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Microencapsulated biofertilizer formulation: product development and effect on growth of green pepper seedlings

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Abstract

Aim of the study: This study aimed to formulate a novel, commercially applicable biofertilizer, to optimize the microencapsulation procedure of *Bacillus subtilis* NCIM 2063 and examine the stability and phytostimulatory effects of obtained formulation.

Area of the study: Southestern Serbia.

Material and methods: Microbial powder formulations were prepared using spray drying with maltodextrin as a carrier. The spray drying conditions were set according to Box-Benkhen experimental desing. The effect of the formulation was tested on green pepper (*Capsicum annuum*) seeds in controled conditions.

Main results: Response surface models were developed. All of the models were statistically significant, adequately fitted and reproducible. The maximum achieved values of viability and yield in a formulation were $1.99 \cdot 10^9$ CFU/g and 96.8%, respectively, whilst the driest formulation had 1.44% moisture. The following optimum conditions were proposed for the spray drying procedure: an inlet air temperature of 133 °C, maltodextrin concentration of 50 g/L and a feed flow rate of 6.5 mL/min. The obtained microbial formulation had a high survival rate after being stored at room temperature over a 1--year period. Its application on green pepper seeds had beneficial effect on plant height, leaf dry weight and chlorophyll content of the seedlings.

Research highlights: *B. subtilis* was successfully microencapsulated on maltodextrin as a carrier. Interaction effects between the process variables were fully explained and statistically significant models were developed. In addition to biocontrol properties formulation had a phytostimulatory effect, excellent stability and satisfactory physical properties.

Additional key words: plant growth promotion; *Bacillus subtilis*; phytostimulation; optimization

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Introduction

Modern agriculture relies on different agrochemicals that have adverse effects on the environment and human health. Given the fact that the current situation is not sustainable, an effective alternative to agrochemicals is needed urgently (Hussain *et al.*, 2017). Microbial fertilizers (biofertilizers) are natural and environmentally safe formulations that are supposed to replace agrochemicals in modern agriculture. These formulations have the potential to stimulate plant growth, assimilate minerals from the soil and increase plant resistance to pathogens. *Bacillus subtilis* has numerous properties that make it suitable for incorporation into microbial fertilizers with the aim to stimulate plant growth and control the diseases. Due to its non-pathogenicity, pronounced antimicrobial and metabolic activity along with the ability to form endospores, this microorganism has significant potential for wide application in various fields, from medicine and pharmacy to agriculture (Mousivand *et al.*, 2012; Chauhan *et al.*, 2015; Hashem *et al.*, 2019; Malićanin *et al.*, 2020). Important features such as growth on inexpensive carbon sources, low nutritional needs, suitability for genetic manipulation, robustness and resistance while performing large-scale fermentation, make this species very attractive for further analysis and optimization for its final application in biocontrol and plant growth stimulation (Hashem *et al.*, 2019).

Commercial application of microbiological fertilizers depends on the properties of the microbial formulation. A good formulation should be simple, functional and stable, exhibiting great efficiency for a long period of time. Experience from the practice often indicates poor product quality, which brings up the demand to improve biotechnological schemes for the production of such formulations (Vassilev *et al.*, 2015; Stamenković *et al.*, 2018; Stamenkovic-Stojanovic *et al.*, 2019). One of the methods to achieve good-quality microbial products is microencapsulation using spray drying.

Spray drying is a promising microencapsulation technology of microorganisms that increases the resistance and durability of the final product. Owing its popularity to the fact that it enables large production capacities and generous yields, this technique is widespread and applied in various industries (Nedović *et al.*, 2013). The final product has low water activity making it stable and long-lasting. It is also easily stored, transported and manipulated (Nedovic *et al.*, 2011; Huang *et al.*, 2017). Using spray drying technology, a liquid culture is converted into a granular powder by the atomization process. Suspension particles are directed into a steam of hot air (150-200 °C) at high speeds (Huang *et al.*, 2017). Atomized droplets are very small and abundant, thus occupying a very large contact area, which allows a short drying time. Thermal inactivation of the microbial culture is possible, affected mostly by inlet and outlet temperature, air and feed flow rate, drying time and nebulizer pressure (Peighambardoust *et al.*, 2011). Most cells survive if drying is performed at lower temperatures, which in turn results in high residual moisture content and poorer product quality. Hence, the appropriate selection of the feed flow rate and inlet air temperature is crucial for the process success. The optimal values of these variables can be found by trial and error method, or by applying some of the statistical optimization methods (Baş & Boyacı, 2007). The design of experiments combined with response surface methodology and Derringer's desirability function (Derringer & Suich, 1980) represents trending multiple criteria statistical methodologies which use a minimum number of experiments to obtain precise data and provide complete information about the studied phenomenon (Rodrigues *et al.*, 2019).

Microencapsulation of different *B. subtilis* isolates have been a subject of a few research groups. So far they examined the influence of different inert ingredients (Yánez-Mendizábal *et al.*, 2012; Meng *et al.*, 2015) on viability and stability of the formulation, with an emphasis on the biocontrol effect. The effect of spray drying conditions have not been investigated yet, neither the possible interaction effects between the factors, which can be very important due to the nature of the process. It remains unknown what spray-drying conditions are required to achieve commercially acceptable products that assert all of the demends of the customers. Is there a compromise solution that will allow high viability along with good powder yield and low moisture content, which in turn is of great importance for the commercialization process? Does the formulation have a phytostimulatory effect in addition to biocontrol properties?

In order to answer the asked questions and fill in the gaps in the previous research, this paper aimed to: (i) apply spray drying to develop novel, comercially competent microbial fertilizers; (ii) asses the individual and combined effects of important spray drying variables using Box-Benkhen experimental design and response surface methodology along with the in depth statistical analysis; (iii) provide statistically significant model equations that simulate the system behaviour; (iv) optimize the spray drying conditions using multiple criteria optimization that will consider both viability and yield, along with moisture content; and (v) examine the shelf-life of the developed product and its phytostimulatory effect on the green pepper seeds.

