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Kinetic analysis of growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 in algae-based medium

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Abstract: An unstructured mathematical model is proposed to describe the fermentation kinetics of growth, lactic acid production, pH and sugar consumption by *Lactobacillus delbrueckii subsp. bulgaricus WDCM 00102* (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) as a function of the buffering capacity and initial dry matter concentration of pretreated biomass of *Spirulina platensis* (*Arthrospira platensis*) ('Simbiotex' Ltd., Sofia, Bulgaria) in the culture media. Initially the experimental data of L. *delbrueckii* subsp. *bulgaricus* WDCM 00102 fermentations in algae-based media with different buffering capacities and dry matter concentrations were fitted to a set of primary models. Later the parameters obtained from these models were used to establish mathematical relationships with the independent variables tested. The models were validated with 6 fermentations of *L. delbrueckii* subsp. *bulgaricus* WDCM 00102 in different algae-based media. In most cases, the proposed models adequately describe the biochemical changes taking place during fermentation and are a promising approach for the formulation of algae-based probiotic foods.

Keywords: *Lactobacillus;* probiotics; growth study; algae; buffering capacity; dry matter concentration

Microalgae are considered to be a rich source of sulfated polysaccharides, and the different types of polysaccharides vary depending on the taxonomic group. *Arthrospira platensis* contains about 13.6% carbohydrates; some of which are glucose, rhamnose, mannose, xylose and galactose. *Arthrospira platensis* biomass consumes nitrogen from the growth medium and releases extracellular carbohydrates and other growth substances that may be responsible for stimulating the growth of lactic acid bacteria (Parada et al. 2014). Foods that combine the characteristics of probiotics and prebiotics are synbiotics that can stimulate and increase the survival of probiotic and autochthonous strains in the intestinal tract (Jain et al. 2014), improve the healthy composition of the colon microbiota and improve the survival of bacteria crossing the upper gastrointestinal tract, thus enhancing their effects in the colon (Tymczyszyn et al. 2011; Novoselova and Stoyanova 2021).

Lactic acid bacteria and bifidobacteria, which use carbohydrates as an energy source, and endogenous carbon sources serve as the ultimate acceptor of electrons instead of oxygen, are used as probiotics in the preparation of functional foods (Tabasco et al. 2014; Mokoena 2017; Mays and Nair 2018). The probiotic effect of these microorganisms includes prevention of gastrointestinal diseases in the elderly people, diarrhoea, stimulation of the activity of the immune system (Novoselova and Stoyanova 2021), lactose intolerance, lowering blood cholesterol levels and cancer prevention (Górska et al. 2019). In addition to these therapeutic benefits, probiotics have an antagonistic effect against many opportunistic human pathogens (O'Sul-

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livan et al. 2010). Restricting the spread of cancer is associated with the binding and degradation of potential carcinogens such as heterocyclic aromatic amines such as 3-amino-1-methyl-5H-pyrido. –4, 3– bisindole, nitrosamines, mycotoxins, polycyclic aromatic hydrocarbons and phthalic acid esters by *Lactobacillus* and *Bifidobacillus* strains, characterised by probiotic activity (Barroso et al. 2016; Mays and Nair 2018). Although the application of the biomass of microalgae of different genera and strains is scarce during the technological production of probiotic foods in the modern literature, which enables his entrapment in the fermentation medium during the cultivation of lactic acid bacteria with potentially probiotic properties.

The establishment of the main relationships describing the kinetics of growth and development of probiotic strains of lactic acid bacteria in media based on processed microalgae biomass is an essential stage of scaling up the process and leads to its production implementation. The growth of lactic acid bacteria when cultivated in media based on cereals is described by unstructured and structured equations (Mora-Villalobos et al. 2020), but information about the kinetic relationships during their development in media from microalgae biomass remains scarce. Higher-order relationships or polynomials containing growth-related parameters are also used to describe changes in other biochemical compounds and physical properties of raw materials during the fermentation process to obtain products with a potential probiotic effect. These changes include concentrations of primary or secondary metabolites, volatile compounds, as well as rheological and textural properties (Terpou et al. 2019).

The relationships under carefully controlled conditions, based on experimental data describing the change of the response variable over time, mathematically summarise the biochemical properties (response variables) with environmental factors (controlling factors) such as temperature, pH, water activity and substrate composition (Mora-Villalobos et al. 2020). Studies on the kinetics of growth and development of probiotic strains of lactic acid bacteria have so far focused on establishing the dependence of growth rate on temperature and pH under pH-controlled conditions (Mora-Villalobos et al. 2020). Very few studies have been done in secondary modelling of growth when pH is not controlled or taking into account other biokinetic parameters, such as lactic acid and bacteriocin production (Hamouda et al. 2022).

Microalgae are considered to be a rich source of sulfated polysaccharides, and the different types of polysaccharides vary depending on the taxonomic group (Górska et al. 2019). The key function of these relatively high molecular weight polysaccharides is that they are rich in hydroxyl (OH) groups, making them hydrophilic. They form intrachain networks of H-bonds, making them solid, rigid and suitable as thickeners. The stability of their structures also promotes their interaction with external ions and interchain H-binding (e.g. gelation) (Tymczyszyn et al. 2011; Novoselova and Stoyanova 2021). The main biochemical characteristics influencing the functionality and quality of foods or food supplements with potential probiotic effects are cell population, lactic acid concentration and pH. The cell concentration in the final product is an indicator of probiotic functionality, lactic acid influences organoleptic properties and acts as a preservative, while pH is the main factor determining the stability and safety of the product during storage (Patel et al. 2021). Although the application of the biomass of microalgae of different genera and strains are scare during the technological production of probiotic foods in the modern literature, which enables his entrapment in the fermentation medium during the cultivation of lactic acid bacteria with potentially probiotic properties.

