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Optimization of *Candida utilis* Biomass Production in Submerged Fermentation Using Citrus Peel as Substrate

Optimización de la Producción de Biomasa de *Candida utilis* en Fermentación Sumergida Utilizando Cáscaras de Cítricos como Sustrato

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Resumen
Los residuos de la industria citrícola generan unos 30 millones de toneladas métricas al año. Estos residuos contienen una alta concentración de azúcares y nutrientes que pueden ser un rico sustrato para generar diferentes productos entre ellos proteína unicelular. La proteína unicelular procede de la biomasa seca de diferentes microorganismos como hongos, bacterias y levaduras. La proteína unicelular puede ayudar a reducir la creciente demanda de alimentos ricos en proteínas porque puede producirse en poco espacio y en poco tiempo. El objetivo de este estudio era encontrar las condiciones adecuadas para la producción de biomasa de *C. utilis* mediante fermentación sumergida utilizando la cáscara de naranja como fuente de carbono. Se comparó el uso de diferentes concentraciones de cascara de naranja (5%, 10%, 15% y 20%) y diferentes fuentes de nitrógeno (extracto de levadura, peptona, NH_4Cl y $(NH_4)_2SO_4$) en un medio. Las fuentes inorgánicas de nitrógeno a una concentración del 10% de cáscara de naranja en el medio presentaron los mejores rendimientos al producir 11.5 g/L de biomasa de *Candida utilis*. Este estudio muestra que la cáscara de naranja cuando se suplementa con fuentes inorgánicas de nitrógeno es un buen sustrato para producir proteína unicelular.
Palabras clave: Cáscara de Naranja, *Candida utilis*, Fermentación Sumergida, Residuos agroindustriales, Single-cell protein.

Abstract
The waste from the citrus industry generates about 30 million metric tons annually. These wastes contain a high concentration of sugars and nutrients that can be a rich substrate for generating different products such as single-cell protein. Single-cell protein comes from the dry biomass of different microorganisms such as fungi, bacteria, and yeasts. Single-cell protein can help to reduce the growing demand for high-protein foods because it can be produced in a small space and in a short time. The goal of this study was to find appropriate conditions for production of *C. utilis* biomass by submerged fermentation using orange peel as carbon source. The use of different concentrations of orange peel (5%, 10%, 15% and 20%) and different sources of nitrogen (yeast extract, peptone, NH_4Cl and $(NH_4)_2SO_4$ in a medium was compared. The inorganic sources of nitrogen at a concentration of 10% orange peel in the medium presented the best yields by producing 11.5 g/L of *Candida utilis* biomass. This study shows that orange peel when supplemented with inorganic nitrogen sources is a good substrate to produce single-cell protein.
Keywords: Orange peel, Candida utilis, Submerged fermentation, Agro-industrial waste, Single-cell protein.

INTRODUCTION

Population growth has caused an increase in the demand for food and energy, causing the food industry generates a large amount of waste annually, which has caused a great contamination problem (Ravindran & Jaiswal, 2016). In 2018, it was estimated that 818 million people suffer hunger and this number is increasing every year, which implies a great challenge to eradicate according with SDG's to 2030 (FAO, 2019). That is why the generation of processes for food production under the concept of circular economy is of vital importance. This is why several strategies have been generated to take advantage of agro-industrial waste for the extraction and production of new food additives and/or foodstuffs.

The citrus industry is one of the largest waste producers, as around 31.2 million metric tons are produced annually worldwide (Mackenzie et al., 2019). Citrus residues contain a large number of carbohydrates such as: glucose, fructose, sucrose, arabinose, xylose and ribose, which are natural substrates for the growth of a wide variety of microorganisms (Bustamante et al., 2020; Chidan Kumar et al., 2011). In parallel to the carbohydrates present in the peel of the orange, it is necessary to use a source of nitrogen since it is one of the most important parts in the synthesis of proteins of the microorganism (Reihani & Khosravi-darani, 2018).

The use of these wastes can be as a substrate for the growth of *C. utilis*. The *C. utilis* biomass can be used as a food supplement for both humans and animals (Reihani & Khosravi-darani, 2018; Santos et al., 2019). Use of microorganisms for biomass production is an option which has several advantages such as the production time, high protein content, the possibility of producing specific amino acids through genetic modifications and the continuous production of this product through fermentation (Magalhães et al., 2018)

Different microorganisms can be used to produce microbial biomass, including yeasts such as *Candida utilis*, which is known to have a high protein content (50% by dry weight), in addition to other nutrients such as vitamin B and minerals (Martínez et al., 2018). *Candida utilis* has been frequently used to produce single-cell protein due to its ability to grow on a wide variety of substrates (Rajoka et al., 2004). This species is also capable of producing ergosterol, a compound that is a promoter of vitamin D.

The aim of this study was to find the best conditions to produce *C. utilis* biomass by submerged fermentation using orange peel as a carbon source.

MATERIALS AND METHODS

Raw Material. The orange peel (Citrus sinensis) variety Valencia was obtained in January 2019 in a local market in the city of Saltillo Coahuila, Mexico. The raw material obtained was cut and dried in an oven at 60°C for 72 h and then was ground in an Oster blender model 6831. The raw material was stored in airtight plastic bags at room temperature in a dry place to prevent it from getting wet.

Proximate analysis of orange peel. Proximate analysis (Ash, crude fiber, and lipids) of orange peel were determined according to. AOAC (AOAC, 2005; AOAC, 920.39, 1990; AOAC, 2003; AOAC, 2005). Crude protein content was determined by Kjeldhal method.

