Coordination of consolidated bioprocessing technology and carbon dioxide fixation to produce malic acid directly from plant biomass in *Myceliophthora thermophila*

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Abstract

**Background:** Consolidated bioprocessing (CBP) technique is a promising strategy for biorefinery construction, producing bulk chemicals directly from plant biomass without extra hydrolysis steps. Fixing and channeling CO<sub>2</sub> into carbon metabolism for increased carbon efficiency in producing value-added compounds is another strategy for cost-effective bio-manufacturing. It has not been reported whether these two strategies can be combined in one microbial platform.

**Results:** In this study, using the cellulolytic thermophilic fungus *Myceliophthora thermophila*, we designed and constructed a novel biorefinery system DMCC (Direct microbial conversion of biomass with CO<sub>2</sub> fixation) through incorporating two CO<sub>2</sub> fixation modules, PYC module and Calvin–Benson–Bassham (CBB) pathway. Harboring the both modules, the average rate of fixing and channeling <sup>13</sup>CO<sub>2</sub> into malic acid in strain CP51 achieved 44.4, 90.7, and 80.7 mg/L/h, on xylose, glucose, and cellulose, respectively. The corresponding titers of malic acid were up to 42.1, 70.4, and 70.1 g/L, respectively, representing the increases of 40%, 10%, and 7%, respectively, compared to the parental strain possessing only PYC module. The DMCC system was further improved by enhancing the pentose uptake ability. Using raw plant biomass as the feedstock, yield of malic acid produced by the DMCC system was up to 0.53 g/g, with <sup>13</sup>C content of 0.44 mol/mol malic acid, suggesting DMCC system can produce 1 t of malic acid from 1.89 t of biomass and fix 0.14 t CO<sub>2</sub> accordingly.

**Conclusions:** This study designed and constructed a novel biorefinery system named DMCC, which can convert raw plant biomass and CO<sub>2</sub> into organic acid efficiently, presenting a promising strategy for cost-effective production of value-added compounds in biorefinery. The DMCC system is one of great options for realization of carbon neutral economy.

**Keywords:** *Myceliophthora*, Metabolic engineering, CBB cycle, CO<sub>2</sub>-fixation, Plant biomass, Malic acid

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source for many industrial applications and green biosynthesis of biofuels and commodity chemicals. However, because of the recalcitrant nature of lignocellulose to enzymatic hydrolysis, the costs of conversion of insoluble lignocellulosic materials to fermentable sugars represent a significant barrier to the production of cost-competitive biofuels [3, 4]. Consolidated bioprocessing (CBP), featuring the hydrolysis and fermentation in a single process step without adding any extra cellulosases, is widely recognized as a promising strategy for cost-effective production of plant biomass-derived biofuels and chemicals [5]. CBP entails microbial engineering by functional expression of cellulosases in a fermentative organism, or incorporation of the desired product-synthesis pathway into a cellulase-producing organism [3, 6]. Recently, cellulytic organisms, such as Trichoderma [7], Aspergillus, Clostridium [8], and Myceliophthora [9, 10] have been tried as CBP strains for direct conversion of plant cell wall materials into biofuels and biochemicals, including ethanol [11], isobutanol [12], itaconic acid [13], and malic acid [9, 10].

CO₂ is also a potentially scalable raw material to produce sustainable fuels and chemicals that could be alternatives to petroleum products. Fixing and channeling CO₂ into the central carbon metabolism of industrial microbes has potential to reduce the CO₂ level in the environment and increase carbon efficiency in the production of value-add compounds, for example, production of succinic or acetic acid from methanol and CO₂ [14]. Several CO₂ fixation pathways, including the Calvin–Benson–Bassham (CBB) cycle, the 3-hydroxypropionate bicycle, and methanol condensation pathways, have been introduced into heterotrophic organisms, such as Escherichia coli, Saccharomyces cerevisiae, Pichia pastoris, and Methylobacterium extorquens [15–22].

The CBB cycle, also known as the reductive pentose phosphate cycle, is the most dominant CO₂ fixation pathway of the seven known natural alternatives [23–25]. The CBB cycle employs 11 enzymes to complete autotrophic CO₂ fixation. Most of these enzymes are also involved in central metabolism, including glycolysis and the pentose phosphate pathway [26]. Ribulose-1,5-bisphosphate carboxylase–oxygenase (RuBisCO) and phosphoribulokinase (PRK) are the two key enzymes of the CBB cycle. PRK catalyzes phosphorylation of ribulose-5-phosphate, a normal intermediate of pentose phosphate pathway, to ribulose-1,5-bisphosphate. RuBisCO is the one enzyme that is specific to the CBB cycle, and catalyzes the carboxylation of ribulose-1,5-bisphosphate with CO₂ to generate two molecules of 3-phosphoglycerate [27]. Functional overexpression of genes encoding RuBisCO and PRK in E. coli resulted in significantly decreased release of CO₂ [20, 22, 28] and the biosynthesis of sugar from CO₂ [29].

