รายงานวิจัยฉบับสมบูรณ์

โครงการ

การพัฒนากระบวนการผลิตเอทานอลจากสารชีวมวลที่มีประสิทธิภาพ โดยใช้เทคนิคการเลี้ยงยีสต์ผสมและแบบจำลองทางจลศาสตร์

Development, optimization and scale-up of a yeast co-culture system using kinetic model for an efficient production of lignocellulosic ethanol

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สัญญาเลขที่ **RSA5880060**

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Development, optimization and scale-up of a yeast co-culture system using kinetic model for an efficient production of lignocellulosic ethanol

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Abstract

The utilization of both C_6 and C_5 sugars is required for economical lignocellulose-based processes. A co-culture system containing multiple strains of the same or different organisms holds promise for conversion of the sugar mixture available in different lignocellulosic feedstock into ethanol. Herein a co-culture kinetic model has been developed to describe *S. stipitis* and *S. cerevisiae* co-cultivation for ethanol fermentation of glucose-xylose mixture. The co-culture kinetic model was implemented to design optimal cell ratios for efficient conversion of lignocellulosic feedstock into ethanol. Ethanol titer and productivity reached by optimized co-culture in batch process was 46 g/L and 0.49 g/L-hr respectively, approximately 30% improvement compared to single-strain cultures. Fed-batch process by the optimized yeast consortium achieved a maximum ethanol titer of 60 g/L and >70% in ethanol yield. Technological and economical potentials of sugarcane bagasse-to-ethanol process using *S. stipitis/S. cerevisiae* consortium were also investigated. Process sensitivity analyses revealed the potentials for further cost reduction up to 44% by reducing enzyme loading and increasing ethanol titer. Hence, the co-culture kinetic modelling described here provided a systematic strategy for designing the optimal cell ratio of yeast consortium, leading to efficient fermentation of the C_6/C_5 sugars available in any biomass feedstock. This study also provided an economically viable prototype for high-titer lignocellulosic ethanol production using *S. stipitis/S. cerevisiae* co-culture which offered better economic and sustainability value than starch-based ethanol production process.

Keywords: Co-culture kinetic model, Yeast consortium technology, Second generation bioethanol, High-solid fed-batch process, Techno-economic analysis

บทคัดย่อ

โครงการวิจัยนี้ทำการศึกษาการเพาะเลี้ยงยีสต์ผสมเพื่อเปลี่ยนน้ำตาล C5 และ C6 ที่ได้จากสารชีวมวลไปเป็นเอทานอล โดยงานวิจัยนี้ได้ใช้แบบจำลองทางจลศาสตร์ของยีสต์ผสม *S. stipitis* และ *S. cerevisiae* เพื่อออกแบบสภาวะการเพาะเลี้ยงยีสต์ผสมและอัตราส่วนของยีสต์แต่ละสายพันธุ์ที่อยู่ในยีสต์ผสมสำหรับการผลิตเอทานอลที่มีประสิทธิภาพ การผลิตเอทานอลด้วยยีสต์ผสมทำให้ได้เอทานอลที่ความเข้มข้น 46 กรัมต่อลิตร และอัตราการผลิตเอทานอลที่ 0.49 กรัมต่อลิตรต่อชั่วโมง ในการหมักแบบกะ ซึ่งมีประสิทธิภาพมากกว่าการหมักแบบกะที่ใช้ยีสต์สายพันธุ์เดี่ยวมากถึง 30% การหมักเอทานอลแบบกึ่งกะด้วยยีสต์ผสมให้เอทานอลที่ความเข้มข้น 60 กรัมต่อลิตร และผลผลิตที่ >70% การคำนวณทางเทคนิคและทางเศรษฐศาสตร์ของกระบวนการแปรรูปเอทานอลจากสารชีวมวลโดยใช้ยีสต์ผสมพบว่าต้นทุนการผลิตเอทานอลจากสารชีวมวลลดลงมากถึง 44% เมื่อเทียบกับต้นทุนการผลิตในกรณีพื้นฐาน

คำสำคัญ: เทคนิคการเพาะเลี้ยงยีสต์ผสม, การหมักเอทานอลจากชีวมวล, แบบจำลองทางจลศาสตร์ของยีสต์ผสม, การคำนวณทางเทคนิคและทางเศรษฐศาสตร์

Executive Summary

Achieving economical lignocellulose-based bioprocess requires efficient utilization of C_6 and C_5 sugar mixture present in biomass feedstock. In this study, we have implemented a model-based strategy to rationally design an optimized *S. stipitis/S. cerevisiae* yeast consortium capable of efficiently converting glucose-xylose mixture into ethanol. The *S. stipitis/S. cerevisiae* co-culture kinetic model was applied to systematically assess ethanol fermentation kinetics under different cell ratio and to predict the optimal cell ratio for maximized ethanol production from different biomass feedstocks. The results proved the efficiency of optimized co-culture based on the model-based design for increasing ethanol titer and reducing fermentation time. The adjustability of co-culture was also an appealing characteristic permitting an efficient fermentation of all types of lignocellulosic biomass feedstock by varying co-culture cell ratio to match with the composition of sugar mixture available. Techno-economic analysis for lignocellulosic ethanol production by yeast consortium was assessed using a fully integrated process flowsheeting model, showing the fed-batch yeast co-culture with minimal ethanol selling price of 26.7 baht/L-ethanol. Hence, the systematic approach based on co-culture kinetic model is useful for guiding yeast consortium design and optimization efforts aimed at rapidly improving efficiency of ethanol fermentation from various lignocellulosic biomass feedstocks. This study also demonstrated an economically viable prototype for lignocellulosic ethanol production using *S. stipitis/S. cerevisiae* yeast co-culture.

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measured values while the lines represent predicted values based on the co-culture kinetic model. For comparison purpose, all experiments are initiated with the same total cell concentration of 0.02 g-cell/g-WIS. The optimized cells ratio for the co-culture as predicted by the model is f_{opt} = 1.94 for sugarcane bagasse.

Figure 6. Effect of yeast cell addition on sugarcane bagasse-to-ethanol conversion. Ethanol titer (A), yield and productivity (B) after 48 hrs of simultaneous saccharification and fermentation of sugarcane bagasse by simultaneous addition of *S. stipitis/S. cerevisiae* and by sequential addition of *S. cerevisiae* followed by every 12 hrs feeding of *S. stipitis* for 48 hrs. The productivity is present as maximum production rate of ethanol within the first 48 hrs. The ethanol yield is based on total ethanol produced per total sugars available.

Figure 7. Ethanol production from sugarcane bagasse in batch and fed-batch saccharification followed by fermentation of *S. stipitis/S. cerevisiae* co-culture. (A) Solid feed profile during fed-batch enzyme hydrolysis at high solid load. Shown are percent of accumulated solid load (dashed line) and accumulated water insoluble solid WIS (solid line) yielding final accumulated sugars at 135.56±5.54 g/l. (B) Ethanol fermentation of sugarcane bagasse hydrolysates obtained from batch or fed-batch by *S. stipitis/S. cerevisiae* consortium. Consumed sugars (triangle) and produced ethanol (diamond) in shake flask batch (green) and shake flask fed-batch process are compared.

Figure 8. Ethanol fermentation of sugarcane bagasse hydrolysates by fed-batch *S. stipitis/S. cerevisiae* consortium in scale-up bioreactor (blue) compared to small-scale shake flask (purple). Consumed sugars (triangle) and produced ethanol (diamond) are shown.

Figure 9. Process diagram for optimal process of fed-batch saccharification followed by fermentation (fed-batch SHF) by *S. stipitis/S. cerevisiae* yeast consortium. The process configuration includes diluted-acid pretreatment (in red dashed square), yeast propagation (in purple dashed square), enzyme hydrolysis and co-fermentation (in green dashed square) steps for the conversion of sugarcane bagasse to ethanol. Detailed downstream processing for ethanol recovery was described in Wingren et al., 2008.

Figure 10. Summary of mass and utility requirements for the conversion of sugarcane bagasse to ethanol using optimized fed-batch saccharification and ethanol fermentation (fed-batch SHF) of

S. stipitis/S. cerevisiae yeast consortium. All values are based on per 1 dry ton sugarcane bagasse.

Figure 11. Cost distribution of optimized fed-batch saccharification followed by fermentation using *S.stipitis/S.cerevisiae* yeast consortium. The values are based on upstream processing only.
Figure 12. Process sensitivity analysis examining impact of enzyme dosage and final ethanol titer on the minimum ethanol selling price (MESP) for fed-batch enzyme hydrolysis and fermentation with *S.stipitis/S.cerevisiae* yeast consortium. Dashed line represents current ethanol selling price from casava-based process (the 2016 average selling price of ethanol in Thai market, www.thaiethanol.com) for comparison purpose.

1. Introduction to the research problem and its significance

The economy, energy security and global climate change are the major factors in favor of an alternative renewable energy policy such as the use of ethanol as automotive fuel in Thailand. Due to insufficient quantities and high price of starch feedstock, it is essential for ethanol production to move away from food feedstock into renewable, plentiful and inexpensive non-food feedstock such as lignocellulosic biomass. Low-priced, abundant and renewable lignocellulosic biomass has become an attractive alternative feedstock to significantly supplement starch as a fermentation feedstock for production of biofuels and biochemicals (Neureiter et al., 2004; Ragauskas et al., 2006). It has been estimated that Thailand produced lignocellulosic biomass from agricultural residues (i.e., rice straw; sugarcane bagasse) approximately 12 million ton/year. Current available quantities of lignocellulose can potentially produce an approximated 300 billion liters of ethanol/year (Slininger et al., 2006; Binod et al., 2010). Although lignocellulosic biomass has many desirable features as an alternative feedstock, the conversion process of biomass into ethanol is challenging.

The hydrolysis of lignocellulosic materials releases a mixture of hexoses (glucose, galactose, mannose) and pentoses (xylose, arabinose) that must be converted by organisms into ethanol. From an economic point of view, a complete and efficient utilization of these lignocellulose-derived sugars both hexoses and pentoses is required for maximizing the profitability of an industrial ethanol production process. However, most organisms, including *Saccharomyces cerevisiae*, *Escherichia coli*, and *Zymomonas mobilis*, currently used in the production of ethanol either are unable to utilize pentoses or consume hexoses and pentoses inefficiently which lead to a prolonged fermentation time for a complete utilization of all sugars. Therefore, it is desirable to design a system of organisms that can ferment all hexoses and pentoses efficiently into ethanol in the shortest fermentation time. We have a hexose-fermenting yeast strain, *Saccharomyces cerevisiae* (e.g. Thermosacc) and a pentose-fermenting yeast strain, *Scheffersomyces stipitis* (e.g. CBS6054 or BCC15191). *S. cerevisiae* and *S. stipitis* are able to produce ethanol from hexose sugars (i.e., glucose) and pentose sugars (i.e., xylose) respectively at relatively high yield. Based on our previous study, these strains are capable of producing ethanol at an approximated yield of 80-90% of the theoretical yield (0.51 g-ethanol/g-sugar). As a result, they are potentially of interest as a biological catalyst system in conversion of hexoses and pentoses from lignocellulosic biomass into ethanol.

The most commonly used co-culture is the combination of *S. stipitis* with *S. cerevisiae* which has been demonstrated as a strategy for efficient conversion of glucose and xylose. The use of *S.stipitis/S.cerevisiae* co-culture has previously shown to enhance ethanol production at a faster rate and a higher titer than single strain culture (Yadav et al., 2011; Li et al., 2011; Suriyachai et al., 2013; Hickert et al., 2013). The fermentation performance of *S. stipitis/S. cerevisiae* co-culture for lignocellulosic ethanol production is strongly dependent on the cell ratio of the two strains. Although, in co-culture, the overall fermentation kinetics can be optimized by varying the relative proportion of each strain in the culture, it still remains unclear how to rapidly determine the optimal cell ratio of co-culture required for optimally handling sugar mixtures available in different types of biomass feedstock. There are few studies that examined the effect of co-culture cell ratio and optimized cell ratio to maximize fermentation performance. However, the cell ratio optimization of co-culture was mostly relied on trial and errors and statistical analysis where large number of experiments is required (Ashoor et al., 2015; Karagöz et al., 2014). This approach is cost and labor intensive as a new set of experiment has to be conducted every time sugar composition in the feedstock changes. Alternative approach to optimize co-culture is to develop the co-culture kinetic model which can be used to identify optimal cell ratio needed for each type of feedstock that contains different sugar composition.

As a result, in this work, we developed a kinetic modeling tool that can describe the fermentation kinetics of a *S. stipitis/S. cerevisiae* co-culture in mixed glucose-xylose fermentation. The developed modeling tool was applied to design optimal cell ratio of *S. stipitis/S. cerevisiae* co-culture enabling improved ethanol productivity and titer compared to single-strain culture of *S. cerevisiae* or *S. stipitis* that is not able to efficiently utilize sugar mixture. The validated model was applied for the design of optimal cell ratio for an efficient ethanol fermentation of sugarcane bagasse by the co-culture. Techno-economic assessment was also performed to evaluate the economic value of yeast consortium process.

2. Objectives

The goal of this work is to develop, optimize and scale-up a co-culture system of *S. stipitis* and *S. cerevisiae* that enables efficient conversion of hexoses and pentoses mixture to ethanol at high yield in short time using systematic approach based on kinetic modeling. The aims of the proposed works include:

1. To develop kinetic modeling of co-culture for fermentation of hexoses and pentoses,
2. To design an optimal co-culture for hexose-pentose fermentation that results in the shortest fermentation time, while maintaining high ethanol yield using the developed kinetic model,
3. To demonstrate the use of co-culture with optimized cell ratio based on kinetic model prediction for efficient production of ethanol from different sugar mixture compositions in various types of biomass feedstock,

To demonstrate the scalability of developed co-culture system that can be suitable for large-scale or commercialized scale production process.

3. Methodology

3.1 Strains and Media

Scheffersomyces stipitis (ATCC58785) and *Saccharomyces cerevisiae* (Thermosacc, Lallemand) were used for the production of ethanol from bagasse hydrolysates. Yeast cell cultivation was performed in a culture medium containing 10% (v/v) molasses, 0.75 g/L $(NH_4)_2SO_4$, 0.35 g/L KH_2PO_4, 0.07 g/L $MgSO_4{\cdot}7H_2O$, and 1 g/L yeast extract with 10% (v/v) inoculum for 24 hrs at 30°C, 200 rpm. Yeast propagation was performed either in incubator shaker or in bioreactor. For co-culture, each yeast strain was prepared separately and inoculated into the bagasse hydrolysates according to the optimized cell ratio identified previously at 1.94 g-cell *S. stipitis*/g-cell *S. cerevisiae*. Yeast cell was harvested from the culture broth by centrifugation at 5,100 rpm for 5-10 mins and resuspended in the culture media prior to use as inoculum.

3.2 Yeast cultivation in synthetic media

Batch fermentation was carried out in YE medium containing 0.1M potassium phosphate buffer, 1g/l yeast extract, 5g/l $(NH_4)_2SO_4$, 0.1g/l $CaCl_2$, 0.1g/l NaCl, 0.5g/l $MgSO_4$, 1g/l KH_2PO_4, 15g/l glucose and 5g/l xylose. The sugars were autoclaved separately and added into the medium prior to use. Fermentation experiments were carried out in 2l Braun bioreactor (Biostat MD, B. Braun Biotech International, Melsungen, Germany) containing 1l of culture media. The yeast cell was grown overnight in YE medium at 30°C with agitation rate of 100 rpm in incubator shaker (Innova 4340, New Brunswick, USA) prior to inoculation into bioreactor. For co-culture, each yeast strain was prepared separately and inoculated into bioreactor according to the specified cell ratio. All batch fermentation experiments were began with the same initial OD_{600} of approximately 0.2, equivalent to an initial cell concentration of 0.1 g-cell/l. The fermentation condition was as previously described in Unrean and Nguyen (2012). Samples were taken periodically for sugars, ethanol and cell concentration measurement.

3.3 Pretreatment

Sugarcane bagasse, collected from Ratchaburi province (Thailand), was prepared by drying in an 80°C oven for 1 day before being cut by milling machine to attain a particle size of 0.25-1 cm. The processed bagasse was steam-pretreated with 0.5% (w/v) H_2SO_4 at 121°C for 30 mins as described earlier (Unrean et al., 2015). Sugar composition in the pretreated bagasse, determined by National Renewable Energy Laboratories (NREL) standard procedures (Hoyer et al., 2009), was 0.37 g-glucose and 0.23 g-xylose per gram bagasse, consistent with the composition previously reported in Canilha et al. (2011). The pretreated bagasse was neutralized with 4M KOH to pH value of 5-6. The suspension slurry was then used for enzyme hydrolysis and fermentation experiments.

3.4 Enzymatic hydrolysis of sugarcane bagasse

Enzymatic hydrolysis of pretreated bagasse was carried out in Erlenmeyer shake flask for small scale and in 10L bioreactor for scale-up. Batch enzyme hydrolysis experiment was conducted by mixing 10% WIS pretreated bagasse (w/w) with Cellic C-TEC2 commercial enzymes (Novozyme, Denmark) at 15 FPU/g-solid. The bagasse-enzyme mixture was supplemented with 0.75 g/L $(NH_4)_2SO_4$, 0.35 g/L KH_2PO_4, 0.07 g/L $MgSO_4 \cdot 7H_2O$ and initially adjusted to pH 5-5.5 using 4M KOH. It should be noted that batch process cannot be operated

at concentration higher than 10% WIS pretreated bagasse due to mixing limitation in a standard shake flask and stirred-tank bioreactor. Fed-batch enzymatic saccharification was carried out in the same initial WIS as batch followed by the pulse addition of pretreated bagasse. All enzymes were added initially. The enzymatic suspension was incubated at 35°C at 200 rpm during batch and 500 rpm during fed-batch for 96 hrs. The hydrolysates were then harvested and used for ethanol fermentation by *S. stipitis/S. cerevisiae* co-culture.

3.5 Batch ethanol fermentation

Batch simultaneous saccharification and fermentation (SSF) was carried out in 250 ml Erlenmeyer flask containing 10% WIS pretreated solid, 0.75g/l $(NH_4)_2SO_4$, 0.35g/l KH_2PO_4, 0.07g/l $MgSO_4 \cdot 7H_2O$, 1 g/l yeast extract, Cellic C-Tec II enzymes (Novozymes, Denmark) and yeast cell. The concentration of WIS content chosen for the experiment is the maximum concentration for each feedstock that can be operated in a standard stirred-tank bioreactor without mixing problem. The pH of the mixture was initially adjusted to 5 using 4M KOH. Yeast cell from seed culture and enzyme were added to the pretreated biomass mixture at 0.02 g-cell/g-WIS cell loading and 25 FPU/WIS enzyme dosage, respectively, to initiate SSF process. Yeast cell used in SSF was cultured in YPD media or in molasse media (10% (v/v) molasses, 0.75g/l $(NH_4)_2SO_4$, 0.35g/l KH_2PO_4, 0.07g/l $MgSO_4 \cdot 7H_2O$, 1g/l yeast extract) for 24 hrs at 30°C with a shaking speed of 200 rpm. Seed culture was harvested by centrifugation at 5,100 rpm for 5 mins and resuspended in the culture media before being used for SSF experiments. The SSF culture was incubated at 35°C, agitation rate of 200 rpm in incubator shaker (Innova R43, New Brunswick, USA) with no pH control during the cultivation. Culture samples were withdrawn periodically for measurement of residual sugars and ethanol product.

3.6 Fed-batch ethanol fermentation

The fermentation of hydrolysates from fed-batch saccharification operations were carried out in Erlenmeyer flask for small scale and in 10L bioreactor for scale-up. The fed-batch bagasse hydrolysates, supplemented with 10% molasses and 1 g/L yeast extract, were inoculated at 0.04 g-cell/g-WIS with *S. stipitis/S. cerevisiae* yeast consortium as specified. It should be noted that after the end of enzyme hydrolysis, the yeast cell and nutrients were added to the hydrolysates to initiate ethanol fermentation process without separation of solid and liquid parts of hydrolysates. The fed-batch fermentation was carried out at 35°C, 200 rpm and initial pH 5-5.5 with no pH control for 96 hrs. Samples were taken periodically for

fermentation kinetics analysis. The samples withdrawn were centrifuged at 13,000 rpm for 10 mins and the supernatant was analyzed for ethanol produced and sugar consumed. All experiments were performed in duplicate.

3.7 Sample analysis

WIS determination: Water insoluble solid (WIS) content for the pretreated bagasse was determined by separating the solid fraction of the pretreated slurry by centrifugation, weighing and washing with excess deionized water before drying in an oven at 80°C for 24 hrs and weighing to determine percent WIS content of pretreated bagasse.

Cell concentration: Concentration of yeast cell was measured via optical density at 600nm wavelength (OD_{600}) using spectrophotometer (DR/2500, Hach Company, Singapore). The cell dry weight was estimated using the correlation: cdw (g/L) = $0.5 \times OD_{600}$. To reduce interference by culture media during OD measurement, the culture sample was centrifuged at 5,100 rpm for 5 mins and the supernatant was discarded. The cell pellet was washed with deionized water before measuring the optical density.

Analysis of sugars and ethanol: Samples were centrifuged at 13,000 rpm for 5 mins and the supernatant was collected and filtered using 0.2 µm sterile filter. The samples were stored at -20°C prior to analysis. Concentration of sugars (glucose and xylose), ethanol and inhibitors (acetic acid and furfural) was measured by HPLC equipped with Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a refractive index detector at 65°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The concentrations were calculated from the calibration curve of standard solution.

Yield and rate calculation: Ethanol yield was calculated by dividing ethanol concentration produced by the concentration of total sugars available in the culture. The ethanol productivity was calculated as the overall rate based on total ethanol produced per total cultivation time.

3.8 Techno-economic analysis

A sugarcane-to-ethanol process was integrated by combining the basic steps of diluted-acid pretreatment, enzymatic hydrolysis, fermentation, downstream process and utilities system. For techno-economic analysis, the demo-scale process model and the overall mass and energy

balance data was calculated using SuperPro Designer software (Intelligen Inc., USA) to determine material flow rates, composition and energy flow for all streams in the integrated process. The process parameters including the sugarcane bagasse components, raw material inputs, yields, rates, processing times and unit operations required for each step was obtained from our experimental studies on lab-scale or scale-up unit in this work. Downstream process for ethanol purification was from the optimal process configuration previously identified by Wingren et al (2008). The equipment sizing, the chemical usage, and the utility usage were determined by the simulation data. The simulated process also considered time for transferring, draining and cleaning. Heat dissipation of the equipment was neglected. The simulated demo-scale plant for economic assessment was operated with the capacity of 123,000 kg of dry sugarcane bagasse during operation 8,000 hrs per year and run with 4 operators per day. The economic evaluation from mass and energy balances consisted of estimating the raw materials, operating, cleaning and labor costs associated with the ethanol production process. The cost of raw materials, utilities and labor together with the byproduct income used in this evaluation is summarized in Table 2 based on the values reported in literatures and in local suppliers in Thailand. The exchange rate of US dollar (USD) and Swedish Kronor (SEK) to Thai Baht (Baht) is set to 33.7 Baht/USD and 4.07 Baht/SEK respectively according to the average 2016 exchange rate (www.exchange-rates.org).

4. ผลการดำเนินงานของโครงการ

4.1 กิจกรรมที่วางแผนไว้/กิจกรรมที่ทำได้จริง

Proposed schedule for the project “Development, optimization and scale-up of a yeast co-culture system using kinetic model for an efficient production of lignocellulosic ethanol” is as follows

Activity	Year 1											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Characterization of fermentation kinetics and Development of co-culture kinetic model												
2. Application of a kinetic model for designing of optimal co-culture system for different biomass feedstock												
3. Optimization of cell ratio for *P. stipitis*/*S. cerevisiae* co-culture to validate co-culture kinetic model												
4. Optimal *P. stipitis*/*S. cerevisiae* co-culture system for efficient production of ethanol from lignocellulosic hydrolysate												
5. Scale-up lignocellulosic ethanol fermentation using optimized *P. stipitis*/*S. cerevisiae* co-culture system												
6. Analyzing sample and data												
7. Preparation of research report, publication and presentation												

Activity	Year 2											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Characterization of fermentation kinetics and Development of co-culture kinetic model												
2. Application of a kinetic model for designing of optimal co-culture system for different biomass feedstock												
3. Optimization of cell ratio for *P. stipitis*/*S. cerevisiae* co-culture to validate co-culture kinetic model												
4. Optimal *P. stipitis*/*S. cerevisiae* co-culture system for efficient production of ethanol from lignocellulosic hydrolysate												
5. Scale-up lignocellulosic ethanol fermentation using optimized *P. stipitis*/*S. cerevisiae* co-culture system												
6. Analyzing sample and data												
7. Preparation of research report, publication and presentation												

แผนการดำเนินงานตาม proposal กิจกรรมที่ได้ดำเนินการจริง

4.2 Development of *S. cerevisiae/S. stipitis* co-culture kinetic modelling

Mixed culture of *S. stipitis* and *S. cerevisiae* can be used to selectively adjust the fermentation kinetics of mixed C_6/C_5 sugars resulting in an optimal co-fermentation of the sugar mixture. Kinetic model describing cell growth and glucose and xylose utilization of *S. cerevisiae/S. stipitis* co-culture was developed based on balance equations. The model included cell growth of *S. stipitis* and *S. cerevisiae,* sugar consumption based on batch fermentation, inhibition effect of glucose on xylose, inhibition effect by ethanol and acetic acid, which is common inhibitor present in lignocellulosic feedstock.

A kinetic model describing the mixed culture of *S. stipitis* (strain 1) and *S. cerevisiae* (strain 2) in a sugar mixture is described as

Strain 1: $$\frac{dX_1}{dt} = \mu_1 X_1 \quad (1)$$

Growth on glucose $$\mu_1(C_G) = \frac{\mu_{\max G,1} C_G}{K_{mu1,G} + C_G} \quad (2)$$

Growth on xylose $$\mu_1(C_{Xy}) = \frac{\mu_{\max Xy,1} C_{Xy}}{K_{mu1,Xy} + C_{Xy}} \quad (3)$$

Strain 2: $$\frac{dX_2}{dt} = \mu_2 X_2 \quad (4)$$

Growth on glucose $$\mu_2(C_G) = \frac{\mu_{\max G,2} C_G}{K_{mu2,G} + C_G} \quad (5)$$

where X_i is the cell concentration of strain i, t is fermentation time, μ_i is specific cell growth rate, $\mu_{\max,i}$ is maximum specific growth rate, K_{mui} is saturation constant and C_G C_{Xy} is the concentration of glucose and xylose, respectively. The subscribe i represents *S. stipitis* (1) and *S. cerevisiae* (2) strain respectively. It should be noted that only *S. stipitis* is able to grow on xylose.

In a co-culture of strain 1 and 2, the initial cell ratio f is represented by

$$X_{1,0} = fX_{2,0} \quad (6)$$

The balance equation for each sugar is given by

Glucose consumption

$$\frac{dC_G}{dt} = q_{G,1}X_1 + q_{G,2}X_2 \quad (7)$$

$$q_{G,i} = \frac{V_{\max G,i}C_G}{K_{mG,i} + C_G}\frac{1}{1+C_{E0}/K_{EG,i}}\frac{1}{1+C_{A0}/K_{AG,i}} \quad (8)$$

where $q_{G,i}$ is specific uptake rate of glucose of strain i which follows Michaelis-Menten kinetics. Inhibition terms are added to represent ethanol inhibition and acetate inhibition on glucose consumption. Kinetic parameters describing glucose consumption are $V_{\max G}$: maximum glucose uptake rate, K_{mG}: saturation constant for glucose uptake, C_{E0}: initial concentration of ethanol, K_{EG}: ethanol inhibition constant for glucose uptake, C_{A0}: initial concentration of acetate, and K_{AG}: acetate inhibition constant for glucose uptake.

Xylose consumption

$$\frac{dC_{Xy}}{dt} = q_{Xy,1}X_1 \quad (9)$$

$$q_{Xy,1} = \frac{V_{\max Xy,1}C_{Xy}}{K_{mXy,1} + C_{Xy}}\frac{1}{1+C_{Xy}/K_{GXy,1}}\frac{1}{1+C_{E0}/K_{EXy,1}}\frac{1}{1+C_{A0}/K_{AXy,1}} \quad (10)$$

where C_{Xy} is the concentration of xylose. $q_{Xy,1}$ is specific xylose uptake rate of *S. stipitis* (strain 1) based on Michaelis-Menten kinetics. Kinetic terms are added to represent glucose repression, ethanol inhibition and acetate inhibition on xylose consumption. Kinetic parameters describing xylose consumption are $V_{\max Xy}$: maximum xylose uptake rate, K_{mXy}: saturation constant for xylose uptake, K_{GXy}: glucose repression constant for xylose uptake, K_{EXy}: ethanol inhibition constant for xylose uptake, and K_{AXy}: acetate inhibition constant for xylose uptake. It should be noted that $q_{Xy,2}$ is zero since *S. cerevisiae* (strain 2) cannot consume xylose.

Ethanol synthesis

$$\frac{dC_E}{dt} = Y_{EG,1}q_{G,1}X_1 + Y_{EG,2}q_{G,2}X_2 + Y_{EXy,1}q_{Xy,1}X_1 \quad (11)$$

$$R_{Etoh} = \frac{C_{E,final}}{t_{exhaust}} \quad (12)$$

where C_E is ethanol concentration and $C_{E,final}$ is the final ethanol concentration after all sugars are exhausted. $Y_{EG,i}$ and $Y_{EXy,i}$ are the ethanol yield on glucose and xylose respectively. R_{Etoh} is the overall ethanol productivity; $t_{exhaust}$ is exhaustion time which is the times when all sugars

are consumed. The exhaustion time for single-strain culture and co-culture can be computed by solving Eq.(1)-(10) simultaneously. Kinetic parameters used in the model are summarized in Table 1 which was obtained either from previous literatures (Hanly *et al* 2014, Unrean *et al* 2015) or from batch fermentation experiment of each strain in this study based on minimization of a weighted sum of the squared errors. This fitting is based on the bisquare weights method which is used for determining the parameters that fit the measured values using the usual least-squares approach, and that minimize the effect of outliers. Concentration profiles for glucose, xylose, and ethanol are obtained by solving the differential equations Eq. (1)-(17) numerically using ODE45 function in MATLAB software (Mathworks, Natick, MA, USA).

