NEW APPROACH TO MODELLING THE KINETICS OF THE FERMENTATION PROCESS IN CULTIVATION OF LACTIC ACID BACTERIA

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ABSTRACT
The present paper reviews the methods for assessing the kinetics of the fermentation process for the cultivation of probiotic strains of lactic acid bacteria in a laboratory bioreactor with stirring. To describe the kinetics of the lactic acid fermentation process, an alternative approach with the use of semi-empirical degree laws that offer a new look into the biological parameters in the description models is proposed.

INTRODUCTION
Probiotic lactic acid bacteria
Lactobacilli belong to the natural microflora of human and animal organisms. They normally exist in the oral cavity, gastrointestinal tract, and vaginal microflora (Adams, 1999). The most common lactobacilli species isolated from the gastrointestinal tract of humans are Lactobacillus brevis, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum and Lactobacillus salivarius (Slover, 2008). The cellular components of certain lactic acid bacteria protect and modulate the immune system of the human body and improve its health status (Pirt, 1975; Salminen and von Wright, 1998a,b). L. delbrueckii ssp. bulgaricus, L. acidophilus, L. casei, L. plantarum are the major microorganisms that regulate the balance of the gastrointestinal microflora (Salminen and Wright, 1998b). Lactic acid bacteria provide the human organism with growth factors, amino acids, vitamins, organic acids, etc. through their metabolism. They have the ability to adhere to the intestinal mucosa, stop the formation of enterobacterial colonies, and prevent the colonization of the gut epithelium by bacteria coming from outside (Pirt, 1975). These regulatory functions are realized by the organic acids, bacteriocins, bacteriocin-like substances (BLIS), and other metabolites produced by lactic acid bacteria through which they inhibit enteropathogens (Fang, 2001).

Lactic acid bacteria and their metabolic products have beneficial effects on the digestive system and positive action during and after antibiotic treatment (O'Brien et al., 1999). The mechanism of action of lactobacilli comprises: a) suppression of microbial putrefaction processes; b) prevention of constipation, colon cancer, etc.; c) prevention and treatment of antibiotic-associated diarrhea; d) stimulation of the immune system; e) suppression of toxic processes in the digestive tract (Pirt, 1975; Fuller, 1986; Salminen and Wright, 1998a,b). Lactic acid bacteria are also characterized by antimutagenic, anticancerogenic and antitumor activity (Hosono et al., 1990). Lactic acid bacteria with proven probiotic properties are included in the composition of probiotic preparations, starter cultures, and in the production of dairy, meat and other products with functional properties. One of the requirements for a strain to be probiotic is that it would allow industrial processes to take place, including cultivation, and accumulation of high concentrations of viable probiotic cells in the cultivation process. One of the main stages in the production of probiotics and starter cultures is the cultivation of the selected strains in industrial bioreactors of different construction. The cultivation process defines mainly the qualitative and quantitative characteristics of the production process as a whole.

Methods of cultivation
A. Batch cultivation. In this method, the microbial population grows in a closed space without changing the medium volume; without the addition of any substrates, and with the addition of substances that correct some of the parameters only. Process parameters are a function of time, and the process is non-stationary. Batch cultivation of lactic acid bacteria is characterized by the following stages (Abdel-Rahman et al., 2013):
• Lag-phase: it occurs immediately after inoculation of the nutrient medium and aims at adapting the microbial population to the conditions of the medium. Cells undergo biochemical processes of synthesis of cellular structures needed for the binary fission. Additionally, if
the medium contains high molecular weight molecules, the cells release extracellular enzymes to break them down to low molecular weight compounds, thereby facilitating their intracellular transport.

- Exponential phase: the cells grow and divide intensively. They multiply at a maximum rate.
- Stationary phase: there is depletion of one or more substances from the growth medium, and the cells stop multiplying. During the stationary phase, the number of newly formed cells is equal to the number of dying cells, so no increase in biomass is observed, but the cells have a preserved metabolic activity.
- Death phase: during this phase, the number of cells that are dying is significantly greater than the number of newly formed cells.

B. Batch cultivation with pH correction.

The constantly increasing concentration of lactic acid during batch cultivation has an inhibitory effect on the growth of lactic acid bacteria. To remove it, pH adjustment is required during the batch fermentation process. The correction is accomplished by the addition of NaOH, KOH, CaCO₃, and ammonia water, and leads to a fuller absorption of the substrate and an increase in the amount of lactic acid produced and accumulated (Hetenyi et al., 2011, Abdel-Rahman et al., 2011a, b, Adsul et al., 2007; Tashiro et al., 2011). According to a number of authors, the optimum pH range for the growth of lactic acid bacteria is between 5 and 6 (Fu and Mathews 1999, Yuwono and Kokugan 2008).

