Bacillus based microbial formulations: Optimization of the production process

Sandra Stamenković Stojanović[1], Ivana Karabegović[1], Vladimir Beškoski[2], Nada Nikolić[1] and Miodrag Lazić[1]

[1]*University of Niš, Faculty of Technology, Bulevar Oslobođenja 124, 16000 Leskovac, Serbia*
[2]*University of Belgrade, Faculty of Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia*

Abstract

Bacillus sp.-based microbial formulations have found wide application in many fields: from pharmacy and medicine to environmental protection and agriculture due to the ability of this species to produce various metabolites and to form endospores. Recently, these products have gained popularity as biopesticidal and phytostimulatory agents, which are a „green" alternative to overused agrochemicals. In order to obtain a high-quality and long-lasting product with desired characteristics, it is necessary to optimize the production process at each stage, which implies coordinating the microbial species, the type and the conditions of microbial cultivation along with formulation technologies. This paper provides a concise overview of the most important findings in this area, regarding characteristics of microbial formulations and specific criteria that need to be met when such a product is formulated. It should serve as a beginning point for everyone starting new research, not just in the field of biofertilization and biological control of plant diseases, but generally in the field of biochemical engineering.

Keywords: microbial formulations; optimization; fermentation; Bacillus sp.

Dostupno na Internetu sa adrese časopisa: http://www.ache.org.rs/HI/

REVIEW PAPER

UDK: 579.64:663.1:615.281:66.011

Hem. Ind. **73 (3)** 169-182 (2019)

1. INTRODUCTION

Microbial formulations based on the *Bacillus* species have wide application in different fields, ranging from medicine and pharmacy to agriculture [1–4]. In recent years, thanks to phytostimulatory and biopesticidal effects of this species, the formulations have become extremely popular in the field of microbiological fertilization and biological control of plant diseases, as an alternative to conventional agrochemicals that pollute the environment [5–8]. Genus *Bacillus* is considered as a microbiological factory for production of an enormous set of antimicrobial substances. Thanks to this feature and the possibility of spore formation, it is contained in about 85% of commercially available biological control agents [9,10].

Although this type of formulations can be produced using simple fermentation and formulation process, commercial application of such processes is limited by the lack of adequate methods for large scale production as well as appropriate technologies that will solve the problem of the short product shelf life [11–13]. Achieving adequate sporulation efficiency is a possible answer to this problem, which is why it is necessary to maintain high spore density. Thus, the whole production process needs to be optimized in an adequate manner that will provide an efficient and good-quality product [6,7,14–16].

In this paper, we discuss *Bacillus*-based formulations: properties and mechanisms of action with a special emphasis on fermentation technology and optimization of formulation production. It should serve as basic knowledge for anyone starting research not only in the field of microbial fertilizers but on every other process that involves fermentation using bioreactors.

2. *BACILLUS* SP.PROPERTIES

In general, *Bacillus* species are considered to be plant growth promoting microorganisms (PGPMs) that have a positive effect on plants. They increase the nitrogen and phosphorus intake (in the processes of nitrogen fixation and

Corresponding author: Sandra Stamenković Stojanović, University of Niš, Faculty of Technology, Bulevar Oslobođenja 124, 16000 Leskovac
E-mail: sandrastamenkovic@tf.ni.ac.rs
Paper received: 14 February 2019
Paper accepted: 23 June 2019
https://doi.org/10.2298/HEMIND190214014S

phosphate solubilization) and release a wide range of metabolites (plant hormones, siderophores, cyanides, antibiotics) that stimulate the plant and protect it from the adverse effects of the environment and pathogens [17–21]. Their antagonistic activity belongs to the ability to induce the systematic resistance in plants and to compete for an ecological niche with pathogenic bacteria [22]. Bacteria from this group also produce a variety of enzymes that are degrading pathogen cell walls [23]. Many of PGPMs are also used for bioremediation of soil and wastewater, as they simultaneously break down contaminants [24–27].

It has been found that lipopeptides such as iturin A, surfactin and fengycins produced by *Bacillus subtilis* show an antifungal effect against phytopathogen *Mycosphaerella fijiensis* and have potential in biotechnological and pharmaceutical applications [28].*Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus circulans, Bacillus pumilis*and *Bacillus altitudins* are synthesizing surfactin which is classified as one of the most powerful biosurfactants [9,29,30]. *Bacillus amyloliquefaciens* also has the ability to solubilize minerals and synthesize antimyotic peptides that inhibit growth of *Botrytis cinerea* [31,32]. *Bacillus cereus* has expressed antagonistic activity against unwanted organisms such as *Fusarium verticillioides* [10]. This microorganism is also used in purification of wastewater contaminated with oil due to the ability to degrade hydrocarbons [33–35]. An overview of biopesticidal and phytostimulatory effects of PGPMs from the *Bacillus* group is presented in Table 1.

Table 1. Biopesticidal and phytostimulatory effects of some species from the genus Bacillus

Microorganism	Activity	Reference
Bacillus subtilis	Production of substances that act as phytostimulators (indole acetic acid) and biopesticides (surfactants, fengycin C, iturin A). Production of enzymes, polysaccharides.	[28,29,36–44]
Bacillus amyloliquefaciens	Solubilization of zinc, phosphorus, etc. Antagonism. Production of substances that enhance plant growth (indole acetic acid, siderophore, gibberellic acid, HCN). Production of enzymes.	[31,37,45]
Bacillus megaterium	Phosphate (phosphorus) solubilization. Production of substances that enhance plant growth: 2-penthylfurane, 2,3-dimethyl-butandinitril, 1-ethenyl-4-methoxy-benzene, 3,5-dimethoxy-toluene, hexadecane. Production of siderophore.	[46–49]
Bacillus cereus	Antagonistic activity. Production of enzymes, polysaccharides.	[50–52]
Bacillus circulans	Antagonistic activity. Production of exopolysaccharides.	[53,54]
Bacillus licheniformis	Antagonistic activity. Production of polysaccharides	[55,56]

The reason that the genus *Bacillus* is often used in biological control of plant diseases is the main feature of this group of microorganisms to form endospores [57]. Endospores are thermo-tolerant structures, with high mechanical strength, resistant to external factors such as drying, ultraviolet radiation, organic solvents or high temperatures [9]. Formation of endospores is, in fact, a way in which bacteria adjust to non-optimal conditions that can be caused by temperature, pH or starvation and lack of nutrients [58]. The process of sporulation is initiated in stressful conditions, but also during the stationary growth phase when nutrients are exhausted. Literature data indicate that a culture initiates sporulation when the cellular density is about 10^8 cells/ml. Under ideal conditions, typical cell density and sporulation efficiency are 10^8 - 10^{10} CFU/ml and 30-80 %, respectively [16]. Microorganisms that form endospores can not only tolerate harmful environmental conditions but can survive aggressive processing steps during large-scale production, guaranteeing the resistance and stability of the formulations and making them ideal biocontrol agents [10]. Thus, it is important to achieve good sporulation and to incorporate spores into the formulation.

3. PRODUCTION PROCESS

Production of microbial formulations is a complex process which can be separated into two main stages:

- Microbial cultivation
- Formulation of the preparation

Both of these stages require a variety of aspects to be considered in order to be optimized in the right manner that provides a high-quality, long-term product, effective for a desired plant.

3. 1.Microbial cultivation

The aim of microbial cultivation is to produce good-quality biomass with desired cell density and high sporulation efficiency, which can be easily incorporated into a formulation [59].

Trial experiments are the first phase in the process of optimization of fermentation conditions. Erlenmeyer flasks are suitable for that purpose since valuable data about the microorganism and its requirements can be obtained at much lower costs. After this phase, the process is gradually scaled up to larger volume bioreactors. Among many of them, the most commonly used are bioreactors with mechanical stirring [60,61].

Optimization of the cultivation process requires adjustment of many different factors and it depends on the microbial culture. After selecting and characterizing the microbial culture, type of fermentation, growth medium and growth conditions should be chosen and optimized.

