Cultivation and Fractionation of Leguminous Biomass for Lactic Acid Production

F. Papendiek[a,*] and J. Venus[b]

[a]Leibniz Centre for Agricultural Landscape Research, Eberswalder Straße 84, D-15374 Müncheberg

[b]Leibniz Institute for Agricultural Engineering Potsdam Bornim, Max-Eyth-Allee 100, D-14469 Potsdam

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Chemical industries are set to increase the proportion of renewable feedstock in their production in the decades ahead. Green Biorefineries that divide fresh green biomass into cakes and juice deliver valuable products for various industrial uses. Press juice can be used to produce lactic acid (LA), a promising building blok for the future. In this study, optimal cultivation and fractionation processes for generating a fermentation medium from legumes for lactic acid production by *Bacillus coagulans* are analyzed. The contents of press juices from alfalfa cultivated on arable land at three different sites and from a clover-grass mixture on a grassland site taken on different sampling dates are compared. In addition, fresh biomass yields from the different biomass samples are examined. This paper focuses on the methods applied, and provides initial results. Yield differences of up to 40 % and 60 % were recorded between different study sites and sampling dates, respectively. Fermentation analysis of the different samples revealed that press juices can supplement the main parts of nutrients for lactic acid bacteria, producing economically interesting amounts of lactic acid. These findings could increase the use of lactic acid in chemical industries and bring about a shift towards a higher proportion of renewables, namely legumes, in the processing chain.

Key words:
alfalfa, lucerne, clover-grass mixture, harvest date, green biorefinery, lactic acid

Introduction

In the decades ahead, biomass is expected to gradually replace fossil resources. The chemical industries will increase the proportion of renewable resources in their production accordingly. It is estimated that from 2012 until 2017 the total chemical sales of biotechnological products will double worldwide.[1] To achieve this, products of a constantly high quality are required, which the concept of biorefinery aims to assure. “Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat).”[2] A special type is the Green Biorefinery, where the focus is on using fresh or ensiled wet biomass as feedstock.[3] Fractionation of this green biomass into press juice and press cakes is essential for the Green Biorefinery, and the new resulting feedstock constitutes valuable products for various industrial uses.

Green Biorefinery facilitates the multiple use of fresh green biomass.[4] Press cakes can be used as solid fuel[5] or fibrous composite materials.[6] In addition, fodder pellets can be produced if raw materials with high protein content, such as legumes, are processed.[4] Legumes, i.e. alfalfa and clover, are typical fodder plants.[4] Originally, press juices were the residue from green fodder pellet production. They were used as fertilizers on fields, but caused eutrophication.[7] Being aware of the valuable contents of press juice, a number of approaches for industrial use were developed. Thomsen *et al.* explored a method for producing a feed concentrate from residual juices that is also suitable for non-ruminants.[5] Chemical industries can use press juices as a universal fermentation medium.[7] Either fresh or silage press juices can be used as such an inexpensive nutrient source. The juices, featuring a variety of nitrogen-containing compounds and inorganic salts, can act as a substitute for synthetic nutrients.[8,9] Already existing processes, like the microbial PHA-production, can become more efficient due to lower feedstock costs.[10] Numerous investigations have been made into alfalfa press juices. These studies demonstrate that alfalfa press juices can act as a substitute for MRS, a synthetic nutrient, in lactic acid production, cutting processing costs.[8,9,11]

[*]Corresponding author: Franka Papendiek;
e-mail: Franka.Papendiek@zalf.de; tel.: + 49 (0)33432 82 441,
fax: + 49 (0)33432 82 223

Lactic acid is a chemical product that can be processed into many different products,[11] including L-Lysin,[12] pollution-free solvents, cosmetics and bioplastics.[13] Bio-based lactic acid (LA) is thought to be an important bulk chemical for the future. Its anticipated production capacity is 216,000 tons per year by 2015, with growing potential in the decades ahead.[14] A carbohydrate-containing raw material, lactic acid bacteria and a fermentation medium, such as alfalfa press juice, are required to produce lactic acid. It is assumed that the demand for press juices will increase in line with the growing market for bioplastics. In this study, therefore, the focus is on the optimal cultivation and fractionation of legumes to generate a high-value fermentation medium for lactic acid production.

