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Sustainable bioethanol production combining biorefinery principles using combined raw materials from wheat undersown with clover-grass

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Abstract To obtain the best possible net energy balance of the bioethanol production the biomass raw materials used need to be produced with limited use of non-renewable fossil fuels. Intercropping strategies are known to maximize growth and productivity by including more than one species in the crop stand, very often with legumes as one of the components. In the present study clover-grass is undersown in a traditional wheat crop. Thereby, it is possible to increase input of symbiotic fixation of atmospheric nitrogen into the cropping systems and reduce the need for fertilizer applications. Furthermore, when using such wheat and clover-grass mixtures as raw material, addition of urea and other fermentation nutrients produced from fossil fuels can be reduced in the whole ethanol manufacturing chain. Using second generation ethanol technology mixtures of relative proportions of wheat straw and clover-grass (15:85, 50:50, and 85:15) were pretreated by wet oxidation. The results showed that supplementing wheat straw with clover-grass had a positive effect on the ethanol yield in simultaneous saccharification and fermentation experiments, and the effect was more pronounced in inhibitory substrates. The highest ethanol yield (80% of theoretical) was obtained in the experiment with high fraction (85%) of clover-grass. In order to improve the sugar recovery of clover-grass, it should be separated into a green juice (containing free sugars, fructan, amino acids, vitamins and soluble minerals) for direct fermentation and a fibre pulp for pretreatment together with wheat straw. Based on the obtained results a decentralized biorefinery concept for production of biofuel is suggested emphasizing sustainability, localness, and recycling principles.

Keywords Biomass · Intercropping · Bioethanol · Wetoxidation · Biorefinery

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Introduction

Bioethanol produced from pretreatment and microbial fermentation of biomass has great potential to become a sustainable transportation fuel in the near future [22]. Using first generation technology Brazil and the United States are accounting for about 90% of world ethanol production using sugarcane (*Saccharum* L.) and corn (*Zea mays* L.) as the primary feedstock, respectively. In 2005 Europe produced only about 2.6% of the world bioethanol production. However, a 10% binding minimum target, decided to be achieved by all EU Member States for the share of biofuels in overall EU transport petrol and diesel consumption by 2020, is expecting to boost both scientific developments and commercial solutions in Europe [4, 5].

Extensive research is carried out to develop second generation bioethanol production from lignocellulosic materials such a cereal straw and corn stover [3, 25], and a successful pilot scale facility have been developed [23]. Lignocellulosic raw materials contain cellulose and hemicellulose polymers built up by long chains of sugar monomers bound together by lignin. After pretreatment and hydrolysis the sugars can be converted into ethanol by microbial fermentation. Different pretreatment methods exist, such as wet-oxidation [3, 16, 19], acid treatment, steam explosion [6], and hydrothermal treatment [23]. The aim of the pretreatment is to open up the lignocellulosic structure to enable enzymatic hydrolysis.

The traditional microorganism used for ethanol production is ordinary baker's yeast, *Saccharomyces cerevisiae*, because it is one of the most tolerant microorganisms towards inhibitors formed during pretreatment [13, 17]. However, it can only convert the cellulosic hexose sugars (such as glucose and mannose), and not the hemicellulosic pentoses (such as xylose and arabinose). Inhibitor formation and pentose fermentation are the main challenge in the development of second generation bioethanol.

In order to secure that bioenergy is produced with limited use of non-renewable fossil fuels the biomass raw materials needs to be integrated into the energy manufacturing steps. Undersowing and intercropping (or mixed cropping) involves growing two or more crop species on the same field lowering the need for fossil fuel based fertilizers and agrochemicals by manipulating plant interactions in time and space [7, 8, 11]. It is a traditional practice and very common before the "fossilisation" of agriculture.

Globally wheat (*Triticum aestivum*) is the second most widely produced crop, just recently surpassed by maize. Clover (*Trifolium repens*)-grass (*Lolium perenne*) pastures are important in many low-input agroecosystems due to: (a) their use, either fresh or as silage, as feed with (b) high dry matter (DM) yields (>10 t ha^{-1} $year^{-1}$) in unfertilised pastures with up to 95% of the nitrogen (N) being supplied by the N_2 fixing clover [11], (c) their roots and stubble contain 60–110 kg N ha^{-1} reducing fertilizer requirements for the succeeding crops [7], and (d) improve ground cover [18] securing efficient uptake of available nutrients [8, 12].

An advantage of intercropping cropping strategies, defined as the growing of two or more species simultaneously on the same area of land, is that the choice of intercrop species and the final composition between components can be designed [7, 8, 11] to produce an almost complete medium for the microbial fermentation containing all essential nutrients, especially N when including leguminous species, whereby addition of e.g. urea and other fossil based fermentation nutrients can be reduced.

