Final Technical Report

Project Title: Engineering yeast consortia for surface-display of complex cellulosome structures

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Executive Summary

As our society marches toward a more technologically advanced future, energy and environmental sustainability are some of the most challenging problems we face today. Biomass is one of the most abundant renewable-feedstock for sustainable production of biofuels. However, the main technological obstacle to more widespread uses of this resource is the lack of low-cost technologies to overcome the recalcitrant nature of the cellulosic structure, especially the hydrolysis step on highly ordered celluloses. In this proposal, we successfully engineered several efficient and inexpensive whole-cell biocatalysts in an effort to produce economically compatible and sustainable biofuels, namely cellulosic ethanol. Our approach was to display of a highly efficient cellulolytic enzyme complex, named cellulosome, on the surface of a historical ethanol producer *Saccharomyces cerevisiae* for the simultaneous and synergistic saccharification and fermentation of cellulose to ethanol.

We first demonstrated the feasibility of assembling a mini-cellulosome by incubating *E. coli* lysates expressing three different cellulases. Resting cells displaying mini-cellulosomes produced 4-fold more ethanol from phosphoric acid-swollen cellulose (PASC) than cultures with only added enzymes. The flexibility to assemble the mini-cellulosome structure was further demonstrated using a synthetic yeast consortium through intracellular complementation. Direct ethanol production from PASC was demonstrated with resting cell cultures. To create a microorganism suitable for a more cost-effective process, called consolidated bioprocessing (CBP), a synthetic consortium capable of displaying mini-cellulosomes on the cell surface via intercellular complementation was created. To further improve the efficiency, a new adaptive strategy of employing anchoring and adaptor scaffoldins to amplify the number of enzymatic subunits was developed, resulting in the creation of an artificial tetravalent cellulosome on the yeast surface and a significant improvement in cellulosic ethanol production. Although this adaptive strategy is ideal for assembling more complex cellulosome for large-scale production of cellulosic ethanol, a substantially larger number of enzymes (up to 10 to 12) is needed to better mimic the natural cellulosome structures for practical usage of the technology.

The objective of this project is to develop a synthetic yeast consortium for direct fermentation of cellulose to ethanol with productivity, yield, and final concentration close to that from glucose fermentation.

Task A1. Secretion of individual dockerin-tagged cellulases
Actual Accomplishments: Demonstrate the successful secretion of dockerin-tagged endoglucanase, exoglucanase and β-glucosidase from three different yeast strains by detecting enzyme activity in the culture medium.

Task A2: Formation of a mini-cellulosome on the yeast surface by a four-population mixed culture
Actual Accomplishments: A synthetic consortium composed of four different yeast strains either displaying the mini-scaffoldin or secreting one of the three enzymes was used to demonstrate the formation of a functional cellulosome structure by intercellular

complementation. The correct assembly of all three cellulases was confirmed by immunofluorescence microscopy.

Task A3. Hydrolysis of cellulose and ethanol production using the mixed population
Actual Accomplishments: By changing the initial inoculation ratios of the different populations, we were able to optimize the presentation of functional cellulosomes in the synthetic consortium. The optimized consortium hydrolyzed PASC and produced 2-fold more ethanol than the initial equal ratio consortium.

Task B1. Construct a yeast strain displaying the anchoring scaffoldin
Actual Accomplishments: An anchoring scaffold composed of two dockerin-cohesin domains from A. cellulolyticus (Ac) and B. cellulosolvens (Bc) was successfully displayed onto the yeast surface. Surface localization of the anchoring scaffoldin was confirmed by immunofluorescence microscopy.

Task B2. Secretion and assembly of adaptor scaffoldins
Actual Accomplishments: We have successfully expressed two adaptor scaffoldins composed of either the dockerin domain from Ac or Bc, flanked by two cohesin domains (f and t) and one cellulose-binding domain (CBM). The resulting adaptors retained the cellulose binding ability as demonstrated by their binding to Avicel. More importantly, the respective dockerin domain on the two adaptor scaffolds was fully functional as demonstrated by the binding of the two adaptors to yeast cells displaying the anchoring scaffoldin (Ac-Bc). Interaction of the two dockerin/cohesin pair was very strong as 100% binding was detected in both cases.

