Original Research Article

Isolation, Identification, Characterization and Enzymatic Profile of the New Strain of Pantoea agglomerans

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ABSTRACT

Enterobacteriaceae of the genus Pantoea are characterized as Gram-negative and have been isolated from a wide variety of environments including soil, water, dust, dairy products, meat, fish, insects, humans and animals. Most of the time they are found associated with a wide variety of plants. The objective of this work was to identify and characterize biochemical, morphological and enzymatic activity, as well as the influence of abiotic factors in a Pantoea isolated from industrial laundry effluent in Pernambuco. The results showed that the bacterium presents characteristics similar to those presented by the species of Pantoea agglomerans; however, the biochemical and morphological tests were not enough to accurately identify the species. However, the isolated species showed production of enzymes such as cellulase, protease and polyphenoloxidase, demonstrating enzymatic biotechnological potential. In addition to tolerance to high concentrations of salinity, they present as mesophyll, cerscendo in optimal temperature of 30 °C and good growth in the pH 7.0 and 8.0.

Keywords
Pantoea, Industrial effluent, Biochemical characteristics, Fatty acids.

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Introduction

The genus Pantoea belongs within the family Enterobacteriaceae and was proposed by Gavini et al., (1983) for two groups of strains that were, at that time, assigned to the Erwinia herbicola–Enterobacter agglomerans complex (Verdonkck, 1987). This complex covered many phena and genomic groups (Brenner at al., 1984), some of which were later designated as new genera (Grimont; Grimont, 2005).

The enterobacterial genus Pantoea currently comprises nineteen species of Gram-negative, yellow or beige pigmented, motile rods
(Kageyama et al., 1992). Grimont and Grimont (2005) stated that the genus Pantoea can be envisioned to include DNA groups I, II, IV and V as determined by Brenner et al., (1984). It was further observed that the species P. citrea, P. punctata and P. terrea, isolated in Japan and described by Kageyama et al., (1992) differed from the “core” Pantoea species in several biochemical or nutritional characteristics. Grimont and Grimont determined the phylogenetic position of all currently recognized Pantoea species and DNA groups of Brenner et al., (1984) using 16S rRNA- and rpoB-sequence comparisons and found that the “Japanese” species constituted a cluster that joined the Pantoea cluster at a lower level. They concluded that more taxonomic work was needed to justify the assignment of these species to the genus Pantoea (Braddy et al., 2008; Braddy et al., 2009; Braddy et al., 2010 a,b).

Identification of plant-pathogenic Pantoea species is difficult, due to the high degree of phenotypic similarity between species of this genus and related Enterobacteriaceae. Pantoea species are typically characterised based on colony morphology, physiological and biochemical tests, and in some cases, fatty acid analysis or quinone composition. This approach has proven to be unreliable though, as identification based solely on phenotypic characteristics has led to the misidentification of many strains belonging to the now obsolete “Erwinia herbicola–Enterobacter agglomerans” complex (Brady et al., 2007).

The genus presents short bacilli, Gram negative, 0.5-1.0 μm in diameter and 1.0-3.0 μm in length, are mobile by peritrichal flagella and many samples produce yellow or beige pigment. They are facultative anaerobes, negative indole, Voges-Proskauer and Simmons citrate positive, the reaction of methyl red is variable. They do not decarboxylate lysine, do not produce H₂S and do not hydrolyze urea (Holt et al., 1994; Camatti-Sartori et al., 2008; Cabral, 2010; Roper, 2011; Nadaeasah; Stavrinides, 2014).

Members of this genus Pantoea have been isolated from a wide range of environments including soil, water, dust, dairy products, meat, fish, insects, humans and animals (Suen et al., 2010; Prakash et al., 2015; Büyükcam et al., 2017). However, Pantoea agglomerans, is not an obligate infectious agent in humans. However, it could be a cause of opportunistic human infections, mostly by wound infection with plant material, or as a hospital-acquired infection, mostly in immunocompromised individuals (Dutkiewicz et al., 2016).

