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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 considered as a waste from pulp and paper industry. Its advantages bring PS as the most potential cellulosic biomass 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 is handling yet in their infancy and suffer from large scale since low productivity. Reviewing many researches that working at the utilization of PS for bio-refinery could encourage the utilization of PS from research at laboratory to be applied in industry. For the reason, PS usage as raw material in industry, it will be effectively solving the environmental problems caused by PS with clean technology. In addition, its conversion to bio-ethanol could offer the alternative solution of 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 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 term for 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) solve these problems and can supply a larger proportion for fuel supply sustainably, affordably, 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

Table 1

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, techno-economic 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

50 production in Europe [7]. This process will be more feasible when subsidies like cellulase
51 production itself also from cellulosic biomass, which means reducing the cost of cellulose-
52 hydrolytic enzymes [5].

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

65 ## WASTE PAPER AS BIORESOURCE

66 ### 1. Waste paper as biomass

67 PS including waste papers, categorizes Industrial waste, is sludge from pulp and paper
68 mills. The sludge is mainly cellulose fiber generated at the pulping process (Fig. 1) prior to
69 entering the paper machine [12]. PS, also known as paper fiber bio-solids, is the residue left

70 Fig. 1

71 over from the paper recycling process. It consists of unusable short fibers, inks and dyes, clay,
72 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].

Tables 2 and 3

Some PS materials also contain non-glucan carbohydrate (Xylan and Mannan) [16]. At 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 10 million tons in 2001 [17]. In the 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, brown, reddish-orange, and were mixed in by bulldozers [13].

An evolution of biomass residue in recent year’s 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.

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

113 **2. Treatment of Waste PS**

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

120 trends in many countries are restricting the amount and types of materials that are disposed
121 by landfill [25].

122 Many research tried to handle the environmental problem of PS. A research to recover
123 Pb, an element in clay from PS, did by employing a hydrothermal reaction at 95–100°C
124 under alkaline conditions. Chemical and physicochemical methods require high temperatures
125 (140–160°C), but it is corrosive in nature and demand neutralization. Moreover such methods
126 offer low yield of carbohydrates and generate inhibitors for further microbial processes [24].
127 This process is energy-intensive and not feasible to be applied for industrial scale [24, 26].

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

136 **CELLULASE**

137 Cellulase is an inducible enzyme complex involving synergistic action of three major
138 types of cellulase: endo-glucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91, CBH) like
139 cellobihydrolase and β-glucosidase (EC 3.2.1.21). These enzymes are produced by a number
140 of bacteria and fungi though species of *Trichoderma* and *Aspergillus* are most reported [29].
141 Another potential fungi, *Acremonium cellulolyticus* C-1 (FERM P-18508) [30], is a hyper
142 cellulase producer mutant from the wild type *A. cellulolyticus* Y-94, and also produces other
143 enzymes like xylanase, amylase and β-1,3-glucanase. Latest research of enzymatic

144 degradation mechanism cellulose by *A. cellulolyticus*, 12 distinct endo-cellulase component
145 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
146 β-glucosidase with naming as β-glucosidase I, I-a, II and III. The key enzyme in the cellulase
147 of *A. cellulolyticus* system is III-A because the high purification of cellulase III-A has potent
148 ability to produce glucose from cellobiose through enzymatic reversion or
149 transcellobiosylation followed by hydrolysis without any participation of β-glucosidase. No
150 evidence for the existence of exo-cellulase was found in the cellulase system of *A.*
151 *cellulolyticus* [31].

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

155 Fig. 2

156 1. Absorption of cellulases onto substrate via the binding domain.
157 a. Endo-cellulase will bind cellulose in the middle at amorphous region become
158 shorter cellulose.
159 b. Cellobiohydrolase (CBH) will bind cellulose from the edge (left and right
160 crystalline region) and breaking it down into very short chain of cellulose like
161 cellobiose, cellotriose
162 c. β-glucosidase breaks down the short chain of cellulose into glucose.
163 2. Location of bond susceptible to hydrolysis on the substrate surface (at chain end will
164 be degrade by CBH, in the middle of chain, usually amorphous region; the cleavable
165 bond will be done by endo-cellulase).
166 3. Formation of enzyme-substrate complex by threading of the chain end into the
167 catalytic tunnel if 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 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 crucial 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.

A research that conducted 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 fibre, 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 was important factors we investigated intensively already. The optimum pH for cellulase production by *A. cellulolyticus* was pH 6.0, in the highest cellulase activity. The mail cause was the highest b-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 2 feedings 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 be still investigated in utilization of PS

213 Clay may immobilize or adsorb cellulase on its surface or pores. To confirm this, the clay
214 was mixed with cellulase solution and precipitated by centrifugation at 4000 rpm for 10 min.
215 It was then washed with buffer and used for saccharification of PS for 60 min. However, the
216 formation of reducing sugars was not detected. Moreover, cellulase activity in the supernatant
217 was not significant different from before mixing. This indicates that the clay constituent of
218 PS had not adsorbed or immobilized the cellulase present in the culture. As the results,
219 cellulase can be produced in pH controlled using PS in the culture of *A. cellulolyticus*, and
220 the enzyme concentration reached 10.96 FPU/mL (Fig. 3) in a fed-batch operation. The
221 produced cellulase can be used for PS saccharification.

