Jennifer Schneiderbanger, Bertram Sacher and Thomas Becker

Determining the temperature optimum of cereal beta-amylase by introduction of the half-life method

To determine the optimum temperature and the thermostability of enzymes in a standardized, accurate way is an elaborate, often time-consuming task. Knowledge of these characteristics of specific enzymes is of enormous importance for many applications, e.g. for optimizing the mashing regimes in breweries. In addition, due to ever-increasing gelatinization temperatures, cereal varieties (especially barley) are being sought and specifically bred whose starch-degrading enzymes exhibit increased optimal temperatures and temperature stabilities. A high-throughput method for the standardized determination of an enzyme's behavior to temperature was needed. Based on enzyme kinetic considerations, the half-life method was developed in which a small number of measuring points can provide a high density of information. This information encompasses the temperature optimum as well as the thermostability of enzymes that degrade high-molecular substrates, which applies to starch-degrading enzymes during mashing. Several cereal malts were analyzed for their beta-amylase temperature optimum and thermostability. In doing so, several prerequisites were established for using the method to obtain valid and comparable results.

Descriptors: Temperature optimum, half-life method, beta-amylase, barley, cereals, malt

1 Introduction

The process of producing beer is only possible because of enzymes. In addition to the complex enzymatically catalyzed metabolic reactions of the yeast cell, above all the formation of ethanol by alcohol dehydrogenase, the enzymatic processes during malting and mashing play a decisive role in beer production. Those processes can be categorized as relating to cytolysis (cell-wall degradation of starch-containing cells of the endosperm), proteolysis (protein degradation to small, middle and high molecular products) and amylolysis (degradation of the main components of starch, i.e. amylose and amylopectin) [4]. Malt contains only small amounts of soluble components such as sugars, small protein degradation products and beta-glucans. They are produced by enzymes already present in the grain and those formed during germination, i.e. malting, to supply the seedling with nutrients. The insoluble fractions of those components need to be transferred into a soluble form by enzymes during mashing. Especially the native starch needs to be converted into fermentable sugars necessary for alcoholic fermentation, which is why amylolytic degradation processes during mashing are of great importance [6, 10, 40].

When an aqueous suspension of starch grains is heated, irreversible swelling of the starch grains occurs above a certain temperature. As a result, the starch loses its semi-crystalline structure and becomes vulnerable to enzymes. The beginning of this process is referred to as gelatinization temperature (often synonymously referred to as pasting temperature) [45]. Only gelatinized starch can be effectively broken down by enzymes.

Starch degradation during mashing is the result of the interaction of four different enzymes: alpha-amylase, beta-amylase, limit dextrinase and α-glucosidase [62]. The effectiveness of the single enzymes in starch degradation changes throughout the mashing process based on their original activities derived of the malt used and their respective thermostabilities. Alpha-amylase (EC 3.2.1.1; 1,4-α-glucan-glucanohydrolase) is an endo-enzyme which is built *de novo* during germination of barley and which is then secreted into the endosperm by the aleurone layer [43]. It cleaves α-1,4-glycosidic bonds within amylose and amylopectin molecules. Beta-amylase (EC 3.2.1.2; 1,4-α-glucan-maltohydrolase) is an exo-enzyme which cleaves maltose units from the non-reducing end of dextrins leaving β-limit dextrins left in the medium. It is already present in ungerminated barley, though mainly in bound form [54]. The bound β-amylase is successively released during germination by cleaving off a sequence of 4 kDa by SH-proteinases [37], which is why in malt the ratio of free to bound beta-amylase has changed with only 10–20 % remaining in its bound form [13, 18, 20]. In barley, there are four different kinds of beta-amylases according to *Eglinton* et al. [17]. *Bmy*1-Sd1, *Bmy*1-SdL2 and *Bmy*1-Sd2H were found in

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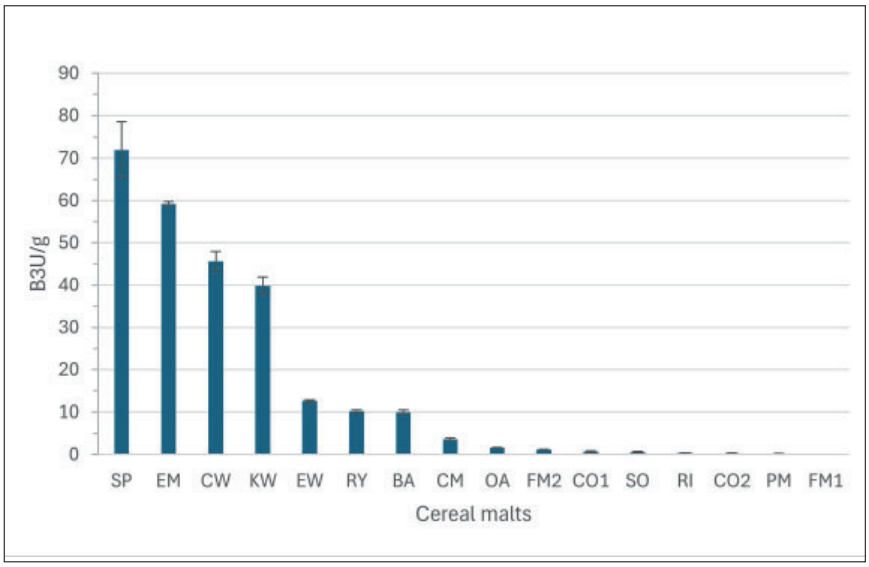

Fig. 1 Beta-amylase activity of the untreated cereal malt samples

cultivated barley with the last allele effecting high beta-amylase thermostability [17, 38, 54]. The fourth kind, *Bmy*1-Sd3, providing for the highest beta-amylase thermostability in comparison, was found in wild barley cultivars of the species *Hordeum vulgare* L. ssp. *spontaneum* only [17]. The residual α-1,6-glycosidic bonds of amylopectin can be cleaved by the endo-enzyme limit dextrinase (EC 3.2.1.142; dextrin α-1,6-glucanohydrolase) generating new substrate for the other enzymes. Limit dextrinase is already present in ungerminated barley, together with its inhibitor protein. Through the action of this inhibitor protein, the activity of the limit dextrinase in mash is partly reduced. The exo-enzyme α-glucosidase (EC 3.2.1.20; α-D-glucoside glucohydrolase) cleaves glucose units from the non-reducing end of oligosaccharides with higher affinities for short molecules.