Based on construction of RuBisCO-dependent E. coli, a RuBisCO mutant with higher activity and better solubility was selected [30, 31]. Recycling CO₂ into the central metabolic network is a promising approach for expanding the use of CO₂ fixation to improve the yield of target metabolites. In S. cerevisiae, parts of the CBB cycle have been integrated into the metabolic network to enable the use of CO₂ as an additional electron acceptor for the reoxidation of NADH and simultaneous recycling of CO₂ released from the decarboxylation of pyruvate, resulting in increased productivity and yield of ethanol [17, 21, 32]. Recently, a non-native CBB cycle has been introduced into heterotrophic organisms to generate autotrophic or mixotrophic organisms, coupling of modification of central metabolic pathway. Antonosky et al. engineered E. coli to hemi-autotrophically grow on CO₂, with reducing power and energy from oxidation of pyruvate [29]. In follow-up studies, E. coli and P. pastoris were converted into autotrophs, which could incorporate CO₂ into biomass, respectively, using formate or methanol as the energy source for a heterologous CBB cycle [18, 33]. Pentoses, including xylose and arabinose, are metabolized by pentose phosphate pathway, where the intermediate ribulose-5-phosphate can serve as the substrate of PRK. Previously, xylose and arabinose were used as the feedstock to drive CO₂-fixation by the CBB cycle in E. coli and S. cerevisiae [17, 20, 32].

Malic acid is widely used in the food industry as the acidulant and flavor enhancer and it was selected as one of the 12 most important building block chemicals available from renewable biomass by the US Department of Energy in 2004. Studies evaluating microbial malate production have attracted much industrial attention. Four native metabolic pathways that produce malic acid from glucose have been identified and analyzed [34]. The reductive tricarboxylic acid (rTCA) pathway, with theoretical yield of 2 mol malic acid/mol glucose, is considered the most efficient pathway, because of the CO₂ fixation during the carboxylation reaction of pyruvate to oxaloacetate catalyzed by pyruvate carboxylase [34]. Filamentous fungi offer great potential advantages in the use of complex carbon sources and production of organic acids at high concentration with yield near the theoretical maximum, and several such species have been engineered as cell factories for producing malic acid, including Aspergillus, Penicillium, Rhizopus, and Myceliophthora [35–38].

The thermophilic filamentous fungus Myceliophthora thermophila (synonym Thermothelomyces thermophila), which is able to secrete a large amount of hydrolytic enzymes and grow robustly on cellulosic materials, is exceptionally attractive for biorefinery construction [39, 40]. A suite of molecular biology tools, including
CRISPR/Cas9 method, are available for *M. thermophila*, which allow rational genetic engineering. Previously, we enhanced the synthetic pathway and export system of malic acid in *M. thermophila* and the resultant transformant was able to produce malic acid by direct conversion of hemicellulose or cellulose without adding extra hydrolase [9]. The major components of plant cells are made from hexose (glucose) and pentoses (xylose and arabinose). Pentose is catabolized via the pentose phosphate pathway and can actuate CO₂-fixation during the CBB cycle.

In this study, the CBB cycle enzymes RuBisCO and PRK were introduced into the cellulolytic fungus *M. thermophila* to form a novel biorefinery system—called DMCC (Direct microbial conversion of biomass with CO₂ fixation) here—to produce a bulk chemical (malic acid) from plant biomass (corn cob) and CO₂ (Fig. 1). The function of the heterologous CBB cycle was demonstrated by growth phenotype, ¹³C-tracer analysis, and increased production of the heterologous CBB cycle was demonstrated by plant cells are made from hexose (glucose) and pentoses (xylose and arabinose). Pentose is catabolized via the pentose phosphate pathway and can actuate CO₂-fixation during the CBB cycle.

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**Results**

**Heterologous expression of genes encoding CBB cycle enzymes in *M. thermophila***

Previously, we performed metabolic modification in *M. thermophila* to generate strain JG207, which can produce malic acid using plant lignocellulose as the carbon source [9]. Efficient utilization of the main components of lignocellulose (glucose, xylose, and arabinose) is a prerequisite for improvement in three major indices of performance (titer, yield and productivity) in the biological production of plant cell-derived biochemicals. In microbial cells, xylose and arabinose are metabolized by pentose phosphate pathway, which can provide the substrate (ribulose-5-phosphate) for PRK of the CBB cycle. The genes encoding CBB cycle enzymes—RuBisCO and PRK—have been introduced into heterotrophic microbes to recycle CO₂ and driven by pentose to improve production of target metabolites [17, 29, 32]. It was reported that prokaryotic form-II RuBisCOs are encoded by a single structural gene [27]. The RuBisCO gene from *R. rubrum* (cbbM) has been functionally overexpressed in *M. extorquens*, *E. coli* and *S. cerevisiae* [17, 19]. Therefore, to improve malate production by *M. thermophila*, cbbM was integrated into the genome of strain JG207, together with *prk* from *S. cerevisiae*, under the control of the strong constitutive promoters of *gpdA* and *pdc*, respectively. After confirmation of the presence of the transgenes by PCR analysis, physiological characterization of the resultant strain, named as CP-1, was conducted using xylose or arabinose as the carbon source.

