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Citric acid production by *Aspergillus spp.* through submerged fermentation using different production mediums containing agroindustrial residues

Produção de ácido cítrico por *Aspergillus spp.* por fermentação submersa utilizando diferentes meios de produção contendo resíduos agroindustriais

Producción de ácido cítrico por *Aspergillus spp.* mediante fermentación sumergida utilizando diferentes medios de producción conteniendo residuos agroindustriales

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Alexandre D'Lamare Maia de Medeiros
ORCID: https://orcid.org/0000-0001-6243-2739
Catholic University of Pernambuco, Brazil
E-mail: alexandredlamare@gmail.com
Thiago Pettrus Maia de Medeiros
ORCID: https://orcid.org/0000-0002-6628-9033
Brazilian University Center, Brazil
E-mail: thiagopettrus@gmail.com

Abstract

The production of secondary metabolites of high added value by microorganisms has been extensively studied, mainly in the formulation of medium containing agro-industrial residues. Citric acid is a tricarboxylic organic acid obtained through submerged fermentation processes. The *Aspergillus* genus is considered an excellent producer of bioactive substances of industrial interest. This study aims to determine the rate of citric acid production using four strains of the microorganism (UCP 1099, 1356, 1357, and 1463) in alternative culture mediums containing citrus pomace (pineapple, orange and lemon) from the food industries in the state of Pernambuco, and to compare the results with the production obtained with the standard production medium using computational statistical tools. Assays were carried out with four samples of *Aspergillus* isolated from Caatinga in three conventional mediums, for 144 h, 37°C, and 180 rpm. The pH variation, sugar consumption, and citric acid production were analyzed. The results showed that the medium-called two e and the sample UCP1357, presented a citric acid production of 7.22g.L^{-1}. After selecting the medium and the best producer microorganisms, production tests were carried out with a medium formulated with citrus fruit residues. The results revealed that all the residues produced citric acid, with the lemon peel being the best of the residues tested (6.91 g.L^{-1}). It appears that there is a possibility of using residues in the formulation of means of production, thus contributing to the reuse of the present nutrients and the reduction of the environmental impact.
Keywords: Alternative medium; Biotechnological potential; Filamentous fungi; Health teaching.

Resumo

A produção de metabólitos secundários de alto valor agregado por microrganismos tem sido amplamente estudado, principalmente na formulação de meios contendo resíduos agroindustriais. O ácido cítrico é um ácido orgânico tricarboxílico obtido através de processos de fermentação submersa. O gênero *Aspergillus* é considerado um excelente produtor de substâncias bioativas de interesse industrial. Este estudo tem como objetivo determinar a taxa de produção de ácido cítrico utilizando quatro cepas do microrganismo (UCP 1099, 1356, 1357 e 1463) em meios de cultura alternativos contendo resíduos de frutas (abacaxi, laranja e limão) provenientes de indústrias alimentícias do estado de Pernambuco, e comparar os resultados com a produção obtida com o meio de produção padrão utilizando ferramentas estatísticas computacionais. Os ensaios foram realizados com quatro amostras de *Aspergillus* isoladas da Caatinga em três meios convencionais, por 144 h, 37°C e 180 rpm. A variação de pH, consumo de açúcar e produção de ácido cítrico foram analisados. Os resultados mostraram que o meio denominado "dois" e a amostra UCP1357, apresentaram uma produção de ácido cítrico de 7,22 g.L^{-1}. Após a seleção do meio e dos microrganismos melhores produtores, foram realizados testes de produção com um meio formulado com os resíduos de frutas cítricas. Os resultados revelaram que todos os resíduos produziram ácido cítrico, sendo a casca de limão o melhor dos resíduos testados (6,91 g.L^{-1}). Verifica-se que existe a possibilidade de utilização de resíduos na formulação de meios de produção, contribuindo assim para o reaproveitamento dos nutrientes presentes e a redução do impacto ambiental.
Palavras-chave: Meio alternativo; Potencial biotecnológico; Fungos filamentosos; Ensino em saúde.

