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Screening of Lypolytic activity of *Lactobacilli* isolates from cow milk

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ABSTRACT

Enzyme Lipase catalyses hydrolysis of triglycerides to produce glycerol and fatty acid. This enzyme have immense importance in several industries like dairy, food and pharmaceutical industries. Lipase of *Lactobacilli* is more important. In the present study, Lipase activity of some isolates of *Lactobacilli* isolated from cow milk was examined. Altogether, 13 isolates belonging to 3 species of *Lactobacilli* were examined. Out of 13 isolates, 6 belongs to *Lactobacillus casei*, 4 belongs to *Lactobacillus acidophilus* and 3 belongs to *Lactobacillus fermentum*. The lipase activity was maximum at a time interval of 15 minutes and it decreased at higher and lower time interval. Among isolates, maximum lipase production was observed in isolate No. LF3 of *L. fermentum* (120 unit) at a time interval of 15 minutes and minimum lipase production was observed in isolate No. LA1 of *L. acidophilus* (112 unit) at a time interval of 15 minutes.

Key Words - Lipase, *Lactobacilli*, Dairy, Food and Pharmaceutical industries.

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INTRODUCTION

Lactobacilli are dominant microflora of milk. They are gram-positive, rod-shaped bacteria capable of producing a variety of compounds such as lactic acid, diacetyl, vitamins, bacteriocin, β-galactosidase and lipase. *Lactobacilli* improves immune system, protect from pathogens and are anticancerous. Due to these features, *Lactobacilli* are being used as probiotics. Probiotics drinks like Yakult, Shrikhand, Lassi, etc. contains different species of *Lactobacilli*. Probiotics drinks in India is prepared and marketed from several industries such as Amul, Nestle, Mother dairy, Yakult Donane, Sudha, etc.. Amul is considered as largest food brand in India. Dahi and Lassi are the common probiotics drinks produced by Amul. In February 2007, Amul developed probiotic ice-cream which was accepted in probiotic category in Feb. 2011. Mother dairy has also launched some probiotic products by the trade name b-active probiotic dahi, b-active probiotic lassi and nutrifit. Yakult Donane India Pvt. Ltd. established in 2007 with the collaboration of Yakult Japan and Donane France. It is producing a probiotic drink with the brand name Yakult. This drink is fermented milk which consist of *Lactobacillus acidophilus* and *Bifidobacterium*. 65ml Yakult contain 6.5 billion bacteria.

More than 30 industries in India are producing drugs from *Lactobacilli*.

Enzyme Lipase produced by *Lactobacilli* are of immense importance in several industries including food, dairy and pharmaceuticals. Enzyme Lipase catalyses hydrolysis of triglycerides to produce glycerol and fatty acid. These enzymes belong to class serine hydrolyses. Several microorganisms like bacteria, fungi and some plants produce this enzyme. Lipase from some bacteria and yeast are

of high quality and may be derived in short time with low cost (Trichel *et al.,* 2010). Lipase of microbial origin have wide application in food, dairy, detergent and pharmaceuticals industries (Hasan *et al.,* 2009). High yield of Lipase is obtained from microbes at optimal growth (Linefield *et al.*, 1990) and factors like nitrogen source, carbon source, pH, aeration and temperature (Gupta *et al.*, 2004).

MATERIAL & METHOD

Milk samples were diluted up to 10^{-5} dilution in distilled water. Diluted samples were inoculated in MRS medium and incubated at 37°C for 24 hours. Culture was sub-cultured repeatedly by transferring single colony in MRS medium to get pure culture. Gram staining, Biochemical test, Sugar fermentation test and KIA tests were performed for each culture. On the basis of these tests *Lactobacilli* were identified up to the species as described in Bergey's manual of Systematic Bacteriology (2012).

Gram Staining: Gram staining technique was developed by Hans Christian Gram and on the basis of this staining technique, Bacteria are divided into two categories: Gram positive and Gram Negative. Two different stains used in this technique are Gentian Violet and Safranin.

