DISSERTATION

THERMAL HYDROLYSIS AND STEAM EXPLOSION PRETREATMENT OF DIFFERENT LIGNOCELLULOSIC AQUATIC WEEDS BY ANAEROBIC DIGESTION

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ABSTRACT

Overgrowth of aquatic weeds has been occurring worldwide, leading to various social and environmental problems. Therefore, it is necessary to harvest and utilise these aquatic weeds. Anaerobic digestion (AD) is considered suitable for treating aquatic plant weeds as it has a lower impact on the environment and can recover energy in the form of biogas; further, the digestate can be used for various purposes. Previous studies pointed out that lignin in the lignocellulose structure of aquatic weeds limits the biodegradability and methane potential of the substrate. Therefore, before AD, a suitable pretreatment is required to make the lignocellulose structure more accessible to produce higher methane. Therefore, in this study, we conducted thermal hydrolysis (TH) and steam explosion (SE) pretreatment on different lignocellulosic aquatic weeds having different chemical compositions. In study 1, the physical and chemical differences of lignocellulosic aquatic weeds pretreated by TH and SE pretreatment will be clarified. The effect of these pretreated aquatic weeds on anaerobic digestion will be discussed in study 2. Therefore, Study 1 and Study 2 compare the TH and SE mechanisms, which have not been studied before. After that, a proposal of an appropriate pretreatment based on biomass type is discussed.

Chapter 2 (Study 1) clarifies the physical and chemical differences between the lignocellulosic aquatic weed pretreated by TH and SE. When results were interpreted using SEM analysis, it was seen that more lignin was condensed on the biomass surface during the SE pretreatment than TH. Also, substrate degradation leads to higher TOC readily available, which increases the amount of inhibitory compounds in the pretreated liquid substrate. For TH, this had an increasing linear trend regardless of the substrate's chemical composition. For SE, as the lignin concentration of the substrate increased, the TOC kept increasing, whereas the phenolic compounds reached an optimum point, leading to a different trend than TH. Also, when the relationship between lignin in the solid fraction and inhibitors in the liquid fraction was observed, it showed an increasingly significant trend. In conclusion, the abovementioned results' effect on anaerobic digestion should be evaluated.

Chapter 3 (Study 2) evaluated the effect of TH and SE on the anaerobic digestion of lignocellulosic aquatic weeds. The study revealed that all the three substrates' methane yield was enhanced after the TH and SE pretreatment. *In Eichhornia crassipes* and *Ludwigia grandiflora* the methane yield enhancement from the untreated condition was much higher for both TH and SE pretreatment than *Hydrilla verticillata*. In this study, the T80 value was calculated, which showed that the T80 values for TH pretreated biomass were lower than the SE pretreated biomass. In conclusion, it was observed that TH pretreatment was sufficient to improve the methane yield for a wide range of lignocellulosic biomasses, whereas SE pretreatment helped disrupt the substrate structure with maximum lignin content.

Lastly, in chapter 4, an appropriate pretreatment based on the biomass type was suggested as a general discussion. After TH and SE pretreatment, the methane yield improvement was enhanced for all the substrates used. The value of the lignin polymer in the untreated substrate can help to evaluate the methane yield improvement yield. The methane yield improvement after the TH pretreatment for *E. crassipes*, *H. verticillata*, and *L. grandiflora* was 89.91, 24.44, and 140.72%, respectively. The methane yield improvement after the SE pretreatment for *E. crassipes*, *H. verticillata*, and *L. grandiflora* was 56.83, 11.33, and 216.13 %, respectively. This evaluation of methane yield improvement can be helpful for new studies/research as only the lignin concentration is used for the evaluation. This is the first study which proposes a methane yield improvement estimation model for both TH and SE pretreatment. In conclusion, when comparing the results with previous studies, it was observed that TH pretreatment could increase the methane yield improvement of substrates at a vaster/broader range of temperature, whereas SE pretreatment improved the methane yield at a lower range of temperature. Techniques such as co-digestion were also discussed to stabilise the methane production process for long-term studies.

1. CHAPTER 1 GENERAL INTRODUCTION

1.1. Overgrowth of aquatic weeds

Aquatic weeds (floating, submerged and emergent) play an essential role in freshwater ecosystems by improving water quality and providing habitat for various life forms. However, within a few decades, due to poor agricultural and waste management practices, the gradual increase in nutrients, especially nitrogen and phosphorus, lead to eutrophication. The leading cause of aquatic weeds growth is eutrophication (Kaur et al., 2018). Aquatic weeds have a rapid growth rate and a potential to spread, leading to various problems in water bodies like lakes, dams, and reservoirs (Figure 1-1). For example, India's most common aquatic weeds are *Pistia stratiotes, Alternanthera philoxeroides*, *Eichhornia crassipes* and *Salvinia molesta*. Due to the increase in food production, the irrigation facilities in India are expanding at a higher rate which is blocked by these aquatic weeds. In India, about a million hectares of interior water channels are at risk of invasive aquatic weeds. Wetlands account for approximately 15.3 million hectares of the land, from which 9% of the total wetland area is having problems due to overgrowth of these aquatic weeds and about 2.06 million hectares in the dry season (before the monsoon) (Space applications centre 2011; Kaur et al., 2018). Similarly in China, the eastern part of Taihu Lake is mainly covered with submerged and floating aquatic weeds, where floating aquatic weeds occupy more than half of the dominated area. In some areas during summer and autumn, floating aquatic weeds (*Nymphoides peltata*) occupy more than 70% of the water surface, blocking water aeration and light from the water columns (Zhu et al., 2019). This excessive aquatic weed develops thick mats on the water bodies, causing greater damages like the evaporation of water, interference with fisheries, dam clogging, flooding, migration of native flora and fauna, and water loss due to irrigation disruptions, and enormous environmental and economic implications (Ban et al., 2019). In Europe, 96 species of exotic invasive aquatic weeds like *Elodea canadensis, Vallisneria twistis* and *Elodea nuttallii* have been found in almost 46 countries (Hussner, 2012). Aquatic weed growth in eutrophic waters is that they collect excess nutrients in the spring and then release them when degraded during autumn (van Donk et al., 1993; O'Hare et al., 2018). Exotic aquatic weeds such as *S. molesta, E. crassipes* and *C. caroliniana* have overgrown in South East Queensland, leading to an increase in mosquito standing sites (O'Sullivan et al., 2010).

Many other countries have also studied the problem of aquatic weeds in many water reservoirs, for example, rice fields and drainage (Abbasi et al., 1990). Japan also has problems with aquatic weeds such as in Lake Biwa. Since 1994, aquatic weeds have been over-infested, primarily in the south of Lake Biwa (Haga and Ishikawa, 2011). Previous studies revealed that the overgrowth of aquatic weeds since 1994 was majorly due to severe water shortages in the hot season, leading to a −1.2m drop in water levels and an increase in light penetration (Haga and Ishikawa, 2011). Currently, aquatic weeds cover about 90% of the Southern Basin (Figure 1-2). Overgrowth of these aquatic weeds causes water stagnation, foul smell, fishing problems, ecosystem changes etc (Haga et al., 2006). Furthermore, algae such as *Spirochetes anabaena* often attach themselves to aquatic weeds producing geosmin, which produces an unpleasant stale odour in water (Sugiura et al., 2004). Also, an invasive emergent aquatic weed, *Ludwigia grandiflora*, is primarily aquatic, but its ability to produce both emergent and floating stems allows it to develop broad and thick mats on open water and exposed mud (Okada et al., 2009). It often builds up monospecific stands in favourable aquatic habitats and outcompetes other species (Dandelot et al., 2005). Lake Biwa is an essential water source for more than 10 million humans in the catchment area and its downstream regions. Recently, it has been outlined that submerged and stuck aquatic weeds and no water movement due to the contamination may increase geosmin concentrations in drinking water from Lake Biwa (Sugiura et al., 2004). Therefore, there is a severe need to harvest and utilize the aquatic weeds in the water bodies.

1.2. Harvesting techniques and treatment methods for excessive aquatic weeds

1.2.1. Conventional techniques to control and harvest aquatic weeds

The harvesting of aquatic weeds has been taking place worldwide, where governments are spending considerable costs to eliminate this risk in the aquatic ecosystem. In Japan, the Ministry of the Environment and Shiga Prefecture recently revised the "Plan for Conservation of Lake Water Quality" and "Mother Lake 21 Plan" to promote the harvesting and treatment of aquatic weeds (Koyama, 2016 (PhD Thesis)). Also in China, to restore the lake ecosystem, the lake management institution started an aquatic weed harvest across 196 km^2 beginning in 2013 (Zhu et al., 2019). The different growth habitats of aquatic weeds can add to harvesting difficulties as each type of weed needs different tools and techniques (Little, 1979). Below are conventional and current techniques for harvesting aquatic weeds (Figure 1-3).

1. **Mechanical harvester and dredging:** A mechanical harvester cuts the upper part of the weed bed, and a dredger is a fork-shaped object which pulls the aquatic weeds, including the roots with bottom sediments, by towing with a boat and then carrying them to an appropriate off-loading site (Knight et al., 2014; Ban et al., 2019). Mechanical harvesting leads to higher costs, around 120K yen ha^{-1} year-^{1} (Agric et al., 2012). One of the disadvantages of mechanical methods is the higher costs due to the capital investment (Kaur et al., 2018).
2. **Manual removal:** In areas sparsely infested, weeds can be removed by hand, where people cut the aquatic weeds with heavy knives and hooks (Van Nes et al., 2002). The yearly cost for the control is about 70K yen ha^{-1}year^{-1}. Manual removal is impractical when area of infestation is large.
3. **Increasing flow velocity:** Hydro-venturi or venturi canal has been used to reduce biomass and aquatic weeds. The increase in the flow of water leads to the increase in the turbidity of the water leading to the removal of aquatic weeds with the roots (Brundu, 2017; Hussner et al., 2017).
4. **Chemical control:** US water bodies annually use various types of aquatic herbicides. Aquatic herbicides fall into two main groups: Systemic herbicides are fed and transported through the plant's vascular system, killing the entire plant. Contact herbicides kill the parts of the plant that come into contact with chemicals, but the roots survive, and the weed can re-grow. Broad-spectrum herbicides put to death the maximum of the plants they come across. The disadvantages

of chemical control are that it can severely harm non-target species (Knight et al., 2014; Kaur et al., 2018).

5. **Biological control:** Biological control uses living organisms (such as fungi or herbivores) to control undesirable forms. Biological controls can be self-supporting as they may skip the winter season and then eat its host the following year. It is relatively slow and not effective in a large infested area.

Even after applying these techniques to remove aquatic weeds, it was reported that regeneration could occur (Haga and Ishikawa, 2011). Removing these aquatic weeds requires a lot of labour and cost, so it is essential to utilize these harvested aquatic weeds by efficient methods.

1.2.2. Treatment methods proposed for the harvested aquatic weeds

After harvesting aquatic weeds, they are kept on the off-loading sites for some days, transferred or utilized by different treatment methods. Several treatment methods are proposed in previous works of literature for the harvested aquatic weeds (Table 1-1). Some treatment methods are incineration, composting, bioethanol fermentation and anaerobic digestion. These are explained below in detail:

1. **Incineration:** The weed can grow just from the slightest remaining buds, making eradication impossible. The only way to eradicate everything is by incinerating it. Incineration is a simple technique in which aquatic weeds are burned without any prior treatment. The high-water content (75-95%-wwt) of aquatic weeds makes the incineration cost-intensive and has adverse environmental effects (Zupančič et al., 2022).
2. **Composting:** Although composting is considered positive for nutrient intake due to its low processing cost, it cannot compete with chemical fertilizers, majorly because of the difference in longer time needed for compost production and the rate of the growth of aquatic weeds (Koyama, 2016 (PhD Thesis)). In addition, aquatic weeds often contain contaminants like fish hooks, which

could be hazardous to farmers who can damage or contaminate crops when using large plant compost. For the same reason, aquatic weeds in animal feed use are limited.

3. **Fermentation:** Fermentation has recently been tested to produce renewable biofuels from aquatic weeds. Bioethanol is advantageous for transportation. However, bioethanol fermentation of lignocellulosic biomass is known to have the major drawback of the high cost of enzyme addition. Unlike bioethanol fermentation of any lignocellulosic biomass, cellulose must be converted to easily degradable sugars, such as xylose, before bioethanol fermentation (Taherzadeh and Karimi, 2008). Bioethanol fermentation was performed using *Eichhornia crassipes*, *Lemna minor* and *Azolla microphylla* and reported that cellulase was required for saccharification (Kaur et al., 2019).
4. **Anaerobic digestion (AD):** AD is a bio-chemical metabolic process in which the biomass's rigid/hardly degradable components are converted to methane (CH_4) and carbon dioxide (CO_2) gas as the major end products (Khalid et al., 2011) (Figure 1-4). Due to the wide range of AD microorganisms involved in fermentation, most organic polymers can be degraded. Since AD does not require expensive enzymes/yeast, the process flow is more straightforward, and the processing cost is lower than bioethanol fermentation. AD has been used commercially to treat various biological wastes such as wastewater, agricultural waste, etc. (Sawatdeenarunat et al., 2015).

1.3. AD for lignocellulose aquatic weeds and enhancement of methane recovery

AD allows the bioenergy recovery from biomass with high water content. Due to this, the application of AD has been popular for effectively treating aquatic weeds. Applying AD to treating aquatic weeds can be very advantageous in energy retrieval and recycling nutrients. Solid and/or liquid residues from AD can be applied as fertilizers due to high nutrient concentration (Liedl et al., 2006; Uysal et al., 2010). The AD of various floating, submerged, and emergent aquatic weeds is summarised in Table 1-2 using previous works. However, it has been specified that CH_4 recovery from these wide varieties of biomasses is primarily restricted by the lignin content of the biomass (Gunaseelan, 2007; Triolo et al., 2011; Frigon et al., 2012).

Lignocellulosic biomass has been considered prospective feedstocks for biomethane recovery in recent years. The lignocellulose matrix comprises cellulose, hemicellulose and lignin (Figure 1-5). Dependent on the type of biomass, cellulose values around 40–50 %, hemicellulose 20–40 %, and lignin 10–40 % of the biomass total solid (McKendry, 2002). Cellulose consists of a linear chain of D-glucose. Hydrogen bonds cover the cellulose chains in plant biomass and form "cellulose microfibril". The major part of cellulose polymer is crystalline although it has crystalline and amorphous regions (Chum et al., 1985). Both types of cellulose have high methane recovery rates, but the degradation rate of high-crystalline cellulose is lower than that of the amorphous one (Jeihanipour et al., 2011). Hemicellulose is a polysaccharide composed of different biopolymers such as pentoses, hexoses and sugar acids. Unlike cellulose, hemicellulose has an amorphous structure that is readily hydrolyzed (Sperry and Forest, 1985). Therefore, due to their easily degradable properties, cellulose and hemicellulose are considered the major carbon source for methane production by AD.

On the other hand, lignin is the most difficult of the three polymers to degrade among the lignocellulose components. The main role of lignin is to provide structural support to plants and protect cellulose and hemicellulose against microbial/enzymatic attack by coating the polysaccharides. Lignin composition significantly varies with plant types and/or tissues (Hedges and Mann, 1979; del Río et al., 2007). Lignin is an amorphous polyphenol that consists of three different phenylpropane units forming a highly complex three-dimensional structure; guaiacyl (G) lignin, syringyl (S) lignin, and *p*-hydroxyphenyl (H) lignin. Generally, hardwoods are characterized by the dominance of S and G lignin, while softwoods contain relatively few S lignin. In addition to these, lignin in herbaceous plant and/or non-woody portion of softwood and hardwood contains hydroxycinnamic acids (ferulic acid and *p*-coumaric acid), which has ester bonds and are cross-linked to polysaccharides and lignin polymer (Sun et al., 1997; Buranov and Mazza, 2008; Sonoda et al., 2010). del Río et al., (2007) confirmed the lignin composition of five non-woody plants and reported that the lignin composition greatly varied with species. Rabamanolontsoa and Saka, 2012; Koyama, 2016 (PhD thesis)) also confirmed that submerged aquatic weeds contain these four types of lignin, although the derivatization method and lignin classification is different. AD microorganisms cannot degrade the lignin (Benner et al., 1984). Therefore, the lignocellulosic component of the substrate's unique nature significantly impacts biodegradability and methane recovery. Therefore, before performing AD, a suitable pretreatment is required to make the lignocellulose structure more accessible for producing higher methane.

1.4. Pretreatment and their characteristics

1.4.1. Various types of pretreatments for the degradation of lignocellulosic biomass

Substrates with high lignin content or lower methane recovery are not suitable for AD. The structural and compositional properties of lignocellulosic biomass include cellulose's crystallinity, degree of polymerization, amount and type of lignin content, lignin composition, and hemicellulose conversion to acidic regulated by various pretreatments types (Biswas et al., 2015). To improve feedstock degradability and microbial metabolic rate, pretreatments have widely been attempted on various plant biomass.

An ideal pretreatment offers high sugars recovery, no chemical use, leads to a minimum amount of degradation products or inhibitors, is cost-effective for large scale, and is valid for a wide type of biomass regardless of the inherent nature of biomass. Generally, these common pretreatments are described as mechanical, chemical, thermal and biological pretreatment. Each pretreatment methods work uniquely to alter/break the rigid structure of the lignocellulose biomass (Figure 1-6). Thus, a varied range of by-products can be obtained from the individual pretreatment method, as each pretreatment has its merits and demerits.

Conversion of biomass is necessary to change the complex biomass into easily degradable sugars and enzymatic hydrolysis to break the hemicellulose and lignin polymers leading to higher cellulose conversion (Sun et al., 2016). Various research has been performed to study different pretreatment methods, including mechanical, chemical (Pu et al., 2013; Kumar et al., 2019) , thermal (Bals et al., 2011) , and biological (Wan and Li, 2012) processes in these years. Some pretreatments are already used for industrial applications; still, some problems like high energy demand or toxic waste production, there is an urgent requirement for environment-friendly methods/research to these challenges (Capolupo and Faraco, 2016; Hassan et al., 2018).

1.4.2. Environmentally friendly approaches for pretreatment of lignocellulosic biomass

The idea of environmentally friendly or green pretreatments is increasing in popularity as an eco-friendly way to the challenge of developing a feasible biorefinery concept. This goal aims to develop a technology that uses raw materials more efficiently, eliminates waste and avoids toxic and hazardous chemicals. Some of the green or environment-friendly methods applied for the pretreatment of lignocellulosic biomass are summarised (Table 1-3). Although these methods are green, some major issues still exist about high production costs and a lack of commercial equipment for industrial scaling. However, more research in this area can lead to the adoption these pretreatments, with decreases in initial capital investment and increasing the scale of operation, which may lead to the popularity of the pretreatment of lignocellulosic biomass (Hassan et al., 2018).

1.4.3. Thermal Hydrolysis (TH) and Steam explosion (SE) pretreatment

Among the pretreatment methods mentioned above, thermal pretreatments can be considered the best choice as a green, competitive and easily scalable pretreatment method for breaking lignocellulosic bonds for biomass such as aquatic weeds (Carrere et al., 2016). TH and SE pretreatment are industrial prospects for lignocellulosic biogas and bioethanol production (Oliveira et al., 2013; Jiang et al., 2016). TH and SE use a combination of chemical and physical processes to degrade the biomass (Cara et al., 2008; López-Linares et al., 2015). This process can only be used with water, pressure and heat and does not use harmful toxic chemicals. Therefore, during TH and SE, the substrate is fed in a vessel and steam with a high temperature and high pressure is supplied for a few seconds to minutes. After the retention time has elapsed, the steam is released suddenly for the SE pretreatment and gradually for the TH pretreatment, and the biomass is quickly brought to room temperature (Hendriks and Zeeman, 2009) (Figure 1-7). Due to the high temperatures and pressures applied, degradation of hemicellulose and lignin transformation occur, enhancing the overall hydrolysis and increasing substrate degradation. TH and SE pretreatment lead to similar autohydrolysis mechanism; the main difference between the two is the pressure release. This decompression forces the substrate to expand rapidly and the fibres and bundles to separate to create a solid fraction with a larger accessible surface area (Ahvazi et al., 2007; Martin-Sampedro et al., 2014), improving the effectiveness of subsequent treatments.

In TH and SE pretreatment, hydronium ions from water act as catalysts to hydrolyze cellulose and hemicellulose to produce acetic acid and other organic acids at high temperatures. These acids help digest biomasses by breaking bonds between lignocellulose polymers (cellulose, hemicellulose and lignin). This reaction is known as autohydrolysis (auto-catalyzed phreatic eruption) (Li et al., 2007). The more severe the process conditions (higher temperature and longer time), the more cellulose and hemicellulose are decomposed, resulting in the production of organic acids that can decompose biomass. This procedure can effectively remove cellulose polymers' fibres and destroy the cell wall's complex structure (Wang et al., 2009). However, if the process is too harsh, the resulting compounds can be overly degraded, leading to various inhibitory compounds (Xu et al., 2011; Wang and Chen, 2014). For lignin, phreatic eruptions can change their structure by depolymerization / repolymerization reactions catalyzed during the acidic phase (Li et al., 2007). This can interfere with the solubilization of lignin as new non-hydrolyzable C-C bonds are formed by severe polymerization reactions. In addition, fractional solubilization of cellulose leads to the production of unwanted degradation products such as dissolved lignin, phenolic compounds, and hydroxymethylfurfural (HMF), which affect the yield of extractable cellulose nanoparticles (Cara et al., 2008).

The pretreatment intensity is mainly stated as a severity factor (SF), used for liquid hot water and steam explosion (Hendriks and Zeeman, 2009).

$$SF = \log\left[t \cdot \exp\left(\frac{T\text{-}100}{14.75}\right)\right]$$

1.4.4. De-coupling/Disintegration of thermal hydrolysis from steam explosion pretreatment

TH and SE pretreatment was initially introduced to treat aspen woodchips for the production of sugars and improve the digestibility of lignocellulosic and non-lignocellulosic biomass due to its intensity and explosion capability (Duque et al., 2016). Also, previous studies revealed that SE had been intensively applied to the lignocellulosic aquatic weeds prior to AD (Bhatia et al., 2020). The trend observed when SE was used in previous studies maximized the substrate degradation. Various studies mention that the concentration of inhibitors can increase with the increase in substrate degradation, which can negatively affect the AD process (Duque et al., 2016). However, as mentioned above, both TH and SE possess a similar mechanism with a difference during the pressure release. The question is whether TH pretreatment will have the same effect on lignocellulosic aquatic weeds as SE pretreatment.

TH and SE pretreatment contributes to the efficiency of the process, but different researchers claim different views (Yu et al., 2012; Pielhop et al., 2016). Brownell et al., 1986 pointed out in 1986 that the importance of explosion in SE pretreatment was minimal when using high temperatures (240 ° C). In this condition, the aspen wood chips were completely disrupted after the SE pretreatment but remained intact during the TH pretreatment. However, there was no change in the yield of reducing sugars. These results can be affected by the high temperatures at which the SE pretreatment was conducted. On the other hand, another study by E.A. DeLong, (1983); Wang et al., (2014) comparing TH and SE showed a significant difference in digestibility of pretreated substrate, and physical force played a role in pretreatment. Therefore, the contribution of SE to the improvement of enzyme digestibility does not seem to be negligible.

SE pretreatment is considered as a dominant or popular pretreatment because of its intensity and explosion ability whereas TH is not given much attention in previous studies. Therefore, this study will de-couple TH from SE pretreatment. Both the pretreatments can be used on the same machine under the same conditions. This is the first study that will compare the effects of TH and SE on the AD of different lignocellulosic aquatic weeds, although few studies (mainly hard and softwoods) have tried comparing these pretreatments with different objectives. These studies are summarized in Table 1-4 with their objectives. The study on wood chips (*E. globulus*) mentioned by Martin-Sampedro et al., (2014), aimed to compare the effects of TH and SE on enzymatic hydrolysis. Both TH and SE improved the solid and liquid fraction degradation, but SE led to higher hemicellulose reduction, which may inhibit the subsequent process. A study done by Regidor Alfageme, 2019 (Master thesis) used *E. crassipes* as a substrate and evaluated the methane yield by both the pretreatments. The limitation of the study was the usage of three temperature conditions and a single type of aquatic weed, making it difficult to conclude the nature of the mechanism. Therefore, the gap in the mechanism of these pretreatments will be studied further for clarification.

1.5. Objective of this study

This PhD thesis aims to reveal the appropriate pretreatment between TH and SE upon the AD of different lignocellulosic aquatic weeds. The specific objectives are: Chapter 2: To clarify the physical and chemical differences of lignocellulosic aquatic weeds pretreated by TH and SE pretreatment. Chapter 3: To evaluate the effect of TH and SE on the anaerobic digestion of lignocellulosic aquatic weeds. Therefore, Chapters 2 and 3 will compare the TH and SE mechanisms, which have not been studied before. After that, a proposal of an appropriate pretreatment based on biomass type in chapter 4 will be discussed.

1.6. Tables

Table 1-1. Identification and treatment type of aquatic weeds

	Treatment type	Area, Country	Reference
Floating Aquatic weed			
Pistia	Composting	India	Gusain et al., 2018
E. crassipes	Incineration	-	Rahman et al., 2011
Lemna spp	Bioethanol fermentation	India	Kaur et al., 2019
E. crassipes	Anaerobic digestion		Kaur et al., 2019
Submerged Aquatic weed			
H. verticillata	Incineration	USA	Charudattan et al.,
H. verticillata	Composting	-	Jain et al., 2018
P. maackianus	Anaerobic digestion	Lake Biwa, Japan	Koyama et al., 2015
Emergent aquatic weed			
L. grandiflora	Anaerobic digestion	Lake Biwa, Japan	Pranshu et al., 2020
Ipomoea carnea	Composting	-	Shyam et al., 2022
Ipomoea aquatica	Bioethanol fermentation	India	Sunil et al., 2015

Table 1-2. CH_4 yields from floating, emergent and submerged aquatic weeds. All data was obtained by batch anaerobic digestion

Aquatic weeds	CH_4 yield (mL g-VS$^{-1)}$	CH_4 yield (mL g-TS^{-1})	Country	Literature
Floating aquatic weed				
Azolla pinnata	132	107	India	Abassi et al. 1990
Cabomba caroliniana	173	-	Australia	O’Sullivan et al. 2010
Ceratopteris sp.	204	164	India	Abassi et al. 1990
Eichhornia crassipes	209	-	India	Chanakya et al. 1993
	140 - 180	120 - 154	USA	Moorhead and Nordstedt 1993
	190	-	Australia	O’Sullivan et al. 2010
	182 - 193	-	India	Patal et al. 1993
Salvinia molesta	242	204	India	Abassi et al. 1990
Submerged aquatic weed				
Elodea nuttallii	333	-	Germany	Muñoz Escobar et al. 2011
Hydrilla verticillata	81	66	India	Abassi et al. 1990
Elodea nuttallii	361	299	Japan	Koyama et al.,2014
Potamogeton malaianus	278	234	Japan	Koyama et al., 2014
Egeria densa	287	234	Japan	Koyama et al., 2014
Potamogeton maackianus	161	136	Japan	Koyama et al., 2014
Emergent aquatic weed				
Cyperus sp.	38	30	India	Abassi et al. 1990
Scirpas sp.	66	53	India	Abassi et al. 1990
Utricularia reticulata	132	108	India	Abassi et al. 1990
Ludwigia grandiflora	86	-	Japan	Pranshu et al., 2019

Table 1-3. Green methods currently being used for pretreatment of lignocellulosic biomass.

Pretreatment methods	Effect	Advantage	Disadvantage	Reference
Supercritical fluids	Cellulose crystallinity reduction and lignin removal	Green solvent is used, it does not cause degradation of sugars, method is suitable for mobile biomass processor	Total utilities costs are high	Daza Serna et al. (2016)
Deep eutectic solvents	Lignin removal and hemicellulose fractionation	Green solvent, biodegradable and biocompatible	Poor Stability under higher pretreatment temperatures,	Zhang et al. (2016b)
Thermal Hydrolysis and Steam explosion	Lignin softening, particle size reduction	Low capital investment, moderate energy requirements and low environmental impacts	It is much less effective for softwood	Pielhop et al. (2016)
Microbes	Lignin and hemicellulose degradation	Environment friendly, selective degradation of lignin and hemicelluloses	Very long pretreatment time (several weeks) due to slow yield	Sun et al. (2016)

Table 1-4. TH and SE pretreatment used on different biomasses

Substrate	Objective	Result	Reference
Hardwood chips (*Eucalyptus globulus*)	Enzymatic hydrolysis	Hydrolysis yields from **TH** were higher	Martin et al., 2014
Aspen hardwood (*Populus tremula*)	Enzymatic digestibility	**SE** enhanced carbohydrates with better enzymatic digestibility	Li et al., 2005
Spruce wood chips (Softwood)	Cellulose digestibility	**SE** enhance its enzymatic cellulose digestibility	Pielhop et al., 2016
Water hyacinth	Methane efficiency	Methane yield from **SE** was higher	Regidor, 2019

1.7. Figures

Fig 1-1. Overgrowth of aquatic weeds around the world

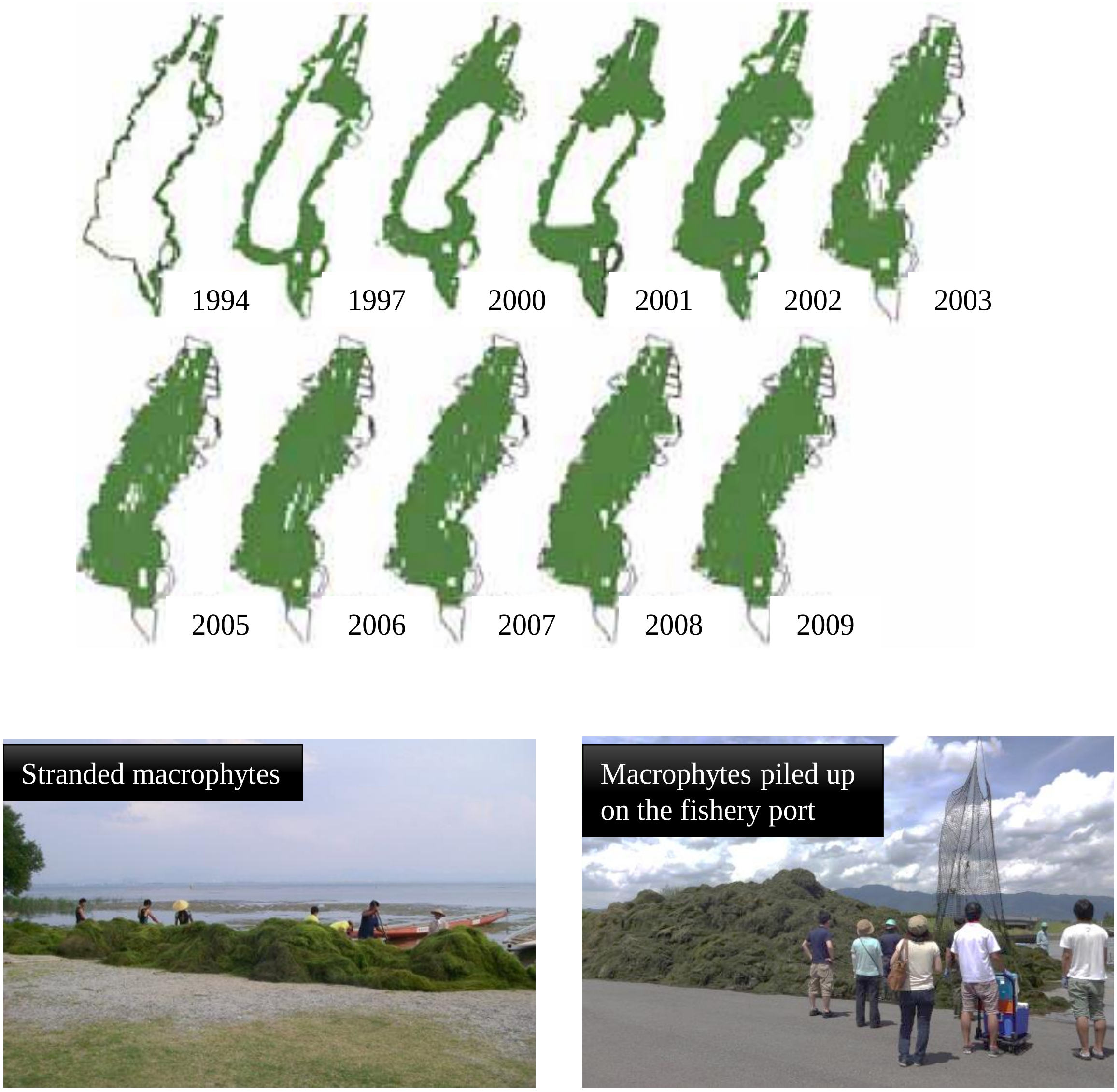


Fig 1-2. Coverage of submerged aquatic weeds in southern basin of Lake Biwa (Haga et al. 2010)

Fig 1-3. Conventional techniques used for the harvesting of aquatic weeds

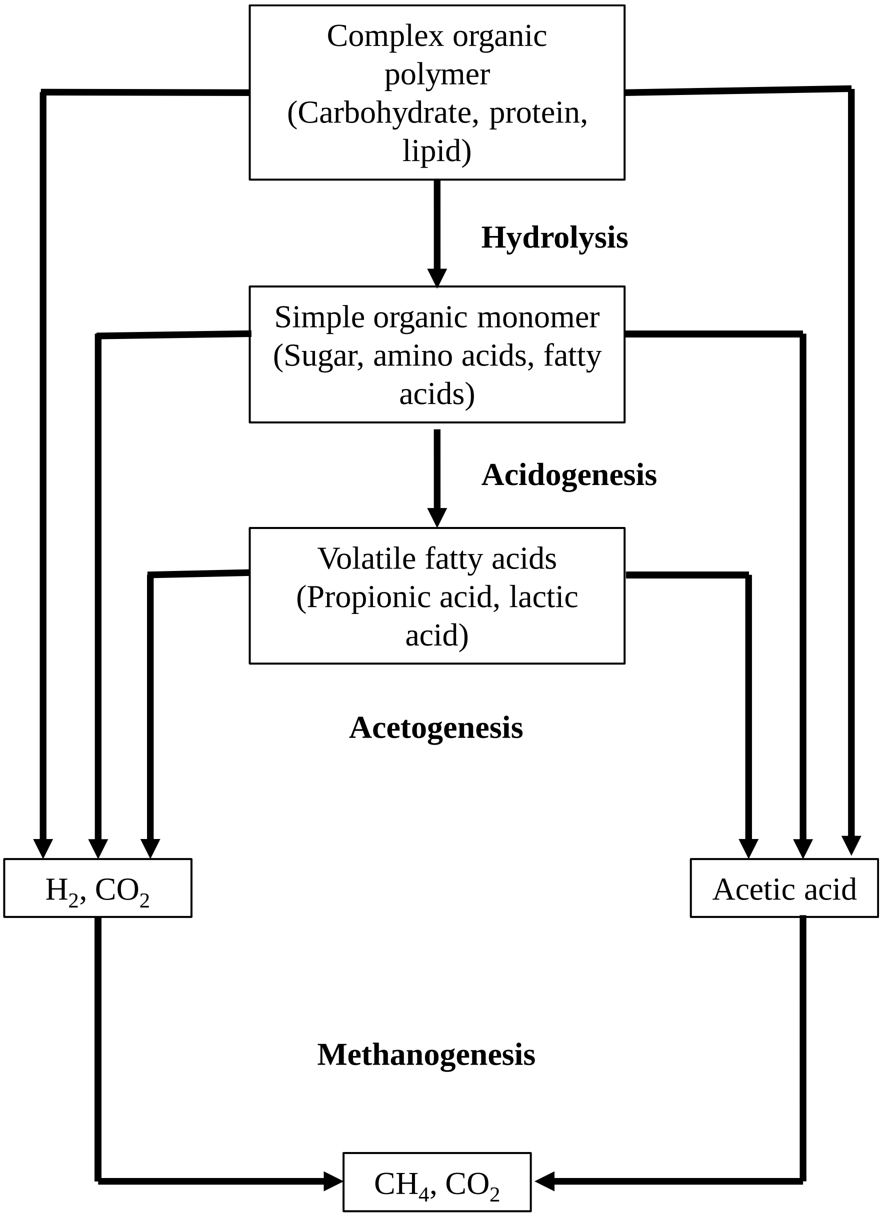


Fig 1-4. The degradation of organics during anaerobic digestion (McCarty and Smith 1986 modified).

