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Isolation, Screening and Characterization of Lactobacilli from Cow Milk

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***Abstract* – *Lactobacillus* species play a major role in fermented dairy products and also contribute to the therapeutic aspects of human health. Raw milk sample was used in this study to isolate and identify the lactobacillus and to find out the incidence of *Lactobacillus* in cow milk. Biochemical *i.e.* IMViC tests were used for identification of isolates of *Lactobacillus* from raw cow milk upto the genus level.**

From the *Lactobacillus* isolates, genomic DNA was extracted by using CTAB method. The size of the DNA was more than 20 Kb. The identification of *Lactobacillus* isolates by phenotypic methods is difficult. As it requires determination of bacterial properties beyond fermentation tests & time consuming therefore Polymerase Chain Reaction (PCR) – was used for identification of *Lactobacillus* at the genus level with genus specific primer. PCR showed positive reaction for isolates of Lactobacilli and amplicon size was 0.5 Kb.

***Keywords* – Cow Milk, DNA isolation, IMViC Test, Lactobacilli, PCR.**

I. INTRODUCTION

Lactobacillus species play a major role in fermented dairy products and food industry and also contribute to the therapeutic aspects of human health [1, 2].

Lactic acid bacteria are gram positive bacteria, non-spore-forming showing a DNA having G+C content less than 50 mol% and organisms of interest in food processing industries because of their typical roles in inhibiting the growth of food spoilage bacteria [3, 4, 5, 6]. Due to their Generally Regarded As Safe [GRAS] status, lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics [7, 8].

The identification of *Lactobacillus* isolates by phenotypic methods is difficult as it requires determination of bacterial properties beyond fermentation tests [9]. The time consuming and ambiguous classical methods used for lactobacilli classification based on phylogenetic and biochemical criteria do not always correspond to the actual genetic relatively of the species and are not discriminative enough to distinguish strains of the same species [10]. Since traditional methods can be time-consuming and often give rise to ambiguous results, more rapid and accurate identification methods are required [11].

Now a day the main focus for the identification has moved from phenotypic to genotypic methods as they yield more sensitive and accurate results, as reported for lactic acid bacteria by several authors [12, 13]. The development of PCR-based methods has opened new possibilities for clear and quick identification of lactic acid bacteria [14]. Nucleic acid template should be free from inhibitory substances for PCR based techniques which offer advantages in terms of sensitivity and specificity [15]. Milk and dairy foods are complex materials containing proteins, fats, salts; a fact that hampers DNA extraction. Although different methods have been reported for milk and dairy foods [16] none of these is ideal and in many cases a technique optimized for one food system or microorganism is not readily adaptable to others. The aim of this study was to find a specific primer for the identification of lactobacilli at the genus level.

II. MATERIALS AND METHODS

The research work was conducted in Department of Animal Biotechnology at K. K. Wagh College of Agricultural Biotechnology, Nashik, Maharashtra.

Isolation and Identification of Lactobacillus:

The samples of fresh milk were collected from local market of Nashik, Maharashtra (India) in sterilized screw capped bottles. Serial dilutions (0.1 ml) of these samples were spread on sterile petri dishes of MRS agar. Inoculation was carried out at 37 ˚C for 24 – 48 hours. Colonies showing typical characteristics of morphology such as color (buff, pale yellow, white), size (less than 1 mm diameter) and shape (biconvex) on MRS agar were selected and purified by streaking at least three times in MRS agar [17 & 18]. All colonies were maintained by weekly subculturing on MRS agar. The morphological characteristics of isolates were examined after staining by Gram stain [19]. It was then followed by motility test, catalase test, indole test, methyl red test, voges proskauer test, citrate test [15]. Morphological and Biochemical test were used for identification of bacteria and all results were compared with Bergey's manual of systematic bacteriology [20].

Isolation of genomic DNA from Lactobacillus:

The isolation of genomic DNA was carried out by the method given in [21] as follows: 1.5 ml of the culture in a micro centrifuge tube was spin for 2 min at 10.000 rpm & supernatant was discarded. The pellet was resuspended in 567µl TE buffer by repeated pipetting. 30 µl of 10% SDS and 3µl of 20 mg/ml proteinase K were added to give a final concentration of 100 µl/ml proteinase K in 0.5% SDS. It was then mixed thoroughly and incubated for1 hr at 37 °C. 100 µl of 5 M NaCl was added and mixed thoroughly. 80 µl of CTAB/NaCl solution was added & upon mixing incubated for 10 min at 65 °C. An

approximately equal volume (0.7 to 0.8 ml) of chloroform: isoamyl alcohol was added, mixed and spined at 12000 rpm for 4 to 5 min in a microcentrifuge. Aqueous viscous supernatant was removed to a fresh microcentrifuge tube leaving the interface behind. Equal volume of phenol: chloroform: isoamyl alcohol was added and spined at 10000 rpm in a microcentrifuge for 5 min. The supernatant was transferred to a fresh tube. Chilled isopropanol (0.6 vol) was added to precipitate the nucleic acids DNA and was washed with 70% ethanol to remove residual CTAB and re-spine 5 min at room temperature to repelleted it. Carefully supernatant was removed and the pellet was dried. The pellet was redissolved in 100 µl TE buffer.

