# Biotechnological parameters determination for cultivation of lactic acid bacteria from goat milk

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**Keywords:**

Milk
Goat
Lactic
Acid
Bacteria
Biotechnological

**Article history:**

Received 23.03.2018
Received in revised form 25.05.2018
Accepted 27.12.2018

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**DOI:**10.24263/2304-974X-2018-7-4-6

## Abstract

**Introduction**. The purpose of research – to establish optimal cultivation conditions to determine of the efficacy of development lactic acid bacteria isolated from goat milk.

**Materials and methods**. Strains cultivation were performed in sterilized goat milk medium at the 30±2 °C. Parameters determination have been established for the periodic cultivation in bioreactor Sartorius Biostat® A plus. Count enumeration of lactic acid bacteria cells was performed using the spread count method.

**Results and discussion**. The slow pH decrease was observed at all selected strains during cultivation time. Lactic acid has been accumulated proportional what shows the intense development of the lactic acid bacteria up to 14 cultivation hours. The active development is characterized through moderate acidity of fermented milk.

Data of strains development dynamics and the biomass accumulation in the medium goat milk-based proved the obtaining to maximum count of lactic acid bacteria cells lg CFU/g.

At the same time the regression analysis of the pH value dinamics and viable cells count were performed for exactly describes results of the experiments. The strains CNMN-LB-73, CNMN-LB-74, CNMN-LB-77 and CNMN-LB-78 has the accurate regression line at pH dinamic and strains CNMN-LB-73, CNMN-LB-74, CNMN-LB-75, CNMN-LB-76 and CNMN-LB-78 at the dynamic of cells development. Strains development stages have observed since 10 hours of cultivation. The decline phase started after 12 hours for CNMN-LB-76, CNMN-LB-77, CNMN-LB-79 strains and after 14 hours for CNMN-LB-73, CNMN-LB-74, CNMN-LB-75, CNMN-LB-78, indicating that they are more resistant to acid conditions that isolated strains reported by other authors, cell multiplication was observed until the pH 4.7.

**Conclusions.** In conditions of periodic cultivation biotechnological parameters for cultivation of lactic acid bacteria from goat milk were determinated. Obtained data demonstrated important biotechnological properties of isolated strains.

## Introduction

Milk is a complete and favorable medium for many microorganisms or a convenient survival medium for other microorganisms and viruses that do not multiply in milk but can pollute it.

The main source of milk contamination is the external environment; the microorganisms reach the milk from the atmosphere due to the lack of hygiene of the shelters and the animal, the way of the milk processing, milking, the way of cooling and transporting of the milk and the water that does not correspond the sanitary-veterinary requirements.

It is worth mention that goats has much lower sanitary-epidemiological risk because they do not suffer from such diseases as brucellosis, tuberculosis and other diseases affecting bovine animals, doing goat's milk consumed raw. Raw goat milk preserve its proteins, lipids, sugars, vitamins, enzymes and mineral salts which have higher antioxidant properties compared to cow's [1, 2]. That is why goat milk products have nutritional and curative properties [3, 4].

There is research demonstrated that goat milk and goat milk products contain more less pathogenic bacteria than other animals' milk [5].

Many authors have published results of the quality and safety research of cheeses made from raw goat milk. These cheeses did not contain pathogenic bacteria of the *Salmonella, Listeria, Escherichia, Staphylococcus* species compared to cheeses made from other animals' milk [6, 7].

It is well known that milk is also a natural source of lactic bacteria strains which are specific microflora and in most cases useful for the dairy industry. These bacteria have important biotechnological properties for the food industry. The starter cultures contained lactic acid bacteria are widely used for dairy products from cow's milk. At the same time goat milk products obtaining needs in the cultures from lactic acid bacteria isolated from goat milk – their natural habitat. In laboratory of food biotechnology investigations were carried at isolation and selection of perspective autochtonous lactic acid bacteria from raw goat milk and determination their biotechnological parameters of cultivation for starter culture creation.

