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Citric acid production by Brazilian garlic *Aspergillus welwitschiae* strains using orange residues

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ABSTRACT. The global production of citrus fruits has expressively grown resulting in large amounts of residue. Orange residues are rich in carbon and could be used as substrates for production of biomolecules such as citric acid. In this study, wild and mutant *Aspergillus welwitschiae* strains isolated from Brazilian garlic were used to produce citric acid from orange residues, using solid-state fermentation. The results showed that the mutant *A. welwitschiae* UELAs 15.262/35 produced great amount of citric acid in the fourth day of solid-state fermentation using total and reducing sugars and producing cellulases. On the other hand, there was no production of citric acid by wild *A. welwitschiae* UELAs 15.262. The use of total and reducing sugars and the production of cellulases by *A. welwitschiae* UELAs 15.262 were detected, suggesting the use of carbon sources for the production of other metabolites. The great use of reducing sugars and cellulases production by *A. welwitschiae* UELAs 15.262/35 indicated the consume of carbon sources from the orange residue to produce citric acid at early times of fermentation. These findings lead to the possibility of biotechnological valorization of orange residues for acid organic production by *A. welwitschiae* strain.

Keywords: Citric acid; Orange residue; *Aspergillus welwitschiae*.

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Introduction

Citriculture occupies a relevant position in Brazil for both the fresh fruit market and the orange juice processing industry (Rivas, Torrado, Torre, Converti, & Dominguez, 2008). However, this great production is responsible for the generation of excessive amounts of residues which may cause serious environmental problems. The orange juice production provides large amounts of residues, as around 50 % of its original weight corresponds to the solid residue generated during processing (Marín, Rivas, García, Castillo, & Alvarez, 2007; Rivas et al., 2008; Leitão et al., 2010). These residues are source of several compounds such as soluble and insoluble sugars, fibers, organic acids, amino acids and proteins, minerals, oils and lipids, flavonoids and vitamins (Lopéz et al., 2004; Shirahigue and Antonini, 2020), that could be used as alternative substrates in bioprocesses to produce valuable biomolecules and help to reduce the environmental impact (Ali, Anwar, Irshad, & Mukhtar, 2019).

Citric acid or hydrogen citrate, officially known as 2-hydroxy-1,2,3 propanotricarboxylic acid, is a weak organic tricarboxylic acid, which is present in most citrus fruits, such as lemons and oranges (Ciriminna, Meneguzzo, Delisi, & Pagliaro, 2017; Uzah, Akani, & Odu, 2020), but also can be produced by microorganisms. In fact, the majority of the citric acid produced in the world is obtained by fermentation. Through solid-state fermentation (SSF), different microorganisms, such as bacteria, fungi or yeasts, can produce several compounds using the industrial residues (Prado, Vanderbergh, Woiciechowski, León, & Soccol, 2005).

The high demand for citric acid is due to its industrial applications such as in personal hygiene products, cosmetics, anticoagulants, in the production of beverages, as a preservative, emulsifiers, antioxidants, buffering and stabilizers and in the environmental industry, as well as in the processes of environmental remediation (Good, Droniuk, Lawford, & Fein, 1985; Prado et al., 2005; Uzah et al., 2020). So, this wide demand requires innovative approaches for production through fermentation such as screening for new producer strains, high yielding mutants, exploitation of low-cost substrates and optimization of the fermentation process. Consequently, citric acid production through fermentation still needs efforts from

researchers. Several agro-industrial wastes that include cassava peel, banana peel, rice straw, orange peel, sugarcane bagasse, chicken feather and pomegranate peel among others have been employed for the microbial production of citric acid (Adeoye, Lateef, & Gueguim-Kana, 2015; Dutta et al., 2019; Ozdal and Kurbanoglu, 2019; Roukas and Kotzekidou, 2020; Sawant, Mahale, Ramchandran, Nagaraj, & Bankar, 2021). Filamentous fungi are extensively used in the fermentation industry for the synthesis of numerous products that include enzymes, functional foods, and citric acid. Studies on the production of citric acid mainly involve fungi and yeasts such as *Aspergillus niger*, *Penicillium luteum*, *Penicillium citrinum, Candida guilliermondii, Saccharomycopsis lipolytica, Trichoderma viridae and Arthrobacter parafineus* (Show et al., 2015; Uzah et al., 2020).

