Scientific Paper

Isolation of *Bacillus* spp. strains with potentialities for agricultural and industrial development, from the bioproduct IHPLUS®

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Abstract

Objective: To determine the potentialities of *Bacillus* spp. isolates obtained from the bioproduct IHPLUS®, as plant growth promoters and with industrial purposes.

Materials and Methods: For the inoculation, 10 mL of the product were inoculated in nutrient broth medium. Afterwards, through thermal shock at 80 ºC during 12 minutes, the vegetative forms were eliminated. Seriated dilutions were carried out, and then planting was performed in nutrient agar medium. The isolates were purified and it was verified that they corresponded to Gram-positive, endospore-producer, bacillary forms. The production of indoleacetic acid and the capacity of the isolates to solubilize tricalcium phosphate were evaluated. The chitinolytic and/or 1,3-β-glucanolytic, lipidic, proteolytic, amylolytic, cellulolytic and mannanolytic activity of such isolates.

Results: Four *Bacillus* spp isolates were obtained. Isolates IH7 and IH5 showed biostimulator and biofertilizer properties, by producing indoleacetic acid (23,55 μg mL^{-1}) and solubilizing tricalcium phosphate, respectively. Isolate IH7 showed potentialities as antagonist agent of phytopathogens, because it produced hydrogen cyanide. The production of 1,3-β-glucanase and/or chitinase (95,55 %) and proteases (43,98 %) was shown. IH5 showed amylolytic and mannanolytic activity; while IH7 produced amylases and cellulases.

Conclusions: The biochemical activities shown by the isolates prove the importance of the bioproduct IHPLUS® as a source for obtaining *Bacillus* spp. strains, with applications in different agricultural and industrial sectors.

Keywords: indoleacetic acid, enzymatic activity, phosphate solubilization.

Introduction

In modern agriculture large quantities of agrochemicals are utilized in order to maximize agricultural productions. Although the effect of these substances is immediate, they constitute a risk for human and animal health, due to the entrance of heavy metals to the trophic chain, which cause death of the biota and soil degradation, because they disturb and damage the soil structure (Zahid *et al*., 2015). The application of chemical fertilizers for long periods frequently reduces soil pH and exchanger bases, which causes the soils to become unproductive for many crops and, consequently, decrease their agricultural yield (Gupta *et al*., 2015).

Among the strategies to solve these problems is the development of new products from rhizosphere microorganisms, which exert direct and indirect beneficial effects on the plants (Thakur *et al*., 2017). The mechanisms shown by these plant growth promoting microorganisms are related to the production of growth regulator substances, such as auxins, gibberellins and cytokinins (Jha and Saraf, 2015). They are also associated with phosphate solubilization (Thanh and Tram, 2018); production of siderophores (Rayavarapu and Padmavathi, 2016) and secretion of antibiotics and lytic enzymes with antagonist and pathogen biocontrol effect (Yamamoto *et al*., 2015).

Bacillus is one of the most studied bacterial genera as growth promoters, due to their easy development in culture media and to their capacity to produce resistant endospores to various unfavorable conditions, which facilitates obtaining bioproducts with stable formulations. There are diverse works (Hauka *et al*., 2016; Robledo-Buriticá *et al*., 2018; Singh, 2018) that report about the properties of this genus, as plant growth-promoting bacteria under normal and environmental stress conditions, especially as biocontrolling agents of the phytopathogens that affect agricultural crops (Méndez-Bravo *et al*., 2018; Sabaté *et al*., 2018). A large group of studies supports the capacity of these microorganisms

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to produce different types of hydrolytic enzymes (mannanases, cellulases and amylases) with applications in the development of the food, pharmaceutical and textile industry; besides referring their utilization in paper, bioethanol and detergent production, among other uses (Singh *et al*., 2015).

In Cuba there are several products based on beneficial microorganisms that promote plant growth. Among them IHPLUS® can be cited, developed by the Pastures and Forages Research Station Indio Hatuey. This product constitutes a microbial complex with wide utilization perspectives in agriculture and in animal feeding and health. Recent studies proved its beneficial effect on the germination and early growth of *Sorghum bicolor* L. (Moench) (Díaz-Solares *et al*., 2019).

