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EFFECT OF DIFFERENT INOCULUM PREPARATION CONDITIONS ON the BIOMASS GROWTH AND ANTIMICROBIAL ACTIVITY OF *BACILLUS* SP.

UTICAJ RAZLIČITIH USLOVA PRIPREME INOKULUMA NA UMNOŽAVANJE BIOMASE I ANTIMIKROBNU AKTIVNOST *BACILLUS SP.*

Ivana PAJČIN[*], Zorana RONČEVIĆ[*], Jelena DODIĆ[*], Siniša DODIĆ[*],
Mila GRAHOVAC[**],Aleksandar JOKIĆ[*], Jovana GRAHOVAC[*]
[*]*Faculty of Technology Novi Sad, 21000 Novi Sad, Bulevar cara Lazara 1, Serbia*
[**]*Faculty of Agriculture, 21000 Novi Sad, Trg Dositeja Obradovića 8, Serbia*
email: paj@tf.uns.ac.rs

ABSTRACT

Plant diseases caused by pathogenic bacteria and fungi can lead to reduced plant growth capacity and significant losses in food production. The use of biopesticides is strongly recommended over applying chemical compounds due to growing concerns about environmental pollution and potential harmful effects to human health. A significant scientific and economic interest lies in the isolation of new Bacillus strains showing the potential for plant disease biocontrol. The purpose of this study is to investigate the effect of different inoculum preparation conditions on the Bacillus sp. biomass growth and antimicrobial activity against the selected phytopathogenic Xanthomonas strains. The results obtained argue the possibility of utilizing the glycerol-based semi-synthetic medium in the second stage of inoculum preparation and reducing the duration of inoculum preparation, which is of great importance to the bioprocess cost attenuation and the development of optimized bioprocess solutions for producing bioactive agents effective against the phytopathogenic Xanthomonas strains.

***Key words**: Bacillus sp., biomass, inoculum, antimicrobial activity, Xanthomonas.*

REZIME

Bolesti biljaka izazvane patogenim bakterijama i gljivama mogu značajno da utiču na porast biljaka ili da nanesu mnogo ozbiljniju štetu dovodeći do smrti biljaka i značajnih gubitaka u proizvodnji hrane. Zbog zagađenja životne sredine i potencijalnih štetnih uticaja na ljudsko zdravlje, umesto upotrebe hemijskih jedinjenja u suzbijanju biljnih bolesti sve više se preporučuje upotreba biopesticida. Biopesticidi privlače značajnu pažnju u suzbijanju biljnih patogena i već dugi niz godina predstavljaju jednu od perspektivnijih alternativa. Iako još uvek čine mali procenat sredstava za suzbijanje patogena, teži se ka njihovoj što većoj upotrebi i postoji trend porasta njihove proizvodnje i primene. Osnovni mehanizmi pomoću kojih bakterije roda Bacillus, koje se najčešće primenjuju kao agensi u biološkom suzbijanju fitopatogena, ispoljavaju antagonističko delovanje jesu kompeticija za prostor i nutrijente potrebne za rast, kao i proizvodnja jedinjenja koja ispoljavaju antimikrobnu aktivnost prema fitopatogenima. Zbog toga je od velikog naučnog i ekonomskog značaja izolacija novih sojeva ovog roda koji pokazuju potencijal za primenu u biološkom suzbijanju, kao i usavršavanje procesa za njihovo umnožavanje i proizvodnju komponenti sa antimikrobnim delovanjem protiv ciljanih fitopatogena. Cilj ovog rada bio je ispitivanje uticaja različitih načina pripreme inokuluma na umnožavanje biomase i antimikrobnu aktivnost proizvodnog mikroorganizma Bacillus sp. Rezultati ovog istraživanja ukazali su na mogućnost uvođenja polusintetičke podloge na bazi glicerola u bar jednu fazu pripreme inokuluma, kao i na mogućnost skraćenja vremena pripreme inokuluma, što predstavlja dobru osnovu za smanjenje troškova bioprocesa i dalji razvoj optimalnog procesnog rešenja za proizvodnju bioaktivnih agenasa efikasnih u biološkom suzbijanju fitopatogenih vrsta roda Xanthomonas.