3. 1. 1. Type of fermentation

The cultivation process is often performed as batch or fed-batch. Batch cultivation owes its popularity to its simplicity and relatively easy implementation as compared to fed-batch, which requires continuous monitoring, higher expenditure but at the same time provides better results [61]. In one of the studies, *Bacillus subtilis* spore cultivation was performed using both batch and fed-batch cultivation where the latter was performed in three phases: batch (lasting 12 hours), semi-continuous (for the next 4 hours) and another batch phase (until the end of fermentation). Both methods achieved high vegetative cell concentrations, although fed-batch cultivation supported prevention of cell lysis and achieved much higher spore concentrations [62]. Those results were confirmed by another study, which proved that the higher glucose concentration in the batch system leads to acetate formation that inhibits bacterial growth, thus recommending the use of a fed-batch system [63]. Similarly, another research group performed cultivation of *Bacillus liceniformis* by shifting periods of batch and fed-batch fermentation in short time intervals. Batch cultivation lasted until stationary conditions were achieved, followed by one more batch and fed-batch shift [64]. The key factor during this kind of procedure is to continuously monitor the amount of substrate in the medium during the entire process. Yet, in a large number of cases, it is difficult to measure substrate concentration directly during fermentation, so other indicators such as pH, CO_2 and organic acids can be used to evaluate whether substrate addition is needed [65].

3. 1. 2. Medium optimization

The usual procedure starts with revitalization of microorganisms before fermentation, which is carried out by forming an inoculum in a small amount of standard media such as nutrient broth or Luria Bertani (LB) medium. Thereafter, a defined amount of inoculum (usually yielding 1-10 %) is transferred to the appropriate growth medium, which is specific for each microorganism, and its composition may vary depending on whether the aim of fermentation is to multiply microorganisms, produce spores, or to obtain some specific metabolite [66]. It should consist of suitable sources of carbon and nitrogen, inorganic salts and additional substances that satisfy the needs of bacterial metabolism [9,58]. For example, Elsayed et. al. showed that the maximal spore concentration of *B. thuringiensis* was achieved with addition of 20 g/L of glucose as a carbon source and 2.5 g/L of ammonium sulfate as a nitrogen source [67]. On the other hand, sporulation of *B. subtilis* is less demanding in terms of the required amount of carbon source. Kardziani et. al. have found out that the maximal spore yield could be achieved at 2 g/L of glucose in the medium, while higher glucose concentrations can even suppress sporulation [68].

Given that microorganisms of the genus *Bacillus* sporulate under adverse environmental conditions, reduction of glucose concentration after reaching the stationary phase induces starvation and the onset of sporulation [57,64]. In one of the studies, *B. subtilis* cells were grown in a defined minimal medium until exponential phase was achieved, where upon 1 % inoculum was transferred in another rich medium. Sporulation was started after reaching the stationary phase by glucose starvation, achieving high sporulation efficiency after 96 h [58]. Apart from the starvation method, a

replacement technique can be also used to trigger sporulation. It implies transferring the bacteria from rich to poor medium after achieving the exponential phase, which provides a more defined starting point [69]. Some studies have shown that *Bacillus subtilis* sporulate better in the presence of vitamins and minerals [62,70], while the increase in ammonium concentration induces the increase inthe spore number [71]. For example, calcium has been proven to enhance sporulation due to its ability to activate genes that are in control of production of polysaccharides, which assemble the outer spore coat layer [72], whilst thiamine was shown to induce the start of sporulation [62].

Different waste materials are a very interesting option when considering solid substrates [73]. Cultivation on a solid growth substrate composed of agricultural waste has been accepted as an environmentally safe method for mass production at low production costs [74]. For example, production of *Bacillus thuringiensis* biomass was carried out on substrates such as wheat bran, rice bran, rice husk, soy powder, molasses and protein hydrolysates [75]. A similar approach was used in another research, in which amylase was obtained from *Bacillus subtilis* cultivated on a waste cotton stalk as a substrate [37]. It is believed that fermentation of agricultural waste results in high-quality spores that can be easily used for seed inoculation or, alternatively, can be incorporated into natural polysaccharide gels. In addition, it was reported that such spores are more efficient than those cultivated by submerge fermentation, exhibit higher adhesion to plant roots and are more resistant to harmful conditions of the environment [73].

In a large number of studies, optimization of growth medium composition is carried out using multifactorial analyses: experimental design and response surface methodology (RSM). Unlike the "one variable at the time" method that requires lot of times and higher costs, RSM allows reduction in number of experiments, while guaranteeing good predictive ability and providing description of the system behavior [76]. Shortly, RSM combines mathematical and statistical methods with the aim of simultaneous optimization of several independent variables (also called factors) that are influencing certain dependent variables (also called responses) at different levels, and provides a suitable statistical model for the system [77–80]. It includes several steps. After selection of the factors, responses and the most appropriate experimental design, experiments are carried out. Results are statistically analyzed and a mathematical model is established and verified [81]. After confirmation of the model adequacy, multicriteria optimization is performed and the optimum combination of independent variables is proposed along with the expected results [82,83]. Although not as much as in the other fields, RSM has been used to optimize fermentation parameters and medium composition of *Bacillus* sp. An overview of the recent research is provided in Table 2.

3. 1. 3. Cultivation conditions

Bacillus sp. belongs to mesophilic microorganisms that grow at temperatures between 20 and 40 °C. A temperature range of 30-37 °C is recommended for optimal growth, whilst the temperature of 85 °C is lethal for vegetative cells and affects spore germination [62,92,93]. Pryor et. al. have found that very good sporulation (92 %) can be achieved even at 27 °C in combination with other factors such as high moisture content and aeration rate, which may help to reduce the energy costs of the process [94]. Spore resistance can be easily enhanced by a cold and high heat shock treatment. It has been reported that a cold shock pretreatment increases the heat resistance of spores to up to 100 °C, due to formation of proteins that protect the spore DNA [95,96]. pH value should be also kept in the constant range of 6 -7 for most of the bacteria. One of the studies reported that alkaline medium enhanced sporulation of *B. amyloliquefaciens*, while, on the other hand, acidic pH suppressed production of spores [97]. Fermentation duration can be up to 72 h, although the period of 48 h is most often used when taking into account economical aspects of the process [61].

Knowing that bacteria from the genus *Bacillus* are aerobic or facultative anaerobic it is clear that oxygen plays a very significant role in the cultivation process, and it is the key factor for achieving efficiency of cultivation [98]. By increasing the concentration of available oxygen, there is an increase in the number of cells, spores and desired metabolites, while its lack or reduced availability can limit the bioprocess and affect the fermentation kinetics [99,100]. For example, it has been found that *Bacillus thuringiensis* does not sporulate at oxygen limited conditions. Among the three aeration rates studied, the highest sporulation was achieved at the highest aeration rate (V/Vm = 2 L L^{-1} min^{-1}) [67]. The same aeration rate was used for cultivation of *B. subtilis* and production of Iturin A achieving excellent yields [88]. Another study also emphasized the importance of adequate aeration, claiming that absence of oxygen may result in lysis of sporangia [101].

