CI&CEQ
Chemical Industry & Chemical Engineering Quarterly

Available on line at
Association of the Chemical Engineers of Serbia *AChE*
www.ache.org.rs/CICEQ

Chem. Ind. Chem. Eng. Q. 27 (2) 131–142 (2021) CI&CEQ

JOVANA GRAHOVAC
IVANA PAJČIN
VANJA VLAJKOV
ZORANA RONČEVIĆ
JELENA DODIĆ
DRAGOLJUB CVETKOVIĆ
ALEKSANDAR JOKIĆ

University of Novi Sad, Faculty of Technology Novi Sad, Novi Sad, Serbia

SCIENTIFIC PAPER

UDC 633.42:632.3:66

Xanthomonas campestris BIOCONTROL AGENT: SELECTION, MEDIUM FORMULATION AND BIOPROCESS KINETIC ANALYSIS

Article Highlights

- Antagonists from 7 genera were tested to select the most promising black rot biocontrol agent
- *B. velezensis* has showed the highest potential for suppression of *X. campestris*
- Glycerol and yeast extract were selected as optimal nutrients in *B. velezensis* cultivation medium
- Cultivation of *B. velezensis* was performed in a laboratory-scale bioreactor (2 L working volume)
- Bioprocess kinetics of biomass growth and carbon source consumption was investigated

Abstract

Black rot, caused by Xanthomonas campestris pv. campestris, is one of the most important diseases of cruciferous crops which causes significant yield losses. Biological control of black rot by microbial biocontrol agents represents a promising alternative to chemical treatments and good agricultural practices which show only limited success. This study was carried out to assess a potential of different antagonists, including Bacillus, Pseudomonas, Lactobacillus, Streptomyces, Saccharomyces and Trichoderma genera, for biological control of black rot. Cultivation broth samples and their filtrates were examined against seven X. campestris strains, isolated from diseased cruciferous plants, using the diffusion-disc method. Bacillus velezensis has showed the highest inhibition zone diameter of 35.62±3.76 mm. Afterwards, different combinations of carbon and nitrogen sources were used in the cultivation medium to maximize antagonistic activity of B. velezensis. The best combinations were glycerol and yeast extract, lactose and peptone, as well as sucrose and yeast extract, suggesting the potential of biodiesel, dairy and sugar industry effluents in the production of bioactive compounds effective against the black rot pathogen. The validation experiment was performed in a laboratory-scale bioreactor, in order to investigate bioprocess kinetics of biomass growth and carbon source consumption, using the cultivation medium containing the optimal carbon and nitrogen source.

Keywords: Bacillus, biological control, black rot, carbon source, cultivation, organic nitrogen source.

Black rot is one of the most economically important diseases of *Brassica* species, other members of *Brassicaceae* family and wild *Capparales* species [1]. The causal agent of this disease is *Xanthomonas campestris* pv. *campestris* (*Xcc*), Gram-negative aerobic bacteria which is known for producing extracellular polysaccharide xanthan. The most important host plants of this pathogen are cabbage, cauliflower, kale, broccoli and Brussels sprouts, where *Xcc* causes significant yield losses [2] because diseased crops have poor market value and rot fast after har-

Correspondence: I. Pajčin, University of Novi Sad, Faculty of Technology Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia.
E-mail: ivana.pajcin@uns.ac.rs
Paper received: 8 May, 2020
Paper revised: 29 July, 2020
Paper accepted: 26 August, 2020

https://doi.org/10.2298/CICEQ200508032G

vest. Black rot is primarily a seed-borne disease, but infected transplants, soil and crop remains could also represent a reservoir of contamination. Bacterial pathogens can be carried over from plant to plant by wind, irrigation water, aerosol, rain, insects, and by equipment and people. *Xcc* enters the plant through hydathodes on leaf margins by reabsorption of guttation droplets into the leaf [2]. Some races can infect the plant entering through stomata and colonizing apoplastic space before penetrating into the vascular tissue. This mode of infection is rare because it requires reduced hydrophobicity, *i.e.*, modification of cuticular waxes around stomata. Other sites of infection include wounds or roots, but in any case, the final destination of the pathogen is vascular tissue [1]. Infection could be latent at low temperatures, where pathogens rest in the vascular system without producing symptoms. When the temperature rises over 25 °C bacteria move through the vascular system, simultaneously producing xanthan, which plugs xylem vessels and restricts water flow, resulting in chlorotic yellow V-shaped lesions on leaves and roots. The name black rot originates from dark veins which ensue due to pathogen movement through vascular tissue. Affected tissues could become necrotic and cause premature leaves falling. Secondary infection by other pathogenic bacteria, such as *Erwinia carotovora* or *Pseudomonas marginalis*, is responsible for the rotting of the diseased plant [2]. Disease management usually includes usage of assayed healthy planting material (seeds and transplants), crop rotation, elimination of other potential inoculum sources (infected crop remains and weeds) and use of resistant cultivars, but these methods have showed only limited success in practice [3]. Reduction of disease spreading is achieved by avoiding overhead irrigation and by usage of chlorine dioxide in the irrigation water. Physical and chemical seed treatments, such as application of hot water, sodium hypochlorite, zinc sulfate, acidified cupric acetate, hydrogen peroxide and antibiotics, are also used, but these treatments are not completely efficient [2]. Therefore, researchers have been looking for alternative ways to control black rot disease, among which biological control represents the promising solution.

Biological control is one of the ecologically acceptable solutions for management of different plant diseases. It represents the usage of beneficial microorganisms and/or their metabolites for control of different plant pathogens by several mechanisms that include competition for nutrients and space, antibiosis, induced plant resistance or parasitism [4]. Utilization of bio-based agents implies reduction of chemical compounds usage in agricultural production, which leads to lower environmental pollution and decreases risks for negative effects of pesticides to human and animal health [5]. Besides, several antagonistic microorganisms used for biological control of plant pathogens show the ability to promote plant growth and induce its defensive mechanisms against pathogens [6]. Root antagonists have also proven to enrich the rhizosphere of plants with essential nutrients, providing a favorable environment for growth and multiplication of beneficial microorganisms. Many of these antagonists have also been investigated for adoption of soil contaminants [7].