RT-qPCR analysis indicated that six copies of *prk* and eight copies of *cbbM* were integrated into the genome of strain CP-1 (Additional file 2: Fig. S2). RuBisCO was active in strain CP-1 when *cbbM* was overexpressed. The RuBisCO activity in the crude extract of this strain achieved 22.3 U/mg protein, while in the parent strain JG207, the activity was undetectable (Fig. 2a). As shown in Fig. 2, simultaneous overexpression of RuBisCO and PRK contributed to malate production from xylose or arabinose. Starting with 75 g/L carbon source, the titer of malic acid reached 37.3 g/L and 54.1 g/L from xylose and arabinose, respectively, 24% and 15% increases compared with parental strain JG207 (malate titers 30 g/L and 47 g/L from xylose and arabinose, respectively). Moreover, increases in cell dry weight of strain CP-1 on xylose (1.38-fold) and arabinose (1.18-fold) were confirmed, compared with strain JG207. These results indicated that integration of the CBB cycle enzymes into the carbon metabolic pathway of a filamentous fungus was beneficial for malate production and cell growth when using pentoses as the feedstock.
Simultaneous CO₂-fixation and branch pathway disruption were propitious to the production of malic acid

Based on the observations described above, we found that the engineered strain containing CBB cycle enzymes had an advantage in malate production. We then applied another metabolic engineering strategy: disruption of the branch points of the synthetic pathway of the target chemical (malic acid) while overexpressing the CBB cycle enzymes. Previous study indicated that in microbes, pyruvate decarboxylase and lactate dehydrogenase, encoded by *pdc* and *ldh*, respectively, use pyruvate as a substrate. These reactions limit organic acid production via the rTCA pathway [9, 41]. Moreover, phosphoenolpyruvate (PEP) carboxykinase (PCK) functions on inverse reaction of PEP to oxaloacetate, the precursor for malate synthesis, in *M. thermophila*, and has been considered as a target of metabolic engineering to improve production of malic acid [9]. In *M. thermophila*, the genes *pdc*, *ldh*, and *pck* show high expression levels in response to various carbon sources [40, 42]. Therefore, here, *prk*, *cbbM*, and the selective marker gene *neo* were integrated into the *ldh*, *pdc*, and *pck* loci, respectively, in the genome of strain JG207, using the CRISPR/Cas9 system. Thus, the CBB cycle enzymes would be heterologously expressed and branch pathways were simultaneously deleted, forming strain CP-51.

When grown on xylose, strain CP-51 displayed 1.40-fold and 1.13-fold increases in malate production compared with the values for strains JG207 and CP-1, respectively. The titer of malic acid was up to 42.1 g/L (Fig. 3b). The dry cell weight of strain CP-51 showed a 59% increase compared with that of the parental strain JG207 (Additional file 2: Fig. S3). Consistently, strain CP-51 was superior to strains JG207 and CP-1 in production of malic acid using arabinose, glucose, and even cellulose (Avicel) as the carbon source. Titer of malic acid produced by strain CP-51 grown on arabinose, glucose and Avicel in flask culture were improved to 58.0 g/L, 70.4 g/L, and 70.1 g/L, respectively. The corresponding yields were 0.77 g/g, 0.94 g/g and 0.93 g/g carbon source, respectively. In addition, due to the disruption of *pck* and *ldh*, ethanol and lactate were undetectable in the culture. These results revealed that the combination of integration of CBB cycle enzymes and disruption of branch pathways contributed to further improvement of malate production from lignocellulose-derived sugars.

