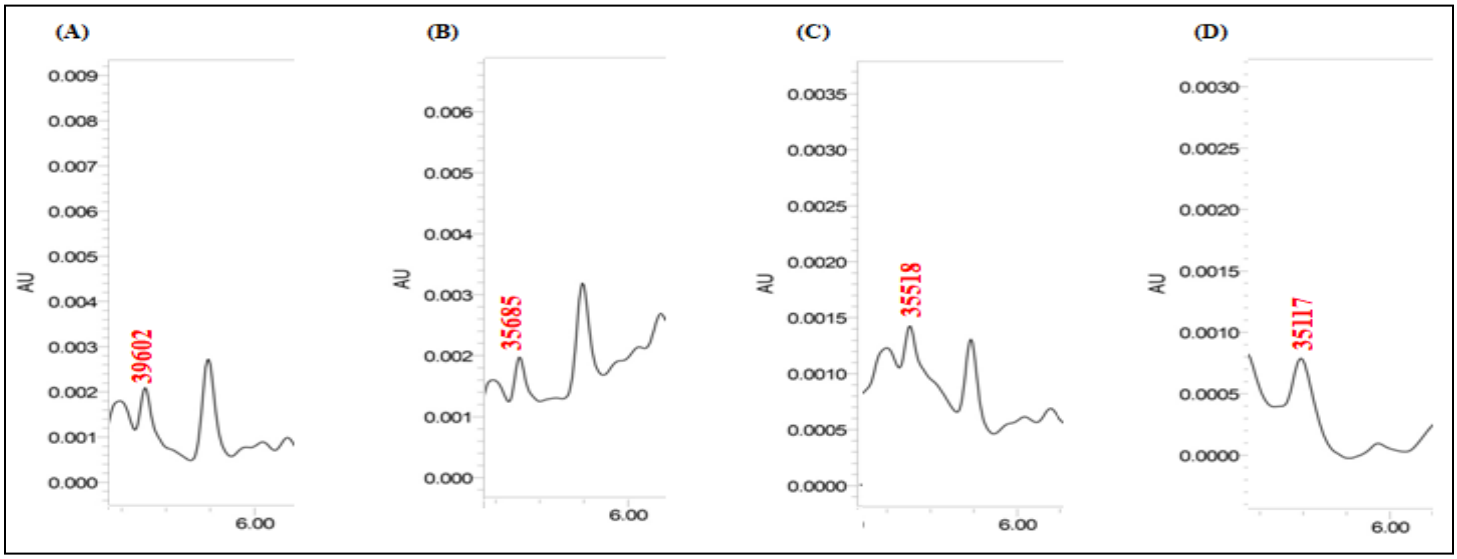

Figure 35: Chromatogram peaks of lactic acid at different fermentation time in BG. (A) 24 (B) 48 (C) 72 and (D) 96 h in 20 % BG + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 22: Retention time (t_R) and area for LA at different hours in BG

Fermentation time (h)	Retention time (t_R) (min)	Area (AU)	LA (g/l)
24	4.773	39602	3.06
48	4.767	35685	1.44
72	4.762	35518	0.21
96	4.972	35117	0.17

In the plotted chromatogram, pigeon pea sample showed maximum LA formation of 6.30 g/l at 24 h inoculated with *Lev. brevis*, while lowest production of 3.06 g/l was observed in black gram sample inoculated with *Lpb. pentosus*. However, decrease in LA was noted after 24 h; in broad beans it went to no detectable level at 96 h. Moreover, decrease in production of LA towards the

(IV)

