Received: 9 February 2021 | Revised: 6 April 2021 | Accepted: 3 May 2021
DOI: 10.1002/btpr.3172

RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Hydrolysis of lignocellulose by anaerobic fungi produces free sugars and organic acids for two-stage fine chemical production with *Kluyveromyces marxianus*

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Funding information
National Science Foundation, Grant/Award Numbers: CBET-1706545, DGE-1333468; U. S. Department of Energy, Grant/Award Numbers: DE-SC0019093, DE-SC0020117

Abstract

Development of the bioeconomy is driven by our ability to access the energy-rich carbon trapped in recalcitrant plant materials. Current strategies to release this carbon rely on expensive enzyme cocktails and physicochemical pretreatment, producing inhibitory compounds that hinder subsequent microbial bioproduction. Anaerobic fungi are an appealing solution as they hydrolyze crude, untreated biomass at ambient conditions into sugars that can be converted into value-added products by partner organisms. However, some carbon is lost to anaerobic fungal fermentation products. To improve efficiency and recapture this lost carbon, we built a two-stage bioprocessing system pairing the anaerobic fungus *Piromyces indianae* with the yeast *Kluyveromyces marxianus*, which grows on a wide range of sugars and fermentation products. In doing so we produce fine and commodity chemicals directly from untreated lignocellulose. *P. indianae* efficiently hydrolyzed substrates such as corn stover and poplar to generate sugars, fermentation acids, and ethanol, which *K. marxianus* consumed while producing 2.4 g/L ethyl acetate. An engineered strain of *K. marxianus* was also able to produce 550 mg/L 2-phenylethanol and 150 mg/L isoamyl alcohol from *P. indianae* hydrolyzed lignocellulosic biomass. Despite the use of crude untreated plant material, production yields were comparable to optimized rich yeast media due to the use of all available carbon including organic acids, which formed up to 97% of free carbon in the fungal hydrolysate. This work demonstrates that anaerobic fungal pretreatment of lignocellulose can sustain the production of fine chemicals at high efficiency by partnering organisms with broad substrate versatility.

KEYWORDS
anaerobic fungi, bioprocessing, esters production, *K. marxianus*, lignocellulose

1 | INTRODUCTION

Renewable plant biomass represents a rich source of trapped carbon poised to accelerate the growth of the bioeconomy.[1,2] These feedstocks are inexpensive, ubiquitous, and rich in carbohydrates (making up to 55%–75% of their composition) that can be used in a broad range of bioprocesses.[3] Current and emerging technologies convert these feedstocks into a variety of products including biogas and biofuels, bioplastics, surfactants, oils and fatty acids, food additives, detergents, adhesives, lubricants, paints, and pigments that together make up a global market of over $48 billion each year.[1,4–6] However, current strategies to degrade recalcitrant plant material rely on

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expensive enzyme cocktails and/or harsh pretreatment techniques that produce compounds inhibitory to subsequent microbial growth and bioproduction.[6–9] On the other hand, fungal pretreatment of biomass could reduce costs and increase efficiency of lignocellulose decomposition because it is low energy, low cost, and does not create inhibitory compounds.[10]

Anaerobic fungi are an appealing solution to this problem as they are able to degrade crude, untreated biomass into fermentable sugars that can be converted into a wide variety of products.[11–13] One example, *Piromyces indianae (Piromyces sp. UH3-1)*, has been shown to degrade a variety of feedstocks including food waste and lignin-rich poplar.[14] This substrate flexibility is due in part to the fact that anaerobic fungi have the largest repertoire of biomass degrading enzymes in the fungal kingdom[15] – even larger than the well-known biomass degraders *Trichoderma reesei*,[9] white rot fungi,[10] and wood decay fungi.[16] More importantly, enzymes produced during cultivation of anaerobic fungi were competitive with commercial *Aspergillus* and *Trichoderma* cocktails on untreated biomass.[17] As native symbionts of the rumen of large herbivores, these fungi have adapted strategies to efficiently degrade the material ingested by their hosts. One such strategy being the ability to tailor the carbohydrate active enzymes (CAZymes) that they express in response to the substrates that they encounter.[13,14,17] These strategies require that they establish synergy with other microbes and archaea present through cross-feeding and niche degradation.[18–20] Until their potential can be realized with genetic engineering tools,[11,21,22] the value of anaerobic fungi lies primarily in their ability to degrade a wide variety of recalcitrant renewable materials.

By pairing the extensive degradative abilities of anaerobic fungi with other microbes that specialize in production of chemicals and fuels, we can create cost-effective, sustainable bioproduction platforms. Previously, in silico modeling was used to explore the range of organisms that can partner with anaerobic fungi in stable coculture and found methanogens and clostridia to be the most suitable partners.[23] However, because of the mismatch in growth rates and metabolic needs of many potential partnering organism, most organisms cannot be cocultured with anaerobic fungi. Instead, a two-stage platform must be pursued where anaerobic fungi hydrolyze lignocellulosic biomass in the first stage into fermentable sugars that microbes in the second stage convert to product. Such a two-stage bioproduction system has been demonstrated for anaerobic fungi partnered with *E. coli* and *S. cerevisiae*.[12,13] These studies, however, focused primarily on the production and utilization of sugars while regarding the anaerobic fungal produced organic acids as byproducts of fermentation. The organic acids, however, represent a significant carbon source, frequently larger than the free sugars, which can be efficiently captured and used by a variety of organisms.

Kluyveromyces marxianus is one such microorganism that can consume lactate, acetate, and ethanol in addition to the liberated sugars.[24–27] Often noted as the fastest growing eukaryote, *K. marxianus* is a facultative anaerobic yeast that produces large amounts of ethyl acetate (EtAc) when metabolizing glucose, whey, and other substrates.[28,29] In addition to EtAc, which can be used as a solvent or scent in perfumes, *K. marxianus* produces a variety of esters and branched alcohols that may serve as next generation biofuels.[30,31] Recent work has demonstrated the biosynthesis of 2-phenyl ethanol (2-PE),[30] a higher alcohol that can be used a food additive or potentially a biofuel, and isoamyl alcohol (IsA),[32] a solvent and flavoring chemical.[33] With a complete gene-editing toolbox[24,30,34,35] and wide range of culturing conditions,[36] *K. marxianus* is an emerging organism for bioproduction.

In this study, we pair *P. indianae* with engineered *K. marxianus* and evaluate the ability of a two-stage system to produce fragrances and advanced biofuels. The substrate versatility of *K. marxianus* enables capture of more available carbon while broadening the range of products generated via anaerobic fungal pretreatment. We demonstrate comparable production yields in a two-stage system relative to *K. marxianus* bioproduction on rich media for EtAc, 2-PE, and IsA, and confirm the use of "waste" fermentation acids such as acetate and lactate. This work provides further evidence for the feasibility of two-stage bioproduction systems with anaerobic fungal pretreatment and demonstrates the production of high-value products directly from inexpensive agricultural wastes.

2 | MATERIALS AND METHODS

2.1 | Strains, media, and culture conditions for feedstock hydrolysis by anaerobic fungi

The anaerobic fungus *P. indianae* (*Piromyces sp. UH3-1*) was previously isolated and characterized by Hooker et al.[14] *Piromyces* cultures were inoculated from 50 ml starter cultures containing corn stover biomass in anaerobic Medium C[37] supplemented with 15% v/v rumen fluid. Medium C is a phosphate and carbonate-buffered media containing some yeast extract and casitone as a nitrogen source, and rumen fluid for additional nutrients. Serum bottles with 12 g/L of substrate in 50 ml of either Medium C or semi-defined minimal Medium B were inoculated with 3 ml of starter culture. Medium B is a semi-defined medium with NH_4Cl replacing some complex nitrogen sources and a complex mix of fatty acids, trace metals, hemin, and vitamins in place of rumen fluid.[38] Substrates used were Sigmacell (Type 20, Sigma Aldrich), corn stover, alfalfa, or poplar. Plant biomass was milled to 20 mesh. Media and substrate were autoclaved at 121°C for 30 min and then supplemented with sterile filtered chloramphenicol dissolved in 60% v/v ethanol (final concentration of 3.5 μg/ml) prior to inoculation with *P. indianae*. After *P. indianae* growth, spent media were centrifuged for 10 min at ≥12,000 g to separate the biomass for analysis from the supernatant for *K. marxianus* cultures. The hydrolysates were stored at −20°C until they were to be used. To assess the effects of substrate loading, the aforementioned substrates were loaded at either 12 or 24 g/L in 10 ml of either Medium B or Medium C. These cultures were treated with antibiotic as above, inoculated with 1 ml of a *Piromyces* starter culture, and monitored by HPLC every 7 days (See *HPLC analysis of supernatants* below).

