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Optimisation of the Action of Commercial Mashing Enzymes on Wort Extracts and Free Amino Nitrogen of the *Safrari* Sorghum Cultivar

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ABSTRACT

The influence of three commercial mashing enzymes (Hitempase 2XL, Bioglucanase TX and Brewers protease) used as sole mashing enzymes on yields of extract and free amino nitrogen (FAN) of the worts of the mashes of unmalted and malted *Safrari* sorghum was studied using the response surface methodology. The study revealed that increasing amounts of Hitempase considerably increased yields of extract during mashing of unmalted *Safrari* grist, while the effect of Bioglucanase was smaller, and that of Brewers protease was insignificant. Extract yields decreased with increasing amounts of the three enzymes during the mashing of malted *Safrari*. This decrease was least expressed in the case of Brewers protease. Yields in FAN amounted to less than 50%, with increasing amounts of both Hitempase and Brewers protease, but constantly decreased to nil for Bioglucanase's action in both unmalted and malted *Safrari* mashes. The milling operation singularly liberated more than 50% of FAN for both mash types and for each of the enzymes. Optimisation of the concerted actions of the three enzymes for extract yields gave a combination of 2,098.5 U, 937.5 BGU and 0 mg (for Hitempase, Bioglucanase and Brewers protease, respectively) for unmalted *Safrari* sorghum mash. This gave a maximal extract yield of 18°P. The combination was 0 U, 28.68 BGU and 0 mg for malted *Safrari* sorghum mash, giving a maximal extract yield of 18.82°P. Optimisation for FAN gave a combination of 2,434.5 U, 0 BGU and 100 mg (for Hitempase, Bioglucanase and Brewers protease, respectively) for unmalted *Safrari* sorghum mash. This gave maximal FAN of 144.77 mg/L. The combination was 2,191.5 U, 0 BGU and 100 mg for malted *Safrari* sorghum mash, giving a maximal FAN of 196.73 mg/L.

Keywords: commercial mashing enzymes, extract, free amino nitrogen, optimisation, *Safrari.*

SÍNTESIS

Se estudiaron, utilizando el método de superficie de respuesta, tres enzimas comerciales de maceración (Hitempase 2XL, Bioglucanase TX y "Brewers protease") con respecto al rendimiento de extracto y de amino nitrógeno libre (FAN) en mostos de macerados de sorgo *Safrari* malteado y no malteado. El estudio reveló que un aumento en la cantidad de Hitempase aumentó considerablemente el rendimiento de extracto de un macerado de *Safrari* no malteado, mientras que el efecto de de la Biogluconase era menor y el efecto de la "Brewers protease" era insignificante. El rendimiento de extracto disminuyó con un aumento en la cantidad de las tres enzimas en el macerado de *Safrari* malteado, siendo menor la disminución con el "Brewers protease". El rendimiento de FAN fue menor del 50 por ciento con el aumento en las cantidades tanto de Hitempase y de "Brewers protease" y con Biogluconase se fue bajando hasta cero, tanto en macerados de *Safrari* malteado como no malteado. La molienda liberó por sí solo más de 50 por ciento del FAN para los dos tipos de macerado y para cada uno de las enzimas. La optimización del uso conjunto de las tres enzimas para aumentar el rendimiento de extracto resultó en la combinación 2.098,5 U, 937,5 BGU y 0 mg (para Hitempase, Bioglucanase y "Brewers protease", respectivamente) para macerados de sorgo *Safrari* no malteado, resultando en un extracto máximo de 18°P. La combinación óptima para *Safrari* malteado fue de 0 U, 28,68 BGU y 0 mg dando un extracto máximo de 18,82°P. La combinación óptima para FAN fue de 2.434,5 U, 0 BGU y 100 mg para *Safrari* no malteado, dando un FAN máximo de 144,77 mg/L, mientras que para *Safrari* malteado fue de 2.191,5 U, 0 BGU y 100 mg, para un FAN máximo de 196,73 mg/L.

Palabras claves: enzimas comerciales de maceración, extracto, amino nitrógeno libre (FAN), optimización, sorgo *Safrari.*

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Introduction

Sorghum in its malted form or as adjuncts has become a potential brewing cereal, particularly in the tropics where barley is not commonly grown[6,14,24,25,26,30,31,32,33,34]. Its low contents of potential mashing enzymes due to their poor development during malting has often triggered the use of commercial mashing enzymes as supplements to achieve higher yields in extracts and other important wort specifications in beer brewing[1,2,3,4,5,7,8,16,17,19,20,28,29,37,38]. The inclusion of commercial mashing enzymes when mashing sorghum malts and adjuncts is important[9,10,11,14,15,22,23,24,27], but the amount of enzymes needed for optimal mashing may vary from one sorghum cultivar to the next. It is also not clear whether the use of commercial mashing enzymes for optimal mashing of malted sorghum is indispensable. In this work, the influence of three commercial mashing enzymes (Hitempase 2XL, Bioglucanase TX and

Table 1. Characteristics of commercial mashing enzyme preparations.

Commercial mashing enzyme	Organism of origin	Activity	Description	Temperature optima	pH optima	Recommended application level in adjuncts	Form
Hitempase 2XL[a]	*Bacillus licheniformis*	4,416.29 ± 19.34 U/ml	α-amylase	60–95°C	4–8	60 U/g	Solution
Bioglucanase TX[a]	*Trichodermareesei*	750 BGU/ml	β-glucanase	60°C	4.5–6.5	0.01 and 0.025% (v/w)	Solution
Brewers protease[b]	*Bacillus amyloliquefaciens*	1,842.2 ± 1.8 mg FAN/min/ml	Protease	45–50°C (denatured at 85°C)	6.5–7.5	0.4–2 g/kg	Solution

[a] Hitempase 2XL and Bioglucanase TX were obtained from Kerry Bioscience; Kilnagleary, Carrigaline, Co. Cork, Ireland.
[b] Brewers protease was obtained from DSM Food Specialities, Cedex France.

