SCREENING ON PROTEOLYTIC ACTIVITY OF LACTIC ACID BACTERIA FROM VARIOUS YOGURTS AND FERMENTED MILK

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Abstract- Proteases are necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Proteases are the key enzymes in industrial application. Microbial protease plays an important role in biotechnological process. In this paper the focus of this study is screening on Proteolytic activity of lactic acid bacteria from various yogurts and fermented Milk. Lactic acid bacteria were isolated from various yogurts and fermented milk by culturing on specific media and pure culture was obtained by sub-culturing. A total of 13 isolated strains was confirmed by Gram's staining and identified by different biochemical tests and their ability to ferment different carbohydrates. They were screened for proteolytic activity on skim milk agar by the agar – well diffusion method. All of isolated strains showed the proteolytic activity. Among them, five isolated strains namely, L_1, L_2, L_6, L_7, L_9 showed the widest clear zones formation after incubation time 24 hrs, 48 hrs, 72 hrs at 37°C. The result obtained in the present study indicated that the widest clear zones were produced at 48 hrs and 72 hrs of incubation period at 37°C. Thus, these bacterial strains were selected for further study.

Index Terms- lactic acid bacteria, protease, proteolytic activity, Yogurt, skim milk

I. INTRODUCTION

Proteases constitute one of the most important groups of enzymes both industrially academically. Proteases are the enzymes that hydrolyse proteins by addition of water across peptide bonds and catalyse peptide synthesis in organic solvents with low water [8]-[4]. Proteases are essential constituents of all forms of on earth, including bacteria, fungi, actinomycetes, plant and animals. Proteases are classified according to their structure or the properties of the activity site such as serine, metallo, carboxyl, acidic, neutral, and alkaline [6]. They are generally used in detergents [4]-[5]-[7] food industries [2]-[5] meat processing, cheese making, silver recovery from photographic film [3]-[5]-[7] production of digestive and certain medical treatments of inflammation and virulent wounds [11].

They also have medical pharmaceutical applications [2]. Lactic acid bacteria are widely distribution in the nature. The general, Lactobacillus, Streptococcous, Lactococcus, Leuconostol, Bifidobacterium, Carnobacterium, Enterococcus and Sporolactobacillus are known as lactic acid bacteria. They can be divided into species, subspecies, variants and strains. Each geneus and species has different characteristics but they are generally Gram positive, non-sporing, cocci or rod, non-motile bacteria that produce lactic acid as a major end-product during fermentation of carbohydrates [9]. Lactic acid bacteria ferment various carbonhydrate mainly lactate and acetate into lactic acid and acetic acid. LAB that only produce lactic acid a an end product are called homofermentative, those that also produce acetic acid, ethanol and carbondioxide are termed heterofermentative. Proteolytic activity is very important characteristic of lactic acid bacteria. Therefore the present research work undertaken to isolate and identify lactic acid bacteria from yogurt and fermented milk and to study the proteolytic activity of lactic acid bacteria [10].

II. MATERIALS AND METHODS

A. Collection of Samples

Yogurt samples were collected from different sources. Then, the samples were stored aseptically in low temperature (-4°C) refrigerator. Fermented milk was prepared by keeping pure fresh milk in a clean bottle. The milk was capped tightly and kept at room temperature for 3 days. Lactic acid bacteria were isolated from various yogurt and fermented milk.

B. Isolation of Lactic Acid Bacteria

The most appropriate media for isolation of Lactobacillus species is deMan-Rogosa-Sharp (MRS) media. The pH of the media used for isolation of Lactobacillus species was adjusted at using 1%HCL solution and NaOH solution. Firstly, one loopfull of yogurt and fermented milk were streaked on the previously prepared isolation media. Then the plate was inverted and incubated under aerobic condition at 37°C for 24 hours. After 24 hours incubation, suspicious colonies having the characteristics of Lactobacillus were picked up and recultivated several times until a pure isolate has been achieved. Finally, the single colony of lactic acid bacteria was isolated by observing their colony morphology and biochemical tests (Gram-staining, motility, catalase) and the culture were maintained at MRS agar at 4°C.Thirteen pure colonies of lactic acid bacteria were isolated from different sources of yogurt and fermented milk.

