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SECOND GENERATION BIOETHANOL PRODUCTION FROM ORANGE PEEL WASTE (BIO/19)

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«Ci sono soltanto due possibili conclusioni: se il risultato conferma le ipotesi, allora hai appena fatto una misura; se il risultato è contrario alle ipotesi, allora hai fatto una scoperta.»

Enrico Fermi

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Extended abstract

In the EU, food industry generates a product volume of about 620 x 10^6 MT each year (Mahro & Timm, 2007) and an amount of wastes of about 175 x 10^6 MT each year (Awarenet, 2004).

These wastes pose increasing pollution problems and represent a loss of valuable biomass and nutrients. In the past they have generally been dumped (Marriott & Gravani, 2008) or used as such for animal feed or as fertilizers. Regrettably, the decline of the livestock farming in industrialized countries disfavors such end use (Mahro & Timm, 2007). In the last few years, owing to the increasing efforts to prevent environmental pollution, as well as to the economic and energetic concerns, new methods and policies have been introduced in the recovery, bioconversion, and utilization of the valuable constituents present in food processing wastes. Lignocellulosic fiber materials, in particular, represent the most abundant fraction of the biogenic wastes of food industry and may be used for a wide variety of applications (Laufenberg et al., 2003).

The present PhD thesis was aimed at assessing the feasibility of the second generation bioethanol production from a few food-processing lignocellulosic residues.

To this purpose, three residues widely produced in the Mediterranean area (orange peel waste, olive pomace and grape pomace) were chemically characterized to select the most suitable one for bioethanol production. Orange peel waste (OPW) appeared to be the most promising matrix, the total fermentable sugars amounting up to about 48% of dry matter and being associated with low amounts of lignin, phenols, and xylose.

Similarly to other lignocellulosic materials, also the structure of OPW polysaccharides is to be preliminary opened so as to make the polysaccharides more accessible to a cellulolytic enzyme pool. In this study, a novel lab-scale direct steam injection apparatus (DSIA) was used to perform an acid-catalyzed steam-explosion (ACSE) pretreatment. Based on previous literature data, four temperature-time conditions were used, that is 200 °C for 90 s; 180 °C for 150 s; 160 °C for 240 s; and 130 °C for 500 s. Acid and solid concentrations were set to 0.5% v/v and 160 g L^{-1}, respectively.

Differently from the conventional lab-scale high-pressure/high-temperature reactors, requiring a too long heating time (even longer than 60 min if the reaction temperature is higher than 175 °C) and leading to extensive degradation of free sugars, the novel lab-scale direct steam injection apparatus (DSIA) used in this study was able to pre-heat the acidic liquid-solid mixture in times of a few seconds. This allowed a stricter control of the pretreatment conditions (temperature and time) in view of their transfer to pilot- and industrial-scale plants. Moreover, its operation

minimized the amounts of raw materials necessary for testing quite accurately numerous combinations of the operating variables. The performance of the ACSE pretreatments was also compared with that of the pretreatment performed in a conventional autoclave at 130 °C for 1 h.

The four acid-catalyzed steam-explosion (ACSE) conditions tested here gave similar results in terms of sugars released and solid solubilization. Some differences were observed in the amount of inhibitors produced, even if these values resulted to be always below the tolerance threshold of the yeast strain used. The most significant difference was that the ACSE conditions at 200 °C for 90 s and 180 °C for 150 s led to a pectin solubilization of about 73%, this value being quite higher than that obtained either in the other ACSE conditions or in the autoclave, with a positive effect on the subsequent enzymatic hydrolysis step.

Some preliminary enzymatic hydrolysis experiments for cellulose depolymerization were carried out in the shaken-flask scale by suspending non-pretreated or autoclave-pretreated (under neutral or mildly acidic conditions) OPW in the liquid (*i.e.*, sodium acetate buffer) recommended for the commercial cellulase preparation used in this study. In this way, it was possible to assess the need of an acidic pretreatment to improve depolymerization, given the discordance in the literature reports, as well as to identify the best cellulase dosage and solid loading. A mild acidic pretreatment was appropriate for the subsequent depolymerization, whereas an enzyme dose of 6 FPU cellulase g^{-1} cellulose represented the best compromise between the needs of yield maximization and cost minimization in view of a scaling-up of this process. The glucose yield was the highest at a solid loading of 5 or 10% (w/v), but tended to decrease slightly when the latter was increased up to 20%. Anyway, at the highest solid concentration, glucose concentration in the liquor after saccharification was also the highest one, this being advantageous for the ultimate ethanol recovery by distillation.

Such optimal values of enzyme (6 FPU cellulase g^{-1} cellulose) and solid (20% w/v) loadings were successfully used to saccharify the ACSE-pretreated OPW residual solids. Actually, the enzymatic hydrolysis performed by suspending the OPW residual solids after ACSE-pretreatment at 200 °C for 90 s in sodium acetate buffer succeeded in releasing 59% of theoretical glucose, this yield being quite higher than that (40%) achieved starting with the OPW residual solids pretreated in the autoclave at 130 °C for 1 h. A similar yield (59%) was also obtained when the former ACSE-pretreated OPW residual solids were re-suspended in their own acidic liquor, so as to utilize even the free-sugar fraction and achieve a higher sugar concentration in view of its conversion into ethanol. Thus, this condition was used for the depolymerization of the remaining ACSE-pretreated solids.

Enzymatic hydrolysis of OPW deriving from pretreatment at 180 °C-150 s and 200 °C-90 s gave the highest glucose yields of about 57%, their difference being statistically insignificant, probably as the result of the highest percentage of pectin solubilization achieved after these pretreatments. Nevertheless, the depolymerization of solids pretreated at 180 °C was faster than that of solids pretreated at 200 °C, this suggesting that the lower temperature-longer time pretreatment led to a more profound destruction of the lignocellulosic crosslinks.

Fermentability of hydrolyzates resulting from ACSE pretreatments and enzymatic hydrolysis was evaluated in the shaken-flask scale under repeated-batch conditions using the industrial strain *Saccharomyces cerevisiae* F15. All the hydrolyzates were efficiently fermented and the highest ethanol yield and productivity were 49.48% (the theoretical value being about 51%) and 4.85 g L^{-1} h^{-1}, respectively. However, no direct correlation between pretreatment conditions and fermentative performances was assessed.

To scale-up the previous results, a new ACSE trial at 180 °C for 90 s was performed by tripling the solid loading, and then submitting the resulting slurry to enzymatic hydrolysis in a 7-L stirred bioreactor, while the repeated batch fermentation was carried out in a 1-L bioreactor.

ACSE pretreatment at 180 °C for 90 s and solid loading of 480 g L^{-1} gave a lower percentage of released glucose and fructose and a higher percentage of inhibitors, in comparison with the same pretreatment performed at a solid loading of 160 g L^{-1}. The lower efficacy of pretreatment at a triple solid loading was counterbalanced by the subsequent enzymatic hydrolysis performed in a 7-L STR reactor. Since most of the pectin was still present in the solid, in this saccharification trial the cellulase preparation was integrated with a commercial pectinase one. In this way, 69.5% release of the theoretical glucose and fructose was obtained, this yield being higher than that (67.8%) obtained in the shaken-flask scale when using the same ACSE-pretreated solids at a density of 160 g L^{-1}.

By transferring the fermentative process from shaken-flasks to a 1-L stirred bioreactor, a slight decrease in ethanol productivity (from 4.85 to 4 g L^{-1} h^{-1}) was observed, probably because of the higher concentration of inhibitors. Differently from productivity, maximum ethanol yield did not differ, being always close to the theoretical value.

Considering the total amount of fermentable sugars obtained through the optimized ACSE pretreatment and the subsequent enzymatic hydrolysis, the overall ethanol yield of the repeated batch process from OPW in 1-L bioreactor was 41.5%.

In conclusion, on the basis of a mass balance including all the glucose and fructose (11.13% dm) released after pretreatment at 180 °C for 150 s with a solid loading of 480 g L^{-1} and the glucose freed after enzymatic hydrolysis (17.86% dm), and accounting for an overall ethanol yield of

41.5%, the overall process yield at 1-L STR reactor scale would amount to about 153 L bioethanol per metric ton dry OPW.

Riassunto

All'interno dell'Unione Europea, l'industria alimentare genera ogni anno una produzione di circa 620 milioni di tonnellate (Mahro & Timm, 2007). Di questa attività gli scarti raggiungono i 175 milioni di tonnellate (Awarenet, 2004).

Questi rifiuti costituiscono un problema di inquinamento sempre crescente e si configurano come una perdita considerevole di biomassa e sostanze nutritive. In passato la soluzione più utilizzata è stata il conferimento in discarica (Marriott & Gravani, 2008); l'alternativa era l'uso di questi rifiuti come fertilizzanti o come mangimi animali. Tuttavia, questo utilizzo è limitato dal declino dell'allevamento nei paesi industrializzati (Mahro & Timm, 2007). Negli ultimi anni, grazie agli sforzi sempre maggiori per prevenire l'inquinamento ambientale e alle crescenti preoccupazioni economiche ed energetiche, sono stati introdotti nuovi metodi per il recupero, la bio-conversione e l'utilizzo delle componenti valorizzabili degli scarti dell'industria alimentare. In particolare, i materiali fibrosi lignocellulosici rappresentano la più importante frazione dei rifiuti del ciclo industriale alimentare e potrebbero essere utilizzati in un'ampia varietà di applicazioni (Laufenberg et al., 2003).

La presente tesi di Dottorato ha come obbiettivo quello di valutare la possibilità di produrre bioetanolo di seconda generazione a partire da residui lignocellulosici dei processi industriali agro-alimentari.

A tale scopo, per trovare il substrato più adatto alla produzione di bioetanolo, sono stati caratterizzati chimicamente tre rifiuti largamente prodotti nell'area mediterranea: il pastazzo di arance, la sansa di olive e le vinacce. Dagli esperimenti effettuati, il pastazzo di arance (OPW) è risultato essere il più promettente tra questi. Infatti in questa matrice il totale degli zuccheri fermentabili ammontava a circa il 48% della materia secca, con basse percentuali di lignina, fenoli e xilosio.

Come per gli altri materiali lignocellulosici, anche l'OPW richiede un pretrattamento per rendere più accessibile la struttura dei polisaccaridi alle cellulasi.

Durante questo studio è stato utilizzato un prototipo a iniezione di vapore su scala di laboratorio (DSIA), per effettuare un pretrattamento di steam-explosion in ambiente acido (ACSE). Sulla base dei dati bibliografici disponibili, sono state applicate quattro differenti condizioni di temperatura-tempo: 200 °C per 90 s; 180 °C per 150 s; 160 °C per 240 s; 130 °C per 500 s. La concentrazione di acido ed il carico di solido sono state fissate a 0.5% v/v e 160 g L^{-1}.

I normali reattori su scala di laboratorio che utilizzano elevate temperature/pressioni, richiedono un lungo tempo di riscaldamento (che può superare i 60 min nel caso di temperature superiori ai

175 °C). Questo fattore porta ad una estensiva degradazione degli zuccheri. Il nuovo apparato a iniezione di vapore (DSIA) ha bisogno al contrario di pochissimi secondi per raggiungere la temperatura richiesta e ciò permette un controllo serrato delle condizioni di pretrattamento (temperatura e tempo), necessario in un processo su scala industriale. Inoltre, comparato a un impianto industriale, il nostro sistema richiede una minore quantità di materiale per ogni esperimento: questo permette di testare un maggior numero di combinazioni delle diverse variabili.

I pretrattamenti di steam-explosion sono stati comparati a pretrattamenti eseguiti in un'autoclave convenzionale a 130 °C per 1 h.

Le quattro condizioni di steam-explosion testate in questo studio hanno dato risultati simili in termini di zuccheri rilasciati e di solubilizzazione del solido. Alcune differenze sono state osservate nella quantità di inibitori prodotti, sebbene questi valori siano comunque al di sotto della soglia di tolleranza del lievito *Saccharomyces cerevisiae*. L'osservazione più importante è stata effettuata durante gli esperimenti alle temperature più alte (200 °C-90 s e 180 °C-150 s), quando si è raggiunta la più alta solubilizzazione della pectina (73.11% della quantità iniziale), con effetti positivi nella successiva fase di idrolisi enzimatica.

Alcuni esperimenti preliminari di idrolisi enzimatica per depolimerizzare la cellulosa sono stati eseguiti in beuta agitata, in tampone sodio acetato (che era la fase liquida consigliata per la cellulasi commerciale utilizzata in questo studio), su OPW non pretrattato e pretrattato in autoclave (in condizioni neutre o blandamente acide). Questa procedura è stata utilizzata per verificare l'utilità di un pretrattamento acido per migliorare la depolimerizzazione (considerata la discordanza presente in letteratura) e allo stesso tempo per definire il miglior carico di cellulasi e di solido. Gli esperimenti hanno evidenziato che le condizioni migliori di pretrattamento per la successiva depolimerizzazione erano quelle blandamente acide. Inoltre 6 FPU di cellullasi g^{-1} di cellulosa si configura come il miglior compromesso tra le necessità di massimizzazione della resa e di abbattimento dei costi in vista di uno scaling-up del processo. Per quanto riguarda il carico di solido, la resa più alta in glucosio è stata ottenuta al 5 e al 10 % (p/v), con un piccolo decremento dal 10 al 20%. In ogni caso, la più alta concentrazione di solido utilizzata (20%) ha dato la più alta concentrazione di glucosio. Questo rappresenta un importante vantaggio, dal momento che nella fase fermentativa potrà essere ottenuta un'alta concentrazione di etanolo, cosa che faciliterebbe la distillazione finale.

Il carico di enzima ottimizzato durante gli esperimenti preliminari (6 FPU di cellullasi g^{-1} di cellulosa), è stato adoperato anche nei campioni di OPW pretrattati con steam-explosion, utilizzando il carico di solido (20% p/v) che rendeva massima la concentrazione di glucosio nel

liquido ottenuto. L'idrolisi enzimatica – eseguita su tampone sodio acetato – su OPW pretrattato con steam-explosion a 200 °C per 90 s, ha dato una resa in glucosio (59% dello zucchero potenzialmente rilasciabile) molto più alta se paragonata all'idrolisi realizzata su OPW pretrattato in autoclave in condizioni blandamente acide (40%). Un risultato simile è stato ottenuto quando l'OPW, pretrattato a 200 °C per 90 s, è stato risospeso nel liquido acido di pretrattamento anziché nel tampone sodio acetato, con il vantaggio che, nel secondo caso, gli zuccheri rilasciati dopo il pretrattamento di steam-explosion sono stati recuperati. In base a questi dati, questa condizione è stata utilizzata per la depolimerizzazione dei rimanenti solidi pretrattati con steam-explosion.

L'idrolisi enzimatica dell'OPW pretrattato a 180 °C-150 s e a 200 °C-90 s, ha dato le più alte rese di glucosio, raggiungendo un valore medio pari a circa il 57%, senza differenze statisticamente significative tra i due dati. Questo risultato potrebbe essere spiegato con la maggiore percentuale di solubilizzazione della pectina che era stata ottenuta dopo questi pretrattamenti, rispetto a quelli eseguiti a temperature più basse. Tuttavia, il solido pretrattato a 180 °C è stato depolimerizzato più velocemente di quello pretrattato a 200 °C: ciò suggerisce che una minore temperatura ed un più breve tempo di pretrattamento portano ad una più profonda distruzione dei legami incrociati della lignocellulosa.

La fermentabilità degli idrolizzati ottenuti dai pretrattamenti di steam-explosion seguiti da idrolisi enzimatica è stata valutata in beuta agitata in condizioni di batch ripetuto usando il ceppo industriale *Saccharomyces cerevisiae* F15. Tutti gli idrolizzati sono stati efficacemente fermentati: la resa più alta di etanolo è stata del 49.48% (su un valore teorico del 51%) e di 4.85 g L^{-1} h^{-1}. Tuttavia, non è stata individuata alcuna correlazione diretta tra le condizioni di pretrattamento e le performance di fermentazione.

Per uno scale up del processo, è stato eseguito un nuovo pretrattamento di steam-explosion a 180 °C per 90 s, triplicando il carico del solido. Il pretrattato è stato sottoposto ad un'idrolisi enzimatica in un bioreattore da 7 L, mentre la fermentazione in bacht ripetuto è stata effettuata in un bioreattore da 1 L.

Il pretrattamento con steam-explosion a 180 °C per 90 s di un carico di solido di 480 g L^{-1}, ha rilasciato una minore percentuale di glucosio e fruttosio ed una maggiore percentuale di inibitori, rispetto allo stesso pretrattamento effettuato con un carico di solido di 160 g L^{-1}. La minore efficacia del pretrattamento a tripla concentrazione è stata controbilanciata dalla successiva idrolisi enzimatica eseguita in un reattore STR da 7 L. Poiché l'analisi del liquido di pretrattamento dimostrava che la maggior parte della pectina era ancora presente nel solido, in questo esperimento alla cellulasi è stata abbinata una pectinasi commerciale. Alla fine del

pretrattamento di steam-explosion a tripla concentrazione e della successiva idrolisi enzimatica, è stato ottenuto il 69.52% del glucosio e del fruttosio potenzialmente rilasciabili. Questo valore risulta essere più alto di quello ottenuto con pretrattamento con steam-explosion a 160 g L^{-1}, seguita da un'idrolisi enzimatica in beuta agitata (67.8%).

Il trasferimento del processo fermentativo dalla beuta agitata ad un reattore STR da 1 L ha portato ad una leggera diminuzione della produttività di etanolo (da 4.85 a 4 g L^{-1} h^{-1}). Questo risultato può essere ascritto all'aumento degli inibitori prodotti dopo il pretrattamento con steam-explosion a triplo carico di solido, comparato a quello a 160 g L^{-1}. A differenza della produttività, la massima resa in etanolo non è cambiata con l'aumento di scala, rimanendo vicina alla resa teorica della fermentazione alcolica. Considerando la quantità totale di zuccheri fermentabili ottenuti attraverso il pretrattamento con steam-explosion ottimizzato e la successiva idrolisi enzimatica, la resa complessiva di etanolo da OPW in un bioreattore da 1 L è stata del 41.52%.

In conclusione, sulla base di un bilancio di massa che include tutto il glucosio ed il fruttosio (11.13% rispetto al peso secco di partenza) rilasciati dopo pretrattamento a 180 °C per 150 s con un carico solido di 480 g L^{-1}, e il glucosio liberato dopo l'idrolisi enzimatica (17.86% rispetto al peso secco di partenza), e considerando una resa complessiva di etanolo del 41.52%, la resa totale del processo in un reattore STR da 1 L ammonterebbe a circa 153 L di bioetanolo per tonnellata di pastazzo di arance secco.

1. INTRODUCTION

In the EU, food industry generates a product volume of about 620 x 10^6 metric tons (MT) each year. Since in most cases only parts of the incoming biomass are actually used for the generation of a particular food product, unavoidable biogenic residues may occur in large quantities during food processing ("product-specific residues"). Although the food industry is a well established industry, it is rather difficult to obtain reliable data on the amounts of waste generated during the various food processing stages. This is due to the fact that either similar waste or by-product fractions are often named and categorized differently or that the production data (incl. waste amounts) are kept confidential (Mahro & Timm, 2007).

The most prominent and detailed analysis of the amount of residues generated in the food processing industry was carried out recently (2001–2004) in the joint European research project Awarenet, in which data from 19 selected different food production processes, including the five main types of food (fish, meat, dairy products, wine and food from vegetables), were analyzed. The analysis is referred to the 15 Eurostat countries and reports an amount of wastes of about 175 x 10^6 MT each year (Awarenet, 2004).

These wastes pose increasing pollution problems and represent a loss of valuable biomass and nutrients. In the past they have often been dumped. Nowadays, this is no more sustainable since the high amounts of carbohydrates, proteins, fats and mineral salts present in these residues cause the formation of bad smells and water browning. For these reasons, a biological stabilization treatment is required prior to their dumping in the environment (Marriott & Gravani, 2008).

Moreover, Council Directive 1999/31/EC on landfill waste compels member states to reduce biodegradable organic waste in landfills by 65% (as compared to 1995 levels) by no later than 2016 (European Union, 1999).

The most common alternative to their disposal is to use them as such for animal feed or as fertilizers. In particular, the residues generated during starch production, like corn germ meal or corn gluten meal, can be easily sold as an animal feed or feed supplement owing to their nutritional value (Owen & Jajasurya, 1989). Regrettably, the decline of the livestock farming in industrialized countries disfavors such end use (Mahro & Timm, 2007).

In the last few years, owing to the increasing efforts to prevent environmental pollution, as well as to the economic and energetic concerns, new methods and policies have been introduced in the recovery, bioconversion, and utilization of the valuable constituents present in food processing wastes (Laufenberg et al., 2003).

Lignocellulosic fiber materials, in particular, represent the most abundant fraction of the biogenic wastes of food industry and may be used for a wide variety of applications.

The present PhD thesis was aimed at improving the process of second generation bioethanol production from food-processing lignocellulosic residues.

Lignocellulosic residues are composed of carbohydrate polymers (mainly cellulose and hemicelluloses), lignin, and a remaining, smaller part, of proteins, acids etc. The majority of the carbohydrates can be hydrolyzed to single sugars and fermented to ethanol. However, in the case of lignocellulose, a particular crosslinking between polysaccharides and lignin creates a barrier to the production and recovery of valuable materials. Thus, the technology used to produce ethanol from this kind of residues is different from the one employed in the conventional starch-to-ethanol industry. An initial pretreatment stage is required to soften the material and break down the lignocellulosic structure in order to make it more susceptible to an enzymatic attack before fermentation (Mtui, 2009). All the pretreatment technologies are usually carried out under severe reaction conditions with a large capital investment, high processing costs and great investment risks (Champagne, 2008; Wheals et al., 1999).

During the high-temperature pretreatment process, degradation compounds are formed from pentoses and hexoses. These components are toxic and can inhibit the succeeding enzymatic and fermentative processes. Therefore, they should be removed or neutralized prior the fermentation. As an alternative, a fermenting organism with high inhibitor tolerance should be used (Palmqvist & Hahn-Hägerdal, 2000a).

After the pretreatment, a consortium of enzymes (endoglucanase, exoglucanase and β-glucosidase) is needed to break down the carbohydrate polymers. Enzymatic hydrolysis can be improved through the optimization of substrate concentration and enzyme dosing (Champagne, 2008).

As for the fermentation, the most frequently used microorganism in industrial processes is *Saccharomyces cerevisiae*, which has proved to be very robust and well suited to the fermentation of hexose sugars from lignocellulosic hydrolyzates (Hahn-Hägerdal et al., 2006).

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. Thus, it is very important to define the optimum conditions to achieve sufficient profitability.

2. STATE OF THE ART

2.1 Valorization of food industry residues

The amount of wastes produced from food-processing industries is continuously increasing and poses serious environmental problems. For this reason many research groups are investigating the opportunities for the valorization of such residues.

The different upgrading options for all the food-processing residues are subdivided into three main groups, as briefly described hereafter. In section 2.2 the attention will be focused on a particular category of food-processing residues, that is the lignocellulosic fiber materials: their potential uses will be described in a more detailed manner.

2.1.1 Separation or extraction of chemical products

Several substances may be separated or extracted from such residues, the most interesting being some special high price compounds, such as polyphenols from pomace to be used as antioxidants. Practically, a huge number of special products (e.g., detergents, gelling agents, cosmetic additives, etc.) can be extracted from all fruit, vegetable, meat or fish processing residues (Healy et al., 1994; Schrieber & Seybold, 1993; Louli et al., 2004; Rezzoug & Louka, 2009). Table 2.1 lists some major compounds that may be recovered from the diverse food processing residues.

Table 2.1 *Chemical products recovered from food processing residues.*

Process residues from	Chemical compounds
fish and crustaceans processing	collagen, gelatin, chitin, fish oil, polyunsaturated fatty acids
meat processing	collagen, gelatin, animal fat, blood plasma
milk and cheese production	Lactose
wine, coffee and cocoa production	natural pigments, tartaric acid, antioxidants, caffeine, tannins, pectin
fruits and vegetables processing	pectin, antioxidants, essential oils, vitamins, lecithin

New additional product lines might be discovered in the future also by applying a more systematic extraction screening for chemical compounds. By applying such a screening approach to cocoa bean shells (a residual waste of chocolate production, generally discarded after thermal treatment), it was possible to extract 4–5 useful products, e.g., polyphenols, a glue, pectins, a pigment, and a flavor (Timm et al., 2006).

2.1.2 Feedstocks for biotechnological processes

Alternatively, food-processing residues can be used as fermentation media in biotechnological processes. Corn-steep liquor (deriving from corn after the extraction of starch and oil), molasses (deriving from beet- and sugar-cane after sucrose extraction), and whey (byproduct of cheese production) have been largely used in biotechnology (Gonzales Siso, 1996; El-Abyad, 1992; Lee et al., 2000). Anyway, their use asks for a careful analysis of their composition since both the selected microorganism and process mostly require a very specific nutrient composition (C/N/P balance), including minor components like vitamins. Furthermore, some additional criteria must be met by biomass resources to be used as a chemical feedstock. In particular:

- availability and quality of the resource must be reliable;
- chemical composition must be suited for transformation in marketable products;
- price of the raw material, its transport and processing must be competitive (Atkinson & Mavituna, 1991).

2.1.3 Bioenergy source

Food processing residues can also be used for producing energy. However, the comparatively high water content of most of them often prohibits their direct use for thermal energy production. To exploit their energetic potential more efficiently, it is necessary to reduce their initial water content by either drying or pressing. Another option is to convert the moist or liquid biowaste fraction directly by methanization or fermentation, thereby generating energy carriers, like biogas, ethanol or hydrogen (El-Refai et al., 1992; Amartey & Leung, 2000).

The situation is different in the case of solid biomass, that can be converted into more useful forms of energy by a number of different technologies. These conversion technologies can be subdivided into two basic categories, such as thermochemical and biochemical processes. Combustion, carbonization/pyrolysis, gasification and liquefaction are considered as thermo-chemical conversion processes, while biochemical conversion processes include two process options, that is digestion and fermentation. When the thermochemical processes are applied, liquid products (wood tar, tar, oil, and pyrolytic oil), gas products (wood gas, pyrolytic gas, and hydrogen), and solid products (char, and charcoal) are produced, while the major products of biological conversion processes are biogas, biohydrogen and ethanol (Klass, 1998).

2.2 Food-processing lignocellulosic wastes: a unique source

Lignocellulosic fiber materials represent the most abundant fraction of the biogenic wastes of food industry and may be used for a wide variety of applications. This section reports the most interesting options for their valorization.

2.2.1 Bioadsorption

Adsorption is a well-known equilibrium separation process and an effective method for water decontamination. It is generally superior to other techniques for water reuse in terms of initial cost, flexibility and simplicity of design, ease of operation, and insensitivity to toxic pollutants. Moreover, no harmful substances are formed (Aksu & Jener, 2001). Several studies dealt with the application of various food processing lignocellulosic residues to this purpose.

Achak et al. (2009) studied the use of banana peel as a biosorbent for removing phenolic compounds from olive mill wastewaters, by investigating the effect of adsorbent dosage, pH and contact time. By increasing the banana peel dosage from 10 to 30 g L^{-1}, the phenolic compounds adsorption rate significantly increased by 60 to 88%. Increase in the pH to above neutrality resulted in the increase in the phenolic compounds adsorption capacity. Such a matrix was applied to remove industrial dyes (Annadurai et al., 2002). An alkaline pH favored the adsorption of dyes, and intraparticle diffusion of dyes within the particle was identified as the rate limiting step. Memon et al. (2009) described the use of banana peel for the removal of Cr(VI) from industrial wastewater by varying several parameters, such as pH, contact time, initial metal ion concentration, and temperature. The binding of metal ions was found to be pH-dependent with the optimal sorption occurring at pH 2.

Chestnut shells were used to remove selected pesticides, such as carbofuran (CF) and methyl parathion (MP) from aqueous solutions (Zuhra Memon et al., 2008), by studying the effect of a few sorption parameters, such as contact time, pH, temperature, and initial pesticide concentration. Maximum percent. sorption (99±1%) was achieved when treating solutions containing $(0.38\text{-}3.80)\times10^{-4}$ and $(0.45\text{-}4.5)\times10^{-4}$ mol $^{dm\text{-}3}$ of MP and CF pesticides, respectively, with 4 g L^{-1} of sorbent at pH 6 for 30 min.

Some food-processing lignocellulosic residues, such as potato peels, coconut shells and hazelnut shells, can also be converted into charcoal or activated carbon and used as biosorbent. Potato peels charcoal (PPC) was tested as an adsorbent of Cu(II) from aqueous solutions by studying the effects of various parameters, such as temperature, pH and solid/liquid ratio. One gram of

PPC allowed 99.8% removal of copper(II) from 100 ml of an aqueous solution at pH 6.0 containing 150 mg l^{-1} of copper (II) under shaking for 20 min (Aman et al., 2008).

Gimba et al. (2009) investigated the adsorption of indigo blue (an industrial dye), lead, cadmium and mercury by activated carbons of different particle size obtained from coconut shells using $FeCl_3$, $ZnCl_2$, $CaCl_2$ and K_2CO_3 at varying concentrations as activating salts. The 250-μm particle size was the most effective one in adsorbing indigo blue up to 98% using $ZnCl_2$-activated carbon, 70% of Pb using $FeCl_3$-activated carbon, and 81 or 83% of Cd or Hg using K_2CO_3-activated carbon.

Impregnated activated-carbon prepared from hazelnut shells using sulfur-containing compounds were tested to adsorb mercuric ions from aqueous solutions (Khalkhali & Omidvari, 2005).

Milenkovic et al. (2009) observed that the removal of Cu(II) ions from aqueous solutions using a granular activated-carbon obtained from hazelnut shells was higher in the presence of ultrasound than in its absence, but ultrasound reduced the kinetic adsorption rate constant.

Since the production of activated carbon represents an additional cost, Ferrero (2007) attempted to remove Methylene Blue and Acid Blue 25 using ground hazelnut shells as such. The adsorption capacity of hazelnut shells toward Methylene Blue was even higher than that reported for activated carbon obtained from the same raw material.

Dye removal was also performed using untreated olive pomace under different process variables, such as initial pH, biosorbent dosage, contact time, temperature, and ionic strength. The highest dye biosorption capacity observed at pH 2, the time needed to reach the biosorption equilibrium being 40 min with a biosorbent concentration of 3.0 g L^{-1} (Akar et al., 2009).

The potential of orange peel to remove lead (II) ions from an aqueous solution was investigated by varying the pH, contact time, amount of sorbent and ions concentration. The sorption capacity of orange peel was comparable to that of other available sorbents (Alzaydien, 2009).

Mercapto-acetic acid-modified orange peel was used to adsorb Cu^{2+} and Cd^{2+} ions, the corresponding maximum adsorption capacities being 70.67 and 136.05 mg g^{-1}, respectively. Moreover, the biosorbent was used for more than five cycles (Liang et al., 2009).

2.2.2 Pectin extraction

Pectins are complex mixtures of polysaccharides containing units of galacturonic acid as the main chain. These molecules have been isolated and extensively studied from various plant tissues, and are widely used in the pharmaceutical, cosmetic and food industries. Commercial

pectins are mostly available from two main sources: apple pomace and citrus peels (Voragen et al., 1995).

Several waste products might be used as unconventional pectin sources.

Emaga et al. (2008) applied a 2^n factorial design to study the influence of pH (1.5 and 2.0), temperature (80 and 90 °C) and time (1 and 4 h) on the extraction yield of pectin from banana peels. The factor pH was the most important parameter influencing pectin yield and chemical composition. Lower pH values negatively affected the galacturonic acid content of pectin, but increased the pectin yield.

The revenue for the pectic extraction from olive pomace by a continuous biphasic extraction system was studied by assessing the gelation potential of extracted pectin via rheological methods. It was inferred that olive pomace might be a potential source of gelling pectic materials, with properties quite similar to those of commercial citrus pectin (Cardoso et al., 2003).

2.2.3 Enzymes production

The main limitation for the extensive industrial application of microbial enzymes is their high cost. Therefore, a good strategy to increase the productivity of the fermentation processes would be the optimization of the culture conditions. Since the nutritive substances employed in the culture medium represent a high percentage of the total production costs, the reduction in the substrate expenses would increase the productivity of the process (Gomez et al., 2005). The composition of food-processing lignocellulosic wastes is usually rich in sugars which, due to their organic nature, are easily assimilated by microorganisms. This makes them very suitable as raw materials for the microbial production of secondary metabolites of industrial significance, such as enzymes (Rodrìguez Couto et al., 2002).

Among the processes used for enzyme production, solid state fermentation (SSF), defined as ‘‘the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water’’ (Perez-Guerra et al., 2003), is an attractive one because it presents higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing with respect to submerged fermentation (SmF) (Pandey et al., 2000a). Some examples of this process applied to food processing lignocellulosic residues are reported below.

Sugar-cane bagasse has been extensively studied for the production of cellulases, laccases and xylanases. Roussos et al. (1992) used a mixture of bagasse and wheat bran (4:1) for the

production of cellulases. They suggested hydraulic pressing as a good technique to leach out the enzymes from the fermented matter. Modi et al. (1994) reported higher yields of cellulase from a strain of *Streptomyces* sp. HM29 when grown on bagasse instead of rice straw, rye straw or corncobs. An extensive study was carried out by Pal et al. (1995) on SSF of sugarcane bagasse using a strain of mushroom fungus and another of white-rot fungus, separately, for 40 days. *Trametes versicolor* produced laccase and manganese-peroxidase activities, while only laccase activity was found with *Flammulina velutipes*. Jain (1995) obtained a high xylanase activity using the thermophilic fungus *Melanocarpus albomyces* IIS-6 on sugarcane bagasse.

Mamma et al. (2008) investigated the production of multienzyme preparations containing pectinolytic, cellulolytic and xylanolytic enzymes by some mesophilic fungi. Under optimal conditions *Aspergillus niger* BTL resulted to be by far the most potent strain in polygalacturonase and pectate lyase production, followed by *Fusarium oxysporum* F3, *Neurospora crassa* DSM 1129 and *Penicillium decumbens*. *N. crassa* DSM 1129 produced the highest endoglucanase activity and *P. decumbens* the lowest one. Comparison of xylanase production revealed that *A. niger* BTL produced the highest activity followed by *N. crassa* DSM 1129, *P. decumbens* and *F. oxysporum* F3. *N. crassa* DSM 1129 and *P. decumbens* did not produce any b-xylosidase activity, while *A. niger* BTL produced approximately 10 times more β-xylosidase than *F. oxysporum* F3. The highest invertase activity was produced by *A. niger* BTL while the lowest ones by *F. oxysporum* F3 and *P. decumbens*.

Osma et al. (2007) studied the potential of banana skin as a support-substrate for the production of extracellular laccase by the white-rot fungus *Trametes pubescens* CBS 696.94, the maximum activity of laccase being 1570 U/l.

Gomez et al. (2005) assessed the potential of two lignocellulosic wastes, chestnut shell and barley bran, for laccase production by the white-rot fungus *Coriolopsis rigida* under solid-state conditions. Barley bran induced the highest laccase activities ($3x10^5$ nkat L^{-1}), about 25-fold higher than the value attained in the chestnut-shell cultures.

2.2.4 Antioxidant production

Increasing interest to replace synthetic antioxidants has led to screen natural sources of antioxidants, especially plant materials. Flavonoids and other polyphenols possess anti-tumoral, anti-allergic, anti-platelet, anti-ischemic, and anti-inflammatory activities, among others, and most of these effects are believed to be due to their antioxidant capacity. Phenolic compounds in edible and non-edible plants have been reported to have antioxidant capacity. Several types of

plant materials (such as vegetables, fruits, seeds, hulls, wood, bark, roots and leaves, spices and herbs, etc.) have been examined as potential sources of antioxidant compounds. These could be used for increasing the stability of foods by preventing lipid peroxidation and also for protecting oxidative damage in living systems by scavenging oxygen radicals (Moure et al., 2001).

Gonzales-Montelongo et al. (2010) optimized the extraction conditions to maximize the antioxidant activity of banana-peel extracts. An aqueous solution of acetone yielded extracts with high antioxidant capacity, quite independent of the banana cultivar.

By leaching chestnut shells with a 2.5% Na_2SO_3 aqueous solution it was possible to maximize the extraction yield and observe a positive linear correlation between the antioxidant activity and total phenols content of the extracts (Vazquez et al., 2008).

2.2.5 Biofuels production

The term biofuel is referred to liquid, gas, and solid fuels that are predominantly produced from renewable biomass. Biofuels include bioethanol, biomethanol, vegetable oils, biodiesel, biogas, bio-synthetic gas (bio-syngas), bio-oil, bio-char, Fischer-Tropsch liquids, and biohydrogen.

There are several reasons for biofuels to be considered as relevant technologies by both developing and industrialized countries. They include energy security reasons, environmental concerns, foreign exchange savings, and socio-economic issues related to the rural sector. Advantages of biofuels are the following:

- biofuels are easily available from common biomass sources;
- their combustion is included in a carbon dioxide cycle;
- biofuels have a considerable environmentally friendly potential;
- biofuels use positively impinges on the environment, economy and consumers;
- they are biodegradable and contribute to sustainability (Demirbas, 2007a).

According to the Directive 2003/30/EC, the share of biofuels consumed in the EU transport sector should have reached the 2% in 2005 and the 5.75% in 2010. Conversely, the current production of liquid biofuels in the EU 25 amounts to about 0.7% of the overall fuel market. Both the high impact of the road transport sector on the global EU energy consumption and its almost complete dependence on fossil fuels have been regarded as the main causes for the EU failure to meet the Kyoto targets (EUR 22066, 2006). Thus, the Directive 2009/30/EC has opened the way to a possible increase in the future level of biofuels to 10%, provided that their production be commercially viable, CO_2-efficient and compatible with vehicle engines (Santi et al., 2012).

To meet these requirements, it has been suggested to obtain biofuels from lignocellulosic residues (*e.g.*, food processing waste biomass) so as to minimize competition for land and food and to develop new integrated refining schemes including co-production of fuels, heat, power and co-products to enhance the overall economy and competitiveness of biofuels (EUR 22066, 2006).

Some examples of biofuels production from food-processing lignocellulosic residues are reported below. Biodiesel is not reported in this list as it's produced through a chemical modification of vegetable oils and is not derived from lignocellulose.

2.2.5.1 Biochar

Demirbas (1999) converted hazelnut shells into charcoal, that was compressed to briquettes using pyrolytic oil or tar as binder. The briquette properties improved by increasing the briquetting pressures and percentages of binder materials, the best charcoal briquettes being obtained at 800 MPa and 400 K.