For the industrial production of dairy products with regulation of legislation should be used pasteurized milk and chosen starter culture which can provide a high product quality and safety with a long shelf life due to active biotechnological properties.

From different regions of Republic of Moldova were studied 150 samples of raw goat milk. As result of cultural, morfological, phisiological and biochemical tests were selected 7 isolates with characteristic features to the species *Lactococcus lactis ssp. lactis*, *Lactococcus lactis* ssp. *lactis biovar diacetylactis*, *Lactococcus lactis ssp.cremoris* and *Streptococcus thermophilus.*

Strains have been characterized by valuable technological properties as protective culture to inhibit the growth of *Salmonella* and *Escherichia* population in goat cheese.

The selection of new lactic acid bacteria strains for the industrial purpose based on pure cultures that have improved organoleptic properties of the product. It is important to establish the optimal conditions for cultivation that to determine the development efficacy [8].

This paper presents the results of biotechnological parameters determination for lactic bacteria cultures, obtained at conditions of periodic cultivation.Based on the obtained data, it is determined the evolution of the pH of the fermented milk, the dynamics of strain development and count of viable lactic acid bacteria cells.

## Materials and methods

### Materials

Strains of the species *Lactococcus lactis ssp. lactis*, *Lactococcus lactis* ssp. *lactis biovar diacetylactis*, *Lactococcus lactis ssp.cremoris* and *Streptococcus thermophilus* have been isolated from the samples of raw goat milk from different regions of the Republic of Moldova. Also identified strains have been stored in the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology.

For the cultivation of bacteria was used medium of skimmed sterilized goat milk. The skimmed milk was sterilized at 1 atm pressure up to 10 minutes [9];

Enumeration of bacterial cells was performed using the spread count method cultured on an agarified medium based on hydrolysed milk. In hydrolysed milk was added 1,5–1,8% agar-agar. After 20–30 min was soften, after that melted at 1 atm up to 15 min. Obtained medium was dispensed into the dishes and sterilized 10 min at 1 atm (121±2) °C [9].

### Methods

#### Assessmentofacidificationactivity

Acidification activity was establish by the pH degree of the milk fermented by the strain. pH measurement was performed by using the electronic pH meter HANNA. The titratable acidity of milk was determined in accordance with ISO 11869: 2012 and measured in turner degrees (T °) [10].

Bioreactor online monitoring: During the bioreactor fermentation, online monitoring parameters of pH have been carried out in the Sartorius Biostat® A plus bioreactor under of periodic cultivation conditions at 30±2 °C. Parameters of cells count have been enumerated and recorded.

#### Regression analysis

Regression analysis are used to determined the relationship between one dependent variable and one or more independent variables. It is designed to find the regression model, which expresses the correct experiments results.

To find stationary points of optimal model must be determined the confidence interval calculation for the regression equation.

#### Confidence interval calculation

The confidence interval (CI) is a type of interval estimate, computed from the statistics of the observed data, that might contain the true value of an unknown area parameter. For CI calculation is used formula (1):

$$CI = (M - t_{\alpha,n-1}\frac{S}{\sqrt{n}}; M + t_{\alpha,n-1}\frac{S}{\sqrt{n}}), \quad (1)$$

where M – the sample mean; $t_{\alpha,n-1}$ – the t value for the desired confidence level α (from the Student's coeficient table); n – samples size; S – the area standard deviation

#### Statistical analysis

The mathematical processing of the obtained experimental data according to experiments matrices type $x^2$ was performed using Microsoft Excel and Advanced Grapher 2.2. software for graphs interpretation of the results.

## Results and discussion

One of the biotechnological *properties* of lactic acid bacteria is the cells viability which plays the crucial for their applications as dairy starters and as probiotics. But the significant count of LAB cells lose activity due to the death of microorganisms during product storage.These applications are conduced to various stress conditions that affect to the biotechnological properties of the bacteria.

The maintenance of the viability presents one of the survival concept. Viability has to be demonstrated by investigation methods.