Resumen
La producción de metabolitos secundarios de alto valor agregado por parte de microorganismos ha sido ampliamente estudiada, principalmente en la formulación de medios que contienen residuos agroindustriales. El ácido cítrico es un ácido orgánico tricarboxílico obtenido mediante procesos de fermentación sumergida. El género Aspergillus es considerado un excelente productor de sustancias bioactivas de interés industrial. Este estudio tiene como objetivo determinar la tasa de producción de ácido cítrico utilizando cuatro cepas del microorganismo (UCP 1099, 1356, 1357 y 1463) en medios de cultivo alternativos que contienen orujo de cítricos (piña, naranja y limón) de las industrias alimentarias del estado de Pernambuco, y comparar los resultados con la producción obtenida con el medio de producción estándar utilizando herramientas estadísticas computacionales. Los ensayos se realizaron con cuatro muestras de Aspergillus aislado de Caatinga en tres medios convencionales, durante 144 h, 37 °C y 180 rpm. Se analizó la variación del pH, el consumo de azúcar y la producción de ácido cítrico. Los resultados mostraron que el medio denominado dos e y la muestra UCP1357, presentaron una producción de ácido cítrico de 7.22g.L-1. Tras seleccionar el medio y los mejores microorganismos productores, se realizaron pruebas de producción con un medio formulado con residuos de cítricos. Los resultados revelaron que todos los residuos produjeron ácido cítrico, siendo la cáscara de limón el mejor de los residuos probados (6.91 g.L-1). Parece que existe la posibilidad de utilizar residuos en la formulación de medios de producción, contribuyendo así a la reutilización de los nutrientes presentes ya la reducción del impacto ambiental.
Palabras clave: Medio alternativo; Potencial biotecnológico; Hongos filamentosos; Enseñanza en la salud.

1. Introduction

Biotechnology has a special role among the technologies that can contribute to sustainable development (Riordon et al., 2019). There are several fields of application for this technology; among them, the production of beverages (Bongaerts et al., 2021), cosmetics (Chikatueva et al., 2021), textile industry (Silva et al., 2021), construction materials (Ramdas et al., 2021), biodegradability of hydrophobic compounds (Medeiros et al., 2022), energy purposes and wastewater treatment (Medeiros et al., 2021; Medeiros et al., 2022b).

This science has been considered for many years as the one that will revolutionize the production and supply of biomaterials to markets (Rizwan et al., 2018; Silva et al., 2019). Further, the possibility of supplying biotechnological materials, the obtaining of bioproducts such as the hydrogen citrate.

Citric acid (CA) is a weak organic tricarboxylic acid, which has a molecular formula $C_6H_8O_6$ (Sawant, 2018; Carsanba et al., 2019), and has several applications in several industrial areas: beverages, drugs, cosmetics, and food (Ali & Haq, 2014; Ciriminna et al., 2017; Magalhães et al., 2019).

More than two million tons of citric acid are produced annually from submerged fermentation since obtaining it chemically is much more expensive than production through fermentative processes (Ozdal & Kurbanoglu, 2018; Tong et al., 2019).

The Aspergillus genus is described as an excellent producer of biotechnological metabolites, responsible for producing 92% of all citric acid worldwide (Perrone et al., 2013; Steiger et al., 2018; Chen et al., 2019). This genus is described in the phylum Ascomycota, in the Eurotiales and family Trichocomaceae (Riordon et al., 2019; Pearce, 2019).

The United Nations Food and Agriculture Organization (FAO) estimates that 1.3 billion tons of food are lost on the planet each year, about 30% of the total produced (Silva et al., 2019, Marenda et al. al., 2019). The reuse of these wastes in industrial production processes commonly presents good environmental and economic aspects for companies (Sant'Anna et al., 2012; Ashour et al., 2014; Sun et al., 2018).

From an environmental perspective, the company would not make inappropriate disposal, in the economic aspect, these residues can be reused in the formulation of medium or used in production lines since they still have high amounts of nutrients, which could be biotransformed by microbial action into high value-added metabolites (Storck, et al., 2013; Araújo, 2019; Marenda et al., 2019).

Brazil occupies a prominent position in food production, being the third largest world producer of fruits and vegetables, thus generating a large amount of waste, which normally does not undergo adequate treatments (Silva et al., 2019, Marenda et al., 2019).