Table 1. Kinetic parameters used for *S. stipitis/S. cerevisiae* co-culture kinetic model

Symbol	Parameter	Strain*		References
Fermentation kinetics		***S. cerevisiae***	***S. stipitis***	
$V_{\max G,i}$	maximum rate for glucose uptake (g/g-hr)	2.90	0.77	This study
$K_{mG,i}$	saturation constant for glucose uptake (g/L)	0.5	0.5	Hanly *et al* 2014
$K_{EG,i}$	ethanol inhibition constant for glucose uptake (g/L)	10	10	Hanly *et al* 2014
$K_{AG,i}$	acetate inhibition constant for glucose uptake (g/L)	7.5	7.5	Hanly *et al* 2014
$V_{\max Xy,i}$	maximum rate for xylose uptake (g/g-hr)	0	0.06	This study
$K_{mXy,i}$	saturation constant for xylose uptake (g/L)	0.25	0.25	Hanly *et al* 2014
$K_{GXy,i}$	glucose repression constant for xylose uptake (g/L)	0.25	0.25	Hanly *et al* 2014
$K_{EXy,i}$	ethanol inhibition constant for xylose uptake (g/L)	4.5	4.5	Hanly *et al* 2014
$K_{AXy,i}$	acetate inhibition constant for xylose uptake (g/L)	0.2	0.2	Hanly *et al* 2014
$\mu_{\max G,i}$	maximum growth rate for glucose uptake (1/hr)	0.3	0.15	This study
$K_{mui,G}$	saturation constant for growth on glucose (g/L)	0.5	0.5	This study
$\mu_{\max Xy,i}$	maximum growth rate for xylose uptake (1/hr)	0.0	0.01	This study
$K_{mui,Xy}$	saturation constant for growth on xylose (g/L)	0.5	0.5	This study

*The subscript i is 1 for *S. stipitis* strain and 2 for *S. cerevisiae*.

4.3 Model validation - Predicting fermentation kinetics of co-culture

The developed co-culture kinetic model was first utilized to predict cell growth and fermentation kinetics of *S. stipitis*, *S. cerevisiae* and *S. stipitis/S. cerevisiae* co-culture in batch fermentation containing glucose-xylose mixture. Glucose represents C_6 sugar whereas xylose

represents C_5 sugar in biomass feedstock. The experiments were performed under the same initial cell concentration of 0.1 g-cell/l for comparison purpose. To validate the co-culture kinetic model, the predictive concentration time profiles of yeast cell, sugars and ethanol was compared with the values observed experimentally in batch fermentation of *S. cerevisiae*, *S. stipitis* and *S. cerevisiae/S. stipitis* co-culture at 1:1 cell ratio (Fig. 1A-C). The experimental results agree well with the model prediction, thus confirming the accuracy of the model in predicting fermentation kinetics of single-strain culture as well as of co-culture. The validated model was then utilized for optimizing cell ratio (f) in co-culture to maximize ethanol productivity and titer such that the co-culture can optimally perform at its best under a given glucose-xylose mixture.

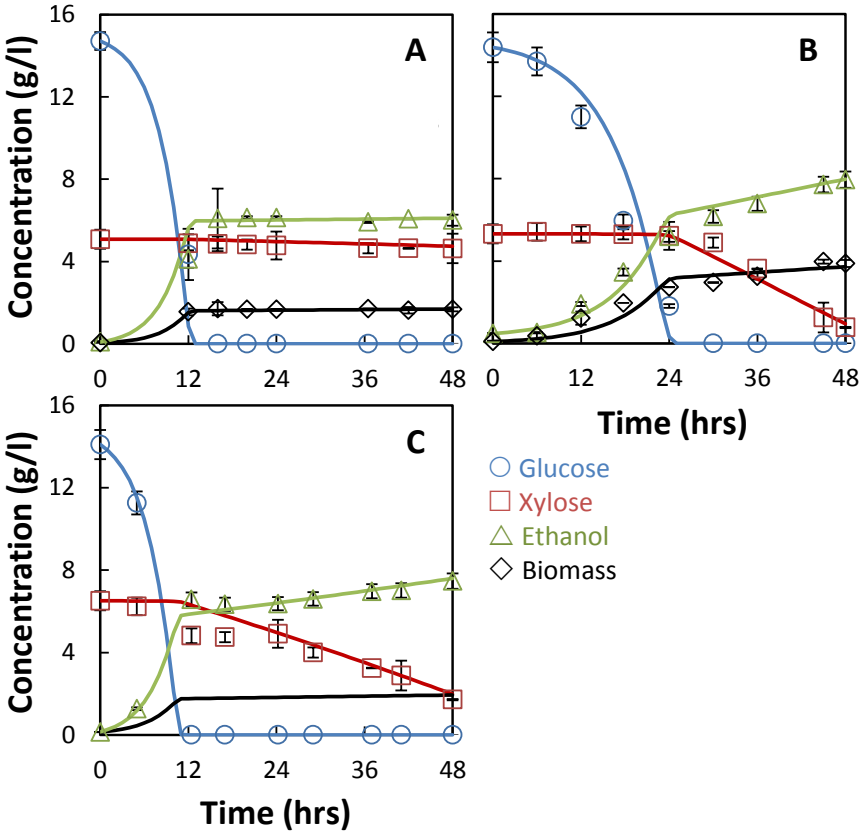

Figure 1. Fermentation kinetics of *S. cerevisiae* (A), *S. stipitis* (B) and co-culture of *S. stipitis* and *S. cerevisiae* (C) in glucose-xylose mixture. Time profiles of glucose (blue), xylose (red), ethanol (green) and biomass (black) in *S. cerevisiae*, *S. stipitis*, and *S. stipitis/S. cerevisiae* co-culture at initial cell ratio f = 1.0 are shown. A good agreement between measured values (symbols) and predicted values (solid lines) validates the kinetic model. The results are based on average of duplicate experiments.

4.4 Model-based design of optimal co-culture system

Unlike single strain culture in which proportion of the cell cannot be adjusted to match with the proportion of each sugar available, co-culture will use shorter time because proportion of each strain used optimally match with proportion of each available sugar. The optimal proportion of each strain in the co-culture would therefore result in the efficient fermentation of mixed sugars. We have implemented the co-culture kinetic model to establish optimized cell ratio of the *S. stipitis/S. cerevisiae* co-culture in order to maximize ethanol production for different composition of glucose and xylose. The model was first applied to simulate the effect of ethanol productivity and titer as a function of the cell ratio (f) of *S. stipitis/S. cerevisiae* co-culture for fermentation of glucose-xylose mixture at a glucose-xylose ratio of 3 (Fig. 2A).

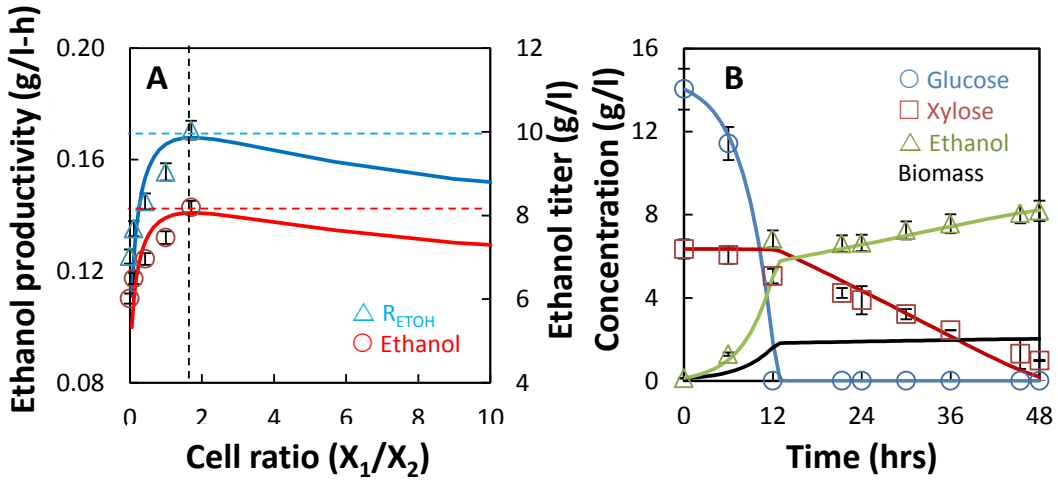

Figure 2. Ethanol production in mixed glucose-xylose fermentation by *S. stipitis/S. cerevisiae* co-culture. (A) Ethanol productivity (cyan) and titer (red) at various cell ratios of *S. stipitis* (X_1)/*S. cerevisiae* (X_2) co-culture in comparison with single-strain culture of *S. cerevisiae*. The optimal cell ratio (f_{opt} = 1.70 g-*S. stipitis*/g-*S. cerevisiae*) indicated by dashed line yields maximum ethanol productivity and titer which is 36% higher than those achieved by single-strain culture. The measured values (symbol) agree well with the values predicted by the co-culture kinetic model (solid line). (B) Fermentation kinetics of *S. stipitis/S. cerevisiae* co-culture at optimal cell ratio (f_{opt} = 1.70). Glucose, xylose, ethanol and biomass are shown in blue, red, green and black, respectively. The symbol represents experimental measurement which is in good agreement with the model prediction

represented by solid line. The results are based on averages of duplicate fermentation experiments in synthetic medium containing 3:1 glucose-xylose mixture.

The results reveal that the ethanol productivity and titer were affected by the co-culture's cell ratio. Comparing fermentation performance after 48 hrs by the co-culture at different cell ratio identified the optimal cell ratio for maximization of ethanol production in both productivity and titer, which was consistent with the model prediction confirming the accuracy of the co-culture kinetic model. The results identified the optimal cell ratio (f_{opt}) of *S. stipitis/S. cerevisiae* to be 1.70 g-*S.stipitis*/g-*S.cerevisiae* for the most efficient ethanol production from a glucose-xylose ratio of 3. The co-culture under optimized cell ratio improved ethanol titer up to 35% compared to the single-strain culture containing *S. cerevisiae* due to a better utilization of mixed glucose-xylose by the co-culture. The ethanol productivity of the optimized co-culture was also enhanced by 36% in comparison to the single-strain culture of *S. cerevisiae* and by 6% in comparison to the single-strain culture of *S. stipitis*, which also closely agreed with the predicted values. Fermentation kinetics of co-culture under the optimal cell ratio (f = 1.70) also agreed well with the kinetic model prediction (Figure 2B). Thus, the model can provide a better insight into the kinetics of mixed sugar fermentation by single-strain culture as well as by co-culture as shown in Fig. 1 and 2.

Kinetics of C_6/C_5 sugar fermentation by *S. stipitis/S. cerevisiae* co-culture shown in Fig. 1C and 2B suggested that by varying cell ratio of co-culture system, the conversion rate of glucose-xylose mixture to ethanol could be adjusted. Thus, the system could be optimized in cell ratio with respect to the change in sugar composition in the biomass feedstock. This was also confirmed in Figure 2A where different cell ratio of co-culture converted the sugar mixture into ethanol at different ethanol production rate. The outperformed productivity of co-culture system relative to single-strain culture in the mixed sugar fermentation is expected since dedicating one strain to consume all sugar mixture would lead to a longer fermentation time for the completed conversion of all sugars compared to having multiple strains. The co-culture kinetic model could be used for optimization of co-culture fermentation by predicting an optimal initial cell ratio of the co-culture in any given sugar mixture. As a result, in the next step, the co-culture kinetic model will then be used for designing optimal cell ratios of co-culture for an efficient conversion of each type of biomass feedstock into ethanol at high titer and productivity.

4.5 Design of optimal co-culture system for different biomass feedstock

The proportion of cell in the co-culture can be adjusted to match with each available sugar. Thus, the optimal cell ratio would be different as the composition of glucose and xylose changes. To demonstrate the flexibility of co-culture system, the kinetic model was utilized to predict the optimal cell ratio required for different biomass feedstock. We implemented the co-culture model for the prediction of cell ratio to optimally match with a given glucose-xylose composition available in major biomass feedstock as summarized in Table 2. The optimal co-culture cell ratio is the ratio which is required for the most efficient conversion of each type of biomass feedstock into ethanol with maximum ethanol productivity and titer.

Table 2. Optimal cell ratio of *S. stipitis/S. cerevisiae* co-culture for maximized ethanol production from different biomass feedstock predicted by co-culture kinetic model. Glucose and xylose ratio for each biomass feedstock is based on Biomass Feedstock Composition and Property Database.

Type of biomass	Sugar ratio	Opt.cell ratio[1]	R_{ETOH}[2] (g/l-hr)		[EtOH][3] (g/l)	
	Glc/Xyl (g/g)	X_1/X_2 (g/g)	Co-culture	Improve (%)[4]	Co-culture	Improve (%)[4]
Rice Straw	3.00	1.70	0.17	35%	8.00	33%
Corn Stover	1.89	1.78	0.17	55%	9.17	52%
Cottonwood	3.23	1.70	0.17	33%	7.86	30%
Sugarcane Bagasse	1.63	1.94	0.18	63%	9.68	61%
Corn Cobs	1.29	1.86	0.19	79%	10.65	77%
Switch grass	1.45	1.86	0.18	71%	10.14	68%
Eucalyptus	4.90	1.63	0.17	23%	7.22	20%
Wheat Straw	1.58	1.94	0.18	65%	9.80	63%

[1] Optimal cell ratio is defined as initial g-cell of *S. stipitis* (X_1) per initial g-cell of *S. cerevisiae* (X_2). The model simulation for each type of feedstock is based on glucose concentration of 15 g/l and xylose concentration according to sugar ratio for each feedstock for comparison purpose.

[2] Rate of ethanol is defined as overall productivity which is total ethanol produced divided by required fermentation time of co-culture under optimal cell ratio. Fermentation time is the time required for completion of all glucose and xylose by co-culture.

[3] Ethanol titer of co-culture under optimal cell ratio is predicted by the model based on total glucose and xylose available and 80% of theoretical yield assumption.

[4] Percent improvement is calculated by comparing the performance of co-culture under optimal cell ratio with that of *S. cerevisiae* based on kinetic model.

According to the model prediction, the co-culture under optimized cell ratio could enhance ethanol fermentation performance by increasing ethanol productivity 23-79% and increasing ethanol titer 20-77% depending on the available sugar composition in each biomass feedstock. The enhancement of ethanol production by co-culture compared to a single-strain culture becomes increasingly evident as the available C_5 sugar content in the biomass feedstock increases. For instance, the co-culture could improve ethanol productivity and titer up to 65% in wheat straw compared to only up to 35% improvement in rice straw due to more availability of C_5 sugar in wheat straw than in rice straw. The results indicate that the use of optimized co-culture system would be a preferred process especially for feedstock with high C_5 sugar content. The application of model-based design of co-culture permits the design of optimal cell ratio of co-culture for efficient ethanol fermentation from any C_6/C_5 available sugars. The model simulation results also emphasize the benefit of using co-culture system in term of its adjustability to match with each type of feedstock composing of different sugar ratio, which could not be achieved if the single-strain culture is used.

4.6 Model-based cell ratio optimization of *S. stipitis/S. cerevisiae* co-culture

Since the yeast cultures in consortium at optimal cell ratio allows efficient fermentation of two sugars available in biomass feedstock, glucose and xylose, and is flexibility as the proportion of cell ratio in the culture can be optimally adjusted to match with the available sugars. The optimal co-culture designed according to the co-culture kinetic model was utilized for simultaneous saccharification and fermentation of sugarcane bagasse as feedstock in order to test the applicability of co-culture system for lignocellulosic ethanol production. The bagasse was chosen as a case study to explore the ethanol production efficiency by the optimized co-culture in biomass feedstock composing of different glucose/xylose ratio. The optimal ratio between two yeast strains was predicted such that the fermentation rate of each sugar was precisely adjusted to match with given glucose-xylose composition available in the feedstock.

Fig. 3 shows the effect of co-culture cell ratio (*S. stipitis/S. cerevisiae*) on ethanol productivity and fermentation time of sugarcane bagasse. The optimal cell ratio predicted by the model for the fermentation of sugarcane bagasse was 1.94 g-cell *S. stipitis*/g-cell *S. cerevisiae*. Effect of cell ratio of co-culture for the fermentation of biomass feedstock illustrated in Fig. 3 also highlights the flexibility of co-culture process for an efficient fermentation of mixed sugars available in a given biomass feedstock by adjusting the operating cell ratio of each strain.

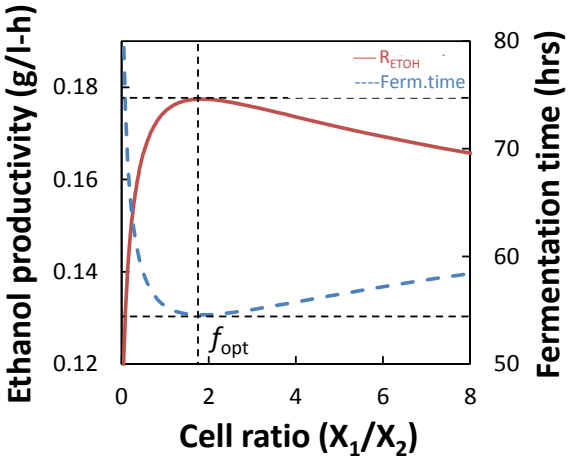

Figure 3. Predicted ethanol productivity (solid line) and fermentation time (dashed line) by co-culture kinetic model for sugarcane bagasse fermentation. The model identifies optimal cell ratio (f_{opt}) of 1.94 g-cell *S. stipitis*/g-cell *S. cerevisiae* for sugarcane bagasse to maximize ethanol fermentation.

To validate the use of co-culture model for lignocellulose-to-ethanol process, we optimized the cell ratio of the *S. stipitis*/*S. cerevisiae* yeast co-culture to efficiently ferment sugarcane bagasse hydrolysates to ethanol in batch culture (Fig. 4). The optimized yeast consortium at cell ratio of 1.94 g-*S.stipitis*/g-*S.cerevisiae* led to the maximum ethanol production with increasing ethanol yield and productivity up to 23% and enhancing ethanol titer by 11% compared to the performance by other cell ratios. The optimal cell ratio determined in this study agrees well with the value predicted by the co-culture kinetic model for efficient sugarcane bagasse-to-ethanol production (Table 2), confirming the accuracy of the model-based design of optimal co-culture.

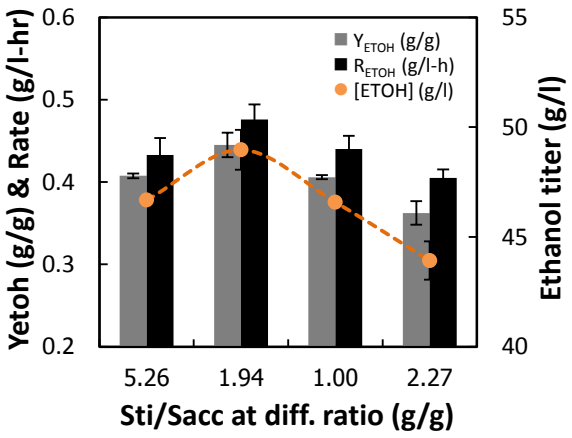

Figure 4. Optimized ratio of *S. stipitis/S. cerevisiae* yeast consortium for enhanced ethanol yield (grey), ethanol productivity (black) and ethanol titer (orange).

4.7 Optimal *S. stipitis/S. cerevisiae* co-culture system for efficient ethanol production

The results in Fig. 5 confirm that the process of co-culture outperformed the process of single-cell culture for the fermentation of sugar mixture in lignocellulosic biomass. Fig. 5 shows ethanol fermentation performance from sugarcane bagasse of *S. stipitis/S. cerevisiae* co-culture in comparison with the single-strain culture of *S. stipitis* and of *S. cerevisiae*. The results reveal that the use of co-culture under optimized cell ratio yielded up to 12% improvement in ethanol titer in pretreated sugarcane bagasse, when compared with the use of single-strain culture of *S. cerevisiae* and of *S. stipitis*. Ethanol yield and productivity achieved by co-culture was also higher than those achieved by single-strain culture. Ethanol fermentation of sugarcane bagasse by co-culture achieved approximately 11% higher ethanol productivity when compared to the performance by single-strain culture. In sugarcane bagasse SSF, the co-culture under optimal cell ratio also produced higher ethanol yield of up to 15% when compared with the yield achieved by single-strain culture. The co-culture kinetic model (as shown in lines) accurately predicted the ethanol production by the single-strain culture and the co-culture in both feedstocks. Optimal operating cell ratio permits the maximization of ethanol production for each type of feedstock. The experimental results showed that the co-culture performance at 1.94 g/g cell ratio, which is the optimal cell ratio designed specifically for sugarcane bagasse feedstock, resulted in higher ethanol production from sugarcane bagasse than those at other cell ratios (Fig. 5). Thus, maximizing ethanol production for each type of feedstock specifically requires different cell ratio according to the available sugars of the feedstock used which could be readily predicted by the developed model.

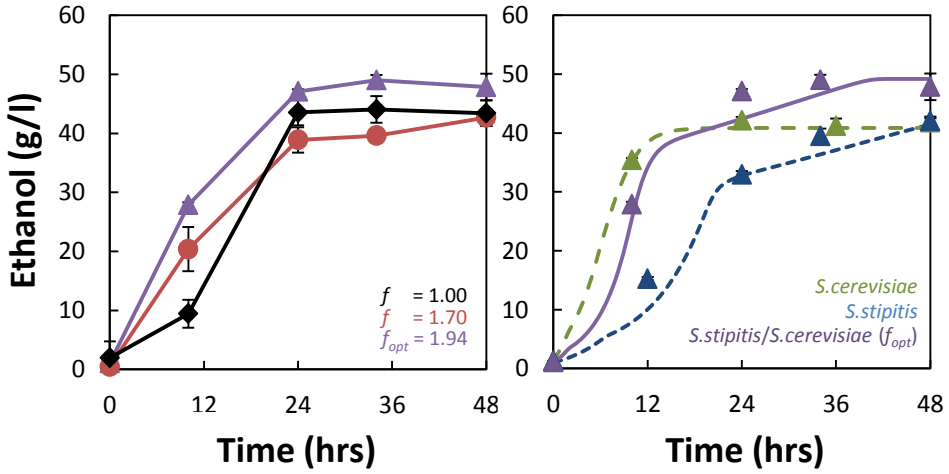

Figure 5. (Right) Saccharification and fermentation of sugarcane bagasse by *S. stipitis/S. cerevisiae* co-culture in comparison with single-strain culture. Time profiles of ethanol in *S. stipitis* (blue), *S. cerevisiae* (green) and *S. stipitis/S. cerevisiae* (purple) culture are compared. (Left) Comparison of ethanol production from sugarcane bagasse at different cell ratio, f = 1.00, f = 1.70 and f = 1.94. Experiments were conducted in SSF configuration in which enzymes and yeast cells are added simultaneously into 10% WIS pretreated sugarcane bagasse. The symbols represent measured values while the lines represent predicted values based on the co-culture kinetic model. For comparison purpose, all experiments are initiated with the same total cell concentration of 0.02 g-cell/g-WIS. The optimized cells ratio for the co-culture as predicted by the model is f_{opt} = 1.94 for sugarcane bagasse.

Lignocellulosic ethanol production performance by co-culture and single-strain culture is summarized in Table 3. Ethanol yield accomplished by co-culture in sugarcane bagasse fermentation was 75% of theoretical yield. Thus far, the highest ethanol titer reached by co-culture in this study was 46.68±0.09 g/l. It should also be noted that reaching higher titer of ethanol in order to meet techno-economic feasibility of industrial scale requires fed-batch co-culture process.

Table 3. Ethanol production performance by single-strain culture of *S. cerevisiae* and *S. stipitis* in comparison with co-culture of *S. cerevisiae/S. stipitis* under optimal cell ratio

Sugarcane bagasse feedstock[1]			
Strains	[EtOH] (g/l)	R_{ETOH}[3] (g/l-hr)	Y_{ETOH}[4] (g/g)
S. cerevisiae	41.81±0.64	0.44±0.01	0.33±0.01
S. stipitis	42.05±0.36	0.44±0.00	0.33±0.01
S. cerevisiae/S. stipitis[2]	46.68±0.09	0.49±0.00	0.38±0.02

[1] Based on batch SSF of sugarcane bagasse at 10%WIS for 96 hrs

[2] Co-culture of *S. cerevisiae* and *S. stipitis* was carried out under optimal cell ratio as predicted by the model (shown in Table 1) for each type of feedstock.

[3] Rate of ethanol is defined as overall productivity which is total ethanol produced divided by fermentation time.

[4] Ethanol yield is based on total sugar available in the feedstock.

4.8 Optimal feeding of *S. stipitis/S. cerevisiae*

The optimal cell ratio at 1.94 g-cell *S. stipitis*/g-cell *S. cerevisiae* was determined based on kinetic modeling and experimental studies. We then investigated the effect of *S. stipitis/S. cerevisiae* addition under optimal cell ratio on ethanol fermentation efficiency. Specifically, two different addition of *S. stipitis/S. cerevisiae* were examined, a simultaneous addition of both *S. stipitis* and *S. cerevisiae* and a sequential addition *S. cerevisiae* followed by feeding of *S. stipitis*. Ethanol fermentation performance by the optimized co-culture in batch hydrolysates was compared between the addition of *S. stipitis* initially (simultaneous addition) and the addition of *S. stipitis* 12-48 hrs after addition of *S. cerevisiae* (sequential addition) as shown in Fig. 6. In the case of sequential addition, *S. cerevisiae* was initially added at the beginning of cultivation, then *S. stipitis* was fed in equal portion every 12 hrs for 48 hrs. Effect of cell addition studies showed no significant improvement in ethanol fermentation in titer, productivity or yield in simultaneous addition of *S. cerevisiae/S. stipitis* compared to sequential addition of *S. cerevisiae* followed by feeding of *S. stipitis*. Thus, initial addition of all yeast cells was selected as a preferred process configuration due to the ease of operation. Based on modeling and experimental studies, the optimum lignocellulosic ethanol production by *S. stipitis/S. cerevisiae* was at 1.94 g-cell *S. stipitis*/g-cell *S. cerevisiae* cell ratio with simultaneous addition of both yeasts at the beginning of fermentation process in order to maximize ethanol production efficiency.

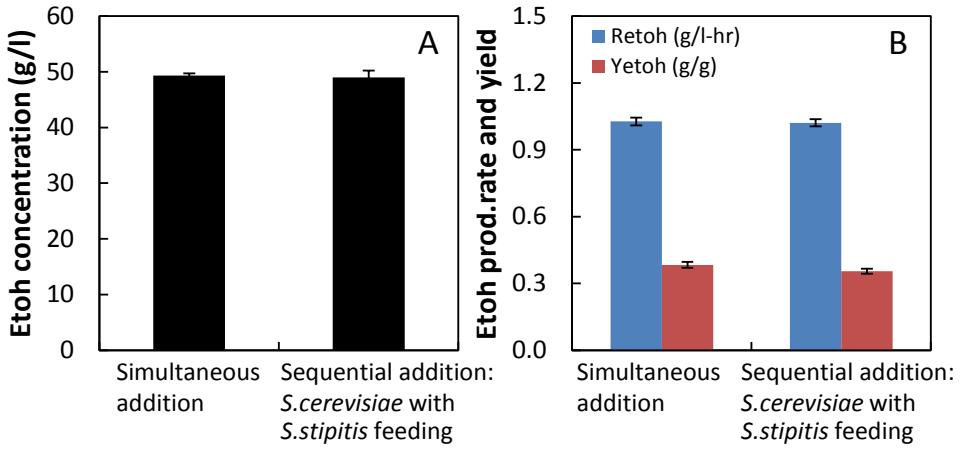

Figure 6. Effect of yeast cell addition on sugarcane bagasse-to-ethanol conversion. Ethanol titer (A), yield and productivity (B) after 48 hrs of simultaneous saccharification and fermentation of sugarcane bagasse by simultaneous addition of *S. stipitis/S. cerevisiae* and by sequential addition of *S. cerevisiae* followed by every 12 hrs feeding of *S. stipitis* for 48 hrs. The productivity is present as maximum production rate of ethanol within the first 48 hrs. The ethanol yield is based on total ethanol produced per total sugars available.

4.9 High-titer ethanol production by optimized *S. stipitis/S. cerevisiae*

High-solid enzymatic saccharification of sugarcane bagasse feedstock is required for the development of high-ethanol-titer process. Challenges of a high-solid enzyme hydrolysis are poor mixing and ineffective heat and mass transfer due to increased viscosity under high concentration of pretreated biomass slurry leading to decreased hydrolysis and fermentation efficiency. A proper design of solid substrate feeding scheme in a fed-batch saccharification process which balances between the rate of enzyme hydrolysis and the addition of solid substrate allows feasible operation of lignocellulose-based process with high solid content. Thus, in this study, we implemented solid feeding designed according to our previous work in order to reach high-solid loading while maintaining mixing sufficiency of sugarcane bagasse. All enzymes were added initially to permit a rapid reduction of viscosity and a better mixing due to faster hydrolysis in the early stage of the process enabling faster feeding of solid substrate. The fed-batch enzymatic hydrolysis with feeding of pretreated sugarcane bagasse is shown in Fig. 7A allowing high-solid operation up to 22% (w/v) WIS of pretreated bagasse with a high concentration of releasing sugars at 135.56±5.54 g/l at 96 hrs, equivalent to 78% yield of the cellulose conversion.

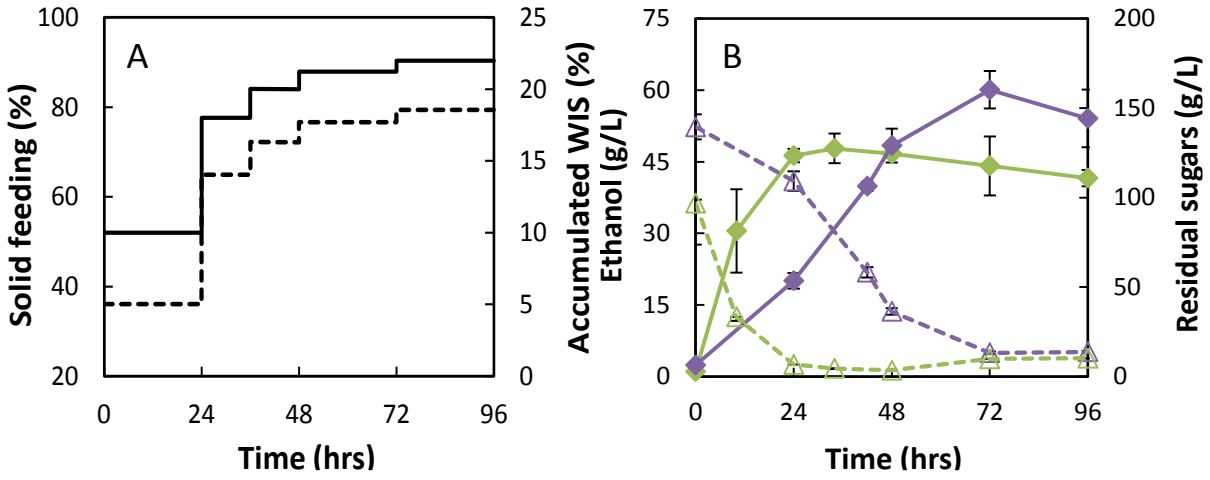

Figure 7. Ethanol production from sugarcane bagasse in batch and fed-batch saccharification followed by fermentation of *S. stipitis/S. cerevisiae* co-culture. (A) Solid feed profile during fed-batch enzyme hydrolysis at high solid load. Shown are percent of accumulated solid load (dashed line) and accumulated water insoluble solid WIS (solid line) yielding final accumulated sugars at 135.56±5.54 g/l. (B) Ethanol fermentation of sugarcane bagasse hydrolysates obtained from batch or fed-batch by *S. stipitis/S. cerevisiae* consortium. Consumed sugars (triangle) and produced ethanol (diamond) in shake flask batch (green) and shake flask fed-batch process are compared.