The activities of starch-degrading enzymes during mashing depend on different characteristics. In addition to the pH optimum of an enzyme [41], the optimal temperatures (T_{opt}) play a decisive role in activity. Those optimal temperatures correlate with the thermostabilities of starch-degrading enzymes in the mashing process which were investigated in the past [12–16, 19, 27–29, 40, 41]. T_{opt} of beta-amylase is stated to be around 50 – 60 °C in starch solution and is, thus, several degrees below T_{opt} of alpha-amylase (60–70 °C) [13, 35, 40, 41, 62]. The generally accepted higher T_{opt} value of barley beta-amylase around 62–64 °C [4, 40] could be related to the fact that sufficient gelatinization of the starch was only observed at this temperature. The consequence for the brewing process is that a maltose rest is performed at 62–64 °C, at which the highest amount of maltose is produced, although this is not necessarily due solely to the highest activity of beta-amylase. Due to the varying T_{opt} of the enzymes, the interplay between the alpha-amylase (degrading starch into dextrins) and beta-amylase (degrading dextrins into fermentable maltose) during the temperature regime of mashing is suboptimal. The pacemaker enzyme alpha-amylase which generates the products for the beta-amylase works best when the activity of beta-amylase is already reduced due to increased temperature. As a result, using malts with higher beta-amylase T_{opt} and subsequent thermostability might help to improve the interplay between the starch-degrading enzymes, potentially leading to a more efficient starch-degradation during mashing and higher fermentability values.

Higher beta-amylase T_{opt} and thermostability could counteract another trend which has arisen during the past decade: the increasing of the barley gelatinization temperatures (GT). GTs of spring barley show a significant positive correlation to the rising climate-related annual temperatures, especially the temperatures during the grain-filling phase of spring barley (June) [50]. GTs > 66 °C can be recorded that are far above the beta-amylase optimum temperature of current barley varieties. Though, it is important to note that GT in most brewing labs is analyzed using a rapid visco analyzer (RVA) which determines GT not in the mash, but outside the mash tun, leading to overestimated values [44]. Nevertheless, it is highly likely that the increase of barley GT will in the future lead to the scenario where the beta-amylase activity is already highly reduced due to temperature while the barley starch swelling (gelatinization) has not yet started to a significant degree – resulting in reduced fermentability recognized by brewers. This could result in insufficient starch breakdown and reduced levels of fermentable sugars. To avoid this scenario and, subsequently, to secure an efficient brewing process, malts with higher beta-amylase optimum temperature and thermostabilities are required.

For breeding barley varieties with higher beta-amylase thermostabilities, the finding and selecting of barley cultivars with promising genetic features is crucial [42]. Consequently, a high number of cultivars of subsequent breedings needs to be evaluated for improved temperature-related characteristics [47]. For this purpose, a high-throughput method for the screening of these traits is necessary. This method should ideally be applicable to several kinds of enzymes inside and outside the brewing environment.

Previous attempts to determine enzymatic characteristics (especially of the starch-degrading enzymes during mashing) towards temperature address either the T_{opt} or the thermostability. T_{opt} is determined by activity measurements in a selected temperature range at selected temperature intervals [12, 13, 35, 55]. In this case it is important to consider that the real T_{opt} might lie in between two measuring points. The determination of thermostability on the other hand relies on two principles: (I) Thermostabilities are determined from (residual) activity measurements in buffer or mash resulting in thermal inactivation curves [12, 13, 55]; (II) thermostability values are determined by a two-point method as a percentage of residual activity (given time at given temperature) versus original activity [14, 15, 17–19, 22, 29, 32, 33, 42, 53, 55, 62]. In this context, one representative value is T_{50}, i.e., the temperature at which only half of the active enzyme is still present (the higher T_{50}, the more thermostable) [55]. The first principle yields accurate thermostability values, but the number of experiments is high, and subsequently the method is only suited to screen a limited number of cultivars. The second principle displays little information apart from the time/temperature scheme chosen and provides no information about the enzyme's optimum temperature, though it might be suitable to screen a high number of cultivars.

Aim of this study was to develop a new method which targets the true optimum temperature of enzymes and their thermostability with a limited number of experiments. In this study, the method is developed using cereal-based beta-amylase as an example. Apart from the characteristics of barley beta-amylase, little is known about the enzymes of other malt types, which is why several cereal-malts were analyzed for T_{opt} and thermostability of their beta-amylases.

2 Materials and Methods

2.1 Enzyme kinetic principles of the method

Enzyme temperature kinetics are based on two distinct opposing reaction mechanisms: (I) The activity or reaction rate of an enzyme is exponentially increased upon temperature increase (known as RGT rule or van't Hoff rule); (II) as the temperature continues to rise, the enzyme undergoes thermal inactivation, resulting in a reduction in activity. An enzyme's T_{opt} is the temperature at which both physical mechanisms are in equilibrium within a specified time interval and at which the product formation rate is the highest [47]. T_{opt} can be evaluated based on activity measurements at different temperatures for set timespans. It is important to note that in this case, the location of T_{opt} can only be narrowed down to the width of the chosen temperature intervals (e.g. 5 K).

