IDENTIFICATION AND EXAMINATION OF SOME PROBIOTIC PROPERTIES OF *LACTOBACILLUS PLANTARUM* F3

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Abstract: *In order to be included in the composition of probiotic preparations each strain has to meet a number of requirements. The strain Lactobacillus F3 from naturally fermented sourdough is identified as a Lactobacillus plantarum strain using molecular-genetic methods (ARDRA and 16S rDNA sequencing). Some of its probiotic properties are examined: ability for industrial cultivation and survival in the model conditions of the gastro-intestinal tract. High concentrations of active cells are retained during cultivation at pH=2 + pepsin, pH=4,5 +pancreatin and pH=7 + pancreatin as well as at different concentrations of bile salts – 0,15%, 0,3%, 0,6%, 1%. The strain allows industrial cultivation with accumulation of high concentrations of viable cells. The results of the studies on some probiotic properties of Lactobacillus plantarum F3 make the strain a potentially probiotic one.*

Keywords: *ARDRA, sequencing, probiotic, batch cultivation, pepsin, pancreatin, bile salts*

1. Introduction

A number of factors influence negatively the interaction between intestinal microorganisms, such as stress and diet. Unfortunately they lead to detrimental effects on human health. There is increasing evidence indicating that consumption of ‘probiotic’ microorganisms helps maintaining a favourable microbial profile as results of which several therapeutic benefits are observed [7].

Probiotics are live microorganisms that confer a beneficial effect on the host when administered in proper amounts [4]. The beneficial effects of probiotic preparations on gastrointestinal infections, the protection of the immune system, the reduction of serum cholesterol, the improvement in inflammatory bowel disease and suppression of *Helicobacter pylori* infection, Crohn's disease, restoration of the microflora in the stomach and the intestines after antibiotic treatment; they are also characterized by anti-cancer properties, antimutagenic action, anti-diarrheal properties are well known [8].

Lactobacilli and bifidobacteria are a natural part of the intestinal microflora of the healthy human. They are included in the composition of probiotics and probiotic foods because of their proven health benefits to the body [6]. But not all strains of lactobacilli and bifidobacteria can be used as components of probiotics and probiotic foods, but only those that exhibit certain properties. Probiotic microorganisms should be of human origin, resistant to gastric acid, bile and to the antibiotics, administered in medical practice, non-pathogenic; they should also have the potential to adhere to the gut epithelial tissue and produce antimicrobial

substances; they should allow the conduction of technological processes, in which high concentrations of viable cells are obtained as well as to allow industrial cultivation, encapsulation and freeze-drying and they should remain active during storage [5]. This leads to the mandatory selection of strains of the genera *Lactobacillus* and *Bifidobacterium* with probiotic properties.

The purpose of this paper is to identify the strain *Lactobacillus* F3, isolated from naturally fermented sourdough, and to examine some of its technological properties – survival in the model conditions of the gastrointestinal tract and ability for industrial cultivation.

2. Experimental

2.1. Microorganisms

The studied *Lactobacillus* strain, *Lactobacillus* F3, is isolated from naturally fermented sourdough.

Reference microorganisms: *Lactobacillus acidophilus* DSM 20079, *Lactobacillus delbrueckii ssp.bulgaricus* DSM 20081, *Lactobacillus casei ssp.casei* DSM 20011, *Lactobacillus casei ssp.paracasei* DSM 20312, *Lactobacillus casei ssp.rhamnosus* LMG 6400, *Lactobacillus fermentum* DSM 20052, *Lactobacillus helveticus* DSM 20075, *Lactobacillus plantarum* DSM 20174.

2.2. Media

Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

LAPTg10-broth medium. Composition (g/dm^3): peptone - 15, yeast extract - 10; tryptone - 10, glucose - 10. pH is adjusted to 6.6 - 6.8 and Tween 80 - $1cm^3/dm^3$ is added. Sterilization - 20 minutes at 121ºC.

LAPTg10-agar. Composition (g/dm^3): LAPTg10-broth medium and 2% agar. Sterilization - 20 minutes at 121ºC.

MRS – broth medium (Scharlau)

2.3.Identification

Isolation of total DNA

The isolation of DNA is performed by the method of Delley et al. [2].

PCR reactions and visualization

All PCR reactions are performed using the PCR kit - Ready To GoTM PCR beads (Amersham Biosciences), in a volume of 25 µl in a Progene cycler (Techne, UK). The resulting products are visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg/ml), using an UVP Documentation System (UK).