222 Fig. 3.

223 **2. Saccharification using cellulase from PS**

224 The saccharification of PS offers many advantages rather than other ligno-cellulosic
225 biomass. In general, the composition of PS is almost the same with paper, but the length of
226 cellulose is shorter. Luckily, the shorter cellulose is the easier to be degraded it into monomer
227 (glucose). Another advantage of PS as carbon source is the lignin content which is negligible.
228 Almost all of lignin removed in bleaching process at pulp and paper industry. Lignin is
229 naturally formed to protect a plant [35]. Removing lignin from cellulosic biomass makes the
230 cellulase more accessible to cellulose. Utilizing PS as carbon source can be done without any
231 pre-treatment. Therefore, the utilization of PS as carbon source is strongly recommended.
232 The PS saccharification has been optimized using the cellulase from *A. cellulolyticus*.
233 The presence of clay in PS did not directly inhibit the hydrolysis of PS organic material
234 (PSOM) but it influenced the pH of the solution. The buffer type was also a key factor in the
235 performance of the *A. cellulolyticus* enzyme. The most effective buffer for this cellulase was
236 maleate buffer [25]. The optimal condition was determined by 3 parameters: PSOM

237 concentration (g/mL), cellulase concentration (FPU/mL) and maleate buffer concentration in
238 Molar. A simulation-computation of saccharification showed that it could be degraded 100%
239 at low concentration of PS but it needs high amount of cellulase (more than 40 FPU/g
240 PSOM).

241 Fig. 4.

242 Unfortunately, the higher concentration of PS is, the less of saccharification is because of
243 mass transfer limitation. Another problem of saccharification is the high concentration of
244 glucose could inhibit the cellulase itself [36].
245 In conclusion, utilization of PS depends on 3 parameters:

246 1. The pH stabilization will depend on the clay amount or type. The more clay will need
247 the higher concentration of buffer. However, the concentration of maleate buffer is
248 limited. At 1 Molar maleate buffer, the buffer is saturated [25].
249 2. The amount of PS. This amount of PS will influence the viscosity and effecting mass
250 transfer limitation. Mass transfer limitation means the sugar, which is release by
251 saccharification process, cannot disperse freely because of the viscosity. This
252 condition can be minimized by agitation. Unfortunately, the higher concentration
253 makes the condition become semi solid.
254 3. The amount of cellulase is of course the key factor. However the effectiveness of
255 saccharification is influence by the other parameters. The amount of PS could cause
256 mass transfer limitation. The mass transfer limitation make the glucose concentration
257 will be collected in certain area. Furthermore, this high glucose will inhibit the
258 cellulase activity. Therefore, the higher concentration of cellulase will not produce
259 glucose linearly event there is enough PS to be hydrolysed.

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 problem and reducing energy crisis as well. Bio-ethanol from PS can reduce the dependence on fossil fuel. In order to overcome fossil fuel crisis and to 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 since its lower cost for the raw material rather and is not compete with human need as in the first generation. The most crucial factor of 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).

Fig. 5.

Fifty grams per litter 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 caused by mass transfer limitation. PSOM is only 25% of PS, meaning that 150 g PSOM is equivalent to 600 g PS/L. It is

283 impossible to mix 600 g/L of PS homogeneously, which decrease performance of enzymatic
284 hydrolysis. This is shown the decreased ethanol yield with the increase in PS concentration.
285 In order to increase ethanol concentration it needs to increase PSOM concentration. In the
286 region from 50 to 165 g PSOM/L the ethanol concentration was in proportional to the initial
287 PSOM concentration. The 165 g PSOM/L was the maximum amount in flask scale operation,
288 and produced 37 g/L of ethanol with the $Y_{e/PSOM}$ of 0.21 g ethanol/g PSOM.
289 The effect of PS concentration on ethanol production is shown in Fig. 6. The higher
290 PSOM concentration is the higher ethanol concentration until certain concentration and time
291 whereas the ethanol becomes toxic to *S. cerevisiae*. To increase ethanol concentration up to
292 40 g/L, there are two options: one is increased cellulase activity for solving glucose
293 limitation; the other one, increased inoculums for activating yeast. Cellulase activity was
294 increase to 35 FPU/g PSOM, which increased the saccharification yield, more than 5%. The
295 ethanol concentration increase from 37 to 40 g/L and the $Y_{e/PSOM}$ also increased from 0.22 to
296 0.23 g ethanol/g PSOM. When 20% of inoculums were used, ethanol concentration and
297 $Y_{e/PSOM}$ increased to 40 g/L and 0.24 g ethanol/g PSOM, respectively.

298 Fig. 6.

299 When 1000 kg of PS is used for bio-ethanol production using the SSF process, which
300 uses cellulase produced by *A. cellulolyticus* utilizing PS as carbon source, 135 kg of PS was
301 used for cellulase production and produced cellulase 3,180 kFPU saccharified the remaining
302 865 kg of PS. In this process produced ethanol amount is 51 kg based on the results of SSF
303 (Fig. 7).

304 Fig. 7.

305 PSOM was used as a carbon source for cellulase production by *A. cellulolyticus* C-1 at
306 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 consist 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. In the experiment containing 170 g/L of PSOM and 35 FPU/g PSOM, culture was not mixed well. In this case, 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.

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, 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]. Unfortunately, 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 difficulties

Fig. 8

of ethanol production from cellulose in a single bioreactor using *A. cellulolyticus* and *S. cerevisiae* cells are 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 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 microorganism.