2.2 | Two-step *K. marxianus* cultivation on spent media

K. marxianus CBS 6556 *ura3Δ his3Δ* was used as the parent strain for the experiments described here and is referred to here as WT-u-h.[30] The strain engineered for high 2-PE production was derived from the parent as described previously.[30] Briefly, WT-u-h was modified by disrupting ARO8, EAT1, and ABZ1, overexpressing feedback insensitive variants of ARO4 and ARO7, and overexpressing wild type alleles of PHA2 and ARO10. Synthetic defined (SD) medium is defined as 6.7 g/L BD Difco™ Yeast Nitrogen Base without amino acids, 0.79 g/L CSM powder (Sunrise Science Products), and 20 g/L D-glucose. *K. marxianus* strains were also cultivated in rich YPD medium (YPD: 10 g/L Gibco™ Bacto™ Yeast Extract, 20 g/L Gibco™ Bacto™ Peptone, 20 g/L D-glucose). All *K. marxianus* strains were precultured in 1 ml YPD medium for 12 h from single colonies, and then inoculated to an initial OD600 of 0.05 in 10 ml of indicated media in 50 ml baffled shake flasks. Spent *P. indianae* hydrolysates were sparged with compressed air for 10 min and sterile filtered before being distributed into 10 ml aliquots in 50 ml baffled shake flasks for culturing of *K. marxianus*. Culturing was conducted at 250 rpm in an INFORS HT Multitron incubation shaker with temperature control set to 30°C.

2.3 | HPLC analysis of supernatants

Supernatants were analyzed for sugars and acids using 1 ml of culture supernatant collected from cultures and controls at indicated time points. The supernatants were stored at −20°C until they were to be analyzed and were then cleared of particulate debris by three cycles of centrifugation at 21,000g for 10 min each cycle. Clarified HPLC samples were kept at 4°C prior to analysis in an Agilent 1260 HPLC with an Aminex HPX 87H anion exchange column using a 50°C, 5 mM H_2SO_4 mobile phase at 0.6 ml/min. An Agilent 1260 Infinity II Refractive Index Detector (Agilent Technologies, Santa Clara, CA) was used to detect analytes over a 30 min run from a 20 μl injection. Concentrations were determined from standard curves of each analyte prepared from commercial standards.

2.4 | GC-FID analysis of *K. marxianus* products

Analysis of 2-phenylethanol (2-PE), 2-phenylethyl acetate (2-PEAc), isoamyl alcohol (IsA), isoamyl acetate (IsAc), and ethyl acetate was carried out on a Shimadzu GC-2010 Plus equipped with a Shimadzu AOC-20s autosampler and a Shimadzu AOC-20i auto-injector. The GC suite was coupled to a flame ionization detector (FID). Compounds were separated on an Agilent J&W DB-WAX Ultra Inert column (length: 30 m; inner diameter: 0.32 mm; film thickness: 0.5 μm). 2-PE and 2-PEAc were detected and separated using a 21 min temperature program as follows: start temperature of 100°C, 20°C/min–140°C, 10°C/min–150°C, 5°C/min–160°C, hold for 2 min then increase by 1°C/min–170°C, hold for 2 min, and finally 25°C/min–220°C. For IsA, IsAc, and ethyl acetate, the temperature was held at 40°C for 2 min, and then increased at 20°C/min–70°C, finally increased to 220°C from 70°C by 50°C/min, and held at 220°C for 2 min.[39] Helium was used as the carrier gas at a flow rate of 1.9 ml/min. The sample injection volume was 1 μl where split mode was used for injection and the ratio was 20:1.

For sample preparation, a 330 μl sample of cell culture was centrifuged for 1 min at 5000g. Three-hundred microliters of supernatant was collected and transferred to a clean 1.5 ml tube with 300 μl of organic extractant. 300 μl of cyclohexane was used as an extractant for 2-PE and 2-PEAc, and 300 μl of hexane was used for IsA, IsAc, and ethyl acetate. Samples were vortexed thoroughly for 30 min, centrifuged at 10,000g for 1 min, and 100 μl of the organic layer was transferred into a 2 ml clear Agilent GC vial with glass insert. Standard curves depicting the linear correlation between the concentration of five compounds (2-PE, 2-PEAc, IsA, IsAc, and ethyl acetate) with the area of peaks from FID were obtained to quantify extracellular metabolite accumulation under different media conditions. A series of YPD solutions with known concentrations were made and extracted by cyclohexane or hexane accordingly following the same procedure which was used to extract these five compounds from the supernatants of cell cultures.

2.5 | Compositional analysis of feedstocks

The sugar composition of raw plant biomass was determined according to NREL methods.[40–42] Carbohydrates were determined using the same HPLC analysis configuration as above, (see *HPLC analysis of supernatants* above), however a temperature of 65°C and run time of 45 min was used for separation of analytes.

The sugar composition of the plant biomass after inoculation with anaerobic fungi was determined by first separating the fermentation liquor from the solids via centrifugation. Samples were centrifuged for 5 min at 4°C at 8000g. The wet pellets containing spent plant biomass and anaerobic fungal biomass were dried to constant weight at 45°C. The resulting dry biomass was used for NREL gravimetric analysis and carbohydrate quantification following standard methods. To correct for the amount of anaerobic fungal biomass present in the pellet at the end of the first stage of fermentation, the amount of formic acid measured in the supernatant (See *HPLC analysis of supernatant* above) was used to calculate the fungal biomass yield and was then subtracted from the dry biomass.[43]

3 | RESULTS AND DISCUSSION

3.1 | Anaerobic fungi pretreatment of lignocellulose supports growth of *K. marxianus*

To evaluate the feasibility of a two-step bioproduction system pairing *K. marxianus* and *P. indianae*, we tested how well the yeast grows on anaerobic fungal media before and after anaerobic fungal hydrolysis

of cellulose-rich substrates. Because *K. marxianus* is not a cellulolytic organism, it was unable to grow on fresh unhydrolyzed anaerobic fungal media containing Sigmacell, a form of cellulose, as a carbon source (Figure 1(a)). In contrast, *K. marxianus* grew on spent *P. indianae* media up to about 60% of that on YPD containing 2% glucose, a standard yeast growth medium. Because spent media sustains *K. marxianus* growth, it suggests the anaerobic fungus liberates enough free carbon to make a two-stage bioprocessing system feasible. To optimize growth of *K. marxianus*, we also investigated the effect of anaerobic fungal media type and lignocellulosic feedstock.

Anaerobic fungi are typically grown on one of two types of media: Medium B[38] or Medium C[37] (Figure S1). Medium C is a richer medium that is rumen fluid based while Medium B is a minimal medium that is more defined and contains only 20% of the undefined yeast extract and 10% of the undefined peptone components compared to Medium C. We evaluated *K. marxianus* growth in Medium B and Medium C supplemented with 12 g/L Sigmacell after *P. indianae* hydrolysis. The richer Medium C hydrolysate allowed *K. marxianus* to grow to an OD_{600} about three-fold higher than Medium B (Figure 1(b)). Growth in Medium B was similar to the synthetic defined (SD) yeast minimal medium containing 20 g/L glucose. However, as Medium C supports more yeast biomass, we selected it as the primary medium for our bioproduction studies.

While *P. indianae* can break down both simple and lignin-rich plant material without any genetic engineering,[14] it is not clear if their hydrolysates from various feedstocks could sustain subsequent yeast cultures. Here, we compared *K. marxianus* growth on a variety of hydrolyzed feedstocks that ranged from simple to complex and lignin-rich. Specifically, we used Sigmacell as an example of crystalline cellulose, as well as three lignocellulosic feedstocks: corn stover, poplar and alfalfa. After 4 weeks hydrolysis of 12 g/L of substrate-supplemented media, each of the resulting hydrolysates supported growth of *K. marxianus* to similar levels (OD_{600} between 25 and 30) in spent Medium C (Figure 1(c)). This suggests that *P. indianae* hydrolysis of both simple and complex cellulosic substrates provided similar levels of usable carbon for sustaining *K. marxianus* growth, or at least enough carbon so that another nutrient became limiting. Similar systems have previously demonstrated that the model organisms *E. coli* and *S. cerevisiae* could be paired for ethanol production on other anaerobic fungus hydrolysates.[12,13] Growth of *K. marxianus* on hydrolysates, however, shows that we can expand this type of bioproduction platform to include non-model organisms that produce various other industrially useful compounds.[29,30,32,36]

3.2 | *K. marxianus* growth is primarily supported by anaerobic fungal generated fermentation products

