PRODUCTION OF BIOCONTROL AGENTS USING *Bacillus* sp. IN A LABORATORY-SCALE BIOREACTOR

PROIZVODNJA AGENASA BIOLOŠKE KONTROLE PRIMENOM *Bacillus* sp. U LABORATORIJSKOM BIOREAKTORU

Ivana PAJČIN, Zorana RONČEVIĆ, Jelena DODIĆ, Siniša DODIĆ, Aleksandar JOKIĆ, Jovana GRAHOVAC
Faculty of Technology Novi Sad, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia
email: paj@tf.uns.ac.rs

ABSTRACT

Biological control by microbial biopesticides, i.e. microbial cells or their metabolites, represents a potentially convenient and environment-friendly alternative to chemical pesticides, the excessive use of which has led to soil deterioration, environmental pollution and considerable concerns for human health. This study has investigated the possibility of biocontrol agent production using *Bacillus* sp. cultivated in a laboratory-scale bioreactor, which ensured temperature regulation (28°C) as well as the appropriate aeration (1 vvm) and mixing (250 rpm) rates of cultivation broth during the cultivation process (96 h). The cultivation broth samples were tested in vitro, using the diffusion-disc method, against three phytopathogenic isolates: two *Xanthomonas campestris* strains isolated from cabbage and the *Xanthomonas campestris* pv. *vesicatoria* strain isolated from pepper. Maximum inhibition zone diameters were obtained after 96 h of cultivation for each isolate, indicating that *Bacillus* sp. has enormous potential for further investigation and possible application as a biocontrol agent against the phytopathogens tested.

Key words: biocontrol, microbial biopesticides, *Bacillus*, *Xanthomonas campestris*, bioreactor

REZIME

Prekomerna upotreba pesticida u zaštiti poljoprivrednih kultura i suzbijanju bolesti biljaka dovela je do narušavanja kvaliteta zemljišta, zagađenja životne sredine i razvoja rezistentnosti patogena prema hemijskim proizvodima koji se uobičajeno primenjuju, što dovodi do potrebe za njihovom primenom u sve većim koncentracijama, a takođe postoji i značajna zabrinutost usled nedovoljno ispitanih i potencijalno štetnih uticaja ovih hemikalija na zdravlje ljudi. Biološka kontrola primenom mikrobioloških biopesticida, to jest ćelija mikroorganizama ili njihovih metabolita, predstavlja pogodnu ekološku alternativu hemijskim pesticidima, koja pritom doprinosi očuvanju životne sredine i omogućava manju toksičnost primenjenih sredstava prema netargetnim organizmima usled veće selektivnosti delovanja, bezbedniju primenu i otvara mogućnost korišćenja u okviru organske poljoprivrede. Cilj ovog rada bio je ispitivanje mogućnosti proizvodnje agenasa biokontrole primenom *Bacillus* sp., izolovanog iz mladog sira, kao proizvodnog mikroorganizma. Sa ciljem obezbeđivanja optimalnih uslova za izvođenje bioprocesa, kultivacija u trajanju od 96 h je vršena u laboratorijskom bioreaktoru, koji omogućava regulaciju temperature (28°C), odgovarajuću brzinu aeracije (1 vvm) i mešanje (brzina mešanja od 250 o/min). Uzorci kultivacione tečnosti su testirani in vitro primenom disk-difuzione metode protiv tri fitopatogena izolata: dva *Xanthomonas campestris* soja izolovana iz kupusa i soja *Xanthomonas campestris* pv. *vesicatoria* izolovanog iz paprike. Maksimalni prečnici zona inhibicije su dobijeni nakon 96 h kultivacije kod svakog izolata, dok su razlike njihovih vrednosti ukazale na različitu osetljivost odabranih izolata prema proizvedenim agensima. Uz prečnike zona inhibicije u opsegu 31-62 mm, *Bacillus* sp. je pokazao značajan potencijal za dalje ispitivanje i moguću primenu u biološkoj kontroli fitopatogenih *Xanthomonas campestris* sojeva.

