JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

A Laboratory Protocol for Determining Glucose and Maximum Ethanol Production from Wheat Grain: Application to a Complete Genetic Set of Near-Isogenic Waxy Lines

Pascale Gadonna-Widehem,*[,†] Clément Debiton,[‡] David Marier,[†] Larbi Rhazi,[†] and Gérard Branlard[‡]

[†]Institut Polytechnique LaSalle Beauvais, 19 rue Pierre Waguet, BP 30313, 60026 BEAUVAIS Cedex, France

[‡]INRA, UMR1095 GDEC—UBP, 234 av du Brezet, 63100 Clermont-Ferrand, France

Supporting Information

ABSTRACT: A laboratory protocol was developed to assess glucose and ethanol yields from wheat. The impact of the analyzed wholemeal flour quantity and the saccharification on the amount of released glucose was estimated. The whole process including the analytical methods (glucose and ethanol) was repeatable and reproducible. This protocol was used to assess the glucose and ethanol yields of six varieties and of a complete set of hexaploid near-isogenic waxy lines of cv. Trémie grown in three locations. As compared to the normal line of Trémie, double null (AnBnD) and triple null (nAnBnD) isogenic lines showed a low Hagberg falling number (218, 65, and 63 s, respectively), a higher grain protein content (10.7, 11.5, and 12.1% DM, respectively), a lower glucose yield (728, 703, and 707 kg/t, respectively), and a lower ethanol yield (463, 453, and 452 L/t, respectively). These values indicate a strong involvement of alleles encoded at *Wx-B1* and *Wx-D1* loci in grain composition.

KEYWORDS: *wheat, starch, glucose yield, maximum ethanol yield, waxy lines*

INTRODUCTION

Many countries (including Brazil and the United States) have focused their energy policy on support for the biofuel sector (biodiesel and bioethanol). In 2003, Europe set a biofuel consumption target of 5.75% for cars by 2010.[1] The proportion of biofuels in total transport energy will increase from 2 to 27% by 2050 according to the roadmap established by the International Energy Agency.[2] The French government encourages the production of biodiesel and bioethanol and aims to reach a 10% share of biofuels used by cars in 2015. Bioethanol is currently mainly produced from sugar cane (*Saccharum officinarum*), beet (*Beta saccharum*), wheat (*Triticum aestivum*), maize (*Zea mays*), triticale (*X-Triticosecale*), or barley (*Hordeum* spp.). Research has demonstrated the potential value of maize and wheat as renewable plant materials for ethanol production. In wheat, ethanol yields of between 300 and 480 L/ton (L/t) of DM have been reported, but yield is affected by the wheat variety, grain protein, starch content, year, and cultivation site [3−7]. For example, Glasgow, a variety recommended for alcohol production in the United Kingdom, had a potential yield of between 423 and 473 L/t (average, 453 L/t).[8] A very high bioethanol yield was recently reported for the wheat variety Dragana (41.16 g ethanol/100 g of dry wheat corresponding to 521 L/t).[9] In addition to differences due to the variety, the above data can also be influenced by the laboratory protocol used. Several parameters such as saccharification (performed separately or simultaneously with fermentation) or the production of ethanol (sample weight, distillation, chromatography, and spectrophotometric analysis) can influence final yields. A highly reproducible laboratory protocol requiring a limited amount of grain for the measurement of ethanol yield is urgently required by breeders to improve wheat for this end use.

High starch and low protein content are the two major characteristics associated with a high ethanol production.[4] The starch content and grain size also affected ethanol yield.[10] In the case of wheat, many grain characteristics remain to be analyzed to evaluate their effects on ethanol yield. The influence of the range of sizes of wheat starch granules (the major kernel component) as well as their amylose and amylopectin contents remains to be characterized to optimize glucose hydrolysis for ethanol production. Amylose-free, or waxy wheats were originally generated to promote new functionalities in food and for chemical or industrial uses[11−13]. Few studies have determined the ethanol yield of waxy wheat to date. In the United States, Zhao et al. demonstrated that nonwaxy soft wheat had an average ethanol yield of 433 L/t, higher than nonwaxy hard wheat and waxy hard wheat (402 L/t).[7] Eight isogenic lines with homozygous null alleles at either one, two, or three loci of the waxy genes in the genetic background of the cultivar Trémie were recently obtained.[14] This kind of material provided an opportunity to test the influence of amylose content on glucose and ethanol yields.

The aim of this study was to design and validate a general procedure to evaluate the ethanol and glucose yields of wheat. The procedure was particularly used to assess a complete set of near isogenic waxy wheat lines, which provided the opportunity to test the effect of amylose content on glucose yield and ethanol yield.