Table2. Medium optimization for cultivation of different Bacillus species using RSM

Microorganism	The aim of the work	Experimental design*	Model p and F value	Optimum proposed factor values	Optimum predicted response	Ref.
Bacillus subtilis	Biomass production	CCD	p = 0.05 F = 6.08	pH 6.72, 0.164 % ammonium citrate and 0.85 % peptone	10.051×10^9 CFU/mL	[84]
Bacillus subtilis	Biomass production	BBD	-	-	$2.03\pm0.21\times10^9$ CFU/mL	[15]
Bacillus coagulans	Biomass production	BBD	-	30 g/L soybean meal, 6 g/L $(NH_4)_2SO_4$, 6 g/L $MgSO_4$	$0.99\pm0.04\times10^9$CFU/mL	[15]
Bacillus licheniformis	Biomass production	BBD	-	-	$0.11\pm0.01\times10^9$CFU/mL	[15]
Bacillus subtilis	Spore production	CCD	-	16.18 g/L corn steep liquor, 17.53 g/L soybean flour, 8.14 g/L yeast extract	$1.52\cdot10^{10}$ spores/mL	[85]
Bacillus subtilis	Biomass production	CCD	p < 0.0001 F = 46,47	13 g/L soybean peptone, 0.05 g/L $CaCl_2$	3.033 g/L (CDW)	[86]
Bacillus subtilis	Cost effective large scale biomass production	CCD	P = 0 F = 32,39	pH 7, *T*=30 °C C/N ratio: 23:1.	0.5 g/L in semi industrial bioreactor	[87]
Bacillus subtilis	Lipopeptide production	CCRD	-	1.098 g/L glucose, 4.01 g/L monosodium glutamate, 0.426 g/Lyeast extract, 0.431 g/L $MgSO_4\times7H_20$, and 0.219 g/L K_2HPO_4	1.501 g/L	[42]
Bacillus subtilis	Lipopeptide production	CCD	-	1.11 % Glucose, 0.7 % corn steep powder	132.23 mg/L	[88]
Bacillus sp. BH072	Iturin production	CCD	-	0.98 % sucrose, 0.94 % soybean meal, and 0.93 % Mg^{2+}.	52.21 mg/ml.	[89]
Bacillus subtilis	Iturin production	CCD	p < 0.0001 F = 91.49	0.998 g/L glucose, 1.83 g/L soybean flour	5.591 mg/kg iturin	[90]
Bacillus subtilis	Carboxy methyl cellulase production	CCD	p = 0.00046 F = 8.78	2 % substrate concentration, 2 % inoculum size, 1 % yeast extract, pH 5.0; incubation T = 50 °C	3.50 ± 0.11 IU/ml	[44]

*BBD- Box-Benkhen design; CCD-Central composite design; CCRD-Central composite rotatable design; FCCCD-Face centered central composite design

Before final optimization of the substrate composition using the RSM method, it is necessary to perform screening experiments in order to determine which of the many compounds have the highest statistical significance. Plackett-Burman designs were used in most of the studies included in this review. In one of the studies, this design was used to determine which of the 10 factors (lactose, peptone, glucose, ammonium citrate, beef extract, sodium acetate, potassium dihydrogen phosphate, sodium chloride, sodium sulfate, pH) were the most significant for Bacillus subtilis biomass production. It was determined that peptone and pH were the most significant variables, and they were selected for further optimization using a central composite design [84]. Using the same experimental design, effects of different medium components were evaluated for biomass and lipase production by the same microorganism, concluding that ammonium chloride, ammonium sulfate and dipotassium hydrogen phosphate were the ones playing the most significant role [91].

Therefore, it is important to measure the amount of available oxygen and maintain it at the desired level. Availability of oxygen in biochemical engineering is expressed by the oxygen transfer rate (*OTR*) [60,102,103]. *OTR* represents the

rate at which oxygen is transferred through the substrate in a gas-liquid system. In aerobic cultures, the oxygen uptake rate (*OUR*) should be lower than *OTR*. It was determined that a maximal *OUR* for *B. subtilis* is 20.3 mmol/L·h at the beginning of the exponential phase after 10 h of cultivation, which is a peak in the oxygen demand [104].

Capacity of oxygen transfer through a vessel is determined on the basis of the oxygen mass transfer coefficient (k_La). In addition to directly applying experimental methods such as sulfite oxidation method, adsorption or a dynamic method, k_La can be predicted by using some of the empirical correlations available, that have shown a satisfactory accuracy [105,106]. For a standard Erlenmeyer flask, existing correlations usually take into account the specific interphase area and the stirring rate [107]. The value of k_La can be also affected by metabolic products that are released in the fermentation broth. Such case was observed during cultivation of *B. subtilis,* which is capable to produce surfactants. Addition of oxygen vectors, on the other hand, increased k_La values at the same time decreasing production of these metabolites [108]. Similarly, Shih et. al. confirmed that the increase in k_La value favored production of cell biomass, but repressed the production of iturin A [109]. Since the presence of such metabolites is often very desirable in the broth due to their antifungal properties, cultivation should be further optimized with controlling the agitation rate [108].

Most often, agitation rate is used as a k_La adjustment parameter, ranging from 100 to 1500 rpm, depending on the process [38]. Tzeng et. al. reported that higher agitation rates may suppress sporulation of bacteria due to higher shear stresses, leading to a recommendation of 200 rpm for achieving efficient sporulation of *Bacillus amyloliquefacies* [97]. On the other hand, higher agitation rates are required for successful biomass production. Correspondingly, Sen et. al. proposed a two-stage strategy, which included production of biomass during the exponential phase under optimal cell growth conditions (pH 6.65, T = 38.3 °C, agitation: 247 rpm, aeration: V/Vm = 1.05 L L^{-1} min^{-1}, and production of spores during the stationary phase under optimal sporulation conditions (pH 6.27, T=41.4 °C, agitation: 115 rpm, aeration: V/Vm = 0.33 L L^{-1} min^{-1}) [110]. Similarly, the multi-stage strategy was also proposed by Man *et. al.* during cultivation of *B. subtilis* along with riboflavin production. The authors implied that the agitation rate should be gradually increased based on the kinetic analysis and specific growth rate of bacteria [111].

3. 1. 4. Scale-up of the system

Once optimization of the cultivation is completed, the system can be scaled up to start industrial production. Since the initial experiments are mainly carried out in small-scale equipment it is necessary to determine whether the same or similar results can be obtained in equipment of larger dimensions [112]. After the laboratory scale, the system is raised to a pilot plant level to further optimize the process and to ensure that the kinetic parameters and process characteristics remain unchanged. Finally, the system is expanded to the production scale, which includes economic aspects of the optimization [105,113].

The described process is certainly not an easy task, knowing that changes in dimensions cause changes in mixing efficiency, affect oxygen supply, and increase possibilities for creation of "dead zones" and uneven distributions of nutrients. According to some authors, key parameters are related to heat and mass transfer, agitation and aeration (along with k_La) as the most important factors that may be affected by the scale change [105]. Then, the scale up process is based on maintaining certain dimensionless groups, that should remain constant during the process. The scale-up process can be also empirical and include previous experiments that simulate desired conditions [114–116].

In a recent paper, authors claim that large scale production of *B. subtilis* is technically feasible, based on high spore yield ($7 \cdot 10^{10}$) achieved while scaling up cultivation from shake flasks to a 7 L stirred tank bioreactor. Although scaling up criteria were not clearly described, it was stated that the most important factors were agitation and aeration rates (300 rpm and V/Vm = 1 L L^{-1} min^{-1}, respectively) [68]. Similar conclusions were made by another group of authors who successfully scaled up a *B. subtilis* spore cultivation system from a shake flask level to a 30 L fermenter. By controlling the agitation rate (400-800 rpm) and dissolved oxygen (30-40 %) they managed to achieve up to $1.52 \cdot 10^{10}$ spores/ml [85]. Aeration rate was also an important parameter for scaling up the cultivation of *B. amyloliquefaciens* to a 20 L bioreactor, that was kept constant at V/Vm = 2.5 L L^{-1} min^{-1}[97].

3.2. Formulation procedure

After optimizing the cultivation conditions and increasing the size of the system, the formulation procedure can be considered regarding the combination of substances that are going to be used and the exact incorporation protocol. Once formulated, the preparation should be tested for its efficiency and longevity [15,16].

