Impact Ionic Liquid [Emim]OAc to Cellulose Hydrolysis Activities of Indigenous Microorganisms (IMO's) on Cassava Peels Substrate

By Heri Satria

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Impact Ionic Liquid [Emim]OAc to Cellulose Hydrolysis Activities of Indigenous Microorganisms (IMO's) on Cassava Peels Substrate

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Abstract

An ionic liquid (IL), *1-ethyl-3-methylimidazolium acetate* ([Emim]OAc), was used as pretreatment catalyst follow by hydrolysis of cellulose on cassava peels using local selected indigenous microorganisms (IMO's). Under 25% (w/v) loading mass of substrate and liquid fermentation condition, cellulolytic of cellulase activities and growth of IMO's were investigated under a series of IL concentration. Optimal temperature and pH were 37°C and 5.5 respectively. Enzyme activity was determined by analyzing the hydrolysis of carboxymethylcellulose (CMC) at 37°C using the dinitrosalicylic acid. In general, 0.1 M of IL did not inhibit cell growth significantly. In addition, the hydrolytic activities of obtained-cellulases were most active in presence of 0.1 M of IL, while 0.5 M of IL pushed down the activity into an half and 1.0M extremely was losing the activity. The optimum experimental conditions for hydrolysis of pretreated cassava peels using 0.1 M of [Emim]OAc was established at 37 °C, for 72 h when yield of glucose rose up to 64.49%.

Keywords: ionic liquid, indigenous microorganisms, cellulose hydrolysis, cassava peels

Introduction

Lampung Province is the largest producer of cassava in Indonesia with an average contribution of cassava production in the period 2012-2016 of 33.93% of the total national contribution of 91.21%. Meanwhile, the trend of cassava production productivity in Indonesia during 1980-2016 rising around 2.64% a year. This condition has a potency to result the surplus of cassava production in 2015 rose to 1,027 million tons. However, the utilization of cassava in the industrial sector remains the cassava peels (26.98%) and onggok (15.87%) as solid waste (Departemen Pertanian and Pangan, 2016). The waste have economic value because cassava peels contains a high starch and cellulose (Cui et al. 2014).

Being substitute starch from first generation to second generation ethanol substrate, cellulose is the most common important natural polymer as a source of carbon in ethanol fermentation. Cellulose is being major component of polymers consists in lignocellulose beside hemicellulose and lignin (Isikgor and Becer 2015). Utilization of lignocellulosic feedstock in ethanol production has promising more advantage for reduction CO_2 emissi than using starch substrate (Marquardt et al. 2010). The abundance of cellulose is valuable as a renewable source of energy. Cellulose degradation and its subsequent utilizations has importance for global carbon sources significantly. This reason has considered cellulose hydrolysis as the subject of intense research and industrial interest (Moreira 2005). Many research objective have purpose to obtaining new microorganisms cellulase producing which have higher specific activities and greater efficiency (Maki, et al. 2010).

Numerous microorganisms which are able to produce cellulase have been isolated and identified. In particular, many studies have focus on fungi because of the abundance and simple procedure to extract, and some of the fungal cellulases have already produced as commercial enzyme (Gusakov and Sinitsyn 2012). On the other hand, various bacteria which produce cellulases are more intensive studied in recently years. Bacterial cellulase properties such as their fast growth, expression of multi-enzyme complexes, and resistance to extreme enviro ments were considered the studies (Liang et al. 2014; Maki, et al. 2010; Rawway, et al. 2018; Shanmugapriya, et al.

2012; Verma, et al. 2012; Waeonukul et al. 2009).

The first step to utilizing cellulose biomass is deconstruction the compact structure of polymers, namely pretreatment process. The pretreatment has primary aim to diminish the crystalline structure of cellulose for efficient hydrolysis the cellulose chemically or biochemically by enhancing enzyme accessibility to the cellulose during hydrolysis step. In addition, the pretreatment accommodates the solubilization major components of biomass (Menon and Rao 2012) and it has impact on hydrolysis to provide a high sugars concentration (Mosier et al. 2005).

The discovery of ionic liquids (ILs), the organic salts which have a melting point below 100 °C, as pretreatment agents are promising technological invention. The ability of ILs to reduce the degree of cellulose crystallization by breaking hydrogen bonds intra- and inter-molecules of cellulose is a paradigm that provides convenience in hydrolysis steps (Wu et al. 2011; Remsing et al. 2008). It has been discovered that 1-ethylpyridiniumchloride ([C2pyr]Cl) can dissolve cellulose. Then, it has been examined that a range of ILs, typically with 1,3-dialkylimidazolium cations, are also effective cellulose solvents (Swatloski et al. 2002; H. Zhang et al. 2005). Clear viscous mixture solutions are resulted, showing the polymer solutions in general and solutions of cellulose in particular (Gericke et al. 2009).