Task B3. Assembly of complex cellulosome
Actual Accomplishments: The functionality of each cohesin on the adaptor was confirmed by docking the endoglucanase CelG-dockerin t (Gt) and the β-glucosidase BglA tagged with a dockerin f (Bglf) onto the adaptor. PASC hydrolysis by resting cells was demonstrated, confirming the functionality of the two enzymes.

Task C1. Hydrolysis of cellulose using the complex cellulosome
Actual Accomplishments: Yeast cells displaying the complete tetravalent cellulosome structure in which enzymes were recruited into both adaptors exhibited a 4-fold increase in ethanol production compared with free enzymes and a 2-fold increase compared to cells displaying only a divalent cellulosome structure. This result again validates the synergistic effect of enzyme proximity on cellulose hydrolysis.

Conclusion: Collectively, our results demonstrated a potentially useful approach to reduce the cost of enzyme hydrolysis by using our synthetic yeast consortia. To make this approach practical, a significantly higher number of enzymes must be assembled. This will require the careful coordination of enzyme and adaptor synthesis based on substrate availability.

Summary of Project Activities

Task A. Surface display of a functional mini-cellulosome by intercellular complementation using a synthetic yeast consortium

In this task, we reported the surface assembly of a functional mini-cellulosome using a synthetic yeast consortium. The basic design of the consortium consisted of four different engineered yeast strains capable of either displaying a trifunctional scaffoldin Scaf-ctf (SC) carrying three divergent cohesin domains from *Clostridium thermocellum* (t), *Clostridium cellulolyticum* (c) and *Ruminococcus flavefaciens* (f) or secreting one of the three corresponding dockerin-tagged cellulases (endoglucanase (AT), exoglucanase (EC/CB) or β-glucosidase (BF)) (Fig. 1). The secreted cellulases were docked onto the displayed Scaf-ctf in a highly organized manner based on the specific interaction of the three cohesin-dockerin pairs employed, resulting in the assembly of a functional mini-cellulosome on the yeast surface. By exploiting the modular nature of each population to provide a unique building block for the mini-cellulosome structure, the overall cellulosome assembly, cellulose hydrolysis, and ethanol production were easily fine-tuned by adjusting the ratio of different populations in the consortium (Fig. 2). The optimized consortium consisted of a SC:AT:CB:BF ratio of 7:2:4:2 produced almost twice the level of ethanol (1.87 g/L) than a consortium with an equal ratio of the different populations (Fig. 2E). The final ethanol yield of 0.475 g ethanol/g cellulose consumed also corresponded to 93% of the theoretical value. This result confirms the use of a synthetic biology approach for the synergistic saccharification and fermentation of cellulose to ethanol using a yeast consortium displaying a functional mini-cellulosome.

