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Research Article

Evaluation of modified MRS media for the selective enumeration of *Lactobacillus casei*

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Abstract

Lactobacillus is an extensive group of Gram-positive, catalase negative and non-spore forming organism belongs to Lactobacillaceae family. *L. casei* is an obligate fermentative bacterium with complex nutritional requirements. It has its novel importance as a bio-preservative agent and enhancing food quality. *Lactobacillus casei* is Generally Recognized as Safe (GRAS). For the isolation and identification, cultural media is one of the most important tools and the methods to distinguish between *Lactobacillus casei* and *Lactobacillus lactis* are based upon various biochemical tests. In general, MRS media is used for the isolation of Lactobacillus species but we have developed the modified selective medium that utilizes the calcium gluconate. Modified media involves a pH indicator, differences in pH can be observed by means of detectable color change. An intervention study was conducted to confirm the capability of modified MRS medium to detect *L. casei* and *L. lactis* recovered from dairy samples. We were able to enumerate circular medium sized predominant yellowish colored colonies morphologically similar to *Lactobacillus casei* while the growth of *Lactobacillus lactis* was inhibited. These colonies were then subjected to PCR to identify *L. casei*.

Keywords: GRAS*; L. casei*; *L. lactis*; MRS; PCR

Introduction

Fermented milk products are made of milk under controlled conditions for producing flavor and acidity at desired level. Organisms used in fermentation mostly belong to Lactic Acid Bacteria (LAB). LAB have specific metabolic, physiological and morphological characteristics. Lactic Acid Producing Bacteria most commonly present in nature as well as necessary organisms in raw milk and fermented yogurt. LAB are Gram positive organisms playing important role in the fermentation of food products. LAB usually are non-sporulating and non-motile organisms which produce lactic acid as an important end product **[1]**. Production of lactic acid is the most important quality of LAB because they prevent the attachment, proliferation and settlement of harmful bacteria. Many components are compulsory for the growth of LAB like nucleic acid, carbohydrates, peptides, vitamins and amino acids **[2]**. Among lactic acid forming organisms *Lactobacillus* are the most dominant organisms consist of various Gram positive, rod shaped, non-pigmented, non-spore forming, microaerophilic to strictly anaerobic organisms **[3-5]**.

Lactobacillus casei (*L. casei*) is considered as the main habitat of gut microflora and is present in the variety of commercial food products that are fermentative in nature

[6]. *L. casei* has shortest growing time among all organisms belongs to Lactobacillus genus [7]. There is no selective media present for the isolation of entire LAB. Isolation media may be altered by change in concentration of reagents which are inhibitory in nature, modifying pH value, adjustment in temperature and time. MRS medium is well known medium for culturing LAB having pH 5.7 to 6.2 [8].

The objective of this research was to optimize the chemically defined medium and to test the suitability of modified MRS medium for the selective enumeration of *L. casei.* The efficacy of modified MRS medium for the selective enumeration of *L. casei* was assessed using commercial yogurt and fermented milk obtained from local supermarkets.

Materials and Methods

Isolation of Lactobacillus

For the isolation of Lactobacillus, commercial yogurt and dairy samples were collected from local market. Samples were serially diluted as reported by Noori and Jafari [9]. Test tubes were labeled with dilution factors as 10^{-1} and 10^{-2}. In each test tube 4.5 ml of normal saline was added. With the help of sterilized pipette, 0.5ml of sample was inoculated in test tube (labeled with 10^{-1}) and mixed thoroughly. To make dilutions 0.5ml of sample from 10^{-1} test tube was shifted to the next test tube, labeled as 10^{-2}. Both test tubes were incubated at 37^{o}C anaerobically (in anaerobic jar) for 48 hours. Commercial MRS agar was rehydrated in distilled water according to manufacturer's instruction. It was divided into portions of 200ml in a flask of 250ml capacity and sterilized in an autoclave at 121^{o}C for 15 minutes at 15psi. Agar was cooled down to 45^{o}C, poured in sterilized petri plates and allowed to solidify.

Enumeration of bacteria

The enumeration was carried out using pour plate technique on MRS medium and anaerobic jar was used for creating anaerobic conditions for 48 hours at 37^{o}C. Plates with 30-300 colonies were enumerated and recorded as colony forming units (CFU/ml) of the bacterial culture.

