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Growth kinetics and pathogenicity of *Photorhabdus luminescens* subsp. *akhurstii* SL0708

María Teresa Orozco-Hidalgo[1], Balkys Quevedo-Hidalgo[2] and A. Sáenz-Aponte[1*]

Abstract

Photorhabdus luminescens subsp. *akhurstii* SL0708 (*Enterobacteriaceae*) is a symbiont of the entomopathogenic nematode (EPN), *Heterorhabditis indica* SL0708 (*Nematoda*: *Rhabditida*), used for insect pest biological control. In the present study, *P. luminescens* subsp. *akhurstii* SL0708 growth kinetic was evaluated considering growth and metabolic phases (phase I, intermediate phase, phase II), as well as pathogenicity. The study can be useful in determining bacterium feeding times in *H. indica* SL0708 production in liquid culture media. The logarithmic phase of the growth of bacterium was from 0 to 24 h, with a specific growth velocity of 0.21 h^{-1}; during this phase, bacterium at metabolic phase I was detected. Maximum bioluminescence was registered at 24 h (3.437 luminescence AU). Finally, it was evidenced that the bacterial metabolic phase had an effect on the greater wax moth, *Galleria mellonella* L., larvae mortality rate. Moreover, biochemical tests were the same for all *P. luminescens* subsp. *akhurstii* SL0708 sampling times. This research is particularly relevant, since no reports are available on this bacterium isolate in Colombia. In the future, this will allow massive *H. indica* SL0708 production, because when pre-incubated with its symbiont, it provides essential nutrients for the EPNs development and reproduction.

Keywords: Entomopathogenic nematodes, Bacterial symbiont, Liquid culture media, Bioluminescence, *Heterorhabditis indica*

Background

For integrated insect pest management, several investigations have demonstrated that entomopathogenic nematodes (EPNs) use is an effective alternative, because it controls different pest species and it is friendly with humans and the environment (Devi and Dhrubajyoti 2017). Of all the nematodes studied for insect biological control, *Heterorhabditis* is one of the most studied genera for EPNs purposes. In Colombia, *Heterorhabditis indica* SL0708 was isolated from *guadua* bamboo soils in Valle del Cauca, demonstrating its high efficacy in the management of various pests in crops of economic importance (San-Blas et al. 2019). Given its pest management use, in vitro EPN production has gained importance in recent years; therefore, it is necessary to meet the demands of the biological controller in the field (Abd-Elgawad et al. 2017). This process consists of designing culture media to supply EPNs nutritional requirements. The bacterial symbiont must digest complex compounds in the media, so the nematode can use them to complete its life cycle (Cho et al. 2011). This production strategy presents various advantages in comparison to traditional in vivo EPNs production; it is less laborious and easier to scale-up to industrial levels (Inman et al. 2012), in search of high infective juveniles (IJs) yields to sufficiently supply for their use in different Colombian crops.

Photorhabdus luminescens SL0708 is a symbiotic bacterium presents in the *H. indica* SL0708 IJs. This bacterium is responsible for EPNs insect host death. *P. luminescens* is a facultative anaerobe, gram-negative *Bacillus* belonging to the luminescent *Enterobacteriaceae* family (Stock et al. 2017). Its life cycle can be divided into three stages: mutualistic association with the IJ, as insect pathogen, and as food source for the nematode (Devi and Dhrubajyoti 2017).

* Correspondence: adriana.saenz@javeriana.edu.co
[1]Departamento de Biología, Laboratory for Biological Control, Biología de Plantas y Sistemas Productivos, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia
Full list of author information is available at the end of the article

To mass produce *H. indica* SL0708, its bacterial symbiont *P. luminescens* SL0708 must be mass produced as well. To grow *P. luminescens*, it must first grow in a nutrient medium up to the stationary phase. After approximately 24 h, IJs which are suspended in an enriched nutrient broth are inoculated into the bacterial culture. To check the entompathogenicity, the greater wax moth, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), was infected by the nematodes along with its symbiont, and its death occurs within 48 h, and the insect pathogenicity of the *H. indica-P. luminescens* combination is confirmed (Salazar-Gutiérrez et al. 2017).

