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ORIGINAL ARTICLE **OPEN ACCESS**

Production of Lactic Acid By Native *Lactobacillus Sp*. From Agricultural and Dairy Wastes

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ABSTRACT
Lactic acid (LA) is one of the oldest microbial metabolites known in fermented foods. It has wide applications in food, beverage, pharmaceutical and chemical industries, primarily as an acidulant, flavour enhancer and preservative. Lactic acid is classified as GRAS (generally recognized as safe) for use as a food additive by FDA in US and other regulatory agencies. There are four genera consisting of; Lactobacillus, Leuconostoc, Pediococcus and Streptococcus under lactic acid bacteria (LAB). Among which Lactobacillus is the largest genus, converts lactose and other sugars to lactic acid and acid tolerant. In present study 7 lactic acid bacterial were isolated from agricultural and dairy wastes of Anand. In vitro lactic acid production efficiency was observed in MRS broth by reduction in pH, titrable acidity and percent lactic acid production at 48, 96 and 144 h interval. Among the seven isolate AAU L2 showed the major lactic acid production 16.2 g/L against L. plantarum MTCC 4461 (12.8 g/L) through HPLC study, 5 days after inoculation in MRS broth. This isolate can utilize different agricultural and dairy wastes viz. orange peels, whey, banana industry waste water and corn steep liquor as carbon source efficiently. This lactic acid producing isolate identified by 16S rRNA gene sequencing as Lactobacillus plantarum AAU L2.
Key words: *Lactobacillus, lactic acid, agricultural waste, dairy waste*

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INTRODUCTION

Microorganisms play significant role in waste disposal with production of useful metabolites. They are important for agricultural waste treatment [1] and production of beneficial compounds for food and pharmaceutical industries [1, 2]. The first report of LAB utilization describing the fermentation of milk dates back to 6000 BC followed by fermentation of meat 1500 BC and vegetable products 300 BC [3]. LABs are fundamental bacteria in effective microorganisms (EM), which are widely used in treating food wastes. Selective use of EM can reduce the offensive odour of fermenting garbage within a week [4]. Moreover LAB enhances the breakdown of organic matter such as lignin and cellulose. Moreover, LAB also has the ability to suppress *Fusarium* propagation which is a harmful microorganism causing disease problem in continuous cropping [5]. Lactic acid (LA) is one of the oldest microbial metabolites known in fermented foods. It has wide applications in food, beverage, pharmaceutical and chemical industries, primarily as an acidulant, flavour enhancer and preservative. Lactic acid is classified as GRAS (generally recognized as safe) for use as a general purpose food additive by FDA in US and other regulatory agencies. Lactic acid is a strong sterilizer, suppresses harmful microorganisms and increases rapid decomposition of organic matter. In recent years, there has been an increasing interest in the manufacture of polylactide based biodegradable plastic from lactic acid. Wastes containing starch generated from food processing plants could be a viable option for meeting the growing demand for lactic acid. It is estimated that in India ~ 700 million tons of organic waste is generated annually which is either burnt or land filled. Fermentation by microbes is the best biotechnology to reduce the load on the treatment and disposal of biodegradable agro wastes. Study of native LAB isolates of from Anand environs can be valuable in the area of agriculture waste utilization followed by production of beneficial metabolite Lactic Acid by fermentation. So present investigation is carried out to isolate and identify the native LAB from various agricultural and dairy waste samples from Anand and Lactic Acid production efficiency of isolates supplemented with different agricultural waste as well as qualitative and quantitative analysis of lactic acid production in liquid media by HPLC.

MATERIAL AND METHODS

Isolation and characterization of native LAB from waste samples:

The representative waste samples (approx. 100 g) were collected from 7 different areas of Anand *viz*. Anand vegetable market, Anand Agricultural University, Anand Fruit Market, Vidhya Dairy, Amul Dairy, Banana waste water from Banana Industry and Potato Industry. These samples were decimally diluted and plated on MRS medium followed by incubation at 37 ± 2°C for 48 hours. All the isolates were identified on the basis of morphological and biochemical characteristics using 9th edition of Bergey's Manual of Determinative Bacteriology and standard literature.

