Sustainable bioethanol production combining biorefinery principles and intercropping strategies

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Abstract

Ethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future. First generation biofuel focus on starch (from grain) fermentation, but in the present study that is regarded as a too important food source. In recent years 2.nd generation technologies are developed utilizing bulk residues like wheat straw, woody materials, and corn stover. However, there is a need for integrating the biomass starting point into the energy manufacturing steps to secure that bioenergy is produced from local adapted raw materials with limited use of non-renewable fossil fuels.

Produced crops can be transformed into a number of useful products using the concept of biorefining, where no waste streams are produced. An advantage of intercropping is that the intercrop components composition can be designed to produce a medium (for microbial fermentation) containing all essential nutrients. Thereby addition of *e.g.* urea and other fermentation nutrients produced from fossil fuels can be avoided.

Intercropping, defined as the growing of two or more species simultaneously on the same area of land, is a cropping strategy based on the manipulation of plant interactions in time and space to maximize growth and productivity. Cereal-legume intercropping data from field trials show the possibility to improve the use of nitrogen resources, because the non fixing species (e.g. wheat) efficiently exploits soil mineral N sources while at the same time atmospheric N from the N_2-fixing species (e.g. pea) enter the cropping system reducing the need for N fertilizer application. Nitrogen fertilization is responsible for more than 85 % of the greenhouse gas emissions from wheat grain production in Denmark. Increase of fertilizer N supply promotes the growth of wheat and results in a decreased pea N accumulation and a different proportion of intercrop components. Intercropping introduce a dynamic change of plant species interactions as a response to the actual growing conditions observed which is not achieved with sole cropping of one species/cultivar. It is also concluded that when growing pea as a sole cropping available soil mineral N reduce N_2 fixation and the full potential of symbiotic nitrogen fixation is not exploited which is regarded as an overall inefficient use of N sources.

Using clover-grass intercropping raw materials, as another potential species combination with equivalent field responses to e.g. pea-wheat intercropping, conversion yields obtained in laboratory experiments show that wet oxidation is an efficient method for fractionating clover, grass, and clover-grass mixtures into a convertible solid cellulose fraction and a soluble hemicellulose fraction. The highest yield of fermentable sugars after enzymatic hydrolysis is achieved in clover-grass (mixed 1:1) pretreated at 195ºC for 10 minutes using 12 bar oxygen. The optimum pretreatment conditions for clover, grass, and clover-grass mixtures is not significantly different from that of wheat, which indicates that wheat straw and clover-grass (from intercropping) could be pretreated in one step. The produced sugars were converted into ethanol by *Mucor indicus* giving good ethanol yields $Y_{E/TS,Aerobic} = 0.37$ and $Y_{E/TS,oxygen\ limited} = 0.41$. It is also concluded that fructans from unheated clover-grass juice can be co-converted into ethanol by natural enzymes and yeast increasing the ethanol production significantly.

Using field data and biomass conversion yields obtained in laboratory experiments a decentralized biorefinery concept for co-production of bioethanol and biogas is described with strong emphasis on sustainability, localness and recycling principles.

1 Introduction

Bioethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future (Thomsen et al., 2003). Brazil and the United States are the largest producers of ethanol for transport, accounting for about 90 percent of world production. Both countries currently produce about 16 billion liters per year with a displacement of 40% of gasoline use in Brazil but only 3% in the United States with sugarcane (*Saccharum* L.) and corn (*Zea mays* L.) as the primary feedstock, respectively (Hazell and Pachauri, 2006). In 2005 Europe produced only about 2.6% of the world bioethanol production, but with a bioethanol sector growing with 70.5% between 2004 and 2005 primarily in Germany and Spain but with new producer countries like Hungary and Lithuania coming up (Eurobserver, 2006).

Recently a 10% binding minimum target was decided to be achieved by all EU Member States for the share of biofuels in overall EU transport petrol and diesel consumption by 2020 (EU 2007). In the U.S. President George W. Bush signed in to law the Energy Policy Act of 2005 creating a national renewable fuel standard (RFS) boosting the bioethanol sector (RFA, 2006). The character of such political activities is appropriate subject to production of biomass being sustainable - in the present study defined as the ability of a farm to produce indefinitely, without causing irreversible damage to ecosystem health.

Intercropping, defined as the growing of two or more species simultaneously on the same area of land (Willey, 1979), is an old traditional practice still widespread in the tropics and common in developed countries before the ‘fossilization’ of agriculture (Crews and Peoples, 2004). This cropping strategy is based on the manipulation of plant interactions in time and space to maximize growth and productivity (Hauggaard-Nielsen et al., 2006). Cereal-legume intercropping data from field trials show the possibility to increase input of leguminous symbiotic nitrogen (N) fixation into cropping systems reducing the need for fertilizer N applications (Jensen, 1996). Moreover, less need for pesticides are obtained due to improved competition towards weeds (Hauggaard-Nielsen et al., 2001; Liebman and Dyck, 1993) and less general damages on intercropped species by pest and disease organisms (Trenbath, 1993). Intercropping is a more adaptive management practice as compared to the present arable crop rotations consisting mainly of sole crops (monocrops, pure stands).

