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# Growth and invertase production by *Neurospora crassa* in submerged and solid-state cultures

# Crecimiento y producción de invertasa por *Neurospora crassa* en cultivos sumergido y sólido

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## ABSTRACT

Filamentous fungi are widely used in industry to produce enzymes, organic acids, and secondary metabolites. *Neurospora crassa* has gained attention due to its flexibility for easy genetic manipulation, fast growth, and non-pathogenic characteristics. This study evaluated the growth of *N. crassa* and invertase production in submerged culture (SC) and solid-state culture (SSC) using pine sawdust (SSC-PS) and polyurethane foam (SSC-PUF) as supports. Modified Vogel's medium with initial sucrose concentrations of 1.5%, 3%, and 5% was used. The specific and maximum $CO_2$ production rates were higher in SSC than SC, particularly with SSC-PS. Protein and invertase production were higher in SSC, with SSC-PS demonstrating the highest sucrose concentration as the inducer and carbon source. The water-holding capacity (WHC) of SSC-PUF was approximately 25 times higher than that of SSC-PS, facilitating a more productive process. SSC-PUF enables higher biomass growth, protein, and invertase production than SC. Moreover, using inert

supports, such as PUF, allows the correct substrates and product assessment without interferences due to the chemical composition and heterogeneity of conventional agro-industrial by-products, such as PS.

**Keywords:** pine sawdust, water-holding capacity, polyurethane foam

## RESUMEN

Los hongos filamentosos son ampliamente utilizados en la industria para producir enzimas, ácidos orgánicos y metabolitos secundarios. *Neurospora crassa* ha llamado la atención debido a su facilidad para ser manipulada genéticamente, su rápido crecimiento y sus características no patógenas. Este estudio evaluó el crecimiento de *N. crassa* y la producción de invertasa en cultivo sumergido (SC) y cultivo en estado sólido (SSC), utilizando aserrín de pino (SSC-PS) y espuma de poliuretano (SSC-PUF) como soportes. Se empleó un medio modificado a partir del medio Vogels con concentraciones iniciales de sacarosa de 1.5, 3 y 5 %. La tasa específica de producción de $CO_2$ y la tasa máxima de producción de $CO_2$ fueron mayores en SSC en comparación con SC, especialmente con SSC-PS. La producción de proteínas e invertasa fue mayor en SSC, siendo SSC-PS el soporte con la concentración de sacarosa más alta como inductor y fuente de carbono. La capacidad de retención de agua (WHC) de SSC-PUF fue aproximadamente 25 veces mayor que el de SSC-PS. SSC-PUF permite un mayor crecimiento de *N. crassa*, producción de proteínas e invertasa en comparación con SC. Además, el uso de soportes inertes como el PUF permite la correcta evaluación de sustratos y productos sin interferencias debidas a la composición química y heterogeneidad de algunos subproductos agroindustriales convencionales como el PS.

**Palabras clave:** aserrín de pino, capacidad de retención de agua, espuma de poliuretano

## 1. Introduction

Filamentous fungi are microorganisms used to produce several industrial proteins and enzymes, such as cellulases, pectinases, proteases, lipases, inulinase, and invertase among others (Sandhya *et al*., 2005; Guerrero-Urrutia *et al.,* 2021; Singh *et al*., 2021; Kumar *et al*., 2023), as well as secondary metabolites, including penicillin and cephalosporin (Fierro *et al.,* 2022). Wild and recombinant strains of *Neurospora crassa* are thought to produce metabolites and enzymes of industrial interest (Dogaris *et al.,* 2009; Roche *et al.,* 2014; Zhao *et al.,* 2018). *N. crassa* is easily genetically manipulated; it grows fast and is non-pathogenic, and for many years, it has been used to obtain relevant information (Davis and Perkins 2002). Additionally, it is well known for its excellent protein secretion capabilities (Lin *et al.,* 2018).