The formulation of means of production in biotechnology, in the area of fermentative processes, mainly using residues for metabolites, has a high added value. (Marcelino et al., 2020; Rodrigues et al., 2019; Romo-Buchelly, Rodríguez-Torres & Orozco-Sánchez, 2019; Merino et.al., 2019; Das & Kumar, 2019).

Several studies have described techniques for reusing these residues in industrial production processes, because, in addition to reducing environmental damage, they present savings for companies, due to the large amounts of macro and micronutrients present in their composition (Sant'anna et al, 2012; Ashour et al, 2014; Sun et al., 2018).

In this study, citric acid production tests were carried out using samples of Aspergillus spp., in a conventional and elaborated medium using residues from the citrus fruit juice industry in the State of Pernambuco

2. Methodology

Tested Samples

Four samples of *Aspergillus spp.* (UCP 1099, 1356, 1357, and 1463) were used. Isolated from the Caatinga of Pernambuco and maintained in the culture bank of the Center for Research and Environmental Sciences and Biotechnology, located at the Catholic University of Pernambuco.

Medium Production

Maintenance

The selected samples were inoculated and kept in test tubes with a Sabouraud medium containing 1 $g.L^{-1}$ of citric acid for maintenance. Then, a spore suspension of 10^7 spores / mL was made. Spore counting was done in a Neubauer chamber under an optical microscope.

Production

The production media were formulated by the authors, modifying the media created by Prescott & Dunn in 1949.

Medium 1 (M1) - $NaNO_3$: 6,0g/L; KH_2PO_4: 1,5g/L; KCl: 0,5g/L; $MgSO_4.7H_2O$: 0,5g/L; $FeSO_4$: 0,01g/L; $ZnSO_4$: 0,01g/L; glucose: 10g/L; peptone: 2,0g/L; yeast extract: 2,0g/L; tiamin: 0,3g; citric acid: 1g/mL.

Medium 2 (M2) - $MgSO_4.7H_2O$: 0,23g/L; KH_2PO_4: 1,0g/L; NH_4NO_3: 1,0g/L; sucrose: 50g/L; urea: 5g/L; peptone: 2,5g/L; $(NH_4)2SO_4$: 2,5g/L; citric acid: 1g/mL.

Medium 3 (M3) - KH_2PO_4: 5,0g/L; trissódic citrate: 5,0g/L; NH_4NO_3: 2,0g/L; $(NH_4)2SO_4$: 4,0g/L; $MgSO_4.7H_2O$: 0,2g/L; peptone: 1,0g/L; glucose: 0,2g/L; yeast extract: 2,0g/L; citric acid: 1g/mL.

Waste Processing

For the formulation of the medium with agro-industrial residues, peels of citrus fruits (pineapple, acerola, orange, and lemon) were collected. The shells were kept for 24 hours in the open air for initial drying and then placed in a drying and sterilization oven model 315-SE by FANEM® for 12 hours at a temperature of 60ºC. After removing the greenhouse, the shells were crushed in an industrial blender and sieved until the standardization of 32 mesh.

Alternative Production Mediums

After selecting the best production medium and sample, the composition of the citric acid present in the control medium was replaced by different concentrations of the selected residues.

Citric Acid production in orthodox medium

Submerged fermentation was initiated using the three-production medium with chemically defined compositions. The medium was distributed in 250 ml Erlenmeyer's, in triplicate. 3% of the spore suspension was inoculated in the flasks containing different concentrations of the selected residues. The submerged fermentation was carried out in an orbital shaker at 37 ° C, 180 rpm, for 144 hours. 15 mL aliquots were collected every 24 hours and stored in a freezer for later analysis of citric acid production.

Citric Acid production in an alternative medium

In this first fermentation stage, the amount of citric acid present in the selected control medium was maintained in the formulation of the alternative medium. Three percent of the spore suspension was inoculated into Erlenmeyer flasks. The production conditions were the same used in the control medium.

