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Characterization of lignocellulosic residues and Acid fermentation of maize silage for the production of carboxylic acids

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ABSTRACT

The potential of various biomasses to produce green chemicals is currently one of the key topics in the field of the circular economy. Especially lignocellulosic feedstock nowadays gained more attention because they are responsible of the formation of the so-called second-generation biofuels.
Characterization of lignocellulosic residues is fundamental to understand their potentiality to become a substrate for further fermentations. In this research the proximate analysis, the chemical structure composition and the carbohydrates content are carried out. Different methods are exploited and compared for this purpose. Every method is applied on four lignocellulosic feedstocks, showing that the type of raw material under study influence each characterization method.
Only one of the four feedstocks characterised is used for the rest of experimental work: maize silage.
Anaerobic digestion is a complex process that involves different phases. The main goal is biogas production. Volatile fatty acids are intermediates in the Anaerobic digestion process, but they have more and useful end-uses respect to biogas. Concentration and distribution of VFAs is the result of acidogenic metabolic pathways, which can be affected by the microenvironment in the digester and by the biogenic phase physiological state.
Different parameters could influence the metabolic pathways, for example. Among these, hydrogen partial pressure was chosen as monitored parameter in this research, showing that it strongly influences the bacteria metabolism. Moreover, the polarizability of the biological phase was analysed at different at different time points with electrooptical analysis. Finally, partial pressure of hydrogen and polarizability of the broth were compared in order to investigate better the cells behaviour.

Since the acid fermentation is investigated, two tests were conducted in order to discern between the most efficient inoculum pre-treatment that have as main goal suppressing methanogenic activity and increase hydrogen and acids production.

SOMMARIO

Introduzione

Al giorno d'oggi, una gran parte del fabbisogno energetico totale del mondo (oltre l'84%) è soddisfatta da risorse fossili non rinnovabili come il carbone, il petrolio e il gas naturale. Lo sviluppo sostenibile in tutto il mondo richiede risorse energetiche alternative, poiché negli ultimi decenni l'uso esagerato dei combustibili fossili ha portato ad un aumento dell'inquinamento. Tutto ciò rende necessaria la presa in considerazione di una transizione graduale verso concetti come l'economia circolare e la bioeconomia. Il concetto di bioeconomia comprende la produzione di risorse biologiche rinnovabili e la conversione di tali risorse, residui, sottoprodotti e flussi secondari in prodotti ad alto valore aggiunto come alimenti, mangimi, bio prodotti o bioenergia. L'enorme crescita della popolazione inoltre è accompagnata dalla generazione di grandi quantità di rifiuti che porta di conseguenza al problema del loro corretto smaltimento. Vi è un rinnovato interesse per la produzione e l'uso di combustibili di origine vegetale o da rifiuti biodegradabili per uno sviluppo sostenibile dell'energia.

In questo contesto si inserisce il concetto di bioraffineria. Molte varietà di biomassa sono oggi utilizzate per la produzione di biofuels. A seconda della materia prima utilizzata i biofuels si suddividono in 4 categorie:

- prima generazione: biodiesel da colture agricole (mais, riso, patate, canna da zucchero). Il principale svantaggio è che la loro produzione compete con quella di mangime o alimenti.
- seconda generazione: da biomassa lignocellulosica tipo residui agricoli o rifiuti (a differenza della prima generazione, le materie prime non sono destinate al consumo umano/animale);
- terza generazione: viene sfruttata la coltivazione di alghe, le quali fissano la CO2 e non sottraggono terreni alla coltivazione;
- quarta generazione: elettro combustibili e combustibili solari fotobiologici (la biomassa non è coinvolta).

Soltanto poche bioraffinerie utilizzano materie prime di seconda generazione, i cosiddetti materiali lignocellulosici, dato che si tratta di materiali rigidi, naturalmente resistenti all'attacco microbico. I materiali lignocellulosici comprendono residui agricoli, residui forestali, erbe, materiali legnosi, ma anche i rifiuti organici rientrano in questa categoria.

La struttura chimica di questo tipo di biomassa è caratterizzata dalla presenza di cellulosa, emicellulosa, lignina, inorganici ed estratti (Figura 2.2). La cellulosa è un polimero del glucosio. L'unità ripetitiva della cellulosa è il cello biosio (unità di due zuccheri). Le fibre di cellulosa sono tenute insieme da legami a idrogeno formando le microfibrille che rendono l'intera struttura cristallina. L'emicellulosa è un polimero costituito da zuccheri esosi e pentosi, principalmente glucosio, galattosio e xilosio. Ha una struttura ramificata (Figure 2.4) e il compito di legare insieme cellulosa e lignina. La lignina è un complesso polimero amorfo, insolubile e principalmente aromatico che agisce come colla, andando a riempire gli spazi tra cellulosa e emicellulosa, dona supporto strutturale ed è la principale responsabile di resistenza agli attacchi microbici. Gli estratti sono un gruppo di composti organici e non che comprendono

resine, cere, alcool, terpeni o acidi grassi, mentre la voce inorganici si riferisce ai sali minerali contenuti nella biomassa.

Nel contesto di bioraffineria, la digestione anaerobica (DA) è una delle tecnologie più versatili e il biogas (CO_2 e CH_4) prodotto con essa è una delle principali alternative ai combustibili fossili. La DA è un processo catabolico che prevede dunque la formazione di energia in seguito alla degradazione della biomassa da parte di microorganismi che secernono cosiddetti enzimi idrolitici. Siccome nessun batterio è in grado di produrre tutti gli enzimi necessari alla degradazione del substrato, una vasta e diversificata comunità di batteri si rende indispensabile.

La digestione anaerobica è un processo a step:

1. la prima fase è detta idrolisi, in cui molecole complesse come carboidrati, proteine e grassi vengono degradate in molecole più semplici (monosaccaridi, aminoacidi e acidi grassi a catena lunga) da batteri idrolitici attraverso la produzione di enzimi (cellulase, lipasi).
2. Durante la seconda fase, l'acidogenesi, queste molecole più semplici sono trasformate in acidi grassi a catena corta C1-C5, alcoli, idrogeno e anidride carbonica da batteri fermentativi.
3. Il terzo stadio, chiamato acetogenesi, comporta la conversione di acidi volatili e alcoli in acido acetico e gas idrogeno. Esiste inoltre un gruppo di batteri chiamati omoacetogeni che convertono H_2/CO_2 in acido acetico.
4. L'ultima fase, ovvero la metanogenesi, coinvolge la presenza di batteri metanigeni. Essi si suddividono in acetotrofici e idrogenotrofi. I primi trasformano acido acetico in metano e CO2, mentre i secondi consumano idrogeno per produrre metano.

Quindi la digestione anaerobica è portata a termine da un consorzio di microorganismi che lavora in maniera sinergica e in cui l'idrogeno è un metabolita chiave per la regolazione dei processi. Il microbioma, in generale può essere classificato in due grandi gruppi: batteri produttori di idrogeno (HPB) e batteri consumatori di idrogeno (HCB). HCB sono batteri anaerobici, mentre gli HPB sono strettamente anaerobi o anaerobi facoltativi, il genere *Clostridium* è stato identificato come il genere dominante tra gli HPB.

Durante il metabolismo, i batteri fermentativi, depositano una parte di elettroni liberati in seguito all'ossidazione del substrato, sullo stesso substrato ossidato o sui protoni, formando metaboliti più ridotti come etanolo e acido acetico oppure idrogeno.

Nella DA del glucosio come substrato, gli HPB convertono il glucosio in piruvato durante la glicolisi producendo ATP e NADH. Il piruvato è ulteriormente ossidato ad acetil-coA e CO_2 mediante un enzima chiamato piruvato: ferredoxina ossidoreduttasi. A seconda del tipo di microorganismo e condizioni ambientali (tipo di substrato, pH, T, p), l'acetil-coA può intraprendere diversi cammini metabolici come mostrato in Figura 2.7. Il cammino che porta alla produzione di acido acetico è quello che libera teoricamente più moli di idrogeno secondo la reazione:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH \text{ (acetic acid)} + 2CO_2 + 4H_2$$

Rese ancora più basse sono ottenute intraprendendo gli altri cammini metabolici.

Tutti i tipi di biomassa che contengono carboidrati, lipidi, proteine, cellulosa e emicellulosa in corretti rapporti C: N possono essere utilizzati come substrato per la fermentazione anaerobica.

Va ricordato però che visto che l'idrolisi diventa il rate-determining step della DA a seconda del tipo di biomassa usata, la degradazione di materiali lignocellulosici è la fase più delicata. Questo perché la struttura è complessa e resistente all'attacco microbico data la presenza di polimeri complessi come la lignina, cellulosa ed emicellulosa. Dunque, per rendere il substrato accessibile e per incrementare la resa dell'idrolisi sono necessari pretrattamenti sul substrato. I pretrattamenti possono essere classificati in:

- Fisici
- Chimici
- Biologici

I pretrattamenti fisici includono tecniche meccaniche come la macinazione, oppure lo steam explosion, l'irradiazione con ultrasuoni o microonde. Tutti questi pretrattamenti hanno lo scopo di diminuire la dimensione delle particelle, o di degradare parzialmente il substrato aumentandone l'area superficiale accessibile dai microorganismi. I pretrattamenti chimici coinvolgono l'uso di acidi o basi che alterano parzialmente la struttura della lignina o dell'emicellulosa, rendendo la cellulosa suscettibile all'attacco enzimatico. I pretrattamenti biologici invece coinvolgono l'aggiunta di enzimi idrolitici. In Tabella 2.4 sono presenti i diversi effetti dei pretrattamenti sulla biomassa.

La fermentazione acida (Dark Fermentation) è una versione abbreviata della fermentazione anaerobica. In questo processo la metanogenesi è repressa e l'idrogeno e gli acidi grassi a catena corta sono i principali prodotti. Ultimamente, l'interesse nella produzione di acidi volatili è aumentato, perché essi hanno un ampio range di applicazioni che va dalle microbial fuel cells alla produzione di bioplastiche (Figura 2.9).

Diversi parametri concorrono alla regolazione dei cammini metabolici dei microorganismi e quindi influenzano la produzione di acidi e idrogeno. Tra questi ritroviamo la temperatura, il pH, il tempo di ritenzione, il contenuto di substrato iniziale e la pressione parziale di idrogeno.

Per aumentare la produzione di acidi e idrogeno diversi pretrattamenti sull'inoculo possono essere applicati in modo da sopprimere l'attività dei metanigeni. Questi pretrattamenti sfruttano le differenze fisiologiche tra HPB e HCB. I batteri appartenenti al genere *Clostridium* sono i principali responsabili per la produzione di idrogeno. Sono anaerobi obbligati, Gram-positivi e possono produrre spore quando le condizioni ambientali sono deleterie alla loro crescita, quindi alta temperatura, condizioni di eccessiva acidità o basicità ecc. le spore diventano di nuovo attive una volta che le condizioni diventano di nuovo favorevoli. Mentre i metanigeni sono incapaci di formare spore e questo li rende più sensibili alle condizioni di stress. Inoltre, essendo gli HPB più attivi crescono più velocemente e sono meno sensibili ai cambiamenti di pH (4.5-7) rispetto ai metanigeni (7-8). I pretrattamenti possono essere classificati sulla base della fonte di stress applicata. Si distinguono:

- Pretrattamenti termici: shock termico o congelamento
- Pretrattamenti acidi o basici: coinvolgono l'utilizzo di acidi o basi forti e un periodo di incubazione.
- Pretrattamenti a onde e radiazioni: ultrasuoni, microonde, radiazioni ultraviolette

- Stress aerobico: consiste nel flussare aria
- Pretrattamento chimico: utilizzo di reagenti chimici come il cloroformio.

Nel contesto delle bioraffinerie, il controllo e il monitoraggio della fase biotica è cruciale per l'ottimizzazione dei processi. Ultimamente, diversi metodi sono stati messi a punto per la misura dello stato fisiologico dei microorganismi nel campo del Process Analytical Technology (PAT). In questo campo ricadono le tecniche elettroottiche che usano come parametro universale per il controllo dell'attività fisiologica lo stato della membrana cellulare dato che rappresenta l'interfaccia con l'esterno e assicura un adatto microambiente interno. Una delle tecniche elettroottiche è la misura dell'anisotropia della polarizzabilità. La polarizzabilità è dovuta ad un'accumulazione di cariche sulla superficie dei materiali con diverse proprietà elettriche. Tra le proprietà elettriche si ricorda la conduttività (σ, in S/m) e la permettività (ε, in F/m), relative alla risposta di un materiale. Solitamente le cellule più attive tendono ad avere un'elevata polarizzabilità. Quando è applicato un campo elettrico di una certa frequenza, questo causa un momento rotazionale sulle cellule, che promuove un'orientazione che è a sua volta usata per il calcolo del tensore di polarizzabilità $a_{ikj}(\omega)$ (Eq. 2.1). Considerando anche il tempo di rilassamento dopo lo spegnimento del campo elettrico, si può avere un'idea della misura delle cellule.

Procedura sperimentale

Il presente lavoro di tesi sperimentale è focalizzato sulla caratterizzazione di materie di origine lignocellulosica e sulla fermentazione acida per la produzione di acidi carbossilici. Gli obiettivi sono i seguenti:

- Definire una metodologia di caratterizzazione delle materie prime
- Valutare il pretrattamento sull'inoculo più efficiente tra pretrattamento acido e termico per la soppressione dell'attività dei metanigeni
- Monitorare la produzione di gas idrogeno e valutare come questa possa influire sui cammini metabolici della fermentazione acida e sullo stato fisiologico della coltura

Dunque, la parte sperimentale si può così suddividere:

1. Selezione delle materie prime
2. Caratterizzazione
3. Test sulla fermentazione acida
    - Test 1 e 2: valutazione efficienza pretrattamento inoculo
    - Test 3: monitoraggio produzione idrogeno

Per quanto riguarda la selezione delle materie prime quattro substrati sono stati scelti per la caratterizzazione: legno, proveniente dalle foreste di Lille in Francia, maize silage, fornito dallo IASP (Institut für Agrar- und Stadtökologische Projekte) a Berlino. Come rappresentanti dei rifiuti organici riso e lattuga sono stati considerati. Per la fermentazione acida soltanto il maize silage è stato preso in considerazione in quanto ritenuto materiale di riferimento.

In questo lavoro le stesse procedure di caratterizzazione sono state applicate sulle 4 materie prime in modo da vedere se è possibile creare un metodo unificato, standard e riproducibile per tutti i tipi di biomassa. I parametri misurati sono: contenuto di umidità MC, solidi totali TS, solidi volatili VS, inorganici, lignina, cellulosa, emicellulosa, carboidrati totali.

Un sommario dei metodi è visibile in **Tabella I**:

Tabella I Sommario metodi caratterizzazione

Parametri	Metodi
TS, MC, VS, inorganici	APHA standard methods
Lignina	Chlorination method Klason-lignin method
Emicellulosa, cellulosa	NaOH hydrolysis
Carboidrati totali	H_2SO_4-UV method Phenol-H_2SO_4 method HPLC

Composizione in massa

La procedura standard APHA è stata applicata per la proximate analysis, ovvero la misurazione del contenuto di umidità, solidi volatili, solidi totali e inorganici. Un'appropriata quantità di materia fresca è stata pesata e sottoposta ad essiccazione in forno a convezione ad 80°C per 24h. Il contenuto di umidità e solidi totali è stato misurato per via gravimetrica. 2g di campione essiccato sono stati pesati e bruciati a 550°C per un minimo di 6h. Il contenuto di VS e inorganici è stato definito sempre per via gravimetrica.

Contenuto di lignina, cellulosa ed emicellulosa

Come riportato dalla Tabella I due metodi sono stati applicati per trovare il contenuto di lignina, il chlorination method e il processo Klason. Il primo permette di solubilizzare l'olocellulosa (cellulosa+ emicellulosa) lasciando come residuo insolubile la lignina, mentre il secondo attraverso un processo di idrolisi acida permette di misurare il quantitativo di lignina solubile e insolubile nell'acido.
Per quanto riguarda il metodo chlorination 2,5g di materia secca sono stati pesati e miscelati con acqua a 70°C, 5mL di acido acetico puro e 1g di clorito di sodio. La miscela è stata mantenuta a temperatura costante di 70°C grazie ad un bagno termostatico per 6-8h. Durante questo tempo 0,5mL di acido acetico e 1g di clorito di sodio sono stati aggiunti ogni ora. La lignina così ottenuta è lasciata in bagno tutta la notte e in seguito filtrata con l'utilizzo di una pompa a vuoto. Il liquido ottenuto è stato usato per la quantificazione di cellulosa e emicellulosa, attraverso il metodo chiamato NaOH hydrolysis. L'olocellulosa è miscelata con idrossido di sodio al 17,5% *w/v* in rapporto solido: liquido 1:5. Quindi ogni cinque minuti per due volte la metà della quantità di NaOH è stata aggiunta. Dopo mezz'ora 33mL di acqua distillata sono stati aggiunti, la soluzione contenente emicellulosa filtrata e la cellulosa quantificata per via gravimetrica previa essiccazione ad 80°C per 24h.

Per quanto riguarda il processo Klason questo si avvale di un doppio step di idrolisi acida, in cui nel primo step vengono aggiunti 0,3g di materia secca a 3mL di H_2SO4 al 72% *v/v*. Dopo un periodo di incubazione di 1h a 30°C, 84mL di acqua sono stati aggiunti e il tutto auto clavato per 1h a 121°C. La soluzione ottenuta è stata filtrata e il contenuto insolubile di lignina calcolato per via gravimetrica, mentre il contenuto solubile di lignina è stato quantificato per via spettrofotometrica ad una lunghezza d'onda dipendente dal tipo di biomassa (tabella 3.2).

Carboidrati totali

Per quanto riguarda il contenuto di carboidrati due metodi di estrazione sono stati applicati:

- Klason extraction
- Ethanol extraction

Il primo è stato descritto nel paragrafo precedente, mentre per l'estrazione con etanolo 5mL di etanolo all'80% *v/v* sono stati fatti bollire a 95°C con 50mg di materia secca per 10 minuti. Dopo i 10 minuti i campioni sono stati centrifugati a 2500rpm per 5min e il liquido prelevato. Tre estrazioni in serie sono state condotte in totale e i liquidi accumulati, per un totale di 15mL di soluzione zuccherina estratta per ogni materia prima.

Per quanto riguarda invece la quantificazione dei carboidrati due metodi sono stati messi in pratica:

- H_2SO_4-UV vis method
- HPLC

La prima procedura sfrutta l'assorbimento di luce ultravioletta da parte dei furfurali prodotti in seguito alla reazione degli zuccheri con acido solforico puro. 1mL di soluzione zuccherina estratta è stato miscelato con 3mL di acido solforico puro. Una volta avvenuta la reazione l'assorbanza è stata registrata a 315nm. Per quantificare gli zuccheri presenti una curva standard è stata costruita seguendo la stessa procedura ma sostituendo la soluzione zuccherina estratta con una soluzione standard di glucosio+ galattosio a diversi fattori di diluizione e quindi diverse concentrazioni.

Per l'HPLC soltanto il liquido proveniente dalla Klason extraction è stato utilizzato. L'analisi è stata condotta usando una 1200-series HPLC system equipaggiata con un rilevatore di indice refrattivo (RID). Cinque standard contenenti zuccheri diversi alla concentrazione di 1g/L sono stati preparati. Il liquido da analizzare è stato prima neutralizzato a pH 7 con carbonato di calcio.

Test sulla fermentazione acida

Tre batch tests sono stati condotti utilizzando maize silage come substrato e un inoculo proveniente da un impianto di biogas per la fermentazione acida. L'apparato sperimentale si avvale di tre batch (Blue-sens flasks) per triplicare la misurazione, con un volume totale di 250mL e un volume di lavoro di 50mL. Tutti e 3 i batch sono stati adoperati in condizioni anaerobiche ottenute flussando azoto per 5-10min, in condizioni mesofiliche (T=35°C), a pH iniziale di 5,2 e con un rapporto substrato: inoculo di 0,5. L'agitazione meccanica di 50rpm è stata ottenuta utilizzando uno shaking water bath. Un tubo per i campionamenti è stato inserito insieme ad un altro tubo per prevenire la sovrapressione (vedi Figura 3.6).

Per il Test1 un pretrattamento alcalino è stato adottato per il substrato. Il maize silage è stato essiccato e diluito con acqua 4:11 e trattato con NaOH al 30% w/w fino a pH 12. La miscela è stata incubata per 24h a 35°C. Il substrato è stato successivamente risciacquato con acqua fino a pH 7. Per quanto riguarda il pretrattamento dell'inoculo, quest'ultimo è stato trattato con HCl al 10% v/v fino a pH 3 e poi incubato per 24h a 35°C. la polarizzabilità prima e dopo il pretrattamento è stata misurato seguendo la procedura di preparazione dei campioni meglio descritta nel paragrafo 3.3.1. Una volta pronti, substrato ed inoculo sono stati miscelati e neutralizzati con NaOH al 30% w/w fino a pH 5,2. La fermentazione è durata 5 giorni e 3 punti

di prelievo sono stati fissati. Le percentuali di metano e anidride carbonica nella fase gassosa sono state monitorate con dei sensori.

Per il Test 2 la stessa procedura è stata applicata fatta eccezione per il pretrattamento dell'inoculo. L'inoculo è stato infatti posto a 70°C per 30min.

Anche per il Test 3 le stesse procedure sono state usate. Come pretrattamento si è scelto quello più efficiente come dimostrato dal Test 1 e Test2. Inoltre, la percentuale di idrogeno in questo caso è stata monitorata invece che quella di metano. la fermentazione è durata 5 giorni durante i quali è stato prelevato un campione ogni 24h. I campioni sono stati usati per la misura della polarizzabilità, del pH, e della produzione di acidi con l'HPLC.

Risultati e conclusioni

Caratterizzazione

È fondamentale trovare la composizione della struttura lignocellulosica. La lignina è la principale responsabile della resistenza, mentre cellulosa e emicellulosa sono le principali fonti di carboidrati. Il contenuto di lignina trovato con il metodo chlorination insieme al contenuto di cellulosa e emicellulosa sono presentati in Figura 4.2. Oltretutto un altro metodo è stato adottato per determinare il quantitativo di lignina, il Klason method. Un confronto tra i due metodi è presentato in Figura 4.3. Sembra evidente come la percentuale di lignina trovata con il chlorination method sia differente dai valori in letteratura. La migliore spiegazione è che il metodo è stato messo appunto per matrici legnose ad alto contenuto di lignina e quindi i risultati sperimentali sono influenzati dal contenuto di estratti e inorganici presenti nel riso, nel maize e nella lattuga. Nel mais e nel riso ad esempio c'è un altissimo contenuto di amido. I valori trovati insieme al confronto con la letteratura sono ripostati in **Tabella II**.

Tabella II Comparison between literature values and experimental data for lignin and cellulose content. t.s. stands for this study.

	Rice		**Lettuce**		**Wood barks**		**Maize silage**	
	Lignin	**cellulose**	**Lignin**	**Cellulose**	**Lignin**	**Cellulose**	**Lignin**	**Cellulose**
Literature [4] [57] [58]	9-20	28-38	15-20	0-6	40-47	16-31	10-15	35-45
t.s (chlorination and NaOH hydrolysis)	81,20 ±2,82	1,00 ±0,28	67,40 ±1,36	24,43 ±0,96	47,87 ±0,13	35,82 ±0,47	74,54 ±4,93	15,13 ±1,75
t.s. (Klason-lignin extraction)	4,32 ±0,52	-	14,37 ±1,18	-	40,98 ±6,70	-	13,86 ±1,91	-

Il legno è quindi la materia prima con più alta % di lignina, seguito dalla lattuga e maize silage.