Beside bioethanol produced from sugar cane primarily in Brazil (producing more than twice the amount of the second largest producer India) the rest of the world production originates from starch fermentation (cereal grains), first generation technology. However, that is regarded as a too important food source in the present study. The emphasis is towards a food and energy approach using second generation technologies developed in recent years and cropping systems where the grain is utilized for food and feed and the remaining residues (straw, undersown grasses, catch crops etc.) is utilized for bioethanol production.

Apart from cellulose (40%), hemicellulose is a main sugar component (25-35%) in the lignocellulosic materials used for 2. generation bioethanol. However, these carbohydrates are closely bound together with lignin in the plant cell wall. Pre-treatment of the lignocellulose is necessary in order to open the structure and make carbohydrates susceptible to enzymatic hydrolysis and bioethanol fermentation. Aqueous pre-treatment at elevated temperature (such as wet-oxidation and steam explosion) result in an insoluble cellulose rich fraction (C-6 sugars) and a soluble fraction containing hemicellulose (C-5 sugars) and degradation products. (Bjerre et al. 1996; Tengborg et al. 2001). After pretreatment sugar polymers can be converted into fermentable sugars by enzymatic hydrolysis. An advantage of intercropping is that the intercrop components composition can be designed to produce a medium (for microbial fermentation) containing all essential nutrients. Thereby addition of e.g. urea and other fermentation nutrients produced from fossil fuels can be avoided.

Biorefineries represent a technology for utilization of renewable resources and natural compounds in form of crops such as ryegrass, alfalfa, clover, and immature cereals from extensive land cultivation and vegetable residues e.g. different kinds of straw and fibres (maize, grain, rape, hemp, flax, etc.), potato and vegetable industry wastes and molasses where all parts of the biomass is transformed into useful products, and no waste streams are produced (Thomsen et al, 2005). In the biorefinery concept crops are converted by means of mechanical and biotechnological methods into useful materials such as food and feed products and additives, as well as materials, organic chemical compounds, and bioenergy. Biotechnology offers several advantages compared to chemical synthesis e.g. high product specificity, low production temperature, and low energy consumption. As a result fermentation is becoming increasingly important in the production of commodity chemicals such as enzymes, antibiotics, biodegradable plastics, organic acids, alcohols, and amino acids.

Using field data and biomass conversion yields obtained in laboratory experiments a decentralized biorefinery concept for co-production of bioethanol and biogas is described with strong emphasis on sustainability, localness and recycling principles.

2 Materials and methods

The intercrop experiment was carried out on a sandy loam on the Experimental Farm of The Royal Veterinary and Agricultural University, Denmark (55^{o}40’N, 12^{o}18’E) in 2002. In spring field pea (*Pisium sativum* L.) and spring wheat (*Triticum sativum* L.) were established as 100% sole crops (SC) and in a 50% pea + 50% wheat intercrop (IC) according to recommended sole crop sowing densities of 90 pea plants and 400 wheat plants m^{-2}. Spring wheat SC and pea-wheat IC were grown at three levels of N supply in the form of urea, i.e. 0 (N0), 4 (N4) and 8 (N8) g N m^{-2} whereas pea SC was only grown at N0 and N4 due to the ability of pea to fix N_2 from the air. Microplots were placed in all fertilized plots and labelled with ^{15}N-urea and used for calculating the proportion of plant N derived from fixation (%Ndfa), fertilizer (%Ndff) and soil (%Ndfs) according to standard procedures (Chalk, 1998). For further information see Ghaley et al. (2005).

The clover-grass mixture (1:1) were cultivated in the experimental fields of Risø National Laboratory, Denmark. The material was harvested and for samples of pure clover and grass - and 1:3 clover-grass mixture - the material was separated by hand. The samples were dried at 50ºC to constant weight and milled to a size of less than 2 mm prior to pretreatment and further analysis. Fresh clover-grass juice was produced by pressing of newly harvested clover-grass in a kitchen fruit-press.

Wet oxidations were performed in a loop autoclave constructed at Risø National Laboratory using 6% dry matter (DM) (Bjerre et al., 1996). After the wet oxidation the pretreated material was separated by filtration into a solid filter cake (containing fibers and lignin) and a liquid fraction (containing soluble sugars and various degradation products). Pretreated liquids were stored at -20ºC until further analysis and use, and the filter cakes were dried and kept in a climate cabinet at 20ºC and 65% relative humidity.

