PREDICTION OF WORT FERMENTABILITY IN TWO-ROWED BARLEY GENOTYPES

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Fermentability is the parameter that describes the ability of yeast to turn sweet wort into alcohol. As the efficiency of this process is related with alcohol yield, it will regulate the amount of beer produced from a given amount of malt. Considering that fermentation efficiency is influenced by the concentration of fermentable sugars in wort, the diastatic power (DP) of malt has long been associated with fermentability. However, research studies have shown that DP alone can not explain much of the observed variability ($R^2 \leq 0.50$) between malt samples. Some reports suggest that the separate determination of the activity and thermal stability of some of the enzymes contributing to DP and some malt modification parameters can considerably improve prediction ($R^2=0.91$). On the other hand, few studies have focused on measuring fermentability variation in barley breeding. The objective of this study was to determine the effect of standard and non-standard malt quality parameters on the fermentability of different barley breeding lines, and to select the best multi-linear regression model for predicting this trait. Malts of 90 barley genotypes, grown in different locations of North Dakota during two years, were used in this study. Assays of the DP enzymes alpha-amylase, beta-amylase, alpha-glucosidase, and limit dextrinase, as well as their thermal stabilities (determined at 65°C for 10 min), were performed and adapted to a microplate reader format. Fermentability was determined as the real degree of fermentation (RDF) considering that the apparent degree of fermentation (ADF) showed strong alcohol dependence ($R^2=0.87$), which could give false differences when comparing breeding lines in a population with high genetic variability. Results showed that, contrary to other reports, DP and beta-amylase and its thermostability are not highly associated with fermentability when the hot water extract (HWE) is used as the mashing method. Alpha-amylase and limit dextrinase though, had a more significant influence. The malt quality parameters: wort glucose, wort

maltose, limit dextrinase, free amino nitrogen, soluble protein, alpha-amylase, and maltotriose, are considered the most significant factors for predicting fermentability as determined by the regression model (R^2=0.71).

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DEDICATION

To my wife, Cinthia, and our son, Julian.

Your love and continuous support, against all odds, made this journey possible. If I had to say thank you, I would not have enough words to express my gratitude.

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CHAPTER 1. INTRODUCTION

One of the main targets of malting barley breeding in the last century has been to increase the amount of soluble solids extracted from the malt of a given barley cultivar (Enari, 1995; Schwarz and Horsley, 1995; Edney et al., 2014). For brewers, the desire for increased extracts was coupled with the assumption that most of these solids are carbohydrates. Thus, it was initially assumed that more solids in wort (i.e. extract) would result in the higher alcohol concentration, and more beer per unit of malt (Aldous, 1890; Bathgate et al., 1978). However, not all carbohydrates in wort are fermentable (MacWilliam, 1968; Willaert, 2007) and some gains in extract have been made with higher levels of soluble protein. As a consequence, extract itself is not an accurate predictor of the fermentative potential (i.e. fermentability) of a malt sample. Diastatic power (DP) later was seen as a good predictor of fermentability (Evans et al., 2003; Bathgate and Bringhurst, 2011), considering that the amount of fermentable carbohydrates in wort will depend on the action and collective efficiency of the DP enzymes (alpha-amylase, beta-amylase, limit-detrinase, and alpha-glucosidase) on gelatinized starch during mashing.

On the other hand, it has been suggested that beta-amylase and its thermostability can better explain fermentability than DP itself, as it is responsible for most of the variation observed in DP (Eglinton et al., 1998; Gibson et al., 1995). Moreover, when mashing at high temperatures (60-75°C), beta-amylase thermostability becomes important in terms of fermentable saccharides in wort. Alpha-amylase and limit-dextrinase are more stable at these temperatures, but the main products of these enzymes are non-fermentable oligosaccharides and limit-dextrins that serve as substrate for beta-amylase (Briggs, 1998; Evans et al., 2003).

In addition to beta-amylase, other researchers have suggested that the level of endosperm modification during malting affects fermentability (Gunkel et al., 2002; Edney et al., 2007). This

is related with the fact that maximum synthesis/release of the DP enzymes will be observed after four to five germination days. Also, adequate breakdown of cell walls will allow proteolytic enzymes to access protein bodies in endosperm cells, resulting amino acids, short-chain peptides, and polypeptides, some of which are used by yeast during fermentation (Briggs, 1998; Stewart, 2009).

As a result of the reported effects of both beta-amylase and endosperm modification on fermentability, Evans and coworkers (2005) developed a model for predicting this trait in commercial malts, which used both standard and non-standard quality parameters. This model suggested that alpha-amylase, limit-dextrinase, Kolbach index, beta-amylase, and beta-amylase thermostability, together can explain most of the variability in fermentability for all-malt (R^2=0.91) worts or 30% rice-70% malt (R^2=0.82) worts. However, when they evaluated other malt sets, alpha-amylase was not important and limit-dextrinase only contributed to small portion of the observed variability (0-5%) in fermentability (Evans et al., 2010a).

These recent findings reflect the complexity of malt fermentability and justify the need to determine what factors are more consistent for its prediction in malting barley breeding programs. The objectives of this research were to determine the main malt factors associated with wort fermentability in a mapping population of two-rowed barley genotypes from North Dakota State University. The population was grown over multiple years and environments in order to increase the fermentability variation. Standard and non-standard malt quality parameters were analyzed, and the importance of using real degree of fermentation (RDF) and apparent degree of fermentation (ADF) in fermentability estimates were determined.

CHAPTER 2. LITERATURE REVIEW

2.1. Barley

2.1.1. Importance

Barley occupies the fifth place of the most-produced crops around the world and it is the fourth most-produced cereal after maize, rice, and wheat (FAO, 2016). One of the reasons for the success of barley cultivation is its wide adaptation to different environments and its tolerance to drought, cold, and salt. Moreover barley is a short-cycle crop, which makes it attractive to farmers who practice crop rotation in some latitudes (Ullrich, 2011).

Currently, barley is mainly used for feed (55%-60%) and brewing purposes (30%-40%), while the rest is used for food (2%-3%) and seed production (5%). Feed barley has been focused on ruminants (e.g. dairy cattle), considering that barley is not well digested by monogastrics (e.g. poultry) due to its fiber content. Barley for food, on the other hand, has acquired renewed interest due to its nutritional value. However other crops with better final product characteristics (e.g. appearance, mouth feel, etc.) such as wheat and rice are still preferred by customers. For brewing though, barley is still the first choice over other crops because it is better suited for industrial processing, providing better quality of final products (McNab and Smithard, 1992; Baik and Ullrich, 2008; Anderson and Schroeder, 2010; Ullrich, 2011; Fernandez et al., 2016).

2.1.2. Grain Composition

Barley grains are harvested covered (with the hull or husk) or naked (hull-less) depending on the cultivar (Takeda et al., 2008). For brewing purposes, covered barley is preferred due to its protection of the growing acrospire, and its function as a filter aid during lautering (Briggs, 1998). In covered grains, the husk is tightly adhering and it is difficult to separate, while in naked barley it can be easily removed during threshing. Similar to other cereals, the covered barley

grain can be divided in three main anatomical parts (endosperm, germ, and bran) plus the husk. However, because of the presence of the husk and the importance of the aleurone layer during germination, bran constituents (i.e. pericarp, testa, and aleurone layer) in barley are usually referred to separately (Briggs, 1998; Delcour and Hoseney, 2010). In terms of total dry weight of barley grain, the husk is 10-12%, pericarp plus testa 2-3%, aleurone layer 4-5%, starchy endosperm 77-82%, and the embryo 2-3% (Palmer, 2006).

The major chemical constituents of the barley grain are carbohydrates (78-83%), proteins (8-15%), and lipids (2-3%). Other minor components such as polyphenols and lipolytic enzymes can have important impacts on beer stability and flavor (Yang and Schwarz, 1995; Briggs, 1998; Siebert, 1999; Gubatz and Shewry, 2011). Most of the barley carbohydrates are concentrated in the starchy endosperm. They are present in the form of starch (50-70%) or cell wall polysaccharides (9-15%). For the brewing process, starch is the substrate of interest since it provides the fermentable saccharides to the yeast. Barley starch is comprised of two molecules. Amylose is essentially linear chains of (1→4)-α-linked D-glucose residues with low number of (1→6)-α-linkages. Amylopectin is a highly branched molecule, consisting mostly of (1→4)-α-linked chains with (1→6)-α-linked branch points. Linkages occur in a ratio of 1/20. The proportion of amylose and amylopectin in a normal barley cultivar is 25% and 75%, respectively. However, there are some barley cultivars with either high amylose (ca. 50%) or high amylopectin content (97-100%) but they are not used in brewing, due to their undesirable pasting properties (Briggs, 1998; Song and Jane, 2000). On the other hand, beta-glucans (1→3,1→4-β-D-glucan) and arabinoxylans (arabino-1→4-β-D-xylan) are the main cell wall polysaccharides in barley (>95%), with the remaining consisting of callose, cellulose, and glucomannan. In the starchy endosperm, beta-glucans constitute 75% and arabinoxylans 20% of the total cell wall

polysaccharides, while in the aleurone cell walls their concentration is 26% and 71%, respectively (Gubatz and Shewry, 2011). When barley is malted, complete degradation of cell walls is one of the main targets due to their negative effects on wort separation and beer filterability during brewing (Sadosky et al., 2002; Kanauchi and Bamforth, 2008).

A second major component of barley grain is protein, and the concentration depends on nitrogen availability, environmental conditions, and genetic factors (Briggs, 1998; Qi et al., 2006; Wei et al., 2009). Similar to other crops, proteins in barley were classified according their solubility as albumins, globulins, prolamins (hordeins), and glutelins (Bishop, 1928; Delcour and Hoseney, 2010). Their concentration in barley grain, in terms of total protein, is albumins, 3-5%; globulins, 10-20%; glutelins, 35-45%; and hordeins, 35-50% (Lásztity, 1995). Barley proteins have been extensively studied due to their effects on barley and malt quality parameters for brewing. Hordeins have been studied in more detail due to their relationship with total protein, beta amylase activity, and malt extract (Howard et al., 1996; Qi et al., 2006; Wei et al., 2009; Gubatz and Shewry, 2011). It is well known that the concentration and distribution of proteins in barley grain as well as their degradation during malting will have determinant effects on many parameters. Some examples are: grain structure and texture (e.g. hordeins, hordoindolines), malting process and malt quality (e.g. effect on water absorption, endosperm modification, kolbach index, free amino nitrogen, soluble protein, Maillard reactions), the brewing process (e.g. hot break formation during wort boiling, yeast nourishment, Maillard reactions), and final beer quality and stability (e.g. protein Z and lipid transfer protein 1 effects on beer foam, beer haze due to protein-polyphenol complexes) (Briggs, 1998; Siebert, 1999; Nierop et al., 2004; Perrocheau et al., 2006; Stanislava, 2007; Nagamine et al., 2009; Steward, 2009; Gubatz and Shewry, 2011).

Lipids, on the other hand, are not as concentrated as carbohydrates and proteins in the barley grain. Nevertheless, they play a key role in malting and brewing in terms of beer quality. The major fatty acids present in barley are unsaturated (C18:1, C18:2, and C18:3), most of them (ca. 70%) present as triacylglycerides. During germination, lipid content will be reduced by about 30% due to the metabolism of free fatty acids and fatty acids released from triacylglycerides. These are needed for the development of the germ. Oxidation products of linoleic and linolenic acids can be perceived in malthouses as the characteristic "green malt" smell of germinating barley (Anness, 1984; Dong et al., 2013). Considering that barley lipids are prone to enzymatic and non-enzymatic oxidations throughout malting and brewing processes, lipolytic enzymes such as lipoxygenases (LOX) have been studied for several decades due to their relationship with beer flavor and stability (Drost et al., 1990; Vanderhaegen et al., 2006). One of the biggest concerns related to lipid reactions is the formation of *trans*-2-nonenal, which is one of the main causes of beer staling and results in a cardboard-like flavor in oxidized beer. LOX are the main enzymes responsible of *trans*-2-nonenal content in wort and beer. These enzymes are synthesized during barley germination, but only 1 to 3% will survive kilning temperatures. Two LOX isoenzymes are present in germinating barley, LOX 1 and LOX2, while only small amounts of LOX 1 can be detected in sound barley (Schwarz and Pyler, 1984; Yang and Schwarz, 1995). Even though the presence of *trans*-2-nonenal can be controlled with processing conditions, brewing companies in collaboration with barley breeders have developed some LOX-less malting barley cultivars (Yu et al., 2014).

2.1.3. Quality Requirements for Malting

Commercial barley is classified as either two-rowed or six-rowed. These terms were coined considering the fertility of spikelets and their arrangement on the spike of barley plants. Single floret spikelets are located in triads (i.e. three flowers) at each node on alternate and opposite sides of the rachis. If these three florets are fertile, the spike will have six rows of grains. However, if only one of them is fertile (i.e. the central floret), the spike will have two rows of grains. In six-rowed barleys the mature central spikelets have symmetrical grains while lateral spikelets are slightly skewed or twisted. In two-rowed, however, grains are more uniform and plumper. These differences between barley types affect the grain quality for malting and brewing, and it is one of the reasons that most of the world barley production for malting has focused on two-rowed. Two-rowed barley is considered to have better processing adaptability, quality, and economic advantages due to the higher malt extract yield. In the past, brewers used to see broad differences in quality between two- and six-rowed barleys, but nowadays, competitive six-rowed barley cultivars are available in some countries as a result of breeding efforts (Briggs, 1998; Schwarz and Horsley, 1995; Schwarz and Horsley, 1997; Muñoz-Amatriaín et al., 2010b).

Compared with other cereals, barley for beer production is a special case because it is purchased on a varietal basis and the seed must be able to germinate. This condition demands specific guidelines for two- and six-rowed malting barley trading and breeding (Table 1). These guidelines are quite similar for breeding malting barley in different countries. Also, there are additional requirements mainly related with grain physiology. For example, it is required that barley matures rapidly with low dormancy and uniform germination. Regarding the grain, the hull should be thin, bright and adhere tightly during harvesting, cleaning, and malting (AMBA,

2014). In other words, besides having high malting and brewing characteristics, barley must be well adapted to the industrial processing conditions.

This evaluation of potential malting barley genotypes is similar around the world by different companies, institutions, or agencies, with some variations (Friedt et al., 2011). In the case of malting barley breeding, a barley genotype must meet or exceed all quality specifications. After several years of a strict screening, it can be considered as a candidate for plant-scale brewing evaluation.

Table 1. Barley and malt quality specifications to barley breeders provided in the United States by the American Malting Barley Association (AMBA), Inc.

	Six-Row	Adjunct Two-Row	All Malt Two-Row
Barley Factors	----------%----------		
Plump kernels (on 6/64)	>80	>90	>90
Thin kernels (thru 5/64)	<3	<3	<3
Germination (4 mL 72h)	>98	>98	>98
Protein	≤13	≤13	≤12
Skinned and Broken Kernels	<5	<5	<5
Malt Factors	----------%----------		
Total protein	≤12.8	≤12.8	≤11.8
On 7/64" sieve	>60	>70	>75
Measures of Malt Modification			
Wort β-glucan (ppm)	<120	<100	<100
F-C extract differences (%)	<1.2	<1.2	<1.2
Kolbach index (%)	42-47	40-47	38-45
Turbidity (NTU)	<10	<10	<10
Wort viscosity (cp)	<1.50	<1.50	<1.50
Congress Wort			
Soluble protein (%)	5.2-5.7	4.8-5.6	<5.3
Extract (% dry basis)	>79	>81	>81
Wort color (°SRM)	1.8-2.5	1.6-2.5	1.6-2.8
Free amino nitrogen (mg L^{-1})	>210	>210	140-190
Malt Enzymes			
Diastatic power (°ASBC)	>150	>120	110-150
α-Amylase (DU at 20°C)	>50	>50	40-70

AMBA, 2014.

2.1.4. Genetics and Quality

In barley, the basic chromosome number is x=7. In the genus *Hordeum*, both diploids (2n=14) and polyploids (2n=4x=28) occur. For commercial production, only diploid barley (*H. vulgare* L.) is used, and includes both two- or six-rowed genotypes. The genes *vrs* (loci 1, 2, 3, and 4) and Intermedium-C are responsible for different spike configurations. However, only the *vrs*1 locus (dominant for two-row) is found in the current commercial malting barley cultivars, and is present in chromosome 2HL (Komatsuda et al., 2007; Bothmer and Komatsuda, 2011; Koppolu et al., 2013). In relation to malt quality, the main effects of the expression of these genes will be observed in some parameters such as total protein and extract (Briggs, 1998).

In the last three decades, multiple loci affecting quantitative traits (i.e. QTLs) have been identified in barley chromosomes as a way to improve agronomic and quality selections during breeding (Table 2). However, not all this information has been used successfully in practice, due to different constraints such as complicated genotype x environment interactions, limited technology, and little understanding of the genetic architecture of complex traits (Ullrich et al., 1997; Heffner et al., 2009; Muñoz-Amatriaín et al, 2010a; Xu et al., 2012; Zhao and Xu, 2012).

Table 2. QTLs reported in barley for several traits.

Chromosome	Number of QTL					Total
	Abiotic stress resistance	Agronomic traits	Biotic stress resistance	Quality	Other	
1H	8	35	9	24	1	77
2H	7	81	16	35	6	145
3H	3	70	17	19	4	113
4H	8	52	15	21	1	97
5H	14	56	14	47	4	135
6H	15	36	13	10	1	75
7H	12	59	19	24	1	115
Total	67	389	103	180	18	757

Hayes et al., 2003.