***Ključne reči**: Bacillus sp., biomasa, inokulum, antimikrobna aktivnost, Xanthomonas*.

INTRODUCTION

One of the major reasons for significant losses in food production are plant diseases caused by pathogenic bacteria and fungi, which can lead to reduced plant growth capacity or even plant death. *Xanthomonas campestris* is well known as the causal agent of the *Brassicaceae* family vegetables decay (*Singha et al., 2016*), as well as the causal agent of pepper and tomato diseases (*Obradović et al., 2004*) associated with significant economic losses. Infections caused by these phytopathogenic bacteria are notoriously complicated to treat, control and prevent, featuring a high incidence of the disease relapse in the seasons following the first infection (*Hassan and Zyton, 2017*). The efficacy of pesticides in controlling the disease is markedly impaired due to the development of pathogen resistance. As there are growing concerns about the present and future environmental consequences and potential harmful effects of these chemicals to human health (*Bursić et al., 2016*), the application of beneficial microorganisms for pathogen control and plant growth promotion becomes an attractive alternative. Biopesticides, primarily microbial biopesticides, based on different types of microorganisms or their products, have gained increasing attention as one of the prospective alternatives (*Seiber et al., 2014; Mnif and Ghribi, 2015*). At present, the market share of biopesticides in the global pest control product market is still minute, but there is a constant increase in their application and production (*Damalas and Koutroubas, 2018*). The bacteria of the genus *Bacillus* are mostly used for pathogen control, hence these bacteria could be found in a majority of commercially available microbial biopesticides. This genus consists of a heterogenic group of gram-positive, aerobic or facultative anaerobic bacteria, with the exceptional ability to rapidly adjust to different ecological and nutritional conditions, as well as to produce a wide spectrum of bioactive metabolites (*Pérez-García et al., 2011*). There are several mechanisms of the antagonistic activity of the *Bacillus* strains against disease-causing phytopathogens: the competition for growth space and nutrients (*Shafi et al., 2017*), the promotion

of plant growth using different mechanisms (*Kumar et al., 2012*) and the production of compounds with the antimicrobial activity against phytopathogens. Some of the *Bacillus* strains can produce over 70 metabolites featuring the antifungal or antibacterial activity such as antibiotics (*Stein, 2005; Zhao et al., 2018*), enzymes (*Shafi et al., 2017*), lipopeptides (*Zhao et al., 2012; Torres et al., 2017*) and biosurfactants (*Gomaa, 2013*). Therefore, a significant scientific and economic interest lies in the isolation of new *Bacillus* strains that show the potential for the biological control of plant diseases caused by pathogenic microorganisms. Moreover, the optimization of the process of producing and multiplying antimicrobial components effective against the targeted phytopathogens is gaining emphasis as well.

The purpose of this study is to investigate the effect of different manners of inoculum preparation on the *Bacillus* sp. biomass growth and antimicrobial activity against the selected phytopathogenic *Xanthomonas campestris* strains.

MATERIAL AND METHOD

Microorganisms

The producing microorganism in this study was *Bacillus* sp., an isolate from fresh cheese, which was grown on a nutrient agar slant at 4 °C. The phytopathogenic isolates tested in this study were the *Xanthomonas campestris* strain Mn 7-2 and the *Xanthomonas campestris* pv. *vesicatoria* strain PAP LIST 1, isolated from diseased plants (cabbage and pepper, respectively). Characterization and identification of the producing microorganism and test strains were performed according to the Bergey's Manual of Systematic Bacteriology (*De Vos et al., 2009*). These isolates were grown on a YMA (yeast maltose agar) slant at the same temperature as the producing microorganism. The composition of the YMA medium is provided in greater detail by *Pajčin et al. (2018)*.

Inoculum preparation

The producing microorganism was incubated on a fresh nutrient agar slant for 48 h in order to regain its physiological activity. Different conditions of inoculum preparation, i.e. the media used and the duration of the preparation process, were examined in this study. The inoculum preparation process was conducted in two stages based using different medium volumes (50 mL in the first stage and 150 mL in the second stage). A combination of media used in both stages of the inoculum preparation could be found in Table 1. Both stages of the inoculum preparation were performed using a laboratory shaker (KS 4000i control, IKA® Werke, Germany) with external mixing (150 rpm) at 28 °C, and their duration was similar (48 h cumulatively in the first experimental phase). After the selection of media combination for inoculum preparation (based on the results of inhibition zone diameters and producing microorganism's biomass content), the effect of different inoculum preparation durations on bioprocess outcomes was examined. The cumulative duration of inoculum preparation (including durations of both inoculum preparation stages) was set to 24 h, 36 h and 48 h.

