Mexican Journal of Phytopathology
ISSN: 2007-8080

Crossref
Open access

Scientific Article

"Nejayote" valorization as a culture medium for *Pseudomonas fluorescens* and production of antifungal extracts

Víctor Manuel Rodríguez-Romero, Ramón Villanueva-Arce, Enrique Durán-Páramo* Laboratorio de Biotecnología Alimentaria, Unidad Profesional Interdisciplinaria de Biotecnología (UPIBI), Instituto Politécnico Nacional (IPN), Av. Acueducto s/n, Barrio La Laguna Ticomán, Ciudad de México, CP 07340, México.

***Corresponding Author**:
Enrique Durán-Páramo
eduran@ipn.mx

Section:
Periodical Issue

Received:
01 May, 2024
Accepted:
30 June, 2024
Published:
16 July, 2024

Citation:
Rodríguez-Romero VM, Villanueva-Arce R and Durán-Páramo E. 2024. "Nejayote" valorization as a culture medium for *Pseudomonas fluorescens* and production of antifungal extracts. Mexican Journal of Phytopathology 42(3): 28. https://doi.org/10.18781/R.MEX.FIT.2405-3

ABSTRACT

Background / Objetive. Nejayote is an alkaline agroindustrial waste that is generated from the nixtamalization process of corn. The purpose of this work was to demonstrate that nejayote can be used as a culture medium for the growth of *Pseudomonas fluorescens* NR113647 and to produce metabolites with antifungal activity for the sustainable management of *Aspergillus niger, Botrytis cinerea* and *Fusarium solani*.

Materials and Methods. Culture media were formulated with nejayote and nejayote with glycerol, with pH 6 and 12. The bacterial biomass was separated by centrifugation and filtration and the antifungal capacity of the extracts against *A. niger, B. cinerea* and *F. solani* was determined. The determination of the metabolites present in the extracts was carried out. *P. fluorescens* NR113647 was able to grow on all media.

Results. The extracts from nejayote at pH 12 showed inhibition of the growth of all the fungi evaluated; at least five metabolites produced by *P. fluorescens* NR113647 and involved in the biocontrol of phytopathogens were identified.

Conclusion. Nejayote can be used as a culture medium for *P. fluorescens* NR113647, to produce biomass and secondary metabolites with antifungal capacity; in addition, nejayote could be used for the cultivation of other microorganisms.

Keywords: Biological control, nixtamalization, antifungal activity.

Introduction

One of the main approaches of modern biotechnology is the use of the biological control of phytopathogens in agriculture, which is defined as the action of effective natural agents to fight diseases in crops, which represent an alternative for the reduction of the use of potentially damaging chemical products to the environment and human health (Thambugala *et al*., 2020). Out of the different types of biological control agents, microbial agents and particularly bacteria of the genus *Pseudomonas* are an excellent option for use in agriculture (Santoyo *et al*., 2012).

Biocontrol mediated by *Pseudomonas* strains is carried out by mechanisms such as direct antibiosis, competition for nutrients and for space, the promotion of plant growth and the induction of systemic resistance in plants (Dimkić *et al*., 2022). Diverse commercial products have been produced from *Pseudomonas* biomass, including BioJect Spot-Less™, Bio-Save® 10LP, AtEze™ and Cedemon (Mark *et al*., 2006). The production of microbial biomass and metabolites in liquid culture media is crucial for the development of products based on bacterial strains such as *Pseudomonas*; however, the use of commercial culture media in the multiplication of microorganisms has a high cost. Therefore, the use of by-products or waste from the food industry is an option to reduce costs associated to the production and recovery of biological control agents, while waste management and conversion of usable products through the application of biotechnological technologies (Khalil *et al*., 2016; Sivakumar *et al.,* 2022; Suryanti *et al*., 2015).

One of the main residues of the food industry comes from the processing of maize (*Zea mays*), which is intimately related to Mexican culture. The tortilla represents the main food derived from the processing of maize (Hellin *et al.*, 2010). In this regard, it is estimated that in Mexico, the current annual consumption of maize per capita is 120 kg (FAOSTAT, 2023). For the production of tortillas, maize grains undergo an alkaline thermal process called nixtamalization, which consists in the cooking of mature maize grains with lime, followed by grinding to obtain a dough and finally process (Serna-Saldivar, 2021). Nixtamalization produces chemical and nutritional changes in the maize, and the main by-product of the nixtamalization process is the 'nejayote,' considered the alkaline water from the cooking of the maize; it is estimated that for every kilogram of maize processed, between 7 and 8 liters of nejayote are obtained (Díaz-Montes *et al*., 2020).

The nejayote is considered one of the most effluent contaminants of the food industry. This liquid contains high concentrations of organic matter dissolved, corresponding to the structures of the maize grain that is detached during cooking (mainly the endosperm and the pericarp) and to the lime used in the process (DQO > 28450 mg L^{-1}). Additionally, the nejayote is characterized for having an alkaline pH with a value of over 10 (Meraz *et al*., 2015). The nejayote represents an important

source of fermentable sugars and compounds such as proteins, fibers, fats, etc. Due to this, the use of the nejayote in other processes or purification of components make it potentially applicable in the biotechnology industry (Xu *et al.*, 2021).

Nejayote has been proven to be usable as an alternative culture medium for the development of different microorganisms such as *Arthrospira maxima*, *Aspergillus oryzae, Bacillus thuringiensis, Chlorella vulgaris, Lactobacillus casei, L. helveticus L. rhamnosus,* as well as for the production of different secondary metabolites such as enzymes, pigments or antibiotics (Bacame-Valenzuela *et al*., 2020; López-Pacheco *et al*., 2019; Ramírez-Romero *et al*., 2013; Salazar-Magallon *et al.*, 2015). The aim of this work was to evaluate the use of nejayote as an alternative culture medium for the growth of *Pseudomonas fluorescens* NR113647 and for the production of secondary metabolites with antifungal activity.

