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Improvement of lactococcal isolates through adaptive laboratory evolution

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Abstract
Most dairy *Lactococcus* stains grow optimally at 30 °C and are not particularly well adapted to the elevated temperatures (37 °C to 39 °C) to which they are often exposed during cheese production. To overcome this challenge, we used adaptive laboratory evolution (ALE). During microbial ALE, a microorganism is cultivated under clearly defined conditions for prolonged periods of time, in the range of weeks to years, which allows the selection of improved phenotypes. A total 60 isolates of *Lactococcus* were isolated and characterized in our lab were used for adaptation studies Isolate RD1 has been improved through ALE. In which mainly 3 adaptation conditions have been studied such as acid, temperature and sugar adaptations when the acid was adapted from 30 °C to 37 °C, which adapted well to 80 subcultures. After that, sugar was adapted from 4% to 9%, as a result of which it adapted well.

Keywords: ALE, lactococci, *dahi* samples, carbohydrate utilization

Introduction
Adaptive laboratory evolution is a frequent method in biological studies to gain insights into the basic mechanisms of molecular evolution and adaptive changes that accumulate in microbial populations during long term selection under specified growth conditions. Lactic acid bacteria are an order of gram-positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring, either rod-shaped (bacilli) or spherical (cocci) bacteria that share common metabolic and physiological characteristics. These bacteria, usually found in decomposing plants and milk products, produce lactic acid as the major metabolic end product of carbohydrate fermentation. Lactic acid bacteria either undergo homo or hetero fermentation to produce their energy for cell functions and produce biomass. The LAB contain the genera *Lactobacillus, Leuconostoc, Enterococcus, Streptococcus, Lactococcus, Pediococcus* etc., mostly associated with dairy environment. Lactococci are used extensively in food fermentation, which represent about 20% of the total economic value of fermented foods produced throughout the world. Lactococci are coccoid Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria, with L-(+)-lactic acid as their predominant end product of glucose fermentation (Li *et al*., 2020) [11].

The common morphology of lactococci consists of 0.5 to 1-µm diameter spheres or ovoid cells that exist in pairs or chains. Cells of lactococci often elongate in the direction of the chain, which makes them difficult to differentiate from lactobacilli. Lactococcal cultures usually grow in the range 10-30 °C, although some species may grow under temperatures as low as 7 °C upon prolonged incubation for 10–14 days. Cultures typically grow in 4.0% (w/v) NaCl; however, *Lac. Lactis* subsp.*cremoris* tolerates only 2.0% NaCl, which is the only known exception. Lactococci grow best at near neutral pH values in media but cease to grow at about pH 4.5.Lactococci are homo-fermentative; when grown in milk, more than 95% of their end product is lactic acid (of the L isomer). Lactococci are typically used for the production dairy products. Within species *Lactococcus lactis*, two sub species *L. lactis subsp. lactis* and *cremoris* are the most widely being used for dairy fermentation. Lactococcal cultures play a key role for determining the quality of fermented dairy products with respect to shelf-life and sensory quality. A high degree of sequence similarity exists between Streptococcaceae, yet they can be found in a broad range of different environmental niches. Strains of *L. lactis* have been isolated from a range of sources including drain water and human vaginal samples (Gao *et al*., 2011; Kato *et al*., 2012) [1, 7]. Although not a common resident of the gastro intestinal tract (GIT), *L. lactis* is capable of surviving gut passage (Kimoto *et al*., 1999; Meyrand *et al*., 2013) [9, 13].

Materials and Methods

Isolation of Lactococci cultures from Dairy Samples

A total 60 isolates of *Lactococcus* were isolated and characterized in our lab were used for adaptation studies. Out of which only one culture named RD1 was given different stress for adaptation studies. Stress is like this acid stress, temperature stress and sugar stress.