Bacteria of the genus *Bacillus* are among the mostly favorable microorganisms for biological control of black rot, due to their high potential for application as biocontrol agents [8-11]. The formation of endospores is one of the desirable characteristics due to their tolerance to high temperatures, desiccation, UV radiation and organic solvents, which imply higher viability compared to vegetative cells, as well as suitability for formulation [11]. One of the most important characteristics of these bacteria is the ability to produce a wide spectrum of antibiotics and other metabolites with antagonistic activity against fungal and bacterial phytopathogens [12]. *Bacillus* species are commonly found in soil, the rhizosphere and the phyllosphere, therefore they are well adjusted to conditions where they should be applied [13]. Bacteria of this genus are known for plant growth-promoting capabilities and they have also showed ability to colonize plants endophytically, which is an important feature for biocontrol of vascular pathogens [11]. Among the *Bacillus* species, the most commonly used are *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus* [14]. *Pseudomonas* species are also suitable candidates for biological control of black rot [15]. Pseudomonads produce several bioactive metabolites, such as antibiotics, siderophores and volatile compounds [16]. There have been several mechanisms proposed for plant growth promoting activity of *Pseudomonas* spp.: stimulation of nutrients adoption, production of phytohormones, antagonistic activity against pathogens and induction of systemic resistance response [17]. *Lactobacillus* spp. have been considered as biocontrol agents due to production of different antimicrobial compounds, such as bacteriocins, organic acids, antimicrobial peptides and hydrogen peroxide [18]. Due to generally regarded as safe (GRAS) status, they usually comply with all recommendations for food and agricultural products. Different lactic acid bacteria are examined for biological control of *Xcc* [19]. Streptomycetes are

also considered as potential candidates for biological control of black rot [20], due to their beneficial trait of producing large number of different metabolites, enzymes and antibiotics with antagonistic activities against microbial pathogens [21]. Different yeasts are also investigated as biological control agents for black rot [22]. Genus *Trichoderma* represents hemibiotrophic fungi, whose main biocontrol traits are antagonism or mycoparasitism against plant pathogens and induction of systemic or localized resistance in plants. Also, fungi of the genus *Trichoderma* produce over 40 secondary metabolites that play a role in plant-microbe interaction and phytopathogen suppression [23]. *Trichoderma viride* [20] and *Trichoderma harzianum* [13] were investigated for biological control of *Xcc*.

The aim of this study was the selection of the antagonistic microorganism with the highest potential for biological control of black rot, whose antimicrobial activity was tested against seven phytopathogenic *Xcc* strains isolated from diseased cruciferous plants. After the selection of the best producing microorganism, another aim of this study was the determination of an optimal combination of carbon and organic nitrogen sources in the cultivation medium for the production of bioactive agents effective against black rot causers. Selected carbon sources were representative for different industrial waste streams in order to evaluate the potential of these waste streams in production of biocontrol agents. Validation experiment was carried out in a laboratory-scale bioreactor using the cultivation medium containing optimal carbon and nitrogen source, where the aim was to investigate bioprocess kinetics. The aims set in this study represent important steps in defining of a techno-economically viable bioprocess solution for the production of biocontrol agent for black rot management, which is a basis for potential commercialization of this product.

EXPERIMENTAL

Pathogens

Test microorganisms used in this study were pathogenic isolates of the genus *Xanthomonas*, isolated from diseased plants of *Brassicaceae* family. The pathogens were isolated using standard phytopathological techniques. All isolates were characterized according to their pathogenic, morphological and ecological characteristics as the members of *X. campestris* species [24]. Microorganisms were stored on YMA (yeast maltose agar) medium (glucose 15.0 g/L, yeast extract 3.0 g/L, malt extract 3.0 g/L, peptone 5.0 g/L and agar 20.0 g/L) at 4 °C and subcultured every four weeks. Working cultures were prepared by subculturing pathogens on YMA medium, and by incubating at 26 °C for 48 h. Test suspensions of pathogens were obtained by suspending microorganisms from YMA medium in 20 ml of sterile NaCl solution (9 g/L).

Antagonists

Antagonistic microorganisms used in this study were: *Bacillus subtilis* ATCC 6633 (M 1), *Bacillus cereus* ATCC 10876 (M 2), *Bacillus amyloliquefaciens* (M 3), *Bacillus* sp. (M 4) and *Bacillus velezensis* (M 5) isolated from soil, *Pseudomonas aeruginosa* ATCC 27853 (M 6), *Pseudomonas aeruginosa* isolated from water (M 7), *Pseudomonas putida* isolated from water (M 8), three isolates of genus *Lactobacillus* isolated from food (M 9-M 11), *Streptomyces hygroscopicus* isolated from soil (M 12), *Saccharomyces cerevisiae* (M 13) and *Trichoderma reesei* QM 9414 (M 14). Antagonists were stored on appropriate media at 4 °C and subcultured every four weeks. The selected antagonist with the highest potential to suppress *X. campestris* was identified using 16S rDNA sequencing, based on query coverage of 100% and sequence homology of 99%.

Media and cultivation conditions for screening of producing microorganisms

Media used for growth and biosynthesis of antimicrobial compounds by antagonistic microorganisms were: nutrient broth (HiMedia, India) for *Bacillus* and *Pseudomonas* strains, MRS (de Man, Rogosa and Sharpe) broth (HiMedia, India) for *Lactobacillus* strains, SM (Sabouraud maltose) broth (HiMedia, India) for *T. reesei*, while *S. hygroscopicus* and *S. cerevisiae* were grown in semi-synthetic media. Medium used for cultivation of *S. hygroscopicus* contained (g/L): glucose (5.0), soybean defatted flour (10.0), $(NH_4)_2HPO_4$ (0.5), K_2HPO_4 (1.0), $CaCO_3$ (3.0), NaCl (3.0) and $MgSO_4$ (0.5). Medium for *S. cerevisiae* cultivation contained (g/L): barley malt extract (14.0) (Malteks®, Prena-M Inžinjering, Serbia), glucose (2.0) and peptone (0.1). Volume of each medium was 50 mL, while the volume of the Erlenmeyer flasks was 200 mL. Inoculation was performed directly from tubes with microorganisms previously grown on the same medium that was used for cultivation, but containing agar (20 g/L) for 48 h at 28 °C, except for *Pseudomonas* strains, which were grown at 37 °C. Cultivation was carried out at 30 °C during 96 h, with aeration on a laboratory shaker (KS 4000i control, IKA® Werke, Germany) at agitation rate of 150 rpm.

Media and cultivation conditions for determination of an optimal carbon-organic nitrogen source combination

Inoculum was prepared by transferring the selected producing microorganism using inoculation loop from nutrient agar to an Erlenmeyer flask containing 50 mL of nutrient broth. Incubation was carried out at 28 °C for 24 h, with spontaneous aeration and agitation (150 rpm) on a laboratory shaker. After 24 h, the liquid culture was poured into an Erlenmeyer flask containing 150 mL of nutrient broth under sterile conditions, which was then incubated at 28 °C for 24 h, with spontaneous aeration and agitation (150 rpm) on a laboratory shaker. Liquid media used for production of bioactive agents by the selected producing microorganism contained (g/L): carbon source (15.0), organic nitrogen source (3.0), $(NH_4)_2SO_4$ (1.5), $MgSO_4 \cdot 7H_2O$ (0.3), K_2HPO_4 (3.0), and pH value was adjusted to 7.0±0.2 prior to sterilization performed by autoclaving at 121 °C and 2.1 bar (20 min). Varied carbon sources were glucose, sucrose, lactose, starch and glycerol, while varied organic nitrogen sources were yeast extract, peptone, soybean meal, L-glutamic acid and urea. Inoculum volume was equal to 10 vol.% of cultivation medium volume. Production of bioactive compounds was carried out in Erlenmeyer flasks (200 mL) containing 50 mL of cultivation medium at 28 °C during 96 h, with an agitation rate of 150 rpm under aerobic conditions on a laboratory shaker (KS 4000i control, IKA® Werke, Germany).

