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Process intensification of cellulase and bioethanol production from sugarcane bagasse via an integrated saccharification and fermentation process

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ABSTRACT

The production of value-added products such as cellulase and ethanol *via* a consolidated bioprocess could be realized by tapping into the multiple actions of a microbial community. For this purpose, an *in situ* saccharification and fermentation process through a sequential co-culture white-rot fungus and *Saccharomyces cerevisiae* on NaOH-pretreated sugarcane bagasse (SCB) was investigated. In the present work, white rot fungus plays a role in the production of cellulase enzymes. With the produced cellulase, an *in situ* saccharification process took place in the reactor to depolymerize pretreated SCB into reducing sugar. The reducing sugar was converted into ethanol *via* fermentation by *S. cerevisiae*, which was added into the system sequentially. White rot fungus *Pycnoporus sanguineus* was selected due to its competency in producing cellulase and reducing sugar production. The operating condition to maximize the production of reducing sugar *in situ* was obtained through a Central Composite Design method. A total of 3.13 g reducing sugar/100 g SCB was obtained when *P. sanguineus* was cultivated at 0.6% inoculum loading, 70% moisture content and 4 days. Subsequently, 4.5 g ethanol/100 g SCB was obtained from the *in situ* saccharification and fermentation system after S. *cerevisiae* was sequentially inoculated.

1. Introduction

Lignocellulosic wastes from the agricultural industry such as sugarcane bagasse, oil palm empty fruit bunches and wheat straws generally have limited commercial applications and the substantial generation of such wastes has created inevitable disposal challenges. Lignocellulosic wastes are rich in cellulose and hemicellulose which can be converted into a variety of value-added products such as ethanol, enzymes and organic acids. A typical conventional scheme of converting lignocellulosic wastes into ethanol is through pretreatment of lignocellulosic waste followed by separate enzymatic saccharification and fermentation processes [1]. During an enzymatic saccharification process, cellulose and hemicellulose in lignocellulosic wastes are depolymerized to sugar monomers. Cellulase enzymes used in saccharification is commonly produced by filamentous fungi. White-rot fungi is a type of filamentous fungi that has the ability to degrade all the components in lignocellulosic substrates by secreting multiple enzymes once the lignocellulosic substrate is colonized [2]. Solid state fermentation (SSF) is preferable for fungal enzymes production instead of submerged fermentation as the former mimics an environment resembling the natural habitat for fungal growth; yielding higher concentration of end products and fermentation productivity [3,4]. In the subsequent fermentation step, sugars produced from enzymatic saccharification can be converted into ethanol by microorganisms such as *Saccharomyces cerevisiae, Zymomonas mobilis* and *Pichia stipites* [5,6].

Process intensification is a useful tool to further increase the production efficiency and to reduce the cost of bioethanol production and purification. Several process intensifying schemes have been proposed to convert lignocellulosic wastes into ethanol. These include integrating the conventional two-step processes of saccharification and fermentation into a simultaneous saccharification and fermentation scheme [7,8]. Besides reducing the number of the required unit operations, the

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end-product inhibition during cellulose saccharification can be overcome *via* a continuous conversion of glucose to ethanol [9]. As a result, the saccharification rate increases [10] with a higher ethanol yield in a shorter processing duration [9]. One of the challenges to the process scheme is that both saccharification and fermentation require different optimum operating conditions; thus, requiring a search for an operating condition that can complement both processes. Other proposed process intensifying schemes include carrying out alkaline pretreatment, saccharification and fermentation sequentially in a fluidized bed reactor, or using a multi-staged membrane-integrated bioreactor system that enabled the recycling of cells and fermentable sugars at higher production efficiencies [11,12].

Another proposed process intensification method is a consolidated bioprocess involving the use of a single microorganism or a microbial community that combines the functions of cellulase production, cellulase saccharification and possibly, ethanol fermentation in a single reactor [7,8,13]. The use of a single microorganism proves difficult since finding a naturally occurring microorganism that can be used to produce both cellulase and ethanol is challenging. Therefore, the use of various microorganisms in a community to directly convert lignocellulosic wastes to ethanol appears to be a good alternative. The production of ethanol by co-culturing filamentous fungi and yeast in an *in situ* saccharification and fermentation system has been investigated and reported feasible [14–17]. In a co-culture consisting of filamentous fungi and yeast, filamentous fungi acts as a producer of cellulase and hemicellulase enzymes that depolymerize the lignocellulosic wastes into sugars in the same reactor while yeast converts the sugar monomers produced from the *in situ* saccharification into ethanol [18].