The objective of this research was to determine the potentialities of *Bacillus* spp. isolates, obtained from the bioproduct IHPLUS®, as plant growth promoters and with industrial purposes.

Materials and Methods

The trials were conducted with lot 31 of liquid inoculant of IHPLUS®, characterized according to the normalized operation procedure (PNO-LB-M-008/2014) of the Pastures and Forages Research Station Indio Hatuey, Perico municipality, Matanzas province, Cuba.

Procedure for the isolation of Bacillus spp. The isolation was carried out in the Microbiology Laboratory, of the Center of Biotechnological Studies of the University of Matanzas. With the primary objective of increasing the bacterial populations, 10 mL of the product IHPLUS® were inoculated in 90 mL of nutrient broth medium, contained in 250-mL Erlenmeyer flasks. The samples were incubated in agitation (130 r.p.m.) at 37 ºC during 72 h (Johnson and Bishop, 1996). Afterwards, the culture media were placed in Bain Marie at 80 ºC during 12 minutes to eliminate all the vegetative forms and start the isolation process from resistant endospores to these conditions (Lobo *et al*., 2018).

Seriated dilutions (10^{-1} and 10^{-5}) were made to the microbial suspensions in saline solution (NaCl 0,9 %). Aliquots of 100 µL were taken from the dilutions between $10^{-1}$and 10^{-5} and were transferred to Petri dishes with nutrient agar medium. Each dilution of the different samples was planted in triplicate. The dishes were placed in an incubator (Boxun®) at 37 ºC during 48 h. Samples were taken from colonies with different morphologies and were planted in culture tubes, wedge-shaped. The isolates were numerically identified and were placed under the same growth conditions during 24 h. Gram staining was performed on all the isolated colonies and the isolates with bacillary morphology and those which were Gram-positive (Ashish *et al*., 2008), were identified. Seventy-two hours after being planted, on the colonies that showed these characteristics malachite green staining was carried out, in order to observe the endospores. The isolates that fulfilled these requisites were purified in Petri dishes with nutrient agar medium through the planting technique by striations or depletion.

Ten milliliters of the product IHPLUS® were inoculated in 90 mL of nutrient broth medium, contained in 250-mL Erlenmeyer flasks. The samples were incubated in agitation (130 r.p.m.) at 37 ºC during 72 h. Afterwards, the culture media were placed in Bain Marie at 80 ºC for 12 minutes to eliminate all the vegetative forms and begin the isolation process from endospores resistant to these conditions.

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Biochemical determinations

Production of 3-indoleacetic acid (IAA). The production of indoleacetic acid was carried out through the method proposed by Brick *et al*. (1991). The bacterial isolates (100 µL 10^{-8} CFU) were inoculated in 25-mL Erlenmeyer flasks with 10 mL of nutrient broth supplemented with L-tryptophan (1 mg mL^{-1}). The culture media were placed under agitation conditions (130 r.p.m.), darkness and 37 ºC during 48 h. Afterwards, the cell suspensions were centrifuged at 6 000 r.p.m. during 10 minutes. The

supernatant was collected for the quantitative determination of 3-indoleacetic acid. The culture medium without inoculant was used as negative control. First, a calibration curve was made from a solution of 100 µg mL^{-1} of IAA, with concentrations of 5, 10, 20, 40, 60 and 80 µg mL^{-1}. From each of the pattern solutions and of the bacterial supernatants 0,5 mL were mixed, with 0,5 mL of Salkowski reagent (50 mL of 35 % perchloric acid, 1 mL of a solution of 0,5 mol L^{-1} FeCl3). The mixtures were left to rest for 30 min at ambient temperature and the absorbance readings were performed at 530 nm in a UV/VIS spectrophotometer (ULTROSPEC, 2000). With the values of the pattern curves, the equation that relates the IAA concentration, with regards to the absorbance, was obtained. The measurements were carried out in triplicate (Sánchez-García, 2013).