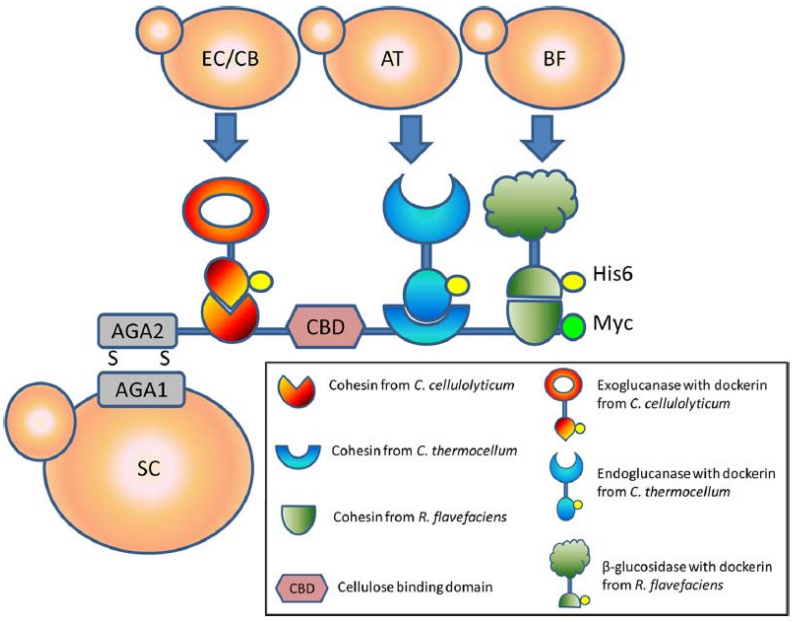
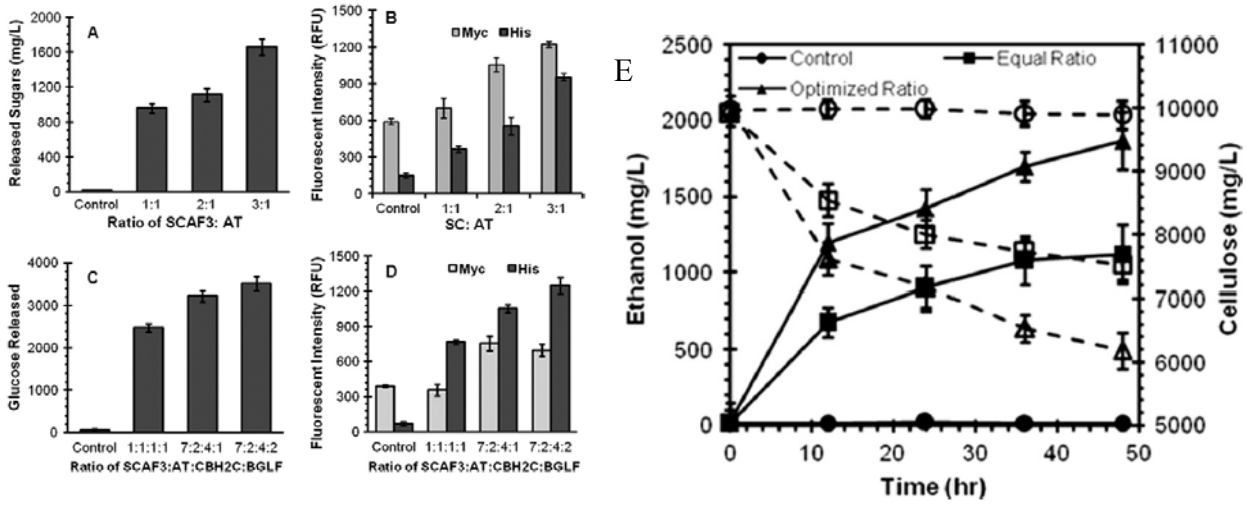


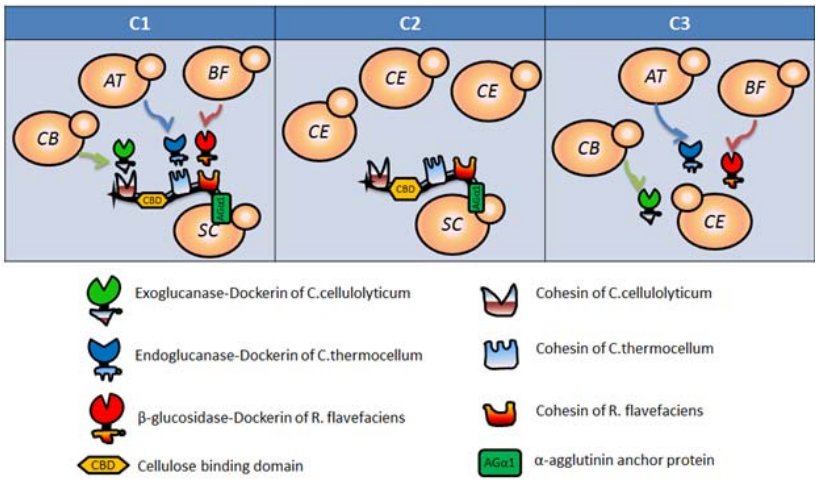
Figure 1. Surface assembly of a functional mini-cellulosome through intracellular complementation using a synthetic yeast consortium. The basic design consisted of four different engineered yeast strains capable of either displaying a trifunctional scaffoldin Scaf-ctf (SC) or secreting one of the three corresponding dockerin-tagged enzymes (endoglucanase (AT), exoglucanase (EC/CB) or β-glucosidase (BF)).

