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Development of a consolidated bioprocess for the production of citric acid using *Aspergillus niger* as biocatalyst

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Abstract: The industrial production of citric acid, an ingredient in beverages, pharmaceuticals, and cosmetics, is based on microbial fermentation of glucose or sucrose. Given the elevated cost of these sugars, lignocellulosic biomass is emerging as a cost-effective and environmentally friendly feedstock for sustainable bioprocesses. However, fermentation of lignocellulosic materials requires that they are first broken down enzymatically. This can be achieved by the filamentous fungus *Aspergillus niger*, which has the ability to secrete hydrolytic enzymes and to produce citric acid. Here, we investigated the production of citric acid using a consolidated bioprocess, in which all conversion steps – from the solid substrate to the final product – occurred in a single process stage. The press cake derived from a perennial ryegrass (*Lolium perenne*) was used as substrate and glucose or the remaining press juice were utilized as an additional carbon source. *Aspergillus niger* produced citrate successfully only when the press cake was supplemented with press juice ($2.1 \pm 0.0\,g\ kg_{DM}^{-1}$) and especially glucose ($84.7 \pm 0.3\,g\ kg_{DM}^{-1}$). Confocal laser scanning microscopy revealed differences in fungal mycelia based on the carbon source supplemented. Overall, the results indicate the successful implementation of solid-state fermentation for the sustainable production of citric acid by *A. niger* fed on press cake. © 2025 The Author(s). *Biofuels, Bioproducts and Biorefining* published by Society of Industrial Chemistry and John Wiley & Sons Ltd.

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Supporting information may be found in the online version of this article.

Key words: perennial ryegrass; filamentous fungi; bioprocessing; solid-state fermentation; confocal laser scanning microscopy

Introduction

Lignocellulosic biomass (LCB) is the most abundant renewable resource on Earth and is attracting increasing interest for the sustainable production of chemicals, materials, and fuels.[1] Given its widespread availability, low cost, and carbon neutral footprint, LCB could overcome environmental and economic concerns related to the exploitation of fossil-based resources.[2] Global annual production of LCB has been estimated at 181.5 billion tons, with output expected to increase to achieve net-zero carbon dioxide emissions by 2050.[3] Lignocellulosic biomass includes residues from agriculture, forestry, industry, and household activities.[4] Conversion from LCB to the targeted product involves several steps, such as pretreatment, saccharification, and fermentation, which can be executed using different process layouts.[5] In separate hydrolysis and fermentation (SHF), enzymatic treatment and fermentation are carried out separately under optimal pH and temperature. However, SHF might not function at industrial scale because enzymes may be inhibited by the release of certain sugars.[6] Separate hydrolysis and fermentation also requires distinct vessels for hydrolysis and fermentation, along with additional heating and cooling steps, which greatly increase operational costs.[7] To overcome these limitations, simultaneous saccharification and fermentation has emerged as an alternative, combining enzymatic hydrolysis and fermentation into a single step. Despite its advantages, this process requires the addition of expensive hydrolytic enzyme mixtures. At 4–20 USD kg^{-1}, the cost of cellulases and hemicellulases significantly impacts overall production expenses.[8,9]

An emerging strategy is consolidated bioprocessing (CBP), whereby the same microorganism accomplishes the production of hydrolytic enzymes, biomass hydrolysis, and fermentation of the released sugars into target product(s), all within a single process step.[6,10,11] This strategy is considered a low-cost, material-and-energy-efficient configuration due to the reduced number of processing steps.[12] Filamentous fungi are particularly well suited for CBP, as they produce large amounts of hydrolytic enzymes and can achieve high yields of target secondary metabolites.[8] One of the latter is citric acid, which has a market potential projected to reach 3.2 billion USD by 2027, due to its low cost and wide range of industrial applications, including food, beverages, pharmaceuticals, and cosmetics.[13,14] An innovative biotechnological approach termed solid-state fermentation (SSF) has been proposed to satisfy the increasing demand for citric acid. In SSF, microorganisms are grown on a solid matrix (inert support or insoluble substrate) in the complete or nearly complete absence of free water.[15,16] It has several advantages, including low water and energy demand, utilization of unexploited LCB, and resistance to catabolic repression by excessive substrate.[15–17] These advantages are estimated to result in tenfold lower production costs than conventional liquid fermentation.[17]