Considering the number and location of QTLs, the selection of genotypes for malt quality during breeding becomes extremely complex due to the fact that variation of quality parameters is highly associated to environmental conditions. Also, and differing from most other crops, germination is a key step when evaluating quality. Not all breeding lines will germinate the same way, especially when they had any stress during grain filling (e.g. frosts, drought, floods) or were grown under conditions that cause pre-harvest sprouted grains or the incidence of detrimental microorganisms on the spike such as *Fusarium graminearum*. These factors can affect germination performance (i.e. low germination energy, high water sensitivity). Besides genetic constitution, this is probably one of the reasons that many QTL's for single quality parameters (Table 3) have been found in different chromosomes (Gusta and O'Connor, 1987; Briggs, 1998; Prom et al., 1999; Chloupek et al., 2003; Fox et al., 2003; Nduulu et al., 2007; Gualano and Benech-Arnold, 2009; Morrison and Linder, 2014).

Table 3. Reported QTLs in barley related with malt quality.

	Chromosome						
Trait	1H	2H	3H	4H	5H	6H	7H
Alpha amylase	2	2	1	3	7	3	2
Beta amylase		1		1			
Barley beta glucans	1	2					1
Malt beta glucans	2		3	1	2		2
Wort beta glucans		2		1	6	1	3
Diastatic power	1	2	1	2	3	2	2
Wort viscosity						1	1
Barley protein	1	5	3	5	5	1	1
Kernel plumpness	2	2	1	3	3		3
Kernel weight				1	2		4
Malt extract	9	5	6	1	5		4
Kolbach index	1	2	1	1	3		1
Test weight	1	3	1	2	3	4	1
Grain hardness		1	1	1	2	1	1
Seed dormancy					1		
Fermentability		3	2	2	3	1	1

Han et al., 1999; Swanston et al., 1999; Meyer et al., 2001; Fox et al., 2003; Fox et al., 2007; von Korff et al., 2008; Szűcs et al., 2009; Gutierrez et al., 2011.

2.1.4.1. Genetic mapping of fermentability

It is estimated that at least 14 genes control malt fermentability. Identified loci are located in all barley chromosomes except 1H; however, few of the QTLs were able to significantly explain the phenotypic variation, either using hot water extract or the Congress Mash as the mashing methods. There are few reports of QTLs affecting malt fermentability compared with reports on mapping other traits associated with fermentability prediction (Swanston et al., 1999; Meyer et al., 2001; Fox et al., 2003; von Korff et al., 2008). Aside from the time and cost of fermentability analysis, one of the reasons for this observation is that the presence of fermentable sugars in wort is the result of the joint action of the enzymes contributing to DP (i.e. alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase) on starch. Moreover, it has been found that beta-amylase (and its thermal stability) is the predominant enzyme affecting fermentability. Alpha-amylase and limit-dextrinase are not considered limiting since both are relatively stable at high mash temperatures (>65°C). Alpha-glucosidase, on the other hand, has variable thermal stability. As its concentration in malt is very low, its real contribution to the final wort carbohydrate profile is questionable (Sissons and MacGregor, 1994; Muslin et al., 2002; Evans et al., 2005; Bathgate and Bringhurst, 2011). However, some researchers observed that thermostable alpha-glucosidase improves fermentability (Muslin et al., 2003). Despite this situation, it is generally accepted that selecting for increased fermentability can be accomplished by analyzing all DP enzymes individually. However, beta-amylase has been looked at as the most reliable enzyme for fermentability prediction (Eglinton et al., 1998; Kihara et al., 1998; Zhang et al., 2004; Evans et al., 2010a, Evans et al., 2010b).

There are three genes coding for beta-amylase in barley, *Bmy1*, *Bmy*2, and *Bmy*3. They are located in chromosomes 4HL, 2HL, and 4HL, respectively. The gene *Bmy*1 is the most

important from a malt quality standpoint since it codes for the two isoenzymes present in malt, Sd1 and Sd2, which are differentiated by a single amino acid substitution (Swanston, 1980; Li et al., 2002; Fox et al., 2003). In terms of beta-amylase thermal stability, there are three endosperm-specific beta-amylase alleles termed *Bmy*1-Sd1, *Bmy*1-Sd2L, and *Bmy*1-Sd2H that have been identified at the *Bmy*1 locus. The corresponding enzymes are referred to as Sd1, Sd2L, and Sd2H and possess intermediate, low, and high relative thermostability, respectively (Eglinton et al., 1998). Initial studies showed that most of the cultivated barley around the world possessed beta-amylase with intermediate to low thermostability. However, the wide variation observed between cultivars (from 0 to 70% thermostability) left an open horizon for barley breeders looking for increased fermentability (Kihara et al., 1998; Kaneko et al., 2000; Kihara et al., 2002; Zhang et al., 2004). Compared with the information available on beta-amylase, allelic variation for the thermostability of alpha-amylase, limit-dextrinase, and alpha-glucosidase is less studied (Henson and Duke, 2008).

It is worth noting that all thermal evaluations between the different reported beta-amylase alleles were carried out at temperatures below 60°C (Eglinton et al., 1998; Kihara et al., 1998; Kihara et al., 2002). Taking in account that current commercial mashing procedures use higher mash-in temperatures (up to 65°C) than the established (45°C) in the Congress Mash (Evans et al., 2011; Yu et al., 2014), the use of beta-amylase as a fermentability predictor during malting barley breeding could become inaccurate. For example, Eglinton et al. (1998) and Kihara et al. (1998) observed that the magnitude of the differences between barley cultivars, in terms of beta-amylase thermostability (e.g. determined at 57.5°C for 30 min), will decrease in response to increased temperatures and incubation times, especially with temperatures above 60°C.

2.1.5. Breeding for Malt Quality

When developing malting barley cultivars, breeders should consider, aside from the agronomic performance of barley plants, many factors such as selecting for two-row vs six-row, or hulled vs hull-less barley (controlled by a single locus *nud* in chromosome 7HL, dominant for covered). These traits are related with technological decisions in malting and brewing processes such as physiological characteristics (e.g. water absorption and distribution rates during steeping, germination time, endosperm modification), or barley biochemistry (e.g. protein and starch ratio, kernel hardness), which impact the final decision of breeders when selecting advances lines based on the brewing industry demands (Briggs, 1998; Cuesta-Marcos et al., 2010; Takeda et al., 2008; AMBA, 2014). For this reason, knowledge on qualitative and quantitative traits, as well as their inheritance, is the corner stone for malting barley breeding.

As observed in Table 4, barley breeding is a long-term process; however, different breeding techniques (e.g. double haploid, male sterile facilitated recurrent selection, marker assisted selection), supported by modern biotechnology, have been adopted and, in many cases, coupled with traditional methods (i.e. pedigree, single seed descent) in order to accelerate cultivars releasing. Even though some researchers have reported the limited success of using the overwhelming QTL information for barley breeding (Fox et al., 2003; Rae et al., 2007; Heffner et al., 2009), it is expected that marker-assisted selection procedures will become a basic tool for selecting superior traits in the mid-term with the improvement of selection techniques. For example, whole-genome strategies are expected to facilitate the molecular marker-assisted breeding procedures (Friedt et al., 2011; Varshney et al., 2012; Xu et al., 2012; Desta and Ortiz, 2014).

Table 4. General description of malting barley breeding.

Year[1]	Generation	Traits evaluated
1	F_1, F_2	Agronomic
2	F_3	Agronomic, quality prediction
3-5	$F_4 – F_8$	Agronomic, quality prediction
6-7	$F_9 – F_{10}$	Agronomic, malt quality
8-11	$F_{11} – F_{12}$	Agronomic, malt quality, Release

[1]Years vary depending on breeding strategies; in this case, years are based on the pedigree method. Friedt et al., 2011; Steffenson and Smith, 2006; Forster et al., 2007; Sleper and Poehlman, 2006.

One of the major advances in the last 50 years of barley breeding has been observed with yield (Garstang et al., 2011), which has steadily increased at different rates depending on the region (Figure 1). On the other hand, considerable improvements in malting quality have been reported by different breeding programs. In the United States, for example, the increase of soluble solids from malt (i.e. extract) has been one of the major successes in six-rowed barley cultivars during the last century (Schwarz and Horsley, 1995). The involvement, collaboration, and funding of different companies, breeding programs, and organizations have been the key for the success of barley breeding around the world (Friedt et al., 2011).

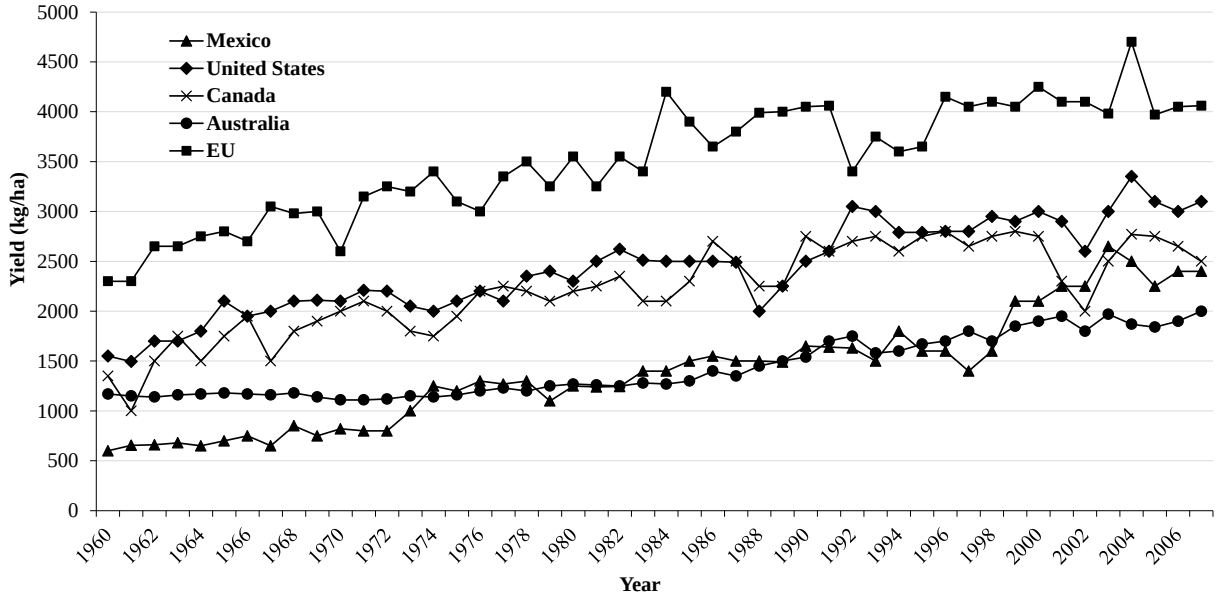

Figure 1. Barley yield in the last 50 years. Elaborated with data of Garstang et al. (2011).

2.1.5.1. Quality selection during breeding

Similar to other crops, formal barley breeding started at the beginning of the 20th century after Gregor Mendel's Laws of Inheritance were rediscovered (Sleper and Poehlman, 2006). However, by that time, barley breeders already had strong knowledge on the heritability of some qualitative traits of the barley plant and some quality parameters due to their observations on some cultivars developed in the second half of the 19th century. Interestingly, the inverse relationship of grain nitrogen and malt extract was known and commonly accepted by brewers as of 1900 (Biffen, 1906; Neumann, 1906; Beaven, 1934). The pioneer work of Biffen (1906) established the importance of evaluating quality during barley breeding. In subsequent decades, with the establishment of industry-sponsored organizations, malt quality evaluations (initially based on nitrogen, extract, and diastatic power) became an essential part of the breeding process (Schwarz and Horsley, 1995; Edney et al., 2014). For example, in 1930 Bishop developed an equation for the prediction of malt extract based on nitrogen content and 1000 kernels weight. Later (1948), and after new cultivars were considered, he corrected his formula with the use of some cultivar constants. In consequence, his formula was discarded for barley breeding, which was one of his initial suggestions.

Following the work of Bishop (1930), many researchers have proposed different techniques or equations for predicting malt quality either for breeding or cultivar differentiation (Allison et al., 1976; Ellis et al., 1979; Gianinetti et al., 2005; Fox et al., 2006; Osborne et al., 2007; Psota et al., 2007; Li et al., 2008; Nagamine et al., 2009). Some of these methods consider the evaluation of physicochemical properties of barley grain (e.g. size, weight, hardness, grinding resistance, and rheology) or some parameters during and after malting (e.g. acrospires growth, viscosity, malt extract, and alpha-amylase activity) with relatively low sample amounts

and at low cost. However, most of these methods are not practical for use in segregating generations due to the required sample amounts or because malting is needed. Also, for advanced generations, breeders prefer confirmatory analyses. Nowadays, the most common quality tests in early generations of barley breeding are grain protein, diastatic power, and those related with grain size and weight, such as test weight, and plumpness (Hertsgaard, 2008). On the other hand, analyses related to brewing such as LOX and fermentability are not commonly carried out in malting barley breeding (Henson and Duke, 2008; Friedt et al., 2011).

It is worth noting that one of the main issues for predicting quality during barley breeding is the small amount of sample available for quality tests (<50g) in early stages. Considering the cost of inbreeding and also heritability estimates, it was initially suggested that quality prediction can be performed after the F_3 generation when the pedigree method is used (Foster et al., 1967). One inherent characteristic of the pedigree method is that only after the F_6 generation there is enough seed to carry out appropriate quality predictions; however, two or three more generations are needed (considering seed used for yield trials) in order to comprehensively evaluate the potential malt quality of any genotype (Lejeune et al., 1951; Cook, 1962; Kulshrestha, 1989). Currently, quality evaluation can be performed earlier due to the introduction of different breeding methods and techniques which can be used alone or in combination with the pedigree method in order to have improved selection and genetic gain. In all cases, predictive tests are still needed for selecting segregating genotypes at early stages of breeding, or after yield trials (Friedt et al., 2011). Another factor that could have induced anticipated quality evaluation is associated with the genetic variability of breeding programs. Most mature breeding programs tend to have reduced genetic variability, which is a common situation linked to the modern breeding practices (Able et al., 2007; Muñoz-Amatriaín et al., 2010a).

2.1.6. Malting

Malting is a controlled germination process that includes three general steps: steeping, germination, and kilning. This process aims the "modification" (i.e. the physical and chemical changes that occur to grains during malting due to the synthesis of different enzymes and the resultant breakdown of the endosperm cell walls) of cereal grain into malt, the main brewing raw material. One of the reasons that barley is preferred over other cereals for beer production, besides its composition and impact on beer flavor, is its adaptation to the malting process that contributes, at the same time, to the economic performance of the malthouse (Briggs, 1998; Briggs, 2002; MacLeod, 2004). At the industrial level, steeping, germination, and kilning steps are physically separated, but they are physiologically interconnected considering that germination starts in steeping and it is completely stopped at initial stages of kilning (Lloyd, 1988; Kuntz and Bamforth, 2007).

2.1.6.1. Steeping

The main objective of steeping is to provide rapid and even uptake of water and oxygen to the barley grain (specifically to the embryo) so it germinates uniformly without generating an excess of roots in a process that is as efficient as possible. A positive side effect of steeping is the removal of materials that could be undesirable in brewing, because of their effects on beer stability. This includes materials such as some polyphenols, but not all of these components are leached (McMurrough et al., 1996; Briggs, 1998; MacLeod, 2004; Miedl et al., 2005).

In commercial steeping, barley is subjected to different immersion and air rest periods. The immersions allow the embryo to absorb water faster, and the air-rest periods allow for effective water distribution (MacLeod, 2004). Even though there is evidence that barley can germinate at low moisture levels (26%), the desired water content in the grain at the end of

steeping is 43-46% which guarantees a consistent and efficient germination (Essery et al., 1954; Brookes et al., 1976; Briggs, 1998). Some variations in water content during malting can be observed when specialty malts are produced (Kunze, 2014).

Steeping regimes depend on many factors related to the barley grain (e.g. size, chemical composition, water sensitivity, etc.) and malthouse conditions (e.g. water availability, water treatment methods, technology, etc.). In the past, steeping times used to be longer (>50 hr) because constant immersion was a common practice (Brookes et al., 1976; Briggs, 1998). Nowadays, water availability and processes focused on energy efficiency in malthouses have driven steeping conditions to a well-balanced relationship between immersion and air rest periods. In fact, air rest periods are longer than the total time barley is immersed. In general, steeping involves 1-4 immersions (1-2 preferred and/or spray steeping in order to reduce water usage) under controlled water temperatures (12-25°C) and constant air injection in order to provide oxygen to the grain (Briggs, 1998; MacLeod, 2004). Even though forced air is provided during all steeping times, oxygen deficiencies are still observed but these have little effect on malt quality (Wilhelmson et al., 2006; Kunze, 2014).

During steeping, very important physiological events take place. Once adequately hydrated, the embryo produces enzymes in order to break down embryonic starch (MacLeod, 2004). Other enzymes are synthesized and released into the starchy endosperm by the scutellum and aleurone layer, the latter being triggered by hormones coming from the embryo (i.e. gibberellic acid, GA_1 and GA_3). The exact proportions and sequence of enzymes released by the scutellum and the aleurone layer are poorly understood. There are estimates that 5-10% alpha-amylase, most of the carboxypeptidase I, and up to 40% of beta-glucanase come from scutellar epithelium, while most of the carboxypeptidase III is released by the aleurone layer (Briggs,

2002). Synthesis of all enzymes involved with cell wall degradation (e.g. xylanase, beta-glucanases, carboxypeptidases, arabinofuranosidase) are initiated in early stages of steeping (Kuntz and Bamforth, 2007; Kanauchi and Bamforth, 2008).