Factorial Planning 2^3

After selecting the best residues for the production of citric acid, a factorial design 2^3, following Singer; Wilensky & McCraven (1956) methodology, was used to obtain the best conditions for the production of citric acid and verify whether or not there was an interaction between the tested residues. The coded matrix used is described in Table 1, where the variables were the residues of citrus fruits used. The matrix generated a total of 12 experiments, with four central points.

Table 1 - Decoded matrix of the weight of the shells of each agro-industrial waste used in grams per liter.

	Matrix code (g.L-1)		
Waste	**-1**	**0**	**+1**
Pinneapple	3	4	5
Orange	2	3	4
Lemon	1	2	3

Source: Authors (2022).

pH Determination

The final pH of the aliquots was measured using a LogRmeter perpHecT model 310 pH reader®

Sugar Consumption

The determinations of sugar concentrations were made using the methodology proposed by Ewing in 1969, the readings were made using a portable refractometer produced by the company Berthold technologies.

Determination of produced citric acid

For the determination of citric acid, the methodology described by Saffran and Denstedt (1948) was used. The amount of citric acid is identified by forming the yellow color in the reaction trichloroacetic acid (TCA) with pyridine and acetic anhydride.

The preparation for reading consists of the introduction of 1.0mL of the samples in a container containing 8.0 mL of acetic anhydride; then, the bottles are heated for 10 minutes in a water bath; after heating, 1.0 mL of pyridine is added; therefore, the tubes are vortexed for 30 seconds, returned to the water bath for another 40 minutes and then taken to a 5 minutes ice bath.

The standard curve was constructed using 1.0 ml of the citric acid solution, dissolving 4 g of citric acid in 100 ml of

15% TCA, with concentrations ranging from 0.04 - 0.4 g.L-1.

For the blank, 1.0mL of TCA (15%) is added to a container with 8.0 mL of acetic anhydride, followed by a water bath for 10 minutes at 60°C. Then, 1.0mL of pyridine is added so that the material is then returned to the water bath for another 40 minutes, followed by an ice bath of 35 minutes. Sample readings were performed on a spectrophotometer at a wavelength of 400 nm.

Statistical Analysis

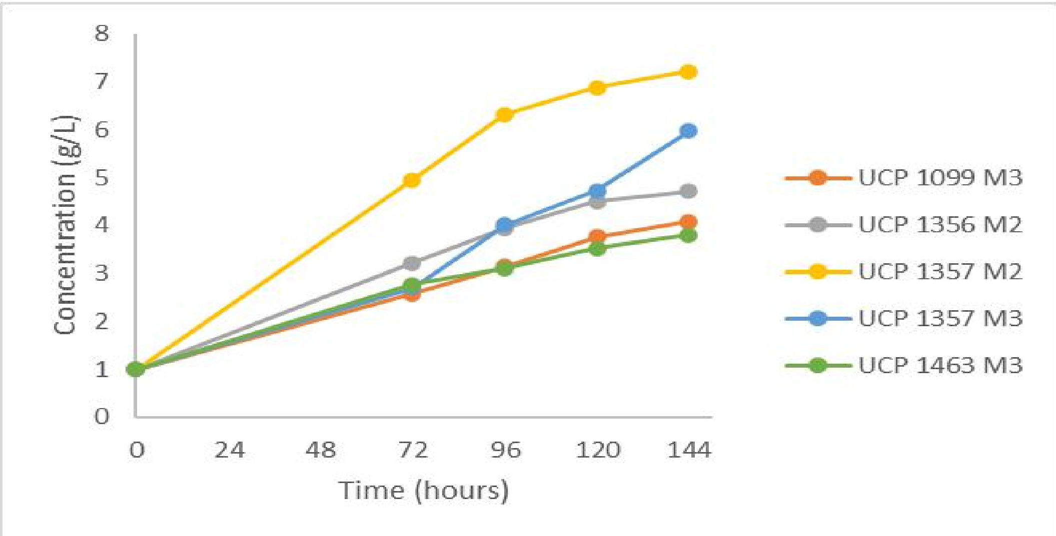
The decoded matrix was taken to the StatSoft® Statistica software for the creation of the coded matrix, determining the necessary conditions for the experiments, following Singer; Wilensky & McCraven (1956) methodology. After performing the tests, the Pareto chart and the 3D response surface were performed in the same software to determine the best production conditions for the CA.

