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KINETICS OF BATCH FERMENTATION IN THE CULTIVATION OF A PROBIOTIC STRAIN *LACTOBACILLUS DELBRUECKII* SSP. *BULGARICUS* B1

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Abstract: A comparative study of kinetic models to describe the dynamics of the fermentation process of culturing of a probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 was performed. The models of Monod, Aiba, Tiessier, Hinshelwood and the equation of the logistic curve combined with the model of Ludeking-Piret were used. It has been found that the different models described the observed fermentation dynamics differently. The conducted comparative study demonstrated that the models of Monod and the equation of the logistic curve combined with the model of Ludeking-Piret were suitable for the description of the fermentation dynamics. The mathematical models showed no significant product and/or substrate inhibition. The culture developed with a low specific growth rate, but nevertheless it accumulated 10^{12}-10^{13} viable cells. The substrate was absorbed primarily from cells in the stationary growth phase rather than cells in the exponential growth phase.

Keywords: probiotics, fermentation, kinetic models

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INTRODUCTION

According to FAO/WHO, 2001 probiotics are defined as live microorganisms when administered in adequate amounts confer a health benefit on the host. There are a number of requirements for probiotic foods including the safety of the products and the content of appropriate probiotic organisms in sufficient numbers at the time of consumption. Therefore, the probiotic strains selected should be suitable for large-scale industrial production and possess the ability to survive and retain their functional and brnrficial properties during production and storage as dried or frozen cultures. Probiotic strains have to survive during food processing, and also in the final food products into which they are formulated (Tripathi and Giri, 2014).

Probiotics provide a number of health benefits mainly through maintenance of the balance of the normal intestinal microflora, enhancement of the immune system (Gilliland, 1990), reduction of serum cholesterol level and blood pressure (Rasic, 2003), protection against gastrointestinal pathogens (D'Aimmo et al., 2007; Lourens-Hattingh & Viljoen, 2001),anti-carcinogenic activity (Rasic, 2003), improved utilization of nutrients and improved nutritional value of food (Lourens-Hattingh & Viljoen, 2001). Probiotics improve the health of the host in several ways: prevention of infantile diarrhea, osteoporosis, urinogenital diseases, food allergy and atopic diseases; alleviation of constipation and hypercholesterolemia; reduction of antibody-induced diarrhea; control of inflammatory bowel diseases; and protection against colon and bladder cancer (Lourens-Hattingh & Viljoen, 2001; Salminen, 1996; Venturi et al., 1999).

These health benefits are suggested to be a result from the growth and action of the probiotics during the manufacturing of functional foods. Other benefits may result from the growth and action of certain probiotic strains in the gastrointestinal tract (Rasic, 2003; Tripathi and Giri 2014).

Cultivation is a key step in the production of probiotic preparations containing lactic acid bacteria. The process of cultivation must be control appropriately to ensure high biomass yield and complete substrate utilization. This can only be achieved through the development of mathematical relationships to describe the kinetics of microbial growth. By the models and their parameters can be developed quality control systems by which the process is maintained in optimal conditions. This in turn ensures the production of high concentrations of probiotic strains and the maintainance of their physiological and functional benefits (Kostov, 2015; Angelov and Kostov, 2011; Pirt, 1975).

The aim of the present work was the study of the process of batch fermentation of a probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus*

B1 and examination of the kinetic models to describe the dynamics of the lactic acid fermentation process.

MATERIALS AND METHODS

Microorganisms

The studies in the present work were performed with *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 with proven probiotic properties, isolated from homemade yoghurt.

Cultural medium

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

Bioreactor and cultivation conditions

The batch fermentation was carried out in LAPTg10-broth without pH adjustment. The medium was sterilized at 121ºC for 20 min. After cooling to 35-37 ºC the medium in the bioreactor was inoculated with 5% (v/v) inoculum from a fresh 24-hour culture of the studied strain. The batch fermentation was performed at 37±1 °C, 150 rpm, without aeration. The duration of the fermentation was 30 hours, taking samples for the determination of the number of viable cells (cfu/cm^3) and the titratable acidity. The used laboratory bioreactor was with a geometric volume of 2 dm^3 and working volume of 1,5 dm^3 and was provided with a control unit "Sartorius A2", which included a control loop for the agitation rate, the temperature, the pH, etc.