Some carbonization experiments using grape seed and chestnut shell samples were carried out to determine the effect of temperature, sweep gas (nitrogen) flow rate and heating rate on the biochar yield (Özçimen and Ersoy-Meriçboyu, 2008). Temperature exerted the strongest effect on the biochar yields in comparison with nitrogen gas flow rate and heating rate. Biochar yields of grape seed and chestnut shell decreased with the increasing temperature, heating rate and sweep gas flow rate.

2.2.5.2 Bio-oil

Bio-oils are liquid or condensable gaseous fuels made from biomass materials, such as agricultural crops, municipal wastes and agricultural and forestry by-products via biochemical or thermochemical processes (Demirbas, 2007b).

A fixed-bed pyrolysis experiment was carried out by Pütün et al. (1999) on a sample of hazelnut shells to evaluate the effects of pyrolysis temperature and sweep gas atmosphere (N_2) on the pyrolysis yields and chemical compositions. The maximum bio-oil yield of 23.1 wt% was obtained in N_2 atmosphere at a temperature of 500 °C and heating rate of 7 K min^{-1}.

2.2.5.3 Biogas

The organic fraction of almost any form of biomass can be broken down through anaerobic digestion into a methane and carbon dioxide mixture, called as ‘‘biogas”. Biogas is an environmentally friendly, clean, cheap, and versatile fuel. Biogas is a valuable fuel which is

produced in digesters filled with a feedstock like dung or sewage. The digestion is allowed to continue for a period ranging from ten days to a few weeks. The activity of the bacteria involved in the process varies with its age, morphology and temperature, with optimal temperature conditions at mesophilic (35 °C) and thermophilic (55 °C) ranges (Demirbas & Balat, 2006).

Martìn et al. (2010) evaluated the anaerobic digestion of orange peel waste at laboratory and pilot scale under mesophilic and thermophilic conditions. The methane production rate and biodegradability were higher at the thermophilic conditions than at the mesophilic ones.

Brooks et al. (2008) developed a technology for the anaerobic digestion of sugar beet pulp (SBP), through lab-scale experiments to confirm the suitability of SBP as substrate for anaerobic bacteria, and pilot-scale experiments for the process optimization and procedures. The first large-scale biogas plant was then put into operation during the sugar processing period 2007 at a Hungarian sugar factory and digested approximately 50% of the SBP.

Bardiya et al. (1996) studied the biomethanation of banana peel and pineapple wastes at various hydraulic retention times (HRTs). The two substrates showed a higher rate of gas production at lower retention times. The lowest possible HRT for banana peel was 25 days, while pineapple-processing waste digesters could be operated at a HRT of 10 days.

2.2.5.4 Biohydrogen

Hydrogen is not a primary fuel because it has a potential only if used in an electricity generating fuel cell. Hydrogen can be produced from biomass by pyrolysis, gasification or biological generation.

Midilli et al. (2000) carried out a pyrolysis of hazelnut shells at temperatures between 500 and 700 °C and were able to separate the pure hydrogen gas from the other combustible gases.

Biological hydrogen production processes are generally more environmentally friendly and less energy intensive than thermochemical and electrochemical processes. Anaerobic fermentation of low-cost substrates rich in carbohydrates, such as organic wastes/wastewater or agricultural residues, is one promising method to produce hydrogen (Demirbas, 2008).

Koutrouli et al. (2009) studied the anaerobic biohydrogen production from olive pulp and the subsequent anaerobic treatment of the effluent for methane production. It was shown that the thermophilic hydrogen production process was more efficient than the mesophilic one in both hydrogen production rate and yield.

2.2.5.5 Bioalcohols

The alcohols that can be used as motor fuels are methanol (CH_3OH), ethanol (C_2H_5OH), propanol (C_3H_7OH) and butanol (C_4H_9OH). However, only the first two are technically and economically suitable as fuels for internal combustion engines (ICEs).

Bioethanol production will be widely discussed in the next sections. As for the methanol, the production of this alcohol from biomass is a cost-intensive chemical process. Therefore, in current conditions, only waste biomass such as old wood or bio-waste is used to produce methanol. Most processes require supplemental oxygen for the intermediate conversion of the biomass into a synthesis gas (H_2 + CO). A readily available supply of hydrogen and oxygen, therefore, should improve the overall productivity of biomass derived methanol (Vasudevan et al., 2005).

2.3 Bioethanol

Bioethanol is ethyl alcohol produced through hydrolysis and fermentation of liquid or solid biomasses. It is the most widely used liquid biofuel and can be blended up to 5% (v/v) with petrol under the EU quality standard EN 228 with no need to modify engines. With engine modification, bioethanol may be used as a primary fuel either in neat (unblended) form or with small amounts of gasoline. E_{100} and E_{85} refer to neat ethanol and to an 85% ethanol-15% gasoline blend, respectively. Combustion of ethanol in internal combustion engines designed for alcohol gives higher thermal efficiency and power than combustion of gasoline in conventional engines, thanks to the higher octane number and to the higher heat of vaporization (Demirbas, 2008).

World ethanol production has increased to $62x10^9$ liters in 2007, approximately 70% of which being produced in the United States and Brazil ((Licht, 2008a).

2.3.1 First-generation bioethanol

Over 90% of the world's bioethanol derives from crops (60% from cane-sugar and beet-sugar while the remainder from grains, mainly corn starch) and is commonly known as "first-generation bioethanol" (Licht, 2008a).

The US ethanol industry uses corn as its main feedstock and the share of corn crop, that is consumed by the ethanol industry, has grown from around 5% to more than 25% in 10 years. In 2009, the US produced $39.5x10^9$ liters of ethanol from this feedstock (Licht, 2008b).

Brazilian ethanol is produced from sugar-cane and in 2004 accounted nearly 18% of the country's automotive fuel needs. In Brazil, ethanol-powered and flexible-fuel vehicles are manufactured for operation with the azeotropic mixture (around 93% v/v ethanol and water 7%). Brazil is the second world largest producer with about $30x10^9$ liters of ethanol from sugar-cane (Petrova & Ivanova, 2010).

Bioethanol production from sugar-cane or corn starch is a relatively simple process (Fig. 2.1), since it is simple to obtain fermentable sugars from such feedstocks.

Sugar-cane (*Saccharum officinarum*) contains 12-17% total sugars on a wet-weight basis with 68-72% moisture (90% sucrose and 10% glucose or fructose). The average extraction efficiency to produce cane juice by crushing is approximately 95% and the remaining solid residue is cane fiber (*bagasse*). For bioethanol production the cane juice is heated up to 110 °C to reduce microbial contamination, decanted, sometimes concentrated by evaporation and then fermented. Sugar-cane juice normally contains sufficient minerals and organic nutrients to be immediately suitable for ethanol production by fermentation with *Saccharomyces cerevisiae* (Wheals et al., 1999).

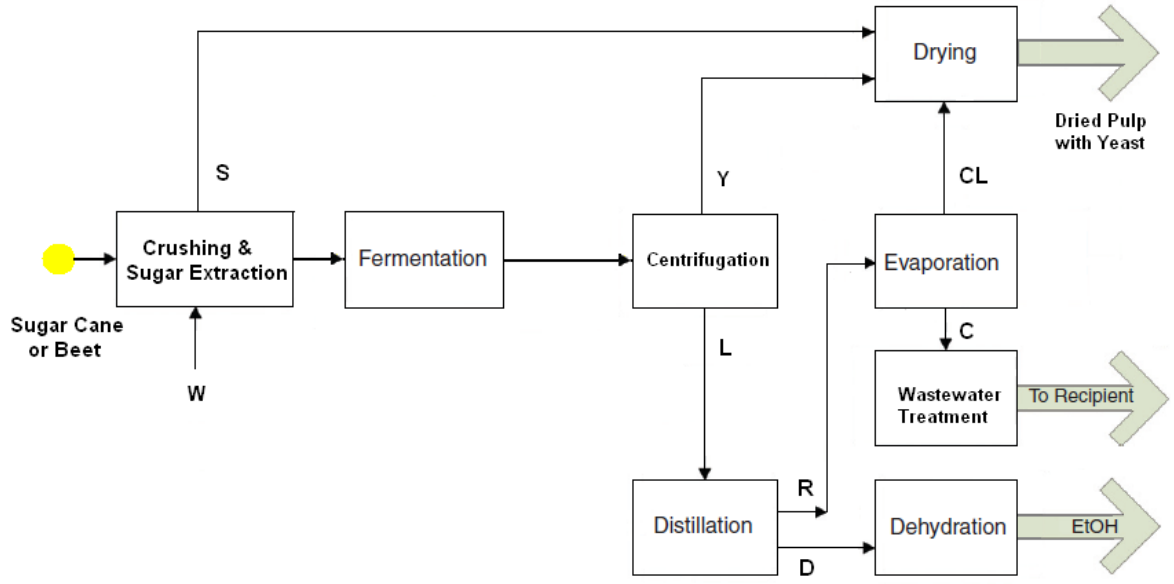


Figure 2.1 Typical flow-sheet of the first-generation bioethanol production from sugar-cane or beet. Item symbol list: C, condensate; CL, concentrate; D, distillate; L, clarified fermented medium;. R, bottom product; S, bagasse; W, leaching solvent; Y, yeast cream.

Maize (*Zea mays*) and other less commonly used grains are usually processed by wet milling. Wet milling was originally devised for the starch industry and adapted for fuel-ethanol production. The grain is soaked (steeped) in water with sulfur dioxide for up to 40 h, before being ground to separate starch and co-products. The starch fraction is gelatinized by cooking.

By adding α-amylase, dextrin oligosaccharides are formed. Further addition of glucoamylase converts dextrin to glucose. The sugar-containing juice that leaves the processor (mash or wort) is essentially sterile, and this is a crucial point in the subsequent successful downstream processing (Bothast & Schlicher, 2005). Fig. 2.2 shows the typical flow-sheet of bioethanol production from starchy materials.

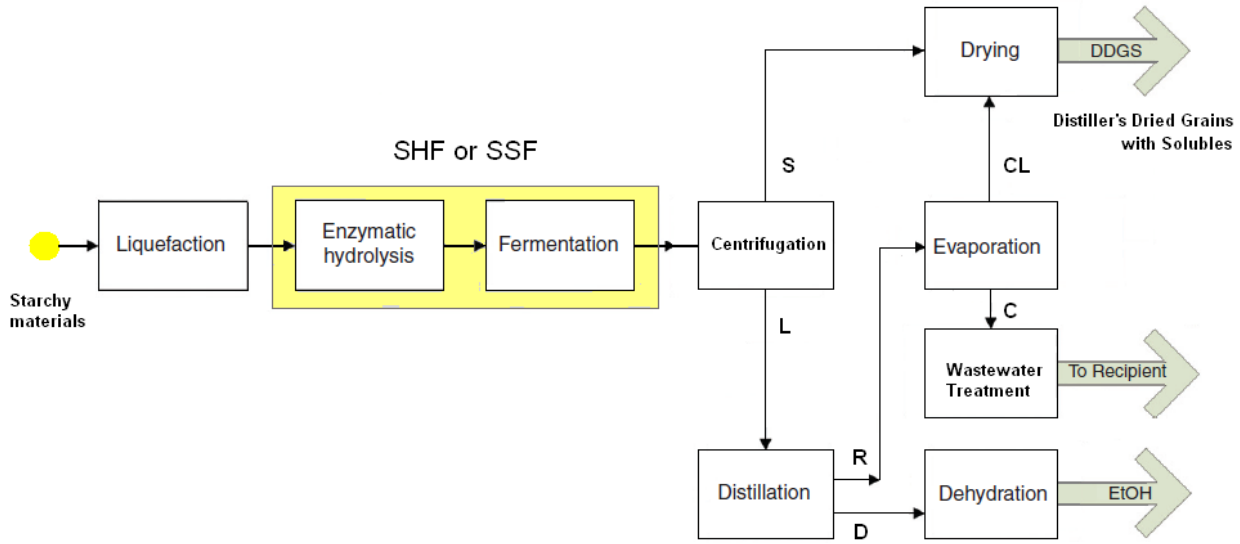


Figure 2.2 Typical flow-sheet of the first-generation bioethanol production from starchy materials. Item symbol list: C, condensate; CL, concentrate; D, distillate; L, clarified fermented medium;. R, bottom product; S, yeast and solid residue; SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation.

First-generation bioethanol also includes the use of food processing residues rich in sugars, like molasses or whey.

The treatment of whey by fermenting lactose to ethanol has received wide attention so far, and various large-scale procedures have been developed. Several distilleries producing ethanol from whey are in commercial operation in Ireland, the USA and, particularly, New Zealand, where 50% of the cheese-whey production is used to produce ethanol (Mawson, 1994).

Although several papers have dealt with the search for microorganisms able to produce ethanol directly from lactose, up to now *Kluyveromyces marxianus (ex fragilis*) is the microorganism of choice for most commercial plants. In batch fermentation *K. fragilis* utilizes more than 95% of the lactose present in raw whey with a conversion efficiency of 80-85% of the theoretical value of 0.538 kg ethanol per kg of lactose consumed (Gonzales Siso, 1996).

El-Refai et al. (1992) evaluated the potentiality of crude beet molasses (CBM) by screening for ethanol production 22 yeast strains. *S. cerevisiae* Y-7 was the strain with the best sugar-ethanol conversion. A sugar concentration of 10.86% gave optimum fermentation efficiency. The

intermittent addition of beet molasses allowed better assimilation of sugars with relatively high biomass and ethanol yields (El-Refai et al., 1992).

Bioethanol production from sugar-rich food processing residues has been successful, but remains marginal in the world bioethanol-production scenarios. Thus, since first-generation bioethanol is mostly obtained from feedstocks that could be used for food production, this process has caused a serious problem of competition between food and energy sector. Expansion of biofuel production in the United States, Europe, and South America has coincided with recent sharp increases in prices for food and feed grains (Palmarola-Adrados et al., 2005; del Campo et al., 2006; Bartle & Abadi, 2010).

Another problem related to first-generation bioethanol is represented by the competitiveness with fossil fuel prices: a renewable fuel may represent a valid alternative to the traditional counterpart only if it is economically convenient. Since the starting material contributes for over the 55% to the final cost, the use of less expensive materials, such as lignocellulosic residues, may represent a solution to make bioethanol competitive on the market (Licht, 2004).

2.3.2 Second-generation bioethanol

Second-generation bioethanol is produced from lignocellulosic feedstocks that cannot be directly used for food production or do not reduce the amount of land destined to food production (Petrova & Ivanova, 2010). Lignocellulosic biomasses represent the biggest renewable source of potentially fermentable sugars on Earth, and bioethanol production from such feedstocks can be accomplished in two ways.

The first most straightforward way is to capture lignocellulosic biomass that is currently treated as waste or that is a co-product of existing production processes with very low or negative current economic value. Examples of waste streams that could potentially be converted into bioethanol include perennial grasses (Digman et al., 2010); a portion of municipal trash and garbage, such as waste paper and waste food scrapes (Stichnothe & Azapagic, 2009); crop residues, such as corn stover (Öhgren et al., 2007), wheat and rice straw (Kaparaju et al., 2009; Huang et al., 2009); macroalgae (Goh & Lee, 2010), and forest residues (Galbe & Zacchi, 2002). Currently, the value of these streams is often negative since consumers and firms must pay for their disposal. New technologies allowing the economic conversion of these feedstocks into bioethanol would offer the double benefit of reducing global waste and generating a valuable transportation fuel. In addition, waste stream upgrading would place no burden on the world's ability to produce food. A recent study estimated that a city of one million people could provide

enough organic waste (i.e., 1,300 tons per day) to produce 430,000 liters of bioethanol/day (Koh & Ghazoul, 2009).

The second way to produce biomass without competing for food land is to use land that unsuitable for food production. There are large areas in the USA and Europe that once produced food crops, but now are converted to pasture or trees. Conversion of these lands to the production of lignocellulosic biomass would not affect food prices. The candidate grass species for cellulosic ethanol production include switch grass, miscanthus (*Miscanthus* spp.), reed canary (*Phalaris arundinacea*), and giant reed (*Arundo donax*).

Most of these crops can be cultivated on marginal or agriculturally degraded lands, and thus may not compete with food production. High-diversity mixtures of grassland species can even provide greater bioenergy yields and green-house gas (GHG) reductions than certain conventional bioethanol production systems (Lewandowsky & Schmidt, 2006; Licht 2008b). The use of arid and desert areas also appears to be a promising approach and in this context plant species that are able to colonize either arid or semiarid environments are of particular interest. The ability of these shrubs, such as those belonging to *Tamarix* spp., to face such ecosystems is normally exploited to counteract wind erosion effects and to control desertification, but could also be exploited for fermentable sugars production (Santi et al., 2012; Zheng et al., 2009).

Lignocellulose is composed of up to 75% carbohydrates (namely cellulose and hemicellulose) and a key issue is to obtain an efficient and cost-effective conversion of these carbohydrates into fermentable sugars. Several schemes for the conversion of lignocellulose into sugars have been demonstrated in laboratory and pilot scales (Thomsen et al., 2006; Wyman et al., 2005).

The general concept involves an initial pretreatment step that increases the digestibility of the material, followed by enzymatic hydrolysis to liberate the monosaccharides that will be fermented (Fig. 2.3). Since lignocellulose is a complex matrix of polymers, an efficient hydrolysis of the carbohydrates to monosaccharides requires not only efficient pretreatment and enzymes but also optimization of both steps in relation to each other (Jørgensen et al., 2007a).

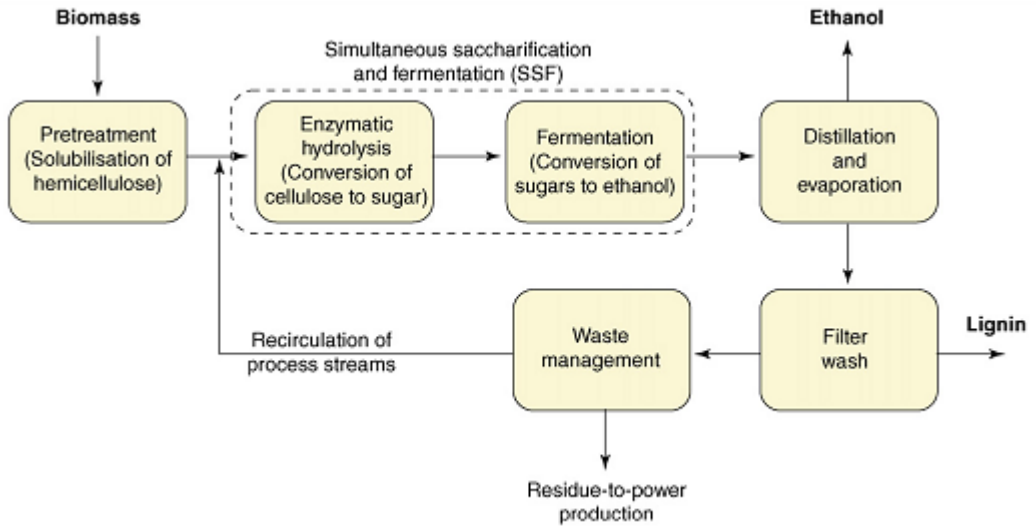


Figure 2.3 Typical flowsheet of second-generation bioethanol production from lignocellulosic materials.

2.3.2.1 The structure of lignocellulose

Lignocellulose is composed of cellulose, hemicelluloses and lignin. These polymers are intimately associated to form the structural framework of the plant cell wall. The composition of lignocellulose depends on plant species, age and growth conditions. Distribution of cellulose, hemicelluloses and lignin, as well as the different sugar content of hemicelluloses, is highly dependent on plant species (Carpita et al., 2001).

Cellulose, the most abundant constituent of the plant cell wall, is a homo-polysaccharide entirely composed of *D*-glucose units linked together by β-1,4-glucosidic bonds and with a degree of polymerization up to 10,000 or higher. The linear structure of the cellulose chain enables the formation of both intra- and inter-molecular hydrogen bonds resulting in the aggregation of chains into elementary crystalline fibrils of 36 cellulose chains. The structure of the elementary fibrils, along with the intermolecular hydrogen bonds, gives cellulose high tensile strength, makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Ward & Moo-Young, 1989). The hydrophobic surface of cellulose results in formation of a dense layer of water that may hinder diffusion of enzymes and degradation products near the cellulose surface (Matthews et al., 2006).

Hemicelluloses are complex heterogeneous polysaccharides, composed of monomeric residues: D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid. Hemicelluloses have a degree of polymerization below 200, side chains, and can be acetylated. Hemicelluloses are classified according to the main sugar in the backbone of the polymer, *e.g.* xylan (β-1,4-linked xylose) or mannan (β-1,4-linked mannose) (Carpita, 1996).

Lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction in lignocellulose. The three monomers in lignin are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol and are joined through alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds. Lignin embeds the cellulose thereby offering protection against microbial and chemical degradation. Furthermore, lignin is able to form covalent bonds to some hemicelluloses, e.g. benzyl ester bonds with the carboxyl group of 4-O-methyl-d-glucuronic acid in xylan. More stable ether bonds, also known as lignin carbohydrate complexes (LCC), can be formed between lignin and arabinose or galactose side groups in xylans and mannans (Kuhad et al., 1997). The structure of lignocellulose is schematically explained in Fig. 2.4.

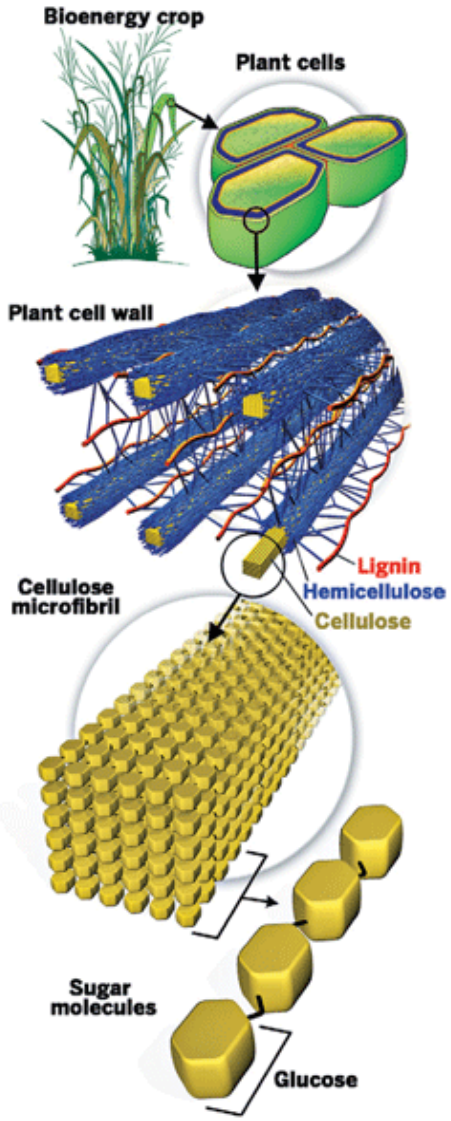


Figure 2.4 The structure of lignocellulose.

2.3.2.2 Pretreatment technologies

Due to the robust structure of lignocellulosic biomass, a pretreatment is required in order to disrupt this structure and make it more susceptible to an enzymatic attack prior to fermentation. Over the years a number of different technologies has been developed for pretreating lignocellulose. There is no ideal pretreatment as it depends on the kind of feedstock, but there is an overall consensus that a successful pretreatment should:

- maximize the enzymatic convertibility;
- minimize loss of sugars;
- maximize the production of other valuable by-products, *e.g.* lignin;
- not require the addition of chemicals toxic to the enzymes or the fermenting microorganisms;
- minimize the use of energy, chemicals and capital equipment;
- be scalable to industrial size (Jørgensen et al., 2007).

In reality, it is difficult to fully accomplish all the aforementioned issues in any process. However, the last two points affect the economical and practical viability of a given industrial process.

Various technologies use different strategies to increase the enzymatic convertibility. The general ideas are to alter or remove hemicelluloses and/or lignin, increase surface area accessible to the enzymes and decrease the crystallinity of cellulose, as shown in Fig. 2.5 (Wyman et al., 2005; Mosier et al., 2005).

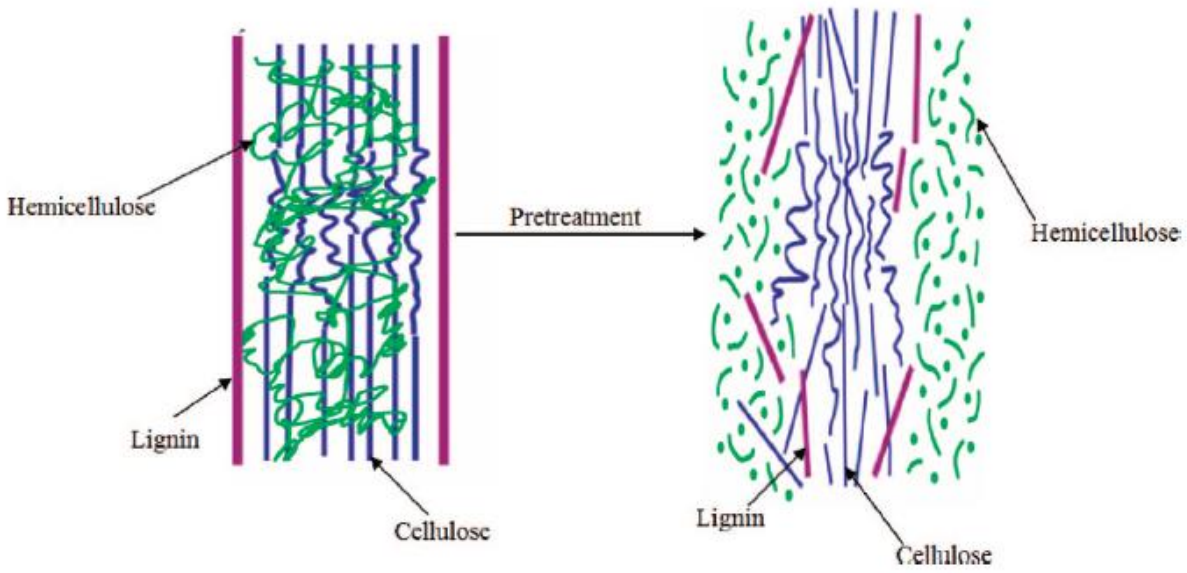


Figure 2.5 Schematic of the role of pretreatment in the conversion of lignocellulosic biomass to bioethanol.

Removal of lignin, and to a less extent hemicelluloses, has been proven to be effective in increasing the cellulose hydrolysis (Chang & Holtzapple, 2000).

Pretreatment methods can be roughly divided into different categories: physical, physicochemical, chemical and biological.

One of the challenges for pretreatment technologies is energy efficiency. Most of the pretreatment technologies presented below in general rely on the principle of heating the material to temperatures ranging from 100 to 200 °C. Pretreatment is therefore energy-intensive. Furthermore, some technologies require or have only been tested with small particle sizes, but grinding or milling is also an energy-intensive unit operation (Schell & Harwood, 1994).

A variety of technologies is on the market or has been described, but most research has so far been aimed at optimizing the technologies towards optimum convertibility of the material and reduced formation of degradation products.

To make biorefineries cost-effective more focus has to be on energy efficiency of the processes and minimized water usage. This means that the processes have to:

- operate with large particle sizes to reduce the energy used for size reduction;
- operate at high solids concentrations to reduce water and energy usage;
- be integrated to use surplus heat/steam from other processes in the pretreatment (Jørgensen et al., 2007a).

Physical methods

- *Mechanical comminution*

Comminution of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping, and 0.2-2 mm after milling or grinding (Sun & Cheng, 2002).

- *Pyrolysis*

Pyrolysis has also been used to pretreat lignocellulosic materials: cellulose rapidly decomposes to gaseous products and residual char, when biomass is treated at temperatures greater than 300 °C. At lower temperatures, the decomposition is much slower, and the products formed are less volatile. Fan et al. (1987) reported that mild acid hydrolysis (1 N H_2SO_4, 97 °C, 2.5 h) of the products from pyrolysis pretreatment resulted in 80-85% conversion of cellulose to reducing sugars with more than 50% glucose.

Physico-chemical methods

- Steam explosion

Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials (McMillan, 1994). In this method, biomass is treated with high-pressure saturated steam; then, the pressure is suddenly reduced to submit the materials to an explosive decompression. Steam explosion is typically carried out at 160-260 °C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Sun & Cheng, 2002). The process causes hemicellulose degradation and lignin transformation.

Removal of hemicelluloses from the microfibrils is believed to increase enzyme accessibility to the cellulose microfibrils (Kabel et al., 2007). Lignin is removed only to a limited extent during the pretreatment, but it is redistributed on the fiber surfaces as a result of melting and depolymerization/ repolymerization reactions (Li et al., 2007). The removal and redistribution of hemicellulose and lignin increase the volume of the pretreated sample. The accessible surface area is also increased by the rapid flashing to atmospheric pressure and the consequence turbulent flow, that cause fragmentation of the material (Duff & Murray, 1996). Depending on the severity of the pretreatment, some degradation of the cellulose to glucose can take place (Jorgensen et al., 2007).

Addition of H_2SO_4 (or SO_2) or CO_2 [typically 0.3-3% (w/w)] in steam explosion can decrease process time and/or temperature, effectively improve hydrolysis, decrease the production of inhibitory compounds, and lead to complete removal of hemicellulose (Ballesteros et al., 2006; Stenberg et al., 1998).

The factors that affect steam-explosion pretreatment are residence time, temperature, chip size, and moisture content (Duff & Murray, 1996; .Wright, 1998).

Steam explosion has been applied to various lignocellulosic materials, such as wheat, barley and oat straws (Viola et al., 2008); pine (Negro et al., 2003); olive tree pruning (Cara et al., 2008); poplar, eucalyptus, *Sorghum* sp. bagasse, wheat straw and *Brassica carinata* residues (Ballesteros et al., 2004); olive pomace (El Asli & Qatibi, 2009); *Tamarix* spp. (Santi et al., 2012).

The advantages of steam-explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs (Holtzapple et al., 1989).

Steam pretreatment with addition of a catalyst is the technology that has been claimed to be closest to commercialization. The pretreatment has been tested extensively for a large number of

different lignocellulosic feedstocks. The technology has been scaled-up and operated at the pilot-plant scale at the Iogen demonstration plant in Canada (Jorgensen et al., 2007).

Limitations of steam explosion include incomplete disruption of the lignin-carbohydrate matrix, and generation of compounds, that might be inhibitory to the microorganisms used in downstream processes (§2.3.2.3).

- *Ammonia fiber explosion (AFEX)*

In AFEX the lignocellulosic material is treated with liquid ammonia at moderate temperatures (90-100 °C) and high pressure (17-20 bar) for 5-10 min. A unique feature of AFEX is to process efficiently material with a dry matter content up to 60% (Teymouri et al., 2005).

The ammonia can be recovered after the pretreatment and, unlike most other methods, no liquid fraction with dissolved products is generated by the AFEX pretreatment, as ammonia is evaporated. Consequently, no lignin or other substances are removed from the material, but lignin-carbohydrate complexes are cleaved and deposition of lignin on the surface of the material is observed. Furthermore, AFEX results in depolymerization of the cellulose and partially hydrolysis of hemicelluloses. Only little degradation of sugars occurs and, therefore, low concentrations of inhibitors are formed.

The AFEX technology has been used for pretreating many lignocellulosic materials, including switchgrass (Alizadeh et al., 2005), miscanthus (Murnen et al., 2007), corn stover (Teymouri et al., 2005) and rice straw (Gollapalli et al., 2002).

- *Dilute acid hydrolysis*

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. In dilute acid pretreatment, the lignocellulosic material is mixed with dilute acid (typically H_2SO_4) and water to form a slurry and heated by steam to the desired temperature. The pretreatment can also be completed with a rapid flashing to the atmospheric pressure. This is in essence very similar to the steam pretreatment and in the literature there is not always a clear distinction between the two methods. Generally, the material used in steam pretreatment is only moist, whereas a slurry with a lower dry matter content down to 5% is applied in dilute acid pretreatment. Sulfuric acid at concentrations usually below 4% (w/v) has been generally used as it is inexpensive and effective (Jorgensen et al., 2007).

The dilute H_2SO_4 pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis. Dilute acid effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis (Esteghlalian et al., 1997).

Recently, dilute acid hydrolysis has been used on a wide range of feedstocks, such as corn stover (Ishizawa et al., 2007; Lu et al., 2007); rice straw (Karimi et al., 2006); wastes from tomato, red pepper, pulse food, artichoke, and cardoon processing (Del Campo et al. 2006); rye straw and bermudagrass (Sun & Cheng, 2005); forest residues (Canettieri et al., 2007).

- *Alkaline hydrolysis*

Some bases can be used for the pretreatment of lignocellulosic materials, and the effect of alkaline pretreatment depends on the lignin content of the materials. Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies, but pretreatment times ranges from hours to days rather than from seconds to minutes, such long residence times being less attractive from an industrial point of view (Mosier et al., 2005).

As opposed to the acid-catalysed methods, the general principle behind alkaline pretreatment methods is the removal of lignin, whereas cellulose and a major part of the hemicelluloses remain in the solid material (Jorgensen et al., 2007).

Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents.

Of these four, sodium hydroxide has been studied the most, and on various substrates, such as sunflower hulls (Soto et al., 1994), corn stover (MacDonald et al., 1983), sugar-cane bagasse (Fox et al., 1989). However, calcium hydroxide (slake lime) has been shown to be an effective pretreatment agent and is the least expensive per kilogram of hydroxide. It is possible to recover calcium from an aqueous reaction system as insoluble calcium carbonate by neutralizing it with inexpensive carbon dioxide (Kumar et al., 2009). Lime has been used to pretreat wheat straw at 85 °C for 3 h (Chang et al., 1998); poplar wood at 150 °C for 6 h with 14 bar of oxygen (Chang et al., 2001); switchgrass at 100 °C for 2 h (Chang et al., 1997); and corn stover at 100 °C for 13 h (Karr & Holtzapple, 1998).

- *Carbon dioxide explosion*

In attempts to develop improved lignocellulose pretreatment techniques, the idea of using supercritical CO_2 explosion, which would have a lower temperature than steam explosion and possibly a reduced expense compared to ammonia explosion, was developed. Supercritical fluid refers to a fluid that is in a gaseous form, but is compressed at temperatures above its critical point to a liquid-like density. It was hypothesized that, because CO_2 forms carbonic acid when dissolved in water, the acid increases the hydrolysis rate. Carbon dioxide molecules are comparable in size to water and ammonia and should be able to penetrate small pores accessible to water and ammonia molecules. Carbon dioxide was suggested to be helpful in hydrolyzing hemicellulose, as well as cellulose. Moreover, the low temperature prevents any appreciable

acidic decomposition of monosaccharides. Upon an explosive release of the carbon dioxide pressure, the disruption of the cellulosic structure increases the accessible surface area of the substrate to hydrolysis (Kumar et al., 2009).

Dale et al. (1982) used the method to pretreat alfalfa and obtained 75% of the theoretical glucose released during 24 h-enzymatic hydrolysis. By comparing CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse, and repulping waste of recycled paper, Zheng et al. (1998) found that CO_2 explosion was more cost-effective than ammonia explosion with limited formation of inhibitory compounds, these occurring in conventional steam explosion.

-*Wet oxidation*

Wet oxidation is a thermal pretreatment at 180-200°C for 5-15 min with addition of an oxidative agent, such as H_2O_2 or over-pressure of oxygen. The pretreatment is performed at 5-20% dry matter, and often the initial pH is increased by the addition of alkali, such as sodium carbonate. In wet oxidation, some of the lignin but also hemicelluloses are partially oxidized to low molecular weight carboxylic acids, CO_2 and water. The degradation products from lignin (phenolics) and sugars (furans), which are inhibitory to microorganisms, can be oxidized to carboxylic acid (Klinke et al., 2002).

Chemical methods

- *Ozonolysis*

Ozone treatment is one way of reducing the lignin content of lignocellulosic wastes. This results in an increase of the *in vitro* digestibility of the treated material, and unlike other chemical treatments, it does not produce toxic residues. The degradation is mainly limited to lignin. Hemicellulose is slightly affected, but cellulose is not. Ozonolysis pretreatment has an advantage that the reactions are carried out at room temperature and normal pressure. Furthermore, the fact that ozone can be easily decomposed by using a catalytic bed or increasing the temperature means that processes can be designed to minimize environmental pollution.

Ozone can be used to degrade many lignocellulosic materials, such as wheat straw (Ben-Ghedalia & Miron, 1981); bagasse, green hay, peanut, pine (Neely, 1984); cotton straw (Ben-Ghedalia & Shefet, 1983); and poplar sawdust (Vidal & Molinier, 1988).

A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive (Quesada et al., 1999).

- *Oxidative delignification*

Lignin biodegradation can be catalyzed by the peroxidase enzyme in the presence of H_2O_2.

The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. By using 2% H_2O_2 at 30 °C for 8 h, about 50% of lignin and most of hemicellulose were solubilized, yielding 95% efficiency of glucose production from cellulose in the subsequent saccharification by cellulase at 45 °C for 24 h (Azzam, 1989).

- *Organo-solvent pretreatment*

In the organo-solvation process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds.

The solvents commonly used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol (Thring et al., 1990). Also some organic acids, such as oxalic, acetylsalicylic, and salicylic acids, can be used (Sarkanen, 1980).

In essence, the organo-solvent process involves simultaneous prehydrolysis and delignification of lignocellulosic biomass supported by organic solvents and, usually, dilute aqueous acid solutions.

This kind of pretreatment has been mostly used on woody biomasses, such as poplar (Chum et al., 1988; Pan et al., 2006) and other mixed softwoods (Arato et al., 2005; Pan et al., 2005).

Biological pretreatment

Biological pretreatment using various types of rot fungi, a safe and environmentally friendly method, is increasingly being advocated as a process that does not require high energy for lignin removal from a lignocellulosic biomass, despite extensive lignin degradation.

In biological pretreatment processes, microorganisms, such as brown-, white-, and soft-rot fungi, are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes, such as peroxidases and laccase. These enzymes are regulated by carbon and nitrogen sources.

White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials (Galbe & Zacchi, 2007). Biological pretreatment has been performed on several feedstocks, such as wheat straw using *Pleurotus ostreatus* (Hatakka, 1983); japanese red cedar using *Pycnoporus cinnabarinus* (Okano et al., 2005); bermudagrass using *Ceriporiopsis subvermispora* and *Cyathus stercoreus* (Akin et al., 1995); japanese red pine using *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis* (Lee et al., 2007).

