ISOLATION AND IDENTIFICATION OF *LACTOBACILLUS* SPP. FROM SAUERKRAUTS AND YOGURTS

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Abstract

The purpose of this study was to investigate *Lactobacillus* species from sauerkrauts and yogurts. Microbiological work was conducted from June 2017 to December 2017 in the Fermentation Department, Pharmaceutical Research Department, Ministry of Industry 1, Yangon Region. In the present study, lactic acid bacteria were isolated from the samples that were collected from three different places (Kamayut Township, Mayangone Township and North Dagon Township). Rogosa and tomato juice agar media were used in the isolation of bacteria by the serial dilution method. Six strains (H-1, 2; K-1, 2 and N-1, 2) from the three sauerkrauts and fifteen strains (HD-1 to 5, KB-1 to 5 and ND-1 to 5) from the three yogurts were isolated, purified and identified. In all samples, five isolates were found to ferment galactose, four isolates showed positive results in maltose and eight isolates exhibited positive results in xylose. Therefore, isolates H-1, H-2, K-1 were nearly the same to the characters of *Lactobacillus fermentum*, isolates K-2 and N-2 were mostly alike to the characters of *Lactobacillus bulgaricus*, isolates HD-5, ND-3 and N-1 were possible to the characters of *Lactobacillus hilgardii*, isolates HD-1 to 4, KB-1 to 5 and ND- 1, 2, 4, 5 were similar to the characters of *Lactobacillus heterohiochii* by morphological, staining methods and biochemical tests according to Bergey's Manual.

Introduction

In the food fermentations, microorganisms play an essential role. Lactic acid bacteria have been well known for centuries about their responsible mainly used in food preservation including dairy, meat, vegetables and bakery products due to their fermentative capacities and safety either separately or in combination with other conventional treatment. Keer *et al.*, 1983; Salminen *et al.*, 1996; Tserovska *et al.*, 2002; Gharaei and Eslamifar, 2011 stated that lactic acid bacteria has been isolated from several foods, sewage, manure animals and humans. Yogurt is a dairy product prepared by fermentation of milk with *Lactobacillus* spp. Boor, 2001 said that therapeutic, prophylactic and nutritional properties of yogurt are widely accepted.

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One of the most popular dairy product, yogurt is used as a therapeutic, diet was an old practice in Myanmar. Yogurt is flavored as a meat tenderizer and also used as curing of meat and fish. Sauerkraut is probable one of the oldest forms of lactic acid fermentation preserved food by the bacteria normally present on the plant. Sauerkraut is the clean, sound product of characteristic flavors obtain by fully fermentation chiefly lactic acid of properly prepared and shredded cabbage in the present of not less than 2% or more than 3% salt. It contain valuable quantities of vitamin A, B and C and the undesirable palatability of a good pack if might will be that the home market has distinct potentialities (Wai, 2001).

An important group from the LAB, especially some *Lactobacillus* spp. are commonly used as probiotics in humans and animals. They help maintain the natural balance of organisms (microflora) in the intestines and a healthy digestive system (Dunne *et al*., 1999). In commercial food products, several *Lactobacillus* strains have been isolated from different sources and used as probiotics (Ashraf *et al*., 2009). One of the major members of the lactic acid bacteria, the genus *Lactobacillus* is a group of gram positive bacteria, catalase-negative bacterial species able to produce lactic acid as a main end-product of the fermentation of carbohydrates (Felis and Dellaglio, 2007). In the classification and identification of bacteria, bacterial cell size is still a useful morphological characteristic (Prescot *et al.*, 1993). The aim of this study is to isolate the lactic acid bacteria in yogurt from different areas and to identify the morphological and biochemical characteristics of lactic acid bacteria from yogurt.

Materials and Methods

Microbiological work was conducted in the Fermentation Department, Pharmaceutical Research Department, Ministry of Industry 1, Yangon Region.

Collection of the yogurt samples from different areas

Samples collected from sauerkrauts and yogurts were obtained from Kamayut Township, Mayangone Township and North Dagon Township.