Lactic Acid production efficiency of isolates:

The production of acid was primarily detected by estimating the pH of the fermented medium by pH meter and confirmed and quantized using the p-hydroxydiphenyl [6]. Lactic acid production in MRS medium supplemented with different agricultural waste also estimated by similar method.

Qualitative and quantitative analysis of Lactic acid production in liquid media by HPLC:

Qualitative and quantitative analysis of Lactic acid was performed by High Performance Liquid Chromatography (HPLC) technique [7].

Molecular identification of selected potential isolate/s:

Molecular identification of selected isolates were performed by sequencing of the 16S ribosomal RNA (rRNA) gene using the set of primers [8] (27 F- 5′AGAGTTTGATCCTGGCTCAG 3′ and 1492 R- 5′ GGTTACCTTGTTACGACTT 3′). The output sequences were subjected to BLAST [Basic Local Alignment Search Tool] analysis to identify the cultures and to determine the nearest match (http://www.ncbi.nlm.nih.gov). A neighbor-joining phylogenetic tree was constructed using Phylogeny.fr software (http://www.phylogeny.fr/).

RESULTS AND DISCUSSION

Isolation and characterization of native LAB from waste samples:

Total 38 bacterial colonies were isolated from different waste sample and out of them seven were subjected to lactic acid production which resembles LAB. All the seven isolates were found Gram positive, straight, non-sporulating, thick rods, occurring in long chains (9, 10, 11). The isolate AAU L2 showed the highest Lactic acid production efficiency 0.76 % with highest pH reduction 4.6 at 144 h compared to other six isolates and standard *Lactobacillus plantarum* MTCC 4461 in MRS medium (Table. 1).

Biochemical characteristics of the isolates were studied and reactions of tests for specific breakdown products and carbohydrate utilization are presented in Table 2. All the isolates successfully fermented lactose to lactic acid. All the isolates were positive for Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trihalose, Melibiose and Sucrose utilization and negative for Lysine utilization, Ornithine utilization, Urease, Phenylalanine deamination, Nitrate reduction and H_2S production tests. Primarily all the isolates showed more or less lactic acid production (Table 1 and Fig. 1).

Table 1. Isolation, lactic acid production and pH reduction by LAB

Sample Location	Total colonies on MRS	LAB like isolates	Lactic Acid % at 144 h	pH		
				48 h	96 h	144 h
Vegetable waste from Anand vegetable market 1	05	AAU L1	0.41^{b}	3.70^{g}	3.13^{f}	2.43^{f}
Vegetable waste from AAU, Anand	09	AAU L2	0.76^{a}	3.00^{h}	2.76^{g}	2.10^{g}
Fruit waste from Anand Market	03	AAU L3	0.26^{d}	4.70^{e}	4.30^{d}	3.40^{d}
Dairy waste from Vidhya Dairy	08	AAU L4	0.44^{b}	4.10^{f}	3.70^{e}	3.03^{e}
Dairy waste from Amul Dairy	06	AAU L5	0.16^{f}	5.10^{d}	4.80^{c}	3.56^{d}
Banana Waste Water from Banana Industry on NH 8B	04	AAU L6	0.22^{e}	5.46^{c}	5.03^{b}	4.26^{c}
Potato waste from Anand vegetable market 2	03	AAU L7	0.23^{de}	5.73^{b}	5.16^{b}	4.60^{b}
***L.plantarum* Std.**			0.32^{c}	4.80^{e}	3.70^{e}	3.13^{e}
S.Em.			**0.01**	**0.07**	**0.08**	**0.07**
C.V. %			**5.12**	**2.41**	**3.05**	**3.28**

