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INVITED REVIEW PAPER

Waste paper sludge as a potential biomass for bio-ethanol production

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Abstract−This review describes the utilization of paper sludge (PS), which is waste from the pulp and paper industry. Its advantages make PS the cellulosic biomass with the most potential for bio-refinery research and applicable for industrial scale. Some of the grain based biofuels and chemicals have already been in commercial operation, including fuel ethanol or biochemical products. Unfortunately, research and application of PS are yet in their infancy and suffer from large scale because of low productivity. Reviewing the many researches that are working at the utilization of PS for bio-refineries could encourage the utilization of PS from laboratory research to be applied in industry. For this reason, PS usage as industrial raw material will be effective in solving the environmental problems caused by PS with clean technology. In addition, its conversion to bio-ethanol could offer an alternative solution to the energy crisis from fossil fuel. Two methods of PS utilization as raw material for bio-ethanol production are introduced. The simultaneous saccharification and fermentation (SSF) using cellulase produced by *A. cellulolyticus* and thermotolerant *S. cerevisiae* TJ14 gave ethanol yield 0.208 (g ethanol/g PS organic material) or 0.051 (g ethanol/g PS). One pot bioethanol production as a modified consolidated biomass processing (CBP) technology gave ethanol yield 0.19 (g ethanol/g Solka floc) and is considered to be the practical CBP technology for its minimizing process.

Key words: Paper Sludge, Cellulase, Bio-refinery, SSF, *Acremonium cellulolyticus*, *Saccharomyces cerevisiae*

BIOMASS

Biomass, in terms of energy, means plant based material. The main difference between biomass and fossil fuels is one of time scale. Biomass takes carbon out of the atmosphere while it is growing, and returns it as it is burned. This process maintains a closed carbon cycle with keeping stable CO_2 levels in atmosphere. There are five basic categories of material [1]: virgin wood, energy crops, agricultural residues, food waste, and industrial waste and co-products.

The first generation ethanol production (1G) is useful, but in many cases there is a limitation above which they cannot produce enough bio-fuel without threatening food supplies and biodiversity. These issues are affecting investor confidence [2]. The second generation bio-ethanol (2G) solves these problems and can supply a larger proportion for fuel supply sustainably, affordability, and greater environmental benefits by using biomass of the residual non-food like agricultural residues, food waste, and industrial waste or its co-product [3]. The structures of ligno-cellulosic biomass (plant) mainly contain cellulose, hemicelluloses and lignin (Table 1). In addition, the lignocelluloses also contain a variety of plant-specific chemicals in the matrix, called extractives (resins, phenolics, and other chemicals), and minerals (calcium, magnesium, potassium, and others). Unfortunately, the production cost of 2G bio-ethanol is still rather high, irrespective of the lingo-cellulosic feedstock used, and the development of a commercially competitive process for 2G technology poses a challenge [5,6]. Recently, the techno-economics of the 2G bio-ethanol has been assessed and the simulation showed that 2G bio-ethanol from sugar cane bagasse and leaves in Brazil is already competitive (without subsidies) with 1G starch-based bio-ethanol production in Europe [7]. This process will be more feasible with subsidies like cellulase production itself also from cellulosic biomass, which means reducing the cost of cellulose-hydrolytic enzymes [5].

By mechanical grinding and phosphoric acid swelling would improve saccharification yield (SY) of biomass and the improvement of SY will elevate the efficiency of ethanol production [8]. To remove hemicelluloses in ligno-cellulosic material, the recycled PS and cotton gin waste were mixed with steam explosion as effective pre-treatment. This pre-treatment method generated toxic compounds to fermentable microorganisms. By mixing recycled PS, which contains calcium carbonate, this over-liming can eliminate the toxic compound [9]. The PS as carbon source to produce bio-ethanol without any pre-treatment is an advantage compared to other ligno-celluloses materials since most of the lignin has already been removed in the pulping process of paper industry. Therefore, no inhibitor like furfural and hydroxymethylfurfural that are derivatives from lignin can be neglected. The presence of those compounds significantly influences the performance of cellulase and ethanol fermentation by yeast [10,11].

WASTE PAPER AS BIORESOURCE

1. Waste Paper as Biomass

PS including waste papers, categorized as industrial waste, is sludge from pulp and paper mills. The sludge is mainly cellulose fiber generated in the pulping process (Fig. 1) prior to entering the paper machine [12]. PS, also known as paper fiber bio-solids, is the residue

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Table 1. Content of cellulose, hemicelluloses and lignin in common agricultural residue and wastes [4]

Lignocellulosicbiomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	45-50	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspapers	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary waste water solid	8-15	NAb	24-29
Swine waste	6.0	28	NAb
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

