

BIOTECHNOLOGICAL PRODUCTION OF PLANT INOCULANTS BASED ON NITROGEN-FIXING BACTERIA

BIOTEHNOLOŠKA PROIZVODNJA BILJNIH INOKULANATA NA BAZI AZOTOFIKSATORA

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ABSTRACT

Nitrogen is one of the essential elements for plant growth and development in terms of DNA and protein synthesis. Its main reservoir in nature is the atmosphere; however, inert molecular nitrogen present in the air isn't a suitable nitrogen form for plants' nutrition. Therefore it has to be chemically transformed to NH_4^+ or NO_3^- ion by the process known as biological nitrogen fixation. Nitrogen fixation is carried out by free-living or symbiotic nitrogen-fixing prokaryotes (diazotrophs), including bacteria, archaea and cyanobacteria. In order to be used as plant inoculants for nitrogen fixation, the biomass of these prokaryotes must be produced and formulated appropriately through different biotechnological processes. The aim of this study is to summarize the main aspects of biotechnological production of plant inoculants based on nitrogen-fixing bacteria in terms of upstream processing, cultivation and downstream processing, with a special emphasis on cultivation media composition, cultivation conditions, biomass separation and formulation techniques.

Keywords: biological nitrogen fixation, upstream, cultivation, downstream, biomass, formulation.

REZIME

Azot je jedan od osnovnih elemenata neophodnih za rast i razvoj biljaka u pogledu sinteze DNK i proteina. Glavni rezervoar azota u prirodi predstavlja atmosfera, međutim inertna molekularna forma azota prisutna u vazduhu nije pogodna za usvajanje od strane biljaka. Stoga je neophodna transformacija molekularnog azota u NH_4^+ ili NO_3^- jone. Ovaj proces je poznat kao biološko fiksiranje azota koje vrše slobodni ili simbiotski prokarioti – azotofiksatori ili diazotrofi. Potrebe biljaka za velikim količinama azota u zemljištu obično se rešavaju dodavanjem hemijskih đubriva na bazi azota. Međutim, procene su da samo 35% dodatog azota iz azotnih đubriva biva iskorišćeno od strane biljaka, dok preostalih 65% završava u atmosferi u vidu zagađujućih gasova (azotnih oksida), u podzemnim vodama ili u zemljištu dovodeći do degradacije njegovog kvaliteta usled smanjenja vrednosti pH. Stoga se biološko fiksiranje azota javlja kao moguće rešenje za održivo povećanje količine asimilabilnog azota u zemljištu. Glavne grupe prokariotskih azotofiksatora čine bakterije, arhea i cijanobakterije. Biomasa ovih prokariota mora biti proizvedena i formulisana na odgovarajući način primenom različitih biotehnoških procesa kako bi se primenila u vidu biljnih inokulanata. Cilj ovog rada je sumiranje glavnih aspekata biotehnoške proizvodnje biljnih inokulanata na bazi bakterijskih azotofiksatora u pogledu pripreme faze proizvodnje, kultivacije i izdvajanja i prečišćavanja. Posebno se ističe poređenje sastava kultivacionih medijuma, uslova kultivacije, metoda separacije biomase i formulacije preparata. Ovaj rad daje koristan pregled dostupnih bioprocenih rešenja za proizvodnju visoko efikasnih biljnih inokulanata, kao jednog od rešenja neophodnih za povećanje održivosti poljoprivredne proizvodnje.

Ključne reči: biološko fiksiranje azota, pripreme faze proizvodnje, kultivacija, izdvajanje i prečišćavanje, biomasa, formulacija.

INTRODUCTION

Plants require nitrogen as one of the essential elements responsible for plant growth and development, whose most important role reflects in DNA and protein synthesis. The atmosphere is the main reservoir of nitrogen in nature. However, inert molecular nitrogen present in the air isn't a suitable nitrogen form for assimilation by plants (Frache et al., 2009). Therefore, molecular nitrogen from the air has to be chemically transformed to NH_4^+ or NO_3^- ions in order to be assimilable by plants. The process responsible for this transformation is known as biological nitrogen fixation and it is carried out by free-living or symbiotic nitrogen-fixing prokaryotes called diazotrophs, which are present freely in the soil or participate in a symbiotic relationship with plants aimed at providing plants with assimilable forms of nitrogen, as well as with growth factors (Bhattacharjee et al., 2008), on one hand, while, on the other hand, symbiotic plants also produce rhizospheric compounds accessible to and usable by symbiotic nitrogen-fixators. High