Microorganism. *Candida utilis* strain was obtained from the National University of Tucuman (Argentina) and grown on PDA medium (Potato Dextrose Agar) for conservation at 30°C.

Preparation of the inoculum. The inoculum medium was prepared in 50 mL of the mineral medium; the composition of medium (g/L); $(NH_4)_2SO_4$ 1.5; KH_2PO_4 0.75; K_2HPO_4 0.75; $MgSO_4 \cdot 7H_2O$ 0.05; anhydrous dextrose 5.0. *C. utilis* was grown at 30 °C and 150 rpm for 48 h. A cell count was carried out using a Neubauer chamber to adjust inoculum concentration at $1x10^6$ cells/mL.

Ratio Carbon:Nitrogen. To calculate the carbon-nitrogen ratio to be used. The total sugars in the medium were calculated using the Dreywood method (1946). Once the concentration of total sugars in the culture medium was known, the C:N ratio was adjusted according to experimental design.

Evaluation of nitrogen source and concentration of orange peel on biomass production. A Box Hunter-Hunter experimental design was carried out being the response variable, the biomass production of *C. utilis*. Two variables were evaluated at two levels (Table 1). The experimental matrix was obtained using the Statistica 7 software (Table 2). The established variables were the concentration of Orange peel with a maximum value of 15% and a minimum value of 5% and the second variable was the carbon-nitrogen ratio (C/N) with a minimum value of 1:30 and a maximum value of 1:10. The design resulted in four experimental units which were carried out in triplicate. This design was applied to compare four nitrogen sources: $(NH_4)_2SO_4$, NH_4Cl, peptone and yeast extract.

The medium was autoclaved at 121°C; 15 psi for 15 min to perform a hydrothermal pretreatment to hydrolyze the substrate and release polysaccharides then, the medium was vacuum filtered through filter paper. Fermentation was carried out in 250 mL Erlenmeyer flasks, with 50 mL of culture medium previously described. The flasks were inoculated with a solution adjusted to a concentration of $1x10^6$ cel/mL of *Candida utilis*. The conditions for fermentation were 35.5°C, 150 rpm and 72 h.

Table 1. Variables to be evaluated in a Box-Hunter & Hunter experimental design

Variables	Minimum -1	Maximum 1
Orange Peel Concentration %	5	15
Ratio C/N	1:10	1:30

Table 2. Box-Hunter & Hunter experimental design matrix

Treatment	Orange Peel Concentration	Ratio C/N
1	-1	-1
2	1	-1
3	-1	1
4	1	1

Optimization of biomass production. For the optimization of biomass production, a Box-Behnken experimental design was used in which two variables were evaluated at three levels each (Table 3). Using the Statistica 7 software an experimental matrix was obtained (Table 4), which resulted in nine experimental units carried out in triplicate. Also, the medium was enriched with the salts used in the mineral medium previously described and NH_4Cl as nitrogen source. The medium was sterilized in an autoclave for 15 minutes at a temperature of 120°C and 15 psi pressure. After sterilization of the medium, as well as hydrothermal hydrolysis of the medium. Afterwards, the medium was vacuum filtered under sterile conditions to separate the orange peel that was not completely hydrolyzed from the rest of the medium obtained. In 250 mL Erlenmeyer flasks, 50 mL of the previously prepared medium and a solution adjusted to a concentration of $1x10^6$ cell/mL of the *C. utilis* yeast was inoculated into it and it was taken to fermentation for 72 h at 35.5°C at 150 rpm. The sampling was carried out at final time of fermentation.

Table 3. Values to be evaluated in the Box-Behnken experimental design.

Variables	Minimum -1	Medium 0	Maximum 1
Orange Peel Concentration %	10	15	20
Ratio C/N	1:40	1:30	1:20

Table 4. Experimental matrix of the Box-Behnken design.

Treatment	Orange Peel Concentration	Ratio C/N
1	-1	-1
2	-1	0
3	-1	1
4	0	-1
5	0	0
6	0	1
7	1	-1
8	1	0
9	1	1

Growth kinetics. A growth kinetic was performed to know the behavior of *C. utilis* during the fermentation process. The kinetics was performed in 250 mL Erlenmeyer flasks containing 10% orange peel medium and a C:N ratio of 1:40 (NH_4Cl) ; a solution adjusted to a cell concentration of $1x10^6$ cells/mL was added and they were placed in an incubator with agitation for 72 h under the conditions of 35.5°C at 150 rpm. Samples were taken every 8 h by removing the corresponding flasks at the established time.

Analytical methods. The biomass production was determined, separating the biomass obtained from the supernatant in a centrifuge under the conditions of 5000 rpm; 10°C for 10 min. The biomass was dried for 12 h at 60°C and weighed on an analytical balance. Subsequently, the total nitrogen content was quantified by the Kjeldahl method and multiplied by 6.25 to obtain the crude protein content. The remaining supernatant was quantified by measuring total sugars and reducing sugars by the anthrone method (Dreywood, 1946) and DNS (Miller, 1959) respectively. Also, the soluble protein present was quantified by the method of Bradford (1976).

RESULTS AND DISCUSSION

Proximate analysis. The results in table 5 show the chemical composition of the orange peel. Moisture, ash, and lipids were 5.8%, 4.25% and 0.78% respectively. The amount of crude protein and crude fiber were 4.8% and 12.9% respectively. So, the orange peel can work as a good substrate for the growth of microorganisms such as *C. utilis* (Carota et al., 2020).