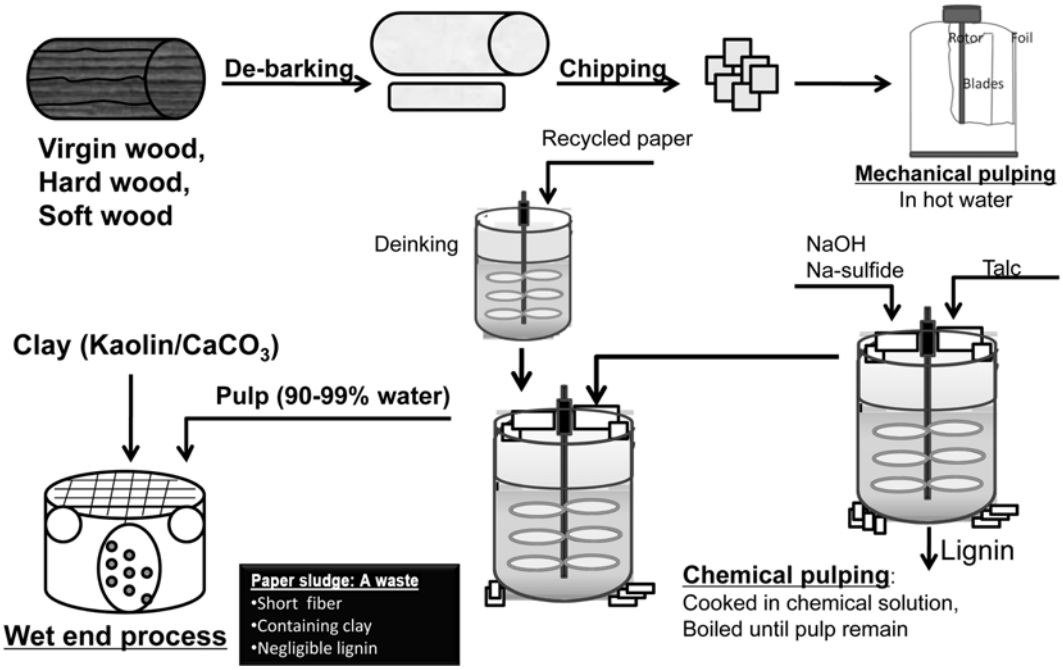

Fig. 1. Paper manufacturing process. Virgin, hard and soft woods are debarked, chipped to produce wood chips. The lignin is removed from mechanical and chemical pulping processes. Remaining fibres are mixed with deinked recycled paper and then sent to wet end process to manufacturing paper, where clay is added. PS is generated from wet end process because of short cellulose fibers.

left over from the paper recycling process. It consists of unusable short fibers, inks and dyes, clay, glues and other residue, along with any chemicals used in the recovery process [13]. In Japan, 5 million tons of PS, consisting of 24.5% cellulose, 10.5% clay, and 65% water (Table 2), is annually discharged from the paper manufacturing industry. More than 40% of the clay contains kaolin and silica together with other elements (Table 3) such as Si, Ti, Al, Fe, Mn, Na, and K [14,15].

Some PS materials also contain non-glucan carbohydrate (Xylan and Mannan) [16]. A single mill in Georgia produced about 100,000 dry tons of solid waste in a year [13]. Estimation of PS produced in fifteen members European countries was more than 10 million tons in 2001 [17]. Pulp sludge waste contains a mix of hundreds of chemicals that can harm the environment. In British Columbia, it took years to get laws that made the mills install secondary treatment to clean up the effluent. A quick survey of PS on the Internet indicates that most safe water advocates considered it an "environmental disaster." Different colors soon appeared in the pile, white,

Table 2. Composition of dry PS [14]

Component	Amount (g/g dry PS)
Total sugar	0.66
Glucan	0.44
Mannan	0.02
Xylan	0.07
Other sugars	0.13
Clay	0.30
Others	0.04

Table 3. Chemical composition of representative PS ash [15]

Ash	Composition (% w/w)
SiO_2	35.7
TiO_2	1.2
Al_2O_3	26.0
FeO*	0.4
MnO	0
MgO	8.0
CaO	25.7
Na_2O	0.1
K_2O	0.1

*Total iron as FeO

brown, reddish-orange, and were mixed in by bulldozers [13].

In the evolution of biomass residue in recent years, considerable attention has been focused on energy conversion. In Turkey, a study demonstrated waste paper could be compacted and utilized as briquetting. Another researcher investigated composting pulp and paper industry solid waste with poultry litter as the amendment has higher level and diversity of micro-nutrients but it needed 30 days of composting to get stability [12]. Alternatively, the PS could be utilized as a raw material for bio-ethanol [18]. Research on cellulosic biomass utilization for biotechnology process is facing problems of the high cost of cellulase production (due to use of pure chemicals in production) coupled with low enzyme activities limits its industrial use [19]. Therefore, efforts are needed to economize cellulase production by media optimization and use of either supplements or additives.

The heat generation when *S. cerevisiae* grows under, respectively, anaerobic and aerobic condition without ethanol formation on a defined medium, releases 8.1 and 165.5 kJ/C-mole glucose. The anaerobic process is almost loss-free since most of the enthalpy from glucose is retrieved in ethanol [20]. In addition, the yeast naturally cannot degrade xylose, which is more than 10% of reducing sugars (RSs) from PS. On an industrial scale, the bioreactor should be controlled at defined temperature using cooling water [21]. Using thermotolerant yeast reduces cooling cost and distillation cost as well. Ethanol concentration is also an important factor for bio-fuel production, and should be at least 40 g/L in order to decrease the energy demand in the ethanol separation and purification processes [22]. To achieve ethanol concentration to 40 g/L, research of ethanol production was conducted in semi continuous fed-batch reactor using a specially designed bio-reactor. However, the starting ethanol concentration was about 20 g/L and after 36 h reached 40 g/L [23]. Solid-state fed-batch fermentation as alternative process was conducted by rotary drum, and gas phase containing ethanol was collected by the condensate at −10 °C as the ethanol product [6]. In this process, external energy was needed to cool the product. Considering the energy balance, this method will be difficult in industrial application.

2. Treatment of Waste PS

Landfills with PS are creating environmental and economic problems. The current legislative trend in many countries is to restrict the amount and types of materials permitted in landfills. Plants with on-site landfills are running out of storage space, and are faced with the environmental concerns and liabilities involving potential ground water contamination from earlier disposal practices [24]. On the other hand, disposing of PS by incineration creates environmental problems, especially contamination of ground water, and legislative trends in many countries are restricting the amount and types of materials that are disposed by landfill [25].

