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Isolation and evaluation of lactic acid production by local isolated Lactic Acid Bacteria

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Abstract

Lactic acid is an organic acid which has numerous applications in the food, pharmaceutical, cosmetics and chemical industries. New application such as biodegradable plastic made from poly (lactic) acid and its production is currently attracting a great deal of research and development.

Lactic acid can be produced commercially by either chemical synthesis or fermentation, but in recent years fermentation process has become more industrially successful because of the increasing demand for naturally produced lactic acid.

This study was embarked upon to obtain laboratory strains of lactic acid bacteria (LAB) from some local raw milk with potential for the production of lactic acid.

Some samples of local raw cow milks collected from different regions of Iran were transferred to research lab under the aseptic conditions.

Lactic acid bacteria were grown on specific culture media (M_{17}, KA, MRS) and a total of 26 isolates were screened and evaluated about acidification on the basis of their milk pH lowering potentials. Finally, *Enterococcus faecium* subsp was determined as the best acidifier LAB. Rich biodiversity of natural environments can be considered as good sources of new LAB species or strains.

Keywords: Lactic Acid Bacteria, acidification, lactic acid, raw milk.

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1. Introduction

Lactic acid (2-hydroxypropionic acid, $C_3H_6O_3$) is an organic hydroxyl acid whose occurrence in nature is widespread. It was discovered and isolated in sour milk [1].

Lactic acid is colorless, sour in taste, odorless and soluble in all proportions in water, alcohol and ether but insoluble in chloroform. It is a weak acid with low volatility, and is one such product that has numerous applications in chemical compound pharmaceutical, cosmetic, technical and especially in food industry [2, 3]. It finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications [4].

New application such as biodegradable plastic made from poly (lactic) acid, have the potential to greatly expand the market for lactic acid which has been widely used in many disposable packaging applications [5]. A review published in 1995 stated that 85% of lactic acid in the USA was used food-related applications, and an emerging application was its use for production of biodegradable and biocompatible polylactate polymers,

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which provided an environmental friendly plastic industry derived from petrochemical materials [1].

Lactic acid exists naturally in two optical isomers: D(−)-lactic acid and L(+)-lactic acid. Since elevated levels of the D-isomer are harmful to humans [6], L(+)-lactic acid is the preferred isomer for food-related and pharmaceutical industries. Lactic acid is the first manufactured on a commercial scale organic acid to be produced by fermentation [7]. It can be produced by microbial fermentation or by chemical synthesis but in recent years fermentative ***lactic acid production*** has become more industrially successful because of the increasing demand for naturally produced lactic acid [7]. Lactic acid producing microorganisms are proprietary. There are numerous species of bacteria and fungi which can produce relatively large amount of lactic acid from carbohydrates [8, 9]. However, in industrial fermentation the use of various species of lactic acid bacteria is preferred because of their higher conversion, yield, rate of metabolism and the ability to adapt and present in different environments [10].

Lactic acid bacteria are widely used in fermentative foods. They can improve flavor, texture and safety perishable raw materials such as milk, meat and vegetable and the presence of LAB in fermented foods improves the health safety [11]. They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid [12, 15].

Lactic acid bacteria are a group of Gram-positive bacteria, non-spore forming, cocci or rods, that excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrate. Lactic acid bacteria have been traditionally defined by the formation of lactic acid as a sole or main end product from carbohydrate metabolism [13].

Lactic acid bacteria are more suited to grow in plant extracts [14]. They are often found in carbohydrate containing substrates such as plants and materials of plant origin [15].

The aim of this study was to evaluate of ability for lactic acid production in local isolated lactic acid bacteria to find local strains for application in food industry.

2. Materials and Methods

2.1 Sample collection

Six samples of raw cows' milk from six cities of Iran were selected. No. 1, 2, 3 and 4 samples collected from different cows of Araak city. No. 5 sample in Semnan and 6 in Isfahan cities. Samples were immediately transported to the lab under the 4ºC and aseptical conditions. A total of six milk samples were subjected to microbiological analysis.

