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Itaconic and Fumaric Acid Production from Biomass Hydrolysates by *Aspergillus* Strains S

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Itaconic acid (IA) is a dicarboxylic acid included in the US Department of Energy's (DOE) 2004 list of the most promising chemical platforms derived from sugars. IA is produced industrially using liquid-state fermentation (LSF) by *Aspergillus terreus* with glucose as the carbon source. To utilize IA production in renewable resource-based biorefinery, the present study investigated the use of lignocellulosic biomass as a carbon source for LSF. We also investigated the production of fumaric acid (FA), which is also on the DOE's list. FA is a primary metabolite, whereas IA is a secondary metabolite and requires the enzyme *cis*-aconitate decarboxylase for its production. Two lignocellulosic biomasses (wheat bran and corn cobs) were tested for fungal fermentation. Liquid hydrolysates obtained after acid or enzymatic treatment were used in LSF. We show that each treatment resulted in different concentrations of sugars, metals, or inhibitors. Furthermore, different acid yields (IA and FA) were obtained depending on which of the four *Aspergillus* strains tested were employed. The maximum FA yield was obtained when *A. terreus* was used for LSF of corn cob hydrolysate (1.9% total glucose); whereas an IA yield of 0.14% was obtained by LSF of corn cob hydrolysates by *A. oryzae*.

Keywords: Lignocellulosic biomass, liquid-state fermentation, itaconic, fumaric, biomass valorization

Introduction

The reduction of fossil resource reserves is currently driving research for viable, renewable, and environmentally friendly alternatives to replace conventional resources as raw material for the production of a large range of chemicals. In this context, the biorefinery concept can be defined as the utilization of plant biomass for simultaneous production of different goods, such as food, feed, materials, energy, and chemicals, with added value. This process should be economically and ecologically sustainable [24]. To also achieve social sustainability, it is important to use inedible parts of plants, such as lignocellulose, which is a low-value byproduct of agriculture or forestry and which represents a non-competitive food resource. This wasted biomass is the most abundant carbon feedstock on Earth [12]. Lignocellulosic biomass is a recalcitrant material that often requires pretreatment to denature its compact structure and liberate and separate its main components (cellulose, hemicellulose, and lignin) [17]. The use of this waste material is an opportunity to develop new, greener processes. In 2004, the US Department of Energy (DOE) studied 300 molecules produced from biomass with industrial and strategic interests and published a final list of 12 molecules with great potential to act as building-block chemicals [34]. During the last decade, different proposals have been submitted to utilize these biobased chemicals and the technologies required for their production [2].

Among these molecules, organic acids represent an interesting group. Most are bioproducts from the metabolic pathways of microorganisms, and their functional chemical groups make them useful platform chemicals for the

industry. Itaconic acid (IA) and fumaric acid (FA) are two biobased organic acids, which were included in the DOE's 2004 list [34]. Both are intermediates of the oxidative phase of the TCA cycle in cells, but FA is also involved in CO_2 fixation during the reductive phase of the TCA cycle [6]. The metabolic pathway for IA and FA synthesis is illustrated in Fig. 1. The production of these acids is modulated by the concentration of glucose and the need for energy (in the form of ATP or GTP) and reducing power (in the form of $NADH_2$ or $FADH_2$). IA and FA are unsaturated dicarboxylic acids formed by five and four carbons, respectively (Fig. 1). The presence of a double bond and two carboxyl groups allows them to polymerize into high-molar-mass compounds [22, 33]. FA is also a valuable intermediate in the preparation of edible products, such as L-malic acid and L-aspartic acid, with increasing usefulness in the production of sweeteners, beverages, and other health foods. The worldwide demand for fumaric acid and its derivatives are projected to grow annually at rate of 5.8% from 2013 to 2018 [36]. IA has a broad application spectrum in the industrial production of resins and is used as a building block for acrylic plastics, acrylate latexes, superabsorbents, and anti-scaling agents [15]. These organic acids can be produced by filamentous fungi at high concentrations [23].