16S rDNA amplification and 16S rDNA ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The method ARDRA involves enzymatic multiplication of the gene encoding the 16S rRNA, using primers complementary to the conservative regions at both ends of the 16S rRNA gene and the product of the multiplication is then restricted with restriction enzymes. The resulting profile is highly specific for the particular studied species.

DNA of the studied strain is amplified using universal primers for the 16S rDNA gene - fD1 and rD1 [9]. The amplification program includes: denaturation - 95°C for 3 minutes, 40 cycles - 93°C for 30 s, 48°C for 60 s, 72°C for 60 s, final elongation - 72°C for 5 min. The resulting PCR product from the 16S rDNA amplification of the tested strain is treated with the endonucleases *Eco* RI, *Hae* III and *Alu* I (Boehringer Mannhem GmbH, Germany). Reactions are carried out according to the following quantities: PCR products - 10µl, enzyme solution - 10 µl (1 µl of the respective enzyme, 2 µl buffer, 7 µl dH_2O). Incubation for 1 night at 37°C is performed. The resulting restriction products are visualized on a 2% agarose gel.

2.4. Purification of the product of the PCR-reaction – 16S rDNA – from TAE-agarose gel

The purification of 16S rDNA is conducted using DNA-purification kit (GFX Microspin™) according to the manufacturer's instructions:

1) Sample capture.

After visualizing the product of the 16S PCR-amplification reaction on a 2% agarose gel with UV light with wavelength 302 nm, the gel is visualized with UV light with wavelength 365 nm. The 16S PCR product is cut from the gel and placed in a DNA-free microcentrofuge tube. Through weighing the microcentrofuge tube before and after the gel fragments are put in them, the weight of the fragments is calculated and 10µl Capture buffer is added to every 10mg of the gel. The microcentrofuge tube are mixed gently and incubated at 60°C for about 20 minutes until the full dissolution of the gel fragments.

2) Sample binding

A GFX Microspin™ column is labelled and placed in a collection tube and the centrofuged (shortspin) samples in the eppendorf tubes from 1) are poured in the GFX Microspin™ columns (no more than 600µl). The GFX Microspin™ columns are allowed to wet for about 60 seconds and centrofuged until the whole volume passes through the column. The liquid from the column is disposed and the GFX Microspin™ column is placed in the same collection tube. If a sample is more than 600µl, all the steps from the sample binding are repeated until the whole sample is eluated.

3) Wash and dry

500 µl of wash buffer type 1 are poured in each GFX Microspin™ column, the columns are centrofuged (shortspin), the collection tubes are disposed and each GFX Microspin™ column is placed in a new 1,5 ml DNAase free microcentrofuge tube.

4) Elution

10-50µl Elution buffer type 4 or type 6 are poured in each GFX Microspin™ column. The column is allowed to wet at room temperature for 60 seconds and the microcentrofuge tubes with the GFX Microspin™ columns are centrofuged for about 60 seconds. The eluate (containing purified 16S rDNA) is collected and freezed at -20°C.

2.5. DNA-sequencing

Sequencing of the gene encoding the 16S rRNA is performed by „Macrogen Europe Laboratory", the Netherlands using the Sanger method for DNA-sequencing.

2.6. Determination of the resistance to low pH in the presence of pepsin and to weakly alkaline pH in the presence of pancreatin [1]

Fresh 24 - hour culture of the studied strain is centrifuged for 15 min at 5,000 x g. The resulting sludge biomass is washed twice with PBS - buffer and resuspended to the initial volume in PBS - buffer. 0.2 cm^3 of the cell suspension are incubated with 5 cm^3 buffer solution with pH = 2 containing 0,5% NaCl and pepsin (at a concentration of 3.2 g/dm^3) (Sigma, 2,500 - 3,500 U / mg protein), buffer with pH = 4,5 + pancreatin and buffer with pH = 7 + pancreatin at a suitable temperature for the studied strain (37°C) for 24h. At the 0, the 2^{nd}, the 4^{th} and the 24^{th} hour aliquots for the determination of the number of viable cells are taken (cfu/cm^3).