plant requirements for nitrogen in the soil are usually addressed by the addition of nitrogen-based chemical fertilizers. However, estimations say that only 35 % of the added nitrogen has been used by plants, while 65 % ends up in the atmosphere as polluting nitrogen oxide gases, as well as in underground waters or the soil, leading to soil degradation, mostly due to pH value decrease (Bhattacharjee et al., 2008). Hence biological nitrogen fixation has emerged as a possible solution for the sustainable increase of assimilable nitrogen form in the soil. Nitrogen fixation is also an entry point of the nitrogen cycle in nature, consisting of nitrogen assimilation by plants, ammonification, nitrification and denitrification. The main groups of nitrogen-fixing prokaryotes include bacteria (genera *Rhizobium*, *Frankia*, *Azotobacter*, *Azospirillum*, *Mycobacterium* and *Bacillus*), archaea (genera *Methanococcales*, *Methanobacteriales* and *Methanomicrobiales*) and cyanobacteria (genera *Anabaena*, *Nostoc*, *Tolypothrix* and *Anabaenopsis*) (Soumare et al., 2020).

The global market of biofertilizers has been projected to grow from \$ 800 million in 2016 to \$ 3 billion in 2024, with a dominant share of nitrogen-fixing biofertilizers of 79 %

(Soumare et al., 2020). Europe holds the second position when it comes to the production of biofertilizers, and leading position, together with Latin America, when it comes to their consumption, mostly due to strict legislative regarding usage of inorganic nitrogen fertilizers. In the Serbian market, nitrogen-fixing biofertilizers make approximately 45 % of the commercially available biofertilizers. Considering 36 registered nitrogen-fixing biofertilizers, one-third of them is imported, while the rest is produced in Serbia. Active components of commercially available nitrogen-fixing biofertilizers could be found in the following bacterial genera: *Rhizobium* – nine products, *Bradyrhizobium* – seven products, *Sinorhizobium* – one product, *Azotobacter* – 25 products, and *Azospirillum* – four products (Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia – Plant Protection Directorate, 2020).

MATERIAL

Azotobacter spp. are Gram-negative, free-living, aerobic soil bacteria with oval or spherical cell shape, able to form thick-walled cysts under unfavorable environmental conditions (Sumbul et al., 2020). Their beneficial effect on plant growth and yield is expressed through several mechanisms of action, including biosynthesis of biologically active substances, stimulation of rhizospheric microbes, producing phytopathogenic inhibitors, modification of plant nutrient uptake and ultimately boosting biological nitrogen fixation (Jnawali et al., 2015). The nitrogen fixation capacity of these heterotrophic and non-symbiotic bacteria holds approximately 20 kg of N/ha per year (Kizilkaya, 2009).

Genus *Azospirillum* includes Gram-negative, free-living nitrogen-fixing bacteria present in the rhizosphere of both cereals and grasses (Moreno-Galván et al., 2012). Twenty species of *Azospirillum* have been described so far (Fukami et al., 2018). Their ability to synthesize auxins, cytokinins, gibberellins, polyamines, ethylene and nitric oxide as plant-growth regulators have contributed to improved root development and hence better water and nutrients uptake, besides nitrogen fixation (Cassán and Díaz-Zorita, 2016). Therefore, plant inoculants based on *Azospirillum* bacterial strains have found application in maize, rice, sugarcane and wheat production (Díaz-Zorita and Fernández-Canigia, 2009; Bashan et al., 2011), leading to yield improvements up to 30 % (Vogel et al., 2013).

Rhizobia are Gram-negative, rod-shaped, aerobic and heterotrophic soil bacteria able to form a symbiosis with leguminous plants, including genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium* (Chhetri et al., 2019). They form specialized organs called nodules on the host stems or roots when it comes to legumes, but they could also act as endophytes of non-legumes as rice and maize. *Rhizobiaceae* family of the bacteria is the most abundant when it comes to bacterial nitrogen fixators making approximately 122 t of nitrogen per year in the biosphere, with a nitrogen-fixing capacity of 30-150 kg N/ha in the cultivation of legumes for grain and 50-250 kg N/ha in the cultivation of green fodder (Niewiadomska and Sawicka, 2005). The genus *Bradyrhizobium*, consisting of 41 bacterial species, is the main rhizobial genus establishing a symbiotic association with soybean, with the species *Bradyrhizobium japonicum* being the most abundant in commercial plant inoculants (Schmidt et al., 2015; Zeffa et al., 2020).

Although nitrogen-fixing bacteria are widely spread in the soil, the strains present in the certain region might not be sufficiently effective or suitable for crops selected to be grown.