Most frequently they are found associated with a broad range of plant hosts, as non-pathogenic endophytes or epiphytes, colonizing the leaves, stems and roots. In this context, some Pantoea strains can be beneficial to the plant host by contributing to growth promotion through processes such as the production of the plant-growth hormone indole-acetic acid (IAA), phosphate solubilization or nitrogen fixation (Mishra et al., 2011; De Maayer et al., 2012). Some Pantoea strains also provide effective protection to plants against various bacterioses as well as fungal diseases and postharvest fruit rots (Smits et al., 2011).

The objective of this study was to isolate from laundry effluent, identification, characterization and enzymatic profile.

Materials and Methods

Sample and Isolation

Bacteria were isolated from industrial laundry effluents. A quantity of 1 ml of water from each of the samples collected was dissolved in
9 ml of sterile distilled water and serial dilutions were made. Each dilution was seeded in Luria Bertani (LB) agar by standard plate spreading method. The plates were incubated at 37 °C for 3 days and the colonies were transferred from the plates to inclined tubes with the same medium. After plaque growth, bacterial colonies were collected according to their morphological characteristics and purified by striations repeated on plates containing nutrient agar and identified with Gram staining. For the characterization, the biochemical and physiological tests were used for the morphology of the colonies. The strain was routinely cultured in LB medium and maintained at 5 °C.

**Biochemical Tests and Morphology**

Bacteria were identified according to macroscopic appearance (colony appearance in solid medium, shape, texture and pigmentation), Gram staining, mobility tests, oxidase, catalase and indole. These tests were done according to Cappuccino and Sherman (1992) and Grimont; Grimont, 2005. The biochemical Tests were done with various sources of carbon, as monosaccharides (D-glucose, D-fructose, D-xylene, D-mannose, D-mannitol and D-gluconase), disaccharides (lactose, maltose, trehalose and sucrose) (Brown; Dilworth, 1975). Proteins (gelatin) were evaluated according to the standard method (Cappuccino; Sherman, 1992).

**Abiotic Stress Factors**

**pH Effect**

The pH effect was tested on Nutrient Broth with values of 4, 5, 6, 7, 8, and 10. The medium was inoculated with 100 μl of the culture, grown to a 0.5 standard on the MacFarland scale, and inoculated at 30 °C / 48h. The experiment was carried out in triplicate. Growth was evaluated using the spectrophotometer at 600nm (Son et al., 2006; Silini-Chérif et al., 2012).

**Temperature Effect**

The effect of different temperatures (4, 30, 37, 41 and 44 °C) on bacterial growth was evaluated. The nutrient broth medium was inoculated with 100μl of the culture, grown to a 0.5 standard on the MacFarland scale. The growth time was 48h and the experiment was performed in triplicate. Growth was evaluated using the spectrophotometer at 600nm (Silini-Chérif et al., 2012).

**Saline Concentration Effect**

The tolerance of the microorganism to an increasing concentration of NaCl (0-100 mM) in Nutrient Broth medium was evaluated. In the medium, 100 μl of the culture was added, grown to a pad of 0.5 on the MacFarland scale. The growth was evaluated at the end of 48h / 30 °C, using the spectrophotometer at 600nm. The method was performed in triplicate (Son et al., 2006; Silini-Chérif et al., 2012).

**Detection of Enzymatic Activity**

**Detection of amylase**

For the detection of aminolytic activity, the methodology described by Hankin and Anagnostakis (1979) was used, using the Nutrient Agar medium containing 0.2% starch, later distributed in Petri dishes. After solidification of the medium, a hole was made in the center of the plate, where a previously prepared bacterial suspension of 100 μL was inoculated with (10^7 / CFU). The plates were incubated at 35 °C for 96 hours with daily monitoring. The enzyme production was evidenced after washing the plates with a lugol solution, by forming an opaque halo
around the colony. All assays were performed in triplicate.

