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1 Executive Summary

This draft report summarizes the results achieved from the batch experiments performed in Task 9.8 – Optimization of co-digestion process of residual fibre and juice residues – for the project period until M36. Since the duration of Task 9.8. has been extended, all results will be presented in D 9.11 – Final report on optimized co-digestion process of residual fibre and juice residues – in M48.

The biomethane potential (BMP) tests were performed by co-digestion of residual fibre and juice fractions of *Salicornia ramosissima* after different upstream fractionation and extraction processes. To find the optimal co-digestion ratio, batch experiments were performed in different co-digestion ratios of fibre and juice fraction.

2 Introduction

In tasks T9.6 – T9.8. of the AQUACOMBINE project the optimal co-digestion ratio of the residual fibre and juice fractions from the AQUACOMBINE biorefinery processes will first be determined in batch experiments. Then, the optimized co-digestion process will be tested in semi-continuous 5-L CSTR lab-scale reactors to determine optimal operation conditions like organic loading rate and hydraulic retention time of the biogas reactor.

In Deliverable 9.3 – Report on the theoretical and practical methane potential of the residual fibre fractions and juice residues – the biomethane potential (BMP) of the different halophyte plant material before and after the biorefining processes was determined in mono-digestion of the different substrates. In conclusion, inhibition of the anaerobic digestion (AD) process was likely for sodium concentrations above 2400 mg/L. In general, the application of different upstream fractionation and extraction processes generates fibre residues with lower salt concentration than in the original halophyte material which imposes less inhibition of the AD process while sodium and other salts may accumulate in the juice fraction. On the other hand, these upstream processes generate residual fibre fractions that are lower in extractives and lignin content and liquid residues with varying lipids and proteins content. Consequently, the residual fibres are characterized by a high C/N ratio which may lead to nutrient deficiency of the AD process while the addition of the residual juice fraction in co-digestion will supply nitrogen and other nutrients to the AD process.

The co-digestion experiments of fibre and juice fractions were performed in order to identify the optimal co-digestion ratio of residual fibres and juice fraction for a stable, non-inhibited AD process with a high methane yield.

3 Material and methods

3.1 Halophyte plant material and residues used for the biogas potential tests

Figure 1 gives an overview of the origin of the different samples of halophyte biomass from the AQUACOMBINE biorefinery used for analysis of the theoretical and practical methane potential (BMP) by FUAS. The residual fibre and juice fractions used for anaerobic co-digestion experiments are shown in Table 1.

Fresh green *Salicornia ramosissima* (1) was grown at Riasearch (RSR) greenhouse facilities equipped with a drip irrigation system with saline water, that is the waste effluent from a recirculating aquaculture system (RAS). Mature dry lignified plants were obtained by drying on mesh trays in the shade for 1 week at room temperature. The fresh green *S. ramosissima* samples were fractionated in juice and fibre fraction (6). Protein was extracted (7) from the nutrient rich juice fraction which generated a residual “brown juice” by UCL. The wet pressed de-juiced green fibre fractions underwent subsequent organosolv pretreatment fractionation (3) by LTU to remove lignin and increase cellulosic content. After screening of different organosolv pretreatment parameters, samples of pre-treated fibres showing the highest delignification and high performance in enzymatic saccharification trials, were supplied by LTU for determining the BMP. The pre-treated fibres that revealed the highest biomethane potential (pretreatment conditions SDJ-1B6, Table 4) were selected as the co-fermentation substrate.

The dry lignified biomass followed Soxhlet extraction with water for removal of phytochemicals (2) by AAU, Esbjerg.