Table 5. Proximate analysis of Orange peel

Constituent	%
Moisture	5.80 ± 0.00
Lipids	0.78 ± 0.04
Crude Protein	4.80 ± 0.88
Crude fiber	12.09 ± 0.53
Ashes	4.25 ± 0.11

Evaluation of nitrogen source and concentration of orange peel on biomass production. Figure 1 shows that *C. utilis* had a lower growth when using organic nitrogen sources such as peptone and yeast extract, in which maximum yields of 4 g/L for peptone and 7.5 g/L for yeast extract were obtained. In comparison with inorganic nitrogen sources, maximum production of about 11.5 g biomass/L was obtained for $(NH_4)_2SO_4$ and similar to NH_4Cl 11.5 g biomass/L. Arous et al., (2016) explains that this may be because the nitrogen present in the ammonium salts has a lower molecular weight than organic sources of nitrogen, which allows an easier assimilation by *C. utilis.*

When comparing the concentration of the orange peel present in the medium, it is observed that the highest biomass production occurs when using 15 % of orange peel in the medium, which indicates that *C. utilis* can grow on the sugars present in the orange peel.

Unlike other microorganisms reported in the literature, *C. utilis* has higher biomass production in a shorter time under similar fermentation conditions. Such is the case reported by (Mahan et al., 2018) who use *Rhodococcus opacus* in orange peel to obtain a biomass production close to 2.96 g/L of biomass after 96 h of fermentation.

When performing statistical analysis, it was observed that there is no significant difference between the biomass produced by using ammonium chloride and ammonium sulfate. Thus, ammonium chloride was selected according to the C:N ratio used (1:30), as it needs less NH_4Cl to produce similar amounts of biomass.

After statistical analysis, it was observed that the concentration of the substrate (orange peel) in the medium was the variable that showed an influence on the growth of the yeast (Figure 2).

Consumption of sugars during fermentation. By comparing sugars' consumption during fermentation, when using inorganic sources of nitrogen, there is a consumption of between 82-92% of the substrate sugars. On the other hand, the use of organic sources of nitrogen, produced a lower consumption as it is observed consumption between 23-87% of sugars. Yeast extract was the source of nitrogen that achieved the lowest consumption (23-38%). This can be reflected in biomass production because the treatment (yeast extract 5%), produced a lower yield of biomass (2.2 g/L). This behavior indicates the importance of the C:N ratio in the medium. Del

Pilar Anzola-Rojas et al., (2015) reports that nitrogen plays a very important role in fermentation, since it is part of proteins, nucleic acids and enzymes that are fundamental in the development of the microorganism. However, an excess of nitrogen in the medium would cause structural changes in the microorganism. An excess of nitrogen in the media influences the carbon-to-nitrogen ratio necessary for adequate yeast growth. An unbalanced C/N ratio can generate stress in the microorganism, strongly impacting its metabolism and limiting its growth.

These high consumption percentages are caused by the hydrothermical hydrolysis to which the medium was subjected. The hemicellulose present in the orange peel is hydrolyzed and taken to their monomeric form which are glucose molecules which are the primary source of carbon in the cells.

