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High cell density cultivation of *Bacillus subtilis* NCIM 2063: modeling, optimization and a scale-up procedure

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Abstract: Bacillus subtilis is a non-pathogenic, sporulating, gram-positive bacteria with pronounced antimicrobial and metabolic activity and great potential for wide application in various fields. The aim of this paper was to determine the optimum *B. subtilis* NCIM 2063 growth conditions and to scale up biomass production from shake flasks to a bioreactor level. The critical growth parameters and their interaction effects were studied using Box-Bekhen experimental design and response surface methodology. Developed model equations were statistically significant with good prediction capability. It was found that during shake flask cultivation glucose should be added in concentration up to 5 g l^{-1} in DSM medium, *OTR* at 10 mol m^{-3}h^{-1} and temperature of 33 °C, to achieve the maximum number of viable cells and spores. To scale up the process from shake flasks to the bioreactor level k_La was used as a main criterion. Scale up effect was evaluated by comparing the growth kinetics in the shake flasks and in a laboratory bioreactor. The total number of cells obtained in the bioreactor was 4.57·10^9 CFU ml^{-1} which is 1.41 times higher than the number of cells in the shake flasks (3.24·10^9 CFU ml^{-1}), proving that the scale-up procedure was conducted successfully.

Keywords: shake flask, bioreactor, microbial biomass.

INTRODUCTION

Bacillus subtilis is a Gram-positive, rod-shaped bacteria with a unique ability to rapidly multiply and to form endospores, thus being resistant to adverse environmental conditions. This strain has the ability to produce industrially important metabolites such as antibiotics, polysaccharides and proteins[1–3] and to degrade different pollutants in the environment.[4] A great diversity of previously published papers has showed that *B. subtilis* is capable of producing various important biotechnological products, such as different enzymes[5,6] (Akcan 2012, Božić, 2011), polysaccharides[7] and surfactants[7,8]. Many

of them, including iturin A and surfactin, have antibiotic, antiviral and immunomodulatory effect when applied on humans. Additionally, *B. subtilis* has wide application in the environmental protection, as it can degrade different trace organic compounds[9] and organoclorine insecticides[10]. It is also attributed to probiotic properties and "GRAS" (generally recognized as safe) status, which makes it safe for human use. Thanks to the fact that it has exceptional physiological properties, it can easily adapt to new environmental conditions and has the ability to produce a large number of metabolites, this species is very attractive for industrial application and commercial biomass production.[3,11]

Microbial biomass production implies cultivation in bioreactors in liquid, semi-liquid or solid nutrient media. At the beginning of the research, cultivation is carried out in shake flasks of smaller volumes, in order to establish optimal production procedures at minimum costs. Different optimization techniques can be applied to determine the optimum medium composition, carbon and nitrogen source concentration, mixing speed, temperature, or pH value. Based on such experiments, valuable data are obtained about the microorganism, it's growth kinetics and nutrient requirements. If modern multifactorial optimization techniques are applied, experimental data are used to develop a predictive model that will describe the further behavior of the system.[12] After that, the scale of the process is gradually increased to a laboratory bioreactor, and further on to bioreactors of larger volumes, to determine whether the same or similar results will be achieved on larger-scale equipment.[13,14] The transition from shake flask to laboratory bioreactor is a critical point of the highest importance in the whole scale-up process. If done properly, it ensures the smooth further continuation of the scale-up procedure from laboratory to pilot and industrial scale bioreactor.[15] When transitioning from a shake flask to laboratory bioreactor particular changes in the vessel geometry and size occur, which cause changes in mixing efficiency, affect oxygen supply, and increase the possibility of creating "dead zones" and uneven nutrient distribution. For that reason, the shake flask-bioreactor transition needs to be carefully optimized and designed. Modeling the complex hydrodynamic behavior is one of the most difficult numerical problems that has fundamental importance in many aspects of engineering. The key parameters are related to mass and heat transfer, mixing and aeration.[16] In order to optimize the performance of the bioreactor, it is necessary to know the local fluid dynamics in the bioreactor, *i.e.* the relation between hydrodynamics and the mass transfer coefficient.[17] Volumetric oxygen mass transfer coefficient (k_{La}) and oxygen transfer rate (OTR) are the key parameters used to describe the efficiency of oxygen utilization. k_{La} is most commonly used as a scale-up criterion in aerobic processes used to estimate the efficiency of the bioreactor.[18,19] This practice is supported by the fact that the main problem in aerobic systems is the adequate supply of oxygen from the gas to the liquid phase. Dimensional analysis helps to