This study aimed to develop a model that would be able to simulate the kinetics of cell growth, lactic acid production, pH drops and sugar consumption in algae-based fermentations with *Lactobacillus delbrueckii subsp. bulgaricus* WDCM 00102 (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) strain. The kinetic parameters of the primary models for these dependent variables were expressed as a function of the initial dry matter concentration of the media and their buffering capacity. The models were built using data from fermentations in media with various levels of dry matter of biomass of *Spirulina platensis* (*Arthrospira platensis*) and different buffering abilities. Finally, the predictability of the models was evaluated using a variety of algae-based fermentations of different concentrations. Thus, the parameters obtained from the models allow for the characterisation of these cultures and could be a preliminary step in the formulation of novel probiotic foods.

MATERIAL AND METHODS

Microorganism and inoculum. *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 was provided by the National Bank for Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) and maintained at 4 °C and sub-cultured monthly on De Man-Rogo-

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sa-Sharpe (MRS) agar slopes. Isolated colonies from MRS agar plates were pre-cultured twice in MRS broth for approximately 24 h at 37 °C. The cells were collected by centrifugation (5 000 × *g*, 10 min, 4 °C), washed twice with sterile quarter-strength Ringer's solution and re-suspended in the same solution. The bacterial suspensions used as inocula for the fermentation studies (1%, v/v) were obtained from 12 h pre-cultured cells.

Culture media and microbiological methods. The microalgae *Spirulina platensis* (*Arthrospira platensis*) was provided by 'Simbiotex' (Ltd., Sofia, Bulgaria). It was grown in the nutrient medium at 28 °C having a common composition of chemicals: $NaHCO_3$, K_2HPO_4, $MgSO_4$, $CaCl_2$, citric acid, Na_2EDTA, Na_2CO_3, and trace metalion solution. *Spirulina platensis* biomass was dried in a vacuum oven at 40 °C until constant weight, then ground and sieved into fractions.

The media used for the studies of the buffering capacity and the effect of dry matter of *Spirulina platensis* (*Arthrospira platensis*) processed biomass on *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 growth is summarised in Table 1. The pH of all media was adjusted to about 5.8 with 1N HCl or 1N NaOH and the media sterilised for 10 min at 121 °C. In all cases the pH was initially adjusted to about 6.0 with 1N·HCl or 1N·NaOH and the media sterilised for 30 min at 121 °C.

Shake-flask fermentations were performed in triplicate using 500 mL screw-capped glass bottles without oxygen control. Bottles were inoculated with 1% (v/v) of lactic acid bacteria and incubated at 150 rpm and 37 °C for 30 or 42 h. The viable cell counting method was used for cell enumeration. Cell growth was monitored by measuring the optical density of the media at 600 nm. The optical density values were transformed to cell counts [log10 colony forming units (CFU)·mL^{-1}] using a pre-established calibration curve. In the fermentation samples pH, reducing sugar (as glucose) and lactic acid content were analysed.

Buffering capacity. The buffering agent used was a 0.2 M acetate buffer stock solution, from which 8 media of different buffering capacities were prepared by dilution (from 1/1 to 1/15). The buffering capacity of the media was measured after the addition of the nutrient substrate. 100 mL of each medium was titrated with 1N·HCl. The values were expressed as the amount of HCl (mmol) required to drop one pH unit per unit volume (1 L).

Analytical methods. The dinitrosalicylic acid (DNS) assay was used to measure the reducing sugar concentration in the supernatants of the fermented algae-based media. A standard curve was made using glucose at various concentrations. Lactic acid was measured using an enzymatic kit for D- and L-lactic acid.

Numerical and statistical methods. All experiments were done on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible independently of the cultures used. Analysis of variance (ANOVA) of the viable counts and the lag times corresponding to the different treatments, was carried out using the statistical program Statistix 8 Software. Differences were tested with paired sample *t*-tests, and if $P < 0.05$ the difference was considered statistically significant.

RESULTS AND DISCUSSION

The present study focused on investigating the relationships describing culture growth (*N*), lactic acid change (*P*), pH and reducing sugar content (*S*) as a function of the duration of cultivation of a strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 in medium obtained on the basis of processed biomass of *Spirulina platensis (Arthrospira platensis)* ('Simbiotex' Ltd., Sofia, Bulgaria) (first-order dependencies). Immediately after this, the search for second-order dependencies of the main factors of the fermentation process was carried out based on the use

Table 1. Composition of the synthetic media used for the development of the model

Controlling factor	Nutrients concentration:
Buffering capacity (mmol HCl·pH^{-1}·L^{-1}): 0.32; 0.36; 0.39; 0.51; 1.20	Proteins: min 70% Reducing sugars: min 12 g·L^{-1}
Dry matter concentration (%) 5; 7.5; 9; 10; 12; 15	Buffering capacity: min 1.0 mmol HCl·pH^{-1}·L^{-1}

All the media are prepared by dispersion of biomass of *Spirulina platensis* (*Arthrospira platensis*) in distilled water, but dried algae biomass contains 78% proteins, 4% lipids and 13.6 g·L^{-1} reducing sugars, determined by Kjeldal's method, Soxhlet s method and dinitrosalicylic acid (DNS) assay in this study.

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of linear or non-linear regression analysis. The results of the study are systematised in Table 2. The exponential increase of the lactic acid bacteria population was significant until 3rd day detecting at the level of almost $9.55 \cdot 10^8$ CFU·g^{-1} during the cultivation of the strains in the medium on the base of Brassica junce leaves under an anaerobic environment at room temperature for 16 days. The lactic acid bacteria population after 3rd day gradually decreased and remained constant after the 8th day at a level of $6.31 \cdot 10^7$ CFU·g^{-1} until the end of the fermentation period (Ghimire et al. 2020). The growth kinetics of lactic acid bacteria during gundruk fermentation in the study of Ghimire et al. (2020) agrees with the assumptions of the Gompertz equation, that the rate of growth is proportional to cell mass and that the growth rate decays exponentially with time due to the inactivation of the bacteria, because the mustard leaves have low sugar content, but is rich in minerals and vitamins have neutral pH and thus provide a natural medium for fermentation by lactic acid bacteria preventing nutrient competition at the early stage.

Growth models. A common model to describe cell population growth is the differential equation proposed by Charalampopoulos et al. (2009), which includes an inhibition factor of growth. By assuming that inhibition of a population N is proportional to N_2, the growth rate is given by the following equation:

$$\frac{dN}{dt} = \mu_m \times N \times \left(\frac{K - N}{K} \right) \quad (1)$$

where: N – cell concertation; K – constant; μ_m – maximum specific growth rate (h^{-1}); t – time.

