I. COVER PAGE

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II. ACCOMPLISHMENTS

a. Major goals and objective

The overall objective of this project is to develop and evaluate the in-planta enzyme engineering technology to reduce lignocellulose deconstruction cost. There are three specific objectives in this Phase I project:
(1) validate the enzyme optimization concept using tobacco plant, a model plant system.
(2) validate the enzyme optimization concept using switchgrass.
(3) techno-economic analysis (TEA) for further scale-up application.

b. What was accomplished under these goals?

Task 1. Validation of in-planta enzyme engineering in tobacco

Task 1.1 Construction and transformation of the expression vector into tobacco

Construction of expression vectors into tobacco. Seven gene constructs encoding cellulase E1cd and laccase Lac2.9 and their derivatives were made in plant expression vector *pBI121* by standard molecular cloning (**Figure 1**). The *E1cd* gene fragment was PCR amplified from the genomic DNA of *A. cellulolyticus* (ATCC 43068). The Lac2.9 gene as reported by Navas et al. was synthesized by GenScript (Piscataway, NJ) and the gene sequence was codon optimized for tobacco expression [1]. Later, the native Lac2.9 gene was also synthesized by GenScript (Piscataway, NJ). The sequence of all the gene constructs was confirmed by DNA sequencing in the DNA Sequencing & Genotyping Facility at the University of Chicago. The DNA sequence of E1cd-$(SP)_{30}$-GPI and Lac2.9-$(SP)_{30}$-GPI are shown in **Appendix 1**.

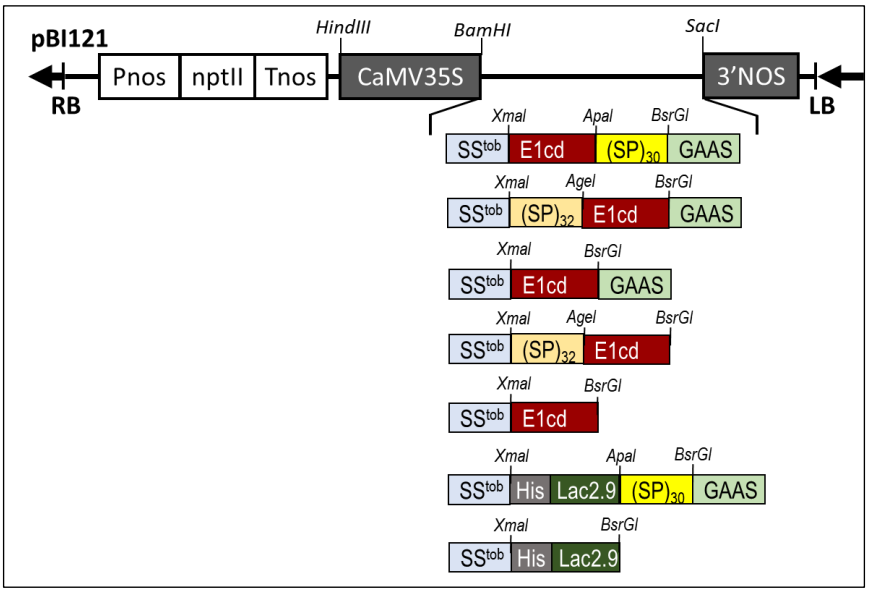

Figure 1. Schematic of the gene expression cassettes in *pBI121* vector. CaMV35S: 35S cauliflower mosaic virus promoter; SS^{tob}: tobacco signal sequence; 3'NOS: Nopaline synthase terminator; GAAS: GPI anchor attachment signal sequence encoding "SGAEKKMLGSLVAGWAVMSWLLF" is derived from tomato arabinogalactan protein-1 (AGP-1).

In order to obtain the enzyme standard for quantification of the recombinant enzyme expression in planta, the E1cd and LAC2.9 enzyme was also expressed in *E. coli* and purified by the Nickle affinity chromatography. The gene expression cassettes for the two enzymes and their purification process were shown in **Figure 2**. Obviously, both enzymes were successfully expressed in *E. Coli*, and the Nickle affinity chromatography partially purified the expressed enzymes. The enzymes were further purified by size exclusion chromatography using a Superdex 200 column.

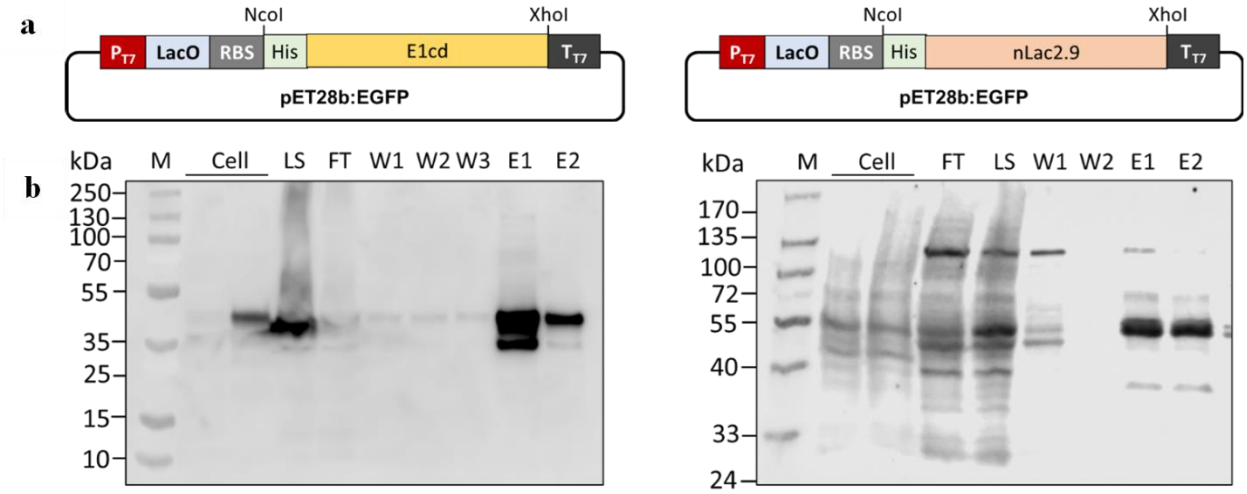

Figure 2. Expression of E1cd and Lac2.9 enzyme in bacteria. (a) Schematic of the gene expression cassettes in *pET28b* vector. P_{T7}: T7 promoter; LacO: Lactose operator; RBS: Ribosome binding site; T_{T7}: T7 terminator. **(b)** Purification of the expressed enzymes by nickel affinity chromatography. The native gene sequence of the two enzymes was used for expression in bacteria. The recombinant E1cd and Lac2.9 was detected by anti-E1cd and anti-6xHis Western blot, respectively. Cell: harvested *E. coli* cells dissolved in 5x loading buffer; LS: cell lysate; FT: flow through; W1, W2: wash solution; E1, E2: elution solution.

Plant transformation into tobacco. The target gene constructs were then transformed into tobacco using the *Agrobacterium*-mediated leaf-disk method. Five transformants were selected based on anti-E1cd Western blotting assay for the transformants expressing E1cd based constructs (**Figure 3a**). Anti-6xHis Western blot was used to detect the recombinant Lac2.9 enzyme expression in tobacco plants. However, no protein expression was detected with Western blot (**Figure 3b**).

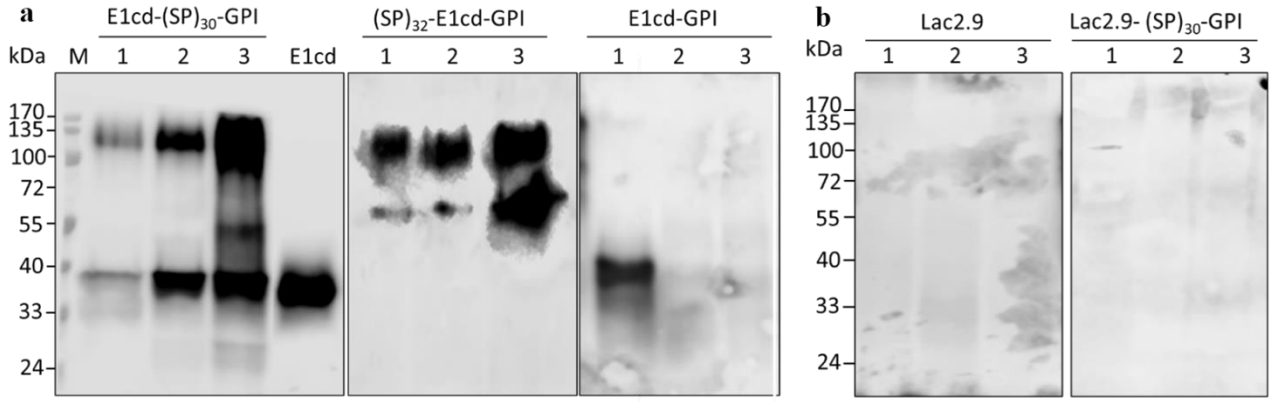

Figure 3. Detection of recombinant E1cd and Lac2.9 enzyme expressed in tobacco plants. (a) Anti-E1cd Western blot detection of E1cd and its derivatives. **(b)** Anti-6xHis Western blot detection of Lac2.9 and its derivatives.