Previously, we conducted studies dedicated to evaluated the stability of biotechnological activity of strains in fermented goat milks samples for 28 days of storage at refrigeration conditions. During experiments the post-acidification was observed in time of cooling and this fact can be explained by the residual metabolic activity of lactic acid bacteria. It is known that activity of β-galactosidase for cleavage of lactose hold active even at the storage refrigeration temperature [11]. This is contributed to the accumulation of lactic acid, acetic acid, citric acid, butyric acid, acetaldehyde and formic acid produced by the starter cultures [12, 13, 14].

The viability of lactic acid bacteria has been affected by acidity. Research results indicated decreasing the count of viable lactic bacteria (CFU/ml) in the first week of storage what is related to the increase of acidity, though at 28 storage day concentrations of lactic bacteria in fermented milk were at the probiotic level ($10^7$ CFU/ml) [15]. Obtained result proves opportunity to obtain a high quality product with probiotic properties and a long shelf life [16]. According to the bibliographic study, the fermented milk has probiotic properties when the lactic acid bacteria in it remain at count min $10^6$ UFC/g at the moments of consumption [17, 18]. The results confirm the maintain stability of biotechnological properties of goat milk strains described by other authors [19].

This research due to biotechnological properties on the dynamics of the strains multiplication and the evolution of the pH value of goats' skim milk were carried out in the bioreactor at the temperature of 30±2 °C.

The confidence intervals of the equation for the pH parameter of a milk fermented by strain CNMN-LB-73 were calculated at using MO Excel (Tabel 1–2).

**Tabel 1**

**Confidence intervals**

| Time,h | ***Lactococcus lactis subsp. lactis biovar. diacetilactis* CNMN-LB-73** | | | | |
|---|---|---|---|---|---|
| | **pH value** | | | | |
| | **x1** | **x2** | **xmed** | **x1-xmed** | **x2-xmed** |
| 0 | 6,7 | 6,6 | 6,65 | 0,05 | -0,05 |
| 2 | 6,58 | 6,62 | 6,6 | -0,02 | 0,02 |
| 4 | 6,40 | 6,35 | 6,37 | 0,03 | -0,02 |
| 6 | 6,17 | 6,22 | 6,2 | -0,03 | 0,02 |
| 8 | 5,86 | 5,94 | 5,9 | -0,04 | 0,04 |
| 10 | 5,62 | 5,71 | 5,66 | -0,04 | 0,05 |
| 12 | 5,10 | 5,08 | 5,09 | 0,01 | -0,01 |
| 14 | 4,77 | 4,88 | 4,82 | -0,05 | 0,06 |

**Tabel 2**

**Confidence intervals**

| (x1-xmed)² | (x2-xmed)² | Σ(xi-xmed)² | Variation | Confidence | Conf 1 | Conf 2 |
|---|---|---|---|---|---|---|
| 0,0025 | 0,0025 | 0,005 | 0,05 | 0,016794 | 6,633206 | 6,666794 |
| 0,0004 | 0,0004 | 0,0008 | 0,02 | 0,006718 | 6,593282 | 6,606718 |
| 0,0009 | 0,0004 | 0,0013 | 0,025495 | 0,008563 | 6,361437 | 6,378563 |
| 0,0009 | 0,0004 | 0,0013 | 0,025495 | 0,008563 | 6,191437 | 6,208563 |
| 0,0016 | 0,0016 | 0,0032 | 0,04 | 0,013435 | 5,886565 | 5,913435 |
| 0,0016 | 0,0025 | 0,0041 | 0,045277 | 0,015207 | 5,644793 | 5,675207 |
| 0,0001 | 0,0001 | 0,0002 | 0,01 | 0,003359 | 5,086641 | 5,093359 |
| 0,0025 | 0,0036 | 0,0061 | 0,055227 | 0,018549 | 4,801451 | 4,838549 |

The results of fermentation process by selected strains during to the cultivation time are presented in Figures 1 and 2. Determined decrease pH values is closely related to high level of lactococci and streptococci, contributed to the faster development of acidity. The high rate of viable lactic acid bacteria cells was maintained during the whole fermentation period and will able to maintain in final product based on the presented mathematical data.