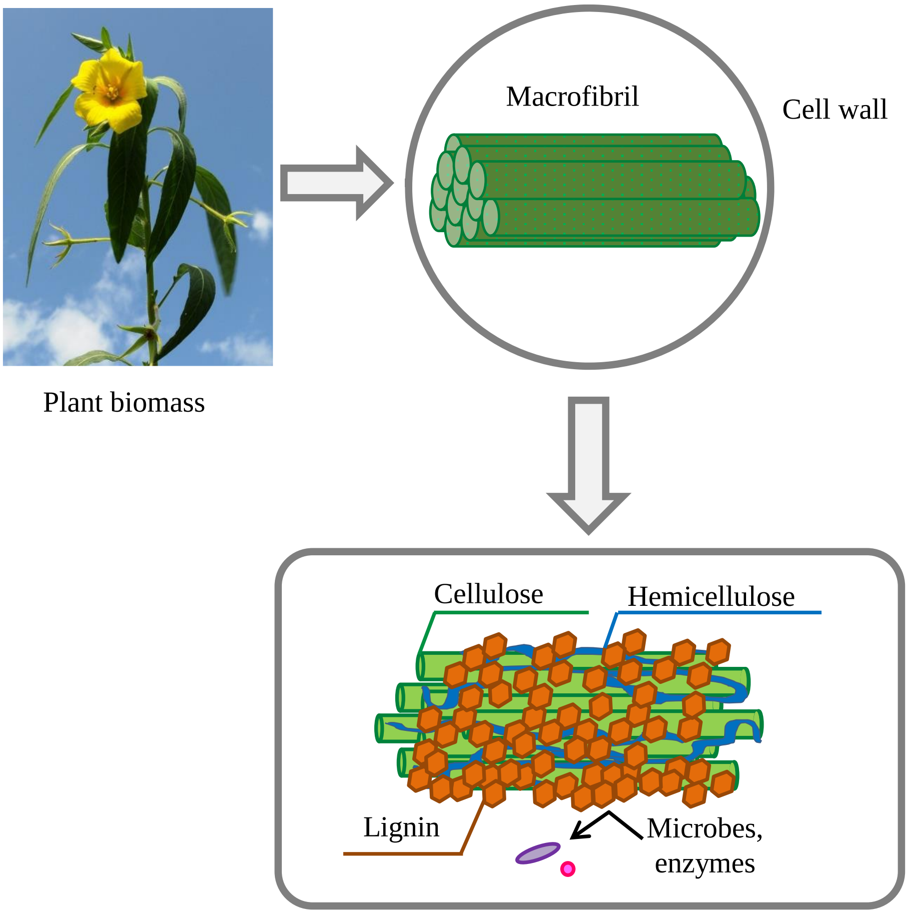


Fig 1-5. Lignocellulose structure of plant cell

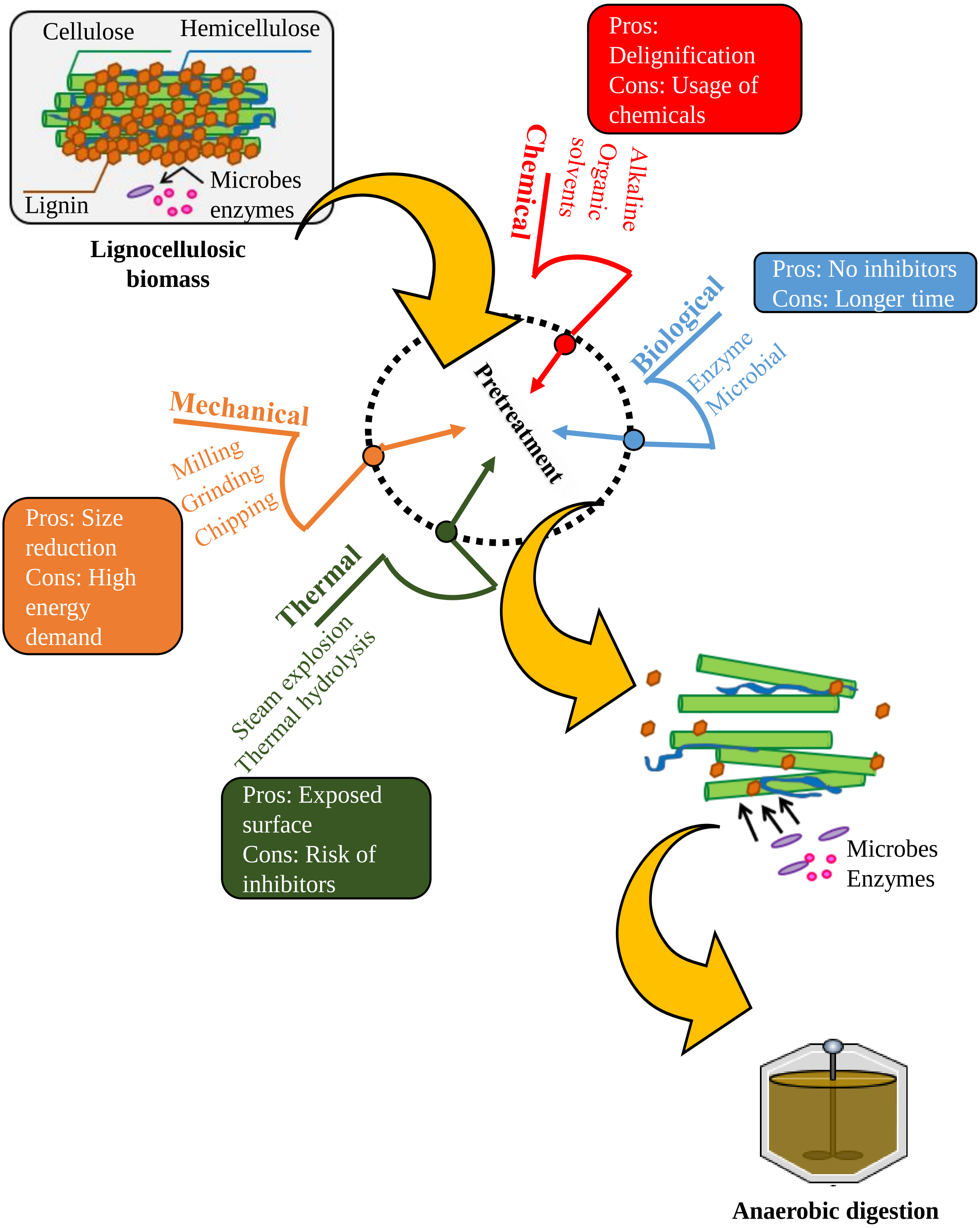


Fig 1-6. Various pretreatments have being applied to different aquatic weeds in previous studies

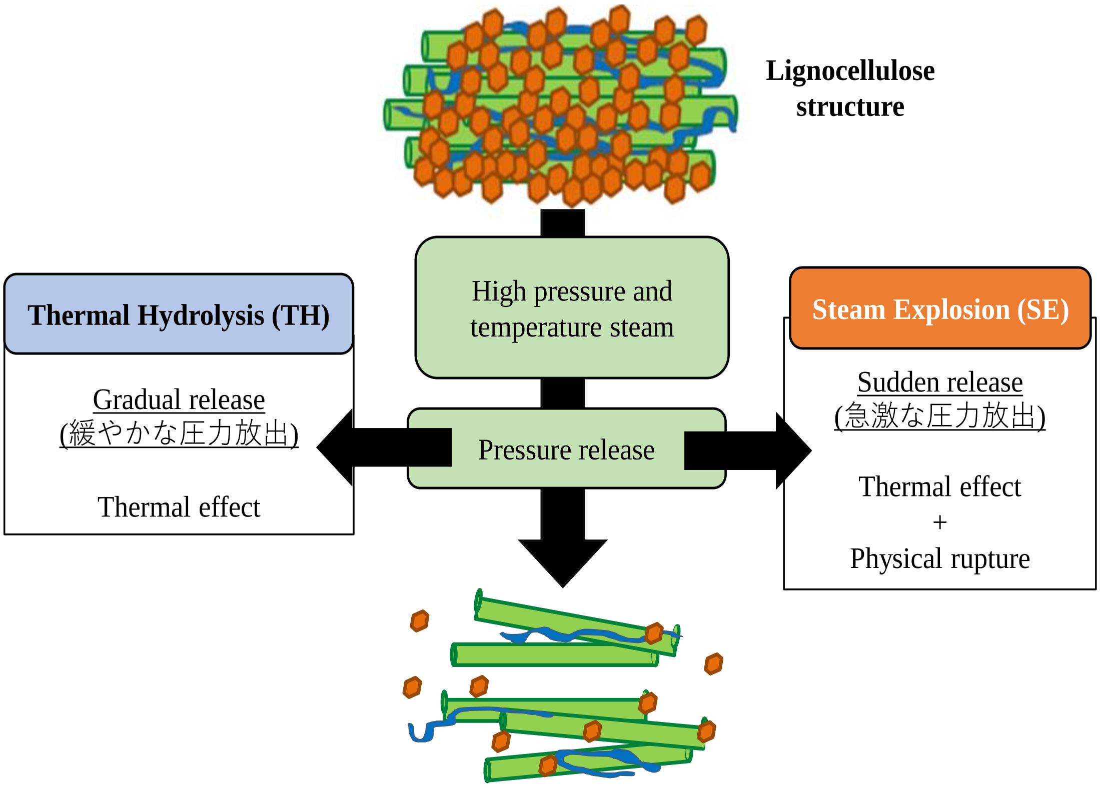


Fig 1-7. Schematic diagram of Thermal Hydrolysis and Steam explosion pretreatment

2. CHAPTER 2 PHYSICAL AND CHEMICAL DIFFERENCE OF SUBSTRATES PRETREATED BY THERMAL HYDROLYSIS AND STEAM EXPLOSION PRETREATMENT

2.1. Introduction

2.1.1. Mechanism of thermal hydrolysis and steam explosion (revealing the difference between two pretreatments)

TH pretreatment consists of a high temperature; pressure cooking stage, whereas SE pretreatment consists of a high temperature; pressure cooking followed by an explosion stage. During the high-temperature cooking stage for both TH and SE pretreatment, cellulose and hemicellulose are hydrolysed by acetic acid derived from the acetyl group and other acids. At the same time, the lignin is degraded, and the binding to cellulose is reduced. However, during the explosion stage for SE pretreatment, the structures of the fibrous material are disrupted by the combined action of explosion and surface expansion, resulting in a change in the physical structure and redistribution of the biomass, thereby altering the biomass both physically and chemically (Yu et al., 2022).

Based on the rules of heat and water transfer, previous studies explained the TH mechanism into three stages and SE pretreatment into four stages, namely steam displacement, steam penetration, steam cooking and steam explosion (only for SE pretreatment), respectively (Fig. 2-1). In the steam displacement stage, liquid water enters the biomass by adsorption and diffusion, softening the fibres. Then, the temperature and pressure increase and the aeration of the steam allows it to penetrate deeper into the biomass at the steam penetration stage, where most of the steam is consumed. This step is followed by a steam cooking stage when the reactor temperature reaches the desired value and hydrolysis of the biomass occurs. For the final explosion stage for SE pretreatment, superheated water abruptly expands the steam volume and biomass is discharged to atmospheric pressure (Yu et al., 2022).

2.1.2. Comparison of characteristics of thermal hydrolysis and steam explosion pretreatment

After the TH or SE pretreatment, condensed water and insoluble solids are recovered using a pretreated material called slurry. The slurry consists of both solid fraction and liquid fraction. The liquid fraction carries easily degradable sugars, mostly hemicellulosic sugars, and relatively all the degradation products/ inhibitors produced during the pretreatment leading to a change in the pH. The amounts of lignocellulose polymers (cellulose, hemicellulose, and lignin) are recovered in the remaining solid fraction. The composition of the liquid and solid fraction depends upon the treatment temperature and retention time during the pretreatment. A study by Barakat et al., (2012) explained that liquid fraction after pretreatment contains the inhibitors released during both the pretreatments which can affect the subsequent processes.

Previous studies reported that during thermal pretreatments at temperatures above the lignin glass transition temperature (180 °C), lignin could coalesce and migrate into the bulk liquid phase. Upon cooling, lignin redeposition on the biomass surface can occur (re-condensed lignin) in the form of droplets, which may negatively impact cellulose hydrolysis (Li et al., 2016). Table 1-4 shows the studies which compare the TH and SE pretreatment. In a study done by Pielhop et al., (2016) when TH and SE pretreated spruce wood chips (softwood), SEM analysis observed a different trend for both the pretreatments. It was seen that the TH pretreated biomass surface was covered with many condensed-like structures or droplets, whereas in SE pretreated biomass, no condensed droplet-like structures were visible, but the exploded biomass surface showed many holes. It, therefore, seems possible that in TH, such structures may have formed upon cooling/condensation due to no explosion, whereas during the explosion in SE, it may not be able to precipitate on the biomass leading to holes in the biomass. In the liquid fractions, the value for total sugar yield was significantly higher for the SE pretreatment than for TH pretreatment (Pielhop et al., 2016). Similarly, a study by (Martin-Sampedro et al., 2014) using *E. globulus* as a substrate shows that the values of inhibitors such as HMF and furfural in the liquid fraction were higher during SE pretreatment.

Therefore, separating the slurry into solid and liquid fractions is essential to check the different effects on biomass solubilisation. The impact of TH and SE on the digestibility of different lignocellulosic feedstock will likely differ due to the inherited chemical composition. Studying the mechanism of TH and SE on the physical and chemical characterisation of different types of aquatic weeds under the same conditions (temperature, time and severity factor) will give more insights into enhancing the effect of both the pretreatments possessing a much similar mechanism.

2.2. Materials and Methods

2.2.1. Substrate selection

For the substrate, three different invasive aquatic weeds were used according to their growing type, as shown in Fig 2-2. Also, a relationship between the untreated methane yield and the lignin content was observed in Fig 2-2. *Eichhornia crassipes* (floating type), *Hydrilla verticillata* (submerged type) and *Ludwigia grandiflora* (emergent type). *E. crassipes* was harvested from a pond in Saitama in December 2019. *H. verticillata* was harvested from the southern of Lake Biwa in September 2020. *L. grandiflora* was harvested from the southern Lake Biwa in July 2018 (for SE pretreatment) and September 2020 (for TH pretreatment) as shown in Fig 2-3. With the help of a milling machine, the substrates were cut down to 3–5 cm. The substrate was stored at −20 °C to be later used in the experiment.

2.2.2. Thermal hydrolysis and steam explosion pretreatment

TH and SE pretreatment were conducted with SE equipment from Nitou Kouatsu Co. Ltd in Soka University, Japan. The reactor consists of a 3-L reactor, electric heater and a flash tank for the substrate collection after the pretreatment (Fig 2-4). TH and SE pretreatments were performed for each substrate under eight conditions (Table 2-1). Pretreatments were performed at temperatures of 150, 165, 180 and 210 °C with two retention times of 10 and 30 min. The severity/intensity of TH and SE is defined through the severity factor (SF) used to evaluate the dual effect of temperature and time. This factor can be seen from Eq. as follow:

$$\mathrm{SF} = \log\left[t \cdot \exp\left(\frac{T\text{-}100}{14.75}\right)\right]$$

Where t refers to the retention time in minutes, T is the heating temperature in degrees centigrade, and the value 14.75 is the activation energy under first-order process kinetics, following the Arrhenius law.

Each milled aquatic weed (consisting of 500 g-wet weight (g-wwt)) and Milli-Q water (750 mL) was added to the reactor boiler to obtain a solid to liquid ratio of 1:1.5 (w/v). The temperature was set on the temperature controller, and steam was supplied from the electric steam boiler at the top of the reactor and heated until the desired temperature for pretreatment was reached. At the end of the retention time for SE pretreatment, the pressure was suddenly released. The pressure was gradually reduced for TH Pretreatment, avoiding the explosion effect (Fig 2-5). The pretreated slurry was transferred into a flash tank. The slurry was thoroughly mixed, weighed, and separated into solid and liquid fractions using a sieve with a mesh opening of 500 μm and stored at −20 °C for future use.

2.2.3. Analytical parameters

For solid and liquid fractions: Total solids (TS) and volatile solids (VS), pH

For solid fraction: Scanning electron microscopy (SEM), Fourier transform infrared spectrophotometry (FTIR), Carbon, nitrogen, protein, and Lignocellulose composition were measured.

For liquid fraction: Chemical oxygen demand (SCOD), Total organic carbon (TOC), Dissolved organic carbon (DOC), Dissolved lignin and Phenolic compounds.

TS and VS in the solid and liquid fractions were measured following the methods from American Public Health Association (1988). The pH was measured using a pH meter (SevenCompact™ pH/Ion meter, S220, Mettler Toledo, Japan. The COD of the liquid fraction was determined colorimetrically (DR-3900, Hach, USA) following dichromate digestion. The TOC and DOC were measured using a total organic carbon analyzer (TOC-L CPH/CPN, Shimadzu, Japan). Carbon and nitrogen contents were measured using a CHN analyser (2400 CHNS/O Series II System, Perkin Elmer). The detergent method (Jančík et al., 2008) and a fibre analyser (ANKOM Technology, A-200, USA) measured the lignocellulose components.

During TH and SE pretreatment, lignin is converted into phenols which can be an inhibitory compound. Therefore, the Folin-Ciocalteau method was used to measure the phenolic compounds by conducting triplicates tests to account for errors (Singleton et al., 1999). Dissolved lignin concentration in the liquid fraction was measured following the same method described in, Koyama et al., (2017) which involves acid precipitation of the dissolved humic fraction and dissolved lignin (both are accounted into the lignin fraction) by measuring SCOD.

For FTIR spectroscopic analysis, the powdered dry samples of each substrate were mixed with KBr (1:200 w/w) and pelletised (KBr Press Model M − 15). Two mg of sample was homogenised with 200 mg of KBr to prepare the translucent pellets, which were then used to obtain the spectra. The absorbance spectrum of 400–4000 cm^{-1} was obtained with a resolution of 2 cm^{-1} and 45 scans (IR Affinity- 1S; Shimadzu, Japan).

For SEM, the powdered biomass samples were sublimated in a vacuum chamber attached to a freeze-drying system (Freeze Dryer FD-6510, SUN Technologies). The samples were then attached to double-sided carbon tape, which was pasted onto a carbon stub. In this study, an osmium coater (HPC-1SW, VACUUM DEVICE, Ibaraki, Japan) was used to make the samples conductive. The cross-sections of samples were observed under a low-vacuum scanning electron microscope (JSM-5600, JEOL, Tokyo, Japan) and/or afield-emission electron microscope (JSM-7500F, JEOL).

2.2.4. Calculations

After the SE pretreatment, the slurry was separated into solid and liquid fractions. Eq. calculated the liquid/solid (L/S) ratio:

$$\text{L/S ratio} = \frac{\text{Liquid fraction (g-wwt)}}{\text{Solid fraction (g-wwt)}}$$

The amount of pretreated slurry obtained after the pretreatment is lower than the total amount of substrate and milli-Q water added because of unavoidable losses during the TH and SE pretreatment. The recovery rate defines the amount of pretreated slurry obtained from the substrate input during SE, which Eq calculated:

$$\text{Recovery rate (\%)} = \frac{[\text{Pre-treated slurry (Solid and liquid fraction)(g-wwt)}]}{1250\ \text{(g-wwt)}} \times 100$$

1250 was the total amount of substrate (500 g-wwt) and Milli-Q water (750 g-wwt) added to the SE reactor.

2.3. Results and Discussions

2.3.1. Mass recovery of the pretreated biomass

The amount of pretreated solid and liquid fraction collected from the slurry was discussed in Table 2-2, 2-3 and 2-4. Table 2-2, 2-3 and 2-4 show the recovery rate for the three substrates used in this study. The recovery rate after TH and SE pretreatment for all the substrates was between 73 to 94% and was not affected by the pretreatment type or its severity. The minimum losses during the pretreatments for all the substrates are defined as the formation of gaseous compounds (volatilisation) during the pretreatment and the residue's adhesion to the pipeline walls, splashing and washing of the reactor, as also discussed in previous studies (Turn et al., 1998; Yang et al., 2004; Wang et al., 2009). Due to the explosion effect in SE pretreatment, it is more challenging to collect the substrate, whereas in TH pretreatment due to no explosion it was relatively easier to collect the pretreated substrate. The reactor used in this study possesses a mechanism in which the substrate is collected with labour work. In contrast, a study by Bhatia et al., (2021) used an industrial-type SE reactor, in which the solid and liquid are collected separately, making it an easier way for L/S separation and collection of the pretreated samples with losses approaching zero and automatic separation of solid and liquid fraction.

Due to the pressure build-up during the TH and SE pretreatment, the formation of gaseous compounds during the pretreatments takes place. Some random conditions were analysed for both pretreatments to check the type of gases formed during the pretreatments. It was observed that only air (comprising of N_2, O_2 and CO_2) was detected for all the conditions selected, with no pattern or trend observed.

The trend observed for solid and liquid fractions is shown in Table 2-2, 2-3 and 2-4 and Fig 2-6. From these Tables and Figures, there was minimal difference between TH and SE pretreatment for *E. crassipes* and *H. verticillata*. The solid fraction for TH pretreated *E. crassipes* ranged from 336 g-wwt to 200 g-wwt and 340 g-wwt to 216 g-wwt for SE pretreated showing a minimal difference. A similar trend was observed for the liquid fraction of pretreated *E. crassipes*. Similarly, for *H. verticillata*, the solid fraction from TH pretreatment ranged from 343 g-wwt to 268 g-wwt and 414 g-wwt to 290 g-wwt for SE pretreated biomass. Fig 2-6 b) shows that both TH and SE had a similar area trend for the solid and liquid fractions. Lastly, the trend from *L. grandiflora* was observed to reveal a clear difference in TH and SE pretreatment (Fig 2-6 c). For the TH pretreated *L. grandiflora*, the solid and liquid fraction trend was relatively stable, and the temperature or severity factor had no effect. When SE was observed, it was seen that with the increase in the severity factor/ temperature, the amount of liquid fraction kept increasing, indicating higher solubilisation/degradation of *L. grandiflora* by SE pretreatment. The liquid fraction for SE ranged from 377 g-wwt at a lower severity factor to 700 g-wwt for the highest severity factor. As mentioned before, SE pretreatment is known for its effective solubilisation for hardwoods/softwoods. Similarly, due to the rigid/woody nature of *L. grandiflora,* SE pretreatment was effective enough to disrupt the structure of *L. grandiflora* due to the intensity and explosion ability of SE pretreatment.

In previous studies, the L/S ratio is defined as a significant factor affecting anaerobic digestion, as the concentration of easily degradable compounds or inhibitors is higher in the liquid fraction after the pretreatment. In contrast, the remaining amounts of lignocellulose polymers are recovered in the solid fraction (Duque et al., 2016). The liquid/ solid ratio was different for the aquatic weeds used. In Figures 2-7, the L/S ratio of the substrates was plotted after TH and SE pretreatments. For the L/S ratio, there was a minimal difference for *E. crassipes* pretreatment for TH and SE, with the L/S ratio increasing for the maximum temperature. Similar to *H. verticillata*, the minimal difference was observed until 180-10 min. After this condition, there was a sudden drop in the L/S ratio of the SE pretreated biomass. For *L. grandiflora*, due to the huge amount of differences in the solid and liquid fractions of TH and SE pretreatment, the L/S ratio also was different. For TH pretreatment, the L/S ratio was stable and ranged from 1.6 to 2.03. Whereas the L/S ratio increased with the severity ($y=0.71x-1.10$, $R^2= 0.87$, $p>0.005$). The highest L/S ratio was 2.19 at SF = 4.2 (210 ◦C for 10 min). This can be described by the fact that the amount of liquid fraction increased with the increase in temperature (Table 2-4), which resulted in a change in the L/S ratio. Therefore, it can be concluded that SE helped *L. grandiflora* have a higher substrate degradation rate than the TH pretreatment.

Some of the reasons explained in previous studies for the difference in the mass recovery of these biomasses are discussed below. One of the main fundamental reasons is the body structure of the biomass. The body structure of each type of biomass is different from others. Submerged aquatic weeds (*H. verticillata*) are more flexible and softer than floating or emergent weeds (Koyama et al., 2014). Whereas for *L. grandiflora*, the body structure is rigid and similar to that of wood, making it a rigid degradable substrate which can be a reason for a successful SE pretreatment for collecting higher amounts of liquid fraction with the increase in SE pretreatment intensity. The second reason can be the particle size and the chemical composition of the biomass. As explained before, each type of biomass has a different chemical composition, leading to a difference in the L/S ratio. For example, one of the reasons for the change in the L/S ratio depends on the raw biomass's moisture content or water retention capacity. In previous studies, it was concluded that for *E. crassipes*, the water retention capacity is high, and 80-90% moisture content was observed, making it biomass with maximum L/S ratio and minimum difference between TH and SE pretreatment (Carvalheiro et al., 2016; Duque et al., 2016).

Fig 2-8 shows the pH of the slurry after the TH and SE pretreatment. One of the main reasons for the pH regulation is the pretreatment intensity and chemical composition. The slurry's pH trend was similar in all the biomasses, leading to a minimal change (*H. verticillata*) and a decrease in the pH value with the increase in the pretreatment intensity (*E crassipes* and *L. grandiflora*). Fig 2-8 shows that the pH range for *E. crassipes* is 4.34-5.51, the values ranged from 5.79-6.63 for *H. verticillata*, and finally, the pH value ranged from 4.66 to 3.89 for *L. grandiflora*. Interestingly, there was no difference in TH and SE pretreatment pH values. One of the reasons for no difference between TH and SE pretreatment is the autohydrolysis mechanism which takes place in both pretreatments. As explained in Chapter 1, the sole difference is the explosion effect, which has no relationship with the pH of the liquid fraction. Secondly, the reason for the decrement of pH for *E. crassipes* and *L. grandiflora* can be a higher amount of organic acids, mainly acetic acids, released during high temperatures (Richard, 1992; Galbe and Zacchi, 2012; Zheng et al., 2016) . Previous studies mentioned that during the autohydrolysis of the lignocellulosic substrate, water could act as an acid at high temperatures (Carvalheiro et al., 2016). Acetic acid is released from the degradation of hemicellulose polymers, and it is considered the main catalyst for the further hydrolysis of the substrate and, if so the case, for glucose and xylose degradation. Besides acetic acid, further degradation of furfural and 5-hydroxymethylfurfural (HMF) may contribute to the efficiency of the pretreatment (Mosier et al., 2005). As *H. verticillata*, pH value was not affected by the pretreatment intensity, which means there was less organic acid or mainly acetic acid released during the pretreatments. pH is an essential factor which can further affect the subsequent processes. A low pH can cause an imbalance in the bacterial population during the AD process.AD is very sensitive to pH; the range for the AD process is between 6-8, as mentioned in Kim et al., (2002). Hydrolysis, acidogenesis, acetogenesis and methanogenesis processes are optimum at a pH of around 7. *L.*

grandiflora and *E. crassipes* had lower pH values for higher conditions, whereas *H. verticillata* had a much higher value of pH, which can be due to no hemicellulose detection in the untreated lignocellulose composition. The low or high pH value can directly affect the AD process.

2.3.2. Effect of thermal hydrolysis and steam explosion pretreatment on the solid fraction of the lignocellulosic biomass

2.3.2.1. Morphological characterisation (Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR))

SEM analysis investigated the morphology of untreated and TH and SE pretreated biomass to better understand the enhancement of biomass digestibility. Image 2-1, 2-2 and 2-3 show the SEM images of the untreated and the pretreated biomass. Untreated *E. crassipes*, *H. verticillata* and *L. grandiflora* exhibited rigid, compact fibrillary morphology with thick-walled fibre cells and fibres constituted by parallel stripes, limiting the cellulose accessibility. From image 2-2, the cell wall for untreated *H. verticillata* could be clearly seen. SEM is a well-adopted technique to visualise the formation of lignin droplets at the surface of the biomasses (x2000) and the separation of bundle fibres to individualise elementary fibre.

The effect of TH and SE pretreatment was seen through SEM in Figure image 2-1,2-2 and 2-3. The effect of conditions after TH and SE conditions was observed. It shows that after the TH pretreatment, the substrates observed slight defibrillation, whereas, for the SE condition, it was seen that there were marked more noticeable changes. Inevitable cracks were observed during the SE pretreatment leading to a more exposed surface which can further increase the accessibility. Several authors have associated the presence and the surface distribution of lignin with an unfavourable effect on the enzymatic hydrolysis of lignocellulosic biomass (Berlin et al., 2006; Hoeger et al., 2012; Martín-Sampedro et al., 2013; Rahikainen et al., 2013). Therefore, it can be concluded that this exposed substrate can increase the accessibility to the AD process, leading to an increase in hydrolysis and further the CH_4 yield.

To investigate the chemical bonds and the changes in the chemical structure were investigated by FTIR analysis (Figure 2-9, 2-10, 2-11) Table 2-5, 2-6, 2-7,2-8,2-9 and 2-10). The graphs plotted show the difference between the untreated substrate and the TH and SE. However, to understand the chemical structural change, specific wavelengths ratios were chosen by using previous studies (Li et al., 2016; Auxenfans et al., 2017), which are shown in Table 2-5, 2-6, 2-7,2-8,2-9 and 2-10. In this study, the absorbent intensity of specific peaks is associated with the 1426cm^{-1} origin from the C-H stretching of cellulose and lignin. The bands are explained below:

1515 cm^{-1} and 1375 cm^{-1}: the frame vibration and C–H stretching of the aromatic ring, which originated from lignin

1125 cm^{-1}: syringyl units in lignin molecules

1726 cm^{-1}: the non-conjugated carbonyl group probably originated from acetyl of the xylan.

1645 cm^{-1}: instability of phenolic acids at a higher temperature

2900 cm^{-1}: C–H stretching of methyl and methylene groups

1426/896: absorption of b-glycosidic bonds between glucose units with the amount of the crystalline structure of cellulose

1508/1600 cm^{-1}: proportion of lignin with condensed and cross-linked structures

Previous studies reported that the SE pretreatment (due to its explosion capability) leads to removing fibres from cellulose and disrupting hemicellulose polymer. During pretreatment, the lignin is redistributed in large fibre extension; lignin and lignin fragments are rearranged and distributed heterogeneously in the cell wall, where they are deposited on the surface or interior of the fibres. Lignin micro-droplets are formed, produced by the coalescence and migration of lignin and progressive collapse of the microfibrils (Donohoe et al., 2008; Araya et al., 2015; Arévalo et al., 2017). Other micro-particles deposited on the surface or interior of the fibre, called pseudo-lignin, consist of modified lignin and carbohydrate degradation products (Sannigrahi et al., 2011; Hu et al., 2012; Kumar et al., 2013). Both lignin micro-droplets and pseudo-lignin deposited on the surface or interior of the fibres reduce the efficiency of enzymatic hydrolysis through nonspecific binding of the enzymes, thus creating a physical barrier that blocks enzyme access to the cellulose. The ratio of the amount of crystalline cellulose to the total sample (includes both crystalline and amorphous) refers to the crystallinity. This is because the molten amorphous cellulose changes to a crystalline state after reaching the glass transition temperature. Crystallinity was also an important parameter that can reveal substrate digestibility (Hu et al., 2013) .

Figure 2-9 shows the FTIR graph and the Table 2-5, 2-6 shows the wavelength ratios for the untreated *E. crassipes* and pretreated sample. Table 2-5, 2-6 shows that the crystallinity index increased from the untreated condition to the condition of 150°C, whereas the crystallinity decreased when the temperature was higher. In contrast, when observing the total crystallinity index, it was seen that biomass had a higher value compared with the untreated condition. The results were similar to previous studies, leading to an increase in crystallinity during TH and SE (Li et al., 2016). Also, as the temperature for this condition is 150°C, there was no evidence for the re-condensed lignin after the pretreatment in both TH and SE pretreatment, whereas when the temperature was 210°C, it was seen that the re-condensed lignin was much higher which can affect the methane yield further.

There was less variation in the graph for *H. verticillata* from Fig 2-10. However, when observing the bond type (Table 2-7, 2-8) for *H. verticillata*, it was seen that the C-O stretching bond type had increased after the TH and SE conditions leading to a clean cellulose morphology in the pretreated biomass and signifying the removal of acetyl groups throughout the pretreatment (Auxenfans et al., 2017; Wang et al., 2017). At the same time, an interesting trend was observed for crystallinity and total crystallinity index, as both indexes were lower than the untreated condition. As crystallinity is considered essential for biomass digestibility, it can be plausible to state that it can affect the results during methane tests.

For *L. grandiflora*, the C-O stretching bonds increased from the untreated condition for both TH and SE pretreatments (Fig 2-11). Interestingly, the values increased for the re-condensed lignin for the TH pretreatment at 210 °C, 10 min (Table 2-9, 2-10). This means that less lignin is being released to the liquid fraction as explained in SEM, that TH is linked with the recondensation due to no explosion, which can lead to the reformation of lignin; this is also seen for *H. verticillata*. These values are also seen in previous studies for wheat straw. FTIR results correspond with the other analysis and recommend that the increase in the SF could improve the degree of morphological alterations of lignin (Yu et al., 2012; Auxenfans et al., 2017).

2.3.2.2. Lignocellulose composition

The lignocellulose composition is one of the essential parameters for this study, as the primary purpose of TH and SE pretreatment is to render the lignin which covers the outer of the cell wall, as explained in Chapter 1. The lignocellulose values in the untreated solid fraction of aquatic weeds were measured in this study (Table 2-11). The untreated *E. crassipes* had cellulose of 9.9%, hemicellulose of 20.7%, and lignin of 21.2%. These values were quite different when compared with previous studies. Usually, *E. crassipes* is considered an aquatic weed with a lower lignin content of around (7-15%), which has been proven by many studies (Ofoefule et al., 2009; Barua and Kalamdhad, 2017; Kist et al., 2018). For example, de Castro et al., (2021) shows that the untreated *E. crassipes* had cellulose= 2.5%, hemicellulose= 34.1% and lignin= 8.6%. Interestingly, a study done by Chanakya et al., (1993) showed a high lignin value of 26.36% for untreated *E. crassipes*, which is even higher when compared with this study. In a study by Barua and Kalamdhad, (2017), when the lignin content of *E. crassipes* stem was examined, it came out to be around 24.94%.

When discussing *H. verticillata*, Kaur et al., (2019) observed a cellulose content of 21.5%, hemicellulose of 50.3% and lignin of 28.2%. The values of cellulose and lignin were much similar to this study which had cellulose of 24.1% and lignin of 29.1%, whereas hemicellulose was not detected in this study. Thermal pretreatments lead to the degradation of hemicellulose, which is then transferred to a liquid fraction as soluble sugars, which can enhance the biodegradability of the biomass, as explained earlier. The values of cellulose and lignin were quite similar in *E. crassipes* used in this study (cellulose of 11.3% and lignin of 21.2%). For the emergent type aquatic plant, *L. grandiflora*, although the type of specie is the same, the sampling time varies as mentioned in the material and methods. Due to this difference in sampling time, the cellulose was different as the *L. grandiflora* used for TH had cellulose of 17.9%, and the value for SE pretreatment was 26.5%. Lignin did not change as the biomass used for TH had a value of 33.4%, and the one used for SE pretreatment had lignin of 34.3%. Fujiwara et al., (2022) revealed that the harvesting time could significantly change the values of these components, which can further affect the anaerobic digestion of these substrates. This difference can be attributed to factors such as harvesting period, growth origin, and degradability, as explained in (Poorter and Bergkotte, 1992; Li et al., 2014; Fujiwara et al., 2022) examined the chemical composition of various wild plant biomass and revealed that the species which grew faster had lower hemicellulose and lignin when compared with other slow-growing species. Lignin content determines the body rigidity of plant biomass. It was indicated that *L. grandiflora* has a more woody and rigid body structure than other species because of its slow-growing property.

As mentioned, some of the solubilised lignocellulose polymers were transferred to the liquid fraction, while the remaining parts of these polymers were transferred to the solid fraction. Figure 2-12 shows the lignocellulose composition after the TH and SE pretreatments in the solid fraction. For *E. crassipes*, hemicellulose was significantly reduced for both TH and SE pretreatment with a similar trend. When comparing TH and SE pretreatment, there was a higher reduction of hemicellulose in the TH pretreatment (2% TS for 210°C-30 min). Interestingly cellulose had a very wavy trend, and it can be seen that more cellulose was retained during TH pretreatment. This can prove that due to no explosion, cellulose is retained during the TH pretreatment due to its less harsh/ rough pretreatment. However, when observing the SE trend, lignin increased for both TH and SE pretreatment, but SE pretreatment had a more direct linear relationship of lignin increase with the temperature. For the effect of pretreatments on *H. verticillata*, cellulose increased with the increase in the temperature for both the pretreatments. The increment of cellulose was higher in the TH pretreatment when compared with the SE pretreatment. Also, the lignin had no trend for the SE pretreatment but had a direct negative relationship with TH pretreatment. For the TH pretreatment of *H. verticillata*, the increase of cellulose leads to a decrease in the lignin inside the solids. Similar results were seen by Hu et al., 2013 when using reed straw as a substrate. For *L. grandiflora*, after the TH pretreatment, it was seen that cellulose had a positive trend with the increase in temperature or the pretreatment severity. Whereas for SE, even though the trend was positive, it was unstable. Similar to *E. crassipes*, TH retained more cellulose during the pretreatments. Similarly, the lignin value for the TH pretreatment was relatively stagnant, but SE showed a positive increase with the increase in temperature.

2.3.2.3. Total solids concentration

Figure 2-13 shows the relationship of total solids (TS) concentration with the severity factor. One of the main differences observed after the TH and SE pretreatment was the reduction of total solids with the increase in severity factor. Previous studies have shown a reduction of solids after the SE pretreatment (Ariunbaatar et al., 2014). Similarly, in this study, when comparing the trend between TH and SE pretreatment, it was seen that SE had a slightly higher reduction of solids when compared with the TH pretreatment for all the substrates used. From Figure 2-13, it can be concluded that the total solids are one of the affecting parameters that had a similar effect on all the substrates after the TH and SE pretreatment. SE pretreatment led to the reduction of the TS compared with TH. The solid fraction recovered after SE pretreatment may be relatively more minor than other hydrothermal pretreatments, as explained in Cara et al., (2008). SE pretreatment leading to a more open structure of the pretreated biomass because of its exploding capability can be the reason for these results (the reduction in TS was higher in SE.)

2.3.2.4. Carbon/Nitrogen (C/N) ratio

The value for untreated *E. crassipes*, *H. verticillata* and *L. grandiflora* were 16.44, 11.49, 27.08 (TH) and 33.23% TS (SE).

The value for untreated *E. crassipes* in previous studies varied compared with this study. For example, previous studies had a C/N ratio of 17.80, 29.0 and 8.2 (Ofoefule et al., 2009; Mathew et al., 2015; Kist et al., 2018), whereas, in this study, the value for untreated biomass was 16.44%TS. Similarly, the C/N ratio for untreated *H. verticillata* in a previous study by Jain and Kalamdhad, (2019) was 14%, which was a little higher than what was observed in this study. Finally, for *L. grandiflora*, as two different substrates were used, the C/N ratio also varied. As there is a lack of study on this emergent aquatic plant, the two different substrates used in this study show the variation in the C/N ratio. Since both carbon and nitrogen are vital for many cellular functions, the proper supply of these two nutrients is critical for plant growth, development, response to various stresses, and eventually for their life cycle (Zheng, 2009). These values can be changed depending on the growth conditions. This can be the main reason for the different values of the C/N ratio for the same species.

The values of the C/N ratio after the TH and SE pretreatment in the solid fraction are explained in Fig 2-14. The Figure explains a similar trend for both *E. crassipes* and *H. verticillata* after pretreatment. The value of the C/N ratio increased after the TH and SE pretreatment, but no relation was found with the severity factor. A previous study also observed the same trend (Vivekanand et al., 2014). The values for *E. crassipes* and *H. verticillata* increased slightly after the TH and SE pretreatment, with minimal difference between TH and SE pretreatment. The reason for no trend is that the total carbon and nitrogen remain constant regardless of the pretreatment and the condition performed. The amount of carbon and nitrogen may differ, but the total amount remains almost unchanged. Whereas the values after the TH and SE pretreatment of *L. grandiflora* were significantly different, as explained in Fig 2-14. Th main reason being the different biomass used for TH and SE. Although the trend was similar, the value varied a lot. For TH pretreated biomass, the value for the C/N ratio was almost 2-3 times higher than the SE pretreated biomass as compared to the untreated biomass. SE pretreatment decreased the value of the C/N ratio, whereas there was a little increment in the TH pretreated biomass.

Both C and N often act as limiting factors. The ideal/optimum ratio in previous studies is between 20-30. Also, it varies with the type of feedstock to be digested (Pavan et al., 2000; Tuomela et al., 2000; Li et al., 2011; Jain et al., 2015). A Low C/N ratio can cause problems, one of them being ammonia accumulation. Previous studies mentioned that conversion rate of C as compared to N in the digestion process is 30–35 times faster, so the optimum ratio of C/N should be 30:1 in the untreated substrate, and *L. grandiflora* as a raw substrate gives a value of 27.08 for TH and 33.23 for SE. Therefore untreated *L. grandiflora* has an optimum value for the C/N ratio, which will help the microbes in the reactor to work efficiently. In contracts, the value of C/N for *E. crassipes* and *H. verticillata* had values of 16.44 and 11.49%, much lower than the optimum value, which can further affect AD. Previous studies reported that the C/N ratio is a vital factor for AD process (Moeller et al., 2018).

