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Enhancement of fermentable sugars production from oil palm empty fruit bunch by ligninolytic enzymes mediator system

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ABSTRACT

The impact of crude ligninolytic enzyme-mediator pretreatment on the bio-modification and partial removal of oil palm empty fruit bunch (OPEFB) lignin prior to cellulose hydrolysis was evaluated. The OPEFB was first treated with ligninolytic enzyme together with mediators and then hydrolysed with commercial cellulase for its fermentable sugars production. Two mediators of laccase, hydroxybenzotriazole (HBT) and azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were compared in the pretreatment of OPEFB whereas manganese sulphate was used as mediator for manganese peroxidase (MnP). Optimum pretreatment conditions with maximum Klason lignin removal were achieved at concentration of 1.5% HBT, 4 mM ABTS and 2 mM manganese (II) with as much as 8.02%, 8.68% and 3.7% respectively as compared to raw OPEFB. lignin was removed from OPEFB by 8.8% at 50 °C and 8.16% at 10% of substrate loading, respectively. Pretreatment verifications at optimal condition was determined by cellulose hydrolysis of pre-treated OPEFB from the combination of HBT-Mn (II) and ABTS-Mn (II) which increased sugar yield by 16%–17% with approximately 30 g/L of fermentable sugars as compared to crude ligninolytic alone with 19.1 g/L, suggesting that mediators had played important roles in modification and partial removal of lignin, thus improved cellulose accessibility.

1. Introduction

Malaysia is the largest exporter of palm oil compared with other agriculture commodities being planted in the country and the palm oil industry generates huge amount of particularly oil palm empty fruit bunch (OPEFB). OPEFB has been an attractive source of biofuel with biomass pretreatment is the most crucial stage in its production (Naik et al., 2010). Pretreatment refers to complete or partial degradation of lignocellulosic biomass lignin for higher exposure to cellulose. Generally, the use of chemical and physical pretreatment technologies have been extensively employed to treat biomass, enhancing the accessibility of enzyme and thus improving enzymatic hydrolysis of cellulose (Mosier et al., 2005) and fermentable sugars production. However, both pretreatments require high technology equipment and chemical usage which involved a detrimental effect to the environment. As an alternative, biological pretreatment of biomass using delignifying enzymes represents another viable approach.

Delignifying enzymes, namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase produced by white rot fungi are the most prominent enzymes for use in lignin degradation processes. They are non-specific enzymes capable of degrading natural aromatic polymers of lignin (Jaqueline et al., 2010; Wong, 2009). Their approach mechanism is by modifying the lignin structure so that the cellulolytic enzyme is able to access cellulose and hemicellulose embedded in the lignin matrix. Generally, there are three major challenges in decomposing lignin, associated with the facts that, 1) lignin is a large polymer, 2) lignin structure is comprised of inter-unit C–C and ether bond C–O–C, therefore the degradation mechanism must be oxidative rather than hydrolytic and 3) lignin polymer is stereo-irregular, therefore the mechanism must be much less specific than degradative enzyme (Dashtban et al., 2010). The characteristics of extracellular, oxidative and unspecific enzymes are very important for catalysing the initial depolymerisation of lignin besides the role of mediator that assist the non-enzyme mechanisms and extended the depolymerisation processes.

Mediators are defined as low-molecular-weight compounds

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that facilitate enzymatic oxidation by generating stable and high-potential intermediates between ligninolytic enzyme and lignin. They are responsible for lignin degradation as actual oxidant and can penetrate deeply into the lignocellulosic matrix due to their small size (Bourbonnais et al., 1997; Morozova et al., 2007). Originally, mediators are used together with delignifying enzymes in pulp bleaching, deinking, dye removal and other applications. A number of mediators with functional groups of −NO, −NOH or HRNOH- (Call and Mucke, 1997) are utilised in the enzymatic process to improve lignin degradation. Nevertheless, there is limited research works on mediator utilization in *in vitro* ligninolytic enzyme pretreatment of biomass. Therefore, in this study, the potential of ligninolytic-mediator system pretreatment for OPEFB were investigated in term of lignin removal and modification to improve fermentable sugars production during cellulose hydrolysis.

2. Materials and methods

2.1. Fungal strain and ligninolytic enzyme production

Locally isolated white rot fungus identified as *Pycnoporus sanguineus* UPM4 was used throughout the study (unpublished data). The culture was grown on potato dextrose agar (PDA) for 7 days and used immediately for mycelia production. The mycelia were scrapped and transferred into pre-culture media and incubated for 5 days and 3 mL of mycelial broth was then transferred into the production medium as inoculum. The compositions of pre-culture media (Elisashvili et al., 2008) were as follows: glucose, 10 g; NH_4NO_3, 1 g; KH_2PO_4, 0.8 g; Na_2HPO_4, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; yeast extract, 2.0 g. The medium was adjusted to pH 6 ± 0.2 and incubated at 30 °C with rotation speed of 150 rpm.