Gram staining technique is as follows:

a) Smear is prepared on clean slide and is fixed by warming on spirit lamp.

b) Gentian violet is flooded over the slide and kept for 1 minute.

c) Iodine is flooded over slide.

d) Slide is now washed with Ethyl alcohol or acetone.

e) Safranin solution is flooded over slide.

f) Now, slide is washed with running water.

g) Slide is air-dried and viewed under microscope.

Bio-Chemical Test:

Milk samples collected from different places was diluted up to 10^{-5} and inoculated in MRS medium. Culture was incubated at 37°C for 24 hours. Single colony from the culture plate was picked up with the help of inoculation needle and transferred in a test tube containing 1ml autoclaved distilled water. It was then inoculated in the fresh medium and incubated at 37°C for 24 hours. Biochemical tests and Sugar fermentation tests were performed.

1. Catalase test: For Catalase test, one loopful culture was taken on a sterile plain slide and one drop of 3% H_2O_2 was added on it. It was mixed thoroughly. Appearance of bubble or froth indicate positive result. If not any bubble or froth appears, the result is negative.
2. Indole test: Some bacteria have Enzyme Tryptophanase which break down Tryptophan to produce Indole. Production of Indole is tested using Kovac's reagent. The formulation of Kovac's reagent is mentioned in Table 1.

   Bacterial inoculum was taken from culture and inoculated in Peptone water in a test tube. Culture was incubated at 37°C for 48 hours. 0.5ml Kovac's reagent was added and shaken gently. A red color ring shows positive result.
3. Oxidase test: Two drops of reagent are dropped in agar plate. The appearance of blue color shows positive result. The reagent for oxidase test is prepared as follow: 1%N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride is dissolved in distilled water.

Kliger's Iron Agar test: For this test, Kliger Iron Agar medium was prepared. Formulation of this medium is recorded in Table 2.

Medium and 15 test tubes were sterilized in autoclave at 15lb/inch^2 pressure for 15 minutes. Slant were prepared in each test tube. Each culture was inoculated in slant by stabbing the butt and steaking the slant. Slant and butt were observed for alkalinity or acidity.

Sugar fermentation test: Sugar fermentation test was performed for the fermentation of glucose, fructose, sucrose, lactose, mannitol, raffinose and arabinose. For sugar fermentation test, 1% sugar

was supplemented in peptone broth and the phenol red was added as indicator. All isolates were separately inoculated in test tube containing medium and incubated for 3-4 days. Color change was observed. If the color changes from pink to yellow, the result was considered as positive otherwise negative.

Table 1- Kovac's reagent

Constituents	Quantity
Amyl alcohol	150ml
p-Dimethyl benzaldehyde	10gm
Conc. HCl	50ml

Table 2- Kliger's reagent

Constituents	Quantity (gm/L)
Peptone	15.00
HM Peptone B	3.00
Yeast extract	3.00
Proteose	5.00
Lactose	10.00
Dextrose	1.00
Ferrous sulphate	0.2gm
Sodium Chloride	5.00
Sodium Thiosulphate	0.30
Phenol red	0.024
Agar	15.00
Distilled water	1000ml
pH	7.4

Detection of Lipase activity:

For the detection of Lipase activity substrate was prepared from Olive oil and polyvinyl alcohol. 50ml of Olive oil and 150ml of polyvinyl alcohol were mixed together and vortexed for 3 minutes. 5ml substrate and 5ml phosphate buffer were added in a conical flask. 1ml pure culture from MRS medium was transferred in a conical flask. The conical flash was then kept at 30^0C for 10 minutes. After 10 minutes 15ml ethanol (95%) was added. Liberated fatty acid was titrated against 0.1M NaOH, phenolphthalein was used as indicator. 1µ mol. Fatty acid is equivalent to 1 unit of enzyme Lipase.

Result

Altogether, 13 isolates belonging to 3 species of *Lactobacilli* were isolated from milk samples. Isolate code and species are listed in Table 3.