Integrating on the base of N_0 –> N and 0 –> t gives the biomass concentration as a function of time:

$$N = \frac{K}{1 + \exp(c - \mu_m \times t)} \text{ which ... } \quad c = \ln\left(\frac{U}{X_0} - 1 \right) \quad (2)$$

where: c – regression parameter; U – initial cell concentration (CFU·mL^{-1}); X – biomass as relative cell population [$\log_{10}(N/N_0)$].

Most of the equations describing the exponential growth curve contain mathematical parameters (a, b, c) rather than biologically meaningful parameters (K, μ_m, and λ). To obtain the inflection point of the curve, the second derivative of the function with respect to t is calculated:

$$X = \lg\left(\frac{N}{N_0} \right) = \frac{A}{1 + \exp\left[2 + \left(4 \times \frac{\mu_m}{A} \right) \times (\lambda - t) \right]} \quad (3)$$

Table 2. Optimum parameter values for the logistic model 3 describing *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) growth in algae biomass-based media of different buffering capacities and glucose concentration

	μ_m	A	λ	F-value	r
Buffering capacity					
0.32	0.281 ± 0.062	3.635 ± 0.820	3.588 ± 0.290	1 000.3	0.963
0.36	0.283 ± 0.059	3.659 ± 0.420	3.569 ± 0.080	925.6	0.980
0.39	0.281 ± 0.077	3.621 ± 0.060	3.564 ± 0.550	561.4	0.988
0.51	0.425 ± 0.081	5.486 ± 0.160	3.569 ± 0.590	1 055.6	0.979
1.20	0.462 ± 0.034	5.960 ± 0.360	3.597 ± 0.330	1 025.3	0.956
Dry matter (%)					
5.00	0.036 ± 0.076	0.457 ± 0.090	1.961 ± 0.780	302.9	0.971
7.50	0.267 ± 0.072	3.449 ± 0.180	2.223 ± 0.930	606.1	0.956
10.00	0.281 ± 0.062	3.635 ± 0.820	2.588 ± 0.290	1 067.3	0.967
12.00	0.467 ± 0.029	6.035 ± 0.510	3.600 ± 0.380	3 028.4	0.991
15.00	0.436 ± 0.046	5.860 ± 0.350	3.375 ± 0.260	3 099.3	0.989

The values are the means with the corresponding confidence intervals ($\alpha = 0.05$); F-values are the results of the F-Fisher test ($\alpha = 0.05$) for 3 model degrees of freedom. μ_m – maximum specific growth rate (h^{-1}); A – maximum relative cell population [$\log_{10}(N/N_0)_{max}$]; λ – growth lag phase (h); r – regression coefficient

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where: N_0 – initial cell concentration (CFU·mL^{-1}); A – maximum relative cell population ($\log_{10}(N/N_0)_{max}$); λ – growth lag phase (h).

The optimum parameters with the 95% confidence intervals and consistency of the equation (Fisher's *F*-test = 0.05) are presented in Table 2. In all cases, the fit of results was statistically satisfactory. The mathematical equations were consistent (Fisher's *F*-test) and the parametric estimations were significant (Student's *t*-test). The values predicted by Equation 3 are highly correlated with the experimental data with a regression coefficient $r > 0.95$.

The growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain, when cultivated in media with different concentrations of biomass of *Spirulina platensis* (*Arthrospira platensis*) ('Simbiotex' Ltd., Sofia, Bulgaria) has an exponential character. The inhibitory effect of cell population growth on the value of μ_m in the range of dry matter content from 10% to 12% is explained by the rapid decrease in the concentration of reducing sugars as a constructive and energetic source in the culture medium or is the result of substrate inhibition at the concentration of dry matter above 12%. Decreasing the buffer capacity of the culture medium also leads to a strong inhibitory effect of pH and a decrease in the value of μ_m.

The highest maximum specific growth rates during the development of *L. fermentum* IMDO 130101 in the the sourdough with carbohydrate content of 10 g·L^{-1} were observed with fructose (0.85 ± 0.02 h^{-1}) and maltose (0.82 ± 0.02 h^{-1}). Glucose supported growth at a maximum specific growth rate slightly inferior (0.72 ± 0.01 h^{-1}) to the ones observed on fructose and maltose as the sole energy sources. Glucose was quantitatively fermented through the heterofermentative pathway. Sucrose was an inferior carbohydrate for the growth of the strain under study. Slow growth (0.52 ± 0.03 h^{-1}) was observed and the maximal cell density was substantially lower 8.8 ± 0.1 log (CFU·mL^{-1}) than on fructose, maltose, or glucose [(9.3–9.4 log (CFU·mL^{-1})] as the sole energy sources (Vrancken et al. 2008).

The value of the biokinetic coefficients μ_m, A and λ in the cultivation of a strain in a biomass-based medium are closely dependent on the buffer capacity and the content of reducing sugars. All the functions were of the form $p(f) = p_{opt}.(f)$, which indicates that an optimum value of the parameter (p_{opt}) is obtained when the controlling factor (f) is also optimum. The function (f) describes the response of the growth to changes in the factor f, with values of f between 0 (no response) and 1 (optimum response). By assuming that the influence of a factor is independent of other factors, the model describing the combined effects of the factors f_1, f_2, f_3,..., f_n would then be f_1, f_2, f_3,..., f_n would then be $p(f_1, f_2, f_3,..., f_n) = p_{opt}.(f_1, f_2, f_3,..., f_n)$.