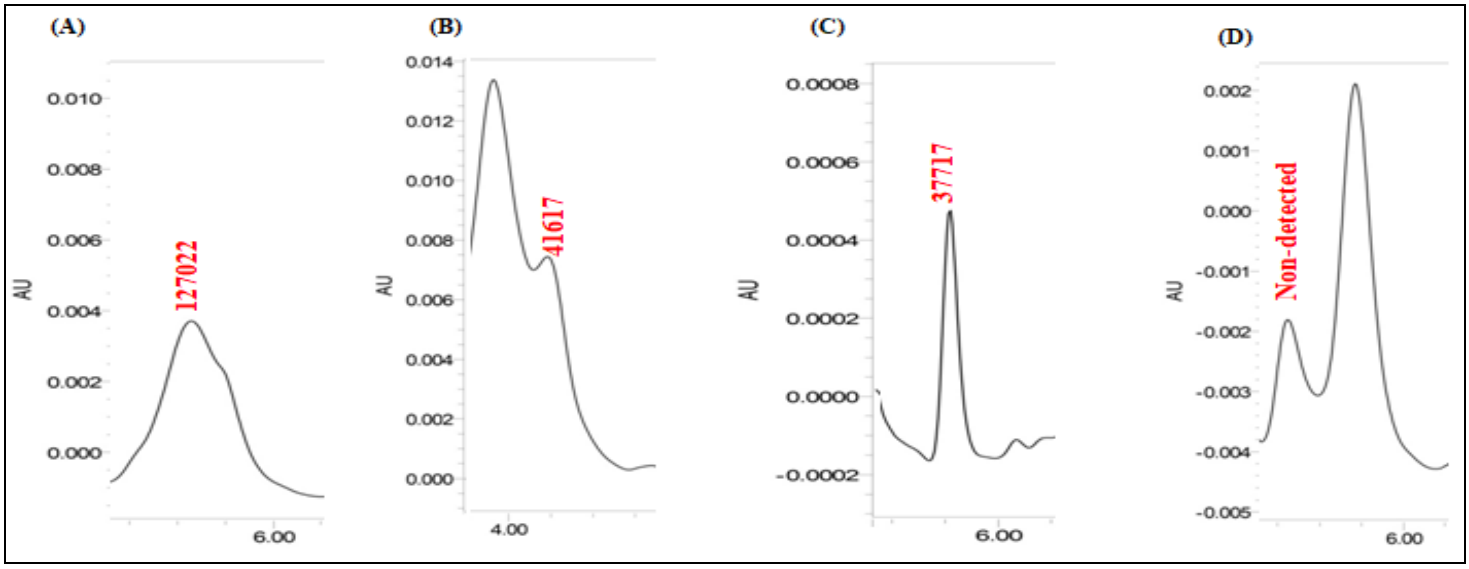

Figure 36: Chromatogram peaks of lactic acid at different fermentation time in BB. (A) 24 (B) 48 (C) 72 and (D) 96 h in 20 % BB + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 23: Retention time (t_R) and area for LA at different hours in BB

Fermentation time (h)	Retention time (t_R) (min)	Area (AU)	LA (g/l)
24	5.144	127022	5.60
48	4.625	41617	2.14
72	5.692	37717	0.28
96	4.608	-	-

end of fermentation is not because of shortage in sugars; the rate of LA production decreased due to presence of high sugar concentration at the end and still was available for microbial consumption [140]. Thus, at the time of fermentation, the amount of viable organism decreases once they achieve highest cell concentration. In comparison to our study, Altaf et al., (2005) reported similar procedure for production of LA; however, moderate amount in LA of 10 g/l was registered by *L. amylophilus* species using pulses flour [132].

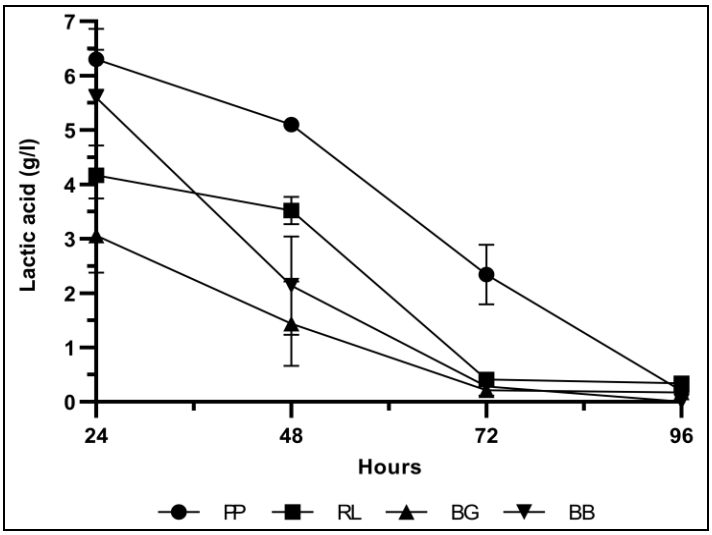

Figure 37: Concentration of LA in pulse-based medium at various time intervals.