2.3.3. Effect of thermal hydrolysis and steam explosion pretreatment on the liquid fraction of the lignocellulosic biomass

2.3.3.1. Total organic carbon (TOC) and dissolved organic carbon (DOC)

Figure 2-15 shows the total organic fraction of the liquid fraction after the pretreatments with the severity factor. TOC concentration of the liquid fraction varied with the different species of aquatic weeds. For *E. crassipes*, the value of TOC for TH pretreatment ranged from 2.10 g L^{-1} (165°C) to 4.07 g L^{-1} (180°C). For SE pretreatment, TOC ranged from 2.79 g L^{-1} (150°C) to 5.43 g L^{-1}, the highest at 180°C. The TOC trend when the SF increased reveals that treatment temperature is the main dependent factor, whereas retention is the next important parameter (both are included in the severity factor equation) and finally by releasing the pressure release as it shows a minor effect on the obtained results. The values for TOC for both TH and SE ($y= -0.43x^2 + 3.87x - 4.30$, $R^2= 0.50$, $p<0.025$) reached an optimum value and decreased when the SF was increased further. When the results of TH and SE pretreatment were observed, it was seen that SE pretreatment had slightly higher TOC values. Some minor differences were gained during earlier studies when performing thermal hydrolysis and steam explosion pretreatment (Kist et al., 2018). *H. verticillata* and *L. grandiflora* had an increasing trend with the SF increase for both TH and SE pretreatment. Firstly for *H. verticillata*, the values for TOC ranged for TH pretreatment ranged from 2.77 g L^{-1} (165°C) to 6.04 g L^{-1} (210°C), whereas for SE pretreatment, the values ranged from 2.49 g L^{-1}at 150°C and 6.66 g L^{-1} at 210°C. During the TOC concentration of *H. verticillata*, it was observed that there was not a significant difference when comparing both pretreatments. Both TH and SE had a linear relationship of TOC with severity factor (TH; $y= 1.01x +0.58$, $R^2=0.50$, $p<0.025$, SE; $y= 1.44x -1.10$, $R^2=0.74$, $p<0.005$). Lastly, for *L. grandiflora* as it also had a linear positive trend with the increase in SF for both TH ($y= 1.84x-2.49$, $R^2=0.81$, $p<0.005$) and SE ($y= 2.88x-1.61$, $R^2=0.88$, $p<0.005$) pretreatment. The TOC values for TH pretreated *L. grandiflora* ranged from the lowest being 1.53 g L^{-1}at 150°C to the highest being 6.27 g L^{-1}at 210°C. Whereas the values for SE pretreatment were almost double those for TH pretreatment. The values of TOC after SE pretreatment ranged from 5.68 g L^{-1} (150°C) to 11.84 g L^{-1} (210°C).

One of the reasons for the lower difference between SE and TH pretreatment for *E. crassipes* and *H. verticillata* can be the composition of the lignin, which is explained by previous studies (Barakat et al., 2012). Koyama et al., (2014; 2015) suggested that the structure of lignin (ratio of guaiacyl (G) and syringyl (S) polymer) after the pretreatment varies for each substrate which can lead to an increase or decrease in the DOC concentration. The high substrate degradation for SE *L.*

grandiflora can be due to its woody type. *L. grandiflora* has a woody structure for which SE pretreatment can be considered a pretreatment that improves the substrate solubilisation much higher than the TH pretreatment.

Figure 2-16 was plotted to check the DOC concentration in the TOC. The Figure shows that most of the part in the TOC comprised of the dissolved fraction for all the substrates. For the TH pretreated biomass, the trend of DOC/TOC was quite unstable, whereas for the SE pretreated biomass, the DOC/TOC had a negative linear relationship with the severity factor for all the substrates. For TH pretreated *E. crassipes*, it was seen that the dissolved fraction ranged from 51% to 94% and for SE pretreated *E. crassipes*, and the values ranged from 57 to 93% (Relationship of SE pretreated DOC/TOC with SF, $y=-0.13x+1.26$, $R^2= 0.72$, $p<0.005$). For *H. verticillata*, the trend was similar with *E. crassipes*; for TH pretreated biomass, the dissolved organic fraction present inside the total organic compounds was ranging from 72 to 96%, whereas for the SE pretreated biomass, the values ranged from 75 to 92% (Relationship of SE pretreated DOC/TOC with SF, $y=-0.066x+1.08$, $R^2= 0.73$, $p<0.005$). Lastly, for *L. grandiflora*, the trend was different for SE and TH. The TH pretreated biomass had a DOC/TOC value ranging between 55 to 97%, whereas, for SE, the values ranged from 57 to 86% (Relationship of SE pretreated DOC/TOC with SF, $y=-0.025x+1.15$, $R^2= 0.63$, $p<0.01$).

2.3.3.2. Formation of inhibitors during the degradation of the pretreated substrate concentration (Dissolved lignin and Phenolic compounds)

Phenolic compounds such as ferulic acid, vanillin, p-coumaric acid and syringaldehyde are derivates of the lignin polymers released during TH and SE pretreatment, as mentioned in previous studies. When the derivatives of lignin, i.e., guaiacyl (G) and syringyl (S), are degraded, the formation of phenolic compounds takes place (Bajaj et al., 2009; Monlau et al., 2014; Auxenfans et al., 2017; Schroyen et al., 2018).

Figure 2-17 shows the concentration of phenolic compounds after the TH and SE pretreatment for different biomasses. The value for phenolic compounds increased with the temperature increase for all the biomasses. This change was divided into two steps for *E. crassipes*. Initially, the values were quite similar for the temperature of 150°C and 165°C. However, from 180°C to 210°C, the value for both TH and SE increased. Although both TH and SE pretreatment had a positive increase with the increase in pretreatment temperature, SE had a higher trend than the TH pretreatment, as shown in Figure 2-17. From Figure 2-18, it can be seen that phenolic compounds had an increasingly positive relationship with the temperature and the severity factor. It also proves that SE ($y= 6.15x - 539.88$, $R^2= 0.63$, $p<0.01$) had a higher positive trend when compared with TH ($y= 5.03x – 432.58$, $R^2= 0.57$, $p<0.025$). Similarly, for *H. verticillata*, the trend was similar. The phenolic compounds kept on increasing with the increase in the temperature. However, when comparing the TH and SE pretreatment values, the concentration of phenolic compounds was not stable. Lastly, for *L. grandiflora*, the phenolic compounds had the maximum concentration. For the TH pretreatment of *L. grandiflora*, a positive relationship was observed with the temperature and severity factor as in the previous biomasses. The overall values were higher for the SE pretreatment, but no trend was observed. The values for phenolic compounds were even higher for lower temperatures of 150°C. Sapci et al., (2013) reported that some lignins are solubilised at ≥ 160 °C during thermal pretreatment. The resulting compounds are usually similar to phenolic compounds (Hendriks and Zeeman, 2009), which could have an inhibitory or toxic effect on bacteria, yeast and methanogens/archaea (Jackowiak et al., 2011).

Michelin et al., (2016)found that phenolic compounds ($\log R_0 = 3.83$, 3.5 mg phenol/mg protein enzyme) from sugarcane bagasse pretreated with liquid hot water had a 20% lower cellulose conversion compared with the control condition, whereas (Substrate= Solka Floc) at higher severity conditions ($\log R_0 = 4.42$, 6.2 mg phenol/mg protein enzyme) yielded 45% lower yield of cellulose. Another study showed that phenol ($\log R_0 = 4.25$, 2 mg phenol/mg protein enzyme) from hardwood pretreated with liquid hot water reduced conversion yields by about 50%. Some researchers say that phenolic compounds are more toxic than other potent inhibitory molecules (furan aldehyde, weak acids, and other degradation products) because they penetrate and damage cell membranes due to their low molecular weight (MW), leading to changes in internal structure and cell morphology. Therefore, removal or reduction of the inhibitory effect was purposed to improve the pretreated substrate's overall efficiency and, in particular, to avoid its inhibitory effect (Michelin et al., 2016; Kim, 2018).

Lignin removed from the lignocellulose matrix was dissolved in the liquid fraction during thermal pretreatments and remained in the digestate during AD because lignin polymer does not degrade quickly (Benner et al., 1984; Tuomela et al., 2000; Barakat et al., 2012). Dissolved lignin polymers such as kraft lignin, organosolv lignin and lignosulfonates are found in the liquid fraction (Barakat et al., 2012). During TH and SE pretreatment, lignin-carbohydrate bonds are broken, and lignin is partially solubilised. Initially, the lignin fraction with low molecular weight is solubilised, increasing dissolved lignin (Biswas et al., 2011; Kumar et al., 2011; Carvalheiro et al., 2016; Koyama et al., 2017b). Therefore, the concentration of dissolved lignin was calculated by assuming that the amount of lignin removed from the solid fraction is equivalent to the dissolved lignin content in the liquid fraction, as explained in (Koyama et al., 2017a). Previous studies reported that inhibitory substance levels increase under harsh conditions (García-Aparicio et al., 2006; Wang et al., 2010). Therefore, their effect on the AD process at different severity levels was evaluated.

From Figure 2-19, the concentration of dissolved lignin was seen, and it was concluded that there was no relationship between dissolved lignin with the severity factor and the treatment temperature. The value of dissolved lignin for *E. crassipes* was the lowest as dissolved lignin was not detected for most conditions. For *H. verticillata*, the trend for dissolved lignin was quite similar for TH and SE pretreatment except for 180°C. The value for *L. grandiflora* reveals that SE pretreatment had relatively higher values for SE samples even at low temperatures, which can further affect AD. The value of dissolved lignin for SE was maximum at 150°C, whereas for TH, it was at around 180°C.

2.3.4. Conclusion: Substrate solubilisation

To check the solubilisation of the liquid fraction by TH and SE pretreatment. The total organic carbon (TOC) relationship with the phenolic compounds was plotted (Figure 2-20). Interestingly, with the increase in the TOC value, the phenolic compounds were increasing. The Figure explains that for *E. crassipes* and *H. verticillata*, the relationship of phenolic compounds increased with the increase in the total organic carbon concentration for both TH and SE. The trend was a little different for *L. grandiflora*; for TH pretreated biomass, it had a positive linear relationship with the increase in the total organic carbon, whereas, for SE, there was no linear relationship with the total organic carbon, but the values of phenolic compounds were even higher at the low temperatures which can also be seen from Figure 2-20. It can be seen that TH for all the substrates had an increasing trend despite the lignin in the untreated substrate kept on increasing. The substrate degradation led to higher TOC readily available, increasing the amount of phenolic compounds in the pretreated liquid substrate. For TH, this had an increasing linear trend regardless of the substrate's chemical composition. For SE, as the lignin concentration of the substrate increased, the phenolic compounds reached an optimum point, whereas the TOC kept increasing, leading to a different trend than TH. In Figure 2-21, all TH and SE pretreated substrates observed a relationship between inhibitors concentration in the liquid fraction (dissolved lignin + phenolic compounds) and lignin concentration in the pretreated biomass. The Figure revealed that if the lignin concentration in the pretreated biomass increases, the inhibitors' concentration in the liquid fraction increases. During thermal pretreatments, lignin is solubilised to mainly dissolved lignin and phenolic compounds. This means higher solubilisation leads to higher inhibitor production but will this affect the AD process? The effect of these inhibitors on the AD process will be discussed in the latter chapters.

2.4. Tables

Table 2-1. Thermal hydrolysis and steam explosion conditions for Experiment 1

Treatment temperature (° C)	Retention time (min)	Severity Factor*
150	10	2.4
150	30	2.9
165	10	2.9
165	30	3.3
180	10	3.3
180	30	3.8
210	10	4.2
210	30	4.7

*: Severity factor (SF) is explained in material and methods.

Table 2-2. Mass recovery of *Eichhornia crassipes* after thermal hydrolysis and steam explosion pretreatment

Condition (Temperature (°C)– Time (min))	**Thermal hydrolysis**			**Steam explosion**		
	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)
150-10	302	823	90	312	814	90
150-30	267	895	93	276	847	90
165-10	244	834	86	275	828	88
165-30	279	708	79	260	812	86
180-10	336	814	92	340	746	87
180-30	295	820	89	340	778	89
210-10	200	874	86	216	854	86
210-30	276	807	87	246	848	88

Table 2-3. Mass recovery of *Hydrilla verticillata* after thermal hydrolysis and steam explosion pretreatment

Condition (Temperature (°C)– Time (min))	**Thermal hydrolysis**			**Steam explosion**		
	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)
150-10	294	826	90	324	741	85
150-30	325	782	89	394	621	81
165-10	327	787	89	318	781	88
165-30	300	778	86	358	740	88
180-10	343	771	89	290	850	91
180-30	291	814	88	414	682	88
210-10	268	846	89	310	598	73
210-30	331	772	88	368	702	86

Table 2-4. Mass recovery of *Ludwigia grandiflora* after thermal hydrolysis and steam explosion pretreatment

Condition (Temperature (°C)– Time (min))	**Thermal hydrolysis**			**Steam explosion**		
	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)	Solid (g-wwt)	Liquid (g-wwt)	Recovery rate (%)
150-10	416	702	89	654	377	83
150-30	326	791	89	603	477	86
165-10	342	737	86	522	641	93
165-30	378	757	91	557	623	94
180-10	343	830	94	511	568	86
180-30	348	796	92	397	702	88
210-10	292	809	88	330	725	84
210-30	366	744	89	350	700	84

Table 2-5. Bond type variation during thermal hydrolysis pretreatment of *Eichhornia crassipes* used in this study

Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	1.05	0.80	1.08	1.02	0.94	0.66	1.11	1.31
150-10	1.17	0.53	1.13	0.97	0.67	0.79	0.89	1.42
150-30	1.00	0.74	1.04	1.01	0.93	0.76	1.29	1.24
165-10	1.17	0.55	1.12	0.98	0.69	0.79	0.89	1.39
165-30	1.09	0.67	1.05	0.98	0.83	0.84	0.98	1.23
180-10	1.16	0.49	1.14	0.94	0.70	0.56	1.12	1.52
180-30	1.19	0.51	1.11	0.94	0.68	0.78	0.86	1.48
210-10	1.20	0.51	1.13	0.95	0.69	0.75	0.85	1.54
210-30	1.13	0.66	1.07	0.95	0.78	0.81	0.91	1.32

Table 2-6. Bond type variation during steam explosion pretreatment of *Eichhornia crassipes* used in this study

Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	1.05	0.80	1.08	1.02	0.94	0.66	1.11	1.31
150-10	1.16	0.53	1.14	0.98	0.66	0.79	0.89	1.40
150-30	0.96	0.75	1.03	1.00	0.92	0.69	1.33	1.19
165-10	1.15	0.56	1.11	0.96	0.70	0.81	0.89	1.38
165-30	1.15	0.55	1.11	0.96	0.71	0.80	0.89	1.38
180-10	1.14	0.54	1.15	0.94	0.68	0.59	1.07	1.46
180-30	1.15	0.62	1.10	0.97	0.75	0.80	0.90	1.34
210-10	1.19	0.51	1.13	0.94	0.67	0.76	0.82	1.53
210-30	1.22	0.46	1.13	0.93	0.64	0.74	0.83	1.63

Table 2-7. Bond type variation during the thermal hydrolysis pretreatment of *Hydrilla verticillata* used in this study

Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	1.11	0.78	1.07	1.09	1.06	0.63	1.20	1.23
150-10	1.47	0.61	1.19	1.20	0.97	0.65	0.92	1.54
150-30	1.46	0.64	1.23	1.20	0.92	0.67	0.90	1.49
165-10	1.74	0.53	1.37	1.29	2.42	0.41	0.80	1.80
165-30	1.35	0.58	1.16	1.13	0.90	0.71	0.91	1.48
180-10	1.24	0.77	1.13	1.13	0.95	0.62	1.01	1.27
180-30	1.25	0.72	1.15	1.12	1.04	0.59	0.93	1.36
210-10	1.23	0.75	1.12	1.06	0.91	0.79	0.93	1.22
210-30	2.04	0.64	1.49	1.33	0.98	0.45	0.84	1.72

Table 2-8. Bond type variation during the steam explosion pretreatment of *Hydrilla verticillata* used in this study

Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	1.11	0.78	1.07	1.09	1.06	0.63	1.20	1.23
150-10	1.55	0.73	1.30	1.27	0.96	0.63	0.87	1.43
150-30	1.64	0.66	1.31	1.28	0.96	0.58	0.81	1.52
165-10	1.54	0.69	1.26	1.27	0.98	0.49	0.90	1.43
165-30	1.76	0.58	1.36	1.31	0.91	0.56	0.82	1.68
180-10	1.25	0.84	1.12	1.15	1.02	0.61	1.07	1.19
180-30	1.30	0.61	1.11	1.01	0.82	0.58	0.89	1.40
210-10	1.89	0.63	1.43	1.33	0.84	0.51	0.75	1.57
210-30	1.71	0.71	1.35	1.23	0.84	0.57	0.81	1.42

Table 2-9. Bond type variation during the thermal hydrolysis pretreatment of *Ludwigia grandiflora* used in this study

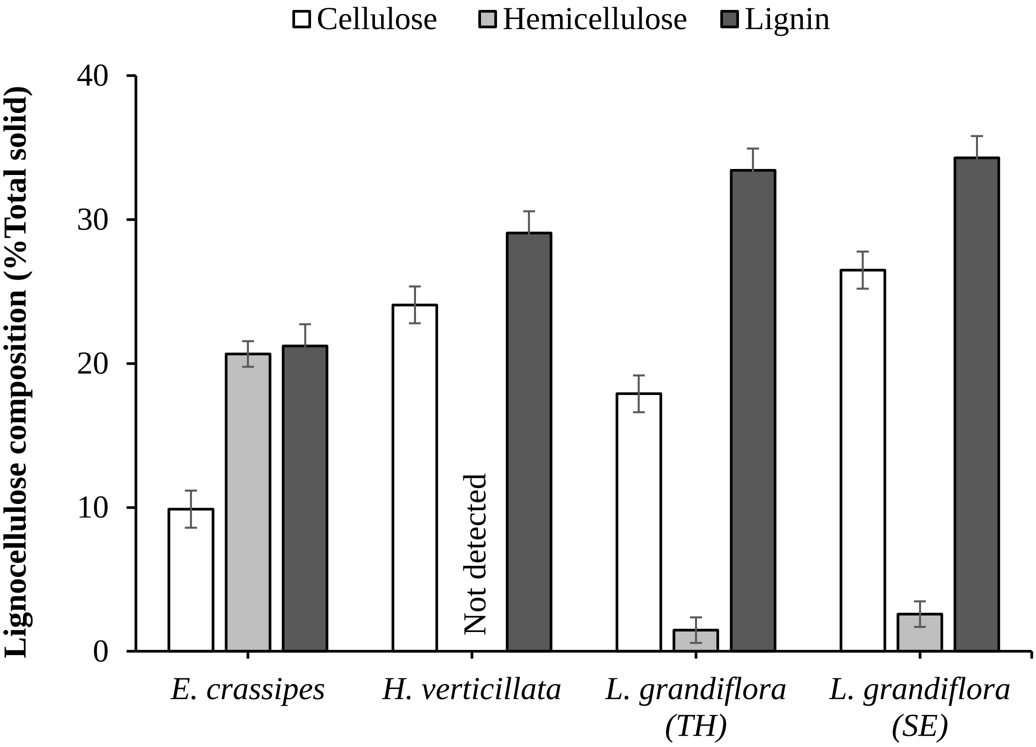
Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	0.99	0.72	1.08	0.96	0.92	0.69	1.10	1.33
150-10	1.07	0.66	1.15	0.94	0.80	0.77	0.84	1.39
150-30	1.14	0.61	1.18	0.95	0.75	0.77	0.81	1.51
165-10	1.06	0.59	1.13	0.93	0.80	0.77	0.79	1.48
165-30	1.21	0.51	1.35	0.87	0.57	0.62	0.78	2.02
180-10	1.05	0.61	1.14	0.93	0.80	0.78	0.78	1.47
180-30	1.09	0.61	1.23	0.88	0.78	0.53	0.70	1.69
210-10	1.02	0.70	1.08	0.95	0.95	0.59	1.05	1.41
210-30	1.10	0.63	1.12	0.93	0.81	0.75	0.77	1.51

Table 2-10. Bond type variation during the steam explosion pretreatment of *Ludwigia grandiflora* used in this study

Bond type	C-O stretching	Phenolic bonds	Aromatic ring	Aromatic ring	Syringyl units (lignin)	Crystallinity Index	Total Crystallinity Index	Re-condensed Lignin
Wavenumber ratio	$\frac{1726}{1426}$	$\frac{1645}{1426}$	$\frac{1515}{1426}$	$\frac{1373}{1426}$	$\frac{1125}{1426}$	$\frac{1426}{896}$	$\frac{1375}{2900}$	$\frac{1508}{1600}$
Untreated	0.93	0.72	1.05	0.98	1.02	0.61	1.35	1.91
150-10	1.11	0.56	1.17	0.92	0.80	0.76	0.79	1.61
150-30	1.14	0.51	1.20	0.88	0.73	0.69	0.73	1.78
165-10	1.10	0.61	1.14	0.93	0.82	0.77	0.81	1.48
165-30	1.18	0.57	1.27	0.87	0.68	0.54	0.69	1.73
180-10	1.14	0.60	1.11	0.92	0.82	0.77	0.80	1.50
180-30	1.27	0.53	1.22	0.87	0.79	0.47	0.62	1.83
210-10	0.97	0.81	1.01	0.98	1.04	0.72	1.12	1.21
210-30	1.12	0.65	1.11	0.93	0.83	0.75	0.79	1.47

Table 2-11. Lignocellulose components of the untreated substrates used in this study

	Cellulose (%TS)	Hemicellulose (%TS)	Lignin (%TS)
Eichhornia crassipes	9.9 ± 1.3	20.7 ± 0.9	21.2 ± 1.5
Hydrilla verticillata	24.1 ± 1.9	Not detected	29.1 ± 2.2
***Ludwigia grandiflora* (used for TH)**	17.9 ± 0.8	1.5 ± 0.0	33.4 ± 0.2
***Ludwigia grandiflora* (used for SE)**	26.5 ± 0.3	2.6 ± 0.3	34.3 ± 5.5



2.5. Figures

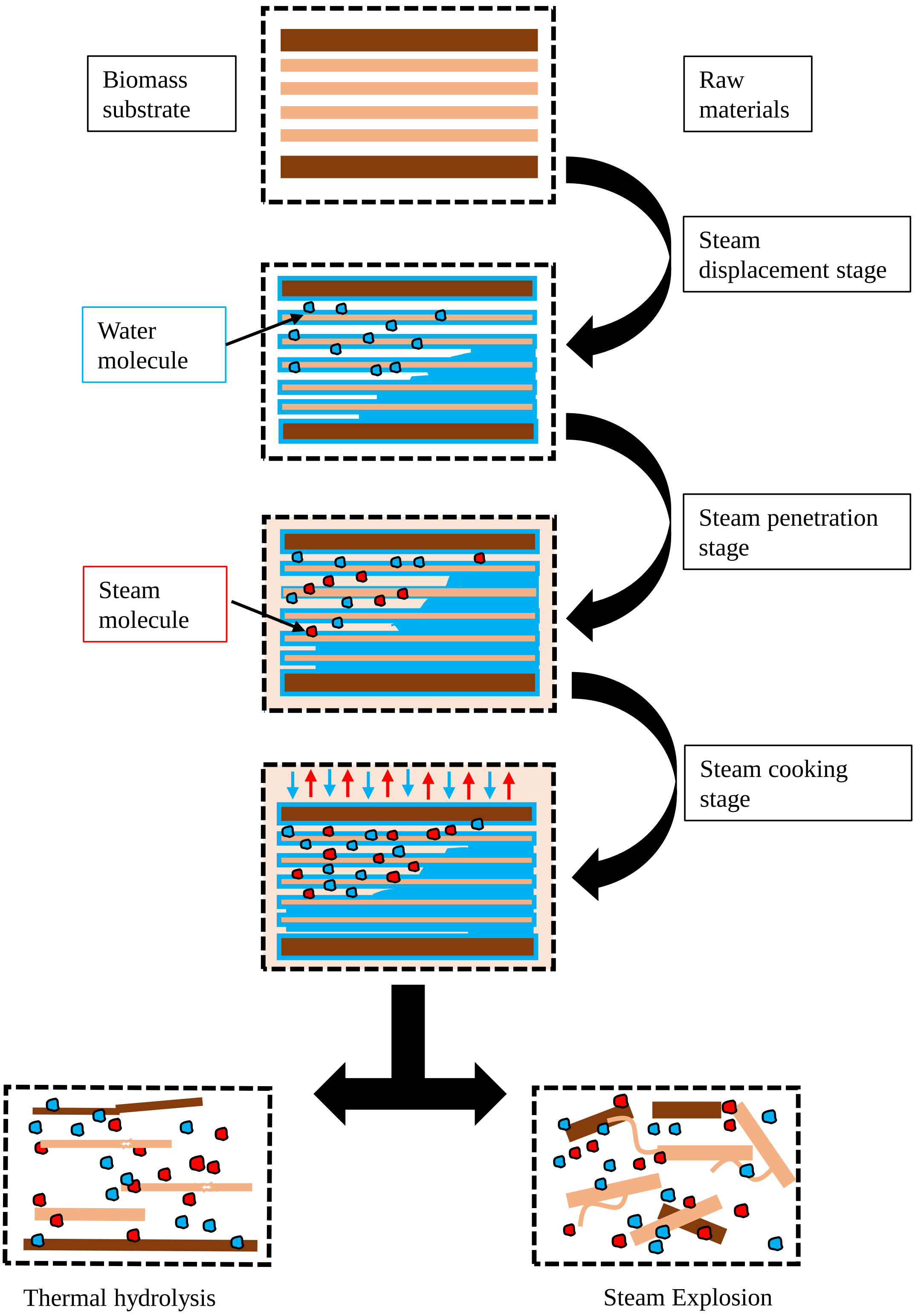


Fig 2-1. Mechanism of thermal hydrolysis and steam explosion explained (Modified by Yu et al., 2022)

1. *Eichhornia crassipes* (ホテイアオイ)

Type: Floating, Invasive
Native country: America
Common name: Water hyacinth

E. crassipes

2. *Hydrilla verticillata* (クロモ)

Type: Submerged, Invasive
Native country : India
Common name: Water thyme

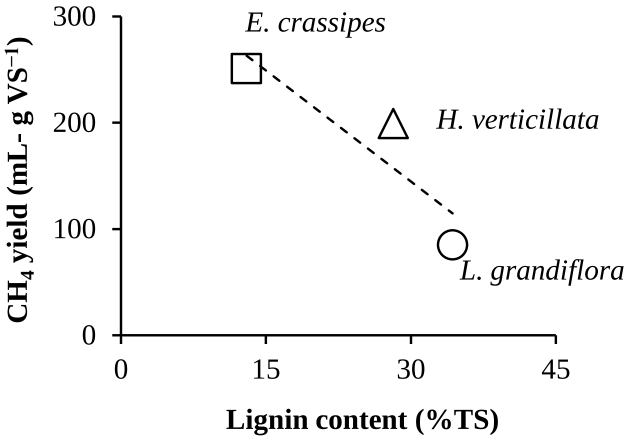
H. verticillata

3. *Ludwigia grandiflora* (オオバナミズキンバイ)

Type: Emergent, Invasive
Native country: America
Common name: Water primrose

L. grandiflora

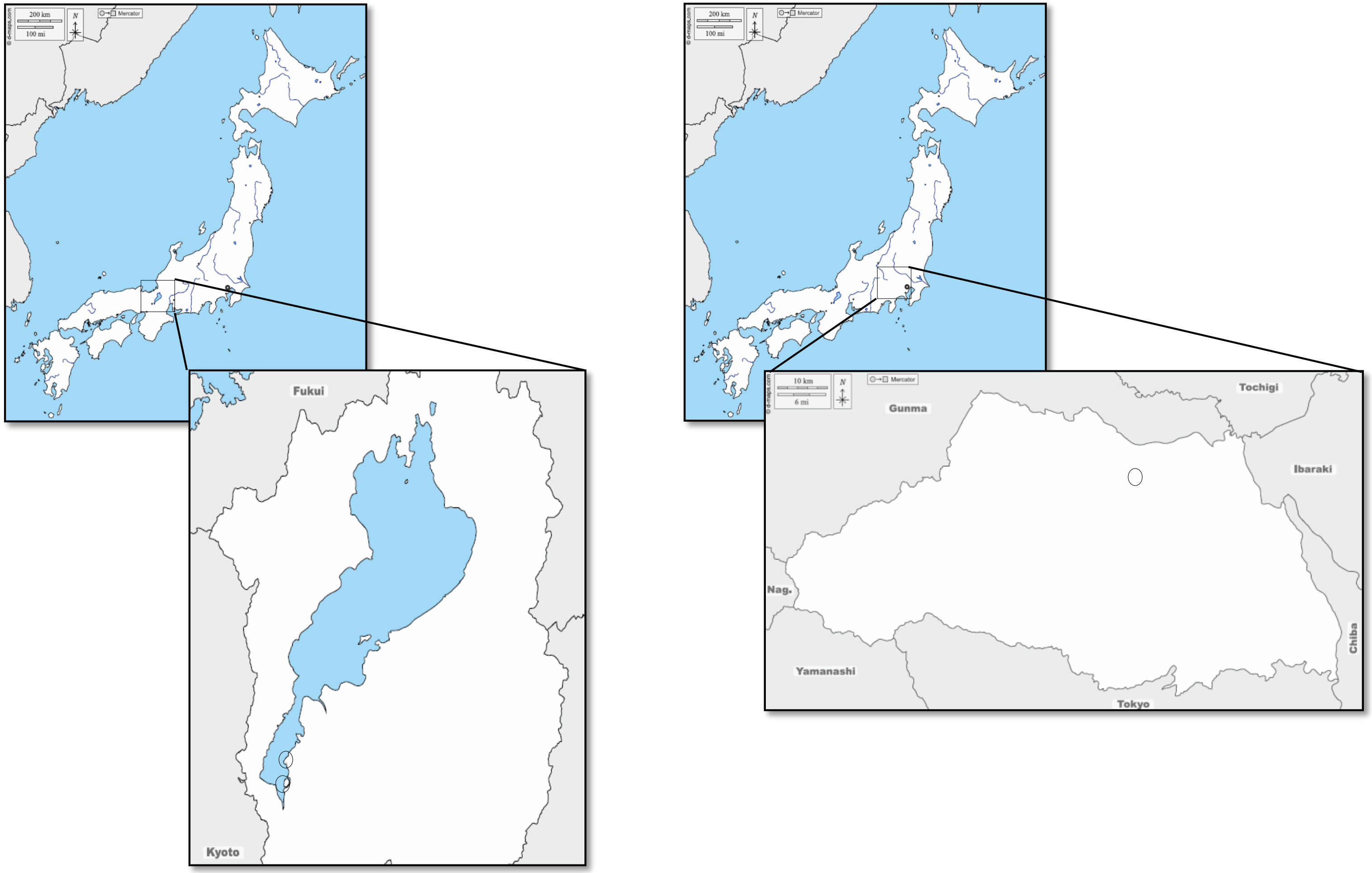
Purpose of choosing these aquatic weeds



1. Enrique 2017 (Master study), Kaur et al., 2018, Fujiwara et al., 2022, Pranshu et al., 2020

Relationship of untreated methane yield with the lignin content using previous studies

Fig 2-2. Aquatic weeds used in this study 1 and study 2



1. Lake Biwa, Shiga prefecture: For *Hydrilla verticillata* and *Ludwigia grandiflora*

2. Suijo park, Gyoda, Saitama prefecture: For *Eichhornia crassipes*

Fig 2-3. Map of sampling place. The sampling stations were marked by ○

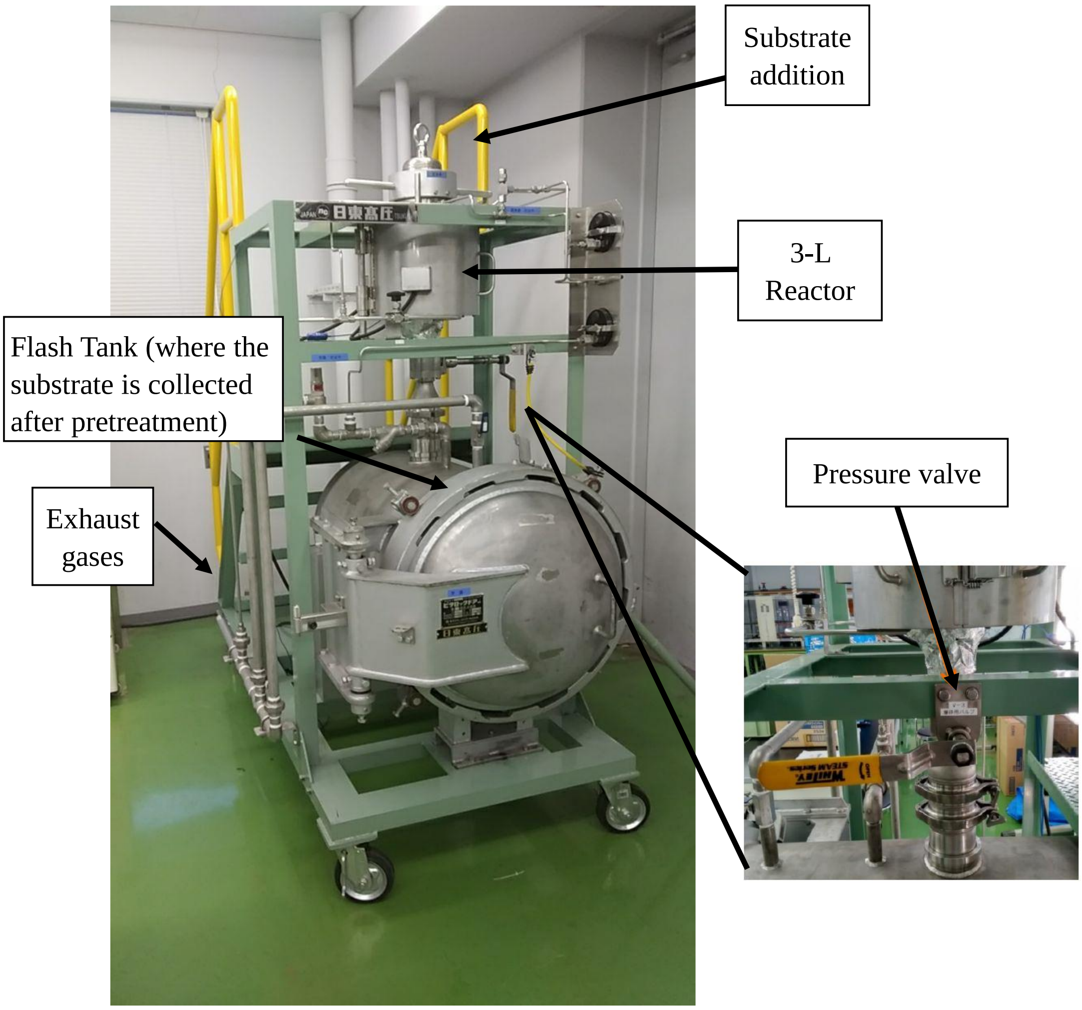
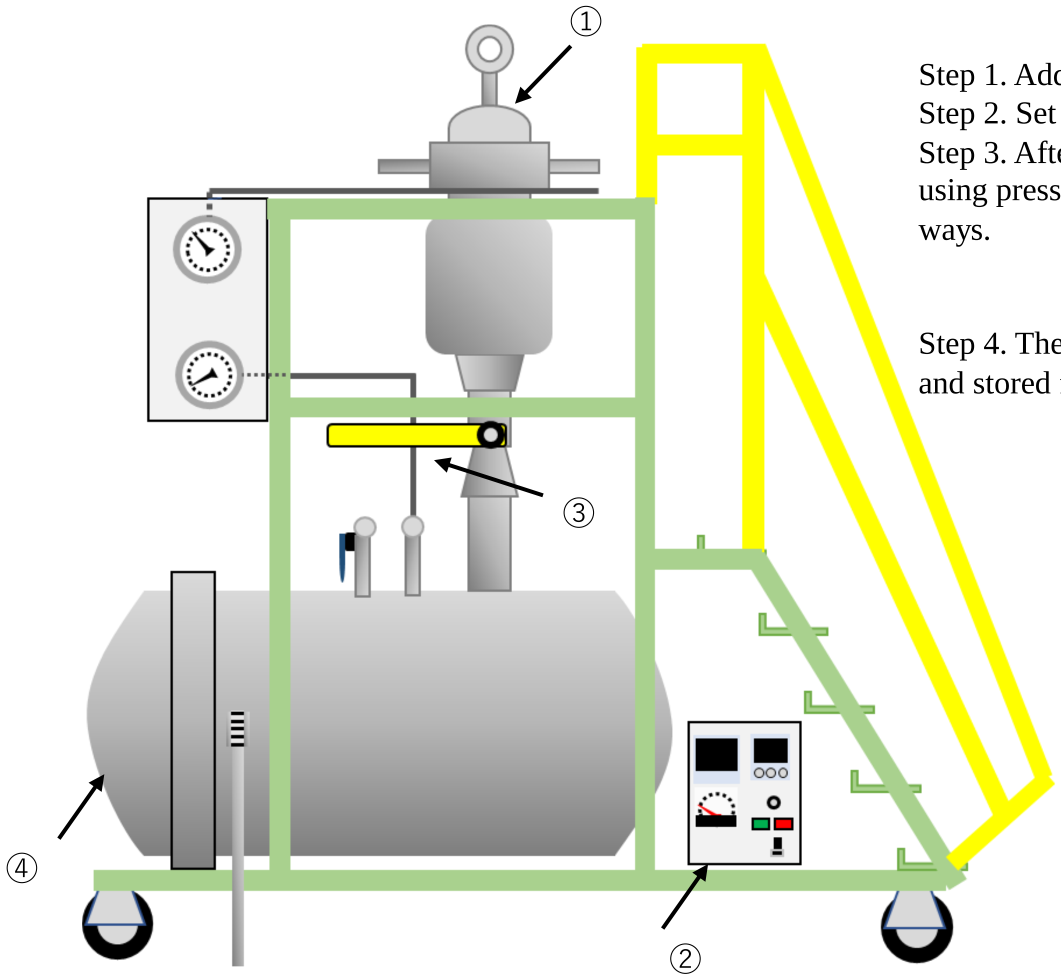


Fig 2-4. Thermal hydrolysis and steam explosion equipment used for study 1 and 2



Step 1. Adding substrate and milli-q water in the reactor ①
Step 2. Set the temperature on the controller ②
Step 3. After the temperature and retention tame has elapsed using pressure valve ③, the pressure can be released in two ways.

<u>1. For thermal hydrolysis: gradually</u>
<u>2. For steam explosion: suddenly</u>

Step 4. The substrate can be collected from flash tank ④ and stored for further experiment.

