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A method for the determination of hop diastatic power – part 1

The enzymatic potential of hops has only recently become a subject of investigation because of a rising interest in hop forward beer styles which are often dry-hopped. In this work, a method for quantification of the diastatic activity of hops was developed. Hops were incubated with a potato starch substrate for 48 hours. Using response surface methodology, the independent factors: starch concentration (1 – 3 % w/v), hop concentration (5 – 15 g/L), and incubation temperature (25 – 35 °C) were varied and optimized. Fructose, glucose, maltose, and maltotriose were analyzed by HPLC after incubation. Because hops possess amyloglucosidase activity, which can degrade one maltose unit to two glucose units, measuring solely maltose or glucose cannot be recommended. Total mono hexoses, as the sum of glucose plus two times maltose, was found to produce more appropriate results and was followed for measuring the diastatic activity of hops. A starch concentration of 3 % (w/v), a hop concentration of 15 g/L, and an incubation temperature of 35 °C were found to be the optimal process parameters for determining hop diastatic activity. 14 different German hop varieties were tested with the optimized methodology and were correlated with the release of fermentable sugars from a lager beer by hop diastatic enzymes. Furthermore, the pH of the starch substrate, which in initial tests was pH 6.6, was adjusted to pH 5.5 or pH 4.5 in subsequent tests. While all substrates showed significant Pearson correlation coefficients for maltose and total mono hexoses, a pH of 6.6 showed the best correlation ($r = 0.849 – 0.853$, $p < 0.001$) and a linear coefficient of determination of $R^2 = 0.72$ was found. Using a beta-limit dextrin substrate instead of potato starch yielded very low and insignificant correlations. Further investigations will aim at looking for even more suitable starch or dextrin substrates. Altogether, this approach shows promise for the development of a reliable and reproducible method to determine hop diastatic activity.

Descriptors: hops, hop creep, amylases, diastatic activity, enzymes

1 Introduction

Like many other plants, hops possess starch-degrading enzymes [1, 2]. These enzymes do not play a role in brewing when hops are added during wort boiling or during the whirlpool rest as the temperature of 80 – 100 °C denatures the enzymes. Carbohydrates like fructose, glucose, maltose, and sucrose are found only in low concentrations, if at all, in beers [3]. Dextrins and oligosaccharides, however, are present in relatively high amounts, as most brewing yeasts cannot assimilate them, because of too long chain lengths (α four glucose units) or branched structure, due to the presence of α-1,6-glycosidic bonds [3, 4]. As described in a study by *Li* et al. [4], fully attenuated beer contains only traces of monosaccharides, while dextrin and oligosaccharides make up to 34.83 % (w/w) and 38.62 % (w/w), respectively. Values in this dimension have already been described earlier by *Buckee* et al. [5]. *Enevoldsen and Schmidt* [6] divided beer dextrins into 4 groups differing in their molecular size and degree of polymerisation. Results indicate that the majority of α-1,6-linkages of malt amylopectin end up in the final beer from which 25 % appear as single-branched oligosaccharides and presumably more than 35 % as multiple-branched oligosaccharides.

When hops are added during fermentation or maturation, hop diastatic enzymes can still be active and may convert beer dextrins in the young beer to fermentable carbohydrates. Yeast cells still present in the green beer can ferment these carbohydrates to carbon dioxide and ethanol. Concurrently, fermentation by-products such as acetaldehyde, diacetyl, ethyl and acetate esters, and higher alcohols can be formed, thus compromising beer quality. Furthermore, the fermentative activity can lead to increased alcohol content, decreased residual extract, and an increased risk of overflowing fermentation tanks or over-carbonated bottles due to the formation of CO_2 [7]. Bursting of bottles or cans may occur in severe cases. This phenomenon is known as 'hop creep' and was first described in 1893 by *Brown and Morris* [8]. They extracted enzymes from hops, hydrolyzed starch solutions and determined the resulting glucose and maltose. A second study on this topic was

