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COMPARATIVE STUDY OF LACTASE PRODUCING BACTERIA FROM VARIOUS ANIMAL AND PLANT SOURCES

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ABSTRACT

Lactase-producing bacteria have several potential health advantages and are used as probiotics. This study aims toward the comparative study of lactase-producing bacteria isolated from different plant and animal sources based on their biochemical characterization. The bacterial isolates were isolated from eight different sources. The colonies were then selected based on morphological characterization. The selected isolates were subjected to X-gal screening to confirm lactase-producing species. The selected 8 isolates were further subjected to biochemical characterization. The isolates showed different growth patterns at different temperatures (30°C to 42°C), salt concentration (3% to 9%) and pH (pH 3 to pH 9). The isolates showed diverse antimicrobial and antibiotic activity. The enzyme activity of the isolates was calculated and influenced by different parameters.

Keywords: Lactase-producing bacteria, Probiotics, Lactose intolerance, Antibiotic susceptibility, antimicrobial activity

1. INTRODUCTION

Lactase is the byproduct of the fermentation of bacteria that makes the enzyme. *Lactobacillus acidophilus* (Yogurt), *Streptococcus cremoris* (sour cream) and *Streptococcus diacetylactis* (butter) are examples of common bacteria found in milk-based products. Lactase cleaves β -1,4glycosidic bond of lactose which yields its monosaccharide units- glucose and galactose, followed by its absorption into blood through intestinal epithelial cell. Glucose is absorbed via SGLT1 (Sodium-D-Glucose cotransporter), a symporter located on apical surface of intestinal epithelial cell; while galactose is absorbed via facilitated diffusion.

Lactose intolerance is one of the prevalent gastrointestinal disorders around the world brought on by lactase deficiency or lactose malabsorption. This happens as a result of a lack of lactase enzyme in the intestines. It can be treated by avoiding foods high in lactose. Recent studies presented the idea that using particular probiotic strains could alter the human gut microbiota. These probiotics contain live bacteria or yeast, which helps to cultivate and regulate the microbiota in the intestine. Due to the abundance of lactase-producing bacteria in the intestine, research into *Lactobacilli* is given top emphasis [1].

Lactic acid bacteria (LAB), which are known for their benefits to nutrition and health, can treat lactose intolerance. In particular, the strains *L. acidophilus*, *L. rahmnosus*, *Bifidobacterium longum*, *Streptococcus thermophilus* are frequently utilized as carriers of Lactose Producing Bacteria (LPB).

Due to their unique physical characteristics of LPB, such as high pH, high solid consistency and high buffering capacity; some fermented products such as cheese, curd, yoghurt and raw milk are proven to be the most probable sources of viable probiotic delivery. The research study highlights plant-based foods including cereals, papaya, apples, soybeans, etc. are rich in *Lactobacilli* species and are involved in manufacturing plenty of products for people who do not consume dairy foods [2] [3]. Plant-based *Lactobacilli* have been observed to colonize the gut and show capability of surviving stomach alterations. These probiotics have unique qualities, which include improved antibacterial activity, immunomodulation, cholesterol lowering, and inflammation reduction. *Lactobacillus plantarum*, a plant-based bacterium that is also found in meat, fish, and dairy is found to be a much secure form of probiotic. These plant-based bacteria

can also be employed as a starter culture for milk fermentation.

2. MATERIALS AND METHODS

Three different types of milk samples were collected from animal sources, such as cow, buffalo, and goat milk from a neighborhood milkman (procured from Sector-46, Chandigarh); milk products such as fermented yoghurt (Epigamia) and soy milk (procured from Sector-32, Chandigarh); and plant sources, such as *Calotropis gigante* from the neighborhood, papaya and apples from the nearby vegetable market. Samples of milk and milk products were collected from dairy farms and milk vending shops early in the morning and were properly labeled and stored at 4°C. *Calotropis gigante*, papaya and apple were washed 5-6 times with distilled water.

2.1 Isolation and Screening of lactase producing bacteria

The samples were serially diluted (10^{-3} to 10^{-6}) in sterile saline solution before being spread out on nutrient agar medium. The solidified agar plates were incubated at 37°C for 24 to 48hrs. Following incubation, colony growth was observed on agar plates and counted using a colony counter. Determination of the CFU/ml of various serially diluted animal and plant samples was done using the formula:

$$CFU \text{ per ml} = \frac{\text{No. of colonies} \times \text{Total Dilution}}{\text{volume of culture plated in ml}}$$

The X-gal plate method was then employed to confirm the presence of the bacteria that produce lactase. The isolates were streaked on lactose agar plates with 50 µl of X-gal (5-bromo-4-chloro-3-indolylβ-D-galactopyranoside) for the purpose of isolating lactase-producing isolates. The incubation duration at 37°C was 24hrs. Later, the pure culture of the blue colonies was generated at 37°C by sub-culturing on lactose agar [4]. For additional research, the chosen lactase-producing bacterial isolates were isolated and maintained as LPB1, LPB2, LPB3, LPB4, LPB5, LPB6, LPB7, and LPB8 at 4°C.

2.2 Morphological characterization of isolated bacteria isolates

The bacterial isolates were morphologically characterized in terms of shape, organization, color, form, elevation, density, surface growth and margins. Gram staining and endospore staining were used to further characterize the various bacterial isolates.

2.3 Biochemical characterization of isolated bacteria isolates

1. Starch Hydrolysis Test

Starch-containing nutritional agar medium, often known as Starch Agar medium, was used to streak the isolates on the plates. For 24 to 48hrs, the plates were incubated at

37°C. The plates were then flooded with iodine solution. After few minutes, extra iodine was removed.

2 IMViC Test (Indole, Methyl red, Voges-Proskauer, and Citrate test)

Indole test

The selected bacterial isolates were inoculated with tryptone broth in the test tubes. The culture was incubated at 37°C for 24hrs. After incubation, 1ml of Kovac's reagent was added and the tubes were shaken for 15–20 minutes.

Methyl Red (MR) and Voges-Proskauer (VP) Test

The bacterial isolates were added to MR-VP broth and allowed to grow for 24- 48hrs at 37°C. The tubes were then split into two sets and filled with broth. One group of tubes received 5 drops of methyl red indicator. Another set of tubes received 11–12 drops of VP reagent 1 and 2–3 drops of reagent 2. After 20 minutes, the tubes were gently shaken for 25-30 seconds and observed.