Materials and Methods

Biological material. The *Pseudomonas fluorescens* NR113647 strain, identified previously based on morphological, cultural and biochemical characteristics of the organism, was used. Molecular identification was carried out with the amplification and sequencing of rRNA 16s and alignment with the Gene Bank of the National Center for Biotechnological Information (Rodríguez-Romero *et al*., 2019). The bacterium was cultivated in Agar King B (KB) (King *et al*., 1954) for 7 days and incubated at room temperature (25 ± 2 °C) for later tests. The *Aspergillus niger, Botrytis cinerea* and *Fusarium solani* strains were isolated and provided by the Postharvest Technology Laboratory of the Biotic Products Development Center of the National Polytechnic Institute of Mexico (CEPROBI-IPN). Molecular identification was performed (Harwood, 1996) in the Comprehensive Plant Safety Diagnose Laboratory (LADIFIT) of the Colegio de Postgraduados. The fungi were cultivated individually in a Potato-Dextrose-Agar medium (PDA, BD Bioxon, Mexico) for 4-7 days and incubated at room temperature (25 ± 2 °C) for later tests.

Obtaining, conditioning of nejayote and formulation of the culture medium. The nejayote used was gathered from the maize processing companies located in La Laguna Ticomán, Mexico City (19° 31’ 12” N, 99° 07’ 45” W and altitude of 2248 masl) and later mixed. To eliminate sedimentable organic matter, the nejayote was centrifuged (Centrífuga Beckman Coulter® J2-MC, California, U.S.A.) at 10000 rpm for 15 min at a temperature of 4 °C, the supernatant was recovered and the pH was measured by electrode immersion (HANNA pHep5®, Romania). To produce the culture media, a completely randomized treatment design was conducted in a factorial arrangement, resulting in a total of four treatments; 1) Nejayote at a pH of

6 (N6), 2) Nejayote at a pH of 12 (N12), 3) Nejayote at a pH of 6 with 10 g L^{-1} of glycerol (Ngly 6) and 4) Nejayote at a pH 12 with 10 g L^{-1} of glycerol (Ngly12). The pH of the nejayote was adjusted with NaOH or HCl 1 N as needed. A King B (KB) culture broth with a pH of 6 was used as a control.

pH and evaluation of the production of *P. fluorescens* biomass. The cultivation was carried out in 250 mL Erlenmeyer flasks with 100 mL of each one of the different treatments; three independent replicates were conducted for each treatment. The beakers were sterilized (Autoclave Yamato Scientific co. Ltd, SM510, Japan) for 15 min at 121 °C, incubated with 10 mL of *Pseudomonas fluorescens* NR113647 (10^6 UFC mL^{-1}) and incubated at 25 ± 2 °C for 72 h shaking constantly (120 rpm). At the end of the cultivation, the pH of the culture media was determined by using an immersion electrode (HANNA pHep5®, Romania) and the biomass development of *P. fluorescens* NR113647 was evaluated using the plate count method. For this purpose, serial dilutions were carried out from each of the culture media, plated on standard plate count agar (ACS, BD Bioxon, Mexico) and incubated at 25 °C for 48 h.

Evaluation of the antifungal *in vitro* activity of the *P. fluorescens* extracts. To obtain the antifungal extracts, after incubation, each one of the treatments were centrifuged (Beckman Coulter® J2-MC Centrifuge, California, U.S.A.) for 15 min at 10015 xg at 4°C, the supernatant was extracted, a filtration was carried out using 0.22 µm sterile membranes (Cole Palmer, U.S.A.). The antifungal effect of the extracts was determined using the poisoned culture medium technique (Erhonyota *et al.,* 2023). For this, Potato-Dextrose-Agar (PDA, BD Bioxon, Mexico) was prepared at double the concentration recommended by the manufacturer (78 g L^{-1}), then sterilized (Yamato Scientific co. Ltd, SM510 Autoclave, Japan) for 15 min at 121 °C. Later, the PDA medium (BD Bioxon, Mexico) was cooled to a temperature of 45 °C, mixed with the filtered supernatant of each one of the treatments in a proportion of 1:1, deposited in Petri dishes (90 x 15 mm) and left to solidify. PDA medium (BD Bioxon, Mexico) was used at the concentration recommended by the manufacturer (39 g L^{-1}) as a control; five independent replicates were performed per treatment. In the middle of the Petri dishes, discs with a diameter of 5 mm were planted with the mycelia of each one of the fungi. The test finished when the growth of the fungi in the negative control reached the edge of the dish. The percentage of inhibition was calculated with the measurement of the diameter of the culture of each experimental unit, followed by the use of the formula: Inhibition (%) = [(Da-Db) / Da] x 100, where Da (mm) is the diameter of growth of the fungi in the dish used as a negative control and Db (mm) is the diameter of growth of the fungi in the test dish (Al-Hetar *et al*., 2011).

Identification of the *P. fluorescens* antifungal metabolites. To identify the metabolites, the nejayote medium extract with the highest *in vitro* antifungal activity was used; the treatment was mixed with ethyl acetate in a 1:1 ratio and shaken for 2 h at 120 rpm. The aqueous fraction was discarded and the organic fraction evaporated at reduced pressure to obtain a dry raw extract. The dry extract was suspended in ethyl acetate at a concentration of 1 mg mL^{-1}. The standards of phloroglucinol and phenazine (Sigma-Aldrich, St. Louis, U.S.A.) were suspended in ethyl acetate at a concentration of 0.5 mg mL^{-1}. Aliquots of 50 µL were taken and subjected to thin-layer chromatography (TLC; silica gel. 60 F254, 20 X 20, 0.5 mm, Merck and Co, Inc) using a hexane:acetone 3:2 solvent system. The plate was dried completely and the compounds were recovered and detected at 254 nm (Suleimana *et al*., 2010).