https://doi.org/10.23763/BrSc21-09wietstock

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only conducted again in the 1940s by *Janicki* et al. [2]. They found that a high hop concentration increases the amount of resulting carbohydrates. Furthermore, no significant difference was found between using a starch solution as a substrate or beer. Up to 5 g/L of fermentable carbohydrates were found after three days of dry hopping. An investigation by *Natsume* et al. [9], researching the hop genome, found 429 glucosidase- and 248 amylase-encoding genes in the DNA sequence of Cascade hops. Of these, it is believed to be the higher-molecular-dextrin degrading enzymes that have the initial impact on re-fermentation [10]. *Kirkpatrick* and *Shellhammer* [11] detected activity of four different enzymes in Cascade hops – alpha and beta amylase, amyloglucosidase, and limit dextrinase. Moreover, in another study, Kirkpatrick and Shellhammer showed that the enzymatic activity differs greatly across different cultivars. It is uncertain to what extent genetic makeup, growing conditions, harvest and processing, and storage have on the enzymatic activity. Studies from *Rubottom* et al. [12] suggest that hop kilning temperature has in fact a major impact and that higher hop kiln temperatures reduce the residual diastatic activity of hops. The enzyme kits used by Kirkpatrick and Shellhammer are designed for measuring malt enzymatic activity. In hops, enzymatic activity is much lower than in malt, and modifications had to be made to adapt the test for measuring hop enzyme activity. Unpublished studies from Claudio Cornaggia from the company Megazymes Ltd. (personal communication, October 5th, 2020), using an optimized protocol for measuring individual enzymatic activities of hops, suggest that e.g. storage time has a major impact. In a more practical approach to estimating the extent of dextrin degradation during dry hopping of different beer types, *Stokholm* et al. [13] recently proposed a benchtop fermentation method which forecasts the terminal gravity post hop creep. Enzyme kits can assess the enzyme activities individually. This is particularly of interest when measuring malt samples or when e.g., aiming to characterize these enzymes independently. However, when measuring hop samples and in the context of the 'hop creep' phenomenon, the activity and interplay of the individual enzymes and the sum of released fermentable carbohydrates is of utmost interest.

Until now, a prediction of the overall diastatic power of hops as the sum and interplay of all the different diastatic enzymes has been imprecise since the enzymatic activity is low and difficult to determine using standard methods. Furthermore, a prediction of the behavior in beer based on a quantitative determination of the enzymes is also inaccurate, as the mechanism and interaction of the individual enzymes is not yet fully understood. Therefore, a standardized method is needed to predict the hop-induced polysaccharide degradation and to enable an investigation of the different factors that may impact the enzymatic activity, such as the hop cultivar, crop year, processing, storage conditions, and treatment before usage in the brewery.

With that in mind, in this study, a novel approach for examining the total hop diastatic activity was developed. Although the congress mashing method is an approved method to produce wort in a standardized way, the resulting wort composition depends on the different malt varieties used and even the crop year. The use of commercial beers as a dextrin source for a standardized method is unfeasible as the quantity and composition of dextrins in different beers as well as other beer attributes such as the pH vary. Consequently, a reproducible and consistent polysaccharide source is needed. As a method for measuring malt diastatic activity is available and validated (MEBAK method R-200.23.031 [14]), this method was used as a base to develop a method for measurement of hop diastatic activity. Applying response surface methodology, the effect and interplay of the independent variables: starch concentration, hop concentration, and incubation temperature were elaborated and subsequently optimized. In a next step, the influence of pH was investigated, and glucose, and maltose, as derived from starch degradation by hop enzymatic activity, were compared to the amount of fermentable carbohydrates from a lager beer which was incubated with hops using the same conditions. This work features new findings with regards to analyzing hop diastatic activity. Outcomes from this work and the second part of the methodology development will allow elucidation of the influences of hop cultivar, crop year, processing, storage conditions, etc. on the activity of hop diastatic enzymes, and will ultimately facilitate greater control of the 'hop creep' phenomenon.

2 Materials and Methods

2.1 Chemicals

The Megazyme Kits beta-amylase 'BETAMYL-3 method' (K-BETA3) and amyloglucosidase (R-AMGR3) were purchased from Romer Labs (Butzbach, Germany). These kits already contain all required buffers. Disodium ethylenediaminetetraacetic acid (EDTA), 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate, tris(hydroxymethyl)aminomethane (TRIS), sodium hydroxide, hydrochloric acid and glacial acetic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Beta-limit dextrin was also purchased from Romer Labs (Butzbach, Germany). Potato starch (CAS 9005-84-9) was obtained from Merck (Darmstadt, Germany), and acetonitrile from VWR international (Darmstadt, Germany). All

Table 1 Hop cultivars used in this study, their abbreviations, and their fructose and glucose concentration in g/L. An amount of 0.15 g of the hop samples were added to 10 mL of double-distilled water and incubated for 48 hours at 35 °C prior to determining carbohydrate concentration by HPLC

hop cultivar	abbreviation	fructose conc. (g/L)	glucose conc. (g/L)
Smaragd	SGD	0.37	0.54
Hersbrucker Spät	HEB	0.38	0.54
Hallertauer Taurus	HTU	0.22	0.35
Herkules	HKS	0.24	0.35
Tettnanger	TET	0.56	0.97
Saphir	SIR	0.41	0.57
Hallertauer Tradition	HTR	0.50	0.77
Cascade	CAS	0.33	0.56
Hallertau Blanc	HBC	0.42	0.60
Huell Melon	HMN	0.34	0.41
Hallertauer Magnum	HMG	0.23	0.28
Spalter Select	SSE	0.44	0.75
Perle	PER	0.45	0.69
Hallertauer Mittelfrüh	HAL	0.34	0.60

aqueous solutions were made with double-distilled water and all chemicals were of analytical grade or higher.

