Technological characterization of lactococci isolated from traditional Chinese fermented milks

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ABSTRACT

To screen lactic acid bacteria for starter cultures in cheese production, 21 *Lactococcus* strains previously isolated from natural fermented milk and koumiss made by herdsman families in the Xinjiang, Gansu, and Qinghai provinces of China were evaluated for optimal growth temperature, acidification activity, proteolytic activity, aminopeptidase activity, and autolytic activity. All isolates presented low acidification rates, and the pH value did not reach 5.3 after 6 h of inoculation in sterile reconstituted skim milk at 30°C. Strains X9C2 and T7C showed the highest proteolytic activity of 24.67 and 23.58 mg of glycine/L of milk, respectively. For aminopeptidase activity, strains X9C2 and T1C2 displayed the highest activities of 30.56 and 27.70 U/mg of protein using L-leucine-*p*-nitroanilide as substrate, respectively. Autolytic activity in simulated cheese-like buffer ranged from 7.45 to 34.76%, and strains Q14C2 and Q16C showed the highest values of 34.76 and 34.20%, respectively. Collectively, one main finding is that some technological characteristics of *Lactococcus* isolates from Chinese traditional fermented products varied greatly. Some isolates with potentially important properties could be valuable for application as starter cultures of cheese or could constitute a mixed culture.

Key words: lactococci, technological characterization, cheese, fermented milk

INTRODUCTION

Lactic acid bacteria (LAB) play a primary role in directly and indirectly affecting most of the biochemical, chemical, and physical processes that occur in curd and cheese (Beresford and Williams, 2004; Parente and Cogan, 2004). Lactic acid bacteria carry out the initial acidification of milk that assists in gelation, and contribute indirectly, via acid production, to syneresis of the curd, expulsion of the whey, and texture of the

cheese. Proteinases and peptidases secreted by LAB could degrade large casein-derived peptides to low-molecular-weight peptides and free amino acids, and play a key role in flavor formation during ripening of cheese (Bartels et al., 1987; Cliffe et al., 1993; Williams and Banks, 1997; Williams et al., 1998). Proteolysis is important in affecting the basic taste of cheese, but its role is more related to the provision of the substrates for enzymes involved in amino acid catabolism, which are often rate-limiting steps in flavor formation (Yvon, 2006). Autolysis of LAB in cheese is of particular importance because it allows key intracellular enzymes involved in cheese ripening to reach their substrates more easily. Therefore, the above properties of LAB are important in their use as starters or adjunct cultures of cheeses. Several studies have been published on the technological properties of lactococci isolated from traditional dairy products (Herreros et al., 2003; Ayad et al., 2004; Lombardi et al., 2004; Aquilanti et al., 2007; Paolo et al., 2008; Nieto-Arribas et al., 2009). These studies on technological characterization showed large intra- and interspecific variability depending on the strains. Some strains showed a distinctive pattern of technological properties and could be good candidates for inclusion as starter cultures in cheese production.

Both raw milk and traditional fermented milk are rich in LAB strains with novel properties (Wouters et al., 2002; Franciosi et al., 2009). Some traditional methods are still used to manufacture natural fermented milks from raw milk without any addition of commercial starter cultures in some herdsman families in Xinjiang, Gansu, and Qinghai provinces in China. After natural domestication for thousands of years, some *Lactococcus* strains contained in traditional fermented dairy products exhibit excellent technological properties. These natural fermented milks are a valuable resource for research and development of cultures. However, studies on screening and characterization of *Lactococcus* strains with potential use as starter cultures for cheese production from traditional fermented milks in China are very limited.

In the present study, 21 *Lactococcus* strains were isolated from Chinese natural fermented milk made

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by herdsman families, and their different technological characteristics were evaluated to allow the selection of strains for starter cultures in cheese production.

MATERIALS AND METHODS

Strains and Culture Conditions

The 21 *Lactococcus* strains tested were isolated from traditional fermented milk, koumiss, and fermented yak milk made in local families in Xinjiang, Gansu, and Qinghai provinces. These isolates were initially identified as *Lactococcus* according to the results of Gram stain reaction, catalase reaction, and sugar fermentation.

Lactococcus lactis ssp. *lactis* SL (ATCC 11454) preserved in the laboratory of Food Science and Engineering at Harbin Institute of Technology (Harbin, China) was used as a standard reference strain.