Adaptive Laboratory Evolution of Isolate RD1 for Acidic Conditions

For adapting the isolate to high acidic conditions by gradual exposure to adaptive acid stress, it was repeatedly sub-cultured in pH adjusted M-17 broth medium with range from pH 6.5 to pH 2.0 for a period of 80 days. The growth at every test pH value was assessed by taking the optical density (OD) at 600nm in a spectrophotometer (Systronics UV-VIS Type-119). The stability of the isolate to grow consistently at adapted pH condition was tested for an extended period of 15 days. The adapted isolate named as RD1 (A) was compared to the original wild type isolate RD1 for growth at low pH conditions.

Adaptive Laboratory Evolution of Isolate RD1 (A) for Temperature

For adapting the isolate to high temperature conditions through adaptive laboratory evolution (ALE), it was sub-cultured in M-17 broth medium with a gradual increase in incubation temperature from 30 °C to 45 °C. These gradual adaptations were achieved by repeated sub culturing for duration of 80 days approximately. The growth at every test Temperature value was assessed by taking the optical density (OD) at 600nm in a spectrophotometer (Systronics UV-VIS Type-119). The stability of the isolate to grow consistently at adapted pH condition was tested for an extended period of 15 days.

Adaptive Laboratory Evolution of Isolate RD1 for Sugar Adaptation

For adapting the isolate to high Sugar conditions through adaptive laboratory evolution, it was sub-cultured in M-17 broth medium with a gradual increase in sugar percentage (4% to 9%). These gradual adaptations were achieved by repeated sub culturing for duration of 80 days approximately. The growth at every test sugar value was assessed by taking the optical density (OD) at 600nm in a spectrophotometer (Systronics UV-VIS Type-119). The stability of the trait was tested finally by growing the culture in M-17 broth at adapted temperature and assessing the viability by plate count method. The isolate RD1 is then compared with the adapted isolate that will come.

Result and Discussions

Adaptation of Acid

Isolate RD1 was subjected to repeat sub-culturing for a period of two months in a medium of decreasing pH. The isolate showed robust growth at pH 6.5 and a gradually decreased growth when grown in a medium of lowered pH. The final growth and viability at every adjusted pH with respect to number of days is shown in the figure 3.1.

The *Lactococcus* isolates were cultivated over the pH range from 6.5 to 4 and the OD value at each pH was determined (Table 3). When sample RD1 was grown to 6.5 pH, it was subcultures gradually for 12 days the average OD value of the sample was 1.93 nm. After that, to adapt it from pH 6.5 to pH 6.0, it was subcultures gradually for 13 days and then its OD value came to 1.66. After being adapted gradually at pH 5.5 for 14 days, in which the OD value came to 1.55 nm. It took 16 days of subcultures to adapt from pH 5.5 to pH 5.0, resulting in an OD value of 1.58 nm. After that again adapted 5 pH in the new environment 4.5 pH for days, whose OD value came to 0.98nm. It was again adapted to pH 4.5 to 4 pH for 60 days, and then saw that it has been adapted in 4.0 pH, whose OD value has came to 0.33 nm. After that got adapted one after the other in 3.5 and 3.0 which did not grow well. As a result, the one that is grown at 4.0 pH is the best grown sample for RD1 (A). The RD1 (A) sample was again sub culturing in 15 days for stability test.

Table 1: Comparison between RD1 and RD1 (A)

S. No.	RD1		RD1(A)	
	pH	OD	pH	OD
1	6.5	1.93	6.5	1.93
2	6.0	0	6.0	1.66
3	5.5	0	5.5	1.55
4	5.0	0	5.0	1.58
5	4.5	0	4.5	0.98
6	4	0	4.0	0.33
7	3	0	3	0.23