The decrease in enzyme activity over time by thermal inactivation can be expressed as a first order reaction by equation (1) [46]:

$$[E](t) = [E_0] \cdot e^{-k \cdot t} \tag{1}$$

The half-life time τ can be determined from equation (1) as:

$$k = \frac{\ln(2)}{\tau} \tag{2}$$

By transforming the Arrhenius equation, the dependence of the rate constant k on temperature θ can be obtained (equation 3) using the constants b' and c:

$$k = b' \cdot e^{-\frac{c}{\theta}} \tag{3}$$

By combining equations (2) and (3) and summarizing the constants b' and $ln(2)$ in b, the temperature dependency of the half-life $\tau(\theta)$ can be expressed as:

$$\tau(\theta) = b \cdot e^{\frac{c}{\theta}} \tag{4}$$

The product formation rate $\dot{P}$ can be described by the Michael-Menten equation with the reaction speed v, the maximum reaction speed v_{max}, the Michael-Menten constant Km and the substrate concentration $[S]$ as follows [46]:

$$v = v_{max} \cdot \frac{[S]}{K + [S]} = [\dot{P}] \tag{5}$$

The product formation rate $\dot{P}$ and the change in substrate concentration $\dot{S}$ correlate through the molar ratio μ as follows:

$$[\dot{P}] = -[\dot{S}] \cdot \mu \tag{6}$$

By inserting (6) in (5), the Michaelis-Menten-equation can be expressed as a first-order differential equation:

$$-[\dot{S}] = \frac{v_{max}}{\mu} \cdot \frac{[S]}{K_m + [S]} \tag{7}$$

The RGT rule (reaction rate-temperature rule) states that when the temperature increases by 10 Kelvin or °C, the reaction rate for enzymatic processes approximately doubles. In other words, the turnover frequency $k_{cat}(\theta)$ of an enzyme doubles with every 10-Kelvin temperature increase, which can be expressed by equation (8):

$$k_{cat}(\theta) = k_{cat0} \cdot 2^{\frac{\theta - \theta_0}{10}} \tag{8}$$

Here, v_{max0} is the product of turnover frequency and the active enzyme concentration at the beginning ($[E_0]$):

$$v_{max0} = k_{cat0} \cdot [E_0] \tag{9}$$

From the combination of equations (1), (2), (7) and (9) follows equation (10) which takes reaction acceleration by factor 2 as well as thermal enzyme inactivation into account:

$$-[\dot{S}] = \frac{v_{max0}}{\mu} \cdot 2^{\frac{\theta - \theta_0}{10}} \cdot e^{-\frac{\ln(2)}{\tau(\theta)} \cdot t} \cdot \frac{[S]}{K_m + [S]} \tag{10}$$

Under consideration that the substrate concentration is not changing for a long period during the degradation of a high-molecular educt, the last term of equation (10) is approximately 1 and therefore negligible. Taking formula (6) into account, it follows:

$$[\dot{P}] = 2^{\frac{\theta - \theta_0}{10}} \cdot e^{-\frac{\ln(2)}{\tau(\theta)} \cdot t} \cdot v_{max0} \tag{11}$$

By integrating from 0 to t and combining all constants in C, the product concentration can finally be formulated as a function of time:

$$[P](t) = C \cdot \tau(\theta) \cdot 2^{\frac{\theta - \theta_0}{10}} \cdot \left(1 - e^{-\frac{\ln(2)}{\tau(\theta)} \cdot t}\right) \tag{12}$$

The half-life τ of an enzyme at different temperatures can be evaluated by determining the constants b and c of equation (4) via linear regression. Under consideration of a specific enzymatic reaction time t and $\tau(\theta)$, the product concentration P can be expressed depending on the reaction temperature θ. This application can be used to determine where the product formation is at its maximum and, accordingly, where the T_{opt} must be. The constant C (see eq. (12)) only increases the amount of product formation, not the position of the optimum and is therefore negligible.

All enzyme kinetic considerations base on the assumptions that

- the enzymes are distributed homogeneously.
- the enzymes are subject to only one dominant inactivation mechanism (e.g. temperature, not pH).
- the rate constant k is independent of time and concentration.
- there is no reactivation or renaturation of the enzymes after exposure to heat.
- additional inhibitors/activators or matrix interactions (e.g. protection by sugars, proteins, viscosity) can be excluded.
- the reaction rate is proportional to the concentration of active enzyme.

2.2 Practical implementation of the half-life method

Half-lives τ for the incubation temperatures θ are calculated using equation (13). This formula is obtained by substituting equation (2) into (1) and solving for τ. There is a direct proportionality between the activity of the enzyme (*[A]*) and the concentration of active enzyme (*[E]*). t_{Ink} is the incubation time of the selected assay, A_0 the activity of the untreated (=without heat inactivation) enzyme solution and A_{Ink} the activity of the enzyme solution after incubation.

$$\tau = \frac{\ln(2)\cdot t_{Ink}}{\ln(A_0) - \ln(A_{Ink})} \quad (13)$$

The calculated half-lives $\tau(\theta)$ for the incubation temperatures θ (in °C) are plotted as $ln(\tau(\theta))$ against $1/\theta$. This application results in a linear dependency in the form of equation (14) which is a transformation of equation (4):

$$\ln(\tau(\theta)) = c \cdot \frac{1}{\theta} + \ln(b) \quad (14)$$

The constants *b* and *c* can be calculated from linear regression for the enzyme under investigation. Subsequently, the half-lives $\tau(\theta)$ of an enzyme for a selected temperature range (here: 50 – 85 °C in 0.5 °C intervals) can be calculated. The reference temperature θ_0 is always the lowest temperature selected (here: 50 °C). The value pairs obtained can be inserted into equation (15) which is based on equation (12) without constant *C*. It is important to note that T_{opt} depends on the selected assay times t_{assay}.