Per la determinazione dei carboidrati due metodi di estrazione sono stati applicati: il metodo Klason basato sull'idrolisi acida e l'estrazione con etanolo basato sulla solubilizzazione dei

carboidrati. Per quanto riguarda invece la quantificazione il metodo H_2SO_4-UV-vis è stato sfruttato. Il confronto tra i due metodi estrattivi è in **Figura I**.

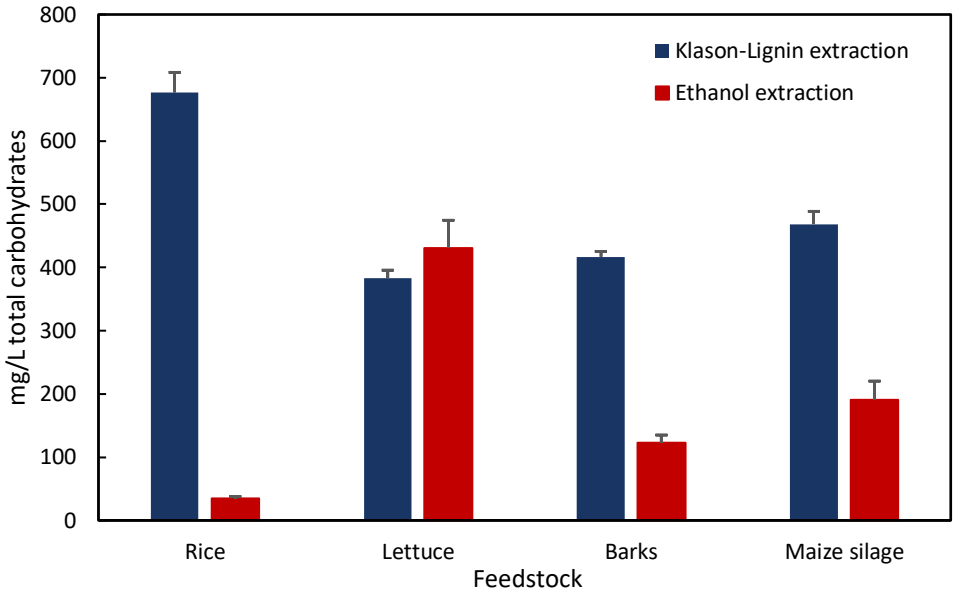

Figura I confronto tra i due metodi estrattivi per la determinazione dei carboidrati totali

Il metodo Klason sembra più efficiente in quanto la quantità di zuccheri estratti ottenuta è maggiore rispetto a quella ottenuta con l'estrazione da etanolo. Questo probabilmente significa che i carboidrati strutturali contenuti in queste materie prime sono più facilmente idrolizzabili che solubili. Questo non appare per la lattuga in quanto la differenza non è sostanziale, forse dovuto al fatto che nemmeno la quantità di carboidrati è molto alta in questa materia prima, dato l'elevato contenuto di umidità e sostanze inorganiche.

Oltre al metodo UV-vis anche l'HPLC è stata investigata. Come si può vedere dalla **Tabella III** le rese ottenute con l'HPLC sono minori. Questo perché l'HPLC è più efficiente per quanto riguarda l'identificazione di singole specie, mentre l'UV-vis porta ad un'identificazione dei carboidrati totali. Le condizioni operative specifiche della colonna dell'HPLC devono essere ottimizzate e monitorate in modo da raggiungere la performance dell'UV-vis. In ogni caso, il riso sembra essere la materia prima con più alto contenuto di zuccheri idrolizzabili, dovuto sicuramente alla presenza di amido.

Tabella III mg sugar/g solidi totali estratti dale quattro materie prime.

mg_{sugar}/g_{TS}	RICE		LETTUCE		WOOD BARKS		MAIZE SILAGE	
	UV-vis	HPLC	UV-vis	HPLC	UV-vis	HPLC	UV-vis	HPLC
KL	813	11	460	3	501	14	563	5
EtOH	41	-	517	-	147	-	228	-

Fermentazione acida:

Test 1 e 2: questi due test sono stati condotti con lo scopo di valutare quale tra i due pretrattamenti applicati fosse il più efficiente per la soppressione dei metanigeni. Nel test 1 un pretrattamento acido è stato scelto, mentre nel Test 2 si è optato per un termico. Uno dei parametri "probe" che portato a discernere tra i due pretrattamenti è la concentrazione di metano registrata con i sensori. L'andamento è mostrato in **Figura II**

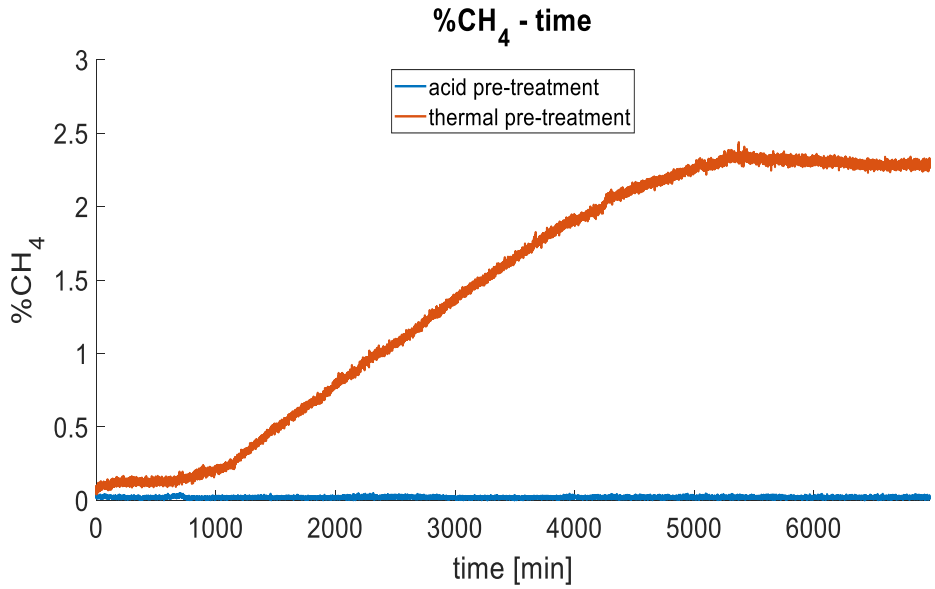

Figura II andamento produzione metano per test 1 e 2

Si può notare una crescita per quanto riguarda il Test 2, infatti è possibile suddividere la curva in tre zone: fase di latenza (16h), fase esponenziale (66h), e fase stazionaria. Quindi il pretrattamento termico non va bene per l'inibizione dei metanigeni a lungo termine, anche perché una parte potrebbe resistere a temperature di 70°C, essendo termofila. Per quanto concerne il Test 1, la quantità di metano registrata oscilla attorno allo zero durante tutta la fermentazione. Un paragone tra i due sistemi indica che la soglia di CH_4 per il Test 2 è 35 volte più alta del Test 1.

Un altro parametro utilizzato per confermare l'ipotesi è la misura di polarizzabilità. Queste mostrano come per il pretrattamento termico lo stato fisiologico delle cellule non ha subito molti cambiamenti, mentre per il pretrattamento acido la polarizzabilità è aumentata probabilmente dovuto ad un cambio di composizione della popolazione. Inoltre, gli HPB sono "più attivi" degli HCB. Queste misure possono quindi parzialmente supportare l'efficienza del metodo acido applicato per la soppressione dei metanigeni.

Test 3: questo test è stato condotto con lo scopo di investigare il ruolo della produzione di idrogeno e la corrispondente produzione degli acidi carbossilici in fase liquida. Infatti, la produzione di idrogeno e acidi è influenzata da molti parametri tra cui la pressione parziale di idrogeno. In figura III è visibile l'andamento della concentrazione di idrogeno e anidride carbonica in fase gassosa.

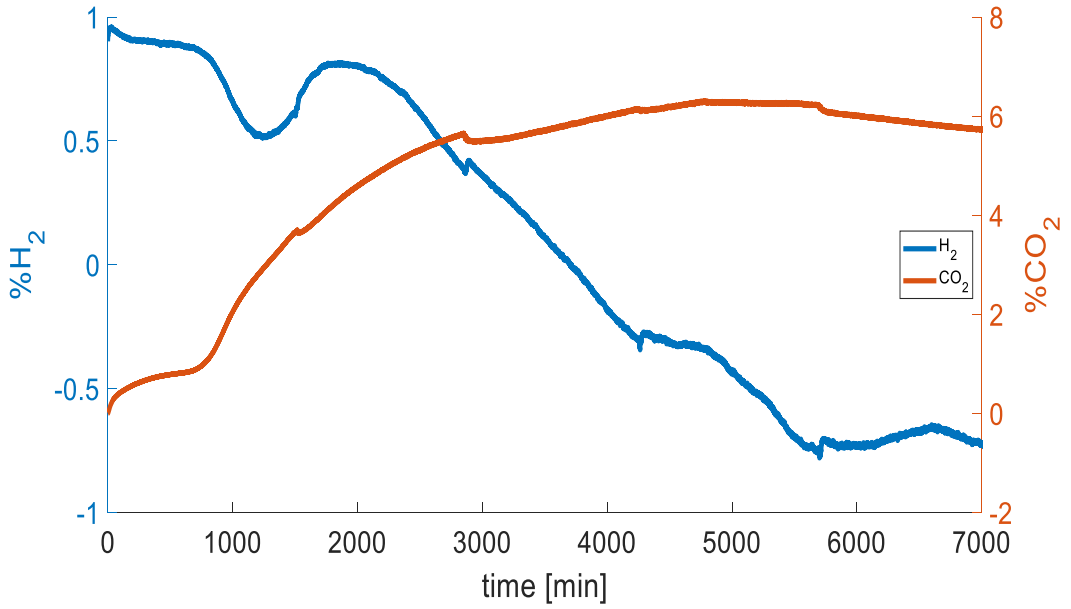

Figura III andamento della fermentazione acida Test 3

Si può notare come l'andamento del grafico può essere diviso in tre zone a diversa pressione parziale di idrogeno. La prima (circa 24h) in cui l'idrogeno inizia ad essere prodotto e la sua pressione parziale è elevata. La seconda zona (circa 60h) in cui la concentrazione di idrogeno da elevata tende a diminuire fino a portarsi a valori costanti alla fine della fermentazione (terza zona). Il motivo probabilmente risiede nel fatto che la coltura si è adattata ad elevate pressioni parziali di idrogeno all'inizio e i cammini metabolici si sono traslati verso quelli che coinvolgono il consumo di idrogeno, diminuendone la concentrazione. Ovviamente ciò che viene influenzato è la produzione di acidi carbossilici, i metaboliti intermedi, di cui se ne può avere un'idea dalla **Tabella IV.**

Tabella IV sommario fasi fermentazione + metaboliti prodotti

Phase	**pH_2**	**Sampling point**	**VFAs and other metabolites detected**
First zone (~24h)	high	I, II (t0, t+24)	Acetate++ Pyruvate++
Second zone (~60h)	low	III, IV (t+48, t+3d)	Pyruvate+ Ethanol++ Acetate+
Third zone	constant	V, VI (t+4d, t+5d)	Pyruvate+

come si può vedere nella seconda zona si può apprezzare la produzione di etanolo, che è uno dei cammini metabolici che coinvolge il consumo di idrogeno. Quindi in conclusione se si desidera produrre acido acetico o butirrico una bassa pressione parziale di idrogeno è raccomandata.

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1 CHAPTER 1: INTRODUCTION

1.1 OBJECTIVES

Recently, the interest in the production of bio-based product from lignocellulosic raw materials is at the basis of the concept of circular economy and bio-economy. This type of raw material can be used in the production of the so-called second-generation biofuels, as their production does not compete with the demands of water or land for the cultivation of food or feed. The main disadvantage is their complex structure that makes them difficult to degrade for microorganisms.
The first aim of anaerobic fermentation is biogas production. Lately there is also an increase of interest in the use of anaerobic fermentation intermediates. These intermediates are short chain carboxylic acids and solvents, which can be used as green and renewable chemical commodities in different sectors and feedstock in production of bioplastics [1].
The valorisation of intermediate liquid products of anaerobic digestion with mixed consortia is still in the upscaling phase. Indeed, acid fermentation from mixed culture is a delicate process where the microorganisms involved are very sensitive to the environmental conditions in which they live. Depending on the environmental conditions, it is possible to have acid production associated with hydrogen (acidogenesis) or a concentration of alcohol and solvents with low hydrogen production (solventogenesis). Furthermore, different inoculum pre-treatments could be used to increase acids and hydrogen production totally or partially suppressing methane production by methanogenic bacteria.
The experimental work is focused on the characterization of different kind of recalcitrant feedstocks and on their test in batch reactors. In this study, the batch anaerobic fermentation is conducted only on one reference feedstock: maize silage.
The main objectives are:

- Evaluate the potential of two methodologies in the characterization of lignocellulose present in four organic materials.
- Evaluate the most efficient inoculum pre-treatment between acid pre-treatment and thermal pre-treatment for the suppression of methanogenic activity.
- Investigate the role of hydrogen production and the correspondent carboxylic acids production which can be influenced by different parameters.

The experimental part of this study was conducted at the Chair of Bioprocess Engineering of the Technische Univesität Berlin, and it is framed within a multidisciplinary project (PASS-BIO). The project aims to establish suitable, cost-efficient bioreactor modules for the flexible conversion of a wide variety of feedstock to produce either short-chain carboxylic acids in an acidic fermentation or methane as alternative.

1.2 THESIS LAYOUT

This thesis starts with Chapter 2 that offers a literature review on the fundamentals of the present experimental work. The review addresses different topics, that range from the definition of a biogeochemical cycle and the reason why recently a shift to circular economy is required to the chemical description of biomass and the key aspects of acid fermentations.
In the introductory part of the Chapter, in section 2.1.1 the definition of carbon cycle is given. Then the chemical and physical properties of biomass are described in section 2.2.
An overview of the different types of biofuels is presented in section 2.3, while the following paragraphs are dedicated to the description of the anaerobic digestion process. More details about the involved microorganisms and relevant metabolic pathways are given, together with an analysis of the possible feedstock than can be used to meet the demand. Since lignocellulosic feedstock are known for their recalcitrant behaviour, a description of all the biomass pre-treatments applied in the research field is presented in section 2.4.3.
The main goal of section 2.5 is to give a detailed background on dark fermentation and acid fermentation, since part of this research is focused on that. The biotechnological relevance of intermediate compounds, such as volatile fatty acids is described together with all the parameters that could influence the metabolic pathways of acidogenic bacteria. And because the main topic is the acid fermentation, an overview of the possible inoculum pre-treatment is given in order to suppress methanogenic activity from methanogenic inocula and enhance the first phases of anaerobic fermentation. At this purpose the physiological differences between hydrogen producing bacteria (HPB) and hydrogen consuming bacteria (HCB) is deeply investigated.
Section 2.6 is useful to understand the theory behind the electrooptical measurements exploited in this research.
Chapter 3, instead, is focused on the experimental part. It provides information about the methodology and materials employed to conduct the experimental part.
Chapter 4 is dedicated to the analysis of results, while in Chapter 5, the conclusions are briefly presented together with the possible future outlooks.

2 CHAPTER 2: THEORETICAL BACKGROUND

2.1 BIOGEOCHEMICAL CYCLES

A biogeochemical cycle is a pathway by which a chemical substance moves through the biotic and abiotic compartment of Earth. The matter which constitutes living organisms is conserved and recycled. Carbon, Nitrogen, Hydrogen, Sulphur, Phosphorus and Oxygen are considered as the six most common elements in organic molecules, and this is the reason why their cycles have been studied very deeply. Those substances can be found in different chemical forms along the cycles and they can be stored for long periods in the ecosystems.
Geologic processes affect those cycles and their role must be considered in biogeochemical cycles balance.
The six elements mentioned above are combined in different forms: oxygen and hydrogen can be found in water and organic molecules. Carbon is found in all organic molecules, Nitrogen is essential for the composition of nucleic acids and proteins. Phosphorus is used to make nucleic acids and phospholipids, main component of cellular membranes whereas Sulphur is important for protein folding and shape [2].

2.1.1 Carbon cycle

The life on Earth is made of carbon. Carbon is the second most abundant element in the Universe, after oxygen. It is present in rocks, oceans, soil, plants, and in the atmosphere. The Carbon cycle is always subjected of continuous modifications due to anthropogenic activities, therefore the study of Climate Change is the study of the global C cycle.
Carbon flows between different reservoirs (**Figure 1.2**) and it is most easily studied as two interconnected sub-cycles: one dealing with rapid carbon exchange among living organism and the other dealing with long-term cycling of carbon through geologic processes [2].
The fast carbon cycle is based on the exchange of atmospheric carbon dioxide (CO_2) between autotrophs and heterotrophs. Terrestrial plants take carbon dioxide directly from the atmosphere, marine autotrophs instead acquire it in the dissolved form (bicarbonate) and during the process called photosynthesis they produce organic substances for their growth. Through food chains, carbon passes to higher trophic levels and during respiration, the organic molecules are broken, and the carbon dioxide is produced and released again in the atmosphere.
The second sub-cycle is complex because carbon flow through lands, soils, water and air are unpredictable and slow. In the sea, the dissolved CO_2 reacts with calcium forming limestone that could be stored in marine floors for some amounts of time.
On land, after the death and decomposition of living organisms or after weathering of rocks the carbon is stored in soil. Some of this carbon can fossilize over millions of years, creating fossil fuels. Fossil fuels are considered a non-renewable energy source because their use rate exceeds their natural rate of formation. This is not the case for biomass, which is hence considered a renewable energy source.

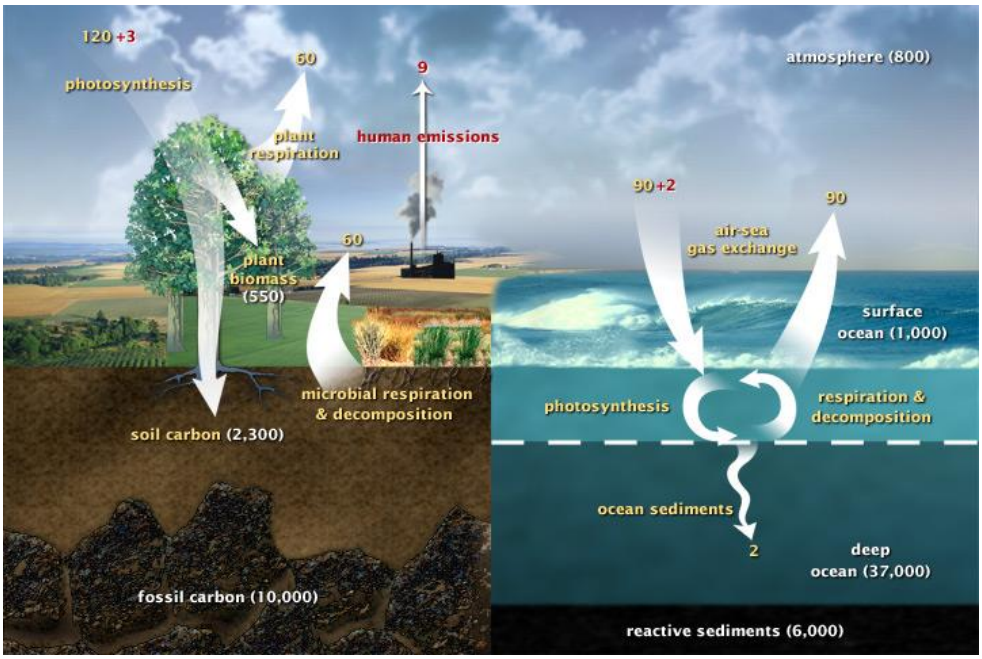


Figure 2.1 Diagram of the fast carbon cycle. Yellow numbers are natural fluxes and red are human contribution. White numbers indicate stored carbon (https://earthobservatory.nasa.gov/features/CarbonCycle)

In the study of carbon cycle, it is essential nowadays to consider human activities. After the Industrial Revolution the energy's demand has risen and humans started to burn more fossil fuels and biomass, causing an increment of CO_2 threshold in the atmosphere during combustion. Therefore, the natural equilibrium is broken and an accumulation of Carbon in the different reservoirs can be registered. Because CO_2 is a greenhouse gas, the Earth's temperature increases as much as CO_2 concentration increases in the atmosphere.

2.2 BIOMASS

Nowadays, a large fraction of the world's total energy demands (more than 84%) is supported by non-renewable fossil resources such as coal, oil and natural gas [3].
Sustainable development around the globe requires alternative energy resources, since the harmful use of fossil fuels have increased pollution in the last few decades [4]. This makes it necessary to consider a gradual transition towards bio-based economies [5]. The bio economy concept encompasses the production of renewable biological resources and the conversion of these resources, residues, by-products and side streams into value added products, such as food, feed, bio-based products, services and bioenergy. Robust increase in human population is also accompanied with the generation of large amounts of different types of wastes and an urgent need to solve the problem of waste disposal has emerged. There is renewed interest in the production and use of plant origin fuels or organic (biodegradable) wastes for sustainable energy development [4].

Biomass can be defined as "recent organic matter originally derived from plants as a result of the photosynthetic conversion process, or from animals, and which is destined to be utilized as a store of chemical energy to provide heat, electricity, or transport fuels" [6].
Biomass resources can be considered as organic matter, in which the energy of sunlight is stored in chemical bonds. When the bonds between adjacent carbon, hydrogen and oxygen molecules

are broken by digestion, combustion, or decomposition these substances release their stored, chemical energy [7].
It is considered a renewable energy source because its inherent energy comes from the sun and because it can regrow in a relatively short time.

Biomass resources include wood from forests, agri-wastes, industrial wastewater, organic fraction (OW) of the municipal solid waste (MSW), food wastes, animal manure, sewage sludge, among others.
In general biomass can be grouped in:

- Lignocellulosic biomass
- Sugars/ starches
- Solid wastes
- Bio-oils
- Animal wastes

In **table 2.1** some details about different categories of biomass resources are shown [4]:

Table 2.1 Categories of Biomass resources [4]

Feedstock type	Resources
Lignocellulosic biomass	Agricultural crop residues, cellulosic crop biomass, food processing wastes, forest residues, mill wastes, urban wood residues, garden residues
Sugars/starches	Agricultural crops (i.e. wheat, rice, maize, sugarcane etc.), food processing residues containing residual sugars and starches.
Solid wastes	Municipal solid waste, furniture construction and demolition wood wastes, non-recycled paper and recycled material
Bio-oils	Agricultural and forestry oil bearing crops and trees, waste oils/fats/grease, algal oil
Other wastes	Animal wastes, waste from waste water treatment, biogas and landfill gas

2.2.1 Biomass composition

Biomass is mostly composed of cellulose, hemicellulose and lignin. There is a small percentage of organic compounds (extractives) and inorganic compounds (ashes) as well.
Table 2.2 shows an example of chemical composition of major agricultural crops.
Lignocellulosic materials including agricultural wastes, forestry residues, grasses and woody materials have great potential for bio-fuel production, as will be explained in 2.3 paragraph. The specific composition of lignocellulosic biomass depends on the plant species, its growth stage, and the environment [8].