2.2 Cell enumeration, isolation of microorganisms and culture conditions

Milk samples were homogenized and serially diluted in peptone water (0.1%) [16]. Dilutions were incubated as follows: microaerophilic condition onto MRS agar (Man-Rogosa-Sharpe agar, Merck KGaA) for 2 days in 30°C for mesophilic and 45°C for thermophilic LAB rods, aerobically onto M_{17} agar (Medium for lactic Streptococci) for 2 days at 30°C for mesophilic LAB cocci; microaerophilic condition onto M_{17} agar for 2 days at 45°C for thermophilic LAB cocci; aerobically onto KA agar (Kanamycin Aesculin, HiMedia Laboratories Pvt. Ltd.) for 2 days at 37°C for enterococci. Total bacterial count (TBC) was abtained on plate count agar added with $1gL^{-1}$ skimmed milk, aerobically incubated at 30°C for 24h (Table 1) [17, 18, 19].

Colonies with the different morphology from types of culture media were selected and analysed about Gram stain and catalase test.

The colonies with the Gram-positive and catalase-negative (To transfer fresh colonies to a glass slide and adding H_2O_2) [20] characterizations were purified by successive sub-culturing on M_{17} and MRS and KA agar. The cultures were maintained at −70°C in glycerol stocks after purity [21].

2.3 Evaluation of acidification

Acidification of milk by starter culture is a crucial step in fresh cheese manufacturing since acid production is predominant in curd formation [16].

To evaluate the acidifying capacity, six mL LAB cultures ($O.D._{600\ nm}$: 0.5) were centrifuged at 8000×g for 5 min, washed with peptone water and inoculated in 10 mL 3% fat ultra-high temperature (UHT) milk, then, were incubated at their optimal growth temperatures as above. Measurement of pH were performed at 2 hours intervals during the first 8 hours and after that at 24 hours and 48 hours after inoculation (Table 3, 4, 5) [16].

2.4 Extraction of genome and 16SrRNA analysis

Bacterial isolates from the best acidification potential were selected for molecular identification as follows: they were grown overnight in LB (Loria-Bertani) broth medium. 1.5 mL were centrifuged at 5000×g

for 3 min and the pellets resuspended in HTE (HCl Tris EDTA) buffer. Extraction of the genomic DNA was performed according to the Sambrook and Russell (2001) protocol [22].

PCR was carried out about 16SrRNA gene with the universal primers (Forward: AGAGTTTGATCCTGGCTC and Reverse: ACGGCTACCTTGTTACGA) for molecular identification. PCR reactions were performed according to the method of Farahani *et al.* (2017) [18]. After sequencing, they were analysed in the GenBank data library of NCBI (National Center for Biotechnology Information). The isolated strain was identified in GenBank using a BLAST (Basic Local Alignment Search Tools) program in the National Center for Biotechnology Information (NCBI) nucleotide database. The molecular classification of the isolate was determined by 16SrDNA sequence analysis [18].

3. Results and discussion

3.1 Microbial cell count and isolation of microorganisms

Cell count are reported in Table 1.

Table 1. Enumeration of microorganisms (cf.mL^{-1}) onto PCA*

Samples	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
PCA	$4.4*10^6$	$1.3*10^5$	$8.6*10^4$	$2.1*10^7$	$1.2*10^7$	$1.0*10^7$

*Medium abbreviations: PCA, plate count agar.

26 isolates were screened onto specific culture media (MRS agar, M_{17} agar and KA agar) based on the morphology of colonies, which are reported in Table 3. Results showed dominance of LAB cocci over LAB rods (Table 2). Same results have obtained by Elena Franciosi *et al.* (2009) and Ioanna-Areti Asteri *et al*. (2009) [23, 24].

On the other hand the mesophilic lactococci are dominant of thermophilic lactococci and also the mesophilic rods are dominant of thermophilic rods [16].

Table 2. Results of screening onto specific culture media (KA, MRS and M_{17} agar)

Culture medium	M_{17} (30°C)	M_{17} (45°C)	MRS (30°C)	MRS (45°C)	KA
Sample Number	1 – 4	5 – 10	12	11,13	14 – 26

3.2 Acidification potential

Acidification findings (Fig. 1, 2, 3) showed that during the first 24 hours pH milk were falling. The enterococci (sample number 14–26) (Table 2) were more than effective the other LAB about to decrease milk pH and the strongest acidifier LAB after 24 hours (Fig. 4) was number 25. We also observed at 8 hours a large range in acid production for thermophilic lactococci (Table 3, 4, 5).