Phosphate solubilization. The capacity to solubilize inorganic phosphates by the bacterial isolates was determined in Pikovskaya medium (Pikovskaya and Pikovskaya, 1948), composed by glucose (10,0 g L^{-1}), $Ca_3(PO_4)_2$ (5,0 g L^{-1}), $(NH_4)_2SO_4$ (0,5 g L^{-1}), KCl (0,2 g L^{-1}), $MgSO_4.7H_2O$ (0,1 g L^{-1}), $MnSO_4$ (0,002 g L^{-1}), $FeSO_4$ (0,002 g L^{-1}), yeast extract (0,5 g L^{-1}) and agar (15,0 g L^{-1}). The isolates were planted on Petri dishes with the culture medium and were incubated at 37 ºC during seven days. The efficiency of the isolates to solubilize phosphates was determined through the power index (PI) (Nguyen *et al*., 1992), which was also used to characterize the production of several hydrolytic enzymes which are described below.

$$IP\ (\%) = \frac{(z-c)}{c} * 100$$

Where:

z-diameter of the hydrolysis halo
c-diameter of the bacterial colony

Lipidic activity. The lipidic activity was determined by planting the isolates in a culture medium composed by peptone (10 g L^{-1}), calcium chloride (0,1 g L^{-1}), sodium chloride (5 g L^{-1}), agar (15 g L^{-1}) and 10 mL of Tween 80 (Omidvari, 2008). Peptone and the salts were dissolved in 990 mL of distilled water. Afterwards, Tween 80 and the culture medium were sterilized separately at 121 ºC during 20 min. Before pouring the culture medium on the Petri dishes, the Tween 80 and the other components of the medium were mixed and homogenized. The dishes with the planted isolates were incubated at 37 ºC during 48 h. The depositions around the bacterial colonies indicated the activity of the lipase enzyme.

Chitinolytic and/or β-*1,3-glucanolytic activity*. The production of β-1,3-glucanase and/or was determined through isolate growth in a culture medium with bread yeast (*Saccharomyces cerevisiae*). An amount of 4,0 g L^{-1} of yeast and 16 g L^{-1} of agar were dissolved in distilled water and the medium was sterilized at 121 ºC for 20 minutes. The isolates were planted in Petri dishes with the culture medium and were incubated at 37 ºC during 48 hours (Swift, 2016).

Proteolytic activity. It was determined according to the method proposed by Chaiharn *et al*. (2008), with modifications. The isolates were planted on Petri dishes with culture medium composed by skimmed milk (15 g L^{-1}) as inducer, yeast extract (0,5 g L^{-1}) and agar (15 g L^{-1}). The dishes were incubated at 37 ºC during 24 h. The diameters of the light halos around the colonies were measured with a millimetric ruler to determine the power index (%) through the above-indicated formula.

Amylolytic activity. It was determined according to the method proposed by Bhojia (2011). The bacterial isolates were planted in a medium composed by NaCl (0,1 %), KH_2PO_4 (0,3 %), K_2HPO_4 (0,6 %), $MgSO_4$ (0,12 %), peptone (0,5 %), yeast extract (0,3 %), soluble starch (1,0 %) and agar (15 g L^{-1}). The dishes were incubated at 37 C during 48 h. The amylolytic activity was observed by staining the culture medium with the Gram iodine solution (2 % iodine and 0,2 % potassium iodide). The presence of a hydrolysis halo around the colony indicated the production of amylase enzymes. The capacity to produce these enzymes was qualitatively evaluated as negative (-) or positive (+).

Cellulolytic activity. The bacterial isolates were planted in culture medium composed by: NaCl (0,1 %), KH_2PO_4 (0,3 %), K_2HPO_4 (0,6 %), $MgSO_4$ (0,12 %), peptone (0,5 %), yeast extract (0,3 %), carboxymethyl cellulose (1,0 %) and agar (15 g L^{-1}). The dishes were incubated at 37 ºC during 48 hours and the cellulolytic activity was determined by revealing with a 0,5 % Congo red solution (Apun *et al*., 2000). The presence of a hydrolysis halo around the colony indicated the production of cellulolytic enzymes. The power index (%) was determined.