Biological pretreatment in combination with other pretreatment technologies has also been studied. Itoh et al. (2003) reported production of ethanol by simultaneous saccharification and fermentation (SSF) from beech wood chips after bio-organosolvation pretreatments by

ethanolysis and white-rot fungi, such as *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Pleurotus ostreatus*, and *Coriolus Versicolor*. The purpose of biotreatments was to reduce the energy input required for the separation of wood components by ethanolysis. The yield was 1.6 times higher than that obtained without the fungal treatments.

Balan et al. (2008) studied the effect of fungal conditioning of rice straw followed by AFEX pretreatment and enzymatic hydrolysis. Treatment of rice straw with the white-rot fungus *Pleurotus ostreatus*, followed by AFEX, gave significantly higher glucan and xylan conversions and less-severe AFEX conditions than the only AFEX treatment.

The advantages of biological pretreatment include low energy requirements and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low (Kumar et al., 2009).

2.3.2.3 Formation of inhibitors

As described above, treatments of lignocellulose at high temperatures under acidic conditions are widely used, even if they lead to the formation and liberation of a range of compounds that are potential inhibitors of the fermenting microorganism. The inhibiting compounds are divided in three main groups based on their origin: weak acids, furan derivatives, and phenolic compounds.

The main degradation pathways are schematically shown in Fig. 2.6.

When hemicellulose is degraded, xylose, mannose, acetic acid, galactose, and glucose are liberated. Cellulose is hydrolysed to glucose. At high temperature and pressure xylose is further degraded to furfural. Similarly, 5-hydroxymethyl furfural (HMF) is formed from hexose degradation. Formic acid is formed when furfural and HMF are broken down. Levulinic acid is formed by HMF degradation. Many different phenolic compounds are generated from partial breakdown of lignin and during carbohydrate degradation (Palmqvist & Hahn-Hägerdal, 2000b).

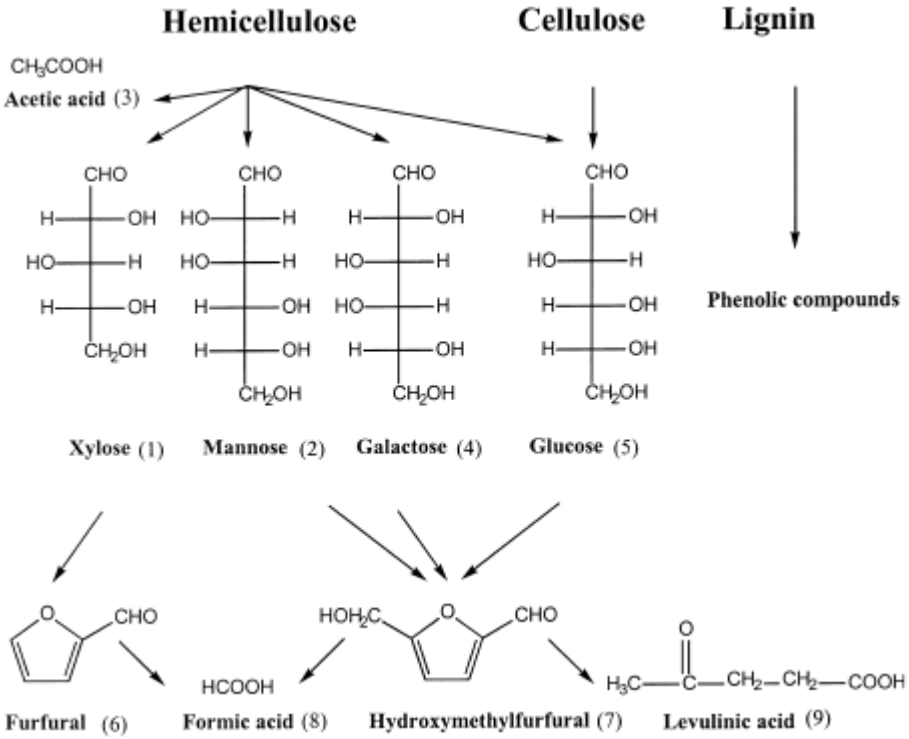


Figure 2.6 Reactions occurring during hydrolysis of lignocellulosic materials.

The generation of fermentation inhibitors is influenced by the hydrolysis temperature and time, as well as acid concentration.

As for the mechanisms of inhibition, undissociated weak acids are liposoluble and can diffuse across the plasma membrane. The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol. In the cytosol, dissociation of the acid occurs due to the neutral intracellular pH, thus decreasing the cytosolic pH and causing damage to the cell. Since the concentration of the undissociated form of an acid depends on the pH, such parameter becomes a crucial variable for the fermentative process (Pampulha & Loureiro-Dias, 1989).

Depending on the microorganism and on their concentration in the medium, furan derivatives (furfural and 5-HMF) can totally inhibit cell growth. At low concentrations, an elongation of the lag-phase can be observed, that might have no consequence on ethanol yield. Hydroxymethylfurfural resulted to be less toxic than furfural (Palmqvist & Hahn-Hägerdal, 2000b).

Phenolic compounds act on the biological membranes causing loss of integrity and thereby affecting their ability to serve as selective barriers and enzyme matrices. Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of

lignocellulosic hydrolyzates, the low molecular weight phenolic compounds being most toxic (Heipieper et al., 1994).

The problem of inhibitors formation in the hydrolyzates can be solved through the use of microorganisms that can tolerate the presence of such compounds. Almeida et al. (2009) used a microplate screening method to assess anaerobic growth of 12 *S. cerevisiae* strains in barley straw, spruce, and wheat straw hydrolyzate. The assay demonstrated significant differences in inhibitor tolerance among the strains. Amartey & Jeffries (1996) found that the adaptation of a *Pichia stipitis* strain to a corn-cob hydrolyzate resulted in a significantly higher fermentation rate, with over 90% of the initial total sugars being utilized, and in an ethanol yield and maximum ethanol concentration of 0.41 g g^{-1} and 13.3 g L^{-1}, respectively. Palmqvist et al., (1999) observed that acetic acid and furfural negatively affected the apparent specific growth rate, ethanol and biomass yields of a *S. cerevisiae* strain.

An alternative to the search of resistant microorganisms is represented by the detoxification of the hydrolyzate itself. To this purpose, various biological, physical and chemical methods have been developed.

Biological methods mainly rely on ligninolytic enzymes or filamentous fungi. By treating a hemicellulose hydrolysate of willow with the enzymes peroxidase and laccase, obtained from the ligninolytic fungus *Trametes versicolor*, the maximum ethanol productivity increased two to three times. Moreover, the laccase treatment led to selective and virtually complete removal of phenolic monomers (2.6 g L^{-1} in the crude hydrolysate) and phenolic acids (Jönsson et al., 1998). A laccase treatment on steam-exploded wheat straw reduced the toxic effect of phenolic compounds by laccase polymerization of free phenols (Jurado et al., 2009).

The filamentous soft-rot fungus *Trichoderma reesei* degraded acetic acid, furfural and benzoic acid derivatives present in a hemicellulose hydrolysate obtained after steam pretreatment of willow, thus increasing around three times the ethanol productivity and four times the ethanol yield (Palmqvist et al., 1997).

Hydrolyzate concentration by vacuum evaporation is a physical detoxification method for reducing the contents of volatile compounds, such as acetic acid, furfural and vanillin, present in the hydrolyzate. However, this method also moderately increases the concentration of non-volatile toxic compounds (extractives and lignin derivatives) and, consequently, the degree of fermentation inhibition (Parajó et al., 1997; Larsson et al., 1999; Silva & Roberto, 1999; Mussatto & Roberto, 2004). Qi et al. (2011) proposed an alternative physical method to remove furfural, using two commercial nanofiltration membranes on a synthetic glucose-xylose-furfural model solution.

Chemical methods include precipitation of toxic compounds and ionization of some inhibitors under certain pH values. Due to the instability of some inhibitors at high pH, a strong detoxification effect was observed by increasing the medium pH to 9-10 and readjusting it to 5.5 (Palmqvist and Hahn-Hägerdal, 2000a). In particular, $Ca(OH)_2$ adjustment of pH has been reported to result in better fermentability than NaOH adjustment, due to the precipitation of toxic compounds (van Zyl et al., 1988). Toxic compounds may also be adsorbed on activated charcoal (Lee et al., 1999; Mussatto & Roberto, 2001), as well as ion-exchange resins (Lee et al., 1999; Larsson et al., 1999; Nilvebrant et al., 2001).

2.3.2.4 Enzymatic hydrolysis: the enzyme system

Cellulose comprises the largest fraction of the sugars in lignocellulose, and glucose is for many microorganisms the preferred carbon source, but the development of microorganisms fermenting hemicellulose sugars efficiently is rapidly progressing (Hahn-Hägerdal et al., 2006; Jeffries, 2006). Since many of the pretreatment methods remove and degrade the hemicelluloses, most focus has traditionally been put on improving cellulases and decreasing the costs associated with the enzymatic hydrolysis of cellulose (Zhang et al., 2006).

Efficient hydrolysis of cellulose requires a number of enzymes. According to the traditional enzyme classification system the cellulolytic enzymes are divided into three classes (Fig. 2.7):

- exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91), which move progressively along the cellulose chain and cleave off cellobiose units from the ends;
- endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4), which hydrolyze internal β-1,4-glucosidic bonds randomly in the cellulose chain;
- 1,4-β-d-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose to glucose and also cleave off glucose units from cellooligosaccharides (Jørgensen et al., 2007a).

Some pretreatment methods leave the hemicelluloses in the matrix; thus, efficient hydrolysis of these materials requires the use of hemicellulases too. Hemicelluloses are heterogeneous with various side groups and as such the hemicellulolytic system is more complex. The hemicellulase system includes among others:

- endo-1,4-β-D-xylanases (EC 3.2.1.8), which hydrolyze internal bonds in the xylan chain;
- 1,4-β-D-xylosidases (EC 3.2.1.37), which attack xylooligosaccharides from the non-reducing end and liberate xylose;
- endo-1,4-β-D-mannanases (EC 3.2.1.78), which cleave internal bonds in mannan and
- 1,4-β-d-mannosidases (EC 3.2.1.25), which cleave mannooligosaccharides to mannose.

The side groups are removed by a number of enzymes; α-D-galactosidases (EC 3.2.1.22), α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72) and feruloyl and p-cumaric acid esterases (EC 3.1.1.73) (Shallom & Shoham, 2003).

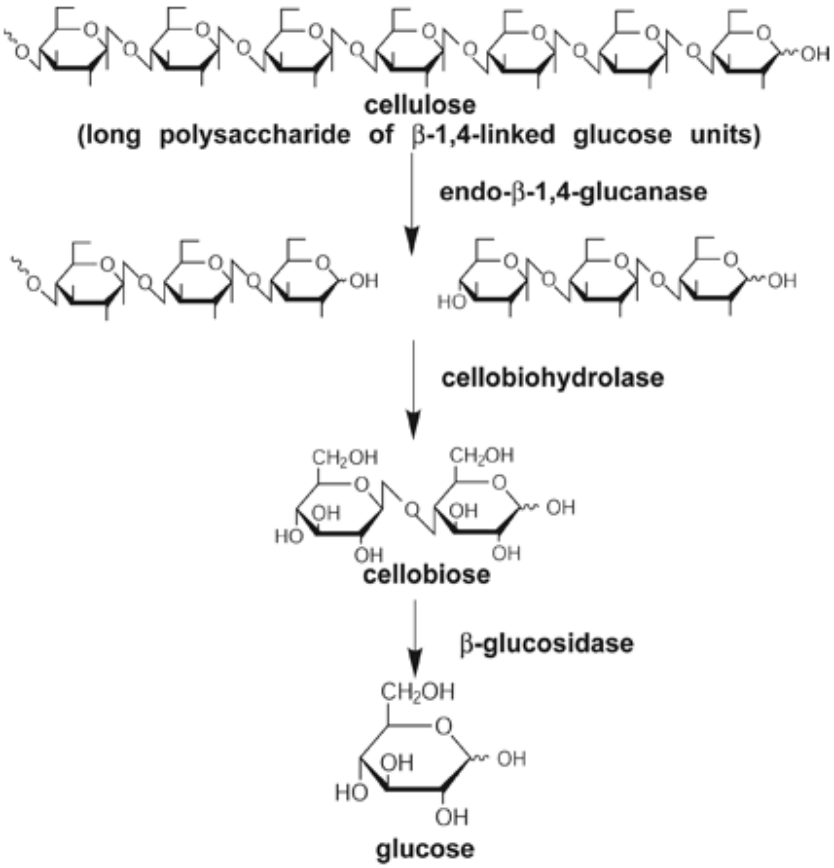


Figure 2.7 **S**chematic presentation of the hydrolysis of cellulose to glucose by cellulolytic enzymes.

Most carbohydrate hydrolases are modular proteins with a catalytic and a carbohydrate-binding module (CBM). CBMs were first discovered on cellulases, but it is now evident that many carbohydrate hydrolases, acting on insoluble, but also soluble polysaccharides, e.g. xylan, mannan and starch, have CBMs. The function of the CBM is to bring the catalytic module in close contact with the substrate and ensure correct orientation. Furthermore, for some CBMs a disruptive effect on the cellulose fibers has also been shown. As cellulose is an insoluble substrate, the adsorption of the cellulases onto the cellulose surface is the first step in the initiation of hydrolysis. Therefore, the presence of CBMs is essential for fast and correct docking of the cellulases on the cellulose (Boraston et al., 2004).

2.3.2.5 Enzymatic hydrolysis: factors of influence

Inhibition

For bioethanol production, high sugar concentrations after the hydrolysis are preferable for the fermentation process. This will increase the product concentration and facilitate the downstream processing and product recovery. However, operating hydrolysis with high initial substrate concentrations has been faced by the problem of product inhibition of especially the cellulolytic enzyme system. The β-glucosidases from typical cellulase-producing microorganisms are to some extent inhibited by glucose (K_i of most β-glucosidases is 1-14 mmol of glucose L^{-1}) (Decker et al., 2000), this causing an accumulation of cellobiose, which is a potent inhibitor of the cellobiohydrolases (Fig. 2.8) (Holtzapple et al., 1990).

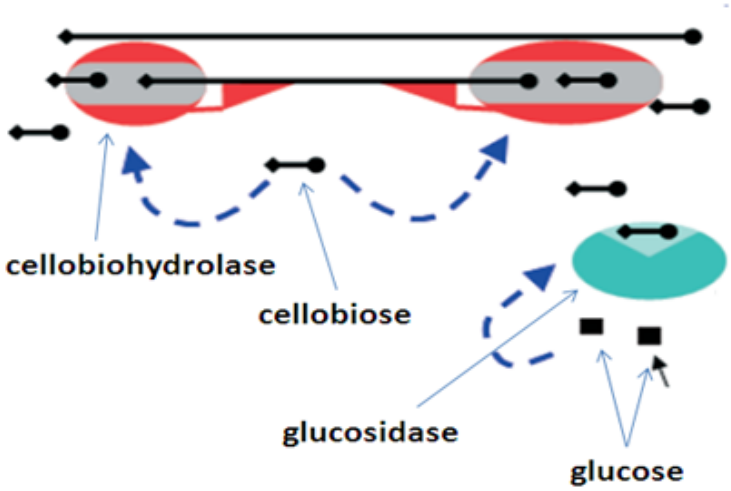


Figure 2.8 Product inhibition of β–glucosidases and cellobiohydrolases by glucose and cellobiose, respectively.

Inhibition of the cellulases by hemicellulose-derived sugars has also been shown (Xiao et al., 2004). The competitive product inhibition of the β-glucosidases can to some extent be overcome by addition of a surplus of β-glucosidase activity.

Another strategy is to screen for β-glucosidases with high glucose tolerance, as those cloned into the cellulase-producing microorganisms and characterized by k_i up to 1400 mmol L^{-1} (Saha & Bothast, 1996).

Removal of end product (glucose) is also one possibility by resorting to the so called simultaneous saccharification and fermentation (SSF) process (§1.4.2.5). The fermentable sugars can also be separated by employing membrane reactors equipped with ultrafiltration membranes with a cut off of 50 kDa or less. In this way, product inhibition from glucose and cellobiose can be reduced and the service life of the enzymes increased (Gan et al., 2002).

The degradation products formed during the raw material pretreatment also inhibit the enzymes. Cellulases were only significantly inhibited by formic acid; whereas phenolic compounds, like vanillic acid, syringic acid and syringylaldehyde in addition to formic acid, caused significant inhibition of xylanases (Panagiotu & Olsson, 2007; Cantarella et al., 2004).
The only washing pretreated material resulted in faster conversion of cellulose due to removal of inhibitors (Tengborg et al., 2001).

Solid concentration

Operating hydrolysis at initial solid concentrations above 10-15% (w/w) has also been technically difficult, especially at laboratory scale. The initial viscosity of the material at these concentrations is very high, which makes mixing difficult and inadequate and increases the power consumption in stirred tank reactors. In pilot scale plants, 15–20% dry matter has often been reported as a maximum level that can be handled. During the initial phase of hydrolysis, the material is liquefied and the viscosity significantly drops (Jørgensen et al., 2007b; Mohagheghi et al., 1992).

Enzyme adsorption

The presence of lignin in the lignocellulosic material is one of the major obstacles in enzymatic hydrolysis. Lignin forms a barrier that prevents the cellulases from accessing the cellulose. Moreover, the lignin is also capable of binding a large part of the enzymes (Fig. 2.9) (Mansfield et al., 1999).

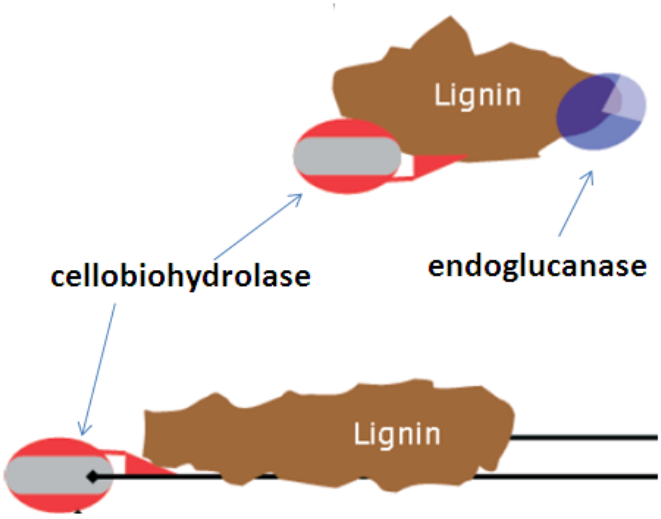


Figure 2.9 Unspecific adsorption of the enzymes onto lignin particles.

The adsorption of cellulases and hemicellulases onto lignin is believed to be due to hydrophobic interaction, but ionic-type lignin-enzyme interaction is also possible (Berlin et al., 2006). After almost complete hydrolysis of the cellulose fraction in lignocellulosic material, up to 60-70% of the total enzyme added can be bound to lignin (Lu et al., 2002; Berlin et al., 2005). Degree of adsorption of various cellulases and their catalytic core is very different and some cellulases appear to have less affinity for lignin (Palonen et al., 2004). This could be exploited in the development of new enzyme preparations. The large fraction of cellulases and hemicellulases unproductively bound to lignin emphasizes that pretreatment methods that remove the majority of the lignin can be advantageous. Removal of 80% of the lignin in steam-exploded soft wood by an alkaline peroxide treatment has been shown to improve the digestibility of the substrate significantly. Furthermore, due to the lower lignin content a six-fold lower enzyme loading was needed to obtain the same degree of conversion (Yang et al., 2002). However, extensive lignin removal and addition of extra steps after steam pretreatment increase the costs of conversion of lignocellulose into fermentable sugars, being also greater the risk of sugar loss or degradation in these subsequent steps.

The value of lignin for other applications, as fuel for combustion, should also be taken into account before performing an oxidative removal of the lignin. The benefit of lignin removal should therefore be carefully evaluated (Jørgensen et al., 2007).

The addition of various compounds to the material before hydrolysis has been shown to improve enzyme performance by reducing unproductive adsorption of enzymes onto lignin.

Addition of other proteins or peptides will bind to the lignin and reduce the binding potential of enzymes by occupying the binding sites (Yang & Wyman, 2006).

Addition of non-ionic surfactants, like Tween 20 or Tween 80, can also reduce unspecific binding of enzymes, thereby improving the hydrolysis rate so that the same degree of conversion can be obtained at lower enzyme loadings (Eriksson et al., 2002).

Ethylene oxide polymers, like poly(ethylene glycol) (PEG), showed a similar effect. PEG is proposed to bind to lignin by hydrophobic and hydrogen bonding, thus preventing enzyme binding to lignin. A stabilizing effect of PEG at elevated temperatures has been shown, apparently due to reduced deactivation through exclusion of enzymes from the lignin surface (Börjesson et al., 2007a).

Unlike Tween 20 or other non-ionic surfactants tested to reduce enzyme adsorption and improve hydrolysis, PEG is a low-cost commodity product. Addition of 2.5–5 g PEG per kg of pretreated material increases the hydrolysis performance by 20–50% for both steam-pretreated spruce and wheat straw (Börjesson et al., 2007b).

The benefit achieved by reducing the enzyme loading should be compared to the costs of adding surfactants (Kristensen et al., 2007).

Enzyme inactivation

Cellulases are stable around pH 5 and temperature of 50 °C for up to 48 h or longer. The presence of substrate can increase the stability even further. However, presence of proteases and shear stress caused by pumps and stirring in the reactor might slowly degrade or denaturate the enzymes. The stability of the enzymes in the presence of substrate is, however, difficult to estimate due to adsorption of the enzymes (Jørgensen et al., 2007a).

Enzyme recovery and recycling

Adsorption and inactivation phenomena largely affect the possibility of recycling the enzymes, this representing an attractive way of reducing enzymatic hydrolysis costs. There are several strategies to recover and reuse the cellulases.

The filtrate obtained after complete hydrolysis of the cellulose fraction can be concentrated by ultra-filtration to remove sugars and other small compounds that may inhibit the action of the enzymes. Depending on the lignin content of the substrate, only up to 50% of the cellulases can be recycled using this approach (Yu et al., 1995). The saving is therefore low, by accounting for the recovery costs.

Another approach takes advantages of the ability of cellulases to adsorb onto the material. By addition of fresh material after hydrolysis, enzymes in solution were readsorbed onto the new material, that could then be separated and hydrolysed in fresh media eventually with supplementation of more enzyme (Tu et al., 2007).

Another method for recycling enzymes is by immobilization, which enables separation of the enzymes from the process flow. The principle of immobilization is to fixate the carbohydrolytic enzymes onto a solid matrix either by adsorption or grafting (Dourado et al., 2002). Apart from extending the service life of a given enzyme, immobilization will also affect activity and stability with regard to temperature and pH optima. Typically broader optima of both pH and temperature are observed. The stability can be significantly increased, but K_m levels are generally higher, thus the specific activity is lower (Gaur et al., 2005).

All of these techniques for recycling and reducing enzyme adsorption have so far only been tested at laboratory scale. Since most of the studies did not evaluate the economic feasibility of adding different compounds to reduce enzyme binding or enzyme recycling, the robustness and feasibility of these techniques are to be established yet (Jørgensen et al., 2007a).

2.3.2.6 Fermentation: microorganisms and their abilities

The most frequently used microorganism for fermenting ethanol in industrial processes is *Saccharomyces cerevisiae*. Among the bacteria, *Zymomonas mobilis* can ferment glucose to ethanol with higher yields, due to the production of less biomass, but is less robust (Galbe & Zacchi, 2002).

Contrary to sucrose- and starch-based ethanol production, lignocellulose-based production is a mixed-sugar fermentation. Lignocellulosic raw materials, in particular hardwood and agricultural raw materials, can contain 5-20% (or more) of pentoses (i.e., xylose and arabinose), which are not fermented to ethanol by wild type *S. cerevisiae* strains. Xylose is by far the most abundant pentose, whereas arabinose can constitute as much as 14-15% in corncob hulls and wheat bran, respectively. Consequently, most research efforts have been devoted to the development of efficient xylose-fermenting microorganisms (Jeffries, 2006).

Xylose-fermenting microorganisms are found among bacteria, yeast and filamentous fungi (Skoog & Hahn-Hägerdal, 1988). Anaerobic bacteria ferment pentoses, but are inhibited at quite low sugar and ethanol concentrations. In addition, the ethanolic fermentation occurs with considerable by-product formation, which reduces the ethanol yield.

Natural xylose-fermenting yeast, notably *Pichia stipitis* CBS 6054, ferments xylose to ethanol with reasonable yield and productivity. Unfortunately, these yeast strains are inhibited by compounds generated during pretreatment and hydrolysis of the lignocellulose material (Hahn-Hägerdal et al., 1994). Filamentous fungi tolerate inhibitors, but are too slow for a competitive industrial process. Therefore, efforts have predominantly been made to obtain recombinant strains of bacteria and yeast able to meet the requirements of industrial lignocellulose fermentation (Hahn-Hägerdal et al., 2006).

Pentose-fermenting *Escherichia coli* and *Klebsiella oxytoca* have been generated by introducing ethanologenic genes from *Zymomonas mobilis* (Burchhardt & Ingram, 1992). At the same time, the first xylose-fermenting *S. cerevisiae* strain was generated through the introduction of genes for xylose-metabolizing enzymes from *P. stipitis* (Kötter & Ciriacy, 1993). Later, xylose-fermenting strains of *S. cerevisiae* were constructed by introducing the genes encoding xylose isomerase from the bacterium *Thermus thermophilus* (Walfridsson et al., 1996) and the anaerobic fungus *Piromyces* sp (Kuyper et al., 2003).

Z. mobilis also efficiently produces ethanol from glucose and fructose, but not from pentoses, even if a xylose-fermenting *Z. mobilis* was generated by introducing a xylose-metabolizing pathway from *E. coli* (Zhang et al., 1995). *E. coli* and *K. oxytoca* naturally metabolize arabinose, such that the ethanologenic strains ferment all lignocellulose- derived sugars (Lindsay et al.,

1995). Xylose- and arabinose-fermenting strains of *Z. mobilis* have been also constructed (Mohagheghi et al., 2002). Because yeasts only ferment arabinose to ethanol in rich media (McMillan & Boyton, 1996), *S. cerevisiae* has been engineered for arabinose use by introducing bacterial genes encoding arabinose-metabolizing enzymes (Becker & Boles, 2003). The functional arabinose-metabolizing pathway has recently been integrated into the diploid xylose-fermenting *S. cerevisiae* strain TMB 3400, and co-usage of xylose and arabinose has been demonstrated (Karhumaa et al., 2006).

2.3.2.7 Process configuration

The steps following the pretreatment, *i.e.* enzymatic hydrolysis and fermentation, can be performed separately or simultaneously.

In *separate hydrolysis and fermentation* (SHF), pretreated lignocellulosic materials are hydrolyzed to glucose and subsequently fermented to ethanol in separate units. The major advantage of this method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimum conditions. The optimum temperature for cellulase is usually between 45 and 50 °C, depending on the cellulose-producing microorganism; while the optimum temperature for most of the ethanol-producing microorganisms is between 30 and 37 °C.

The main drawbacks of SHF are inhibition of cellulase activity by the released sugars and contamination. In fact, since the hydrolysis process is rather long, *e.g.* one to four days, and a dilute solution of sugar always has a risk of microbial contaminations, even at rather high temperature, such as 45-50 °C. A possible source of contamination could be the enzymes. In practice, it is difficult to sterilize the cellulase in large scale, since it should be filtered because of its deactivation in an autoclave (Taherzadeh & Karimi, 2007).

Simultaneous saccharification and fermentation (SSF) is another process option for producing ethanol from lignocellulose. It consolidates hydrolysis of cellulose with the direct fermentation of the produced glucose. Both hydrolysis of cellulose by the cellulase complex and fermentation of sugars by the ethanologenic microorganism are coupled in one vessel (Kang et al., 2010).

The principal benefits of SSF are the decrease in end-product inhibition of the enzymatic hydrolysis, as in contamination risk and investment costs. The main disadvantage of SSF in comparison to SHF is that the optimum temperature for enzymatic hydrolysis is typically higher than that of fermentation – at least when using yeast as the fermenting organism. In SHF, the temperature for the enzymatic hydrolysis can be optimized independently from the fermentation temperature, whereas a compromise must be found in SSF. Finally, the yeast cannot be reused in

SSF due to the problems of separating the yeast from the matrix after fermentation (Olofsson et al., 2008).

In SSF processes the pentoses resulting from hemicellulose degradation during the pretreatment are separated from the solid cellulose and fermented in another vessel, while in simultaneous saccharification and co-fermentation (SSCF) the hydrolysis of the cellulose and co-fermentation of pentose and hexose sugars by xylose- and glucose-fermenting microorganisms is performed in one vessel. This is a great advantage as a unique biomass production setup is required (Taherzadeh & Karimi, 2007).

In the processes considered up to this point, a separate enzyme production unit is required, or the enzymes should be provided externally.

Consolidated bioprocessing (CBP) is a system in which cellulase production, substrate hydrolysis, and fermentation are accomplished in a single process step by cellulolytic microorganisms in microbial community. CBP offers the potential for lower biofuel production costs due to simpler feedstock processing, lower energy inputs, and higher conversion efficiencies than the other processes (Xu et al., 2009).

Two potential paths have been identified to obtain organisms suitable for CBP.

The first path involves modification of excellent ethanol producers, so that they also become efficient cellulase producers; while the second one involves modification of excellent cellulase producers, so that they also become efficient ethanol producers (Lynd et al., 2005).

2.4 Bioethanol production from food-processing lignocellulosic residues

In this section the state of the art of second generation bioethanol production from different kinds of lignocellulosic residues generated by food-processing industries is described in detail. Each paragraph also contains information about composition, annual production and current uses of the feedstock.

2.4.1 Olive pulp

Olive pulp (OP) is the semi-solid (approximately 70% water content) residue generated from the two-phase processing of the olives to extract olive oil. Olive pulp is a biomass with a considerable carbohydrate concentration depending on the variety and maturity of olives used (Guillen et al., 1992).

According to the literature, the main components of olive pulp are: lignin (22-28%), glucose (16-20%), xylose (12-20%), and arabinose (1,9-2%) on a dry-weight basis (Georgieva et al., 2007; El Asli et al., 2009).

Southern Europe (i.e., Spain, Italy and Greece) is the world's largest producer of olive oil (79% of world olive oil production) and thus generates approximately 8 million tons of OP per year (UNCTAD, 2007: http://www.unctad.org/infocomm).

At present, OP is either rejected to the environment or combusted with low calorific value. Although combustion offers the possibility of recovering energy in the form of steam and/or electricity, it requires suitable and expensive power plants, the combustion process itself resulting in high operating costs to control air emissions and dispose off residual ash.

Composting of OP as an alternative to chemical fertilizer for crop production is another possible treatment of this agro-industrial waste leading to its detoxification and stabilization. However, the high water content of OP requires large amounts of bulking materials, resulting in prohibitively large volumes of compost to be handled (Filippi et al., 2002).

Utilization of OP carbohydrates (cellulose and hemicelluloses) for ethanol production could be an attractive and sustainable solution to dispose of this agro-industrial waste. Although utilization of OP for production of bioethanol might not remove the main pollutants (phenolic compounds), the production could still be advantageous.

Pretreatment of OP using 1.75% (w/v) sulfuric acid at 160 °C for 10 min yielded a fermentation medium cultured using an engineered strain (*E. coli* FBR5), containing 8.1 g of ethanol/L (El Asli & Qatibi, 2009).

A wet oxidation pretreatment, followed by enzymatic hydrolysis, was applied by Haagensen et al. (2009). In this work different concentrations of enzymes and enzymatic durations were tested. Both wet oxidation and enzymatic treatment were evaluated on the basis of the ethanol obtained in a subsequent fermentation step by *S. cerevisiae* and *Thermoanaerobacter mathranii*. A four-day hydrolysis was adequate for a satisfactory release of glucose and xylose. The combination of wet oxidation and enzymatic hydrolysis resulted in a glucose and xylose concentration increase of 138 and 444%, respectively, compared to 33 and 15% when only enzymes were added. However, the highest ethanol production was obtained when using the only enzymatic pre-treatment, this implying that wet oxidation cannot be recommended for olive pulp processing at the conditions tested. By increasing the solid content in the slurry, there was no negative effect on simple sugar release, indicating that the cellulose and xylan content of the olive pulp was easily available.

Ballesteros et al. (2001) reported the production of ethanol by a simultaneous saccharification (by the addition of cellulases) and fermentation process, using the two main components of olive mill wastes (stones and olive pulp) as substrates. Pre-treatment of fragmented olive stones by sulfuric-acid-catalyzed steam-explosion (210 °C for 4 min with 0.5% w/v H_2SO_4) increased the enzymatic digestibility. On the other hand, in the case of olive pulp only, it was demonstrated that the steam- explosion process when adding no sulfuric acid was not ineffective. In fact, although 72% of the hemicelluloses and 67% of the cellulose present in the pulp dissolved during the pretreatment, practically no free sugars were detected in the filtrate, indicating almost total degradation of the dissolved sugars. Thus, a pretreatment of the olive pulp was not necessary in order to improve the enzymatic hydrolysis. The yield of the enzymatic hydrolysis (expressed as glucose obtained in the enzymatic hydrolysis divided by potential glucose in the raw material) was in the range 38–49%, and concentrations of >10 g l^{-1} of glucose after the hydrolysis could be obtained. It was also demonstrated that the SSF process was better when the fed-batch procedure was used than when substrate was added all at once. In this way, *Kluyveromyces marxianus* was capable of fermenting at high substrate concentrations with minimum inhibitory effect by phenols and tannins present in the feedstock.

Pre-treatment of olive mill wastes with hot water (200-250 °C) in a stirred autoclave, combined with the use of fed-batch procedure is another option to improve the production of ethanol. In particular, pretreatment of OP slurry at 20% (w/v) at 210 °C resulted in the best yield of 12 g ethanol/L. Simultaneous saccharification and fermentation (SSF) of untreated olive pulp resulted in ethanol yields close to 67% of the theoretical value (Ballesteros et al., 2001). Higher SSF yields (about 80%) were achieved using hot-water-pretreated olive pulp with the same microbial strain. The increase in SSF yields is due to higher enzymatic hydrolysis yield (90%) and higher fermentation efficiency. Thus, hot-water pretreatment was effective for two reasons. It increased, from one side, the cellulose accessibility of olive pulp to enzymatic attack, while, from the other one, it removed some fermentation-inhibiting compounds, such as tyrosol and hydroxytyrosol (Ballesteros et al., 2002).

A steam pretreatment (200°C for 5 min) was also tested by Rodriguez et al. (2007) on alperujo, the two-phase olive-mill waste. This process reduced appreciably the hemicellulose concentrations (75-88%) and removed a substantial portion of Klason lignin and protein (50%). Cellulose was very resistant to autohydrolysis and acid-catalyzed hydrolysis, thus representing the main component of OP (15-25 g/100 g of dry and defatted steam-treated alperujo). The steam-treated material was efficiently saccharified with commercial cellulases.

The enzymatic hydrolysis of untreated OP slurry at different dry matter levels, as well as the subsequent glucose fermentation by baker's yeast, was evaluated to optimize ethanol production from OP (Georgieva & Ahring 2007). The enzymatic hydrolysis increased the glucose concentration by 75%. Moreover, glucose liberation was almost independent from the slurry solid content and enzyme loading used. The fermentation step using *S. cerevisiae* was carried out without nutrient addition and gave rise to a maximum ethanol production of 11.2 g L^{-1}, revealing yeast tolerance to OP toxicity.

Despite OP is one of the most investigated food processing residues for bioethanol production, there is some discordance among the various studies about the need of a pretreatment step. Owing to the high percentage of lignin, deriving from stone fragments, that is present in this matrix it can be stated that in most of the cases a high temperature and pressure pretreatment could help the depolymerization of the cellulose. Thus, since the process variables seem to be already optimized, the main issue to be solved is to make the pretreatment as cheap as possible, in view of a scaling up of the process.

2.4.2 Sugarbeet pulp

Processing of pectin-rich crops generates residues abundant in carbohydrates which can be fermented to ethanol. Sugar-beet pulp is the solid residue of sugar production from sugar-beet. On a dry weight basis, over 1.6 x 10^6 tons of sugar beet pulp (SBP) remain after extraction of sucrose. Sugar beet pulp is primarily composed of polymers of cellulose (20-24%), hemicellulose (25-36%), pectin (19-25%), and lignin (1-5.6%) on a dry weight basis (Wen et al., 1988). Over 400 million tons of sugar-beet are produced annually worldwide (Clarke & Edye, 1996). During sucrose extraction, a pressed form of sugar-beet pulp (75% moisture) is generated and is dried and pelletized (10% moisture) for sale as cattle feed. (Sutton et al., 2001).

High carbohydrate and low lignin content make sugar-beet pulp an attractive substrate for ethanol production. However, the polymers must be broken down into simpler carbohydrates. After reducing the polymers to monomeric carbohydrates, beet pulp contains primarily arabinose, glucose, and galacturonic acid, along with smaller amounts of other sugars (Micard et al., 1996).

Sutton et al. (2001) evaluated ethanol production from sugar beet pulp by a bioengineered ethanologenic *Klebsiella oxytoca*. Fermentations were conducted with and without fungal enzyme supplementation. Without fungal enzyme supplementation, *K. oxytoca* produced 5.4 g ethanol L^{-1} from pelletized pulp (106 g dw L^{-1}), and 7.0 g ethanol L^{-1} from pressed pulp (106 g

dw L^{-1}). Inclusion of fungal enzymes (60 mg cellulase and 30 mg pectinase/g dw SBP) increased ethanol production to 15.5 g ethanol L^{-1} using pelletized pulp, while fermentation of pressed pulp produced 18.3 g ethanol L^{-1}. A pre-incubation step at 42 °C and pH 5.0 before fermentation of pressed beet- pulp with cellulose and pectinase decreased viscosity, increased mixing, and increased ethanol production from 18.3 g L^{-1} after 120-h fermentation to 21 g ethanol L^{-1} after 96-h fermentation.

The higher levels of ethanol produced from pressed rather than from pelletized forms of sugar-beet pulp without any mechanical treatment were economically advantageous, also because pulp drying and pelletizing increased the total annual energy costs by 25 to 40%. There was moreover no need for additional nutrient supplementation to sustain ethanol production from beet pulp (Grohmann & Bothast, 1994a).

In conclusion the finding that the high content of mineral nutrients in such substrate makes the addition of nutritional supplementation unnecessary shows promise for a successful scaling up of the process, since the use of water, without mineral nutrients, in large-scale fermentations would reduce overall costs in ethanol production from biomass.