Thanks to their metabolic versatility, *Aspergillus* species are used in biotechnology for the production of a variety of products, such as organic acids, pharmaceuticals, and enzymes. Since 1960, the production of IA has been achieved by fermentation of liquid sugar-containing media by *Aspergillus terreus*. The strain NRRL1960 achieves production yields of 80–90 g/l [16]. In recent decades, investigations have focused on the production of IA from renewable biomass sources, at first from starchy materials such as cornstarch, molasses, or grains. Nonetheless, these primary biomasses are expensive and used in the food industry. Consequently, lignocellulosic feedstocks are more attractive for biorefinery [21]. In contrast, the industrial production of FA from biomass is still in its early stages and is not competitive with chemical synthesis from petrochemical feedstock. However, *Rhizopus* species were shown to be the best producers of FA using renewable biomass as an energy source [35]. However, some research efforts are still necessary to produce IA and FA from waste biomass with sufficiently high yields to be competitive with current industrial processes [25].

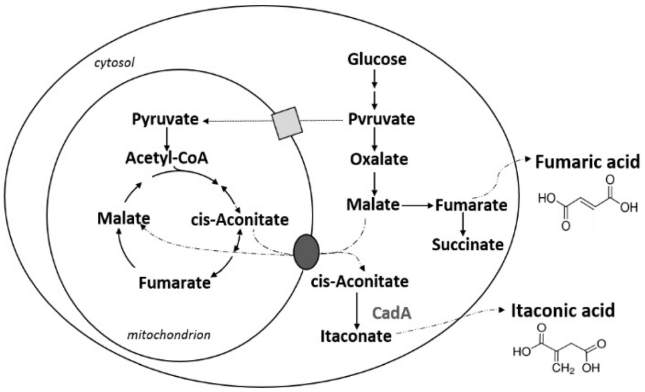

Fig. 1. Simplified metabolic pathway of itaconate and fumarate production.

The aim of this study was to determine the abilities of four *Aspergillus* strains (*A. terreus* 826, *A. terreus* 62071, *A. tubingensis*, and *A. oryzae*) to produce IA and FA from liquid-state fermentation (LSF) of two lignocellulosic biomasses (wheat bran and corn cobs). Two different biomass treatments (acid and enzymatic) were tested.

Materials and Methods

Microorganisms

The *A. terreus* strains (DSM 826 and DSM 62071) were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) Culture Collection. The strains were revived on potato dextrose broth medium for 5–6 days at 25°C. *A. oryzae* (UMIP 1042.72) was provided by the Fungal Culture Collection of the Pasteur Institute (France). *A. tubingensis* (IMI 500512) was isolated in our laboratory from agricultural residues and identified by CABI Bioscience (UK). The microorganisms were maintained on potato dextrose agar (PDA) and Czapek dox agar (CDA). The spore suspensions were harvested from 5/6 days old PDA and CDA plates with 0.2% (v/v) Tween-80. The spores were counted using a Malassez counting chamber and stored at –20 ± 1°C.

Substrate and Pretreatment

Two agro-industrial waste biomasses, wheat bran and corn cobs (Comptoir Agricole, France), were used as substrates to evaluate their suitability for the production of IA and FA but also for the production of enzyme cocktails. The lignocellulosic material was crushed (SX 100, Resch) to obtain particles that were 0.5–1 mm in size. Moreover, the liquid extracts that resulted from these biomasses after pretreatment were used in LSF. The physicochemical characterizations of the extracts are provided in Table 1. The chemical analyses were performed by SOCOR (France) and CSIC (Spain).

Dilute acid pretreatment. Wheat bran and corn cob slurries were made with 1% dilute H_2SO_4 (42 g dry solid substrate in 230 ml dilute acid, 18.26% (w/v)), preheated at 150°C for 25 min and then cooled down to room temperature according to previously described methods [19]. The liquid was collected by centrifugation (8,000 ×*g* for 30 min) and then adjusted to the desired pH with 5 M

Table 1. Hydrolysate composition after acid and enzymatic treatments of wheat bran (Wb) and corn cob (Cc) biomasses.