Brewers protease) was modelled and optimised for yields of extracts and free amino nitrogen during mashing of unmalted and malted *Safrari* sorghum cultivar using the response surface methodology (RSM).

Materials and Methods

Enzymes

The characteristics of the commercial enzymes used (Hitempase 2XL, a thermo stable α-amylase from *Baccillus licheniformis;* Bioglucanase TX, an enzymatic composition of β-glucanase and hemicellulases from *Trichodermareesel;* and Brewers protease from *Bacillus amyloliquefaciens*) are presented in Table 1.

Sorghum Cultivar

The *Safrari* sorghum cultivar was obtained from the Institute of Research and Agronomic Development (IRAD) Maroua, Cameroon.

Modelling

Modelling was carried out as previously described[15]. A Doehlert matrix design with three factors representing Hitempase (X_1), Bioglucanase (X_2) and Brewers protease (X_3) at ranges of 0–3,000 U, 0–937.5 BGU and 0–100 mg, respectively, was used. The transformed matrix of coded variables to an experimental matrix and desired responses (extract and free amino nitrogen) are shown in Table 2. The coefficients of the models and the models were obtained using the Systat version 12 software (Systat Software, Inc., San Jose, USA). This software also gives a statistical analysis on the model. Lastly, the curves were plotted using Sigmaplot version 11 build 11.0.0.77 software (WPCubed, GmbH, Germany).

Validation of Models

The models were validated using two procedures. The first consited of coupling the method earlier described[15] to the absolute average deviation (AAD) method[13]. The second procedure consisted of applying the method described[12,36].

Malting

About 1 kg of *Safrari* sorghum cultivar grains were washed three times using 3 litres of distilled water to remove dirt and other foreign bodies. The grains were steeped in 3 litres of distilled water for 48 h at room temperature (≈25°C) with three changes of water at intervals of 12 h before steep out. Germination was carried out for 4 days in a Heraeus type oven (D-63450 Hanau, Germany) at a temperature of 25°C with water sprinkled on the grains on daily basis. The malt was then air dried at 40°C for 4 days using a CKA 2000 AUF-type dryer (Ngaoundere, Cameroon). The malt was rubbed-off of its rootlets and stored until further use.

Mashing

Two hundred and fifty ml of distilled water were put into a 600 ml beaker and 50 g of sorghum (malted or unmalted) flour (Ø < 1 mm) added with continuous stirring until a homogenous mixture was obtained. This mixture was incubated at 45°C for 1 h in a water bath with intermittent stirring at intervals of 5 min. The mix was allowed to decant and 50 ml of the supernatant withdrawn and kept aside. The temperature of the mash was then raised to boiling so as to gelatinise sorghum starch during 40 min with intermittent stirring at intervals of 5 min before cooling to 65°C. The 50 ml of supernatant, to which commercial enzymes are added according to the Doehlert matrix design of three factors, were added to the mash and mashing continued for 1 h and 30 min with intermittent stirring at intervals of 10 min. The mash was cooled and filtered at 25°C for 1 h and 30 min using Whatmann paper no. 42.

Determination of Extract

Extract was determined as described by analytica-EBC[18].

Determination of Free Amino Nitrogen

Free amino nitrogen (FAN) was determined using the Ninhydrin method as described by analytica-EBC[18].

Optimisation of Models

Models were optimised as previously described[15]. The optimal zone of intersection of the curves was highlighted.

Results and Discussion

Optimisation of the action of mashing enzymes on the two key mashing parameters, extract and FAN, was carried out by modelling the experimental design required for laboratory purposes. Table 2 shows the results obtained for extracts and free amino nitrogen (FAN) after mashing unmalted and malted *Safrari* using the commercial mashing enzymes Hitempase (α-amylase), Bioglucanase (β-glucanase) and Brewers protease (protease).

The mathematical models obtained for extracts after mashing unmalted and malted *Safrari* were as follows, respectively:

$$Y_{SafEX}(X_1, X_2, X_3) = 14.62 + 5.478X_1 + 1.226X_2 - 0.881X_3 + 0.527X_1X_2 + 0.589X_1X_3 - 2.008X_2X_3 - 6.569X_1^2 - 0.935X_2^2 - 0.496X_3^2$$

$$Y_{SafMEX}(X_1, X_2, X_3) = 16.566 + 1.076X_1 - 1.377X_2 - 0.469X_3 + 4.774X_1X_2 + 2.338X_1X_3 - 1.326X_2X_3 - 0.901X_1^2 - 3.117X_2^2 - 0.180X_3^2$$

With: $Y_{SafEX}(X_1, X_2, X_3)$ representing the mathematical model for unmalted *Safrari;* $Y_{SafMEX}(X_1, X_2, X_3)$, the model for

Table 2. Matrices of Doehlert coded and transformed experimental values.