Mannanolytic activity. It was determined by planting the bacterial isolates in the culture medium composed by: NaCl (0,1 %), KH_2PO_4 (0,3 %), K_2HPO_4 (0,6 %), $MgSO_4$ (0,12 %), peptone (0,5 %), yeast extract (0,3 %), supplemented with locust beans gum

(LBG) (0,5 %) and agar (15 g L^{-1}). The dishes were incubated at 37 ºC during 48 h and the mannanolytic activity was observed through the application of Congo red to the culture medium (0,5 %). The presence of a hydrolysis halo around the colony indicated production of mannanases (Carder, 1986). The power index (%) was determined.

Hydrogen cyanide production (HCN). The method proposed by Lorck (1948) was used to determine the hydrogen cyanide production capacity of the bacterial isolates. The nutrient agar medium was supplemented with 4,4 g L^{-1} of glycine and was put in autoclave at 121 ºC during 20 min. The isolates were planted by striations in Petri dishes with the aid of a planting handle. Afterwards, a layer of filter paper (Whatman No. 1) was moisturized in a solution composed by 2 % sodium carbonate (Na_2CO_3) and 0,5 % pycric acid. The moisturized paper was placed on the top lid of the Petri dish, and then sealed with parafilm. The Petri dishes were incubated at 37 ºC during four days. The color change from yellow to reddish orange indicated the production of hydrogen cyanide. A Petri dish with culture medium without inoculation was used as control.

Statistical processing. Data fit to normal distribution was determined through Kolmogorov-Smirnov test and variance homogeneity, by Bartlett tests, the data were processed by Kruskal-Wallis non-parametric analysis. The differences among the average ranges of the variables, in each of the established treatments, were determined through Mann-Whitney's U-test (Sigarroa, 1985). All the biochemical analyses were conducted in triplicate. The data were processed with the program SPSS®, version 18.

Results and Discussion

Isolation of the bacterial colonies. The results of the bacillus colony isolation process are shown in table 1. Twenty isolates were obtained in total, among sporulated bacilli (SB), non-sporulated bacilli (NSB), cocci (C) with different groupings (diplococci, tetracocci, sarcins, among others), as well as other forms, such as actynomycetes. Four bacillary isolates with sporulating capacity (IH4, IH5, IH7 and IH9) were observed, which represented 20,0 % with regards to the total isolated forms. The fact that few isolates were obtained in number and diversity can be related to the acid pH of the product IHPLUS® (3,87), which affects the growth of *Bacillus* spp. The isolates with capacity to form endospores were selected for the chemical essays.

Production of indoleacetic acid (IAA). Indoleacetic acid constitutes a natural auxin produced by plants and by a wide range of soil microorganisms. Figure 1 shows the IAA production by the bacterial isolates. IH7 showed the highest values, with concentration of 23,58 µg mL^{-1}; while IH4, IH5 and IH9 produced lower contents, without significant differences among them.

These results are in agreement with the reports by other authors, who observed the production of this auxin by *Bacillus* spp. isolates, obtained from the rhizosphere of different plant species, such as *Aloe vera* L. (Thakur *et al*., 2017), *Arachis hipogea* L. (Thakur and Parikh, 2018) and *Piper nigrum* L. (Thanh and Tram, 2018). Particularly, isolate IH7 can constitute a potential candidate as plant growth biostimulator, because in diverse studies with IAA-producing *Bacillus* spp. strains, an increase of root development was observed in such species as *Zea mays* L. (Mike-Anosike *et al*., 2018), as well as the increase of germination and growth of wheat (*Triticum aestivum* L.) and broad beans [*Vicia faba* L.] (Yousef, 2018). However, it is necessary to test the effect of this isolate on the germination and growth process of agriculturally important crops, because plant response depends on the concentration of this regulator in the medium and on plant genotype, among other factors (Vrbničanin *et al*., 2011).

Phosphate solubilization. Isolate IH4 was the only one that showed the capacity to solubilize $Ca_3(PO_4)_2$ with power index of 48,25 % (figure 2). This coincides with other authors, who obtained rhizospheric isolates of *Bacillus* spp. with similar efficiency to that observed

Table 1. Results of the isolation of *Bacillus* spp from the product IHPLUS®.