Constituents	Acid hydrolysates		Enzymatic hydrolysate *A. tubingensis*		Enzymatic hydrolysate *A. oryzae*	
	Wb	Cc	Wb	Cc	Wb	Cc
Sugars (g/l)						
Reducing sugars	19.8	6.9	24.1	17.3	36.5	27.2
Glucose	3.2	4.2	8.4	4.7	12.9	7.7
Total nitrogen (% dry wt)	0.34	0.25	0.79	0.48	2.87	1.53
Metal (mg/l)						
Calcium	121.9	241.9	3.6	1.2	18.2	21.8
Iron	10.1	19.5	4.7	4.4	4.5	1.5
Magnesium	286.5	33.5	327.8	112.6	516.9	292.8
Manganese	2.5	2.5	8.3	2.8	11.5	5.9
Potassium	1,608.5	1,083.5	2,650.6	2,244.9	2,749.1	2,555.2
Sodium	4,892.1	6,099.2	2,203.8	2,211.9	1,892.5	1,975.9
Zinc	1.5	5.1	5.5	3.7	4.7	2.7
Anions (mg/l)						
Orthophosphates	478.5	107.5	3,760.2	3,240.3	3,970.1	3,330.2
Phenolic compounds (mg/l)						
Determined at 280 nm	970.2	945.7	627.8	677.1	904.3	917.7
Determined at 320 nm	573.9	585.9	287.8	368.1	454.4	486.1

NaOH and finally filter-sterilized with a 0.22 µm filter.

Enzymatic pretreatment. Both biomasses were treated with a liquid enzymatic cocktail of *A. tubingensis* and *A. oryzae* produced by solid-state fermentation of wheat bran. These cocktails displayed endoxylanase, amylase, and cellulase activities (data not shown). The hydrolysates were obtained as follows: 60 ml of the enzymatic cocktail was added to 5 g of dry biomass in 125 ml baffled Erlenmeyer flasks, and the flasks were incubated at 37°C with 80 rpm shaking. Sugar formation was estimated at regular intervals for 216 h. The hydrolysates were sterilized by filtration after a 9-day incubation period before they were used for fermentation.

Liquid State Fermentation

Fifty milliliters of the liquid hydrolysates (acid pretreatment and enzymatic pretreatment) were poured into 125 ml baffled Erlenmeyer flasks. The hydrolysates were inoculated with spore suspensions at 10^5 spores/ml. The flasks were incubated on a rotatory shaker (Infors Multitron, Switzerland) for 7 days at 33°C with 120 rpm shaking. The hydrolysates from wheat bran and corn cobs were set at two different pH values for fermentation (pH 3–4 and pH 6–7). Unless specified otherwise, these fermentation conditions were maintained throughout the study. All experiments were conducted in duplicates. Samples of 500 µl were taken at regular intervals, filtered with a 0.2 µm membrane and stocked at –20°C for HPLC analysis and additional studies. Diluted hydrolysates from acid and enzymatic pretreatments were also used in LSF. These diluted solutions were tested with and without the addition of glucose and/or metals up to the level of the optimized medium (used as a positive control).

LSF of optimized culture medium. The optimized IA production medium contained 180 g glucose, 0.1 g KH_2PO_4, 3 g NH_4NO_3, 1 g $MgSO_4{\cdot}7H_2O$, 5 g $CaCl_2{\cdot}2H_2O$, 0.00167 g $FeCl_3{\cdot}6H_2O$, 0.008 g $ZnSO_4{\cdot}7H_2O$, and 0.015 g $CuSO_4{\cdot}7H_2O$ per liter of medium [12]. The pH was adjusted to 3.1 with 1 M H_2SO_4 before addition of the $CaCl_2$ solution. The solutions were autoclaved for 20 min at 121°C and 1.4 bars/min. The $FeCl_3$ solution was not autoclaved but was filter sterilized. The fermentations were performed in 500 ml baffled Erlenmeyer flasks with a working volume of 100 ml. The experiments were conducted on a rotatory incubator shaker (Infors Multitron, Switzerland) at 33°C with 120 rpm shaking and an initial spore concentration of 10^5 spores/ml. The conditions for sampling and analysis were the same as those used for LSF. The fermentations were conducted in duplicates.

Analytical Procedures

Sugar assays. The reducing sugars present in the different hydrolysates were determined by the DNS method with spectrophotometric measurements at 550 nm [25]. Glucose was measured by a colorimetric method measured at 420 nm [27].