Paolo Piraino et al. (2008) [25] studied on pH in different temperatures and times. In this study we obsereved that relatively the highest acid producing ability at 24 hours are enterococci (Fig. 4) and Paolo Piraino et al. (2008) also were observed a relatively large range in acid production for enterococci at 24 hours. They also were observed a large range in acid production for enterococci and lactococci at 6 hours.

Table 3: Evaluation of acidification of LAB isolated from raw caw milk (first test)

Samples	0h	2h	4h	6h	8h	24h	48h
Control	6.600	6.618	6.759	6.865	6.818	6.392	5.919
1	6.622	6.879	6.884	6.873	6.942	6.330	5.200
2	6.572	5.790	5.681	5.454	5.383	4.464	4.371
3	6.542	6.219	6.129	5.980	5.938	4.930	4.629
4	6.500	5.709	5.776	5.591	5.522	4.292	4.393
5	6.565	5.939	5.766	5.676	5.423	4.428	4.661
6	6.566	5.998	5.755	5.598	5.468	4.724	4.630
7	6.411	5.989	5.665	5.497	5.390	4.676	4.648

8	6.589	6.050	5.763	5.623	5.447	4.763	4.680
9	6.495	6.118	6.067	5.948	5.848	5.178	4.662
10	6.549	6.159	6.043	5.908	5.842	5.210	4.645
11	6.652	6.579	6.426	6.240	6.061	5.440	5.354
12	6.672	6.061	5.761	5.478	5.365	4.620	4.291
13	6.628	6.588	6.450	6.341	6.218	5.547	5.098
14	6.662	6.733	6.620	6.582	6.620	3.408	4.615
15	6.663	5.730	5.566	5.479	5.378	4.685	4.569
16	6.600	5.708	5.630	5.467	5.387	4.732	4.569
17	6.570	6.367	6.060	5.728	5.630	4.891	4.595
18	6.669	5.805	5.607	5.455	5.337	4.718	4.549
19	6.668	5.814	5.676	5.552	5.465	4.895	4.647
20	6.663	5.848	5.627	5.646	5.348	4.750	4.535
21	6.666	5.727	5.496	5.330	5.242	4.576	4.514
22	6.662	6.466	6.185	5.855	5.688	4.068	4.687
23	6.669	5.863	5.570	5.278	5.332	3.167	4.538
24	6.660	5.774	5.579	5.354	5.256	4.535	4.583
25	6.661	5.617	5.450	5.260	5.220	3.231	4.729
26	6.667	6.394	5.888	5.538	5.369	2.868	4.647

Table 4: Evaluation of acidification of LAB isolated from raw caw milk (second test)

Samples	0h	2h	4h	6h	8h	24h	48h
Control	6.628	6.581	6.686	6.640	6.729	6.113	5.157
1	6.719	6.803	6.946	7.007	7.088	6.219	5.293
2	6.598	6.160	6.066	5.974	5.989	5.317	4.829
3	6.667	6.564	6.501	6.545	6.477	5.893	5.205
4	6.300	6.000	5.678	5.575	5.526	5.126	4.580
5	6.663	6.461	6.219	6.128	5.982	5.514	5.194
6	6.610	6.203	5.646	5.425	5.315	5.003	4.937
7	6.570	6.157	5.615	5.354	5.251	4.984	4.920
8	6.615	6.573	6.644	6.649	6.604	5.212	4.529
9	6.636	5.779	5.351	5.151	4.976	4.376	4.328
10	6.571	6.121	5.781	5.554	5.497	4.875	4.758
11	6.668	6.534	6.341	6.150	5.964	5.213	5.061
12	6.647	6.465	6.203	5.717	5.542	4.858	4.769
13	6.514	6.499	6.191	6.081	5.926	5.325	5.097
14	6.701	6.765	6.755	6.696	6.687	6.065	5.353
15	6.671	6.528	6.224	6.058	5.896	5.153	4.977
16	6.719	6.599	6.257	5.984	5.845	5.176	5.007
17	6.682	6.701	6.547	6.276	6.104	5.213	5.338
18	6.632	6.545	6.196	5.996	5.833	4.991	4.848
19	6.523	6.466	6.227	6.049	5.892	5.175	4.928
20	6.681	6.506	6.265	6.045	5.818	4.998	4.917
21	6.701	6.502	6.158	5.975	5.861	5.060	4.928
22	6.582	6.487	6.274	6.050	5.915	5.171	5.069
23	6.630	6.611	6.266	5.931	5.826	5.053	4.985
24	6.579	6.465	6.167	5.956	5.807	4.887	4.782
25	6.565	6.296	6.114	5.787	5.670	5.190	4.866