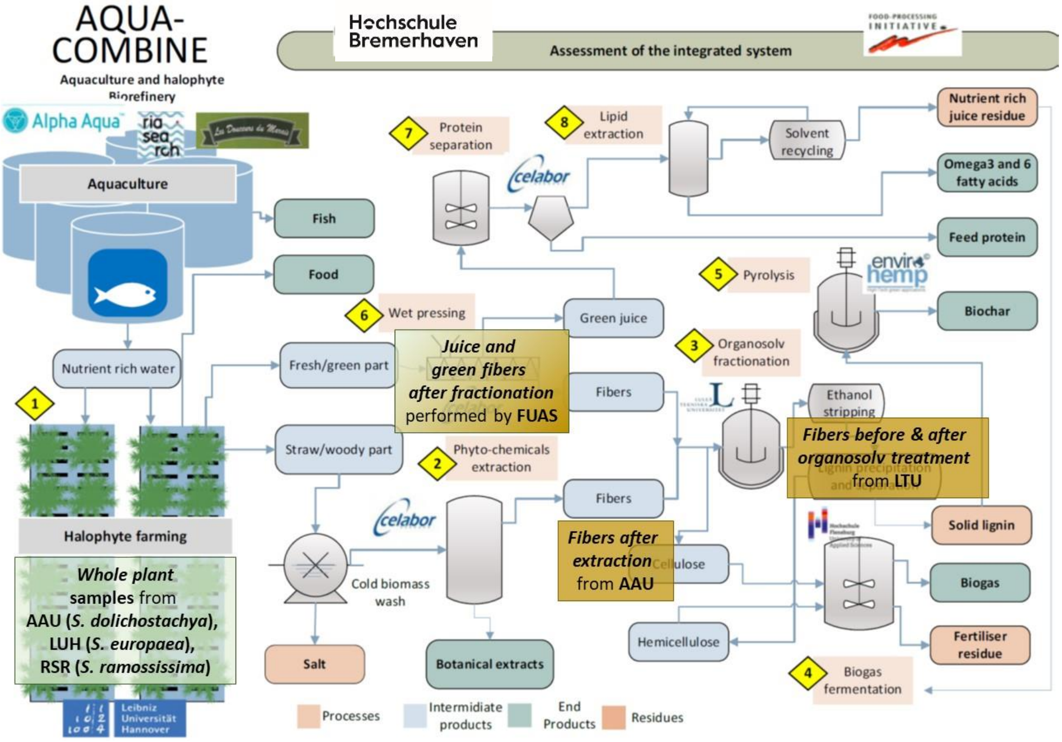

Figure 1: Overview of samples taken for BMP analysis within the AQUACOMBINE biorefinery

Table 1. Samples of S. ramosissima residual fractions used for anaerobic digestion experiments

S. ramosissima fractions	Process applied	Performed by	Residual fraction
Juice fraction	Protein extraction	UCL	Brown juice
Green fibre	Organosolv fractionation	LTU	SDJ-1B6 pre-treated fibres
Lignified plant	Soxhlet extraction with deionized H_2O: extraction of HCA	AAU	Extractive free lignified plant

3.2 *Inoculum source*

Inoculum for the biomethane potential (BMP) tests was sludge taken from an anaerobic digester of the Wastewater Treatment Plant (WWTP) in Flensburg, Germany, that operates under mesophilic conditions (38-42^{o}C). The fresh inoculum was incubated at 37±2°C for up to 5 days to minimize the production of background gas and influence of the blanks. On average, the inoculum used in this study had 2 wt% total solids (TS) and 60 wt% volatile solids (VS) of TS determined according to standard procedures (DIN EN 12880 and DIN EN 12879, respectively). The pH of the inoculum was about 8, which stayed stable during the AD experiments.

3.3 *Biomass compositional analysis*

The TS, VS and ash content were determined for the halophyte plant biomass, according to standard methods (DIN EN 12880 and DIN EN 12879, respectively). The TS content was determined by drying at 105°C for 24 hours. The VS content was measured by subsequent incineration of the TS at 550°C for 3 hours in a muffle furnace. The extractive free lignified fibres and de-juiced fibres post organosolv pretreatment at optimal pretreatment conditions (SDJ-1B6) of *S. ramosissima* were analysed in terms of cellulose, hemicellulose, and lignin composition according to the National Renewable Energy Laboratory protocol for the determination of structural carbohydrates and lignin in biomass (analysed by LTU). An elemental analysis of carbon (C), hydrogen (H), nitrogen (N) and oxygen (O) was performed on the dried and ground plant material, using a CHNS-O FlashSmart elemental analyser (Thermo Scientific). Elemental analysis was used to determine the crude protein content and C/N ratio of the samples. Crude protein content was calculated by a mass factor of 6.25 based on the nitrogen content determined in the elemental analysis. The theoretical BMP (TBMP) was determined based on the analysed content of lignocellulose and crude protein of the biomass.