A microbial formulation consists of one or more PGPMs and different ingredients that enhance the product quality [117]. To attain a pure culture, cells should be separated from the broth, which is usually performed by centrifugation at 4 °C, followed by further purification [68]. Tavares *at al.* have described a purification procedure for *B. subtilis*, which consisted of multiple centrifugations (at 10000xg at 4 °C) and subsequent washing with distilled water, followed by suspending the pellets in a Tris-HCl and lysozyme solution for 1h incubation and resuspension in sodium dodecyl sulfate (SDS). This protocol is a simplified version of the one proposed earlier which additionally contained repeated washings of the pellet with KCl and NaCl [92]. Still, some authors omit the separation and purification step and use cells along with the fermentation broth since it contains valuable metabolites which are often a very desirable part of the final product [10,118,119].

For microbial formulations to be commercially competent it is necessary to provide a shelf life of minimum 6 months to a year, which means that a total number of cells and their properties should remain unchanged during that period. It is also important to ensure avoidance of any competition between the cells if the formulation consists of a large number of different microbial species [118,120].

Formulations can be either solid or liquid, obtained by different formulation methods. Solid formulations can be produced by several drying processes: spray-, freeze-, vacuum- or fluidized-bed drying [121–123]. Although liquid formulations are easier and cheaper to form, they have a disadvantage of a shorter shelf life, as well as packing and storing problems [14].

Since *Bacillus* species form endospores, they are suitable to be prepared as solid formulations, with the addition of different carriers, stabilizers, protectants and other supplements. Review of different *Bacillus* based formulations is presented in Table 3.

Table 3. Different types of Bacillus sp. formulations

Microorganism	Formulation type	Formulation method	Added substances	Ref.
Bacillus meghaterium	Granules	Wet granulation technique using the granulator	Lactose monohydrate PVP K-30, Sodium alginate	[14]
Bacillus meghaterium	Powder formulation	Air drying	Talc, clay and cellulose; CMC, Sodium benzoate, $CaCO_3$, Glucose, sucrose, mannitol, yeast, peptone.	[120]
Bacillus amyloliquefacines	Powder formulation	Freeze drying	Sucrose, powder skimmed milk, $MgSO_4$	[119]
Bacillus amyloliquefacines	Liquid formulation	Homogenization	Sucrose, powder skimmed milk, $MgSO_4$	[119]
Bacillus amyloliquefacines	Powder formulation	Fluidized bed spray drying	$MgSO_4$	[119]
Bacillus amyloliquefacines	Powder formulation	Freeze drying	Glucose, trehalose and xylitol	[124]
Bacillus cereus	Powder formulation	Freeze drying	Glucose, fructose, D-galactose, sucrose, trehalose, cellobiose, glutamic acid, soluble starch, glycerol, sorbitol, peptone, nonfat skimmed milk.	[125]
Bacillus cereus	Powder formulation	Oven drying	Talc, CMC, $CaCO_3$, Glucose.	[10]
Bacillus subtilis	Powder formulation	Freeze drying	Soybean flour	[126]
Bacillus subtilis	Liquid formulation	Homogenization	Groundnut oil, Pongamia oil and sunflower oil; Glycerol	[127]
Bacillus subtilis and Bacillus licheniformis	Powder formulation	Air drying	Natural zeolite Synthetic zeolite	[118]

Carriers used may be solid or liquid, organic or inorganic. Talk, clay, zeolite, cereal flour, and vegetable oils can be used as carriers, and the effect of each of them should be specifically investigated for the desired species [120,128]. A good carrier should be ecologically acceptable, cheap and suitable for the microorganism [117]. The carrier choice depends on the formulation type and incorporated bacterial cultures. If the formulation consists of two or more

competitive species, the carrier must enhance viability for each of them without adverse effects. For example, there is a competition between the vegetative form of *Bacillus subtilis* and *Bacillus licheniformis*, which negatively affects the formulation itself and prevents expression of desired results. This problem can be solved by using synthetic and natural zeolites [118]. Similarly, it has been found that *Bacillus licheniformis* induces a lethal effect on *Bacillus cereus*, so it is not recommended to combine these two bacterial species in the same formulation [129].

Apart from carriers, stabilizers such as sodium benzoate or lactose are added to the formulation along with additional nutrients (glucose, molasses, peptone), thickeners (xanthan gum), surfactants, desiccants (silica gel) and many other substances that aim to make the product more durable and more efficient [124]. In a previous study it was found that addition of adjuvants, additives and surfactants enhanced the shelf-life and efficacy of *Bacillus meghaterium,* concluding that the combination of polyvinyl pyrrolidone, carboxymethyl cellulose and polysorbate provides the best results in liquid formulations [70].

Since the shelf life and product stability are among the major problems that limit applications of microbiological formulations, additional research in this area needs to be directed to develop a clear picture of constituents of a formulation for each microorganism or a microbial combination [10].

4. CONCLUSION

Previous practice of using chemical fertilizers and agrochemicals has proven unsustainable and has contributed to disturbing the ecological balance and fertility of lands, as well as to environmental pollution. Excessive treatment of plant crops with agrochemicals is a global problem causing many health issues, causing a desperate need for finding a safer and greener alternative solution. A natural and ecologically acceptable approach to this problem is to replace agrochemicals with microbial formulations, which can simultaneously increase yields, protect plants and regenerate soil. Application of microbial formulation ssupports sustainable agriculture, environmental protection and production of health-safe foods. Consequently, research in this area has become a focus throughout the world.

Thanks to its phytostimulatory and biopesticidal properties, genus *Bacillus* has a great potential to be incorporated in microbial formulations that can be used to protect plants and stimulate their growth. To formulate good quality products, suitable bacterial culture or consortium of cultures should be selected along with the proper cultivation and formulation method, which implies optimizing the cultivation conditions to enhance sporulation and production of some antifungal metabolites, if desired. Cost-effectiveness can be accomplished by applying multifactorial analyzes like experimental design and RSM for process optimization. Choice of a formulation type and delivery system also plays a very important role, especially when it comes to the commercial application of the product. Different protective substances are added to the product, to ensure viability, longevity and success on fields.

Although this idea is not new and there is plenty of research on the subject, there are many parts of the process that are yet to be improved and fully understood. The fact that biological control of disease and plant stimulation has not yet come to life, convincingly suggests that further research in this field is more than necessary.

Acknowledgements: *This research is part of the project: Simultaneous Bioremediation and Solidification of Degraded Areas to Preserve Natural Resources of Biologically Active Substances, and Development and Production of Biomaterials and Dietetic Products, No: III 43004, funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.*

REFERENCES

[1] Shahcheraghi S, Ayatollahi J, Lotfi M. Applications of *Bacillus subtilis* as an important bacterium in medical sciences and human life. *Trop J Med Res*. 2015; 18(1): 1-9.

[2] Mercado-Flores Y, Cárdenas-Álvarez IO, Rojas-Olvera AV, Pérez-Camarillo JP, Leyva Mir SG, Anducho-Reyes MA. Application of *Bacillus subtilis* in the biological control of 359 the phytopathogenic fungus *Sporisoriumreilianum*. *Biol Control.* 2014; 76: 36-40.

[3] Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol*. 2004;50(1):1-17.

[4] Govedarica M, Nada M, Mirjana J, Simonida Đ, Zora J, Janja K, Snežana Đ. Primenabiofertilizera, biostimulatora i biopesticida u poljoprivrednoj proizvodnji. Naučni Inst za Ratar i Povrt Novi Sad, ZbornikRadova 2002; 37: 85-95. (in Serbian)

[5] Benhamou N, Kloepper JW, Tuzun S. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: Ultrastructure and cytochemistry of the host response. *Planta*. 1998;204(2):153-168.

[6] Wei Z, Yang X, Yin S, Shen Q, Ran W, Xu Y. Efficacy of *Bacillus*-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. *Appl Soil Ecol*. 2011;48(2):152-159.

[7] Yazdani M, Bahmanyar MA, Pirdashti H, Ali M. Effect of Phosphate Solubilization Microorganisms (PSM) and Plant Growth Promoting Rhizobacteria (PGPR) on Yield and Yield Components of Corn (*Zea mays* L .). *World Acad Sci Eng Technol*. 2009;25(1):90-92.