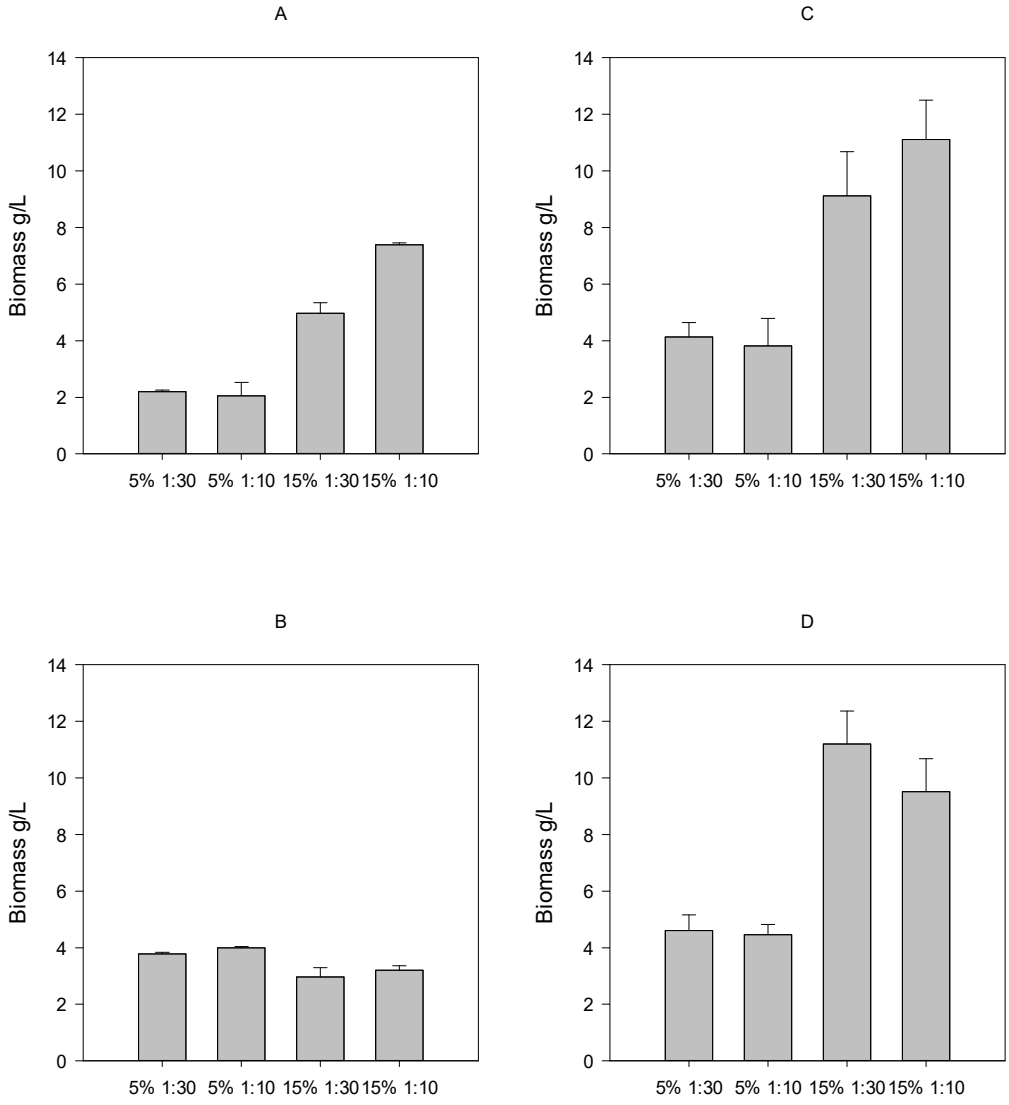

Figure 1. Comparison of biomass production end-time using different concentrations of orange peel and different sources of nitrogen (A.Yeast Extract, B.Peptone, C.Ammonium Sulphate, D. Ammonium Chloride)

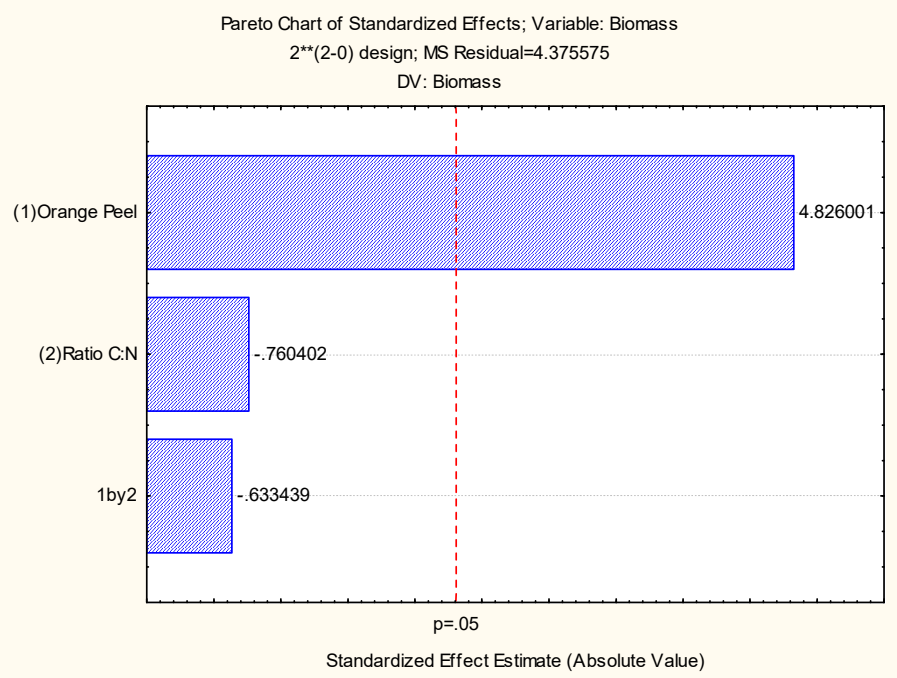

Figure 2. Pareto diagram of the effects of ammonium chloride as a source of nitrogen in biomass production

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Table 6. Sugar consumption in fermentation using different concentrations of orange peel and different sources of nitrogen

Treatment	Initial (g/L)	S/D	Final (g/L)	S/D	% Consumed
5% Yeast Extract 1:30	63.19	±4.04	48.12	±6.44	23.83
5% Yeast Extract 1:10			39.00	±4.87	38.26
5% Peptone 1:30			8.45	±0.22	86.62
5% Peptone 1:10			7.71	±0.09	87.79
15% Yeast Extract 1:30	143.66	±17.50	21.78	±1.48	84.83
15% Yeast Extract 1:10			22.66	±0.72	84.22
15% Peptone 1:30			52.34	±7.28	63.56
15% Peptone 1:10			51.95	±13.45	63.83
5% NH_4Cl 1:30	63.19	±4.04	7.35	±0.18	88.36
5% NH_4Cl 1:10			4.98	±0.078	92.11
5% $(NH_4)_2SO_4$ 1:30			9.57	±1.56	84.84
5% $(NH_4)_2SO_4$ 1:10			7.67	±0.46	87.85
15% NH_4Cl 1:30	143.66	±17.50	22.6	±0.12	82.26
15% NH_4Cl 1:10			24.09	±0.34	83.22
15% $(NH_4)_2SO_4$ 1:10			22.36	±0.10	84.42
15% $(NH_4)_2SO_4$ 1:30			18.65	±0.26	87.01

Soluble Protein Production. Protein production under submerged fermentation using nitrogen sources of organic origin was similar between the 5% concentration of orange peel in the medium using yeast extract at a ratio of 1:30 and the 15%

concentration of orange peel in the medium supplemented with peptone at a ratio of 1:10 in which about 18g/L were produced (Figure 3).