2.2 Hop samples

14 different dried hop cone samples from crop 2018 were obtained from Hopsteiner, Germany. Table 1 features the different hop cultivars used. The hops were stored in vacuum-sealed packaging at −18 °C. Hops were then milled prior to usage to give the following particle size distribution: > 0.5 mm, 3 %; 0.25−0.5 mm, 23 %, 0.125−0.25 mm, 43 %; < 0.125 mm, 31 %. To test how much fructose and glucose are brought in from hops during the assay, fructose and glucose concentrations were assessed by adding 0.15 g of the hop samples to 10 mL of double-distilled water, and incubating them for 48 hours at 35 °C. They were subsequently analyzed by HPLC (Table 1).

2.3 Base beer characterization

A commercially available lager beer was obtained as a base beer for determining the release of fermentable carbohydrates from beer residual dextrins. The beer was characterized according to the Mitteleuropäische Brautechnische Analysenkommission e.V. (MEBAK) methodologies [15]: Extract (2.10.3), alcohol (2.10.7), color (2.13.2), pH (2.14), foam stability (2.19), total nitrogen (2.8.1), free amino nitrogen (2.8.4.1), total polyphenols (2.17.1), and bitter units (2.22.1). The numbers in parentheses indicate the method used. Carbohydrates were analyzed by HPLC. The beer data are presented in table 2.

Table 2 Analytical parameters for the base beer used in this study

parameter	value
real extract (% w/w)	4.05
gravity (% w/w)	11.62
alcohol (% v/v)	4.98
apparent attenuation [%]	81.4
real attenuation [%]	66.5
pH	4.42
color [°EBC]	6.1
bitter units [IBU]	28
fructose (g/L)	0.03
glucose (g/L)	0.09
maltose (g/L)	1.02

2.4 HPLC determination of fructose, glucose, and maltose concentration

HPLC analysis was performed according to the MEBAK, method 2.7.1. [15] with slight modifications and by using an Agilent 1100 HPLC system equipped with an AppliChrom OTUAmino-RP (250 mm x 4.6 mm). The flow rate was 1 mL/min and the mobile phase consisted of 23 % (v/v) water and 77 % (v/v) acetonitrile. The column temperature was set to 80 °C. A five-point calibration for the carbohydrates fructose, glucose, maltose, and maltotriose with $R^2 > 0.99$ was applied for determining the concentration in the samples.

2.5 Determination of beta-amylase and amyloglucosidase activity of hops by using Megazyme enzyme kits

The activity of beta-amylase and amyloglucosidase was measured according to the Megazyme kits BETAMYL-3 and R-AMGR3 with modifications according to Kirkpatrick et al. [10, 11]. As the hop enzymatic activity is much lower than that of barley malt, the incubation time was increased from 10 minutes to 48 hours.

2.6 Determining the diastatic activity of hops – method development and optimization

To develop a method for measuring the activity of hop diastatic enzymes, the existing method for malt diastatic activity R-200.23.031 from MEBAK was modified [14]. Response surface methodology (RSM) was further employed to investigate the relationships and interactions between the independent factors: starch concentration, hop concentration, and incubation temperature on glucose and maltose concentration. Fructose and maltotriose were not further investigated and are not reported here because they were found to be very low in all treatments when the concentrations already contained in the hops were subtracted. This is in accordance with findings from Kirkpatrick and Shellhammer [10, 11]. As maltose may also be degraded to glucose by hop amyloglucosidase activity, the response 'total mono hexoses' which represents the sum of glucose plus two times maltose concentration (1 maltose = 2 glucose units) was additionally implemented. The experimental design used was a central composite rotatable design (CCRD). The design matrix was a factorial three-level, three factor design with 6 central points to calculate the pure error. To maintain rotatability of the design matrix, 8 axial points were selected with $\alpha = \pm 1.414$. The concentrations of each component were coded at the levels ($-\alpha, -1, 0, +1, +\alpha$) (Table 3). The complete RSM experimental design consisted of 20 experimental points and is provided in table 4.