The *Aspergillus welwitschiae* is included in the *Aspergillus* section *Nigri*. In this section, there is some species with similar morphological characteristics that are difficult to identify, named as "niger aggregate", comprising *A. welwitschiae* (Samson et al., 2014). *A. welwitschiae* has been isolated from onion bulbs, garlic and others sources. Some strains of *A. welwitschiae* does not produce Fumonisin B2 and Ochratoxin A and can then be used in biotechnological purposes (Gherbawy et al., 2015; Massi et al., 2016; Massi et al., 2020; Yang, Lübeck, & Lübeck, 2017; Vanzela et al., 2020).

The present study aims to evaluate the production of citric acid by wild and mutant *A. welwitschiae* strains using orange waste by solid-state fermentation and verify whether the mutant *A. welwitschiae* strain produces a greater amount of citric acid in orange residue. The wild *A. welwitschiae* strain was previously isolated and selected as no-producing potential fumonisin B2 and ochratoxin A by Vanzela et al. (2020). The mutant *A. welwitschiae* strain was selected by ultraviolet light-induced random mutation from the wild-type strain (unpublished data). The results showed the production of citric acid by a mutant *A. welwitschiae* UELAs 15.262/35 strain at early times of fermentation using orange agro-industrial residues. These results lead to the possibility of biotechnological valorization of orange wastes for citric acid production by *A. welwitschiae* strain.

Material and methods

Microorganisms

The strains were isolated from garlic marketed in Brazil and identified as *A. welwitschiae* and characterized as non-producing potential Ochratoxin A and Fumonisin B2 by Vanzela et al. (2020). In addition to the wild strain, *A. welwitschiae* UELAs 15.262/35 strain was introduced in this study to evaluate the production of citric acid. *A. welwitschiae* UELAs 15.262/35 is a mutant strain obtained from the *A. welwitschiae* UELAs 15.262 wild strain, by random mutation induced by ultraviolet light (Sartori, Ribeiro, Castilho, Bossa, & Amador, 2021).

Orange residue

The orange residue was supplied by Natú, located in Londrina, PR. After being collected, it was immediately processed and used as a substrate for the production of citric acid. Initially, the bark with the albedo was manually separated from the remains of the endocarp and seeds. The material was distributed on a tray and dried at 50 ºC in an oven with air circulation. Then, the dry residue was crushed in a mill A10 Basic 23 (IKA® Werke, IKA A10) and sieved to a particle size of 0.59 nm (Solotest, ABNT 30, Tyler 80). Initially, the amount of total and reducing sugars in the orange residue was evaluated before and after exposure to 121 ºC, 1 atm for 15 minutes, with the aim of verifying the existence of drastic changes in the composition of the residue that could interfere with the production of citric acid. They were then placed in 50 mL Erlenmeyer flasks containing 1.4 g of orange residue introduced before and after exposure to 121 ºC, 1 atm for 15 minutes. Then, 30 mL of distilled water was added to the orange residue, followed by resting for 1 hour and subsequent vacuum filtration to collect the crude extract and analysis of total and reducing sugars.

Solid-state fermentation

A. welwitschiae strains were previously grown on Potato Dextrose Agar (PDA) for 7 days at 28 ºC and then the kinetics of citric acid production under solid-state fermentation for a period of 10 days were performed in experimental triplicate. Initially, a suspension of 10^7 conidia/mL from each strain was prepared in 0.5 % Tween 80. Erlenmeyer flasks (50 mL) containing 1.4 g of orange residue were inoculated with *A. welwitschiae* UELAs 15.262 or UELAs 15.262/35 strains and the system was moistened with 3.0 mL of distilled water and 1.0 mL of Prescott & Dunn medium (10 g L^{-1} sucrose; 5 g L^{-1} of peptone, 5 g L^{-1} ammonium sulfate, 1 g L^{-1} ammonium nitrate, 1 g L^{-1} monopotassium phosphate, 0.23 g L^{-1} magnesium sulfate heptahydrate, pH 4.0).

The flasks were incubated at 35ºC for 10 days and every 2 days the culture was moistened with 500 µL of distilled water. In addition, every two days an experimental triplicate of each strain was collected to obtain the crude extract and to determine total and reducing sugars, citric acid and cellulases activity.