Anaerobic fungus release carbon in the form of free glucose, xylose, lactate, formate, acetate, and ethanol from lignocellulosic feedstocks.[12] However, it is unclear how these products vary with substrates or are used by *K. marxianus*. We tracked sugar and organic acid levels as a function of time in both stages to determine what carbon was being released by *P. indianae* and which was used by *K. marxianus*. With anaerobic fungi, growth phase is tracked by monitoring culture pressure (Figure 2(a)), as particulate lignocellulosic biomass interferes with conventional OD measurements.[44] Accumulation of free carbon in the medium does not occur until approximately the mid-late log stage of *P. indianae*'s growth (Figure 2(b),(c)). In stationary phase (≥96 h), the organic acid production by the fungus does not show a statistically significant increase although its CAZymes continue to liberate free sugars from the feedstocks. Enzymatic hydrolysis continues long after *P. indianae* growth, up to at least 3 weeks after *P. indianae* entered stationary phase, consistent with observations in other anaerobic fungi.[12,13] After 4 weeks of *P. indianae* hydrolysis, there was up to 8.7 g/L glucose or 96 mM acetyl-CoA equivalent metabolites from

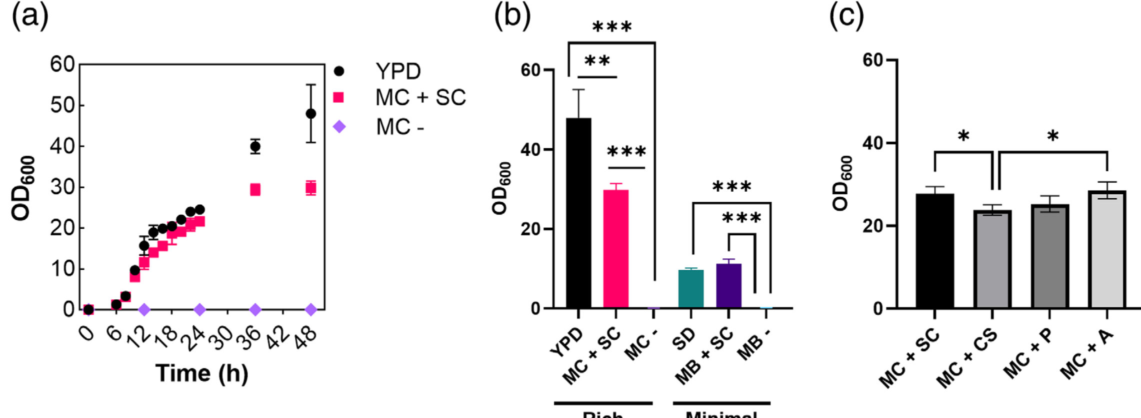


FIGURE 1 Aerobic growth of *K. marxianus* on anaerobic fungal-treated lignocellulosic feedstocks. (a) Growth curves of *K. marxianus* on standard yeast media (YPD) and anaerobic fungal media with Sigmacell before (Medium C, MC -) and after *P. indianae* hydrolysis (MC + SC). (b) Final OD of *K. marxianus* after growth for 48 h in rich medias: YPD and MC and minimal medias: synthetic defined medium (SD) and Medium B (MB). (c) Final OD of *K. marxianus* after growth for 48 h as a function of feedstock used in the spent fungal media. OD 1 represents 10^7 cells/ml of *K. marxianus* cells. Error bars represent standard deviation of triplicate cultures. MC, Medium C, MB, Medium B, SC, Sigmacell, CS, corn stover, P, Poplar, A, Alfalfa. Results from Student's *T*-tests are indicated by: $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$

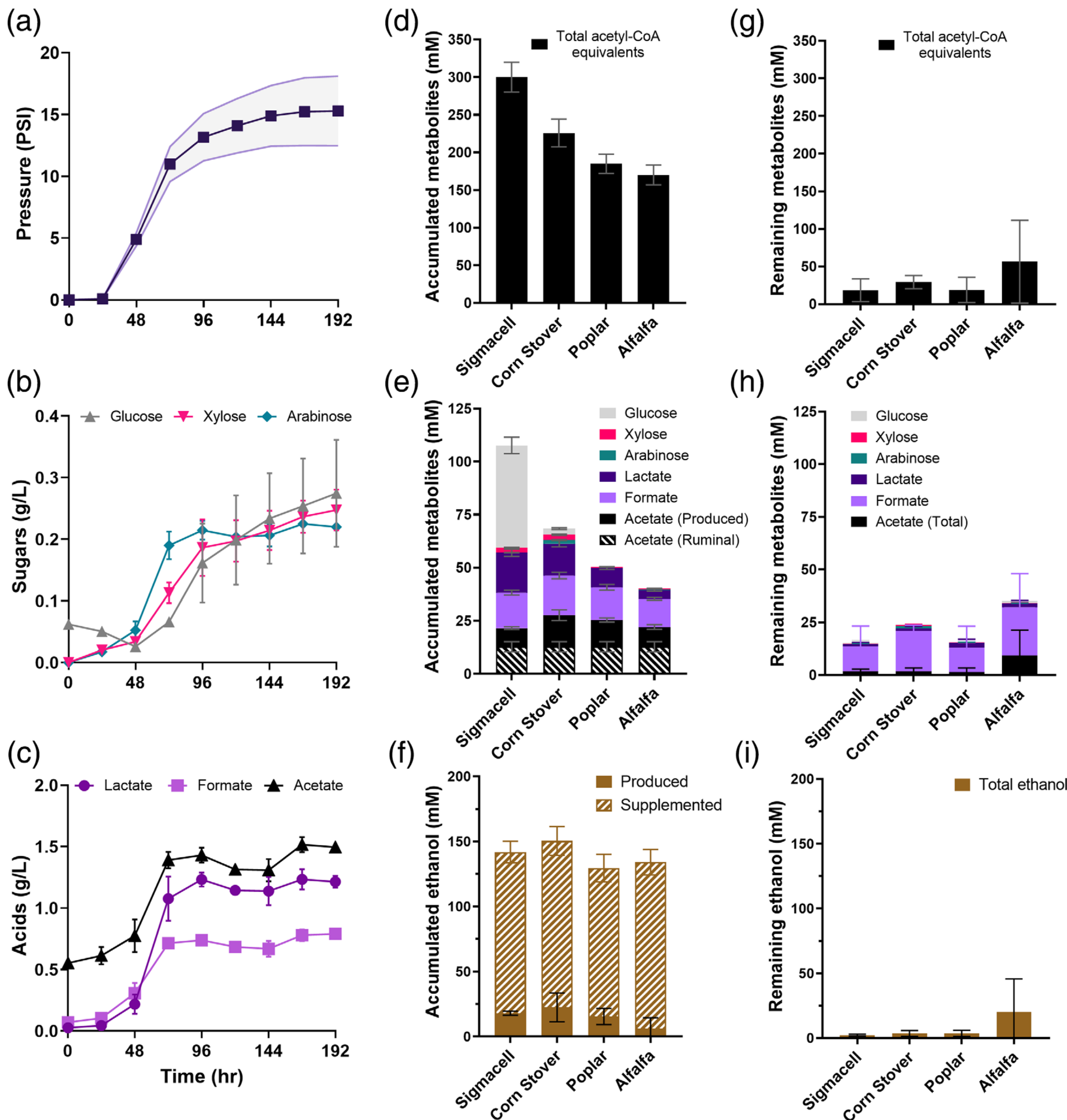


FIGURE 2 Composition of *P. indianae*-treated corn stover Medium C before and after growth by *K. marxianus*. (a) Representative 8-day *P. indianae* growth curve where pressure is used as a proxy for fungal growth[44]; the shaded confidence interval represents the standard deviation. (b) Free sugars released by *P. indianae* hydrolysis. (c) Fermentation acids produced by *P. indianae* growth. (d) Acetyl-CoA equivalents liberated from diverse feedstocks after 28 days, where the total carbon is a sum of the organic acids, sugars, and ethanol. One millimeter glucose = 2 mM acetyl-CoA equivalents (e) Free sugars and organic acids released from diverse feedstocks after 28 days; ruminal acetate = media baseline. (f) Ethanol accumulated in *P. indianae* cultures on diverse feedstocks after 28 days; produced ethanol = total ethanol − supplemented ethanol; supplemented ethanol = media background ethanol (measured) + antibiotic supplemented ethanol (calculated). (g) Acetyl-CoA equivalents remaining after *K. marxianus* CBS6556 WT-u-h growth on feedstock hydrolysates (h) Remaining acids and sugars, and (i) ethanol after 48 h of *K. marxianus* growth. Error bars, standard deviation. Acetyl-CoA equivalents are based on stoichiometric conversions of metabolite to Acetyl-CoA via central metabolism

glucose and up to 200 mM of acetyl-CoA equivalent metabolites in the form of xylose, arabinose, lactate, formate, acetate, and ethanol (Figure 2(d)).

