

Article

Natural Grasslands as Lignocellulosic Biofuel Resources: Factors Affecting Fermentable Sugar Production

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Abstract: Semi-natural grassland habitats are most often limited to animal grazing and low intensity farming. Their potential in bioenergy production is complicated due to the heterogeneity, variation, accessibility, and need for complex pre-treatment/hydrolysis techniques to convert into valuable products. In this research, fermentable sugar production efficiency from various habitats at various vegetation periods was evaluated. The highest fermentable sugar yields (above 0.2 g/g volatile solids) over a period of 3 years were observed from habitats “xeric and calcareous grasslands” (Natura 2000 code: 6120) and “semi-natural dry grasslands and scrubland facies on calcareous substrates” (Natura 2000 code: 6210). Both had a higher proportion of dicotyledonous plants. At the same time, the highest productivity (above 0.7 t sugar/ha) was observed from lowland hay meadows in the initial stage of the vegetation. Thus, despite variable yield-affecting factors, grasslands can be a potential resource for energy production.

Keywords: fermentable sugar; enzymatic hydrolysis; lignocellulosic biomass



Citation: Mezule, L.; Strazdina, B.; Dalecka, B.; Skripsts, E.; Juhna, T. Natural Grasslands as Lignocellulosic Biofuel Resources: Factors Affecting Fermentable Sugar Production. *Energies* **2021**, *14*, 1312. <https://doi.org/10.3390/en14051312>

Academic Editor: Alberto Coz

Received: 4 January 2021

Accepted: 23 February 2021

Published: 28 February 2021

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1. Introduction

Worldwide attention towards application of waste materials for energy and high-value chemical production has become a standard. Extensive use of agricultural and wood processing waste in lignocellulosic biofuel production increases the overall turnover of this industry annually. Furthermore, the use of lignocellulosic biomass for biofuel production is now facilitated by the European Union (EU) Renewable Energy Directive 2018/2001 [1]—the resource is included as alternative raw material under Annex IX. Regrettably, biomass recalcitrance towards saccharification is often the major limitation in the conversion of the resource to valuable end-products. Effective and economically feasible extraction of fermentable sugars is closely linked to the selection of an appropriate pre-treatment/hydrolysis technique and to the type of biomass used. A tremendous amount of studies have been performed to evaluate the potential of certain biomass resources, e.g., wheat or barley straw, corn stover, with various technologies and their combinations [2,3], resulting in an extensive amount of data and laboratory scale research. Furthermore, it has been demonstrated that the combination of climate, soil fertility, and grassland biomass type can influence the overall bioenergy potential, i.e., hydrolysis efficiency and fermentable sugar yields [2,4].

Currently in the EU, more than 61 million hectares are occupied by permanent grasslands [5] where temperate semi-natural grasslands with a long extensive management history represent the richest species ecosystems on earth. At a small spatial scale, their vascular plant diversity exceeds tropical rainforests, which are normally considered as global maxima [6]. Ref. [7] described the trend of grassland management abandonment due to economic reasons in Europe, leaving huge amounts of this resource unused. The abandoned areas are predominantly semi-natural and nature conservation grasslands, bearing

a large variety of plant and animal species. Most of these grasslands are characterized by low productivity, but the optimal management regime includes low-intensity agricultural practices. In many cases, this means controlled grazing or late seasonal harvest that leads to the creation of patchiness, selection of particular species, or high amounts of lignin and cellulose in the biomass, respectively. Thus, forage quality is reduced [8,9]. Therefore, it is necessary to find alternative management regimes to maintain the biodiversity in European manmade landscapes [10] and at the same time to facilitate sustainable use of this resource. Unfortunately, semi-natural grasslands cannot be evaluated on a species level due to the high diversity and variability of the vegetation. Species composition and, especially, the coverage and distribution of particular species can vary even within one vegetation class or small grassland plot. Furthermore, it is influenced by environmental conditions [11–13], management [14,15], surrounding areas [16], land use history [17], and other factors. Thus, it is crucial to investigate and perform proper evaluation of the local grassland variations, their productivity and variability to estimate the costs and possible yields of biomass that can be further converted into high value chemicals, including biofuels [18].