Viable bacteria count in sauerkraut and yogurt samples (Brugger *et al.*, 2012)

The number of bacteria in sauerkraut and yogurt were estimated using colony- forming unit (cfu). The CFU/ml can be calculated using the formula:

$$\text{Colony-forming unit (cfu) / ml} = \frac{\text{(no . of c olo nies x dil utio n fac to r)}}{\text{volume of culture plate}}$$

Isolation of *Lactobacillus* species by serial dilution method and streaking method

(Collin *et al.*, 1995; Dubey and Maheshwari, 2002)

Dilution of sample were plated on Rogosa and tomato juice agar media, and incubated at 37°C for 1-3days. The separate colonies appear and the different types of colonies were cultured in test tubes. The slants of media were repeatedly sub-cultured to obtain pure cultures.

Rogosa Medium (Sharpe, 1960)

Composition per liter	
Tryptone	10.000 g
Yeast extract	5.000 g
Glucose	20.000 g
Sorbitan mono-oleate `Tween 80'	1.000 ml
Potassium dihydrogen phosphate	6.000 g
Ammonium citrate	2.000 g
Sodium acetate, anhydrous	17.000 g
Magnesium sulphate	0.575 g
Manganese sulphate	0.120 g
Ferrous sulphate	0.034 g
Agar	20.000 g
Final pH (at 25℃)	5.4

Tomato Juice Medium (Atlas, 1993)

Composition per liter	
Tomato juice	20.000 g
c10.000 g	
Dextrose	10.000 g
Dipotassium phosphate	0.500 g
Monopotassium phosphate	0.500 g
Magnesium sulphate	0.200 g
Manganese sulphate	0.010 g
Ferrous sulphate	0.010 g
c	0.010 g
Agar	20.000 g
Final P^H (at 25°C)	6.7

Identification of isolated *Lactobacillus* species (Breed *et al.*, 1957; Buchanam and Gibbon, 1974)

(i) Oxygen requirement of the isolated bacteria (aerobic/anaerobic)

5 ml of broth media tubes were inoculated with 1% of *Lactobacilli* cultures. The development of turbidity in culture tubes was recorded as aerobic or anaerobic.

(ii) Staining methods for bacteria

1. Gram staining (Harley and Prescott, 2002)
2. Spore staining (Harley and Prescott, 2002)
3. Acid-fast staining (Bisen and Verma, 1998)

(iii)Motility test (Atlas, 1993)

(iv)Biochemical tests

1. Citrate utilization test (Cruickshank *et al.*, 1968)
2. Indole testone (Hucker, 1948)
3. Nitrate reduction test (Cowan, 1974)
4. Methyl red test (Bisen and Verma, 1998)
5. Voges-Proskauer test (Cruickshank *et al.*, 1968)
6. Hydrogen Sulphide (H_2S) production medium – Triple Sugar Iron (TSI) test (Bisen and Verma, 1998)
7. Gelatin liquefaction test (Hucker, 1948)
8. Catalase test (Salle, 1948)
9. Urease test (Dubey and Maheshwari, 2002)
10. Starch hydrolysis test (Dubey and Masheshwari, 2002)
11. Sugar fermentation test (Cruickshank *et al.*, 1968)

Results

Isolation and identification of *Lactobacillus* species from yogurt samples

The isolation and identification of *Lactobacillus* species from sauerkrauts and yogurts were conducted. Seven bacteria strains (HD-1 to HD-5), (H-1 and H-2) from the Kamayut Township, the other seven bacteria strains (KB-1 to KB-5), (K-1 and K-2) from the Mayangone Township, and next seven bacteria strains (ND-1 to ND-5), (N- 1 and N-2) from the North

Dagon Township were isolated, purified and identified. (HD- 1 to HD-4), (KB-2 to KB-5) and (ND-2 to ND-5) were isolated from Rogosa medium and, (HD-5, KB-1, ND-1) and (H-1, H-2, K-1, K-2, N-1, N-2) were isolated from tomato juice agar medium.

Viable bacteria count in yogurt samples

In sauerkraut samples, total viable counts of bacteria in Kamayut Township was 1.15 x 10^5 cfu/ml, in Mayangone Township was 1.43 x 10^5 cfu/ml and in North Dagon Township was 7.9 x 10^5 cfu/ml. In yogurt samples, total viable counts of bacteria in Kamayut Township was 1.67 x 10^5 cfu/ml, in Mayangone Township was 1.4 x 10^5 cfu/ml and in North Dagon Township was 2.0 x 10^5 cfu/ml.