26	6.660	6.553	6.303	5.938	5.783	5.198	5.151

Table 5: Evaluation of acidification of LAB isolated from raw caw milk (Third test)

Samples	0h	2h	4h	6h	8h	24h	48h
Control	6.550	6.600	6.553	6.564	6.560	6.050	5.138
1	6.566	6.385	6.130	6.051	6.040	5.641	5.068
2	6.705	6.745	6.714	6.692	6.623	5.771	5.566
3	6.685	6.643	6.641	6.568	6.464	5.536	3.806
4	6.565	6.586	6.594	6.150	6.555	6.249	6.030
5	6.570	6.171	5.607	5.294	5.240	5.021	4.914
6	6.628	5.902	5.485	5.354	4.936	4.512	4.909
7	6.663	5.167	4.984	5.001	4.947	4.850	4.804
8	6.610	6.601	6.514	6.312	6.124	5.229	5.225
9	6.572	6.139	5.703	5.493	5.401	4.851	4.743
10	6.589	5.920	5.729	5.449	5.213	4.859	4.670
11	6.680	6.390	6.362	6.053	4.996	5.125	4.942
12	6.500	6.675	6.576	6.584	6.486	5.703	5.242
13	6.663	6.492	6.560	6.214	6.132	5.628	5.027
14	6.651	6.626	6.637	6.571	6.530	6.314	4.944
15	6.411	6.288	6.644	5.975	5.908	4.744	3.108
16	6.500	6.241	5.442	5.901	5.896	5.253	4.802
17	6.647	6.196	5.961	5.832	5.794	3.687	4.629
18	6.662	6.277	5.907	5.815	5.590	4.067	4.714
19	6.615	6.355	6.109	5.875	5.896	5.089	4.726
20	6.589	6.345	6.122	5.952	5.944	3.298	4.892
21	6.501	6.274	6.027	5.850	5.689	5.054	4.669
22	6.571	6.441	6.126	5.979	5.872	5.216	4.889
23	6.598	6.214	6.162	5.915	5.799	5.329	4.784
24	6.597	6.433	6.301	6.033	5.880	4.072	4.802
25	6.601	6.191	6.072	5.773	5.686	3.630	4.900
26	6.692	6.732	6.664	6.420	6.070	4.976	4.911

