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Isolation and Identification of Wild Strains of Lactic Acid Bacteria for Yoghurt Preparation from Indigenous Dahi

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Abstract: Lactic acid bacteria are commonly found in fermented dairy products. Their role in the production of value added products of milk like yoghurt, cheese and butter etc has got great significance. In this study thirty-five samples of dahi were collected randomly from the local markets of Rawalpindi. A total of 69 isolates were identified phenotypically and divide into three genera *Lactococcus* (36 isolates), *Lactobacillus* (15 isolates) and *Streptococcus (*18 isolates*)*. Out of 69 isolates 26% were of *S. thermophilus* followed by 22% *L. bulgaricus*, 16% *L. acidophilus*, 9% *L. lactis* and 9% *L. casei*, respectively. After identification, potential of strains for lactic acid production after 6 hrs, 12 hrs and 24 hrs were also determined. In addition to acid production diacetyl production was also observed at 37°C for 8 hrs with two hours intervals. There were generally increasing trend for diacetyl production. The study showed that there was a variety of lactic acid bacteria in our environment which has potential to produce quality yoghurt.

Key words: Lactic acid bacteria, fermented dairy products, isolation, identification, acid production

INTRODUCTION

Starter culture organisms used in fermentations belongs to a family of bacteria collectively known as the lactic acid bacteria (LAB). These LAB are united by a constellation of morphological, metabolic and physiological characteristics. There are several factors, which influence the quality of yoghurt. These include type of milk, processing conditions, storage conditions etc., however quality of starter culture is the most important factor that influence in the development of quality yoghurt.

LAB is widely employed in traditional fermented milk, in industrial fermentation processes and as starter cultures in the dairy industry. Some strains of lactic acid bacteria have importance in general health, providing a beneficial microflora in the intestinal tract. LAB plays an important role in food fermentation, as the products obtained with their aid are characterized by hygienic safety, storage stability and attractive sensory properties (Salminen *et al*., 1998).

The ability to produce lactic acid from lactose is probably the most important property of dairy LAB, known as lactic acid fermentation. It helps to reduce the pH, which increases the expulsion of whey from the curd, thus lowers the moisture content and helps in preventing microbial spoilage (Cogan *et al*., 1997). Fermentation is one of the oldest methods of food preservation known to mankind and today fermented foods represent a very significant part of the food processing industry. The major function of a starter culture is the production of lactic acid at a suitable rate to ensure a consistent and successful fermentation (McKay and Baldwin, 1990). The LAB carries out the initial acidification of the milk, which assists in gelation. In fact, its ability to produce acid rapidly is probably the most important property of starter bacteria (Cogan *et al*., 1997).

It is well recognized that technological properties of yoghurt, such as acidification, flavour production and viscosity in great extent are strain dependent (Accolas and Auclair, 1977). Lactic acid is used today by food industry as acidulent and preservative for the production of sour curd cheese and yoghurt (Linkater and Griffin, 1971) but Lipinsky (1981) has emphasized on the potential importance of biotechnologically produced lactic acid as chemical feedstock via lactonitrile and lactides. Lactococci are the major mesophilic bacteria used for acid production in dairy fermentations and used as starter cultures in the manufacture of a vast range of dairy foods including fermented milks, lactic butter, cheese and lactic casein (Ward *et al*., 2002).

Available methods for quantifying the starter activity usually have been based on pH changes or lactic acid production, measured after a fixed time by titration with NaOH (Accolas and Auclair 1977).

The dairy industry has developed considerably, thanks to the use of selective lactic acid bacteria, the choice being based on their production of lactic acid, aromatic compounds, bacteriocins and their resistance to the phages (Herrero *et al*., 1996). Industrialization of the biological transformation of foodstuffs has increased the economic importance of lactic acid bacteria, because they play crucial role in the sensorial and safety aspects of fermented products.

The aim of the present work was to identify the strains of lactic acid bacteria isolated from fermented milk products and to study their technological characteristics

in order to select strains of lactic acid bacteria used as lactic acid starter in the manufacturing of fermented dairy products and which are suitable to local conditions.

