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Research Article

DETERMINATION OF MORPHOLOGICAL, PHYSIOLOGICAL, BIOCHEMICAL AND FERMENTATIVE PROFILES OF LACTIC ACID BACTERIAL ISOLATES FROM HORSE GRAM (*Macrotyloma uniflorum*)

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Abstract- The present study aimed at isolation and identification of lactic acid bacterial isolates from horse gram. A total of 13 isolates were considered as presumptive lactic acid bacteria after screening for Gram positive, catalase negative, nonmotile and asporogenous. They were further screened for physiological and biochemical characteristics. Results revealed that, majority of the isolates (76.92 %) were cocci shaped, with varied arrangements like independent, pairs / short chains, while only 23.07% of isolates were rod shaped. Based on their physiology, all isolates were confirmed as mesophilic in nature with optimum growth at 4.0 -6.5 pH and 2- 4% NaCl concentration. Biochemical characterization disclosed that three of isolates showed dextran producers, 84.6 % of them performed mixed acid fermentation and three isolates showed acetoin production, 23.07 % hydrolyzed starch, 30.76 % hydrolyzed gelatin and none of them could hydrolyze lipid. Finally, isolates were studied for sugar fermentation profiles and results confirmed that majority of them could metabolize diversified sugars such as galactose, rhamnose, glucose, lactose, sucrose, mannose and trehalose. Only 46.15 % isolates fermented sorbitol and none of them utilized xylose.

Keywords- *Horse gram, Lactic acid bacteria, Catalase reaction, Biochemical characterization, Sugar fermentation*

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Introduction

Pulses are considered as one of the cheapest sources of proteins for lower income groups and for vegetarians. They are known to have good amounts of functional and bioactive substances, have been frequently reported to improve human health by preventing / curing various diseases. Although they are proven with nutraceutical properties, only a few of them are commercially popularized, leaving remaining as undervalued. The Horse gram (*Macrotyloma uniflorum*) is one of such undervalued pulse crops with high protein, minerals and vitamin content compared to other commonly consumed legumes [1]. In spite of nutritional significance and medicinal properties, still its usage is restricted due to poor cooking quality and presence of antinutritional factors that interfere with mineral bioavailability and protein digestibility [2]. Though different processing techniques such as soaking, roasting, cooking and germination are available at domestic level, fermentation is proven to be the efficient way in terms of reducing antinutritional factors and improves nutritional quality of product, thereby enhancing texture, flavor and taste compared to its raw form [3].

Pulse based fermented foods are ubiquitous and consumed for decades in developing nations. They are quite popular with some being served as breakfast recipes, snacks, meal or spices in Indian sub-continent [4]. The microbial intervention may be natural or by using starter cultures. The common microbial groups involved in pulse-based fermentations are bacteria, fungi (possibly yeasts), may act parallel or sequentially. However, lactic acid bacteria (LAB), are reported to be dominant microflora in these fermentations which is heterogeneous, fastidious and diversified group having probiotic properties [5,6]. The growth characteristics, functionality and activity of these bacteria are going to be varied depending upon type of substrate and physico-chemical environment as well. Majority of these fermented foods are involved with indigenous or traditional preparations, successful at domestic level, but they are not commercialized so far except for a few foods like tempeh and dhokla *etc* [7]. Since, there is a huge demand from consumers for novel pulse based fermented foods and on the other hand, there is no scientific information available on lactic acid bacterial population from horse gram, an attempt was made to isolate, identify and determine biochemical properties of lactic acid bacteria from horse gram to develop a fermented product of horse gram.

Material and Methods

Sample collection

Horse gram (*Macrotyloma uniflorum*) seeds were purchased from National Seed Project (NSP), GKVK, Bangalore and were thoroughly washed, cleaned and dried before analysis.

