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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

*Correspondence: pornkamol.unr@biotec.or.th
National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park Phahonyothin Road, Klong Nueng, Klong Luang, Pathum Thani 12120, Thailand

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

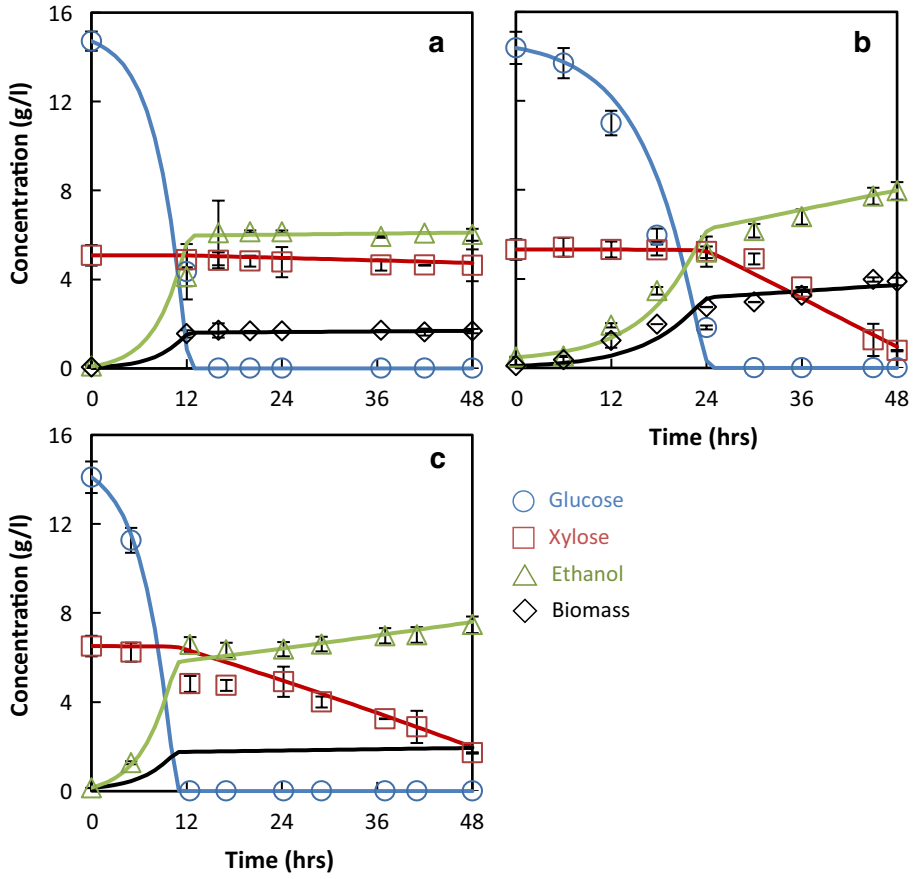

Fig. 1 Fermentation kinetics of *S. cerevisiae* (**a**), *S. stipitis* (**b**) and co-culture of *S. stipitis* and *S. cerevisiae* (**c**) in glucose–xylose mixture. Time profiles of glucose (*blue*), xylose (*red*), ethanol (*green*) and biomass (*black*) in *S. cerevisiae*, *S. stipitis*, and *S. stipitis*/*S. 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

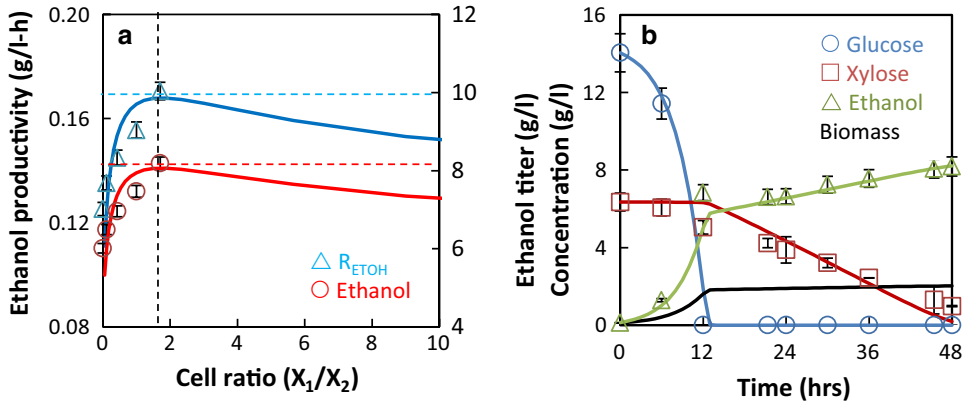

Fig. 2 Ethanol production in mixed glucose–xylose fermentation by *S. stipitis*/*S. cerevisiae* co-culture. **a** Ethanol productivity (*cyan*) and titer (*red*) at various cell ratios of *S. stipitis* (X_1)/*S. cerevisiae* (X_2) co-culture in comparison with single-strain culture of *S. cerevisiae*. 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