To produce the base starch solution, a defined weight of potato starch was initially stirred in cold, double-distilled water, and subsequently mixed with 800 mL of double-distilled boiling water. After 5 minutes of boiling, the solution was cooled to 20 °C, and the volume was made up to 1000 mL. Hallertauer Magnum hops were used for the optimization trials as it showed a high enzymatic activity in pre-trials. The hops were milled prior to addition to the starch solution, and the mixture was incubated at the temperatures given in table 5 using a temperature-controlled water bath. After incubation for 48 hours, the samples were centrifuged at 3600 g for 10 minutes. An aliquot of 2 mL of the supernatant was then mixed with 2 mL of acetonitrile to precipitate starch residues, and the aqueous phase was subsequently filtered using a 0.45 µm syringe filter prior to HPLC analysis. Fructose and glucose concentrations already contained in the hops (cp. Table 1) were subtracted from the sample concentration. The statistical significance of the different factors and their interaction was determined using analysis of variance (ANOVA). A backward elimination was applied to the data in which all blocks and forced terms were fit to the data first. According to the principle of hierarchy, non-significant terms were kept in the model if they were contained in other interaction terms that were found to be significant. This procedure is considered to

be a robust choice since all model terms will be given a chance to be included in the model [16]. Design-Expert software, version 11.1.0.1 was used for creating the experimental design and for data evaluation.

After employing the experiments, the optimal conditions for maximizing the carbohydrate concentration were determined by using the numerical optimization feature from the Design-Expert software. The following settings were used for optimization: temperature, hop concentration, and starch concentration. These were set to be in the range of the experimental design space (hop concentration, 5–15 g/L; starch concentration, 1–3 % (w/v); temperature, 25–45 °C); total mono hexose concentration as a measure for the total amount of fermentable carbohydrates was set to be maximized.

2.7 Hop diastatic activity in starch matrix at different pH values vs. beer matrix

To elaborate and compare the release of fermentable carbohydrates using the optimized method parameters, 10 mL of a 3 % (w/v) potato starch solution, or 10 mL of beer were incubated with 0.15 g of ground hop samples from 14 different German hop cultivars (Table 1). Incubation was carried out for 48 hours at 35 °C. After incubation, the samples were treated as described above and fructose, glucose, maltose, and maltotriose concentrations were then analyzed by HPLC. For every sample, initial fructose and glucose concentrations in the hops were subtracted from the sample concentration. After incubation (and subtracting fructose which was already contained in the hops) both fructose and maltotriose concentrations were very low and were therefore not taken into consideration. The response total mono hexose concentration was calculated again as the sum of glucose plus two times maltose.

The starch solution initially used for the trials had a pH of 6.6. To test the effect of pH on the release of glucose and maltose from the starch source, the pH of the starch solution was adjusted to 4.5 or 5.5 by addition of glacial acetic acid prior to carrying out the methodology as described above. All treatments were carried out in duplicate. Glucose, maltose, and total mono hexose concentrations as released from beer or from the different starch solutions after incubation were ultimately correlated. Pearson correlation coefficients with a significance level of $\alpha = 0.05$, and coefficients of determination (R^2) were calculated by using XLSTAT software, version 2016.02.27444 (Andernach, Germany).

2.8 Hop diastatic activity using beta-limit dextrin as substrate

Beta-limit dextrin is produced by fully hydrolyzing corn starch by a beta-amylase. To test how this substrate performs in the assay, a 3 % (w/v) beta limit dextrin solution was prepared, and the assay was carried out as described before. The release of total mono hexose from the substrate was again compared and correlated to the release of total mono hexoses from beer.

Table 3 Levels used in the response surface methodology study

	desired levels for CCRD				
variables	**−α**	**−1**	**0**	**1**	**+α**
starch concentration (% w/v)	0.32	1.00	2.00	3.00	3.68
hop concentration (g/L)	1.59	5.00	10.00	15.00	18.41
incubation temperature (°C)	18.18	25.00	35.00	45.00	51.82

Table 4 Response surface design with CCRD points, sequence numbers, and concentrations. The numbers in parentheses represent the coded levels (−1), (0), (+1), (+α), and (−α)