The form of the available carbon varied dramatically with feedstock composition. Free sugars were only released from Sigmacell, a more amorphous synthetic cellulose that is >90% glucose, and corn stover (45.0 ± 1.1% glucan, 26.4 ± 0.04% xylan, 4.1 ± 0.9% arabinan, 17.7 ± 0.4% lignin) (Figure 2(e)).[14] Sigmacell was hydrolyzed better than the other feedstocks with the hydrolysate reaching about 8.7 g/L (or 48 mM) of free glucose. Hydrolysis of lignocellulose, however, seemed to be hindered by the complex structure (Table S1). For example, only about 1 g/L (or 7 mM) glucose, xylose, and arabinose are made available from corn stover. In poplar (48.4 ± 0.3% glucan, 23.6 ± 0.6% xylan, 0% arabinan, 23.9 ± 0.5% lignin) and more lignin-

rich alfalfa (28.5 ± 4.4% glucan, 9.5 ± 1.8% xylan, 3.9 ± 0.1% arabinan, 77.2 ± 1.6% lignin), released sugars decreased an order of magnitude to <0.5 mM despite comparable glucan fractions to corn stover. This may potentially be attributed to some CAZymes becoming deactivated by lignin[45] through irreversible binding of the CAZymes onto the feedstocks,[9] or reduced hydrolysis due to lignin and other structural polymers blocking access to hydrolysable carbon. Nonetheless, the dominant carbon source for *K. marxianus* growth was not free sugars but the organic acids generated by the anaerobic fungus. Up to 60 mM of lactate, formate and acetate were produced from anaerobic fungal hydrolysis (Figure 2(e)). Yields for more complex poplar and alfalfa were slightly reduced at 50 mM and 40 mM, respectively, perhaps reflecting reduced substrate hydrolysis. Interestingly, hydrolysis of lignocellulosic feedstocks produced mainly formate and acetate, whereas lactate was the dominant product on Sigmacell. Finally, ethanol was produced at around 0.3–0.4 g/L (15–22 mM) from Sigmacell, corn stover, and poplar, but only about 0.1 g/L (or 6 mM) from alfalfa (Figure 2(f)). It should also be noted that there is a sizeable amount of ethanol present (~125 mM) in the media - some comes from the crude rumen fluid but the majority is from the antibiotic solution that is supplemented to keep the cultures sterile (Figure 2(f)). Despite the variations in the form of available carbon, however, the total carbon available for *K. marxianus* is similar.

We next evaluated the ability of *K. marxianus* to use the different forms of available carbon. >95% of the free acetyl-CoA equivalents generated by *P. indianae* were consumed by *K. marxianus* (Figure 2(g)). All metabolites except formate were completely consumed from the Sigmacell, corn stover, and poplar hydrolysates (Figure 2(h),(i)). Formate is a *K. marxianus* end-product[25] and a small amount is produced (2–9 mM).[46] Since *K. marxianus* can utilize nearly all of the carbon forms released into the *P. indianae* hydrolysate, we pose that pairing these two organisms will allow us to increase the amount of carbon captured in the final products and reduce the amounts that go to unwanted byproducts.

3.3 | *K. marxianus* converts spent fungal media to high-value commodity chemicals with high efficiency

Since all feedstock hydrolysates supported growth of *K. marxianus*, we assessed bioproduction from each spent media and compared the titers to those on rich YPD media. Here, we evaluated two engineered *K. marxianus* strains: WT-u-h and ENGR. The WT-u-h parent strain is a derivative of a high-producing EtAc CBS 6556 strain[29] with URA3 and HIS3 knockouts. The high-producing 2-PE strain (ENGR) is a derivative of the parent strain that contains an EAT1 knockout that suppresses *K. marxianus*' ability to produce ethyl acetate and overexpresses *ARO4*^{K221L}, *ARO7*^{G141S}, *PHA2*, and *ARO10* to increase flux to the 2-PE biosynthesis pathway (Figure 3(a)).[30] The EAT1 knockout also prohibits *K. marxianus* from forming 2-phenylacetate (2-PEAc) or isoamyl acetate (IsAc), acetylated derivatives of 2-PE and IsA, respectively. Depending on whether we want to produce EtAc, 2-PE, or IsA, we can select or create new strains that enhance production of a particular product from our spent media.

Ethyl acetate is commodity chemical used in several industrial processes as a solvent and fragrance, and it is a product made by wildtype *K. marxianus* strains from ethanol and acetate (Figure 3(a)). We found that the parent WT-u-h strain of *K. marxianus* made 2.4 g/L EtAc on the Sigmacell hydrolysate in Medium C (see Figure S2 for data on Medium B). This represented a 33% increase from the 1.8 g/L made on YPD, likely due to the high levels of ethanol and free sugars and acids present in spent media. WT-u-h cultures from the lignocellulosic feedstock hydrolysates that contained lower levels of free sugars, however, produced less EtAc reaching about 0.75 g/L for corn stover and around 0.2 g/L for alfalfa and poplar (Figure 3). This reduction cannot be explained by the relative abundance of acetyl-CoA equivalents. Lignocellulosic hydrolysates with low sugar levels (corn stover, poplar, alfalfa) exhibited reduced EtAc yields (50%–90% reduction) suggesting that EtAc production requires high sugar (Figure 3(b)). Due to *EAT1* knockout in the ENGR strain, this strain did not produce any EtAc and likely redirected the flux of acetyl-CoA equivalents to other pathways.

In contrast to the EtAc that is formed directly from central metabolism, 2-PE is a product of the Shikimate and Ehrlich pathways. As a fine chemical, 2-PE is a valuable food additive and next generation biofuel due to its increased energy density.[31] Recently, the *K. marxianus* ENGR strain was created to pull flux through the Shikimate pathway and increase titers produced in rich media to the g/L scale.[30] We found that the both the WT-u-h and ENGR strain were able to produce 2-PE from spent media; however, the *EAT1* *k*nockout boosted the yield four- to five-fold (Figure 3(a)). For the WT-u-h strain, 2-PE titers scaled with available acetyl-CoA equivalents (Figure 3(b),(c)). That is, spent fungal media resulted in comparable production yields of 2-PE in WT-u-h strains. For the ENGR strain, however, rich YPD media produced more 2-PE (908 mg/L) than the richest hydrolysate (551 mg/L). This did not scale with available acetyl-CoA equivalents, potentially due to the formation of alternate products.

IsA is valuable solvent, scent, and flavoring produced from the Ehrlich pathway reactions (Figure 3(a)). While the strains here were not specifically engineered for enhanced IsA production, it is made as a byproduct of both WT-u-h and ENGR metabolism (Figure 3(a), Figure S2 for Medium B). In YPD medium, the ENGR strain makes about twice as much IsA as the WT-u-h strain, which is consistent with the fact that the *EAT1* knock-out suppresses acetylation from IsA to IsAc, and the overexpression of *ARO10* enhances the downstream pathway biosynthesis from α-ketoisocaproate to IsA. Interestingly, we observed that the IsA levels do not vary between the WT-u-h and ENGR strains when they are grown on the lignocellulosic hydrolysates (Figure 3(b),(c)) suggesting there is no change in the flux to IsA when grown in spent media. Overall, our results demonstrate that not only can *K. marxianus* capture and use the released sugars and fungal degradation products, but that they may be converted to product with high efficiency.