Secondary modelling of μ_m. Figure 1 shows the effect of concentrations of dry matter of *Spirulina platensis* (*Arthrospira platensis*) biomass ('Simbiotex' Ltd., Sofia, Bulgaria) (*S*) on μ_m during cultivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain. The data show that μ_m increases with the dry matter concentration from 6 to 10%, while between 10 and 15% μ_m remains approximately constant. The hyperbolic shape of the trend was described using the Monod model:

$$\mu_m = \mu_{optS} \times \frac{S}{K_s + S} \quad (4)$$

where: μ_{optS} – maximum specific growth rate at the optimum sugar concentration (h^{-1}); S – sugar concentration (g·L^{-1}); K_S – half-saturation constant (g·L^{-1})

The fit of Equation 4 to the μ_m values (Table 2) was performed by the non-linear least-squares method. The fit was satisfactory and the derived parameters were 0.469 h^{-1} for μ_{optS} and 1.5 g·L^{-1} for K_S (Table 3). In the study of Charalampopoulos et al. (2009) μ_m increases in the process of cultivation of *L. plantarum* NCIMB 8826, isolated from human saliva, in the synthetic media with the glucose concentration from 0 to 6 g·L^{-1}, while between 6 and 20 g·L^{-1} μ_m remains approximately constant. The hyperbolic shape of the trend was described using the Monod model (Charalampopoulos et al. 2009).

The small K_S value suggests that the sugar requirement of this strain is relatively low, and the strain could be used to ferment media of low sugar content. It must be pointed out that little information is available in the literature regarding the kinetics of lactic acid bacteria growth in a fermented food product. Most published works study the kinetics of the fermentation process for the optimisation of the production of lactic acid in industrial applications. In these cases fermentations are carried out under pH control using media with very high dry matter of biomass concentrations (12–15%). This is the reason why K_S is considered to be very small and usually is neglected.

It can be also observed from the data presented in Table 3 that μ_m increases with the buffering capacity of the medium. This can be attributed to the fact that in media with low buffering capacity, the pH drop was greater.

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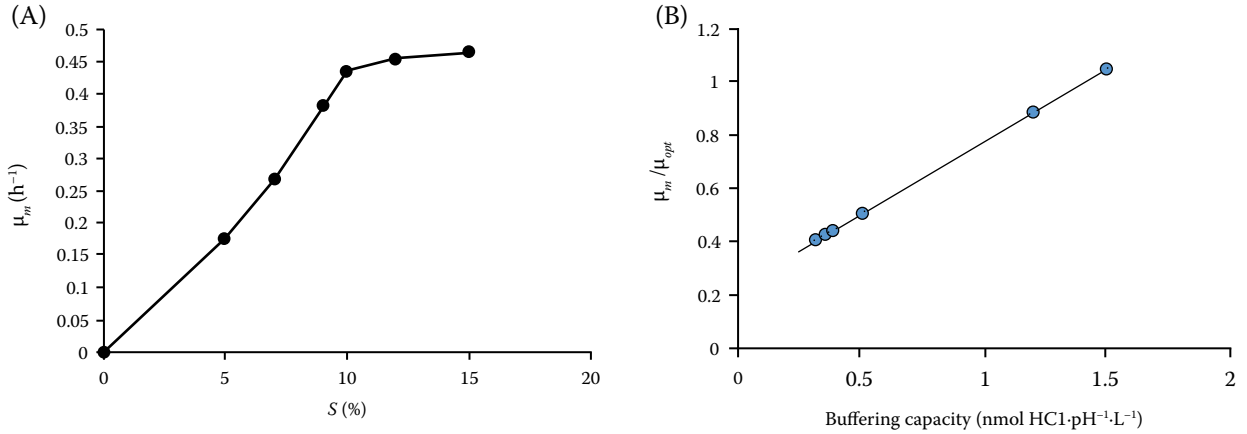

Figure 1. Maximum specific growth rate (μ_m) as a function of the initial algae-biomass concentration (S) of the medium (A) and as a function of their buffering capacity (B)

The inhibitory effect of pH takes place earlier in the fermentation process, resulting in lower m values. A linear relationship was observed between m and the buffering capacity, and the m value corresponding to the higher buffering capacity (1.5 mmol HCl·pH^{-1}·L^{-1}) was considered to be the optimum value (μ_{optB} = 0.469 h^{-1}) for these working conditions. The relative m values (μ_m/μ_{optB}) were then plotted against the buffering capacity (B) in order to obtain an equation of the form $\mu_m = \mu_{optB}\cdot\gamma$ (Figure 1).

The following equation was used to test the adequacy of the relationship between maximum growth rate and dry matter concentration in the culture medium:

$$\frac{\mu_m}{\mu_{optB}} = \alpha_1 + \beta_1\left(B - 0.57\right) \quad (5)$$

where: α_1 – regression parameter; β_1 – regression parameter (pH·lmmol^{-1}·HCl); B – buffer capacity (mmol HCl·pH^{-1}·L^{-1})

The value of the statistical coefficients (r = 0.965; F-value = 427.50, Table 3) obtained in the course of the study determined the adequacy of Equation (5), which also gives the relationship of the maximum growth rate from the buffer capacity of the environment.

Systematising the influence of dry matter and the buffer capacity of the medium based on processed biomass on the maximum growth rate of a strain by integrating the two Equations (4) and (5), the dependence is reached:

$$\mu_m = \mu_{optS} \times \left(\frac{S}{K_s + S}\right)\left[\alpha_1 + \beta_1 \times \left(B - 0.57\right)\right] \quad (6)$$

where: μ_{opt} (h^{-1}) – μ_m value at the conditions where both controlling factors are at their optimum, which occurs when B – 1.09 mmol HCl·pH^{-1}·L^{-1} and S is much greater than K_S.

According to Equation (6) at S = 10% and B = 1.09 mmol HCl·pH^{-1}·L^{-1} the value of μ_{opt} is 0.469 h^{-1}, which is slightly higher than the μ_{optB} value of 0.61 h^{-1} in the recent study. Of the three possible μ_{opt} values (μ_{opt} obtained from Equation 6, μ_{optS} and μ_{optB}), the one with the smaller coefficient of variation is μ_{optS}, and this value was the one considered in the equation describing the joint effect (Equation 7):

$$\mu_m = \mu_{optS} \times \left(\frac{S}{K_s + S}\right)\left[\alpha_1 + \beta_1 \times \left(B - 0.57\right)\right] \quad (7)$$

Secondary modelling of *A*. Figure 2 illustrates the dependence of the maximum relative colony forming units (A) on the dry substance concentration (S). The A values increase with the initial dry matter concentration of the medium until a stationary value is reached. This growth curve is similar to μ_m and therefore a Monod-type equation is used to describe the relationships between relative colony forming units A and the dry substance concentration.