Culturing *P. luminescens* in vitro has allowed to identify different phases associated with its metabolism and pathogenicity. Metabolic phase I is associated with the insect and IJ intestines. It is characterized by producing an array of pathogenic factors, toxin complexes (Tc's), and *Photorhabdus* insect-related (Pir) toxins and makes caterpillars floppy toxins (Mcf), *Photorhabdus* virulence cassettes (PVC), and bioluminescence (Hu and Webster 2000; Lang 2010; Nouh and Hussein 2015). Phase II is the other metabolic phase related to in vitro bacterium culture. It is characterized by loss of pathogenicity factors associated with phase I. Similarly, an intermediate phase has been identified between the two phases associated with in vitro culture, where the bacterium starts to lose its pathogenic factors (Han and Ehlers 2001). Change between phase I to intermediate phase and phase II entails nourishment depletion, therefore, a loss of capability to associate with the nematode, when it enters into IJ stage. The latter directly affects pathogenicity, because without bacteria, the nematode cannot kill the insect host (Turlin et al. 2006). In vitro bacterium characterization has been performed by NBTA media (nutrient agar supplemented with bromothymol blue and triphenyltetrazolium chloride), where bacteria in phase I are bioluminescent in the dark. Additionally, they grow into blue-green round colonies, as they absorb bromothymol blue stain, with mucous or creamy texture. Phase II bacteria do not present bioluminescence in the dark and has reddish-brown colonies with a sticky texture (Nouh and Hussein 2015). Phases can also be verified by qualitative enzyme activity tests, such as lipolytic activity (tributyrin agar media), proteolytic (egg yolk and gelatin media), and hemolytic activities (media with sheep blood), for which all are positive for phase I and negative for phase II (Salazar-Gutiérrez et al. 2017). Accordingly, to produce nematodes in vitro, bacterium must be at phase I. It is therefore a requirement to establish when the other phases take place, thus, allowing to supply IJ demand for use in the field.

Therefore, the present study aimed to evaluate *P. luminescens* subsp. *akhurstii* SL0708 growth kinetics and pathogenicity in *H. indica* SL0708 under in vitro production media.

Materials and methods

Culture media

The culture media are as follows:

- NBTA agar medium: 0.025 g/l bromothymol blue, 0.04 g/l 2, 3, 5-triphenyltetrazolium chloride, 20 g/l nutrient agar, pH 7.
- LB medium (Luria Bertani): 10 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone, 12 g/l agar-agar, pH 7 (Sezonov et al. 2007).
- *H. indica* SL0708 in vitro liquid production media: 10 g/l yeast extract, 10 g/l peptone, 3 g/l soy flour, 30 g/l soy oil, 6 egg yolk, 3 g/l glucose, 4 g/l NaCl, 0.3 g/l $CaCl_2$, 0.2 g/l $MgSO_4$, 0.05 g/l $FeSO_4$, 0.35 g/l KCl, pH 7 (Johnigk et al. 2004).

Microorganism and inoculum

The strain of the bacterial symbiont, *P. luminescens* SL0708, was obtained from the collection of microorganisms of Pontificia Universidad Javeriana (CMPUJ), conserved in glycerol stocks at − 80 °C. It was suspended in LB and NBTA media and incubated for 8 h at 28 °C, to be inoculated later; 100-ml Erlenmeyer flasks were filled with 90 ml *H. indica* SL0708 in vitro production media. Media was inoculated with 10 ml *P. luminescens* subsp. *akhurstii* SL0708 culture, containing bioluminescent colonies corresponding to approximately 10^8 cells per ml; Erlenmeyer flasks with inoculum were incubated at 28 °C and 150 rpm for 24 h.