3.4 *BMP tests*

The BMP of the residual fibre and juice fractions was first determined in mono-digestion by an automated methane potential test system (AMPTS II, Bioprocess Control, Sweden AB) while the BMP of de-juiced *S. ramosissima* fibres was analysed in a batch fermentation assay conducted in triplicates in 0.5 L batch flasks under mesophilic conditions 37±2°C according to German Standard, VDI 4630, using an inoculum to substrate ratio (ISR) ≥ 2 (based on VS) and a TS concentration in the batch assay of $\leq$ 10wt% to ensure sufficient mass transfer (VDI 4630, 2016). The batch tests were operated until the daily gas production was less than 1 vol% of the accumulated gas production on three consecutive days. The BMP values are expressed as the volume of methane produced per gram of organic matter (volatile solids, VS) added to each batch flask at the beginning of the experiment.

In the co-digestion batch tests, the BMP was analysed for different co-digestion ratios of residual fibres to brown juice. The co-digestion of extractive free lignified fibre and brown juice was performed using mixing ratios of 50:50, 70:30 and 80:20 (based on VS). The mixing ratios of optimally organosolv pre-treated fibres (SDJ-1B6) with brown juice were analysed using ratios of 70:30 and 80:20. All co-digestion batch test were conducted using an I/S ratio of 2 (gVS/gVS).

4 Results and Discussion

4.1 Biomass composition

The TS, VS, ash and lignocellulosic content of the different residual juice and fibre fractions of *Salicornia* biomass material used in this study is shown in Table 2. The results of the ash content show that approximately 50% of inorganic remains in the brown juice after protein extraction of the green juice which initially composed of 58% TS (Cayenne et al 2022). The ash content of extractive free lignified fibres after Soxhlet extraction with deionized water was lower than 1wt%, indicating that salt removal was very efficient using deionized water as earlier found by Cybulska et al 2014. Likewise, the de-juiced fibres of *S. ramosissima* had an ash content 12wt% (Cayenne et al 2022) and 4wt% following subsequent organosolv pretreatment.

Table 2. TS, VS, ash and lignocellulosic content of residual juice and fibre fractions of S. ramosissima samples

Residual fractions	TS	VS	Cellulose	Hemi-cellulose	Lignin	Extractives	Ash	TBMP*
	wt% of FM	wt% of VS_{FM}	wt% of TS	wt% of TS	wt% of TS	wt% of TS	wt% of TS	mL-CH_4 /g-VS
Extractive-free lignified fibres	94.89 (0.02)	94.03 (0.04)	25.8	27.98	23.81	17.66	0.90 (0.01)	253
SDJ-1B6*	97.17 (0.01)	93.12 (0.32)	36.5	7.6	15.9	20.8	4.17 (0.33)	234
Brown juice	4.99 (0.02)	3.69 (0.01)	n.d	n.d	n.d	n.d	26.08 (0.02)	n.d

*TBMP theoretical BMP based on the analysed content of lignocellulose and crude protein of the biomass

From the elemental analysis of C, H, N, and O (Table 3), the results of nitrogen (N) and crude protein (CP) show that the brown juice contain significantly more protein than the residual fibre fractions, resulting in a much lower C/N ratio.

Table 3. Elemental analysis of residual juice and fibre fractions, standard deviation in brackets

Residual fractions	C	H	N	O	C/N ratio	Crude Protein
		wt% TS			wt/wt	
Extractive Free lignified fibres	46.1 (0.4)	6.2 (0.1)	0.9 (0.1)	45.5 (0.5)	51.2	5.5
SDJ-1B6	47.1 (0.5)	6.3 (0.1)	1.3 (0.1)	40.7 (0.3)	36.2	8.4
Brown Juice	35.1 (0.5)	5.2 (0.1)	3.0 (0.1)	30.1 (0.2)	11.7	18.8

4.2 *Biomethane potential of residual fractions in anaerobic co-digestion*

The BMP of *S. ramosissima* biomass material: extractive free fibres post Soxhlet extraction, de-juiced fibres before and after organosolv pretreatment and of the brown juice are shown in Table 4. The lowest BMP of 172.4 mL-CH_4/g-VS was found for the extractive free lignified fibres; the methane yield of the wet-presses green fibres was significantly higher at 243.6 mL-CH_4/g-VS and increased after organosolv pretreatment at conditions SDJ-1B6 to 288.6 mL-CH_4/g-VS. The brown juice revealed the highest BMP of 324.3 mL-CH_4/g-VS.