To quantify the sugar polymers in the raw material and the solid fraction after wet oxidation a two step acid hydrolysis was performed. The first hydrolysis step was performed at 30ºC for 60 min. with 1.5 ml of H_2SO_4 (72%) for 0.16 g DM. Then 42 ml water was added and the second step was performed at 121ºC for 60 min. The hydrolyzate was filtered and the dried filter cake subtracted for ash content is reported as Klason lignin. In order to quantify the sugar content in the liquid fraction a weak hydrolysis was performed at 121 ºC for 10 min using 4% H2SO4, in duplicate. The amounts of released sugar monomers in the hydrolyzate as well as concentrations of ethanol, malic acid, succinic acid, glycolic acid, formic acid and acetic acid were determined by HPLC (Shimadzu) using a Rezex ROA column (Phenomenex) at 63ºC and 4 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min. A refractive index detector (Shimadzu Corp., Kyoto, Japan) was used.

The enzymatic hydrolysis was carried out at 50ºC, pH 4.8 and with 2% DM and an enzyme load of 30 FPU/g DM. The enzyme used was Cellubrix L, (Novozymes, Denmark) and the amounts of hydrolyzed sugars were determined by HPLC as described above. The experiments were carried out in triplicates for each solid pretreatment fraction.

Shake-flask fermentations of clover-grass with *Mucor indicus* were run in 250-ml erlenmeyer flasks containing 100 ml of clover-grass enriched with glucose to obtain a total of 16 g glucose/litre. One ml of a spore suspension in sterile water was inoculated in the medium and the flasks were incubated at 30^{o}C with shaking (130 rpm). Oxygen-limited fermentations were run in 32-ml flasks containing 30 ml of medium and equipped with cannulas for sampling and CO_2 removal. Fermentation of fresh clover-grass juice was also performed in in 250-ml erlenmeyer flasks containing 100 ml of medium. 0.5 g of dry commercial yeast (Malteserkors tørgær, De Danske Spritfabrikker A/S, Denmark) was added to the flasks together with the clover-grass, no nutrients were added. Glucose, xylose, and ethanol in fermentation broths were analysed by HPLC as described above. Flasks were incubated at 30^{o}C with shaking (130 rpm).

3 Results and discussions

Biomass cultivation

Wheat SC significantly increased dry matter (DM) production at increased rates of fertilizer nitrogen whereas pea-wheat IC and pea SC did not respond to fertilizer N (Figure 1). In N0 plots, pea SC and the pea-wheat IC produced more than twice the amount of DM as compared to wheat SC. With N4, no significant difference was observed between wheat and pea SC and total intercrop DM yield. However, doubling the fertilizer N rate (N8), sole cropped wheat accumulated significantly higher amounts of DM. In general, growing N_2 fixing species like pea as sole crops is considered an inefficient way of utilizing soil N resources, as the legume is able to fix N_2 and may only need a small amount of soil inorganic N in the establishment phase to overcome any N-deficiency after seed-N sources have been exhausted.

Land Equivalent Ratio (LER) can be used as a measure of the crop stands ability to capture environmental resources for growth (Mead and Willey, 1980). When using the total crop dry matter production in the calculation (Figure 1) the highest LER value was 1.26 in IC N0 indicating that 26% more land would have to be used when sole cropping in order to obtain the same yield, if each sole crop was allocated to 50% of land. Thus, in a future with availability of cultivated land as a potential limiting resource such increasing efficiencies in local resource use are of high importance. Increasing fertilization decrease LER to 0.97 (N4) and down to 0.85 (N8) indicating more efficient utilizations of environmental resources for growth by sole crops than by intercrops. Improving N supply by fertilization stimulates the wheat component, which thus suppresses the growth of the legume and indirectly the interspecific complementarity.

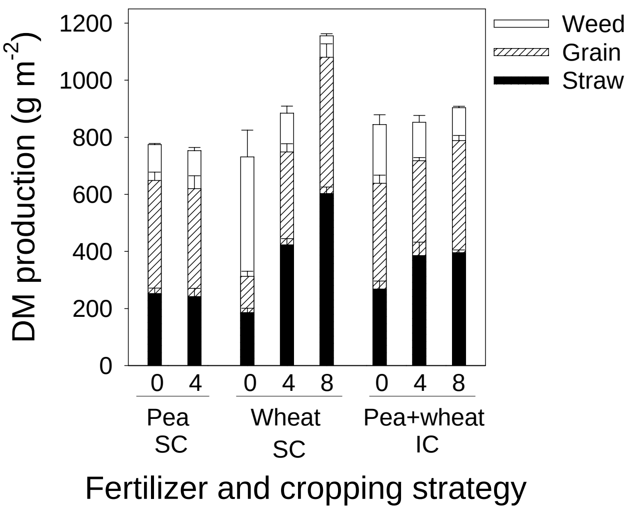

Figure 1. Average straw, grain and weed aboveground dry matter (DM) production in sole crops (SC) and intercrops (IC) of pea and wheat without (0) and with 4 and 8 g N m^{-2} application, respectively. Values are the mean (n=3) ± S.E. From Ghaley et al. 2005