Identification of isolates

Colonies were observed macroscopically which were obtained after 48 hours of incubation. Cultural examination included texture, size, edges and colony color. Microscopic characterization was done after Gram's staining and Lactobacillus was observed as Gram positive rods. For further identification biochemical profiling was done by citrate test, catalase test, Methyl red test and Voges Proskauer test. *Lactobacillus casei* and *Lactobacillus lactis* were used in this study. *L. casei* was used to optimize the composition of modified MRS medium while the *L. lactis* was used to validate the effectiveness of modified medium.

Modification of MRS media

Modifications in MRS media were carried out to improve its selectivity and specificity for the enumeration of *L. casei.* For this, glucose was substituted with Calcium gluconate and several concentrations were tested (0.5, 1.0, 1.5, 2.0 g/100ml) at 37^{o}C temperature with *L. casei* and *L. lactis*. Media was prepared by the following method (Table 1).

Different concentrations of calcium gluconate were added (5g, 10g, 15g, 20g/L) in the above composition for the preparation of modified MRS medium. The pH was adjusted to 7.0 ± 0.2 by using 1 *M* HCl and 10 *M* NaOH. The medium was autoclaved for 15 minutes at 121^{o}C before pouring. Previously isolated and identified fresh colonies of *L. casei* and *L. lactis* were inoculated on different concentration of the modified MRS medium and incubated at 37^{o}C anaerobically for 48 hours.

Results

Enumeration of bacteria on modified MRS medium

Experiments were carried out to compare the growth of *L. casei* and *L. lactis* on the modified culture media at different

concentrations. Significant white colonies with irregular edges of *L. casei* were observed on the modified MRS medium (Fig. 1) containing 1.5% calcium gluconate as compared to 0.5, 1.0 and 2.0% concentration/100ml of the medium while all the concentrations inhibited the growth of *L. lactis* which was unable to ferment calcium gluconate (Fig. 2). The DNA extraction and PCR based identification was carried out on all the isolates of *L. casei*.

Table 1. Preparation of Modified MRS media

Ingredients	**g/L**
Peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Polysorbate 80	1.0 ml
Ammonium Citrate	2.0 g
Sodium acetate	5.0 g
Magnesium Sulfate	0.1 g
Manganese Sulphate	0.05 g
Di-potassium Phosphate	2.0 g
Agar	15.0 g
Phenol red	indicator

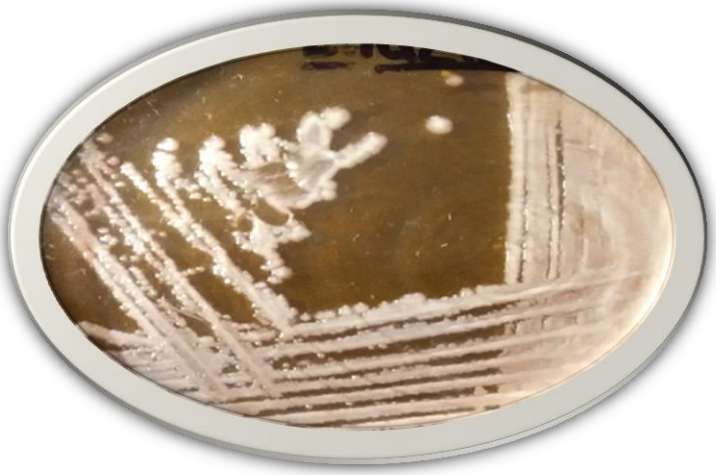

Figure 1. Growth of *Lactobacillus casei* on modified MRS media

Figure 2. Growth pattern of *Lactobacillus casei* on one side while the inhibition of *Lactobacillus lactis* on other side of the modified MRS media

Molecular identification of the isolates

Aliquots of 10 ng of DNA extracted from the pure culture of each isolate of *L. casei* (from modified MRS medium plates) were subjected to molecular identification by PCR using 16sRNA primers. F: TGGATCACCTCCTTTCTA, R: GTGCGCCCTTTATTAACTT.

Thermocycling described by (Sajid *et al*, 2020) in 50µl reaction mixture containing 25µl of master mix, 2µl of each primer, 5µl of genomic DNA and 18µl of nuclease free water. Random amplification was performed using PCR conditions of initial denaturation at 94^{o}C for 4 minutes, followed by 30 cycles consisted of denaturation at 94^{o}C for 30 sec, annealing at 56^{o}C for 30 sec, extension at 72^{o}C for 45 sec and final extension at 72^{o}C for 7 minutes [10]. The resulted bands of 450bp were observed through gel documentation system after electrophoresis and *Ethidium Bromide* staining (Fig. 3).