Ključne reči: biološka kontrola, mikrobiološki biopesticidi, *Bacillus*, *Xanthomonas campestris*, bioreaktor

INTRODUCTION

Chemical pesticides are still considered the most common means of suppressing different plant diseases caused by microbial pathogens. However, their excessive utilization has resulted in soil quality deterioration, environmental pollution and the development of pathogen resistance to commonly applied agents (Mnif and Ghribi, 2015; Bursić et al., 2016), thus increasing the agent concentrations required. Considerable concern has been raised over the insufficiently examined and potentially harmful effects of these chemicals to human health and welfare (Czaja et al., 2015). Therefore, in the last two decades, an emerging initiative has been designed to seek alternative means of suppressing microbial plant pathogens. Biological control by microbial biopesticides, i.e. microbial cells or their metabolites, represents a potentially convenient and environment-friendly alternative to chemical pesticides (Chandler et al., 2008). The advantages of biopesticides over convenient chemical pesticides include a reduced toxicity to

non-target organisms, safer application, shorter persistence in the environment, and the possibility of application in the organic agriculture (Seiber et al., 2014). Different bacteria and fungi could be used as biological control agents such as the genera *Bacillus*, *Pseudomonas*, *Trichoderma*, *Streptomyces* etc. The market of biopesticides has indicated significant growth, with a constantly increasing demand for these products (a projected annual growth rate of 16.1 % has produced a global market of \$5.2 billion in 2017) (Villaverde et al., 2016). Consequently, a growing number of products for commercial application emerge based on different microorganisms.

The bacteria of the genus *Bacillus* have shown the great potential for biological control due to their ecological and physiological traits such as the sporulation ability, short reproduction cycle, ability to induce systemic resistance in plants, production of wide spectra of different metabolites (Pérez-García et al., 2011), and remarkable ability to adjust to different ecological conditions. One of the major advantages of these bacteria, regarding their application as biocontrol agents in the field, is the fact that their main habitat is soil (Hong et al.,

2009). Accordingly, these bacteria are well adjusted to the conditions where they should be applied. There are several commercial products in the European market based on the genus *Bacillus* bacteria, containing the following strains: *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus subtilis*, *Bacillus firmus* and *Bacillus thuringiensis* (Czaja et al., 2015). Some of these bacteria also show the ability to colonize plant endophytically (Card et al., 2015), which is a desirable trait for the biological control of vascular plant pathogens such as phytopathogenic *Xanthomonas campestris* strains.

Phytopathogenic strains from the genus *Xanthomonas* are well known to cause different plant diseases. Black rot of cruciferous crops and bacterial leaf spot of pepper and tomato (caused by *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *vesicatoria*, respectively) are amongst the most important and the most destructive plant diseases, causing significant crop and thereby economic losses (Obradovic et al., 2004; Singha et al., 2016). The bacteria of the genus *Bacillus* have demonstrated an intense antagonistic activity against the phytopathogenic *Xanthomonas campestris* strains, and thus represent the potential agents for the biological control of these phytopathogens (Card et al., 2015).

The purpose of this study is to investigate the possibility of producing agents for biological control of the phytopathogens from the genus *Xanthomonas* by *Bacillus* sp., under controlled conditions in a laboratory-scale bioreactor. During the cultivation process, the cultivation broth samples were analyzed to determine the residual nutrients content, biomass content and *in vitro* antimicrobial activity of the produced bioagents against the phytopathogenic *Xanthomonas* strains isolated from the diseased cabbage and pepper samples.

MATERIAL AND METHOD

Microorganisms

The producing microorganism used in this study was *Bacillus* sp. (isolated from fresh cheese). The test microorganisms were phytopathogenic isolates from diseased plants: two *Xanthomonas campestris* strains (xp 3-1 and Mn 7-2) isolated from cabbage and *Xanthomonas campestris* pv. *vesicatoria* (PAP LIST 1) isolated from pepper. The producing microorganism has been maintained on nutrient agar slant, whereas the test microorganisms have been maintained on YMA (yeast maltose agar) medium, the composition of which is shown in Table 1. All microorganisms have been stored at 4 °C and subcultured every two months.

Table 1. Composition of YMA medium

Component	Concentration [g/L]
Glucose	15
Yeast extract	3
Maltex®	3
Peptone	5
Agar	20

Inoculum preparation

Bacillus sp. was subcultured on nutrient agar slant and incubated at 28 °C during 48 h. The inoculum for the cultivation phase was prepared in two stages. The first stage included suspending the producing microorganism from agar slant to liquid medium (nutrient broth, 50 mL), and a 24-h incubation at 28 °C on a laboratory shaker (KS 4000i control, IKA® Werke, Staufen, Germany) with an agitation rate of 150 rpm. In the second stage, the inoculum from the first stage was transferred to

a larger volume of nutrient broth (150 mL) and incubated under the previously described conditions.