$$[P'](\theta) = \tau(\theta) \cdot 2^{\frac{\theta-\theta_0}{10}} \cdot \left(1 - e^{\frac{\ln(2)}{\tau(\theta)}\cdot t_{assay}}\right) \quad (15)$$

T_{opt} of an enzyme is determined as the temperature θ where the transformed product concentration $[P'](\theta)$ reaches its maximum at a selected assay time t_{assay}. Relative enzyme activities are calculated by relating the single values $[P'](\theta)$ to the maximum obtained at the enzyme's T_{opt}.

2.3 Important notes on implementation of the method

There are certain general challenges concerning the half-life method that need to be considered:

- The incubation temperatures must be selected in such a way that the presumed optimum temperature of the respective enzyme lies within the represented temperature range. Additionally, a wide temperature range should be represented in order to achieve high accuracy in linear regression.
- The combination of incubation temperatures and times must ensure that the enzyme activity of the extract incubated with the lowest heat input is below the activity of the untreated sample. Additionally, the activity of the extract incubated with the highest heat input should be > 0 and be within measuring range.
- The extinction of the untreated samples must not be too low. If this is neglected, extinction values of the incubated samples will potentially fall below the measuring range or reach the lower limit even before the end of incubation time. As a result, the half-lives and thus the optimum cannot be determined correctly.
- To cope with the challenge above, the extraction buffer and dilution solution volumes as well as the weighed-in sample quantities may need to be adjusted.

Additional specific considerations concerning the determination of the beta-amylase temperature optimum using the K-BETA3 Megazyme kit encompass:

- During the extraction of beta-amylase, the ratio of sample weight to extraction solution (5 ml) could only be increased from the original 0.5 g to a maximum of 2 g due to excessive viscosities.
- Extraction and dilution solutions of the K-BETA3 Megazyme kit are not interchangeable due to different compositions and different pH values. The dilution ratio cannot be adjusted arbitrarily.

2.4 Malt samples

To determine the beta-amylase T_{opt} of different malts, the cereals shown in table 1 were micro-malted. Additionally, two commercial malts (barley and common millet) were analyzed. The steeping time for all micro-maltings was 2 days. For breaking dormancy, the first steeping of several cereals (see table 1) was done using a H_2O_2 solution (0.75 %) based on MEBAK method R-110.27.611 [39].

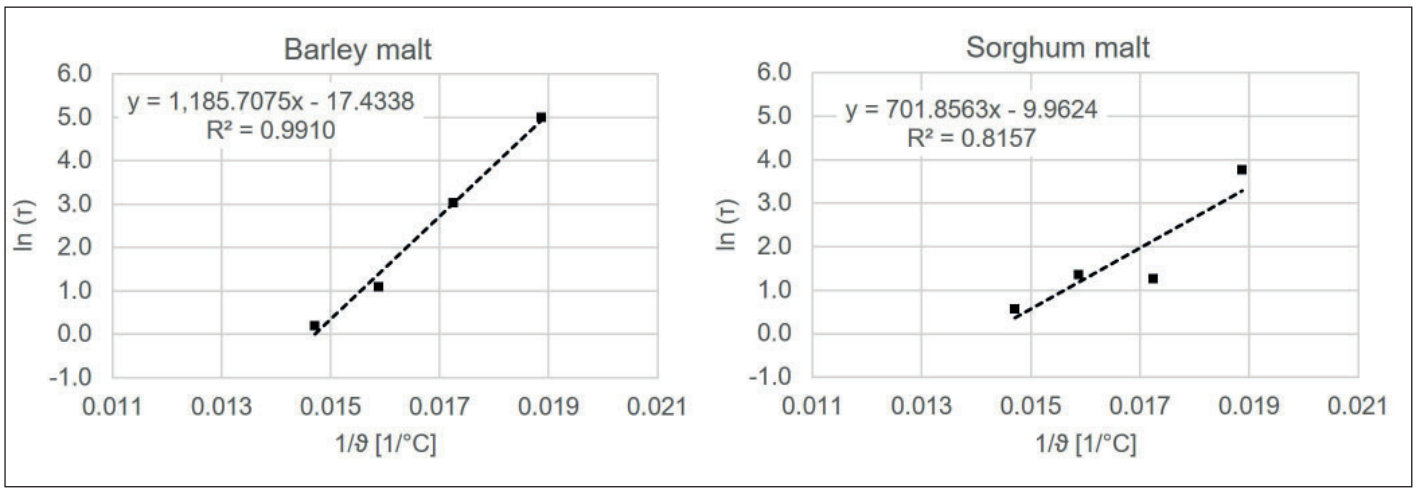

Fig. 2 Linear regression of ln(τ) as a function of 1/θ [1/°C] using the example of barley (left) and sorghum (right) beta-amylase activities