[8] Iqbal Hossain M, Sadekuzzaman M, Ha S-D. Probiotics as potential alternative biocontrol agents in the agriculture and food industries: A review. *Food Res Int*. 2017;100:63-73.

[9] de Carvalho ALU, de Oliveira FHPC, Mariano R de LR, Gouveia ER, Souto-Maior AM. Growth, sporulation and production of bioactive compounds by *Bacillus subtilis* R14. *Brazilian Arch Biol Technol*. 2010;53(3):643-652.

[10] Martinez-Alvarez JC, Castro-Martinez C, Sanchez-Pena P, Gutierrez-Dorado R, Maldonado-Mendoza IE. Development of a powder formulation based on *Bacillus cereus*sensulato strain B25 spores for biological control of *Fusarium verticillioides* in maize plants. *World J Microbiol Biotechnol*. 2016;32(5):1-10.

[11] Wang S., Zhong JJ. Bioreactor engineering. In: Yang ST, ed. *Bioprocessing for Value-Added Products from Renewable Resources*. Amsterdam: Elsevier; 2007.

[12] Najafpour GD. *Biochemical Engineering and Biotechnology*. Amsterdam: Elsevier; 2007.

[13] Doran PM. *Bioprocess Engineering Principles: Second Edition*. London: Academic Press; 2013.

[14] Chumthong A, Kanjanamaneesathian M, Pengnoo A, Wiwattanapatapee R. Water-soluble granules containing *Bacillus megaterium* for biological control of rice sheath blight: Formulation, bacterial viability and efficacy testing. *World J Microbiol Biotechnol*. 2008;24:2499.

[15] Cho J-H, Kim Y-B, Kim E-K. Optimization of culture media for *Bacillus* species by statistical experimental design methods. *Korean J Chem Eng*. 2009;26(3):754-759.

[16] Posada-Uribe LF, Romero-Tabarez M, Villegas-Escobar V. Effect of medium components and culture conditions in *Bacillus subtilis* EA-CB0575 spore production. *Bioprocess Biosyst Eng*. 2015;38(10):1879-1888.

[17] Bjelić D. Karakterizacija i efektivnost bakterija promotora biljnograsta izolovanih iz rizosfere kukuruza. Doctoral dissertation, Univerzitet u NovomSadu, Poljoprivredni 395 fakultet. 2014. (in Serbian)

[18] Garcia-Fraile P, Menendez E, Rivas R. Role of bacterial biofertilizers in agriculture and forestry. *AIMS Bioeng*. 2015;2(3):183-205.

[19] Abhilash PC, Dubey RK, Tripathi V, Gupta VK, Singh HB. Plant Growth-Promoting Microorganisms for Environmental Sustainability. *Trends Biotechnol*. 2016;xx:1-4.

[20] Owen D, Williams AP, Griffith GW, Withers PJA. Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. *Appl Soil Ecol*. 2015;86:41-54.

[21] Cerozi B da S, Fitzsimmons K. Use of *Bacillus* spp. to enhance phosphorus availability and serve as a plant growth promoter in aquaponics systems. *Sci Hortic (Amsterdam)*. 2016;211:277-282.

[22] Fira D, Dimkić I, Berić T, Lozo J, Stanković S. Biological control of plant pathogens by *Bacillus* species. *J Biotechnol*. 2018;285:44-55.

[23] Hashem A, Tabassum B, Fathi Abd E. *Bacillus subtilis:* A plant-growth promoting rhizobacterium that also impacts biotic stress. *Saudi J Biol Sci*. 2019 (article in press)

[24] Milić J, Beškoski V, Ilić M, Ali S, Gojgić-Cvijović G, Vrvić M. Bioremediation of soil heavily contaminated with crude oil and its products: Composition of the microbial consortium. *J Serbian Chem Soc*. 2009;74(4):455-460.

[25] Beškoski VP, Gojgić-Cvijović GĐ, Milić JS, Ilić MV, Miletić SB, Jovančević BS, Vrvić MM. Bioremedijacija zemljišta kontaminiranog naftom i naftnim derivatima: mikroorganizmi, putanje razgradnje, tehnologije. Hem Ind. 2012; 66(2): 275-289. (in Serbian)

[26] Beškoski VP, Gojgić-Cvijović G, Milić J, Ilić M, Miletić S, Šolević T, Vrvić MM. Ex situ bioremediation of a soil contaminated by mazut (heavy residual fuel oil) – A field experiment. *Chemosphere*. 2011;83(1):34-40.

[27] Gojgić-Cvijović GD, Milić JS, Šolevic TM, Beškoski VP, Ilić M V., Djokić LS, Narančić TM, 414 Vrvić MM. Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: A laboratory study. *Biodegradation*. 2012;23(1):1-14.

[28] Mosquera S, González-Jaramillo LM, Orduz S, Villegas-Escobar V. Multiple response optimization of *Bacillus subtilis* EA-CB0015 culture and identification of antifungal metabolites. *Biocatal Agric Biotechnol*. 2014;3(4):378-385.

[29] Guez JS, Chenikher S, Cassar JP, Jacques P. Setting up and modelling of overflowing fed-batch cultures of *Bacillus subtilis* for the production and continuous removal of lipopeptides. *J Biotechnol*. 2007;131(1):67-75.

[30] Goswami M, Deka S. Biosurfactant production by a rhizosphere bacteria *Bacillus altitudinis*MS16 and its promising emulsification and antifungal activity. *Colloids Surfaces B Biointerfaces*. 2019;178:285-296.

[31] Sharma S, Ramesh A, Johri B. Isolation and characterization of plant growth-promoting *Bacillus amyloliquefaciens* strain sks_bnj_1 and its influence on rhizosphere soil properties and nutrition of soybean (*Glycine max* L. Merrill). *J Virol Microbiol*. 2013;2013(2013):1-19.

[32] Xia Zhang Q, Zhang Y, Ling He L, Lin Ji Z, Hui Tong Y. Identification of a small antimycotic peptide produced by *Bacillus amyloliquefaciens*. *PesticBiochem Physiol.* 2018; 150: 78-82.

[33] Banerjee A, Ghoshal AK. Biodegradation of an actual petroleum wastewater in a packed bed reactor by an immobilized biomass of *Bacillus cereus. J Environ Chem Eng*. 2017;5(2):1696-1702.

[34] Jovančićević B, Antić M, Pavlović I, Vrvić M, Beškoski V, Kronimus A, Schwarzbauer J. Transformation of Petroleum Saturated Hydrocarbons during Soil Bioremediation Experiments. *Water Air Soil Pollut*. 2008;190(1-4):299-307.

[35] Ramadan MMMA, Šolević Knudsen T, Antić M, Beškoski VP, Vrvić MM, Schwarzbauer J, Jovančidević B. Degradability of n-alkanes during ex situ natural bioremediation of soil contaminated by heavy residual fuel oil (mazut). *J Serbian Chem Soc*. 2013;78(7):1035-1043.

[36] Abdullah R, Kiran S, Iqtedar M, Kaleem A, Saleem F, Iftikhar T, Cheema JS, Naz S, Random mutagenesis and process optimization of bacterial co-culture for hyperproduction of 1,4-α-D-glucan glucanohydrolase using submerged fermentation. *Hem Ind*. 2018;72(6):329-339.

[37] Akcan N, Serin B, Uyar F. Production and Optimization Parameters of Amylases from *Bacillus subtilis* RSKK96 Under Solid State Fermentation. 2012;26(3):233-239.

[38] Božić N, Ruiz J, López-Santín J, Vujčić Z. Optimization of the growth and α-amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. *J Serbian Chem Soc*. 2011;76(7):965-972.

[39] Cagri-Mehmetoglu A, Kusakli S, van de Venter M. Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium. *J Dairy Sci*. 2012;95(7):3643-9.

[40] Yeh MS, Wei YH, Chang JS. Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis.Process Biochem*. 2006;41(8):1799-1805.

[41] Grobelak A, Napora A, Kacprzak M. Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecol Eng*. 2015;84:22-28.

