

Fungal Fermentation of Lignocellulosic Biomass for Itaconic and Fumaric Acid Production ^S

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The production of high-value chemicals from natural resources as an alternative for petroleum-based products is currently expanding in parallel with biorefinery. The use of lignocellulosic biomass as raw material is promising to achieve economic and environmental sustainability. Filamentous fungi, particularly *Aspergillus* species, are already used industrially to produce organic acid as well as many enzymes. The production of lignocellulose-degrading enzymes opens the possibility for direct fungal fermentation towards organic acids such as itaconic acid (IA) and fumaric acid (FA). These acids have wide-range applications and potentially addressable markets as platform chemicals. However, current technologies for the production of these compounds are mostly based on submerged fermentation. This work showed the capacity of two *Aspergillus* species (*A. terreus* and *A. oryzae*) to yield both acids by solid-state fermentation and simultaneous saccharification and fermentation. FA was optimally produced at by *A. oryzae* in simultaneous saccharification and fermentation (0.54 mg/g wheat bran). The yield of 0.11 mg IA/g biomass by *A. oryzae* is the highest reported in the literature for simultaneous solid-state fermentation without sugar supplements.

Keywords: Lignocellulosic biomass, solid-state fermentation, submerged fermentation, *Aspergillus oryzae*

Introduction

Itaconic acid (IA) and fumaric (FA) acid are products of the metabolic pathways of microorganisms and are intermediates of the oxidative portion of the TCA cycle [1]. Owing to their carboxylic functions, these diacids are considered key platform chemicals (or building blocks). For instance, both acids were among the top 12 biomass-derived platform chemicals selected by the US Department of Energy with several high-value applications and emerging markets for fine chemicals, pharmaceuticals, and materials [2].

IA and FA can be commonly produced by filamentous fungi in high concentrations [3]. These microorganisms stand out for their diverse portfolio of products, such as enzymes, organic acids, alcohols, single-cell oils, proteins (amino acids), biopolymers (chitin/chitosan and glucans), antibiotics, and other bioactive compounds. The genus *Aspergillus* is

particularly interesting, with more than 250 species, including commercially exploited species (e.g., *A. niger*, *A. oryzae*, *A. sojae*, and *A. terreus*). *Aspergillus* can be used for solid-state fermentation (SSF) or submerged fermentation (SmF). Some fermentation protocols have been established for large-scale industrial processes. Since 1960, the industrial production of IA has been achieved by fermentation with *A. terreus* in liquid glucose-containing media (>100 g/l) [4, 5]. The strain NRRL1960 has demonstrated the highest yield: 129 g/l [6]. In contrast, FA bioproduction is still in its early stages, and the corresponding reported yields [7] have yet to be competitive compared with equivalent chemical synthesis from petrochemical feedstock.

Currently, research is focused on viable, renewable, and environmentally friendly alternatives to replace conventional resources (i.e., fossil fuel derivatives) for the production of chemicals. In this context, the biorefinery concept can be defined as the full integration of incoming biomass for

simultaneous production of different compounds such as food, feed, materials (fibers, papers, *etc.*), energy, and chemicals with added value. Corresponding biomass streams should be economically and ecologically sustainable [8]. To also achieve social sustainability, lignocellulose, an inedible low-value byproduct of the agriculture or forestry industries, could be used as a valuable resource [9, 10]. This biomass is the most abundant carbon feedstock on Earth [11]. However, lignocellulosic biomass is a recalcitrant material that often needs pretreatment to achieve effective disentanglement of its complex multiphase structure, separating the main components (cellulose, hemicellulose, and lignin) [12].