The batch and fed-batch bagasse hydrolysates were subsequently fermented to ethanol using *S. stipitis/S. cerevisiae*, and the fermentation kinetics of sugarcane bagasse hydrolysates by the co-culture is compared in Fig. 7B. The highest ethanol titer of 46.68±0.09 g/L and 60.09±3.92 g/L were achieved in batch and fed-batch operation by *S. stipitis/S. cerevisiae* co-culture respectively. Fed-batch process brought about approximately 1.3-folds and 1.2-folds increment in the ethanol titer and the ethanol productivity, respectively, compared to batch process (Table 4). The enhanced ethanol production performance from fed-batch operation made the process more industrially realistic. High ethanol yield up to 76% of the theoretical was obtained during the fermentation of fed-batch hydrolysates which was lower than the yield observed in the fed-batch SSF process from our previous work. The reduction in ethanol yield is likely due to high concentration of inhibitors at high substrate content leading to the loss of cell viability and fermentation activity. Nevertheless, the results confirm that the fed-batch process of *S. stipitis/S. cerevisiae* yeast consortium outperformed both batch processes by single-strain and co-culture in both ethanol titer and productivity.

Table 4. Ethanol production performance by *S. cerevisiae/S. stipitis* under optimal cell ratio in batch and fed-batch processes

	[Ethanol] (g/l)	Maximum R_{ETOH} (g/l-h)[2]	Overall R_{ETOH} (g/l-h)[3]	Ethanol Yield (g/g)[3]
S. stipitis/S. cerevisiae[1] in batch shake flask	46.68±0.09	0.97±0.00	0.49±0.00	0.38±0.02
S. stipitis/S. cerevisiae in fed-batch shake flask	60.09±3.92	1.01±0.07	0.56±0.06	0.39±0.04
S. stipitis/S. cerevisiae in fed-batch bioreactor	56.08±0.82	0.85±0.02	0.58±0.01	0.36±0.04

[1] A *S. stipitis* and *S. cerevisiae* yeast cultures in consortium is performed under optimized cell ratio as determined previously.
[2] Maximum ethanol productivity is the production rate of ethanol within the first 48 hrs.
[3] Overall ethanol productivity is determined from total ethanol produced divided by total fermentation time whereas ethanol yield is based on total ethanol produced per total sugars available.

4.10 Scaling up lignocellulosic ethanol fermentation using *S. stipitis/S. cerevisiae*

The scale-up of high-solid loading fed-batch process in 10L stirred tank bioreactors produced 56.08±0.82 g/L ethanol, corresponding to 70% of the theoretical value, based on the total sugar content in the sugarcane bagasse. The overall ethanol yield was 250 kg-ethanol per dry ton sugarcane bagasse. The scale-up result was relatively consistent with the result in lab-scale shake flask confirming the scalability of the optimized high-solid yeast consortium process (Fig. 8). Table 4 summarizes ethanol fermentation performance from sugarcane bagasse hydrolysates by yeast consortium in scale-up fed-batch process compared to small-scale shake flask. The results in Table 3 and 4 confirm that the fed-batch process of *S. stipitis/S. cerevisiae* yeast consortium outperformed batch processes by both single-strain and co-culture in both ethanol titer and productivity and was scalable.

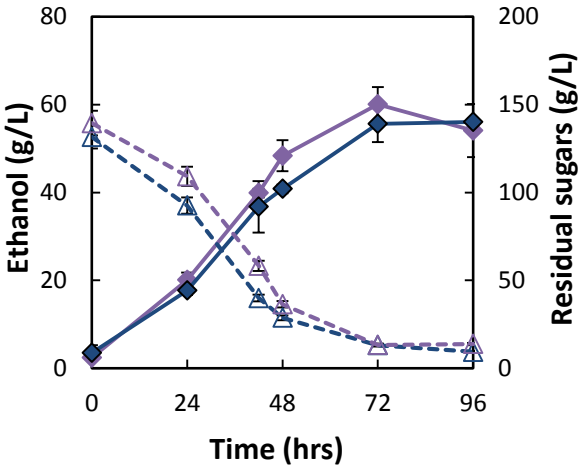

Figure 8. Ethanol fermentation of sugarcane bagasse hydrolysates by fed-batch *S. stipitis/S. cerevisiae* consortium in scale-up bioreactor (blue) compared to small-scale shake flask (purple). Consumed sugars (triangle) and produced ethanol (diamond) are shown.

4.11 Optimal yeast consortium fed-batch process integration

The optimized fed-batch process with high titer of ethanol using *S. stipitis/S. cerevisiae* yeast consortium was examined for its potentials for industrialization and economic feasibility through process integration and techno-economic analysis. The integrated sugarcane bagasse to ethanol process as depicted in Fig. 9 began with diluted-acid pretreatment and the pH of the pretreated slurry was adjusted with KOH (amount based on experimental data) and diluted to a water-insoluble solids (WIS) concentration as designed based on the experimental work before enzyme hydrolysis with the addition of enzyme converting biomass into sugar monomers followed by the addition of required nutrients and yeast cells for fermentation of sugars into ethanol.

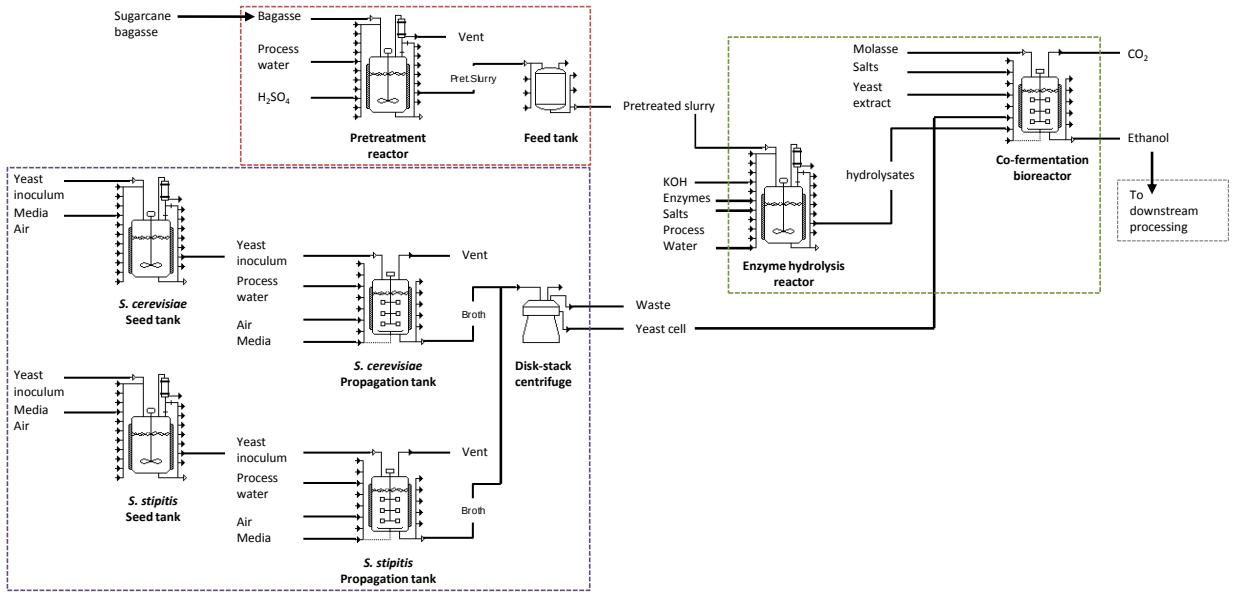

Figure 9. Process diagram for optimal process of fed-batch saccharification followed by fermentation (fed-batch SHF) by *S. stipitis/S. cerevisiae* yeast consortium. The process configuration includes diluted-acid pretreatment (in red dashed square), yeast propagation (in purple dashed square), enzyme hydrolysis and co-fermentation (in green dashed square) steps for the conversion of sugarcane bagasse to ethanol. Detailed downstream processing for ethanol recovery was described in Wingren et al., 2008.

The process conditions for each step were described in Method section. The fed-batch saccharification was pulse-fed with pretreated bagasse slurry at desired feed profile (Fig. 7A). The *S. stipitis/S. cerevisiae* consortium was inoculated at optimized cell ratio to start fermentation. In our designed process no solid–liquid separation after the pretreatment, thereby no wastewater pretreatment was required in this step and in fermentation step. Dilute acid pretreatment with sulfuric acid as a catalyst was utilized since it is considered an economically viable technology to solubilize hemicellulose and increase the digestibility of cellulose in enzymatic hydrolysis (Canilha et al., 2011). Enzymatic hydrolysis of the whole pretreated slurry was carried out by C-TEC2 cellulase enzymes to produce monomeric sugars for fermentation. The yeasts were produced on-site in separate aerated propagation tanks using molasses and salts, which is commonly available in a sugar factory that provides sugarcane bagasse feedstock, to obtain a satisfactory yeast cell concentration. The yeast cultures of *S. stipitis* and *S. cerevisiae* were separated in a continuous centrifuge, re-suspended in culture media, and added into fermentation reactor at optimized cell ratio. Since the solid-liquid slurry after the enzymatic hydrolysis unit was directly used for fermentation, in practice enzyme hydrolysis and

fermentation process could be carried out in one tank in a sequential manner. However, the process diagram of fed-batch saccharification and fermentation units was depicted in separate tank for clarification purpose (Fig. 9). The output stream from the fermentation unit was transferred to the downstream operations to recover the ethanol solution at 94% (w/w) and the remaining solid and liquid waste was separated out after downstream processing. The downstream steps are obtained from the previously optimized process by Wingren et al (2008). In techno-economic evaluations of sugarcane bagasse-to-ethanol production process, the conversion yield, titer and processing time assumed for yeast propagation, enzyme hydrolysis and fermentation processes were based on experimental data in this work. The purpose of the fully integrated process simulations and the economic evaluation is to evaluate techno-economic feasibility of the fed-batch yeast consortium process.

4.12 Techno-economic analysis of fed-batch yeast consortium process

We analyzed the profitability of the fed-batch yeast co-culture process for ethanol production from sugarcane bagasse based on operating costs and minimal selling prices. The cost estimation of all the process steps at different configurations was estimated from the economic data reported in our case study and others as summarized in Table 5.

Table 5. Raw materials and utilities cost used in the techno-economic evaluation of sugarcane bagasse-to-ethanol process by yeast co-culture

	Cost	Unit[1]	Sources
Raw materials			
Sulfuric acid	1.18	Baht/kg	Kazi et al 2010
$MgSO_4$	18	Baht/kg	Wingren et al 2008
Molasses	4.07	Baht/kg	Sassner et al 2008
Enzymes	4.08	Baht/kg	Geraili et al 2014
$(NH_4)_2SO_4$	28	Baht/kg	Local supplier
KOH	8.5	Baht/kg	Local supplier
KH_2PO_4	19	Baht/kg	Local supplier
Yeast extract	480	Baht/kg	Local supplier
Utilities and labor			
Electricity	0.28	Baht/MJ	Wingren et al 2008
Process water	0.006	Baht/L	Wingren et al 2008
Chilled water	0.013	Baht/L	Wingren et al 2008
Cooling water	0.010	Baht/L	Wingren et al 2008

Cleaning agent	16.28	Baht/kg	Wingren et al 2008
Steam	0.41	Baht/kg	Local supplier
Labor	37.5	Baht/hr	Local supplier
Byproducts income			
CO_2	0.12	Baht/kg	Wingren et al 2008

[1] The values reported in literatures are converted to Baht using the following exchange rate, 33.7 Baht/USD or 4.07 Baht/SEK.

The fed-batch process using *S. cerevisiae/S. stipites* consortium culture yielded the minimal ethanol selling price (MESP) of 26.7 Baht/L. With fed-batch strategy, the MESP was decreased by 22% compared to batch process (Table 6). This is due to the higher ethanol titer reached by increasing the solid content using fed-batch and the efficient utilization of mixed sugars by yeast consortium which had a significant positive effect on the process economy.

Table 6. Techno-economic analysis of sugarcane-to-ethanol process by yeast co-culture

Process configurations	Ethanol yield (kg-etoh/ton-bagasse)	Minimal ETOH selling price (Baht/L)[1]
S. cerevisiae/S. stipitis batch process	193.7	32.6
S. cerevisiae/S. stipitis fed-batch process	250.0	26.7

[1] Ethanol selling price is estimated based on total production cost per total ethanol produced. Downstream processing cost is calculated from the previously reported value of required energy for ethanol purification, 10.2 MJ/L ethanol (Wingren et al., 2008).

Although the estimated minimum ethanol selling price in this study is higher than the previously reported MESP of 19.58 Baht/L (approximated 2.2 USD per gal) (Zhang et al., 2010. Vicari et al., 2012), the MESP in our process may not be comparable with their processes due to the difference in process flowsheet, production scale (demo-scale vs. industrial scale), feedstock composition and raw materials used (sugarcane bagasse vs. corn stover). Nevertheless, the techno-economic framework reveals that the estimated minimal ethanol selling price of yeast consortium fed-batch process was relatively closed to the current selling price of ethanol from cassava-based process (27.19 baht/L, www.thaiethanol.com). Thus, the high-solid, fed-batch process platform using *S. stipitis/S. cerevisiae* consortium technology is suitable to meet the economic demand of large-scale ethanol production process, thereby replacing the cassava-based ethanol production process in Thailand. It should be noted that the fully integrated process simulation should extend beyond upstream and downstream steps by incorporating all

unit operations for processing biomass feedstock prior to pretreatment (e.g. preprocessing, transportation and storage), waste treatment, waste re-utilization after end-product recovery including energy and heat integration of lignin processing step (e.g. combustion of solids/lignin waste for electricity generation).

4.13 Material and energy requirements of optimized fed-batch yeast co-culture

An overview of materials and utilities requirements per 1 dry ton sugarcane bagasse for the fed-batch saccharification and fermentation process with *S. stipitis/S. cerevisiae* co-culture is shown in Table 7 and Fig. 10.

Table 7. Raw material and utility requirements for the conversion of 1 dry ton sugarcane bagasse to ethanol by *S. stipitis/S. cerevisiae* yeast consortium in optimized fed-batch saccharification and ethanol fermentation process.

Raw materials	**Per 1 dry ton bagasse**	
Process water	13.13	ton
H_2SO_4	0.021	ton
KOH	0.041	ton
Molasses	2.05	ton
Enzymes	0.18	ton
Salts	0.014	ton
Yeast extract	0.002	ton
Utility requirements		
Cooling water	178	ton
Chilled water	201.78	ton
Steam	4.13	ton
Electricity	8.48	GJ
Cleaning water	12.18	ton
Products		
Ethanol	0.25	ton
CO_2[1]	2.13	ton
Waste water	11.55	ton
Ethanol yield[2]	250 kg-etoh/ton-bagasse	

[1] CO_2 is sale as byproduct income.

[2] The value is estimated from upstream process only thereby excluding the loss during downstream steps.

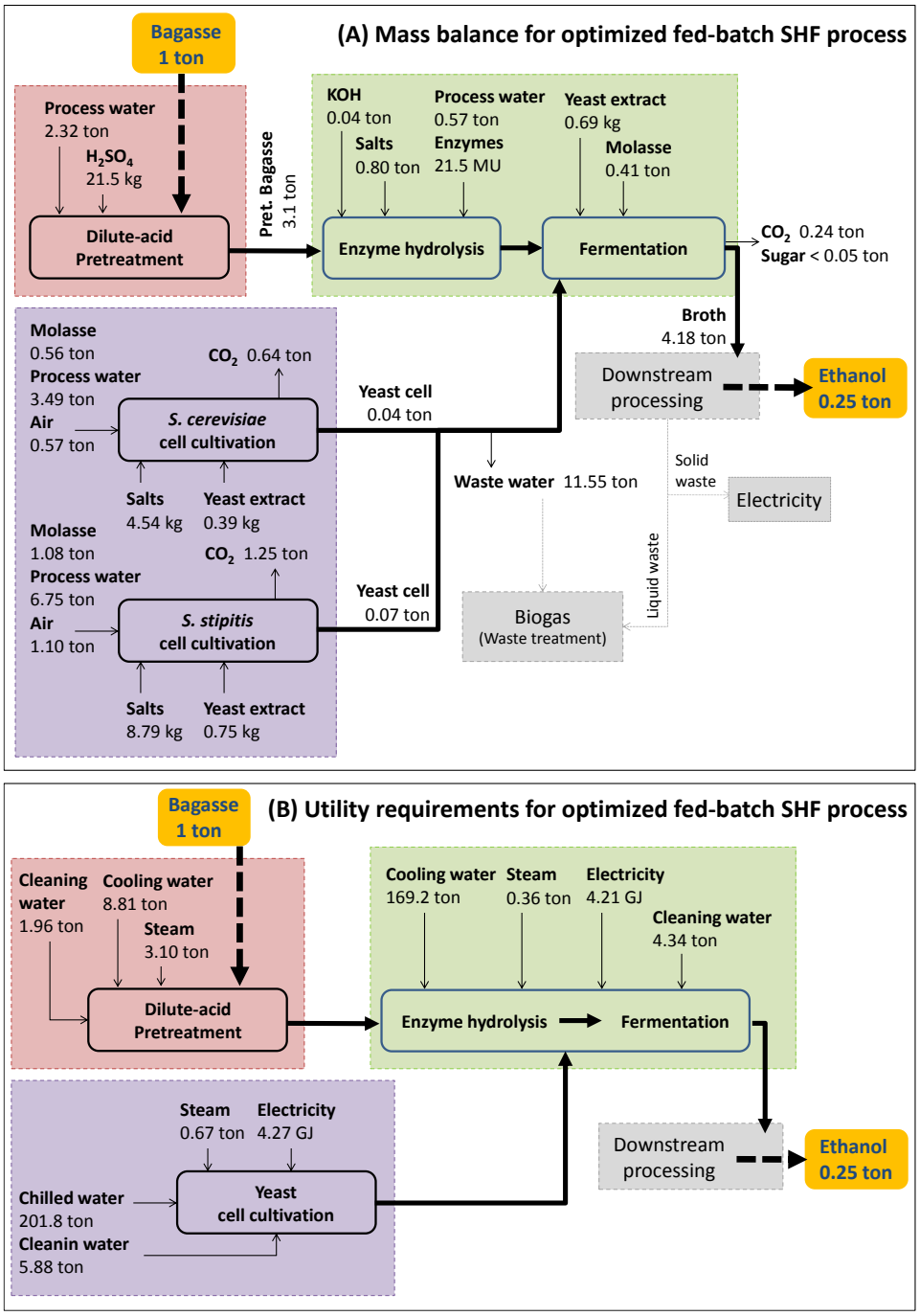

Figure 10. Summary of mass and utility requirements for the conversion of sugarcane bagasse to ethanol using optimized fed-batch saccharification and ethanol fermentation (fed-batch SHF) of *S. stipitis*/*S. cerevisiae* yeast consortium. All values are based on per 1 dry ton sugarcane bagasse.

For every one ton of dry bagasse being processed, 2.13 tons of carbon dioxide was generated (Table 7). Therefore, the co-product credit would have a significant effect on the overall process economy. Upgrading the CO_2 byproduct into valued-added chemicals through catalytic or biological conversion process would increase income and profit margin of the current integrated process. In addition, based on material flow balance, 13.13 ton of process water is required for upstream processing of 1 ton of raw sugarcane bagasse.

To reduce the plant makeup water and further improve process economic, major part of the process water required could be replaced with steam condensates without affecting ethanol production yield. Integrating the wastewater treatment would also reduce the utility cost for steam and electricity which are the two major cost of the process (Fig. 11B) making the sugarcane bagasse-to-ethanol process more cost effective and energy efficient in the economic outcome. Spent and other liquid waste from the yeast propagation and downstream steps could be used for biogas (e.g. methane) production in anaerobic digestion which can be used for steam generation. The solid waste containing the yeast could also be utilized as cattle feed. Furthermore, the solid, lignin-rich waste obtained from the downstream process is a co-product that can be dried and re-used as a solid fuel for electricity generation, underlining the importance of lignin recovery.

4.14 Cost distribution analysis

Cost distribution analysis for identifying various process bottlenecks that decrease the efficiency of yeast consortium fed-batch process economic is demonstrated in Fig. 11. In Fig. 11A, the cost of enzymes constituted 45% of the raw material costs, to which alkaline used for detoxification was also the second contributor of 22% as has also been observed in several previous reports (Wingren et al., 2003; Zhuang et al., 2004). Since the enzyme cost is the main concern for the ethanol production from sugarcane bagasse, the on-site enzyme production or reducing enzyme usage in the process seems most reasonable to offer economic advantages. The production of enzymes on-site could be done using fungus cell or genetic engineered yeasts (Kovacs et al., 2009; Puseenam et al., 2015). The advantage of the production of enzymes on-site includes eliminating transportation and the need to add stabilizers to reduce enzyme degradation during storage. However, multiple steps for on-site enzyme production would add to the overall process cost including operating cost, cost of cell removal, enzyme concentrating and purifying steps. Hence, techno-economic feasibility analysis is required to demonstrate what yield and production cost tradeoffs occur when enzymes are produced on-

site. Increasing enzymes activity using enzyme enhancer or recycling enzymes for reuse are options for reducing enzyme loading. Future research and development should, therefore, be focused on engineering high-activity and robust enzymes to reduce enzyme loading or facilitate enzyme recycling. The cost of alkali also contributes significantly to the cost of cellulosic ethanol. Thus, from the process economics perspective, the improvement in cell robustness against the inhibitors present in pretreated biomass slurry is a prerequisite to reduce cost on detoxification step. Development of robust yeast cell factory could be accomplished through metabolic engineering or adaptive evolution approaches as being demonstrated in several studies (Cheng et al., 2015; Wallace-Salinas et al., 2013). The robust yeast strains would also make it possible to run enzymatic hydrolysis and fermentation at higher solid loading since one of major limitations of high-solid operation is high concentration of inhibitors which hamper yeast cell growth and fermentation (Unrean et al., 2015).

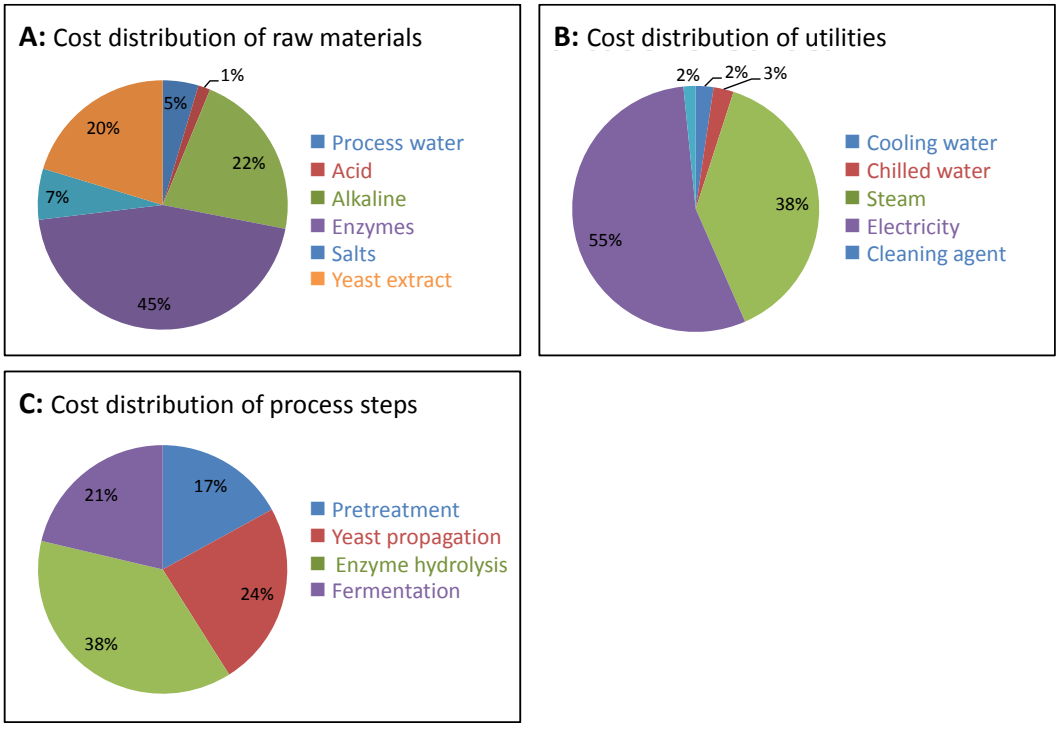

Figure 11. Cost distribution of optimized fed-batch saccharification followed by fermentation using *S. stipitis/S. cerevisiae* yeast consortium. The values are based on upstream processing only.

The cost of electricity and steam energy covers more than 90% of the overall utility cost (Fig. 11B), thereby reducing production could be done by lowering utility cost. Several

alternatives for lower utility cost are recycling steam condensates for increasing energy efficiency, utilizing lignin and other solid wastes as energy source, and integrating biogas production from liquid waste into the process for electricity and steam generation. The analysis in Fig. 11C also points to enzyme hydrolysis as the most expensive processing steps within the conversion of sugarcane bagasse to ethanol process due to high enzyme cost. Comparing among process steps, yeast propagation process was the second largest cost distribution. Therefore, production of yeast from molasses or other waste products is necessary for cost saving. On-site yeast production is also more economical compared to purchasing dried yeast for use in the process (results not shown). Further reduction of yeast propagation cost should be implementing high-cell-density fermentation to maximize yeast production while minimize operating cost. Engineering robust cell that could grow and propagate in high solid lignocellulose-based process is another process option to reduce the amount of yeast addition into the process.

4.15 Process sensitivity analysis

Techno-economic process simulation was made to study and understand process sensitivity. Sensitivity analyses on two major contributor to the overall process cost, enzyme loading and final ethanol titer, were performed to provide information on potential cost reduction for each parameter. Fig. 12 shows the relative effects of enzyme loading and ethanol titer on the overall process economy in term of minimal ethanol selling price (MESP). Assuming the same levels of enzyme hydrolysis yield and other parameters remain unchanged, reducing the amount of enzyme during the fed-batch enzymatic saccharification from 15 FPU/WIS to 4 FPU/WIS reduced MESP by 6% leading to a 4-fold increase in profit margin when compared with the current selling price of ethanol in the local market (Fig. 12A). The result is another confirmation that reduction of enzyme loading is necessary for economic feasibility of the sugarcane bagasse-to-ethanol process.

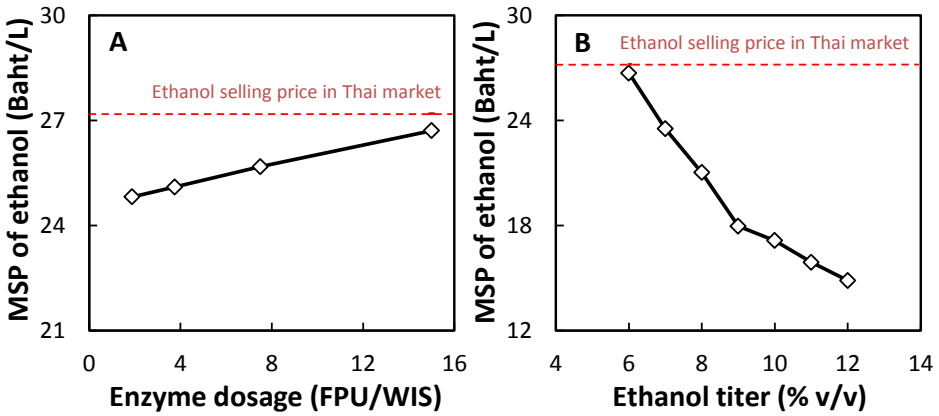

Figure 12. Process sensitivity analysis examining impact of enzyme dosage and final ethanol titer on the minimum ethanol selling price (MESP) for fed-batch enzyme hydrolysis and fermentation with *S. stipitis/S. cerevisiae* yeast consortium. Dashed line represents current ethanol selling price from casava-based process (the 2016 average selling price of ethanol in Thai market, www.thaiethanol.com) for comparison purpose.

An increase in the final ethanol titer from 6% to 12%, while all other parameters are kept constant, would reduce the MESP significantly up to 44% (Fig. 12B). Reducing the production cost and MESP can be explained by the lower energy demand in downstream processing at higher titer of ethanol. As been studied previously, doubling the ethanol titer from 2.5 to 5% could reduce the energy required in distillation by 33% (Sassner et al., 2008). It should be noted that increasing final ethanol titer shows a larger decrease in MESP than reducing enzyme usage indicating that the ethanol titer is the factor with the most control over the overall production cost. Higher ethanol titer could be accomplished either by improving ethanol yield in the yeast cell through genetic or evolutionary engineering or by increasing concentration of solid loading in enzyme hydrolysis followed by fermentation. One option of engineering yeast with improved ethanol yield could be based on the utilization synthetic biology or systems metabolic engineering to redirect more fluxes towards ethanol synthesis (Unrean et al., 2012; Trinh et al., 2008). It is also expected that the ethanol yield may be decreased when the solid concentration is increased due to increasing toxicity and stress caused by high viscosity of high-solid content as reported previously (Koppram et al., 2014). Thus, having an efficient and robust yeast cell is desirable and essential trait for economical lignocellulose-based process. Additionally, as the concentration of solid-content increases, it is the authors' belief, that a

specialized reactor for high-solid operation may be necessary. This has been demonstrated in experimental results (He et al., 2014; Palmqvist et al., 2011; Zhang et al., 2010) showing stirred tank reactor with helical impellers could provide sufficient mixing at solid load as high as 30% (w/v). In addition, uncertainty from various sources such as change in market or operational parameters should be included into the future process integration model for evaluation of the impact of uncertainties affecting the overall production cost in order to improve a degree of realism of the simulated process (Vicari et al., 2012; Morales-Rodriguez et al., 2011).