In order to verify the early production of citric acid, the kinetics of citric acid production by the *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains for 96 hours (4 days) were also performed under the same conditions. Crude extract was collected every 24 hours.

Assessment of total and reducing sugars

The amount of total sugars were determined through sulfuric phenol, using 1 mL of the crude extract of *A. welwitschiae* strains, according to Dubois, Gilles, Hamilton, Rebers and Smith, (1956). The reducing sugars were evaluated according to Somogyi, (1952) and Nelson, (1944), using 1 mL of the crude extract.

Assessment of citric acid

The quantification of citric acid was evaluated by pyridine-acetic anhydride according to Marrier and Boulet, (1958). To 1 mL of the crude extract from each *A. welwitschiae* strain, 1.3 mL of pyridine was added, followed by rigorous stirring. To the mixture, 5.7 mL of acetic anhydride was added and at 32 ºC for 30 min. The citric acid was identified at A_{405nm} in spectrophotometer (Biochrom Libra S22) and compared with a standard curve.

Control of cellulases activity

Cellulases activity was determined according to Periyasamy et al., (2017) using 0.5 mL of the crude extract.

All results obtained in this study were subjected to comparison of means and evaluation using the Tukey test with 5% significance.

Results and discussion

The initial amount of total and reducing sugars in the orange residue was 8.81 g L^{-1} and 10.26 g L^{-1}, respectively. After exposition of residue to 121ºC, 1 atm for 15 minutes, the total and reducing sugars quantification values was 8.40 g L^{-1} and 10.03 g L^{-1}, respectively. These results confirm the absence of significant changes in the orange residues composition, allowing their use as substrates for citric acid production in *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains.

The results showed a significant increase in citric acid production by mutant *A. welwitschiae* UELAs 15.262/35 in the fourth day of fermentation (4.88 g L^{-1}) (Figure 1A), corresponding to an increase of 41.86 % when compared to control (day 0). The citric acid detected in the day zero is due to the citric acid present in the fruits. A significant amount of citric acid was detected on the 10th day of fermentation (5.20 g L^{-1}), corresponding to 51.2 % of increase when comparing to control. On the other hand, citric acid was not detected during the 10 days of solid-state fermentation by wild *A. welwitschiae* 15.262 (Figure 1A). The production of citric acid in *A. welwitschiae* UELAs 15.262 remained at baseline until the sixth day of fermentation, comparing to the control.

The abundance of orange residue in the processing of citric fruits is important for the production of citric acid. *A. niger* strains present great ability to produce citric acid (Kadooka et al., 2020; Pires, Vasconselos, Ambrozim, & Pinheiro, 2020). The increase of citric acid production by *A. niger* strains is consequence of the duplication of some genes such as *citrate synthase* (Andersen et al., 2011). However, some *A. niger* strains are potential producers of fumonisin B2 and/or ochratoxin A (Abarca, Bragulat, Castellá, & Cabañes, 1994; Frisvad, Smedsgaard, Samson, Larsen, & Thrane, 2007). Recently, our group described the isolation and identification of *A. welwitschiae* strains that do not have a genotype for mycotoxins production and have a potential for the production of hydrolytic enzymes and organic acids (Vanzela et al., 2020).

The increase in the production of citric acid by fermentation is dependent on abiotic factors such as the availability of nutrients, temperature, pH and other parameters (Prado et al., 2005; Uzah et al., 2020; Miyamoto et al., 2020). In addition, mutant strains may result in the increase of citric acid production (Parvez et al., 1998; Haq et al., 2001; Conte and Marin, 2003; Lotfy et al., 2007; Javed, Asgher, Sheikh, & Nawaz, 2010; Adeoye et al., 2015). In this study, *A. welwitschiae* UELAs 15.262/35, a mutant strain obtained from the *A. welwitschiae* UELAs 15.262 wild strain (Sartori et al., 2021), showed high potential for citric acid production.