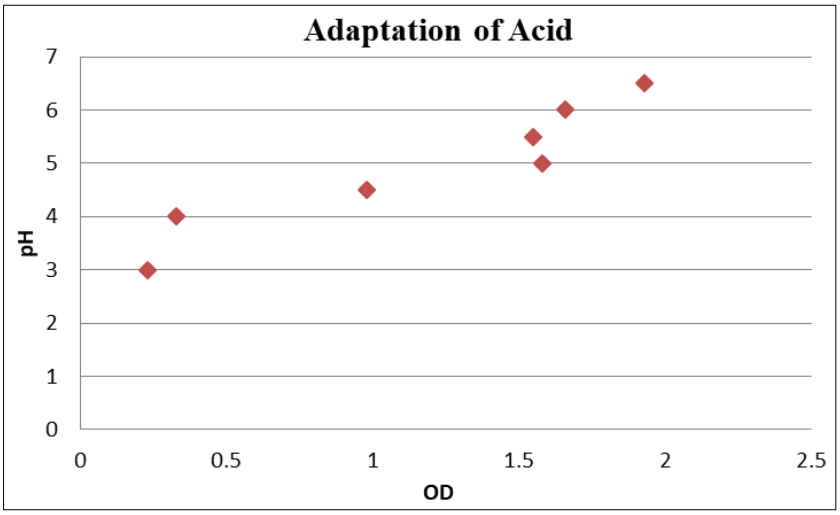

Fig 1: The Graphical Image of Adaptation of Acid

Adaptation of Temperature

The Lactococci isolates were cultivated over the temperature from 30 °C to 42 °C and the number of viable cells at each temperature was determined in (Table 3). The number of viable cells increased significantly at 30 °C. When the temperature was raised from 30 °C to 32 °C gradually for 8 days, it was observed that the OD value was 1.88nm. After well growth, again raised the temperature from 32 °C and did 20 subcultures which resulted in the OD value was 1.66nm. Bringing the temperature of 35 °C, for which the temperature was gradually raised and 28 subcultures were done, which resulted in the OD value was 1.55nm. Again the temperature was brought down from 37 °C. Then gradually increased the subculture to 45 subcultures. In which the OD value came to 1.47nm. When the temperature was shifted 39 °C there was a slight increase the OD was 0.93, indicating that the cells were still growing slowly at 39 °C temperature and also growing slowly in 42 °C there OD was 0.94 nm. The Sample RD1 (T) was sub-cultured for 20 days for stability test.

Table 2: Comparison between RD1 and RD1 (T)

S. No.	RD1		RD1(T)	
	Temperature	OD	Temperature	OD
1	30 °C	1.88	30 °C	1.88
2	32 °C	0	32 °C	1.66
3	35 °C	0	35 °C	1.55
4	37 °C	0	37 °C	1.47
5	39 °C	0	39 °C	0.93
6	42 °C	0	42 °C	0.94

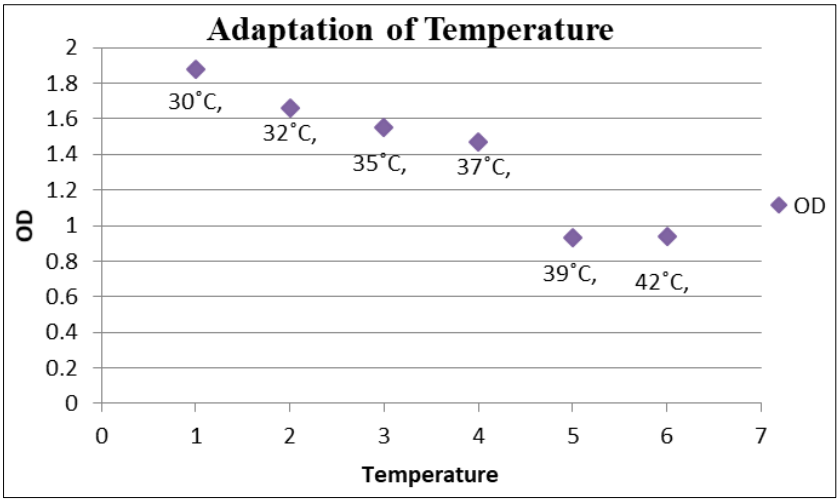

Fig 2: The Graphical Image of Adaptation of Temperature

Adaptation of Sugar

The Lactococci isolates were cultivated over the sugar percentage from 4% to 9% and the number of viable cells at each sugar percentage was determined in (Table 3). Isolate RD1 was grown with 4% sugar followed by 7 subcultures, with the OD value was 1.93nm. Again the isolate was adapted from 4% to 5% in which 9 subcultures were done; resulting in the OD value was 1.84nm. Again the percentage of sugar was increased from 5% to 6% percent and again it was subjected to 11 subcultures, as a result of which the OD value came to 1.71nm. After that the sugar was increased again from 6% to 7% and was sub-cultured to 12, in which the OD value came to 1.63 nm. Again when adapted in 7% to 8% then 36 subcultures were done whose OD value came to 0.96 nm.