CCRD point	run sequence	starch conc. (% w/v)	hop conc. (g/L)	incubation temp. (°C)
central point	1, 5, 6, 7, 12, 14	(0) 2.00	(0) 10.00	(0) 35.00
factorial point	2	(−1) 1.00	(−1) 5.00	(+1) 45.00
factorial point	4	(−1) 1.00	(−1) 5.00	(−1) 25.00
factorial point	8	(+1) 3.00	(+1) 15.00	(−1) 25.00
factorial point	11	(+1) 3.00	(+1) 15.00	(+1) 45.00
factorial point	15	(+1) 3.00	(−1) 5.00	(+1) 45.00
factorial point	16	(+1) 3.00	(+1) 15.00	(−1) 25.00
factorial point	17	(−1) 1.00	(−1) 5.00	(-1) 25.00
factorial point	18	(−1) 1.00	(+1) 15.00	(+1) 45.00
axial point	3	(0) 2.00	(0) 10.00	(+α) 51.82
axial point	9	(0) 2.00	(+α) 18.41	(0) 35.00
axial point	10	(0) 2.00	(−α) 1.59	(0) 35.00
axial point	13	(0) 2.00	(0) 10.00	(−α) 18.18
axial point	19	(+α) 3.68	(0) 10.00	(0) 35.00
axial point	20	(−α) 0.32	(0) 10.00	(0) 35.00

3 Results and Discussion

Applying the modified enzyme kits according to Kirkpatrick and Shellhammer yielded partly erroneous results, and in some cases, the absorbance values of the blanks exceeded the values of the test sample (data not shown). The tests were then repeated up to nine times to exclude possible errors during the performance. Despite these many repetitions, it was not possible to obtain reproducible results. It is conceivable that some cultivars produce problems with the enzyme kits and others do not. This may explain why Kirkpatrick and Shellhammer [10, 11] were able to receive reliable data in their studies.

In a first step to develop a new method for measuring hop diastatic activity, the effect and interplay of the independent factors starch concentration, hop concentration, and incubation temperature on the dependent responses glucose, maltose, and total mono hexose concentrations (as the sum of glucose plus two times maltose) were investigated by using a RSM and a factorial design. Fructose and maltotriose were

not further examined as their concentrations were very low in the samples after the treatment. In table 5 the experimental data from all trials are shown. When processing the response, all factor interaction model terms and the quadratic term hop concentration × hop concentration were eliminated from the model because they were insignificant at $\alpha > 0.1$. For all remaining independent variables, a quadratic model was found to represent the experimental data best and a significant model F-value of 26.00 was found ($p < 0.001$). The factors starch concentration, hop concentration, temperature, starch concentration^2, and temperature^2 were significant model terms with $p < 0.05$, while starch concentration and hop concentration had the highest model F-values of 47.43 and 44.37, respectively (Table 6). The coefficient of determination (R^2) was calculated to be 0.9028 which implies that the sample variation of 90.28 % can be attributed to the significant independent variables, and only 9.72 % of the total variation was not explained by the model (Table 7). These data demonstrate a satisfactory adjustment of the model to the experimental data. The adequate precision as measured by the signal to noise ratio of 17.16 indicates an adequate signal and thus further supports the applicability of the model and 'goodness' of the response signals. The predicted R^2 of 0.7698 is in reasonable agreement with the adjusted R^2 of 0.8681. The R^2 adjusted corrects the 'raw' R^2 for the sample size and number of terms in the model and should be close to R^2 which was given for both models [17].

In figure 1, the response from the different independent variables incubation temperature and starch concentration are plotted. The temperature clearly shows a maximum at 30 – 35 °C, while for both variables, starch concentration, and hop concentration (not shown),

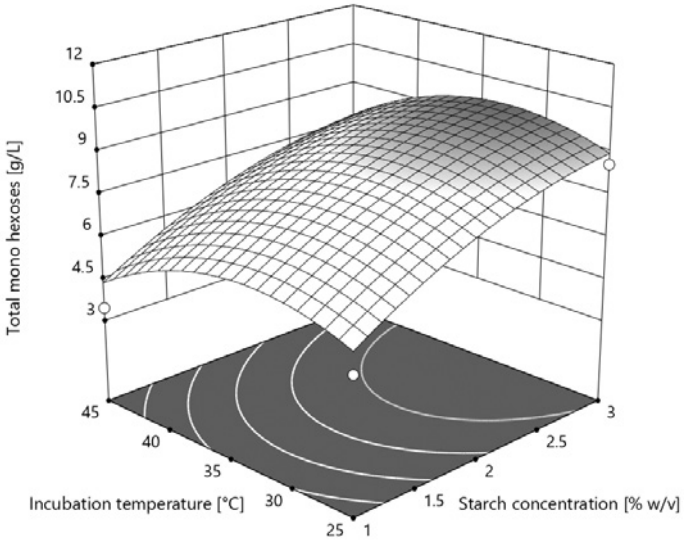

Fig. 1 3D response surface diagram for the response total mono hexose concentration and the effect of the independent factors incubation temperature and starch concentration; hop concentration was set at 15 g/L

a higher concentration yields a higher response. For method optimization, all independent variables were kept in the range of the experimental design and total mono hexose concentration was maximized, yielding the following optimal experimental conditions: starch concentration, 3 % (w/v); hop concentration, 15 g/L, and incubation temperature, 33.67 °C. For simplicity, a temperature of 35 °C was chosen.