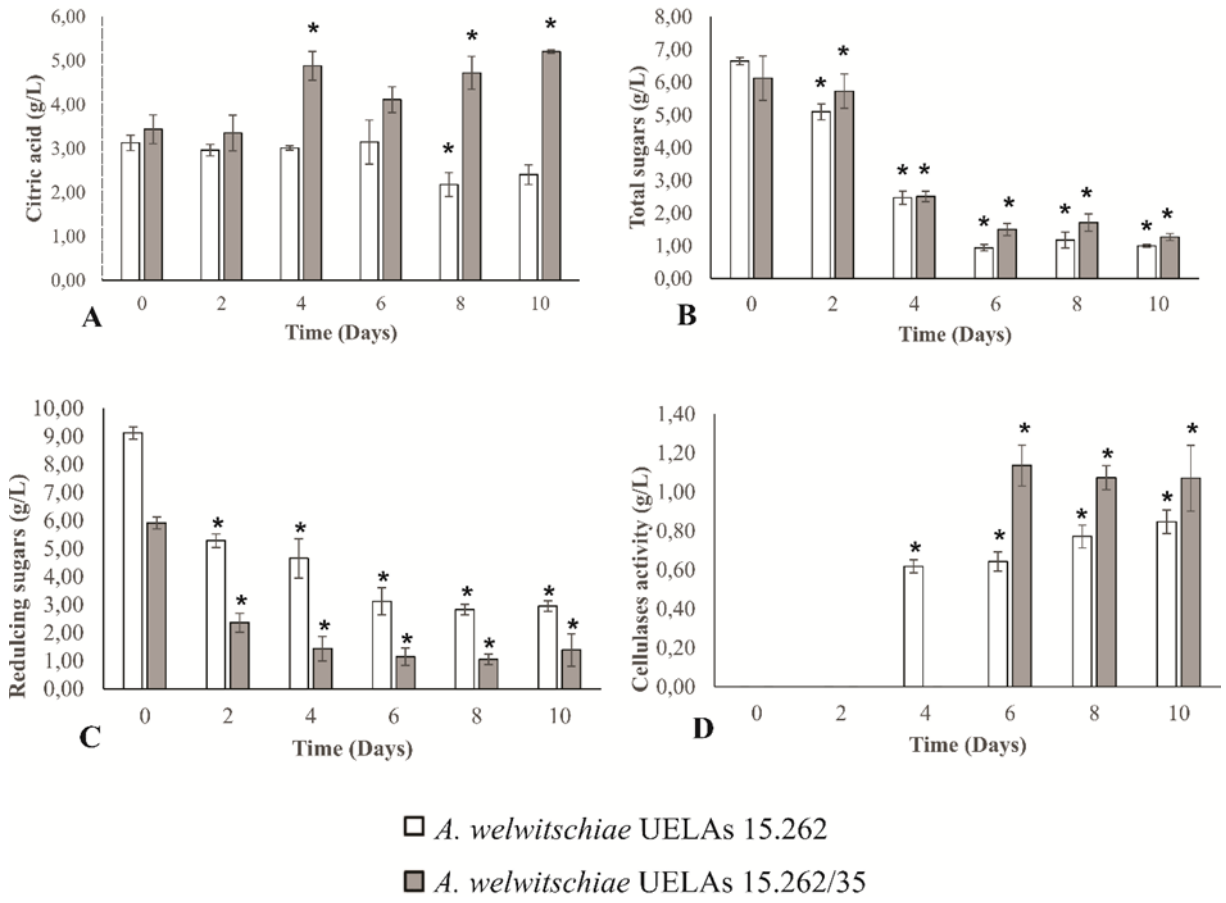

Figure 1. Kinetics of citric acid production by *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains using orange residue, during 10 days. (A) Citric acid production, (B) Total sugars (C) Reducing sugars (D) *Cellulases* activity. * Indication of significant differences (Tukey $p < 0.05$).

The use of total sugars was detected by both *A. welwitschiae* strains (Figure 1B). Although, the production of citric acid remained unchanged over the days for the *A. welwitschiae* UELAs 15.262 strain, a significant consumption of total sugars was detected (2.47 g L^{-1}, corresponding to 62.8 % comparing to control). Similar results were observed on UELAs 15.262/35 (2.45 g L^{-1}, corresponding to 60 % comparing to control). Both *A. welwitschiae* strains continued to use total sugars until the tenth day of fermentation.

Regarding the use of reducing sugars (Figure 1C) by *A. welwitschiae* strains the significant consumption of these sugars became evident from the second day of fermentation (UELAs 15.262 - 5.28 g L^{-1}, corresponding to 42.1 % of use of these sugars and UELAs 15.262/35 - 2.36 g L^{-1}, corresponding to 60.1 % of use of these sugars, comparing to control). The use of reducing sugars by *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 increased significantly until the eighth day.