develop correlations that will ensure a constant value of k_{La} corresponding to the desired *OTR*. The success of the scale-up process is confirmed experimentally when it is shown that the same or similar results can be achieved in a bioreactor under the same conditions as in shake flasks.[17] Therefore, the aim of this work was to produce good-quality biomass of desired high cell and spore density, and to:

- assess the individual and combined effect of critical *B. subtilis* NCIM 2063 growth parameters: oxygen transfer rate, mixing speed, temperature and glucose concentration;
- determine the optimum growth conditions for *B. subtilis* NCIM 2063 on a shake flasks level using response surface methodology (RSM);
- provide -statistically significant model equations for the shake flasks level;
- scale-up the cultivation of *B. subtilis* NCIM 2063 from shake flasks to batch bioreactor using k_{La} as scale-up criterion;
- evaluate the success of the scale-up procedure by bioreactor cultivation with additional analysis of kinetic and stoichiometric parameters.

EXPERIMENTAL

Inoculum preparation

Sporogenic Gram-positive bacterium *B. subtilis* 2063 from the NCIM collection was provided by a private company Fertico d.o.o. located in Nis, Serbia. The bacterial culture was stored at -80 °C in vials and at 4 °C on agar plates. 300 ml Nutrient broth was inoculated with a single loop of *B. subtilis* NCIM 2063 and incubated at 37 °C for 24 h in shake flasks at 150 rpm. A 1% (v/v) overnight culture was used as inoculum for further cultivation.

Shake flask cultivation study

To optimize the *B. subtilis* growth conditions in the shake-flasks, the Box-Benken experimental design (BBD) was used (Table 1). 1 % *B. subtilis* 2063 overnight culture was used to inoculate sterilized nutrient DSM medium (8g nutrient broth, 10 ml 10% (w/v) KCl, 10 ml 1.2% (w/v) MgSO$_4$, 1 ml 1 M Ca(NO$_3$)$_2$, 1 ml 0.01 M MnCl$_2$, 1 ml 1 mM FeSO$_4$) in a 500 ml Erlenmayer vessel. DSM is a commonly used medium for *Bacillus* cultivation, owing its popularity to its simplicity, high biomass yield and sporulation efficiencies [20–24]. The three factors varied on three levels were: temperature (25-37 °C), *OTR* (2-10 mol m^{-3}h^{-1}) and glucose concentration (0-20 g l^{-1}). The factors and their levels were selected based on the preliminary experiments and available literature data [22,25–27]. Cultivation was performed for 24 h on a rotary shaker at 150 rpm according to the design matrix. The total number of viable vegetative cells and the number of spores were chosen as dependent variables. The viable cell count was determined using the spread plate method. Spores were counted using the same method, but the samples were previously heated at 80 °C for 10 min.

Table 1 Coded and non-coded values of process factors according to BBD design

Symbol	Factor	Low level (-1)	Middle level (0)	High level (+1)
A	Temperature, °C	25	31	37
B	*OTR*, mol/m3h	2	6	10
C	Glucose concentration, g/L	0	10	20

Bioreactor cultivation

1% *B. subtilis* NCIM 2063 inoculum was used to inoculate the laboratory bioreactor containing a sterile DSM medium. The cultivation was performed at the optimum conditions determined in the shake flasks and after the scale-up procedure. The bioreactor cultivation was performed in a bioreactor KLFM, BioEngineering, Wald, Switzerland (working volume: 2.5 l, total volume 3.7 l), equipped with two Rashtan impellers with 6 blades (48 mm diameter) and 4 baffles. Sterile air supplied from an external compressor was used for aeration with a specific air flow of 0.3 v.v.m. The bioreactor is equipped with a pH (BioEngineering 4695) and an oxygen electrode (Mettler Toledo 3420036) and connected to the BioScada software system, which monitors the process parameters.

