Single mutation at a highly conserved region of chloramphenicol acetyltransferase enables isobutyl acetate production directly from cellulose by *Clostridium thermocellum* at elevated temperatures

Hyeongmin Seo¹,³†, Jong-Won Lee²,³†, Sergio Garcia¹,³ and Cong T. Trinh¹,²,³*  

Abstract

**Background:** Esters are versatile chemicals and potential drop-in biofuels. To develop a sustainable production platform, microbial ester biosynthesis using alcohol acetyltransferases (AATs) has been studied for decades. Volatility of esters endows high-temperature fermentation with advantageous downstream product separation. However, due to the limited thermostability of AATs known, the ester biosynthesis has largely relied on use of mesophilic microbes. Therefore, developing thermostable AATs is important for ester production directly from lignocellulosic biomass by the thermophilic consolidated bioprocessing (CBP) microbes, e.g., *Clostridium thermocellum*.

**Results:** In this study, we engineered a thermostable chloramphenicol acetyltransferase from *Staphylococcus aureus* (CAT₅₉₄) for enhanced isobutyl acetate production at elevated temperatures. We first analyzed the broad alcohol substrate range of CAT₅₉₄. Then, we targeted a highly conserved region in the binding pocket of CAT₅₉₄ for mutagenesis. The mutagenesis revealed that F97W significantly increased conversion of isobutanol to isobutyl acetate. Using CAT₅₉₄ F97W, we demonstrated direct conversion of cellulose into isobutyl acetate by an engineered *C. thermocellum* at elevated temperatures.

**Conclusions:** This study highlights that CAT is a potential thermostable AAT that can be harnessed to develop the thermophilic CBP microbial platform for biosynthesis of designer bioesters directly from lignocellulosic biomass.

**Keywords:** Alcohol acetyltransferase, Thermostability, Chloramphenicol acetyltransferase, Isobutyl acetate, Esters, Consolidated bioprocessing, *Clostridium thermocellum*
Due to high volatility of esters, ester production at elevated temperatures can benefit downstream product separation and hence reduce the process cost. Interestingly, it has recently been shown that for the same total carbon chain length, short-chain esters are less toxic to microbial health than alcohols, which is potentially beneficial for ester fermentation [8]. However, most of the AATs known to date are isolated from mesophilic microbes or plants [9–12], and none of them has been reported to be active at elevated temperatures (> 50 °C). The highest temperature reported for ester production is 42 °C in a thermotolerant yeast [13]. Hence, finding and developing a thermostable AAT are crucial to produce esters at elevated temperatures.

Chloramphenicol acetyltransferase (E.C. 2.3.1.28, CAT) is another acetyltransferase class that has been found in various microbes [14]. This enzyme acetylates chloramphenicol, a protein synthesis inhibitor, by transferring the acetyl group from acetyl-CoA. The acetylation of chloramphenicol detoxifies the antibiotic compound and confers chloramphenicol resistance in bacteria. Recent studies have implied that CATs likely recognize a broad substrate range for alcohols and acyl-CoAs [7]. In addition, high thermostability of some CATs enables them to be used as selection markers in thermophiles [15–17]. Therefore, CAT can function or be repurposed as a thermostable AAT suitable for ester biosynthesis at elevated temperatures.

In this study, we engineered a CAT from *Staphylococcus aureus* (CAT Sa) for isobutyl acetate production at elevated temperatures. First, we investigated a broad alcohol substrate range of CAT Sa. Protein homology modeling along with sequence alignment was performed to identify the binding pocket of CAT Sa as a potential target for protein engineering to enhance condensation of isobutanol and acetyl-CoA. In silico mutagenesis discovered a variant (F97W) of CAT Sa that was then experimentally validated for improved catalytic activity towards isobutanol. As a proof-of-concept, the engineered CAT Sa was successfully expressed in *Clostridium thermocellum*. We further demonstrated a F97W CAT Sa-overexpressing *C. thermocellum* for consolidated bioprocessing (CBP) to produce isobutyl acetate directly from cellulose without a need for external supply of cellulases. To our knowledge, this study presents the first demonstration of CAT engineering to enable ester production directly from cellulose at elevated temperatures.

**Results and discussion**

**In silico and rapid in vivo characterization of a thermostable chloramphenicol acetyltransferase(s) for broad alcohol substrate range**

To develop a thermophilic microbial ester production platform, a thermostable AAT is required. Unfortunately, the AATs known to date are isolated from mesophilic yeasts or plants [9–12], and none of them has been reported to be active at a temperature above 50 °C. To tackle this problem, we chose CATs to investigate their potential functions as a thermostable AAT, because some thermostable CATs have been successfully used as a selection marker in thermophiles [17–21] and others have been shown to perform the acetylation for not only chloramphenicol but various alcohols like AATs [7, 22–25] (Fig. 1a, Additional file 1: Figure S1A). As a proof-of-study, we investigated CAT Sa, classified as Type A-9, from...
the plasmid pNW33N for a broad range of alcohol substrates as it has been widely used for genetic engineering in *C. thermocellum* at elevated temperatures (≥ 50 °C) [17–19].

