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ORIGINAL RESEARCH

High citric acid production in solid-state fermentation by *Aspergillus brasiliensis* on polyurethane foam

Alta producción de ácido cítrico en fermentación en estado sólido por *Aspergillus brasiliensis* sobre espuma de poliuretano

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ABSTRACT

A statistical approach based on fractional and complete factorial designs to increase the citric acid yield was used. Polyurethane foam (PUF) was used as inert support for solid-state fermentation (SSF) due to its high-water retention capacity (up to 60-fold of its weight), resulting in the recovery of concentrated citric acid by squeezing it. The maximum yields for citric acid (0.62 Cmol/Cmol glucose) and polyols (0.11 Cmol/Cmol glucose) are obtained by growing *Aspergillus brasiliensis* in a well-defined culture medium (at 30°C for 120 h). Citric acid, polyols, and glucose consumption are negatively affected by a high glucose concentration (250 g/L). In contrast, enrichment of the culture medium with yeast extract only enhances citric acid production yield (0.54 Cmol/Cmol glucose). This study demonstrates that the citric acid production yield is negatively affected by phosphate (> 4.54 g/L) and magnesium (> 0.26 g/L). The PUF for SSF is an inexpensive and useful support for the commercial production of citric acid. The utilization of low-cost inert supports through SSF can contribute to achieving industrially viable and sustainable citric

acid production. As far as we know, this work reports the highest citric acid production yield using SSF on an inert support such as PUF soaked with a defined medium.

Keywords: *Aspergillus brasiliensis*, citric acid production, defined culture medium, inert support, polyurethane foam, solid-state fermentation.

RESUMEN

Para aumentar el rendimiento de ácido cítrico se utilizó una serie de diseños factoriales fraccionados y completos, usando espuma de poliuretano (PUF) como soporte inerte para la fermentación en medio sólido (SSF), por su alta capacidad de retención de agua (60 veces su peso), resultando en la recuperación del ácido cítrico concentrado al exprimirlo. El rendimiento máximo de ácido cítrico (0.62 Cmol/Cmol glucosa) y polioles (0.11 Cmol/Cmol glucosa) se obtuvo cultivando *Aspergillus brasiliensis* en un medio de cultivo definido (30 °C durante 120 h). La producción de ácido cítrico, polioles y el consumo de sustrato son afectados negativamente por la concentración de glucosa (250 g/L). Adicionalmente, la adición de extracto de levadura aumenta la producción de ácido cítrico (0.54 Cmol/Cmol glucosa). Este estudio demuestra que la producción de ácido cítrico es afectada negativamente por el fósforo (> 4.54 g/L) y magnesio (> 0.26 g/L). El PUF es un soporte económico y útil para la producción de ácido cítrico. El uso de soportes inertes de bajo costo a través de SSF puede contribuir a lograr una alta producción de ácido cítrico que sea industrialmente viable y sostenible. Reportamos el mayor rendimiento de producción de ácido cítrico usando PUF como soporte inerte.

Palabras clave: *Aspergillus brasiliensis,* espuma de poliuretano, fermentación en medio sólido, medio de cultivo definido, producción de ácido cítrico, soporte inerte.

1. INTRODUCTION

Filamentous fungi are extensively used in industry to produce organic acids, heterologous proteins, and industrial enzymes (Dhillon *et al*., 2011). Citric acid is one of the major organic acid produced at industrial level since it is used in food, beverages, chemicals, detergents, pharmaceuticals, cosmetics, and other industries (Majumder *et al*., 2010; Campanhol *et al.*, 2019). The food industry consumes about 70% of the global production of citric acid and it is expected that the market will reach USD 3.9 billion by 2024 (Behera *et al*., 2021; Reena *et al.,* 2022). Citric acid is produced industrially using *Aspergillus niger* strains and its production is highly dependent on the composition of the culture medium (Show *et al*., 2015). This composition includes different variables, such as, the type and concentration of the carbon source, oxygen limitations, suboptimal concentrations of phosphate and magnesium and trace elements (Guilherme *et al.,* 2008; Diano *et al.,* 2009; Ozdal & Kurbanoglu, 2019). Particularly, oxygen limitation during the production of citric acid leads to the production of polyols; mainly glycerol, erythritol, mannitol, and arabitol (Diano *et al.,* 2006). Nevertheless, other polyols like xylitol, ribitol, and dulcitol are produced under specific conditions. For example, a sudden decrease in oxygen availability drives the production of mannitol and the use of xylose as a substrate causes a large production of xylitol (David *et al*., 2003; Ruijter *et al.,* 2003; Diano *et al.*, 2009).