Since it is vital that industries are supplied with high-quality preproducts,[15] the composition of biomass contents must be stable and impartial to variation. Pilot plants that work with green biomass analyze the advantages and drawbacks of different feedstock. The main challenge in Green Biorefinery is the seasonal availability of the feedstock while it is not storable.[16] Uncontrolled fermentation processes start directly after harvesting.[5] Therefore, the whole plant or the cake and juice need to be preserved, i.e. with lactic acid bacteria.[5] Most of the planned or existing Green Biorefinery plants in Europe focus on biomass from grassland. The reason for this is that pressure on arable land is already high, and the demand for forage grassland has continued to decline, creating new potential for exploitation.[6,16–18] This study investigates leguminous biomass from both grassland and arable land. Arable land is included in the study due to the positive effect of legume cultivation on the soil fertility and the importance of legumes in crop rotations.[19] Green Biorefinery pilot and demonstration plants show how the concept can be implemented successfully on the industrial scale.[4,5,13,16] However, no systematic analysis of biomass cultivation for Green Biorefineries has been published to date, and only little information is available about fractionation.[20] Typically, screw presses are used for the fractionation process.[20] The first qualitative measurements on the fractionation process into cakes and juice were undertaken and published by King *et al.*[20] In our study, the contents of press juices from alfalfa cultivated on arable land and from a clover-grass mixture cultivated on grassland on different sampling dates are analyzed. The results enable assessment of the best harvest time for the production of a fermentation medium for lactic acid. In addition, valuable results on decisive characteristics of biomass for the pressing process and for calculating biomass potentials are obtained. Besides reporting some results generated from the study, this paper focuses on the methods that were used.

Methods

Cultivation and harvest

Field trials were established on four different sites in north Brandenburg (Germany). We cultivated alfalfa on arable land at three different sites. The fourth field was a clover-grass mixture cultivated on grassland. The climate in Brandenburg is continental; the average annual rainfall over the past 20 years was about 550 mm for all sites, and the average annual temperature was between 9.1 °C at Müncheberg/Steinbeck and 9.6 °C at Paulinenaue.[21,22] Table 1 gives information on soil conditions, pretreatment and fertilizer input on the study sites. In Table 2, weather conditions for the sampling dates are given. Further details about the sites are given below.

Table 1 – *Sampling dates in 2012*

	Early	Middle	Late
1st cut	May 22	June 4	June 6
2nd cut	July 10	July 23	July 31
3rd cut	August 28	September 10	September 18

Table 2 – *Weather conditions during the 2012 harvest period*

Study site	Date	Daily average temperature in °C (2 m above surface)	Precipitation in mm on the day of harvest and the day before
Müncheberg/Steinbeck	May 22	22.6	0.0
Müncheberg/Steinbeck	June 4	11.4	4.3
Müncheberg/Steinbeck	June 11	16.6	0.0
Müncheberg/Steinbeck	July 10	19.1	0.0
Müncheberg/Steinbeck	July 23	16.7	0.0
Müncheberg/Steinbeck	July 31	16.1	0.0
Müncheberg/Steinbeck	Aug 28	15.9	0.5
Müncheberg/Steinbeck	Sept 10	20.0	0.1
Müncheberg/Steinbeck	Sept 18	16.9	0.0
Paulinenaue	May 22	22.9	3.0
Paulinenaue	June 4	11.8	10.7
Paulinenaue	June 11	17.5	0.2
Paulinenaue	July 10	19.0	4.2
Paulinenaue	July 23	18.2	0.0
Paulinenaue	July 31	16.9	0.7
Paulinenaue	Aug 28	15.3	0.5
Paulinenaue	Sept 10	18.2	0.0
Paulinenaue	Sept 18	15.5	1.0