Figure 1. Twenty-one isolated strains from yogurt and six isolated strains from sauerkraut

Oxygen requirement of the isolated bacteria (aerobic/anaerobic)

In the present study, all isolated bacteria were anaerobes

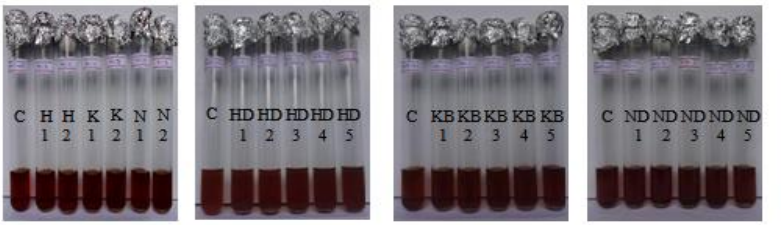


Figure 2. Oxygen requirement of the isolated bacteria (aerobic/anaerobic)

Morphological and microscopic characteristics of *Lactobacillus* species from sauerkraut and yogurt samples

Twenty-one bacterial strains were isolated and purified from the samples. The colony characters of isolates from sauerkraut samples were cream, white, creamish white colours, circular shapes and most isolated strains were entire margin and convex elevation (Table 1 and Figures 3, 4 and 5). The colony characters of isolates from yogurt samples were cream, white, creamish white, grayish white colours, circular and irregular shapes, and most isolated strains were filiform margin and umbonate elevation, HD-5 and ND-3 were entire margin and convex elevation (Table 2).The sizes of isolates were (0.5-1.0) × (1.0-6.0) µm from sauerkraut samples and (0.6-1.0) × (2.0- 5.0) µm from yogurt samples. All of these isolates were gram positive, non-spore forming and unstained in acid-fast staining.

Table 1. Colony morphology of *Lactobacillus* species from sauerkraut samples

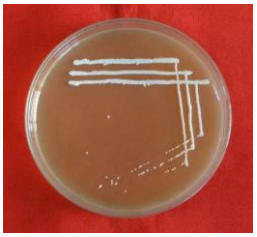
Sample No.		Colony Morphology				Cell Morphology
		Colour	Shape	Margin	Elevation	Cell Size (µm) Width x Length
H	1	Cream	Circular	Entire	Convex	0.5 - 1.0 x 1 - 5
	2	Creamish white	Circular	Entire	Convex	0.5 - 0.8 x 2 - 5
K	1	Creamish white	Circular	Entire	Convex	0.6 - 1.0 x 2 - 6
	2	Cream	Circular	Entire	Convex	0.5 - 1.0 x 2 - 6
N	1	Cream	Circular	Entire	Convex	0.5 - 0.8 x 2 - 5
	2	Creamish white	Circular	Entire	Convex	0.6 - 1.0 x 2 - 5

H - Kamayut Township, K - Mayangone Township, N - Northgon Township

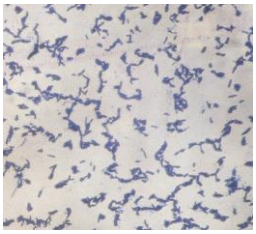
Table 2. Colony morphology of *Lactobacillus* species from yogurt samples

Sample No.		Colony Morphology				Cell Morphology
		Colour	Shape	Margin	Elevation	Cell Size (µm) Width x Length
HD	1	White	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 5
	2	Creamish wh	Irregular	Filiform	Umbonate	0.6 - 1.0 x 2 - 4
	3	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	4	White	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 5
	5	White	Circular	Entire	Convex	0.6 - 1.0 x 2 - 4
KB	1	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	2	Creamish white	Irregular	Filiform	Umbonate	0.6 - 1.0 x 2 - 5
	3	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 5
	4	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	5	White	Irregular	Filiform	Umbonate	0.6 - 1.0 x 2 - 5
ND	1	Grayish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	2	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	3	Cream	Circular	Enire	Convex	0.7 - 1.0 x 2 - 4
	4	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4
	5	Creamish white	Irregular	Filiform	Umbonate	0.7 - 1.0 x 2 - 4