3. Results and Discussion

During the period of citric acid production by different *Aspergillus* samples, it can be observed that none of the tested samples obtained significant production results in “medium 1”.

Two of the four samples tested (UCP 1099 and 1463) had a higher production in medium 3: 4.08 and 3.81 $g.L^{-1}$ respectively, while the other two (UCP 1356 and 1357) had a better response in medium 2, obtaining a maximum production value of 4.71 and 7.22 $g.L^{-1}$ as shown in Figure 1.

Figure 1. Citric acid production with different Aspergillus samples.



Source: Authors (2022).

Of the four samples used, in the three tested medium, the association that achieved the highest concentration of CA in the final period of fermentation was that of the microorganism called UCP1357 in "medium 2", with an average production of 7.22 g.L-1 of CA after 144 hours of fermentation (Table 2).

Table 2 - Citric acid concentration in UCP1357 tests every 24 hours (Φ).

UCP1357	**Production over time (g.L^{-1})**				
	0h	**72h**	**96h**	**120h**	**144h**
Medium 1	1	1,22	1,53	1,67	1,78
Medium 2	1	4,96	6,32	6,88	7,22
Medium 3	1	2,70	4,02	4,73	5,97

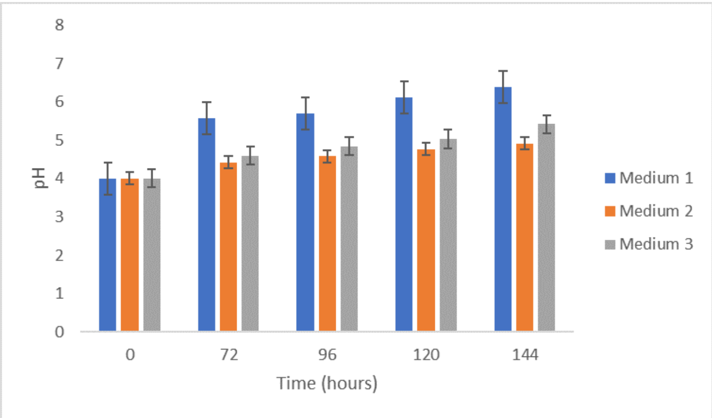
Source: Authors (2022).

The fermentation with the UCP1357 sample showed results similar to those found in the literature, Pastore; Hasan & Zempulski (2011), using the Prescott & Dunn medium ($MgSO_4.7H_2O$: 0.23g/L; KH_2PO_4: 1.0g/L; NH_4NO_3: 2,23g/L; sucrose: 140g/L; pH adjusted between 1.6 and 2.2) reached its highest productivity at 144h of fermentation with a value of 12.9g/L, using the fungus *Aspergillus niger*.

According to the author, it is not always easy to point out which factors influenced production, as individual factors should not be considered without the influence of other factors. It is of paramount importance, however, to introduce nitrogen sources to the fermentation medium since there is no evidence of any known filamentous fungus that can fix the nitrogen present in the atmosphere.

As for the pH, it can be observed that the higher the CA production, the more stable the potential remains, as can be seen in Figure 2, which demonstrates the readings of the UCP1357 sample.

Figure 2. Citric UCP1357 sample pH variation during 144 h of fermentation.



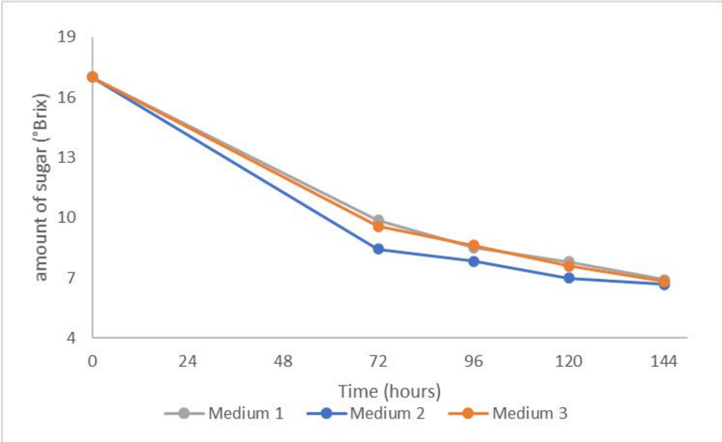
Source: Authors (2022).