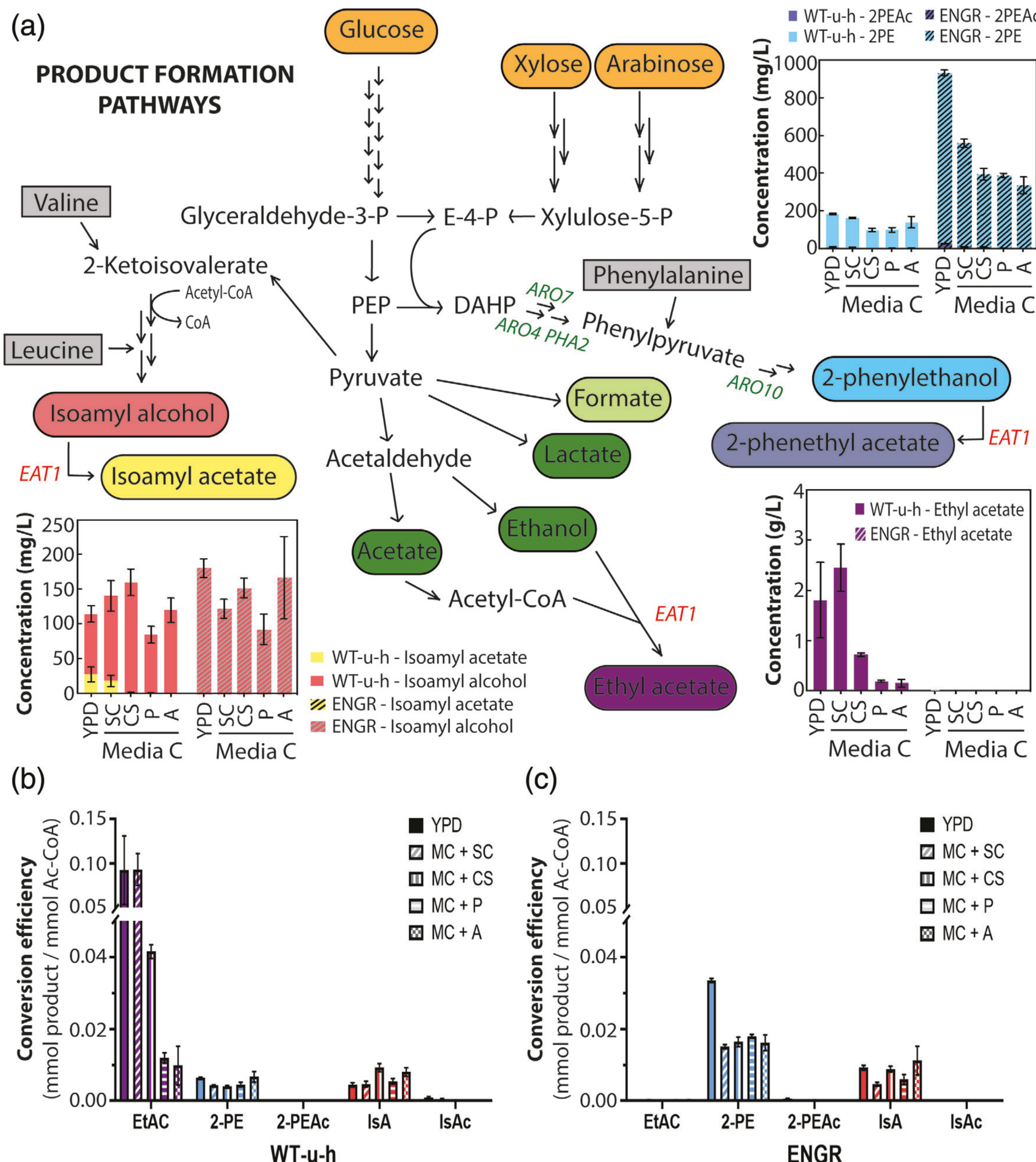


FIGURE 3 *K. marxianus* synthesis of acetate esters and alcohols from *P. indianae* hydrolyzed biomass. Orange indicates sugars in *P. indianae* hydrolysates, while dark green indicates organic acids and alcohols in the hydrolysates that are consumed by *K. marxianus*. Formate (light green) is also present in the hydrolysate. Product titers from *K. marxianus* cultures are shown next to their biosynthetic pathways for EtAc (dark purple) 2-PE (blue), 2-PEAc (light purple), IsA (red), and IsAc (yellow). WT-u-h (solid) and ENGR (striped) values are shown. Genes knocked out in the engineered strain (ENGR) are shown in red and overexpressed genes are shown in green. Alternate substrates are shown in gray. (b) Conversion efficiencies of *K. marxianus* WT-u-h and (c) *K. marxianus* ENGR to produce bioproducts from the various medias. Conversion efficiency is calculated using the product yields and total free acetyl-CoA in the hydrolysates (i.e., generated acid, ethanol, and sugar + native acids and supplemented ethanol). Error bars, standard deviation

3.4 | *K. marxianus* captures "lost" carbon from anaerobic fungal pretreatment increasing efficiency

To determine the overall efficiency of a two-stage process, we then evaluated how much of the feedstock hydrolyzed by *P. indianae* was converted to product by *K. marxianus*. Compositional analysis of the substrates before and after anaerobic fungal growth revealed that nearly all (92%) of the loaded Sigmacell biomass was hydrolyzed (Table S1). For lignocellulosic feedstocks, between 20% and 50% of the feedstocks were hydrolyzed (Figure 4(a)) although in all cases, this encompassed more than 55% of all the available sugars in the substrate (Figure 4(b)). This compares well to bacterial-based biomass hydrolyzing *Clostridium thermocellum* which solubilized 24%–65% of the glucose and xylose of switchgrass biomass before

pretreatment.[47,48] However, despite the high utilization of feedstocks, 47%–98% of the cleaved sugars were metabolized by *P. indianae* (Figure 4(c)) resulting in the low observed sugar yields (Figure 2(e)). As little as 2% of this sugar was made available for direct conversion to product (Figure 4(d)). Similarly, *C. thermocellum* biocatalysis systems, which are often loaded with ~5–15g of biomass/L,[47,48] also metabolize the majority of solubilized sugars at this loading and may make them poorly suited as a coculture partners. That is, current 2-stage bioproduction systems that rely on sugar transfer lose a significant amount of the carbon released from plant biomass. Nonetheless, *K. marxianus* is able to recover this metabolized carbon by using the produced lactate, acetate, and ethanol to grow and generate product. Despite low sugar yields, our two-stage system makes 37%–72% of the hydrolyzed carbon available depending on substrate (Table 1). The usable acid and ethanol from lignocellulosic hydrolysates account for 78%–97% of the available carbon or 3.7–54 times the amount of carbon from sugars alone demonstrating the potential that these substrates possess if they are presented to the right organism such as *K. marxianus*.

Ultimately, the exogenous and liberated substrates manifest in product titers of EtAc, 2-PE, and IsA up to 0.20, 0.046, and 0.014g product/g feedstock hydrolyzed in our two-stage system (Table S2). Similarly, the efficiency of formation of these various products on a per-gram-substrate basis are greater than the efficiency of product formation from the glucose in YPD – 0.15, 0.015, and 0.007 for EtAc, 2-PE, and IsA, respectively (Table S2). These titers also compare reasonably well with the yield of ethanol per gram biomass of other two-stage platforms (0.22 g/g biomass)[49] including those with

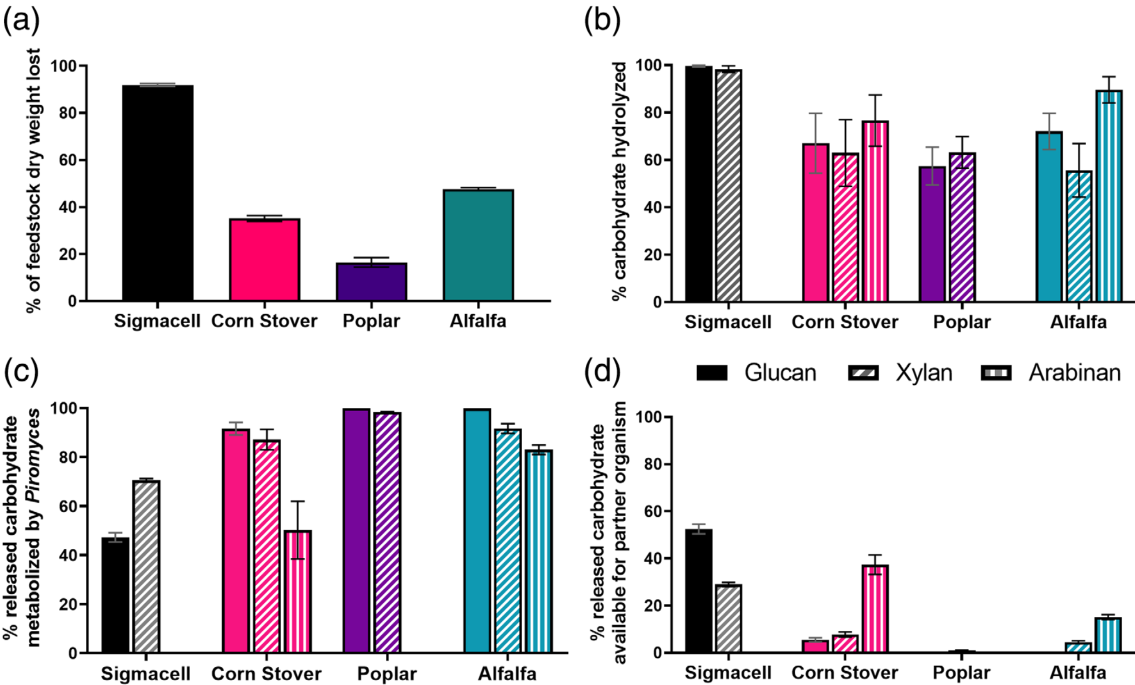


FIGURE 4 Hydrolysis of untreated feedstock by *P. indianae*. (a) Feedstock biomass lost due to hydrolysis. (b) Percentage of glucan, xylan, and arabinan hydrolyzed. (c) Percentage of sugar that was metabolized by *P. indianae* (e.g., was hydrolyzed but not released into the supernatant). (d) Percentage of sugar released into the supernatant. Error bars, standard deviation

TABLE 1 Carbon released by anaerobic fungi and made available to *K. marxianus*[a]

		Sigmacell	Corn Stover	Poplar	Alfalfa
Stage 1	% hydrolyzed	99.5%	69.7%	59.0%	70.0%
	Released carbon (mmol)	6.68	3.50	3.04	1.10
Stage 2	Available carbon (mmol)	4.84	1.87	1.12	0.67
	Sugars (mmol)	3.40	0.40	0.02	0.04
	Lactate and acetate (mmol)	0.98	0.83	0.64	0.41
	Ethanol (mmol)	0.46	0.63	0.45	0.22
	% hydrolyzed carbon available	72%	53%	37%	61%
	Ratio of avail. Acids + ethanol to sugars	0.42	3.65	54.50	15.75

[a]Amounts of hydrolyzed carbon reported in Acetyl-CoA equivalents.