Received: October 26, 2011
Revised: December 21, 2011
Accepted: December 21, 2011
Published: December 21, 2011

dx.doi.org/10.1021/jf204383u | *J. Agric.Food Chem.* 2012, 60, 985−990

MATERIALS AND METHODS

Plant Material. Seven hexaploid wheat cultivars, Ambrosia, Astuce, Crousty, Glasgow, Rytmic, Soissons, and Trémie, were grown with two replicates at the INRA plant breeding station in Clermont Ferrand, France, with a normal nitrogen supply (165 kgN/ha) and full fungicide protection in a field trial in 2009. The eight near-isogenic waxy lines (NIWLs) (Table 1) of Trémie with zero, one, two, or three null granule binding starch synthase (GBSS) alleles[14] were also grown under normal field trial conditions with two replicates in three locations in France in 2009. The harvested grains were air cleaned to remove foreign matter, and then, 500 g of each sample was used for technological analyses and 400 g was stored at −20 °C for glucose and ethanol determination.

Table 1. Name and Allelic Composition of the Eight Different Isogenic Lines for Each of the Three Waxy Null Alleles in the French Wheat Cultivar Trémie

		allelic composition		
form	name	Wx-A1	Wx-B1	Wx-D1
normal	ABD	a (+)	a (+)	a (+)
single null	ABnD	a (+)	a (+)	b (−)
	ADnB	a (+)	b (−)	a (+)
	DBnA	b (−)	a (+)	a (+)
double null	AnBnD	a (+)	b (−)	b (−)
	BnAnD	b (−)	a (+)	b (−)
	DnAnB	b (−)	b (−)	a (+)
triple null	nAnBnD	b (−)	b (−)	b (−)

Analytical Methods. Thousand-kernels weight (TKW), test weight (TW), and dry matter (DM) moisture content were measured according to standard French AFNOR 1997 protocols NF V 03-702,[15] NF V 03-719,[16] and NF ISO 712,[17] respectively. The grain protein content (GPC) and grain hardness (GH) were evaluated by near-infrared spectroscopy using wholemeal flour ground in a laboratory mill 3100 and sieved to 0.75 mm (Perten, United States) according to AACC 39-25[18] and 39-70A,[19] respectively. The Hagberg falling number (HFN) was assessed on wholemeal flour using the AFNOR NF V03-703 method.[20]

The amylose content was determined using the dual wavelength iodine binding technique.[21] Briefly, 10 mL of 1 N sodium hydroxide solution was added to 60 mg of wholemeal flour. After 1 h of agitation at 20 °C, 1 mL of this solution was added to 51 mL of distilled water. The pH value was adjusted to neutral with 0.1 N hydrochloric acid. Then, 2 mL of 0.2% iodine solution (containing 2 g of potassium iodide and 0.2 g of iodine diluted to 100 mL with distilled water) was added, and the sample volume was adjusted to a final fixed volume of 80 mL with distilled water. The solution was allowed to settle for 30 min at 20 °C for the color to fully develop; the difference between absorbance values at 620 and 510 nm was then used for amylose determination. Standard curves were established using different fractions of amylose and amylopectin. These two products were purchased from Fluka Analytical (Sigma Aldrich, St. Louis, MO).

The starch concentration was determined with a total starch assay (Megazyme, Ireland) using 100 mg of wholemeal flour according to the AOAC 996.11 method.[22] Glucose concentrations were determined using the Gopod procedure (K-GLUC assay) (Megazyme). After 10% (v/v) dilution in distilled water, the weighed samples were vortexed and centrifuged at 4000 rpm for 25 min at 10 °C. One milliliter of supernatant was weighed and diluted in deionized water before being analyzed in triplicate using the Gopod kit. Briefly, 3 mL of Gopod enzymatic solution was added with 0.1 mL of sample or 0.1 mL of deionized water (as the blank). After 20 min at 50 °C, the absorbance was measured at 510 nm. Calibration was carried out by replacing the sample volume with 0.1 mL of glucose calibration solution in deionized water (range 0.1−1 g/L).

For ethanol determination, each 1 mL of sample collected during fermentation was weighed, then 9 mL of deionized water was added, and the mixture was vortexed and then centrifuged at 4000 rpm for 25 min at 10 °C. Into a preweighed vial, 100 μL of methanol at 3% w/w was added as an internal standard, and then, 1 mL of supernatant was poured and weighed; this operation was performed in triplicate. The vials were rapidly sealed with a silicone-septum cap and placed on the autosampler. Ethanol analysis was by gas chromatography using a Focus GC (ThermoScientific, Rodano, Italy) equipped with a flame ionization detector operating at 280 °C. The column used was a 30 m × 0.25 mm id., 0.25 μm, Polyethylene Glycol SolGel-wax BP 20 (SGE-Europe LTD, Courtabœuf, France), and hydrogen at 1.3 mL/min was used as the carrier gas. One microliter of sample was placed in the head chamber by an automatic injector. A carrier gas flow of 200 mL/min was used to sweep the sample into the column.