Many researchers have tried to handle the environmental problem of PS. A research to recover Pb, an element in clay from PS, did so by employing a hydrothermal reaction at 95-100 °C under alkaline conditions. Chemical and physicochemical methods require high temperatures (140-160 °C), but it is corrosive and demands neutralization. Moreover, such methods offer low yield of carbohydrates and generate inhibitors for further microbial processes [24]. This process is energy-intensive and not feasible for industrial scale [24,26].

In terms of PS function, its high calcium in PS could be used as a liming agent and adds to the organic matter levels of soil. Therefore, PS ash, which contains lime more than 10%, is valuable as a liming agent in agricultural applications. This PS was treated by combustion to produce PS ash (PSA). Seventy percent of PSA is sold to end users and 30% of it is recycled in landfills since PSA acts as a liming agent and adds to the organic matter levels of soil [27]. However, combustion of PSA is energy intensive and one of reasons for increased carbon dioxide evolution. Therefore, finding alternative uses for PS would be of economic benefit to paper mills and would have a positive environmental effect [28].

CELLULASE

Cellulase is an inducible enzyme complex involving synergistic action of three major types of cellulase: endo-glucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91, CBH) like cellobihydrolase and β-glucosidase (EC 3.2.1.21). These enzymes are produced by a number of bacteria and fungi, though species of *Trichoderma* and *Aspergillus* are most reported [29]. Another potential fungus, *Acremonium cellulolyticus* C-1 (FERM P-18508) [30], is a hyper cellulase producer mutant from the wild type *A. cellulolyticus* Y-94, and also produces other enzymes like xylanase, amylase and β-1,3-glucanase. Latest research of enzymatic degradation mechanism cellulose by *A. cellulolyticus*, 12 distinct endo-cellulase component with naming as cellulase I, I-a, I-b, I-c, I-d, III-c, III-d, III-e, III-f, III-A, III-B and IV and 4 β-glucosidase with naming as β-glucosidase I, I-a, II and III. The key enzyme in the cellulase of *A. cellulolyticus* system is III-A because the high purification of cellulase III-A has potent ability to produce glucose from cellobiose through enzymatic reversion or transcellobiosylation followed by hydrolysis without

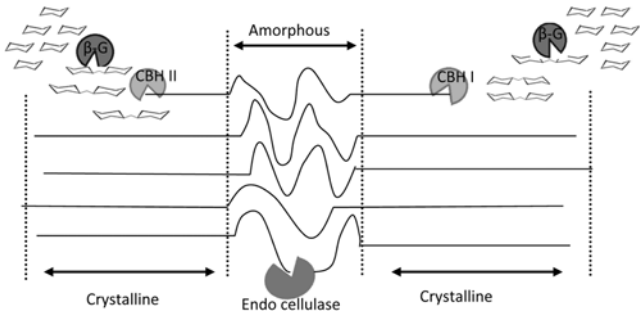

Fig. 2. Mechanism of cellulase in breaking down cellulose. Endo-cellulase cleaves in the middle part of cellulose in amorphous region perform shorter cellulose. Exo-cellulose (CBH I and II) cleaves cellulose from the edge and degrades cellulose into single cellulose (cellobiose, cellotriose) both in crystalline region and amorphous region. β-glucosidase cleaves the single cellulose and degrades it to glucose.

any participation of β-glucosidase. No evidence for the existence of exo-cellulase was found in the cellulase system of *A. cellulolyticus* [31].

The concept of incorporation of adsorption of cellulase on cellulosic substrate due to its heterogeneous nature involves more steps than classical enzyme kinetics. The major steps are described in Fig. 2 and explained as follows [32]:

1. Absorption of cellulases onto substrate via the binding domain.
   a. Endo-cellulase will bind cellulose in the middle at amorphous region and become shorter cellulose.
   b. Cellobiohydrolase (CBH) will bind cellulose from the edge (left and right crystalline region) and break it down into very short chain of cellulose like cellobiose, cellotriose
   c. β-glucosidase breaks down the short chain of cellulose into glucose.
2. Location of bond susceptible to hydrolysis on the substrate surface (at chain end will be degraded by CBH; in the middle of the chain, usually amorphous region; the cleavable bond will be done by endo-cellulase).
3. Formation of enzyme-substrate complex by threading of the chain end into the catalytic tunnel of CBH, to initiate hydrolysis.
4. Hydrolysis of β-glycosidic bond and simultaneous forward sliding of the enzyme along the cellulase chain.
5. Hydrolysis of cellobiose to glucose is done by β-glucosidase.

In addition, the mechanism of β-glucosidase, which was explained according to Mata [33], involves the first step protonation of the anomeric oxygen atom by an acidic group of the enzyme to give the aglyconemoiety of the substrate. The glycosyl-enzyme intermediate could be either a stabilized carbocation or a covalent intermediate. Recent studies point to a covalent glycopyranosyl intermediate. As a result, a group of the enzyme is involved in a general base catalysis, and a hydroxy group is stereospecifically added to the glycosyl moiety of the substrate. Water, alcohols or some other hydroxy compound can be involved as hydroxy-group donor.

Cellulase has an important role in the environmentally friendly utilization of cellulolytic biomass. The effectiveness of the cellulase performance was determined by the synergistic combination of these three enzymes. Therefore, if the breaking and cleaving reactions of cellulose are performed at an acidic pH, but the hydrolytic reaction to produce the monosaccharide is accomplished at a neutral pH, then the saccharification yield may be improved.