Table 3: Comparison between RD1 and RD1 (S)

S. No.	RD1		RD1(T)	
	Sugars	OD	Sugars	OD
1	4%	1.93	4%	1.93
2	5%	0	5%	1.84
3	6%	0	6%	1.71
4	7%	0	7%	1.63
5	8%	0	8%	0.96
6	9%	0	9%	0.93

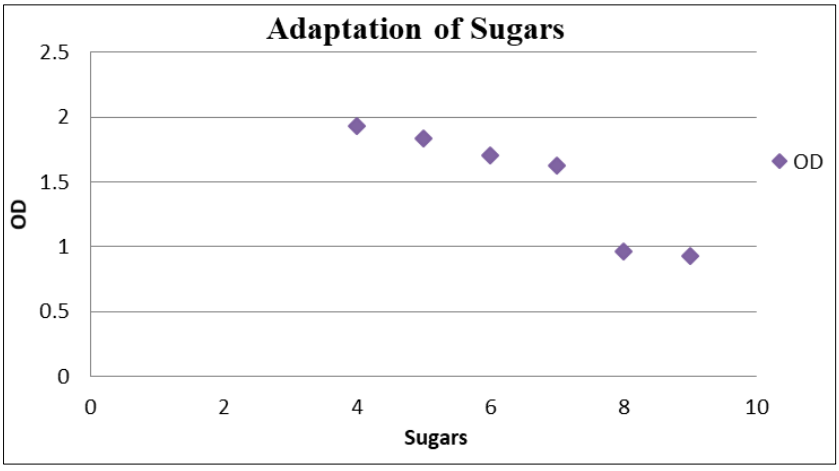

Fig 3: The Graphical Image of Adaptation of Sugars

Gonzalez *et al*. (2017) [3] evaluated the salt tolerance of *Lactococcus lactis* R-604 as influenced by exposure to various stressed conditions. The culture was exposed to 10% v/v ethanol for 30 minutes, 15 mM of hydrogen peroxide for 30 minutes, mild heat at 52 °C for 30 minutes and UV light (245 nm) for 5 minutes, starvation (no lactose in M-17 broth) for 24 hours or prior osmotic adaption (3% w/v NaCl in M17 broth) for 24 h aerobically at 30 °C. Results demonstrated that salt tolerance of *Lactococcus lactis* R-604 was enhanced after ethanol or mild heat exposure at 5% w/v NaCl on days 3, 4 and 5; after hydrogen peroxide exposure at 5% w/v NaCl on days 4 and 5; and after lactose starvation at 3% w/v NaCl on day 3. Growth of *L. lactis* R-604 was not negatively affected by any of the stress conditions applied at salt concentrations of 0, 1 and 3% w/v.