Standard curves were drawn using the ethanol calibration solution (at concentrations of from 0.2 to 1.6 g/L) and methanol as an internal standard (100 μL for 1 mL of each solution). Starch and glucose data were converted into computed ethanol data. The ethanol yield from glucose was computed from the known equation where 1 molecule of glucose is converted into 2 molecules of ethanol (corresponding to 0.51 g or to 0.675 mL of ethanol per g of glucose).[23] For a wheat grain containing 69% starch and 3% sugar on a DM basis, a potential yield of 800 kg of glucose, and hence of 518 L of alcohol per ton of grain, was obtained.[4,23] All results were given on a DM basis.

Hydrolysis Procedure. To measure the impact of wheat amount, hydrolysis was performed in a 500 mL Schott Duran bottle containing 16, 38, or 80 g of wholemeal flour (Perten Laboratory Mill 3100, sieve 0.75 mm, Perten). Boiled hot water was added to wholemeal flour to obtain a final mass of 380 g (corresponding to final concentrations of 4.2, 10, and 21% w/w wet basis), and the bottles were closed with a membrane screw cap and were autoclaved for 15 min at 121 °C to limit bacterial contamination and ensure starch gelatinization. The mixture (mash) was cooled to 82 °C at room temperature, liquefied by adding 2.85% w/w of α-amylase from *Bacillus amyloliquefaciens* (250 U/g, Sigma Aldrich) per gram of flour, and then placed in a shaking water bath (82 °C, 145 rpm).

The mixtures were cooled to 58 °C at room temperature, and the pH was adjusted to 4.8 with sulfuric acid (0.1 N). For saccharification, each of the two enzymes, pullulanase from *Bacillus acidopullulyticus* (100 U/mL, Sigma Aldrich) and amyloglucosidase from *Aspergillus niger* (3260 U/mL, Megazyme), was added at a rate of 2.85% w/w of enzyme per gram of flour, and the mixture was placed in a shaking water bath (58 °C, 130 rpm).

To measure the impact of amyloglucosidase, an experiment was carried out using a mixture of 4.2% of wholemeal flour during which starch was liquefied in the presence of 1.2 mg of α-amylase per gram of mash; 0.3, 0.625, and 1.25 μL of amyloglucosidase was added per gram of wholemeal flour, and the released glucose was measured after 1, 3, and 5 h. At the end of the incubation, samples (1 mL in triplicate) were taken from the reaction mixture and rapidly cooled to 0 °C on ice to stop the reaction and stored at −20 °C for glucose determination.

Fermentation. After hydrolysis, the mixtures were cooled to 30 °C at room temperature. Dried baker's yeast (*Saccharomyces cerevisiae*, Francine brand, from Nutrixo, France) was rehydrated with sterile water to a final concentration of 3×10^7 CFU/mL (determined by plating appropriate dilutions on Sabouraud agar, Difco). To limit bacterial contamination, the mixtures were supplemented with a solution of potassium disulfite (0.275 M; CAS number: 16731-55-8) to obtain a final concentration of 0.27% v/w.

All fermentations were performed at 30 °C for 68 h using an orbital shaker agitated at 90 rpm. Samples (1 mL in triplicate) were taken after 24 or 68 h of fermentation and stored at −20 °C for ethanol determination. At the end of fermentation, microbiological analysis was carried out to determine the purity of the culture by plating a sample on plate count agar (Difco).

Simultaneous Saccharification and Fermentation Procedure. Liquefaction of Glasgow wholemeal flour (4.2% w/w of sample) was performed as described above. Saccharification and fermentation were carried out at the same time (SSF) at 30 °C after

Table 2. Evaluation of Repeatability on Glucose or Ethanol Assays Using Standards of, Respectively, 1.09 and 1.58 g/L, on Glucose Yield Determination after Hydrolysis of Different Mashes (%Wholemeal Flour Wet Basis) and on Ethanol Yield Determination after 24 or 68 h during Separate or SSF Obtained by Two Technicians

	n^a	min^b	max^c	means	SE^d	CV^e	P value^f
				glucose (kg/t except for standard g/L)			
standard	13	1.08	1.10	1.09	0.01	0.9	
mash (% w/w)							
4.2	7	697.7	716.2	708.2	7.5	1.1	0.79
10.0	7	676.6	750.4	703.1	24.5	3.5	
21.0	7	631.5	722.3	698.8	36.9	5.3	
repeatability							
technician 1	11	703.9	719.0	712.8	4.9	0.7	0.557
technician 2	11	711.3	715.0	711.9	1.0	0.1	
				ethanol (L/t except for standard g/L)			
standard	13	1.57	1.59	1.58	0.01	0.6	
technician 1 (24 h)	11	402.5	428.1	412.4	7.0	1.7	0.599
technician 2 (24 h)	11	394.8	435.5	414.5	11 .0	2.7	
technician 1 (68 h)	11	458.4	472.4	465.4	3.6	0.8	0.152
technician 2 (68 h)	11	450.1	469.8	462.3	5.9	1.3	
SSF (68 h)	2	458.7	466.7	462.3	4.0	0.9	

^{a}Represent numbers of sample. ^{b}Represent the minimal value. ^{c}Represent the maximal value. ^{d}For standard errors. ^{e}Coefficient of variation (%). ^{f}The P value was determined after T student or ANOVA.

simultaneous addition of amyloglucosidase, pullulanase, potassium bisulfate, and yeast. The ethanol yield was determined after 68 h of reaction.