Coded values			Transformed experimental values			*Safrari*											
						Unmalted						Malted					
						Extract			FAN			Extract			FAN		
Hit X_1	Bio X_2	Brew prot X_3	Hit (U) X_1	Bio (BGU) X_2	Brew prot (mg) X_3	Exp[a]	Theo[b]	Res[c]	Exp	Theo	Res	Exp	Theo	Res	Exp	Theo	Res
0.000	0.000	0.000	1,500	468.75	50	14.81	14.62	0.19	95	91.78	3.22	16.43	16.57	–0.14	140	136.97	3.03
1.000	0.000	0.000	3,000	468.75	50	14.21	13.53	0.68	88	90.76	–2.76	16.64	16.74	–0.10	132	131.32	0.68
0.500	0.866	0.000	2,250	937.5	50	15.89	16.31	–0.42	110	97.91	12.09	15.60	15.42	0.18	165	151.42	13.58
–0.500	–0.866	0.000	750	0.00	50	8.75	8.70	0.05	100	106.23	–6.23	16.02	16.72	–0.70	149	154.06	–5.06
0.500	–0.866	0.000	2,250	0.00	50	13.40	13.73	–0.33	135.60	128.72	6.88	14.56	13.67	0.89	189	182.34	6.66
–0.500	0.866	0.000	750	937.5	50	10.73	10.37	0.36	63	70.41	–7.41	9.36	10.21	–0.85	94.50	101.93	–7.43
0.500	0.289	0.816	2,250	615.18	100	14.29	14.79	–0.50	118.33	124.00	–5.67	16.64	17.05	–0.41	177.50	186.44	–8.94
–0.500	–0.289	–0.816	750	312.32	0.0	9.98	10.04	–0.06	66.33	51.87	14.46	17.16	17.53	–0.37	99.50	77.78	21.72
0.500	–0.289	–0.816	2,250	312.32	0.0	14.55	14.88	–0.33	61.67	66.12	–4.46	14.56	15.32	–0.76	92.50	100.32	–7.82
0.000	0.577	–0.816	1,500	781.07	0.0	16.44	16.35	0.09	38.33	43.54	–5.21	16.33	15.62	0.71	57.50	64.42	–6.92
–0.500	0.289	0.816	750	615.18	100	9.06	8.68	0.38	91.92	88.27	3.65	13.52	12.69	0.83	137.88	131.22	6.66
0.000	–0.577	0.816	1,500	156.43	100	13.61	13.50	0.11	126	123.99	2.01	16.02	16.44	–0.42	178	175.72	2.28
0.000	0.000	0.000	1,500	468.75	50	14.50	14.62	–0.12	85.33	91.78	–6.45	16.64	16.57	0.07	128	136.97	–8.97
–1.000	0.000	0.000	0	468.75	50	1.80	2.57	–0.77	45	40.78	4.22	14.56	14.59	–0.03	55	53.55	1.45
–1.000	–0.866	–0.816	0	0.0	0.0	1.02	0.72	0.30	54	58.80	–4.80	19.24	18.81	0.43	81	87.97	–6.97
0.000	0.000	0.000	1,500	468.75	50	14.60	14.62	–0.02	91.67	91.78	–0.11	16.64	16.57	0.07	137.50	136.97	0.53
0.000	0.000	0.000	1,500	468.75	50	15.00	14.62	0.38	88.33	91.78	–3.45	17.16	16.57	0.59	132.50	136.97	–4.47

[a] Experimental result values.
[b] Theoretical values (values coming from mathematical models).
[c] Residue.

Table 3. Estimation of regression coefficients for the extracts of umalted and malted *Safrari.*

Effects	Coefficient		Std. deviation		t-statistics		P-value	
	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted
CONSTANT	14.62	16.566	0.271	0.396	54.039	41.882	0.000	0.000
X_1	5.476	1.076	0.278	0.407	19.683	2.644	0.000	0.033
X_2	1.226	–1.377	0.243	0.355	4.374	–3.361	0.003	0.012
X_3	–0.881	–0.469	0.229	0.335	–3.136	–1.141	0.016	0.291
X_1^2	–6.569	–0.901	0.477	0.698	–13.768	–1.291	0.000	0.238
X_2^2	–0.935	–3.117	0.347	0.508	–2.020	–4.604	0.083	0.002
X_3^2	–0.496	–0.18	0.300	0.438	–1.103	–0.274	0.307	0.792
X_1*X_2	0.527	4.774	0.545	0.796	0.839	5.192	0.429	0.001
X_2*X_3	–2.008	–1.326	0.479	0.701	–2.960	–1.336	0.002	0.223
X_1*X_3	0.589	2.338	0.568	0.83	0.846	2.299	0.425	0.055

malted *Safrari;* X_1, Hitempase; X_2, Bioglucanase and X_3 Brewers protease.

These mathematical models are polynomials having several variables with correlation coefficients $R^2 = 0.993$ for unmalted *Safrari* and $R^2 = 0.930$ for malted *Safrari*. These coefficients, coupled to AAD values of 0.026 and 0.031 for unmalted and malted *Safrari,* respectively, allowed for the validation of the models for the wort extract yields. In addition, a bias factor of 1, coupled to exactitude factors of 1.06 and 1.03 for both unmalted and malted *Safrari,* respectively, also allowed for validation of the models according to the method described[12,36]. The factors of the models were linear or of first degree (X_1, X_2 and X_3), quadratic or of the second degree (X_1^2, X_2^2 and X_3^2) and of interacting form (X_1X_2, X_1X_3 and X_2X_3). They were statistically considered significant or not if the probability (P) of increasing yields of extracts was ≤0.05 or ≥0.05, respectively (Table 3).

The impact of Hitempase as sole mashing enzyme on the yields of extract of unmalted and malted *Safrari* is shown in Figure 1A. Extract yield increased with increasing concentration of enzyme for unmalted *Safrari* mash to attain a maximum level (≈13°P) at about 2,000 U, followed by a slight and steady decrease thereafter. It was already maximal (≈18.5°P) even in absence of Hitempase for malted *Safrari* mash. Indeed, increasing amounts in enzyme concentration instead induced a steady decrease of extract yield for malted *Safrari* mash. However, when the mathematical models were applied to predict the impact of supplements of Bioglucanase and Brewers protease at concentrations of 400 BGU and 60 mg, respectively, it was observed that the profile of extract remained fairly constant with increasing amounts of Hitempase for malted *Safrari* mash (Fig. 1B). Starch is indeed the main macromolecule of cereals and the main substrate of α-amylase. It is therefore expected that this enzyme contributes the greatest amount of soluble materials that could be found in resulting worts due to its action on starch[15,35]. Figures 1A and B also showed that supplements of Hitempase in malted *Safrari* mash was of no importance. The steady decrease in yields of extract of malted *Safrari* mash could be attributed to Maillard reactions between soluble nitrogenous compounds and reducing sugars earlier produced during malting (Fig. 1A). These results also indicated that efficient mashing was obtainable in the presence of the key mashing enzymes developed during malting (Fig. 1A). From the mathematical model, it was shown that in its linear form (X_1), Hitempase contributed 29% and 7% of extract yields for unmalted and malted *Safrari,* respectively (Figs. 2A and B). Statistical analyses also showed that this contribution was significant (P = 0.000 and 0.033 for unmalted and malted