$k_L a$ and OTR determination

$k_L a$ values in the bioreactor were determined using the absorption method, while the *OTR* values in shake flasks and in the bioreactor were calculated according to equations 1 and 2, respectively [28]:

$$OTR = 7.23 \cdot 10^{-4} \cdot \left(\frac{V_L}{V_F}\right)^{-0.845} \cdot C \cdot N \qquad (1)$$

$$OTR = k_L a \cdot (C^* - C) \qquad (2)$$

where: V_L is a liquid volume (ml), V_F is Erlenmayer flask volume (ml), C is dissolved oxygen concentration (mg l^{-1}), N is shaking speed (s^{-1}), and C^* oxygen solubility in the medium at a given temperature.

Kinetic and stoichiometric parameters

The yield coefficients $Y_{x/o}$ and $Y_{x/s}$ were calculated according to the following equations:

$$Y_{x/o} = \frac{X}{OTR \cdot t} \qquad (4)$$

$$Y_{x/s} = \frac{s_0 - s}{x - x_0} \qquad (5)$$

where: s_0 is initial glucose concentration (g l^{-1}), s is final glucose concentration, x is final biomass concentration (g l^{-1}), x_0 is initial biomass concentration (g l^{-1}), X is the biomass concentration at the moment t, *OTR* is oxygen transfer rate (mol m^{3}h^{-1}), and t is time (s). $Y_{x/0}$ was calculated under the assumption that all of the oxygen that was transferred to the medium was also consumed by the microorganism.

Specific growth rate μ_m and generation time t_d were calculated using the following equations[29]:

$$ln \frac{x}{x_0} = \mu_m \cdot t \qquad (6)$$

$$t_d = \frac{\ln 2}{\mu_m} \tag{7}$$

where x is the biomass concentration at the moment t, and x_0 is the initial biomass concentration (g l^{-1}).

Statistical analysis

Each experiment was run three times in parallel, and the findings were reported as the mean value of three repetitions ± standard deviation. The program Origin 6.0. Excel 2013 and Expert Design 7.0 were used for the statistical processing of experimental data. RSM and Derringer's Desirability function were used for modeling and optimization. The adequacy of the response surface model was assessed using the analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The reliability and durability of microbial biomass formulation largely depend on the number of living cells, particularly the spores, which is why it is important to enabling the microorganism to reach a high cell density and to sporulate. Three independent factors (temperature, oxygen transfer rate and initial glucose concentration) were varied at three levels according to the Box-Benken experimental design, and their effect on total vegetative cell and spore count after 24 h of cultivation is shown in Table S1 (supplementary file). By applying the nonlinear regression method to the obtained experimental data the following mathematical models were proposed for the total number of *B. subtilis* vegetative cells and spores, respectively:

$$X = 9.04 + 0.01 \cdot A - 0.29B + 0.02C + 6.04 \cdot 10^{-3}AB - 8.75 \cdot 10^{-4}AC +$$
$$0.01BC - 81.87 \cdot 10^{-4}A^2 + 0.01B^2 - 5.36 \cdot 10^{-3}C^2 \tag{8}$$
$$Y = 7.05 + 0.06A - 0.18B + 0.105C + 0.02B^2 - 7.43 \cdot 10^{-3}C^2 \tag{9}$$

where *X* is vegetative cell count, *Y* is spore count, *A* is the temperature (°C), *B* is *OTR* (mol m^{-3}h^{-1}) and *C* is initial glucose concentration (g l^{-1}). The significance and reliability of the models were assessed by comparing the predicted and experimental values and by conducting ANOVA analysis (Table 2). It can be observed that each of the individual factors in the model that predicts the number of viable vegetative cells is statistically significant with a degree of reliability of 99 %. The calculated *p*-value for the lack of fit (0.219) for this model is statistically insignificant and indicates a good fit of the experimental data for both of the models. The highest significance was recorded for initial glucose concentration and *OTR* (in both their individual and quadratic form). Individual temperature term, as well as temperature - *OTR* interaction term are also found to significantly influence the number of *B. subtilis* vegetative cells. Since *OTR* and the initial glucose concentration are significant on a quadratic level, a small variation in their values will greatly affect the growth rate and the final number of cells[21].