The aim of this study was to assess whether the press cake from a perennial ryegrass (*Lolium perenne*) obtained using mechanical pretreatment could be employed as a substrate for the production of citric acid using SSF (Fig. 1). The filamentous fungus *Aspergillus niger* was selected as

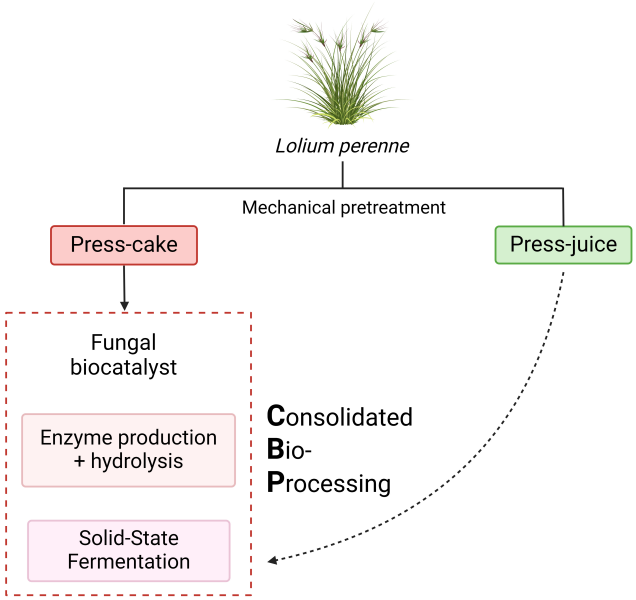

Figure 1. Overall process for citric acid production investigated in the present study. *Lolium perenne* was mechanically pretreated using a screw press to separate the press cake (solid fraction) and press juice (liquid fraction). The press cake was used as substrate in CBP for citric acid production. The fungal biocatalyst *Aspergillus niger* secreted the enzymes necessary to hydrolyze the solid substrate and then fermented it to citric acid using SSF. The remaining press juice was used as additional carbon source.

biocatalyst because of its ability to grow on different LCBs and to produce large volumes of citric acid. Initially, the grass press cake was intended as the only source of nutrients, but was then supplemented with glucose or press juice to provide additional carbon, along with nitrogen, phosphate, potassium, and methanol. To determine whether *A. niger* used the press cake as solid support or as insoluble substrate, β-glucosidase activity was used as an indicator of cellulolytic hydrolysis of the press cake. Interactions between the press cake and the fungus, along with fungal morphology, were evaluated by confocal laser scanning microscopy (CLSM) at both micro- and macroscopic level.

Materials and methods

Production of the grass press cake

The perennial ryegrass *L. perenne* was provided by the Julius-Kühn Institute (Braunschweig, Germany). The conditions used for pretreatment of the raw material can be found elsewhere.[18] Briefly, mechanical pretreatment using a screw press was carried out to separate the press cake (solid fraction) from the press juice (liquid fraction). The resulting press cake was sealed in vacuum bags and stored at −20 °C until further use.

Compositional analysis of the press cake

The compositional analysis of the press cake was performed according to the National Renewable Energy Laboratory/TP-510–42 618 protocol.[19] Dry mass content was measured in triplicates at 105 °C using a drying oven (Memmert GmbH, Schwabach, Germany)[20].

Microorganism and spore solution preparation

The filamentous fungus *Aspergillus niger* ATCC 1015 was obtained from LGC Standards (Teddington, UK) and was reactivated following the supplier's instructions. Spore solution agar plates were prepared with 24 g L^{-1} potato dextrose broth and 15 g L^{-1} agar (Carl Roth GmbH + Co KG, Karlsruhe, Germany). After approximately 7 days of incubation at 32 °C, spores were harvested using 10 mL sterile 0.9% (w/v) NaCl solution and scraped from the plate with a sterile spatula. To determine their concentration, spores were counted under a light microscope (B1 Series, Motic, Hong Kong, China) using an improved Neubauer chamber. The spore concentration was calculated as the ratio between the average number of counted spores and the volume of the counting chamber.

