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Direct fungal fermentation of lignocellulosic biomass into itaconic, fumaric, and malic acids: current and future prospects

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Abstract Various economic and environmental sustainability concerns as well as consumer preference for bio-based products from natural sources have paved the way for the development and expansion of biorefining technologies. These involve the conversion of renewable biomass feedstock to fuels and chemicals using biological systems as alternatives to petroleum-based products. Filamentous fungi possess an expansive portfolio of products including the multifunctional organic acids itaconic, fumaric, and malic acids that have wide-ranging current applications and potentially addressable markets as platform chemicals. However, current bioprocessing technologies for the production of these compounds are mostly based on submerged fermentation, which necessitates physicochemical pretreatment and hydrolysis of lignocellulose biomass to soluble fermentable sugars in liquid media. This review will focus on current research work on fungal production of itaconic, fumaric, and malic acids and perspectives on the potential application of solid-state fungal cultivation techniques for the consolidated hydrolysis and organic acid fermentation of lignocellulosic biomass.

Keywords Fungi · Solid-state fermentation · Biomass · Bio-based chemicals · Simultaneous hydrolysis and fermentation · Mixed cultures · Valorization

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Introduction

Research activities and commercialization efforts on microbial fermentation processes for the production of commodity chemicals that could otherwise be obtained from petroleum continue to attract varying levels of interest. This is despite the fact that chemical methods using petroleum-based raw materials have been more widely applied than biological production routes due to the abundance and low cost of crude oil and their well-established chemical processing technologies. While fears of peak oil supply and depletion could for now be set aside due to the current shale oil boom in the United States and increased petroleum exploration in many parts of the world, concerns regarding environmental sustainability of manufacturing products continue to prevail. These issues include the effects of greenhouse gas (GHG) and volatile organic compound (VOC) emissions on global warming and air quality as well as the effects of solid and liquid chemical manufacturing wastes on water and soil quality. Additionally, current trends in consumer preference towards natural, biodegradable, and environmentally friendly products have also fueled a recent resurgence of research interest in this field. Furthermore, the increased interest in utilizing lignocellulosic biomass waste materials from various agricultural, foods, and forestry industry sectors as well as domestic/municipal solid wastes have added both economic and environmental sustainability dimensions. Specifically, this involves land disposal reduction and *valorization* or value addition of wastes as raw materials for the microbial production of marketable products.

A significant amount of current science and engineering efforts have been focused on the replacement of petroleum-based refineries with *biorefineries* that utilize

microbial systems to convert renewable biomass-based feedstock into fuels and commodity chemicals as environmentally sustainable alternatives to their petrochemical analogs. Research activities have mainly focused on the development of fermentation technologies, robust microbial strains, and improvement of downstream processes for product recovery. Compared with bacteria, yeasts, and other molds, filamentous fungi stand out with its much more diverse portfolio of products such as enzymes, organic acids, alcohols, singe-cell oils, proteins (amino acids), biopolymers (chitin/chitosan and glucans), antibiotics, and bioactive compounds with numerous high-value biotechnological applications [32]. The research literature on biorefining is dominated by the application of submerged fermentation (SmF) technologies for bacteria, yeasts, and filamentous fungi, which utilize liquid-phase media for microbial growth and metabolism. Although SmF technologies are highly developed with standardized engineering design and scale-up methods due to its widespread application, its premise is contrary to the natural habitat and growth conditions of filamentous fungi, which grow on moist solid matrices like wood, barks, leaves, and decaying organic matter for support and sustenance [118]. Additionally, due to the fact that fermentation occurs in the liquid state, solid agro-industrial biomass substrates need to undergo a variety of physicochemical pretreatment and hydrolysis processes to break down polymeric carbohydrates into more readily assimilable and fermentable monosaccharide sugars that are soluble in liquid-phase media. Fortunately, nature has bestowed fungi with the capacity ability to synthesize and excrete a number of hydrolytic enzymes, such as amylases, pectinases, cellulases, xylanase, and ligninases [24, 119]. These enzymes could be used by fungi to break down polymeric carbohydrates such as starch and lignocellulose to release the more readily assimilable mono- and disaccharide sugars as carbon sources for growth and metabolism. This capability allows many fungi strains to directly ferment not only pure sugars such as glucose and sucrose but also complex carbon sources ranging from starchy crops to lignocellulosic agro-industrial biomass residues. Furthermore, fungi in their natural habitats typically grow in symbiotic associations with other fungal strains on solid substrates and use metabolic synergisms among the different components of the fungal consortia for the complete degradation of lignocellulosic biomass into carbon and nutrients for growth and sustenance and the production of various metabolites [48]. Given these advantages, it appears counterintuitive that SmF is currently the preferred and more widely applied technology over solid-state fermentation for bioprocesses involving filamentous fungi. Unlike SmF in which microbial cells are fully suspended in a liquid medium, solid-state fermentation (SSF) involves the immobilization and growth of microbial cells on a moist solid-substrate matrix in the absence or near absence of free water [97]. The substrate should have sufficient bound moisture to support the growth and metabolism of the fungi which serve as biocatalysts for the bioconversion of the same substrate into specific target products [96]. The substrate could serve solely as a growth support for the microorganisms and be supplemented by a broth solution containing both carbon and nutrients or it can serve both functions as the growth support and sole carbon and nutrient source without supplementation of additional carbon and nutrients in a broth solution. The latter case could exploit the capability of fungi to release hydrolytic enzymes in conjunction with other downstream metabolic pathways for the direct bioconversion of polymeric carbohydrates such as lignocellulose to metabolic products such as organic acids of the tricarboxylic acid (TCA) cycle as shown in Fig. 1. Furthermore, several operational and technical challenges in submerged fermentation processes involving filamentous fungi exist which hinder its economical commercial-scale adoption for the production of bio-based products could be alleviated with the use of SSF technology.

The goal of this review paper is to discuss the current technologies based on submerged fermentation and the potential of applying fungal solid-state direct fermentation for the production of platform chemicals leading to valorization of renewable waste lignocellulosic biomass from agro-industrial sectors. The target products are multifunctional dicarboxylic organic acids that have been identified as part of the top twelve biomass-derived platform chemicals by the U.S. Department of Energy with numerous high-value applications and emerging markets; namely itaconic, fumaric, and malic acids [131]. The first part of the paper summarizes the current research, development, commercialization efforts, and existing biotechnological production technologies for these organic acids, which are mostly based on fungal SmF, as well as the challenges and limitations that they face. The second part of the paper considers the potential of SSF technology for the production of the target organic acids by filamentous fungi from waste agro-industrial biomass materials. Emphasis is given on direct bioconversion, which integrates hydrolysis and acid fermentation using mixed fungal cultures. The use of mixed cultures of lignocellulolytic and acid fermenting fungi in theory could exploit the synergistic capabilities of different filamentous fungal strains with different enzymatic hydrolysis and biosynthetic capacities to directly ferment complex carbohydrate substrates into high-value metabolites in a consolidated bioprocessing step. The overarching theme is to develop an innovative bioprocess technology based on the concepts discussed above for the valorization of

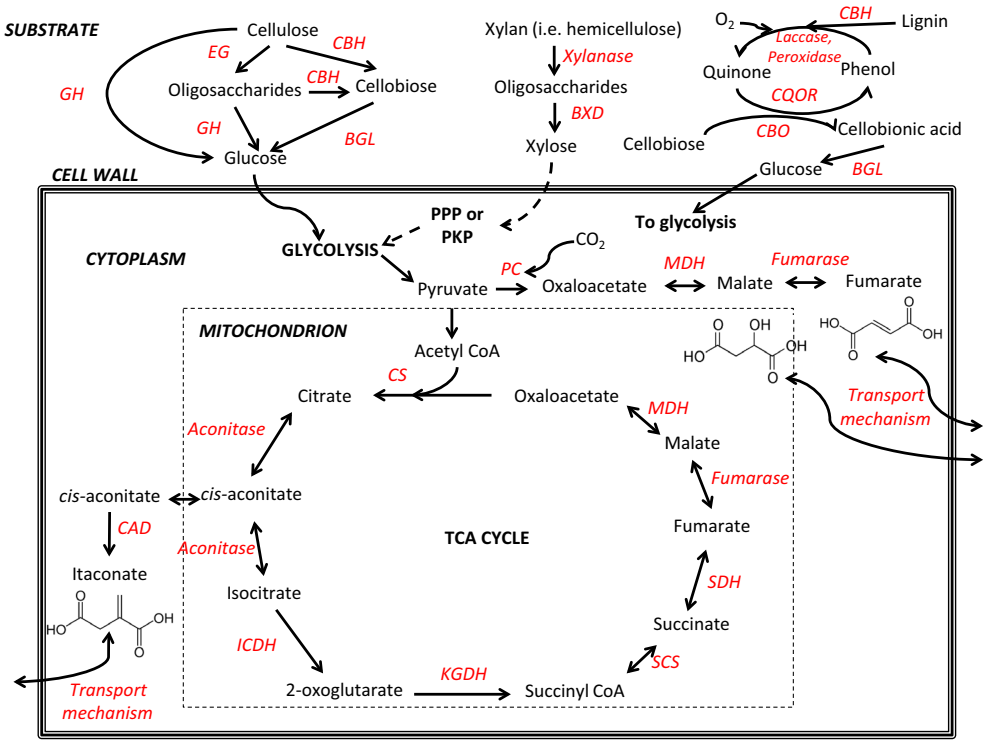

Fig. 1 Biochemical pathways and key enzymes for the direct bioconversion of lignocellulose to itaconic, fumaric, and malic acids by filamentous fungi: *EG* endo-β-1,4-glucanase; *GH* glucohydrolase (exoglucanase); *CBH* cellobiohydrolase; BGL, β-1,4-glucosidase; *CS* citrate synthase; *CAD* cis-aconitate decarboxylase; *ICDH* isocitrate dehydrogenase; *KGDH* α-ketoglutarate dehydrogenase complex; *SCS* succinyl-CoA synthase; *SDH* succinate dehydrogenase; *PC* pyruvate decarboxylase; *MDH* malate dehydrogenase; *PPP* pentose phosphate pathway; *PKP* phosphoketolase reaction pathway; *CQOR* cellobiose-quinose oxidoreductase; *CBO* cellobiose oxidase. Glycolysis and xylose metabolic pathways (*dashed lines*) are abbreviated to emphasize lignocellulose degradation and acid fermentation biochemical routes [4, 9, 100]