Transgenic tobacco growth. Transgenic and wild-type plants were moved to greenhouse and their growth and phenotype was detected. All the plants showed a similar growth rate and started flowering 6 to 7 weeks after being transferred into soil. There were no significant differences in the phenotype observed (stem height and leaf size and shape) (**Figure 4a**). This indicated that neither the target recombinant enzyme (E1cd) nor the functional modules, $(SP)_n$ and GPI anchor, engineered *in planta* had a pronounced impact on plant growth and development. Expression of E1cd products in transgenic plants was validated with Anti-E1cd Western blot (**Figure 4b**).

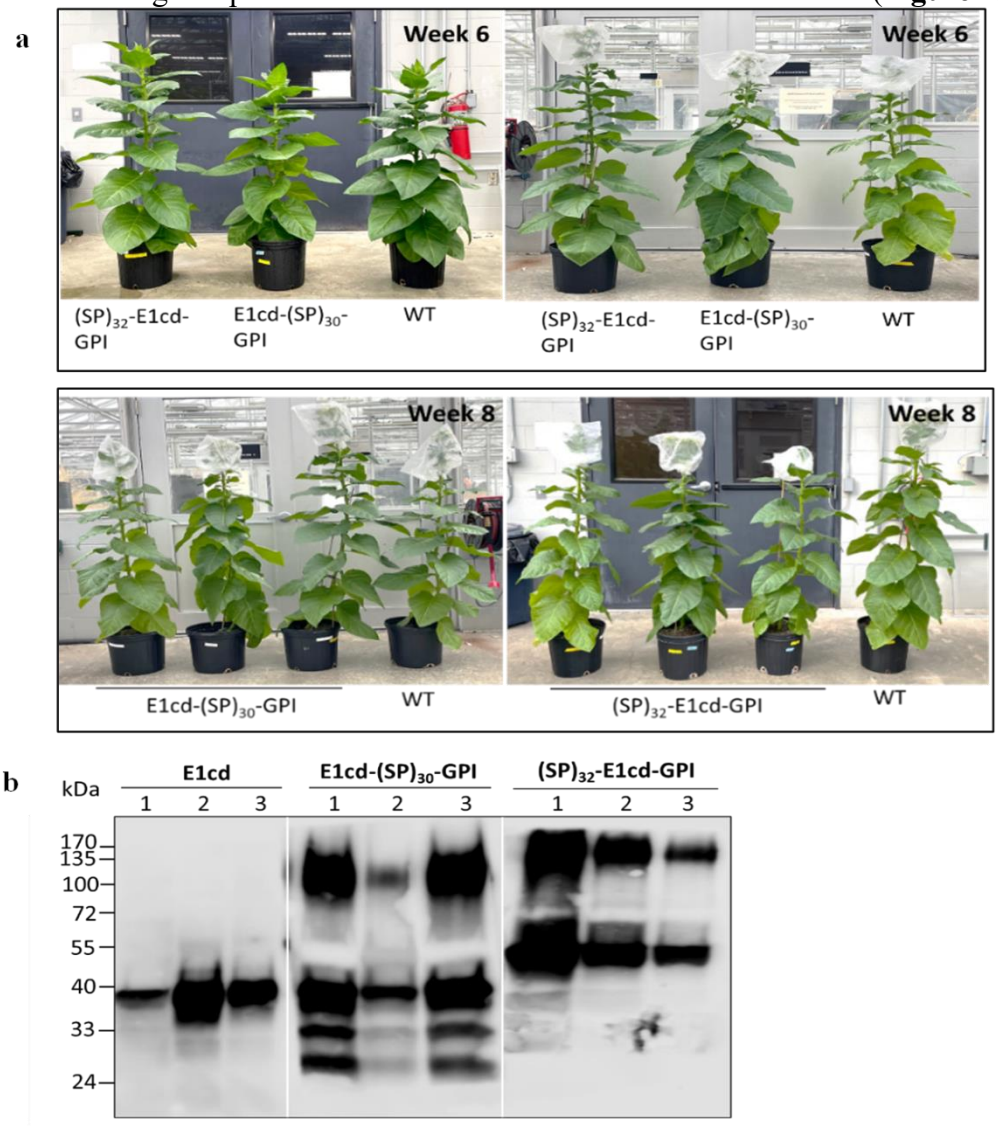

Figure 4. Characterization of T_0 tobacco transgenic plants grown in greenhouse. (a) Growth of T_0 tobacco transgenic plants and wild-type (WT) plants 6 and 8 weeks after transferring into soil. No significant difference in the phenotype was observed (stem height and leaf size and shape); **(b)** Anti-E1cd Western blot detection of the transgene products accumulated in tobacco leaves (6 weeks)

The accumulation of E1cd products in transgenic plants was further quantified. As seen in **Table 1**, the engineered $(SP)_n$ module generally improved the accumulation of the fused enzyme (E1cd) in the transgenic plants grown in the greenhouse. Plant biomass will be harvested, with the stems separated from the leaves, and dry weights determined for both.

Table 1. Accumulation of recombinant E1cd products in the leaves of transgenic plantlets grown in a greenhouse. The top-expression transgenic lines for each gene construct were analyzed. Each data point represents the mean of three measurements ± standard deviation (*SD*). Recombinant E1cd products were quantified by densitometry based on anti-E1cd Western blot. The blot images were captured on the Li-Cor Odyssey Fc imaging system (Li-Cor Biosciences, NE) and the target protein products were quantified with the Li-Cor's Image Studio™ Software or Image J (NIH).

Time	E1cd	E1cd-GPI	$(SP)_{32}$-E1cd-GPI	E1cd-$(SP)_{30}$-GPI
4 weeks	70.8±2.9	32.1±4.3	84.8±2.4	128.4±6.3
8 weeks	71.5±3.5	32.5±2.8	86.3±3.5	125.5±4.8

Besides, the seeds collected from different plants can successfully germinate, although germination rates varied (Figure 5a). Both fusion proteins were expressed in the T1 plants at high yields, higher than in T_0 plants (Figure 5b).

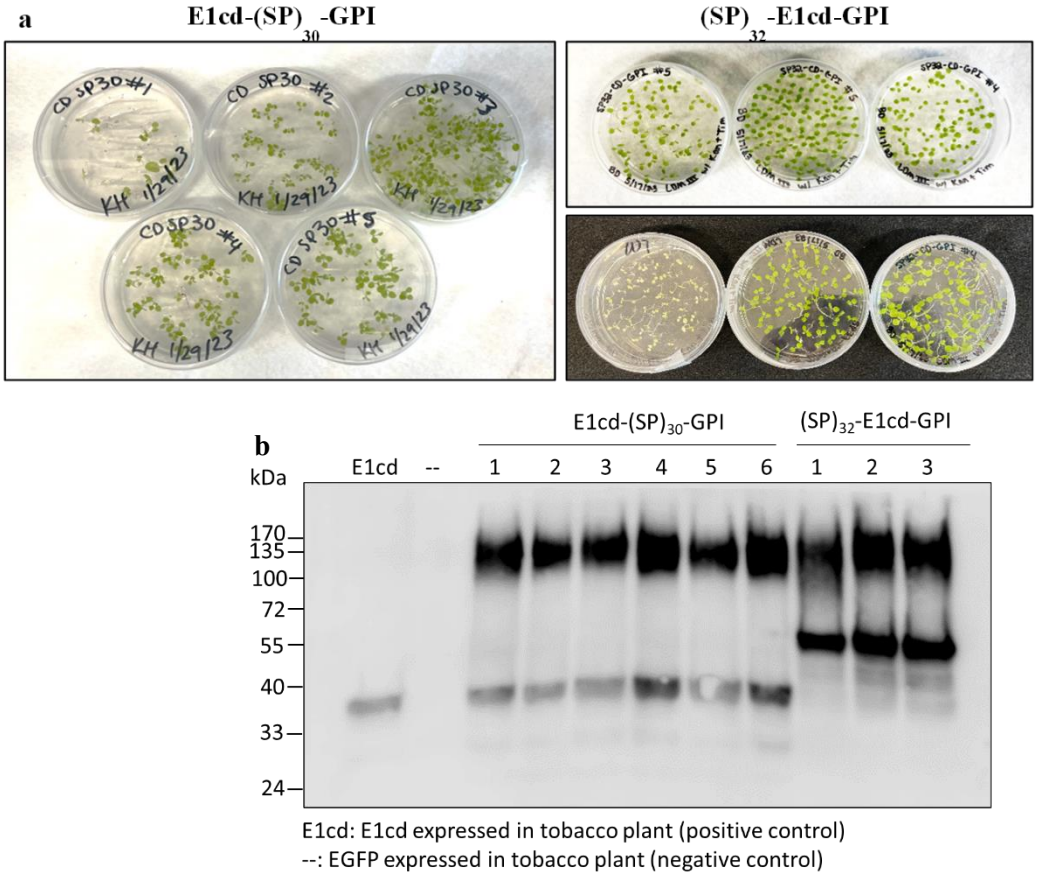

Figure 5. Gemination of transgenic tobacco seeds (from T_0) and enzyme expression. (a) Growth from T_0 tobacco seeds on agar plates; (b) Anti-E1cd Western blot detection of fusion proteins in geminated seedlings (T_1).