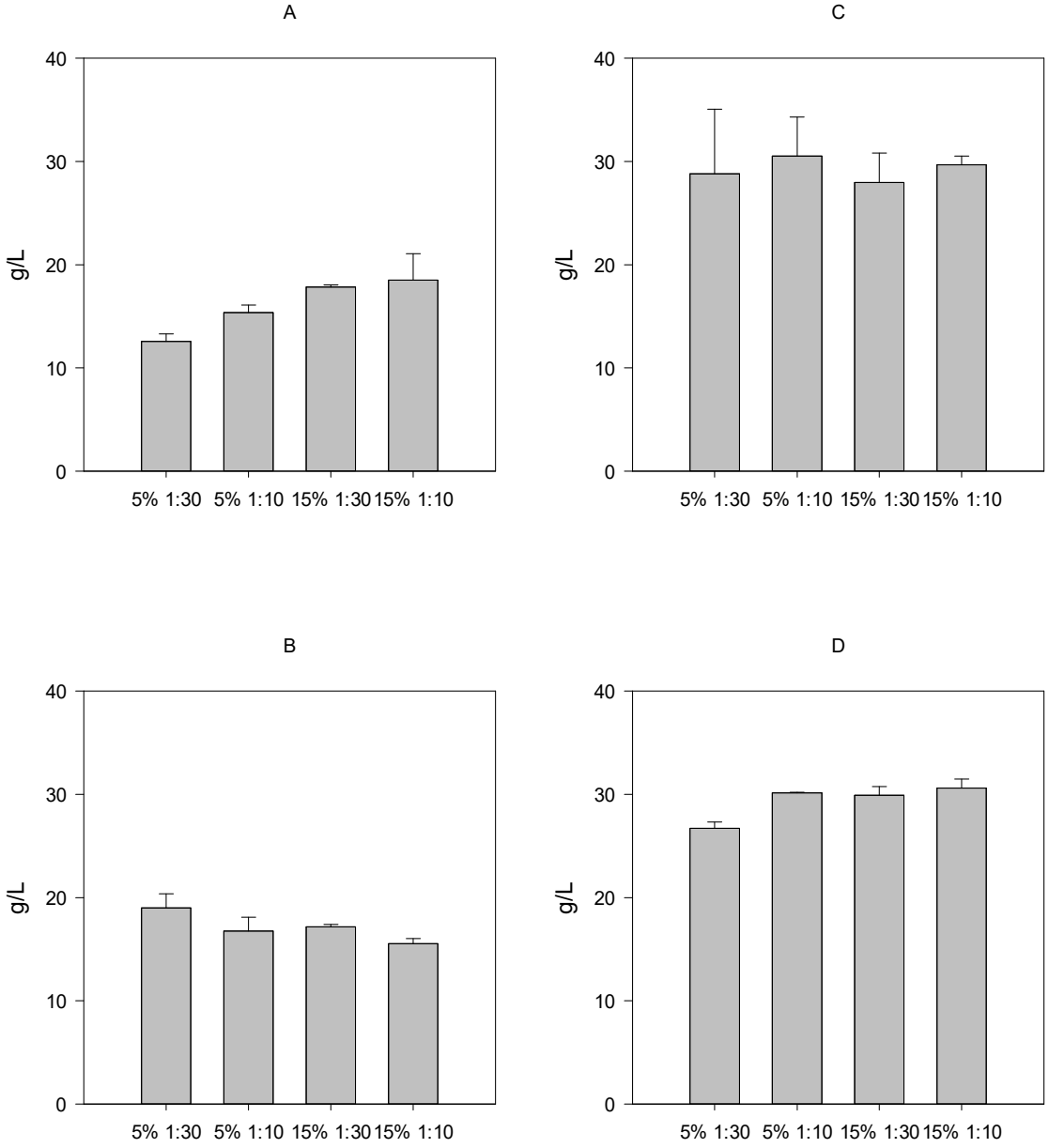

Figure 3. Comparison of soluble protein production obtained from biomass using different concentrations of orange peel and different sources of nitrogen (A. Yeast Extract, B. Peptone, C. Ammonium Sulphate, D. Ammonium Chloride)

Inorganic sources of nitrogen produced more protein than organic sources of nitrogen. Inorganic sources produced about twice as much protein, with which 30 g/L was obtained in both concentrations (5% and 15%) and in both C:N ratios (1:10 and 1:30) (Figure 3). Unlike what is reported by (Akintomide et al., 2012), who used a coculture of the fungus *Aspergillus niger* and the yeast *Saccharomyces cerevisiae*, in a medium composed of sweet potatoes and supplemented with $(NH_4)_2SO_4$ and NH_4Cl, they obtained soluble protein yields between 16-20 g/L. Higher

production of protein may occur when using an inorganic source of nitrogen because the ammonium ion is chemically more available than organic sources.

Optimization of biomass production. It is observed that of the nine treatments of the experimental matrix, treatments 1, 2 and 3 (Fig. 4), which corresponded to a concentration of 10% of orange peel in the medium, presented the highest biomass production of about 10 g/L of *C. utilis* biomass as opposed to the other treatments which produced between 6-8 g/L of biomass. However, when evaluating the C:N ratio, there is no significant difference in yeast growth, which indicates that the C:N ratios evaluated is not a variable with a significant influence on yeast growth. According to the Pareto chart from the statistical analysis (Fig. 5), the variable with influence on biomass production is the carbon source concentration (orange peel). Ezekiel & Aworh (2018) used yam as a carbon source to produce single-cell protein under similar fermentation conditions (30°C 150 rpm); however, they obtained a biomass production of 1.6 g/L. When comparing the results obtained with the literature, it is observed that there is a greater growth of biomass using orange peel as a carbon source. Another residue that has been used in the production of unicellular protein is the stillage from the cachaça; however, like the yam, the production of biomass from this residue is lower than that obtained from the orange peel since (Martínez et al., 2018) and (Santos et al., 2019) report production of 2.3 g/L.