Filamentous fungi can be grown on renewable resources such as lignocellulosic biomass owing to their capacity to hydrolyze biopolymers to yield easily assimilable energy sources [13]. In the last few decades, some investigations have focused on the production of IA from renewable biomass, first from starchy materials such as corn starch, molasses, or grains, achieving the production of 0.36 g of IA/g of sago starch [14] or 48.70 g/l from *Jatropha* seed cake hydrolysates [15]. *Rhizopus* species were shown to be the best producers of FA. For instance, FA was produced from corn straw with a yield of 0.35 g of FA/g of consumed sugar [16].

Filamentous fungi are particularly adapted to SSF because the substrate also provides a support for the growth of microorganisms via intimate contact between the organism and the biomass [17]. This phenomenon is abundantly used for the production and excretion of enzyme cocktails [18]. Furthermore, several operational and technical challenges exist with SmF processes involving filamentous fungi, hindering economical and commercial-scale adoption. These drawbacks could possibly be alleviated by the use of SSF technology [7, 19, 20].

The aim of this study was to investigate and optimize the use of wheat bran and corn cobs as cheap and common lignocellulose sources for the microbial production of two dicarboxylic acids, IA and FA, respectively. Four strains of *Aspergillus* were employed, and the fermentation strategy comprised SSF and SmF. Additionally, a simultaneous saccharification and fermentation process was investigated to verify the efficiency of enzyme cocktail production by SmF and SSF.

Materials and Methods

Microorganisms

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). *A. terreus* strains (DSM 826 and DSM 62071) were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The strains were revived in potato dextrose broth medium for 5–6 days at 25°C. The microorganisms were then grown and sporulated on potato dextrose agar (PDA). The spore suspensions were harvested from 5–6-day-old PDA plates with 0.2% (v/v) Tween-80. The spores were counted using a Malassez counting chamber and stored at –20°C.

Substrates

The agricultural waste biomasses used as carbon sources were wheat bran and corn cobs obtained from Comptoir Agricole (France). The lignocellulosic material was crushed (SX 100; Retsch) to obtain particles 0.5–1 mm in size. The results of the physicochemical characterizations of these solid substrates are given in Table 1. The chemical elementary analyses were performed by SOCOR (France). The water activity (A_w) was measured with 1 g of dry substrate with a Fast-lab A_w meter (GBX, France).

Solid-State Fermentation

In a glass flask, 5 g of solid substrate was autoclaved at 121°C

Table 1. Water content and chemical analysis of raw wheat bran and corn cobs.

	Wheat bran	Corn cobs
Dry substrate A_w	0.629	0.391
Hydrated substrate A_w^a	0.982	0.984
Carbohydrate composition (% dry wt)	[25]	[33]
Cellulose	10.5–14.8	32.3–45.6
Hemicellulose	35.5–39.2	39.8
Lignin	8.3–12.5	6.7–13.9
Sugars (mg/g of dry wt)		
Reducing sugars	387.1	389.2
Glucose	261.2	57.5
Total nitrogen (% dry wt)	2.95	0.44
Metals (mg/g of dry wt)		
Calcium total	1,078.9	280.4
Iron total	120.61	119.05
Magnesium total	4,988.4	275.6
Manganese total	167	8
Potassium total	14,414.6	7,094.4
Sodium total	53.13	11
Zinc total	89	20
Anions (mg/l)		
Orthophosphates	15,300	669

^a120% (w/v) water for wheat bran and 90% (w/v) water for corn cobs. A_w , water activity; wt, weight.

and 3 bars for 20 min. The substrates were inoculated with spore suspensions to achieve an initial content of 10^6 spores/ml. Initial moisture was adjusted to 120% for wheat bran and 90% for corn cobs (both corresponding to an A_w close to 1). After thorough mixing, the flasks were covered with gas exchange film and incubated at 30°C for 6 days. Unless otherwise specified, these fermentation conditions were maintained throughout the study. All the experiments were conducted in duplicates. After fermentation, the samples were recovered by mixing the fermented substrate with sterilized distilled water (7 ml/g of initial dry substrate). The preparations were centrifuged (8,000 $\times g$ for 15 min) to eliminate residual solids. Then, another round of centrifugation was performed on the supernatants to remove mycelia and spores (13,600 $\times g$ for 30 min). The resulting liquid was finally filtered through a 0.2 μm membrane. The samples were then analyzed by high-performance liquid chromatography (HPLC) or stored at -20°C for additional analysis.