It is beneficial to provide suitable inoculant for a crop on new land or in areas where effective nitrogen-fixing bacteria are not present. Therefore, there is a necessity to introduce commercial microbial inoculants based on nitrogen-fixing bacteria in order to increase productivity and reduce the usage of nitrogen-based inorganic fertilizers through biofertilization. Simultaneously, biological nitrogen fixation also contributes to the reduction of greenhouse gas emissions and energy consumption for the production of inorganic nitrogen fertilizers (Soumare et al., 2020). Furthermore, it is recommended to use autochthonous strains with genetic adaptations to the local agri-climatic conditions in the region where they should be applied in the form of microbial inoculant to achieve better results of biological nitrogen fixation (Pastor-Bueis et al., 2019). In order to be used as plant inoculants for nitrogen fixation, the biomass of bacterial nitrogen-fixators must be produced and formulated appropriately through different biotechnological processes. To achieve maximal efficiency of the microbial inoculant under field conditions, an inoculant must be simple to apply, contain the highest possible number of bacteria (at least 10^9 CFU/g), survive after introduction to soil, colonize plant rhizosphere under various soil conditions, and be compatible with farmer practices (Howieson and Dilworth, 2016). Therefore, the aim of this study is to give a useful overview of the available solutions when it comes to biotechnological production of microbial inoculants based on nitrogen-fixing bacteria, with a special perspective on upstream processing (inoculum and cultivation media preparation), cultivation conditions and downstream processing (biomass separation and product formulation). Special emphasis is given to the most abundant genera of bacterial nitrogen fixators, including *Azotobacter*, *Azospirillum*, *Rhizobium* and *Bradyrhizobium*, which are present in the majority of commercially available microbial inoculants in the Serbian, but also in the global market of nitrogen-fixing inoculants.

DISCUSSION

Considering the complexity of the biotechnological processes, it is usually recommended to observe process technology divided into three phases: upstream, cultivation and downstream processing. Upstream processing refers to preparative operations in biotechnological production, including preparation of pure culture in the form of inoculum, preparation of the medium for inoculum and cultivation, as well as sterilization of cultivation medium, process equipment and necessary consumables. Since sterility is one of the major requirements in microbial bioprocesses, sterilization methods applied in the upstream processing are not separately analyzed in this study. However, preparation of the medium for inoculum, inoculation (in terms of the ratio between inoculum and cultivation medium volume), as well as preparation of cultivation medium are important phases in upstream processing, therefore, these phases are thoroughly analyzed. As it could be observed, upstream processing is analyzed simultaneously with cultivation conditions, considering their tight correlation. Cultivation conditions in terms of temperature, pH value, agitation and aeration rate, as well as bioprocess duration, usually could be found only in the scientific papers dealing with the cultivation of nitrogen-fixing bacteria at a laboratory scale. On the other hand, scarce data regarding cultivation parameters at a pilot or industrial scale could be found due to the protection of the proprietary information. Therefore, the lack of data is the most obvious in terms of aeration conditions, considering that spontaneous aeration is usually applied at a laboratory scale. After cultivation, it is necessary to separate and purify the final

desired product (complete cultivation broth or biomass of the selected nitrogen-fixator) and to formulate it appropriately to provide high product quality in terms of efficiency and shelf life, which in this case reflects in preserving the viability of nitrogen-fixing bacteria. Different techniques were discussed when it comes to biomass separation, as well as different suggested approaches for the formulation of the final product in solid or liquid form.

Upstream processing and cultivation conditions

When it comes to cultivation media used in the production of nitrogen-fixing microbial inoculants at a laboratory scale, it could be observed that usually common synthetic or semi-synthetic media were applied, with mannitol, malic acid and glutamate as the main carbon sources, while yeast extract is usually applied as an organic nitrogen source, followed by conventional sources of phosphorus and micronutrients in the form of inorganic salts. In order to make biotechnological production cost-effective at a larger scale, the introduction of cheaper natural substrates as alternative cultivation medium components, with a special emphasis on waste materials or by-products of other technologies, was investigated. Besides the reduction of the overall bioprocess cost, utilization of cultivation media based on the waste of by-products also contributes to the minimization of the bioprocess environmental footprint. The overall temperature range during the cultivation of nitrogen-fixing bacteria corresponds to their mesophilic nature (25-30 °C). When it comes to pH value, it could be observed that its range was close to neutral values (6-8). Mixing and aeration of the liquid cultivation medium during submerged cultivation are mandatory for efficient mass and heat transfer in order to provide a sufficient amount of nutrients for biomass growth and multiplication, therefore agitation and aeration were adjusted accordingly. Bioprocess duration is usually determined by the growth phase desired to be achieved, and it also defines the type of inoculant (biomass or cultivation broth). Inoculants based on cultivation broth of nitrogen-fixing bacteria are biological formulations that combine a stable microorganism population and various types of compounds produced and released during cultivation, such as phytohormones and plant growth regulators, which are usually secondary metabolites produced during the lag growth phase, requiring longer bioprocess duration (Cassán et al., 2009). On the other hand, biomass as a product requires bioprocess termination at the end of the log growth phase, therefore these bioprocesses are characterized by shorter duration. Furthermore, bioprocess duration significantly affects overall productivity and cost, therefore it usually should be optimized to achieve maximal biomass concentration and final product activity in as short a time as possible.