Statistical Analyses. The response surface for starch hydrolysis was obtained using JMP Software (version 6, SAS, Cary, NC). To determine reproducibility and repeatability, glucose and ethanol assays were carried out 11 times by two technicians on the Glasgow variety as a wheat reference. The reproducibility (variation between the two technicians in our case) was calculated by adding the between-technicians variance to the repeatability variance. The P value was determined for differences between the technician means after T student test or analysis of variance (ANOVA). Distribution parameters, ANOVA, and Pearson correlation analyses were performed using the Statgraphics Centurion software version XVI (StatPoint Technologies, Warranton, VA). The genotypic factor (Gen), the location factor (Loc), and the interaction (Gen × Loc) were tested in ANOVA for each technological trait (Y), using the following model: $Y = m + \text{Gen} + \text{Loc} + \text{Gen} \times \text{Loc} + \text{err}$, where m and err are the general mean and the residual error, respectively. Each trait had normal distribution, and Pearson correlation coefficients were computed between traits for each growing location.

RESULTS

Validation of the Protocol. *Effect of Wheat Quantity on Glucose Yield.* The effect of wheat concentration ranging from 4.2 to 21% w/w of wholemeal flour on glucose yield was evaluated (Table 2). Whatever the wheat amount in mash, glucose concentrations did not differ significantly ($p = 0.79$) on the three concentrations tested. Considering that the values were slightly more dispersed when the quantity of wholemeal flour was increased, the smaller quantity (16 g of wholemeal flour per assay) was used for all of the experiments, thus limiting the amount of wheat required for the test.

Effect of the Quantity of Amyloglucosidase and Saccharification Time. Results of the experiment showed that both the amount of amyloglucosidase and the hydrolysis time strongly influenced the glucose yield (Figure 1). Glucose liberation was strongly affected by the quantity of amyloglucosidase added when the hydrolysis time was short (1 h). When a large quantity of amyloglucosidase was used, glucose liberation was less influenced by saccharification time. For this study and with the aim of limiting saccharification time, a high concentration of amyloglucosidase (1 μL per g of mash) coupled with an average reaction time of 2.5 h was chosen, corresponding to 93% of released glucose.

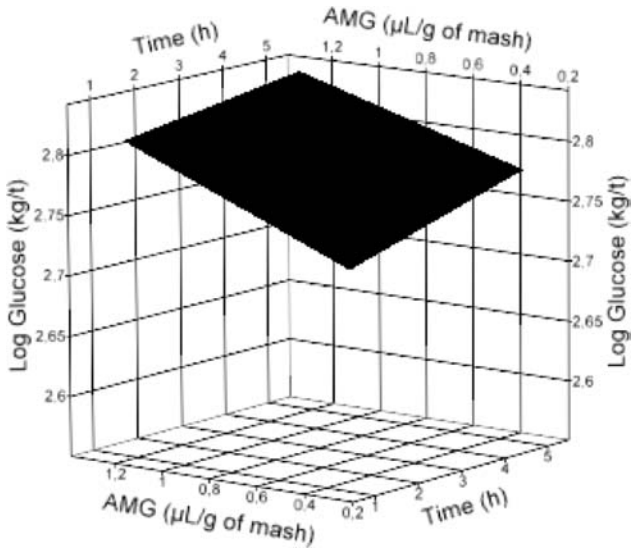

Figure 1. Response surface obtained after hydrolysis of starch from wholemeal flour at different times with different concentrations of AMG.

Repeatability and Reproducibility of the Glucose and Ethanol Assays. A preliminary repeatability experiment using the glucose standard (1.09 g/L) and the ethanol standard (1.58 g/L) resulted in variation coefficients inferior to 1%. The maximum coefficient obtained for ethanol, after 24 or 68 h of fermentation, was 2.7 and 1.3%, respectively. In addition, there was no significant difference ($p > 0.05$) in mean values in both the glucose and the ethanol determinations between the technicians (Table 2). For reproducibility, the coefficient obtained for ethanol yield at 68 h was inferior to 1.5%, by two different technicians who performed the protocol 11 times independently using the same Glasgow wholemeal flour. The

Table 3. Ethanol Yield on Six Cultivars Using the Proposed Protocol: Coefficient of Variation of Four Replicates and Conversion Rate as Compared with Theoretical Values Based on Glucose Measurements