Table 2 ANOVA for the models obtained for *B. subtilis* NCIM 2063 viable cell and spore count

Parameter	Viable cell count		Spore count	
	F value	p-value	F value	p-value
Model	41.32	< 0.0001	20.86	**< 0.0001**
A	13.62	0.0078	5.84	**0.0363**
B	109.04	<0.0001	0.99	0.3425
C	126.23	< 0.0001	91.22	**< 0.0001**
AB	5.03	0.0599	-	-
AC	0.66	0.4436	-	-
BC	40.2	0.0004	9.26	**0.0124**
A^2	0.011	0.9177	-	-
B^2	6.5	0.0381	4.13	**0.0696**
C^2	75.52	<0.0001	14.52	**0.0034**
Lack of fit	2.3	0.2186	3.59	0.1182
R^2	0.982		0.926	
Adj R^2	0.958		0.882	
Pred R^2	0.802		0.697	
C.V., %	1.47		5.10	
PRESS	1.25		6.59	
MRPD, %	0.1		1.52	

Values of p less than 0.05 (p < 0.05) indicate model terms are significant.

On the other hand, sporulation was highly affected by initial glucose concentration (individual and quadratic term) which is the most significant factor. Apart from glucose, temperature, quadratic term of *OTR* and interaction between *OTR* and initial glucose concentration were also significant. The spore-predicting model was also statistically significant (with an *F* value of 21.01 and 0.0002 for the *p*-value), with an insignificant lack of fit. The reliability of the models was additionally assessed by analyzing the values of R^2, *Adj* R^2 and *Pred* R^2, which are in accordance with the requirements that R^2 and *Adj* R^2 should be reasonably close to 1, and that the difference between *Adj* R^2 and *Pred* R^2 should not exceed 0.2.[30] Obtained values imply that both developed regression equations have a good fit and that they can successfully predict system responses.

The regression equation is also graphically represented in two-dimensional contours, visualizing the relationship between the response and each of the independent variables in the system (Figure 1).

a)   **b)**
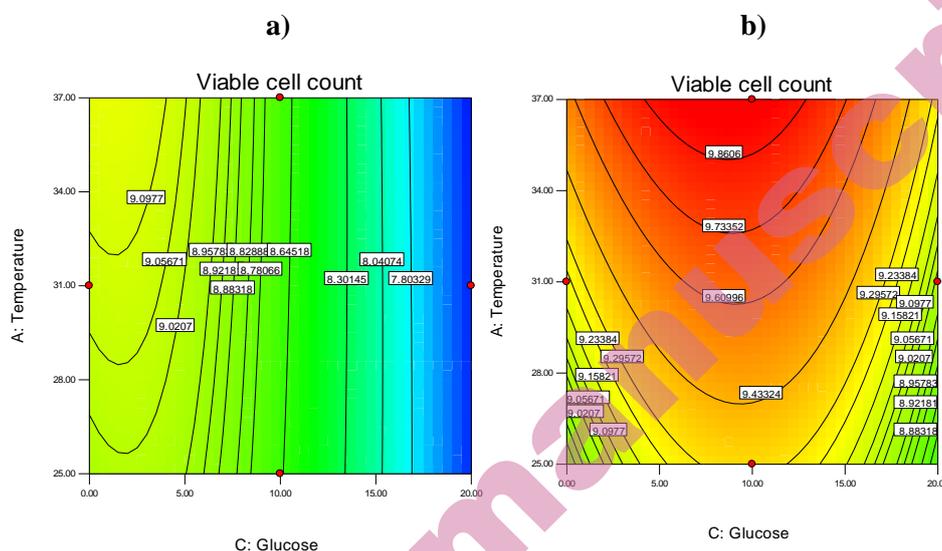
Fig 1 Contour plots representing the total number of *B. subtilis NCIM 2063* viable cells as a function of glucose concentration, g l^{-1}, temperature (°C), and OTR 2 mol m^{-3}h^{-1} (a), 10 mol m^{-3}h^{-1} (b)