4.8.2 Estimation of acetic acid and ethanol in sample using GC-FID

The GC-FID method was able to quantify acetic acid and ethanol exists in the fermentation medium. Separate peaks for each component were clearly visible in standard (Figure 38) and samples. The retention time of standard acetic acid and ethanol is summarized in Table 24.

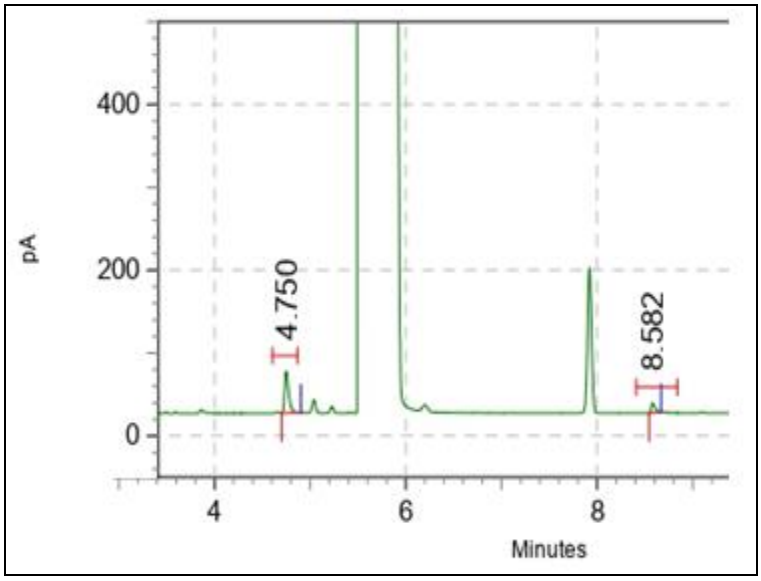

Figure 38: Chromatogram of standard acetic acid and ethanol. (100ppm mix)

Table 24: Retention time (min) and area of standard acetic acid and ethanol

Standards	Retention time (t_R) (min)	Area (AU)
Acetic acid	8.582	255185
Ethanol	4.750	1347036

(I)

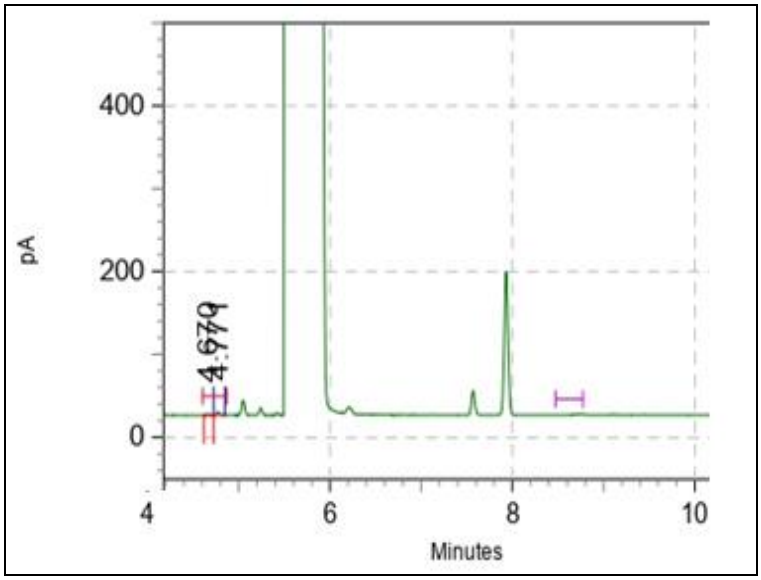

Figure 39: Chromatogram peaks of acetic acid and ethanol in PP. Fermentation time, 24 h in 20 % PP + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 25: Retention time (t_R) and area of acetic acid and ethanol in PP

