British Microbiology Research Journal
3(4): 743-752, 2013

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Characterization of Lactic Acid Bacteria from Raw Milk Samples of Cow, Goat, Sheep, Camel and Buffalo with Special Elucidation to Lactic Acid Production

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Author's contributions

This work was carried out in collaboration between all authors. Author RS and BSS were involved in experimental work, authors GST, PJ and SP were associated with analyses of the study, writing first draft of the manuscript, and authors AS and PSB were associated with study design, execution and manuscript writing. All authors read and approved the final manuscript.

Research Article

Received 13th May 2013
Accepted 27th August 2013
Published 6th September 2013

ABSTRACT

Aims: Selective enumeration, isolation and characterization of lactic acid bacteria from different raw milk sources with special elucidation to lactic acid production.
Study Design: Serial dilution for strain isolation from raw milk samples to be done followed by complete morphological, biochemical and molecular characterization aiming to study the microbial behavior to ferment different sugar and producing lactic acid as end product.
Place and Duration of Study: Microbiology Biotechnology Laboratory, Tropilite Foods Pvt. Ltd. Gwalior (M.P)-INDIA from Sep 2012 to April 2013.
Methodology: Milk samples from cow, sheep, goat, camel and buffalo were collected from the surrounding area of Gwalior district of Madhya Pradesh, India, from local milk suppliers. 13 potent strains in terms of lactic acid production were selected for analysis. Gram

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Staining, Catalase activity, Sugar fermentation, growth at high (45ºC) and low (10ºC) temperatures along with growth in different NaCl concentrations was observed with all isolates. Significant molecular characterization was done to determine the homology between different isolates of lactic acid bacteria.

Results and Conclusion: Five potent lactic acid bacteria strains *Streptococcus thermophilus* from goat milk, *Lactococcus lactis* from buffalo milk, *Streptococcus gallolyticus* from camel milk, *Streptococcus thermophilus* from cow milk, *Lactobacillus delbrueckii* from sheep milk were identified capable of producing lactic acid in generous amount. Also all isolated strains from goat milk were found efficient in terms of lactic acid production when compared to other raw milk sources.

Keywords: Lactic acid bacteria; goat milk; lactic acid; sugar fermentation.

1. INTRODUCTION

Milk and milk products provide a wealth of nutrition benefits with their healthy contents along with the micro-flora these products carry. There are a variety of bacteria that can be present in milk. Among other differences, they vary in the temperatures at which they will grow optimally. Some are described as psychrophilic, which means that they grow best at cold temperatures, while others are severely retarded by being in the refrigerator and grow rapidly only at warmer temperatures. Lactic acid bacteria (LAB) have the property to convert natural sugars into lactic acid by fermentation. When camel milk samples are left to stand, acidity increases due to the presence of LAB [1], same is the result with cow, goat, sheep and buffalo milk. This acidity is due to the presence of lactic acid which was firstly discovered in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as milk component which was in 1857 corrected by Louis Pasteur as a fermentation end product [2]. The production of lactic acid has been used for a long time in food production (e.g., yogurt, cheese, sauerkraut, sausage,). Since the 1970s, the popularity of fermented foods such as kefir and tofu that were formally confined to certain ethnically oriented cuisines has greatly increased. The biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources [3]. Lactic acid can be produced either by chemical synthesis or by microbial fermentation. Chemical synthesis from petrochemical resources always results in racemic mixture of DL-lactic acid, which is a major disadvantage of this approach [4]. The fermentation process is becoming more relevant because the raw materials used in fermentation are renewable in contrast to petrochemicals. Furthermore, the fermentation process could produce optically pure isomers of lactic acid by selecting an appropriate strain [5]. Pure isomers, L or D lactic acid, are more valuable than the racemic DL form because each isomer has its own applications in the cosmetics and pharmaceuticals industries. For example, the ratio of L- and D-lactic acids influences the properties and the degradability of poly-lactic acids [6].

Lactic acid is under increasing demand in Food, Pharmaceutical and Chemical Industries and for production of Poly lactic acid polymers, which possess outstanding biomedical applications. The global manufacture of this organic acid is estimated to be 45 million kilogram/yr and is expected to grow by 8.6% annually [7]. Lactic acid is currently manufactured either through chemical or by microbial fermentation mode. In India, the annual production capacity of lactic acid is 6000 tons and estimate gaps of 2300 tons in

supply by the year 2015 have been predicted, if the present level of production is not increased [8].