We first conducted alcohol docking simulations using the homology model. Remarkably, the model predicted binding affinities of short-to-medium-chain length alcohols (e.g., ethanol, propanol, isopropanol, butanol, and isobutanol) and aromatic alcohols (e.g., benzyl alcohol and phenethyl alcohol) to the binding pocket. The change in the protein’s Gibbs free energy upon the substrate binding was ordered as follows: 2-phenethyl alcohol > benzyl alcohol > isobutanol > butanol > propanol > ethanol > isopropanol (Fig. 1b).

To quickly evaluate the in silico docking simulation results experimentally, we next performed in vivo characterization of a CAT<sub>Sa</sub>-overexpressing *E. coli* and screened for acetate esters production. Acetyl-CoA was derived from glycolysis, while various alcohols were externally supplied to the medium. Remarkably, the results exhibited the same trend of specificities of CAT<sub>Sa</sub> towards alcohols as predicted by the in silico docking simulation (Fig. 1b). The CAT<sub>Sa</sub>-overexpressing *E. coli* produced all the expected acetate esters including ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, benzyl acetate, and 2-phenethyl acetate at titers of 1.12 ± 0.07, 2.30 ± 0.28, 0.08 ± 0.02, 9.75 ± 1.57, 17.06 ± 6.04, 152.44 ± 29.50, and 955.27 ± 69.50 mg/L and specific ester production rates of 0.02 ± 0.00, 0.05 ± 0.01, 0.00 ± 0.00, 0.19 ± 0.03, 0.34 ± 0.12, 3.02 ± 0.57, and 19.27 ± 1.32 mg/gDCW/h, respectively. We observed that the specific ester production titers and rates are higher for aromatic alcohols than linear, short-chain alcohols likely because the hydrophobic-binding pocket of CAT<sub>Sa</sub> has been evolved towards chloramphenicol [26], an aromatic antibiotic (Fig. 1c). Specifically, the bulky binding pocket of CAT<sub>Sa</sub> likely contributes to more interaction with the aromatic substrates than the short, linear-chain alcohols (Additional file 1: Figure S1B, C).

Overall, thermostable CATs, e.g., CAT<sub>Sa</sub>, can have broad range of substrate specificities towards linear, short-chain, and aromatic alcohols and hence can be harnessed as AATs for novel ester biosynthesis at elevated temperatures.

**Discovery of a CAT<sub>Sa</sub> variant improving conversion of isobutanol and acetyl-CoA into isobutyl acetate**

Since the in vivo activity of CAT<sub>Sa</sub> is more than 50-fold higher for the aromatic alcohols than isobutanol, we asked whether its activity could be improved for isobutyl acetate biosynthesis. Using the in silico analysis, we started by examining whether any modification of the binding pocket of CAT<sub>Sa</sub> could improve the activity towards isobutanol. According to the homology model, the binding pocket consists of Tyr-20, Phe-27, Tyr-50, Thr-88, Ile-89, Phe-90, Phe-97, Ser-140, Leu-141, Ser-142, Ile-143, Ile-144, Pro-145, Trp-146, Phe-152, Leu-154, Ile-166, Ile-167, Thr-168, His-189, Asp-193, Gly-194, and Tyr-195, where the His189 and Asp193 are the catalytic sites (Fig. 2a). Since chloramphenicol resistance is likely a strong selective pressure throughout evolution, we expected all CATs to exhibit a common binding pocket structure. Unsurprisingly, conserved sequences in the binding pocket were observed by protein sequence alignment of CAT<sub>Sa</sub> with other CATs of Type A (Additional file 1: Figure S2A). Especially, Pro-85 and Phe-97 were highly conserved in CATs of not only Type A but also Type B (Fig. 2b and Additional file 1: Figure S2B).

Based on the binding pocket identified, we performed docking simulation with alanine and residue scans using the acetyl-CoA–isobutanol–CAT<sub>Sa</sub> complex to identify potential candidates for mutagenesis (Additional file 1: Figure S3A, B). Remarkably, the top three variant candidates were suggested at the Phe-97 residue (i.e., F97Y, F97W, and F97V). This residue is involved in the formation of a tunnel-like binding pocket [26]. Motivated by the analysis, Phe-97 was chosen for site-saturated mutagenesis, and the variants were screened in *E. coli* for isobutyl acetate production by external supply of isobutanol.

Among the variants characterized, the F97W variant exhibited the best performance (Fig. 2c), with the similar protein expression levels in *E. coli* (Additional file 1: Figure S4). As compared to the wild type, the F97W variant enhanced the isobutyl acetate production by fourfold. Subsequent in silico analysis showed that the mutation created a CH–π interaction between the hydrogen of isobutanol and the indole ring of F97W (Fig. 2d). The model also indicated no change in distance between the isobutanol and active site (His-189) in F97W. Therefore, the CH–π interaction is likely responsible for the improved activity of F97W variant towards isobutyl acetate biosynthesis.

**In vitro characterization of CAT<sub>Sa</sub> F97W**

Before deploying CAT<sub>Sa</sub> F97W for isobutyl acetate biosynthesis in the thermophilic CBP organism *C. thermocellum*, we checked whether the F97W mutation affected thermostability of the enzyme. We overexpressed and purified both the wild-type CAT<sub>Sa</sub> and CAT<sub>Sa</sub> F97W variant (Fig. 3a). The SDS-PAGE analysis confirmed the expression and purification of the enzymes by bands with the expected monomer size (25.8 kDa). Thermostability assay revealed that the F97W variant slightly lowered the wild-type melting point from 72 to 68.3 °C (Fig. 3b). Since CAT<sub>Sa</sub> F97W maintained high melting
point, it is possible that CAT_{S_a} F97W still maintains its functionality at high temperature (≥ 50 °C), but needs to be thoroughly characterized.