In view of the benefits associated with the *in situ* saccharification and fermentation system, white-rot fungus and yeast, *S. cerevisiae* was sequentially co-cultured in an integrated *in situ* saccharification and fermentation system in the present study. White-rot fungus cultivated on sugarcane bagasse will produce cellulase enzymes and these enzymes would saccharify the sugarcane bagasse to reducing sugar *in situ*, which takes place in the same reactor. To achieve this objective, a suitable white-rot fungus based on its capability to produce cellulase and reducing sugar *in situ* was first determined. After the selection of a suitable fungal strain, the production of reducing sugar *in situ* by the white-rot fungus was maximized. Sequentially, *S. cerevisiae* was inoculated to convert the reducing sugar produced into ethanol. This system taps upon the benefits of both the chosen white-rot fungus and *S. cerevisiae,* that are naturally occurring and are widely available, as well as applying unsterilized substrates for cost effective purposes.

2. Materials and methods

2.1. Materials

Sugarcane bagasse (SCB) collected from Purecane Manufacturing Sdn. Bhd., Malaysia was thoroughly washed, dried and ground. SCB was then pretreated by using a 0.62 M NaOH solution under an optimum condition of 128 °C and 30 min at 9% (g/g) solid loading [19]. After NaOH pretreatment, SCB contained 55.9% of cellulose, 27.1% of hemicellulose and 9.9% of lignin.

2.2. Inoculum preparation of white-rot fungi and yeast

White-rot fungi *Pycnoporus sanguineus* (DSM 3023), *Phanerochaete chrysosporium* (DSM 1556), *Phlebia radiata* (DSM 5111) and *Pleurotus sajor-caju* (DSM 8265) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The fungal strains were maintained on a malt extract-peptone agar at 4 °C and subcultured monthly. The inoculum of white-rot fungus was prepared by washing the surface of the fungus grown on an agar plate with 5 mL of sterilized water. The mycelia suspension obtained was transferred to a 50 mL of 2% malt extract medium and incubated at 30 °C at 100 rpm for 5 days. The fungal pellets obtained were washed and homogenized. Homogenized fungal cells were suspended into a sterile Mandel's medium prior to usage. The Mandel's medium consists of 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 0.3 g/L $CaCl_2.2H_2O$, 0.3 g/L $MgSO_4.7H_2O$, 1 g/L peptone, 0.2% (v/v) Tween 80, 5 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4.2H_2O$, 1.4 mg/L $ZnSO_4.7H_2O$, and 2 mg/L $CoCl_2.6H_2O$ [20].

Yeast *Saccharomyces cerevisiae* (S41) was supplied by the Institute of Biological Sciences, University of Malaya, Malaysia. The yeast strain was maintained on the yeast extract-peptone-glucose agar at 4 °C and subcultured every fortnightly. Inoculum of *S. cerevisiae* was prepared by adding one inoculation loop of actively grown cells into 50 mL of yeast extract-glucose-peptone medium. The *S. cerevisiae* was cultivated for a day at 150 rpm before usage.

2.3. Screening of white-rot fungus for in situ cellulase and reducing sugar production

Four white-rot fungal strains (*P. sanguineus*, *P. chrysosporium*, *P. radiata* and *P. sajor-caju*) were screened according to their reported potential in cellulase production [21–25]. The most suitable white-rot fungus was selected based on its best ability to produce cellulase and reducing sugar *in situ*. The production of cellulase and reducing sugar by white-rot fungus was conducted in an Erlenmeyer flask with 2 g of NaOH-pretreated SCB, and inoculated with white-rot fungus mycelia suspension in a ratio of 0.5% g dry fungal weight/g substrate [26]. After fungal inoculation, Mandel's medium was supplemented to SCB to achieve the moisture content of 70%. Fungal cultivation was then carried out at room temperature (approximately 30 °C) for 8 days.

The content of the flask was extracted with 20 mL of citrate buffer (50 mM, pH 4.8) after the fungal cultivation. The extract was agitated at 150 rpm for an hour before being centrifuged at 4 °C, 3500 rpm for 20 min. The supernatant obtained from the centrifugation was filtered through a Whatman No.1 filter paper. The filtrate collected was analyzed for cellulase activities and reducing sugar concentration.

2.4. Determination of operating condition to maximize reducing sugar production by selected white-rot fungus

The operating condition to maximize reducing sugar production by the selected white-rot fungus was determined by using Design Expert 6.0.6 software (STAT-EASE Inc., Minneapolis, USA). Central Composite Design was applied and the effects of inoculum loading, moisture content and fermentation duration on the amount of reducing sugar obtained were examined (Table 1). The method used for fungal cultivation by means of solid state fermentation similar to the conditions used in Section 2.3. The statistical significance of the model developed was evaluated by using an analysis of variance (ANOVA) method.