5. Output ที่ได้จากโครงการ

In this study, we have implemented a model-based strategy to rationally design an optimized co-culture capable of efficiently converting glucose-xylose mixture into ethanol. Specifically, a consortia consisting of two yeast strains of *S. stipitis* and *S. cerevisiae* was modeled. The *S. stipitis*/*S. cerevisiae* co-culture kinetic model was applied to systematically assess ethanol fermentation kinetics under different cell ratio and to predict the optimal cell ratio for maximized conversion of sugarcane bagasse to ethanol. The results prove the efficiency of optimized co-culture based on the model-based design for increasing ethanol titer and productivity. The adjustability of co-culture is also a very appealing characteristic permitting an efficient fermentation of all types of lignocellulosic biomass feedstock by varying co-culture cell ratio to match with the composition of sugar mixture available. Through model-based design of feed profiles in fed-batch process, high insoluble solid loading up to 22% (w/v) has also been reached, which otherwise could not be achieved in batch process. Ethanol fermentation following the fed-batch enzyme hydrolysis using optimal cell ratio of *S. stipitis* and *S. cerevisiae* yeast consortium led to high ethanol titer up to 60 g/l, a 1.4-fold improvement compared to single-strain batch process. In addition, a mass and energy balance based on process flowsheet simulation for design of a sugarcane bagasse-to-ethanol process suitable for large scale production was performed. The flowsheet process modeling pointed to a potential ethanol production cost reduction via future improvements to yeast robustness, reduction of enzyme cost (e.g. on-site production), low enzyme usage (e.g. increasing specific activity or enzyme recycling) and increment of ethanol titer during fermentation by increasing solid content which will make the sugarcane bagasse-to-ethanol conversion process using fed-batch and yeast consortium technology economic feasible for industrialization. Hence, this work validates the application of kinetic modeling tool to aid the design and optimization of yeast consortium fed-batch process for efficient lignocellulosic ethanol fermentation. Techno-economic analyses also provided insights into the effect of operational conditions on process economics and possible process integrations of yeast consortium for minimized total ethanol production cost.

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6. ภาคผนวก

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Model-based optimization of *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* co-culture for efficient lignocellulosic ethanol production

Pornkamol Unrean* and Sutamat Khajeeram

Abstract

Background: The utilization of both C_6 and C_5 sugars is required for economical lignocellulosic bio-based processes. A co-culture system containing multiple strains of the same or different organisms holds promise for conversion of the sugar mixture available in different lignocellulosic feedstock into ethanol.

Results: Herein a co-culture kinetic model has been developed which can describe the co-cultivation of *S. stipitis* and *S. cerevisiae* for ethanol fermentation in mixed C_6/C_5 sugars. The predicted fermentation kinetics and ethanol production performance agreed well with experimental results, thus validating the model. The co-culture kinetic model has been implemented to design the optimal cell ratio for efficient conversion of rice straw or sugarcane bagasse feedstock into ethanol. The results reveal that the optimal co-culture system could enhance ethanol titer by up to 26 %, and ethanol productivity by up to 29 % compared to a single-strain culture. The maximum ethanol titer and productivity reached by the optimized co-culture was 46 and 0.49 g/l h, respectively.

Conclusion: The co-culture model described here is a useful tool for rapid optimization of *S. stipitis*/*S. cerevisiae* co-culture for efficient and sustainable lignocellulosic ethanol production to meet the economic requirements of the lignocellulosic ethanol industry. The developed modeling tool also provides a systematic strategy for designing the optimal cell ratio of co-culture, leading to efficient fermentation of the C_6/C_5 sugars available in any biomass feedstock.

Keywords: Systematic co-culture optimization, Co-culture kinetic model, Second generation bioethanol, Mixed sugar fermentation

Background

Low-priced, abundant and renewable lignocellulosic biomass has become an attractive alternative to significantly supplement corn and starch as a fermentation feedstock for production of bioproducts (FitzPatrick et al. 2010; Kircher 2012). The sustainable use of lignocellulose resources for production of ethanol as transportation fuel would not only promote the bio-based economy but also provide energy security and environmental protection (Binod et al. 2010; Lopes 2015). Although lignocellulosic biomass has many desirable features as an alternative feedstock, the conversion process of biomass into ethanol is challenging. The hydrolysis of lignocellulosic materials releases a mixture of C_6 (mainly glucose) and C_5 (mainly xylose) sugars that must be converted by organisms into ethanol (Sun and Cheng 2002). In addition, the glucose and xylose composition in biomass feedstock can be varied between 30–50 % and 10–25 % of dry weight, respectively, depending on the type of biomass feedstock (http://www.afdc.energy.gov/biomass/progs). The fluctuation of sugar composition in lignocellulosic biomass

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strongly affected the fermentation performance since an organism may not be able to optimally adjust its fermentation capacity to match the change in sugar composition. The economic success of lignocellulose-based ethanol production would, therefore, require a culture system able to handle the variation of sugar composition and efficiently ferment the sugar mixture into ethanol at high titer and productivity to meet the technical and economic requirements of the ethanol industry.

Saccharomyces cerevisiae, which is currently used in the ethanol production process, is unable to utilize xylose effectively. Despite many attempts of genetic engineering the yeast cell for C_6/C_5 co-fermentation, many genetically engineered strains, as reported in several cases, suffer from limited enzyme activity in the pentose metabolism resulting in undesired production of side products and low yield and productivity of ethanol (Wisselink et al. 2007; Bera et al. 2010; Konishi et al. 2015). On the contrary, a mixture of two yeast strains, one capable of fermenting C_6 and another capable of fermenting C_5 is expected to act in concert leading to an efficient C_6/C_5 co-fermentation. The lignocellulosic ethanol production using co-culture strategy is a promising technology for industrial application as it can enhance ethanol titer and yield, shorten fermentation time, and reduce production cost (Chen 2011; Wan et al. 2012). We have previously shown that the co-culture of multiple strains is preferred over the culture of a single strain for mixed sugar fermentation (Unrean and Srienc 2010; Suriyachai et al. 2013). Unlike the single-strain culture, the co-culture containing two C_6- and C_5-fermenting strains can be adjusted in the cell inoculum ratio of each strain used for efficient ethanol fermentation. This makes the co-culture an adjustable system to efficiently ferment C_6/C_5 sugar mixture at minimal fermentation time and at high titer and productivity, thereby resulting in less production time and cost.

The most commonly used co-culture is the combination of *S. stipitis* with *S. cerevisiae* which has been demonstrated as a strategy for efficient conversion of glucose and xylose. The use of *S. stipitis/S. cerevisiae* co-culture has previously shown to enhance ethanol production at a faster rate and a higher titer than single-strain culture (Yadav et al. 2011; Li et al. 2011; Suriyachai et al. 2013; Hickert et al. 2013). The fermentation performance of *S. stipitis/S. cerevisiae* co-culture for lignocellulosic ethanol production is strongly dependent on the cell ratio of the two strains. Although, in co-culture, the overall fermentation kinetics can be optimized by varying the relative proportion of each strain in the culture, it still remains unclear how to rapidly determine the optimal cell ratio of co-culture required for optimally handling sugar mixtures available in different types of biomass feedstock. There are few studies that examined the effect of co-culture cell ratio and optimized cell ratio to maximize fermentation performance. However, the cell ratio optimization of co-culture was mostly relied on trial and errors and statistical analysis where large number of experiments is required (Ashoor et al. 2015; Karagöz and Özkan 2014; Suriyachai et al. 2013). This approach is cost and labor intensive as a new set of experiment has to be conducted every time sugar composition in the feedstock changes. Alternative approach to optimize co-culture is to develop the co-culture kinetic model which can be used to identify optimal cell ratio needed for each type of feedstock that contains different sugar composition.

As a result, in this work, we developed a kinetic modeling tool that can describe the fermentation kinetics of a *S. stipitis*/*S. cerevisiae* co-culture in mixed glucose–xylose fermentation. The developed modeling tool was applied to design optimal cell ratio of *S. stipitis*/*S. cerevisiae* co-culture enabling improved ethanol productivity and titer compared to single-strain culture of *S. cerevisiae* or *S. stipitis* that is not able to efficiently utilize sugar mixture. The validated model was applied for the design of optimal cell ratio for an efficient ethanol fermentation of rice straw and sugarcane bagasse by the co-culture.

Methods

Strain and media

Saccharomyces cerevisiae (Thermosacc® Dry yeasts; Lallemand, Milwaukee, WI, USA) and *Scheffersomyces stipitis* CBS6054 (ATCC 58785) was used in this study. The culture was maintained at 4 °C on YPD agar plate consisting of 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose and 25 g/l agar. Lignocellulosic biomass used in this study was steam pretreated with 0.5 % (w/v) H_2SO_4 at 121 °C for 30 min. The pretreated biomass slurry was used for all SSF experiments.

Batch fermentation

Batch fermentation was carried out in YE medium containing 0.1 M potassium phosphate buffer, 1 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 0.1 g/l $CaCl_2$, 0.1 g/l NaCl, 0.5 g/l $MgSO_4$, 1 g/l KH_2PO_4, 15 g/l glucose and 5 g/l xylose. The sugars were autoclaved separately and added into the medium prior to use. Fermentation experiments were carried out in 2-l Braun bioreactor (Biostat MD, B. Braun Biotech International, Melsungen, Germany) containing 1 l of culture media. The yeast cell was grown overnight in YE medium at 30 °C with agitation rate of 100 rpm in incubator shaker (Innova 4340, New Brunswick, USA) prior to inoculation into bioreactor. For co-culture, each yeast strain was prepared separately and inoculated into bioreactor according to the specified cell ratio. All batch fermentation experiments were began with the same initial OD_{600} of approximately 0.2, equivalent to an initial

cell concentration of 0.1 g cell/l. The fermentation condition was as previously described in Unrean and Nguyen (2012). Samples were taken periodically for sugars, ethanol and cell concentration measurement.

Simultaneous saccharification and fermentation

Batch simultaneous saccharification and fermentation (SSF) was carried out in 250-ml Erlenmeyer flask containing 10 % WIS (for pretreated sugarcane bagasse) or 6 % WIS (for pretreated rice straw), 0.75 g/l $(NH_4)_2SO_4$, 0.35 g/l KH_2PO_4, 0.07 g/l $MgSO_4{\cdot}7H_2O$, 1 g/l yeast extract, Cellic C-Tec II enzymes (Novozymes, Denmark) and yeast cell. The concentration of WIS content chosen for the experiment is the maximum concentration for each feedstock that can be operated in a standard stirred-tank bioreactor without mixing problem. The pH of the mixture was initially adjusted to 5 using 4 M KOH. Yeast cell from seed culture and enzyme were added to the pretreated biomass mixture at 0.02 g cell/g WIS cell loading and 25 FPU/WIS enzyme dosage, respectively, to initiate SSF process. Yeast cell used in SSF was cultured in YPD media or in molasses media [10 %(v/v) molasses, 0.75 g/l $(NH_4)_2SO_4$, 0.35 g/l KH_2PO_4, 0.07 g/l $MgSO_4{\cdot}7H_2O$, 1 g/l yeast extract] for 24 h at 30 °C with a shaking speed of 200 rpm. Seed culture was harvested by centrifugation at 5100 rpm for 5 min and resuspended in the culture media before being used for SSF experiments. The SSF culture was incubated at 35 °C, agitation rate of 200 rpm in incubator shaker (Innova R43, New Brunswick, USA) with no pH control during the cultivation. Culture samples were withdrawn periodically for measurement of residual sugars and ethanol product.

Analysis

Cell concentration Concentration of yeast cell was measured via optical density at 600 nm wavelength (OD_{600}) using spectrophotometer (DR/2500, Hach Company, Singapore). The cell dry weight was estimated using the correlation: cdw (g/l) = 0.5 × OD_{600}. To reduce interference by culture media during OD measurement, the culture sample was centrifuged at 5100 rpm for 5 min and the supernatant was discarded. The cell pellet was washed with deionized water before measuring the optical density.

Analysis of sugar and ethanol Samples were centrifuged at 5100 rpm for 5 min and the supernatant was collected and filtered using 0.2 μm sterile filter. The samples were stored at −20 °C prior to the analysis. Concentrations of sugar (glucose and xylose) and ethanol were measured by HPLC equipped with Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a refractive index detector (RID-10A) at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The concentration of sugar and ethanol was calculated from calibration curve correlating area and concentration of standard solution.

Yield and rate calculation

Ethanol yield was calculated by dividing gram of total ethanol produced by gram of total sugar available in the culture. The ethanol productivity was determined as the overall rate by dividing total ethanol concentration produced by time of its production.

Theory

Kinetic model of co-culture containing S. stipitis and S. cerevisiae

A kinetic model describing the mixed culture of *S. stipitis* (strain 1) and *S. cerevisiae* (strain 2) in a sugar mixture was developed. The kinetic growth of each strain is described as

Strain 1:

$$\frac{dX_1}{dt} = \mu_1 X_1 \tag{1}$$

Growth on glucose

$$\mu_1(C_G) = \frac{\mu_{\max G,1} C_G}{K_{\mathrm{mu1},G} + C_G} \tag{2}$$

Growth on xylose

$$\mu_1(C_{\mathrm{Xy}}) = \frac{\mu_{\max \mathrm{Xy},1} C_{\mathrm{Xy}}}{K_{\mathrm{mu1,Xy}} + C_{\mathrm{Xy}}} \tag{3}$$

Strain 2:

$$\frac{dX_2}{dt} = \mu_2 X_2 \tag{4}$$

Growth on glucose

$$\mu_2(C_G) = \frac{\mu_{\max G,2} C_G}{K_{\mathrm{mu2},G} + C_G} \tag{5}$$

where X_i is the cell concentration of strain i, t is fermentation time, μ_i is specific cell growth rate, $\mu_{\max,i}$ is maximum specific growth rate, $K_{\mathrm{mu}i}$ is saturation constant and C_G and C_{Xy} are the concentrations of glucose and xylose, respectively. The subscript i represents *S. stipitis* (1) and *S. cerevisiae* (2) strain, respectively. It should be noted that only *S. stipitis* is able to grow on xylose.

In a co-culture of strain 1 and 2, the initial cell ratio f is represented by

$$X_{1,0} = fX_{2,0} \tag{6}$$

The balance equation for each sugar is given by
Glucose consumption

$$\frac{dC_G}{dt} = q_{G,1} X_1 + q_{G,2} X_2 \tag{7}$$

$$q_{G,i} = \frac{V_{\max G,i} C_G}{K_{mG,i} + C_G} \frac{1}{1 + C_{E0}/K_{EG,i}} \frac{1}{1 + C_{A0}/K_{AG,i}} \tag{8}$$

where $q_{G,i}$ is specific uptake rate of glucose of strain i which follows Michaelis–Menten kinetics. Inhibition terms are added to represent ethanol inhibition and acetate inhibition on glucose consumption. Kinetic parameters describing glucose consumption are $V_{\max G}$ maximum glucose uptake rate, K_{mG} saturation constant for glucose uptake, C_{E0} initial concentration of ethanol, K_{EG} ethanol inhibition constant for glucose uptake, C_{A0} initial concentration of acetate, and K_{AG} acetate inhibition constant for glucose uptake. Xylose consumption

$$\frac{dC_{Xy}}{dt} = q_{Xy,1}X_1 \tag{9}$$

$$q_{Xy,1} = \frac{V_{\max Xy,1}C_{Xy}}{K_{mXy,1} + C_{Xy}} \frac{1}{1 + C_{Xy}/K_{GXy,1}} \frac{1}{1 + C_{E0}/K_{EXy,1}} \frac{1}{1 + C_{A0}/K_{AXy,1}} \tag{10}$$

where C_{Xy} is the concentration of xylose. $q_{Xy,1}$ is specific xylose uptake rate of *S. stipitis* (strain 1) based on Michaelis–Menten kinetics. Kinetic terms are added to represent glucose repression, ethanol inhibition and acetate inhibition on xylose consumption. Kinetic parameters describing xylose consumption are $V_{\max Xy}$ maximum xylose uptake rate, K_{mXy} saturation constant for xylose uptake, K_{GXy} glucose repression constant for xylose uptake, K_{EXy} ethanol inhibition constant for xylose uptake, and K_{AXy} acetate inhibition constant for xylose uptake. It should be noted that $q_{Xy,2}$ is zero since *S. cerevisiae* (strain 2) cannot consume xylose.

Ethanol synthesis

$$\frac{dC_E}{dt} = Y_{EG,1}q_{G,1}X_1 + Y_{EG,2}q_{G,2}X_2 + Y_{EXy,1}q_{Xy,1}X_1 \tag{11}$$

$$R_{Etoh} = \frac{C_{E,final}}{t_{exhaust}} \tag{12}$$

where C_E is ethanol concentration and $C_{E,final}$ is the final ethanol concentration after all sugars are exhausted. $Y_{EG,i}$ and $Y_{EXy,i}$ are the ethanol yield on glucose and xylose, respectively. R_{Etoh} is the overall ethanol productivity; $t_{exhaust}$ is exhaustion time which is the times when all sugars are consumed. The exhaustion time for single-strain culture and co-culture can be computed by solving Eqs. (1)–(10) simultaneously.

To simulate co-culture in SSF process, the developed co-culture kinetic model was integrated with enzymatic hydrolysis model. The hydrolysis model used in this study was modified based on the model proposed by South et al. (1995). The model includes adsorption of enzyme, hydrolysis of cellulose and xylan with inhibition by glucose and time profiles of releasing glucose and xylose. The enzyme adsorption was described by second-order kinetics as follows,

$$\frac{dE_{ad}}{dt} = k_{ad}\left(E_{load}\frac{IS_0}{IS} - E_{ad}\right)^2 \tag{13}$$

where E_{load} and E_{ad} are the total enzyme and the absorbed enzyme on water insoluble solid substrate (WIS); k_{ad} is the adsorption rate constant; IS_0 and IS are initial WIS concentration and WIS concentration at time t, respectively.

Hydrolysis rate of cellulose to glucose is represented by a conversion-dependent rate equation reflecting cellulase–cellulose complex dependent hydrolysis,

$$\frac{dC_{Cellulose}}{dt} = k_H\left(\frac{E_{ad}C_{Cellulose}}{1 + C_G/K_G}\right) \tag{14}$$

where $C_{Cellulose}$ is the concentration of cellulose. k_H and K_G are cellulose hydrolysis rate constant and inhibition constant of cellulose hydrolysis by glucose, respectively.

The hydrolysis of xylan to xylose is based on the following correlation,

$$\frac{dC_{Xylan}}{dt} = k_H\frac{C_{Xylan}}{C_{Cellulose}}\left(\frac{E_{ad}C_{Cellulose}}{1 + C_G/K_G}\right) \tag{15}$$

Based on hydrolysis rate, the kinetic rate of releasing glucose and xylose are

$$\frac{dC_G}{dt} = 1.111k_H\left(\frac{E_{ad}C_{Cellulose}}{1 + C_G/K_G}\right) \tag{16}$$

$$\frac{dC_{Xy}}{dt} = 1.136k_H\frac{C_{Xylan}}{C_{Cellulose}}\left(\frac{E_{ad}C_{Cellulose}}{1 + C_G/K_G}\right) \tag{17}$$

All kinetic parameters describing enzyme hydrolysis, cell growth and fermentation of sugars used in this simulation are summarized in Table 1. These parameters are obtained from literatures (Hanly and Henson 2014; Unrean and Franzen 2015) or from experimental data by adjusting the values with minimized weighted sum of the squared errors such that the predictive concentration time profiles of yeast cell, sugars and ethanol are in agreement with the values observed experimentally in batch fermentation of single-strain culture (Fig. 1a, b). This fitting is based on the bisquare weights method which is used for determining the parameters that fit the measured values using the usual least-squares approach, and that minimize the effect of outliers. Concentration profiles for glucose, xylose, and ethanol are obtained by solving the differential equations Eqs. (1)–(17) numerically using ODE45 function in MATLAB software

Table 1 Kinetic parameters used in co-culture kinetic model

Symbol	Parameter	Strain[a]		References
Fermentation kinetics		***S. cerevisiae***	***S. stipitis***	
$V_{maxG,i}$	Maximum rate for glucose uptake (g/g h)	2.90	0.77	This study
$K_{mG,i}$	Saturation constant for glucose uptake (g/l)	0.5	0.5	Hanly and Henson (2014)
$K_{EG,i}$	Ethanol inhibition constant for glucose uptake (g/l)	10	10	Hanly and Henson (2014)
$K_{AG,i}$	Acetate inhibition constant for glucose uptake (g/l)	7.5	7.5	Hanly and Henson (2014)
$V_{maxXy,i}$	Maximum rate for xylose uptake (g/g h)	0	0.06	This study
$K_{mXy,i}$	Saturation constant for xylose uptake (g/l)	0.25	0.25	Hanly and Henson (2014)
$K_{GXy,i}$	Glucose repression constant for xylose uptake (g/l)	0.25	0.25	Hanly and Henson (2014)
$K_{EXy,i}$	Ethanol inhibition constant for xylose uptake (g/l)	4.5	4.5	Hanly and Henson (2014)
$K_{AXy,i}$	Acetate inhibition constant for xylose uptake (g/l)	0.2	0.2	Hanly and Henson (2014)
$\mu max_{G,i}$	Maximum specific growth rate for glucose uptake (1/h)	0.3	0.15	This study
$K_{mui,G}$	Saturation constant for growth on glucose (g/l)	0.5	0.5	This study
$\mu max_{Xy,i}$	Maximum specific growth rate for xylose uptake (1/h)	0.0	0.01	This study
$K_{mui,Xy}$	Saturation constant for growth on xylose (g/l)	0.5	0.5	This study
Symbol	**Parameter**	**Strain[a]**		**References**
Enzyme hydrolysis kinetics		**Rice straw**	**Bagasse**	
k_{ad}	Adsorption rate constant (g/FPU h)	0.43	0.43	This study
k_H	Hydrolysis rate constant (g/FPU h)	0.06	0.02	This study
K_G	Inhibition constant (g/L)	1.47	1.47	This study

[a] The subscript *i* is 1 for *S. stipitis* strain and 2 for *S. cerevisiae*

(Mathworks, Natick, MA, USA). The co-culture kinetic model was utilized for predicting and optimizing co-culture fermentation. Specifically, the model was used to determine optimal cell ratio to maximize ethanol production for each type of biomass feedstock containing different composition of glucose and xylose.

Results and discussion

An efficient co-fermentation of glucose and xylose can be achieved by selecting a proper combination of cell ratio in co-culture system. Since the consumption of C_6/C_5 sugar mixture depends on the composition of the two strains used in the culture, the optimal cell ratio of co-culture can be varied with varying composition of sugars available in different sources of biomass feedstock. A kinetic model is, therefore, a convenient tool for designing optimum co-culture cell fraction. In this study, a kinetic model for *S. stipitis*/*S. cerevisiae* co-culture has been developed and implemented to systematically design co-culture system capable of efficiently converting sugar mixture from rice straw or sugarcane bagasse feedstock into ethanol.

Development of co-culture kinetic modeling

Mixed culture of *S. stipitis* and *S. cerevisiae* can be used to selectively adjust the fermentation kinetics of mixed C_6/C_5 sugars resulting in an optimal co-fermentation of the sugar mixture. Kinetic model describing cell growth and glucose and xylose utilization of co-culture was developed based on balance equations as described in Theory section. The model included cell growth of *S. stipitis* and *S. cerevisiae*, sugar consumption based on batch fermentation, inhibition effect of glucose on xylose, inhibition effect by ethanol and acetic acid, which is common inhibitor present in lignocellulosic feedstock. Kinetic parameters used in the model are summarized in Table 1 which were obtained either from previous literatures (Hanly and Henson 2014; Unrean and Franzen 2015) or from batch fermentation experiment of each strain in this study based on minimization of a weighted sum of the squared errors. The developed model was first utilized to predict cell growth and fermentation kinetics of *S. stipitis*, *S. cerevisiae* and *S. stipitis*/*S. cerevisiae* co-culture at cell ratio (f) of 1.0 in batch fermentation containing glucose–xylose mixture (Fig. 1). Glucose represents C_6 sugar whereas xylose represents C_5 sugar in biomass feedstock. The experiments were performed under the same initial cell concentration of 0.1 g cell/l for comparison purpose. The results revealed higher ethanol titer by *S. stipitis*/*S. cerevisiae* co-culture as compared with the single-strain culture of *S. cerevisiae* as xylose was not being utilized by *S. cerevisiae*. No significant improvement in ethanol productivity and titer was observed between the co-culture and the single-strain culture of *S. stipitis*. This is expected

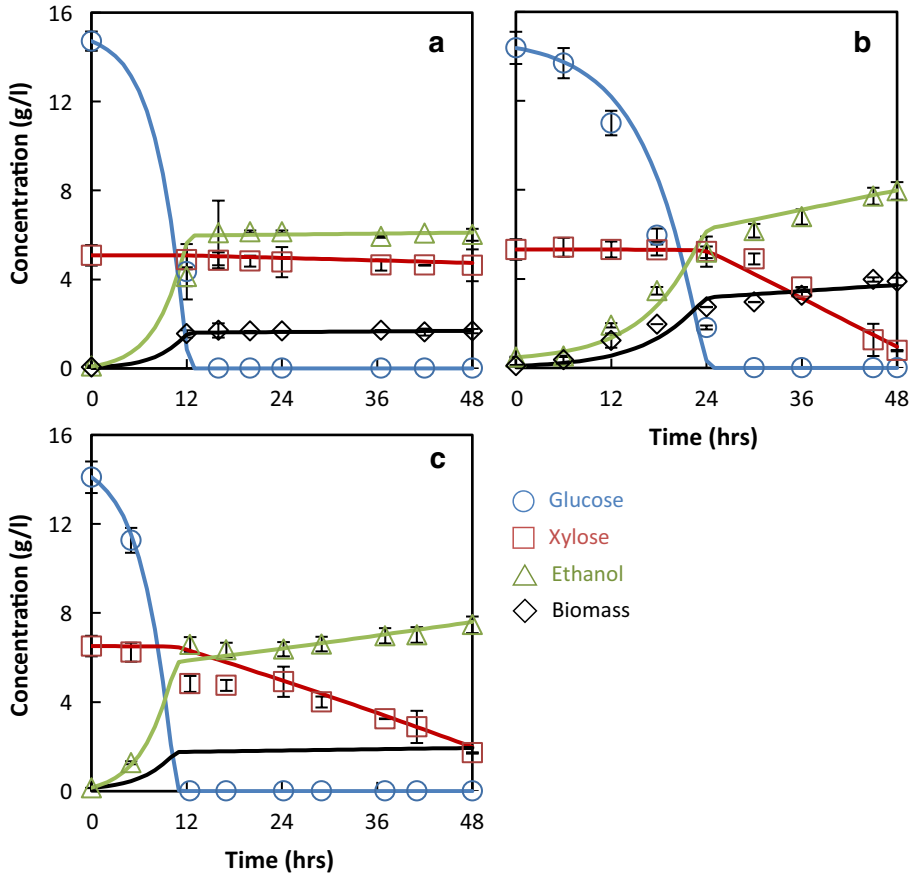

Fig. 1 Fermentation kinetics of *S. cerevisiae* (**a**), *S. stipitis* (**b**) and co-culture of *S. stipitis* and *S. cerevisiae* (**c**) in glucose–xylose mixture. Time profiles of glucose (*blue*), xylose (*red*), ethanol (*green*) and biomass (*black*) in *S. cerevisiae*, *S. stipitis*, and *S. stipitis*/*S. cerevisiae* co-culture at initial cell ratio $f = 1.0$ are shown. A good agreement between measured values (*symbols*) and predicted values (*solid lines*) validates the kinetic model. The results are based on average of duplicate experiments

as the co-culture was not at the optimal cell ratio for available glucose–xylose mixture present. To validate the co-culture kinetic model, predicted fermentation time profiles of *S. cerevisiae*, *S. stipitis* and co-culture based on the developed model were compared with measured values. The experimental results agree well with the model prediction, thus confirming the accuracy of the model in predicting fermentation kinetics of single-strain culture as well as of co-culture. The validated model was then utilized for optimizing cell ratio (f) in co-culture to maximize ethanol productivity and titer such that the co-culture can optimally perform at its best under a given glucose–xylose mixture.

Model-based design of optimal co-culture system

The optimal proportion of each strain in the co-culture would result in the efficient fermentation of mixed sugars. We, therefore, implemented the co-culture kinetic model to establish optimized cell ratio of the *S. stipitis*/*S. cerevisiae* co-culture for improving ethanol production efficiency. The model was first applied to simulate the effect of ethanol productivity and titer as a function of the cell ratio (f) of *S. stipitis*/*S. cerevisiae* co-culture for fermentation of glucose–xylose mixture at a glucose–xylose ratio of 3 (Fig. 2a). The results reveal that the ethanol productivity and titer were affected by the co-culture's cell ratio. Comparing fermentation performance after 48 h by the co-culture at different cell ratio identified the optimal cell ratio for maximization of ethanol production in both productivity and titer, which was consistent with the model prediction confirming the accuracy of the co-culture kinetic model. The results identified the optimal cell ratio (f_{opt}) of *S. stipitis*/*S. cerevisiae* to be 1.70 g *S. stipitis*/g *S. cerevisiae* for the most efficient ethanol production from a glucose–xylose ratio of 3. The co-culture under optimized cell ratio improved ethanol titer up to 35 % compared to the single-strain culture containing *S. cerevisiae* due to a better utilization of mixed

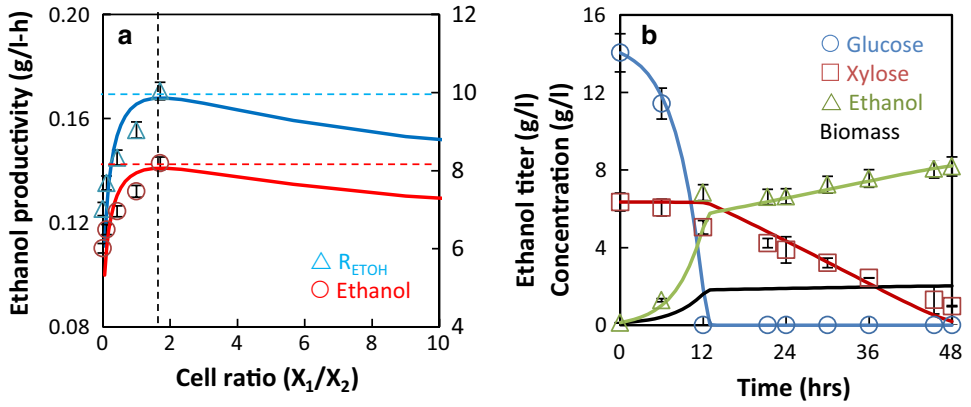

Fig. 2 Ethanol production in mixed glucose–xylose fermentation by *S. stipitis*/*S. cerevisiae* co-culture. **a** Ethanol productivity (*cyan*) and titer (*red*) at various cell ratios of *S. stipitis* (X_1)/*S. cerevisiae* (X_2) co-culture in comparison with single-strain culture of *S. cerevisiae*. The optimal cell ratio ($f_{opt} = 1.70$ g *S. stipitis*/g *S. cerevisiae*) indicated by *dashed line* yields maximum ethanol productivity and titer which is 36 % higher than those achieved by single-strain culture. The measured values (*symbol*) agree well with the values predicted by the co-culture kinetic model (*solid line*). **b** Fermentation kinetics of *S. stipitis*/*S. cerevisiae* co-culture at optimal cell ratio ($f_{opt} = 1.70$). Glucose, xylose, ethanol and biomass are shown in *blue*, *red*, *green* and *black*, respectively. The *symbol* represents experimental measurement which is in good agreement with the model prediction represented by *solid line*. The results are based on average of duplicate fermentation experiments in synthetic medium containing 3:1 glucose–xylose mixture

glucose–xylose by the co-culture. The ethanol productivity of the optimized co-culture was also enhanced by 36 % in comparison to the single-strain culture of *S. cerevisiae* and by 6 % in comparison to the single-strain culture of *S. stipitis*, which also closely agreed with the predicted values. Fermentation kinetics of co-culture under the optimal cell ratio ($f = 1.70$) also agreed well with the kinetic model prediction (Fig. 2b). Thus, the model can provide a better insight into the kinetics of mixed sugar fermentation by single-strain culture as well as by co-culture as shown in Figs. 1 and 2.

