Lactic Acid Bacteria Isolated from Local Fermented Milk and Their Proteolytic Activity

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Abstract

Lactic acid bacteria were isolated from traditionally made curd samples. The isolates were characterized thoroughly for cultural, morphological, physiological and biochemical characters and were identified as strains of *Streptococcus* and of *Lactobacillus*. Of the 25 *Streptococci* 1,4 were *S.cremoris*, 6 were *S. faecalis* and 1 each were *S. lactis* and *S. lactis* subspecies diacetylactis. Out of 12 strains of *Lactobacillus*, 1 was *L. bulgaricus*, 3 were *L. helveticus* and 1 was *L. lactis*. From the efficiency tests, 3 strains each from *Lactobacillus* and *Streptococcus* were selected for extracellular protease activity. All the 3 lactobacilli and one strain of *Streptococcus* showed their highest protease activity at acidic p^H. The other two showed the same at alkaline p^H. The protease activity was the highest at 40°C for all the strains of *Lactobacillus* and *Streptococcus*.

Introduction

Curd (Dadhi) is a fermented milk product which is the sub-continental (Indian) equivalent of yogurt. It is made traditionally where cows or buffaloes milk is allowed to ferment with lactic acid bacteria (LAB), predominantly *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. But, yet its microbiology is extremely variable[16] since, other species such as *S. lactis*, *S. lactis* subspecies *diacetylactis*, *L. plantarum*, *L. helveticus* etc. are also used[3,8]. The traditional curd is made in mud pots and its taste and appearance are also variable from place to place. In spite of such variation in quality the product is not yet studied scientifically. Although in India some studies have been made on different scattered aspects[4,22,25,29,30] but in Bangladesh, except a very few[12,28] little attention has been paid to this product. Hence, it is necessary to know the lactic acid bacteria (LAB) associated with the curds and its variable microbiology.

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Curds with good organoleptic qualities require good growth of bacteria in milk. But the growth requirements of LAB are complex. The simple enzyme complements of LAB made them unable to synthesize many amino acids[17]. The lactic group of both streptococci and lactobacilli have extensive requirements of free amino acids for their growth[14,19,23,26] but free amino acid concentration in milk are not sufficiently high to permit commercially useful growth. To overcome this nutritional problem, dairy lactic acid bacteria use their proteolytic enzymes to make (milk) protein-bound amino acids available for growth. Exterkate[10] observed that actively growing or metabolizing cells released a constant amount of proteinase in the medium. The sysnthesis of these enzymes is controlled by the factors dependent on temperature and p^H as informed by Mills and Thoma[17] and Exterkate (1976). To some extent, most of the dairy lactic acid bacteria possess proteolytic ability. But for better activity suitable physiological conditions, specially temperature and p^H of the media are necessary.

In the present study, the extra-cellular proteases of the selected LAB were separated and detected and for the activity, optimum temperature and p^H of the media were also determined.

Materials and Methods

Isolation and Identification

Lactic acid bacteria were isolated from traditionally prepared curd samples. Yeast Glucose Lemco Agar (YGLA), Rogosa and MRS (deMann, Rogosa and Sharpe) agar were used for *Streptococcus* and *Lactobacillus* respectively. The isolate were studied for their cultural, morphological, biochemical and physiological characters carefully. To obtain the pure culture the discrete colonies were picked-up from YGLA and Rogosa or MRS agar plates and subcultured on the slopes of the same media. The cultures from the YGLA and Rogosa or MRS agar were considered as presumptive streptococci and lactobacilli respectively. For the identification of streptococci the following tests were performed : growth at 10°C, 40°C and 45°C; growth in 2%, 4% and 6.5% NaCl; survival at 60°C for 30 min, production of NH_3 from arginine and acid from different sugars e.g. glucose, arabinose, lactose, sucrose,

raffinose and salicin. These were followed according to Swartling[31] and Reiter and Madsen[24].