Table 1: Micro-malted cereals and the respective malting schemes

Cereal	Abbreviation	Botanical name	Steeping/Germination temperature [°C]	Germination time [d]	H_2O_2	Kilning regime
Common wheat	CW	Triticum aestivum ssp. aestivum	15	4	–	MEBAK
Kamut	KW	Triticum turgidum ssp. turanicum	15	4	–	MEBAK
Rye	RY	Secale cereale	15	4	–	MEBAK
Oat	OA	Avena sativa	15	5	–	MEBAK
Foxtail millet 1	FM1	Setarica italica	22	3	+	MEBAK
Corn 1	CO1	Zea mays	22	3	+	MEBAK
Sorghum	SO	Sorghum bicolor	22	2	–	ISO
Foxtail millet 2	FM2	Setarica italica	22	3	+	ISO
Pearl millet	PM	Pennisetum glaucum	22; 15	3	+	ISO
Corn 2	CO2	Zea mays	22	3	+	ISO
Spelt	SP	Triticum aestivum ssp. spelta	15	5	+	ISO
Einkorn wheat	EW	Triticum monococcum	22; 15	11	–	ISO
Emmer wheat	EM	Triticum turgidum ssp. dicoccum	15	5	+	ISO
Rice	RI	Oryza sativa	22	3	+	ISO
Barley	BA	Hordeum vulgare	ready-to-use malt			
Common millet	CM	Panicum miliaceum	ready-to-use malt			

(+ = first steep with H_2O_2 addition; - = first steep without H_2O_2 addition; MEBAK = Kilning regime according to method R-110.00.008 [34]; ISO = Kilning for 24 h at 50 °C)

The corn and foxtail millet samples CO1 and FM1 were kilned according to MEBAK first and analyzed for their beta-amylase content. As the beta-amylase activity in the malts was very low (0.64 B3U/g and 0.00 B3U/g, respectively), a second micro-malting CO2 and FM2 was done using moderate kilning at 50 °C for 24 h.

2.5 Water content determination

The water content was determined in duplicates. 5 g ground sample (hammer mill 3100, 0,8 mm screen width, Perten Instruments; exception: Einkorn wheat was ground using a mortar) were dried for 3 h at 108 °C and the water content calculated according to MEBAK method R-100.01.020 [39].

2.6 Activity measurement of beta-amylase

Beta-amylase activity was measured using the Beta-Amylase Assay Procedure (K-BETA3; Betamyl-3 method) of Megazyme in quadruplicates and the original test and malt standard reagents of the company [57]. The samples were ground by a hammer mill or mortar (*see Water content determination*), weighed in, extracted for 1 h in 5 ml extraction buffer, centrifuged (10 min at 3000 U/min) and diluted 1:20. The sample quantities were adjusted according to amylase activity (0.5 g for all malts; exceptions 2.0 g for samples RY, SO, FM2, PM, CO2, RI). To achieve a higher throughput, the method was scaled down to microtiter format using 96-well plates, a pipet robot (Precision XY Automated Sample Processor PRC384/1M, Bio-Tek Instruments) and a multiplate-reader at 400 nm (Synergy H1, BioTek Instruments).

The extracts (2 ml each) were measured directly (= untreated sample) or incubated for a pre-defined period at 4 different temperatures at intervals of 5 °C in a water bath (Thermolab, Typ GFL-1070). After incubation, the vials were quickly cooled in an ice water bath. Enzyme extracts showing turbidity were centrifuged for 5 min. The incubated samples (n=4) as well as the untreated sample (n=1) were then analyzed for their activity using the enzyme kit K-BETA3 and 10 min of assay time.

It is worth noting that due to the reducing effect of the cysteine contained in the extraction buffer used, both the free and bound forms of beta-amylase are extracted [1, 10, 57].

2.7 Incubation settings

Due to different activities of the untreated cereal malt samples and their different thermostabilities, the time/temperature settings for the cereal malts were chosen as follows (see table 2):

Table 2: Temperature / time combinations for all cereal malt samples except for CW, KW and RY

Temperature [°C]	53	58	63	68
Incubation times [min]	30	10	5	3

Table 3: Beta-amylase T_{opt}, T_{50} values, regression coefficients R^2 and the slope of the linear regression lines of different cereal malts

Malt sample	T_{opt} [°C]	T_{50} [°C]	R^2	Slope of linear regression
CW	57.0	65.0	0.9974	983.24
KW	58.0	64.5	0.9999	1318.06
RY	55.5	67.0	0.9864	720.33
OA	57.5	64.0	0.9308	1363.42
FM1	indeterminable	-	-	-
CO1	indeterminable	-	-	-
SO	57.5	70.5	0.8157	701.86
FM2	indeterminable	-	-	-
PM	indeterminable	-	-	-
CO2	indeterminable	-	-	-
SP	59.0	67.5	0.9985	1031.62
EW	54.5	62.5	0.9881	940.45
EM	60.5	68.5	0.9961	1149.18
RI	59.5	68.0	0.9167	1042.16
BA	58.5	66.0	0.9910	1185.71
CM	60.0	72.0	0.9377	768.95

The incubation schemes for the common wheat and the Kamut malt were altered to display 15 min at 58 °C and 10 min at 63 °C. An alteration in the rye malt incubation scheme was a 3-min period at 63 °C. The time/temperature scheme can be freely selected as long as the above-mentioned challenges are met.

3 Results

3.1 Beta-amylase activity of the untreated cereal malt samples

The highest activity of beta-amylase was determined in the spelt malt sample (72.02 B3U/g; see fig. 1). In descending order, this was followed by Emmer wheat, common wheat and the Kamut samples with activities of 59.21, 45.61 and 39.82 B3U/g, respectively. The Einkorn wheat, rye and barley malt samples had moderate beta-amylase activities of 12.62, 10.16 and 9.97 B3U/g, respectively. All other cereal malt samples yielded beta-amylase activities < 4 B3U/g (see fig. 1).