Figure 2. Systematic optimization of different cell populations in the synthetic consortium. Effects of SC to AT ratio on (A) reducing sugars production and (B) At binding. (C) Effects of SC:AT:CB:BF ratio on (C) glucose production and (D) enzyme binding. Enzyme binding was determined by immunofluorescence microscopy. Cells were probed with either anti-C-myc or anti-C-His6 sera and fluorescently stained with a goat anti-mouse IgG conjugated with Alexa Fluor 488. Whole cell fluorescence was determined using a fluorescent microplate reader. (E) Cellulose hydrolysis (dashed line) and ethanol production (solid line) from PASC by the optimized consortium.



Task B. Simultaneous cell growth and ethanol production from cellulose by an engineered yeast consortium displaying a functional mini-cellulosome

In this task, we engineered a yeast consortium capable of displaying a functional mini-cellulosome for the simultaneous growth and ethanol production on phosphoric acid swollen cellulose (PASC). A yeast consortium composed of four different populations was engineered to display a functional mini-cellulosome containing an endoglucanase, an exoglucanase and a β-glucosidase. The resulting consortium was demonstrated to utilize PASC for growth and ethanol production. The final ethanol production of 1.25 g/L corresponded to 87% of the theoretical value and was 3-fold higher than a similar yeast consortium secreting only the three cellulases (Fig. 3). Quantitative PCR was used to enumerate the dynamics of each individual yeast population for the two consortia (Fig. 4). Results indicated that the slight difference in cell growth cannot explain the 3-fold increase in PASC hydrolysis and ethanol production. Instead, the substantial increase in ethanol production is consistent with the reported synergistic effect on cellulose hydrolysis using the displayed mini-cellulosome.