[42] Eswari J, Anand M, Venkateswarlu C. Optimum culture medium composition for lipopeptide production by *Bacillus subtilis* using response surface model-based ant colony optimization. *Sadhana - Acad Proc Eng Sci*. 2016;41(1):55-65.

[43] Stein T. Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Mol Microbiol*. 2005;56(4):845-857.

[44] Irfan M, Mushtaq Q, Tabssum F, Shakir HA, Qazi JI. Carboxymethyl cellulase production optimization from newly isolated thermophilic *Bacillus subtilis* K-18 for saccharification using response surface methodology. *AMB Express*. 2017;7:29.

[45] Vučetić J, Veljković VB, Vrvić MM, Lazić ML. *Mikrobiološke Sinteze Polisaharida*. Beograd: Naučnaknjiga Beograd; 1995. (In Serbian).

[46] Velineni S, Brahmaprakash GP. Survival and phosphate solubilizing ability of *Bacillus megaterium* in liquid inoculants under high temperature and desiccation stress. *J Agric Sci Technol*. 2011;13(5):795-802.

[47] Zou C, Li Z, Yu D. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran. *J Microbiol*. 2010;48(4):460-466.

[48] Santos S, Neto IFF, Machado MD, Soares HMVM, Soares E V. Siderophore Production by *Bacillus megaterium:* Effect of Growth Phase and Cultural Conditions. *Appl BiochemBiotechnol*. 2014;172(1):549-560.

[49] Liu M, Liu X, Cheng B Sen, Ma XL, Lyu XT, Zhao XF, Ju YL, Min Z, Fang YL. Selection and evaluation of phosphate-solubilizing bacteria from grapevine rhizospheres for use as biofertilizers. *Spanish J Agric Res*. 2016;14(4):e1106.

[50] Vasiee A, Behbahani BA, Yazdi FT, Moradi S. Optimization of the production conditions of the lipase produced by *Bacillus cereus* from rice flour through Plackett-Burman Design (PBD) and response surface methodology (RSM). *MicrobPathog*. 2016;101:36-43.

[51] Zhao J-L, Zhou L-G, Wu J-Y. Promotion of *Salvia miltiorrhiza* hairy root growth and tanshinone production by polysaccharide–protein fractions of plant growth-promoting rhizobacterium *Bacillus cereus*. *Process Biochem*. 2010;45(9):1517-1522.

[52] Lizárraga-Sánchez GJ, Leyva-Madrigal KY, Sánchez-Peña P, Quiroz-Figueroa FR, Maldonado-Mendoza IE. *Bacillus cereus* sensulato strain B25 controls maize stalk and ear rot in Sinaloa, Mexico. *F Crop Res*. 2015;176:11-21.

[53] Vidhyalakshmi R, Valli NC, Narendra Kumar G, Sunkar S. B*acillus circulans* exopolysaccharide: Production, characterization and bioactivities. *Int J Biol Macromol*. 2016;87:405-414.

[54] Fontaine T, Wieruszeski JM, Talmont F, Saniez MH, Duflot P, Leleu JB, Fournet B. Exopolysaccharide structure from *Bacillus circulans*. *Eur J Biochem*. 1991;196(1):107-13.

[55] Kekez B, Gojgić-Cvijović G, Jakovljević D, Pavlović V, Beškoski V, Popović A, Vrvić MM, NikolićV.Synthesis and characterization of a new type of levan-graft-polystyrene copolymer. *CarbohydrPolym*. 2016;154:20-29.

[56] Kekez BD, Gojgić-Cvijović GD, Jakovljević DM, Stefanović Kojić JR, Marković MD, 492 Beškoski VP, Vrvić MM. High Levan Production by *Bacillus licheniformis* NS032 Using Ammonium Chloride as the Sole Nitrogen Source. *Appl BiochemBiotechnol*. 2015;175(6):3068-3083.

[57] Castillo HFD, Reyes CF, Morales GG, Herrera RR, Aguilar C. Biological Control of Root Pathogens by Plant- Growth Promoting *Bacillus* spp. In: Saloneski S, Larramnedy M, eds. *Weed and Pest Control - Conventional and New Challenges*. InTech; 2013:80-103.

[58] Abhyankar W, Beek A Ter, Dekker H, Kort R, Brul S, de Koster CG. Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat protein fraction. *Proteomics*. 2011;11(23):4541-4550.

[59] Öztürk S, Çalık P, Özdamar TH. Fed-Batch Biomolecule Production by *Bacillus subtilis*: A State of the Art Review. *Trends Biotechnol*. 2016;34(4):329-345.

[60] Galaction A-I, Oniscu C, Cascaval D. Studies on oxygen mass transfer in stirred bioreactors 2: Suspensions of bacteria, yeasts and fungis. *Hem Ind*. 2003;57(6):276-287.

[61] Stamenković S, Beškoski V, Karabegović I, Lazić M, Nikolić N. Microbial fertilizers: A comprehensive review of current findings and future perspectives. *Spanish J Agric Res*. 2018;16(1):e09R01.

[62] Monteiro SMS, Clemente JJ, Carrondo MJT, Cunha AE. Enhanced Spore Production of *Bacillus subtilis* Grown in a Chemically Defined Medium. *Adv Microbiol*. 2014;4(8):444-454.

[63] Matar SM, El-Kazzaz SA, Wagih EE, El-Diwany AI, Moustafa HE, Abo Zaid GA, Hafez EE. Bioprocessing and scaling-up cultivation of *Bacillus subtilis* as a potential antagonist to certain plant pathogenic fungi, III. *Biotechnology*. 2009;8(1):138-143.

[64] Reis A, Da Silva TL, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ. Monitoring population dynamics of the thermophilic *Bacillus licheniformis* CCMI 1034 in batch and continuous cultures using multi-parameter flow cytometry. *J Biotechnol*. 2005;115(2):199-210.

[65] Glick BR. *Beneficial Plant-Bacterial Interactions*. Springer International Publishing Switzerland; 2015.

[66] Shaikh SS, Sayyed RZ. Role of Plant Growth-Promoting Rhizobacteria and Their Formulation in Biocontrol of Plant Diseases. In: Arora NK, ed. *Plant Microbes Symbiosis: Applied Facets*. New Delhi: Springer India; 2015:337-351.

[67] Elsayed AE, Othman NZ, Malek RA, Awad HM, Wu K, Azizi R, Wadaan HM, Enshasy H. Bioprocess Development for High Cell Mass and Endospore Production by *Bacillus thuringiensis* var *israelensis* in Semi-Industrial Scale. *J Pure Appl Microbiol*. 2014;8(4):2773-2783.

[68] Khardziani T, Kachlishvili E, Sokhadze K, Elisashvili V, ChikindasML, Chistyakov V. Elucidation of *Bacillus subtilis* KATMIRA 1933 Potential for Spore Production in Submerged Fermentation of Plant Raw Materials. *Probiotics Antimicrob Proteins.* 2017; 9 (4): 435-443.

[69] Piggot PJ, Coote JG. Genetic aspects of bacterial endospore formation. *Bacteriol Rev*. 1976;40(4):908-962.

[70] Leo Daniel A, Venkateswarlu B, Suseelendra D, *et al.* Effect of Polymeric Additives, Adjuvants, Surfactants on Survival, Stability and Plant Growth Promoting Ability of Liquid Bioinoculants. *J Plant PhysiolPathol*. 2013;1(2):1-5.

[71] Monteiro SM, Clemente JJ, Henriques AO, Gomes RJ, Carrondo MJ, Cunha AE. A procedure for high-yield spore production by *Bacillus subtilis*. *Biotechnol Prog*. 2005;21(4):1026-1031.

[72] Brul S, van Beilen J, Caspers M, O'Brien A, de Coster C, Oomes S, Smelt J, Kort R, Ter Beek A. Challenges and advances in systems biology analysis of *Bacillus* spore physiology; molecular differences between an extreme heat resistant spore forming *Bacillus subtilis* food isolate and a laboratory strain. *Food Microbiol*. 2011;28(2):221-227.