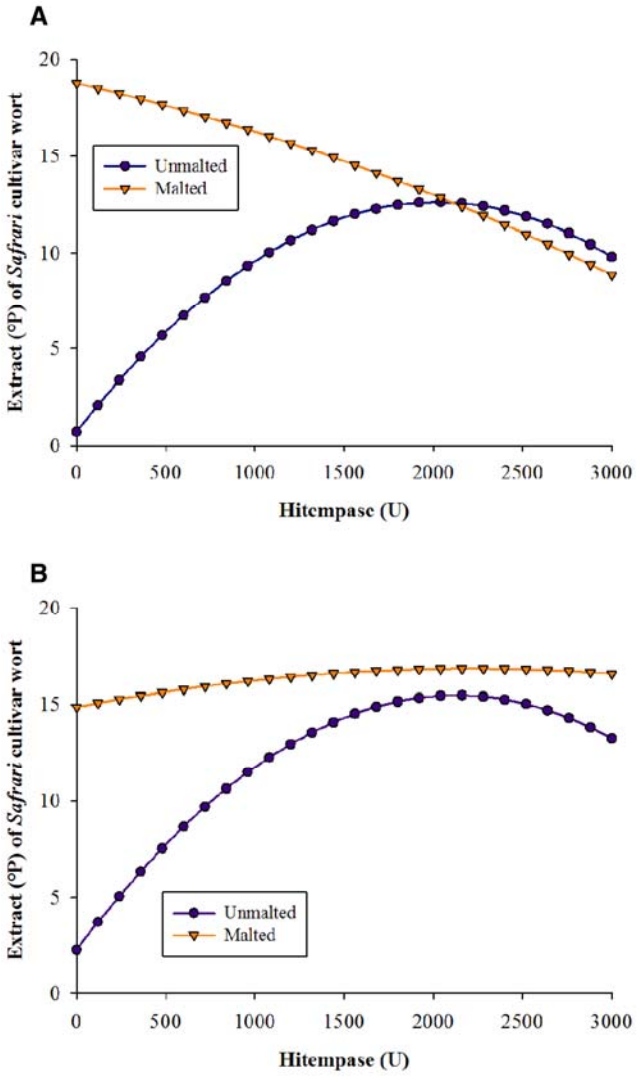

Figure 1. A, Effect of concentration of Hitempase (α-amylase) as sole mashing enzyme (concentrations of Bioglucanase and Brewers protease set at 0) on yield of wort extract of sorghum cultivar *Safrari.* **B,** Effect of concentration of Hitempase (α-amylase) in the presence of fixed concentrations of Bioglucanase (400 BGU) and Brewers protease (60 mg) on yield of wort extract of sorghum cultivar *Safrari.*

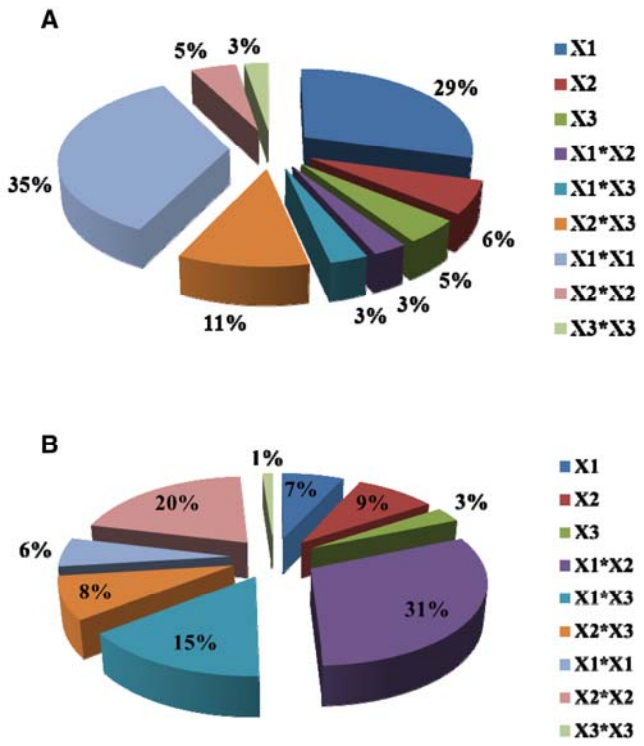

Figure 2. A, Contribution to yield of wort extract (°P) of each factor in its linear, quadratic and interaction (combined) forms for unmalted sorghum cultivar *Safrari*. **B,** Contribution to yield of wort extract (°P) of each factor in its linear, quadratic and interaction (combined) forms for malted sorghum cultivar *Safrari*.

Safrari, respectively) (Table 3). In its quadratic form (X_1^2), Hitempase remained statistically significant for mashing unmalted *Safrari,* but not for malted *Safrari* (P = 0.000 and 0.238, respectively). This confirmed the above biological observation according to which supplements of this enzyme in malted mashes of *Safrari* were of no importance. Its contribution to increasing extract yields in its quadratic form (X_1^2) (excess of α-amylase in principle) is indeed 35% and 6% for unmalted and malted *Safrari,* respectively (Figs. 2A and B).