C. Continuous fermentation.

Continuous cultivation systems provide increased productivity and reduce the inhibitory effect of lactic acid on cell growth. They are classified as open and closed continuous cultivation systems. The most important factors for the production of probiotic products in continuous mode are the closed continuous cultivation systems. The targeted management of the lactic acid fermentation process is an important indicator for ensuring the quality of lactic acid foods and liquid probiotic preparations. The main point in the technological process is the provision of optimal conditions for the growth of the microbial cells, ensuring the accumulation of high active flora concentration, and creating conditions for obtaining standardized starters with constant properties and biochemical activity (Driessen et al., 1977).

Kinetic models for lactic acid fermentation description.

The description of the kinetics of microbial growth is done through a number of models. Currently, different types of dependencies are used in practice, many of which are based on Monod's classic equation, but there are other types of models that offer a new look at the fermentation process.

The Monod equation expressing the specific growth rate dependence on the concentration of the limiting substrate is analogous to the Michaelis-Menten equation (Bouguettoucha et al., 2011; Abboud et al., 2010; Dey and Pal, 2013; Ghaly et al., 2005):

\[
\mu = \mu_{\text{max}} \frac{S}{K_s + S} \quad (1)
\]

where: \( \mu \) - specific growth rate, h⁻¹; \( \mu_{\text{max}} \) - maximum specific growth rate, h⁻¹; \( K_s \) - saturation constant, kg/m³; \( S \) - substrate concentration kg/m³. The saturation constant is equal to the substrate concentration at which the specific growth rate is half of its maximum value (\( \mu = 0.5\mu_{\text{max}} \)).

High concentrations of the substrate can lead to cell growth inhibition. This process should be taken into account when developing the mathematical model. The Andrews-Halden model is one of the most commonly used models for description of the inhibitory action of high substrate concentrations (Abboud et al., 2010, Dey and Pal, 2013; Bouguettoucha et al., 2011):

\[
\mu = \mu_{\text{max}} \frac{S}{K_s + S + \frac{S^2}{K_i}} \quad (2)
\]

where: \( K_i \) - inhibition constant.

Another model for describing the inhibitory action of high substrate concentrations is the Edward model (Abboud et al., 2010; Dey and Pal, 2013; Bouguettoucha et al., 2011):

\[
\mu = \mu_{\text{max}} \frac{S}{(K_s + S) + (1 + \frac{S^2}{K_i})} \quad (3)
\]

The specific growth rate depends not only on the concentration of the limiting substrate but also on other factors, primarily the concentration of the metabolic products. Yerusalimski proved that this dependence is described by the non-competitive inhibition equation (Abboud et al., 2010; Dey and Pal, 2013; Bouguettoucha et al., 2011):

\[
\mu = \mu_{\text{max}} \frac{K_p}{K_p + P} \quad (4)
\]

where: \( P \) - concentration of the inhibitory product, kg/m³; \( K_p \) - constant, kg/m³. \( K_p \) is the concentration of the inhibitory product in which the specific growth rate is equal to half of its maximum value (\( \mu = 0.5\mu_{\text{max}} \)).

All growth parameters in the previous models have no clear biological meaning. The biological requirement of the model parameters is satisfied by the Verhulst equation (Bouguettoucha et al., 2011):

\[
\frac{dX}{d\tau} = \mu X - \beta X^2 = \mu X - \frac{\mu}{X_e} X^2 \quad (5)
\]

where: \( X \) – biomass concentration, kg/m³; \( X_e \) – final biomass concentration, kg/m³; \( \beta \) - coefficient of internal population competition, kg/(kg.h)

SEMI-EMPirical MODEls: A NEW APPROACH TO THE DESCRIPTION OF MICROBIAL KINETICS

Differential equations used in practice for describing the lactic acid process are rarely solved with high accuracy, largely due to the presence of too many ambiguous variables. This is particularly true of the more complex models describing the kinetics of the fermentation.
process. Thus, a purely numerical solution can hardly be found, and in some cases the solution to a task is semi-empirical rather than analytical (Tishin and Fedorov, 2016).

