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Simultaneous production of cellulase and reducing sugar through modification of compositional and structural characteristic of sugarcane bagasse

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ABSTRACT

This study examined the potential of untreated and alkali-pretreated sugarcane bagasse (SCB) in cellulase, reducing sugar (RS) and fungal biomass production via solid state fermentation (SSF) using *Pycnoporus sanguineus*. The impact of the composition, structure and cellulase adsorption ability of SCB on the production of cellulase, RS and fungal biomass was investigated. From the morphological and compositional analyses, untreated SCB has relatively more structural changes with a higher percentage of depolymerisation on the cellulose, hemicellulose and lignin content compared to alkali-pretreated SCB. Thus, untreated SCB favoured the production of cellulase and fungal biomass whereas alkali-pretreated SCB yielded a higher amount of RS. The composition and morphology of untreated SCB did not encourage RS production and this suggested that RS produced during SSF might be consumed in a faster rate by the more abundantly grown fungus. Besides that, alkali-pretreated SCB with higher cellulase adsorption ability could have adsorbed the cellulase produced and resulted in a lower cellulase titre. In short, the production of specific bioproducts via SSF is dependent on the structure and composition of the substrate applied.

1. Introduction

Agricultural residues with their lignocellulosic nature are rich in cellulose, hemicellulose and lignin [1]. They appear as prominent feedstocks for the production of a wide range of bioproducts such as enzymes, fermentable sugars and bioethanol [2,3]. To convert the lignocellulosic agricultural residues into bioproducts, bioconversion processes such as enzymatic hydrolysis and fermentation are commonly applied.

Solid state fermentation (SSF) is one of the most extensively studied fermentation methods. This method offers more advantages as compared to the conventional submerged fermentation (SmF) method. SSF is carried out in the absence of free flowing water and thus, a lower amount of wastewater is generated from SSF [2]. The production cost of bioproducts via SSF can be lowered since the cost of purification could be reduced due to higher concentration of end products and higher fermentation productivity obtained [4]. SSF is also particularly favourable for the cultivation of fungi since it is conducted under the conditions that are closer to the natural habitat of filamentous fungi [5]. Higher yield of bioproducts can be achieved due to the better growth of filamentous fungi during SSF as the filamentous fungi are better enzyme producer under SSF compared to SmF [6].

The solid substrate employed in SSF plays a crucial role in anchoring the filamentous fungi during their growth [7,8]. Substrate that contains sufficient nutrients to supplement the fungal growth is preferable [7]. The types and the nature of lignocellulosic substrates used also have a great impact on SSF [7]. However, some of the lignocellulosic substrates could not support the growth of certain types of filamentous fungi. For instance, Orzua et al. [9] stated that out of ten other types of substrates tested in their study, only apple pomace, lemon peel, orange peel, and coconut husk have great potential to support the growth of *Aspergillus niger* in SSF. Furthermore, Hong et al. [8] reported that *Phanerochaete chrysosporium* could not grow well on substrates such as paddy husk, coconut fibre, wood dust, coconut meal, palm kernel cake, sugarcane bagasse and oil palm trunk. They further concluded that substrates with structure that limits diffusion often do not support fungal growth and impede the production of bioproducts such as enzymes and fermentable sugars.

Apart from that, some researchers pretreat the lignocellulosic substrate before SSF. Pretreatment is performed to alter the structure of the lignocellulosic substrate with the aim to increase the surface area of cellulose and hemicellulose, to remove lignin and also to reduce the degree of polymerization and crystallinity of cellulose [10]. As suggested by Limayem and Ricke [11], pretreatment

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of lignocellulosic substrate has major effects on the downstream processes and thus, appropriate pretreatment techniques should be applied to improve the yield of the desired products via SSF. Alkali pretreatment is one of the most commonly applied and effective pretreatment techniques for lignocellulosic substrate. There are diverse opinions on the impact of alkali pretreatment on the production of bioproducts such as cellulase and reducing sugar (RS) from the pretreated biomasses via fermentation. Some researchers stated that alkali-pretreated substrate was able to enhance the yield of bioproducts produced during SSF whereas some reported otherwise [7,12,13]. Untreated substrates are known to be compositionally and structurally different from alkali-pretreated substrates [14,15]. These criteria might also contribute to the different ability in enzyme adsorption for both untreated and pretreated substrates [16,17].

In this study, the impact of structural and compositional changes of SCB resulted from alkali pretreatment on the production of bioproducts such as cellulase, RS and fungal biomass via SSF was investigated. The production of these bioproducts and fungal biomass was performed by a WRF, *Pycnoporus sanguineus* via SSF. The relationship between composition, structural characteristic and cellulase adsorption ability of SCB with the production of bioproducts and fungal biomass during SSF was also highlighted.

2. Materials and methods

2.1. Fungal culture and inoculum preparation

Pycnoporus sanguineus was determined to be the most suitable WRF to be cultivated on SCB based on screening test. *P. sanguineus* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and the fungus was maintained on malt extract peptone agar at 4 °C. The inoculum was prepared by growing *P. sanguineus* in 50 mL of 2% malt extract medium for 5 days at 100 rpm. After the incubation period, mycelial pellets obtained was washed and homogenized prior to be used in SSF.

2.2. Substrates and pretreatment

SCB was collected from Purecane Manufacturing Sdn. Bhd., Malaysia. The SCB was thoroughly washed, dried and ground before being subjected to alkali pretreatment or SSF. Alkali pretreatment of SCB was carried out under the optimized condition (128 °C, 30 min and 0.62 M NaOH) [18]. The substrate was then washed with water and citrate buffer until the wash water of pH 5 was obtained. The washed substrate was then dried at 60 °C and used in SSF.