The total and reducers sugars of orange residue based-medium was 8.4 g L^{-1} and 10.03 g L^{-1}, respectively. The results showed that prior to the production of citric acid by *A. welwitschiae* UELAs 15.262/35, total sugars were used, becoming accentuated from the fourth day of fermentation, while the use of reducing sugars was already observed on the second day of fermentative process. Therefore, the enzymatic apparatus of *A. welwitschiae* UELAs 15.262/35 promotes the release of sugars from the orange residue for citric acid production.

The greatest production of citric acid occurs with the availability of substrates rich in sugars (Wang et al., 2017). In this sense, the production of citric acid using orange residue is an alternative to reduce the production costs and also can contribute to decrease the environmental impact provided by the disposal of this residue in the nature. The orange residue holds a large amount of sugars such as glucose, fructose and sucrose, and organic acids such as citric acid (Zafiris, Tzia, Oreopoulou, & Thomopoulos, 1994; Kuforiji and Koboye, 2011; Mantzouridou, Paraskevopoulou, & Lalou, 2015). Then, the composition of orange waste is promising to the production of citric acid.

The cellulases activity (Figure 1D) of *A. welwitschiae* UELAs 15.262 strain was significantly detected from the fourth day (0.62 U mL^{-1}), increasing until the tenth day (0.85 U mL^{-1}) of fermentation. However, the activity

of cellulases by *A. welwitschiae* UELAs 15.262/35 was significantly detected from the sixth day (1.22 U/mL) and remain constant until the tenth day of fermentation.

The fact that cellulases activity and consequent glucose availability have been detected from the sixth day of fermentation corroborates with the ability of *A. welwitschiae* UELAs 15.262/35 to use sugars released from this nutritional source for the production and accumulation of citric acid. In fact, one of the ways used by fungi for the release of reducing sugars is the release of cellulolytic enzymes (Prajapati, Suryawanshi, Agrawal, Ghosh, & Kango, 2018; Pires et al., 2020).

On the other hand, the production of citric acid by *A. welwitschiae* UELAs 15.262 was not observed probably for this strain of *A. welwitschiae* requires a different nutritional source from *A. welwitschiae* UELAs 15.262/35. Such fact can be observed by the consumption of sugars by *A. welwitschiae* UELAs 15.262, where total sugars were consumed, but the use of reducing sugars was lower, when compared to the use of sugars by *A. welwitschiae* UELAs 15.262/35. The activity of cellulases by *A. welwitschiae* UELAs 15.262 on the fourth day of fermentation, indicative of the availability of glucose and a subsequent decrease in the production of citric acid, shows that *A. welwitschiae* UELAs 15.262 probably does not use glucose as the main nutritional source for citric acid synthesis. The consumption of total and reducing sugars and the production of cellulases by *A. welwitschiae* UELAs 15.262 also could indicate the use of carbon sources for the production of other metabolites.

The kinetics of citric acid production (Figure 2A) by both *A. welwitschiae* strains showed a significant decreased of 21.2 % (2.63 g L^{-1}) in the amount of citric acid by the *A. welwitschiae* UELAs 15.262 strain in 96 h compared to the control (0 hour). However, 15 % (4.07 g L^{-1}) of significant increase in citric acid accumulated with 96 h of fermentation was detected by *A. welwitschiae* UELAs 15.262/35 strain.

Analyzing the Figure 2B, it was observed in 72 h and 96 h, a significant utilization of 26.9 % (2.93 g L^{-1}) and 63.8 % (1.45 g L^{-1}), of total sugars in *A. welwitschiae* UELAs 15.262 strain, respectively. In the same time period, *A. welwitschiae* UELAs 15.262/35 strain significantly used up 35.8 % (4.92 g L^{-1}) and 42.9 % (4.38 g L^{-1}) of total sugars, respectively.

A. welwitschiae 15.262 strain significantly used up 46.7 % (4.05 g L^{-1}) and 47.6 % (3.98 g L^{-1}) of reducing sugars in 72 h and 96 h, respectively (Figure 2C). Under the same conditions, the use of reducing sugars by *A. welwitschiae* UELAs 15.262/35 was 30.2 % (2.31 g L^{-1}) and 72.8 % (0.90 g L^{-1} - significantly) in 72 h and 96 h, respectively.