Material and methods

Microorganism

Bacillus subtilis strain NCIM 2063, obtained from the National Collection of Industrial Microorganisms (NCIM, Pune, India), was used for microencapsulation using spray drying. The bacterial strain was stored at -80 °C in a cryovial and at 4 °C on agar plates. A pre-inoculum was formed

from a frozen stock by transferring a single loop to nutrient agar medium (Torlak, Serbia), comprising 0.5% peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, and incubating at 37 °C. Erlenmeyer flask containing 300 mL of nutrient broth (Torlak, Serbia) comprising 0.5% peptone, 0.3% beef extract, 0.5% NaCl and 0.03% KH_2PO_4, was inoculated with a single colony and grown for 24 h in a rotary shaker at 37 °C and 150 rpm to form inoculum, 1% of which was then used for bioreactor inoculation.

Bioreactor cultivation

NCIM 2063 isolate was cultivated in a 2.5 L bioreactor (KLFM, BioEngineering, Wald, Switzerland) containing sterilized DSM medium: nutrient agar, 10% (w/v) KCl, 1.2% (w/v) $MgSO_4$ with addition of 1 mL of filter-sterilized solutions: 1 M $Ca(NO_3)_2$, 0.01 M $MnCl_2$ and 1 mM $FeSO_4$. The pH was adjusted by adding 0.01 M NaOH. The culture was grown for 24 h at constant conditions: 33 °C, agitation rate of 440 rpm, airflow rate of 0.3 vvm.

Microencapsulation by spray drying

After 48 h of cultivation, fermentation broth containing *B. subtilis* NCIM 2063 culture was exposed to a thermal shock for 1 h at 54 °C. Fermentated broth samples were mixed with different amounts of maltodextrin (according to the Box-Benkhen experimental design) and incubated for 10 min to form a homogenous suspension. The initial concentration of viable cells was confirmed by plating on nutrient agar. Each suspension was spray dried in a laboratory scale spray drier Büchi mini B-290 (Flavil, Switzerland). The airflow rate and atomization pressure had constant values of 600 L/h and 0.55 bar, respectively. The inlet temperature and the feed flow rate were set according to the Box-Benkhen experimental design.

Experimental design

The optimization of the spray drying procedure was based on the Box-Benkhen experimental design coupled with response surface technology and Deringer's desirability function (Derringer & Suich, 1980). Seventeen experiments were conducted in total, with 3 factors at 3 levels. Three independent process variables: maltodextrin concentration (10-50 g/L), inlet air temperature (110-140 °C), and feed flow rate (6-10 mL/min), were determined based on a literature search and preliminary experiments. Dependent variables: moisture content (%), product yield (%), and number of viable cells (CFU/g), were determined in triplicate; mean values were used for the regression analyses using Design Expert software package (Trial version 7.0.0, STAT-EASE Inc., Minneapolis, MN, USA). Experimental data were fitted to the model proposed by the software. Obtained model equation was used to calculate the values predicted by the model. Model adequacy, statistical significance and deviation from the experimental results were evaluated by ANOVA. Optimization of spray drying parameters was performed using Derringer's desirability function, which is used in complex systems requiring simultaneous optimization of several factors. Based on a optimization criteria it transforms all the system responses on a scale from 0 to 1. By combining individual functions of desirable responses, the total function of preferred responses (D) is calculated with a maximum value ranging from 0 (which is an unwanted answer) to 1 (desired response), and then combined into a mutual response that should have a maximum value under previously defined optimization criteria. As the value of D is approaching 1, the system is being closer to the global optimum value (Derringer & Suich, 1980).

Viability and shelf life

The number of viable microencapsulated cells was determined immediately after spray drying, after six months and one year of storage at room temperature, respectively. Powder formulation (0.1 g) was rehydrated in 0.9% saline solution (NaCl, distilled water), shaken vigorously, and allowed to rehydrate for 12 h. Viable cell concentration was determined using the spread plate method.

Moisture content determination

To determine the moisture content, 2 g of the powder formulation obtained after spray drying was transferred to an aluminum dish and dried at 105 °C to a constant weight. The residual moisture content was calculated according to the following equation:

$$moisture\,(\%) = 100\ \frac{w_f - w_i}{w_i} \qquad (1)$$

where w_f and w_i are weighed mass of the formulation prior to and after drying at 105 ºC, respectively.

Product yield

The yield of the powder formulation obtained after spray drying was calculated using the following equation:

$$Y\,(\%) = (W_m\ 100)/W_p \qquad (2)$$

where W_m is the weight of the recovered powder and W_p is the dry weight of the initial suspension in addition to maltodextrin.

Encapsulation efficacy

Encapsulation efficacy was calculated using the following equation:

$$EE = 100 \frac{N_r}{N_f} \quad (3)$$

where N_r is log cfu/mL before spray drying and N_f is log cfu/mL after spray drying.

Characterization and morphology of the formulation

Powder formulation obtained under optimum conditions was analyzed for hygroscopicity, solubility dissolution time and morphology of the microparticles. Hygroscopicity and solubility were determined according to the method described earlier (Bakar *et al.*, 2013), while the dissolution time was calculated as the time required for total dissolution of 1 g of powder in 50 mL distilled water using a magnetic stirrer at 829 rpm (Bhagwat *et al.*, 2020). The morphology of the formulation obtained at optimum conditions was investigated using a JEOL JSM-6610LV scanning electron microscope (30 kV accelerating voltage) with an energy-dispersive X-ray spectrometer (SEM/EDS; XMax Large Area Analytical Silicon Drift connected with INCAEnergy 350 Microanalysis System). Dried powder was coated with a thin gold film at 20 kV voltage to obtain a higher quality secondary electron image for further SEM examination.