Batch fermentation

Eighteen milliliters of sterile production media were placed into 100-ml flask and inoculated with 2 ml *P. luminescens* subsp. *akhurstii* SL0708 inoculum. Media was incubated at 28 °C. Flasks were shaken in an orbital shaker incubator (New Brunswick Scientific™ Innova™ 42, Thermo Fisher Scientific Inc. Waltham, MA, USA) at 150 rpm for 36 h. Biomass content of each flask was monitored by sampling at 0, 8, 12, 24, 32, and 36 h (it is taken until this time, because the kinetics of *P. luminescens* subsp. *akhurstii* SL0708 allows to establish the time of inoculation of infective juveniles in vitro cultures of *H. indica* SL0708). Cells were separated from media by centrifugation (Sorvall RC-6 plus Thermo Scientific Co Waltham, MA, USA) at 10,800×*g* for 20 min at 4 °C. Obtained supernatant was used to evaluate pH and reducing sugar concentration, equivalent to glucose. Reducing sugars concentration was quantified by 3,5-dinitrosalicilic acid (DNS) technique (Miller 1959), employing an Evolution 60 UV–VIS Thermo Scientific Co. spectrophotometer (Waltham, MA, USA). Values were determined based on 0.5 to 2 g/l glucose standard curve.

Growth and bioluminescence analyses

Due to the presence of microemulsion droplets in the system, biomass concentration could not be quantified by spectrophotometric methodologies. Microbial growth was determined from colonies formed (colony-forming unit CFU) on agar plates in NBTA media. Colonies were described based on their morphology, color, and bioluminescence for each collected sample. Each experiment was repeated three times. The bioprocess parameters duplication time (td) and specific growth rate (μ) were calculated from the experimental data (Eqs. 1–3) during the logarithmic phase of the growth (from 0 to 24 h).

The specific growth rate (μ, h^{-1}) of the microorganism was calculated from Eq. (1).

$$\frac{dX}{dt} = \mu X \tag{1}$$

where X is biomass at time t (h), and CFU/ml μ_x was calculated by integrating Eq. (1) with conditions, $X = X_0$ at $t = t_0$

$$\mu = \frac{lnX - \ln X_0}{t - t_0} \tag{2}$$

μ was determined by plotting ln (X/X_0) vs. time. Doubling time (t_d), is the time required for the microorganism to double its population, calculated from Eq. (3).

$$t_d = \frac{\ln 2}{\mu} \tag{3}$$

Bioluminescence was quantitated by assaying in FluostarOptima luminescence plate reader (BMG labtech, Germany) with a 355-nm excitation and 460-nm emission filters. To this end, 250-μl fresh culture media were placed in 96-well COSTAR plate. Results are reported as luminescence arbitrary units (LAU).

Bacterium metabolic phases

Phase I was determined for bacterium during growth kinetics. To this end, morphological characteristics in NBTA media, bioluminescence, and pathogenicity were taken into account (Table 1).

Biochemical assays

To determine biochemical characteristics at different times of *P. luminescens* subsp. *akhurstii* SL0708 culture, an API 20NE (BioMerieux, Inc. Durham, NC) was used following manufacturer's instructions.

Galleria mellonella pathogenicity assays

Bacteria colonies were obtained from NBTA cultures at 24, 48, 72, and 96 h and re-suspended in 0.85% (w/v) saline solution at 1×10^4 CFU/ml. Additionally, *G. mellonella* last instar larvae were previously disinfected with 1% hypoclorite and rinsed with distilled water. Larvae were then infected by 10-μl culture suspension with 1 mL BD ultra-fine syringe and incubated at 28 °C for 96 h. Mortality percentage, color, and larvae bioluminescence were registered every 24 h.

Statistical analysis

Results are presented as mean ± SD ($n = 3$) with three replicates per fermentation. Mortality percentage is the average of three replicas ± SD. To determine statistical differences for mortality percentages, one-way ANOVA analysis was performed with data from bacterial infections at 24, 48, 72, and 96 h.