lignocellulosic wastes from municipal and agro-industrial sectors to value-added products without expending high costs due to substrate pretreatments and hydrolysis prior to fermentation. Future perspectives, research needs, and suggested technological innovations for improving the productivity and economic feasibility of the proposed solid-state direct bioconversion technology are presented.

Bio-based itaconic, fumaric, and malic acids as platform chemicals: economics, uses, and markets

Itaconic, fumaric, and malic acids belong to a class of multifunctional organic acids classified as dicarboxylic acids due to the presence of two carboxylic groups. The presence of multiple functional groups makes these organic acids as ideal platform chemicals or starting materials and building blocks for the manufacture of products in various industries such as food, feed, agriculture, pharmaceuticals, fine chemicals, and biodegradable polymers [142]. Citric and lactic acids also belong to this class of compounds but are more widely studied and produced since they have fully developed bio-based production technologies using fungal fermentations [122]. Increased interest has been afforded in recent years on itaconic acid, fumaric acid, and malic acid, particularly on the development and/or improvement of biological production methods.

Itaconic acid (CAS#: 97-65-4), also known as methylenesuccinic acid or 2-methylidenebutanedioic acid, is a five-carbon dicarboxylic acid that is used as a key building block for the manufacture of various synthetic resins, coatings, and polymers and has emerging applications as super-absorbents, phosphate-free detergents and cleaners, and bioactive compounds [93, 134]. Itaconic acid has an aqueous solubility of 8.31 g/100 mL (at 20 °C) and has pK_a values of 3.84 and 5.55 [142]. Although chemical methods such as pyrolysis of citric acid to form anhydrides and hydrolyzing the anhydrides, or decarboxylation of aconitic acid have been tested, it is the biological method involving fermentation of pure sugars (glucose and sucrose) or cheaper carbohydrate sources such as corn syrup and beet or sugarcane molasses by fungi such as *Aspergillus terreus* in submerged cultures that is the current commercial-scale production approach [93, 134]. The most recent (2009)

selling price estimate for itaconic acid was $2 per kg or $0.91 per lb and the current worldwide production was around 80,000 tons annually [93]. Although still considered a high-value commodity at this cost, the price of IA has actually dropped from US $4.3 per kg (US $1.82 per lb) in 2000 [9]. Over that time span, the production capacity has exceeded the demand, as its relatively high price as a raw material has been cost-prohibitive to its intended applications. As shown in Fig. 1, biosynthesis of itaconic acid (as the anionic form itaconate) involves the decarboxylation of the TCA cycle intermediate *cis*-aconitate through the action of the *cis*-aconitate decarboxylase (CAD) enzyme [7, 135]. Studies have suggested that the CAD enzyme, and hence the reaction for itaconate formation from *cis*-aconitase, could be localized in the cytoplasm; thus mechanisms should be available to transport *cis*-aconitase from the mitochondrion where the TCA cycle occurs in the cytoplasm for the conversion reaction [50]. Furthermore, the mechanism for the transport of itaconate across the fungal cell wall from the cytoplasm to the culture medium is still not fully elucidated, although it is suspected to follow the same mechanism as citrate. The theoretical yield of itaconic acid from glucose according to the combined glycolysis and TCA cycle pathways is 1 mol of itaconate per mole of glucose.

Fumaric acid (CAS#: 110-17-8), also known as (*E*)-2-butenedioic acid or *trans*-1,2-ethylenedicarboxylic acid, is a four-carbon dicarboxylic acid that has been applied as a chemical intermediate for other platform chemicals such as succinic and maleic acids, and as monomers or building blocks for the manufacture of polymers for use as plasticizers, paper resins, alkyl resins, and other unsaturated polyester resins [112]. Unlike itaconic acid, fumaric acid has a low aqueous solubility of 0.63 g/100 mL (at 20 °C) and pK_a values of 3.03 and 4.44 [142], which makes bioprocessing and downstream recovery processes slightly different from itaconic acid. The multifunctional chemical structure of fumaric acid (Fig. 1) shows its high reactivity and amenability for polymerization and esterification reactions. Additionally, fumaric acid is non-toxic and has been commonly used as a food acidulant and beverage ingredient [127] as well as a treatment for the skin condition psoriasis [87]. It has also been suggested as a cattle feed additive to reduce emissions of the greenhouse gas methane of up to 70 % [79]. Fumaric acid is currently only produced commercially via catalytic isomerization of petroleum-derived maleic acid, having actual yields of up to 90 % [142]. Maleic acid is produced from maleic anhydride, which is produced via the catalytic oxidation of hydrocarbons such as benzene, butane, and butane-butene mixtures [74]. According to data cited by Roa Engel et al. [112] from an online source, roughly 3 % of the 1.8 M tons annual production of maleic anhydride is utilized for the production of fumaric acid, which amounts to roughly 90,000 tons per year. Petroleum-based fumaric acid prices were also reported to be around $1.6–1.8 per kg or $0.72–0.82 per lb, roughly 10 % higher than its raw material maleic anhydride [112]. As shown in Fig. 1, fumaric acid (as fumarate) could also be biologically produced as an intermediate of the TCA cycle which is present in most aerobic organisms, specifically through the reversible conversion of malate to fumarate catalyzed by the fumarase enzyme [127]. However, the fumarate generated by the mitochondrial TCA cycle is mainly utilized for the biosynthesis of cellular components and thus cannot accumulate to significant amounts during active cell growth [142]. Further investigations on the biosynthetic pathways have shown that the primary source of fumarate accumulation in biological systems is a cytoplasm-based version of the reductive TCA pathway involving pyruvate carboxylation with CO_2 fixation, oxaloacetate dehydrogenation, and malate hydration catalyzed by the cytoplasm-localized fumarase enzyme with a high theoretical yield of 2 mol/mol glucose [136]. The accumulated fumarate is then transported from the cytoplasm across the fungal cell wall into the culture through mechanisms that are yet to be fully elucidated although research on transport of dicarboxylic acids in yeasts suggested the transport of the anionic form by means of an accumulative dicarboxylate proton symporter similar to citric acid [112].

Malic acid (CAS# 6915-15-7) or hydroxybutanedioic acid is another four-carbon dicarboxylic acid that has D- and L-isomers. Malic acid has a relatively higher solubility in water than IA and MA at 55.8 g/100 mL (at 20 °C) and has pK_a values of 3.40 and 5.20 [142]. In the past, malic acid was prepared via extraction from apple juice, which contains 0.4–0.7 % of the acid [5]. The current commercial-scale manufacturing method for malic acid production is via the catalytic hydration of maleic or fumaric acid, which produces a racemic mixture of D- and L-isomers. Another production method involves the enzyme-catalyzed conversion of fumaric acid to malic acid using immobilized cells of the bacteria *Brevibacterium flavum* with high fumarase enzyme activity, although this is not done commercially [142]. The current worldwide production of malic acid is estimated at 40,000 metric tons annually [41]. The L-isomer is the natural form that is found in fruits and produced by biological systems. DL mixtures of malic acid classified as GRAS (generally recognized as safe) are mainly used as additives in food, beverages, and confectionaries imparting tart and sour taste, while non-food applications include metal cleaning and finishing, textile finishing, electroless plating, and pharmaceuticals [41]. MA also has a potential market as a raw material for the production of polymalic acid, a novel biodegradable polymer analogous to polylactic acid; however, this is contingent upon the economic

feasibility of the production process [42]. Various online bulk retailers of malic acid report costs of around $1.80–2.00 per pound. Malic acid is closely related to fumaric acid, since they share a common biosynthetic pathway as shown in Fig. 1. Malate is an intermediate of the mitochondrion-based TCA cycle formed via reversible dehydration of oxaloacetate and hydration of fumarate. Similar to fumaric acid, malic acid accumulation in microbial cells could be attributed to the cytosol-localized reductive TCA pathway with CO_2 fixation that is thought to be exclusive to filamentous fungi followed by transport of accumulated malate across the cell wall.