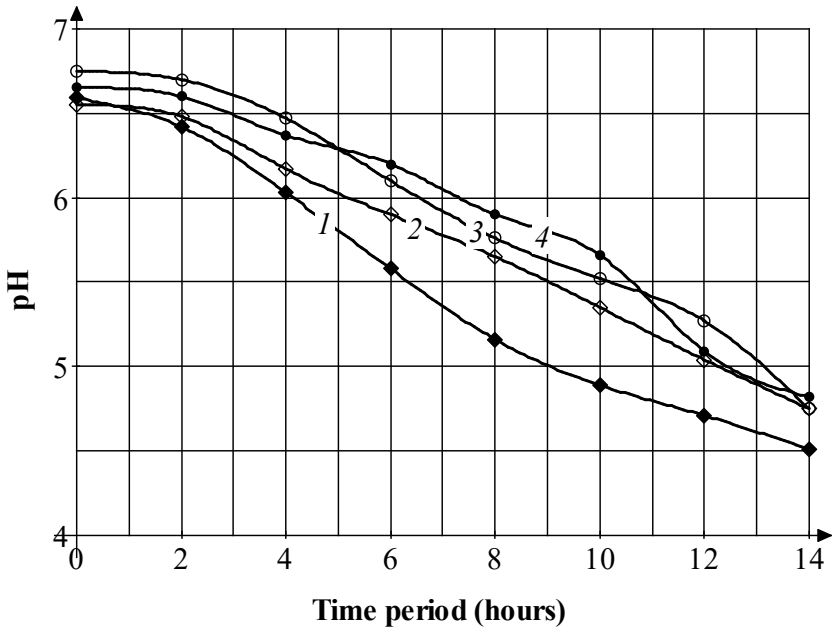


**Figure 1a. Modification of pH value of goat milk fermented by lactic acid bacteria at a temperature of 30±2 °C**
1 - CNMN-LB-79; 2 - CNMN-LB-78; 3 - CNMN-LB-75; 4 - CNMN-LB-73

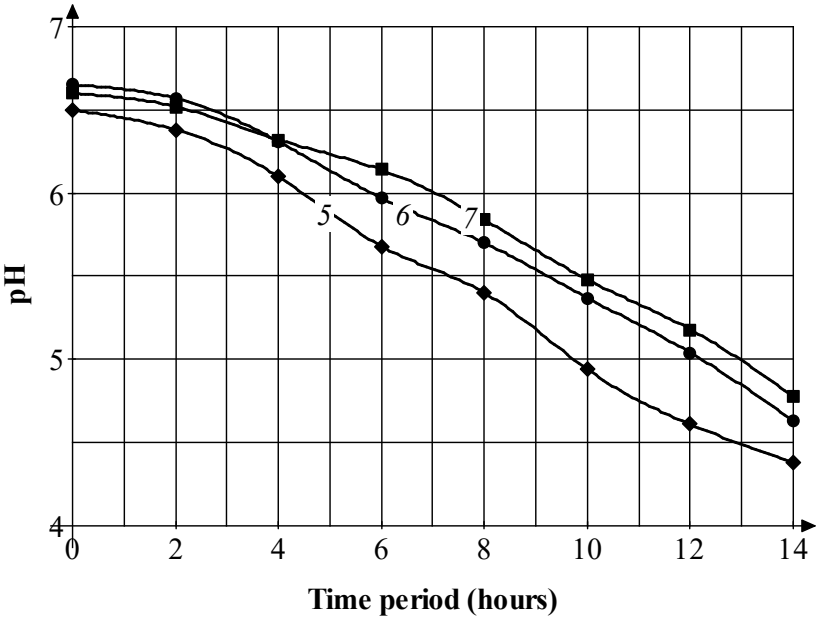


**Figure 1b. Modification of pH value of goat milk fermented by lactic acid bacteria at a temperature of 30±2 °C**

5 - CNMN-LB-76; 6 - CNMN-LB-77; 7 - CNMN-LB-74;

Based on mathematical data processing and regression equations obtained by the order $x^2$ was describe the dependence of pH during of cultivation time which allow to predict the acidogenesis process. The regression analysis is presented in Figures 1-2.