Results and discussion

Growth kinetics, bacterium metabolic phases, and bioluminescence

P. luminescens subsp. *akhurstii* SL0708 growth in production media, reducing sugars consumption, and pH are presented in (Fig. 1). Up to 24 h, a logarithmic phase of the growth was observed with a respective specific growth rate of 0.21 h^{-1} (R^2= 0.99) and duplication time (t_d) 3.3 h. The biomass in this phase ranged between 2.4×10^7 CFU/ml and 5.8×10^9 CFU/ml. After 24 h, it was constant up to 36 h (stationary phase), with a biomass yield obtained from glucose ($Y_{x/s}$) of 2.4 $\times 10^{12}$ CFU/g. At this time, (77.4%) of the reducing sugars were consumed, equivalent to the available glucose in the media. Regarding the pH profile during fermentation, it has a tendency to increase towards alkalinity from the beginning to the end of the culture. Possibly, the microorganism could have used available amino acids and proteins as an energy source and, consequently,

Table 1 *Photorhabdus luminescens* subsp. *akhurstii* SL0708 metabolic characteristics

Parameter	Phase I	Intermediate phase	Phase II
NBTA colony culture	Greenish with light green-yellow halo.	Brown with small translucent zone surrounding the colony, it can be green or yellow.	Dark red, does not present translucent zone surrounding the colony.
NBTA colony morphology	Mucous or creamy texture, convex elevation, entire colony margin, round, and shiny.	Mucous or creamy texture, translucent margin, burgundy center, convex elevation, entire colony margin, round, and shiny.	Sticky texture, high adhesiveness to culture media, flat, round, defined regular margin, opaque.
Bioluminescence	Yes, evidenced in the dark.	Partial, it cannot be seen at simple eyesight in the dark.	Slight or absent, it cannot be seen at simple eyesight in the dark.

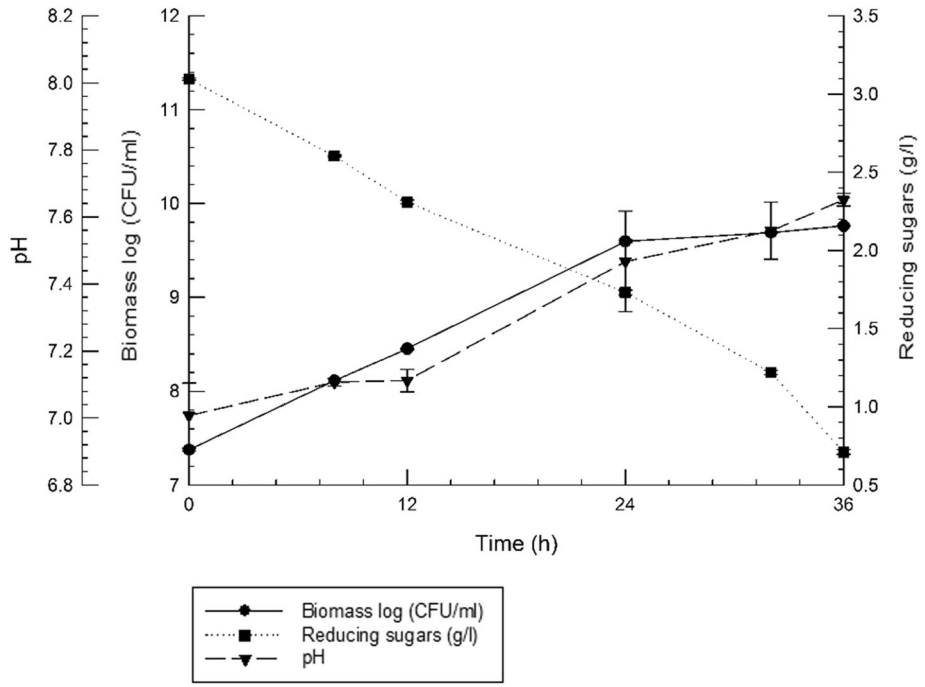

Fig. 1 Growth kinetic of *Photorhabdus luminescens* subsp. *akhurstii* SL0708

ammonium ions were produced (Belur et al. 2013). Specific growth velocity (μ) differs from previously reported ($\mu = 0.36\ h^{-1}$ $t_d = 2.1$ h) (Singh et al. 2012; Belur et al. 2013), which could be attributed to different media composition, culture conditions, and bacterial isolates.