In their works, Tishin and Fedorov, 2016; Tishin et al., 2015 suggest the use of a different principle for describing microbial kinetics. It is based on various assumptions about continuous cell division and the proportionality of biomass accumulation in time, its concentration in the culture medium and the cultivation time. In this case, cell multiplication can be described with the following dependence:

$$dX = kX^n \tau$$  \hspace{1cm} (6)

where: $k$ - a proportionality factor, $m$ and $n$ - degree indicators. Depending on the values of the degree indicators, different mathematical models can be obtained for the same fermentation process.

For example, at $n = 0$ the step model (7) is obtained, and the coefficient $k_{s}$ is analogous to the specific growth rate $\mu$ (Tishin and Fedorov, 2016).

$$dX = k_{s}X^m$$  \hspace{1cm} (7)

In real form, after integration, equation (7) acquires the form:

$$X^m - 1 = \left(\frac{1}{1 - m}\right)k_{s}X^{m - 1} \tau$$  \hspace{1cm} (8)

Equation (8) can be converted by entering parameter $\delta$ and assuming that:

$$m_i = \frac{1}{1 - m}$$  \hspace{1cm} (9)

where:

$$X_{b} = \left(1 + \delta \tau\right)^{m_i}$$  \hspace{1cm} (10)

According to Tishin et al., 2015, parameter $\delta$ is the average specific growth rate for the entire cultivation process.

In the second case, the differential equation can be solved if $m = 0$ and $n = 1$ are known:

$$dX = k_{s}X^n$$  \hspace{1cm} (11)

After integrating the obtained equation and dividing by $X_{b}$:

$$X_{b} = 1 + \frac{k_{s}}{(n + 1)}X^{\gamma(n+1)}$$  \hspace{1cm} (12)

If the parameter $\gamma$, which is the ratio to the right side of equation (12), is introduced, the following is obtained:

$$X_{b} = 1 + \left(\frac{1}{\gamma \tau}\right)^{n}$$  \hspace{1cm} (13)

$\gamma$ can be considered an average specific growth rate, but it has a much clearer meaning since it can easily be shown that $1/\gamma$ represents the doubling time of the cell population (Tishin and Fedorov, 2016 Tishin et al., 2015).

Using Equations (8) and (13), equations for the substrate consumption during the fermentation process can also be derived. The detailed solution is presented in Tishin and Fedorov, 2016; Tishin et al., 2015, where dependencies of the following type are obtained:

$$S = \frac{1}{(1 + \delta \tau)^{\gamma}}$$  \hspace{1cm} (14)

$$S = \frac{1}{(1 + \gamma \tau)^{\gamma}}$$  \hspace{1cm} (15)

Tishin and Fedorov, 2016; Tishin et al., 2015 show that equations (13) and (15) have a clearer biological sense of their parameters and are therefore preferable when describing the kinetics of the fermentation process.

On the basis of equations (6) to (15), other types of process equations are also proposed, which also include the knowledge of kinetics when using the classical equations (1) to (5) (Tishin and Fedorov, 2016):

- exponential degree model
  $$X_{b} = a(d - e^{-\mu \tau})$$  \hspace{1cm} (16)

- modified empirical model of the logistic curve
  $$X_{b} = \mu \frac{1}{1 + be^{-\mu \tau}}$$  \hspace{1cm} (17)

- Weibull's equation for describing lactic acid biosynthesis
  $$K_{T} = c_{m} - c_{m}^{\gamma(q \tau)^{n}}$$  \hspace{1cm} (18)

where $X_{b}$ - biomass in a dimensionless form; $\mu$ - specific relative (average) growth rate of biomass at a time interval from $\tau = 0$ to $\tau = \tau_{e}$; $\mu_{m}$ - maximum specific growth rate, h$^{-1}$; $a$, $b$ and $d$ - empirical coefficients carrying certain biological meaning; $c_{m}$ - maximum value of titratable acidity, °T; $c$ - coefficient equal to the difference between the maximum and the initial titratable acidity, °T; $q$ - specific rate of acid formation, °T/cfu.cm$^{-3}$.h; $\delta$ - an indicator defining the change in the curve shape or the change in the rate of lactic acid accumulation over time; $\tau$ - time of cultivation, h.

The advantage of this type of model lies in the fact that it can easily be solved by simple methods and does not require complicated numerical solution programs, but also allows for a different kind of interpretation of the biological processes observed in the cultivation of microorganisms.