1. Cellulase Production by *A. cellulolyticus* Utilizing Waste PS

PS is a waste material that should be recovered and reused. It is cheap and abundant, but its disposal is a problem in environmental terms. Therefore, it would be useful to bio-convert PS to the high-value bio-product, cellulase. Utilizing of PS to produce cellulase is a key step in order to utilize cellulosic biomass because of cost efficiency.

Research on cellulase production from the waste of cellulosic biomass, PS, has been conducted. This work can answer the bottleneck of the utilization of the waste cellulosic biomass as carbon source for research in bio-refinery. The problem of the price for cellulase can be minimized. In additional, this work also solves environmental problem. The usual PS was collected off primary clarifier sludge dewatering process for the production of virgin wood fiber, which is a mixture of pine, cypress and eucalyptus. Therefore microorganisms that can consume celluloses from several origins are essential for cellulase production utilizing PS. *A. cellulolyticus* cells were potential cellulase producer and applied to produce cellulase from PS.

This product, cellulase, can be used for the saccharification of any cellulosic biomass, including PS itself, without any pre-treatment. In the study, dissolved oxygen concentration (DO), PS amount, feeding time, pH, buffer, and nutrients affected cellulase production. Referring to DO, minimum DO level in different pH-controlled culture was higher than 30%, suggesting that DO is not a limiting factor in cellulase production. Since pH and buffer were important factors, we investigated intensively. The optimum pH for cellulase production by *A. cellulolyticus* was pH 6.0, in the highest cellulase activity. The main cause was the highest β-glucosidase activity at this condition [34]. Feeding time, nutrient and PS amount are also the most significant factors. The feeding time and nutrient can be controllable, but the PS amount causes problem, which were encountered due to the viscosity of the culture. The viscosity resulted in mass transfer limitations. However, these could be overcome by fed-batch culture. Unfortunately, when more than two feedings were added, it resulted in a very high increase in the amount of clay (more than 30%), which affected cell growth. The effect of clay on cellulase production will be still investigated in utilization of PS.

Clay may immobilize or adsorb cellulase on its surface or pores. To confirm this, the clay was mixed with cellulase solution and precipitated by centrifugation at 4,000 rpm for 10 min. It was then washed with buffer and used for saccharification of PS for 60 min. However, the formation of reducing sugars was not detected. Moreover, cellulase activity in the supernatant was not significantly different from before mixing. This indicates that the clay constituent of PS had not adsorbed or immobilized the cellulase present in the culture. As the results, cellulase can be produced in pH controlled by using PS in the culture of *A. cellulolyticus*, the enzyme concentration reached 10.96 FPU/mL (Fig. 3) in a fed-batch operation. The produced cellulase can be used for PS saccharification.

2. Saccharification Using Cellulase from PS

The saccharification of PS offers many advantages rather than other ligno-cellulosic biomass. In general, the composition of PS is almost the same with paper, but the length of cellulose is shorter. Luckily, the shorter cellulose is the easier to degrade into monomer (glucose). Another advantage of PS as carbon source is the lignin

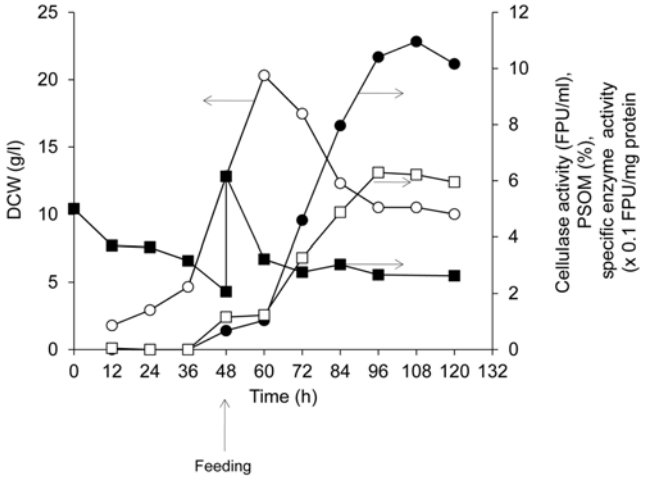

Fig. 3. Cellulase production using the culture of *A. cellulolyticus* using optimized medium. Symbols: closed squares, residual PSOM concentration; closed circles, cellulase activity; open circles, DCW; open squares, specific enzyme activity.

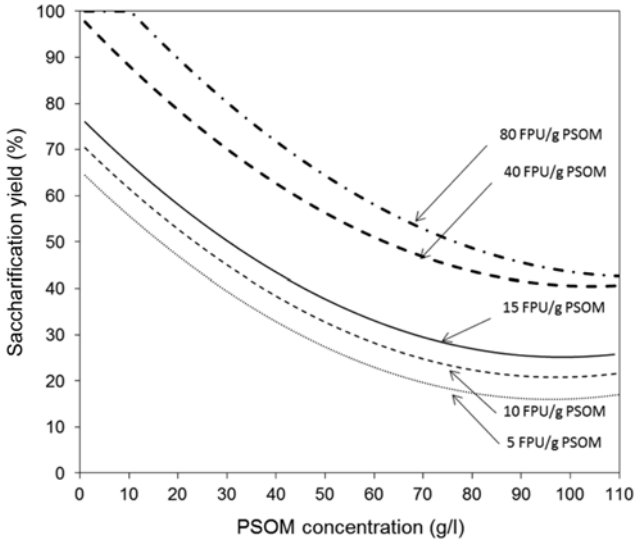

Fig. 4. Simulation of the saccharification with 0.8 M maleate buffer in various cellulase and PSOM concentration.

content, which is negligible. Almost all of the lignin is removed in the bleaching process in the pulp and paper industry. Lignin is naturally formed to protect a plant [35]. Removing lignin from cellulosic biomass makes the cellulase more accessible to cellulose. PS can be used as carbon source without any pre-treatment. Therefore, the utilization of PS as carbon source is strongly recommended.