Solid-state fermentation

Solid-state fermentation was carried out in 500 mL Erlenmeyer flasks. In all experiments, the total reaction mass was 10 g, and moisture content was adjusted to 75% (w/w) prior to inoculation with sterile MilliQ water. In the experiments with nutrient addition, the press cake was supplemented with 25% (v/w) of either one of the following mixtures: 1) glucose (100 g L^{-1}), ammonium sulfate (1 g L^{-1}), potassium dihydrogen phosphate (0.05 g L^{-1}) or 2) press juice (1:1 with glucose), ammonium sulfate (1 g L^{-1}), potassium dihydrogen phosphate (0.05 g L^{-1}). The nutrient composition of the press juice used can be found elsewhere.[21] All chemicals were purchased from Carl Roth GmbH + Co KG, except glucose, which was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). In all cultivations, 4% (w/v) methanol was added, and the inoculum contained 1×10^7 spores g_{DM}^{-1} (dry matter). Growth was carried out at 32 °C and 140 rpm in a shaking incubator (Ecotron, Infors AG, Bottmingen, Switzerland).

Downstream process

After 5 days of cultivation, 100 mL MilliQ water was added to the flasks and cultures were incubated for 1 h at 32 °C and 140 rpm. Then, the solid and liquid fractions were separated by centrifugation at 4500 rpm for 20 min (Z38K, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was filtered to remove any remaining fungal mycelia using Whatman filter paper (grade 4; Sigma-Aldrich), and then stored at −20 °C for further analyses.

Analytical methods

The dry mass content of the press cake was measured in triplicate as dry weight after overnight incubation in an oven at 105 °C (Memmert GmbH). The pH of the supernatant was measured using a pH meter (Microprocessor pH 211, Hanna Instruments GmbH, Vöhringen, Germany). The citric acid concentration was determined by high-performance liquid chromatography (HPLC) using a Merck Hitachi L-6200 pump (Merck KGaA, Darmstadt, Germany), Midas cool autosampler (Spark Holland B.V., Emmen, Netherlands), and an RI 101 refractive index detector (Shodex, Kawasaki, Japan). The HPLC was equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) at 80 °C. The mobile phase was 2.5 mmol L^{-1} H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The instrument and data evaluation were managed with Clarity software (Data Apex, Prague, Czech Republic). Protein content was determined using the Bradford assay (Pierce Coomassie Bradford Protein Assay kit,

Thermo Scientific, Waltham, MA, USA) and bovine serum albumin as internal standard. Absorbance was measured at 595 nm using a Cary 60 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

β-Glucosidase activity assay

The *β*-glucosidase activity was determined as described previously,[22] with adjustments according to Caputo *et al.*[23] Briefly, 50 µL of supernatant was mixed with 50 µL of 2.0 mmol L^{-1} para-nitrophenyl-*β*-D-glucopyranoside (in 50 mmol L^{-1} sodium acetate buffer, pH 5.0). The assay was performed in 96-well plates with a 200 µL reaction volume at 50 °C. The samples were incubated for 10 min at pH 5 using 50 mmol L^{-1} sodium acetate buffer. The reaction was stopped with 1 mol L^{-1} Na_2CO_3 and absorbance of both the calibration curve and samples was measured at 405 nm. One unit of enzyme activity (U) corresponded to 1 µmol of para-nitrophenol produced per minute.

Confocal laser scanning microscopy

Samples for microscopy analysis were cut in small pieces and fixed in 10% (v/v) formaldehyde (100 g L^{-1}). Then, they were washed with 50 mmol L^{-1} sodium carbonate–bicarbonate buffer (pH 9.2), immersed in 0.01 g L^{-1} Calcofluor White solution (Sigma-Aldrich), and incubated for 5 min. Images were captured with a Nikon A1 (Nikon Instruments Inc., Melville, NY, USA) using a 40×/0.75NA objective with 414 nm excitation and 420–460 nm emission.