The aim of the present work was to apply these types of models to describe the process of cultivation of probiotic lactic acid bacteria under static and dynamic (cultivation in a bioreactor) conditions, and to present the possibilities for interpreting the obtained results and the biological meaning of the variables.

**MATERIALS AND METHODS**

- Microorganisms
  The study was conducted with two strains of different lactobacilli species: *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB2 isolated from spontaneously fermented dairy products, and *Lactobacillus plantarum* BZ3 isolated from spontaneously fermented vegetables.
  Nutrient media (ISO 7889:2005)
  - MRS - broth;
  - MRS-agar;
  - Saline solution.

Methods of analysis
- Determination of titratable acidity (ISO/TS 11869:2012);
• Number of viable lactobacilli cells (ISO 7889:2005).
  Batch cultivation
  • Under static conditions. Cultivation was carried out in flasks thermostated at 37±1°C;
  • Cultivation in a bioreactor. Cultivation was carried out in the laboratory bioreactor shown in Fig. 1. The apparatus has a geometric volume of 2 dm³ and a working volume of 1.5 dm³ and is equipped with a Sartorius A2 control device, which includes all the measuring instruments for the fermentation process: temperature, pH, dissolved oxygen, etc. The fermentation process was carried out at a stirring speed of 150 rpm at 37±1°C.

Models for describing the kinetics of the fermentation process

The description of the kinetics was made using equations (16) to (18). The process parameters were defined using TableCurve2D and Excel. The software was also used for conducting statistical evaluation of the obtained models.

RESULTS AND DISCUSSION

The results of the studies on the dynamics of the fermentation process under static and dynamic conditions are presented in Fig. 2 and Fig. 3, and in Table 1 and Table 2. Biomass data are displayed in dimensionless form relative to the initial cellular concentration immediately after inoculation of the medium.

In the cultivation of L.delbrueckii ssp. bulgaricus TAB2, shortening of the lag-phase from 6 to 3 hours during dynamic cultivation was observed. At the same time, cultivation in a bioreactor led to the accumulation of one order higher concentration of viable lactobacilli cells, and as a result, about 10^{12} cfu/cm³ were accumulated in the apparatus compared to static cultivation (Fig. 2.). The titratable acidity values were close and were in the range between 170-190°T (Fig. 2). The data on the static and dynamic cultivation of L. plantarum BZ3 were similar (Fig. 3). About 10^{13}cfu/cm³ of L. plantarum cells were grown in the bioreactor. At the end of the process, comparable acidity values for L. plantarum BZ3 grown in a bioreactor and under static conditions were observed: 219°T and 214 °T, respectively.

The statistical analysis of the two models (part of the results are summarized in Table 1 and Table 2) showed that the semi-empirical models used described the kinetics of the fermentation process extremely accurately, and their accuracy was comparable to the classical kinetic models (data not presented). This was confirmed by the high correlation coefficient as well as by the low identification error of the model.

According to the exponential model for strain L. delbrueckii ssp. bulgaricus TAB2, a relatively higher specific growth rate was observed in its cultivation in the bioreactor (μ = 0.062 h⁻¹) compared to the same parameter during the static process (μ = 0.057 h⁻¹). Factor a is about 30% higher in bioreactor cultivation, indicating increased biochemical activity in the cells due to better stirring in the bioreactor compared to static cultivation. This was due to the presence of dissolved oxygen in the apparatus. It is well known that L. delbrueckii ssp. bulgaricus is the most sensitive lactic acid bacteria species in relation to oxygen, and in order to overcome the presence of oxygen in the apparatus, the oxid-peroxide system of the cells is activated (Table 1). There was a similar trend in the cultivation of L. plantarum BZ3. Again, coefficient a was 22% higher, which also reflected the higher specific growth rate in the bioreactor cultivation (μ = 0.110 h⁻¹) compared to the value of the same parameter during static cultivation (μ = 0.103 h⁻¹). The reason for the observed difference was once again the dissolved oxygen, but unlike its impact on strains of the L. delbrueckii ssp. bulgaricus species, the presence of oxygen had another impact on L. plantarum strains. Although they do not contain the cytochromes involved in transferring electrons from the substrate to the oxygen, these lactic acid bacteria are microaerophilic, and thanks to their flavoprotein systems, the flavoprotein oxidases, in particular, they can oxidize different substrates in the presence of oxygen (Kwasnik and Nesterenko 1975). Probably, through its flavoprotein oxidases, L. plantarum BZ3 manages to incorporate dissolved oxygen as the terminal acceptor of electrons in the oxidation of some substrates from the medium.