Table 3 – *Composition of liquid phases in the press juice at the Müncheberg site*

	Sampling date	Dry matter 105 °C (%)	Disaccharide (g L^{-1})	Glucose (g L^{-1})	Fructose (g L^{-1})	Nitrogen (g L^{-1})	Crude Protein (g L^{-1})	Phosphorus (g L^{-1})
	May 22	7.26	3.7	9.44	9.01	2.69	16.81	0.41
1st cut	June 4	5.88	5.4	7.94	5.77	1.88	11.75	0.3
	June 11	8.21	4.6	11.71	6.7	2.48	15.5	0.48
	July 10	7.37	2.72	5.32	6.6	3.82	23.88	0.47
2nd cut	July 23	11.54	2.22	4.67	5.83	4.21	26.31	0.53
	July 31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Aug 28	9.26	4.55	12.4	8.04	4.22	26.38	0.38
3rd cut	Sept 10	8.4	3.22	11.0	10.2	3.68	23.0	0.18
	Sept 18	6.61	2.86	10.1	7.2	2.96	18.49	0.12

n.d. not detected

In the study region, fodder legumes are typically cut three times within the harvest period. In order to determine the optimal harvest time for industrial use, three sampling dates (early, middle, late) for each cut were set. These sampling dates, which were dependent on weather conditions and took into account weekends, were approximately at 10-day intervals (Table 3). Samples of alfalfa and clover-grass were harvested and chaffed at the study site, and then transported for analysis. At Leibniz Institute for Agricultural Engineering Potsdam-Bornim (ATB), the fresh biomass was pressed using a screw press Cv (VETTER Maschinenfabrik GmbH & Co.KG, Kassel/Germany) (Fig. 1). The press had a 7.5 kW engine and a flow rate of 600 to 800 kg h^{-1} depending on the feedstock. The sieve size was ø 1.1 mm. Maximum temperature of the juice outflow was 55 °C. The gap width was 25 and 35 mm, respectively, for the last two sampling dates (Fig. 2). The biomass was pressed such that there were sufficient soluble components in the press cake for potential use as feed.

Fig. 1 – *Screw press*

Fig. 2 – *Screw press gap width*

Samples of the fresh biomass, press cakes and press juice were taken for analytical purposes. Biomass and its press juice decay very quickly after harvesting, due to uncontrolled fermentation processes.[5] Therefore, in this study, the raw material was always pressed and conserved close to where it was cultivated just a few hours after harvesting.

Site 1 – Leibniz Centre for Agricultural Landscape Research (ZALF) Müncheberg field station (coordinates: 52.516045, 14.124929): a fully randomized block design was created for the alfalfa site at Müncheberg field station. One hectare was divided to enable each cut to be harvested on three sampling dates (Table 3).

Yields were determined using a HALDRUP F-55 grass harvester, and afterwards harvested using a Maral 125 forage harvester. Biomass was directly chaffed into 5 to 10 cm pieces in the harvesting process, and transported to ATB laboratories for pressing and analysis.

A problem arose on July 31, 2012 when the forage harvester broke down, making it impossible to harvest the biomass. For this reason, only one sample of a few kilograms of fresh biomass was taken and analyzed, as opposed to a representative sample.

Site 2 – Steinbeck (coordinates: 52.713809, 13.909371): a farmer from Steinbeck allowed us to use part of his alfalfa fields for our study. The 400 m^2 area was divided into three plots. Alfalfa was in the fourth year of cultivation and plants were heavily overgrown with dandelion. In order to determine yield, the farmer's decision to harvest and the sampling date in the study had to be synchronized.