To identify hydrogen cyanide (HCN), the method described by Verma *et al*. (2007) was used. Petri dishes containing soybean casein digest agar (AST, BD Bioxon, Mexico) were inoculated with 100 μL of the extract from the nejayote medium with the highest antifungal activity, KB medium extract (positive control) and distilled water (negative control). Filter paper (Whatman no. 1) impregnated with 0.5% pyric acid and 2% sodium carbonate were placed on top of the dishes. The dishes were sealed and incubated at 30 °C for 24 h. After incubation, a color change in the filter paper indicated a positive HCN production.

To identify siderophores, the method used was the CAS-agar plate assay method (Schwyn and Neilands, 1987). The Chrome Azurol S (CAS)/Fe3+/ Hexadecyltrimethylammonium bromide (HDTMA) complex was used as the indicator. To prepare 1 L of blue agar, 60.5 mg of CAS were dissolved in 50 mL of water mixed with 10 mL of Fe III (1 mM $FeCl_3 \cdot 6H_2O$ in 50 mL of 10 mM HCl), then mixed with 900 mL bacteriological agar (of 15 g L^{-1}) adjusted to a pH of 7. The dishes were inoculated with 1mL of the nejayote extract with the highest antifungal activity, 1 mL of KB extract (positive control) and 1 mL of distilled water (negative control). The dishes were incubated for 6 days. Siderophore production was classified visually in the culture medium in four categories: 1. No production, 2. Low production (light yellow), 3. High production (moderate yellow) 4. Very high production (yellow).

Statistical analysis. The experimental data were analyzed using the SAS® Studio 3.82 software (SAS Institute, Cary, NC). The analysis of variance (ANOVA) and Tukey's test (*=$p \leq 0.05$) were used to compare the means of the treatments.

Results and Discussion

pH and evaluation of the production of *P. fluorescens* biomass. Approximately 40 L of de nejayote were gathered, and the pH after centrifuging was 12.21, therefore it was adjusted to the corresponding values for each treatment. *P. fluorescens* NR113647 was able to grow in all the culture media of this study. The concentration of biomass produced in the different culture media was variable, where the highest cell development was obtained in the KB medium (control) and there were no statistical differences in relation to the media 3 and 4; the three media were supplemented with glycerol in their initial formulation. Media 1 and 2 (without the addition of glycerol) presented a lower concentration of biomass than the control and the media with nejayote, which contained added glycerol (Table 1). *Pseudomonas* has simple nutritional requirements due to its ability to degrade a large variety of organic substrates, from aromatic compounds to halogenated derivatives and other organic residues (Sah *et al*., 2021). In this sense, the ability of the nejayote has been proven for use as a culture medium, since it contains fermentable sugars at an approximate concentration of 148.6 mg L^{-1} (García-Depraect *et al*., 2017). On the other hand, *Pseudomonas* is able to use glycerol as a source of carbo for the production of biomass (Poblete-Castro *et al*., 2020). Furthermore, *Pseudomonas* has been observed to produce a greater concentration of biomass in the media containing glycerol as a source of carbon in comparison to those with other carbon sources such as glucose or succinate (Nikel *et al*., 2014).

Table 1. Evaluation of the growth of *P. fluorescens* NR113647 and pH values of the different treatments after 72 h of incubation.

Treatment	Culture media	Biomass (*Ln* UFC mL^{-1})	Final pH
Control	KB6	20.52±0.03 [a]	6.93±0.05 [a]
1	N6	19.55±0.17 [b]	8.40±0.06 [b]
2	N12	19.72±0.25 [b]	8.46±0.06 [b]
3	Ngly6	20.17±0.11 [a]	8.02±0.09 [c]
4	Ngly12	20.22±0.03 [a]	8.03±0.03 [c]

Results expressed with the mean ± standard deviation for each sample (n = 3). *Different letters in each column indicate a significant difference (*=$p \leq 0.05$) according to Tukey's test.

The pH values varied during cultivation in all the culture media used. In the treatments that began with a pH of 6.0, an increase was observed in the pH value, whereas in the cultures that began with a pH of 12, it decreased (Table 1). In media

1 and 2, in which there was only nejayote, no statistical differences were observed (*=p ≤ 0.05). The pH value between treatments 3 and 4 displayed no statistical differences either (*=p ≤ 0.05). On the other hand, the culture medium used as a control maintained a low and significantly different pH in comparison with the other treatments (Table 1).

P. fluorescens, and bacterial cells in general, crucially depend on the pH of the medium in which they develop. pH values closer to 7 are adequate for most bacteria. The self-regulation of pH, which includes the detection and adaptation to external pH, is an important mechanism for microbial development. In this regard, numerous adaptations have been identified that contribute to homeostasis in an alkaline pH (Sánchez-Clemente, 2018). When bacteria are found in alkaline conditions (pH<10), the active transport of protons or the motor force of protons is crucial for their adaptation to their ecological niche; the mechanism includes high levels of transporters and enzymes that promote the capture and retention of protons (ATP synthase and proton/monovalent cation antiporters). Additionally, metabolic changes take place, leading to increased acid production and changes in the in the cell surface layers that contribute to protein retention in the cytoplasm (Krulwich *et al.,* 2011; Padan *et al*., 2005). Tolerance to the alkaline medium and the reduction of the initial pH (from 12 to 8, approximately) is important, considering that in Mexico it is established that the permissible pH range for wastewater discharged into urban or municipal sewer systems is between 5.5 and 10.0 (NOM-002-ECOL-1996). Therefore, nejayote should not be deposited in the municipal sewer system, but rather, on the contrary, it could be used as a culture medium for the development of biomass and the production of metabolites, considering that the final culture broth used would have a pH value in accordance with Mexican regulations and could be discarded.

