Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 1421-1424
Received: 03-07-2018
Accepted: 04-08-2018

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Morphological and biochemical studies for the identification of *Lactobacillus plantarum* sp. nov., and *Lactobacillus fermentum* sp. nov., from municipal waste

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Abstract
Two predominant strains of Lactobacillus were isolated from the municipal waste in Kashmir valley. The cells were non-motile rod cells, Gram-positive, non-spore-forming, catalase, Oxidase and urease negative. Colonies were smooth and round and nitrate reduction test showed positive results in case of LAB33 while as colonies were creamy-white, circular, smooth, low convex and nitrate reduction-negative in case of LAB45. Ammonia from Arginine, Acid from mannitol and gas is produced from glucose fermentation by LAB33 while as no ammonia from Arginine, no acid from mannitol and no gas is produced from glucose by LAB45. Both the strains fermented L- arabinose, D-fructose, galactose, glycerol, lactose, maltose, mannitol, salicin, sorbitol, sucrose, trehalose, D-xylose as carbon source. Rhamnose is not fermented by LAB33 but showed positive results by LAB45. Lactic acid was the exclusive product from glucose fermentation. LAB33 and LAB45 were biochemically characterized and identified by the methods in Bergey's Manual of Systematic Bacteriology, Vol. 2 as *Lactobacillus fermentum* and *Lactobacillus plantarum* respectively.

Keywords: Gram- positive, Lactobacillus, *Lactobacillus fermentum*, *Lactobacillus plantarum*, Rhamnose

Introduction
India has different consumption and waste generation pattern because of varied geographic and climatic conditions. With the doubling of population and changing lifestyle pattern of the inhabitants the quantity of municipal waste generated is increasing in an alarming rate. An Indian city produces about 0.8 to 1 kg solid wastes per capita per day as studied on waste management at military station Tezpur in 2009 (Sarkar *et al*; 2011) [9]. Lactic acid bacteria comprise an ecologically diverse group of microorganisms united by formation of lactic acid as the primary metabolite of sugar metabolism (Liu and Dong, 2002) [6]. They are a group of rod shaped, lactic acid-producing, phylogenetically heterogeneous organisms (Kandler & Weiss, 1986) [6]. Lactobacilli are widely distributed in nature and are found frequently in fermented foods such as dairy products, beverages, fish, pork and vegetables and in sewage (Stiles & Holzapfel, 1997) [10]. Some species are believed to be members of the commensal flora of the intestinal tract of humans and are beingused as probiotics against diseases (Zhang *et al.*, 2015) [12]. Kandler and Weiss have classified Lactobacillus isolates from temperate regions according to their morphology, physiology and molecular characters (Kandler & Weiss, 1986) [6]. Lactic acid bacteria from food and their current taxonomical status have been described by many (Gonzalez *et al.*, 2000) [4]. Lactic acid bacteria are characterized as Gram - positive, usually non-motile, non - sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism. The aim of the study was the isolation and identification of Lactic acid bacteria from municipal waste based on the morphological and biochemical characteristics.

Material and Methods
The investigation was carried out at Crop Pathology Laboratory, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar during 2017. Ten composite samples of biodegradable wastes (soil mixed with waste) five each from Srinagar and Gulmarg were collected and brought to the laboratory in sterile zip-lock plastic bags maintaining aseptic conditions, stored at 4°C and marked accordingly to their source and site (Table 1). Purposive method of Sampling was used as a design of the survey.