Improved competition with weeds has been emphasised as one of the benefits of intercrops (Liebman and Dyck, 1993) because of increased interspecific competition as compared to sole cropping (Willey, 1979) assumed to result in a more dynamic crop response to a variety of growth conditions including temporal and spatial heterogeneity in growth of weeds throughout a growing season. When including weeds in the total DM production the variability comparing treatments (Figure 1) is rather limited with a coefficient of variation (CV) averaging 15% - also when taking into account the general availabilities included in every field study. Thus, for future cropping systems reducing external inputs any part of the soil surface that is not occupied by the crop plants is potentially subject to invasion by weedy species. When combining appropriate crop species within an intercrop instead of sole crops increased efficiency in utilising environmental sources for plant growth can be achieved improving the competitive ability towards weeds using soil N and other important growth resources for crop dry matter production instead of weed biomass.

Looking at the entire ethanol production cycle, biomass production and thereby management is a very prominent source of GHG emissions independent of whether it is first or second generation technologies with 60-70 % of total LC emissions for wheat grain ethanol and 30-45 % for wheat straw based ethanol when utilizing the Danish IBUS concept (Maarschalkerweerd, 2006). Nitrogen fertilization is responsible for the main part of GHG emissions from wheat grain production in Denmark primarily caused by energy intensive production of N-fertilisers and soil emissions of N_2O (LCA Food 2006).

When intercropped, the N derived from fertilizer in intercropped wheat was significantly higher (10 - 21%) than in intercropped pea (1-3%). In SCs with fertilizer N, soil N accounted for 62-78% of the total N in wheat and 17% in pea. With N0, the total amount of soil N accumulation was significantly higher in the pea sole crop (7.9 g N m^{-2}) compared to wheat (2.9 g N m^{-2}) and the combined intercrop (6.7 g N m^{-2}) (Figure 2). However, with fertilizer N, sole and intercropped wheat accumulated a greater amount of soil N compared to pea. As the fertilizer N input increase the percentage of N derived from N_2-fixation in pea both when sole cropped and intercropped peaked at N4 (90%) followed by a decrease with N8 (79%). The greatest amount of N_2-fixation was achieved in pea SC (10.3 g N m^{-2}) with N4 followed by the pea IC and pea SC with N0. The amount of fixed N_2 by pea IC was similar to pea SC when no N was applied. When increasing fertilizer N inputs, there was proportionate decrease in the amount of fixed N_2 in the intercrop due to the reduction of the pea intercrop proportion.

When developing sustainable plant production systems with a limited use of external inputs the present pea-wheat intercrop study show how crop interactions change dynamically over time, due to the species ability to exploit different resources and thereby secure capture of available plant growth resources. Cereals like wheat is strong competitors towards soil N. Pea-wheat IC without or with a low amount of fertilizer N supply offers an opportunity to maximise total DM production and on the same time increase N_2-fixation without compromising the yield levels. When enhancing fertilizer N LER decreased because the complementarity between the two species was decreased with wheat recovering up to 90% of the total intercrop fertilizer N acquisition and decreased the proportion of pea in the intercrop. A high degree of complementarity are important for resilient cropping strategies with the capacity for self-regulation to recover from biotic and abiotic stress when reducing external inputs and thereby energy use with less fertiliser and pesticide inputs.

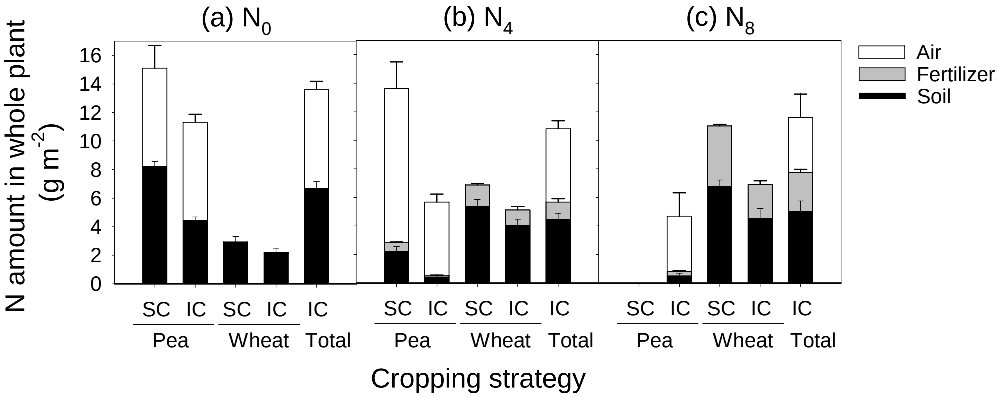

Figure 2. Amount of nitrogen (N) derived from soil, fertilizer and air in pea and wheat when grown as sole crops (SC) and as pea-wheat intercrop (IC) with 0 (N_0), 4 (N_4) and 8 (N_8) g N m^{-2} application. Values are the mean (n=3) + S.E. From Ghaley et al. 2005

The growing demand for bioenergy crops may create further competition for land and water and could result in additional negative environmental pressures from cultivating bioenergy crops (EEA, 2006). The environmental impact of bioenergy production depends to a large extent on the selection of areas that are used for bioenergy production, the crops cultivated and the farming practice. There is a need for integrating the biomass starting point into the energy manufacturing steps to secure that bioenergy is produced from local adapted raw materials with limited use of non-renewable fossil fuels. Chemical quality for conversion to secure efficiency in bioethanol production needs to go hand-in-hand with the development of ecologically benign farming systems in order to fulfil the aim of sustainable bioethanol production.