Lactic acid bacteria are group of Gram positive bacteria. Other main characteristics include non-respiring, non-spore forming, cocci or rods, and produce lactic acid as the major end product from the fermentation of carbohydrates. In general, LAB occurs in habitats with a rich nutrition supply. They occur on decomposing plant material and fruits, in dairy products, fermented meat and fish, beets, potatoes, mash, sauerkraut, sourdough, pickled vegetables, silage, beverages, plants, water, juices, sewage and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, all fermented milks, cassava (to produce gari and fufu), and most fermented vegetables. The production of this fermented food is still largely a traditional art associated with poor hygiene, inconsistent quality presentation and short shelf life which is due to acidity development. But the acidity development also plays a crucial role in majority of food fermentations by the impact of time duration and amount produced. Lactic acid bacteria are good organisms for lactic acid fermentation. The intention of the present investigation was to study growth and multiplication of lactic acid producing micro-organisms from different milk sources and to observe the differences in between these organisms specifically for acid production from different environments, temperatures, and pH etc.

2. MATERIALS AND METHODS

2.1 Sample Collection

Milk samples from cow, sheep, goat, camel and buffalo were collected from the surrounding area of Gwalior district of Madhya Pradesh, India. The sources of milk samples from cow, buffalo and goat were urban dairy farms while as sheep and camel milk samples were obtained from local milk suppliers. Raw milk samples were collected in sterile tubes and maintained in chilled condition (8-10^{o} C) during their transport to Microbiology Biotechnology Laboratory, Tropilite Foods Pvt. Ltd. Gwalior, for further analysis and research.

2.2 Isolation of Lactic Acid Bacteria

Serial dilutions of all raw milk samples in 0.1% peptone saline were used for microbial isolation on MRS (DE MAN, ROGOSA and SHARPE) and M17 media agar plates. Plates were incubated for 24 hours at 15º, 32º, 38º and 45ºC followed by picking the distinguishable colonies by sterile loop [9]. A total of 88 strains were isolated from five samples, 56 were observed coccus in chains and others were bacillus. Isolation methods followed were similar to those recommended by Van den Berg et al. [10]. With raw milk samples from cow, goat, sheep, camel and buffalo the isolates were divided into five groups from group I to group V starting from the sample of their origin respectively. All the isolates were further cultured to obtain purity. Purification of the isolates was confirmed by Gram Staining and pure isolated were maintained MRS agar plates at 4ºC.

2.3 Culture Identification

Gram Staining, Catalase activity, Sugar fermentation and growth in 5% NaCl was observed with all isolates [11], which leads the research on a way from where only 13 LAB strains with Gram positive and Catalase negative results were shortlisted for further analysis [12]. The

strains were named on the first letter of the source of their isolation. The Selected isolates were inoculated in MRS and M17 broth at pH 6.5 to check the maximum lactic acid productivity which was measured by pH droppage after incubation of 24 hours.

2.4 Lactic Acid Analysis

A 10ml sample was collected during fermentation aseptically produced from 100mg of sugar. Centrifugation of fermented broth samples was carried out at several intervals at 12000 RPM for 10 minutes and supernatant was analyzed by HPLC. The cultured medium was filtered through 0.45 µm membrane filter. Organic acids were analyzed by HPLC (Merck–Hitachi). Five microliters of the sample was injected into the HPLC system equipped with an Aminex HPX-87 H column and RI detector. The column temperature was maintained at 65ºC. The mobile phase was 10 mM H2SO4 at flow rate of 0.6 mL/min [13].