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References

1. Gao Y, Hamid N, Gutierrez-Maddox N, Kantono K, Kitundu E. Development of a probiotic beverage using breadfruit flour as a substrate, Foods (Basel, Switzerland). 2019;8(6):214.
2. Gao Y, Lu Y, Teng KL, Chen ML, Zheng HJ, Zhu, *et al*. Complete genome sequence of Lactococcus lactis subsp. lactis CV56, a probiotic strain isolated from the vaginas of healthy women. Journal of bacteriology. 2011;193(11):2886-2887.
3. Gonzalez EE, Olson D, Aryana K. Salt tolerance of Lactococcus lactis R-604 as influenced by mild stresses from ethanol, heat, hydrogen peroxide, and UV light. Journal of Dairy Science. 2017;100(6):4290-4293.
4. Harris LJ, Fleming HP, Klaenhammer TR. Characterization of two nisin-producing Lactococcus lactis subsp. lactis strains isolated from a commercial sauerkraut fermentation. Applied and environmental microbiology. 1992;58(5):1477-1483.
5. Harun-ur-Rashid M, Togo K, Ueda M, Miyamoto T. Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk Dahi in Bangladesh. World Journal of Microbiology and Biotechnology. 2007;23(1):125-133.
6. Itoi S, Abe T, Washio S, Ikuno E, Kanomata Y, Sugita H. Isolation of halo tolerant Lactococcus lactis subsp. lactis from intestinal tract of coastal fish. International journal of food microbiology. 2008;121(1):116-121.
7. Kato H, Shiwa Y, Oshima K, Machii M, Araya-Kojima T, Zendo, *et al*. Complete genome sequence of *Lactococcus lactis* IO-1, a lactic acid bacterium that utilizes xylose and produces high levels of L-lactic acid. Journal of bacteriology. 2012;194(8):2102-2103.
8. Kelly WJ, Davey GP, Ward LJ. Characterization of lactococci isolated from minimally processed fresh fruit and vegetables. International Journal of Food Microbiology. 1998;45(2):85-92.
9. Kimoto H, Kurisaki J, Tsuji NM, Ohmomo S, Okamoto T. Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. Letters in Applied Microbiology. 1999;29(5):313-316.
10. Lancefield RC. A serological differentiation of human and other groups of hemolytic streptococci. Journal of experimental medicine. 1993;57(4):571-595.
11. Li W, Ren M, Duo L, Li J, Wang S, Sun Y, *et al*. Fermentation characteristics of *Lactococcus lactis* subsp. lactis isolated from naturally fermented dairy products and screening of potential starter isolates. Frontiers in microbiology. 2020;11:1794.
12. Mannu L, Paba A, Pes M, Scintu MF. Genotypic and phenotypic heterogeneity among lactococci isolated from traditional Pecorino Sardo cheese. Journal of applied microbiology. 2000;89(2):191-197.
13. Meyrand M, Guillot A, Goin M, Furlan S, Armalyte J, Kulakauskas S, *et al*. Surface proteome analysis of a natural isolate of Lactococcus lactis reveals the presence of pili able to bind human intestinal epithelial cells. Molecular & Cellular Proteomics; c2013. p. 113.
14. Mitra S, Chakrabartty PK, Biswas SR. Production of nisin Z by Lactococcus lactis isolated from Dahi. Applied biochemistry and biotechnology. 2007;143(1):41-53.
15. Mugula JK, Nnko SAM, Narvhus JA, Sørhaug T. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. International journal of food microbiology. 2003;80(3):187-199.
16. Ponce AG, Moreira MR, Del Valle CE, Roura SI. Preliminary characterization of bacteriocin-like substances from lactic acid bacteria isolated from organic leafy vegetables. LWT-Food Science and Technology. 2008;41(3):432-441.
17. Saeed AA, Hasoon MF, Mohammed MH. Isolation and molecular identification of Salmonella typhimurium from chicken meat in Iraq. J. World's Poult. Res. 2013;3(2):63-67.
18. Sangami R, RadhaiSri S. Probiotic oats milk drink with microencapsulated Lactobacillus plantarum an alternative to dairy products. Nutrition & Food Science. 2020;7(8):14912-14915.
19. Salama MS, Musafija-Jeknic T, Sandine WE, Giovannoni SJ. An Ecological Study of Lactic Acid Bacteria: Isolation of New Strains of Lactococcus Including Lactococcus lactis subspecies cremoris. Journal of Dairy Science. 1995;78(5):1004-1017.
20. Shah NP. Functional cultures and health benefits, International Dairy Journal. 2007;11:1262-1277.
21. Terzaghi BE, Sandine W. Improved medium for lactic streptococci and their bacteriophages. Applied microbiology. 1975;29(6):807-813.
22. Tomovska J, Gjorgievski N, Makarijoski B. Examination of pH, titratable acidity and antioxidant activity in fermented milk. Journal of Materials Science and Engineering, 2016;6(11):326-333.
23. Wichchukit S, Omahony M. The 9-point hedonic scale ranking in food science: some reappraisals and alternatives. Journal of the Science of Food and Agriculture. 2015;95(11):2167-2178.