A biorefinery concept for sustainable production of bioethanol from wheat straw and clover-grass cuts in autumn and early spring, before sowing the subsequent spring crop, is suggested, where the wheat grains are going to the traditional food and feed markets. It is taken into account that these raw materials have similar optimal pretreatment conditions [3, 15, 23]. Three relative proportions of wheat straw and clover-grass (15:85, 50:50, and 85:15) are tested and pretreated by wet oxidation. According to the results obtained a theoretical biorefinery concept is visualised and discussed.

Materials and methods

Raw materials

In the present study the emphasis is conversion of alternative raw materials and not evaluation of specific cropping strategies. However, in order to mimic agricultural practises the raw materials used was produced on a farmer's field where clover-grass was undersown in winter wheat [24].

The wheat, clover, and grass raw materials were produced in 2005–2006 on a farmer's field at Risø-DTU (55°41′N, 12°05′E). The 25 year mean annual rainfall is 550 mm, mean annual air temperature is 8 °C with maximum and minimum daily means of 16 °C (July) and −1 °C (February). The area meets temperate conditions. The soil at the site is sandy loam (Typic Hapludalf) topsoil (0–25 cm). The field has been cropped previously with a wheat–barley–oilseed rotation, with optimum application of inorganic fertilizers. Winter wheat was sown (3 cm depth) ultimo September followed by distribution of clover-grass seeds on the soil and a gentle incorporation into the topsoil (1.5 cm) using a mechanical finger weeder. Winter wheat was produced using recommended optimum N, phosphorus (P) and potassium (K) fertilization (150 kg N–30 kg P–50 kg K ha^{-1}). Pest and diseases were controlled with appropriate agrochemicals.

The wheat crop was combine harvested medio August yielding 8 tons grain DM and 5 tons straw DM ha^{-1} indicating high wheat interspecific competitive ability and no yield reduction caused by the undersowing of clover-grass when comparing the yields to previous years. The clover-grass growth was increased rapidly shortly after removal of the straw from the field. Using conventional grass cutter machinery the first cut were taken in late September (1.5 tons DM ha^{-1}) and the second in late October (1.5 tons DM ha^{-1}). A final third cut were taken medio April (1 tons DM ha^{-1}) just before sowing subsequent spring crop. A subsample of the fresh clover-grass pasture was separated in clover and grass species. Part of the clover-grass was pressed and divided into a juice and a pulp fraction. Another part was dried at 50 °C to constant weight.

Pretreatment

The pretreatment method used in this study was wet oxidation (WO). Three relative proportions of wheat straw (WS) and clover-grass (CG) (WO1 = CG15:WS85, WO2 = CG50:WS50, and WO3 = CG85:WS15) were mixed and used in wet oxidation experiments. The wet oxidation pretreatments were performed in a loop autoclave constructed at Risø-DTU using 6% DM [3]. Pretreatment conditions were chosen close to the optimum pretreatment conditions for clover-grass and wheat straw

(195 °C, 10 min, 12 bar, 2 g l^{-1} Na_2CO_3) found in other published studies [3, 15]. After pretreatment the material was separated by filtration into a solid filter cake (containing fibres and lignin) and a liquid fraction (containing soluble sugars and various degradation products). Pretreated liquids were stored at −20 °C until further analysis and use, and the filter cakes were dried and kept in a climate cabinet at 20 °C and 65% relative humidity.

Simultaneous saccharification and fermentation

After pretreatment 8 g DM of the solid fraction (filter cakes) were mixed with 60 ml of filtrate and the raw sample was mixed with 60 ml of water (pH 4.8) in 250 ml fermentation flasks. All experiments were done in duplicate. Liquefaction was performed at 50 °C with an enzyme (Cellubrix L) load of 15 FPU g^{-1} DM for 24 h. After cooling to room temperature, 15 FPU g^{-1} DM enzymes (Cellubrix L), and 0.2 g active dried yeast (95% DM) commercial yeast (Malteserkors tørgær, De Danske Spritfabrikker A/S, Denmark) were added. The head space in the flasks was flushed with N_2, and the flasks were equipped with yeast locks filled with glycerol. The flasks were then incubated at 32 °C and the amount of produced ethanol was determined as weight loss caused by CO_2 liberation. The final ethanol concentrations were determined by HPLC.

Analysis methods

Dry matter and ash content

Duplicates of 0.5 g solid material or 10 ml of liquid sample were dried at 105 °C over night to determine DM. The samples were then heated to 550 °C for 3 h to determine the total ash content.