Fig 2-5. Schematic diagram and the step guide of the reactor used in this study

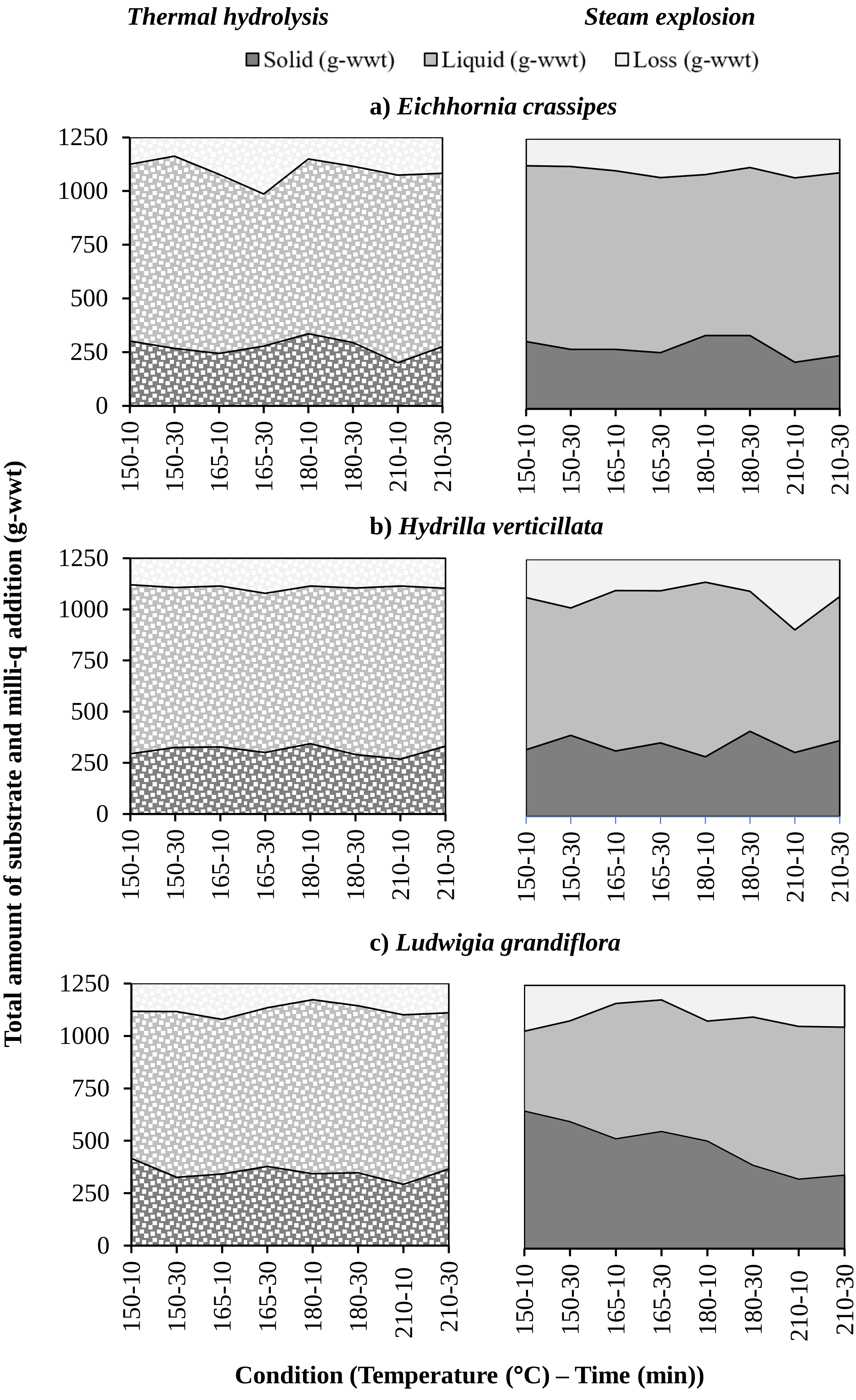
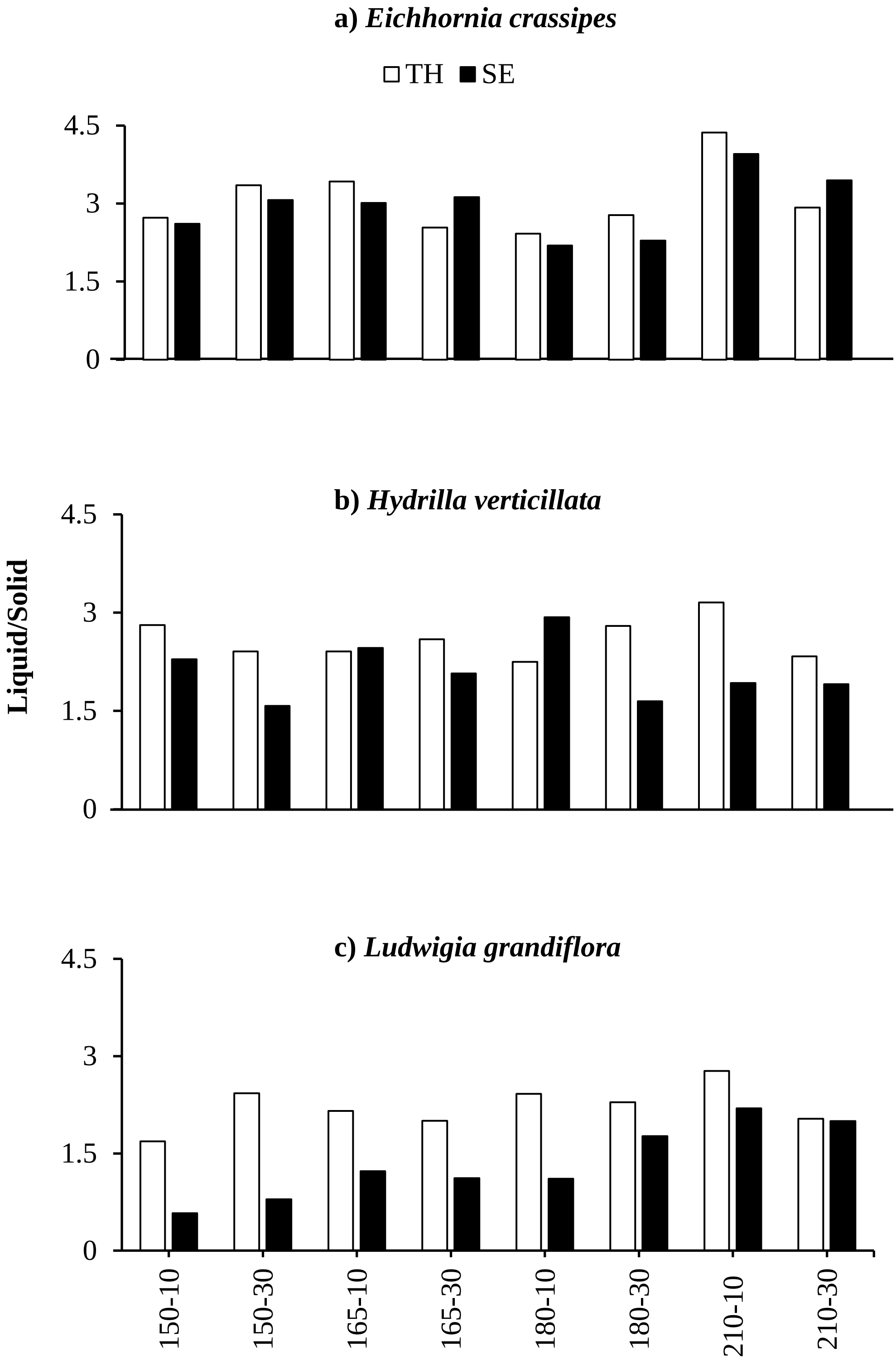


Fig 2-6. 2D area graph for the mass recovery of the thermal hydrolysis and steam explosion pretreated substrates

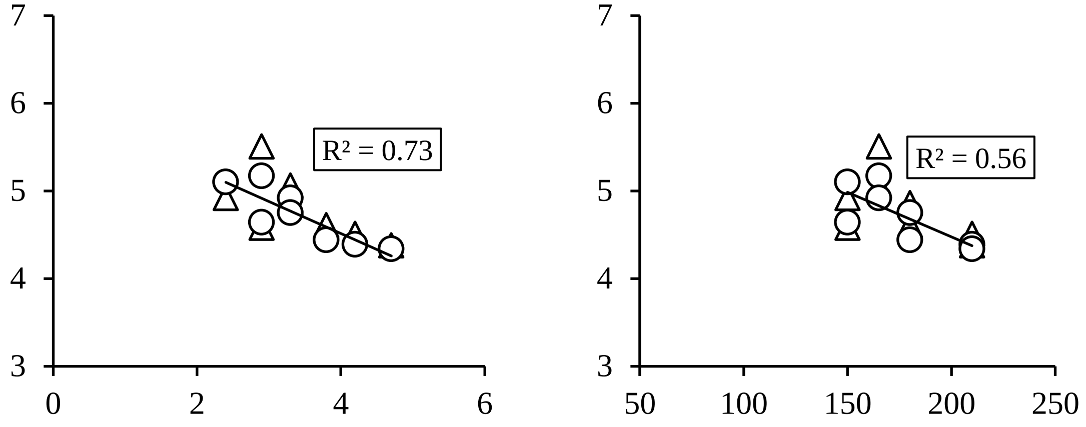


Condition (Temperature (°C) – Time (min))

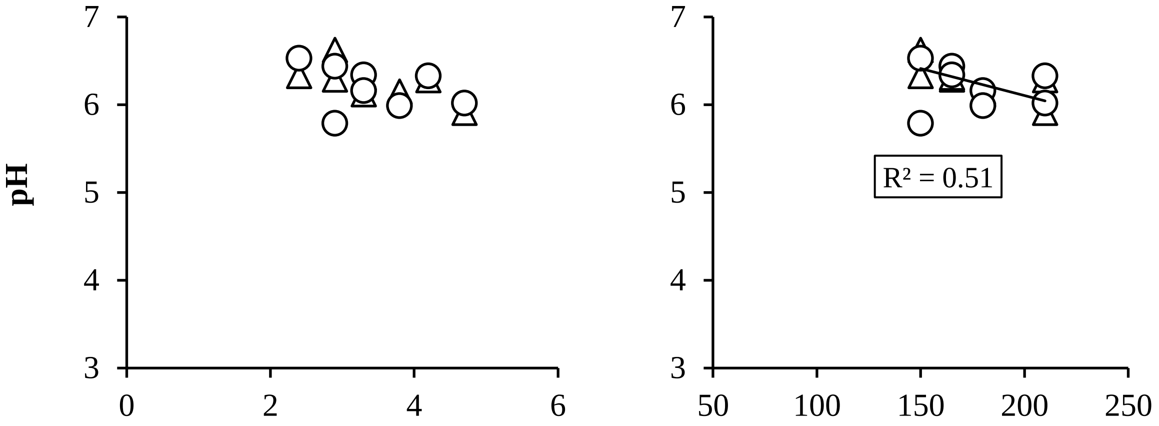
Fig 2-7. Liquid/Solid ratio of the thermal hydrolysis and steam explosion pretreated substrates

Thermal hydrolysis △ TH ○ SE ***Steam explosion***

a) ***Eichhornia crassipes***



b) ***Hydrilla verticillata***



c) ***Ludwigia grandiflora***

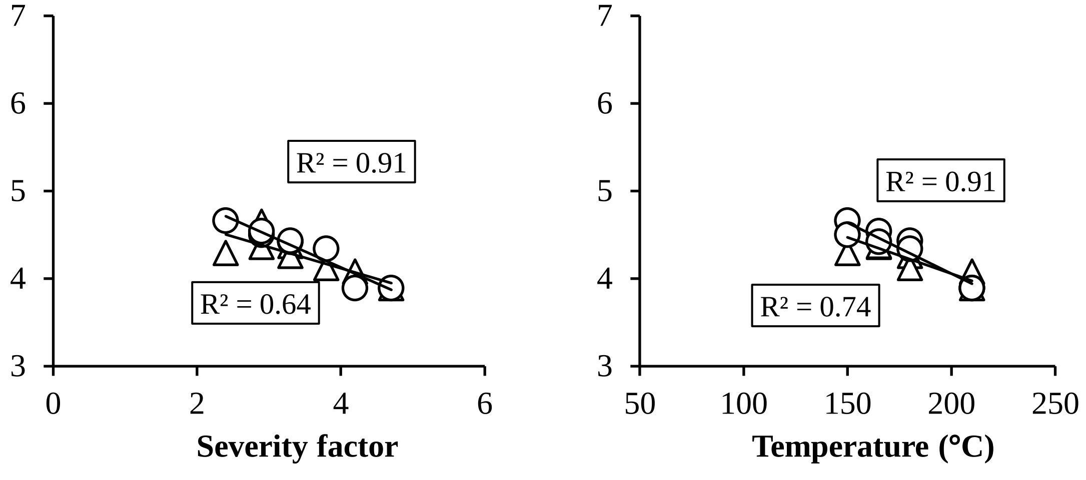
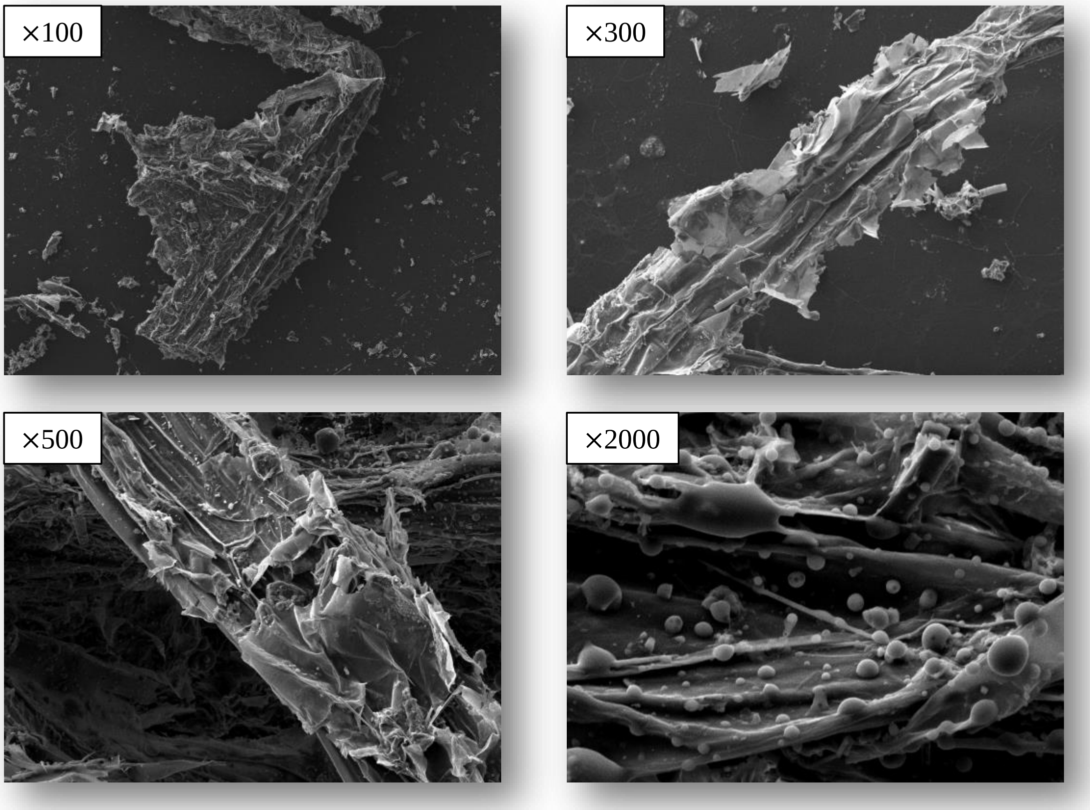
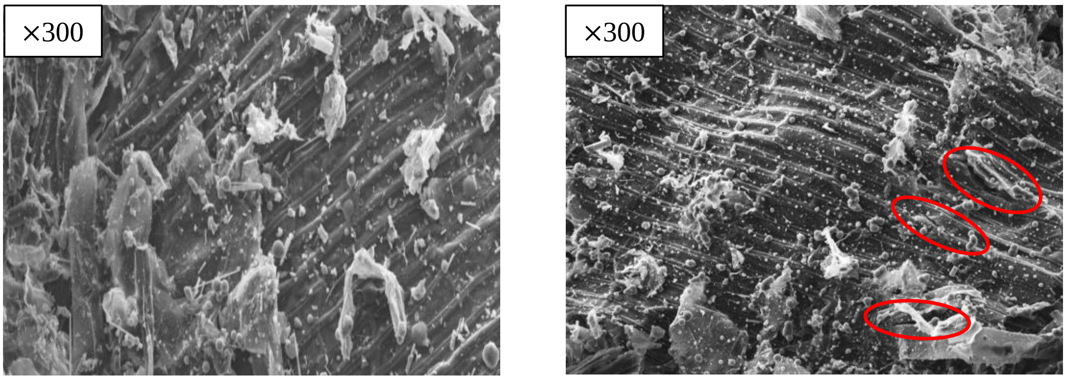


Fig 2-8. pH of slurry after the thermal hydrolysis and steam explosion of the pretreated substrates

Untreated



Pretreated

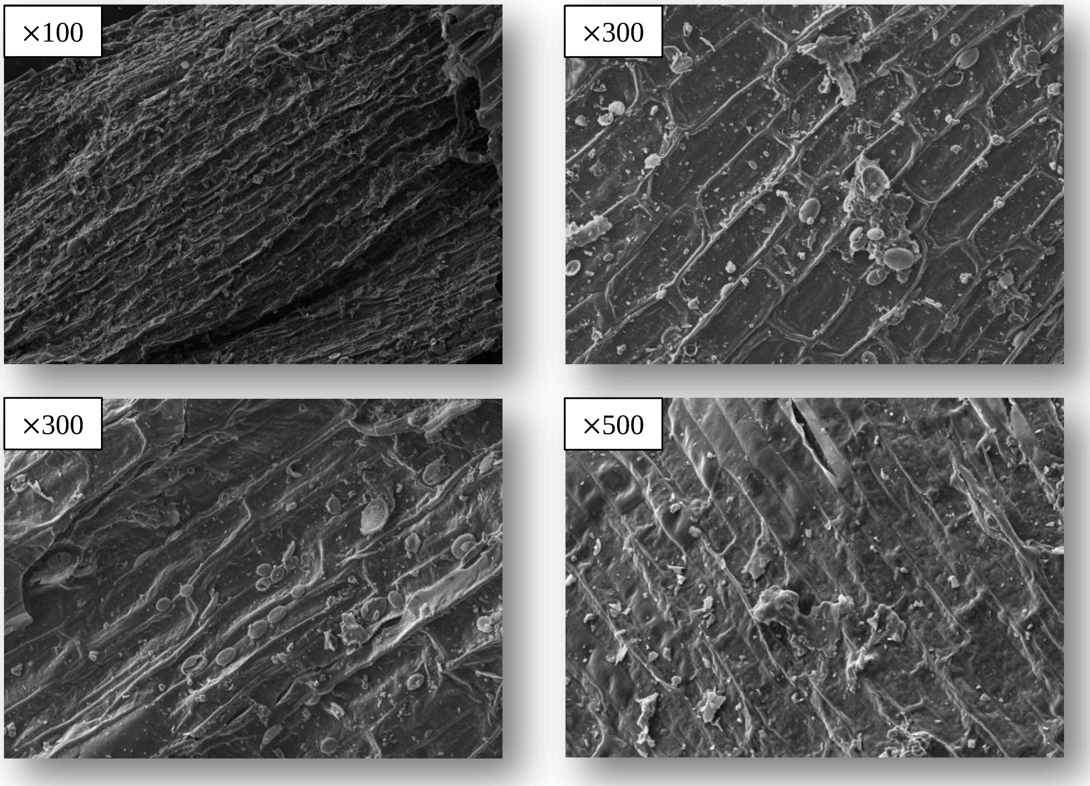


180°C-10 min, TH

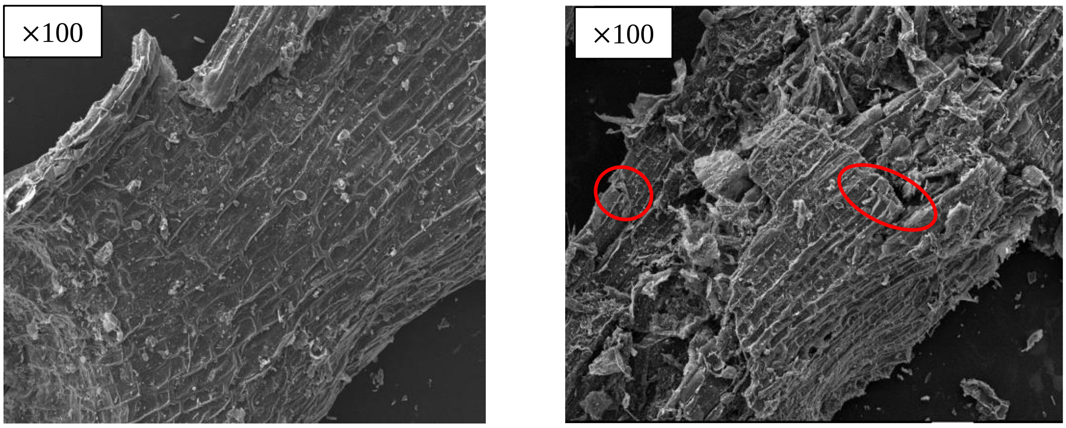
180°C-10 min SE

Image 2-1. SEM image for untreated and pretreated *Eichhornia crassipes*

Untreated



Pretreated

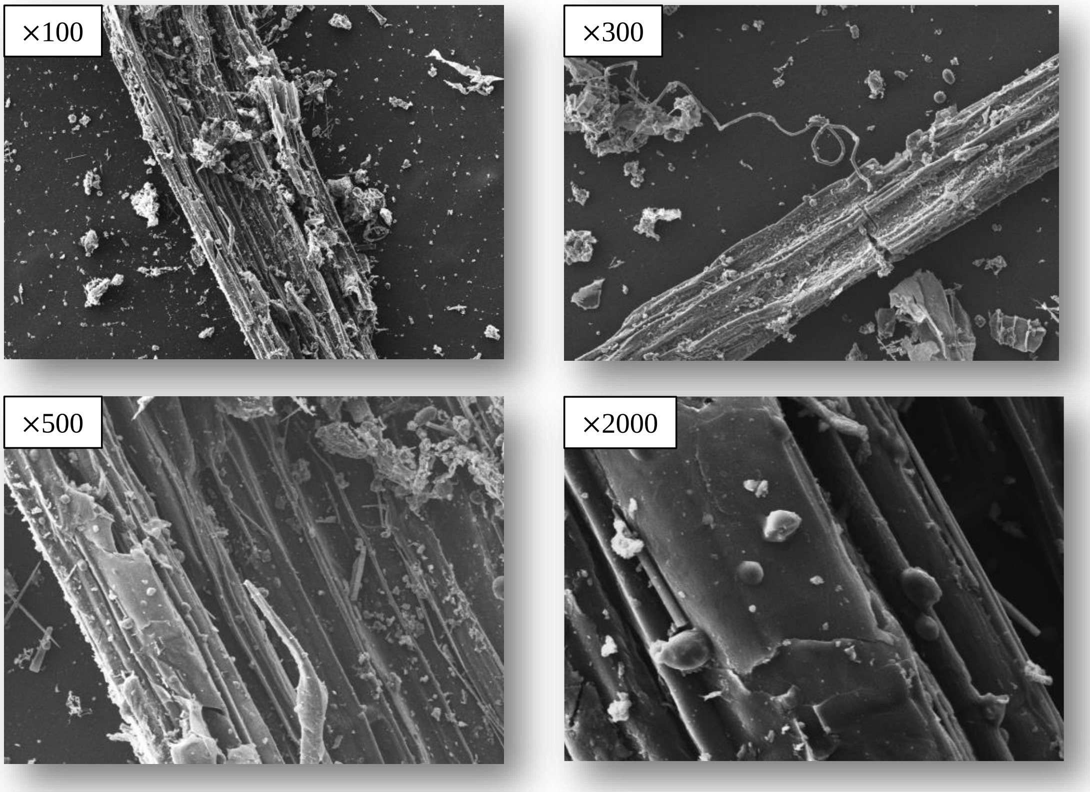


180°C-30 min, TH

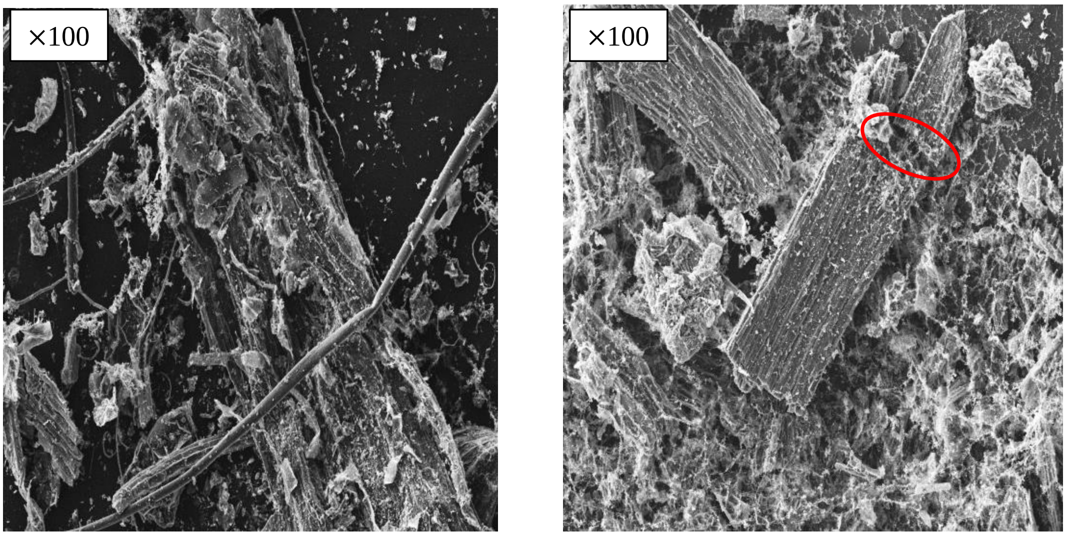
180°C-30 min, SE

Image 2-2. SEM image for untreated and pretreated *Hydrilla verticillata*

Untreated



Pretreated



165°C-30 min, SE

165°C-30 min, TH

Image 2-3. SEM image for untreated and pretreated *Ludwigia grandiflora*

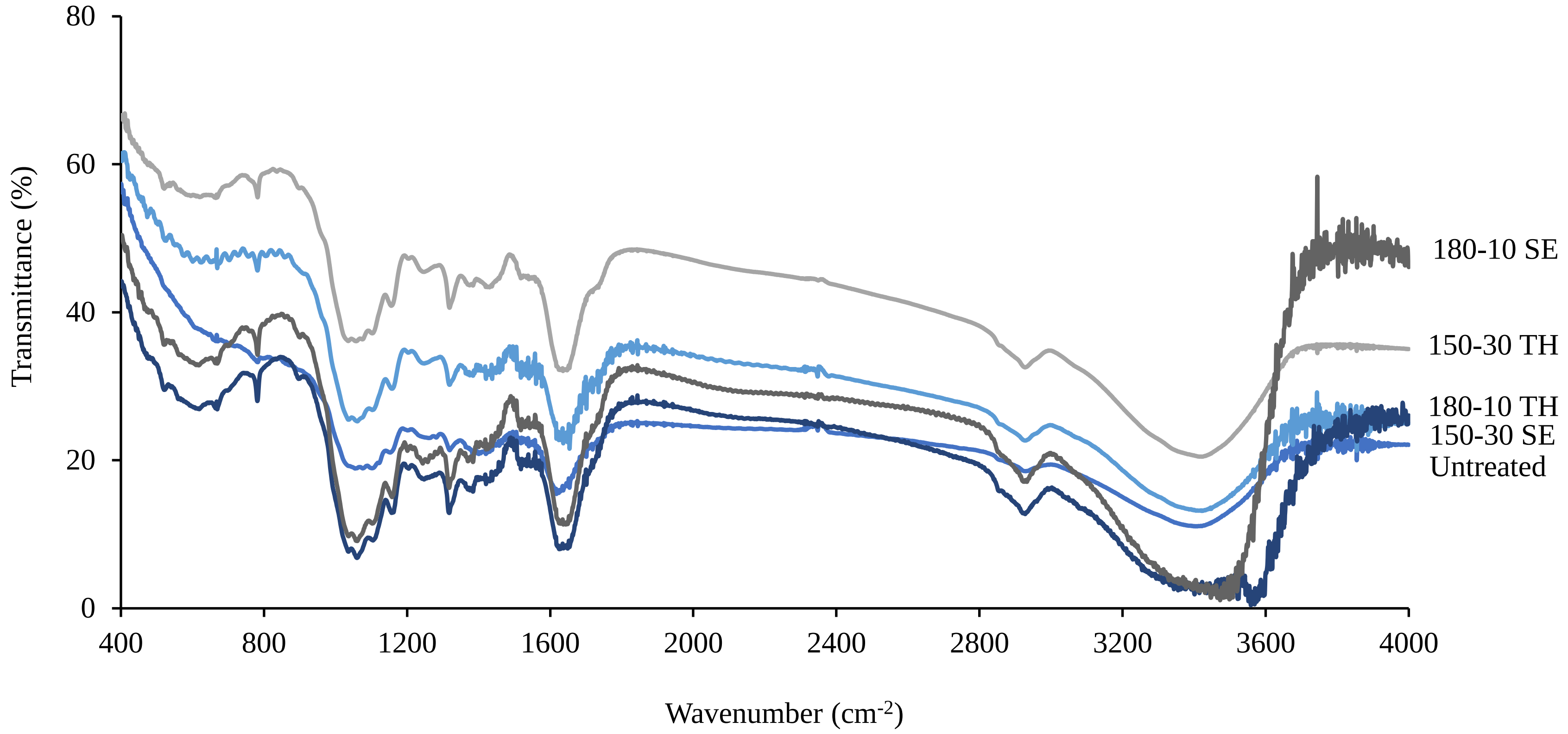


Fig 2-9. FTIR graphs during the thermal hydrolysis and steam explosion pretreatment of *Eichhornia crassipes* used in this study

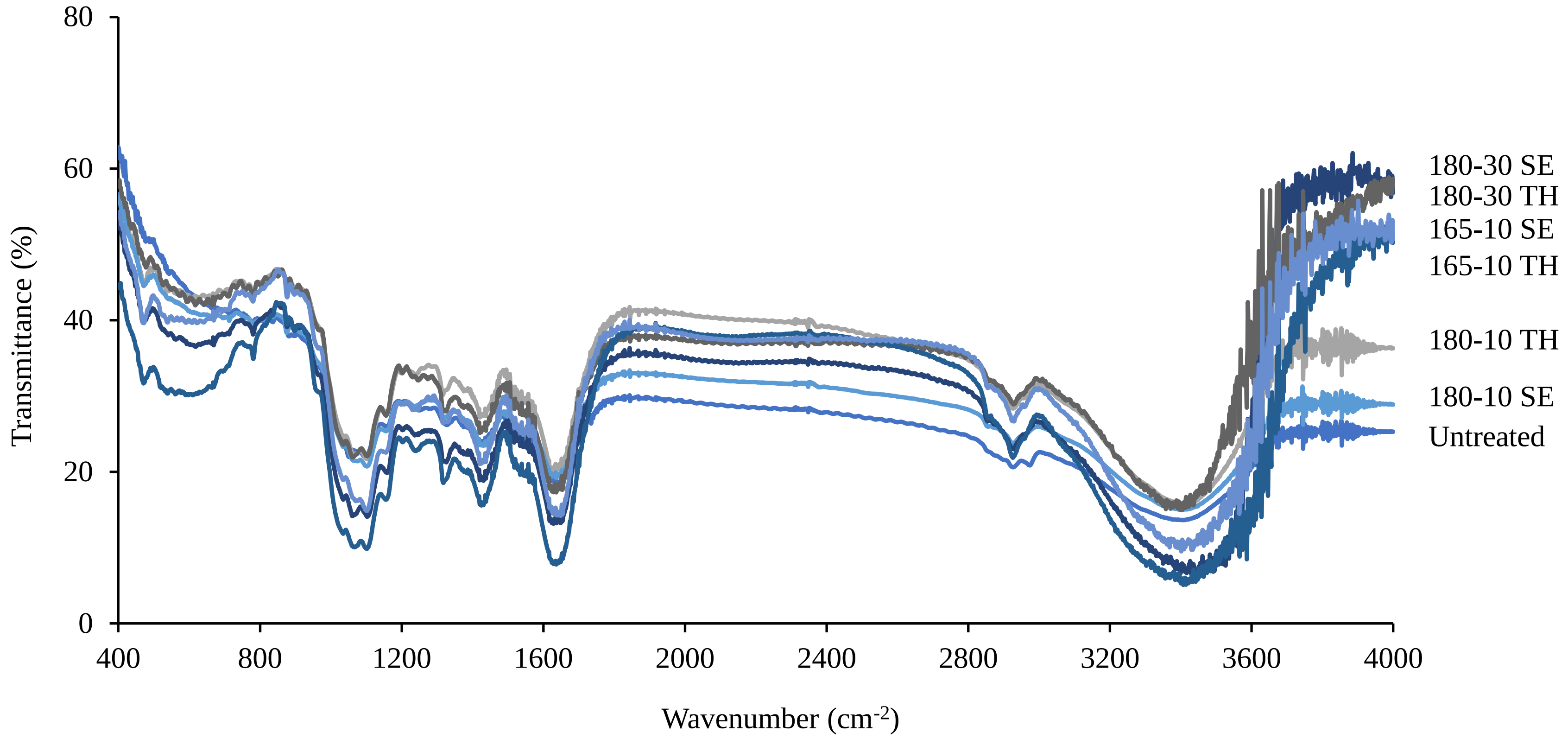


Fig 2-10. FTIR graphs during the thermal hydrolysis and steam explosion pretreatment of *Hydrilla verticillata* used in this study

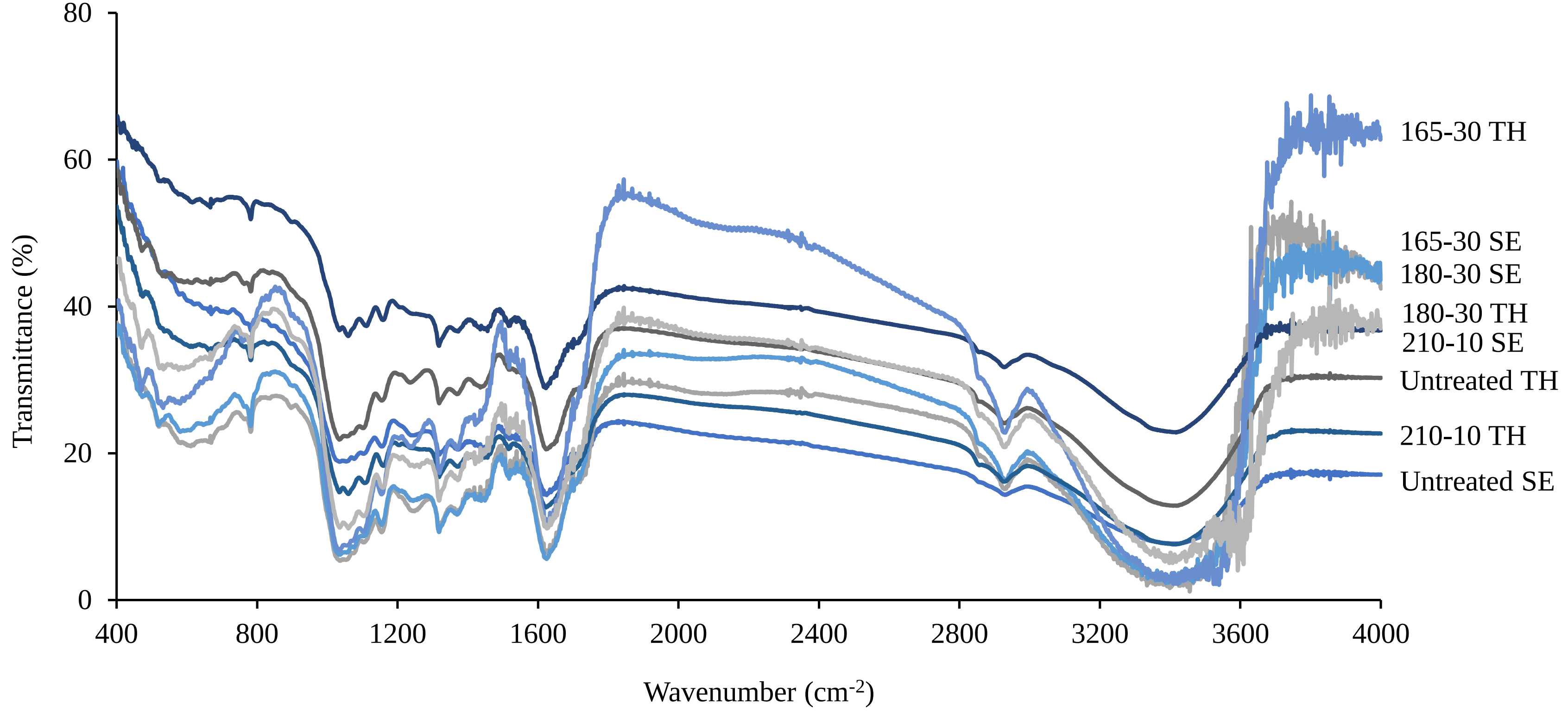


Fig 2-11. FTIR graphs during the thermal hydrolysis and steam explosion pretreatment of *Ludwigia grandiflora* used in this study

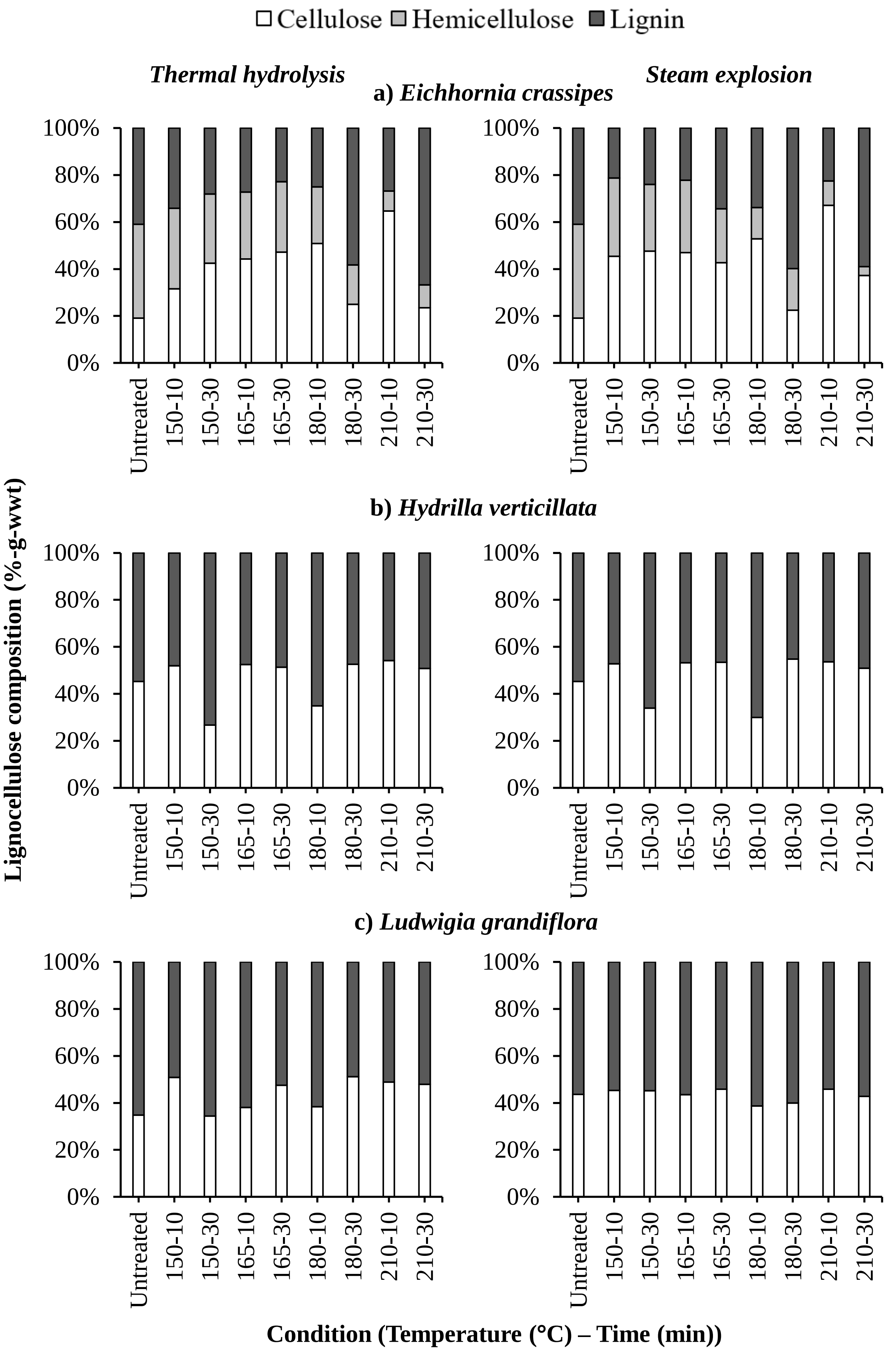


Fig 2-12. Composition of lignocellulose with the condition of thermal hydrolysis and steam explosion pretreatment for the substrates used in this study