Previous studies with this fungus uncovered a relationship between enzymes and genes (Beadle and Tatum 1941); after that, invertase (Metzenberg, 1963; Trevithick and Metzenberg 1964) and endoxylanase (Mishra *et al*., 1984) production were evaluated by submerged culture (SC), and cellulase and glucosidase production by solid-state culture

(SSC) using wheat straw as support (Macris *et al.,* 1987). Alhomodi *et al.* (2022) demonstrated that the production of β-glucosidase and cellulase with *N. crassa* grown in SSC with canola seed is higher than that obtained in SC. Enzyme production by *N. crassa* in SSC has been widely studied. Some of the enzymes evaluated include α-amylase from coffee waste (Murthy *et al.,* 2009), cellulases and hemicellulases from wheat straw and bran (Dogaris *et al.,* 2009), proteases from soy pulp (Zheng *et al.,* 2020), and lipase from mustard meal (Arooj *et al.,* 2023). Advantages of SSC over SC include higher biomass and enzyme production and lower proteolysis (Viniegra-González *et al.,* 2003). Additionally, enzyme production in SSC is faster and higher than in SC; catabolite repression and substrate inhibition are lower in SSC than in SC (Aguilar *et al.,* 2001). Due to these advantages, different variables have been evaluated to optimise enzyme production conditions, such as water content, type of support, pH, aeration, substrate type, and substrate concentration. It is well known that substrate concentrations as high as 15% enhance enzyme production in SSC (Aguilar *et al.,* 2001). Initial glucose concentrations as high as 450 g/l were used to evaluate the growth of *Aspergillus niger* by SSC with amberlite as an inert support (Favela *et al.,* 1998). For initial glucose concentration from 60–180 g/l, hyphae branching and extracellular protein production by *Aspergillus brasiliensis* in SSC increase up to 1.5-fold and 3.3-fold, respectively (Salgado-Bautista *et al.,* 2020). Similar behavior was observed for invertase production with sucrose (Guerrero-Urrutia *et al.,* 2021). The lower holding capacity of typically agro-industrial products, such as rice (Méndez-González *et al*., 2022) and soybean (Lio and Wang 2012), and by-products, such as wheat bran (Hussain, 2019) and sugar cane bagasse (Yadegary *et al*., 2013), among others (Reena *et al*., 2022) used as substrates is a disadvantage for SSC since, in most cases, water must be added to recover the product. That is, enzyme production is higher in SSC than in SC, but extracting the product from the solid matrix used in SSC requires adding water, leading to a more diluted product. Therefore, support for SSC with high water holding capacity (WHC) will improve productivity without diluting the product during the recovery steps.

This study aimed to compare the production of biomass and invertase by *N. crassa* under SC and SSC with polyurethane foam (SSC-PUF) and pine sawdust (SSC-PS) as supports.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

Erlenmeyer flasks (250 mL) containing 50 mL of modified Vogel´s medium (MVM) with sucrose as the carbon source and inducer for invertase production (see below) and 1.5% agar were inoculated with *N. crassa* FGSC#9717 (Δmus-51::bar+; mat A his-3) and incubated at 30 °C for four days. Conidia were recovered with 50 mL of sterile 0.05% (v/v) Tween 80. Two hundred microliters of the conidia suspension were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of MVM with 1.5% sucrose and 1.5% agar. After five days at 30 °C, the conidia were harvested and used as the inoculum for the SSC and SC cultures.

## 2.2. Culture medium

SCs and SSCs were carried out using MVM. The culture medium was prepared as previously reported (Vogel, 1956) with sucrose as the sole carbon source and the addition of histidine at 20 μg per 15 mg of sucrose. Concentrated culture media were prepared to attain the indicated initial sucrose concentration; this helps to maintain the same ratio of all the culture medium components.

## 2.3. Supports for SSC

Pine sawdust (PS, particle size 2.38–1.19 mm) and Polyurethane foam (PUF, with a density of 17 Kg/$m^3$) were used as supports for SSC. The PUF was cut into cylinders (diameter 5.4 cm, height 3 cm, and 2.47 g ± 0.06 g dry weight). The supports were washed exhaustively with tap water and finally rinsed with distilled water. Afterwards, they were dried at 60 ºC and stored under dry and dark conditions.

## 2.4. SC

Erlenmeyer flasks (250 mL) with 40 mL of inoculated culture medium (1 x $10^6$ conidia/mL) were connected to the respirometric device (see below) and incubated at 30 ºC and 150 rpm until the end of culture, determined by the lack of $CO_2$ production. Each experimental condition was evaluated in triplicate.