Results and discussion

Citric acid production in SSF

Citric acid was produced using the press cake obtained from mechanical pretreatment of *L. perenne* with a screw-press. The results of the compositional analysis are shown in Supporting Information, Fig. S1. The possibility of producing citric acid using the press cake as sole nutrient source was assessed. However, after 10 days of cultivation, no fungal growth or citric acid was detected (data not shown). The lack of growth could be explained by the complex nutrient requirements of *A. niger*, and the specific cultivation conditions required. Based on this, it is highly likely that cultivation with press cake alone did not meet the requirements.

Several factors influence citric acid production, including medium pH, microorganism morphology, aeration, phosphate limitation, and nitrogen limitation, and the concentration and type of carbon source.[24] Only sugars that are metabolized rapidly by the fungus can contribute to good citric acid yields, which means that non-hydrolyzed polysaccharides do not support high conversion rates.[25] To overcome such limitations, the press cake was supplemented with a 25% (v/w) mixture of glucose or press juice from perennial ryegrass as carbon source, along with ammonium sulfate as nitrogen source, potassium dihydrogen phosphate as potassium source, and methanol. The latter was added based on previous reports of its positive effect on citric acid production. Dhillon *et al.*[26] demonstrated that supplementing apple pomace waste with methanol resulted in 113 g of citric acid per kg of dry apple pomace. When using pomegranate peel waste as substrate, addition of up to 3% (w/w) methanol correlated positively with citric acid yields.[27] Similarly, addition of methanol to molasses nearly doubled citric acid titers, possibly because methanol increased cell permeability.[28] *Aspergillus niger* supplemented with two different carbon sources (Supporting Information, Fig. S2A,B), and with nutrients, was cultivated for 5 days, after which pH and citric acid production were determined (Table 1).

The pH is a crucial parameter in citric acid production. Spores germinate at pH ≈ 5; however, during citric acid production, the pH drops as a result of ammonia uptake and the consequent release of protons by the spores.[25] The lower pH of the culture broth favors citric acid production,[24] reduces the risk of contamination, and inhibits the production of side products, such as gluconic and oxalic acids.[24,25] Here, the starting pH was 4.4 with glucose and 4.9 with press juice. During cultivation, the pH dropped to 2.3 and 3.1, respectively. This decrease in pH could be explained by increased citric acid production, which reached 84.7 g kg_{DM}^{-1} and was a direct consequence of more fungal cells. Their greater numbers would be favored by the addition of glucose but would also result in greater ammonium sulfate consumption. In line with the above hypothesis, experiments performed with press juice yielded only 2.1 g kg_{DM}^{-1} citric acid. The press juice may have contained compounds

Table 1. Production of citric acid by *Aspergillus niger* after 5 days of solid-state fermentation (SFF) cultivation. The SSFs were carried out at 32 °C, 140 rpm.

	pH	Citric acid (g kg_{DM}^{-1})
Press cake +25% (v/w) glucose	2.3	84.7 ± 0.3
Press cake +25% (v/w) press juice	3.1	2.1 ± 0.0

Values represent the means ± standard deviations of two replicates.

that limited citrate production. For instance, an excess of heavy metals and alkaline metals can inhibit citric acid production.[25,29] Yet, 0.3 ppm zinc and 1.3 ppm iron promote citric acid output.[30] Mattey and Bowes[31] reported that 10 mg L^{-1} manganese reduced citric acid production by 50%. As reported by Boakye-Boaten, press juice from the perennial ryegrass *Miscanthus* × *giganteus* (MxG) contained various metal ions.[32] Due to the biomass similarities, it is likely that the press juice from *L. perenne* contained a comparable metal ion composition.