Table 1 shows the in vitro enzymatic activities of both the wild-type CAT_{S_a} and CAT_{S_a} F97W at 50 °C. The turnover number (k_{cat}) of CAT_{S_a} F97W was two times higher than that of the wild type. The increased turnover number of CAT_{S_a} F97W led to 1.9-fold increase in enzymatic efficiency (k_{cat}/K_M, 4.08 ± 0.62, 1/M/s), while the mutation did not result in significant change in K_M. The improved enzymatic efficiency of CAT_{S_a} F97W agrees with the enhanced isobutanol production observed in the in vivo characterization using the CAT_{S_a}-overexpressing E. coli (Fig. 2c).

Based on the rigidity of the binding pocket, we originally presumed that mutagenesis on the binding pocket would result in activity loss towards chloramphenicol. Surprisingly, CAT_{S_a} F97W retained the activity towards chloramphenicol (Table 1). The F97W mutation decreased k_{cat} but also lowered K_M resulting in a compensation effect. Turnover number of CAT_{S_a} (k_{cat} 202.97 ± 3.36, 1/s) was similar to the previously reported value by Kobayashi et al. [16], but K_M (0.28 ± 0.02, mM) was about 1.75-fold higher. The difference might attribute to the experimental condition and analysis performed. Kobayashi et al. used chloramphenicol in a range of 0.05–0.2 mM for the assay and the Lineweaver–Burk method for analysis, while we used a 0–1.0 mM range with a non-linear regression analysis method. Interestingly, affinity towards acetyl-CoA was independent of the alcohol co-substrates (Additional file 1: Table S2), suggesting that the alcohol affinity would be likely the main bottleneck for microbial production of isobutyl acetate.
Taken altogether, the F97W mutation not only resulted in 1.9-fold higher enzymatic efficiency towards isobutanol but also retained thermostability of CAT$_{Sa}$. Thus, CAT$_{Sa}$ F97W variant can serve a starting candidate to demonstrate direct biosynthesis of isobutyl acetate at elevated temperatures by $C.\thermocellum$.

**Isobutyl acetate production from cellulose at elevated temperatures by an engineered $C.\thermocellum$ overexpressing CAT$_{Sa}$ F97W**

We next investigated whether $C.\thermocellum$ overexpressing CAT$_{Sa}$ F97W could produce isobutyl acetate at elevated temperatures. This thermophile was chosen, because it has a high cellulolytic activity suitable for CBP, a one-step process configuration for cellulase production, cellulose hydrolysis, and fermentation for direct conversion of lignocellulosic biomass to fuels and chemicals [27]. Furthermore, studies have demonstrated that the wild-type $C.\thermocellum$ has native metabolism capable of endogenously producing precursor metabolites for ester biosynthesis, such as acetyl-CoA, isobutyryl-CoA, as well as ethanol [28] and higher alcohols (e.g., isobutanol) under high cellulose loading fermentation [29–31] (Fig. 4a, Additional file 1: Figure S5A).

We started by generating two isobutyl acetate-producing strains, HSCT0101 and HSCT0102, by introducing the plasmids pHS0024 (harboring the wild-type CAT$_{Sa}$) and pHS0024_F97W (harboring the CAT$_{Sa}$ F97W variant) into $C.\thermocellum$ DSM1313. Colonies were isolated on antibiotic-selective plates at 55 °C. Successful transformation clearly indicated that CAT$_{Sa}$ F97W conferred the thiamphenicol resistance and hence maintained CAT activity. This result agrees with the in vitro enzymatic activity of CAT$_{Sa}$ F97W (Table 1).

We next evaluated whether the $C.\thermocellum$ strains could synthesize isobutyl acetate from cellobiose. Since the endogenous isobutanol production from a typical cellobiose concentration (5 g/L) is low [31], we supplemented the medium with 2 g/L isobutanol. Both HSCT0101 and HSCT0102 could produce isobutyl acetate at 55 °C as expected. Like the in vivo characterization in $E.\coli$ (Fig. 2c), HSCT0102 outperformed HSCT0101 with 3.5-fold increase in isobutyl acetate production (Fig. 4b). Interestingly, we also observed the parent $C.\thermocellum$ M1354 produced a trace amount of isobutyl acetate (<0.1 mg/L), even though this strain does not harbor a CAT (Additional file 1: Figure S5). This phenomenon was only observed when hexadecane overlay was used during fermentation for ester extraction. One possible explanation is the endogenous activity of esterases in $C.\thermocellum$ might have been responsible for low isobutyl acetate production, while the organic phase overlay...
helps to extract the target ester. It should be noted that the esterase reaction is reversible and more thermodynamically favorable for ester degradation than biosynthesis.

Finally, we tested whether HSCT0102 could endogenously produce isobutyl acetate directly from cellulose at elevated temperatures (55 °C). After 72 h, cell mass, containing 550 mg/L of pellet protein, reached 1.04 g/L, and 17 g/L of cellulose were consumed (Fig. 4c). About 103 mg/L of isobutanol were produced for the first 48 h and further increased up to 110 mg/L for additional 24 h.