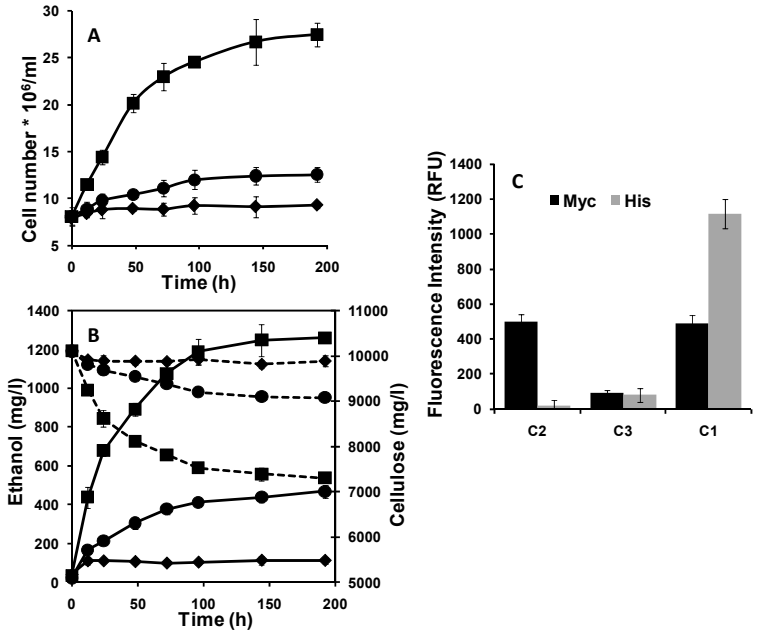


Figure 3. A schematic of the different consortia used in this study. Cell growth and ethanol production by the cell consortia. (A) Cell growth and (B) PASC hydrolysis (dotted line) and ethanol production (solid line) by the different yeast consortia, i.e., consortium C2 without secreting enzymes (♦), consortium C3 only secreting enzymes (●) and consortium C1 forming the cellulosome structure (■). (C) Surface display of the mini-cellulosome was probed with either anti-C-myc sera for the displayed scaffoldin or anti-C-His6 sera for the three cellulases docked on the scaffoldin and fluorescently stained with a goat anti-mouse IgG conjugated with Alexa Fluor 488. Whole cell fluorescence was determined using a fluorescent microplate reader. Data shown are the mean values (± standard deviation) obtained from 3 independent experiments.

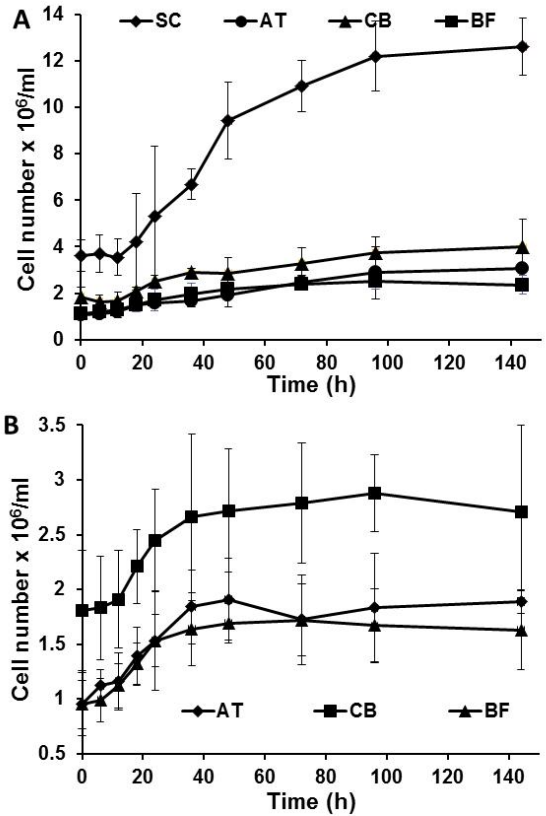


Figure 4. Growth dynamics of individual populations in (A) consortium C1 that could form cellulosome structure and (B) consortium C3 that can only secret enzymes. Changes in cell number of individual yeast populations were probed by qPCR. Data shown are the mean values (± standard deviation) obtained from 3 independent experiments.

Task C. Functional display of complex cellulosomes on the yeast surface via adaptive assembly

In this task, a new adaptive strategy was developed for the *ex vivo* assembly of a functional tetravalent designer cellulosome on the yeast cell surface. The design is based on the use of (1) a surface-bound anchoring scaffoldin composed of two divergent cohesin domains, (2) two dockerin-tagged adaptor scaffoldins to amplify the number of enzyme loading sites based on the specific dockerin-cohesin interaction with the anchoring scaffoldin, and (3) two dockerin-tagged enzymatic subunits (the endoglucanse Gt and the β-glucosidase Bglf) for cellulose hydrolysis (Fig. 5). Cells displaying the tetravalent cellulosome on the surface exhibited a 4.2-fold enhancement in the hydrolysis of phosphoric acid swollen cellulose (PASC) than free enzymes (Fig. 6). More importantly, cells displaying the tetravalent cellulosome also exhibited a ~2-fold increase in ethanol production than cells displaying a divalent cellulosome using a similar enzyme loading. These results clearly indicate the more crucial role of enzyme proximity than just simply increasing the enzyme loading on the overall cellulosomal synergy. To the best of our knowledge, this is the first report that exploits the natural adaptive assembly strategy in creating artificial cellulosome structures. The unique feature of the anchoring and the adaptor scaffoldin strategy to amplify the number of enzymatic subunits can be easily extended to more complex cellulosomal structures to achieve an even higher level of enzyme synergy.