Polyols have important roles across different cellular functions: they can act as carbohydrates reserve, have a role in osmoregulation, store reducing power and acts as translocation compounds (Lewis & Smith, 1967). Indeed, the accumulation of erythritol is linked to high osmotic pressure and high carbon source concentration; glycerol is linked to an increase in osmotic pressure, and the role of the enzyme mannitol-1-phosphate dehydrogenase in the reoxidation of NADH when the final electron acceptor, oxygen, is limiting (Diano *et al.*, 2009). Additionally, the pathways for polyol biosynthesis and catabolism can differ in *Aspergillus* spp. in comparison to other organisms. These pathways have mainly been studied in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Aspergillus niger* under submerged culture conditions (Hohmann, 2002; Diano *et al.,* 2006). Medium composition impacts citric acid production as well as the fermentation process. In industry, citric acid is produced by submerged fermentation (SmF); however, this type of culture depends, to a large extent, on the medium composition. On the other hand, the production of citric acid by solid-state fermentation (SSF) is more robust in nutritional terms, favoring the growth of *Aspergillus spp.* and allowing the development of more productive processes (Max *et al.,* 2010; Swain *et al.*, 2012). Some of the major advantages of SSF over SmF are its higher production yield of enzymes such as invertases, pectinases, and tannases (Viniegra-González *et al*., 2003; Soccol *et al*., 2006; Swain *et al*., 2012); SSF requires fewer pretreatment steps than SmF (Show *et al*., 2015); and citric acid production by SSF is less sensitive to the presence of trace elements than SmF (Berovic & Legisa, 2007). However, up to now, SmF has shown a higher citric acid production yield than SSF (Rodrigues *et al*., 2013). Many studies have tried to increase the yield of citric acid in SSF conditions; however, none of them has reached citric acid production yields above 0.55 Cmol/Cmol carbon source; in average, citric acid yield in SSF is 0.30 Cmol/Cmol carbon source (Gutiérrez-Rojas *et al.,* 1995; Pintado *et al*., 1998; Vandenberghe *et al*., 2000; Kuforiji *et al*., 2010). Another attractive advantage of citric acid production in SSF is the use of economical and widely available agro-industrial waste as substrate, which can be used for making processes environmentally friendly. The disadvantage is that the heterogeneity and complexity of agro-industrial waste (pineapple waste, sweet potato, sugar cane bagasse, corn grains, cassava bagasse, coffee husk, kiwifruit peel, banana peel) lead to operational problems related to the chemical composition of the culture medium and analytical problems for analysis of substrate, biomass, and byproducts (Kumar *et al.,* 2022). Therefore, an important alternative is the use of inert supports such as polyurethane foam (PUF), since it has a high water-holding capacity (up to 60-fold of its weight) because the liquid is dispersed and retained in thin layers formed by capillarity within the polymer. This allows the recovery of concentrated products since there is no need to add extraction agents as usually happens with SSF with agro-industrial materials such as sugar cane bagasse (Yadegary *et al.,* 2013) and wheat bran (Hussain, 2019), among others (Reena *et al.,* 2022). To maintain this high water-holding capacity, PUF should not be milled, since milling breaks the structure of the polymer. One of the major advantages of using PUF as the support, over SmF, is the increase in the area-to-volume ratio (up to 300 cm^{-1}) with respect to SmF (1 cm^{-1}); this property facilitates dispersion of the culture, increasing the oxygen transfer in it (Núñez-Reyes *et al.,* 2022). Here, the use of an inert support such as PUF impregnated with a defined medium in SSF studies allows the experimental design of accurate culture conditions to produce citric acid. This study aimed to determine the effect of the culture medium composition on the production of citric acid and polyols with *Aspergillus*

brasiliensis supported on PUF, taking the advantages of SSF by using a well-defined culture medium. To evaluate the effect of the components of the culture medium on citric acid and polyol production, a series of fractional and complete factorial designs were used.

2. MATERIALS AND METHODS

2.1. Microorganisms

The strain *Aspergillus brasiliensis* ATCC 9642, previously known as *A. niger* ATCC 9642 (Volke-Sepulveda *et al*., 2016), was used. It was maintained in cryotubes (TSC Technical Service Consultants cat. no. TS/73-YM 25) with pearls containing a hypertonic cryopreservative solution at −20 °C and it was also lyophilized.

2.2. Preparation of conidial inoculum

Pearls from one cryotube were inoculated on slanted tubes with YPDA medium containing (in g/L): glucose, 20; casein peptone, 5; yeast extract, 5; and 10 agar. The culture tubes were incubated at 30 °C for 7 days; subsequently, sterile 0.05% (v/v) Tween 80 was used for spore recovery. The spore suspension was used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of YPDA medium. The flasks were incubated at 30 °C for 7 days; the produced spores were used as inoculum. The inoculum size was 2.7×10^7 spores per gram of inert support.

2.3. Support and experimental unit

PUF with a density of 20 kg/m^3 was used as the inert support. The PUF was cut into cylinders (diameter 3.5 cm and height 1 cm). As a pretreatment, the PUF was: (i) washed with deionized water, (ii) washed with a solution of HCl 10% (v/v) for 10 min and washed with deionized water, (iii) washed with a solution of NaOH 4% (w/v) for 10 min and washed with deionized water. Finally, it was dried at 60 °C for 24 h (Tomasini *et al.*, 1997). Small cylindric amber bottles (35 mL) were used as the experimental units.

2.4. Culture media

The composition of the cultivation medium (macronutrients and micronutrients) is presented in the statistical analysis section (Table 1). The culture media contained glucose, $NaNO_3$, K_2HPO_4, and yeast extract as macronutrients, and $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, and $NaMoO_4.2H_2O$ as trace elements solubilized in deionized water.