Table 2: Biochemical characteristics of the native *LAB* isolates

Sr. No.	Substrate utilization/ Sugar fermentation	Bacterial Isolates							
		AAU L1	AAU L2	AAU L3	AAU L4	AAU L5	AAU L6	AAU L7	Std.
1	Lactose	+	+	+	+	+	+	+	+
2	Xylose	+	+	+	+	+	+	+	+
3	Maltose	+	+	+	+	+	+	+	+
4	Fructose	+	+	+	+	+	+	+	+
5	Dextrose	+	+	+	+	+	+	+	+
6	Galactose	+	+	+	+	+	+	+	+
7	Raffinose	+	+	+	+	+	+	+	+
8	Trehalose	+	+	+	+	+	+	+	+
9	Melibiose	+	+	+	+	+	+	+	+
10	Sucrose	+	+	+	+	+	+	+	+
11	L- Arabinose	+	+	+	+	+	+	+	+
12	Mannose	+	+	+	+	+	+	+	+
13	Inulin	+	-	+	+	+	+	+	-
14	Glycerol	+	+	+	+	+	+	+	+
15	Salicin	+	+	+	+	+	+	+	+
16	Sorbitol	+	-	+	+	+	+	+	-
17	Mannitol	+	+	+	+	+	+	+	+
18	Adonitol	+	-	+	+	+	+	+	-
19	α- Methyl- D-glucoside	+	-	+	+	+	+	+	-
20	Rhamnose	+	+	+	+	+	+	+	+
21	ortho-Nitrophenyl-β-galactoside	-	+	-	-	-	-	-	+
22	D- Arabinose	+	+	+	+	+	+	+	+
23	Citrate utilization	-	-	-	-	-	-	-	-
24	Malonate utilization	-	-	-	-	-	-	-	-
25	Sorbose	+	-	+	+	+	+	+	-
26	Lysine utilization	-	-	-	-	-	-	-	-
27	Ornithine utilization	-	-	-	-	-	-	-	-
28	Urease	-	-	-	-	-	-	-	-
29	Phenylalanine Deamination	-	-	-	-	-	-	-	-
30	Nitrate reduction	-	-	-	-	-	-	-	-
31	H_2S production	-	-	-	-	-	-	-	-
32	Vogus Proskauer	-	-	-	-	-	-	-	-
33	Methyl red	-	-	-	-	-	-	-	-
34	Indole	-	-	-	-	-	-	-	-
35	Oxidase	+	+	+	+	+	+	+	+

Lactic Acid production efficiency of isolates on different agricultural and dairy waste:

Modified MRS medium having orange peel hydrolysate, whey, Banana waste, Corn steep liquor as an agricultural waste was inoculated with LAB isolates and estimated the titrable acidity, residual reducing sugar and lactic acid production. From the results, it was clearly observed that the isolate AAU L2 produced the highest titrable acidity (1.17 % at 144 h), utilized the highest sugar with the lowest residual reducing sugar in broth at 144 h (5.8 mg/ml) and highest LA production 0.47, 0.66 and 0.67 % at 48, 96 and 144 h respectively, as compared to standard (Table 3) on MRS medium with orange peel hydrolysate.

As shown in table 4 modified MRS medium having whey as dairy waste was inoclualted with LAB isolates. All the isolates showed significantly high titrable acidity as compared to control. Among them isolate AAU L2 gave the highest titrable acidity 0.97 % followed by AAU L4 (0.94 %) at 48 h. Similarly, at 96 h, AAU L2 reported the highest acidity 1.01 % followed by AAU L4 (0.97 %) and AAU L1 (0.95 %). Finally at 144 h, AAU L2 reported the highest acidity 1.02 % followed by AAU L4 (0.99 %), AAU L1 (0.97 %) and *L. plantarum* standard (0.95 %).

Reducing sugar was estimated by DNSA method at 48, 96 and 144 h after inoculation. All the isolates showed significantly low reducing sugar as compared to un-inoculated control. Among them isolate AAU L2 gave the lowest reducing sugar (8.30 mg/ml) at 144 h followed by AAU L1 (9.82 mg/ml), AAU L4 (10.22 mg/ml) and *L. plantarum* Std. (11.27 mg/ml) as compared to control (44.50 mg/ml). Similarly, isolate AAU L2 showed significantly the highest LA production 0.47, 0.51 and 0.53 % at 48, 96 and 144 h followed by AAU L4 (0.42, 0.51 and 0.51 %).