***In vitro* evaluation of the antifungal activity of the *P. fluorescens* extracts.** The incubation period, until the fungi in the negative control (PDA, BD Bioxon, Mexico) reached the rim of the Petri dish, was of 9 days for *Aspergillus niger* and *Botrytis cinerea*, whereas for *Fusarium solani* it lasted 14 days. Table 2 shows the growth (mm) and inhibition values (%) generated by the different treatments in fungi.

The fungi used in this study and cultivated in a PDA medium had a radial growth. The extracts obtained from the KB6 medium (positive control) produced the highest levels and percentages of *in vitro* inhibition of all treatments. *Botrytis cinerea* was the most sensitive, since its growth was inhibited by 100%. Other studies observed different inhibition values ranging from 60% up to 75-99%, with extracts obtained from a nutritional broth (Mikani *et al.*, 2008; Wallace *et al.*, 2019).

Table 2. Evaluation of the antifungal effect of the extracts obtained from *P. fluorescens* NR113647, on the mycelial growth and the inhibition of *Fusarium solani, Botrytis cinerea* and *Aspergillus niger.*

	F. solani		*B. cinerea*		*A. niger*	
Culture media	Growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)
PDA	90.0±0.0 [a]	0.0	90.0±0.0 [a]	0.0	90.0±0.0 a	0.00
KB6	30.5±1.4 [b]	66.0	0.0±0.0 [e]	100.0	48.6±2.5 [d]	45.9
N6	90.0±0.0 [a]	0.0	90.0±0.0 [a]	0.0	90.0±0.0 [a]	0.00
N12	31.9±1.4 [b]	64.5	21.0±4.5 [d]	76.6	66.0±3.6 [c]	26.5
Ngly6	90.0±0.0 [a]	0.0	60.9±1.6 [b]	32.3	90.0±0.0 [a]	0.00
Ngly12	67.4±2.3 [c]	25.0	27.3±2.2 c	69.5	76.9±2.16 [b]	14.5

Negative control=PDA. Positive control=KB6. Results expressed with the mean ± standard deviation for each sample (n = 5). Different letters in each column indicate a significant difference (*=$p \leq 0.05$) according to Tukey's test.

In the treatments of the positive control for the *Fusarium solani* strain, an inhibition of 66% was obtained, in comparison with other studies in which *P. fluorescens* strains displayed an inhibition ranging from 43% to complete inhibition (100%) of the mycelial growth of *Fusarium* spp. (Al-Fadhal, 2019; Trejo-Raya *et al.,* 2021). The *Aspergillus niger* strain developed the greatest growth, with an inhibition of 45%. However, other studies have reported that *P. fluorescens* displayed an inhibition of 20 to 30% in comparison with the control (Akocak *et al.,* 2015) and a mycelial growth of up to 78.77% (Deshmukh, 2015).

The extracts obtained from the N6 medium did not inhibit the growth of any of the fungi evaluated *in vitro* in this study. This behavior was similar for the extracts obtained from the Ngly6 medium, which did not inhibit the growth of neither *Aspergillus niger* nor *Fusarium solani,* although for *Botrytis cinerea*, an inhibition of approximately 32% was recorded. On the other hand, the extracts obtained from Ngly12 generated inhibition values ranging from 14 to 25% for *Aspergillu niger* and *Fusarium solani*, although an inhibition of nearly 70% was observed in *Botrytis cinerea*. The extracts obtained from N12 for the fungi *Botrytis cinerea* and *Aspergillus niger* generated numerically similar inhibition values, but significantly different to those obtained with the extracts of the positive control (KB6). For the fungus *Fusarium solani* there were no statistical differences between the KB6 and N12 media.

Because the composition of the nejayote media is basically the same in all treatments, the results of this study may suggest that the differences in the inhibition effect is linked to the initial pH values and the addition of glycerol to the culture media. Saleh-Lakha *et al.* (2009) determined that the genic expression values for

the production of metabolites by *Pseudomonas mandelii* were 539 and 6190 times lower in media with a pH of 5 than for pH values of 8. On the other hand, Zhou *et al.* (2012) observed that for a *Pseudomonas* strain to express antifungal activity, the pH value of the culture medium must be of at least 10. The genes involved in the production of metabolites are directly related with the transcription of genes involved in the protection against stress, as well as damage in the cell membrane and the cell wall. These genes are a part of the global regulatory system for the activation of the synthesis of antibiotics and cyanide (GaC) and the secondary metabolite repressor system (RsM), which are positively influenced by environmental signals, in which pH values of 8 stand out (Anderson *et al.,* 2017; Xue *et al*., 2013). Regarding the addition of glycerol to the medium, Nikel *et al*. (2014) concluded that the growth of *Pseudomonas* in the presence of glycerol alters the transcription of around 104 genes that codify metabolic and non-metabolic activities, which may be involved in the production of antifungal metabolites. In the case of this study, it is probable that the cultivation of *P. fluorescens* NR113647 at a pH of 12 may increase the expression of the production of metabolites related to biocontrol.

Identification of the metabolites involved in the antifungal activity. The thin-layer chromatography of the dry *P. fluorescens* NR113647 raw extracts cultivated in the N12 culture medium displayed the presence of at least five compounds, which were compared with the standards of phenazine and phloroglucinol (Table 3). The elution paths of the components could indicate that the dry raw extract contains at least one antifungal compound that may be a derivative of phloroglucinol and which corresponds to stain 1. Phloroglucinol is a metabolite with numerous derivatives and is widely known for its antifungal capacity. Some

Table 3. Retention factors of the *P. fluorescens* NR113647 extracts obtained from treatment N12, comparison against standard compounds.