Figure 3A shows the effect of mashing unmalted and malted *Safrari* using Bioglucanase as sole mashing enzyme on yields of extract. There was a slight and constant increase in yields of extract as enzyme concentration increased for unmalted *Safrari* mash. The yields of extract for malted *Safrari* mash was maximal even in the absence of Bioglucanase and steadily decreased with an increase in enzyme concentration. The small yields of extract due to Bioglucanase action in unmalted *Safrari* mash can be attributed to its ability to hydrolyse β-glucans into glucose and other soluble carbohydrates. Bioglucanase was therefore not a backbone enzyme for extract production during mashing. A similar application of the mathematical models as carried out above for Hitempase's action, using 60 mg of Brewers protease and 2,000 U of Hitempase, predicted that supplements of these two key mashing enzymes could provide similar results in extract yields for both unmalted and malted *Safrari* mashes (Fig. 3B). Hitempase once more demonstrated that it was the backbone enzyme contributing to most of the extract yields. Figure 3B indeed showed that in the absence of Bioglucanase, but in the presence of Hitempase, yields of extract became comparable for both unmalted and malted *Safrari* mashes. These observations were all statistically confirmed. Indeed, in its linear form (X_2), Bioglucanase's action was significant (P = 0.003 and 0.012 for unmalted and malted *Safrari,* respectively) (Table 3). Figures 2A and B showed that this enzyme contributed for 6% and 9% of extract yield for unmalted and malted *Safrari,* respectively. In its quadratic form (X_2^2) (excess of enzyme in principle), Bioglucanase contributed for 5% and 20% of extract yield for unmalted and malted *Safrari,* respectively (Figs. 2A and B). These contributions were not statistically significant for unmalted *Safrari* mash, but were for malted *Safrari* mash (P = 0.083 and 0.002) (Table 3). Once more, the observed 1:4 ratio difference of extract yield between unmalted *Safrari* mashes as to malted *Safrari* mashes could be attributed to the impact of enzymes developed during malting and not to Bioglucanase supplements as such.

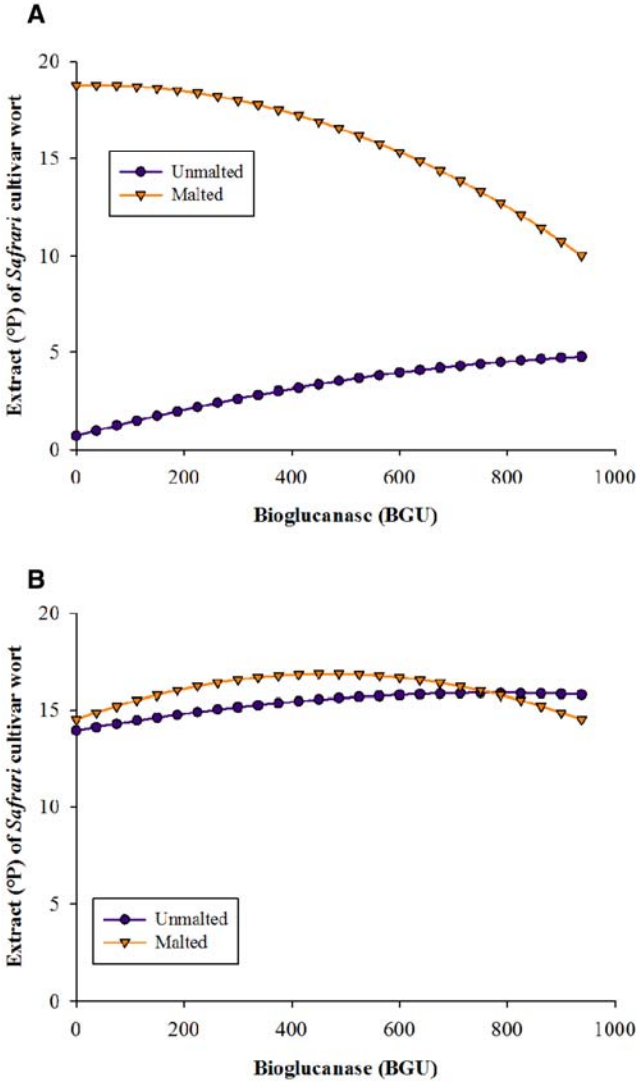

Figure 3. A, Effect of concentration of Bioglucanase (β-glucanase) as sole mashing enzyme (concentrations of Hitempase and Brewers protease set at 0) on yield of wort extract of sorghum cultivar *Safrari*. **B,** Effect of concentration of Bioglucanase (β-glucanase) in the presence of fixed concentrations of Hitempase (2,000 U) and Brewers protease (60 mg) on yield of wort extract of sorghum cultivar *Safrari*.