The samples were analyzed after due processing. Lactic acid bacteria were isolated by standard methods (Kandler & Weiss, 1986) [6]. One gram of sample was soaked in ten ml of sterile water treated with 0.85% NaCl for two minutes in a stomacher blender. The suspension so obtained was serially diluted and pour plated on the Man Rogosa and Sharpe (MRS) agar medium plates and then incubated at 26 °C for 48 to 72 hours. Three replications of 10^{-6} and 10^{-7} dilutions were plated from each sample. The pure white well isolated small colonies (2-3 mm diameter) with entire margins were picked from each plate and transferred to new MRS plates for further purification. The selected Lactic acid bacterial colonies were purified by four way streak plate method on GYP agar plates containing $CaCO_3$ (0.5%) and incubated under anaerobic conditions. The isolated pure cultures were kept on MRS agar slants at 4 °C for further use. The pure cultures were tested for utilization of diammonium phosphate as a nitrogen source and those who showed negative response were selected as lactic acid bacteria (Jusoh, *et al*., 2013) [5]. The composition of selective medium used for Lactic acid bacteria isolation is given in Table 2. All the selected isolates of lactic acid bacteria were examined for the colony morphology, cell shape, mobility, gram reaction and spore formation ability as per the standard procedures given by Ahmad and Zargar (2017) [1]; Anonymous (1957) [2] and Barthalomew and Mittewer (1950) [3]. Colony morphology were studied with the help of magnifying lens and cell shape of the isolates under microscope. The isolates were biochemically characterized by catalase test, oxidase test, urease test, motility test and nitrate reduction test (Zaved *et al*., 2008) [11]. Carbohydrate fermentation tests of the selected isolates of lactic acid bacteria were conducted in MRS fermentation broth with 0.004% bromocresol purple containing 2% of tested sugars (Kandler and weiss, 1986) [6]. The identification of the selected isolates of lactic acid bacteria were carried out by following the methods in Bergey's Manual of Systematic Bacteriology, Vol. 2.

Table 1: Location of samples collected from Kashmir valley for analysis

S. No.	Survey area	Site	Sample Site Coordinates	Sample Code
1.	Gulmarg	Gulmarg Market	34º 3/ 13.16//N 74º23/ 49.03//E	G1
2.		Near Hotel Hill Top	34º 2/ 46.57//N 74º23/ 15.82//E	G2
3.		Kangdoor Gandola Station	34º 1/ 57.41//N 74º22/ 1.41//E	G3
4.		Gulmarg Meadow	34º 4/ 9.08//N 74º22/ 24.93//E	G4
5.		Khilanmarg	34º 2/ 52.83//N 74º23/ 5.75//E	G5
6.	Srinagar	Dargah Hazrat Bal Sharief	34º 7/ 38.16//N 74º50/ 18.06//E	S1
7.		Achan Landfill Area	34º 9/ 18.41//N 74º49/ 0.20//E	S2
8.		Forest Lane, Lal Chowk	34º 4/ 11.78//N 74º48/ 41.93//E	S3
9.		Hyderpora	34º 2/ 14.64//N 74º47/ 26.85//E	S4
10.		Opposite Noora Hospital, HMT	34º 6/ 40.86//N 74º43/ 31.24//E	S5
	Total Composite Sample=			10

Table 2: Composition of different media used for isolation of Effective Microorganisms

S. No.	Effective Microorganisms	Media used	Composition/ litre of Distilled H_2O
1.	Lactic acid bacteria	1. Man Rogosa & Sharpe (MRS)	Yeast extract = 4 g; Beef extract = 8 g; Bactopeptone = 10 g; Glucose = 20 g; Tris Sodium Citrate = 2 g; Sodium Citrate = 5 g; K_2HPO_4 = 2 g; Mg SO_4. $7H_2O$ = 0.2 g; Mn SO_4. $5H_2O$ = 0.05 g; Tween 80 = 1 ml; Malic acid to adjust pH = 5
		2. Glucose Yeast Peptone Agar (GYP)	Glucose=10 g; yeast extract = 5 g; peptone = 5g; KH_2PO_4 = 0.25 g; K_2HPO_4 = 0.25 g; Salt sol. = 10 ml; Agar = 15 g Salt Solution composition= Mg SO_4. $7H_2O$ = 400 mg Mn SO_4. $5H_2O$ = 20 mg Fe SO_4. $7H_2O$ = 20 mg Na Cl = 20 mg