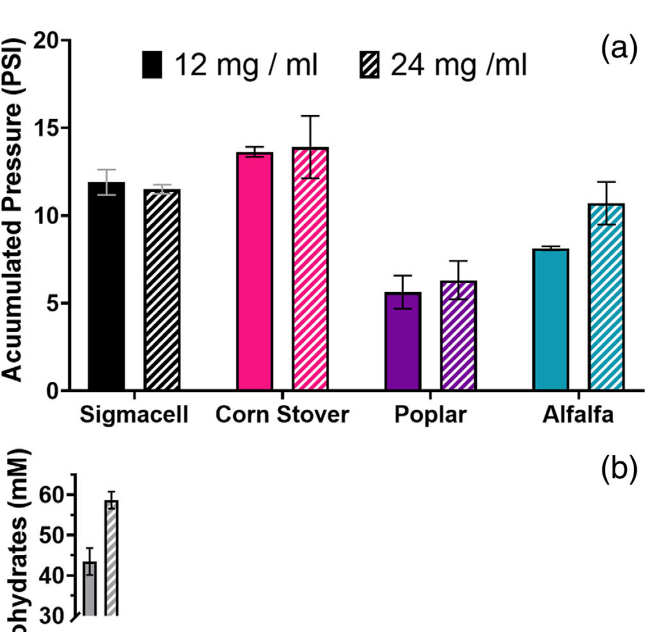
anaerobic fungi (0.04–0.14 g/g biomass).[12,13] However, to our knowledge, this is the first report of a two-stage system for producing esters and higher alcohols.

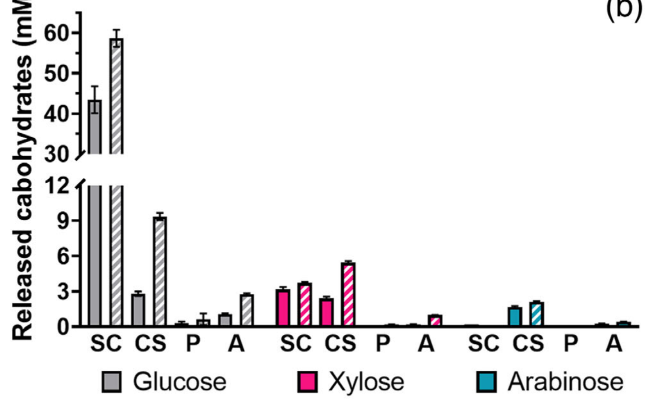
A similar approach was recently used to create 82 μg of poly-α-olefins per g of substrate from the hydrolysate of *Clostridium cellulolyticum* and subsequent culture of *Acinetobacter baylyi* ADP1.[50] The olefin production phase was sustained from a hydrolysate that reached a maximum concentration of ~5 mM glucose, 5 mM acetate, and 7 mM lactate produced from crystalline cellulose (Avicel) via *C. cellulolyticum*. While *C. cellulolyticum* grows slightly more quickly than our isolate, at the same culture time (7 days) anaerobic fungi had produced almost 5 mM in glucose, xylose and arabinose, 16 mM acetate and 13 mM lactate at roughly half the substrate loading. Our anaerobic fungal hydrolysates are able to make available at least twice the carbon available on a more complex substrate with reduced substrate loading – ~22 mM Acetyl CoA equivalents for *C. celluloyticum* from Avicel versus ~39 mM for *P. indianae* from untreated corn stover. Thus, anaerobic fungi can hydrolyze even crude untreated agricultural residues to release more carbon than more established strains on purified substrates.

Based on the initial glucan, xylan, and arabinan content, we calculated that our system reached up to 41% EtAc, 12% IsA, and 17% 2-PE of the theoretical yield for various feedstocks (Table S2B). While the theoretical yield is not achievable due to carbon needed for cell biomass and energy, these hydrolysates produced more EtAc, 2-PE, and IsA than would theoretically be yielded by sugars alone for the more complex lignocellulosic feedstocks (Table S2C). Taken together, our system increases efficiency of chemical production from lignocellulose by capturing carbon that would otherwise be discarded or viewed as unusable.

3.5 | Increasing substrate loading enhances hydrolysis and results in higher yields of liberated sugars

As formation of EtAc and other products appeared sugar-limited, we assessed whether increasing substrate loading can increase the sugar and acid yields. We doubled the feedstock loading (from 12 to 24 g/L) for all four feedstocks and tracked the metabolites from *P. indianae*. When we doubled the loading of Sigmacell, corn stover, and poplar, the growth of *P. indianae* was unaffected while alfalfa growth increased about 20% (Figure 5(a)). We suspect that the alfalfa cultures were carbon limited under 12 g/L loading conditions because the glucan and xylan content of alfalfa is so low. However, the amount of free glucose from double loaded alfalfa increased 2.53-fold suggesting that it is no longer carbon limited and can generate free sugar. Glucose levels in the double loaded Sigmacell and corn stover hydrolysates also increased from about 8–10.7 g/L (43–58 mM; 1.28-fold) and from about 0.5–1.7 g/L (2.9–9.1 mM; 3.26-fold), respectively (Figure 5(b)). Similar trends were obtained for hydrolysates in Medium B, albeit at lower yields (Figure S3). All double-loaded feedstock hydrolysates, however, had increased amounts of free xylose and arabinose, for those with measurable arabinan content (Figure 5 (b)). Interestingly, these did not appear to be maximum yields as sugar release did not taper off after 21 days (Figure S4). In comparison to other anaerobic fungi, at 50 g/L loading of reed canary grass *Anaeromyces robustus* and *Neocallimastix californiae* also yielded up to 2 g/L glucose, as well as up to 1 g/L xylose and 1 g/L arabinose.[13] Here, *P. indianae* hydrolyzed similar levels of glucose and xylose in half the loading of corn stover (24 g/L; Figure 5(b)). The similar sugar yields for our fungi under conditions with half the substrate suggest that





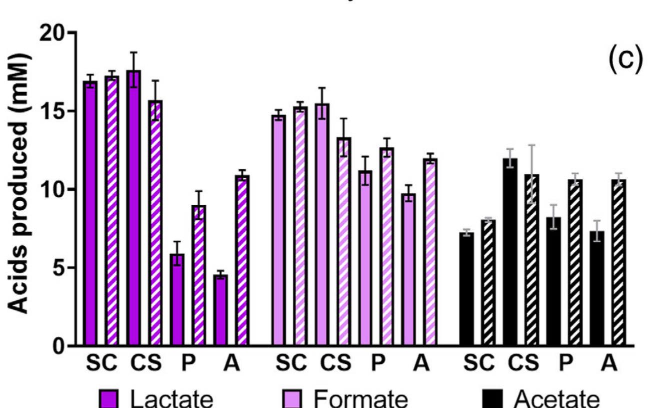


FIGURE 5 Effects of substrate loading on *P. indianae* hydrolysis. (a) Growth of *P. indianae* on Media C with 12 or 24 g/L substrate loading. (b) Released sugars from *P. indianae* hydrolysis with 12 or 24 g/L loading. (c) Organic acids produced from *P. indianae* hydrolysis with 12 or 24 g/L loading. Error bars represent the standard deviation or triplicate experiments

yields are a complex function of feedstock composition, substrate loading, and fungal strain. Interestingly, acid production by *P. indianae* did not change with loading suggesting that given more feedstock, the excess is hydrolyzed to sugars and not metabolized by the fungus (Figure 5(c)). Thus, increasing substrate loading can produce excess free sugars and should be optimized to improve product formation efficiency in the second stage.