Determination of the titratable acidity

For the determination of the acid-forming ability of the lactic acid bacteria, the titratable acidity (expressed as Toerner degree, 1 °T) was determined by titration of 10 cm^3 sample with 0.1N NaOH to a pale pink endpoint using phenolphthalein as an indicator (1 °T=0.009 g lactic acid) (Macrae et al., 1993; Madigan et al., 2000).

Determination of the viable counts of lactobacilli

Appropriate tenfold dilutions of the samples were prepared according to the method of serial dilutions in saline solution. The last three dilutions were spread plated on MRS-agar medium and the Petri dishes were incubated for 3 days at 37±1 °C, until the formation of single colonies (Macrae et al., 1993; Madigan et al., 2000).

The samples were decolorized with activated carbon, then deproteinated with lead acetate (Ivanov et al., 1979). After that they were diluted to the proper level with distilled water. 50 µl of each sample were mixed with 1 cm^3 PAHBAH reagent. A blank containing only PAHBAH reagent was prepared as well. The tubes with the samples and the blank were incubated in a boiling water bath for 6 min, cooled and the absorbance at 410 nm was measured (Barry & Murphy, 2000).

Kinetic models and identification of the model parameters

The kinetics of the lactic acid fermentation process were examined by the system of differential equations (Birol et al., 1998; Kostov et al., 2012):

$$\begin{aligned} \frac{dX}{dt} &= f(X,S,P) \\ \frac{dP}{dt} &= f(X,S,P) \\ \frac{dS}{dt} &= f(X,S,P) \end{aligned} \qquad (1)$$

The used kinetic equations (Birol et al., 1998; Kostov et al., 2012) through which the system (1) acquires a certain type are presented in Table 1.

Table 1. Mathematical models for description of the kinetics of the fermentation process

№	Model	dX/dt	dP/dt	dS/dt
2	**Monod**	$\mu_{\max}\left(\frac{S}{K_{sx}+S}\right)$	$q_{P\max}\left(\frac{S}{K_{sp}+S}\right)X$	$-\frac{1}{Y_{x/s}}\frac{dX}{dt}-\frac{1}{Y_{p/s}}\frac{dP}{dt}$
3	**Tiessier**	$\mu_{\max}\left(1-\exp\left(\frac{S}{K_{sx}}\right)\right)X$	$q_{P\max}\left(1-\exp\left(\frac{S}{K_{sP}}\right)\right)X$	
4	**Hinshelwood**	$\mu_{\max}\left(\frac{S}{K_{sx}+S}\right)(1-K_{PX}P)X$	$q_{P\max}\left(\frac{S}{K_{sp}+S}\right)(1-K_{PP}P)X$	
5	**Aiba**	$\mu_{\max}\left(\frac{S}{K_{sx}+S}\right)\exp(-K_{PX}P)X$	$q_{P\max}\left(\frac{S}{K_{sp}+S}\right)\exp(-K_{PP}P)X$	
6	**Ghose and Tyagi**	$\mu_{\max}\left(1-\frac{P}{P_{x\max}}\right)$	$q_{P\max}\left(1-\frac{P}{P_{p\max}}\right)$	
7	**Logistic equation and Ludeking-Piret model**	$[\mu_{\max}-\beta X]X$	$q_{p0}X+K\frac{dX}{d\tau}$	$\gamma\frac{dX}{d\tau}+\delta X$

Parametric identification of the models was carried out in MATLAB environment (Kostov et al., 2012; Mitev and Popova, 1995; Popova 1997). The sum of squared errors of the model output data:

$$F(r)=\left(X\left(k_1,k_2,...,k_n\right)-X^e\right)^2+\left(S\left(k_1,k_2,...,k_n\right)-S^e\right)^2+\left(P\left(k_1,k_2,...,k_n\right)-P^e\right)^2 \quad (1)$$

was minimized. For that purpose the function "fmincon" was applied. Its input vector was:

$$V=\left[X\left(k_1,k_2,...,k_n\right),S\left(k_1,k_2,...,k_n\right),P\left(k_1,k_2,...,k_n\right)\right]^T \quad (2)$$

The output is vector of model parameters: $k=\left[k_1,k_2,...,k_n\right]$. For that purpose to the ordinary differential equations model are added the following complimentary differential equations:

$$\frac{dk_1}{dt}=0;\ \frac{dk_2}{dt}=0;\ \frac{dk_3}{dt}=0\ ...\ \frac{dk_n}{dt}=0 \quad (3)$$

because $k_1,k_2,...,k_n$ are constants. The overall differential equations system the function "ode45" was used.