	Ambrosia	Astuce	Bagou	Crousty	Rytmic	Soissons
starch ± SD[a] (%)	69.0 ± 1.8	67.9 ± 1.7	69.5 ± 1.6	69.1 ± 2.0	65.8 ± 1.0	68.4 ± 3.4
converted in EthY[b]	496.0	488.4	499.8	496.8	473.0	491.4
GluY[c] ± SD[a]	734.2 ± 11.4	701.9 ± 7.6	735.9 ± 11.7	737.6 ± 12.7	704.6 ± 9.7	729.1 ± 9.9
converted in EthY[b]	475.4	454.5	476.5	477.6	456.2	472.1
EthY[b] ± SD[a]	473.5 ± 4.4	451.0 ± 6.4	473.2 ± 4.7	472.1 ± 0.8	443.5 ± 9.4	470.3 ± 6.5
CV[d] (%)	1.1	1.4	1	0.2	2.1	1.4

[a]Represent standard error. [b]Ethanol yield (L/t). [c]Glucose yield (kg/t). [d]Coefficient of variation.

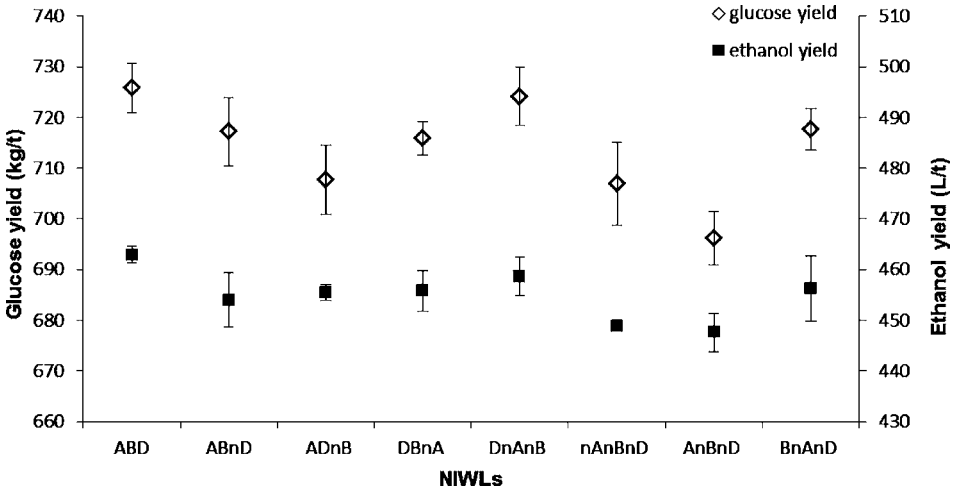

Figure 2. Mean values and standard error bars of glucose yield (◊) and ethanol yield (■) for the eight NIWLs grown in three locations.

protocol was thus considered to be reproducible even if different technicians performed it.

Fermentation. With Glasgow, the ethanol yield obtained after 24 h of fermentation was 413 L/t, which represented 89% of the final ethanol yield (462 L/t DM). In our conditions (mash with 4.2% of flour), no difference in Glasgow ethanol yield was observed using simultaneous saccharification fermentation (SSF) or separate protocols (Table 2).

Converting Starch and Glucose into Ethanol. The ethanol yield resulting from glucose hydrolysis and chromatography analysis was first measured using six cultivars with four independent replicates each. The average ethanol yield ranged from 443.5 to 473.5 L/t DM with a coefficient of variation inferior to 2.1% over the four replications (Table 3). Unlike the ethanol yield computed from starch results, the measured ethanol yield was very similar to the theoretical ethanol yield computed from glucose yield measured after hydrolysis.

Influence of Waxy Starch on Glucose and Ethanol Yields. *Variability of Technological Traits.* The eight NIWLs of Trémie grown in three locations exhibited a high potential for grain yield (GY from 9.2 to 11.4 t/ha) and a rather high TKW (from 46.1 to 57.1 g). These two traits were significantly influenced ($p < 0.001$) by the growing locations. These NIWLs, which were mainly distinguished by amylose content (from 1.67 to 20.7% DM), also exhibited a wide range of HFNs from 62 (waxy line carrying the three null granule binding starch synthase alleles) to 360 s. Amylose and HFN were the only two traits exhibiting highly significant genetic × location interaction. Normal nitrogen fertilization resulted in GPCs ranging from 9.9 (for the normal Trémie) to 12.6% DM (for waxy line carrying the three null GBSS alleles). Both HFN and GPC were influenced by genetic effects and location.

The GH also significantly varied between these NIWLs and between locations (from 42.9 to 81.8). The glucose yield was significantly influenced by NIWLs and locations, whereas ethanol yield was significantly influenced by locations. For these two important traits glucose yield and ethanol yield, the phenotypic Min−Max differences were, however, limited: 65 kg/t and 38 L/t, respectively (Figure 2). Considering the field yield, the differences found between the normal Trémie (highest values) and the waxy form (lowest values) were rather large: 1661 kg/ha and 1203 L/ha for glucose yield in the field and ethanol yield in the field, respectively.