2.3. Solid state fermentation (SSF)

SSF was conducted with 2 g of untreated or alkali-pretreated SCB and inoculated with 1 mL of homogenized mycelial suspension. Mandel's medium with pH 5 was supplemented to the inoculated SCB until the final moisture content of 70% was obtained. Mandel's medium consisted of 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 0.3 g/L $CaCl_2{\cdot}2H_2O$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L peptone, 0.2% (v/v) Tween 80, 5 mg/L $FeSO_4{\cdot}7H_2O$, 1.6 mg/L $MnSO_4{\cdot}2H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$ and 2 mg/L $CoCl_2{\cdot}6H_2O$. SSF was carried out at room temperature for 1–8 days.

After fermentation, the content of the flask was extracted with 20 mL of citrate buffer (50 mM, pH 4.8). The flask was then agitated at 150 rpm for 1 h and subsequently, the content was centrifuged at 4 °C and 3500 rpm for 20 min. The supernatant obtained from centrifugation was filtered and thereafter, the filtrate was analyzed for cellulase activities, total soluble protein content, and reducing sugar (RS) concentration.

2.4. Compositional analysis

The chemical composition of SCB was determined according to the method suggested by National Renewable Energy Laboratory (NREL) [19]. The compositions of cellulose, hemicellulose and lignin of SCB were reported in this study.

2.5. Fourier transform infrared (FTIR) analysis

The chemical structure of SCB was examined with FTIR spectrometer (IFS66v/S, Bruker, USA). The dried solid samples were mixed with potassium bromide (KBr) and the mixtures were pressed into a disc. The samples were then scanned in the range of 400 cm^{-1} to 4000 cm^{-1} with a resolution of 0.1 cm^{-1}.

2.6. Scanning electron microscopy (SEM)

The morphology of untreated and alkali-pretreated SCB before and after SSF was examined with scanning electron microscope (Quanta 200 FESEM, FEI, USA). The solid samples were first mounted on the aluminium sample stubs with double sided carbon tape. Following that, images were acquired with 10 kV acceleration voltage.

2.7. Cellulase adsorption

The procedures for determining cellulase adsorption on untreated and alkali-pretreated SCB was carried out as described by Du et al. [20] with some modifications. Cellulase solution (Cellulase Onozuka R-10 from *Trichoderma viride*, Merck, Germany) with an activity of 30 FPU/g SCB was added to the untreated and alkali-pretreated SCB with equal cellulose loading. The reaction was maintained at 30 °C in a water bath for 2 h. After the reaction, citrate buffer was added into the mixture followed by shaking the content of the flask at 150 rpm for 1 h. The mixture was then centrifuged and filtered before subjected to total soluble protein analysis according to micro Lowry Peterson's modification method. The percentage of cellulase adsorption was calculated based on Eq. (1).

$$\text{percentage of cellulase adsorption} = \frac{C_{\text{initial}} - C_{\text{unadsorbed}}}{C_{\text{initial}}} \times 100\% \quad (1)$$

where $C_{initial}$ = initial amount of cellulase dosage applied (μg) and $C_{unadsorbed}$ = amount of unadsorbed cellulase in filtrate (μg).

2.8. Analytical methods

The reducing sugar (RS) concentration was determined by using DNS method [21]. Fungal biomass was quantified by total soluble protein content. The total soluble protein content was determined by using TP0300 total protein kit based on Micro Lowry Peterson's modification method (Sigma–Aldrich, USA).

The procedure reported by Ghose [22] was applied to determine the cellulase activities that were expressed in terms of filter paper activity (FPase), carboxylmethyl cellulase activity (CMCase) and β-glucosidase activity. FPase, CMCase and β-glucosidase activity represent the total cellulase activities, endoglucanase activity and β-glucosidase activity respectively [23]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol mL^{-1} min^{-1} of glucose (2 μmol mL^{-1} min^{-1} of glucose in the case of β-glucosidase) from the particular substrate under the assay conditions [22]. All experiments were conducted in duplicate and statistical analysis was performed by applying the analysis of variance (ANOVA) method. Confidence level of 95% was applied to test the significance of the data obtained.

3. Results and discussion

3.1. Compositional analysis on untreated and alkali-pretreated SCB

The chemical composition of both untreated and alkali-pretreated SCB was determined and the results are tabulated in Table 1. It is worth noting that alkali-pretreated SCB consists of higher amount of cellulose and hemicellulose with lower lignin content compared to the untreated SCB. This correlates well with other studies whereby lignin in lignocellulosic biomass is significantly removed when alkaline solution is employed to pretreat the biomass [14,23].

As stated by Kuhad and Singh [24], WRF are capable of degrading all the components in lignocellulosic biomass. Hence, *P. sanguineus* should have the ability to depolymerise all the three main chemical components (cellulose, hemicellulose and lignin) in SCB during SSF. The cellulose and hemicellulose content decreased during SSF for both untreated and alkali-pretreated SCB as presented in Table 1 and this implies that enzymes cellulase and hemicellulase were produced during SSF and these enzymes play a role in the depolymerisation of cellulose and hemicellulose in SCB. The percentage of cellulose removal from alkali-pretreated and untreated SCB during SSF were 2.8% and 14.1% respectively. The higher percentage of cellulose removal from untreated SCB might be attributed to the higher amount of cellulase produced from untreated SCB via SSF.

Besides that, a higher amount of hemicellulose compared to cellulose and lignin was removed from both untreated and alkali-pretreated SCB during SSF. Hemicellulose was prone to be removed