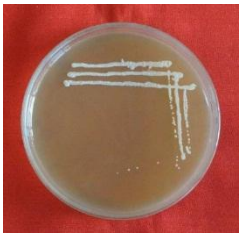
HD - Kamayut Township, KB - Mayangone Township, ND - North Dagon Township



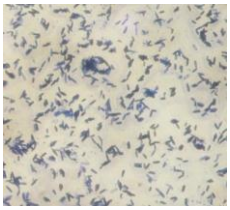
Isolate H-1



Isolate H-1 (1000x)

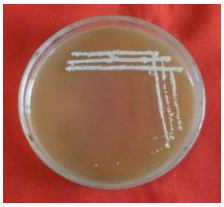


Isolate H-2

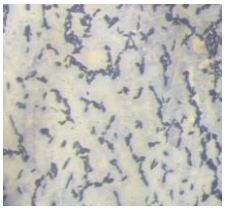


Isolate H-2 (1000x)

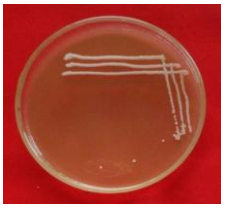
Figure 3. Colony morphology and gram staining of isolates H-1 and H-2 from sauerkraut samples (Kamayut Township)



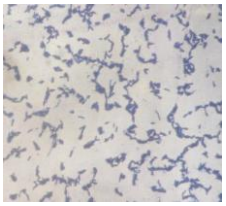
Isolate K-1



Isolate K-1(1000x)



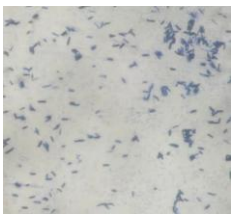
Isolate K-2



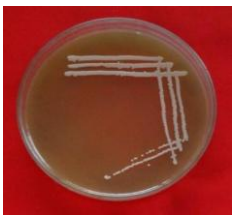
Isolate K-2(1000x)

Figure 4. Colony morphology and gram staining of isolates K-1 and K-2 from sauerkraut samples (Mayangone Township)

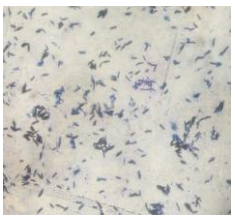
Isolate N-1



Isolate N-1(100x)



Isolate N-2



Isolate N-2(100x)

Figure 5. Colony morphology and gram staining of isolates N-1 and N-2 from sauerkraut samples (North Dagon Township)

Motility test

All isolated *Lactobacillus* species were negative results in motility test shown in Figure 6.

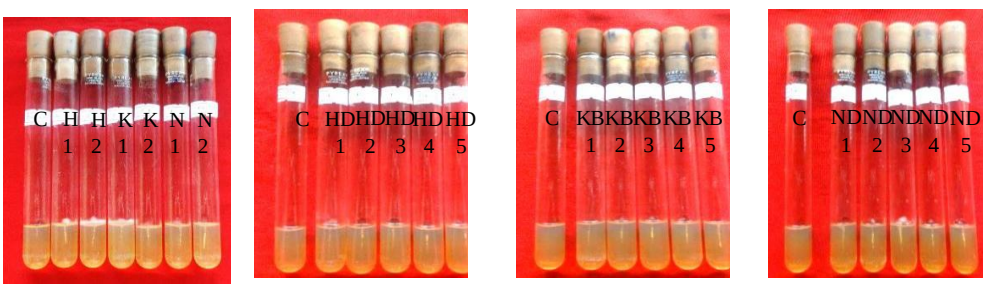


Figure 6. Motility test of isolates

Biochemical characteristics of *Lactobacillus* species from sauerkraut and yogurt samples

The identification of genus level, twenty-one isolated bacteria were carried out by biochemical tests. All isolated *Lactobacillus* species from sauerkrauts and yogurts were negative results in motility test, citrate utilization test, indole test, nitrate reduction test, methyl red test, Voges-Proskauer test, triple sugar iron (TSI) test, H_2S production test, gelatin liquefaction test, catalase teat, urease test and starch hydrolysis test. These results were shown in Table 3 and 4.