The fermentations for enzyme cocktail preparation were performed under equivalent conditions, with the exception that the inocula were prepared at pH 10 with Tris buffer. The incubation temperature was 25°C. Samples were analyzed to determine corresponding enzyme activities for the four *Aspergillus* strains. As activities were much higher (see Results), large-scale enzyme cocktail production was performed in plates containing 200 g of wheat bran, using *A. tubingensis* and *A. oryzae*. One liter of enzyme cocktail was recovered for each experiment.

Submerged Fermentation

Autoclaved corn cobs (5 g) or wheat bran (5 g) were inoculated with spore suspensions at 10^5 spores/ml in 125 ml Erlenmeyer flasks with 60 ml of H₂O. The flasks were incubated on a rotatory shaker (Infors Multitron, Switzerland) for 7 days at 33°C with shaking at 120 rpm. Samples of 500 μl were taken at regular intervals, filtered through a 0.2 μm membrane and stored at -20°C for further analyses. At 96 h, samples were collected to measure enzyme activities. All experiments were conducted in duplicates.

Simultaneous Saccharification and Fermentation

Two conditions, (i) a simultaneous SmF and (ii) a simultaneous SSF, were employed, both with the enzyme cocktails from *A. tubingensis* and *A. oryzae*. The fermentative conditions were equivalent to those described above for SmF and SSF with the exception that the enzyme cocktails were used for spore inoculation and biomass humidification instead of water. The use of enzyme cocktails allowed biomass saccharification thanks to their cell wall-degrading enzymes.

Analytical Procedures

Enzyme assays. Enzyme cocktail activities were measured using chromogenic substrates, specifically azurine-crosslinked (AZCL) polysaccharides such as AZCL-HE-cellulose, AZCL-xylan, AZCL-xyloglucan, or AZCL-amylose (Megazyme, Ireland). The corresponding activities were determined by monitoring the

solubilization of dyed compounds and measuring the absorbance of the supernatant at 595 nm. The substrates were prepared at 0.4% (w/v) in 0.1 M sodium acetate buffer at pH 5. The reactions were performed with 950 μl of AZCL substrates and 50 μl of diluted enzyme cocktail samples for 30 min and incubated with overhead rotation at 25°C. The supernatants were isolated by centrifugation at 13,600 $\times g$ for 1 min. All spectrophotometric measurements were performed using a Genesys 10 Bio spectrophotometer (Thermo, USA). Given that the molar extinction coefficient of AZCL was unknown, the enzyme activities were expressed in arbitrary units corresponding to optical density variations per minute and per milliliter of the supernatant.

Sugar assays. To determine the total available sugar contents in wheat bran and corn cobs, 0.5 g of solid substrates was subjected to acid hydrolysis using incubation in 5 ml of 0.4 M HCl at 100°C for 2 h [21]. Glucose was measured by a colorimetric method at 420 nm [22]. Reducing sugars were determined by the dinitrosalicylic acid method, with spectrophotometric measurements at 550 nm [23].

Organic acid assays. Chromatographic separation was achieved on a hypercarb porous graphitic carbon LC column (150 \times 3.0 mm i.d., 3 μm ; Thermo Scientific, USA) at 70°C. HPLC analysis was performed using a 616 pump, a 2996-photodiode array detector operating in a range from 200 to 450 nm, and a 717 Plus autosampler controlled with Empower 2 software (all from Waters, USA). 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: (i) 0% B (0–5 min), (ii) 0–27% B (5–18 min), and (iii) 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 . The calibration was performed using commercial IA and FA with 99.9% purity (Sigma-Aldrich, USA). Each sample was supplemented with 10 ppm IA or FA as an internal standard, and the organic acids were detected at 205 nm.