The Titrable acidity**,** Reducing sugar content and lactic acid was measured of modified MRS medium having banana industry waste. All the isolates showed significantly higher titrable acidity compared to control (Table 5). Among them, isolate AAU L2 gave the highest titrable acidity (1.10 %) followed by AAU L4 (1.06 %), AAU L1 (1.02 %) at 144 h and lowest reducing sugar (5.7 mg/ml) at 144 h followed by AAU L1 (5.85 mg/ml), AAU L4 (6.1 mg/ml) and *L. plantarum* Std. (6.3 mg/ml) compared to control (38 mg/ml). Among all isolates AAU L2 produced the highest Lactic Acid (0.51 % at 144 h) followed by AAU L4 (0.49 % at 144 h), AAU L1 (0.48 % at 144 h) and *L. plantarum* Std. (0.48 % at 144 h).

Table 3: Titrable acidity, Reducing sugar content and lactic acid content in modified MRS medium supplemented with Orange peels hydrolysate

Isolates	Titrable acidity (%)			Reducing sugar content (mg/ml)			Lactic Acid (%)		
	48 h	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**
L.plantarum std.	1.03^{b}	1.04^{b}	1.08^{b}	31.30^{d}	22.60^{d}	10.16^{d}	0.30^{d}	0.48^{c}	0.48^{c}
AAU L1	0.83^{d}	0.89^{d}	0.91^{d}	33.58^{c}	24.41^{c}	11.67^{c}	0.37^{c}	0.45^{c}	0.44^{c}
AAU L2	1.10^{a}	1.13^{a}	1.17^{a}	21.72^{e}	11.92^{e}	5.80^{e}	0.47^{a}	0.66^{a}	0.67^{a}
AAU L4	0.92^{c}	0.93^{c}	0.98^{c}	40.70^{b}	31.90^{b}	21.20^{b}	0.42^{b}	0.52^{b}	0.52^{b}
Control	0.03^{e}	0.05^{e}	0.07^{e}	50.00^{a}	50.00^{a}	50.00^{a}	0.009^{e}	0.006^{d}	0.007^{d}
S.Em.	**0.01**	**0.09**	**0.01**	**0.12**	**0.30**	**0.12**	**0.004**	**0.01**	**0.01**
C.V. %	**1.61**	**1.44**	**1.36**	**0.66**	**2.17**	**1.24**	**2.79**	**5.35**	**5.88**

Table 4: Titrable acidity, Reducing sugar content and lactic acid content in modified MRS medium supplemented with whey

Isolates	Titrable acidity (%)			Reducing sugar content (mg/ml)			Lactic Acid (%)		
	48 h	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**
L.plantarum std.	0.88^{c}	0.93^{b}	0.95^{c}	29.70^{b}	18.07^{b}	11.27^{b}	0.31^{d}	0.46^{c}	0.48^{c}
AAU L1	0.90^{bc}	0.95^{ab}	0.97^{bc}	27.65^{bc}	16.62^{bc}	9.82^{bc}	0.37^{c}	0.49^{b}	0.46^{d}
AAU L2	0.97^{a}	1.01^{a}	1.02^{a}	26.90^{c}	15.45^{c}	8.30^{c}	0.47^{a}	0.51^{a}	0.53^{a}
AAU L4	0.94^{ab}	0.97^{ab}	0.99^{ab}	28.45^{bc}	17.55^{b}	10.22^{bc}	0.42^{b}	0.51^{a}	0.51^{b}
Control	0.03^{d}	0.04^{c}	0.06^{d}	45.00^{a}	45.00^{a}	44.50^{a}	0.008^{e}	0.007^{d}	0.006^{e}
S.Em.	**0.02**	**0.03**	**0.01**	**0.75**	**0.68**	**0.79**	**0.002**	**0.003**	**0.01**
C.V. %	**4.99**	**6.48**	**3.03**	**4.76**	**6.00**	**9.34**	**1.37**	**1.74**	**2.83**