It is observed that UCP1357 medium 2 is also the medium that has the greatest stability of pH readings reaching a maximum potential of 4.91.

It can be seen that these results corroborate the studies by Ali (2014) and Steiger (2018), showing that the pH of the metabolic liquid during fermentation remains proportional to the amount of CA produced, that is, the more the pH remains. In an acidic region, greater use of the microorganism for the production of the bioactive.

After the pH readings, tests were performed to determine the amount of sugars present in the medium to demonstrate the glucose consumption of microorganisms (Figure 3).

Figure 3. UCP1357 test aliquots sugar consumption (Φ).

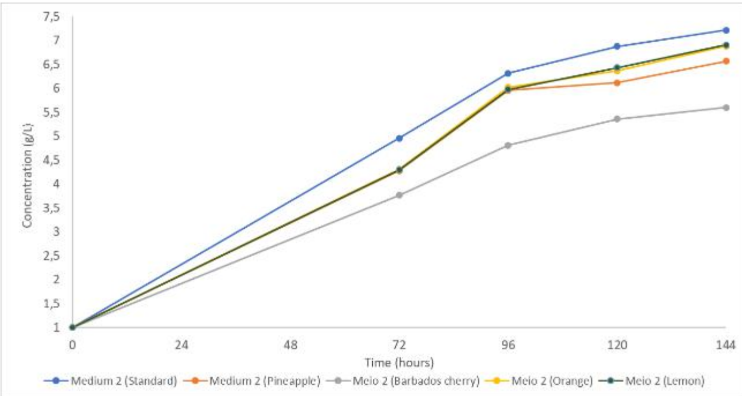


Source: Authors (2022).

Through the observation of sugar consumption, we can also see that medium 2 showed the greatest pH stability, was also the medium in which there was the greatest consumption of sugar during the first 72 hours. According to Bier et al. (2007) and Moraes et al. (2012), the proportion rate of substrate consumption by the microorganism, demonstrates a better supply of nutrients, thus generating better use of conversion in the desired bioproduct.

After all these tests, it was proven that medium 2 has the best growing conditions for microorganisms to consume the extracts and produce citric acid. With that, the selection of residues was then started. After the formulation of the new medium, new submerged cultivation was carried out. The results of the fermentation can be seen in Figure 4.

Figure 4. Citric acid production from UCP1357 in alternative medium (Φ).



Source: Authors (2022).

In these results, it can be observed that all the medium formulated with the residues of citrus peels obtained solid results and close to the CA concentration obtained in the standard medium. However, the medium with lemon peel was the one that obtained the best quantity of production, reaching an average of 6.91g.L^{-1}, being only 0.31 g.L^{-1} less than the conventional medium.

After determining the quantity of each type of residue through the decoded matrix and the fermentation of the 12 tests, a new chemical determination was performed. These results were taken to the software (Table 3) to analyze the results using the Pareto chart and response surface.

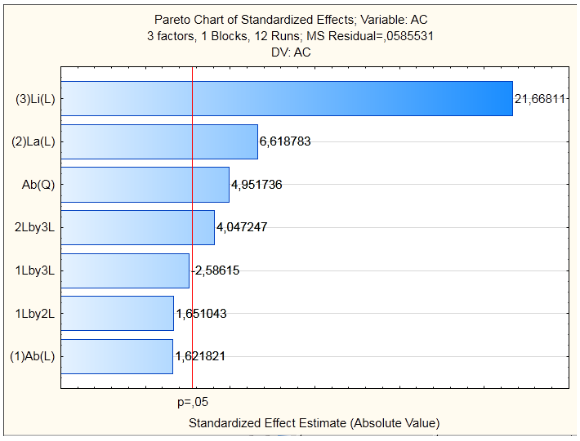
Table 3. Results of the proposed tests on the decoded matrix.