Grass co-digestion with other waste streams to produce biogas has been shown to be efficient [19]. It is estimated that 8–17% of the current grassland biomass could provide up to 1% of EU transport fuel [20]. However, the high effect of area-specific biomass diversity, cutting time, accessibility, and need for pre-treatment have limited the potential use of grass in biogas production at an industrial level [21,22]. As an alternative to methane production via complex anaerobic digestion process, the use of lignocellulosic grassland biomass has been demonstrated for fermentable sugar production [23], which is an intermediate stage to produce various liquid biofuels, e.g., bioethanol or biobutanol, high value chemicals, used as an additional feedstock in biogas stations or regarded as a first step towards biorefinery [20]. The aim of this study was to evaluate fermentable sugar yields and overall productivity potential from various grassland habitats that are common in a temperate climate and classified under EU habitat codes. To aid towards biorefinery, non-commercial enzymes extracted from white rot fungi were used in the hydrolysis. The assessment involved not only the evaluation of habitat type but also seasonality, cutting time, species diversity, and solid content in the biomass. In-house made enzymes were preferred to commercial products due to their potential onsite production capacity and, thus, minimization of manufacturing costs. To the best of the authors' knowledge, this is the first study where the Natura 2000 grassland habitat classification [24] is linked with fermentable sugar productivity in the Baltic region, thus offering new grassland management practices by facilitating the use of these resources for high value chemical production.

2. Materials and Methods

2.1. Biomass Sampling

In total, 162 grass biomass samples were collected from 67 randomly selected semi-natural grassland plots in Sigulda and Ludza municipalities (Latvia) over a 3 year period (Supplementary Materials Annex 1), corresponding to 6 habitat types of Community importance (the most common habitat types within these municipalities), and classified under the EU (Table 1).

Table 1. Description of analyzed semi-natural grassland habitats.

European Union (EU) Habitat Type [24]	National Variants of EU Habitat Type [25]	PAL. CLASS. [26]	Dominant Species [25]	Typical Species [25]
6120 Xeric sand calcareous grasslands	6120_2	34.12	<i>Poa angustifolia</i> , <i>Festuca ovina</i> , <i>Festuca rubra</i>	<i>Jasione montana</i> , <i>Hylotelephium</i> spp., <i>Pilosella officinarum</i> , <i>Sedum acre</i> , <i>Thymus</i> spp., <i>Veronica spicata</i> , <i>Viscaria vulgaris</i> 6210_2: <i>Agrimonia eupatoria</i> , <i>Carex caryophylla</i> , <i>Centaurea scabiosa</i> , <i>Pimpinella saxifraga</i> , <i>Polygala comosa</i> , <i>Thymus ovatus</i>
6210 Semi-natural dry grasslands and scrubland facies on calcareous substrates	6210_2, 6210_3	34.31 to 34.34	6210_2: <i>P. angustifolia</i> , <i>F. rubra</i> , <i>Fragaria vesca</i> 6210_3: <i>Helictotrichon pubescens</i> , <i>F. rubra</i> , <i>Fragaria viridis</i> ,	6210_3: <i>Filipendula vulgaris</i> , <i>Medicago falcata</i> , <i>Plantago media</i> , <i>P. angustifolia</i> , <i>Polygala comosa</i> , <i>Potentilla reptans</i> , <i>Trifolium montanum</i> 6270_1: <i>Alchemilla</i> spp., <i>Dianthus deltoideus</i> , <i>Leontodon hispidus</i> , <i>Leontodon autumnalis</i> , <i>P. media</i> , <i>Plantago lanceolata</i> , <i>Primula veris</i> , <i>Prunella vulgaris</i> , <i>Rhinanthus minor</i> , <i>Trifolium repens</i>
6270 Fennoscandian lowland species-rich dry to mesic grasslands	6270_1, 6270_3	35.1212, 35.1223, 38.22, 38.241	6270_1: <i>Agrostis tenuis</i> , <i>Anthoxanthum odoratum</i> , <i>Briza media</i> , <i>Cynosurus cristatus</i> , <i>F. rubra</i> 6270_3: <i>Deschampsia caespitosa</i> , <i>F. rubra</i> , <i>Holcus lanatus</i>	6270_3: <i>Filipendula ulmaria</i> , <i>Galium boreale</i> , <i>Geum rivale</i> , <i>Geranium palustre</i> , <i>Hierochloa odorata</i> , <i>Lychnis flos-cuculi</i> , <i>Scirpus sylvaticus</i> , <i>Carex cespitosa</i> , <i>Lysimachia nummularia</i> <i>Carex buxbaumii</i> , <i>Carex flacca</i> , <i>Carex hartmanii</i> , <i>Carex hostiana</i> , <i>Carex panicea</i> , <i>Galium boreale</i> , <i>Inula salicina</i> , <i>Polygala amarella</i> , <i>Potentilla erecta</i> , <i>Scorzonera humilis</i> , <i>Succisa pratensis</i>
6410 Molinia meadows on calcareous, peaty, or clayey-silt-laden soils	6410_4	37.31	<i>Molinia caerulea</i> , <i>Festuca arundinacea</i> , <i>Filipendula ulmaria</i> , <i>H. pubescens</i> , <i>D. caespitosa</i>	<i>Carex rostrata</i> , <i>Carex vulpina</i> , <i>Stellaria palustris</i> , <i>Lathyrus palustris</i> , <i>Lythrum salicaria</i> , <i>Veronica longifolia</i>
6450 Northern boreal alluvial meadows	6450_1	- *	<i>Carex acuta</i> , <i>Carex acutiformis</i> , <i>Carex appropinquata</i> , <i>Carex elata</i> , <i>Carex paniculata</i> , <i>Carex vesicaria</i> , <i>Calamagrostis canescens</i> , <i>Phalaris arundinacea</i>	<i>Crepis biennis</i> , <i>Heracleum sibiricum</i> , <i>Knautia arvensis</i> , <i>Pastinaca sativa</i> , <i>Tragopogon pratensis</i> , <i>Campanula patula</i> , <i>Centaurea jacea</i> , <i>Carum carvi</i> , <i>Galium album</i> , <i>Lathyrus pratensis</i>
6510 Lowland hay meadows	6510_1	38.2	<i>Arrhenatherum elatius</i> , <i>Bromopsis inermis</i> , <i>Festuca pratensis</i> , <i>H. pubescens</i>	