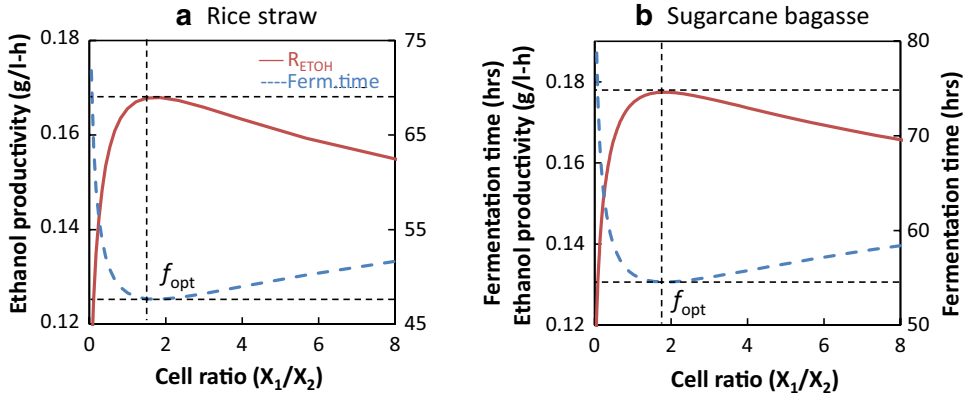

Fig. 3 Predicted ethanol productivity (*solid line*) and fermentation time (*dashed line*) by co-culture kinetic model for **a** rice straw and **b** sugarcane bagasse fermentation. The model identifies optimal cell ratio (f_{opt}) of 1.70 g cell *S. stipitis*/g cell *S. cerevisiae* for rice straw and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

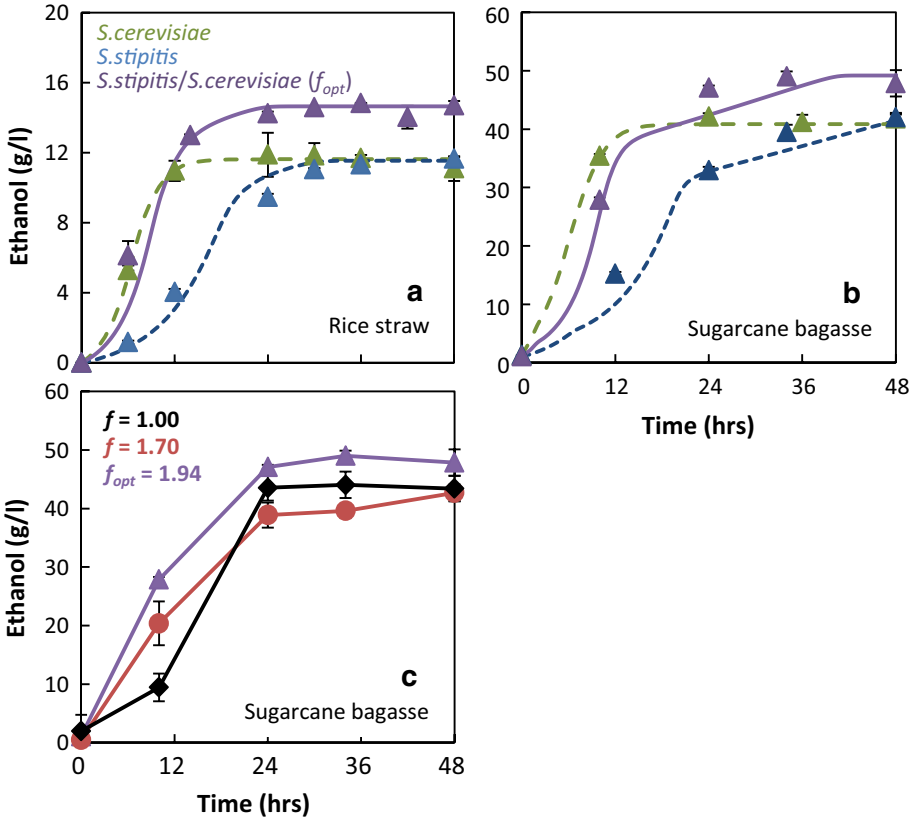

Fig. 4 Saccharification and fermentation of rice straw (**a**) and sugarcane bagasse (**b**) by *S. stipitis*/*S. cerevisiae* co-culture in comparison with single-strain culture. Time profiles of ethanol in *S. stipitis* (*blue*), *S. cerevisiae* (*green*) and *S. stipitis*/*S. cerevisiae* (*purple*) 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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References