Compound	Rf
Spot 1	0.20
Spot 2	0.31
Spot 3	0.47
Spot 4	0.52
Spot 5	0.92
*Phenazine	0.83
*Phloroglucinol	0.21

*Standards [0,5 mg mL^{-1}] and *P. fluorescens* NR113647 extracts [1 mg mL^{-1}]. Hexane-acetone system (2:1). These compounds were detected using UV at 254 nm.

Pseudomonas strains produce 2,4-diacetylfluoroglucinol and its intermediates monoacetylphluoroglucinol and phloroglucinol (Biessy and Filion, 2021). On the other hand, stain 5 is the nearest to the phenazine standard, which may indicate the presence of a derivative of this component, since the genus *Pseudomonas* is known to produce phenazine-1-carboxylic acid and 2-hydroxyphenazine, which are metabolites with antifungal activity against phytopathogenic fungi (Castaldi *et al.,* 2021). Despite not being able to identify all the metabolites present in the dry raw extract, Zhang *et al.* (2020) observed that *Pseudomonas* harbors genes involved in the production of more than 10 metabolites, such as diacetylphluoroglucinol, pyoverdines, pyochelins, pyrrolnitrin, HCN, etc., which could correspond to the rest of the eluted components in the thin-layer chromatography assay. However, the results might not be entirely extrapolated, and in-depths studies are required for the determination of metabolites in this study.

To identify the production of HCN (Figure 1), in the control dish (A), no color change was observed. The extracts obtained from the KB6 medium (control) displayed an orange color on the periphery, indicating that *P. fluorescens* NR113647 produces HCN in this culture medium. The plate of the extract from the N12 medium showed a light orange-yellow color, so it can be assumed that there is also a presence of HCN, though in smaller amounts compared to KB6. The production of HCN by microorganisms plays an important role in inhibiting the growth of various fungi and other biotic phytopathogenic agents, including weeds, insects and nematodes. It has a broad spectrum, since it directly interferes with cell respiration in eukaryotic pathogens, so it can be widely used as a biocontrol metabolite (Sehrawat *et al.,* 2022).

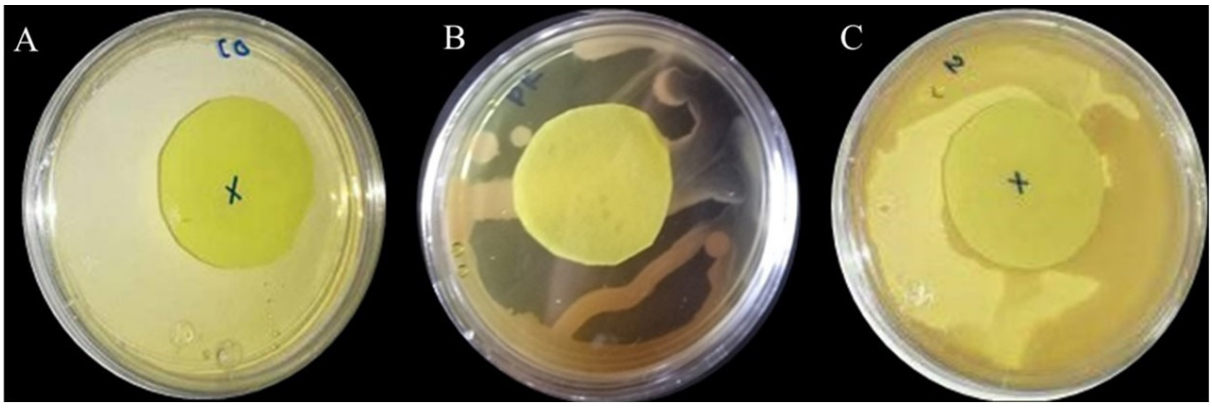

Figure 1. Identification of the production of hydrogen cyanide in a Petri dish. Negative control of distilled water (A), extracts free of *P. fluorescens* NR113647 cells in a KB6 medium (B) and extracts free of *P. fluorescens* NR113647 cells in a N12 medium (C).

Regarding the production of siderophores, according to the trial in CAS-agar plates described in the methodology (Figure 2), no changes in color were observed in the Petri dish with sterile distilled water. In the KB6 medium, an intense yellow color was observed that displays a large proportion of these metabolites. In the case of the N12 medium, a pale-yellow color was observed, which displays a limited production of siderophores. These metabolites are reported as one of the main biological control compounds with an antifungal effect produced by *P. fluorescens*, which allow the acquisition of Fe from the medium or colonization site and thus maintain the normal growth of the bacterium (Rizzi *et al.,* 2019). However, this study showed that the conditions of the N12 medium did not promote the production of siderophores, due to the low production in comparison with the control and therefore were not the main biocontrol metabolites.