Although chemical pathways for the synthesis of these compounds from petrochemicals have been more readily available for application in the commercial scale, numerous attempts have been made to exploit the biochemical pathways for synthesis of these organic acids to provide for non-petroleum derived and sustainably produced alternative bio-based products. The ability for overproduction and accumulation of organic acids including itaconic, fumaric, and malic acids has been widely documented in filamentous fungi, particularly those belonging to the *Aspergillus* and *Rhizopus* spp [41, 76, 127, 142]. Research and technology development efforts have focused on attaining the key performance indicator that in order for bio-based itaconic, fumaric, and malic acids to be competitive with their petrochemical-based counterparts especially for its applications in emerging potentially addressable markets, the cost attributed to fermentation processing should be at most US $0.25 per pound [131].

Current biotechnological production trends

Submerged fermentation using filamentous fungi

Among the three dicarboxylic acids identified in this paper, only itaconic acid is currently manufactured via large-scale fermentation and bioprocesses as the chemical production route is deemed impractical for commercial-scale application [134, 142]. The current commercial-scale itaconic acid production process utilizes SmF technology. Despite its establishment, further studies have been conducted more recently in the attempt to further improve productivity, titer, and the economics of the process. Studies on the development of fungal fermentation processes based on SmF for fumaric and malic acid production have continued despite the establishment of more economical petrochemical conversion routes [41, 112]. The vast research literature on this field features investigations and reviews that focused on screening and identifying the appropriate fungal strains for production of a particular acid; identifying the appropriate fermentation media composition; and elucidating the effects of and determining the optimum levels of various fermentation and bioprocessing conditions for maximum fungal growth and biosynthetic productivity. A few of these findings are summarized in this section. Additionally, several technical challenges of current SmF technology for filamentous fungal fermentations that could be solved more appropriately by employing SSF have been identified through the course of the discussion.

Fungal strains

Itaconic acid production by fungi has been reported as early as 1931 by the group of Kinoshita using cultures of the fungus *Aspergillus itaconicus* [66] and 1939 by Calam et al. [14] in *A. terreus*. In these studies, surface and submerged fermentation runs of *A. terreus* NRRL 1960 for the production of itaconic acid from glucose and product recovery from aqueous media by crystallization were conducted. The surface fermentation cultures that they used consisted of shallow aluminum pans with containing a thin layer of liquid glucose media with a low pH of 2.0 in which *A. terreus* spores were inoculated and incubated under static conditions up to 54.3 % fermentation yield of itaconic acid (w/w of glucose consumed) and productivity of 0.17 g/L h (calculated based on available data) [73]. In another study, pilot scale SmF runs in 300- and 600-gallon fermenters using the same *A. terreus* strain obtained a 57.9 % yield (w/w glucose consumed) and 0.31 g/L h productivity at optimum fermentation conditions of initial pH 5.0, 115 RPM agitation rate, and 6.03 % (w/w) initial glucose [105]. Additionally, fungal strains other than *Aspergillus* spp. such as *Pseudozyma antartica* [70] and *Ustilago maydis* [67] have also been shown to produce itaconic acid although at relatively lower levels than *A. terreus*.

Fumaric acid production has been shown to occur mostly in filamentous Mucoralean fungi, particularly those belonging to the *Rhizopus* genera such as *Rhizopus nigricans*, *R. arrhizus*, *R. oryzae*, and *R. formosa* [36]. Other Mucoralean fungi such as *Cunninghamella* and *Cirnella* spp., and non-Mucoralean fungi such as *Penicillum griseofulvum*, *Aspergillus glaucus*, and *Caldariomycels fumago* were also identified as fumaric acid producers as well. Fumaric acid production by *Rhizopus* fungi has been found to be frequently accompanied by one or more acids such as lactic, formic, malic, and acetic acids and ethanol; thus studies have aimed to reduce the co-formation of these by-products [124]. Fumaric acid production using glucose as the sole carbon source in shake flask and stirred tank batch submerged fermentation cultures has been previously studied using *R. nigricans* [36, 114], *R. arrhizus* [37, 62, 91, 109–111], and *R. oryzae* [143, 144]. Production titers ranged from 14.7 to 107 g/L with glucose conversion yields

of 33–82 % (w/w of glucose consumed) and productivities ranging from 0.25 to 1.22 g/L h.

Malic acid production via glucose fermentation has been observed mainly in *Aspergillus* strains along with the production of other organic acids but the research literature is relatively scarce compared with itaconic and fumaric acids [41]. The earliest fungal fermentation process involving *Aspergillus flavus* was first patented by Abe et al. in 1962 [1]. In another study, SmF cultures containing *A. flavus* ATCC 13697 produced a 36 g/L of L-malic acid after 6–8 days of cultivation in a chemically defined medium containing 10 % glucose [102]. Later studies include *Aspergillus* sp. N1-14′ cultures yielding an L-malic acid titer of 106 g/L and productivity of 0.88 g/L h [145]. Another *A. flavus* strain culture with a 120 g/L initial glucose concentration resulted in a maximum titer of 113 g/L L-malic acid, a 128 % molar glucose conversion yield, and 0.59 g/L h productivity in a stirred tank fermenter [5]. However, it was shown that *A. flavus* also produced significant quantities of succinic acid, another TCA cycle intermediate, and to a much lesser extent, fumaric acid. The production of these organic acid co-products reduces the carbon flux towards malic acid production. The fungus also produced toxic aflatoxin rendering the malic acid products unsuitable for food and pharmaceutical applications.

Substrates

With regard to the fermentation medium composition, of particular interest is the type of fermentation substrate/carbon source used, since it comprises a significant fraction of production cost in fermentation processes. Glucose is the most preferred and utilized carbon source for growth and organic acid production by filamentous fungi, since it offers the most direct biochemical pathway towards organic acid production. Other pure sugars such as xylose and sucrose have been used as the sole carbon source for itaconic and fumaric acid production. However, in most cases, yields and productivities were relatively lower than when glucose was used under the same fermentation conditions and bioreactor configurations. This is most likely due to the additional biochemical pathways needed to link xylose and sucrose with the glycolysis and TCA cycle pathways as shown in Fig. 1 [58–61]. In a few cases, non-sugar carbon sources that are precursors of the target acids in their biosynthetic pathways have been tested to stimulate the fermentation process such as citrate for the production of itaconic acid [9] and pyruvic and fumaric acid for malic acid production [1]. However, the use of pure sugars or chemical intermediates as fermentation carbon sources could significantly increase the production costs of the target organic acid products, particularly in the commercial scale. As a result, many succeeding efforts have tested various cheap, nutrient-rich sugary and starchy materials as the fermentation carbon source in an attempt to lower production costs attributed to the substrate. Examples include sugar or beet molasses [57, 92, 103], corn starch [138], Jatropha seed cake [89], wheat flour, potato flour, cassava flour, sorghum starch, and sweet potato [86, 104]. Lignocellulosic materials from agro-industrial residues and food processing wastes have been tested as well. Examples include cassava bagasse [18], dairy manure [71], corn straw [137], eucalyptus wood [113], and thin stillage [133]. However, despite their low to zero costs, these materials require pretreatment and hydrolysis using a variety of mechanical, acid, alkali, or enzymatic methods to release fermentable monosaccharide sugars from the polymeric carbohydrate structures and solubilize them in liquid-phase culture media for direct assimilation by the microbial cells in SmF cultures. These additional processing steps for carbon source preparation could add to the production costs and generate potentially inhibitory or toxic byproducts such as furans and short-chain organic acids that could hinder fungal growth and metabolism and necessitate detoxification prior to fermentation [94].

Effect of SmF parameters

The effect of culture conditions and operational variables that are specific to SmF processes has been investigated in numerous previous studies with the intention of optimizing fermentation and bioprocessing conditions for maximum fermentation yields and productivity for the target bio-based platform chemical products. In terms of media composition, a high C:N ratio (that is, an excess of carbon and limitation of nitrogen levels) in the culture medium was found to be essential for the overproduction and accumulation of dicarboxylic acids within the TCA-related biosynthetic pathways [41]. Under these conditions, cell growth and multiplication cease due to the limitation of nitrogen required for the biosynthesis of cellular materials. Certain filamentous fungi strains respond to this environmental stress by diverting the excess carbon towards organic acid production. However, very high C:N ratios could cause substrate inhibition and loss of cell productivity and viability over time. According to one study, this phenomenon could be attributed to the repression of the α-ketoglutarate dehydrogenase (KGDH) enzyme complex in the TCA cycle (Fig. 1) under high sugar concentrations [100]. For the same reasons of limiting cell growth in favor of promoting acid accumulation, inorganic nitrogen sources such as urea and ammonium salts are also preferred over organic nitrogen sources like yeast extract, which promote cell growth [142]. The level of the phosphorus source should also be low enough relative to the carbon source. High phosphorus levels have been shown to reduce the carbon flux towards

acid synthesis in the same manner as nitrogen for the assembly of cellular materials and decrease CO_2 fixation in the cytosolic reductive TCA pathway for fumaric and malic acid production [100, 134].