Isolation and purification of Lactic acid bacteria

A known quantity (10 g) of presoaked horse gram seeds were transferred to 100 ml sterile saline (0.85 %), homogenized and a 10 ml aliquot from this suspension was serially transferred to the next saline blanks to get 10-5 dilution. Aliquots (1ml) of each dilution was transferred to sterile Petri plates and de Man Rogosa Sharpe (MRS) agar was transferred and the plates were incubated at 35°C for 48h. The colonies with different morphology were randomly selected and streaked on MRS agar and the pure bacterial isolates obtained after successive transfers were preserved in 20 % glycerol stocks.

Preliminary identification of lactic acid bacteria

Initially, the pure isolates were tested for gram reaction, motility, catalase activity and endospore formation [8].

Table-1 *Morphological characteristics of lactic acid bacterial isolates [+ Positive, - Negative]*

Isolates	Colony Characteristics			Morphological Characteristics		
	Size	Shape	Color	Cell shape	Cells arrangement	Endospore formation
HLB-1	Medium	Round	Creamy white	Cocci	Independent	-
HLB -2	Small	Round	Dull white	Cocci	In pairs	-
HLB -3	Tiny	Round	Creamy white	Cocci	In pairs	-
HLB -4	Large	Round	Creamy white	Bacilli	Independent	-
HLB -5	Small	Round	Dull white	Bacilli	Chain form	-
HLB -6	Tiny	Round	Milky white	Cocci	Independent	-
HLB-7	Large	Round	Milky white	Cocci	In pairs	-
HLB -8	Tiny	Round	Dull white	Cocci	In groups	-
HLB-9	Small	Round	Milky white	Cocci	Independent	-
HLB-10	Small	Round	Dull white	Bacilli	Independent	-
HLB-11	Medium	Round	Creamy white	Cocci	In chains	-
HLB-12	Small	Round	Milky white	Cocci	In pairs	-
HLB-13	Tiny	Round	Milky white	Cocci	In pairs	-

Table-2 *Growth of lactic acid bacterial isolates at different temperature, pH and NaCl concentrations [+ Positive, - Negative]*

Isolates	Temperature (°C)				pH			NaCl concentration (%)		
	15	30	45	50	2.5	4.0	6.5	2.0	4.0	6.5
HLB-1	+	+	-	-	-	-	+	+	+	-
HLB -2	+	+	-	-	-	-	+	+	+	-
HLB -3	+	+	-	-	-	-	+	+	+	-
HLB -4	+	+	-	-	-	+	+	+	+	-
HLB -5	-	+	+	-	-	+	+	+	-	-
HLB -6	-	+	+	-	-	+	+	+	+	-
HLB-7	+	+	-	-	-	+	+	+	+	-
HLB -8	+	+	-	-	-	-	+	+	+	-
HLB-9	+	+	-	-	-	+	+	+	+	-
HLB-10	+	+	-	-	-	-	+	+	+	-
HLB-11	-	+	+	-	-	+	+	+	-	-
HLB-12	+	+	-	-	-	+	+	+	+	-
HLB-13	+	+	-	-	-	+	+	+	+	-

Table-3 *Biochemical characterization of Lactic acid bacterial isolates [+ Positive, - Negative]*

LAB isolates	Gram Reaction	Catalase	Starch Hydrolysis	MR	VP	Dextran production	Gelatin Hydrolysis	Lipid Hydrolysis	Casein Hydrolysis
HLB-1	+	-	-	+	-	-	-	-	-
HLB -2	+	-	-	+	-	-	-	-	-
HLB -3	+	-	-	+	-	+	+	-	-
HLB -4	+	-	-	+	-	-	-	-	-
HLB -5	+	-	-	+	-	-	-	-	-
HLB -6	+	-	-	+	+	-	-	-	-
HLB -7	+	-	-	+	-	-	-	-	+
HLB -8	+	-	+	-	+	-	+	-	-
HLB-9	+	-	-	-	+	-	-	-	-
HLB-10	+	-	+	+	-	+	+	-	+
HLB-11	+	-	-	+	-	-	+	-	-
HLB-12	+	-	+	+	-	+	-	-	-
HLB-13	+	-	-	+	-	-	-	-	+

Table-4 *Sugar fermentation profiles of Lactic acid bacterial isolates [+ Positive, - Negative]*