Analysis of sugar polymers in solid fraction

To quantify the sugar polymers in the raw material and the solid fraction after wet oxidation a two-step acid hydrolysis was performed in duplicate. The first hydrolysis step was performed at 30 °C 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 121 °C for 60 min. The hydrolyzate was filtered and the dried filter cake subtracted for ash content is reported as Klason lignin.

Analysis of carbohydrates in liquid fraction

In order to quantify the sugar content in the liquid fraction a weak hydrolysis was performed at 121 °C for 10 min using 4% H_2SO_4, in duplicate. The concentrations of sugar monomers were determined by HPLC.

HPLC analysis

The amounts of released sugar monomers in the hydrolysate, furans, and produced ethanol in simultaneous saccharification and fermentation (SSF) were determined by HPLC (Shimadzu) using a Rezex ROA column (Phenomenex) at 63 °C and 4 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}. A refractive index detector (Shimadzu Corp., Kyoto, Japan) was used.

Analysis of minerals

Dried samples of wheat straw and clover grass were digested in a microwave oven using a mixture of 65% HNO_3 and 40% HF. The digested samples were diluted with water and following analysed by Varian Vista AX-ICP AES (optical emission spectrometry with inductively coupled plasma) using argon gas as carrier gas. The plasma temperature was 7,000 °C at which temperature most elements emit light of characteristic wavelengths, which can be measured and used to determine the concentration a by light-sensitive detector at 167–785 nm.

Calculations

Recovery of sugars after pre-treatment were calculated according to (Eq. 1).

$$\text{Recovery} = \frac{[\text{sugar in filtrate (g/100 g)} + \text{sugar in solid (g/100 g)}]}{[\text{sugar in raw material (g/100 g)}]} \times 100\% \quad (1)$$

The ethanol (EtOH) yield in SSF experiments was calculated as percentage of the theoretical ethanol production based on the glucan content of the fibre fraction and glucose in the filtrates (Eq. 2). Theoretically, the production yield of *Sahharomyces cerevisiae* is 0.511 for EtOH and 0.489 for CO_2 [2].

$$\text{EtOH yield} = \frac{\text{EtOH}_{\text{Gravimetric/HPLC}}}{\text{glucan in solid} \times 0.51 + \text{glucose infiltrate} \times 0.51} \times 100 \quad (2)$$

The theoretical ethanol production based on the actual pretreatment and hydrolysis yields (in SSF) was calculated according to (Eq. 3).

$$\text{Theoretical ethanol production} = \text{TSC*} \times 0.51 + \text{TSH*} \times 0.51 \quad (3)$$

*TSC = Total sugar from cellulose (based on yield obtained in SSF experiments) (g/100 g raw material).

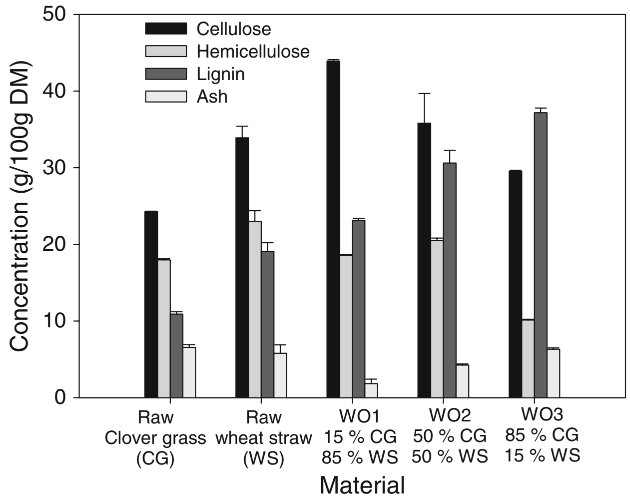

Fig. 1 Composition of raw clover-grass, raw wheat straw, and the pretreated fibres from wet oxidation of three different wheat straw and clover-grass mixtures

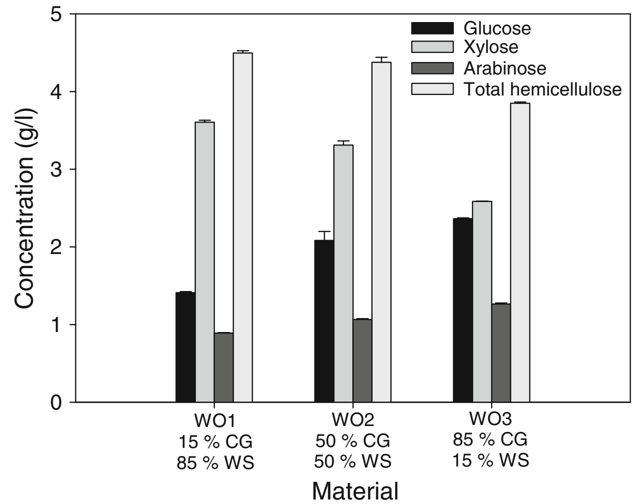

Fig. 2 Sugars composition of liquid fractions from wet oxidation pretreatment of three different wheat straw and clover-grass mixtures