Phytostimulatory effect of microencapsulated cells – Pot experiments

Microencapsulated *B. subtilis* NCIM 2063 formulation was evaluated for promoting growth of green pepper (*Capsicum annuum*). The experiments were conducted in growth chamber in Leskovac (Serbia) during March-April, 2019. Pepper seeds were disinfected by immersion in a mixture (1:1) of 30% hydrogen peroxide and 70% ethanol for 10 minutes, subsequently washed by immersion in distilled water several times. Seed inoculation was performed by immersion in a resuspended culture of *B. subtilis* NCIM 2063 with a cell density of 9.1 LOG (CFU/g), after which sowing was performed in previously sterilized plastic pots containing 200 g of dry sterilized fertile chernozem soil. Treatment was performed in 5 replications, 10 pots per replication. The seeds immersed in sterilized water served as a control. The plants were grown for 8 weeks in an incubation chamber at a constant temperature (22 °C), average relative humidity 50-60%, exposed to light of 140 µmol/m^2·s wiht a 16 h photoperiod. Vegetative tissue was analyzed for: stem height, root length, number of leaves, leaves and root dry weight and leaf chlorophyll content. Dry weights were determined after drying plant tissues at 65 °C for 24 h, and then at 110 °C until constant weight (Garcia *et al.*, 2011).

The leaf chlorophyll content was determined using the Hiscox & Israelstam´s (1979) method. Briefly, 100 mg of leaves were immersed in 7 mL of DMSO and incubated at 65 °C for 30 min. After adding DMSO to a total volume of 10 mL, 1 mL was transferred to a cuvette and the absorbance was measured at 645 and 663 nm (DMSO blank) using a UV/VIS spectrophotometer (UV/Vis Spectrophotometer - Pye Unicam Ltd, Cambridge England). The chlorophyll content was calculated using Arnon's equations:

$$\text{Chla (g/L)} = 0.0127 \times A663 - 0.00269 \times A645; \quad (4)$$
$$\text{Chlb (g/L)} = 0.0229 \times A645 - 0.00468 \times A663; \quad (5)$$
$$\text{Tot Chl (g/L)} = 0.0202 \times A645 + 0.00802 \times A663. \quad (6)$$

Statistical analyses

All experiments were performed in three parallel replications, and the results were presented as mean value of three repetitions ± standard deviation. The programs Origin 6.0, Excel 2013 and Expert Design 7.0 were used for statistical processing, modeling and graphical analysis of experimental data. Multicriteria optimization was performed by applying the Box-Benkhen experimental design, Response surface methodology and Deringer's desirability function. The adequacy of the response surface model was assessed using the analysis of variance (ANOVA).

Results

The influence of three process factors: maltodextrin concentrations (*A*, 10, 30 and 50 g/L), inlet air temperature (*B*, 110, 125 and 140 °C), and feed flow rate (*C*, 6; 8 and 10 mL/min) on spray drying of *B. subtilis* NCIM 2063 was assessed using Box-Benkhen experimental design. The full matrix of the experimental design with the experimental and predicted values of the response variables is shown in Table 1, while the results of ANOVA are represented in Table 2.

Based on multiple regression of the experimental results an empirical relationship was developed for all tested dependent and independent variables in the form of second-order polynomial model equations. Non-significant model terms were eliminated from the model. ANOVA results revealed that it is possible to plot the response surface for the experimental design (Table 2). All of the models were statistically significant, indicated by the high F values of the model, small p values (p<0.05) and insignificant lack of fit. The values of coefficient of determination (R^2) and adjusted R^2 (Adj R^2) were close to 1 for all models, denoting that experimental data are adequately fitted and deviating minimally from the predicted values.

Table 1. Box-Benkhen experimental design and obtained responses during the spray drying of *B. subtilis* NCIM 2063

Run	Uncoded factors[1]			Responses					
	A	B	C	Viability, LOG (CFU/g)		Yield,%		Moisture content,%		Encapsulation efficacy,%
				EXP[2]	PRED[3]	EXP	PRED	EXP	PRED	
1	50	140	8	8.83±0.06	8.83	93.51±1.13	94.35	2.43±0.13	2.40	89.61
2	50	125	6	9.19±0.13	8.97	96.80±2.26	97.20	3.20±0.1	3.01	93.26
3	30	125	8	8.70±0.1	8.68	75.83±0.99	77.62	3.51±0.18	3.46	88.29
4	30	125	8	8.55±0.08	8.68	81.38±0.85	77.62	3.60±0.04	3.46	86.77
5	30	125	8	8.79±0.07	8.68	80.65±0.57	77.62	3.01±0.3	3.46	89.20
6	10	140	8	7.90±0.00	7.94	80.35±0.99	80.25	2.40±0.19	2.18	80.17
7	30	110	6	8.56±0.07	8.67	86.09±1.27	85.59	5.30±1.13	5.12	86.83
8	30	125	8	8.69±0.05	8.68	77.70±1.98	77.62	3.51±0.14	3.46	88.15
9	50	110	8	9.30±0.00	9.41	68.36±0.99	68.46	5.88±0.12	6.48	94.38
10	10	125	6	8.04±0.14	8.08	89.11±0.55	90.46	4.13±0.14	4.33	81.59
11	30	140	6	8.30±0.07	8.38	95.16±1.27	93.92	1.44±0.07	0.58	84.23
12	50	125	10	8.93±0.07	8.96	86.15±0.85	84.80	8.90±0.64	9.86	90.62
13	10	110	8	8.65±0.12	8.52	47.33±0.21	46.48	6.30±0.16	6.37	87.82
14	10	125	10	8.09±0.11	8.07	55.87±1.27	55.47	5.19±0.07	5.52	82.10
15	30	110	10	8.94±0.13	8.95	39.15±0.10	40.39	9.10±1.29	8.46	90.72
16	30	125	8	8.69±0.06	8.68	72.53±0.28	77.62	3.71±1.03	3.46	88.24
17	30	140	10	8.10±0.11	8.08	91.23±2.26	91.73	3.42±0.14	3.54	82.20

[1] A: maltodextrin concentration. g/L. B: inlet temperature (°C). C: feed flow rate. mL/min. [2] EXP: experimental values. [3] PRED: values predicted by the model