2.8. Determining the tolerance to bile salts [3]

Fresh 24 - hour culture of the studied strain is centrifuged for 15 min at 5,000 x g. The resulting sludge biomass is washed twice with PBS - buffer and resuspended to the initial volume in PBS - buffer. 0.2 cm^3 of the cell suspension are incubated with 5 cm^3 of the MRS-broth medium with different concentrations of bile salts - 0%, 0.15%, 0.3%, 0.6% and 1% - for 24h at the optimum temperature for the strain (37°C), and aliquots for the determination of the number of viable cells (cfu/cm^3) at the 0, the 2^{nd}, the 4^{th}, the 6^{th}, the 8^{th} and the 24^{th} hour are taken.

2.9. Batch cultivation in a bioreactor with continuous stirring and in a thermostat at static conditions

The laboratory cultural vessel (Fig.1) is a cylinder with geometric volume of 2 dm^3 and displacement – 1,5 dm^3.

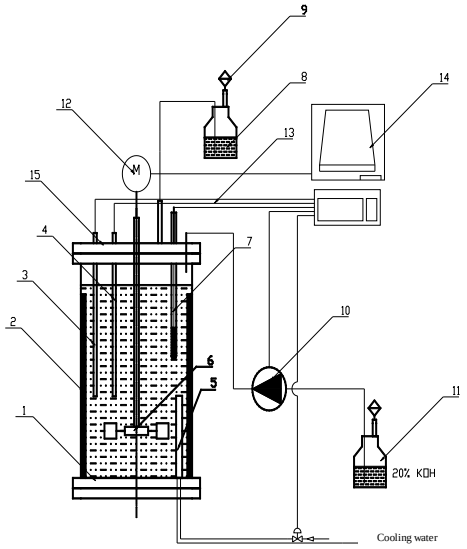

Figure 1. Scheme of the laboratory bioreactor 1 - vessel with geometric volume of 2 dm^3; 2- four repulse devises; 3–thermo-strength Pt100; 4–heater; 5-heat exchanger for cold water ; 6–turbine stirrer; 7–pH electrode; 8–exit for CO_2; 9–filter; 10–peristaltic pump for pH correction; 11– reagent for pH correction – 20% KOH; 12–motor; 13-control links; 14–control device "Applikon".

The periodic cultivation processes are conducted in MRS-broth without pH adjustment. The medium is sterilized at 118ºC for 15 min. After cooling to 39-40ºC the prepared medium in the bioreactor (MRS-broth) is inoculated with 5% (v/v) inoculum. The process of cultuvation is conducted at 37ºC, stirring speed of 100 rpm, without air supply. During the cultivation pH, Eh, number of colony-forming units and tirable acidity are examined.

Along with the carried out periodical cultivation with constant stirring (in a bioreactor), static cultivation (in an incubator) under the same conditions is carried out as well.

The number of viable cells of *Lactobacillus plantarum* F3 is determined through appropriate tenfold dillusions of the samples and plating on coloured LAPTg10 – agar medium. The Petri dishes are cultivated for 72 hours at 37°C until single colonies can be counted. The titratable acidity is determined using 0,1N NaOH. 5 cm^3 of each sample are mixed with 10 $cm^3 dH_2O$ and titrated with 0,1N NaOH, using phenolphtalein as an indicator, until the appearance of pale pink colour, which retains for 1 minute. The value for the titratable acidity is obtained by multiplying the millilitres 0,1N NaOH by the factor of the 0,1N NaOH and the number 20.

3. Results and Discussion

The strain *Lactobacillus* F3 is isolated from naturally fermented sourdough.

Identification of Lactobacillus F3

The identification of *Lactobacillus* F3 is performed using ARDRA analysis, followed by sequencing of the gene encoding the 16S rRNA.

ARDRA analysis. As a result of the ARDRA analysis with the enzymes *Eco* RI (Fig.1), *Hae* III (Fig. 2) and *Alu* I (Fig. 3) the studied strain is determined to be a representative of the species *Lactobacillus plantarum*.

DNA-sequencing of *Lactobacillus* F3 is conducted by Macrogen Europe Laboratory, the Netherlands by the method of chain termination (method of Sanger). After careful comparison of the obtained sequence with the public online nucleotide BLAST database, the strain *Lactobacillus* F3 is confirmed to be a *Lactobacillus plantarum* strain (Fig. 4).