MATERIALS AND METHODS

35 samples of indigenous dahi were collected from the local markets of different areas of Rawalpindi, in sterilized sample bottles and were immediately transferred to laboratory for further analysis.

Isolation of lactic acid bacteria: Selective media M-17 and MRS was used for the isolation of wild strains of Lactic acid bacteria. The samples were inoculated through a streak plate method. After inoculation plates were incubated at 37°C for 24-48 hours.

Phenotypic characterization: Phenotypic characteristics were carried out by the following tests.

Gram's staining: Gram's staining was performed according to procedure described by Collins and Lyne (1980).

Catalase test: The test was conducted to check the production of enzyme catalase. A drop of 3 percent hydrogen peroxide was placed on a clean microscopic slide. A visible amount of bacterial growth was added aseptically with the help of an inoculating loop. Both were mixed and observed for gas bubble production.

Identification tests: The strains showing Gram positive and Catalase negative were identified by the following tests.

Sugar fermentation tests: One percent solution of lactose, fructose, galactose, glucose, maltose and mannitol was prepared. One gram of sugar was dissolved in 10ml of distilled water and was sterilized by passing through 0.45µm filter. Nutrient broth was prepared by dissolving 0.8g in 100ml distilled water and 1ml of phenol red was added in it and was autoclaved at 121°C for 15 minutes and cooled at room temperature. 5ml of broth and 100 micro liters of sugars were taken into sterilized test tubes and labeled properly and were placed at room temperature for 24 hours to check for contamination. After 24 hours the purified colonies were inoculated into test tubes and incubated at 37°C for 48 hours. Incase of fermentation, the color of sugar was changed from red to yellow, reflecting the test as positive.

Arginine test: Culture was incubated in arginine broth for 24-48 hours and few drops of Nessler reagent was added in it. A brown color indicated hydrolysis Dissolved by heating and pH was adjusted to 7.0. 5-10ml of arginine broth was taken in tubes and autoclaved at 115°C for 10 minutes. For arginine breakdown of Lactobacilli, MRS broth was used in which the ammonium citrate was replaced with 0.3 percent arginine hydrolysis.

Sodium chloride utilization test: For sodium chloride utilization test, NaCl solution was prepared at different concentrations. Colonies were inoculated in MRS broth containing NaCl in test tubes. Test tubes were incubated at 28-30°C for 48-72 hrs.

Technological evaluation of lactic acid bacteria: Selected strains of lactic acid bacteria were used for the preparation of yoghurt. Yoghurt was further analyzed for pH, Titratable acidititiy, diacetyl production as well as organoleptic properties.

pH: pH was measured by pH meter according to AOAC method No.981.12 (1990).

Titratable acidity: Acidity was determined by AOAC method No. 967.16 (1990).

Diacetyl determination: Diacetyl was determined by the method of Pack *et al.* (1964).

Organoleptic evaluation: a panel of judges using 9 points Hedonic scale carried out organoleptic evaluation

Statistical analysis: Data obtained for given parameters was statistically analyzed using the Analysis Of Variance (ANOVA) technique in two-factor factorial Completely Randomized Design (CRD) using M STAT C statistical software to compare the means according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Isolation and Identification: Colonies in the form of mosaic of bacteria were observed on the surface of M17 and MRS plates. More than one colony was observed in most of the cases. Cultural and morphological characteristics were examined with the help of microscope. Different types of microorganisms were observed, majority of them belonged to Gram + ve rods and cocci shaped bacteria. The purification of isolates was done by transferring Gram+ve rods and cocci shaped bacteria to the plates of selective media MRS and M-17, respectively. These isolates were further sub cultured until pure isolates were obtained.

Total of 69 lactic acid bacterial cultures were isolated from 35 tested samples. After initial identification, 33 of them were determined as representative of the genus *Lactococcus* and rest were referred to genus *Lactobacillus*, respectively. The number and type of lactic acid bacteria's isolated from dahi samples are given in Table 1. The strains were than identified to species level.