Effect of spray drying on viability and encapsulation efficacy

Various factors showed a significant effect on product viability, and thus the efficacy of encapsulation. The experimental data log values were subjected to a nonlinear regression analysis and fitted to a second-order polynomial model function that predicts the viability of cells in spray dried powder:

$$Viability = 3.01 + 0.02A + 0.02B + 1.25C - 4.86 \cdot 10^{-3}BC - 0.04C^2 \quad (7)$$

The results of *B. subtilis* viability affected by spray drying inlet temperature, feed flow rate and maltodextrin concentration are visually represented in Fig. 1 in the form of three-dimensional (3D) response surfaces. It can be seen that an increase in concentration of maltodextrin had a positive effect on the viability of microorganisms in the final product, regardless of inlet temperature. Although in most cases an increase in temperature reduced the cell viability, Fig. 1a clearly shows that increasing the feed flow rate can reduce the negative impact of temperature. Encapsulation efficacy undergoes the same dependancy pattern as viability. A maximum number of viable cells within the tested range ($1.99 \cdot 10^9$ CFU/g) and a maximum encapsulation efficacy (94.38%) were achieved at the lowest temperature (110° C), medium flow rate (8 mL/min) and the maximum concentration of maltodextrin (50 g/L).

Effect of spray drying on the product yield

The yield of dry powder after spray drying varied from 39.2 to 96.8%. The influence of selected variables on product yield was determined by the response surface method. Regression analysis of experimental data presents the response equation:

$$Yield = 109.53 + 0.23 \cdot A + 3.5 \cdot B - 74.73 \cdot C + 6.55 \cdot 10^{-3} AB - 0.14 \cdot AC - 0.36 \cdot BC + 1.47 \cdot 10^{-3} \cdot A^2 - 0.02 \cdot B^2 + 1.23 \cdot C^2 \quad (8)$$

Based on response surface analysis (Fig. 2), it can be concluded that the maximum yield was achieved by a simultaneous increase of maltodextrin concentration and temperature. The influence of maltodextrin concentration

Table 2. ANOVA for the model equations obtained for viability. yield and moisture content in the spray-dried formulation of *B. subtilis* NCIM 2063

Source of variation	Viability		Yield		Moisture	
	***F* value**	***p* value**	***F* value**	***p* value**	***F* value**	***p* value**
Model	40.8	< 0.0001	54.99	<0.0001	40.85	<0.0001
A	132.10	< 0.0001	51.50	0.0002	0.1	0.76
B	55.84	< 0.0001	92.86	<0.0001	35.02	0.0006
C	3.1	0.106	174.44	<0.0001	22.57	0.0010
AB	-	-	1.76	0.2260	34.26	0.0006
AC	-	0.0223	14.54	0.0066	0.17	0.6909
BC	7.06	0.0120	52.69	0.0002	2.77	0.1401
A^2	-	-	0.16	0.7005	12.67	0.0092
B^2	-	-	10.39	0.0146	1.94	0.2059
C^2	9.01	-	11.72	0.0111	16.47	0.0048
Lack of fit	1.99	0.2643	0.23	0.8724	2.06	0.2477

Factor	R^2	*Adj* R^2	*Pred* R^2	CV%	MRPD%
Viability	0.9488	0.9256	0.8527	1.28	0.6
Yield	0.9861	0.9681	0.9488	3.82	1.79
Moisture	0.9813	0.9573	0.8069	7.08	4.63

CV%: coefficient of variation. MRPD: mean relative percentage deviation.

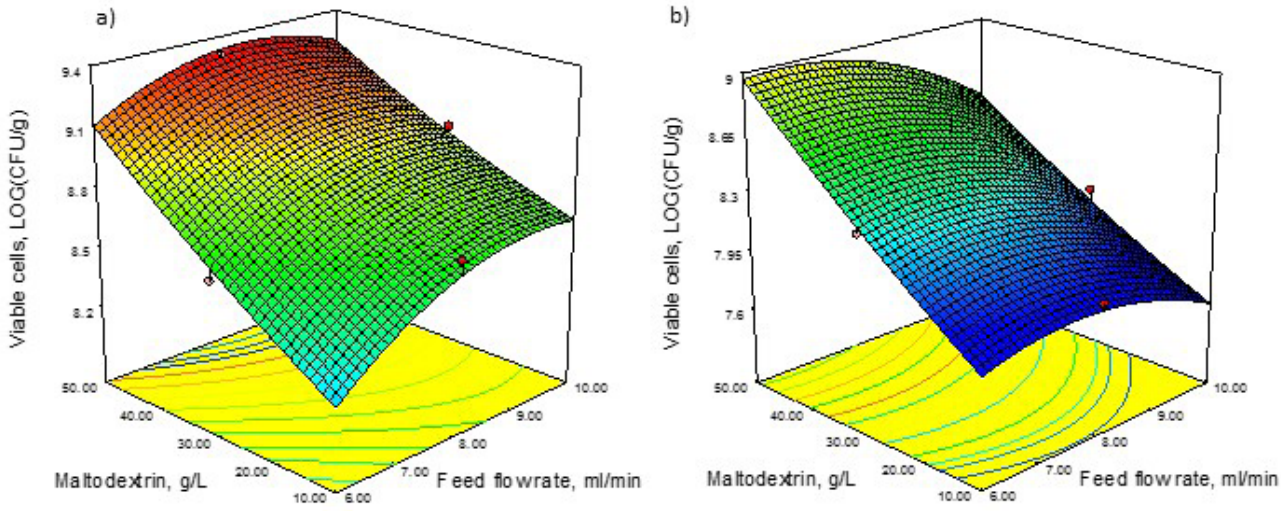

Figure 1. Response surface plots for *B. subtilis* NCIM 2063 viability after spray drying at different temperatures: a) 110 °C; b) 140 °C.

was most pronounced at the highest tested temperatures, while at lower temperatures, a decrease in feed flow rate weakened the influence of maltodextrin. Increasing the feed flow rate generally reduced the yield of the product, but as the temperature increased, the influence of the flow rate on the yield decreased.