T_1 plants were further grown in green house and their growth and phenotype was detected (**Figure 6a**). Expression of E1cd products in transgenic plants was validated with Anti-E1cd Western blot (**Figure 6b**). All T_1 plants also showed a similar growth rate and started flowering 6 to 7 weeks after being transferred into soil. There were no significant differences in the phenotype observed (stem height and leaf size and shape) (**Figure 6a**). This further indicated that neither the target recombinant enzyme (E1cd) nor the functional modules, (SP)n and GPI anchor, engineered in planta had a significant impact on plant growth and development.

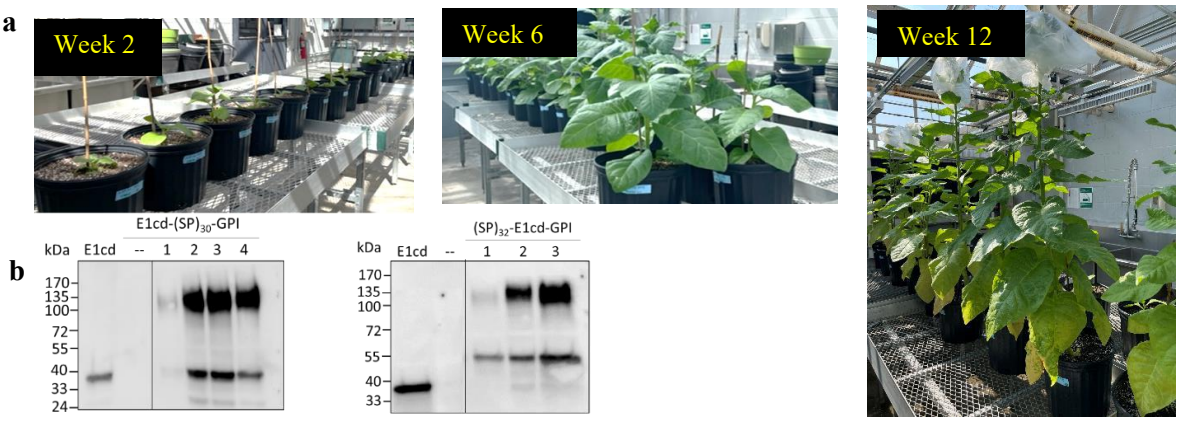

Figure 6. Characterization of T_1 transgenic plants grown in greenhouse. (a) Growth of T_1 transgenic plants and wild-type (WT) plants 2, 6, and 12 weeks after transferring into soil. No significant difference in the phenotype was observed (stem height and leaf size and shape); **(b)** Anti-E1cd Western blot detection of the transgene products accumulated in tobacco leaves (6 weeks)

Task 1.2 Analysis of expressed enzymes in tobacco

The activity of the recombinant E1cd or E1cd derivatives was assayed by its ability to cleave 4-methylumbelliferyl-β-D-cellobioside (4-MUC) to produce the fluorophore, 4-methylumbelliferone (4-MU). One unit of enzyme activity was defined as the amount of the enzyme that generates one nanomole of 4-MU per minute at 80 °C and pH 5.0. The Lac2.9 activity was determined by monitoring its ability to oxidate 2,6-dimethoxyphenol (DMP). One unit of Laccase is the amount of enzyme that produces 1 µmol of oxidized product per minute at pH6 at 30ºC. As shown in **Table 2**, The specific enzyme activity was determined as 39.2 U/µg for the E1cd control and 35.5-38.5 U/µg for the E1cd with the two functional modules. As predicted, the $(SP)_n$ module and GPI anchor exhibited minimal effect on the enzyme activity of the cellulase. The specific enzyme activity for the Lac2.9-$(SP)_{30}$-GPI was 141.3 U/g TSP over 2 times that of wild type (67.7±21.5 U/g TSP). This indicates expression of Lac2.9-$(SP)_{30}$-GPI in Tobacco.

Table 2. Specific enzyme activity of plant expressed E1cd, E1cd-$(SP)_{30}$-GPI, $(SP)_{32}$-E1cd-GPI, and Lac2.9-(SP)30-GPI. E1cd equivalent amount was used for the calculation. Each data point represents the mean of three replicate measurements with the standard deviation (SD). *(U/µg); **U/g TSP

Enzymes	E1cd	E1cd-GPI	$(SP)_{32}$-E1cd-GPI	E1cd-$(SP)_{30}$-GPI	Lac2.9-$(SP)_{30}$-GPI
Specific activity	39.6±2.3*	38.5±1.8*	35.5±3.4*	37.6±2.5*	141.3±25.1**

The activity of designer E1cd enzymes was further analyzed for their sensitivity to temperature and pH. As shown in **Figure 7**, the designed enzymes displayed a broader sub-optimum temperature range (70 °C to 85 °C) than E1cd (70 °C to 80 °C) (**Figure 7**). This was presumably due to the presence of the glycosylated $(SP)_n$ module that could stabilize the enzyme.

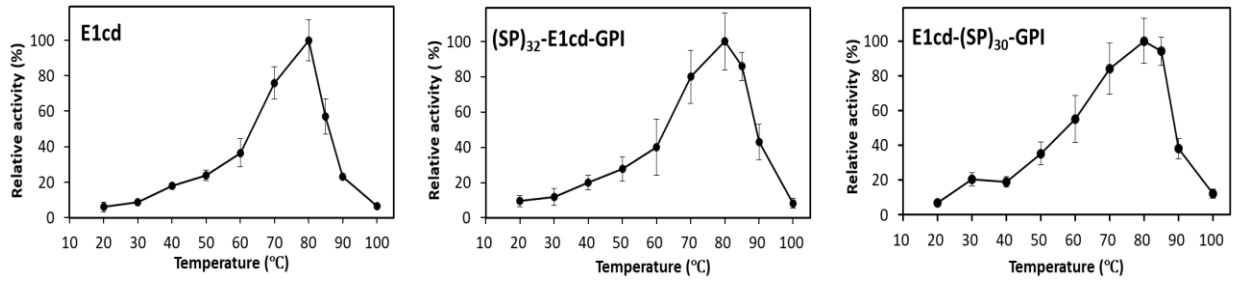

Figure 7. Effect of temperature on the recombinant enzyme activity. The activity was determined at the optimal pH value (pH5). Error bars represent the standard deviation (*SD*) of three replicate measurements.

The effect of pH on the designer enzyme activity is illustrated in **Figure 8**. The designed enzymes showed comparable stability to E1cd with a sub-optimum pH range from 5 to 10.

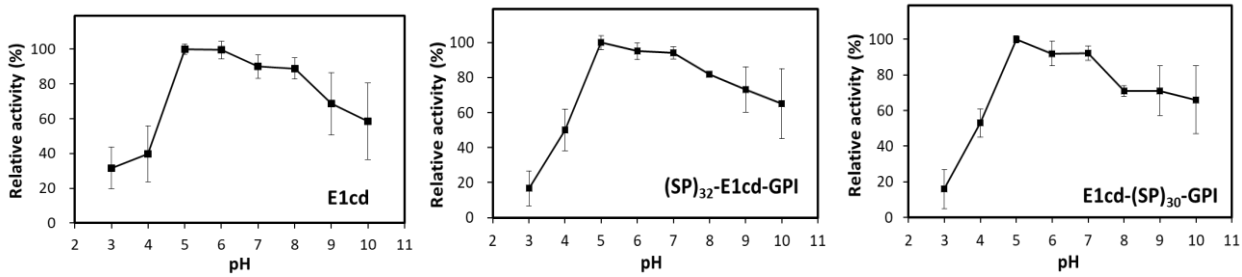

Figure 8. Influence of pH on the recombinant enzyme activity. The activity was determined at the optimal temperature 80℃. Error bars represent the standard deviation (SD) of three replicate measurements.

Task 1.3 Evaluation of biomass digestibility of transgenic tobacco

Biomass digestibility of transgenic tobacco and wild type was evaluated *via* saccharification (**Figure 9**). Compared to wild type, T_0 tobacco plants with $(SP)_{32}$-E1cd-GPI, E1cd-$(SP)_{30}$-GPI, E1cd-GPI and E1cd improved the saccharification efficiency by 3.2, 2.5, 1.6 and 1.6 times, respectively (**Figure 9a**). As a result, T_1 tobacco plants with $(SP)_{32}$-E1cd-GPI and E1cd-$(SP)_{30}$-GPI were further tested for biomass digestibility (**Figure 9b**). Saccharification efficiency of transgenic plants with $(SP)_{32}$-E1cd-GPI and E1cd-$(SP)_{30}$-GPI varied from 16% to 46%, indicating the importance of selecting seeds for a consistent performance.