2.5. Production of ethanol via in situ saccharification and fermentation system

After cultivation of selected fungus for 4 days as determined from Section 2.4, the system was subject to 50 °C for 2 days to enhance the *in situ* saccharification process for reducing sugar production. This is

Table 1
Levels of independent variables applied in maximizing reducing sugar production.

Variable	Coding	Units	Levels		
			−1	0	1
Inoculum loading	A	%	0.2	0.6	1.0
Moisture content	B	%	60	70	80
Cultivation duration	C	day	2	4	6

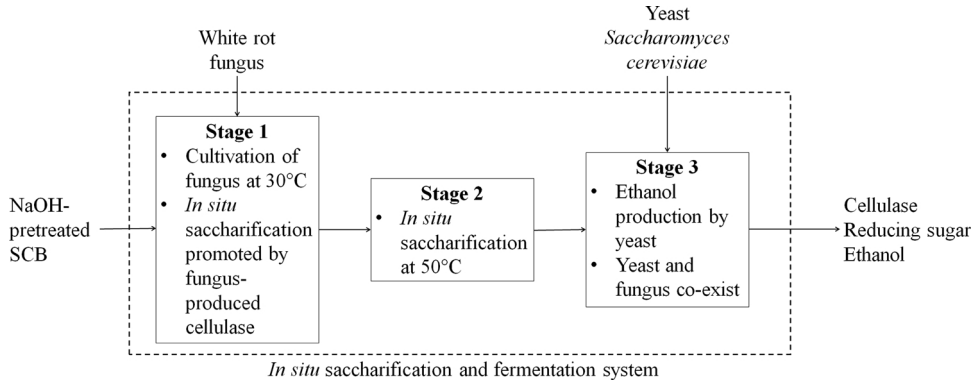

Fig. 1. Experimental procedures of the *in situ* saccharification and fermentation system.

because cellulase saccharification is often reported to be optimum at 50 °C. Subsequently, *S. cerevisiae* was inoculated after 6 days. 50 mL of nutrient solution containing 6 g/L yeast extract, 0.233 g/L $CaCl_2.H_2O$, 1.5 g/L KH_2PO_4, 4 g/L $(NH_4)_2SO_4$ and 1 g/L $MgSO_4.7H_2O$ was added. The production of ethanol by *S. cerevisiae* was carried out under anaerobic condition without agitation. The experimental procedure for ethanol production *via in situ* saccharification and fermentation system is depicted in Fig. 1. The production profile of reducing sugar and ethanol throughout the *in situ* saccharification and fermentation processes was analyzed.

2.6. Analytical methods

The total cellulase, endoglucanase and β-glucosidase activities represented by filter paper activity (FPase), carboxymethyl cellulase activity (CMCase), and β-glucosidase activity, respectively, were determined according to reported protocols [27,28]. Reducing sugar concentration was determined by the 3,5-dinitrosalicyclic acid (DNS) method while ethanol concentration was analyzed using High Performance Liquid Chromatography (HPLC) (Waters, United States) equipped with a refractive index detector (Waters 410, United States). Hi-Plex H column (7.7 mm x 300) (Agilent, USA) was used with the column temperature maintained at 65 °C. Filtered ultrapure water was used as the mobile phase in the HPLC analysis.

3. Results and discussion

3.1. Screening of white-rot fungus for in situ cellulase and reducing sugar production

The first stage of the *in situ* saccharification and fermentation system involves the selection of a suitable white-rot fungus. One of the criteria to select a suitable white-rot fungus is through the examination of the cellulase and reducing sugar production profiles, depicted in Figs. 2 and 3. Cellulase enzymes were quantified based on three methods: filter paper activity (FPase), carboxymethyl cellulase activity (CMCase) and β-glucosidase activity in order to measure the total cellulase, endoglucanase and β-glucosidase activities respectively. Cellulase system with high FPase, CMCase and β-glucosidase can effectively break down cellulose in sugarcane bagasse into glucose. Three types of cellulase enzymes, namely endoglucanase, exoglucanase and β-glucosidase must act synergistically to break down cellulose to glucose. Endoglucanase saccharifies cellulose chains to form new chain ends and these chain ends are further broken down into cellobiose and cello-oligosaccharides by exoglucanase. Cellobiose is then depolymerized into glucose by β-glucosidase.