Isolates with tendency to ferment sugar into lactic acid efficiently were further allowed to multiply individually and concentrations of lactic acid productivity was determined (Table 2) by using HPLC and the yield was calculated by the equation (1).

$$Percentage\ yield\ = \frac{Grams\ of\ Lactic\ acid\ produced}{Grams\ of\ Total\ sugar\ consumed}\ X\ 100 \quad (1)$$

2.5 Genotypic Characterization

The genetic interrelationships of members of the lactic acid bacteria have been studied extensively in 16S rDNA sequence, and DNA-DNA hybridization experiments. Total genomic DNA of 13 strains was prepared by using the following procedure [14]. DNA (214 ng/µl) was subjected to PCR utilizing the primer 1 (AGA GTT TGA TCC TGG CTC AG) and primer 2 BOX A1R (CTA CGG CAA GGC GAC GCT GAC G) as Versalovic et al. [15] described. Each 27 µl PCR reaction contained 5 µl 5× Gitschier buffer (1 M (NH4)2SO4, 1 M Tris-HCl (pH 8.8), 1 M MgCl2, 0.5 M EDTA (pH 8.8) and 14.4 M β-mercapto-ethanol add double distilled water till 200 ml), 0.6 mg/ml BSA (Sigma, A-7906), 100% DMSO (Sigma, D-8418), 0.2 mM dNTP (Sigma, D7295), 0.5 µM oligonucleotide primer, 1 units of Taq DNA polymerase (Sigma, D1806) and distilled water. PCR amplifications were performed in a DNA thermal cycler with an initial denaturation step (95ºC, 7 min), followed by 30 cycles of denaturation (94ºC, 1 min), annealing (53ºC, 1 min) and extension (65ºC, 8 min), and a single final extension step (65ºC, 16 min). The amplified fragments were fractionated on a 1.5% w/v agarose gel during 200 min at a constant voltage of 40 V in 0.5×TAE (Tris-Acetat EDTA) at 4ºC. A 10-kb reference marker (Sigma, D7058) was used to allow standardization, followed by staining with ethidium bromide and visualization.

3. RESULTS AND DISCUSSION

3.1 Screening of Isolates

A total of 13 Isolates with high percentage of lactic acid production were analyzed according to the methods described in Bergey's Manual [16]. The cultures were examined microscopically by staining and morphological characteristics were noted. Cultural, Physiological and biochemical characteristics specifically for lactic acid bacteria were also studied [17]. Growth characteristics at different temperatures were also monitored on daily basis at 10ºC and 45ºC in MRS medium over 7 days.

Table 1. Morphological and Biochemical characterization of selected isolates

	Code of Isolates					
Characteristics	**C1**	**C2**	**C7**	**G1**	**G7**	**G22**
Cell Morphology	Cocci in chains	Short Rods	Cocci	Cocci in chains	Rods	Cocci
Gram staining	+	+	+	+	+	+
Spore Formation	-	-	-	-	-	-
Catalase Activity	-	-	-	-	-	-
Fermentation Type	Homo	Hetero	Homo	Hetero	Hetero	Homo
Glucose Fermentation	+	+	+	+	+	+
Nitrate Reduction	-	-	-	-	-	-
Casein Hydrolysis	+	+	+	+	+	+
Growth at 10^{o} C	-	-	-	-	-	-
Growth at 45^{o} C	+	-	-	+	-	+
Growth with 1.5% NaCl	+	-	+	-	-	+
Growth with 3% NaCl	-	-	-	-	-	-
Growth with 5% NaCl	-	-	-	-	-	-
At pH 4.2	-	-	-	+	-	-
`						Cont.

	Code of Isolates						
Characteristics	**S2**	**S5**	**S16**	**CAM3**	**CAM7**	**B4**	**B9**
Cell Morphology	Cocci in chains	Long Rods	Rods	Cocci	Short Rods	Cocci in bunches	Rods
Gram staining	+	+	+	+	+	+	+
Spore Formation	-	-	-	-	-	-	-
Catalase Activity	-	-	-	-	-	-	-
Fermentation Type	Hetero	Hetero	Homo	Hetero	Hetero	Homo	Hetero
Glucose Fermentation	+	+	+	+	+	+	+
Nitrate Reduction	-	-	-	-	-	-	-
Casein Hydrolysis	+	+	+	+	+	+	+
Growth at 10	-	-	-	-	-	-	-
Growth at 45	+	-	-	+	-	-	+
Growth with 1.5% NaCl	+	-	+	-	+	-	-
Growth with 3% NaCl	-	-	-	-	-	-	-
Growth with 5% NaCl	-	-	-	-	-	-	-
At pH 4.2	-	-	-	+	+	-	-