Table 5: Titrable acidity, Reducing sugar content and lactic acid content in modified MRS medium supplemented with Banana waste

Isolates	Titrable acidity (%)			Reducing sugar content (mg/ml)			Lactic Acid (%)		
	48 h	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**
L.plantarum std.	0.93^{c}	0.96^{b}	1.00^{c}	26.3^{b}	17.55^{b}	6.30^{b}	0.38^{a}	0.46^{c}	0.48^{c}
AAU L1	0.95^{bc}	1.00^{ab}	1.02^{bc}	25.52^{bc}	14.67^{bc}	5.85^{b}	0.39^{a}	0.46^{c}	0.48^{bc}
AAU L2	1.02^{a}	1.06^{a}	1.10^{a}	24.42^{c}	12.27^{c}	5.70^{b}	0.42^{a}	0.50^{a}	0.51^{a}
AAU L4	0.97^{b}	1.04^{a}	1.06^{ab}	26.12^{bc}	16.17^{bc}	6.10^{b}	0.40^{a}	0.48^{b}	0.49^{b}
Control	0.03^{d}	0.05^{c}	0.07^{d}	38.00^{a}	41.00^{a}	38.00^{a}	0.005^{b}	0.005^{d}	0.005^{d}
S.Em.	**0.01**	**0.02**	**0.02**	**0.57**	**0.50**	**0.39**	**0.01**	**0.003**	**0.004**
C.V. %	**3.33**	**5.27**	**4.97**	**4.05**	**4.93**	**6.24**	**9.05**	**1.79**	**2.09**

Table 6: Titrable acidity, Reducing sugar content and lactic acid content in modified MRS medium supplemented with Corn steep liquor (CSL)

Isolates	Titrable acidity (%)			Reducing sugar content (mg/ml)			Lactic Acid (%)		
	48 h	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**	**48 h**	**96 h**	**144 h**
L.plantarum std.	0.88^{c}	0.92^{b}	0.96^{b}	32.10^{b}	21.00^{b}	11.80^{b}	0.37^{d}	0.46^{b}	0.46^{d}
AAU L1	0.91^{bc}	0.94^{ab}	0.98^{ab}	29.92^{d}	16.82^{d}	7.07^{d}	0.38^{c}	0.46^{ab}	0.47^{c}
AAU L2	0.95^{a}	0.98^{a}	1.01^{a}	28.70^{e}	14.27^{e}	5.67^{e}	0.39^{a}	0.47^{a}	0.48^{a}
AAU L4	0.94^{ab}	0.96^{a}	1.00^{ab}	30.67^{c}	18.17^{c}	9.87^{c}	0.39^{b}	0.47^{a}	0.47^{b}
Control	0.05^{d}	0.05^{c}	0.06^{c}	42.02^{a}	42.02^{a}	42.00^{a}	0.009^{e}	0.008^{c}	0.009^{e}
S.Em.	**0.01**	**0.01**	**0.01**	**0.23**	**0.14**	**0.15**	**0.002**	**0.002**	**0.002**
C.V. %	**2.83**	**3.44**	**3.55**	**1.41**	**1.24**	**1.91**	**1.09**	**1.18**	**0.88**

Modified MRS medium having corn steep liquor was showed significantly higher titrable acidity compared to control. Among them, the isolate AAU L2 gave the highest titrable acidity (1.01 %) at 144 h followed by AAU L4 (1.00 %), AAU L1 (0.98 %) as compared to *L. plantarum* standard (0.96 %). The results showed that isolate AAU L2 reported the lowest reducing sugar (5.67 mg/ml) at 144 h (Table 6) and highest LA production (0.48 % at 144 h).

Qualitative and quantitative analysis of Lactic acid production in liquid media by HPLC

At 5 DAI AAU L2 produced lactic acid as major product (16.2 g/l) as *L. plantarum* standard (12.8 g/L) (Fig 2).