[73] Vassilev N, Vassileva M, Lopez A, Martos V, Reyes A, Maksimovic I, Elcher-Lobermann B, Malusa E. Unexploited potential of some biotechnological techniques for biofertilizer production and formulation. *Appl Microbiol Biotechnol*. 2015;99(12):4983-4996.

[74] Singhania RR, Patel AK, Soccol CR, Pandey A. Recent advances in solid-state fermentation. *BiochemEng J*. 2009;44(1):13-18.

[75] Morris ON, Kanagaratnam P, Converse V. Suitability of 30 Agricultural Products and By-Products as Nutrient Sources for Laboratory Production of *Bacillus thuringiensis* subsp. *aizawai* (HD133). *J InvertebrPathol*. 1997;70(2):113-20.

[76] Sánchez Blanco A, Palacios Durive O, Batista Pérez S, Díaz Montes Z, Pérez Guerra N. Simultaneous production of amylases and proteases by *Bacillus subtilis* in brewery wastes. *Brazilian J Microbiol*. 2016;47(3):665-674.

[77] Baş D, Boyacı İH. Modeling and optimization I: Usability of response surface methodology. *J Food Eng*. 2007;78(3):836-845.

[78] Montgomery DC. *Design and Analysis of Experiments*. John Wiley & Sons, Inc; 2001.

[79] Rushing H, Karl A, Wisnowski J. *Design and Analysis of Experiments by Douglas Montgomery: A Supplement for Using JMP(R)*. Cary, North Carolina, USA: SAS Institute Inc.; 2013.

[80] Nwabueze TU. Basic steps in adapting response surface methodology as mathematical modelling for bioprocess optimisation in the food systems. *Int J Food Sci Technol*. 2010;45(9):1768-1776.

[81] Karabegović IT, Stojičević SS, Veličković DT, Nikolić NČ, Lazić ML. Optimization of Microwave-Assisted Extraction of *Cherry Laurel* Fruit. *Sep Sci Technol*. 2014;49(3):416-423.

[82] Lundstedt T, Seifert E, Abramo L, *et al.* Experimental design and optimization. *ChemomIntell Lab Syst*. 1998;42(1-2):3-40.

[83] Ameer K, Bae S, Jo Y, Lee H, Ameer A, Kwon J. Optimization of microwave-assisted extraction of total extract, stevioside and rebaudioside-A from *Stevia rebaudiana* (Bertoni) leaves, using response surface methodology (RSM) and artificial neural network (ANN) modelling. *Food Chem*. 2017;229:198-207.

[84] Sreekumar G, Krishnan S. Enhanced biomass production study on probiotic *Bacillus subtilis* SK09 by medium optimization using response surface methodology. *African J Biotechnol*. 2010;9(45):8078-8084.

[85] Chen Z-M, Li Q, Liu H-M, Yu N, Xie TJ, Yang MJ, Shen P, Chen XD. Greater enhancement of *Bacillus subtilis* spore yields in submerged cultures by optimization of medium composition through statistical experimental designs. *Appl Microbiol Biotechnol*. 2010;85(5):1353-1360.

[86] Anh NQ. *Development of Bacillus Subtilis Spores and Cells for Surface Display of Proteins*. Bayreuth: der FakultätfürBiologie, Chemie und Geowissenschaften der Universität Bayreuth; 2010.

[87] Ghasemi S, Ahmadzadeh M. Optimisation of a cost-effective culture medium for the large-scale production of *Bacillus subtilis* UTB96. *Arch Phytopathol Plant Prot*. 2013;46(13):1552-1563.

[88] Shih IL, Lin CY, Wu JY, Hsieh C. Production of antifungal lipopeptide from *Bacillus subtilis* in submerged fermentation using shake flask and fermentor. *Korean J Chem Eng*. 2009;26(6):1652-1661.

[89] Zhao X, Han Y, Tan X qian, Wang J, Zhou Z jiang. Optimization of antifungal lipopeptide production from *Bacillus* sp. BH072 by response surface methodology. *J Microbiol*. 2014;52(4):324-332.

[90] Mizumoto S, Shoda M. Medium optimization of antifungal lipopeptide, iturin A, production by *Bacillus subtilis* in solid-state fermentation by response surface methodology. *Appl Microbiol Biotechnol*. 2007;76(1):101-108.

[91] Cheng S., Y.F. W, F.F. L. Optimization of Medium Compositions Using Statistical Experimental Design to Produce Lipase by *Bacillus subtilis*. *Chem BiochemEng Q*. 2011;25(3):377-383.

[92] Tavares MB, Souza RD, Luiz WB, Cavalcante RCM, Casaroli C, Martins EG, Ferreira RCC, 584 Ferreira LCS.*Bacillus subtilis* endospores at high purity and recovery yields: Optimization of growth conditions and purification method. *Curr Microbiol*. 2013;66(3):279-285.

[93] Bashan Y, Trejo A, de-Bashan LE. Development of two culture media for mass cultivation of *Azospirillum*spp. and for production of inoculants to enhance plant growth. *Biol Fertil Soils*. 2011;47(8):963-969.

[94] Pryor SW, Gibson DM, Hay AG, Gossett JM, Walker LP. Optimization of spore and antifungal lipopeptide production during the solid-state fermentation of *Bacillus subtilis*. *Appl BiochemBiotechnol*. 2007;143(1):63-79.

[95] Movahedi S, Waites W. Cold shock response in sporulating Bacillus subtilis and its effect on spore heat resistance. *J Bacteriol*. 2002;184(19):5275-81.

[96] Pandey R, Pieper GH, Beek A Ter, Vischer NOE, Smelt JPPM, Manders EMM, Brul S. Quantifying the effect of sorbic acid, heat and combination of both on germination and outgrowth of *Bacillus subtilis* spores at single cell resolution. *Food Microbiol*. 2015;52:88-96.

[97] Tzeng Y-M, Rao YK, Tsay K-J, Wu W-S. Effect of cultivation conditions on spore production from *Bacillus amyloliquefaciens* B128 and its antagonism to *Botrytis elliptica*. *J Appl Microbiol*. 2008;104(5):1275-1282.

[98] Naseva O, Stamenković I, Banković-Ilić I, Lazić M, Veljković V, Skala D. Sadržaj gasa u bioreaktoru sa vibracionom mešalicom - tečnafaza je nenjutnovski fluid. *HemInd*. 2002;56(5):198-203. (in Serbian).

[99] Suresh S, Srivastava VC, Mishra IM. Techniques for oxygen transfer measurement in bioreactors: A review. *J Chem Technol Biotechnol*. 2009;84(8):1091-1103.

[100] Wu Z, Du G, Chen J. Effects of dissolved oxygen concentration and DO-stat feeding strategy on CoQ 10 production with *Rhizobium radiobacter. World J Microbiol Biotechnol*. 2003;19:925-928.

[101] Sarrafzadeh MH, Schorr-Galindo S, La H-J, Oh H-M. Aeration effects on metabolic events during sporulation of *Bacillus thuringiensis*. *J Microbiol*. 2014;52(7):597-603.

[102] Suresh S, Srivastava VC, Mishra IM. Critical analysis of engineering aspects of shaken flask bioreactors. *Crit Rev Biotechnol*. 2009;29(4):255-278.

[103] Cascaval D, Galaction A-I, Oniscu C, Ungureanu F. Modeling of mixing in stirred bioreactors 4. Mixing time for aerated bacteria, yeasts and fungus broths. *Hem Ind*. 2004;56(3):128-137.

[104] Richard A, Margaritis A. Rheology, oxygen transfer, and molecular weight characteristics of poly(glutamic acid) fermentation by *Bacillus subtilis*. *BiotechnolBioeng*. 2003;82(3):299-305.

[105] Garcia-Ochoa F, Gomez E. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotechnol Adv*. 2009;27(2):153-176.