For the identification of different species of *Lactobacillus*, the tests followed, were given by Buchanan and Gibbons[6] and Sharpe[27] which were the production of CO_2 from glucose, NH_3 from argenine, growth at 15°C and 45°C temperature and acid formation from fermentation of glucose, arabinose, galactose, lactose, maltose, sucrose, raffinose and salicin.

Selection of the potential isolates

For the selection of the efficient isolates, efficiency tests were carried out for the determination of the amount of the formed whey, amount of lactic acid, drop of p^H, dry weight of curd and time of curdling. Acid producing activity (Harrigans and McCance, 1976) was also conducted for the purpose[11]. From the tests, six potent isolates were selected for the determination of extracellular protease activity. For the preparation of protease, Mao et al's method was followed. Activity of alkaline protease was determined by slightly modification of casein digestion method of Kunitz[13].

Enzyme Assay

The reaction mixture consisting of 0.5 ml of enzyme solution and 1 ml of 1.0% casein at different buffer i.e 0.2 M Na_2HPO_4 and 0.2M glycine NaOH with the final p^H as 6.5, 7.0,8.2 and 10.6 (for strains PC26,YY37, CG38); 5.9, 6.4 and 10.0 (for strains RMY 23, RCG38, RB 44) was incubated at 37°C for 1 hour. The reaction was stopped by the addition of 1 ml of 10% TCA. The control was prepared in the same quantity but the enzyme was added to the mixture of substrate and TCA. The resulting precipitate was removed by filtration and absorbence of the filtrate was measured at 660 nm. The protease activity was expressed as the difference of absorbance at 660 nm between the control and test samples.

Activity of the acidic protease was determined by the slight modification of haemoglobin digestion method of Anson[2]. For this purpose 1 ml of 1% haemoglobin (BDM) at different buffers (0.2M KC1 & 0.2M Glycine, NaCl,

0.2M Na_2HPO_4) and 0.5 ml of enzyme was mixed together and the final p^H was recorded as 3.3, 4.5, and 6.0 (for the strains PC26, YY37, CG38) and 2.8, 3.7 and 5.8 (for the strains RMY23, RCG38, RB44)[2]. A standard curve was constructed with each experiment using BSA (Bovine Serum Albumen).

Effect of temperature on protease activity was determined selecting two strains from the above experiment. For the purpose, the enzyme activity was conducted at 17, 30, 40, 50, and 60°C.

Results

For the characterization of the isolates, the cultural and morphological characters were studied and biochemical and physiological tests were performed.

Table 1 shows the characters of 25 presumptive streptococci, of which 14 isolates were identified as *Streptococcus cremoris* as described by Swartling[31] and Reiter and Madsen[24], 6 isolates as *Streptococcus thermophilus*, 3 as *Streptococcus faecalis*, 1 as *Streptococcus* lactis and one more was identified as *S. lactis* subspecies *diacetylactis*.

From the presumptive lactobacilli (Table 2), 9 isolates resembled with *Lactobacillus bulgaricus* as given by Buchanan and Gibbons[6] and Sharpe[27], 2 isolates were confirmed as *L. helveticus* and 1 agreed with the characters of *L. lactis*.

From the efficiency tests, S. *thermophilus (PC 26, YY 37)* and a strain of *S. cremoris* (CG 38) and *L. bulgaricus* (RMY 23, RB 44) and a strain of *L. helveticus* (RCG 38)) were found most efficient (Table 3) which were selected for the protease activity. The activity of the protease towards casein and haemoglobin at different p^H levels has been determined. It could be seen from Table 4 that protease of *S. thermophilus* (PC 26) was the most active at p^H 4.5 and 6.0. However, little activity was found in alkaline p^H. Whereas, protease of *S. thermophilus* (YY37) and S. *cremoris* (CG 38) showed highest activiy at alkaline p^H (8.2). In case of the species of *Lactobacillus*, the protease activity, demonstrated by L. bulgaricus (RMY23) was the highest at p^H 3.7, that of *L. bulgaricus* (RB 44) was at p^H 2.8 and of *L. helveticus* (RCG 38) was at 5.3.