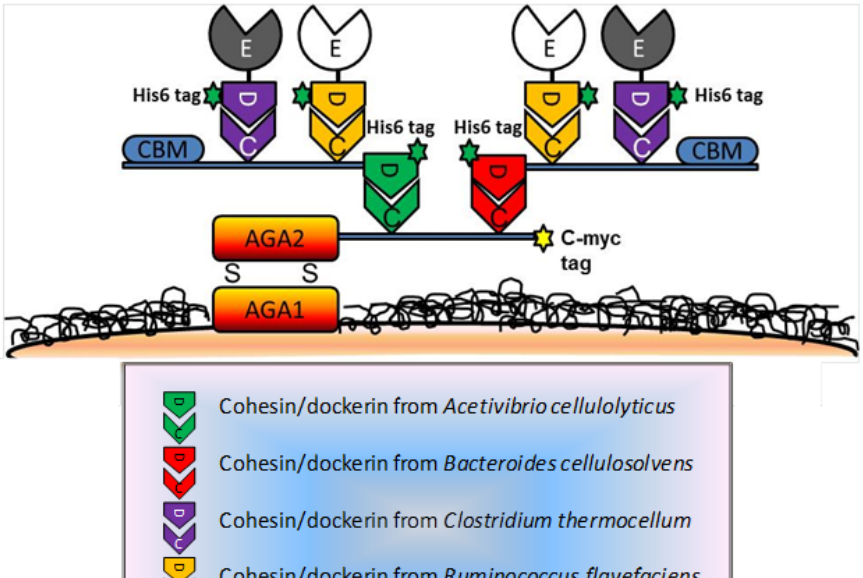


Figure 5. A schematic diagram of the complex cellulosome assembled on the yeast surface using the adaptive assembly. The two adaptor scaffoldins served as templates for enzyme recruitment to the yeast surface via specific interaction with the surface-displayed anchoring scaffoldin.