Table 3- List of species and its isolate code

Species	Isolate Code
L. casei	LC1, LC2, LC3, LC4, LC5, LC6
L. acidophilus	LA1, LA2, LA3, LA4
L. fermentum	LF1, LF2, LF3

All isolates were Gram positive, Catalase, Oxidase and Indole negative. KIA test reveals that all isolates showed yellow acidic in initial test and red alkaline in final test. The result is mentioned in Table 5. In Sugar fermentation tests, all isolates were Glucose, Lactose, Mannitol and Raffinose positive, Fructose and Sucrose negative. The result is mentioned in Table 4.

Table 4- Sugar fermentation test

Species	Isolates	Glucose	Lactose	Sucrose	Fructuse	Mannitol	Raffinose
L. casei	LC1	+ve	+ve	-ve	-ve	+ve	-ve
	LC2	+ve	+ve	-ve	-ve	+ve	-ve
	LC3	+ve	+ve	-ve	-ve	+ve	-ve
	LC4	+ve	+ve	-ve	-ve	+ve	-ve
	LC5	+ve	+ve	-ve	-ve	+ve	-ve
	LC6	+ve	+ve	-ve	-ve	+ve	-ve
L. acidophilus	LA1	+ve	+ve	-ve	-ve	+ve	-ve
	LA2	+ve	+ve	-ve	-ve	+ve	-ve
	LA3	+ve	+ve	-ve	-ve	+ve	-ve
	LA4	+ve	+ve	-ve	-ve	+ve	-ve
L. fermentum	LF1	+ve	+ve	-ve	-ve	+ve	-ve
	LF2	+ve	+ve	-ve	-ve	+ve	-ve
	LF3	+ve	+ve	-ve	-ve	+ve	-ve

Table 5- Cell morphology, Gram staining, Biochemical test and KIA test

Species	Isolates	Cell Shape	Gram Staining	Catalase	Oxidase	Indole	KIA Test	
							Initial	Final
L. casei	LC1	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LC2	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LC3	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LC4	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LC5	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LC6	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
L. acidophilus	LA1	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LA2	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LA3	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LA4	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
L. fermentum	LF1	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LF2	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline
	LF3	Rod	+ve	-ve	-ve	-ve	Yellow/ Acidic	Red/ Alkaline

Lipase Activity: Lipase activity was maximum at a time interval of 15 minutes and it decreased at higher and lower time interval. Among isolates, maximum lipase production was observed in isolate No. LF3 of *L. fermentum* (120 unit) at a time interval of 15 minutes and minimum lipase production was observed in isolate No. LA1 of *L. acidophilus* (112 unit) at a time interval of 15 minutes. The result is shown in Fig. 1 to 3.