Table 5 Summary data of the response surface methodology experiment

run #	space type	starch conc. (% w/v)	incubation temperature (°C)	hop conc. (g/L)	glucose conc. (g/L)	maltose conc. (g/L)	total mono hexoses (g/L)
1	center	2	35	10	2.27	2.31	7.16
2	factorial	1	45	5	1.42	0.41	2.37
3	axial	2	51.82	10	1.97	< 0.1	2.19
4	factorial	1	25	15	1.81	1.3	4.76
5	center	2	35	10	2.38	2.38	7.35
6	center	2	35	10	2.25	2.25	6.98
7	center	2	35	10	2.28	1.87	6.22
8	factorial	3	25	15	2.36	3	8.57
9	axial	2	35	18.41	4.46	2.96	10.73
10	axial	2	35	1.59	0.62	0.85	2.44
11	factorial	3	45	5	1.90	1.33	4.65
12	center	2	35	10	2.48	2.33	7.36
13	axial	2	18.18	10	1.17	1.6	4.58
14	center	2	35	10	2.69	2.21	7.29
15	factorial	3	45	15	4.05	1.48	7.29
16	factorial	3	25	5	1.11	1.81	4.84
17	factorial	1	25	5	0.89	1.22	3.44
18	factorial	1	45	15	2.47	0.36	3.45
19	axial	3.68	35	10	2.48	3.09	8.86
20	axial	0.32	35	10	0.93	0.2	1.64

Table 6 **ANOVA data for the dependent significant factors of the response surface reduced quadratic model; insignificant model terms were eliminated by backward selection at α > 0.1**

	total mono hexoses	
factor	**F-value**	**p-value[a]**
starch concentration	47.73	< 0.0001
hop concentration	44.37	< 0.0001
incubation temperature	5.33	0.0367

[a] probability of the F-value. It is the probability of getting an F-value of this size if the term did not have an effect on the response

Table 7 **ANOVA model data for response surface reduced quadratic model**

values	total mono hexose response
R2	0.9028
adjusted R2	0.8681
predicted R2	0.7798
adequate precision	17.1615
model F-value	26.00
lack of fit F-value	6.47

In a next step, the optimized hop diastatic assay was assessed with respect to the release of fructose, glucose, maltose, and maltotriose. 14 different hop samples were incubated for 48 hours with 3 % (w/v) potato starch at T = 35 °C, or with beer, at a hop dosage of 15 g/L. To elaborate the effect of pH, additional experiments were conducted where the pH of the starch solution (pH 6.6) was adjusted to pH 5.5 or 4.5 by using acetic acid. When substracting out the fructose concentration as already contained in the different hop samples, remaining concentrations were again very low and fructose was therefore not taken into consideration. Maltotriose was also very low and was therefore not included in subsequent calculations. Total mono hexose concentration were calculated again.

The carbohydrate concentrations after incubation and analysis are shown in table 8. Glucose and maltose concentrations in beer after incubation with hops ranged from 1.22 to 2.05 g/L and 2.80 to 4.58 g/L, respectively. When using the starch substrate at pH 6.6, glucose and maltose ranged from < 0.1 g/L to 2.31 g/L and 2.01 to 5.31 g/L. When lowering the pH to 5.5 or 4.5, in individual trials, glucose concentrations increased and maltose concentrations decreased. This is an indication that there is amyloglucosidase activity present among the hop varieties which appears to be more active at a lower pH. The slightly acidic pH optimum of amyloglucosidase is in accordance with literature reports [18]. This observation is furthermore a hint that maltose concentration alone may not be good predictor of hop diastatic activity, as 'uncontrolled' degradation to glucose may occur which would not be considered.

When correlating the carbohydrates found after incubation from beer and from the starch substrates at different pH values, maltose and total mono hexoses at pH 6.6 showed the highest significant Pearson correlation coefficient of 0.809 – 0.853 ($p < 0.001$) (Table 9). Lowering the pH of the starch substrates yielded also lower Pearson correlation coefficients. However, the correlation was still significant for maltose and total mono hexoses at $p < 0.05$. Glucose was not a good predictor and only showed significant correlation at pH 5.5 with the beer data set.