Samples in Steinbeck were only harvested but not chaffed on the first sampling dates. Problems arising when pressing long material necessitated chaffing. For this reason, we transported all samples taken from Steinbeck after June 4, 2012 to Müncheberg, where they were chaffed using a Maral 125.

Due to dandelion overgrowth, yields on the alfalfa fields in Steinbeck were so low that the farmer ploughed up the field after we had completed the second cut (July 31, 2012). For this reason, no samples or data are available for the final three cuts in Steinbeck.

Sites 3 and 4 – Leibniz Centre for Agricultural Landscape Research (ZALF) Paulinenaue field station (coordinates 52.683381, 12.685897): At the research station in Paulinenaue, one hectare of arable land was tilled with alfalfa and another hectare of existing perennial grassland site was resown with a perennial ryegrass and white clover mixture. On the first sampling date, alfalfa and the clover-grass mixture were harvested using a Splendimo 240 disc mower. Yields were determined in the same way as on the other sampling dates. The biomass was not chaffed but cut with a disc spacing of 8 to 10 cm, pressed into bales and transported to the laboratories. On the next two sampling dates (June 4, 2012; June 11, 2012), the biomass was also hackled and pressed into bales but not chaffed. However, the disc mower was unable to produce chaffs (average chop length of 20 cm) that were comparable to those produced by the forage harvester. This reduced the quality available for pressing. Consequently, from the second cut biomass was chaffed using a Claas Jaguar forage harvester. The loose material with an average chop length of 2 cm was transported to the laboratories.

Biomass samples from all of the study sites arrived in Potsdam at least 5 hours after harvesting and were pressed and conserved at least after 8 hours. As an exception, however, on the third sampling date of the first cut (June 11, 2012), the press broke down after pressing samples from Paulinenaue. The two remaining series of biomass samples from Müncheberg and Steinbeck were pressed later on, but immediately conserved in the freezer at –20 °C. It must be said that a number of irregularities occurred in the sampling due to the lack of an established sampling procedure; for the time being, these will have to be accepted.

Analytical determination

After pressing, a sample of each press juice was conserved in a freezer at –20 °C. In order to determine the dry matter (DM) value, a sample of each press juice was dried to a constant weight at 105 °C.

Organic acid and sugar concentrations were measured by HPLC using an ultimate 3000 from the company DIONEX (column: Eurokat H (300 x 8 mm, 10 µm), company KNAUER; mobile phase: 0.005 mol L^{-1} sulfuric acid; flow rate: 0.8 mL min^{-1}; sample volume: 10 µL). The single components were detected using an RI-71 detector (SHODEX) with a minimum detection limit of 0.01 g L^{-1} and a maximum limit of 5 g L^{-1}.

Total Kjeldahl nitrogen was analyzed using standard-method Vapodest apparatus from Gerhardt by digestion using a selenium catalyst. In order to calculate the content of raw protein, nitrogen values were multiplied by a factor of 6.25. The colorimetric technique was used to measure total phosphorus by applying the molybdenum blue method (according to DIN EN ISO 15681).

Batch fermentation

Batch fermentations with the strain *Bacillus coagulans* (internal ATB no. A107) were carried out in a 3-litre stirred tank reactor under the condition of both temperature (52 °C) and pH value (6.0) control. The medium used was a mixture of glucose stock solution (600 mL) and different press juices (1400 mL) with no other nutrients. The volume of a common inoculum was divided into three parallel cultivations to ensure the same bacteria activity at the beginning of the experiments. The preculture was used after 15 hours shake flask cultivation with a cell density of $2.7 \cdot 10^7$ CFU mL^{-1}. Aliquots of the fermentation liquid were taken periodically to determine the concentration of lactate and sugars, which were measured after biomass separation and dilution by HPLC as described above.