2.4.3 Sugarcane bagasse

One of the major lignocellulosic materials found in great quantities to be considered, especially in tropical countries, is sugarcane bagasse (SCB), the fibrous residue resulting from juice extraction from sugar cane (*Saccharum officinarum*) in the sugar production process (Martín et al., 2007). SCB is primarily composed of lignin, cellulose and hemicelluloses, representing 20-30%, 40-45% and 30-35% on dry-weight basis, respectively (Peng et al., 2009).

In general, 1 ton of sugar-cane generates 280 kg of bagasse, and 5.4 x 10^8 dry tons of sugarcane are processed annually throughout the world (Cerqueira et al., 2007). About 50% of this residue is used in distillery plants as a source of energy (Pandey et al., 2000b), while the remainder is stockpiled. Therefore, because of the importance of SCB as an industrial waste, there is great interest in developing methods for its biological conversion into biofuels (Adsul et al., 2004; Cardona et al., 2010).

SCB has proven to be a feasible raw material for fuel ethanol production due its relative low lignin content and high production of sugars by appropriate pretreatments. Some of the more currently advances in fuel ethanol production using bagasse reported alcohol yields up to 48% (% w/w of reducing sugars). Hernández-Salas et al. (2009) have pretreated the whole SCB and different fractions of it by dilute acid (HCl) and alkaline pretreatment (NaOH). Selected

hydrolysates were fermented with a non-recombinant strain of *S. cerevisiae* with a maximum alcohol yield of 32.6%.

By using *Pachysolen tannophilus* DW06 to ferment a SCB hydrolysate obtained from acid pretreatment (H_2SO_4) and detoxified with electrodialysis, the ethanol yield reached 34% (Cheng et al., 2008). Higher yields were obtained by Chandel et al. (2007) with *C. shehatae* NCIM 3501 fermenting hydrolysates of SCB obtained with dilute acid (HCl) pretreatment and different detoxification methods: 48% with an industrial ion exchange resin, 42% with activated charcoal and 37% with laccase (from *C. stercoreus*). Lower ethanol yields were obtained with overliming (30%) and neutralization (22%).

Ethanol production from a hydrolysate of sugar-cane bagasse by *Pichia stipitis* DSM 3651 was studied. The medium was composed by raw (non-detoxified) hydrolysate or by hydrolysates detoxified by pH alteration followed by active charcoal adsorption or by adsorption into ion-exchange resins. An ethanol yield of 20% was obtained using the non detoxified hydrolysate, while the detoxification by pH alteration and active charcoal, and the detoxification by adsorption into ion-exchange resins provided a yield of 30% (Canilha et al., 2009).

Carrasco et al. (2010) obtained an ethanol yield of 36% from xylose- and glucose-rich liquid resulting from SO_2-catalyzed steam pretreatment, using the recombinant *S. cerevisiae* strain TMB3400. The best xylose conversion was achieved by the natural xylose-utilizing yeast *Pichia stipitis* CBS 6054.

In conclusion, the main problem related to the use of SCB is that the cost of the end-product is relatively high based on current technologies, mainly because of the presence of high lignin levels that require harsh pretreatments. The main challenge is to reduce hydrolysis costs to make SCB a cheaper substrate, like molasses and other directly fermentable materials. Furthermore, the conversion of xylose is obviously crucial, since it represents about 1/3 of the totally mass of sugars, thus a suitable microorganism is needed that efficiently ferments both pentoses and hexoses (Cardona et al., 2010).

2.4.4 Grape pomace

Grape pomace (GP) is the solid residue resulting from the extraction of grape juice. It makes up for 11-15 % of grapes crushed and has a humidity of about 70%. One ton of pomace contains about 249 kg of stalks, 225 kg of grape seeds and 425 kg of grape pellicles. The main chemical components of GP are carbohydrates, phenols and lipids. The amount of carbohydrates can range from 12 to 48%. The great differences in GP carbohydrate content depends on the moisture left

in pressed GP. The juice that is left in GP, in fact, contains sugars which will adhere to the skins when they are dried and thus increase the carbohydrate content. Phenols and lipids content range from 3.9 to 8.2 and from 4.4 to 5.2 % on dry weight basis, respectively. The annual production of grape pomace is estimated to range from 5 to 14.5 million tons in Europe, where the 59% of world wine production takes place (Kammerer et al., 2004). In the past, the common practice of direct incorporation of grape pomace into agricultural land has caused serious problems, since degradation products could inhibit root growth. An alternative to overcome such disadvantages and to recycle wastes is composting or using GP as fermentation medium to produce a variety of compounds, such as enzymes, polyphenols, citric acid, gluconic acid, bioethanol, etc. (Nerantzis & Tataridis, 2006).

Ethanol production by solid-state fermentation (SSF) of grape (GP) and sugar beet (SBP) pomaces using *S. cerevisiae* was compared to that in submerged fermentation of sugar-beet juice (Rodrìguez et al., 2010). The ethanol yields in SSF were equal to or greater than those in liquid fermentations. Maximum ethanol concentrations were achieved after 48 h both for GP and SBP. Ethanol concentration in SSF was higher than in liquid fermentation, this making its recovery less expensive. An important decrease in waste mass was attained in SSF.

Bioethanol production from GP asks for further studies before attempting to scale-up such process.

2.4.5 Banana peels

The banana processing industry generates a huge amount of solid wastes, mostly composed of peels. The main components of banana peels are fibers (31.7%), glucose (16%), sucrose (50%), ash (8.5%), lipids (1.7%), and proteins (0.9%) (Anhwange et al., 2009). Banana peels represent 30% of the fruit and total annual world production is estimated at 95.6 million tons of fruits (FAO, 2006). Banana peels are often dumped in landfills, rivers, oceans and unregulated dumping grounds. Therefore, their reutilization would help to diminish the pollution problems caused by their disposal. This kind of waste offers many possibilities of reuse, such as matrix for toxic compounds removal, feedstock for pectin, antioxidants, enzymes or biofuels.

Little research has been done about bioethanol production from banana peel waste.

Manikandan et al. (2008) used five mutant strains of *S. cerevisiae* and evaluated the effect of temperature, pH and initial substrate concentration on ethanol yield. By pretreating banana peels with 2 ml/g of 67% sulphuric acid as a catalyst and steaming the slurry at 10% by weight for 60

min, the best ethanol-producing strain produced 9.8 g ethanol/L. The optimum temperature and pH for this strain were 33 °C an 4.5, respectively.

Sharma et al. (2007) used steam-pretreated banana peels together with kinnow waste as substrate in the ratio 4:6 (kinnow waste: banana peels) to evaluate the role of some fermentation parameters, like inoculum concentration, temperature, incubation period and agitation time on ethanol production by simultaneous saccharification and fermentation using cellulase and co-culture of *S. cerevisiae* and *Pachysolen tannophilus*. Best ethanol production was observed by setting temperature to 30°C, inoculum size to 6% (v/v) for *S. cerevisiae* and 4% (v/v) for *Pachysolen tannophilus*, incubation period to 48 h, and agitation for the first 24 h. The pretreated steam- exploded biomass after enzymatic saccharifycation containing 63 g L^{-1} reducing sugars was fermented with both hexose and pentose fermenting yeast strains under optimized conditions resulting in ethanol production, yield and fermentation efficiency of 26.84 g L^{-1}, 0.426 g g^{-1} and 83.52% respectively.

In conclusion, the fermentation parameters for bioethanol production from banana peels have already been optimized at lab scale. Further scaling-up to a pilot-scale is need to make the process more cost effective.

2.4.6 Corncobs

Corncobs are the central cores of maize ears and form about 30% of the corn stover, that amounts to about 80-100 million tons per year (Kadam & Mc Millan, 2003). Corncobs are composed of about 45% cellulose, 40% hemicellulose and 15% lignin (Zhang et al, 2010).

The cobs produced from corn are underutilized, being mostly used as manure for agricultural production or burnt as fuel in households (Yah et al., 2010). Utilization of corncobs for second generation bioethanol production has been reported in several studies and represents a valid opportunity for the utilization of such feedstock.

De Carvalho Lima et al. (2002) used the recombinant *Escherichia coli* KO11 for the fermentation of corncob hydrolyzates containing xylose as the dominant sugar. When inoculum was grown on LB medium containing glucose, fermentation of the hydrolysate was completed in 163 h and the maximum ethanol yield (0.50 g ethanol g^{-1} sugar) was obtained.

Chen et al. (2007) investigated enzymatic hydrolysis of corncob after pretreatment with 1% H_2SO_4 at 108 °C for 3 h and ethanol fermentation from cellulosic hydrolysate. Pretreated solid was hydrolyzed by cellulase from *Trichoderma reesei* ZU-02 and cellobiase from *Aspergillus niger* ZU-07 and the hydrolysis yield was 83.9%. Further fermentation of cellulosic hydrolysate

containing 95.3 g L^{-1} glucose was performed using *Saccharomyces cerevisiae* 316, and 45.7 g L^{-1} ethanol was obtained within 18 h.

Zakpaa et al. (2009) used *Aspergillus niger* isolated from soil to hydrolyze corncobs into simple sugars. Filtrate obtained from corncobs broth fermented by *A. niger* was then used as crude enzyme in optimization tests on corncobs powder suspended in 50 mM citrate buffer pH 5.0. Optimum temperature, pH and substrate concentration for saccharification were 40 °C, 4.0 and 6% respectively. *Saccharomyces cerevisae* was subsequently added to *A. niger* filtrate to cause fermentation of the corncobs. The highest ethanol concentration of 0.64 g L^{-1} was recorded over the 24 h fermentation period.

Zhang et al. (2010) pretreated corncob with acid and alkali to remove non-cellulose components, and then performed a simultaneous saccharification and fermentation (SSF) with cellulose loading of 22.8 FPU/g glucan and dry yeast (*Saccharomyces cerevisiae*) loading of 5 g L^{-1}. An ethanol concentration as high as 69.2 g L^{-1} with 19% dry matter (DM) was achieved, resulting in an 81.2% overall ethanol yield.

Another recent study (Yah et al., 2010) reports dilute sulphuric acid and enzymatic hydrolysis methods for sugar extraction from corncobs, the acid hydrolysis giving a higher amount of sugars than enzymatic hydrolysis. Optimal temperature and time for sugar fermentation by two yeast strains (*S. cerevisiae* and *P. Stipitis*) were approximately 25°C and 50 h, respectively. Bioethanol produced at this temperature was 11.99 mg mL^{-1}. Data obtained revealed that xylose level decreased from 27.87-3.92 mg mL^{-1} during the first 50 h of fermentation and complete metabolism of glucose was observed during this time.

Bioethanol production from corncobs has only been optimized at lab-scale. However, from the aforementioned reports it can be concluded that acid pretreatments are a good option to maximize sugars yield, and xylose fermentation is crucial for the final ethanol yield of the process, given the high concentration of this sugar in corncob hydrolyzates.

2.4.7 Orange peel waste

Orange peel waste (OPW) is the solid residue left after juice extraction from oranges. This kind of waste consists of peel, rag (segment membranes and cores), juice sacs, and seeds and accounts for approximately 44% of the wet fruit mass (Braddock, 1999). The dried OPW (10% moisture) is composed of sugars (30-40%), pectin (15-25%), cellulose (8-10%) and hemicellulose (5-7%) (Grohmann et al., 1995). Citrus fruits are among the most important fruits grown and consumed

all over the world. Oranges alone account for about 55% of the global citrus fruit production, that is reported to be over 88 million tons per year (Marin et al., 2007).

Despite being rich in nutrients, citrus fruit residues do not find any commercial importance and are largely disposed in municipal dumps or dried to produce cattle feed (Tripodo et al., 2004). Since drying OPW consumes a large amount of energy, the profit of making cattle feed is marginal or negative, particularly when the energy cost is high. The presence of low lignin levels makes such a substrate ideal for fermentation-based products, such as ethanol.

Enzymatic hydrolysis of OPW is highly sensitive to the complex structures of polysaccharides (pectin, cellulose and hemicellulose). Grohmann & Baldwin (1992) investigated the hydrolysis of polysaccharides in comminuted orange peel by commercial cellulase and pectinase enzymes and found that high levels of conversion to monomeric sugars were observed after treatment with pectinase enzyme, but cellulase enzyme alone achieved only limited solubilization. The combination of cellulase and pectinase, instead, appeared to be the most efficient system for enzymatic hydrolysis of polysaccharides in orange peel.

Grohmann et al. (1994b) evaluated the fermentability of enzymatic hydrolyzates of OPW by the yeast *S. cerevisiae* and concluded that such a process was technically simple but from the economic point of view was hampered by the small increase in the yield of fermentable sugars after enzymatic hydrolysis.

Grohmann et al. (1996) investigated the fermentability of orange peel enzymatic hydrolyzate supplemented with nutrients by the recombinant bacterium *Escherichia coli* KO11, and found that the addition of supplements resulted in an increase of ethanol yield (maximum value amounted to about 75-85%).

Wilkins et al. (2005) studied the effect of seasonal variation on enzymatic hydrolysis of OPW and concluded that arabinose and galacturonic acid yields were affected by the harvest season.

A recent study (Wilkins et al. 2007c) reports the hydrolysis of another citrus peel waste (grapefruit peel waste) but was included in this paragraph for the sake of simplicity. On this feedstock different loadings of commercial cellulase and pectinase enzymes and pH levels were tested. It was found that 5 mg pectinase and 2 mg cellulase g^{-1} peel dry matter were the lowest loadings to yield the most glucose (about 22% dm).

A problem related to the fermentation of OPW hydrolyzates is the presence of limonene, the main component of orange peel oil, which is reported to be an inhibitor of yeast fermentation. Wilkins et al. (2007a) assessed the minimum peel oil concentration inhibiting ethanol production by *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* after 24, 48 and 72 h. Minimum

inhibitory peel oil concentrations for ethanol production were 0.05% at 24 h, 0.10% at 48 h, and 0.15% at 72 h for both yeasts.

Wilkins (2009) performed a similar experiment using *Zymomonas mobilis* and assessed that minimum inhibitory orange peel oil concentrations for ethanol production at 30 °C were 0.05% after 24 h, 0.10% after 48 h, 0.15% after 72 h, and 0.20% after 96 h, while minimum inhibitory orange peel oil concentrations for ethanol production at 37 °C were 0.05% after 24 h, 0.10% after 48 h, and 0.20% after 72 h. Orange peel oil did not inhibit ethanol production after 96 h at a temperature of 37 °C.

Since limonene is a valuable co-product, pretreatments to maximize limonene removal for its economical recovery are to be preferred. Because limonene is a volatile compound, immiscible with water, steam stripping has been considered an effective pretreatment to remove and recover limonene from OPW (Odio, 1996).

The following studies report various pretreatment methods aimed at maximizing sugar yield and/or separate limonene from OPW.

Grohmann et al. (1995) studied the fractionation and pretreatment of OPW by dilute acid hydrolysis. In particular, solubilization and depolymerization of carbohydrates by treatment of orange peel with dilute (0.06 and 0.5%) sulfuric acid at 100, 120 and 140 °C was investigated. Only soluble sugars and sugars derived from hydrolysis of hemicelluloses were efficiently released by the treatment with hot dilute sulfuric acid, while cellulose and segments of pectin containing galacturonic acid units were very resistant to acid-catalyzed hydrolysis. Anyway, the treatment with dilute sulfuric acid had a positive effect on the rate of subsequent enzymatic hydrolysis of orange peel by a mixture of cellulolytic and pectinolytic enzymes. Thus, the compact structures and crosslinks of polysaccharides could hinder the enzymatic hydrolysis of unpretreated OPW. Thus, similar to the enzymatic hydrolysis of other cellulosic materials, appropriate pretreatments are needed to open the structure of citrus polysaccharides and make them more accessible to enzymes.

Wilkins et al. (2007b) evaluated the effects of *D*-limonene concentration, enzyme loading, and pH on ethanol production from simultaneous saccharification and fermentation (SSF) of OPW by *S. cerevisiae* at 37 °C. Prior to SSF, OPW underwent a steam-explosion process to remove more than 90% of the initial *D*-limonene present in the peel waste. Ethanol concentrations after 24 h reduced as the initial D-limonene concentration was greater than or equal to 0.33% (v/v). Ethanol production was also reduced when enzyme loadings were (IU or FPU/g peel dry solids) less than 25 for pectinase; 0.02 for cellulase; and 13 for β-glucosidase. Fermentations with an initial pH of 6.0 produced more ethanol than fermentations with initial pH of 5.6, 5.0, and 4.4.

The pH after 48-h fermentations with an initial pH of 6.0 was 4.9, which is ideal for both *S. cerevisiae* and the cellulase enzyme used in the study.

Talebnia et al. (2008) investigated the effects of time, acid concentration, temperature and solid concentration on dilute-acid hydrolysis of OPW and found that hydrolysis up to 210 °C was not able to hydrolyze pectin to galacturonic acid, while the sugar polymers were hydrolyzed at very low temperature. The best results (i.e., total sugars of 41.8% dry matter with 2.6% of total hexoses degraded to hydroxymethyl furfural) were yielded by operating at 116°C, 0.5 % (v/v) sulphuric acid concentration, 6% solid fraction, and 12.9 min retention time.

Widmer et al. (2010) applied steam-explosion for pretreating OPW under various conditions of pH, process temperature, and time to determine their effects on limonene removal, solubilization, carbohydrate composition, and ethanol yields after a simultaneous saccharification and fermentation process. Pretreatments at 160 °C for longer than 4 min with steam purging were needed to remove limonene to below 0.1%. The same pretreatment allowed a total solubilization of hemicelluloses, while just 70% of the pectin was solubilized in natural OPW compared to over 80% after pretreatments using acid modified OPW (pH 2.8). Pretreatments at 160 °C on alkaline modified OPW (initial pH 6.8) were also tested. Although this kind of pretreatment quickly destroyed pectin, and had significantly lower dissolved solids, the resulting material was excessively viscous. The SSF process, performed by *S. cerevisiae* with the addition of cellulase, pectinase and β-glucosidase, was not affected by acid or alkaline adjustment of OPW. Ethanol yields (based on sugar content after enzymatic hydrolysis) ranged from 76% to 94% of the theoretic yield after 48 h simultaneous saccharification and fermentation.

Boluda-Aguilar et al. (2010) applied a steam explosion pretreatment, followed by SHF or SSF, to another citrus peel waste, that is mandarin peel wastes. As the study reporting enzymatic hydrolysis of grapefruit peel waste (Wilkins et al. 2007c), this study was also reported in this paragraph for the sake of simplicity. It was concluded that SHF processing of steam-exploded mandarin peel waste gave higher sugar yields than the same process applied on non-pretreated mandarin peel waste, despite the higher enzyme loading used in the second case.

Oberoi et al. (2010) assessed the feasibility of a two-stage dilute-acid hydrolysis pretreatment to produce sugars from orange peel powder (OPP), to minimize the formation of inhibitory compounds [acetic acid, hydroxymethylfurfurals (HMFs), and phenolic compounds], and optimize ethanol productivity. Primary hydrolysis of OPP was carried out at acid concentrations ranging from 0 to 1.0% (w/v) at 121 °C and 15 psi for 15 min. It was found that when the acid level was beyond 0.5% (w/v), the production of HMF and acetic acid was higher and the sugar concentration decreased. Secondary hydrolysis of the residual biomass after primary hydrolysis

was carried out at 0.5% (w/v) acid. On the basis of the results obtained from the response surface methodology, a validation test was carried out in a 2 L batch fermenter at pH 5.4 and a temperature of 34 °C for 15 h. Because the sugar concentration in the hydrolysate obtained from primary and secondary hydrolysis was significantly different, separate fermentation was performed. Fermentation beyond 9 h in both cases did not result in any significant increase in ethanol concentration. Fermentation variables optimized through RSM were successful in producing ethanol with a yield of 0.46 g/g on a substrate-consumed basis and a high volumetric productivity of 3.37 g L^{-1} h^{-1} in a batch fermenter.

Martin et al. (2008) used wet oxidation as a pretreatment method prior to the enzymatic hydrolysis of orange waste. The pretreatment, performed at 195 ºC and an oxygen pressure of 1.2 MPa, for 15 min, in the presence of Na_2CO_3, increased the cellulose content of the materials, gave cellulose recoveries of approximately 90% and enhanced the susceptibility of cellulose to enzymatic hydrolysis. Ethanol yield from total reducing sugars during the fermentation of the filtrate of OPW resulted to be 0.40 g/g.

Zhou et al. (2008) assessed the techno-economical feasibility of the entire process of bioethanol production from OPW with pilot-plant studies. In particular, continuous pretreatment and batch SSF were successfully tested in a pilot-plant scale (10,000-gal mash/batch fermentation) using *S. cerevisiae*. Pretreatment (steam explosion) removed more than 90% of the limonene in OPW at temperatures of 150 to 160 °C. Using SSF, a fermented mash containing 4% to 5% ethanol by volume could be achieved in less than 20 h. It was also demonstrated that ethanol could be stripped off the fermented OPW by single-stage batch distillation. Although ethanol production and limonene recovery from OPW together with subsequent drying of the residues left after distillation for use as a cattle feed resulted to be feasible, the long-term economic viability of this process might be greatly improved by recovering additional high-value co-products from the distillation residues.

Pourbafrani et al. (2010) investigated the production of ethanol, biogas, pectin and limonene in an integrated process from dilute-acid hydrolyzed orange peel waste. The best sugar yield (0.41 g/g of the total dry citrus waste (CW) was obtained by dilute-acid hydrolysis at 150 °C and 6-min residence time, while an ethanol yield of 0.43g/g of fermentable sugars was obtained using a wild-type *S. cerevisiae* strain.

By referring to the experimental data by Pourbafrani et al. (2010), Lohrasbi et al. (2010) attempted to design an industrial-scale process and estimate its revenue on the investment. An overview of the process flow-sheet is shown in Fig. 2.10.

The plant receives citrus wastes that are conveyed to the hydrolysis reactors. The hydrolyzates are then explosively flashed-out to an expansion tank to cool down at atmospheric pressure. The vapors leaving the expansion vessel strip off almost 99% of the limonene content of CW, and reduce its concentration in the hydrolyzate to 0.004% (v/v). Limonene-containing vapor is condensed, and the organic limonene phase is recovered from water phase in a decanter. A filter press is employed to separate solid residues from the hydrolyzate slurry. Solids are then washed with recycled water from distillation. It results in approximately 96% sugar recovery from the solid materials. The supernatant hydrolyzate is then neutralized with lime in a mixing-vessel and then fed to the fermentor. A continuous fermentation process with cell recycling and a flocculating yeast strain of *S. cerevisiae* is employed to produce ethanol. The effluent from the fermentor is settled in a cone-shaped settler with 30-min retention time. The settler has two outlets: a down-flow line which continuously brings the yeast back to the bioreactor, and an up-flow line placed on the top of settler which transfers the ‘‘beer” to the distillation columns. In order to keep the cells productive, the recycled cells are aerated in a vessel prior to being added to the reactor. The ‘‘beer” is fed to the distillation columns, in which 99% of the ethanol is recovered. The solid residues of the hydrolyzates are mixed with stillage of the distillation columns, and fed to anaerobic digesters. The retention time inside the digesters is 20 days and a biogas with composition of 41% methane and 50% CO_2 is produced. The anaerobic digester effluents are introduced to the wastewater treatment section. The sludge including suspended solids and cell mass is first settled and removed by gravity sedimentation and filtration to reduce the COD load. The supernatant effluent, with estimated COD load of 3,230 mg L^{-1}, is then treated in a conventional wastewater treatment process with activated sludge wherein 60% of incoming COD is converted to CO_2 and water, and 30% of the COD is converted to sludge. The remaining COD level in the treated water is estimated to be lower than 325 mg L^{-1} (10% of the total COD load), which could be drained into a domestic sewage system (the typical COD load of domestic sewage is 250–1000 mg L^{-1}). The total cost of ethanol for base case process with 100,000 tons/year CW capacity was calculated as 0.91 USD/L, assuming 10 USD/ton handling and transportation cost of CW to the plant.

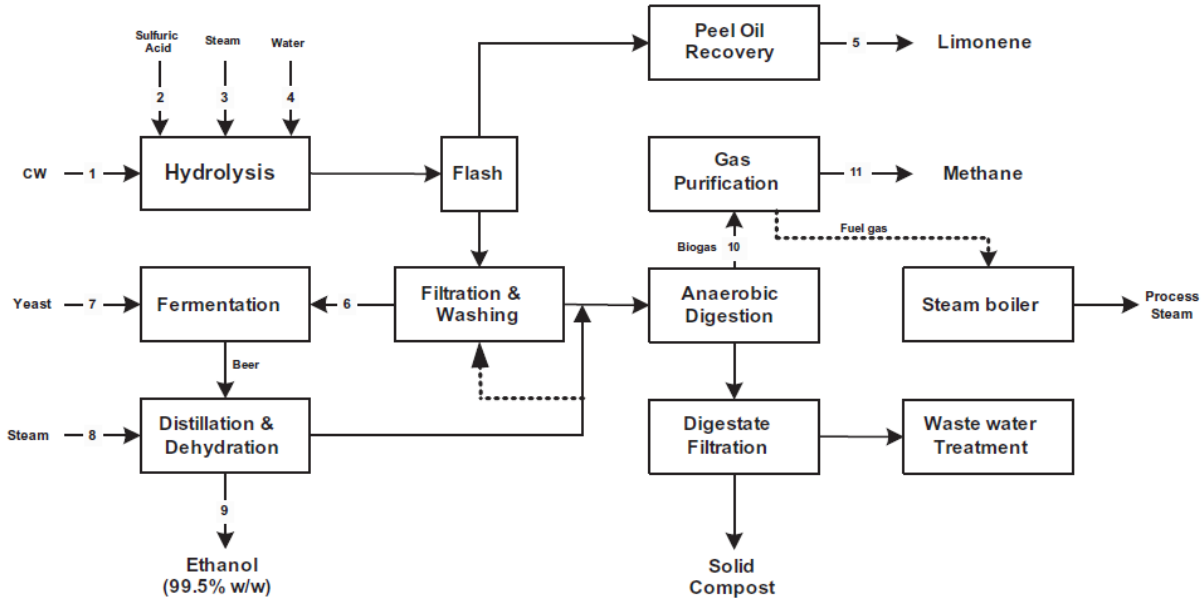


Figure 2.10 Overview of a hypothetic industrial-scale citrus waste biorefinery to ethanol, biogas and limonene.

Coll et al. (2009) also presented a project, called ATENEA, for bioethanol production from OPW at a semi-industrial scale by pretreatment + SSF (the pretreatment technology not being specified, Fig. 2.11), through the adaptation of a pre-existing demonstration plant.

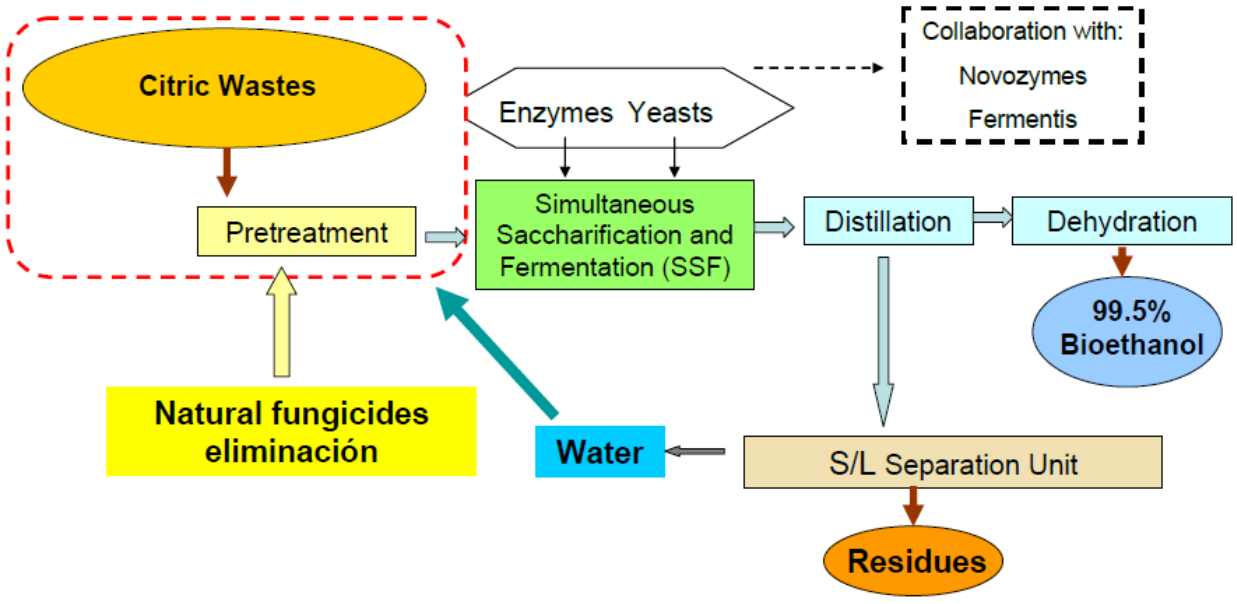


Figure 2.11 Flow-sheet of the process described in the ATENEA project for bioethanol production from OPW at semi-industrial scale.

Most of the papers dealing with bioethanol production from orange peel waste seem to agree on steam-explosion as best pretreatment prior to the enzymatic hydrolysis of such feedstock. As for

the other substrates, this is the critical point of the entire process. The above mentioned process design also highlights that the major costs are associated to the energy consumption. Thus, more research has to be made in this direction, in order to reduce the energetic requirements, at the same time maintaining or improving the overall yield of the process.

3. AIMS OF THE STUDY

Recovery and re-use of solid by-products and wastes generated by food-processing industries may be an option to face the environmental problems that these residues are causing (Mahro & Timm, 2007). Lignocellulosic fiber materials, in particular, are the most abundant fraction of the biogenic wastes produced by food industries and represent an interesting resource of biomass for second generation bioethanol production.

The crucial point of bioethanol production from lignocellulose is represented by the pretreatment step, that is required to improve the enzymatic depolymerization of the biomass. All the pretreatment technologies are usually accomplished under severe reaction conditions (high temperatures and pressures), this involving a large capital investment, high processing costs and great investment risks. These are also affected by lignin and hemicellulose content, waste materials porosity and cellulose crystallinity. Thus, no ideal method has been so far identified since each kind of residue requires specific pretreatment conditions so as to maximize the susceptibility of the solid to enzymatic attack and to minimize the formation of degradation products hindering the fermentative process (Mtui, 2009).

The main aims of this PhD thesis project were the following:

- To characterize chemically three residues that are widely produced in the Mediterranean area (*i.e.*, orange peel waste, olive pomace, and grape pomace) to select the most suitable one for second generation bioethanol production. Although some information about the composition of such materials is available in the literature, their natural heterogeneity made necessary to determine the exact concentration of each component in the samples tested.
- To assess the ability of a novel lab-scale direct steam injection apparatus (DISA) to heat the selected feedstock up to the reaction temperature in very short times compared to the conventional lab-scale high-pressure/high-temperature reactors, where the too long heating times is the main cause of sugar degradation.
- To evaluate the effect of acid-catalyzed steam-explosion (ACSE) pretreatment, performed in the DSIA at different times and temperatures, on sugar release, pectin solubilisation and inhibitor formation.
- To optimize the depolymerization yield in the shaken-flask scale with respect to the cellulase and solid loadings for the saccharification of the cellulose present in the solids resulting from different combinations of ACSE times and temperatures.
- To assess the influence of the different combinations of ACSE times and temperatures on the ethanol fermentability of the enzymatic hydrolyzates by the industrial strain *Saccharomyces cerevisiae* F15 in the repeated batch mode.

- To scale-up the best ACSE-pretreatment conditions using a triple solid loading, carrying out the enzymatic hydrolysis in a 7-L stirred bioreactor and the fermentation of the resulting hydrolyzate in a 1-L STR reactor under repeated batch mode using the aforementioned yeast strain.
- To estimate the overall process yield in L ethanol per MT of dry feedstock and propose a hypothetical process flow sheet for converting orange peel wastes into bioethanol.

4. MATERIALS AND METHODS

4.1 Raw Materials

In this work, dried orange peel waste (OPW) deprived of the essential oils, olive pomace (OP) and grape pomace (GP) were provided by Agrumigel s.n.c. (Barcellona P.G., ME, Italy), Frantoio di Viceno (Castel Viscardo, TR, Italy), and Barberani Vallesanta Farm (Orvieto, TR, Italy), respectively. OP and GP were frozen at -20 °C and lyophilized. All freeze-dried samples were ground to particle size smaller than 1 mm using a lab-scale MF109 miller (IKA, Staufen, Germany).

4.2 Compositional analysis of the feedstocks

Any freeze-dried and ground material was analyzed to assess the following components:

- pectin by using the method of Sudhakar & Maini (2000; §4.6.9);
- ash, gravimetrically determined after ignition at 500 °C for 4 h;
- phenols and tannins, once extracted according to a modification of the method of Makkar et al. (1993; §4.6.10) and determined using the method by Swain & Hills (1959; §4.6.11);
- condensed tannins by means of the method of Porter et al. (1985; §4.6.12);
- total nitrogen via the Kjeldahl method (Mariani et al., 1995; §4.6.13).

For the determination of the water-soluble fraction the feedstocks were submitted to water extraction in Soxhlet using the Extraction System B 811 (Büchi, Switzerland). Once the receiving vessel had been charged with 200 ml of deionized water, the thimble with about 6 g of sample, and the heating mantle had been adjusted to provide a minimum of 4-5 siphon cycles per hour, each sample was refluxed for about 6 hours. A sequential second extraction was performed by leaving the water extractives-free sample into the thimble and replacing the water containing the extracted compounds with a mixture of toluene and ethanol (2:1 v/v) in lieu of ethanol 100%, according to a modification of the method NREL/TP-510-42619. All the other operating conditions of the water extraction were kept unaltered (Sluiter et al., 2005). The amounts of water-soluble and toluene-ethanol extractives were gravimetrically determined. In particular, an aliquot of the water extract was dried using a boiling-water bath, while the toluene-ethanol extract was dried using the Rotavapor EL-130 (Büchi, Switzerland). The resulting extracts were then further dried at 100 °C to constant weight.

The aqueous extract was also analyzed for reducing sugars (§4.6.1), total sugars (§4.6.2), glucose and fructose (§4.6.3), uronic acids (§4.6.5) and proteins (§4.6.6).

The final extractives-free solid residue was analyzed to determine the cellulose, hemicellulose and lignin content (§4.6.7) and the total monosaccharides content (§4.6.8).

The analytical procedures for feedstock characterization are summarized in Fig. 4.1.

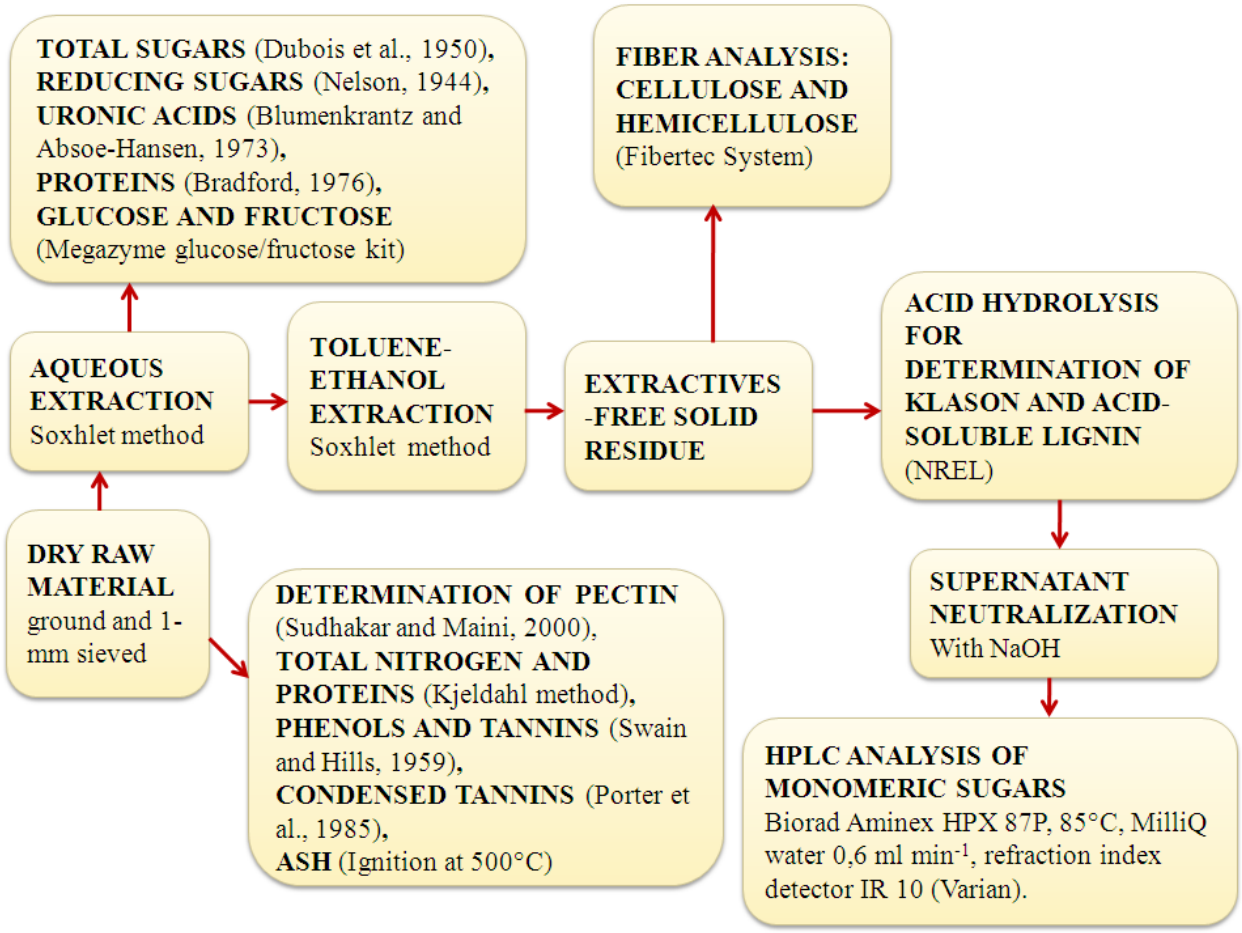


Figure 4.1 Flow-sheet of the procedure used to characterize of the feedstocks.

4.3 Steam explosion pretreatment of orange peel waste

4.3.1 Description of the apparatus

The flow-sheet of the lab-scale Direct Steam-Injection Apparatus (DSIA) is shown in Fig. 4.2. It was independently designed and constructed by DiMa e.c.d. Engineering Consulting and Development (Fiumicino, Italy).

