Influence of the composition of the culture medium on the growth and sporulation of the probiotic strain *Bacillus amyloliquefaciens* M

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Abstract. *Bacillus amyloliquefaciens* M is a non-pathogenic microorganism with probiotic properties isolated from mineral waters in the village of Yabalkovo, Haskovo region, for inclusion in the composition of probiotics and probiotic foods. It has high antimicrobial activity against pathogenic bacteria, causing food toxic infections and intoxications, as well as against saprophytic microorganisms - bacteria and fungi, which makes it suitable as a biological preparation for plant protection. In order to perform these functions, it is necessary to obtain a spore concentrate. The composition of the nutrient medium for the cultivation and sporulation of the strain has been determined: MP nutrient broth with the addition of salts, in which the strain grew well and a high sporulation rate of 99% was achieved. Cultivation in a bioreactor has been carried out and the time to achieve a high degree of sporulation has been determined. The kinetic parameters of growth and sporulation of *Bacillus amyloliquefaciens* M in aerobic batch cultivation with a high concentration of spore forms have also been determined. It has been shown that the sporulation process in this strain starts after 12 h from the cultivation start.

1 Introduction

The balance of the gastrointestinal microbiota is of particular importance for the health of the body, as intestinal bacteria take part in nutritional, immunological and physiological activities [1]. Disturbance of the balance in the gastrointestinal tract causes chronic diseases, such as inflammation of the intestinal mucosa, violation of its selective selectivity and semi-permeability, irritable bowel syndrome [2]. Maintaining balance in the gastrointestinal tract is achieved by taking up concentrates containing a significant amount of viable cells of beneficial microorganisms known as probiotics. Some strains of the genera *Lactobacillus*, *Bifidobacterium* and also some representatives of *Propionibacterium* sp. are included in the composition of probiotics and foods due to their health effects [3, 4]. Probiotic microorganisms contribute to the restoration of the intestinal balance, play an important role in maintaining health and improve the quality of some foods in which they are included [5, 6]. It has been found that orally ingested *Bacillus* spores can proliferate in the intestine for a certain period [7, 8]. Probiotic adhesion is related to stability, such as survival of strains that have been exposed to the gastrointestinal tract, autoaggregation, and hydrophobicity [9]. *Bacillus* probiotics in spore form can survive extreme environmental conditions, allowing long-term survival in conditions that would otherwise kill vegetative bacteria [10]. Spores of *Bacillus* probiotic bacteria have been shown to germinate, grow and resporulate in the gastrointestinal tract [11, 12]. Probiotics produce antimicrobial substances, including bacteriocins, short-chain fatty acids, and organic acids, and modulate gastrointestinal disorders through antimicrobial and anti-adhesive effects against pathogenic strains [9].

Probiotics are also known to have immunomodulatory roles, anticancer effects and promote cholesterol lowering. Their function depends on their metabolites such as bacteriocins, biosurfactant, exopolysaccharide and siderophore [13]. *Bacillus* probiotics have been shown to temporarily reside as symbiotic organisms in the host [14]. *Bacillus amyloliquefaciens* belongs to the superkingdom of bacteria, family *Bacillaceae* and genus *Bacillus*. It is Gram-positive, endospore-forming, rod-shaped with chain-forming cell morphology [15]. The US Food and Drug Administration (FDA) considers *Bacillus amyloliquefaciens* to be safe [16, 17] for application in food and pharmaceutical preparations. It has also been mentioned that its metabolic activity is considered non-toxigenic [18]. In addition to being considered safe, its widespread distribution in various raw materials [19, 20], as well as its endospore-forming abilities allow it to survive in extreme living conditions. This, in turn, determines its wide application in various areas of the

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food industry: in food production (enzyme synthesis, prebiotics and probiotics and functional and bioactive foods), in pharmaceuticals (antimicrobial, anticancer and diabetic pharmaceuticals), in agriculture (for the prevention of plant and animal diseases), for environmental protection (waste treatment and biofuels) and others [21]. The ability of *Bacillus amyloliquefaciens* to hydrolyze various plant and animal products [15, 22]; to synthesize various enzymes, proteins and carbohydrates [15, 16]; to exert prebiotic and probiotic properties [23] makes it relevant for the food industry. It can be used to produce enzymes, antimicrobials, insecticides, and biochemicals, exopolysaccharides (EPS), vitamins, purine nucleosides, and poly gamma glutamic acid [15, 24, 25]. In order for its curative-prophylactic probiotic and insecticidal action to be effective, it is necessary for the concentration of spores to exceed 10^6 cfu/g in preparations and functional foods, as well as in plant protection agents [26, 27].

The aim of the present work was to investigate the influence of the composition of the nutrient medium on the growth and sporulation of *Bacillus amyloliquefaciens* M.

2 Materials and methods

2.1 Microorganisms

Bacillus amyloliquefaciens M, isolated from mineral waters in the village of Yabalkovo, Haskovo region and identified to species level by sequencing the gene for 16S rRNA, was used in the present research. The strain was stored at a temperature of 4±2°C and was subcultured every 60 d.