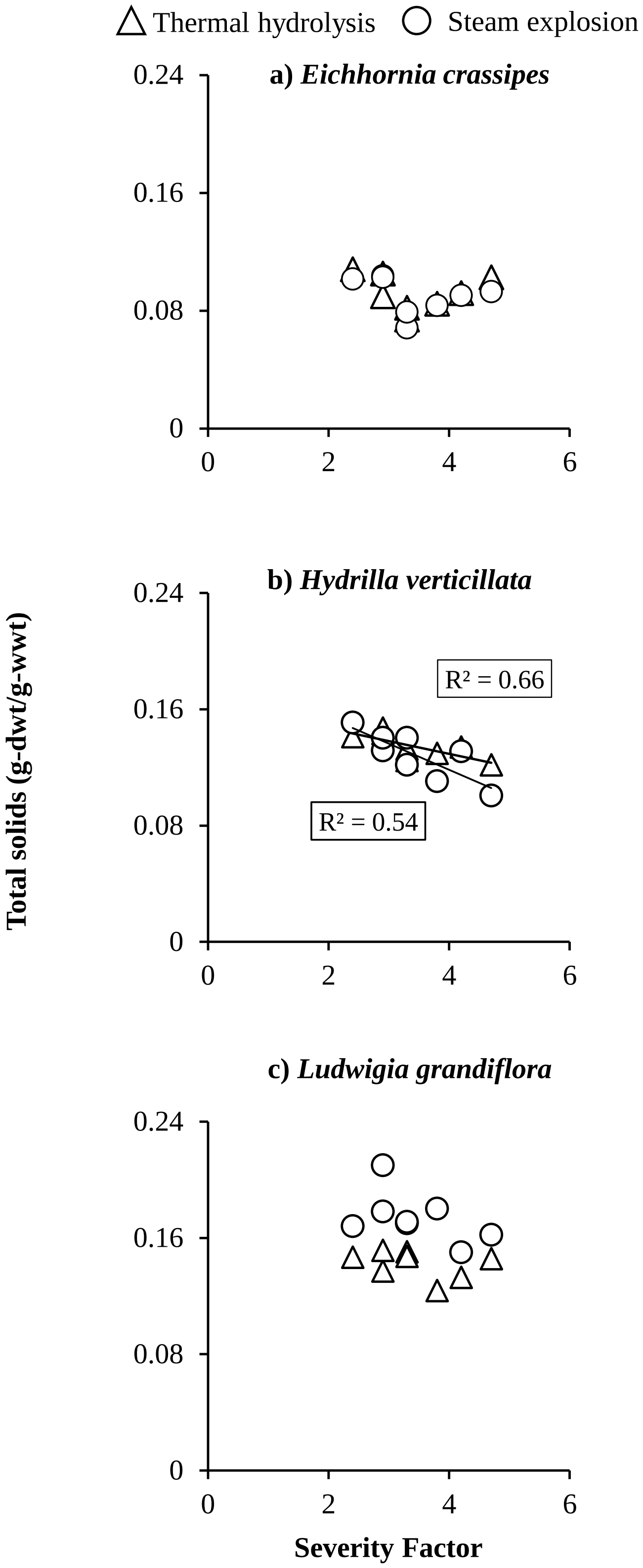


Fig 2-13. Total solids concentration in the solid fraction after thermal hydrolysis and steam explosion pretreatment relationship with the severity factor

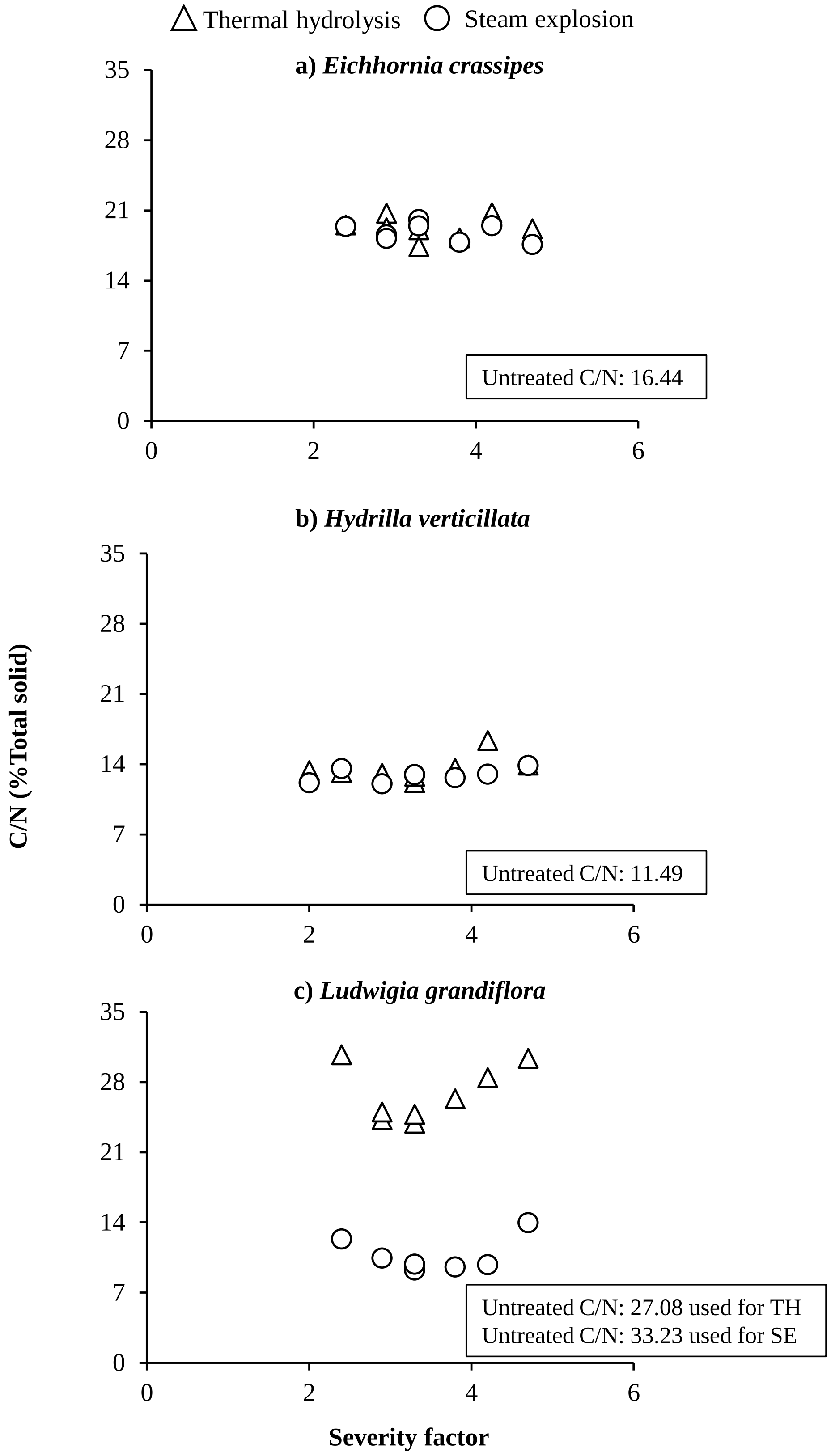


Fig 2-14. C/N ratio in the solid fraction after the thermal hydrolysis and steam explosion pretreatment relationship with the severity factor

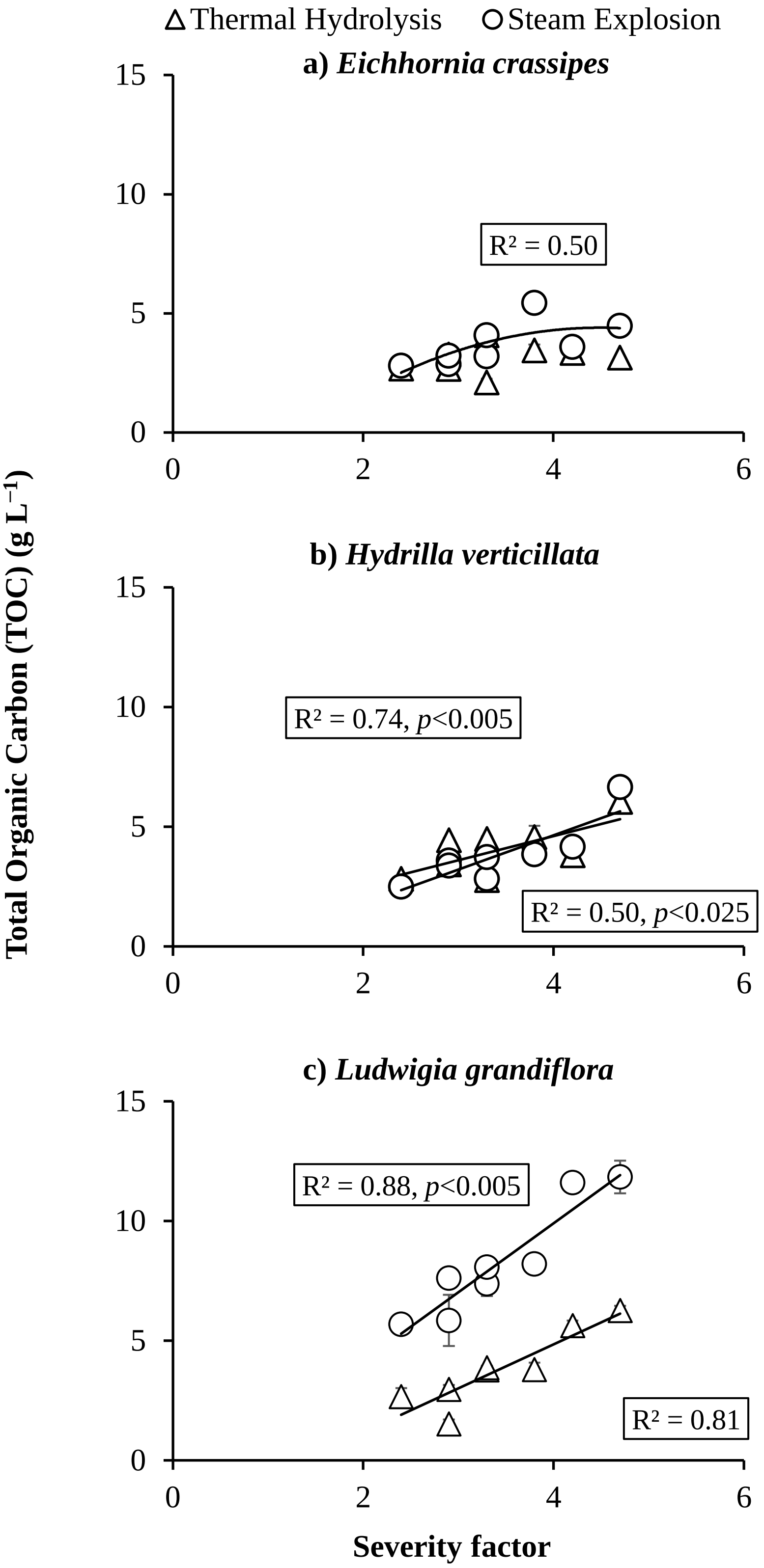


Fig 2-15. Total organic carbon in the liquid fraction after the thermal hydrolysis and steam explosion pretreatment relationship with the severity factor

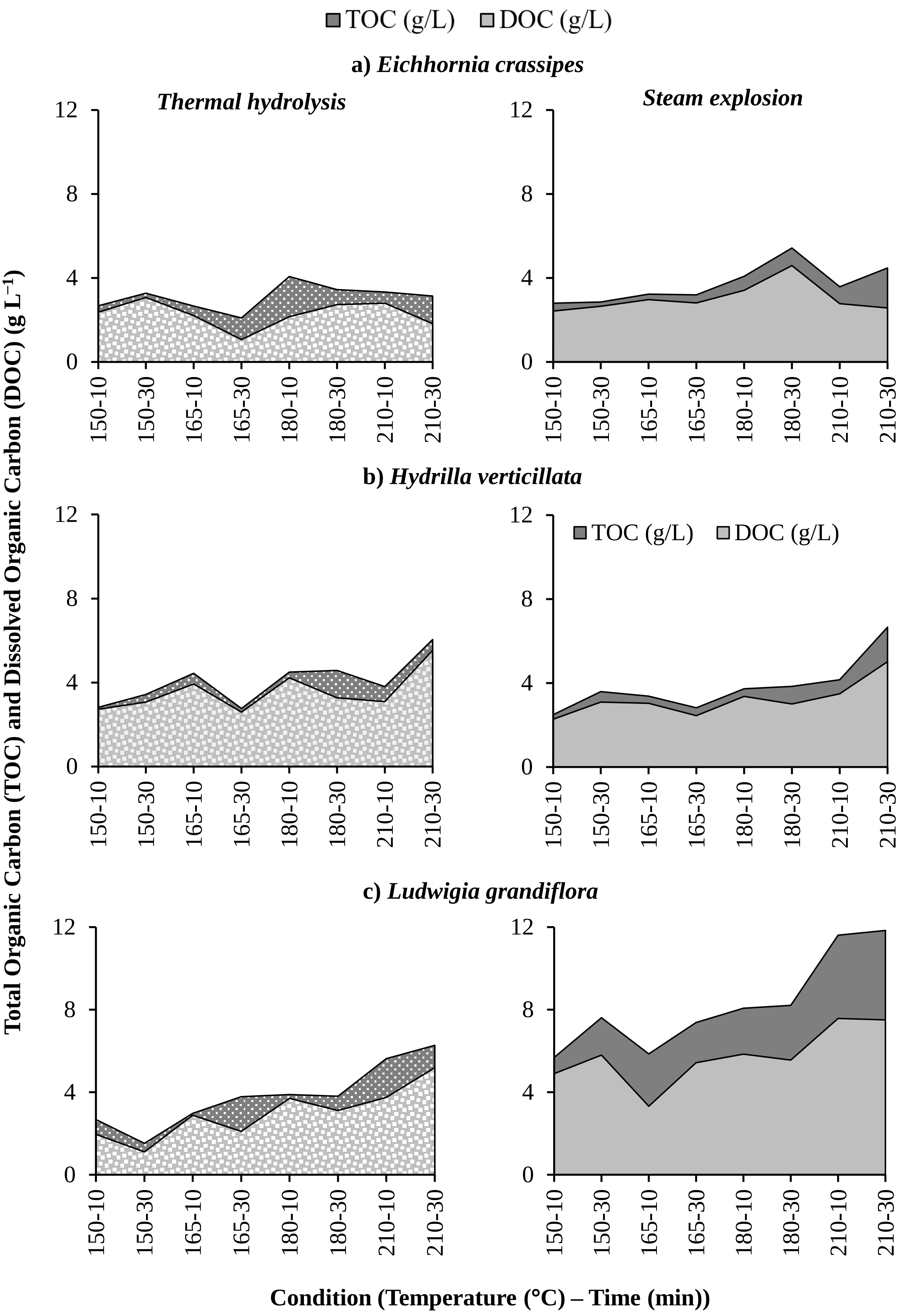


Fig 2-16. Total organic carbon and dissolved organic carbon concentration in the liquid fraction after thermal hydrolysis and steam explosion pretreatment

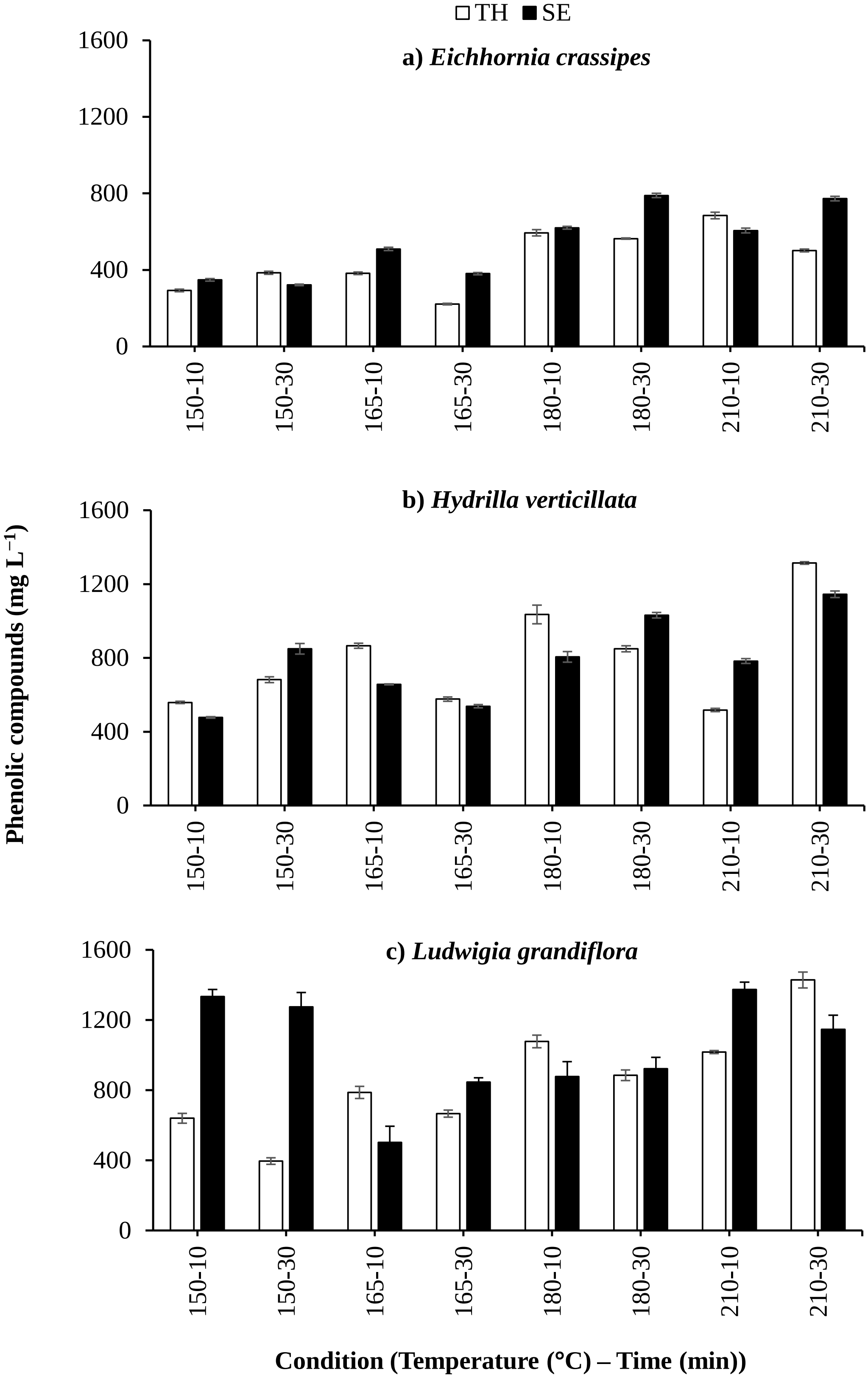


Fig 2-17. Phenolic compounds concentration during the thermal hydrolysis and steam explosion pretreatment

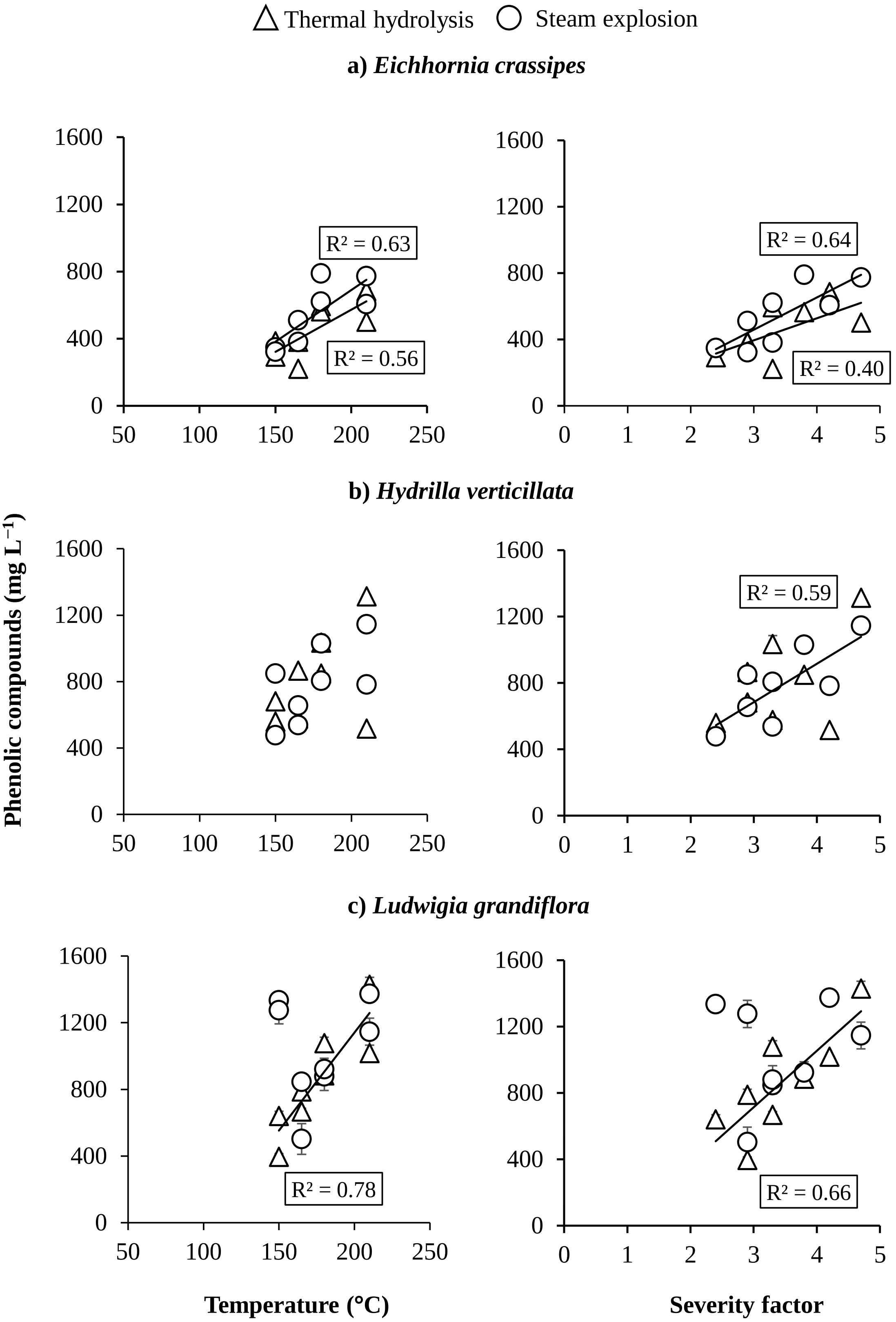


Fig 2-18. Phenolic compounds concentration relationship with temperature and severity factor during thermal hydrolysis and steam explosion pretreatment. a) *Eichhornia crassipes* , b) *Hydrilla verticillata*, c) *Ludwigia grandiflora*

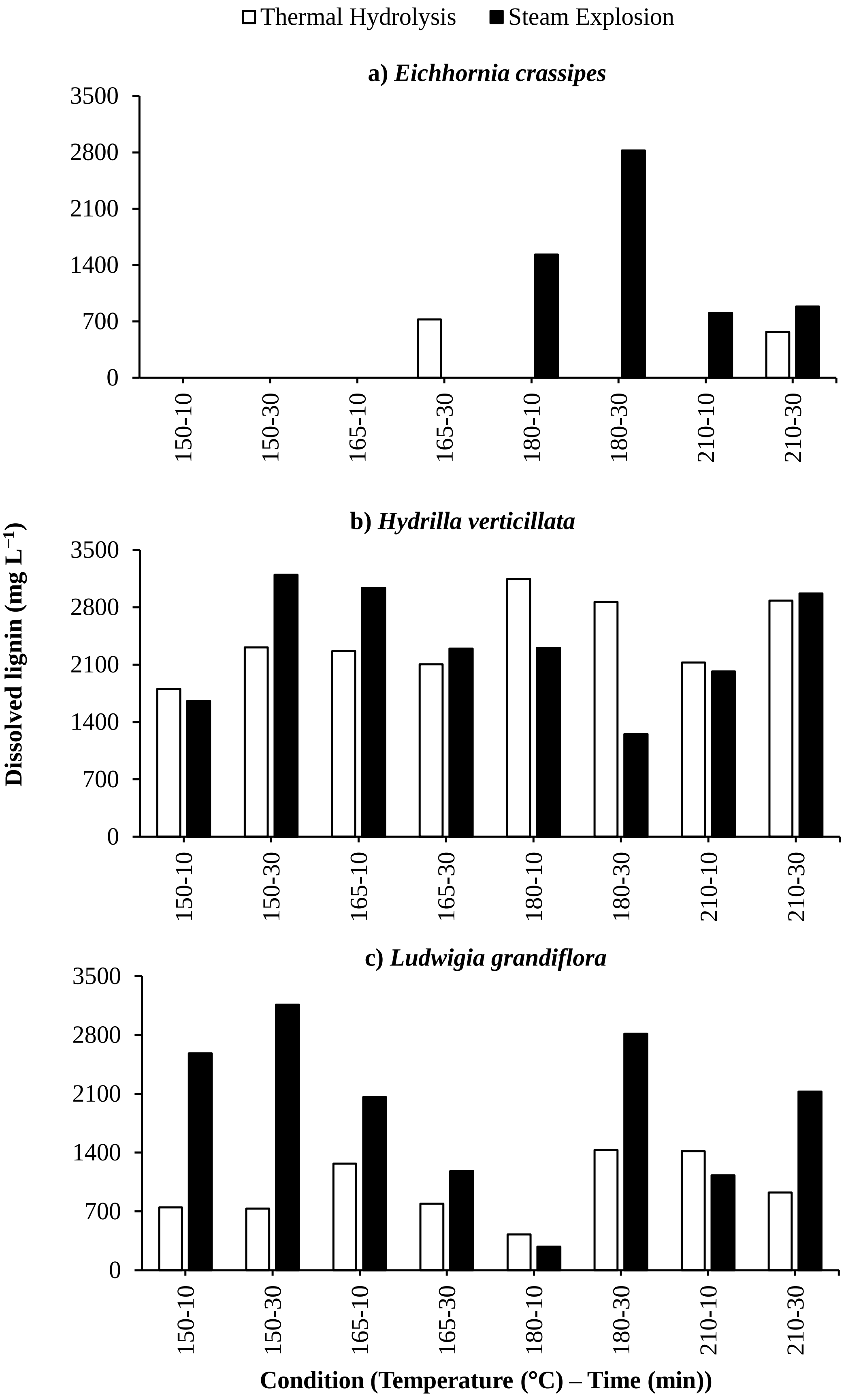


Fig 2-19. Dissolved lignin concentration during the thermal hydrolysis and steam explosion pretreatment

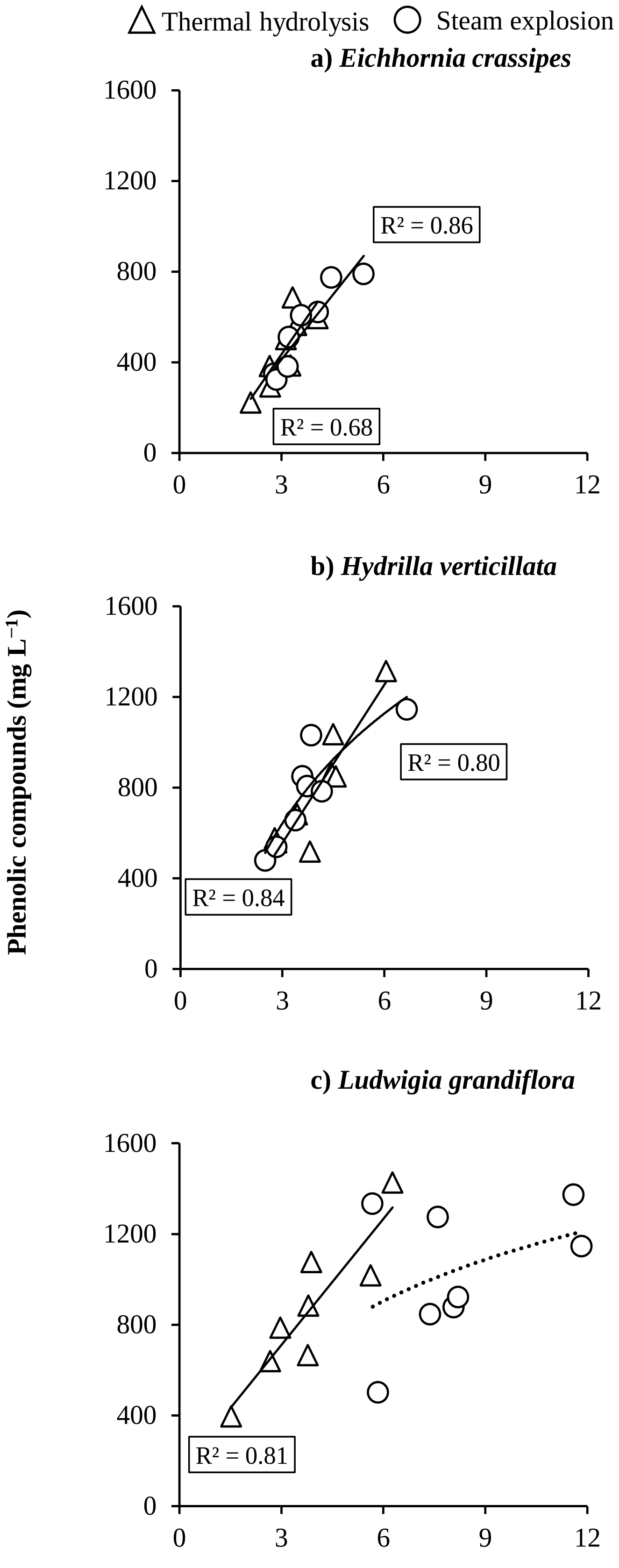


Fig 2-20. Phenolic compounds concentration relationship with total organic carbon in liquid fraction during thermal hydrolysis and steam explosion pretreatment

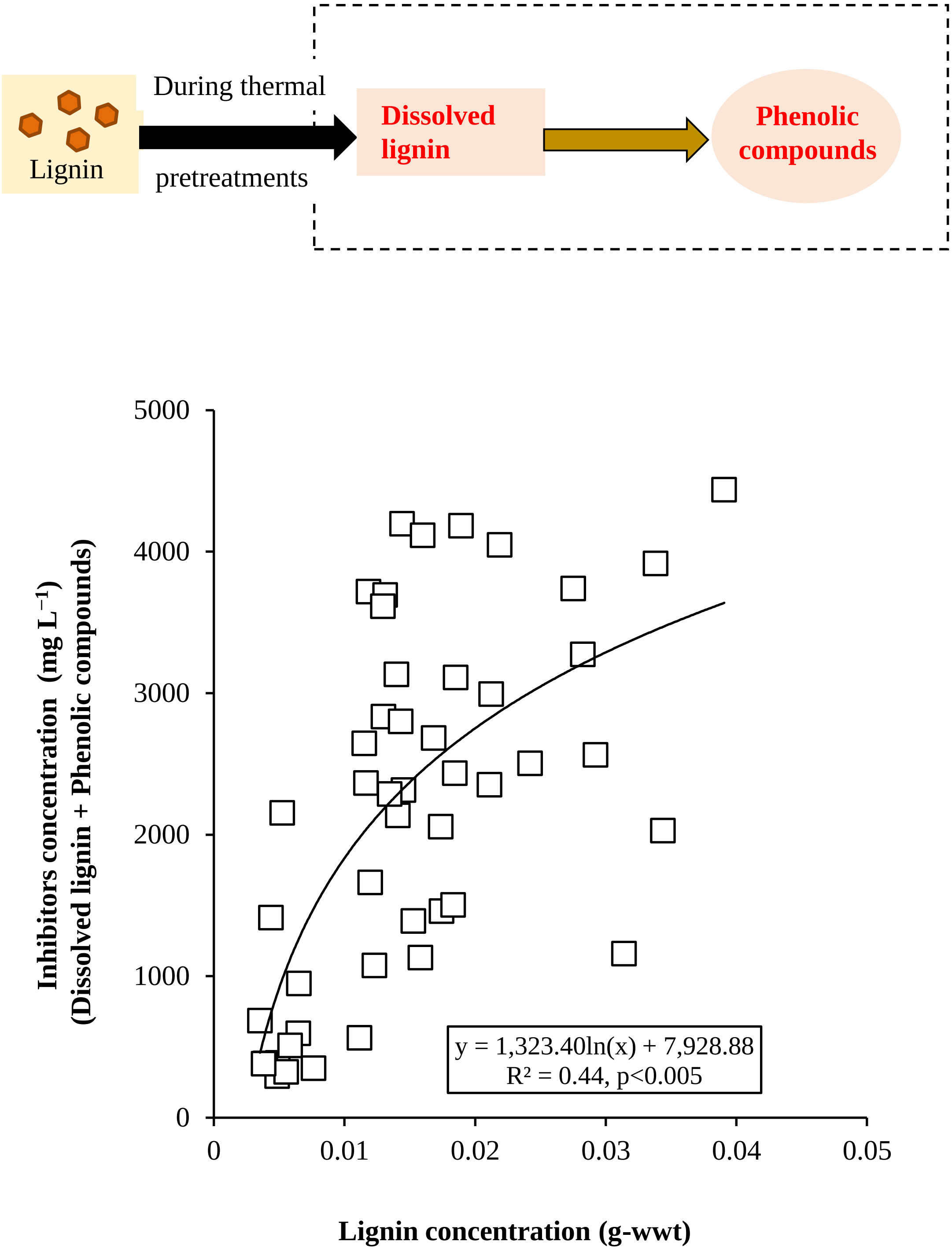


Fig. 2-21. TH and SE pretreated substrates observed a relationship between inhibitors concentration in the liquid fraction (dissolved lignin + phenolic compounds) and lignin concentration in the pretreated substrate

3. CHAPTER 3 EFFECT OF PRETREATMENTS ON THE ANAEROBIC DIGESTION OF PRETREATED LIGNOCELLULOSIC AQUATIC WEEDS

3.1. Introduction

3.1.1. Anaerobic digestion of different substrates with steam explosion as a pretreatment

SE pretreatment is widely used in previous studies on various lignocellulosic aquatic weeds, which are explained in Table 3-1. In a study by Vivekanand et al., (2014), when bagasse was used as a substrate. The lignin value in the untreated bagasse was 26.3% TS. The untreated methane yield for bagasse was around 160 mL-g VS^{-1}. The SE pretreatment was performed at severity factors 3.8, 4.1, 4.4, and 4.6. The methane yield after the SE pretreatment was maximum at the highest severity factor of 4.6 (225°C-10 min) with a value of 216 mL- g VS^{-1}.

On the other hand, wheat was used as a substrate (Ferreira et al., 2014) with a lignin content of 28.3% TS, higher than the bagasse explained before. This study performed the SE pretreatment at a lower SF, starting from 2.65 to 4.23. The untreated methane yield for wheat was 226 mL- g VS-1. The maximum methane after the SE pretreatment was for the severity factor of 3.53 at 273 mL- g VS-1. As per the basic understanding, maximum SF produces maximum methane, according to Vivekanand et al., 2014, but this study proves that a higher SF dose does not ensure a higher methane yield. Also, the lignocellulose structure (physical and chemical composition) and the pretreatment conditions (treatment temperature and retention time) can affect the methanogenesis process. Therefore, the effect of different substrates pretreated with TH and SE at different pretreatment conditions on AD will be evaluated.

3.2. Material and Methods

3.2.1. Substrate and Inoculum

The substrates after the TH and SE pretreatment in Chapter 2 will be used for evaluating the effect of the pretreatment on anaerobic digestion. The inoculum for the batch test was mesophilic AD sludge obtained from a biogas plant (6800 m^3) that treats domestic sewage from Hokubu Sludge Treatment Centre in Yokohama, Japan. The sludge was degassed at 37 °C for four days to remove the residual organic material in the inoculum batch before performing AD tests. The total solids (TS) and the volatile solids (VS) contents in the sludge were 1.8%-wwt and 1.3%-wwt, respectively.

3.2.2. Biomethane potential tests (BMP)

The Biomethane potential tests were performed for around three weeks (20 days) at a mesophilic temperature of 37 ± 1 °C. A combination of solid and liquid fractions (1.7 g-VS) was added to a 500-mL medium bottle. The substrate to inoculum ratio in the reactor was 1:2 based on the VS content. Milli-Q water was added to the medium bottle to adjust the volume of the reactor, respectively. The VS content of 1.7 g was adjusted using the mixed VS and L/S ratio for every condition of the BMP reactor. A control condition was tested without a substrate to quantify the methane production of the inoculum. All conditions were conducted in triplicates to evaluate the error result. The medium bottles were purged with argon/nitrogen gas to create an anaerobic environment. The BMP tests were performed by an automated AD system (AMPTS II, Bioprocess Control AB, Sweden) with agitation/no agitation cycles of 10 s (Figure 3-1). The biogas produced in the digester was transferred to a CO_2 absorption unit filled with 3M NaOH.

3.2.3. Calculations

The cumulative methane yield of each test was fitted to a modified Gompertz equation to investigate the anaerobic digestibility of aquatic weeds:

$$M = P \left\{ -\exp \left[\frac{Rm \cdot e}{P} (\lambda - t) + 1 \right] \right\}$$

where M (t) is the measured cumulative methane production (mL g- VS^{-1}) at a given time t (days), P refers to the ultimate methane yield (mL g-VS^{-1}), R_m is the maximum methane production rate (mL g-VS^{-1} day^{-1}), λ represents the lag phase to produce methane (days), t stands for the digestion time (days), and e is equal to 2.7183. The least-square Fitting and t-test were performed using the solver add-in function in Excel 2016. Differences with $p < 0.05$ were considered significant.

T80 representing the required days to finish 80% of the ultimate methane yield, was calculated from the obtained parameters. Multiple regression analysis was performed to provide a statistical model for predicting anaerobic digestibility parameters.

The methane yield improvement was calculated using the equation given below:

$$\text{Methane yield improvement (\%)} \frac{\text{Methane yield}_{\text{pretreated}} \text{-Methane yield}_{\text{untreated}}}{\text{Methane yield}_{\text{untreated}}} \times 100$$

3.3. Results and Discussions

3.3.1. Methane yield of untreated biomass

The present study shows the total untreated methane yield for the aquatic weeds used in this study (Table 3-2).

The methane yield for the untreated *E. crassipes* is 166.57 mL g-VS^{-1}, compared with a study done by Kist et al., (2018), which shows that the methane yield (140-193 mL g-VS^{-1}) is similar to this study. Interestingly, there was a significant difference for *H. verticillata*. The value for the untreated *H. verticillata* in this study was 231.81 mL g-VS^{-1} as shown in Table 3-2, whereas in a previous study, the value was 81 mL g-VS^{-1} (Abbasi et al., 1990). This difference can be attributed to factors such as harvesting period, growth origin, and degradability as explained in (Li et al., 2014; Fujiwara et al., 2022). Fujiwara et al., (2022) mention the difference in lignocellulosic components due to the harvesting time, affecting methane yield. The higher methane yield in this study may be due to overly degraded substrate leading to a difference in this study from the previous study. The untreated methane yield for *L. grandiflora* was 85.84 mL g-VS^{-1}. *L. grandiflora* is considered an emergent aquatic plant with a rigid body because it emerges from the water (Asaeda et al., 2005). *L. grandiflora* has a lignin amount of 34.3%TS. Lignin strengthens a plant's cell walls; thus, rigidity can be shown by lignin content (Kumar and Gupta, 1992) which may have affected lowering the methane yield.