Azospirillum. According to Bashan and de-Bashan (2015), there are four media suitable for cultivation (propagation) of *Azospirillum* spp.: OAB medium or modified NFb medium (in g/l: DL-malic acid 5, K₂HPO₄ 6, KH₂PO₄ 4, NaOH 3, MgSO₄·7H₂O 0.2, CaCl₂ 0.02, NaCl 0.1, NH₄Cl 1, yeast extract 0.1, FeCl₃ 0.01, in mg/l: NaMoO₄·2H₂O 2, MnSO₄ 2.1, H₃BO₃ 2.8, Cu(NO₃)₂·3H₂O 0.04, ZnSO₄·7H₂O 0.24, pH 6.8), modified TYG medium (in g/l: tryptone 5, yeast extract 5, D-glucose 5, NaCl 1.2, MgSO₄·7H₂O 0.25, K₂HPO₄ 0.13, CaCl₂ 0.22, K₂SO₄ 0.17, Na₂SO₄ 2.4, NaHCO₃ 0.5, Na₂CO₃ 0.09, Fe(III)-EDTA 0.07, pH 7.0), and BTB-1 and BTB-2 medium (glucose in TYG medium is replaced by sodium gluconate (5 g/l) and glycerol (8 ml/l), respectively). Osmotic stress-tolerant strain *Azospirillum* sp. Az19 was grown in the modified Nfb medium supplemented with 0.3 g/l of NH₄Cl for two days at 30 °C under agitation (180 rpm) to produce liquid microbial inoculant for seed treatment of maize (García et al., 2017). *Azospirillum brasilense* Az39 was

grown at 32±1 °C with an agitation rate of 140 rpm for three days using the modified NFb medium (Larraburu et al., 2016). Vinasse is a significant by-product of a sugar-ethanol industry, being generated at a rate of 9-14 l per one liter of ethanol produced (Silva et al., 2020). It is usually valorized as a fertilizer in sugarcane fields due to its nutritional characteristics, which may reduce requirements for nitrogen fertilization, especially if associated with diazotrophic bacteria. The study by Silva et al. (2020) investigated the possibility to use a vinasse-based medium (100 % vinasse, or vinasse: distilled water ratios 75:25, 50:50, 25:75) for biomass production of *Azospirillum brasilense*. The cultivations were carried out at room temperature under agitation conditions (120 rpm) for seven days. The best results in terms of bacterial growth were obtained using a medium consisting of 100 % vinasse, surpassing the cultivation in the commercial nutrient broth medium. Moreno-Galván et al. (2012) have applied sequential statistical design to develop an alternative medium for cultivation and mass-production of *Azospirillum brasilense* C16. Cultivation experiments were carried out at 28±2 °C with an agitation rate of 120 rpm for two days, while the optimized composition of the ABRA medium was as follows (g/l): glutamate 28.33, yeast extract 2.92, K₂HPO₄·3H₂O 1.34, MgSO₄·7H₂O 0.5 and FeCl₃ 0.02. The same medium and the same strain were used in the study performed by Cortés-Patiño and Bonilla (2015) under slightly different cultivation conditions: bioprocess duration was set to 27 h, and 1 % of inoculum was applied for inoculation of the ABRA medium. *Azospirillum brasilense* Sp245 was grown in the 5 l-benchtop bioreactor (working volume 3 l) for 37 h at 30 °C, pH 6.8, with dissolved oxygen concentration (DO) 3 %, and agitation rate 50–500 rpm (adjusted automatically to maintain the set DO value), while inoculum (100 ml) was prepared for 14 h at 30 °C and 180 rpm (Ona et al., 2005). Trujillo-Roldán et al. (2013) successfully performed a scale-up of the *Azospirillum brasilense* cultivation aimed at the production of liquid microbial inoculant from laboratory scale (10 l-shake flask) to pilot scale (1000 l-bioreactor).