The following pathogenic microorganisms were used as test microorganism: *Escherichia coli* ATCC 25922, *Salmonella* sp. (clinical isolate), *Salmonella abony* NTCC 6017, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 19111, *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus cereus* and saprophytic microorganisms - yeast *Candida utilis* ATCC 42402, *Saccharomyces cerevisiae* ATCC 9763 and phytopathogenic fungi *Aspergillus niger* ATCC 1015, *Penicillium chrysogenum* ATCC 28089, *Aspergillus flavus* ATCC 9643, *Fusarium moniliforme* ATCC 38932. The following nutrient media were used for conducting the experimental studies

2. 1. MP nutrient broth (g/dm^3): peptone – 10; NaCl – 5; meat extract – 3; glucose – 10. The medium was autoclaved for 25 min at 121 °C.

2. 2. LBG-agar (g/dm^3): tripton - 10; yeast extract - 5; NaCl – 10; glucose-10; agar – 15. The medium was autoclaved for 25 min at 121 °C.

2. 3. Fermentation medium with molasses (g/dm^3): molasses - 20; peptone - 10; corn extract – 3; $CaCl_2$ - 0.22; $MgSO_4$ - 0.11; K_2HPO_4 - 0.24. The medium was sterilized three times in a Koch apparatus for 30 min each time, at a temperature of 98°C.

2. 4. Fermentation medium with malt (g/dm^3): malt – 20; corn extract – 3; molasses - 20; corn extract – 3; $CaCl_2$- 0.22; $MgSO_4$ - 0.11; K_2HPO_4 - 0.24. The medium was sterilized three times in a Koch apparatus for 30 min each time, at a temperature of 98°C.

2. 5. Fermentation medium (g/dm^3): peptone - 10; NaCl – 10; meat extract – 5; $CuCl_2$- 0.001; $MgSO_4$ - 0.5. The medium was autoclaved for 25 min at 121°C.

2.2 Methods of analysis

2.2.1 Cultivation of Bacillus amyloliquefaciens M

Cultivation of the strain was carried out in 500 cm^3 Erlenmeyer flasks with 10 cm^3 of nutrient medium with aeration and statically at a temperature of 37°C for 24-48 h on a rotary shaker (220 min^{-1}). Inoculation was carried out with 1% (v/v), with 18 h of vegetative inoculation material.

Preparation of sterile filtrate from the culture medium. The culture medium was centrifuged at 3500 min^{-1} for 15 min to separate the biomass. The supernatant was separated and filtered through a membrane filter (0.45 μm). The biomass was washed twice with physiological solution and resuspended to the original volume (2.5 cm^3).

2.2.2 Determination of antimicrobial activity

To determine the antimicrobial activity of the studied strain against pathogenic and saprophytic microorganisms, culture suspension (CS), biomass in physiological solution (BMPS) and cell-free supernatant (CFS) obtained from a 24-h culture of the strain were used. The antimicrobial activity was tested against the following test microorganisms: *Escherichia coli* ATCC 25922, *Salmonella* sp. (clinical isolate), *Salmonella abony* NTCC 6017, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 19111, *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus cereus, Candida utilis* ATCC 42402, *Saccharomyces cerevisiae*, *Aspergillus niger* ATCC 1015, *Penicillium chrysogenum* ATCC 28089, *Aspergillus flavus*, *Fusarium moniliforme* ATCC 38932. Prior to preparation of the fungal spore suspensions, the fungal strains were grown in an incubator at 30°C on LBG-agar for 3 to 7 d. Suspensions of each of the test microorganisms (10^6-10^7 cfu/cm^3) were prepared and used to inoculate the petri dishes with LBG-agar medium and after solidification of the medium, wells (d=6 mm) were prepared. 0.06 cm^3 of CS, BMPS and CFS were pipetted into the wells and the petri dishes with the test microorganisms were incubated at 30°C or 37°C for 24 h to 48 h. The antimicrobial activity was determined by measuring the zones of inhibition in mm.

2.2.3 Determination of the number of viable cells and spores of Bacillus amyloliquefaciens M

The number of viable cells of *Bacillus amyloliquefaciens* M was determined by preparation of tenfold dilutions and spread plating on LBG-agar medium. The Petri dishes were thermostated for 24 h at 37±1°C until the appearance of single colonies.

The same procedure was performed for the determination of the number of spores, but the samples were treated at a temperature of 80°C for 20 min to destroy the vegetative forms before the preparation of the tenfold dilutions.

2.2.4 Batch cultivation in a laboratory bioreactor with a volume of 2 dm³

The cultivation of *Bacillus amyloliquefaciens* M was carried out in a laboratory bioreactor with a geometric volume of 2 dm^3 and a working volume of 1.5 dm^3 (Fig. 1). Management and monitoring of the main fermentation parameters was done by a Sartorius A2 control unit, which included control loops for stirring rate, temperature, pH, etc.

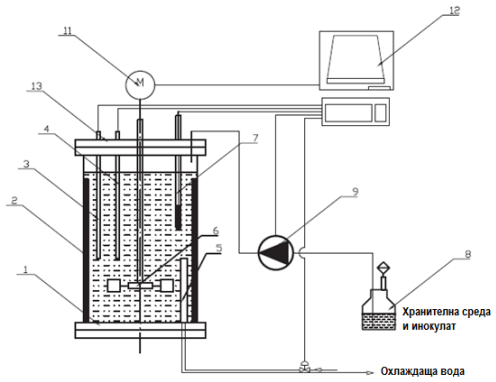

Fig. 1. Laboratory bioreactor
1 – apparatus with a geometric volume of 2 dm^3; 2 – breakers; 3 – temperature probe /thermometer/; 4 – cooling/heating device (water jacket); 5 – additional cooling/heating device; 6 – turbine mixer; 7 – pH/Eh electrode; 9 – fermentation medium/inoculum/medium for pH correction; 9 – filter; 10 – peristaltic pump; 11 – stirring drive; 12 – control "Sartorius A2"; 13 - cover;

