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Camel milk: a potential source of therapeutic lipolytic lactic acid bacteria

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ABSTRACT

Dairy products such as curd, cheese, butter, goat, buffalo, camel and cow milk were screened for lipolytic bacteria. Of the twelve isolates screened five were found to be lactic acid strains as they secrete true lipase, producing free fatty acids from tributyrin. On the basis of microscopic and biochemical characteristics these isolates were identified as *Streptococcus* (MRLa) and *Streptococcus* (MRLb), *Lactobacillus* (MRLc), *Lactobacillus* (MRLd), *Lactobacillus* (MRLe). Cell free extract prepared from stock culture of above mentioned strains was used as crude lipase prepration and free fatty acids released were determined titrimetrically. Among these strains MRLd showed maximum lipase activity followed by MRLe, both isolated from camel milk, and MRLc, MRLb and MRLa, all three isolated from cow's milk . Results indicate that camel milk can be used as a potential source of lipolytic bacteria that have therapeutic and commercial value.

Key words: Lactic acid bacteria, lipolytic activity, camel milk, free fatty acid, crude lipase.

INTRODUCTION

Lactic acid bacteria are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. This group includes representatives of the genus *Lactobacillus, Lactococcus, Pediococcus*, *Leuconostoc* and *Streptococcus* (Guessas *et al*, 2004) which are used in the production of fermented food products such as yogurt, cheese, sauerkraut and sausage (Kamaly *et al*, 1989, Nowroozi *et al*., 2004, Tserovska *et al*.,2002, Conter *et al*.,2005). Lactic acid bacteria are natural inhabitants of human gastrointestinal tract (GIT) and are also considered as a probiotic. These bacteria also support digestion and immune function enhancement in addition to controlling the pH of the large intestine through the liberation of lactic and acetic acid, which in turn restricts the growth of many potential pathogens (Nowroozi *et al*., 2004). In GIT these bacteria are capable of delivering enzymes and other substances into the intestine which possibly help to control intestinal microflora (Collins *et. al*., 1999). Lactic acid bacteria also produce biologically active peptides or protein complexes that display a bactericidal mode of action almost exclusively toward gram positive bacteria (Mojgani *et al*., 2006). Lactic acid bacteria naturally found in dairy products have been implicated in lipid degradation also (Kamaly *et al*., 1989; Marta *et al*., 2002; Kun *et al*., 2004; Andrews *et al*., 2006).

Since sedentary life style has given rise to the increased incidence of coronary heart problems such as atherosclerosis, high blood pressure etc which are consequences of increase in triglyceride content of blood. Isolated bacterial strains could be helpful to derive lipase preparation that could find therapeutic advantage in serum triglyceride reduction as well as for those peoples produce inadequate pancreatic lipase. Enzyme preparation

could also have other applied benefits such as flavour and aroma enhancer in various dairy products and other fermented food products. It may also find application in development of probiotics which in today's health- conscious scenario will not only help in natural reduction of serum triglycerides but also maintain gastrointestinal tract microflora that prevent infection and adhesion of pathogenic bacteria due to bacteriocin production and antagonistic activity.

Thus, the need of the hour is to identify such food and dairy products which can either be consumed directly or used to isolate these bacteria. Hence, in the present study samples of various dairy products were screened for the presence of and isolation of lactic acid bacteria.

MATERIAL AND METHODS

Sample collection

21 dairy samples viz. curd, cheese, milk (goat, buffalo, cow, camel) were used in this study. These samples were collected from local (domestic and commercial) sources of Udaipur (Rajasthan) in presterlized ampoules at room temperature and screened on same day of collection.

Isolation of lipolytic bacteria

Isolation of lipolytic strains was done by enrichment culture according to the modified method of Vargas *et al*, 2004. The (g/l) composition of enrichment medium was as follows: lipid substrate (tributyrin and castor oil); 20 g, K_2HPO_4 ; 2.5 g, $(NH_4)_2$ SO4;1.3 g, $MgSO_4$; 0.5 g, yeast extract; 0.5 g. 6.5ml of filter sterilized urea (200 g/l stock) were added in media after autoclaving. pH was adjusted to 8.5 by addition of sterile sodium carbonate : bicarbonate mixture (3:1) to the medium. 10% (v/v) dilutions of samples were inoculated in enrichment medium and incubated in orbital shaker incubator at 220 rpm for 24h at 37 °C.

Lipolytic bacteria were isolated by streaking the enriched culture on tributyrin agar media containing fatty substrate. The components of media include 0.5% peptone, 0.3% yeast extract, 2% agar and1% fatty substrate. Lipolytic activity was observed as zone of clearance around bacterial colonies.