Results and Discussion

Solid State Fermentation

Raw biomasses were fermented by four *Aspergillus* strains. Total available glucose, determined from the liberated glucose after acid hydrolysis, was found to be 261.5 mg of glucose per gram of wheat bran and 57.5 mg per gram of corn cobs (Table 1). These results were corroborated by the mycelial development of the four strains, in which mycelia were clearly larger on wheat bran compared with corn cobs. FA was produced by the four strains from both biomasses (Fig. 1). When yields were calculated with respect to the total glucose, corn cobs gave better results for the production of FA for all strains (*i.e.*, *A. oryzae* produced 0.31% and 0.03% FA from total glucose with corn cobs and

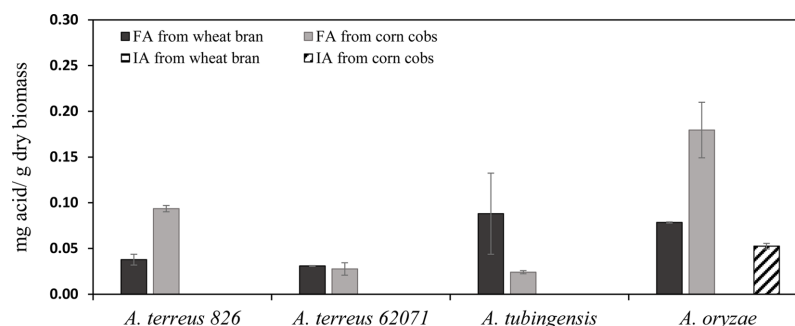


Fig. 1. Production of itaconic and fumaric acids using solid-state fermentation by four *Aspergillus* strains on the biomasses.

wheat bran, respectively). FA is a primary metabolite, so it is not surprising that this acid was concomitantly produced as the four strains grew. Conversely, IA is a secondary metabolite and was produced only by *A. oryzae* and only with corn cobs, with a yield of 0.05 mg of IA/g of biomass. IA production was previously demonstrated to be optimal under phosphate limitation [5]. The phosphate content was 23 times lower in corn cobs compared with wheat bran (Table 1). This gap explains the highest results obtained with corn cobs, as already demonstrated with the hydrolysates in liquid-state fermentation [24]. *A. oryzae* also produced the highest quantity of FA at 0.18 mg/g of corn cobs. The strain *A. terreus* 826, even though it is industrially employed as an IA producer, did not yield IA under these conditions but produced 0.09 mg of FA/g of corn cobs. Thus, the combination of *A. oryzae* and corn cobs produced the most promising results, converting 0.31% FA and 0.09% IA from the total available glucose.

IA production is dependent on *cis*-aconitate decarboxylase (CAD), an enzyme found in *A. terreus* [4]. The production of IA by *A. oryzae* suggests the presence of CAD in this species. A thorough search of the *A. oryzae* genomic database indicated a gene-encoding protein (AO090010000161) exhibiting 54% identity and 69% similarity to CAD of *A. terreus* (ATET_09971), supporting this hypothesis.

Submerged Fermentation

Submerged fermentation was performed with the four strains and produced varied results for acid production (Fig. 2). *A. tubingensis* gave the best results for FA production with the two biomasses, yielding 0.19 and 0.11 mg/g of biomass for wheat bran and corn cobs, respectively. Both *A. terreus* strains produced FA only with corn cobs. *A. oryzae* produced FA with wheat bran (0.065 mg of FA/g) and demonstrated a lower yield with corn cobs (0.011 mg of FA/g).