**Detection of Urease**

For the detection of urease activity, the method of Hankin and Anagnostakis (1979) was used, using the nutrient agar medium (lower layer), with addition of 5% urea. The top layer was made with phosphate buffer agar plus 5% urea solution and 5% bromothymol blue solution. After solidification of the culture medium, a hole was made in the center of the Petri dish, with a diameter of 0.8 cm, in which 100 μL of the previously prepared bacterial suspension was inoculated. Plates were incubated at 28 and 37 °C for 96 hours with daily monitoring. After the period of microbial growth, a light yellow halo around the colony indicated the presence of urease. All assays were performed in triplicate.

**Detection of Tanase**

For the detection of tannic acid activity, a medium having the following composition was used: 3 g of sodium nitrate, 1 g of dibasic potassium phosphate, 0.5 g of magnesium sulfate, 0.5 g of Potassium chloride, 20 g of agar, 4 g of tannic acid, 0.04 g of bromophenol blue and 1000 ml of distilled water (Sharma et al., 2000). Another medium with the same composition was also prepared, however, by removing the bromophenol blue and increasing the tannic acid concentration to 20 g. After sterilization in autoclave (121 °C, 1 atm, 20 min.), These media were poured into Petri dishes, solidified and inoculated using triplicates and control (uninoculated).

After solidification of the culture medium, a hole was made in the center of the 0.8 cm diameter Petri dish, where 100 μL of the bacterial suspension (10^7 / CFU) was inoculated. The plates were incubated at 35 °C for 96 hours, with daily monitoring (dark). The degrading activity was evaluated by the appearance of clear zones around the bacterial growth (Hankin; Anagnostakis, 1975).

**Detection of Polyphenoloxidase**

The colonies were aseptically removed and transferred to the center of the Petri dish containing modified nutrient agar medium (5g meat extract, 10g peptone, 5g sodium chloride, 750ml distilled water, pH 6.0, Tannic acid 5 g to 125 ml water and gallic acid 5 g to 125 ml distilled water) and incubated for 24 h / 35 °C. The enzymatic activity was observed by the brown halo formation around the colony on the rest of the plaque (Harkin; Obst, 1973).

**Detection of Lipase**

For the detection of lipolytic activity a medium was used with the following composition: 10 g peptone, 5 g sodium chloride, 0.1 g calcium chloride bihydrate, 20 g agar, 20 ml tween 20 And tween 80 and 1000 mL of distilled water. The tween was autoclaved separately in flowing steam and added to the medium before dispensing into Petri dishes. After sterilization in autoclave (121 °C, 1 atm, 20 min.), These media were poured into Petri dishes, solidified and inoculated using triplicates and control (without tween). Methodology described by Hankin and Anagnostakis (1979).

**Results and Discussion**

**Isolation and Characterization of Bacteria**

The isolated bacteria were cultured in nutrient agar (AN) for 24h at 30 °C. The colonies obtained had the following macroscopic characteristics: circular, smooth colonies, regular and flat borders, 1 mm in diameter and yellow pigment. Microscopic
examination revealed to be a Gram-negative bacillus with rounded ends. They were presented alone or in pairs. It is mobile, catalase positive, facultative anaerobic, non-fermenting glucose (Table 1).

The results obtained corroborate those found by Silini-Cherif and collaborators (2012) in the identification of a strain of *Pantoea agglomerans* IMA2 isolated from wheat rhizosphere. Fujikawa and Akimoto (2011) also show similar results for Pantoea agglomerans. The biochemical characteristics presented by the bacterium isolated from industrial laundry residue are also similar to the strains of *P. ananatis* and *P. stewartii* (Delétoile et al., 2009).

Gavini et al., (1989) and Mergaert (1993) describe the genus *Pantoea* as bacilli of 0.5-1.3 × 1.0-3.0μm. Non-encapsulated and non-spore forming. Most of the strains are mobile by means of perimeter, Gram-negative flagella and colonies when grown on nutrient agar are smooth, translucent with convex or heterogeneous margins in whole consistency and adhering to agar.

The colonies are yellow, beige or non-pigmented, facultative anaerobes. The optimum temperature of growth is around 28 and 30 °C. Oxidase negative.