2.5. Fermentation

All the experiments were carried out at 30 ± 1 °C for 120 h using 35 mL cylindric amber bottles containing one PUF cylinder (PUFc). The amber bottles with the PUFc (0.2 ± 0.02 g) and the culture medium were separately sterilized at 93.1 °C for 15 min. After cooling at room temperature, the culture medium was inoculated with the spore suspension and

added to the amber bottle containing the PUFc at a ratio of 15 mL of inoculated culture medium per gram of PUF. Before sterilization, the pH of the culture medium was adjusted to 3.5 with concentrated H_2SO_4.

2.6. Analytical methods

2.6.1. Sampling and biomass estimation

Twenty milliliters of deionized water were added to each experimental unit. Then PUFc was squeezed with a syringe plunger (diameter 1 cm) to obtain the liquid media. Afterwards, by decantation, we obtained the extracellular extract. The wet PUFc was vacuum filtered (Whatman No 1441-070) and washed with 60 mL of deionized water. Finally, it was dried at 60 °C until constant weight and biomass was gravimetrically determined.

2.6.2. Quantification of glucose and metabolites

Glucose, citric acid, erythritol, and glycerol in the extracellular extract were analyzed using a high-performance liquid chromatography (HPLC) system. An isocratic method was used. An Aminex HPX-87H ion-exclusion column (BioRad, Hercules, CA, USA) was eluted at 60 °C with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Metabolites were detected with a refractive index (IR) detector (Perkin Elmer LC-30).

2.7. Yield determination

The biomass and metabolites yields were calculated on a Cmol basis (Eq. 1).

$$Yield = \left(\frac{metabolite\ (Cmol)}{consumed\ glucose\ (Cmol)}\right) \quad \text{(Eq.1)}$$

2.8. Statistical analysis

To determine the effect of the factors affecting the citric acid and polyol yields, two experimental designs were performed. All treatments were performed in quintuplicate. Experimental data were assessed by the analysis of variance (ANOVA) using STATGRAPHICS Centurion XVI statistical software. The significance level was set at $p < 0.05$, indicating that the variables were significantly different from zero at the 95% confidence level.

2.9. Experimental design

The experimental designs were evaluated by a fractional factorial design (FFD) followed by a factorial design (FD). The composition of the culture media is presented in Table 1. The influence of the composition of the culture medium was evaluated in the first stage by a FFD with six independent variables: glucose, $NaNO_3$, yeast extract, K_2HPO_4, $MgSO_4$, and mineral solution. In the second stage a FD was used to determine the influence of a mineral solution containing glucose, K_2HPO_4, and $MgSO_4$. Each variable was studied at two different levels (−1, +1). The $NaNO_3$ concentration was not modified in the second FD.

Table 1. Experimental designs to determine the effect of the composition of the culture medium (first stage) and determine the influence of a mineral solution containing glucose, K_2HPO_4, and $MgSO_4$ (second stage) on citric acid production by *A. brasiliensis* by solid-state fermentation.

Factors	First stage			Second stage		
	Code	Low (-1)	High (1)	Code	Low (-1)	High (1)
Glucose	A	150	250		125	150
$NaNO_3$	B	16.5	24.75		16.5	16.5
Yeast extract	F	0.05	0.08		0.1	0.15
$CuSO_4.5H_2O$	E	1.50E-02	2.25E-02	A	1.50E-02	2.25E-02
$ZnSO_4.7H_2O$		1.66E-02	2.49E-02		1.66E-02	2.49E-02
$MnSO_4.H_2O$		8.00E-03	1.20E-02		8.00E-03	1.20E-02
$NaMoO_4.2H_2O$		1.00E-02	1.50E-02		1.00E-02	1.50E-02
K_2HPO_4	C	5.5	8.25	B	4.54	5.95
$MgSO_4.7H_2O$	D	0.28	0.43	C	0.26	0.38

The concentration of macronutrients and micronutrients (factors) was in g/L. Code A to F mean the factors, respectively. Low and high mean the level of the factors.

3. RESULTS

3.1. Composition of the culture medium

The influence of the culture medium composition on citric acid and polyols production during the growth of *Aspergillus brasiliensis* on PUFc was evaluated, an FFD was used for this purpose. In the first stage (Table 1), two culture media with different concentrations of glucose (factor A), $NaNO_3$ (factor B), K_2HPO_4 (factor C), $MgSO_4$ (factor D), mineral solution (MS) based on $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, and $NaMoO_4.2H_2O$ (factor E), and yeast extract (factor F) were assayed.

The Fig. 1 shows the magnitude of the estimated effects obtained in the FFD, which can be attributed to the change in the concentration of some nutrients in the culture. According to this, only glucose (factor A) and yeast extract (factor F) have a significant effect ($p < 0.05$) on the citric acid yield and glucose consumption; however, only yeast extract has a positive influence on the citric acid yield. On the other hand, in the evaluated interval, factors B to E do not have a significant effect on metabolite production.