The cultivation was carried out in the following order: the apparatus was washed, then filled with 0.3% neomycin solution for cold sterilization for 24 h. After the sterilization time has expired, the apparatus was washed several times with sterile physiological solution or distilled water, after which it was ready to perform the fermentation process. The nutrient medium was sterilized and then it was loaded into the apparatus sterilely, using a peristaltic pump. The batch cultivation process was carried out in nutrient medium without pH adjustment. The nutrient medium was sterilized at 121°C for 20 min prior to loading in the bioreactor. After cooling to 37°C, the medium was inoculated with 1% (v/v) inoculum of a fresh 24 h culture of *Bacillus amyloliquefaciens* M, grown on MPB. The cultivation process was carried out at 37°C, with stirring rate of 450 rpm, with aeration. The duration of cultivation was 48 h, with periodic sampling of the culture liquid for analysis of total number of viable cells and spores of *Bacillus amyloliquefaciens* M (cfu/cm^3).

2.2.5. Modeling of process kinetics and identification of model parameters

The modeling of the growth kinetics of *Bacillus amyloliquefaciens* M cultivated in the different fermentation media was done using the classical logistic curve equation (1), and the sporulation kinetics of the strain was modeled with the exponential growth model (2):

$$\frac{dX_b}{d\tau} = \mu_m X_b - \beta {X_b}^2$$
$$\mu_m X_b - \frac{\mu_m}{X_{bk}} {X_b}^2 \quad (1)$$
$$\mu_m \left(1 - \frac{X_b}{X_{bK}}\right) X_b$$

$$\frac{dX_{bs}}{d\tau} = kX_{bs} \Longrightarrow X_{bs} = {X_0}_{bs} e^{k\tau} \quad (2)$$

where: μ_m - maximum specific growth rate of vegetative cells, h^{-1} ; X_b and X_{bK} – current and final concentration of vegetative cells in dimensionless form; k – maximum rate constant of sporulation; X_{bs} and X_{0bs} - current and initial spore concentration in dimensionless form; β – coefficient of intra-population competition, $Xb/m^3.h$.

The differential equations were solved by the 4th order Ringe-Kutta method in Excel. The general and discretized forms of the equation were:

$$\frac{dy}{dx} = F(x,y)$$
$$\frac{y_{i+1} - y_i}{x_{i+1} - x_i} = \frac{y_{i+1} - y_i}{h} = F(x,y)$$

where h is the step of the solution.
For a system of ordinary differential equations (k=1,...,.K equations):

$$\frac{dy_1}{dx_1} = F_1(x, y_1, y_2, \cdots\cdots\cdots, y_k)$$
$$\frac{dy_2}{dx_2} = F_2(x, y_1, y_2, \cdots\cdots\cdots, y_k)$$
$$\frac{dy_k}{dx_k} = F_1(x, y_1, y_2, \cdots\cdots\cdots, y_k)$$

The algorithm for solving ordinary differential equations by the Runge-Kutta method of the 4th order is as follows:

$$y_{k,i+1} = y_{k,i} + \frac{1}{6}\left(k_{k,1} + 2 \cdot k_{k,2} + 2 \cdot k_{k,3} + k_{k,4}\right)$$
$$k_{k,1} = hF_k\left(x_i, y_{1,i}, y_{2,i}, \cdots\cdots\cdots y_{k,i}\right)$$

$$k_{k,2} = hF_k\left(x_i + \frac{h}{2}, y_{k,i} + \frac{k_{k,1}}{2}\right)$$

$$k_{k,3} = hF_k\left(x_i + \frac{h}{2}, y_{k,i} + \frac{k_{k,2}}{2}\right)$$

$$k_{k,4} = hF_k\left(x_i + h, y_{k,i} + k_{k,3}\right)$$

The Solver function in Excel was used to identify the parameters of the models. The model parameters were determined by the minimization of the square of the difference between the experimental data and the data obtained from the corresponding model.

2.3 Processing of the results

Data from triplicate experiments were processed using MS Office Excel 2013 software, using statistical functions to determine the standard deviation and maximum error of estimate at $p<0.05$ significance levels.

3. Results and discussion

Bacillus species with proven antifungal and antibacterial properties are part of biological preparations both for agriculture (such as plant protection agents, for example phytosporin and probiotics for animal husbandry - biosporin) and for human purposes. The activity of these forms depends on the concentration of substances with antimicrobial activity produced by the selected strains during cultivation, and also on the amount of spores obtained. The type and concentration of substances with antimicrobial action and the amount of spores depends on the composition of the fermentation medium and on the cultivation conditions. In order for a preparation to be used in one direction or another, it is necessary to contain a high concentration of spores of the relevant strain (not less than 10^8-10^9 cfu/g). Therefore, it is of prime importance to investigate the antimicrobial activity and growth and sporulation kinetics in the batch cultivation of the investigated strain in different fermentation media.

A batch cultivation process of *Bacillus amyloliquefaciens* M was carried out in shaker flasks, as well as in a laboratory bioreactor in three different fermentation media - medium with molasses, medium with malt and MP nutrient broth with addition of salts. The antibacterial and antifungal activity of the biomass and cell-free supernatant was determined, and the results of these studies are reflected in Table. 1 and Table 2.