Glucose dehydrogenase and gluconate dehydrogenase are produced and are active without an added cofactor. Lysine and ornithine are not decarboxylated, urease negative, does not degrade pectin, H$_2$S is not produced from thiosulfate. Most of the strains are Voges-Proskauer-positive and indol-negative. The acid is produced from the fermentation of L-arabinose, D-ribose, D-xyllose, D-galactose, D-fructose, Lramnose, D-mannitol, N-acetylglucosamine, maltose and trehalose. The sources of carbon used at 28 °C (Biotype-100) are D-glucoside, D-fructose, D-galactose, trehalose, D-mannose, celloobiase, 1-O-methyl β-D-glucopyranoside, L-arabinose, Glycerol, and L-serine. The sources of unused carbon at 28 °C (Biotype-100) are L-sorbose, palatinose, melezitose, maltitol, turanose, tricarballylate, 4-hydroxybenzoate, gentisate, methyl 3-hydroxybenzoate, methyl benzoate, 3-phenylpropionate, M-cammarate, histamine, caprate, caprylate, glutarate, 5-aminovalerate, ethanolamine, tryptamine, itaconate, 3-hydroxybutyrate, propionate and L-tyrosine. Reference strains were isolated from plants, seeds, fruits, soils and water, and from humans (urine, blood, wounds, internal organs) and other animals. Strains of various species are phytopathogenic in a wide range of facilities and agricultural machinery. The G + C content of the DNA varies from 52.7-60.6 mol% (Deletoile et al., 2009; Duron et al., 2016).

**Biochemical Characterization**

The results of several biochemical tests were listed in Table 1. The *Pantoea* sp. Degraded some carbon sources such as D-mannitol, D-mannose, D-glucose, D-gluconase, D-fructose and sucrose. And also the gelatin protein. He presented H$_2$S production and was positive for the Voges-Proskauer test, methyl red and lactose. According to Delétoile et al., (2009) and Mergaert et al., (1993) these are characteristics of the strains of *Pantoea agglomerans*.

**Responses to Abiotic Stress**

*Pantoea* sp. Demonstrated great growth ability over a broad pH range, ranging from pH 4.0 to pH 8.0. There was inhibition of growth for alkaline pHs (pH 9.0 and 10.0). The bacteria showed optimum growth at pH 7.0 (Figure 1). The results were similar to those reported by *Pantoea agglomerans* CPA-2 and *Pantoea agglomerans* IMA2 in works.
presented by Costa et al., (2002), Son et al., (2006) and Silini-Chérif et al., (2012), respectively. Other studies have reported that the highest development of Pantoea occurs in culture medium with pH values ranging from 6.0 to 7.0 (Costa et al., 2002).

**Table 1** Biochemical tests and acid production to bacterial identification

| Biochemical Tests | Bacterial strain | Acid production | Bacterial strain |
|-------------------|------------------|-----------------|-----------------|
| Motility          | +                | D-glucose        | +               |
| Yellow Pigment    | +                | D-fructose       | +               |
| Catalase          | +                | D-xylose         | -               |
| Citrate           | -                | D-mannose        | +               |
| Lactose           | +                | D-mannitol       | +               |
| Methyl red        | +                | D-gluconase      | +               |
| Glucose           | -                | Lactose          | -               |
| Indol production  | -                | Maltose          | -               |
| Production H2S    | +                | Trehalose        | +               |
| Reaction Voges-Proskauer | +     | Sucrose          | +               |

+: Positive Test; -: Negative Test.

**Fig. 1** Effect of different pH in nutrient broth medium on the growth of *Pantoea* sp. at 48 h of incubation
Table 2 Enzyme production by bacterial strain isolated from laundry effluent

| Enzyme Activity     | Bacterial strain |
|---------------------|------------------|
| Urease              | _                |
| Protease            | _                |
| Amylase             | _                |
| Tanase              | +                |
| Polyphenoloxidase   | +                |
| Cellulase           | +                |

The turbidity measurement, for growth at different temperatures, showed a significant result at 30 °C, indicating that the tested Pantoea lineage is mesophilic (Figure 2). Similar results were found by Camatti-Sartori et al., (2008) when evaluating the influence of temperature on the growth of Pantoea agglomerans and obtained the best results at temperatures of 30 °C. The microorganism showed good viability of growth at high temperatures of 40 and 44 °C (Figure 2), which could explain the presence of these organisms in arid regions (Silini-Chérif et al., 2012).