Table 1 Kinetic parameters of the wild-type CATSa and mutant CATSa F97W

| Substrates | CATSa                                      | CATSa F97W                                 |
|------------|--------------------------------------------|--------------------------------------------|
|            | Chloramphenicol                            | Isobutanol                                 | Chloramphenicol | Isobutanol |
| Kₐ₀ (mM)   | 0.28 ± 0.02                                | 138.66 ± 28.92                             | 0.18 ± 0.01     | 144.77 ± 23.65 |
| kcat (1/s) | 202.97 ± 3.36                              | 0.30 ± 0.03                                | 102.63 ± 2.04   | 0.59 ± 0.05   |
| kcat/Kₐ₀ (1/M/s) | 7.37 ± 0.48 × 10⁵ | 2.16 ± 0.45                                | 5.77 ± 0.49 × 10⁵ | 4.08 ± 0.62 |

The reactions were performed at 50 °C. The co-substrate, acetyl-CoA, was supplied at the saturated concentration of 2 mM. Melting temperature (Tm) of CATSa and CATSa F97W is 72.0 ± 0.8 and 68.3 ± 1.2 °C, respectively.

Fig. 4 Isobutyl acetate production in the engineered C. thermocellum. a A simplified isobutyl acetate production pathway from cellulose in C. thermocellum. b Biosynthesis of isobutyl acetate of the wild-type and engineered C. thermocellum strains at 55 °C from MTC medium with 5 g/L cellobiose and external supply of 2 g/L isobutanol. Isobutyl acetate was measured after 24 h from the hexadecane layer of cell cultures. Initial OD of each cell culture was in a range of 0.8–1.0. The error bars represent standard deviation of five biological replicates. Statistical analysis: t test, **p value < 4 × 10⁻⁴, t = − 6.475, df = 7. c Kinetic profiles of cell growth and residual cellulose of HSCT0102. HSCT0102 was cultured in C-MTC medium with 20 g/L Avicel PH-101. The error bars represent standard deviation of three biological replicates. d Kinetic profiles of isobutanol and isobutyl acetate production by HSCT0102 in C-MTC medium with 20 g/L Avicel PH-101. The error bars represent standard deviation of three biological replicates. KOR 2-ketoisovalerate ferredoxin oxidoreductase, ADH alcohol dehydrogenase
(Fig. 4d). Besides isobutanol, C. thermocellum also produced other fermentative metabolites, including ethanol, formate, acetate, and lactate, as expected (Additional file 1: Figure S6A, B). For the target isobutyl acetate production, HSCT0102 did not produce isobutyl acetate for the first 24 h but started accumulating the target product for the next 48 h. The observed profile of isobutyl acetate production could be attributed to the low substrate affinity of CAT Sa F97W (Table 1). The final titer of isobutyl acetate reached 1.9 mg/L.

Besides isobutyl acetate, we also observed that HSCT0102 produced other detectable esters such as ethyl acetate, ethyl isobutyrate, and isobutyl isobutyrate (Additional file 1: Figure S6A, C, D). Endogenous biosynthesis of these esters could be explained from the complex redox and fermentative metabolism of C. thermocellum [30, 32]. Specifically, C. thermocellum can endogenously synthesize the precursor metabolites, acetyl-CoA and ethanol via the ethanol biosynthesis pathway, as well as isobutyryl-CoA and isobutanol via the valine biosynthesis pathway (Additional file 1: Figure S6A). With the availability of these four precursor metabolites, C. thermocellum could make ethyl acetate, ethyl isobutyrate, isobutyl acetate, and isobutyl isobutyrate as observed experimentally (Additional file 1: Figure S6C, D).

Taken altogether, C. thermocellum overexpressing CAT Sa F97W successfully produced the target isobutyl acetate from cellulose at elevated temperatures (55 °C). However, the low titer and conversion rate require optimization to improve isobutyl acetate production in future studies. One of the key metabolic engineering targets is to enhance enzymatic efficiency of CAT Sa. In contrast to S. cerevisiae-derived ATF1 that has high specificity towards isobutanol [6] and can be expressed in E. coli to achieve a high titer of 17.5 g/L isobutyl acetate and 80% theoretical maximum product yield [7], CAT Sa F97W exhibits a relatively low affinity towards isobutanol. The rationale for utilizing CAT Sa instead of ATF1 is that CAT Sa is thermostable, and this study is the first ever to report its function for ester production at elevated temperatures. Tuning gene expression in C. thermocellum is another challenge that needs to be addressed for enhanced ester production. Since CAT Sa F97W still retains the activity towards chloramphenicol, adaptive evolution strategies such as chemically induced chromosomal evolution (CICHe) can offer a promising strategy to improve the gene expression level [33]. Finally, model-guided optimization at system levels should be implemented for the most effective conversion of cellulose into isobutyl acetate to achieve high production of isobutyl esters and other class of esters [34–36].