## 2.5. SSC

Erlenmeyer flasks (250 mL) containing one cylinder of dry PUF (dPUF) were added with 9 ± 1, 17 ± 1, and 34 ± 1 mL of inoculated culture medium (2 x $10^7$ conidia/g dPUF) to have the volume of culture medium to mass of dPUF ratios of 5, 10, and 15 mL/g, respectively. Regarding pine sawdust, 20 mL of inoculated culture medium was added to Erlenmeyer flasks (250 mL) containing 8 g of dry pine sawdust (dPS; 2 x $10^7$ conidia/g dPS). Erlenmeyer flasks with inoculated culture medium were connected to the respirometric device (see below) and incubated at 30 ºC until the end of culture, determined by the lack of production of $CO_2$. Before inoculation, half of the culture medium was added to Erlenmeyer flasks containing one cylinder of PUF per flask, and the other half was placed in test tubes. They were sterilised at 120 ºC for 15 min. After sterilisation, the inoculums were added to the culture medium in the test tubes and then transferred to the Erlenmeyer flasks containing dPUF cylinders. Each experimental condition was evaluated in triplicate. All the response variables were tested by one-way ANOVA. The means comparison was done using Duncan's multiple range test ($\alpha = 0.05$). Statistical differences ($p < 0.001$) between means are indicated with different letters. Analyses were performed using the SAS software (SAS Institute Inc. Cary, USA).

## 2.6. On-line monitoring of $CO_2$ production

SCs and SSCs were aerated at flow rates from 15 to 30 mL/min, and the $CO_2$ content in the exhaust flow was analysed online. Fungal growth in both SSC and SC was indirectly

assessed through continuous monitoring of $CO_2$ production. Data were collected and used to estimate parameters such as the lag phase time, specific $CO_2$ production rate ($\mu CO_2$), maximum $CO_2$ production rate, and final $CO_2$ production (Volke-Sepulveda *et al*., 2016).

## 2.7. Biomass determination

The produced biomass was gravimetrically determined. The final culture medium obtained by SC was filtered (Whatman™ Grade 1), washed with distilled water, and dried at 60 ºC. To determine the biomass concentration in SSC-PUF, the PUF cylinder contained in each Erlenmeyer flask was squeezed with a syringe plunger (diameter 1.824 ± 0.001 cm) to obtain the extracellular fraction used for all determinations (e.g., pH, sucrose, extracellular protein, and invertase activity). Then, the exhaust PUF cylinder was transferred to the Büchner funnel with filter paper (Whatman™ Grade 1) and filtered under vacuum; 50 mL of distilled water was used to rinse the Erlenmeyer flask and the PUF cylinder. The rinsed PUF cylinder with the filter paper was dried at 60 ºC until constant weight. Biomass weight corresponds to the total weight minus the filter paper and the PUF cylinder dry weights. The biomass determination procedure from SSC-PS was like the one described for SSC-PUF. Biomass estimation was done in triplicate.

## 2.8. Total sugars and pH determination

Initial and final total sugar concentrations were determined using the phenol-sulfuric acid method (Dubois *et al.,* 1956). The pH values in the extracellular fractions were measured by potentiometry (PHS-3BW, BANTE Instruments). Sugar and pH analyses were done in triplicate.

## 2.9. Quantification of extracellular protein

The total protein concentration in the extracellular fractions was determined using the Bradford protein assay protocol (Bio-Rad, Hercules, CA). Briefly, 0.2 mL of crude extract was combined with 0.8 mL of dye reagent concentrate. After a 5 min incubation at 25 °C, absorbance was measured at 595 nm on a Spectrophotometer UV/vis (Shimadzu UV-1800). The total protein was quantified from a standard calibration curve prepared with bovine serum albumin (BSA) as the standard. Extracellular protein was measured in triplicate.

## 2.10. Enzymatic activity assay for invertase

The enzymatic assay was performed as reported by Guerrero-Urrutia *et al.* (2021). The amount of reducing sugars (RS) released was quantified as described by Miller *et al*. (1960). The invertase unit (U) is defined as the amount of enzyme required to liberate 1 µmol of RS per minute under assay conditions. Invertase activity was assayed in triplicate.

## 2.11 Critical moisture content and water-holding capacity (WHC)

The critical moisture content was obtained from a drying curve of water-saturated samples of dPUF and PS at 130 ºC. The WHC was determined as reported by Nelson *et al.* (2024).

## 3. Results

SC and SSC were compared to determine the best growth conditions for protein and invertase production by *N. crassa.* Additionally, SSC studies were carried out with a low WHC support (PS) and a high WHC support (PUF). Initial sucrose concentrations of 1.5%, 3%, and 5% were evaluated for both culture types. To maintain the same ratio of the culture medium constituents of the MVM, all mineral salts, biotin, and histidine were increased proportionally to the sucrose concentration.