According to data described in (Table 1), *P. luminescens* subsp. *akhurstii* SL0708 metabolic phases were determined in the following manner: phase I, from 0 to 36 h; intermediate phase, between 36 and 72 h; and phase II from 72 to 96 h (Fig. 2). Maximum bioluminescence value was at 24 h (3,436.7 LAU), with a 21% decrease until reaching a value of 2709.8 LAU at 36 h, corresponding to the stationary phase. Obtained results are in agreement for the growth phase with those reported by Belur et al. (2013), who affirmed very high bioluminescence observed in a defined medium during the early stationary phase, and it was maintained throughout the stationary phase. On the contrary, they differ from other reports, demonstrating minimal bioluminescence until cells reach late logarithmic or stationary phase (Schmitz et al. 1999).

Likewise, an intermediate and phase II gene expression were established, resulting in a more active metabolism, greater production of oxidative stress proteins, and "stock" proteins. These mechanisms are designed to facilitate adaptation to new conditions, favoring an increase in growth velocity (Turlin et al. 2006). Further, phase II reduced levels of dye absorption, pigmentation, production of antibiotic substances and degradative enzymes, occurrence of crystalline inclusion proteins, and bioluminescence (Ffrench-Constant et al. 2003). Possibly, the above can be attributed to the fact that this gave the bacteria a strategy to adapt to more than one particular environment, especially low osmotic resistance or anoxic environments.

When glucose was added to EPNs production media, it had a positive effect on phase I maintenance, which was of interest for production processes. Furthermore, constant supplementation of this carbohydrate significantly increased (up to twice as much) biomass production and antibiotic production (140% increase) in phase I (Jeffke et al. 2000). This not only hinders contamination in culture media, but also benefits recovery percentage and final IJs yield, since a greater bacterial biomass at phase I results in a higher accumulation of the "feeding signal" (Gil et al. 2002). This molecule induces IJ's mouth and anus opening to start the feeding process. Moreover, it prompts the IJ to molt to the next stage and start its life cycle, resulting in new IJ generations (Aumann and Ehlers 2001).

Bacterium metabolic phase I is required for EPN production, particularly due to four metabolic characteristics: (1) capacity to degrade media into compounds that can be assimilated and necessary for IJs, distinctively sterols by lytic enzymes; (2) secretion of "feeding signals" to induce development and production of new IJs generations; (3) capacity to establish symbiosis or re-associate/adherence to the nematode's intestines; and (4) secretion of antibiotic substances, avoiding contamination in the media (Ehlers 2001; Salazar-Gutiérrez et al. 2017). Consequently, it is most important to maintain phase I during in vitro EPNs