IA was produced by *A. oryzae* from corn cobs with a yield

of 0.076 mg of IA/g, which was 1.5 times higher than production in SSF. Furthermore, SmF facilitated IA production by the two strains of *A. terreus* in contrast with SSF. As with *A. oryzae*, these *A. terreus* strains produced IA only from corn cobs. This is likely due to phosphate availability [5]. Furthermore, the higher cellulose content in corn cobs (roughly 3 times more than in wheat bran; Table 1) eases the liberation of glucose from this biomass, as already reported [25].

Enzymatic Treatment of Biomasses

The biological treatment of lignocellulosic biomasses represents an interesting approach for the better solubilization of sugar from native biopolymers [26, 27]. Such enzymatic treatments employ cocktails of enzymes containing different types of cellulases and hemicellulases that are able to convert biopolymers into assimilable sugars.

To generate an appropriate enzyme cocktail adapted to the complexity of the biomasses of interest, and also to test the feasibility of coupled biomass valorization (IA/FA and enzyme production), fermentation was performed with the four strains and two biomasses under investigation in SSF and in SmF conditions. Enzyme activities obtained by SmF (Table S1) were lower for the four microorganisms than

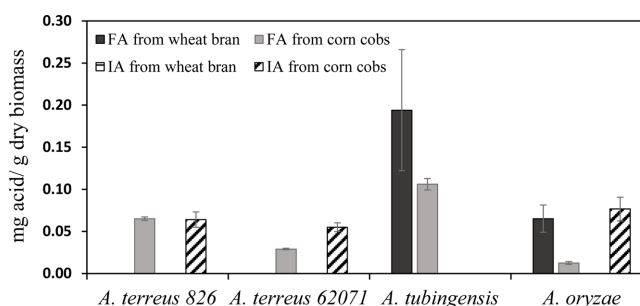


Fig. 2. Production of itaconic and fumaric acids using submerged fermentation by four *Aspergillus* strains on the biomasses.

Table 2. Enzymatic activities of *Aspergillus* strain cocktails in solid-state fermentation on wheat bran and corn cobs.

	<i>A. terreus</i> 826		<i>A. terreus</i> 62071		<i>A. tubingensis</i>		<i>A. oryzae</i>	
	Wb	Cc	Wb	Cc	Wb	Cc	Wb	Cc
α -Amylase activity	1.63	0.18	1.59	0.22	24.90	0.17	76.25	0.22
Cellulase activity (cellulose)	0.23	0.03	0.75	0.00	2.70	0.01	2.47	0.00
Endoxylanase activity	1.75	0.00	2.10	0.00	72.81	0.33	63.70	0.07
Cellulase activity (xyloglucan)	0.19	0.01	0.27	0.02	3.10	0.05	1.96	0.02

All experiments were performed at pH 5 and at 25°C.

Activity in OD/g·min.

those from SSF (Table 2). These results are consistent with previous studies [17]. As a result, SSF was chosen to produce the enzyme cocktails. Differential growth with wheat bran and corn cobs was evident (Fig. 3). After 2 days of cultivation, fungal mycelia and spores were formed with wheat bran. In contrast, fermentation of corn cobs clearly resulted in delayed development for the four strains. In relatively good agreement, all enzymatic activities (Table 2) obtained with corn cob fermentation were null or very low, in contrast to the enzyme activities obtained with wheat bran. *A. tubingensis* and *A. oryzae* exhibited higher xylanolytic activities, which were more than 30 times greater than the activities of *A. terreus*. Endoxylanase activity, which is crucial

for sugar release from the hemicellulose fraction, was higher for *A. tubingensis*. In contrast with the *A. tubingensis* cocktail, the *A. oryzae* cocktail exhibited higher amylase activity. However, starch constitutes only a small portion of these agricultural resources.