Effect of spray drying on moisture content

Quality formulation should contain less than 5% moisture after spray drying (Peighambardoust *et al.*, 2011), although some authors indicate that up to 12% moisture is also acceptable for bacterial formulation. Low moisture

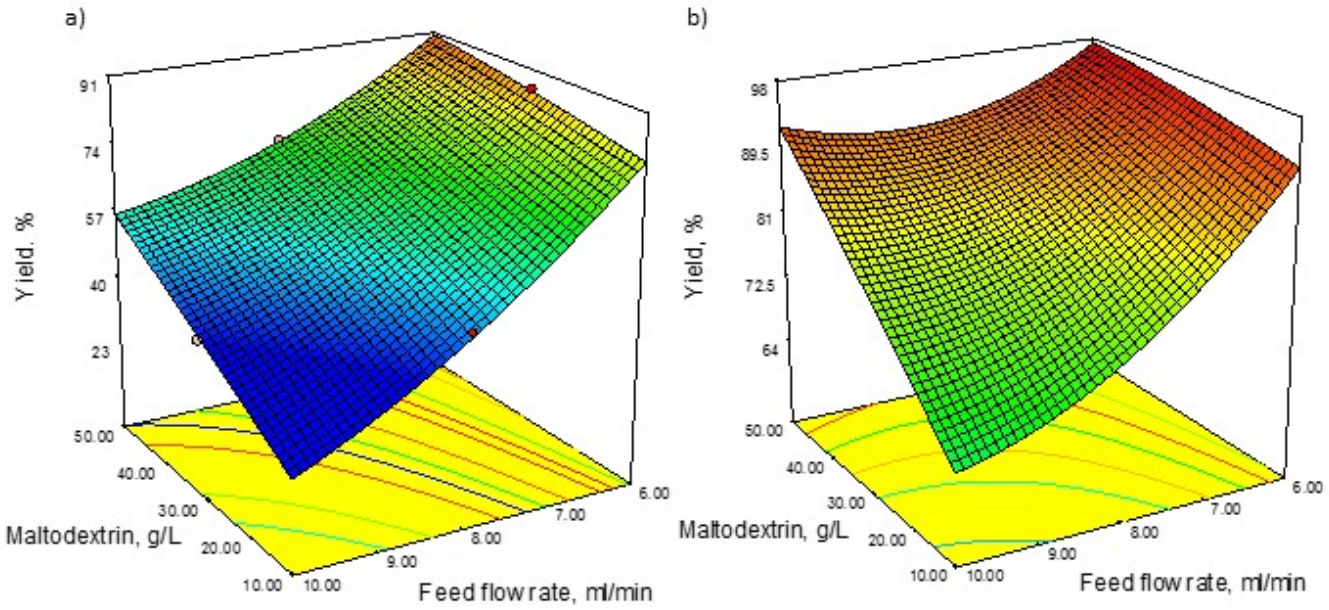

Figure 2. Response surface plots for product yield after spray drying at different temperatures: a) 110 °C; b) 140 °C.

content prevents contamination of the product and provides longer shelf life (Keswani *et al.*, 2016). Hence, the combination of factors that reduce the moisture content was considered most desirable. The following model equation was proposed:

$$lnY = 7.19 - 0.072\,A + 0.16\,B - 1.09\,C + 6.75 \cdot 10^{-5}\,AB + 4.96 \cdot 10^{-3}\,AC + 2.71 \cdot 10^{-3}\,BC + 4.23\,10^{-4}\,A^2 - 2.95 \cdot 10^{-4}\,B^2 + 0.05\,C^2 \quad (9)$$

where Y is moisture content (%).

An increase in temperature and decrease in feed flow rate decreased the moisture content (Fig 3). However, the influence of maltodextrin concentration is insignificant. Although maltodextrin concentration dose did not affect moisture content significantly, the influence of maltodextrin concentration became more pronounced at a lower feed flow rate in the examined range. Hence, when maltodextrin concentration was increased along with the low feed flow rate, the moisture content gradually decreased. Still, at higher feed flow rates, maltodextrin did not play a crucial role for this system response.

Multiple criteria optimization of spray drying procedure

Spray drying variables were optimized using Derringer's desirability function in order to obtain a microbial formulation of maximum viability and yield with a minimum residual moisture.

According to desirability criteria (Table 3) the following conditions were proposed for spray drying of *B. subtilis* NCIM 2063 with D=0.87: temperature 133 °C, maltodextrin concentration 50 g/L and feed flow rate 6.5 mL/min.

The optimized solution (Fig. 4) proposed in this study was validated in laboratory conditions (Table 4), which confirmed the minimum deviation of experimental and software predicted values, as well as the high survival rate of encapsulated bacteria after being stored at room temperature for six months and one year.

Physical characterization and morphology of the obtained particles

The morphology of microcapsules (Fig. 5) can be described as amorphous glassy structure, varying from flat and deflated to round spherical particles with porous surface. The diameter of spherical particles obtained in this study ranged from 5 to 300 μm. The dimensions of the particles are not of crucial importance as they can be modifed subsequently, by granulation adjustment methods. The outer surfaces of the microcapsules were characterized by the presence of indentation, with a few cracks on the surface. On the other hand, variables such as hygroscopicity, dissolution time and solubility (Table 4) showed that the powder has enhanced stability and a potential to be stored long-term, which was also proven by high number of surviving cells after one-year period.