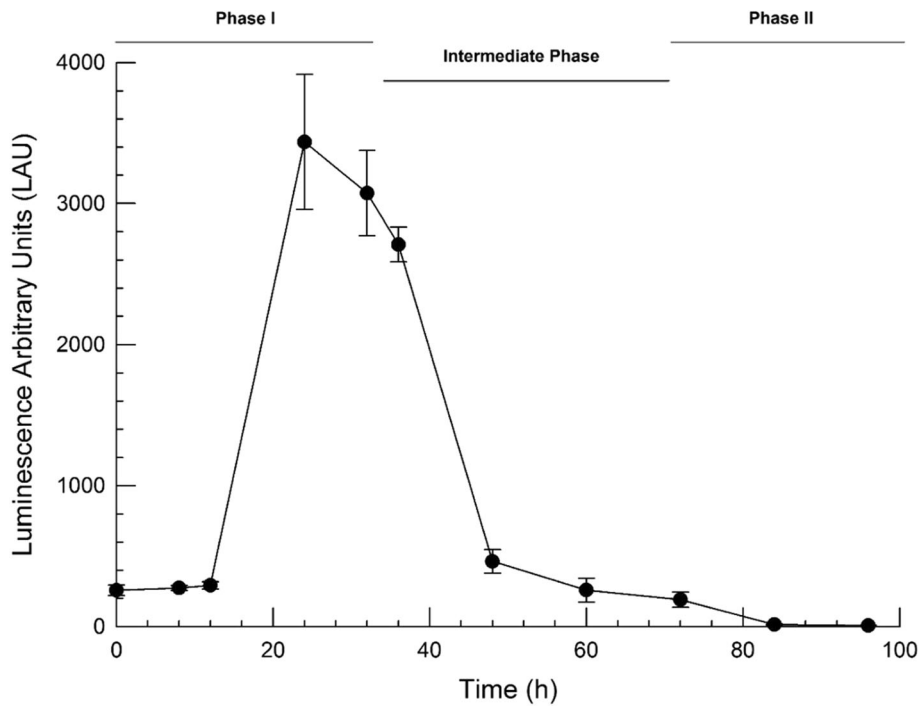

Fig. 2 *Photorhabdus luminescens* subsp. *akhurstii* SL0708 luminescence arbitrary units (LAU)

production to increase recovery percentage and to obtain high IJ yield during the production process (Stock et al. 2017). Therefore, it is possible to achieve this under strictly controlled culture conditions, optimized media composition, and knowledge of isolated bacterial growth kinetics in EPN production media.

In this study, it was observed under the evaluated culture conditions (media composition, batch culture, 28 °C, 150 rpm 20% effective work volume) that glucose was a determinant factor phase I in maintenance. Thus, IJs inoculation should be performed, when glucose has not been totally consumed, i.e., up to 36 h. During this time lapse, media was degraded and it can be assimilated by EPNs. Accumulation of "feeding signaling" takes place, favoring EPN development. Moreover, bacteria present are capable of establishing symbiosis with new IJs generations (Cho et al. 2011).

Regarding *P. luminescens* subsp. *akhurstii* SL0708 growth kinetics and metabolic phases, it was reported that phase I was present in late exponential phase or at the start of the stationary phase, suggesting population density was a determinant factor in phase I variation to intermediate or phase II (Aumann and Ehlers 2001; Yoo et al. 2001; Cho et al. 2011). However, in this study, it was observed that metabolic phase I was at the beginning of the exponential phase (Fig. 1). This suggests that for the media studied, the evaluated conditions, and the bacterium isolate were related to the bacterial population density and the metabolic phase, and it was primarily influenced by the media composition for in vitro *H. indica* SL0708 production.

P. luminescens akhurstii SL0708 pathogenicity

Regarding *P. luminescens* subsp. *akhurstii* SL0708 pathogenicity obtained from cultures at 24, 48, 72, and 96 h, they presented 100% of *G. mellonella* larvae mortality percentage during the first 24 h of exposure (Fig. 3).

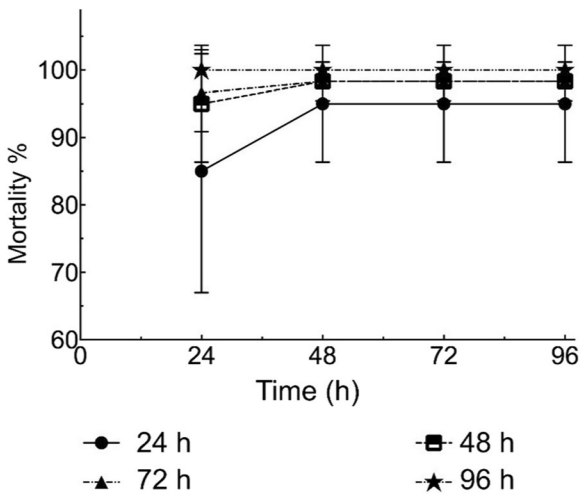

Fig. 3 *Galleria mellonella* larvae mortality percentage over the course of 4 days after infection with bacteria obtained from cultures at 24 (black circle), 48 (black square), 72 (black triangle), and 96 (black star) hours

Insignificant differences were observed among bacterium pathogenicity from cultures at 24, 48, 72, and 96 h and infection time course, i.e., 24, 48, 72, and 96 h of exposure ($F = 0.934$; df = 15; $p = 0.5339$).