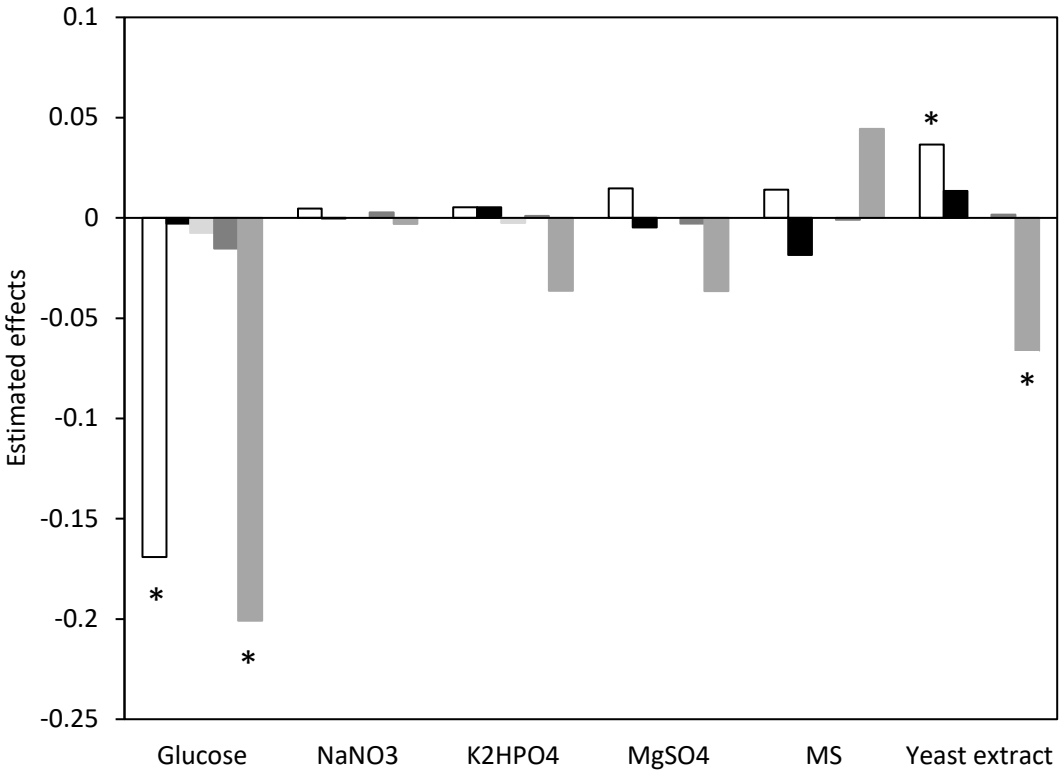

Fig. 1. Estimated effects obtained from the fractional factorial design, on citric acid (□), biomass (■), erythritol (▫), glycerol (▪) yields, and glucose consumption (▪) according to the experimental result. MS: mineral solution. Asterisks (*) indicate significant statistical effect (95% confidence level). The effect of the factor glucose on glucose consumption was divided by one hundred.

Based on these results, we can see that the citric acid yield is higher in the treatments with a low glucose concentration (150 g/L) and high concentration of yeast extract (0.08 g/L); reaching a maximum value of 0.54 (Cmol/Cmol glucose). Additionally, glucose consumption is higher, around 90%, in the treatments with a low glucose concentration (150 g/L) and low concentration of yeast extract (0.05 g/L) except for the treatment 15. Under these conditions, the highest citric acid yield is 0.47 (Cmol/Cmol glucose). The treatments with a high glucose concentration (250 g/L) allow a maximum citric acid production yield of 0.15 Cmol/Cmol glucose. Here, the biomass yield is similar (less than 0.35 Cmol/Cmol glucose) for all treatments, except for treatment 15 (0.41 Cmol/Cmol glucose) with a glucose consumption of only 37%. Under the conditions studied, polyols represent around 10% of the consumed carbon.

Table 2. Carbon balance in Cmol per Cmol glucose obtained in fractional factorial design 2^{6-2} and variables responses obtained.

Treatment	Factor						Variables response				
	A	B	C	D	E	F	Citric acid (Cmol/Cmol)	Biomass (Cmol/Cmol)	Erythritol (Cmol/Cmol)	Glycerol (Cmol/Cmol)	Glucose consumption (%)
1	-1	-1	-1	-1	-1	-1	0.13 ± 0.11	0.23 ± 0.02	0.02 ± 0.01	0.07 ± 0.02	95.15 ± 5.84
2	1	-1	-1	-1	1	-1	0.15 ± 0.08	0.28 ± 0.08	0.03 ± 0.01	0.04 ± 0.04	57.20 ± 8.19
3	-1	1	-1	-1	1	1	0.40 ± 0.05	0.28 ± 0.02	0.02 ± 0.00	0.06 ± 0.01	95.54 ± 0.61
4	1	1	-1	-1	-1	1	0.10 ± 0.03	0.35 ± 0.04	0.01 ± 0.00	0.05 ± 0.00	36.83 ± 0.64
5	-1	-1	1	-1	1	1	0.50 ± 0.04	0.25 ± 0.04	0.03 ± 0.00	0.06 ± 0.01	87.87 ± 7.13
6	1	-1	1	-1	-1	1	0.09 ± 0.01	0.32 ± 0.11	0.02 ± 0.01	0.05 ± 0.04	30.97 ± 8.52
7	-1	1	1	-1	-1	-1	0.41 ± 0.03	0.29 ± 0.01	0.03 ± 0.00	0.07 ± 0.00	97.91 ± 1.62
8	1	1	1	-1	1	-1	0.06 ± 0.02	0.25 ± 0.06	0.01 ± 0.00	0.04 ± 0.00	49.41 ± 4.74
9	-1	-1	-1	1	-1	1	0.54 ± 0.00	0.27 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	90.49 ± 6.89
10	1	-1	-1	1	1	1	0.09 ± 0.04	0.28 ± 0.04	0.01 ± 0.01	0.04 ± 0.01	31.6 ± 6.55
11	-1	1	-1	1	1	-1	0.47 ± 0.02	0.26 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	87.8 ± 10.10
12	1	1	-1	1	-1	-1	0.04 ± 0.01	0.22 ± 0.05	0.01 ± 0.01	0.02 ± 0.01	56.24 ± 1.07
13	-1	-1	1	1	1	-1	0.37 ± 0.07	0.25 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	89.93 ± 5.48
14	1	-1	1	1	-1	-1	0.04 ± 0.02	0.29 ± 0.05	0.01 ± 0.00	0.03 ± 0.01	40.95 ± 5.69
15	-1	1	1	1	-1	1	0.49 ± 0.02	0.41 ± 0.08	0.04 ± 0.01	0.1 ± 0.00	37.67 ± 8.13
16	1	1	1	1	1	1	0.04 ± 0.01	0.18 ± 0.02	0.00 ± 0.00	0.02 ± 0.01	57.83 ± 4.57