The PS saccharification has been optimized using the cellulase from *A. cellulolyticus*. The presence of clay in PS did not directly inhibit the hydrolysis of PS organic material (PSOM), but it influenced the pH of the solution. The buffer type was also a key factor in the performance of the *A. cellulolyticus* enzyme. The most effective buffer for this cellulase was maleate buffer [25]. The optimal condition was determined by three parameters: PSOM concentration (g/mL), cellulase concentration (FPU/mL) and maleate buffer concentration in molar. A simulation-computation of saccharification showed that it could be degraded 100% at low concentration of PS but it needs high amount of cellulase (more than 40 FPU/g PSOM).

Unfortunately, the higher the concentration of PS is, the less of saccharification is because of mass transfer limitation. Another problem of saccharification is the high concentration of glucose could inhibit the cellulase itself [36].

In conclusion, utilization of PS depends on three parameters:

1. The pH stabilization will depend on the clay amount or type. The more clay will need a higher concentration of buffer. However, the concentration of maleate buffer is limited. At 1 Molar maleate buffer, the buffer is saturated [25].

2. The a mount of PS. This amount of PS will influence the viscosity and effecting mass transfer limitation. Mass transfer limitation means the sugar, which is released by the saccharification process, cannot disperse freely because of the viscosity. This condition can be minimized by agitation. However, the higher concentration makes the condition become semisolid.

3. The amount of cellulase is of course the key factor. However, the effectiveness of saccharification is influenced by the other parameters. The amount of PS could cause mass transfer limitation. The mass transfer limitation makes the glucose concentration collected in a certain area. Furthermore, this high glucose will inhibit the cellulase activity. Therefore, the higher concentration of cellulase will not produce glucose linearly even if there is enough PS to be hydrolyzed.

For example, the maximum RS for PS using cellulase of PS is 38.4 g/L using 75.6 g/L of PSOM, in 1.06 M maleate buffer (pH 5.2) and cellulase 20 FPU/L. This condition can be different for different PS.

3. Simultaneous Saccharification and Fermentation (SSF)

Utilizing the PSOM as carbon source to produce ethanol as renewable energy means solving environment problems and reducing the energy crisis as well. Bio-ethanol from PS can reduce the dependence on fossil fuel. To overcome the fossil fuel crisis and slow global warming, bio-ethanol produced from PS is as an alternative energy. Utilizing feedstock PS, which is considered as a waste in industry [37,38], is economically feasible to produce bio-ethanol in second generation because of the lower cost for the raw material and not competing with human need as in the first generation. The most crucial factor in ethanol production from PS depends on how efficient saccharification is: the amount of sugar produced and how fast the sugar produced.

Using cellulase from PS needs only simple separation such as removing insoluble materials like clay and other biomass is required. The performance of SSF was much more effective compare to separated hydrolysis and fermentation (SHF) (Fig. 5).

Fifty grams per liter of PSOM was used, the ethanol yield based on initial PS organic material ($Y_{e/PSOM}$) of SHF and SSF were 0.12 and 0.23 (g ethanol/g PSOM), respectively, but ethanol concentration with SSF was 11.4 g/L. However, when the PSOM concentration was increased the ethanol concentration increased to nearly 40 g/L, but the $Y_{e/PSOM}$ was decreased. The reason why the ethanol yield was decreased may be due to mass transfer limitation. PSOM is only 25% of PS, meaning that 150 g PSOM is equivalent to 600 g PS/L. It is impossible to mix 600 g/L of PS homogeneously, which decreases performance of enzymatic hydrolysis. This is shown in the decreased ethanol yield with the increase in PS concentration.

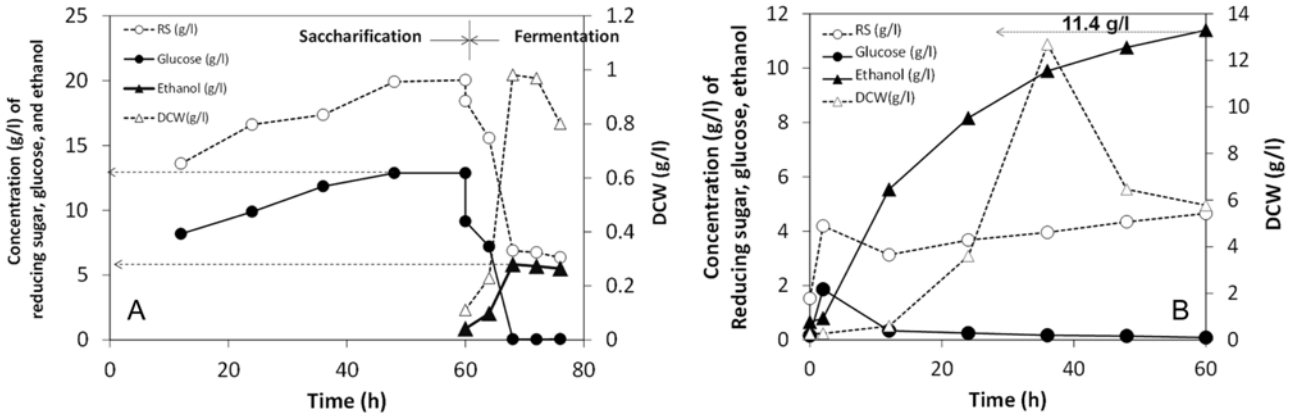

Fig. 5. The ethanol production from PS using SHF (a) and SSF (b) methods. (a) Saccharification in SHF was done cellulase from PS origin for 60 h and inoculated by *S. cerevisiae* TJ14 afterward for 12 h for ethanol production. (b) SSF was carried out by adding cellulase from PS origin and culture of *S. cerevisiae* TJ14 into the SSF at the beginning of fermentation.