Confirmation of CO₂-fixation by ¹³C-tracer analysis

To confirm CO₂ fixation during production of malic acid, ¹³C-tracer analysis was performed to detect the relative abundances of malic acid with ¹³C atom after fermentation was over. In malic acid-producing strain JG207, malic acid can be synthesized via rTCA pathway, where pyruvate is catalyzed to oxaloacetate by pyruvate carboxylase (PYC), companied by CO₂ fixation. In order to estimate the efficiency of CO₂ fixation by PYC module, ¹³C-tracer analysis was carried out in strain JG207 when grown on xylose, glucose or Avicel. As shown in Fig. 4, the contents of ¹³C molecular were up to 0.34, 0.65, and 0.55 mol/mol malic acid on xylose, glucose and Avicel, respectively, after 8 days of fermentation. The corresponding average rates of fixating and channeling ¹³CO₂ into malic acid achieved 17.3, 71.1, and 62.0 mg/L/h, CO₂ fixation by rTCA. These data indicated that when grown on xylose, efficiency of CO₂ fixation was lower than that on glucose and Avicel. Malic acid was mainly produced by rTCA pathway under glucose and Avicel conditions.
When using engineered strains CP-1 and CP-51 to produce malic acid, we found that the average rates of fixing and channeling $^{13}\text{CO}_2$ into malic acid achieved 32.2 mg/L/h and 44.4 mg/L/h on xylose, representing 1.86- and 2.57-fold higher than that of strain JG207 (Fig. 4a). This enhancement led to 50% and 83% increases in contents of $^{13}\text{C}$ in malic acid, achieving 0.51 and 0.62 mol/mol malic acid in strain CP-1 and CP-51, respectively (Fig. 4b). The relative abundance of malic acid with one $^{13}\text{C}$ atom on xylose was improved by 73.2% and 120%, respectively, compared to the parental strain JG207. Meanwhile, the relative abundance of malic acid with two $^{13}\text{C}$ atoms produced by strains CP-1 and CP-51 exhibited 12.3% and 22.7% more than that by strain JG207 (Fig. 4c).

When grown on glucose and cellulose (Avicel), enhancement of CO$_2$ fixation was also observed after integration of CBB cycle enzymes (Fig. 4d–g). The average rates of fixing and channeling $^{13}\text{CO}_2$ into malic acid in strains CP-1 and CP-51 were increased by 17.2% and 27.6%, respectively, and up to 83.3 mg/L/h and 90.7 mg/L/h, respectively, compared to strain JG207 on glucose (Fig. 4e). The corresponding contents of $^{13}\text{C}$ in malic acid achieved 0.73 and 0.75 mol/mol malic acid, respectively. When using cellulose (Avicel) as the feedstock, content of $^{13}\text{C}$ in malic acid produced by strain CP-51 was up to 0.67 mol/mol malic acid (Fig. 4g), indicating that the production of 1 mol of malic acid is accompanied by the fixation of at least 0.67 mol CO$_2$. The average rate of fixing $^{13}\text{CO}_2$ for synthesizing malic acid achieved 80.7 mg/L/h. In addition, it was observed that $^{13}\text{CO}_2$ fixation rates of all three strain on glucose and Avicel were much higher than that on xylose. These data indicated that enhancement of CO$_2$ fixation resulted from combined effort of pyruvate carboxylation by pyruvate carboxylase and the CBB cycle during malate production. It was noteworthy that the relative abundance of malic acid without $^{13}\text{C}$ atoms was high in the cultures.
of the three strains, which might result from that more malic acid were synthesized by other pathways without CO$_2$ fixation on xylose, such as the mitochondrial TCA cycle and the glyoxylate pathway.

**Enhanced pentose uptake facilitates pentose fermentation**

The capability for CO$_2$-fixation in engineered strain CP-51 is related to pentose phosphate pathway activity, which can be driven by pentose metabolism. However, xylose use is inhibited by the presence of glucose in the feedstock [43]. Enhancement of pentose uptake can alleviate this inhibition and facilitate improved pentose use and elevated co-fermentation rates of hexose and pentose [44]. Previous study showed that N376F mutation in the Gal2 protein, a galactose/glucose transporter from *S. cerevisiae*, led to improved affinity for xylose and
Gal2-N376F becomes a glucose inhibition-free xylose transporter [45]. To further improve the use of pentose to provide the precursor for the CBB cycle, the gene encoding Gal2-N376F (Gal2M), driven by the strong constitutive promoter of elf (encoding elongation initial factor), was introduced into strain CP-51. RT-qPCR analysis indicated that eight copies of gal2M were integrated into the genome of the resultant strain Gal-1. As we expected, increases in the transport rate of xylose (1.20-fold) and arabinose (1.26-fold) by strain Gal-1 were observed, compared with strain CP-51. The glucose uptake rate was similar to that in the parental strain CP-51 (Fig. 5a–c).

To test the benefits of gal2M overexpression on the use of lignocellulosic sugar, strain Gal-1 was incubated in Vogel's minimal medium supplemented with single or multiple sugars, including glucose, xylose, and arabinose. Enhanced consumption rates of xylose and arabinose were observed in strain Gal-1 compared with strain CP-51 when they were grown with a single sugar as the carbon source. The utilization rate of glucose by strain Gal-1 was similar to that by strain CP-51. When growth in Vogel’s minimal medium supplemented with the mixture of sugars, the level of sugar consumption in strain Gal-1 was considerably higher than that in strain CP-51 (Additional file 2: Fig. S5). When growth on malate-producing medium containing 40 g/L glucose, 20 g/L xylose, and 20 g/L arabinose, which is similar to the components of the hydrolysate of plant biomass, the substrate consumption rate of strain Gal-1 was faster than that of the parental strain CP-51 (Fig. 5d). After 10 days of fermentation, strain Gal-1 achieved a malate titer of 76.1 g/L, a 1.22-fold increase compared with strain CP-51 (62.5 g/L). The corresponding yield was up to 1.01 g/g carbon source in flask cultivation (Fig. 5e). These results indicate that improvement of pentose uptake can facilitate pentose utilization and improve co-fermentation rates of hexoses and pentoses for production of malic acid.