The coefficients of determination (R^2) were highest for maltose and total mono hexoses with 0.72 at pH 6.6 and were almost similar (Table 10). Because of the hops' amyloglycosidase activity, solely analyzing maltose can not be recommended with these data as it may also be degraded to glucose if there is higher amyloglycosidase activity present. Measuring solely glucose also showed very low coefficients of determination and can likewise not be

Table 8 **Data summary of carbohydrate concentration increase after 48 hours of incubation; Glc = glucose, Mal = maltose, Hex = total mono hexoses, for hop abbreviations see table 1; all values are given in g/L**

hop cultivar	beer			starch, pH 4.5			starch, pH 5.5			starch, pH 6.6		
	Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex
SGD	1.85	3.68	9.22	3.17	2.19	7.56	1.51	1.66	4.83	2.31	2.85	8.01
HEB	1.96	3.51	8.98	2.91	1.01	4.93	1.50	1.19	3.89	0.77	2.47	5.71
HTU	1.39	3.78	8.95	2.69	1.58	5.84	1.43	1.81	5.04	1.04	2.80	6.64
HKS	1.22	3.56	8.34	2.30	1.31	4.91	1.05	1.29	3.62	1.49	2.61	6.71
TET	1.63	3.30	8.22	3.84	1.83	7.50	1.35	1.58	4.52	0.58	3.76	8.10
SIR	1.93	3.45	8.83	4.11	1.77	7.65	1.53	1.28	4.09	1.99	2.72	7.44
HTR	1.75	4.10	9.94	3.81	2.03	7.87	1.45	1.95	5.34	0.60	4.67	9.94
CAS	1.90	4.43	10.76	2.76	1.62	6.00	1.77	2.73	7.23	0.81	4.60	10.02
HBC	1.58	3.01	7.59	2.87	1.18	5.24	1.40	1.14	3.68	< 0.1	2.01	3.41
HMN	1.36	4.58	10.53	3.28	2.48	8.24	0.86	2.50	5.86	0.66	4.73	10.13
HMG	1.90	4.55	11.00	4.08	3.64	11.36	2.41	4.65	11.71	2.15	5.31	12.78
SSE	2.05	2.80	7.65	3.28	1.22	5.72	1.31	1.48	4.27	1.50	2.46	6.43
PER	1.93	3.42	8.77	4.10	1.91	7.92	1.72	1.76	5.23	1.88	3.49	8.86
HAL	1.92	3.83	9.58	3.72	1.38	6.48	1.28	1.77	4.83	1.28	3.14	7.57

Table 9 **Pearson correlation coefficients of carbohydrate concentration increase after 48 hour incubation with beer or starch substrates at pH 4.5, 5.5, or 6.6. Glc = glucose, Mal = maltose, Hex = total mono hexoses**

		beer			starch, pH 4.5			starch, pH 5.5			starch, pH 6.6		
		Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex
beer	Glc	**1**	−0.154	0.086	0.524	0.027	0.201	**0.550**	0.113	0.195	0.372	0.020	0.136
	Mal	−0.154	**1**	**0.971**	0.082	**0.665**	**0.545**	0.255	**0.761**	**0.712**	0.052	**0.841**	**0.809**
	Hex	0.086	**0.971**	**1**	0.209	**0.677**	**0.598**	0.390	**0.795**	**0.765**	0.142	**0.853**	**0.849**
starch, pH 4,5	Glc	0.524	0.082	0.209	**1**	0.519	**0.747**	0.409	0.306	0.340	0.348	0.407	0.493
	Mal	0.027	**0.665**	**0.677**	0.519	**1**	**0.956**	**0.542**	**0.860**	**0.849**	0.421	**0.776**	**0.864**
	Hex	0.201	**0.545**	**0.598**	**0.747**	**0.956**	**1**	**0.562**	**0.774**	**0.777**	0.447	**0.743**	**0.841**
starch, pH 5,5	Glc	**0.550**	0.255	0.390	0.409	**0.542**	**0.562**	**1**	**0.657**	**0.750**	0.418	0.380	0.490
	Mal	0.113	**0.761**	**0.795**	0.306	**0.860**	**0.774**	**0.657**	**1**	**0.991**	0.269	**0.827**	**0.864**
	Hex	0.195	**0.712**	**0.765**	0.340	**0.849**	**0.777**	**0.750**	**0.991**	**1**	0.308	**0.791**	**0.843**
starch, pH 6,6	Glc	0.372	0.052	0.142	0.348	0.421	0.447	0.418	0.269	0.308	**1**	0.022	0.335
	Mal	0.020	**0.841**	**0.853**	0.407	0.776	**0.743**	0.380	**0.827**	**0.791**	0.022	**1**	**0.950**
	Hex	0.136	**0.809**	**0.849**	0.493	**0.864**	**0.841**	0.490	**0.864**	**0.843**	0.335	**0.950**	**1**