Qualitative and quantitative assessment of DNA:

DNA concentration was determined by recording the absorbance at 260 nm (A_{260}) using a biospectrophotometer (Eppendorf, USA). The purity of DNA was determined from A_{260}/A_{280} ratio. The quality of the isolated DNA was also evaluated by 0.8 % agarose gel electrophoresis. The type of band pattern indicates the quality of the DNA isolated [18].

Polymerase chain reaction analysis:

All PCR reactions were done in gradient cycler (BioEra, Pune). PCR analysis with *Lactobacillus* genus specific primers shown in Table 1. The PCR reaction was performed with 25 µl of reaction mix containing 2.5 µl of PCR reaction buffer, 1.5 µl $MgCl_2$, 2.0 µl dNTP mix, 1 µl Forward primer, 1 µl Reverse primer, 0.5 µl *Taq* DNA Polymerase, 1.0 µl Template DNA, 15.5 µl Nuclease free water. The reaction mixture was subjected to an initial denaturation at 94 ^oC for 4 min., then denaturation at 94 ^oC for 45 sec, annealing at 56 ^oC for 45 sec, primer extension at 72 ^oC for 1 min. and final extension at 72 ^oC for 5 min. The cycle was repeated 29 times.

The PCR profiles were visualized after staining with ethidium bromide under UV transilluminator and later images were saved using Uvitech Gel doc. A DNA molecular weight marker was used to evaluate the weight. To assess the reproducibility of the genus specific PCR procedure three separate trails starting from the same DNA preparation and using the same PCR reagents were performed.

Table 1: List of the genus specific primers used in the identification of lactobacilli

S. No.	Primer Name	5'-3' Sequence
1	LF 1	5'-GTA AGG TGG CGA TGT GTA CCT C-3'
2	LR 1	5'-CAC CGC TAC ACA TGG AG-3'

III. Results

Isolation of Lactobacillus

Raw milk sample was spread on MRS media and after incubation, the bacterial colonies are observed on plates as shown in Fig. 1.

Fig.1. Bacterial Colonies observed on MRS plates.

Morphological and biochemical properties

The isolated bacteria were characterized using the morphological and biochemical tests. The results are shown in Table 2. These results were compared with Bergey's manual of Bacteriology and isolated bacteria was characterized as *Lactobacillus* genus.

Table 2: Results of morphological and biochemical tests of isolated bacteria.

Characteristic	Result
Color	Yellow
Size	< 1mm
Shape	Round
Gram staining	Positive
Motility	Non motile
Indol test	Negative
Methyl Red test	Negative
Voges Proskauer	Positive
Citrate	Negative
Catalase	Negative

DNA yield and quality

Chromosomal DNA was isolated from lactobacillus isolates by CTAB method and estimated spectrophotometrically by measuring the absorbance at 260 nm (A_{260}). The amount of DNA isolated from these isolates was 39.8 µg/ml and the ratio of A_{260}/A_{280} was found to be 1.7. The integrity of the purified genomic DNA was also analyzed by agarose gel electrophoresis. Intact high molecular weight of DNA was found to be greater than 20 Kb. This confirms that the purified genomic DNA from gram positive *Lactobacillus* is of a high quality.

Genus specific PCR from lactobacillus isolates

DNA was amplified with genus specific primers LF 1 and LR 1. 25 mM $MgCl_2$ was used in PCR mix. The amplicon size was found to be 500 bp (0.5kb).

IV. Discussion

In the present study, cow milk was used for *Lactobacillus* isolation and morphological & biochemical characteristics were used for the identification. Results were compared with Bergeys manual of Bacteriology & isolates were characterized as *Lactobacillus* genus. Similarly [4] isolated & identified from raw cow milk, white cheese and rob in sudan and they found 3 genera *i.e.*

Lactobacillus, Lactococcus & Pediococcus. In the present study, genomic DNA was isolated by using CTAB method. The concentration of DNA was 39.8 µg/ml and absorbance at 260 nm/280nm ratio of DNA sample was 1.7. Isolated DNA was used for molecular identification. For that *Lactobacillus* genus specific primers LF 1 and LR 1 were used and PCR showed positive results. The amplicon size was found to be 0.5 Kb & it was concluded that isolate was *Lactobacillus*. Similar findings were reported by using raw milk sample and the amplicon size was found to be 0.4 Kb for genus specific markers [1].

V. CONCLUSION

In this study, Lactobacilli were identified at molecular level and the rapid identification of *Lactobacillus* was possible due to polymerase chain reaction [PCR] method. The development of PCR based methods has opened new possibilities for clear and quick identification of lactic acid bacteria.

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