The adequacy of the experimentally obtained dependence with Equation (7) is established in the present research on the cultivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in a medium based on processed *Spirulina platensis* (*Arthrospira platensis*) biomass ('Simbiotex' Ltd., Sofia, Bulgaria), regardless of the deviation in the K_S value presented in Table 4. During batch cultivation of *Lactobacillus del-*

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Table 3. Parameters for the secondary models expressing μ_m, A and λ as a function of sugar concentration and buffering capacity

Factor	Equation	Parameter	Value	*F*-value	*r*
Buffering capacity	4	μ_{optS} (h^{-1})	0.469 ± 0.029	407.90	0.955
		K_S ($g{\cdot}L^{-1}$)	0.231 ± 0.005	–	–
Dry matter (%)	5	μ_{optS} (h^{-1})	0.475 ± 0.078	427.50	0.965
		α_1	0.352 ± 0.067	–	–
		β_1	0.021 ± 0.089	–	–
Buffering capacity	4	A_{optS}	6.050 ± 0.130	671.90	0.973
		K_S ($g{\cdot}L^{-1}$)	0.217 ± 0.043	–	–
Dry matter (%)	8	A_{optS}	6.120 ± 0.670	1 287.50	0.971
		α_2	0.414 ± 0.019	–	–
		β_2	0.024 ± 0.033	–	–

F-values are the results of the *F*-Fisher test ($\alpha = 0.05$); μ_{optS} – maximum specific growth rate at the optimum sugar concentration (h^{-1}); K_S – half-saturation constant ($g{\cdot}L^{-1}$); α – regression parameter; β – regression parameter ($pH{\cdot}L{\cdot}mmol^{-1}{\cdot}HCl$); A_{optS} – maximum A value at the optimal glucose concentration; r – regression coefficient

brueckii subsp. *bulgaricus* WDCM 00102 strain, where the buffering capacity of processed *Spirulina platensis* (*Arthrospira platensis*) was the main controlling factor, the A values ranged from 2.46 to 6.03 [$\log10(N/N_0)_{max}$], suggesting that the effect of buffering capacity was less significant than that of dry substance of microalgae biomass. The A values increase during cultivation of *L. plantarum* in synthetic media with the initial sugar concentration of 1.2 $g{\cdot}L^{-1}$ to 8.0 $g{\cdot}L^{-1}$ and buffering capacity of 0.4 to 1.2 mmol $HCl{\cdot}pH^{-1}{\cdot}L^{-1}$ until a stationary value is reached (Charalampopoulos et al. 2009). Similar relationships were exterminated during fermentation experiments by the participation of *Lactobacillus helveticus* in the whey permeate/yeast extract medium (Schepers et al. 2002). This behaviour was similar to μ_m and therefore a Monod-type equation was used to describe the dependence of A with the sugar concentration. The achievement of a high level of viable cell titer when the pH value of the medium is lowered is due to the high concentration of reducing sugars. The value of A increases in proportion to the buffering capacity of the culture medium, with an optimal value of A_{optB} = 6.05 reported at 1.5 mmol $HCl{\cdot}pH^{-1}{\cdot}L^{-1}$. Based on the experimental data, the following equation was derived:

$$A = 6.05 \times \left(\frac{S}{0.22 + S}\right) \times \left[0.42 + 0.024 \times \left(L - 0.57\right)\right] \quad (8)$$

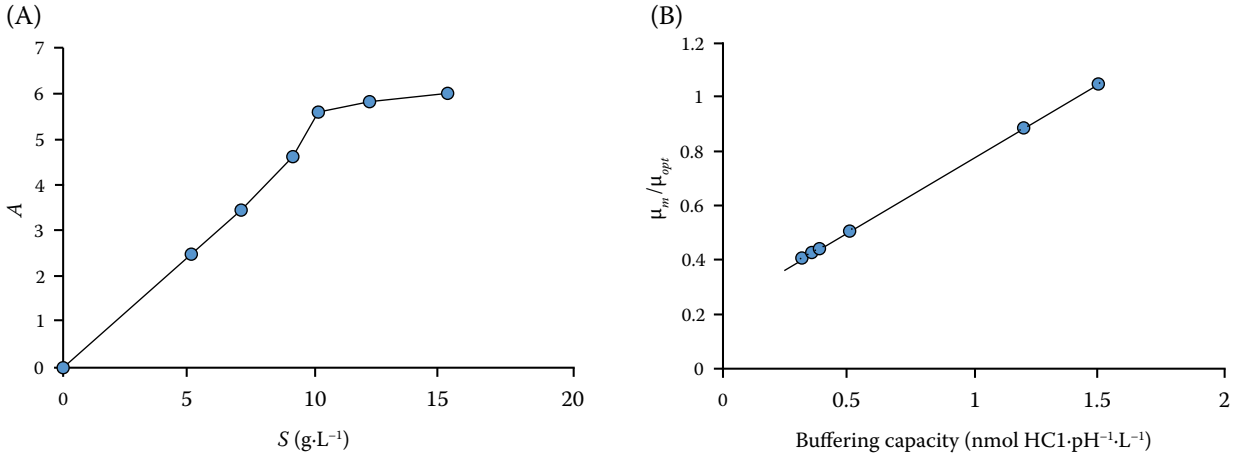

Figure 2. Maximum relative cell concentration (A) as a function of initial algae-biomass concentration (S) of the medium (A) and as a function of their buffering capacity (B)

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Table 4. Optimum parameters according to Equation 14 describing lactic acid production during *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) growth in algae biomass-based media of different buffering capacities and sugar concentrations