As a result of the strains growth the $\mu$ pH value = 4.6 at 14 hours of cultivation.

Analyzing the data from Figure 1a and 1b, it is noted slow pH decrease during cultivation time to all strains. Thus, after 14 hours the ΔpH of the strains was 2.37 units, indicating the accumulation of lactic acid by the intense and proportional development of the lactic bacteria.

Based on regression equations shower on Figure 1a and 1b the results strongly indicate that the confidence intervals are calculated correctly. The data show the moderate acidity and sufficient biochemical activity of these strains.

Thus, the following regression equation of the pH value of goat milk fermented by selected strains is obtained:

| | |
|---|---|
| *L. lactis biovar diacetylactis CNMN-LB-73* | $Y(x)=-0.0063X^2-0.0474X+6.68$ |
| *L. lactis CNMN-LB-74* | $Y(x)=-0.0058X^2-0.0504X+6.6208$ |
| *L. lactis CNMN-LB-75* | $Y(x)=-0.0042X^2-0.0865X+6.8125$ |
| *L. lactis CNMN-LB-76* | $Y(x)=-0.0020X^2-0.1348X+6.5854$ |
| *L. cremoris CNMN-LB-77* | $Y(x)=-0.0042X^2-0.0893X+6.6991$ |
| *L. cremoris CNMN-LB-78* | $Y(x)=-0.0025X^2-0.0987X+6.6037$ |
| *Str. thermophilus CNMN-LB-79* | $Y(x)=0.0035X^2-0.2098X+6.7079$ |

The next point was to determine biotechnological parameters of selected lactic strains. Date obtained on Figure 2a and 2b shows development kinetics at the beginning of the logarithmic phase through the polynomial model of *x2* order also. These graphs help to determine the absolute maximum point of the function.

Methods of mathematical analysis were used according to the optimal model. The critical points were found by formation the experimental dependencies (the cells count during cultivation time), in which the absolute maximum values of the function were determined. Extremes obtained according to mathematical models coincide with the extremes obtained in the experiment, which confirms that the experiment was performed exactly.

The comparative evaluation of the extremes of the dependency models at order ($2^2$) is presented in Table 3.

**Tabel 3**

**Extreme points of lactic acid bacteria count at temperature 30±2 °C**

| Strain | Regression equations | Experiment extremes | | Mathematical model extremes | |
|---|---|---|---|---|---|
| | | x | y | x | y |
| CNMN-LB-73 | $y = -0,0253x^2 + 0,7473x + 3,2583$ | 14,78 | 8,89 | 14,77 | 8,77 |
| CNMN-LB-74 | $y = -0,0221x^2 + 0,6435x + 4,1985$ | 14,31 | 8,92 | 14,56 | 8,88 |
| CNMN-LB-75 | $y = -0,0285x^2 + 0,7941x + 3,1202$ | 13,71 | 8,66 | 13,93 | 8,65 |
| CNMN-LB-76 | $y = -0,0237x^2 + 0,5796x + 4,7508$ | 11,69 | 8,30 | 12,23 | 8,29 |
| CNMN-LB-77 | $y = -0,0251x^2 + 0,6437x + 3,8808$ | 12,49 | 8,06 | 12,82 | 8,00 |
| CNMN-LB-78 | $y = -0,0236x^2 + 0,6299x + 4,5895$ | 13,29 | 8,75 | 13,34 | 8,79 |
| CNMN-LB-79 | $y = -0,0596x^2 + 1,3449x + 1,3035$ | 11,31 | 9,00 | 11,28 | 8,89 |

The strain development and biomass accumulation are presented on Figures 2a and 2b. Also at the these figures the regression equations obtained to each strain were used to determine the extremes. Knowing the factors that influence to the lactic acid bacteria growth, according to the table it can be chosen an optimum. Based on showed regression equations the results strongly indicate that the confidence intervals are calculated correctly.