Conclusions

This study demonstrated that a CAT can function and/or be repurposed as an AAT for novel biosynthesis of designer esters at elevated temperatures. Both in silico and in vivo characterization discovered a broad alcohol substrate range of the thermostable chloramphenicol acetyltransferase from S. aureus (CAT Sa). Discovery of the F97W mutation of CAT Sa by model-guided protein engineering enhanced isobutyl acetate production. This study presented the consolidated bioprocessing of cellulose into ester(s) by the thermophilic CBP organism C. thermocellum harboring an engineered thermostable CAT Sa F97W. Overall, this research helps to establish a foundation for engineering non-model organisms for direct conversion of lignocellulosic biomass into designer bioesters.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2. Clostridium thermocellum DSM1313 Δhpt (M1354) strain was used as a host for the ester production at elevated temperatures. It should be noted that the deletion of hypoxanthine phosphoribosyltransferase gene (hpt, Clo1313_2927) in the wild-type DSM1313 allows genetic engineering by 8-azahypoxanthine (8-AZH) counter selection; this deletion does not have any known adverse effect on cell growth and metabolism [37, 38]. The plasmid pNW33N, containing CAT Sa is thermostable and was used to express various CATs in C. thermocellum. The pET plasmids were used for molecular cloning and enzyme expression in E. coli.

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (MO, USA) and/or Thermo Fisher Scientific (MA, USA), unless specified elsewhere. For molecular cloning, restriction enzymes and T4 ligase were obtained from New England Biolabs (MA, USA). Phusion Hot Start II DNA polymerase was used for polymerase chain reaction (PCR).

Media and cultivation

For molecular cloning and protein expression, E. coli strains were grown in lysogeny broth (LB) containing appropriate antibiotics unless noted otherwise. For in vivo characterization of CAT Sa in E. coli, M9 hybrid medium [5] with 20 g/L glucose was used. For C. thermocellum culture, MTC minimal medium or CTFuD-NY medium [38] was used as specified in the experiments.
Optical density (OD) was measured by a spectrophotometer at 600 nm wavelength (Spectronic 200+, Thermo Fisher Scientific, MA, USA).

**Multiple sequence alignment analysis**

Multiple sequence alignment (MSA) analysis was performed using MEGA7 [39]. Protein sequences were aligned by ClustalW [40] and visualized by ESPript 3.0 ([http://espript.ibcp.fr](http://espript.ibcp.fr)) [41]. The key features in protein structures of 3U9F [42], 4CLA [43], and 2XAT [44] were extracted from CAT_SALTI, CAT3_ECOLIX, and CAT4_PSEAE, respectively.

**Molecular modeling and docking simulations**

**Three-dimensional (3D) structures**

The 3D structure of CAT_{Sa} and alcohols of interest was first generated using Swiss-Model [45] and the ‘Builder’ tools of MOE (Molecular Operating Environment software, version 2019.01), respectively. The 3D structure of the dual substrate-bounded CAT_{Sa} complex (i.e., acetyl-CoA–isobutanol–CAT_{Sa}) was obtained by extracting an isobutanol from the isobutanol–CAT_{Sa} complex and then adding it to the acetyl-CoA–CAT_{Sa} complex. All the structures were prepared by the ‘QuickPrep’ tool of MOE with default parameters and further optimized by energy minimization with the Amber10:EHT force field.

**Docking simulation**

To perform docking simulations, the potential binding pocket was searched using the ‘Site Finder’ tool of MOE. The best-scored site, consistent with the reported catalytic sites [46], was selected for further studies. Docking simulations were performed as previously described [47]. Briefly, acetyl-CoA and each alcohol were docked using the induced fit protocol with the Triangle Matcher placement method and the London ΔG scoring function. After the docking simulations, the best-scored binding pose, showing the crucial interaction between the residue and the substrate at root-mean-square-deviation (RMSD) < 2 Å, was selected. As an example, for the acetyl-CoA docking, the binding pose exhibiting the hydrogen bond between the hydroxyl of Ser-148 and the N71 of the CoA was chosen [48]. For the alcohol docking, the binding pose showing the hydrogen bond between the N3 of His-189 and the hydroxyl of alcohol was selected [26].

**In silico mutagenesis analysis**

In silico mutagenesis analysis of the acetyl-CoA–isobutanol–CAT_{Sa} complex was carried out as previously described [47]. Specifically, the ‘alanine scan’ and ‘residue scan’ tools of MOE were used to identify the potential residue candidates for mutagenesis.

**Molecular cloning**

**Plasmid construction**

Plasmids were constructed by the standard molecular cloning technique of ligase dependent method and/or Gibson assembly [49] using the primers listed in Additional file 1: Table S1. The constructed plasmids were

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**Table 2  Plasmids and strains used in this study**

| Name      | Descriptions                                                                 | Source                        |
|-----------|------------------------------------------------------------------------------|-------------------------------|
| Plasmids  |                                                                              |                               |
| pNW33N    | Bacillus–E. coli shuttle vector, CmR, pBC1 ori for Gram-positive strains, pBR322 ori for E. coli, source of CAT_{Sa} | Bacillus Genetic Stock Center |
| pETDuet-1 | pBR322 ori, AmpR, lacI, T7lac promoter                                        | Novagen                       |
| pET_CAT_{Sa} | CAT_{Sa} wild-type encoding gene between BamHI, SacI site, pETDuet-1 backbone, 6× Histag at N-terminus | This study                    |
| pET_CAT_{Sa}F97W | F97W site-directed variant, pET_CAT_{Sa} backbone                           | This study                    |
| pHS0024   | CAT_{Sa} wild-type gene under C. thermocellum PgpdDH promoter, downstream of Clo1313_2927 for the transcription terminator, tdk operon under cbp promoter substituting with the native cat selection marker, pNW33N plasmid backbone | This study                    |
| pHS0024_F97W | CAT_{Sa}F97W site-directed mutated from pHS0024                             | This study                    |
| Strains   |                                                                              |                               |
| E. coli Top10 | Host for molecular cloning, mcrA, Δmrr-hsdRMS-mcrBC, Phi80lacZ(del)M15, ΔacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1, rpsG | Invitrogen                    |
| E. coli BL21 (DE3) | E. coli B dcm, ompT, hsdR(B-M-B), gal | Invitrogen                    |
| M1354     | C. thermocellum DSM1313 Δhpt                                                  | [37]                          |
| HSCT0101  | M1354 harboring pHS0024                                                      | This study                    |
| HSCT0102  | M1354 harboring pHS0024, F97W                                                | This study                    |