Citrate Utilization test:

The bacterial isolates were stab streaked in the citrate slants with the use of an inoculating loop. The tubes were incubated at 37° for 48hrs. After incubation, color change was observed.

3 Catalase Test

The bacterial isolates were picked and placed on the glass slide with the help of sterile inoculating loop. Approximately 2-3 drops of 3% H₂O₂ were put on the slide. The bacterial culture was then examined for changes.

4 Casein Hydrolysis

The bacterial isolates were streaked on skimmed milk agar media plates. The plates were incubated at 37°C for 24-48hrs. The presence of casein hydrolysis was checked next on the plates.

5 Protease Test

The bacterial isolates were streaked on the gelatin agar medium plates. Following that, the plates were incubated for 24hrs at 37°C. The streaked plates were gently flooded with mercuric chloride after the incubation and left to stand for 5 to 6 minutes. The plates were then analyzed.

2.4 Growth curve

50ml of lactose broth was inoculated with 2.5 ml of 24hr old lactose broth culture of selected bacterial colonies to determine the growth pattern and were kept at 37°C in a shaker incubator at 120 rpm. Absorbance was recorded at 600nm after every 30 minutes.

2.5 Bile salt tolerance test

MRS media was prepared containing 0.3% bile salt and MRS media without bile salt was used as a control. Both media were inoculated with LPB isolates and incubated

at 37°C in a shaker incubator with speed of 120rpm. The bacterial growth was monitored by measuring the absorbance at 600nm for 4hrs and 24hrs [5].

2.6 Effect of temperature, pH and salt tolerance tests

The isolate's ability to grow in the nutrient broth medium was evaluated at various temperatures, including 27°C, 37°C, and 42°C, as well as at various pH values, including pH 5, pH 7, pH 8, and pH 9 [6]. The isolates were further evaluated for bacterial stability in sterile nutritional broth media at various NaCl concentrations (3%, 6%, and 9%) [7].

2.7 Antibiotic susceptibility test

The 24hr old lactose broth cultures LPB isolates were tested against 4 different antibiotics i.e., gentamycin, kanamycin, ampicillin and nalidixic acid. The antibiotic discs were aseptically placed on the inoculated plates and incubated at 37°C for 24hrs and zone of inhibition was measured.

2.8 Antimicrobial well diffusion assay

Escherichia coli isolated from water sample collected from Ghaggar River near Chandigarh. Water sample was spread on nutrient agar plates to determine the antimicrobial activity of selected isolates. Wells of 5mm diameter were made aseptically in the nutrient agar plates and

20µl of cell free supernatants; obtained by centrifuging 5ml of 24 hr old LPB cultures at 8000rpm for 5mins; were added to the wells. The plates were incubated at 37°C for 24 hrs.

2.9 Determination of lactic acid production

20ml of cell free supernatant of 24 hr old culture was taken in a conical flask; few drops of phenolphthalein (1%) were added as an indicator. Titrimetric estimation was performed against 0.1M NaOH. 1ml of 0.1M NaOH for neutralization of acid is equivalent to 90.08mg of lactic acid (as per standards provided by A.O.A.C. 1990).

2.10 Lactase enzyme assay

Lactase enzyme assay was carried out for 72 hrs lactose broth culture using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate. Assay was carried out using 100 µl enzyme sources and 200 µl of 3mM ONPG substrate solution in PBS and incubating them in a water bath at 40°C for 15 mins. The reaction was stopped using 700µl of 1M Na₂CO₃. The absorbance was recorded at 420nm. Lactase activity was calculated for each bacterial isolate.

Enzyme activity (U/ml) was calculated by following formula:

$$\text{Enzyme activity (U/ml)} = \frac{a \cdot DF \cdot \text{volume of reaction}}{\text{enzyme volume}}$$

Where, a= absorbance; DF= dilution factor and volume of reaction and enzyme volume is noted in ml; U/ml= enzyme units per ml

Statistical Analysis: The assays were carried out in triplicate and the results were expressed as mean values and standard deviation (SD).

3. RESULTS AND DISCUSSIONS

3.1 Isolation and screening of lactase producing bacteria

The colony growth of serially diluted animal's milk sample such as cow, buffalo, and goat milk, were observed on nutrient agar media and plant samples such as *Calotropis gigante*, papaya and apple on MRS agar media after the incubation of 24-48 hrs at 37°C (**Figure 1**). Then, the CFU/ml of various serially diluted animal and plant samples were calculated (**Table 3.1**).

The lowest value was of *Calotropis gigante* sample, serial dilution 10^{-2} (6×10^3 CFU/ml) while the highest value was found in cow sample, serial dilution of 10^{-6} (7×10^7 CFU/ml).

In another study, *Lactobacillus* spp. were found to be high (2.3×10^8 CFU/ml) in yoghurt and low (4.5×10^7 CFU/ml) in raw milk; similarly, a larger load of *Lactococcus* and *Bifidobacteria* spp. was detected in yoghurt [8].

X-gal plate method:

The colonies from different sources were isolated and screened on lactose agar medium containing X-gal after the incubation

(**Figure 2**). The blue colonies were observed on total of 8 isolates LPB1, LPB2, LPB3, LPB4, LPB5, LPB6, LPB7 and LPB8 and these bacterial isolates were maintained as pure culture at 4°C (**Table 3.2**).

The X-gal technique for screening was also reported by Sreekumar Krishnan, 2010 [9].

3.2 Morphological characterization

The morphological characterization of selected isolates was done considering several parameters like shape, arrangement, colonies, pigmentation, form, elevation, density, surface growth, margin, clouding, etc. LPB5 was round in shape while rest seven colonies were spherical. The color of the 4 colonies, LPB1, LPB4, LPB6, LPB7 were cream, whereas LPB2 and LPB8 were off-white in color and LPB3 and LPB5 were milky white in color. The yellow pigmentation was seen in isolates LPB1, LPB4, LPB6. LPB3 and LPB4 were elevated while others were flat. All the isolates were regular in form except LPB2. Density of all the isolates was opaque, and margins were lobate as shown in **Table 3.3**.