Equation (7) was solved analytically by the procedure described in Wang et al., 2007.

RESULTS AND DISCUSSIONS

Dynamics of the lactic acid fermentation process

A batch fermentation process with the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 was performed. The data for the dynamics of the fermentation process is presented on Figure 1.

The fermentation process was characterized by a quick start, the lag-phase was about 3-4 hours. In this period, the accumulation of cells was delayed, a reduction in the redox potential of the medium was observed as well. After the 3rd hour of cultivation the redox potential retained its value within 2-3 hours. It can be considered that at the end of this period the lag phase has ended and the biomass gradually entered the exponential growth phase between the 3rd and the 6th hour.

The active accumulation of biomass started with the end of the lag-phase. High concentration of viable cells - between 10^{12} - 10^{13} cfu/cm^3 were accumulated within 12 to 15 hours from the beginning of the fermentation. During the exponential growth phase the redox potential increased. The pH decreased with a rate close to the rate of lactic acid production. At the end of the exponential phase, a gradual deceleration of acid production, which resulted in a change in the rate of the pH reduction, was observed.

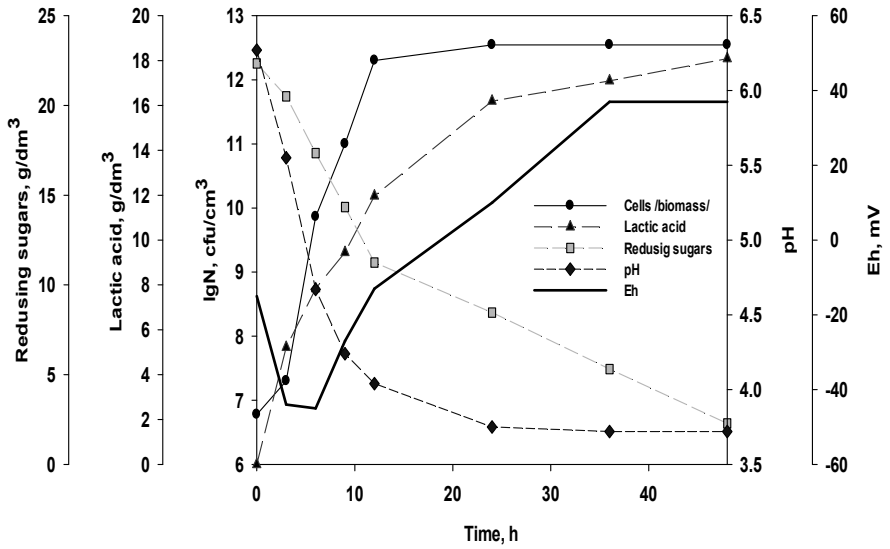

Figure 1. Dynamics of the batch lactic acid fermentation process of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1

The cells entered the stationary growth phase between the 12th and the 15th hour. This is the period of slow exponential growth. After this period began the establishment of most process parameters to constant values. pH maintained a constant value around the 24th hour, while the redox potential of the period maintained a constant value after the 36th hour. This was due to the reduction of the sugars in the medium. It is noticeable that the utilization of sugars was also characterized by two rates. In the exponential growth phase the consumption was faster, while in the stationary phase was delayed.

Kinetic models for describing the dynamics of the fermentation process

For the description of the kinetics of the fermentation process the models in Table 1 were used. They reflect different biological relations - substrate and product inhibition, internal population competition. The comparison of the models with the experimental data is presented on Figure 2 to Figure 4. The identified kinetic characteristics are presented in Table 2.