* Includes several vegetation types which vary according to the moisture (flooding) gradient: *C. acuta* or *C. aquatilis*-alluvial meadows, *Calamagrostis*-alluvial meadows, *Phalaris*-alluvial meadows, *Deschampsia caespitosa*-alluvial meadows.

Most of the samples (89) were collected in June–August of 2014. Thirty-nine and 34 samples were collected in 2015 and 2016, respectively (Table S1). Sampling in June (almost half of the samples) corresponded to a vegetation period when grassland biomass has the highest fodder value. August samples: period of late mowing.

The selection of semi-natural grassland sampling plot locations was based on visual assessment of the area. One most representative 1×1 m vegetation plot was selected and biomass was clipped at 2 cm above the ground level within the 1×1 m square using hand shears (Figure 1). First samplings were performed before the first cut or at the beginning of the grazing period (late June or early July). The second sample was collected in late July or August in sites managed by late mowing. In unmanaged sites, the third sample was also collected in September 2015 (9 samples in total). To evaluate the fermentable carbohydrate potential of early biomass, one sample from each habitat was collected in early June (season of 2015).

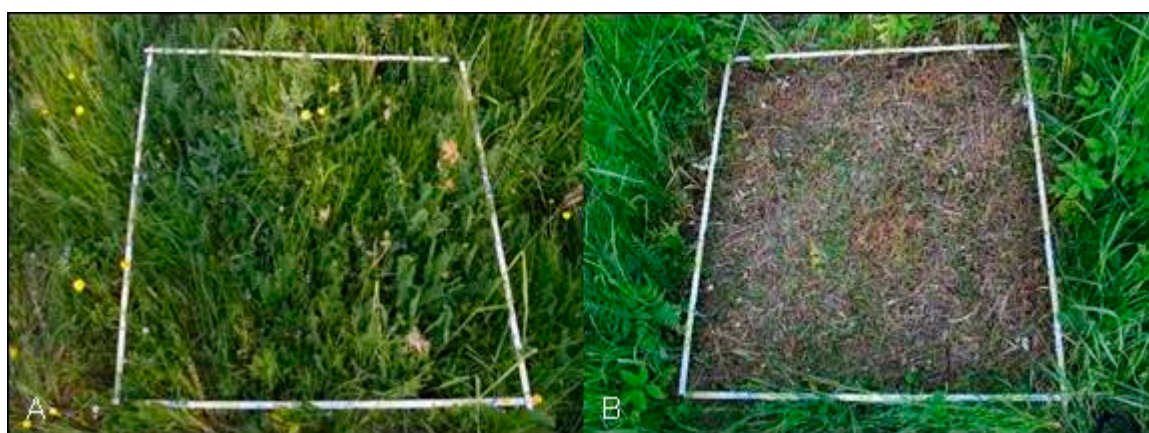


Figure 1. 1×1 m square frame sampling plots before (A) and after (B) collection of grass samples.