3.3 Gram- staining:

The morphological characterisation was done by performing Gram staining of selected bacterial isolates. All the isolates were observed as Gram-positive. In all the isolates short rods were observed but in LPB6 long

rods were observed (Figure 3). In another study by Venkateswarulu and Ahmed *et al*, reported lactase producing *B. subtilis* was Gram positive with different short and long rod arrangement [10] [11].

3.4 Endospore staining:

The endospore staining of the isolates with malachite green were done to differentiate the vegetative cells from spores. Every isolate showed negative results i.e., no spore was observed. All the cells were in vegetative state and appear as pink/reddish in colour. T.C. Venkateswarulu reported pale green spores produced by selected isolates [10]. Casula and Cutting reported that spores of *B. subtilis* species were utilised as probiotics and competitive exclusion agents [12].

3.5 Biochemical characterization

3.5.1 Starch hydrolysis test

The test was done to determine the ability of bacterial isolates to produce enzymes like starch α -amylase and oligo-1, 6-glucosidase to hydrolyse starch. After addition of iodine solution in the 24 hrs incubated starch containing NAM plates, a clear zone was observed around the line of growth. 5 isolates out of 8 were able to hydrolyse starch and hence gave positive results. LPB5 and LPB7 gave negative results whereas LPB4 showed no growth in the medium (Figure 4).

In other study by Timothy and Dotun, isolates were isolated from milk and fermented food, and all showed positive results for starch hydrolysis [13].

3.5.2 IMViC Test

Indole test:

Indole test was done to determine the indole production in anaerobes and non-fermenters. Positive results were obtained when indole react with the aldehyde present in the Kovac's reagent to give a red color. All the isolates gave negative results except LPB6 (Figure 5A and 5B). In other studies, *B. subtilis* strain gave negative results for indole test [10].

Methyl Red (MR) and Voges -Proskauer (VP) test:

MR test conducted to determine the capabilities of isolates to ferment glucose and retain an acid end product. The red color indicates a positive outcome by addition of methyl red. Only LPB5 produced negative result; all other isolates had positive results (Figure 5C and 5D). In the study, by T.C.Venkateswarulu there was no color change in the culture tube which indicated negative results [10].

The VP test was utilized to identify butylene glycol producers. The MR-VP broth was supplemented with (1-naphthol) and (40% KOH). No creation of the color red was

observed. This test came out negative for all isolate.

Citrate utilization test:

The isolates who were able to utilize citrate as their carbon source gave positive results. The change in the color of Simmon's citrate agar slant from green to blue after the inoculation indicates positive result. 5 isolates namely LPB3, LPB4, LPB6, LPB7, LPB8 were able to utilize citrate (**Figure 5E**).

3.5.3 Catalase test

This test was used to differentiate catalase-positive (*Staphylococci*) from catalase-negative (*Streptococci*). Only 3 isolates namely LPB5, LPB7 and LPB8 were catalase positive as they showed the formation of gas bubbles due to the breakdown of hydrogen peroxide (**Figure 9**).

Catalase degrades hydrogen peroxide produced during utilisation of carbohydrates for energy production, so its presence or absence indicates that the isolates may belong to the *Lactobacilli* group [14].

3.5.4 Casein Test

This test was used to determine the ability of isolates to degrade casein into amino acids. All the isolates gave negative results except isolates LPB4 and LPB5 as they were able to produce casein enzyme. Therefore, no clear

zone was observed around the streaked isolates except LPB4 and LPB5.

3.5.5 Protease Test

The incubated gelatin agar plates were flooded with mercuric chloride and observed for clear zone after 5 minutes. The isolates LPB4, LPB5, LPB6, and LPB7 gave positive results (**Figure 7**).

3.6 Growth curve

Over the period of 3 hrs, the growth characteristics of selected bacterial isolates were investigated. During the incubation, the absorbance at 600 nm was measured every 30 minutes with a colorimeter to determine the cell mass concentration. During the early log phase of incubation, a steady increase in cell mass concentration was noted.

When compared to the other test isolates, LPB1 grew in a uniform manner. LPB3 grew the fastest and had the greatest increase in cell mass concentration after a 2-hour interval. LPB8 had the lowest cell mass concentration of all the test isolates after 1.5 hours, but LPB2 and LPB6 had the same level of rise in cell mass concentration. **Figure 11** depicts the growth curves of all of the selected bacterial isolates.

3.7 Bile Salt Tolerance Test

LPB4 isolate grew most rapidly in 0.3% bile salt concentration with an OD of 0.04 that demonstrates isolate LPB4 shows the highest

level of medium survival at both 4 hrs and 24 hrs, i.e., the strongest bile salt tolerance, whereas LPB6 and LPB7 growth was suppressed. After 24 hours of incubation, every isolate had a decline in growth, indicating that the bile salt concentration of the isolates had decreased. In terms of absorbance at 4 hr and 24 hr intervals, figure 12 displays the cell mass concentration in bile salt-containing solutions.

Bile decreases the likelihood of bacteria surviving by damaging the lipids and fatty acids in the cell membrane [15]. According to Nath's research, *L. plantarum* strain GCC 19M1 was significantly more viable in 0.3% bile, with an 83.70% survival rate [16].

3.8 Effect of Temperature, pH and Salt tolerance tests

At 30°C LPB1 showed the maximum growth whereas LPB5 showed maximum growth at 37°C. LPB2 and LPB4 were recorded to have the maximum growth at temperature 42°C (Figure 10).

In one research, two temperatures i.e., 10°C and 42°C were used to investigate the effect of temperature on the culture of *Lactobacillus* isolated from curd samples. When cultures were cultured in MRS broth at 10°C for 7 days, the turbidity in the broth was used as an indication of microbial development in which except *Lactobacillus*

acidophilus, *Lactobacillus bulgaricus*, and *Lactobacillus lactis*, rest all showed good turbidity, while at 42°C turbidity was observed in tubes containing *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus lactis* isolates after 24-48 hours of incubation [14].

The maximum growth was shown by LPB 2 at pH5 and by LPB 5 at pH7, pH8, pH9. The LPB 5 was the most stable isolate showing maximum growth alkaline pH whereas LPB 2 was the most stable isolate in acidic pH (Figure 11).

The effect of pH on enzyme activity revealed that activity increased gradually from pH 5.0 to 7.0, then decreased gradually as pH values increased. In a research Venkateswarulu studied several dairy samples, the maximal enzyme activity was observed to be 15.04/ml at pH 7.0 [10].