Many other species than wheat and pea are potential intercrop components, each suiting different purposes and cropping conditions (Willey, 1979). Reviewing published intercropping studies Connolly et al. (2001) listed crops includes as intercrop component with the most common species first: corn (Zea mays), cowpea (*Vigna unguiculate* L.), groundnut (*Arachis hypgaea*), wheat, millet (*Pennisetum glaucum*), clover cultivars (*Trifolium spp.*), beans (*Phaseolus vulgaris*), pigeonpea (*Cajanus cajan*), other beans (*Vicia faba*), barley (*Hordeum vulgare*) and pea, with 80% of published intercrop research conducted in developing counties in Africa and Asia. However, increasing demand for bioenergy in Europe and United States may create new uses for e.g. grass cuttings on marginal land, new bioenergy cropping systems and perennials might also add diversity and require less pesticide or fertiliser input than in current intensive agricultural systems, like shown for the present pea-wheat intercropping example.

Biomass conversion

At present cereal straw and corn stover is the typical biomass to be used in production of 2. generation bioethanol. Pre-treatment, hydrolysis, and ethanol fermentation of these materials are well studied and have been optimised in both laboratory scale (Bjerre et al. 1994, Scmidt & Thomsen 1998) and pilot scale (Thomsen et al., 2005). However, production of bio-ethanol in a larger scale may also require the use of alternative forms of biomasses e.g. pea, grass, or clover as described above.

Since clover and grass are rich in carbohydrates, mainly cellulose and hemicelluloses, they can be considered as substrates for bioethanol production. Clover grass pastures can be harvested several times a year and the green biomass can be collected and processed to bioethanol. Furthermore, clover grass is suitable for intercropping with wheat (just as pea in the example given above) (Thorsted et al. 2006). An interesting feature of clover grass mixtures is their high mineral, especially nitrogen, content, which is very useful in down-stream processing, since the utilisation of mineral nutrients in the fermentation step can be reduced or even avoided.

In this study clover, grass, and a mixture of clover-grass were pretreated by wet oxidation in order to examine the suitability of clover-grass to be used in bioethanol production alone or in combination with e.g. wheat straw. Table 1 shows the composition of the materials compared to wheat straw.

Table 1 Composition of raw materials.

Raw material	Cellulose (g/100 g DM)	Hemicellulose (g/100 g DM)	Lignin (g/100 g DM)
Wheat straw*	33.9	23.0	19.1
Clover	15.6	10.5	14.4
Grass	23.9	17.5	12.8

*Thomsen et al., 2006

Pretreatment of clover, grass and clover-grass mixed 1:1 and 1:3 were performed at 195ºC for 10 minutes using 12 bar of oxygen pressure and 2 g/l of Na_2CO_3, which have been shown to give the optimal pre-treatment of wheat straw. Furthermore, pre-treatment of the 1:1 mixture of clover-grass where studied at 175ºC and 185ºC with and without addition of Na_2CO_3, and with high (12 bar) and low (3 bar) oxygen pressure. Figure 3 shows the sugar and lignin content of the fibre-fraction after pre-treatment and figure 4 shows the composition of the liquid fraction. The pretreated grass fibres have higher glucan content than clover (Figure 3), and the grass-liquid also has a higher content of hemicellulose (Figure 4), which is due to the different in the two materials (Table 1).

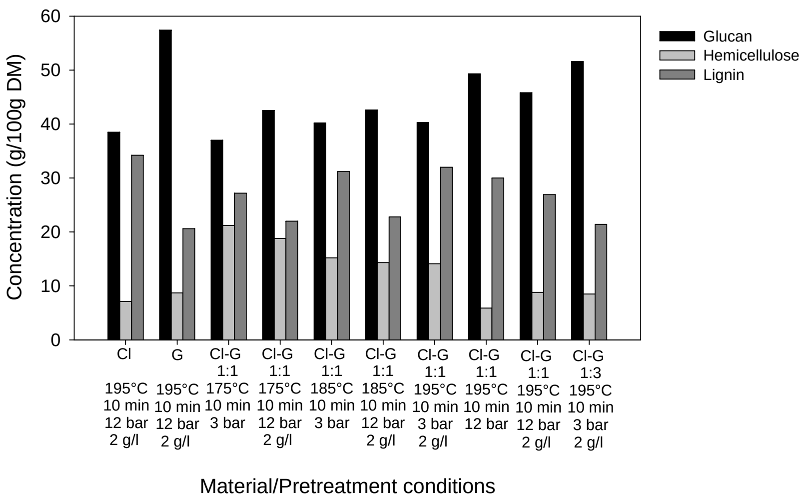

Figure 3 Glucan, hemicellulose, and lignin content in the fiber fraction of wet oxidised clover (Cl), grass (G), and clover-grass mixtures (Cl-G).