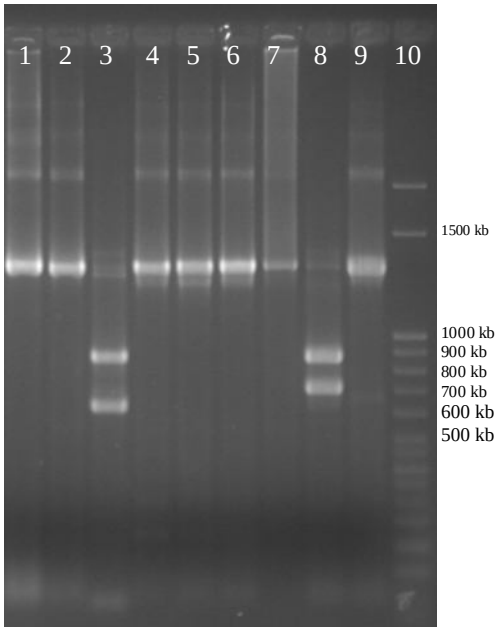

Fig. 1. Restriction profile of the 16S rDNA with *Eco*RI
1. *Lactobacillus* F3
2. *Lactobacillus acidophilus* DSM 20079
3. *Lactobacillus delbrueckii ssp.bulgaricus* DSM 20081
4. *Lactobacillus casei ssp.casei* DSM 20011
5. *Lactobacillus casei ssp.paracasei*
6. *Lactobacillus casei ssp.rhamnosus*
7. *Lactobacillus fermentum* DSM 20052
8. *Lactobacillus helveticus* DSM 20075
9. *Lactobacillus plantarum* DSM 20174
10. M

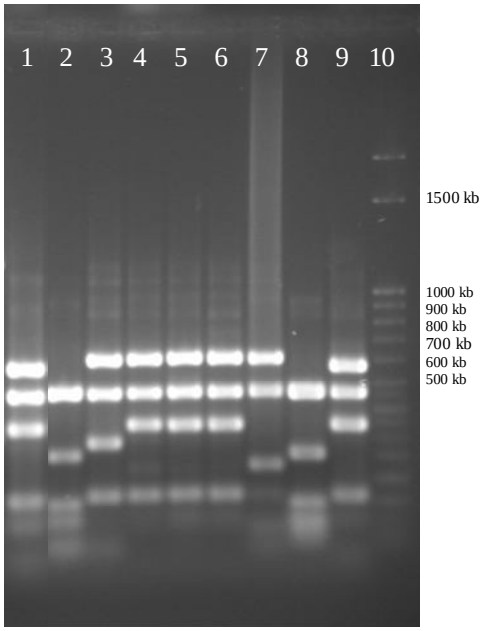

Fig. 2. Restriction profile of the 16S rDNA with *Hae*III
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2. *Lactobacillus acidophilus* DSM 20079
3. *Lactobacillus delbrueckii ssp.bulgaricus* DSM 20081
4. *Lactobacillus casei ssp.casei* DSM 20011
5. *Lactobacillus casei ssp.paracasei*
6. *Lactobacillus casei ssp.rhamnosus*
7. *Lactobacillus fermentum* DSM 20052
8. *Lactobacillus helveticus* DSM 20075
9. *Lactobacillus plantarum* DSM 20174
10. M

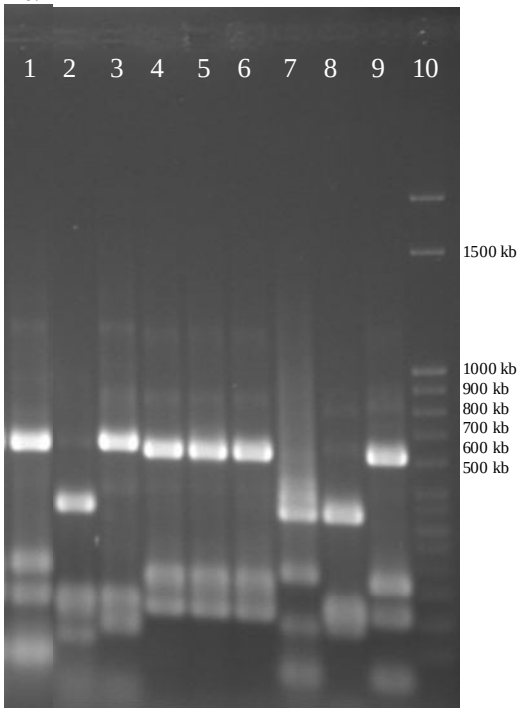

Fig. 3. Restriction profile of the 16S rDNA with *Alu* I
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2. *Lactobacillus acidophilus* DSM 20079
3. *Lactobacillus delbrueckii ssp.bulgaricus* DSM 20081
4. *Lactobacillus casei ssp.casei* DSM 20011
5. *Lactobacillus casei ssp.paracasei*
6. *Lactobacillus casei ssp.rhamnosus*
7. *Lactobacillus fermentum* DSM 20052
8. *Lactobacillus helveticus* DSM 20075
9. *Lactobacillus plantarum* DSM 20174
10. M