Protein concentration and enzymatic activity

Filamentous fungi play a critical role in the degradation of LCB as a result of their ability to secrete a wide range of extracellular enzymes, which break down polysaccharides into sugar monomers. The hydrolysis of cellulose requires three different cellulases, which act synergistically and concurrently.[33,34] *Trichoderma reesei* is known for its high production of exo- and endoglucanase, while *A. niger* is particularly effective in producing *β*-glucosidase.[35–39] This enzyme is crucial for the complete hydrolysis of cellulose, as it converts cellobiose and cellooligomers to glucose monomers. Removal of cellobiose is important as it prevents product inhibition on other cellulolytic enzymes. In this work, *β*-glucosidase activity was used as an indicator of cellulolytic hydrolysis of the press cake. The use of *β*-glucosidase activity as an indicator of cellulolytic hydrolysis of the press cake was based on the assumption that if cellobiose is hydrolyzed into glucose monomers, then the initial hydrolysis of cellulose into cellobiose by cellulases (endo- and exoglucanases) must have occurred. Following the sequential nature of cellulose hydrolysis, *β*-glucosidase activity can therefore serve as an indirect measure of upstream cellulolytic activity.

To gain insight into protein production during cultivation, the supernatant resulting after downstream processing was tested for protein content (Fig. 2). The protein concentration was twice as high when the press cake was supplemented with glucose ($10.51 \pm 2.49\,\mu g\,mL^{-1}$) compared to press juice ($5.04 \pm 0.99\,\mu g\,mL^{-1}$). In contrast, *β*-glucosidase activity was highest following supplementation with press juice ($1.15 \pm 0.07\,U\,g_{DM}^{-1}$) as opposed to glucose ($0.89 \pm 0.02\,U\,g_{DM}^{-1}$) (Fig. 2). This finding suggested that *A. niger* might have been compelled to hydrolyze the press cake to access sugars. Accordingly, growth conditions and/or cultivation parameters might influence the ability to produce enzymes necessary to hydrolyze the press cake. Indeed, biomass composition, pH, temperature, dissolved oxygen, and salts have been shown to influence production of cellulolytic

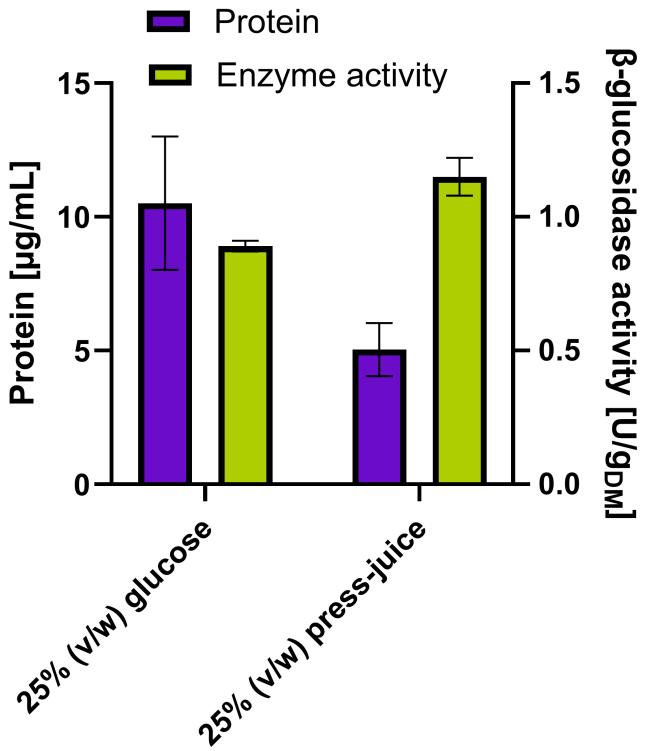

Figure 2. Protein concentration and *β*-glucosidase activity of the supernatant after SSFs supplemented with either 25% (v/w) glucose or press juice. One unit of enzyme activity (U) corresponded to 1 µmol of para-nitrophenol produced per minute. Error bars represent the average value of two replicates.