Cultivation

Bacillus sp. was cultivated in a laboratory-scale bioreactor (Biostat® Aplus, Sartorius AG, Göttingen, Germany) with a working volume of 2 L. The composition of the medium used for the biosynthesis of biocontrol agents is shown in Table 2. The cultivation medium pH value was adjusted to 7.0 ± 0.2 , and the medium was sterilized by autoclaving (2.1 bar, 121 °C, 20 min). The inoculation was performed under sterile conditions, and the inoculum volume amounted to 10 % of the cultivation medium volume. The following process parameters were maintained constant and automatically regulated during the cultivation process: temperature (28 °C), agitation rate (250 rpm) and aeration rate (1 vvm). The duration of cultivation was 96 h. During the first 48 h of cultivation, the broth samples were obtained from the bioreactor at 6-h intervals, whereas the sampling intervals were 12 h between 48 h and 96 h of cultivation. The samples were used to determine the biomass and residual nutrient contents, as well as to assess their antimicrobial activity against the *Xanthomonas campestris* phytopathogenic isolates.

Table 2. Composition of the medium for the production of biocontrol agents using *Bacillus* sp.

Component	Concentration [g/L]
Glycerol	15
Yeast extract	3
(NH ₄) ₂ SO ₄	1.5
K ₂ HPO ₄	3
MgSO ₄ ·7H ₂ O	0.3

Residual nutrient contents determination

The samples of cultivation broth were centrifuged at 10,000 rpm for 10 min (Rotina 380R, Hettich, Tuttlingen, Germany) to separate biomass and cultivation medium residues. The supernatants obtained after centrifugation were further analysed. The glycerol content was determined using the HPLC method. The HPLC instrument (Thermo Scientific Dionex UltiMate 3000 series) was equipped with the pump HPG-3200SD/RS, the autosampler WPS-3000(T)SL (10 µL injection loop), the column Zorbax NH2 (250 mm×4.6 mm, 5 µm) and the refractive index detector (ERC RefractoMax520, Germany). The mobile phase was 70 % acetonitrile and the following experimental conditions were established: an eluent flow rate of 1 mL/min, an elution time of 20 min and a temperature of 30 °C. The total nitrogen content was assessed using the Kjeldahl method (Herlich, 1990), whereas the total assimilable nitrogen content was determined using the Formol titration method (Zoecklein et al., 1999). The spectrophotometric method with ascorbic acid was employed to determine the total phosphorus content (Gales et al., 1966).

Determination of biomass content

The cell number in the cultivation samples, expressed as CFU/mL, was determined using the plate count method. The samples of cultivation broth (1 mL) were serially diluted to 10⁻⁷ dilution. The dilutions 10⁻⁵, 10⁻⁶ and 10⁻⁷ (1 mL) were subcultured on the nutrient agar plates and incubated at 28 °C during 72 hours, followed by counting the number of colonies.

Antimicrobial activity assaying

The diffusion-disc method (Bauer et al., 1966) was applied for antimicrobial activity testing. The test suspensions of three phytopathogenic isolates were obtained by transferring the microorganisms from YMA slant (previously incubated at 26 °C for 48 h) to saline. The test media were prepared by transferring 1 mL of the test suspensions to the melted and tempered

(50±1°C) YMA media, which were poured into Petri dishes, homogenized and left for 30 min to solidify. Sterile discs for antibiotic susceptibility testing were placed subsequently on the media in the Petri dishes (three discs per plate) and the samples of cultivation broth (15 µL) were deposited onto them. The incubation was carried out at 26 °C during 72 h, followed by the measurement of inhibition zone diameters.

Statistical data analysis

All experiments were conducted in triplicate tests, under the same experimental conditions. The results obtained are presented as average values with standard deviations, calculated using Microsoft® Excel 2010 software (MS Office, Microsoft Corporation, USA). The statistical analysis of the experimental data was performed using the Statistica 13 software (Dell Inc., USA). The Levene's test was applied to test the hypothesis of variance homogeneity. The one-way ANOVA and post-hoc testing, using the Duncan's multiple range test, were also performed. Using all the tests applied, the statistical analysis was carried out at a significance level of 0.05.