As a result of these complex events, shoot and root growth is initiated. This can be observed at the end of the steeping process with a high proportion of "chit" counts. This parameter measures the consistency and uniformity of a given sample when the proportion of grains with a visible white protrusion (chit) in the basal region is determined (MacLeod, 2004). In technical terms, this protrusion is the elongating coleorhiza (a multicellular embryonic tissue that covers the seminal roots of grass seeds) at initial germination stages as a result of imbibition before root emergence (Barrero et al., 2009).

2.1.6.2. Germination

As the term implies, germination has the objective of initiating all metabolic processes related with the development of a new plant (Bewley, 1997) and, from a maltsters point of view, to achieve adequate endosperm modification without significantly losing dry matter (Briggs, 1998). While these events start during steeping, the maximum rate of enzyme synthesis and its effects are observed in the germination step. There are many enzymes synthesized and/or activated during barley grain germination but the most important, for brewing purposes, are alpha-amylase, beta-amylase, limit-dextrinase, alpha-glucosidase, beta-glucanases, xylanase, lipases, arabinofuranosidase, and endo- and exo-proteinases (MacLeod, 2004; Kuntz and Bamforth, 2007; Kanauchi and Bamforth, 2008; Bamforth, 2009). Even though some of these enzymes are regulated in response to endogenous hormones, exogenous gibberellic acid (GA_3) is often added after steeping in commercial malting in order to improve process efficiency. The major effects of adding GA_3 on malt quality are observed in low malt losses, increased soluble

nitrogen, malt extracts, and fermentability with no considerable changes in wort viscosity (Briggs, 1998; Briggs, 2002).

Once the synthesis and release of different enzymes has started, their movement across the endosperm is readily noticeable by degraded cell walls. Adequate water distribution across the endosperm is the key for acceptable endosperm modification at this stage. A well modified endosperm has its cell walls evenly and fully (or mostly) degraded with little or no starch granule degradation (MacLeod, 2004). This is commonly observed after 96 hr germination at constant grain moisture (e.g. 45%). The exact pattern of enzymes movement across the endosperm is not well defined since it is a parameter dependent on cultivar characteristics. Malting barley cultivars have different endosperm types which can include porous, mealy, hard or vitreous, and are due to chemical composition and distribution of some constituents, such as proteins and beta glucans. Differences can also be observed in the same cultivar from different harvest years or locations (Chandra et al., 1999; Ferrari et al., 2010). These conditions directly affect the diffusion of water and enzymes across the endosperm, and it is likely one of the reasons for discrepancies between some 20th century scientists when trying to define a universal endosperm modification pattern (Palmer et al., 1985; Briggs, 1998; Gianinetti, 2009; Mayolle et al., 2012).

2.1.6.3. Kilning

Once the barley germination process is complete, the "green malt" is transferred to the kiln in order to reduce moisture content, halt germination, preserve most of enzyme activity, and develop color and flavors. Green malt is usually dried for 20-30 hr in order to decrease moisture content from >40% to 4-5% which is a stable moisture level for storage, transport, and further processing conditions. A secondary objective of kilning is to stabilize colors, aromas, and flavors by promoting some chemical reactions (e.g. Maillard) and removing undesirable chemical

compounds (e.g. dimethyl sulfide) (Lloyd, 1988; Briggs, 1998; Yang et al., 1998; MacLeod, 2004). Since kilning aims to preserve enzyme activity, initial kiln temperatures are low (40-60°C) and the air flow is high. As the moisture content decreases, the temperature is increased gradually in a stepwise manner up to 80-105°C (curing stage) with reduced air flow. There are three physical stages during kilning, that includes the removal of free water (>40% to 20% grain moisture), an intermediate stage (20% to 10% grain moisture), and a bound-water or curing stage (10% to 4-5% grain moisture). Depending on the difference between air temperatures entering and leaving the bed of "green malt", as well as the relative humidity in the air leaving the bed, air can be recirculated in final stages of kilning (Lloyd, 1988).

Kilning can also be classified in three sequential biochemical stages. The first is an extended germination step with continued enzyme synthesis and increased enzymes reactions, while the second is strictly enzymatic as temperature increases and moisture levels are still adequate. However as temperature continues increasing, there is inactivation and partial denaturation of some enzymes. This third or "malt curing" stage is also where changes in color and flavor (Maillard reactions) are accelerated by high temperatures (Lloyd, 1988; MacLeod, 2004).

The major effects of changing kiln schedules and kilning environmental conditions will be observed on the final enzyme concentration, flavor, and color in malt. Also, the concentration of some undesirable components in beer flavor such as dimethyl sulfide (DMS) and its precursors (S-methyl methionine, SMM; dimethyl sulfoxide, DMSO) will be impacted (Lloyd, 1988; Karababa et al., 1993; Yang et al, 1998).

The kilning conditions previously described are those related with the production of pale malt. For specialty malts (e.g. caramel, roasted malts), higher kiln or roasting temperatures (100-

200°C) with high water levels, in some cases, are used in order to increase color and flavor development through the Maillard and/or caramelization reactions (Briggs, 1998; MacLeod, 2004; Coghe et al., 2006).

2.2. Malt

2.2.1. Quality

After water, malt is the most abundant ingredient in beer production. The remainder are cereal adjuncts (not used in all beers), hops, and yeast (Harrison, 2009). As a consequence of different beer types and brewing processes, it is not possible to precisely define malt quality without considering the brewing process. In fact, in the malt trade, laboratory evaluation of malt quality was initially only used to determine the amount of extract to be recovered in the brewery (Aldous, 1890), and one of the main complaints with current and historical laboratory malt quality results is that they do not predict the real brewing performance of a malt. However, when one considers the wide variety of brewing processes, it is very unlikely that any standard procedure will predict malt performance under all conditions of different breweries. Even though the reported quality values by a malthouse are used as a reliable reference for brewers, some considerable changes or additions to the current malt quality evaluation techniques are expected in the long term as brewery production methods are accelerated and made less flexible (e.g. high gravity brewing, differences with commercial mashing) (Wainwright and Buckee, 1977; Henson and Duke, 2008; Stewart, 2009; Stewart, 2010; Evans et al., 2011).

The main targets of large brewing companies are to efficiently convert fermentable sugars into alcohol and to obtain high quality products with extended shelf life. Malt is expected to provide the biochemical factors associated to these processes (Willaert, 2007; Harrison, 2009). Briefly described, the brewing process is divided into: brewhouse operations, fermentation, cellar

operations, and packaging (Schwarz and Li, 2011). From a malt quality standpoint, most of the common quality parameters are related mainly with the brewhouse operations and fermentation efficiencies. However, as it was mentioned in previous sections, some components in malt will play a key role in beer stability and quality (e.g. proteins, polyphenols). These components can be reduced or stabilized in cellar operations (Bamforth, 2003; Willaert, 2007), but techniques associated with beer clarification, maturation, and stabilization, will not be discussed in this document.

Brewhouse operations include malt milling, mashing, lautering (i.e. wort separation or filtration), and wort boiling. If malt samples are well modified, particle size reduction during milling will be consistent due to a more friable endosperms, and as a result, uniform substrates for the action of different enzymes during mashing will be available (Briggs, 2002). At mashing, the enzymes developed during malting will mainly degrade starch to fermentable and non-fermentable carbohydrates (i.e. glucose, maltose, fructose, maltotriose, dextrins), and protein to polypeptides and amino acids (Willaert, 2007). Some lipases will also be active during mashing but their impact, in terms of final wort composition, is neglectable (Bamforth, 2003). At the end of mashing, separation of wort from the insoluble spent grains is performed in the lauter tun or mash filter. Once the wort is clear and the desired gravity (% Plato) is adjusted, it is boiled. Wort boiling has many objectives, but the main ones are to extract hops components, coagulate proteins (hot break formation), remove undesirable volatile aroma compounds, form color, completely inactivate enzymes, and to sterilize the wort prior to fermentation (Willaert, 2007). Adequate conversion of wort components to alcohol will depend on fermentation conditions (e.g. oxygen, temperature), and the concentration of amino acids and peptides (Stewart, 2009). After

fermentation, cellar operations are intended to mature or stabilize flavor and the colloidal stability of beer before packaging (Schwarz and Li, 2011).

2.2.1.1. Mashing conditions used for malt quality evaluations in barley breeding

The operations described above illustrate the brewing process in a general and simple sequence. However, it provides a perspective to discuss the importance of some malt quality parameters related with this process.

One of the main laboratory operations when evaluating malt quality in advanced breeding lines is mashing. Before discussing malt quality parameters, the two most common laboratory mashing methods will be described considering that they significantly impact the final wort composition. These methods are the Congress Mash and Hot Water Extract (HWE). The Congress Mash method consists of ramped temperatures from 45 to 70°C where ground malt is mashed in an excess of water with constant stirring. The first step, or mashing-in at 45°C for 30 min, is often termed the "protease or beta-glucanase rest". It is intended to ensure additional degradation of proteins and remaining cell walls. After this first 30 min, the temperature is increased to 70°C at a rate of 1°C/min. This temperature is maintained for 1 hr, after which the mash is cooled to 20°C and adjusted to a final malt/water concentration of 1/8. The mash is then filtered, and the resultant solution is called "wort".

The characteristics of solids present in the mash at different temperatures clearly reflect the action of DP enzymes. For example, beta-amylase and alpha-glucosidase will be more stable at intermediate temperatures (50-60°C) of the Congress mash, while alpha-amylase and limit-dextrinase will work better at slightly higher temperatures (60-70°C). The Congress Mash temperatures ensure uniform starch gelatinization, which enables efficient starch hydrolysis by the joint action of all DP enzymes (Briggs, 1998; Evans et al., 2005).

On the other hand, the HWE mash is isothermal at 65 °C. These conditions better emulate the mashing procedures traditionally used in brewing (Evans et al., 2011). It has been observed that worts prepared with the HWE method are more viscous; contain more beta-glucans, and lower free amino nitrogen compared with the Congress Mash. This is mainly related to the action of beta-glucanases and some proteases; which are not active at higher temperatures of the HWE mash. Also, wort has lower extract and its carbohydrate profile shows a considerable increase in maltose (Schwarz et al., 2007; Evans et al., 2005; Evans et al., 2011). Interestingly, and considering that beta-amylase has a major impact on maltose concentration in wort, it is known that this enzyme retains very low activity under HWE mash conditions. For example, Henson et al. (2008, 2014) reported that only 30% of beta-amylase is retained after 1 hr at 63°C, while at 68°C its activity is close to zero. Alpha-amylase though retains 50-100% of its activity under both conditions. Limit-dextrinase behaves similar to alpha-amylase, while alpha-glucosidase tends to behave similar to beta-amylase. Evans et al. (2011) reported some changes in malt extract, fermentability, and lautering performance when the Congress Mash and HWE procedures were carried out on same samples. They concluded that these differences can potentially result in bias during routine malt analyses.

2.2.1.2. The diastatic power enzymes

As it was mentioned before, the estimation of the diastatic activity of a malt sample is called DP. Brewers use DP as a key parameter of malting quality and consider it an estimate of the capacity of the malt to degrade starch into fermentable sugars, especially when large quantities of starch from un-malted adjunct are included in the grist. Therefore, DP has always been one of the main malt quality parameters during barley breeding and in the malt trade (Wainwright and Buckee, 1977; Briggs, 1998; Evans et al., 2003). The method for determining

DP is carried out by measuring the concentration of carbohydrate reducing groups after a malt enzyme extract is incubated with soluble starch. The enzymes that mainly contribute to DP are alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase (Briggs, 1998).

Alpha-amylase is an endo-acting enzyme that attacks the α-1,4-glucose linkages at random within the starch chains. It can also attack short-chain dextrins but its activity is slower. Alpha-amylase action stops in the immediate vicinity of α-1,6-branch points in amylopectin and branched dextrins. Alpha-amylase acting on its own can generate a complex mixture of sugars from starch such as oligosaccharides, or limit-dextrins containing α-1,4- and α-1,6-linked glucose residues. In gelatinized starch, the action of alpha-amylase is faster if beta-amylase or alpha-glucosidase are present (Briggs, 1998; Evans et al., 2003).

Beta-amylase is an exo-acting enzyme that catalyzes the hydrolysis of the α-1,4-glucose linkages penultimate to the non-reducing ends, releasing the disaccharide β-maltose plus an oligosaccharide (or polysaccharide) shortened by two glucose residues. In the absence of other enzymes such as alpha-amylase, beta-amylase is unable to degrade native starch granules. However it is able to hydrolyze linear chains (e.g. amylose, linear zones of dextrins) in a stepwise manner if they are in solution. Beta-amylase will not attack α-1,6-branch points or α-1,4-linkages immediately adjacent to them (Briggs, 1998; Evans et al., 2003).

Limit-dextrinase, also known as debranching enzyme, is an endo-acting enzyme that catalyzes the hydrolysis of α-1,6-linkages in amylopectin, dextrins, and oligosaccharides. Thus, it removes branch-points which allow further hydrolysis by beta-amylase and alpha-glucosidase. On the other hand, alpha-glucosidase is an exo-acting enzyme that primarily cleaves α-1,4-linkages to produce glucose, although it can also hydrolyze α-1,6-linkages. This is one of the reasons that alpha-glucosidase, similar to alpha amylase, is able to attack granular (native) starch.

However, during mashing, the primary activity of alpha-glucosidase appears to be against oligosaccharides (Briggs, 1998; Evans et al., 2003).

2.2.1.3. Extract

Malt extract is the total amount of solids from malt that can be solubilized in mashing, and is probably the most important quality parameter for maltsters and brewers when selecting or purchasing malting barley (Li et al., 2008). Laboratory methods (Congress Mash and HWE) for extract determination provide an estimate of the theoretical maximum value in a malt sample (Schwarz and Li, 2011). Extract is expressed as a percentage of the malt on a dry-weight basis. Extract in pale malts typically ranges from 79% to 82% depending on barley cultivar, malting conditions, and mashing methods. The main components of extract in wort from an all-malt mash are carbohydrates (90-92%), and about 80% are fermentable. Other minor components are nitrogenous compounds (protein fractions, polypeptides, amino acids), polyphenols, salts, and many other substances (Willaert, 2007; Kunze, 2014). Considering that laboratory extract is a maximum theoretical value that is seldomly obtained at the industrial level, brewers use extract numbers as a reference when doing beer formulations according their brew-house efficiencies (Schwarz and Li, 2011).

2.2.1.4. Soluble protein and related parameters

Soluble protein is the sum of all nitrogenous compounds in wort, and includes amino acids, peptides, and polypeptides. It is worth noting that the yield of soluble nitrogen is influenced by mashing conditions. For example, thick brewery mashes generally yield more soluble nitrogen than thin laboratory mashes (Briggs, 1998). Soluble protein in malt when expressed as the ratio (%) of the total protein present in barley (also known as the Kolbach index), indicates the extent of modification of nitrogenous compounds during malting. This is a

very important indicator of endosperm modification during malting and, also, a good predictor of nitrogen available for yeast nourishment during fermentation when barley protein content is in the adequate range before malting (e.g. 11-13%). However, when high-nitrogen and low-nitrogen barley cultivars are malted and they give similar Kolbach indices, the worts will contain different concentrations of various nitrogenous fractions. This will affect fermentation patterns during brewing and beer properties (Wainwright and Buckee, 1977). Kolbach indices can range from 30 to 50% in malts with different degrees of endosperm modification. Values below 35% are generally considered undermodified, 35-41% as well modified, and values higher than 41% as highly modified (Schwarz and Li, 2011; Kunze, 2014).

Nitrogenous compounds in wort play an important role related to yeast performance during fermentation. The level of free amino nitrogen (FAN) has always been considered as a good predictor of healthy yeast growth, viability, vitality, fermentation efficiency, and consequently beer quality and stability. FAN is mainly composed of amino acids, ammonium ions, and small peptides. FAN levels higher than 150 ppm in wort are required to ensure appropriate yeast nutrition. While it has been observed that different concentrations of oligopeptides can trigger protease synthesis by yeast, this event does not significantly change its performance, mainly due to yeasts preference for amino acids and ammonia in solution (Wainwright and Buckee, 1977; Lekkas et al., 2009).

2.2.1.5. Wort consistency and related parameters

Wort parameters related to its appearance, consistency, and potential brewhouse difficulties include color, viscosity, and beta-glucan content. The official method for wort color determination was developed to measure the characteristic color of pale beers that dominate the market (Schwarz and Li, 2011). In fact, for barley breeding, light wort colors (1.5-3.0 °SRM) are

generally desired. This parameter has always been criticized since it is measured after the wort is filtered under standard laboratory mashing methods. Considering that wort is boiled for variable times during brewhouse operations, with concomitant Maillard reactions, it has been observed that there is little or no relationship between wort color and the color of beer produced from it. However, wort color determined in different types of malt included in a beer recipe will show consistent increase or decrease in beer colors according malts proportions (Bremner, 1963; Wainwright and Buckee, 1977; Coghe et al., 2005).