Prior to clipping, a description of the vegetation (vascular plant species richness) in each square was prepared. Then, the collected material was stored in pre-weighed plastic bags and brought to the laboratory for further analyses. If the biomass was not processed within one day, the samples were cut to fractions <20 cm, manually homogenized, and kept frozen (-18 °C) in sealable bags.

2.2. Dry Matter and Ash Content Analyses

A representative set of grass biomass was cut to pieces below 10 mm. Total dry weight (DW) was determined as weight after drying of sample at $+105$ °C (laboratory oven 60/300 LSN, SNOL, Utena, Lithuania) for 24 h. Total ash content was measured according to a modified EN ISO 18122 [27]. In brief, the samples were heated at $+550$ °C for 2.5 h (Laboratory furnace 8, 2/1100, SNOL). Volatile solid (VS) percentage was calculated as the difference between total dry matter and ash.

2.3. Enzymatic Hydrolysis

For enzymatic hydrolysis, a previously described method was used [23]. In brief, all biomass samples (fresh or frozen) were ground (Retsch, Grindomix GM200) to fractions below 0.5 cm. Then, 0.05 M sodium citrate buffer (mono-sodium citrate pure, AppliChem, Germany) was added to the biomass samples (final concentration, 9% w/v wet biomass) and mixed by vortexing. Then, the samples were boiled for 5 min (1 atm) to eliminate any indigenous microorganisms. After cooling to room temperature, a laboratory prepared enzyme (0.2 FPU/mL, obtained from white rot fungi *Irpex lacteus* (Fr.) Fr.) was added to the samples and incubated on an orbital shaker (New Brunswick, Innova 43) for 24 h at 30 °C and 150 rpm. Enzyme efficiency was compared with a commercial enzyme

product (Viscozyme, Novozymes) and substrate control—hay (obtained in Latvia, 2015, DW $92.8 \pm 1.3\%$).

Samples for reducing sugar measurements were collected after the addition of sodium citrate buffer, prior enzyme addition (both as zero time controls), and after 24 h of hydrolysis. All biomass samples were analyzed in six repetitions.

2.4. Reducing Sugar Analyses

The Dinitrosalicylic Acid (DNS) method was used to estimate the reducing sugar quantities in the collected samples [28]. First, the samples were centrifuged ($6600 \times g$, 10 min). Then, 0.1 mL of the supernatant was mixed with 0.1 mL of 0.05 M sodium citrate buffer and 0.6 mL of DNS (SigmaAldrich, Taufkirchen, Germany). Distilled water was used as blank control. To obtain the characteristic color change, the samples were boiled for 5 min and transferred to cold water and supplied with 4 mL of distilled water. Absorption measurements were performed with a spectrophotometer (Camspec M501, Leeds, UK) at 540 nm. For absolute concentrations, a calibration curve against glucose was plotted.

2.5. Statistical Analyses

For data analysis, MS Excel 2013 *t*-test (two tailed distribution) and ANOVA single parameter tool (significance level ≤ 0.05) were used for analysis of variance on data from various sample setups.

3. Results and Discussion

3.1. Assessment of Biomass Resources

Biochemical parameters such as total solids (TS), volatile solids (VS), and ash content were analyzed for grass biomass samples collected from 6 habitats to evaluate the overall composition of the biomass and its changes over time. These parameters characterize the biomass as a potential energy source and indicate its absolute energetic value. Fast growing biomass can have ash content above 20%; woody biomass has typically 1% ash content. Each 1% increase in ash translates roughly into a decrease of 0.2 MJ/kg of heating value, making it an unpopular resource for combustion [29]. At the same time, the presence of inorganic chemicals can be a good source of microelements along with sugars in the fermentation processes.

The average dry matter from grassland samples in respective Community Importance habitats ranged roughly from 1.0 to 6.0 t/ha (Figure 2) and $93 \pm 2\%$ from the dry matter were volatile solids. The highest average yields were obtained from Lowland hay meadows (6510), but the lowest were from Xeric sand calcareous grasslands (6120). That corresponds to yields from semi-natural grasslands in Estonia [30], central Germany [31], and Denmark [32].

The harvesting time had a significant impact on the total amount of the biomass. On average, 5% to 32% less biomass was harvested in June than in July and 17.5 to 42.6 % less in June than in August.