Validation experiment

Cultivation of the selected producing microorganism was carried out in a laboratory-scale bioreactor (Biostat® Aplus, Sartorius AG, Germany) using cultivation medium containing selected optimal carbon and organic nitrogen sources. Inoculum in this experimental stage was prepared in the same way as in the previous stage. Working volume of the bioreactor was 2 L, where 10 vol.% of inoculum was added compared to cultivation medium volume. Cultivation parameters were set to temperature of 28 °C, agitation rate of 250 rpm and aeration rate of 1 vvm (volume of air/volume of medium/min) using sterile air. During 96 h of cultivation, cultivation broth was sampled at predefined time intervals (12 h) to determine biomass concentration, carbon source content and antimicrobial activity of the produced bioactive agents, as well as the kinetics of microbial growth and carbon source consumption.

In vitro assaying of antimicrobial activity

In this study, antimicrobial activities of cultivation broth samples and their filtrates obtained after cultivation of the aforementioned antagonists were examined against seven *X. campestris* pathogenic isolates using the diffusion-disc method. Suspensions containing phytopathogens were prepared using sterile saline to achieve 10^8 CFU/mL. Biomass concentration in these suspensions was measured using the calibration curve between plate count and spectrophotometric measurement at 600 nm (UV 1800, Shimadzu, Japan). Each cultivation broth was filtered through nylon filter (Agilent Technologies, Germany) with pore diameter of 0.22 μm to obtain biomass-free filtrates. Commercial discs containing 30 μg of streptomycin (Torlak, Serbia) (K) were used as control to compare antimicrobial activity of the tested antimicrobial agents. Cultivation media used for antimicrobial activity assaying were prepared by adding suspension of pathogens (1 mL) to melted YMA media, which were previously tempered at 50±1 °C. After homogenization, prepared media were poured into Petri dishes. Every cultivation broth sample and its filtrate were examined against each pathogenic strain using three diffusion discs in triplicate tests. Each sample volume was 15 μL. Petri dishes were incubated at 26 °C during 72 h, and afterwards inhibition zone diameters were measured using a ruler for accurate zone reading (Antibiotic ZoneScale, Himedia®, India). *In vitro* assaying of antimicrobial activity of bioactive agents produced by the selected producing microorganism was carried out in triplicate tests against each pathogenic isolate using cultivation broth samples (15 μL) with different combinations of carbon and organic nitrogen sources.

Quantification of glycerol content

Supernatants obtained after centrifugation of cultivation broth samples at 10000 rpm during 10 min (Rotina 380R, Hettich, Germany) were used for determination of glycerol content. Glycerol content was determined using the HPLC instrument (Thermo Scientific Dionex UltiMate 3000 series, Thermo Fisher Scientific, MA, USA), which 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) (Agilent Technologies, Germany) and the refractive index detector (ERC RefractoMax520, Germany). The mobile phase was mixture of acetonitrile and bidistilled water (70:30 volume ratio) and the conditions of analysis were: eluent flow rate 1 mL/min, elution time 20 min and temperature 30 °C.

Quantification of biomass content

Biomass content was determined using DCW (dry cell weight) method. Cultivation broth samples

(20 mL) were centrifuged (10000 rpm, 10 min) to separate biomass from the liquid phase. The obtained biomass pellets were dried at 105 °C until reaching constant weight. Biomass concentration (g/L) was calculated based on the measured dry cell weight and initial volume of cultivation broth sample (20 mL).

Experimental data analysis

In vitro assaying of the produced antimicrobial agents' activity was performed in triplicate tests, and mean values of inhibition zone diameters along with standard deviations were calculated using Microsoft Excel 2010 (Microsoft Corporation, WA, USA). Obtained results were analyzed using several different statistical tests. Levene's test was applied for testing homogeneity of variances, followed by ANOVA and Duncan's multiple range tests. Statistical analysis using all applied tests was carried out at significance level of $\alpha = 0.05$ in Statistica software 13.0 (StatSoft, OK, USA).

Bioprocess kinetics investigation

Bioprocess kinetic parameters were determined using the GraphPad Prism software, v. 8.1.0 (GraphPad Software, Inc., CA, USA). Biomass growth kinetics was described using the Gompertz model (Eq. (1)):

$$X(t) = X_{\max}\left(\frac{X_0}{X_{\max}}\right)^{e^{-\mu t}} \tag{1}$$

where biomass concentration $X(t)$ during cultivation could be calculated using initial biomass concentration X_0, maximal biomass concentration $X_{\max}$, and specific growth rate μ.

Glycerol consumption was described using modified Luedeking-Piret equation (Eq. (2)):

$$S(t) = S_0 - \gamma X_0\left(\frac{e^{\mu t}}{1-\frac{X_0}{X_{\max}}\left(1-e^{\mu t}\right)}-1\right) - \delta\frac{X_{\max}}{\mu}\ln\left(1-\frac{X_0}{X_{\max}}\left(1-e^{\mu t}\right)\right) \tag{2}$$

where glycerol concentration $S(t)$ during cultivation could be calculated using initial glycerol concentration S_0, kinetic parameters previously determined using the Gompertz equation (X_0, $X_{\max}$ and μ), as well as by using γ and δ, considered as glycerol consumption constants related to growth and metabolites production, respectively [25].

RESULTS AND DISCUSSION

Analysis of antimicrobial activity of antagonistic agents against phytopathogenic *X. campestris* isolates

Production of preparations based on different microorganisms and their products for protection of agricultural crops has been gaining increasing interest in the last decade. Since microorganisms are an almost inexhaustible source of different bioactive compounds, great efforts are still invested in the development of new and efficient preparations. Therefore, this research investigates the possibility of applying different microbial antagonist for suppressing phytopathogenic isolates of *X. campestris* species. Cultivation broth samples obtained after cultivation of 14 antagonists and their filtrates, as well as commercial streptomycin discs, were tested against seven *X. campestris* isolates. Mean values of inhibition zone diameters obtained due to antimicrobial activity of cultivation broth samples and filtrates were calculated. Experimental results were analyzed using several statistical tests in order to adequately asses which preparation is the most efficient against phytopathogenic *X. campestris* isolates.

Results of the Levene's test for inhibition zone diameters measured after testing of cultivation broth samples have confirmed homogeneity of variances ($p = 0.9970$). Considering that the antagonistic effect can be expressed by both microorganisms' cells and its metabolites, inhibition zone diameters formed by the cultivation broth samples and their filtrates were compared (Figure 1). Filtration was carried out using filters with pore diameter of 0.22 μm in order to remove cells of antagonistic microorganisms.