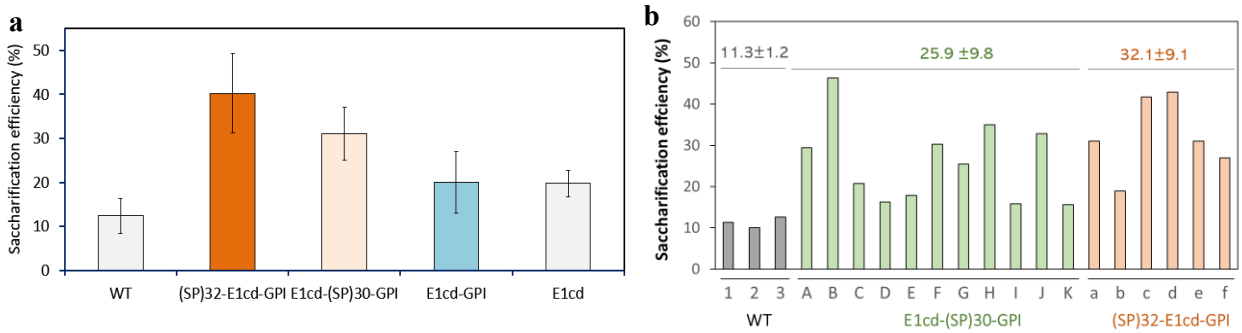

Figure 9. Saccharification efficiency of transgenic plants. (a) T_0 transgenic plants and wild-type (Error bars represent the standard deviation of three replicate measurements); **(b)** T_1 transgenic plants and wild-type

❖ *Significant findings from Task 1*

- E1cd with or without functional modules, (SP)n and GPI anchor, was successfully expressed in tobacco.
- Neither E1cd nor the functional modules engineered in planta had a significant impact on plant growth and development.
- E1cd with functional modules showed comparable enzyme activity and better enzyme stability than E1cd.
- Transgenic tobacco plants with $(SP)_{32}$-E1cd-GPI and E1cd-$(SP)_{30}$-GPI showed higher biomass digestibility than wild type.

Task 2. Validation of in-planta enzyme engineering in switchgrass

Task 2.1 Construction and transformation of expression vector into switchgrass

Construction of expression vectors into switchgrass. The *E1cd-(SP)$_{30}$-GAAS* and *Lac2.9-(SP)$_{30}$-GAAS* gene fragment was amplified by PCR from the corresponding *pBI121* vector as shown in **Figure 1**, and subcloned into the *pANIC6A* binary vector (obtained from Arabidopsis Biological Resource Center, Columbus, OH) by Gateway cloning to create the expression vector shown in **Figure 10**.

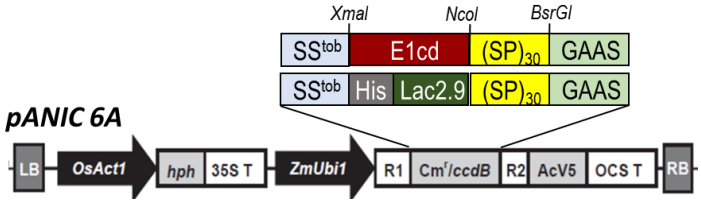

Figure 10. Schematic of the gene cassette in expression vector pANIC6A. OsAct1: rice actin 1 promoter; hph: hygromycin B phosphotransferase coding region; 35S T: 35S terminator; Zmubi1: maize ubiquitin 1 promoter; OCS T: octopine synthase terminator.

Plant transformation into switchgrass. The two expression vectors were sent to the Boyce Thompson Institute (BTI) at Cornell University for switchgrass transformation. The two constructs were stably transformed into switchgrass cultivar, Alamo, using the *Agrobacterium*-mediated method, , and the integration of transgenes was detected using direct PCR (**Figure 11**).

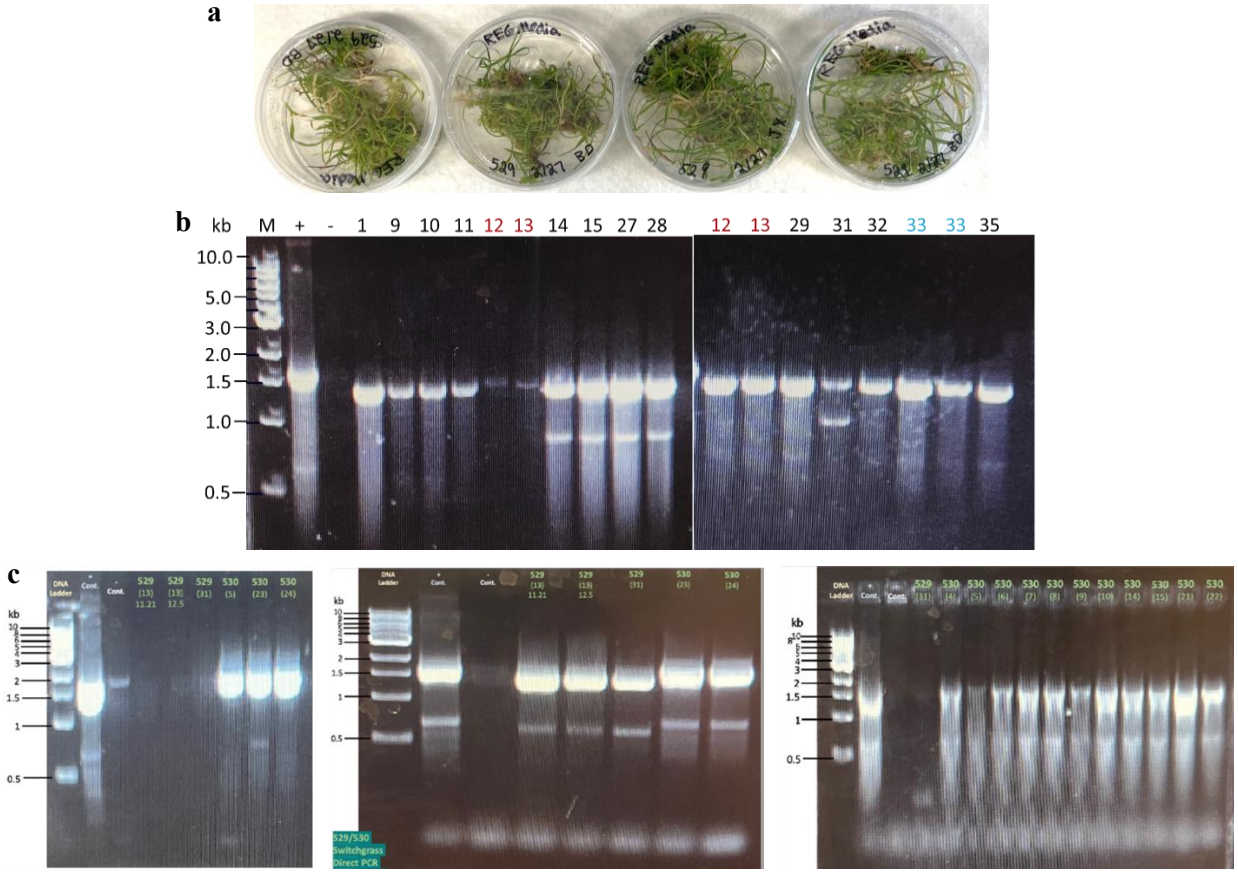

Figure 11. Detection of transgene in transgenic switchgrass plantlets using direct PCR. (a) transgenic switchgrass plantlets; **(b)** detection of E1cd-(SP)$_{30}$-GPI gene construction (529); **(c)** detection of Lac2.9-(SP)30-GPI gene construction (530).

Expression of E1cd-$(SP)_{30}$-GPI and Lac2.9-$(SP)_{30}$-GPI was further checked with Anti-E1cd Western blot (**Figure 12**). More than 8 plants were found to express E1cd-$(SP)_{30}$-GPI (**Figure 12a**), but no expression of Lac2.9-$(SP)_{30}$-GPI was detected using an anti-6xHis antibody (**Figure 12b**).

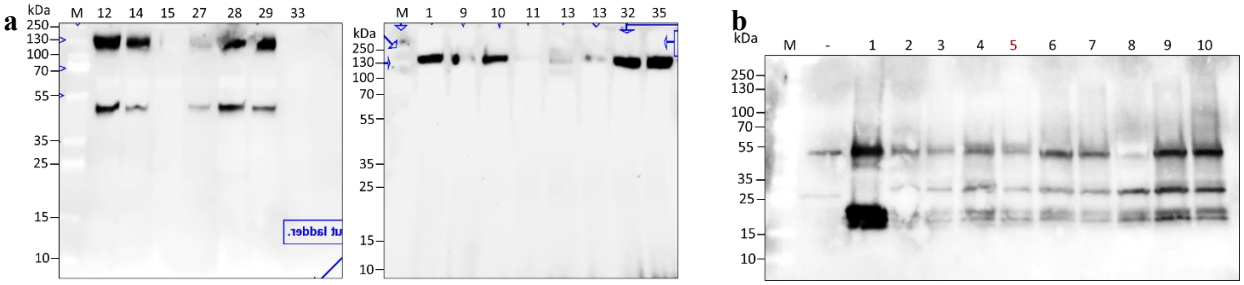

Figure 12. Western blotting detection of transgenes in switchgrass plantlets. (a) detection of E1cd-$(SP)_{30}$-GPI expression using an anti-E1cd antibody; **(b)** detection of Lac2.9-$(SP)_{30}$-GPI expression using an anti-6xHis antibody

Transgenic switchgrass growth. Transgenic switchgrass plants were moved to greenhouse and their growth and phenotype was detected. All the plants showed a similar growth rate after being transferred into soil. There were no significant differences in the phenotype observed (stem height and leaf size and shape) (**Figure 13a**). Expression of E1cd products in E1cd-$(SP)_{30}$-GPI plants was validated with anti-E1cd Western blot (**Figure 13b**). Again, the expression of Lac2.9-$(SP)_{30}$-GPI could not be detected by the anti-6xHis Western blotting (**Figure 13c**).