Factor A: glucose; B: $NaNO_3$; C: K_2HPO_4; D: $MgSO_4.7H_2O$; E: $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $NaMoO_4.2H_2O$; F: yeast extract. Low (-1) and high (1) mean the level of the factors. *Average: The standard deviation after the average metabolite production is indicated.

The results obtained from the first stage of the FFD show that the best conditions for citric acid production (0.54 Cmol/Cmol glucose) are: (i) a low initial glucose concentration and

(ii) a high yeast extract concentration. To increase glucose consumption without a negative effect on the citric acid production yield, glucose was fixed at the highest concentration (150 g/L) and the yeast extract content was increased (from 0.08 to 0.15 g/L). The fact that the other nutrients do not have a significant effect ($p > 0.05$) on the citric acid production yield might be due to the extremely strong effect of glucose. Therefore, we evaluated the effect of a mineral solution containing glucose, K_2HPO_4, and $MgSO_4$ in an FD.

3.2. Influence of a mineral solution containing glucose, K_2HPO_4, and $MgSO_4$

The influence of a mineral solution containing glucose, K_2HPO_4, and $MgSO_4$ on metabolite production during the growth of *A. brasiliensis* on PUFc was evaluated, an FD being used for this purpose. In the second stage (Table 1), two culture media with different concentrations of a Mineral Solution Containing Glucose (MSCG) based on $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $NaMoO_4.2H_2O$, $NaNO_3$, yeast extract, and glucose (factor A), K_2HPO_4 (factor B), and $MgSO_4$ (factor C) were assayed.

According to Fig. 2, all the evaluated factors have a significant effect ($p < 0.05$) on the citric acid yield; however, the MSCG (factor A) and the interaction $K_2HPO_4 \times MgSO_4$ have a positive influence. Additionally, the biomass production yield is positively affected by the MSCG and the interaction $K_2HPO_4 \times MgSO_4$; however, K_2HPO_4 (factor B) alone has a negative effect. For erythritol and glycerol production yields, the MSCG and the interactions MSCG $\times$ K_2HPO_4 have a positive effect; however, only K_2HPO_4 alone has a negative effect. This made it clear that MSCG is the factor that favors the production of polyols. Moreover, the MSCG, $MgSO_4$, and the interactions MSCG $\times$ $MgSO_4$ and MSCG $\times$ K_2HPO_4 affect glucose consumption; however, the increase of $MgSO_4$ favors the consumption of glucose whilst the increase of MSCG led to a decrease in the glucose consumption.

According to this stage, an MSCG with a high concentration of glucose in the medium (150 g/L) has a positive effect on the citric acid production yield; moreover, the citric acid yield is higher in the treatments with low concentrations of K_2HPO_4 (4.54 g/L) and $MgSO_4$ (0.26 g/L); obtaining a maximum value of 0.62 (Cmol/Cmol glucose). The biomass yield is lower than the obtained in the previous stage; reaching a maximum value of 0.15 (Cmol/Cmol glucose), these values are obtained with a low concentration of K_2HPO_4 and $MgSO_4$.