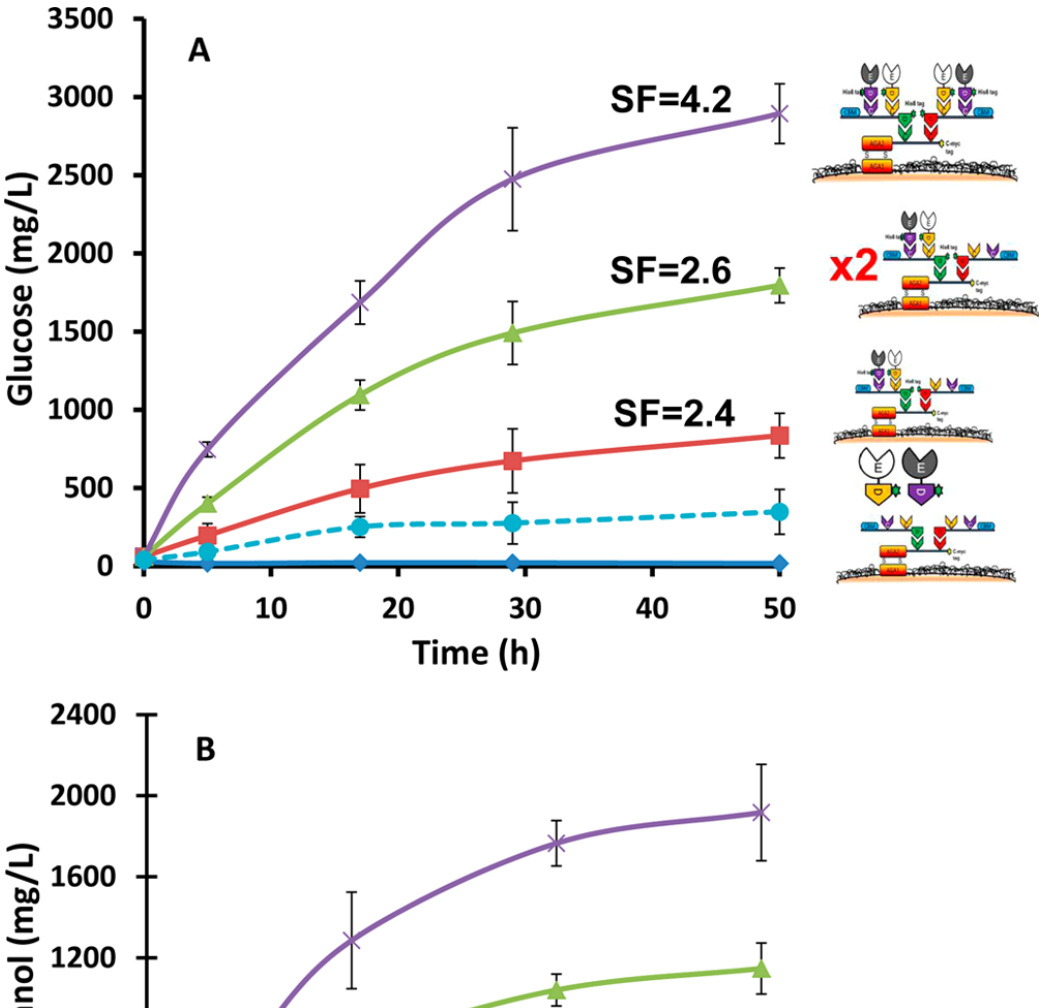


Figure 6. Production of glucose (A) and ethanol (B) from PASC using yeast cells displaying different cellulosome structures. (♦) S1 – no enzymes, (■) S2 – 50% cells composed of the divalent cellulosome, (▲) S3 – 100% cells composed of the divalent cellulosome, (x) S4 – 50% cells composed of the tetravalent cellulosome, (●) the same amount of free enzymes as in S2. Other than S3, the rest of the population was composed of cells displayed only the anchoring scaffoldin AnScf. The synergy factor (SF), cellulosome activity/free enzyme activity, for each cellulosome structure is listed.

Publications

1. Shen-Long Tsai, Miso Park, **Wilfred Chen**, Size-modulated synergy of cellulase clustering for enhanced cellulose hydrolysis, *Biotechnol. J*., **8**, 257–261, 2013.

2. Shen-Long Tsai, Nancy A. DaSilva, and **Wilfred Chen**, Functional display of complex cellulosomes on the yeast surface via adaptive assembly, *ACS Synthetic Biology*, **2**, 14-21, 2013.

3. Garima Goyal, Shen Long Tsai, Bhawna Madan, Nancy A. DaSilva, and **Wilfred Chen**, Simultaneous cell growth and ethanol production from cellulose by an engineered yeast consortium displaying a functional mini-cellulosome, *Microb. Cell Factories*, **10**, 89, 2011.

4. Shen-Long Tsai, Garima Goyal, and **Wilfred Chen**, Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production, *Appl. Environ. Microbiol*., **76**, 7514-7520, 2010.

5. Shen-Long Tsai, Jeongseok Oh, Shailendra Singh, Ruizhen Chen**,** and **Wilfred Chen**, Functional assembly of mini-cellulosomes on the yeast surface for cellulose hydrolysis and ethanol production, *Appl. Environ. Microbiol*., **75**, 6087-6093, 2009.

Inventions/Patent Applications, licensing agreements:

1. Engineering of yeast for cellulosic ethanol production
   United States Patent Application 20110306105-A1