Ashoor S, Comitini F, Ciani M (2015) Cell-recycle batch process of *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* co-culture for second generation bioethanol production. Biotechnol Lett. doi:10.1155/2015/26198848

Bera AK, Sedlak M, Khan A, Ho NW (2010) Establishment of L-arabinose fermentation in glucose/xylose co-fermenting recombinant *Saccharomyces cerevisiae* 424A(LNH-ST) by genetic engineering. Appl Microbiol Biotechnol 87(5):1803–1811

Binod P, Sindhu R, Singhania RR, Vikram S, Devi L, Nagalakshmi S, Kurien N, Sukumaran RK, Pandey A (2010) Bioethanol production from rice straw: An overview. Bioresour Technol 101(13):4767–4774

Chen Y (2011) Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review. J Ind Microbiol Biotechnol 38(5):581–597

FitzPatrick M, Champagne P, Cunningham MF, Whitney RA (2010) A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour Technol 101(23):8915–8922

Hanly TJ, Henson MA (2014) Dynamic model-based analysis of furfural and HMF detoxification by pure and mixed batch cultures of *S. cerevisiae* and *S. stipitis*. Biotechnol Bioeng 111(2):272–284

Hickert LR, de Souza-Cruz PB, Rosa CA, Ayub MA (2013) Simultaneous saccharification and co-fermentation of un-detoxified rice hull hydrolysate by *Saccharomyces cerevisiae* ICV D254 and *Spathaspora arborariae* NRRL Y-48658 for the production of ethanol and xylitol. Bioresour Technol 143:112–116

Karagöz P, Özkan M (2014) Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture in batch and continuous system. Bioresour Technol 158:286–293

Kircher M (2012) How to turn industrial biotechnology into reality. N Biotechnol 29(2):243–247

Konishi J, Fukuda A, Mutaguchi K, Uemura T (2015) Xylose fermentation by *Saccharomyces cerevisiae* using endogenous xylose-assimilating genes. Biotechnol Lett 37(8):1623–1630

Li Y, Park JY, Shiroma R, Tokuyasu K (2011) Bioethanol production from rice straw by a sequential use of *Saccharomyces cerevisiae* and *Pichia stipitis* with heat inactivation of Saccharomyces cerevisiae cells prior to xylose fermentation. J Biosci Bioeng 111(6):682–686

Lopes MS (2015) Engineering biological systems toward a sustainable bioeconomy. J Ind Microbiol Biotechnol 42(6):813–838

South CR, Hogsett DA, Lynd LR (1995) Modeling simultaneous saccharification and fermentation of lignocellulose to ethanol in batch and continuous reactors. Enz Microb Technol 17:797–803

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83(1):1–11

Suriyachai N, Weerasaia K, Laosiripojana N, Champreda V, Unrean P (2013) Optimized simultaneous saccharification and co-fermentation of rice straw for ethanol production by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture using design of experiments. Bioresour Technol 142:171–178

Unrean P, Franzen CJ (2015) Dynamic flux balancing elucidates NAD(P)H production as limiting response to furfural inhibition in *Saccharomyces cerevisiae*. Biotechnol J. doi:10.1002/biot.201400833

Unrean P, Nguyen NH (2012) Rational optimization of culture conditions for the most efficient ethanol production in *Scheffersomyces stipitis* using design of experiments. Biotechnol Prog 28(5):1119–1125

Unrean P, Srienc F (2010) Continuous production of ethanol from hexoses and pentoses using immobilized mixed cultures of *Escherichia coli* strains. J Biotechnol 150(2):215–223

Wan P, Zhai D, Wang Z, Yang X, Tian S (2012) Ethanol production from nondetoxified dilute-acid lignocellulosic hydrolysate by cocultures of *Saccharomyces cerevisiae* Y5 and *Pichia stipitis* CBS6054. Biotechnol Res Int. doi:10.1155/2012/656371

Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, Van Dijken JP, Pronk JT, Van Maris AJ (2007) Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Appl Environ Microbiol 73(15):4881–4891

Yadav KS, Naseeruddin S, Prashanthi GS, Sateesh L, Rao LV (2011) Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. Bioresour Technol 102(11):6473–6478