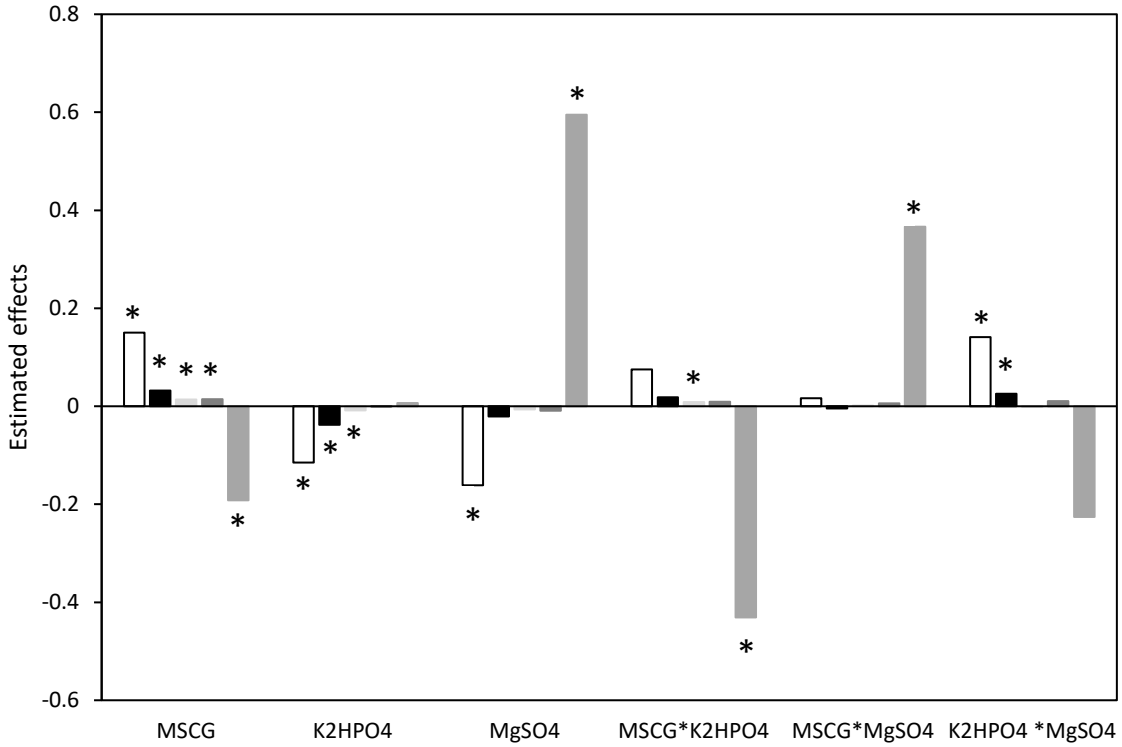

Fig. 2. Estimated effects obtained from the factorial design, on citric acid (□), biomass (■), erythritol (▫), glycerol (▪) yields, and glucose consumption (▪) according to the experimental result. MSCG: mineral solution containing glucose. Asterisks (*) indicate significant statistical effect (95% confidence level).

In this stage, the erythritol and glycerol represent up to 10% of the consumed carbon source. However, the polyol yields are higher with high MSCG than those obtained with low MSCG. Finally, a high concentration of glucose in the medium (150 g/L) has a negative effect on glucose consumption (high MSCG); however, the glucose consumption is less than 80%.

A further increase in the concentration of $MgSO_4$ reduces the citric acid production yield, from 0.62 to 0.17 Cmol/Cmol glucose. The results obtained from the second stage of the FD show that the best conditions for citric acid production (0.62 Cmol/Cmol glucose) are: (i) a high initial MSCG concentration, (ii) a low initial a K_2HPO_4 concentration, and (iii) a low initial $MgSO_4.7H_2O$ concentration.

Table 3. Carbon balance in Cmol per Cmol glucose obtained in factorial design 2^3 and variables responses obtained.

Treatment	Factor			Variables response				
	A	B	C	Citric acid* (Cmol/Cmol)	Biomass* (Cmol/Cmol)	Erythritol* (Cmol/Cmol)	Glycerol* (Cmol/Cmol)	Glucose consumption* (%)
1	-1	-1	-1	0.54 ± 0.16	0.12 ± 0.04	0.03 ± 0.01	0.08 ± 0.01	74.16 ± 5.83
2	1	-1	-1	0.62 ± 0.13	0.15 ± 0.02	0.04 ± 0.01	0.07 ± 0.00	56.46 ± 5.89
3	-1	1	-1	0.22 ± 0.04	0.05 ± 0.02	0.02 ± 0.00	0.04 ± 0.01	81.65 ± 1.59
4	1	1	-1	0.42 ± 0.04	0.09 ± 0.04	0.03 ± 0.00	0.06 ± 0.00	53.62 ± 4.06
5	-1	-1	1	0.24 ± 0.04	0.09 ± 0.02	0.02 ± 0.01	0.04 ± 0.01	79.57 ± 0.27
6	1	-1	1	0.31 ± 0.05	0.08 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	67.48 ± 3.44
7	-1	1	1	0.17 ± 0.09	0.04 ± 0.03	0.00 ± 0.00	0.03 ± 0.01	80.83 ± 2.71
8	1	1	1	0.43 ± 0.18	0.10 ± 0.02	0.03 ± 0.01	0.07 ± 0.03	61.83 ± 6.03

Factor A: glucose, $NaNO_3$, yeast extract, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, $NaMoO_4.2H_2O$; B: K_2HPO_4; C: $MgSO_4.7H_2O$. Low (-1) and high (1) mean the level of the factors. *Average: The standard deviation after the average metabolite production is indicated.

4. DISCUSSION

Studies over several decades have shown that carbon, nitrogen, phosphorus, magnesium, trace elements, and alcohols affect the organic acid and polyol yield directly by *A. niger* (Max *et al*., 2010; Show *et al*., 2015; Behera *et al*., 2021). Particularly, the type and concentration of the carbon source are probably two of the most important parameters for the successful production of citric acid; some authors have reported accumulation of 30–40 g/L of citric acid, even if conditions for all other compounds of the culture medium are far from optimal (Alekseev *et al*., 2015). In contrast, when the concentration of carbon source is less than 50 g/L, citric acid is not formed, even if the other parameters correspond to the optimal value (Kubicek & Röhr, 1986; Xu *et al*., 1989).