It can be concluded that at higher initial glucose concentrations, an increase in *OTR* has a positive effect on the total number of viable vegetative cells, while temperature has no significant effect. By reducing the concentration of glucose, the effect of temperature becomes more pronounced, with the largest number of cells being achieved in a medium with 10 g l^{-1} glucose. In contrast, in a glucose-free medium, increasing the *OTR* has little effect on the cell number. This effect is diminished by an increase in temperature. Such moderate interaction of *OTR* and temperature was also previously confirmed through the calculated *p*-value. Similarly, the significance of this interaction effect was observed in a study optimizing the growth of *Bacillus coagulans* using the RSM method, with maximum biomass yield obtained by a combination of high temperatures and specific airflow.[31] The graphic analysis confirmed the importance of oxygen availability, which is explained by the fact that lack of oxygen reduces the pH of the substrate, leading to rapid lysis of the cell and the initiation of new metabolic pathways.[32]

a)   b)
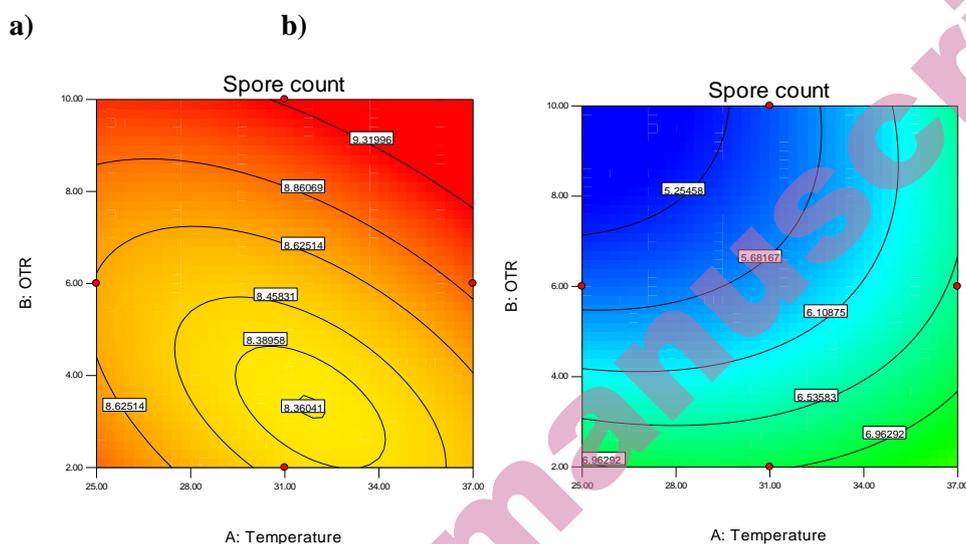
Fig 2 Contour plots for the number of *B. subtilis* NCIM 2063 spores as a function of *OTR*, temperature and initial glucose concentration 0 g l^{-1} (a), 20 g l^{-1} (b)

Figure 2 shows the visualized relationship between dependent and independent variables for the spore-predicting model. The maximum number of spores was obtained in a glucose-free medium at $OTR = 10$ mol m^{-3}h^{-1}, while the effect of temperature was negligible. As expected, nutirent deprevation inititated sporulation, which allows *B. subtilis* to enter a dormant state, preserving its genetic material and resistance to harsh conditions until favorable growth conditions are restored[24]. The effect of temperatures higher than 30 °C is most pronounced at the maximum applied initial glucose concentration. In a glucose-free medium, increasing the *OTR* increases the number of sporulated cells. As the initial glucose concentration increases, the influence of *OTR* on the sporulation is reduced with the increase in the initial glucose concentration in the medium, although cultivation at higher temperatures reduces the effect of glucose. It can be explained by the fact that increasing the initial glucose concentration increases the viscosity of the medium, which creates greater resistance of the liquid film and thus reduces the real *OTR* value. Increasing the temperature increases the solubility of the components of the nutrient medium, which diminishes the negative effect of glucose in this interaction. The literature data agree with the experimental results achieved here. It was found that the maximum yield of spores is achieved at low initial glucose concentrations and that bacterial culture begins to sporulate when the cell density is about 10^8 CFU ml^{-1}.[33] The reason for this is the characteristic of cells to sporulate in unfavorable environmental conditions, ie. in conditions when nutrients are not available in excess.[34] As a result of that, the microorganism can

undergo metabolic shifts, activating alternative metabolic pathways, or emploing strategies such as gluconeogenesis to sustain growth using non-carbohydrate carbon sources. The gluconeogenesis pathway involves a series of enzymatic reactions that convert non-carbohydrate precursors into glucose-6-phosphate, which are metabolized through glycolysis for energy production or used as a precursor for biosynthesis [35].