Table 3. Biochemical characteristics of *Lactobacillus* species from sauerkraut samples

Code Number	H		K		N	
	1	2	1	2	1	2
Citrate utilization test	-	-	-	-	-	-
Indole test	-	-	-	-	-	-
Nitrate reduction test	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	-
TSI	-	-	-	-	-	-
H_2S production test	-	-	-	-	-	-
Gelatin liquefaction test	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-
Urea	-	-	-	-	-	-
Starch hydrolysis test	-	-	-	-	-	-

+ = positive reaction, - = negative reacti

Table 4. Biochemical characteristics of *Lactobacillus* species from yogurt samples

Code Number	HD					KB					ND				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Citrate utilization test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TSI test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = positive reaction, - = negative reaction

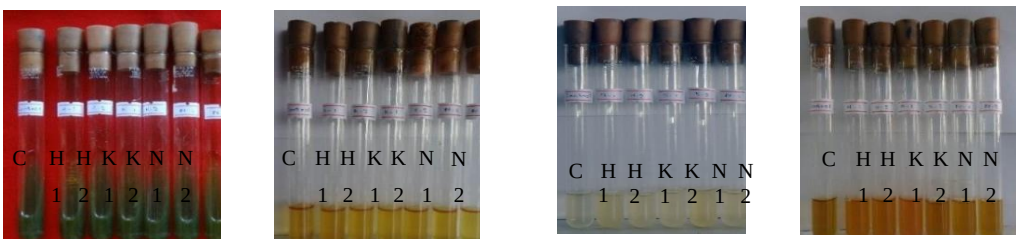


Figure 10. Citrate utilization test, indole test, nitrate reduction test and methyl red test of isolates from sauerkrauts

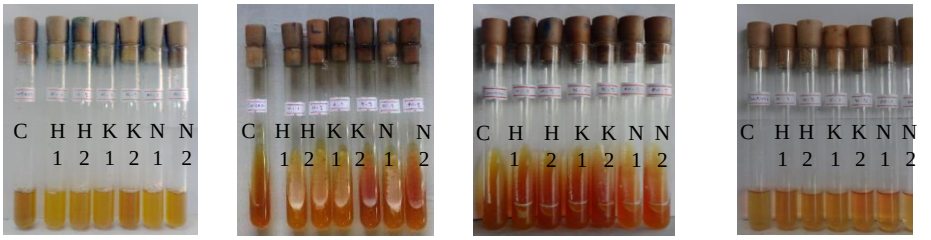


Figure 11. Voges-Proskauer test, Triple sugar iron (TSI) test, Hydrogen Sulphide (H_2S) production test and urease test of isolates from

Figure 12. Gelatin liquefaction test and catalase of isolates from sauerkrauts

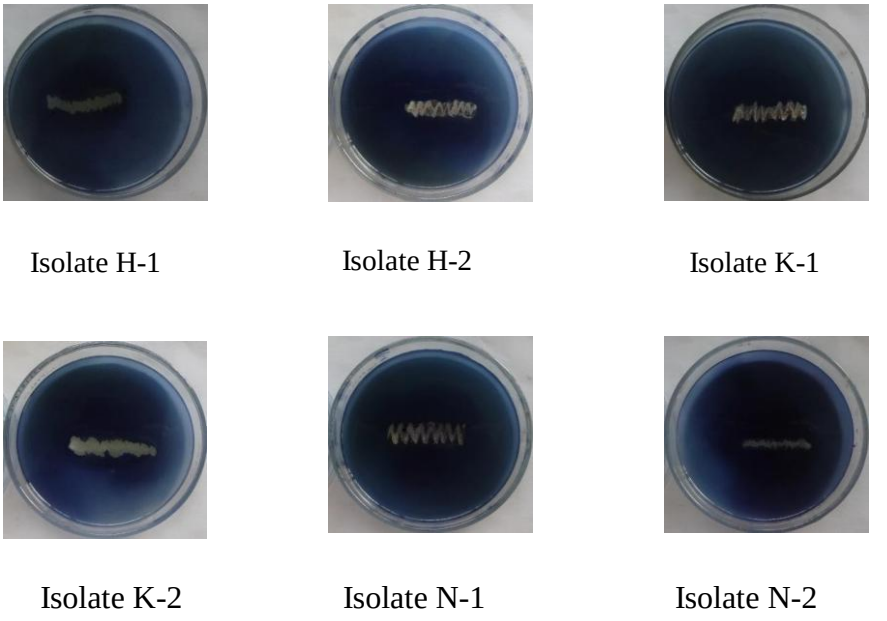


Figure 13. Starch hydrolysis test of isolates from sauerkrauts (H1, H-2, K-1, K-2, N-1 and N-2)