Simultaneous Saccharification and Fermentation

Previous studies have shown that simultaneous saccharification and fermentation appears to be an interesting process for the optimization of biomass valorization [28]. In this process, the enzyme cocktail is directly added to wet the biomass, becoming the liquid phase during fermentation. Simultaneous saccharification and fermentation were

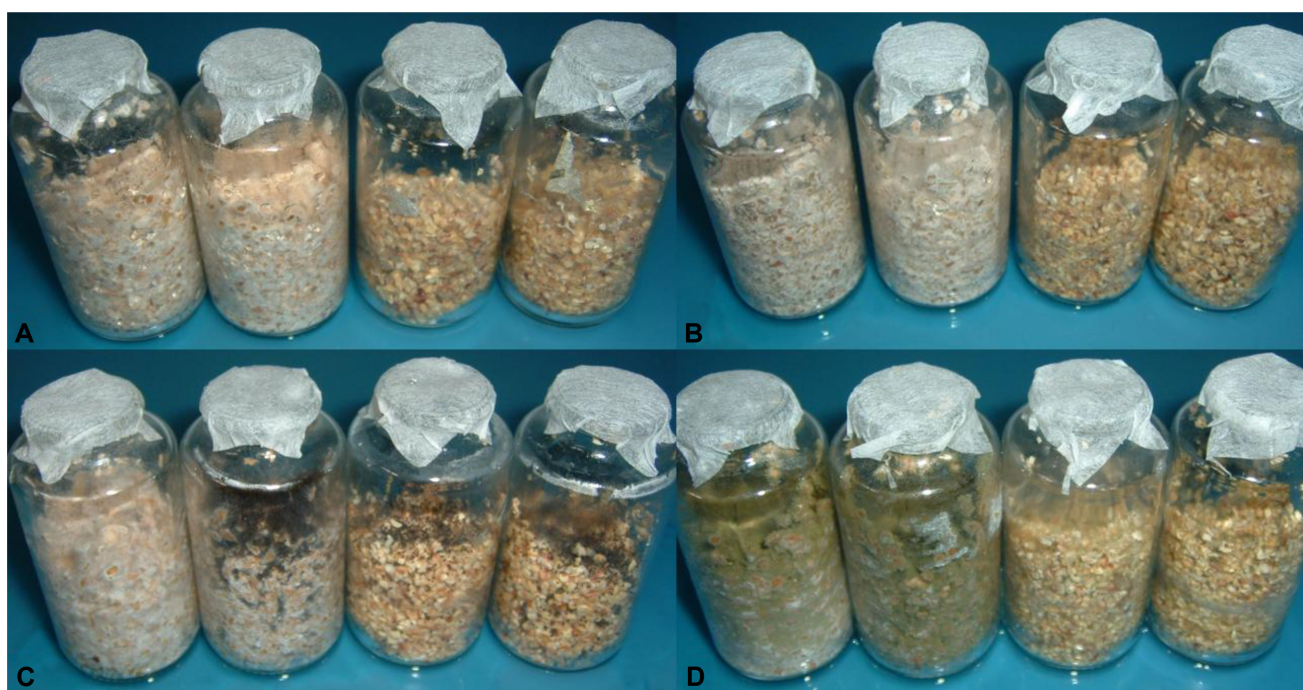


Fig. 3. Pictures of SSF for enzymatic cocktail production with the two biomasses (two left glass flasks: wheat bran; two right flasks: corn cobs).

(A) *A. terreus* 826. (B) *A. terreus* 62071. (C) *A. tubingensis*. (D) *A. oryzae*.

performed in a submerged manner (SmF) and in SSF. In both cases, the enzyme cocktails of the *Aspergillus* strains (*A. tubingensis* or *A. oryzae*) prepared as described in Materials and Methods were used as the liquid phase.

Fermentations Using *A. tubingensis* Enzyme Cocktail

The production of FA was generally similar for all conditions in simultaneous SSF (Fig. 4A). The FA yields were quite similar to those from SSF with raw biomasses. The yields calculated from total glucose indicated that *A. tubingensis* gives the highest overall yield for FA production, with 0.09% recovery from simultaneous SSF of corn cobs. When production was determined in relation to the biomass weight, higher FA levels were obtained using SmF of wheat bran (Fig. 4B).