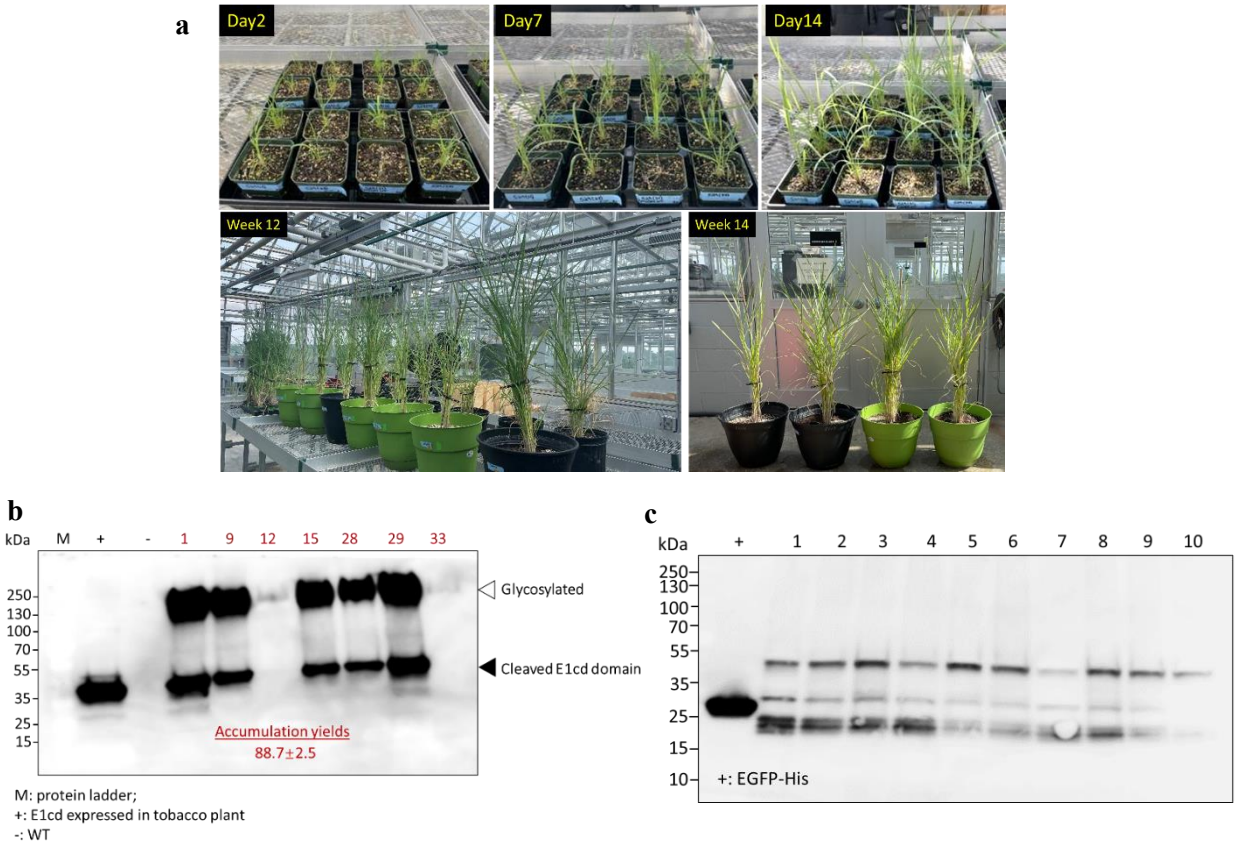

Figure 13. Characterization of T_0 transgenic switchgrass plants grown in greenhouse. (a) Growth of T_0 transgenic switchgrass plants from day 2 to week 14 after transferring into soil. No significant difference in the phenotype was observed (stem height and leaf size and shape) between the E1cd-$(SP)_{30}$-GPI and Lac2.9-$(SP)_{30}$-GPI plants; **(b)** Anti-E1cd Western blot detection of E1cd-$(SP)_{30}$-GPI accumulated in switchgrass leaves (14 weeks); **(c)** Anti-6xHis Western blot detection of Lac2.9-$(SP)_{30}$-GPI accumulated in switchgrass leaves (14 weeks).

Besides, switchgrass plants can re-grow after the top parts are cut off (**Figure 14**). This is beneficial for providing a stable biomass supply allowing frequent harvesting.

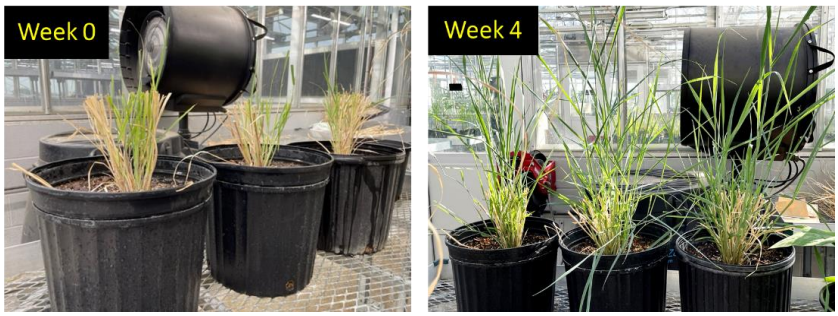

Figure 14. Regrowth of transgenic switchgrass plants

The switchgrass biomass was harvested after 16 weeks of culture (**Figure 15**). The measured biomass yields of E1cd-$(SP)_{30}$-GPI plants are lower than those of Lac2.9-$(SP)_{30}$-GPI plants. However, there is no statistical difference between these two groups. As we do not have wild-type (WT) lines regenerated from calli like the two transgenic plant lines, we are currently growing WT switchgrass from seeds to compare plant growth between the transgenic and WT lines. In addition, the cultivated transgenic switchgrass produced empty seed pods that were incapable of germinating in soil. To address this issue, we cut the tops of the plants and allowed them to regrow in soil enriched with nutrients, particularly nitrogen and phosphate. This approach has resulted in increased seed production, with seeds exhibiting robust filling. For future study, we will harvest the seeds and test their gemination potential.

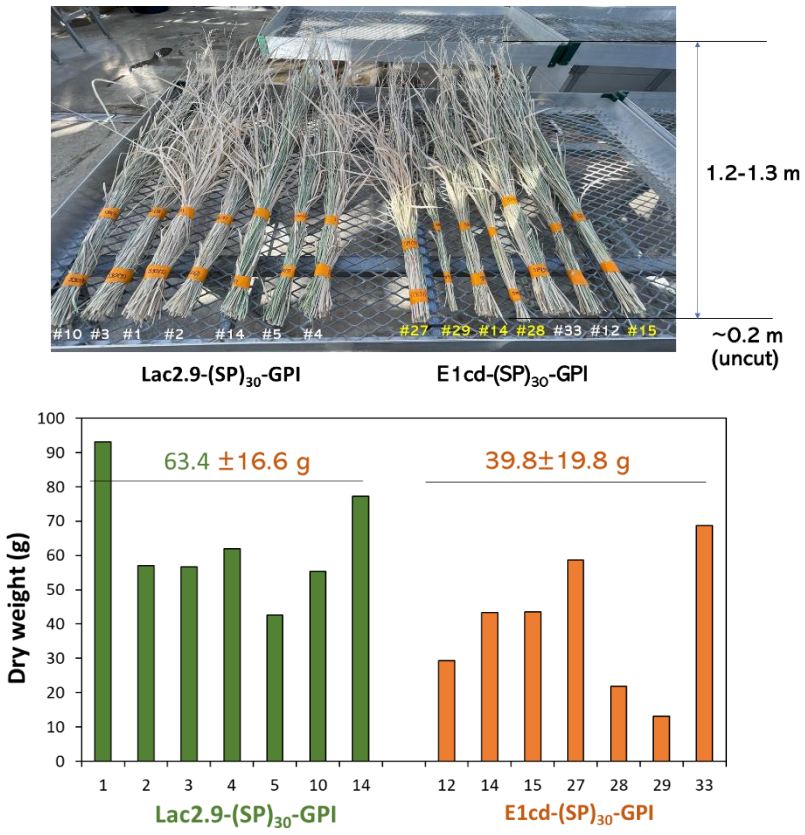

Figure 15. Biomass yields of transgenic switchgrass plants.