On the other hand, viscosity and beta-glucans are somehow related with endosperm modification during malting. Thus, elevated values indicate that a malt is under-modified or unevenly modified (Briggs, 1998). Viscosity of wort is mainly due to the presence of dextrins. However, as dextrin content generally does not vary within a specific beer, variations in viscosity are attributed to the levels of soluble cell wall polysaccharides, arabinoxylans and beta-glucans, in different samples. The molecular weights of arabinoxylans and beta-glucans have a major effect on beer filterability (Sadosky et al., 2002; Schwarz and Li, 2011). Currently, malt specifications focus on beta-glucans concentration rather than their molecular weight. The measurement of arabinoxylans, on the other hand, is not an established malt quality parameter.

2.2.1.6. Fermentability

Fermentability is sometimes referred to as the apparent attenuation limit (AAL) of beer, considering that it is the most common method used for its determination. It basically describes the ability of yeast to turn sweet wort into alcohol. As the efficiency of this process is related with alcohol yield, it will regulate the amount of beer produced from a given amount of malt and adjunct grain (Evans and Hamet, 2005). Distillers require maximum wort fermentability to maximize spirit yield, while brewers require moderate to high fermentabilities when making

normal and low-carbohydrate beers (Briggs, 1998). Fermentability is a complex parameter, and is dependent on several factors that complicate its measurement (Edney et al., 2007). As a consequence, it is difficult to breed barley for fermentation potential.

Fermentability is not routinely measured in the malt quality laboratory. This trait can be determined on the standard laboratory mash (i.e. Congress Mash) but this mini-fermentation test is time consuming, expensive to conduct, and does not reflect brewery conditions. For example, laboratory mashing conditions and the excess of yeast used in this accelerated fermentation differ from brewery practice. In addition, fermentation assays require more malt than is commonly available during the early generation testing of breeding lines (Evans et al., 2010a).

Many studies have demonstrated significant correlations between the AAL and DP, beta-amylase and DP, and beta-amylase and AAL. This reflects the long-held belief that beta-amylase drives DP values and influences fermentability. Others have reported that alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase activities are significantly correlated with AAL and DP. However, brewers have generally considered beta-amylase and its thermal stability to be the most important factor that contributes to DP values and, in consequence, fermentability (Kaneko et al., 2000; Evans et al., 2003; Evans et al., 2005; Evans et al., 2010a). Consistent with these observations, Evans et al. (2005) reported that the prediction of malt AAL can be improved from R^2=0.54 with DP alone to R^2=0.91 when other variables were included in a stepwise regression procedure. These variables were: limit-dextrinase, alpha-amylase, beta-amylase, beta-amylase thermostability, and Kolbach index. However, when they evaluated other malt sets, including commercial malts and micro-malted genotypes from different barley breeding programs (with different malting conditions), alpha-amylase was not important and limit-dextrinase only contributed to small portion of the observed variability (0-5%) in fermentability.

Also, the inclusion and order of significant malt quality parameters in the regression model varied with the source of the malt (Evans et al., 2010a).

These discrepancies observed by Evans et al. (2010a) compared with their initial model (2005) could have been related with differences between commercial malting and laboratory malting, considering that they only used commercial malts for developing that model. There are mainly three differences between commercial malting and laboratory malting that affect malt quality: gibberellic acid (GA3), processing pressures, and malt blending. The use of exogenous GA_3 in many malt-houses is a common practice. Small amounts of GA_3 are added at initial barley germination stages in order to increase the effects of the naturally produced hormones by the germinating seed; resulting in improved malting performance and malt quality. On the contrary, differences in mechanical and hydraulic pressures observed in commercial malting can lead to considerable differences in malt quality. For example, high pressures will restrict germination and negatively affect fermentability due to poorly-modified endosperms. Considering the antagonistic effects of GA_3 addition and processing pressures during malting, it is worth noting that they can reduce the observed differences between commercial and laboratory malts; however, subtle but significant differences can still be present (Yoshida et al., 1979; Briggs, 1998; Briggs, 2002; MacLeod, 2004). Malt blending, on the other hand, is a common practice in malthouses in order to comply with brewers specifications. It has been reported that some synergisms can be observed in some quality parameters when two malts with different qualities are blended. Fermentability presents this behavior, probably due to an enzymatic compensation considering the observed differences of DP enzymes (Evans, 2012).

A second, but not minor, reason for those discrepancies is related with the mashing conditions used during experiments. When Evans et al. (2005) developed a model for predicting

fermentability, they used a 65°C mash-in protocol, while in the second study (Evans et al., 2010a) the Congress Mash was used. Supporting this assumption, Evans et al. (2005) reported that the optimum temperature for determining the maximum fermentability potential is a 65°C mash-in protocol. Also, they observed that the magnitude of fermentability differences between barley samples is not parallel at different mash-in temperatures.

The observations of Evans and coworkers (2003, 2005, 2010a, 2010b, 2011) reflect the complexity of malt fermentability and justify the need to determine what quality parameters are more consistent for its prediction, especially during malting barley breeding. Many factors need to be considered; however, it is a fact that increasing fermentability variation between malt samples gives a better understanding of this trait. On the other hand, the adequate selection of the mashing method is determinant on the results.

CHAPTER 3. MATERIALS AND METHODS

3.1. Barley Samples

A population of 81 experimental two-rowed malting barley genotypes from the North Dakota State University (NDSU) two-rowed barley breeding program, plus three two-rowed cultivars (Conlon, Pinnacle, and Rawson) and six-rowed cultivars (Lacey, Quest, Rasmusson, Robust, Stellar-ND, and Tradition) were used in this study. All samples were provided by the NDSU barley breeding program. All entries were grown at three North Dakota locations including Fargo (2013 and 2014), Nesson Valley (2013 and 2014), and McVille (2014). Each entry at a location was replicated twice in a randomized complete block design. However, for malting, seed of each entry was combined across replicates.

3.2. Barley Analysis

Analyses of barley protein, moisture, test weight, and assortments were carried out according to the Official Methods of the American Society of Brewing Chemists (ASBC, 2009). Barley protein and moisture were determined on whole grain by near infrared reflectance (NIR) using a FOSS Infratec^{TM} Grain Analyzer 1241 (FOSS, Hillerød, Denmark). Test weight was performed according the method Barley-2B using a ¼ pint cylinder. Assortments (7/64 in, 6/64 in, 5/64 in) were done according to method Barley-2C by using a Eureka-Niagara sample barley grader (S. Howes Inc., Silver Creek, NY). Plump kernels are reported as the total grain weight (%) retained on the sieve 6/64 in x 3/4 in (i.e. the sum of 6/64 plus 7/64).

3.3. Malting

Pilot-steeping of all samples was performed before malting in order to determine the time needed for samples to reach 43.7% moisture according NDSU Barley Laboratory Standard Procedures (Banasik et al., 1956a and 1956b; Karababa et al., 1993). This value considers a total

dry weight loss of 2.4% during steeping and germination due to leaching and respiration, respectively; hence, the real moisture in the grain at the end of germination is expected to be 45%. Ten grams dry basis (db) were pilot-steeped at 16°C for 72 hr in constant immersion with 1 hr air rests every 12 hr. Moisture measurements were taken by weight at 24 hr, 48 hr, and 72 hr. Total steep time was calculated after determining the linear equation from the logarithmic plot of time vs moisture.

For malting, 160 g db were steeped under the defined conditions and germinated in beakers for 96 hr at 16°C and 95% relative humidity. To avoid matting, samples were turned by hand every 24 hr. After steeping and every day during germination, weight of samples was adjusted to 43.7% moisture by adding distilled water when needed. Kilning was performed according the kiln schedule III (24 hr, 49-85°C) described by Karababa et al. (1993). After kilning, samples were cleaned by hand on a sieve in order to remove rootlets.

3.4. Malt Quality Analysis

Conventional malt analyses were carried out according to the ASBC Official Methods (2009). Malt moisture was determined in a forced draft oven at 104°C for 3.5 hr according the method Malt-3. Friability was performed according the Method Malt-12 by using a Pfeuffer friabilimeter (Pfeuffer GmbH, Kitzingen, Germany). Alpha-amylase and DP were analyzed from the same extract (Malt-6A). Assays were performed in a flow automated analyzer with standard samples of known alpha-amylase and DP determined by the reference methods Malt-7A and Malt-6A, respectively (Karababa et al., 1993).

3.4.1. Mashing and Wort Analysis

Mashing was performed at constant temperature (65±0.5°C) according to the hot water extract (HWE) method 4.6 of the European Brewery Convention (EBC, 1998). However,

considering that water/malt proportions are practically the same that for the ASBC congress mash, the extract (% dry basis) was calculated according the ASBC method Malt-4 for extract fine grind. Wort specific gravity, color, soluble protein, free amino nitrogen, fermentable saccharides, beta-glucans, and viscosity were analyzed according the ASBC methods (2009) Wort-2B, Wort-9B, Wort-17, Wort-12, Wort-14B, Wort-18, and Wort-13, respectively. Extract (%Plato) in wort was calculated by using the specific gravity values and the polynomial equation (1) derived by Cutaia et al. (2009) based on ASBC Tables for extract in wort and beer.

$$\begin{aligned} \textit{Wort Extract } (\%\textit{Plato or } g/100g) = {} & (256.899*(SG_{wort}-1)) + (6.7126*(SG_{wort}-1)^2) \\ & -(14481.6*(SG_{wort}-1)^3) + (517578*(SG_{wort}-1)^4) - (10746400*(SG_{wort}-1)^5) \\ & +(130110000*(SG_{wort}-1)^6) - (850786000*(SG_{wort}-1)^7) \\ & +(2323100000*(SG_{wort}-1)^8) \end{aligned} \quad (1)$$

Where: %Plato= Degree Plato or grams of extract in 100g of wort; SG_{wort}= Specific gravity of wort.

3.4.2. Analysis of the DP Enzymes

All samples were milled to pass 0.5 mm screen in a Udy Cyclone Mill (Udy Corporation, Fort Collins, CO). Analyses of alpha-amylase, alpha-glucosidase, beta-amylase, and limit-dextrinase were adapted to a microplate reader format considering Evans (2008) technical suggestions. However, modifications were made on extraction and dilution conditions. All assays and thermal treatments were carried out in 2.2 mL VWR® 96-well deep well plates with sealing mats (VWR International, Radnor, PA). In order to avoid trapped air under deep well plates and provide adequate heat transfer during assays, 1-mm holes were drilled on the top of their edges.

Alpha-glucosidase, alpha-amylase, and beta-amylase were assayed from the extract obtained in the Betamyl-3 method of Megazyme for the beta-amylase assay (Megazyme, Bray, Ireland). The extraction was performed at room temperature (22±1°C) in 80 mg of malt flour

with 800 μL of extraction buffer (0.05 M Tris-HCl plus 1 mM EDTA, pH 8.0; with 100 mM cysteine HCl added right before extraction) placed in 1.2 mL covered microtiter tubes (Biotube™ 96-tube rack, Simport Scientific, QC, Canada) with 3 mm ball bearings inside (placed before flour weighing in order to get adequate mixing) during 1 hr at constant shaking (20 rpm) on a VWR® 3-D Rotator Waver (VWR International, Radnor, PA). After the set time, racks with tubes were centrifuged at 3,700 x *g* for 10 min at 4°C.

Alpha-glucosidase was assayed with 4-Nitrophenyl α-D-glucopyranoside (pNPG, Sigma Cat. N1377, St. Louis, MO) as the substrate (Sissons and MacGregor, 1994; Evans et al., 2005). For total activity assay, the undiluted extract (50 μL) was incubated with 50 μL of 10 mM pNPG (in 0.1 M sodium acetate buffer plus 0.1% BSA, pH 4.6) for 30 min at 37°C. The reaction was stopped with 750 μL of 1% w/v trisodium phosphate (pH 11.0) and the absorbance was measured at 400 nm. Under these conditions, one unit of alpha glucosidase is defined as the amount of enzyme required to produce one μmole of *p*-nitrophenol per minute. Thermal stability of the enzyme was determined by placing 50 μL of extract in a water bath at 65°C. The extracts were removed after 10 min, and immersed in water at 0-4°C for cooling. Once cooled, the samples were assayed for alpha-glucosidase activity. Total activity and thermal stability were calculated with the average absorbance of two different extractions. Calculations were done by considering 18.1 as the extinction coefficient of *p*-nitrophenol in trisodium phosphate according the Ceralpha Megazyme method (Megazyme, Bray, Ireland) for alpha-amylase assay. Alpha-glucosidase is reported in Units kg^{-1} (ΔE_{400} x 313.08) and thermal stability as the activity (%) remaining after thermal treatment.

Beta-amylase was determined according the Betamyl-3 method of Megazyme (Megazyme, Bray, Ireland; McCleary and Codd, 1989) after a suitable dilution of the extract to

1:21 with 0.1 M MES buffer plus 1 mM EDTA, 1.0 mg mL^{-1} BSA, and 0.02% w/v sodium aside. The Betamyl-3 method employs *p*-nitrophenyl-β-D-maltotrioside (PNPβ-G3) as the substrate in the presence of β-glucosidase, which will release *p*-nitrophenol from *p*-nitrophenyl-β-D-glucose after β-amylase action. For total activity assay, 50 μL of diluted extract were incubated with 50 μL of Betamyl-3® Substrate Solution for 10 min at 40°C. The reaction was stopped with 750 μL of 1% w/v Trizma Base (pH 8.5) and the absorbance was measured at 400 nm. For the thermal stability assay, the thermal treatment (65°C) procedure was similar to that described for the alpha-glucosidase assay. Once the samples were cooled, they were assayed for beta-amylase activity. Total activity and thermal stability were calculated with the average absorbance of two different extractions. Under these assay conditions, one unit of beta-amylase is defined as the amount of enzyme needed to release, in an excess of thermostable beta-glucosidase, one μmole per minute of *p*-nitrophenol from PNPβ-G3.

Alpha-amylase was determined as suggested in the Betamyl-3 method of Megazyme (Megazyme, Bray, Ireland) by doing a further dilution of the 1:21 diluted extract for beta-amylase assay to a new 1:336 dilution with Ceralpha extraction buffer (1 M sodium malate, 1 M sodium chloride, 40 mM calcium chloride, 0.1% w/v sodium azide). The new diluted extract was assayed according the Megazyme Ceralpha method for alpha-amylase (Megazyme, Bray, Ireland; McCleary and Sheehan, 1987; McCleary et al., 2002). The Ceralpha method employs a blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) as the substrate in the presence of thermostable alpha-glucosidase, which will release *p*-nitrophenol from fragments of *p*-nitrophenyl maltosaccharide after alpha-amylase action. For total activity assay, 50 μL of diluted extract (1:336) were incubated with 50 μL of Amylase HR Reagent for 10 min at 40°C. The reaction was stopped with 750 μL of 1% w/v trisodium phosphate (pH 11.0) and the absorbance

was measured at 400 nm. For the thermal stability assay, the thermal treatment (65°C) procedure was similar to that described for the alpha-glucosidase assay. Once the samples were cooled, they were assayed for alpha-amylase activity. Total activity and thermal stability were calculated with the average absorbance of two different extractions. Under these assay conditions, one unit of alpha-amylase is defined as the amount of enzyme needed to release, in an excess of thermostable alpha-glucosidase, one μmole per minute of *p*-nitrophenol from BPNPG7.

Limit-dextrinase was determined using the Limit-Dextrizyme method of Megazyme (Megazyme, Bray, Ireland; McCleary, 1992; MacGregor et al., 2002; Mangan et al., 2015). This method employs Azurine-crosslinked-pullulan (in tablet form) as the substrate which, after limit dextrinase action, is degraded to water soluble dyed fragments. The rate of release of these fragments is directly related with the enzyme activity. The extraction of limit dextrinase was performed in a water bath at 40°C in 50 mg of malt flour with 800 μL of extraction buffer (100 mM sodium maleate plus 0.02% sodium azide, pH 5.5; with 25 mM dithiothreitol added right before extraction) and 20 μL of amyloglucosidase (Megazyme Cat. E-AMGDF, Megazyme, Bray, Ireland) placed in 1.2 mL covered microtiter tubes (Biotube^{TM} 96-tube rack, Simport Scientific, QC, Canada) with 3 mm ball bearings inside (placed before flour weighing in order to get adequate mixing) during 5 hr with occasional mixing (4-5 times). For adequate heat transfer during extraction, the base of the rack was removed and 1-mm holes were drilled in the top of the edges of the rack and in the top of its cover in order to release trapped air and water vapor. After the set time, the base was placed back, and racks with tubes were centrifuged at 2,500 x *g* for 10 min at 22°C. For total activity assay, 100 μL of the extract were incubated with 11.6±0.3 mg of ground Limit Dextrizyme substrate for 10 min at 40°C. The reaction was stopped with 1 mL of 1% w/v trizma base (pH 11.0) and, after covering the deep well plate with its sealing mat,

it was centrifuged at 3,700 x *g* for 15 min at 22°C in order to precipitate the unreacted substrate. Absorbance of supernatant was read at 590 nm and calculation of activity was done according to the reference standard curve provided by the manufacturer. For the thermal stability assay, the thermal treatment (65°C) procedure was similar to that described for the alpha glucosidase assay with the difference that this time 100 μL of extracts were heat treated. Once the samples were cooled, they were assayed for limit dextrinase activity. Total activity and thermal stability were calculated with the average absorbance of two different extractions. Under these assay conditions, one unit of limit dextrinase is defined as the amount of enzyme needed to release one μmole of glucose reducing-sugar equivalents per minute from pullulan.