Several studies stated that lignin content influences methane recovery explained in chapter 2 (Koyama et al., 2014). In the present study, the lignin content of all the substrates is much higher than other feedstocks. Figure 3-2 states the relationship between lignin value and the CH_4 yield from previous studies. From the Figure, it was clear that the methane yield decreases with the increase in the lignin content. It is found that *E. crassipes*, *H. verticillata*, and *L. grandiflora* lignin content is higher and needs a compelling TH or SE pretreatment to enhance the total methane yield of these untreated substrates.

3.3.2. Effect of thermal hydrolysis and steam explosion pretreatment on AD process

3.3.2.1. Cumulative methane yield

The cumulative methane yield is shown in Figure 3-3 and its relationship with the severity factor is shown in Figure 3-4. Firstly, the methane yield for *E. crassipes* is shown in Figure 3-3. For all the conditions from 150°C-10 min to 210°C-30 min for both TH and SE pretreatment, the values were higher than the untreated substrate meaning that TH and SE effectively enhanced the methane yield. The CH_4 yield after TH pretreatment shows no trend, the lowest value being at 165°C-30 min (169.85 mL g-VS^{-1}) and the highest value at a condition of 180°C-10 min (316.33 mL g-VS^{-1}). The value at 180°C-10 min was 1.9 times higher than the untreated condition. The methane yield after the SE pretreatment also has no trend; the lowest value was at the same condition as TH, i.e., 165°C-30 min (168.28 mL g-VS^{-1}), whereas the highest value was at a condition of 180°C-30 min (256.73 mL g-VS^{-1}). The methane yield was 1.5 times higher than the untreated condition. From Figure 3-4, the relation with the severity factor can be seen, suggesting that *E. crassipes* methane yield has no trend with the severity factor. When TH and SE were performed by Regidor Alfageme, 2019 (Master thesis), the maximum methane yield for TH was 293 mL g-VS-1 at 170°C-60 min, whereas for SE pretreatment, the maximum methane yield was around 347 mL g-VS-1 at 170°C-60 min. A temperature of 170 °C was the optimum temperature for both TH and SE pretreatment whereas in this study the optimum temperature was 180 °C for TH and SE. Also, in Regidor Alfageme, 2019 (Master thesis), only three different treatment temperatures were used, making it difficult to understand the mechanism for both TH and SE.

The methane yield of pretreated *H. verticillata*, was mainly lower than the untreated condition for both TH and SE pretreatment. The methane yield after the TH pretreatment was enhanced only at one condition of 180°C-30 min (288.47 mL g-VS^{-1}). This was 1.24 times higher than the untreated condition. For the SE pretreatment, the values of methane yield were lower than the untreated condition, with only one condition enhancing the methane yield at 165°C-10 min (258.08 mL g-VS^{-1}). One of the reasons for the low enhancement of methane yield for both TH and SE pretreatment is the higher methane yield of the untreated substrate, as discussed before in 3.3.1. Also, from Figure 3-4, it can be seen that for both TH and SE, the methane yield values reached an optimum value and then started to decrease at the maximum SF which can be due to some inhibitory compounds which are produced during high temperatures.

Lastly, for *L. grandiflora*, the methane yield was enhanced for both TH and SE pretreatment in most conditions. After the TH pretreatment, the methane yield was lowest at 150°C-30 min (107.77 mL g-VS^{-1}) and highest at a condition of 180°C-30 min (206.63 mL g-VS^{-1}), enhancing the methane 1.46 times higher than in the untreated condition. There was no trend seen for the TH pretreatment methane yield. Similarly, after the SE pretreatment, the lowest value of methane yield was at a condition of 150°C-30 min (80.82 mL g-VS^{-1}) and the highest at a condition of 165°C-30 min (271.37 mL g-VS^{-1}). The enhancement of methane yield was 3.16 times higher than in the untreated condition.

When observing Fig 3-4, it was seen that SE pretreated biomass, methane yield values reached an optimum point and started decreasing after a specific severity factor. Bhatia et al., (2020) mentioned that at a lower temperature (120–150 °C), the hydrolysis acted as a limiting step, while at a temperature range of 165–210 °C, the inhibitory effect had a relatively significant effect.

3.3.2.2. **Methane production rate**

Fig 3-5 shows the methane production rate of the untreated substrates used in this study. It was seen that the methane production rate of *E. crassipes* and *L. grandiflora* was relatively low. The methane production rate for *E. crassipes* was around 28.5 mL g-VS^{-1} day^{-1} maximum on day 2. The value started to decrease after day 2. Similarly, for *L. grandiflora*, the maximum methane production rate was observed on day 4 (17.3 mL g-VS^{-1} day^{-1}). Whereas the value for *H. verticillata*, the maximum methane rate was on day 3 with a value of 74.3 mL g-VS^{-1} day^{-1}; which is relatively higher than other substrates used in this study.

Fig 3-6 shows the methane production rate of *E. crassipes* after TH and SE pretreatment under different conditions. TH and SE enhanced the methane production rate of *E. crassipes*, with TH being more effective in all the conditions. As mentioned in previous studies, hydrolysis of *E. crassipes* was enhanced by both TH and SE pretreatment. Anaerobic microbes metabolized cellulose solubilized by both thermal pretreatments. Therefore, it is revealed that TH and SE improve the hydrolysis step, which helps improve the CH_4 production rate by giving higher values in the initial days. For almost all the conditions, the methane production was finished within the first five days of the AD, leading to a faster AD process.

For *H. verticillata*, as the methane production rate of untreated biomass was much higher, only a few conditions could surpass the value from the untreated condition (Fig. 3-7). For TH, the conditions which enhanced the methane production rate were 150°C-30, 165°C -10, and 180°C -30, whereas for the SE pretreated biomass, even though the values of methane yield were lower than the untreated condition, the methane production rate of some conditions which were improved are 165°C-30, 180°C-30 and 210°C-10 min. This indicates the initial hydrolysis improvement, which is improved after the TH and SE pretreatment.

Finally, the maximum methane rate of *L. grandiflora* pretreated with SE was 95.5 mL g-VS^{-1} day^{-1} on the first day when the pretreatment condition was 30 min, 165°C, and SF = 3.3 (Fig. 3-8). A relatively high value (95.3 mL g-VS^{-1} day^{-1}) was obtained after the first day with a condition of 10 min at 180°C and SF = 3.3. Therefore, the initial methanogenesis rate of *L. grandiflora* was enhanced by SE. From day one onwards, the methanogenesis rate declined rapidly under all the tested conditions, indicating that methane generation is nearly completed at the early stages. The rapid methane production can be due to the decomposition of soluble organic matter. Therefore, SE helps to increase methane production by enhancing the hydrolysis step and increasing the rate during the first few days. Similarly, TH also enhanced the methane production rate of *L. grandiflora* but, when compared with SE, was much lower, proving that substrate like *L. grandiflora*, which has a woody structure and a high lignin content, requires SE as a pretreatment to increase the methane yield faster and more efficiently.

3.3.2.3. Kinetic parameters obtained by modified Gompertz equation

Kinetic parameters by TH and SE were calculated by fitting to the modified Gompertz model in Table 3-3, 3-4, and 3-5.

For *E. crassipes*, the P values for untreated and pretreated by TH and SE were similar to the experimental data attained by the AD tests. The value of R_m was improved from the untreated condition, the R_m for the untreated condition was 13.69 mL g-VS^{-1} day^{-1}. This value ranged from 41.05-86.71 mL g-VS^{-1} day^{-1} for TH pretreatment and 32.39-87.85 mL g-VS^{-1} day^{-1} for SE pretreatment. This increase in the R_m means both TH and SE pretreatment improved the hydrolysis rate. The value for the lag phase for both TH and SE suggested *E. crassipes* was an easily degradable substrate. After the fitting of R^2 was high, the fitting of the modified Gompertz was a success. When Regidor Alfageme, 2019 (Master thesis) used Gompertz modeling, shows that the 170ºC (R_m= 42-45 mL g-VS^{-1} day^{-1}) and the 120ºC (R_m= 36-52 mL g-VS^{-1} day^{-1}) conditions were initially faster than the untreated condition (R_m= 35). However, the 210ºC conditions were slower than the untreated. This may be because of inhibitors or recalcitrant substances produced (Taherzadeh and Karimi, 2008) or because of the degradation of readily biodegradable compounds during the pretreatment since the temperature is high (potentially being the reason for the low productivity for 210°C samples). These recalcitrant compounds are produced by condensing substances between polymers not affected by the pretreatment, creating a bond (Taherzadeh and Karimi, 2008). The value of T80 for the untreated condition was 12.28 days, which is high when compared with a previous study (T80= 8.4 days) done by (Kist et al., 2018). It was also observed that TH pretreatment had slightly lower T80, which means that TH pretreatment leads to faster methane production. This proved that this study helped improve the R_m, lag phase and T80 when compared with previous studies.

In Table 3-4, the results for the modeling done on *H. verticillata* can be observed. The value for the untreated condition of P was 231.47 mL g-VS^{-1}, R_m was 70.20 mL g-VS^{-1} day^{-1}, lag phase was 0.06 days, and T80 was 3.18 days. When this was compared to the previous study, it was seen that the value of P ranged from 199.1-216.6 mL g-VS^{-1}, which was slightly lower than this study. At the same time, the value of R_m ranged from 81-86.1 mL g-VS^{-1} day^{-1}, which is higher than in this study. The lag phase in the previous study ranged from 0.37-0.47 days (Fujiwara et al., 2022). The values of R_m after the TH and SE pretreatment in some conditions were lower than the untreated condition, which can be seen in the cumulative methane yield and methane production rate shown above. When *H. verticillata* was co-digested with rice straw and cow dung (Kainthola et al., 2019) and modified Gompertz was performed, it was seen that even after the co-digestion, the value of R_m ranged from lowest to 61.38 mL g-VS^{-1} day^{-1} to maximum at 87.63 mL g-VS^{-1} day^{-1}. This proves that the *H. verticillata* used in this study were overly degraded, and the harvesting season had a significant effect.

Table 3-5 shows the results of untreated and pretreated *L. grandiflora*. The value for R_m for the untreated condition was 15.01 mL g-VS^{-1} day^{-1}. This value increased significantly after TH and SE pretreatment ranging from 21.45-71.33 mL g-VS^{-1} day^{-1}, increasing the hydrolysis rate. *L. grandiflora* also showed good fitting results as the value of R^2 was higher than 0.915. The lag phase was higher for the untreated condition (0.22 day), which was reduced after the TH and SE pretreatment. Similar to *E. crassipes*, the values of T80 were higher for the SE pretreatment, suggesting TH pretreatment was a faster pretreatment.

3.3.3. Conclusion: Methane yield improvement after TH and SE pretreatment

The methane yield improvement was evaluated after TH and SE pretreatment for all the substrates used. The optimum value of methane yield and the improvement are shown in Table 3-6. The methane yield for *E. crassipes* was improved by 89.91% (TH) and 56.83% by SE pretreatment. Compared with Enrique 2019 (master study), the methane yield was improved by 16.3% (TH) and 37.9% by SE pretreatment. For *H. verticillata*, the improvement was 24.44% for TH and 11.33 for SE pretreatment. For both *E. crassipes* and *H. verticillata*, the improvement was greater for thermal hydrolysis pretreatment, but as the lignin concentration increased to 34.2% for *L. grandiflora*, the methane yield improvement was higher for SE (216.13%) than TH (140.72%) pretreatment. This is further discussed in the general discussion.

3.4. Tables

Table 3-1. Anaerobic digestion in batch mode of steam exploded lignocellulose biomass in previous studies

Substrate	Condition*1			CH_4 yield (mL g-VS^{-1})		Increment ratio (%)	References
	Temperature (°C)	Retention time (min)	Severity factor	Un-treated	Pre-treated*2		
Bagasse	225	10	4.6	160	216	35	Vivekanand et al. (2014)
Bamboo	248	5	4.9	almost 0	215	-	Kobayashi et al. (2004)
Bulrush	198	6	3.7	165	205	24.2	Wang et al. (2010)
Hay	175	10	3.2	243	281	15.6	Bauer et al. (2014)
Miscanthus	220	10	4.5	84	374	345.2	Menardo et al. (2013)
Miscanthus	198	3	3.4	182	274	50.5	Li et al. (2016)
Reed	200	15	4.1	188	355	88.8	Lizasoain et al. (2016)
Wheat straw	180	15	3.5	276	331	19.9	Bauer et al. (2009)
Wheat straw	220	1	3.5	226	273	20.8	Ferreira et al. (2013)
Wheat straw	140	60	3.0	276	286	3.6	Theuretzbacher et al. (2015)

*1: Best steam explosion condition that highest CH_4 yield was obtained.
*2: CH_4 yield pretreated at optimum condition.

Table 3-2. Untreated methane yield of the substrates used in this study

	Methane yield (mL g VS^{-1})
Eichhornia crassipes	166.57 ± 25.26
Hydrilla verticillata	231.81 ± 8.86
Ludwigia grandiflora	85.84 ± 22.7

Table 3-3. Summaries of estimated parameters from modified Gompertz equation for *Eichhornia crassipes*

Condition TH	P	R_m	λ	R^2	T80
Untreated	177.25	13.69	0.00	0.930	12.28
150-10	186.68	52.64	0.00	0.972	3.32
150-30	266.10	73.04	0.00	0.961	3.41
165-10	232.48	73.84	0.00	0.970	2.92
165-30	171.30	70.40	0.10	0.991	2.48
180-10	305.81	86.71	0.00	0.975	3.32
180-30	239.78	82.49	0.04	0.994	2.78
210-10	177.93	41.05	0.03	0.973	4.59
210-30	264.76	61.92	0.00	0.985	3.99

Condition SE	P	R_m	λ	R^2	T80
150-10	194.37	52.71	0.00	0.966	3.45
150-30	250.22	78.83	0.00	0.964	2.94
165-10	233.84	80.70	0.00	0.979	2.72
165-30	179.07	31.10	0.00	0.959	6.50
180-10	227.98	54.21	0.00	0.962	4.40
180-30	254.88	87.85	0.02	0.992	2.76
210-10	179.85	33.68	0.07	0.974	5.40
210-30	239.09	32.39	0.00	0.928	7.08

Table 3-4. Summaries of estimated parameters from modified Gompertz equation for *Hydrilla verticillata*

Condition TH	P	R_m	λ	R2	T80
Untreated	231.47	70.20	0.06	0.993	3.18
150-10	180.74	48.15	0.00	0.989	3.52
150-30	203.96	92.24	0.23	0.996	2.38
165-10	224.79	102.98	0.21	0.999	2.32
165-30	186.09	93.69	0.34	0.998	2.29
180-10	161.63	57.90	0.29	0.992	2.90
180-30	262.12	100.86	0.14	0.993	2.63
210-10	210.36	80.39	0.32	0.996	2.80
210-30	188.22	67.44	0.14	0.987	2.78

Condition SE	P	R_m	λ	R^2	T80
150-10	168.72	45.57	0.00	0.989	3.46
150-30	162.73	62.25	0.44	0.997	2.92
165-10	252.06	92.73	0.10	0.992	2.68
165-30	187.45	104.66	0.21	0.999	1.93
180-10	209.99	91.78	0.30	0.998	2.52
180-30	217.95	52.03	0.03	0.978	4.07
210-10	211.36	89.30	0.19	0.995	2.47
210-30	214.15	36.20	0.00	0.960	5.69

Table 3-5. Summaries of estimated parameters from modified Gompertz equation for *Ludwigia grandiflora*

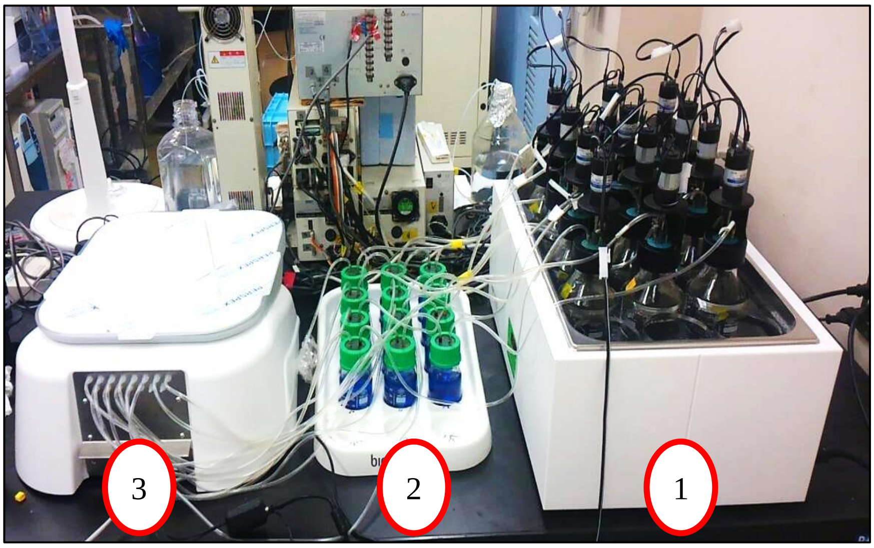
Condition TH	P	R_m	λ	R^2	T80
Untreated	88.85	15.01	0.27	0.996	5.80
150-10	159.24	58.84	0.00	0.987	2.64
150-30	110.71	42.11	0.10	0.994	2.71
165-10	110.48	44.48	0.09	0.993	2.47
165-30	174.17	43.05	0.00	0.965	5.25
180-10	131.05	54.84	0.03	0.993	2.29
180-30	206.41	71.33	0.00	0.987	2.83
210-10	130.05	30.76	0.00	0.935	4.56
210-30	178.52	50.42	0.00	0.989	3.31

Condition SE	P	R_m	λ	R^2	T80
150-10	104.82	25.87	0.00	0.952	3.84
150-30	75.85	21.45	0.00	0.915	3.29
165-10	246.30	50.26	0.00	0.952	4.55
165-30	258.46	60.86	0.00	0.955	3.94
180-10	243.98	53.11	0.00	0.933	4.25
180-30	177.55	44.37	0.00	0.946	3.74
210-10	181.21	60.57	0.00	0.942	2.87
210-30	200.85	45.77	0.00	0.951	4.07

Table 3-6. Optimum values of methane yield of TH and SE pretreated aquatic weeds with their respective methane yield improvement

Substrate	TH pretreated CH_4 yield (mL-g VS $^{-1}$)	CH_4 yield improvement for TH (%)	SE pretreated CH_4 yield (mL-g VS $^{-1}$)	CH_4 yield improvement for SE (%)
Eichhornia crassipes	316.33 (180 °C- 10 min)	89.91	261.23 (150 °C- 30 min)	56.83
Hydrilla verticillata	288.47 (180 °C- 30 min)	24.44	258.08 (165 °C- 10 min)	11.33
Ludwigia grandiflora	206.63 (180 °C- 30 min)	140.72	271.37 (165 °C- 30 min)	216.13

3.5. Figures



① Sample incubation unit

② CO_2 absorption unit

③ Gas volume measuring device

Fig 3-1. Automatic Methane Potential Test System (AMPTSII, Bioprocess) used in Experiment.

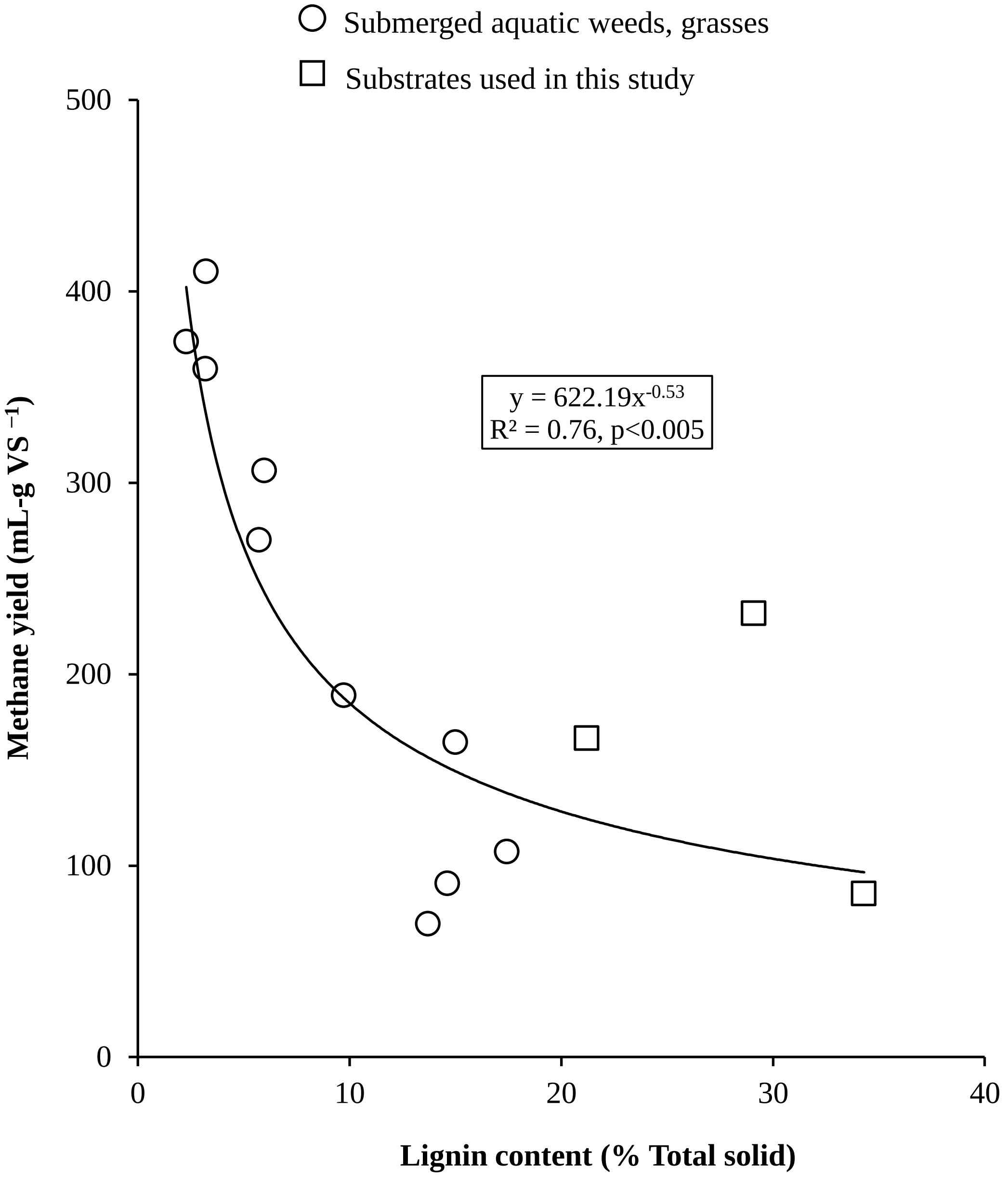


Fig 3-2. Comparing the relationship of methane yield in batch modes with lignin content of different substrates with previous studies. Untreated methane yield of the substrates used in this study (Koyama et al., 2014 modified)

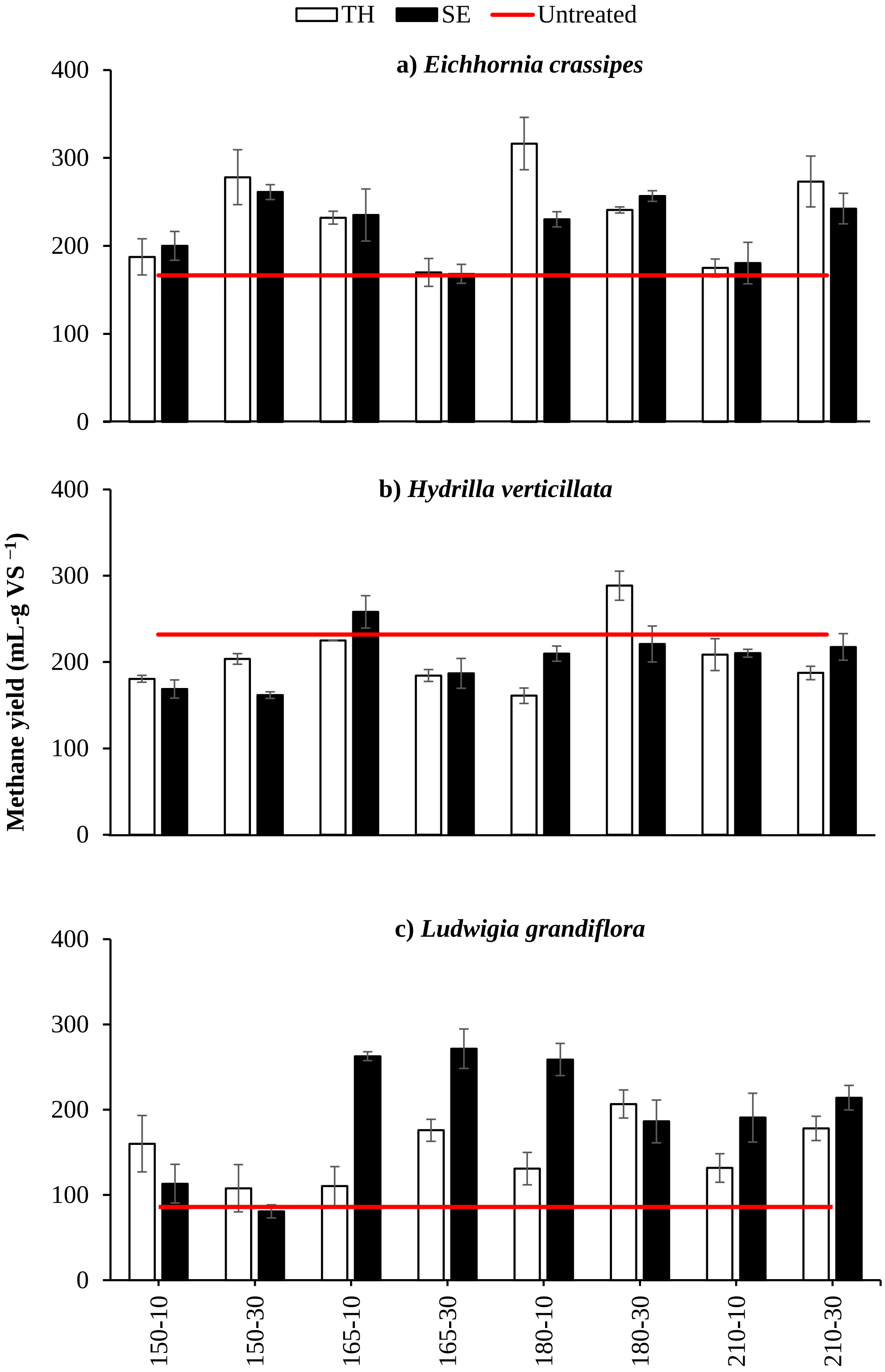


Fig 3-3. Methane yield after the TH and SE pretreatment relationship with the pretreated condition for the substrates used in this study

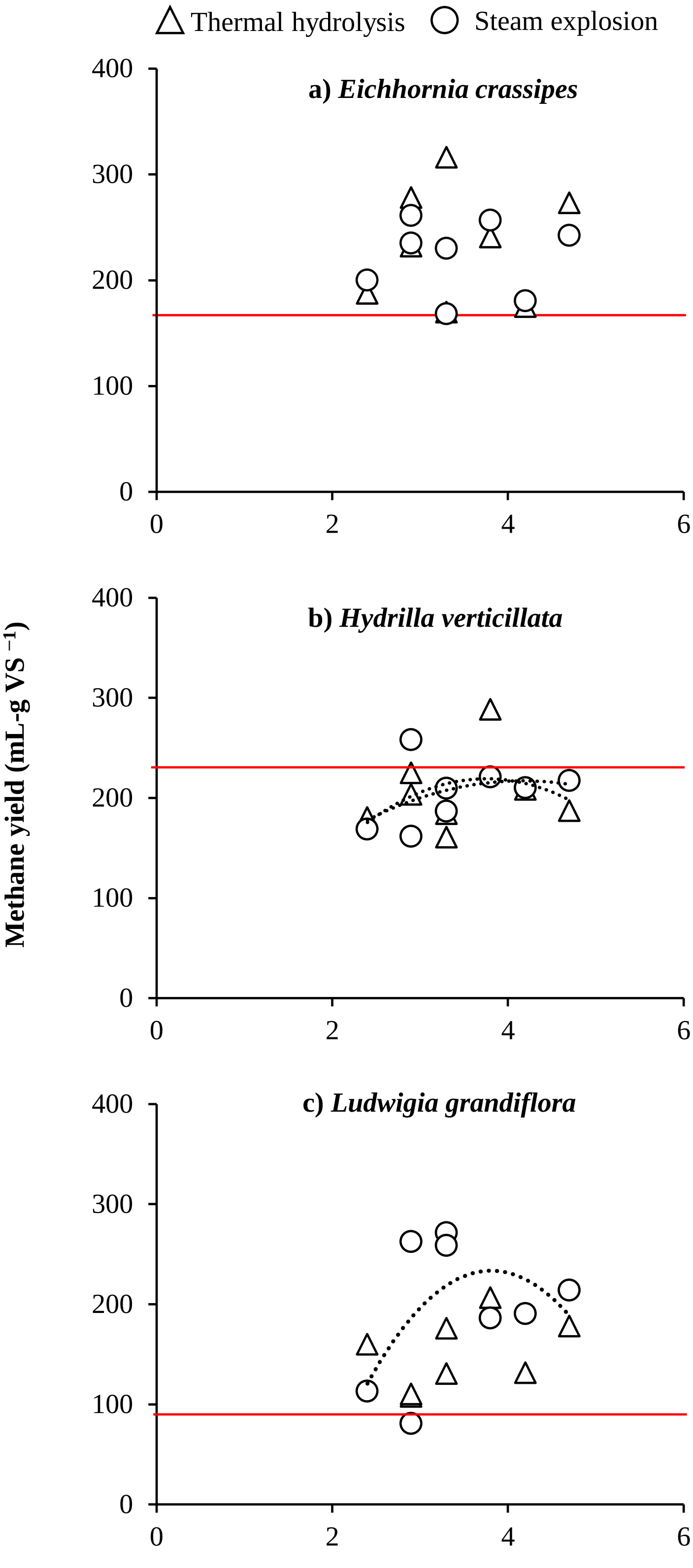


Fig 3-4. Methane yield after the TH and SE pretreatment relationship with the severity factor

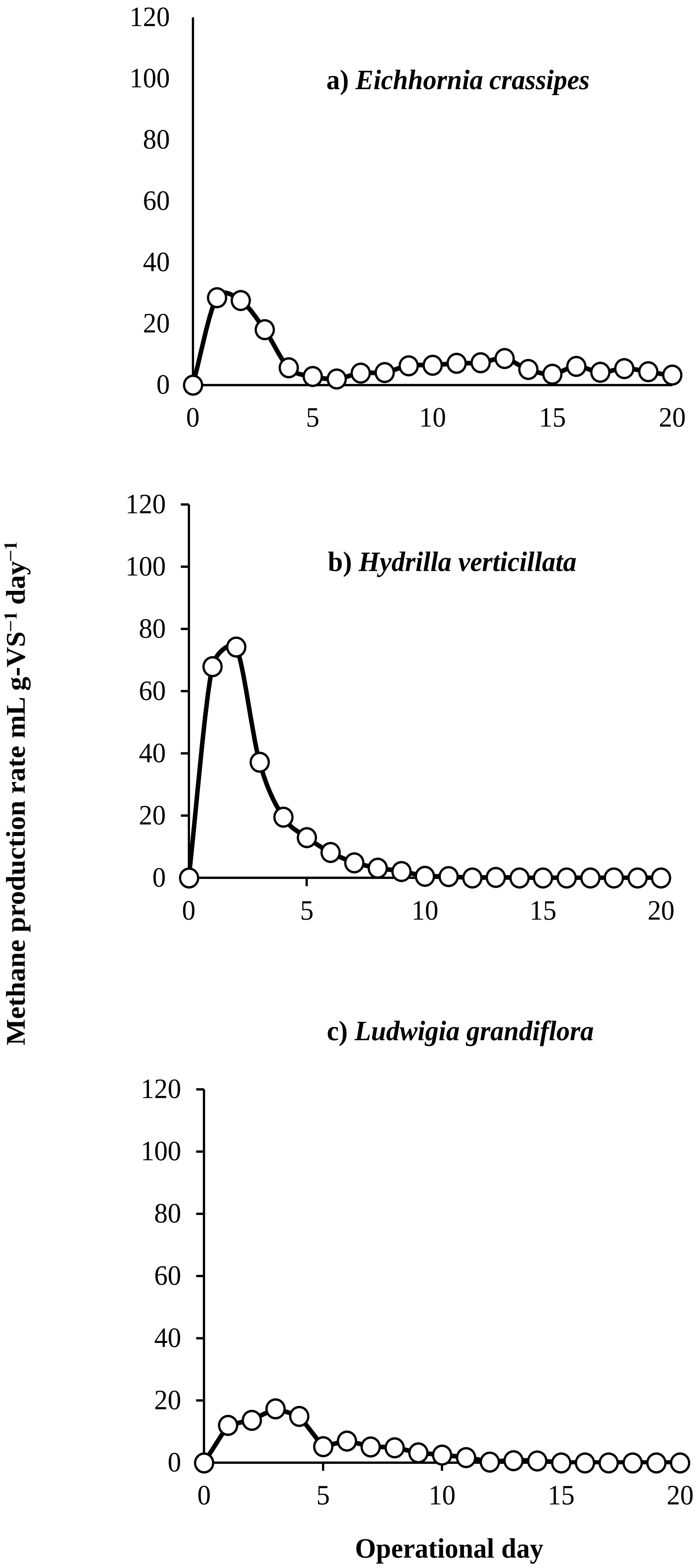


Fig 3-5 . Methane production rate of untreated aquatic weeds used in this study

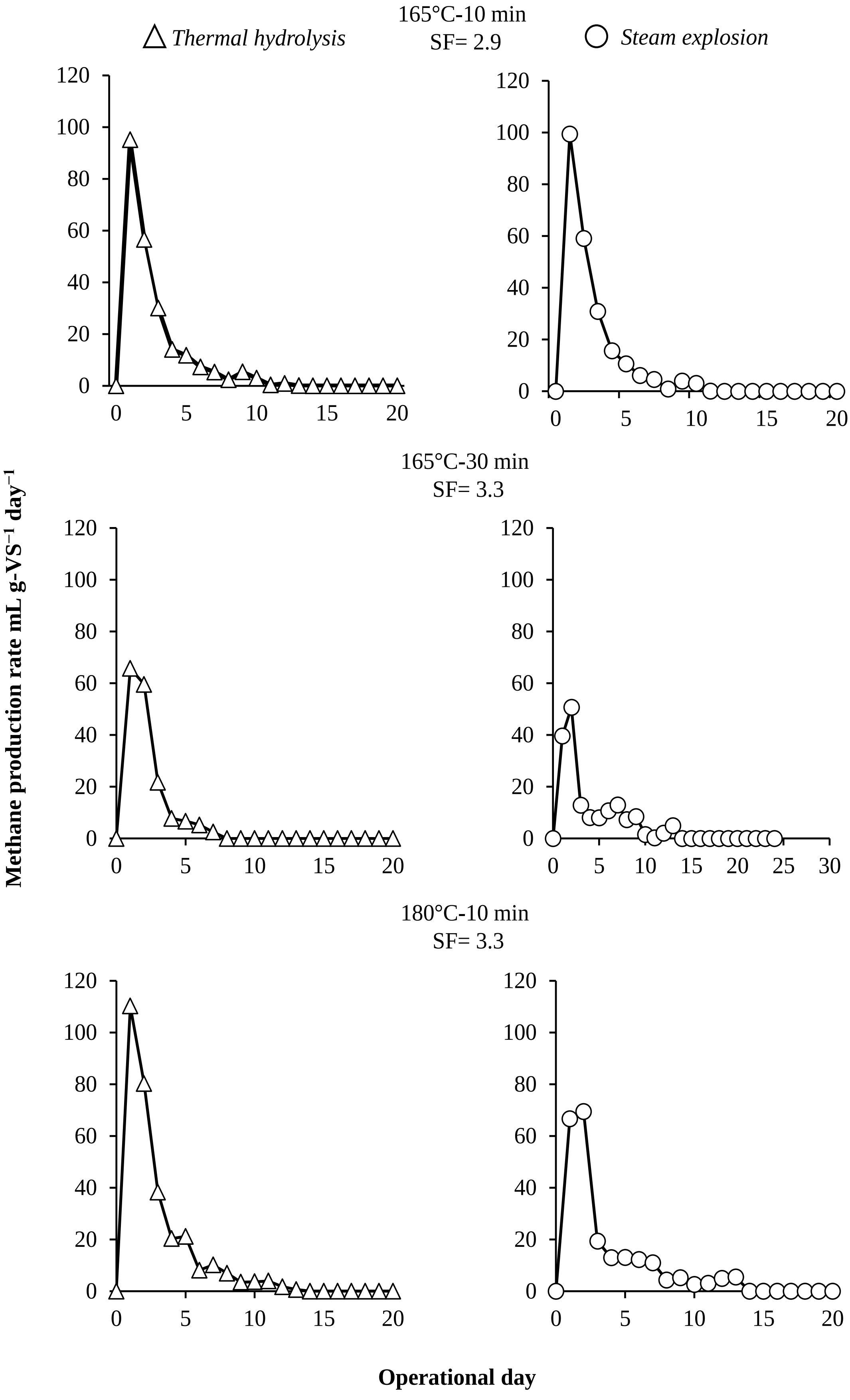


Fig 3-6 . Methane production rate of pretreated *Eichhornia crassipes* (continued)