Filtrates obtained by filtration of cultivation broth after cultivation of *B. subtilis* ATCC 6633 (M 1), *B. cereus* (M 2), *P. aeruginosa* ATCC 27853 (M 6), *Lactobacillus* sp. (M 10), *S. hygroscopicus* (M 12) and *S. cerevisiae* (M 13) didn't show antimicrobial activity against tested phytopathogenic isolates, which clearly indicates that the bioactive compound in these preparations is the biomass of cultivated antagonists. Monteiro *et al.* [11] and Issazadeh *et al.* [26] have showed similar antimicrobial activity of *B. subtilis* and *B. cereus* cultivation broths against *X. campestris* phytopathogenic strains. Research of *P. aeruginosa* strains activity has shown that bioactive compounds effective against *Xcc* [15] are metabolites contained in biomass-free filtrates or supernatants of cultivation broth, which is opposite to the results of this research. Different *Lactobacillus* strains were examined for their antimicrobial activity against pathogenic *Xantho-*

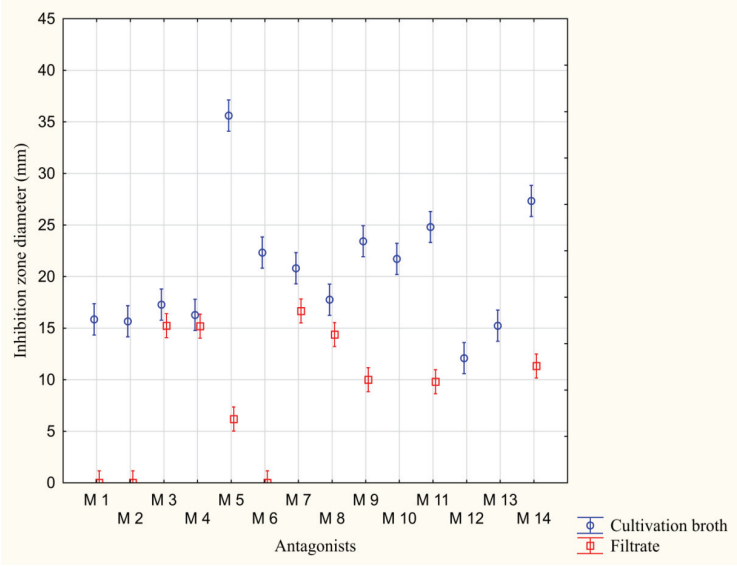

Figure 1. Inhibition zone diameters against seven Xanthomonas campestris strains for cultivation broth samples and filtrates obtained after cultivation of 14 antagonists.

monas strains. Kannan *et al.* [27] demonstrated antimicrobial activity of extracellular bioactive compounds of *Lactobacillus* against *X. campestris*. Encheva-Malinova *et al.* [28] demonstrated that cultivation broths of different *Streptomyces* isolates show antimicrobial activity against few *Xanthomonas* species. Biomass of *T. harzianum* has been shown to suppress growth of *Xcc* [13], while Deivamani and Muthamilan [20] showed antagonistic activity of different *Trichoderma* species' spores against black rot pathogen. The same authors have shown antagonistic activity of *S. cerevisiae* against *Xcc* [20].

Filtrates of cultivation broth samples obtained by cultivation of other antagonists showed certain antimicrobial activity, but in case of each antagonist, inhibition zone diameter obtained by cultivation broth testing was larger than the inhibition zone diameter obtained by filtrate testing. Statistical analysis of the presented results has showed that only for strain M 4 (*Bacillus* sp.) there wasn't statistically significant difference between inhibition zone diameters resulting from cultivation broth and filtrate activity (p = 0.2919). These results indicate that the bioactive compound is synthesized extracellular antimicrobial metabolite. On the other hand, preparations based on *Bacillus* spp. (M 3, M 5), *P. aeruginosa* from water (M 7), *P. putida* (M 8), *Lactobacillus* spp. (M 9, M 11) and *T. reesei* QM 9414 (M 14) have showed statistically significant larger inhibition zone diameters when applied as unfiltered cultivation broth, which is confirmed by p-values of 0.0023, 0.0956, 0.0051, 0.0182, 0.0000, 0.0000 and 0.0039, respectively. Higher antimicrobial activity of cultivation broth could suggest that biomass is more likely to be the bioactive agent against phytopathogenic *X. campestris* isolates.

Homogenous groups of antagonists and significance of differences were established using Duncan's multiple range test (Table 1).

Table 1. Mean values of inhibition zone diameters obtained by assaying cultivation broth samples of tested antagonists against Xanthomonas campestris isolates; values marked with the same superscript letter are at the same level of significance with confidence level of 95% (Duncan's test)

Antagonist	Inhibition zone diameter, mm
M 12	12.10±3.28[a]
M 13	15.24±3.40[b]
M 2	15.67±3.32[bc]
M 1	15.86±3.31[bc]
M 4	16.29±3.49[bc]
M 3	17.29±3.36[bc]
M 8	17.76±3.85[c]
M 7	20.81±3.72[d]
M 10	21.71±3.48[de]
M 6	22.33±3.29[de]
M 9	23.43±3.44[ef]
M 11	24.81±3.72[f]
M 14	27.33±3.80[g]
M 5	35.62±3.76[h]
K[a]	36.10±3.53[h]

[a]Commercial streptomycin disks

Results presented in Table 1 show that cultivation broth samples for each examined antagonist have manifested antimicrobial activity against tested phytopathogenic *X. campestris* isolates and that there

was statistically significant difference between their activities. The antagonist that has showed the weakest antimicrobial activity (the smallest inhibition zone diameter) against tested phytopathogens was *S. hygroscopicus* (M 12), while the best antagonist for this purpose was *B. velezensis* (M 5), which has shown the highest potential for suppressing infections caused by phytopathogenic *X. campestris* strains. In order to confirm antagonistic activity of biomass of *B. velezensis*, centrifugation of the cultivation broth sample was performed (10000 rpm, 10 min). The obtained solid phase after the supernatant had been discarded was resuspended in the same volume of sterile saline and homogenized using vortex. Antimicrobial activity of resuspended biomass was also tested using the diffusion-disc method against the tested phytopathogenic *X. campestris* strains under the previously defined conditions. Mean value and standard deviation of the obtained inhibition zone diameters was 32.85±3.82 mm, suggesting that the most of *B. velezensis* antimicrobial effect arises from biomass activity. Furthermore, presented results point out that there was no statistically significant difference between antimicrobial activity of this antagonist and bactericidal activity of commercial streptomycin discs (K) (p = 0.6615), indicating that *B. velezensis* has a great potential for application in biological control of diseases caused by phytopathogenic *X. campestris* strains.

