OBTAINING PROBIOTIC CONCENTRATES OF LACTOBACILLI OF HUMAN ORIGIN

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Abstract: Batch cultivation in a bioreactor with continuous stirring and at static conditions of the strains with probiotic properties of the genus *Lactobacillus*: *Lactobacillus acidophilus* A2 and *Lactobacillus acidophilus* Ac in skimmed milk is conducted. It is shown that better conditions for the development of the lactobacilli are created in the bioreactor which allows obtaining concentrates of viable cells in a shorter time at lower acidity than in cultivation under static conditions. The *Lactobacillus* strains influence the redox potential of the system through their metabolic activity. The redox potential is strain specific. The resulting concentrates are liquid probiotic concentrates and can be kept at 4±2°C up to 20-30 days and used as probiotic beverages.
Key words: *Lactobacillus*, probiotic, cultivation, bioreactor, concentrates

Introduction

Probiotics are live microorganisms that confer a beneficial effect on the host when administered in proper amounts [1, 2]. Their beneficial effects on gastrointestinal infections, the reduction of serum cholesterol, the protection of the immune system, anti-cancer properties, antimutagenic action, anti-diarrheal properties, 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, etc. are proven by addition of selected strains to food products [3, 4, 5, 6].

Lactobacilli and bifidobacteria are normal components of the healthy human intestinal microflora. They are included in the composition of probiotics and probiotic foods because of their proven health effects on the body [7, 8, 9]. They are the main organisms that maintain the balance of the gastrointestinal microflora [10].

Not all strains of lactobacilli and bifidobacteria can be used as components of probiotics and probiotic foods, but only those that are of human origin, non-pathogenic, resistant to gastric acid, bile and to the antibiotics, administered in medical practice; 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 [11, 12]. This requires the mandatory selection of strains of the genera *Lactobacillus* and *Bifidobacterium* with probiotic properties. Moreover, the concentration of viable cells of microorganisms in the composition of probiotics should exceed 1 million per gram [13] in order for the preparation to exhibit a therapeutic and prophylactic effect.

One of the requirements for a strain to be probiotic is to allow industrial cultivation with accumulation of high concentrations of viable cells, which are maintained during freeze-drying and storage.

The control of the lactic acid fermentation is an important indicator of the quality assurance of lactic acid products and liquid probiotic preparations.

A major milestone in the technological process is to ensure optimal conditions for the growth of microbial cells, ensuring the accumulation of high concentration of active flora. This is achieved through batch or continuous cultivation of lactic acid bacteria in suitable media. Their cultivation in a bioreactor allows better development of the microbial cells and creates opportunities for cultivation of mixed cultures. Conditions for obtaining standardized starter cultures with homogeneous properties and biochemical activity are created [14].

Pirt [15] indicates that during batch cultivation of lactic acid bacteria each microbial species increases its biomass at a rate which is a function of the chemical and the physical conditions of the environment. In co-cultivation the production of substances that affect the development of other species is a factor as well.

The purpose of the present article is cultivation of strains of the species *Lactobacillus acidophilus* of different origin - *Lactobacillus acidophilus* A2, *Lactobacillus acidophilus* Ac and *Lactobacillus acidophilus* Z10 in an incubator and in a bioreactor with continuous stirring and obtaining concentrates with high numbers of viable cells and moderate titrable acidity, which are maintained during storage.

Materials and methods

1. Microorganisms

The studies in this work are performed with two strains of *Lactobacillus acidophilus* of human origin: *Lactobacillus acidophilus* A2 and *Lactobacillus acidophilus* Ac.

2. Media:

2.1. Sterile skimmed milk with titrable acidity 16-18ºT. Composition (g/dm^3): skimmed milk powder (Scharlau). The medium is sterilized for 15 minutes at 118ºC.

2.2. Saline. Composition (g/dm^3): NaCl - 5 g; distilled water - 1l. Sterilize 20 minutes at 121 º C.

2.3. MRS – broth medium (medium of De Man, Rogosa & Sharpe). Composition (g/dm^3): peptone from casein - 10 g; yeast extract - 4 g; meat extract - 8 g; glucose - 20 g; K_2HPO_4 - 2 g; sodium acetate - 5 g; diammonium citrate - 2 g; $MgSO_4$ - 0.2 g; $MnSO_4$ - 0.04 g; Tween 80-1 ml; pH = 6.5. The medium is sterilized for 15 min at 118ºC.

2.4. MRS – agar medium. Composition (g/dm^3): MRS - broth +2% agar. The medium is sterilized for 15 min at 118ºC.

3. Cultivation and storage of microorganisms studied

The studied strains of microorganisms are cultivated in a liquid medium (MRS-broth) and on agar medium (MRS-agar) at 37°C. The tested strains are isolated from a single colony and are cultivated on MRS-broth medium for 24 hours. The strains are stored as stock-cultures in MRS-broth with 20% v/v glycerol at -20ºC.