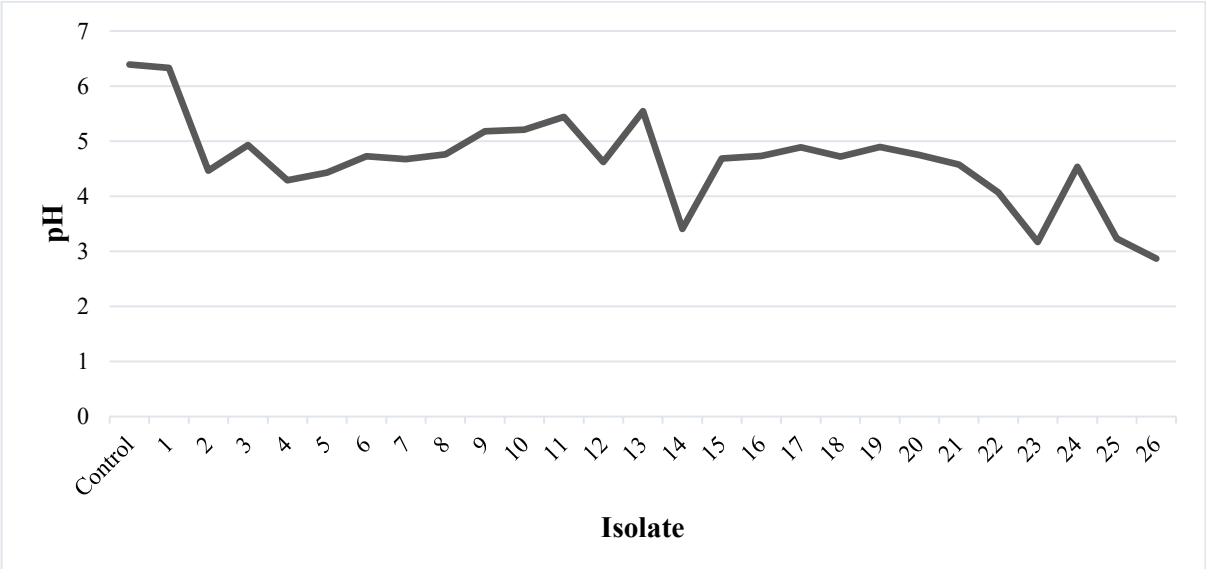

Fig. 1. Milk pH of LAB isolated from raw caw milk after 24 hours on first test

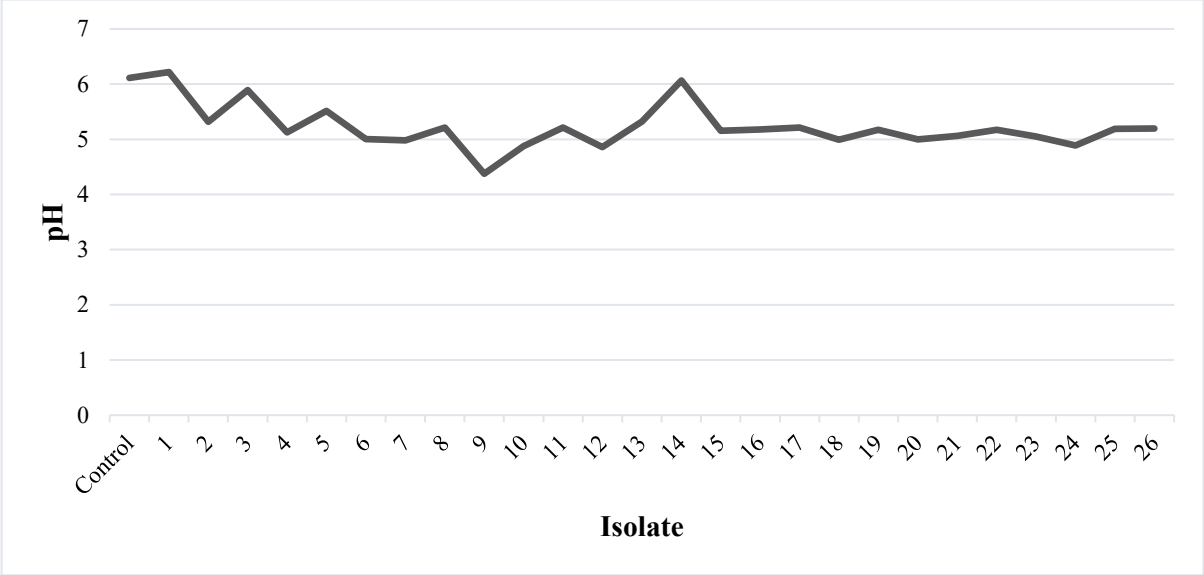

Fig. 2. Milk pH of LAB isolated from raw caw milk after 24 hours on second test

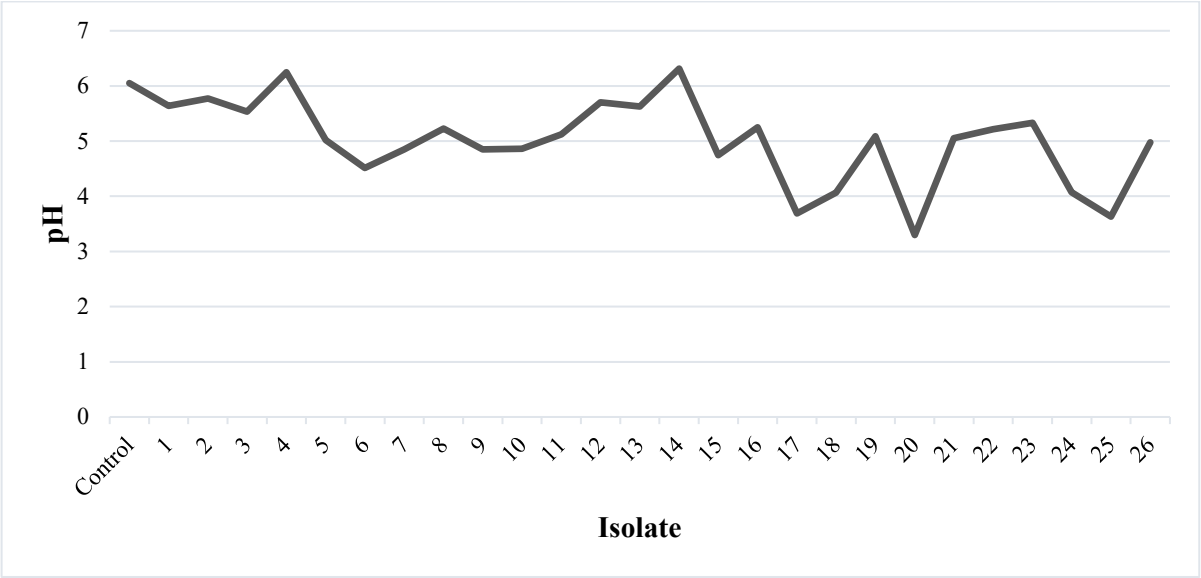

Fig. 3. Milk pH of LAB isolated from raw caw milk after 24 hours on third test

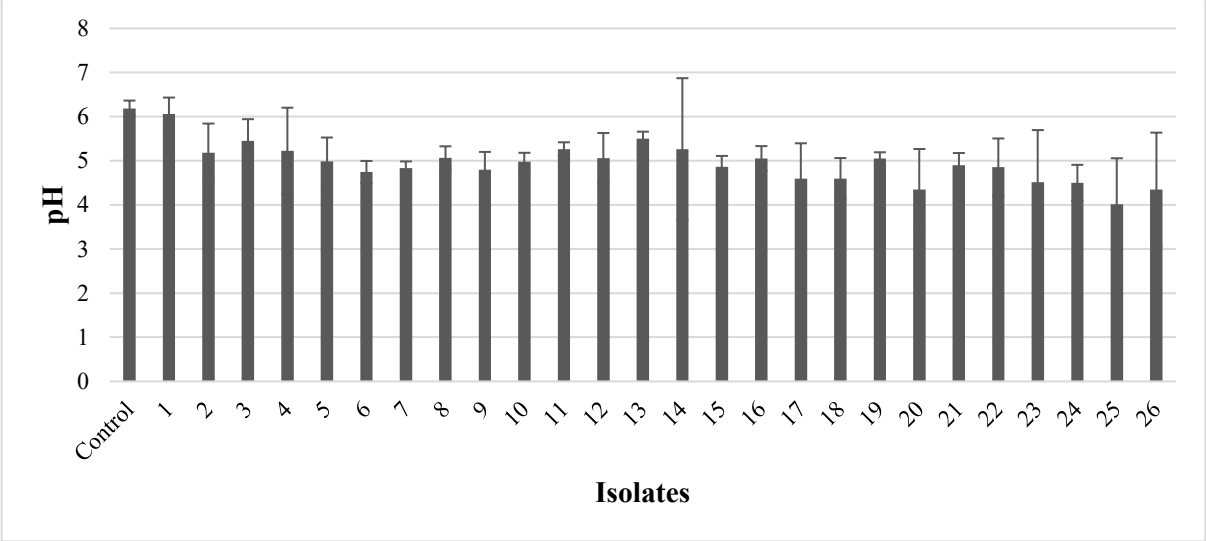

Fig. 4. Milk pH of LAB isolated from raw caw milk after 24 hours (Mean tests)

3.3 Extraction of genome and 16SrRNA analysis

Whereas the number of 25 was the strongest acidifier LAB after 24 hours (Fig. 4), it was extracted and then 16SrRNA gene amplified with PCR and to analyse it in NCBI have shown it is *Entrococcus faecium.* So, in this study the *Entrococcus faecium* was the highest acid producing strain [18].

4. Conclusion

This study provides evidence that natural environments are rich in biodiversity and can be considered as proper sources of new strains and species within the LAB group. Since the screening and finding local strains has been considered in the food industry, with this view we can focus on pleasant local dairy products for finding the new strains and apply them in food industry. The *Entrococcus* is one of the best genus about lactic acid production, so we can focus on this genus to find better strains for lactic acid production in the industry.

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