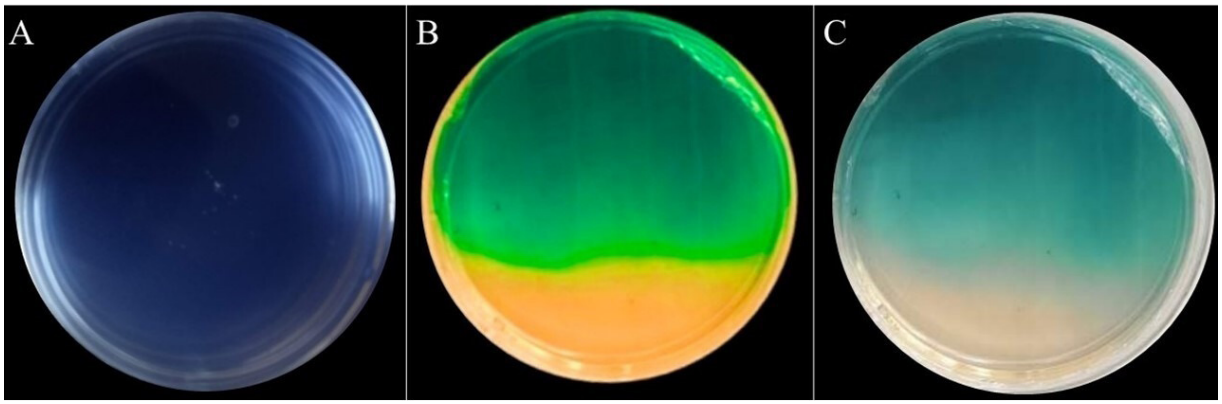

Figure 2. Identification of siderophores, Petri dish with distilled water as a control (A) and extracts free of *P. fluorescens* NR113647 cells (B) (Trejo-Raya *et al*., 2021) and extracts free of *P. fluorescens* NR113647 cells in a N12 medium (C).

The *Pseudomonas fluorescens* complex includes more than fifty species of taxonomically assigned strains, most of which have potential applications for biocontrol. A high proportion of these strains have not been completely sequenced and display a high molecular homology. In addition, new species are frequently being described (Garrido-Sanz *et al.,* 2016); therefore, the results of this investigation cannot be directly correlated for all *P. fluorescens* populations. However, the use of *P. fluorescens* NR113647 and this culture medium proposed could be useful to value the metabolisms of other strains and/or genetic populations of the *P. fluorescens* complex.

Conclusions

This study investigated the potential use of nejayote as an alternative culture medium for *Pseudomonas fluorescens* NR113647. The strain was able to grow in all treatments containing nejayote; among the extracts evaluated, the Nejayote extract with a pH of 12 displayed the best results, since it inhibited the mycelial growth *in vitro* of the fungi *Botrytis cinérea*, *Fusarium solani* and *Aspergillus niger* by 76.66, 64.55 and 26.56%, respectively. In the analysis of antifungal metabolites of the Nejayote extract with a pH of 12, the production of hydrogen cyanide and siderophores was identified, as well as the possible production of compounds derived from phenazine and phloroglucinol. The valorization of the nejayote and its use at a pH of 12 without the addition of glycerol represents a viable and eco-friendly alternative for its use as a culture medium for *P. fluorescens* NR113647 in the production of biomass and extracts containing secondary metabolites of interest in the agriculture industry for the control of diseases caused by phytopathogenic fungi.

Literature Cited

Akocak P B, Churey J J, and Worobo R W. 2015. Antagonistic effect of chitinolytic *Pseudomonas* and *Bacillus* on growth of fungal hyphae and spores of aflatoxigenic *Aspergillus flavus*. Food Bioscience 10, 48–58. https://doi:10.1016/j.fbio.2015.01.005

Al-Fadhal F A, Al-Abedy A N, and Alkhafije D A. 2019. Isolation and molecular identification of *Rhizoctonia solani* and *Fusarium solani* isolated from cucumber (*Cucumis sativus L*.) and their control feasibility by *Pseudomonas fluorescens* and *Bacillus subtilis*. Egyptian Journal of Biological Pest Control 29: 1-11. https://doi.org/10.1186/s41938-019-0145-5

Al-Hetar M Y, Zainal Abidin M A, Sariah M, and Wong M Y. 2011. Antifungal activity of chitosan against *Fusarium oxysporum* f. sp. *cubense*. Journal of applied polymer science 120(4), 2434-2439. https://doi.org/10.1002/app.33455

Anderson A J. Kang,B R, and Kim Y C. 2017. The Gac/Rsm signaling pathway of a biocontrol bacterium, *Pseudomonas chlororaphis* O6. Research in Plant Disease 23(3): 212-227.https://doi.org/10.5423/RPD.2017.23.3.212

Bacame-Valenzuela F J, Pérez-Garcia J A, Figueroa-Magallón M L, Espejel-Ayala F, Ortiz-Frade L A, and Reyes-Vidal Y. 2020. Optimized production of a redox metabolite (Pyocyanin) by *Pseudomonas aeruginosa* nej01r using a maize by-product. Microorganisms 8(10): 1–17. https://doi.org/10.3390/microorganisms8101559

Biessy A, and Filion M. 2021. Phloroglucinol derivatives in plant-beneficial *Pseudomonas spp*.: biosynthesis, regulation, and functions. Metabolites 11(3): 182. https://doi.org/10.3390/metabo11030182

Castaldi S, Masi M, Sautua F, Cimmino A, Isticato R, Carmona M, and Evidente A. 2021. *Pseudomonas fluorescens* showing antifungal activity against Macrophomina phaseolina, a severe pathogenic fungus of soybean, produces phenazine as the main active metabolite. Biomolecules 11(11): 1728.

Deshmukh S, Bishi S, and Vakharia D. 2015. *Pseudomonas fluorescens* modulate *in-vitro* lytic enzyme production and inhibit the growth of collar rot pathogen (*Aspergillus niger*) in groundnut (*Arachis hypogaea L*.). J Pure Appl Microbiol 9: 1531-1538. https://www.cabidigitallibrary.org/doi/full/10.5555/20173288182

Díaz-Montes E, Barragán-Huerta B E, and Yáñez-Fernández J. 2020. Identification and Evaluation of Antioxidant Activity of Hydroxycinnamic Acids Extracted by Ultrafiltration from Three Varieties of Mexican Maize. Waste and Biomass Valorization 11(5): 1799–1808. https://doi.org/10.1007/s12649-018-0420-9

Dimkić I, Janakiev T, Petrović M, Degrassi G, and Fira D. 2022. Plant-associated *Bacillus* and *Pseudomonas* antimicrobial activities in plant disease suppression via biological control mechanisms - A review. In Physiological and Molecular Plant Pathology (Vol. 117). Academic Press. https://doi.org/10.1016/j.pmpp.2021.101754

Erhonyota C, Edo G I, and Onoharigho F O. 2023. Comparison of poison plate and agar well diffusion method determining the antifungal activity of protein fractions. Acta Ecologica Sinica 43(4): 684-689. https://doi.org/10.1016/j.chnaes.2022.08.006

FAO (Food and Agriculture Organization of the United Nations). 2023. FAOSTAT Statistics Database. http://www.fao.org/faostat/ (February 2023).