Table 2.2 Cellulose, hemicellulose and lignin contents in common agricultural biomass from reference [4]

Lignocellulosic biomass	Cellulose, %	Hemicellulose, %	Lignin, %
Corn cobs	33.6	37.2	19.3
Cotton seed hairs	80–95	5–20	0
Oat straw	39.4	27.1	20.7
Grasses	25–40	35–50	10–30
Hardwood stems	40–55	24–40	18–25
Leaves	15–20	80–85	0
Newspaper	40–55	25–40	18–30
Nut shells	25–30	25–30	30–40
Wheat straw	30, 39.2	50, 26.1	15, 21.1
Maize stover	37.5	30	10.3
Rice straw	44.3	35.5	20.4
Rice husk	34.4	29.3	19.2
Corn straw	42.6	21.3	10–20
Sugarcane bagasse	45	20	30

The lignocellulosic biomass cell walls mainly consist of sugar-based polymers that are combined with lignin (see **Figure 2.2**). The major carbohydrate portion of wood is composed of holocellulose (cellulose + hemicellulose) with a minor amount of other sugar polymers such as starch and pectin.

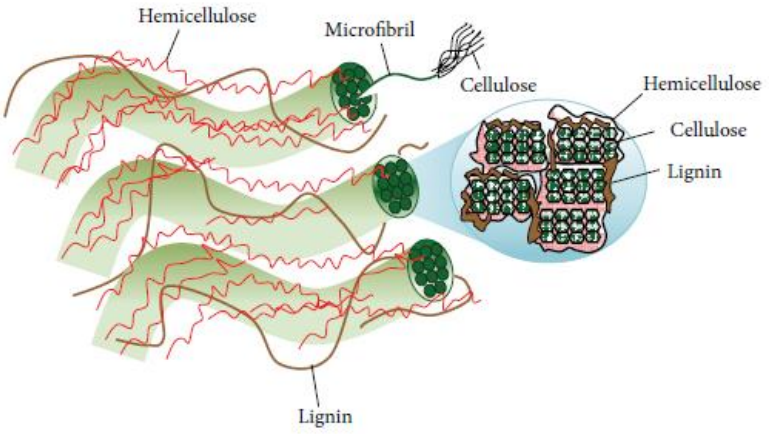


Figure 2.2 Plant cell wall structure and microfibrils cross-section (strands of cellulose molecules embedded in a matrix of hemicellulose and lignin), from reference [9].

Cellulose is the most abundant organic chemical on the face of Earth. It is a polymer of glucose, mainly D-glucopyranose units, which are linked together by (1,4)-glucosidic bonds (see **Figure 2.3**). Actually, the building block for cellulose is cellobiose since the repeating unit in cellulose is a two sugars unit. The number of glucose units in a cellulose molecule is referred as the degree of polymerization. In wood, for example, the degree of polymerization is between 9000-10000. Cellulose molecules are held together by intermolecular hydrogen bonds, because of which it is packed into microfibrils, making the macromolecule highly crystalline and difficult to hydrolyse. There are several types of cellulose in wood: crystalline and non-crystalline,

accessible and non-accessible. Only the surfaces of the crystalline cellulose are accessible. Most of the non-crystalline cellulose is accessible but part is covered with both hemicellulose and lignin [10].

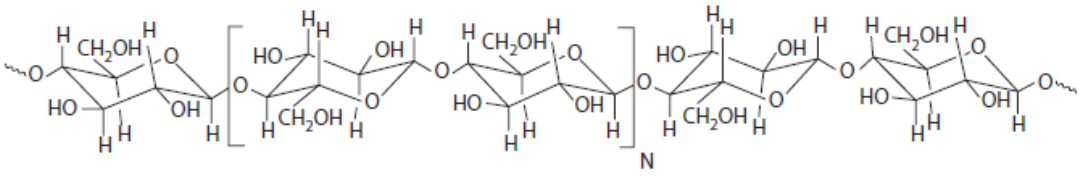


Figure 2.3 Structure of cellulose

Hemicellulose is a polymer of hexose sugars like D-glucose, D-galactose, D-mannose and pentose sugars like D-xylose, D-arabinose, and some organic acids like acetic and glucuronic acid. Hemicellulose has a linear and branched structure with backbone of repeated units of the same sugar (homopolymer) or a mixture of different sugars (heteropolymer). An example of hemicellulose structure is reported in **Figure 2.4**. They are classified by the sugars which are contained, such as like galactoglucomannan, arabinogalactan, among others. Hemicelluloses are soluble in alkali and easily hydrolysed by acids, and compared with cellulose, they present lower degree of polymerization [4]. Hemicellulose serves to link cellulose with lignin and, as stated before, it restricts access to cellulose.

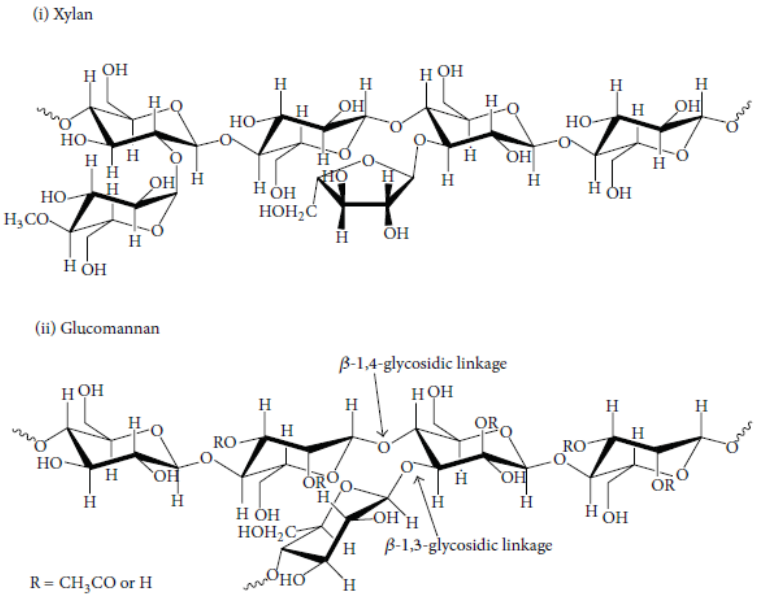


Figure 2.4 Chemical structure of hemicellulose compound (xylan and glucomannan are the most existing biopolymer). From reference [9]

Lignin is an amorphous, insoluble, highly complex, mainly aromatic, polymer of phenylpropane units. The precursors of lignin biosynthesis are: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (see **Figure 2.5**). They form a complex matrix of substituted phenolic units, therefore does not have a single repeating unit like cellulose [10]. Lignin acts like a glue by filling the gap between and around the cellulose and hemicellulose complexion.

Lignin provides structural support to the cell wall, as well as low permeability and resistance against oxidative stress and attacks by microorganism [11].

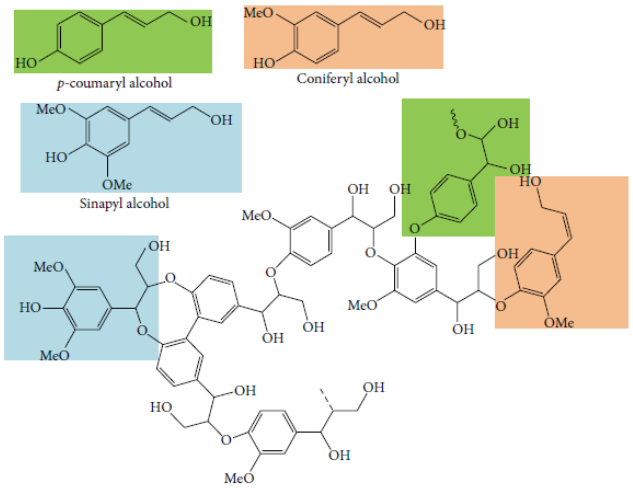


Figure 2.5 Chemical structures of lignin (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol). From reference [9].

The extractives are chemicals in the biomass, which, as the name suggests, can be extracted using different solvents. The extractives are a group of cell wall chemicals mainly consisting of fats, fatty acids, terpenes, alcohols, phenols, resin acids, waxes and many other organic compounds. These chemicals exist as monomers, dimers or polymers. Some are responsible of smell, colour, durability.
The inorganic content of wood is usually referred to its ash content which is an approximate measure of the mineral salts and other inorganic matter. This small amount of inorganic material contains a wide variety of elements like Na, K, Ca, Mg, Mn, Zn and P [10].

2.2.2 Biomass properties

It is the inherent properties of the biomass source that determines both, the choice of conversion process and any subsequent processing difficulties that may arise.
The main chemical properties related to:

- Moisture content
- Ash content
- Volatile matter content
- Fixed carbon

Moisture content represents the amount of free water in biomass, expressed as a percentage of the material weight (range 4-5% to 90-96%). It has a strong influence on harvesting, preparation, transport or storage of biomass [12]. Moisture content can be divided in extrinsic and intrinsic. The latter does not depend on weather conditions and is the only one applicable under laboratory conditions [7].

Ash content represents the quantity of the solid residue left after the biomass sample is completely burnt. The primary ingredient of biomass ash is the oxide form of calcium, magnesium, sodium, potassium and so on. Dependent on the magnitude of the ash content, the available energy of the fuel is reduced proportionately.
The volatile matter of biomass is the condensable vapour and permanent gas released from biomass when it is heated at standard conditions.
Fixed carbon is the solid combustible residue that remains after biomass is heated and the volatile solids expelled. It is determined subtracting the moisture, ash and volatile solids content [12].
The significance of volatile solids and fixed carbon contents is that they provide a measure of the ease with which the biomass can be ignited or oxidised. This type on analysis is relevant for biological conversion processes only once the fuel is produced, enabling a comparison among different biomass source [7].

The main physical biomass properties are:

- Heating value
- Particle size
- Density

The heating value (HV) of a material is an expression of the energy content. It is measured as heat of combustion, which is the total energy released as heat when the biomass undergoes complete combustion with oxygen under standard conditions. There are two heating value types frequently used, higher heating value (HHV) and lower heating value (LHV). The HHV is defined as the total amount of heat that is available in biomass including the latent heat of vaporization of water in the fuel and the reaction products. The LHV does not include the latent heat of vaporization of water. The heating value of a lignocellulosic biomass type can vary significantly with the climate and soil conditions. The HHV values of most woody biomasses fall in the range of 18.5–22.5 kJ mol−1, whereas the HHV values for most herbaceous biomasses are about 15.5–19.5 kJ mol−1.
The shape and size of biomass feedstock particles affect the mixing and fluidization, surface area for heat and mass transfer and the flow behaviour of biomass particles. In general, lignocellulosic biomass is irregular in shape, which results in difficulties in accurate dimension measurements of length, width, and thickness [12]. The particle size is a fundamental property to investigate during biological process because it is an index of the degradation of biomass [13].
The bulk density is the ratio of the mass of biomass particles to the total volume of biomass particles including the pore space volume between and within the biomass particles. It is directly related to the technical aspects concerning biomass storage and handling. Extremely variable parameters and depending on the biomass moisture content [14].

2.3 BIOFUELS AND BIOREFINERIES

Biorefinery is a vital component of the future bio economy, defined by the International Energy Agency (IEA Bioenergy-Task 42 2014b) as a "sustainable processing of biomass into a spectrum of marketable food and feed ingredients, bio-based products (chemicals, materials) and bioenergy (biofuels, power and/or heat)" [15].
In 2017 there were 224 biorefineries operating across Europe. Different types of biomass are fully utilised and transformed into a large variety of chemicals and materials [16].
Biofuels can be classified according to the type of biomass from which they come from: first-, second-, third- and fourth-generation biofuels. Hereafter a brief description [17] [18].

1. First-generation biofuels are those that come from agricultural crops (corn, rice, potatoes, sugarcane). Biofuels obtained from this type of biomass present advantages, since they use the highest technological development, they have competitive cost and scalable process. The main disadvantage is the competition in terms of land and water requirement for food/feed production.
2. Second-generation biofuels are produced from lignocellulosic biomass derived from agricultural wastes, municipal and industrial waste, therefore do not compete with food/feed production. This type of biomass is low cost and abundant, but it needs to overstep some technological barriers. In fact, pre-treatment steps are required to disrupt the complex recalcitrant structure and the production cost increases.
3. Third-generation biofuels are those that are produced from Algae feedstock. These photosynthetic organisms fix inorganic carbon (CO_2) or simple compounds directly into higher organic compounds. The use of microalgae presents several advantages: they can grow faster than terrestrial plants and they have lignin-deficient cell wall that made them less recalcitrant compared with lignocellulosic biomass. Moreover, fixing CO_2, they contribute to mitigate environmental pollution. On the other hand, there are many challenges that need to be overcome, like the high cost of cultivation, harvest and operation or simply microbial cultivation problems.
4. Fourth-generation biofuels take advantage of synthetic biology of algae and cyanobacteria, which comprises the design and construction of new biological parts, devices and systems. For the first, second and third generation biofuels, the raw material is either biomass or waste, but fourth generation biofuels will be based on raw materials that are inexhaustible, cheap and widely available.

181 of the biorefineries are so-called "first generation facilities". Just 43 are "second generation facilities" which use more sustainable lignocellulosic feedstock [19]. From here the importance to invest in the research field to find efficient methods to overcome the refractory properties of lignocellulosic feedstock, especially in Anaerobic Digestion for Biogas production.

2.4 ANAEROBIC DIGESTION AND BIOGAS PLANT

In the context of biorefineries, Anaerobic digestion is one of the most versatile technology. Bioenergy, and especially biogas (BioH_2 and BioCH_4) produced through Anaerobic digestion (AD) of renewable feedstock, is one of the highly promising alternatives to fossil fuels. Anaerobic digestion is a four-step biological process that take place in absence of oxygen (**Figure 2.6**). In the first step, called hydrolysis, complex insoluble molecules like carbohydrates, lipids, proteins and nucleic acid, contained in organic wastes, are hydrolysed in simplex soluble molecules (monosaccharides, amino acids and long chain fatty acids) by hydrolytic bacteria. Hydrolytic bacteria excrete enzymes like cellulase, cellobiase, xylanase and lipase and they can be strict anaerobes such *Clostridia* and some facultative anaerobes (e.g. *Streptococci*). Then acidogenesis occurs and these molecules are degraded in volatile fatty acids (e.g. lactate, propionate, butyrate, valerate, acetate), alcohols like ethanol, hydrogen and carbon dioxide by fermentative bacteria. Acidogenesis is followed by acetogenesis where organic acids and alcohols are converted by acetogens (e.g *Acetobacterium woodii* and *Clostridium aceticum*) into acetate as well as carbon dioxide and hydrogen. Moreover, the group called homoacetogenic bacteria is able to convert H_2 and CO_2 into acetate. The last step is called methanogenesis and involve methanogenic bacteria. There are two groups of methanogenic bacteria: acetotrophic bacteria, which degrade acetate into methane and carbon dioxide and hydrogenotrophic methanogens, that consume hydrogen and carbon dioxide to produce methane [20]. Therefore, anaerobic digestion is carried out by a mixed microbe consortium that works synergistically and in which H_2 is a key intermediate metabolite and regulator for methane production process.

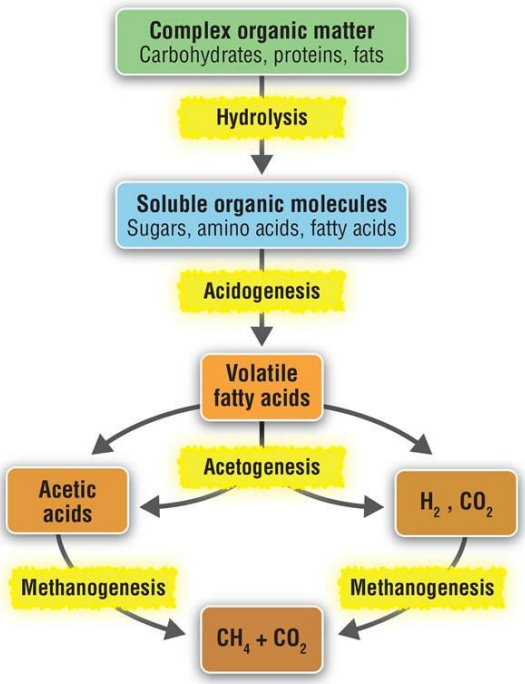


Figure 2.6 Biological process of Anaerobic digestion.
https://www.biocycle.net/2016/11/10/managing-digester-feedstocks/ (last access 02/09/2019)

2.4.1 Microorganism and their metabolism

In biochemistry, all the involved reactions are oxidations and reductions, therefore they include an electron flow between a donor and an acceptor. Chemical energy is the energy released when organic or inorganic compounds are oxidized. One of the most common metabolic energy carriers involved in the operation of the cell is ATP. Electron transfer from the donor to the acceptor in a cellular redox reaction involves the participation of electron carriers, like the coenzyme NAD^+/NADH. Fermentation is an ATP-regenerating metabolic process in which degradation products or organic substrates are either electron donors or electron acceptors, while during respiration O_2 is the final electron acceptor. During their metabolic pathway, fermentative bacteria will deposit a portion of the liberated electrons on the oxidized substrate or protons and hence they will form reduced metabolites such as ethanol and acetate or H_2. In the case of Anaerobic Digestion, as stated before, biogas is produced from organic matter by a syntrophic interaction in a mixed culture. The microbiome, in general, can be classified in two large groups: Hydrogen Producing Bacteria (HPB) and Hydrogen Consuming Bacteria (HCB) based on the relation between the key metabolite H_2 and the biotic phase. HCB are strict anaerobes like methanogens, while HPB are facultative anaerobes and obligate anaerobes [11]. Clostridia (obligate anaerobes) have been identified as the dominant hydrogen producing microorganisms, followed by Enterobacteria, Streptococci or Lactobacilli (facultative anaerobes) [21].
The production of hydrogen is associated with the presence of an iron-sulfur protein, ferredoxin (Fd), which is a carrier of electrons at low redox potential. The transfer of electrons from Fd to H^+ is catalysed by the enzyme hydrogenase [11]. In the AD of glucose as a model substrate, HPB convert glucose to pyruvate during glycolysis producing ATP and NADH. Pyruvate is further oxidised to acetyl coenzyme A (acetyl Co-A) and CO_2 by means, another enzyme, pyruvate: ferredoxin oxidoreductase (PFOR). Depending on the type of microorganism and environmental conditions (substrate type, loading rate, pH, T), acetyl Co-A might be converted to acetic acid, butyric acid, formic acid and ethanol by others metabolic reactions. For example, pyruvate can be also converted to lactate if bacteria from *Lactobacillus* genus are present [22]. The oxidation of pyruvate into acetyl Co-A requires the reduction of Fd by PFOR, which is then oxidised by a hydrogenase that regenerates Fd_{ox} and hydrogen gas. Additional hydrogen can be produced from the NADH that is generated during glycolysis. The NADH is oxidised by Fd reduction and a NADH-Fd reductase (**Figure 2.7**).

Acetate and butyrate are the most common products of the acidogenesis phase. Their biochemical reactions can be written as:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH \text{ (acetic acid)} + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH \text{ (butyric acid)} + 2H_2$$

Then, the highest theoretical yield of hydrogen, 4 mol hydrogen per mol glucose, is produced when acetic acid is the only fermentation end-product, whereas the yield of H_2 is 2 mol for a mol of glucose when the final product is butyric acid. Lower H_2 yields are associated with others reduced end-products such as lactic acid or propionic acid due to the stoichiometry and metabolic pathways involved.

The group of HCB, such as hydrogenotrophic methanogens, consume the acetate and H_2 produced during acetogenesis, and produce CH_4 and H_2O [23].

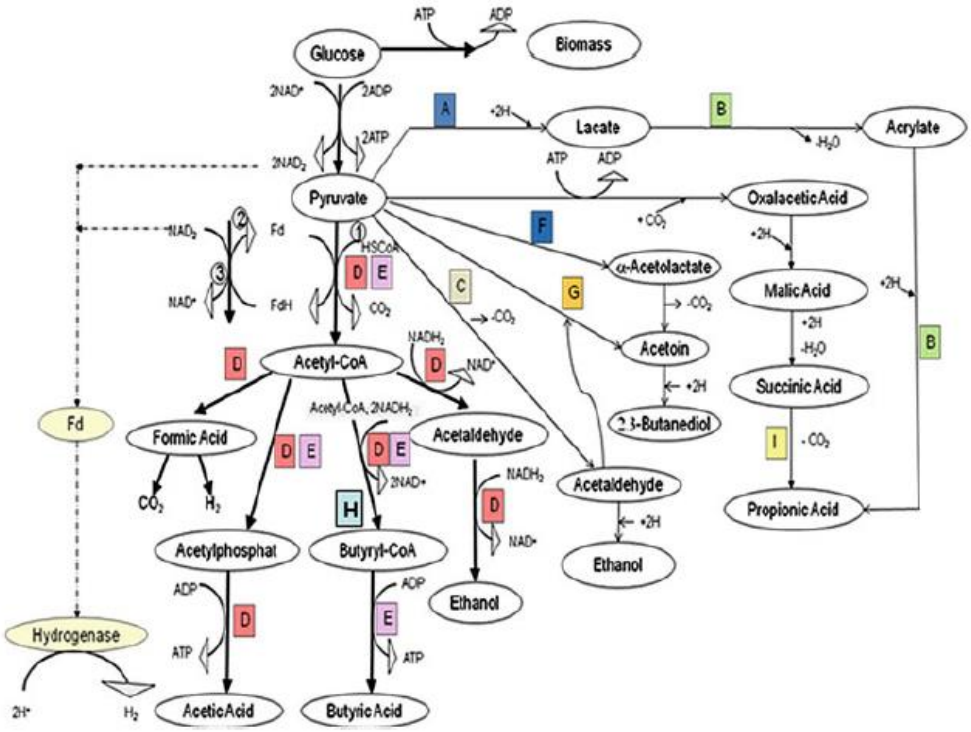


Figure 2.7 Metabolic pathway of glucose by HPB under anaerobic conditions. ① Pyruvate ferredoxin oxidoreductase (PFOR); ② Hydrogenase; ③ NADH ferredoxin oxidoreductase. Letters indicate organisms which conduct these reactions as follows: A Lactic acid bacteria; B Clostridium propionicum; C Yeast, Acetobacter, Zymomonas; D Enterobacteriaceae; E Clostridia; F Aerobacter; G Yeast; H Clostridia; I Propionic acid bacteria. From reference [11].

2.4.2 Biomass feedstock for AD

All sorts of biomass containing carbohydrates, lipids, proteins, cellulose, hemicellulose, as main components, are suitable to be used as substrates for biogas production. The substrate plays an important role in the final yield, production rate and overall economic balance of the process.

Table 2.3 shows the typical composition (%) of biogas from different biomass sources. The theoretical gas yield varies with the content of carbohydrates, proteins and lipids. Lipids provide the highest biogas yield but require long retention time due to their slow biodegradability, whereas carbohydrates and proteins show faster conversion rates but lower gas yields [24].