The effect produced due to the concentration and type of carbon source is not restricted to glycolytic flow activity, other processes are triggered. Torres *et al*. (1996) reported two types of glucose transporters in *A. niger*, high-affinity glucose transporters when grown

at a low glucose concentration (1% w/v) and low-affinity glucose transporters (permeases) when grown at a high glucose concentration (15% w/v). A high concentration of the carbon source in the medium induces the synthesis of permeases in *A. niger* which in turn leads to an increase in carbon consumption (Torres *et al*., 1996). A rapid consumption of carbon causes a high flux in the glycolytic pathway, which is related to the high production of citric acid. Additionally, the transcriptomic analysis of *A. niger* during the fermentation process confirms that permeases maintained high transcript levels, while the high-affinity glucose transporters did not (Yin *et al*., 2017). This physiological state of *A. niger* can explain the limited citric acid yield and the low glucose consumption in the medium with *A. brasiliensis*. Therefore, the glucose transporters are the limiting point for citric acid production (Xue *et al*., 2021). Thus, the impact of the type and concentration of the carbon source on the accumulation of citric acid depends more on the influence of the metabolic activity of *Aspergillus* spp. (Alekseev *et al*., 2015).

Xu *et al*. (1989) demonstrated that in a culture with glucose as carbon source, the maximal yield of citrate is reached at a glucose concentration of 7.5% (w/v); above that, the citric acid production yield drastically decreases. This behavior coincides with that observed in the present work and demonstrates that the concentration of glucose has an important effect on the production of citric acid by *Aspergillus brasiliensis*. Additionally, increasing the glucose concentration (150 to 250 g/L) has a negative effect on glucose consumption; de Oliveira *et al.* (2022) reported similar behavior: at 120 h of fermentation there was around 50% of total sugars in SSF conditions. Several studies have shown the presence of residual sugars after 288 h of incubation under SSF conditions (Roukas & Kotzekidou, 2020); this probably means that the heterogeneous conditions in the medium contribute to lower glucose consumption. Kuforiji *et al.* (2010) and Pintado *et al.* (1998) reported maximal citric acid production yields of 0.45 and 0.52 Cmol/Cmol carbon source, respectively. In this study, the treatments with a high glucose concentration (250 g/L) allows a maximum citric acid production yield of 0.15 Cmol/Cmol glucose. Previous work showed that an increase in glucose concentration in the medium (300 to 400 g/L) decreases citric acid production under SSF conditions (Gutiérrez-Rojas *et al.,* 1995). A low concentration of glucose in the medium (150 g/L) has a positive effect on the citric acid production yield; the apparent increase in citrate biosynthesis may be due to rapidly absorbed glucose in the medium caused by an increase in glycolytic flow activity (Alekseev *et al*., 2015). However, the concentration of citrate affects regulatory enzymes in the Embden-Meyerhof-Parnas (EMP) pathway, such as phosphofructokinase which is inhibited by citrate at physiological concentrations of 1 to 5 mM (Habison *et al*., 1983). An increase in the carbon source concentration is accompanied with an increase in the concentration of fructose 2-6- biphosphate, which is an activator of phosphofructokinase (Kubicek-Pranz *et al*., 1990). Usually, citric acid production is carried out at high initial concentration of the carbon source which involves an osmotic shock effect in *Aspergillus* spp.; to counter this effect, it produces erythritol and glycerol acting as osmoregulatory metabolites (Diano *et al*., 2006).

Average biomass yield from *A. niger* under SSF conditions has been reported with values close to 0.31 Cmol/Cmol carbon source (Leangon *et al*., 1999). In this work, considerable biomass production yields are obtained when $NaNO_3$ is used as a nitrogen source; assays without nitrogen limitation (C/N = 24.5) produce a higher biomass yield (Gutiérrez-

Rojas *et al.,* 1995). Additionally, the use of $NaNO_3$ as a nitrogen source involves the consumption of two moles of NADPH to reduce one mole of nitrate. This cofactor is produced mainly in the pentose phosphate (PP) pathway. Once glucose-6-phosphate enters the PP pathway, the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reduce two NADP molecules. Poulsen *et al*. (2005) showed evidence of the activity of three enzymes belonging to the PP pathway when nitrate is used as a nitrogen source in the medium: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transketolase. In consequence, the accumulation of erythritol in this work, might be the result of using nitrate as a nitrogen source, as the precursors for the erythritol syntheses, are intermediates in the PP pathway. Under the conditions studied, polyols represented around 10% of the carbon consumed, the ratio between erythritol and glycerol depending on the carbon flux in the EMP and PP pathway (Diano *et al*., 2006). Additionally, erythritol and glycerol yields of 0.01 and 0.08 (Cmol/Cmol carbon source) respectively were obtained during similar cultivation conditions, using 100 g/L of glucose (Diano *et al*., 2006). Similar values were obtained in this study.