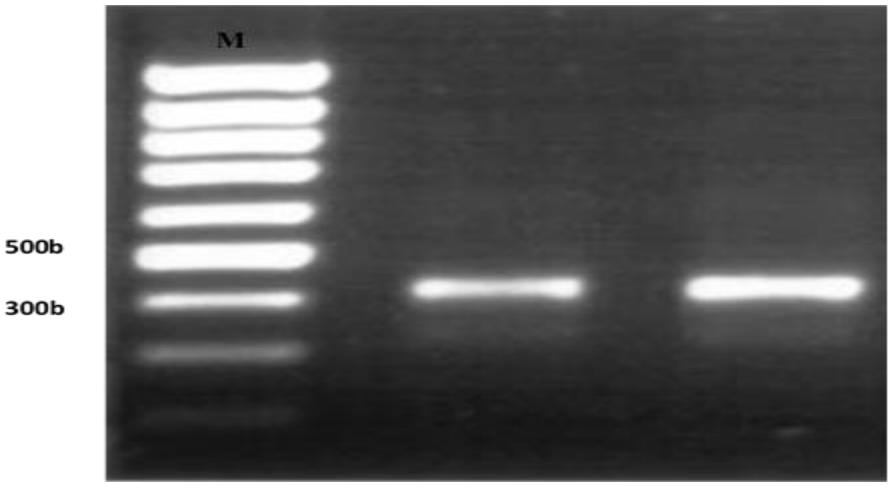

Figure 3. Molecular characterization of *L. casei* showing 450bp band after PCR amplification

Discussion

LAB is a fundamental group of bacteria having Gram positive property and catalase negative in nature. They produce major end product which is lactic acid and ferment carbohydrates. Lactobacilli are the most authoritative organisms in human nutrition and food microbiology. They contribute in fermentation of food products and commonly known as probiotic. According to taxonomy, Lactobacillus comes under family called Lactobacillaceae [11]. The culture of Lactobacillus is important for commercial purposes include dietary adjuncts and fermentative starters [12].

In this study, we modified the MRS medium for the reliable and selective enumeration of *L. casei*. Initially we assay *Lactobacillus casei* and *Lactobacillus lactis* using MRS medium. The MRS medium was chosen since it is widely recognized as an optimal medium for the growth and enumeration of Lactobacilli under both aerobic and anaerobic conditions, due to its nutritional components and acidity. On the basis of previous studies, MRS agar exhibits a low capability of discrimination among *L. casei* and *L. lactis*. To improve the selectivity of this medium, we have modified its formulation by substituting the glucose with calcium gluconate to void the growth of *L. lactis*. The selectivity of this modified medium was tested at different concentrations of calcium gluconate with the isolated colonies of *L. casei* and *L. lactis* anaerobically. Thus, modified MRS with 1.5% calcium gluconate allows the optimal growth of all the isolates of *L. casei* at 37^{o}C after 48 hours of incubation anaerobically but none of the *L. lactis* could grow in any concentration of calcium gluconate containing modified MRS medium. The concentration more than 1.5% may have deleterious effects even on the growth of *L. casei.*

Different types of bacteria which have ability to gain energy in the form of organic molecules which are not usual nutrients for

the huge group of bacteria. For instance, *L. casei* and enterococci grow best in the presence of malate, pentitols and calcium gluconate. These organisms contain specialized and novel type of system called phosphotransferase system which metabolizes these substrates.

Conclusion

Initially *Lactobacillus casei* and *Lactobacillus lactis* was isolated using MRS medium but MRS agar exhibits a low capability of discrimination among *L. casei* and *L. lactis.* To improve the selectivity of this medium, a modified MRS medium with calcium gluconate was used. Modified MRS with 1.5% calcium gluconate allows the optimal growth of all the isolates of *L. casei* at 37^{o}C after 48 hours of incubation anaerobically but none of the *L. lactis* could grow in any concentration of calcium gluconate.

Authors' contributions

Conceived and designed the experiments: SU Rahman, Performed the experiments: I Farzand, Analyzed the data: S Sajid, Contributed materials/ analysis/ tools: S Nayab, Wrote the paper: I Farzand & S Sajid.

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