Kinetics of C_6/C_5 sugar fermentation by *S. stipitis*/*S. cerevisiae* co-culture shown in Figs. 1c and 2b suggested that by varying cell ratio of co-culture system, the conversion rate of glucose–xylose mixture to ethanol could be adjusted. Thus, the system could be optimized in cell ratio with respect to the change in sugar composition in the biomass feedstock. This was also confirmed in Fig. 2a where different cell ratio of co-culture converted the sugar mixture into ethanol at different ethanol production rate. The outperformed productivity of co-culture system relative to single-strain culture in the mixed sugar fermentation is expected since dedicating one strain to consume all sugar mixture would lead to a longer fermentation time for the completed conversion of all sugars compared to having multiple strains as illustrated in several previous studies (Ashoor et al. 2015; Karagöz and Özkan 2014; Yadav et al. 2011; Suriyachai et al. 2013). The co-culture kinetic model could be used for optimization of co-culture fermentation by predicting an optimal initial cell ratio of the co-culture in any given sugar mixture. It is worth noting that although co-culture strategy has been previously implemented for utilization of C_6 and C_5 sugar, the kinetic model that can describe fermentation performance of the co-culture has not yet been developed. The model developed in this study is considered a useful tool to provide a thorough understanding of the effect of cell ratio of co-culture on ethanol fermentation as well as to identify the optimal operating cell ratio of co-culture that can maximize efficiency of lignocellulosic ethanol fermentation process.

Optimized co-culture system for different biomass feedstock

Unlike single-strain culture, the proportion of cell in the co-culture can be adjusted to match with each available sugar. Thus, the optimal cell ratio would be different as the composition of glucose and xylose changes. To demonstrate the flexibility of co-culture system, the kinetic model was utilized to predict the optimal cell ratio required for different biomass feedstock. We implemented the co-culture model for the prediction of cell ratio to optimally match with a given glucose–xylose composition available in major biomass feedstock as summarized in Table 2. The optimal co-culture cell ratio is the ratio which is required for the most efficient conversion of each type of biomass feedstock into ethanol with maximum ethanol productivity and titer. According to the model prediction, the co-culture under optimized cell ratio could enhance ethanol fermentation

Table 2 Optimal cell ratio of *S. stipitis*/*S. cerevisiae* co-culture for maximized ethanol production from different biomass feedstock predicted by co-culture kinetic model

Type of biomass	Sugar ratio	Opt. cell ratio[a]	R^{b}_{ETOH} (g/l h)		[EtOH][c] (g/l)	
	Glc/Xyl (g/g)	X_1/X_2 (g/g)	Co-culture	Improve (%)[d]	Co-culture	Improve (%)[d]
Rice straw	3.00	1.70	0.17	35	8.00	33
Corn stover	1.89	1.78	0.17	55	9.17	52
Cottonwood	3.23	1.70	0.17	33	7.86	30
Sugarcane bagasse	1.63	1.94	0.18	63	9.68	61
Corn cobs	1.29	1.86	0.19	79	10.65	77
Switch grass	1.45	1.86	0.18	71	10.14	68
Eucalyptus	4.90	1.63	0.17	23	7.22	20
Wheat straw	1.58	1.94	0.18	65	9.80	63

Glucose and xylose ratio for each biomass feedstock is based on biomass feedstock composition and property database

[a] Optimal cell ratio is defined as initial g cell of *S. stipitis* (X_1) per initial g cell of *S. cerevisiae* (X_2). The model simulation for each type of feedstock is based on glucose concentration of 15 g/l and xylose concentration according to sugar ratio for each feedstock for comparison purpose

[b] Rate of ethanol is defined as overall productivity which is total ethanol produced divided by required fermentation time of co-culture under optimal cell ratio. Fermentation time is the time required for completion of all glucose and xylose by co-culture

[c] Ethanol titer of co-culture under optimal cell ratio is predicted by the model based on total glucose and xylose available and 80 % of theoretical yield assumption

[d] Percent improvement is calculated by comparing the performance of co-culture under optimal cell ratio with that of *S. cerevisiae* based on kinetic model

performance by increasing ethanol productivity 23–79 % and increasing ethanol titer 20–77 % depending on the available sugar composition in each biomass feedstock. The enhancement of ethanol production by co-culture compared to a single-strain culture becomes increasingly evident as the available C_5 sugar content in the biomass feedstock increases. For instance, the co-culture could improve ethanol productivity and titer up to 65 % in wheat straw compared to only up to 35 % improvement in rice straw due to more availability of C_5 sugar in wheat straw than in rice straw. The results indicate that the use of optimized co-culture system would be a preferred process especially for feedstock with high C_5 sugar content. The application of model-based design of co-culture permits the design of optimal cell ratio of co-culture for efficient ethanol fermentation from any C_6/C_5 available sugars. The model simulation results also emphasize the benefit of using co-culture system in terms of its adjustability to match with each type of feedstock composing of different sugar ratio, which could not be achieved if the single-strain culture is used.

Lignocellulosic ethanol fermentation performance by co-culture system

The optimal co-culture designed according to the co-culture kinetic model was utilized for simultaneous saccharification and fermentation of rice straw or sugarcane bagasse as feedstock to test the applicability of co-culture system for lignocellulosic ethanol production. These feedstocks were chosen as a case study to explore the ethanol production efficiency by the optimized co-culture in biomass feedstock composing of different glucose/xylose ratio. The optimal ratio between two yeast strains was predicted such that the fermentation rate of each sugar was precisely adjusted to match with given glucose–xylose composition available in the feedstock. Figure 3 shows the effect of co-culture cell ratio (*S. stipitis*/*S. cerevisiae*) on ethanol productivity and fermentation time of rice straw and sugarcane bagasse. Due to different sugar composition, the two feedstocks require different optimal cell ratio. The optimal cell ratio predicted by the model for the fermentation of rice straw and for the fermentation of sugarcane bagasse was 1.70 and 1.94 g cell *S. stipitis*/g cell *S. cerevisiae*, respectively. Effect of cell ratio of co-culture for the fermentation of biomass feedstock illustrated in Fig. 3 also highlights the flexibility of co-culture process for an efficient fermentation of mixed sugars available in a given biomass feedstock by adjusting the operating cell ratio of each strain.

The results in Fig. 4 confirm that the process of co-culture outperformed the process of single-cell culture for the fermentation of sugar mixture in lignocellulosic biomass. Figure 4a, b shows ethanol fermentation performance from sugarcane bagasse of *S. stipitis/S. cerevisiae* co-culture in comparison with the single-strain culture of *S. stipitis* and of *S. cerevisiae*. The results reveal that the use of co-culture under optimized cell ratio yielded up to 26 and 12 % improvement in ethanol titer in pretreated rice straw and in pretreated sugarcane bagasse, respectively, when compared with the use of single-strain culture of *S. cerevisiae* and of *S. stipitis*. Ethanol yield and productivity achieved by co-culture was also higher than those achieved by single-strain culture. Optimal co-culture in rice straw SSF process reached ethanol

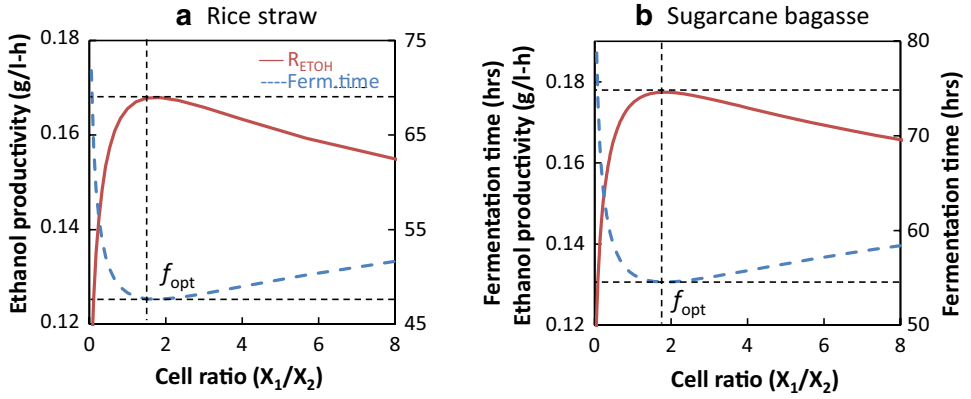

Fig. 3 Predicted ethanol productivity (*solid line*) and fermentation time (*dashed line*) by co-culture kinetic model for **a** rice straw and **b** sugarcane bagasse fermentation. The model identifies optimal cell ratio (f_{opt}) of 1.70 g cell *S. stipitis*/g cell *S. cerevisiae* for rice straw and 1.94 g cell *S. stipitis*/g cell *S. cerevisiae* for sugarcane bagasse to maximize ethanol fermentation

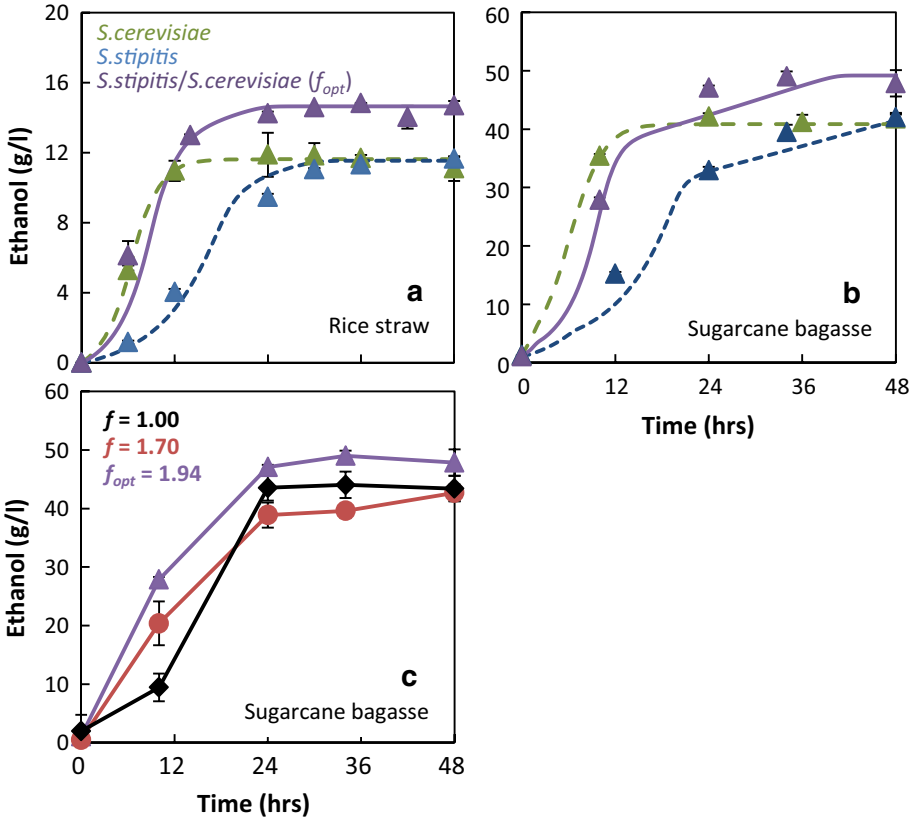

Fig. 4 Saccharification and fermentation of rice straw (**a**) and sugarcane bagasse (**b**) by *S. stipitis*/*S. cerevisiae* co-culture in comparison with single-strain culture. Time profiles of ethanol in *S. stipitis* (*blue*), *S. cerevisiae* (*green*) and *S. stipitis*/*S. cerevisiae* (*purple*) culture are compared. The *symbols* represent measured values while the *lines* represent predicted values based on the kinetic model. **c** Comparison of ethanol production in sugarcane bagasse at different cell ratio, $f = 1.00$, $f = 1.70$ and $f = 1.94$. Experiments were conducted in SSF configuration in which enzymes and yeast cells are added simultaneously into 6 % WIS pretreated rice straw or 10 % WIS pretreated sugarcane bagasse. For comparison purpose, all experiments are initiated with the same total cell concentration of 0.02 g cell/g WIS. The optimized cells ratio for the co-culture as predicted by the model for each type of feedstock is $f_{opt} = 1.70$ for rice straw and $f_{opt} = 1.94$ for sugarcane bagasse

Table 3 Ethanol production performance by single-strain culture of *S. cerevisiae* and *S. stipitis* in comparison with co-culture of *S. cerevisiae*/*S. stipitis* under optimal cell ratio

Strains	[EtOH] (g/l)	R^{c}_{ETOH} (g/l h)	Y^{d}_{ETOH} (g/g)
Rice straw feedstock[a]			
S. cerevisiae	11.10 ± 0.71	0.23 ± 0.01	0.41 ± 0.03
S. stipitis	11.64 ± 0.12	0.24 ± 0.00	0.39 ± 0.00
S. cerevisiae/*S. stipitis*[b]	14.69 ± 0.27	0.31 ± 0.01	0.46 ± 0.00
Sugarcane bagasse feedstock[a]			
S. cerevisiae	41.81 ± 0.64	0.44 ± 0.01	0.33 ± 0.01
S. stipitis	42.05 ± 0.36	0.44 ± 0.00	0.33 ± 0.01
S. cerevisiae/*S. stipitis*[b]	46.68 ± 0.09	0.49 ± 0.00	0.38 ± 0.02

[a] Based on batch SSF of rice straw at 6 % WIS for 48 h and sugarcane bagasse at 10 % WIS for 96 h

[b] Co-culture of *S. cerevisiae* and *S. stipitis* was carried out under optimal cell ratio as predicted by the model (shown in Table 2) for each type of feedstock

[c] Rate of ethanol is defined as overall productivity which is total ethanol produced divided by fermentation time

[d] Ethanol yield is based on total sugar available in the feedstock

yield and productivity at 12 and 29 % higher than those reached by single-strain culture. Similarly, ethanol fermentation of sugarcane bagasse by co-culture achieved approximately 11 % higher ethanol productivity when compared to the performance by single-strain culture. In sugarcane bagasse SSF, the co-culture under optimal cell ratio also produced higher ethanol yield of up to 15 % when compared with the yield achieved by single-strain culture. The co-culture kinetic model (as shown in lines) accurately predicted the ethanol production by the single-strain culture and the co-culture in both feedstocks. Optimal operating cell ratio permits the maximization of ethanol production for each type of feedstock. The experimental results showed that the co-culture performance at 1.94 g/g cell ratio, which is the optimal cell ratio designed specifically for sugarcane bagasse feedstock, resulted in higher ethanol production from sugarcane bagasse than those at other cell ratios (Fig. 4c). Thus, maximizing ethanol production for each type of feedstock specifically requires different cell ratio according to the available sugars of the feedstock used. Lignocellulosic ethanol production performance by co-culture and single-strain culture is summarized in Table 3. Ethanol yield accomplished by co-culture was 90 and 75 % of theoretical yield in rice straw and in sugarcane bagasse fermentation, correspondingly. The highest ethanol titer reached by co-culture in this study was 46.68 ± 0.09 g/l. It should also be noted that reaching higher titer of ethanol to meet techno-economic feasibility of industrial scale requires fed-batch co-culture process which is left for future investigation.

Conclusion

Achieving economical lignocellulose-based bioprocess requires efficient utilization of C_6 and C_5 sugar mixture present in biomass feedstock. In this study, we have implemented a model-based strategy to rationally design an optimized co-culture capable of efficiently converting glucose–xylose mixture into ethanol. Specifically, a consortia consisting of two yeast strains of *S. stipitis* and *S. cerevisiae* was modeled. The *S. stipitis*/*S. cerevisiae* co-culture kinetic model was applied to systematically assess ethanol fermentation kinetics under different cell ratio and to predict the optimal cell ratio for maximized batch ethanol production in two biomass feedstocks, rice straw and sugarcane bagasse. The model prediction was validated with fermentation and SSF experiments. The results prove the efficiency of optimized co-culture based on the model-based design for increasing ethanol titer and reducing fermentation time. The adjustability of co-culture is also a very appealing characteristic permitting an efficient fermentation of all types of lignocellulosic biomass feedstock by varying co-culture cell ratio to match with the composition of sugar mixture available. Thus, this study demonstrates the utility of systematic approach based on co-culture kinetic model for guiding bioprocess design and optimization efforts aimed at rapidly improving efficiency of ethanol fermentation from lignocellulosic biomass. The modeling tool could also be useful for designing optimal cell ratio of co-culture for other lignocellulosic bio-based processes.

Authors' contributions

PU planned the research study, constructed the model, performed the experiment and analysis, interpreted the results and wrote the paper. SK performed the experiment and analysis and interpreted the results. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Optimization and techno-economic assessment of high-solid fed-batch saccharification and ethanol fermentation by *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* consortium

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ABSTRACT

In the present work, technological and economical potentials of sugarcane bagasse-to-ethanol process using *Scheffersomyces stipitis*/*S. cerevisiae* consortium were investigated. A fed-batch enzyme saccharification followed by fermentation (SHF) using optimized yeast consortium achieved a maximum ethanol titer of 60 g/L with ethanol yield exceeding 70% of theoretical. Techno-economic analysis was assessed using a fully integrated process flowsheeting model, showing the optimized fed-batch yeast co-culture as the most cost-effective configuration with the ethanol yield of 250 kg-ethanol/ton-bagasse. The minimal ethanol selling price was 26.7 baht/L-ethanol, closed to the current ethanol selling price from cassava-based process. Process sensitivity analyses revealed the potentials for further cost reduction up to 44% by reducing enzyme dosage and increasing ethanol titer. Hence, this study provides an economically viable prototype for high-titer lignocellulosic ethanol production using *S. stipitis*/*S. cerevisiae* consortium which may offer better economic value than starch-based process.

1. Introduction

The finite nature of fossil fuels and the concerns about their environmental impact have propelled the world's efforts to develop and industrialize biofuels and bio-based chemicals from lignocellulosic feedstock. Lignocellulose such as agricultural residues is attractive owing to its relatively low cost and abundance, renewable nature and sustainable availability. Production of cellulosic ethanol from lignocellulosic biomass could reduce the dependence on crude oil and minimize the competition between crops for food and fuel production. Hence, there arises a need for developing lignocellulose-to-ethanol production process that can be scaled up efficiently and techno-economically. Conversion of lignocellulose to fuel ethanol requires multiple processing steps. After pretreatment, enzymatic saccharification and fermentation is performed for hydrolyzing pretreated biomass into mixed sugars and for converting released sugars into ethanol, respectively [1]. Owing to the interdependency of each processing step, turning lignocellulosic ethanol production towards a successful industrial scale is only possible by optimizing and defining optimal integrated process option meeting the techno-economic feasibility requirements [2]. The question then becomes, which lignocellulose-based processes and conversion technologies are economically viable for industrialization.

Due to the structure of lignocellulose which is composed of a mixture of hexose and pentose sugars, the utilization of both sugars is required for economical lignocellulosic ethanol process. We have previously demonstrated the outperformance of yeast consortium technology containing two organisms, *Scheffersomyces stipitis* and *Saccharomyces cerevisiae*, for an efficient fermentation of sugarcane bagasse hydrolysates with enhanced ethanol titer and productivity when compared with single-strain process [3,4]. Maximizing cost effectiveness of lignocellulosic ethanol production process requires high ethanol titer with reduced water consumption which is highly beneficial for the overall process economy by greatly reducing the energy demand and cost up to 80% in distillation and evaporation steps [5–7]. The high-ethanol-titer process could only be achieved at a higher substrate load in the saccharification and fermentation steps as demonstrated in recent studies [8,9]. Previous study has shown that increasing the solid substrate concentration from 7% to 15% could reduce the energy demand and operating cost by as much as 50% [7]. As shown previously [9], high substrate loading

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with high process efficiency could be accomplished in fed-batch under optimal feeding of pretreated bagasse and enzymes to maintain the low level of viscosity and sufficient mixing. High-solid operation using fed-batch strategy has several economic advantages over conventional batch process including lower operating and labor costs [10]. Although earlier reports have applied fed-batch strategy to achieve high cumulative solid loading of lignocellulose-based process thus improving its working capability [9,11–13], no high-solid enzyme hydrolysis and fermentation using yeast consortium approach has been studied.

Additionally, with the fully-integrated process analyses the implicit correlations between each unit operation and across the entire process can be assessed to determine comparative process profitability of various process configurations based on process parameters, operating cost and energy consumption from mass and energy balance. The flowsheet modeling framework, which provides dynamic simulation of plant wide operation, facilitates the optimization of integrated process based on economic objective and the identification of key process requirements to improve overall process economy [7,14–17]. Yet, no techno-economic assessment and optimization of lignocellulose-based process to maximize process profitability using yeast consortium technology has been reported.

Thereby, in this study, we optimized *S. stipitis* and *S. cerevisiae* consortium in fed-batch operation of sugarcane bagasse using the model-based optimal cell ratio and solid feed profile developed in our previous works [3,9] to enhance the total solid content which eventually resulted in high ethanol titer. The performance of high-solid process with yeast consortium and its scaling-up were described. Techno-economic assessment of the optimized yeast consortium process was also evaluated and compared with other process configurations using conventional batch or single-strain culture to prove its techno-economic feasibility for industrialization as well as to identify process bottlenecks.

2. Methods

2.1. Strain and media

Scheffersomyces stipitis CBS6054 (ATCC 58785) and *Saccharomyces cerevisiae* (Thermosacc® Dry yeasts; Lallemand, Milwaukee, WI) was maintained on Yeast Extract-Peptone-Dextrose (YPD) agar plate consisting of 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose and 25 g/l agar. The yeast inoculum for use in ethanol fermentation studies was grown in a culture medium containing 10% (v/v) molasses, 0.75 g/L $(NH_4)_2SO_4$, 0.35 g/L KH_2PO_4, 0.07 g/L $MgSO_4 \cdot 7H_2O$, and 1 g/L yeast extract with 10% (v/v) inoculum for 24 h at 30 °C and 200 rpm. Yeast propagation was performed either in incubator shaker (Innova 4340, New Brunswick, USA) or in 2L Braun bioreactor (Biostat, Germany). For co-culture, each yeast strain was prepared separately and inoculated into the bagasse hydrolysates according to the specified cell ratio. Yeast cell was harvested from the culture broth by centrifugation at 5100 rpm for 5–10 min and resuspended in culture media prior to use as inoculum.

2.2. Pretreatment

Sugarcane bagasse, collected from Ratchaburi province (Thailand), was prepared by drying in an 80 °C oven for 1 day before being cut by milling machine to attain a particle size of 0.25–1 cm. The processed bagasse was mixed with 0.5% (w/v) H_2SO_4 at the concentration of 30% solid (based on dried mass) and was steam-pretreated at 121 °C for 30 min as described previously [9]. Sugar composition in the pretreated bagasse, determined by National Renewable Energy Laboratories (NREL) standard procedures [25], was 0.37 g-glucose and 0.23 g-xylose per gram bagasse, consistent with the composition previously reported in Canilha et al. (2011). The pretreated bagasse was neutralized with 4M KOH to a pH value of 5–5.5. The suspension slurry was then used for enzyme hydrolysis and fermentation experiments.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated bagasse was carried out in a 500 mL Erlenmeyer shake flask for small scale and in 10L Braun bioreactor (Biostat, Germany) for scale-up. Batch enzyme hydrolysis experiment was conducted by mixing 10% water-insoluble solid (WIS) pretreated bagasse (w/w) with Cellic C-TEC2 commercial enzymes (Novozyme, DK) at 15 FPU/g-solid, equivalent to approximately 20 mg-protein/g-solid. The bagasse-enzyme mixture was supplemented with 0.75 g/L $(NH_4)_2SO_4$, 0.35 g/L KH_2PO_4, 0.07 g/L $MgSO_4 \cdot 7H_2O$ and initially adjusted to pH 5–5.5 using 4M KOH. It should be noted that batch process cannot be operated at a concentration higher than 10% WIS pretreated bagasse in a standard shake flask and stirred-tank bioreactor due to mixing limitation. Fed-batch enzymatic saccharification as shown in Fig. 1B was carried out at the same initial WIS as batch followed by the pulse addition of pretreated bagasse according to the model-based design of substrate feed profile described in Unrean et al. (2016). The C-TEC2 enzymes were added initially. The enzymatic suspension was incubated at 35 °C at 200 rpm during batch and 500 rpm during fed-batch for 96 h. The hydrolysates was then harvested and used for ethanol fermentation. Samples were withdrawn at the end, centrifuged at 5100 rpm for 5–10 min and the supernatant was analyzed for concentration of released sugars.

2.4. Ethanol fermentation

The fermentation of both the hydrolysates from batch and fed-batch saccharification operations were carried out in a 500 mL Erlenmeyer flask for small scale and in 10L Braun bioreactor (Biostat, Germany) for scale-up. The batch and fed-batch bagasse hydrolysates, supplemented with 10% molasses and 1 g/L yeast extract, were inoculated at 0.04 g-cell/g-WIS with *S. cerevisiae* or with *S. stipitis*/*S. cerevisiae* consortium as specified. It should be noted that after the end of enzyme hydrolysis, the yeast cell and nutrients were added to the hydrolysates to initiate ethanol fermentation process without separation of solid and liquid parts of hydrolysates. The fermentation was carried out at 35 °C, 200 rpm and initial pH 5–5.5 with no pH control for 96 h. Samples were taken periodically for fermentation kinetics analysis. The samples withdrawn were centrifuged at 5100 rpm for 5–10 min and the supernatant was analyzed for ethanol produced and sugar consumed. All experiments were performed in duplicate.

2.5. Sample analysis

WIS determination. Water insoluble solid (WIS) content for the pretreated bagasse was determined by separating the solid fraction of pretreated slurry through centrifugation, weighing and washing with excess deionized water before drying in an oven at 80 °C for 24 h and weighing to determine percent WIS content of pretreated bagasse.

Cell concentration. Cell concentration in seed culture was measured via optical density at 600 nm. The cell dry weight was estimated using following correlation: cdw (g/L) = $0.5 \times OD_{600}$. To reduce interference by culture media during OD measurement, the culture sample was centrifuged at 5100 rpm for 5 min and the supernatants were discarded. The cell pellet was washed with

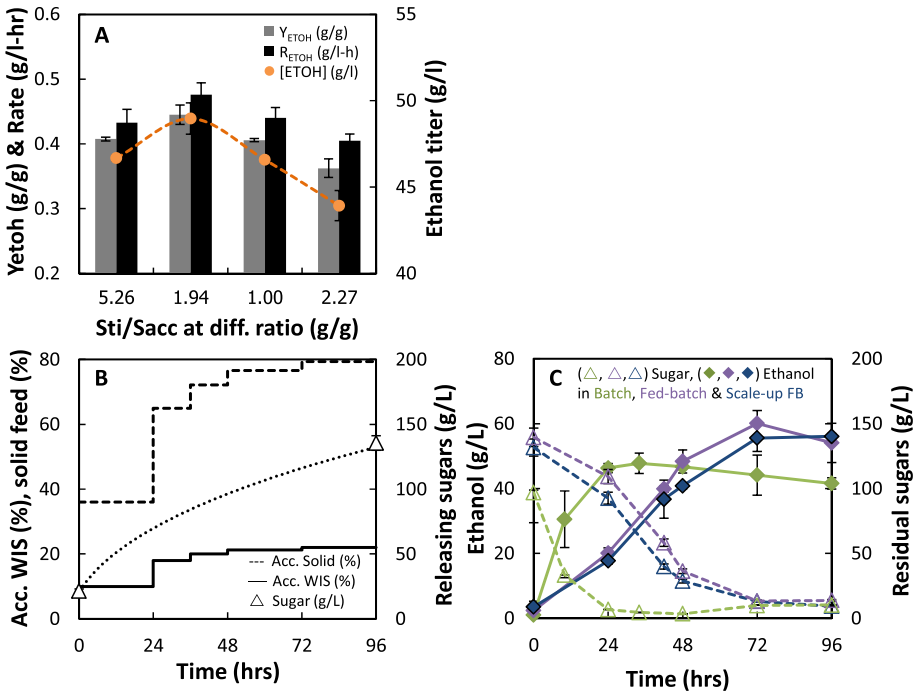

Fig. 1. Fed-batch saccharification followed by fermentation (fed-batch SHF) for high-titer ethanol production from sugarcane bagasse. (A) Optimized ratio of *S. stipitis*/*S. cerevisiae* consortium for enhanced ethanol yield (grey), ethanol productivity (black) and ethanol titer (orange) in batch SHF (B) Solid feed profile during fed-batch enzyme hydrolysis at high solid load. Shown are percent of accumulated solid load (dashed line), accumulated water insoluble solid WIS (solid line) and total releasing sugars (triangle). (C) Ethanol fermentation of sugarcane bagasse hydrolysates obtained from batch or fed-batch by *S. stipitis*/*S. cerevisiae* consortium. Consumed sugars (triangle) and produced ethanol (diamond) in shake flask batch SHF (green), shake flask fed-batch SHF (purple) and scale-up fed-batch SHF (blue) are compared. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deionized water before measuring OD_{600}. The concentration of viable cell was measured by plating 1 mL of sample after appropriated serial dilution on YPD plate, incubating for 24 h before counting colony forming units (CFU).

Analysis of sugars and ethanol. Samples were centrifuged at 5100 rpm for 5–10 min and the supernatant was collected and filtered using 0.2 μm sterile filter. The samples were stored at −20 °C prior to analysis. Concentration of sugars (glucose and xylose), ethanol and inhibitors (acetic acid and furfural) was measured by HPLC equipped with Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a refractive index detector at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The concentrations were calculated from the calibration curve of standard solution.

Yield and rate calculation. Ethanol yield was calculated by dividing ethanol concentration produced by the concentration of total sugar available in the culture from both in molasses and in pretreated bagasse. The ethanol productivity was calculated as the overall rate based on total ethanol produced per total cultivation time.