Isolates	Bacillary forms		cocci	others	total
	NSB	SB			
IH	5	4	6	5	20
%	25	20	30	25	100

NSB: Non-sporulated bailli, SB: sporulated bacilli

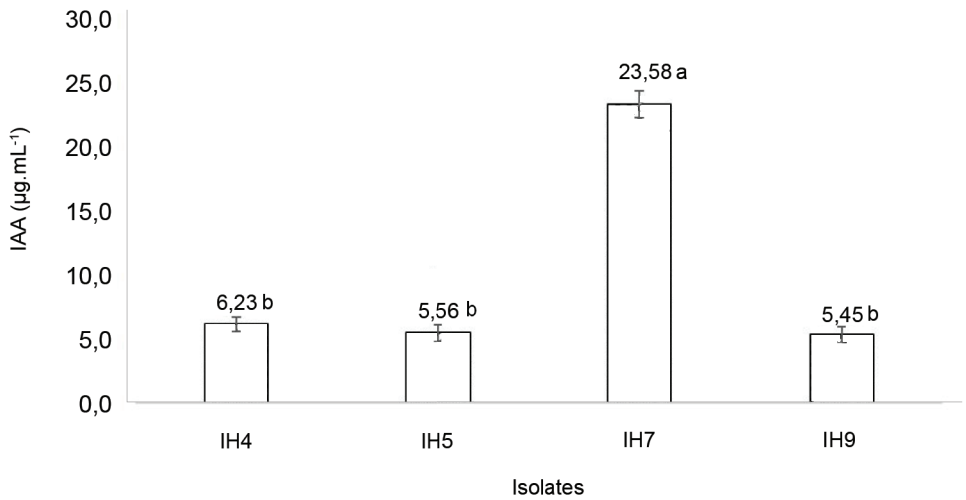

Figure 1. Production of 3-indoleacetic acid (IAA) by the isolates.
Different letters indicate significant differences among isolates, according to the *Kruskal-Wallis* test ($p \leq 0,05$)

in IH4 (Thanh and Tram, 2018). Thus, IH4 could contribute to increase crop growth and development. The utilization of insoluble phosphate solubilizing bacteria increases the availability of phosphorus for the crops. Phosphorus has vital cell functions, being a structural component of nucleic acids, phospholipids and ATP molecules, besides increasing seed and fruit production and stimulating root growth (Ranjbar-Moghaddam and Aminpanah, 2015).

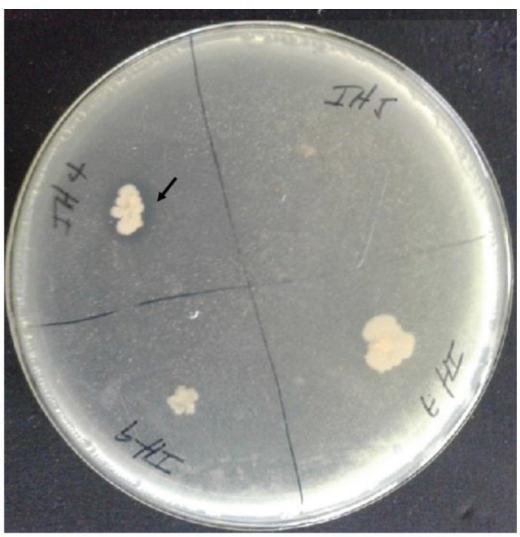

Figure 2. Solubilization of tricalcium phosphate by the bacterial isolates.
The arrow indicates the light halo around the isolate, which shows the solubilizing activity of phosphate.

Hydrogen cyanide production. Hydrogen cyanide production is part of the defense system of some microorganisms. The study of the production capacity of this gaseous metabolite showed that only isolate IH7 is capable of synthesizing this compound in low proportions, which was proven by the color change of the filter paper, from yellow to brown. In similar studies, several authors referred hydrogen cyanide production by rhizospheric isolates (Thakur and Parikh, 2018). The biocontrolling effect of HCN was associated to the capacity of this compound to bind with iron, which affects the available quantity of this element for phytopathogens. However, recent studies suggest that the main contribution of biogenic HCN is metal capturing and, consequently, the direct increase of nutrient availability that causes the increase of plant growth (Rijavec and Lapanje, 2016). The HCN function was compared with other similar activities such as phosphate solubilization and siderophore function, which allow to increase the availability of mineral elements for the plants (Sagar *et al*., 2018). The use of bacterial strains that produce HCN in nutrient-deficient soils can constitute an alternative for the development of sustainable agriculture in the future.