Table 1-Physiological and Biochemical Charaoteristics of the isolated LAB (Presumptive *Streptococcus* species)

Isolates	Growth at			Growth in NaCl soln.			Survival at 60°C for 30 min.	NH_3 Production from Arginine	Production of acids from sugars							Indentified species
	10°C	40°C	45°C	2%	4%	6.5%			G	S	F	R	A.	L	S	*S. faecalis*
IIMC4	-	+	+	+	+	+	+	+	+	-	+	-	-	+	-	*S. thermophilus*
IIIMC4	-	+	+	+	+	-	+	+	+	+	±	-	-	+	-	*S. thermophilus*
JCR8	-	+	+	+-	+	-	+	-	±	-	+	-	-	+	-	*S. thermophilus*
GS15	+	+	-	+	-	-	+	-	+	-	±	-	-	+	-	*S. thermophilus*
TCS21	-	+	+	+	-	-	-	+	-	-	-	-	-	+	-	*S. cremoris*
TCB21	-	+	+	+	+	-	-	+	+	-	-	-	-	+		*S. cremoris*
MC22	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	*S. faecalis*
NY24	-	+	+	+	+	+	+	+	+	-	+	-	-	+	-	*S. thermophilus*
PC26	-	+	+	+	+	-	+	+	+	+	+	-	-	+	-	*S. cremoris*
GC27	-	+	-	+	+	-	-	-	+	+	+	-	-	+	-	*S. lactis*
AC28	-	+	+	+	+	-	-	+		+	+	+	+	+	-	*S. faecalis*
ZC29	-	+	-	+	+	+	-	+	+	+	-	-	-	+	-	*S. cremoris*
FC30	-	+	-	+	-	-	-	-	+	+	+	-	+	-	-	*S. cremoris*
SY31	-	+	-	+	-	-	-	+	-	-	+	-	-	+	-	*S. crenoris*
SMS33	-	+	-	+	-	-	-	+	+	+	-	-	-	-	-	*S. subsp. diacetylactis*
SMB33	-	+	-	+	-	-	-	+		-	+	+	-	-	-	*S. cremoris*
FS35	-	+	+	+	-	-	-	-	+	+	+	-	-	+	-	*S. thermophilus*
FB35	-	+	+	+	-	-	-	-	+	+	+	-	-	+	-	*S. cremoris*
HC36	-	+	-	+	-	-	-	+	+	+	+	+	-	-	-	*S. thermophilus*
YY37	-	+	+	+	-	-	+	+	+	+	+	-	+	+	-	*S. cremoris*
CG38	-	-	-	+	-	-	-	-	+	+	+	-	+	+	-	*S. cremoris*
CE39	–	+	-	+	+	-	-	-	+	+	+	-	-	+	-	*S. cremoris*
CF40	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	*S. cremoris*
YA41	-	+	-	-	-	-	-	-	+	+	+	-	-	-	-	*S. cremoris*
YC42	-	+	-	+	+	-	-	-	+	+	+	-	+	+	-	*S. cremoris*

G = Glucose, S = Sucrose, F = Fructose, R = Raffinose, A = Arabinose, L = Lacose, S = Salicin.