Table 1: Frequency distribution of isolates of Lactic Acid Bacteria from Indigenous Dahi samples

	No. of Isolates							
Description	No. of samples	*S. thermophilus*	*Lactococcus lactis*	*Lb. lactis*	*Lb. acidophilus*	*Lb. bulgaricus*	*Lb. casei*	Total
Indigenous Dahi Samples	35	18	15	6	11	13	6	69

The strains were phenotypically characterized on the basis of their morphological, cultural, physiological and biochemical characteristics by the procedure described in Bergey's manual (Williams and Wilkins, 1984) and Collins and Lyne (1980).

The results of phenotypic characterization, identification and biochemical characteristics are given in Table 2.

These 69 isolates were identified phenotypically and divided into three genra: *Lactobacillus, Lactococcus* and *Streptococcus*. These isolates were gram positive and Catalase negative. 36 isolates belonged to genera *Lactobacillus,* fifteen in *Lactococcus* and eighteen in *Streptococcus*.

It was evident from the result that the lactic acid bacteria dominated the microbial flora of dahi. It might be due to the reason that two specific media MRS and M-17 agar were used to study the morphological characteristics of rods and cocci isolates, respectively. This selective media allows only specific type of microorganisms to grow therefore the ability of bacterial species to grow on specific media is regarded as an important characteristic in identification. MRS and M17 media are the best suitable media for the isolation of lactic acid bacteria as reported earlier by Ghoddusi (2002).

The presence of such bacteria has been reported in earlier studies (Naeem and Rizvi, 1983). Moreover it was further observed that all the isolated bacteria from indigenous dahi were thermophillic and mesophillic in nature (Jay, 1978). This diversity of species is relative and dependent primarily on the nature of the material isolated and different criteria used for each study as reported by Masud *et al.* (1991).

Out of 69 isolates 26% of *Streptococcus thermophilus* followed by 22% *Lactococcus lactis*, 18% *Lactobacillus bulgaricus*, 16% *Lactobacillus acidophilus*, 9% *Lactobacillus lactis,* 9% *Lactobacillus casei,* respectively. It was further observed that *S. thermophilus* and *L. lactis* along with *L. bulgaricus* constituted the dominant microflora of dahi. Thus these species play an important role for the preparation of this fermented milk product, as noted by Warsy (1983).

The presence of high number of *S. thermophilus* as recorded in the present investigation could be attributed to the presence of old inoculums containing number of *S. thermophilus*. Similar views are expressed by Mohanan *et al.* (1983). They are mainly used for acid flavor production in yoghurt making (Kosikowski, 1982).

The presence of *Lactococcus lactis* in our study is of great importance. Koiskowski (1982) reported that these organisms are used for the preparation of different types of cheese. However, they may be used for the preparation of yoghurt where multi-strains starter culture is used to produce the desired acidity. The role of these strains have not been yet characterized, however, they may play a role in the preparation of this product in winter season. Different studies reported that these strains have ability to produce slime characteristics, which may be used to improve the quality of the final product.

Only 11 strains of *Lactobacillus acidophilus* are recorded in the present study. These strains are considered to produce higher titrable acidity and result in the production of low pH that may be considered objectionable (Naeem and Rizvi, 1983). However, the results of studies reported that these strains have the ability to produce bacteriocins. Its importance is well documented by Isani *et al.* (1986). Therefore, it is proposed to look its role in dahi with reference to public health significance.

Potential of lactic acid bacteria: After identification than these isolates were analyzed for their rate of acid production at 30°C, the strains, which produced acidity more than 0.7% after 6 hrs interval, were selected for lactic acid production i.e. the selection was done on the basis of time and rate of acid production. The strains were also characterized into fast, medium and slow on the basis of acid production at 30°C after 6 hrs. of interval as shown in Table 3.

Based on these technological properties of individual strains, among 18 strains of *Streptococcus thermophilus* examined, 17 were considered to be fast and 1 was found medium. While in case of *Lactococcus lactis*, 13 were fast and 2 were slow. Whereas all 6 strains of *Lactobacillus lactis* were reported to be fast. Similarly all 11 strains of *Lactobacillus acidophilus* were also reported to be fast. In case of *Lactobacillus bulgaricus*, 10 were found to fast, 1 was medium and 2 were reported to be slow, while in case of *Lactobacillus casei*, 5 were fast and 1 was medium.