On the other hand, citric acid yield is higher in the treatments with low concentrations of K_2HPO_4 (4.54 g/L) and $MgSO_4$ (0.26 g/L); a similar observation was found by Mostafa & Alamri (2012) and Ozdal & Kurbanoglu (2019). They reported that phosphorus limitation can have a positive effect on the citric acid yield, while a high concentration of phosphorus leads to a lower CO_2 production, which in turn increases the production of sugar acids as the carbon is deviated through these reactions. An excess of phosphate increases the metabolic activity, and it is observed with an increase in CO_2 production, which in turn is related to growth rate (Zhang & Roehr, 2002; Mostafa & Alamri, 2012). Several authors have described similar observations of the effect of K_2HPO_4 and $MgSO_4$ on citric acid and polyols, considering K_2HPO_4 to be a growth-enhancing and buffering agent which maintains the desired values of pH; therefore, a reasonable amount should be used to keep the pH within the desired ranges (Lotfy *et al*., 2007; Ali *et al*., 2012). $MgSO_4$ is considered essential, and it affects the rate of sugar consumption by the action of the variety of enzymes required for both fungal growth and the production of citric acid (Mostafa & Alamri, 2012). Additionally, phosphorus is metabolized as phosphate, and the limitation of this ion when nitrogen is not limited leads to the formation of citric acid (Kubicek & Röhr, 1977). The phosphate is used for the generation of energy, usually in the form of ATP, this molecule works as a cofactor for enzymes in EMP and Krebs cycle pathways; this limitation of ATP may cause growth inhibition, diverting carbon to the formation of citric acid.

A high concentration of magnesium ions inhibits citrate synthase which results in a decrease in the yield of citric acid (Ikram-Ul *et al*., 2004). It has also been reported that several hexokinase enzymes such as glucokinase are inhibited by citrate because of chelation with Mg^{2+} (Papagianni, 2007). The effect of magnesium with sulfate ions leads to a decrease in glucose consumption by the action of the enzymes required for fungal growth and the production of citric acid (Mostafa & Alamri, 2012). A wide range of citric acid yields by *A. niger* using glucose as a carbon source have been reported (Adeoye *et al*., 2015). The citric acid yield achieved in the present study with *A. brasiliensis* (0.62 Cmol/Cmol glucose) is greater than those previously reported under SSF (Papagianni &

Mattey, 2004; Upton *et al.,* 2017; Steiger *et al.,* 2019; Perwitasari *et al.,* 2021). The culture of *A. brasiliensis* on PUFc appears most suitable for citric acid and polyol production. A similar result reported by Lee *et al*. (1989) used a bubble column with PUF cubes (0.2 cm); however, the yield is lower (0.44 Cmol/Cmol carbon source) than the one obtained in this study. Considering this fact, control of the culture medium is a critical factor.

Trace elements are reported to have a negative effect on citric acid and polyol accumulation since they act as activators of certain enzymes. For this reason, in SmF they are added in small concentrations or even not added at all; therefore, there is an elevated production of citric acid only if there is a rigorous control of trace element availability (Bizukojc & Ledakowicz, 2004; Soccol *et al*., 2006). In the first stage, we did not observe any negative effects caused by the mineral concentrations of copper, zinc, manganese, molybdenum, and magnesium. The second stage of the FD did not clearly show the possible effect of micronutrient concentrations on the citric acid and polyol yields. Several authors have reported that the yields obtained under SSF conditions are higher than those obtained under SmF (Show *et al*., 2015). Moreover, some reports indicate that the presence of trace elements does not affect the production of citric acid in a harmful way as it does under SmF conditions. Therefore, a substrate treatment is not required for the control of trace elements (Soccol *et al*., 2006; Dhillon *et al.,* 2011; Börekçi *et al.,* 2021). Additionally, this study showed that yeast extract increases the citric acid production yield and decreases glucose consumption.

The use of PUFc gives the advantage of being able to be squeeze it since it is a porous elastic material, facilitating the extraction of citric acid, polyols, and biomass. López-Pérez & Viniegra-González (2015) reported a system with 1 cm PUF cubes; however, they mentioned that these are difficult to make and leak the broth when they are piled up because gravity overcomes the capillarity forces holding liquid to the polymer. A similar system proposed by Pintado *et al.* (1998), with cylindrical PUF particles (with a diameter and height of 0.6 cm), reported a similar citric acid yield (0.52 Cmol/Cmol carbon source). It seems that PUF geometry is an important factor to consider for producing metabolites such as citric acid and polyols.

The use of a statistical approach based on FD allows a high citric acid production yield to be obtained, that represents up to 62% of the carbon consumed by *A. brasiliensis* from a defined culture medium on PUF and demonstrates some differences in the behavior of metabolism of polyols among the environmental conditions studied. This is due to the complex regulation of citric acid and polyols as they can fulfill different functions. Citric acid and polyol production was positively affected by an initial glucose concentration lower than 250 g/L; in contrast, glucose consumption was negatively affected. Moreover, this study confirms that K_2HPO_4 and $MgSO_4$ negatively affect citric acid production at concentrations greater than 5.95 and 0.38 g/L, respectively; however, only erythritol was negatively affected by K_2HPO_4. Additionally, enrichment of the culture medium with yeast extract enhances the production of citric acid. This study demonstrates that within the studied conditions, trace elements (Cu^{2+}, Zn^{2+}, Mn^{2+}, and Mo^{2+}) do not influence the production of citric acid, polyols, or biomass. It should be mentioned here that the influence of trace elements could not be determined, because it was raised as a single factor and the individual effect of each nutrient could not be visualized. Furthermore, the

use of non-milled PUF allowed us to carry out SSF on an inert support with a high water-holding capacity (15-fold its weight) that can be increased up to 60-fold its weight. This feature will allow an increase in the process productivity to produce high-value products.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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