Antagonistically, the synergic of ILs for the fermentation process is faced by ILs toxicity to microbial fermentation. Some imidazolium ILs that have great pretreatment abilities such as Emim Acetat ([Emim] OAc) and 1-butyl-3-methylimidazolium chloride ([C_4C_1im] Cl) are toxic while some ILs are biocompatible (Ouellet et al. 2011; Ganske and Bornscheuer 2006; X. Zhang et al. 2016).

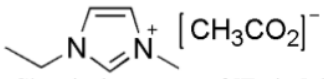

Fig.1. Chemical structure of [Emim] OAc

The aim of this study are to observe growth of indigenous bacteria isolates and their cellulase activities in presence of [Emim]OAc. The chemical structure of [Emim] OAc is shown in Figure 1.

Materials and Methods

Pretreatment of Cassava Peels.

The cassava peels was collected then rinse in tap water for overnight to reduce HCN content. After rinsing period, the cassava peels were collected then, and were dried at 65 °C. Dried cassava peels then were grinded and meshed using 250 µm mesh sieve. The obtained cassava peels powder (5.0 g) then were immersed in a series of ILs weight and were stirred gently at 50 °C for 24 h (Cui et al. 2014).

Isolation and Screening of Bacterial.

The cassava peels were sliced and were immersed in sterile nutrient broth (NB; Difco) then it was incubated at 37 °C for 24 h. After incubation period, a dilution series of cultivated bacteria from cassava peels on NB were done in 0.85% physiological solution and were poured on to nutrient agar (NA; Difco). The cultivated NA then were incubated at 37 °C for 48 h. Bacterial colonies that appeared on NA medium were picked using needle and grown onto enriched NA containing 1% CMC (Sigma). The isolate that resulted clear zone after 0.1% congo red (Merck) pored followed by 1% NaCl (Merk) was selected as cellulase isolate (Yin 2010).

Growth Assay

The assay medium contains NB medium enriched by 25% of cassava peel powder in a series of IL's concentrations (0.00; 0.01; 0.05; 0.10; 0.50; 1.00 M) was prepared. Pre-culture of isolated bacteria was prepared by inoculation 1 colony of isolate into 5.0 mL NB aseptically and was incubated in a shaker incubator at 160 rpm, 37°C, for 18-24 hours. The density of pre-cultured cells was measured after 18-24 hours by using a spectrophotometer, and the absorbance at 600 nm then was defined as OD_{600}. The appropriate volume of pre-cultured isolate was taken to set up initial OD_{600} at 0.1 in the 2.0 mL assay medium and was transferred into micro-tube and then was centrifuged at 15,000rpm, 4°C, for 5 minutes. The filtrate was then discharged, and the cells were re-

suspended using the assay medium. Suspended of inoculum cells was inoculated into assay medium, then was incubated in a shaker incubator at 160 rpm, 37ºC, for 24 hours. The measurement of OD_{600} was done at 6, 12, and 24 hours regularly. Relative OD_{600} 24h then was calculated using the following equation:

$$ROD_{600}\ 24h = \frac{OD_{600} \text{ of sample the IL at 24 hours}}{OD_{600} \text{ of 0.00 M at 24 hours}}$$

(Kuroda et al. 2017)

Enzyme Production.

Appropriate NB were added into pretreated cassava peels mixture aseptically to obtain the series concentration of ILs. The loading mass of cassava peels in the solution mixture was 20 g/L. The bacterial isolates were pre-culture overnight in NB medium at 37 °C and 160 rpm. Pre-cultured isolates then collected into centrifuge tube and appropriate volume of inoculum were then used to give initial OD 1 in the series concentration of ILs in the cassava peels solution mixture. The mixtures then were incubated at 37 °C and 160 rpm for 96 h (Bayitse et al. 2015).

Enzyme Assay

Enzyme production during cultivation was assayed at 24 h intervals up to 4 days. The culture were centrifuged at 10.000 rpm for 15 min at 4 °C. The supernatants were collected as crude enzyme for enzyme assay. Cellulase activities was determined using the 3,5-dinitrosalicyclic acid (DNS). The buffer used for dissolving or resuspending substrate (CMC) was 200 mM sodium citrate buffer (pH 5.5). The reaction system was prepared as follows 250 μL of crude enzyme mixed 250 μL of 2% (w/v) of CMC. The mixtures were incubated at 50∘C for 30 min. Then, the reactions were stopped by adding 1ml of DNS reagent. All the mixtures were heated in boiling water for 5 min for color development. Subsequently, 500 μL of each sample was transfered to cuvet and the absorbance at 550 nm were determined using *uv-vis* spectrophotometer (Adney and Baker 2008).