Grain Characteristics Associated with Glucose and Ethanol Yields. Pearson correlations were computed for each experimental location since the location effect was highly significant for the majority of the technological traits. As expected, grain yield strongly influenced the glucose yield in the field and ethanol yield in the field in each of the three locations, but ethanol yield in the field was significantly associated with glucose yield and ethanol yield in only one location. These last two traits were negatively influenced ($p < 0.01$ to $p < 0.001$) by GPC in all trials. Starch composition, as revealed by HFN, had a strong positive influence on glucose yield, while amylose content was positively associated with glucose yield and ethanol yield in only one location.

■ DISCUSSION

In industrial facilities, because of limited water addition and increased productivity, alcohol processes currently operate with concentrations ranging from 21 to 28 g of dissolved solids per 100 g.[23] However, a very viscous mash was obtained at these concentrations, possibly causing handling errors in the laboratory. Lemuz et al.[24] showed that the yield of ethanol

from dry ground maize was affected by the solid contents when these were greater than 35%. These authors attributed this to insufficient mixing during fermentation. As we observed no difference in glucose yields, we chose to carry out all experiments at a final sample concentration of 4.2% w/w (wet basis).

Repeatable values were obtained in glucose and ethanol measurements. When we tested a standard sample of ethanol (n = 13), we obtained 0.6%. In their works on whole blood ethanol analysis by gas chromatography, after automatic injection of a known control, Szymanowicz et al. obtained a coefficient of variation of 0.66% (n = 20).[25] No differences in mean values were observed between the two technicians, and the results were thus considered to be reproducible (CV < 1.5% for ethanol at 68 h). Considering the maximum average ethanol yield obtained in France, 462 versus 453 L/t in the United Kingdom, we confirm that Glasgow wheat has a high potential for alcohol production.[4]

The ethanol yield obtained in industrial plants will probably be similar to the ethanol yield measured using the laboratory procedure for 24 h. After 24 h, any additional ethanol yield will become more expensive (immobilization of the fermentation tank, energy required etc.) particularly for a cultivar like Glasgow with a high potential, which yielded 89% of ethanol in 24 h of fermentation. The laboratory measurement of ethanol yield after 24 h was more dispersed, 2.7 as compared to 1.3% for 68 h (Table 3), because of the fact that the transformation reaction was not completed in 24 h, in particular because of the physiological state of the yeast.

Ethanol yield calculated from starch (AOAC method) was higher than the yield calculated from the hydrolysis of glucose (Table 3). This difference can be attributed to the duration of hydrolysis and to the hydrolytic capacity of the enzymes used. The AOAC method allows the quantity of starch and of free glucose to be rapidly measured using large quantities of high-performance enzymes; hydrolysis performed in our protocol was achieved using 150 times less amyloglucosidase and 15 times less α-amylase than in the AOAC method (the amount was reduced by 23%).

The most frequently cited conversion efficiencies of glucose to ethanol in the literature ranged between 89 and 93.9% because some of the glucose released was used by the yeast during fermentation or transformed into other products (glycerol, etc.).[7] This is why, in our experimental design, we chose to measure the performance of glucose when 93% of hydrolysis was completed, whereas hydrolysis actually ended during fermentation. In consequence, the duration of hydrolysis (liquefaction and saccharification) was 4.5 h.

On the SSF often used to save time in industrial processing, we found that when saccharification and fermentation were performed separately or simultaneously, there was no impact on the yield of ethanol in our conditions. In the laboratory protocol developed here, separating the hydrolysis and fermentation (SHF) processes allowed the operator to check if saccharification was correctly performed or not. This means that in the case of poor ethanol yield, the respective influence of starch hydrolysis and glucose fermentation can be checked.

In the protocol described here, a high concentration of yeast was preferred to increase the glucose consumption rate and ethanol productivity throughout the fermentation process. A high yeast cell density, at the beginning of fermentation, increased fermentation capacity, which is advantageous for industrial ethanol fermentation [24,26].