2.6. Techno-economic analysis

A sugarcane bagasse-to-ethanol conversion process was integrated by combining the basic steps of diluted-acid pretreatment, enzymatic hydrolysis, fermentation, downstream process and utilities system. For techno-economic analysis, demo-scale process model and overall mass and energy balance data was calculated using SuperPro Designer software (Intelligen Inc., USA) to determine material flow rates, composition and energy flow for all streams in the integrated process. Process parameters including sugarcane bagasse components, raw material inputs, yields, rates, processing times and unit operations required for each step was obtained from our experimental studies on lab-scale or scale-up unit in this work as well as in the previously published works [3,9]. Downstream process for ethanol purification was from the optimal process configuration previously identified by Wingren et al. (2008). The equipment sizing, chemical usage, and utility usage were determined by the simulation data. The simulated process also considered time for transferring, draining and cleaning. Heat dissipation of the equipment was neglected. The simulated demo-scale plant for economic assessment was operated with the capacity of 123,000 kg of dry sugarcane bagasse during operation 8000 h per year and run with 4 operators per day. The economic evaluation from mass and energy balances consisted of estimating raw materials, operating, cleaning and labor costs associated with the ethanol production process. The cost of raw materials, utilities and labor together with byproduct income used in this evaluation is based on the values reported in literatures and from local suppliers in Thailand (Table 2). The exchange rate of US dollar (USD) and Swedish Kronor (SEK) to Thai Baht (Baht) is set to 33.7 Baht/USD and 4.07 Baht/SEK respectively, according to the average 2015 exchange rate (www.exchange-rates.org).

3. Results and discussion

3.1. Optimization of S. stipitis/S. cerevisiae consortium

Conversion of sugarcane bagasse to ethanol requires enzyme saccharification and fermentation. In the present study, separate enzymatic hydrolysis and fermentation (SHF) is chosen for use with *S. stipitis*/*S. cerevisiae* consortium due to the ease of determining releasing sugar mixture as the optimized cell inoculation ratio of yeast consortium is dependent on the available sugars in

hydrolysates [3,4,20]. Unlike single-strain culture, the yeast co-culture at optimal cell ratio allows efficient fermentation of sugars available in biomass feedstock, glucose and xylose, and is flexibility as the proportion of cell ratio in the culture can be optimally adjusted to match with the mixed sugars. Thus, we optimized the cell ratio of *S. stipitis*/*S. cerevisiae* co-culture to efficiently ferment sugarcane bagasse hydrolysates to ethanol in batch culture (Fig. 1A). The result highlights the flexibility of yeast consortium process for an efficient conversion of lignocellulosic hydrolysates to ethanol by adjusting the operating cell ratio of each strain. The optimized yeast consortium at cell ratio of 1.94 g-*S. stipitis*/g-*S.cerevisiae* led to the maximum ethanol production with increasing ethanol yield and productivity up to 23% and enhancing ethanol titer by 11% compared to the performance by other cell ratios. The optimal cell ratio determined in this study also agrees well with the value predicted by the co-culture kinetic model reported previously for efficient sugarcane bagasse-to-ethanol production [3], confirming the accuracy of the model-based design of optimal co-culture. The *S. stipitis*/*S. cerevisiae* consortium under optimized cell ratio was then implemented in fed-batch enzymatic hydrolysates for high-titer ethanol fermentation.

3.2. High-titer ethanol production in separate fed-batch saccharification and fermentation

High-solid enzymatic saccharification of sugarcane bagasse feedstock is required for the development of high-ethanol-titer process. Challenges of a high-solid enzyme hydrolysis are poor mixing and ineffective heat and mass transfer due to increased viscosity under high concentration of pretreated biomass slurry leading to decreased hydrolysis and fermentation efficiency [13,21,22]. Other major challenges of high-solid operation also include accumulation of inhibitors generated during pretreatment inhibiting enzymes and yeast cell as well as inactivation of enzymes by irreversible binding to accumulated lignin [9,39]. A proper design of solid substrate feeding scheme in a fed-batch saccharification process which balances between the rate of enzyme hydrolysis and the addition of solid substrate allows feasible operation of lignocellulose-based process with high cumulative solid content. We have previously developed enzyme hydrolysis and fermentation kinetic modeling which was implemented for determining optimal solid feeding profile to reach high-solid loading while maintaining mixing sufficiency of sugarcane bagasse. Briefly, the modeling was used to simulate the hydrolysis profile and predict the amount of solid substrate to be added at discrete time during the fed-batch enzymatic hydrolysis to ensure the residual, un-hydrolyzed solid content not exceeding threshold that could alleviate high viscosity and mixing problems. Thus, in this study, the optimal solid feed profile designed according to the previous model was utilized for a fed-batch enzyme hydrolysis at high-solid loading to enhance the concentration of sugars in enzyme saccharification of pretreated sugarcane bagasse (Fig. 1B). The enzymes were added initially to permit a rapid reduction of viscosity and a better mixing due to faster hydrolysis in the early stage of the process enabling faster feeding of solid substrate. The fed-batch enzymatic hydrolysis was carried out at elevated solid loading up to 22% (w/v) WIS of pretreated bagasse with a total releasing sugars of 135.56 ± 5.54 g/L at 96 h, equivalent to 87% yield of cellulose conversion. Total sugars obtained in fed-batch bagasse hydrolysates were approximately 1.6-folds higher than that obtained during batch enzyme hydrolysis (result not shown).

The batch and fed-batch bagasse hydrolysates were subsequently fermented to ethanol using *S. stipitis*/*S. cerevisiae* consortium, and the fermentation kinetics of bagasse hydrolysates by the co-culture is compared in Fig. 1C. The highest ethanol titer of 46.68 ± 0.09 g/L and 60.09 ± 3.92 g/L were achieved in the fermentation of hydrolysates obtained from batch and fed-batch operation, respectively. Fed-batch process brought about approximately 1.3-folds and 1.2-folds increment in ethanol titer and productivity, respectively, compared to batch process. Enhanced ethanol production performance from fed-batch operation made the process more industrially realistic. High ethanol yield up to 76% of the theoretical was obtained during the fermentation of fed-batch hydrolysates which was lower than the yield observed in the fermentation of batch hydrolysates. The reduction in ethanol yield is likely due to higher ethanol accumulation and high concentration of inhibitors at high substrate content leading to the loss of cell viability and fermentation activity as observed in previous work [9]. The decreased ethanol yield, titer and productivity during high-solid operation were also previously reported [23–25]. Scale-up of high-solid loading fed-batch process in 10L stirred tank reactors produced 56.08 ± 0.82 g/L ethanol, corresponding to 70% of the theoretical, based on total sugar content in the sugarcane bagasse. Overall ethanol yield was 315 L-ethanol per dry ton sugarcane bagasse. The scale-up result was relatively consistent with lab-scale shake flask result confirming scalability of the optimized high-solid yeast consortium process. Table 1 summarizes ethanol fermentation performance from sugarcane bagasse hydrolysates by yeast consortium and single-strain culture in either batch or fed-batch process. The results confirm that the fed-batch process of *S. stipitis*/*S. cerevisiae* consortium outperformed both batch process by single-strain culture in both ethanol titer and productivity.

3.3. Process integration of optimized fed-batch using yeast consortium for conversion sugarcane bagasse to ethanol

The optimized fed-batch process with high ethanol titer using *S. stipitis*/*S. cerevisiae* consortium was examined its potentials for industrialization and economic feasibility through process integration and techno-economic analysis. The integrated sugarcane bagasse to ethanol process as depicted in Fig. 2 began with diluted-acid pretreatment then pH of the pretreated slurry was adjusted with KOH (amount based on experimental data) and diluted to a water-insoluble solid (WIS) concentration as designed based on the experimental work before enzyme hydrolysis with the addition of enzymes converting biomass into sugar monomers followed by the addition of required nutrients and yeast cells for fermentation of sugars into ethanol. Process conditions for each step were described in Methods section. Fed-batch saccharification was pulse-fed with pretreated bagasse slurry at desired feed profile (Fig. 1B). The *S. stipitis*/*S. cerevisiae* consortium was inoculated at optimized cell ratio to start fermentation. In our designed process, no solid–liquid separation after the pretreatment, thereby no wastewater pretreatment was required in this step and in fermentation step. Dilute acid pretreatment with sulfuric acid as a catalyst was utilized since it is considered an economically viable technology to solubilize hemicellulose and increase the digestibility of cellulose in enzymatic hydrolysis [19]. Enzymatic hydrolysis of the whole pretreated slurry was carried out by C-TEC2 cellulase enzymes to produce monomeric sugars for fermentation. The yeasts were produced on-site in separate aerated propagation tank using molasses and nutrients, commonly available in a sugar factory that provides sugarcane bagasse feedstock, to obtain a satisfactory yeast cell concentration. The yeast cultures of *S. stipitis* and *S. cerevisiae* were separated in a continuous centrifuge, re-suspended in culture media, and added into fermentation reactor at optimized cell ratio. Since solid-liquid slurry after the enzymatic hydrolysis unit was directly used for fermentation, in practice enzyme hydrolysis and fermentation process could be carried out in one tank in a sequential manner. However, the process diagram

Table 1
Ethanol production performance comparison by single-strain and consortium cultures.

	[Ethanol] (g/l)	Maximum $R_{Ethanol}$ (g/l-h)[b]	Overall $R_{Ethanol}$ (g/l-h)[c]	Ethanol yield (g/g)[c]
S. cerevisiae in batch shake flask	42.17 ± 0.65	0.87 ± 0.03	0.40 ± 0.01	0.44 ± 0.02
S. stipitis/*S. cerevisiae*[a] in batch shake flask	46.68 ± 0.09	0.97 ± 0.00	0.46 ± 0.02	0.46 ± 0.03
S. stipitis/*S. cerevisiae* in fed-batch shake flask	60.09 ± 3.92	1.01 ± 0.07	0.56 ± 0.06	0.39 ± 0.04
S. stipitis/*S. cerevisiae* in fed-batch bioreactor	56.08 ± 0.82	0.85 ± 0.02	0.58 ± 0.01	0.36 ± 0.04

[a] A *S. stipitis* and *S. cerevisiae* yeast culture in consortium is performed under optimized cell ratio as determined in Fig. 1A.
[b] Maximum ethanol productivity is the production rate of ethanol within the first 48 h.
[c] Overall ethanol productivity is determined from total ethanol produced divided by total fermentation time whereas ethanol yield is based on total ethanol produced per total sugars available.

of fed-batch saccharification and fermentation units was depicted in separate tank for clarification purpose (Fig. 2). The output stream from fermentation unit was transferred to downstream operations to recover ethanol solution at 94% (w/w) and the remaining solid and liquid waste was separated out after downstream processing (Fig. 4). The downstream steps are obtained from previously optimized process by Wingren et al. (2008). In techno-economic evaluations of sugarcane bagasse-to-ethanol production process, the conversion yield, titer and processing time assumed for yeast propagation, enzyme hydrolysis and fermentation processes were based on experimental data in this work and previous works [3,9]. The carbohydrates content of sugarcane bagasse consisted of 23.5% hemicellulose and 37.6% cellulose based on dry basis as measured in previous studies [9,19]. The purpose of the fully integrated process simulations and the economic evaluation is to compare different process configurations on their techno-economic feasibility and not to determine an absolute ethanol production cost and the exact selling price.

3.4. Techno-economic comparison of process configurations for ethanol production from sugarcane bagasse

The effectiveness of enzyme saccharification and fermentation conditions contributes significantly to the economics of overall process. To select an optimal process configuration for sustainable and economical lignocellulose-based bioprocess, four possible integrated process scenarios which have been studied in previous section were simulated and compared for their techno-economics: (1) *S. cerevisiae* single-strain culture in batch SHF process, (2) *S. stipitis*/*S. cerevisiae* consortium in batch SHF process, (3) *S. cerevisiae* single-strain culture in fed-batch SSF process and (4) *S. stipitis*/*S. cerevisiae* consortium in fed-batch SHF process. The process scenarios under study are summarized in Fig. 3. The evaluation was mainly focused on the process conditions in enzyme hydrolysis and fermentation that have been optimized in the experimental studies to examine how each of these conditions affects the overall energy demand and production cost of the process. The downstream processing cost estimation was according to previous investigation [14]. We analyzed the profitability of the process based on operating cost and minimal ethanol selling price (MESP). The cost estimation of all the process steps at different configurations was estimated from the economic data reported in our case study and others [7,14,15,17] as summarized in Table 2. Based on comparative process techno-economic summarized in Table 3, the chosen process configuration for economic viability is fed-batch separate hydrolysis and fermentation (SHF) using

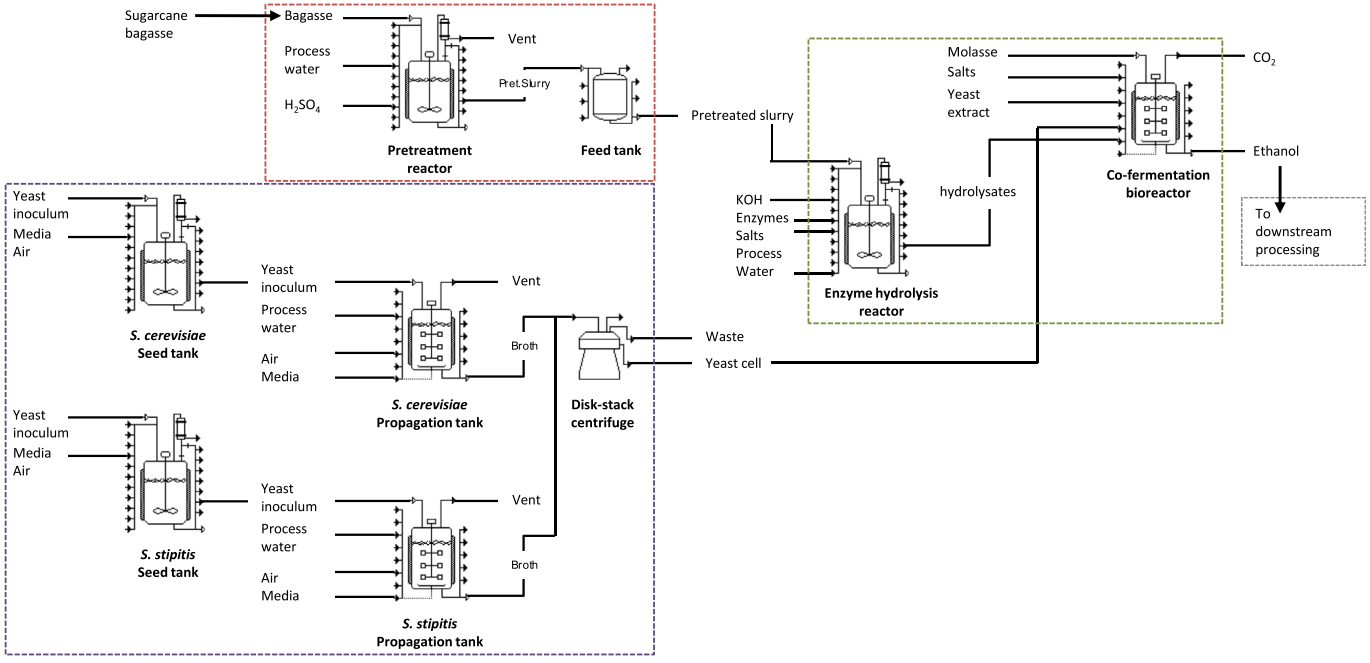

Fig. 2. Process diagram for optimized fed-batch saccharification followed by fermentation (fed-batch SHF) by *S. stipitis*/*S. cerevisiae* consortium. The process configuration includes diluted-acid pretreatment (in red dashed square), yeast propagation (in purple dashed square), enzyme hydrolysis and co-fermentation (in green dashed square) steps for the conversion of sugarcane bagasse to ethanol. Detailed downstream processing for ethanol recovery was described in Wingren et al., 2008. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

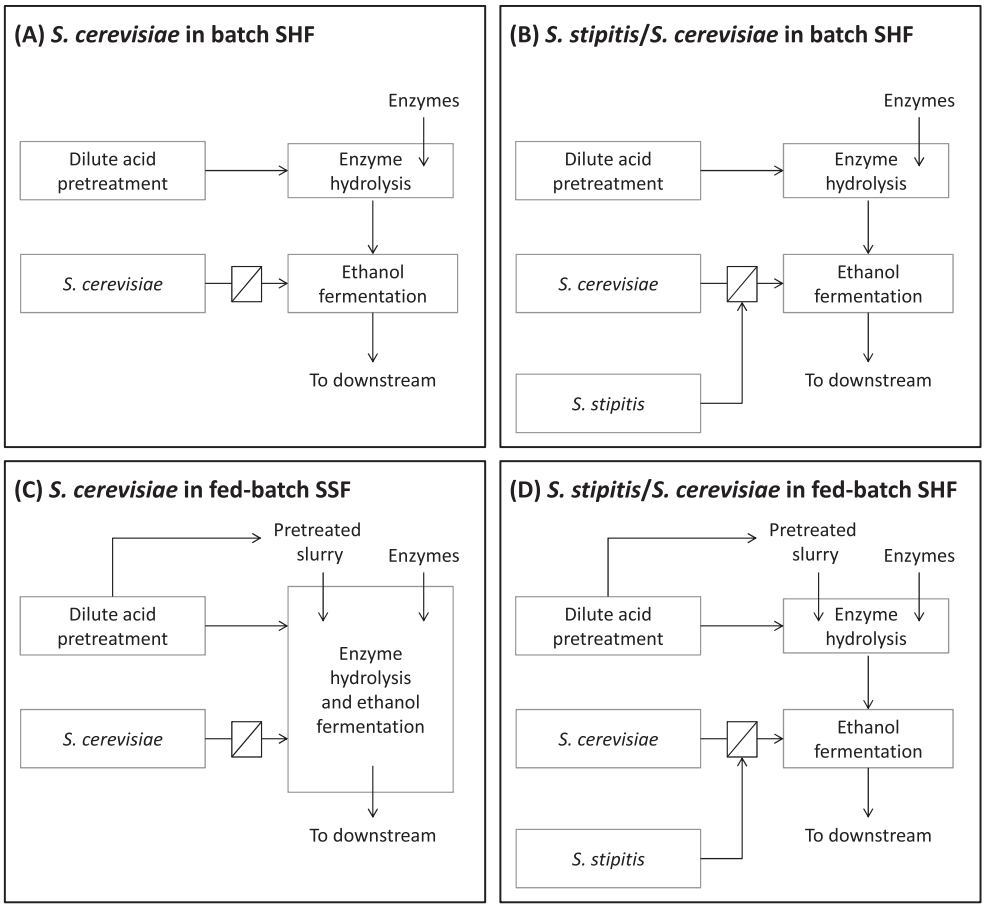

Fig. 3. Four process scenarios for the conversion of sugarcane bagasse to ethanol under techno-economic evaluation: (A) *S. cerevisiae* in batch separate hydrolysis and fermentation (Single-B-SHF), (B) *S. stipitis*/*S. cerevisiae* in batch separate hydrolysis and fermentation (Co-B-SHF), (C) *S. cerevisiae* in fed-batch simultaneous hydrolysis and fermentation (Single-FB-SSF), and (D) *S. stipitis*/*S. cerevisiae* in fed-batch separate hydrolysis and fermentation (Co-FB-SHF). The conditions and performance of Single-FB-SSF process was described in previous study (Unrean et al., 2016).

S. cerevisiae/*S. stipites* consortium culture as depicted in Fig. 2 with the lowest MESP of 26.7 Baht/L. This is due to the higher ethanol titer reached by increasing the solid content using fed-batch and the efficient utilization of mixed sugars by yeast consortium which had a significant positive effect on process economy.

Using *S. stipitis*/*S. cerevisiae* consortium could reduce the minimal selling price by 7% hence increasing profit margin compared to when single-strain of *S. cerevisiae* was used in batch process. With fed-batch strategy, the MESP was decreased further by 22% compared to batch process. Comparison between batch and fed-batch process also revealed that an increase in solid loading from 10% WIS to 22% WIS in enzyme hydrolysis followed by fermentation also reduced the process cost by 13%. An increase in solid content from 10 to 22% lowered the MESP by 5.9 baht/L ethanol (Table 3). The similar trend was also reported in previous studies [5,14,26]. Thus, the process options with single-strain or with batch process should not be considered for practical applications because of their high cost. To the best of the authors' knowledge, this is the first techno-economic report of lignocellulosic ethanol production process at high solid operation using yeast consortium technology. Although the estimated minimum ethanol selling price in this study is higher than the previously reported MESP of 19.58 Baht/L (approximated 2.2 USD per gal), the MESP in this process may not be comparable with the previously published processes [26,27] due to the difference in process flowsheet, production scale (demo-scale vs. industrial scale), feedstock composition and raw materials used (sugarcane bagasse vs. corn stover). Nevertheless, the techno-economic framework reveals that the estimated MESP of fed-batch yeast consortium process was relatively closed to the current selling price of ethanol from cassava-based process (27.19 baht/L, www.thaiethanol.com). Thus, the high-solid, fed-batch process platform using *S. stipitis*/*S. cerevisiae* consortium technology is suitable to meet the economic demand of large-scale ethanol production process, thereby replacing the cassava-based ethanol production process in Thailand. It should be noted that the fully integrated process simulation should extend beyond upstream and downstream steps by incorporating all unit operations for processing biomass feedstock prior to pretreatment (e.g. pre-processing, transportation and storage), waste treatment, waste re-utilization after end-product recovery including energy and heat integration of lignin processing step (e.g. combustion of solids/lignin wastes for electricity generation).

3.5. Material and energy requirements of optimized fed-batch process with yeast consortium

An overview of materials and utilities requirements per 1 dry ton sugarcane bagasse for the fed-batch saccharification and fermentation process using *S. stipitis*/*S. cerevisiae* co-culture is shown in Fig. 4 and Table 4. Table 5 shows cost summary of the optimized fed-batch yeast consortium process which resulted in approximately 38,969 L of cellulosic fuel ethanol production per year with an annual income of 1,091,659 baht based on the demonstration scale of 3 ton dry bagasse per batch. For every one

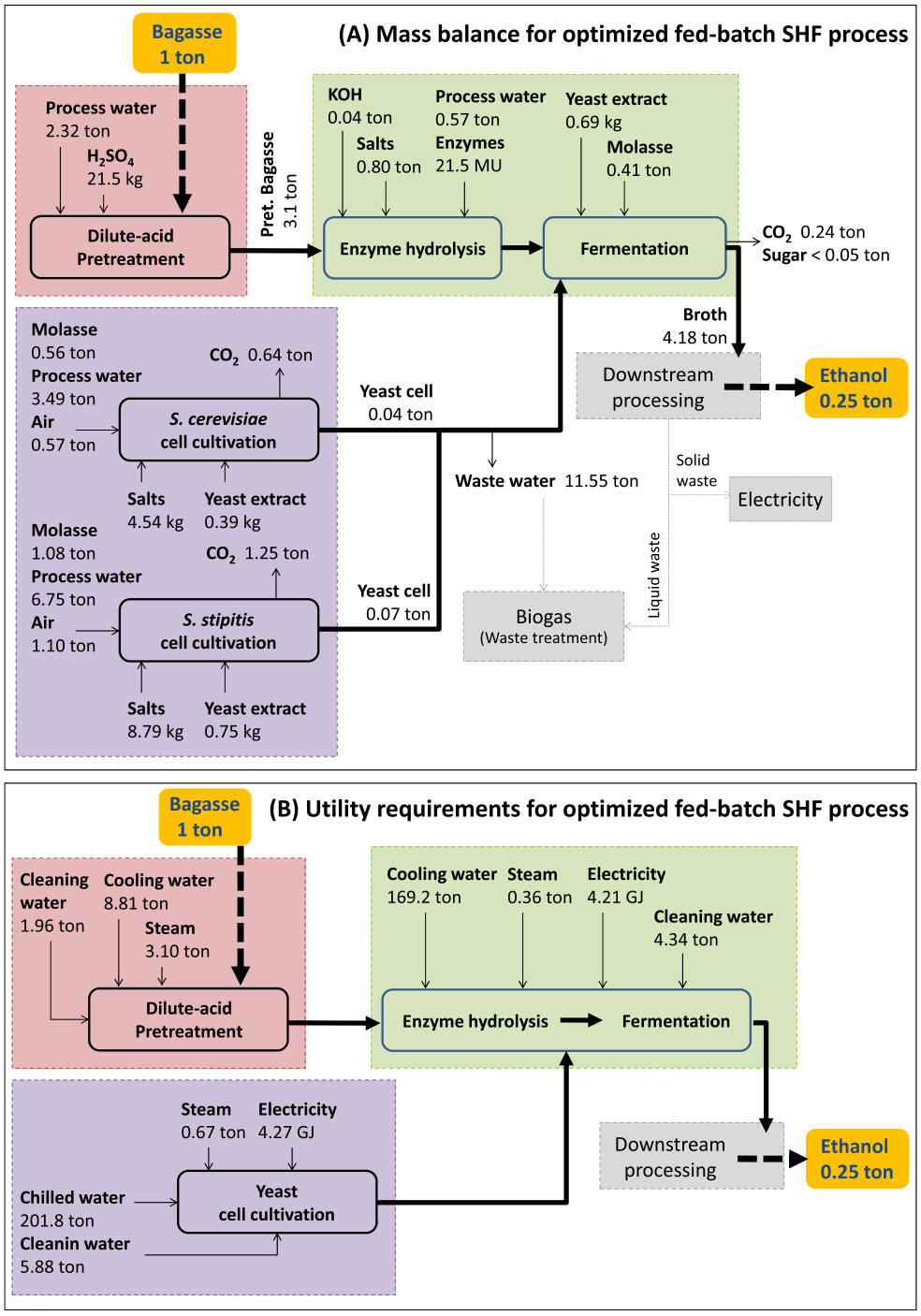

Fig. 4. Summary of mass and utility requirements for the conversion of sugarcane bagasse to ethanol using optimized fed-batch saccharification and ethanol fermentation (fed-batch SHF) of *S. stipitis*/*S. cerevisiae* consortium. All values are based on 1 dry ton sugarcane bagasse.

ton of dry bagasse being processed, 2.13 tons of carbon dioxide was generated (Table 4). Therefore, the co-product credit would have a significant effect on the overall process economy. Upgrading the CO_2 byproduct into valued-added chemicals through catalytic or biological conversion process would increase income and profit margin of the current integrated process. In addition, based on material flow balance, 13.13 ton of process water is required for upstream processing of 1 ton of raw sugarcane bagasse. To reduce the plant makeup water and further improve process economic, major part of the process water required could be replaced with steam condensates without affecting ethanol production yield. Integrating the wastewater treatment would also reduce the utility cost for steam and electricity which are the two major cost of the process (Fig. 4 and Table 5) making the sugarcane bagasse-to-ethanol process more cost effective and energy efficient in the economic outcome. Spent and other liquid wastes from the yeast propagation and downstream steps could be used for biogas (e.g. methane) production in anaerobic digestion which can be utilized for steam generation. The solid waste containing the yeasts could also be utilized as cattle feed. Furthermore, the solid, lignin-rich

Table 2
Raw materials and utilities cost used in the techno-economic evaluation of sugarcane bagasse-to-ethanol process.

	Cost	Unit[a]	Sources
Raw materials			
Sulfuric acid	1.18	Baht/kg	Kazi et al., 2010
$MgSO_4$	18	Baht/kg	Wingren et al., 2008
Molasse	4.07	Baht/kg	Sassner et al., 2008
Enzymes	4.08	Baht/kg	Geraili et al., 2014
$(NH_4)_2SO_4$	28	Baht/kg	Local supplier
KOH	8.5	Baht/kg	Local supplier
KH_2PO_4	19	Baht/kg	Local supplier
Yeast extract	480	Baht/kg	Local supplier
Utilities and labor			
Electricity	0.28	Baht/MJ	Wingren et al., 2008
Process water	0.006	Baht/L	Wingren et al., 2008
Chilled water	0.013	Baht/L	Wingren et al., 2008
Cooling water	0.010	Baht/L	Wingren et al., 2008
Cleaning agent	16.28	Baht/kg	Wingren et al., 2008
Steam	0.41	Baht/kg	Local supplier
Labor	37.5	Baht/hr	Local supplier
Byproducts income			
CO_2	0.12	Baht/kg	Wingren et al., 2008

[a] The values reported in literatures are converted to Baht using the following exchange rate, 33.7 Baht/USD or 4.07 Baht/SEK (the 2015 rate average, www.exchange-rates.org).

Table 4
Raw material and utility requirements for the conversion of 1 dry ton sugarcane bagasse to ethanol by *S. stipitis*/*S. cerevisiae* consortium in optimized fed-batch saccharification and ethanol fermentation (fed-batch SHF).

Raw materials	Per 1 dry ton bagasse	
Process water	13.13	ton
H_2SO_4	0.021	ton
KOH	0.041	ton
Molasse	2.05	ton
Enzymes	0.18	ton
Salts	0.014	ton
Yeast extract	0.002	ton
Utility requirements		
Cooling water	178	ton
Chilled water	201.78	ton
Steam	4.13	ton
Electricity	8.48	GJ
Cleaning water	12.18	ton
Products		
Ethanol	0.25	ton
CO_2[a]	2.13	ton
Waste water	11.55	ton
Ethanol yield[b]	315 L-ethanol/ton-bagasse	

[a] CO_2 is sale as byproduct income.
[b] The value is estimated from upstream process only thereby excluding the loss during downstream steps.

waste obtained from the downstream process is a co-product that can be dried and re-used as a solid fuel for electricity generation, underlining the importance of lignin recovery. Investigating potentials for increasing income from by-products and solid-liquid wastes generated during the production is left for future study.

3.6. Cost distribution analysis

The annual production cost for the conversion of sugarcane bagasse to ethanol using fed-batch saccharification then fermentation with *S. stipitis*/*S. cerevisiae* co-culture is summarized in Table 5. The main costs in fed-batch yeast consortium process contribute to raw materials and utilities. Capital cost was not included in this simulation since our aim is to use already established cassava-based production plant for lignocellulosic ethanol production, thereby no capital cost is required. It should be noted that the cost analysis has not yet considered the cost due to mixing energy consumption when the process is operated at high solid condition (25% solid or more). Thus, a balance for achieving the optimal energy cost between the increased mixing energy cost and the reduced distillation energy cost needs to be thoroughly evaluated and account for in the future during process integration at higher solid loading. Cost distribution analysis for identifying various process bottlenecks that decrease the efficiency of fed-batch yeast consortium process economic is demonstrated in Fig. 5. In Fig. 5A, the cost of enzymes constituted 45% of the raw material costs, to which alkaline used for detoxification was also the second contributor of 22% as has also been observed in several previous reports [5,28]. Since the enzyme cost is the main concern for the ethanol production from sugarcane bagasse, the on-site enzyme production or reducing enzyme usage in the process seems most reasonable to offer economic advantages. The production of enzymes on-site could be done using fungus cell or genetic engineered yeasts [29,30]. The advantage of the production of enzymes on-site includes eliminating transportation and the need to add stabilizers to reduce enzyme degradation during storage. However, multiple steps for on-site enzyme production would add to the overall process cost including operating cost, cost of cell removal, enzyme concentrating and purifying steps. Hence, techno-economic feasibility analysis is required to demonstrate what yield and production cost tradeoffs occur when enzymes are produced on-site. Increasing enzymes activity using enzyme enhancer or recycling enzymes for reuse are options for reducing enzyme loading. Minimizing enzyme irreversible binding to lignin is also essential for enzymatic hydrolysis efficiency which could be achieved through addition of surfactants [39]. Future research and development should, therefore, be focused on engineering high-activity and robust enzymes to reduce enzyme loading or facilitate enzyme recycling. Additionally, removal of enzyme inhibitors present in hydrolysates or engineering enzymes able to withstand

Table 3
Techno-economic comparison of different sugarcane-to-ethanol process configurations.