3.5. Analysis of Wort Fermentability

Wort samples (200 mL) previously heated at 35±1°C were pitched with 3 g of dried yeast (Fermentis Safale^{TM} S-04, Fermentis Division of S.I. Lesaffre, Marcq-en-Barœul, France). After 15 min, worts were cooled in a water bath at 20°C and fermented for 24 hr at room temperature (22±1°C) with constant shaking (150 rpm). The selected pitching temperature (35±1°C) allows a consistent fermentation in a broad range of wort gravities when dry yeast is used (Evans and Hamet, 2005). On the other hand, fermentation time (24 hr) was determined in preliminary trials with worts at two different original gravities (based on expected specific gravities in this research) at different fermentation times (16, 18, 20, 22, 24, 26, 28, and 30 hr) in order to get complete attenuation. Complete attenuation was considered when the real extract (analyzed by distillation according the ASBC method Beer-5A) difference between a fermenting wort with original gravity of 8.9% Plato and a fermenting wort+glucose solution with original gravity of 9.9% Plato (prepared by the addition of 1% Plato with anhydrous glucose to the wort with 8.9% Plato) was less than 0.1% Plato, and the difference within any of these samples was less than

0.02% Plato compared with the same sample fermented for two more hours. After fermentation, samples were vacuum filtered through 110 mm Whatman™ GF/C™ (Cat. 1822) glass microfiber filters (GE Healthcare Life Sciences, Chicago, IL), and specific gravity (SG) was determined by using an Anton Paar DMA 5000 density meter (Anton PAAR GmbH, Graz, Austria). Alcohol content was determined by HPLC with the same operation conditions specified in the ASBC method Wort-14B for fermentable saccharides analysis. Samples were prepared as directed in the method; however, the alcohol content (%v/v) was calculated from the linear equation obtained after plotting response areas of different alcohol (0-7%) standards. Alcohol by weight was calculated with equation (3). The real extract of each sample was calculated with specific gravity and alcohol values according the equation (2), which accounts for water-alcohol and ethanol-sucrose interactions in samples from 0-7% w/w alcohol and 0-10% Plato apparent extract calculated with equation (1) (Cutaia et al., 2009).

$$\begin{aligned} RE\,(\%Plato\ or\ g/100g) = {} & (0.496815689 * A_{w/w}) + (1.001534136 * AE) \\ & - (0.000591051 * A_{w/w} * AE) - (0.000294307 * AE^2) \\ & - (0.0084747 * A_{w/w}^2) + (0.000183564 * A_{w/w}^3) \\ & + (0.000011151 * AE^3) + (0.000002452 * A_{w/w}^2 * AE^2) \end{aligned} \quad (2)$$

Where: RE=Real extract; $A_{w/w}$= Alcohol (% w/w); AE= Apparent extract (% Plato).

$$A_{w/w} = \frac{A_{v/v} * 0.78924}{SG_{fermented\ wort} * 0.9982343} \quad (3)$$

Where: $A_{w/w}$= Alcohol (% w/w); $A_{v/v}$= Alcohol (% v/v); $SG_{fermented\ wort}$= Specific gravity of the fermented wort or alcohol/water mixture; 0.78924 = Density (g mL^{-1}) of alcohol at 20°C according the International Bureau of Legal Metrology (1973); 0.9982343= Density (g mL^{-1}) of water at 20°C according the ASBC Tables for extract in wort and beer (ASBC, 2009).

Considering that the observed SG in the fermented worts ranged from 0.99901 to 1.00861 with alcohol contents of 3.15-5.24% v/v, and assuming that samples with SG<1 have no "apparent" extract, the validity of using equation (2) with these values was tested. After preparing several model solutions with different alcohol contents (0-5% v/v) and extract (Figures 2 and 3), it was observed that equation (2) is valid for SG<1 only when apparent extract is calculated by equation (1), which yields "negative" apparent extracts. While negative extract is not possible in reality, the results are reasonable considering that the equation (2) was developed with a response surface methodology based on results of a routine that accounted for ethanol-sucrose interactions with SGs as low as 0.9877 (Cutaia et al., 2009; Hackbarth, 2009). Even though some fermented worts have SG slightly out of scope (i.e. SG<1), negative values for apparent extract allow a good projection of the formula when alcohol content is in range. In fact, when real extracts of all fermented worts were determined by Tavari's equation, it was observed that, even with slightly higher values than those obtained by equation (2), there is almost a perfect linear relationship (r=0.9999) between these formulas. This is not surprising considering that the impact of low alcohol by weight (<16%) on ethanol/sugar interactions is marginal (Hackbarth, 2009).

The model solutions prepared by weight at 20°C, with three replicates each, consisted of 0, 1, 2, 3, 4, and 5 mL absolute ethanol (purity ≥ 99.91%) and 100, 99, 98, 97, 96, and 95 mL of sucrose solutions at 1 % and 5% Plato, respectively. After mixing, and considering that volume is not additive in water/ethanol mixtures (Chang, 2005), the real alcohol by volume was calculated based on the real weight of 100 mL of the mixture (from the SG of the mixture determined in an Anton Paar DMA 5000 density meter) in order to know the total mL prepared

(which sugar concentration was recorded by weight). The range of SG covered with these model solutions was 0.99651-1.01843.

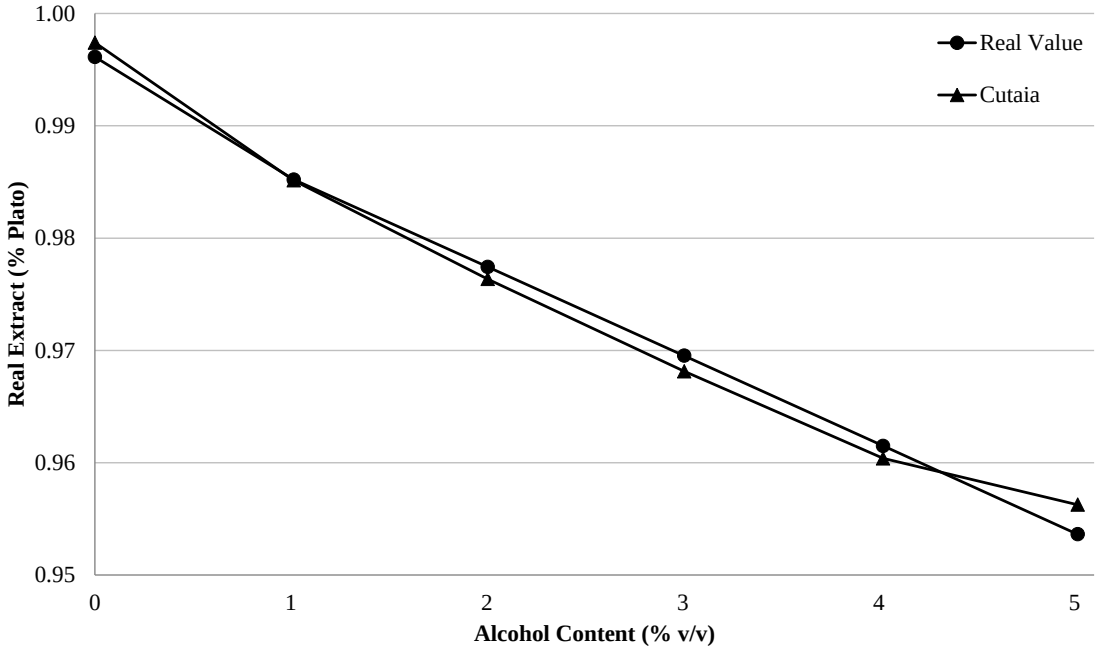

Figure 2. Predicted real extract by Cutaia's equation (2009) in a 1% Plato (real value= 0.9961) model solution and its dilutions to different alcohol contents.

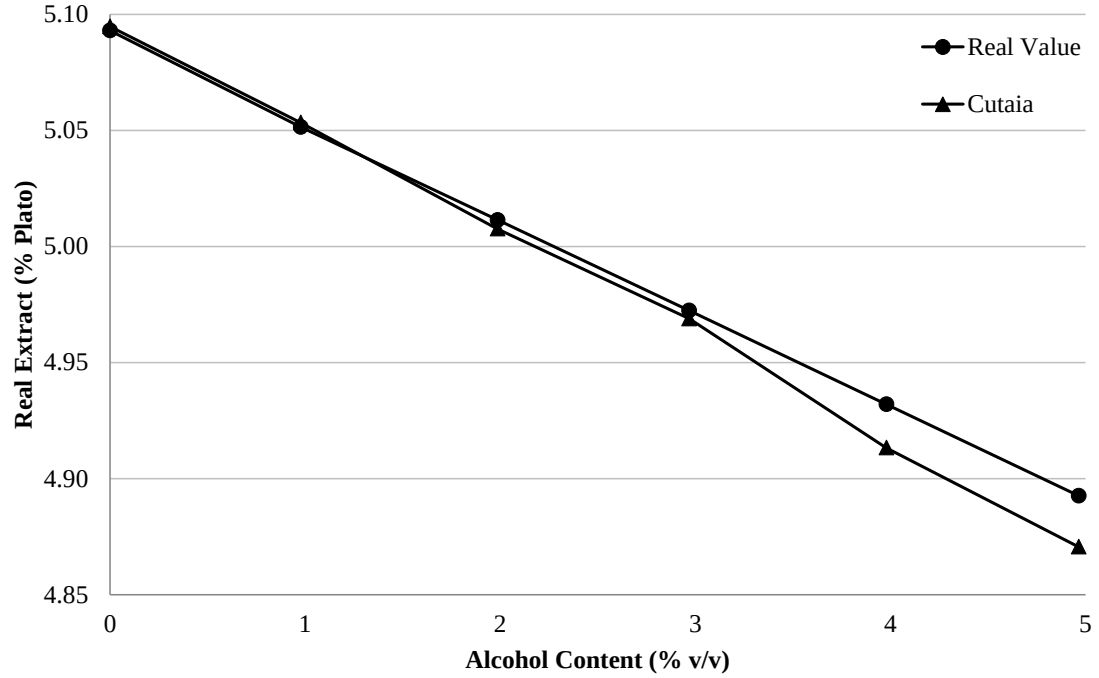

Figure 3. Predicted real extract by Cutaia's equation (2009) in a 5% Plato (real value= 5.0930) model solution and its dilutions to different alcohol contents.

Once the real extract in fermented wort was determined, fermentability was calculated with equation (4) and reported as real degree of fermentation (RDF) according the ASBC method Beer-6B. This equation considers a correction due to the mass lost by CO_2 evolution and yeast uptake during fermentation (Cutaia and Munroe, 1979).

$$RDF\ (\%) = \left[\frac{100*(OE-RE)}{OE}\right]*\left[\frac{1}{1-(0.005161*RE)}\right] \quad (4)$$

Where: RDF= Real degree of fermentation; OE= Original extract (%Plato); RE= Real extract (% Plato).

The reason to using the real degree of fermentation instead of apparent degree of fermentation (ADF; or apparent attenuation limit, AAL) was based on the effect of alcohol content of fermented samples (with 2.47-4.14% alcohol w/w) on calculated ADF values (Figure 4). It was observed that the difference between ADF-RDF increases as alcohol does. ADF and RDF were highly correlated (r=0.96); however, due to the observed alcohol dependence, the use of ADF for selecting (and comparing) breeding materials is questionable.

3.6. Statistical Analysis

Statistical analyses (correlations, PCA) were performed in the SAS software version 9.3 (Cary, NC) at the significant level α=0.05. For the regression analysis, only the means of all variables for each line across locations and years were used. The regression analysis consisted of a forward stepwise regression, with α to enter=0.15, in order to determine the variables that explain most of the fermentability variation and to develop the best model for its prediction (Evans et al., 2005; Schwarz et al., 2007; SAS Institute Inc., 2011).

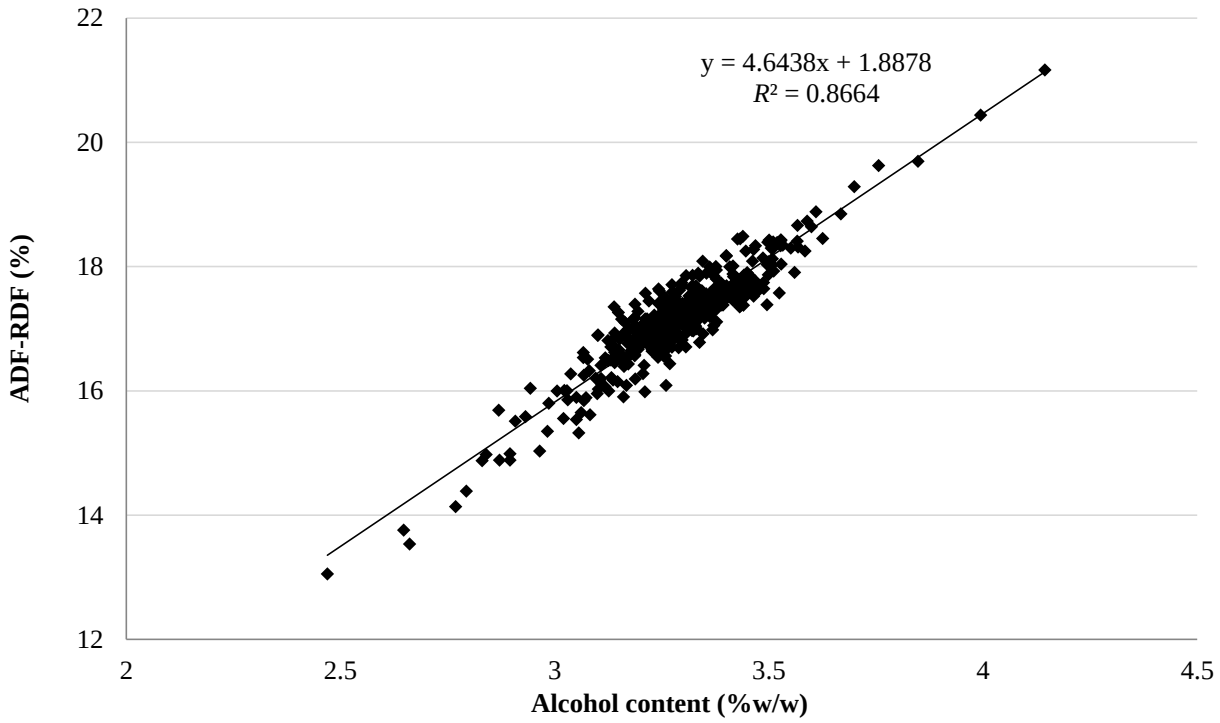

Figure 4. Difference between apparent degree of fermentation (ADF) and real degree of fermentation (RDF) in samples with different alcohol content (n=450).

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Barley and Malt Quality

The evaluation of the different genotypes in several environments showed broad variation in most of the barley and malt quality parameters as indicated by their ranges. However, small standard deviations were also observed for most (Table 5; Appendix A1, A2, A3).

Table 5. Barley and malt quality of two-rowed malting barley genotypes grown at three locations in North Dakota during 2013 and 2014.

Quality parameter[a]	Mean	Std. Dev.	Min	Max
Barley protein (% db)	11.8	0.9	9.6	14.8
Test weight (kg hL^{-1})	65.1	2.0	54.6	69.0
Plump kernels (%)	91.4	8.3	32.9	99.2
Hot water extract (% db)	79.9	1.5	74.8	84.6
Wort viscosity (cP)	1.52	0.06	1.32	1.99
Soluble protein (% db)	5.19	0.5	3.8	7.9
Wort color (°SRM)	2.4	0.7	1.7	9.1
DP (°ASBC, db)	118	20.3	62.9	181.0
Beta glucans in wort (mg L^{-1})	461	154	127	802
Maltotriose in wort (g 100 mL^{-1})	1.01	0.14	0.70	1.88
Maltose in wort (g 100 mL^{-1})	4.54	0.31	3.11	5.19
Glucose in wort (g 100 mL^{-1})	0.82	0.12	0.58	1.25
Fructose in wort (g 100 mL^{-1})	0.11	0.05	0.04	0.87
FAN in wort (mg L^{-1})	200	25.1	112.6	336.6
Kolbach index (%)	44	4.6	33.7	68.1
Friability (%)	72.1	7.5	43.6	88.3
Partly unmodified kernels (%)	9.8	5.6	1.6	31.1
Malt glassy kernels (%)	1.2	1.2	0.0	8.5
Alpha-amylase (U g^{-1} db)	233	31.1	77.9	315.6
Limit dextrinase (U kg^{-1} db)	154	34.8	76.2	254.5
Beta-amylase (U g^{-1} db)	15	3.4	5.3	23.7
Alpha-glucosidase (U kg^{-1} db)	38	12.8	2.9	82.0
Alpha-amylase thermostability (%)[b]	55	13.7	8.3	100.0
Limit dextrinase thermostability (%)[b]	24	6.0	10.4	49.9
Beta-amylase thermostability (%)[b]	3	2.2	0.0	17.5
Alpha-glucosidase thermostability (%)[b]	17	21.0	0.0	97.5
RDF (%)	82.4	2.6	62.31	88.1

[a]db=Dry basis; cP=Centipoise; DP=Diastatic power; ASBC=American Society of Brewing Chemists; FAN=Free amino nitrogen; RDF=Real degree of fermentation; SRM=Standard Reference Method. [b]Thermostabilities of alpha-amylase, beta-amylase, limit dextrinase, and alpha-glucosidase are expressed as the percentage of the total activity remaining after 10 min at 65°C.