Thus, the following regression equation of the lactic acid bacteria growth is obtained:

| | |
|---|---|
| *L. lactis biovar diacetylactis CNMN-LB-73* | $Y(x)=-0.0253X^2+0.7473X+3.2583$ |
| *L. lactis CNMN-LB-74* | $Y(x)=-0.0221X^2+0.6435X+4.1985$ |
| *L. lactis CNMN-LB-75* | $Y(x)=-0.0285X^2+0.7941X+3.1202$ |
| *L. lactis CNMN-LB-76* | $Y(x)=-0.0237X^2+0.5796X+4.7508$ |
| *L. cremoris CNMN-LB-77* | $Y(x)=-0.0251X^2+0.6437X+3.8896$ |
| *L. cremoris CNMN-LB-78* | $Y(x)=-0.0236X^2+0.6299X+4.5895$ |
| *Str. thermophilus CNMN-LB-79* | $Y(x)=-0.0596X^2+1.3449X+1.3035$ |

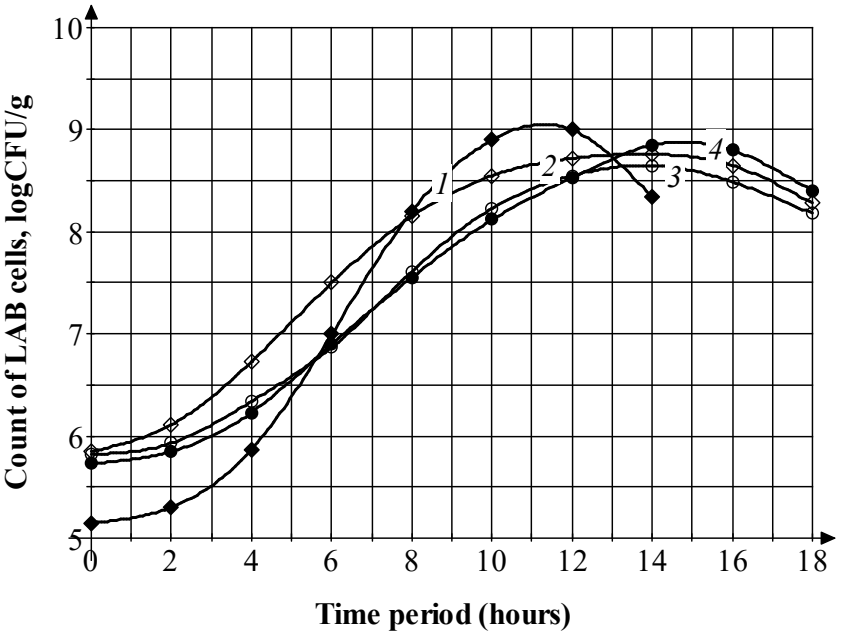


**Figure 2a. Lactic acid bacteria growth at temperature 30±2 °C**
1 - CNMN-LB-79; 2 - CNMN-LB-78; 3 - CNMN-LB-75; 4 - CNMN-LB-73