**Conversion of plant biomass and CO₂ into malic acid using M. thermophila**

In real-word applications, direct utilization of raw plant biomass as the feedstock is the greatest advantage of CBP technique and critical to overcome the remaining
barriers to cost-effective production of biofuels and commodities. To test the combined effects of incorporation of the CBB cycle, disruption of branch points of the malate synthesis pathway, and enhancement of substrate uptake on production of malic acid from lignocellulosic biomass, we tested the use of pulverized raw corn cob without pretreatment by alkali, acid, or hydrolytic enzymes as the feedstock for malate production. Starting with 75 g/L raw corn cob, the titer of malic acid in strain Gal-1 was up to 40 g/L, 10.4% more than that in the original strain JG207 [9]. The yield of malic acid from raw corn cob were up to 0.53 g/g total plant biomass (Fig. 6a). These data represent the highest yield of malate production yet reported from raw plant biomass. ¹³C-tracer analysis indicated when grown on raw corn cob, the average rate of ¹³CO₂ fixation for malic acid synthesis achieved 33.8 mg/L/h and the content of ¹³C atom in malic acid was up to 0.44 mol/mol malic acid (Fig. 6b), suggesting that 1 t of malic acid could be produced from 1.89 t of biomass with 0.14 t of CO₂ fixation. Our results clearly show the synergy between lignocellulosic biomass conversion and CO₂-fixation for producing organic acids.

As expected, the titer and yield of malic acid on corn cob shown less than that on crystalline cellulose (Avicel), as more complicated structure and multiple components of plant biomass. Lignin acts as a barrier for depolymerization of plant cell wall and its degradation products are known to be toxic to microorganisms [46, 47]. Moreover, xylose occupies a large proportion of the components of plant biomass [9, 48]. However, the average rate of fixing and channeling CO₂ into product malic acid in engineered strains on xylose was approximately 1.7-fold less than that on glucose (Fig. 4). Therefore, the further engineering of DMCC in M. thermophila is needed to improve overall bioconversion from plant biomass, such as speeding up pentose utilization and reducing the negative effect of lignin.

**Discussion**

Plant biomass and CO₂ have many desirable features as industrial raw material to decrease reliance on fossil fuels. Direct conversion of plant lignocellulose and fixing CO₂ into the central carbon metabolism of industrial microbes are promising for cost-effective production of value-add compounds in biorefinery. The thermophilic and cellulolytic M. thermophila has been engineered to produce malic acid using raw plant biomass as the feedstock without addition of hydrolytic enzyme [10]. In this study, using M. thermophila, the DMCC system (direct microbial conversion of biomass with CO₂ fixation), a novel strategy for biorefinery from plant cell wall and CO₂, was constructed to convert plant biomass and CO₂ into malic acid efficiently.

For fixing and channeling CO₂ into carbon metabolism for producing value-added compounds, there are two different strategies. One approach is the integration of exogenous biosynthetic production pathways into naturally existing carbon-fixing organisms, such as cyanobacteria and algae. Autotrophic microbes have been engineered to produce chemicals and biofuels from CO₂, such as 2,3-butanediol, lactic acid and malic acid [49–51]. However, production performance remains far below industrial feasibility. The other option is to equip heterotrophic fermentation strains with efficient CO₂-fixation pathway. Recently, Calvin–Benson–Bassham (CBB) pathway was constructed in heterotrophic microbes to recycle released CO₂ into central metabolic pathway for improved carbon efficiency [20, 22, 28] and even synthesize sugars and other major biomass components from CO₂ [29]. An efficient energy supply is required for biological CO₂ fixation. Engineered E. coli and P. pastoris could incorporate CO₂ into cell components via heterologous CBB pathway, requiring reducing power and energy from the supplements, such as pyruvate, formate, and methanol [18, 29, 33]. Using sustainable plant biomass as energy source and actuate CO₂-fixation would be a promising strategy for cost-effective bio-manufacturing. In addition, the main components of lignocellulose comprise glucose, xylose, and arabinose. Intermediate ribulose-5-phosphate of pentose catabolism via pentose phosphate pathway can serve as the substrate of PRK. In E. coli and S. cerevisiae, xylose and arabinose were used to drive CO₂-fixation of the CBB cycle [17, 20, 32]. In this work, the CBB cycle enzymes were successfully introduced into the cellulolytic fungus M. thermophila to producing CO₂ fixation system, combined with native PYC module to produce malic acid using plant biomass and CO₂ as the carbon sources. The proportion
of carbon atoms from fixed CO₂ in the total carbon of malic acid was significantly increased and the average rates of fixing and channeling ¹³CO₂ into malic acid achieved 44.4 mg/L/h, 90.7 mg/L/h and 80.7 mg/L/h in strain CP-51, when grown on xylose, glucose and Avicel, respectively (Fig. 4). With raw corn cob as the feedstock, the yield of malic acid produced by the final engineered strain Gal-1 achieved 0.53 g/g total plant biomass, representing a 10.4% increase over the highest reported yield (0.48 g/g) [9] and the content of ¹³C atom in malic acid was up to 0.44 mol/mol malic acid. Furthermore, this strategy of synergistic conversion of plant biomass and CO₂ can be utilized for the production of other chemicals, such as fumaric and succinic acid.