Results and discussion

Influence of study site and harvest date on biomass yield

Analysis of all samples revealed major differences in biomass yield between study sites and sampling dates. Fig. 3 shows the total of fresh biomass yields from the first, second, and third cut for the study sites, with the exception of Steinbeck, which was no longer included in the study. Due to dandelion overgrowth, the site generated low yields on each sampling date (3 and 7 tons of fresh biomass per hectare) and was ploughed up after the second cut in 2012. Dandelion overgrowth is a typical progression for three- to four-year-old alfalfa if no herbicides are used.[23] Very few pesticides are available for alfalfa because the development and approval process is expensive and less rewarding for minor crops such as legumes.[24]

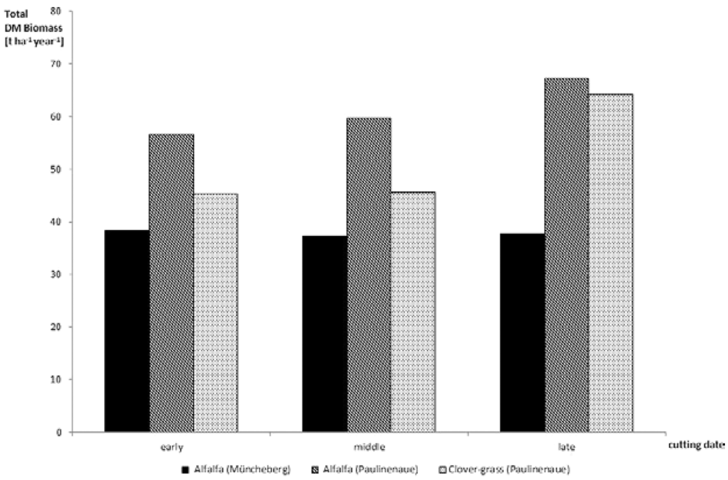

Fig. 3 – *Total biomass production at the Müncheberg and Paulinenaue study sites in 2012*

Alfalfa developed better in Müncheberg than in Steinbeck, in spite of poorer soil conditions (Fig. 3). Total yields of nearly 40 t ha^{-1} are an adequate yield for the sandy soils that are not well suited for alfalfa cultivation. Kreil *et al.* analyzed yield data for alfalfa in the former GDR, and for sands with a clay layer in deeper soils, as they occur in Müncheberg, found a typical yield span between 37 and 48 t ha^{-1}.[23] Müncheberg exhibited no significant differences in total fresh biomass yields between the early, middle and late sampling dates (Fig. 3). However, there were significant yield ranges between the sampling dates of one cut. The range was particularly high in the first and second cuts (Fig. 4).

The study site at Paulinenaue exhibited much higher yields than in Müncheberg for alfalfa (Fig. 3). The sand humus gleys at Paulinenaue are very well suited for alfalfa cultivation and therefore can deliver yields of more than 60 t ha^{-1} fresh biomass.[23] Yield ranges within one cut were even higher than in Müncheberg. For alfalfa in Paulinenaue, the highest margins were 36 % in the first cut. The clover-grass mixture at Paulinenaue also delivered high yields but exhibited ranges of 60 % in the first cut, 40 % in the second and even 18 % in the third. Comparable yield data for clover-grass are generally given as dry matter. In 2011, the average yield for pastures and meadows, including clover-grass, was 6.5 t ha^{-1} in Germany,[25] compared to the annual dry matter yields of 9.7 to 13.6 t ha^{-1} recorded at Paulinenaue in this study. Accordingly, the study site delivered above-average yields for grassland for all harvest periods. It is difficult to find current and comparable yield data for legumes, especially for fodder legumes cultivated conventionally on arable land. The reason for this is that protein plants are relatively rare in many European Union Member States[26] due to cheap protein feed such as soybean meal imported mainly from Latin America.[27] Comparing the alfalfa yield data from Kreil *et al.* and the findings of this study, yields did not increase over the last 30 years. A main reason is the breeding arrear.[24] Legumes are therefore underrepresented in crop rotations. The positive effect they have on succeeding crops[28] and their high nutritional value[29] therefore remain unexploited, which, in the context of our study, turns out to be a lost advantage. The multiple use of press cakes and juice generated by biorefineries could revitalize the cultivation of legumes in European agricultural systems and the unexploited breeding potential can even increase the economic potential of legumes.