Values in bold are different from 0 with a significance level $\alpha = 0.05$

Table 10 **Coefficient of determination (R^2) of carbohydrate concentration increase after 48 hour incubation with beer or starch substrates at pH 4.5, 5.5, or 6.6; Glc = glucose, Mal = maltose, Hex = total mono hexoses**

		beer			starch, pH 4.5			starch, pH 5.5			starch, pH 6.6		
		Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex	Glc	Mal	Hex
beer	Glc	**1**	0.024	0.007	0.274	0.001	0.040	0.303	0.013	0.038	0.138	0.000	0.018
	Mal	0.024	**1**	0.943	0.007	0.443	0.298	0.065	0.579	0.507	0.003	0.708	0.655
	Hex	0.007	0.943	**1**	0.044	0.459	0.358	0.152	0.632	0.585	0.020	0.728	0.721
starch, pH 4,5	Glc	0.274	0.007	0.044	**1**	0.269	0.558	0.167	0.094	0.115	0.121	0.166	0.243
	Mal	0.001	0.443	0.459	0.269	**1**	0.914	0.294	0.740	0.720	0.178	0.602	0.746
	Hex	0.040	0.298	0.358	0.558	0.914	**1**	0.316	0.599	0.603	0.200	0.553	0.707
starch, pH 5,5	Glc	0.303	0.065	0.152	0.167	0.294	0.316	**1**	0.432	0.563	0.175	0.145	0.240
	Mal	0.013	0.579	0.632	0.094	0.740	0.599	0.432	**1**	0.983	0.072	0.683	0.746
	Hex	0.038	0.507	0.585	0.115	0.720	0.603	0.563	0.983	**1**	0.095	0.626	0.710
starch, pH 6,6	Glc	0.138	0.003	0.020	0.121	0.178	0.200	0.175	0.072	0.095	**1**	0.001	0.112
	Mal	0.000	0.708	0.728	0.166	0.602	0.553	0.145	0.683	0.626	0.001	**1**	0.902
	Hex	0.018	0.655	0.721	0.243	0.746	0.707	0.240	0.746	0.710	0.112	0.902	**1**

recommended. Again, a lower pH of the starch substrate resulted in lower R^2 values. Taking together all data, using a starch substrate at pH 6.6 appears to be a promising strategy for determining hop diastatic activity.

A beta-limit dextrin substrate was also tested. This product is made of corn starch which is fully digested with solely beta-amylase. The usage of beta-limit dextrin instead of a starch substrate at a concentration of 3 % (w/v) resulted in very low and insignificant Pearson correlation coefficients of −0.085 ($p = 0.773$) for total mono hexose concentration when correlating it with the beer data set. Also, the R^2 value for this data set was very low ($R^2 = 0.007$) and no correlation for any of the carbohydrate responses was found (data not shown). Beta-limit dextrin is hence not a suitable substrate for this application. This furthermore indicates that beta-amylase activity plays a major role in the release of fermentable carbohydrates by hop diastases.

4 Conclusion

A new assay for the determination of hop diastatic activity was developed. Potato starch was used as a substrate and a starch concentration of 3 % (w/v), a hop concentration of 15 g/L, and an incubation temperature of 35 °C were found to be optimal experimental conditions for maximizing total mono hexose concentration. Within the scope and parameters tested in this study, a pH of 6.6 was found to be more appropriate than pH 4.5 or pH 5.5 when correlating carbohydrate release from the lager beer used and the starch substrate. It should be considered that different starch or dextrin sources and other beer matrices may behave differently which demands further testing. A beta-limit dextrin was found to be an unsuitable substrate. Even though using potato starch as a substrate showed good results, in future work, the method will be further optimized by testing the applicability of different starch and dextrin substrates for measuring hop diastatic activity. Furthermo-

re, once a final methodology is established, method ruggedness and further validation procedures such as the reapeatability will be elaborated. Ultimately, a reliable and reproducible method for determining hop diatatic activity will be proposed.

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Received 9 June, 2021, accepted 27 July, 2021