Analysis of different carbon and organic nitrogen sources' effects on antimicrobial activity of *B. velezensis* against phytopathogenic *X. campestris* isolates

Since *B. velezensis* has arisen as the best potential antagonistic agent among tested antagonists, the next step was to determine the optimal combination of carbon and organic nitrogen sources in the medium for its cultivation. Cultivation broth samples of this isolate were tested against phytopathogenic *X. campestris* strains in further assays, since they have shown a larger inhibition zone diameter compared to filtrate. The chosen carbon sources were selected to represent the main nutrients in effluents of different food industries in order to estimate the potential of application of different waste streams in production of bioactive agents effective against black rot pathogens. Glucose was selected as the main carbon source in effluents from fruits and vegetables processing, as well as in vinery wastewaters, simultaneously being most widely used sugar in biotechnological production. Industrial waste containing high levels of glucose was used for biosurfactant production by *B. subtilis* [29]. Sucrose is the main component of molasses, a by-product of sugar beet and sugar cane processing. Molasses has been mainly used as cultivation medium for biosurfactants production by different *Bacillus* strains [30]. Płaza *et al.* [31] investigated antifungal activity of Bacilli cultivated on different agro-industrial wastes, including molasses. Lactose is mainly distributed in waste streams from the dairy industry. Gomaa [32] investigated antimicrobial activity of biosurfactant produced by *B. licheniformis* M104 using whey as cultivation medium. Starch can be found in wastewaters and effluents of grains, cassava and potato processing. Cassava wastewater was proved as good cultivation medium for production of surfactant by *B. subtilis* [33]. Starch processing water was used for production of *B. thuringiensis* biopesticide [34]. Glycerol is obtained in significant amount as by-product of continuously growing biodiesel industry and there has been significant scientific interest in its conversion to different value-added products using microorganisms [35]. Crude glycerol from biodiesel synthesis was used as carbon source in cultivation medium for production of biosurfactants by *B. subtilis* ATCC 6633 [36]. Examined organic nitrogen sources were commonly used yeast extract, peptone, soybean flour, L-glutamic acid and urea.

Two-way ANOVA was employed to determine whether there were significant differences between inhibition zone diameters obtained by assaying cultivation broth samples of *B. velezensis* against seven *X. campestris* phytopathogenic isolates, as a result of using different carbon and organic nitrogen sources in the cultivation medium. ANOVA results have showed that the effects of carbon and organic nitrogen sources, as well as their interaction, were significant (p-values less than 0.05, Table 2).

Table 2. Two-way ANOVA of inhibition zone diameter obtained by assaying Bacillus velezensis cultivation broth samples against Xanthomonas campestris isolates; SS - sum of squares; DF - degree of freedom; MS - mean square

Source of variability	SS[1]	DF[2]	MS[3]	F	p-value
Carbon source	717.7	4	177.9	13.63	0.0000
Nitrogen source	1506.0	4	376.5	28.85	0.0000
Carbon source*Nitrogen source	1954.1	16	122.1	9.36	0.0000
Error	1631.5	125	13.1	-	-

In order to determine which combination of carbon and organic nitrogen source is the most suitable for production of bioactive agents effective against tested *X. campestris* isolates by *B. velezensis*, Figures 2 and 3 were generated, which present the mean values of inhibition zone diameters for each carbon and organic nitrogen source, respectively. As it can be seen in Figure 2, the best carbon sources were glycerol and lactose, followed by starch and sucrose, while the presence of glucose in the cultivation medium has resulted in the lowest inhibition zone diameter. These results indicate the high potential of glycerol, as the by-product of biodiesel production, and dairy waste streams, which contain lactose, for production of bioactive agents by *B. velezensis* for biological control of black rot. Considering that the ability to degrade β-1→4 glycosidic bond of lactose is not a common characteristic among microorganisms [37], utilization of dairy industry waste streams by microbial conversion is therefore limited. Since the dairy industry generates one liter of wastewater per one liter of produced milk [38], the fact that *B. velezensis* metabolize lactose as a carbon source opens up a new chapter of possibilities for dairy industry waste streams utilization for the production of value-added microbial products. On the other hand, the ability of *B. velezensis* to metabolize glycerol presents a basis for utilization of raw glycerol from biodiesel industry. This effluent represents a major problem since it has been generated in large amounts (10% compared to the produced biodiesel amount) and contains very high concentrations of different impurities arising from raw materials used for biodiesel production, as well as from the biodiesel production process itself [39]. There is an ongoing scientific challenge to reveal possible routes for raw glycerol exploitation, where microbial conversion into different microbial products represents one of the attractive solutions [39]. Therefore, further research of production of biocontrol agents by *B. velezensis* will include the investigation of suitability of raw glycerol as a carbon source in this bioprocess.

When it comes to organic nitrogen sources (Figure 3), yeast extract was shown to be the best solution, followed by peptone, L-glutamic acid and urea. Since amino acids are the preferred nitrogen source for the most *Bacillus* species, yeast extract, that contains variety of amino acids, and peptone, which contains peptides and amino acids, were shown to be the best nitrogen sources for *B. velezensis*. Although yeast extract has been considered an expensive nitrogen source, further optimization of cultivation medium composition in terms of nutrients' content would show the required amount of this nitrogen source and the economical profitability of its application in cultivation medium. Also, the usage of complex carbon sources, such as dairy wastewater and molasses, could satisfy producing microorganism's nutritional requirements for amino acids, therefore optimization could show that the required amount of organic nitrogen source could be low. Soybean flour, although the economically most payable organic nitrogen source, was shown to be the least suitable solution in this particular case.

Mean values of inhibition zone diameters obtained by testing *B. velezensis* cultivation broth

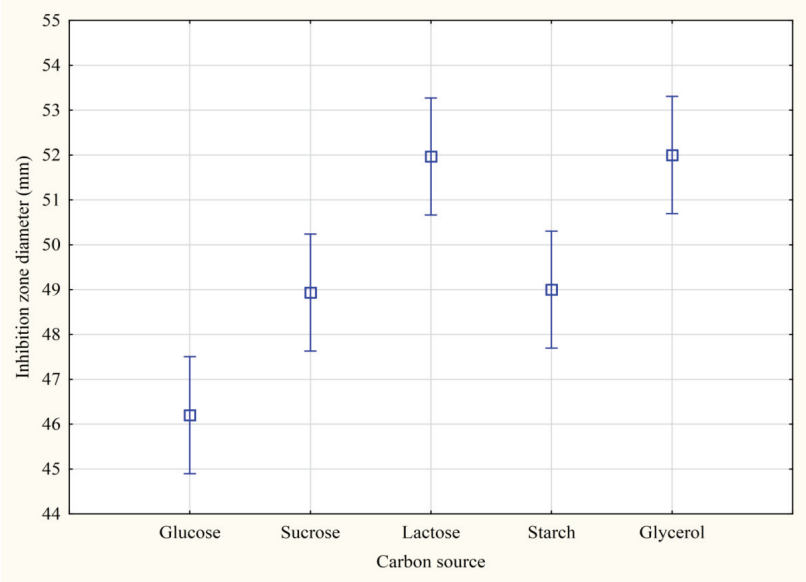

Figure 2. Inhibition zone diameters against seven Xanthomonas campestris strains obtained using different carbon sources in cultivation medium for Bacillus velezensis.