Table 2.3 Typical composition % of biogas from different feedstock. Adapted from [25]

Component	Agricultural wastes	Landfills	Industrial wastes
Methane - CH_4	50-80	50-80	50-70
Carbon dioxide - CO_2	30-50	20-50	30-50
Hydrogen sulphide - H_2S	0.70	0.10	0.80
Hydrogen - H_2	0-2	0-5	0-2
Nitrogen - N_2	0-1	0-3	0-1
***Water* -H_2O**	saturation	saturation	saturation

Protein-rich wastes (animal manure or meat processing factories) present low carbon to nitrogen ratio (C/N ratio), so there is a high nitrogen content in AD systems. This could lead to ammonia accumulation that is an inhibition factor for anaerobic treatments. Therefore, feedstocks require a balanced C/N ratio. The suggested optimum C/N ratio for anaerobic digestion is in the range of 20/1–30/1 [24]. As reported by B. Bharathiraja *et al*. [20], a practical strategy is the addition of co-substrates in order to increase the organics content and thus achieve higher biogas yield.
Lignocellulosic wastes are an economically viable and renewable source of second-generation biofuels. These include plant biomass waste (rice straw, wheat straw), food waste and food processing wastes.
Carbohydrates are the main component of food waste and collected organic fraction of municipal solid waste from household or markets. Food waste has been used extensively in the research field since AD has been proposed as the most suitable treatment option for wastes. It is composed of raw and cooked food discarded before or during food preparation.
Anyway, there are limitations in the utilization of these valuable resources, as will be explained in the next section.

2.4.3 Biomass pre-treatments

Hydrolysis step is the most delicate step in AD process of lignocellulosic biomass. Hydrolysis occurs for the presence of hydrolytic bacteria which excrete enzymes like cellulase, cellobiase, lipase, protease to degrade the substrate. For example, the enzymatic hydrolysis of cellulose is carried out by enzymes called cellulase. Cellulase is composed of a pool of enzymes such as endoglucanase, exoglucanase, β-glucosidase. The degradation process, which is an heterogeneous reaction, is composed by two steps as reported by Fenila F. *et al*. [26]: at the beginning endoglucanase and exoglucanase bind the substrate by adsorption. The bound fraction converts cellulose in cellobiose. The unbounded fraction of β-glucosidase converts cellobiose to glucose. The typical operating temperature for cellulose hydrolysis is between 40 and 55°C and pH around 4,5-5,5. Hydrolysis is known as the rate-determining step since is the slowest part of the whole process. Indeed, the degradation of lignocelluloses into biogas is complicated since lignocelluloses have a recalcitrant structure which has naturally evolved to resist and to prevent enzymatic degradation [27]. Pre-treatments are necessary to make most complex organic sources available to microorganism. A pre-treatment step can enhance the bio-

digestibility of the wastes and the accessibility of the enzymes to the material, improving the yield of the bioreaction. Since lignocellulosic materials are usually made of cellulose, hemicellulose and lignin and cellulose is the major source of glucose for microorganisms, the main purpose of a pre-treatment is to change the intrinsic structure, breaking the lignin and disrupting the crystalline structure of cellulose in order to prepare the materials for enzymatic degradation (see **Figure 2.8**).

There are several factors that should be considered while employing a pre-treatment strategy for a particular biomass residue before it is used as a substrate for fermentation. In general pre-treatments should lower the capital cost and the operational cost. Besides all these factors, other parameters that affects lignocellulose degradation need to be taken into account. Rajeev Ravindran *et al*. in their review [28], give a detailed description of the influence of these parameters.

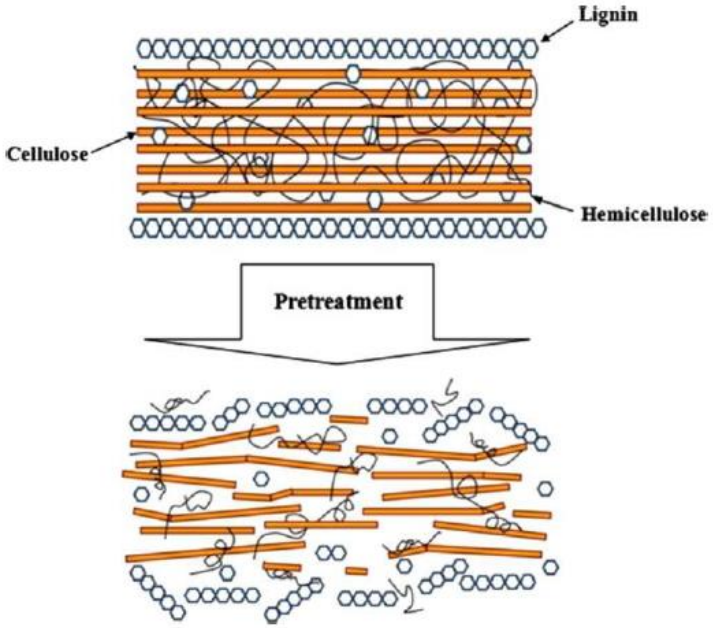


Figure 2.8 Schematic pre-treatment of lignocellulosic material. From reference [27].

First, crystallinity of cellulose, its accessible surface area, the degree of cellulose polymerization and the degree of acetylation of hemicellulose are recognised to be the main factors that affect the hydrolysis rate. Crystalline cellulose exists in different polymorphs and is seen in microfibrils in plant cell wall. These microfibrils are bonded together by hydrogen bonds which resist enzymatic and microbial attack. It has been shown that cellulase hydrolyses first the accessible amorphous part of cellulose and then the crystalline cellulose. Reducing the crystallinity of cellulose might help in increasing the digestibility. Pre-treatments are depolymerisation process of cellulose. The higher the degree of polymerisation is, the higher the degradation time. The process of hydrolysis greatly depends upon the adsorption of enzymes on the substrate. This is determined by the accessible surface area which depends on particle size, porosity and pore volume. An increase in the latter increases the mass transfer rate, which in turns increases the enzymatic reaction. Acetyl groups form the backbone of hemicellulose in plant cell wall. The acetyl group may inhibit the enzyme activity by interfering the hydrogen bond formation between cellulose and cellulose binding domains. According to Hendriks *et al*. [29], the lignin content also limits the rate of enzymatic hydrolysis by acting as a shield,

allowing inaccessible the parts of the substrate to be hydrolysed. It is accepted that delignification process could enhance biogas production but during delignification part of hemicellulose is also hydrolysed leading to different effects.

Pre-treatments can be classified in three categories:

- Physical
- Chemical
- Biological

In the next sub-paragraphs, a brief description of each will be provided.

2.4.3.1 Physical pre-treatments

Physical pre-treatments techniques include mechanical comminution (e.g. milling, grinding), steam explosion, liquid hot water, extrusion and irradiation (ultrasound and microwave). Mechanical comminution of lignocellulosic biomass is utilized to reduce particle size. Particle size reduction increases the accessible surface area, reduces the degree of cellulose crystallinity and the degree of cellulose polymerisation. Comminution of biomass can be accomplished by using milling or grinding machines, including knife, hammer, attrition mills. Different comminution methods produce various particle size. For example, chipping and milling can yield final particle size of 10-30 mm and 2-0,2 mm, respectively. Size reduction has been shown to be more efficient when combined with other pre-treatments methods. One disadvantage is the cost of the process, since requires a lot of energy, mainly electrical for the comminution step. Also, it is unable to remove lignin [13].

In the steam explosion, biomass particles are heated with high-pressure saturated steam, then the pressure is reduced to terminate the reaction causing a decompression. The advantages are the efficiency, low pollution levels and no recycling cost for waste stream, but this technique is one of the most cost-effective [28]. Non-ionizing radiation is a physical pre-treatment that include microwave and ultrasound. Microwave irradiation is an alternative to the simple heating process as it rapidly alters the cellulose structure and partially degrades hemicellulose and lignin, leading to energy savings. Ultrasound pre-treatment can disrupt cell structure, increasing the accessible surface area. According to [30] ultrasound has been extensively studied and it has been shown that they could improve biogas yield from sludge by 34%.

2.4.3.2 Chemical pre-treatments

Chemical pre-treatments techniques have received a lot of attention among all other categories. Typical examples include acid and alkaline pre-treatment, by far the most applied methods. Alkaline pre-treatment involves the use of alkaline solutions such as sodium hydroxide (NaOH), lime ($Ca(OH)_2$) or ammonia to remove lignin and part of the hemicellulose structure. The mechanism of alkaline hydrolysis is the saponification and cleavage of lignin-carbohydrate linkages. The removal of the cross-links leads to an increase in porosity, decrease of degree of polymerisation and crystallinity and damage of the lignin structure. The effectiveness of the treatment is associated with the lignin content of biomass materials and the concentration of the employed reagents.

Acid pre-treatment can be conducted with dilute acids (e.g. 0,1%) and high temperature (~230°C) or with concentrated acids (30-70%) and low temperature (~40°C). Both inorganics or organics acids can be used, including sulfuric acid H_2SO_4, hydrochloric acid HCl, nitric acid HNO_3, acetic acid CH_3COOH have been used but sulfuric acid is the most common. Concentrated acids are highly effective but corrosive and hazardous, therefore diluted acids are preferred. This pre-treatment can potentially hydrolyse up to 100% of the hemicellulose into sugars and disrupt lignin, but it is not effective in dissolving lignin. Surely, it makes cellulose susceptible to enzyme degradation [30].

2.4.3.3 Biological pre-treatment

Biological pre-treatments include fungal pre-treatment, microbial consortium pre-treatment and enzymatic pre-treatment. Compared with the physical and chemical ones, biological pre-treatment shows low energy input, no chemical requirement, therefore no by-products formation and mild environmental conditions. However, most of the research is conduct at laboratory-scale.
Compared with the other methods, the cellulose quantity at the end of the process is higher and this can improve biogas production. Thus, the main goal of biological pre-treatments is to minimize carbohydrates loss and maximize lignin removal.
For this purpose, several fungi classes, including brown-, white- and soft-rot fungi have been studied as well as microbial consortium screened from natural environments. While the former has the potential to degrade better lignin and hemicellulose, the latter has high cellulose and hemicellulose degradation ability. Another option is adding before or directly during AD hydrolytic enzymes (cellulase and hemicellulase) to help with digestibility of lignocellulosic biomass.
Table 2.4 shows the effect effects of the different pre-treatments on the physical/chemical composition or structure of lignocellulose.

Table 2.4 Effect of pre-treatment on compositional and structural alteration of lignocellulosic biomass. Adapted from [29] and [30].

	Increase accessible area	Decrystallization cellulose	Solubilisation hemicellulose	Solubilisation lignin	Alteration lignin structure
Mechanical	+	+	-	-	-
Steam-explosion	+	-	+	+/-	+
Irradiation	+	+/-	+/-	-	-
Liquid hot water	+	ND	+	+/-	+/-
Alkaline	+	-	+/-	+	+
Acid	+	-	+	+/-	+
Biological	+	ND	+	+	+

+ major effect – no effect +/- minor effect ND not determined

2.5 DARK FERMENTATION AND VFA PRODUCTION

Dark fermentation (DF or acidogenic fermentation) is an abbreviate version of Anaerobic fermentation where methanogenesis is repressed and H_2 and volatile fatty acids (VFAs) are the two most important value-added intermediates. DF is the most promising and studied technology for bioH_2 production, a clean, ideal and renewable energy source.
Recently, the interest in VFAs intermediates has increased as they have high-value possibilities for their end-uses.
The term VFAs is used for short-chain carboxylic acids consisting of six or fewer carbon atoms. Due to their functional group, they are extremely useful as building blocks of various organic compounds including alcohols, aldehydes, ketones, esters, among others [31]. At present, commercial production of VFAs is mostly accomplished by chemical routes, oxidation or carboxylation of chemicals precursor, such as aldehydes or alkenes deriving from the oil-processing. In biotechnological applications, chemical-grade sugars have been commonly employed, but the research is currently shifting to VFA production from lignocellulosic feedstock digestion by mixed cultures.

2.5.1 Biotechnological relevance of VFA

These acids have a wide range of applications. Some possible applications and DF process integration are shown in **Figure 2.9**.
Waste-derived VFAs is an inexpensive energy source that can be used to produce different forms of energy. For example, direct electricity generation is possible through Microbial Fuel Cell [32]; coupling DF with heterotrophic cultivation of algae which produce lipids, carbohydrates and proteins is a promising and innovative solution for developing sustainable biorefineries [33]; furthermore, hydrogen production from the waste-derived VFA can be achieved by photo-fermentation. Dark fermentation has been coupled to Anaerobic digestion for biogas production in a system called two-stage AD (TSAD). There are two digesters, the first operates at acidic pH and low retention time to cultivate acidogens, while the second has neutral pH and higher retention time to enrich the culture in slow-growing methanogens. In this way, both hydrogen and methane are produced: the former in the first digester and the latter in the second [34].
Other interesting applications are bioplastic production and biological nutrient removal from wastewater.

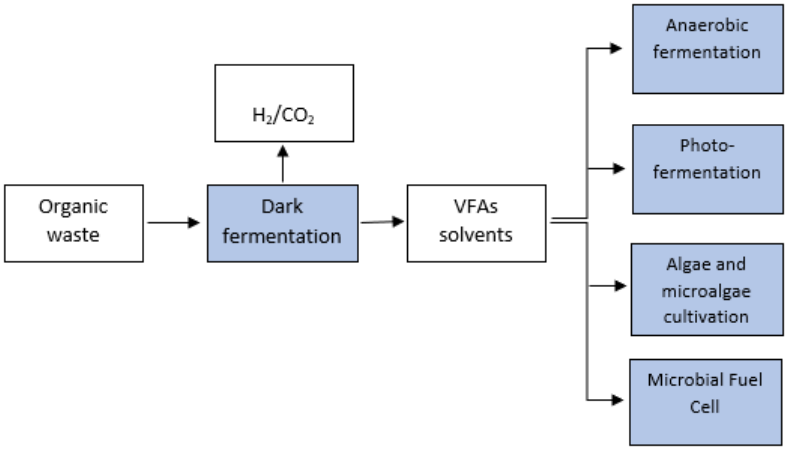


Figure 2.9 Possible application of DF intermediates. Adapted from [35]

2.5.2 Factors affecting VFA production

Operational parameters, such as temperature, pH, hydraulic retention time, organic loading rate and H_2 partial pressure can be used to regulate metabolic pathways of hydrogen producing bacteria, hence they have important effects on VFAs production from DF. In addition, substrate types and their pre-treatment methods, bioreactor configurations and inoculum sources also influence the biohydrogen and VFA production. Although these parameters have a synergic effect, researchers investigated their influence one at a time. Regarding the inoculum source, lately, mixed cultures have been employed to increase the production yields. On the other hand, methanogens and acetogens activity must be inhibit and pre-treatments should be applied (see section 2.5.3).

The effect of pH on VFAs production is significantly marked, since acidogens cannot survive in extremely acidic (pH 3) or alkaline (pH 12) environments. A study carried out by Dahiya *et al.* [31] emphasizes the relationship between initial pH and individual VFAs obtained from acidogenic fermentation. They fermented food waste in bench-scale batch reactors. The pH was adjusted at the beginning of the experiments to 5, 6, 7, 8, 9, 10, and 11. The maximal total VFAs concentration was detected in the reactor operating with an initial pH of 10, followed by pH 9, 6, 5, 7, 8 and finally pH 11. Therefore, the optimal values are in the range 5.25-11, but the specific ranges are dependent on the type of waste used and the inoculum. In general, a slightly acid-neutral conditions show positive effects on microbial metabolism. In addition, pH can also affect the type of VFA produced from acidogenic fermentation, especially acetic, propionic and butyric acid [36].

Temperature is directly involved in bacteria growth and metabolism. Increasing the temperature within the psychrophilic and mesophilic temperature ranges is good for VFA concentration, rate of production and yield. And further increase from mesophilic to thermophilic might still improve the VFA production.

Another parameter is the hydraulic retention time. Applying higher HRT could be advantageous because the microorganisms have more time to react with the substrate. However, prolonged HRT could lead to stagnant VFA production. Longer retention time also means larger reactor volumes or decreasing the feed, which has economic implications

The organic loading rate (OLR) indicates the amount of wastes (which can be expressed in terms of COD or VS) fed into the reactor daily or per unit reactor volume. OLR should be

abundant enough to provide an adequate amount of carbon source, according to working temperature. In fact, under mesophilic conditions an OLR higher than 5 g_{TS}/L/d may increase viscosity, reducing mass and heat transfer and subsequentially substrate conversion into VFAs [37]. The OLR applied in acidogenic fermentation has a strong influence on VFAs composition. Higher loading rates tend to result in higher acetic acid concentrations [36].
Another relevant parameter that affects hydrogen production, and therefore VFA production, is the H_2 partial pressure.
During the fermentation process, hydrogenases are involved in reversibly oxidizing and reducing ferredoxin. If the hydrogen concentration in the liquid phase increases, the oxidation of ferredoxin becomes less favourable and the reduction of ferredoxin takes place, thus reducing the H_2 production [23]. H_2 production is directly linked to VFAs production, since all the metabolic pathways that lead to acid production involve NADH production and consumption. Indeed, the reaction related to H_2 formation is:

$$NADH + H^+ \longrightarrow NAD^+ + H_2$$

The ΔG of the reaction that produces hydrogen depends on the hydrogen concentration in the liquid phase, which is linked to the partial pressure of the gas phase in equilibrium, as stated by Henry's law. Several studies lead to the conclusion that a decrease in partial pressure is one of the best approaches towards improvement of H_2 and VFAs productivity.

2.5.3 Physiological differences between HCB and HPB

As stated at the beginning, VFAs production occurs during the acidogenic phase of Anaerobic digestion by acidogenic bacteria also called hydrogen producing bacteria (HPB), therefore no methanogenesis should begin if the final goal is to achieve high yield in VFA production. Indeed, methanogens and homoacetogenic bacteria (which belong to the hydrogen consuming bacteria group, HCB) consumes VFA and H_2 to produce biogas, thus to improve the production of VFA, methanogenic bacteria in inoculum must be inhibited. Several methods have been used for this purpose and they exploit the physiological differences between HPB and HCB.
Bacteria belonging to the genus *Clostridium* are the main responsible for H_2 production. They are obligate anaerobes, Gram-positive and rod-shaped. They can produce spores when the environmental conditions are too deleterious for their growth, for instance high temperatures, extreme acidity or alkalinity, freezing, among others. These spores are metabolically inactive until the environmental conditions are again favourable. Methanogens, instead, are coccoid rods or rod-shaped bacteria, strictly anaerobes and incapable of forming spores, therefore much more sensitive than HPB. Moreover, HPB have faster growth kinetics than HCB and they can grow in broader pH range (4.5-7) compared to HCB (7-8) [11].

2.5.3.1 Inoculum pre-treatment

Most of the inoculum pre-treatment are based on the ability of HPB to form spores in unfavourable environmental conditions. They can be classified on the base of the stress source which is applied [11]:

- Thermal pre-treatment: heat shock (70-110°C) for a short time (20-60 min), boiling, sterilization or freezing/thawing (-20/25 °C for 6h in two cycles).

- Wave and radiation pre-treatment: ultrasonication, microwave and ultraviolet radiation.
- Acid and alkaline pre-treatment: they cause a pH stress. They are carried out by adding a strong acid or base until a set pH value and incubating for relative short-periods, for example, 24h in anaerobic conditions. Usually the pH set is 3-5 for acid pre-treatment and 10-12 for alkaline pre-treatment. Treatment at pH 3 gives the best results [38].
- Aerobic stress: consist of flushing air for a certain amount of time. But this pre-treatment in not working well since the methanogenic activity is not totally suppressed.
- Use of chemicals: chloroform, sodium 2-bromoethansulfonate and iodopropane. They selectively inhibit methanogenic activity without affecting H_2 production. But the use of these strong chemicals is against the sustainability of the process.

2.6 CELL VIABILITY OF MIXED CULTURES

In the case of biorefineries, the control and monitoring of the biotic phase, for instance the physiological status of microbial cultures, is crucial for process optimization.
The determination of viability for pure cultures is made through different methods such as plate count method, image analysis [39], or through optical density measurement. Meanwhile, in mixed cultures, these methods are much more difficult to apply because of the population heterogeneity and complexity of fermentation broths.
Nowadays, new methods are emerging for the measurement of cell viability in the field of Process Analytical Technology (PAT). This field involves the application of on-line, off-line and at-line controls for monitoring chemical or physical parameters that cannot be derived from conventional physical variables like temperature or pressure [40]. For instance, electro-optical techniques are a common method for estimating cell viability, which focus on key features of bacterial populations. An interesting parameter, which can be used for viability measurements is the state of the cell membrane. Indeed, the most universal parameter to quantify and study cell viability is the state of the cellular membrane since represents the interface with the outside, and an intact and functional one is able to maintain a suitable intracellular environment. As a matter of fact, all living organisms consist of cells that have a similar structure consisting of cytoplasm surrounded by a membrane and life is ensured by a correct ions exchange between the internal and external environment.
One recently developed electro-optical technique is based on the measurement of the frequency-dependent polarizability anisotropy of cell suspensions.

2.6.1 Frequency-dependent polarizability anisotropy (FDPA)

When an electric field is applied to a material, energy in the field is lost for charge motion and turned into heat or stored by polarization of the material's components. Polarization can be due to charge accumulation at the surfaces between materials with different electrical properties or to dipole orientation. The response of a material to an applied electric field is described by its conductivity (σ, in S/m) and permittivity (ε, in F/m). The conductivity gives a measure of the ability to conduct, whereas the permittivity gives a measure of the polarizability of the material, that is to store charge. Generally, the permittivity falls as frequency increases. These steps are

called dispersions and each of them reflects the loss of a particular polarization process [41]. In the case of biological cell suspensions, it shows different polarization mechanism. Each of them produces a dielectric dispersion in an appropriate frequency range.
According to [42] the three main dielectric dispersions are:

- α- dispersion: mainly due to the ionic character of the constituents, the cytoplasm and the cell suspension medium, that is responsible of the polarization of the ionic atmosphere around the cell surface, and of a surface conductivity, which causes a surface electrical current. These are low-frequency dispersions.
- β- dispersion: this one originates from the differences in dielectric properties at the cell membrane-external medium interfaces causing the so-called Maxwell-Wagner effect or interfacial polarizability.
- γ- dispersion: originates from the orientation polarization of water molecules. Found at higher frequencies (~20GHz).

The electrooptical analysis of the anisotropy of polarizability represents a method to monitor the viability of microorganism. The cell polarizability is referred to the Maxwell-Wagner polarization, therefore it relies, as said before, on the accumulation of electric charge at the interface between the cell wall and medium. The intracellular ion balance changes during a cultivation due to the substrate consumption, product formation and the metabolic response of the cell to environmental stresses. This has an impact on the interfacial polarization, and cells with high viability tend to exhibit a larger polarizability.