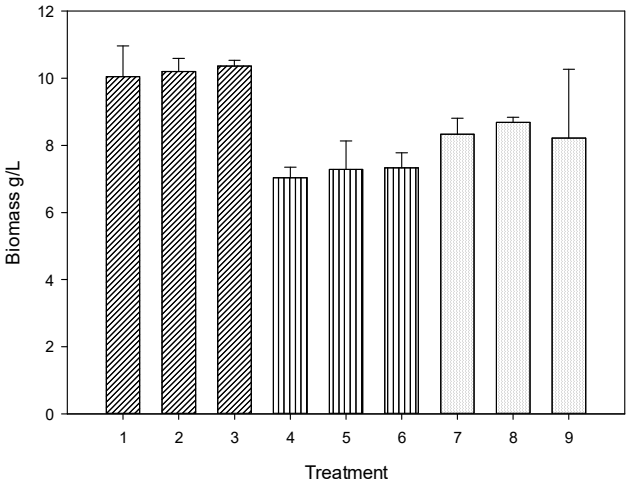

Figure 4. Biomass production to different treatments. (Diagonal pattern 1-3: 10% orange peel concentration) (Vertical pattern 4-6: 15% orange peel concentration) (Dotted pattern 7-9: 20% orange peel concentration)

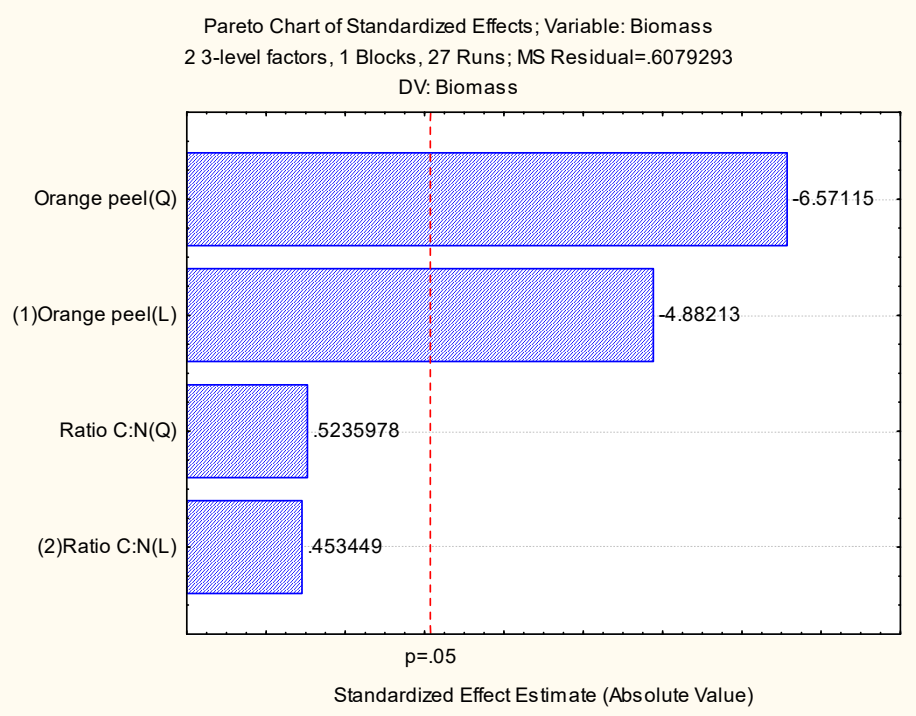

Figure 5. Pareto diagram of significant variables in biomass production.

Figure 6 shows the response surface diagram (Fig. 16) of the nine treatments of the Box Behnken design obtained by analyzing the data obtained when analyzing the data obtained, shows that the highest production of biomass is found in the minimum levels of concentration of orange peel in the medium (10%). Adoki (2008) reports that to have a good biomass yield, the substrate concentration must be kept low because otherwise, the substrate can oxidize and form carbon dioxide and heat, affecting the growth of *C. utilis*.

When the statistical analysis of soluble protein growth behavior is performed, the Pareto diagram (Figure 7) also shows that the orange peel concentration is a significant variable in the development of soluble protein. However, the Pareto chart's values about the carbon source are positive, which indicates that a higher concentration of this source will obtain higher values of protein. (Moftah et al., 2012) mentions that by increasing the concentration of the substrate, *C. utilis* increases the production of protease enzymes.