Enzyme production. Table 2 shows the β-1,3-glucanolytic and/or chitinolytic, lipidic and proteolytic activities of the isolates. Isolates IH4 and IH7 showed β-1,3-glucanolytic and/or chitinolytic activity. The latter had higher effectiveness (95,55 %) than IH4 (43,98 %). Lipidic activity was not shown

in any of the isolates; while only IH7 expressed proteolytic activity, with power index of 43,98 %.

Different authors (Majumdar and Chakraborty, 2017; Dar *et al*., 2018) have referred to the enzymatic activities proteases and chitinases in *Bacillus* spp. strains. The proteolytic activity obtained with IH7 was higher than the one observed in other *Bacillus* spp. isolates, extracted from the rhizosphere of *Aloe vera* L., with power index between 11,3 and 21,8 % (Thakur *et al*., 2017).

These results suggest the possible use of IH7 and IH4 as biocontroller of phytopathogens, because the production of these enzymes causes lysis or disturbs the components of the fungi cell walls (Haldar and Sengupta, 2015) and decreases the incidence of fungal pathogens, such as *Macrophomina phaseolina* (Hernández *et al*., 2017) and *Pythium myriotylum* (Jimtha *et al*., 2016). In addition, the proteolytic activity of IH7 can contribute with the nitrogen cycle in the rhizosphere, by accelerating the degrading processes of organic matter, which increases soil fertility (Singh *et al*., 2018).

It is important to state that the isolates obtained from the product IHPLUS® also have the capacity to tolerate acid pH lower than 4, for which it is likely that they have tolerant enzymes to high concentrations of hydronium ions. This suggests the possible use of the identified isolates in acid soils.

The amylolytic, cellulolytic and mannanolytic activity of the isolates is shown in table 3 and in figure 3. Isolates IH5 and IH7 expressed amylase enzymes. In IH4 the amylolytic activity could not be determined, because this isolate secreted ceric substances of hydrophobic nature, which covered the culture medium, for which it was not possible to reveal the activity by staining the culture medium. Regarding the production of cellulases and mannanases, IH7 showed remarkable cellulolytic activity (132,06 %) and IH5 was efficient in the mannanolytic activity, with power index equal to 94,44 %.

These results coincide with those from other works that refer amylolytic, celluloytic and mannanolytic activities in *Bacillus* spp. (Singh *et al*., 2016). Several species of this genus are widely used for obtaining α-amylases with industrial purposes, such as *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* (Saini *et al*., 2017).

The cellulolytic activity of IH7 allows to see its possible applications in different agricultural areas in the biocontrol of phytopathogen fungi, such as *Phytophthora* (Naing *et al*., 2014) and in the decomposition of the cellulosic organic matter present in the rhizosphere, which increases nutrient availability for the plants. Cellulases also have different applications in the food and textile industries, as well as in paper and detergent production (Singh *et al*., 2016).

The high mannanolytic activity of isolate IH5 also indicates its high potential use in paper and detergent industries (Singh *et al*., 2016). In addition, in the animal husbandry it can be used to improve nutrient digestibility and absorption in animals that are fed from plant fibrous waste, such as sugarcane

Table 2.β-1,3-glucanolytic and/or chitinolytic, lipidic and proteolytic activities of the isolates.

Isolate	Power index, %		
	β-1,3-glucanolytic and/or chitinolytic ± SE	Lipidic ± SE	Proteolytic ± SE
IH4	23,2 ^{b} ± 0,92	NE	NE
IH5	NE	NE	NE
IH7	95,6 ^{a} ± 3,61	NE	44,0 ± 4,75
IH9	NE	NE	NE

Different letters indicate differences among isolates for the same activity, according to Kruskal-Wallis test ($p \leq 0,05$). ± SE: standard error. NE: negative for the biochemical test

Table 3. Amylolytic, cellulolytic and mannanolytic activity of the evaluated isolates.