García-Depraect O, Gómez-Romero J, León-Becerril E, and López-López A. 2017. A novel biohydrogen production process: Co-digestion of vinasse and Nejayote as complex raw substrates using a robust inoculum. International Journal of Hydrogen Energy 42(9): 5820-5831. https://doi.org/10.1016/j.ijhydene.2016.11.204

Garrido-Sanz D, Meier-Kolthoff J P, Göker M, Martín M, Rivilla R, and Redondo-Nieto M. 2016. Genomic and genetic diversity within the *Pseudomonas fluorescens* complex. PloS one 11(2): e0150183. https://doi.org/10.1371/journal.pone.0150183

Harwood, A. J. (1996). Basic DNA and RNA protocols. Methods in molecular biology (ISSN 1064-3745: 58. https://www.cabidigitallibrary.org/doi/full/10.5555/19960103443

Hellin J, Keleman A, and Bellon M. 2010. Maize diversity and gender: Research from Mexico. Gender and Development 18(3): 427–437. https://doi.org/10.1080/13552074.2010.521989

Khalil S, Ali T A, Skory C, Slininger P J, and Schisler D A. 2016. Evaluation of economically feasible, natural plant extract-based microbiological media for producing biomass of the dry rot biocontrol strain *Pseudomonas fluorescens* P22Y05 in liquid culture. World Journal of Microbiology and Biotechnology 32(2). https://doi.org/10.1007/s11274-015-1984-1

King E O, Ward M K, and Raney D E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. The Journal of laboratory and clinical medicine 44(2): 301-307. https://doi.org/10.5555/uri:pii:002221435490222X

Krulwich T A, Sachs G, and Padan E. 2011. Molecular aspects of bacterial pH sensing and homeostasis. Nature Reviews Microbiology 9:5 330–343. https://doi.org/10.1038/nrmicro2549

López-Pacheco I Y, Carrillo-Nieves D, Salinas-Salazar C, Silva-Núñez A, Arévalo-Gallegos A, Barceló D, Afewerki S, Iqbal H M N, and Parra-Saldívar R. 2019. Combination of nejayote and swine wastewater as a medium for *Arthrospira maxima* and *Chlorella vulgaris* production and wastewater treatment. Science of the Total Environment 676: 356–367. https://doi.org/10.1016/j.scitotenv.2019.04.278

Mark G L, Morrissey J P, Higgins P, and O'Gara F. 2006. Molecular-based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. FEMS Microbiology Ecology 56:2 167–177. https://doi.org/10.1111/j.1574-6941.2006.00056.x

Meraz S, Karla A, Vargas P, Trinidad J, Maldonado L, Manuel J, Bravo C, Oropeza M T, Alberto E, and Maldonado L. 2015. Eco-friendly innovation for nejayote coagulation-flocculation process using chitosan: Evaluation through zeta potential measurements. Chemical engineering journal 284. https://doi.org/10.1016/j.cej.2015.09.026

Mikani A, Etebarian H R, Sholberg P L, O'Gorman D T, Stokes S, and Alizadeh A. 2008. Biological control of apple gray mold caused by Botrytis mali with *Pseudomonas fluorescens* strains. Postharvest Biology and Technology 48:1, 107–112. https://doi.org/10.1016/j.postharvbio.2007.09.020

Nikel P I, Kim J, and de Lorenzo V. 2014. Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putid*a KT2440. Environmental Microbiology *1*6(1), 239–254. https://doi.org/10.1111/1462-2920.12224

NORMA Oficial Mexicana NOM-002-ECOL-1996, Que establece los límites máximos permisibles de contaminantes en las descargas de aguas residuales a los sistemas de alcantarillado urbano o municipal. Diario Oficial de la Federación https://dof.gob.mx/nota_detalle.php?codigo=4881304&fecha=03/06/1998#gsc.tab=0)

Padan E, Bibi E, Ito M, and Krulwich T A. 2005. Alkaline pH homeostasis in bacteria: New insights. Biochimica et Biophysica Acta - Biomembranes 1717:2 67–88. https://doi.org/10.1016/j.bbamem.2005.09.010

Poblete-Castro I, Wittmann C, and Nikel P I. 2020. Biochemistry, genetics and biotechnology of glycerol utilization in *Pseudomonas* species. Microbial Biotechnology 13:1 32–53 https://doi.org/10.1111/1751-7915.13400

Ramírez-Romero G, Reyes-Velazquez M, and Cruz-Guerrero A. 2013. Study of nejayote as culture medium for probiotics and production of bacteriocins. Revista Mexicana de Ingeniera Quimica 12(3), 463–471. https://www.scielo.org.mx/scielo.php?pid=S1665-27382013000300009&script=sci_abstract&tlng=en

Rizzi A, Roy S, Bellenger J P, and Beauregard P B. 2019. Iron homeostasis in *Bacillus subtilis* requires siderophore production and biofilm formation. Applied and Environmental Microbiology 85(3), e02439-18. https://doi.org/10.1128/AEM.02439-18

Rodríguez-Romero V M, Villanueva-Arce R, Trejo-Raya A B, y Bautista-Baños S. 2019. Quitosano y extractos de *Pseudomonas fluorescens* para el control de *Alternaria alternata* en jitomate (*Solanum lycopersicum*). Revista Mexicana de Fitopatología 37:2 202-219 https://doi.org/10.18781/r.mex.fit.1812-2

Sah S, Krishnani S, and Singh R. 2021. *Pseudomonas* mediated nutritional and growth promotional activities for sustainable food security. Current Research in Microbial Sciences 2:100084.https://doi.org/10.1016/j.crmicr.2021.100084

Schwyn B, & Neilands J. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry 160 (1), 47-56.