The strains were routinely propagated in M17 broth (Beijing Land Bridge Technology Co., Ltd., Beijing, China) and stored at -80°C in M17 broth containing 20% (vol/vol) glycerol. The strains were successively subcultured twice in the same broth at 30°C for 18 h before use.

Optimum Growth Temperature of Isolates

The isolates were inoculated (2%, vol/vol) in M17 broth and cultured at 25°C , 31°C , 37°C and 43°C , respectively. The optical density at 650 nm (OD_{650}) was measured using a UV spectrophotometer (Ultrospec 1100 pro, Amersham/GE Healthcare, Piscataway, NJ) after 24 h of incubation.

Acidification Activity

The strains were subcultured in M17 broth overnight at 30°C . The microbial culture was inoculated at a level of 1 mL/100 mL in sterile reconstituted skim milk (100 g/L, Nestlé, Shuangcheng, China). The pH was determined at 0, 6, and 24 h of incubation at the optimal growth temperature using pH meter (AG PB-10 pH meter, Sartorius, Beijing, China).

Proteolytic Activity

The activated strains were inoculated in reconstituted skim milk cultures as described previously. After incubation, 5 mL of the culture was removed, and the proteins were precipitated using 10 mL of 12% trichloroacetic acid. The concentration of free amino groups was estimated using the Cd-ninhydrin method as described by Folkertsma and Fox (1992). The results were calculated from a standard curve obtained from

dilution of glycine in distilled water and expressed as milligrams of Gly per liter of milk.

Aminopeptidase Activity

The strains were inoculated (2%, vol/vol) in M17 broth (80 mL) using an active stock culture. After 16 h of incubation, cells were harvested by centrifugation ($8,000 \times g$, 15 min, 4°C). The obtained pellets were washed twice in sterile 0.85% (wt/vol) saline solution, and resuspended in 4 mL of Tris-HCl buffer (50 mM, pH 7.0). This suspension was precooled to 4°C and mechanically disrupted by vortexing with 150- to 212- μm glass beads for 30 cycles (30 s/cycle), with each cycle being followed by 30 s of cooling (Aquilanti et al., 2007). The supernatants were centrifuged ($8,000 \times g$, 15 min, 4°C), and cell debris was removed to obtain cell-free extracts (CFE) used for enzyme assays.

Aminopeptidase (AP) activity in the CFE was measured using L-leucine-*p*-nitroanilide (Sigma, St. Louis, MO) as a substrate according to El-Soda et al. (2003). One unit of AP activity was defined as the amount of enzyme giving an absorbance increase of 0.01 in 1 min at 410 nm and 30°C . The specific activity was expressed as the number of activity units per milligram of protein in the CFE. The protein concentration was determined by the method of Bradford (1976), and BSA (Sigma) was used as a standard for calibration.

Autolytic Activity

The obtained pellets were resuspended in sodium citrate buffer (50 mM, pH 5.5) containing 0.5 M NaCl and were diluted to $\text{OD}_{650} = 1.0$ (UV spectrophotometer, model Ultrospec 1100 pro, Amersham/GE Healthcare). The autolytic activity was determined as the percentage decrease in the absorbance at 650 nm after 48 h of incubation at 30°C as described by Ouzari et al. (2002).

Statistical Analysis

Each strain was tested twice for optimal growth temperature and thrice for other technological properties; all trials were repeated 3 times. One-way ANOVA was applied to the result of technological properties, using the Student-Newman-Keuls test for comparison of the means ($P < 0.05$). The SPSS software package (version 16.0, SPSS Inc., Chicago, IL) was used for this purpose.

RESULTS

Optimal Growth Temperature

The OD_{650} values of the medium at 24 h of inoculation at different temperature were compared to deter-

Table 1. Optical density at 650 nm values of the medium after 24 h of inoculation at different temperatures