A table cast-iron boiler type GVE 2 kW (Magnabosco Srl, Zugliano, Italy), mounted on a skid and powered by electricity, was used to produce saturated steam up to 30 bar. A plug valve EV1 allowed the automatic regulation of the steam inlet flow necessary to reach the set reaction temperature. The 4-dm^3 stainless steel reactor R (diameter and height of 250 and 105 mm, respectively) was internally lined with a hollow cylinder of polytetrafluoroethylene (Teflon) (internal diameter: 190 mm; thickness: 30 mm) to protect the reactor from acid corrosion, and closed with two flat heads. It was mechanically stirred using an axial flow impeller (diameter of

150 mm). To avoid stagnant particles near the reactor wall and bottom, the impeller consisted of two thin stainless steel rectangles (width, height and thickness of 55, 35 and 3 mm, respectively), that were welded to the agitator shaft along their wider side, cut at mid-height so as to bend the two fins at 90° in the reverse directions.

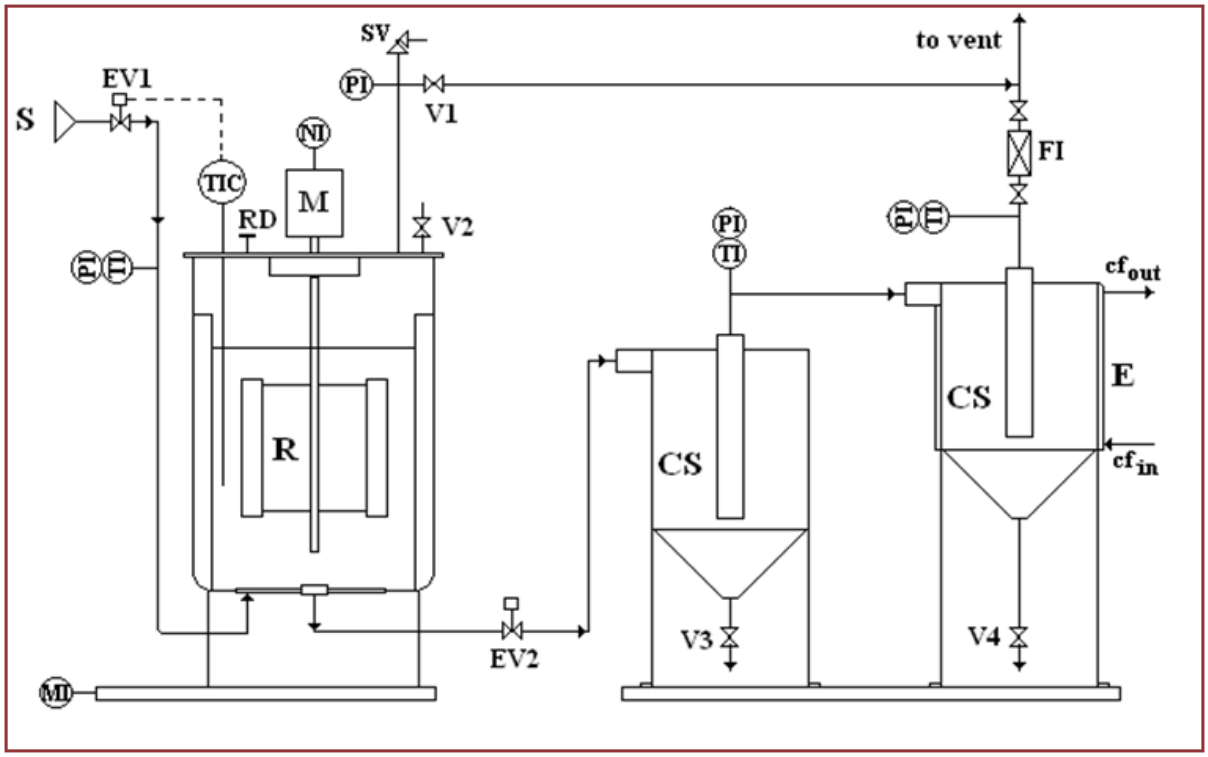


Figure 4.2 Process flow-sheet of the lab-scale Direct Steam Injection Apparatus (DSIA), designed by Prof. M. Moresi. Equipment and ancillary fluid identification items: **cf** – cooling fluid; **CS**- cyclone separator; **E** – jacket heat exchanger; **EV** – electrovalve; **FI** - filter; **M** - electric motor; **MI** – mass indicator; **NI** – stirrer speed indicator; **PI** - manometer; **R** – mechanically-stirred reactor; **RD**, rupture disk; **S** - high pressure steam; **SV** – safety valve; **TIC** - temperature indicator and controller; **V** - manual ball valve.

The impeller speed (N) ranged from 0 to 300 rpm and was operated using a 0.75 kW, 4-pole, three-phase alternating current asynchronous motor EFF2 type MS80B-4 (Melco-Able Motori Elettrici, Milan, Italy), piloted via a frequency inverter. On the lower flat head, an annular manifold (external and internal diameters of 123 and 153 mm) provided with a ring plug was used to sparge the high-pressure steam. On the same end cap an automatic piston valve EV2 was centrally positioned to flash-cool suddenly the reaction medium after a prefixed reaction time in the range of 5 s to 30 min. The top head was equipped with a safety-valve (SV) to release automatically the vapor from the vessel when the pressure exceeds a preset limit of 35 bar, an opening endowed with the ball valve V2 to perform preliminary reactor cleaning after any trial, and sensors to monitor the reaction temperature (T_R) and pressure (P_R) with top scale values of 300 °C and 40 bar and precision accuracy of 0.1 °C and 0.1 bar, respectively.

All the apparatus was mounted over an extensiometric strain gauge load cell type 500 K (Imsystem, Cagliari , Italy) with a top scale of 150 kg and accuracy of 0.1 g, so as to monitor the initial mass of the slurry, and, after zero resetting, that of the steam injected throughout the trial.

Once the electrovalve EV2 had been automatically opened, the mixture was dispatched into the first cono-cylindrical cyclone separator CS (volume of 12 dm^3) via a rectangular opening, tangential to the upper part of the cylindrical section so as to induce a vorticous spinning flow and centrifuge residual particles and liquor towards the external cylindrical wall and collect both phases downward along the wall of the conical section. By opening the sphere valve V3, it was possible to collect the resulting slurry for further essay as reported below.

The steam was significantly recondensed in the second CS which is thermostated at low temperatures by an external jacket circulated by a cooling fluid (cf).

The remaining vapor phase, released at atmospheric pressure, was discharged through the cylindrical tube of the second CS (diameter of 87 mm), that is called *overflow pipe* or *vortex finder* and is fixed in the centre of the top cover. The vapor is then fed to the bulb condenser to be partially condensed and collected into the stainless steel reservoir. All incondensable gases and residual vapors were discharged through an air vent via the filter FI to avoid dispersing the entrained finer particles in the atmosphere. A cooling fluid consisting of ethylene glycol at -5 °C was provided by a FL 300 Recirculating Cooler/Chiller (JULABO Labortechnik GmbH, Seelbach, Germany). By opening the ball valve V4, it was possible to recover the condensate to check for its mass, chemical composition and suspended matter content. In Fig. 4.3 (a and b) two pictures of the apparatus are reported.

a)

b)

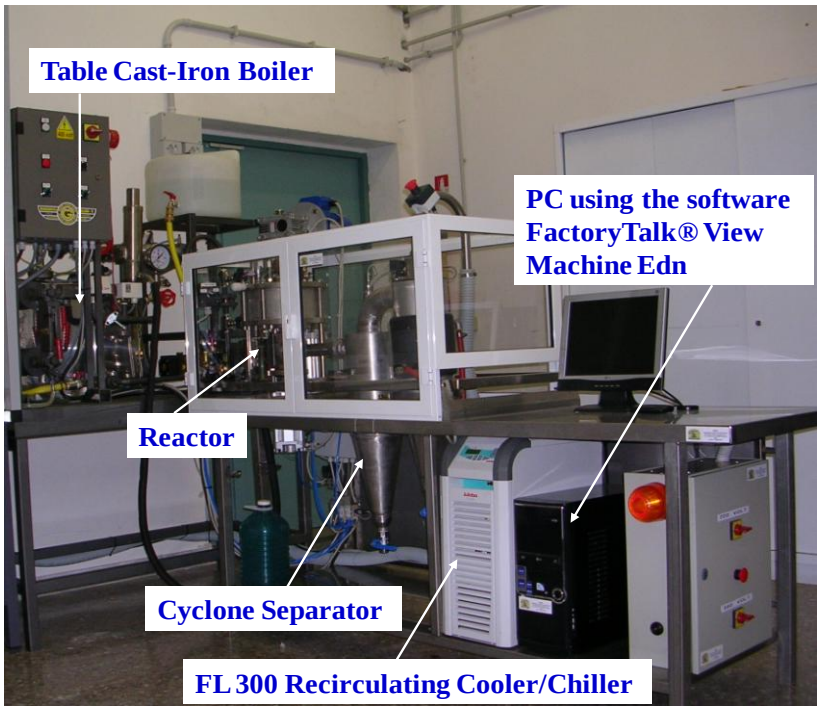


Figure 4.3 Pictures of the laboratory-scale Direct Steam Injection Apparatus used in this work: a) front view of boiler and reactor; b) overall view of the equipment.

The operating parameters (i.e., the amounts of de-ionized water, m_L, acid, m_A and biomass particles, m_{SSo}, charged into R; premixing time, t_m, and stirrer speed, N; reaction temperature, T_R, pressure, P_R, and time, t_R; cyclone top temperature, T_C, and pressure, P_C) were monitored and/or controlled via a personal computer using the software FactoryTalk® View Machine Edition (ME) (Rockwell Automation, Milwaukee, WI, USA).

4.3.2 Operating procedure

Once the top cover of the reactor R had been automatically opened, it was possible to charge 0.75 dm^3 of an aqueous solution of sulfuric acid at 0.5% v/v and 120 or 360 g of ground material and weigh them using the strain gauge load cell. After the top head had been closed, the process was started. After premixing the slurry at 200 rpm for 2 min, the automatic valve EV1 was opened to inject high-pressure steam and heat the slurry. Four different conditions of reaction temperature and time were tested: 200 °C for 90 s, 180°C for 150 s, 160°C for 240 s and 130°C for 500 s. Thereafter, the electrovalve EV2 was opened to flash the slurry to the atmospheric pressure. The reacted slurry was collected via the ball valve V3, while the condensed fraction of the vapor discharged via V4.

All the liquors extracted from the bottom of the second cyclone were discarded, being clear with no visible particle matter and an overall volume smaller than 50 cm^3.

The hydrolytic performance of DSIA was compared to that obtained in a laboratory steam autoclave type B0 (International PBI Srl, Milan, Italy) by treating 0.1 dm^3 of the same slurries at 130 °C for 60 min under acidic conditions (H_2SO_4 0.5% v/v).

Slurries arising from DSIA and conventional autoclave were thoroughly stirred to collect a homogeneous sample, that was vacuum filtered through pre-weighed glass filter (2.7-µm Sartorius Stedim MGD disc). The retained solid particles were washed twice with de-ionized water, dried at 105 °C overnight and their mass and cellulose amount determined (§4.6.7).

The liquid hydrolyzate was analyzed for glucose and fructose (§4.6.3), galactose (§4.6.4), reducing sugars (§4.6.1), phenols (§4.6.11), galacturonic acid (§4.6.14), acetic acid, formic acid, furfural and 5-hydrolymethylfurfural (5-HMF; §4.6.15).

4.4 Enzymatic hydrolysis of steam-treated orange peel waste residual solids

Enzymatic hydrolysis of orange peel waste was performed by re-suspending the solid residues, arising from autoclave (after pretreatment at 130 °C for 60 min in deionized water or in H_2SO_4 0.5% v/v) or DSIA, in the acidic liquor (after pH adjustment to 5.0 using NaOH 10% w/v) or in 50 mM sodium acetate buffer pH 5.0. For the depolymerization a few solid concentrations (i.e., 2, 5, 10 and 20% w/v) were tested at 50 °C for different incubation times (i.e., 24, 48, and 72 h) using 250-ml Erlenmeyer flasks under orbital shaking (150 rpm). Reactions were initiated by adding appropriate amounts of the commercial cellulase Cellic® CTec 2 (Novozymes A/S, Bagsvaerd, Denmark) so as to set the initial activity loading at 2, 4, 6 or 30 Filter Paper Unit

(FPU) per each g of cellulose present in pretreated material. Cellulase activity was determined as described by Ghose (1987; §4.6.16).

An enzymatic hydrolysis experiment was also carried out in a 7-L bench-top stirred-tank reactor (Applikon, Schiedam, Netherlands), jacketed and equipped with two four-blade fan turbines (diameter, 70 mm), and a pH probe. The operating conditions chosen were the following: incubation temperature, 50 °C; pH, 5.0; stirrer speed, 500 rev min^{-1}; antifoam (Antifoam A, Sigma Chemical Co., St Louis, MO, USA), 0.05% (v/v). In this test, the hydrolytic reaction was initiated by adding not only the commercial cellulase preparation mentioned above, but also the pectinase Pectinex Ultra SPL (Sigma Aldrich, St Louis, MO, USA), the corresponding enzyme loadings having been set to 12 FPU/g cellulose and 25 UI/g dry matter, respectively. Pectinase was added as suggested by their supplier.

At different incubation times, aliquots of the reaction mixtures were collected, centrifuged (6000 x g, 10 min) and subsequently analyzed for residual solids (by gravimetric determination), glucose (§4.6.3) and galactose (§4.6.4). The results were expressed in relation to the initial cellulose and galactose content of pretreated material, respectively.

4.5 Repeated-batch fermentation of the hydrolyzates

The slurry arising from the enzymatic hydrolysis was centrifuged at 6000xg for 15 min and the supernatant was used to prepare the hydrolyzate fermentation medium by adding (g L^{-1}): yeast extract, 1.0; ammonium sulfate, 1.0; $MgSO_4$, 0.5; KH_2PO_4, 1.0. Inoculation was performed with 24 h-old *Saccharomyces cerevisiae* F15 precultures so as to yield and initial absorbance at 600 nm of 2.0.

Fermentations were performed at 30 °C and pH 5.0 in 100-ml Erlenmeyer flasks containing 80 ml medium under orbital shaking (90 rpm).

A fermentation experiment was carried out in a bench-top 1-L stirred-tank reactor (Applikon, Schiedam, the Netherlands), thermoregulated through a heat-conductive stainless steel coil in which thermostated water was re-circulating, and equipped with two six-blade Rushton turbines (diameter, 45 mm), and dissolved oxygen and pH probes. Throughout any fermentation trial dissolved oxygen and pH were monitored, while pH was automatically controlled by adding 1M NaOH via the Bio Controller ADI 1030 (Applikon, Schiedam, Netherlands). The volumetric fraction of CO_2 in the gaseous outflow was measured using the gas analyzer Binos 100 2M (Rosemount Analytical, Solon, OH, USA). The operating conditions chosen were the following: incubation temperature, 30 °C; pH, 5.0; stirrer speed, 300 rev min^{-1}; aeration rate, 1 L $(L\ min)^{-1}$

for the first batch, and 0.2 L (L min)$^{-1}$ for the subsequent batches; antifoam (Antifoam A, Sigma Chemical Co., St Louis, MO, USA), 0.05% (v/v). At the end of any batch, the exhausted broth was recovered using the peristaltic pump Masterflex L/M Economy Drive (Cole Parmer, Milan, Italy) and centrifuged at 6000xg. Thereafter, the biomass recovered was re-suspended in 1 L of fresh medium and reintroduced in the reactor. In the 1-L STR experiment the first batch was carried out with a "model" medium, prepared by replacing the hydrolyzate with deionized water and integrating the aforementioned supplements with glucose (50 g L^{-1}).

A preliminary fermentation experiment, in which the aforementioned model medium was used instead of the hydrolyzate at each recycle, was performed in a 2-L stirred-tank reactor (Applikon Schiedam, the Netherlands), equipped as the aforementioned reactor with the exception that the thermoregulation was guaranteed by an external jacket. Fermentation conditions were the same as reported above.

At different incubation times, aliquots of any fermenting broth were collected to determine dry weight, using 2.7-µm MGD glass filters (Sartorius Stedim). The filtrate was subsequently analyzed for glucose and fructose (§4.6.3), galactose (§4.6.4) and ethanol (§4.6.17) contents.

4.6 Analytical methods

4.6.1 Reducing sugars (Nelson, 1944)

Principle

The reducing sugars when heated with alkaline copper tartrate to reduce the copper from the cupric to cuprous state and thus form cuprous oxide. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place.

Reagents

- Solution A was prepared by dissolving in 1 L of distilled water: 25 g anhydrous sodium carbonate, 20 g sodium bicarbonate, 25 g potassium sodium tartrate and 200 g anhydrous sodium sulphate.
- Solution B was prepared by dissolving 30 g copper sulfate and adding 4 drops of sulfuric acid in 200 mL of distilled water.
- Solution C was prepared by dissolving 25 g ammonium molybdate in 450 ml water and adding 21 ml sulfuric acid. Then 3 g disodium hydrogen arsenate dissolved in 25 mL water were added to the previous solution.
- Solution D was prepared by mixing 25 ml of solution A and 1 ml of solution B.

Procedure

200 µl sample were added to 800 µl deionized water and 1 ml of solution D. The mix was incubated in a boiling-water bath for 20 min. Blank was prepared using 1 ml de-ionized water. After the incubation, the mix was kept at room temperature for 5 min and then 1 ml of solution C was added. Samples were mixed for 15 s and therefore 20 ml water were added. After mixing absorbance was read at 520 nm. Calibration curve was made using glucose (0.05-0.5 g L^{-1}) as standard.

4.6.2 Total sugars (Dubois et al., 1950)

Principle

The method is based on the hydrolysis of glycosidic bonds caused by concentrated sulfuric acid, and on the following dehydratation of monosaccharides. Dehydrated products react with phenol to give an orange color spectro-photometrically detectable at 490 nm.

Reagents

- phenol 5% (w/v);
- concentrated sulfuric acid.

Procedure

Reaction was started by adding the sample (200 µl), phenol (200 µl) and sulfuric acid (1 ml). Samples were then mixed and after 30 min the absorbance at 490 nm was read. Calibration curve was made using glucose (0-0.1 g L^{-1}) as standard.

4.6.3 Glucose and fructose

Glucose and fructose were analyzed using the K-GLUCOSE/FRUCTOSE kit (Megazyme International Ireland Ltd, Wicklow, Ireland).

Principle

D-Glucose and D-fructose are phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP):

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{G-6-P} + \text{ADP} \qquad (1)$$

$$\text{D-Fructose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{F-6-P} + \text{ADP} \qquad (2)$$

In the presence of glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH):

$$\text{G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-phosphate} + NADPH + H^+ \quad (3)$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. It is, in fact, the NADPH which is measured by the increase in absorbance at 340 nm.

On completion of reaction (3), F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI):

$$\text{F-6-P} \underset{\text{(PGI)}}{\rightleftharpoons} \text{G-6-P} \quad (4)$$

The G-6-P formed reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance, that is stoichiometric with the amount of D-fructose.

The entire assay procedure is reported in the Data Booklet (http://www.megazyme.com/downloads/en/data/K-FRUGL.pdf).

4.6.4 Galactose

Galactose was analyzed using the K-RAFFINOSE/GALACTOSE kit (Megazyme International Ireland Ltd, Wicklow, Ireland).

Principle

Inter-conversion of the α- and β-anomeric forms of D-galactose is catalyzed by galactose mutarotase (GalM):

$$\alpha\text{-D-Galactose} \xrightarrow{\text{(GalM)}} \beta\text{-D-galactose.} \quad (5)$$

The β-D-galactose is oxidized by NAD^+ to D-galactonic acid in the presence of β-galactose deyhydrogenase (β-GalDH) at pH 8.6:

$$\beta\text{-D-Galactose} + NAD^+ \xrightarrow{\text{(β-GalDH)}} \text{D-galactonic acid} + NADH + H^+ \quad (6)$$

The amount of NADH formed in this reaction is stoichiometric with the amount of D-galactose released. It is the NADH which is measured by the increase in absorbance at 340 nm.

The entire assay procedure is reported in the Data Booklet (http://www.megazyme.com/downloads/en/data/K-RAFGA.pdf).

4.6.5 Uronic acids (Blumenkrantz & Absoe-Hansen, 1973)

Reagents

- m-hydroxydiphenyl 0.15% w/v in NaOH 0.5% w/v;
- Sodium tetraborate 0.0125 M in concentrated sulphuric acid.

Procedure

1.2 ml of sulfuric acid/sodium tetraborate solution were added to the sample (200 µl). After mixing the solution was cooled on ice for 2 min, and then vortexed and heated up to 100 °C for 5 min. The reaction mixture was then cooled on ice again for 5 min and therefore 20 µl m-hydroxydiphenyl in NaOH were added. After mixing the absorbance was read at 520 nm.

Since carbohydrates produce a pink color in presence of sulfuric acid/sodium tetraborate at 100 °C, a blank was prepared by adding NaOH without m-hydroxydiphenyl, and its absorbance was subtracted to the absorbance of the sample.

The calibration curve was obtained by using galacturonic acid (0-0.1 g L^{-1}) as standard.

4.6.6 Proteins (Bradford, 1976)

Principle

The Bradford assay, a colorimetric protein assay, is based on a color shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed.

Procedure

The reactive (200 µl) was added to the sample (800 µl), and the solution was mixed. After 15 min the absorbance was read at 595 nm. The calibration curve was obtained by using Bovine Serum Albumine (1 to 20 µg) as standard.

4.6.7 Cellulose, hemicellulose and lignin (NDF, ADF, ADL; van Soest, 1963)

Principle

Neutral Detergent Fiber (NDF) includes cellulose, hemicellulose and lignin as the major components, while Acid Detergent Fiber only includes cellulose and lignin. The Acid Detergent Lignin (ADL) is the insoluble lignin residue remaining after acid hydrolysis with H_2SO_4.

NDF reagent composition and preparation:

- sodium dodecyl sulphate, 30 g L^{-1};
- EDTA sodium salt, 18.61 g L^{-1};
- Sodium borate decahydrate, 6.81 g L^{-1};

- disodium phosphate anhydrous, 4.56 g L^{-1};
- 2-ethoxyethanol, 10 ml L^{-1}.

Sodium borate decahydrate was dissolved in 100 ml de-ionized water (sol. A). Disodium phosphate anhydrous was dissolved in 100 ml deionized water (sol. B). After mixing 100 ml water and solutions A and B and then adding the mixture to EDTA and SDS, the resulting solution was stirred and heated at 50-60 °C. 500 ml deionized water were then added and after complete solubilization 2-ethoxyethanol was added and the volume was adjusted to 1 L.

ADF reagent composition and preparation

- 1 N sulfuric acid;
- cetyltrimethylammonium bromide (CTAB).

CTAB (40 g) was solubilized by slow addition of 300 ml 1 N sulfuric acid and heating. Then 1 L acid was added and after complete dissolution the volume was adjusted to 2 L.

Determination of the Neutral and Acid Detergent Fiber

About 700 mg extractive-free material were put into previously calibrated filter crucibles. The digestion was performed using the Tecator Fibertec System M 1020 Hot Extractor (PBI international, Italy) for 1 h after addition of 100 ml ADF or NDF reagent. After the digestion, reagents were eluted and crucibles were incubated at 110 °C for 24 h and weighted. This quantity represents the ADF or NDF fraction of the material. Thereafter, crucibles were incubated at 500 °C for 4 h and subsequently weighted. The ash content must be subtracted to the ADF or NDF filtration residue.

The difference between NDF and ADF fraction represents the hemicellulose amount of the sample.

Acid-insoluble and acid-soluble lignin determination

For *acid-insoluble lignin* determination (ADL) about 0.5 g of the three extractive-free matrices under study were incubated at 30 °C for 60 min with the addition of 3 cm^3 of an aqueous solution of H_2SO_4 at 72% w/w. Thereafter, the mixture was diluted with de-ionized water to adjust the acid concentration at 2.5% w/w and then autoclaved at 121 °C for 60 min (Sluiter et al., 2008). The mixture was then vacuum filtered through a calibrated sintered glass crucible with standard porosity 2 (PBI, Milan, Italy). Crucibles were incubated at 110 °C for 24 h and weighted. This quantity represents the *acid detergent lignin* or a*cid-insoluble lignin* fraction of the material. Thereafter, crucibles were incubated at 500 °C for 4 h and subsequently weighted. The ash content must be subtracted to the ADL filtration residue.

The difference between ADF and ADL represents the cellulose amount of the sample.

Acid soluble lignin determination was performed by UV spectrophotometry at 205 nm (blank was prepared using 0.3 ml 72% sulfuric acid and 8.4 ml deionized water). The filtrate was diluted with de-ionized water in order to give an absorbance between 0.5 and 0.8. The amount of acid soluble lignin was calculated as follows:

$$ASL\% = \frac{OD\ V_f\ DF}{C\ EP} \times 100$$

where OD is the absorbance at 205 nm; V_f the final volume (L); DF the dilution factor; CE the extinction coefficient (105 L g^{-1} cm^{-1}); and P is the extractives free biomass weight.

4.6.8 Total monosaccharides

The acid hydrolyzate resulting from lignin determination was also used for total monosaccharides analysis after pH was adjusted to 6.5 by adding solid $CaCO_3$. The neutralized filtrate was then centrifuged (6000xg, 15 min) and passed through a 2.7-µm MGD glass filter (Sartorius Stedim). Total monosaccharides contained in the acid hydrolyzate were then analyzed using a Varian 9010 HPLC system, with a refraction index detector IR10 (Varian) and an Aminex 87P (7,8 mm x 350 mm) column (Biorad) equilibrated at 85 °C with MilliQ water (0.6 mL min^{-1} flow rate).

4.6.9 Pectin (modification of the method of Sudhakar & Maini, 2000)

For pectin extraction 20 g raw material was submitted to water extraction using 200 ml deionized water (solid-liquid ratio 1:10) at room temperature under orbital shaking (150 rpm) for 1 h. The suspension was then centrifuged (6000xg, 10 min) and the pellet re-suspended in the same initial volume, and centrifuged in the aforementioned conditions. The supernatant was discarded and the pellet was incubated at 45 °C to constant weight.

The extractive-free material (5 g) was extracted with 50 ml HCl 0.05 M for 1 h in boiling-water bath. During the incubation the tubes were mechanically stirred every 15 min for 3 min. At the end of the incubation the reaction mixture was allowed to cool down to room temperature and centrifuged in the aforementioned conditions. The supernatant was then separated from the residue and the solid residue underwent to a second acid extraction using the same conditions. The two supernatants were collected together and the volume was 4-fold reduced using a Rotavapor EL-130 (Büchi, Switzerland).

The extracted pectin was then precipitated using 50 ml ethanol 95% (v/v) containing HCl 0.05 M, centrifuged under the same conditions mentioned above and washed with ethanol 65%

containing 0.05 M HCl. After washing the pectin was collected using 2.7-µm MGD pre-weighted glass filters (Sartorius Stedim) and dried at 45 °C to constant weight.

4.6.10 Phenols and tannins extraction (Makkar et al., 1993)

The method was modified as follows: the raw material was extracted with a mixture of methanol and acetate buffer 10 mM pH 5 (50:50 v/v) at a 1:10 ratio (w/v) for 4 h under orbital shaking (200 rpm) at 50 °C. The suspension was then filtered using GF/C glass filters (Whatman, USA) and 10 mg ml^{-1} Polyvinylpolypyrrolidone (PVPP) were added to the supernatant. The reaction mixture was incubated under orbital shaking (200 rpm) for 30 min at room temperature and then centrifuged (6000xg, 10 min). The PVPP additionwas repeated another time, while the subsequent centrifugation was carried out at 6000 x g for 20 min. Total phenol amount was determined according to Swain and Hills (1959) (§4.6.11) before (total phenols) and after (non tannic phenols) precipitation with PVPP. The difference between the two values gave the amount of total tannins.

4.6.11 Phenol determination (Swain & Hills, 1959)

Principle

Folin-Ciocalteau (FC) colorimetry is based on the chemical reduction of the reagent (a mixture of tungsten and molybdenum oxides).

Procedure

Folin-Ciocalteau reagent was diluted 1:10 in water and 1 ml was added to 200 µl sample. Then, 800 µl sodium carbonate 7.5% (w/v) were added and the reaction mixture was incubated in the dark for 2 h. Absorbance was read at 760 nm. Calibration curve was obtained using tannic acid (0.01-0.1 g L^{-1}) as standard.

4.6.12 Condensed tannins (Porter et al., 1985)

Principle

Condensed tannins or proanthocyanidins are a subgroup of the flavonoid class of polyphenols. The Porter assay is an acidic hydrolysis, which splits larger chain units into single unit monomers and oxidizes them. This leads to a color change that can be spectro-photometrically determined.

Reagents

Butanol-HCl (95 ml n-butanol + 50 ml concentrated HCl);

iron reagent (2% ferric ammonium sulfate in 2 N HCl).

Procedure

3 ml butanol-HCl reagent and 1 ml iron reagent were added to 500 µl sample and mixed. The sample was then incubated in a boiling-water bath for 50 min. After cooling the volume was adjusted to 12.5 ml using the butanol-HCl reagent. Absorbance was read at 550 nm. Calibration curve was obtained using delphinidin (0-150 µg) as standard.

4.6.13 Total nitrogen (Kjeldahl Method; Mariani et al., 1995)

Principle

The method is based on the conversion of the nitrogen present in the sample into ammonia, that is determined by back titration.

Reagents

- Concentrated sulfuric acid;
- catalyst (K_2SO_4: $CuSO_4$: Se in proportion 100:10:1);
- H_3BO_3 2% containing the indicators (30 mg L^{-1} Bromocresol Green and 15 mg L^{-1} Methyl red, dissolved in 50 m EtOH);
- NaOH 30%;
- HCl 0,1 M.

Mineralization

Mineralization was performer using a Digestor 430 (Büchi, Switzerland). Sulfuric acid (6 ml) and catalyst (0.5 g) were added to 0.5 g sample and the temperature was increased up to 500 °C. The mineralization is finished when the solution inside the tubes is clear and colorless. In this phase the sulfuric acid decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulfate.

Distillation

When the digestion tubes were cooled down to room temperature, 6 ml de-ionized water and 30 ml NaOH were added to the samples, in order to convert the ammonium salt into ammonia. Distillation was performed using the Distillation Unit 321 (Büchi, Switzerland).

The distillate was collected in a flask containing 20 ml of the boric acid solution. The distillation is finished when the pH of the distillate ranges from 5 to 6.

The collected ammonia was then backtitrated using 0.1 M HCl and total nitrogen was determined using the following formula:

$$\% N = \frac{(ml\ HCl - ml\ HCl\ blank)\ \ M\ HCl\ x14}{g\ sample\ x\ 1000} x100$$

where *ml HCl* is the volume of HCl used for titrating the sample; *ml HCl blank* the volume of HCl used for the blank; M is the molarity of HCl; 14 is the atomic mass of the nitrogen; 1000 is used to convert the concentration of HCl from moles per liter to moles per ml.
Total proteins were determined using the N × 6.25 conversion factor.

4.6.14 Galacturonic acid (Taylor & Buchanan-Smith, 1992)

Reagents

- Carbazole 0.1% (w/v) in absolute ethanol;
- concentrated sulfuric acid.

Procedure

Sulfuric acid (1.5 ml) and carbazole reagent (50 µl) were added to 100 µl sample and mixed. Samples were then incubated at 60 °C in a water bath for 1 h and cooled to room temperature in another water bath. The absorbance of the pink-to-red-colored sample was read at 530 nm. Since carbohydrates produce a brown color in presence of concentrated sulfuric acid, for each sample a blank was prepared by adding to the sample absolute ethanol without carbazole, and the absorbance was subtracted to the absorbance of the sample containing carbazole. Calibration curve was made by using galacturonic acid (0-0.5 g L^{-1}) as standard.

4.6.15 Degradation products resulting from the pretreatment

Acetic acid, formic acid, furfural and 5-hydrolymethylfurfural (5-HMF) contained in the steam explosion or autoclave-pretreated material were determined using a Varian 9010 HPLC system, with a refraction index detector IR10 (Varian) and an Aminex 87H (7,8 mm x 350 mm) column (Biorad) equilibrated at 65 °C with H_2SO_4 50 mM, (flow rate, 0.6 ml min^{-1}).

4.6.16 Cellulase activity (Ghose, 1987)

Cellulase activity was determined by using 6x1 cm filter paper Whatman #1 (Whatman, Maidstone, UK) strips as the substrate. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min.

Procedure

Strips were incubated with 1 ml sodium acetate buffer 0.05 M pH 4.8 for 30 min at 50 °C in a water bath. Thereafter, 0.5 ml of the enzyme solution were added and the samples were incubated at 50 °C for 15 min.
Liberated sugars were determined using the DNSA method (3.6.14).
The Filter Paper Units were calculated as follows:

$$FPU/ml = \frac{\frac{g/L\ sugar}{\frac{15}{180.16}} \times 1000 \times 0.5}{0.167}$$

Where 15 is the incubation time, expressed in min; 180.16 is the molecular weight of the glucose; 1000 is the conversion factor from moles per liter to μmoles per ml; 0.5 (ml) is the sample volume used for the DNSA assay; 0.167 (ml) is the enzyme volume, because the enzyme 0.5 ml was added to 1 ml buffer, but only 0.5 ml of the reaction mixture were used for the DNSA, and this volume contained 0.167 ml cellulase.

4.6.17 Ethanol

Ethanol was determined using a DANI Master fast gas chromatography system with a Teknokroma Meta WAX capillary column (30 m x 0,25 mm x 0,5 μm); oven temperature was programmed from 50 to 180 °C at 15 °C/min; split ratio was 50 ml/min, injector temperature 180 °C and FID temperature 250 °C. Carrier gas was helium at a flow rate of 1 ml/min.

4.7 Statistics

Results are the means of duplicate experiments ± standard deviation. Data were analyzed by one-way ANOVA and Tukey test at a significance level of 0.05, using the software Sigmastat 3, Jandel Corp (San Rafael, Ca, USA).

5. RESULTS AND DISCUSSION

Food processing wastes pose increasing pollution problems besides representing a loss of biomass and nutrients. In the last years new methods, including bioethanol production, have been introduced for the recovery, bioconversion, and utilization of the valuable constituents present in food processing wastes (Laufenberg et al., 2003).

At the beginning of the present PhD thesis three residues (orange peel waste, olive pomace and grape pomace) were chosen among others since their disposal in the Mediterranean area represents a serious environmental problem.

5.1 Chemical characterization of the feedstocks

The main chemical compositions in terms of sugars, phenols and polymers content of orange peel waste (OPW), olive pomace (OP) and grape pomace (GP) was comparatively determined in order to assess their adequacy as potential feedstocks for second generation bioethanol production. Some information about the composition of such materials can also be found in the literature. However, given the heterogeneity and the importance of the composition for the hydrolysis and fermentation experiments and analysis, it was essential to determine the exact concentration of each component in the samples that were used in this study.

Particular attention was focused on cellulose determination, since this polymer represents the main source of fermentable sugar in lignocellulosic residues. In this respect, an initial aqueous extraction was needed in order to remove all the soluble compounds, that would affect the fiber determination. The solvent extraction was performed with a toluene-ethanol mixture in lieu of ethanol 100% for a better quantification of the lipophylic component.

All the characterization data are expressed as percentage with reference to the initial dry matter (dm).

5.1.1 Main chemical composition

Table 5.1 shows that the chemical compositions of the wastes under study were significantly different.

GP and OP showed the highest (35.44) and the lowest (7.24) percentages of aqueous extractives, respectively, that will be further described in Table 5.2.

As expected, OP exhibited the highest lipophylic extractives amount (7.62% w/w), this being mainly due to the presence of residual olive oil in the sample examined. On the contrary, OPW presented the lowest percentage of toluene-ethanol extractives, probably because the sample tested was deprived of the essential oils during the orange juice production process. This is

certainly advantageous, orange peel oil exhibiting an inhibitory effect on ethanol-producing yeasts (Wilkins et al., 2007; Wilkins, 2009).

The greatest cellulose content (35.1% w/w) was found in OP and resulted to be higher than that reported in another study, where cellulose accounted for the 27.9% of the dry matter (Felizón et al., 2000). Unfortunately, OP also exhibited the highest amount of lignin (36.4% w/w), in accordance with the value reported by Felizón et al. (2000), this component representing a physico-chemical barrier to the accessibility of cellulolytic enzymes to the cellulose fibers and thus decreasing the saccharification yields.

In OPW a considerable cellulose amount (26.1% w/w) was associated with the lowest lignin content (1.9% w/w). Pourbafrani et al. (2010) reported a similar lignin amount (2.19%), but a lower cellulose amount (22%). Alternately, Oberoi et al. (2010) quoted cellulose and lignin as the 14.2% and 0.59% of OPW, respectively.

GP contained the lowest cellulose amount (13.52% w/w), but a very high lignin amount (33.75% w/w). The high lignin percentage found in OP and GP might be due to the presence of stones or seeds in the samples under study.

As concerning the hemicellulose component, the highest percentage was found in OP (17.77% w/w), this being in accordance Felizón et al. (2000). No significant difference in its content between OPW and GP was detected.

Pectin represented the 16.96% (w/w) of OPW dry matter, and this value is either lower or similar to those reported for the same feedstock, that is 25% (Pourbafrani et al., 2010) or 18% w/w (Oberoi et al., 2010). OP exhibited a low pectin amount, probably lost during the olive oil extraction process (Felizón et al., 2000).

As concerning the pectin and cellulose present in GP, the values determined here were in accordance with those by Bravo & Saura-Calixto (1998), where the GP indigestible fraction, being targeted as non-starch polysaccharides (NSP), that is mainly cellulose and pectin, ranged from 17 to 21% of dry matter.

Table 5.1 Chemical compositions of orange peel waste (OPW), olive pomace (OP) and grape pomace (GP). Data are expressed as percent contents on dry matter.

Parameter	% dm		
	OPW	**OP**	**GP**
Aqueous extractives	30.80±0.49[b]	7.24±0.36[a]	35.44±1.79[c]
Toluene-EtOH extractives	1.33±0.11[a]	7.62±0.43[c]	4.66±1.15[b]
Ash	7.43±0.41[c]	0.47±0.05[a]	2.08±0.15[b]
Acid soluble lignin	0.09±0.03[a]	1.81±0.03[b]	2.82±0.04[c]
Acid insoluble lignin	1.85±0.71[a]	34.55±3.01[b]	30.93±1.08[b]
Cellulose	26.09±2.12[b]	35.05±2.65[c]	13.52±0.24[a]
Hemicellulose	11.88±0.83[a]	17.77±0.54[b]	9.82±2.89[a]
Pectin	16.96±0.90[c]	2.12±0.19[a]	4.81±0.28[b]
Total nitrogen	0.89±0.02[b]	0.71±0.04[a]	0.65±0.02[a]
Phenols	1.19±0.01[a]	1.34±0.09[a]	4.30±0.09[b]
Tannins	0.65±0.05[a]	1.26±0.21[b]	0.98±0.08[ab]
Condensed tannins	0.22±0.01[a]	0.18±0.02[a]	0.79±0.06[b]

5.1.2 Characterization of the aqueous extractives

Table 5.2 shows that a valuable amount of free sugars was recovered by leaching both OPW and GP with water. In particular, GP contained the highest percentage not only of free glucose and fructose (17.1 and 15.9% w/w, respectively), but also of phenols (4.3% w/w, see Table 4.1). The latter however was lower than that (7.6% w/w) reported by Starnes Saunders et al. (1982). Since a significant aliquot of phenols is water-soluble, they affect yeast growth and fermentation (Palmqvist & Hahn-Hägerdal, 2000b), and this negatively counterbalances the considerable amounts of readily fermentable sugars.