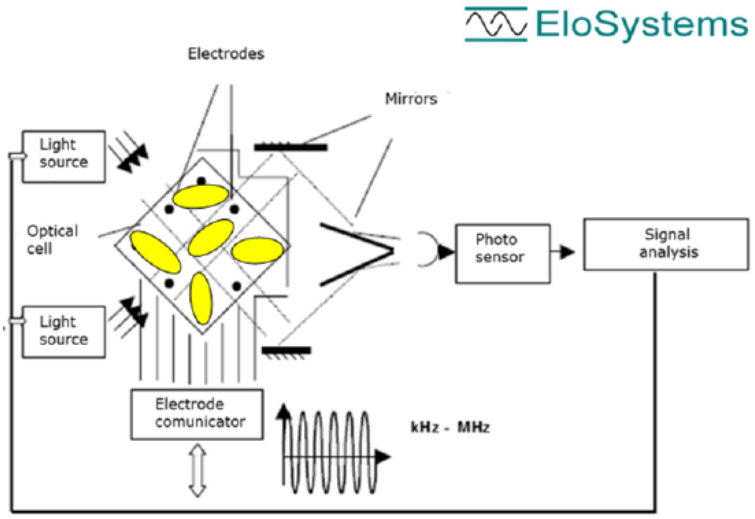


Figure 2.10 Scheme of electrooptical measurements. From [43]

As showed in Figure 2.10, an optical cell is placed, and a cell suspension of appropriate concentration is subject to an electric field at low/medium frequencies. Charges on the cell structure are induced and this causes a rotational momentum on cells which promotes an orientation effect. The change in cell orientation alters the adsorption of orthogonally located light beams, so it also affects light scattering. The signal is captured by photosensors and converted into units of FDPA[44]. Mathematically, polarizability is described by the tensor of polarizability $a_{ikj}(\omega)$ where i is the longitudinal component, j and k are the transversal components and ω is the electric field frequency. It is a quantitative parameter that describe the

spatial configuration of the induced charges and their absolute values. The difference between the longitudinal component of polarizability and the transversal component is the definition of anisotropy of polarizability (Eq. 2.1)

$$da(\omega) = a||(\omega) - a \perp (\omega) \qquad [Eq. 2.1]$$

The length of the bacterial cell can also be determined by analysing the time of relaxation when the electric field is shut off [44].
Several studies have tried to prove that electrooptical measurements can follow the cell viability during cultivation. For example, Pellicer-Alborch *et al.* [45] and Junne *et al.* [44] showed that the use of automatic sampling during cultivation allowed high-throughput data to be obtained and the differentiation among growth phases and stress response were detected in *E.coli* batch cultures and *L. plantarum* batch and fed-batch fermentations, respectively. Also Junne *et al.* [46] were able to show that this method is able to predict fermentation shifts between acidogenesis and solventogenesis in *C.acetobutylicum* cultures.

3 CHAPTER 3: EXPERIMENTAL PROCEDURE

The entire work of this thesis is focused on the characterization and fermentation of lignocellulosic feedstock to produce carboxylic acids (VFA). The experimental work is divided in two parts and the followed steps are:

1. selection of feedstock;
2. standard characterization;
3. feedstock pre-treatment;
4. inoculum pre-treatment;
5. acid fermentation;
6. HPLC and viability measurements.

3.1 SELECTION OF FEEDSTOCK

As discussed in Chapter 2, lignocellulosic feedstocks are very promising to produce bio-based products, because they belong to the second-generation feedstock group and their use do not affect the food/feed network. Moreover, they have a high carbohydrates content, thus they are suitable fermentation substrates. The main problem is that the lignocellulose structure is recalcitrant and naturally made to avoid enzymatic attack, hence the hydrolysis step for these materials is very slow and delicate. Lignin is the most recognized factor in the recalcitrance of lignocellulosic materials, since cellulose and hemicellulose are cemented together by lignin and this limits the enzymatic accessibility. Moreover, these feedstocks present high crystallinity, high degree of polymerization and particle size and porosity as well might affect their accessibility. This problem can be overcome with the application of pre-treatments.

Selected feedstock

For this study four feedstock have been chosen for the characterization: **Wood barks** (cypress) collected from Lille's forest in France, **Maize silage** provided from the IASP (Institut für Agrar- und Stadtökologische Projekte) in Berlin. **Lettuce** and **rice** have been chosen to better represent food waste and come from one supermarket in Berlin. Then, fermentation was carried out using maize silage as substrate. It is one of the most used crops from which a high yield of methane formation can be obtained. A recent study showed that corn is the most common co-substrate used with manure in biogas plants [17], but unfortunately it is not a sustainable material since its use would lead to a competition in the food/feed market. Here it is used as a reference material.

3.2 METHODS FOR LIGNOCELLULOSIC DETERMINATION

Content determination is an important step in the study of the feedstock and their potentiality to become a substrate for further fermentations. It is fundamental for the identification of the most suitable pre-treatment to increase hydrolysis rates. Moreover, it is useful for the calculation of the VFA production yield. Indeed, the yield is usually calculated on the volatile solids base or on the total solids base. The same methods are applied on the four feedstocks in order to create a standard and reproducible method for the characterization of complex organic matrices. Furthermore, different methods for measuring the same parameter are applied in order

to identify the most suitable and efficient that can be used for different feedstocks. The parameters considered are:

- total solids (TS),
- moisture content (MC),
- volatile solids (VS),
- ashes,
- lignin,
- holocellulose,
- hemicellulose
- cellulose
- total carbohydrates content.

An overview of the methods can be seen in **Table 3.1** and in the following sections a detailed description is presented.

Table 3.1 Overview characterization methods

Parameters	Methods
TS, MC, VS, ashes	APHA standard methods
Lignin	Chlorination method Klason-lignin method
Hemicellulose, cellulose	NaOH hydrolysis
Total carbohydrates	H_2SO_4-UV method Phenol-H_2SO_4 method HPLC

3.2.1 Mass composition

Every fresh biomass is composed of a mass of water (moisture content or MC) and a dry mass (Total solids or TS) which could be divided in the organic matter (volatile solids or VS plus fixed carbon) and the small percentage of inorganics (ashes) as shown in **Figure 3.1**. Before the proximate analysis, the samples of the four feedstock are prepared according to the method described in [47]. All the analyses are performed in duplicates. First, an appropriate amount of material is weighted in a pre-weighted glass plate and dried in a convection oven at 80°C for 24h. The final weight is registered and used for the calculation of the moisture and total solids content according to the APHA standard [48] or [47]. Then, the sample is grinded in a laboratory batch mill IKA A10 (IKA-Werke, Staufen, Germany) to decrease the particle size and transform it into a medium-size particles powder (max. 4mm particles). For the other selected feedstocks, different variations of the protocol applied: rice is previously boiled (100°C; 13min), and lettuce is cut with a kitchen knife before drying.

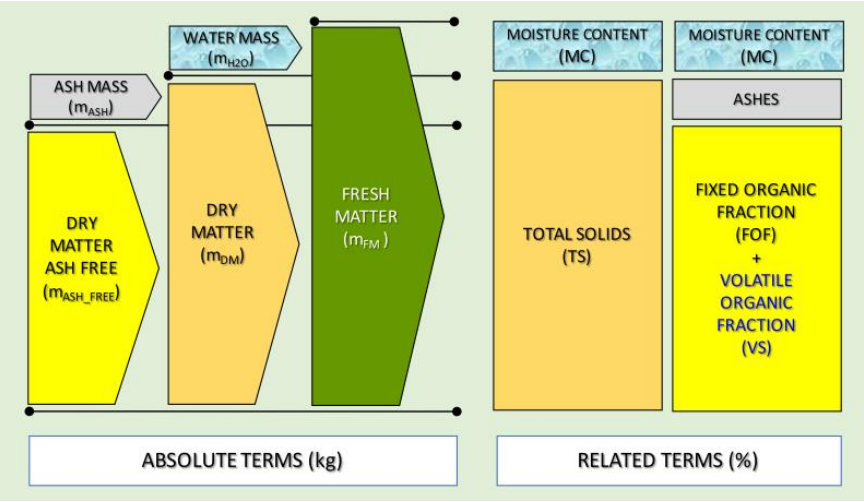


Figure 3.1 Mass composition. From [14]

The utilized equations for dry matter and moisture content calculations are [48]:

$$TS\% = \frac{W_f - W_t}{W_i - W_t} \qquad \text{[Eq.3.1]}$$

$$MC\% = 100 - TS\% \qquad \text{[Eq.3.2]}$$

With:

- W_f =weight glass plate/crucible + dry weight sample (g)
- W_i =weight glass plate/crucible + sample (g)
- W_t =weight glass plate/crucible (g)
-

The same notation will be maintained for all the other equations. All the experiments are done on dried samples and the content will be expressed in function of the dried value.
Following APHA standard, the volatile solids and ash content for each feedstock was determined. The determination of volatile solids in the samples was performed by placing 2g of sample into a pre-weighted crucible and then ignite at 550°C for at least 6h in a muffle. After ignition the crucibles are cooled down in a desiccator and then weighted. The inorganics can be estimated from the residue left after ignition, while the volatile solids are the condensable vapours and permanent gas released. Hereafter the equations utilized for the calculation of ashes and volatile solids:

$$ASH\% = \frac{W_f - W_t}{W_i - W_t} * 100 \qquad \text{[Eq.3.3]}$$

$$VS\% = 100 - ASH\% \qquad \text{[Eq.3.4]}$$

3.2.2 Lignin and cellulose determination

As discussed before, lignin is the most recognized components that made the feedstock recalcitrant to microbial attack. Therefore, it is very important find its content in order to choose the most correct biomass pre-treatment. Apart from lignin, hemicellulose and cellulose, extractives belong to lignocellulose structure and they are a group of cell wall chemicals

consisting of fatty acids, phenols, terpenes, steroids, resin that are extracted with the help of different solvents. It is not convenient remove the extractives because it is energetically expensive. Lignin determination is carried out with the chlorination method discussed by [10]. This method consists in the holocellulose extraction which is defined as the water-insoluble carbohydrate fraction of lignocellulose materials.
The protocol establishes 2,5g of dry sample (W) to be mixed with hot distilled water (70°C), 0,5 mL of acetic acid and 1g of sodium chlorite ($NaClO_2$) in a 250mL round-bottomed flask covered with glass plugs and heated at 70°C until complete delignification in a water bath (~ 6 to 8 h). The tests are conducted using plastic balls to avoid water evaporation (see **Figure 3.2**). To obtain complete delignification during the set interval (~ 6 to 8 h), 0,5 mL of acetic acid and 1g of sodium chlorite are added to the system every hour. Sodium chlorite is aimed to destroy lignin, while acetic acid serves as buffer agent to avoid excess of chloriting. The sample is left still overnight at 70°C. The holocellulose is then vacuum-filtered on a 589/1 paper filter (Whatman, Sigma Aldrich) and washed with water and acetone until the yellow colour disappeared. The holocellulose is put in a glass plate and then in an oven at 80°C until constant weight. The lignin and holocellulose content are calculated from the following equations:

$$HoC\% = \frac{W_f - W_t}{W\ (2{,}5g)} * 100 \qquad \text{[Eq.3.5]}$$

$$L\% = 100 - HoC\% \qquad \text{[Eq.3.6]}$$

However, the calculate percentage of lignin includes also the extractives content, and this must be considered in the results evaluation.

Figure 3.2 Holocellulose extraction apparatus

The dried holocellulose is used for hemicellulose extraction and cellulose isolation following the method reported in [10]. The cellulose content should be estimated before in order to

calculate the quantity of enzymes (e.g. cellulase) to be added before fermentation takes place. The term hemicellulose is defined as the cell wall components that are readily hydrolysable by hot dilute mineral acids, hot diluted alkali or room temperature 5% sodium hydroxide. The holocellulose, is the present case is treated with sodium hydroxide and acetic acid. Hence, the soluble fraction corresponds to the hemicellulose fraction. The holocellulose is mixed with 17,5% *w/v* sodium hydroxide solution (1:5 solid-liquid ratio) and washed 2 times every 5 minutes with half the starting volume of the same solution of sodium hydroxide (1:2,5 solid-liquid ratio). Then, 33 mL of distilled water are added after 30 minutes. The sample is kept at 20°C for 1h. The solution is vacuum-filtered, washed 2 times with 50 mL of 8,3% of sodium hydroxide solution and water. Without suction, 15 mL of 10% of acetic acid are added and after three minutes the cellulose is finally washed with water. The filtrate is captured before washing and the remaining cellulose is dried in oven at 80°C until constant weight in a glass plate. **Figure 3.3** shows a cellulose sample after hemicellulose extraction.

$$C\% = \frac{W_f - W_t}{W\,(2{,}5g)} \qquad \text{[Eq.3.7]}$$

Figure 3.3 Cellulose after hemicellulose extraction and filtration

3.2.3 Klason-lignin method

This method was developed in order to find structural carbohydrates and lignin compositions in biomass, especially wood barks and here it is applied to compare results from the previous extractions and to find total carbohydrates by HPLC (see section 3.3). This procedure exploits a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified as described by [49] and [50]. The lignin fractionates into acid insoluble materials and acid soluble material. The generally small amount of lignin soluble in the acid solution can be

quantified by UV-vis spectrophotometry and is termed acid soluble lignin (ASL). Gravimetric analysis of the solid residue is applied to determine the acid insoluble lignin (AIL). During hydrolysis, polymeric carbohydrates are hydrolysed in their monomeric constituents, that are soluble in the hydrolysis liquor. Hereafter a description of the method followed in the laboratory and an explicative figure (**Figure 3.4**). First, 0,3 g of sample (W) are placed in a 250mL borosilicate bottle, together with 3mL of 72% *v/v* H_2SO_4. The bottle is placed in a water bath at 30°C for 1h. The second step consisted in slowly adding 84mL of distilled water (until a 4% H_2SO_4 dilution is obtained) and autoclaving for 1h at 121°C. The bottle is cooled down until room temperature and then the solution is filtrated with the help of a vacuum pump on paper filters (previously weighted). The filtrate is stored at -20°C or utilised within 6h, and the solid residue is washed with 50 mL of ultra-deionised water, provided by an Easypure II RF device (Barnstead, Iowa, USA), and dried until constant weight at 80°C. Finally, a gravimetric analysis is done to find the Klason Lignin. All the experiments are made in duplicate for each feedstock.

$$KL\% = \frac{W_f - W_t}{W\ (0{,}3g)} \qquad \text{[Eq.3.8]}$$

Where:

- W_f =weight dried sample + filter
- W_t =weight filter

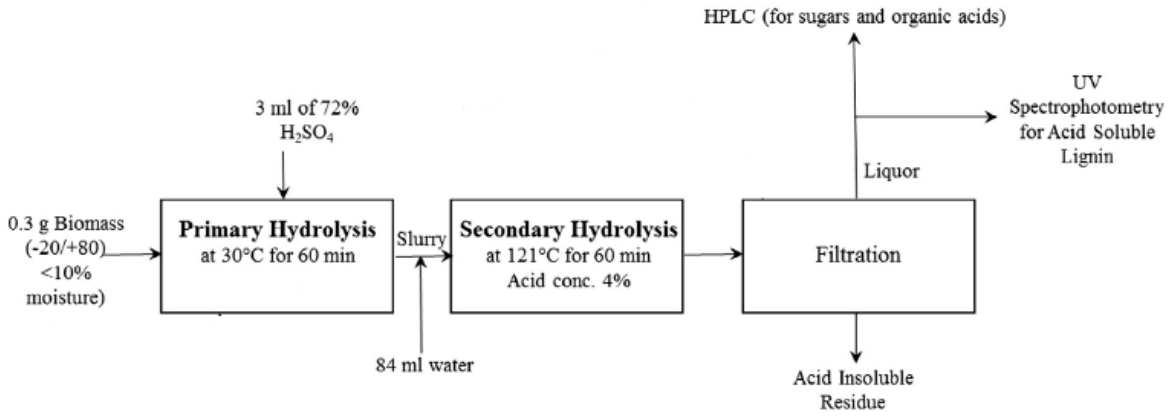


Figure 3.4 Experimental design. Modified from [50]

For the acid soluble lignin determination, an UV-vis spectrophotometer is used (Ultrospec 3300 pro, Amersham Bioscience). Distilled water is used as a blank, however, the standard procedure was modified (i.e. no dilution was performed), since the absorbance of the liquor is already in the range 0,7-1, even lower for some cases. The following equation is used to determine the ASL content:

$$ASL\% = \frac{DV(A_s - A_b)}{aW} \qquad \text{[Eq.3.9]}$$

Where:

- D =dilution factor (in this case=1)
- V=sample volume (0,087 L)
- A_s =sample absorbance
- A_b =solvent absorbance =0
- a =lignin absorptivity [L/(g·cm)] (see **Table 3.2** below)
- W =dried sample weight = 0,3 g

Table 3.2 Absorbance and absorptivity values for different types of feedstock. In yellow the values used in this study. Modified from [51]

Biomass type	Lambda max (nm)	Absorptivity at lambda max (L/g cm)	Recommended wavelength (nm)	Absorptivity at recommended wavelength (L/g cm)
Pinus Radiata	198	25	240	12
Bagasse	198	40	240	25
Corn Stover	198	55	320	30
Populus	197	60	240	25
Food Waste	190	25	240	20

The preparation of samples and the determination of carbohydrates from the Klason Lignin liquor by HPLC is detailed described in the next section.

3.2.4 Total Carbohydrates determination

Several extraction and measurement methods have been employed in literature for the determination of total sugars in biomass. Here two methods are compared for carbohydrates extraction in the different matrices under analysis:

- Ethanol extraction
- Klason lignin extraction method

The latter has been widely explained in the previous section. The liquor from the extraction and autoclavation is captured and used for the analysis. The ethanol extraction method is taken from [52] and described below:
In plastic centrifuge tubes, 50mg of each feedstock are placed and weighted with the precision balance (Sartorius, Germany). Then, 5mL of 80% ethanol solution are added. The centrifuge tubes are closed (i.e. non-hermetically) with plastic cups and boiled in a 95°C water bath for 10min. For the water bath, a borosilicate Becher is filled with water and put on a heater plate IKA HS7 (IKA Werke, Staufen, Germany); the temperature is monitored with a precision thermometer IKA D5 (IKA Werke, Staufen, Germany). The tubes are centrifuged (3K30, Sigma Aldrich) at 2500 rpm for 5 min and the supernatants are collected in different plastic tubes. Then, again 5mL of the aforementioned ethanol solution are added in each tube which is boiled, centrifuged and the supernatants collected together (see **Figure 3.5**). This procedure is repeated three times in total in order to obtain at the end ~15mL of supernatants to be analysed

for each feedstock. The extractions are made in duplicate and the tubes are stored at -20°C prior to carbohydrates determination.
However, for carbohydrates determination three methods have been investigated:

- Sulphuric Acid-UV method
- Phenol-Sulphuric Acid method
- High Pressure Liquid Chromatography (HPLC)

Figure 3.5 On the left the experimental set-up for EtOH extraction. On the right the ethanol extracted carbohydrates solution of the four feedstocks.

3.2.4.1 Sulphuric Acid UV-method vs Phenol-Sulphuric Acid method

One of the most versatile, cheap and relatively easy approach for determination of carbohydrate concentrations is the colorimetric method developed by Dubois *et al*. [53]. It is based on reaction between hydrolysed carbohydrate solution and a colouring reagent that develops colour that is detectible in the visible range of the electromagnetic spectrum. Reagents commonly used for colour development include phenol. The phenol-sulphuric acid method depends on dehydration of hydrolysed saccharides to furfural derivatives during reaction with concentrated sulphuric acid. Further reaction of the furfural derivatives with phenol forms coloured complexes that absorb light. Anyway, phenol and its vapour are hazardous and corrosive to skin, eye and respiratory system and its waste disposal could create problems to the environment. Thus, different alternatives have been studied and the sulphuric acid-UV method seems to be an adequate alternative as described by [54]. In the present work, both methods have been tested on the four feedstocks and the results compared. The description of the methods is given below:

Phenol-Sulphuric acid method by [53]:

Both the Klason lignin liquor and ethanol extracted solution are used in order to compare the extraction methods. Each measure is done for each extracted duplicate.

In 15mL Falcon tubes covered with plastic cups, 2mL of extracted sample and 50 µL of 80% phenol (prepared by adding 1mL of water and 4g phenol reagent grade) are added. Then, 5mL of sulphuric acid reagent grade are rapidly added directly on the surface to increase mixing. The tube is let stand for 10min in the dark, shaken and placed for 10 min in a water bath at 25°C. After this step, 2mL are transferred into special closed glass cuvettes and the absorbance is read at 490nm in a UV-vis spectrophotometer. The control blank is obtained in the same way but substituting the carbohydrate solution with distilled water. The results are compared with the standard curve.

The procedure for the standard curve is given below, glucose and galactose are chosen as standard sugars:

1. A glucose standard solution is prepared with ultra deionised water (100mg/L)
2. A galactose standard solution is prepared with ultra deionised water (100mg/L)
3. Different dilution solutions are created as reported in **Table 3.3**
4. A mixed sugars solution is created combining the standard solutions in a 1:1 ratio using the same dilution factor
5. The same procedure described above of phenol-sulphuric acid method is applied on 2mL of each diluted solution and the absorbance is read at 490nm. The blank is prepared at the same way substituting the sugars solution with the 0mg/L of glucose, galactose and mix standard solution. Point 5 is repeated three times for each diluted solution.

Table 3.3 Diluted solutions for creation of the standard curve phenol-H2SO4 method

	mg/L glucose or galactose					
	0	10	20	30	40	50
mL Glc or Gal stock solution	0	0,4	0,8	1,2	1,6	2
mL milliQ water	4	3,6	3,2	2,8	2,4	2

Sulphuric Acid-UV method by [54]:

This procedure is a non-hazardous alternative of the previous method and exploits the UV-light absorption properties of the furfural solution produced after the reaction with sulphuric acid. In a glass tube covered with cups, 1mL of extracted carbohydrate solution is added and rapidly mixed with 3mL of sulphuric acid reagent grade. The solution is mixed for 30 seconds with the help of a Vortex Mixer and cooled on ice for 2 min until room temperature. The liquid is transferred into cuvettes of 2mL and the absorbance is read in a UV-vis spectrophotometer at 315nm. The blank is obtained using instead distilled water. The results are compared with the standard curve.

The procedure for the standard curve is given below, glucose and galactose are chosen as standard sugars:

1. A glucose standard solution is prepared ultra deionised water (100mg/L)
2. A galactose standard solution is prepared ultra deionised water (100mg/L)
3. Different dilution solutions are created as reported in **Table 3.4**

4. A mixed sugars solution is created combining the standard solutions in a 1:1 ratio using the same dilution factor
5. The same procedure described above of sulphuric acid-UV method is applied on 1mL of each diluted solution and the absorbance is read at 315nm. The blank is prepared at the same way substituting the sugars solution with the 0mg/L of glucose, galactose and mix standard solution. Point 5 is repeated three times for each diluted solution.

Table 3.4 Diluted solutions for the creation of the standard curve H2SO4-UV method

	mg/L glucose or galactose					
	0	20	40	60	80	100
mL Glc or Gal stock solution	0	2	4	6	8	10
mL milliQ water	10	8	6	4	2	0

3.2.4.2 Carbohydrates determination by chromatography (HPLC)

High pressure liquid chromatography is an analytical technique used to separate, identify and quantify each component in a mixture. HPLC relies on pumps to pass a pressurized liquid (mobile phase) and a sample mixture through a column filled with adsorbent (stationary phase), leading to the separation of the sample components due to their different degrees of interaction with the adsorbent particles. The mobile phase is typically a mixture of solvents. Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic, dipole–dipole and ionic or more often a combination of them. The system is equipped with a detector which generates a signal proportional to the amount of sample component emerging from the column. The result is a chromatogram with peaks at different retention times. The area under the peak can be linked to each component concentration.
In this thesis the liquor from Klason Lignin extraction of the four feedstocks is used to quantify the carbohydrates composition with HPLC column.
The analysis is performed using a 1200-series HPLC system (Agilent technologies, Waldbronn, Germany), equipped with a Refractive Index Detector (RID) and a 300x7.7mm, 8µm Hiplex-Ca (Varian Inc.) operated at 85°C and using ultra deionised water as mobile phase, at a flow rate of 0,6 mL/min.
Five calibration standards are prepared containing the compounds that needed to be quantified. **Table 3.5** reports the type of standard sugar chosen and its concentration.