The FPase recorded was at lower titre compared to CMCase and β-glucosidase. This is because the substrate used in quantification for FPase which is filter paper is more difficult to be depolymerised to

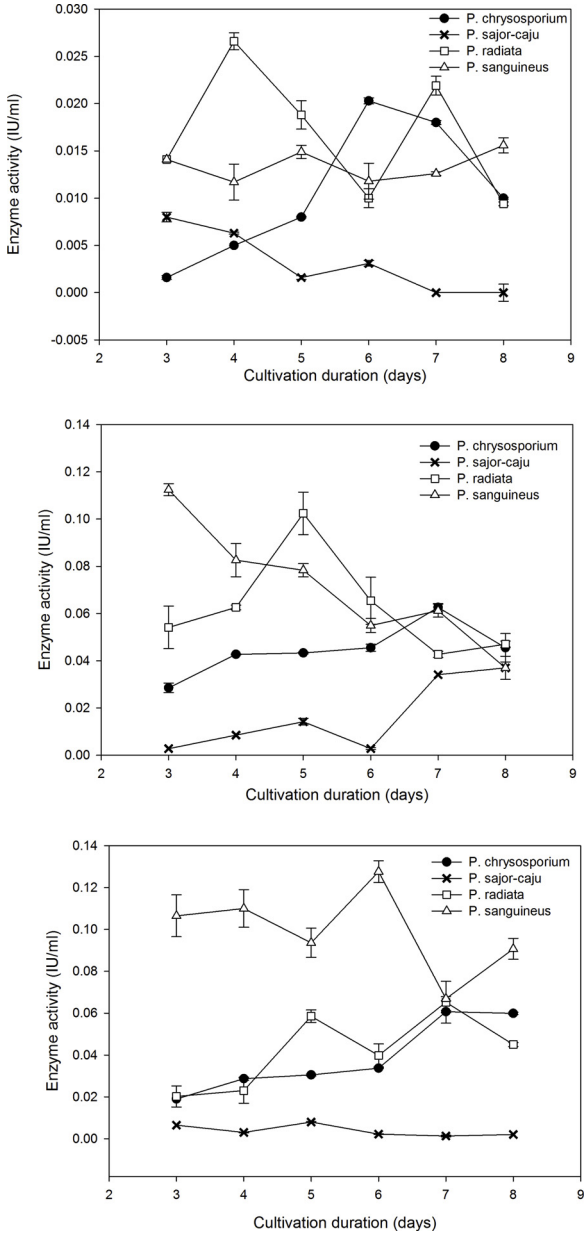

Fig. 2. Cellulase production by white-rot fungi from NaOH-pretreated SCB expressed in terms of (a) filter paper activity (FPase), (b) carboxymethyl cellulase activity (CMCase) and (c) β-glucosidase activity.

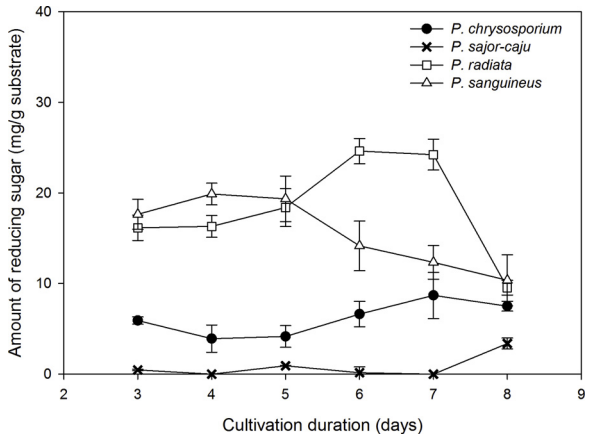

Fig. 3. Reducing sugar production from *in situ* saccharification by white-rot fungi from NaOH-pretreated SCB.

glucose compared to CMC and cellobiose used in CMCase and β-glucosidase quantifications respectively. Generally, *P. radiata* and *P. sanguineus* were producing a higher amount of cellulase compared to *P. chrysosporium* and *P. sajor-caju*. The total cellulase activities obtained in the present study are found to be slightly lower than those reported using combinations of white-rot fungi and lignocellulosic substrate such as *P. chrysosporium* cultivated on cotton stalks and rice straw *via* SSF [29,30]. On the other hand, certain combinations of fungus and substrate such as *Pleurotus ostreatus* with banana leaf and *Pleurotus sajor-caju* with banana pseudostem [31] were found to be unsuitable for cellulase production; suggesting that white-rot fungus is substrate specific and that the composition of lignocellulosic substrate determines the success of fungal cultivation.