The percentage content of fructose (6.67% w/w) and glucose (6.81% w/w), as detected in the OPW under study, was slightly lower than that reported by Pourbafrani *et al*. (2010: 8.1 and 12% w/w, respectively) or Grohmann *et al*. (1995: 10.73 and 8.99% w/w, respectively).

The very low percentages of free sugars and pectin detected in OP were probably due to their leaching in the oil vegetation water during the olive oil extraction process (Felizón *et al*., 2000).

Proteins represented a small water soluble fraction in all of the three feedstocks, their total amount corresponding to 5.56% in OPW, 4.43% in OP and 4.06% in GP.

Table 5.2 Chemical components in the water soluble extracts of orange peel waste (OPW), olive pomace (OP), and grape pomace (GP). Data are expressed as percent contents on dry matter.

Parameter	% dm		
	OPW	**OP**	**GP**
Total sugars	15.27 ± 0.26^{b}	1.70 ± 0.11^{a}	34.12 ± 0.78^{c}
Reducing sugars	14.48 ± 0.75^{b}	1.11 ± 0.14^{a}	32.08 ± 0.91^{c}
Uronic acids	0.18 ± 0.01^{ab}	0.08 ± 0.01^{a}	0.21 ± 0.08^{b}
Proteins	0.20 ± 0.01^{a}	0.58 ± 0.04^{b}	0.72 ± 0.02^{c}
Glucose	6.67 ± 0.69^{b}	0.95 ± 0.03^{a}	17.10 ± 1.32^{c}
Fructose	6.81 ± 0.36^{a}	n.d.†	15.90 ± 1.31^{b}

† n.d., not detected.

5.1.3 Determination of monomeric sugars in extractives-free materials

The cellulose and hemicellulose percentages resulting from fiber analysis (Table 5.1) were confirmed by the HPLC analysis of the supernatant resulting from the concentrated-acid hydrolysis of extractives-free materials (Table 5.3). In each feedstock the main sugar resulted to be glucose (mostly deriving from cellulose depolymerization), representing the 37.5% (w/w) of dry mass in OP, 28.23% in OPW and 14.42% in GP.

The remaining sugars derived from the hemicellulosic fraction of any substrates. In particular, a substantial amount of xylose (12.6% w/w) was detected in OP in agreement with Georgieva & Ahring (2007), while the lowest one in OPW. In this respect, a low xylose amount is desirable since it is easily degraded to furfural during physico-chemical pretreatments, the latter negatively affecting the fermentation step (Palmqvist & Hahn-Hägerdal, 2000b). In addition, the low xylose level in OPW makes unnecessary the need for pentose-fermenting microorganisms when OPW is used to produce ethanol. This makes such a process significantly advantageous with respect to the production of bioethanol from conventional lignocellulosic materials, where the pentose fermentation is considered one of the "bottlenecks" of the process (Hahn-Hägerdal et al., 2006). Moreover, the lower amount of glucose in OPW than in OP is counterbalanced by a significant percentage of galactose (6.0% w/w), another easily fermentable hexose. Galactose and arabinose amounts in OPW are similar to those (5.0 and 7.1% w/w, respectively) assessed by Talebnia et al. (2008).

Table 5.3 Concentrations of structural carbohydrates in extractive-free orange peel waste (OPW), olive pomace (OP) and grape pomace (GP). Percent values related to the initial dry raw material.

Parameter	% dm		
	OPW	**OP**	**GP**
Glucose	28.23±0.72[b]	37.5±1.71[c]	14.42±0.25[a]
Xylose	1.40±0.41[a]	12.61±0.95[c]	3.77±0.13[b]
Arabinose	6.02±0.60[c]	1.46±0.15[a]	2.81±0.21[b]
Galactose	6.04±0.54[c]	1.17±0.38[a]	2.37±0.14[b]

In conclusion, of the three agro-food residues examined OPW appeared to be the most promising substrate for ethanol production. Total fermentable sugars, in fact, amounted up to about 48% of the dry matter and were associated with low amounts of lignin, phenols, and xylose. Owing to the low pentose content, it is possible to resort to a wild-type *Saccharomyces cerevisiae* strain with no significant loss in ethanol yield.

5.2 Autoclave and steam-explosion pretreatments of orange peel waste

Similarly to other lignocellulosic materials, OPW requires a pretreatment step to open the structure of polysaccharides and make them more accessible to the cellulolytic enzyme pool. The operating conditions of these pretreatments may exert opposing effects on the yield of fermentable sugars (*e.g.*, glucose and fructose). Although pretreatments can release and hydrolyze polysaccharides from the cell walls and increase dry matter solubility, the use of extreme temperatures and pH values may degrade these sugars into toxic compounds, such as furfural, hydroxymethylfurfural, and volatile acids), all inhibiting subsequent saccharification and fermentation steps (Grohmann et al., 1995).

In this study, a novel lab-scale direct steam injection apparatus (DSIA) was used to perform an acid-catalyzed steam-explosion (ACSE) pretreatment of OPW.

As a result of a literature survey, the use of diluted acid solutions in steam explosion appears to be an effective physico-chemical pretreatment step (Aden et al., 2002; Kumar et al., 2009; Sun & Cheng, 2002), even if the ranges of acid concentration, and reaction temperature (T_R) and time (t_R) are not only rather different, but also matrix-specific. For instance, only dilute acid hydrolysis of several lignocellulosic materials has been so far investigated by varying T_R and t_R from 90 °C for 30-90 min in the case of 0.5 (% v/w) H_2SO_4-impregnated cotton stalks (Silverstein et al., 2007) to 210 °C for 2 min in the case of 0.175 (% w/w) H_2SO_4-impregnated

chips of *Eucalyptus grandis* (Emmel et al., 2003). These temperature-time conditions generally resulted in strong hemicellulose degradation and small lignin and cellulose removal (Canettieri et al., 2007). Thus, the equipment types used for such tests have ranged from Pyrex bottles operating at atmospheric pressure or conventional steam autoclaves operating up to 3 bar (Lee et al., 1997) to high pressure/high temperature Parr reactors (Moline, IL USA), as those used for instance by Yat et al. (2008) and Zheng et al. (2007).

Despite the extensive range of temperature-time conditions used to perform the dilute acid hydrolysis of several timber varieties [i.e., aspen, balsam, basswood, red maple (Yat et al., 2008), and *Eucalyptus grandis* (Canettieri et al., 2007)], as well as switchgrass, wheat straw and corn stover (Yat et al., 2008), the kinetics of xylose formation was generally found to be of the first-order with respect to hemicellulose with an activation energy of 49-179 kJ mol^{-1} (Canettieri et al., 2007; Yat et al., 2008). To grasp better the significance of such a finding, the well known concepts of the *decimal reduction time* (D_T) and *thermal resistance constant* (z) were used. In particular, D_T is defined as the treatment time at a given temperature (T) required to reduce hemicellulose concentration by a factor of 10, while z is the temperature increase with respect to T needed to reduce the treatment time to the tenth part of D_T (Ibarz & Barbosa-Cánova, 2003). In this way, the dilute acid hydrolysis of the aforementioned matrices resulted to be characterised by decimal reduction times at 160 °C and thermal resistance constants of 7-42 min and 29-30 °C (i.e., aspen, basswood, and *Eucalyptus grandis*) or 72-91 °C (i.e., balsam, red maple, and switchgrass), respectively.

As concerning the citrus wastes, among the investigations so far carried out (Oberoi et al., 2010; Pourbafrani et al., 2010; Talebnia et al., 2008; Widmer et al., 2010) the following can be pointed out.

Pourbafrani et al. (2010) suspended raw citrus processing wastes in sulphuric acid at 0.5% (v/v) at a total solid content of 15% (w/v) and submitted the resulting slurries to thermal treatments at different temperatures and residence times in the ranges of 130-170 °C and 3-9 min, respectively. The best sugar yield (0.41 g/g of total dry mass) was obtained by dilute-acid hydrolysis at 150 °C and 6-min residence time.

Widmer et al. (2010) pre-treated raw citrus processing waste at different times, temperatures, and pH in the ranges of 2-10 min, 120-160 °C, and 2.2-8.2, respectively. Pre-treatments at 160 °C for longer than 4 min with steam purging were needed to remove limonene, an inhibitor for fermentation, to below 0.1%. Over 80% of hemicelluloses was solubilised after pretreatments at pH 2.8. Whatever the pH level, total sugars fermentable by *Saccharomyces cerevisiae* were not changed after pretreatment at 160 °C for up to 8 min.

Talebnia et al. (2008) yielded the best results (i.e., total sugars of 41.8% dry matter with 2.6% of total hexoses degraded to hydroxymethyl furfural) by operating at 116°C, 0.5 % (v/v) sulphuric acid concentration, 6% solid fraction, and 12.9 min retention time.

Thus, it was assumed that the dilute acid hydrolysis for the orange peel matrix examined here approximately yielded 90% degradation of initial hemicellulose when operating under the following conditions:

T_1=116 °C; D_1= 12.9 min (Talebnia et al., 2008)

T_2=160 °C; D_2= 4.0 min (Widmer et al., 2010)

This allowed the two aforementioned residence times to be regarded as coinciding with the *decimal reduction times* (D_i) of the hemicellulose degradation kinetics at the corresponding temperatures T_i. Thus, the *thermal resistance constant* (z) of this hydrolytic process resulted as equal to:

$$z = \frac{T_1 - T_2}{\log_{10}(D_2/D_1)} = \frac{116-160}{\log_{10}(4/12.9)} \approx 86.5\,°C$$

By assuming that the dilute-acid hydrolysis of orange peel was characterised by D_T at 160 °C and z of the order of 4 min and 86.5 °C, respectively, the following temperature-time conditions were estimated:

T= 116 °C; $D = 4 \times 10^{-\frac{T-160}{86.5}} = 12.9$ min = 774 s

T= 130 °C; D = 8.9 min = 533 s

T= 150 °C; D = 5.2 min = 313 s

T= 160 °C; D = 4.0 min = 240 s

T= 170 °C; D = 3.1 min = 184 s

T= 180 °C; D = 2.3 min = 141 s

T= 200 °C; D = 1.4 min = 93 s

It is worthy of noting that at 150 and 170 °C the estimated retention times did not differ from those (6 and 3 min) tested by Pourbafrani et al. (2010). In conclusions, for the sake of simplicity, the following four temperature-time conditions were used:

200 °C for 90 s; 180 °C for 150 s; 160 °C for 240 s; 130 °C for 500 s

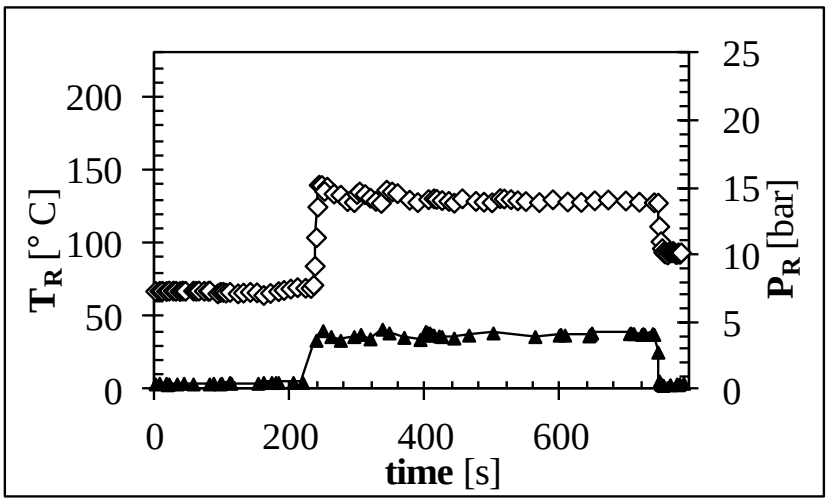
to test the validity of the pseudo-first order kinetic model assumed to describe hemicellulose degradation in the dilute-acid hydrolysis of the orange peel matrix used in this work.

Acid concentration was set to 0.5% v/v, since higher concentrations are reported to cause fructose degradation (Grohmann et al., 1995).

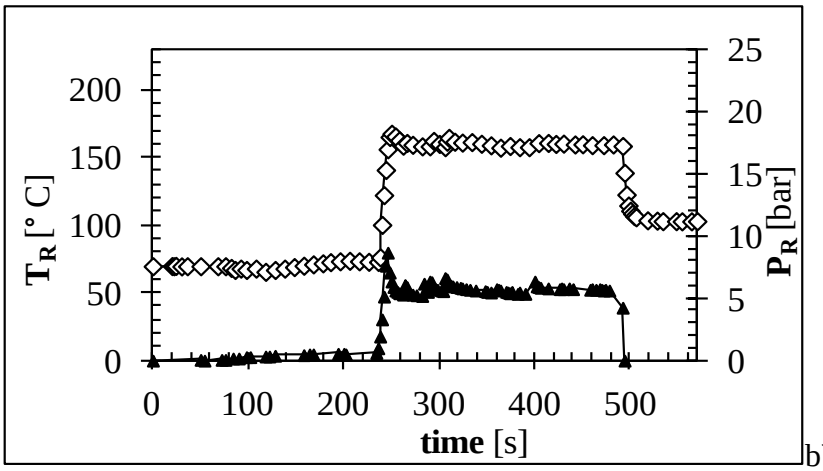
Solid loading was set to 160 g L^{-1} by referring firstly to the experimental results extracted from Talebnia et al. (2008) and Pourbafrani et al. (2010). Secondly, since a future pilot-scale testing of this process will be in all probability carried out in the continuous steam-injection heater developed by ProSonix Co. (Milwaukee, WI, USA), it was noted that such equipment may present potential pumping issues when fed with slurries at solid fractions greater than 14% (w/v), even if it has been applied to cook starch slurries up to 35% (w/w) solid content and high temperatures (104-160 °C) or cellulosic slurries at 20-25% (w/w) solid content and temperatures greater than 150 °C at flow rates ranging from about 0.067 to 630 dm^3 h^{-1} (http://www.pro-sonix.com).

5.2.1 Time course of the steam explosion pretreatment

Fig. 5.1 shows the typical time course of the ACSE pretreatment process. It can be noted that, in some cases, at the beginning of the reaction the temperature inside the reactor showed some slight fluctuations, while it was quite stable during the second part of the incubation time. In all cases, after the flash cooling temperature immediately decreased down to 100 °C. The reactor pressure also showed some fluctuations due to the small automatic puffs of high-pressure steam that were needed to keep the temperature stable during the process. Moreover, it is worth noting that the reaction temperature was reached in less than 15 s in any experiment. This is an important advantage since most of the current biomass research work has so far focused on laboratory techniques to assess the effects of several variables (*e.g.*, temperature, pressure, acid concentration, etc.) on the polysaccharide hydrolysis rates, but the heating time required by conventional lab-scale high-pressure/high-temperature reactors is by far longer, this leading to sugar degradation (Yat et al., 2008).



a)



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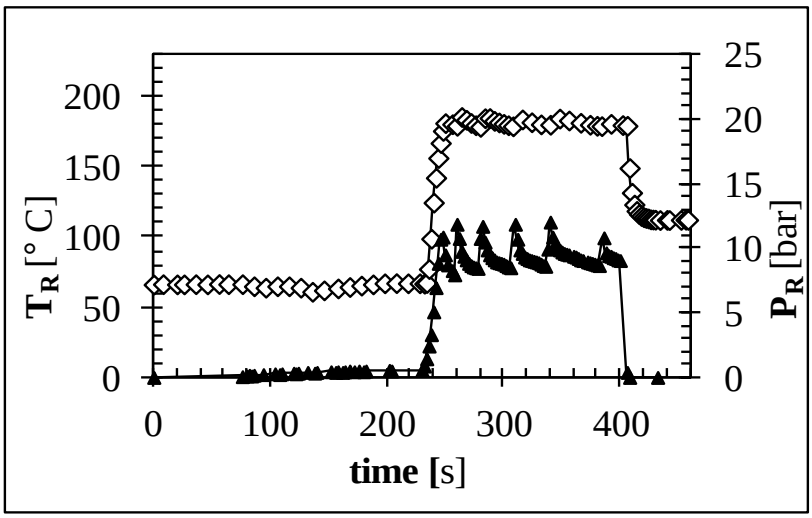
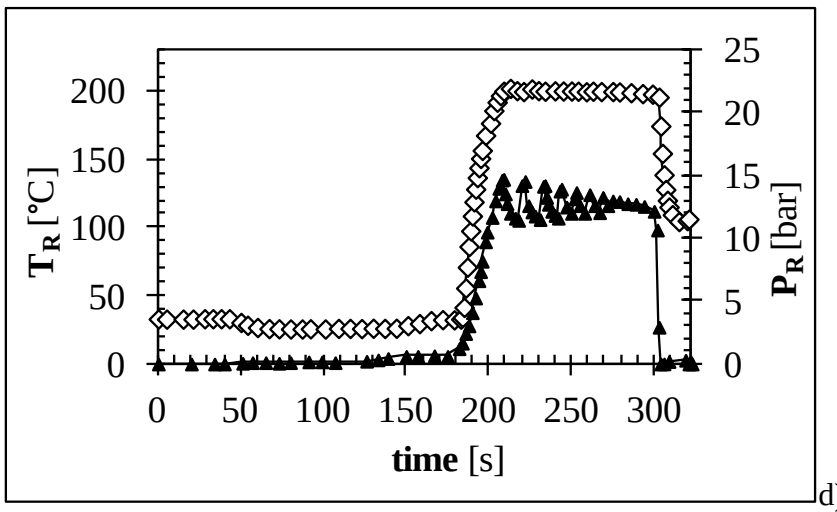


c)



d)

Figure 5.1 Typical time course of the ACSE pretreatment process performed at different temperature and time conditions (130 °C-500 s, A; 160 °C-240 s, B; 180 °C-150 s, C; 200 °C-90 s, D) in the DSIA when feeding an aqueous solution of H_2SO_4 (0.5 % v/v) enriched with 160 g L^{-1} of OPW: reaction temperature (T_R: ◇), reaction pressure (P_R: ▲).

Fig. 5.2 shows the time course of an acid-hydrolysis experiment of basswood particles as performed in a conventional lab-scale reactor (Yat et al., 2008). In this case, the reaction

temperature was reached in as quick as about 70 min. During this time interval the xylose concentration not only reached its maximum value, but also started to decrease owing to its degradation to furfural.

On the contrary, the novel lab-scale DSIA used here allowed the heating time to be extremely reduced, this contributing to reduce sugar decomposition and to make easier to transfer the operating conditions of the process from a laboratory to a pilot- and then to an industrial-scale steam-injection reactor.

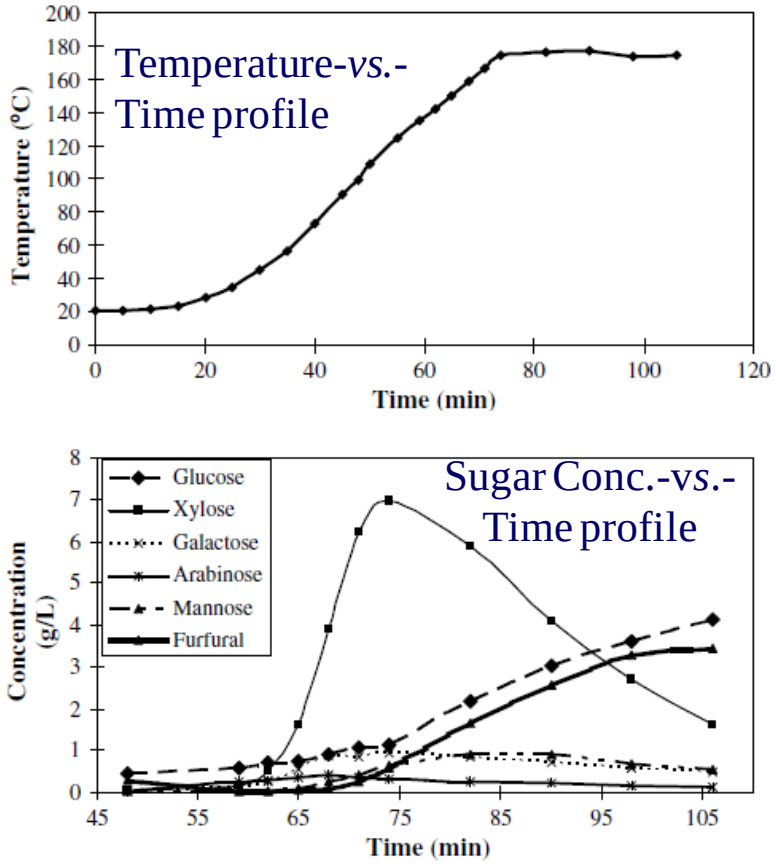


Figure 5.2 Time course of a basswood acid hydrolysis pretreatment performed in a conventional lab-scale reactor (Yat et. al., 2008).

5.2.2 Analysis of the acidic liquors resulting from ACSE and autoclave pretreatments

In this PhD thesis, the acidic liquors resulting from ACSE pretreatments, as well as that resulting from a conventional autoclave pretreatment at 130 °C for 1 h under the same acid and solid loadings, were analyzed to assess the amounts of monomeric sugars, reducing sugars, galacturonic acid and degradation products. The residual solids remaining after pretreatment were also quantified.

Table 5.4 shows the analyses of glucose, fructose, galactose, galacturonic acid and reducing sugars, expressed as percentages of the dry matter (% dm) and as percent yield compared to the initial amount of the single compound.

Since arabinose is not fermentable by the great majority of ethanologenic yeasts, its presence does not affect ethanol yield. For this reason, its accurate determination after the acid and enzymatic hydrolyses was not pursued. As concerning the glucose recovered in the acidic liquor, its amount ranged from 7.60 (130 °C-500 s ACSE) to 10.64% w/w (autoclave pretreatment), this corresponding to the 21.15 and 31.01% of the initial total glucose content (*i.e.*, the free sugar plus that present in the cellulose). At the higher temperature pretreatments (160, 180 and 200°C), the difference in glucose yield was statistically insignificant and approximately corresponded to the 8.8% of the initial dry matter. These values are not much higher than those detected in the water-soluble extractives of OPW (Table 5.2). Thus, the great majority of cellulose was preserved in the residual solid after each pretreatment, in accordance with Grohmann *et al.* (1995).

On the contrary, practically all fructose (about 100% of the initial content) was recovered in the acidic liquid, the differences assessed being insignificant whatever the pretreatment conditions.

As regarding the galactose, in OPW it mostly derives from hemicellulose and, in smaller amounts, from pectin. Thus, it can be considered as a parameter for indirect determination of hemicellulose solubilization. All of the four ACSE pretreatments yielded a very low galactose release yield, ranging from 2.32 (at 130 °C-500 s) to 6.33% (at 200 °C-90 s) of the total galactose present in the sample, this suggesting that the hemicellulosic fraction of the OPW was partially hydrolysed by the steam-explosion reaction. This finding is discordant with respect to Pourbafrani et al. (2010)'s observation of 69.48% hemicellulose solubilization under similar conditions. Unexpectedly, the autoclave pretreatment led to a quantitative liberation of the galactose, thus, the residence time seemed to be influential on hemicellulose degradation. Basing on these results, the pseudo-first order kinetic model used to fix the residence time at a given temperature was unable to predict hemicellulose degradation and consequently galactose formation. Further tests are to be carried out to fully assess the validity of the real hemicellulose kinetics.

Galacturonic acid is the major component of pectin (Mohnen, 2008) and its determination allows to estimate the extension of pectin solubilisation. The highest galacturonic acid percentage (12.38, corresponding to the 73.11% of the total pectin amount) was obtained after 200 and 180 °C ACSEs, and was considerably lower in the case of 160 and 130 °C ACSEs, as well as in the autoclave pretreatment. Pectin solubilisation was significantly affected by the reaction

temperature and by the rapid flash cooling performed in the DSIA: galacturonic acid percentage after 130 °C ACSE (3.31) was significantly higher than that obtained after autoclave pretreatment at the same temperature (1.29%). Pourbafrani et al. (2010) reported that no pectin was converted to galacturonic acid after pretreatment at 150 °C for 6 min, while in our case a similar condition (160°C for 4 min) led to the liberation of about 38.36% of the pectin, in accordance with Widmer et al. (2010).

Globally, the amount of liberated reducing sugars ranged from 22.75 to 27.76% dm (in 130 °C-500 s and autoclave pretreatment, respectively).

Basing on these data, the autoclave pretreatment yielded the highest fermentable sugars amount, but it is worth noting that the presence of residual pectin in the pretreated solids prevents the cellulolytic enzymes from reaching the cellulose fibers, thus reducing the enzymatic hydrolysis yield and making necessary the addition of considerable pectinase amounts, with an increase in the overall processing costs.

Table 5.4 Effect of the temperature-time conditions of diluted acid hydrolysis trials in DSIA and autoclave on Glucose, fructose, galactose, galacturonic acid and reducing sugars recovered in the acidic liquor. Numbers in bold represent weight percentages of the dry matter (dm), whereas numbers in italics represent t weight percentages of the initial amount of any single compound.

% dm *% single compound*	**ACSE**				**Autoclave**
	200°C 90 s	**180°C 150 s**	**160°C 240 s**	**130°C 500 s**	**130°C 1h**
Glucose	**8.93±0.78^{b}**	**8.67±0.04^{b}**	**8.73±0.35^{b}**	**7.60±0.14^{a}**	**10.64±0.51^{c}**
	25.58±2.23	*24.83±0.11*	*25.00±1.00*	*22.15±0.4*	*31.01±1.48*
Fructose	**6.71±0.22^{a}**	**6.83±0.19^{a}**	**6.87±0.17^{a}**	**6.62±0.33^{a}**	**6.85±0.21^{a}**
	98.53±0.81	*100.29±0.73*	*100.87±0.88*	*96.36±2.05*	*100.56±1.76*
Galactose	**0.38±0.03^{b}**	**0.41±0.01^{b}**	**0.19±0.01^{a}**	**0.14±0.04^{a}**	**6.1±0.20^{c}**
	6.33±0.50	*6.83±0.16*	*3.16±0.16*	*2.32±0.66*	*101.8±3.31*
Gal. acid	**12.38±0.53^{d}**	**12.38±0.72^{d}**	**8.25±0.35^{c}**	**3.31±0.47^{b}**	**1.29±0.44^{a}**
	73.11±3.12	*73.11±4.25*	*48.72±2.06*	*19.54±2.77*	7.61±2.59
Reducing sugars	**25.93±0.64^{ab}**	**26.24±0.37^{b}**	**24.07±2.88^{ab}**	**22.75±0.52^{a}**	**27.76±0.58^{c}**
	39.74±1.02	*41.82±0.58*	*38.36±4.59*	*36.25±0.82*	*44.23±0.92*

Table 5.5 reports the percentages on dry matter basis of residual solids and degradation products formed during the pretreatment reaction. Concentration of degradation products is also important, controlling the inhibitory effect on the subsequent steps of the process. Anyway, in

the present study this value depended on the dilution caused by the injection of steam in ACSE pretreatments, and was heavily affected by the different amounts of steam needed to keep the temperature constant during each trial. For this reason, concentration values are not considered significant to describe the trend of the process, and are not reported in the table, but the highest concentration value of each compound is always reported in the text.

Altogether, about half of the solids was dissolved during all ACSE pretreatments, with no statistically significant difference, this amount being higher than that resulting from the autoclave pretreatment.

The amount of phenols increased with the increase of the reaction temperature in spite of the progressively shorter residence times, while that freed in the autoclave pretreatment was not statistically different from the amounts freed at 180 and 160 °C ACSEs.

Unlike conventional lignocellulosic biomasses, where phenolics deriving from lignin degradation have an adverse effect on the cell membrane integrity (Palmqvist & Hahn-Hagerdal, 2000b), most of the phenolics present in citrus peel consist of flavonoids, such as naringin, hesperidin, isoflavanone, etc., which at concentrations below 3 g L^{-1} exert no antimicrobial activity (Oberoi et al., 2010). In the acidic liquors analyzed in this thesis their concentration was always lower than 2.2 g L^{-1}.

As concerning HMF, that derives from the degradation of hexose sugars, Talebnia et al. (2008) detected a yield of 3-3.5% after a pretreatment at 116 °C for 12.9 min, while in this work the maximum HMF yield resulted to be 1.23% at the highest temperature pretreatment (200 °C ACSE). This parameter seemed to be affected by the reaction temperature, the lowest amount being found after 130 and 160°C ACSE, and by reaction time, since the autoclave pretreatment yielded a higher HMF percentage compared to the ACSE pretreatment at the same temperature for a shorter time. Alves et al. (1998) observed that the growth of *Saccharomyces cerevisiae* and its fermentation ability were drastically affected at HMF concentrations higher than 1 g L^{-1}. In this work the measured HMF concentration was always lower than 0.83 g L^{-1}.

Throughout the tests in the DSIA no formation of furfural was revealed, probably because of the stability of arabinose that, with the exception of very small amounts of xylose, was the only pentose present in OPW. Pentose sugars are degraded into furfural through a first-order reaction, where the reaction constant is affected by both acid concentration and temperature. Talebnia et al. (2008) observed that among the various pentose sugars exposed to the acid for furfural formation, arabinose showed the lowest reactivity with a small reaction rate constant. This would explain the lack of furfural after short-time pretreatments (ACSEs) and its presence in the

autoclave pretreatment supernatant (0.35% w/w dm, corresponding to a concentration of 0.32 g L^{-1}) owing to the longer residence time of 1 h.

Formic acid derives from the degradation of furfural and HMF. The highest amount of formic acid (1.45% dm, 0.5 g L^{-1}) was detected in the 200 °C-ACSE supernatant, while the amounts detected in the other four conditions tested did not differ from one another.

Acetic acid generally originates from the break down of the acetyl bonds in hemicellulose under high pressure, temperature and acidic conditions (Lawford et al., 1998). In the present work its formation (about 0.8% dm) appeared to be unaffected by the pretreatments used and its concentration was always lower than 0.72 g L^{-1}. Weak acids toxicity is more dependant on the pH of the solution than on their concentration, since only the undissociated form (prevailing at pH values lower than 5.0) may diffuse across the plasma membrane and inhibit cell growth and viability (Palmqvist & Hahn-Hagerdal, 2000b).

Table 5.5 Percentages on dry matter basis of residual solids, phenols, acetic acid, formic acid, 5-hydroxymethylfurfural (HMF), and furfural resulting from ACSE and conventional autoclave pretreatments.

% dm	ACSE				Autoclave
	200 °C-90 s	180 °C-150 s	160 °C-240 s	130 °C-500 s	130 °C-1h
Residual solids	51.12±3.01^{b}	52.42±1.5^{b}	52.69±4.82^{b}	54.25±2.33^{b}	46.12±1.04^{a}
Phenols	2.89±0.14^{d}	2.4±0.01^{c}	2.11±0.28^{b}	1.73±0.02^{a}	2.31±0.06^{bc}
HMF	1.23±0.04^{d}	0.73±0.01^{b}	0.69±0.08^{a}	0.63±0.04^{a}	0.90±0.01^{c}
Furfural	n.d. †	n.d. †	n.d. †	n.d. †	0.35±0.01
Formic acid	1.45±0.21^{b}	0.84±0.03^{a}	0.77±0.04^{a}	0.67±0.02^{a}	0.74±0.10^{a}
Acetic acid	0.88±0.09^{a}	0.79±0.08^{a}	0.77±0.07^{a}	0.69±0.05^{a}	0.78±0.06^{a}

† not detected

Such a complete analysis of the acidic liquors formed during an ACSE pretreatment of OPW cannot be found in literature. However, Oberoi et al. (2010) analyzed the supernatant resulting from an acid hydrolysis performed in two stages (at 121 °C for 15 and 30 min, respectively) to assess the sugars yield and the presence of HMF, acetic acid and phenolics. The sugars yield was similar to that found in the present study, but was associated with a lower HMF, acetic acid and phenols yield, if compared to the 130 °C ACSE experiment and to the autoclave pretreatment. From such results it might be concluded that a milder pretreatment is sufficient for fermentable sugars production from OPW. In this respect, it is worth noting that Oberoi et al. (2010) did not

submit the pretreated solid to an enzymatic hydrolysis before fermentation. Thus, the effect of the pretreatments on the subsequent depolymerization efficiency cannot be compared.

In conclusion, given the quite similar sugar yields and low concentrations of inhibitors obtained at the 4 different temperature-vs.-time conditions, it was impossible at this stage to identify the optimal conditions for the ACSE pretreatment. The only significant difference was that higher temperature pretreatments yielded a higher solubilization of pectin: in order to verify the relation between pectin solubilization and subsequent depolymerization yield, all the residual solids deriving from the aforementioned pretreatments underwent enzymatic hydrolysis, as described in the following section.

5.3 Enzymatic hydrolysis of OPW in shaken flasks

Several studies have so far dealt with the enzymatic hydrolysis of citrus peel wastes so as to maximize monomeric sugars content without any pretreatment (Grohmann & Baldwin, 1992; Grohmann et al., 1994; Wilkins et al., 2005; Wilkins et al., 2007c). Nevertheless, other authors (Grohmann et al., 1995; Talebnia et al., 2008; Widmer et al., 2010; Boluda-Aguilar et al., 2010) demonstrated the need of a neutral or acid hydrolysis to improve the rate of the subsequent enzymatic step. Finally, some studies described ethanol production from OPW in the absence of the enzymatic hydrolysis (Pourbafrani et al., 2010; Oberoi et al., 2010).

In this PhD thesis several enzymatic hydrolysis experiments were performed on non-pretreated, autoclave-pretreated (under neutral and acidic conditions) and ACSE-pretreated OPW, in order to assess the efficacy of the DSIA under study in comparison with the conventional methods.

5.3.1 Effect of enzyme loading on non-pretreated and autoclave pretreated OPW

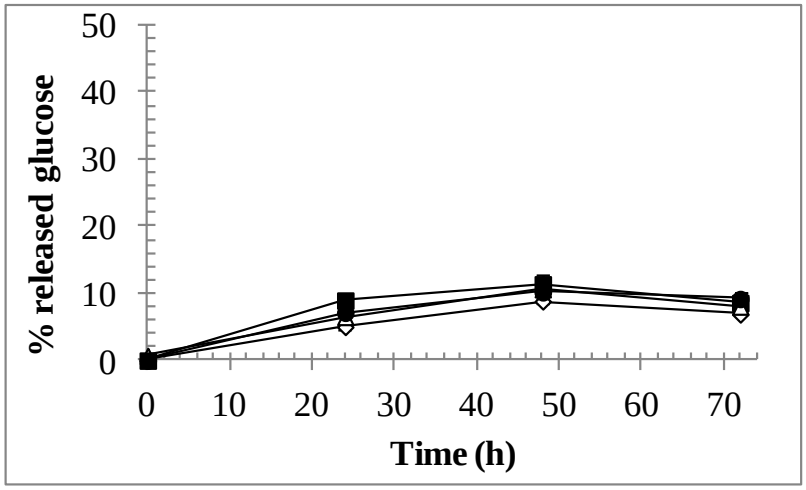
For a preliminary investigation of the enzymatic hydrolysis step, a series of experiments was performed on non-pretreated and autoclave pretreated, under neutral or mild acidic conditions (H_2SO_4 0.5% v/v), OPW, in order to assess the effect of different pretreatments and enzyme loadings. To this purpose the material was suspended in 50 mM sodium acetate buffer (pH 5) at a solid concentration of 2% w/v. Sodium acetate buffer is the recommended liquid phase for the commercial cellulase used (Cellic Ctec2, Novozymes, Bagsvaerd, Denmark), that was added at the following loadings: 2, 4, 6, 30 FPU g^{-1} cellulose. Solid and enzyme loadings were selected on the basis of some previous tests (Grohmann et al., 1995; Wilkins et al., 2007c). Figure 5.3 (a b and c) shows the hydrolytic process, expressed in terms of percent glucose yield referred to the total glucose potentially releasable from the solid undergoing the depolymerization.

Fig. 5.3a shows that untreated OPW was quite recalcitrant to the enzymatic hydrolysis whatever the enzyme loading applied, no more than 10% of the glucose being liberated. On acid-pretreated material (Fig. 5.3c), the maximum yield (about 45%) was obtained with the highest enzyme loading of 30 FPU after 24 h of incubation. After this time, the enzymatic reaction proceeded very slowly, the glucose yield remaining practically constant. This observation agrees with previous studies, where the enzymatic hydrolysis was stopped after 24 h (Grohmann et al., 1995; Wilkins et al., 2007c). From Fig. 5.3b it was noted that the neutral pretreatment was inadequate to depolymerize effectively the cellulose, the glucose yield being lower than 30%.

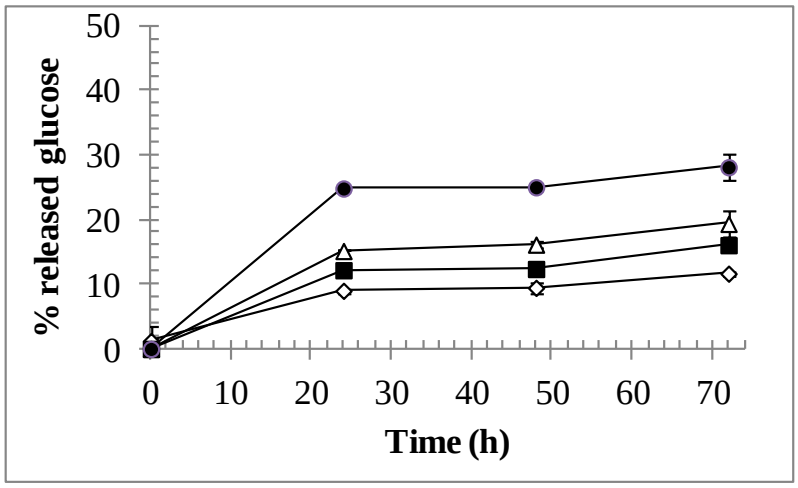
Thus, autoclave incubation with 0.5% sulfuric acid yielded the best results, this confirming and supporting previous studies recommending a mild acidic pretreatment, despite the low lignin amount of OPW, to promote the enzymatic attack (Pourbafrani et al., 2010; Oberoi et al., 2010; Grohmann et al., 1995).