Strain	Temperature			
	25°C	31°C	37°C	43°C
Q9C2	1.484 ± 0.035 ^a	1.523 ± 0.021 ^a	1.538 ± 0.000 ^a	1.554 ± 0.219 ^a
Q11C	1.399 ± 0.100 ^a	1.310 ± 0.025 ^a	1.421 ± 0.016 ^a	1.339 ± 0.054 ^a
Q14C2	1.106 ± 0.105 ^a	1.052 ± 0.028 ^a	1.127 ± 0.057 ^a	1.074 ± 0.033 ^a
Q16C	1.825 ± 0.041 ^a	1.886 ± 0.000 ^a	2.072 ± 0.036 ^b	1.839 ± 0.021 ^a
Q17C	0.980 ± 0.035 ^a	1.369 ± 0.057 ^b	1.310 ± 0.000 ^b	1.329 ± 0.040 ^b
M30C2	1.276 ± 0.011 ^b	1.280 ± 0.006 ^b	1.264 ± 0.017 ^b	1.229 ± 0.000 ^a
X5C	1.018 ± 0.089 ^a	1.022 ± 0.077 ^a	1.069 ± 0.025 ^a	0.982 ± 0.064 ^a
X8C	1.233 ± 0.006 ^a	1.241 ± 0.050 ^a	1.297 ± 0.006 ^b	1.191 ± 0.034 ^a
X9C2	0.854 ± 0.050 ^b	1.021 ± 0.042 ^c	0.916 ± 0.018 ^b	0.697 ± 0.018 ^a
X17C	1.629 ± 0.013 ^a	1.648 ± 0.014 ^a	1.658 ± 0.028 ^a	1.690 ± 0.045 ^a
X18C2	1.368 ± 0.043 ^{ab}	1.438 ± 0.024 ^b	1.337 ± 0.134 ^a	1.306 ± 0.019 ^a
X21C	1.168 ± 0.009 ^a	1.186 ± 0.052 ^a	1.188 ± 0.029 ^a	1.212 ± 0.206 ^a
X20C	1.253 ± 0.033 ^b	1.194 ± 0.010 ^{ab}	1.219 ± 0.035 ^{ab}	1.171 ± 0.013 ^a
T1C2	1.772 ± 0.004 ^b	1.803 ± 0.001 ^c	1.776 ± 0.002 ^b	1.630 ± 0.002 ^a
T1C	1.740 ± 0.001 ^d	1.702 ± 0.004 ^c	1.605 ± 0.000 ^b	0.909 ± 0.001 ^a
T2C	1.777 ± 0.002 ^b	1.806 ± 0.001 ^c	1.780 ± 0.005 ^b	1.647 ± 0.004 ^a
T2C2	1.754 ± 0.003 ^b	1.814 ± 0.003 ^c	1.782 ± 0.000 ^b	1.653 ± 0.004 ^a
T5C	1.699 ± 0.001 ^d	1.662 ± 0.015 ^c	1.591 ± 0.007 ^b	0.853 ± 0.001 ^a
T5C2	1.676 ± 0.002 ^d	1.605 ± 0.017 ^c	1.411 ± 0.018 ^b	0.487 ± 0.009 ^a
T7C	0.775 ± 0.002 ^b	1.809 ± 0.003 ^c	1.780 ± 0.001 ^b	1.609 ± 0.011 ^a
T8C2	1.734 ± 0.003 ^d	1.691 ± 0.006 ^c	1.611 ± 0.005 ^b	0.783 ± 0.007 ^a
SL	1.190 ± 0.100 ^b	1.079 ± 0.184 ^b	1.204 ± 0.015 ^b	0.731 ± 0.077 ^a

^{a-d}Values (mean ± SD) in each row without a common superscript differ significantly ($P < 0.05$).

mine the optimum growth temperature of *Lactococcus* isolates (Table 1). All isolates showed a wide range of optimum growth temperatures, but most isolates (14 of 21) could grow well at 30°C. The optimum growth temperature for isolates Q16C and X8C was found to be 37°C, and for isolates X20C, T1C, T5C, and T5C2 it was 25°C.

Acidification Activity

As shown in Table 2, all isolates could ferment skim milk. Significant differences ($P < 0.05$) in acidifying activity at 6 h were observed among strains, with pH values ranging from 5.63 to 6.06. None of the *Lactococcus* strains were found to be fast acid producers at 6 h because the pH did not decrease to 5.3. All isolates except X9C2 and T2C2 displayed similar behavior after 24 h. Strain X9C2 showed high acidifying capacity, which was similar ($P > 0.05$) to that of strain *L. lactis* SL throughout the incubation time.

Proteolytic Activity

The results showed that proteolytic activity of *Lactococcus* isolates ranged from 18.29 to 24.67 mg of Gly/L of milk, and significant differences ($P < 0.05$) were observed among all isolates (Table 3). Strains X9C2 and T7C showed the highest release of free amino groups (expressed as mg of Gly/L of milk) with values of 24.67 and 23.58 mg of Gly/L of milk, respectively, after 24

h of fermentation. Proteolytic activity of strain X9C2 was similar to that of the reference strain SL ($P > 0.05$).