Process configurations	Ethanol yield (kg-etoh/ton-bagasse)	Production capacity (L-etoh/year)[a]	Production cost (Baht/Batch)[b]	Minimal ETOH selling price (Baht/L)[c]
S. cerevisiae batch process	173.5	27,041	20,147	34.8
S. cerevisiae/*S. stipitis* batch process	193.7	30,190	20,658	32.6
S. cerevisiae fed-batch process[d]	267.3	41,664	25,761	27.5
S. cerevisiae/*S. stipitis* fed-batch process	250.0	38,969	23,545	26.7

[a] Production capacity is based on demonstration scale operating at 3 dry tons sugarcane bagasse per batch and 41 batches annually.
[b] Production cost is estimated from upstream processing with 3 dry tons sugarcane bagasse per batch. Estimated cost is based on the local or reported cost for raw materials and utilities as summarized in Table 2.
[c] Ethanol selling price is estimated based on total production cost per total ethanol produced. Downstream processing cost is calculated from the previously reported value of required energy for ethanol purification, 10.2 MJ/L ethanol (Wingren et al., 2008).
[d] Process conditions for fed-batch of *S. cerevisiae* are according to experimental data reported in Unrean et al., 2016.

Table 5
Cost summary of optimized fed-batch saccharification followed by fermentation using *S. cerevisiae*/*S. stipitis* consortium for the production of ethanol from sugarcane bagasse.

	Annual cost (Baht)[a]
Raw materials/Chemicals	190,134
Process water	9206
Cooling water	12,475
Chilled water	14,142
Steam	206,287
Electricity	294,613
Cleaning	8536
Total expense	735,393
Total income[b]	1,091,659

[a] Annual cost is estimated from upstream processing in demonstration scale operated at 3 dry tons raw bagasse per batch and 41 batches per year.
[b] Estimated income is from annual ethanol production capacity and the current selling price of ethanol at 27.19 Baht/L (the 2015 average selling price of ethanol in Thai market, www.thaiethanol.com).

high accumulation of inhibitors is also necessary for higher solid operation.

The cost of alkali also contributes significantly to the cost of cellulosic ethanol. Thus, from the process economics perspective, improvement in cell robustness against the inhibitors present in pretreated biomass slurry is a prerequisite to reduce cost on detoxification step. Development of robust yeast cell factory could be accomplished through metabolic engineering or adaptive evolution approaches as being demonstrated in several studies [31,32]. The robust yeast strains would also make it possible to run enzymatic hydrolysis and fermentation at higher solid loading since one of major limitations of high-solid operation is high concentration of inhibitors which hamper yeast cell growth and fermentation [9,33]. The cost of electricity and steam energy covers more than 90% of the overall utility cost (Fig. 5B), thereby reducing production cost could be done by lowering utility cost. Several alternatives for lower utility cost are recycling steam condensates for increasing energy efficiency, utilizing lignin and other solid wastes as energy source, and integrating biogas production from liquid waste into the process for electricity and steam generation. The analysis in Fig. 5C also points to enzyme hydrolysis as the most expensive processing steps within the conversion of sugarcane bagasse to ethanol process due to high enzyme cost. Comparing among process steps, yeast propagation process was the second largest cost distribution. Therefore, the production of yeast from molasses or other waste products is necessary for cost saving. On-site yeast production is also more economical compared to purchasing dried yeast for use in the process (results not shown). Further reduction of yeast propagation cost should be implementing high-cell-density fermentation to maximize yeast production while minimize operating cost. Engineering robust cell that could grow and propagate in high solid lignocellulose-based process is another process option to reduce the amount of yeast addition into the process.

3.7. Process sensitivity analysis

Techno-economic process simulation was made to study and understand process sensitivity. Sensitivity analyses on two major contributors to the overall process cost, enzyme loading and final ethanol titer, were performed to provide information on potential cost reduction for each parameter. Fig. 6 shows the relative effects of enzyme loading and ethanol titer on the overall process economy in term of MESP. Assuming the same levels of enzyme hydrolysis yield and other parameters remain unchanged, reducing the amount of enzymes during the fed-batch enzymatic saccharification from 15 FPU/WIS (equivalent to 20 mg-protein/WIS) to 4 FPU/WIS (equivalent to 5 mg-protein/WIS) reduced MESP by 6% leading to a 4-fold increase in profit margin when compared with the current selling price of ethanol in the local market (Fig. 6A). The result is another confirmation that reduction of enzyme loading while maintaining high enzymatic activity is necessary for economic feasibility of the sugarcane bagasse-to-ethanol process.

An increase in the final ethanol titer from 6% to 12%, while all

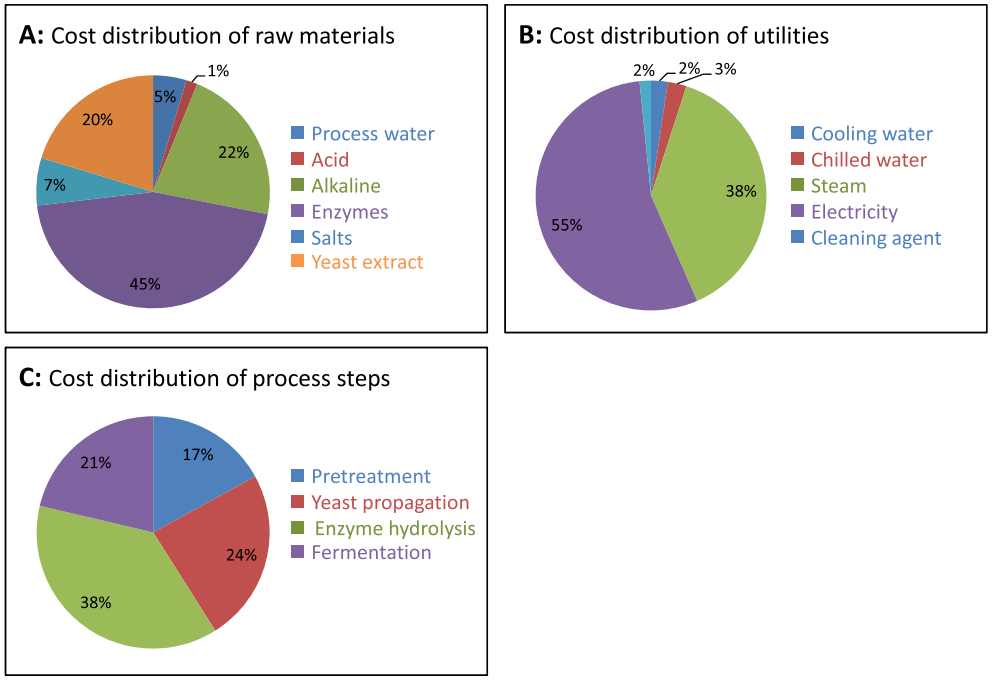

Fig. 5. Cost distribution of optimized fed-batch saccharification followed by fermentation (fed-batch SHF) using *S. stipitis*/*S. cerevisiae* consortium. The values are based on upstream processing only.

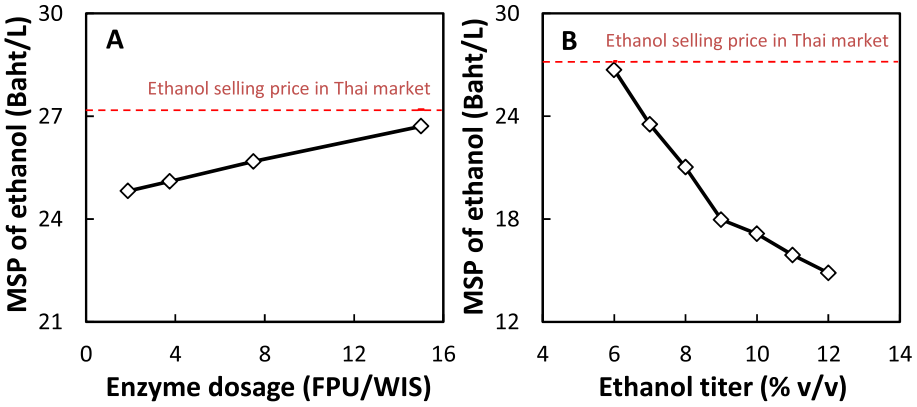

Fig. 6. Process sensitivity analysis examining impact of enzyme dosage and final ethanol titer on the minimum ethanol selling price (MESP) for fed-batch enzyme hydrolysis and fermentation with *S. stipitis*/*S. cerevisiae* consortium. Dashed line represents current ethanol selling price from casava-based process (the 2015 average selling price of ethanol in Thai market, www.thaiethanol.com) for comparison purpose.

other parameters are kept constant, would reduce the MESP significantly up to 44% (Fig. 6B). Reducing the production cost and MESP can be explained by the lower energy demand in downstream processing at higher titer of ethanol. As been studied previously, doubling the ethanol titer from 2.5 to 5% could reduce the energy required in distillation by 33% [7]. It should be noted that increasing final ethanol titer shows a larger decrease in MESP than reducing enzyme usage indicating that the ethanol titer is the factor with the most control over the overall production cost. Higher ethanol titer could be accomplished either by improving ethanol yield in the yeast cell through genetic or evolutionary engineering or by increasing concentration of solid loading in enzyme hydrolysis followed by fermentation. One option of engineering yeast with improved ethanol yield could be based on the utilization synthetic biology or systems metabolic engineering to redirect more fluxes towards ethanol synthesis [33,34]. It is also expected that the ethanol yield may be decreased when the solid concentration is increased due to increasing toxicity and stress caused by high viscosity of high-solid content [9,21]. Thus, having an efficient and robust yeast cell is desirable and essential trait for economical lignocellulose-based process. Additionally, as the concentration of solid-content increases, it is the authors' belief, that a specialized reactor for high-solid operation may be necessary. This has been demonstrated in experimental results [35–37] showing stirred tank reactor with helical impellers could provide sufficient mixing at solid load as high as 30% (w/v). In addition, uncertainty from various sources such as change in market or operational parameters should be included into the future process integration model for evaluation of the impact of uncertainties affecting the overall production cost in order to improve a degree of realism of the simulated large-scale production process [16,38].

4. Conclusions

This study exploits a previously developed kinetic modeling of enzyme hydrolysis and fermentation [12] and a mass and energy balance based on process flowsheet simulation for the design of a sugarcane bagasse-to-ethanol process with improved techno-economic characteristics suitable for large-scale production. Through model-based design of feed profiles in fed-batch process, high insoluble solid loading up to 22% (w/v) has been reached, which otherwise could not be achieved in batch process. Ethanol fermentation following the fed-batch enzyme hydrolysis using optimal cell ratio of *S. stipitis* and *S. cerevisiae* consortium led to high ethanol titer up to 60 g/l, a 1.4-fold improvement compared to single-strain batch process. Hence, this work validates the application of kinetic modeling tool to aid the design and optimization of fed-batch yeast consortium process for efficient lignocellulosic ethanol fermentation. Techno-economic was analyzed to provide insights into the effect of operational conditions on process economics and possible process integrations of yeast consortium for minimized total ethanol production cost. Among four process scenarios considered, the fed-batch saccharification followed by fermentation with *S. stipitis*/*S. cerevisiae* consortium provided a cost effective process option with MESP of 26.7 Baht/L-ethanol. The flowsheet process modeling also pointed to a potential ethanol production cost reduction via future improvements to yeast robustness, reduction of enzyme cost (e.g. on-site production), low enzyme usage (e.g. increasing specific activity or enzyme recycling) and increment of ethanol titer during fermentation by increasing solid content which would make the sugarcane bagasse-to-ethanol conversion process using fed-batch and yeast consortium technology economically feasible for industrialization.

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Techno-economic assessment of high-solid simultaneous saccharification and fermentation and economic impacts of yeast consortium and on-site enzyme production technologies

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ABSTRACT

An efficient simultaneous saccharification and fermentation (SSF) at high-solid loading are keys to the successful commercialization of lignocellulose-based process. In the present work, technological and economical potentials of high-solid SSF for sugarcane bagasse-to-ethanol conversion process [6] was analyzed based on process flowsheet simulation for an estimation of the minimal ethanol selling price (MESP). Based on techno-economic assessment a high-solid SSF process platform for a low-cost lignocellulosic ethanol production was designed composing of (1) yeast consortium for C_5 and C_6 sugars co-fermentation and (2) cellulase/on-site hemicellulase enzyme mixtures acting synergistically for efficient saccharification. Implementing the integrated SSF process with on-site enzymes and yeast consortium, the MESP could be reduced to as low as 15.7 Baht/L equivalent to 1.66 USD/gal which is a 6% lower than the current market selling price of 1.76 USD/gal. Thus, the on-site enzymes together with cellulase-hemicellulase synergism to lower enzyme demand as well as the yeast consortium technology to increase ethanol titer from C_5/C_6 co-fermentation would provide economic feasibility for the future cellulosic ethanol production in the industrial scale. Such process platform is also an important strategy for the development of low-cost biorefinery industry that can outperform the current sugar-based process for the production of biofuels.

1. Introduction

The use of lignocellulosic biomass for the production of biofuels and biochemicals has received great attention due to its low cost, renewable and abundance. The production of lignocellulosic ethanol as an alternative energy source could lower the dependency on crude oil and minimize the competition between crops for food and fuel production [1]. Sugarcane bagasse, which is the solid residue left after sugar processing steps, is one of the major lignocellulosic feedstock that could potentially be used for biorefinery process. The development of an integrated simultaneous saccharification and fermentation (SSF) process at high solid loading of bagasse for high-titer ethanol production is thus of great interest. The high-titer lignocellulosic ethanol process could maximize the overall process economy by lowering water consumption and greatly reducing (>80%) the cost for downstream processing steps [2–5]. We have previously developed a fed-batch SSF at high-solid loading up to 22% WIS, thereby reaching high ethanol titer [6].

Owing to the interdependency of each processing step, turning lignocellulosic ethanol production towards a successful industrial scale is only possible by optimizing and defining optimal integrated process option meeting the techno-economic feasibility of cellulosic biomass conversion technologies [7]. The flowsheet modeling framework could provide dynamic simulation of plant wide operation, facilitate the optimization of integrated SSF process based on economic objective as well as determine key process requirements to improve the overall process economy [8–11]. As a result, to evaluate the high-solid SSF process economy, the techno-economic model of fully-integrated SSF process containing various interdependent steps of pretreatment, yeast propagation, enzymatic hydrolysis and fermentation and downstream processing in a demonstration ethanol plant was developed in the present work. Using process flowsheet simulation, an economic of high-solid SSF

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process was evaluated through minimal ethanol selling price (MESP) to assess its cost competitiveness with starch- and sugar-based processes [12].

Sugarcane bagasse is typically composed of 35–40% cellulose, 25–30% hemicellulose and 30–40% lignin. Conversion of cellulose and hemicellulose to ethanol requires cellulase and hemicellulase enzymes, respectively, for hydrolysis and yeast cells for co-fermentation of C_5/C_6 sugars. The efficiencies of enzymatic hydrolysis and co-fermentation of C_5 and C_6 sugars are critical process variables in high-solid SSF process which directly affect MESP and its economic feasibility [13,14]. We have previously demonstrated the implementation of yeast consortium containing two yeasts, *Scheffersomyces stipitis* (C_5-fermenting yeast) and *Saccharomyces cerevisiae* (C_6-fermenting yeast) for high ethanol titer and productivity [15,16]. Incorporating experimental results reported previously, the effect of yeast consortium for C_5/C_6 co-fermentation on MESP was studied using the techno-economic model of integrated high-solid SSF process in the present work.

Making cellulosic ethanol process economically viable also requires high saccharification efficiency at high sugar yield and low enzyme load for achieving a competitive MESP. Enhancement of the hydrolytic capacity of cellulases to increase sugar yield could be accomplished through a supplementation of hemicellulases due to the synergistic effect of enzymes mixture [17–20]. The presence of hemicellulases such as xylanase removes hemicellulose hence improving the accessibility and the digestibility of cellulases to the cellulose leading to higher sugar production and lower overall process cost [21]. Additionally, the use of on-site or near-site enzyme production was proposed as a promising way to the significant reduction of enzyme cost up to 30–70% owing to its simplified purification and logistics [22–24]. Due to the cost benefit of on-site enzyme, on-site hemicellulase production was modeled together with the integrated high-solid SSF process. A commercial cellulase preparation was employed due to its present low-cost [4,25,26] and the effect of varying dosage of cellulase and on-site hemicellulase enzyme mixtures on the MESP was investigated.

In the present study, the MESP was evaluated for the high-solid SSF process and the process economy was studied under the varying process scenario e.g. with yeast consortium for co-fermentation, on-site enzyme supply mode as well as enzyme loading. The economic impact of these process schemes on the commercialization were discussed based on the MESP calculation. Eventually, the design of integrated SSF process with cost-competitive MESP to the current selling price was proposed.

2. Methods

2.1. Process simulation

A sugarcane bagasse-to-ethanol demonstration plant was simulated by integrating the basic steps of diluted-acid pretreatment, yeast propagation, enzymatic hydrolysis and fermentation, downstream process and utilities system as shown in Fig. 1. Briefly, sugarcane bagasse at the size of 0.25–1 cm was steam-pretreated with 0.5% (w/v) H_2SO_4 at 121 °C for 30 min. The pretreated bagasse was then neutralized to pH 5 using KOH prior to use in SSF process where yeast cells suspension from propagation step, enzymes and pretreated bagasse were mixed at dosage as specified in Results and Discussion section and incubated at 35 °C, 120 h for ethanol production. For reference case of high-solid SSF using *S. cerevisiae* with purchased cellulase enzymes (SSF-Sacc), yeast cells, enzymes and solid loading were 0.02 g-cell/g-WIS cell dosage, 25 FPU/g-WIS enzyme dosage and 22% WIS solid loading respectively. Downstream process for ethanol purification was from the optimal process configuration previously identified [26]. Sugar composition in pretreated bagasse, determined by National Renewable Energy Laboratories (NREL) standard procedures, was 0.37 g-glucose and 0.23 g-xylose per gram bagasse. The process parameters including water insoluble solid (WIS) content for the pretreated bagasse, raw material inputs, yields, rates, processing times and unit operations required for each step was obtained from previous experimental studies [6,16,19,27]. The simulated demo-scale ethanol plant for economic assessment was operated with the capacity of 123,000 kg of dry sugarcane bagasse during operation 8000 h per year. The equipment sizing, the chemical usage, and the utility usage were determined by the simulation data. The simulated process also considered time for transferring, draining and cleaning. Heat dissipation of the equipment was neglected.

2.2. Techno-economic analysis

The demo-scale process model and the overall mass and energy balance data was calculated using SuperPro Designer software (Intelligen Inc., USA) to determine material flow rates, composition and energy flow for all streams in the integrated process. Techno-economic evaluation from mass and energy balances consisted of estimating the raw materials, operating, cleaning costs associated with the production process. The cost of

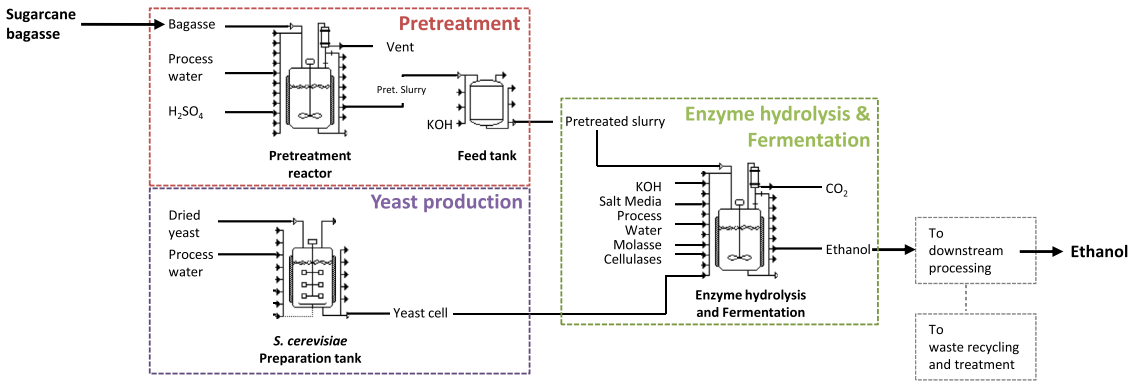

Fig. 1. Process diagram for fed-batch simultaneous saccharification and fermentation with minimum waste using dried yeast and commercial cellulase enzymes. The process configuration includes diluted-acid pretreatment (in red dashed square), dried yeast preparation (in purple dashed square), enzyme hydrolysis and fermentation (in green dashed square) steps for the conversion of sugarcane bagasse to ethanol. The optimized fed-batch SSF process at high-solid load present here was described in Ref. [6]. The SSF process was carried out with a total WIS content of 22%. Detailed downstream processing for ethanol recovery was described in Ref. [8]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

raw materials and utilities together with the byproduct income used in this evaluation is summarized in Table 3 based on the values reported in literature and in local suppliers in Thailand. The downstream processing cost estimation was according to previous investigation [26]. Fixed capital investment (FCI) costs were not included in production cost estimation since our aim is to use already established cassava-based production plant in Thailand for lignocellulosic ethanol production, thereby no capitol cost is required. All costs are presented in Thai Baht using the exchange rate of 0.028 US dollar (USD) and 0.24 Swedish Kronor (SEK) to Thai Baht (Baht) according to the average 2015 exchange rate (www.exchange-rates.org). MESP is determined as total production cost divided by liter of ethanol produced referring to the ethanol price where production cost and income of selling ethanol are equal.

2.3. Comparative process design

Techno-economic process simulation was applied to examine process economy and sensitivity. Sensitivity analyses on two major contributor to the overall process cost, the use of yeast consortium for co-fermentation of C_5/C_6 sugars and the use of on-site hemicellulase and cellulase enzyme mixtures for saccharification, were performed. The evaluation was mainly focused on the impact of yeast consortium and the effect of supplementation of hemicellulase to cellulase enzyme hydrolysis on process economy. Three integrated process configurations, denoted with A-C, were investigated in the techno-economic model of SSF process (Fig. 4). The process scenarios under comparison were (1) SSF-Sacc: *S. cerevisiae* in fed-batch, high-solid SSF using commercial cellulases, (2) SSF-Co: *S. stipitis*/*S. cerevisiae* in fed-batch, high-solid SSF using commercial cellulases, and (3) SSF-Co-Enz: *S. stipitis*/*S. cerevisiae* in fed-batch SSF using commercial cellulases and on-site hemicellulase enzymes production. The three processes differed in the yeast and enzyme supply mode. In scenario Fig. 4A, *S. cerevisiae* which is a C_6-fermenting yeast was used for SSF, while in scenario Fig. 4B and C, the yeast consortium of *S. cerevisiae* (C_6-fermenting yeast) and *S. stipitis* (C_5-fermenting yeast) was applied for co-fermentation of C_5/C_6 sugar mixture. Mixture of the liquid fraction and molasses was used for yeast propagation. In scenario Fig. 4C, hemicellulase enzyme was prepared on-site and added to the SSF process to examine economic effect when cellulases and on-site hemicellulases was used together compared to other scenarios with only cellulases enzyme. Effect of cellulases and hemicellulase ratio on MESP in scenario Fig. 4C was also investigated. The MESP was used as the indicator to show the economic impacts of different process design and process conditions.

3. Results and Discussion

We have previously developed a fed-batch, high-solid SSF process based on enzyme hydrolysis kinetics and dynamic metabolic modeling [6]. The optimized fed-batch SSF process was implemented using sugarcane bagasse substrate, purchased cellulases and *S. cerevisiae* yeast cell resulting in a total accumulated WIS content of 22% and a high ethanol titer up to 65 g/L which is equivalent to an ethanol yield of 267.3 kg-ethanol/ton-bagasse. In our designed SSF process, waste was minimized as the whole slurry of pretreated bagasse was used with no solid-liquid separation after the pretreatment and dried yeast of *S. cerevisiae* was used, thereby no wastewater pretreatment was required in the pretreatment and yeast cultivation steps. The fed-batch SSF process with minimized waste was therefore evaluated for its techno-economic feasibility.

3.1. Integrated SSF process for conversion sugarcane bagasse to ethanol using S. cerevisiae

Operation with high biomass loadings of 15% (w/v) or higher is preferred for higher sugar concentrations and greater ethanol titers, which in turn require less energy and smaller equipment for a given throughput [28]. The integrated sugarcane bagasse to ethanol process as depicted in Fig. 1 began with diluted-acid pretreatment. pH of the pretreated slurry was then adjusted with KOH and diluted to a water-insoluble solids (WIS) concentration as designed based on the previous experimental work. After that, the simultaneous enzyme hydrolysis and fermentation (SSF) with the addition of required nutrients, yeast cells and cellulases was performed to convert pretreated bagasse to ethanol. Molasses, commonly available in a sugar factory that provides sugarcane bagasse feedstock, was provided as nutrients in the SSF process. Mineral salt solution composing of 0.75 g/L $(NH_4)_2SO_4$, 0.35 g/L KH_2PO_4, 0.07 g/L $MgSO_4 \cdot 7H_2O$ and yeast extract (1 g/L) were also supplied to the SSF process. Dilute acid pretreatment with sulfuric acid as a catalyst was utilized since it is considered an economically viable technology to increase the digestibility of cellulose in enzymatic hydrolysis [29]. The fed-batch SSF was pulse-fed with pretreated bagasse slurry and yeast cells at desired feed profiles. The output stream from the SSF unit was transferred to the downstream operations as previously optimized [8] to recover the ethanol solution at 94% (w/w) concentration. The remaining solid and liquid waste was separated out after downstream processing for electricity and biogas production. The process conditions for each step in term of conversion yield, titer and processing time were based on experimental data previously described [6]. These data were then used in techno-economic evaluations of the fed-batch SSF process. The purpose of the fully integrated process simulation and the economic evaluation is to evaluate economic feasibility of the developed fed-batch SSF as well as to explore other process alternatives for further improvement on process economy. It should be noted that the techno-economic assessment is by no mean to determine an absolute ethanol production cost and the exact selling price.

3.2. Techno-economic assessment of high-solid SSF process

The process scheme for fed-batch, high-solid SSF using *S. cerevisiae* and purchased cellulases (SSF-Sac) was simulated using SuperPro Designer platform and the previously reported ethanol production performance [6] as shown in Table 1 and Fig. 1. An overview of material and utility requirements of the SSF-Sac

Table 1
Techno-economic evaluation of sugarcane-to-ethanol process in fed-batch, high-solid SSF using *S. cerevisiae* with minimized waste.

	Fed-batch SSF using *S. cerevisiae*	References
Ethanol titer (g/L)	65.43 ± 3.86	[6]
Ethanol yield (g/g)	0.43 ± 0.03	[6]
Ethanol yield (kg-etoh/ton-bagasse)	267.3	This study
Production capacity (L-etoh/year)[a]	41,664	This study
Production cost (Baht/Batch)[b]	20,361	This study
Minimal ETOH selling price (Baht/L)[c]	22.2	This study

[a] Production capacity is based on demonstration scale operating at 3 dry tons sugarcane bagasse per batch and 41 batches annually.
[b] Production cost is estimated from upstream processing with 3 dry tons sugarcane bagasse per batch. Estimated cost is based on the local or reported cost for raw materials and utilities as summarized in Table 1.
[c] Ethanol selling price is estimated based on total production cost per total ethanol produced. Downstream processing cost is calculated from the previously reported value of required energy for ethanol purification, 10.2 MJ/L ethanol [8].

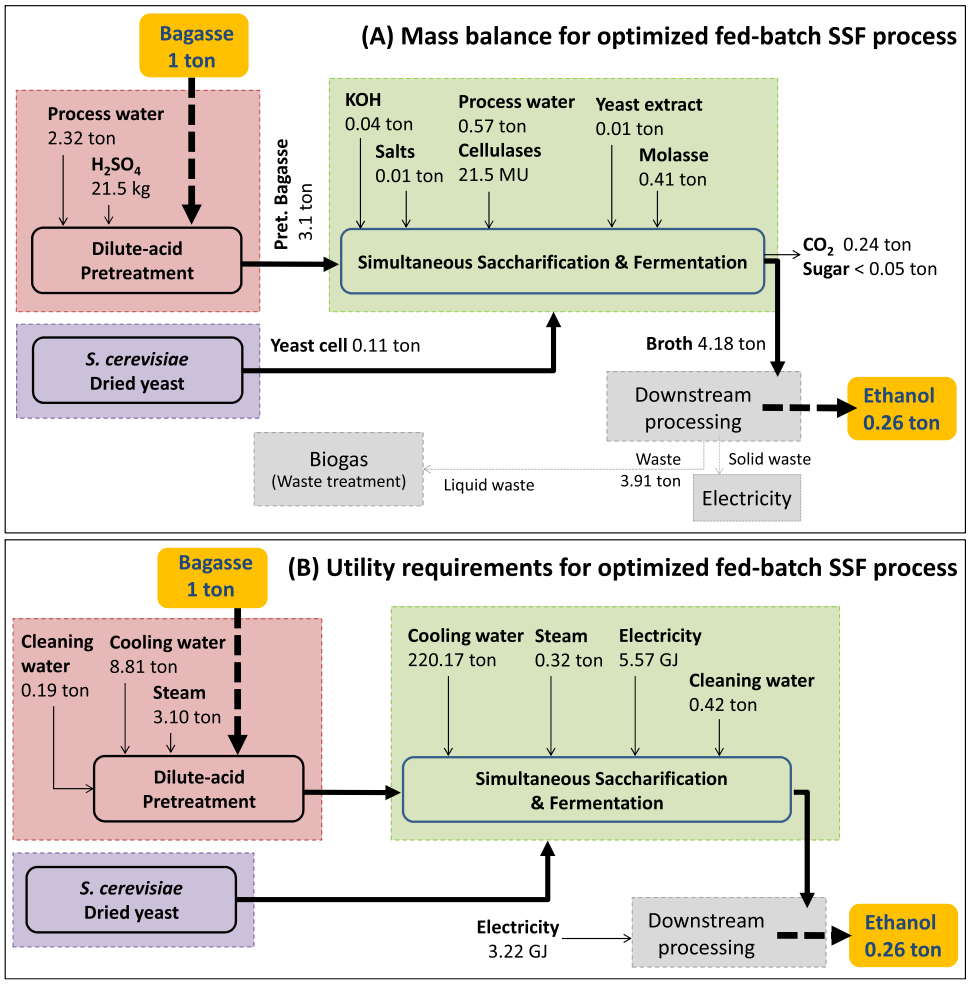

Fig. 2. Overall materials (A) and utilities (B) required for the conversion of sugarcane bagasse to ethanol using optimized, high-solid fed-batch saccharification and fermentation of *S. cerevisiae* with minimized waste [6]. The SSF process was carried out with a total WIS content of 22%. All values are calculated using SuperPro Designer platform based on per 1 dry ton sugarcane bagasse.

process per 1 dry ton sugarcane bagasse is summarized in Fig. 2 and Table 2. We economically evaluated the SSF-Sac process based on operating costs and minimal selling prices. The cost analysis of all the process steps for MESP determination was estimated based on raw materials and utilities costs from the economic data reported in our case study and others [2,4,9,11] as summarized in Table 3. The SSF-Sacc process demonstrates economic viability with the ethanol production yield of 267.3 kg-ethanol/ton-bagasse and the MESP of 22.2 Baht/L which is equivalent to 2.4 USD/gal (Table 1). The SSF-Sacc process in fed-batch mode for high ethanol titer also reduced the MESP by 16.5% and the production cost by 27.5% hence increasing profit margin compared to batch process (result not show). Similar trend was reported in previous studies [2,8,12], confirming a significant positive effect of high-solid, fed-batch SSF on the process economy. Although the MESP in this study is higher than the previously reported MESP of 19.58 Baht/L (approximated 2.1 USD/gal) [12,30], the MESP in our process may not be comparable with their processes due to the difference in process flowsheet, production scale (demo-scale vs. industrial scale), feedstock composition and raw materials used (sugarcane bagasse vs. corn stover). Nevertheless, the techno-economic framework reveals that the estimated minimal ethanol selling price of high-solid, fed-batch process was still below the current selling price of ethanol from cassava-based process (27.19 baht/L, www.thaiethanol. com). Thus,

Table 2
Raw material and utility requirements for the conversion of 1 dry ton sugarcane bagasse to ethanol by *S. cerevisiae* in optimized fed-batch SSF process with minimized waste.