4.1.1. Relationship between Quality Parameters

Malt quality is the result of the joint effect of different quality parameters determined in barley and malt. Many of these parameters are related with the endosperm modification of barley during the malting process. Not surprisingly, the final wort composition will be influenced by processing conditions, and genetic makeup and phenotypic variation of barley (Briggs, 2002; Fox et al., 2003). For this reason, and considering that fermentability is mostly affected by wort composition (Paik et al., 1991; Edney et al., 2007), knowledge of the interaction of different malt quality parameters helps to understand critical points when developing a predictive model for different quality traits.

It is well known that there is an inverse relationship between barley protein and malt extract, and a positive relationship between barley protein and DP. Correlations between other parameters associated with endosperm modification (e.g. kolbach index – KI, free amino nitrogen – FAN, Friability) have been reported as well. These interactions have been considered by many authors in order to predict malt quality in single cultivars, global malt quality based on scoring systems, or simply monitoring barley performance during malting (Molina-Cano et al., 1997; Briggs, 1998; Edney et al., 2007; Wentz et al., 2004; Gianinetti et al., 2005; Li et al., 2008). In this study, the most significant relationships observed between common malt quality/endosperm modification parameters were soluble protein (SP) and FAN (r=0.73), SP and KI (r=0.74), wort beta-glucans and viscosity (r=0.57), malt friability and wort beta-glucans (r=-0.60), malt friability and hot water extract (HWE) (r=0.54), and malt friability and barley protein (r=-0.66). The rest of quality parameters were not highly associated (Table 6, Appendix A4). However, the positive or negative correlation coefficients in most of them reflect their expected roles and relationships in malting and mashing (Arends et al., 1995; Briggs, 1998).

Table 6. Correlation coefficients[b] (*r*) of malt quality parameters determined in two-rowed malting barley genotypes grown in North Dakota (2013 and 2014) at three locations.

Trait[a]	BP	DP	HWE	SP	BG	FAN	WV	KI	Fria	PK
WC	-0.27	-0.50	0.26	0.44	-0.36	0.54	ns	0.65	0.15	0.13
BP		0.50	-0.47	0.36	0.33	0.23	0.23	-0.36	-0.66	ns
DP			-0.12	ns	ns	ns	ns	-0.32	-0.16	0.13
HWE				ns	-0.53	0.10	-0.17	0.37	0.54	0.53
SP					-0.26	0.73	ns	0.74	ns	-0.10
BG						-0.38	0.57	-0.50	-0.60	-0.39
FAN							ns	0.57	ns	0.15
WV								-0.18	-0.48	-0.17
KI									0.41	ns
Fria										0.21

[a]WC=Wort color (°SRM); BP=Barley protein (% dry basis, db); DP=Diastatic power (°ASBC); HWE=Hot water extract (% db); SP=Soluble protein (% db); BG=Wort beta glucans (mg L^{-1}); FAN=Free amino nitrogen (mg L^{-1}); WV=Wort viscosity (cP); KI=Kolbach index (%); Fria=Friability (%); PK=Barley plump kernels (% on sieve 6/64 in).
[b]All correlations were significant at $P<0.05$; ns=Nonsignificant.

Although endosperm modification parameters have usually been utilized to monitor the malting performance of barley samples, their role in the final wort composition is recognized. For example, the relationship observed between KI, SP, and FAN, besides reflecting the effect of proteolytic enzymes during malting and mashing, also can help to provide an estimation of FAN when only KI is determined. The relationship between friability, wort beta-glucans, and extract, reflects the characteristic physical changes of barley endosperm during malting as a result of cell walls degradation (i.e. reduced beta-glucans in wort). If adequate endosperm modification is attained, the access of different enzymes to starch and protein during malting and mashing will result in higher extracts (Briggs, 1998; Briggs, 2002). In consequence, this event is very important for brewing purposes considering that about 90% of wort solids are carbohydrates, and 5% are nitrogenous compounds (MacWilliam, 1968; Willaert, 2007). Edney et al. (2007) studied the effect of endosperm modification on wort fermentability, and observed that high wort viscosities, slowed starch release during mashing, increased starch hydrolysis due to better beta-

glucan breakdown, and increased FAN levels, affect this trait. In fact, a model for predicting fermentability (apparent attenuation limit, AAL) reported by Evans et al. (2005) considers KI as an important factor. Later (2010a), they observed that FAN and Friability also play a marginal but important role in predicting AAL, although their effect was not the same in malt sets from different sources.

4.2. Fermentability Prediction

4.2.1. Feasibility of Using a Published Model

In order to determine the applicability of the model developed by Evans et al. (2005) to predict fermentability (expressed as AAL), appropriate calculations were done to compare the results of the published model with the experimental AAL of the corresponding fermented worts; although in the present study, fermentability is expressed as the real degree of fermentation (RDF). As shown in Figure 5, the malt quality parameters considered by the published model are alpha-amylase activity, limit-dextrinase activity, KI, beta-amylase, and beta-amylase thermostability. Considering that enzyme units for beta-amylase included in their model were based on an older beta-amylase assay which used the Betamyl® reagent (*p*-nitrophenyl-α-D-maltopentaoside, PNPG5, plus alpha-glucosidase), and that enzyme units for beta-amylase in this study are based on the Betamyl-3® reagent (*p*-nitrophenyl-β-D-maltotrioside, PNPβ-G3, plus beta-glucosidase), appropriate conversions were done as suggested in the Megazyme method K-BETA3 10/10 (Betamyl-3 method) for beta-amylase assay (Megazyme, Bray, Ireland). On the other hand, AAL was calculated with specific gravities of fermented samples according the EBC method 8.6 (EBC, 1998). The results showed that the model suggested by Evans et al. (2005) was not applicable for the samples used in this experiment considering that it only explained about 6% of the variability in the experimental AAL (Figure 5). One of the main possible

reasons of the observed differences is that they used commercial malt samples for developing the model. A second, and probably minor, reason is that 3 g of dried yeast was used in this experiment instead of 1 g used by Evans and coworkers.

The commercial malts used by Evans et al. (2005) came from six companies from Australia and Japan, and included samples from 43 batches. Malting conditions vary between companies and barley cultivars, which in turn impact malt quality parameters. Moreover, malt blending with malts from different cultivars is a common practice in industry. When comparing commercial malt samples with laboratory malt samples (e.g. in a barley breeding program), many aspects have to be considered. Three large differences between commercial malting and laboratory malting that affect malt quality include the potential use of gibberellic acid (GA_3) in industry, hydrostatic pressure effects in large steep tanks, and malt blending.

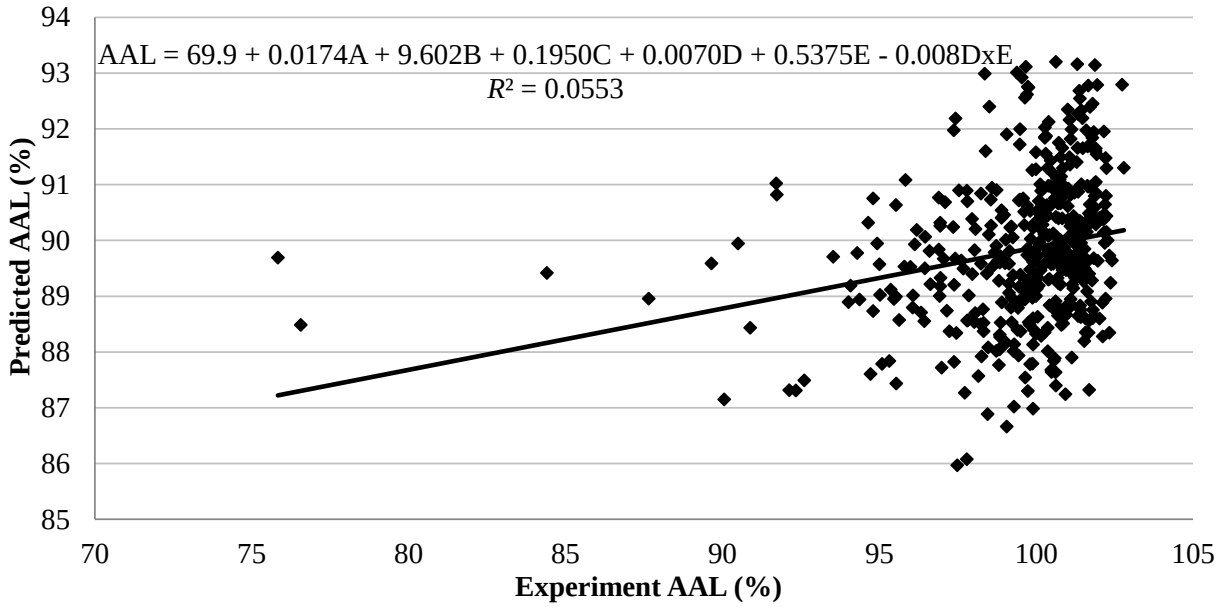

Figure 5. Comparison of the experimental apparent attenuation limits (AAL) determined in 450 malt samples with results calculated using the predictive model published by Evans et al. (2005), which included alpha-amylase (A), limit-dextrinase (B), Kolbach index (C), beta-amylase (D), and beta-amylase thermostability (E).

Exogenous GA_3 is used in many malt-houses when allowed by customer specifications. Small amounts (0.005-0.25 mg kg^{-1} barley) of GA_3 are added at initial barley germination stages (usually after the steeping step) in order to increase the effects of the hormones naturally produced by the germinating seed. The main effects of using GA_3 during malting can be reduced processing times, and increased values for parameters such as extract, friability, KI, and fermentability; with no significant, to sometimes positive, effects on malt losses and wort viscosity (Briggs, 1998; Briggs, 2002; MacLeod, 2004). Considering this, endosperm modification and the pattern of enzymatic synthesis during laboratory malting (with no GA_3 used) would not be the same to that observed in commercial malting; which would result in different malt qualities.

Differences in mechanical and hydraulic pressures observed in commercial malting, especially during steeping, can lead to considerable differences in malt quality. These pressures, contrary to the effects of GA_3, will impact negatively the malting process and, in some cases, can delay germination up to a day. Pressure events can happen when transporting barley in a mixture with water to the steep vessel, or in steeping, depending on the bed depth. For example, a 5 m column of grain is sufficient to cause damage (Briggs, 1998). Currently, in modern malt-houses, steeping and germination are carried out in flat-bottomed vessels with grains beds less than 2 m; however, many old malt-houses still operate under high-pressure conditions. The main effects of pressure in malt quality have been reported as restricted germ growth, abnormal germination, and subsequent reduced and inconsistent wort fermentability. The pressure treatment (level and time) effects on fermentability were different between barley cultivars (Yoshida et al., 1979; Briggs, 1998; Briggs, 2002; MacLeod, 2004). Considering the antagonistic effects of processing pressures and GA_3 addition during malting, it is worth noting that this can reduce differences

between commercial and laboratory malts; however, subtle but significant characteristics could still be present in malt.

In the case of malt blending, Evans (2012) reported that some synergisms can occur when malts with different quality are blended. These effects were observed in quality parameters such as lautering times and fermentability when poor and good quality malts were blended at ratios of 40% and 60%. Lautering performance was linked to the marked differences in beta-glucans and wort viscosity of the blended malts, whereas that fermentability was linked to KI, DP enzymes, and beta-amylase thermostability. Blending was also observed to impact malt extracts, but the effects were not as pronounced.

4.2.2. Developing a Predictive Model

Correlation coefficients between malt enzymes, enzyme thermostability and fermentability (RDF) are shown in Table 7. These results show that, consistent with previous reports, DP is not a strong fermentability predictor (Evans et al., 2003; Evans et al., 2005; Evans et al., 2010a). However, alpha-amylase, beta-amylase, and limit-dextrinase, were positively correlated with RDF. Even though alpha-glucosidase was not significantly associated with RDF, it was correlated with DP and the other three DP enzymes.

Contrary to other reports (Eglinton et al., 1998; Evans et al., 2005; Evans et al., 2010a, Evans et al., 2010b), beta-amylase thermostability was not associated with RDF in the present study. However, it should be considered that most of the previous reports on beta-amylase thermostability used temperatures ≤60°C, based on temperature ranges of different mashing methods that allow an adequate enzymatic window (50-60°C) for beta-amylase. In fact, experiments for the characterization of the barley beta-amylase alleles based on thermostability in malt (*Bmy*l-Sdl, -Sd2L, -Sd2H) were carried out by measuring the remaining activity after

thermal treatments in the range of 55-60°C for different times (Eglinton et al., 1998; Kihara et al., 1998; Kihara et al., 2002; Zhang et al., 2004). In the current study, thermal stabilities of DP enzymes were determined at 65°C, considering the isothermal condition of the mashing method used (i.e. HWE). However, when the HWE method, or other mashing methods with mash-in temperatures of 65°C or higher are used, studying the association between beta-amylase thermostability (determined at low temperatures, e.g. incubated at 57.5°C for 30 min) and the fermentability of these worts could lead to biased results. For example, Eglinton et al. (1998) observed that differences between barley cultivars, in terms of beta-amylase thermostability (at 60°C for 2.5-20 min), are relative considering that residual beta-amylase activity depends on incubation time and its response is not uniform across cultivars. Kihara et al. (1998) reported similar results with the same incubation time, but varying temperatures. They observed that beta-amylase activity, in either low- or high-thermostable genotypes (determined at 57.5°C), was practically nonexistent after 30 min at 62.5°C. Interestingly, the loss of beta-amylase activity was almost parallel between different genotypes in the temperature range 55.0-57.5°C.

Table 7. Correlation coefficients[b] (*r*) between fermentability and malt enzymes determined in barley genotypes grown in North Dakota during 2013 and 2014.

Trait[a]	DP	AA	BA	LD	AG	AAT	BAT	LDT	AGT
RDF	0.24	0.41	0.20	0.31	ns	0.12	ns	-0.41	-0.10
DP		0.11	0.88	ns	0.17	0.22	-0.37	ns	0.20
AA			0.16	0.47	0.23	0.22	0.11	-0.33	ns
BA				-0.15	0.16	0.19	-0.35	0.11	0.17
LD					0.19	0.11	0.23	-0.82	0.19
AG						ns	ns	-0.10	0.37
AAT							-0.11	ns	-0.14
BAT								ns	ns
LDT									-0.14

[a]RDF=Real degree of fermentation; DP=Diastatic power (°ASBC); AA=Alpha-amylase (U g^{-1}); BA=Beta-amylase (U g^{-1}); LD=Limit-dextrinase (U kg^{-1}); AG=Alpha-glucosidase (U kg^{-1}); AAT=Alpha-glucosidase thermostability (%); BAT=Beta-amylase thermostability (%); LDT=Limit-dextrinase thermostability (%); AGT=Alpha-glucosidase thermostability (%). [b]All correlations were significant at $P<0.05$; ns=Nonsignificant.

One of the major challenges when developing predictive models for quality traits in malting barley breeding lines is that the variability of different parameters change across growing locations or years, or due to interactions such as genotype x location, genotype x year, or location x year (Molina-Cano et al., 1997; Ullrich et al., 1997; Molina-Cano et al., 2001; Molina-Cano et al., 2002; Zhao and Xu, 2012). In this study, for example, the observed variation of fermentability for different crop years and locations was explained by different parameters (Table 8). The parameters alpha-amylase, limit dextrinase, and some fermentable sugars (glucose, maltose) were consistent across locations but with different R^2 values (Appendix A5). One of the main possible reasons of the differences observed between 2013 and 2014, in terms of cumulative R^2s for fermentability prediction, could have been associated with the adverse environmental conditions presented at Fargo in 2013, which resulted in broad variation in plump kernels (32-93%) and extract values as low as 74.8%; although DP variation was similar across locations and years (Appendix A1, A2, A3). Due to this situation, and considering that one of the main targets of malting barley breeding is to obtain stable genotypes in different locations, the mean values for genotypes were used across locations and years for the regression analysis.

Table 8. Results of the stepwise regression, by year, for the prediction of fermentability in malted samples of barley genotypes grown in North Dakota.

2013			2014		
Parameter[a]	Cum. R^2	*Pr > F*	Parameter	Cum. R^2	*Pr > F*
Maltose (g 100 mL^{-1})	0.0613	0.0008	WC (°SRM)	0.4813	<0.0001
AA (U g^{-1})	0.1184	0.0009	LD (U kg^{-1})	0.5957	<0.0001
Glucose (g 100 mL^{-1})	0.1942	<0.0001	SP (%)	0.6682	<0.0001
Friability (%)	0.2408	0.0013	WV (cP)	0.6993	<0.0001
LD (U kg^{-1})	0.2518	0.1135	AA (U g^{-1})	0.7280	<0.0001
PUG (%)	0.2686	0.0482	Maltotriose (g 100 mL^{-1})	0.7343	0.0132
WV (cP)	0.2817	0.0791	Glucose (g 100 mL^{-1})	0.7390	0.0310
WC (°SRM)	0.2913	0.1312	BG (mg L^{-1})	0.7414	0.1191
BAT (%)	0.3001	0.1456			

[a]AA=Alpha-amylase; LD=Limit-dextrinase; PUG=Partly unmodified grains; WC=Wort color, SP=Soluble protein; WV=Wort viscosity; BAT=Beta-amylase thermostability; SP=Soluble protein.