Acid assays. A chromatographic system, made of a 616 pump, a 2996 photodiode array detector operating in a range of 200 to 450 nm, and a 717 Plus autosampler (Waters, France) controlled with Empower 2 software (Waters) was used to analyze the samples. Chromatographic separation was achieved on a hypercarb porous graphitic carbon LC column (150 × 3.0 mm i.d., 3 µm;

Thermo Scientific, USA) at 70°C. The mobile phase consisted of water:formic acid (99.9:0.1 (v/v), phase A) and acetonitrile:formic acid (99.9:0.1 (v/v), phase B) at a flow rate of 0.55 ml/min. Elution was performed with a gradient as follows: 0% B (0–5 min), 0–27% B (5–18 min), and 27–27% B (18–26 min). Finally, phase B was decreased to its initial concentration (0%) in 1 min, and the column was re-equilibrated for 14 min. The injection volume was 20 µl. For the analysis of samples from LSF on optimized culture medium, the temperature was 60°C and the gradient was as follows: 10–34% B (0–8 min), 34–10% B (8–9 min), and 10% B (9–23 min) to re-equilibrate the column. The columns were calibrated using commercial IA and FA with 99.9% purities (Sigma-Aldrich, USA) and UV measurement at 205 nm. Each sample was supplemented with 10 ppm IA or FA to confirm the acid production.

Phenolic compound assays. The pretreatment, which is useful for increasing the yield of available sugars, can also release metabolism inhibitors [9, 11]. The specific inhibitors released from the lignin portion after treatment were measured to compare the acid and enzyme treatments. These compounds with an aromatic ring liberated from the lignin solubilization can be detected between 280 and 320 nm by photometry [18]. The acid and enzymatic hydrolysates were filtered with 0.22 µm filters before their respective absorbances were measured with an Ultrospec 7000 spectrophotometer (GE Healthcare, UK).

Results

LSF in the Optimized Culture Medium

A. terreus is the most frequently used fungus to produce IA in large-scale formats, but it is particularly sensitive to the cultivation conditions. IA production is strongly affected by several components, including the type and concentration of carbon source; levels of nitrogen, phosphate, trace minerals, dissolved oxygen, and carbon dioxide; pH; and temperature [16].

In this study, the four *Aspergillus* strains were tested in the optimized medium to check their IA production. This medium was optimized for *A. terreus* DSM 23081 to produce IA with high yield [16]. This condition could be considered as a positive control for the IA production in this study. All of the strains grew quickly in this medium, but IA was only produced by the two *A. terreus* strains (Fig. 2). FA was not produced by any *Aspergillus* strains under these conditions. The final concentration of IA for *A. terreus* 826 was 33.2 g/l and for *A. terreus* 62071 was 7.8 g/l. The IA production yield based on the initial total glucose content was 180 mg/g glucose (18%) for *A. terreus* 826.

LSF of Acid Hydrolysates

The pretreatment releases reducing sugar and glucose from the two biomasses (Table 1). In the wheat bran hydrolysate, 3.2 g of glucose/l hydrolysate was released, and in the corn cob hydrolysate, 4.2 g of glucose/l hydrolysate was released. However, IA was not produced during LSF of the hydrolysates from the diluted acid pretreated biomasses.

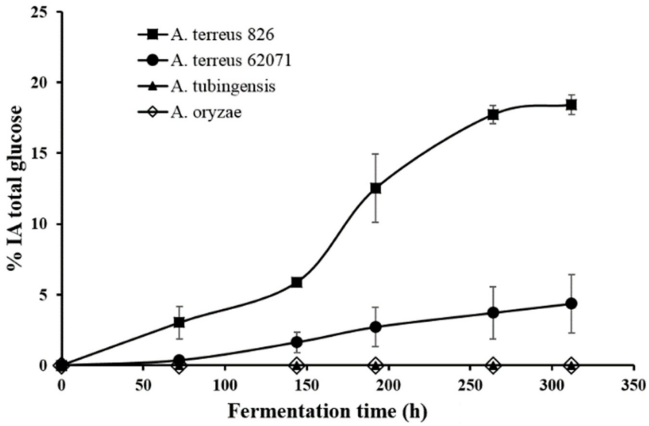

Fig. 2. IA production by *Aspergillus* strains in optimized culture medium.

The *A. terreus* strains produced the highest levels of FA, especially in the corn cob hydrolysate at pH 6 with a yield of approximately 2% of FA from the initial glucose (Fig. 3). *A. oryzae* produced 8-times less FA, and *A. tubingensis* did not produce FA at all from the corn cob hydrolysate. On the contrary, these two strains showed better FA yields from wheat bran hydrolysate at pH 3.