Table 1. Antifungal activity of *Bacillus amyloliquefaciens* M

	C. utilis	***S. cerevisisae***	***A.niger***	***A.flavus***	***F.moniliforme***	***Penicillium* sp**
medium with molasses						
BMPS	37	39	25	40	20	32
CFS	40	26	20	30	18	28
medium with malt						
BMPS	30	25	30	30	18	20
CFS	26	27	20	32	20	18
MP nutrient broth with addition of salts						
BMPS	37	30	22	37	20	15
CFS	32	24	22	25	12	12

Table 2. Antibacterial activity of *Bacillus amyloliquefaciens* M

	E.coli	***Salmonella* sp.**	***S. abony***	***St.aureus***	***Ps.aeruginosa***	***L.monocytogenes***	***B.cereus***
medium with molasses							
BMPS	18	-	9	26	20	32	18
CFS	18	-	9	23	18	28	20
medium with malt							
BMPS	18	-	9	15	20	33	13
CFS	20	-	9	28	28	26	17
MP nutrient broth with addition of salts							
BMPS	12	-	9	25	20	20	13
CFS	10	-	-	12	9	18	17

From the data presented in Table 1, it can be seen that the biomass and the cell-free supernatant of the studied strain exhibited high antifungal activity against the used test microorganisms. The zones of inhibition in the samples from the cultivation of *Bacillus amyloliquefaciens* M in the three nutrient media were comparable and ranged from 12 to 40 mm for the tested fungi and yeasts. The close values of the zones of inhibition for fungi and yeasts for the three different media testified to the production of substances with close antifungal activity.

The data presented in Table 2 indicated that *Bacillus amyloliquefaciens* M did not exhibit antibacterial activity against *Salmonella* sp. and had weak activity against *Salmonella abony* NTCC 6017, which was confirmed by the small zones of inhibition (9 mm). All other pathogens

showed highly pronounced antibacterial activity, with zones of inhibition ranging from 12 to 33 mm for the individual strains.

In order to investigate the influence of the composition of the nutrient medium on the growth of *Bacillus amyloliquefaciens* M, batch cultivation in flasks on a rotary circular shaker was carried out and the dynamics of biomass accumulation, the sporulation process, as well as the percentage of sporulation during cultivation were monitored. The results of these studies are presented in Fig. 2, Fig. 3 and Fig. 4.

The results presented in Fig. 2 show that the total number of viable cells of *Bacillus amyloliquefaciens* M continuously increased up to the 36th h during its cultivation in the three different nutrient media, reaching a value of about 10^{13}-10^{14} cfu/cm^3. After that the concentration of the total number of cells remained relatively constant. The presence of spore forms was not established until the 12th h from the beginning of the process. Spore formation in *Bacillus amyloliquefaciens* M starts from the 12th h from the start of the cultivation process of the strain in the three fermentation media (Fig. 2).

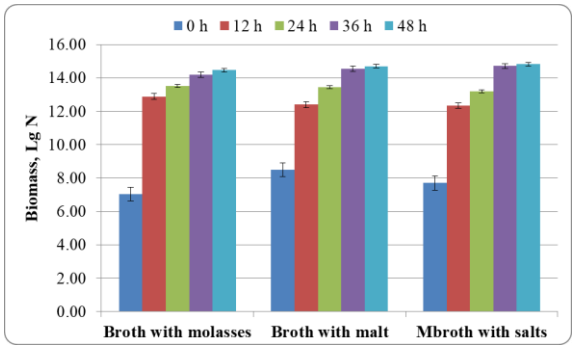

Fig. 2. Dynamics of the changes in the concentration of viable cells of *Bacillus amyloliquefaciens* M during its batch cultivation in different nutrient media
Mbroth with salts – MP nutrient broth with salts

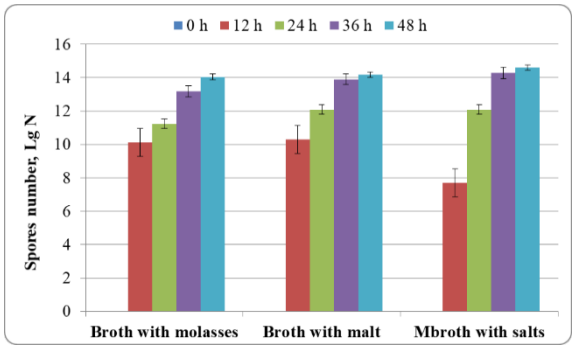

Fig. 3. Dynamics of the changes in the concentration of spores of *Bacillus amyloliquefaciens* M during its batch cultivation in different nutrient media
Mbroth with salts – MP nutrient broth with salts

The experimental data shown in Fig. 3 show that the concentration of spores in the medium with molasses and the one in the medium with malt are higher (about 10^{10} cfu/cm^3) compared to that in the MP nutrient broth medium with the addition of salts, in which the amount of spore forms was about 10^7 cfu/cm^3. The amount of spore forms in the three nutrient media continuously increased until the end of the cultivation process, reaching a concentration of 1.0 × 10^{14} cfu/cm^3, 1.5 × 10^{14} cfu/cm^3 and 4.0 × 10^{14} cfu/cm^3 for the medium with molasses, the medium with malt, and the MP nutrient broth with the addition of salts, respectively.