As already observed for SSF and SmF in this study, IA was produced by *A. oryzae*. However, the highest yield was observed for simultaneous SSF (Fig. 4A) with 0.11 mg of IA/g corn cobs, which is more than twice that obtained with SSF (0.05 mg of IA/g; Fig. 1). In simultaneous SSF, *A. oryzae* demonstrated a yield of 0.19% IA from total available glucose, more than twice the production observed in SSF with corn cobs (0.09% IA from total glucose). These results show the large effects of the enzyme cocktail for the liberation of sugars (Table S2) to facilitate microbial fermentation and therefore enhance organic acid production, as reported previously for *Aspergilli* [29]. For the same reason, the enzymatic treatment of biomasses in simultaneous SSF allowed IA production for *A. terreus* 826, whereas IA was not produced in SSF (Fig. 1). However, glucose availability is not sufficient to explain all of the obtained results. Indeed, both strains of *A. terreus* produced IA from corn cobs in SmF (Fig. 2) but not in simultaneous SmF (Fig. 4B). It could be that inhibitory compounds are released by the action of enzymes and solubilized in the aqueous environment to disturb the metabolism [29]. This is another

illustration of the versatile nature of secondary metabolite production.

Additionally, simultaneous fermentation permits a significant reduction in process duration in comparison with current processes for liquid-state fermentation of biomass hydrolysates, which involve two successive unitary operations of biomass hydrolysis and subsequent fermentation; for instance, 4 days instead of 6 days for IA production from corn starch [4].

Fermentations Using *A. oryzae* Enzyme Cocktail

To further investigate IA production, and considering the results obtained with the *A. tubingensis* cocktail, we decided to use the enzymatic cocktail produced by *A. oryzae* for the two strains (*A. terreus* 826 and *A. oryzae*) that demonstrated the ability to produce IA.

Experiments with the *A. oryzae* enzymatic cocktail resulted in the highest FA yield observed in this study by SSF on wheat bran (Fig. 5), with 0.54 mg/g of dry biomass. This was 12 times the yield obtained under the same conditions with the cocktail of *A. tubingensis* (0.045 mg of FA/g). This result can be explained by the use of a more specific and adapted cocktail for this biomass, as it was produced by the same strain. Moreover, the use of this cocktail facilitated IA production with wheat bran in SSF and in SmF for *A. oryzae*, which was not observed before (0.010 and 0.012 mg/g of biomass, respectively). Conversely, the yields from the corn cob biomass were lower than the yields obtained with the *A. tubingensis* cocktail: 5 and 2 times less than SSF and SmF, respectively. IA was also produced by *A. terreus* 826, both in simultaneous SSF and simultaneous SmF.

Corn grains have previously been used as a resource for FA production [30]. In comparison, our experiments with SSF produced lower yields. However, cobs are sustainable by-product resources and do not compete with food. Concerning IA production by SSF, the literature is poor,