Rapid utilization of all components of the hydrolysate of plant biomass is the prerequisite for efficient production of biochemical. However, due to the preference of microbes for glucose, pentose utilization is inhibited by glucose presented in the culture, which led to two-stage utilization of sugar mixture and low productivity of target products. It was suggested that D-glucose impairs the simultaneous utilization of pentose mainly by inhibition of pentose uptake [43]. Enhancement of pentose uptake can alleviate this inhibition and facilitate improved pentose utilization and elevated co-fermentation rates of hexose and pentose [44, 52]. Recently, transporter engineering and directed evolution have been used for rewiring substrate specificity to obtain glucose-insensitive xylose transporters [45, 53]. Herein, a glucose inhibition-free xylose transporter was integrated into engineered strain CP-51 for facilitating pentose utilization to actuate CO₂-fixation of heterologous CBB pathway and improving co-fermentation rates of hexoses and pentoses for production of malic acid. Titer and yield of malic acid were increased to 76.1 g/L and 1.01 g/g carbon source, respectively, by conversion of a mixture of sugars derived from plant biomass. In addition, although xylose and arabinose are both pentose, there are dramatic differences in transcriptomic profiles in filamentous fungus when exposed to them in a previous study [9], indicating that regulation network of xylose catabolism is different from that of arabinose catabolism. Here, it was also observed that titer of malic acid on xylose was obviously below that on arabinose. The exact molecular basis of the two pentose metabolism needs more investigation in the future.

In this study, CBB cycle enzymes, RuBisCO and PRK, were integrated into the metabolic network of the thermophilic fungus *M. thermophila* for enhanced fixation efficiency of CO₂. A novel biorefinery system named DMCC was designed and constructed, which can produce 1 t of bulk chemicals (such as malic acid) using less than 2 t of plant biomass, accompanied by the fixation of 0.14 t CO₂. This study provides a novel strategy for producing biochemicals and operating carbon neutral.

### Materials and methods

#### Strains and culture conditions

*Myceliophthora thermophila* strain JG207 and its mutants were propagated on 1 × Vogel’s minimal medium plates supplemented with 2% glucose at 35 °C to obtain conidia after 8 d, and corresponding antibiotic was added when needed for transformant screening. *Escherichia coli* DH5α was employed for vector construction, and was cultivated in Luria–Bertani medium with 100 µg/mL ampicillin or 50 µg/mL kanamycin for plasmid selection.

#### Plasmid construction

For the construction of plasmids overexpressing target genes, *cbbm* (GenBank no. X00286.1) from *Rhodospirillum rubrum* was codon-optimized on the basis of *Neurospora crassa* codon frequency (http://www.kazusa.or.jp/codon/), artificially synthesized, and inserted between the SpeI and BamHI sites of plasmid pAN52-PtrpC-neo-PMtgpdA [9] carrying the neo selectable marker to form overexpression plasmid PpgpdA-cbbM-neo, using the NEB Gibson Assembly Kit. Similarly, codon-optimized *prk* (GenBank no. X07654.1) from *Spinacia oleracea*, under control of the strong constitutive promoter of *pdc* (*Mycth*_112121), was inserted between the BglII and BamHI sites of pAN52-PtrpC-neo-PMtgpdA to generate the vector Ppdc-prk-neo.

The strong constitutive promoter of *eif* (*Mycth*_2297659) was employed to efficiently overexpress pentose transporter genes. Site-directed mutation of sugar transporter gene gal-2 from *S. cerevisiae* was performed using a fusion PCR strategy to generate Gal2M with mutation of residue N376 to phenylalanine (N376F). With the aid of the NEB Gibson Assembly Kit, the amplicons were ligated between the SpeI and BamHI sites of plasmid pAN52-PpgpdA-bar [10] to generate the corresponding plasmid Peif-gal2M-bar.