Multicriteria optimization using Derringer's desirability function

Derringer's desirability function is used in complex multivariate processes in which variables that need to be optimized are influenced by multiple factors simultaneously. Based on multicriteria optimization (Table S2, supplementary file), several combinations of process conditions have been proposed to obtain the maximum value of the desirability function as well as of both response variables. Taking into account the response surface analysis and knowing that the increase in temperature does not decisively affect the increase in the total number of viable cells, it was decided to maximize the cell growth and provide the necessary conditions for sporulation, while achieving energy savings. According to that, the following optimum conditions for the cultivation of *B. subtilis* NCIM 2063 were proposed: T = 33 °C, *OTR* = 10 mol m^{-3}h^{-1}, and initial glucose concentration 4.89 g l^{-1}. Under these conditions, the model predicts a maximum viable vegetative cell concentration of 9.66 log (CFU ml^{-1}) and spores of 9.19 log (CFU ml^{-1}) with a high desirability function value (0.931). The experimental cell density for the total number of cells and spores obtained under the given conditions was 9.51 ± 0.09 log (CFU ml^{-1}) and 9.08 ± 0.06 log (CFU ml^{-1}), respectively. The experiment was performed in triplicate, and the relative error between predicted and obtained values for vegetative cells and spores was 1.5% and 1.2%, respectively, which confirms that there is a good agreement between predicted and experimental values.

A scarce number of previously published studies is dedicated to the topic of multicriteria optimization of *B. subtilis* growth conditions. Most of the available research deals with optimizing the media composition,[36–40] while a small number of them optimize growth conditions. A group of authors conducted a screening of the influence of volumetric airflow and mixing rate on cell density and sporulation of *B. subtilis* EA-CB0575 using a central composite experimental design.[31] As optimal conditions, a mixing speed of 432 rpm and a volumetric airflow of 12 l/min at a temperature of 30 °C are given.[41] The same experimental design was used to optimize the sporulation of *B. subtilis* in a solid medium. It was found that temperature and volumetric air flow have an impact on sporulation, with 27 °C and 1.2 l min^{-1} being recommended as optimal values for solid medium, respectively.[42]

Scale up from shake flask to laboratory bioreactor

   k_La is a key parameter for scaling and optimization in mechanical mixing systems, where the rate of oxygen mass transfer between the gas and liquid phases is an essential phenomenon for process control.[43] Hence, k_La was chosen as the basic criterion for the scale-up procedure to the bioreactor level. The main goal of the scale-up process was to define the values of process conditions at the bioreactor level that will enable the same value of k_La established for shake flasks: namely to define the mixing speed that will provide the desired oxygen transfer from gas to a liquid phase.

   Firstly, k_La was measured at different mixing speeds in the DSM medium at the bioreactor level (Table 3). As expected, reducing the mixing speed also affects the reduction of the oxygen mass transfer rate. An increase in the stirrer speed from 100 rpm to 400 rpm, causes an exponential increase in the k_La value. Such a result is in accordance with the literature data, since in the medium with 10 g l⁻¹ of glucose at a specific air flow of 1 v.v.m, an increase in the value of k_La from 25.2 h⁻¹ to 104.4 h⁻¹ was detected when the mixing speed was increased from 300 rpm to 600 rpm.[44] At higher mixing speeds the air bubbles break into small bubbles, which increases the gas-liquid interfacial surface to transfer the oxygen in the medium, thus increasing the k_La.[45]

Table 3 Influence of mixing speed on the k_La values in DSM medium at bioreactor level

Mixing speed, rpm	100	200	300	400
kLa, h-1	5.26±0,02	6.51±0,11	11.88±0,15	45.35±0,25
OTR, mmol m-3h-1	1.15	1.42	2.59	9.88

   After applying regression analysis to experimental data obtained by the absorption method, the following empirical equation was developed to describe the relationship between the mixing speed N and k_La:

$$k_L a = \exp\left(7.1 \cdot 10^{-3} N + 0.7\right) \tag{9}$$

   Based on the obtained correlation it was calculated that at given conditions in a laboratory bioreactor containing DSM medium (33 °C and 0.3 v.v.m air flow rate) the mixing speed should be set to 452 rpm in order to achieve the required k_La value (45.35 h⁻¹).