Task 2.2 Analysis of expressed laccase enzyme

Since the expressed Lac2.9-$(SP)_{30}$-GPI could not be detected using anti-6xHis Western blotting, the laccase enzyme activity retained in the switchgrass leaves was subsequently analyzed. In comparison to the transgenic switchgrass expressing the E1cd-$(SP)_{30}$-GPI (control), the Lac2.9-$(SP)_{30}$-GPI plant leaves exhibited a significant increase in enzyme activity of nearly two-fold **(Figure 16)**. This suggests successful expression of Lac2.9-$(SP)_{30}$-GPI in switchgrass. However, the anti-6xHis antibody failed to recognize the N-terminal 6xHis tag, likely due to its loss from the fusion protein.

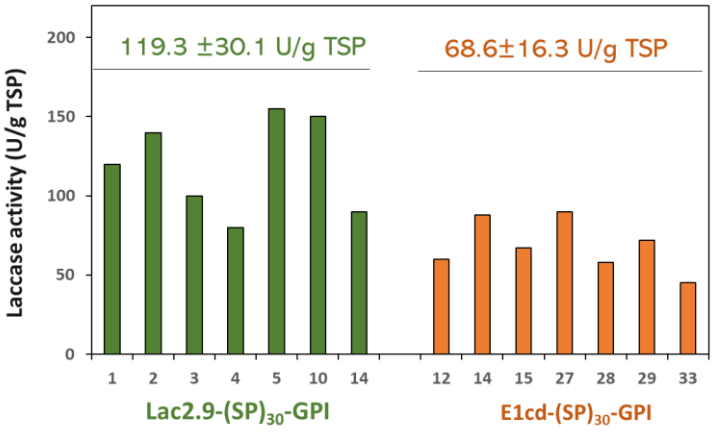

Figure 16. Laccase activity of transgenic switchgrass

Task 2.3 Evaluation of biomass digestibility of transgenic switchgrass

Biomass digestibility of transgenic switchgrass was evaluated *via* saccharification (**Figure 17**). As the WT control is currently unavailable, the saccharification efficiency between the two types of transgenic switchgrass lines was compared. The E1cd-$(SP)_{30}$-GPI exhibited a significantly higher saccharification efficiency than Lac2.9-$(SP)_{30}$-GPI plants. Interestingly, the enzymatic saccharification efficiency correlated with the enzyme accumulation level in the E1cd-$(SP)_{30}$-GPI plants. The lower saccharification efficiency detected in plants 12 and 33 corresponded to their lower enzyme accumulation levels **(Figure 13b)**. This finding supports the potential of genetic modification of switchgrass with strategically designed enzymes for improved biomass processibility.

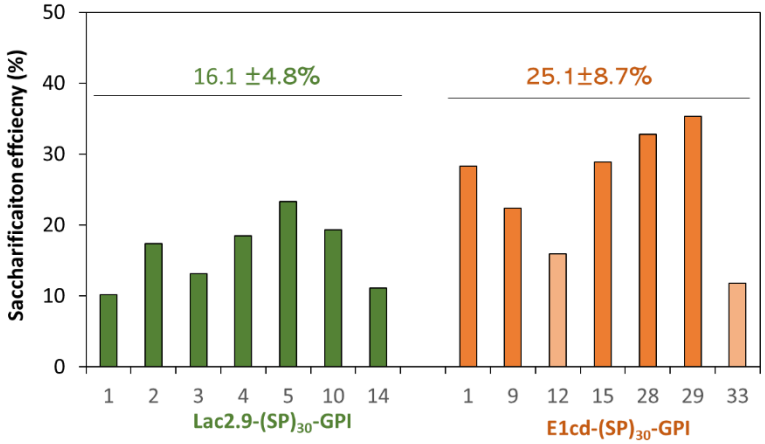

Figure 17. Saccharification efficiency of transgenic switchgrass

❖ ***Significant findings from Task 2***

- E1cd and Lac2.9 with functional modules, $(SP)_{30}$ and GPI anchor, were successfully expressed in switchgrass, respectively.
- The measured biomass yields of Lac2.9-$(SP)_{30}$-GPI plants are higher than those of E1cd-$(SP)_{30}$-GPI plants in average.
- The E1cd-(SP)30-GPI exhibited a significantly higher saccharification efficiency than Lac2.9-(SP)30-GPI plants.
- There are huge variations in biomass yield and saccharification efficiency among individual plants.

Task 3. TEA of PHA production from switchgrass

Process overview

Two models were developed for biomass to Polyhydroxyalkanoate (PHA) process using different biomass deconstruction technologies (baseline technology and proposed technology) as presented in **Figure 18**. Baseline technology is based on dilute-acid pretreatment and exogenous enzymatic deconstruction reported by NREL [2]. The proposed technology is a one-step self-deconstruction method based on in-planta enzyme engineering in this project.

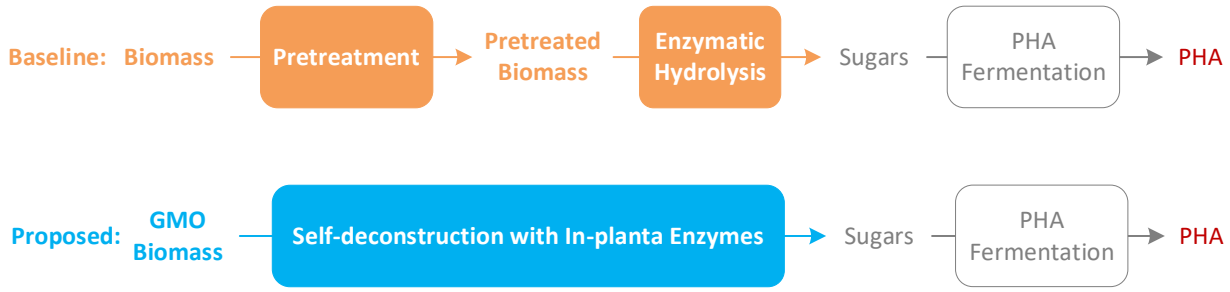

Figure 18. Comparison of different approaches for production of PHA from lignocellulose

Process design, cost, and profitability

(1) Biomass production

Switchgrass is native to North America from Canada southwards in the U.S. and Mexico. Switchgrass biomass production involves multiple activities, mainly including establishment and maintenance, seeding, soil fertility, weed control and harvesting. Biomass production cost is estimated using a fact sheet of switchgrass budget based on a 15-year timeline [3]. Average annual costs of those activities during the 15-year period are calculated and listed in Table 3. Soil fertility and harvesting contribute most of the biomass production cost (48% and 45% respectively) with minimal cost (<7%) for other activities (3.3% for establishment and maintenance, 1.9% for seeds and 1.5% for weed control) (**Table 3**). One strategy for reducing the fertility cost is to recover the nutrients (NPK) that are remained during the biomass to PHA process.

Table 3. Biomass production cost breakdown

Items	Values
Establishment and maintenance cost ($/acre/year)	9.48
Brush mowing ($/acre/year)	1.66
Moldboard plow ($/acre/year)	1.59
Disking ($/acre/year)	2.33
Soil finish ($/acre/year)	2.52
Drill ($/acre/year)	1.37
Seed cost ($/acre/year)	5.33
Seed Price ($/lb)	10
Seed Amount-15 year (lb/acre)	8
Soil fertility cost ($/acre/year)	135.58
N ($/acre/year)	35.84
P_2O_5 ($/acre/year)	16.13
K_2O ($/acre/year)	46.08
Fertilizer application ($/acre/year)	9.89
Lime ($/acre/year)	26.50
Soil testing ($/acre/year)	1.13
Weed control cost ($/acre/year)	4.36
Burndown ($/acre/year)	0.43
Postemergence ($/acre/year)	1.63
Sprayer ($/acre/year)	2.30
Harvesting cost ($/acre/year)	129.12
Mowing/conditioning ($/acre/year)	15.12
Baling (large, round) ($/acre/year)	114.00

Table 4 summaries the overall biomass production cost, revenue, and profit. A 1,000-acre farm was selected as the base case for growing Switchgrass based on a 15-year timeline. The biomass yields are expected to be 0 in the 1st year, 5 dry ton/acre/year for the 2nd year, and 7 dry ton/acre/year for the next 13 years (or an average yield of 6.4 dry-ton/acre/year) [3]. Therefore, the overall biomass production cost is estimated to be $44.35 per dry ton biomass or $283,868 per year (**Table 4**). The farm-gate price of switchgrass biomass is assumed to be $55.00 per dry ton biomass [3]. As a result, the net revenue of biomass would be $10.65 per dry ton biomass (**Table 4**). The biomass production profit is estimated to be $68.13 per acre per year (**Table 4**), which is much lower than those of growing corn ($449 per acre per year) or soybean ($442 per acre per year) [4]. Therefore, switchgrass can hardly be more competitive than corn or soybean in terms of profit, which could also limit growing switchgrass to margin lands that are not suitable for growing corn or soybean. For example, switchgrass could be more profitable than corn on land that produces less than 60 bushels per acre [5]. It should be noted that the biomass production profit is sensitive to the biomass price which could be higher (e.g., $95 per dry ton, up to $150 per dry ton or higher) [5]. Higher biomass price could make growing switchgrass much more competitive with increased profit of $324.13 per acre per year (up to $676.13 per acre per year) (**Table 4**). On the other hand, the increased price will affect the economics of biorefineries that use switchgrass biomass as feedstock for PHA production, which is also a driving force for using advanced technology to reduce other costs of biomass to PHA process.