Table 2. Physiological and Biochemical characteristics of the isolated LAB (Presumptive *Lactobacillus* species[a])

Isolates	NH_3 from Arginine	Growth at 45°C	Acid Production from Sugars																		Indentified Species
			G		S		F		R		G		M		A		L		S		
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
RTC 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	*L. bulgaricus*
RMY 23	-	+	+	+	-	-	+	-	-	-	+	+	-	-	-	-	+	+	-	-	*L. bulgaricus*
RMY 24	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	*L. bulgaricus*
RGC 27	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	*L. bulgaricus*
RAB 28	+	+	+	+	-	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	*L. lactis*
RAS 28	+	+	+	+	-	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	*L. helveticus*
RZC 29	-	-	-	-	-	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	*L. bulgaricus*
REY 30	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	*L. bulgaricus*
RSY 31	+	+	±	±	-	-	+	+	-	-	-	-	-	-	±	±	+	+	-	-	*L. bulgaricus*
RCG 38	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	-	*L. helveticus*
RCD 43	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+	+	-	-	*L. bulgaricus*
RB 44	-	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+	+	-	-	*L. bulgaricus*

a = All the isolates studied could not produce CO_2 from glucose and showed no growth at 15°C
1 = Peptone water fermentation media
2 = MRS fermentation media

G = Glucose
S = Sucrose
F = Fructose
R = Raffinose

G = Galactose
M = Maltose
A = Arabinose
L = Lactose
S = Salicin.

Table 3. Efficiency of the strains in curdling and acid production activity test.

Species of the LAB	Whey (ml)	pH of Curd[a]	Titratable Acidiy (N)/100 ml culure	Dry wt. of curd (gm)
S. faecalis	6.8	4.0	0.55	0.30
S. thermophilus	6.7	3.8	0.70	0.28
S thermophilus	6.6	3.9	0.40	0.30
S thermophilus	7.4	4.5	0.35	0.14
S. cremoris	7.0	5.0	0.35	0.12
S. cremoris	6.8	5.0	0.30	0.25
S. faecalis	6.2	5.0	0.35	0.29
S thermophilus	6.8	4.5	0.60	0.29
S cremoris	6.0	3.5	0.75	0.35
S thermophilus	6.9	5.0	0.30	0.18
S. cremoris	6.8	4.0	0.35	0.26
S. lactis	7.0	5.0	0.25	0.17
S.faecalis	6.8	5.0	0.25	0.15
S.cremoris	6.4	5.0	0.35	0.32
S.cremoris	6.2	5.0	0.35	0.20
S. cremoris	6.1	5.0	0.30	0.29
S. lactis subsp. diacelytactis	6.9	3.5	0.65	0.36
S. cremoris	5.5	3.2	0.40	0.28
S.thermophilus	8.0	5.0	0.30	0.12
S. cremoris	6.7	4.2	0.80	0.36
S. thermophilus	8.2	2.5	0.80	0.42
S. cremoris	6.2	3.0	0.50	0.39
S. cremoris	6.2	4.8	0.30	0.20
S. cremoris	6.8	4.1	0.55	0.26
S. cremoris	7.0	2.5	0.55	0.33
S. cremoris	7.0	5.5	0.30	0.16
L. bulgaricus	6.9	3.0	0.75	0.29
L. bulgaricus	6.2	5.0	0.30	0.20
L. bulgaricus	7.1	5.0	0.35	0.14
L. bulgaricus	7.0	5.0	0.30	0.17
L. lactis	6.9	2.7	0.65	0.34
L. helveticus	6.0	5.0	0.35	0.20
L. bulgaricus	6.3	5.0	0.50	0.33
L. bulgaricus	6.0	5.0	0.50	0.26
L. bulgaricus	6.9	2.5	0.85	0.36
L. helveticus	7.0	5.0	0.30	0.16
L. helveticus	6.9	2.5	0.75	0.30

a. pH of whole milk was 7.6

Table 4. Effect of p^H (final) on the protease activity ($\times 10^{-2}$) of the six LAB.