Table 3.5 Standard solutions and their concentrations

Standard sugar	Concentration g/L
Glucose	10
Galactose	1
Fructose	1
Arabinose	1
Xylose	1

The liquor from Klason-lignin extraction of the four feedstocks is previously prepared, since is very acid and therefore dangerous for the HPLC column activity. The procedure is taken from [49]. Approximately 1 mL of liquor is transferred in a Becher and diluted 1:100 with distilled water. Then, the liquid is neutralized with calcium carbonate ($CaCO_3$) to pH 5-6. The calcium carbonate is added slowly after reaching a pH of 4. The sample is swirled frequently with the help of a magnetic bar and a magnetic stirrer. After reaching a pH of 5-6, the sample is allowed to settle. The pH of the liquid after settling is 7. The neutralized sample is passed through a 0,2µm nylon filter with the help of a 1mL syringe into an autosampler glass vial (Carl RothGmbH, Germany) and closed with cups. The standard solutions are filtrated as well with the 0,2µm nylon filter and placed into glass vials.
The chromatogram is obtained. For the samples various peaks corresponding to a defined sugar at different retention time are detected. The area under each peak is integrated. In order to find the sugars concentration this area is divided by the standard area of the corresponding sugar, obtained by integrating the peak of the standard solutions of known concentration.

3.3 ACID FERMENTATION TEST

The main goal of this part of the work was monitoring the state of the acid fermentation with parameters to be controlled, in order to evaluate the behaviour of the culture subjected to different operational parameters and stress conditions. Three batch tests are carried out, in which corn silage is used as substrate and a treated AD sludge from an on-going plant as inoculum. The reason why corn silage is used is explained at the beginning of this chapter. Since it is known as the most studied feedstock for anaerobic digestion, in this work it is used as a reference material.
The goals of the three batch tests conducted in this thesis are explained below, but deeply investigated in the next sections, together with the experimental protocols:

- Test 1: the goal is to evaluate the efficiency of an acid pre-treatment on biogas plant inoculum for acid fermentation.
- Test 2: the goal is to evaluate the efficiency of a thermal pre-treatment on biogas plant inoculum for acid fermentation.
- Test 3: the goal of this test is to monitor biohydrogen production in order to check how the partial pressure could influence metabolic pathways.

The experimental set-up of the acid fermentation system consists in three batch bioreactors (BlueSens, Herten, Germany) with a total volume of 250mL and a working volume of 50mL for each bioreactor. All the three flasks are operated under anaerobic conditions achieved by flushing N_2 for 5 minutes into the fermentation broth, at mesophilic conditions of 35°C and with mechanic agitation. Indeed, the three flasks are placed in a shaking water bath at 50rpm (1086, GFL). A tube for sampling is placed reaching the bottom of the flask and a device to prevent overpressure is placed on the top of the flasks and filled with water (see **Figure 3.6**).
The initial pH is set to 5,2, the dry matter ratio between substrate and inoculum is fixed at 0,5:1
In all the tests four parameters are monitored:

- pH
- Frequency-dependent polarizability anisotropy with an Elotrace device (EloSystems, Berlin, Germany)
- $BioCH_4$, $BioH_2$ and CO_2 production with specific sensors (BlueSens, Herten, Germany).
- Acid production with HPLC (Agilent technologies, Waldbronn, Germany)

The first two are at-line measurements, the gas production is an on-line control, while the last is an off-line analysis.

Figure 3.6 Experimental set-up of acid fermentation tests. On the right the BlueSens flask with the overpressure preventing device and sampling tube. On the left the final set-up with sensors fixed on the flaks.

The corn silage and the inoculum are provided by IASP (Institut für Agrar- und Stadtökologische Projekte) in Berlin. The inoculum is an AD sludge from an on-going corn silage plant for biogas production.

3.3.1 Test 1: efficiency of inoculum acid pre-treatment for acid fermentation

The goal of this test is proving the efficiency of an acid pre-treatment on the inoculum. As explained in Chapter 2, different pre-treatments could be applied to create an acidogenic inoculum, enriched in spore-forming Hydrogen Producing Bacteria (HPB) in order to avoid methane formation and acid consumption by Hydrogen Consuming Bacteria (HCB). In this way the acid production yield would be higher.

The sludge is first thawed from -20°C on ice for 1 day and then incubated at 35°C for 48 hours. Then, the thawed sludge is treated with 10% *v/v* HCl solution until pH 3 and incubated for 24 hours in a water bath at 35°C. The polarizability before and after pre-treatment is measured following the procedure described in the section: "Anisotropic polarizability (AP) measurement".
The dry matter percentage (DM%) of the inoculum is determined before and after pre-treatment. 1mL of sludge is taken and put in a previously weighed Eppendorf tube. The tube with the sludge is weighted and centrifuge 15 minutes at 1500g. The supernatants are discarded carefully, and the pellets are washed with distilled water. The tube is again centrifuged, and the washing steps repeated until the water seemed clean. The sample is then dried at 80°C in a convection oven for 24 hours until constant weight. The DM% is calculated following Eq. 3.10.

$$\mathrm{DM\%} = \frac{W_f - W_t}{W_i - W_t} * 100 \qquad \text{[Eq. 3.10]}$$

With:
- W_t = weight tube
- W_i = weight tube + inoculum
- W_f = weight tube + dried inoculum

The procedure is done in triplicate before and after pre-treatment.

Substrate pre-treatment
For corn silage a mechanical and alkaline pre-treatments are chosen according to [35]. Raw substrate (50-70g) is weighted and dried at 80°C for 24 hours in order to find the dry matter percentage (DM%) before pre-treatment. Then, it is grinded with a laboratory batch mill IKA A10 (IKA-Werke, Staufen, Germany) to decrease the particle size (see **Figure 3.7**), and thus facilitate mixing during the fermentation process and simplify the hydrolysis step. Then, the substrate is diluted 4:11 *w/w* with tap water and treated with 30% *w/w* NaOH solution until pH 12 and incubated at 35°C for 24 hours in a water bath.
Once ready, the substrate is filtrated and rinsed with tap water until pH 7 measured with pH indicators papers.
The DM% is found after pre-treatment taking three times 1g of substrate and drying it always at 80°C 24 h.

Figure 3.7 Mechanical pre-treated corn silage

Cultivation set-up

Once the inoculum and the substrate are ready, the correct amount is measured three times and split into three beakers. The broth is then neutralized until pH 5,2 with 30% NaOH solution and mixed for homogenization purposes with the help of a magnetic bar and magnetic stirrer. The broth is transferred into the BlueSens flasks and flushed 5 minutes with N_2. The temperature and shaking velocity are set-up and the sensors switched on. The cultivation of Test 1 takes 5 days, samples from each flask were taken at the beginning (t0), in the middle (t+3d) and at the end (t+5d) of fermentation with the help of a syringe and prepared for HPLC analysis and Elotrace analysis. Also, the pH is measured each time.

Sensors set-up

The composition of the gas output produced during fermentation is detected with two gas sensors (BlueSens, Herten, Germany) of CO_2 and in the case of the first test CH_4. BPC-CO_2 and BPC-CH_4 are based on infrared beam weakening measurement principle. Before fermentation they are calibrated one by one with the help of a calibration software, flushing N_2 for at least 1 h in the closed BlueSens flask.

Quantification of Volatile Fatty Acids (VFA) production

The analysis of the produced VFA is performed using a 1200-series HPLC system (Agilent technologies, Waldbronn, Germany) equipped with a Refractive Index Detector (RID) and a 300x7,7mm, 8µm Hiplex-Ca column (Varian, Inc.) operated at 85°C and using ultra deionised water provided by an Easypure II RF device (Barnstead, Iowa, USA) at a flow rate of 0,6 mL/min. The samples are carefully prepared in 1,5mL Eppendorf tubes following this protocol:

- The sample (1,5mL) is vortexed thoroughly and centrifuged (Eppendorf, Hamburg, Germany) at 17000g for 15min at 4°C
- The supernatants are carefully transferred and centrifuged again at 17000g for 10min at 4°C
- 800µL of the supernatant are taken and 10 µL of 1-propanol (0,1g/L, 99,7% from Sigma-Aldrich) were added
- 200 µL of Carrez solution I from the protein precipitation kit (Merck KGaA) are added and the solution is homogenised by vortexing
- 200 µL of Carrez solution II from the same kit are added and sample is vortexed thoroughly
- When the solution gets turbid, the tube is again centrifuged at 17000g for 15min at 4°C
- The supernatants are transferred and centrifuged again at 17000g for 10min
- The supernatants are transferred in 1,5mL glass vial (Carl Roth GmbH, Karlsruhe, Germany) and the insert is filled up to 180µL.
- The samples are stored in freezer at -20°C prior to the analysis

Anisotropic polarizability (AP) measurements

The protocol for samples preparation is taken and adapted from [35]. Samples from each flask (2mL) are initially filtrated with the help of a vacuum pump with 125 µm nylon filters (Carl Roth GmbH, Karlsruhe, Germany) in order to remove large particles and diluted with 3mL of distilled water. The filtrate (5mL) is centrifuged at 4°C and 600rpm for 5 minutes. 2mL of supernatants are taken and diluted with 18mL of ultra deionised water and centrifuged at

6000rpm for 2 minutes. The supernatants are discarded, and the pellets washed with 30mL of ultra deionised water to adjust the initial electrical conductivity to an adequate range compatible with AP measurements. The AP measurements are conducted at-line with an Elotrace device (EloSystems, Berlin, Germany) (see **Figure 3.8**). The AP measures are acquired at four different frequencies: 2100, 900, 400, 210 KHz. Triplicate analyses are performed for each sample and the mean values are presented.

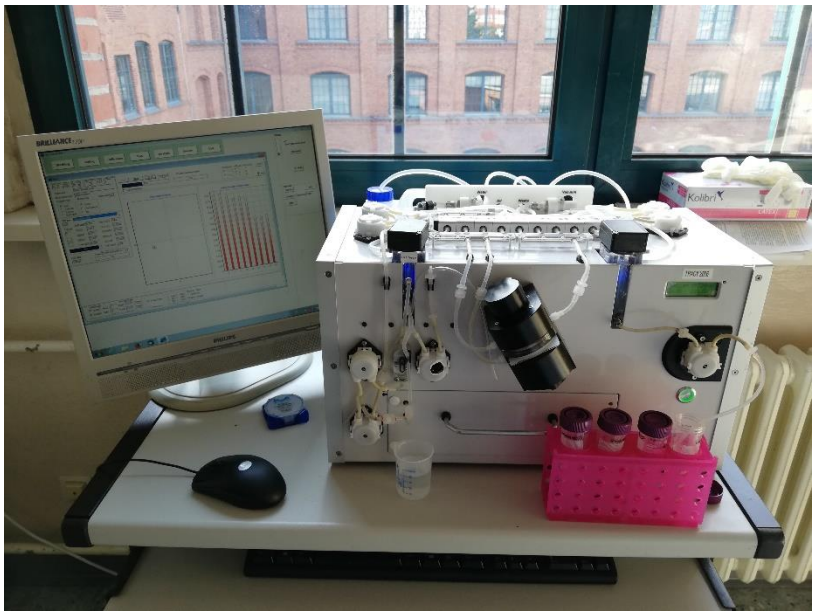


Figure 3.8 Elotrace device

3.3.2 Test 2: efficiency of inoculum thermal pre-treatment for acid fermentation

The goal of this test is proving the efficiency of the thermal pre-treatment on the inoculum for acid fermentation.
The inoculum is incubated at 70°C for 30min in a water bath. The DM% and the polarizability are measured before and after pre-treatment in the same way of the previous Test 1.
The same cultivation set-up applied in Test 1 is replicated here. Also, methane and CO_2 production are registered with the sensors and compared. The mechanical and alkaline pre-treatments are applied on the corn silage.
The fermentation takes 5 days and the sampling point are fixed at the beginning (t0), in the middle (t+3d) and at the end of fermentation (t+5d). The same protocols for HPLC and Elotrace sample preparation are followed.

3.3.3 Test 3: bioH2 production as an on-line tool for the control of acid fermentation

Differently from the earlier Tests (Test 1 and Test 2), Test 3 is conducted in order to check if the production of biohydrogen that occurs during acid fermentation could strongly influence the metabolic pathways that lead to the VFAs production and their composition in the liquid

phase. Hence, in the experimental set-up, CH_4 sensors are replaced with H_2 sensors. The BCP-H_2 sensor functioning is based on thermal conductivity difference to estimate the amount of H_2. The obtained results from Test 1 and 2 were the criterion to select the pre-treatment for this fermentation Test. Therefore, the acid pre-treatment is applied. Again, DM% before and after pre-treatment is found in order to calculate the correct amount of corn silage needed. The mechanical and alkaline pre-treatments are applied on the substrate.
The fermentation takes 5 days and the sampling point are fixed every 24 hours. Therefore, at t0, t+24h, t+48h, t+72h, t+4d and t+5d to better monitor the acid production detected with the HPLC measurements and the viability of the cells with the Elotrace device.
All the results are presented in the following chapter.

4 CHAPTER 4: RESULTS AND DISCUSSION

4.1 METHODS FOR LIGNOCELLULOSIC FEEDSTOCK CONTENT DETERMINATION

4.1.1 Mass composition

The physical and chemical characteristics of the organic waste are important information for designing and operating anaerobic digesters, because they affect VFA and biogas production and process stability during anaerobic digestion [55]. A comprehensive characterization of biomass includes Proximate Analysis and Ultimate Analysis. The proximate analysis serves as a simple means for determining the behaviour of a solid biomass when it is heated. From it, different fractions can be identified, as the contents of moisture (MC), total solids (TS), volatile solids (VC) and ash, therefore the mass composition. On the other hand, the main purpose of an ultimate analysis is to determine the elemental composition of the solid substance. The main elements of solid biomass include carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O) but for some solid biomass fuels chlorine (Cl) and other elements may also be of interest [56]. In this work the results from proximate analysis are presented, since the ultimate analysis was not carried out.

The average MC, TS, VS and ashes are presented in **Table 4.1**, together with standard deviation and the comparison between different feedstock, which is also showed in **Figure 4.1**. The ratio between volatile solids and total solids VS/TS has been calculated since it is an index of the organic content of biomass available to be fermented by microorganisms. All the values are reported on a wet weight basis. The MC ranged from 26 to 95% for wood barks and lettuce respectively, emphasising that lettuce is the feedstock with higher moisture content and less organic matter, and this could be seen from VS/TS ratio that is 90% compared to the others. The feedstock that shows the higher VS/TS ratio is rice, followed by wood barks and maize silage. In general, the analysed feedstocks seem suitable for anaerobic digestion, since the moisture content is relatively high, hence they are fermentable in wet fermentation processes (TS<15%).

Table 4.1 Proximate analysis results with standard deviation

Feedstock / Parameters	Rice	Lettuce	Wood barks	Maize silage
Total solids TS%	24,81±0,12	5,36±0,07	73,89±0,05	42,69±0,37
Moisture content MC%	75,19±0,12	94,64±0,07	26,11±0,05	57,31±0,37
Volatile solids VS%	24,66±0,12	4,85±0,06	72,42±0,05	41,19±0,39
Inorganics (ashes)%	0,15±0,01	0,51±0,01	1,48±0,01	1,50±0,03
VS/TS	0,99	0,90	0,98	0,96

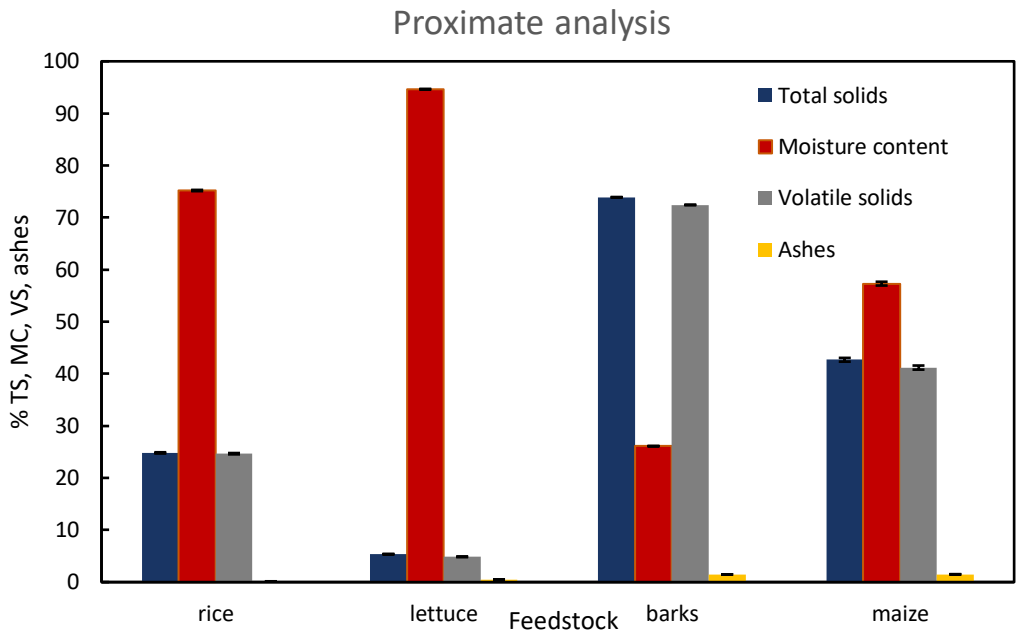


Figure 4.1 Proximate analysis distribution for each feedstock

4.1.2 Lignin and cellulose determination

An important step towards the utilisation of different biomass matrices is the development of robust methodologies which can provide information about the main constituents, which can also serve as baseline for comparison. In the case of fermentation processes, as introduced in the previous section, it is fundamental to find the composition of the lignocellulosic structure. Lignin is the main responsible of the recalcitrant behaviour, while cellulose and hemicellulose are the main source of carbohydrates, which can be used as substrate for microorganism to growth. The accessibility to carbohydrates is hindered by the presence of lignin and therefore pre-treatments are necessary prior to fermentation to increase their availability. The determination of lignin content is a determinant factor in the selection of the most suitable pre-treatment. Feedstocks with higher lignin generally tend to require stronger pre-treatment steps. On the other hand, the determination of the cellulose content is also important for the selection of the enzyme or microbial consortia as well as the necessary amounts to be supplemented for biological pre-treatments. In **figure 4.2** lignin, hemicellulose and cellulose contents are showed for each feedstock under analysis with the error bars representing the correspondent standard deviation.

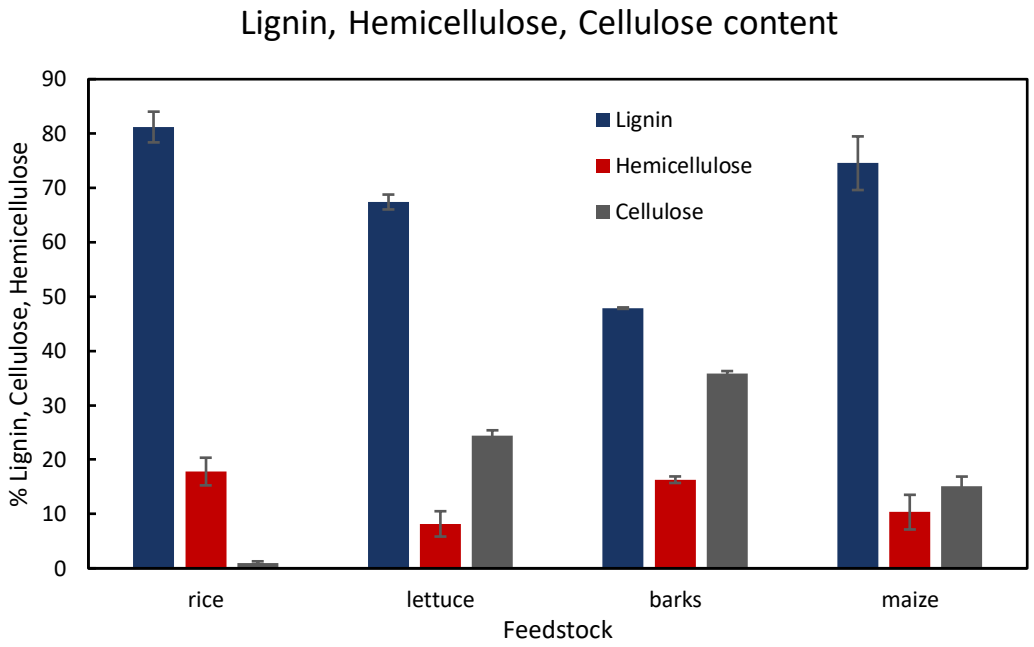


Figure 4.2 Percentage of lignin (chlorination method), hemicellulose and cellulose for rice, lettuce, wood and maize with error bars

The lignin content in **Figure 4.2** is determined with the chlorination method and calculated with Equation 3.6. In this method, $NaClO_2$ breaks the lignin structure that will be solubilized in water. The solid residue is constituted by holocellulose, the sum of hemicellulose and cellulose. Cellulose and hemicellulose are calculated by gravimetric analysis using Equation 3.7 after NaOH hydrolysis that is aimed to disrupt the hemicellulose bonds, leaving free the cellulose. The results are compared with the one found in literature (see **Table 4.2**).

Table 4.2 Comparison between literature values and experimental data for lignin and cellulose content. t.s. stands for this study.

	Rice		**Lettuce**		**Wood barks**		**Maize silage**	
	Lignin	**cellulose**	**Lignin**	**Cellulose**	**Lignin**	**Cellulose**	**Lignin**	**Cellulose**
Literature [4] [57] [58]	9-20	28-38	15-20	0-6	40-47	16-31	10-15	35-45
t.s (chlorination and NaOH hydrolysis)	81,20 ±2,82	1,00 ±0,28	67,40 ±1,36	24,43 ±0,96	47,87 ±0,13	35,82 ±0,47	74,54 ±4,93	15,13 ±1,75
t.s. (Klason-lignin extraction)	4,32 ±0,52	-	14,37 ±1,18	-	40,98 ±6,70	-	13,86 ±1,91	-

It seems evident that the percentages of lignin and cellulose found experimentally with the chlorination method and subsequently with the NaOH hydrolysis are different from the percentages found in literature. In fact, the chlorination method for lignin determination is mostly applied to high-lignin content biomass, such as wood or high-grade pulp and provides a

measure of the total lignin content. When biomass samples present high percentages of extractives content and inorganics (see **section 2.2.1**), total lignin determination via the chlorination method tends to be biased. In this respect, since two of the tested substrate, rice and maize silage present high starch content, about 70-90% [59] and 60-70% [57], respectively, the measurement of lignin tends to deviate and include these shares. Starch is removed together with lignin since it is a hydrophilic polymer at high temperature. The other biomass tested, lettuce, present a lower content of extractives and almost non-existent content of starch, but the lignin content is also low, hence the chlorination method proved unsuitable.

The other methodology regards the determination of acid-insoluble lignin, which is also known as the Klason method. The percentage of lignin with the Klason method is obtained adding the %KL (acid insoluble lignin) found by gravimetric analysis (Equation 3.8) and the %ASL (acid soluble lignin) found by spectrophotometric analysis (Equation 3.9) as reported in Chapter 3. Although the Klason method is also recommended for the quantification of lignin in wood-based matrices it proved to be more reliable for all samples under analysis. In this method, in addition to the cellulose and hemicellulose disruption, also the acid hydrolysis of starch occurs and that is the reason why the lignin percentage is lower (compared to the obtained values from the chlorination method) especially for rice and maize silage. A graphical comparison between the two methods is shown in **Figure 4.3** together with the error bars.
In general, it is worth it to underline that is difficult to develop a standard procedure which can be applied to different biomass sources. However, it is worth noting that among the studied biomass matrices, the feedstock with higher lignin content is wood barks. The difference among the methodologies for the determination of lignin content in wood barks yielded an error of about 7 %, which is the best result obtained from the analysed matrices, with a higher standard deviation corresponding to the Klason method. Since the two methodologies were initially developed to quantify the lignin content in with wood-based matrices, the obtained results seem in agreement with literature values.