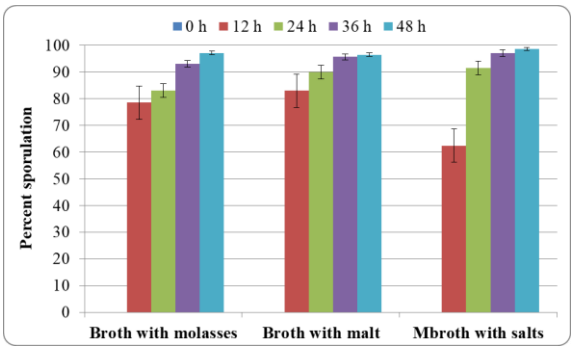

Fig. 4. Dynamics of the changes in the sporulation percentage during batch cultivation of *Bacillus amyloliquefaciens* M in different nutrient media
Mbroth with salts – MP nutrient broth with salts

The percentage of sporulation was monitored from the beginning of sporulation to the end of the fermentation process. These studies are reflected in Fig. 4. At the 12th h the percentage of sporulation was 78.52, 82.91 and 62.38%, for the medium with molasses, the medium with malt and for the MP nutrient broth with the addition of salts, respectively. During the cultivation period, the sporulation percentage for the molasses and malt media changed with a lower intensity compared to that of the MP nutrient broth with the addition of salts, reaching at the end of the process a sporulation percentage of 97.09%, 96.44 % and 98.58 % for the for the medium with molasses, the medium with malt, and the MP nutrient broth with the addition of salts, respectively. These results show that the highest sporulation percentage of 99% was observed in the MP nutrient broth medium with the addition of salts.

Knowing the kinetics of the process is essential for scaling-up the process in different volume bioreactors and its management. For this reason, basic kinetic parameters of growth and sporulation of *Bacillus amyloliquefaciens* M in the three selected fermentation media were calculated. When determining the kinetic parameters of the process, the total number of active cells and the spore forms are reduced to a dimensionless form so that the kinetic curves to be modeled to start from the same point.

The obtained results of the parametric identification of the kinetic models are presented in Table 3.

The logistic curve model used to describe the growth kinetics was characterized by high correlation coefficients between the experimental data and those of the model.

Table 3. Kinetic parameters of growth of *Bacillus amyloliquefaciens* M from the logistic curve model

Strain	Medium with molasses			Medium with malt			MP nutrient broth with salts		
	μ	β	$X_{кb}$	μ	β	$X_{кb}$	μ	β	$X_{кb}$
	h^{-1}	$X_b/cm^3.h$	-	h^{-1}	$X_b/cm^3.h$	-	h^{-1}	$X_b/cm^3.h$	-
B.amyloliquefaciens M	0.115	0.0548	2.10	0.105	0.0596	1.75	0.115	0.0601	1.91
R^2	0.9973			0.9995			0.9990		
E	0.20			0.14			0.17		

This coefficient varied in the range from 0.9973 to 0.9995 (Table 3). Quite impressive were the low values of the identification error, which were significantly below 1 – 0.20, 0.14 and 0.17 for the different fermentation media. These low values of the identification error and the high correlation coefficients testified that the model described the kinetics of the process with high accuracy and adequacy and could be used to predict the process in the studied time interval. *Bacillus amyloliquefaciens* M grew with the same maximum specific growth rate - 0.115 h^{-1} in the medium with molasses and in the MP nutrient broth with the addition of salts, and this value was higher than that of the medium with malt - 0.105 h^{-1}. Furthermore, the predicted theoretical maximum concentrations of active cells in dimensionless form for the three media were 2.10, 1.75, and 1.91 for the molasses medium, the malt medium, and the MP nutrient broth with the addition of salts, respectively.

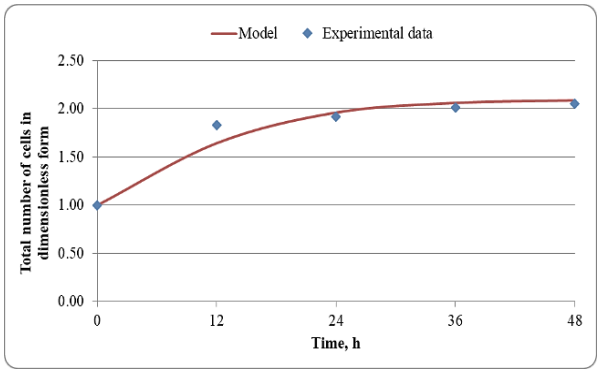

Fig. 5. Comparison of experimental data with logistic model data for *Bacillus amyloliquefaciens* M grown in medium with molasses

These values were very close to the experimentally established ones, which for the three media were 2.05, 1.73 and 1.92, which once again proved the adequacy of the model. The mathematical model gave comparable values of the intrapopulation competition coefficient - 0.0548 $Xb/cm^3.h$, 0.0596 $Xb/cm^3.h$ and 0.0601 $Xb/cm^3.h$ for the medium with molasses, the medium with malt and the MP nutrient broth with the addition of salts, respectively. These coefficients were significantly lower than 1, which indicated that the composition of the nutrient media used, the stirring rate and the culture temperature were appropriately selected and the strain grew without experiencing inhibitory influences that would lead to cell death in their competition for nutrients and oxygen. The values for the total number of cells in dimensionless form for *Bacillus amyloliquefaciens* M agreed very well with the experimental values (Fig.5 to Fig. 7). A comparison between the experimental results for the total number of active cells in dimensionless form and those from the model was made (Fig. 5, Fig. 6 and Fig. 7). Analogous studies were carried out to calculate the maximum rate constant of sporulation (Table 4).