RESULTS AND DISCUSSION

The production of biocontrol agents using *Bacillus* sp. was carried out in a laboratory-scale bioreactor under the previously defined conditions of temperature, agitation and aeration rate during 96 h. During the cultivation process, the samples of cultivation broth (obtained at defined periods) were analyzed in order to determine the biomass and residual nutrient contents, as well as the antimicrobial activity of produced biocontrol agents. The antimicrobial activity of the cultivation broth samples was determined *in vitro* using the disc-diffusion method and the phytopathogenic *Xanthomonas campestris* and *Xanthomonas campestris* pv. *vesicatoria* strains (isolated from diseased plants). Inhibition zone diameters, as direct indicators of antimicrobial activity, were measured after 72 h of incubation. The Levene's test confirmed no significant differences between the variances of the parameters examined ($p > 0.05$). The shifting of biomass content during the cultivation process is shown in Fig. 1. After inoculation, the biomass content at the beginning of cultivation was $4 \cdot 10^6$ CFU/mL. During the cultivation process, the biomass content increased until the end of the cultivation process. In the first 12 hours of cultivation, the biomass content increase was slow due to the adaptation of the producing microorganism to the cultivation medium and conditions in the bioreactor. After 12 hours of cultivation, the biomass content increased significantly until 36 h of cultivation, indicating an intensive growth of the producing microorganism in the exponential growth phase. Subsequently, the biomass content was augmenting until the end of the cultivation process, but at a slightly slower growth rate, which complies with the stationary growth phase in which the microorganism continues to reproduce, but most of the nutrient uptake is used for the production of secondary metabolites. The final biomass content after 96 h of cultivation amounted to $3 \cdot 10^8$ CFU/mL, with a biomass content increase of 2 log units during the cultivation process. The residual contents of main nutrients, i.e. glycerol, total nitrogen, assimilable nitrogen and total phosphorus, determined at defined sampling periods during the cultivation process, are shown in Fig. 2. The residual nutrient contents were determined from the supernatants obtained after the centrifugation of the cultivation broth samples (in order to remove the producing microorganism biomass and thus prevent analysis errors that could arise from the nutrients incorporated in the cells of *Bacillus* sp). As can be seen in Fig. 2, the residual glycerol content decreased from 15.37 g/L at the beginning of

cultivation to 10.43 g/L at the end of cultivation (a decrease of 33.33%).

Fig. 1. Content of *Bacillus* sp. biomass during the cultivation process

A slow consumption of glycerol by the producing microorganism is recorded during the first 12 hours of cultivation, responding to the lag growth phase. Faster glycerol consumption rates could be noticed between 12 and 24 h of cultivation, which indicated a utilization of this nutrient for cell reproduction. Compared to the glycerol consumption, the total nitrogen content showed a larger decrease between 18 and 30 h of cultivation, whereas after 36 h of cultivation there were no significant changes recorded in the total nitrogen content until the end of the cultivation process ($p = 0.0516$). The total nitrogen content was reduced by 12.09 %, whereas the assimilable nitrogen content was reduced by 39.02 %. With regard to phosphorus sources, the total phosphorus content decreased from 0.70 g/L to 0.51 g/L (27.14 %), with the most significant decrease occurring between 18 and 24 h of cultivation ($p = 0.0030$). A significant decrease in the quantity of each nutrient during the exponential phase indicates that larger amounts of nutrients were utilized for growth and cell reproduction than for the synthesis of secondary metabolites during the stationary growth phase (Sanchez and Demain, 2002). Considering that only a small share of each nutrient was depleted by the producing microorganism and used for growth and metabolic activity, and the fact that the applied nutrients remained, for the most part, in the cultivation broth after the cultivation process, it can be concluded that further optimization of the cultivation medium composition should be reviewed. The optimization of the cultivation medium composition in this case implies the reduction of the initial nutrient contents in order to decrease the residual nutrient contents and improve bioprocess productivity, as well as to lower total bioprocess costs, which are conditioned by the reduction in the cultivation medium price and the costs of necessary effluent treatments (Rončević et al., 2014). Furthermore, a techno-economic analysis relative to the bioprocess duration should be performed in order to determine whether the bioprocess costs could be reduced by shortening the cultivation time. The antimicrobial activity of the cultivation broth samples, expressed as inhibition zone diameters against the phytopathogenic *Xanthomonas* isolates, is shown in Fig. 3.