Enzymatic Treatment of Biomasses

Sugar release from biomasses. Fungi enzymatic cocktails are well known to produce a large diversity of cell-wall-degrading enzymes [28]. High cellulase activity is crucial

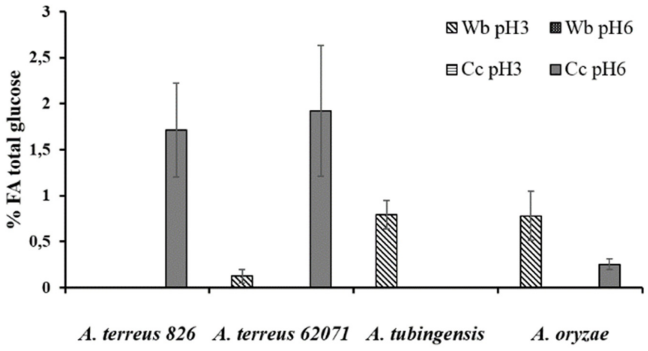

Fig. 3. Fumaric acid production in liquid-state fermentations of acid-pretreated biomass hydrolysates.

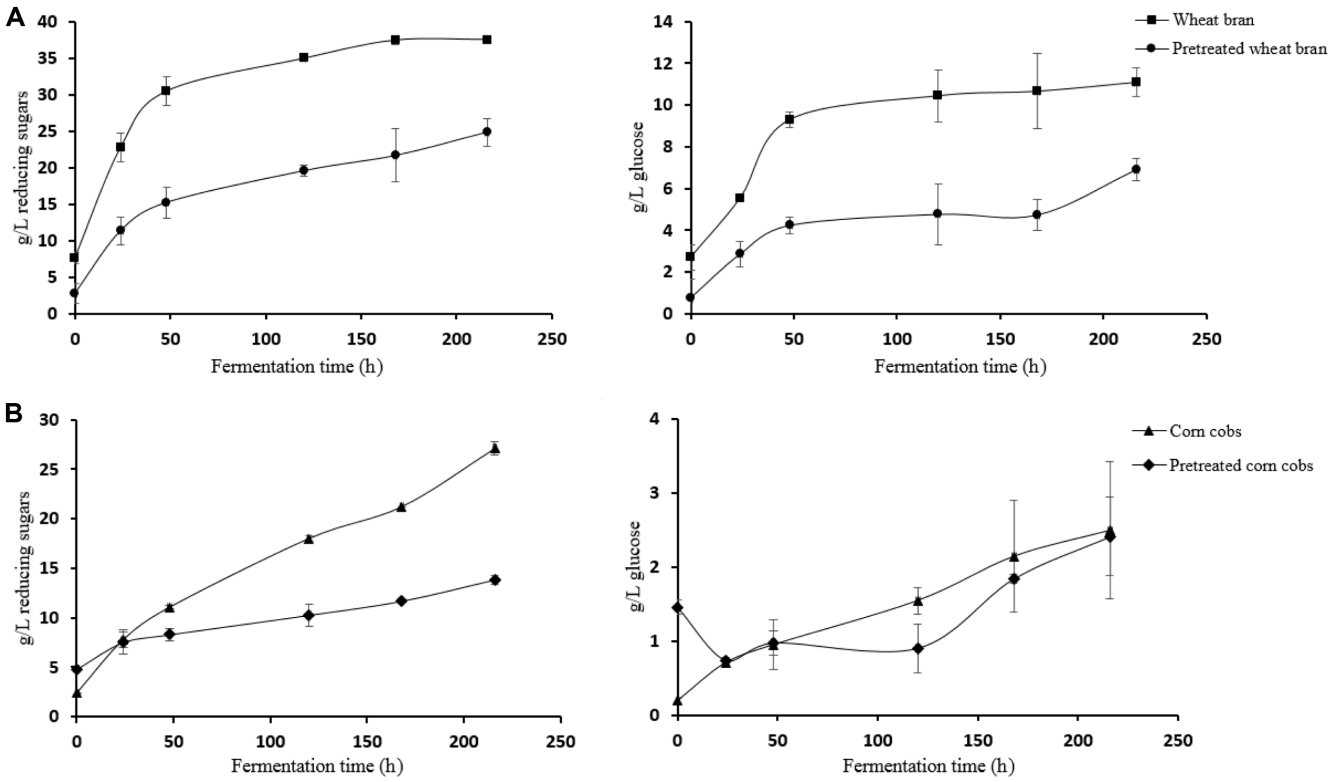

Fig. 4. Sugars released from biomasses after enzymatic treatment (216 h) with an *A. tubingensis* cocktail.
(**A**) From wheat bran. (**B**) From corn cobs.

for glucose release from biomasses [3]. Consequently, the *A. tubingensis* enzyme cocktail that displayed the highest cellulase activity (data not shown) was chosen for this study.