*TSH = Total sugar from hemicellulose (based on hemicellulose recovery) (g/100 g raw material)

Results and discussion

Biomass conversion

Three different mixtures of wheat straw and clover-grass: (WO1) 15% clover-grass mixed with 85% wheat straw, (WO2) 50% of each crop, and (WO3) 85% clover-grass and 15% wheat straw were pretreated by wet oxidation. The composition of the pretreated fibres was compared with the composition of raw wheat straw and raw clover-grass (Fig. 1). The results indicate that wheat straw has a higher content of glucan, hemicellulose, and lignin than clover-grass. As expected the highest ash content was found in WO3 with the highest fraction of clover-grass. Likewise, as expected, the highest glucan content was found in WO1 mixtures and visa versa for WO3. However, the concentration of hemicellulose sugars was slightly higher in WO2 as compared to WO1, even though the total raw material contains more hemicellulose (Fig. 1). This could be due to loss of hemicellulose sugars during pretreatment or due to a higher extraction of the pentoses into the liquid fraction in WO1. As expected from the chemical composition (Fig. 1), the hemicellulose content was much lower in WO3. It is surprising to see the strong tendency of increasing lignin content with decreasing wheat straw content, because wheat straw contains more lignin that clover-grass (Fig. 1). An important observation in these experiments was that no fructose could be found in the raw clover-grass (after drying and strong acid hydrolysis)—or in any of the other samples—indicating that fructan might be transformed to other compounds during drying.

The sugar-composition of the liquid fractions from the three pretreatment experiments is shown in Fig. 2. The amount of glucan, which is extracted during pretreatment, increases with decreasing wheat straw addition, even though wheat straw contains more glucan than clover-grass (Fig. 1). The reason could be that the cellulosic bound glucose is only extracted into the liquid phase to a very low extent in wet oxidation pretreatments [3], whereas clover-grass probably contain some more available glucose oligomers [1], which are extracted into the liquid during pretreatment and hydrolysed in the weak acid hydrolysis that is used in the analysis of the liquid fraction. The direct HPLC-analysis of all three liquids showed no traceable levels of free sugars (data not shown), showing that it is not glucose (monomers) which are being extracted. The amount of xylose extracted into the liquid was highest in the experiments with high fraction of wheat straw, as expected, due to the higher content of hemicellulose in wheat straw compared to clover-grass. However, more arabinose is extracted in the experiments with the highest fraction of clover-grass, which is due to the fact that the clover-grass hemicellulose contains significant amount of arabinan (approx. 3%—results not shown), whereas wheat straw hemicellulose consists primarily of xylose. The highest total sugars concentration was found in WO2 (6.5 g l^{-1}) with equal amounts of wheat straw and clover-grass in the raw material (Fig. 2).

Very high sugars recoveries were found, especially in the experiment with 85% wheat straw (WO1) with recovery rates of 98% for glucose and 86% for hemicellulose sugars (Fig. 3). The glucose recovery is decreasing as the content of clover-grass in the mixture is increased. This

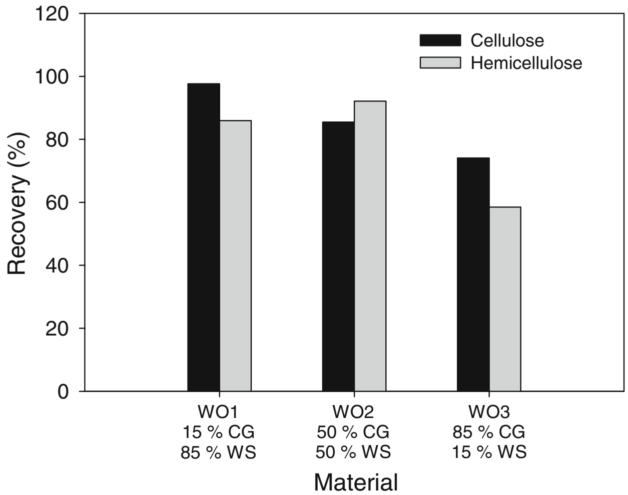

Fig. 3 Total recovery of glucose and hemicellulose sugars in materials (solid + liquid phase) of wet oxidised mixtures of wheat straw and clover-grass