Similar with cellulase production, the production of reducing sugar *in situ* was also influenced by a combination of lignocellulosic wastes and white-rot fungus. The production of reducing sugar was possible when NaOH-pretreated bagasse was inoculated with *P. radiata*, *P. sanguineus* and *P. chrysosporium*, shown in Fig. 3. The present work finds that *P. radiata* produced the highest amount of reducing sugar at 2.5 g/100 g SCB after 6 days of cultivation while *P. sanguineus* produced 2 g/100 g SCB at a shorter duration of 4 days. The production of reducing sugar by *P. chrysosporium* was in the range of 0.4 g/100 g SCB to 0.9 g/100 g SCB. The higher cellulase activities especially in the form of CMCase and β-glucosidase have contributed to higher reducing sugar production from *P. radiata* and *P. sanguineus*. On the other note, although the enzymes activities recorded from *P. radiata* and *P. sanguineus* were not significantly different, but the trend in reducing sugar production for both microbes were different. The higher amount of reducing sugar produced by *P. sanguineus* at initial stage could be due to the higher titre of CMCase and β-glucosidase at the beginning of cultivation. The higher amount of CMCase and β-glucosidase aid to depolymerise cellulose into glucose which is measured as reducing sugar at a faster rate. The depletion of reducing sugar produced by *P. sanguineus* after Day 5 could be due to many factors. Besides reduction in cellulase activities, reducing sugar produced at the beginning could be consumed by fungus to support its growth. Among the fungi examined, *P. sajor-caju* did not produce a significant amount of reducing sugar during SSF; indicating that there were insufficient cellulase activities to promote cellulose saccharification *in situ* when *P. sajor-caju* was cultivated on NaOH-pretreated bagasse (Fig. 2).

Comparing the findings obtained between *P. radiata* and *P. sanguineus*, it was found that *P. sanguineus* was able to produce reducing sugar in a shorter duration but with a slightly lower reducing sugar yield. Shorter cultivation duration is favorable as the risk of contamination can be reduced, particularly when unsterilized lignocellulosic substrate is applied. Hence, *P. sanguineus* was selected for the subsequent study on ethanol production when *S. cerevisiae* was sequentially co-cultured.

Table 2
Experimental design matrix and the results of reducing sugar production *via in situ* saccharification.

Run	A: Inoculum loading (%)	B: Moisture content (%)	C: Cultivation duration (days)	Y: Amount of reducing sugar (g/100 g SCB)
1	0.2	80	6	1.28
2	0.2	60	2	0.58
3	0.2	60	6	2.68
4	0.2	80	2	0.27
5	0.2	70	4	2.27
6	0.6	70	4	3.06
7	0.6	70	4	2.85
8	0.6	70	4	3.40
9	0.6	70	4	3.42
10	0.6	70	6	2.04
11	0.6	70	4	3.15
12	0.6	60	4	2.62
13	0.6	70	4	2.87
14	0.6	70	2	0.35
15	0.6	80	4	3.00
16	1.0	80	2	1.69
17	1.0	60	6	0.73
18	1.0	60	2	0
19	1.0	80	6	1.90
20	1.0	70	4	1.97

3.2. Effect of inoculum loading, moisture content and cultivation duration on the production of reducing sugar via in situ saccharification

Following the selection of white-rot fungus, the production of reducing sugar from *in situ* cellulase saccharification by *P. sanguineus* was examined. It is important to maximize the reducing sugar produced so that it may facilitate ethanol production in the *in situ* saccharification and fermentation system. The effect of inoculum loading (A), moisture content (B) and fermentation duration (C) on reducing sugar production is presented in Table 2. The maximum reducing sugar was obtained at the midpoints of the investigated parameters (0.6% inoculum loading, 70% moisture content and 4 days). Of note, reducing sugar was not detected at 1% inoculum loading, 60% moisture content and 2 days.