Sample	Retention time (t_R) (min)	Area (AU)	Concentration (g/l)
Acetic acid	8.582	Not detected	-
Ethanol	4.771	83166	0.0047

(II)

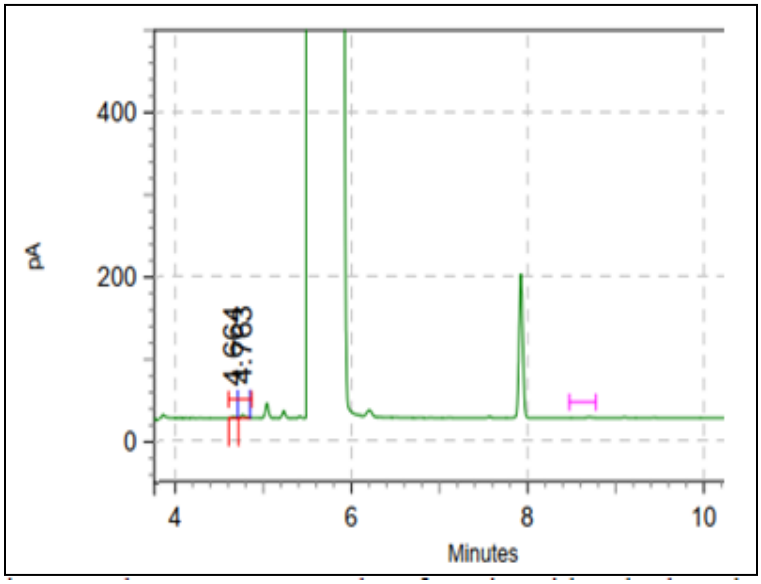

Figure 40: Chromatogram peaks of acetic acid and ethanol in RL. Fermentation time, 24 h in 20 % RL + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 26: Retention time (t_R) and area of acetic acid and ethanol in RL

Sample	Retention time (t_R) (min)	Area (AU)	Concentration (g/l)
Acetic acid	8.582	Not detected	-
Ethanol	4.771	80223	0.0045

(III)

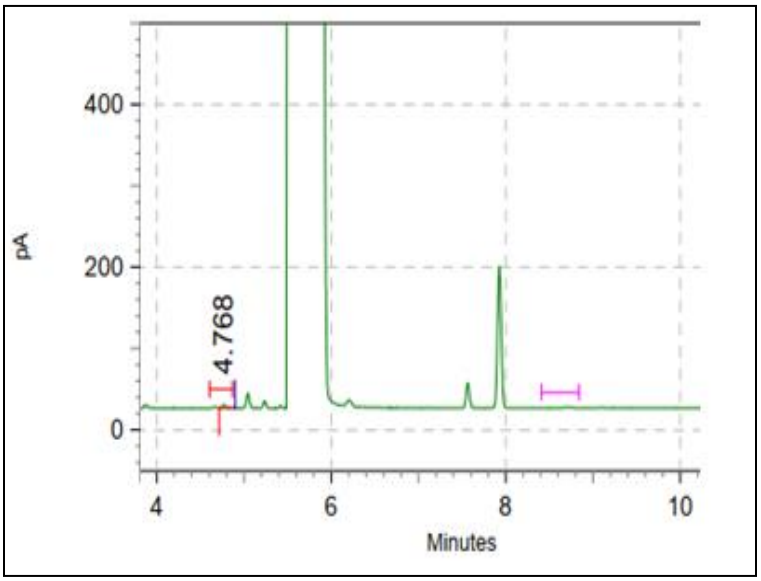

Figure 41: Chromatogram peaks of acetic acid and ethanol in BG. Fermentation time, 24 h in 20 % BG + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 27: Retention time (t_R) and area of acetic acid and ethanol in BG