The model of Monod described best the kinetics of accumulation of biomass. This model described all three growth phases very well (Figure 2) - the specific growth rate was relatively low (0,062 h^{-1}) at a very good relationship between the substrate and the cell population (K_{SX} = 2.182 g/dm^3). The lower growth rate was probably due to the prolonged stationary phase. The models of Aiba and Hinshelwood showed product inhibition differently. These two models described satisfactory the cells growth during the fermentation. No product inhibition was observed, the values of the inhibition constants were

much lower than the specific growth rate and ranged between 0,048-0,0795 g/dm^3. The Aiba's model assumed exponential inhibition; therefore higher specific growth rate - 0,237 h^{-1} was observed. For the model of Hinshelwood a characteristic linear inhibition was established, thus the growth rate of the population was closer to the value of the model of Monod - 0,089 h^{-1}. The model of Tiessier implied substrate inhibition, which was not observed in the fermentation dynamics. This model gave the highest specific growth rate - 0,517 h^{-1}, but the error was quite high. This model was not suitable for the description of this type of fermentation. The model of the logistic curve also described the accumulation of biomass very precisely - the population was characterized by a low specific growth rate (0,0312 h^{-1}), but the low rate of internal population competition compensated that effect. For this reason, low cell loss, which is an advantage for the probiotic strains, was observed.

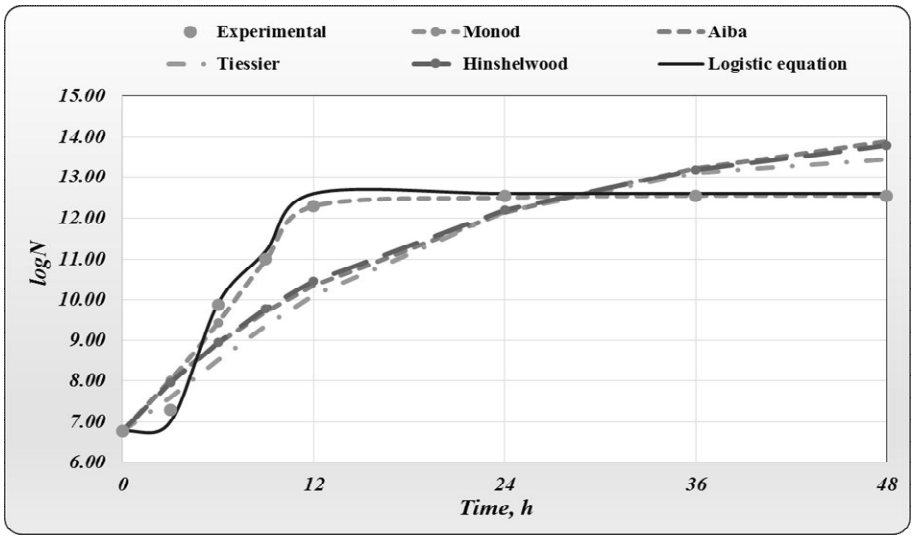

Figure 2. Comparison of the experimental data on the accumulation of viable cells with the kinetic models in the culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1

The models of Monod and the logistic curve were the most suitable models for the description of the accumulation of the biomass.

Naturally, for a general conclusion it is necessary to take into account the processes of accumulation of lactic acid and absorption of sugars.

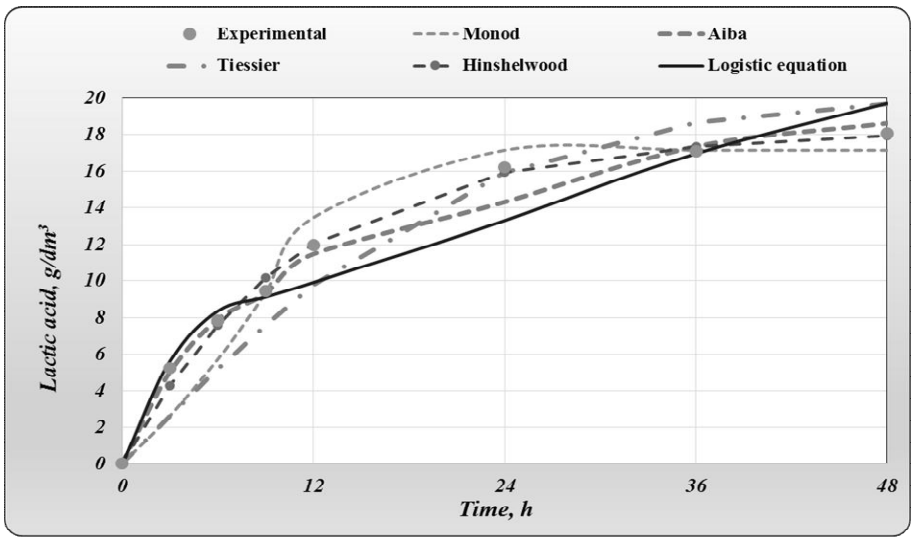

Figure 3. Comparison of the experimental data on the accumulation of lactic acid with the kinetic models in the culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1