Moreover, variations were observed among the harvesting years. The amount of the biomass (t/ha) in 2016 was 33% to 19% less than in 2015 and up to 27% less than in 2014 (Table 2). Assessment of average daily temperature did not present any significant fluctuations among the years (Figure S1). At the same time, total precipitation in both sampling locations during the summer months was lower in 2015 when compared to 2014 and 2016 (Figure S2). This, to some extent, could explain the differences between these years. A similar influence of annual weather conditions on yield in multi-species grassland has been reported from Estonia and Denmark [30,33].

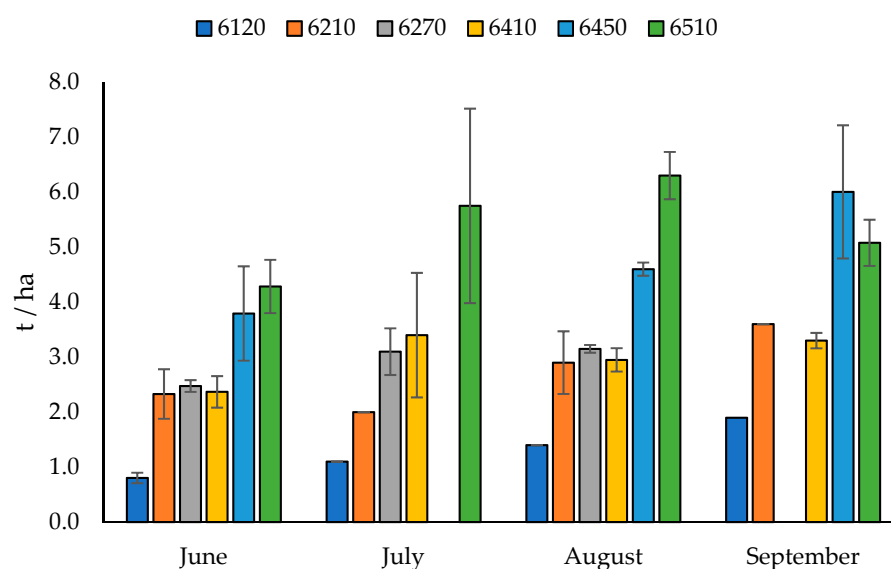


Figure 2. The average biomass dry matter (t/ha) collected from various grassland habitats at different sampling months over a three year period.

Table 2. The average quantity of biomass (t/ha as dry matter) collected from grassland habitats at various sampling years.

Habitat Type	Average Dry Matter, t/ha		
	2014	2015	2016
6120 Xeric sand calcareous grasslands	1.0	1.2	0.8
6210 Semi-natural dry grasslands and scrubland facies on calcareous substrates	2.1	3.0	2.1
6270 Fennoscandian lowland species-rich dry to mesic grasslands	2.8	3.2	2.6
6410 Molinia meadows on calcareous, peaty, or clayey-silt-laden soils	3.0	2.9	2.2
6450 Northern boreal alluvial meadows	4.5	5.1	3.5
6510 Lowland hay meadows	4.4	5.7	3.9

The ash content ranged from 3.84 to 9.62% from DW. The lowest ash content was observed in samples from Xeric sand calcareous grasslands (6120) ($5.72 \pm 1.03\%$) and the highest for semi-natural dry grasslands and scrubland facies on calcareous substrates (6210) ($7.41 \pm 1.10\%$, $p < 0.05$ among other biotopes). This corresponds to the results of other studies—the highest ash concentrations are typically identified in samples from the habitats with larger proportion of dicotyledonous plant species. Typically, ash content is associated with the concentration of minerals in plant organs [34] and dicotyledonous plants tend to accumulate greater quantities of minerals compared with monocotyledonous plants [33].

3.2. Enzyme Potential to Release Carbohydrates

Prior to application of a non-commercial enzyme from *I. lacteus*, its efficiency to release fermentable sugars from hay was compared with a commercial enzyme product. The results demonstrated that a commercial preparation was able to release 0.39 ± 0.05 g/g hay DW after 24 h of incubation. Due to the variable species composition, the amount of cellulose and hemicellulose in hay can vary from 35–45% and 30–50%, respectively [35]. However, prolonged incubation (48 h) did not yield any significant increase ($p > 0.05$) and reached only 0.409 ± 0.048 g/g DW. At the same time, a crude non-commercial product (un-concentrated, un-purified) yielded 0.183 ± 0.03 g/g DW after 24 h and 0.199 ± 0.045 g/g DW after 48 h. In both cases, the amount of sugar released after mechanical and thermal pre-treatment was not significant. Despite lower yields ($p < 0.05$), the observed extractable sugar concentration was still higher than reported for various grass materials [36]. Due to lower costs and

potential wide scale application, a non-commercial preparation was used for all future tests and 24 h incubation was set as the optimal.