At 3% salt concentration, LPB3 grew the most (0.620 ± 119), whereas LPB7 grew the least (0.100 ± 100). LPB5 showed the greatest growth at salt concentrations of 6% and 9%. The LPB5 isolation grew the fastest (0.920 ± 105) in 9% salt concentration and hence had the best salt tolerance of any isolate. In the 6% salt concentration, LPB 5 isolate showed the greatest development (0.620 ± 115) (Figure 12).

Venkateswarulu *et al.* performed a salt tolerance test to investigate the growth efficiency of the isolate from several dairy samples in a salt-rich environment, and the isolate was discovered to be able to survive at high salt concentrations (8% NaCl). In the 6% salt concentration, the isolated strain grew significantly [10].

3.9 Antibiotic susceptibility test

The antibiotic susceptibility of the selected isolates to different antibiotics including ampicillin, nalidixic acid, gentamycin, and kanamycin was investigated. The isolates' growth was inhibited in zones around the antibiotic discs. Except for LPB4, all of the test isolates were ampicillin resistant. LPB2, LPB3, LPB4, and LPB6 were likewise resistant to nalidixic acid. Gentamycin sensitivity was highest in LPB1. LPB2 exhibited nearly same sensitivity to gentamycin and kanamycin. In the case of gentamycin, LPB3, LPB4, LPB5, LPB6, LPB7, and LPB8 demonstrated the greatest zone of inhibition. Out of all the test isolates, LPB6 had the smallest zone of inhibition in the presence of kanamycin. The diameter of zone of inhibition of various test isolates against various antibiotics is shown in **Table 3.5**.

Zhang *et al.* assessed the susceptibility of 20 *Lactobacillus* strains to 11 different

antibiotics in 2016; 14 were sensitive to chloramphenicol, 11 were sensitive to rifampin, 13 were sensitive to ampicillin, and 14 were sensitive to tetracycline, 19 were resistant to CPFX, and 17 were resistant to penicillin [17].

3.10. Antimicrobial well diffusion assay

The selected isolate's cell free supernatant was evaluated for antibacterial activity against *E. coli* as the test organism. Three of the test isolates exhibited a zone of inhibition on growth surrounding the well with cell free supernatant. LPB7 (1.6mm) isolate had the highest activity, followed by LPB5 (1.5mm) isolate and LPB8 (1.4mm) isolate.

The antimicrobial action of the isolates is attributed to the synthesis of antimicrobial metabolites such as hydrogen peroxide, lactic acid, and bacteriocin etc. Six strains of yoghurt were tested for their antimicrobial activity, and all of them demonstrated antimicrobial activity against *E. coli* on nutrient agar plates, with LB3 having the largest zone of inhibition at 2.2 cm [18].

3.11. Lactic acid fermentation

Titration with 0.1 M NaOH and 1 percent phenolphthalein was used to assess the lactic acid production of the recovered bacterial isolates. The color changed from lime yellow to pink (**Figure 15**). The quantity of lactic acid was calculated by multiplying the

amount of NaOH used during titration by 90.08.

1ML NaOH (0.1M) =90.08mg lactic acid

LPB4 produced the most lactic acid (i.e., 10.8mg), whereas LPB6, LPB7, and LPB8 produced the least amount of lactic acid (i.e., 1.8mg) (**Figure 16**).

3.12 Lactase Enzyme Assay

The lactase enzyme activities of all chosen isolates were determined using ONPG as a substrate. The enzyme assay was performed on a 72 hrs old lactose broth culture of the

test bacterial isolates. After 36 hours of incubation, LPB8 had the greatest lactase enzyme activity (71.5 U/ml) while LPB7 had the lowest enzyme activity (20.9 U/ml). The enzyme test was computed using ONPG as a substrate and a 72 hrs old lactose broth culture. Vekateshwarulu *et al.* investigated isolates isolated from dairy effluent and chosen on X-gal plates. Following 36 hrs of incubation, one strain of *Bacillus subtilis* produced the greatest activity among all blue isolates, measuring 15.10 U/ml [10].

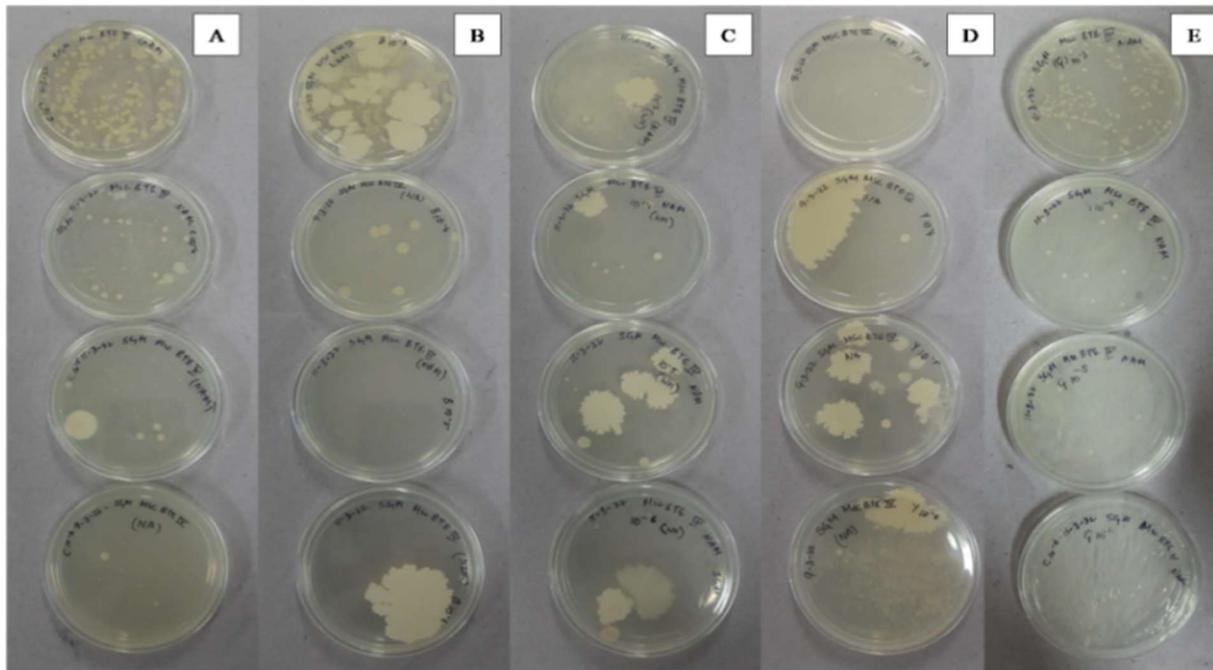


Figure 1: The colony growth of serially diluted samples

Table 3.1: Calculation of CFU (colony forming unit)/ml of animal and plant samples

