Bacillus based microbial formulations: Optimization of the production process

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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.

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

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

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 $10^9$ 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, CO₂ 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
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 in the 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 albeit 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].
Table 2. Medium optimization for cultivation of different Bacillus species using RSM

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>The aim of the work</th>
<th>Experimental design*</th>
<th>Model p and F value</th>
<th>Optimum proposed factor values</th>
<th>Optimum predicted response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Biomass production</td>
<td>CCD</td>
<td>p = 0.05 F = 6.08</td>
<td>pH 6.72, 0.164 % ammonium citrate and 0.85 % peptone</td>
<td>10.051×10⁹ CFU/mL</td>
<td>[84]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Biomass production</td>
<td>BBD</td>
<td>-</td>
<td>30 g/L soybean meal, 6 g/L (NH₄)₂SO₄, 6 g/L MgSO₄</td>
<td>0.99±0.04×10⁹ CFU/mL</td>
<td>[15]</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>Biomass production</td>
<td>BBD</td>
<td>-</td>
<td>16.18 g/L corn steep liquor, 17.53 g/L soybean flour, 8.14 g/L yeast extract</td>
<td>1.52×10¹⁰ spores/mL</td>
<td>[85]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Biomass production</td>
<td>CCD</td>
<td>p &lt; 0.0001 F = 46.47</td>
<td>13 g/L soybean peptone, 0.05 g/L CaCl₂</td>
<td>3.033 g/L (CDW)</td>
<td>[86]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Biomass production</td>
<td>CCD</td>
<td>P = 0 F = 32.39</td>
<td>pH 7, T=30 °C C/N ratio: 23:1.</td>
<td>0.5 g/L in semi industrial bioreactor</td>
<td>[87]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Lipopeptide production</td>
<td>CCRD</td>
<td>-</td>
<td>1.098 g/L glucose, 4.01 g/L monosodium glutamate, 0.426 g/L yeast extract, 0.431 g/L MgSO₄×7H₂O, and 0.219 g/L K₂HPO₄</td>
<td>1.501 g/L</td>
<td>[42]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Lipopeptide production</td>
<td>CCD</td>
<td>-</td>
<td>1.11 % Glucose, 0.7 % corn steep powder</td>
<td>132.23 mg/L</td>
<td>[88]</td>
</tr>
<tr>
<td><em>Bacillus sp. BH072</em></td>
<td>Iturin production</td>
<td>CCD</td>
<td>-</td>
<td>0.98 % sucrose, 0.94 % soybean meal, and 0.93 % Mg²⁺</td>
<td>52.21 mg/ml.</td>
<td>[89]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Iturin production</td>
<td>CCD</td>
<td>p &lt; 0.0001 F = 91.49</td>
<td>0.998 g/L glucose, 1.83 g/L soybean flour</td>
<td>5.591 mg/kg iturin</td>
<td>[90]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Carboxymethyl cellulase production</td>
<td>CCD</td>
<td>p = 0.00046 F = 8.78</td>
<td>2 % substrate concentration, 2 % inoculum size, 1 % yeast extract, pH 5.0; incubation T = 50 °C</td>
<td>3.50 ± 0.11 IU/ml</td>
<td>[44]</td>
</tr>
</tbody>
</table>

*BBD- Box-Benken 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 (kLa). In addition to directly applying experimental methods such as sulfite oxidation method, adsorption or a dynamic method, kLa 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 kLa 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 kLa values at the same time decreasing production of these metabolites [108]. Similarly, Shih et. al. confirmed that the increase in kLa 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 kLa 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 amyloliquefaciens* [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, \( T = 38.3 \, ^\circ \text{C} \), agitation: 247 rpm, aeration: \( \text{V/Vm} = 1.05 \, \text{L}^{-1} \text{min}^{-1} \), and production of spores during the stationary phase under optimal sporulation conditions (pH 6.27, \( T = 41.4 \, ^\circ \text{C} \), agitation: 115 rpm, aeration: \( \text{V/Vm} = 0.33 \, \text{L}^{-1} \text{min}^{-1} \)) [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 kLa) 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 (7·10⁸) 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 \( \text{V/Vm} = 1 \, \text{L}^{-1} \text{min}^{-1} \), 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 1.52·10¹⁰ 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 \( \text{V/Vm} = 2.5 \, \text{L}^{-1} \text{min}^{-1} \) [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 et 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**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Formulation type</th>
<th>Formulation method</th>
<th>Added substances</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus meghaterium</td>
<td>Granules</td>
<td>Wet granulation technique</td>
<td>Lactose monohydrate, PVP K-30, Sodium alginate</td>
<td>[14]</td>
</tr>
<tr>
<td><em>Bacillus meghaterium</em></td>
<td>Powder formulation</td>
<td>Air drying</td>
<td>Talc, clay and cellulose; CMC, Sodium benzoate, CaCO₃, Glucose, sucrose, mannitol, yeast, peptone.</td>
<td>[120]</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefacines</em></td>
<td>Powder formulation</td>
<td>Freeze drying</td>
<td>Sucrose, powder skimmed milk, MgSO₄</td>
<td>[119]</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefacines</em></td>
<td>Liquid formulation</td>
<td>Homogenization</td>
<td>Sucrose, powder skimmed milk, MgSO₄</td>
<td>[119]</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefacines</em></td>
<td>Powder formulation</td>
<td>Fluidized bed spray drying</td>
<td>MgSO₄</td>
<td>[119]</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefacines</em></td>
<td>Powder formulation</td>
<td>Freeze drying</td>
<td>Glucose, trehalose and xylitol</td>
<td>[124]</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Powder formulation</td>
<td>Freeze drying</td>
<td>Glucose, fructose, D-galactose, sucrose, trehalose, cellobiose, glutamic acid, soluble starch, glycerol, sorbitol, peptone, nonfat skimmed milk.</td>
<td>[125]</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Powder formulation</td>
<td>Oven drying</td>
<td>Talc, CMC, CaCO₃, Glucose</td>
<td>[10]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Powder formulation</td>
<td>Freeze drying</td>
<td>Soybean flour</td>
<td>[126]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Liquid formulation</td>
<td>Homogenization</td>
<td>Groundnut oil, Pongamia oil and sunflower oil, Glycerol</td>
<td>[127]</td>
</tr>
<tr>
<td><em>Bacillus subtilis and Bacillus licheniformis</em></td>
<td>Powder formulation</td>
<td>Air drying</td>
<td>Natural zeolite, Synthetic zeolite</td>
<td>[118]</td>
</tr>
</tbody>
</table>

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 megaterium, 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 supports 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.

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SAŽETAK

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 mikrobiološke vrste, tipa i uslova gajenja kao i tipa formulacije i formulacionih tehnologija. Cilj 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 biohemijiškog inženjerstva.

Ključne reči: mikrobiološke formulacije, optimizacija, fermentacija, Bacillus