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Microbial production of organic acids by endophytic fungi

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ABSTRACT

The microbial production of organic acids has been considered as a promising strategy to obtain building-block chemicals from renewable feedstocks such as lignocellulosic agro-industrial wastes. This approach is in accordance with the biorefinery concept where the production of biofuels is integrated to higher-value platform chemicals. However, finding a suitable microbial source and the optimum cultivation conditions remains a challenge for the implementation of such process. Endophytic microorganisms have been explored as a source of novel biochemical compounds for biotechnological applications, but the production of organic acids by endophytic fungi remains to be investigated. Here, the potential of using endophytic fungi for organic acids production has been evaluated by carrying out a screening of 35 fungal strains isolated from the leaves and branches of trees inhabiting two mangroves in the state of Sao Paulo, Brazil. The cultivation of a selected *Aspergillus awamori* 09 (4) strain under solid-state fermentation (SSF) using a mix of wheat bran and sugarcane bagasse (1:3) resulted in 135.5 mg/g of organic acids, which represents around 7-fold increase when compared to the use of sugarcane bagasse alone. These results indicate the potential of mangrove-associated endophytic fungi for organic acid production under SSF using agro-industrial wastes as feedstock, being compatible with the current bioeconomy demands.

1. Introduction

The microbial production of organic acids has been considered a promising strategy to obtain building-block chemicals from renewable feedstocks, being compatible with the biorefinery concept (Becker et al., 2015; Chen and Nielsen, 2016; Kamm and Kamm, 2004; Sauer et al., 2008). Such multi-product biorefinery concept comprises the efficient conversion of different renewable feedstocks into fuels, chemicals and novel materials, where the co-production of higher-value products such as organic acids are integrated to biofuels and contributes to the overall process economic feasibility (Cheali et al., 2015). Among the twelve sugar-based building-block chemicals identified by the US Department of Energy as the top value-added chemicals from biomass, nine are organic acids (Werpy and Petersen, 2004). Due to their functional groups, organic acids can be used as raw materials for the chemical industry, with applications in the production of biodegradable polymers, potentially replacing petroleum-based or synthetic chemicals (Sauer et al., 2008). Citric, oxalic, gluconic, fumaric, malic, and succinic acid are examples of organic acids with multiple industrial applications in the food, pharmaceutical, among others sectors (Ciriminna et al., 2017; Goldberg et al., 2006; Sauer et al., 2008; Yang et al., 2017). Furthermore, the production of organic acids in nature is related to the solubilization of soil minerals and release of nutrient ions to plants, thus suggesting a potential role of organic acids in the manufacture of biofertilizers as well (Klaic et al., 2017; Vassilev et al., 2015).

Although the microbial production of organic acids can be carried out by bacteria and yeasts, the fungi are well known for their production of high amounts of various organic acids (Max et al., 2010; Yang et al., 2017). In particular, the filamentous fungus *Aspergillus niger* has been considered as the workhorse for the industrial production of organic acids such as citric acid (Yang et al., 2017). Nevertheless, recent studies exploring the microbial biodiversity have demonstrated the potential of new sources of organic acids producers (Liaud, 2014). However, finding a potential strain and the optimized cultivation conditions to produce high yields of organic acids remains a critical challenge for the industrial implementation of this biotechnological process.

The endophytic fungi represent a potential source of microorganisms for organic acids production. Endophytic fungi inhabit the internal tissues of plants without causing any negative effects, being considered a potential source of novel biochemical compounds for biotechnological applications (Correa et al., 2014; Liu et al., 2017; Robl et al., 2013; Sebastianes et al., 2013, 2017). Comparative studies of the diversity of

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endophytic fungal isolated from the leaves and branches of trees inhabiting two mangroves in the state of São Paulo, Brazil, revealed a large reservoir of fungal diversity (Sebastianes et al., 2013, 2017). Therefore, it is of great interest to investigate the potential of endophytic fungi from such diversified communities for organic acids production.

Microbial cultivation processes for the industrial production of organic acids has been mostly carried out using submerged fermentation (Max et al., 2010). However, the cultivation of filamentous fungi under solid-state fermentation (SSF) has received increasing attention due to the inherent advantages of this cultivation system (Farinas, 2015; Soccol et al., 2006). In fact, several studies have described the use of SSF to produce organic acids, as recently reviewed by (Mondala, 2015). SSF is particularly advantageous for the cultivation of filamentous fungi, since it simulates the natural habitat of these microorganisms. From the environmental perspective, the benefit of SSF is related to the use of agro-industrial wastes as solid substrate, acting as sources of both carbon and energy (Cunha et al., 2017; Farinas et al., 2011; Farinas, 2015; Rodriguez-Zuniga et al., 2013).

Here, the collection of endophytic fungi isolated from the Brazilian mangrove tropical forests was assessed for the potential of organic acids production. For that, 35 fungal strains were initially screened using a plate assay and the selected strains were further evaluated for their production of organic acids by cultivation under SSF. The effect of the type of agro-industrial residue used as feedstock was also investigated by using a combination of sugarcane bagasse and wheat bran.