Isolation of lipolytic lactic acid bacteria

Isolation of lactic acid bacteria was done on selective medium i.e. MRS agar and M-17 agar (Tserovska *et al*., 2000). These isolates once again checked for lipolytic activity.

Identification of the bacterial strains

Identification of the strains as lactic acid bacteria was done on the basis of their morphological, cultural, physiological and biochemical characteristics as suggested in Bergey's Manual (Bergey's Manual**,** 1984).

Lipolytic activity

Lipolytic activity was determined by agar well diffusion assay (Blake *et al*,1996). 12 mm sized punched well in tributyrin agar plate were filled with 200 µl of cell free extract prepared from 4×10^6 CFU. These plates were incubated at 37 °C for 24h. A clear zone around the well indicated lipid hydrolysis. The size of clear zone was measured with the help of Hi- Antibiotic zone scale-C. Nutrient broth was used as negative control and pancreatic lipase as positive control.

Free fatty acids (FFAs) Titration

Titration method of Deeth *et al*.,1975 was used to measure the amount of FFA released by the activity of crude lipase. Overnight culture of isolates, developed in basal medium containing tributyrin, were centrifuged at 5000 rpm and filtered to obtain cell free extract which was used as crude lipase preparation. The reaction mixture containing crude lipase, tributyrin and phosphate buffer (0.2 M, pH 7.2) in 1:1:2 ratio respectively was placed in a 25 ml glass stoppered test tube and incubated for different time duration of 6h, 12h, 24h, 30h, 36h, 48h, 54h, 60h and 72h at 37 ^{0} c temperature. After specified time of incubation, 2 ml of reaction mixture were mixed with 10 ml of extraction mixture (40:10:1, vol/vol/vol, isopropanol: petroleum ether: 4N H_2SO_4), 6 ml of petroleum ether and 4 ml of water. The mixture was shaken vigorously. The layers were allowed to separate for 10 min. at 25^0 c. and 5 ml of the upper layer were then titrated with 0.02 N methanolic KOH using 50 µl of methanolic phenolphthalein as an indicator. Each time FFA content was determined using the formula $(TN/PV)x10^3$, where T is the net titration volume, N is the normality of the methanolic KOH , P is the

proportion of the upper layer titrated, and V is the volume (in millilitres) of sample.

Protein Determination

The protein content of cell free extract was determined according to Bradford *et al*., 1976 with bovine serum albumin as a standard.

Statistical analysis

The statistical analysis of data was done by using software origin 6.1. The data were analysed by one way ANOVA and also by determining linearity of lipase activity with time.

RESULTS AND DISCUSSION

Enrichment culture allows the selective growth of strains with desired characteristics hence in the present study enrichment culture technique was used to isolate lipolytic bacteria. Five lipolytic strains of lactic acid bacteria were isolated from enriched samples. Identification of strains was done by studying morphological and biochemical characteristics as indicated in table 1. These strains were identified as *Streptococcus* (MRLa) , *Streptococcus* (MRLb), *Lactobacillus* (MRLc), *Lactobacillus.* (MRLd) and *Lactobacillus* (MRLe).

Lipolytic activity of crude lipase, obtained from isolated lactic acid bacteria, was determined by agar well diffusion method (Blake *et al*, 1996). The basic principle behind this method is the hydrolysis of lipid substrate present in the media by the microbial enzyme which can be observed as a clear zone around the bacterial colonies or wells containing crude lipase preparation. The size of zone of clearance indicates the magnitude of activity. Results indicate (Table 2) that maximum zone of clearance (14mm) was produced by crude lipase from *Lactobacillus* (MRL d) while minimum (3mm) was produced by *Streptococcus* (MRLa). Lipolytic activity has been reported in lactic acid bacteria by several workers (Kamaly *et al*, 1990, Woo *et al*, 1984). Lipase enzyme responsible for this activity may be extracellular, intacellular or it may be cell membrane bound (Mourey., 1981, Lee and Lee., 1989 and Large *et al*., 1999). Presence of extracellular lipase in cell free extract is therefore possibly responsible for the lipolytic activity of the isolates (Kalogridou-Vassiliadou,1984).