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partial sequence
Length=1474

 Score =  1977 bits (1070),  Expect = 0.0
 Identities = 1072/1073 (99%), Gaps = 0/1073 (0%)
 Strand=Plus/Minus

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Query  131   TCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTG  190
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Figure 4. Comparison of the nucleotide sequences of the 16S rDNA of *Lactobacillus* F3 and the partial sequence of the 16S rDNA of *Lactobacillus plantarum NRRL B-14768*.

Probiotic properties of *Lactobacillus plantarum* F3

Survival in the model conditions of the gastrointestinal tract

The resistance of the cells of *Lactobacillus plantarum* F3 in the model conditions of the gastro - intestinal tract is examined: survival at pH = 2 + pepsin, at pH = 4,5 + pancreatin and at pH = 7 + pancreatin. The results of the experimental studies are presented on Fig. 5.

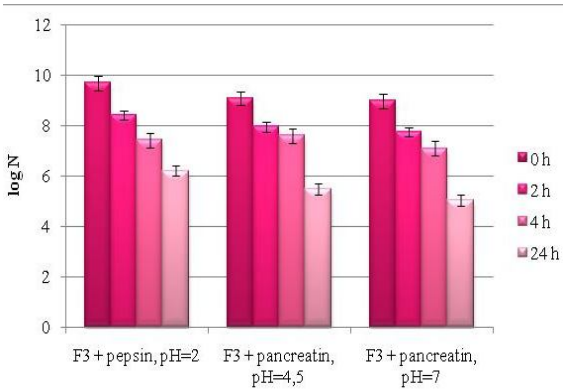

Figure 5. Survival of the cells of the strain *Lactobacillus plantarum* F3 at pH = 2 + pepsin, at pH = 4,5 + pancreatin and at pH = 7 + pancreatin.

It is observed that the sensitivity of *Lactobacillus plantarum* F3 to pH = 2 + pepsin, pH = 4,5 + pancreatin and pH = 7 + pancreatin is comparable – the reduction of the number of viable cells is about 4logN (Fig. 5) by the 24th hour of cultivation. But the concentration of active cells even by the 24th hour remains high – 1,6x10^{6}cfu/cm^{3} at pH = 2 + pepsin, 3x10^{5}cfu/cm^{3} at pH = 4,5 + pancreatin, 1,1x10^{5}cfu/cm^{3} at pH = 7 + pancreatin, which makes the strain appropriate for incorporation in probiotics.

Another factor of great importance that influences the survival of probiotic strains in the gastrointestinal tract are bile salts. About three hours after ingestion of food the concentration of bile salts in the small intestine reaches about 0.3%. This requires study of the influence of different concentrations of bile salts on the survival of *Lactobacillus plantarum* F3 in MRS-broth medium with different concentrations of bile salts, 0%, 0.15%, 0.3%, 0.6% and 1% for 24 hours of incubation. The number of viable cells of *Lactobacillus plantarum* F3 starts decreasing since the beginning of the cultivation of the strain in MRS-broth medium with different concentrations of bile salts (Fig. 6).

The degree of reduction is different at the different concentrations of bile salts – it is greater at concentrations 0,6% and 1% and smaller at 0,15% and 0,3% bile salts. At 0,15% bile salts the reduction is 1,3logN and at 0,3% it is 1logN. At 0,6% bile salts the degree of reduction is considerably higher – 3logN and at 1% bile salts it is about 3,5logN.

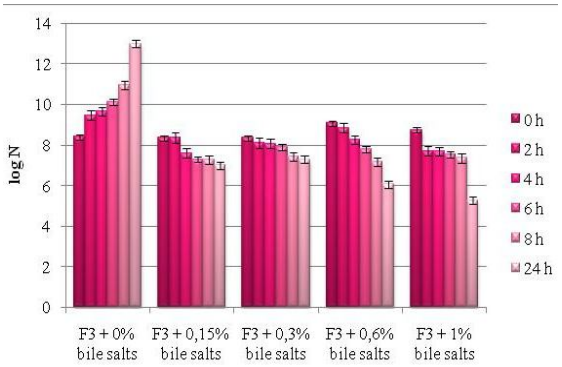

Figure 6. Survival of the cells of *Lactobacillus plantarum* F3 at different concentrations of bile salts.