In the ethanol production from Solka Floc (SF), 100% cellulose, *A. cellulolyticus* and *S. cerevisiae* cells consume glucose both for productions of cellulase and ethanol, respectively, and for their cellular maintenances, which cause the ethanol yield based on SF ($Y_{e/SF}$) decreased. It is better to keep in anaerobic condition in the ethanol production phase, but it was necessary to some extent agitation rate to avoid a precipitation of SF inside the reactor. In 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 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–

355 0.18 (g ethanol/g SF), respectively. In 3-L fermentor with 50–300 g SF/L, the ethanol
356 concentration and yield were 9.5–35.1 g/L with their yields of 0.12–0.19 (g/g) respectively,
357 demonstrating that the one-pot bio-ethanol production is a reproducible process in a scale-up
358 bioconversion of cellulose to ethanol.

359 Fig. 9

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

364 **CONCLUSION**

365 Knowledge about cellulosic biomass is very important in order to its utilization. Global
366 warming and grain price hikes can be avoided by switching bio-fuel raw materials from grain
367 and plant-oil sources to cellulosic biomass waste in the beginning. The extending volatile
368 fatty acid-platform technology can be gradually moved to ordinary woody ligno-cellulosic
369 biomass or energy crops in the future [41]. By recognizing its characteristic, biomass can be
370 used it optimal. PS gives many advantages rather than other cellulosic biomass since the
371 negligible lignin and unrequired pretreatments. Therefore, many researchers tried using this
372 PS for cellulosic biomass. One of the most important research figured out that the PS from
373 virgin wood can be used to produce cellulase by *A. cellulolyticus*. This invention broke up the
374 bottleneck of any research, which tried to use cellulosic biomass waste because of the price
375 of commercial cellulase. Beside the utilization of PS to produce cellulase, the sugar, which
376 was produced from the saccharification of PS, could be converted to bio-ethanol by *S.*
377 *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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463 **Legend for figures**

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

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

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

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

480 **Fig. 5. The ethanol production from PS using SHF (A) and SSF (B) methods. (A)**
481 **Saccharification in SHF was done cellulase from PS origin for 60 h and**
482 **inoculated by *S. cerevisiae* TJ14 afterward for 12 h for ethanol production. (B)**
483 **SSF was carried out by adding cellulase from PS origin and culture of *S.***
484 ***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.

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.

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

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).

Fig. 1, Joni and Park

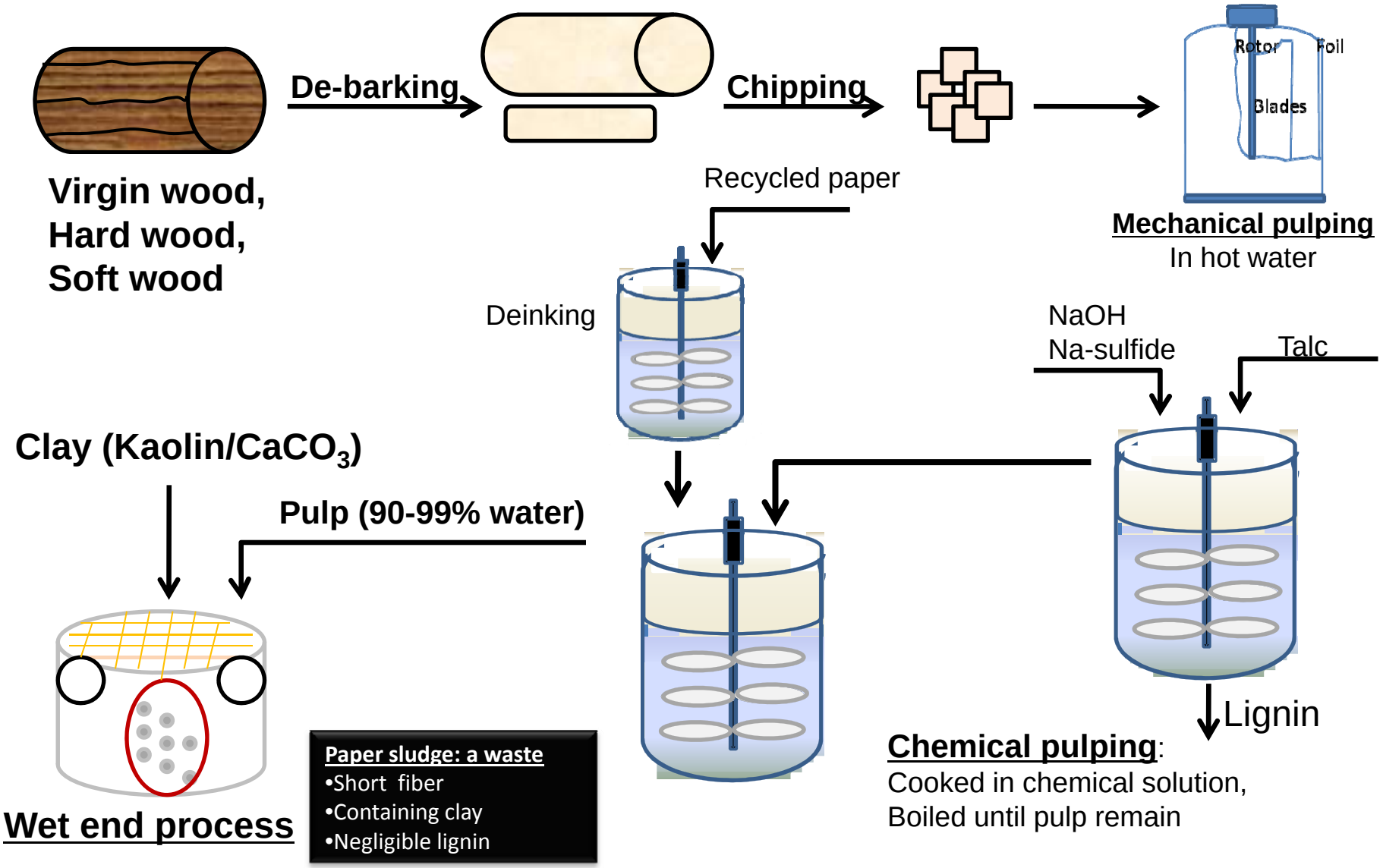
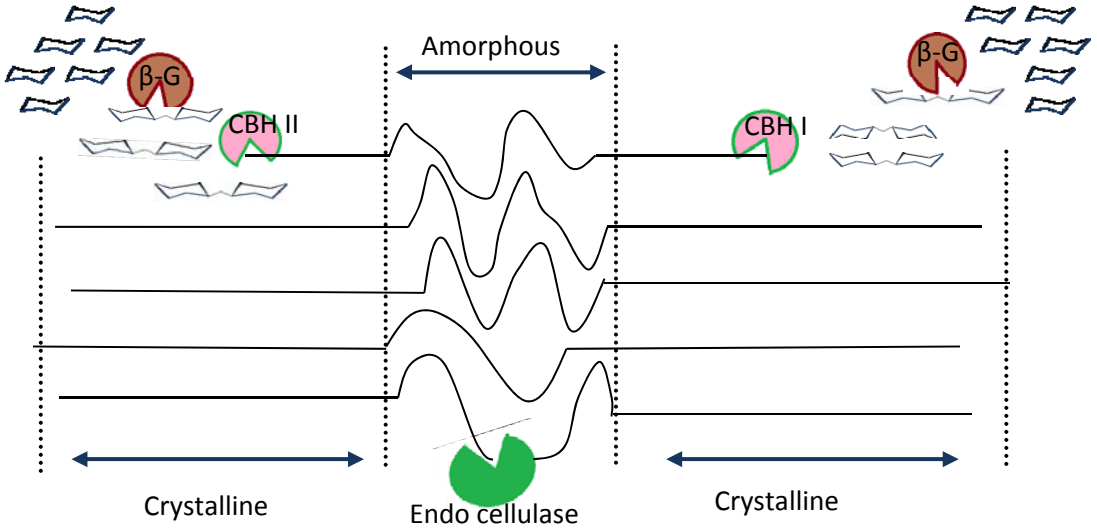