SAMPLE	SERIAL DILUTION	No. of colonies	CFU/ml
Cow	10^{-3}	114	114×10^4 CFU/ml
	10^{-4}	17	17×10^5 CFU/ml
	10^{-5}	5	5×10^6 CFU/ml
	10^{-6}	7	7×10^7 CFU/ml
Buffalo	10^{-3}	20	20×10^4 CFU/ml
	10^{-4}	7	7×10^5 CFU/ml
	10^{-5}	No growth	-
	10^{-6}	No growth	-
Goat	10^{-3}	132	132×10^4 CFU/ml
	10^{-4}	21	21×10^5 CFU/ml
	10^{-5}	7	7×10^6 CFU/ml
	10^{-6}	3	3×10^7 CFU/ml
Yogurt	10^{-3}	1	1×10^4 CFU/ml
	10^{-4}	2	2×10^5 CFU/ml
	10^{-5}	5	5×10^6 CFU/ml
	10^{-6}	1	1×10^7 CFU/ml
<i>Calotropis gigante</i>	10^{-2}	6	6×10^3 CFU/ml
	10^{-3}	3	3×10^4 CFU/ml
	10^{-4}	2	2×10^5 CFU/ml
	10^{-5}	1	1×10^6 CFU/ml
	10^{-6}	No growth	-
Papaya	10^{-2}	294	294×10^3 CFU/ml
	10^{-3}	32	32×10^4 CFU/ml
	10^{-4}	12	12×10^5 CFU/ml
	10^{-5}	7	7×10^6 CFU/ml
	10^{-6}	1	1×10^7 CFU/ml
Apple	10^{-2}	232	232×10^3 CFU/ml
	10^{-3}	27	27×10^4 CFU/ml
	10^{-4}	16	16×10^5 CFU/ml
	10^{-5}	4	4×10^6 CFU/ml
	10^{-6}	No growth	-

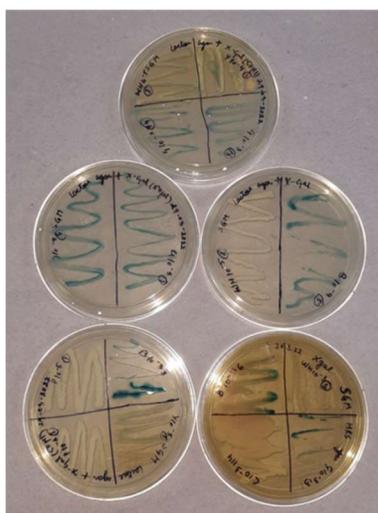
Figure 2: Blue colored colonies showing the presence of *Lactobacillus* by using X-gal method

Table 3.2: The isolated colony which were selected for further studies

Sample name, serial dilution and colony no.	Buffalo	Buffalo	Goat	Goat	Yogurt	Giant <i>Calotropis</i> PC 10 ⁻³	Apple A 10 ⁻²	Papaya P 10 ⁻³
	B10 ⁻³	B10 ⁻⁴	G10 ⁻¹	G10 ⁻⁵	Y10 ⁻⁴			
	(8)	(6)	(13)	(1)	(1)	(1)		
LPB	LPB 1	LPB 2	LPB 3	LPB 4	LPB 5	LPB 6	LPB 7	LPB 8
Result (Blue colonies)	+	+	+	+	+	-	-	-

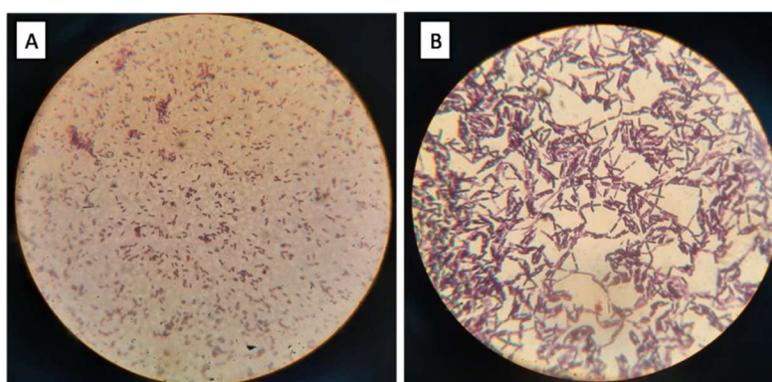


Figure 3: Gram-positive isolates. A) Showing Gram-positive short rods isolate. B) Showing Gram-positive long rods isolate LPB 6

Table 3.3: LPB isolates showing different morphological characteristics

LPB	LPB 1	LPB 2	LPB 3	LPB 4	LPB 5	LPB 6	LPB 7	LPB 8
Morphological Characteristics								
Shape	Spherical	Spherical	Spherical	Spherical	Round	Spherical	Spherical	Spherical
Arrangement	Single	Single	Single	Single	Single	Single	Single	Single
Colonies	Cream	Off-white	Milky white	Cream	Milky white	Cream	Cream	Off-white
Pigmentation	Pigment	No pigment	No pigment	Pigment	Pigment	No pigment	No pigment	No pigment
Form	Regular	Irregular	Regular	Regular	Regular	Regular	Regular	Regular
Elevation	Flat	Flat	Elevated	Elevated	Flat	Flat	Flat	Flat
Density	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Surface growth	None	None	None	None	None	None	None	None
Margin	Lobate	Lobate	Lobate	Lobate	Lobate	Lobate	Lobate	Lobate
Gram Staining	+	+	+	+	+	+	+	+
Endospore Staining	-	-	-	-	-	-	-	-