Table 4. Maximum specific sporulation rate

Strain	Medium with molasses	Medium with malt	MPB + salts
	k, h^{-1}	k, h^{-1}	k, h^{-1}
B.amyloliquefaciens M	0.183	0.199	0.371
R^2	0.9992	0.9975	0.9987
e	0.26	0.26	0.23

The exponential model describing the kinetics of the sporulation process of *Bacillus amyloliquefaciens* M cultivated in the three fermentation media was characterized by high correlation values - 0.9992, 0.9975 and 0.9987 (Table 4), as well as low identification error values of 0.26 and 0.23. This shows that this model also very accurately agreed with the experimental data and could be used to predict the sporulation process in the selected cultivation time interval.

The highest sporulation rate of the studied strain was observed in the fermentation medium MP nutrient broth with the addition of salts - 0.371 h^{-1}, which could explain almost the 99% for the sporulation of the strain. An intermediate place in relation to the maximum rate of sporulation was occupied by the medium with malt - 0.199 h^{-1}, and the lowest rate constant of sporulation was found in the medium with molasses - 0.183 h^{-1} (Table 4).

Experimental data on the amount of spore forms in dimensionless form were compared with those from the model (Fig. 8, Fig. 9 and Fig. 10).

The values for the spore forms of *Bacillus amyloliquefaciens* M from the exponential model agreed very well with the experimental values (Fig. 8, Fig.9, Fig. 10).

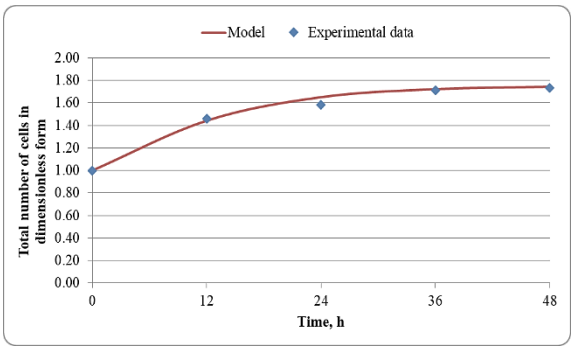

Fig. 6. Comparison of experimental data with logistic model data for *Bacillus amyloliquefaciens* M grown in medium with malt

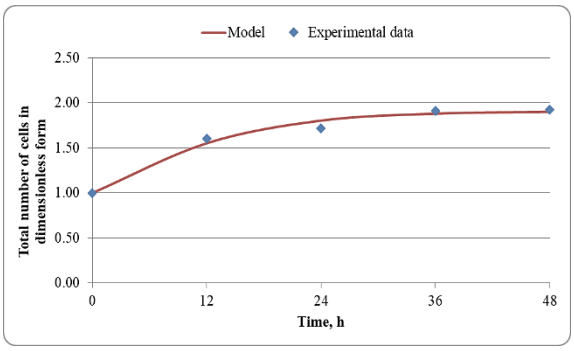

Fig. 7. Comparison of experimental data with logistic model data for *Bacillus amyloliquefaciens* M grown in MPB with the addition of salts

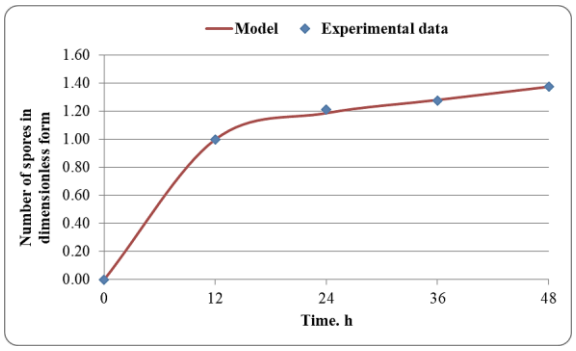

Fig. 8. Comparison of experimental data with that of the exponential model for *Bacillus amyloliquefaciens* M grown in medium with molasses

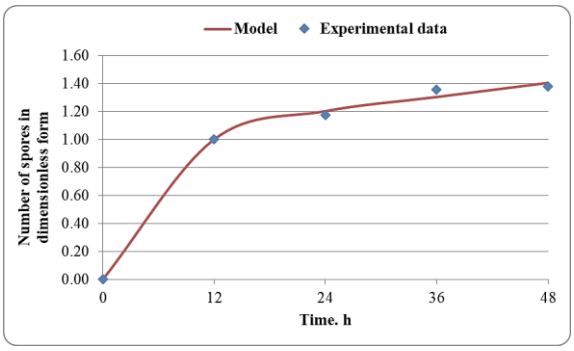

Fig. 9. Comparison of experimental data with that of the exponential model for *Bacillus amyloliquefaciens* M grown in medium with malt

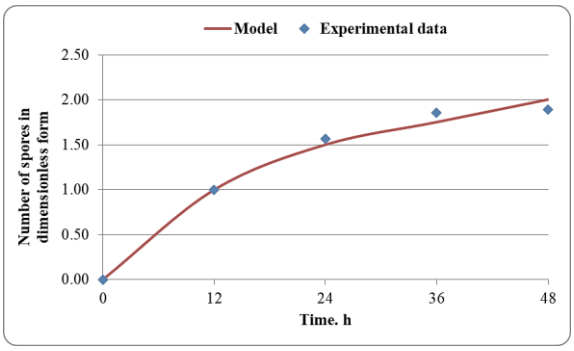

Fig. 10. Comparison of experimental data with that of the exponential model for *Bacillus amyloliquefaciens* M grown in MP nutrient broth with the addition of salts