But by the end of the experiment the concentration of viable cells remains between 1,9x10^{5} cfu/cm^{3} (at 1% bile salts) and 1x10^{7}cfu/cm^{3} (at 0,15% bile salts), which allows the inclusion of *Lactobacillus plantarum* F3 in the composition of probiotic preparations.

Batch cultivation in a bioreactor with continuous stirring and at static conditions of Lactobacillus plantarum F3

The strain *Lactobacillus plantarum* F3 is cultivated in MRS-broth at 37ºC in a laboratory bioreactor with continuous stirring and in a thermostat. It is observed that the time to reach high concentration of viable cells during cultivation in the bioreactor with continuous stirring is reduced in comparison to cultivation at static conditions (Fig. 7, Fig. 8).

At the 6th hour the number of cells reaches 8,3x10^{10}cfu/cm^{3} (Fig. 7), while under static conditions, the same concentration of cells is reached at the 12th hour from the beginning of the process (Fig. 8). The number of active cells of *Lactobacillus plantarum* F3 obtained in cultivation in a

bioreactor with continuous stirring and at static conditions by the 24th hour is comparable – $3x10^{12}$cfu/cm^3 in the bioreactor and $7,8x10^{12}$cfu/cm^3 at static conditions. The titratable acidity of the medium in the bioreactor increases from 46,2°T to 241,7°T, while at static conditions it reaches 240,1°T by the 24th hour and 248,3°T by the 48th hour.

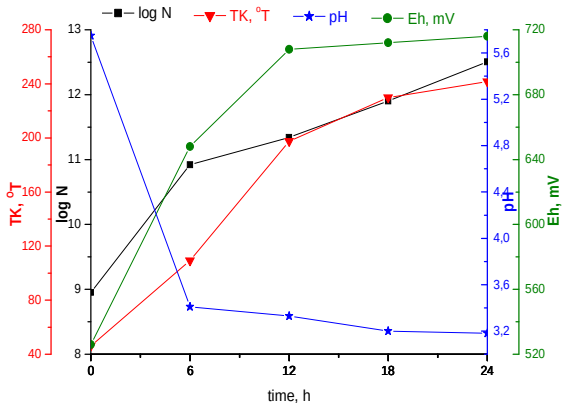

Figure 7. Batch cultivation of *Lactobacillus plantarum* F3 in MRS-broth in a bioreactor with constant stirring.

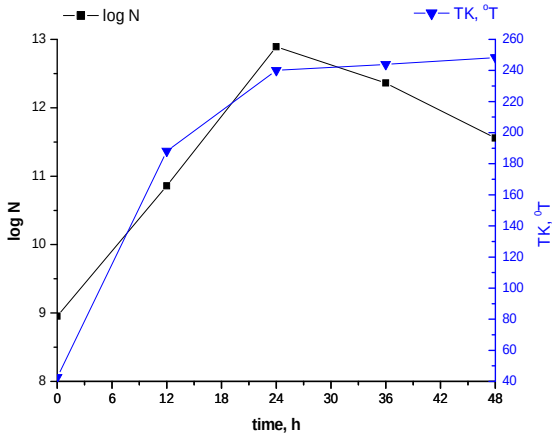

Figure 8. Static cultivation of *Lactobacillus plantarum* F3 in MRS-broth

The redox potential of the system starts increasing since the beginning of the batch process. It starts from +526mV and reaches +716 mV (Fig. 7).

The strain *Lactobacillus plantarum* F3 allows industrial cultivation with accumulation of high concentrations of viable cells.

4. Conclusion

The strain *Lactobacillus* F3 is identified as belonging to the species *Lactobacillus plantarum*. *Lactobacillus plantarum* F3 has the ability to survive in the model conditions of the gastro - intestinal tract and allows industrial cultivation with accumulation of high concentrations of viable cells. Thus, it can be defined as a potential probiotic culture, which after further research can be incorporated in the composition of probiotic preparations for treatment and prevention.

5. References

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