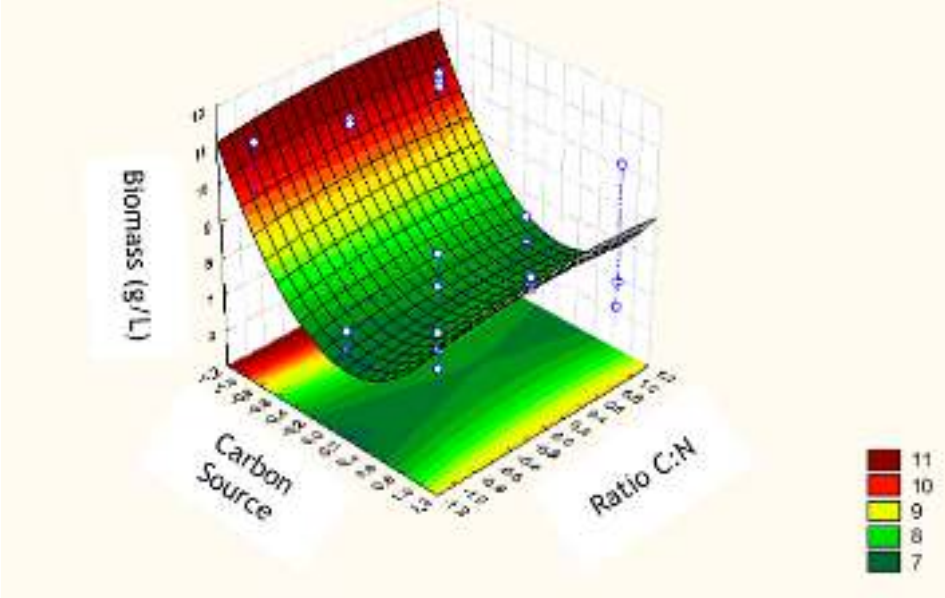

Figure 6. Biomass production response surface diagram.

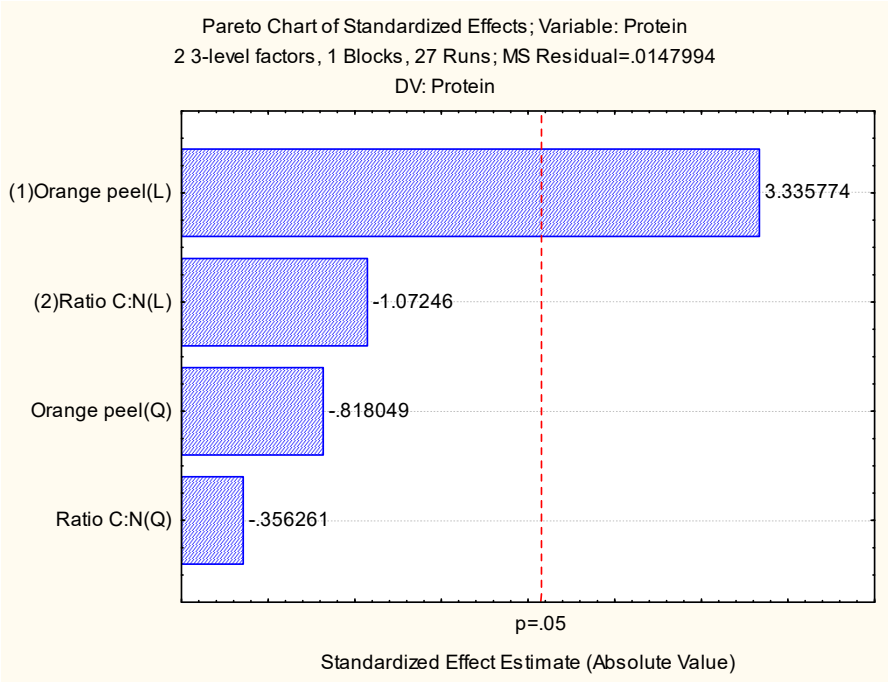

Figure 7. Pareto diagram of significant variables in Protein production.

Growth kinetics. Figure 8 shows the kinetic behavior of *C. utilis* biomass production, using orange peel as a fermentation substrate. The biomass production reaches a production of 12 g/L after 72 h of fermentation. When comparing this biomass production with the production of other types of yeasts such as *Rhodosporidium toruloides* (7 g/L) and *Candida laurentii* (9 g/L) after 72 h of fermentation using orange peel as substrate (Carota et al., 2020), *Candida utilis* produces a greater quantity of biomass.

As for sugars' consumption can be seen in Figure 9, an initial concentration close to 93 g/L which decreased to 30 g/L

after 16 hours of fermentation, then an increase is observed, resulting in a final concentration of 45 g/L of sugars. Similar to those reported by (Jha et al., 2019) who using *Saccharomyces cerevisiae* in a medium added with orange peel obtained sugars consumption close to 30%. This indicates a high affinity between the sugars present in the orange peel with *C. utilis.*

Table 7. Kinetics parameters of *C. utilis* growth

Parameters	**Results**
Yx/s (Yield Biomass/Substrate)	0.2284677 g/L
Qs (Rate of substrate consumption)	0.04528133 g/L h
Yp/s (Yield product/Substrate	0.2284677 g/L
Yp/x (Yield Product/Biomass)	1 g/L
Qp (Rate of product production)	0.19819574 g/L h
P	0.16342593g/L h