Experiment	Pineapple (g/L)	Orange (g/L)	Lemon (g/L)	Citric Acid (g/L)
1	3,00	2,00	1,00	9,86
2	3,00	2,00	3,00	13,63
3	3,00	4,00	1,00	10,33
4	3,00	4,00	3,00	14,86
5	5,00	2,00	1,00	10,61
6	5,00	2,00	3,00	12,87
7	5,00	4,00	1,00	11,02
8	5,00	4,00	3,00	15,29
9	4,00	3,00	2,00	11,72
10	4,00	3,00	2,00	11,47
11	4,00	3,00	2,00	11,50
12	4,00	3,00	2,00	11,61

Source: Authors (2022).

Before taking the results to the software to obtain the Pareto graph (Figure 5), it is possible to see that the amount of CA produced in test 8 was more than twice the amount produced by the standard means of CA production with only chemical substrates.

Figure 5. Pareto chart of interactions between agro-industrial residues used in the experiments.

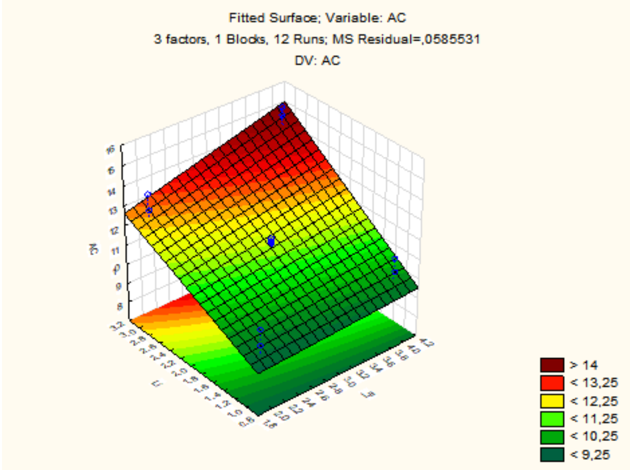


Source: Authors (2022).

From these results, it is possible to see that the variance is significant in the residues independently. However, good interaction between the residues occurs only between lemon and orange peels (residues that produced better individually).

In this same graph, it can also be noted that the residue from the lemon peel is what most accentuates the production of CA in the medium and that there was only significant interaction with the mixture of lemon and orange residues (Figure 6). However, the graph also demonstrates that pineapple and lemon residues obtained a result very close to the study's significance margin.

Figure 6. 3D response surface of interactions between orange and lemon residues.



Source: Authors (2022).

In this graph, we can identify that the margin with a more greenish coloration of the interactions is positioned between the two analyzed variables (orange and lemon), thus showing the great interaction between the two residues. With this, it can be observed that all the alternative sources provided significant concentrations of citric acid for the microorganisms and replaced the chemical source with perfection both individually and together.

It is also possible to determine that the interaction between the residues, even not being considered significant in the statistical study, obtained substantially higher CA production results (greater than twice as much) than the medium with citric acid PA e, accounting for 2.39 $g.L^{-1}$ a more than the study carried out by Pastore; Hasan & Zempulski (2011) using the industrial environment formulated exclusively with chemical substrates, thus demonstrating that agro-industrial residues are an excellent choice to reduce the cost of production of industrial CA.

4. Conclusion

It was observed in this study that the conventional medium 2 provided the best condition for the adaptation and the initial growth of the microorganism, since the initial consumption of sugars in the medium was prominent in this medium. In the studies of agro-industrial residues, it can be seen that all alternative sources of citric acid replaced the chemical source of CA well. However, lemon peel had the best average of results, producing only 0.31 g/L unless the default medium.

In studies of interactions between sources of citric acid, it can be seen that all combinations between residues generated concentrations of CA above those obtained with the use of the PA chemical. However, the combination that showed the greatest interaction was that of the peels—of lemon and orange, obtaining results significantly superior to those presented in the literature for medium formulated with chemical compounds.

The results of the work offered enough data to support that the alternative media used can be produced on a large scale for an effective production above the laboratory level of citric acid. Therefore, further studies are needed to determine the concentrations of nitrogen and carbon (glucose) introduced to the fermentation medium and salts need to be analyzed in further study in response to citric acid productivity, as well as the scale change of this experiment.

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