[106] Venkatachalam S, Palaniappan A, Kandasamy S, Kandasamy K. Prediction of gas holdup in a combined loop air lift fluidized bed reactor using Newtonian and non-Newtonian liquids. *Chem Ind Chem Eng Q*. 2011;17(3):375-383.

[107] Veljković VB, Nikolić S, Lazić ML, Engler CR. Oxygen transfer in flasks shaken on orbital shakers. *Hem Ind*. 1995;49:265-272.

[108] Hbid C, Jacques P, Razafindralambo H, Mpoyo MK, Meurice E, Paquot M, Thonart P. Influence of the production of two lipopeptides, Iturin a and Surfactin S1, on oxygen transfer during *Bacillus subtilis* fermentation. *Appl BiochemBiotechnol*. 1996;57-58(1):571-579.

[109] Shih I-L, Lin C-Y, Wu J-Y, Hsieh C. Production of antifungal lipopeptide from *Bacillus subtilis* in submerged fermentation using shake flask and fermentor. *Korean J Chem Eng*. 2009;26(6):1652-1661.

[110] Sen R, Babu KS. Modeling and optimization of the process conditions for biomass production and sporulation of a probiotic culture. *Process Biochem*. 2005;40(7):2531-2538.

[111] Man Z-W, Rao Z-M, Cheng Y-P, Yang TW, Zhang X, Xu MJ, Xu ZH. Enhanced riboflavin production by recombinant *Bacillus subtilis* RF1 through the optimization of agitation speed. *World J Microbiol Biotechnol*. 2014; 30(2): 661-667.

[112] Wang DIC, Cooney CL, Demain AL, Dunnill P, Humphrey AE, Lilly MD. Translation of laboratory, pilot, and plant scale data. *Ferment Enzym Technol Wiley, New York*. 1997:194-211.

[113] Seletzky JM, Noak U, Fricke J, Welk E, Eberhard W, Knocke C, Buchs J. Scale-up from shake flasks to fermenters in batch and continuous mode with *Corynebacterium* glutamicum on lactic acid based on oxygen transfer and pH. *Biotechnol Bioeng*. 2007;98(4):800-811.

[114] Trujillo-Roldán MA, Valdez-Cruz NA, Gonzalez-Monterrubio CF, Acevedo-Sanchez EV, Martínez-Salinas C, García-Cabrera RI, Gamboa-Suasnavart RA, Marín-Palacio LD, Villegas J, Blancas-Cabrera A. Scale-up from shake flasks to pilot-scale production of the plant growth-promoting bacterium *Azospirillum brasilense* for preparing a liquid inoculant formulation. *Appl Microbiol Biotechnol*. 2013;97(22):9665-9674.

[115] Maier U, Losen M, Büchs J. Advances in understanding and modeling the gas-liquid mass transfer in shake flasks. *BiochemEng J*. 2004;17:155-167.

[116] Meier K, Klöckner W, Bonhage B, Antonov E, Regestein L, Büchs J. Correlation for the maximum oxygen transfer capacity in shake flasks for a wide range of operating conditions and for different culture media. *BiochemEng J*. 2016;109:228-235.

[117] Malusá E, Sas-Paszt L, Ciesielska J. Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. *Sci World J*. 2012;2012:1-12.

[118] Chung S, Lim JH, Kim SD. Powder formulation using heat resistant endospores of two multi-functional plant growth promoting rhizobacteria *Bacillus* strains having phytophtora blight suppression and growth promoting functions. *J Appl Biol Chem*. 2010;53(4):485-492.

[119] Gotor-Vila A, Usall J, Torres R, Abadias M, Teixidó N. Formulation of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 using different approaches: liquid, freeze-drying and fluid-bed spray-drying. *BioControl*. 2017;62(4):545-555.

[120] Omer AM. Bioformulations of bacillus spores for using as Biofertilizer. *Life Sci J*. 2010;7(4):124-131.

[121] Schoebitz M, López MD, Roldán A. Bioencapsulation of microbial inoculants for better soil-plant fertilization. A review. *Agron Sustain Dev*. 2013;33 (4): 1-10.

[122] Morgan CA, Herman N, White PA, Vesey G. Preservation of micro-organisms by drying; A review. *J Microbiol Methods*. 2006;66(2):183-193.

[123] Morgan C, Vesey G. Freeze-Drying of Microorganisms. *Encycl Microbiol*. 2009; 162-173.

[124] Han L, Pu T, Wang X, *et al.* Optimization of a protective medium for enhancing the viability of freeze-dried *Bacillus amyloliquefaciens* B1408 based on response surface methodology. *Cryobiology*. 2018.

[125] Zhan Y, Xu Q, Yang MM, *et al.* Screening of freeze-dried protective agents for the formulation of biocontrol strains, *Bacillus cereus* AR156, *Burkholderiavietnamiensis* B418 and *Pantoeaagglomerans* 2Re40. *Lett Appl Microbiol*. 2012;54(1):10-17.

[126] Mahidsanan T, Gasaluck P, Eumkeb G. A novel soybean flour as a cryoprotectant in freeze-dried *Bacillus subtilis* SB-MYP-1. *LWT - Food Sci Technol*. 2017;77:152-159.

[127] Jayasudha SM, Kirankumar KC, Mesta RK, Ippikoppa R. Liquid Formulation Using Different Oils and Shelf Life Study of Effective Bacterial Bio-Agents. *IntJCurrMicrobiolAppSci*. 2018;7(4):317-324.

[128] Schisler DA, Slininger PJ, Behle RW, Jackson MA. Formulation of *Bacillus* spp. for Biological Control of Plant Diseases. *Phytopathology*. 2004;94(11):1267-1271.

[129] Kim S-Y, Kim H-E, Kim Y-S. The potentials of *Bacillus licheniformis* strains for inhibition of *B. cereus* growth and reduction of biogenic amines in cheonggukjang (Korean fermented unsalted soybean paste). *Food Control*. 2017;79:87-93.

SAŽETAK

Mikrobne formulacije sa bakterijama iz roda *Bacillus*: Optimizacija procesa proizvodnje

Stamenković Stojanović Sandra[1], Karabegović Ivana[1], Beškoski Vladimir[2], Nikolić Nada[1] i Lazić Miodrag[1]

[1]*Univerzitet u Nišu, Tehnološki fakultet, Bulevar Oslobođenja 124, 16000 Leskovac*
[2]*Univerzitet u Beogradu, Hemijski fakultet, Studentski trg 12-16, 11000 Belgrade*

(Pregledni rad)

Mikrobiološke formulacije bazirane na vrsti *Bacillus*, zahvaljujući svojim osobinama da produkuju raznovrsne antimikrobne metabolite i formiraju endospore, imaju široku primenu na različitim poljima: od farmacije i medicine do zaštite životne sredine i poljoprivrede. U poslednje vreme, posebnu popularnost stekle su kao biopesticidni i fitostimulatorni agensi, koji predstavljaju „zelenu“ alternativu prekomerno korišćenim agrohemikalijama koje zagađuju životnu sredinu. Kako bi se dobio kvalitetan i dugotrajan mikrobni proizvod željenih karakteristika, potrebno je izvršiti optimizaciju proizvodnog procesa u svakoj njegovoj fazi, što podrazumeva usaglašavanje mikrobne vrste, tipa i uslova gajenja kao i tipa formulacije i formulacionih tehnologija. Ciilj ovog rada je da omogući sažet i jasan pregled najbitnijih saznanja u ovoj oblasti, koja se tiču karakteristika mikrobnih preparata i specifičnih kriterijuma koje treba dostignuti tokom njihove formulacije. Trebalo bi da posluži kao polazna osnova svima koji započinju novo istraživanje, ne samo na polju biofertilizacije i biološke kontrole biljnih bolesti, već generalno na polju biohemijskog inženjerstva.

Ključne reči: mikrobne formulacije, optimizacija, fermentacija, *Bacillus*