Azotobacter. Gutiérrez-Rojas et al. (2011) have chosen sucrose and yeast extract as the optimal carbon and nitrogen sources, respectively, for the growth of *Azotobacter chroococcum*. The same nitrogen fixator was cultivated in Ashby medium (in g/l: mannitol 10, K₂HPO₄ 0.2, MgSO₄·7H₂O 0.2, NaCl 0.2, CaCO₃ 5, pH 6.8) for the production of liquid inoculant for chickpea (*Cicer arietinum* L.). Quiroga-Cubides et al. (2017) have performed scale-up of the *Azotobacter chroococcum* (strains Ac1 and Ac10) cultivation from the laboratory to the pilot scale, simultaneously with shifting of operation mode from batch to fed-batch, using the medium previously described by Moreno-Galván et al. (2012) at each scale. Cultivation in the laboratory-scale stirred tank bioreactor (total volume 5 l, working volume 3.5 l) was performed at 30±0.5 °C, airflow rate of 1 vvm (sparger ring), stirring rate of 500 rpm (Rushton turbine with two impellers) and final fermentation time of 30 h and 72 h for the strains Ac1 and Ac10, respectively. Inoculum for laboratory scale (45 ml) was prepared by suspending nitrogen fixators biomass from agar plate in sterile saline. Fed-batch cultivation was performed in the same laboratory-scale bioreactor with an initial medium volume of 1 l and with two feeding pulses to achieve a final volume of 3 l, which were applied when carbon source concentration was reduced to half of the initial value. Stirring rate reduced by 60 % compared to batch mode was applied, while other cultivation conditions remained the same. Pilot-scale cultivation was carried out in 70 l-stirred tank bioreactor (50 l working volume) equipped with the same type of air sparger and stirrer in the fed-

batch mode (starting with 80 % of the working volume with a unique pulse feeding strategy) under the following conditions: temperature 30 ± 1 °C, airflow rate 1 vvm, stirring speed adjusted according to fluid dynamic behavior scale-up criteria, cultivation duration 26 h. Inoculum for this phase was prepared in the previously described laboratory-scale bioreactor (30 ± 0.5 °C, 200 rpm, 24 h) (Quiroga-Cubides et al., 2017). In the study by Din et al. (2019), inoculum of *Azotobacter* SR-4 (50 ml) was produced at 30 °C (360 rpm, 24 h) using the following medium (g/l): sucrose 2, K_2HPO_4 0.06, KH_2PO_4 0.016, NaCl 0.02, $MgSO_4$ 0.02, yeast extract 0.05, K_2SO_4 0.01, pH 7). The same medium was used during cultivation in the laboratory-scale bioreactor (working volume 1 l) for six days. Mukhtar et al. (2018) have found that mannitol (2.5 %) as carbon source and $(NH_4)_2SO_4$ (2 %) as nitrogen source support optimal growth of *Azotobacter vinelandii* and *Azotobacter* IIB-3, while optimal cultivation conditions include temperature 30 °C, pH value 8.0, bioprocess duration two days, and inoculum volume 1 % (v/v; 24 h old culture).

Rhizobium. YMB (yeast mannitol broth) medium was used by Argaw and Akuma (2015) for the cultivation of *Rhizobium leguminosarum* bv. *viciae* (pH 6.8) under the following conditions: temperature 28 °C, agitation rate 150 rpm, bioprocess duration five days, to achieve the middle or the late exponential growth phase of the selected nitrogen-fixing strain. Slightly different conditions, but the same medium, were used for the cultivation of *Rhizobium leguminosarum* bv. *viciae* STDF-Egypt 19 by Abd-Alla et al. (2014): temperature 28 °C, agitation rate 120 rpm, bioprocess duration three days. *Rhizobium leguminosarum* bv. *trifolii* strains TA1 and CC275e were grown in the same medium at 27 °C for two days under an agitation rate of 100 rpm (Baena-Aristizábal et al., 2019). Bekele et al. (2013) have proven the possibility to replace mannitol with beet molasses and yeast extract with baker's yeast to reduce the cost of the standard YMB medium. Martyniuk and Oron (2011) have applied media based on potato extract and glucose or sucrose (1 %) inoculated with 4 % (v/v) of inoculum for the cultivation of *Bradyrhizobium japonicum* B3S, *Bradyrhizobium* sp. LZ, *Rhizobium leguminosarum* bv. *viciae* GGL and *Sinorhizobium meliloti* 330 at 28 °C with agitation rate of 150 rpm for three days. Pastor-Bueis et al. (2019) have used a pilot bioreactor (5 l) to produce microbial inoculant based on *Rhizobium leguminosarum* bv. *phaseoli* LCS0306. Cultivation was carried out using the YMB at 28 °C with 10 % of the dissolved oxygen for five days. The same bioreactor was employed for the production of inoculant based on *Rhizobium leguminosarum* bv. *phaseoli*, *Azotobacter chroococcum* and *Pseudomonas brassicacearum* subsp. *neaurantiaca* (Pastor-Bueis et al., 2021). The inoculum was prepared at 28 °C using the YM (yeast mannitol) medium for *Rhizobium leguminosarum* bv. *phaseoli* (five days), TS (tryptic soy) medium for *Pseudomonas brassicacearum* subsp. *neaurantiaca* (two days) and Ashby medium for *Azotobacter chroococcum* (five days). The growth media consisted of 2.3 % (v/v) of sugar beet molasses and 1.5 % (v/v) of beer vinasse, while cultivation was carried out at 28 °C for five days.