Table 1: Morphological ,culture and biochemical characteristics of isolates used for study

S. No.	Isolates	Cultural Characteristics				Biochemical Characteristics							Identification
		Gram's reaction	Cell Morphology	Colony morphology	Colony colour	A*	B*	C*	D*	E*	F*	G*	
1.	MRL a	+ve	Coccoid	wide rough	White Gray	-ve	+ve	-ve	*	-ve	*	-ve	*Streptococcus sp.*
2.	MRL c	+ve	Rods	Wide, smooth, glossy	Cream coloured	+ve	+ve	*	+ve	+ve	+ve	+ve	*Lactobacillus sp.*
3.	MRL b	+ve	Coccoid	Smooth opalescent	White	-ve	+ve	-ve	*	-ve	*	-ve	*Streptococcus sp.*
4.	MRL d	+ve	Rods	Small sized, pinpointed	Creamish white	+ve	+ve	*	+ve	+ve	+ve	+ve	*Lactobacillus sp.*
5.	MRL e	+ve	Rods	Small sized	White	+ve	+ve	*	+ve	+ve	+ve	+ve	*Lactobacillus sp.*

A* Catalase test, B* Nitrate Reduction test, C* Vogus Proskar test, D* Gelatinase test, E* T.S.I. Reduction test, F* Litmus test, G* Methyl Red test

Table 2: Zone of hydrolysis produced by LAB isolates on Tributyrin agar media

S. No	Lipolytic lactic acid bacteria	Zone of clearance (mm)*
1	*Lactobacillus* (MRLc)	9
2	*Lactobacillus* (MRL d)	14
3	*Lactobacillus* (MRL e)	11
4	*Streptococcus* (MRL a)	3
5	*Streptococcus* (MRL b)	4
6	Control	N.D.
7	Pancreatin	15

*values are mean of three replicates
N.D. = Not Determined

Table 3: Statistical analysis of data given in figure1, 2, 3, 4 and 5

S.No.	Isolates	R value	P value
1.	MRL a	0.9886	<0.0001
2.	MRL b	0.9674	<0.0001
3.	MRL c	0.9945	<0.0001
4.	MRL d	0.9951	<0.0001
5.	MRL e	0.9876	<0.0001

Further quantitative assay of lipase activity was done by titrimetric method. In this method lipase activity was measured as a release of free fatty acids during specific time duration by using phenolphthalein as end point indicator. This enzyme hydrolyzes the lipid into fatty acids and glycerol. Hydrolysis of tributyrin results in increase in acidity of reaction mixture which can be measured titrimetrically (Deeth *et.al.*,1975).

Lipase assays measure fatty acid released in periods less than an hour (Chen *et al.,* 2003; Deeth & Touch, 2000). However, some authors, in attempting to detect trace levels of lipase have performed longer incubations (Blake *et al.,* 1996; Deeth & Touch, 2000). Hence, for each strain release of free fatty acids was determined over 72 hours and was found to be linear with time (Figs. 1,2,3,4 and 5). After 72 h of incubation of reaction mixture, maximum lipase activity was observed for strain MRLd followed by MRL e, MRL c, MRL b and MRL a respectively.

Factors like environmental conditions at the time of sample collection, type of sample, chemical and physical nature of sample , substrate specificity etc. might be responsible for variations in lipase activity among lipolytic lactic acid bacterial strains. Either single or cumulative effect of above mentioned factors may affect the expression of gene coding for lipase degrading principle and subsequently in the amount of FFAs release by each strain. On the basis of this results obtained, it can be suggested that physical and chemical nature as well as environmental condition may be responsible for better enzymatic activity in the strain isolated from camel milk as compare to cow milk .

The value of correlation coefficient (R) for each strain was found to be significant (Table 3). In addition to these, values of coefficient (P<0.0001) were also found to be more significant for each strain. Analysis of data by one -way ANOVA at 0.05 level of significance also indicates that this work is significant.

Of the various dairy samples screened, strains isolated from camel milk show best results. Therefore it can be suggested that camel milk can be a potential source for isolation of lipolytic lactic acid bacteria as compared to cow milk.

Apart from this it can also be suggested that consumption of camel milk can be used for natural enhancement of inherent gastrointestinal lipolytic flora and therefore it can be a part of natural therapy for hypertriglyceridemia.

Since lipolytic activity of crude lipase preparation of MRLd is comparable with that of pure lipase preparation (pancreatin) and hence there is a possibility that pure lipase prepration might have greater activity than pancreatin.