In turn clover has a higher content of lignin. The hemicellulose content of the fibres is dependent on the pre-treatment temperature, at higher temperatures more hemicellulose is extracted from the fibres (Figure 3), giving a higher hemicellulose concentration in the pretreatment liquids (Figure 4). Also a high oxygen pressure seems to have an effect on hemicellulose extraction, and the three experiment with clover-grass (1:1) performed at 195ºC indicates that the extraction is highest when no Na_2CO_3 is added. Clover and grass hemicellulose consist significant amount of both xylose and arabinose (Figure 4) in contrast to wheat straw hemicellulose with is 86 % xylose (Gong et al., 1981). The arabinose concentration is highest in liquid pretreated at low temperature whereas the opposite tendency is observed for xylose. This could indicate that arabinose is more susceptible to thermal degradation than xylose.

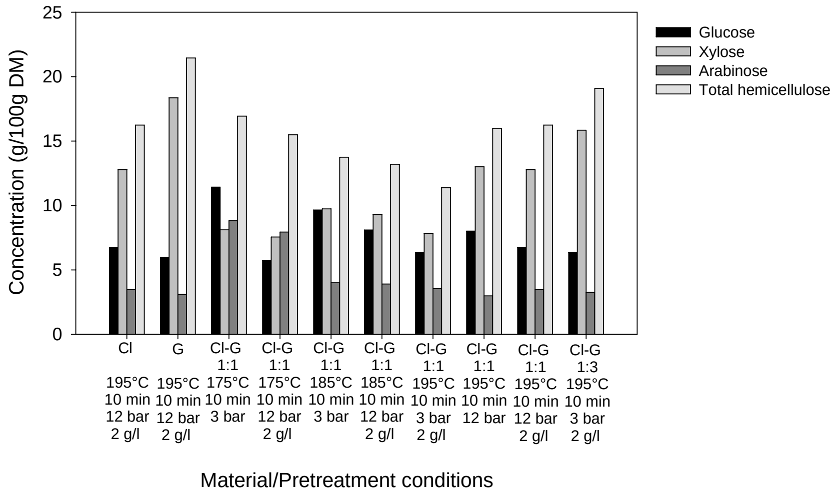

Figure 4 Glucose, xylose, arabinose and total hemicellulose content in the liquid fractions of wet oxidised clover (Cl), grass (G), and clover-grass mixtures (Cl-G).

Figure 5 shows the results of the enzymatic hydrolysis of the pretreated fibers. When pretreated at identical conditions (195ºC, 10 min, 12 bar, 2 g/l Na2CO3) grass gives a higher sugar yield than clover, which could be due to the higher lignin content in clover, since lignin acts as the glue that binds the sugar polymers together in the cell wall materials (kilde). At 175ºC only approximately 40% of the glucose and 30% of the xylose in the grass-clover mixture can be converted to fermentable sugars. At higher temperatures the convertibility of the fibers are significantly improved, and the optimal treatment of the clover-grass is found at 195ºC using high oxygen pressure and no addition of Na_2CO_3 where the glucose yield is 94 % and the xylose yield is 66% - Arabinose?

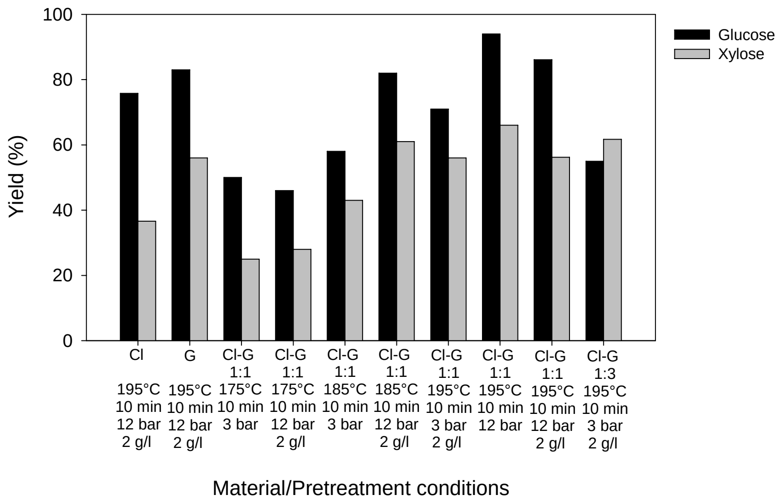

Figure 5. Glucose and xylose yield in enzymatic hydrolysis of fibers from wet oxidation of clover (Cl), grass (G), and clover-grass mixtures (Cl-G).