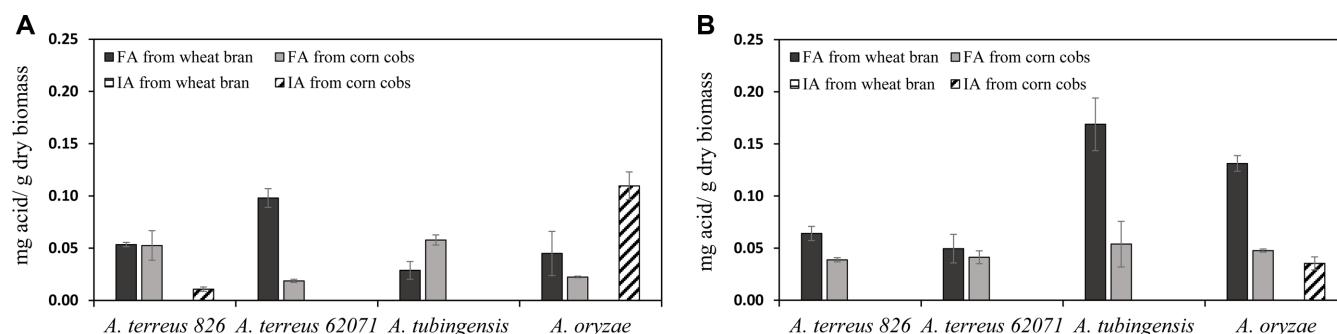


Fig. 4. Simultaneous saccharification and fermentation with the enzymatic cocktail of *A. tubingensis*. Acid production by four *Aspergillus* strains. (A) solid-state fermentation and (B) submerged fermentation.

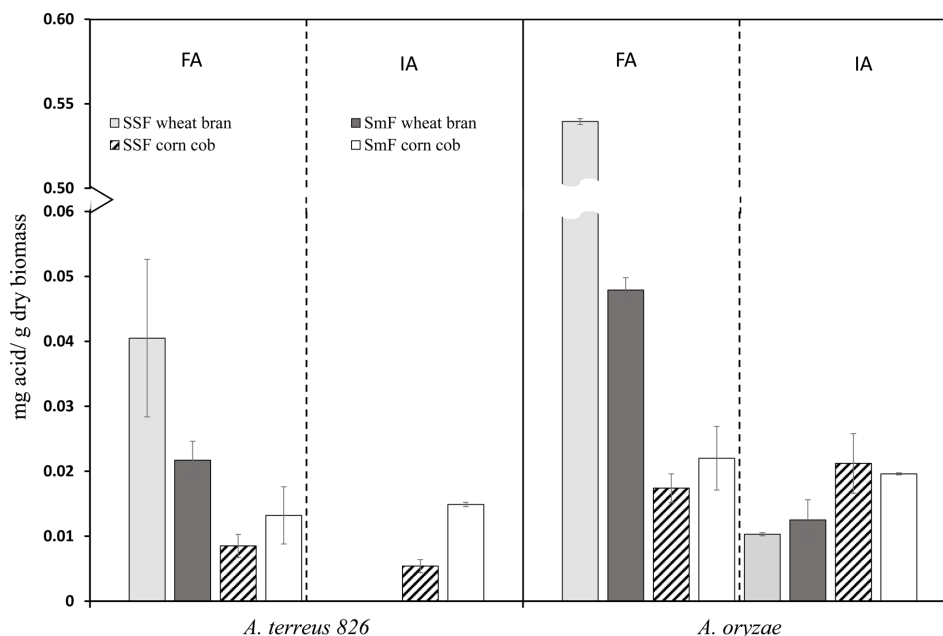


Fig. 5. Simultaneous saccharification and fermentation with the enzymatic cocktail of *A. oryzae*. Acid production by *A. terreus* 826 and *A. oryzae*. SSF, solid-state fermentation; SmF, submerged fermentation.

and the only data available indicate the production of 0.036 mg/g of sugarcane press mud (0.004% total glucose; Tsai *et al.* 2001. Method for the production of itaconic acid using *Aspergillus terreus* solid state fermentation. US6171831). In that paper, however, IA production was supported by a supply of glucose (100 g/l), nitrate, and other salts. Peeled sugarcane press mud was added to support growth. Our system (*A. oryzae*/corn cobs) proved to be more efficient for IA production in simultaneous SSF with the *A. tubingensis* cocktail: 0.11 mg/g of corn cobs and 0.03% of total glucose. Conditions for the optimal growth of *A. oryzae* in SSF and SmF have been described in different studies [31, 32]. However, enzyme and organic acid production by this fungus could be further improved.

In conclusion, the most promising strategy for the production of itaconic and fumaric acids with lignocellulosic biomass is simultaneous SSF, and the entire process can be achieved with a single fungal species, *A. oryzae*.

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