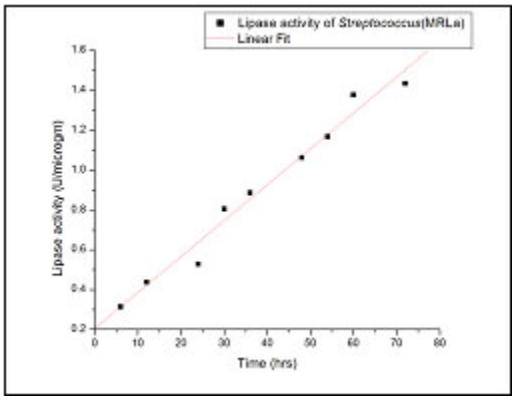

Fig. 1: Linearity of specific activity of lipase (U/microgm of protein) with time (hours) for *Streptococcus* (MRLa) (R = 0.9886; P<0.0001)

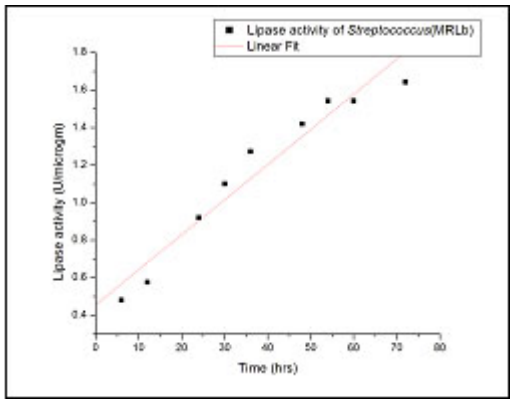

Fig. 2: Linearity of specific activity of lipase (U/microgm of protein) with time (hours) for *Streptococcus* (MRLb) (R = 0.96742; P<0.0001).

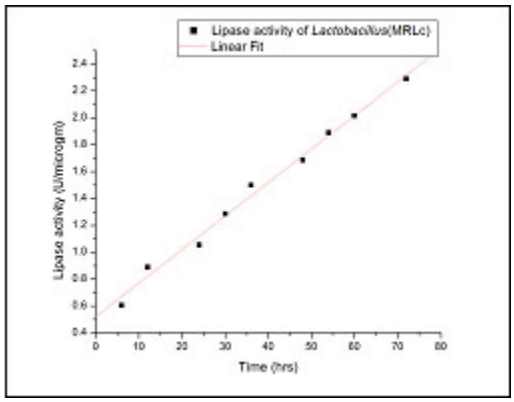

Fig. 3: Linearity of specific activity of lipase (U/microgm of protein) with time (hours) for *Lactobacillus* (MRLc) (R =0.99456; P<0.0001)

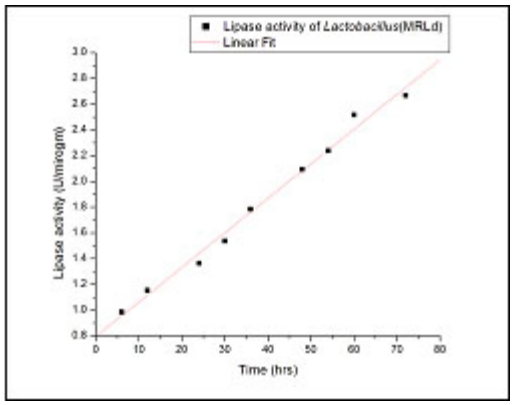

Fig. 4: Linearity of specific activity of lipase (U/microgm of protein) with time (hours) for *Lactobacillus* (MRLd) (R =0.9951; P<0.0001).

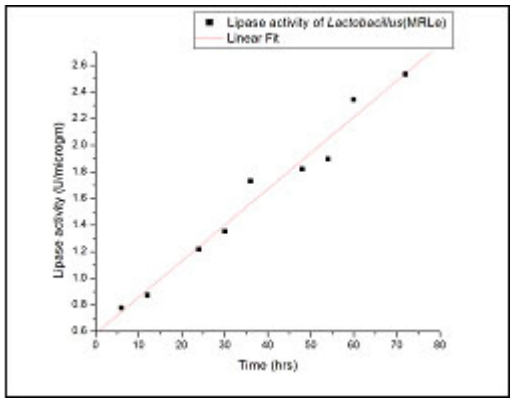

Fig. 5: Linearty of specific activity of lipase (U/ microgm of protein) with time (hours) for *Lactobacillus* (MRLe) (R = 0.98768; P<0.0001)