Plasmids for sgRNA expression were constructed as described previously [54]. Briefly, specific sgRNA target sites in target genes (*pck*, *Mycth*_2315623; *ldh*, *Mycht*_110317; and *pdc*, *Mycht*_112121) were identified using the sgRNACas9 tool [55] and the *M. thermophila* genome sequence and target gene sequences as the input. Oligos with no off-target probability were selected. The *M. thermophila* U6 promoter and a target-directed sgRNA fragment were amplified from the U6-sgRNA plasmid [54], assembled by overlapping PCR, and cloned into blunt cloning vector pJET1.2 to generate the plasmids U6-pck-sgRNA, U6-ldh-sgRNA, and U6-pdc-sgRNA.
A vector carrying donor DNA was constructed to perform genomic modification. The 5’- and 3’-flanking fragments of pck were amplified from the M. thermophila genome. These fragments and selectable marker cassette PTrpC-neo from plasmid p0380-neo [56] were assembled using the NEB Gibson Assembly Kit and cloned into pPK2BarGFDP digested with SpeI and EcoRV to generate the donor DNA sequence donor-pck-neo.

Codon-optimized prk and cbbM were amplified from plasmids Ppdc-prk-neo and PgpDA-cbbM-neo, respectively, using paired primers. prk and cbbM were knocked-in to the ldh and pdc loci of the M. thermophila genome, respectively, controlled by in situ promoters. 5’- and 3’-flanking fragments of the ldh and prk codon sequences were assembled to generate donor-ldh-prk. Similarly, 5’- and 3’-flanking fragments of pdc and cbbM codon sequences were assembled to generate donor-pdc-cbbM.

All vectors were constructed using E. coli DH5α and the target genes cloned into shuttle vectors were sequenced to verify the authenticity of the plasmid construction.

**Myceliophthora transformation**
Polyethylene glycol-mediated transformation of M. thermophila protoplasts was performed as described previously [57]. For gene overexpression, 10 µg linearized plasmid were transformed into M. thermophila protoplasts. Putative transformants were selected on agar plates supplemented with corresponding antibiotics and confirmed via PCR amplification of the transgene with paired primers.

For multiple gene replacement involving the pck, pdc, and ldh loci, sgRNA and donor expression cassettes for neo, prk, and cbbM were mixed with Cas9-expression PCR cassette and co-transformed into strain JG207. Putative transformants were selected with 100 µg/mL G418 followed by sequential identification via PCR with paired primers.

**Malate-production medium**
Shake-flask cultivation was performed with 50 mL of medium inoculated with mature spores at a final concentration of 2.5 x 10⁵ spores/mL in 250-mL Erlenmeyer flasks to evaluate the malic acid production capabilities of M. thermophila. The culture was incubated at 45 °C with shaking at 150 rpm and samples (1 mL) were taken at different intervals. Each liter of the cultivation medium contained 75 g of carbon source, 0.15 g of KH₂PO₄, 0.15 g of K₂HPO₄, 0.1 g of MgSO₄·7H₂O, 0.1 g of CaCl₂·2H₂O, 8 g of Bacto peptone, 1 mL of biotin (0.1 g/L), and 1 mL of trace element of Vogel's salt, and was sterilized by autoclaving. Subsequently, sterilized CaCO₃ was used as a neutralizing agent at a final concentration of 80 g/L to keep the pH at approximate 6.0. When corn cob was used as the feedstock, mechanical pulverization was carried out as follows: corn cob was chopped into pieces, pulverized by grinding mill, and then passed through the 80-mesh size sieve.

**Metabolite analysis**
To detect organic acid titer in culture broth, 1 mL of 2 M sulfuric acid was added into 1-mL well-mixed sample in a 15-mL tube and the mixture was incubated at 80 °C for 30 min. The mixture was vortexed at intervals to resolve the malate adequately. Subsequently, 2 mL of distilled water was added, mixed, and an aliquot was used for metabolite analysis.

Malic acid titer was determined by high-performance liquid chromatography (HPLC) using an instrument (e2695; Waters, Manchester, United Kingdom) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 35 °C and a Waters 2489 UV detector at 40 °C; 5 mM H₂SO₄ was used as the mobile phase with a constant flow rate of 0.5 mL/min. Sugar concentrations were monitored with a Waters 2414 refractive index detector and an Aminex HPX-87P column (Bio-Rad) with distilled water as the mobile phase. Data analysis was performed using a Waters e2695 separation module.

**Enzyme assays**
A 50-mL sample was poured into a Büchner funnel equipped with four pieces of gauze, washed with distilled water until most of the CaCO₃ was removed, and subsequently collected. Then, mycelia were immediately homogenized in liquid nitrogen and ground into a powder in a prechilled mortar with a prechilled pestle. The paste was transferred into 1 mL phosphate-buffered saline (pH 7.4). After centrifugation for 10 min at 4 °C, clear supernatant was used for protein qualification and enzyme assay.