The addition of trace metal ions and vitamins in the fermentation medium was found to be beneficial for fungal growth and metabolism. Metal ions such as Mg^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, and Mn^{2+} and vitamins such as biotin and riboflavin are used as cofactors or activators for some enzymes involved in catabolic and biosynthetic pathways such as the TCA cycle. In particular, Mg^{2+} and Mn^{2+} assist in substrate binding and stabilization of the reaction intermediate in the conversion of isocitrate to α-ketoglutarate catalyzed by ICDH as shown in Fig. 1 [142]. Biotin is an activator of the pyruvate carboxylase (PC) enzyme which catalyzes the condensation of pyruvate with CO_2 fixation step in the cytosol-localized reductive TCA cycle for malate and fumarate synthesis, while riboflavin is a component of the cofactors FAD and FMN which play key roles in energy metabolism [142]. Thus, the presence of optimum levels of these supplements could improve fungal growth and acid productivity. However, the direct addition of pure vitamins into fermentation media is not usually done due to economic limitations. These vitamins could be supplied through the addition of relatively cheap nutrient-rich materials such as corn steep liquor or yeast extract.

Fermentation parameters and operating variables such as pH, dissolved oxygen level, and incubation temperature significantly affect carboxylic acid fermentations involving submerged fungal cultures and thus should be carefully monitored and controlled. Among these three variables, the effect of incubation temperature on fungal growth and dicarboxylic acid yield and productivity has not been particularly investigated due to the wide range and strain specificity of the optimal growth temperature of filamentous fungi, which is usually between 30 and 37 °C [142]. The pH of the culture broth could affect yields and productivities during fungal dicarboxylic acid production. Culture broth pH affects the activities of metabolic and biosynthetic enzymes as well as the transport of the produced acids across the fungal cell membrane back and forth between the cytoplasm and the culture medium. Although the transport mechanism for itaconic, fumaric, and malic acids in filamentous fungal submerged fermentation cultures has not been researched yet, previous studies on citric acid production by *A. niger* and malic acid in yeasts point to possible similarities in terms of active proton symport-dependent systems. These involve the transport of the accumulated anionic forms of the dicarboxylic acid from the cytosol across the cell membrane to the culture medium by means of pH gradients [90, 112]. Studies on itaconic acid production via fungal submerged fermentations have found that a low pH is required for maximum productivity and yield. A possible reason based on the above-mentioned pH gradient-dependent acid transport mechanism is that since fungal cells excrete itaconic acid as its anionic form itaconate in the culture medium, a low pH or high hydronium ion concentration in the culture broth must be needed to shift the dissociation equilibrium of itaconate towards the protonated form and to maintain a continuous flux of itaconate from the fungal cell to the culture. The culture pH is usually allowed to drop in the absence of buffers and neutralizing agents and maintained between 2.8 and 3.1 to prevent the formation of itartaric acid which could reduce itaconic yield [68]. The low pH could also prevent the formation of by-products such as gluconic and oxalic acids, which could complicate the itaconic acid recovery process at pH levels greater than 2.0 due to the inactivation of mycelial-bound glucose oxidase enzymes [81]. Additionally, the low culture pH could limit excessive mycelial growth to increase the carbon flux towards itaconate biosynthesis and lower the possibility of contamination by inhibiting the growth of other microorganisms in the medium.

In contrast, the effect of culture pH varies differently for fumaric and malic acids' production, which requires the addition of a neutralizing agent to maintain a culture pH level of around 6 and cause the immediate precipitation, separation, and recovery of the acids from the liquid culture as fumarate and malate salts [112]. Unlike with itaconic acid, the accumulation of the protonated fumarate (or malate) resulting from the decrease in the culture pH could cause a passive diffusion of some of the free acids back into the cell through the cell membrane, which in turn could decrease the intracellular pH and cause progressive inhibition of the fumarate (or malate) biosynthetic pathway [20, 111]. The inhibition of fumaric acid production could also be due to the decrease in the permeability of the fungal cell membrane brought about by a decrease in the culture pH, thus causing a build-up of undissociated acid inside the cell and a decrease in intracellular pH [20]. As for the choice of neutralizing agents, $CaCO_3$ has been the most frequently used since it also releases CO_2 during the neutralization reaction. The released CO_2 is then channeled into the cytosolic-based reductive TCA pathway shown in Fig. 1 wherein pyruvate is converted to oxaloacetate with CO_2 fixation through the catalytic action of the pyruvate carboxylase (PC) enzyme. The oxaloacetate is subsequently converted to malate and fumarate. However, the resulting calcium fumarate salts have low aqueous solubility and have the tendency to aggregate with fungal cells, causing high culture broth viscosity leading to agitation, aeration, and mass transfer problems in submerged cultures [112, 142]. Another problem related to the use of $CaCO_3$ is the use of sulfuric acid to release fumarate and malate and neutralize excess $CaCO_3$. This process generates insoluble $CaSO_4$, which is of little industrial use and is a persistent

environmental pollutant. Thus, alternatives to $CaCO_3$ have been tested by different investigators to circumvent these problems such as Na_2CO_3, $NaHCO_3$, $Ca(OH)_2$, and $(NH_4)_2CO_3$ [37, 111, 146]. However, the use of these neutralizing salts resulted in lower fumaric acid yields and productivity compared with $CaCO_3$. On one hand, the use of sodium salts releases sodium ions in the fermentation broth that could negatively impact cell metabolism. Moreover, sodium fumarate has a higher water solubility, which could cause product inhibition but nevertheless could facilitate separation and recycling of fungal cell biomass in succeeding batch fermentation steps [146].

Mycelial growth and production of the itaconic, fumaric, and malic acids by filamentous fungi require aerobic conditions, thus it is crucial to maintain a sufficient dissolved oxygen (DO) level in the culture broth to meet the oxygen requirements of the fungi for cell growth and metabolism. In SmF processes, the DO level is controlled by the combined effects of agitation and aeration. Aeration is the direct injection of high-pressure air into the liquid culture as gas bubbles but agitation is necessary to disperse these bubbles and break them into smaller ones to increase the oxygen transfer rate to the fungal cells. However, high agitation rates could produce high shear forces that can damage fungal cells. Air-lift bioreactors were then applied for these fermentations to eliminate shear forces due to mechanical mixing by impellers and instead relied on the force produced by air bubbles to induce bulk movement of the liquid culture [26, 60, 138]. For itaconic acid production, a sufficient and uninterrupted supply of oxygen is especially critical as found in a previous study where pausing aeration for 5 min caused a complete cessation in IA production, which was only restored slowly within 24 h after aeration was resumed [45]. In contrast, too high aeration or agitation rates could reduce levels of dissolved CO_2 that is needed for the cytosol-localized reductive TCA pathway involving CO_2 fixation for fumaric and malic acids production. For this reason, two-stage batch cultures with high DO level in the growth phase to promote cell growth and reduced DO levels in the acid accumulation phase were applied and resulted in improved FA yield and productivity [72]. Another operational issue related to DO is foaming due to excess aeration, which could require increased amounts of antifoam materials or construction of mechanical defoamers for commercial-scale fermenters that could add to capital costs.

Aside from dicarboxylic acid yield and productivity, these culture conditions and fermentation variables also affect the type of fungal morphology produced in submerged cultures. The main types of fungal morphology observed specifically for fungal organic acid fermentations are filamentous growth with evenly dispersed hyphae, pellets, and mycelial clumps and aggregates [142]. Each of these morphologies presents their own set advantages and operational issues. Mycelial masses and large aggregates could lead to high broth viscosity, thus imposing difficulties in mixing and aeration and imposing mass and oxygen transfer limitations, especially in the inner portions of the mycelial clump. The formation of fungal pellets alleviates high broth viscosity problems and biofilm build-up in the internal surfaces of the bioreactor and allows for the recovery and recycle of fungal cells for subsequent fermentation batches. However, pellets having large diameters above a critical size could pose oxygen and mass transfer limitations within the pellet cores causing autolysis and a decline in fermentation performance. Several authors have studied the effect of fermentation variables and culture conditions on filamentous fungal morphology. For instance, a high C:N ratio promoted mycelial growth in *A. niger* [30] but produced pellets for *R. arrhizus* and vice versa [13]. In terms of the type and level of nitrogen and phosphate source, high phosphate concentrations were found to induce branching and increase filament lengths in mycelial clumps for *A. niger* [99]. For *R. chinesis*, smooth compact pellets are obtained by the addition of ammonium sulfate as nitrogen source, loose fluffy pellets were obtained with corn steep liquor, dispersed mycelia were obtained with peptone, and entangled filaments were obtained with yeast extract [27]. The levels of trace metals such as Zn, Mg, Fe, Cu, and Mn were also found to significantly affect the morphology of *R. oryzae* as discussed elsewhere [144]. Inoculum spore concentration also influenced fungal morphology of *R. oryzae* for fumaric acid production in which a low spore concentration led to pellet formation while high spore concentration caused filamentous growth [71]. In terms of agitation, *A. niger* pellet size was found to decrease with increasing agitation rate but above a critical level, and hyphal cell growth with fragmentation is promoted [29]. For incubation temperature, pellet formation was induced by low temperature but high temperature induced filamentous growth due to increased spore swelling and germination in *A. niger* [30]. For the culture pH, filamentous mycelia of *A. nidulans* were produced at a culture pH of 3.0–5.0 and pellets at pH levels above 5.0 [8]. On the other hand for *R. oryzae*, pellet formation was found at low pH levels of 2.6–3.36 and large mycelial clumps at pH 4–5 [146]. Thus, various SmF parameters and culture conditions not only affect fermentation performance in terms of organic acid yield, titer, and productivity but also culture rheology which could affect operational power requirements and bioreactor design and capital costs.