The plasmids containing mutagenized genes are presented in Additional file 1: Table S1.

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introduced into *E. coli* TOP10 by heat shock transformation. Colonies isolated on a selective plate were PCR screened and plasmid purified. The purified plasmids were verified via Sanger sequencing before being transformed into *E. coli* BL21 (DE3). Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis protocol with reduced overlap length [50] or Gibson assembly method [49]. For the *C. thermocellum* engineering, the plasmid pHS005 was constructed first and then modified to pH50024. pHS0024 has no hpt and then modified to pHS0024. pHS005 is identical to pHS005.

**Transformation**

The conventional chemical transformation and electroporation methods were used for transformation of *E. coli* [51] and *C. thermocellum* [38], respectively. For *C. thermocellum*, the method, however, was slightly modified as described here. First, *C. thermocellum* M1354 (Table 2) was cultured in 50 mL CTFuD-NY medium at 50 °C inside anaerobic chamber (Bactron300, Sheldon manufacturing Inc., OR, USA). The cell culture with OD in a range of 0.8–1.0 was cooled down at room temperature for 20 min. Beyond this point, all steps were performed outside the chamber. The cooled cells were harvested at 6500 × g and 4 °C for 20 min. The cell pellets were washed twice with ice-chilled Milli-Q water and resuspended in 200 μL of the transformation buffer consisting of 250 mM sucrose and 10% (v/v) glycerol. Several 30 μL aliquots of the electrocompetent cells were immediately stored at −80 °C for further use. For electroporation, the electrocompetent cells were thawed on ice and incubated with 500–1000 ng of methylated plasmids [52] for 10 min. Then, the cells were transferred to an ice-chilled 1-mm gap electroporation cuvette (BTX Harvard Apparatus, MA, USA) followed by two consecutive exponential decay pulses with 1.8 kV, 350 Ω, and 25 μF. The pulses usually resulted in a 7.0–8.0 ms time constant. The cells were immediately resuspended in pre-warmed fresh CTFuD-NY and recovered at 50 °C under anaerobic condition (90% N₂, 5% H₂, and 5% CO₂) inside a rubber capped Balch tube. After 0–12 h of recovery, the cells were mixed with molten CTFuD-NY agar medium supplemented with 15 μg/mL thiamphenicol. Finally, the medium-cell mixture was poured on petri dish and solidified inside the anaerobic chamber. The plate was incubated at 50 °C up to 1 week until colonies appeared. Transformation efficiency was 2–100 colony-forming units per μg plasmid (CFU/μg plasmid).

**In vivo characterization of CATsa and its variants in E. coli**

For in vivo characterization of CATsa and its variants in *E. coli*, high-cell density cultures were performed as previously described [53] with an addition of 2 g/L of various alcohols. For in situ extraction of esters, each tube was over-laid with 25% (v/v) hexadecane. To confirm the protein expression of CATsa and its variants, 1% (v/v) of stock cells were grown overnight at 37 °C and 200 rpm in 15 mL culture tubes containing 5 mL of LB medium and antibiotic. Then, 4% (v/v) of the overnight cultures were transferred into 1 mL of LB medium containing antibiotic in a 24-well microplate. The cultures were grown at 37 °C and 350 rpm using an incubating microplate shaker (Fisher Scientific, PA, USA) until OD reached to 0.4–0.6 and then induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h with a BreathEasy Sealing Membrane to prevent evaporation and cross contamination (cat# 50-550-304, Research Products International Corp., IL, USA). The protein samples were obtained using the B-PER complete reagent (cat# 89822, Thermo Scientific, MA, USA), according to the manufacturer’s instruction and analyzed by SDS-PAGE.

**Enzyme characterization**

**His-tag purification**

For enzyme expression, an overnight culture was inoculated with a 1:50 ratio in fresh LB medium containing 1 mM IPTG and antibiotic, followed by 18 °C overnight incubation (up to 20 h) in a shaking incubator at 200 rpm. The induced cells were harvested by centrifugation at 4 °C, and 4700 × g for 10 min. The cell pellet was then washed once with Millipore water and resuspended in the B-PER complete reagent. After 30 min incubation at room temperature, the mixture was centrifuged at 17,000 × g for 2 min. The supernatant was collected and designated as crude extract. For His-tag purification, the crude extract was incubated with HisPur Ni-NTA superf low agarose in a batch as the manufacturer recommends. Then, the resin was washed with at least three volumes of wash buffer, consisting of 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.1 mM EDTA. The resin bound proteins were eluted by 300 μL elution buffer containing 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 300 mM imidazole, and 0.1 mM EDTA. The eluted sample was then desalted and concentrated via an Amicon filter column with 10 kDa molecular weight cut-off. Finally, the protein sample was suspended in 200 μL of 20 mM Tris–HCl buffer (pH 8.0). Protein concentration was measured by the Bradford assay [54] with bovine serum albumin (BSA) as the reference protein.