could be connected to the fact that the analysis of the liquid fraction showed that WO3 contained more extractable glucose-oligomers. When the sugars are extracted into the liquid phase during pretreatment, the sugars become more vulnerable to thermal degradation, and are converted to organic acids and furans [13, 14]. This is in agreement with analysis of organic acids in the liquids where the highest concentration of formic acid is found in WO3 (Table 1). Very low concentrations of furans are present in all three liquid fractions (Table 1), probably due to the fact that in wet oxidations furfural and 5 hydroxy-2-methylfurfural (5-HMF) can be oxidized to formic acid [13, 14]. The hemicellulose recovery was similar in WO1 and WO2, although tending to be higher in WO2 probably due to the higher concentration of hemicellulose found in the fibre-fraction from this experiment (Fig. 1). However, it can be concluded that the higher the fraction of clover-grass in the pretreated material, the lower the overall sugar recovery. Fresh clover-grass contains significant amount of free sugars [1], and since no free sugars could be found in any of the pretreated liquids, it is likely that the free sugars

Table 1 Content of fermentation inhibitors (organic acids and furans) in the liquid fractions

Inhibitor	WO1 15% CG 85% WS	WO2 50% CG 50% WS	WO3 85% CG 15% WS
Glycolic acid (g l^{-1})	0.30	0.24	0.22
Formic acid (g l^{-1})	0.61	0.78	0.99
Acetic acid (g l^{-1})	1.65	1.55	1.38
5-HMF (g l^{-1})	0	0	0
Furfural (g l^{-1})	0.03	0.03	0.02

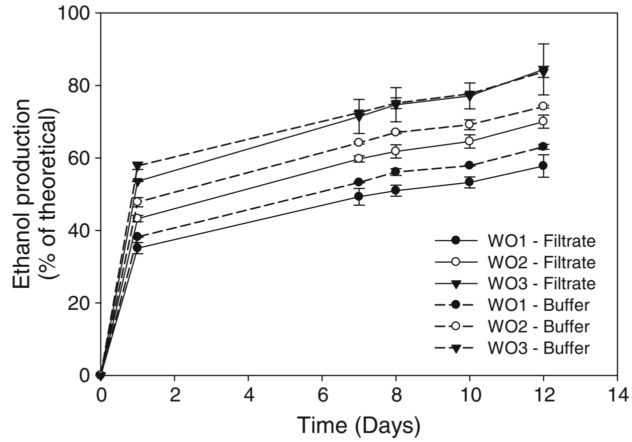

Fig. 4 Ethanol production in simultaneous saccharification and fermentation (SSF) of fibre fraction suspended in buffer-solution (*dashed line*) and in the liquid phase from pretreatment (*solid line*)

have been degraded to other compounds. Clover-grass contains both free sugars and amino acids and while heated up in the pretreatment step it is likely that formation of Maillard compounds [21] are the reason of the low recovery in the experiment with 85% clover-grass (WO3). The Maillard reactions result in formation of fermentation inhibitors and reduce the amount of available amino acids and carbohydrates in the liquids [21]. This should be studied further also to determine if the Maillard products could precipitate as Klason lignin in the strong acid hydrolysis, explaining why the lignin content of the pretreated samples increase with increasing clover-grass content (Fig. 1). Due to the content of free sugars and fructan in the fresh clover-grass [1] it might be better to integrate a first generation ethanol step in the raw material processing, where the clover-grass is pressed into an easy fermentable green juice containing free sugars, fructan, amino acids, vitamins and soluble minerals [1] and a fibre pulp, which can then be pretreated together with the wheat straw but without the loss of the free sugars.

The enzymatic convertibility of the fibres was examined by SSF (using *S. cerevisiae*) in order to avoid product inhibition of the enzymes. SSF of the fibre-fraction was performed both with the fibres suspended in a buffer-solution (to give the convertibility of the pure fibres) and with the fibres suspended in the liquid phase (to examine the fermentability of the hydrolysates) (Fig. 4). All six fermentations started without a lag-phase showing that the fermentability of the fibres and liquids were good and indicating that inhibitor levels were low. The results show that the higher the fractions of clover-grass in the medium, the higher the final ethanol yield could be achieved, with a WO3 ethanol yield of 80% of the theoretical based on sugars in the pretreated material. In all experiments the productivity was highest in the experiment where SSFs