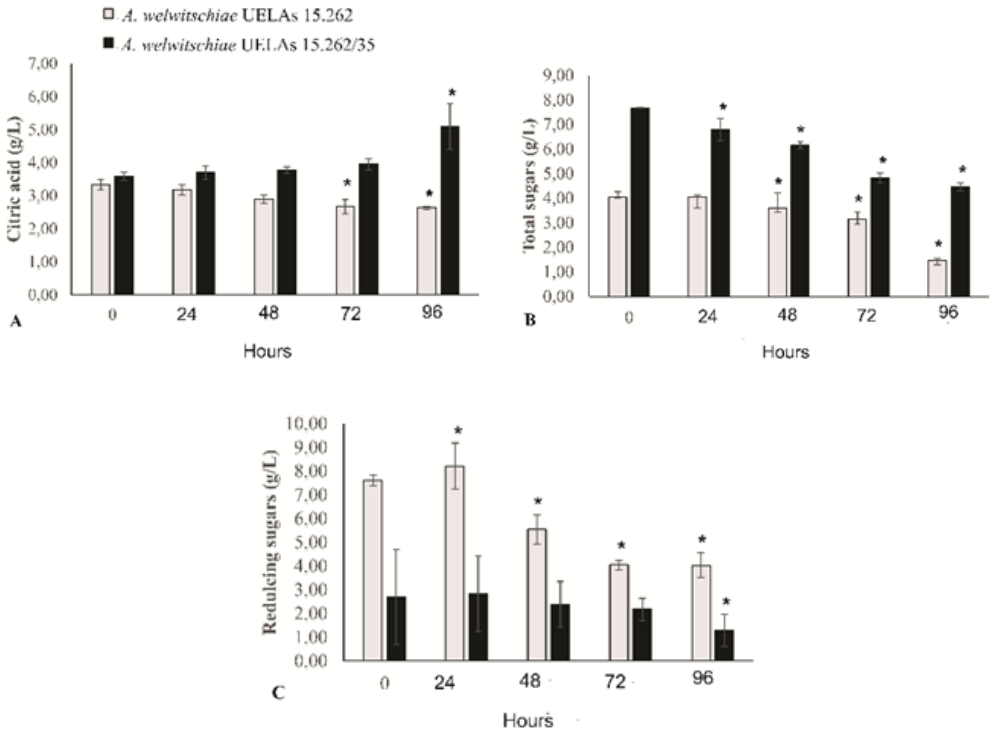

Figure 2. Kinetics of citric acid production by *A. welwitschiae* UELAs 15.262 and UELAs 15.262/35 strains, from orange residue, during 96 hours. (A) Citric acid production, (B) Total sugars (C) Reducing sugars. * Indication of significant differences (Tukey $p < 0.05$).

The use of fermentation medium nutrients to produce citric acid is highly related to the production time of this organic acid. As observed in the present study, there are reports in the literature about the production of citric acid in four days of fermentation (Ayeni et al., 2019). In contrast, other studies have shown citric acid production in a longer period of time (Lofty et al., 2007). The production of citric acid in a short period of time is favorable to reduce the production costs of fermentation process. In this sense, due to the fact that *A. welwitschiae* UELAs 15.262/35 started the production of citric acid on the fourth day of fermentation, the evaluation of the production of citric acid every 24 hours was important to detect the beginning of the production of citric acid. In fact, the production of citric acid by *A. welwitschiae* UELAs 15.262/35 started with 72 hours of fermentation, following the same pattern observed previously of greater use of reducing sugars by *A. welwitschiae* UELAs 15.262/35 comparing to *A. welwitschiae* UELAs 15.262.

Conclusion

The production of citric acid from orange residues using the isolated Brazilian garlic *A. welwitschiae* strain, thereby increasing the biotechnological value of wastes from orange processing industry. Solid-state fermentation has been a more cost-effective alternative to the fermentation process using waste as a substrate. In this study, the mutant *A. welwitschiae* strain showed great potential for the production of citric acid by solid-state fermentation using orange residue as substrate. This is the first report of citric acid production by *A. welwitschiae* UELAs 15.262/35 strain using residues from orange industry.

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