	α_p	β_p	*F*-value	*r*
Buffering capacity				
0.32	0.330 ± 0.048	0.0190 ± 0.0042	1 267.0	0.991
0.36	0.341 ± 0.004	0.0200 ± 0.0094	1 654.9	0.993
0.39	0.403 ± 0.097	0.0230 ± 0.0012	1 763.4	0.996
0.51	0.304 ± 0.007	0.0170 ± 0.0087	2 320.5	0.997
1.20	0.302 ± 0.086	0.0180 ± 0.0026	2 775.2	0.992
Dry matter (%)				
5.00	1.517 ± 0.066	–0.1890 ± 0.0046	115.0	0.961
7.50	0.246 ± 0.042	0.0250 ± 0.0044	120.6	0.976
10.00	0.412 ± 0.086	0.0370 ± 0.0017	715.2	0.993
12.00	0.372 ± 0.076	0.0310 ± 0.0023	1 063.7	0.995
15.00	0.401 ± 0.024	0.0230 ± 0.0072	2 036.9	0.979

The values are the means with the corresponding confidence intervals (α = 0.05); α – regression parameter; β – regression parameter (pH·L·mmol^{-1}·HCl); *r* – regression coefficient

Secondary modelling of λ. The relationship between the logarithmic phase growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain (λ) and fermentation process factors such as temperature and pH were established in the present study based on the search for an inversely proportional relationship between λ and μ_m. As shown in Figure 3, there is a strong linear correlation between $\lambda \times \mu_m$ and μ_m (r = 0.95, F-value = 1667.5), which can be described by the relationship:

$$\mu_m \times \lambda = \alpha_3 + \beta_3 \times \mu_m \quad (9)$$

The values of the regression parameters are α_3 = 0.36 ± 0.17 and β_3 = –0.21 ± 0.02 h. Equation (9) can then be used to predict the lag phase of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain by introducing the value of μ_m obtained from Equation (7) in the recent study. Their values during fermentation of synthetic media with a sugar concentration of 1.2 g·L^{-1} to 20.0 g·L^{-1} and buffering capacity of 0.4 to 1.2 mmol HCl·pH^{-1}·L^{-1} by *Lactobacillus plantarum* NCIMB 8826 strain were α_3 = –0.26 ± 0.14 and β_3 = 4.17 ± 0.32 h (Charalampopoulos et al. 2009).

Lactic acid production models. Homofermentative lactic acid bacteria degrade reducing sugars from the composition of the medium mainly to lactic acid via the Emben-Meyerhof-Parnas pathway, and the necessary cellular energy in the form of adenosine triposphate (ATP) for their growth and development is accumulated as a result of oxidative phosphorylation and electron transfer through the cytoplasm membrane. The formation of lactic acid at the end of the fermentation process when the strain is cultivated in a medium based on has an inhibitory effect that can be visualised with the Luedeking–Piret dependence. This model suggests that the product (*P*) formation rate depends on the growth rate (*dN/dt*) and the cell concentration (*N*). The cell concentration in terms of the relative cell population (*X*):

$$\frac{dP}{dt} = (\alpha_p \times \frac{dX}{dt}) + (\beta_p \times X) \quad (10)$$

Integration of Equation (10) between P_0 –> P, 0 –> X and 0 –> t gives:

$$P = P_0 + \frac{\alpha_p \times A}{1 + \exp\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]} + \frac{\beta_p \times A^2}{4 \times \mu_m} \times \ln\left[\frac{\exp\left(2 + \left(4 \times \mu_m \times \frac{\lambda}{A}\right)\right) + \exp\left(4 \times \mu_m \times \frac{t}{A}\right)}{1 + \exp(2 + \left(4 \times \mu_m \frac{\lambda}{A}\right))}\right] \quad (11)$$

In Table 4 the parameters of this equation with their confidence intervals (α= 0.05), the *F*-value and correlation coefficients are summarised. Similar results

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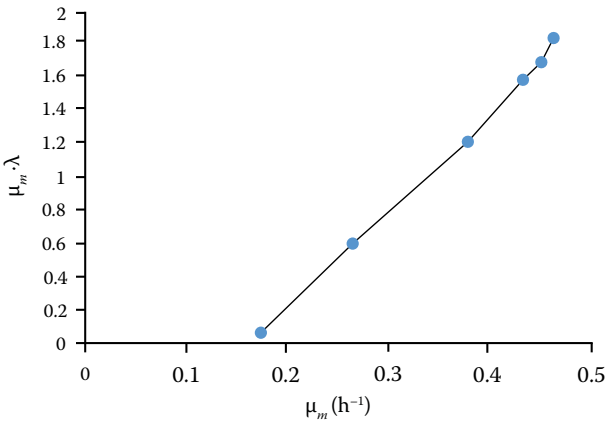

Figure 3. Maximum specific growth rate (μ_m) and growth lag phase (λ) as a function of maximum specific growth rate

were obtained during the cultivation of *L. plantarum* in the synthetic medium with a concentration of glucose in the range of 0.4–20 g·L^{-1} (Charalampopoulos et al. 2009). Lactic acid production was modelled with the Luedeking and Piret model in the experiments by *Lactobacillus helveticus* R211 strain and the estimated α_p and β_p values were used as response variables. The second-order response surface for α_p was not significant because of their dependence of the pH of the whey permeate/yeast extract medium (Schepers et al. 2002).

Secondary modelling of parameter α_p. The regression coefficient α_p, which accounts for the growth of the strain when it is cultivated in the base medium of capacity (Table 4). However, no linear relationship between the parameter α_p and the buffering capacity of the medium was observed in the conducted study. The value of the coefficient, which takes into account the influence of the dry matter content of the culture medium, varies between 0.24 and 0.40 g of lactic acid $[\log_{10}(N/N_0)^{-1}]^{-1}$ and increases proportionally to the amount of dry matter by reaching a polynomial of the 3rd or 4th order (r = 0.95), i.m. the mean α_p value in all causes was used to describe the growth associated lactic acid production [α_p = 0.302 ± 0.05 g lactic acid $[\log_{10}(N/N_0)^{-1}]^{-1}$]. The values for the growth associated constant α_p varies between 0.64 and 0.84 g lactic acid $[\log_{10}(N/N_0)]^{-1}$·L^{-1} during cultivation of *L. plantarum* strain in synthetic media, where buffering capacity was the controlling factor, but The values of α_p in the batches where sugar was the controlling factor varied between 0.42 and 0.82 g lactic acid $[\log_{10}(N/N_0)]^{-1}$·L^{-1} (Charalampopoulos et al. 2009). The growth-associated lactic acid production parameter on growth and lactic acid production by *Lactobacillus helveticus* during pH-controlled batch cultures was constant, while the nongrowth-associated production parameter b, estimated during growth and early stationary phase, was a linear function of pH (Schepers et al. 2002).