Fig. 2, Joni and Park



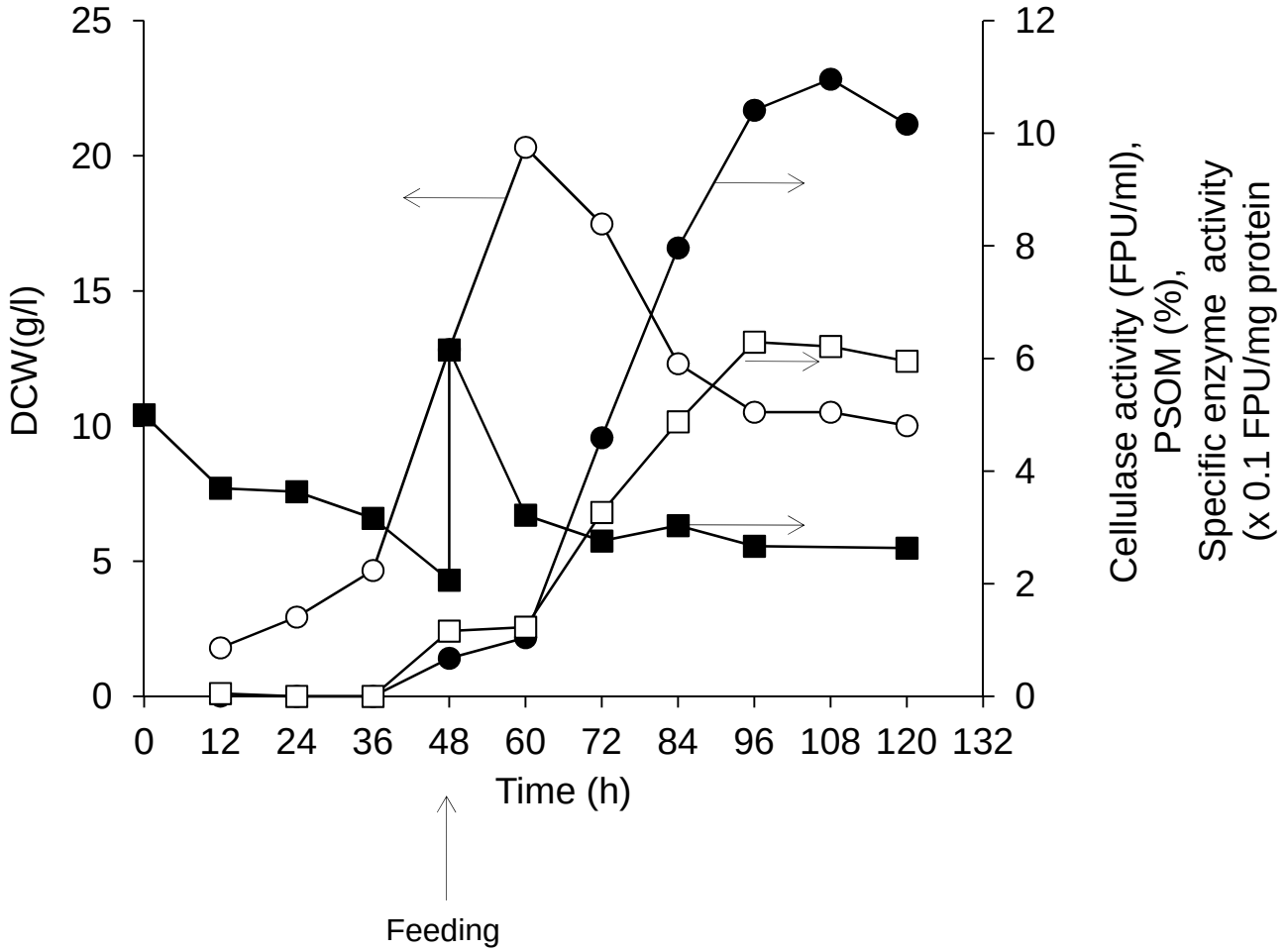

25
20
15
10
5
0
DCW(g/l)
12
10
8
6
4
2
0
Cellulase activity (FPU/ml),
PSOM (%),
Specific enzyme activity
(x 0.1 FPU/mg protein
0 12 24 36 48 60 72 84 96 108 120 132
Time (h)
Feeding