3.3. Fermentable Sugar Yields

To evaluate the amount of fermentable sugar released from various grassland biomass resources, enzymatic hydrolysis with the non-commercial enzyme product at optimal conditions was performed. The results of 2014 showed significantly higher ($p < 0.05$) sugar yields (w/w) in June than in July or August (Table 3, Figure 3).

The length of the vegetation season had an overall tendency to decrease the amount of produced sugar. This was observed for all habitats in both 2014 and 2015 sampling seasons where June produced the highest sugar yields ($p < 0.05$) when compared to August or September. The samples from August and September demonstrated no significant sugar yield difference ($p > 0.05$).

Semi-natural dry grassland and scrubland facies on calcareous substrates (6210) and Lowland hay meadow (6510) samples produced the highest fermentable carbohydrate yields in 2014, e.g., 0.235 and 0.165 g per g VS, respectively. In 2015, the highest sugar yields were attributed to Xeric sand calcareous grasslands (6120) and 6210, but the lowest ones were in the samples of 6510 and Northern boreal alluvial meadows (6450) collected in September (Table 3). This slightly contradicted the results obtained in 2014, when from 6210, the highest yield (w/w) was obtained. One of the reasons for this could be the higher proportion of dicotyledonous plants in samples from 6210 collected during 2014. Similarly, as observed before, biomass with dominant monocotyledonous plant proportion showed lower carbohydrate yields due to higher crystallinity, lower hydrolysability, and potential presence of enzyme activity interfering substances [37].

The assessment of the overall producible sugar quantity from one ha exhibited a high potential of 6510 which from all tested habitats had the highest productivity in all vegetation periods, and in June, more than 0.7 t of fermentable sugar per ha could be produced. Other habitats that have demonstrated high sugar yields had lower productivity, e.g., 6210 having only 0.45 t/ha in June (Figure 3, Table 3) and 6120 even having below 0.2 t/ha.

In 2016, samplings were performed only in June with the aim to determine if there was any trend in-between habitats over the years. Again, the highest sugar yields (w/w) were produced from the habitat 6120, followed by *Molinia* meadows on calcareous, peaty, or clayey-silt-laden soils (6410) and 6510. Assessment of the total sugar quantity per 1 ha revealed that 6510 was able to generate more than 0.78 t of sugar per ha; however, 6120, only 0.186 t/ha. Similarly, as in previous seasons, this difference was due to the low total biomass quantity in 6120; thus, low correlation between fermentable sugar yield (per g biomass) and total amount of sugar per ha of habitat was observed.

The evaluation of the vegetation period showed a strong decrease in sugar yields with increasing vegetation time (Figure 3). No significant decrease ($p > 0.05$) was observed only between the samples collected in August and September. Similar observations have been made for methane yields in biogas production, where the increase in crude fiber at the end of the vegetation period has been set out as one of the main factors influencing the methane production [38]. Others have pointed out that to grasses harvested after October, an extra carbohydrate source must be added if applied for energy production purposes [36]. No influence of specific habitat type has been observed or recorded previously.

Table 3. The Reducing sugar yield (mg/g volatile solid (VS) or t/ha) that can be produced from natural grassland habitats at various sampling period.

EU Habitat Code	2014						2015						2016			
	June		July		August		June		July		August		September		June	
	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha	mg/g VS	t/ha
6120	147.61 ± 29.69	0.133	107.48 ± 23.29	0.118	n/d	n/d	225.46 ± 19.90	0.180	n/d	n/d	n/d	n/d	84.58 ± 13.56	0.161	233.35 ± 109.1	0.186
6210	235.49 ± 68.20	0.447	114.80 ± 24.75	0.230	81.71 ± 25.21	0.204	176.44 ± 22.80	0.493	158.00 ± 19.10	n/d	n/d	n/d	n/d	n/d	181.26 ± 28.61	0.380
6270	n/d	n/d	81.20 ± 24.88	0.227	101.34 ± 22.09	0.314	n/d	n/d	115.49 ± 31.47	0.393	n/d	n/d	109.87 ± 10.01	n/d	147.06 ± 26.92	0.382
6410	n/d	n/d	88.49 ± 13.33	0.265	103.56 ± 6.64	0.321	139.66 ± 6.40	0.377	142.48 ± 44.60	0.369	97.87 ± 11.77	0.274	90.23 ± 1.68	0.298	203.67 ± 50.59	0.447
6450	157.08 ± 46.71	0.659	94.10 ± 3.94	0.489	n/d	n/d	152.32 ± 9.38	0.669	n/d	n/d	92.84 ± 11.9	0.427	56.16 ± 18.3	0.337	161.98 ± 37.01	0.564
6510	164.74 ± 50.59	0.725	90.55 ± 25.80	0.498	n/d	n/d	166.66 ± 5.44	0.783	105.48 ± 15.17	0.738	n/d	n/d	69.71 ± 4.83	0.356	201.88 ± 36.01	0.784