Sugar fermentation tests of Lactobacillus species from sauerkraut and yogurt samples

In the results of sugar fermentation tests in sauerkraut samples, all isolates were found to ferment fructose, glucose, lactose and xylose but could not ferment mannitol. In the sugar fermentation test of cellobiose, galactose, raffinose and sucrose, H-1, H-2, K-1 showed positive results. H-1, H-2, K-1 and N-1 showed positive results in maltose sugar fermentation test. In yogurt samples, all isolates were found to ferment fructose and glucose but could not ferment cellobiose, lactose, sucrose, mannitol and raffinose. In the sugar fermentation test of galactose, maltose and xylose, all isolates showed negative results except HD-5 and ND-3. The results of sugar fermentation test were presented in Tables 5 and 6.

Table 5. Sugar fermentation tests of *Lactobacillus* species from sauerkraut samples

Fermentable sugars	H		K		N	
	1	2	1	2	1	2
Cellobiose	+	+	+	-	-	-
Fructose	+	+	+	+	+	+
Galactose	+	+	+	-	-	-
Glucose	+	+	+	+ (G)	+ (G)	+
Lactose	+	+	+	+	+	+
Maltose	+	+	+	-	+	-
Mannitol	-	-	-	-	-	-
Raffinose	+	+	+	-	-	-
Sucrose	+	+	+	-	-	-
Xylose	+	+	+	+	+	+

+ = able to ferment sugar, - = not able to ferment sugar, (G) = gas produced
H - Kamayut Township, K - Mayangone Township, N - North Dagon Township

Table 6. Sugar fermentation tests of *Lactobacillus* species from yogurt samples

Fermentable sugars	HD					KB					ND			
	1	2	3	4	5	1	2	3	4	5	1	2	3	4
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	+	-	-	-	-	-	-	-	+	-
Glucose	+	+	+	+	+ (G)	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	+ (G)	-	-	-	-	-	-	-	+	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	+	-	-	-	-	-	-	-	+	-

+ = able to ferment sugar, - = not able to ferment sugar, (G) = gas produced
HD - Kamayut Township, KB - Mayangone Township, ND - North Dagon Township

Figure 14. Cellobiose, fructose, galactose, glucose and lactose sugarfermentation tests of isolates from sauerkrauts

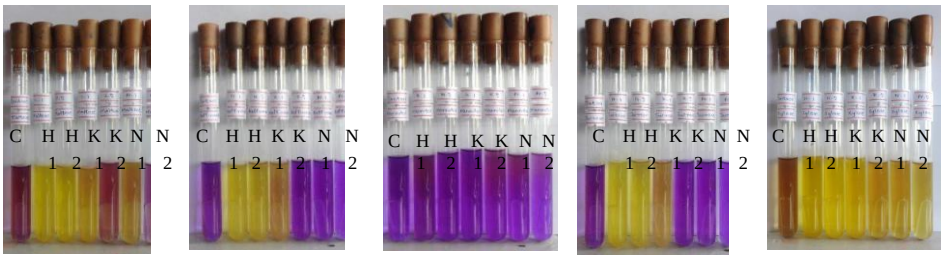


Figure 15. Maltose, raffinose sugar fermentation tests, mannitol fermentation test, sucrose and xylose sugar fermentation tests of isolates from sauerkrauts

Discussion and Conclusion

In the present study, fifteen bacteria strains (HD-1 to 5, KB-1 to 5, ND-1 to 5) were isolated by using Rogosa and tomato juice agar media, six strains (HD-1, 2; K-1, 2 and N-1, 2) were isolated by using tomato juice agar medium from sauerkraut and yogurt samples that were collected from three different areas. These strains were identified by morphological, cultural and biochemical characteristics using Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957; Buchanam and Gibbon, 1974).