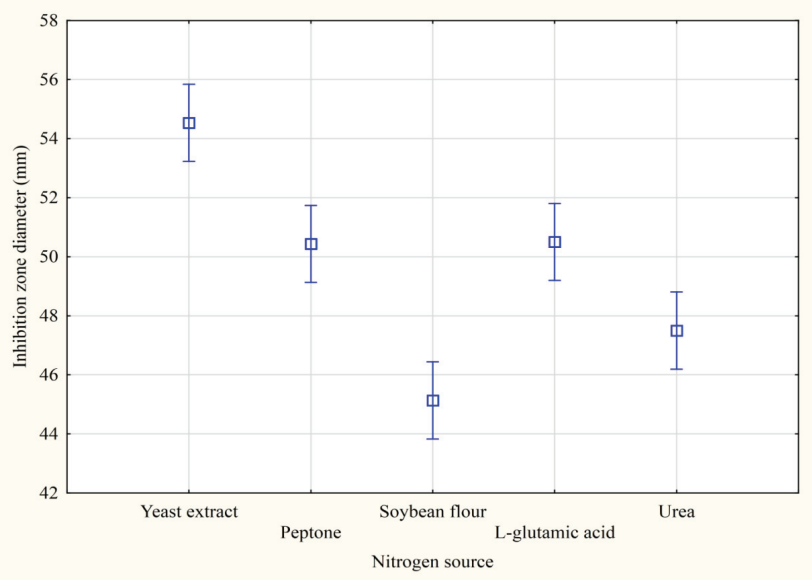

Figure 3. Inhibition zone diameters against seven Xanthomonas campestris strains obtained using different nitrogen sources in cultivation medium for Bacillus velezensis.

samples against seven *X. campestris* isolates for each investigated combination of carbon and organic nitrogen sources and the results of Duncan's test are given in Table 3. Duncan's test was performed to examine whether any combinations of carbon and organic nitrogen sources are at the same level of statistical significance.

As the results presented in Table 3 show, the lowest antimicrobial activity of *B. velezensis* was evinced when sucrose and L-glutamic acid were used as carbon and organic nitrogen sources in the cultivation medium. The highest inhibition zone diameter was obtained when a combination of glycerol and yeast extract was used, but data also suggests that combinations of lactose and peptone, as well as sucrose and yeast extract, are at the same level of statistical significance, indicating that the potential of these carbon sources, *i.e.*, dairy effluents and molasses, is only slightly lower than the potential of glycerol when it comes to the production of biocontrol agents. Since molasses, as a sucrose source, and dairy wastewater, as a lactose source, contain significantly smaller amounts of amino acids compared to standard organic nitrogen sources, as yeast extract, these cultivation media should be supplemented with yeast extract or peptone as the preferred nitrogen sources by *B. velezensis*.

Bioprocess kinetics

Since the previously discussed experimental results suggest that biomass of *B. velezensis* presents the major biocontrol agent against phytopathogenic *X. campestris* strains, it is of a great importance

Table 3. Mean values of inhibition zone diameters obtained by assaying Bacillus velezensis cultivation broth samples against Xanthomonas campestris isolates, obtained due to usage of different carbon and organic nitrogen sources in cultivation medium; values marked with the same superscript letter are at the same level of significance with confidence level of 95% (Duncan's test)

Carbon source	Nitrogen source	Inhibition zone diameter, mm
Sucrose	L-Glutamic acid	40.83 ± 0.41^{a}
Glucose	Soybean flour	41.33 ± 2.66^{ab}
Lactose	Urea	44.00 ± 1.26^{abc}
Starch	Soybean flour	44.83 ± 0.75^{abc}
Sucrose	Soybean flour	44.83 ± 0.98^{abc}
Glucose	Yeast extract	45.83 ± 1.33^{bcd}
Glycerol	Peptone	45.83 ± 1.33^{bcd}
Glucose	Peptone	46.50 ± 3.08^{cde}
Starch	Urea	46.67 ± 2.25^{cde}
Lactose	Soybean flour	46.83 ± 4.31^{cde}
Glucose	Urea	47.50 ± 0.84^{cdef}
Glycerol	Soybean flour	47.83 ± 5.31^{cdef}
Starch	Peptone	48.67 ± 1.21^{cdef}
Sucrose	Urea	48.83 ± 0.98^{cdef}
Glucose	L-Glutamic acid	49.83 ± 4.75^{defg}
Glycerol	Urea	50.50 ± 3.56^{defgh}
Starch	L-Glutamic acid	51.33 ± 5.20^{efghi}
Sucrose	Peptone	51.67 ± 0.52^{fghi}
Starch	Yeast extract	53.50 ± 6.32^{ghi}
Lactose	Yeast extract	54.00 ± 5.55^{ghi}
Glycerol	L-Glutamic acid	55.00 ± 2.61^{hij}
Lactose	L-Glutamic acid	55.50 ± 1.76^{ijk}
Sucrose	Yeast extract	58.50 ± 3.87^{jkl}
Lactose	Peptone	59.50 ± 7.15^{kl}
Glycerol	Yeast extract	60.83 ± 6.21^{l}

to investigate the kinetics of biomass growth, as well as the kinetics of carbon source (*i.e.*, glycerol) consumption, and to establish kinetic models suitable for description of these bioprocess outcomes. Data required for determination of bioprocess kinetic parameters are biomass concentration and glycerol content in different periods during the bioprocess. These data were gathered by sampling the cultivation broth during a validation experiment in a laboratory-scale bioreactor at predefined time intervals. The obtained kinetic parameters using the Gompertz model for biomass growth and modified Luedeking-Piret Equation for glycerol consumption are given in Table 4.

Experimental data fitting using the obtained parameters of Gompertz biomass growth model and modified Luedeking-Piret equation for glycerol consumption are given in Figures 4 and 5, respectively.

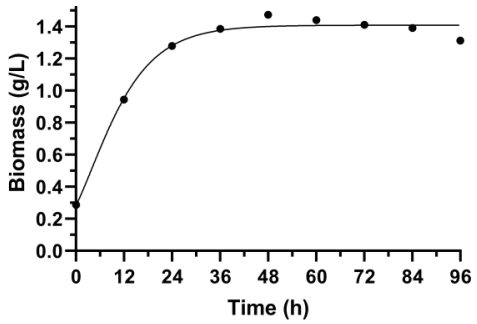

Figure 4. Experimental data fitting for Bacillus velezensis biomass concentration using Gompertz growth model.

Initial biomass concentration obtained using the Gompertz growth model was 0.282 g/L, which is in accordance with the experimental value of 0.287 g/L. Exponential growth phase could be observed until the 48th hour of cultivation, when the predicted value of maximal biomass concentration was 1.409 g/L (Figure 4). Afterwards, a slight decrease of biomass content was noticed until the end of the bioprocess. Calculated average specific growth rate for *B. velezensis* was 0.118 1/h. Coefficient of determination for the Gompertz growth model was 0.9860, indicating very good fitting of the experimental data using this equation. Since the maximal biomass content was observed at 48th hour of cultivation, there is a possibility to shorten bioprocess duration from 96 to 48 h. In this way, significant savings could be made when it comes to the bioprocess cost, especially considering the possibility of bioprocess scale-up to pilot or industrial scale, which would be the ultimate goal of bioprocess development.