Submerged fungal cultures using immobilized cells

Control of fungal morphology and improvement of fermentation yields and productivity of fungal organic dicarboxylic

acids have been attempted with the use of immobilized fungal cells. The immobilized cells were either in constant or intermittent contact with liquid media but were not suspended in the culture broth unlike in conventional SmF processes. Several studies on the use of immobilized *A. terreus* cells for itaconic acid production have been conducted in an attempt to improve fungal biomass handling and separation and itaconic acid productivity. Live *A. terreus* cells were entrapped or anchored in materials such as polyacrylamide gels [49], calcium alginate beads, Celite, agar gel cubes [58], stainless steel wire mesh disk [55], and polyurethane foam [59, 129]. These immobilized cell cultures were utilized in repeated batch and continuous cultures using pure sugars such as glucose, xylose, and sucrose resulting in itaconic acid fermentation yields and titers similar to conventional SmF but with increased productivity of up to 1.2 g/L h, the highest reported so far [58]. Immobilized fungal cells of *Rhizopus* spp. fungi were tested for fumaric acid production from glucose such as in a previous study employing a rotary biofilm contactor bioreactor with self-immobilized biofilms of *R. oryzae* ATCC 20344 resulting in high volumetric FA productivities of 3.78 and 4.25 g/L h [15, 16]. Malic acid production using immobilized fungal cells has not been reported yet although studies using immobilized cells of the yeast *Saccharomyces cerevisiae* [33] and the bacterium *Brevibacterium ammoniagenes* [139] have been conducted previously. Despite these improvements, several issues such as the mass transfer limitations into the interior of the immobilized mycelial mass could result into low productivity. The materials typically used as immobilization matrices and carriers could be mechanically weak and highly susceptible to shear forces, making it difficult to perform industrial scale-up. Immobilized cell systems in submerged fermentation cultures are also based on available surface area for cell immobilization; therefore, the challenge is to prepare support matrices that would expose fungal cells to the liquid culture media without compromising its attachment to the matrix and cell integrity [142].

Challenges and limitations of SmF technology for fungal fermentations

Based on the preceding discussion, the key technical and economic challenges and limitations facing the application of SmF technology in filamentous fungal fermentation processes for the production of bio-based platform chemicals can be summarized into two main aspects: (1) mass and oxygen transfer limitations and culture broth viscosity issues brought about by difficulty in controlling fungal morphology and formation of mycelial aggregates with insoluble salts in the case of fumaric and malic acid production; and (2) for utilizing lignocellulosic agro-industrial biomass residues as cheap carbon source in place of pure sugars, the necessity for pretreatment and hydrolysis steps to release fermentable sugars in aqueous solution for use in submerged cultures, which could add to production costs and produce by-products that are potentially toxic towards growth and metabolism by fungi. These issues could be alleviated through the use of solid-state fermentation, a technology that is more suited for filamentous fungi in simulating their natural growth conditions and exploiting this towards the development of sustainable biotechnological processes for the production of bio-based fuels and chemicals.

Future prospects: solid-state fermentation for direct bioconversion of biomass substrates to platform chemicals by filamentous fungi

Solid-state fermentation basics and applications

Solid-state fermentation is defined as a fermentation process that involves a moist solid-substrate matrix in the absence or near absence of free water [97]. The substrate should possess sufficient bound moisture to support the growth and metabolism of the fungi, which serve as biocatalysts for the bioconversion of the substrate into specific target products [96]. A few authors have extensively reviewed the historical roots, research and engineering progress and development, and application of SSF technology for the production of various bio-based fuels, commodity chemicals, enzymes, bioactive compounds, and food and pharmaceutical products [48, 80, 83, 96, 98, 118]. Historical records indicate that SSF technologies have already been applied mainly to food preparation by the ancient Egyptian and East Asian cultures. During the early eighteenth to twentieth century, SSF has been applied for vinegar production, leather tanning, and composting and solid waste treatment. It was not until the 1940s during the discovery of penicillin that SmF technologies have begun to expand in popularity and outpace SSF in terms of scientific research and standardization of process engineering and equipment design methods. Despite this, work on SSF has continued behind the scenes, and has only found niche applications in the fields of bioremediation and biodegradation of hazardous compounds, biotransfomation for nutritional enhancement of crop residues, biopulping, and production of bioactive secondary metabolites such as antibiotics, biopesticides, aroma compounds, spores, and plant growth factors [96]. SSF has also been applied as the preferred method for the production and isolation of hydrolytic enzymes such as cellulases, xylanases, proteases, amylases, and lipases; but is sparsely applied for the production of a few organic acids such citric, lactic, oxalic, and gluconic acids [48, 95, 122]. Another interesting application of SSF

is in air biofiltration, which involves uptake and removal or detoxification of atmospheric pollutants by microbial biomass immobilized in porous solid substrates [25, 63].

Solid-state fermentation is theoretically better suited for cultivating filamentous fungi than SmF as it simulates the natural habitat and growth conditions of these organisms on moist solids in the environment. Furthermore, SSF technology could alleviate or eliminate entirely the key technical issues inherent with SmF involving filamentous fungi mentioned earlier. There is minimal concern with regard to the control of fungal morphology and selection of product recovery methods to minimize culture broth viscosity and improve mass and oxygen transfer since the growing fungal biomass is immobilized and exposed to the gas phase. Furthermore, it is potentially more advantageous and environmentally sustainable than SmF when faced with the task of value addition and direct bioconversion of a broad spectrum of solid waste-type substrates such as agro-industrial biomass residues and municipal solid wastes. This could reduce the amounts of these wastes disposed in the environment and provide added economic incentives for reuse and recycling by the agriculture, food, and forestry products processing and manufacturing industry. SSF processes in most cases require equipment with smaller footprint than SmF due to the use of solid media and thus could result into lower capital investments, lower energy requirements, minimal water usage, and wastewater generation. Other added benefits of SSF that have been cited from literature include minimal catabolite repression, higher fermentation productivity and titer, higher product stability, cultivation of microorganisms specialized for water-insoluble substrates, the possibility of applying mixed fungal cultures, and lower sterility demands due to lower water activities in solid media [48].

Despite the mentioned advantages that SSF has over SmF, SSF technologies have recently been mostly relegated to low volume-high cost products such as enzymes, bioactives, and biopharmaceuticals unlike SmF processes that have been widely used for high volume–low cost production application such as biofuels and bio-based chemicals [96]. SSF processes have been applied to a lesser extent than submerged fermentation for the production of organic acids. The production of citric acid, which is closely related to itaconic acid via the TCA cycle intermediate *cis*-aconitate, by SSF using various strains of *A. niger* has been widely studied for many years [122]. Reported citric acid production titers range from 82 to 816 g of citric acid per kg of substrate, which varies widely depending on the type of substrate used, type of SSF bioreactor configuration applied, and SSF conditions. Lactic acid, which is closely related to fumaric and malic acids directly via pyruvate, has also been produced via solid-state fermentation using *Rhizopus* spp. fungi. One study involved the use of glucose-impregnated sugarcane bagasse as a substrate for *R. oryzae* NRRL 395 in a semi-solid fermentation culture [121]. In the presence of the neutralizing agent $CaCO_3$, 137 g/L of lactate was produced by the fungus grown on the solid substrate impregnated with a 180-g/L glucose solution at a productivity of 1.43 g/L. A more recent study utilized thermally treated cassava bagasse as a sole carbon source without supplementation of additional sugar and nutrients for *R. oryzae* for direct solid-state fermentation to L-lactic acid resulting in a yield of 206.20 g per kg of dry substrate [106]. With regard to the production of itaconic, fumaric, and malic acids via solid-state fermentation, the research literature is very scant. An SSF process for itaconic acid production by a mutant strain *Aspergillus terreus* ATCC 10020 using sugarcane pressmud as substrate has been patented previously [126]. In this process, the substrate was utilized merely as a fungal growth support, since the remaining sucrose in the sugarcane pressmud was supplemented with added amounts of sugars such as glucose, fructose, sucrose, or starch hydrolyzate; and nitrogen and mineral salts to be used as carbon and nutrients for the fungus. The authors reported maximum sugar conversion yields of 44–55 % (wt./wt. of residual sugar consumed); however, the calculated productivities (approximately 0.0003 g/kg h) were at least a hundred times lower than those reported from SmF processes utilizing soluble sugars in liquid media. Fumaric acid was produced via SSF of corn distillers' grains with solubles using three ATCC strains of *Rhizopus oryzae:* 10260, 20344, and 52918 [132]. Similarly, the intention was to allow to fungus to utilize the remaining fermentable sugars and starch in the substrate as carbon source for growth bioconversion to fumaric acid. Among the three strains, *R. oryzae* ATCC 52918 produced the highest fumaric acid titer of 5.04 g/kg of substrate over a period of 10 days with a calculated productivity of 0.021 g of fumarate per kg of substrate per h. However, this was obtained only after pretreating the substrate with mild acid hydrolysis (1.0 % w/w H_2SO_4 at 121 °C and 17 psi for 20 min). As of the time of writing, there is no known published work involving SSF for the production of malic acid.