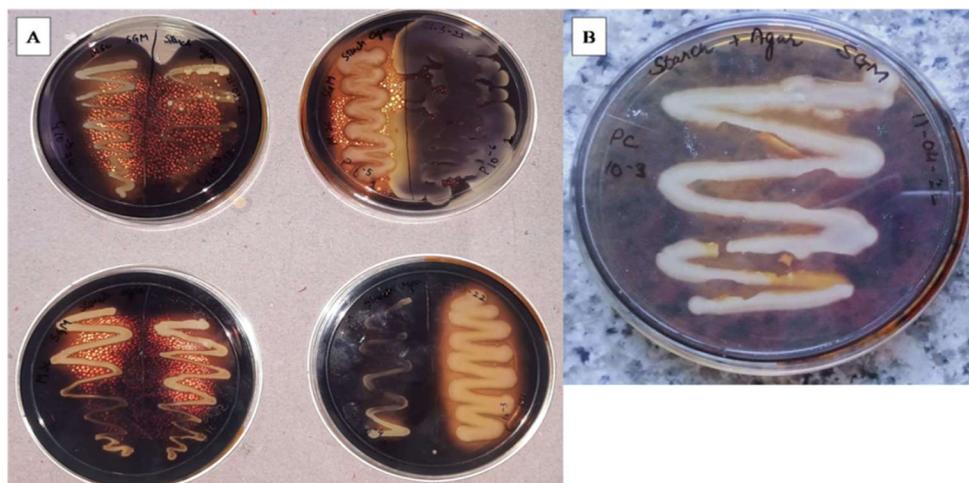


Figure 4: After the addition of iodine solution on the 24hours incubated starch containing NAM plates, a clear zone was observed around the line of growth of LPB1 LPB2 LPB3 LPB6 and LPB8 isolates. Hence, they gave positive results for starch hydrolysis test

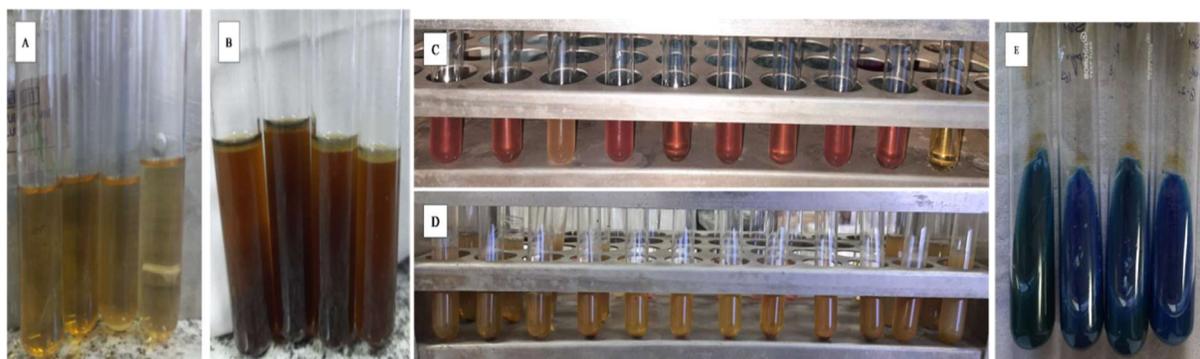


Figure 5: (A) Indole test before the addition of Indole Kovac's reagent. (B) After the addition of Indole Kovac's reagent all the isolates gave negative results except LPB6 i.e., no red coloration was observed (C) MR-VP Test: By adding the methyl red indicator in the MR-VP broth after the incubation of 24-48 hrs at 37°C, the red color change was observed in all isolates except LPB 5 which changed into red/ pink colour i.e., gave positive result. (D) By adding the VP reagent-I (α -naphthol) and VP reagent-II (40% KOH) to the MR-VP broth, there was no color change was observed, therefore, they all were tested negative. (E) In Citrate Utilization test, the change in color from green to blue was observed in the Simmon's citrate agar slants of LPB3, LPB4, LPB6, LPB7, and LPB8 isolates



Figure 6: After the addition of hydrogen peroxide on the smear of the isolates gas bubble formation was observed on LPB5, LPB7 and LPB8. Hence, they were tested catalase positive. While in rest no gas bubble formation was observed. Hence, they were tested catalase negative



Figure 7: After flooding mercuric chloride on the isolates streaked on the gelatin agar medium, a clear zone was observed around the isolates of LPB 4, LPB 5, LPB 6, and LPB 7. Hence, they were tested protease positive. While other isolates were tested protease negative

Table 3.4: Showing results of different biochemical tests

LPB	LPB 1	LPB 2	LPB 3	LPB 4	LPB 5	LPB 6	LPB 7	LPB 8
Biochemical tests								
Starch hydrolysis	+	+	+	No growth	-	+	-	+
Indole test	-	-	-	-	-	+	-	-
Methyl red test	+	+	+	+	-	+	+	+
IMViC test								
Voges proskauer test	-	-	-	-	-	-	-	-
Citrate utilisation test	-	-	+	+	-	+	+	+
Catalase test	-	-	-	-	+	-	+	+
Casein test	-	-	-	+	+	-	-	-
Protease test	-	-	-	+	+	+	+	-