Table 4. Biomass production cost, revenue, and profit

Items	Values		
Land area (acre)	1,000		
Switchgrass yield (dry ton/acre/year)	6.40		
Biomass production cost			
Biomass production cost ($/dry ton biomass)	44.35		
Biomass production cost ($/acre/year)	283.87		
Biomass production cost for 1,000 acres ($/year)	283,868		
Biomass production revenue with different prices			
Switchgrass price ($/dry ton biomass)	55.00	95.00	150.00
Biomass production revenue ($/acre/year)	352.00	608.00	960.00
Biomass production revenue for 1,000 acres ($/year)	352,000	608,000	960,000
Biomass production profit with different prices			
Biomass production profit ($/dry ton biomass)	10.65	50.65	105.65
Biomass production profit ($/acre/year)	68.13	324.13	676.13
Biomass production profit for 1,000 acres ($/year)	68,132	324,132	676,132

(2) Biomass to PHA

The base-line biomass deconstruction process involves multiple steps, mainly including deacetylation, dilute-acid treatment, conditioning, enzyme production and enzymatic hydrolysis (**Figure 19**) [2]. Briefly, acetate and other non-fermentable components in the biomass are solubilized and removed by an alkaline deacetylation step. Then, a dilute-acid treatment (sulfuric acid at a high temperature for a short time) is used to release hemicellulose sugars and partially break down the biomass. A pH neutralization step is conducted to adjust the pH of pretreated slurry to about 5 before the enzymatic hydrolysis. Cellulase enzyme prepared onsite is used for enzymatic hydrolysis. The pretreated slurry is partially hydrolyzed in a high-solids continuous reactor and transferred to one of several parallel batch reactors to complete the hydrolysis with a total hydrolysis time of 3.5 days. The hydrolyzed slurry is filtrated by a vacuum filter press to separate insoluble solids (lignin) from liquids (sugars). The solids fraction from the filter press is sent to a combustor and the liquid fraction is concentrated by an evaporator. The concentrated sugar liquid from the evaporator is cooled before using for PHA fermentation.

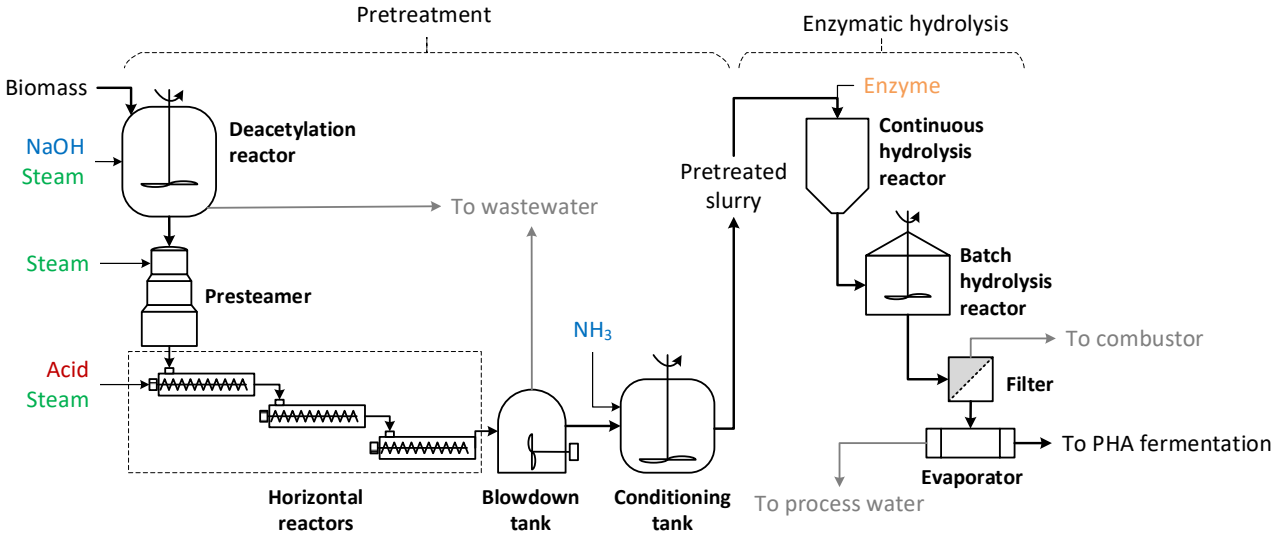

Figure 19. Flow diagram of base-line biomass deconstruction technology

In contrast, the proposed technology involves a one-step biomass deconstruction process, since the genetically engineered biomass contains both enzymes for lignin modification and cellulose hydrolysis (**Figure 20**). As a result, all steps from deacetylation to conditioning tank in the base-line process can be removed by using the genetically engineered biomass (**Figures 19 and 20**).

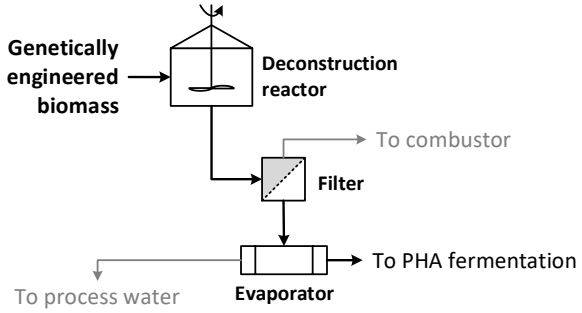

Figure 20. Flow diagram of proposed deconstruction technology (simultaneous pretreatment and enzymatic hydrolysis of genetically engineered biomass)

Table 5 compares conversion efficiencies and yields during biomass-to-PHA processes between baseline and proposed technologies. C6 and C5 contents are calculated from switchgrass composition that are average values of published results [6]. For the baseline technology, sugar recovery efficiencies are 100% (C6) - 95% (C5) during pretreatment process, and 90% (C6 and C5) during enzymatic hydrolysis [2]. For the proposed technology, sugar recovery efficiencies are expected to be at least 90% [2]. C6 to PHA yield of 0.40 kg/kg and C5 to PHA yield of 0.11 kg/kg are used for both baseline and proposed technologies [7]. Baseline and proposed technologies are expected to obtain similar PHA production rates of 966,135 kg/year and 973,751 kg/year, respectively (**Table 5**).

Table 5. Mass balance of PHA production from biomass

Items	Baseline	Proposed
Biomass supply		
Land area (acre)	1,000	
Biomass yield (dry ton/acre/year)	6.40	
Biomass composition		
C6 (%-dry)	39.3	
C5 (%-dry)	26.5	
Sugar production		
Overall C6 recovery (%)	90.0	90.0
Overall C5 recovery (%)	85.5	90.0
C6 recovery during pretreatment	100	n/a
C5 recovery during pretreatment	95	n/a
C6 recovery during hydrolysis	90	n/a
C5 recovery during hydrolysis	90	n/a
PHA production		
C6 to PHA yield (kg/kg)	0.40	
C5 to PHA yield (kg/kg)	0.11	
PHA production rate (kg/year)	966,135	973,751

Table 6 summarizes biomass to PHA production costs based on per kg of PHA with comparison between baseline and proposed technologies for biomass deconstruction. The two approaches have similar biomass costs which vary with the biomass process ($0.361-$0.364/kg PHA for $55 per dry ton; $0.624-$0.629/kg PHA for $95 per dry ton; $0.986-$0.994/kg PHA for $150 per dry ton) (**Table 6**).

Capital costs for biomass deconstruction are estimated based on values in the NREL report with adjustments using the 0.6 rule according to biomass flow rate [2]. Operational costs for biomass deconstruction are estimated also based on the NREL report [2]. Since the proposed deconstruction technology doesn't require pretreatment process and additional enzymes, the cost is reduced from \$0.664/kg PHA to \$0.177/kg PHA (**Table 6**).

PHA fermentation cost includes operational (chemical and labor) and capital costs. Chemical cost is \$0.249/kg PHA as reported previously [8]. Labor cost is \$0.421/kg PHA according to Leong et al., 2017 [9]. The operational cost is calculated to be \$0.670/kg PHA. Capital costs (15-year period) is estimated using the following equation based on PHA production rate [8]. Basically, the higher the PHA production rate, the lower the capital cost. Proposed biomass deconstruction technology would obtain a slightly higher PHA production rate (973,751 kg PHA/day) than the baseline technology (966,135 kg PHA/day) (**Table 3**). Therefore, the PHA fermentation cost (\$0.931/kg PHA) associated with the proposed technology is slightly lower than that of the baseline technology (\$0.932/kg PHA).

$$\text{Capital cost [\$/(kg PHA/year)]} = 36{,}438 \times [(\text{PHA production rate kg PHA/year})/1000]^{-0.324}/1000 \quad (1)$$

Using the baseline biomass deconstruction technology, biomass to PHA costs could be from \$1.961 to \$2.590/kg PHA, which is close to or higher than that of petroleum-based polymers (< \$2.0/kg) (**Table 6**). In contrast, the proposed biomass deconstruction technology could decrease the biomass to PHA cost to \$1.470/kg PHA (with a relatively low biomass price of \$55 per dry ton), or \$1.732/kg PHA (with a relatively high biomass price of \$95 per dry ton) (**Table 6**). As a result, the biomass-based PHA can possibly be competitive to petroleum-based polymers in terms of price, while still achieving an attractive profit up to \$516,466 per year (**Table 6**).