A one-way analysis of variance (ANOVA) was used to determine the significance of the results, shown in Table 3. A significant model term, together with an insignificant lack of fit term, were obtained. In addition, the R-squared value (0.9798) obtained is close to 1; further proving the goodness of the model developed in fitting the experimental data. The ANOVA results also imply that cultivation duration and the interaction between inoculum loading and moisture content have great impact on reducing sugar production. All the factors

Table 3
ANOVA of the reduced quadratic model for reducing sugar production *via in situ* saccharification.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Probability > F
Model	22.13	8	2.77	54.50	< 0.0001
A	0.062	1	0.062	1.23	0.3195
B	0.23	1	0.23	4.61	0.0739
C	3.29	1	3.29	64.92	< 0.0001
A^2	1.08	1	1.08	21.19	0.0026
C^2	7.02	1	7.02	138.35	< 0.0001
AB	2.61	1	2.61	51.44	< 0.0001
AC	0.59	1	0.59	11.60	0.0078
BC	0.32	1	0.32	6.38	0.0324
Residual	0.46	9	0.051		
Lack of fit	0.40	6	0.066	3.22	0.1827
Pure error	0.061	3	0.020		

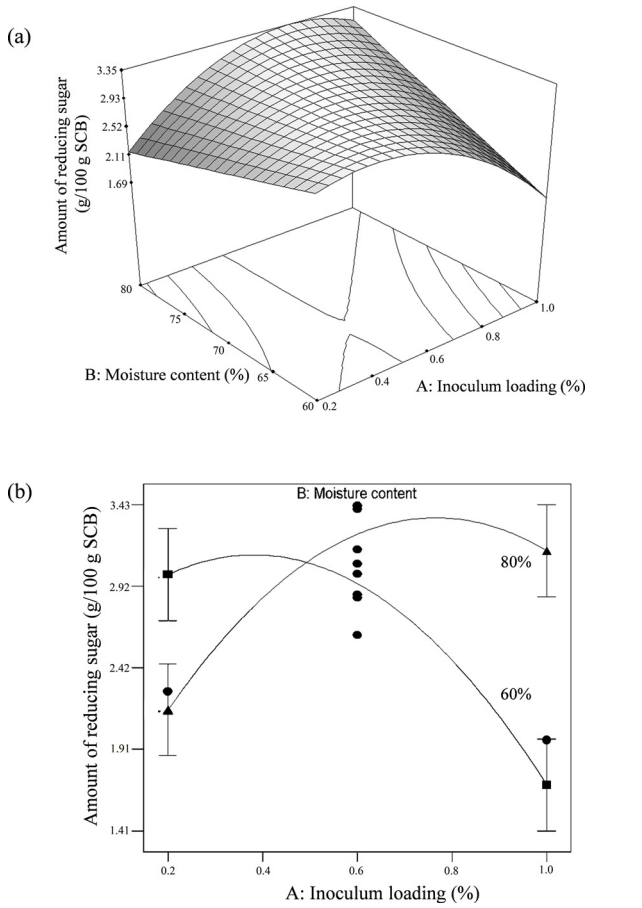

Fig. 4. (a) Three-dimensional response surface plot and (b) interaction plot of the effect of inoculum loading and moisture content on reducing sugar production on day 4 of cultivation.

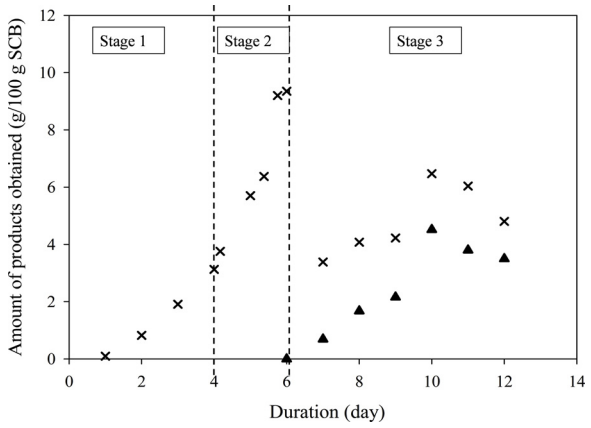

Fig. 5. Profile of reducing sugar and ethanol production from the *in situ* saccharification and fermentation system. Stage 1: Cultivation of *P. sanguineus* and *in situ* saccharification at room temperature (30 °C), Stage 2: *In situ* saccharification at 50 °C, Stage 3: Sequential inoculation of *S. cerevisiae* (× represents the amount of reducing sugar and▲ represents the amount of ethanol).

examined were found to exhibit certain degree of interactions with each other, based on the significant interaction terms (AB, AC and BC) with Probability > F values less than 0.05.