4. Bioreactor and cultivation conditions

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

The periodic cultivation processes are conducted in skimmed milk without pH adjustment. The medium is sterilized at 118ºC for 15 min. After cooling to 39-40ºC the prepared medium in the bioreactor (skimmed milk) is inoculated with 2,5% (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.

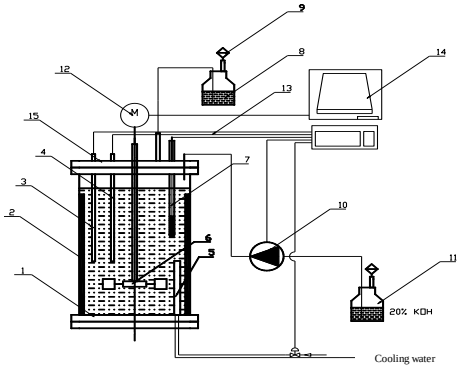

Fig. 1. Scheme of the laboratory bioreactor
1 - vessel with geometric volume of 2 dm^3; 2-four repulse devises ; 3–thermo-strength Pt100 ; 4–heater ; 5-heat exchanger for cold water ; 6–turbine stirrer ; 7–pH electrode ; 8–exit for CO_2; 9–filter ; 10–peristaltic pump for pH correction ; 11– reagent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

5. Method for determining the number of colony-forming units [cfu/cm^3]: After tenfold dillutions of each sample in 0,5% saline solution, MRS-agar medium is inoculated with the corresponding dillutions. After incubation at 37°C for 48-72 hours the number of colony-forming units is determined.

6. Method for detetrmining titrable acidity (TA) [°T]: 10 cm^3 of the sample are dilluted in 20 cm^3 distilled H_2O and the solution is titred with 1,01N NaOH. 1°T is the amount of 1,01N NaOH, necessary for the neutralization of the acids in 100 cm^3 of the sample.

Results and discussion

1. Batch cultivation in a bioreactor with continuous stirring and at static conditions of *Lactobacillus acidophilus* A2

In the cultivation of *Lactobacillus acidophilus* A2 in skimmed milk in a laboratory bioreactor with continuous stirring at 37ºC the time to reach high concentration of viable cells is reduced compared with cultivation at static conditions (Fig. 1a), b)). At the 5^{th} hour the number of cells reaches 10^{10}cfu/cm^3, while under static conditions, the same concentration of cells is reached at the 24^{th} hour from the beginning of the process (Fig. 1b)). During cultivation in a bioreactor with continuous stirring higher concentration of viable cells is obtained and the titrable acidity of the medium increases from 55.4 to 61.1°T, while at static conditions it reaches 186.9°T. These studies confirm the results obtained by Schiraldi et al., 2003 that products with higher concentration of viable cells at lower acidity are produced in a bioreactor in the presence of oxygen.

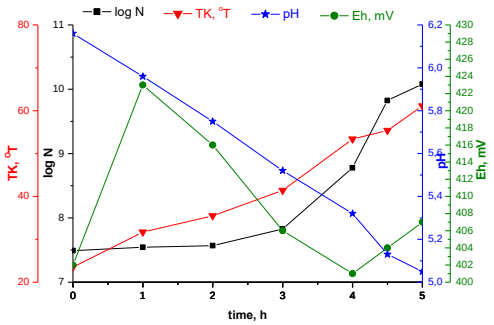

Fig. 1 a) Batch cultivation of Lactobacillus acidophilus A2 in skimmed milk in a bioreactor with constant stirring

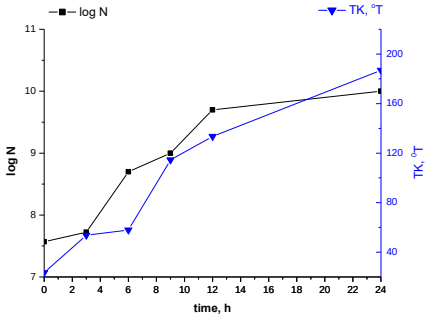

Fig. 1 b) Static cultivation of Lactobacillus acidophilus A2 in skimmed milk

The redox potential of the system increases during the first hour from the start of the batch process and reaches +423 mV, then decreases during the logarithmic phase to +401 mV and continues to grow smoothly as the culture passes from the exponential to the stationary growth phase (Fig. 1a)).

The concentrate from the bioreactor is stored in a refrigerator at 4 ± 2°C for 30 days. The changes in the titrable acidity and the number of viable cells during storage are traced. The results of these studies are shown on Fig. 3.