Table 4. Biomethane potential (BMP) of S. ramosissima extractive-free lignified fibres, green fibres

Biomass samples	BMP (mL-CH_4/g-VS)
Extractive-free lignified fibre	*172.4 ± 3.0*
Untreated wet-pressed de-juiced fibres of green S. ramosissima	*243.6 ± 10.4*
After pretreatment SDJ-0C6 (200 °C, 45 min, EtOH 60% v/v)	*248.1 ± 5.4*
After pretreatment SDJ-0B6 (200 °C, 30min, EtOH 60% v/v)	*259.3 ± 13.2*
After pretreatment SDJ-1B6 (180 °C, 30min, EtOH 60% v/v)	*288.6 ± 12.7*
Brown juice	*324.3 ± 3.8*

In co-digestion with brown juice, the BMP of the extractive free lignified fibres increased significantly to 220.8 – 234.7 mL-CH_4/g-VS (Figure 2). Accordingly, the BMP of the co-digestion of extractive free fibres and brown juice was similar and higher than the respective calculated BMP value (BMPcalc) that was determined based on the experimental BMP in mono-digestion and the VS% share of fibres and juice in the co-digestion mixture (Table 5). This shows that the addition of brown juice has a positive effect on the biogas process of the extractive free lignified *S. ramosissima* fibres.

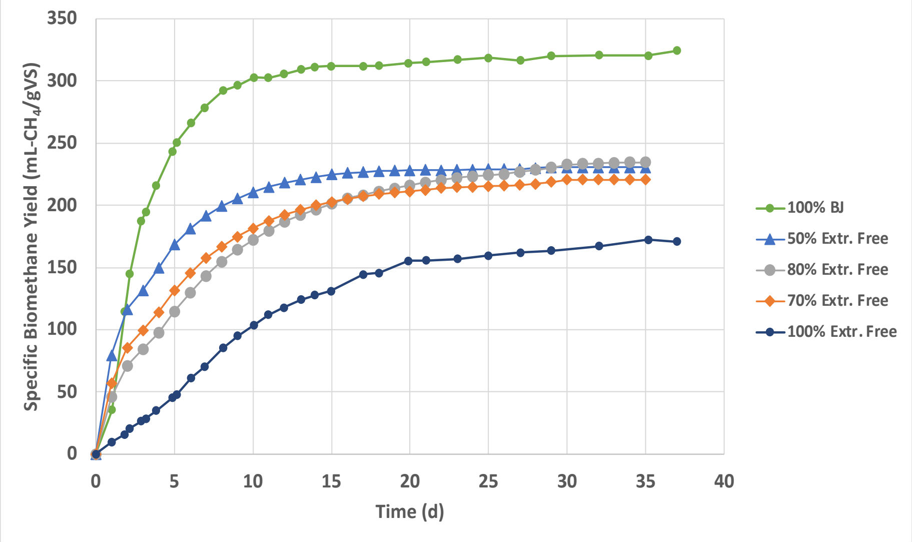

Figure 2: BMP of extractive free lignified S. ramosissima fibres (Extr. Free) in different co-digestion ratios with brown juice (BJ)

In contrast, the BMP of organosolv pre-treated green fibres SDJ-1B6 decreased with the addition of brown juice to 214 – 226 mL-CH_4/g-VS (Figure 3) and the biomethane yield was significantly lower than the calculated BMP values (Table 5). The lower BMP of the co-digested substrates to calculated BMP indicated process inhibition.