Table 1. Combination of media used for inoculum preparation in the first experimental phase

Experiment number	Stage 1	Stage 2
1	Nutrient broth	Nutrient broth
2	Nutrient broth	Glycerol-based medium
3	Glycerol-based medium	Glycerol-based medium

Cultivation

The producing microorganism was cultivated in Erlenmayer flasks with a cultivation media volume of 100 mL. The glycerol-based medium used for cultivation contains the following components: yeast extract (3 g/L), $(NH_4)_2SO_4$ (3 g/L), K_2HPO_4 (1 g/L) and $MgSO_4 \cdot 7H_2O$ (0.3 g/L), besides glycerol (10 g/L). This cultivation medium was inoculated using different amounts of previously prepared inocula from the first experimental phase (5% and 10%, v/v). The cultivation of each inoculated medium was carried out using a laboratory shaker (KS 4000i control, IKA® Werke, Germany) under the following conditions: a temperature of 28 °C, an agitation rate of 150 rpm and a duration period of 96 h. During cultivation, portions of the cultivation broth were sampled at predefined time intervals (24 h) in order to assess the optical density and antimicrobial activity of the cultivation broth samples.

Antimicrobial activity assaying

The samples of cultivation broths prepared using different inocula for the inoculation of cultivation media were tested for their antimicrobial activity against two phytopathogenic isolates: *X. campestris* Mn 7-2 and *X. campestris* pv. *vesicatoria* PAP LIST 1. The test microorganisms were subcultivated on a YMA slant at 26 °C for 48 h. Subsequently, the suspensions of test microorganisms' biomass were made using sterile saline. The YMA medium was also used for the antimicrobial activity testing, inoculated with 1 mL of suspension of each phytopathogen after melting and tempering at 50 ± 1 °C. After the solidification of the test medium in Petri dishes, three discs for antimicrobial activity testing were placed in each Petri dish. Therefore, the samples of cultivation broths (10 μL) were tested in triplicate. Upon incubation (lasting 72 h at 26 °C), the inhibition zone diameters were measured.

Spectrophotometry

The biomass contents of the cultivation broth samples were monitored by optical density measurements using a spectrophotometer (UV 1800, Shimadzu, Japan) at a wavelength of 600 nm. The blank medium was used for the cultivation of the producing microorganism (glycerol-based medium).

Statistical data analysis

All the experiments were conducted in triplicate under the same experimental conditions. The obtained results of the inhibition zone diameters are presented in the form of average values with a standard deviation, calculated using the Microsoft® Excel 2010 software (MS Office, Microsoft Corporaton, USA). 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 ANOVA and post-hoc testing procedures, using the Duncan's multiple range test, were also performed. The statistical analysis was performed at a significance level of 0.05.

RESULTS AND DISCUSSION

The first experimental phase of this study examined different media used in the first and the second stage of inoculum preparation, as well as amounts of inoculum added during the inoculation of cultivation media. The monitored bioprocess outcomes were the biomass content of the producing microorganism, measured using the spectrophotometric method, and inhibition zone diameters, obtained by assaying the antimicrobial activity of the cultivation broth samples against the phytopathogenic strains *X. campestris* Mn 7-2 and *X. campestris* pv. *vesicatoria* PAP LIST 1.

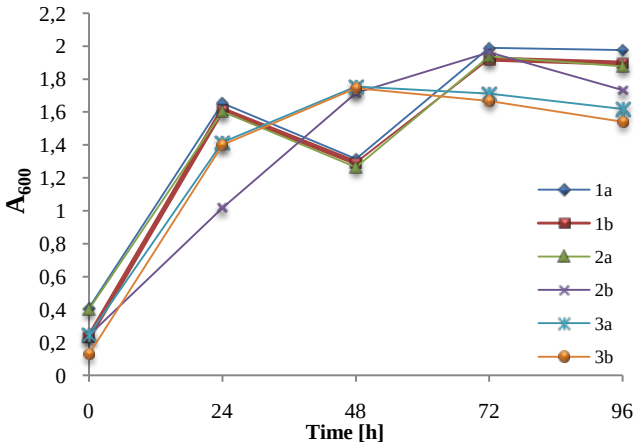

Fig. 1. Results of the spectrophotometric analysis of the cultivation broth samples obtained using different media for inoculum preparation and different inoculum amounts for cultivation media inoculation.
1-3 – combinations of media used for inoculum preparation (Table 1);
a – an inoculum amount of 10 % (v/v), b – an inoculum amount of 5 % (v/v)