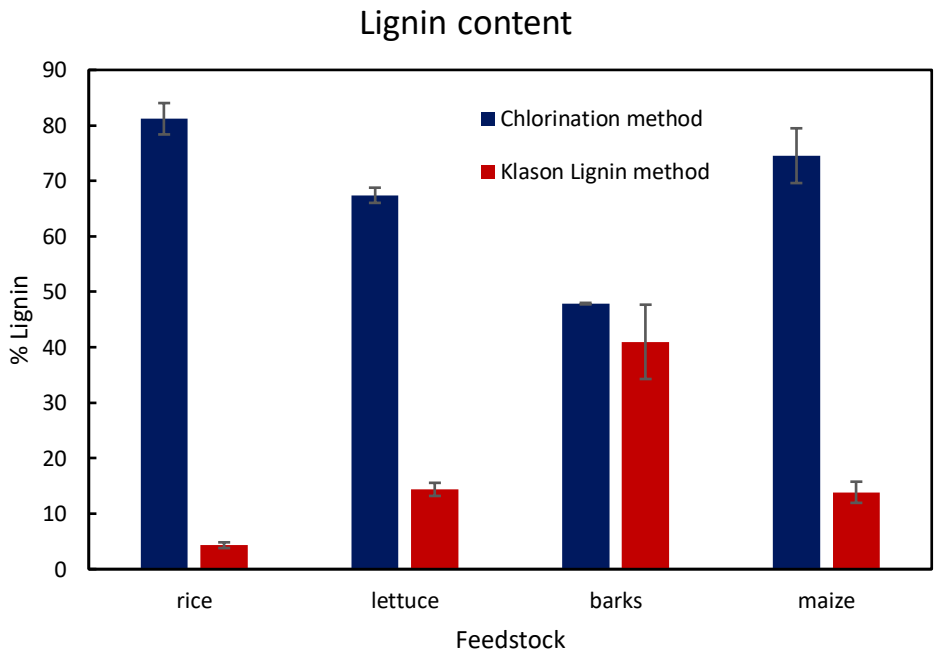


Figure 4.3 Comparison between chlorination and Klason method for lignin determination

4.1.3 Carbohydrates determination

Carbohydrates concentration must be measured as part of a comprehensive biomass analysis. Moreover, knowing the sugar content is fundamental to compare different feedstocks and to study their potentiality as substrate or co-substrate for fermentation processes. As described in Chapter 3, two extraction methods have been exploited in order to have a liquor rich in carbohydrates to be detected. These two methods are the Klason-lignin method that is based on an acid hydrolysis of structural carbohydrates and the ethanol extraction that is based on the solubilization of sugars in alcohol. Then, this liquor was analysed applying three detection methods for carbohydrates content. These methods are: H_2SO_4-UV-vis method, phenol-H_2SO_4-UV-vis method and HPLC.

4.1.3.1 Comparison between H_2SO_4-UV-vis method and phenol-H_2SO_4-UV-vis method

Spectrophotometric quantification of sugars needs a reference. At this purpose standard curves are built using a sugar solution at different dilution factor as a reference. As described in Chapter 3 three standard curves are built for each detection method. The first curve using glucose as standard sugar, the second curve using galactose as standard sugar and the third one built using a mixture 1:1 of glucose and galactose. The latter is used as a reference for the estimation of the total carbohydrates content since represents better the thermodynamic behaviour of a mixture of sugars. Hereafter in **Table 4.3** are shown the results of the linear interpolation of the experimental data at different dilution factors of the standard mixture of sugars. Meanwhile **Figure 4.4** shows the curves trend.

Table 4.3 Linear interpolation results of the standard curve total sugars

Method	Slope	Intercept	R^2
H_2SO_4- UV	0,0068	-0,0064	0,9886
Phenol-H_2SO_4	0,0166	-0,0028	0,9978

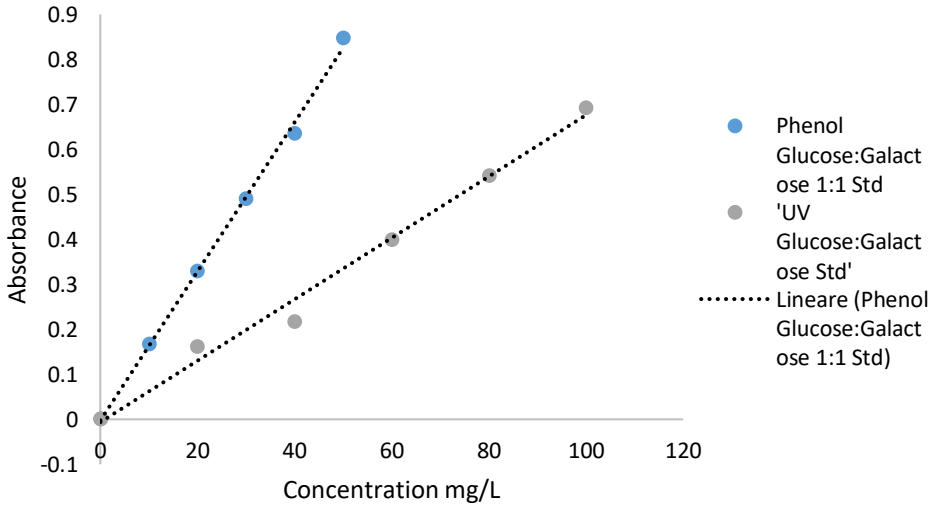


Figure 4.4 Standard curves Glucose: Galactose 1:1, phenol-H_2SO_4 and H_2SO_4-UV method

Using the equations of the standard curve the carbohydrates content for each feedstock is determined, since the absorbance of the extracted solution is measured with the spectrophotometer and, hence the concentration can be determined. In order to compare the experimental sugar concentrations obtained with the two methods, a normalization factor must be taken into account. Indeed, the working volume of the two procedures is different. 4mL (1mL sugar solution) for H_2SO_4-UV method and 7,05mL (2mL sugar solution) for phenol-H_2SO_4-UV method.

$$normalization\ factor1 = 1{:}1{,}1347$$

Moreover, another normalization is necessary since the initial amount of feedstock is different for the two extraction methods. 0,3g in 87mL for Klason-Lignin extraction and 50mg in 15mL for ethanol extraction.

$$normalization\ factor2 = 1{:}\,0{,}966$$

In **Table 4.4** are shown the results.

Table 4.4 Total carbohydrates content in mg/L

Experimental normalization of the Total Carbohydrates Content (mg/L)				
	On Klason Lignin		On EtOH extraction	
	H_2SO_4-UV	Phenol-H_2SO_4	H_2SO_4-UV	Phenol-H_2SO_4
Rice	676,84±31,73	255,86±36,43	34,21±3,38	23,51±0,75
Lettuce	383,27±12,45	88,46±11,78	430,68±43,97	159,68±25,90
Wood barks	416,79±8,63	147,55±17,03	122,37±12,57	77,92±16,14
Maize silage	468,49±20,28	73,51±21,82	190,38±29,85	176,14±34,16

A statistical analysis was done between H_2SO_4-UV and Phenol- H_2SO_4 in order to find a correlation between the two methods and see if the traditional method of phenol can be substituted with the non-hazardous method with H_2SO_4. The Pearson coefficient is reported below in **Table 4.5**. It represents the linear correlation between two variables X and Y, in this case the two methods. It is a number between -1 and +1, where 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation. The Pearson coefficient is defined as reported in Equation 4.1:

$$Pearson\ coefficient = \frac{cov\ (X, Y)}{\sigma_x \sigma_y}$$

Where:

- cov (X, Y) is the covariance
- σ_x is the standard deviation of X
- σ_y is the standard deviation of Y

Table 4.5 Statistical analysis of phenol and sulfuric acid method

σ_x=St. Deviation H_2SO_4-UV (mg/L)	210,1764
σ_y =St. Deviation Phenol- H_2SO_4 (mg/L)	73,6337
Covariance σ_{xy}	9298,5494
Pearson coefficient	0,6008

In this analysis the Pearson coefficient obtained lied between 0 and 1. This means that there is a weak correlation among the analysed population, and hence the linear trend is not enough to validate the UV-sulfuric acid method for total carbohydrates content determination. Hence, further efforts should be performed to improve the process towards the reduction of the phenol method. Although a positive correlation was found, this is not the only parameter to be validated (Pearson coefficient=0.6 is definitely not enough).

4.1.3.2 Comparison between Klason lignin extraction and EtOH extraction

Due to the experimental difficulties associated to the use of phenol for the UV-vis determination, H_2SO_4 was used as reagent, bearing in mind the considerations of section 4.1.3.1. The next step regards the comparison between the two-extraction methods. In **Figure 4.5,** the difference between the total carbohydrates yield is presented while in **Table 4.4** it is possible to read the quantity of sugars in mg/L. For rice, wood barks and maize silage the difference among the two methods is clear. The Klason-lignin extraction seems more efficient since the quantity extracted and detected with the UV method is higher than the quantity obtained from ethanol extraction. This could probably mean that the structural carbohydrates contained in these feedstocks are more easily hydrolysable than solubilized. That does not appear for lettuce where the discrepancy is not very sharp, however, due to its high moisture content, the similarity in the carbohydrates yield among the methods could be due to the less amount of carbohydrates (totally) and the selected titres of the reagents.

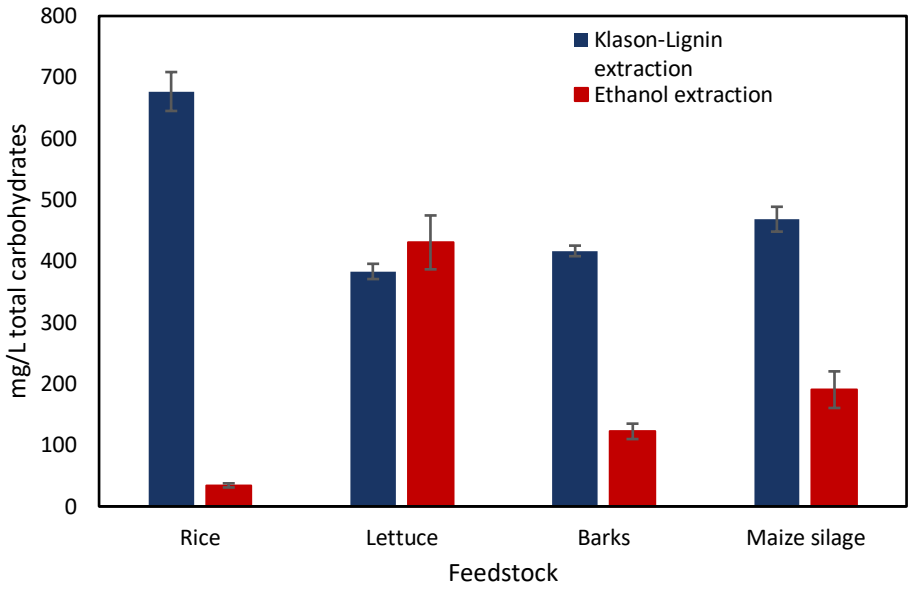


Figure 4.5 Total carbohydrates content lignocellulosic feedstock with H_2SO_4-UV method- comparison of two extraction method

4.1.3.3 Yield in sugars of the four different feedstocks

In **Table 4.6** is possible to see the yields in sugars extracted and detected from the different methods previously presented. Also, the yields from KL extracted liquor detected with HPLC are presented. Analysing the obtained numbers, it is evident that KL extraction is more efficient than EtOH extraction for all the feedstock except for lettuce. Indeed, from 1g of feedstock more sugars can be extracted following the KL extraction procedure. Obviously, a sustainability analysis should be done to see the energetic consumption for each extraction method [60]. In general, compared to the others feedstock rice present the higher yield regarding the hydrolysed sugars, that is because in rice there is a high starch content that overcome to the acid hydrolysis. Instead the lower yield can be noted regarding the solubilised sugars (EtOH method).

Regarding the HPLC results from KL liquor, the yields obtained are lower respect to the yields with H_2SO_4 UV-vis. However, the HPLC technique present more robustness towards single-species identification and quantification, while the UV-vis yields an indication regarding total content of carbohydrates. The sensitivity of the HPLC is strongly influenced by the species in the liquid phase, as well as the process parameters and the selectivity of the bed for the specific components. In the present study, column parameters, as temperature or flow rate of the eluent were not modified and monitored, nor adapted to the specific liquor under analysis but instead a standard calibration for fermentation broths was used. Hence, it is valid to state that the specific conditions of the HPLC analysis should be improved in order to meet the performance of UV-vis for carbohydrates determination.

Table 4.6 mg sugar/g total solids extracted for the four feedstocks.

mg_{sugar}/g_{TS}	RICE		LETTUCE		WOOD BARKS		MAIZE SILAGE	
	UV-vis	HPLC	UV-vis	HPLC	UV-vis	HPLC	UV-vis	HPLC
KL	813	11	460	3	501	14	563	5
EtOH	41	-	517	-	147	-	228	-

4.2 ACID FERMENTATION

4.2.1 Comparison of Test 1 and Test 2: efficiency of inoculum pre-treatment

The main goal of Test 1 and 2 is to evaluate the efficiency of a pre-treatment on the inoculum from an on-going biogas plant. The purpose of a pre-treatment is to stop methanogens activity and therefore avoid methane production to ensure a higher yield in hydrogen and volatile fatty acids production. Only the first three phase of an anaerobic digestion (hydrolysis, acidogenesis and acetogenesis) should occur. As explained in Chapter 2, the selected pre-treatments exploit the physiological difference between HPB and HCB. When these kinds of mixed consortia are exposed to stress sources, there could be high-order changes which induce microbial population shifts. Different pre-treatment can be applied, but in this study an acid pre-treatment and a thermal pre-treatment are applied and compared. The *probe parameter* for the comparison is the production of CH_4 in the systems (% CH_4 detected in the gas phase by the *ad-hoc* sensors place in the flaks). In fact, methane production is ubiquitous to methanogens, hence it can used as parameter to determine the efficiency of the aforementioned pre-treatments.

The experimental set-up is able to measure the composition of the gas phase in the flasks. Since N_2 was flushed at the beginning of the tests to bring the system into anaerobic conditions, it is the most present in the gas phase at the beginning of fermentation tests. The average CH_4 composition of the systems during fermentation for the three flasks of each pre-treatment is showed in **Figure 4.6**. First, the %DM of inoculum and substrate are showed in **Table 4.7**, together with the total %DM of the fermentation for each test. The substrate pre-treatment chosen is the alkaline pre-treatment with NaOH.

Table 4.7 %DM inoculum and substrate before and after pre-treatments. Last column indicates the total %DM in each flask.

	%DM inoculum		%DM substrate		%total DM
	Before pre-treatment	After pre-treatment	Before pre-treatment	After pre-treatment	
Test 1	2,37±0,05	3,55±0,02	46,66±1,24	13,72±1,66	3,55
Test 2	2,18±0,29	1,83±0,12	41,42±1,32	16,12±0,39	3,26

The lower %DM content after substrate pre-treatment indicates that the NaOH reacted well with the feedstock that was partially degraded. While the total %DM, below 8% in 100mL of broth indicates that the fermentation proceeded in wet fermentation (%TS<15%) conditions, hence mass-transfer phenomena should not hinder the rate of the process. Moreover, a ratio $gTS_{substrate}$: $gTS_{inculum}$ =0,5 was selected.

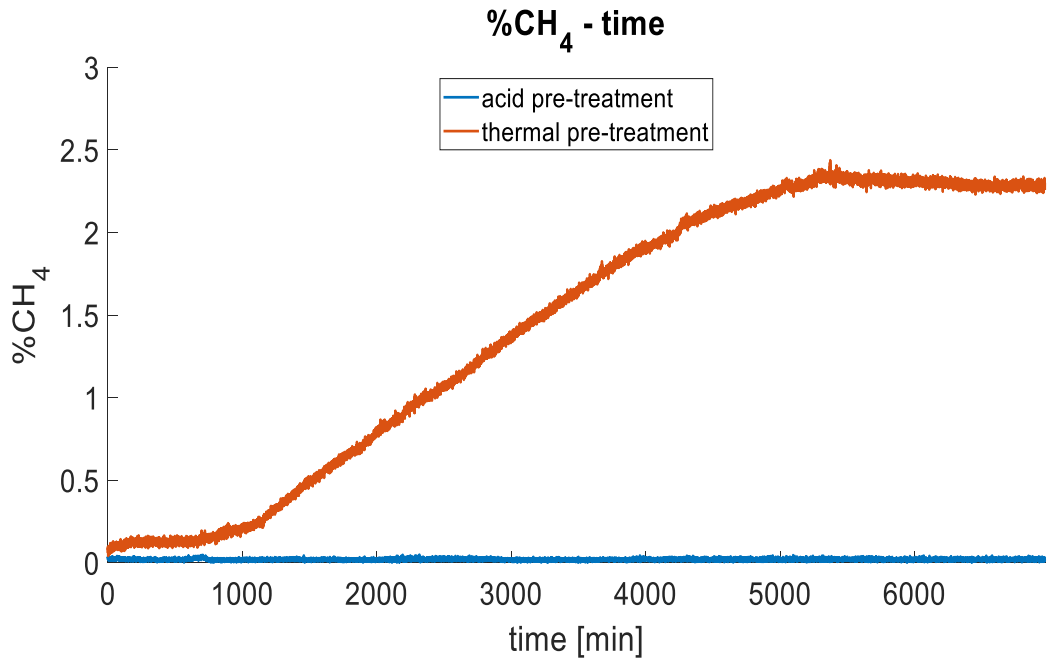


Figure 4.6 %CH4 over time detected from fermentation with the different inoculum pre-treatments

Figure 4.6 shows the fermentation trend for each test, that is the methane variation detected over time. An average between the three flasks is presented for each pre-treatment applied, since the results between the three-batch exhibited very good reproducibility. The fermentation system where the thermal pre-treatment was applied (**TEST 2**) indicates that methanogenic growth was not suppressed, since methane production can be appreciated, even at different rates. In fact, it is possible to discern between lag phase (i.e. first 16 h), an exponential phase (c. 66 h) and stationary phase at the end of tests, probably due to substrate exhaustion. Hence, the thermal pre-treatment is not suitable for long-term inhibition of methanogenic bacteria, since a share might be also thermophilic, able to resist at 70°C. Since the methanogenic inoculum was provided by an ongoing-methane plant, which tend to be very robust microbial consortia, a probable explanation is the adaptation of microorganisms to stress conditions of high temperature (i.e. fermentative and methanogens).

For the acid-treatment (**TEST 1**), the share of detected methane during the fermentation is neglectable, oscillating around zero during the whole duration of the fermentation tests. A comparison among the two systems (Test 1 and Test 2) indicates that the CH_4 threshold for the system 2 are 35 times higher than system 1, which confirmed that the acid pre-treatment is suitable to produce HPB from methanogenic sludges.

Another key parameter, which was measured during the experiments is the anisotropy of polarizability before and after pre-treatment. These measurements of anisotropy of polarizability are aimed to check the viability of the cells together with other key electro-optical parameters (Chapter 2). The results of electrooptical polarizability measurements (triplicates) before and after pre-treatments are reported in **Figure 4.7** and **4.8** together with error bars.

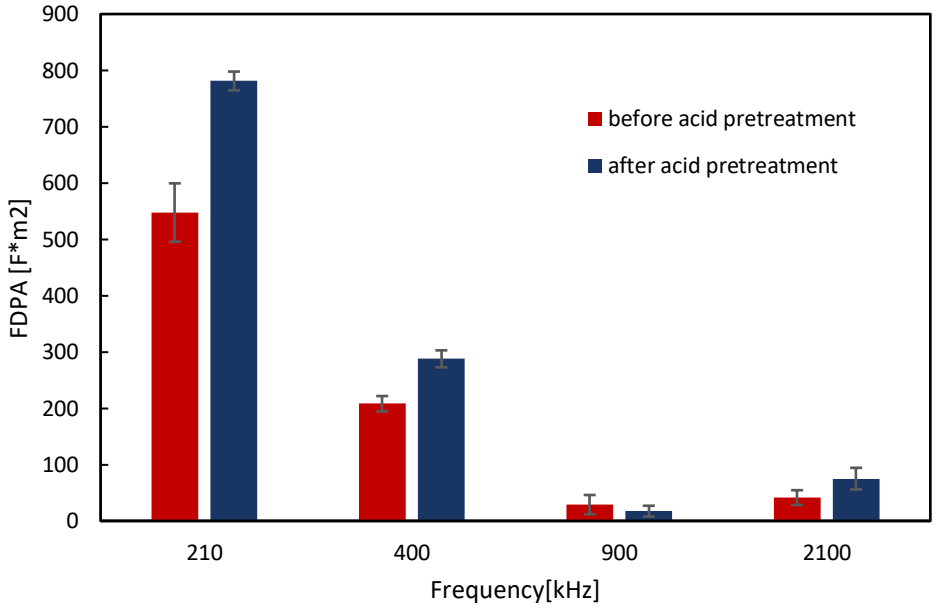


Figure 4.7 Electrooptical measurements of cell polarizability at 210, 400, 900 and 2100 kHz of inoculum before and after acid pre-treatment (Test 1)

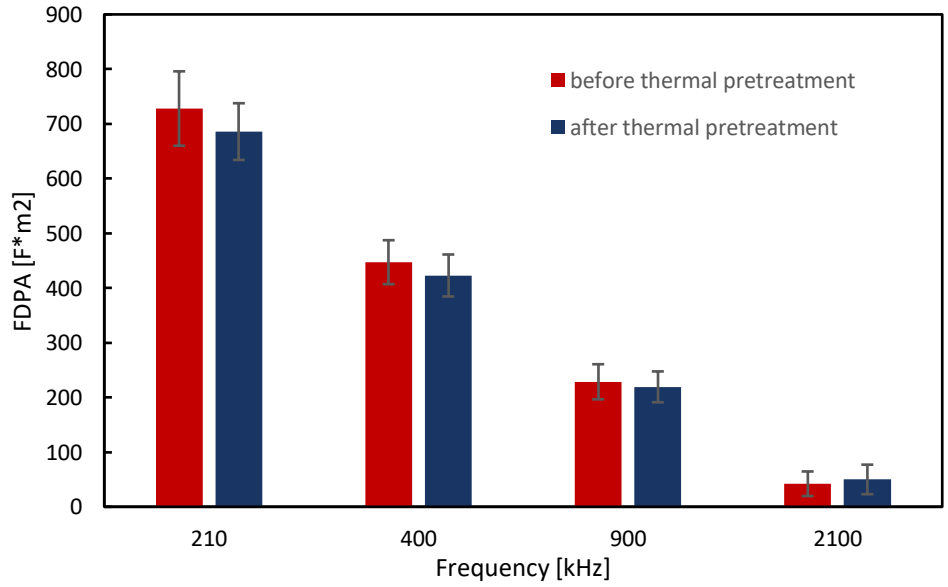


Figure 4.8 Electrooptical measurements of cell polarizability at 210, 400, 900 and 2100 kHz of inoculum before and after thermal pre-treatment (Test 2)

Regarding the thermal pre-treatment, the polarizability thresholds before and after pre-treatment are almost always the same at the four frequencies, even considering the reported uncertainties. Since no significant differences were observed, it can be said that the thermal treatment did not have a clear effect on the physiology of the mixed culture.