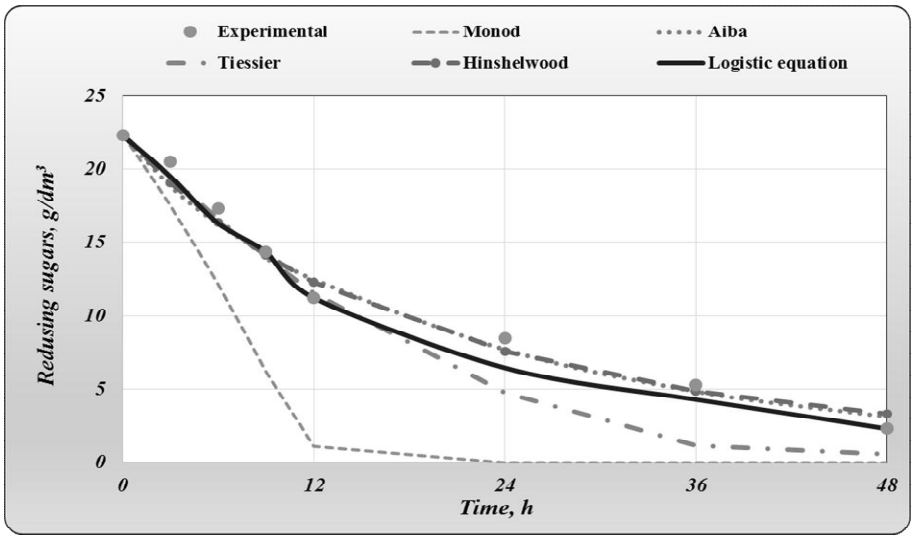

Figure. 4. Comparison of the experimental data for the substrate absorption with the kinetic models in the culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1

All five tested mathematical model described the accumulation of lactic acid with high accuracy. Depending on the model, the specific rate of lactic acid production ranged from 0,12 g/(lgN.h) to 2,25 g/(lgN.h). There was a less pronounced product inhibition, but the constants in the models of Aiba and Hinshelwood were from 5 to 24 times lower than the rate of accumulation of the product, and therefore can be neglected. It should be noted that the

equation of Luedeking-Piret showed that lactic acid was accumulated mainly by cells in the stationary growth phase and not by cells in the exponential growth phase. This means that the main amount of substrate was used for the accumulation of cells, which is important for the preparation of large amounts of biomass of a probiotic strain. With regard to the accumulation of acid no preference can be given to any of the models (Figure 3).
Interesting results were obtained by the comparison of the experimental data for the absorption of the substrate (Fig. 4). The model of Monod demonstrated that the sugars were consumed within 12-15 hours, while at the same time the cell growth and accumulation of the acid continued. This was probably due to differences in the rates of substrate consumption and transformation, but this could not be recognized by the model. The other models described the fermentation dynamics very well. The yield coefficients $Y_{P/X}$ and $Y_{P/S}$ varied in an extremely wide range (Table 2). They may be viewed as generalized rate constants that take into account the cumulative growth of the population. Interesting results were obtained from the model of Luedeking-Piret for the absorption of sugars. The parameters of this model (Table 2) showed that only cells in the stationary growth phase digested the substrate meaning that the cells had absorbed the substrate and only then they began the process of cellular division. This confirmed the results obtained from the model of Monod, indicating that the cells first utilized the substrates and only then they started the processes of division and production of lactic acid.