Sample	Retention time (t_R) (min)	Area (AU)	Concentration (g/l)
Acetic acid	8.582	Not detected	-
Ethanol	4.771	98443	0.0056

(IV)

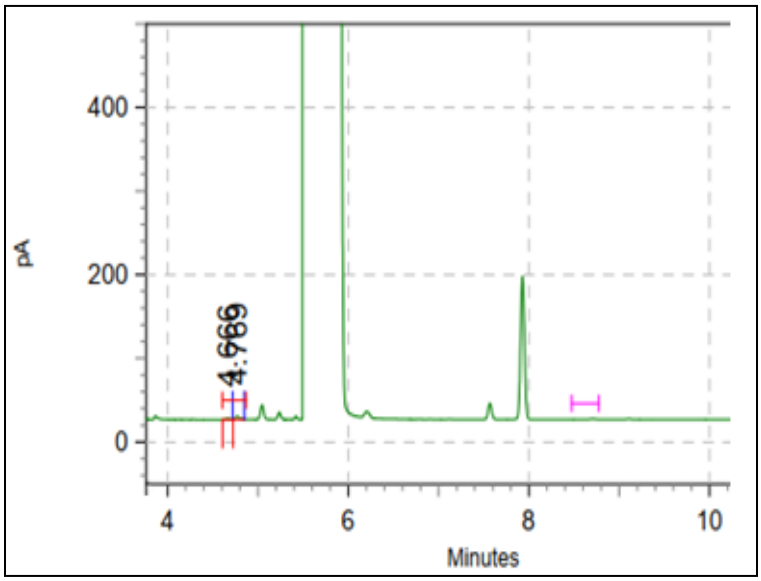

Figure 42: Chromatogram peaks of acetic acid and ethanol in BB. Fermentation time, 24 h in 20 % BB + 20 % YE + 0.05 g/l of $MnSO_4$ medium incubated at 37˚C.

Table 28: Retention time (t_R) and area acetic acid and ethanol in BB

Sample	Retention time (t_R) (min)	Area (AU)	Concentration (g/l)
Acetic acid	8.582	Not detected	-
Ethanol	4.771	86154	0.0047

Acetic acid was not detected at 24 h in any samples (Figure 39-42). The accumulation of these acids is mainly depending on the metabolism of carbohydrate and substrate supply of starter culture [141]. The result corresponds well to literature that produced AA with kimchi fermentation by inoculating *L. mesenteroides*, but not with *Lpb. plantarum* fermentations [142]. Another author reported that concentration of acids is strain dependent in lactobacilli species but

in some strains, level of AA detected are lower in concentration than LA or sometimes are at no detectable level [142]. Although low amount of LA in hetero-fermentative organism could lead to increase in concentration of other by-products such as ethanol, propionic acid, butyric acid and succinnic acid [143]. Contrary to this result, Alves de Oliveira et al., (2018) reported low AA concentration with lactobacilli strain in samples [144]. In fact, AA in some cases appeared after 48 h mostly through citrate metabolism or by LA degradation which may justify depletion of LA concentration inoculated with *Lev. brevis* and *Lpb. plantarum* [145].

pH of the medium has always played a significant impact on the ethanol concentration [146]. A reduction in final ethanol produced was noted as the medium pH decreased. Lowering pH could reduce cell growth and metabolism of spoilage bacteria in the medium [147]. Significantly, this reduces the efficiency of bacteria in converting carbohydrate to ethanol, ultimately leading to reduced ethanol yield [148]. However, maximum ethanol production can be achieved by increasing carbohydrate content with optimal pH of 5.0 to 5.5 [147]. Taking into account, this study could produce LA with higher concentration and productivity than the reported concentration making it more promising for future study and process improvement.

4.9 Change in anti-nutritional factors in pulses

There was a remarkable reduction in anti-nutrients of fermented pulses throughout fermented period (Table 9). A representative image of fermented pulses inoculated with respective organism is shown in Figure 43. Effect of various processing methods such as soaking, dry roasting, dehulling were applied to eliminate tannins and saponins in pulses beans. Further, using lactobacilli reduction in anti-nutrients was carried out to check its effect on fermentation.