Molecular identification of selected potential isolate/s:

The isolate AAU L2 was identified as *Lactobacillus plantarum* with 99 % similarity and 99 % query coverage to strain *Lactobacillus plantarum* WCFS1. The phylogenetic tree (Fig 3) indicated AAU L2 belongs to Firmicutes and grouped with other *Lactobacillus* sp. *Lactobacillus plantarum* isolated from calf ruminal liquid, pig stomach and silage [8].

Fig 1: Primary estimation of Lactic Acid production

Fig 2: Lactic Acid profile of promising isolates and standard by HPLC

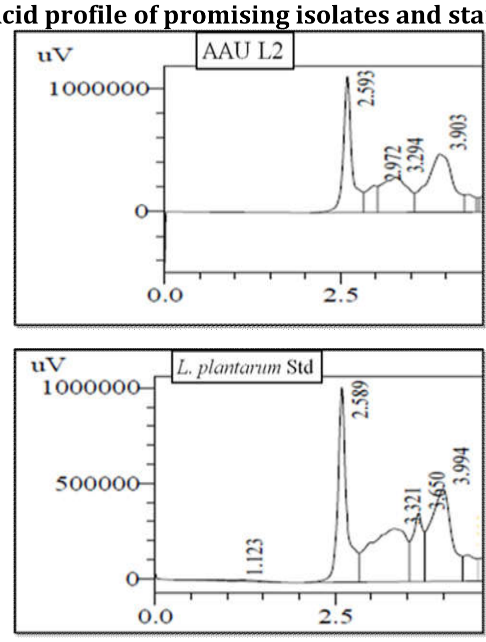
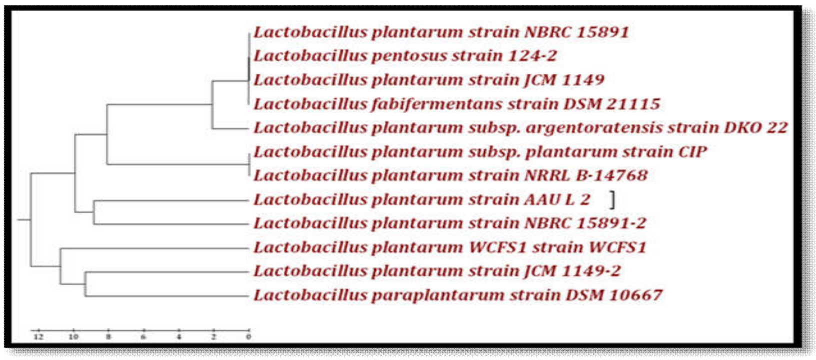


Fig 3: Phylogenetic tree based on 16*S rRNA* sequence of LAB isolate AAU L2



CONCLUSION

Totally seven native lactic acid bacteria were isolated from agricultural and dairy waste samples of Anand city of Middle Gujarat which showed typical *Lactobacillus* like colonies on Nutrient and MRS agar. The organisms were gram positive, non-spore forming bacilli. All the isolates were negative for catalase production. All the 7 isolates were positive for lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trihalose, melibiose and sucrose utilization. These were negative for utilization of lysine, ornithine, phenylalanine, urease and H_2S production, nitrate reduction *etc.*

The isolate AAU L2 showed the highest Lactic acid production efficiency 0.76 % with highest pH reduction 4.6 at 144 h compared to other six isolates and standard *Lactobacillus plantarum* MTCC 4461 in MRS medium. In further experiments, with agricultural and dairy waste samples, the isolate AAU L2 showed the highest Lactic acid production efficiency in modified MRS broth supplemented with hydrolysates of orange peels, whey, banana waste water from banana Industry and Corn steep liquor i.e. 0.66 %, 0.53 %, 0.51 % and 0.48 % and the highest pH reduction 4.6, 4.4, 4.82 and 4.7 at 144 h, respectively, compared to two isolates and standard *L. plantarum* MTCC 4461. On the basis of partial 16S r*RNA* gene sequencing isolate AAUL2 was identified as *Lactobacillus plantarum* AAU L2 (NCBI Acc. no. KJ396071) can be exploited as a starter culture for lactic acid production utilizing agro and dairy waste.

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