The results of this preliminary study shows that the optimum pretreatment conditions for clover, grass, and clover-grass mixtures is not significantly different from that of wheat straw (195ºC, 10 min, 12 bar, 2 g/l Na_2CO_3), even though the composition of the raw material is different (Table 1). Both clover, grass, and clover-grass mixtures give glucose yields close to and above 80% when pretreated at these conditions, which indicates that wheat straw and clover-grass could be pretreated in one step if it was cultivated together in order to achieve the benefits described in the previous section about intercropping. However the effect of the Na_2CO_3 catalyst should be examined in experiments with straw and clover-grass pretreated together in order to decide if the catalyst should be added, since in the clover-grass mixture the highest yield is achieved without addition of the catalyst.

When pretreating biomass at these high temperatures some thermal degradation of sugar and lignin components is inevitable, resulting in formation of fermentation inhibitors. The fermentability of the clover-grass liquid fraction produced at optimal conditions (195ºC, 10 min, 12 bar) in this study was examined by ethanol fermentation with the filamentous fungus *Mucor indicus* (Figure 6). The avantage of using *Mucor indicus*, instead of the traditional ethanol producer Bakers yeast (*Saccharomyces cerevisiae*), is that it is capable of utilising the hemicellulose sugars.

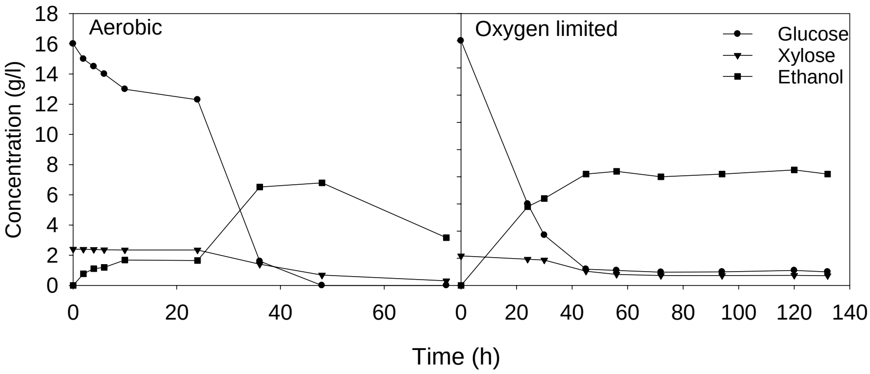

Figure 6. Ethanol formation and free sugar consumption during aerobic and oxygen-limited fermentation of a glucose enriched clover-grass hydrolysate by *M. indicus*.

Mucor indicus was successfully adapted to the clover-grass hydrolysat, showing that the inhibitor level in the hydrolysates was acceptable. Ethanol was the main product formed during fermentation, but a considerable formation of cell biomass was also detected, especially under aerobic conditions. Good ethanol yields were obtained (calculated from total sugar consumed): YE/TS,Aerobic = 0.37 and YE/TS,oxygen limited = 0.41. Glucose was completely consumed in both experiments. Xylose consumption started only when most of the glucose was consumed. 80% of the free xylose was consumed under aerobic conditions.

Biorefinery concepts

In the experiments described in the previous section clover-grass was dried before pre-treatment as it would be the case if clover-grass were undersown in a wheat field and harvested and dried on the field together with the wheat straw. But when heating the material to 195ºC valuable components of the clover-grass such as enzymes and free sugars are lost. Figure 7 shows the result of yeast fermentation of fresh (non-heat-sterilised) clover-grass juice. After 24 hours of fermentation all glucose present (12 g/l) in the juice is consumed, and approximately 15 g/l of ethanol is produced. From 12 g/l glucose only approx. 6 g/l of ethanol can be produced, which shows that other sugars in the juice is utilised for ethanol production.

Grass and clover contains significant amount of fructans; approx. 166 g/kg DM and 111 g/kg DM respectively (Thomsen et al., 2006). Fructans are polymeric carbohydrates consisting of variable numbers of fructose molecules with terminal sucrose. Fructans can be decomposed to free carbohydrates by enzymes in the crops that are activated after harvesting and pressing (Hirst, 1957). Plant fructan hydrolases are reported to be most active between pH 4.5 to 5.5 and to have temperature optimum ranging from 25 to 40°C (Simpson and Bonnett, 1992), which means they could be active during yeast fermentation at 32ºC and pH 4-6.

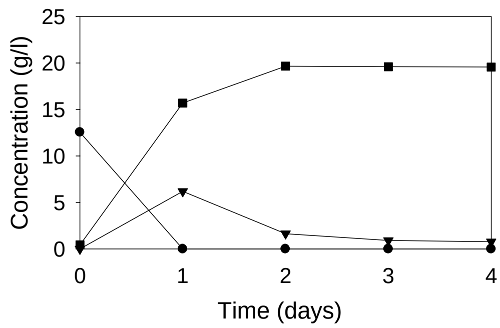

Figure 7. Yeast fermentation of fresh clover-grass juice.