Bradyrhizobium. In the study performed by Egamberdieva et al. (2018) the strain *Bradyrhizobium japonicum* USDA110 was used to investigate the interactive effects of nitrogen, phosphorus, and magnesium on the symbiotic performance with soybean and its root architecture under hydroponic conditions. This strain was cultivated for 5 days using YEM (yeast extract mannitol) medium containing (g/l): mannitol 10, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1 and yeast extract 1. Research conducted by Kuwamat and Sharma (2015) has shown that

mannitol in the YEM medium could be replaced by sucrose or sorbitol with slightly lower biomass production, as well as that the optimal NaCl concentration for biomass growth and exopolysaccharide production by three *Bradyrhizobium japonicum* strains was 2 %. Rodríguez-Navarro et al. (2003) have used Alvarez medium for cultivation of *Bradyrhizobium japonicum* USDA 110 and *Sinorhizobium fredii* strains, with the following composition (g/l): K_2HPO_4 0.4, KH_2PO_4 0.4, $CaCl_2$ 0.1, KNO_3 0.7, $(NH_4)_2HPO_4$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.005, yeast extract 1, glycerol 10 ml/l. In the study by Chhetri et al. (2019) starter culture of *Bradyrhizobium japonicum* was prepared in 100 ml of YEM broth at 30 °C for four days. Mass production of *Bradyrhizobium japonicum* cultivation broth was performed in two steps using the same culture medium (pH 6.5-7) at 30 °C with the duration of 3-4 days and 4-9 days with the increase of the culture volume, respectively. Tewari et al. (2020) have used the same medium for the cultivation of *Bradyrhizobium* sp. IC-4059 under the following conditions: ratio of inoculum and cultivation medium volume 1:1000, temperature 30 °C, agitation rate 150 rpm, bioprocess duration 4 days. Albareda et al. (2008) have used several media (YEM, Alvarez and Bergersen (Bergersen, 1961)) for the production of liquid inoculants based on *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* strains at 28 °C with an agitation rate of 180 rpm. Investigation of the effect of different pH and temperature values on growth and exopolysaccharide production of three *Bradyrhizobium japonicum* strains has shown that the optimal pH value of the cultivation medium was in the range 6-7, while the temperature optimum was in the range 25-30 °C (Kuwamat and Sharma, 2015).

Downstream processing – biomass separation and product formulation

Classical techniques applied in biotechnological processes for bacterial biomass separation include centrifugation and microfiltration (Jokić et al., 2019; Jokić et al., 2020). Furthermore, flocculation as a biomass separation technique could be employed, considering that some of the nitrogen-fixing bacteria show the tendency to flocculate or aggregate. Flocculation and aggregation are usually followed by the modification of the entire vegetative cells, which form an additional cell wall or capsule and accumulate intracellular polymers, such as polyhydroxybutyrate, useful to being utilized as carbon and energy source under stress conditions (Joe et al., 2012). These methods provide a protective microenvironment against physical and chemical stresses improving bacterial survival and later release.

Most of the microbial inoculants based on nitrogen-fixing bacteria are being formulated as solid preparations, by adsorption of cultivation broth or separated bacterial biomass on different solid carriers, such as peat, clay, charcoal, cellulose powder, lignite, soil compost mixtures, or crop residues (Chhetri et al., 2019). Peat as the most widespread carrier is usually combined with different bacterial protectors and adhesives as additives (Atieno et al., 2018). However, the most abundant peat formulations don't provide effective protection of immobilized bacteria against water and temperature stress (Ardakani et al., 2010). Also, the limited availability of peat considering it is a non-renewable resource has driven a search for suitable alternative carriers. Biochar has been investigated as a possible carrier for nitrogen-fixing plant inoculants since it has been used as a soil amendment to increase soil fertility and plant growth. This fine-grained substrate rich in organic carbon is produced by pyrolysis or by heating biomass in a low-oxygen environment (Lehmann et al., 2009). The porous structure of biochar could contribute to retention of the soil moisture and improves

aeration, thus providing a habitat for bacterial proliferation after colonization of biochar micropores (Egamberdieva et al., 2017). In recent years, different formulations based on biomass immobilization using polymers have been proposed. These formulations protect immobilized cells and provide their controlled release in the soil simultaneously with polymer degradation. Therefore, the main reasons for encapsulation as an immobilization technique include preservation of cells' viability and prevention of contamination (Chhetri et al., 2019).