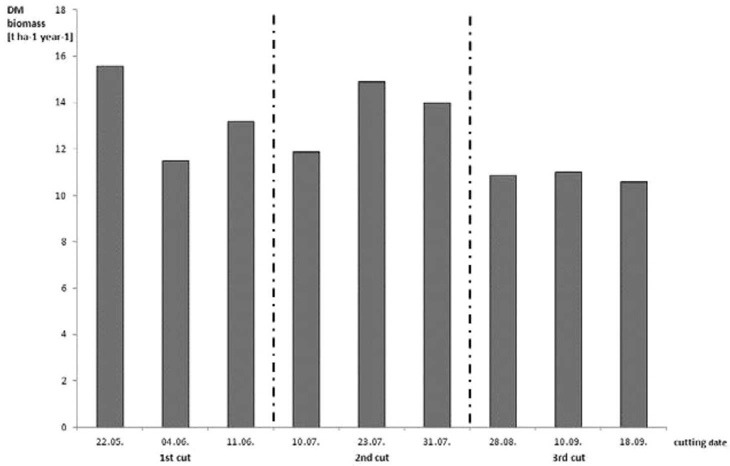

Fig. 4 – *Biomass production on the individual harvest dates at the Müncheberg study site in 2012*

The yield data recorded in the study show that the study site and sampling date play a major role. Although alfalfa is a perennial plant, it only delivers acceptable yields for two to three years after sowing.[29] Nonetheless, even marginal sites, such as at Müncheberg, which has a low soil quality index, can produce good yields over the year. For better soils, the harvest date plays an even greater role. A much higher yield range between harvest dates occurred in Paulinenaue than in Müncheberg. The field station in Paulinenaue is a fen site where ground frost appears well into spring, thus delaying the harvest. This influences calculations on biomass potential, since this high variance is often not taken into consideration.

Influence of study site and harvest date on the composition of press juices

In this study, the task was to test whether protein components from juice can be used as a source of nitrogen and other nutrients for lactic acid production for further polymerization towards PLA. The objective was to substitute expensive yeast and meat extracts as well as peptone with proteins contained in the green juice.

Fig. 4 shows the total dry matter (DM) content and the content of individual press juice components with regard to later application for fermentation processes, in particular to produce lactic acid. Data for the Müncheberg site is given. Carbohydrates contained in pressed juices for different sampling dates are shown. Green juice is unable to act as a source of carbon for lactic acid production due to the small quantities of total sugars (water soluble carbohydrates (WSC) content is usually less than 30 g L^{-1}). In a fermentation process that uses juice or its components containing nitrogen, the carbon source must be an external raw material such as starchy biomass, lignocellulosic feedstock or residues in order to enhance fermentable sugars and the efficiency of the entire process.

After strain selection, based on previous research, the aim was to investigate the suitability of complex agricultural materials such as alfalfa and clover-grass juice for the fermentation process. As noted above, green juice cannot be used as a carbon source in lactic acid fermentation. Therefore, sugar (glucose monohydrate) was used as the main carbon source. However, green juice contains a series of nitrogen-containing compounds and inorganic salts, which are essential for cell growth and a number of mineral salt components that microorganisms require for growth. Depending on the type and concentration of individual impurities (e.g. metal ions), these mineral salt components can cause problems in lactic acid purification when high-purity lactic acid is to be produced.