C. Identification of Lactic Acid Bacteria

The isolated bacteria were identified as lactic acid bacteria by observing their morphological characteristic and by means of biochemical tests. They were;

(1) Gram staining

(2) Motility
(3) Catalase
(4) Citrate Utilization
(5) Methyl Red
(6) Voge's Proskaver
(7) Indole
(8) Litmas Milk
(9) Tri Sugar Iron
(10) Acid Production and
(11) Carbohydrate Fermentation

D. Study on the Proteolytic Activity of Isolated Lactic Acid Bacteria

All the thirteen bacterial cultures were screened for their ability of protease production on skim milk agar plate (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skim milk powder 2.8% and agar 1.5%). In this test all bacteria isolates (LAB) were incubated in MRS broth after 24 hrs, 48 hrs and 72 hrs of incubation time at 37°C for agar well diffusion assay. Well of 7 mm in diameter were prepared on skim milk agar medium. To each of wells, 50 µl from each broth was introduced into them with the help of micropipette. The bacterial culture were incubated at various hours (24, 48, 72 hrs) at 37°C. After incubation, the diameter of each clear zone were measured and recorded in mm.

III. RESULTS

E. Isolation of Lactic Acid Bacteria

In this study, a total of 13 lactic acid bacterial strains were isolated and subcultured on MRS medium. The sources of isolated strains were shown in Table I.

TABLE I
Source of Isolation of Lactic Acid Bacteria

Isolated Strains	Source	Common Medium
L_1	Yogurt	MRS
L_2	Yogurt	MRS
L_3	Yogurt	MRS
L_4	Yogurt	MRS
L_5	Yogurt	MRS
L_6	Fermented Milk	MRS
L_7	Fermented Milk	MRS
L_8	Fermented Milk	MRS
L_9	Yogurt	MRS
L_{10}	Yogurt	MRS
L_{11}	Yogurt	MRS
L_{12}	Yogurt	MRS
L_{13}	Yogurt	MRS

B. Biochemical Characterization and Identification of Lactic Acid Bacteria

The morphology and biochemical characteristics of Lactic acid bacterial isolates were studied. Isolated bacterial strains were identified by biochemical tests and the results are shown in Table II. Carbohydrate utilizations were carried out and the results are shown in Table III. Colonial morphology of isolated bacterial strains was shown in Fig.1. Lactic acid production of bacteria isolates using MRS-$CaCO_3$ medium are shown in Fig.2.

TABLE II
Biochemical Tests of Selected Isolated LAB

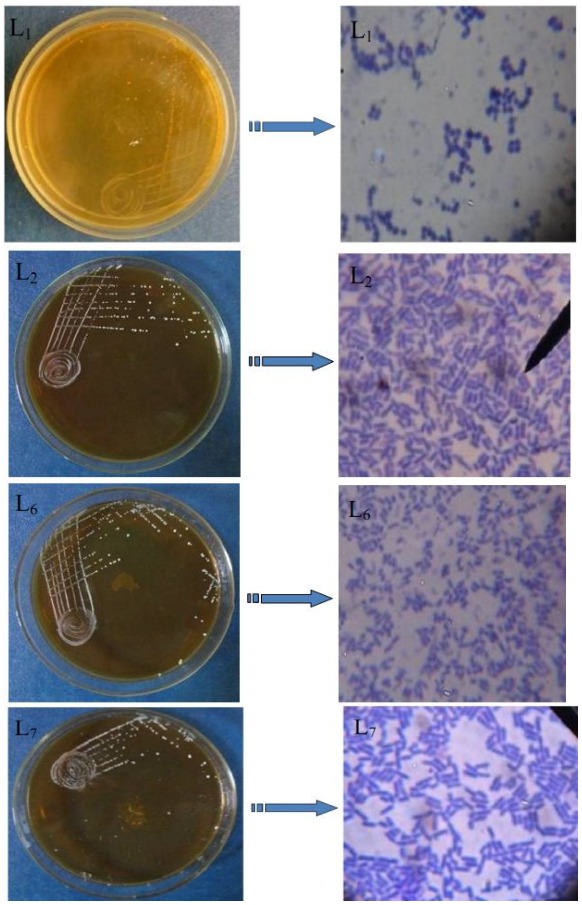
Biochemical Test	L_1	L_2	L_6	L_7	L_9
Shape	Cocci	Rod	Rod	Rod	Rod
Gram Straining	+	+	+	+	+
Motility	–	–	–	–	–
Catalase Activity	–	–	–	–	–
Citrate Utilization	–	–	–	–	–
Methyle Red	+	+	–	+	+
Voge's Proskaver	–	–	–	–	–
Lactic Acid	+	+	+	+	+
Indole	–	–	–	–	–
Litmas Milk	+	+	+	+	+
TSI	+	+	+	+	+