Based on these observations it is concluded that among the examined strains, 62 are considered to be the fast acid producing strains followed by 3 medium acid producing strains and 4 slow acid producing strains.

The possible reason for this behavior may be their optimum growth condition such as temperature, composition of medium and other environmental conditions along with their genetic make-up.

On the basis of these observations, 10 fast acid producing species were randomly selected for their

Table 2: Morphological, Cultural, Physiological and Biochemical Characteristic of the Isolated Strains

	Lactococci		Lactobacilli			
Group	1	2	1	2	3	4
No. of Isolates	18	15	6	11	13	6
Gram stain reaction	+	+	+	+	+	+
Catalase activity	-	-	-	-	-	-
Sugar fermentation lactose	+	+	+	+	+	+
Glucose	4	-	2	6	9	+
Mannitol	-	-	-	-	3	-
Galactose	+	+	+	+	7	+
Maltose	+	-	-	5	+	+
Fructose	-	+	+	+	+	+
Argnine Test	+	+	+	-	-	+
Sodium Chloride utilization Test	-	-	+	-	+	+

Table 3: Technological activities of isolates

Species	Fast	Medium	Slow
Streptococcus thermophilus	17	1	-
Lactococcus lactis	13	-	2
Lactobacillus lactis	6	-	-
Lactobacillus acidophilus	11	-	-
Lactobacillus bulgaricus	10	1	2
Lactobacillus casei	05	1	-

lactic acid production after 2 hour interval up to 8 hours at 37°C. The growth of these was examined on the basis of pH, acidity and diacetyl production at respective temperatures.

Technological evaluation of lactic acid bacteria:

pH: The pH of the selected strains was noted at different time interval at 37°C. Their interaction of time and temperature was presented in Table 4. The 10 different high acid producing strains of lactic acid bacteria were selected on the random basis. Interaction of temperature and time was determined and is expressed in Table 4. The pH was determined after 2-hrs interval up to 8 hours at temperature of 37°C. The Table showed that there is a general trend of decrease in pH with the passage of time. The lower the pH more will be the acid produced and H + ion concentration that express the efficiency of strains.

Titratable acidity: The data regarding the acidity produced by 10 selected strains is given in Table 4. The acidity was measured at 37°C with 2-hr interval up to 8 hrs. The strain that produced high acidity is the most efficient. The table showed that there is general increase in acidity of the strains with increase of time. More the acid produced, the higher is the performance of the strain. The statistical analysis reflects that there is a significant effect of interaction between temperature and time on acidity with respect to time interval. The coefficient of variation of these strains was 1.03 percent.

Diacetyl determination: Diacetyl produced by the lactic acid bacteria is responsible of the characteristic taste of the end product. The data pertaining to the diacetyl determination of these selected strains is given in Table 5. Interaction of temperature on the production of diacetyl was determined after 2 hrs intervals up to 8 hrs at 37°C. It is cleared that there is a significant difference among different treatments at different intervals. After 2 hrs the diacetyl of all the strains was found low might be due to the lag phase of strain as in this phase of strain is going to adjust itself with the environment. As the time passes production of diacetyl of these strains increases.

Among the examined strains S6 strain at 37°C produced higher rate of diacetyl after 8 hrs. The results of present study about diacetyl determination of lactic acid producing microorganisms are in close agreement with those of Boumerdassi *et al*. (1997), who reported the effect of citrate on diacetyl production by *Lactococcus lactis*.

Organoleptic evaluation: All the selected strains were evaluated organoleptically just after the preparation for color, texture, taste, flavour and overall acceptability. The panel of five judges did sensory evaluation of the prepared yoghurt.

Color: Data pertaining to color scores of yoghurt are given in Table 6. The average color point for yoghurt L2 was highest followed by, S17, L1, S7, F13, LT1, R20, S3, S6, J10 respectively. The results obtained are in line with the findings of Kristinapa and Namburdipad (1982) who stated that *S. thermophilus* and *L. bulgaricus* when used gave a good quality product and satisfactory color. DMR test applied on color scores also displayed that the yoghurt prepared using S6, S7, S3, S17, R20, LT1, L1, L2, F13 and J10 showed significant difference among each other.