Phytostimulatory effect of obtained formulation on green pepper

The effect of pepper seeds treatment with a spray-dried microbial formulation of *B. subtilis* NCIM 2063 on growth variables is shown in Table 5. The application of formula-

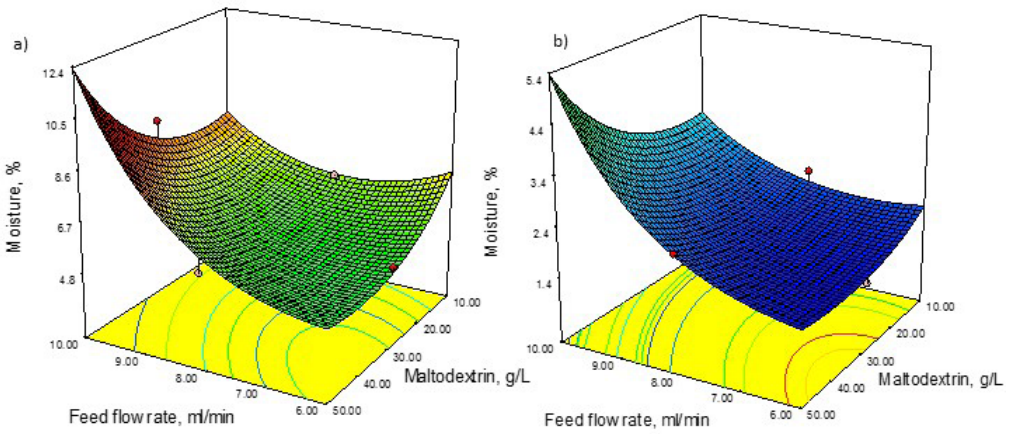

Figure 3. Response surface plots for product moisture content after spray drying at different temperatures a) 110 °C; b) 140 °C

tion resulted in a significant increase in the leaf dry weight and plant height, while root weight and leaf number did not differ significantly from the control. Particularly, plants treated with the formulation were about 40% higher and had almost 3 times larger weight of the leaves, than the control.

It is known that chlorophyll plays a key role in the process of photosynthesis and that its content is an indicator of the capacity of photosynthesis and plant health. Table 5 shows a significant positive effect of *B. subtilis* NCIM 2069 isolate on chlorophyll content in treated pepper seedlings. Compared to the control, the application of the formulation significantly increased the content of chlorophyll *a* (by 76.77%) and chlorophyll *b* (by 52.5%). This in turn contributed to an increase in the total chlorophyll content in peppers by 57.5% compared to the control.

Discussion

Statistical analyses and model fitting

Quadriatic model equation was found to be adequate and statistically significant for all three of the tested variables. Apart from *F*, *p* and lack of fit values, goodness of fit was also confirmed by an adequate mean relative percent deviation (MRPD) value. More than 94% of variation can be explained by the models and their adequacy was confirmed by the compliance of the predicted R^2 (Pred R^2) and Adj R^2, which differ in less than 0.2. Adequate values of the coefficient of variation (CV) confirmed that the models are reproducible.

The linear effect of inlet temperature was found to be significant for all response variables at 99% confidence interval. This factor also demonstrated a strong interaction effect with the feed flow rate affecting viability and yield, along with maltodextrin concentration affecting moisture content. Among other interaction effects, the interaction of maltodextrin concentration with feed flow rate was found to be significant at a 95% confidence level for moisture content. Maltodextrin concentration also exhibited a positive linear effect on viability and yield, while the linear effect of the feed flow rate was found to be significant for the yield and the moisture. The positive linear effect of a dependent variable reveals that increase in its value also causes increase in the response. Although feed flow rate did not show statistical significance for viability as an individual linear term, squared and interacting with temperature, this factor nev-

Table 3. Multicriteria optimization goals and ranges

Variables	Low level	High level	Significance	Criteria
Temperature, °C	110	140	3	In range
Maltodextrin concentration, g/L	10	50	3	In range
Feed flow rate, mL/min	6	10	3	In range
Number of viable cells, CFU/g	7.9	9.3	5	Maximum
Yield, %	39.15	96.8	3	Maximum
Moisture, %	1.43	9.03	3	Minimum

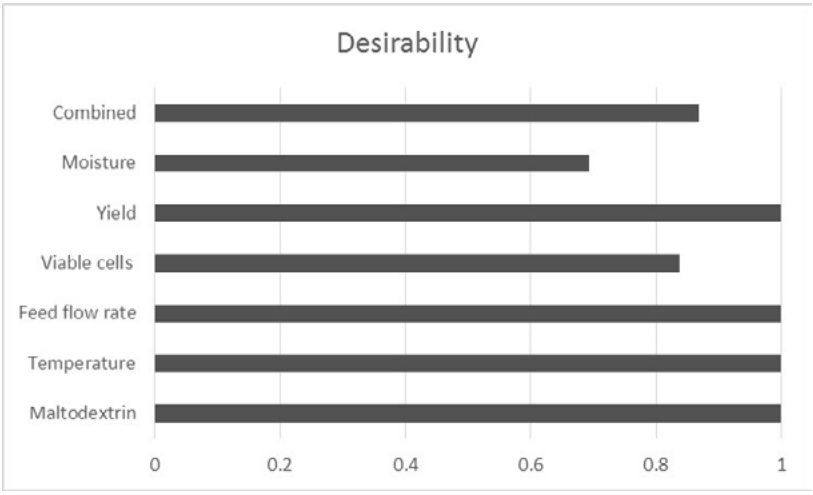

Figure 4. Optimization using Deringer's desirability function: desirability bar graph.