Note: (+) = Positive reaction, (−) = Negative reaction

TABLE III
Carbohydrate Fermentation of Selected Isolated LAB

Sugar Fermentation	L_1	L_2	L_6	L_7	L_9
Glucose	+	+	+	+	+
Dextose	+	+	+	+	+
Lactose	+	+	+	+	+
Fructose	+	+	+	+	+
Maltose	+	+	+	+	+
Raffinose	+	+	+	+	+
Galactose	+	+	+	+	+
Xylose	+	+	+	+	+

Note: (+) = Positive reaction, (−) = Negative reaction



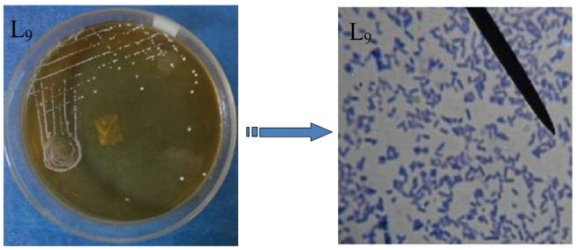


Fig.1 Cultural Morphology and Microscopic Morphology of L_1, L_2, L_6, L_7 and L_9 Strains on MRS Medium

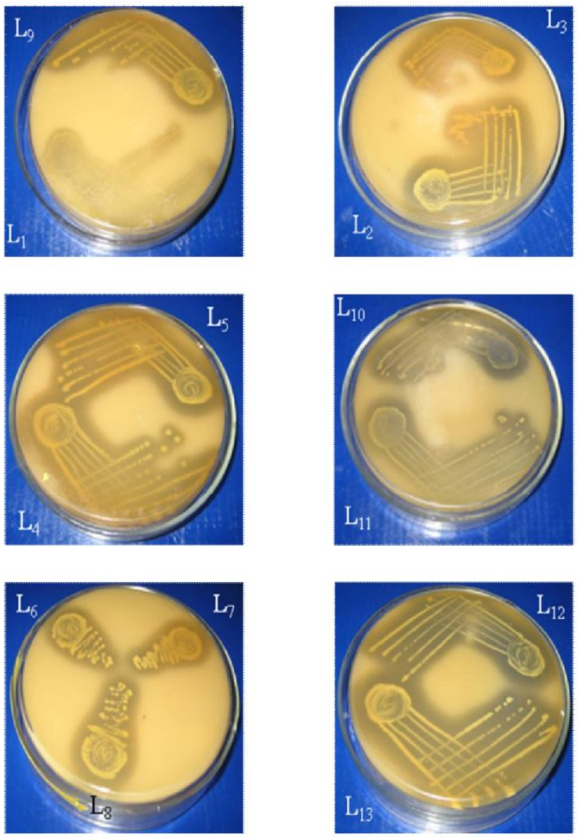


Fig.2 Acid Production on $CaCO_3$ of Isolated LAB

C.Screening for Protease Activity

All the isolated bacterial colonies were screened for their protease activity. Using skim milk agar, proteolytic activity could be determined by observing the presence of the clear zone. The results showed that the proteolytic activity of bacterial strains were assayed on skim milk agar and exhibited as diameter of clear zone in Table IV. Isolated bacterial strains were screened on skim milk agar after 24 hrs, 48 hrs, 72hrs and the results were shown in Fig.3, 4, 5 and 6. Five isolated strains will be chosen to produce peptides because of their largest zone than other strains.

TABLE IV
Zone Diameters (mm) of Selected LAB on Skim Milk Agar Plate (50µl/well)

Hours	L_1	L_2	L_6	L_7	L_9
24 hrs	24mm	23mm	23mm	21mm	22mm
48 hrs	25mm	23mm	22mm	22mm	22mm
72 hrs	21mm	22mm	23mm	22mm	26mm