Monitoring of the bioprocess course relative to the biomass content (Fig. 1) showed differences between the medium based on glycerol and the commercial medium (nutrient broth) for inoculum preparation. The use of nutrient broth in at least one stage of inoculum preparation (combinations 1 and 2) led to an increase in the initial biomass content during the first 24 hours of cultivation, after which the optical density of the observed cultivation broth samples decreased (from 24 h to 48 h of cultivation). This trend underscores a typical shift from the consumption of fermentable sugars (contained in the nutrient broth as the main carbon sources) during the first 24 hours of cultivation to the consumption of glycerol, which is less desirable from the nutritional and metabolic perspectives (*Li et al., 2013*). The producing microorganism requires a certain period of time to adjust its metabolic pathways to the consumption of glycerol as the main carbon source, accompanied by the biomass content decrease (from 24 h to 48 h of cultivation). Subsequently, the biomass content increased again and reached the maximum value after 72 hours of cultivation for the combinations 1 and 2, indicating the end of the exponential growth phase. Relative to the use of glycerol-based medium in the both stages of inoculum preparation (the combination 3), it can be observed that there was no decrease in the biomass content until 48 hours of cultivation, whereas a slight decrease in the biomass content was recorded by the end of the cultivation. Higher biomass contents were achieved using the combinations 1 and 2 than that achieved using the combination 3, where the glycerol-based medium was used in both stages of inoculum preparation. These results indicate that fermentable sugars, as well as peptides with different chain lengths, contained in the nutrient broth of the commercial medium represent more suitable nutrients for the biomass growth and multiplication of *Bacillus* sp. than glycerol, which is in accordance with the previously reported data on the metabolic activity of different *Bacillus* spp. (*Sanchez and Demain, 2002*).

A two-way ANOVA was employed in order to assess the statistical significance of the effects of media used for inoculum preparation and initial inoculum amounts on the inhibition zone diameters against the tested phytopathogenic *Xanthomonas* isolates. The ANOVA results indicate a significant effect of the both investigated parameters (*p*-value less than 0.05). The media used for inoculum preparation were more significant than the initial inoculum volume, whereas the interaction of these parameters was statistically non-significant (Table 2).

Table 2. Two-way ANOVA of the inhibition zone diameters of different media used for inoculum preparation and different inoculum amounts used for cultivation media inoculation

Effect	SS	MS	DF	F-value	*p*-value
Inoculum media	165.26	82.63	2	15.518	**0.000024**
Inoculum amount	49.00	49.00	1	9.202	**0.004954**
Inoculum media*Inoculum amount	21.13	10.56	2	1.984	0.155215
Error	159.75	5.33	30		
SS – sum of squares, MS – mean squares, DF – deg. of freedom					

Using the Duncan's multiple range test, the post-hoc testing was employed to examine the effect of these two factors on the inhibition zone diameters against the tested *Xanthomonas* strains. The results of the Duncan's test indicate that the highest inhibition zone diameters were obtained using the combinations 1a and 2a, with both combinations at the same level of statistical significance (Table 3). These results suggest the possibility of using the glycerol-based medium in the second stage of inoculum preparation (2a) instead of the nutrient broth (1a), which directly reduces bioprocess costs with regard to the inoculum medium price. Furthermore, an initial inoculum volume of 10 % (v/v) showed better results than the cultivation medium volume, confirming once again that reductions in the inoculum medium price could be economically significant.

Table 3. Mean values of the inhibition zone diameters obtained using different media for inoculum preparation (reference to Table 1) and different amounts of inoculum for inoculation of cultivation media

Inoculum media	**Inoculum amount**	**Inhibition zone diameter [mm]**
3	b – 5 % (v/v)	20.58 ± 1.96^{a}
3	a – 10 % (v/v)	21.00 ± 2.00^{ab}
1	b – 5 % (v/v)	23.55 ± 2.59^{bc}
2	b – 5 % (v/v)	23.83 ± 2.16^{bc}
2	a – 10 % (v/v)	26.25 ± 0.88^{cd}
1	a – 10 % (v/v)	27.67 ± 3.46^{d}
Values marked with the same superscript letter are at the same level of significance with a confidence level of 95% (Duncan's test)		

The second experimental stage was aimed at examining the effect of inoculum preparation durations on the biomass content and inhibition zone diameters against the phytopathogenic strains *X. campestris* Mn 7-2 and *X. campestris* pv. *vesicatoria* PAP LIST 1 during the cultivation of the producing microorganism *Bacillus* sp. The inoculum preparation was performed under the same conditions as in the first experimental phase. The inoculum preparation media were the nutrient broth and the glycerol-based medium in the first and second stages, respectively. Relative to the biomass content of the producing microorganism (using inocula prepared during 36 h and 48 h), the cultivation course showed similar trends to those observed in the first experimental phase. The best results regarding biomass content were obtained using the inoculum prepared during 36 h, with only slightly lower values of absorbance than those obtained for the inoculum prepared during 48 h. When using the inoculum prepared during 24 h, the biomass content reached the maximum after 24 hours of cultivation.