The study of the eight NIWLs showed that the presence of either one, two, or three null alleles responsible of the starch composition in the grain had a significant effect on each grain characteristics, the ethanol yield excepted. As expected, the most important genotypic variation was observed for the amylose content. As compared to normal line of Trémie, double null (AnBnD) and triple null (nAnBnD) isogenic lines showed the lowest amylose contents (average over the three locations: 18.8, 6.4, and 3.6% DM, respectively). The HFN, as compared to Trémie (ABD), was also very low for the double null (AnBnD) and triple null (nAnBnD) isogenic lines (on average 218, 65, and 63 s, respectively). Hence, an important genotypic effect was revealed for HFN. Very low HFN was also reported for the triple null waxy line[12] and was shown independent from the α-amylase activity. The GPC was also more important for double null (AnBnD) and triple null (nAnBnD) isogenic lines as compared to the normal line Tremie (on average over the three locations: 11.5, 12.1, and 10.7% DM, respectively). This means that starch quantity in the waxy grain is lower than the one in normal line of Tremie. Consequently, the glucose and ethanol yields of the waxy triple null line were lower, confirming the observations already made.[7] The negative influence of GPC on both glucose yield and ethanol yield (respectively, $y = -15.645x + 0.725$, $R^2 = 0.72$ and $y = -7.965x + 547.6$, $R^2 = 0.74$ computed on average values), reported by ref 4 for normal wheat cultivars, was confirmed using our NIWLs.

The amylose content was positively correlated to yield of glucose ($y = 1.319x + 700.0$, $R^2 = 0.86$) and ethanol yields in only one location (Clermont-Ferrand, CFD) out of three, where the correlations were at $p < 0.10$. The higher temperatures that occurred at CFD, as compared the two other locations, during grain formation and accumulation, could explain differences in starch composition.

The three different null alleles, which are responsible for the absence of granule binding starch synthase, do not have the same effect on glucose yield. The *Wx-D1b* and *Wx-B1b* alleles appear to have a major effect on glucose yield because the isogenic line AnBnD (*Wx-A1a*, *Wx-B1b*, and *Wx-D1b*) had a glucose yield similar to the triple null form (average values 703.4 and 707.6 kg/t, respectively). Moreover, the double null form DnAnB (*Wx-A1b*, *Wx-B1b*, and *Wx-D1a*) had a glucose yield that was as high as the single null form BDnA and the normal form of Trémie (725.3, 722.9, and 728 kg/t, respectively). The major effect associated with the normal allele *Wx-D1a* and *Wx-B1a* on amylose quantity was already reported.[27] These two alleles were reported to express higher amounts of granule binding starch synthase per grain than the *Wx-A1a* allele.[14]

The protocol that we developed allowed us to measure both the amounts of glucose and the maximum ethanol successively produced in the separate hydrolysis and fermentation processes. The repeatability of ethanol yield measurements allowed accurate evaluation of bioethanol yield of a wide range of genetic resources including isogenic waxy lines. The NIWLs revealed an effect of the different waxy null alleles on glucose and ethanol yields with a major effect of the *Wx-D1b* and *WxB1b* alleles. The lines carrying these alleles had higher GPCs and are consequently not suitable for ethanol production. GPC was not the only limiting factor on ethanol yield. Studies of the environmental and genetic factors that influence glucose and ethanol yield can now be carried out using the protocol presented here.

■ ASSOCIATED CONTENT

Supporting Information

Table of statistical parameters and ANOVA, table of Pearson correlations, figure of general protocol used to study saccharification and fermentation of whole flour meal, and figure of regression for the eight different isogenic forms of Trémie. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +33-344-067-600. Fax: +33-344-062-526. E-mail: pascale.gadonna@lasalle-beauvais.fr.

■ ACKNOWLEDGMENTS

We thank Véronique Rame, Houda Turki, and Charlène Le Gall for their technical support and François Xavier Oury for his help. The GNIS (Groupement National Interprofessionel des Semences) who financed the French FSOV bioethanol program as well as CETAC (Centre d'Etude technique pour l'Amélioration des Céréales) breeders are gratefully acknowledged.

■ REFERENCES

(1) EC, Directive 2003/30/EC of 8 May 2003 on the promotion of the use of biofuels or other renewable fuels for transport. *Off. J. Eur. Union* **2003**, *L123*, 42−46.

(2) IEA. *Technology Roadmap: Biofuels for Transport*; OECD/IEA: Paris, 2011; p 56.

(3) Rosenberger, A. Identification of top-performing cereal cultivars for grain-to-ethanol operations. *Zuckerindustrie* **2005**, *130*, 697−701.

(4) Smith, T. C.; Kindred, D. R.; Brosnan, J.; Weightman, R.; Shepherd, M.; Sylvester-Bradley, R. *Wheat as a Feedstock Alcohol Production*; Research Review No. 61; Home-Grown Cereals Authority: London, 2006; p 89.

(5) Agu, R. C.; Bringhurst, T. A.; Brosnan, J. M. Production of grain whisky and ethanol from wheat, maize and other cereals. *J. Inst. Brew.* **2006**, *112*, 314−323.

(6) Kindred, D. R.; Verhoeven, T. M. O.; Weightman, R. M.; Swanston, J. S.; Agu, R. C.; Brosnan, J. M.; Sylvester-Bradley, R. Effects of variety and fertiliser nitrogen on alcohol yield, grain yield, starch and protein content, and protein composition of winter wheat. *J. Cereal Sci.* **2008**, *48*, 46−57.