2. Materials and methods

2.1. Microorganism

The evaluation of the organic acids production was performed by screening 35 strains of endophytic fungi that had been previously isolated from two mangrove areas in the state of São Paulo, Brazil (Sebastianes et al., 2013). To preserve the collection of fungi, the fungal strains were grown on potato dextrose agar (PDA) medium and small samples were put in sterile distilled water and stored at room temperature. The fungal strains assessed belong to six different genera (*Aspergillus*, *Diaporthe*, *Fusarium*, *Hypocrea, Penicillium* and *Xylaria*).

2.2. Plate assay for screening of organic acid production

All 35 fungal strains were initially screened qualitatively for the production of organic acids using a plate assay based on pH change. For that, the endophytic fungi were inoculated in the center of Petri dishes containing Czapek-Dox agar medium (30.0 g/L sucrose, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 2.0 g/L $NaNO_3$, 15.0 g/L agar), surfactant triton X-100 (1000 μL/L), and bromocresol green (0.1 g/L) as pH indicator. The initial pH of the medium was adjusted to 6. After inoculation, the plates were incubated at 30 °C for 4–7 days. After this period, the diameter of the yellow zone formed was measured and used as an indicative of the strain ability for organic acid production.

2.3. Solid-state fermentation

Production of organic acids was carried out by cultivation of the previously selected endophytic fungi strains under solid-state fermentation (SSF). The cultivations were carried out in 500 mL Erlenmeyer flasks containing 3 g of dry substrate (sugarcane bagasse with particle size of $1.0 \leq dp \leq 1.5$ mm and wheat bran), with the humidity adjusted to 75% (w/v) by addition of a nutrient medium adapted from (Kumar et al., 2003). The composition of the medium (% w/v) was as follows: 20% sucrose, 0.25% $(NH_4)_2SO_4$, 0.1% de KH_2PO_4, 0.025% de $MgSO_4{\cdot}7H_2O$, 0.004% de $CuSO_4$, pH 5.5. The moist substrate was sterilized at 121 °C for 60 min to provide proper cooking of the substrate and to increase its susceptibility to microbial attack. After autoclaving, 4% methanol was added to the medium. A concentration of 10^7 spores/g of dry substrate was inoculated and the cultivations were conducted under static conditions at 30 °C for up to 6 days. The organic acids were extracted by the addition of 1:15 (w/v) distilled water, with 200 rpm agitation at 30 °C for 30 min. The final acid extracts were vacuum-filtered, centrifuged at 10,000 rpm for 15 min at 4 °C, and used for the analytical assays. A control flask without inoculation was used for all the different cultivation conditions. For these control flasks, all the experimental steps were carried out similarly to the inoculated samples and the organic acids value measured in the control was subtracted from the corresponding sample. All cultivation experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

2.4. Determination of total reducing sugars

The inversion of sucrose was carried out by acid hydrolysis, where 1 mL of 2 N HCl was added to test tubes containing 1 mL of the diluted sample. The tubes were incubated at 100 °C for 5 min, followed by ice cooling for 5 min. After that, 1 mL of 2 N NaOH was added to neutralize the samples and the total reducing sugar concentrations were determined by the DNS method (Miller, 1959). All experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

2.5. Determination of organic acids

The quantification of total organic acids was carried out by measuring the volumetric titratable acidity using 1% phenolphthalein as indicator. For that, a sample containing 3 mL of the extract acid and 7 mL of distilled water was titrated with a 0.01 M NaOH solution. The quantitation of organic acids was performed by using citric acid as the equivalent factor in grams. The concentrations of gluconic, oxalic and citric acids were determined by HPLC (Waters Co system HPLC W515 pumps, W717 Injector and W486 UV reader), using an Aminex HPX-87H column (Bio-Rad), 5 mM sulfuric acid solution as the mobile phase (at a flow rate of 0.6 mL/min with isocratic pumping and the organic acid kit (Sigma-Aldrich) as standard. The injector and column temperature were 4 °C and 65 °C, respectively. The organic acids were detected at 210 nm. All experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

3. Results and discussion

3.1. Screening of fungal strains for organic acid production

Table 1 presents a list of 35 endophytic fungal strains screened for their potential of organic acids production. The fungal strains belong to six different genera (*Aspergillus*, *Diaporthe*, *Fusarium*, *Hypocrea, Penicillium* and *Xylaria*), which had been previously isolated from either branches or leaves of different plants collected from the mangroves of São Paulo state, Brazil (Sebastianes et al., 2013). Initially, each of these 35 fungal strains was assessed for organic acid production by using a plate assay with a pH indicator. The formation of a yellow zone around the inoculation was used as an indication of the formation of organic acids. For the strains that showed a positive result, the yellow zone diameter was measured and used for a qualitative classification (Table 1). Among the 35 strains evaluated, five presented a significant yellow zone in the plate screening assay. The four fungal strains of the genus *Aspergillus* showed the most significant yellow zones, followed by the *Penicillium* strain (Fig. 1). Therefore, these five strains (*A. awamori* 09(4), *A. awamori* 82(4), *A. awamori* 108(4), *A. niger* 56(3), *P. minioluteum* 24(4)) were selected to be further evaluated in terms of their organic acid production under solid-state fermentation (SSF) cultivation.