Texture: It was observed that texture mean score was highest for L2 and S7 followed by S17, LT1, S6, F13, S3, L1, J10, R20.The results are in concordant with the findings of Kristinapa and Namburdipad (1982) as they stated that *Streptococcus thermophilus* and *Lactobacillus bulgaricus* when used together gave a good quality product and a satisfactory texture. DMR test applied on texture scores also displayed that the yoghurt prepared

Table 4: Interaction of temperature and time on pH of high acid producing strains at 37°C.

	PH				Acidity			
Strains	2hr	4hr	6hr	8hr	2hr	4hrs	6hr	8hr
S. thermophilus S6	5.12^{f}	4.20^{q}	4.30^{p}	4.05^{r}	0.68^{mno}	0.89^{hi}	1.050^{d}	1.36^{a}
S. thermophilus S7	4.85^{ij}	4.60^{l}	4.52^{mn}	4.49^{n}	0.8^{ijk}	0.90^{hi}	1.040	1.24^{b}
L. lactis S3	5.20^{e}	4.95^{h}	4.83^{ij}	4.75^{k}	0.66^{mnop}	0.87^{i}	0.900^{efg}	1.10^{c}
L. lactis S17	5.23^{e}	4.85^{j}	4.54^{mn}	4.38^{o}	0.65^{nopq}	0.85^{ij}	0.98^{fg}	1.07^{cd}
L. acidophilus R20	4.88^{i}	4.20^{q}	3.95^{s}	3.72^{t}	0.62^{pq}	0.90^{hi}	1.02^{def}	1.24^{b}
L. acidophilus LT1	4.80^{ijk}	4.30^{p}	4.05^{r}	3.98^{s}	0.64^{opq}	0.80^{ijk}	0.89^{hi}	0.94^{gh}
L. bulgaricus L1	5.57^{b}	5.25^{e}	4.40^{o}	4.07^{r}	0.40^{t}	0.71^{lm}	0.90^{hi}	0.99^{efg}
L.balgaricus L2	5.60^{b}	5.15^{f}	4.55^{lm}	4.27^{p}	0.50^{s}	0.66^{mnop}	0.85^{ij}	1.04^{de}
L. casei F13	5.35^{d}	4.95^{h}	4.60^{l}	4.39^{o}	0.56^{r}	0.70l^{mn}	0.800^{ijk}	0.89^{hi}
L. casei J10	5.60^{g}	5.75^{a}	5.45^{c}	5.24^{e}	0.4^{t}	0.600^{qr}	0.7500^{kl}	0.85^{ij}

*Results are means of three replications. *Means having same letters are non-significant at 5 % rejection level

Table No 5: Interaction of temperature and time on diacetyl determination of high acid producing strains at 37°C

	Diacetyl			
Strains	2hr	4hr	6hr	8hr
S. thermophilus S6	1.5^{b}	1.7^{b}	1.9^{a}	2.2^{a}
S. thermophilus S7	1.4^{c}	1.6^{c}	1.9^{a}	2.1^{b}
L. lactis S3	1.6^{a}	1.8^{a}	1.9^{a}	2.0^{c}
L. lactis S17	1.3^{d}	1.5^{d}	1.6^{d}	1.8^{e}
L. acidophilus R20	1.6^{a}	1.8^{a}	1.9^{a}	2.1^{b}
L. acidophilus T1	1.5^{b}	1.6^{c}	1.6^{d}	1.7f
L. bulgaricus L1	1.3^{d}	1.5^{d}	1.6^{d}	1.8^{e}
L. balgaricus L2	1.3^{d}	1.5^{d}	1.8^{b}	1.9^{d}
L. casei F13	1.4^{c}	1.6	1.7^{c}	1.8^{e}
L. casei J10	1.3^{d}	1.4^{e}	1.5^{e}	1.6^{d}

*Results are means of three replications. *Means having same letters are non-significant at 5 % rejection level

using S6, S7, S3, S17, R20, LT1, L1, L2, F13 and J10 showed non-significant difference among each other.