### 3.1. Kinetic studies

For the same initial sucrose concentration (1.5%, 3%, and 5%), cultures grew faster in SSC-PS than in SSC-PUF, and these were faster than those in SC (Table 1).

The lag time increases with the increase in the initial sucrose concentration, with the shortest lag phase observed in SSC-PS cultures and the longest in SC cultures. At the three sucrose concentrations evaluated, the total $CO_2$ production was similar in SC and SSC-PUF, while it was slightly higher in SSC-PS (Table 1).

The total $CO_2$ production was higher in SSC-PS compared to SSC-PUF and SC. Additionally, the specific $CO_2$ production rate strongly depended on the culture type and slightly decreased as the initial sucrose concentration increased.

Maximum $CO_2$ production rates were higher with SSC-PS compared to SSC-PUF, which was also higher than with SC (Table 1). Sucrose consumption was above 98% in SC and SSC-PUF; however, in SSC-PS, it ranged from 85% to 97% for an initial sucrose concentration of 15–50 g/L.

### 3.2. Impact of the culture conditions on biomass, extracellular protein, and invertase activity

There were no statistically significant differences in $CO_2$ production between SC and SSC-PUF. However, biomass production in SSC-PS was approximately six times higher than SC and three times higher than SSC-PUF (Fig. 1a). Furthermore, extracellular protein concentration in SSC-PS were 13 and 30 times higher compared to SSC-PUF and SC, respectively (Fig. 1b). Similarly, invertase production in SSC-PS was six and ten times higher than SSC-PUF and SC, respectively (Fig. 1c). Conversely, specific invertase activity in SC was two and three times higher than SSC-PUF and SSC-PS, respectively (Fig. 1d). These findings underscore the substantial impact of the culture conditions on growth, protein production, and invertase activity, highlighting the potential of SSC-PS as a support for protein production. Initial and final pH values range between 5.7–6.5 and 5.2–7.5, respectively.

**Table 1.** Kinetic parameters obtained for *N. crassa* in submerged (SC) and in solid-state culture (SSC) using polyurethane foam (PUF) or pine sawdust (PS) as support*.

| Type of culture / Initial sucrose concentration | SC | | | SSC-PUF | | | SSC-PS | | |
|---|---|---|---|---|---|---|---|---|---|
| (%, w/v) | 1.5 | 3 | 5 | 1.5 | 3 | 5 | 1.5 | 3 | 5 |
| Culture time (h) | 50 | 65 | 70 | 40 | 50 | 60 | 25 | 30 | 35 |
| Lag time (h) | 10.03 ± 0.48$^{c}$ | 11.76 ± 0.97$^{b}$ | 14.26 ± 0.69$^{a}$ | 8.73 ± 0.04$^{d}$ | 9.35 ± 0.15$^{cd}$ | 11.28 ± 0.34$^{b}$ | 6.17 ± 0.36$^{f}$ | 7.32 ± 0.13$^{e}$ | 7.58 ± 0.01$^{e}$ |
| $CO_2$ production (mg/mL) | 5.71 ± 0.98$^{f}$ | 12.17 ± 2.39$^{d}$ | 18.02 ± 2.02$^{c}$ | 6.58 ± 0.22$^{ef}$ | 13.01 ± 0.89$^{d}$ | 22.47 ± 0.7$^{b}$ | 8.45 ± 0.07$^{e}$ | 19.05 ± 0.32$^{c}$ | 29.83 ± 1.24$^{a}$ |
| Total $CO_2$ productivity (mg/mL h) | 0.114 ± 0.00$^{f}$ | 0.187 ± 0.03$^{e}$ | 0.257 ± 0.00$^{d}$ | 0.165 ± 0.01$^{e}$ | 0.260 ± 0.02$^{d}$ | 0.375 ± 0.01$^{c}$ | 0.338 ± 0.01$^{c}$ | 0.635 ± 0.01$^{b}$ | 0.852 ± 0.04$^{a}$ |
| $CO_2$ maximum production rate (mg/mL h) | 0.29 ± 0.04$^{f}$ | 0.48 ± 0.05$^{e}$ | 0.53 ± 0.09$^{e}$ | 0.52 ± 0.03$^{e}$ | 0.81 ± 0.04$^{d}$ | 1.15 ± 0.17$^{c}$ | 1.04 ± 0.05$^{c}$ | 2.06 ± 0.18$^{b}$ | 3.30 ± 0.18$^{a}$ |
| $CO_2$ specific production rate (1/h) | 0.15 ± 0.01$^{d}$ | 0.11 ± 0.02$^{f}$ | 0.09 ± 0.01$^{g}$ | 0.18 ± 0.00$^{c}$ | 0.17 ± 0.01$^{c}$ | 0.12 ± 0.01$^{e}$ | 0.30 ± 0.00$^{a}$ | 0.18 ± 0.01$^{c}$ | 0.20 ± 0.00$^{b}$ |