Coefficient d in the exponential model showed the influence of the culture conditions on the rate of the biochemical processes occurring in the cell. For both strains examined, higher values of this parameter were observed (2.263 for L.delbrueckii ssp. bulgaricus TAB2 and 2.462 for L.plantarum BZ3) compared to the values of the same parameter during static cultivation (1.994 and 2.218, respectively). The observed difference was a result of the delayed diffusion of nutrients to the surface of the cell; slow diffusion of secreted metabolic products from the cell into the culture medium; uneven temperature and pH distribution throughout the culture medium that put the microorganisms in the different microvolumes of the medium under different growth conditions. This was due to the lower kinetic parameter values for the studied strains cultivated under static conditions.
From the results presented in Table 1 for the logistic curve model, it can be seen that the relative and maximum specific growth rates for the two strains studied were higher in their cultivation in the bioreactor compared to static cultivation. For *L. delbrueckii* ssp. *bulgaricus* TAB2, \( \mu = 0.108 \text{ h}^{-1} \) and \( \mu_{\text{m}} = 1.818 \text{ h}^{-1} \) in dynamic cultivation, and \( \mu = 0.096 \text{ h}^{-1} \) and \( \mu_{\text{m}} = 1.637 \text{ h}^{-1} \) under static conditions. For *L. plantarum* BZ3, the values of these kinetic parameters when grown in a bioreactor with mechanical stirring and under static conditions were \( \mu = 0.175 \text{ h}^{-1} \) and \( \mu_{\text{m}} = 1.719 \text{ h}^{-1} \) and \( \mu = 0.156 \text{ h}^{-1} \) and \( \mu_{\text{m}} = 1.585 \text{ h}^{-1} \), respectively.

From the experimental data presented in Table 1, it appears that the exponential model yields lower relative growth rates for the two strains in their cultivation both in the bioreactor and in the static process compared to the logistic model. An analogous trend was also observed for the coefficient \( b \) in the logistic model showing the difference in the intensity of the biochemical processes occurring in the cells. For both strains tested, the value of this parameter was higher in cultivation in a bioreactor than in static cultivation: 0.956 and 0.725, respectively, for *L. delbrueckii* ssp. *bulgaricus* TAB2, and 0.739 and 0.649, respectively, for *L. plantarum* BZ3 (Table 1).

The results on the lactic acid formation are also interesting. The data from Table 2 show that the type of cultivation did not affect the degree of acid formation of *L. delbrueckii* ssp. *bulgaricus* TAB2. In the cultivation of this strain under both dynamic and static conditions, almost the same acid formation rate was observed: \( q = \)
In L. plantarum BZ3, the type of cultivation method had an effect on the degree of acid formation. Upon cultivation of the strain in a bioreactor, a slower acidification rate of \( q = 0.020 \) °T/cfu.cm\(^3\).h and a lower value of \( \delta = 1.96 \) was observed compared to the values of these parameters in static cultivation: \( q = 0.037 \) °T/cfu.cm\(^3\).h and \( \delta = 2.64 \). These results make it possible to conclude that the dissolved oxygen in the L. plantarum BZ3 cultivation in the bioreactor had a greater influence on acid formation, while in the L. delbrueckii ssp. bulgaricus TAB2, cultivation the dissolved oxygen did not affect the acid formation. This was the reason for the equalization of the titratable acidity values at the end of the process during L. plantarum BZ3 static and dynamic cultivation. Almost the same amounts of lactic acid at the end of the two processes were accumulated as a result of the more intensive acid formation process under static conditions, although during the lag phase in static cultivation, a retention in the titratable acidity values followed by a more intensive process of acid formation was observed.

CONCLUSION

The present paper reviews a new approach to the description of the fermentation kinetics of lactic acid bacteria cultivation. For this purpose, semi-empirical dependencies were used that allow the differential equations of the fermentation process to be solved analytically and without the help of complex mathematical procedures for the identification of process parameters. The proposed model was applied to the description of the lactic acid process in the cultivation of representatives of the Lactobacillus delbrueckii ssp. bulgaricus and Lactobacillus plantarum species. Through the described process kinetics, it was shown that the presence of dissolved oxygen in the culture medium led to differences in the cultivation process and hence to substantial differences in kinetic parameters. The parameters of the proposed models also revealed differences in the biochemical intensity of the processes occurring in the lactic acid bacteria cells.

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