3.2 Determination of beta-amylase optimum temperatures

After thermal treatment of the extracts at specific temperature/time combinations (see table 2), the residual beta-amylase activities were determined. From equation (13) the half-lives τ can be calculated using the original beta-amylase activity (see fig. 1) as A_0. Using the transformed half-life values ($ln(\tau)$), the incubation temperatures θ and equation (14), a linear regression can be created (example see figure 2).

The regression coefficients R^2 were determined as > 0.98 for several malt samples except for rice, oat and common millet malt (0.9167, 0.9308 and 0.9377, respectively; see table 3). The sorghum malt had the lowest R^2 of 0.8157. In this case, a higher number of measurement points might have improved the regression, but it is evident that there is a correlation between low activities of the untreated samples (see fig. 1) and low regression coefficients (see table 3). For the corn, foxtail millet and pearl millet malt samples, no regression line and no temperature optimum could be established. This was because some of the prerequisites for implementing the method (see section: *Important notes on implementation of the method*), i.a. sufficient beta-amylase activities of the untreated samples, were not met. In summary, it seems reasonable to assume that, based on the present data, the initial beta-amylase activities should be above a certain threshold (here: > 3.5 B3U/g) to receive highly valid results using the half-life method.

From the linear regressions (examples see fig. 2), the half-lives of a wide temperature range can be calculated. Using equation (15), the product concentrations at selected intervals (e.g. 0.5 °C) can be determined. The maximum product concentration is where the enzyme reaches its temperature optimum. The specific product concentration within the chosen temperature range can be related to the maximum product concentration and displayed in a diagram (see fig. 3).

The result of the half-life method is a typical shifted (left-skewed) bell-shaped curve. The product formation accelerates at low temperatures until the increase in temperature results in the maximum product concentration where the opposing mechanisms (I) higher turnover rates due to higher temperatures and (II) the reduction of enzyme activity due to thermal inactivation are optimally balanced. As the temperature rises further, the product concentration quickly drops. At 62 °C where the typical beta-amylase rest in the brewhouse takes place, only 87.6 % of relative product formation can be expected with this malt beta-amylase (see fig. 3). 50 % of relative product concentration is reached at 66 °C, and at 70 °C only 23.8 % of product formation is left.

It is also important to note that the curve displayed in figure 3 approaches the x-axis at high temperatures, but very slowly. This allows us to conclude that there is a residual activity even at temperatures > 80 °C which confirms the findings of other researchers that this might be due to additional thermostable beta-amylase isoform [12].

3.3 Temperature optima of cereal malt samples

The beta-amylase T_{opt} of the analyzed malts display values between 54.5 °C (Einkorn wheat; EW) and 60.5 °C (Emmer wheat, EM) (see fig. 4 and table 3). The malts mainly used in brewing, barley and wheat, had a T_{opt} of 58.5 °C and 57.0 °C, respectively.

Not only T_{opt} is of interest, but also the response of the enzyme's

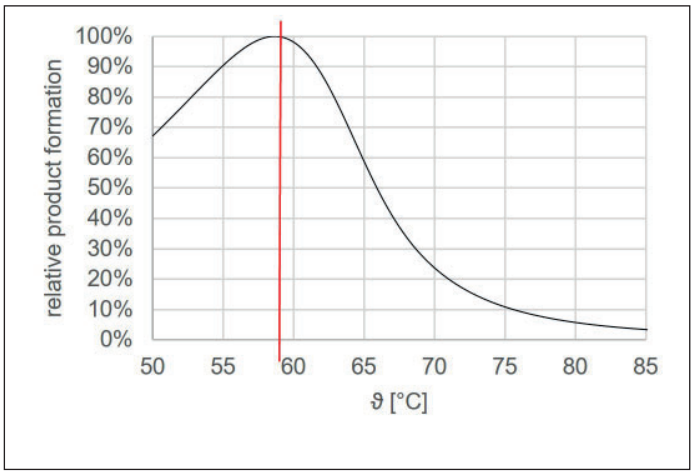

Fig. 3 **Relative product formation of a barley malt beta-amylase in a temperature range of 50 to 85 °C (red line: maximum product concentration = T_{opt}; here: 58.5 °C)**

activity to temperatures above the optimum. In figure 4 it can be seen that the common millet, the sorghum and the rye malt samples showed significantly higher activity values above 70 °C. These curves are caused by the lower linear regression gradients compared to the others (see table 3). A lower slope signifies that the enzyme reacts less to changing temperatures and, therefore, indicates higher thermostability.

4 Discussion

4.1 Half-life method for enzymatic optimum temperature determination

The presented half-life method requires a small number of experiments (4 time/temperature settings + enzyme activity without heat treatment) to determine the product formation curves of an enzyme displaying the acceleration of activity below T_{opt}, the highest product formation at T_{opt}, and the response to temperatures above it (=thermostability). Thus, it is important to note that T_{opt} is only valid for the specific assay time chosen (here: 10 min). For screening purposes, it is highly recommended to keep this assay time constant. This also affects considerations regarding the implementation of an optimal mashing schedule: An extension of the mash rest times should be accompanied by a reduction in temperature in order to accommodate the optimum temperatures of the target enzymes.

To use this method as a high-throughput method for screening purposes, the obtained T_{opt} values can be related to the highest T_{opt} found within the panel under investigation or to known cultivars with the target feature (e.g. cultivars with thermostable beta-amylase isoform) and calculated as "levels of expression" (e.g. levels from 1 (low T_{opt}) – 9 (high T_{opt})).

The method can be easily transferred to other enzymes if the following conditions are met: (I) An incubation apart from substrate and potential auxiliary enzymes is possible, (II) a selective target enzyme activity determination method is available and (III) educts of the enzyme under investigation are macromolecules that are available in excess and are not subject to substrate limitations. If condition (III) is not met, the Michaelis-Menten term of equation (10) is ≠1 and simplification of the subsequent equations is no longer possible.