This experiment show that fructans in the un-heated juice can be converted to ethanol by natural enzymes and yeast (or maby other microorganisms in the non-sterilised medium) increasing the ethanol production significantly. The fiber fraction form the pressing (which contains the lignocellulosic sugars) can be pretreated together with e.g. wheat straw in the biorefinery for maximum utilization of biomass components. Figure 8 shows the concept of utilization of straw and an N-fixating crop e.g. clover-grass for ethanol production in a biorefinery.

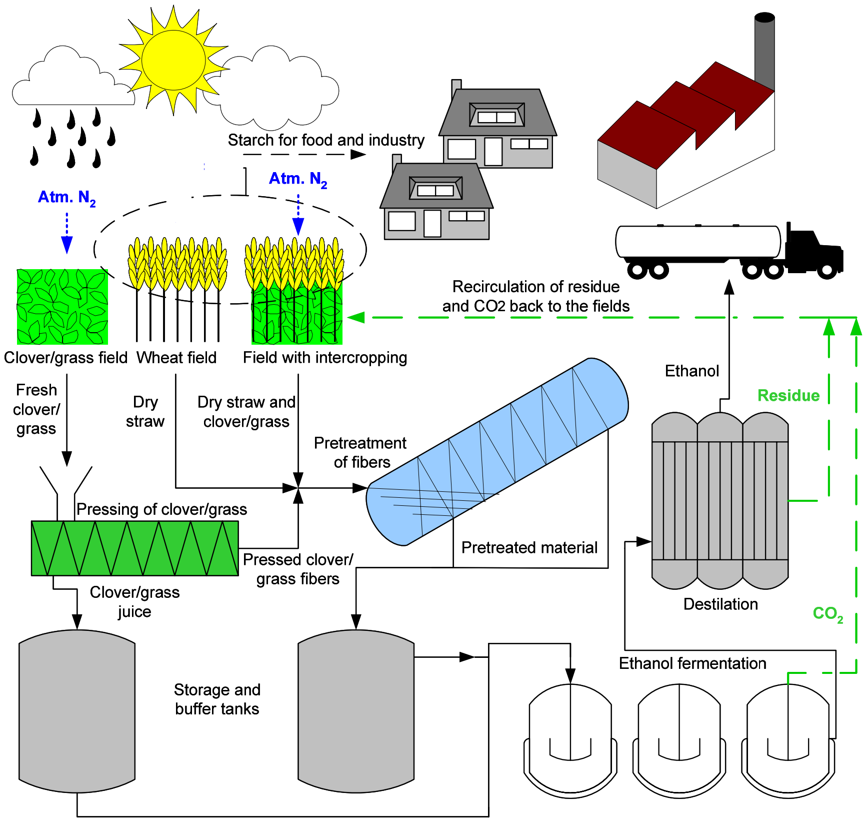

Figure 8. Biorefinery concept for utilization of N-fixating and carbohydrate rich crops.

The next step in this research would be to examine the pretreatment of clover-grass and straw in one step as well as examine co-fermentation of pretreated fibers and fresh clover-grass juice.

4 Conclusions

A legume-cereal intercrop like pea-wheat seems to be an optimal cropping strategy in relation to the use of N resources, because wheat efficiently exploits soil mineral N sources while at the same time fixed N_2 from pea enter the cropping system.

Increase of fertilizer N supply promotes the growth of wheat and results in a decreased pea N accumulation and a different proportion of intercrop components possibly influencing the conversion requirements.

Dynamic change of plant species interactions as a response to the actual growing conditions is not achieved with sole cropping of one species/cultivar. Furthermore, in the pea sole crop situation available soil mineral N reduce N_2 fixation and the full potential of symbiotic nitrogen fixation is not exploited which is regarded as an overall inefficient use of N sources.

Wet oxidation is an efficient method for fractionating clover, grass, and clover-grass mixtures into a convertible solid cellulose fraction and a soluble hemicellulose fraction.

The highest yield of fermentable sugars after enzymatic hydrolysis is achieved in clover-grass (mixed 1:1) pretreated at 195ºC for 10 minutes using 12 bar oxygen.

The optimum pretreatment conditions for clover, grass, and clover-grass mixtures is not significantly different from that of wheat, which indicates that wheat straw and clover-grass (from intercropping) could be pretreated in one step.

The produced sugars were converted into ethanol by *Mucor indicus* giving good ethanol yields $Y_{E/TS,Aerobic} = 0.37$ and $Y_{E/TS,oxygen\ limited} = 0.41$.

Fructans from unheated clover-grass juice can be co-converted into ethanol by natural enzymes and yeast increasing the ethanol production significantly.

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