Fig. 4, Joni and Park

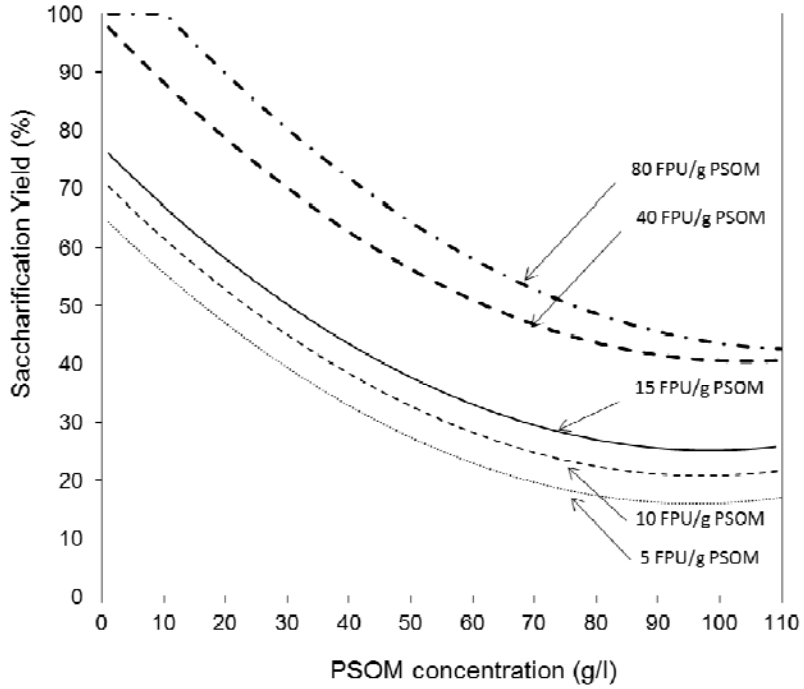


Fig. 5, Joni and Park