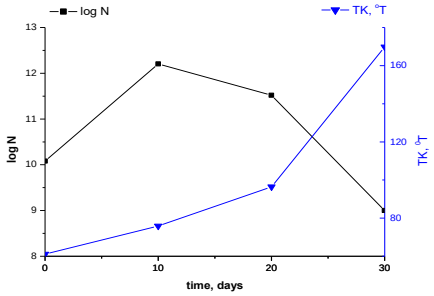

Fig. 2. Changes in the titrable acidity and the concentration of viable cells in the milk concentrate of Lactobacillus acidophilus A2 during storage

In the first 10 days of storage of the milk concentrate an increase in the number of active cells (2,2 logN) and in the titrable acidity is observed. The latter increases up to the 30th day reaching 169,81°T and the concentration of viable cells decreases slightly (less than 1 logN by the 20th day and with around 3,2 logN by the 30th day). These results suggest that the probiotic liquid concentrate of *Lactobacillus acidophilus* A2 can be stored and applied as a probiotic beverage for 20 days under refrigerated conditions, maintaining high concentration of viable cells at moderate titrable acidity (Fig. 2).

2. Batch cultivation in a bioreactor with continuous stirring and at static conditions of *Lactobacillus acidophilus* Ac

Similar studies are conducted with the strain *Lactobacillus acidophilus* Ac. With this strain higher concentration of living cells at higher titrable acidity (66.6°T) (Fig. 3 a)) is achieved for 6 hours of cultivation in a bioreactor with continuous stirring in skimmed milk.

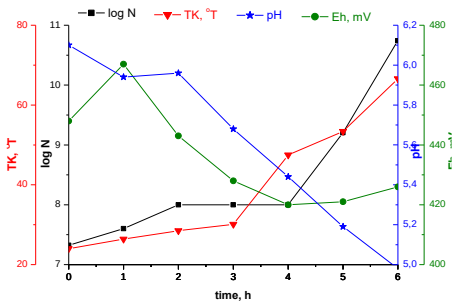

Fig. 3 a) Batch cultivation of Lactobacillus acidophilus Ac in skimmed milk in a bioreactor with constant stirring

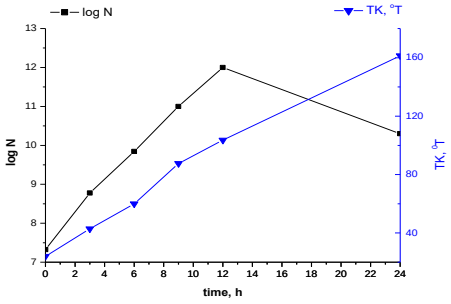

Fig. 3 b) Static cultivation of Lactobacillus acidophilus Ac in skimmed milk

Higher levels of viable cells under static conditions on the 12th hour are determined. The number of active cells reaches 10^{12} cfu/cm^3 (Fig. 3b)). Concentrates with high content of living cells for shorter time are obtained after cultivation in a bioreactor.

The curve, reflecting the change in the redox potential of the system shows that Eh increases during the lag-phase and decreases during the logarithmic growth phase and then gradually increases up to +426 mV (Fig. 3a)).

The obtained concentrate is stored at refrigeration temperature 4 ± 2°C for 30 days and the changes in the concentration of viable cells and in the titrable acidity are traced.

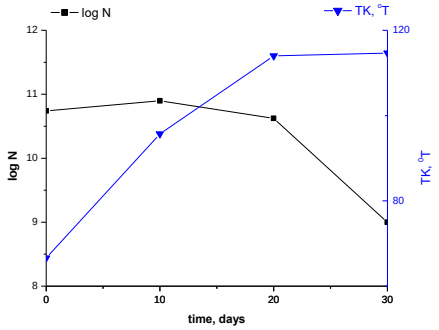

Fig. 4. Changes in the titrable acidity and the concentration of viable cells in the milk concentrate of Lactobacillus acidophilus Ac during storage.

The concentration of active cells of the strain *Lactobacillus acidophilus* Ac is retained during storage and on the 20th day it is 4,2.10^{10}cfu/cm^3. The titrable acidity reaches 114°T and this value is maintained up to the 30th day when the concentration of viable cells is reduced by about 1,6 logN. The results indicate that the concentrate can be stored and used as a probiotic beverage for 30 days at 4±2°C, maintaining a high concentration of viable cells at moderate titrable acidity (Fig. 4).

Conclusion

The results show that better conditions for the propagation of the *Lactobacillus* strains are created in the bioreactor and concentrates with high concentrations of viable cells are obtained in shorter time. The fermentation process is strain specific. The two strains allow industrial cultivation with the accumulation of high concentrations of viable cells, which almost do not change during storage, making them potentially probiotic strains.

The resulting concentrates of the probiotic *Lactobacillus acidophilus* strains maintain the concentration of viable cells and titrable acidity during storage at 4 ± 2°C for 20 (*Lactobacillus acidophilus* A2) or 30 days (*Lactobacillus acidophilus* Ac) and can be applied as probiotic beverages (liquid probiotics).

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