The isolate *Xanthomonas campestris* xp 3-1 was the most sensitive to the antimicrobial agents produced by *Bacillus* sp., whereas *Xanthomonas campestris* pv. *vesicatoria* PAP LIST 1 exhibited the highest resistance to the produced agents.

Fig. 2. Residual contents of nutrients during the cultivation of *Bacillus* sp.

Fig. 3. Inhibition zone diameters of the cultivation broth samples against the *Xanthomonas campestris* phytopathogenic isolates

As in previous cases, the most significant increase in the inhibition zone diameters for each isolate was observed during the first 30 h of cultivation, indicating that the biomass of *Bacillus* sp. and extracellular products of its primary metabolism could be considered the dominant antimicrobial agents. A further increase in the inhibition zone diameters for all isolates recorded continued until the end of the cultivation process, but at a significantly slower rate for isolates xp 3-1 and Mn 7-2, which underscores that the production of secondary metabolites failed to demonstrate a significant impact on the antimicrobial activity against these two phytopathogenic strains isolated from cabbage. Conversely, a constant increase in inhibition zone diameters against *Xanthomonas campestris* pv. *vesicatoria* PAP LIST 1 during the complete cultivation process could be attributed to the antimicrobial activity of the *Bacillus* sp. biomass, as well as to its primary and secondary metabolites. This difference in the

sensitivity to the products of the secondary metabolism of *Bacillus* sp. between the *Xanthomonas* strains isolated from different sources confirms the difference between their defense and accommodation mechanisms, originating from different plant hosts and different ecological and physiological conditions to which they were adapted. Such difference is also confirmed by a different relative increase in the inhibition zone diameters during the cultivation process amounting to 14.52 %, 27.97 % and 42.59 % for xp 3-1, Mn 7-2 and PAP LIST 1 isolates, respectively. Significantly smaller inhibition zones of the biocontrol agents produced under various conditions were observed in the studies relative to *Xanthomonas campestris* pv. *campestris* (Xcc). Luna et al. (2002) have tested several *Bacillus* isolates against Xcc, and the inhibition zone diameters ranged from 13 to 17 mm. *Bacillus subtilis* R14 indicated an inhibition zone diameter of 13.6 ± 0.2 mm against Xcc (de Carvalho et al., 2010), whereas the antimicrobial activity of different *Bacillus* isolates ranged from 15 to 28 mm of the inhibition zone diameter against the same test microorganism (Issazadeh et al., 2012). In the study conducted by Deivamany and Muthamilan (2015), the inhibition zone diameter of *Bacillus subtilis* against Xcc was 9.8 mm. Markedly elevated values of the inhibition zone diameters for each isolate at the end of cultivation in this study, ranging from 54 to 62 mm, indicated an intense antimicrobial activity of the bioactive agents produced by *Bacillus* sp. and the great potential of this producing microorganism to produce biocontrol agents that could be used for the control of plant diseases commonly caused by the tested phytopathogens such as black rot of cruciferous crops and bacterial leaf spot of peppers and tomatoes.

CONCLUSION

The results obtained in this study indicate the successful production of biocontrol agents using *Bacillus* sp. in a laboratory-scale bioreactor. The biocontrol agents produced exhibited an intense antagonistic activity against the phytopathogenic *Xanthomonas campestris* isolates. High values of the inhibition zone diameters recorded against the tested phytopathogens indicate the considerable potential of the applied producing microorganism to produce biological control agents, requiring further *in vivo* testing of the agents produced. A successful application of the cultivation medium based on glycerol has increased the knowledge of raw glycerol use, i.e. the main byproduct of the biodiesel industry, as the potential carbon and energy source. A microbial conversion of the raw glycerol would reduce its increscent quantity, thus offering additional benefits in obtaining value-added products. One of the future research aims should involve the optimization of the cultivation medium composition relative to the initial and residual nutrient contents in order to

lower the total bioprocess costs by reducing the cultivation medium price and the costs of necessary effluent treatments.

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