The enzymatic hydrolysis was conducted on both the raw and the acid-pretreated biomasses. As shown in Fig. 4, the biological treatment released more reducing sugars and glucose from the raw biomasses. Glucose concentration after 216 h from the raw wheat bran (11.09 g/l) was 4.6-times greater than that for the corn cobs (2.49 g/l), and the reducing sugars were 1.4-times higher in the raw wheat bran (37.53 g/l vs. 27.12 g/l).

LSF of enzymatic hydrolysates. The use of two fungal cocktails (see Materials and Methods) produced liquid hydrolysates from raw lignocellulosic biomasses with their respective sugar concentrations shown in Table 1. Almost 2-times more glucose was liberated from the biomasses treated with the *A. oryzae* enzymatic cocktail. The enzymatic hydrolysates were collected as previously described to perform LSF with the four *Aspergillus* strains. The LSF lasted for 168 h, and the acids produced are shown in Fig. 5. Whereas FA was produced by the four strains, IA was produced by only three (not by *A. tubingensis*). *A. terreus* 826 and *A. oryzae* produced 0.12% and 0.14% IA from the initial glucose, respectively, from the corn cob hydrolysate produced by treatment with the *A. tubingensis* enzymatic cocktail.

FA was produced at the highest concentration by *A. terreus* 826 in the enzymatic hydrolysate of raw wheat bran from the *A. oryzae* enzyme cocktail, with a yield of 1.07% from the initial glucose (14.9 g/l).

LSF of Diluted Hydrolysates

Based on the comparison between the optimized culture medium and analyses shown in Table 1, some constituents in the hydrolysates were too concentrated. The more concentrated metals, mostly in the acid hydrolysates, such as calcium and sodium, must be reduced to approach the appropriate concentrations in the optimized culture medium to facilitate IA production. The dilution of the hydrolysates also reduces the presence of potential inhibitors such as phenol compounds.

Unfortunately, the fermentation of different acids and enzymatic hydrolysates after dilution did not result in

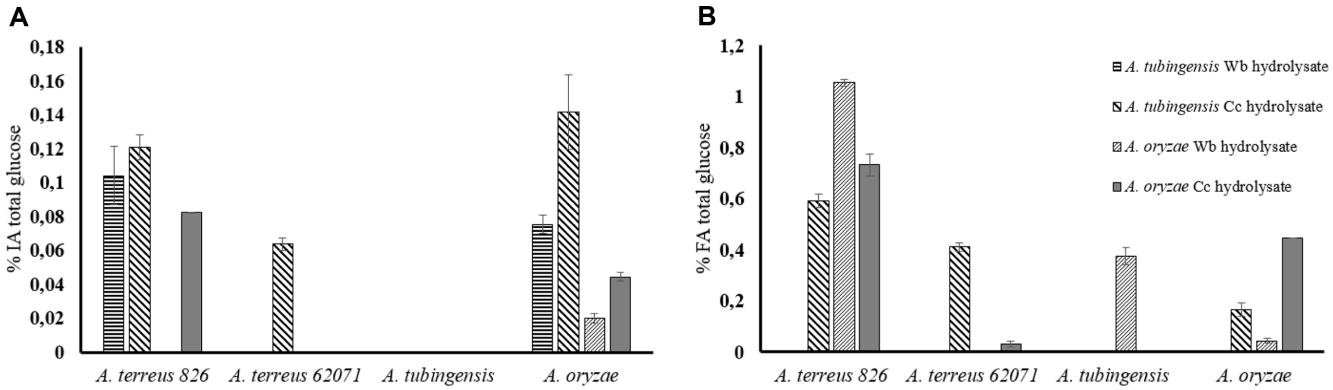

Fig. 5. Production of IA and FA in the enzymatic hydrolysates. (**A**) IA production and (**B**) FA production.

increased acid production.