Bioreactor cultivation

   The success of the scale-up procedure was evaluated after the cultivation of *B. subtilis* NCIM 2063 at determined optimum conditions at the bioreactor level. It was concluded that, at the end of the cultivation period, the total number of viable cells in the bioreactor was 9.65 ± 0.05 log (CFU ml⁻¹), which is 1.4 times more than the number of cells achieved in shake flasks (9.51 ± 0.09 log (CFU ml⁻¹) under the same conditions. Growth kinetic and stoichiometric analysis lead to the same conclusion (Fig 3, Table 4). After 24 h cultivation, the biomass concentration was

higher in the bioreactor than in the shake flasks, although similar values of specific growth rate were recorded in both systems. The higher cell density at the end of cultivation in the bioreactor can be explained by better oxygen saturation of the medium, which is confirmed by a higher biomass yield on oxygen ($Y_{x/o}$) in the bioreactor (Table 4). Namely, in shake flasks, gas induction is based only on surface aeration. Initially, the substrate is saturated with air and the amount of oxygen is sufficient for microbe growth. After the exponential phase, a sharp drop in the oxygen concentration occurs in shake flasks. In the case of bioreactors, mixing and a constant supply of fresh air provide sufficient levels of oxygen, which can contribute to greater multiplication of cell mass, or lead to prolongation of the exponential phase.[46] This once again confirms that aeration and mixing play a very important role in the metabolic activity of microorganisms. Given that a satisfactory number of cells was achieved in the bioreactor at the end of the cultivation period, it is concluded that the scale-up process was successfully implemented, which created the basic condition for further scale-up to the semi-industrial level.

Table 4 Specific growth rate (μ_m), generation time (t_d), final biomass concentration (X) and biomass yield from oxygen consumption ($Y_{x/o}$) during shake flask and bioreactor cultivation of *B. subtilis* NCIM 2063

Cultivation	μ_m, h^{-1}	t_d, h	X, g l^{-1}	$Y_{x/o}$, g g^{-1}
Bioreactor	0.44±0.08	1.57±0.58	7.24±0.00	0.81±0.00
Shake flask	0.41±0.03	1.71±0.26	5.6±0.51	0.71±0.31

The success of the k_La-based scale-up process strategy has been shown earlier in the literature. k_La was used as a criterion for increasing the scale of phenyl acetyl carbinol production using the yeast *Saccharomyces cerevisiae*. In that research, the k_La value was first estimated by the absorption method in shake flasks, based on which appropriate correlations were developed. Similar k_La values and higher yield of the desired product were achieved in a 5 l bioreactor.[46] An analogous scale-up strategy was applied for the cultivation of *Corynebacterium glutamicum* and the production of lactic acid using adapted empirical models obtained by the sulfite method.[47] The optimal value of k_La (31 h^{-1}) was also the leading parameter for adjusting the mixing speed and air volume flow in order to obtain a quality *Azospirillum brasilense*-based product for pathogen biocontrol at the semi-industrial level.[18]
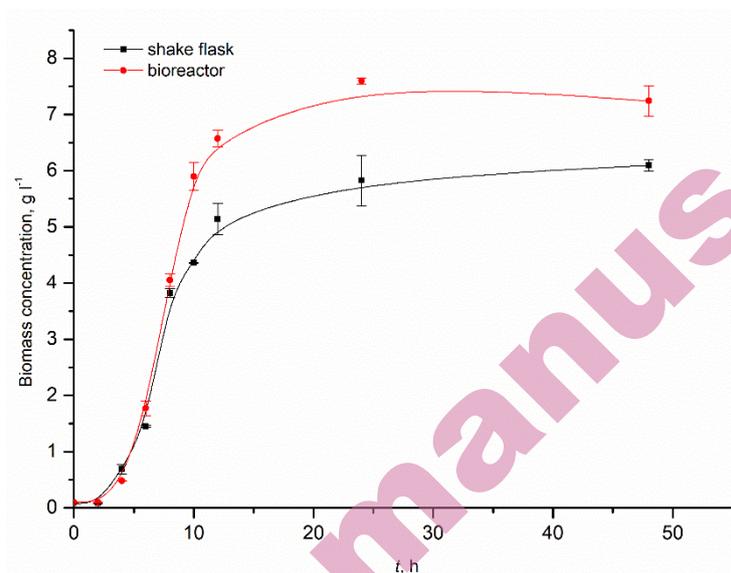
Fig. 3. *B. subtilis* NCIM 2063 growth kinetics at bioreactor and shake flask level at DSM medium containing 4.89 g l⁻¹ glucose at bioreactor level under the optimum conditions: 33 °C and 452 rpm