	p^H						
Name of the LAB	**3.3**	**4.5**	**6.0**	**6.5**	**7.0**	**8.2**	**10.6**
	Specific protease activity						
1.S.thermophilus (PC 26)	1.858	5.583	4.48	2.90	1.50	0.833	0.7692
2. S thermophilus (YY37)	1.897	3.86	4.236	4.24	4.80	5.09	1.212
3. S. cremoris (CG 38)	0.55	2.77	3.512	5.061	6.12	8.88	6.23
	p^H						
	2.8	3.7	5.3	5.9	6.4	8.8	10.0
4. L. bulgaricus (RMY 23)	3.127	7.134	3.216	2.5146	1.766	1.461	1.361
5. L. helveicus (RCG 38)	2.045	2.628	3.03	2.424	2.272	1.818	0.757
6. L. bulgaricus (RB44)	8.9937	5.6289	3.21	2.389	2.201	1.886	0.490

Table 5. Effect of temperatures on the protease activity ($\times 10^{-2}$) of the six LAB.

Name of the LAB	**17°C**	**30°C**	**40°C**	**50°C**	**60°C**
	Specific protease activity				
1. S. cremoris (CG 38)	3.148	4.753	8.88	2.962	2.3456
2. L. bulgaricus (RB 44)	1.25	1.88	8.9937	4.465	0.7546

The strains which showed highest activity such as *S. cremoris* (CG 38) and *L. bulgaricus* (RB 44) were selected for the effect of temperatures on the activity of extracellular proteases as shown in Table 5.The specific activity of extracellular proteases were highest at 40°C and lowest at 60°C.

Discussion

The strains of LAB isolated from local curds were identified and classified according to the criteria described by Swartling (1951), Reiter and Madsen[24] and by Buchanan and Gibbons[6] and Sharpe[27], (Table 1 & 2). From acid producing activity test, *S. thermophilus* (PC 26, YY 37), *S, cremoris* (CG 38), *Lactobacillus bulgaricus* (RMY 23, RB 44) and L. helveticus (RCG 38) were found very efficient. Significant correlations were also found between curd formation and drop of p^H and titratable acidity.

Protease activity is a part of the metabolism of lactic acic bacteria. The temperature and p^H are the important factor which influence the enzymatic activity[14].

According to Mills and thomas[18], *S. cremoris* and *S. lactis* release part of their proteolytic enzymes, without lysing cells, if they are suspended in buffer which is temperature and p^H dependent. However, the present experiment also supports the effect of temperature and p^H on he bacterial protease activities. *S. cremoris* (CG38) showed its optimum proteolytic activity at alkaline p^H 8.2

From Table 4, it could be seen that the protease activity of the 3 isolates of *Lactobacillus* (RMY 23,RCG 38 and RB 44) were optimum at acidic p^H. The results agreed with those of Burlingame-Fray et al (1993). But in case of *Streptococcus* (PC 26,CG 38 and YY 37), it was at alkaline p^H with an exception of *S. thermophilus* (PC 26) which showed its activity at acidic p^H (4.5).

However, at acidic p^H (2.8) the highest activiy was shown by a strain of *Lactobacillus* (RB 44) at p^H 2.8, where as, at alkaline p^H (8.2) the highest activity was recorded in case of *S. cremoris* (CG38). This was an exceptional observation since no such reference was found in the literature.

The overall highest protease activity was however, shown by *L. bulgaricus* (RB 44). The results are supported by Tourneur[32].

The protease activity was the highest at 40°C in case of both *Lactobacillus* and *Streptococcus* spp. which was related with the coagulation of milk during the fermentation of milk. Similar results with *L. bulgaricus* were obtained by Abraham et al[1] and Burlingame Frey et al[7]. They observed the proteolytic activity at 40-42°C.

This study evidenced the variable microbiology of the traditionally made curds. Inconsistent use of lactic cultures as starters for curds was observed in the present investigation. The study also revealed that different species of LAB are used as starter of curds of different places. Better strains could be selected out of these isolates and better curds could be produced in order to develop better organoleptic characters through sereening and selection by efficiency tests. The results of proteolytic activity of the starter cultures (LAB) could be employed during manufacturing practices keeping relation with the p^H and temperature of the media.

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