enzymes.[40] Both oligosaccharides and cellulose analogues can induce cellulase production,[41] whereas nitrogen limitation has the opposite effect.[42] So far, *β*-glucosidase activity has been shown to be affected more by acidic than alkaline pH.[43] The presence of metal ions can also influence *β*-glucosidase. In particular, Mn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2} and Co^{+} have a negative effect on its activity.[36,39,43,44]

Morphological analysis

The morphology of filamentous fungi such as *A. niger* varies significantly depending on the desired product and the bioprocess used. In submerged cultivation, filamentous fungi can grow in dispersed or pelletized form.[45] In SSF, a dispersed mycelium is the most frequent morphological type observed. The dispersed morphology can be described as a branched pattern where a hypha emerges from the spore and elongates at the tip by creating new branches. The process continues until a three-dimensional network of hyphae called mycelium is formed. The mycelium is particularly suitable for growth

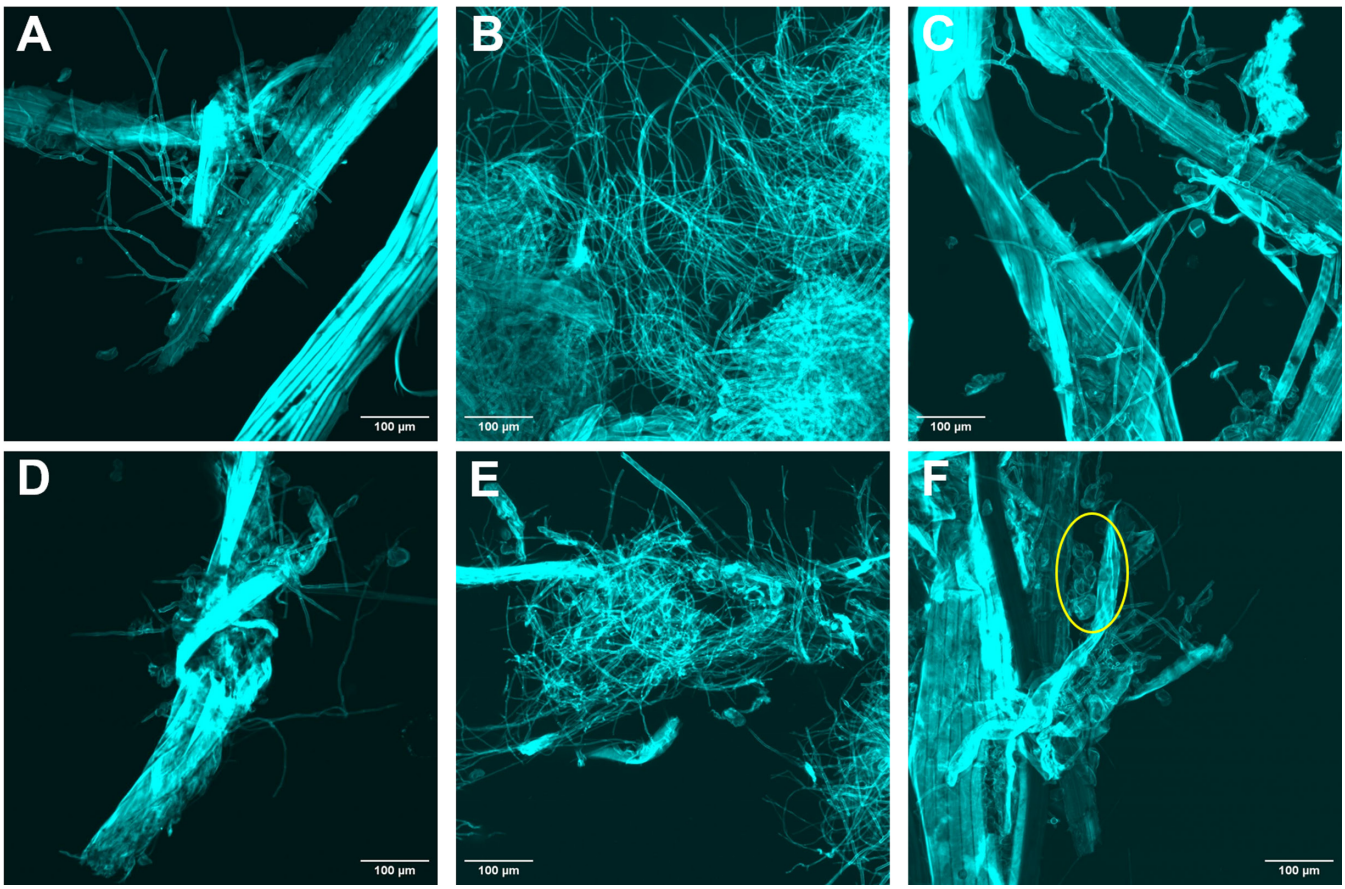

Figure 3. Confocal laser scanning microscopy micrographs of *A. niger* after solid-state fermentation using glucose (a–c) or press juice (d–f) as an additional carbon source. The figure depicts the fungal mycelia growing on the press cake (a, c, and d), branching degree of fungal mycelia (b and e), and the presence of bulbous cells (yellow circle in f). Color was assigned arbitrarily.

on solid substrates, as it allows filamentous fungi to colonize and penetrate such environments in search of nutrients. At present, SSF has been applied for the production of enzymes, antibiotics, organic acids, biosurfactants, and aroma compounds.[15]

To understand the interaction between *A. niger* and the press cake, cultivations were imaged by CLSM. A close interaction between *A. niger* and the insoluble substrate was observed in cultures supplemented with either glucose (Fig. 3(a)–(c)) or press juice (Fig. 3(d)–(f)). In both cases, the mycelial hyphae were growing in and around the biomass surface (Fig. 3(a), (c), and (d)). However, the choice of carbon source led to differences at the microscopic level. When *A. niger* was cultivated on the press cake supplemented with glucose, the degree of ramification increased, suggesting higher enzyme production (Fig. 3(b)). This result is in accordance with the findings by Novy *et al.*,[46] who reported that a higher degree of branching boosted protein production in *T. reesei*. Intact, robust, and thick hyphae could also be observed, evidencing favorable growth conditions for the fungus.