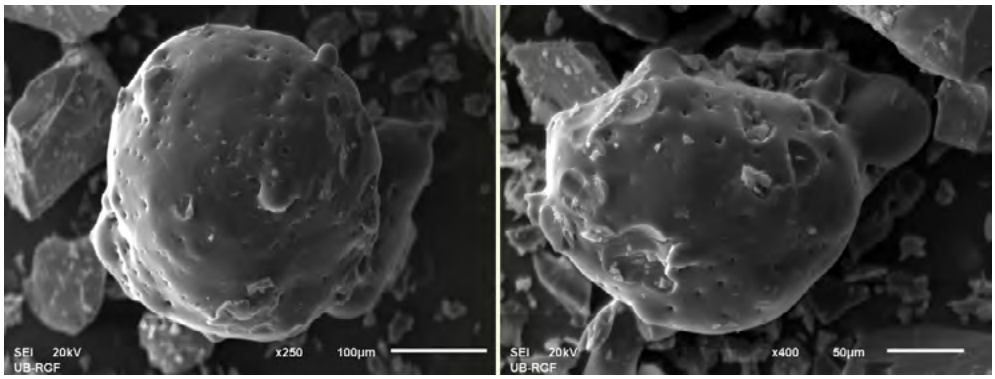

Figure 5. SEM microphotogrpaphs of microcapsules obtained at optimum spray drying conditions.

ertheless shows statistical significance at a 95% confidence level, which denotes that the optimum level is within the experimental region, and not at the extremes (Table 2) (Baş & Boyacı, 2007). Similarly, in the case of spray dried *Bacillus thuringiensis*, Adjallé *et al.* (2011) found that linear terms of temperature and feed flow rate had a statistically significant positive effect on the viability, their quadratic terms had a negative effect, while interaction terms were not statistically significant. Wang *et al.* 2018) also found that inlet air temperature and feed flow rate had the greatest effect on the survival of spray-dried *Sphingomonas* sp. in the formulation. The same factors, particularly their linear, quadratic and interaction effects were also found statistically significant by Seth *et al.* (2017) for the moisture content in the spray drying process of *Streptococcus thermophilus* and *Lactobacillus delbrueckii bulgaricus*.

Effect of spray drying conditions on product quality

Processes of mass multiplication and formulating procedures are the most important steps for the effectiveness of the obtained formulation. Therefore, to obtain a commercially competent, stable and effective product, the influence of crucial drying varibles on product properties should be carefully analyzed and explained (Vassilev *et al.*, 2015). Product yield, the number of viable cells per gram of the product and mositure content are the three variables that denote the quality of the product: its potential to colonize the plant's root, durability and economical profitability. Hence, spray drying should be conducted with a combination of variables that will enable high yield powder formulation with good cell viability and minimum moisture content.

Although in most cases an increase in temperature affects the reduction of cell viability, the research has shown that increasing the feed flow rate can reduce the negative impact of temperature. Namely, increasing the feed flow rate leads to a decrease in the temperature on the surface of the droplets, which causes changes in heat transfer thus reducing the physical damage to the bacteria cell membranes (Behboudi-Jobbehdar *et al.*, 2013). In the present work, we noticed that the feed flow rate influence was more pronounced at lower and medium temperatures within the examined range. Nevertheless, feed flow rate

Table 4. Validation of optimum spray drying factor values in laboratory conditions and characterization of obtained formulation

Variable	Experimental value	Predicted value
Viability after spray drying, LOG (CFU/g)	9.18 ± 0.01	9.07
Viability after six months, LOG (CFU/g)	8.93 ± 0.03	-
Viability after one year, LOG (CFU/g)	8.45 ± 0.21	-
Yield, %	92.6 ± 1.98	96.8
Moisture content, %	2.90 ± 0.28	2.53
Hygroscopicity (g H_20/g powder)	0.21 ± 0.02	-
Solubility, %	0.311 ± 0.32	-
Dissolution time, s	79.00 ± 5.00	-

values in the range 6-8 mL/min had practically no impact on the survival of microorganisms in the formulation when applied in combination with higher temperatures. Maxiumum number of viable cells achived in this study was $1.99 \cdot 10^9$ CFU/g, which is consistent with previous results of spray drying *B. subtilis* CPA-8 when the viability of $3.3 \cdot 10^9$ CFU/g was achieved at feed flow rate of 8 mL/min with the addition of $MgSO_4$ (Yánez-Mendizábal *et al.*, 2012). The difference in the number of surviving cells can be attributed to the difference in carriers and the cultivation conditions applied before spray drying (Peighambardoust *et al.*, 2011).

The results of this study indicate that the maximum yield was achieved by a simultaneous increase of maltodextrin concentration and temperature. Such effect is explained by a fact that a higher concentration of the carrier in the feed increases the viscosity, thus reducing the radial velocity of the particles. This in turn reduces the possibility of collision with the walls of the drying chamber and increases the number of particles reaching the collecting vessel which directly increseas the yield (Schuck, 2009). Decrease in feed flow rate also increases the yield of the product. The same effect was noticed for the moisture content of the formulation. High temperatures combined with low feed flow rate guarantee a low moisture content. An increase in inlet air temperature creates a larger temperature gradient between atomized particles and hot air, which enables a stronger driving force to remove moisture. That in turn reduces the likelihood of agglomeration of microparticles and the deposit formation on the walls of the drying chamber, which directly affects the increase in yield (Amiet-Charpentier *et al.*, 1998; Adhikari *et al.*, 2005). On the other hand, an increase in feed flow rate affects the formation of larger droplets, which makes evaporation more difficult, resulting in higher residual moisture and lower yield (Seth *et al.*, 2017). This conclusion is consistent with previous spray drying results of *Lactobacillus acidophilus* (Behboudi-Jobbehdar *et al.*, 2013) and *Pseudomonas putida* (Amiet-Charpentier *et al.*, 1998) which state that the yield was increased significantly after a simultaneous increase in air inlet temperature and feed flow rate. Seth *et al.* (2017) also concluded that in the spray drying process of *Streptococcus thermophilus* and *Lactobacillus delbrueckii bulgaricus*, temperature and flow rate had the greatest influence on the moisture content, whose linear, quadratic and interaction effects were statistically significant. Other authors examined the influence of different carriers (skimmed milk and $MgSO_4$) on the moisture content of the *B. subtilis* CPA 8 formulation obtained by spray drying. The lowest moisture content (6%) was achieved at 150 °C and 8 mL/min by applying 10% skimmed milk powder and 10% $MgSO_4$ (Yánez-Mendizábal *et al.*, 2012). Given that the lowest moisture content achieved in the present work was 1.4%, it is concluded that maltodextrin in combination with the high inlet air temperature achieves a significantly better effect.