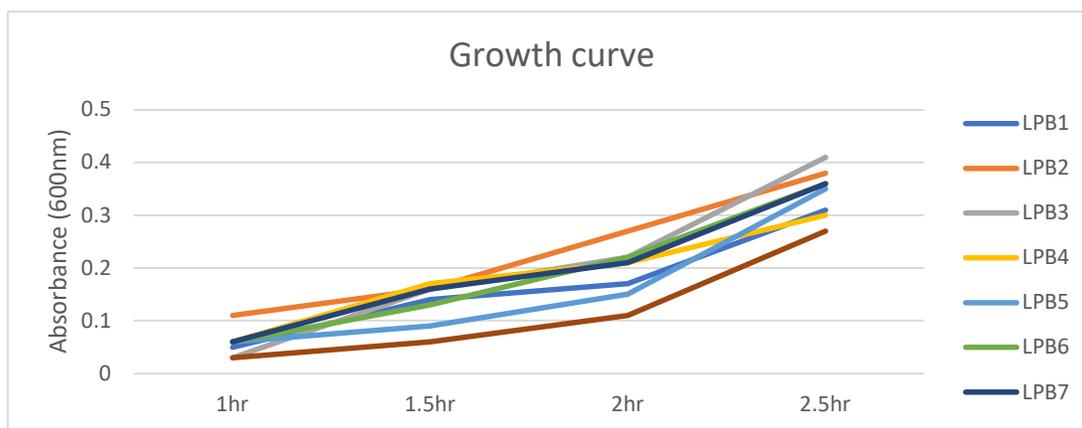


Figure 8: Growth curve analysis of selected LPB isolates

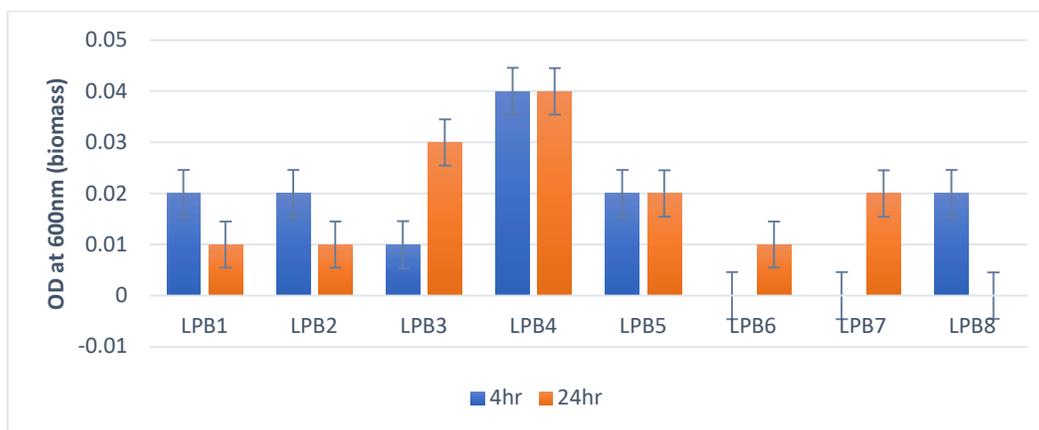


Figure 9: The cell mass concentration in bile salt containing media in terms of absorbance at 4 hr and 24 hr time intervals

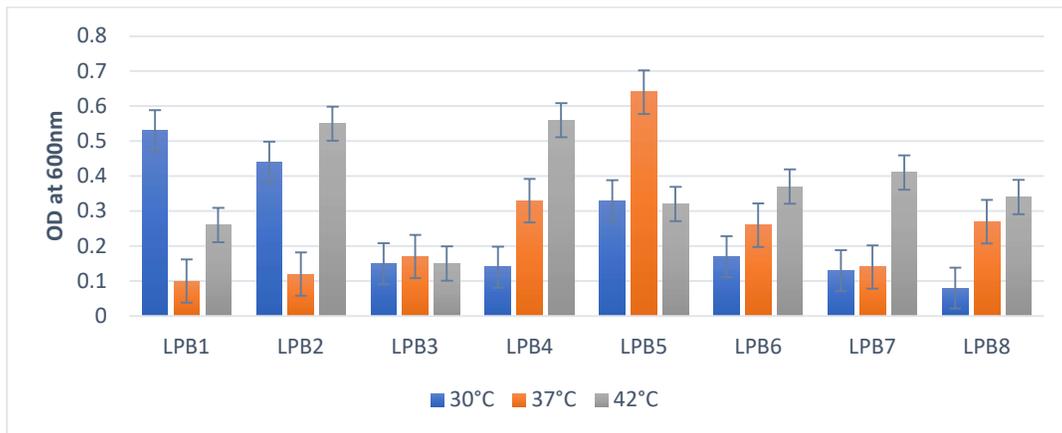


Figure 10: Comparison of the absorbance and temperature condition provided to selected isolates

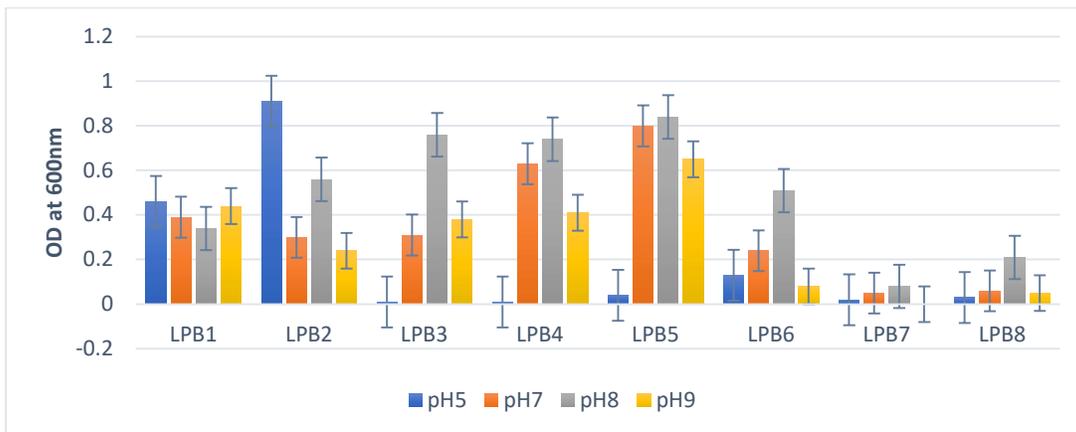


Figure 11: Comparison of the absorbance and different pH provided to selected isolates

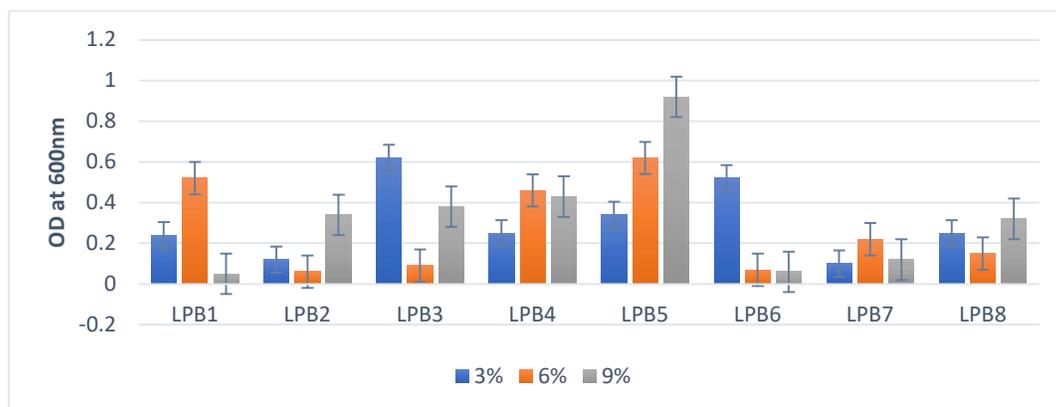


Figure 12: Comparison of the absorbance and different salt conc. of selected LPB isolates