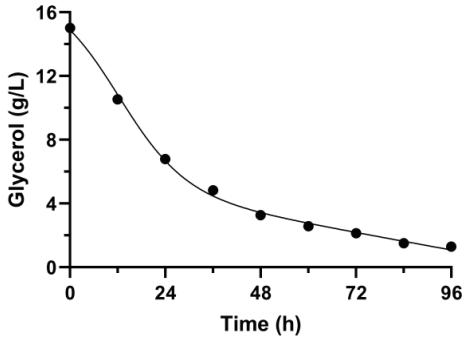

Figure 5. Experimental data fitting for glycerol consumption by Bacillus velezensis using modified Luedeking-Piret equation.

When it comes to the usage of modified Luedeking-Piret Equation for describing glycerol consumption during batch cultivation of *B. velezensis* (Figure 5), it could be concluded that the given equation has shown satisfying fitting ability of the obtained experimental data, with coefficient of determination of 0.9979. Also, initial glycerol content predicted by this equation was 14.89 g/L, which is in a very good accordance with 15 g/L of glycerol initially added during cultivation medium preparation. Glycerol consumption constant related to growth (γ), being significantly higher than constant related to metabolites production (δ), has also suggested that the major amount of glycerol was utilized for growth of *B. velezensis*, while only a minor amount of glycerol was used for metabolites production. These kinetic parameters confirm previously discussed results, where only slight difference of inhibition zone diameters could be observed during testing of antimicrobial activity of cultivation broth samples and *B. velezensis*

Table 4. Bioprocess kinetic parameters and model predicted values for biomass growth and glycerol consumption during cultivation of Bacillus velezensis in the laboratory-scale bioreactor

Model	Parameter	Value of the kinetic parameter	R^2 value
Gompertz – biomass concentration	X_{max} (g/L)	1.409	0.9860
	X_0 (g/L)	0.282	
	μ (1/h)	0.118	
Modified Luedeking-Piret – glycerol concentration	S_0 (g/L)	14.89	0.9979
	Y (g_S/g_X)	8.916	
	δ ($g_S/g_X \cdot h^{-1}$)	0.032	

biomass, indicating that biomass of *B. velezensis* is the main biocontrol agent effective against tested phytopathogenic *X. campestris* strains.

Further steps in bioprocess development should include optimization of cultivation medium composition in terms of main nutrients' quantity aiming at maximal yield of *B. velezensis* biomass, as well as investigation of cultivation parameters effect on bioprocess outcomes. Also, since the literature data suggest that *B. velezensis* shows supreme ability to synthesize a wide range of different antimicrobial metabolites [40], including lipopeptide antibiotics, further research will include analysis of cultivation broth supernatants using LC-MS and MALDI-TOF in order to identify and characterize antimicrobial compounds produced by this strain. The reason for further investigation in this field is the fact that the biomass-free filtrate from the first experimental stage in this study has also shown antimicrobial activity against tested phytopathogenic *X. campestris* strains isolated from plants with black rot symptoms, suggesting the presence of some antimicrobial compounds biosynthesized by *B. velezensis*. Further optimization of cultivation medium composition and cultivation conditions could also be aimed at directing metabolic activity of *B. velezensis* towards synthesis of targeted antimicrobial compounds in larger amounts. Formulation of final biocontrol product could be adjusted for biomass of the producing microorganism or targeted, separated and purified antimicrobial compounds, but as well as for product containing both biomass and bioactive compounds, depending on the requirements of eventual product application in the field.

CONCLUSION

B. velezensis has shown the greatest potential among tested antagonists to be used as biocontrol agent against phytopathogenic *X. campestris* strains, causing black rot of cruciferous crops. The optimal combinations of nutrients in the cultivation medium for production of bioactive compounds by *B. velezensis* were glycerol and yeast extract, lactose and peptone, as well as sucrose and yeast extract. Kinetic study performed during the validation experiment in the laboratory-scale bioreactor, using optimal carbon and nitrogen sources in the cultivation medium, has confirmed the results which indicate that biomass of *B. velezensis* presents the major bioactive agent effective against phytopathogenic *Xanthomonas* spp. Since the biomass-free filtrate has also shown antimicrobial activity against tested phytopathogens, further research will also include LC-MS and MALDI-TOF analysis to identify the produced antimicrobial compounds. The results of this study confirm biocontrol potential of *B. velezensis* and represent a basis for further bioprocess development in terms of optimization of cultivation medium composition and cultivation parameters, including investigation of different industrial waste streams, ultimately aimed at defining of a techno-economically viable bioprocess solution applicable at industrial scale.

Acknowledgement

This research was supported by the Ministry of education, science and technological development of the Republic of Serbia, project: 451-03-68/2020-14/200134.

REFERENCES

[1] A. Aires, C.S.P. Dias, R. Carvalho, M.H. Oliveira, A.A. Monteiro, M.V. Simões, E.A.S. Rosa, R.N. Bennett, M.J. Saavedra, Sci. Hortic. 129 (2011) 503–510

[2] J.G. Vicente, E.B. Holub, Mol. Plant Pathol. 14 (2013) 2–18

[3] J.D. Taylor, J. Conway, S.J. Roberts, D. Astley, J.G. Vicente, Phytopathology 92 (2002) 105–111

[4] C.A. Nunes, Eur. J. Plant Pathol. 133 (2012) 181–196

[5] R.R. Sharma, D. Singh, R. Singh, Biol. Control 50 (2009) 205–221

[6] A. Pérez-García, D. Romero, A. de Vicente, Curr. Opin. Biotechnol. 22 (2011) 187–193

[7] S. Compant, B. Duffy, J. Nowak, C. Clément, E.A. Barka, Appl. Environ. Microbiol. 71 (2005) 4951–4959

[8] C.L. Luna, R.L.R. Mariano, A.M. Souto-Maior, Braz. J. Chem. Eng. 19 (2002) 133–140

[9] E.G. Wulff, J.W.L. van Vuurde, J. Hockenhull, Plant Soil 255 (2003) 463–474

[10] S.M.S. Massomo, C.N. Mortensen, R.B. Mabagala, M.A. Newman, J. Hockenhull, J. Phytopathol. 152 (2004) 98–105

[11] L. Monteiro, R.D.R. Mariano, A.M. Souto-Maior, Braz. Arch. Biol. Technol. 48 (2005) 23–29

[12] P. Zhao, Y. Xue, W. Gao, J. Li, X. Zu, D. Fu, X. Bai, Y. Zuo, Z. Hu, F. Zhang, Peptides 101 (2018) 10-16

[13] I. Nikolić, Ž. Ivanović, J. Blagojević, S. Živković, T. Popović, Zašt. Bilja 64 (2013) 189–197

[14] E.G. Wulff, C.M. Mguni, C.N. Mortensen, C.L. Keswani, J. Hockenhull, Eur. J. Plant Pathol. 108 (2002) 317–325