* Data correspond to mean values of at least three replicates per response variable ± the corresponding standard deviation (SD). Different letters indicate significative differences between treatments ($p < 0.001$).

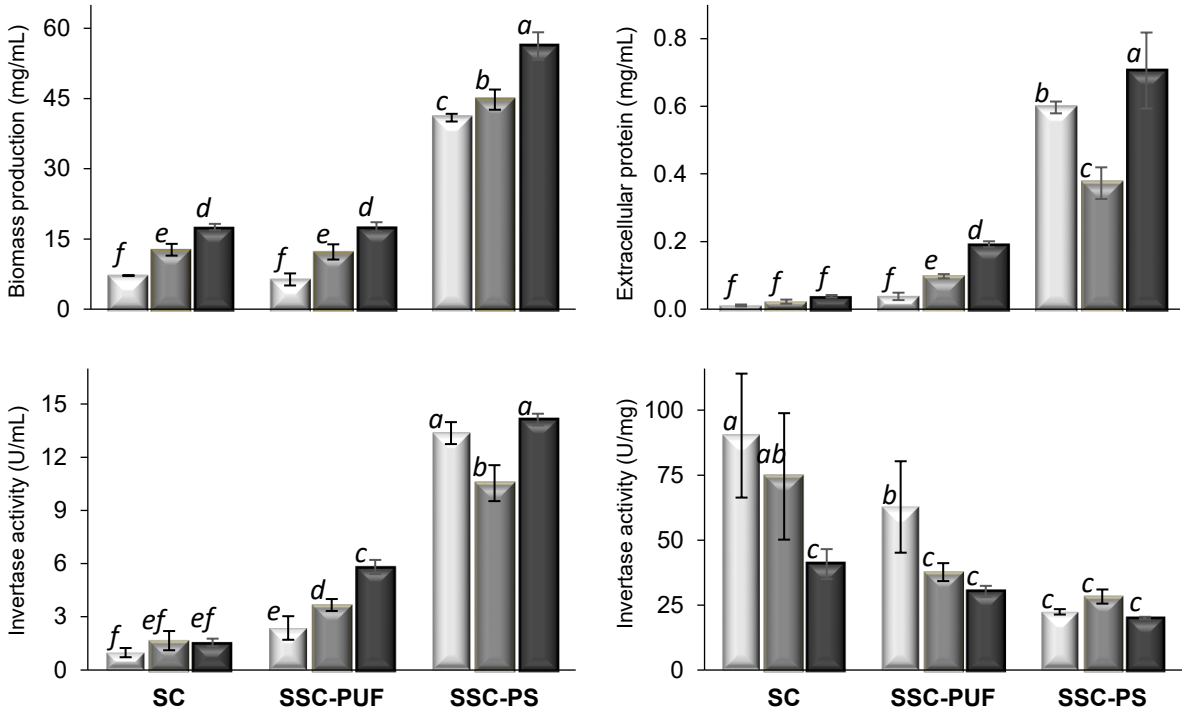


**Figure 1.** Effect of the initial sucrose concentration (1.5 [white bars], 3 [grey bars] and 5% [black bars]), the type of culture (liquid [SC] or solid [SSC]), and the support (polyurethane

foam [PUF] or pine sawdust [PS]) on biomass, extracellular protein and invertase production by *N. crassa.* Different letters indicate significative differences ($p < 0.001$).