A wide variety of agro-industrial residues have been applied as SSF substrates. These are mainly processing wastes from starchy and fruit crops, which contain substantial amounts of residual monosaccharide sugars adhering to the substrate surface as well as the starchy and lignocellulosic fraction that could be used as carbon and energy sources by the fungus. However, in these processes, the fungi utilized only surface adhered residual or supplemented fermentable sugars as their carbon and energy source and not the lignocellulose fraction of the substrate.

The requirement for supplementation of sugar and nutrient broths could add to the production cost and reduce the economic feasibility of SSF processes. Therefore, a technically and economically feasible method for completely harnessing the carbon-rich lignocellulosic fraction of biomass substrates for microbial growth and metabolism with minimal physicochemical, chemical, or enzymatic pretreatment and hydrolysis is desired.

Various SSF bioreactor configurations have also been utilized in numerous previous studies including static cultures in Erlenmeyer flasks, glass incubators, and trays; or aerated/agitated cultures in rotating horizontal drum bioreactors or packed-bed columns. The choice between static or aerated/agitated beds could depend on the nature of the fungal strain and its sensitivity to shear forces as forced aeration and agitation could destroy mycelial growth in the solid substrate. In a previous study on SSF of cassava bagasse by *A. niger* LPB 21 for citric acid production, maximum citric acid yields were obtained with an aerated column fermenter operated at the optimum aeration rate of 30 mL/g min and humidity of 60 % [128]. Respirometric assays indicated the importance of having an optimum aeration rate to maintain a low-oxygen and high-CO_2 atmosphere, which limits fungal growth and stimulates citric acid accumulation. An optimal humidity of the air stream is necessary to prevent desiccation of the substrate and fungal biomass. For itaconic acid production, an oxygen-rich continuous air stream through the substrate bed may be necessary according to biochemical findings in SmF studies. However, SSF studies are severely lacking and need to be investigated to definitively make this conclusion.

Minimal pretreatment methods that mainly involve autoclaving substrate moistened with water or with dilute acid or alkali are usually conducted to reduce the crystallinity of starchy and lignocellulosic substrates and render them more susceptible to attack by hydrolytic enzymes secreted by the filamentous fungi. The effect of various fermentation parameters such as incubation temperature, pH, humidity, moisture content, aeration, and nutritive composition of the solid substrate is also critical for optimal organic acid production via SSF. For SSF processes on citric acid production using *A. niger*, the solid substrates are usually moistened to about 70 %, initial pH adjusted to 4.5–6, and the cultures were incubated at 28–30 °C [122]. The substrate moisture content is crucial to provide the appropriate water activity essential for the growth of the fermenting fungus. Similar to SmF, the initial pH of the solid substrate must be adjusted to the proper level to induce acid production by the fungus. For itaconic acid production, SmF studies recommended adjusting the initial pH to around 5.5 and allowing levels to drop to around 3, after which the pH must be maintained within this vicinity. Control of pH to this specified range in SSF processes for itaconic acid production is a challenging task, especially considering that with static solid substrates; large pH gradients may exist due to the heterogeneity of the solid medium. For fumaric and malic acids, bioprocess considerations for the use of SSF of biomass substrates for the production of these acids could be inferred from studies involving SSF for lactic acid production since these acids share the same intermediate pyruvate. Control of the substrate pH to around 6.0 is critical for optimal lactic acid yields to prevent inhibition of the fermentation due to end-product accumulation [106]. For this reason, continuous neutralization and/or washing and recovery of the product lactic acid from the substrate could be necessary for maximum fermentation yields. Optimal aeration rates have been found to be critical to attaining maximal lactic acid yields in SSF fungal cultures as aeration controls not only a balanced oxygen supply for growth of fungal growth and acid production but also humidity and temperature of the solid medium [121, 141]. Other important factors with which optimum levels are necessary for maximum lactic acid yields in solid-state fermentation cultures are inoculum spore concentration and sugar levels in the nutrient solution used to moisten the fermentation substrate [121].

The literature findings on SSF for citric and lactic acid production could suggest similar approaches for SSF production of itaconic, fumaric, and malic acids. However, more extensive fermentation studies specific to these target products with bioprocess parameter optimization and kinetic modeling need to be conducted to make more precise generalizations. Although no direct comparison on product yields and productivities can be made between SmF and SSF processes for the production of organic acids, SmF processes have generally had higher substrate conversion efficiencies and production rates per unit of substrate (L in SmF and kg in SSF), at least in numerical magnitude terms. The lower fermentation productivity of SSF could be attributed to the longer fermentation periods requried to allow the fungi to utilize some of the readily available and assimilable residual carbon and nutrients adhering to the solid substrate surface for growth and assembly of hydrolytic enzymes to break down the polymeric carbohydrates and release more of the fermentable mono- and disaccharide sugars for further metabolism. It is only after a certain critical concentration level of hydrolyzed sugars has been achieved would the fungi be able to utilize the released carbon source for further growth and the biosynthesis of metabolic enzymes for production of organic acids and other metabolites. To make SSF process technically and economically competitive for the production of platform chemicals, fungal bioconversion and organic acid production rates and efficiencies must be improved. This could be achieved through the enhanced and efficient integration of the biomass hydrolysis and fermentation using a consolidated bioprocessing approach that

could involve either supplementation of solid-state pure fungal cultures with commercial enzyme preparations or using mixed fungal cultures.

Harnessing fungal lignocellulolytic capabilities for direct fermentation

SSF processes could be classified according to the intended function and usage of the solid substrate. The first type, which is the most commonly applied, involves the use of the substrate solely as an inert growth support for the microorganisms. Carbon and nutrients are either obtained from the residual adhering sugars on the substrate surface such as in the case of various sugary crops and food processing wastes; or supplied externally in the form of aqueous solution that is also used to reconstitute the substrate to the appropriate moisture content. In the latter type, the substrate serves both functions as the growth support and sole carbon and nutrient source without supplementation of additional carbon and nutrients in a broth solution. This process then relies on the innate capability of the fungi to produce extracellular hydrolytic enzymes to break down the polymeric carbohydrates and other nutrient factors in the solid substrate into more readily assimilable forms for growth and metabolite formation. This type of process is referred to in different publications by different terms such as direct fermentation, direct bioconversion, simultaneous saccharification and fermentation, or consolidated bioprocessing [17].

In this paper, the focus is on the direct bioconversion of lignocellulose in agro-industrial residues to the platform chemicals itaconic, fumaric, and malic acids. The overall metabolic pathways for this process are summarized in Fig. 1. The first stage of the direct bioconversion process involves the breakdown of the lignocellulose to the fermentable sugars by a complex mix of enzymes reflecting the complexity of the substrate. The cellulose fraction of the lignocellulose is hydrolyzed by the cellulase enzyme complex consisting of exocellulases, endocellulases, and β-glucosidases [4, 24]. Exocellulases such as cellobiohydrolase (CBH) hydrolyze β-1,4-glycosidic bonds at the cellulose chain ends, producing cellobiose as the main product. Endocellulases or endo-1,4-β-glucanases (EG) attack the amorphous regions of cellulose to produce oligosaccharides with new chain ends that are more susceptible to exocellulase attack. Glucose is released via the action of gluco- or glycoside hydrolases (GH), which attack the chain ends of cellulose and oligosaccharides; and β-1,4-glucosidase, which hydrolyze cellobiose and other cellodextrins to glucose. The hemicellulose fraction, such as xylans, is presumed to be degraded with an analogous enzyme system which hydrolyzes β-1,4 linkages to produce xylooligosaccharides and subsequently to the sugar monomers such as xylose. Degradation of the lignin fraction is carried out via an oxidative degradation mechanism involving "ligninase" enzymes that include phenol oxidase (lacasse) and peroxidases. The action of these enzymes leads to the production of highly reactive free radicals that not only facilitate the oxidative degradation of lignin through a phenol–quinone redox cycling process but also an alternative oxidative degradation pathway for cellulose degradation through oxidative attack on cellobiose to produce cellobionic acid, which is then converted to glucose via β-1,4-glucosidase action [4]. The released hexose sugars, mostly glucose, are assimilated by the fungal cell across the cell membrane and shuttled into the glycolytic pathway for conversion to pyruvate, which could be then metabolized to itaconate, fumarate, and malate through the mitochondrial or cytoplasmic (in the case of fumarate and malate) TCA cycle-related pathways [142]. Pentose sugars such as xylose released through hemicellulose degradation are first converted to glyceraldehyde-3-phosphate (G-3-P) either via the pentose phosphate pathway or phosphoketolase reaction pathway, after which G-3-P is shuttled to the glycolytic pathway in which it is an intermediate [140].