In sauerkraut samples, total viable counts of bacteria in Kamayut Township was 1.15 x 10^5 cfu/ml, in Mayangone Township was 1.43 x 10^5 cfu/ml and in North Dagon Township was 7.9 x 10^5 cfu/ml. In yogurt samples, total viable counts of bacteria in Kamayut Township was 1.67 x 10^5 cfu/ml, in Mayangone Township was 1.4 x 10^5 cfu/ml and in North Dagon Township was 2.0 x 10^5 cfu/ml. These results are found nearly relevant to the findings of Hoque *et al.*, 2010, Amin *et al.*, 2011. The colony characters of isolates were cream, white, creamish white, grayish white

colours, circular and irregular shapes, and most isolated strains were filiform and entire margins, umbonate and convex elevations. All of these isolates were anaerobes, gram positive, non-motile, non-spore forming and negative in acid-fast staining. Therefore, all isolates might be identified as *Lactobacillus* species according to Schleifer and Ludwig (1995), Kadere and Kutima (2012).

The identification of genus level, the isolated bacteria was carried out biochemical tests. All isolated *Lactobacillus* species provided negative results in motility test, citrate utilization test, indole test, nitrate reduction test, methyl red test, Voges-Proskauer test, triple sugar iron (TSI) test, H_2S production test, gelatin liquefaction test, catalase teat, urease test and starch hydrolysis test. These results were in according with those revealed in Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957; Buchanam and Gibbon, 1974). Therefore, all isolates might be identified as *Lactobacillus* species according to Breed *et al.*, (1957), Buchanam and Gibbon (1974), Batt (2000), Holzapfel *et al.*, (2001), Axelsson (2004), Kadere and Kutima (2012), Arimah and Ogunlowo (2014). The *Lactobacillus* isolate exhibited negative pattern of H_2S formation, starch hydrolysis, nitrate reduction and urease activity. These are the common characters of *Lactobacillus* species. Similar results were observed by Schleifer and Ludwig (1995), Forouhandeh *et al.*, (2010), Chakraborty and Bhowal (2015).

In sauerkraut samples, all isolates were found to ferment fructose, glucose, lactose and xylose but could not ferment mannitol. In the sugar fermentation test of cellobiose, galactose, raffinose and sucrose, H-1, H-2, K-1 showed positive results. H- 1, H-2, K-1 and N-1 showed positive results in maltose sugar fermentation test. In the results of sugar fermentation tests in yogurt samples, all isolates were found to ferment fructose and glucose but could not ferment cellobiose, lactose, sucrose, mannitol and raffinose. In the sugar fermentation test of galactose, maltose and xylose, all isolates showed

negative results except HD-5 and ND-3. These results were also similar to the result showed in Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957; Buchanam and Gibbon, 1974). All these results are found relevant to the findings of Bhardwaj *et al.,* 2012; Chowdhury *et al.*, 2012. They reported that the sugar fermentation test of most *Lactobacillus* species were acid production without gas from glucose.

In Bergey's Manual, the characters of *Lactobacillus* species are anaerobic or facultative, non-motile, gram-positive, non-sporing, catalase negative, gelatin not liquefied, indole and H_2S not produced (Breed *et al*., 1957; Buchanam and Gibbon, 1974). According to the above characters, in yogurt samples, isolates HD-1 to HD-4, KB-1 to KB-5 and ND-1, 2, 4, 5 were very similar to the characters of *Lactobacillus heterohiochii*, isolates HD-5, ND-3 were nearly the same to the characters of *Lactobacillus hilgardii*. In sauerkraut samples, isolates H-1, H-2, K-1 were mostly alike to the characters of *Lactobacillus fermentum,* isolate N-1 were nearly the same to the characters of *Lactobacillus hilgardii,* and K-2, N-2 were indistinguishable to the characters of *Lactobacillus bulgaricus* (Wai, 2001).

It could be concluded that *Lactobacillus heterohiochii, Lactobacillus hilgardii, Lactobacillus fermentum and Lactobacillus bulgaricus* were possible in the samples of present work and their morphological, biochemical characteristics were investigated from yogurt and sauerkraut samples.

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