[15] S. Mishra, N.K. Arora, World J. Microbiol. Biotechnol. 28 (2012) 693–702

[16] F.R. Spago, C.S.I. Mauro, A.G. Oliveira, J.P.O. Beranger, M.V.T. Cely, M.M. Stanganelli, A.S. Simionato, J.A.B. San Martin, C.G.T.J. Andrade, J.C.P. Mello, G. Andrade, Crop Prot. 62 (2014) 46–54

[17] Shivalingaiah, S. Umesha, Can. J. Physiol. Pharmacol. 1 (2013) 147–153

[18] R. Vaitkevičienė, D. Žadeikė, E. Bartkienė, V. Krunglevičiūtė, V. Baliukoniene, S. Supronienė, G. Juodeikienė, Zemdirbyste 106 (2019) 59–64

[19] R. Trias, L. Baneras, E. Montesinos, E. Badosa, Int. Microbiol. 11 (2008) 231-236

[20] M. Deivamani, M. Muthamilan, J. Innov. Agric. 2 (2015) 1-9

[21] T. Veličković, Z. Rončević, J. Grahovac, A. Jokić, I. Mitrović, B. Bajić. J. Dodić, Chem. Ind. Chem. Eng. Q. 24 (2018) 399-410

[22] S.M.P. Assis, R.L.R. Mariano, S.J. Michereff, G. Silva, E.A.A. Maranhão, Rev. Microbiol. 30 (1999) 191-195

[23] N. Ortuño, J. A. Castillo, C. Miranda, M. Claros, X. Soto, Renew. Agr. Food Syst. 32 (2016) 366-375

[24] G.S. Saddler, J.F. Bradbury, in Bergey's Manual® of Systematic Bacteriology, Vol. 2: The Proteobacteria, Part B: The Gammaproteobacteria, Springer-Verlag GmbH, Wien, 2005, pp. 63-122

[25] A. Mohsin, K. Zhang, J. Hu, Salim-ur-Rehman, M. Tariq, W. Q. Zaman, I. M. Khan, Y. Zhuang, M. Guo, Carbohydr. Polym. 181 (2018) 793-800

[26] K. Issazadeh, S.K. Rad, S. Zarrabi, M.R. Rahimibashar, Afr. J. Microbiol. Res. 6 (2012) 1615-1620

[27] N. M. Kannan, Abhiramy, P. Rajvanshi, IJAPBC 3 (2014) 1016-1027

[28] M. Encheva-Malinova, M. Stoyanova, H. Avramova, Y. Pavlova, B. Gocheva, I. Ivanova, P. Moncheva, Biotechnol. Biotechnol. Equip. 28 (2014) 721-727

[29] J.F.F. Secato, D.F. Coelho, N.G.J. Rosa, L.D.L. Costa, E.B. Tambourgi, Chem. Eng. Trans. 49 (2016) 103-108

[30] S.N. Al-Bahry, Y.M. Al-Wahaibi, A.E. Elshafie, A.S. Al--Bemani, S.J. Joshi, H.S. Al-Makhmari, H.S. Al-Sulaimani, Int. Biodeterior. Biodegradation 81 (2013) 141-146

[31] G.A. Płaza, E. Król, M. Pacwa-Płociniczak, Z. Piotrowska-Seget, L.R. Brigmon, Acta Sci. Pol. Hortorum Cultus 11 (2012) 169-182

[32] E.Z. Gomaa, Braz. Arch. Biol. Technol. 56 (2013) 259--268

[33] M. Nitschke, G.M. Pastore, Bioresour. Technol. 97 (2006) 336-341

[34] M. Chang, S.G. Zhou, N. Lu, J.R. Ni, World J. Microbiol. Biotechnol. 24 (2008) 441-447

[35] C. Li, K. L. Lesnik, H. Liu, Energies 6 (2013) 4739-4768

[36] M. de Sousa, I.T. Dantas, A.K.N. Felix, H.B. de Sant'Ana, V.M.M. Melo, L.R.B. Gonçalves, Braz. Arch. Biol. Technol. 57 (2014) 295-301

[37] Sumithra, A.V.N. Swamy, V.V. Basava Rao, IJAR 4 (2016) 2196-2200

[38] M. Priyadharshini, R.S. Kumar, IJMTST 3 (2016) 133-138

[39] V.K. Garlapati, U. Shankar, A. Budhiraja, Biotechnol. Rep. 9 (2016) 9-14

[40] L. Chen, H. Shi, J. Heng, D. Wang, K. Bian, Microbiol. Res. 218 (2019) 41-48.

JOVANA GRAHOVAC
IVANA PAJČIN
VANJA VLAJKOV
ZORANA RONČEVIĆ
JELENA DODIĆ,
DRAGOLJUB CVETKOVIĆ
ALEKSANDAR JOKIĆ

Univerzitet u Novom Sadu, Tehnološki fakultet Novi Sad, Novi Sad, Srbija

NAUČNI RAD

BIOKONTROLNI AGENS PATOGENA *Xanthomonas campestris*: ODABIR, FORMULACIJA HRANLJIVE PODLOGE I ANALIZA KINETIKE BIOPROCESA

Crna trulež uzrokovana patogenom Xanthomonas campestris pv. campestris je jedna od najznačajnijih bolesti biljaka iz porodice kupusnjača, koja izaziva značajne gubitke prinosa. Biološka kontrola crne truleži mikrobiološkim biokontrolnim agensima predstavlja obećavajuću alternativu hemijskim tretmanima i dobroj poljoprivrednoj praksi, koji pokazuju samo ograničenu uspešnost u suzbijanju bolesti. U ovom radu ispitan je potencijal različitih antagonista iz rodova Bacillus, Pseudomonas, Lactobacillus, Streptomyces, Saccharomyces i Trichoderma za biološku kontrolu crne truleži. Antimikrobna aktivnost uzoraka kultivacione tečnosti i njihovih filtrata testirana je protiv sedam sojeva Xanthomonas campestris, izolovanih iz obolelih biljaka, primenom disk-difuzione metode. Najveći prečnik zone inhibicije (35,62±3,76 mm) pokazao je izolat Bacillus velezensis. Nakon toga su ispitane različite kombinacije izvora ugljenika i azota u kultivacionom medijumu u cilju maksimizacije antagonističke aktivnosti odabranog soja. Najbolje kombinacije su bile glicerol i ekstrakt kvasca, laktoza i pepton, kao i saharoza i ekstrakt kvasca, što ukazuje na potencijal efluenata industrije biodizela, mleka i šećera kao sirovina za proizvodnju bioaktivnih komponenti efikasnih protiv izazivača crne truleži. Validacioni eksperiment je izvršen u laboratorijskom bioreaktoru u cilju ispitivanja kinetike bioprocesa u pogledu rasta biomase i potrošnje izvora ugljenika, primenom kultivacionog medijuma koji sadrži optimalni izvor ugljenika i azota.

Ključne reči: Bacillus, biološka kontrola, crna trulež, izvor ugljenika, kultivacija, organski izvor azota.