This experimental set of data was compared to those obtained by Grohmann et al. (1995). By resorting to a cellulase loading of 0.6 FPU g^{-1} dm, the residual solids of OPW pretreated under mildly acidic conditions under similar temperature and time produced a glucose amount of about 14% dm, including the free glucose fraction. In this case, at a cellulase loading of 4 FPU g^{-1} cellulose (corresponding to about 0.66 FPU g^{-1} of dm), a similar glucose yield of about 14.5% dm was observed, by accounting for the free glucose fraction (6.7%) and the glucose fraction liberated after enzymatic hydrolysis (30% of the initial glucose amount, corresponding to about 7.8% of the initial dry matter).

By increasing the enzyme loading from 2 to 6 FPU g^{-1} cellulose, the glucose yield increased from about 30 to 40% of the potentially releasable glucose. Under an excess of the enzyme (30 FPU/g), the glucose yield exhibited a moderate increase from about 40 to 45%, this not justifying the use of such a high enzyme loading.



a)



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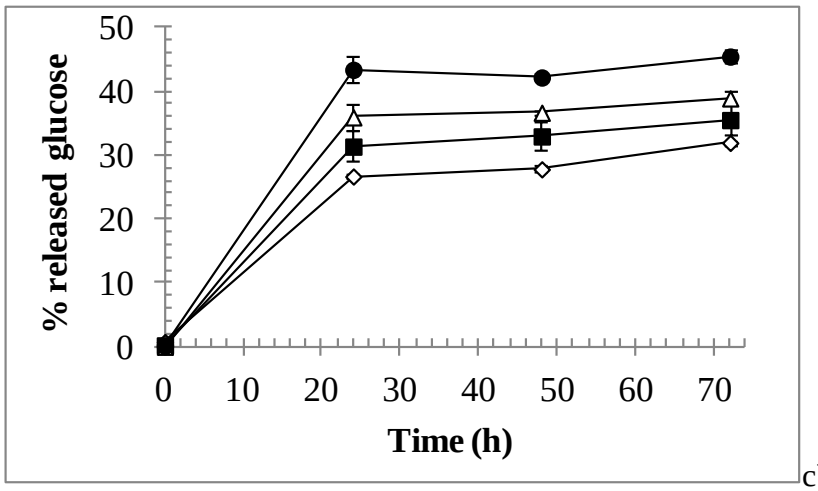


c)

Figure 5.3 Time course of OPW enzymatic hydrolysis performed under different cellulase loadings on untreated raw material (a) and after autoclave pretreatment under neutral (b) or acidic (0.5% H_2SO_4, c) conditions: ◇ 2 FPU g^{-1} cellulose; ■ 4 FPU g^{-1} cellulose; △6 FPU g^{-1} cellulose; ● 30 FPU g^{-1} cellulose. Values are expressed as glucose % yield, with respect to the total glucose potentially releasable from the solid subjected to depolymerization.

5.3.2 Effect of solid loading on OPW pretreated in autoclave under mildly acidic conditions

On the basis of these preliminary results (§5.3.1) autoclave pretreatment under acidic conditions was chosen as the best condition for further optimization of the enzymatic hydrolysis. Moreover, owing to the little gain in glucose yield observed when increasing the enzyme loading from 6 to 30 FPU g^{-1} cellulose, the subsequent set of experiments was performed at 6 FPU/g, since such loading seemed to represent a right compromise between yield maximization and cost minimization in view of a further scaling-up of this process. Thus, such a loading was tested by setting the solid concentration of autoclaved biomass under mild acid conditions at 2, 5, 10, and 20%, the last being the highest concentration allowing a sufficient mixing of the slurry in shaken-flask scale.

Fig. 5.4 shows that at 5 and 10% solid concentration the glucose yield after 48 h reached 52% and 48% of the initial amount, respectively. After the same time, about 40% of glucose was liberated using a solid loading of 2 and 20%.

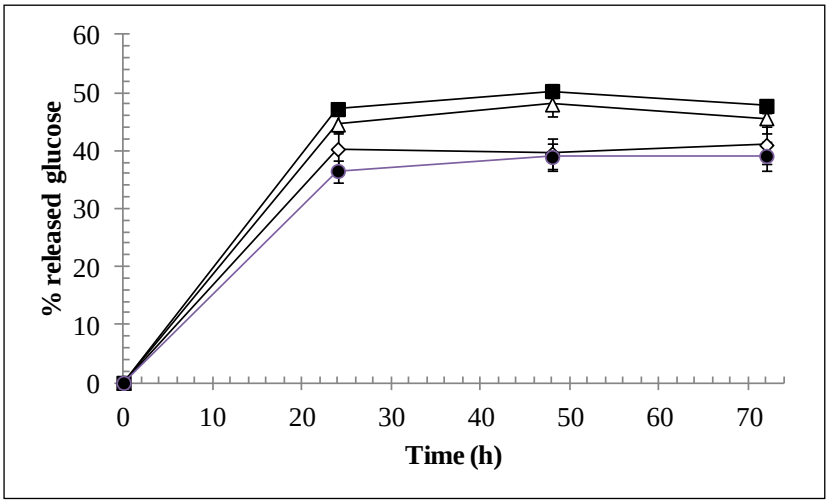


Figure 5.4 Enzymatic hydrolysis of OPW pretreated under mildly acidic conditions (H_2SO_4 0.5% v/v) using 6 FPU cellulase g^{-1} cellulose at different solid concentrations (w/v): ◇ 2%; ■ 5%; △10%; ●20%. Values are expressed as glucose % yield, with respect to the total glucose potentially releasable from the solid subjected to depolymerization.

The slight decrease in yield observed at 20% solid loading, highlighted in Fig. 5.5, might depend on the unspecific adsorption of the enzyme on the matrix. Anyway, it is worth noting that a higher solid concentration would mean a higher sugar and ethanol concentration, that would facilitate the final distillation. In this case, the final glucose concentration was 17 g L^{-1} at 20% and 10 g L^{-1} at 10% solid concentration. In this sense, the optimal condition can be hardly defined since it depends on what parameter (*i.e.*, yield or concentration) is to be optimized.

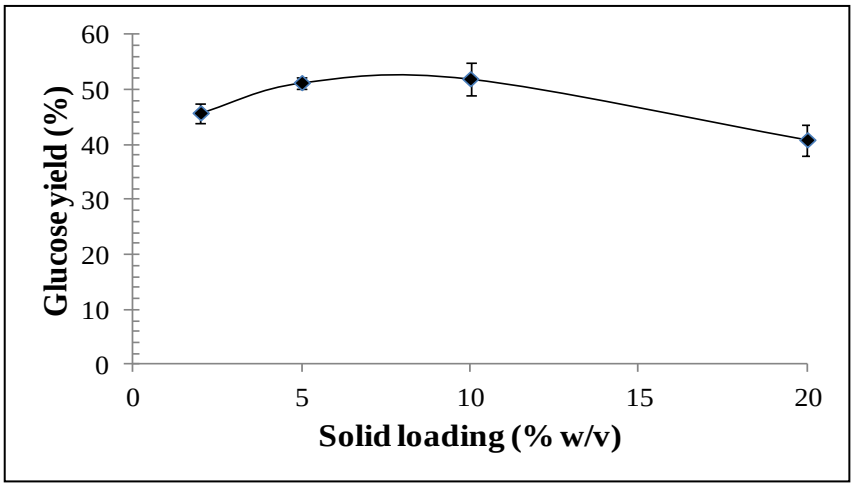


Figure 5.5 Maximum glucose yields against solid loadings after enzymatic hydrolysis at 6 FPU cellulase g^{-1} cellulose using OPW pretreated under mildly acidic conditions (H_2SO_4 0.5% v/v). Values are expressed as glucose % yield, with respect to the total glucose potentially releasable from the solid subjected to depolymerization.

5.3.3 Enzymatic hydrolysis of ACSE pretreated OPW

The enzyme loading optimized in preliminary experiments (6 FPU cellulase g^{-1} cellulose) was also applied on ACSE pretreated OPW samples, using the solid loading (20% w/v, §5.3.2) yielding the maximum glucose concentration in the resulting liquor.

Grohmann et al. (1992, 1995) and Wilkins et al. (2007c) corroborated the efficacy of a combined enzymatic treatment involving the use of cellulases, pectinases, and β-glucosidases to improve glucose liberation from cellulose. The commercial cellulase Cellic Ctec2 (Novozymes, Bagsvaerd, Denmark) used in this study comprised even the β-glucosidase activity. Since in the ACSE treatments at 200 and 180 °C most of the pectin had been solubilised, the ability of the commercial enzyme mixture was tested with no extra pectinase addition, since this would represent an interesting opportunity to reduce the overall processing costs of this process.

In the first experiment, 200 °C-90 s pretreated OPW residual solids were suspended in sodium acetate buffer (§5.3.1) to compare the efficiency of the ACSE pretreatment with the autoclave pretreatment.

Fig. 5.6 shows that the hydrolysis of ACSE-pretreated material continued after the first 24 h and reached a plateau after 52 h. A significantly higher glucose yield (59.28%) was obtained, compared to that (~40%) obtained starting with the autoclave-pretreated material. This result demonstrates the higher effectiveness of steam explosion with respect to a conventional pretreatment, and may be related to the corresponding different percentages of pectin solubilised (table 5.4), as the presence of residual pectin prevents cellulase from reaching cellulose fibers (Grohmann & Baldwin, 1992; Wilkins et al., 2007c). In fact, in this case the percentage of residual pectin was 26.89% after 200°C steam explosion and 92.39% after autoclave pretreatment.

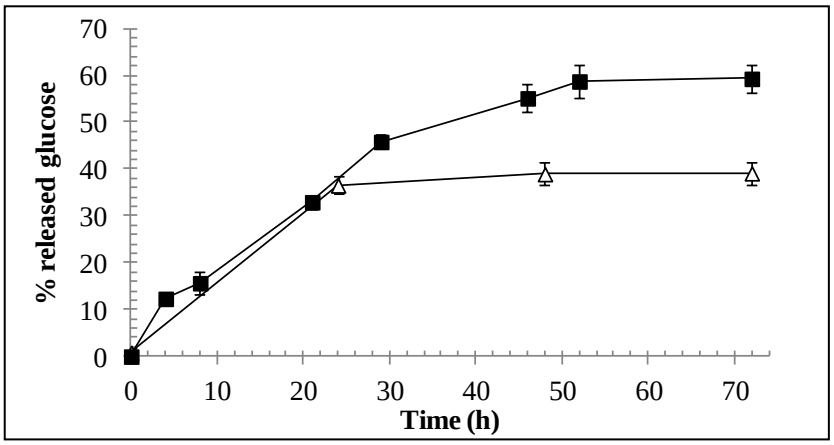


Figure 5.6 Time course of the enzymatic hydrolysis of 200 °C-90 s ACSE pretreated (■) or autoclave- pretreated (△) residual solids suspended in sodium acetate buffer at 6 FPU cellulase g^{-1} cellulose and 20% total solid loading. Values are expressed as percent glucose yield referred to the total glucose potentially releasable from the raw solids.

A subsequent experiment was performed by resuspending the 200 °C-ACSE pretreated residual solids at the same solid loading (20%) in the steam-exploded liquid fraction (its pH having been adjusted to 5.0 with NaOH) instead of the sodium acetate buffer so as to utilize even the free-sugar fraction and achieve a higher sugar concentration in view of its conversion into ethanol.
Fig. 5.7 compares the hydrolytic performance in the two cases tested.
To establish a correct comparison, glucose yield was calculated without considering the initial amount of free glucose present in the acidic supernatant, but only the glucose liberated during the enzymatic hydrolysis. In both cases, not only the time course was very similar, but also the final glucose yield (about 58%) practically coincided. This suggested that the inhibitors formed during the pretreatment and collected in the steam-explosion supernatant exerted no significant inhibitory effect on the enzymatic hydrolysis step.

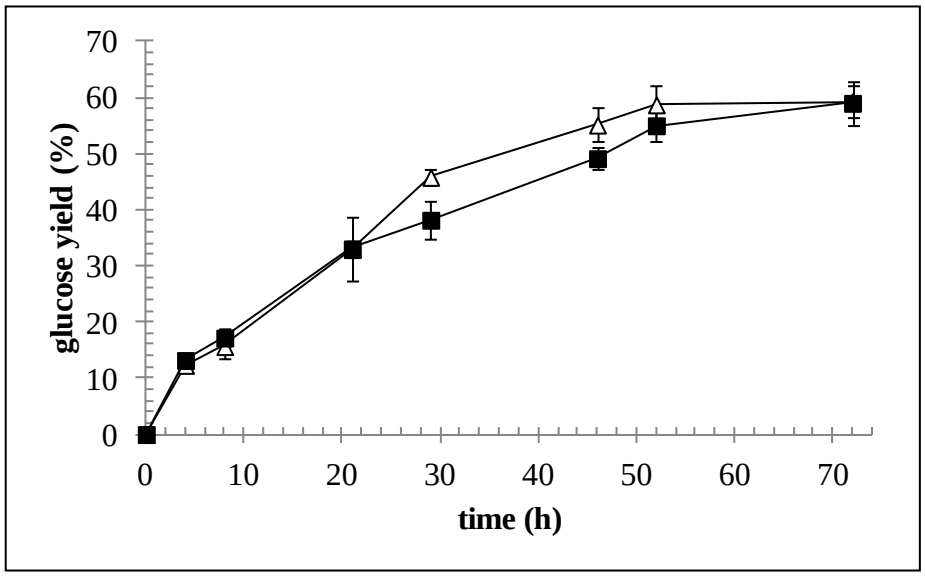


Figure 5.7 Time course of the enzymatic hydrolysis of the 200 °C-90 s pretreated biomass, once re-suspended in the acidic supernatant (■) or in 50 mM Na-Acetate buffer (△) at an enzyme loading of 6 FPU cellulase g $^{-1}$ cellulose, 20% solid loading, and 50°C. Values are expressed as percent glucose yield referred to the total glucose potentially releasable from the solid undergoing depolymerization.

The most relevant observation was that in the experiment using the ACSE supernatant, the fermentable sugars concentration (practically accounting for the glucose and fructose liberated during the acidic pretreatment only, the amount of galactose being negligible) started from 4.4 g L^{-1} and increased to about 26 g L^{-1} as a result of the glucose freed from cellulose hydrolysis. On the contrary, the final sugar concentration in the slurry obtained by resuspending the residual solids in sodium acetate buffer was about 21 g L^{-1}, the simple sugars released by the pretreatment being lost. Thus, the efficiency of the two processes was similar even when the concentration of free glucose was taken into account.

For these reasons, such procedure was applied to assess the depolymerization performance of all the solids remaining after ACSE pretreatments at 180 °C for 150 s, 160 °C for 240 s, and 130 °C for 500 s.

Table 5.6 shows that the solids pretreated at 130 and 160 °C exhibited the lowest susceptibility to enzymatic attack, without any significant difference in glucose yield after 72 h (about 45%). This finding agrees with that reported by Boluda-Aguilar (2010) for mandarin peel waste pretreated with a steam-explosion process at 160 °C for 5 min. In this specific case, the glucose yield was about 15.34% of the dry matter and was obtained by inoculating a slurry at 3% dry solid loading with an enzymatic cocktail composed of cellulase (0.62 FPU g^{-1} cellulose), β-glucosidase (8 IU g^{-1} cellulose), and pectinase (380 IU g^{-1} dm). In this work, the glucose yield after ACSE-pretreatment at 160 °C for 4 min followed by enzymatic hydrolysis with 6 FPU cellulase g^{-1} cellulose was 46.94% of the cellulose content, corresponding to about 13% of dry matter (glucose representing about the 26.5% of pretreated solids). The main operating differences were the followings: no addition of pectinase, use of a cellulose activity 10-fold higher; and a solid concentration of 20% dm instead of 3%, this resulting in a higher glucose concentration (§5.3.2).

Anyway, in the present study the highest yields (about 58%) were obtained using the solids ACSE-pretreated at 180 and 200 °C, their difference being statistically negligible.

The higher efficiency of the enzymatic hydrolysis after these pretreatments might be attributed to the higher percentage of pectin solubilisation with respect to that obtained at the lower temperature pretreatments, as demonstrated by the percentages of galacturonic acid detected in the impregnation liquors (table 5.4).

In these experiments the amount of galactose freed corresponded to about 18% of its initial content (excluding the negligible amount of galactose liberated during the ACSE pretreatments) with no statistically significant difference among the pretreatments (Table 5.6). In the circumstances, it was possible to assume that the commercial cellulase used had a partial effect on the hemicellulosic fraction still present in the solids.

Table 5.6 Glucose and galactose yields, expressed as % with respect to the total amount of sugar potentially releasable from the solid undergoing depolymerization, of enzymatic hydrolyses of ACSE pretreated materials (6 FPU cellulase g^{-1} cellulose, 20% solid loading).

ACSE conditions	**200 °C-90 s**	**180 °C-150 s**	**160 °C-240 s**	**130 °C-500 s**
Glucose Yield (%)	59.01 ± 3.95^{b}	55.73 ± 3.34^{b}	46.94 ± 1.80^{a}	43.86 ± 4.92^{a}
Galactose Yield (%)	18.04 ± 1.88^{a}	16.96 ± 0.71^{a}	17.86 ± 0.61^{a}	18.8 ± 1.58^{a}

As concerning the time course of the process shown in Fig. 5.8, it was similar. However, even if the final yields for the solids pretreated at 180 and 200 °C were practically coincident, the solids pre-treated at 180°C were depolymerized faster than the others. Thus, the lower temperature-longer time pretreatment appeared to have caused a more intense destruction of lignocellulosic crosslink, the amount of pectin solubilised being the same after ACSE pretreatments at 180 and 200 °C (Table 5.4).

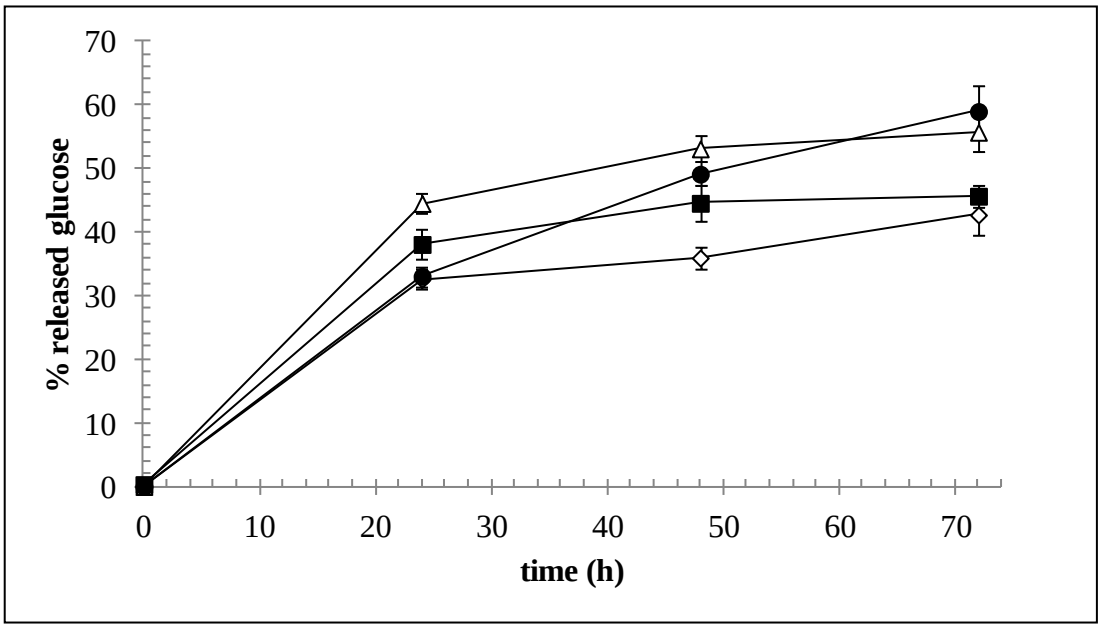


Figure 5.8 Time course of enzymatic hydrolysis (6 FPU cellulase g $^{-1}$ cellulose, 20% solid loading) of ACSE-pretreated solids: 130 °C-500 s ◇; 160 °C-240 s ■; 180 °C-150 s △; 200 °C-90 s ●. Values are expressed as percent glucose yield referred to the total glucose potentially releasable from the solid undergoing depolymerization.

Thus, the ACSE pretreatment at 180 °C and 150 s appeared to be the best one among those examined and was used to estimate the global efficiency of the bioethanol production process from OPW (§5.5).

5.4 Fermentation of OPW hydrolyzates in shaken flasks

In this section the conversion of the fermentable sugars released during acidic pretreatment and enzymatic hydrolysis into ethanol will be examined. Fermentation of OPW hydrolyzate was investigated in several studies, dealing with simultaneous saccharification and fermentation (SSF) (Grohmann et al., 1994; Wilkins et al., 2007b; Widmer et al., 2010) or separate hydrolysis and fermentation (SHF) (Boluda-Aguilar et al., 2010; Pourbafrani et al., 2010; Oberoi et al., 2010). SSF processes are generally performed at 37 °C, as a compromise to maintain the activity of hydrolytic enzymes (their optimum being at about 50 °C) and allow *S. cerevisiae* to still

ferment sugars (§2.3.2.7). However, the ideal temperature for most strains of *S. cerevisiae* is approximately 30 °C and greater temperatures can make the yeast more susceptible to other stresses, such as the presence of ethanol, with the overall result of reducing yield and productivity (Oberoi et al., 2010).

For these reasons, in the present study SHF method was applied and, contrary to previous studies always performed in single fermentation batches, in this PhD thesis the repeated-batch fermentation mode was chosen to improve ethanol yield and productivity. These factors, in fact, play a decisive role in the commercial exploitation of the process under study.

Having assessed the higher efficiency of ACSE, compared to the autoclave pretreatment, especially in terms of susceptibility of residual solids to the enzymatic attack, only the enzymatic hydrolyzates deriving from the four different ACSE pretreatments examined were submitted to fermentation trials. In all cases, a progressive decrease of the time needed to consume fermentable sugars was observed, and this was due to biomass growth and to its adaptation to the hydrolyzate. During the third cycle, in fact, after 4-5 h glucose and fructose were completely consumed, with ethanol reaching its maximum concentration, yield and productivity. The best results (Table 5.7) were obtained when using the hydrolyzate deriving from 180 °C-150 s pretreatment: at the end of the third batch ethanol productivity was 4.85 g L^{-1} h^{-1} while ethanol yield 49.48%, the latter being very close to the theoretical yield of ethanol fermentation (51% w/w). Actually, in these trials there was a very low consumption of sugars to sustain yeast growth, since the fermentative process had been approached like a biotransformation. The fact that the highest ethanol yield and productivity were obtained from the hydrolyzate pretreated at 180 °C cannot be explained on the basis of the different ACSE conditions, since the only influence that such pretreatments may exert on the fermentation pattern is related to the amount of inhibitors formed. However, the ACSE at 180 °C led to higher inhibitor concentrations than the pretreatments at 160 and 130 °C, and in all cases inhibitor concentrations were below the tolerance threshold of the yeast. Thus, since no direct correlation between pretreatment conditions and fermentative performances can be established, the differences in yields and productivities are more probably due to the higher initial concentration of fermentable sugars exhibited by the hydrolyzate pretreated at 180 °C, with respect to these in the other hydrolyzates. In other words, since a constant percentage of fermentable sugars is assimilated but not fermented by the yeast, the higher is the total amount of sugars available, the higher is the quantity of those sugars that can be converted into ethanol. This would explain why the proportion of sugar converted into ethanol is higher in the hydrolyzate pretreated at 180 °C than in the others.

Table 5.7 Main results of the repeated-batch fermentations based the four hydrolyzates deriving from ACSE and enzymatic depolymerization in the shaken-flask scale: glucose and fructose consumption (ΔS), ethanol production (ΔE), yield (Y_E) and productivity (P_E) at the end of each batch. As for consumed sugars and ethanol concentration, statistical analysis was only carried out among batches of the same category; as for ethanol yield and productivity, different lower case letters indicate statistically significant difference among batches within the same category, while different capital letters indicate statistically significant difference among the four theses within the same batch.

Pretreatment	batch n.	ΔS (g L^{-1})	ΔE (g L^{-1})	Y_E %*	P_E (g L^{-1} h^{-1})
130 °C-500 s	1	23.80 ± 1.31^{ab}	9.11 ± 0.29^{a}	38.80 ± 0.71^{aC}	0.60 ± 0.03^{aAB}
	2	21.57 ± 1.24^{a}	9.70 ± 0.34^{a}	44.96 ± 0.25^{bC}	1.61 ± 0.09^{bA}
	3	23.82 ± 0.72^{b}	10.8 ± 0.15^{b}	45.34 ± 0.2^{bA}	2.71 ± 0.11^{cA}
160 °C-240 s	1	23.13 ± 0.36^{a}	7.89 ± 0.46^{a}	34.11 ± 0.21^{aA}	0.52 ± 0.04^{aA}
	2	23.81 ± 0.75^{a}	9.11 ± 0.19^{b}	38.26 ± 0.88^{bA}	1.51 ± 0.11^{bA}
	3	24.01 ± 0.88^{a}	10.98 ± 0.17^{c}	45.73 ± 1.04^{cA}	3.66 ± 0.09^{cB}
180 °C-150 s	1	27.81 ± 0.79^{a}	10.12 ± 0.45^{a}	36.40 ± 0.37^{aB}	0.67 ± 0.04^{aB}
	2	27.40 ± 0.37^{a}	12.13 ± 0.28^{b}	44.28 ± 0.93^{bC}	2.02 ± 0.41^{bA}
	3	29.01 ± 0.40^{b}	14.55 ± 0.20^{c}	49.48 ± 1.05^{cB}	4.85 ± 0.16^{cC}
200 °C-90 s	1	23.87 ± 0.42^{a}	8.12 ± 0.33^{a}	34.01 ± 1.04^{aA}	0.54 ± 0.03^{aA}
	2	24.93 ± 0.68^{a}	10.31 ± 0.13^{b}	41.35 ± 0.89^{bB}	1.71 ± 0.11^{bA}
	3	24.52 ± 0.45^{a}	11.12 ± 0.45^{c}	45.35 ± 1.54^{cA}	2.78 ± 0.09^{cA}

* g etOH g^{-1} consumed sugars x 100

Some wild *S. cerevisiae* strains are also reported to metabolize galactose, in absence of glucose (Timson, 2007). Anyway, galactose was not assimilated by the *S. cerevisiae* strain used in this study in shaken flask, even after the complete consumption of the other hexoses (data not shown). This had no significant effect on the ethanol yield, since the galactose concentration was always lower than 1 g L^{-1}.

However, these results demonstrate that OPW hydrolyzates deriving from steam-explosion pretreatment can be efficiently fermented. In conclusion, the overall ethanol yield, estimated by accounting for the total amount of ethanol produced in three cycles in shaken-flask scale from the OPW hydrolyzate deriving from ACSE at 180 °C-150 s and enzymatic hydrolysis, was 43.69%. This value is very similar to that (43%) reported by Pourbafrani et al. (2010) after fermentation in a 1-L reactor of OPW hydrolyzate deriving from dilute-acid pretreatment at 150 °C for 6 min. Therefore these results, obtained in 100-ml Erlenmeyer flasks, will be further checked for in the following exercise (§5.5).

5.5 Scaling-up experiment

To scale up the previous results, a new ACSE trial at 180 °C for 90 s was performed by tripling the solid loading, and the treated slurry was submitted to enzymatic hydrolysis in a 7-L bioreactor, while the repeated batch fermentation was carried out in a 1-L bioreactor.

5.5.1 ACSE pretreatment at triple solid concentration

The solid loading during the steam explosion was tripled (480 g L^{-1}) in order to increase sugar concentration in the resulting liquor and assess the inhibitor effect at a theoretical triple level. All the other conditions were maintained (§5.2.2). This choice was motivated by the fact that any increase in sugar concentration would increase the ethanol concentration and facilitate its final recovery by distillation.

Results, show that, although percentages of measured glucose and fructose were significantly lower after the pretreatment at 480 g L^{-1} solid loading (6.46 and 4.67% dm, respectively), compared with the pretreatment at lower concentration (where represented 8.67 and 6.83% dm, respectively, Table 5.8), the concentrations of released sugars were higher (data not shown). Talebnia et al. (2008) also reported that increase in total solids concentration led to a decreased percentage of sugars released. A partial explanation is that, given the higher concentration, a larger part of the sugars dissolved in the impregnation liquor was converted to degradation products. In this respect, in fact, as a consequence of the increase of solid loading, a higher percentage (with respect to the initial dry matter) of phenols, acetic acid and formic acid was formed, the amount of the latter compound being about twice the amount produced in the lower concentration pretreatment (1.61 compared to 0.84% dm). Thus the decrease of glucose and fructose percentage would depend on their conversion into formic acid (§1.4.2.3). The other partial explanation might be that, given the higher solid loading, a larger part of the sugars remained adsorbed onto the matrix. This hypothesis was confirmed by the higher percentage of residual solids resulting from the higher concentration pretreatment.

The presence of a significantly lower percentage of galacturonic acid after the pretreatment at 480 g L^{-1} demonstrates that the increase of solid loading negatively affected the efficiency of ACSE in solubilizing pectin. This result, as the hypothesis of sugars adsorption onto the matrix, was also confirmed by the higher amount of solids that remained insolubilized (61.8% dm) after the pretreatment at 480 g L^{-1} in comparison with the lower solid concentration pretreatment (52.42% dm). The difference in residual solids between the two pretreatments is 9.38%, and the difference in galacturonic acid is 7.55%, this suggesting that most of the residual solids surplus,

found after the higher concentration pretreatment, is represented by insolubilized pectin, and the remaining part is ascribable to glucose and fructose adsorbed onto the matrix.

Galactose release, that was already low at 160 g L^{-1}, was further hindered by the increase of OPW concentration.

Regarding the remaining inhibitors, as in the previous experiments no furfural was found after pretreatment at 480 g L^{-1} solid loading. HMF percentage, instead, resulted to be lower after the higher concentration pretreatment (0.3% dm) than after the previous experiment (0.69% dm). This might depend on a higher conversion of HMF, formed as a consequence of sugars decomposition, into formic acid, that, in fact, resulted to be higher than in the previous experiment.

Table 5.8 Glucose, fructose, galactose, galacturonic acid, reducing sugars resulting from ACSE performed at 180 °C for 150 s at 160 and 480 g L^{-1} solid concentration. Numbers in bold represent percentages calculated on dry matter basis; numbers in italics represent percent yields with respect to the initial amount of the single compound.

% dm *% single compound*	**solid loading**		**% dm**	**solid loading**	
	160 g L^{-1}	**480 g L^{-1}**		**160 g L^{-1}**	**480 g L^{-1}**
Glucose	**8.67±0.04^{b}**	**6.46±0.24^{a}**	Residual solids	**52.42±1.5^{a}**	**61.8±4.9^{b}**
	24.83±0.11	*18.82±0.69*			
Fructose	**6.83±0.19^{b}**	**4.67±0.05^{a}**	Phenols	**2.4±0.01^{a}**	**3.36±0.06^{b}**
	100.29±0.73	*68.55±0.73*			
Galactose	**0.41±0.01^{b}**	**0.08±0.01^{a}**	HMF	**0.69±0.01^{b}**	**0.3±0.01^{a}**
	6.83±0.16	*1.33±0.16*	Furfural	**n.d. †**	**n.d. †**
Galacturonic acid	**12.38±0.72^{b}**	**4.83±0.31^{a}**	Acetic acid	**0.79±0.08^{a}**	**1.13±0.01^{b}**
	73.11±4.25	*28.49±1.82*			
Reducing sugars	**26.24±0.37^{b}**	**19.34±0.29^{a}**	Formic acid	**0.84±0.03^{a}**	**1.61±0.11^{b}**
	41.82±0.58	*30.81±0.46*			

† not detected

5.5.2 Enzymatic hydrolysis in 7-L bioreactor

In order to recover all of the acidic liquor resulting from ACSE pretreatment, in this experiment the slurry resulting from DSIA was transferred into the 7-L reactor (Fig. 5.9) without changing the solid-liquid ratio, that corresponded to about 10% w/v.

The reactor system allowed a continuous pH monitoring and a better mixing of the slurry than the shaken flask. In this experiment the commercial cellulase Cellic Ctec2 (Novozymes,

Bagsværd, Denmark) was used at the previously optimized enzyme loading of 6 FPU g^{-1} cellulose (§5.3.1 and 5.3.2). Anyway, since the impregnation liquor analysis demonstrated that most of the pectin was still present in the solid, the use of cellulase was associated with the addition of the commercial pectinase Pectinex Ultra SPL (Sigma Aldrich, St Louis, MO, USA) at an enzyme loading of 25 IU g^{-1} dry matter (Wilkins et al., 2007). The higher volume of material subjected to hydrolysis, compared to the shaken-flasks experiment, also allowed a higher volume of sampling, through which it was possible to follow the solubilization of the solid, together with the increase in glucose and galactose.

Figure 5.9 7-L Stirrer tank reactor used for enzymatic hydrolysis experiment

Fig. 5.10 shows the time course of the hydrolytic process with reference to the glucose and galactose release (expressed as percentage of the potentially releasable sugar), and solid solubilization (expressed as percentage of the dry matter subjected to enzymatic hydrolysis). As in the above reported experiments (§5.3), also in this case the depolymerization reaction was very fast in the first 24 h. During this time, about half of the final glucose and galactose

solubilization, and almost all of the solid solubilization, took place. Fructose amount remained constant along the process (data not shown).

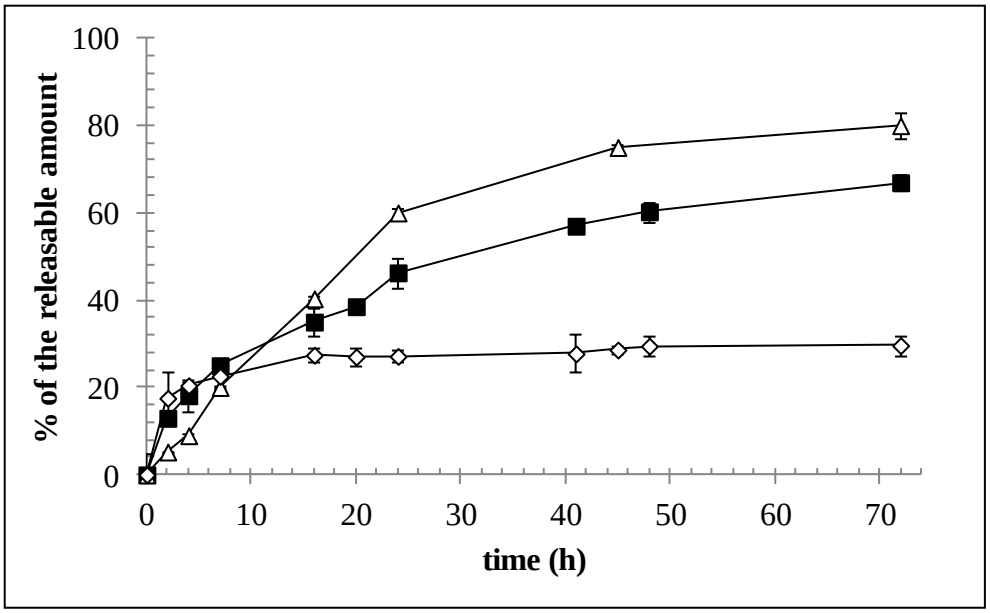


Figure 5.10 Time course of the enzymatic hydrolysis of solids pretreated at 180 °C-150 s in a 7-L reactor. Values are expressed as percentages with respect to the potentially releasable amount: solids solubilization ◇; glucose ■; galactose △.

Table 5.9 shows a comparison between enzymatic hydrolysis in shaken flask and in 7-L bioreactor of solids pretreated at 180 °C-150 s, with reference to glucose and galactose yield. After incubation in bioreactor, both glucose and galactose yields were much higher than those resulting from the shaken-flask experiment. In particular, glucose increased from 55.73 to 67.16% and galactose from 16.96 to 80.72%, a fourfold higher value. This result is ascribable not only to the better efficiency of the bioreactor system, but also to the lower solid concentration (10% instead of 20% dm) and also to the positive contribution to depolymerization deriving by the addition of pectinase to the sample.

Table 5.9 Glucose and galactose yields, expressed as percentage with reference to the potentially releasable amount of sugar, after enzymatic hydrolysis of OPW pretreated at 180 °C-150 s in shaken flask or in 7-L bioreactor (after 72 h).

Parameter	shaken flask	bioreactor
Glucose Yield (%)	**55.73±3.34**[a]	**67.16±0.51**[b]
Galactose Yield (%)	**16.96±0.71**[a]	**80.72±3.07**[b]

As for fermentable sugars concentration, at the end of such process the final glucose concentration was about 27 g L^{-1}. Despite the higher yield, this value was not statistically different from that (26 g L^{-1}) obtained in the shaken-flask experiment, and this was probably because of the lower solid (10%) used in this enzymatic hydrolysis trial. On the other hand, fructose concentration (7 g L^{-1}) was almost twice that measured in the previous experiment (4 g L^{-1}). This is due to the higher solid loading at which the steam explosion pretreatment was carried out. The amount of fructose reached, instead, is practically unaffected by the extent of the subsequent enzymatic hydrolysis.

Anyway, to assess the efficacy of the scaling-up exercise, the overall glucose and fructose yield was calculated by accounting for the total percentage of these sugars (29.0%) released after ACSE pretreatment (11.13% dm) and enzymatic hydrolysis (17.87% dm), this corresponding to 69.52% of the potentially releasable glucose and fructose. The same estimation referred to the ACSE pretreatment at 180 °C and 160 g L^{-1} for 90 s and enzymatic hydrolysis in shaken-flasks, resulted in an overall release of 67.8% of the glucose and fructose theoretically present.

5.5.3 Repeated-batch fermentation at bioreactor scale

In a preliminary phase of the present study, the fermentative ability of the strain *Saccharomyces cerevisiae* F15 was tested in a 2-L STR bioreactor (Fig. 5.11) using a model medium enriched with 40 g of glucose per liter so as to mimic orange peel hydrolyzate. In this way it was possible to assess the effect of some process variables (such as pH, stirring and aeration rate) and the behavior of the yeast strain in the bioreactor scale, in absence of limiting and disturbing factors deriving from OPW hydrolyzate.