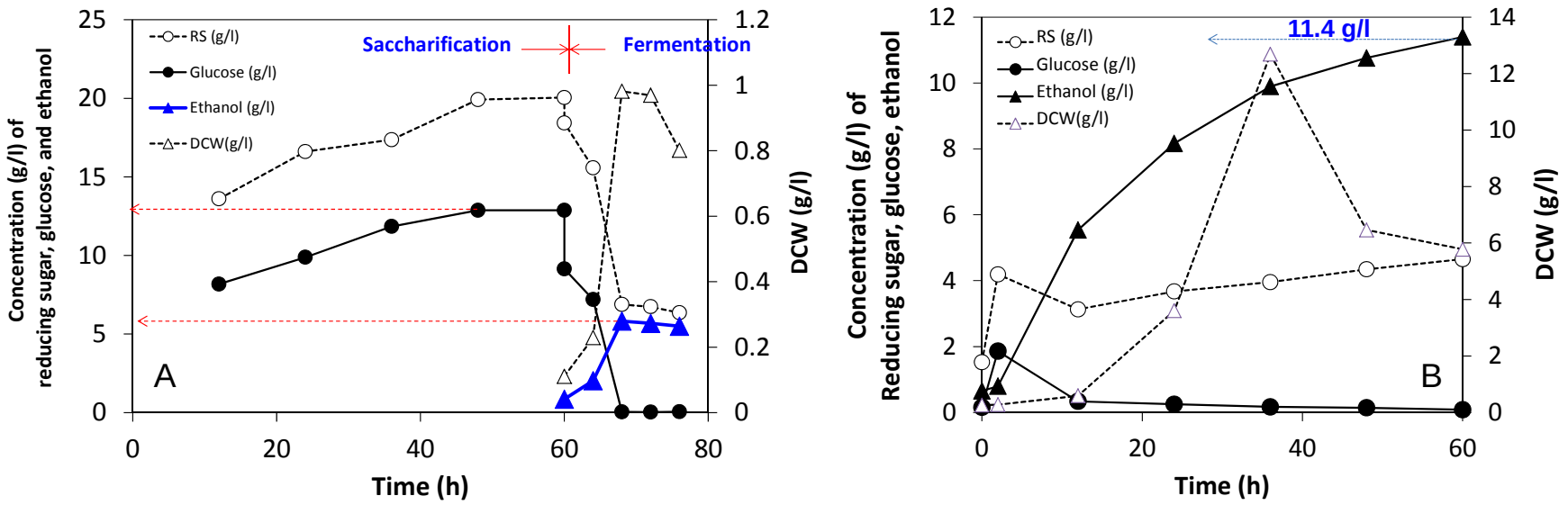
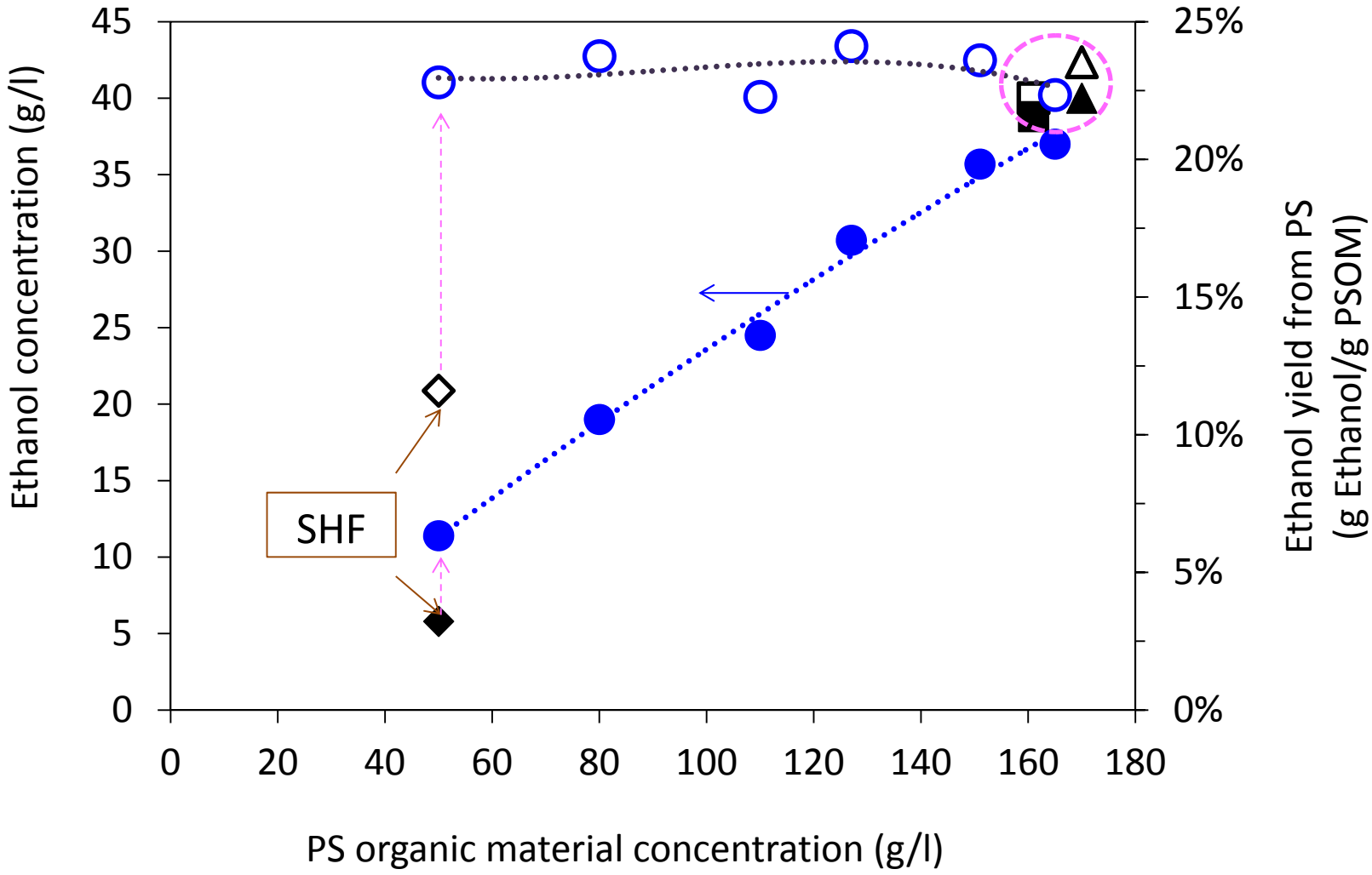