4.9.1 Total tannin content in pulses

Tannins are anti-nutrient, a major concern in plant foods [149]. Tannin has ability to bind with protein to reduce solubility of protein and make less susceptible protein to proteolytic attack than protein alone [150]. It has ability to reduce iron content and increase overall quality of foods [151]. The effect of soaking on tannin content in PP, RL, BG and BB is shown in Table 29. The tannin content in the raw pigeon pea was 5.32 mg/100 g (Table 29). This was lower to reported

tannin content in PP beans; while highest content was obtained in BB with 18.26 mg/100 g (Table 29).

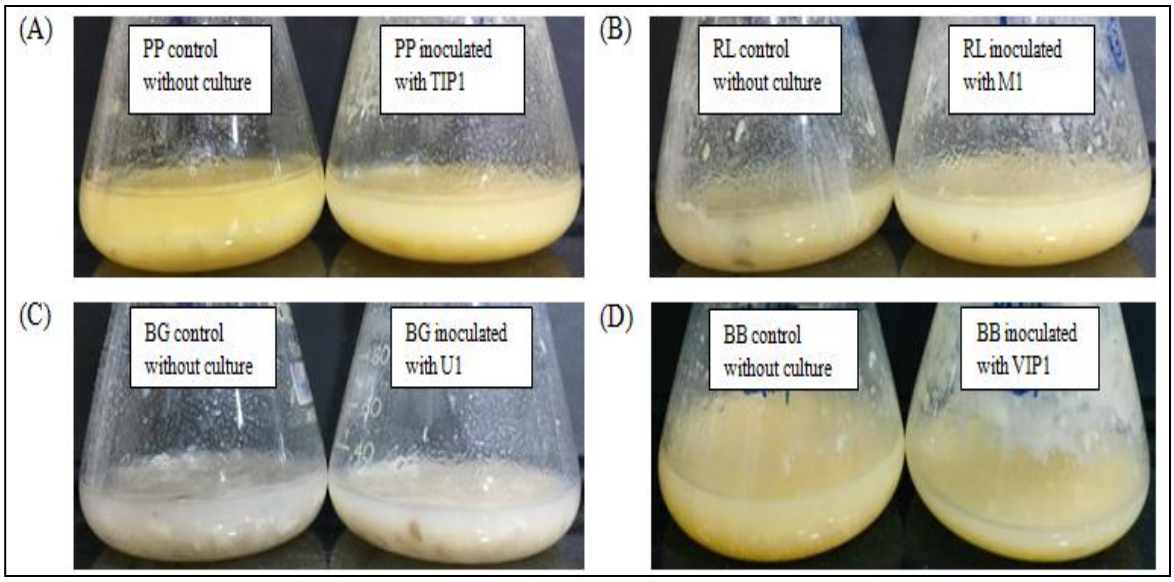

Figure 43: Flask containing different beans inoculated with respective cultures. Without inoculation (left) and test flask inoculated (right) with strains such as *Lev. brevis* TIP1 in PP (A) *Lpb. plantarum* M1 in RL (B) *Lpb. pentosus* U1 in BG (C) *Lpb. plantarum* VIP1 in BB (D) and kept at 37˚C incubation for 36 h.

This difference in value can be related to varietal differences in seeds [151]. Soaked beans showed tannin content reduction and this was in agreement with the findings that soaking can eliminate percentage of tannins [149]. After roasting treatment, the processed bean showed significant effect on the nutritional quality with effective reduction in anti-nutritional factors. The results are in agreement with other studies using lentils were roasting proved to be effective in reducing anti-nutrients [152]. Hence using combination of processing methods such as soaking and roasting can be a useful strategy to overcome problem of anti-nutrient in beans. Significant reduction of tannins was observed by dehulling process implying that presence of tannins was mainly in seed coats [34]. Results are contrary with the work of Egounlety and Aworh (2003) reporting elimination level of tannins in beans [153].