Secondary modelling of parameter β_p. The study found no correlation between the value of the parameter and the buffer capacity of the medium, while the change of dry matter in the culture medium determined β_p values from 0 to 0.037 g lactic acid $[\log_{10}(N/N_0)^{-1}]^{-1}$·h^{-1} (Table 4). At a dry matter content of 5%, the value of the β_p parameter had a negative value, and a linear relationship (r = 0.99, F-value = 135.1) was established between the three values, which is described by the equation:

$$\beta_p = 0.006 \times S + 0.019 \quad (12)$$

In the cultivation of *Lactobacillus plantarum* strain in synthetic media where the buffering capacity was the controlling factor, when β_p was plotted against buffer capacity (B, mmol HCl·pH^{-1}·L^{-1}) not a clear trend was observed and β_p was considered to be independent of the buffering capacity. The values of β_p, when sugar was the controlling factor, ranged from 0 to 0.021 g lactic acid $[\log_{10}(N/N_0)^{-1}]$·L^{-1} (Charalampopoulos et al. 2009). The response surface model for the nongrowth-associated production parameter β_p was significant without significant lack of fit during fermentation of whey permeate-yeast extract medium by *Lactobacillus helveticus* R211 strain, but the linear relationship of the nongrowth-associated production parameter β_p as a function of pH was confirmed the importance of the pH of the medium on the value of n (nongrowth-associated parameters) (Schepers et al. 2002).

pH model. The decrease in pH during cultivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 is exclusively due to the accumulation of lactic acid. In the initial stages of the exponential growth phase, the pH decreased more rapidly in media with lower buffering capacity, and the final pH value was always lower in media with lower buffering capacity. Another relationship was then investigated where the decrease in pH due to fermentation of sugars to form lactic acid is directly proportional to pH minus final pH (pH_{min}):

$$\frac{d\text{pH}}{dP} = k \times (\text{pH} - \text{pH}_{min}) \quad (13)$$

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Integration of Equation (13) with initial conditions P_0 and pH_0 yields:

$$pH = pH_{min} + (pH_0 - pH_{min}) \times e^{-k.(P-P_0)} \quad (14)$$

The values of these regression coefficients in studies of the influence of the concentration of lactic acid formed and pH in the cultivation of the strain in media with different buffer capacities were reported based on checking the adequacy of Equation (14) using the non-linear method of least squares. The pH_{min} values did not change proportionally to buffer capacity (B) and the mean value of 4.21 ± 0.16 was used to express pH_{min} in all cases. The k values decrease with increasing buffer capacity, which is illustrated in Figure 4. This trend is adequately described by a 2nd order polynomial equation (r = 0.957, F-value = 865.9) and k can be expressed by:

$$k = 1.42 - 2.60 \times B + 1.47 \times B^2 \quad (15)$$

Finally, a full model of pH can be obtained by introducing Equation (11) in Equation (14) (see Equation16).

The pH_{min} values did not show any obvious dependency on the buffering capacity and the mean value 3.21 ± 0.06 was used to express the pH_{min} in all cases of the cultivation of *L. plantarum* in synthetic media with the initial sugar concentration of 1.2 g·L^{-1} to 8.0 g·L^{-1}. The k values decrease with increasing buffering capacity from 0.4 to 1.2 mmol HCl·pH^{-1}·L^{-1} (Charalampopoulos et al. 2009). The results of Schepers et al. (2002) show that interactions between culture conditions pH, whey permeate concentration and yeast extract concentration are important for maximum specific growth rate and maximum biomass concentration of *Lb. helveticus* in pH-controlled batch cultures.

Sugar consumption model. Reduction of reducing sugars when culturing *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in processed *Spirulina platensis* (*Arthrospira platensis*) biomass media ('Simbiotex' Ltd., Sofia, Bulgaria) is closely related to growth rate, the concentration of lactic acid formed, and rate of substrate uptake to maintain cell viability:

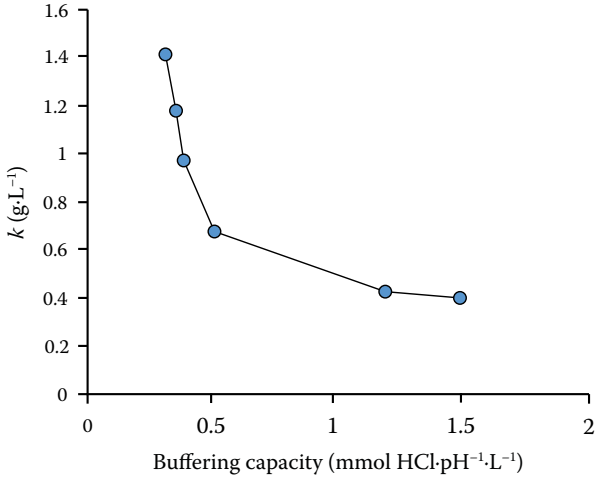

Figure 4. Parameter k as a function of buffering capacity

$$\frac{dS}{dt} = \left(-\frac{1}{Y_{\frac{p}{s}}} \times \frac{dP}{dt}\right)\left(-\frac{1}{Y_{\frac{p}{s}}} \times \frac{dX}{dt}\right)(-m_s \times X) \quad (17)$$

where: $Y_{p/s}$ – yield of lactic acid production on the substrate [g (lactic acid)·g^{-1} (sugar)]; m_s – relative cell maintenance coefficient [g (sugar)·L^{-}·[log$_{10}$(N/N_0)]$^{-1}$·h^{-1}]