Fig. 1- Lipase activity of isolates of *L. casei* at different time interval

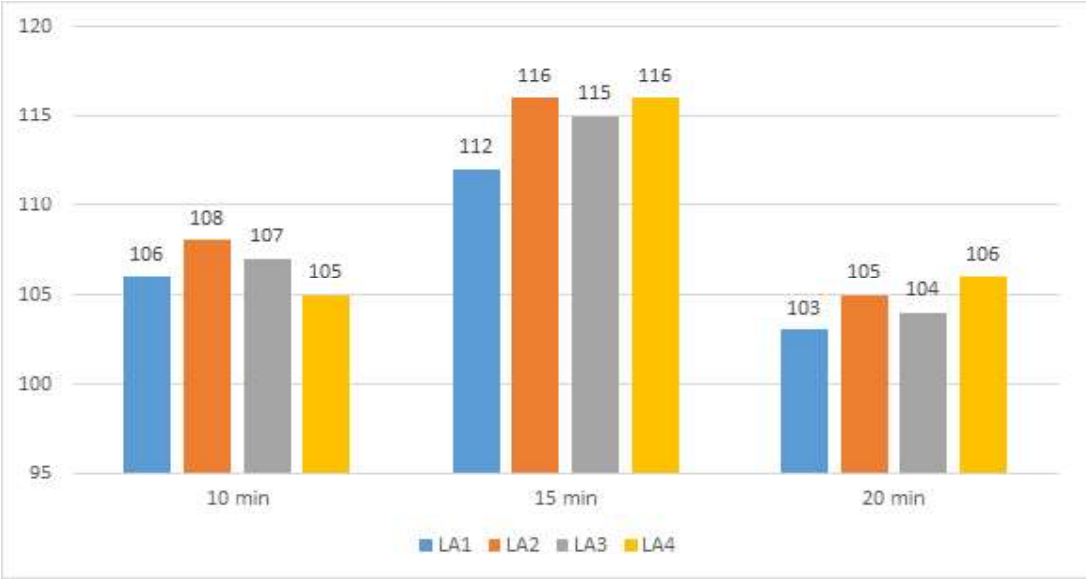

Fig. 2- Lipase activity of isolates of *L. acidophilus* at different time interval

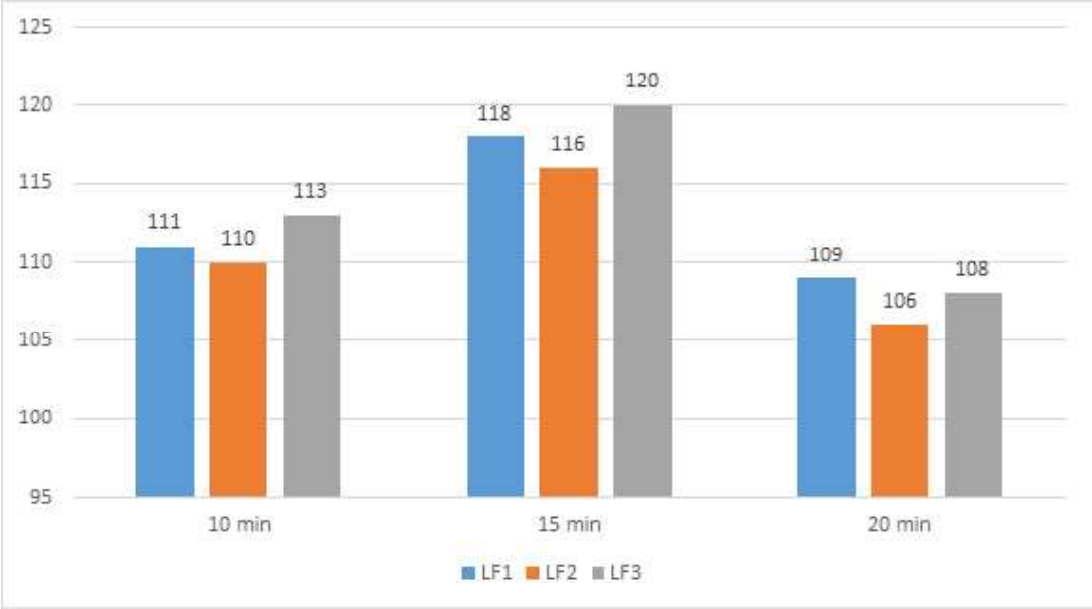

Fig. 3- Lipase activity of isolates of *L. fermentum* at different time interval

CONCLUSION

In the present study, lipase activity of *Lactobacilli* was examined. Altogether 13 isolates belonging to 3 species of *Lactobacilli* were isolates from milk samples. All isolates were rod shaped, Gram positive, Catalase, Oxidase and Indole negative. Among 13 isolates, 6 belongs to *Lactobacillus casei*, 4 belongs to *Lactobacillus acidophilus* and 3 belongs to *Lactobacillus fermentum*.

Enzyme Lipase catalyses hydrolysis of triglyceride. The enzyme Lipase from *Lactobacilli* are most important for industries like food, dairy and pharmaceutical. The lipase activity was maximum at a time interval of 15 minutes and it decreased at higher and lower time interval. Among isolates, maximum lipase production was observed in isolate No. LF3 of *L. fermentum* (120 unit) at a time interval

of 15 minutes and minimum lipase production was observed in isolate No. LA1 of *L. acidophilus* (112 unit) at a time interval of 15 minutes.

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