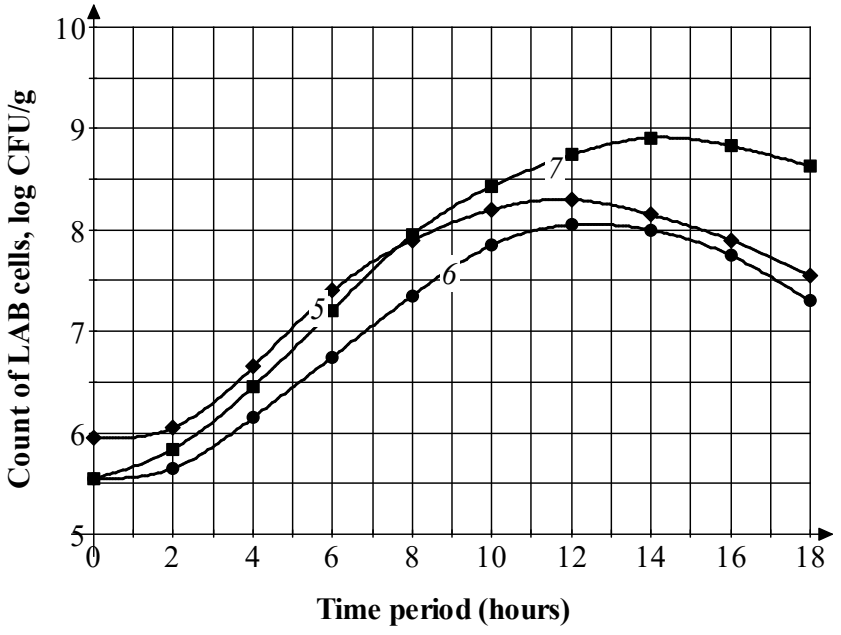


**Figure 2b. Lactic acid bacteria growth at temperature 30±2 °C**
5 - CNMN-LB-76; 6 - CNMN-LB-77; 7 - CNMN-LB-74

The obtained regressions showed on Figure 2a and 2b described enabled accurate determination of fermentation process related to biological activity of the strains. The CNMN-LB-74, CNMN-LB-75 and CNMN-LB-78 amount the maximum of the logarithmic phase of growth at 14 hours of cultivation, CNMN-LB-73 at 15 hours, CNMN-LB-76 and CNMN-LB-77 at 12 hours of cultivation and the strain CNMN-LB-79 amount the maximum of the logarithmic phase of growth at 11,5 hours of cultivation, what is characterized to the cultures of this specie.

Figure 2a and 2b showed the graphs of parameters monitoring for growth of selected lactic cultures, respectively, that have been carried out for 18 h of fermentation. The cultivation of the strains showed exactly the phase of the exponential multiplication and the phase of decline. According to mathematical models, the extremes have been obtained, which can determine the number of viable cells during cultivation - the absolute maximum of the function in the researched area at the moment (Table 3).

Although the technologically important parameters like optimal pH and temperature for industrially used strains are well known, the behaviour of the quantitative growth characteristics like specific growth rate, lactic acid production rate and growth with changing pH, temperature and other environmental factors are relatively poorly studied. Methods based on the measurement of pH or acid formation are used to determine the temperature optimum and acidifying activity of LAB.The method is optimal and convenient to determination technological parameters the acid formation rate and biomass concentration in food environment.

Several authors have studied the capacity of lactic bacteria to survive at low pH values. They have demonstrated the resistance of lactococcus at the environment with pH no lower that 4.8 [20, 21].

The obtained results showed that the decline phase of the selected strains started after 12 hours to the CNMN-LB-76, CNMN-LB-77, CNMN-LB-79 strains, after 14 hours CNMN- LB-74, CNMN-LB-75, CNMN-LB-78 and after 15 hours to the CNMN-LB-73 indicating that the selected strains are more resistant to acidic conditions and cell multiplication is observed until the pH = 4.7.

## Conclusion

In conclusion, from the parameters controlled during the fermentation in the bioreactor, showed high count of viable cells even at influence factor of pH value with acidic condition. The process become more productive in periodic cultivation condition in goat milk based medium. After storage period at refrigeration conditions selected strains demonstrated high regeneration capacity in goat milk medium rich in nutrients and exhibit specific biochemical properties. Obtained data allow to modificate cultural medium which will have a significant stimulating effect on viability of strains isolated from goat milk. Defining of cultivation conditions allow the production of autochthonous cultures will be used as starters in traditional dairy-making from goat milk.

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