P. indianae produced similar glucose yields of free glucose from *untreated* plant biomass as *T. reesei*, a more common and faster growing aerobic fungus, on a pretreated substrate. *P. indianae* enzymes released ~0.09g glucose/g corn stover versus ~0.11g glucose/g sorghum by *T. reesei*.[51] However, *T. reesei* hydrolysis required alkaline pretreatment to barely exceed the performance of anaerobic fungi. Although *T. reesei* produced these maximum sugar yields on pretreated biomass in 36 h, hydrolysis longer than 36 h resulted in lower sugar yields due to consumption by *T. reesei*. In contrast, anaerobic fungal sugar yields increase monotonically with time (Figure 2(b)). Moreover, the substrate loading for *T. reesei* was 100 g/L or more than four times higher than the highest loading tested with anaerobic fungi. Our data suggests that anaerobic fungal sugar yields scale nonlinearly with substrate loading; for example, doubling corn stover substrate more than tripled the glucose released on corn stover (Figure 5 (c)). Thus, yields from anaerobic fungi may exceed those of *T. reesei* systems at comparable substrate loadings.

In addition to optimizing system parameters such as substrate loading, efficiency could perhaps be tuned further with a suite of genetic tools for anaerobic fungi to control CAZyme expression and organic acid production. Specifically, the development of gene-editing tools, like CRISPR-Cas9 that allow knock-ins and knock-outs would allow us to exchange weak promoters for stronger ones that overexpress hydrolytic enzymes to enhance feedstock conversion. In addition, these tools could be used to knockout xylose metabolisms genes that may hinder yields in anaerobic fungi. Advanced synthetic biology tools could create kill-switch constructs that stop *P. indianae* metabolism of the feedstock while allowing the hydrolysis to reach higher yields. As we work to advance these organisms for biotechnology and the bio-based economy, these tools will be extremely useful to optimize feedstock conversion and potentially bioproduction by anaerobic fungi.

4 | CONCLUSIONS

We built a two-stage bioprocessing system pairing *P. indianae* and *K. marxianus* to produce fine and commodity chemicals from lignocellulosic feedstocks. We first assessed the ability of *P. indianae* to metabolize a variety of lignocellulosic biomass to generate free sugars, organic acids, and ethanol. While the simpler feedstocks generated more free carbon, we found that even the most complex substrates could produce enough carbon to support *K. marxianus* growth. Recently engineered strains of *K. marxianus* were shown not only to grow on the hydrolyzed media from *P. indianae* but also to produce ethyl acetate, 2-phenyl ethanol, and isoamyl alcohol. In comparison to commonly used yeast media (YPD), the hydrolysates performed at comparable efficiencies for ethyl acetate and isoamyl alcohol and at half the efficiency for phenyl ethanol. Despite the low sugar content of some hydrolysates, the organic acids and ethanol was converted to useful product by *K. marxianus*. Finally, we identified substrate loading as a limiting factor for efficient sugars yields that could improve our system. While this two-stage bioproduction system is unoptimized, this work demonstrates the adaptability of this platform to incorporate effective degrader and producer specialists to produce a wide array of chemicals from lignocellulosic waste.

ACKNOWLEDGMENTS

This project was supported in part by the Office of Biological and Environmental Research through the DOE Office of Science Early Career Program, Contract No DE-SC0020117 (KS) and through the Genomic Science Program, Contract No DE-SC0019093 (IW). This material is also based upon work supported by the National Science Foundation Graduate Research Fellowship to CAH under Grant No DGE-1333468 and through NSF-CBET Grant No 1706545 to IW. We express our gratitude to C3Bio at Purdue University, Dr. Rich Meilan, and Carl Huettemann for providing the poplar materials. We also thank Lewis Hooker, Clifford Selby, and Dr. Cliff Weil for providing corn stover, alfalfa, and sorghum, respectively. The Purdue Lab for Renewable Resource Engineering (LORRE), and Dr. Klein Ileleji provided the equipment necessary to prepare and mill these feedstocks, respectively. We also thank Blaine Brown and the Boiler Butcher Block at Purdue University for providing the rumen fluid necessary to culture this organism.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

1. Rogers JN, Stokes B, Dunn J, et al. An assessment of the potential products and economic and environmental impacts resulting from a billion ton bioeconomy. *Biofuels Bioprod Biorefin*. 2017;11:110-128. https://doi.org/10.1002/bbb.1728.
2. Anwar Z, Gulfraz M, Irshad M. Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *J Radiat Res Appl Sci*. 2014;7(2):163-173. https://doi.org/10.1016/j.jrras.2014.02.003.
3. Mosier N, Wyman C, Dale B, et al. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol*. 2005; 96(6):673-686. https://doi.org/10.1016/j.biortech.2004.06.025.
4. de Jong E, Higson A, Walsh P, Wellisch M. Task 42 Biobased Chemicals - Value Added Products from Biorefineries; 2011.
5. Diaz AB, Blandino A, Caro I. Value added products from fermentation of sugars derived from agro-food residues. *Trends Food Sci Technol*. 2018;71:52-64. https://doi.org/10.1016/j.tifs.2017.10.016.