Table 6. Biomass-based PHA production cost, revenue, and profit

Items	Baseline	Proposed
Biomass price of \$55 per dry ton		
Feedstock cost (\$/kg PHA)	0.364	0.361
Deconstruction cost (\$/kg PHA)	0.664	0.177
PHA Fermentation cost (\$/kg PHA)	0.932	0.931
Biomass to PHA cost (\$/kg PHA)	1.961	1.470
PHA production cost (\$/year)	1,894,330	1,431,035
PHA production revenue at \$2/kg (\$/year)	1,932,269	1,947,501
PHA production profit at \$2/kg (\$/year)	37,939	516,466
Biomass price of \$95 per dry ton		
Feedstock cost (\$/kg PHA)	0.629	0.624
Deconstruction cost (\$/kg PHA)	0.664	0.177
PHA Fermentation cost (\$/kg PHA)	0.932	0.931
Biomass to PHA cost (\$/kg PHA)	2.225	1.732
PHA production cost (\$/year)	2,150,330	1,687,035
PHA production revenue at \$2/kg (\$/year)	1,932,269	1,947,501
PHA production profit at \$2/kg (\$/year)	-218,061	260,466
Biomass price of \$150 per dry ton		
Feedstock cost (\$/kg PHA)	0.994	0.986
Deconstruction cost (\$/kg PHA)	0.664	0.177
PHA Fermentation cost (\$/kg PHA)	0.932	0.931

Biomass to PHA cost ($/kg PHA)	2.590	2.076
PHA production cost ($/year)	2,502,330	2,039,035
PHA production revenue at $2/kg ($/year)	1,932,269	1,947,501
PHA production profit at $2/kg ($/year)	-570,061	-91,534

Figure 21 further points out the advantage of the proposed technology over the baseline technology. Compared to the baseline technology, the proposed technology doesn't need multiple steps or separated reactors for pretreatment and enzymatic hydrolysis, and doesn't need additional enzymes, which reduces the biomass deconstruction cost from 33% to 9% of PHA revenue (**Figure 21**).

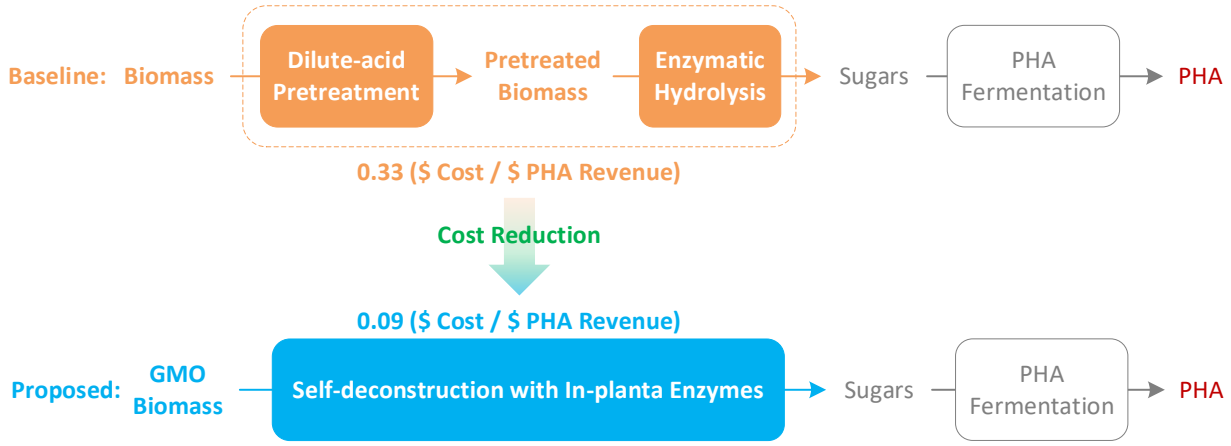

Figure 21. Comparison of different approaches for production of PHA from lignocellulose

❖ ***Significant findings from Task 3***

- Switchgrass biomass production cost is mainly (>93%) attributed to by fertility and harvesting. Biomass production profit can increase up to 10-fold (from $68K to $676K per year based on 1,000 acre) depending on biomass price (from $55 to $150 per dry ton biomass). The PHA production profit is also sensitive to the biomass price.
- Using the proposed biomass deconstruction technology, the PHA production profit can be up to $260K-$516K per year about 13%-26% of the PHA production revenue.
- The proposed technology could potentially reduce the biomass deconstruction cost from 33% to 9% of PHA revenue, making the biomass-based PHA competitive to petroleum-based polymers even in case of relatively high biomass price of $95 per dry ton biomass.

Conclusions

In-planta enzyme engineering was validated in both tobacco and switchgrass plants, with improved enzyme activity and saccharification efficiency. In-planta engineering of Lac2.9 is more challenging than E1-cd, in terms of expression detection. Lac2.9-(SP)30-GPI and E1cd-(SP)30-GPI improved biomass yield and saccharification efficiency of switchgrass, respectively. It is promising to express both genes in switchgrass for optimized overall performance. Cultivation of the genetically engineered self-deconstruction switchgrass for PHA production could benefit switchgrass grower and PHA producer with attractive profits for both sectors.

c. What opportunities for training and professional development has the project provided?

Participating students at the Arkansas State University has been trained in the study of biofuels, plant molecular biology, *Agrobacterium*-mediated plant transformation, Western blotting, enzyme activity analysis, and enzymatic saccharification of plant biomass. These students were also provided with the opportunity to present the results at national and local meetings.

d. How have the results been disseminated to communities of interest?

The teams are working on manuscripts for peer-reviewed journal articles. The PI and Co-PI will also present the work at professional conferences and workshops.

e. What do you plan to do during the next reporting period to accomplish the goals and objectives?

No.

III. PRODUCTS

We have one manuscript under development for journal publication.

IV. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Dr. Xumeng Ge (PI) at Quasar Energy Group (QEG) has been working on project activity management for 3.3 months. Dr. Yebo Li at QEG has worked on TEA for 2.7 months. Mr. Steve Smith, VP for project development at QEG, has worked on commercialization plan for 0.1 month. Dr. Wachiranon Chuenchart at QEG has worked on biomass characterization and data analysis for 0.3 months. Joshua Andre, Jacob Epifano and Daniel Redick at QEG have worked on sample handling for 0.2 months in total.

Dr. Jianfeng Xu (Co-PI), Professor at Arkansas State University (ASU), worked on the design and construction of expression vectors for tobacco and switchgrass transformation while overseeing project activities at A-State for 1 month annually. Dr. Xu also supervised undergraduate student Berry Dick to work on the planting of transgenic plants in greenhouse, detection of transgene expression, and harvesting plant biomass and seeds for 12 months, supervised undergraduate student Daniela PerezLaguna to work on biomass yields detection and enzymatic saccharification for 8 months, and supervised undergraduate student Jonathan Trejo-Martinez to work on assaying enzyme activity and its sensitivity to pH and temperature for 6 months. Uddhab Karki, a research associate at ASU, conducted research on molecular cloning and recombinant enzyme expression for 3 months.

Collaborated with individual in foreign country: No.

Traveled to foreign country: No.

V. IMPACT

The development of novel enzyme optimization approaches represents a technological leap forward in the field of bio-industries. This advancement can pave the way for more efficient and sustainable processes in converting energy crops into renewable products. The transition from food crops to energy crops as raw materials aligns with broader sustainability goals by reducing

competition for agricultural land and resources. Additionally, the production of renewable products from energy crops helps mitigate greenhouse gas emissions and contributes to overall environmental stewardship. The project contributes to the goals of the Biden Administration, particularly in achieving net-zero emissions by 2050, underscores its relevance and importance within the current policy landscape. Progress in this project can support broader policy initiatives aimed at transitioning to a clean energy future.

VI. CHANGES/PROBLEMS

Gene construction and transformation in switchgrass was much longer than expected, which delayed the research progress.

VII. DEMONGRACHIC INFORMATION

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VIII. SPECIAL REPORTING REQUIREMENTS

No

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Appendix 1

E1cd-$(SP)_{30}$-GPI sequence

CCCCTTCACCGGATCCGCAATGGGAAAAATGGCTTCTCTATTTGCCACATTTTTAGTGGTTTTAGTGTCA
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CACCCTCACCCTCACCATCTCCTTCGCCATCACCCTCACCCTCACCATCTCCTTCGCCATCACCCTCACC
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Lac2.9-$(SP)_{30}$-GPI sequence

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