4.2 Initial beta-amylase activities of different cereals

As was mentioned before, the germination of cereals should not affect beta-amylase activities to a significant extent as the extraction buffer used to determine activity contains cysteine to also extract bound forms. But the temperature applied during kilning is assumed to have a great impact (protein denaturation) [20]. The very low initial beta-amylase activities of the corn and foxtail millet samples (CO1 and FM1) could not be significantly increased by careful isothermal kilning at 50 °C (CO2 and FM2, see fig. 1). However, the highest beta-amylase activities were observed in gently dried samples (SP and EW, see fig. 1). The activity values determined in this study for the different wheat malt samples EW, KW, CW, SP and EM are in line with the findings of *Hildago* et al. [30] and *Bailliere* et al. [5], but differ in some respects significantly from other literature sources [8, 23]. Especially the initial beta amylase activity of spelt samples was determined significantly lower in previous studies with values of 27 B3U/g [23] and 16 B3U/g [8]. Whether this deviation is due to different kilning schemes or whether there is considerable variation within the individual varieties of spelt cannot be conclusively determined. It is worth noting that, in general, differing enzyme activities can be the result of variation of determination methods applied or even a change in the artificial substrate in the Megazyme kits [19]. The low activity of the oat malt sample (1.67 B3U/g) might be a result of the high proportion of husks as polyphenols can influence enzyme activities [11]. The low beta-amylase activities of

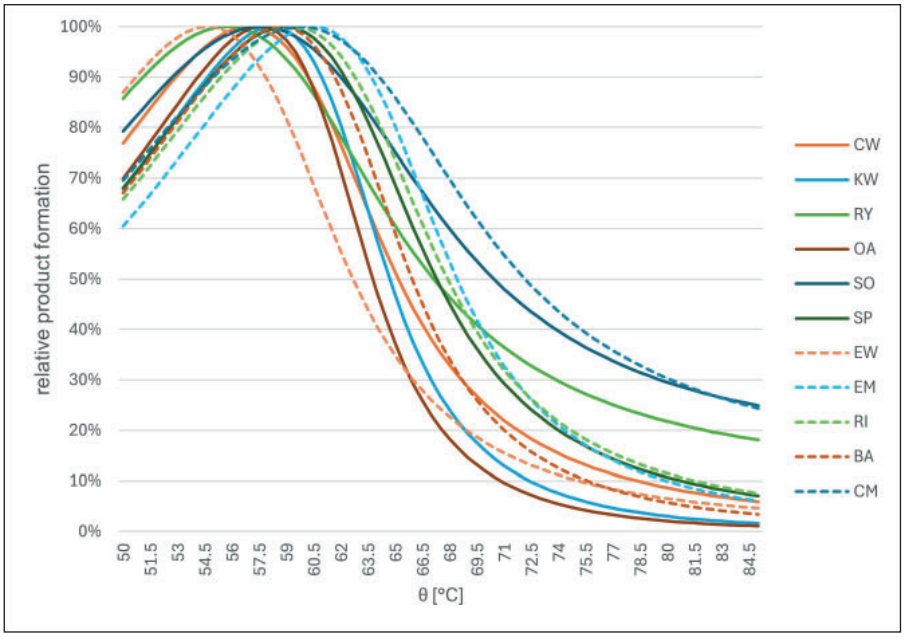

Fig. 4 **Optimum temperatures of various cereal malt samples determined by half-life method**

the sorghum, millet and rice malts are in line with other literature sources [8, 36, 40, 49]. The moderate kilning at 50 °C for 24 h did not result in significant higher beta-amylase activity values of the corn and foxtail millet samples CO2 and FM2 in comparison to the MEBAK-kilned CO1 and FM1 samples.

4.3 Beta-amylase optimum temperature and thermostability of different cereals

Comparing the results concerning T_{opt} of starch-degrading enzymes of other literature sources is often difficult due to the performance of analysis under varying conditions (e.g. time, temperature, matrices, dilution, original enzyme activity). The deviations of T_{opt} proclaimed by different authors might be a result of different determination methods [12, 13, 19, 42, 55, 62]. Especially the information about the reaction time used to analyze T_{opt} is crucial as was stated before, but often not stated. Additionally, information about T_{opt} and thermostability of enzymes derived of other cereals than barley is scarce.

De Schepper et al. [13] determined the beta-amylase T_{opt} of six barley malts as ranging from 45–50 °C when incubated for 5 min at 12 selected temperatures within a range of 30 to 65 °C. A similar T_{opt} of 50 °C was found by *Osman* et al. [41] for barley malts using natural substrates, though the chosen broad temperature intervals of 10 °C do not allow for a more precise determination. One value often presented as an indication of thermostability, T_{50}, is plausible, but provides little information regarding the enzyme's activity curve apart from that specific temperature where activity is 50 %. T_{50} values for barley beta-amylase were found to vary between 55.8 °C and 62.1 °C by different researchers [38, 55, 62]. In this study, barley beta-amylase's T_{50} value was determined as 66.0 °C. The problem using this value alone as a measurement for thermostability is that it only displays a small section of the enzyme curve (compare fig. 4 and table 3). From the T_{50} values shown in table 3, the obvious higher thermostability of the rye malt sample (see fig. 4) is not apparent.