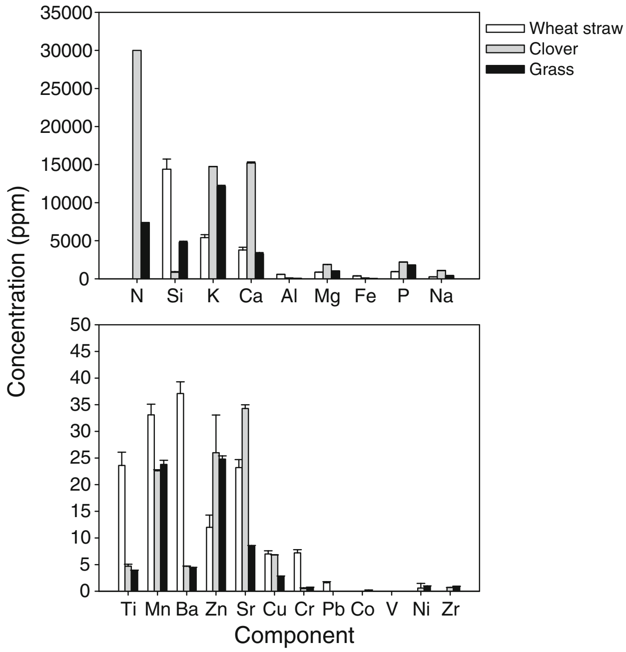

Fig. 5 Mineral composition of wheat straw, clover (*Trifolium repens*), and ryegrass (*Lolium perenne*)

were performed in buffer-solution, indicating that the liquids did contain some inhibitory compounds. However, in the experiment with 85% clover-grass the final ethanol yield was the same in buffer as in the liquid phase, even though the sugar recovery was lowest in this experiment—and it should be expected that the inhibitor levels were higher. This is probably due to the high nutrient content of the clover-grass compared to wheat straw (Fig. 5). It has been shown, that *S. cerevisiae* can tolerate higher inhibitor levels when wheat straw hydrolysates were supplemented with yeast extract (unpublished data). The results show that supplementing wheat straw with clover-grass has a positive effect on the ethanol yield in SSF experiments, especially in whole slurry fermentations (fibre and liquids) and the effect is more pronounced in more inhibitory substrates. This is a very important advantage of combined wheat straw and clover-grass raw materials compared to conversion of wheat straw alone.

First generation technology in a second generation factory

Besides free and lignocellulosic carbohydrates fresh clover-grass contains significant amounts of fructans [1], which are polymeric carbohydrates consisting of variable numbers of fructose molecules and terminal sucrose. Fructans have differences in molecular structure and in molecular weight, and they may be classified in three main types: the inulin group, the phlein group, and the branched

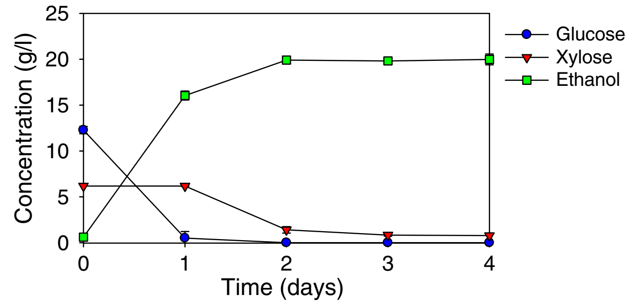

Fig. 6 Yeast fermentation of fresh clover-grass juice added 10 g l^{-1} of glucose

group [10]. Cutting and drying of the clover-grass result in loss of fructane, because it activates enzymes in the cytoplasm which break down the fructan [9]. Actually, no fructose could be found when analyzing the dried clover-grass, neither before nor after weak acid hydrolysis. Instead the clover-grass was separated into a juice for direct fermentation and a fibre pulp for pre-treatment. The non-heat-sterilised clover-grass juice was fermented directly by addition of *S. cerevisiae* (no nutrients or sugars were added) and incubated at 32 °C. After 24 h all glucose present (12 g l^{-1}) in the juice was consumed, and approximately 15 g l^{-1} of ethanol was produced (Fig. 6). From 12 g l^{-1} glucose only approximately 6 g l^{-1} 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; approximately 166 and 111 g kg^{-1} DM, respectively [1]. Plant fructan hydrolases are reported to be most active between pH 4.5–5.5 and to have temperature optimum ranging from 25 to 40 °C [20], which means they could be active during yeast fermentation at 32 °C and pH 4–6. This experiment shows that fructans in the un-heated juice can be converted to ethanol by natural enzymes and yeast (or maybe other microorganisms in the non-sterilised medium) increasing the ethanol production significantly.