The interactive effect between inoculum loading and moisture content (AB term) was the greatest among the interaction terms examined. Subsequent discussion on the effect of the AB term on the production of reducing sugar would be based on the three-dimensional response surface plots and the interaction plots, shown in Fig. 4. The cultivation of *P. sanguineus* on NaOH-pretreated bagasse at a lower inoculum loading (0.2%) and lower moisture content was found to enhance the reducing sugar production. However, when an inoculum loading of greater than 0.5% was applied in an environment with lower moisture content, a reduction in the reducing sugar production was observed. The explanation for the observation is as follows. In an environment where moisture was supplied at a rate lower than the required level, the dissolution of the nutrients would be limited by the moisture content and therefore, hindered the effective uptake of nutrients by the fungi [26]. This is in contrast to an environment of excess moisture content where the substrate particles may be surrounded by a thick layer of water that promotes the agglomeration of substrate particles thereby, limiting gas exchanges between the particles and the surrounding [32]. Hence, fungal growth is restricted in part, from an anaerobic environment that is unfavorable to growth. The preceding discussion explains the need to increase moisture content at a higher inoculum loading to improve the production of reducing sugar in the context of the present study.

A numerical optimization study was conducted in which the maximum reducing sugar was found to be at 0.6% inoculum loading, 70% moisture and 4 days. Under this condition, the predicted and the average experimental yields of reducing sugar obtained were 3.0 g/100 g SCB and 3.1 g/100 g SCB, respectively. The small deviation between the predicted and experimental values implies that the model is reliable in predicting reducing sugar yield based on the range of operating conditions examined.

The amount of reducing sugar obtained in the present study is comparable with the work of Khalil et al. [23] in which 3.25 g/100 g SCB of reducing sugar was produced when *Pleurotus ostreatus* was cultivated on sterilized NaOH-pretreated sugarcane bagasse. However, when Khalil et al. [23] employed *Plerotus sajor-caju*, a mere 0.4 g/100 g SCB of reducing sugar was obtained. The results suggest that in maximizing the reducing sugar production, the synergistic effect arising between the pretreatment methods together with the types of lignocellulosic substrate and fungal strain, ought to be taken into consideration. Hong et al. [22] and Shrestha et al. [15] have also conducted similar studies by cultivating *Phanerochaete chrysosporium* on sterilized oil palm fronds and sterilized NaOH-pretreated corn fiber, respectively. Both studies yielded reducing sugar productions of approximately 5.5 g/100 g substrate. In contrast, the reducing sugar concentration obtained from the present work is lower. The lower reducing sugar concentration obtained may be due to the unsterilized NaOH-pretreated SCB used in the present work, as opposed to the sterilized substrates used in the works of Hong et al. [22] Khalil et al. [23] and Shrestha et al. [15]. Sterilization under high pressure and temperature could have depolymerized carbohydrates in the biomass into reducing sugar; thereby, contributing to a higher reducing sugar yield when sterilized substrates were used. In a nutshell, the present study demonstrates that the production of reducing sugar *via* an *in situ* saccharification without the involvement of a sterilization process is viable. Sidestepping the sterilization process could potentially lead to a reduced unit operations and a lowering of production costs.

3.3. In situ saccharification and fermentation system for reducing sugar and ethanol production

The feasibility of adopting an *in situ* saccharification and fermentation system for reducing sugar and ethanol production was examined at three stages. In Stage 1, *P. sanguineus* was cultivated on sugarcane bagasse for an *in situ* production of cellulase and reducing sugar. The operating condition for Stage 1 was obtained from Section 3.2. Subsequently, the system was subjected to 50 °C to further facilitate cellulose saccharification at Stage 2. By adopting Stage 2, reducing sugar production was enhanced and more feedstock was available for subsequent ethanol production. *S. cerevisiae* was then inoculated into the system

Table 4
Different lignocellulosic substrates applied for ethanol production *via in situ* saccharification and fermentation system.

Substrate	Operating conditions	Amount of ethanol	Reference
Corn stover	• Microorganisms: Gloeophyllum trabeum and Escherichia coli K011 • Temperature: 37 °C • Fermentation duration: 8 days	4.79 g/100 g substrate	Vincent et al. [16]
Corn fiber	• Microorganisms: Gloeophyllum trabeum and Saccharomyces cerevisiae • Temperature: 30 °C • Fermentation duration: 8 days	4 g/100 g substrate	Rasmussen et al. [14]
Filter paper	• Microorganisms: Trichoderma reesei and Saccharomyces cerevisiae • Temperature: 37 °C • Fermentation duration: 9 days	5.13 g/100 g substrate	Vincent et al. [17]
Corn fiber (pretreated with NaOH)	• Microorganisms: Trichoderma reesei and Saccharomyces cerevisiae • Temperature: 37 °C • Fermentation duration: 10 days	5.5 g/100 g substrate	Shrestha et al. [15]
Sugarcane bagasse (pretreated with NaOH)	• Microorganisms: Pycnoporus sanguineus and Saccharomyces cerevisiae • Temperature: room temperature (30 °C) • Fermentation duration: 10 days	4.5 g/100 g substrate	This study

with *P. sanguineus* cultivation to convert reducing sugar into ethanol in Stage 3. In Stage 3, *P. sanguineus* and *S. cerevisiae* coexist with both having different functions. The profiles of reducing sugar and ethanol in all three stages of *in situ* saccharification and fermentation system are presented in Fig. 5.