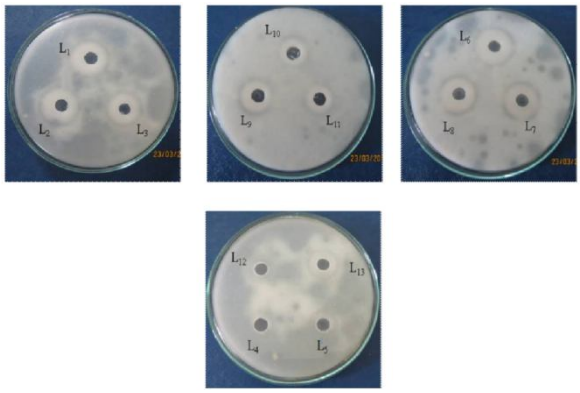


Fig.3 Screening of Proteolytic Activity on Skim Milk Agar Plate after 24 Hours

Fig.4 Screening of Proteolytic Activity on Skim Milk Agar Plate after 48 Hours

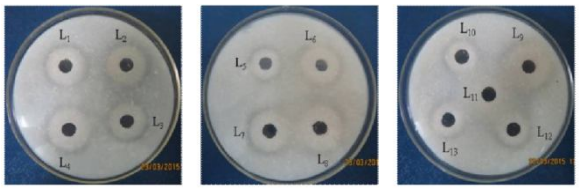


Fig.5 Screening of Proteolytic Activity on Skim Milk Agar Plate after 72 Hours

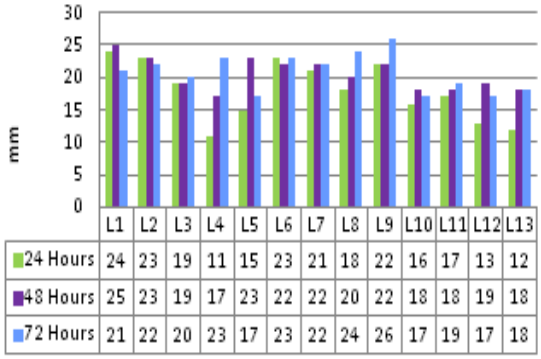


Fig.6 Comparison of Zone Diameters on Skim Milk Agar Plate at Different Hours (50µl/well)

DISCUSSIONS

Proteins are the basis of structure and function of composed of twenty amino acids the building blocks: organized into primary, secondary, tertiary, quaternary structure and classified as simple, conjugated and derived proteins. The peptides and proteins are the polymers of amino acid. Two or more amino acid molecules can be covalently joined through a peptide bond, to yield a dipeptide, oligopeptide and polypeptide. Proteins are polymers composed of linear chains of amino acids linked by peptide bonds. Proteases are enzymes that catalyse the hydrolysis of peptide bonds in proteins and polypeptides. Protease or peptides constitute the largest group of enzymes in

bio-industry with a long array of uses. They play an invincible role in industrial biotechnological such as pharmaceutical, food, detergent, leather and bioremediation processes. Proteolytic activity is very important characteristic of lactic acid bacteria. In this research, MRS media was used to isolate lactic acid bacteria as a selective medium under aerobic condition. Lactic acid bacteria cannot grow on the nutrient agar medium. These strains were biochemical identified as lactic acid bacteria. Gram positive, non-spore forming, cocci or rod, non-motile and negative tests of catalase activity obtained by the isolated strains would be confirmed that they were lactic acid bacteria. The transparent zone appearing on $CaCO_3$ (chalk agar) culture indicated the production of lactic acid by bacterial isolates. Subsequently, these lactic acid bacteria were found to ferment sugars consisting glucose, dextose, lactose, fructose, maltose, raffinose, galactose and xylose.

Lactic acid bacterial isolates were screened for proteolytic activity detected by agar-well diffusion method. All of isolated bacterial strains showed the proteolytic activity on skim milk agar. Among them, five isolated strains namely L_1, L_2, L_6, L_7, L_9 showed highest proteolytic activity as indicated by the widest clear zone. Moreover, lactic acid bacterial isolates were cultured in MRS broth at 37°C for various hours and effects of daily culture were determined. LAB culture broth of 48 hrs and 72 hrs incubation times were more effective than 24 hrs incubation time. The results obtained in the present study revealed that five selected bacterial strains of 48 hrs and 72 hrs incubation times showed the widest zones formation for proteolytic activity.

So, L_1, L_2, L_6, L_7, L_9 will be selected for further experiments.

CONCLUSION

In this research, 13 strains of lactic acid bacteria were isolated and characterized by morphological, biochemical and sugar fermentation tests. All of these strains have the property of proteolytic activity. Among them, five isolated strains were more effective in proteolytic activity. This review is to highlight lactic acid bacteria to produce bioactive peptides.

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