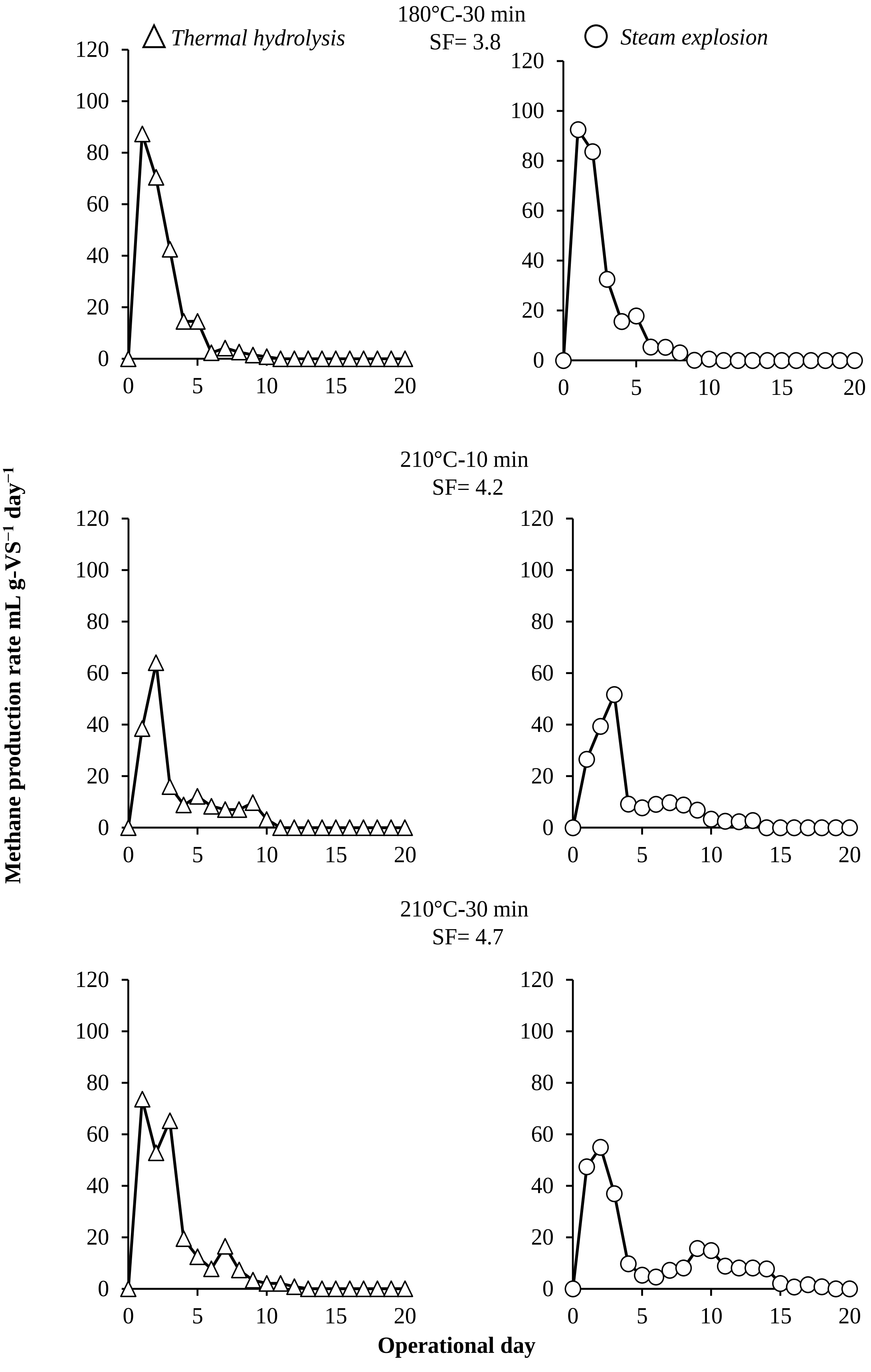


Fig 3-6 . Methane production rate of pretreated *Eichhornia crassipes*

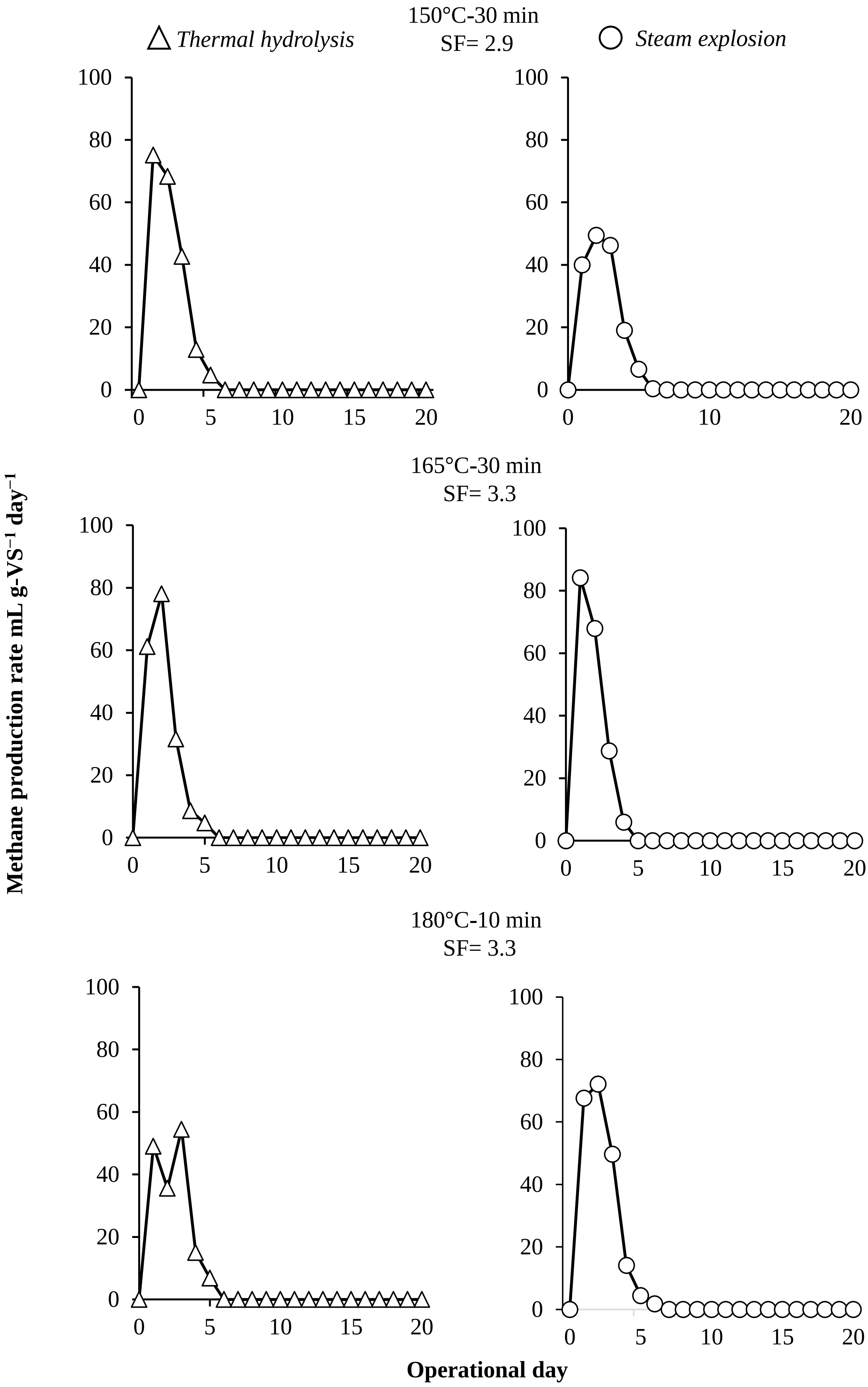


Fig 3-7 . Methane production rate of pretreated *Hydrilla verticillata* (continued)

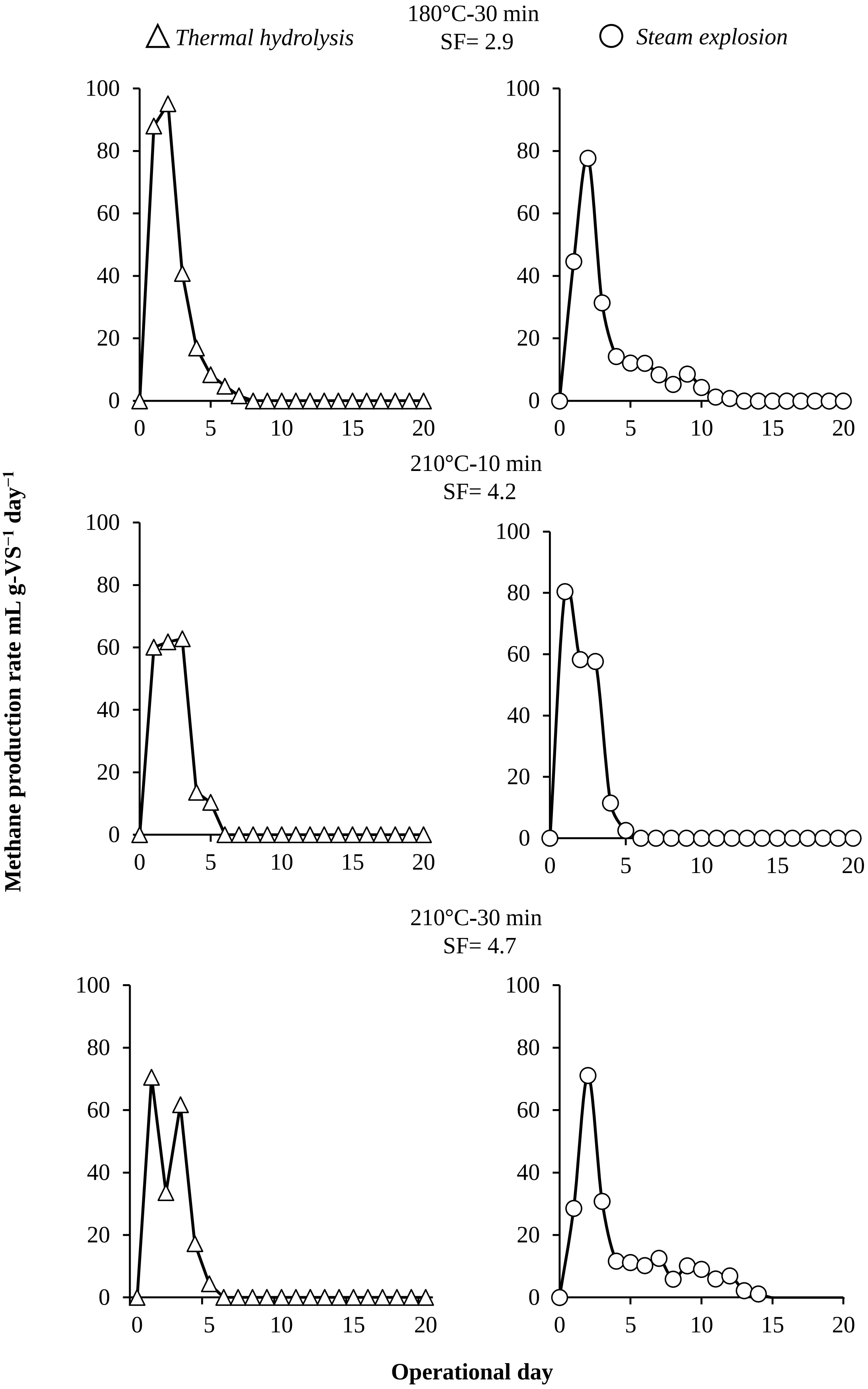


Fig 3-7 . Methane production rate of pretreated *Hydrilla verticillata*

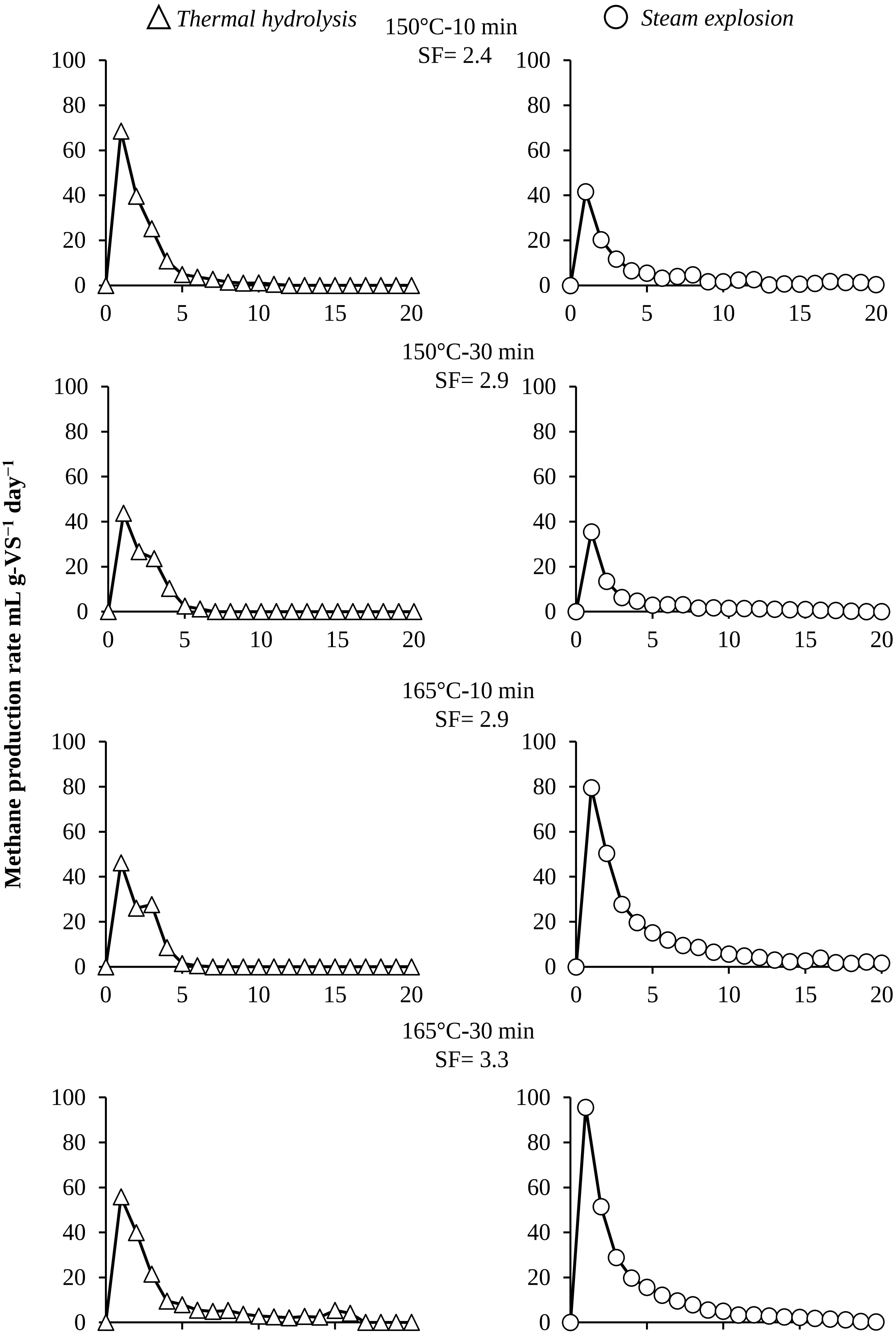


Fig 3-8. Methane production rate of pretreated *Ludwigia grandiflora* (continued)

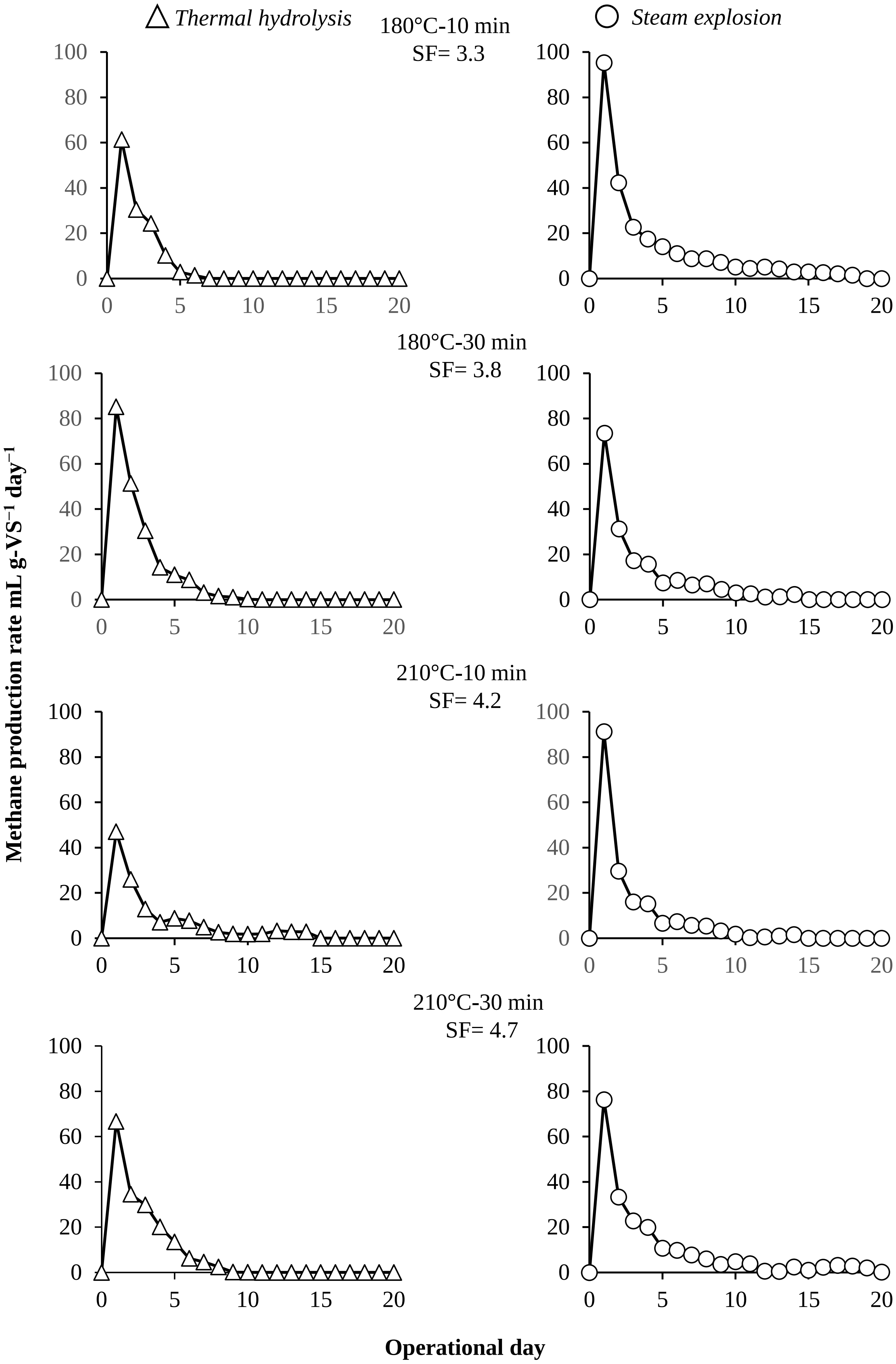


Fig 3-8 . Methane production rate of pretreated *Ludwigia. grandiflora*

4. CHAPTER 4 GENERAL DISCUSSION

4.1. Summary of the findings and proposal of an appropriate pretreatment depending on the lignocellulose composition

An appropriate pretreatment between thermal hydrolysis and steam explosion pretreatment on different lignocellulosic aquatic weeds was attempted in this study. In Chapter 2, the effect of TH and SE was observed on the physical and chemical composition of different lignocellulosic aquatic weeds. The results indicated that the SE had a higher reduction in solids when comparing the TH pretreatment. The substrate degradation led to higher TOC readily available, which also increases the amount of phenolic compounds in the pretreated liquid substrate. For TH, this had an increasing linear trend regardless of the substrate's chemical composition. For SE, as the lignin concentration of the substrate increased, the phenolic compounds reached an optimum point, whereas the TOC kept increasing, leading to a different trend than TH. Also, when the relationship between lignin in the pretreated substrate and inhibitors in the liquid fraction was observed, it showed an increasingly significant trend. In Chapter 3, the methane yield was evaluated after the TH and SE pretreatments. It was seen that both TH and SE effectively improved the methane yield for all the substrates used, as discussed in chapter 3. The optimum conditions are shown in Table 3-5. Also, by using Modified Gompertz modelling, TH and SE pretreatment helped increase the hydrolysis rate by reducing the T80 value. It was seen that TH had a lower T80 value than the SE pretreatment.

A summary Figure was concluded from all these results mentioned above (Figure 4-1). Figure 4-1A) compares the methane yield improvement with the lignin concentration of different types of lignocellulosic biomass from previous studies (Dereix et al., 2006; Bauer et al., 2009; 2014; Wang et al., 2010; Vivekanand et al., 2012; Perez-Elvira et al., 2015; Theuretzbacher et al., 2015; Li et al., 2016; Sapkaite et al., 2017; Kamdem et al., 2018; Mulat et al., 2018; Regidor Alfageme, 2019). The methane yield improvement trend for TH and SE pretreatment was similar. The methane yield improvement for the TH pretreated biomass had an exponential trend; y= 14.62e0.05x, R^2 = 0.47

($p<0.05$) whereas for the SE pretreated biomass the value; $y = 10.47e0.07x$, $R^2 = 0.63$ ($p<0.005$). The methane yield improvement after the TH pretreatment for *E. crassipes*, *H. verticillata* and *L. grandiflora* was 89.91, 24.44 and 140.72% and after SE pretreatment was 56.83, 11.33 and 216.13% respectively. The Figure clearly shows that both TH and SE helped in enhancing the methane yield improvement. The methane yield improvement was greater for TH pretreatment when lignin concentration was lower at 21.2% for *E. crassipes* and 29.1% for *H. verticillata*. In contrast, when the lignin concentration was a maximum of 34.3% for *L. grandiflora*, SE pretreatment significantly enhanced the methane yield improvement (216.13 %), which can be seen in Figure 4-1A). One of the reasons for such a high improvement of *L. grandiflora*, when pretreated with SE pretreatment, can be the TOC concentration in the liquid fraction, as shown in Fig 4-1B). It was observed that the TOC values were quite similar between TH and SE pretreatment for *E. crassipes* and *H. verticillata* with the increasing severity factor for which the reasons were mentioned in Chapter 2. The value of TOC was much different for TH and SE pretreated *L. grandiflora*, leading to a greater improvement in methane yield. The values of TOC in SE pretreated liquid fraction for *L. grandiflora* were almost double than in TH pretreated. *L. grandiflora* had a very woody/rigid structure that needed SE pretreatment to disrupt its structure, leading to a significantly higher value of the TOC. Steam exploded samples have a more accessible surface as a result of the sudden decompression, which forces the fibrous material to come apart, separating fibres and fibre bundles (Martin-Sampedro et al., 2014).

As mentioned above, treatment temperature is also an essential factor which can affect the methane yield or other subsequent processes. Figures 4-2 evaluated the effect of lignin concentration and the optimum temperature at which the biomass achieved the maximum methane yield. As mentioned in Figure 4-1, both TH and SE pretreatment helped improve the methane yield with the lignin concentration. However, when checking the effect of temperature and lignin, it was observed that TH was an effective treatment for a broader range of temperatures ranging from 140 °C to 210 °C.

Higher temperature does not affect the methane yield improvement negatively during the TH pretreatment. For the SE pretreatment 3-D graph, it was seen that in SE pretreatment, methane yield improvement was much greater than TH, but the range of temperature seems to be effective at a lower value. When the temperature is increased, it seems the improvement is lower. This can be due to the severity of inhibitory compounds formed during higher temperatures. Previous studies mentioned that the amount of inhibitors was maximum during SE pretreatment at high temperatures whereas as TH is considered less harsher pretreatment, possibly a broader range of temperatures can help to improve the subsequent process (Martin-Sampedro et al., 2014).

Both TH and SE pretreatment enhanced the methane yield of aquatic weeds significantly. TH pretreatment was sufficient to improve the methane yield for a wide range of lignocellulosic biomasses, whereas SE pretreatment helped disrupt the substrate structure with maximum lignin content (*L. grandiflora*= 34.2%TS). The value of the lignin polymer in the untreated substrate can help to evaluate the methane yield improvement yield. This evaluation of methane yield improvement can be helpful for new studies/research as only the lignin concentration is used for the evaluation. This is the first study which proposes a methane yield improvement estimation model for both TH and SE pretreatment. Also, as no equipment is needed for TH pretreatment, its application/feasibility needs to be studied further.

4.1.1. Mechanism of TH and SE and effect of different parameters on the methane yield

To fully understand the process mechanism which took place during both TH and SE pretreatment, Figure 4-3 was explained (Li et al., 2007; 2015; Gourlay et al., 2012; Martin-Sampedro et al., 2014; Biswas et al., 2015; Duque et al., 2016; Pielhop et al., 2016; Troncoso-Ortega et al., 2021). It can be said that optimal severity in thermal pretreatments requires a compromise between generating sugar accessibility (which increases with the severity of the pretreatment) and sugar degradation (which becomes more pronounced under more severe conditions). Hydrolysis yields may be lowest after the harsher thermal pretreatments because of the following: (1) the presence of degradation products and/or the higher content of lignin and (2) the chemical changes induced in lignin by the pretreatment. Several authors have associated the presence and the surface distribution of lignin with an unfavourable effect on the enzymatic hydrolysis of lignocellulosic biomass (Berlin et al., 2006; Hoeger et al., 2012; Martín-Sampedro et al., 2013; Rahikainen et al., 2013; Martin-Sampedro et al., 2014). During pretreatment, the lignin is redistributed in large fibre extension; lignin and lignin fragments are rearranged and distributed heterogeneously in the cell wall, where they are deposited on the surface or interior of the fibres. Lignin micro-droplets are formed, produced by the coalescence and migration of lignin and progressive collapse of the microfibrils (Donohoe et al., 2008; Araya et al., 2015; Arévalo et al., 2017). Other micro-particles deposited on the surface or interior of the fibre, called pseudo-lignin, consist of modified lignin and carbohydrate degradation products (Sannigrahi et al., 2011; Hu et al., 2012; Kumar et al., 2013). Both lignin micro-droplets and pseudo-lignin deposited on the surface or interior of the fibres reduce the efficiency of enzymatic hydrolysis through nonspecific binding of the enzymes, thus creating a physical barrier that blocks enzyme access to the cellulose. Two possible mechanisms of inhibition have been suggested: (1) physical or steric hindrance of the cellulosic surfaces (Mooney et al., 1998; Chandra et al., 2009); and (2) reversible/irreversible adsorption of cellulases onto lignin, with the consequent loss of active enzymes (Berlin et al., 2006; Hoeger et al., 2012). Similar to thermal pretreatments, this study de-couples the thermal pretreatments to TH and SE pretreatment. The effect of TH and SE on the substrate is different, as observed in this study. This inhibition effect of lignin can be seen by SEM and FTIR analysis which was also performed and explained in this study. It was explained that during TH pretreatment, the moisture remained inside the biomass due to its mechanism. Hence, when the substrate is cooled, there are chances that lignin can condensate or precipitate on the biomass, whereas during the SE pretreatment, the moisture evaporates due to the explosion, which can lead to the distribution of lignin in solid and liquid fractions, as explained in Figure 4-3. Similarly, in this study, as observed in Figure 4-3, it can be seen that in the SEM image, it can be seen that during TH pretreatment, fewer lignin droplets were observed on the surface, whereas, in the comparison, SE observed more droplets in the substrate. Also, the FTIR analysis had different values for both TH and SE pretreatment. Therefore, some critical variables were selected to explain the correlation with the methane yield.

Principal component analysis (PCA) was used to analyze the correlation among explanatory variables and their correlations with methane yield. As the PCA biplot shows in Figure 4-4 and 4-5, each vector represents an individual variable, and the correlation between any two variables is determined by the cosine value of the angle between the two vectors. Two vectors pointing toward similar directions are highly positively correlated (cosine → 1); two vectors pointing toward opposite directions are highly inversely correlated (cosine → −1); while two vectors with an angle close to 90° are highly independent (cosine → 0) (Appels et al., 2011; Gabriel, 1971). The PCA analysis includes different variables from solid fraction (Cellulose, lignin and re-condensed lignin) and liquid fraction (TOC, SCOD, dissolved lignin and phenolic compounds) on the methane yield. The length of a variable vector represents its weight on each axis (principal component) and indicates the relative importance of each principal component.

From Figure 4-4, it was seen that lignin had the most strong and inverse correlation with the methane yield, followed by cellulose and re-condensed lignin, whereas dissolved lignin had a strong and positive correlation with the methane yield. The strong relationship between methane yield and lignin might be attributed to the degradation of lignin during the TH pretreatment. The re-condensed lignin and cellulose affected the methane yield due to the formation of lignin droplets, which can block the enzymes from degrading the cellulose. The positive correlation of dissolved lignin with the methane yield can be due to the acclimatization of dissolved lignin to the AD process. A similar case was seen before in a long-term study when dissolved lignin acclimatizes to the AD process leading to an increase in the methane yield (Koyama et al., 2017a). The acclimatization to AD against dissolved lignin has not been investigated, although numerous reports of acclimatization to phenol under mesophilic conditions (Rosenkranz et al., 2013). The acclimatization of AD against dissolved lignin should be accompanied by the change in the microbial community and the saturation of lignin's adsorption capacity. Most adsorbed cellulolytic enzymes on lignin are not desorbed and form a strongly-bonded "lignin-enzyme complex" on the surface of lignin (Funaoka, 1998). Palonen et al., (2004) reported that the binding affinity of added enzymes on lignin is decreased because large amounts of cellulolytic enzymes are already attached to lignin and reduce the active binding site. A decrease in binding affinity, i.e., hydrophobicity, indicates that the toxicity of lignin to a microorganism could be decreased. Thus, the toxic effect of dissolved lignin on the AD process may be alleviated once the adsorption of cellulolytic enzymes is saturated. Therefore, dissolved lignin can "temporarily" inhibit AD, particularly during the start-up period, although acclimatization to dissolved lignin may occur through the adaptation of microorganisms, a saturation of enzyme adsorption on dissolved lignin that recovers stable AD, or both.

Figure 4-5 shows the effect of SE pretreatment. It can be seen that dissolved lignin and re-condensed lignin is strongly and inversely correlated with the methane yield. As mentioned in previous studies done on SE, it was seen that re-condensed lignin could be one of the major inhibitors of various subsequent processes. Similarly, the possible mechanism of inhibition from dissolved lignin is likely the direct toxicity of dissolved lignin to bacteria and archaea (Vidal and Diez, 2005), irreversible adsorption of cellulolytic enzymes on dissolved lignin, or both (Palonen et al., 2004; Koyama et al., 2017a,b) leading to inhibition for the AD process. Koyama et al., (2017a) investigated the inhibitory effect of dissolved lignin of alkali pretreated *P. maackianus* on three AD steps (hydrolysis, acidogenesis and methanogenesis) in batch mode and found that methanogenic and hydrolytic activities are inhibited by dissolved lignin at 5.0 g L^{-1} and 1.0 g L^{-1} respectively.

Based on this PCA analysis, some less correlated explanatory variables, such as the TOC, SCOD and phenolic compounds in the liquid fraction, can be ignored in further analysis. Also, an assumption using various previous literature was made to explain the process of lignin solubilization during the TH and SE pretreatment, which can be seen in Figure 4-6.

4.1.2. Fate of lignin during the TH and SE pretreatment

Figure 4-6 explained the process of lignin solubilization during both the pretreatments, the glimpse of which was seen in the PCA analysis. During thermal pretreatments, both TH or SE pretreatment lignin gets degraded and converted/separated into two different forms as a solid and liquid fraction. The solid fraction contains the insoluble lignin, which mainly accounts for inhibitory compounds such as re-condensed lignin, kraft lignin, etc., whereas the liquid fraction contains the soluble lignin (inhibitory compounds) mainly known as dissolved lignin or phenolic compounds. However, the amount of lignin solubilization varies for TH and SE pretreatment because of their different mechanisms (Li et al., 2005; Martin-Sampedro et al., 2014; Pielhop et al., 2016; Kist et al., 2018). An assumption was made based on this process. If 100% of the lignin is solubilized during both TH and SE pretreatment, it was assumed that 50% of lignin was transferred to a solid fraction and the other 50% was transferred to a liquid fraction due to no explosion involved during the TH pretreatment. Whereas for SE pretreatment, due to its explosion capabilities, it was assumed that 25% of the lignin remained in the solid fraction, and the other 75% was transferred to the liquid fraction. In the PCA analysis results above, the lignin in the solid fraction was already discussed as it had a similar correlation with the methane yield, especially the re-condensed lignin but the lignin present inside the liquid fraction seems to have a different trend for TH and SE. One of the reasons the previous works of literature stress can be the lignin composition/type shown in Figure 4-7. The lignin composition was also briefly explained in chapter 1. For instance, even though the amount of dissolved lignin can be similar for both pretreatments, the ratio of the lignin type can be different for every condition of thermal pretreatments, as mentioned in previous studies (Wu et al., 2013; Zhu et al., 2015). For example, an alkaline pretreatment was performed by Koyama et al., (2015); it was seen that the S/G lignin ratio of aquatic weeds ranged from 0.2 to 0.9 depending on the species. These S/G ratios are similar to flax (0.4) and hemp (0.8), but these values are considered to be pretty low as compared with other plant biomass such as jute (1.7), abaca (2.9) and sisal (3.4) (del Río et al., 2007). A low S/G lignin ratio implies a lower delignification rate and higher alkali consumption during the alkaline delignification process, suggesting that lignin polymers of aquatic weeds are relatively resistant to alkaline pretreatment. The measurement and the optimum ratio of these different lignin types after thermal pretreatments should be measured, and their effect on the methane yield should be evaluated in future work.

4.1.3. Can TH pretreatment be the new prominent pretreatment?

As mentioned above, TH significantly improved the methane yield of all the substrates despite the lignin content. TH was also performed in this study using the steam explosion reactor. A SE reactor requires a flash tank to withstand the high pressure released during the pretreatment, making the SE reactor an expensive method. The capital investment needed for SE is higher because of the reactor design. For instance, a lab-scale reactor used by Forgács et al., (2012) was estimated to be around 1 million USD. Similarly, a pilot scale SE reactor used by Baral and Shah, (2017) was around 119 million USD. As TH pretreatment releases the pressure gradually, it does not require a flash tank. Therefore, a new reactor design without the flash tank can be a vital way to reduce capital investment costs, making TH pretreatment more cost-effective. A cost-effective TH pretreatment reactor can be easily taken to industrial scale from lab scale, which can help to treat the more considerable amount of harvested aquatic weeds leading to management of water bodies such as Lake Biwa and others around the world.

4.1.4. Removal of inhibitors before performing anaerobic digestion

Inhibitors produced during the breakdown of lignocellulosic materials have the potential to limit and deactivate cellulolytic enzyme activity and microbial biodegradability. Many studies have attempted to evade or reduce inhibition problems before or after the pretreatment process.

Enzyme inhibition by non-productive binding by inhibitory compounds such as phenol, lignin, and lignin degradation products highly depend on the pretreatment, biomass composition, chemical structure, and lignin content. Lignin inside the lignocellulose is so rigid that it must be degraded and removed prior to enzymatic hydrolysis. In recent years, attention has been focused on recovering lignin from lignin-rich wastewater such as black liquor, pulp industry wastewater, and pretreatment liquor of plant biomass. Potential lignin uses include automotive brakes, wooden panel products, bio-disperses, polyurethane foams and epoxies (Lora and Glasser, 2002). Various lignin recovery techniques, such as chemical coagulation, filtration, acid precipitation and electrocoagulation, have been investigated to develop a low-cost and energy-efficient lignin biorefinery process (Das and Patnaik, 2000; Ibrahim et al., 2004; Uğurlu et al., 2008). As a result, combining the lignin recovery process and anaerobic digestion of thermally pretreated biomass may be more beneficial in terms of bioenergy recovery and additional benefits from recovered lignin. However, dissolved lignin and anaerobic digestion effluent (ADE) should not be mixed when considering a lignin refinery due to the reduced lignin recovery efficiency due to lignin adsorption on sludge (Hernandez and Edyvean, 2008) and contamination by the microorganism or undesirable pathogens (Chen et al., 2012). Accordingly, to reduce the risk of lignin contamination by ADE, the lignin recovery process should be preceded by anaerobic digestion.

4.2. Further efficient ways of improving anaerobic digestion

4.2.1. Why Co-digestion is important (Semi-continuous experiment (Bhatia et al., 2021))

In a study conducted at the University of Shiga prefecture, the anaerobic digestibility of *L. grandiflora* was assessed by a long-term experiment. This study aimed to perform a long-term (98 days) AD process for treating *L. grandiflora* with high lignin using a continuous stirred tank reactor (CSTR). The steam explosion was performed prior to AD for the solubilization of the substrate, and the effect of steam-exploded *L. grandiflora* on AD was evaluated. SE was carried out at 180 °C for 30 minutes with an SF of 3.8. Industrial equipment from Yasujima Co. Ltd., Ishikawa, Japan, was used. A temperature of 180°C can benefit in assessing the effect of inhibitors produced during the pretreatment. Therefore, in this study, using *L. grandiflora* as a substrate, the solubilization of the substrate and the effect of inhibitors were evaluated. This was the first study to use *L. grandiflora* and perform a semi-continuous AD operation for further industrial applications.

The lignin content of untreated biomass was 25.22% and increased to 29.15%TS after the SE pretreatment. The C/N ratio of the untreated substrate was 22.12% TS, and after the SE pretreatment, the C/N remained at 8.25 in the solid fraction, and 10.82% was moved to the liquid fraction. The carbon content of the untreated biomass was 40.51 %TS, and the nitrogen content of the untreated biomass was 1.83 ± 0.1%TS. The carbon values changed after the pretreatment solid and liquid fraction to 42.90 ± 0.5 and 28.03 ± 0.5, respectively. Contrariwise, the nitrogen value increased after the pretreatment (increased to 5.28 ± 0.7 for the solid fraction and 2.60%TS for the liquid fraction).

AD showed a fluctuating biogas production trend (Figure 4-9). The average biogas production during the operation was 265 mL g-VS^{-1}. The highest biogas production was 400 mL g-VS^{-1} on day 49, and the lowest biogas production (71.4 mL g-VS^{-1}) was recorded on day 67. The initial OLR and HRT were 0.9 g-VS L^{-1} day^{-1} and 30 days, as shown in Figure 4-8. The addition of NaOH from day 67 to the final operation on day 98 did not show an upward trend in the pH but showed a significant change in biogas production. The gas production tended to be less fluctuating and more stable towards the end of the operation. The average value of the methane content in this study was 49.04%. The low methane content in the biogas could be due to the high carbon in the SE pretreated *L. grandiflora*. The reason for the low biogas production, its fluctuation and low methane content in this study can be the high lignin content of 29.15 ± 0.5%TS in the pretreated *L. grandiflora*. The lignin amount in the effluent on the last operational day 98 was 17.0 ± 1.0%TS, which is higher than that of other untreated plant biomasses. Furthermore, *L. grandiflora* has a woody structure; it does not entirely degrade even after SE pretreatment at 180 ◦C. Lignin contains 60–65% of carbon in the plant biomass (Bengtsson et al., 2019). This high carbon content can lead to nutrient imbalances. Therefore, another evidence could be a high amount of residual carbon inside the reactor, owing to which the biogas production fluctuated and had a lower value.

One of the main reasons for the lower biogas produced during the AD process is possibly the residual carbon in the CSTR. Therefore, the carbon mass balance was evaluated during the AD process (Fig. 4-10). The carbon fed inside the reactor was present in the solid and liquid fractions of SE pretreated *L. grandiflora*. The carbon content in the pretreated solid and liquid fractions was 42.9 ± 0.5%TS and 28.0 ± 0.5%TS, respectively. The carbon mass balance during the AD operation can be seen in Figure. The feeding in the CSTR reactor was performed every two days, leading to an increase in the carbon amount inside the reactor. However, because of the lower degradability and high lignin of *L. grandiflora*, as discussed above, the carbon content in the biogas and effluent was significantly less compared with that in the fed carbon. Therefore, the carbon accumulated inside the digester is measured as residual carbon. The carbon kept accumulating (43.1−49.7%) in the reactor, indicating a large amount of undigested carbon, which could be a potential source for stable AD processes.

Therefore, changes such as co-digestion are considered an essential step in improving the stability and efficiency of the reactor when using *L. grandiflora* as a substrate. Food and municipal waste are highly biodegradable and often used for co-digestion (Heo et al., 2004). Co-digestion can be achieved in a variety of ways, including optimization of the C/N ratio (Yen and Brune, 2007), improving buffering capacity (Mshandete et al., 2004) and providing micro/macronutrient (Alatriste-Mondragón et al., 2006). In a previous study, a steam explosion of citrus waste was performed at 150 °C, 20 min, and later it was co-digested with municipal solid waste (Forgács et al., 2012). The co-digestion helped to increase the methane yield, and the value reached around 560 ± 15 mL $g\text{-}VS^{-1}$. This proves that for making the thermal pretreatment process industrial scale, co-digestion can be the necessary modification to improve the yield in the AD reactor.