The low acid production following hydrolysate dilution could be due to the reduced sugar concentration. Therefore, glucose was added to reach to the initial glucose concentration of the optimized culture medium (180 g/l). The fermentation was performed for the acid and enzymatic hydrolysates with a 10-times dilution and glucose added. No IA was formed with any of the strains tested. FA was produced from the wheat bran diluted acid hydrolysate by *A. tubingensis* and *A. oryzae* (0.04% and 0.03% total glucose, respectively), and the four strains produced FA from the corn cob diluted acid hydrolysate, with the highest yield of 0.09% total glucose by *A. terreus* 826. In the same way, this strain produced the maximum FA from the corn cob diluted enzymatic hydrolysate with 1.21% total glucose. As expected, the addition of metals but not glucose did not allow for any acid production.

To complete this experiment, other concentrations of sugars and metals were added to the diluted hydrolysates in order to mimic the optimized culture medium. For the diluted acid hydrolysates with additions, the production yields are shown in Fig. 6. All of the strains produced FA from the two biomasses, but the corn cob diluted acid hydrolysate displayed the best IA production (0.018% total glucose). The *A. terreus* strains also produced IA from wheat bran diluted acid hydrolysate, but these yields were 900-times and 130-times lower than those from the corn cob hydrolysates for *A. terreus* 826 and 62071, respectively. The most surprising result was the IA production by *A. oryzae* (1.6% total glucose) because this strain could not produce IA during LSF of optimized culture media. The introduction of corn cob hydrolysate into the fermentation medium contributed to fulfill the requirement for *A. oryzae* to produce IA.

Discussion

In the present study, FA was produced by four different *Aspergillus* strains (*A. terreus 826*, *A. terreus 62071*, *A. oryzae*, and *A. tubingensis*). In contrast, IA was only produced by the *A. terreus* strains and *A. oryzae*. Furthermore, although the production of FA was quite general, IA was only

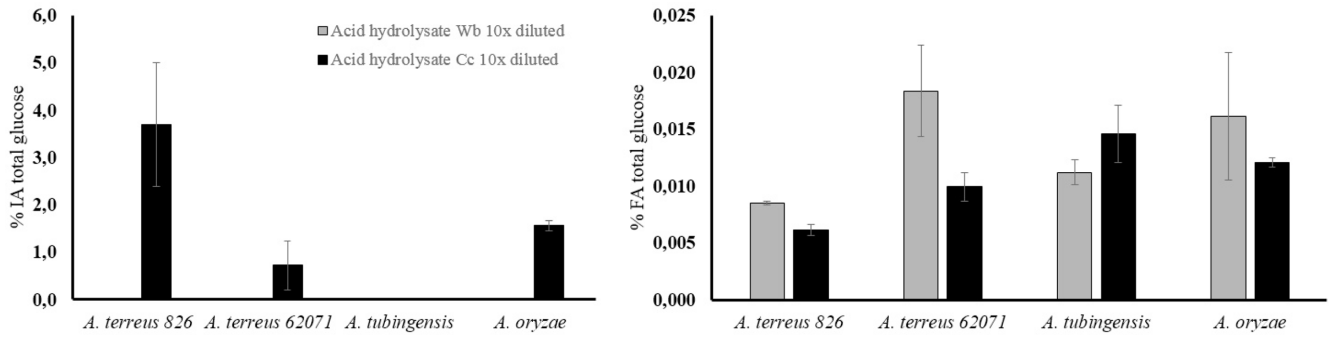

Fig. 6. IA and FA production from 10-times diluted acid hydrolysates with the addition of glucose and metals.

produced in a few specific fermentation conditions. These results are quite surprising because a recent study reported that these three species were not found previously as FA producers [20]. Moreover, the capacity to produce IA was only shown by *A. terreus* [20]; nonetheless, our study proves the IA production by *A. oryzae* also.

IA is produced by *cis*-aconitate decarboxylase (CAD, Fig. 1), an enzyme found and characterized in *A. terreus* [5]. Recently, some studies have focused on cloning this enzyme to produce IA in host microorganisms such as *E. coli* or *A. niger* [13, 32]. In our work, the production of IA by *A. oryzae* is described for the first time, suggesting the presence of CAD in this species. An examination of the *A. oryzae* genome revealed a gene encoding a protein (AO090010000161) displaying 54% identity and 69% similarity with CAD of *A. terreus* (ATET_09971), supporting this hypothesis.