Azospirillum. Centrifugation was applied as a solid-liquid separation technique for harvesting *Azospirillum* Az19 cells (8142 g, 10 min) by García et al. (2017). Flocculation of *Azospirillum brasilense* MTCC125 was performed as follows: cultivation broth prepared in nutrient broth medium was mixed with 2 % sodium alginate solution (30 °C, 1 h). Alginate beads solidification took place for 30 min in 0.1 M CaCl₂ solution. After washing with saline, alginate beads were transferred to the nutrient broth for additional cultivation (2 h at 30±2 °C at 100 rpm) in order to achieve bacterial concentration prior to flocculation (Joe et al., 2012). A slightly different procedure was applied by Gonzalez et al. (2018). After centrifugation of cultivation broth at 2817 g for 5 min, biomass pellet was resuspended in sterile saline and used for the preparation of alginate beads by mixing with 1.4 % sodium alginate solution in the ratio 1:4. Beads formation was performed by the microbeads-producing device in 2 % CaCl₂ solution (1 h). After polymerization and filtration to separate liquid phase, beads were inoculated in BTB-1 medium and cultivated for 18 h at 30 °C and 120 rpm to maintain the same concentration of bacteria in the microbeads that are reduced by the polymerization process. Beads were consequently dried at 37 °C for 24 h. Cortés-Patiño and Bonilla (2015) have investigated several polymers and protectants and their protective activity in terms of *Azospirillum brasilense* C16 survival at 45 °C: carrageenan (1.5 %), sodium alginate (1 %), trehalose (10 mM), polyvinylpyrrolidone (2 %) and glycerol (10 mM). Each polymer or protectant was added to cultivation broth in the amount of 10 % (v/v) prior to incubation at 45 °C for 15 days. The best results obtained using sodium alginate (1 %) and carrageenan (0.75 %) indicated the possibility of their utilization in liquid formulations of *Azospirillum brasilense* C16.

Azotobacter. Centrifugation at 8000 g was employed to increase the concentration of *Rhizobium leguminosarum* bv. *phaseoli* cultivation broth by one order of magnitude in the study published by Pastor-Bueis et al. (2019), as well as for biomass separation of *Azotobacter chroococcum* (Pastor-Bueis et al., 2021). Rojas-Tapias et al. (2013) have investigated three preservation methods for *Azotobacter chroococcum* C26 and *Azotobacter vinelandii* C27: cryopreservation, immobilization in dry polymers and freeze-drying, which has emerged as the best technique to maintain viability and nitrogen-fixing activity in combination with S/BSA (20 % of sucrose and 10 % of bovine serum albumin) as a protective agent. Their following study (Rojas-Tapias et al., 2015) has shown that carrageenan (1.5 %) was the best polymer compared to sodium alginate (1 %) and hydroxypropyl methyl-cellulose (0.5 %) for dry preservation of *Azotobacter chroococcum* C26 since 900 days are required at 4 °C to reduce its viability in two log units. *Azotobacter vinelandii* NDD-CK-1 solid inoculants were prepared by mixing cultivation broth with the following solid carriers in ratio 0.75:1, giving the order of suitability in terms of bacterial survival: peat mixed with golden flamboyant leaf compost, peat mixed with corn stubble compost and peat mixed with mushroom waste compost (Phiromtan et al., 2013). *Azotobacter chroococcum* 76A biomass was harvested by centrifugation (3293 g) and

resuspended in 5 % sucrose solution in the ratio of 1:5 before freeze-drying and addition of quartz sand to produce solid microbial inoculant for tomato (Van Oosten et al., 2018). Abd El-Fattah et al. (2013) have investigated several formulations of *Azotobacter chroococcum* A101 (peat moss, mixture of peat moss plus vermiculite 1:2 (w/w), wheat bran, rice husk, clay, and sodium alginate), where formulations based on wheat bran and sodium alginate have shown the highest bacterial density after six months of storage.

Rhizobium. Pastor-Bueis et al. (2019) have investigated several possible combinations of carriers in the formulation of *Rhizobium leguminosarum* bv. *phaseoli*: perlite, compost and biochar from pyrolysis. All carrier materials were ground, sieved (80 µm sieve) and sterilized by autoclaving (120 °C, 20 min, while compost was sterilized for 40 min) prior to their mixing. Previously prepared bacterial culture was mixed with a cell protector (1 % (w/v) of locust bean and 1 % (w/v) of trehalose) before mixing with carriers to achieve final moisture of 50 % for peat as control and 33 % for formulations based on other carriers. The best results in terms of bacterial viability after the shelf-life assessment were obtained for a combination of 25 % perlite and 75 % biochar. Argaw and Akuma (2015) have used decomposed filter mud to formulate microbial inoculant based on *Rhizobium leguminosarum* bv. *viciae* (carrier:cultivation broth=2:1, final moisture 35 % (w/w)). Albareda et al. (2008) have tested several inorganic (perlite, attapulgite, sepiolite and amorphous silica) and organic (compost of grape bagasse and compost of cork industry residues) carriers for *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* strains. Soybean inoculated with cork compost and perlite formulations produced seed yields that were comparable to those produced by standard peat-based inoculants. Microencapsulation as a formulation technique was tested for *Rhizobium leguminosarum* bv. *trifolii* strains TA1 and CC275e (Baena-Aristizábal et al., 2019). Cultivation broths of two selected strains were mixed with peat or diatomaceous earth (Celite® 289) in the following ratio – 1:1.5:0.75 (cultivation broth: solid carrier: distilled water). Microencapsulation of the inoculated solid carriers, used as a core, was performed using guar gum and spray drying with a single-cyclone separator for collecting dry powder.