Production medium was prepared accordingly to Matsubara and co-workers with slight modifications by the addition of ammonium tartrate and Tween 80 (Matsubara et al., 2006). It consists of raw OPEFB (approximately 10 mm size), 1% (w/v); KH_2PO_4, 2 g; $MgSO_4{\cdot}7H_2O$, 0.5 mg; $CaCl_2{\cdot}2H_2O$, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.1 mg; $CuSO_4{\cdot}5H_2O$, 0.02 mg; $ZnSO_4{\cdot}7H_2O$, 0.01 mg; ammonium tartrate, 2.3 g/L and Tween 80 (5% v/v), 10 mL. The cultures were then incubated at 30 °C at 150 rpm for 6 days. The air was flushed for 10 min at 24 h interval. The supernatant was collected and employed as crude ligninolytic enzymes for the enzymatic pretreatment.

2.2. Ligninolytic enzyme analysis

The manganese peroxidase activity was assayed at 465 nm with molar extinction coefficient, $\varepsilon = 12{,}100\ M^{-1}cm^{-1}$ using the method described by Li et al. (2009). Laccase determination was also done by spectrophotometry at 420 nm with molar extinction coefficient, $\varepsilon = 36{,}000\ M^{-1}cm^{-1}$ from the method described by Bourbonnais and Paice (1988). The kinetic of the graph slope values were calculated to determine the activities of enzyme in Unit per millilitre (U/mL). The crude enzyme collected were kept in −20 °C to maintain its catalytic activity, though this may lead to less than 10% drop in activity over time (Snajdr and Baldrian, 2007) before proceeded with the pretreatment.

2.3. Preparation of OPEFB as substrate

OPEFB was obtained from Seri Ulu Langat palm oil mill, Dengkil, Selangor, Malaysia. The raw OPEFB were washed, dried at 60 °C and ground to 1 mm size. Further removal of extractive were done according to Abdul Khalil et al., (2008) using Soxhlet extraction with ethyl alcohol and benzene as the solvent at (1:1 v/v) ratio. The extraction was done in 12 h with rotary evaporator and the samples were dried in oven at 60 °C for 48 h and kept at room temperature for further use.

2.4. Pretreatment of OPEFB

In this study, the ability of crude enzymes containing laccase and MnP to remove lignin was investigated in the presence of mediators whereas the presence of LiP was considered negligible due to very low enzyme activity. OPEFB was pre-treated with three mediators at different concentrations of mediators (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (ABTS), 1-hydroxybenzotriazole (HBT) and manganese sulphate ($MnSO_4$), whereas additional factors affecting overall efficiency of lignin degradation such as cofactor (hydrogen peroxide), substrate loading concentrations and temperatures were also investigated. The effect of each factor on lignin removal and modification were studied according to the following conditions: 1–6 mM of ABTS, 1–3.5% of HBT, 0.5–2.5 mM of manganese sulphate, 5–25 mM of hydrogen peroxide, substrate loading percentage was conducted from 5% to 20% and temperatures ranging from 30 to 60 °C and substrate loading percentage was conducted ranging from 5% to 20%. All the experiments were carried at 150 rpm for 48 h. The residual substrates were dried and used for determination of Klason and acid soluble lignin. The best conditions for each of the investigated parameters were further combined and the pretreated OPEFB were applied for cellulose hydrolysis.

2.5. Cellulose hydrolysis and fermentable sugar analysis

Cellulose hydrolysis was carried out using commercial cellulase from *Acremonium cellulolyticus* supplied from Meiji Seika Co. Ltd., Japan. The experiments were performed in 50 mL Erlenmeyer flasks using 1% of substrate concentration in 0.05 M of sodium acetate buffer (pH 4.8) containing 0.01% of sodium azide and cellulase activity was fixed at 25 FPU/ml and incubated at 50 °C for 72 h. Samples were then centrifuged and the supernatant was kept for sugar determination. Cellulose enzymatic hydrolysis percentage of pre-treated OPEFB were determined using equation below according to Latif et al. (1994).

$$\text{Cellulose Hydrolysis}(\%) = \frac{\text{Reducing sugar}\,(g/L) \times 0.9}{\text{Substrate}\,(g/L) \times \text{potential sugar}\,(g/g)} \times 100 \quad (1)$$

where the amount of reducing sugar in the liquid after enzymatic hydrolysis, OPEFB fibre used and the amount of initial cellulose and hemicellulose in gram per gram OPEFB. The amount of total sugars released during cellulose hydrolysis was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). The monosaccharides of glucose and xylose present were analysed in a Shimadzu high performance liquid chromatography (HPLC) system with an NH_2 column (Merck) and Jasco RI-1530 refractive index detector, using 80% acetonitrile and 20% distilled water as mobile phase at 0.6 mL/min flow rate.

2.6. Klason lignin and acid soluble lignin determination

The amount of Klason lignin was determined by TAPPI, Tappi, (2002) which is used to determine the amount of insoluble lignin at 72% sulphuric acid (H_2SO_4) in which 150 mg of fibres were impregnated with 3 mL of 72% sulphuric acid and placed in a water bath with controlled temperature of 30 °C for 1 h. Following this,