LAB isolates	Lactose	Galactose	Trehalose	Mannose	Sucrose	Fructose	Sorbitol	Rhamnose	Xylose
HLB-1	+	+	+	+	+	+	-	+	-
HLB -2	+	+	+	+	+	+	+	+	-
HLB -3	+	+	+	+	+	+	-	+	-
HLB -4	+	+	+	+	+	+	-	+	-
HLB -5	+	+	+	+	+	+	+	+	-
HLB -6	+	+	+	+	+	+	+	+	-
HLB -7	+	+	+	+	+	+	+	+	-
HLB -8	+	+	+	+	+	+	+	+	-
HLB-9	+	+	+	+	+	+	+	+	-
HLB-10	+	+	+	+	+	+	-	+	-
HLB-11	+	+	+	+	+	+	-	+	-
HLB-12	+	+	+	+	+	+	-	+	-
HLB-13	+	+	+	+	+	+	+	+	-

Motility test

The motility of the isolates was checked by hanging drop technique [9]. It was performed by adding a drop of fresh culture on cover slip (smeared with adhesive) and was placed on cavity slide, so that drop should hang freely in cavity. These slides were observed under microscope for motility.

Endospore staining

Endospore staining of isolates was done by [10] using 30days old culture. The smears were prepared, dried, kept on hot water bath and flooded with malachite green (0.5% aqueous solution) frequently to avoid drying of slides. Then, slides were washed and counter stained with safranin for 30 seconds and observed under microscope.

Catalase activity
A loop full of each bacterial isolate was placed on sterile glass slide followed by addition of hydrogen peroxide (3%). The production of effervescence indicates positive result for the test [11].

Physiological characterization
The presumptive lactic acid bacterial isolates were further screened for their growth at different temperatures (10, 15, 40 and 50°C), NaCl concentrations (2 %, 4 % and 6.5 %) and pH levels (2.5, 4.5 and 6.5). There were frequently used criteria for classification of cocci, belonging to LAB group [12].

Biochemical characterization

Dextran production
The dextran production capacity of isolates was checked using sucrose agar medium. The isolates were streaked on sterile sucrose agar plates, incubated for three days and observed for presence of mucoid secretions on agar surface [13].

Acid and gas production
The bacterial isolates were inoculated into sterilized glucose broth tubes with Durham's tubes inserted in an inverted position and phenol red was used as pH indicator. The tubes were incubated for 48h and observed for color change and gas accumulation in Durham's tubes [14].

Methyl Red (MR) and Voges-Proskauer's (VP) test
The bacterial isolates were inoculated to MRVP broths and incubated at room for 48h. After incubation, five drops of Methyl red indicator were added and observed for color change. Acetoin production was tested by adding Naphthol and 40 % potassium hydroxide. Development of pink color indicates positive result [15].

Gelatin Hydrolysis
Nutrient gelatin stabs were inoculated with isolates and incubated at room temperature for 4 days, followed by placing them in refrigerator at 4°C for 15 min. The gelatin tubes with liquefaction were considered as positive and tubes remained in solid state as negative [16].

Starch hydrolysis
The starch agar plates were inoculated with isolates and incubated for 48 h. After incubation, plates were flooded with iodine solution wherein, the presence of clear zone around the line of growth was confirmed as positive for the test [9].

Casein hydrolysis
All the isolates were inoculated on skimmed milk agar medium and incubated at room temperature for 48 h. A clear zone around the colony was considered as positive for extracellular caseinase production [9].

Lipid hydrolysis
The test was performed by inoculating isolates on tributyrin agar medium plates and the presence of transparent clear zone around the colonies was indicated as positive result [9].

Carbohydrate fermentative profiles of lactic acid bacterial isolates
All the bacterial isolates were inoculated in MRS broth tubes with respective sugars (Glucose, lactose, sucrose, mannose, galactose, trehalose, rhamnose, xylose and sorbitol) and 0.01 % phenol red indicator and incubated for 48h. The change in broth color from red (neutral) to yellow (acidic) indicates the acid production [17].