Likewise, *G. mellonella* observed signs were the same for all evaluated times, consistent with reports for this isolate (Saenz-Aponte et al. 2014). Moreover, independent of bacterium metabolic phase in culture media, when injected into the larva, it generated pathogenicity. This could be attributed to the bacterium capacity to revert its metabolic phase, from phase II to phase I, when it entered into a controlled condition (larva), with specific composition, favoring metabolite expression characteristic of phase I (Turlin et al. 2006).

The bacterium presented the same biochemical profiles, regardless to time of culture (24, 48, 72, and 96 h) and metabolic phase (phase I, intermediate phase, or phase II), as was evidenced by biochemical test result profile (API 20NE) (Table 2). Results are in agreement with previous reports of this isolate (Saenz-Aponte et al. 2014). Enzyme production could be induced by the substrate present in the test or bacterium did not present variations against all biochemical characteristics depending on culture conditions.

Conclusion

P. luminescens subsp. *akhurstii* was cultured in *H. indica* SL0708 in vitro liquid production media, allowing to establish that the growth phase had a direct relationship with the bioluminescence. On the other hand, the bacterium metabolic phase change had no effect on *G. mellonella* larvae pathogenicity. Based on the results, the inoculation at 36 h of culture possibly assured the highest *H. indica* SL0708 IJ yield and recovery percentage. Therefore, given the glucose importance as a substrate to maintain it in the metabolic phase I, future studies should evaluate high glucose concentrations or addition of a carbohydrate source during the different time points of the process to IJ *H. indica* SL0708 culture media to prolong or sustain this metabolic phase and plausibly obtain a greater recovery and IJ yield at the end of the process in comparison to in vivo results.

Table 2 *Photorhabdus luminescens* subsp. *akhurstii* SL0708 biochemical characteristics (API 20NE) for bacteria obtained at 24, 48, 72, and 96 h of culture

Biochemical characteristics	24 h	48 h	72 h	96 h
Nitrate to nitrite reduction (NO_3)	–	–	–	–
Nitrate reduction to N_2 (N_2)	+	+	+	+
Indole production (tryptophan enzyme assay) (TRP)	+	+	+	+
Glucose fermentation (GLU)	+	+	+	+
Arginine dehydrolase (ADH)	+	+	+	+
Urease test (URE)	+	+	+	+
β-glucosidase (ESC)	+	+	+	+
Protease (GEL)	+	+	+	+
β-galactosidase (PNPG)	–	–	–	–
Glucose assimilation (GLU)	+	+	+	+
Arabinose assimilation (ARA)	–	–	–	–
Mannose assimilation (MNE)	+	+	+	+
Mannitol assimilation (MAN)	+	+	+	+
N-acetylglucosamine assimilation (NAG)	+	+	+	+
Maltose assimilation (MAL)	+	+	+	+
Potassium gluconate assimilation (GNT)	+	+	+	+
Capric acid assimilation (CAP)	+	+	+	+
Adipic acid assimilation (ADI)	–	–	–	–
Malate assimilation (MLT)	+	+	+	+
Trisodic citrate assimilation (TCI)	+	+	+	+
Phenyl acetic acid assimilation (PAC)	–	–	–	–
Oxidase (OX)	–	–	–	–

+ positive, – negative

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Authors' contributions
MTOH conducted research experiments. BQH and ASA supervised the project and wrote the paper. ASA submitted the paper. All the authors approved the submission of this original article, which has not been submitted or is not under consideration for publication elsewhere. All authors understood and agreed that the published article will not be published elsewhere in the same form, in any language including electronically, without the consent of the copyright holder. All authors read and approved the final manuscript.

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Availability of data and materials
All data of the study have been presented in the manuscript, and the materials, which are used in this study, are of high quality and grade.

Ethics approval and consent to participate
Not applicable for this section.

Consent for publication
Not applicable for this section.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Departamento de Biología, Laboratory for Biological Control, Biología de Plantas y Sistemas Productivos, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia. [2]Departamento de Microbiología, Laboratory Applied Biotechnology, Facultad de Ciencias, Grupo de Biotecnología Ambiental e Industrial, Pontificia Universidad Javeriana, Bogotá 110561, Colombia.

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