### 3.3. Impact of VCM/MS ratio on kinetic parameters and metabolic activities in SSC-PUF

To determine the effect of volume of culture medium to mass of support (VCM/MS) on the kinetic parameters associated with $CO_2$ production, biomass, and extracellular protein and invertase production by SSC-PUF, three VCM/MS ratios (e.g., 5, 10, and 15 mL/g) were evaluated (Table 2 and Fig. 2). Although extracellular protein production slightly decreased as a function of the VCM/MS ratio, both biomass (Fig. 2a) and the extracellular protein production (Fig. 2b) were similar across the three VCM/MS levels evaluated (18.86 ± 0.23 mg/mL and 0.19 ± 0.1 mg/mL, respectively). In contrast, invertase production, and therefore, specific invertase production, decreased as the VCM/MS ratio increased (Fig. 2c, d).

These findings suggested a low VCM/MS ratio favours extracellular protein and invertase production (Fig. 2).

**Table 2.** Effect of the volume of culture medium/mass of support ratio on the kinetic parameters of *N. crassa* in solid-state culture using polyurethane foam (PUF) as support*.

| | Culture medium/PUF ratio (mL/g) | | |
|---|---|---|---|
| | **5** | **10** | **15** |
| Culture time (h) | 45 | 55 | 65 |
| Lag time (h) | 8.61 ± 0.6$^{a}$ | 9.06 ± 0.1$^{a}$ | 11.19 ± 0.3$^{b}$ |
| $CO_2$ production (mg/mL) | 20.60 ± 0.26$^{a}$ | 22.38 ± 0.47$^{b}$ | 22.52 ± 0.69$^{b}$ |
| Total $CO_2$ productivity (mg/mL h) | 0.57 ± 0.01$^{a}$ | 0.49 ± 0.00$^{b}$ | 0.42 ± 0.02$^{c}$ |
| $CO_2$ maximum production rate (mg/mL h) | 1.57 ± 0.18$^{a}$ | 1.39 ± 0.08$^{ab}$ | 0.99 ± 0.02$^{b}$ |
| $CO_2$ specific production rate (1/h) | 0.19 ± 0.01$^{a}$ | 0.16 ± 0.00$^{b}$ | 0.12 ± 0.01$^{c}$ |
| $CO_2$ production (mg/g dPUF) | 104 ± 0.01$^{a}$ | 229 ± 0.01$^{b}$ | 342 ± 0.04$^{c}$ |
| Extracellular protein (mg/g dPUF) | 1.0 ± 0.1$^{a}$ | 1.9 ± 0.1$^{b}$ | 2.8 ± 0.0$^{c}$ |
| Invertase activity (U/g dPUF) | 43.5 ± 0.06$^{a}$ | 68.9 ± 0.19$^{b}$ | 75.5 ± 0.13$^{c}$ |

* Data correspond to mean values of at least three replicates per response variable ± the corresponding standard deviation (SD). Different letters indicate significative differences between treatments ($p < 0.001$).