Results and Discussion
The bacterial isolates from horse gram were further subjected to biochemical characterization and sugar fermentation profiles and the lactic acid bacteria were identified by comparing with Bergey's manual and published research data.

Isolation and purification of lactic acid bacteria
The isolation of Lactic acid bacteria from horse gram was attempted by following routine microbiological procedure using MRS agar medium, which has been reported earlier as the best suitable medium [18]. Majority of them were found to be subsurface and a few were colonized on surface of agar medium. Initially, a total of 18 isolates were observed with distinguishable colony characteristics, which were purified and preserved after repetitive sub culturing. The isolates varied in size from tiny to large and color from creamy white to dull white [Table-1].

Preliminary identification of lactic acid bacteria
In general, lactic acid bacteria were reported as Gram+ve, non-motile and catalase -ve [19]. Based on this, the pure isolates were screened for morphological characterization, where 13 isolates were Gram+ve, non-motile and catalase -ve.
In the present study, Gram staining differentiated ten isolates as *Lactococcus* genus and three isolates as *Lactobacillus* genus. However, the isolates were with varied cell arrangement from independent cocci / bacilli, in pairs, groups or in short chains [Table-1]. These 13 isolates were considered as presumptive lactic acid bacteria and were further studied for physiological and biochemical characteristics.

Physiological characteristics
The growth of bacterial isolates at different temperatures classified all cocci (10) into mesophilic group, they could grow between 15 and 30°C and was ceased to multiply between 45 and 50°C. Among bacilli, growth was observed at 30 to 45°C [Table-2]. All the isolates grew at pH of 6.5, whereas some of them grew at pH of 4.0 and none of the isolates grew at 2.5. All the 13 isolates exhibited growth at 2 % and 4 % NaCl (except HLB-5, HLB-11) and no growth was observed at 6.5 % salt concentration. The results obtained in the present study are in accordance with the published data [19,20].

Biochemical characterization
The lactic acid bacterial isolates were further studied for biochemical characteristics [Table-3]. All the isolates fermented glucose with acid production and no gas formation confirming them as homofermenters, except HLB-12 which is a heterofermenter. Out of all isolates, three (HLB-3, HLB -10 and HLB-12) were dextran producers, three (HLB-3, HLB-10 and HLB-12) hydrolyzed starch, four hydrolyzed gelatin (HLB-3, HLB-8, HLB-10 and HLB-11), three hydrolyzed casein (HLB-7, HLB-10 and HLB-13) and none of them had lipolytic activity. Isolates (11) were positive for Methyl red test indicating that they could perform mixed acid fermentation, while 3 isolates showed positive for Voges-Proskauers test confirming the acetoin production.

Sugar fermentation profile
The isolates were found to possess diversified sugar fermentation capabilities, wherein they were proven to be capable of fermenting glucose, lactose, sucrose, mannose, trehalose, galactose and rhamnose with acid and no gas production [Table-4]. In case of sorbitol, seven isolates (HLB-2, HLB-5, HLB-6, HLB-7, HLB-8 and HLB-9 and HLB-14) showed positive results with acid production which were in accordance with results obtained by [20], while no bacterial isolate was found to ferment xylose.

Conclusion
The present study was attempted to isolate and identify lactic acid bacteria from horse gram. Initially, a total of 18 lactic acid bacterial isolates were obtained based on cultural characteristics. Out of them, a total of 13 isolates were Gram +ve, catalase -ve and non-endospore formers. The Gram stain results identified the bacterial isolates up to genus level as *Lactococcus* (10) and *Lactobacillus* (3). Further, the physiological and biochemical characteristics revealed that they were mesophilic homofermenters with mixed acid / acetoin production and some of them identified with dextran production, starch, gelatin and casein hydrolysis and were capable of utilizing diversified sugar sources.

Application of research: Studies related to Pulse based fermented products developed by using Lactic acid bacteria.

Research Category: Fermentation Microbiology

Abbreviations: LAB-Lactic Acid Bacteria, MRS- de Man Rogosa Sharpe Agar

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Cultivar/ Variety Name: Horse gram (*Macrotyloma uniflorum*)

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