CONCLUSIONS

Cultivation of a probiotic strain of lactic acid bacteria in order to determine the kinetics of the microbial process was performed. It was found that under optimal conditions the culture accumulated significant amount of viable cells - 10^{12}-10^{13} cfu/cm^3 at the end of the fermentation process. From the studies with the different kinetic models it has been established that there was no product and substrate inhibition of the growth of the biomass. Among the studied kinetic dependencies the equation of Monod and the model of the logistic curve combined with the equations of Luedeking-Piret were the most suitable to describe the dynamics of the fermentation process. From these two models it was found that the cells utilized the substrate primarily for the formation of new cells and only then lactic acid was produced and accumulated in the medium as an end product of the metabolism. The models of Tiessier, Aiba and Hinshelwood described the cultivation process quite

precisely, but due to the low coefficient of substrate and product inhibition can easily be reduced to the equation of Monod.

Table 2. Parameters of the kinetic models to describe the lactic acid fermentation process

Monod								
PARAMETERS OF THE MODEL								**ERROR**
μ_{max}	$K_{S/X}$	q_{pmax}	$K_{S/P}$	$Y_{X/S}$	$Y_{P/S}$	K_{px}	K_{pp}	
h^{-1}	g/dm^3	g/(lgN.h)	g/dm^3	-	-	g/dm^3	g/dm^3	
0.062	2.182	0.12	0.007	0.278	11.34	-	-	10.16
μ_{max} - maximum specific growth rate; Ks/x - Monod constant for the cells; q_{pmax} -maximum specific rate of accumulation of lactic acid; $K_{S/P}$ – Monod constant for the product; Yx/s – yield coefficient of cells from one unit of substrate; $Y_{P/S}$ - yield coefficient of lactic acid from one unit of substrate;								
Aiba								
PARAMETERS OF THE MODEL								**ERROR**
μ_{max}	$K_{S/X}$	q_{pmax}	$K_{S/P}$	$Y_{X/S}$	$Y_{P/S}$	K_{px}	K_{pp}	
h^{-1}	g/dm^3	g/(lgN.h)	g/dm^3	-	-	g/dm^3	g/dm^3	
0.237	50	0.517	10	0.409	10	0.0795	0.163	18.02
μ_{max} - maximum specific growth rate; Ks/x - Monod constant for the cells; q_{pmax} -maximum specific rate of accumulation of lactic acid; $K_{S/P}$ – Monod constant for the product; Yx/s – yield coefficient of cells from one unit of substrate; $Y_{P/S}$ - yield coefficient of lactic acid from one unit of substrate; Kpx, Kpp - constants of inhibition of the growth and the accumulation of lactic acid								
Hinshelwood								
PARAMETERS OF THE MODEL								**ERROR**
μ_{max}	$K_{S/X}$	q_{pmax}	$K_{S/P}$	$Y_{X/S}$	$Y_{P/S}$	K_{px}	K_{pp}	
h^{-1}	g/dm^3	g/(lgN.h)	g/dm^3	-	-	g/dm^3	g/dm^3	
0,089	10,61	1,28	100	0,374	100	0,0485	0,0526	15,67
μ_{max} - maximum specific growth rate; Ks/x - Monod constant for the cells; q_{pmax} -maximum specific rate of accumulation of lactic acid; $K_{S/P}$ – Monod constant for the product; Yx/s – yield coefficient of cells from one unit of substrate; $Y_{P/S}$ - yield coefficient of lactic acid from one unit of substrate; Kpx, Kpp - constants of inhibition of the growth and the accumulation of lactic acid								
Tiessier								
PARAMETERS OF THE MODEL								**ERROR**
μ_{max}	$K_{S/X}$	q_{pmax}	$K_{S/P}$	$Y_{X/S}$	$Y_{P/S}$	K_{px}	K_{pp}	
h^{-1}	g/dm^3	g/(lgN.h)	g/dm^3	-	-	g/dm^3	g/dm^3	
0,517	250	1,51	250	0,33	10	-	-	80,1
μ_{max} - maximum specific growth rate; Ks/x - Monod constant for the cells; q_{pmax} -maximum specific rate of accumulation of lactic acid; $K_{S/P}$ – Monod constant for the product; Yx/s – yield coefficient of cells from one unit of substrate; $Y_{P/S}$ - yield coefficient of lactic acid from one unit of substrate;								
Equation of the logistic curve and model of Luedeking-Piret								
PARAMETERS OF THE MODEL								**R^2**
μ_{max}	**β**	q_{pmax}	**K**		**δ**		**γ**	
h^{-1}	$dm^3/(lgN.h)$	g/(lgN.h)	g/(lgN.h)		-		-	
0.0312	0.00248	2.25	0.0095		6.23		0	0.76