Results and Discussion

The selected isolates were characterized and tentatively identified up to genus level based on morphological and biochemical properties. During the investigation of lactic acid bacteria in the waste samples, we isolated two predominant Lactobacillus strains (LAB33 & LAB45) along with other three strains from them that showed distinguishing characteristics including their fermentation patterns. These lactic acid bacterial isolates were purified, characterized, identified and maintained for further use. Only Gram positive, catalase negative and Oxidase negative strains were selected from mesophiles for further studies. The isolates were classified into the genera *Lactobacillus* and *Pediococcus* based on their morphology and biochemical characters (Saha and Santra, 2014). The predominant *Lactobacillus sp.* and *Pediococcus sp.* were further classified to the species level (Kandler and Weiss, 1986) [6]. The species of mesophillic Lactic acid bacteria identified were *Lactobacillus plantarum* (18 isolates), *L. acidophilus* (8), *L. casei* (12), *L. bulgaricus* (8) and *L. fermentum* (15) and the results are presented in Table 3 and Table 4.

Table 3: Morpho-biochemical characteristics of Lactic acid bacterial isolates

Characteristics	LAB1	LAB8	LAB19	LAB33	LAB45
No. of isolates	12	08	08	15	18
Colony Morphology	White to very light yellow, smooth, lens or diamond shaped	Cream- white, circular	Cream- white, circular	Smooth, round	Creamy-white, circular, smooth, low convex
Cell shape	Rod	Rods with rounded ends	Rod	Rod	Rods with sub-terminal ellipsoidal spores
Mobility	Non motile	Non motile	Non motile	Non motile	Non motile

Gram staining	+	+	+	+	+
Spore formation ability	-	-	-	-	-
Catalase test	-	-	-	-	-
Oxidase test	-	-	-	-	-
Urease test	-	-	-	-	-
Motility test	-	-	-	-	-
Nitrate Reduction test	-	-	-	+	-

Positive: (+) ; Negative : (-)

Table 4: Differentiating characteristics of Lactic acid bacterial isolates

Characteristics (Carbohydrate fermentation)	**LAB1**	**LAB8**	**LAB19**	**LAB33**	**LAB45**
L- arabinose	-	+	-	+	+
NH_3 from Arginine	-	-	-	+	-
Citrate	-	-	-	-	-
D-fructose	+	+	-	+	+
Galactose	+	-	+	+	+
D-Glucose	-	+	+	+	+
Gas production :	-	-	-	+	-
Glycerol	-	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	+	+	-	+	+
Acid from Mannitol	+	-	-	+	-
Rhamnose	-	-	-	-	+
Salicin	+	-/+	-	+	+
Sorbitol	+	-	-	+	+
Sucrose	+	+	-	+	+
Trehalose	+	+/-	+	+	+
D-xylose	-	-	-	+	+
Probable LAB	*Lactobacillus casei*	*L. acidophilus*	*L. bulgaricus*	*Lactobacillus fermentum*	*Lactobacillus plantarum*

Positive (+): Utilization; Negative (-) :Non Utilization

Conclusion

Lactobacillus fermentum (LAB33) and *Lactobacillus plantarum* (LAB45) are non-motile rod cells, Gram-positive, non-spore-forming, catalase, Oxidase and urease negative. Colonies are smooth and round and nitrate reduction test showed positive results in case of *Lactobacillus fermentum* (LAB33) while as colonies are creamy-white, circular, smooth, low convex and nitrate reduction is negative in case of *Lactobacillus plantarum* (LAB45). Ammonia from Arginine and gas is produced from glucose fermentation by *Lactobacillus fermentum* (LAB33) while as no ammonia from Arginine and no gas is produced from glucose by *Lactobacillus plantarum* (LAB45). Rhamnose is not fermented by *Lactobacillus fermentum* (LAB33) but showed positive results by *Lactobacillus plantarum* (LAB45). Both the strains showed assimilation of carbohydrates as carbon source like L- arabinose, D-fructose, galactose, glycerol, lactose, maltose, mannitol, salicin, sorbitol, sucrose, trehalose, D-xylose. No acid is formed from mannitol by *Lactobacillus plantarum* (LAB45).

Acknowledgement

The authors are thankful to the Hon'ble Vice Chancellor and Director Research, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir for providing technical and research facilities.

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