Table 2. Percentage yield of lactic acid production from selected LAB isolates

Code of Isolate	Source of Isolate	Percentage yield of Lactic acid production
C1	RAW COW MILK	31.45
C2	RAW COW MILK	27.66
C7	RAW COW MILK	22.13
G1	RAW GOAT MILK	82.66
G7	RAW GOAT MILK	56.34
G22	RAW GOAT MILK	72.91
S2	RAW SHEEP MILK	12.22
S5	RAW SHEEP MILK	17.23
S16	RAW SHEEP MILK	21.36
CAM3	RAW CAMEL MILK	34.67
CAM7	RAW CAMEL MILK	33.21
B4	RAW BUFFALO MILK	57.61
B9	RAW BUFFALO MILK	54.29

Table 3. Sugar fermentation results from different selected isolates

Isolate code	Sugar fermentation							
	Sucrose	**Lactose**	**Maltose**	**Dextrose**	**Ribose**	**Sorbitol**	**Arabinose**	**Mannose**
G1	+	+*	+	+	+	+	+	+
B4	+*	+	+	+		-	+	-
CAM3	+	+	+	+*	-	-	-	+
C1	+	+*	-	+	-	-	-	-
S16	+*	+	+	+	-	+	-	-

+ indicates the specific sugar involved in maximum acid production for particular isolate*

Salt tolerance capability of the strains was accessed with 1.5%, 3% and 5% concentrations followed by casein hydrolysis and nitrate reductase test (Table 1). On the basis of lactic acid production from the isolated strains, only high acid production efficient strains were targeted with at least one strain from each milk source to obtain a comparative data. For further analysis, strain C1 from cow milk sample, strain G1 from goat milk sample, S16 from sheep milk sample, CAM3 from camel milk sample and B4 from buffalo milk sample were selected for sugar fermentation and genotypic testing. It was found interesting to observe that strains isolated from goat milk showed high acid productivity compared to other milk sources. Strain G1 and G22 specifically target lactose as sugar source to convert into lactic acid by fermentation. While as strain B4, B9 and G7 use sucrose to efficiently produce lactic acid at favorable temperatures (Table 3).