**Thermal shift assay**

To measure protein melting temperature (Tm), a thermostability assay was employed with SYPRO Orange [55]. About 10–250 μg of His-tag purified protein was mixed with 5× SYPRO Orange in a 50 μL final volume in a
96-well qPCR plate. The plates were sealed with PCR caps before running the assay. The StepOne real-time PCR machine (Applied Biosystems, CA, USA) was used to run the assay with the following parameters: ROX reporter, 1 °C increment per cycle, 1-min hold at every cycle, and temperature range from 20 to 98 °C. The data were collected, exported, and processed to calculate Tm.

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay

Reaction rate for each CAT was determined by a DTNB assay [56] in a 384-well plate. Total reaction volume was 50 μL with the reaction buffer comprising of 50 mM Tris–HCl (pH 8.0). Concentrations of acetyl-CoA (CoALA Biosciences, TX, USA) and alcohols were varied as specified in each experiment. Final enzyme concentrations of 0.05 μg/mL and 10 μg/mL were used for the reactions towards chloramphenicol and alcohols, respectively. Reaction kinetics was collected by measuring absorbance at 412 nm every minute for 1 h at 50 °C in a microplate reader (Synergy HTX microplate reader, BioTek). The reaction rate was calculated using the extinction coefficient from a standard curve of free coenzyme A (MP Biomedicals, OH, USA) under the same condition. It should be noted that since the maximum operating temperature recommended for the plate reader is 50 °C, the high-throughput enzyme assay for CAT at elevated temperatures was only performed to determine enzyme kinetics parameters.

Calculation of kinetic parameters for reaction rates

The parameters of Michaelis–Menten rate law (Eq. 1) were calculated for each enzyme as follows. First, linear regression was performed on data collected from a microplate reader to identify initial reaction rates, $v_i$, at different initial substrate concentrations, $s_i$, where $i = 1, 2, ..., n$ is the number of data points collected. Then, these initial reaction rates and associated initial substrate concentrations for all replicates were simultaneously fit to the Michaelis–Menten model (Eq. 1) using robust non-linear regression (Eq. 2) with a soft-L1-loss estimation methods, robust non-linear regression provides the most precise parameter estimate for the Michaelis–Menten model [59].

The least-squares problem determines the parameters $K_M$ and $v_{\text{max}}$ by minimizing the difference between the model predicted reaction rates $v_i$ and measured reaction rates $y_i$ (Eq. 2). A smoothing function $\rho(z)$ is used to make the least square problem resistant to outliers (Eq. 3). Due to the unbiased resistance to outliers and the avoidance of errors resulting from conventional linearization methods, robust non-linear regression provides the most precise parameter estimate for the Michaelis–Menten model [59].

Isobutyl acetate production in C. thermocellum

Cellulose fermentation

Isobutyl acetate production from cellobiose in C. thermocellum strains was performed by the two-step bioconversion configuration. Cells were first cultured in MTC minimal medium [38] containing 5 g/L cellobiose in a rubber capped Balch tube until OD reached 0.8–1.0. The cells were cooled down at room temperature for 20 min and centrifuged at 4700 x g and 4 °C for 20 min. After removing the supernatant, cells were resuspended in the same volume of fresh MTC minimal medium containing 2 g/L isobutanol in an anaerobic chamber. The cell suspension was then divided into 800 μL in a 2.0 mL screw cap microcentrifuge tube with a 200 μL hexadecane overlay. The cells were incubated at 55 °C for 24 h followed by analysis of gas chromatography coupled with a mass spectrometer (GC/MS) to quantify the amount of isobutyl acetate produced.

Cellulose fermentation

For the cellulose fermentation, modified MTC medium (C-MTC medium) was used. 20 g/L of Avicel PH-101 was used as a sole carbon source instead of cellobiose, and 10 g/L of MOPS was added to increase buffer capacity. Initial pH was adjusted to 7.5 by 5 M KOH and autoclaved. In an anaerobic chamber, 0.8 mL of overnight cell culture was inoculated in 15.2 mL of C-MTC medium (1:20 inoculation ratio) with 4 mL of overlaid hexadecane. Each tube contained a small magnetic stirrer bar to homogenize cellulose. The rubber capped Balch tube was incubated in a water bath connected with a temperature controller set at 55 °C and a magnetic stirring system. Following pH adjustment with 70 μL of 5 M KOH injection, 800 μL of cell culture and 200 μL of hexadecane layer were sampled every 12 h. Culture pH was maintained within a range of 6.4–7.8 during the fermentation.

Cell growth was monitored by measuring pellet protein. The cell–cellulose pellet from 800 μL sampling volumes was washed twice with Milli-Q water and suspended by 200 μL lysis buffer (0.2 M NaOH, 1% SDS) followed by an hour incubation at room temperature. Then, the solution was neutralized with 50 μL 0.8 M HCl and
diluted by 550 μL water. The mixture was centrifuged at 17,000×g for 3 min. Protein concentration from the supernatant was analyzed by the detergent-compatible Bradford assay (Thermo Scientific, WA, USA). The residual pellet was boiled in a 98 °C oven for an hour before quantifying residual cellulose.