Using different substrates also needs to be considered. Most of the recent studies used the K-BETA3 kit of Megazyme for determining beta-amylase activity. Though using natural substrates such as native starch reflect the enzyme's reality more accurately, the usage of artificial substrates such as para-Nitrophenyl-β-D-maltotriosid contributes to increased standardization. If using native starch, the type of the starch used (e.g. granular or soluble, originating from potato or cereal), its gelatinization temperature and the temperature-dependent physical changes in starch structure need to be considered and specified accordingly [24, 25, 45]. In addition, the substrate as well as potential auxiliary enzymes should not be subjected to higher temperatures than specified by the manufacturer (e.g. 40 °C) to ensure proper functionality [13]. This issue is addressed in the presented half-life method by external incubation of enzyme extracts. No further pretreatment of the extract is necessary, even when determining several starch-degrading enzymes in parallel (compare *De Schepper* et al. [13]).

In the brewing industry, the T_{opt} of barley beta-amylase proposed by *Narziß* is often referred to (60–65 °C) [4, 35, 40] and used in generalized mashing regimes as the beta-amylase or maltose rest temperature. It was also often stated that an isothermal mash at 60-65 °C would achieve maximal malt attenuation [26, 48, 52]. Though, it is not clear, if the high(est) activity in this temperature range is actually the result of the enzyme's optimum temperature or the result of the fact that the malt starch has undergone sufficient gelatinization only at these temperatures.

T_{opt} of the commercial barley malt used in this study was determined as 58.5 °C. Taking into account that the results were obtained in a buffer surrounding, this value might fit to the values found in literature for beta-amylase T_{opt} values (see above) obtained in mash [26, 40, 48]. Concerning the other cereals under investigation, little information can be found in literature concerning their optimum temperatures. Several research groups determined very different values for beta-amylase T_{opt} in common millet (also known as Proso millet), 62.0 °C, 55 °C and 40 °C [51, 58, 61]. The result determined in this study for common millet (T_{opt} = 60.0 °C, see table 3) is similar to the temperatures postulated by *Yamasaki* and *Sebestyén* et al. [51, 58]. The deviations of T_{opt} proclaimed by different authors might be a result of different determination methods. The standardized, mathematically justified half-life method might narrow the differences.

4.4 Considering the transfer to mashing

As was stated before, the presented half-life method uses an upstream enzyme extraction using the optimized buffer solutions provided by Megazyme. Then, an external incubation period takes place, after which the residual activities are determined. Several factors, such as performing an extraction using a buffer as well as using an artificial substrate, deviate from the real action of an enzyme during mashing. Transfer of the enzyme's T_{opt} and thermostability determined in buffer to a matrix as complex as mash is not readily possible. The complexity of the matrix mash is based on changing parameters and molecular composition over time (e.g. mashing-in temperatures, temperature at begin and end of enzyme rests, time span of the enzyme rests, pH of mash, mash concentration, physical digestion support (decoction) or only enzyme modification (infusion), mashing equipment, addition of exogenous enzymes in non-purity-law countries).

What was found before is that enzymes are stabilized by the soluble components such as proteins and sugars of mash [2, 3, 14, 27, 36], especially by the amount of maltose present [14, 28]. Maltose was described as thermoprotectant [28], but also as a competitive inhibitor (*Ki* ≈ 20 – 30 mM) of cereal-derived beta-amylase [37]. Due to the high dilution factor as part of the activity measurement with the K-BETA3 kit of Megazyme, the maltose concentration in the sample will drop below relevant concentrations [19]. Several studies state that the enzyme's stability is significantly higher in a mashing environment than in buffer [7, 9, 31, 34] and correlates positively with thicker mashes [19]. Another study found no differences between the activities in 1:6 and 1:2.5 mash thicknesses [12]. It remains challenging to project an enzyme's stability and activity in this highly complex matrix mash, especially as thermal inactivation studies in undiluted solutions are scarce [13]. Further factors such as cell wall material and proteins in the mash could also influence enzyme-substrate interactions [60]. To determine T_{opt} under mashing conditions, analyses should be extended to a mash and/or wort-like matrix using the newly developed half-life method.

5 Conclusion

The determination of optimum temperatures and thermostabilities of starch-degrading enzymes is highly relevant to optimize the process of mashing, e.g. by adapting the timespans and temperatures of certain enzyme rests to meet the respective enzyme's intrinsic characteristics. Using the presented half-life method under varying incubation conditions and in a varying mash environment (e.g. different malts, different mash thicknesses) might be a promising path for these optimizations. Another important application of the half-life method is the screening of breeding lines for improved T_{opt} and thermostability of cereal-based enzymes. The low number of activity measurements (n = 5; 4 after incubation + 1 without heat treatment) per sample allows high throughput.

As was stated before, the half-life method can be transferred to other enzymes that have high molecular weight substances as educts which are not subject to substrate limitation for a long period of time. This is the case for all 4 starch-degrading enzymes described previously. To optimize fermentability of malt, it might be profitable to search for genetic features providing for a higher limit dextrinase T_{opt} and thermostability [21, 56, 59]. The search for those genetic features in breeding lines as well as the adaptation of the mashing regime to meet the enzyme's temperature characteristics can be performed using the presented method.

One further breeding goal might be the optimization of malt-originating endo- and exo-glucanases, that break down high-molecular weight β-glucans derived from the cell-walls resulting in lower viscosities and less lautering and filtration problems. T_{opt} of those glucanases of modern cultivars (40-45 °C) is far below their pacemaker enzyme β-glucan solubilase (62-65 °C) [4, 6, 7, 10, 40]. The latter dissolves high-molecular beta-glucans from the cell walls at higher temperatures, when these can no longer be broken down by glucanases. Barley varieties with higher glucanase thermostability could have a positive influence on the improved cooperation between the two enzyme types mentioned.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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