Multicriteria optimization of spray drying procedure

B. subtilis NCNIM 2063 powder formulation had the best viability when spray dried on low temperatures and high feed-flow rates in the tested intervals. Contrary to that, high temperature and low feed flow rate valus are needed to obtain the highest yield and the lowest moisture content of the same formulation. To obtain products in satisfactory yield, viability and moisture content at the same time, multicriteria optimization was performed. The results of multicriteria optimization revealed that the ideal spray drying temperature value is 133 °C, while maltodextrin concentration and feed flow rate should be set to 50 g/L and 6.5 mL/min, respectively. A much higher temperature (180 °C) was used by Adjallé *et al.* (2011) for microencapsulation of *B. thuringiensis* by spray drying at a feed flow rate of 0.29 g/min, which resulted in $2.2 \cdot 10^8$ viable cells in the dry formulation. Behboudi-Jobbehdar *et al.* (2013) achieved the maximum desirability of Deringer's function

Table 5. Phytostimulatory effect of *B. subtilis* NCIM 2063 formulation when applied onto green pepper seeds

	B. subtilis **NCIM 2603**	**Control**
Plant height, cm	16.66±2.88a	11.97±2.41b
Number of leaves	3.66±0.58a	3.33±0.57a
Leaf dry weight, mg	100.47±20.7a	38.33±12.58b
Root dry weight, mg	4.86±0.3a	4.1±0.60a
Chlorophyll *a*, g/L	10.2±1.85a	5.77±0.46b
Chlorophyll *b*, g/L	3.66±0.57a	2.9±0.65b
Total chlorophyll, g/L	13.66±3.05a	8.67±1.53b

Values (mean ± SD) with different letter in the same row denote significant differences at $p < 0.05$ (Tukey's range test)

for spray drying of *L. acidophilus* at 133.54 °C at a feed flow rate of 7.14 mL/min, using maltodextrin in combination with glucose and whey protein as a carrier; under these optimal conditions, the viability of 8.59 CFU/g and a water activity of 0.171 were achieved. The differences in optimized values of spray drying are mainly attributed to differences in the used strains and their properties, which is why it is necessary to perform optimization for each microbial species separately. As far as *Bacillus* is concerned, one-factor optimization was performed by Ma *et al.* (2015) for spray drying of *B. subtilis* with maltodextrin and gum arabic; under the proposed optimal conditions (temperature 140 ºC and feed flow rate 9.1 mL/min), 4.83-5.35·10^8 CFU/g was reached. Barcelos *et al.* (2014) dealt with spray drying of biosurfactants obtained from *B. subtilis* LBBMA RI4914, used as a carrier maltodextrin at a concentration of 250 g/L. Our study showed that it is possible to use lower inlet air temperatures in combination with a lower feed flow rate to achieve better viability of the bacterial culture. Significant savings can also be achieved from the aspect of consumed material, considering that significantly lower concentrations of maltodextrin are sufficient for the achievement of satisfactory results. Morphology of the particles is also in accordance with previous literature data. While the presence of indentation is usual for polysaccharide carries, cracks can be attributed to a long storage time (Campos *et al.*, 2014). Maltodextrin as a carrier can particularly influence the morphology of the particles (Behboudi-Jobbehdar *et al.*, 2013).

Phytostimulatory effect of the formulation

Microencapsulated *B. subtilis* NCIM 2063 proved the ability to adhere to the pepper seed surface and positively influence the growth of the seedlings in the early period. The positive effect of the isolate is a result of its ability to produce growth hormones and siderophores, affect nitrogen fixation, and phosphorus solubilization (Ben Khedher *et al.*, 2021). Similarly, *B. subtilis* SL-13 isolate increased pepper leaf area (Tao *et al.*, 2019), *B. subtilis* CAS 15 contributed to an increase in yield, height and weight of pepper (Yu *et al.*, 2011), while *B. subtilis* V26 promoted the growth of potato seedlings (Ben Khedher *et al.*, 2021). Yu *et al.* (2011) explained that microbial siderophores, which are produced by this microbe, stimulte plant growth as they increase the availability of the iron in the soil. Additionaly, *B. subtilis* produces indole acetic acid, which stimulates plant cell division and allows better adsorption of minerals and water, which in turn results in better growth parameters (Swain *et al.*, 2007).

The increase in leaf dry weight observed in this study, is in a correlation with the increased content of chlorophyll *a* and *b* and the total chlorophyll in the leaves. The contents of chlorophyll *a* and *b* achieved in this study was almost 3.5 times higher than the one achieved by the isolate *B. subtilis* SL-13 (Tao *et al.*, 2019). Multiple increases in chlorophyll content is explained by the ability of this isolate to modulate endogenous signaling molecules for glucose and abscisic acid, which have a regulatory role in the process of photosynthesis (Zhang *et al.*, 2008). Very often, in addition to chlorophyll, an increase in content of other pigments, such as α-tocopherol and β-carotene can be observed (Sonbarse *et al.*, 2020). A mechanistic study confirmed that applying *B. subtilis* to pepper seeds improves the efficiency of photosystem II and positively affects photosynthesis by increasing the amount of chlorophyll and the rate of carbon dioxide assimilation. Such an effect is explained by the increase in the electron transfer rate in the thylakoid membrane due to the action of the bacterium (Samaniego-Gámez *et al.*, 2016).

In conclusion, this research proved that *B. subtilis* NCIM 2063 can be succesffuly microencapsulated with maltodextrin using spray drying procedure at following conditions: temperature 133 °C, maltodextrin concentration 50 g/L and feed flow rate 6.5 mL/min. Additionaly, the influence of spray drying process variables on the most important formulation characteristics is determined

and explained, which greatly facilitates the selection of drying conditions with other plant growth-promoting microorgansms and encourages future research on this subject. Obtained formulation was stable over a 1-yer period and had several positive plant growth-promoting traits, facilitating the growth of green pepper seedlings in controled conditions. The next step of the research shall be the confirmation of the phytostimulatory effect in field conditions, after which commercialization of the product can be started.

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