Table 2. Acidification activity of whole cells of wild *Lactococcus* strains

Strain	pH	
	6 h	24 h
Q9C2	5.71 ± 0.08 ^{ef}	4.94 ± 0.09 ^b
Q11C	5.71 ± 0.07 ^{ef}	5.02 ± 0.10 ^b
Q14C2	5.63 ± 0.06 ^f	4.88 ± 0.10 ^b
Q16C	5.70 ± 0.06 ^{ef}	4.95 ± 0.09 ^b
Q17C	5.75 ± 0.11 ^{cdef}	5.07 ± 0.09 ^b
M30C2	5.78 ± 0.03 ^{cde}	4.97 ± 0.07 ^b
X5C	5.73 ± 0.02 ^{def}	4.91 ± 0.08 ^b
X9C2	5.74 ± 0.02 ^{def}	3.79 ± 0.05 ^c
X8C	5.71 ± 0.03 ^{ef}	5.14 ± 0.06 ^b
X17C	5.75 ± 0.04 ^{cdef}	4.97 ± 0.13 ^b
X18C2	5.80 ± 0.01 ^{cde}	5.01 ± 0.10 ^b
X20C	5.70 ± 0.10 ^{ef}	5.06 ± 0.09 ^b
X21C	5.94 ± 0.02 ^b	5.32 ± 0.15 ^b
T1C2	5.77 ± 0.04 ^{cdef}	4.93 ± 0.14 ^b
T1C	5.76 ± 0.03 ^{cdef}	5.20 ± 0.06 ^b
T2C	5.86 ± 0.01 ^{bcd}	5.06 ± 0.08 ^b
T2C2	6.06 ± 0.03 ^a	5.80 ± 0.09 ^a
T5C	5.74 ± 0.03 ^{def}	4.99 ± 0.11 ^b
T5C2	5.82 ± 0.02 ^{cde}	4.92 ± 0.15 ^b
T7C	5.88 ± 0.01 ^{bc}	4.95 ± 0.12 ^b
T8C2	5.74 ± 0.03 ^{def}	5.13 ± 0.10 ^b
SL	5.78 ± 0.04 ^{cde}	3.76 ± 0.06 ^c

^{a-f}Values (mean ± SD) in the same column without a common superscript differ significantly ($P < 0.05$).

Table 3. Proteolytic activity of whole cells of wild *Lactococcus* strains

Strain	Proteolysis (mg of Gly/L of milk)
Q9C2	19.85 ± 1.15 ^{def}
Q11C	18.64 ± 0.78 ^{ef}
Q14C2	21.98 ± 0.23 ^c
Q16C	19.71 ± 3.13 ^{def}
Q17C	20.47 ± 3.43 ^{cde}
M30C2	18.72 ± 1.07 ^{ef}
X5C	19.95 ± 1.93 ^{def}
X9C2	24.67 ± 3.34 ^{ab}
X8C	20.07 ± 3.03 ^{cde}
X17C	19.07 ± 4.09 ^{ef}
X18C2	17.80 ± 0.12 ^f
X20C	21.77 ± 0.27 ^c
X21C	19.39 ± 3.17 ^{def}
T1C2	21.33 ± 2.42 ^{cd}
T1C	18.80 ± 1.82 ^{ef}
T2C	19.07 ± 3.22 ^{ef}
T2C2	19.41 ± 1.32 ^{def}
T5C	18.29 ± 0.35 ^f
T5C2	19.87 ± 2.45 ^{def}
T7C	23.58 ± 2.75 ^b
T8C2	19.91 ± 0.049 ^{def}
SL	25.52 ± 6.15 ^a

^{a-f}Values (mean ± SD) in the same column without a common superscript differ significantly ($P < 0.05$).

AP Activity

The AP activity of the CFE of the tested strains is shown in Figure 1. Significant differences ($P < 0.05$) in AP activity in CFE were found among isolates, with values ranging between 1.67 and 30.56 U/mg of protein. Strains X9C2 and T1C2 showed higher AP activities at 30.56 and 27.70 U/mg of protein, respectively, followed by strains X18C2 and T2C with 19.85 and 19.75 U/mg of protein, respectively. The AP activities of strains

X9C2, T1C2, X18C2, and T2C were significantly higher than that of strain SL ($P < 0.05$).