Bradyrhizobium. Encapsulation of *Bradyrhizobium japonicum* in polymeric beads could be successfully performed using sodium alginate (2 %) as a carrier, 1-3 % of sucrose as an additive and 0.1 M CaCl₂ solution as the hardening agent. Immobilized bacteria have remained viable for more than 190 days, while the increase of sucrose concentration to 5 % or 10 % has resulted in cells' survival for only five months (Chhetri et al., 2019). Another way of formulation of soybean inoculant based on *Bradyrhizobium japonicum* includes usage of perlite beads, which were mixed with cultivation broth in ratio 1:3 (solid: liquid) (Rahmani et al., 2009). Sterilized sugar mill filter mud was also used as a carrier for plant inoculant based on rhizobial strains, with 16 % gum arabic adhesive, in the field experiment with soybean performed by Waswa et al. (2014). In the study performed by Egamberdieva et al. (2017), downstream processing of *Bradyrhizobium* sp. before was performed by centrifugation (10000 rpm, 10 min). Different types of biochar were investigated as carriers for *Bradyrhizobium* sp.: hydrochar from maize silage, pyrolysis biochar from maize and pyrolysis biochar from wood. The final formulation was prepared by mixing the cultivation broth with biochar carrier (ratio 1:2, respectively). The results of this study have shown that the hydrochar from maize silage has proven as a suitable carrier for the selected nitrogen-fixing bacterium, improving its survival, as well as growth, nutrient uptake and symbiotic performance with

lupin under drought stress. Talc as a solid carrier for *Bradyrhizobium* sp. IC-4059 cells was investigated in the study performed by Tewari et al. (2020). Briefly, the final product formulation consisted of talc, cultivation broth, CMC solution (1 %) and glycerol, following the ratio: 1:1.5:0.25:0.25. Furthermore, formulations of the culture supernatant and extracted exopolysaccharides prepared in the same way as previously described were investigated separately and mixed with the cell-based formulation. The best results in field experiments with pigeon pea were obtained using the combination of all three agents, proving the thesis that more than one mechanism of action is desirable when it comes to the selection of agents for plant growth-promoting or biocontrol activities (Pajčin et al., 2020). Khavazi et al. (2007) have investigated several carriers for *Bradyrhizobium japonicum* strain CB1809: perlite with pH adjusted with calcium carbonate or charcoal, 1:4 mixtures of perlite and malt residue, sugarcane bagasse, coal, and rice husk. The results have shown that perlite-based carriers can produce high-quality inexpensive inoculants, maintaining the viability of the bacterial population for at least 6 months.

CONCLUSION

Considering a rising problem of soil contamination and degradation due to intensive farming practices, there is a necessity to replace or reduce the usage of agrochemicals – chemical pesticides and inorganic fertilizers. Biological nitrogen fixation has emerged decades ago as a suitable alternative for inorganic nitrogen fertilization. Traditional microbial inoculants are usually formulated using peat as a solid carrier. However, recent trends reflecting the necessity to replace non-renewable resources with available alternatives and burning problems regarding the rising amount of municipal and industrial waste have dictated a shift in microbial inoculants production technology. Furthermore, novel studies regarding genetic diversity and detailed characterization of nitrogen-fixing bacteria reveal the necessity to develop technology for the production of microbial inoculants based on native or autochthonous strains to be applied in a certain region, due to their better adaptability to the soil conditions and therefore higher nitrogen-fixing and plant growth-promoting activity. This study gives a useful overview of the main phases and principles of biotechnological production in order to be able to define a cost-effective bioprocess solution for the production of highly-efficient microbial plant inoculants based on nitrogen-fixing bacteria. Trends in terms of bioprocess cost reduction and increase in efficiency of the final product due to preserved bacterial viability and improved formulation, replacement of synthetic medium ingredients with readily available complex natural substrates and peat as a non-renewable carrier material, are also addressed, simultaneously with traditional approaches in the production of microbial inoculants based on nitrogen-fixing bacteria, in order to better understand existing and provide the basis for the development of novel biotechnological processes aimed at obtaining of high-quality microbial inoculants to be applied in sustainable agricultural production.

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