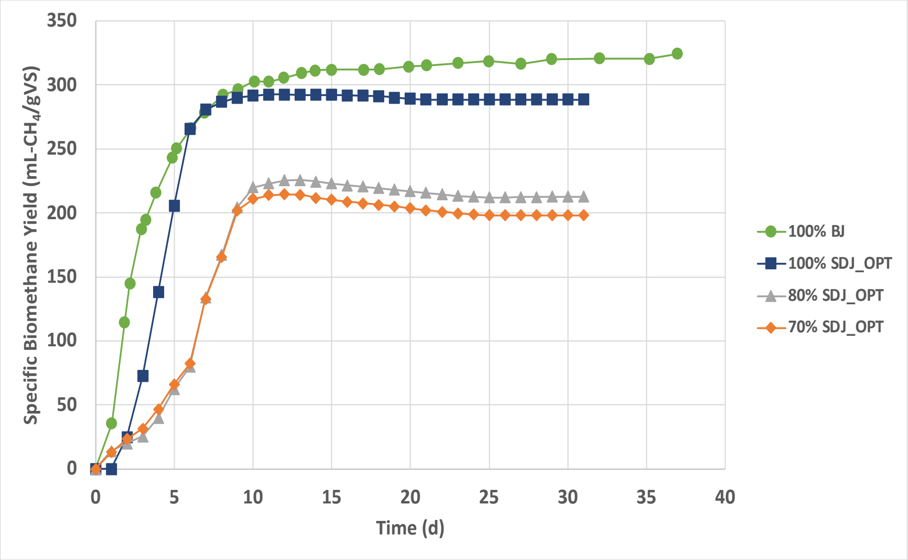

Figure 3: BMP of S. ramosissima de-juiced fibres post organosolv pretreatment (SDJ-1B6) in different co-digestion ratios with brown juice (BJ)

Table 5. Co-digestion ratios, I/S ratio, experimental and calculated biomethane potential

Co-digestion ratio of *S. ramosissima* fractions	**Inoculum to substrate ratio (I/S)**	**BMP$_{EXP}$**	**BMP$_{CALC}$**	**C/N of co-substrate mix**
	(gVS/g/VS)	mL-CH$_4$/g-VS		wt/wt
Extractive-free lignified fibres : brown juice (%VS)				
50:50	2	231 ± 3.7	248	18.6
70:30	2	220.8 ± 3.1	222	24.5
80:20	2	234.7 ± 5.3	203	29.7
SDJ-1B6 : brown juice (%VS)				
70:30	2	214 ± 24.6	300	21.5
80:20	2	226 ± 4.3	296	25.6

The C/N ratio ranged in the co-digestion set-up with brown juice from 18.6 -29.7 and 21.5 - 25.6 for extractive free fibres and wet-pressed green fibres, respectively, which is within the optimal C/N ratio of 10-30, confirmed by Turcios et al 2021 to be applicable also for halophytic biomass. Chen et al 2010 found for the co-digestion of the halophyte *Spartina alterniflora* with varying co-digestion ratios of cow manure that the C/N ratio dropped from 21.82 to 14.19 and improved the biodegradation of *Spartina alterniflora*, with lower VFAs concentrations and higher methane yields of 7 to 44%. At low C/N ratios a high release of ammonia during anaerobic digestion of biomass could lead to inhibition of the methanogenic activity, resulting in a decrease in biomethane production and an accumulation of volatile fatty acids (VFA). On the other hand, the high C/N ratio like that of extractive free lignified fibres and fibres post organosolv pretreatment can cause nitrogen deficiency in the whole anaerobic microbial consortium, thus decreasing the methane production potential when digested as mono-substrate.

5 Conclusions and Outlook

The investigations on the biomethane potential of the co-digestion process of residual juice and fibre fractions from different upstream fractionation pretreatment and extraction processes of the AQUACOMBINE biorefinery reveal the following main findings:

- Residual juice and fibre fractions have significantly lower salt concentration after pretreatment and extraction processes as reflected in the ash content of the residual biomass.
- Co-digestion of extractive free lignified fibres together with brown juice had a positive effect on the biogas process of this halophyte fibre material.
- Co-digestion of de-juiced green fibres post organosolv pretreatment with the brown juice showed, however, significantly lower anaerobic degradability and biomethane yields than calculated BMP.
- The difference in the co-digestion performance of both fibre materials could not be explained by the C/N ratio, which was for all co-digestion set-ups within the optimal ratio for a stable AD process of 10-30.

This means, more investigations are necessary to identify the optimal co-substrate and co-digestion ratios for halophytic plant material. Furthermore, the optimized co-digestion process will be tested in continuous lab-scale reactors experiments to determine optimal operation conditions like organic loading rate and hydraulic retention time of the biogas reactor.

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