The LSF in optimized culture medium confirmed the ability of *A. terreus* strains to produce IA. *A. terreus* 826 produced 90 g/l of IA in a totally controlled bioreactor with 180 g/l of glucose, resulting in a yield of 0.5 g IA/g total glucose [16]. Our experiments were conducted in flasks in an uncontrolled atmosphere. This could explain the lower production yields (0.18 g IA/g total glucose) as pH and aeration control is necessary for high yields [7, 10, 29]. *A. terreus* 62071 displayed a lower production. In contrast, FA was not formed by any strain. This could be because the medium was optimized for IA production by *A. terreus*, and the other species have different requirements.

When the medium was prepared with diluted acid hydrolysates and with addition of glucose and metals (Fig. 6), the four strains produced FA. The addition of wheat bran and corn cob hydrolysates induced similar FA production. Nevertheless, IA was produced at greater concentrations when diluted acid corn cob hydrolysates were used, even if the metals and phosphates were at the same concentrations. A possible explanation for this is that each hydrolysate has a particular composition that affects the fungal growth and interferes with IA production [8].

Biomass treatment, which permits sugar release from the polysaccharide chains of lignocellulose, also released some inhibitors of microorganism metabolism and interfered with the fermentation yield [31, 37]. The performance of biomass hydrolysates as fermentation media varies owing to the presence of inhibitory compounds and their concentrations. After acid treatment, the phenolic compounds were more concentrated in the wheat bran hydrolysate than in the corn cob hydrolysate (Table 1). Indeed, IA production was impaired after this treatment, although FA production was unaffected. Unlike IA, FA is a primary metabolite, and as the four strains grow in the acid hydrolysate, it is not surprising that FA was produced.

FA displayed the highest production yield (1.9% total glucose) for *A. terreus* in LSF of the corn cob acid hydrolysate. A possible explanation for this is that the corn cob hydrolysate is richer in glucose than is the wheat bran hydrolysate, with 4.2 and 3.2 g /l, respectively.

The enzymatic treatment study showed that raw biomasses are better alternatives than acid-treated ones. The comparison of two fungal enzyme cocktails displayed an unexpectedly greater extent of sugar liberation with the *A. oryzae* enzyme cocktail, which supported the highest FA yield (1.07% total glucose). This cocktail also released higher manganese concentrations from both biomasses. However, a manganese ion concentration less than 3 ppb seems necessary to obtain the greatest IA yield [14]. This requirement is close to fulfilled with the corn cob hydrolysate treated with the *A. tubingensis* enzyme cocktail, and thus the IA production yield was higher under these conditions.

In the same way, the acid treatment of biomasses liberated higher concentrations of sodium, 5 g/l from wheat bran and 6 g/l from corn cobs, compared with the enzymatic hydrolysates (Table 1). However, the optimized culture medium did not contain any sodium source, and the presence of sodium could be deleterious for IA production.

This production of IA could be compared with the production in the optimized medium by *A. terreus* 826, the positive control. The IA yield production was 18% from the initial glucose; that is, 160-times the production from the enzymatic corn cob hydrolysate. However, a high sugar concentration is a critical parameter for IA production by *A. terreus*, and the corn cob enzymatic hydrolysate had 50-times less glucose than did the optimized culture medium. Therefore, the results obtained for IA and FA production were lower than current production by fermentations with glucose as the carbon source [26].

In summary, the use of lignocellulosic biomass with enzymatic treatment brings us closer to potentially renewable production of IA and FA. Furthermore, the identification of *A. oryzae* as an IA and FA producer leaves open the possibility of a simultaneous saccharification and fermentation, thanks to the great enzymatic capacity of this fungal strain [4, 30]. This option is better suited for an industrial process because the treatment time could be reduced, and the cellulosic enzymes formed by the microorganism are also commercial products [1]. Furthermore, the results obtained could boost future research about the organic acid metabolism and metabolic engineering (gene expression,

metabolic fluxes, coenzyme availability, *etc*.) in this species. The biorefinery concept can become economically and environmentally more interesting by the generation of multiple products with the optimal utilization of renewable resources.

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