Fungal species with extracellular lignocellulolytic enzyme production capabilities have been found to be widespread among the Ascomycetes and Basidiomycetes groups [24]. Cellulase and xylanase enzymes have been produced mainly by Ascomycete fungi, such as *Trichoderma reesei*, *T. harzanium*, and *Aspergillus niger* grown in solid-state cultures with agro-industrial biomass residues as substrates [19, 65, 69, 101, 120]. Other *Aspergillus* species such as *A. niger*, *A. oryzae*, and *A. phoenicis* have been shown to generate substantial amounts of extracellular cellulolytic enzymes that could be utilized for enzymatic hydrolysis of cellulose to soluble sugars for fermentation [24, 78, 108]. Interestingly, strains of the itaconic acid-producing fungus *Aspergillus terreus* have also been shown to demonstrate cellulose degradation capabilities, which indicate its high potential for application for direct itaconic acid fermentation from biomass [22, 38, 53]. Oxidative ligninase enzymes are limited to the basidiomycetes including white-rot fungi and brown-rot fungi such as *Phanerochaete chrysosporium*, *Fusarium palustris*, *Coloris versicolor*, and *Trametes versicolor* [12, 47]. Numerous studies have shown that different fungi produce different levels and activities of the different hydrolytic enzymes. *Trichoderma* spp. was found to produce higher levels of endoglucanase and cellobiohydrolases but low levels of β-glucosidase [24, 64]. This could lead to inhibition of further cellulolytic activity due to build-up of the degradation products cellobiose and cellodextrins. On the other hand, *Aspergillus* species have been shown to have higher production and activities of β-glucosidase enzymes, which could alleviate inhibition of endo- and exocellulase activities, in addition

to endoglucanase and cellobiohydrolase [24]. Furthermore, hydrolytic enzyme expression and activities in one fungal strain could be affected by bioprocess conditions and environmental parameters. Thus, to achieve high performance of direct bioconversion of lignocellulose to organic acids, it is necessary to maximize the activities of all types of hydrolytic enzymes to minimize inhibition caused by build-up of hydrolysis products and intermediates and maintain a continuous flux of fermentable sugars for fungal growth and organic acid biosynthesis.

Consolidated bioprocessing: integrated hydrolysis and fermentation for improved direct bioconversion

Previous investigations have attempted to improve the performance in terms of substrate conversion yield and productivities of microbial fermentation processes particularly those utilizing solid starchy and lignocellulosic agro-industrial biomass residues as the carbon source in both SmF and SSF processes by utilizing an integrated (simultaneous) biomass hydrolysis (saccharification) and fermentation approach. The main premise of this technology is coupling the two separate processes of (1) hydrolysis of polymeric carbohydrate substrates to fermentable monosaccharide and disaccharide sugars, and (2) utilization of the derived sugars for microbial growth and metabolite production (in this case, acid fermentation) in a single bioprocessing step [141]. This method could eliminate the need for separate harsh physicochemical treatments for complete hydrolysis of carbon sources prior to fermentation, particularly in SmF processes, and minimize substrate pretreatment steps for solid-state fermentation applications. Additionally, inhibition of enzymatic hydrolysis caused by the accumulation of hydrolysis products could be avoided due to the continuous flux of carbon towards metabolic utilization for growth and product formation. This could lead to an increase in hydrolysis rate, which could consequently increase productivity and reduce required bioreactor volumes and capital costs [3]. The integrated direct fermentation approach could be applied to fungal fermentations for organic acid production from agro-industrial biomass substrates using either of these two strategies: (1) by adding commercially available hydrolytic enzymes to the fermentation culture to supplement and enhance the enzymatic activity of the fungal cultures, or (2) fermentation using mixed cultures of fungal strains with synergistic hydrolysis and fermentation activities.

Supplementation with commercial hydrolytic enzymes

The first strategy for integrated hydrolysis and fermentation involves supplementation of the fungal cultures with commercially available enzyme preparations such as cellulases and amylases. This approach has been applied previously for the production of lactic acid by *Rhizopus oryzae* in solid-state and semi-solid cultures using wheat straw [116], processed wood [2], paper mill sludge [88], and cassava pulp [106] as substrates, which demonstrated improved acid productivity, titer, and yield. In the production of lactic acid through a solid-state simultaneous saccharification and fermentation of cassava pulp by *Rhizopus oryzae*, the culture doubled its lactic acid titer from 206.20 mg per g dry substrate to 463.18 mg per g dry substrate following supplementation of the culture with commercial cellulase and glucoamylase [106]. Another study utilized carboxymethyl cellulase, a component of the cellulase enzyme complex and xylanase for the production of lactic acid using the same integrated hydrolysis and lactic acid fermentation with *R. oryzae* on corncob [115]. In another study on *R. oryzae* lactic acid fermentation by simultaneous saccharification and fermentation of processed wood, the build-up of cellobiose caused inhibition of endo- and exocellulases and addition of beta-glucosidase improved hydrolysis and fermentation kinetics and performance by [2]. However, the build-up of lactic acid caused inhibition of cellulase activity, thus continuous removal of the product acid is also necessary using methods such as passing the fermentation media through an anion-exchange resin column. Other strategies for reducing inhibition include multiple substrate addition (fed batch fermentation), and supplementation of fresh nutrients and enzymes. This approach has demonstrated much promise for improving the performance of solid-state fungal fermentation cultures. However, using expensive commercial enzyme preparations could add to the production costs, especially in the industrial scale.

Using mixed fungal cultures

Another strategy for performing simultaneous lignocellulose hydrolysis and organic acid fermentation of solid-state cultures involves co-culturing two or more different but compatible fungal strains in mixed consortia to exploit the metabolic synergisms among different fungi with different levels and activities of hydrolytic and biosynthetic enzymes. This approach has been applied previously for the enhancement of cellulase activity by lignocellulolytic fungi to minimize physicochemical pretreatments and supplementation of the culture with expensive commercial cellulolytic enzyme preparations. As mentioned earlier, none of the fungal strains are able to produce high levels of the different hydrolytic enzymes at the same time. *Trichoderma* fungi have been shown to exhibit high exo- and endocellulase production and activities but low on β-glucosidase while *Aspergillus* strains usually have higher β-glucosidase production and activities. Furthermore, other

fungal strains such as *Polyporus versicolor*, *Schizophyles commune*, and *Linzites saepiria* have been shown to produce hemicellulolytic enzymes such as β-mannanase and endoxylanase and exhibit a higher affinity towards hydrolyzing the hemicellulose fractions of biomass [54]. As a result, mixed cultures of *Trichoderma* and *Aspergillus* have been studied to synergistically enhance growth and the production and activity of cellulolytic enzymes using SSF cultures with biomass-based substrates such as bagasse [28], waste paper [56], paper mill sludge [77, 130], oat straw and wheat bran [123], soybean hull [10, 11], and corn stover [75]. Production of xylanases and mannanases from pure and mixed cultures of fungi has been reported as well [39, 43, 52, 64]. Direct production of organic acids from biomass substrates using fungal-mixed cultures has been tried previously for the production of lactic acid from lignocellulosic corn cob [85]. In the said study, lactic acid-producing *Rhizopus oryzae* MK-96-1196 was co-cultured with a cellulase-producing Ascomycete fungus *Acremonium thermophilus* ATCC 24622 resulting in improved lactic acid titer. This method has not been employed for the production of itaconic, fumaric, and malic acids. Itaconic acid-producing *A. terreus*, in itself able to express cellulase enzymes [22, 38, 53], could be co-cultured with *T. reesei* and/or *A. niger* in solid-state cultures for potentially improved direct bioconversion of lignocellulosic biomass to itaconic acid. The same could be done with fumaric acid-producing *R. oryzae* and malic acid-producing *A. flavus*. Another possibility is co-culturing with fungal strains producing biosynthetic intermediates other than sugars. In particular, cellulase- and citrate-producing *A. niger* could be co-cultured with itaconate-producing *A. terreus*, which could utilize the citrate as it is a direct substrate for itaconic acid biosynthesis [9]. The same could be applied for fumaric and malic acid producing strains, *Rhizopus* spp. and *A. flavus*, respectively, since each strain produces compounds that are direct biochemical intermediates of each other in the same pathway.