Biorefinery concept

The illustrated biorefinery (Fig. 7) is based on a combination of raw materials to utilize the complementarity gained by combining different species from both a crop production (annuals and perennials) and an energy conversion point of view. Different crop production options are possible like (a) wheat straw from the traditional sole cropping cultivation with clover-grass biomass collected from a separate field as compared to (b) the present practise where clover-grass is undersown in winter wheat in order to enhance the stability of the agroecosystems lowering the need for fossil fuel based fertilizers and agricultural chemicals [12, 18]. In the present study the intercrop concept used was though as a one-year option for a traditional rotation system, but obviously when the clover-grass pasture is established it can be harvested several times over 2–3 subsequent years producing a high quality biomass with very limited use of fossil inputs [11].

The basic idea is that the clover-grass is subjected to a wet separation process resulting in a juice and a pulp fraction in order to utilize the fructan and free sugars (Fig. 6) which will otherwise be degraded during drying and pretreatment (Fig. 2). The juice is fermented directly in order to utilize all available sugars and at the same time minerals, vitamins, and amino acids in the juice will act as nutrient supplement for the yeast (Fig. 5). The remaining clover-grass fibre-pulp can be pretreated together with the wheat straw in order to make lignocellulosic sugars available for enzymatic hydrolysis and ethanol fermentation. The pretreated N-rich clover-grass contains important essential nutrients for the microbial fermentation, as compared to straw alone, and thereby addition of e.g. urea and other fossil based fermentation nutrients can be reduced. Furthermore, the fermentation residue after ethanol distillation containing yeast, residual nutrients, and lignin can be separated into a high quality protein rich feed and an organic fertilizer to be returned to the fields.

The use of biological interactions in the crop production step through intercropping strategies should likewise, as a principal, be integrated in the bioenergy manufacturing. Farming systems in the not too distant past were diversified with integrated animal and crop production in close proximity that allowed recycling of agricultural residues and accompanying nutrients essentially within the farming unit. Even though the present conditions for farmers to make business is different the way forward is to try and work in close cycles—potentially by creating biorefinery solutions where biomass is delivered and in return organic matter are received as an essential component of long-term sustainability of the soil-plant system in agricultural production.

Theoretical ethanol production and production of raw material

When produced on a sandy loam soil, like the present raw material, in an intensive sole cropping system with optimum use of fertilizers and agrochemicals and under Danish climatic conditions the yield of wheat straw would be around 5–7 tons DM ha^{-1} and for clover-grass cropped in a separate field approximately 10–12 tons ha^{-1} (Table 2). When undersown the actual harvest values of these raw materials were in the present study 5 tons wheat straw ha^{-1} and 4 tons clover-grass biomass ha^{-1}. This is not a matter of reduced yield due to the undersowing practise but more a picture of site specific conditions including annual climatic variation. The present clover-grass is growing in the time of the year with low temperatures lowering plant productivity, but it is also leaving space for a second crop. From a rotational point of view the gab between a winter crop and the following spring crop is filled out with the undersown clover-grass pasture securing that the local available environmental resources for plant growth are captured in biomass and not for instance lost through