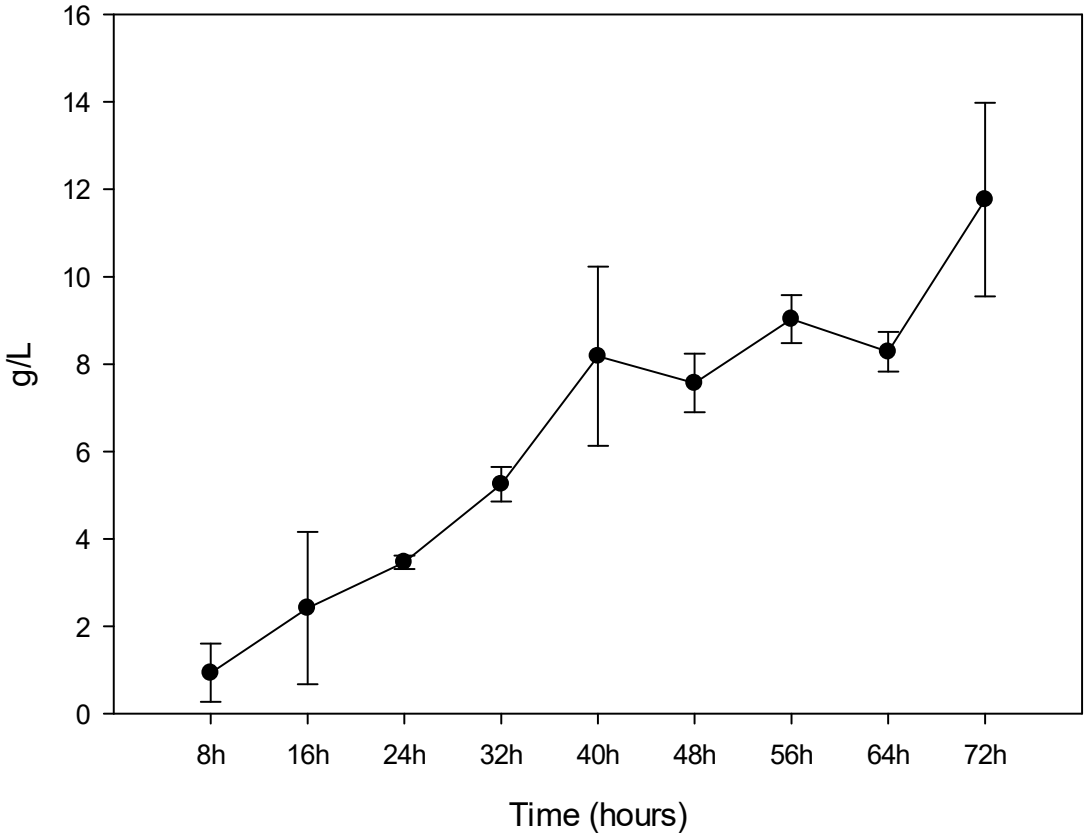

Figure 8. Growth kinetic of biomass production from *C. utilis*

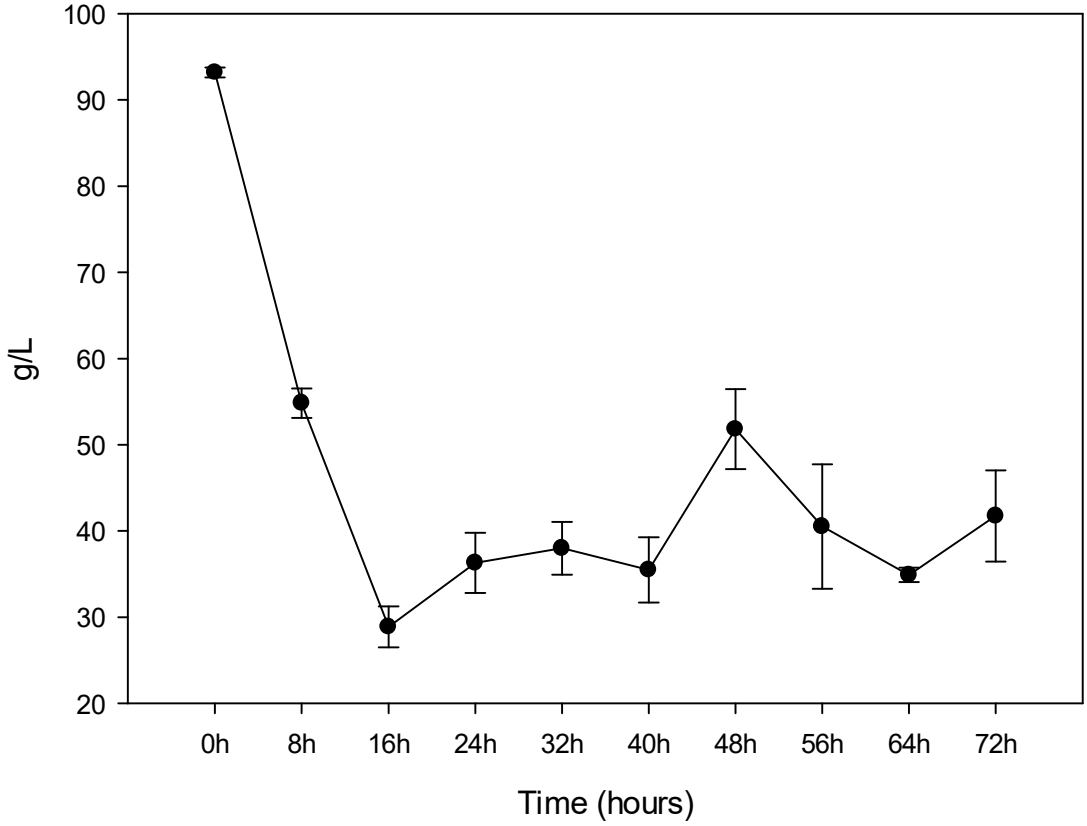

Figure 9. Behavior of total sugar consumption during submerged fermentation

However, total protein production obtained in the biomass (4.7%) is lower than reported by other authors such as, (Luisa et al., 2010) reporting 49.95% and (Zhao, Zhang, & Zhang, 2010) reporting 48.2%. Zhou et al. (2019) report that it is possible that an increased consumption of sugars, cellulases and pectinases, inhibit protein production. Another probable cause of the low percentage of protein may be due to the production of proteases. Moftah et al. (2012) reported that by increasing the concentration of substrate *C. utilis* increased proteases' production.

CONCLUSIONS

Unlike organic sources of nitrogen such as peptone or yeast extract, orange peel in 10% concentration combined with an inorganic nitrogen source as ammonium chloride in the medium proved to have a high affinity with the *Candida utilis* yeast as it was where the highest biomass production exists.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical Statement

N/A

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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