Taste: It was observed that taste mean score was highest for S7, followed by S17, LT2, LT1, F13, L1, S6, S3, J10, S6, respectively. The results are in line with the findings of Lee *et al.* (1988), who concluded that the starter mixture had better growth and acid production and produced superior yoghurt in term of taste. The values obtained could be related to the findings of Martin *et al.* (1999) as the association of thermophilic lactic acid bacteria gave best acid taste according to strain association. Instrumental parameters correlated best with acid taste and mouth coating.

DMR test applied on taste scores also displayed that the yoghurt prepared using S6, S7, S3, S17, R20, LT1, L1, L2, F13 and J10 showed significant difference among each other.

Flavor: It was observed that flavor mean score was highest for S17, followed by LT1, S7, L2, S3, S6, R20, L1, F13 and J10, respectively. The results obtained were in line with the finding of Jay (1978) as *L. bulgaricus* produces more acetaldehyde that is chief volatile component in yoghurt. Similarly the results were same as the findings of Bylund (1995) too, who reported that acetaldehyde is attributed to *Lactobacillus bulgaricus*. In the association growth of *S. thermophillus* and *L. bulgaricus*, the rate of acetaldehyde production is considerably increased. DMR test applied on flavor scores also displayed that the yoghurt prepared using S6, S7, S3, S17, R20, LT1, L1, L2, F13 and J10 showed significant difference among each other.

Overall acceptability: It was observed that overall acceptability mean score was highest for L2, followed by S17, S7, LT1, L1, F13, S3, R20, S3 and J10 respectively. The results obtained were in line with the finding of Beal *et al.* (1999) who concluded that consumer acceptance of stirred yoghurt depends on acidity, aroma perceptions, and textural properties of the product. Lactic acidification is the result of lactose fermentation by the associative growth of two *thermophilic*, homofermentive lactic acid bacteria. *Streptococcus thermophilus* and

Table 6: Effect of different stains on organoleptic of yoghurt

Strains	Color	Texture	Taste	Flavor	Over all acceptability
S thermophilus S6	6.04^{i}	6.40^{c}	6.00^{f}	6.40^{e}	6.23^{f}
S. thermophilus S7	56.54^{d}	7.30^{a}	7.50^{a}	7.40^{c}	7.18^{b}
L. lactis S3	6.10^{h}	6.30^{cd}	6.00^{f}	6.40^{e}	6.20^{f}
L. lactis S17	7.30^{b}	7.10^{b}	7.00^{b}	7.60^{a}	7.25^{a}
L. acidophilus R20	6.20^{g}	6.30^{cd}	6.10^{e}	7.60^{a}	6.23^{f}
L. acidophilus T1	6.30^{f}	7.10^{a}	6.90^{c}	7.50^{b}	6.95^{c}
L bulgaricus L1	7.20^{c}	6.30^{cd}	6.10^{e}	6.30^{f}	6.48^{d}
L.balgaricus L2	7.50^{a}	7.30^{a}	7.00^{b}	7.30^{d}	7.28^{d}
L. casei F13	6.50^{e}	6.40^{c}	6.20^{d}	6.30^{f}	6.35^{e}
L. casei J10	6.00^{j}	6.30^{cd}	6.00^{f}	6.20^{g}	6.12^{g}

*Results are means of three replications. *Means having same letters are non-significant at 5 % rejection level

Lactobacillus bulgaricus and is influenced by the quality of milk, the strains used and the incubation temperature. DMR test applied on Overall acceptability scores also displayed that the yoghurt prepared using S6, S7, S3, S17, R20, LT1, L1, L2, F13 and J10 showed significant difference among each other.

Conclusion: The study revealed that there is a variety of lactic acid bacteria present in our environment, which has a potential to perform equally good like exotic strains. In this study, we were able to isolate and explore the maximum potential of LAB strains. Now it is the need of time to develop some sophisticated system for the isolation, propagation and preservation of the best performing indigenous LAB strains. This study provides the basis for the development of starter culture bank, which will be used for the future investigations and their ultimate utilizations in our dairy industry.

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