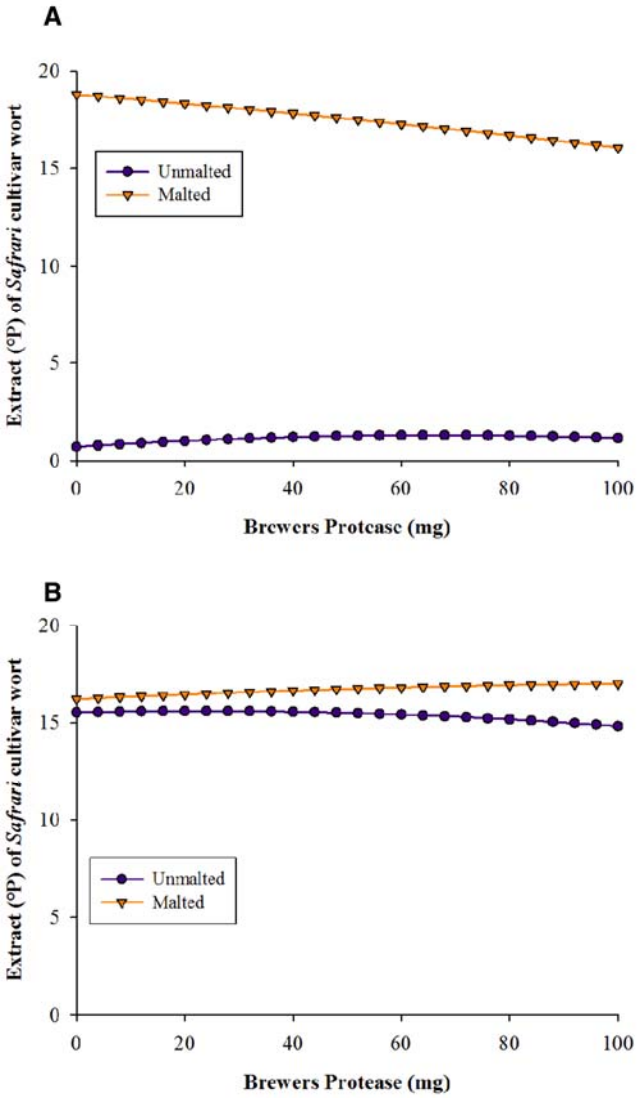

Figure 4. A, Effect of concentration of Brewers protease as sole mashing enzyme (concentrations of Bioglucanase and Hitempase set at 0) on yield of wort extract of sorghum cultivar *Safrari.* **B,** Effect of concentration of Brewers protease in the presence of fixed concentrations of Hitempase (2,000 U) and Bioglucanase (400 BGU) on yield of wort extract of sorghum cultivar *Safrari*.

The effect of mashing unmalted and malted *Safrari* on yields of extract using as sole mashing enzyme, Brewers protease, is shown in Figure 4A. This enzyme contributed little or no extracts in mashes of unmalted *Safrari* as compared to Hitempase and even Bioglucanase. Once more, the maximal extract observed when mashing with malted *Safrari* even in the absence of Brewers protease supplements is due to the virtues known to the malting process as explained earlier. These results confirmed earlier observations[15]. The steady and slight decrease of extract yield with increase of Brewers protease for the malted *Safrari* mash could once more be attributed to reactions between nitrogenous functions and some of the soluble sugars resulting from the malting process. The mathematical model was once more used to predict the yield in extract as carried out above for Hitempase and Bioglucanase actions. Thus, using 2,000 U of Hitempase and 400 BGU of Bioglucanase with increasing amounts of Brewers protease, the results once more showed that adding these mashing enzymes could provide similar results in extract yields for both unmalted and malted *Safrari* mashes (Fig. 4B). These observations were statistically confirmed using the mathematical model. In its first degree form (X_3), the impact of Brewers protease was significant for unmalted *Safrari* mash but not for malted *Safrari* mash (P = 0.016 and 0.291, respectively) (Table 3). Its contribution to extract yields was barely 5% and 3%, respectively, for both mash types (Figs. 2A and B). The impact of the enzyme in its quadratic form (X_3^2) was not significant for both mashes (P = 0.307 and 0.792, respectively) (Table 3). Its contribution to extract yield was 3% and 1%, respectively (Figs. 2A and B).

The models were further exploited to predict the impacts of the interactions (X_1X_2, X_1X_3 and X_2X_3) of these enzymes on yields of extract. The results are shown in Figures 2A and B. Globally, they were statistically not significant for unmalted

Table 4. ANOVA for the extracts of umalted and malted *Safrari.*

		Sum square		Mean square		F-value		P-value	
Source	**DDL**	**Unmalted**	**Malted**	**Unmalted**	**Malted**	**Unmalted**	**Malted**	**Unmalted**	**Malted**
Regression	9	333.038	65.429	37.004	7.27	113.241	10.408	0.000	0.003
Linear	3	240.289	18.625	80.096	6.208	245.112	8.888	0.000	0.009
Quadratic	3	89.659	7.736	29.886	2.579	91.459	3.692	0.000	0.070
Interactions	3	3.090	39.069	1.030	13.023	3.152	18.645	0.095	0.001
Residual error	7	2.287	4.889	0.327	0.698				
Total error	16	335.326	70.319						

Table 5. ANOVA for comparing extracts of unmalted and malted *Safrari* worts.

Source	**DF**	**Sum of squares**	**Mean of squares**	**F-value**	**P-value**
Inter-groups	1	130.693	130.693	9.21	0.004
Intra-groups	32	454.199	14.193		
Total	33	584.892			

Safrari mashes (P = 0.095), but were for malted *Safrari* mashes (P = 0.001) (Table 4). The interaction X_1X_2 (Hitempase/Bioglucanase) had no significant impact on unmalted *Safrari* mash, but did for malted *Safrari* (P = 0.429 and 0.001, respectively) (Table 3). It contributed merely 3% of extract for unmalted *Safrari* mash but up to 31% for malted *Safrari* mash (Figs. 2A and B). However, it is important to underline that this significant contribution could be attributed to the intrinsic virtues that malting offers when mashing with malted *Safrari,* and not to the Hitempase/Bioglucanase interaction as such. Though known to be the backbone starch hydrolysing enzyme, the action of Hitempase is best exploited when the cell walls of cereal grains are broken down by β-glucanases, hemicellulases and cellulases to liberate starch granules. This sequence of events during malting was confirmed by the mathematical models above. The interaction X_1X_3, corresponding to the couple Hitempase/Brewers Protease, also had no significant impact on both the extract yields of unmalted *Safrari* and malted *Safrari* mashes (P = 0.425 and 0.055, respectively) (Table 3). Its contribution to extract yields was 3% and 15%, respectively (Figs. 2A and B). This result was once more in conformity with the biological sequence occurring during malting. Efficient starch hydrolysis by α-amylase indeed occurs only after the breakdown of cell walls by β-glucanase, followed by liberation of starch granules due to proteolysis of the protein matrix enrobing them. The interaction Bioglucanase/Brewers protease (X_2X_3) had a significant impact on extract yields for unmalted *Safrari* mash but not for malted *Safrari* mashes (P = 0.002 and 0.223, respectively) (Table 3). Its contribution to extract yields was 11% and 8%, respectively, for both mash types (Figs. 2A and B). These low contributions by the couple (Bioglucanase/Brewers protease) were expected, as the two enzymes only play a supporting role in starch hydrolysis during mashing[15]. Table 5 statistically confirmed the observation that *Safrari* sorghum malted type samples were more potential mashing materials than unmalted *Safrari* sorghum adjuncts to which commercial enzymes are supplemented for the production of worts of higher extract yields (P = 0.004).