Glucose yields were calculated using following equation:

$$\text{Glucose Yield (\%)} = \frac{W_{glucose}\ (g)}{W_{potential\ glucose}\ (g)} \times 100\%$$

where $W_{glucose}$ is weight of obtained glucose, and $W_{potential\ glucose}$ is weight of cellulose (g) × 180/(180 − 18) (Tsai and Bayer 2014).

One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 min of reaction (Rawway et al. 2007).

Result and Discussion

A total of 45 cellulose-degrading aerobic bacterial strains were isolated from local cassava peels in Bandar Lampung, Indonesia, which were cultured in agar medium containing pretreated cassava peels powder as the sole carbon source. Out of these strains, 3 isolates showed hydrolyzing zones on agar plates containing CMC-Na after Congo-red staining (Figure 2). The isolates, SCWb-3, SCWb-13 and SCWb-17, were cultivated to produce cellulase in presence of [Emim]OAc. The crude cellulases of each isolate have activities at 22.25 U/mL, 23.97 U/mL, and 23.59 u/mL, respectively. These crude enzymes were produced in medium containing cellulose from cassava peels. The production system allowed 5 g of cassava peels powder loading into 200 mL of medium solution. It means the loading mass in this study was 25 g/L.

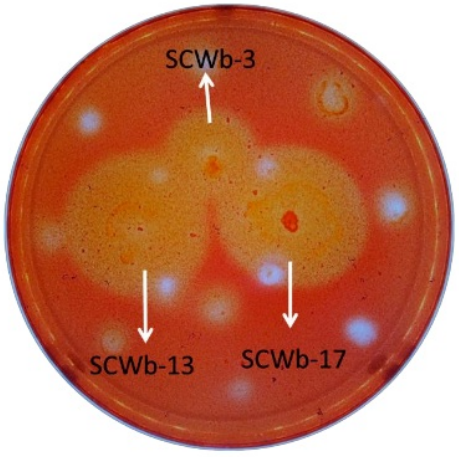

Fig.2. Hydrolyzing zones produced by bacterial isolates on agar plate containing CMC after Congo-red staining. The cellulase positive were given by SCWb-3, SCWb-13

and SCWb-17 and were indicated by clear zone appearance.

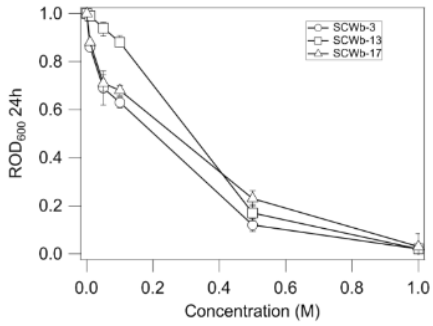

Fig.3. ROD_{600} 24h of isolate SCWb-3, SCWb-13 and SCWb-17 t describe growth of isolat in medium consist of [Emim] OAc

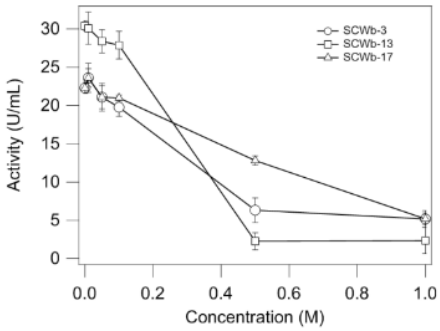

Fig.4. Cellulase activities solate SCWb-3, SCWb-13 and SCWb-17 in the presence of [Emim]OAc

To determine whether [Emim]OAc was the cause of growth inhibition and cellulase activity, the isolates were grown in NB medium cultures were supplemented with 0-1.0 M of [C2mim]OAc. Fig-3 expresses the growth of isolate compare with control when they cultivate in medium. It shows that the presence of [Emim]OAc below 0.5 M inhibite the grow of isolate less than 50%, since the concentration above 0.5 M the inhibition effects are increasing dramatically. Effect of ILs on cellulase activity during cultivation of isolated presents in Fig-4. It appears that the trend between the growth of isolate and enzyme activites are almost similar. Firstly, [Emim]OAc pushed down the cellulase vities for all of isolate cellulases, starting in the presence of 0.01 M of [Emim]OAc the isolate cellulases activities were placed under the control (un-presence of [Emim]OAc). However, cellulase activitiy of SCWb-13 was higher than others. When the concentration of [Emim]OAc increasing to 0.5 M, the cellulase activities decrease almost an half of initial isolate cellulase activity. It seems that [Emim]OAc presence was the main cause of inhibition.