For the first evaluation of differences between the three cuts on the typical (middle) harvest date, three juices from Müncheberg were used as a nutri-

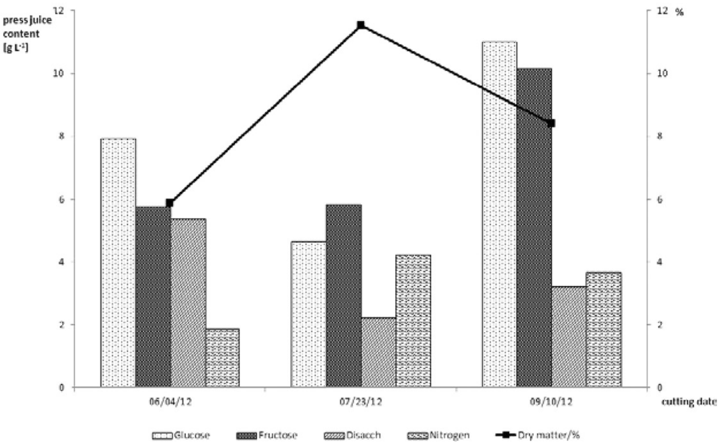

Fig. 5 – *Characteristic parameters of alfalfa press juices at Müncheberg*

ent supplement for a glucose-based broth. For the sugar and nitrogen content on the three typical cut dates (June 4; July 23; Sept 10), the tendency of the juice composition could not be correlated clearly with the dates, since the weather conditions were not really comparable (e.g. higher water content for the harvest date in June due to heavy rain). Nonetheless, the different time courses of lactic acid concentration and substrate consumption (Fig. 5) reveal a number of basic findings. The results for the green juice with the lowest nitrogen content (sample 06/04/2012) indicate speedier product formation but incomplete sugar consumption. The maximum volumetric lactate productivity was achieved after 20 hours (2.5 g L^{-1} h^{-1} together with a yield of produced lactate out of sugars $Y_{P/S}$ 0.66 g g^{-1}), whereas the sugar conversion at the end of the fermentation was calculated at 87.8 %. Due to the relatively high concentration of disaccharides, it is difficult to distinguish between magnitude of influence (e.g. inadequate nitrogen content and/or delay of sugars besides glucose and fructose). There is also a delay in the performance of samples with a higher nitrogen concentration (07/23/2012 and 09/10/2012). However, after passing the logarithmic phase, the final titer of lactate results in a higher level of about 80 g L^{-1}, together with nearly fully consumed sugars. The maximum volumetric productivity reached 2.65 g L^{-1} h^{-1} after 28 hours for the juice containing 4.21 g L^{-1} nitrogen, and 2.8 g L^{-1} h^{-1} after 24 hours for the juice containing 3.68 g L^{-1} L nitrogen, respectively. There is no negative impact on the fermentation process in general, and the final lactate concentration is in the same range as for all batch runs. Considering the partially different sugar concentration, lactic acid yield (product concentration in relation to the substrate concentration used) exhibits slightly higher values ($Y_{P/S}$ 0.74 and 0.71 g g^{-1}) for experiments conducted using press juice containing higher levels of nitrogen. At present, it can be summarized that green juice supplements can act as a substitute for most standard nutrients (mineral salts, complex nitrogen sources such as yeast extract, peptones) for lactic acid bacteria. These first runs have been done without replications due to the limit of raw material samples. With regard to the significance of the differences between the results, it should be stated that the fermentations were based on the same preculture at a time. From long-term experiences, the activity and behaviour of the inoculum is known as a key factor for the comparison of several process parameters. In this context, the composition of the fermentation broth (i.e. nitrogen content) can be assumed as the main influence on the product formation and sugar consumption, respectively.

The results concerning the composition of press juices can be compared to those determined by Kromus *et al.*[16] Crude protein content is similar to that in press juices from Müncheberg; WSC with 12 % of the DM are much less concentrated than in the samples taken in this study. The results generated by King *et al.*[20] cannot be compared properly with the ones in this study because they used silage rather than fresh biomass. When compared, however, fresh press juices from alfalfa achieve far higher crude protein contents in most samples than the 100 to 155 g kg^{-1} DM yielded in grassland analyzed by King *et al.,* as well as larger amounts of WSC (Table 3). In this study, the pressure applied in the pressing process was set so that additional juice would occur in press cakes, enabling them to be used as a feed source. In contrast, King *et al.* sought to gain the maximum quantity of juices.