The mathematical models obtained for FAN for mashing unmalted and malted *Safrari* were as follows, respectively:

$$Y_{SafAAL}(X_1, X_2, X_3) = 91{,}781 + 24{,}992X_1 - 19{,}234X_2 + 35{,}693X_3 + 2{,}892X_1X_2 + 12{,}142X_1X_3 + 14{,}822X_2X_3 - 26{,}014X_1^2 + 20{,}723X_2^2 - 11{,}921X_3^2$$

$$Y_{SafMAAL}(X_1, X_2, X_3) = 136{,}969 + 38{,}884X_1 - 23{,}975X_2 + 51{,}247X_3 + 12{,}243X_1X_2 + 15{,}682X_1X_3 + 31{,}393X_2X_3 - 44{,}536X_1^2 + 28{,}806X_2^2 - 17{,}579X_3^2$$

With: Y_{SafAAL} (X_1, X_2, X_3) representing the mathematical model for unmalted *Safrari;* $Y_{SafMAAL}$ (X_1, X_2, X_3) for malted *Safrari;* X_1, Hitempase; X_2, Bioglucanase and X_3, Brewers protease. These mathematical models were once more polynomials having several variables with correlation coefficients R^2 = 0.944 for unmalted *Safrari* and R^2 = 0.955 for malted *Safrari*. These coefficients, coupled to AAD values of 0.073 and 0.060 for unmalted and malted *Safrari,* respectively, allowed for the validation of the models for assessment of the wort free amino nitrogen content. In addition, a bias factor of 1, coupled to exactitude factors of 1.08 and 1.06 for both unmalted and malted *Safrari,* respectively, also allowed for validation of the models according to the method described[12,36]. The factors of the models were once more linear or of first degree (X_1, X_2 and X_3), quadratic or of the second degree (X_1^2, X_2^2 and X_3^2) or of interaction form (X_1X_2, X_1X_3 and X_2X_3). They were statistically considered significant or not if the probability (P) of increasing yields of FAN was ≤0.05 or ≥0.05, respectively (Table 6).

The impact of Hitempase as sole mashing enzyme on yields of FAN for unmalted and malted *Safrari* is shown in Figure 5A. FAN content of wort gradually increased with increasing enzyme concentration to reach maxima of about 85 and 150 mg/L at about 1,750 U for both unmalted and malted *Safrari* mashes, respectively. This was followed by slight decreases for the unmalted *Safrari* mash as compared with malted *Safrari* mash. The curves showed that either for unmalted *Safrari* or malted *Safrari* mash types, the FAN content at origin was well above zero (60 mg/L and 90 mg/L, respectively). This suggests that the milling operation was at the basis of a good amount of the FAN present at the beginning of mashing. Although Hitempase is not a protein hydrolyzing enzyme, it exposes more free amino nitrogen functions upon acting on starch granules. This could explain the slight increase in FAN observed with increase in enzyme concentration. The higher FAN content for malted *Safrari* mash as compared to unmalted *Safrari* mash is once more to be attributed to the natural virtues that the grains incur during the malting process. Use of the models to predict the profile of FAN content of worts if the mashing enzymes Bioglucanase (at 400 BGU) and Brewers protease (at 60 mg) were coupled to Hitempase's action showed no remarkable difference. A balance between the amounts of enzymes needed to obtain maximal FAN contents and its disappearance in the medium due to reactions with sugars needs to be known to clearly understand this indifference. The models also showed that Hitempase (X_1), in its first degree form, contributed 15% of the FAN content of both the unmalted and malted *Safrari* mashes (Figs. 6A and B). This contribution was statistically significant for the two mash types (P = 0.001 and 0.000), re-

Table 6. Estimation of regression coefficients for free amino nitrogen of umalted and malted *Safrari.*

Effects	Coefficient		Std. deviation		T-statistics		P-value	
	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted
CONSTANT	91.781	136.969	4.75	6.148	19.321	22.278	0.000	0.000
X_1	24.992	38.884	4.886	6.325	5.115	6.148	0.001	0.000
X_2	–19.234	–23.975	4.261	5.515	–3.909	–3.765	0.006	0.007
X_3	35.693	51.247	4.026	5.211	7.234	8.025	0.000	0.000
X_1^2	–26.014	–44.536	8.377	10.843	–3.105	–4.107	0.017	0.005
X_2^2	20.723	28.806	6.097	7.891	2.549	2.738	0.038	0.029
X_3^2	–11.921	–17.579	5.259	6.807	–1.509	–1.72	0.175	0.129
X_1*X_2	2.892	12.243	9.563	12.378	0.262	0.857	0.801	0.420
X_2*X_3	14.822	31.393	8.418	10.895	1.244	2.036	0.253	0.081
X_1*X_3	12.142	15.682	9.966	12.899	0.994	0.992	0.353	0.354