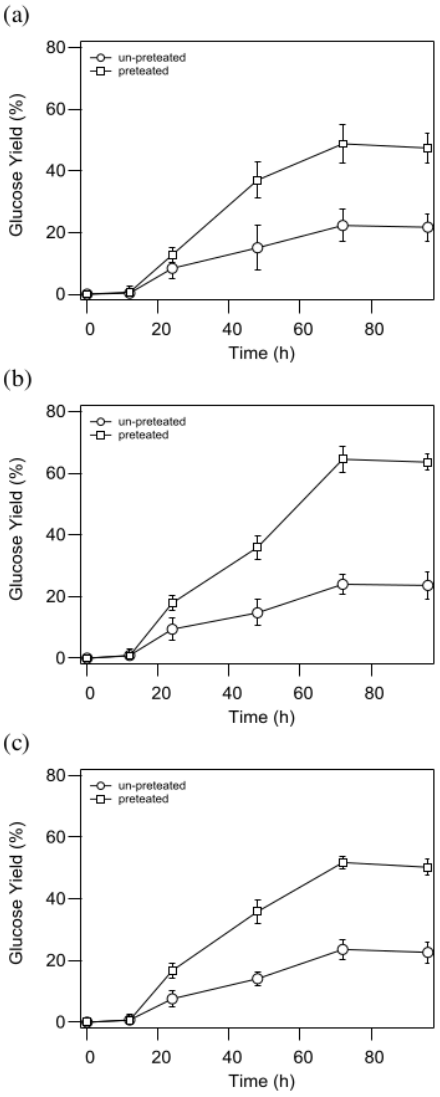

Fig.5. Effect of pretreatment [Emim]OAc on cassava peels to the yield of glucose resulted from cellulase activities of isolate SCWb-3 (Fig-5a), SCWb-13 (Fig-5b) and SCWb-17

(Fig-5c).

Ouellet et al. (2011) were observed that cation $Emim^+$ has the primary source of inhibition than anion OAc^- when ionic liquid [Emim]OAc interact with fermentated microbial. Since the pH in this study were detected between 5.07-6.57 was not a factor explaining the inhibition observed.

When we evaluated the amount of producing glucose that expressed as yield of glucose in this study, using 0.1 M of [Emim]OAc as pretreatment agent of cassava peels, it was appeared that the pretreatment gave positive impact into hydrolysis of substrate significantly. It was seen clearly in Fig-5 that after 24 h cultivated there were differences between pretreated cassava peels and un-pretreated ones in glucose yield. It is known that [Emim]OAc is one of effective ILs using in pretreatment. It work to break down the crystallinity of cellulose via destruct hydrogen bonding intra- and intermolecular of cellulose structure. In nature, cellulose is microcrystalline and its native cellulose I structure is recalcitrant to enzymatic hydrolysis. In the cellulose I lattice form, cellulose chains align in a parallel position fashion via hydrogen bonding and van der Waals forces to form a high compact and ordered micro-fibril highly, which consists of crystalline and amorphous regions. The others form of cellulose detected from recent progress involving experimental studies and computer simulations have shown that other forms of cellulose such as amorphous cellulose, cellulose II, and cellulose III are less recalcitrant (Cheng et al. 2012).

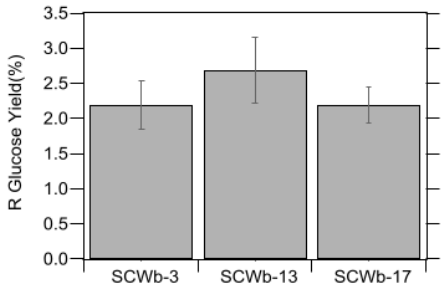

Fig.6. The glucose yield relative given by hydrolysis cassava peels during 72h cultivation using isolate SCWb-3, SCwb-13 and SCWb-17.

We consider that the glucose yield of un-pretreated substrate was lower than pretreated ones. To find out how high the difference in glucose yield, in particular calculation of relative glucose yield made from the data in Figure 5. The assumed was made based on comparation the glucose yield during the 72h hydrolysis process between preteated and un-pretreated substrate. The calculation resulted that after 72h cultivation the pretreated substrate hydrolyzed almost more two times effective than un-pretreated substrate (Figure 6). Moreover, the SCWb-13 isolate has the highest point which it showed 2.5 times more effective to hydrolysis pretreated cassava peels compare to control.

Conclusions

Three isolate of indigenous bacteria showed hydrolysis of cellulose ability. All of isolates gave the cellulase activity in presence of [Emim]OAc up to 0.1 M while 0.5 M of IL decreasing the activity into an half. Pretreated cassava peels using 0.1 M of [Emim] OAc was effective to increase the yield of glucose relative more than two times compare by un-pretreated ones.

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