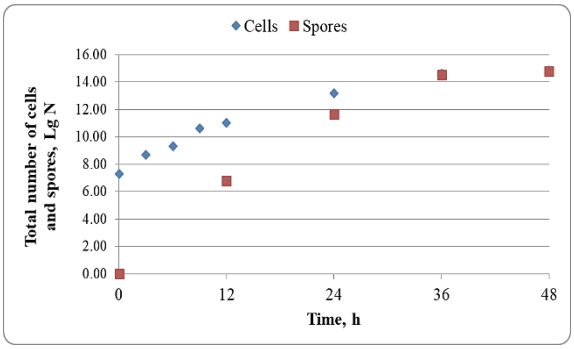

Fig. 11. Dynamics of changes in the total number of cells and spores of *Bacillus amyloliquefaciens* M cultivated in a laboratory bioreactor with mechanical stirring in MP nutrient broth with the addition of salts at 37°C

Due to the high maximum sporulation rate, high sporulation percentage and comparable antimicrobial activity of the strain cultured in MP nutrient broth medium with the addition of salts, this medium was chosen to conduct a batch cultivation process in a bioreactor with mechanical stirring. The growth dynamics of the total number of viable cells and spore forms, the dynamics of changes in the sporulation process were monitored and the main kinetic parameters of the process carried out in the laboratory bioreactor were calculated (Fig. 11, Fig. 12 and Fig. 13).

The duration of the lag phase was about 3 h, after which intensive growth of the culture during the exponential phase with a continuous increase in the total number of cells until the culture entered the stationary growth phase was observed. The culture entered the stationary growth phase on the 36th h from the beginning of the process, reaching a concentration of 4.3×10^{14} cfu/dm^3. During further cultivation, this value remained relatively constant and at the end of the process a total cell count of 7.0×10^{14} cfu/dm^3 was established.

The data presented in the figure show that the sporulation process of the studied strain cultivated in the

bioreactor began at the 12th h from the start of the process, analogous to that in the flasks. The concentration of the spore forms continuously increased until the 36th h from the start of the process, and a spore concentration of 3.2 × 10^{14} cfu/dm^3 was reached. It remained relatively constant and at the end of the process 6.5 × 10^{14} cfu/dm^3 spores were found.

The dynamics of changes in the percentage of sporulation during cultivation were also monitored (Fig. 12).

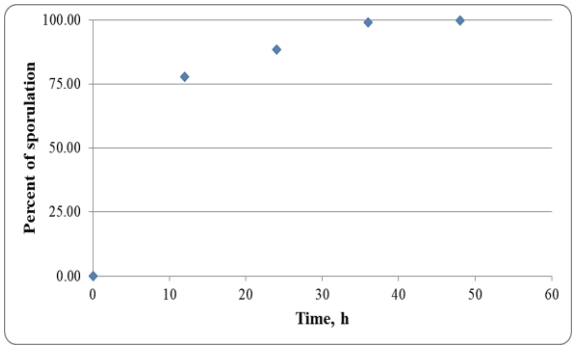

Fig. 12. Dynamics of changes in the percentage of sporulation in *Bacillus amyloliquefaciens* M cultivated in a laboratory bioreactor with mechanical stirring in MP nutrient broth with the addition of salts at 37°C

The percentage of sporulation was higher than that obtained when the strain was cultivated in the same medium on shaker flasks (62.38 %) (Fig. 13). An exponential increase in the sporulation percentage from the 12th to the 36th h of cultivation was observed and it reached 99.12%. Then the percentage increased relatively smoothly and at the end of the process it reached a value of 99.78%.

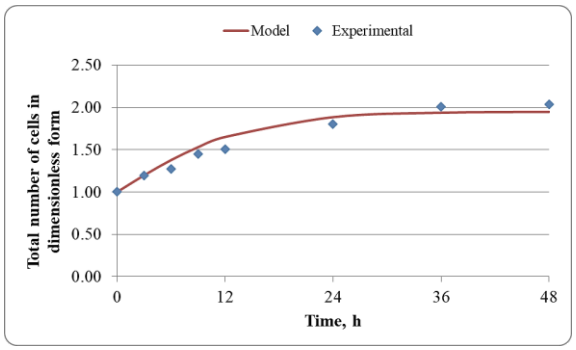

Fig. 13. Comparison of experimental data with data from the logistic curve model for the total viable cell counts for *Bacillus amyloliquefaciens* M grown in MP nutrient broth with the addition of salts in a mechanically stirred laboratory bioreactor

Growth and sporulation kinetic parameters were calculated, and again the concentration of total cells and spore forms was presented in dimensionless form (Table 5). Both models – the growth model and the sporulation model were characterized by high correlation coefficients and low values of identification errors, which made them adequate and they could describe and predict the studied processes (of growth and sporulation) with high accuracy (Table 5). Moreover, in the laboratory bioreactor *Bacillus amyloliquefaciens* M developed with a higher maximum specific growth rate - 0.138 h^{-1} compared to that obtained when it was cultivated in shaker flasks - 0.115 h^{-1}. This, in turn, unequivocally indicated that the hydrodynamic conditions in the laboratory bioreactor significantly improved the mass exchange processes, and hence the growth of the strain. When cultivating *Bacillus amyloliquefaciens* M in a laboratory bioreactor with mechanical stirring, the model gave a close value of the maximum theoretical concentration of the total number of active cells in dimensionless form – 1.95, compared to the experimentally determined value – 2.03. The low value of the coefficient of intrapopulation competition (0.0708 Xb/cm^3.h) confirmed the fact that optimal conditions for the growth of the studied strain were created in the bioreactor (Table 5).