Results shown in Table 9 suggest wort color, KI, and plump kernels as the main parameters explaining more than 60% of the fermentability variation. With the inclusion of other parameters, the model R^2 was 0.76. However, wort color is not a parameter that likely can explain fermentability itself. Rather it is likely a correlative parameters as it is affected by carbohydrates, nitrogenous compounds, and the Maillard reaction during mashing and boiling (MacWilliam, 1968; Wainwright and Buckee, 1977; Schwarz and Li, 2011; He et al., 2014). Besides the known differences in wort color changes due to different operations in the brewing process, wort color is also affected by external factors such as light exposure, or aging (Bremner, 1963; Wainwright and Buckee, 1977; Kunze, 2014). If wort color were included in the model as suggested by this regression analysis, fermentability prediction would be limited to worts with color values similar to this experiment (2.44±0.67 °SRM) considering its instability to different factors. Thus, wort color is not a reliable parameter for quality prediction purposes.

Table 9. Results of the stepwise regression for the prediction of fermentability in malted samples of barley genotypes grown in North Dakota during 2013 and 2014.

Parameter[a]	Partial R^2	Cum. R^2	$Pr > F$
WC (°SRM)	0.3855	0.3855	<0.0001
KI (%)	0.1940	0.5794	<0.0001
PK (%)	0.0484	0.6279	<0.0001
AA (U g^{-1})	0.0302	0.6581	0.0001
FAN (mg L^{-1})	0.0322	0.6903	<0.0001
LDT (%)	0.0196	0.7098	0.0008
Maltotriose (g 100 mL^{-1})	0.0199	0.7297	0.0005
AG (U kg^{-1})	0.0095	0.7392	0.0135
AGT (%)	0.0078	0.7470	0.0235
Fructose (g 100 mL^{-1})	0.0039	0.7509	0.1037
Maltose (g 100 mL^{-1})	0.0040	0.7550	0.0982
Glucose (g 100 mL^{-1})	0.0039	0.7589	0.1010

[a]WC=Wort color, KI=Kolbach index; PK=Plump kernels, AA=Alpha-amylase; FAN=Free amino nitrogen; LDT=Limit-dextrinase thermostability; AG=Alpha-glucosidase, AGT=Alpha-glucosidase thermostability.

In order to determine if wort color was directly associated with fermentability, or whether some other parameters were involved, data were subjected to principal component analysis (PCA). As observed with the interaction of the main three components, it was difficult to define clusters due to de dispersion of quality parameters, which also reflects the complex behavior of malt quality in barley breeding. However, results of PCA clearly suggest that wort color is not directly associated with fermentability (Figure 6). Wort color is linked to protein degradation parameters, such as SP, FAN, and KI, as well as to, the extract-related parameter Friability. It was also related to some fermentable sugars. Fermentability, on the other hand, was closer to some of the DP associated enzymes and maltose, than to color. For this reason, it was decided to remove wort color from the regression analysis.

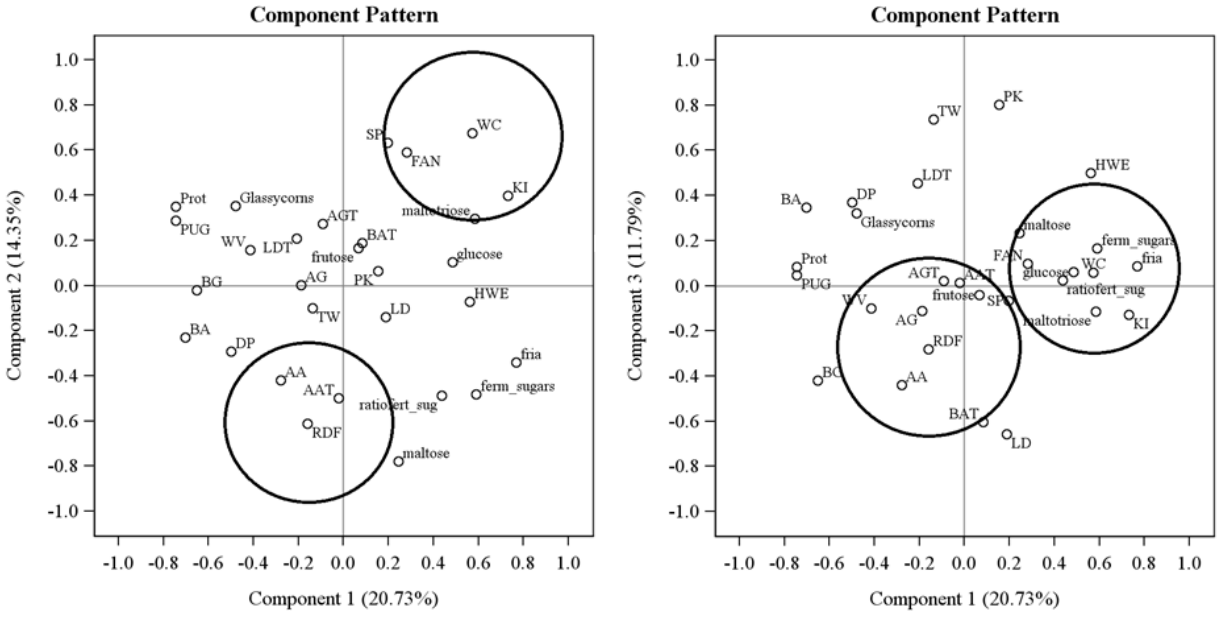

Figure 6. Dispersion of malt quality parameters and their relationship with fermentability (RDF) and wort color (WC) in the first three components of a PCA with data from 90 barley genotypes evaluated in five different environments.

Regression analysis, with wort color excluded, suggested that glucose, maltose, and limit-dextrinase could explain almost 60% of the variability observed in fermentability. This represents no considerable reduction in the predictive power observed when wort color was included (Appendix A6). In the suggested equation, glucose had a negative coefficient while maltose was positive. This suggests that the role of fermentable sugars in wort is not necessarily additive for explaining fermentability. For example, it was observed that the ratio glucose/maltose in the sample with the highest fermentability (RDF=88.08) was 0.15, while it was 0.28 in the sample with the lowest fermentability (RDF=62.31). Also, maltose content in the first sample (4.92 g 100 mL^{-1}) was higher than the one observed in second sample (3.11 g 100 mL^{-1}). The remainder of parameters suggested in this model were plump kernels, FAN, soluble protein, alpha-amylase, alpha-glucosidase, and limit-dextrinase thermostability.

The importance of each parameter (excluding glucose, maltose, and limit-dextrinase) in terms of explaining the variability in fermentability, need to be explained in terms of both the practicability of their evaluation during barley breeding, and their real effect in the final wort composition. For example, limit-dextrinase activity is likely more important than its thermostability during mashing, when one considers that the results of Stenholm and Home (1999) suggested that the enzyme will be more than 50% active after 1 hr at 65°C. However, in the extracted form, in which thermostability is commonly assayed, there is no activity after 20 min at the same temperature. The protective effect of mash thickness has also been observed with other DP enzymes such as alpha-amylase and beta-amylase (Muller, 1991). This could be one of the reasons for the very low partial R^2 value (0.0062) for the contribution of limit-dextrinase thermostability to fermentability under the mashing conditions of this study (1 hr, 65°C) even though its relationship with total activity was high (Table 7). In addition to these

observations, Evans et al. (2010a) reported that limit-dextrinase thermostability is not important in predicting fermentability after evaluating different malt sets including commercial malts and breeding lines. However, in that study they fermented worts from Congress Mashes. This was probably one of the reasons that FAN, KI, and beta-amylase and its thermostability, together explained about 70% of the variation in fermentability. However, KI (27-49%) explained 65% of the variation in a malt set where FAN was not significant, while FAN (71-202 mg L^{-1}) explained 43% in a malt set where KI was not significant.

The evaluation of plump kernels in barley breeding is used to predict, to some degree, the potential extract yield of barley genotypes. The parameter plump kernels was significantly correlated with malt extract in the current study (Table 6). Nevertheless, in terms of fermentability, this parameter did not contribute much to the observed variation (R^2=0.0411). One of the reasons that plump kernels may have had some importance in explaining fermentability was because barley samples were cleaned on the sieve 5/64 x ¾ in (0.1984 x 1.905 cm). Barley yield and plumpness was poor at some locations, and in order to have adequate seed, it was thus necessary to use grain that remained on top of the 5/64 sieve rather than the 6/64 sieve (0.2381 x 1.905 cm).

As a consequence, kernel plumpness was excluded from the regression analysis, as were limit-dextrinase thermostability and alpha-glucosidase. Alpha-glucosidase had a very low effect on fermentability (R^2=0.012). It was not included in the regression analysis as its thermal stability is similar to beta-amylase and its role on the final concentration of fermentable sugars in wort is still under discussion. As it was expected (Appendix A6, Table 10), the effect of removing these three parameters from the regression analysis was minimal according to the R^2 values (from 0.74 to 0.71).

The final regression analysis led to a new model (equation 5), which includes the previously mentioned parameters plus maltotriose (Table 10). This result indicates that fermentability is a trait dependent of two main factors: fermentable sugars and wort nitrogen. Fermentable sugars are related to the joint action of different DP enzymes during mashing, and wort nitrogen to protein degradation parameters. The balance of these factors will lead to high fermentabilities when appropriate amounts of the involved parameters are present (Table 11).

$$RDF(\%) = 65.71054 - (6.32812 * G) + (3.34969 * M) + (0.02333 * LD) - (0.05355 * FAN) + (1.55135 * SP) + (0.01601 * AA) + (2.07022 * MT) \quad (5)$$
$$R^2 = 0.71$$

Where: RDF=Real degree of fermentation (%); G=Glucose (g 100 mL^{-1}); M=Maltose (g 100 mL^{-1}); LD=Limit-dextrinase (U kg^{-1}); FAN=Free amino nitrogen (mg L^{-1}); SP=Soluble protein (%); AA=Alpha-amylase (U g^{-1}); MT=Maltotriose (g 100 mL^{-1}).

Table 10. Results of the stepwise regression (WC, PK, AG, and LDT out) for the prediction of fermentability in malted samples of barley genotypes grown in North Dakota during 2013 and 2014.

Parameter[a]	Partial R^2	Cum. R^2	$Pr > F$
Glucose (g 100 mL^{-1})	0.2867	0.2867	<0.0001
Maltose (g 100 mL^{-1})	0.2434	0.5301	<0.0001
LD (U kg^{-1})	0.0686	0.5987	<0.0001
FAN (mg L^{-1})	0.0351	0.6338	<0.0001
SP (%)	0.0531	0.6869	<0.0001
AA (U g^{-1})	0.0208	0.7077	0.0006
Maltotriose (g 100 mL^{-1})	0.0051	0.7129	0.0815

[a]WC=Wort color; LD=Limit-dextrinase; PK=Plump kernels, AA=Alpha-amylase; FAN=Free amino nitrogen; LDT=Limit-dextrinase thermostability; AG=Alpha-glucosidase; SP=Soluble protein.

Table 11. Characteristics of some parameters[a] determined in malt of nine two-rowed barley genotypes with high, intermediate, and low fermentability (RDF).

RDF	HWE	DP	AA	LD	BA	BAT	M	G	WC	SP	FAN
88.08	82.94	135	253	174	17.30	2.21	4.92	0.76	2.09	5.47	191
87.40	81.93	139	313	171	18.98	1.72	4.80	0.65	2.00	5.18	199
87.26	79.78	120	216	221	13.16	3.85	4.61	0.70	2.36	5.83	217
79.21	80.31	119	207	109	15.73	4.63	4.25	0.69	1.91	4.88	179
79.12	76.72	119	245	125	18.91	3.66	3.88	0.73	2.06	5.02	199
79.30	80.64	127	227	112	16.86	1.35	4.64	0.94	2.12	5.14	210
62.31	82.57	63	85	84	5.27	7.12	3.11	0.86	9.14	7.55	337
63.52	79.44	67	78	86	7.73	4.60	3.25	1.07	6.09	7.91	321
70.03	81.27	69	116	113	6.89	8.80	3.71	1.12	6.16	6.66	289

[a]RDF=Real degree of fermentation (%); HWE=Hot water extract (%); DP=Diastatic power (°ASBC db); AA=Alpha-amylase (U g^{-1}); LD=Limit-dextrinase (U kg^{-1}); BA=Beta-amylase (U g^{-1}); BAT=Beta-amylase thermostability (% remaining after 10 min at 65°C); M=Maltose (g 100 mL^{-1}); G=Glucose (g 100 mL^{-1}); WC=Wort color (°SRM); SP=Soluble protein (%); FAN=Free amino nitrogen (mg L^{-1}).

4.2.3. Discussion of Factors Involved in Fermentability Prediction

4.2.3.1. Endosperm modification

The importance of different parameters associated to the prediction of fermentability has been discussed by many authors (Paik et al., 1991; Briggs, 2002; Gunkel et al., 2002; Evans et al., 2003; Evans et al., 2005; Edney et al., 2007; Evans et al., 2010a). Besides the genetic makeup that differentiates barley genotypes, all reports agree in some way that endosperm modification plays a determinant role in the fermentation potential of a given malt sample. This is in consideration of the fact that all starch-degrading enzymes as well as different proteinases will be either released or synthesized in the germinating seed. However, most of the dry matter profile (ca. 90%) in wort will depend exclusively on the action of the DP enzymes. For example, the model developed by Evans et al. (2005) included alpha-amylase, beta-amylase and its thermostability, limit dextrinase, and KI, as the main malt quality parameters that explained fermentability (R^2=0.91) in worts from commercial malts, and malt/rice-adjunct mashes (R^2=0.82). Their models were intended to improve the estimates of fermentability that was based

on DP. DP alone was able to explain a large portion (e.g. 54%) of the variability in fermentability variation, although this value varies considerable depending on malt sets (Evans et al., 2010a). In the current study, DP explained only 6% of the variation in fermentability and, unexpectedly, beta-amylase and its thermostability were not important in explaining this trait. However, when observing extreme differences in fermentability values of different barley genotypes (Table 11), it is clear that DP and beta-amylase have to be considered when discriminating barley breeding lines with low fermentability. The possible reasons of differences observed between the model of Evans et al. (2005) and the results of this study were discussed in a previous section.

On the other hand, the results of the current study agree with those of Evans and coworkers (2005, 2011) in that alpha-amylase, limit-dextrinase, and one or more protein-degradation parameters (KI, FAN, SP) are useful for fermentability prediction. Again, this supports the importance of barley endosperm modification during malting. Protein-degradation parameters are associated with the amount of nitrogenous compounds present in wort (amino acids, di-peptides, tri-peptides, ammonium ions), which are utilized by yeast during fermentation (Leekas et al., 2009). When adequate amounts of FAN are present in fermenting wort, yeast growth will be consistent and fermentation will be efficient. However, it is important to consider that once the minimum FAN requirement is accomplished (150-200 mg L^{-1}), increasing its concentration will not considerably improve fermentation conditions (Paik et al., 1991). In this study, FAN values ranged from 113 to 337 mg L^{-1} (mean=201 mg L^{-1}), which may help, in part, explain the small negative coefficient for FAN (equation 4). For example, wort from barley genotypes with high and intermediate fermentability (Table 11) had on average 200 mg L^{-1} of FAN, while genotypes with extremely low fermentability had >300 mg L^{-1}. These findings agree

with the fact that breeding barley for FAN targets a narrow range (150-200 mg L^{-1}) instead of increased amounts. On the other hand, even though FAN is highly correlated (r=0.73) with SP, the latter had a positive coefficient in the developed equation. However, similar to that observed for FAN, fermentability decreased as SP increased. This behavior was particularly observed in samples with low fermentability (RDF<78%), and high FAN and SP values. Samples with intermediate and high fermentability were more stable to FAN and SP changes, especially when these parameters were in the ranges of 150-250 mg L^{-1} and 4-5.5%, respectively; which included 95% of the samples. Nevertheless, when wort has low concentrations of nitrogenous compounds, a different trend can be expected. For example, Evans et al. (2010a) observed that FAN explained 43% of the variability in the fermentability of a group of Australian malts. Interestingly, FAN values ranged from 71 to 202 mg L^{-1}, with mean of 128 mg L^{-1}. Thus, it is very likely that the low amount of nitrogenous compounds in those worts was one of the reasons of the fermentability dependence on FAN. For example, Paik et al. (1991) reported that 200 mg L^{-1} of FAN is close to the optimum for fermenting a 12% Plato wort, which is consistent with the FAN values (140-190 mg L^{-1}) specified by the American Malting Barley Association for breeding two-rowed barley (AMBA, 2014).