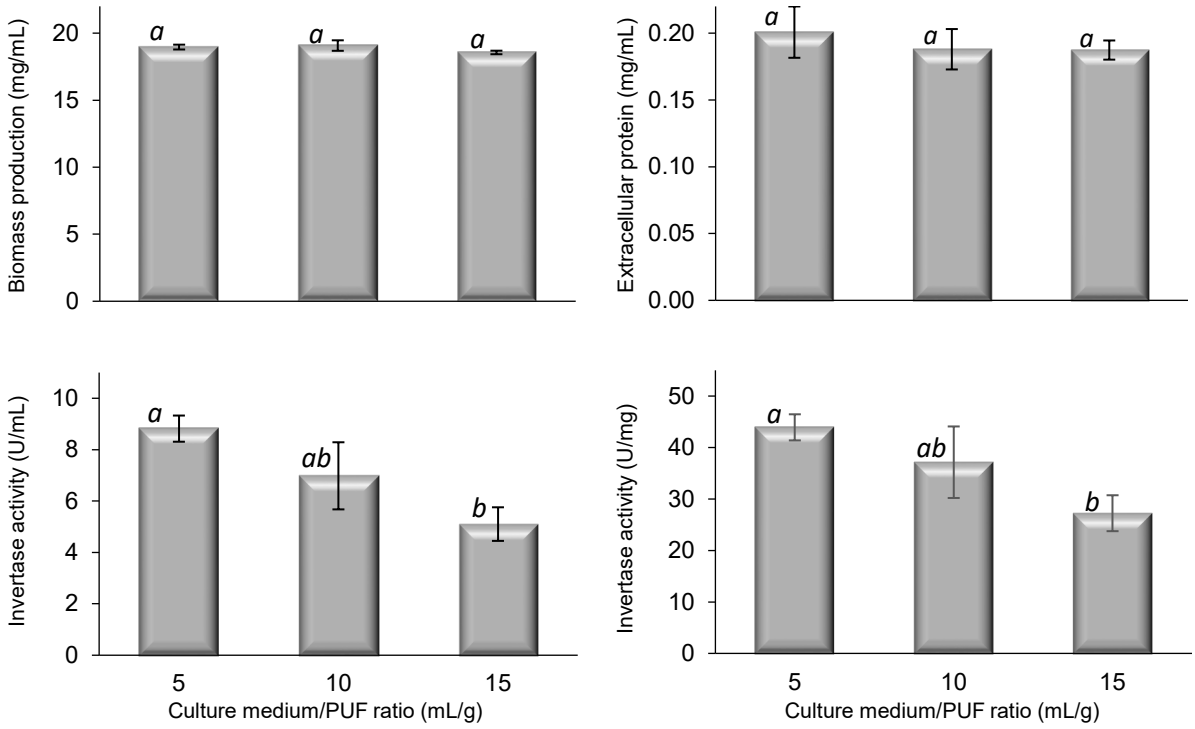


**Figure 2.** Effect of the volume of the culture medium/mass of support ratio on the biomass, extracellular protein and invertase production by *N. crassa* in solid-state culture with polyurethane foam as support (SSC-PUF). Different letters indicate significative differences ($p < 0.001$).

## 4. Discussion

### 4.1. Kinetic studies

*N. crassa* is a potentially helpful microorganism for protein and enzyme production. Genetically modified strains produce heterologous plant and human proteins and vaccines (Havlik *et al.,* 2017). The method of culture dramatically affects the production of extracellular metabolites by filamentous fungi (Iwashita, 2002). There is strong evidence that SSC reduces catabolite repression and substrate inhibition, allowing higher protein and enzyme production than SC (Aguilar *et al*., 2002). The statistical analysis of the kinetic parameters related to $CO_2$ production indicated faster growth with SSC-PS than with SC and SSC-PUF. These results agree with previous reports from studies comparing SSC and SC (Salgado-Bautista *et al.,* 2020; Guerrero-Urrutia *et al.,* 2021). Differences in $CO_2$ production between SSC-PUF and SSC-PS might be due to the fungi using a small fraction of PS as a carbon source.

### 4.2. Impact of the culture conditions on biomass, extracellular protein, and invertase activity

The statistical analysis showed that differences in the specific activity production were due to the low extracellular protein produced in SC. Our results suggest that increased substrate concentration helps produce higher levels of total secreted protein or specific proteins, given they were higher in SSC cultures with high substrate concentrations. Similar behaviour was observed for *A. brasiliensis* with perlite as an inert support (Salgado-Bautista *et al*., 2020). Differences in protein secretion in SC and SSC are well documented (Aguilar *et al.,* 2001; Oda *et al*., 2006); SSC favours protein secretion and could improve invertase secretion. The higher protein secretion might be related to the higher branching level under SSC (Salgado-Bautista *et al*., 2020). The above results show that *N. crassa* growth, protein, and invertase production are faster and higher in SSC-PS and SSC-PUF and, therefore, more productive than SC. No advantage of SC, in terms of growth rate nor concentration of products or sucrose consumption, was observed. On the other hand, SSC-PS is more productive in both (i.e., growth rate and product concentration) than SSC-PUF.

The higher biomass production in SSC-PS compared to SSC-PUF can be only explained by the partial enzymatic hydrolysis of PS. Part of the fibre (e.g., cellulose or hemicellulose) present in the SSC-PS is enzymatically hydrolysed by enzymes produced by *N. crassa* (Sun *et al.,* 2012; Wang and Arioka 2021), leading to the formation of soluble products. Therefore, the final weight of the SSC-PS used as support is lower than the initial one. The loss of the initial dry weight cannot be determined since a fraction of the initially insoluble dry weight was enzymatically hydrolysed and converted into a soluble fraction contained in the extracellular extract at the end of culture. This apparent increase in biomass production in SSC-PS is not due to the presence of additional carbon sources generated by the enzymatic hydrolysis of PS since the production of $CO_2$ was similar to SSC-PS and SSC-PUF. The carbon balance in both SSC-PS confirms these arguments. The biomass/sucrose yield expressed in C-mol basis were 4.9, 2.5, and 1.8 for SSC-PS cultures with 1.5%, 3%, and 5% of initial sucrose, respectively. The aberrancy of these values confirms that the biomass concentration was overestimated. This overestimation can be only due to the loss of dry matter associated with the PS used as support.