For the acid pre-treatment, a marked increase in polarizability can be seen after pre-treatment at the four frequencies. This raise can be due to the change in the population composition. Indeed, hydrogen producing bacteria can be seen as "more active" than hydrogen consuming bacteria, which can lead to an increase in cells viability. This fact can partially support the effectiveness of the acid-method applied to suppress methanogenic activity.

The ELOTRACE machine, besides the polarizability measurements, can also provide a qualitative measure of the cell size. After the application of the electrical field the cells are in an excitement state, when they come back to the non-excited state, the time they employ can be related to their cell size. In the case of a pure culture, the measure is more accurate, while for mixed cultures the time employed reflects rather an average relaxation time, which is biased due to different morphologies of the bacterial cells within mixed cultures. In **Table 4.8** the results relative to the cells size are shown together with the standard deviations:

Table 4.8 average size cells before and after pre-treatments

SIZE CELLS (µm)	before pre-treatment	after pre-treatment
acid pre-treatment	5,32 ± 1,02	7,88± 0,92
thermal pre-treatment	6,46 ± 0,94	6,94 ± 1,11

The results suggest that while for the thermal pre-treatment no significant difference was observed in the "cell size" (i.e. considering also the standard deviations), in the case of the acid pre-treatment an increase is reported. In general, we can hypothesize, knowing that methanogens tend to be smaller than fermentative bacteria, that when they are eliminated from the inoculum an apparent increase in the "cell size" of the culture is observed. The acid pre-treatment seems to be more efficient because the difference is prominent, while there is just a small difference visible after thermal pre-treatment.

4.2.2 How H_2 production influence metabolic pathways for VFA production (Test 3)

During anaerobic fermentation from mixed cultures different steps occurs at the same time and millions of microorganisms works synergistically to degrade. In this study the acid fermentation is conducted. This means that a pre-treatment is applied in order to suppress methanogenic activity from the inoculum and hence to prevent methane formation. This exploratory test is aimed to investigate the role of hydrogen and the correspondent carboxylic acids production, which can be influenced by different parameters. These parameters influence the metabolic pathways that are related to the bacteria growth and sustenance (section 2.4.1). Since it is a

mixed culture composed of millions of microorganisms, the metabolic pathways involved are complex and very connected to each other. Ruggeri *et al.* [11] suggest that the optimal conditions for H_2 production are T=35°C and a pH of 5,5. In this study the same temperature is used and an initial pH of 5,2 is set.

First, the %DM of inoculum and substrate are showed in **Table 4.9**, together with the total %DM of the fermentation for each test. The substrate pre-treatment chosen is the alkaline pre-treatment with NaOH. The inoculum pre-treatment chosen is the acid pre-treatment since the previous tests demonstrated its efficacy over the thermal pre-treatment.

Table 4.9 %DM inoculum and substrate before and after pre-treatments. Last column indicates the total %DM in each flask.

	%DM inoculum		**%DM substrate**		**%total DM**
	Before pre-treatment	After pre-treatment	Before pre-treatment	After pre-treatment	
Test 3	1,97±0,10	3,11±0,21	41,42±1,32	16,12±0,39	2,96

4.2.2.1 Course of fermentation and metabolic end products

The amount of H_2 synthesized during a fermentation process is related to the oxidation level of the organic end-products released. When end-products that are more reduced than acetate are formed, fewer electrons are available for release as H_2 as is deeply described in Chapter 2. Although the highest theoretical yields of H_2 are associated with the synthesis of acetate and CO_2 as the major fermentation end-products, in practice, protons and organic inter-mediates compete for electrons, leading to the formation of mixtures of end-products which are more reduced than acetate (propionate, butyrate and longer aliphatic acids, lactate, formate, alcohols and ketones), plus CO_2, thus lowering the yield of produced hydrogen [22].
The results of this fermentation are shown in **Figure 4.9**. The variation of the relative concentrations of H_2 and CO_2 along the fermentation time are presented. They are an average between the three flasks since the results were reproducible.

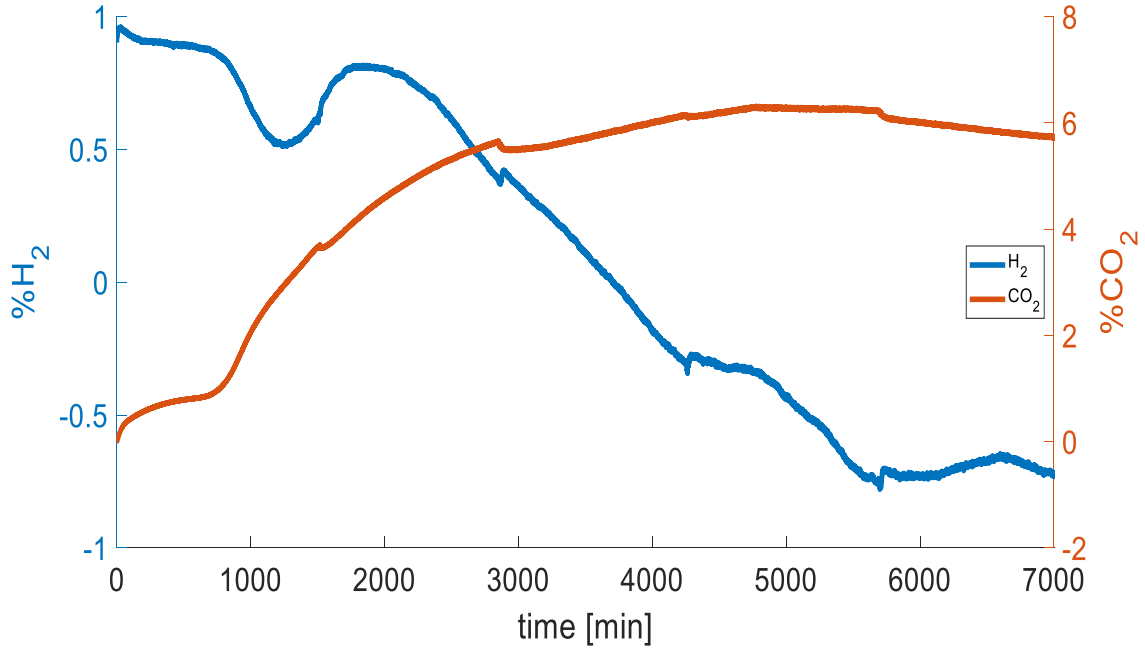


Figure 4.9 Development of acid fermentation. In blue the %H2, in red the %CO2. The grey lines divide the fermentation phases.

In the fermentation course in **Figure 4.9** can be divided into three parts depending on the $\%H_2$ detected, hence on the threshold of hydrogen partial pressure. The first zone ranging from the beginning of the measurement to during the first 24h (first two sampling point) is characterised by a higher hydrogen partial pressure, while the fraction of CO_2 also is growing during time. In this phase, acetate and pyruvate production is observed with HPLC measurements. This could indicate that hydrolysis and the first fermentation (acidogenesis) take place, degradation sugars to pyruvate couple to the production of biohydrogen and acetic acid according to the reactions:

$$C_6H_{12}O_6 + 2NAD^+ \longrightarrow 2CH_3COCOOH \text{ (pyruvate)} + 2NADH + H^+$$

$$C_6H_{12}O_6 + 2H2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

Then, the second part ranging from 24h to 3 days (third and fourth sampling point) is characterised by a decrescent trend of $\%H_2$, hence lower partial pressure, while CO_2 continue growing. During this phase, an initial production of H_2 and organic acid occurs. Pyruvate has been detected with HPLC together with a decreasing amount of acetate. Then, the influence of H_2 partial pressure is strongly visible. pH_2 is one of the most important parameters that affect the metabolic pathways of microorganisms. In this study, the H_2 was not continuously removed in the headspace, therefore it remains solubilized in the liquid phase according to the Henry's law. Apparently, the accumulation of H_2 in the liquid phase took place. Then, the culture has adapted itself to high H_2 partial pressure and the metabolic pathways seem to change. In particular, the metabolic pathways shifted to the ones that consumes hydrogen, which is reflected as a continuous decrease of H_2 detected in gas phase. Hydrogen can be consumed in two forms [61]:

- As reducing equivalents ($NADH_2$)

e.g. $NADH + H^+ \longrightarrow NAD^+ + H_2$

- As molecular H_2

e.g. $C_6H_{12}O_6 + 2\ H_2 \longrightarrow 2CH_3CH_2COOH$ (propionic acid) $+ 2\ H_2O$
e.g. $2CO_2 + 4\ H_2 \longrightarrow CH_3COOH + 2\ H_2O$ (homoacetogenesis)

In the first case most of the biochemical reactions involved consumes and produce NADH. If reactions that consume NADH happen than there is less NADH available for H_2 production. For example, the reaction that leads to ethanol formation [11]:

$Glucose + 2NADH + 2H^+ \longrightarrow CH_3CH_2OH + HCOOH + 2NAD^+$

belongs to this category, hence electrons are subtracted for hydrogen production by hydrogenase. Moreover, reactions that involved CO_2 as reagent can be placed in this category. CO_2 indeed compete with H_2 for electrons from NADH. In this study neither CO_2 is removed from the headspace therefore can be hypothesised that reactions that lead to fumaric and succinic acid production occurred [11], supported also by ethanol production observed after 48h.

While in the second case, the reactions falling in the homoacetogenesis category consumes directly molecular hydrogen, thus lowering its yield. After 72h, again acetate production occurred, and this could be related to reaction:

$2CO_2 + 4\ H_2 \longrightarrow CH_3COOH + 2\ H_2O$

Furthermore, after 72h ethanol was no longer present in the analysed liquid phase, probably due to the reverse transformation in pyruvate and then in acetate.

Indeed, since biochemical reactions that take place in microorganism are very linked together and, at some extend they are also reversible, it possible that acetyl-CoA is carbonylated again forming pyruvate.

At the end of fermentation (fifth and sixth sampling point), in the third part of the graph, a constant $\%H_2$ and $\%CO_2$ is detected.

In synthesis, the metabolic production and especially composition of VFA in the liquid phase is strongly influenced by pH_2, since according to the analysis of the liquid phase, hydrogen consuming reactions and hydrogen producing reactions took place at different stages. If the carboxylic acids of interest are acetic or butyric acid, then low hydrogen partial pressure is recommended, which can be achieve by implementing degassing systems. Considering the reactions that lead to acetic or butyric formation, removing hydrogen means that the reaction shifts to the right, further increasing the carboxylic acids yield. In anaerobic digestion, pH_2 is kept low thanks to the activity of methanogens that consume hydrogen to produce methane but in acid fermentation other strategies must be applied.

In the following **Table 4.10**, a brief summary of the different zones and relative qualitative VFAs production detected with HPLC are shown.

Table 4.10 summary of fermentation phases and metabolites detected

Phase	pH_2	Sampling point	VFAs and other metabolites detected
First zone (~24h)	high	I, II (t0, t+24)	Acetate++ Pyruvate++
Second zone (~60h)	low	III, IV (t+48, t+3d)	Pyruvate+ Ethanol++ Acetate+
Third zone	constant	V, VI (t+4d, t+5d)	Pyruvate+

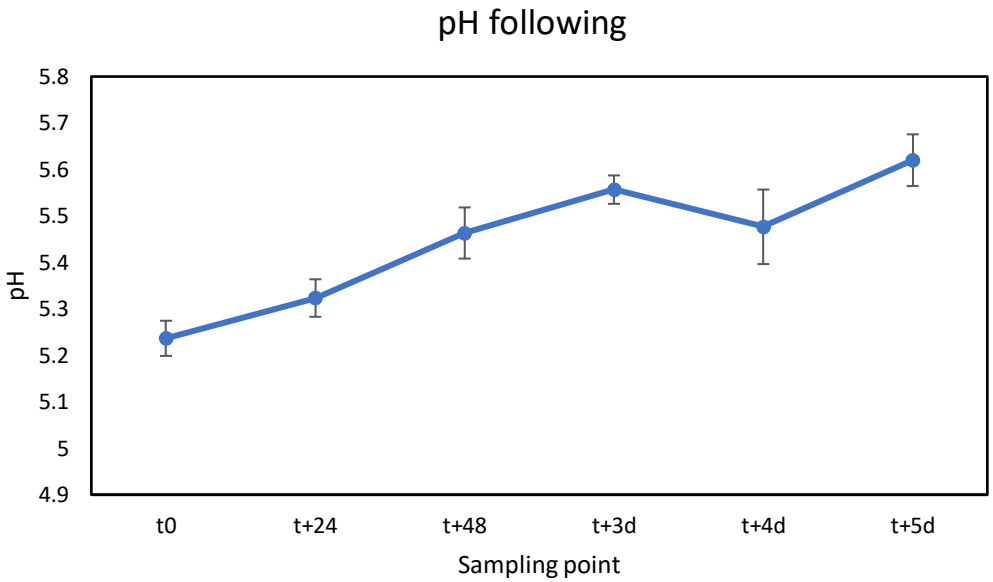


Figure 4.10 pH following acid fermentation

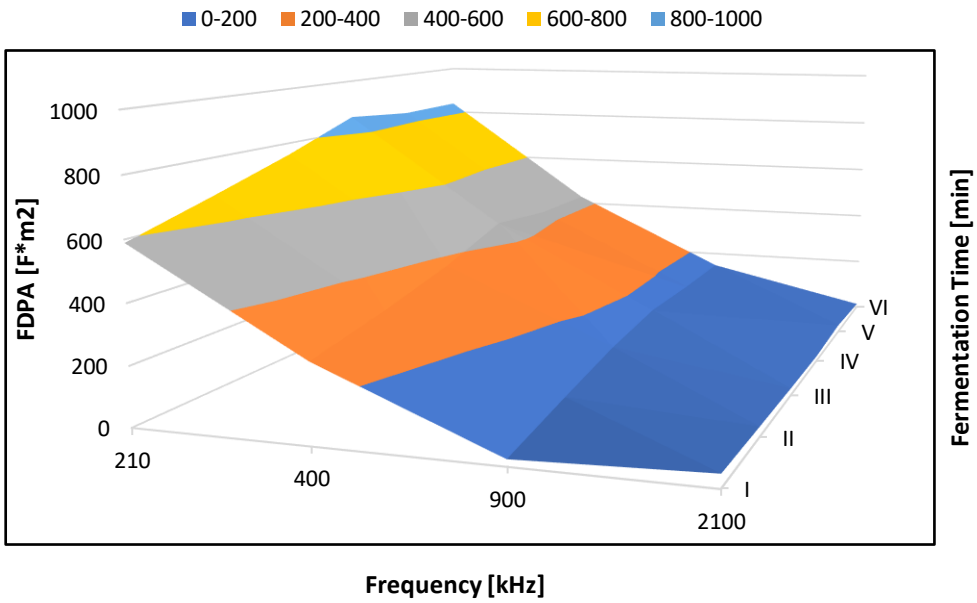
Regarding the pH following, it remained almost constant during fermentation.

4.2.2.2 Anisotropic polarizability (AP) measurements

The results of electrooptical measurements of cell polarizability throughout fermentation can be observed in **Figure 4.11**. It should be noted that AP measurements are very sensitive to the presence of ions. Typical fermentation broths exhibit high conductivities; thus, this parameter is constantly monitored to avoid osmotic stress on biomass. Especially at high frequencies, the electrical conductivity of the media could have an important effect on the measurements [35]. A washing procedure was first applied in order to decrease the conductivity in the range 2-3 μS/cm.

Theoretically the reactions that involved H_2 production tends to be thermodynamically favoured, e.g. acetic and butyric acid production. Therefore, the associated polarizability of the cells should be high. While the metabolic pathways that consumes H_2 are energetically disadvantaged, and the polarizability should be lower since the cells use their energy mainly for maintenance rather than growth and metabolite synthesis.

In a mixed culture it is more complicated and different behaviour can take place. In general, in this study also the polarizability results can be divided in the same three parts. In fact, in the first zone (sampling point I, II) the culture started producing hydrogen, and the polarizability increases. In the second zone (sampling point III, IV), characterised at the beginning by a high hydrogen partial pressure that decrease along time, the polarizability should decrease because reactions that involve hydrogen consumption take place. However, the polarizability continues increasing and that is probably because the culture has adapted to high hydrogen partial pressure shifting the metabolic pathways to the one that consume H_2 as discussed in the previous section. In the third zone (sampling point V, VI) however, there is a slightly decrease in the polarizability confirming that the fermentation is almost over, and the culture is undergoing other type of stress, starvation. Hence, the metabolism is shifted to maintain homeostasis rather than growth and synthesis of metabolites.



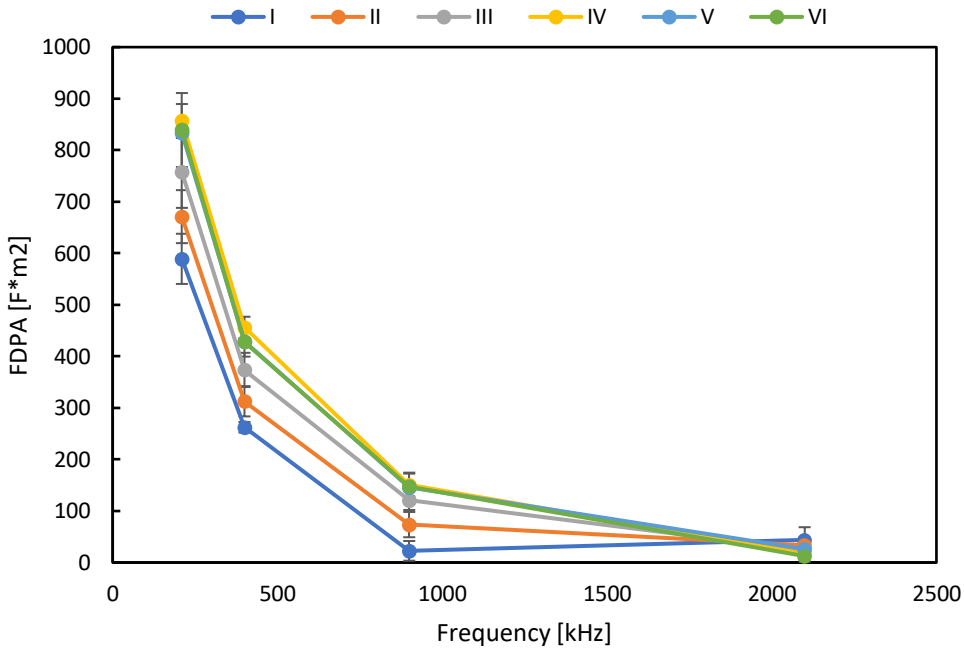


Figure 4.11 3D and 2D electrooptical measurements of cell polarizability at 210, 400, 900 and 2100 kHz of acid fermentation

5 CHAPTER 5: CONCLUSIONS AND OUTLOOKS

This thesis work can be split in two parts that have as common thread: the interest in the potential of different acidic fermented raw materials to produce valuable carboxylic acids and solvents. Obviously comparing different feedstock means thoroughly analysing how they are constituted. A proximate analysis together with the structural composition and carbohydrates content is carried out on four feedstocks: rice, lettuce, wood barks and maize silage. Different methods have been applied and compared, since each method strongly depends on the feedstock used. Furthermore, before comparing different feedstock in terms of VFAs production potentiality, an analysis of the possible parameters that could influence acid fermentation must be done.

Regarding the feedstock characterization, a proximate analysis and chemical composition analysis is done, together with carbohydrates content determination.

Lignin, cellulose and hemicellulose content is found by applying different methods. Chlorination method and NaOH hydrolysis for lignin and cellulose respectively. Furthermore, the Klason-lignin method was applied to determine lignin content and compared to the chlorination method. The results show that even if it is difficult to develop a standard procedure which can be applied to different biomass sources, the Klason lignin extraction method is more reliable with respect to the chlorination method because the results of the latter are biased by the extractives and inorganics content. Since the methodologies were initially developed to quantify the lignin content in wood-based matrices, the obtained results seem in agreement with literature values.

Carbohydrates content was determined applying two different extraction methods: Klason-lignin and ethanol extraction. The liquor from the two extraction was then used to quantify the sugars content with H_2SO_4-UV vis method. Furthermore, the UV-vis method is compared with the phenol-H_2SO_4-UV vis, that is the traditional procedure. Results show that there is a weak correlation among the analysed population, and hence the linear trend is not enough to validate the UV-sulfuric acid method for total carbohydrates content determination. Hence, further efforts should be performed to improve the process towards the reduction of the phenol method. In addition to the UV-vis method also the HPLC is used to determine sugars content starting from the Klason-lignin extracted liquor. The yields calculated confirm that UV-vis seems to be more efficient than HPLC. Lower yields can be related to the type of carbohydrates extracted and column affinity problems.

The results obtained from UV-vis quantification allow to affirm that the Klason-lignin extraction is more efficient than EtOH extraction, maybe because the structural carbohydrates are more easily hydrolysable than solubilized. Conclusions that are confirmed also by the yields of sugar extracted calculated on a dry matter basis. Obviously, all these comparisons are made on the four feedstocks used, showing that depending on the raw material each method reacts in different ways. For example, KL and EtOH gives the same results for lettuce, probably due to the less amount of carbohydrates in it.

Regarding the acid fermentation part, three tests were conducted.

The first two are made to compare acid and thermal inoculum pre-treatments. The conclusion is that the thermal pre-treatment is less efficient than the acid one because the $\%CH_4$ detected

was around zero for the acid pre-treatment, while methane production was observed in the thermal pre-treated culture after 16 h. Furthermore, the polarizability results show no clear differences in viability before and after thermal pre-treatment, while an increase is visible in the acid pre-treatment, probably due to the change in population composition.

The last test aim is to investigate the role of hydrogen and the correspondent carboxylic acid production, which can be influenced by different parameters. In this case the main parameter identified is the hydrogen partial pressure. Gas phase composition (H_2 and CO_2) is detected with on-line sensors; polarizability measurements are done at-line to monitor the viability of the broth during fermentation and volatile fatty acids production is analysed with HPLC.
The result is that the hydrogen partial pressure strongly influences the metabolic pathways of fermentative bacteria, hence the composition of VFAs in the liquid phase. The culture has adapted itself to high pH_2 and the metabolic pathways seem shifted to the ones that consumes hydrogen, which is reflected as a continuous decrease of H_2 detected in the gas phase. Also, polarizability measurements confirm that the culture has adapted to the presence of hydrogen in the liquid phase. Consuming hydrogen should lower the viability of the microorganism because the reactions involved are thermodynamically unfavoured, but in this study the polarizability is increased showing how the culture rapidly adapted to these conditions.

This work is the first step of a major project. More research could be done to improve characterization methods and to study more deeply acid fermentation. The future outlooks should include:

- Evaluation of the effective efficiency of the Klason-lignin extraction method and ethanol method in terms of energy consumed, moving forward with a sustainability analysis.
- Act on the HPLC column in term of column material, temperature profile, eluent or eluent flow to see how they could influence the detected carbohydrates.
- Improve the mixing in the acid fermentation tests to enhance mass transfer and hydrogen release in the gas phase.
- Measure the particle size distribution of the fermentation broth to check the hydrolysis of substrates.
- Carry on the same presented study on the three characterised feedstocks, comparing their behaviour and potentiality in terms of VFA production. Moreover, it is necessary to identify hydrolysis time yield after different substrate pre-treatments.

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