Isolate	Power index, %		
	Amylolytic	Cellulolytic ± SE	Mannanolytic ± SE
IH4	ND	NE	NE
IH5	+	NE	94,4 ± 5,56
IH7	+	132,10± 14,23	NE
IH9	NE	NE	NE

SE: standard error. +: positive test, NE: negative for the biochemical essay. ND: undetermined

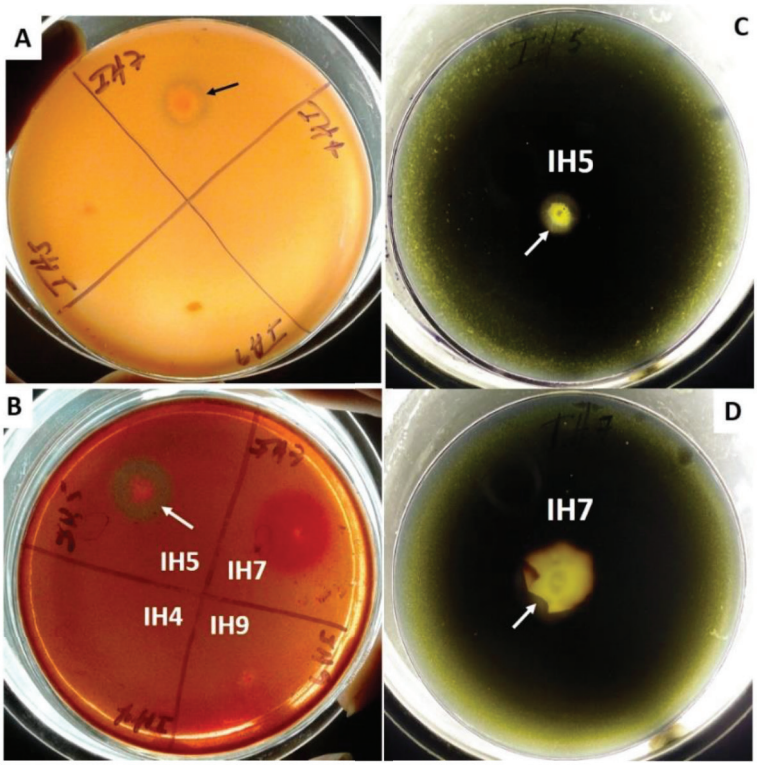

Figure 3. Production of hydrolytic enzymes by the isolates.
A: β-1,3-glucanases and/or chitinases from IH7, B: mannanases from IH5, C: amylases from IH5, D: amylases from IH7. The arrows indicate the activity halos.

bagasse and corn straw (Matos *et al*., 2018). The use of IH5 in fermentation processes with these plant materials could contribute to the partial digestion of different cell wall components, because mannanases hydrolyze the β-1,4-mannanosidic bonds present in carbonated skeletons of mannans, galactomannans, glucomannans and galactoglucomannans (Song *et al*., 2017). The released products constitute mostly oligosaccharides, which increase the availability and assimilation of energy nutrients in monogastric animals; they can also show prebiotic activity or be used as nutritional additives (Scapini *et al*., 2018).

Conclusions

The bioproduct IHPLUS® constitutes a potential source for the isolation of bacilli with different biological activities and possible applications in the agricultural and industrial sectors.

Isolates IH7 and IH5 showed biostimulator and biofertilizer properties, by producing indoleacetic acid (23,55 μg mL^{-1}) and solubilizing tricalcium phosphate, respectively. IH7 also showed potentialities as antagonist agent of phytopathogens, because it produced hydrogen cyanide and β-1,3-glucanolytic and/or chitinolytic and proteolytic. Meanwhile, isolate IH5 constitutes a promising candidate for it use with different industrial purposes, given the efficient production of mannanases.

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Authors' contribution

- Yunel Pérez-Hernández. Design and execution of the experiments, statistical processing, manuscript writing and final revision.
- Maykelis Díaz-Solares. Research design, manuscript writing and final revision.
- Ana Julia Rondón-Castillo. Design and execution of some essays, manuscript writing and final revision.

- Leticia Fuentes-Alfonso. Statistical processing, manuscript writing and final revision.
- Liliet González-Sierra. Manuscript writing and final revision.
- Ángel Monserrate Guzmán-Cedeño. Experiment design and final revision.

Conflict of interests

The authors declare that there are no conflicts of among them.

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