When the press cake was supplemented with press juice, however, the hyphae appeared thinner, fragmentated, and less compact (Fig. 3(e)). As shown in Fig. 3(f) (yellow circle), in some spots, a bulbous cell growth was observed. This growth is generally associated with nutrient deficiency. It is possible that the scarcity of sugar in press juice and/or the presence of inhibitory compounds such as metal ions had a negative effect on fungal growth.

Conclusion

The present study explored the use of grass press cake for the production of citric acid by *A. niger* using CBP. When the press cake was used as the sole source of nutrients, neither fungal growth nor citric acid production were observed.

However, supplementing the press cake with an additional carbon source in the form of glucose or press juice, along with nitrogen, phosphate, potassium, and methanol, led to successful citric acid production. These findings highlight the importance of optimizing process conditions such as nutrient availability in SSF processes. Although the fermentation setup did not allow differentiation between the supplemented glucose and the glucose released from the press cake hydrolysis, β-glucosidase activity was used as indicator of the press cake consumption. The authors hypothesized that the detection of β-glucosidase activity would confirm the hydrolysis of the press cake by the fungus. Interestingly, β-glucosidase activity was higher when press juice was used as additional carbon source, suggesting that the lower sugar content of this supplement prompted *A. niger* to hydrolyze the press cake to access sugar. Qualitative information about the interaction between the fungus and the press cake was obtained by CLSM analysis. At a macroscopic level, close interaction between *A. niger* and the press cake was observed, regardless of the carbon source. At a microscopic level, differences in mycelial appearance and degree of ramification could be observed with respect to carbon source. Overall, these findings reveal that filamentous fungi such as *A. niger* are particularly suitable for developing CBP, in which production of hydrolytic enzymes, biomass hydrolysis, and fermentation occurs simultaneously in a single system.

Conflict of interest

The authors declare no conflicts of interest.

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