Figure 5.11 2-L STR reactor used in repeated batch fermentation with a model medium.

Fig. 5.12 shows the time course of the repeated batch fermentation trials. As expected, the first batch accounted for yeast growth too, having set the aeration rate at 1 L (L min)$^{-1}$, and involved a lower ethanol production. During the subsequent batches the aeration rate was reduced to 0.1 L (L min)$^{-1}$ in order to favor the anaerobic metabolism. Despite the lower aeration used, a significant cell growth was still observed along each batch, the final biomass concentration being about 20 g L^{-1}. From the second batch onward, the glucose consumption became faster, and the sugar was exhausted in as short as 3.5 h during the last cycle.

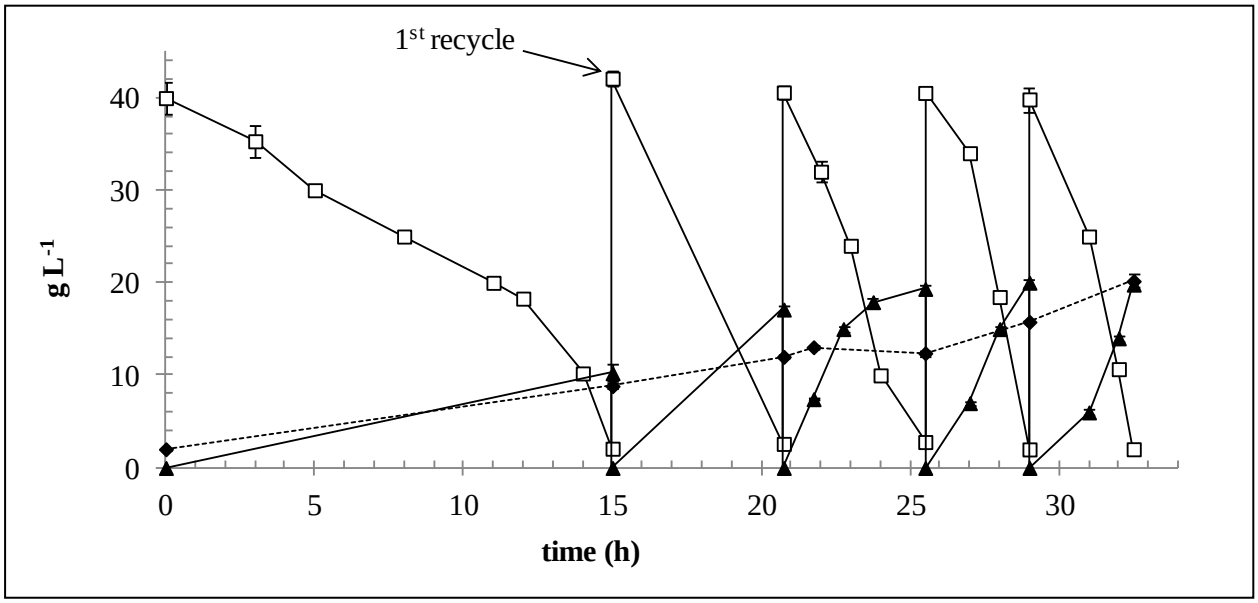


Figure 5.12 Time course of repeated batch fermentation in a 2-L STR bioreactor using a model medium: glucose (□), ethanol (▲) and biomass (dry weight ◆) concentration (g L^{-1}) versus time.

Fig. 5.13 shows the volumetric fraction of CO_2 in the gaseous effluent during the fermentation, together with sugar consumption. In particular it was observed that, from the second batch onward, CO_2 evolution, that is proportional to bioethanol production, reached its maximum immediately after the recycle, this suggesting that the yeast started fermenting the sugar a high rate. Its dramatically decrease corresponded to sugar exhaustion, this indicating the end of the fermentation activity. Thus, in the subsequent fermentation experiments using OPW hydrolyzate, such parameter was considered as an indicator of the right time to perform the medium recycle, without any check on the actual sugar concentration.

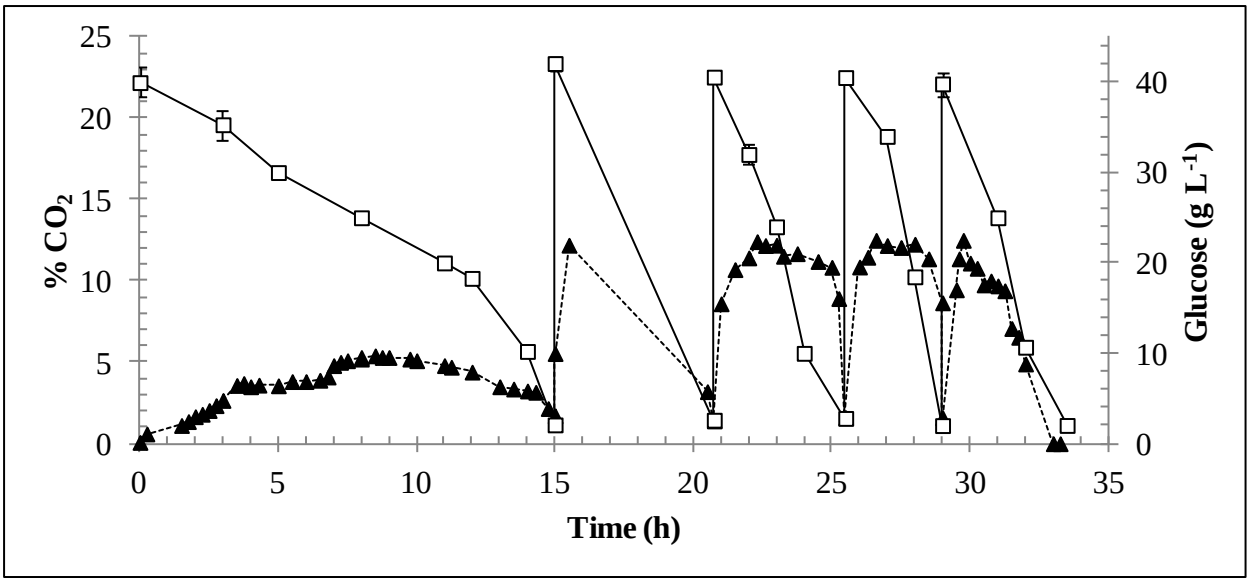


Figure 5.13 Time course of repeated-batch fermentation in a 2-L stirred bioreactor using a model medium: volumetric fractions of CO_2 (▲) in the gaseous outflow and sugar concentration (□) in the medium versus time.

In conclusion, use of the model medium resulted in a maximum ethanol yield and productivity (at the end of the fourth and fifth batches) of about 49.5% and 5.7 g L^{-1} h^{-1}, respectively (see also Table 5.10).

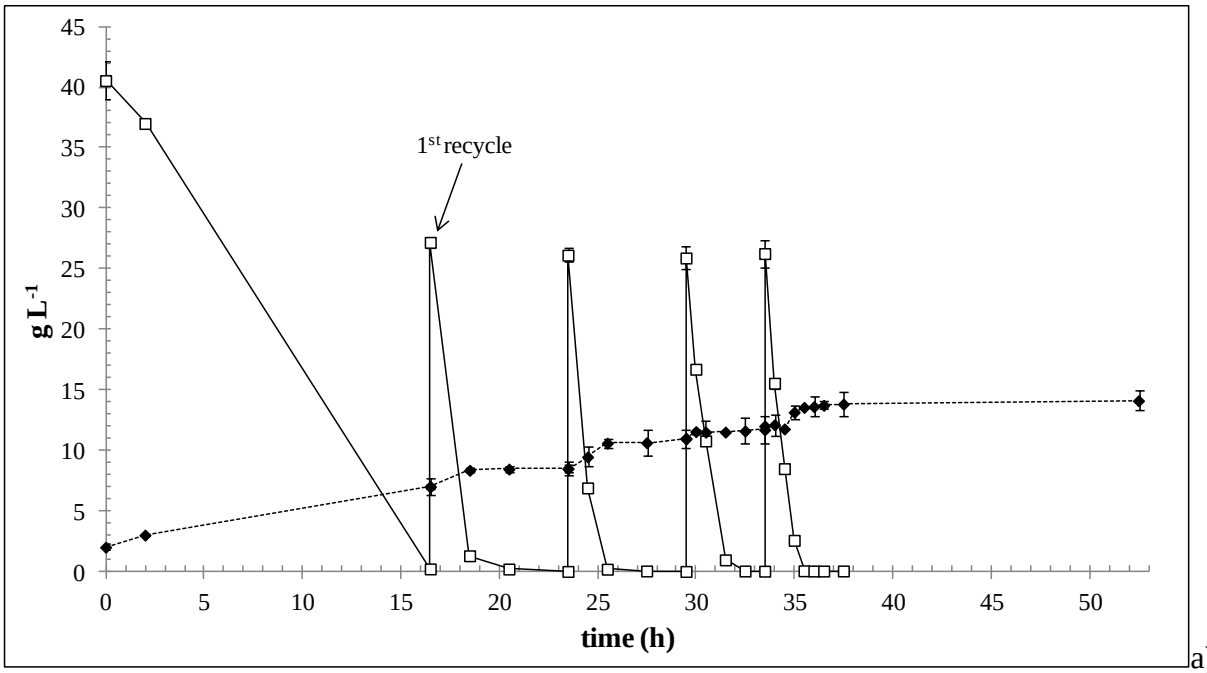
Having also ascertained the fermentation capability of the yeast strain chosen on the OPW hydrolyzate after acidic steam-explosion and enzymatic saccharification in the shaken-flask scale (§5.4), a final test was performed using the liquor resulting from ACSE pretreatment at 180 °C-150 s with a solid loading of 480 g L^{-1} and enzymatic hydrolysis in the 7-L STR bioreactor. Differently from the fermentation performed using the model medium, this fermentation trial was carried out in a 1-L STR bioreactor (Fig. 5.14), to assess the feasibility and efficiency of the process over as more recycles as possible. In this test, the repeated-batch process (4 batches of the hydrolyzate) was preceded by a batch culture carried out using the model medium to grow the yeast biomass.

Figure 5.14 Repeated batch fermentation in 1-L STR reactor using OPW hydrolyzate: recycle.

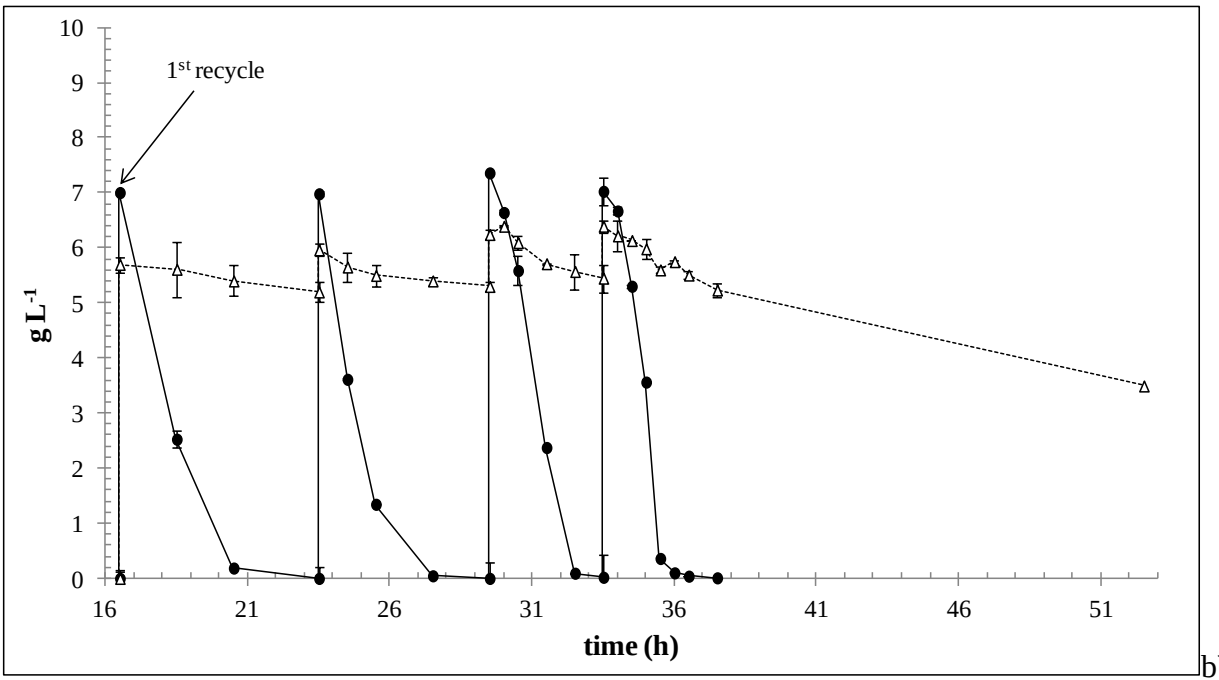
Fig. 5.15 (a and b) shows the time course of the repeated-batch experiment performed in a 1-L bioreactor, in terms of instantaneous concentration of glucose, fructose, galactose and biomass. The initial trend of the process was very similar to that obtained in the previous experiment, the first batch having been performed with the same medium and in the same conditions (with the exception of reactor volume). Moreover, as in the previous experiment, after the first recycle (when OPW hydrolyzate fermentation started) the time required for sugar consumption became progressively shorter. Glucose and fructose, in fact, were exhausted after 2-2.5 h in the fourth and fifth cycle. The overall consumption rate of these sugars was also satisfying, even if OPW hydrolyzate was harder to be fermented than the model medium: the time required for the completion of five batches, in fact, was only 5 h longer (about 38 h for complete glucose and fructose consumption) than that required in the previous experiment (about 33 h).

In shaken-flask experiments, *S. cerevisiae* F15 wasn't able to assimilate galactose (§5.4), even after finishing the other hexoses. In the reactor experiment, the last batch was left on many hours after the end of fermentation, in order to monitor the eventual consumption of galactose. Effectively, after about 15 h from the end of glucose and fructose a decrease in galactose concentration was observed (from 6 to about 4 g L^{-1}, Fig. 5.15 b). Anyway, such a long time is

not compatible with an industrial process, thus galactose consumption amount and time were not considered in the calculation of yield and productivity, respectively, the maximum ethanol concentration being obtained at the end of glucose and fructose consumption. Even if *S. cerevisiae* F15 strain demonstrated a high efficiency in fermenting glucose and fructose, it would be important to exploit also the fraction of galactose release, thus for future experiments on OPW hydrolyzates a galactose fermenting yeast belonging to the genus *Kluyveromyces* will also be tested.



a)



b)

Figure 5.15 Time course of repeated batch fermentation in a 1-L STR bioreactor using OPW hydrolyzate after a first cycle carried out with a model medium: glucose (□) and biomass (dry weight ◆) concentration (g L^{-1}) (a); fructose (●) and galactose (△) concentration (g L^{-1}) (b).

Fig. 5.16 reports the outflow of CO_2 during the fermentation, together with ethanol production. As expected basing on the previous experiments, ethanol concentration increased during the subsequent cycles, with the exception of the first batch, where it depended on sugar concentration of the model medium. As for OPW hydrolyzate fermentation, ethanol reached a maximum concentration (about 16.3 g L^{-1}) at the end of the last two cycles (see also Table 5.10).

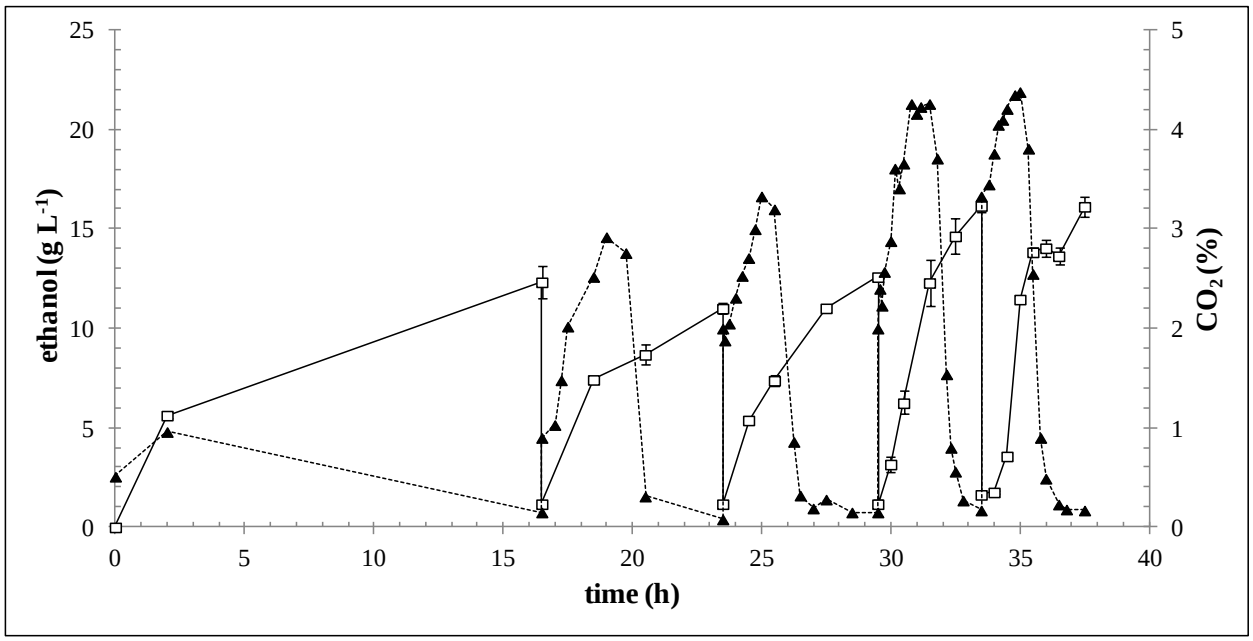


Figure 5.16 Time course of repeated batch fermentation in a 1-L bioreactor with OPW hydrolyzate after a first cycle carried out with a model medium: volumetric fraction of CO_2 (▲) in the gaseous outflow and ethanol concentration (□) in the medium versus time.

Ethanol production also became progressively faster along each batch, this having a positive repercussion on the final volumetric productivity, as shown in Table 5.10 where consumed sugars and ethanol concentration, yield and productivity resulting from model medium and OPW hydrolyzate fermentation are compared. During the first three cycles, ethanol yields obtained from model medium fermentations were higher than those obtained from OPW hydrolyzate. Anyway, maximum yields, that in both cases were obtained at the fourth and fifth cycle, were not statistically different, the average value corresponding to about 48.6%. On the other hand, as expected, maximum volumetric ethanol productivity resulting from model medium fermentation was sensitively higher (about 5.7 g L^{-1} h^{-1}) than that resulting from OPW hydrolyzate (about 4 g L^{-1} h^{-1}), that was more difficult to be fermented.

Table 5.10 Repeated batch fermentations in STR bioreactors with model medium and OPW hydrolyzate: biomass (ΔX), sugars (glucose and fructose) consumption (ΔS), ethanol production (ΔE), yield (Y_E) and productivity (P_E) at the end of each batch are reported. As for consumed sugars and ethanol concentration, statistical analysis was only carried out among the batches of the same thesis; as for ethanol yield and productivity, lower case letters indicate statistical significance among the batches within the same thesis, while capital letters indicate statistical significance among the two thesis within the same batch.

Culture Medium	batch n.	ΔS (g L^{-1})	ΔE (g L^{-1})	Y_E %*	P_E (g L^{-1} h^{-1})
Model medium	1	39.95 ± 1.66^{ab}	10.23 ± 0.19^{a}	25.60 ± 0.17^{aB}	0.68 ± 0.03^{aB}
	2	42.06 ± 0.78^{b}	17.11 ± 0.43^{a}	40.67 ± 0.85^{bB}	2.97 ± 0.09^{bB}
	3	40.56 ± 0.71^{a}	19.34 ± 0.35^{b}	47.68 ± 1.32^{cB}	4.07 ± 0.11^{cB}
	4	40.51 ± 0.49^{a}	20.01 ± 0.41^{b}	49.39 ± 0.66^{dA}	5.71 ± 0.09^{dB}
	5	39.81 ± 1.31^{a}	19.80 ± 0.45^{b}	49.73 ± 1.51^{dA}	5.65 ± 0.05^{dB}
OPW	1	40.34 ± 0.43^{b}	12.33 ± 0.80^{b}	30.56 ± 0.21^{aA}	0.74 ± 0.04^{aA}
	2	32.82 ± 0.75^{a}	11.01 ± 0.27^{a}	33.53 ± 0.48^{bA}	1.57 ± 0.11^{bA}
	3	34.06 ± 0.88^{a}	12.59 ± 0.11^{c}	36.96 ± 1.04^{cA}	2.09 ± 0.09^{cA}
	4	33.56 ± 1.11^{a}	16.17 ± 0.30^{d}	48.17 ± 0.72^{dA}	4.04 ± 0.12^{dA}
	5	34.90 ± 0.65^{a}	16.43 ± 0.49^{d}	47.07 ± 1.65^{dA}	4.03 ± 0.07^{dA}

Transferring the process from the shaken-flask to a 1-L STR reactor, a slight decrease in ethanol productivity was observed (from 4.85 to 4 g L^{-1} h^{-1}). This result can be ascribable to the increased amount of inhibitors produced after ACSE at triple solid loading, in comparison with ACSE at 160 g L^{-1} (from which the hydrolyzate used in the shaken flasks derived, Table 5.11). Differently from productivity, maximum yield remained similar after the scale increase, the two highest percentages in shaken flask and in 1-L bioreactor (49.48 and 48.17%, respectively) not showing any significant difference, with the average value (about 48.8%) being always close to the theoretical yield of ethanol fermentation (about 51%).

Table 5.11 Maximum ethanol yield and productivity resulting from repeated batch fermentation of OPW hydrolyzate in shaken flask and in 1-L STR bioreactor.

Parameter	shaken flask	1-L bioreactor
maximum ethanol Yield (%)	49.48 ± 1.05^{a}	48.17 ± 0.72^{a}
maximum volumetric productivity (g L^{-1} h^{-1})	4.85 ± 0.16^{b}	4.04 ± 0.12^{a}

Considering the total amount of fermentable sugars obtained through the optimized ACSE pretreatment and the subsequent enzymatic hydrolysis, overall ethanol yield, from OPW in 1-L bioreactor was 41.52%.

Fig. 5.17 summarizes the whole process for bioethanol production from orange peel waste using our lab-scale direct steam injection apparatus. Ground OPW is suspended in H_2SO_4 (0.5% v/v) and incubated in the DSIA. After flash cooling and pH adjustment the acidic slurry undergoes enzymatic hydrolysis. After saccharification the solid is separated from the liquid and the lignin residue can be recovered, while the remaining solid can be used in a steam/energy generator. Glucose rich liquor resulting from saccharification undergoes reverse osmosis for glucose concentration, while the retentate is used for ethanol production via fermentation. At the end of the fermentative process biomass is separated from the alcoholic solution, and a part of it can be recycled, while a part can be submitted to autolysis to produce the yeast extract needed to support the yeast growth during the process and the remainder disposed off via the steam energy generator. The ethanol-containing liquor is then distilled and the resulting stillage can be anaerobically digested for biogas production, while the azeotropic mixture is dewatered through a molecular sieve to obtain anhydrous ethanol.

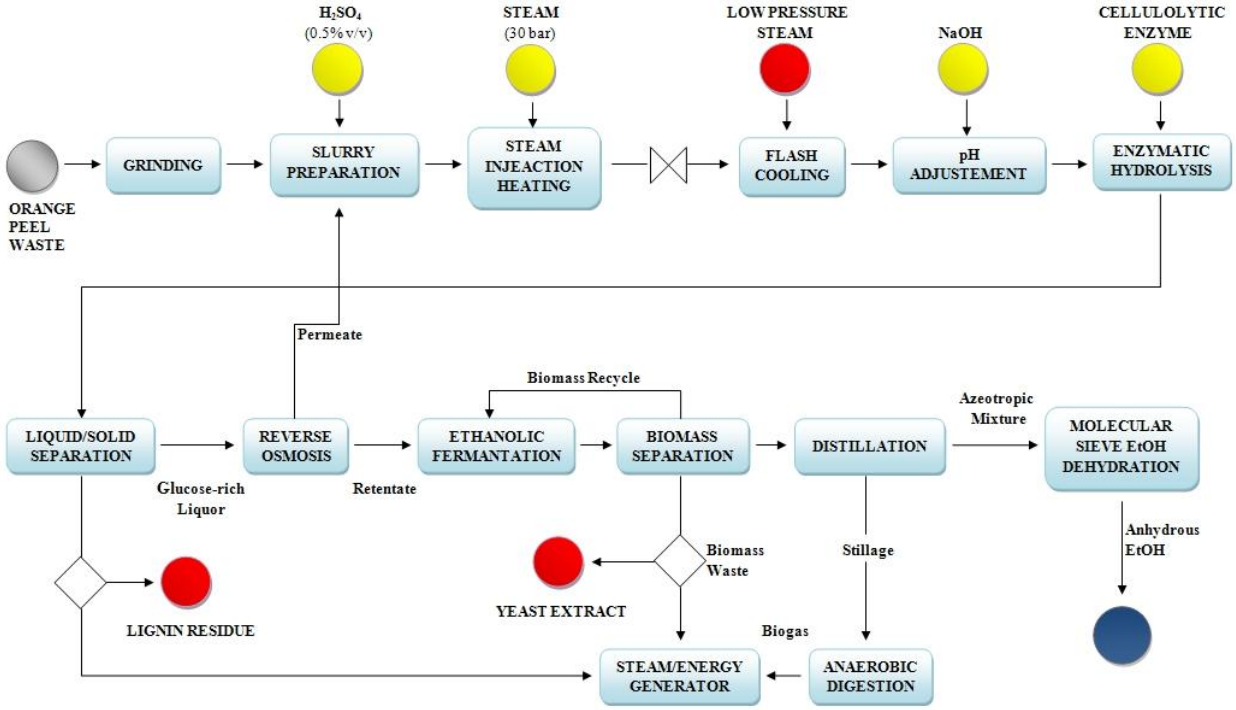


Figure 5.17 Process flowsheet to produce bioethanol from orange peel waste.

In conclusion, on the basis of a mass balance including all the glucose and fructose (11.13% dm) released after pretreatment at 180 °C for 150 s with a solid loading of 480 g L^{-1} and the glucose

freed after enzymatic hydrolysis (17.86% dm), and accounting for an overall ethanol yield of 41.52%, the overall process yield at 1-L STR reactor scale would amount to about 153 L bioethanol per metric ton dry OPW. This resulted much higher than that (60 L ton^{-1}) obtained by Boluda-Aguilar et al (2010) using mandarin peel waste, but 22.5% less than that (198 L ton^{-1}) reported by Pourbafrani et al. (2010). Probably such a higher ethanol yield was due to the fact that the dilute acid hydrolysis pretreatment was performed at a solid loading of 150 g L^{-1}. However, no information was reported about the final ethanol concentration. This should have been at least three times smaller than that (16.43 g L^{-1}) obtained in the present scaling-up experiment carried out with a solid loading of 480 g L^{-1}. In the circumstance, the specific energy consumption to distillate the ethanol from the exhausted fermentation media would have been certainly higher (Leeper, 1986).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The amount of wastes produced from food-processing industries is continuously increasing and poses serious environmental problems. For this reason, many research groups are investigating the opportunities for upgrading such residues. The present PhD thesis was aimed at assessing how to convert a food-processing lignocellulosic residue such as orange peel waste (OPW) into bioethanol.

From the experimental results it can be concluded that:

- Among the feedstocks chemically characterized, OPW resulted to be the most promising source for bioethanol production owing to its high total glucose and fructose content associated with low lignin and phenols amounts.
- Differently from the conventional lab-scale high-pressure/high-temperature reactors, requiring a too long heating time (even more than 60 min if the reaction temperature is higher than 175 °C) and leading to extensive degradation of free sugars, the novel lab-scale direct steam injection apparatus (DSIA) used in this study was able to pre-heat the acidic liquid-solid mixture in times of a few seconds. This allowed a stricter control of the pretreatment conditions (temperature and time) in view of their transfer to pilot- and industrial-scale plants. Moreover, its operation minimized the amounts of raw materials necessary for testing quite accurately numerous combinations of the operating variables.
- The four acid-catalyzed steam-explosion (ACSE) conditions tested here gave similar results in terms of sugars released and solid solubilization. Some differences were observed in the amount of inhibitors produced, even if these values resulted to be always below the tolerance threshold of the yeast strain used. The most significant difference was that the ACSE conditions at 200 °C for 90 s and 180 °C for 150 s led to a pectin solubilization of about 73%, this value being quite higher than that obtained either in the other ACSE conditions or in the autoclave with a positive effect on the subsequent enzymatic hydrolysis step.
- The enzyme loading of 6 FPU cellulase per g of cellulose present in the ACSE residual solids was chosen, being the best compromise between the needs of yield maximization and cost minimization in view of a further scaling-up of this process. In the circumstances, ACSE-pretreated solids were depolymerized with higher sugar yields than the autoclave-pretreated ones. Moreover, there was no need to suspend the pretreated solids in sodium acetate buffer, being possible to use the acidic liquor resulting from ACSE pretreatment, once its pH had been adjusted to 5.0. The free sugars dissolved in such liquor were therefore available for being converted into ethanol in the subsequent fermentation step.

- The hydrolyzates obtained after ACSE-pretreatments and enzymatic depolymerization gave interesting ethanol yields in the shaken flasks using an industrial *S. cerevisiae* strain, with no direct correlation between fermentation performance and pretreatment conditions;
- The scaling-up exercise, carried out under the best ACSE pretreatment conditions (180 °C-90 s), but at a triple solid concentration (480 g L^{-1}), and followed by enzymatic hydrolysis in a 7-L stirred bioreactor, yielded a higher percentage of glucose and fructose than that resulting from the same ACSE pretreatment at 160 g L^{-1} and enzymatic hydrolysis in shaken flask. The sugars released, except galactose, were efficiently fermented in a 1-L stirred bioreactor by *S. cerevisiae* in the repeated-batch mode.
- By accounting for the fermentable sugars released and ethanol produced in the above mentioned scaling-up exercise, the overall process yield amounted to about 153 L of bioethanol per metric ton of dry OPW.

In the present study attention was mainly focused on the development and optimization of pretreatment and enzymatic hydrolysis steps, the fermentation step being only used to assess the overall feasibility of the process. Future perspectives are to be directed:

1) to increase the sugar concentration in the liquor resulting from the saccharification step by resorting to nanofiltration or reverse osmosis (as shown in the hypothetical process flow sheet) to maximize the ethanol concentration in the fermentation exhausted media and minimize ethanol recovery costs by distillation;
2) to improve the repeated batch procedure when feeding a high-sugar level medium by increasing yeast density via biomass recycle or, alternatively, via cell immobilization to attempt to perform the fermentation process in the continuous mode;
3) to replace the *S. cerevisiae* strain used here with another one (i.e., *Kluveromyces marxianus*) capable of fermenting the galactose freed from hemicellulose degradation.

In conclusion, the process outlined in this PhD thesis may be regarded as technically feasible. In order to assess its economical feasibility, all the steps examined here are to be also tested in a pilot-scale plant. In any case, this process might represent a valid alternative to minimize the present carbon footprint of citrus juice production, that is heavily burdened by orange peel waste disposal.

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APPENDIX - LIST OF PUBLICATIONS

- **Santi G.**, D'Annibale A., Petruccioli M., Crognale S., Ruzzi M., Valentini R., Moresi M. (2012) Development and testing of a novel lab-scale direct steam-injection apparatus to hydrolyse model and saline crop slurries. *Journal of Biotechnology* 157: 590-597.

  Abstract

  In this work, a novel laboratory-scale direct steam-injection apparatus (DSIA) was developed to overcome the main drawback of the conventional batch-driven lab rigs, namely the long time needed to heat fiber slurry from room to reaction temperatures greater than 150 ◦C. The novel apparatus mainly consisted of three units: (i) a mechanically-stirred bioreactor where saturated steam at 5–30 bar can be injected; (ii) an automatic on–off valve to flash suddenly the reaction medium after a prefixed reaction time; (iii) a cyclone separator to recover the reacted slurry. This system was tested using 0.75 dm3 of an aqueous solution of H2SO4 (0.5%, v/v) enriched with 50 kg m−3 of either commercial particles of Avicel® and Larch xylan or 0.5 mm sieved particles of Tamarix jordanis. Each slurry was heated to about 200 ◦C by injecting steam at 28 bar for 90 s. The process effi-ciency was assessed by comparing the dissolution degree of suspended solid (YS), as well as xylose (YX), glucose (YG), and furfural (YF) yields, with those obtained in a conventional steam autoclave at 130 ◦C for 30 or 60 min. Treatment of T. jordanis particles in DSIA resulted in YS and YG values quite similar to those obtained in the steam autoclave at 130 ◦C for 60 min, but in a less efficient hemicellulose solubilization. A limited occurrence of pentose degradation products was observed in both equipments, suggesting that hydrolysis predominated over degradation reactions. The susceptibility of the residual solid fractions from DSIA treatment to a conventional 120 h long cellulolytic treatment using an enzyme loading of 5.4 FPU g−1 was markedly higher than that of samples hydrolysed in the steam autoclave, their corre-sponding glucose yields being equal to 0.94 and 0.22 g per gram of initial cellulose, respectively. Thus, T. jordanis resulted to be a valuable source of sugars for bioethanol production as proved by preliminary tests in the novel lab rig developed here.

- **Santi G.** (2011) Bioethanol production from agri-food lignocellulosic residues. In: *Proc.s of the 16th Workshop on the Developments on the Italian PhD Research on Food Science Technology and Biotechnology.* University of Milan, Lodi, Sept. 21-23, pp. 171-175.

Abstract

In this PhD thesis orange peel waste (OPW), that is the main citrus processing residue, was converted into bioethanol by resorting to the three subsequent steps of acid-catalysed steam explosion, enzymatic hydrolysis of residual solids and alcoholic fermentation. These tests were carried out in laboratory scale equipments and the overall process yield was about 173 L of bioethanol per metric ton of dry OPW.

- D'Annibale A., Moresi M., Petruccioli M., Ruzzi M., Calisti C., Crognale S., **Santi G.**, Valentini R. (2011) Bioethanol Production from Xerophilic Tamarix Spp.: Chemical Characterization and Performance of Hydrolysis and Fermentation Steps. In: Proc.s Book Science & Technology of Biomasses: Advances and Challenges. From Forest and Agricultural Biomasses to High Added Value Products: Processes and Materials, Viterbo (Italy), Sept., 5-8, 2011. Rome: omgrafica srl, p. 157-160 (ISBN 978-88-95688-65-7).

  Abstract

  A laboratory-scale study was carried out to assess the technical feasibility of converting *Tamarix* spp. biomass, cultivated in a desert area, into bio-ethanol. Of the four *Tamarix* varieties (namely, *T. gallica*, *T. jordanis*, *T. aphylla* var. *erecta* and *T. aphylla*) chemically characterized, *T. jordanis* was selected owing to its higher cellulose and lower phenol content, and used to outline a hypothetical process flow sheet for bioethanol production including the following steps: steam-injection heating of the liquid-solid mixture, enzymatic hydrolysis of cellulosic fractions, and ethanolic fermentation.

- **Santi G.**, Crognale S., D'Annibale A., Moresi M., Petruccioli M. (2011) Conversion of Food Processing Lignocelllulosic Residues into Fermentable Sugars for Bioethanol Production. In: Book of Proceedings of the 5th European Conference on Bioremediation, Chania, Crete (Greece), July, 4-7, 2011, p. ID096-1-ID096-6.

  Abstract

  Recovery and re-use of solid by-products and wastes generated in the food industry represent a valid option to obtain raw materials and biomasses as resource for bioenergy production. In this paper, three different food processing residues, such as olive pomace, orange peel waste, and grape pomace were chemically characterized and evaluated for the possible bioconversion into fermentable sugars for bioethanol production. Orange peel waste (OPW) was the most suitable for studies of pretreatment and enzymatic hydrolysis, being characterized by the lowest phenols and lignin content (1.19 and 1.89% w/w, respectively).

After steam explosion pretreatment, enzymatic hydrolysis and preliminary fermentation tests, a yield of about 173 L bioethanol/ton OPW was obtained.

- **Santi G.**, D'Annibale A., Petruccioli, Ruzzi M., Crognale S., Valentini R., Moresi M. (2011) Hydrolysis of Saline Crop (Tamarix Jordanis) Slurries Using a Novel Lab-Scale Direct Steam Injection Apparatus. In: Proc.s Book Science & Technology of Biomasses: Advances and Challenges. From Forest and Agricultural Biomasses to High Added Value Products: Processes and Materials, Viterbo (Italy), Sept., 5-8, 2011. Rome: omgrafica srl, p. 234-237 (ISBN 978-88-95688-65-7).

  Abstract

  A novel laboratory-scale Direct Steam-Injection Apparatus (DSIA) was developed. It was tested using an aqueous solution of H2SO4 (0.5 % v/v) enriched with 50 g l-1 of 0.5-mm sieved particles of *Tamarix jordanis*. Each sample was heated to about 200°C by injecting high-pressure steam for 90 s. The process efficiency was expressed by assessing the dissolution degree of suspended solids, and xylose, glucose, and furfural yields. The residual solid fractions from DSIA treatment resulted to be very susceptible to a conventional 120-h long enzymatic treatment using an enzyme loading of 5.4 FPU g-1, the corresponding glucose yield being equal to 0.94 g per g of initial cellulose.

- **Santi G.** (2010) Bioethanol production from agri-food lignocellulosic residues. In: *Proc.s of the 15thWorkshop on the Developments on the Italian PhD Research on Food Science Technology and Biotechnology*. University of Naples-Federico II, Portici, Sept. 15-17, pp. 297-298 (ISBN 978-88-95028-62-0).

  Abstract

  In order to evaluate the suitability of various lignocellulosic wastes as possible substrates for hydrolysis and ethanol fermentation, the chemical characterization of a first residue, olive pomace, was performed. Lignin, cellulose and hemicellulose content was determined. Secondly, various ethanol-producing yeast strains were tested in synthetic media under different culture conditions, thus assessing their fermentative potential on hydrolyzates of agri-food residues.

- **Santi G.** (2009) Bioethanol production from agri-food lignocellulosic residues. Poster communication in: *Proc.s of the 14thWorkshop on the Developments on the Italian PhD Research on Food Science Technology and Biotechnology*. University of Sassari,Oristano, Sept. 16-18, pp. 442-443.

Abstract

This PhD thesis research project is aimed at converting various agri-food lignocellulosic residues into bioethanol through fermentation. To this end, different ethanol-producing yeast strains and different hydrolysis methods will be assessed. The final purposes are the optimization and the scaling-up of the fermentative process with the selected strain and residue.