CONCLUSION

In this study, the conditions for batch cultivation of *B. subtilis* NCIM 2063 were optimized to maximize viability and sporulation. The individual and combined effects of k_La, temperature and glucose concentration were assessed and explained. Glucose and k_La have the greatest statistical significance (both as an individual and as a quadratic term) for the number of viable cells, followed by the interaction factor of these two terms, the individual temperature factor and the interaction of temperature and k_La. When it comes to the total number of spores, the concentration of glucose (individual and quadratic term), temperature, and the interaction of k_La and glucose have the greatest influence on this response. Statistically significant quadratic models were developed with an insignificant lack of fit, which is confirmed by a good agreement between experimentally obtained and predicted data. Using Derringer's desirability function the following optimum conditions were proposed for a DSM medium: T=33 °C, k_La =50 mol m⁻³h⁻¹ and glucose concentration 4.89 g l⁻¹. Scale-up from shake flasks to a batch bioreactor was performed using k_La as a scale-up criterion. An empirical equation was developed to calculate the exact stirring speed needed to achieve the desired k_La. The success of the scale-up procedure was evaluated by bioreactor cultivation with additional analysis of kinetic and stoichiometric parameters. Given that a satisfactory number of cells has been achieved in the bioreactor and that the scale-

up process was successfully implemented, a prerequisite is created to further scale up the process to semi-industrial and industrial levels in further research.

SUPPLEMENTARY MATERIAL

Supplementary Materials are available electronically from https://www.shd-pub.org.rs/index.php/JSCS/article/view/12350, or from the corresponding authors on request.

ИЗВОД

КУЛТИВАЦИЈА *BACILLUS SUBTILIS* NCIM 2063: МОДЕЛОВАЊЕ, ОПТИМИЗАЦИЈА И ПРОЦЕДУРА ПОВЕЋАЊА РАЗМЕРЕ

САНДРА СТАМЕНКОВИЋ СТОЈАНОВИЋ, ИВАНА КАРАБЕГОВИЋ, БОЈАНА ДАНИЛОВИЋ, СТОЈАН МАНЧИЋ И МИОДРАГ ЛАЗИЋ

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Bacillus subtilis је непатогена, грам-позитивна бактерија која спорулише и има изражену антимикробну и метаболичку активност, а самим тим и велики потенцијал за примену у различитим областима. Циљ овог рада био је одредити оптималне услове раста за *B. subtilis* NCIM 2063 и повећати размере процеса са ерленмајера на ниво биореактора. Критични параметри раста и ефекти њихове интеракције су изучавани применом Бокс-Бенкеновог експерименталног дизајна и методе одзивних површина. Развијене једначине модела биле су статистики значајне. Током култивације у ерленмајерима са мешањем, глукозу треба додати у концентрацији до 5 $g\ l^{-1}$ при концентрацији *OTR* од 10 $mol\ m^{-3}h^{-1}$ и на 33 °C, како би се постигао максималан број ћелија и спора. За повећање размере процеса са нивоа ерленмајера на ниво биореактора k_La је коришћен као главни критеријум. Ефекат повећања размере утврђен је поређењем кинетике раста у ерленмајерима и у биореактору. Укупан број ћелија добијен у биореактору био је 4,57·10^9 CFU ml^{-1} што је1,41 пута више у одосу на број ћелија добијен у ерленмајеру, 3,24·10^9 CFU ml^{-1}, доказујући да је процедура повећања размере успешно спроведена.

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