Salazar-Magallon J A, Hernandez-Velazquez V M, Alvear-Garcia A, Arenas-Sosa I, and Peña-Chora G. 2015. Evaluation of industrial by-products for the production of *Bacillus thuringiensis* strain GP139 and the pathogenicity when applied to *Bemisia tabaci* nymphs. Bulletin of Insectology 68:1 103–109. http://www.bulletinofinsectology.org/pdfarticles/vol68-2015-103-109salazar-magallon.pdf

Saleh-Lakha S, Shannon K E, Henderson S L, Goyer C. Trevors J T, Zebarth B J, and Burton D. L. 2009. Effect of pH and temperature on denitrification gene expression and activity in Pseudomonas mandelii. Applied and environmental microbiology 75(12), 3903-3911. https://doi.org/10.1128/AEM.00080-09

Sánchez-Clemente R, Igeño M I; Población A G, Guijo M I, Merchán F, and Blasco R. 2018. Study of pH Changes in Media during Bacterial Growth of Several Environmental Strains. Proceedings 2, 1297. https://doi.org/10.3390/proceedings2201297

Santoyo G, del Orozco-Mosqueda M C, and Govindappa M. 2012. Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: A review. Biocontrol Science and Technology. 22:8 855–872. https://doi.org/10.1080/09583157.2012.694413

Schwyn B, and Neilands J B. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry 160(1), 47-56. https://doi.org/10.1016/0003-2697(87)90612-9

Sehrawat A, Sindh S. S, and Glick B R. 2022. Hydrogen cyanide production by soil bacteria: Biological control of pests and promotion of plant growth in sustainable agriculture. Pedosphere 32(1), 15-38. https://doi.org/10.1016/S1002-0160(21)60058-9

Serna-Saldivar S O. 2021. Understanding the functionality and manufacturing of nixtamalized maize products. Journal of Cereal Science 99: 103205. https://doi.org/10.1016/j.jcs.2021.103205

Sivakumar D, Srikanth P, Ramteke P W, and Nouri J. 2022. Agricultural waste management generated by agro-based industries using biotechnology tools. Global Journal of Environmental Science and Management 8(2), 281-296. https://doi.org/10.22034/GJESM.2022.02.10

Suleimana M M, McGaw L J, Naidoo V, and Eloff J N. 2010. Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. Afr J Tradit Complement Altern Med 7:64–78. https://doi.org/10.4314/ajtcam.v7i1.57269

Suryanti V, Marliyana S D, and Wulandari A. 2015. Biosurfactant production by *Pseudomonas fluorescens* growing on molasses and its application in phenol degradation. AIP Conference Proceedings 1699. https://doi.org/10.1063/1.4938318

Thambugala K M, Daranagama D A, Phillips A J L, Kannangara S D, and Promputtha I. 2020. Fungi vs. Fungi in Biocontrol: An Overview of Fungal Antagonists Applied Against Fungal Plant Pathogens. Frontiers in Cellular and Infection Microbiology 10:604923. Frontiers Media S.A. https://doi.org/10.3389/fcimb.2020.604923

Trejo-Raya A B, Rodríguez-Romero V M, Bautista-Baños S, Quiroz-Figueroa F R, Villanueva-Arce R, and Durán-Páramo E. 2021. Effective *in vitro* control of two phytopathogens of agricultural interest using cell-free extracts of *Pseudomonas fluorescens* and chitosan. Molecules 26(21), 6359. https://doi.org/10.3390/molecules26216359

Verma R, Naosekpam A S, Kumar S, Prasad R, and Shanmugam V. 2007. Influence of soil reaction on diversity and antifungal activity of fluorescent pseudomonads in crop rhizospheres. Bioresource technology 98(7), 1346-1352. https://doi.org/10.1016/j.biortech.2006.05.030

Wallace R L, Hirkala D L, and Nelson L M. 2019. *Pseudomonas fluorescens* and low doses of chemicals inhibit postharvest decay of apples in commercial storage. Canadian Journal of Plant Pathology 41:3, 355–365. https://doi.org/10.1080/07060661.2019.1605539

Xu C, Fu Y, Liu F, Liu Z, Ma J, Jiang R, Song C, Jiang Z, and Hou J. 2021. Purification and antimicrobial mechanism of a novel bacteriocin produced by *Lactobacillus rhamnosus* 1.0320. Lwt 137:600, 110338. https://doi.org/10.1016/j.lwt.2020.110338

Xue Wei, Xianqing Huang, Lulu Tang, Daqiang Wu, and Yuquan Xu. 2013. Global Control of GacA in Secondary Metabolism, Primary Metabolism, Secretion Systems, and Motility in the Rhizobacterium *Pseudomonas aeruginosa* M18. Journal of bacteriology 195(15), 3387-3400. https://doi.org/10.1128/jb.00214-13

Zhang Q X, Kong X W, Li S Y, Chen X J, and Chen X J. 2020. Antibiotics of *Pseudomonas protegens* FD6 are essential for biocontrol activity. Australasian plant pathology 49, 307-317. https://doi.org/10.1007/s13313-020-00696-7

Zhou T, Chen D L C, Sun Q, Li L, Liu F, and Shen B. 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiological research 167(7), 388-394. https://doi.org/10.1016/j.micres.2012.01.003