Despite the immense economic potential for applying mixed fungal cultures for direct fermentation of lignocellulosic biomass, the main technical challenge associated with simultaneous hydrolysis and fermentation in solid-state cultures of acid-producing fungi either using either of the two strategies is the expected difference in optimum conditions such as temperature and pH required for maximum activity, stability, and viability of hydrolytic enzymes versus acid biosynthesis enzymes, possibly even within the same fungal strain. Differences in optimum growth and metabolic conditions for different fungal strains in the mixed consortium could also exist [141]. Therefore, characterization of the microbial and biochemical kinetics and determination of the optimum bioprocessing conditions benefiting both substrate hydrolysis and acid fermentation processes are of high importance. For instance, studies on biochemical kinetics of simultaneous saccharification and fermentation for lactic acid production by *R. arhizzus* 36017 and *R. oryzae* 2062 with respect to growth pH, temperature, and substrate found the optimal growth conditions of initial starch concentration 20 g/L, pH 6.0, and 30 °C favorable for both starch saccharification and lactic acid fermentation [51]. Another factor to consider with regard to mixed fungal cultures is culture phasing, which involves determining the appropriate delay time between inoculation of each of the fungal strains comprising the fungal consortium into the fermentation substrate as well as the volumetric ratio of their respective inocula. Typically, the sequence of inoculation involved *T. reesei* preceding the addition of *Aspergillus* spp. by 36–48 h to ensure maximum synergism of cellulase activity [43, 44]. The effect of culture phasing and inoculum volume ratio on total cellulolytic activity of a mixed culture of *T. reesei* and *A. niger* has been studied in depth in the past. One investigation found optimum delay time of 48 h for *A. niger* addition and 5:1 of *T. reesei* and *A. niger* inoculum volume ratio [31]. Another study found an optimum phasing time of 15 h and inoculum ratio of 1:4 (*T. reesei* to *A. wentii*) for inoculation of *A. wentii* following *T. reesei* resulting into maximum cellulase and xylanase activities but no enhancement in β-glucosidase activity [40]. The same study also tested reversing the order of inoculation and found a decrease in the activities of cellulase, xylanase, and β-glucosidase with increasing delay time compared with the control (*T. reesei* to *A. wentii* inoculated simultaneously). The findings appear to suggest the eventual domination of the co-culture by the *Aspergillus* spp. fungi, regardless of the order of inoculation or inoculum ratio since these fungi produce organic acids that lower the pH of the culture, which restrict the growth of *T. reesei* or inhibit the activity of the hydrolytic enzymes. Finding the appropriate culture phasing time and inoculum ratio that will result in fermentation pH profiles that represent an optimum level for all fungal hydrolytic and biosynthetic enzyme components is thus a critical task not only for improving biomass hydrolysis performance but also for the direct bioconversion of lignocellulosic biomass to multifunction organic acid products. It is noteworthy to mention that majority of these studies have been conducted using submerged fermentation cultures. The performance of mixed cultures for direct acid fermentation by filamentous fungi in solid-state cultures has not been explored in depth yet, especially for itaconic, fumaric, and malic acid production.

Challenges, limitations, and research needs

The fact that direct biomass fermentation and solid-state fermentation in general are not more widely applied to bulk platform chemical production could be attributed to the technical and engineering challenges that come with

attempts to scale-up SSF processes. In practice, most of the superior benefits and advantages of SSF over SmF mentioned earlier were found to be applicable only in the bench scale [48]. This is due to scale-up issues that have been attributed to the build-up of temperature, pH, O_2, substrate, and moisture gradients in the solid substrate most especially for SSF processes involving static and intermittently agitated cultures. The presence of these gradients raises some challenging engineering problems that can be summarized into two main aspects: (1) sensing, monitoring, and control of critical bioprocess variables; and (2) fermentation kinetics modeling. These two aspects are interrelated with each other as successful modeling of SSF relies on precise and accurate measurement and collection of process variable data. Moreover, the design of bioreactors with appropriate monitoring and control systems for a process as complex as SSF in which extreme gradients in environmental parameters exist depends on precise and accurate mathematical modeling of the process.

The application of appropriate sensors to monitor and control critical environmental (pH, O_2, temperature, and moisture content) and carbon cycle (biomass growth, substrate concentration, CO_2) parameters is crucial to the successful scale-up of fungal SSF processes [6]. Unlike SmF cultures in which the liquid medium is fully mixed and homogeneous, SSF cultures are mostly heterogeneous and complex. Thus obtaining precise and accurate measurements of variables that represent the bulk substrate is difficult to obtain. In particular, the quick measurement and online monitoring of fungal biomass growth is identified as one of the most challenging tasks in SSF technology. Direct determination of fungal biomass growth in solid substrates is difficult due to the tight adherence and hyphal penetration of filamentous fungal mycelia into the solid substrate, making it almost impossible to separate and differentiate between the inert and bioactive solids [82]. Data on fungal growth is necessary for determining fermentation and bioreactor residence times and for mathematical modeling of the fermentation process. Respirometric methods, i.e., measuring O_2 uptake and/or CO_2 production rates and correlating them with biomass production, are commonly applied to estimate microbial growth in SSF, but are limited to systems with forced aeration [117]. For SSF cultures without forced aeration, off-line chemical assays of cellular components such as glucosamine, ergosterol, nucleotide acids, and proteins have been used. However, these assays are tedious, time-consuming, susceptible to interference by other cellular components, and preclude further analysis of samples for enzymatic activities and concentration of metabolites [82]. Other methods for measurement of fungal biomass growth, and the topic of SSF bioprocess sensing and monitoring as a whole have been widely researched and reviewed by other sources and are beyond the scope of this paper. For this purpose, the reader is directed elsewhere [6, 21, 84, 98, 125]. For fungal production of itaconic, fumaric and malic acids, precise monitoring and control of pH and O_2 and CO_2 to the optimum levels specific for each type of acid for maximum productivity and yield are required. Quick and precise online methods for measuring fungal biomass growth, substrate composition, product formation, and enzymatic activities are desirable since the process relies on nitrogen limitation to trigger acid overproduction and accumulation by the fungi. Ultimately, the purpose is to obtain the appropriate amount of kinetic data with the highest accuracy and precision as possible to be used for mathematical modeling and design of SSF bioreactors.

The development of mathematical models that describe the kinetics of the solid-state fermentation process is another important aspect that needs significant attention if the goal is to facilitate the scale-up of bench scale technologies to economically feasible commercial processes. The objective of mathematical or kinetic modeling is to develop mathematical expression that establishes and describes relationships among two or more different process variables that characterize the system; i.e., an equation relating biomass growth with incubation time and/or substrate concentration. As with any chemical or biochemical process, mathematical models with established parameters values for SSF processes are important in such a way that they provide a means to simulate the process using computer tools without performing numerous costly, time-consuming, and laborious experiments; a basis for the evaluation of the techno-economic feasibility of the process; and criteria for process scale-up and design of equipment and control systems [118]. However, there is not enough available information and standardized kinetics modeling rules and methods specific to SSF unlike with SmF technology, which could be attributed again to the structural and nutritional heterogeneity and complexity of solid media [96]. Kinetics of microbial fermentations are extremely sensitive to variations in environmental conditions such as pH, temperature, O_2, and moisture content. In SSF cultures gradients of these culture conditions are commonplace which nullifies a well-mixed assumption inherent with the commonly used models in SmF. Existing models in SmF mainly describe substrate conversion and diffusion and the consequent microbial growth but neglect many of the significant phenomena occurring in the complex interfaces of solid–gas–liquid biomass in SSF cultures. A few studies have sought to develop mathematical models for SSF that consider the effect of variations of bioprocess parameters such as temperature, moisture content, and air velocity on the kinetics of microbial growth in solid-state cultures [23, 34, 35, 46]. Mathematical modeling approaches in SSF that account

for diffusion and transport of substrate, concentration gradients as the primary driving force in SSF due to the absence of bulk convection as in SmF, and the generation of numerous side products have been reviewed previously [107]. Moreover, the unavailability of a suitable method for direct measurement of growth of the microorganisms in solid substrate cultures as mentioned earlier further complicates mathematical modeling tasks where microbial growth is a key variable.

These issues have not been adequately addressed leading to significant gaps in scientific and engineering knowledge on the subject of SSF. Research work has focused mainly on the general applicability of SSF for the production of various bio-based products such as enzymes, metabolites, and spores, and that many different solid substrates mainly as agro-industrial wastes have been combined with many different fungi and the productivity of each fermentation reported [48]. As a result, engineering concepts and design methods have yet to be fully standardized for a more successful commercial scale-up of SSF-based biotechnological processes for the production of value-added bio-products. Additionally, very little if any work has been done to elucidate molecular dynamics, gene expressions, and physiological properties on the different behavior of microorganisms, in particular fungi, when cultivated in liquid or solid media, thus can be regarded as the current "black box" of SSF biotechnology [48].

Concluding remarks

Solid-state fermentation, in particular direct bioconversion or integrated hydrolysis and fermentation of lignocellulosic biomass by filamentous fungi, has an immense potential to be a key biorefining technology in the future for the production of value-added platform chemicals. It also has a high economical and environmental sustainability component with regards to the value addition or valorization of low cost agro-industrial and municipal solid wastes and reduction of their landfill disposal. Although a significant amount of scientific knowledge and engineering background are already available for the conversion of various biomass materials to bio-based fuels and chemicals, these findings are obtained mostly from SmF studies and are not necessarily applicable to SSF. The use of SSF could minimize, if not eliminate entirely the need for physicochemical pretreatment and hydrolysis of lignocellulosic substrates to release soluble fermentable sugars in liquid media. Instead, the proposed SSF technology will rely entirely on the natural capabilities and growth conditions of filamentous fungi for the direct bioconversion of polymeric carbohydrates to platform chemicals such as itaconic, fumaric, and malic acids. Exploratory studies to determine the appropriate fungal-mixed culture composition and culture phasing, testing of different biomass substrates, optimization of bioprocess and downstream conditions, collection of biochemical kinetic data (growth, acid production, substrate degradation/consumption, enzymatic activities, etc.), development of precise and accurate mathematical models, and application of molecular methods for tracking the composition of fungal consortia in addition to mechanisms of gene expressions are highly desired. Overcoming these technological challenges could be considered as the next frontier in sustainable biorefining for many years to come.

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