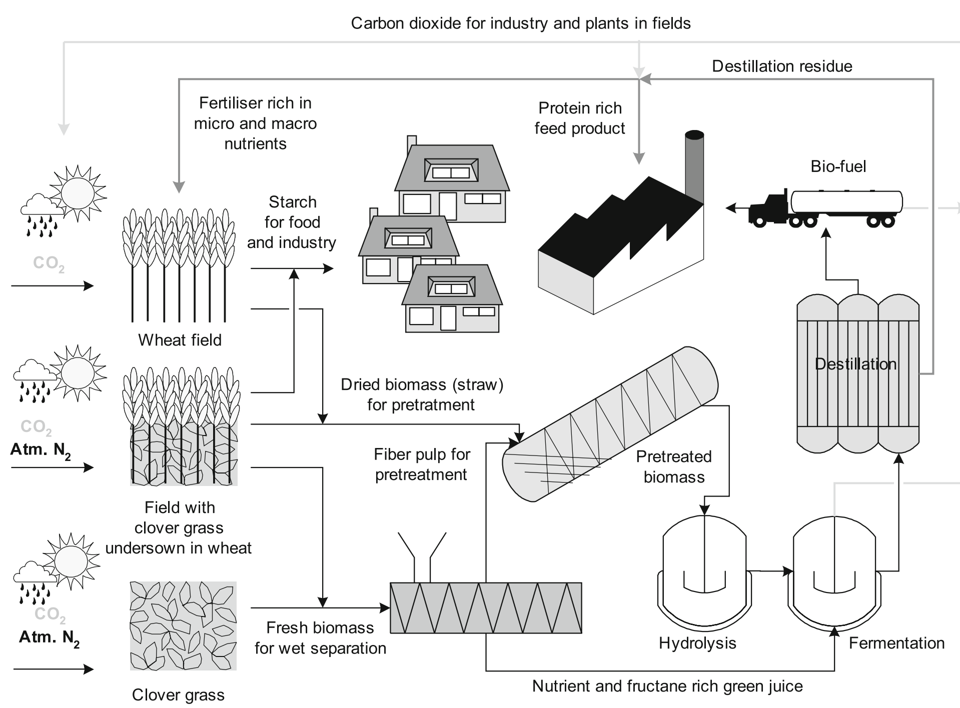

Fig. 7 Concept of decentralized biorefinery for production of biofuel from wheat straw and clover-grass with emphasize on sustainability, localness and recycling principles

Table 2 Parameters used in calculation of ethanol yield per hectare in the different cropping scenarios (sole crop and undersowing)

Parameter	Unit	Wheat straw	Clover-grass	References
Cellulose	kg ton^{-1} DM	340	203	[15, 23]
Hemicellulose	kg ton^{-1} DM	230	140	[15, 23]
Fructan	kg ton^{-1} DM	0	138	[1]
Yield[a] cellulose	%	95	94	[15, 23]
Yield[a] hemicel.	%	68	66	[15, 23]
Ethanol production[b]	kg ton^{-1} DM	270	241	
Cultivation yield, sole crop	Ton DM ha^{-1}	5	10	Actual harvest
Cultivation yield, undersowing	Ton DM ha^{-1}	5	4	Actual harvest

[a] Yield of lignocellulosic sugar after pretreatment and enzymatic hydrolysis, according to references

[b] Theoretical ethanol production calculated from sugar content and yields (Eq. 3)

nitrate leaching. Finally, it is important to stress that the present study highlight principal possibilities using alternative raw materials in complex biorefinery solutions. The exact values in every step can be optimised and probably increased in future improvement steps.

When wheat and clover-grass are cultivated on separate fields 1.35 ton ha^{-1} of ethanol can be produced from wheat straw (270 kg ton^{-1} DM × 5 ton DM ha^{-1}) and 2.4 tons ha^{-1} of ethanol from clover-grass (241 kg ton^{-1} DM × 10 ton DM ha^{-1}) (Table 2). Using the present actual harvest from wheat undersown with clover-grass 1.35 + 0.96 = 2.3 tons ethanol ha^{-1} can be produced. In the suggested system wheat grain (starch) is sold for direct human consumption or maybe utilised for on farm for feed purposes and on the same time 1,000 kg more ethanol is produced than from wheat straw originating from a wheat sole cropping strategy, making optimal use of available farm land. When changing agricultural practice from the present food and feed approach to include bioenergy more fast-growing crop grown simultaneously with, or between successive plantings of a main crop, exemplified by clover-grass in the present study, may be introduced gaining not only biomass to be used for bioenergy, but also environmental advantages.

Conclusions

The results showed that supplementing wheat straw with clover-grass had a positive effect on the ethanol yield in SSF experiments, and the effect was more pronounced in inhibitory substrates. The highest ethanol yield (80% of theoretical) was obtained in the experiment with high fraction (85%) of clover-grass. Optimally, the clover-grass should be subjected to a wet separation process resulting in a juice and a pulp fraction in order to utilize the fructan and free sugars which will otherwise be degraded during drying and the subsequent pretreatment. The wet separation process fractionates the clover-grass into a green juice (containing free sugars, fructan, amino acids, vitamins and soluble minerals) for direct fermentation and a fibre pulp for pretreatment together with wheat straw.

When comparing undersowing of clover-grass in wheat to clover-grass production in separate fields, approximately the same ethanol yield per ha can be achieved, with the additional gain of grains for food or feed. Comparing the undersowing to wheat sole cropping yields approximately 1,000 kg ethanol ha^{-1} in surplus, and at the same time clover increases input of symbiotic fixation of atmospheric N reducing the need for fertilizer applications for subsequent crops. The conclusion is that undersowing of clover-grass in wheat gives a more efficient use of available farm land, which will be a limited resource in meeting the bioenergy production targets set up for the coming years. When utilised in the biorefinery as concluded above—the clover grass also reduces the need for addition of urea and other fermentation nutrients produced from fossil fuels in the whole ethanol manufacturing chain. The value of this is additional, and is illustrating the value of integrating the bioenergy processing with the raw material production unit, a farm, in order to secure the best possible resource use efficiency throughout the full bioenergy manufacturing chain.

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