Residual cellulose was quantified by the phenol–sulfuric acid method [60] with some modifications. The boiled sample was washed twice with Milli-Q water and suspended in 800 μL water to make equivalent volume to the original. The sample was homogenized by pipetting and vortexing for 10 s, and 20 μL of the homogenized sample was transferred to a new 2.0 mL microcentrifuge tube or 96-well plate and dried overnight in a 55 °C oven. The dried pellet was suspended in 200 μL of 95% sulfuric acid and incubated for an hour at room temperature. After the pellet was dissolved completely, 20 μL of 5% phenol was added and mixed with the sulfuric acid solution. After 30 min incubation at room temperature, 100 μL of the sample was transferred to a new 96-well plate, and the absorbance at 490 nm was measured. The absorbance was converted to cellulose concentration by the standard curve of Avicel PH-101 treated by the same procedure.

Analytical methods
High-performance liquid chromatography (HPLC)
Extracellular metabolites were quantified using a high-performance liquid chromatography (HPLC) system (Shimadzu Inc., MD, USA). 800 μL of culture samples were centrifuged at 17,000×g for 3 min, and then, the supernatants were filtered through 0.2 μm filters and run with 10 mM H₂SO₄ mobile phase at 0.6 mL/min on an Aminex HPX-87H (Biorad Inc., CA, USA) column at 50 °C. Refractive index detector (RID) and ultra-violet detector (UV) at 220 nm were used to monitor concentrations of sugars, organic acids, and alcohols.

Gas chromatography coupled with mass spectroscopy (GC/MS)
Esters were measured by GC (HP 6890, Agilent, CA, USA) equipped with an MS (HP 5973, Agilent, CA, USA). For the GC system, the Zebon ZB-5 (Phenomenex, CA, USA) capillary column (30 m × 0.25 mm × 0.25 μm) was used to separate analytes, and helium was used as the carrier with a flow rate of 0.5 mL/min. The oven temperature program was set as follows: 50 °C initial temperature, 1 °C/min ramp up to 58 °C, 25 °C/min ramp up to 235 °C, 50 °C/min ramp up to 300 °C, and 2-min bake-out at 300 °C. 1 μL of sampled hexadecane layer was injected into the column in the splitless mode with an injector temperature of 280 °C. For the MS system, selected ion mode (SIM) was used to detect and quantify esters with the following parameters: (i) ethyl acetate, m/z 45.00 and 61.00 from 4.2 to 4.6 min retention time (RT), (ii) isopropyl acetate, m/z 45 and 102 from 4.7 to 5.0 min RT, (iii) propyl acetate, m/z 59 and 73 from 5.2 to 5.8 min RT, (iv) ethyl isobutyrate, m/z 73 and 116 from 6.1 to 6.6 min RT, (v) isobutyl acetate, m/z 61 and 101 from 6.6 to 7.6 min RT, (vi) butyl acetate, m/z 61 and 116 from 7.7 to 9.2 min RT, (vii) isobutyl isobutyrate, m/z 89 and 129 from 10.1 to 12.5 min RT, (viii) benzyl acetate, m/z 108 and 150 from 13.1 to 13.8 min RT, and (ix) 2-phenethyl acetate, m/z 104 and 121 from 13.8 to 15.5 min RT. Isoamyl alcohol and isoamyl acetate were used as the internal standard analytes. The esters were identified by RT and quantified by the peak areas and standard curves. Standard curves were determined using pure esters diluted into hexadecane at concentrations of 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.5 g/L, and 1 g/L.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13068-019-1583-8.

Additional file 1. Additional Figures S1–S6 and Tables S1, S2.

Abbreviations
AAT: alcohol acetyltransferase; CBP: consolidated bioprocessing; CAT: chloramphenicol acetyltransferase; CFU: colony-forming unit; PCR: polymerase chain reactions; MSA: multiple sequence alignment; DCW: dried cell weight; DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid); GC: gas chromatography; HPLC: high-performance liquid chromatography; IPTG: isopropyl β-1-thiogalactopyranoside; kDa: kilo Dalton; MOE: Molecular Operating Environment software; MS: mass spectrometry; OD: optical density; RMSD: root-mean-square-deviation; RT: retention time; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis; 8-AZH: 8-azahypoxanthine; Tm: melting temperature.

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Authors’ contributions
CTT initiated and supervised the project. HS, JWL, and CTT designed the experiments, analyzed the data, and drafted the manuscript. HS and JWL performed the experiments. SG calculated enzyme kinetic parameters and edited the manuscript. All authors read and approved the final manuscript.

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Availability of supporting data
One additional file contains supporting data.

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Consent for publication
All the authors consent for publication.
Competing interests
The authors declare that they have no competing interests.

Author details
1. Department of Chemical and Biomolecular Engineering, The University of Tennessee, Knoxville, TN, USA.
2. Bredesen Center for Interdisciplinary Research and Graduate Education, The University of Tennessee, Knoxville, TN, USA.
3. Center for Bioenergy Innovation (CBI), Oak Ridge National Laboratory, Oak Ridge, TN, USA.

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