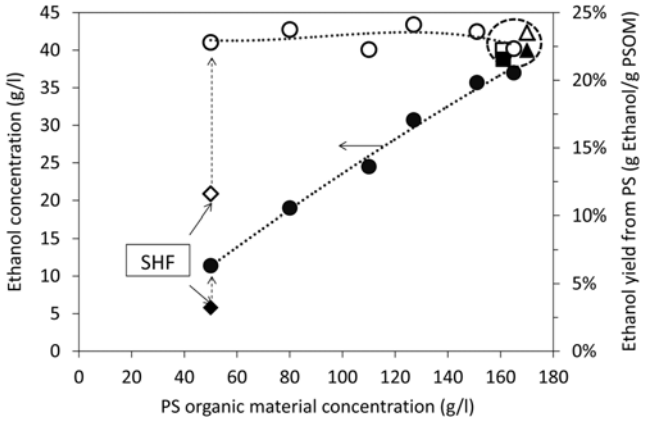

Fig. 6. $Y_{e/PSOM}$ and concentrations for various PSOM concentrations in SSF. Closed and open circles denote ethanol concentration and $Y_{e/PSOM}$, respectively. Open and closed triangles denote $Y_{e/PSOM}$ and ethanol concentration when increased PSOM with 35 FPU/g PSOM was used, respectively. Open and closed squares denote $Y_{e/PSOM}$ and ethanol concentration when increased inoculum was used, respectively. Open and closed rhombuses denote $Y_{e/PSOM}$ and ethanol concentration in SHF, respectively.

To increase ethanol concentration requires increasing PSOM concentration. In the region from 50 to 165 g PSOM/L the ethanol concentration was proportional to the initial PSOM concentration. The 165 g PSOM/L was the maximum amount in flask scale operation, and produced 37 g/L of ethanol with the $Y_{e/PSOM}$ of 0.21 g ethanol/g PSOM.

The effect of PS concentration on ethanol production is shown in Fig. 6. The higher PSOM concentration is the higher ethanol concentration until certain concentration and time, whereas the ethanol becomes toxic to *S. cerevisiae*. To increase ethanol concentration up to 40 g/L, there are two options: one is increased cellulase activity for solving glucose limitation; the other one, increased inoculums for activating yeast. Cellulase activity was increased to 35 FPU/g PSOM, which increased the saccharification yield, more than 5%. The ethanol concentration increased from 37 to 40 g/L and the $Y_{e/PSOM}$ also increased from 0.22 to 0.23 g ethanol/g PSOM. When 20% of inoculums were used, ethanol concentration and $Y_{e/PSOM}$ increased to 40 g/L and 0.24 g ethanol/g PSOM, respectively.

When 1,000 kg of PS was used for bio-ethanol production using the SSF process, which uses cellulase produced by *A. cellulolyticus* utilizing PS as carbon source, 135 kg of PS was used for cellulase production and produced cellulase 3,180 kFPU saccharified the remaining 865 kg of PS. In this process produced ethanol amount is 51 kg based on the results of SSF (Fig. 7).

PSOM was used as a carbon source for cellulase production by *A. cellulolyticus* C-1 at 28 °C. Culture broth containing cellulase was separated from *A. cellulolyticus* culture and used for saccharification of PS in SSF at 42 °C. Ethanol fermentation was simultaneously carried out by yeast inoculation with saccharification of PS in SSF. After SSF ethanol solution was separated from SSF culture broth, SSF medium compositions consisted of PSOM, 5 g/L yeast extract, 10 g/L poly peptone and 4 g/L KH_2PO_4. Initial PSOM concentrations were 50, 80 and 110 g/L. After medium sterilization, 15 FPU/g PSOM of PS cellulase and 10% inoculums were added in 500 mL Erlenmeyer flask with working volume of 100 mL. However, in the experiment containing 170 g/L of PSOM and 35 FPU/g PSOM, culture was not mixed well. To overcome this problem the initial concentration of PSOM was 85 g/L and 35 FPU/g PSOM. After 8 hours, another 8.5 g PSOM (34.7 g PS) with cellulase 35 FPU/g PSOM was added, and final PSOM concentration in the culture was 170 g/L. By this method, the ethanol production reached 40.1 g/*l*.

ONE-POT ETHANOL PRODUCTION

Consolidated bioprocessing (CBP) is considered as the most ideal process since its simplification of the conversion process of cellulose to bio-ethanol [39,40] but SSF is the most appropriate strategy in practice. One-pot bio-ethanol production, including cellulase production, saccharification of cellulose, and ethanol production, was already investigated for bio-ethanol by co-culture of two different microorganisms such as a hyper cellulase producer, *Acremonium cellulolyticus* C-1 and an ethanol producer *Saccharomyces cerevisiae*.