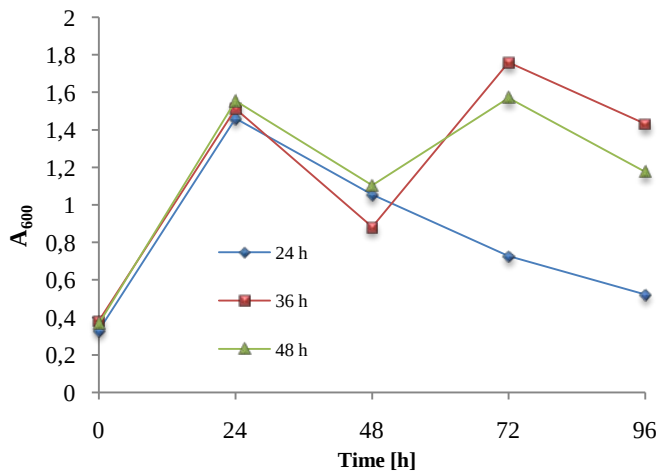

Fig. 2. Results of the spectrophotometric analysis of the cultivation broth samples obtained using different inoculum preparation durations

However, a decreasing trend in the biomass content ensued until the end of the bioprocess (Fig. 2), suggesting that an inoculum preparation period of 24 h is not long enough to produce sufficient amounts of bacterial cells required for the inoculation of cultivation medium This observation is in accordance with the results of the study conducted by Korsten and Cook (1996), who reported that the utilization of nutrient broth has resulted in the maximum biomass content of *Bacillus subtilis* after 32 hours of cultivation. A one-way ANOVA was employed to examine the effect of inoculum preparation durations on the inhibition zone diameters obtained by testing the cultivation broth samples against the phytopathogenic *Xanthomonas* isolates. The ANOVA results obtained confirmed the statistical significance of the inoculum preparation duration relative to the producing microorganism's antimicrobial activity (Table 4).

Table 4. One-way ANOVA of the inhibition zone diameters for different inoculum preparation durations

Effect	SS	MS	DF	F-value	*p*-value
Inoculum preparation duration	73.58	36.79	2	20.129	**0.000057**
Error	27.42	1.83	15		
SS – sum of squares, MS – mean squares, DF – degree of freedom					

The Duncan's multiple range tests were performed subsequently in order to form homogenous groups of the same statistical significance. The best inhibition zone diameter values were obtained for the cultivation broth samples inoculated using the inocula prepared during 48 h and 36 h (Table 5). As both inoculum preparation durations were at the same level of statistical significance, these results indicate that the duration of inoculum preparation could be reduced from standard 48 h to 36 h, leading to significant reductions in the overall bioprocess costs.

Table 5. Mean values of the inhibition zone diameters obtained using different inoculum preparation durations

Inoculum preparation duration [h]	Inhibition zone diameter [mm]
24	25.33 ± 1.17^{a}
36	29.25 ± 1.64^{b}
48	29.92 ± 1.20^{b}
Values marked with the same superscript letter are at the same level of significance with a confidence level of 95 % (Duncan's test)	

CONCLUSION

Bacillus sp., isolated from fresh cheese, exhibited the significant antimicrobial potential for producing bioactive agents effective against microbial causers of cabbage and pepper diseases belonging to the genus *Xanthomonas*. The results obtained indicate that the best results regarding the biomass content of the producing microorganism and its antimicrobial activity against the tested phytopathogens were obtained when the commercial medium (namely nutrient broth) was used in both stages of inoculum preparation, or with a combined use of the commercial and glycerol-based media (better results were achieved in both cases using an initial inoculum content of 10 %). Furthermore, the possibility of reducing the duration of inoculum preparation from usual 48 h to 36 h was found viable. The utilization of a semi-synthetic medium based on glycerol as the main carbon source in the second stage of inoculum preparation and reductions in the duration of inoculum preparation were found to be of paramount importance to bioprocess cost attenuation and the development of optimized bioprocess solutions for producing bioactive agents effective against the phytopathogenic Xanthomonas strains.

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