(7) Zhao, R.; Wu, X.; Seabourn, B. W.; Bean, S.; Guan, L.; Shi, Y.; Wilson, J. D.; Madl, R.; Wang, D. Comparison of waxy vs. nonwaxy wheats in fuel ethanol fermentation. *Cereal Chem.* **2009**, *86*, 145−156.

(8) Clarke, S.; Kindred, D.; Weightman, R.; Dyer, D.; Sylvester-Bradley, R. *Growing Wheat for Alcohol and Bioethanol Production in the North East*; ADAS: Cambridge, 2008; p 51.

(9) Pejin, D.; Mojovic, L. J.; Vucurovic, V.; Pejin, J.; Dencic, S.; Rakin, M. Fermentation of wheat and triticale hydrolysates: A comparative study. *Fuel* **2009**, *88*, 1625−1628.

(10) Lacerenza, J. A.; Martin, J. M.; Talbert, L. E.; Lanning, S. P.; Giroux, M. J. Relationship of ethanol yield to agronomic and seed quality characteristics of small grains. *Cereal Chem.* **2008**, *85*, 322−328.

(11) Be Miller, J. N.; Whistler, R. L. Carbohydrates. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker, Inc.: New York, 1996; pp 157−204.

(12) Graybosch, R. A.; Guo, G.; Shelton, D. R. Aberrant falling numbers of waxy wheats independent of α-amylase activity. *Cereal Chem.* **2000**, *77*, 1−3.

(13) Patil, S. K.; Meschi, M. A. Formulating for texture and viscosity. *Prepared Foods* **2008**, *177*, 112−124.

(14) Debiton, C.; Bancel, E.; Chambon, C.; Rhazi, L.; Branlard, G. Effect of the three waxy null alleles on enzymes associated to wheat starch granules using proteomic approach. *J. Cereal Sci.* **2010**, *52*, 466−474.

(15) AFNOR. *NF V03-702 Détermination de la Masse de 1000 Grains*; AFNOR Association Française de Normalisation: Paris la Défense, 1997; pp 131−133.

(16) AFNOR. *NF V03-719 Détermination de la Masse Volumique, Dite ≪ Masse à l'Hectolitre≫*; AFNOR Association Française de Normalisation: Paris la Défense, 1997; pp 187−201.

(17) AFNOR. *NF ISO-712 Détermination de la Teneur en eau (Méthode de Référence Pratique)*; AFNOR Association Française de Normalisation: Paris La Défense, 1997; pp 143−147.

(18) AACC. *Near Infrared Method for Protein Content in Whole-Grain Wheat*; *39−25*; AACC: St. Paul, MN, 1999; Vol. 2, pp 1−3.

(19) AACC. *Wheat Hardness As Determined by near-Infrared Reflectance*; *39−70A*; AACC: St. Paul, MN, 1999; Vol. 2, pp 1−3.

(20) AFNOR. *NF V03-703 Détermination de l'Indice de Chute Selon Hagberg-Perten*; AFNOR Association Française de Normalisation: Paris la Défense, 1997; pp 285−295.

(21) Zhu, T.; Jackson, D. S.; Wehling, R. L.; Geera, B. Comparison of amylose determination methods and the development of a dual wavelength iodine binding technique. *Cereal Chem.* **2008**, *85*, 51−58.

(22) McCleary, B. V.; Gibson, T. S.; Mugford, D. C. Measurement of total starch in cereal products by amyloglucosidase−α-amylase method: Collaborative study. *J. AOAC Int.* **1997**, *80*, 571−579.

(23) Thomas, K. C.; Hynes, S. H.; Ingledew, W. M. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochem.* **1996**, *31*, 321−331.

(24) Lemuz, C. R.; Dien, B. S.; Singh, V.; McKinney, J.; Turnbleson, M. E.; Rausch, K. D. Development of an ethanol yield procedure for dry-grind corn processing. *Cereal Chem.* **2009**, *86*, 355−360.

(25) Szymanowicz, A.; Magdinier, S.; Cuenca, S.; Neyron, M.-J.; Denis, I. Étude comparative des alcoolémies obtenues par la méthode officielle par chromatographie en phase gazeuse sur sang total versus méthode enzymatique-ADH automatisée Integra 800 sur plasma. *Immuno-anal. Biol. Spécialisée* **2007**, *22*, 329−338.

(26) Ding, M.-Z.; Tian, H.-C.; Cheng, J.-S.; Yuan, Y.-J. Inoculum size-dependent interactive regulation of metabolism and stress response of *Saccharomyces cerevisiae* revealed by comparative metabolomics. *J. Biotechnol.* **2009**, *144*, 279−286.

(27) Yamamori, M.; Quynh, N. T. Differential effects of Wx-A1, -B1 and -D1 protein deficiencies on apparent amylose content and starch pasting properties in common wheat. *Theor. Appl. Genet.* **2000**, *100*, 32−38.