Figure 13: Zones of inhibition shown around the antibiotic discs by the isolates

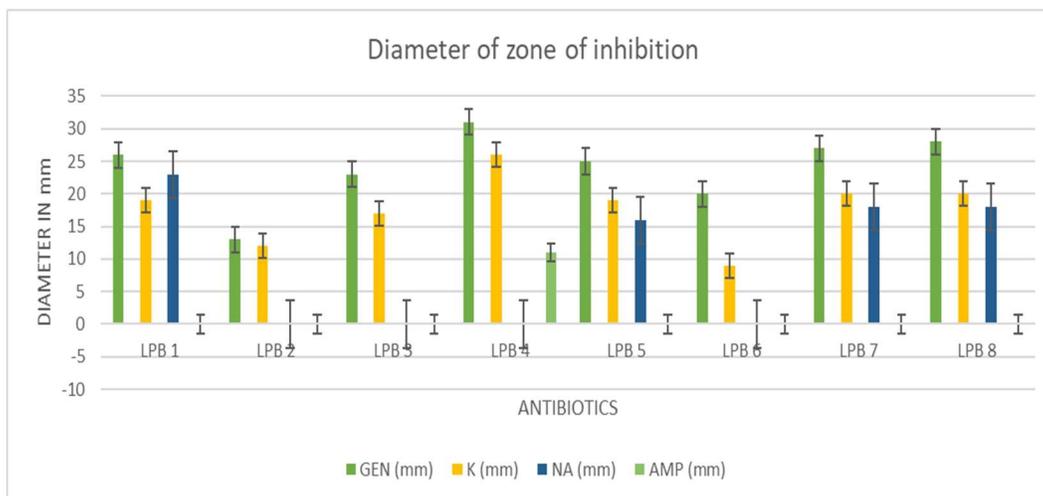


Figure 14: Diameter of zone of inhibition obtained by selected isolates



Figure 15: Color change from lime yellow to pink while performing lactic acid fermentation

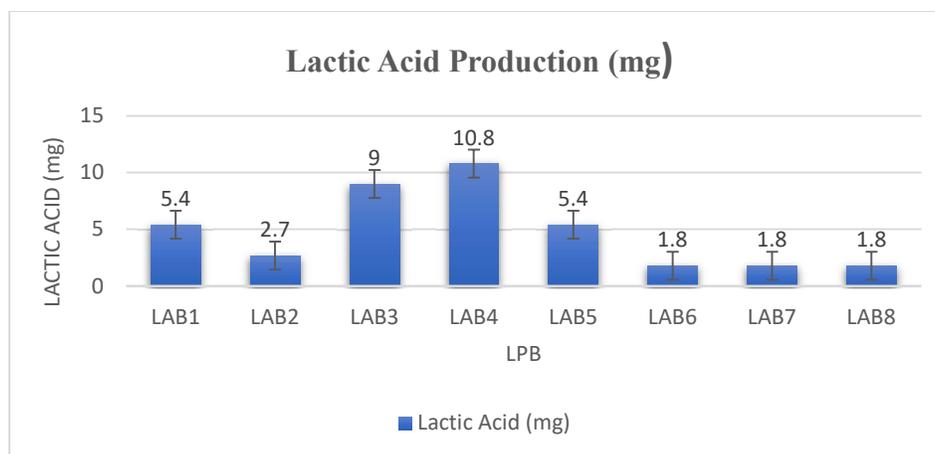


Figure 16: Lactic acid production of selected bacterial isolates

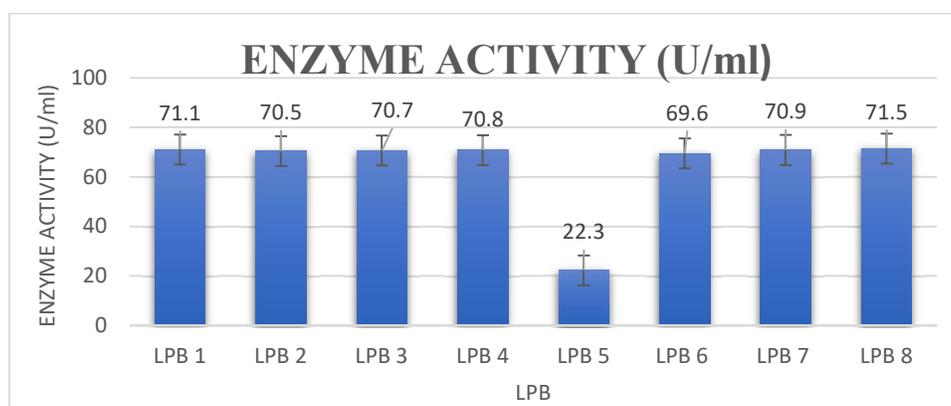


Figure 17: Enzyme activities of different bacterial isolates

CONCLUSION

Lactase is a commercially available enzyme used in the dairy and pharmaceutical sectors. The present study concludes that both plant and animal sources have lactase producing bacteria but the enzyme is produced in rather low quantity and therefore cannot be readily consumed by lactose intolerant people. Since selected isolates produce lactase enzyme, they can be commercially exploited to meet the need of lactose intolerant people by producing low lactose or lactose free milk or enzyme supplements such as Lacteeze and Lactaid. These supplements can break down

lactose in milk to make it suitable for people with lactose intolerance. Therefore, in future a lactase enzyme can come into use which can work at lower pH and higher temperature for lactose intolerant people. Isolated bacterial strains also possess probiotic properties. The enhanced lactase enzyme production can be accomplished by using conventional and statistical methodologies to optimize various process parameters of the selected bacterial isolates.

Lactase enzyme can also be used in production of frozen yoghurt and ice-creams that are creamier and better tasting. Lactase

can additionally accustom screen for Blue-white colonies within the multiple biological research sites of assorted inclusion body vectors in *E. coli* or different organisms. An important application of lactase is lactose hydrolysis which converts whey lactose into a number of suitable products such as sweet syrups that are used in bakery and confectionary industries.

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