Raw materials	SSF fed-batch Per 1 dry ton bagasse	
Process water	2.90	ton
H_2SO_4	0.021	ton
KOH	0.041	ton
Molasse	0.41	ton
Enzymes	0.18	ton
Mineral salts	0.014	ton
Yeast extract	0.012	ton
Dried yeast	0.11	ton
Utility requirements		
Cooling water	228.98	ton
Steam	3.42	ton
Electricity	8.79	GJ
Cleaning water	0.61	ton
Products		
Ethanol	0.26	ton
CO_2[a]	0.24	ton
Waste water	3.91	ton
Ethanol yield[b]	267.3 kg-etoh/ton-bagasse	

[a] CO_2 is sale as byproduct income.
[b] The value is estimated from upstream process only thereby excluding the loss during downstream steps.

Table 3
Raw materials and utilities cost used in the techno-economic evaluation of fed-batch SSF process for the production of ethanol from sugarcane bagasse.

	Cost	Unit[a]	Sources
Raw materials			
Sulfuric acid	1.18	Baht/kg	[9]
$MgSO_4$	18	Baht/kg	[8]
Molasse	4.07	Baht/kg	[4]
Cellulases	408	Baht/kg	[11]
$(NH_4)_2SO_4$	28	Baht/kg	Local supplier
KOH	8.5	Baht/kg	Local supplier
KH_2PO_4	19	Baht/kg	Local supplier
Yeast extract	480	Baht/kg	Local supplier
Utilities and labor			
Electricity	0.28	Baht/MJ	[8]
Process water	0.006	Baht/L	[8]
Chilled water	0.013	Baht/L	[8]
Cooling water	0.010	Baht/L	[8]
Cleaning agent	16.28	Baht/kg	[8]
Steam	0.41	Baht/kg	Local supplier
Byproducts income			
CO_2	0.12	Baht/kg	[8]

[a] The values reported in literatures are converted to Baht using the following exchange rate, 0.028 USD/Baht or 0.24 SEK/Baht (the 2015 rate average, www.exchange-rates.org).

Table 4
Cost summary of optimized fed-batch saccharification and fermentation using *S. cerevisiae* for the production of ethanol from sugarcane bagasse in a demonstration scale.

	Annual cost (Baht)[a]
Raw materials/Chemicals	192,046
Process water	7321
Cooling water	19,781
Chilled water	13,604
Steam	203,272
Electricity	385,498
Cleaning	6650
Total expense	**828,171**

[a] Annual cost is estimated from upstream processing in demonstration scale operated at 3 dry tons raw bagasse per batch and 41 batches per year.

the high-solid, fed-batch process platform could potentially be suitable to meet the economic demand of large-scale ethanol production process, thereby replacing the starch-based ethanol process platform. It should be noted that the exact MESP could be determined using the fully integrated process simulation that is extend beyond upstream and downstream steps by incorporating all unit operations for processing biomass feedstock prior to pre-treatment (e.g. pre-processing, transportation and storage), waste treatment, waste re-utilization after end-product recovery including energy and heat integration of lignin processing step (e.g. combustion of solids/lignin waste for electricity generation).

3.3. Cost distribution analysis

Annual production cost for the conversion of sugarcane bagasse to ethanol using fed-batch, high-solid SSF with *S. cerevisiae* is summarized in Table 4. The cost summary of the SSF-Sacc process resulted in approximately 41,664 L of cellulosic fuel ethanol production per year with an annual income of 1,164,776 baht based on the demonstration scale of 3 ton dry bagasse per batch. The process main costs contributed to raw materials and utilities which accounted for 23% and 71% of total production cost respectively. For every one ton of dry bagasse being processed, 0.24 tons of carbon dioxide was generated (Table 2). Therefore, the co-product credit would have a significant effect on the overall process economy. Upgrading the CO_2 byproduct into valued-added chemicals through catalytic or biological conversion process would increase income and profit margin of the current integrated process. In addition, based on material flow balance, 2.90 ton of process water was required for upstream processing of 1 ton of raw sugarcane bagasse. To reduce the plant makeup water and further improve process economic, major part of the process water required could be replaced with steam condensates without affecting ethanol production yield. It should be noted that the cost analysis has not yet considered the cost due to mixing energy consumption when the process is operated at high solid condition (25% solid or more). Thus, a balance for achieving the optimal energy cost between the increased mixing energy cost and the reduced distillation energy cost needs to be thoroughly evaluated and account for in the future during process integration at higher solid loading.

Cost distribution analysis for identifying various process bottlenecks that decrease the efficiency of SSF-Sacc process economic is demonstrated in Fig. 3. In Fig. 3A, the cost of enzymes constituted 44% of the raw material costs, to which alkaline used for detoxification was also the second contributor of 21% as has also been observed in several previous reports [2,31]. Thus, from the process economics perspective, the improvement in cell robustness against the inhibitors present in pretreated biomass slurry is a prerequisite to reduce cost on detoxification step [32,33]. Engineering robust yeast cell that could grow and propagate in high-solid SSF could also reduce the amount of yeast addition into the process since major limitation of high-solid operation is high concentration of inhibitors which hamper yeast cell growth and fermentation [6,16].

The cost of electricity and steam energy also covered more than 90% of the overall utility cost (Fig. 3B). Several alternatives for lower utility cost are recycling steam condensates for increasing energy efficiency, utilizing lignin and other solid wastes as energy source,

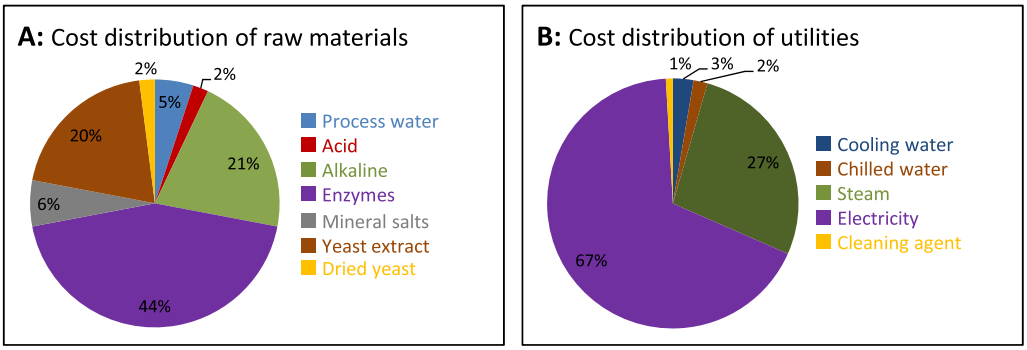

Fig. 3. Cost distributions of raw materials (A) and utilities (B) in fed-batch, high-solid simultaneous saccharification and fermentation (fed-batch SSF) using *S. cerevisiae* and purchased cellulase enzymes [6]. The values are based on upstream processing only. No labor, maintenance and capital investment cost was accounted for in the production process.

and integrating biogas (e.g. methane) production from liquid waste into the process for electricity and steam generation. Integrating the wastewater treatment would also reduce the utility cost for steam and electricity which are the two major cost of the process (Fig. 3, Table 4) making the sugarcane bagasse-to-ethanol process more cost effective and energy efficient in the economic outcome. Liquid waste from the downstream steps could be used for biogas production in anaerobic digestion which can be used for steam generation. The solid waste containing the yeast could also be utilized as cattle feed. Furthermore, the solid, lignin-rich waste obtained from the downstream process is a co-product that can be dried and re-used as a solid fuel for electricity generation, underlining the importance of lignin recovery. Investigating potentials for increasing income from by-products and solid-liquid wastes generated during the production is left for future works.

3.4. Process design and sensitivity analysis

The effectiveness of enzyme saccharification and fermentation conditions contributes significantly to the economics of the overall process. Co-fermentation of C_5 and C_6 sugars which could be accomplished by yeast consortium of *S. stipitis* and *S. cerevisiae* is essential to achieve high ethanol yield for economic advantages. According to the techno-economic evaluations (Fig. 3A), the enzyme cost was also the main concern for the ethanol production from sugarcane bagasse. Since enzyme hydrolysis is the main contributors to the overall costs of producing ethanol from bagasse, on-site or near-site enzyme production is desirable in order to further reduce the MESP to be competitive with the current selling price. The production of enzymes on-site could be done using fungus cell or genetic engineered yeasts [27,34]. Increasing enzymes activity using enzyme synergism is also one of the options for reducing enzyme loading and enhancing efficiency of enzyme hydrolysis.

(1) Impact of yeast consortium utilization on MESP

The carbohydrates content of sugarcane bagasse consisted of 37% C_6 sugars (e.g. glucose) from cellulose and 23% C_5 sugars (e.g. xylose) from hemicellulose based on dry basis as measured in previous studies [6,29]. These C_5/C_6 sugars must be converted to ethanol in order to achieve the economic feasibility of lignocellulosic ethanol production process. We have previously demonstrated an approach for C_5/C_6 sugar fermentation using a yeast consortium of *S. stipitis* (C_5-fermenting yeast) and *S. cerevisiae* (C_6-fermenting yeast) which led to 26% enhancement in ethanol titer compared to when only *S. cerevisiae* is used [16]. The yeast cultures of *S. stipitis* and *S. cerevisiae* were separated in a continuous centrifuge, re-suspended in culture media, and added into fermentation reactor at optimized cell ratio as previously reported. Techno-economic feasibility analysis to evaluate the yield and production cost tradeoffs for the use of yeast consortium was performed based on the previous experimental study. Comparing between process configurations in Fig. 4A and B, the impact of yeast consortium on MESP revealed up to 11.6% reduction of MESP when the yeast consortium technology was utilized (Fig. 5A). This trend is consistent with previous study showing up to 30% production cost reduction if both C_6 and C_5 sugars are converted to ethanol compared to only C_6 sugar conversion [13]. The result, therefore, validated the yeast consortium technology for economical biomass-to-ethanol conversion. Additionally, impact of cellulase enzyme dosage on the process economy and MESP was examined in high-solid SSF using *S. cerevisiae*/*S. stipitis* yeast consortium.

(2) Impact of cellulase enzyme loading on MESP

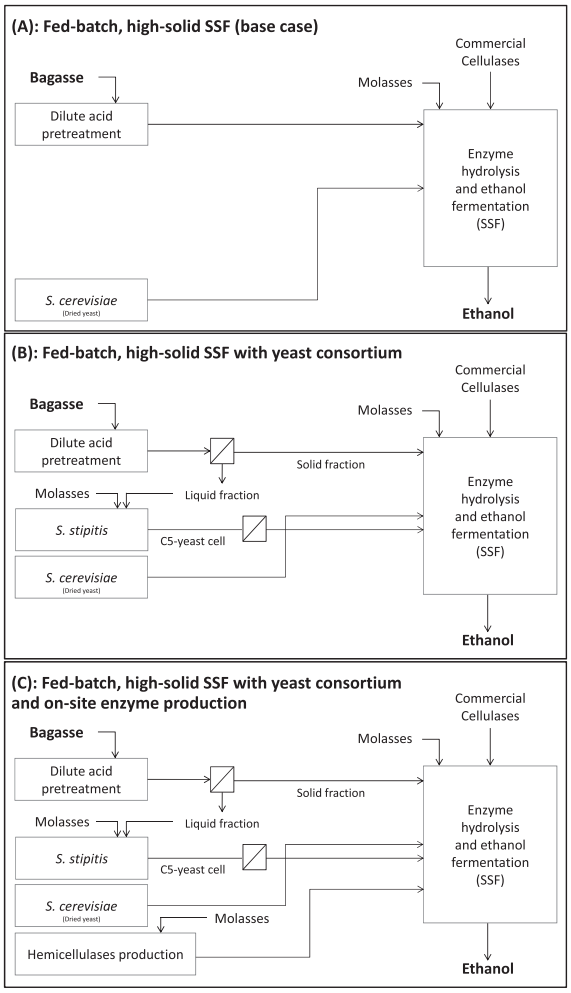

Fig. 4. Three process scenarios for the conversion of sugarcane bagasse to ethanol under techno-economic evaluation: (A) SSF-Sacc: *S. cerevisiae* in fed-batch, high-solid simultaneous saccharification and fermentation (SSF) using commercial cellulases, (B) SSF-Co: *S. stipitis*/*S. cerevisiae* in fed-batch, high-solid SSF using commercial cellulases, and (C) SSF-Co-Enz: *S. stipitis*/*S. cerevisiae* in fed-batch SSF using commercial cellulases and on-site hemicellulase enzymes production. SSF-Sacc is the reference case using only C_6-fermenting yeast, purchased cellulase enzymes and no on-site hemicellulase enzymes production. SSF-Co is the reference case with the addition of C_5-fermenting yeast, while SSF-Co-Enz is the reference case with the addition of C_5-fermenting yeast and on-site produced hemicellulase enzymes supplementation. The condition and performance of SSF-Sacc process and yeast consortium was described in previous study [6,16]. The production of hemicellulase enzymes and hydrolysis yield of cellulases supplemented with hemicellulases are as previously described [19,27].

Based on cost analysis (Fig. 3), cellulase enzyme cost was major cost distribution. Thus, the MESP could be further reduced to make the cellulose ethanol market competitive if enzyme loading can be further reduced and the sugar yield from enzyme saccharification process can be further improved. Based on experimental SSF studies using co-culture with different cellulase loading, MESP was calculated. Fig. 5A shows the relative effects of enzyme loading on the overall process economy in term MESP. Reducing cellulase enzyme load during the fed-batch, high-solid SSF from 25 FPU/WIS to 15 FPU/WIS increased MESP by 9%. Although at low enzyme loading the cost of purchased enzyme was reduce naturally [10], the release sugar yield of enzyme hydrolysis was also decreased when lower enzyme load was used resulting in lower final titer of ethanol fermentation. The same trend was also observed in Fig. 5B where the MESP linearly increased when cellulase enzyme loading reduced. Reducing enzyme load from 25 FPU/WIS to 2.5 FPU/WIS

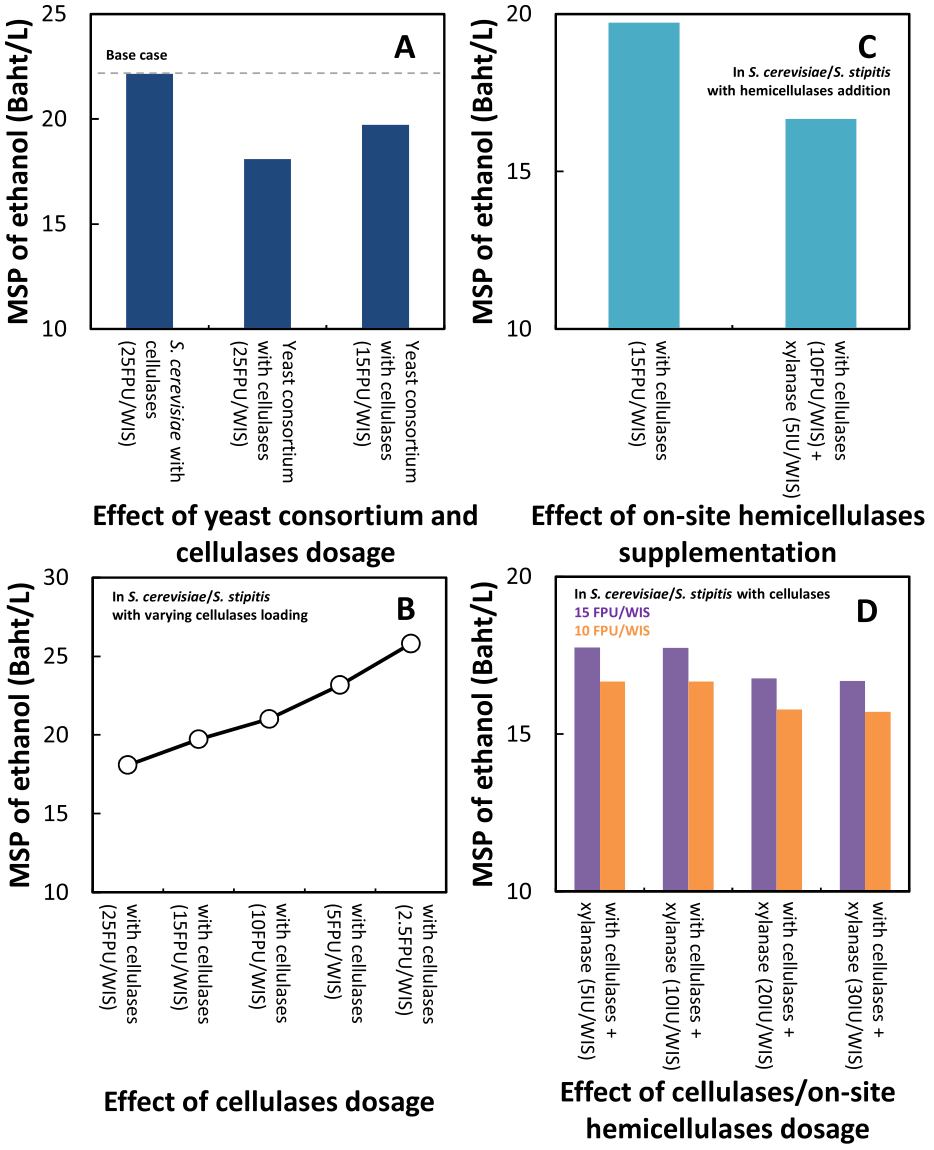

Fig. 5. Process sensitivity analysis examining impacts of yeast consortium (A), cellulase enzymes dosage (B), and cellulase/on-site hemicellulase enzymes loading (C, D) on the minimum ethanol selling price (MESP) for fed-batch, high-solid SSF of sugarcane bagasse to ethanol process. Reference case is fed-batch, high-solid SSF of sugarcane bagasse using single-strain culture of *S. cerevisiae* with purchased cellulase enzymes. The condition and performance of SSF-Sacc process and yeast consortium using commercial cellulase enzymes was as described in previous study [6,16]. The production of hemicellulase enzymes and hydrolysis yield of cellulases supplemented with hemicellulases are as previously described [19,27].

(based on unpublished experimental data, Supplementary S1) yielded an increased MESP from 18.1 Baht/L to 25.8 Baht/L, equivalent to more than 30% increase. The results indicated that the performance of enzyme hydrolysis had higher impact on MESP than the cost of enzyme usage.

(3) Impact of cellulase/hemicellulase enzyme synergism on MESP

The synergistic action of the cellulases/xylanase mix has been reported to increase >50% superior saccharification yield and ethanol production compared to the cellulases enzymes alone [18,19,31,37]. Thus, the production economics of lignocellulosic ethanol is largely dependent on the usage of cellulase and hemicellulase enzymes [21]. On-site enzyme production can significantly decrease the overall MESP up to 40% compared to the use of purchased enzymes [13,35], providing a promising alternative approach for economical cellulosic ethanol production. The obvious advantages of on-site enzyme production include no additional cost on enzyme concentrating and purifying steps, storage and transportation cost, thereby providing a significant cost advantages to the process [34,36,37]. Thus, we examined economic impact of on-site xylanase supplementation on lignocellulosic ethanol production process. The cost analysis was evaluated based on unpublished SSF experiments with varying cellulases and on-site hemicellulases dosage (Supplementary S1 and S2). The performance of SSF experiments was from using *S. cerevisiae*/*S. stipitis* yeast consortium and purchased cellulases mixed with various load of crude xylanase. The production of crude xylanase was simulated based on the performance of recombinant strain of *S. stipitis* expressing xylanase as previously reported [27]. The impact of the on-site hemicellulase enzymes supplementation on MESP of the high-solid SSF process is shown in Fig. 5C and D. Adding 5 IU/WIS xylanase produced on-site to SSF process with 15 FPU/WIS

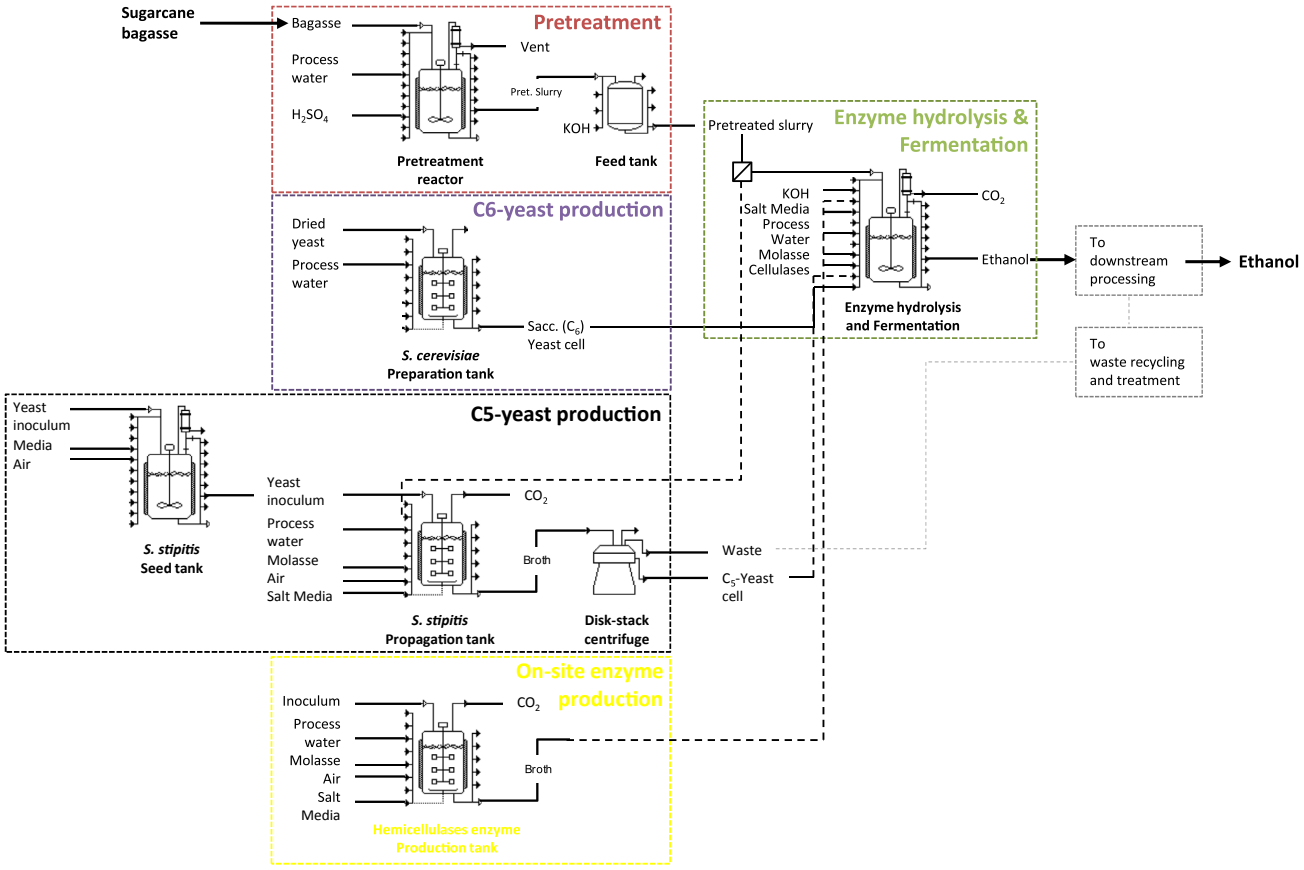

Fig. 6. Proposed process diagram of fed-batch, high-solid SSF by *S. stipitis*/*S. cerevisiae* cell consortium and on-site hemicellulase enzyme production for low-cost ethanol production from sugarcane bagasse. The sugarcane bagasse-to-ethanol process includes diluted-acid pretreatment (in red dashed square), yeast propagation for *S. cerevisiae* (in purple dashed square) and *S. stipitis* (in black dashed square), on-site hemicellulase production (in yellow dashed square), and simultaneous enzyme hydrolysis and co-fermentation (in green dashed square) steps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purchased cellulase dosage led to a 15% reduction in MESP compared to SSF process with only purchased cellulases (Fig. 5C). Xylanase hydrolyzes xylan in the pretreated bagasse into xylose allowing more accessibility of cellulose by cellulases yielding increased glucose release. Higher C_5 (xylose) and C_6 (glucose) sugars released during hydrolysis in cellulases/hemicellulases mixture was then converted to ethanol by the yeast consortium resulting in higher ethanol yield and titer. Thus, this result demonstrated the impact of enzyme synergism on the lignocellulosic process economy. Varying ratio of cellulases and xylanase enzyme load also affected the economics of bagasse-to-ethanol production process. As shown in Fig. 5D, increasing xylanase dosage from 5 to 30 IU/WIS lowered the MESP by up to 15% under 15 FPU/WIS cellulase dosage and by 25% under 10 FPU/WIS cellulase dosage in fed-batch, high-solid SSF process with *S. cerevisiae*/*S. stipitis* yeast consortium. Thus, a proper combination of cellulase and hemicellulase enzyme mixtures is one of important factors for the sugarcane bagasse-to-ethanol process economy. The result is another confirmation that the dosage ratio between cellulases and hemicellulases mixture is necessary for economic feasibility of the sugarcane bagasse-to-ethanol process.

(4) Low-cost lignocellulosic ethanol production by high-solid SSF

Based on techno-economic analysis of different process schemes, key features that must be reached for economic feasibility of sugarcane bagasse-to-ethanol process are (1) high sugar yield of enzyme hydrolysis with optimized cellulase and hemicellulase enzyme mixtures, (2) reduction of enzyme cost through on-site production, (3) efficient conversion of both C_5 and C_6 sugars to ethanol using yeast consortium and (4) enhanced ethanol titer through fed-batch, high-solid SSF process. Comparing all process schemes under investigation, the lowest MESP achieved was 15.7 Baht/L (equivalent to 1.66 USD/gal based on an exchange rate 0.028 USD/Baht) which was obtained from the high-solid SSF process scheme with *S. cerevisiae*/*S. stipitis* yeast consortium using 10 FPU/WIS purchased cellulases supplemented with 30 IU/WIS on-site crude xylanase. This MESP was a 29% lower compared to that obtained from the reference case using *S. cerevisiae* and purchased cellulases. Under this condition, the ethanol cost is also below the current market price of 1.76 USD/gal (as of December 2016, http://www.tradingeconomics.com/commodity/ethanol), thus the commercialization feasibility exists. The estimated MESP under the best process configuration will generate up to 6% increase in profit margin based on the current selling price of fuel ethanol reported. The proposed process scheme for low-cost ethanol production process from sugarcane bagasse is shown in Fig. 6.

To the best of authors' knowledge, this study is the first to demonstrate the techno-economic impacts of *S. stipitis*/*S. cerevisiae* yeast consortium, on-site enzyme production and cellulase/hemicellulase enzyme synergism on process economy and MESP of lignocellulosic ethanol production process at high solid operation. Overall, addition of optimized cellulase and xylanase enzyme loading which is produced on-site together with optimal yeast consortium of *S. stipitis* and *S. cerevisiae* should permit a highly

efficient and low-cost simultaneous enzyme hydrolysis and fermentation for the production of ethanol from sugarcane bagasse. A proposed process with fed-batch, high-solid SSF using yeast consortium and cellulases/on-site hemicellulase mixture still requires the validation of industrial-scale operations for the future cellulosic ethanol industry. Potential economic benefit with higher activity enzymes (e.g. through supplementation of enzyme enhancer, addition of surfactants to minimize enzyme irreversible binding to lignin [42]) and more robust yeast cell is not yet considered in the MESP, and will be addressed in the future. In addition, uncertainty from various sources such as change in market or operational parameters should be included into the future process integration model for evaluation of the impact of uncertainties affecting the overall production cost in order to improve a degree of realism of the simulated process [10,38].

4. Conclusion

In this study, techno-economic of the previously developed high-solid SSF process [6] was analyzed based on process flowsheet simulation for an estimation of the minimal ethanol selling price (MESP) under current process condition. Techno-economic assessment also provided insight into the possible process design for an economical-viable sugarcane bagasse-to-ethanol process. The estimated MESP for fed-batch, high-solid SSF process was 22.2 Baht/L which is equivalent to 2.4 USD/gal. The process cost analysis pointed to a potential ethanol production cost reduction via (1) lowering enzyme demand of SSF through on-site production and enzyme synergism for high saccharification efficiency as well as (2) increasing ethanol titer through yeast consortium for C_5/C_6 co-fermentation. Integrating the high-solid SSF process with the use of yeast consortium and optimized cellulase and on-site hemicellulase enzyme mixtures, the MESP could be reduced to as low as 15.7 Baht/L equivalent to 1.66 USD/gal. This was a 6% lower than the current market selling price of ethanol of 1.76 USD/gal providing economic feasible for industrialization. Techno-economic analysis obtained in this study could provide basis for cost comparisons with those obtained by other SSF processes. Future research and development would include engineering high-activity enzymes to further reduce enzyme load as well as developing robust yeast cell to facilitate higher ethanol titer under higher solid operation for the lower MESP.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.energy.2017.01.090.

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