However, Camatti-Sartori et al., (2008) presented discordant results regarding the temperature of 40 °C, because there was a significant decrease in the development of the microorganism. Son et al., (2006) worked with insoluble phosphate solubilization by Pantoea agglomerans and obtained good microorganism growth results in a temperature range between 5 and 45 °C. With best results at 25-35 °C. Temperature is one of the most important factors governing the physiology and growth of microorganisms, as reported by Rahman et al., (2006).

Fig. 2 Evaluation of temperatures (°C) of incubation on the growth of Pantoea spin in the nutrient broth medium at 48h
Growth of the nutrient broth strain with variations in salt concentration from 0 to 1000 mM showed a high tolerance capacity at high salt concentrations. The high point of growth was with 100 mM. However, it presented excellent results at concentrations of 400, 800 and 1000 mM NaCl (Figure 3). According to Borneman et al., (1996), soil salinity plays an important role in the microbial selection process. Several studies indicate that bacteria isolated from saline environments are better able to survive inhibition concentrations compared to non-saline isolates (Sharma, 2006). Similar results were found by Silini-Chérif et al., (2012) when working with a strain of Pantoea agglomerans.

**Enzymatic Profile of Pantoea agglomerans**

Microbial enzymes have an essential role in the processes related to soil quality, since it is through them that the soil microorganisms degrade complex organic molecules in simple and that can be assimilated. In addition to allowing microorganisms to have access to the energy and nutrients present in complex substrates, extracellular enzymes are responsible for the decomposition and mineralization of nutrients in the soil, also making them available to plants and promoting the cycling of nutrients in the soil (Makol; Ndakidemi, 2008). The bacterium of the genus Pantoea sp was submitted to enzyme activity detection tests for amylase, urease, protease, tannase, cellulase and polyphenoloxidase. Pantoeaagglomerans isolated from laundry effluent showed positive results for enzymes tanase, polyphenoloxidase and cellulase (Table 3). Tannase is an enzyme that hydrolyzes esters and side bonds of hydrolysable tannins producing glucose and gallic acid (Banerjje et al., 2001). It is mainly used for the production of gallic acid, instant teas, wine color stabilization, leather treatment process, food detanification, antioxidant production and effluent treatment in the leather industry (Battestin et al., 2004;).

Madhukar et al., (1996) and Souza and Magalhãs (2010 detected the enzymatic activity of amylase and cellulase by Pantoea agglomerans isolated from pea leaves (Lathyrus maritimus L). Cellulases are used in many biotechnological applications. In the
textile industry, these enzymes are used to give better finishing to the fabrics, making them smoother, smoother and with better trim. Cellulases are also used in the beverage industry for the production of fruit juices and in winemaking processes. Also, they play an important role in animal nutrition, detergent manufacturing, and the paper pulp industry, making the paper whiter and smoother. However, interest in these enzymes has increased greatly due to their use in the ethanol production process (Castro; Pereira, 2010).

Phenol oxidase-catalyzed reactions are related to changes in cell wall properties (increased impermeability and hydrostatic resistance), intracellular interactions and removal / detoxification of certain secondary metabolites (Mayer; Harel, 1979; Mardaneh; Dallal, 2013; Dushyanthie to al., 2014).

The biochemical and morphological tests showed that the microorganism isolated is of the Pantoea genus and presents characteristics similar to those presented by Pantoea agglomerans. The Pantoea strain studied showed tolerance to high salt concentrations, optimum growth in mesophilic temperature codons, 30, 37 °C, and alkaline pH 7 and 8 indicating an enzymatic biotechnological potential for cellulase, tannase and polyphenoloxidase.

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