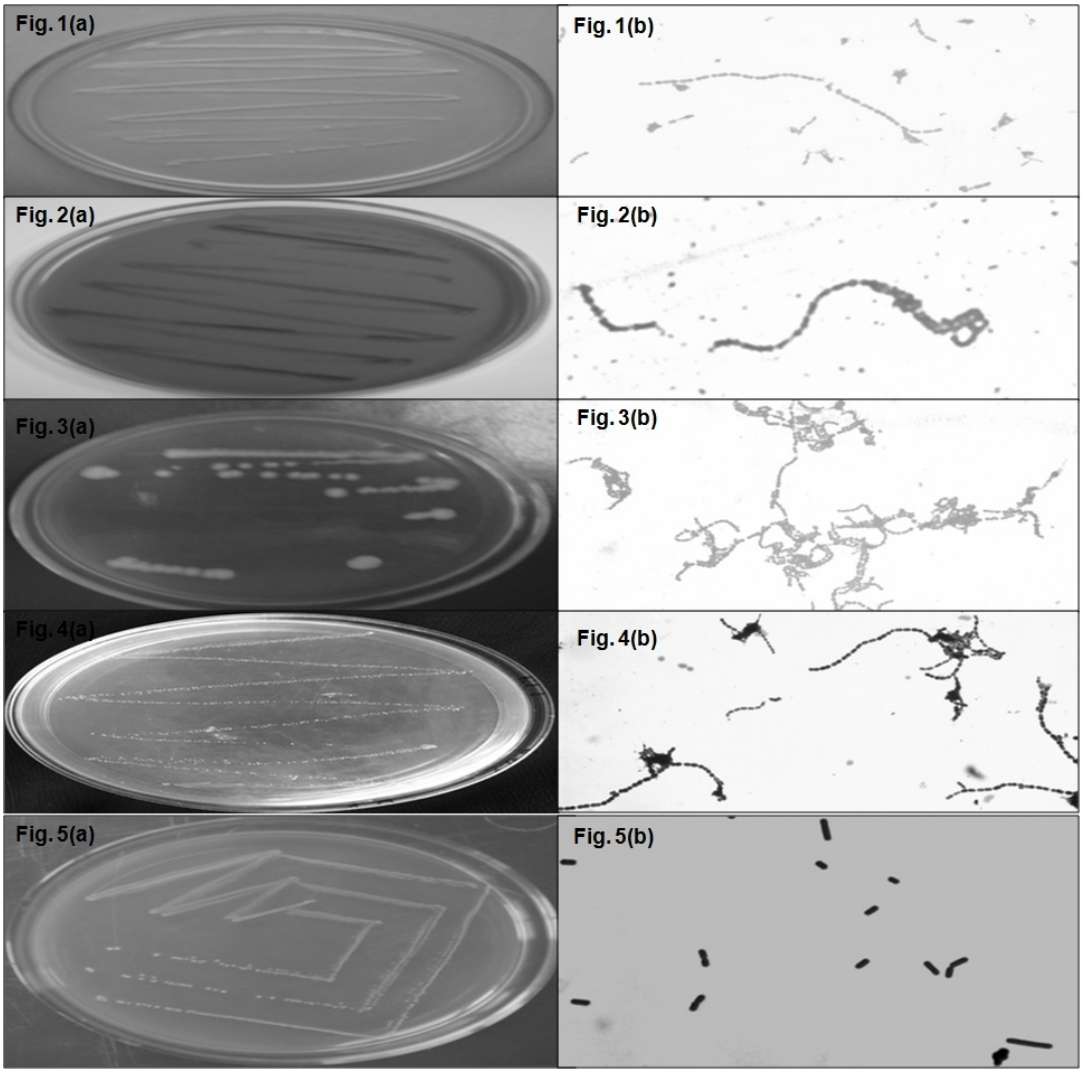

Fig. 1(a), 2(a), 3(a), 4(a) and 5(a) presenting the growth of G1, B4, CAM3, C1 and S16 on MRS agar plates and on the right side is the morphological presentation of the same strains respectively in figures 1(b), 2(b), 3(b), 4(b) and 5(b)

All the phenotypic testing which involves identification on the basis of physiological, biochemical and chemotaxonomic methods [18] are sufficient for preliminary identification and the results concluded from this identification were found enough to classify all the five strains G1, B4, CAM3, C1 and S16 (Fig 1(a)(b) to Fig 5(a)(b) respectively) under lactic acid bacteria group. But the fact remains that four out of five strains were found *Streptococcus* with homology in their fermentation and other characteristics, which creates difficulty in identifying closely related type strains.

The distribution of lactic acid bacteria species are related to molecular studies, therefore it was found necessary to demonstrate significant molecular characterization. Despite the wide application of lactic acid bacteria, they are currently differentiated by the determination of DNA-DNA similarity, their G+C content and, with more difficulty, by physiological characterization [19]. PCR amplification and subsequent sequencing of the 16S rDNA genes directly from selected isolates was done and the strains were identified on the basis of 16S rDNA homologies with entries in the GenBank NCBI databases (Table 4)

Table 4. Homology comparisons of isolated strains from database

Isolate code	Compared with database	
	Strain homology	**Percentage similarity**
G1	*Streptococcus thermophilus* strain ATCC 19258 16S ribosomal RNA, complete sequence	96%
B4	*Lactococcus lactis* strain IMAU40136 16S ribosomal RNA gene, partial sequence	98%
CAM3	*Streptococcus gallolyticus* UCN34 strain UCN34 16S ribosomal RNA, complete sequence	94%
C1	*Streptococcus thermophilus* MN-ZLW-002 strain MN-ZLW-002 16S ribosomal RNA, complete sequence	95%
S16	*Lactobacillus delbrueckii* strain SDa4-56 16S ribosomal RNA gene, partial Sequence	93%

4. CONCLUSION

Lactic acid obtained by biotechnological process is preferred for industrial applications, especially, bioplastic industry. Based on the current finding it was observed that the lactic acid bacteria strains isolated from goat milk are efficient in terms of lactic acid production in amassed quantities and fast acid producing also as compared to raw milk samples from cow, sheep, camel and buffalo. It was also observed that strains isolated from camel raw milk are comparatively more tolerant to acids. The microorganisms isolated from goat milk could therefore play an important role for the commercial lactic acid producing industries for the production of pure isomers of lactic acid.

COMPETING INTERESTS

Authors declare that there is no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=246&id=8&aid=1985