During Stage 1, reducing sugar produced by *P. sanguineus* increased steadily from Day 1 to Day 4. Following that, depolymerization of cellulose by the action of cellulase was further enhanced at a higher temperature, contributed to the increased in reducing sugar production in Stage 2. It was found that although *P. sanguineus* had been exposed to 50 °C for 2 days, the fungus was still viable and able to co-exist with *S. cerevisiae* in Stage 3. In Stage 3, reducing sugar production decreased substantially at the beginning of the co-culture process between Day 6 and Day 7. The reduction of reducing sugar can be correlated with the increase in ethanol production as shown in Fig. 5. The depletion of reducing sugar could also be used to support the growth of white-rot fungus and yeast. After the drastic drop, reducing sugar was maintained in the range of 3.4 g/100 g SCB to 6.5 g/100 g SCB.

A similar trend on the continuous reducing sugar production during co-culture of filamentous fungi and yeast was reported by [16,17]. Reducing sugar did not deplete to an insignificant level throughout Stage 3 and this could probably be attributed to two factors. First, both the cellulase production and saccharification of cellulose were still active and are responsible for the continuous production of reducing sugar in Stage 3. Another possible explanation could be that part of the reducing sugar which contained cellobiose and xylose was not readily to be fermented by yeast [18].

The end product, ethanol was only produced in Stage 3 with the highest amount of 4.5 g ethanol/ 100 g SCB on Day 10 when *S. cerevisiae* was co-cultured with *P. sanguineus*. This indicates that *S. cerevisiae* was robust in ethanol production even with the presence of *P. sanguineus*. Similar findings showing good compatibility between white-rot fungus and *S. cerevisiae* were observed [14,33]. In the current study, *P. sanguineus* demonstrated its cellulase producing ability by recording carboxylmethyl cellulase activities in the range of 0.04 IU/mL to 0.09 IU/mL even during Stage 3. The production of ethanol by *S. cerevisiae* from a continuous uptake of reducing sugar might have alleviated the feedback inhibition cellulase enzymes where its action was suppressed by the accumulation of glucose in the system [34].

Considering the advantages offered by co-culturing filamentous fungi and yeast, several researchers have adopted a similar scheme of ethanol production from various lignocellulosic substrates *via in situ* saccharification and fermentation system. As tabulated in Table 4, the amount of ethanol produced from this study was comparable with those from other studies. The combination of *P. sanguineus* and *S. cerevisiae* adopted by this study works equally well compared with other combinations of filamentous fungi and yeast. The duration necessary for ethanol production *via in situ* saccharification and fermentation system was in the range of 8 days to 10 days.

Ethanol production of sequential-co-culturing *P. sanguineus* and *S. cerevisiae* in the *in situ* saccharification and fermentation system is a combination that has never been reported. *P. sanguineus* cultivated on pretreated sugarcane bagasse played a role in cellulase production and promoted *in situ* saccharification; leading to the production of reducing sugar. Following the enhancement of the reducing sugar production, *S. cerevisiae* was inoculated; playing a role in converting the reducing sugar into ethanol. Continuous production of ethanol and cellulase was feasible due to the alleviation of the end-product inhibition when both microbial strains co-exist. In brief, the proposed *in situ* saccharification and fermentation scheme is feasible to convert pretreated sugarcane bagasse into multiple value-added products including cellulase, reducing sugar and ethanol *via* sequential-co-culture of *P. sanguineus* and *S. cerevisiae*.

4. Conclusion

By strategizing the roles of *P. sanguineus* and *S. cerevisiae*, the production of multiple value-added products including cellulase enzymes, reducing sugar and ethanol *via* an *in situ* saccharification and fermentation scheme is deemed feasible. Integration of cellulase production, cellulose saccharification and ethanol production in a single reactor reduces the unit operations thereby improving the economic viability of the system. In addition, sequential-co-culturing of both the naturally occurring microbial strains on unsterilized sugarcane bagasse further improve the cost effectiveness of the *in situ* saccharification and fermentation.

Conflict of interest

Authors declare that no conflict of interest exists.

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