Table 29: Effect of processing methods on tannin content in some pulses beans (mg/100g)

Sample	Pigeon pea	Red lentil	Black gram	Broad beans
Raw	5.32 ± 0.27	11.66 ± 0.58	9.30 ± 0.10	18.26 ± 0.84
Dry roasting	4.08 ± 0.03	9.00 ± 0.15	7.67 ± 0.12	15.28 ± 0.71
Soaked	1.70 ± 0.11	8.42 ± 0.35	6.42 ± 2.56	14.44 ± 0.16
Dehulled	3.56 ± 0.51	8.12 ± 0.50	6.30 ± 0.17	14.07 ± 1.24
Fermentation				
12	2.47 ± 0.92	6.76 ± 0.25	4.40 ± 0.06	12.62 ± 0.23
16	2.36 ± 0.46	6.24 ± 0.36	4.10 ± 2.03	11.13 ± 0.48
18	2.30 ± 0.05	6.18 ± 1.83	3.82 ± 0.09	10.89 ± 0.76
24	1.86 ± 0.66	5.11 ± 0.36	3.05 ± 0.08	9.37 ± 0.69
36	1.22 ± 0.20	3.60 ± 0.00	1.79 ± 0.11	6.02 ± 0.35

Values are means of triplicate ± SEM. Means in the same column are not significantly different $P < 0.05$

In this study, fermentation resulted in drop of tannin content in all fermented batches. From 24 h to 36 h of fermentation, higher tannin reduction was observed (Table 29). This comes in agreement with Adeniran et al., (2013) where reduction of tannin can be associated to polyphenolic compound hydrolysis present in tannin at the time of fermentation [154].

4.9.2 Total saponins content in pulses

Saponins are present in considerable amount in pulses. Saponins have carbohydrate moiety attached to steroid aglycon with health benefits such as anti-cholestrol and anti-cancer activities in humans [149]. Table 30 presents saponin content in raw and processed samples. A range of 50-230 mg/100g saponin contents in pigeon pea was found by Anaemene (2020) [155]. Saponins are heat stable, but soaking, dehulling and cooking can deduct saponins levels, possibly due to leaching effects [156]. Dehulled soaked seeds (6 h) expressed loss in saponin content and

reduction in values of saponin content significantly differed ($P < 0.05$) among processed beans (Table 30). Saponin content declined from 142.18 mg/100 g to 42.60 mg/100 g at 36 h. Saponin losses was between 24th h and 36th h of pulses bean fermentation (Table 30). Francis et al., (2001) recommended heat treatment like dry roasting could remove almost saponin from food due to high solubility of saponin in water and aqueous extraction [152].

Table 30: Effect of processing methods on saponin content in some pulses beans (mg/100g)

Sample	**Pigeon pea**	**Red lentil**	**Black gram**	**Broad beans**
Raw	59.20 ± 0.44	142.18 ± 0.93	42.67 ± 0.13	94 ± 0.00
Dry roasting	58.67 ± 0.28	136.60 ± 1.62	41.33 ± 0.41	90.11 ± 0.39
Soaked	55.41 ± 0.26	133.22 ± 1.02	38.80 ± 0.43	88.63 ± 0.97
Dehulled	54.88 ± 0.06	131.98 ± 2.10	38.86 ± 0.07	87.61 ± 1.05
Fermentation				
12	50.55 ± 0.38	123.30 ± 2.14	30.70 ± 0.19	85.23 ± 0.75
16	48.50 ± 0.54	119.41 ± 0.01	28.19 ± 0.22	83.22 ± 0.03
18	48.21 ± 0.73	118.60 ± 2.37	27.22 ± 0.24	81.51 ± 1.85
24	45.79 ± 0.65	115.17 ± 1.81	25.92 ± 0.15	79.57 ± 1.72
36	42.60 ± 0.46	111.86 ± 0.22	23.32 ± 2.99	76.82 ± 0.40

Values are means of triplicate ± SEM. Means in the same column are not significantly different $P < 0.05$