Table 5. Kinetic parameters of growth and sporulation of *Bacillus amyloliquefaciens* M cultivated in a laboratory bioreactor

Growth kinetics				
μ	**β**	**$X_{кb}$**	**R^2**	**e**
h^{-1}	***X_b*/cm^3.h**	**cfu/cm^3**	**-**	**-**
0.138	0.0708	1.95	0.9725	0.13
Sporulation kinetics				
-	$k_,$	R^2	e	-
-	h^{-1}	-	-	-
-	0.430	0.9963	0.41	-

The strain cultivated in the laboratory bioreactor retained high values of the maximum sporulation rate constant - 0.430 h^{-1}, which was significantly higher than that obtained during cultivation in shaker flasks - 0.371 h^{-1}. This was also due to intensification of the processes as a result of the improved hydrodynamic conditions in the apparatus, compared to those in the flasks (Table 5). A comparison of the experimental data with data obtained from the models used for the kinetics of growth and sporulation was made (Fig. 13 and Fig. 14).

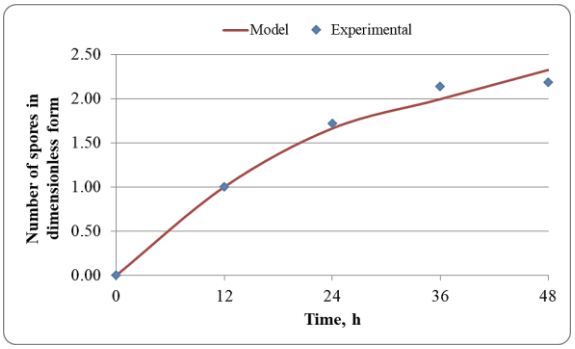

Fig. 14. Comparison of experimental data with data from the exponential model for the number of *Bacillus amyloliquefaciens* M spores formed diring culturing in MP nutrient broth with the addition of salts in a mechanically stirred laboratory bioreactor

The values calculated by the selected models for modeling the kinetics of growth and sporulation of *Bacillus amyloliquefaciens* M in a laboratory bioreactor with mechanical stirring in MP nutrient broth with the addition of salts agreed very well with the experimental ones (Fig. 13 and Fig. 14).

The dynamics of the oxidation-reduction potential as well as the pH during the fermentation process were monitored (Fig. 15).

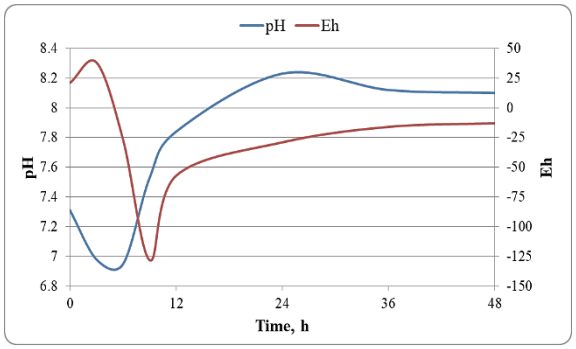

Fig. 15. Dynamics of changes in the pH and the redox potential during the cultivation of *Bacillus amyloliquefaciens* M in a laboratory bioreactor with mechanical stirring in MP nutrient broth with the addition of salts

There was a decrease in the pH in the initial stage of the process, followed by an increase in the pH during the exponential phase up to pH = 8.2. When the culture entered the stationary growth phase (at the 36th h), a slight reduction in pH to about pH = 8.10 was observed, and this value was maintained until the end of the process. The redox potential during the lag phase rose to +38 mV, then began to decrease until the beginning of the sporulation and was 128 mV, and from the moment of initiation of sporulation its value began to increase. This trend was maintained until the end of the cultivation, reaching a value of –13 mV.

4. Conclusion

A nutrient fermentation medium composition for growth and sporulation of *Bacillus amyloliquefaciens* M was determined. It was found that the strain grew well and sporulated in medium with malt, medium with molasses and medium with MP nutrient broth and addition of salts. The sporulation process started at the 12th h for all three fermentation media, and at the end of the processes the percentage of sporulation was - 97.09%, 96.44% and 98.58% for the medium with malt, medium with molasses and medium with MP nutrient broth and addition of salts, respectively. It is clear that the highest sporulation percentage of 99% was observed for the MP nutrient broth medium with the addition of salts. The highest rate of growth and sporulation for *Bacillus amyloliquefaciens* M cultivated in shaker flasks was found in MP nutrient broth with addition of salts - μ_m = 0.115 h^{-1} and k = 0.371 h^{-1}, thus this medium was chosen for fermentation in a laboratory bioreactor with mechanical stirring. When *Bacillus amyloliquefaciens* M was cultivated in a laboratory bioreactor with mechanical stirring in MP nutrient broth, the strain grew with a higher maximum specific growth rate - 0.138 h^{-1}, and a similar trend was observed for the sporulation rate constant (0.430 h^{-1}). *Bacillus amyloliquefaciens* M exhibited pronounced high antibacterial and antifungal activity, which value was comparable for the three fermentation media in which it was cultivated.

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