Introducing the logistic growth model (3) and the lactic acid production model (11) into Equation (17) and integrating, a model describing the change of sugar concentration with time could be obtained. This equation was used to fit the experimental data using the nonlinear least-squares in order to estimate the parameters $Y_{p/s}$, $Y_{x/s}$ and m_s. Though the iterative process converged to a final solution, the parameters obtained were not realistic (and not significant) and depended on the initial values of the estimated parameter. Reducing sugars from the composition of the medium obtained as a result of biomass processing of *Spirulina platensis* (*Arthrospira platensis*) ('Simbiotex' Ltd., Sofia, Bulgaria), are converted to a greater extent to lactic acid and relatively little are included in the constructive metabolism of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain. Excluding the growth and maintenance terms from

$$pH = pH_{min} + (pH_0 - pH_{min}) \times \exp\left\{-k \times \left[\frac{\alpha_p \times A}{1 + e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]}} + \frac{\beta_p \times A^2}{4. \times \mu_m} \times \ln\left[\frac{e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]} + e^{\left(4 \times \mu_m \times \frac{\lambda}{A}\right)}}{1 + e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]}}\right]\right]\right\} \quad (16)$$

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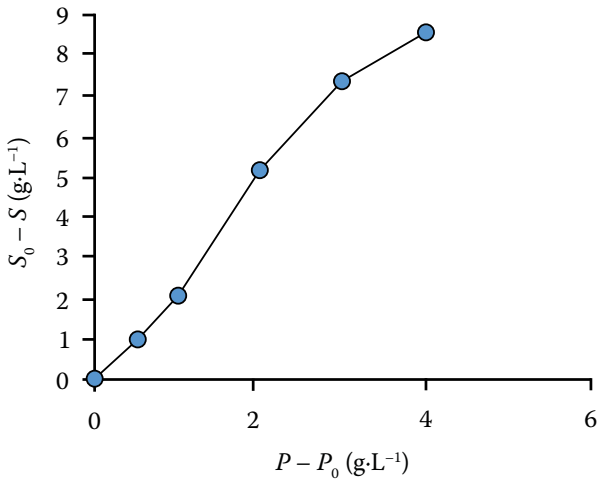

Figure 5. Biomass concentration (S_0–S) as a function of production (P–P_0)

Equation (17) and integrating with initial conditions S_0 and P_0 gives:

$$S - S_0 = \frac{1}{Y_{\frac{p}{s}}} \times (P - P_0) \quad (18)$$

In order to estimate $Y_{p/s}$ (g of lactic acid produced per g of glucose consumed) the glucose and lactic acid concentrations were determined at regular time intervals for a number of batch cultures. The results are shown in Figure 5 and indicate that the glucose uptake and lactic acid formation are linearly correlated (r = 0.96, F-value = 893.2). The estimated $Y_{p/s}$ value was 0.196 ± 0.02 g·g^{-1}, which is considerably lower than other reported values for lactic acid bacteria at optimum conditions with pH control and nutrition-rich media. The low $Y_{p/s}$ obtained is probably due to the lack of pH control.

At pH 3.5 glucose and fructose (converted into mannitol) were depleted after 10 h of fermentation of sourdough by *L. fermentum* IMDO 130101 strain, but maltose was no longer consumed. At pH 7.5 (Figure 5), the maltose concentration decreased from 30 to 25 mM, while the glucose concentration remained constant. The fructose concentration decreased slightly and this decrease could be completely accounted for by mannitol production. Fructose was exclusively used for reduction into mannitol in all fermentations, which was shown by the fact that the rate of substrate uptake equalled one under all conditions tested (Vrancken et al. 2008). The glucose uptake and lactic acid formation are linearly correlated during the cultivation of *L. plantarum* in a synthetic medium. The estimated $Y_{p/s}$ value was 0.51 ± 0.02 g·g^{-1} and is greater than the value in the recent study due to the lack of pH control (Charalampopoulos et al. 2009).

Introducing the model for lactic acid production (11) into Equation (18) a full model for sugar consumption can be obtained (Equation 19).

CONCLUSION

In the present study, the kinetics of growth of viable cell titer, concentration of formed lactic acid, pH drop and uptake of reducing sugars in the cultivation of *Lactobacillus delbrueckii* subsp. *bulgaricus* WDCM 00102 strain in *Spirulina platensis* (*Arthrospira platensis*) ('Simbiotex' Ltd., Sofia, Bulgaria) biomass-based medium as a function of initial dry matter concentration and buffer capacity were determined. The growth of the studied culture is described by differential equations, the regression coefficients of which are established by applying non-linear regression analysis. The maximum specific growth rate (m) and the relative maximum cell concentration (A) depend on the buffer capacity and sugar concentration. The effect of dry matter concentration in the culture medium on strain growth followed the Monod equation, while a linear relationship was found for the effect of buffer capacity. The lag phase (λ) was considered in the present study as the inverse of the maximum specific growth rate. The coefficient accounting for the formation of lactic acid due to the fermentation of reducing sugars when the strain was cultivated in algae-based medium was characterised by a constant value, while the regression coefficient, which did not take into account the increase in viable cell titer, varied linearly with the buffer capacity. The coefficient k, expressing the dissociation constant of lactic acid to hydrogen ions, depends on the buffer capacity, while the minimum pH coefficient (pH_{min}) is constant. The yield of lactic acid

$$S = S_0 - \frac{1}{Y_{p/s}} \times \exp\left\{\left[\frac{\alpha_p \times A}{1 + e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]}} + \frac{\beta_p \times A^2}{4 \times \mu_m} \times \ln\left[\frac{e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]} + e^{\left(4 \times \mu_m \times \frac{\lambda}{A}\right)}}{1 + e^{\left[2 + \left(4 \times \frac{\mu_m}{A}\right) \times (\lambda - 1)\right]}}\right]\right]\right\} \quad (19)$$

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on sugar ($Y_{p/s}$) did not depend on the dry matter content of the medium or the buffer capacity.

The present study is a first step in the development of new functional foods based on microalgae biomass, which serve as a suitable medium for the growth and development of lactic acid cultures with probiotic properties. A further stage of the research will lead to establishing the impact of biomass on the cryotolerance of a strain in the lyophilisation process, which represents a second essential stage of the production cycle.

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