n/d—not determined; VS—volatile solids.

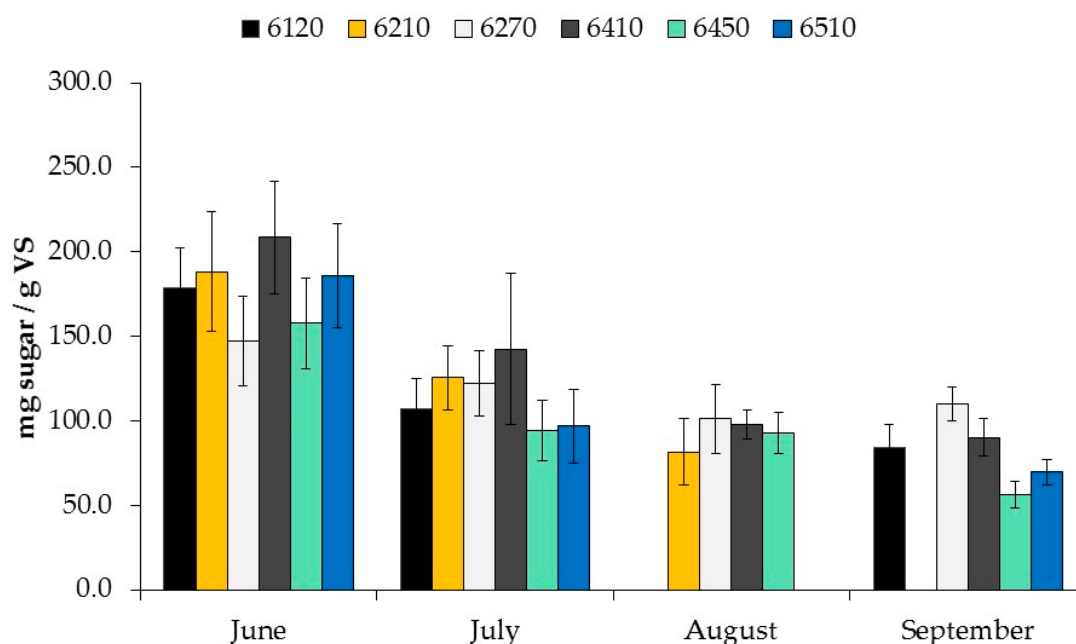


Figure 3. The amount of fermentable sugar produced per g volatile solid (VS) from biomass collected at various community importance habitats during 2014–2016 vegetation periods. Each bar represents the average value from at least two samplings with six individual measurements of reducing sugar.

In some cases, discrepancies from general observations have been detected. *Molinia* meadows on calcareous, peaty, or clayey-silt-laden soils (6410) did not produce the observed decrease in sugar yields with the progression of the vegetation season. This could be linked to the fact that 6410 includes Molinion grasslands, grasslands with low height sedge species like *Carex flacca*, *Carex hartmanii*, *Carex hostiana*, *Carex panicea*, *Carex buxbaumii*, as well as grasslands lacking any predominant species. Usually these habitats are represented with high species diversity and located in periodically drying soils [25]. One of the possible explanations can be related to the fact that in July 2014 and June 2015, the samples were collected mainly in sedge grasslands, while in August 2014 and July 2015, in *Molinia* grasslands. Furthermore, both sugar yield and productivity in 6270 was higher in August 2014 than in July—0.081 and 0.101 g/g VS or 0.22 and 0.31 t/ha, respectively. Apart from the general view (the increase in biomass and carbohydrate yields progresses with the vegetation time) that is challenged within this study, we hypothesize that the observed trend in 6270 is more linked to the environmental conditions, species composition in each individual sampling plot, and vegetation structure in general. Even in one habitat, multiple subtypes with diverse plant communities can be found. Nevertheless, to give the precise explanations of these variations, a more sophisticated classification and evaluation of species compositions would be required.