The CBP was categorized into CBPs I and II. Category I CBP is an engineering method of a cellulase producer to be ethanologenic,

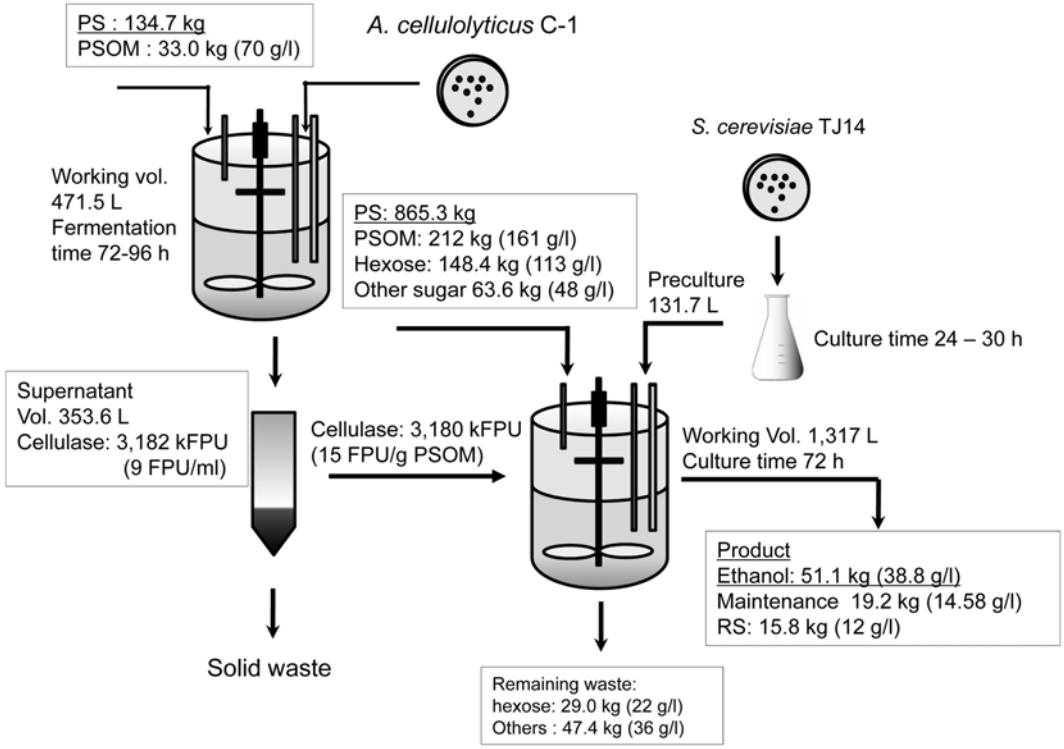

Fig. 7. Mass balance for ethanol production using 1 tons of PS in SSF. One hundred thirty five kg and 865 kg of PS were used for cellulase and ethanol productions, respectively. Theoretical ethanol yields on hexose basis ($Y_{e/hex}$) of 63.4% and the $Y_{e/PSOM}$ of 24% were based for estimation of ethanol production. The saccharification yield in SSF was estimated 64% based on experimental data in 3-L reactor.

while category II CBP of an ethanologen to be cellulolytic. Those microorganisms can produce ethanol from cellulose, followed by the fermentation of the resulting sugars to ethanol in anaerobic growth conditions [14]. However, their ethanol tolerances are low due to the low expression of the relevant genes involved in ethanol fermentation or to the low activity of the enzymes encoded by these genes so that the ethanol yield and productivity is low. These bottlenecks can be solved by improving the feasibility of the modified CBP in a single reactor using two microorganisms, cellulase producer and ethanol conversion (Fig. 8). The most difficulty of ethanol production from cellulose in a single bioreactor using *A. cellulolyticus* and *S. cerevisiae* cells is the co-culture condition, because *A. cellulolyticus* is aerobic microorganism, while *S. cerevisiae* is facultative anaerobic microorganism. In addition, *A. cellulolyticus* and *S. cerevisiae* cells grow in different media. For successful one-pot process for ethanol production, the characteristic of oxygen consumption of both microorganisms is the key factor especially for *A. cellulolyticus* as cellulase producer. Timing for inoculation of each microorganism and substrate addition should be managed exactly to get synergism of both microorganisms.

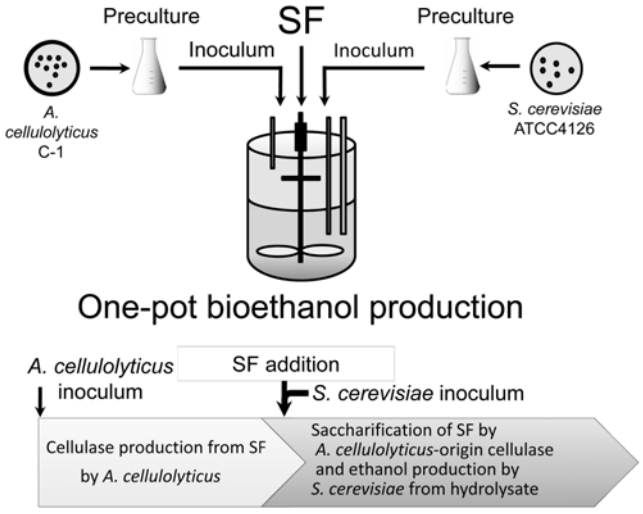

Fig. 8. Schematic diagram of one-pot bio-ethanol production. One-pot bio-ethanol production was carried out by two steps in a single reactor: first step, cellulase production by *A. cellulolyticus* cells; second step, simultaneous saccharification of SF by the addition of *S. cerevisiae* inoculum and SF.