The positive result for using different green juices in lactic acid fermentation correlates well with previous observations (Leiß *et al.*[9], Vodnar *et al.*[30]). For more scientific conclusions much more experiments and observations on the cultivation and fractionation of leguminous biomass for lactic acid production are needed. In the following research years, the results of this study have to be verified. In addition, experiments on the quantity and composition of press juice caused by different chop length and gap widths in the press must be analyzed and an economic evaluation of legume cultivation and fractionation conducted.

Influence of chop length and weather conditions on pressing process

Biomass from the Müncheberg and Paulinenaue sites were sent to the laboratories with different chop lengths, depending on chaffing methods and machines. Unchaffed material from Steinbeck as well as the first samples from Paulinenaue delivered low amounts of juice. The fibers wrapped around the screw and material was scraped instead of pressed. In general, the shortest chop length of 2 cm generated the best results in the press with regard to

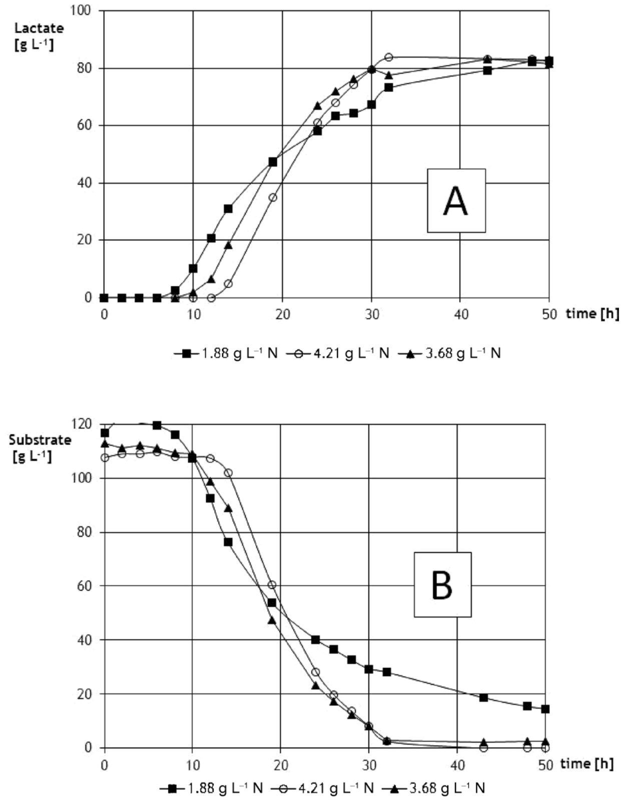

Fig. 6 – *Product formation (A) and substrate consumption (B) with different nitrogen concentrations at the Müncheberg site*

quantities of press juice and throughput speed. However, the chop length had no significant impact on the composition of press juices.

The second sampling date was already postponed from June 1 to 4, due to unfavorable weather conditions but still the biomass had to be harvested and chaffed wet (Table 2). Whenever the biomass was harvested and chaffed wet, the short chop length increased the risk of the press becoming clogged. The gap width therefore must always be adjusted to the specific biomass characteristics.

Conclusions

In this study on fermentation media generation for lactic acid production from leguminous press juices, the influence of different parameters such as study site and harvest date on their use as substitutes in chemical industries was analyzed. New methods were established to obtain sound information on the first link in the supply chain for industries based on renewable resources. Initial results given in this paper reveal important differences for feedstock. These recent findings can influence calculations of biomass potential and also change processing methods in the chemical industries, generating an increased demand for legumes.

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