The average amount of the fermentable sugars highly varied not only seasonally, but also among the years. Sugar yields from the biomass harvested in June 2016 (a month with the most comprehensive data set) were 3% to 58% higher than in those collected in June 2014 and June 2015 for all habitats except 6210 (Table 3). Furthermore, it was estimated that the sugar yields tend to fluctuate ($p < 0.05$) even on a monthly basis, e.g., samples collected within the first ten days of June and at the end of June. The rationale for these differences within one habitat can be explained by the habitat's heterogeneity. The habitats listed in the annexes of the EU Habitats Directive are not classified in a single hierarchical system. Habitats can be separated by the phytosociological classification of plant communities or by habitat groups that include several similar habitats. These can be

further divided by specific environmental conditions. Moreover, weather conditions could affect the productivity in single habitat on a yearly basis.

The management of natural grasslands in Natura 2000 classified territories is generally restricted to low-intensity agricultural practices and strict regulations related to grazing, mowing, and cutting [9]. Despite grazing being seen as one of the simplest strategies, follow up on over- or under-grazing, formation of patchiness, preference of certain species by animals, or maintenance of cattle are limiting factors. Mowing at the same time requires the selection of correct timing and frequency; e.g., late mowing is preferred to protect animal species and late-flowering plants. At the same time, early cutting and removal of cut grass help to maintain low nutrient levels, keep plant diversity, and avoid alien species [9,39]. On average, the amount of sugar produced from the various grassland habitats at various vegetation periods was comparable to the data obtained with hay (~0.2 g/g DW) and the strategy was shown to be applicable in both high productivity grasslands and at early cutting periods. Upgraded enzymes, adjustment of the technology, e.g., introduction of more intense pre-treatment, could further facilitate the release of the energy stored into grassland biomass. Nevertheless, as demonstrated by this study, multispecies presence, quantities, and applicability under variable conditions set grassland resources as highly sustainable when fermentable carbohydrate production is foreseen.

4. Conclusions

A simple pre-treatment/hydrolysis technique with non-commercial enzymes made from *I. lacteus* was demonstrated to be efficient for the production of fermentable sugars from the biomass of community important grassland habitats classified under Natura 2000 that have to follow restricted farming practices.

The results showed that fermentable sugar yields from semi-natural grassland habitats are closely linked to vegetation period and plant species variation (monocotyledonous/dicotyledonous species proportion). Dicotyledonous plant rich habitats (6120, 6210) at the beginning of vegetation generated the highest amount of fermentable sugar per mass of biomass—above 0.2 g per g VS. At the same time, habitats rich in total biomass (6510) yielded higher sugar quantities per ha. The lowest yield and productivity in all habitats were observed in August–September, indicating potential bottlenecks of bioenergy production when biomass is collected at a late vegetation period. Overall, the study demonstrated that fermentable carbohydrate production from multispecies biomass of natural and semi-natural grasslands can be used as an alternative management strategy to currently practiced grazing. Thus, fuel production technologies can be merged with sustainable environment management.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1996-1073/14/5/1312/s1>, Annex 1: Location of biomass sampling plots, Table S1: Number of collected biomass samples per sampling year and habitat type; Figure S1: Average daily temperature in sampling months of 2014, 2015 and 2016 at 2 locations; Figure S2: Total precipitation (mm) in sampling months of 2014, 2015 and 2016 at 2 locations and the whole period (Total).

Author Contributions: Conceptualization, writing, and data analysis, L.M.; validation, T.J.; formal analysis and data collection, B.D., E.S.; sampling, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the IPP3: INNO INDIGO Programme Project B-LIQ ‘Development of an Integrated Process for Conversion of Biomass to Affordable Liquid Biofuel’, No. ES/RTD/2017/18, and the National Research Programme “Energetics” Project “Innovative solutions and recommendations for increasing the acquisition of local and renewable energy resources in Latvia” No. VPP-EMAER-2018/3-0004.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We acknowledge the EU LIFE+ Nature & Biodiversity program Project “GRASSSERVICE”—Alternative use of biomass for maintenance of grassland biodiversity and ecosystem services (LIFE12 BIO/LV/001130) for access to biomass samples and data on sampling plots.

Conflicts of Interest: The authors declare no conflict of interest.

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