4.3. Figures

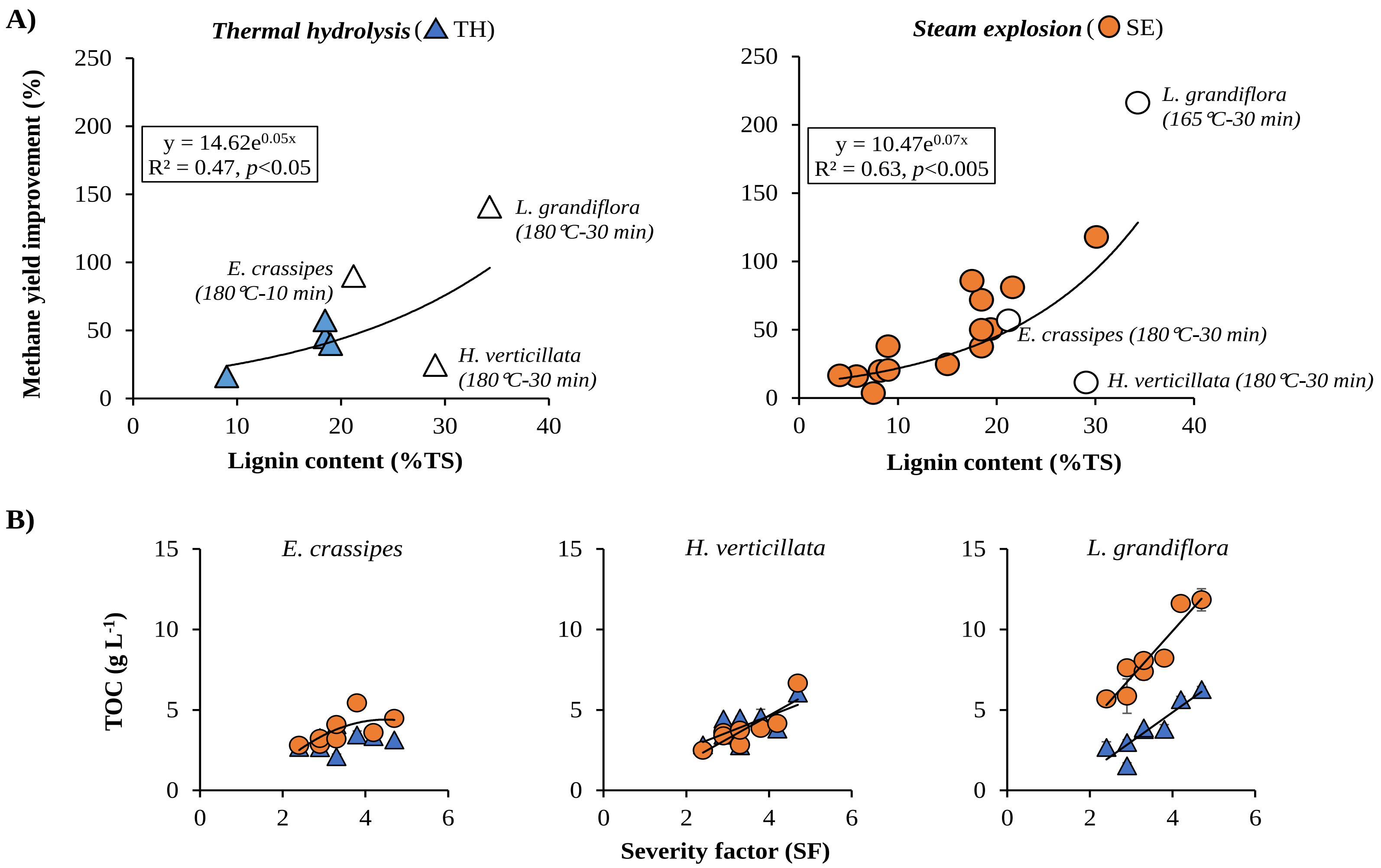
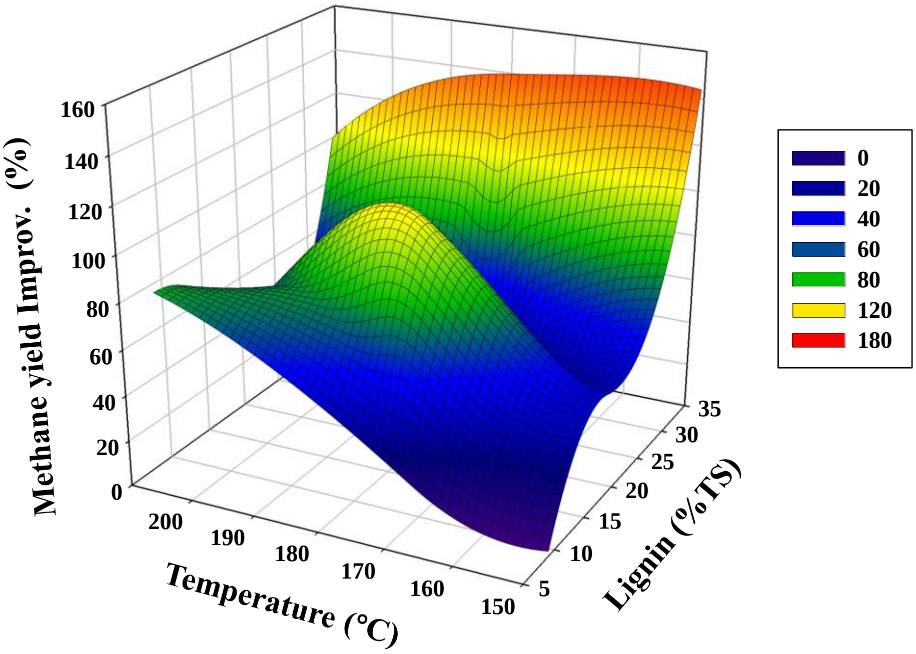


Fig 4-1. The methane yield improvement relationship with the lignin concentration of different lignocellulosic biomass from previous studies (Enrique 2019 (master study), Sapkaite et al., 2017, Elvira et al., 2015, Bauer et al., 2009, Theuretzbacher et al., 2015, Mulat et al., 2018, Vivekanand et al., 2012, Bauer et al., 2014, Li et al., 2016, Wang et al., 2010, Suzuki 2019 (master study), Dereix et al., 2006, Kamdem et al., 2018)

Thermal hydrolysis



Steam explosion

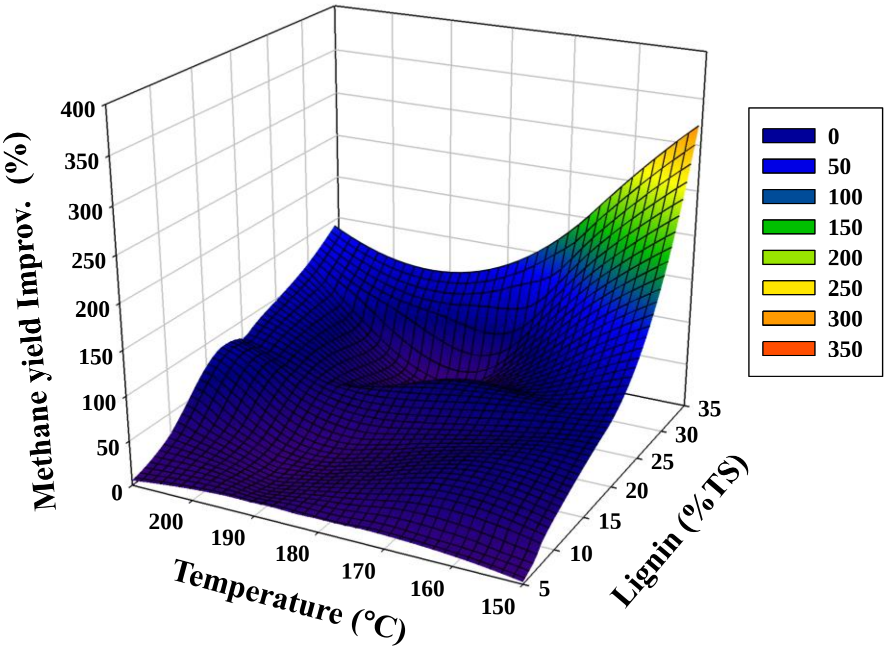
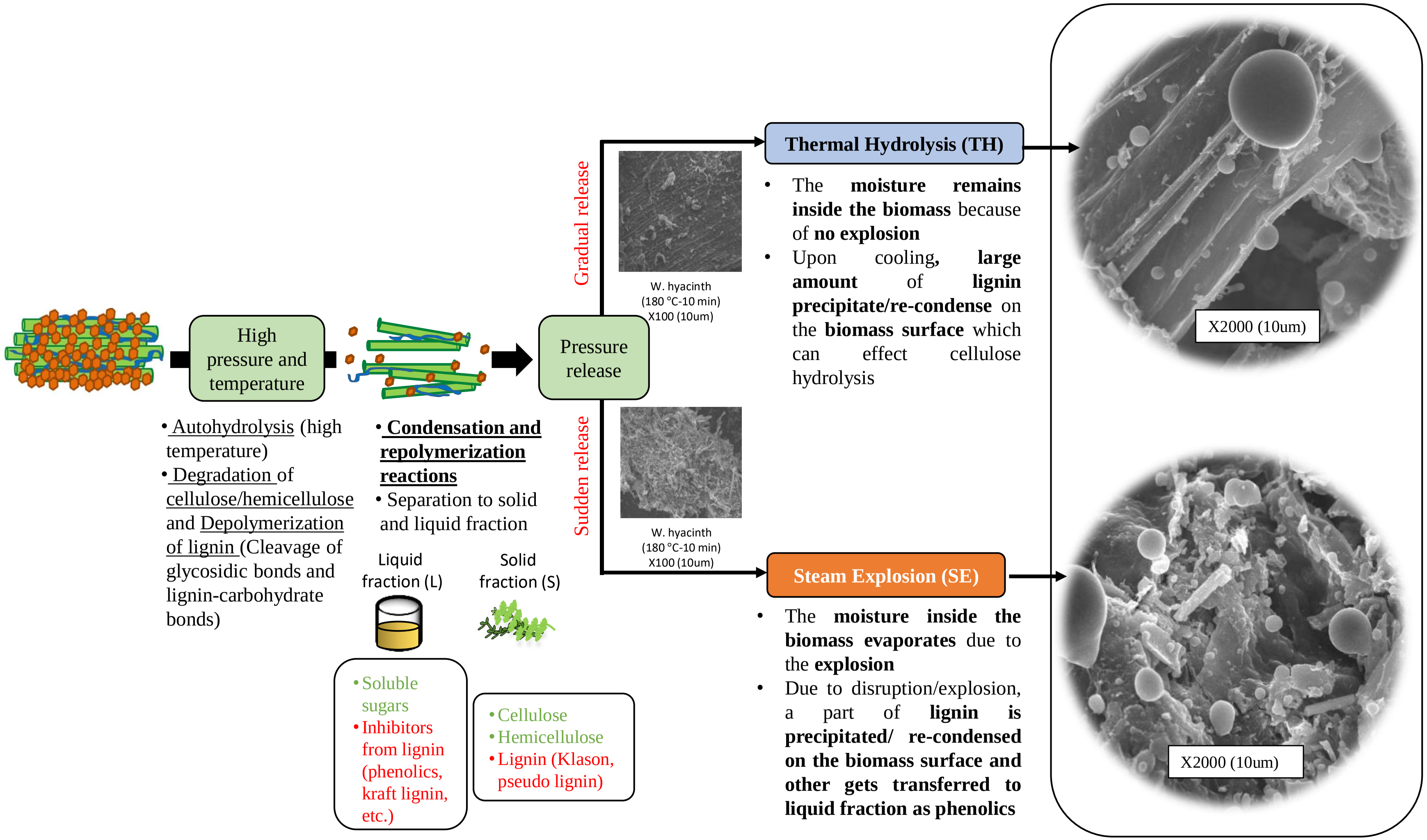


Fig 4-2. The methane yield improvement relationship with the lignin concentration and optimum temperature of different lignocellulosic biomass from previous studies



- Autohydrolysis (high temperature)
- Degradation of cellulose/hemicellulose and Depolymerization of lignin (Cleavage of glycosidic bonds and lignin-carbohydrate bonds)

- **Condensation and repolymerization reactions**
- Separation to solid and liquid fraction

- Soluble sugars
- Inhibitors from lignin (phenolics, kraft lignin, etc.)

- Cellulose
- Hemicellulose
- Lignin (Klason, pseudo lignin)

- The **moisture remains inside the biomass** because of **no explosion**
- Upon cooling**, large amount of lignin precipitate/re-condense** on the **biomass surface** which can effect cellulose hydrolysis

- The **moisture inside the biomass evaporates** due to the **explosion**
- Due to disruption/explosion, a part of **lignin is precipitated/ re-condensed on the biomass surface and other gets transferred to liquid fraction as phenolics**

Li et al., 2007, Gourlay et al., 2012, Martin et al., 2014, Biswas et al., 2015, Li et al., 2015, Duque et al., 2016, Pielhop et al., 2016, Ortega et al., 2021.

Fig 4-3. Schematic figure for the mechanism of TH and SE pretreatment

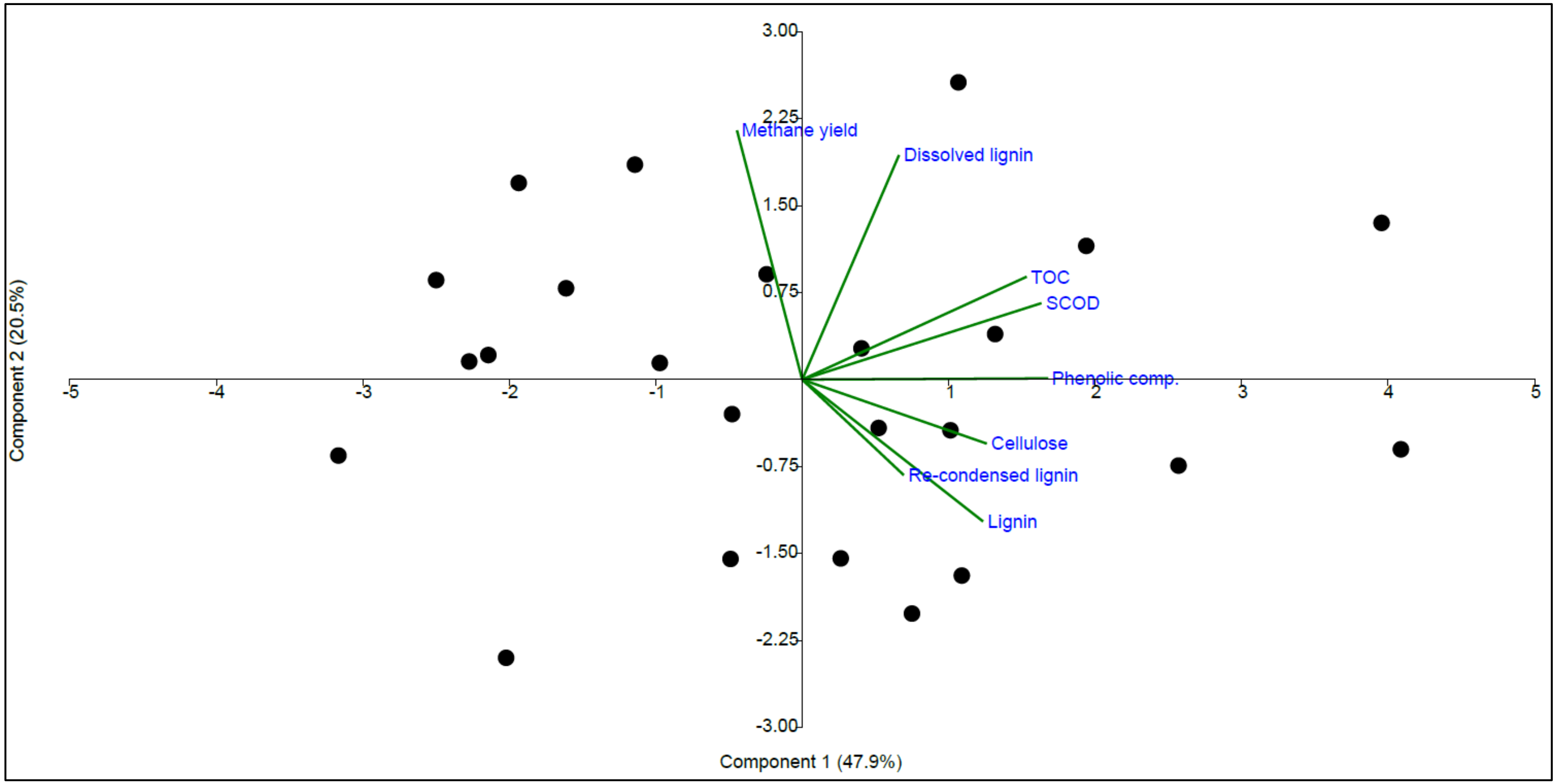


Fig 4-4. PCA analysis for evaluating the effect of different parameters on the methane yield pretreated by thermal hydrolysis

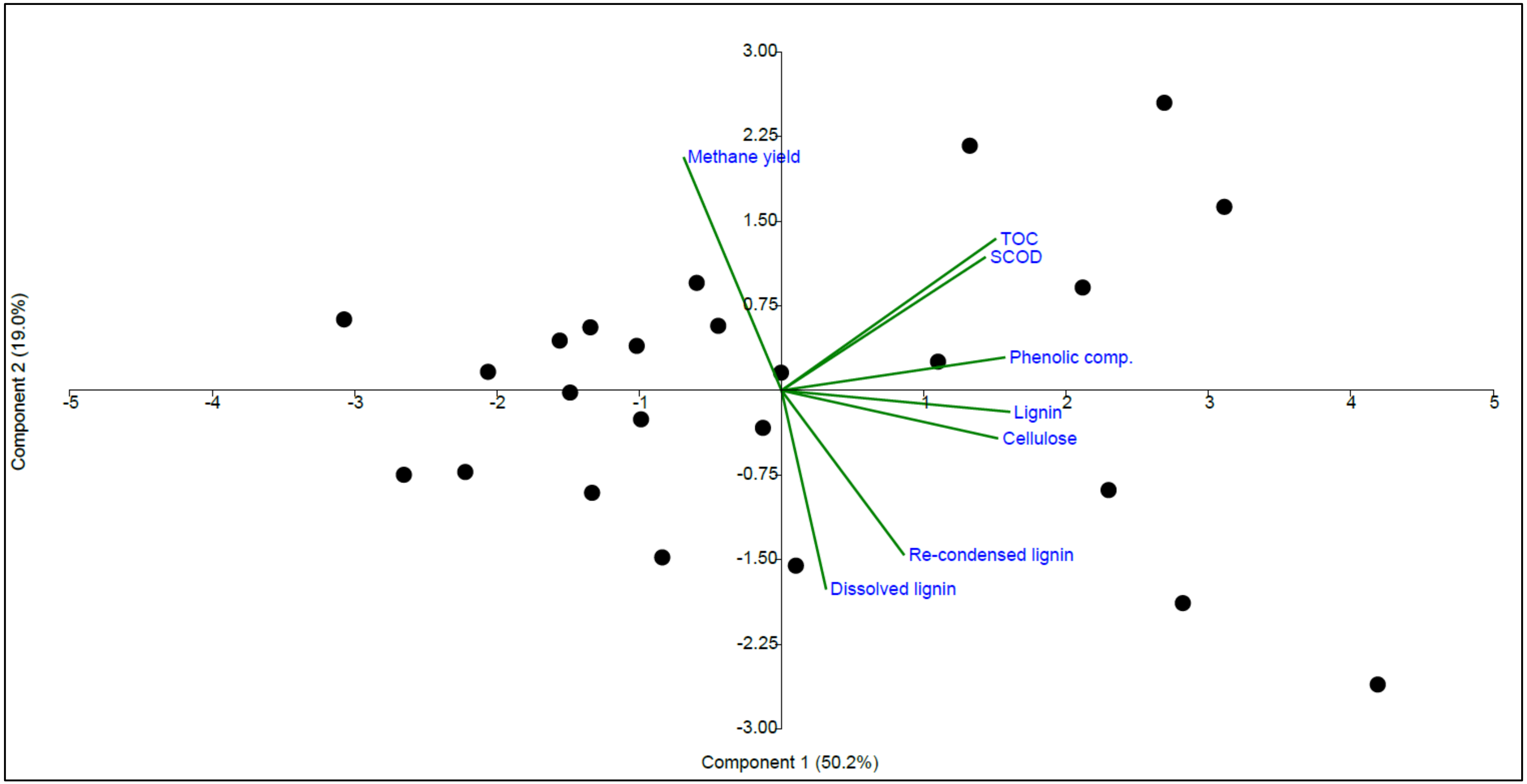


Fig 4-5. PCA analysis for evaluating the effect of different parameters on the methane yield pretreated by steam explosion

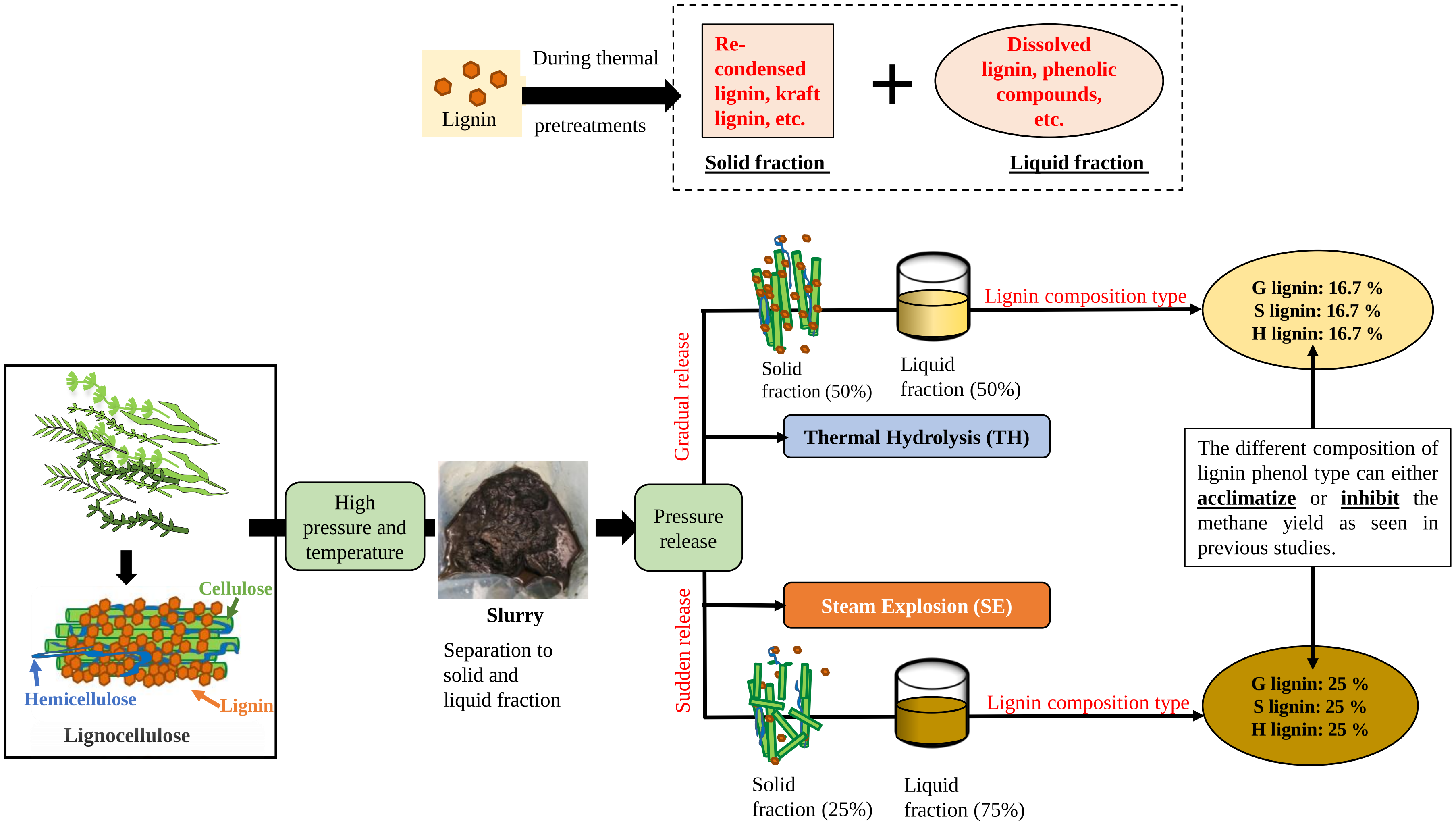


Fig. 4-6. Assumption made for the fate of lignin during TH and SE pretreatment

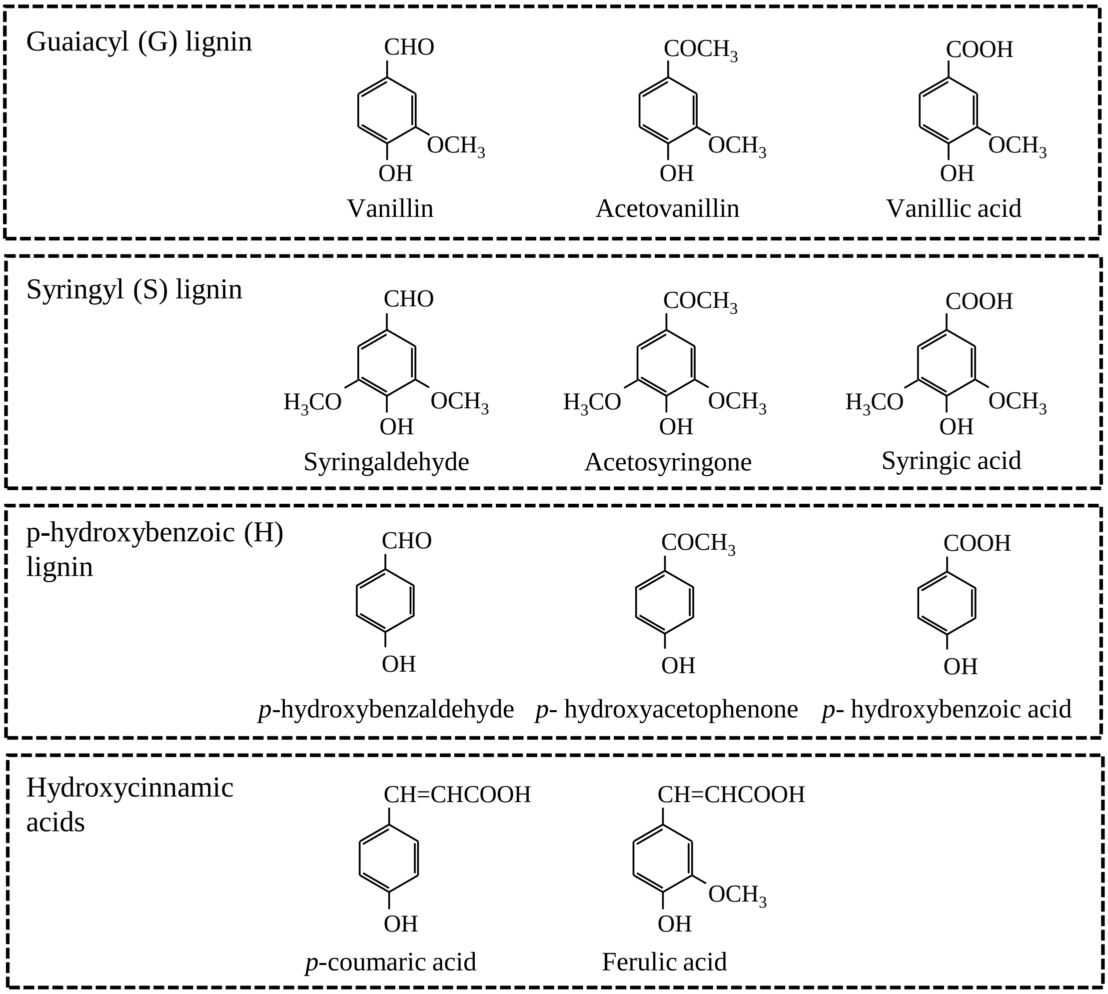


Fig. 4-7. Lignin phenols existing in plant biomass

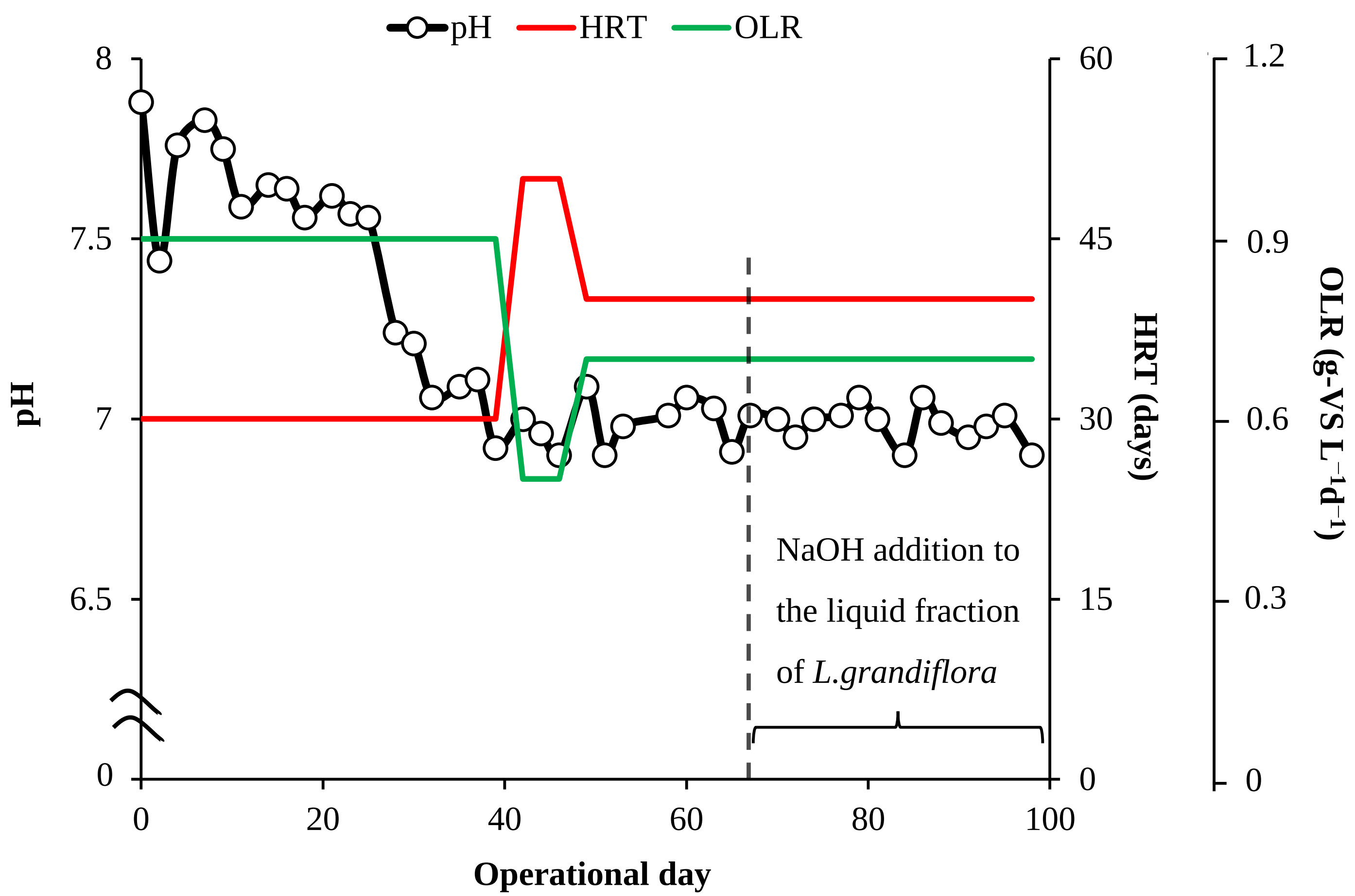


Fig 4-8. Relationship of the pH with HRT and OLR during the AD process

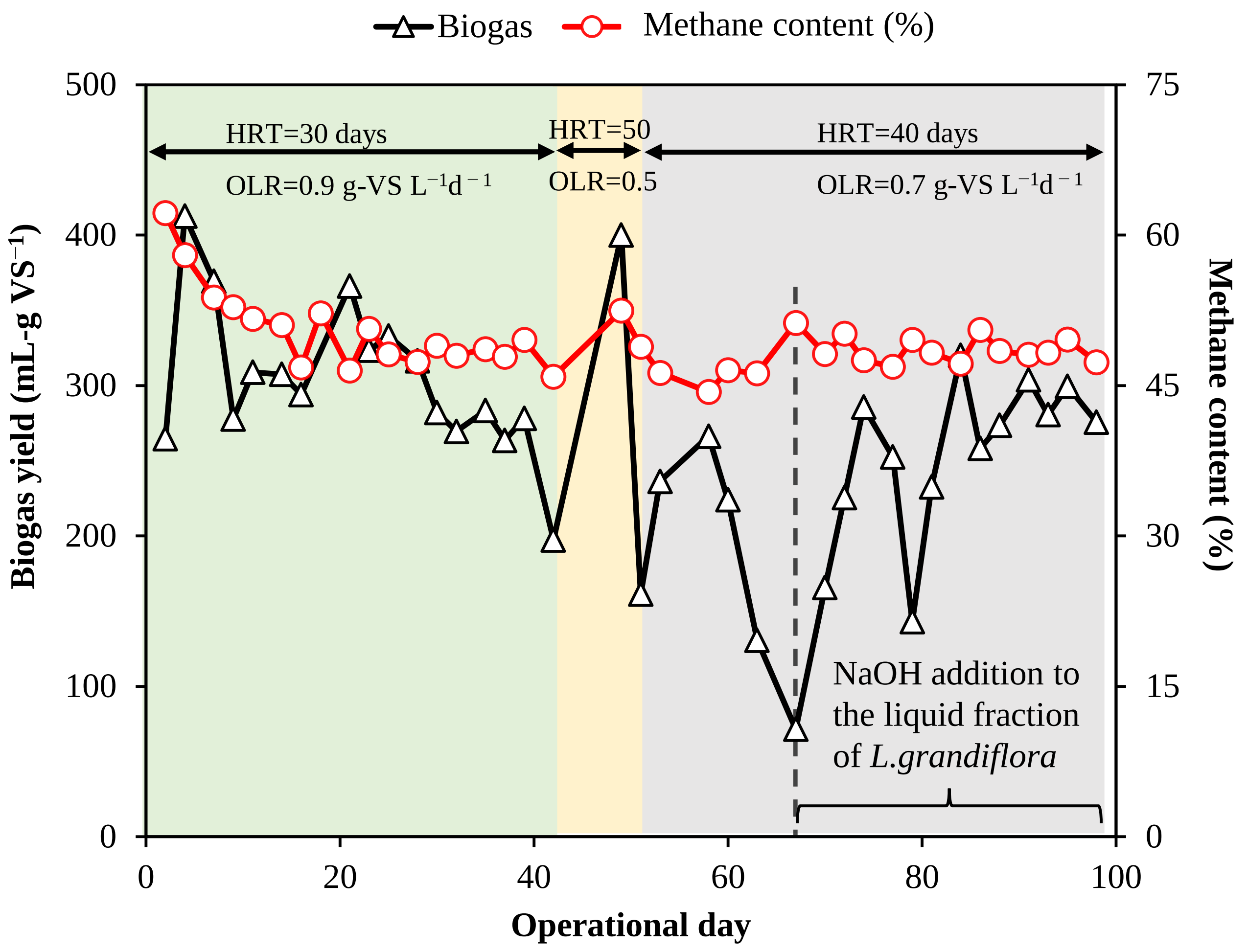


Fig 4-9. Relationship of the biogas yield and methane content in the produced biogas

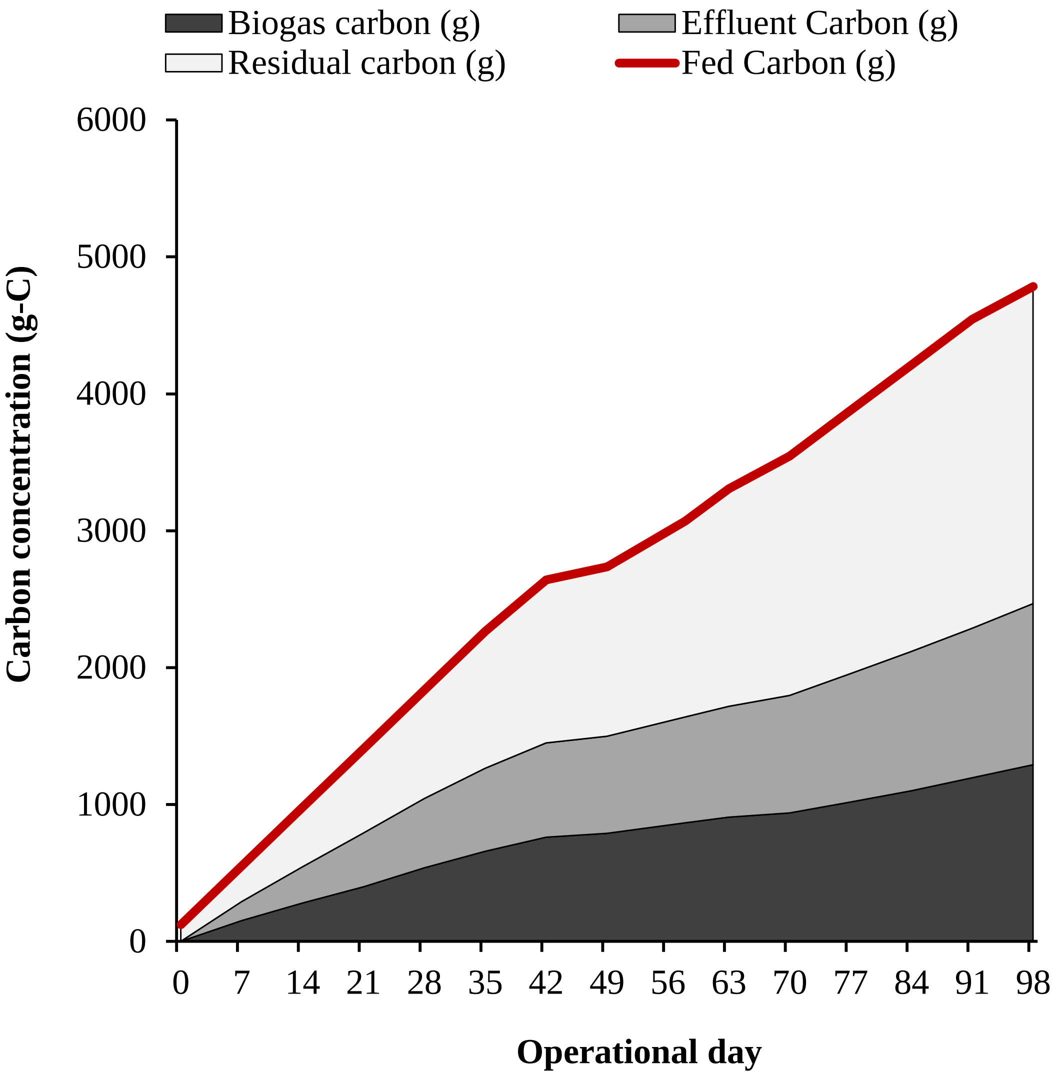


Fig 4-10. Carbon mass balance during the AD process

Biogas carbon = the carbon content in methane and carbon dioxide.
Effluent carbon = the carbon content in the digestate removed from the AD reactor.
Residual carbon = the remaining carbon in the AD reactor;
[∑Fed carbon (solid + liquid fraction) −∑[Biogas carbon + Effluent carbon]].
Fed carbon = the carbon content in the solid and liquid fractions of the pretreated *L. grandiflora*.

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