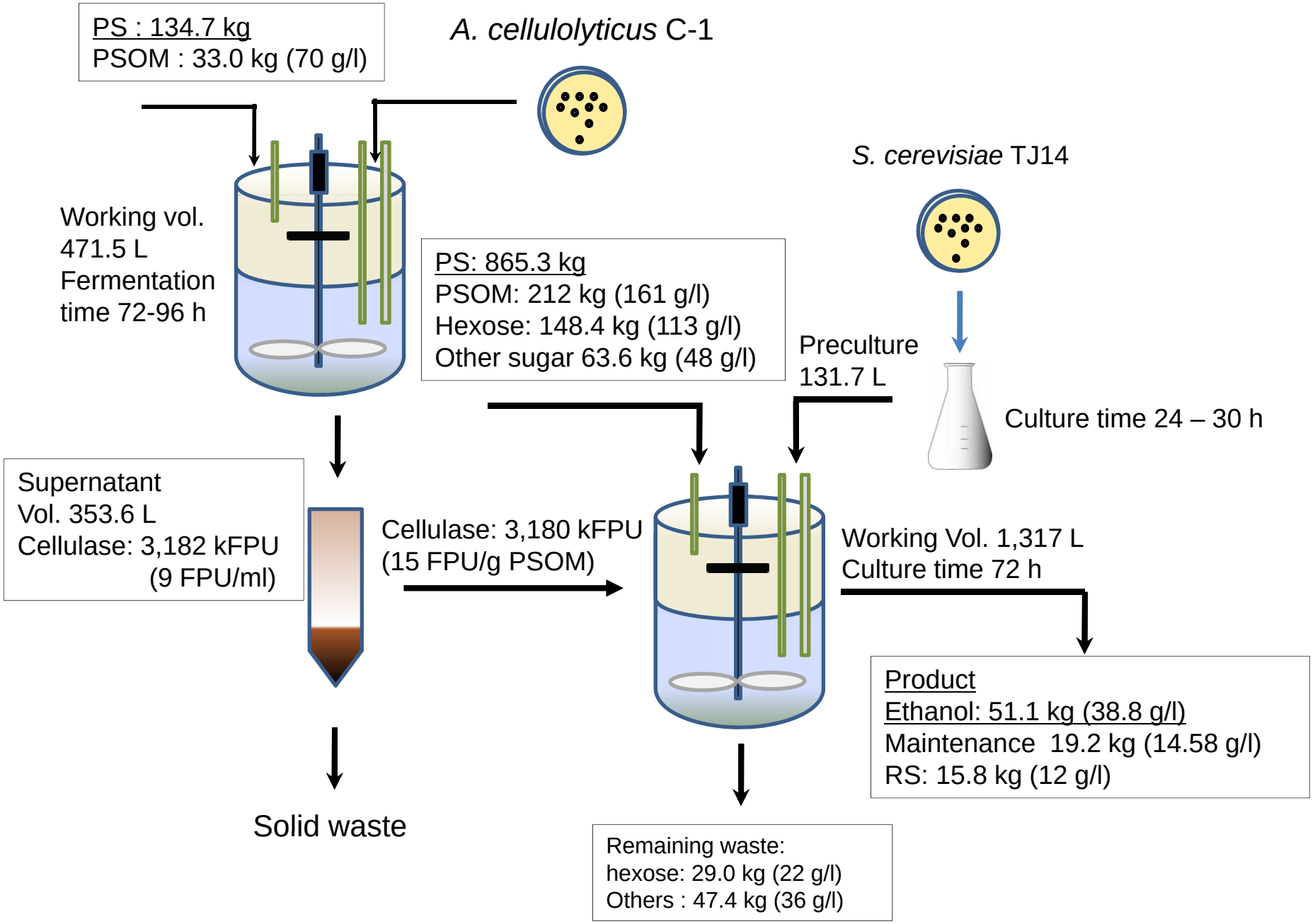
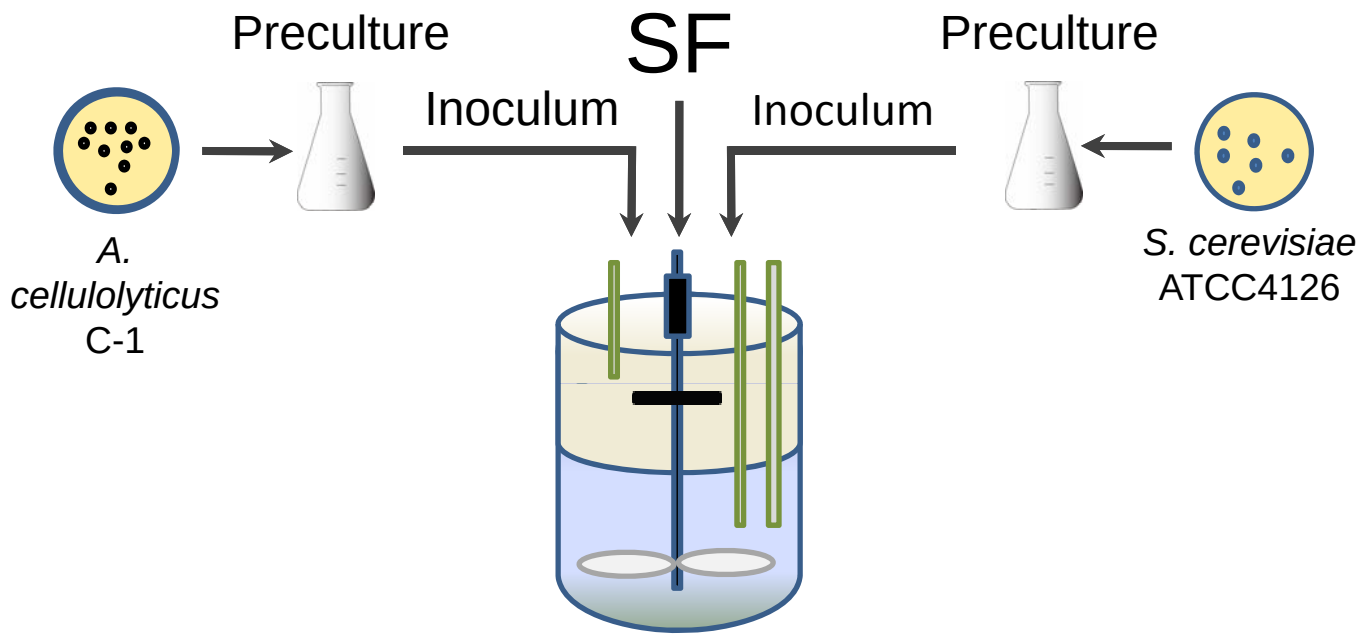