Autolytic Activity

The results in Figure 2 show that autolysis rate varied greatly among strains, with values ranging from 7.45 to 34.76%. Strains Q14C2 and Q16C showed the highest rates of autolysis, with values of 34.76 and 34.20%, respectively, followed by Q17C, X8C, X20C, T2C, and T2C2. Strains Q14C2, Q16C, X8C, and T2C showed high autolytic activity, which was similar to that of strain SL ($P > 0.05$).

DISCUSSION

The acid-producing capacity of starters plays an important role in cheese making and can directly affect the production time of cheese. In the present study, all isolates presented low acidification rates, which was in accordance with results reported by Durlu-Ozkaya et al. (2001). The results in this study are also in agreement with those of Ayad (2001) and Ayad et al. (1999), who reported that wild lactococci from traditional Egyptian dairy products had low acidifying activity. It is worth noting that strain X9C2 showed an acidifying capacity similar to that of standard reference strain *L. lactis* SL throughout the incubation time. A rapid decrease in pH is crucial during the initial step of cheese manufacture, because it is essential for coagulation and for prevention or reduction of the growth of adventitious microflora. Thus, the strains with high acidifying capacity in this study may be suitable for cheese starter selection.

Figure 1. Aminopeptidase specific activity of crude cell-free extract of *Lactococcus* strains. One unit of aminopeptidase activity was defined as the amount of enzyme giving an absorbance increase of 0.01 in 1 min at 410 nm after incubation. Aminopeptidase specific activity was expressed as the number of activity units per milligram of protein in the cell-free extracts. Color version available in the online PDF.

Figure 2. Autolytic activity of *Lactococcus* strains from Chinese traditional dairy products. Autolytic activity was determined as the percentage decrease in the absorbance at 650 nm after 48 h of incubation in sodium citrate buffer (50 mM, pH 5.5) containing 0.5 M NaCl at 30°C. Color version available in the online PDF.

Proteolytic activity is involved in the development of some organoleptic characteristics in cheese (Christensen et al., 1999). Strains X9C2 and T7C showed the highest proteolytic activity of 24.67 and 23.58 mg of Gly/L of milk, respectively. Proteolytic activity is a significant property for cultures because it can provide the substrate for enzymes involved in amino acid catabolism, which are often rate-limiting for flavor development (Yvon, 2006). The proteolytic strains X9C2 and T7C might be most suitable for use as starter cultures.

The production of high-quality fermented dairy products is usually dependent on proteolytic systems of strains. Peptidase activity of starter has been extensively studied because peptidase plays a key role in the hydrolysis of bitter peptides and flavor formation during cheese ripening (Bartels et al., 1987). As for AP activity, strains X9C2 and T1C2 displayed the highest activity of 30.56 and 27.70 U/mg of protein, respectively. Ayad et al. (2004) reported that lactococci isolated from traditional Egyptian dairy products with AP activity between 20 and 40 U/mg protein could be classified as high-aminopeptidase strains. According to this standard, the isolated strains X9C2, T1C2, X18C2, and T2C could be regarded as the high-aminopeptidase strains.

Autolysis of lactococci is of special interest regarding their use as starters in fermented dairy foods. The ability to cause autolysis and subsequent release of intracellular enzymes is a desirable trait during the ripening of cheese, and the degree of autolysis is strain-dependent (Wilkinson et al., 1994; El-Soda et al., 2000). Ayad et al. (2004) reported that lactococci isolated from traditional Egyptian dairy products with autolytic activity between 25 and 37% could be classified as high-autolytic-activity strains. In the present study, strains Q14C2,

Q16C, Q17C, X8C, X20C, T2C, and T2C2 could also be regarded as high autolytic strains. Moreover, autolytic activities of strains Q14C2, Q16C, X8C, and T2C were similar to that of strain *L. lactis* SL. These results are comparable to the findings of Ayad (2001), who reported that several wild lactococci strains were found to be stable during cheese ripening, in contrast to industrial strains. It is generally accepted that one of the most effective ways to accelerate cheese ripening is addition of highly autolytic strains. The effect of cell lysis on cheese ripening, particularly the increase of free amino acids due to early lysis and the decrease in bitterness through the hydrolysis of large hydrophobic peptides, has been reported previously (Crow et al., 1995; Lepeuple et al., 1998).

CONCLUSIONS

Our results showed that some technological characteristics of *Lactococcus* isolates from Chinese traditional fermented products varied greatly. Some isolates might be valuable for practical applications as starter cultures for cheese making, such as strain X9C2 because of its desirable acidifying capacity, and high proteolytic and AP activities. Strains T1C2 and X18C2 could also be included because of their high AP activity.

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