4.2.3.2. Mashing conditions

Temperature, as well as mash thickness, will affect the activity of different enzymes during mashing, when one considers that DP enzymes have different thermal stabilities (Muller, 1991; Stenholm and Home, 1997). Alpha-glucosidase and beta-amylase will be rapidly inactivated at temperatures above 60°C compared to alpha-amylase and limit-dextrinase, which are stable even at higher temperatures. This is one of the most important parameters to consider when evaluating fermentability in different malt samples. The HWE and Congress Mash

methods are the most common procedures for the laboratory evaluation of malt. The HWE involves isothermal extraction at 65°C (1 hr), while mash-in for the Congress method is at 45°C (30 min), after which temperature is ramped to 70°C. The Congress mash involves 50 min at temperatures below 65°C, and 65 min at higher temperatures. Evans et al. (2011) for example, determined that mash-in temperatures of 65°C increased (7%) wort fermentability compared with the Congress Mash (45°C mash-in). However, they observed slight reductions in extract, FAN, and SP with the 65°C mash. One of the main reasons of higher fermentabilities at high mash-in temperatures is the increase of maltose content in wort and the slight concomitant reduction of glucose compared with worts from the Congress Mash. Some researchers have attributed this event to the thermal stability of beta-amylase, which would be lower at the 70°C rest of the Congress Mash (Evans et al., 2005; Schwarz et al., 2007). In the current study, maltose in wort (4.54 g 100 mL^{-1}) was similar to the values reported by Evans et al. (2005), 4.72 g 100 mL^{-1}, and Schwarz et al. (2007), 4.76 g 100 mL^{-1}, at the same mash-in temperatures (65°C) but with mashing schedules slightly different from the HWE. On the other hand, when the Congress Mash is used, up to 30% less maltose in wort can be observed (Schwarz et al., 2007). Evans et al. (2005) observed that AAL steadily increased with mash-in temperatures ranging from 45 to 65°C, but after 65°C AAL rapidly decreased. The same trend was observed with maltose, which rapidly decreased after 65°C. Thus, they suggested that the best mash-in temperature for determining the maximum potential of malt fermentability is 65°C. The reason for increased maltose content in worts obtained by mashing protocols with mash-in temperatures up to 65°C is still unclear. However, considering that alpha-amylase and limit dextrinase activities will increase the amount of oligosaccharides under these conditions, the presence of beta-amylase (even with low activity remaining) could at least partially explain these results. In

relation to the protective effects of wort gravities on the activity of DP enzymes (Muller, 1991; Stenholm and Home, 1999), Duke and Henson (2016), and Henson and Duke (2016) reported that the concentration of maltose can also be a factor for stabilizing enzyme activities at high isothermal mashing temperature, which is more significant for beta-amylase. Although beta-amylase total activity is reduced at higher temperatures, the rate of reaction (catalytic cycles/unit time/enzyme molecule) will be much higher, especially if a significant Q_{10} is observed. The temperature coefficient (Q_{10}) (Peterson et al., 2007; Daniel and Danson, 2013) for enzymatic reactions generally falls between 2-3, which indicates reactions will double or triple, with a 10°C increase in temperature. As temperature continues to increase, beta-amylase activity will rapidly decrease until all enzyme has been completely inactivated. The effect of temperature on enzyme activity typically appears as a bell shape curve, and reflects the effects of increased reaction rate and enzyme denaturation. A similar bell shaped trend has been observed with AAL and maltose at different mash-in temperatures (Muller, 1991; Wolfenden et al., 1999; Evans et al., 2005; Henson et al., 2014). Although Evans et al. (2005) did not include maltose in the model for predicting fermentability (apparently not considered for the regression analysis), the role of maltose, and beta-amylase, seems to be determinant in explaining fermentability at different mash-in temperatures. In the current study, beta-amylase (in solution) retained <5% of its activity at 65°C. However, considering the protective role of mash thickness, it can be assumed that beta-amylase contributes to the maltose content in wort under the HWE conditions, resulting in increased total fermentable sugars as observed by Schwarz et al. (2007). This is consistent with the amounts of maltose and the glucose/maltose ratios observed in malts with extremely low fermentabiliy (Table 11).

CHAPTER 5. CONCLUSIONS

The effect of different malt quality parameters on wort fermentability was determined in this study. It was observed that some starch- as well as some protein-degradation related parameters together explain most of the variation observed for fermentability. For developing a model, all possible interactions of these parameters as well as their real role on the final wort composition were considered. As a result, the resultant stepwise regression model suggested that glucose, maltose, limit-dextrinase, free amino nitrogen, soluble protein, alpha-amylase, and maltotrisose, are the most important malt quality parameters. Together these explained 71% of the variation of fermentability observed in the population. Considering the result, it is clear that while highly-predictive models are still needed, the broad, and usual, environmental variability of malt quality during barley breeding presents a huge challenge for the development of a universal model.

On the other hand, and contrary to previous reports, beta-amylase and its thermostability were not important in terms of the fermentability model. However, the high maltose content observed in worts from the Hot Water Extract, compared with some reports on worts obtained from the Congress Mash, suggests that beta-amylase activity be still considered for future experiments. The observed differences in maltose content between high- and low-fermentability malts support the need of determining the real role of beta-amylase at high temperatures. Studies on its catalytic activity at different temperatures (e.g. K_{cat}, K_{inact}, Q_{10}) and mash thicknesses, either in the presence or absence of other DP enzymes, would lead to unraveling the origin of high maltose content in worts obtained with higher mash-in temperatures.

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APPENDIX

Table A1. Barley and malt quality of 90 malting barley genotypes grown at three locations during two years in North Dakota.

	2013			2014		
Quality parameter[a]	Mean	Min	Max	Mean	Min	Max
Barley protein (% db)	11.83	10.00	14.40	11.79	9.60	14.80
Test weight (kg hL^{-1})	64.59	54.60	69.00	65.48	61.50	68.50
Plump kernels (%)	86.72	32.90	99.20	94.43	82.30	98.60
Hot water extract (% db)	79.91	74.79	84.23	79.94	76.71	84.57
Wort viscosity (cP)	1.54	1.32	1.99	1.51	1.42	1.73
Soluble protein (% db)	5.42	4.54	6.86	5.03	3.79	7.91
Wort color (°SRM)	2.29	1.72	3.54	2.53	1.68	9.14
DP (°ASBC, db)	118.72	82.30	181.00	117.56	62.90	117.50
Beta glucans in wort (mg L^{-1})	488.32	127.00	802.00	442.44	131.00	747.90
Maltotriose in wort (g 100 mL^{-1})	1.06	0.85	1.34	0.98	0.70	1.88
Maltose in wort (g 100 mL^{-1})	4.59	4.06	5.16	4.51	3.11	5.19
Glucose in wort (g 100 mL^{-1})	0.76	0.58	1.05	0.87	0.62	1.25
Fructose in wort (g 100 mL^{-1})	0.09	0.04	0.26	0.13	0.04	0.87
FAN in wort (mg L^{-1})	195.16	117.00	253.00	204.09	112.59	336.60
Kolbach index (%)	45.92	37.60	56.80	42.82	33.67	68.12
Friability (%)	72.10	50.60	88.28	72.13	43.62	84.88
Partly unmodified kernels (%)	9.98	1.72	29.00	9.75	1.62	31.10
Malt glassy kernels (%)	1.27	0.00	5.88	1.21	0.10	8.48
Alpha-amylase (U g^{-1} db)	230.10	161.92	315.59	234.23	77.85	290.44
Limit dextrinase (U kg^{-1} db)	157.80	77.33	242.78	151.61	76.15	254.50
Beta-amylase (U g^{-1} db)	15.29	8.61	22.78	15.38	5.27	23.72
Alpha-glucosidase (U kg^{-1} db)	33.76	2.93	66.01	41.61	15.25	82.04
Alpha-amylase thermostability (%)[b]	53.27	8.25	100.00	56.07	19.49	77.28
Limit dextrinase thermostability (%)[b]	22.46	10.41	41.10	24.73	14.57	49.91
Beta-amylase thermostability (%)[b]	3.83	0.08	11.10	3.06	0.00	17.46
Alpha-glucosidase thermostability (%)[b]	14.25	0.00	93.08	19.52	0.00	97.54
RDF (%)	84.03	80.53	88.08	81.32	62.31	85.69

[a]db=Dry basis; cP=Centipoise; DP=Diastatic power; ASBC=American Society of Brewing Chemists; FAN=Free amino nitrogen; RDF=Real degree of fermentation; SRM=Standard Reference Method. [b]Thermostabilities of alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase are expressed as the percentage of the total activity remaining after 10 min at 65°C.

Table A2. Barley and malt quality of 90 malting barley genotypes grown in 2013 at two locations in North Dakota.

	Fargo			Nesson Valley		
Quality parameter[a]	Mean	Min	Max	Mean	Min	Max
Barley protein (% db)	11.62	10.00	13.30	12.03	10.50	14.40
Test weight (kg hL^{-1})	62.98	54.60	67.90	66.20	62.70	69.00
Plump kernels (%)	78.41	32.90	93.10	95.03	86.70	99.20
Hot water extract (% db)	78.72	74.79	82.54	81.11	78.84	84.23
Wort viscosity (cP)	1.55	1.44	1.99	1.53	1.32	1.77
Soluble protein (% db)	5.36	4.56	6.23	5.49	4.54	6.86
Wort color (°SRM)	2.32	1.80	3.54	2.27	1.72	3.28
DP (°ASBC, db)	107.06	82.30	150.00	130.39	95.60	181.00
Beta glucans in wort (mg L^{-1})	574.53	162.00	802.00	402.11	127.00	715.00
Maltotriose in wort (g 100 mL^{-1})	1.08	0.90	1.34	1.05	0.85	1.31
Maltose in wort (g 100 mL^{-1})	4.55	4.06	5.16	4.63	4.20	5.11
Glucose in wort (g 100 mL^{-1})	0.78	0.62	1.05	0.73	0.58	0.92
Fructose in wort (g 100 mL^{-1})	0.10	0.05	0.18	0.08	0.04	0.26
FAN in wort (mg L^{-1})	193.36	117.00	235.00	196.97	160.00	253.00
Kolbach index (%)	46.22	38.60	56.80	45.61	37.60	55.10
Friability (%)	70.81	51.50	88.28	73.38	50.60	84.44
Partly unmodified kernels (%)	9.83	1.72	28.00	10.12	3.14	29.00
Malt glassy kernels (%)	0.67	0.00	4.40	1.87	0.22	5.88
Alpha-amylase (U g^{-1} db)	249.53	183.49	315.59	210.68	161.92	313.24
Limit-dextrinase (U kg^{-1} db)	185.23	90.70	242.78	130.37	77.33	209.96
Beta-amylase (U g^{-1} db)	13.56	8.61	19.65	17.03	11.57	22.78
Alpha-glucosidase (U kg^{-1} db)	38.48	22.09	66.01	29.04	2.93	65.98
Alpha-amylase thermostability (%)[b]	55.24	26.65	76.80	51.31	8.25	100.00
Limit-dextrinase thermostability (%)[b]	20.76	14.75	41.10	24.16	10.41	39.82
Beta-amylase thermostability (%)[b]	5.46	0.58	11.10	2.18	0.08	5.26
Alpha-glucosidase thermostability (%)[b]	16.61	0.00	71.50	11.89	0.00	93.08
RDF (%)	84.04	81.63	87.26	84.02	80.53	88.08

[a]db=Dry basis; cP=Centipoise; DP=Diastatic power; ASBC=American Society of Brewing Chemists; FAN=Free amino nitrogen; RDF=Real degree of fermentation; SRM=Standard Reference Method. [b]Thermostabilities of alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase are expressed as the percentage of the total activity remaining after 10 min at 65°C.

Table A3. Barley and malt quality of 90 malting barley genotypes grown in 2014 at three locations in North Dakota.

	Fargo		Nesson Valley		McVille	
Quality parameter[a]	Mean	SD	Mean	SD	Mean	SD
Barley protein (% db)	12.45	0.90	12.01	0.74	10.89	0.61
Test weight (kg hL^{-1})	65.36	1.14	66.32	0.93	64.77	1.28
Plump kernels (%)	93.34	2.78	94.68	2.62	95.28	1.81
Hot water extract (% db)	79.19	1.25	79.52	0.92	81.11	0.98
Wort viscosity (cP)	1.50	0.04	1.53	0.05	1.49	0.04
Soluble protein (% db)	5.05	0.51	5.01	0.44	5.03	0.70
Wort color (°SRM)	2.23	0.32	2.14	0.20	3.23	1.07
DP (°ASBC, db)	119.64	18.65	132.17	17.12	100.86	14.38
Beta glucans in wort (mg L^{-1})	530.33	116.54	445.87	135.87	351.11	118.32
Maltotriose in wort (g 100 mL^{-1})	0.98	0.13	0.87	0.10	1.10	0.14
Maltose in wort (g 100 mL^{-1})	4.22	0.24	4.78	0.21	4.52	0.35
Glucose in wort (g 100 mL^{-1})	0.84	0.12	0.85	0.10	0.91	0.11
Fructose in wort (g 100 mL^{-1})	0.14	0.09	0.12	0.05	0.13	0.04
FAN in wort (mg L^{-1})	198.68	24.81	208.09	21.98	205.50	34.42
Kolbach index (%)	40.59	3.20	41.69	3.38	46.18	5.51
Friability (%)	69.72	7.28	71.29	7.52	75.37	5.78
Partly unmodified kernels (%)	11.94	5.58	9.85	5.77	7.47	4.31
Malt glassy kernels (%)	1.55	1.73	1.08	0.91	1.00	0.80
Alpha-amylase (U g^{-1} db)	242.02	24.41	240.82	25.31	219.84	35.71
Limit dextrinase (U kg^{-1} db)	155.05	32.87	149.78	28.66	149.99	24.86
Beta-amylase (U g^{-1} db)	16.14	3.08	17.85	2.90	12.14	2.38
Alpha-glucosidase (U kg^{-1} db)	43.48	12.17	47.30	12.03	34.06	11.12
Alpha-amylase thermostability (%)[b]	46.62	8.77	64.96	7.92	56.64	11.11
Limit dextrinase thermostability (%)[b]	24.79	6.34	24.65	5.31	24.76	5.57
Beta-amylase thermostability (%)[b]	4.74	2.64	1.67	0.53	2.76	1.29
Alpha-glucosidase thermostability (%)[b]	22.28	23.27	22.01	18.10	14.28	21.01
RDF (%)	81.98	1.48	81.61	1.87	80.35	3.83

[a]db=Dry basis; cP=Centipoise; DP=Diastatic power; ASBC=American Society of Brewing Chemists; FAN=Free amino nitrogen; RDF=Real degree of fermentation; SD=Standard deviation; SRM=Standard Reference Method. [b]Thermostabilities of alpha-amylase, beta-amylase, limit-dextrinase, and alpha-glucosidase are expressed as the percentage of the total activity remaining after 10 min at 65°C.

Table A4. Correlation coefficients[b] (*r*) of fermentability and malt quality parameters determined in two-rowed malting barley genotypes grown in North Dakota at three locations during 2013 and 2014.

Trait[a]	BP	DP	HWE	SP	BG	FAN	WV	KI	WC	PK
RDF	ns	0.24	ns	-0.16	0.13	-0.40	ns	-0.16	-0.64	-0.29
BP		0.50	-0.47	0.36	0.33	0.23	0.23	-0.36	-0.27	ns
DP			-0.12	ns	ns	ns	ns	-0.32	-0.50	0.13
HWE				ns	-0.53	0.10	-0.17	0.37	0.26	0.53
SP					-0.26	0.73	ns	0.74	0.44	-0.10
BG						-0.38	0.57	-0.50	-0.36	-0.39
FAN							ns	0.57	0.54	0.15
WV								-0.18	ns	-0.17
KI									0.65	ns
WC										0.13

[a]RDF=Real degree of fermentation (%); BP=Barley protein (% dry basis, db); DP=Diastatic power (°ASBC); HWE=Hot water extract (% db); SP=Soluble protein (% db); BG=Wort beta-glucans (mg L^{-1}); FAN=Free amino nitrogen (mg L^{-1}); WV=Wort viscosity (cP); KI=Kolbach index (%); WC=Wort color (°SRM); PK=Barley plump kernels (% on sieve 6/64 in). [b]All correlations were significant at $P<0.05$; ns=Nonsignificant.

Table A5. Results of the stepwise regression for the prediction of fermentability in malted samples of two-rowed barley genotypes grown in two different environments of North Dakota.

Nesson Valley 2013			McVille 2014		
Parameter[a]	Cum. R^2	Pr > *F*	Parameter	Cum. R^2	Pr > *F*
AA (U g^{-1})	0.2054	<0.0001	WC (°SRM)	0.7492	<0.0001
WC (°SRM)	0.2721	0.0059	WV (cP)	0.8024	<0.0001
HWE (%)	0.3365	0.0049	AA (U g^{-1})	0.8330	0.0001
BA (U g^{-1})	0.3781	0.0193	TW (kg hL^{-1})	0.8536	0.0009
AAT (%)	0.4149	0.0241	PK (%)	0.8726	0.0007
Glucose (g 100 mL^{-1})	0.4383	0.0660	SP (%)	0.8813	0.0155
Maltose (g 100 mL^{-1})	0.4544	0.1243	DP (°ASBC)	0.8867	0.0526

[a]AA=Alpha-amylase; HWE=Hot water extract; BA=Beta-amylase, AAT=Alpha-amylase thermostability; TW=Test weight; PK=Plump kernels; WC=Wort color, SP=Soluble protein; WV=Wort viscosity; DP=Diastatic power.

Table A6. Results of the stepwise regression (WC out) for the prediction of fermentability in malted samples of two-rowed barley genotypes grown in North Dakota during 2013 and 2014.

Parameter[a]	Partial R^2	Cum. R^2	Pr > F
Glucose (g 100mL^{-1})	0.2867	0.2867	<0.0001
Maltose (g 100mL^{-1})	0.2434	0.5301	<0.0001
LD (U kg^{-1})	0.0686	0.5987	<0.0001
PK (%)	0.0411	0.6397	<0.0001
FAN (mg L^{-1})	0.0307	0.6704	<0.0001
SP (%)	0.0300	0.7004	<0.0001
AA (U g^{-1})	0.0177	0.7181	0.0012
AG (U kg^{-1})	0.0117	0.7298	0.0073
LDT (%)	0.0062	0.7360	0.0471

[a]WC=Wort color; LD=Limit-dextrinase; PK=Plump kernels, AA=Alpha-amylase; FAN=Free amino nitrogen; LDT=Limit-dextrinase thermostability; AG=Alpha-glucosidase; SP=Soluble protein.