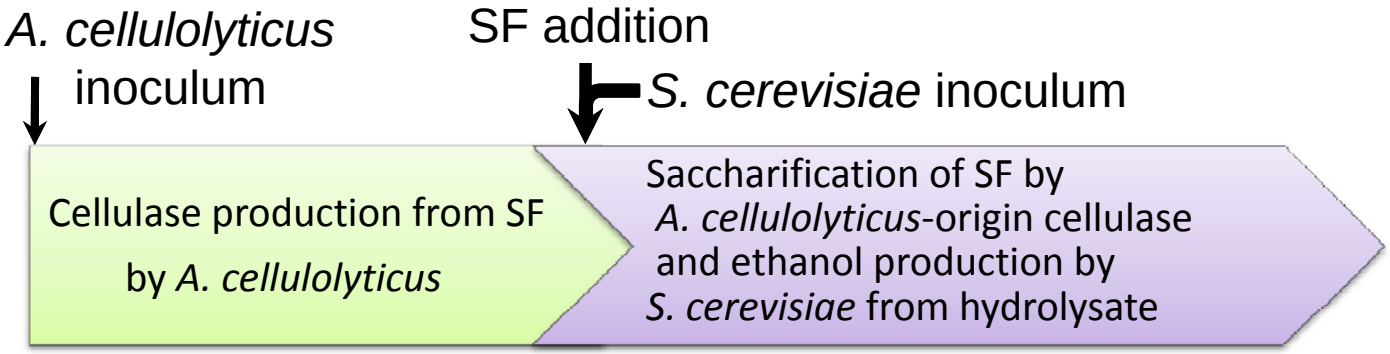
Fig. 6, Joni and Park




PS : 134.7 kg
PSOM : 33.0 kg (70 g/l)
A. cellulolyticus C-1
Working vol.
471.5 L
Fermentation
time 72-96 h
PS: 865.3 kg
PSOM: 212 kg (161 g/l)
Hexose: 148.4 kg (113 g/l)
Other sugar 63.6 kg (48 g/l)
S. cerevisiae TJ14
Preculture
131.7 L
Culture time 24 – 30 h
Supernatant
Vol. 353.6 L
Cellulase: 3,182 kFPU
(9 FPU/ml)
Cellulase: 3,180 kFPU
(15 FPU/g PSOM)
Working Vol. 1,317 L
Culture time 72 h
Product
Ethanol: 51.1 kg (38.8 g/l)
Maintenance 19.2 kg (14.58 g/l)
RS: 15.8 kg (12 g/l)
Solid waste
Remaining waste:
hexose: 29.0 kg (22 g/l)
Others : 47.4 kg (36 g/l)



One-pot bioethanol production



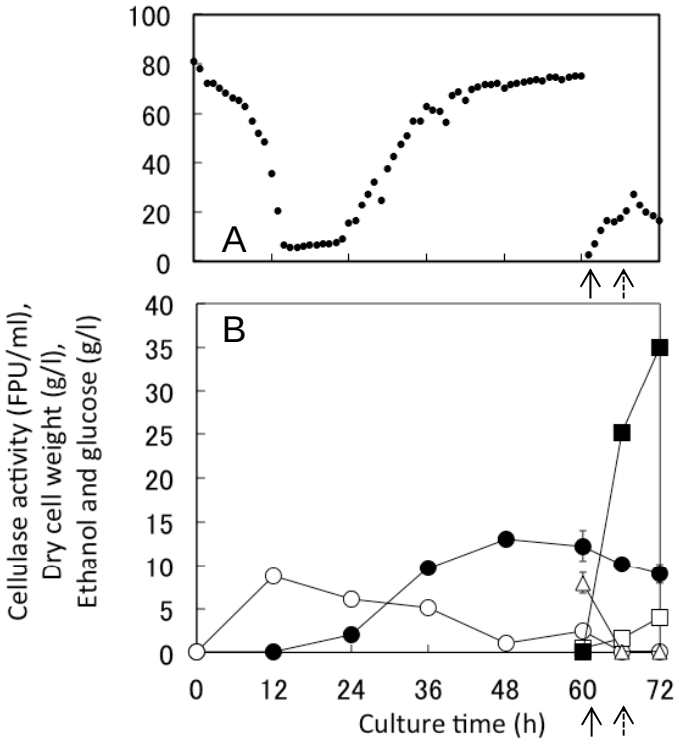

A
B
Cellulase activity (FPU/ml),
Dry cell weight (g/l),
Ethanol and glucose (g/l)
Culture time (h)
0
12
24
36
48
60
72
0
20
40
60
80
100
0
5
10
15
20
25
30
35
40

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

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