In the ethanol production from Solka Floc (SF), 100% cellulose, *A. cellulolyticus* and *S. cerevisiae* cells consume glucose both for production of cellulase and ethanol, respectively, and for their cellular maintenances, which cause the ethanol yield based on SF ($Y_{e/SF}$) to decrease. It is better to keep in anaerobic condition in the ethanol production phase, but it was necessary to some extent of agitation rate to avoid precipitation of SF inside the reactor. In the one pot system, the dissolved oxygen level in the ethanol production phase increased to 20%, which might decrease the carbon flux from glucose to ethanol. It is necessary to optimize the dissolved oxygen both for maximizing ethanol production and for maintaining *A. cellulolyticus* cells actively. So far, this one-pot bio-ethanol production is an alternative strategy as a mimic of CBP, because cellulase production, saccharification of carbohydrate, and ethanol fermentation

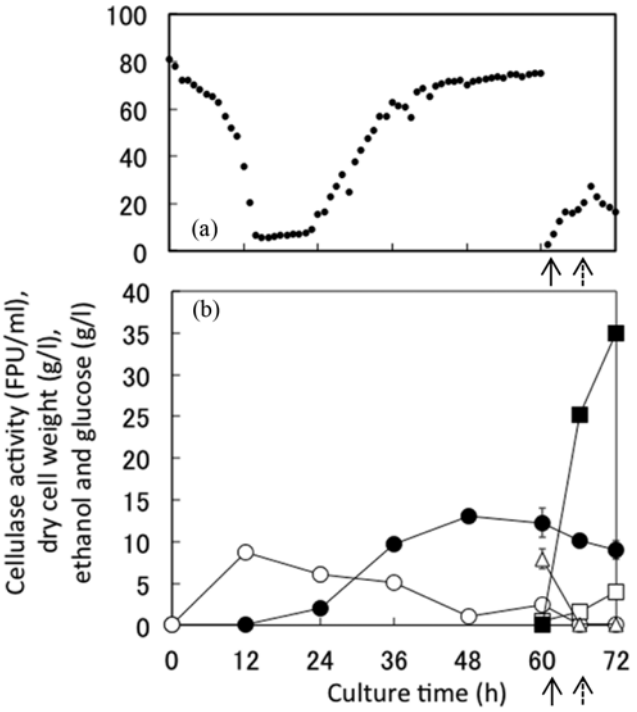

Fig. 9. Co-culture of *A. cellulolyticus* and *S. cerevisiae* in one-pot bio-ethanol production. Symbols in B: open circles, dry cell weight of *A. cellulolyticus*; closed circles, cellulase activity; open triangles, glucose concentration; open squares, dry cell weight of *S. cerevisiae*; closed squares, ethanol concentration. Arrows indicate SF-addition times. Error bars denote standard deviation (n=3).

occur in a single reactor. Cellulase activity remained 8-12 FPU/mL throughout the one-pot process. Using 50-300 g SF/L was used in 500 mL Erlenmeyer flask scale, the ethanol concentration and yield based on initial SF were as 8.7-46.3 g/L and 0.15-0.18 (g ethanol/g SF), respectively. In 3-L fermentor with 50-300 g SF/L, the ethanol concentration and yield were 9.5-35.1 g/L with their yields of 0.12-0.19 (g/g), respectively, demonstrating that the one-pot bio-ethanol production is a reproducible process in a scale-up bioconversion of cellulose to ethanol.

Based on the research above, PS can be used to produce cellulase by *A. cellulolyticus,* and the sugar from PS can be converted by *S. cerevisiae* TJ14 to ethanol. Both microorganisms can tolerate other compounds in PS (Tomoegawa Ltd, Shizuoka, Japan). Therefore, the work of one pot bio-ethanol production from SF is applicable for PS.

CONCLUSION

Knowledge about cellulosic biomass is very important for its utilization. Global warming and grain price hikes can be avoided by switching bio-fuel raw materials from grain and plant-oil sources to cellulosic biomass waste in the beginning. The extended volatile fatty acid-platform technology can be gradually moved to ordinary woody ligno-cellulosic biomass or energy crops in the future [41]. By recognizing its characteristics, biomass can be used optimally. PS gives many advantages rather than other cellulosic biomass because of negligible lignin and unrequired pretreatments. Therefore, many researchers tried using this PS for cellulosic biomass. One of the most important researches figured out that the PS from virgin wood can be used to produce cellulase by *A. cellulolyticus*. This invention broke up the research bottleneck, which tried to use cellulosic biomass waste because of the price of commercial cellulase. Beside the utilization of PS to produce cellulase, the sugar, which was produced from the saccharification of PS, could be converted to bio-ethanol by *S. cerevisiae* TJ14. It means other compounds in PS can be conditioned and tolerated by both microorganisms, *A. cellulolyticus* and *S. cerevisiae* TJ14. Some strategies of PS utilization were conducted to produce bio-ethanol in order to answer energy crises of fossil fuel. SHF, SSF and one pot bio-ethanol production using PS as cellulosic biomass were tried to produce ethanol. SSF was much more effective rather than SHF. One pot bio-ethanol production is already applied using SF, 100% cellulose. Therefore, it should be applicable using PS as cellulosic biomass. As comparison, using PSOM, the yield of ethanol is 0.208 (g ethanol/g PSOM) or 0.051 (g ethanol/g PS), while one pot bio-ethanol was almost the same 0.19 g ethanol/g SF.

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