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REFERENCES

1. Angelov M., Kostov G. (2011). *Bioengineering aspects of fermentation processes with free and immobilized microbial cells.* Agency 7Д.
2. Barry V. M. & Murphy A. (2000). Measurement of total fructan in foods by enzymatic/spectrophotometric method: collaborative study. *Journal of AOAC International*, 83(2), 356-364.
3. Birol G., Doruker P.; Kardar B., Onsan Z., Ulgen K. (1998). Mathematical description of ethanol fermentation by immobilised *Saccharomyces cerevisiae*, Process Biochemistry, 33, 763-771.
4. D'Aimmo, M. R., Modesto M., & Biavati B. (2007). Antibiotic resistance of lactic acid bacteria and Bifidobacterium spp. Isolated from dairy and pharmaceutical products. *International Journal of Food Microbiology*, 115(1), 35–42.
5. FAO/WHO. (2001). Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. from ftp.fao.org/es/esn/food/probioreport_en.pdf
6. Gilliland, S. E. (1990). Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiology Letters*, 87(1–2), 175–188.
7. Ivanov, T., Gerov S., Yankov A., Bambalov G., Tonchev T., Nachkov D., Marinov M. (1979). *Practical course in wine technology*, Hristo G. Danov, Plovdiv.
8. Kostov G., Popova S., Gochev V., Koprinkova-Hristova P., Angelov M., Georgieva A. (2012). Modeling of batch alcohol fermentation with free and immobilized yeasts *Saccharomyces cerevisiae* 46 EVD. *Biotechnology & Biotechnological Equipment*, 26(3), 3021-3030, DOI: 10.5504/bbeq.2012.0025
9. Kostov G. (2015). *Intensification of fermentation processes with immobilised biocatalysts*. Thesis for Doctor of Sciences, University of Food Technologies, Plovdiv, Bulgaria.
10. Lourens-Hattingh, A., & Viljoen B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal*, 11(1–2), 1–17.
11. Macrae, R., Robinson R. K., Sadler M., Encyclopaedia of Food Science, Food Technology and Nutrition, Vol. 5, Academic Press, San Diego 1993, p. 3082.
12. Madigan, M. T., Martinko J. M., Parker J., Brock Biology of Microorganisms, Prentice-Hall, Upper Saddle River 2000, 142–143
13. Mitev S.V., Popova S. (1995). A model of yeast cultivation based on morphophysiological parameters. J. *Chemical and Biochemical Engineering Quarterly*, 3, Zagreb, 119-121.
14. Pirt S.J. (1975). *Principles of microbe and cell cultivation.* Wiley, p. 274

15. Popova S. (1997). Parameter identification of a model of yeast cultivation process with neural network, *Bioprocess and Biosystems Engineering*, 16(4), 243-245, DOI: 10.1007/s004490050315.
16. Rasic J. L. (2003). Microflora of the intestine probiotics. In B. Caballero, L. Trugo, & P. Finglas (Eds.), *Encyclopedia of food sciences and nutrition* (pp. 3911–3916). Oxford: Academic Press.
17. Salminen S. (1996). Uniqueness of probiotic strains. *IDF Nutrition Newsletter*, *5*, 16–18.
18. Tripathi M.K., S.K. Giri (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of functional foods*, 9: 225–241. DOI:10.1016/J.JFF.2014.04.030
19. Venturi, A., Gionchetti P., Rizzello F., Johansson R., Zucconi E., & Brigidi P. (1999). Impact on the composition of the faecal flora by a new probiotic preparation: Preliminary data on maintenance treatment of patients with ulcerative colitis. *Alimentary Pharmacology & Therapeutics*, 13(8), 1103–1108.
20. Wang X., Xu P., Yuan Y., Liu C., Zhang C., Yang Z., Yang C., Ma C. (2006). Modeling for Gellan Gum Production by *Sphingomonas paucimobilis ATCC 31461* in a Simplified Medium. *Applied and Environmental Microbiology*, 3367–3374