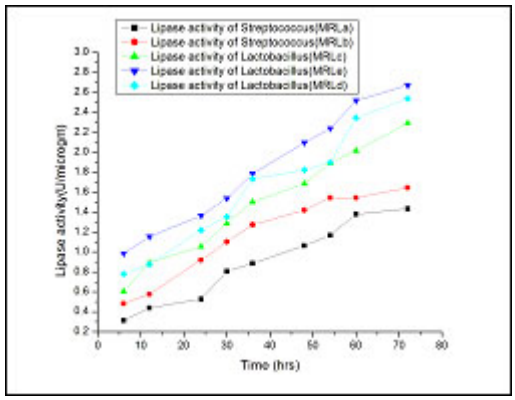

Fig. 6: Specific activity of lipase enzyme of Lactic acid bacteria isolates

REFERENCES

1. Guessas B. and Kihal M., Characterization of lactic acid bacteria isolated from algerian arid zone raw goat's milk. *African Journal of Biotechnology* **3**(6): 339-342 (2004).
2. Kamaly KM, Takayama K and Marth EH., Lipase activities of *Streptococcus* bacteria and their mutants. *J Dairy Sci.***73**: 280-290 (1989).
3. Nowroozi J, Mirzali M and Norouzi M., Study of *Lactobacillus* as probiotic bacteria. *Iranian J Publ Health* **33**(2): 1-7 (2004).
4. Tserovska L, Stefanova S and Yordanova T., Identification of lactic acid bacteria isolated from katyk, goat's milk and cheese. *Journal of culture collection* **3**: 48-52 (2002).
5. Conter M, Muscariello T, Zanardi E, Ghidini S, Vergara A, Campanini G and Ianieri A., Characterization of lactic acid bacteria isolated from an Italian dry fermented sausage. *Ann. Fac. Medic. Vet. di Parma* **25**: 167-174 (2005).
6. Collins M, Glenn D and Gibson R., Probiotics, prebiotics and symbiotics: approaches for modulating the microbial ecology of the gut. *American J Clin Nutri* **69**(5): 1052s- 57s (1999).
7. Mojgani N, Ashtiani MP and Khanian SE., Plasmid associated lactocin RN 78 production in a *Lactobacillus* RN 78 strain isolated from a dairy sample in Iran. *Medical J. of Islamic World Academy of sciences* **16**(1): 19-24 (2006).
8. Marta K, Roxana M, Silvia G and Guillermo O., Esterolytic and lipolytic activities of lactic acid bacteria isolated from ewe's milk and cheese *J. food prot.* **65**: 1997-2001 (2002).
9. Kun HC and Ondul E., Free fatty acid accumulation by mesophilic lactic acid bacteria in cold stored milk. *J of Micro* **42**: 133-138 (2004).
10. Andrews P, Baldwin A, Broome A, Hill B, Holand R, Mills O and Neusetead D., Detection of lipase in skim and whole milk powders using triheptonin as a substrate International dairy J.**17**: 587-595 (2006).
11. Vargas VA, Delgado OD, Hatti-Kaul R and Mattiasson B., Lipase-producing microorganisms from a kenyan alkaline soda lake. *Biotechnology Letters* **26**: 81-86 (2004).
12. Bergey's Manual of Systematic Bacteriology., N. Krieg (Ed), vol. 1, 2, Baltiore: Williams and Wilkins (1984).
13. Blake MR, Koka R, and Weimer BC., A semi automated reflectance colorimetric method for the determination of lipase activity in milk. *Journal of Dairy Science* **79**: 1164-1171 (1996).
14. Deeth HC, Fitz- Gerald CH and Wood A., A convenient method for determining the extent of lipolysis in milk. *Aust . J. Dairy Techno.* **30**: 109 (1975).
15. Bradford MM., A rapid and sensitive method for quantification of microgram quantity of protein utilizing the principles of protein dye binding. *Anal. Biochem* **72**: 248-254 (1976).
16. Woo AH and Lindsay RC., Characterization of lipase activity in cold-stored butter. *J Dairy Sci* **67**: 1194-1198 (1984).
17. Mourey A., Lipolytic activity of *Bacillus pumilus. Rev. Fr. Corps Gras* **28**(2): 55-8 (1981).
18. Lee SY and Lee BH., Production and characterization of esterase-lipase of *Lactobacillus casei* subsp. *pseudoplantarum* LE2 *Biotechnol. Appl. Biochem* **11**: 552-563 (1989).
19. Large KP, Mirjalili N, Peacock MOLM, Zormpaidis V, Walsh M, Cavanagh ME, Leadlay PF and Ison AP., Lipase activity in Streptomycetes. *Enzym. Microb. Technol.* **25**(7): 569-575 (1999).
20. Kalogridou-Vassiliadou D., Lipolytic activity and heat resistance of extracellular lipases of some gram negative bacteria. Milchwissenschati 39501 (1984).
21. Chen L, Daniel RM and Coolbear T., Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal*, **13**: 255-275 (2003).
22. Deeth HC and Touch V., Methods for detecting lipase activity in milk and milk products. *Australian Journal of Dairy Technology* **55**: 153-168 (2000).