### 4.3. Impact of VCM/MS ratio on kinetic parameters and metabolic activities in SSC-PUF

The critical moisture content (CMC) of the SSC-PUF and the SSC-PS is similar (1.77 g and 1.75 g $H_2O$ per gram of dry matter, respectively). In contrast, the WHC of the SSC-PUF (60.12 g/g dm) is higher than that of the SSC-PS (2.48 g/g dm). That is, the WHC of the SSC-PUF is nearly 25-fold that of the SSC-PS. This difference in WHC affects the product recovery strategy. Most of the agro-industrial by-products (e.g., straw or wheat bran, corn, soybean, coffee pulp, among others) or products (e.g., grains, such as soy or rice) typically used as substrates for SSC processes have low WHC (< 3 g $H_2O$ per gram of dry matter) (Sadh *et al.,* 2018). This WHC value is close to the CMC of these products and by-products. Therefore, when WHC is close to CMC, the fermentation products must be obtained after a liquid-solid extraction process; that is, a certain amount of solvent (e.g.,

water or buffer) should be added to extract the product obtained at the end of culture. This step will dilute the product in the liquid fraction (i.e., the extract). In contrast, supports with a high WHC/CMC ratio enable recovery of the product from the solid matrix by just pressing or squeezing the wet solid material, resulting in higher product concentration in the liquid extract and higher productivity per unit of volume.

It is well known (Viniegra-González and Favela-Torres 2006) that the characteristic low content of free water in SSCs reduces substrate inhibition and catabolic repression. These reductions are mainly due to the lower substrate diffusion when low free-water content is used. Then, the VCM/MS ratio affects the kinetic parameters and metabolic activities of the culture. In this study, cultures with PUF and PS were carried out with VCM/MS of 15 and 2.5 mL/g, respectively. This might be another reason why kinetic parameters and protein and invertase production with PS are higher than those obtained with PUF.

Since PS is not an inert support and its WHC is considerably lower than the WHC of PUF, PUF was used to determine the effect of the VCM/MS (5, 10, and 15 mL/g) on the kinetic parameters associated with $CO_2$ production, and biomass, extracellular protein, and invertase production were evaluated.

The increase of the VCM/MS significantly correlated with the increase in the cultivation time and the lag time. Since the initial sucrose concentration was similar, the maximum $CO_2$ production was also similar (22.45 ± 0.5 mg $CO_2$/mL) for the three VCM/MS ratios evaluated (e.g., 5, 10, and 15 mL/g). The maximum and specific $CO_2$ production rates decreased with increasing VCM/MS. Therefore, these results corroborate the negative effect of VCM/MS on the parameters associated with $CO_2$ production that are directly related to the kinetic parameters of growth.

These results confirm that the low free water content in the culture medium enhances enzyme production. Nevertheless, the product concentration, based on the mass of inert support (which is equivalent to the operational volume of the bioreactor) instead of the volume of the culture medium, is considerably higher when the VCM/MS ratio increases. It is an important detail when the VCM/MS can be a criterium design, and it occurs only with SSC supports with a high WHC/CMC.

## 5. Conclusion

SSCs present advantages over SC in the growth and protein and invertase production by *N. crassa*. An important characteristic of supports used for SSC is the culture medium holding capacity (CMHC). The increase in the volume of the culture medium to the support mass leads to a significantly higher product formation without affecting the incubation period required to obtain the maximum product concentration. Therefore, the supports for highly productive SSCs should have a high CMHC to CMC ratio, and the volume of culture medium to support mass ratios should be as high as possible. The increase in substrate concentration enhances the amount of total secreted protein or specific proteins, given they were higher in SSCs with high substrate concentrations. To our knowledge, this is the first study evaluating $CO_2$ production and using this respirometric variable to determine kinetic parameters associated with *N. crassa* growth under SC and SSC.

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## Author contribution

C. J Mora-Pérez: experimental, writing and editing. M. C. Sánchez-Ruiz: experimental. E. Favela-Torres: conceptualization, supervision, experimental, resources, data curation and analysis, writing and editing. D. Salgado-Bautista: data analysis, discussion and writing. U. Carrasco-Navarro: writing and review.

## Conflict of interest

The authors declare no conflict of interest.

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