6. Lopes AM, Ferreira Filho EX, Moreira LRS. An update on enzymatic cocktails for lignocellulose breakdown. *J Appl Microbiol*. 2018;125(3): 632-645. https://doi.org/10.1111/jam.13923.
7. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng*. 2012;109(4):1083-1087. https://doi.org/10.1002/bit.24370.
8. Sun Y, Cheng JJ. Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. *Bioresour Technol*. 2005;96(14): 1599-1606. https://doi.org/10.1016/j.biortech.2004.12.022.
9. Palonen H, Tjerneld F, Zacchi G, Tenkanen M. Adsorption of Trichoderma reesei CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. *J Biotechnol*. 2004;107 (1):65-72. https://doi.org/10.1016/j.jbiotec.2003.09.011.
10. Wan C, Li Y. Fungal pretreatment of lignocellulosic biomass. *Biotechnol Adv*. 2012;30(6):1447-1457. https://doi.org/10.1016/j.biotechadv.2012.03.003.
11. Flad V, Young D, Seppälä S, et al. The biotechnological potential of anaerobic gut fungi. In: Benz JP, Schipper K, eds. *Genetics and Biotechnology*. Cham: Springer International Publishing; 2020:413-437. https://doi.org/10.1007/978-3-030-49924-2_17.
12. Ranganathan A, Smith OP, Youssef NH, Struchtemeyer CG, Atiyeh HK, Elshahed MS. Utilizing anaerobic fungi for two-stage sugar extraction and biofuel production from lignocellulosic biomass. *Front Microbiol*. 2017;8:635. https://doi.org/10.3389/fmicb.2017.00635.
13. Henske JK, Wilken SE, Solomon KV, et al. Metabolic characterization of anaerobic fungi provides a path forward for bioprocessing of crude lignocellulose. *Biotechnol Bioeng*. 2018;115(4):874-884. https://doi.org/10.1002/bit.26515.
14. Hooker CA, Hillman ET, Overton JC, et al. Hydrolysis of untreated lignocellulosic feedstock is independent of S-lignin composition in newly classified anaerobic fungal isolate, Piromyces sp. UH3-1. *Biotechnol Biofuels*. 2018;11(1):1-14. https://doi.org/10.1186/s13068-018-1292-8.
15. Seppälä S, Wilken SE, Knop D, Solomon KV, O'Malley MA. The importance of sourcing enzymes from non-conventional fungi for metabolic engineering and biomass breakdown. *Metab Eng*. 2017;44:45-59. https://doi.org/10.1016/j.ymben.2017.09.008.
16. Skyba O, Douglas CJ, Mansfield SD. Syringyl-Rich lignin renders poplars more resistant to degradation by wood decay fungi. *Appl Environ Microbiol*. 2013;79(8):2560-2571. https://doi.org/10.1128/AEM.03182-12.
17. Solomon KV, Haitjema CH, Henske JK, et al. Early-branching gut fungi possess large, comprehensive array of biomass-degrading enzymes. *Science*. 2016;351(6278):1192-1195. https://doi.org/10.1126/science.aad1431.
18. Gilmore SP, Lankiewicz TS, Wilken SE, et al. Top-down enrichment guides in formation of synthetic microbial consortia for biomass degradation. *ACS Synth Biol*. 2019;8(9):2174-2185. https://doi.org/10.1021/acssynbio.9b00271.
19. Hagen LH, Brooke CG, Shaw CA, et al. Proteome specialization of anaerobic fungi during ruminal degradation of recalcitrant plant fiber. *ISME J*. 2020;15:421-434. https://doi.org/10.1038/s41396-020-00769-x.
20. Teunissen MJ, Kets EPW, Op den Camp HJM, Huis in't Veld JHJ, Vogels GD. Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. *Arch Microbiol*. 1992;157(2):176-182. https://doi.org/10.1007/BF00245287.
21. Hooker CA, Lee KZ, Solomon KV. Leveraging anaerobic fungi for biotechnology. *Curr Opin Biotechnol*. 2019;59:103-110. https://doi.org/10.1016/j.copbio.2019.03.013.
22. Hillman ET, Readnour LR, Solomon KV. Exploiting the natural product potential of fungi with integrated -omics and synthetic biology approaches. *Curr Opin Syst Biol*. 2017;5:50-56. https://doi.org/10.1016/j.coisb.2017.07.010.
23. Wilken SE, Saxena M, Petzold LR, O'Malley MA. In silico identification of microbial partners to form consortia with anaerobic fungi. *Processes*. 2018;6(1):1-14. https://doi.org/10.3390/pr6010007.
24. Löbs AK, Engel R, Schwartz C, Flores A, Wheeldon I. CRISPR-Cas9-enabled genetic disruptions for understanding ethanol and ethyl acetate biosynthesis in Kluyveromyces marxianus. *Biotechnol Biofuels*. 2017;10(1):1-14. https://doi.org/10.1186/s13068-017-0854-5.
25. Fonseca GG, Gombert AK, Heinzle E, Wittmann C. Physiology of the yeast Kluyveromyces marxianus during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Res*. 2007;7(3): 422-435. https://doi.org/10.1111/j.1567-1364.2006.00192.x.
26. Sakihama Y, Hidese R, Hasunuma T, Kondo A. Increased flux in acetyl-CoA synthetic pathway and TCA cycle of Kluyveromyces marxianus under respiratory conditions. *Sci Rep*. 2019;9(1):1-7. https://doi.org/10.1038/s41598-019-41863-1.
27. Mo W, Wang M, Zhan R, Yu Y, He Y, Lu H. Kluyveromyces marxianus developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways. *Biotechnol Biofuels*. 2019; 12(1):1-15. https://doi.org/10.1186/s13068-019-1393-z.
28. Löser C, Urit T, Gruner E, Bley T. Efficient growth of Kluyveromyces marxianus biomass used as a biocatalyst in the sustainable production of ethyl acetate. *Energy Sustain Soc*. 2015;5(1):1-15. https://doi.org/10.1186/s13705-014-0028-2.
29. Löbs AK, Lin JL, Cook M, Wheeldon I. High throughput, colorimetric screening of microbial ester biosynthesis reveals high ethyl acetate production from Kluyveromyces marxianus on C5, C6, and C12 carbon sources. *Biotechnol J*. 2016;11(10):1274-1281. https://doi.org/10.1002/biot.201600060.
30. Li M, Lang X, Moran Cabrera M, et al. CRISPR-mediated multigene integration enables Shikimate pathway refactoring for enhanced 2-phenylethanol biosynthesis in Kluyveromyces marxianus. *Biotechnol Biofuels*. 2021;14(1):1-15. https://doi.org/10.1186/s13068-020-01852-3.
31. Keasling JD, Chou H. Metabolic engineering delivers next-generation biofuels Jay. *Nat Biotechnol*. 2008;26(3):298-299. https://doi.org/10.1038/ng.85.
32. İşleten HM. Study of increasing the production of volatile flavor compounds by the yeast Kluyveromyces marxianus through optimization of carbon and nitrogen sources. *Food Heal*. 2018;4(2):112-123. https://doi.org/10.3153/fh18011.
33. Yuan J, Mishra P, Ching CB. Engineering the leucine biosynthetic pathway for isoamyl alcohol overproduction in *Saccharomyces cerevisiae*. *J Ind Microbiol Biotechnol*. 2017;44(1):107-117. https://doi.org/10.1007/s10295-016-1855-2.
34. Rajkumar AS, Varela JA, Juergens H, JMG D, Morrissey JP. Biological parts for Kluyveromyces marxianus synthetic biology. *Front Bioeng Biotechnol*. 2019;7:1-15. https://doi.org/10.3389/fbioe.2019.00097.
35. Lang X, Besada-Lombana PB, Li M, Da Silva NA, Wheeldon I. Developing a broad-range promoter set for metabolic engineering in the thermotolerant yeast Kluyveromyces marxianus. *Metab Eng Commun*. 2020;11:e00145. https://doi.org/10.1016/j.mec.2020.e00145.
36. Fonseca GG, Heinzle E, Wittmann C, Gombert AK. The yeast Kluyveromyces marxianus and its biotechnological potential. *Appl Microbiol Biotechnol*. 2008;79(3):339-354. https://doi.org/10.1007/s00253-008-1458-6.
37. Lowe SE, Griffith GG, Milne A, Theodorou MK, Trinci APJ. The life cycle and growth kinetics of an anaerobic rumen fungus. *J Gen Microbiol*. 1987;133(7):1815-1827. https://doi.org/10.1099/00221287-133-7-1815.
38. Lowe SE, Theodorou MK, Trinci APJ, Hespell RB. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *J Gen Microbiol*. 1985;131(9):2225-2229. https://doi.org/10.1099/00221287-131-9-2225.

39. Löbs AK, Schwartz C, Thorwall S, Wheeldon I. Highly multiplexed CRISPRi repression of respiratory functions enhances mitochondrial localized ethyl acetate biosynthesis in Kluyveromyces marxianus. *ACS Synth Biol*. 2018;7(11):2647-2655. https://doi.org/10.1021/acssynbio.8b00331.
40. Hames B, Ruiz R, Scarlata C, Sluiter A, Sluiter J, Templeton D. Preparation of samples for compositional analysis. *Natl Renew Energy Lab*. 2008;12. https://www.nrel.gov/docs/gen/fy08/42620.pdf.
41. Sluiter A, Hames B, Ruiz R, et al. Determination of structural carbohydrates and lignin in biomass. *Natl Renew Energy Lab*. 2012;TP-510-42618:17.
42. Sluiter A, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of extractives in biomass. *Natl Renew Energy Lab*. 2008;TP-510-42619:838-852. https://doi.org/10.1016/j.rmr.2016.02.006.
43. Lowe SE, Theodorou MK, Trinci APJ. Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. *Appl Environ Microbiol*. 1987;53(6):1216-1223. https://doi.org/10.1128/aem.53.6.1216-1223.1987.
44. Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim Feed Sci Technol*. 1994;48(3):185-197. https://doi.org/10.1016/0377-8401(94)90171-6.
45. Vermaas JV, Petridis L, Qi X, Schulz R, Lindner B, Smith JC. Mechanism of lignin inhibition of enzymatic biomass deconstruction. *Biotechnol Biofuels*. 2015;8(1):1-16. https://doi.org/10.1186/s13068-015-0379-8.
46. Geertman JMA, Van Dijken JP, Pronk JT. Engineering NADH metabolism in *Saccharomyces cerevisiae*: Formate as an electron donor for glycerol production by anaerobic, glucose-limited chemostat cultures. *FEMS Yeast Res*. 2006;6(8):1193-1203. https://doi.org/10.1111/j.1567-1364.2006.00124.x.
47. Paye JMD, Guseva A, Hammer SK, et al. Biological lignocellulose solubilization: comparative evaluation of biocatalysts and enhancement via cotreatment. *Biotechnol Biofuels*. 2016;9(1):1-13. https://doi.org/10.1186/s13068-015-0412-y.
48. Holwerda EK, Worthen RS, Kothari N, et al. Multiple levers for overcoming the recalcitrance of lignocellulosic biomass. *Biotechnol Biofuels*. 2019;12(1):1-12. https://doi.org/10.1186/s13068-019-1353-7.
49. Zuroff TR, Xiques SB, Curtis WR. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic clostridium phytofermentans/yeast co-culture. *Biotechnol Biofuels*. 2013;6(1):1-12. https://doi.org/10.1186/1754-6834-6-59.
50. Salmela M, Lehtinen T, Efimova E, Santala S, Santala V. Towards bioproduction of poly-α-olefins from lignocellulose. *Green Chem*. 2020;22(15):5067-5076. https://doi.org/10.1039/d0gc01617a.
51. Syadiah EA, Haditjaroko L, Syamsu K. Bioprocess engineering of bioethanol production based on sweet sorghum bagasse by co-culture technique using Trichodermareesei and *Saccharomyces cerevisiae*. *IOP Conf Ser Earth Environ Sci*. 2018;209(1):012018. https://doi.org/10.1088/1755-1315/209/1/012018.

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How to cite this article: Hillman ET, Li M, Hooker CA, Englaender JA, Wheeldon I, Solomon KV. Hydrolysis of lignocellulose by anaerobic fungi produces free sugars and organic acids for two-stage fine chemical production with *Kluyveromyces marxianus*. *Biotechnol Progress*. 2021;e3172. https://doi.org/10.1002/btpr.3172