Protein concentration in supernatants was measured using a Bio-Rad protein assay kit. RuBisCO activity was measured by the modification of the method described by Xia et al. [17]. The reaction mixture containing 100 mM Tris (pH 7.4), 10 mM MgCl₂, 20 mM NaHCO₃, 10 mM KCl, 1 mM dithiothreitol, 2 mM oxaloacetate, 5 mM creatine phosphate, 10 U 3-phosphoglycerate kinase, 10 U glyceraldehyde 3-phosphate dehydrogenase, 10 U creatine phosphokinase, 0.2 mM NADH, and crude enzyme solution was incubated for 15 min at 30 °C. The assay was started by the addition of 0.5 mM ribulose-1,5-bisphosphate and was immediately monitored at 340 nm for 5 min. RuBisCO activity was defined as the amount of enzyme required to produce 1 nM product per min.
Sugar uptake assays in *M. thermophila*

Strains were incubated in 100 mL of 1 × Vogel’s medium containing 2% glucose at 45 °C for 18 h, then washed three times in 1 × Vogel’s salts without any carbon source. Subsequently, the mycelia were transferred to Vogel’s salts containing 0.5% sugar (glucose, xylose, or arabinose) for induction for an additional 4 h. After that, the mycelia were washed again as above and resuspended in uptake buffer [1 × Vogel’s salts plus 10 mM sugar (glucose, xylose, or arabinose) and 10 μg/mL cycloheximide] for 20 min. The amount of residual sugar in the supernatant was determined and the fungal biomass was blotted dry and then completely dried at 105 °C to determine the dry weight for data normalization.

**Quantitative real-time PCR analysis**

To assay copy numbers of genes ectopically inserted into the *M. thermophila* genome, fungal genomic DNA was extracted from transformants as described previously [58] and used as the template for real-time qPCR (RT-qPCR). Quantitative PCR was carried out with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) and a CFX96 real-time PCR detection system (Bio-Rad), according to the manufacturer’s instructions. The reaction mixture (with three replicates) included 1 μL of template DNA, 0.4 μL of each primer (10 μM), 10 μL of RNA-direct SYBR® Green Realtime PCR Master Mix, and 8.2 μL of H2O. The actin gene (MYCTH_2314852) was used as an internal control. The primers for each gene were optimized to obtain amplification efficiency between 95 and 105% and only one melting temperature on the melting curve. The primers used for RT-qPCR are listed in Additional file 1: Table S1.

**Statistical significance tests**

Unless otherwise noted, statistical significance was tested using a one-tailed homoscedastic (equal variance) t-test. All p-values were generated using Microsoft Excel 2013 (Microsoft Corporation). n.s. indicates no statistical significance; * represents a p-value < 0.05; ** represents a p-value < 0.01; and *** represents a p-value < 0.001.

**Abbreviations**

CBP: Consolidated bioprocessing; CBB cycle: Calvin–Benson–Bassham cycle; PRK: Phosphoribulokinase; RuBiCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase; rTCA pathway: Oxaloacetate carboxylase; CRISPR: Clustered regularly interspaced short palindromic repeats; DMCC: Direct microbial conversion of biomass with CO₂ fixation; PVC: Pyruvate carboxylase.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02042-5.

**Additional file 1: Table S1.** List of PCR primers used in this study.

**Additional file 2: Figure S1.** PCR analysis of the mutants of *M. thermophila* generated in this study. **Figure S2.** Copy number assay by RT-qPCR, prk and cbbM in the genome of strain CP-1; prk and cbbM in strain CP-1 genomic DNA; gal2M in the genome of strain CP-1. The values and error bars represent means and standard deviations. **Figure S3.** Dry cell weight of strain CP-1 grown on xylose for 4 days. The values and error bars represent means and standard deviations. **Figure S4.** Titer of malic acid produced by strains JG607, CP-1, and CP-51, when grown on glucose and Avicel. Titer of malic acid was determined after 8 days of fermentation. The values and error bars represent means and standard deviations. **Figure S5.** Sugar utilization of strain Gal-1 when growth in Vogel’s minimal medium supplemented with single or multiple sugars derived from lignocellulose: a 40 g/L xylose; b 40 g/L arabinose; c 40 g/L glucose; d 40 g/L glucose and 20 g/L xylose; e 40 g/L glucose and 20 g/L arabinose; f 20 g/L xylose and 20 g/L arabinose; g 40 g/L glucose, 20 g/L xylose, and 20 g/L arabinose. The values and error bars represent means and standard deviations.

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**Authors’ contributions**

JL and CT conceived the project and wrote the manuscript. JL, BC, SG, ZZ, TS, YZ, TW, and WS performed metabolic engineering experiments. JL, QL, DL, and CT analyzed the data. JL and CT wrote the initial paper draft. All authors read and approved the final manuscript.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its Additional files.
Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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