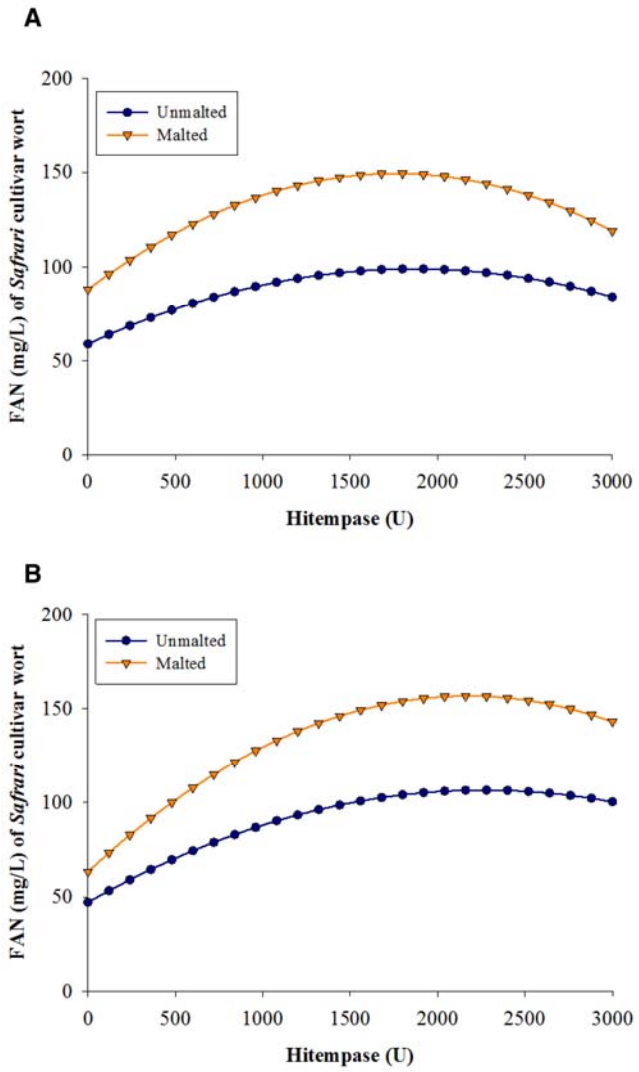

Figure 5. A, Effect of concentration of Hitempase (α-amylase) as sole mashing enzyme (concentrations of Bioglucanase and Brewers protease set at 0) on yield of wort free amino nitrogen of sorghum cultivar *Safrari.* **B,** Effect of concentration of Hitempase (α-amylase) in the presence of fixed concentrations of Bioglucanase (400 BGU) and Brewers protease (60 mg) on yield of wort free amino nitrogen of sorghum cultivar *Safrari*.

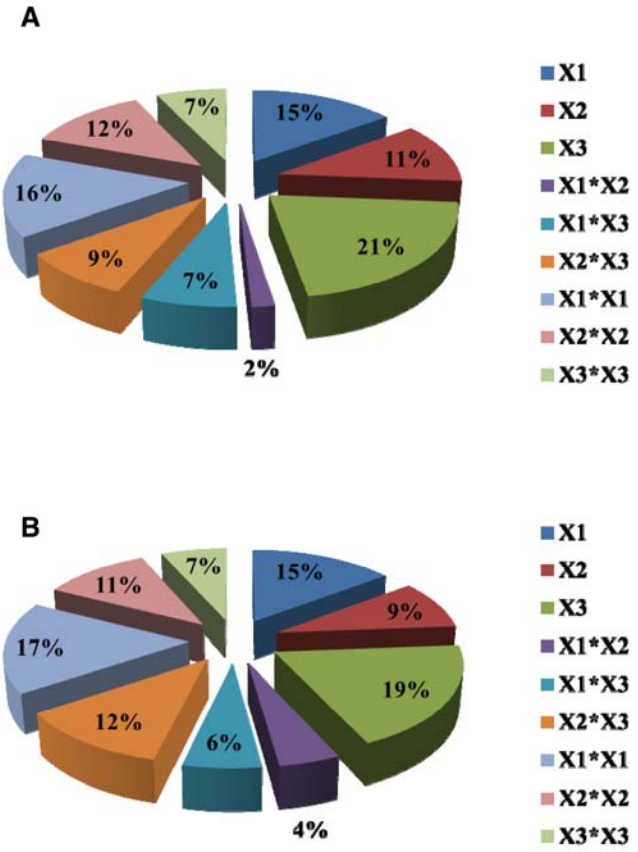

Figure 6. A, Contribution to yield of wort free amino nitrogen (FAN) of each factor in its linear, quadratic and interaction (combined) forms for unmalted sorghum cultivar *Safrari*. **B,** Contribution to yield of wort free amino nitrogen (FAN) of each factor in its linear, quadratic and interaction (combined) forms for malted sorghum cultivar *Safrari*.

spectively (Table 6). Similarly, in its quadratic form (X_1^2), Hitempase's effect remained significant for the two mash types (P = 0.017 and 0.005, respectively) (Table 6). Its contribution in this form was 16% and 17%, respectively (Figures 6A and B). The small difference in FAN content contribution (±2%) between its linear and quadratic (enzyme supplemented in excess) forms suggests that supplementing the enzyme in excess is of no technological use.

Figure 7A shows the effect of Bioglucanase on the FAN content as sole mashing enzyme in unmalted and malted *Safrari*. This content decreased to zero with increasing enzyme concentration in the mashes, indicating that Bioglucanase is not a protein hydrolysing enzyme. Mashing with the enzyme rather seemed to be of no use as the FAN was exposed to reactions with reducing sugars. Upon using the model to predict the amounts of FAN if mashed in the presence of Hitempase (at 2,000 U) and Brewers protease (at 60 mg), it was observed

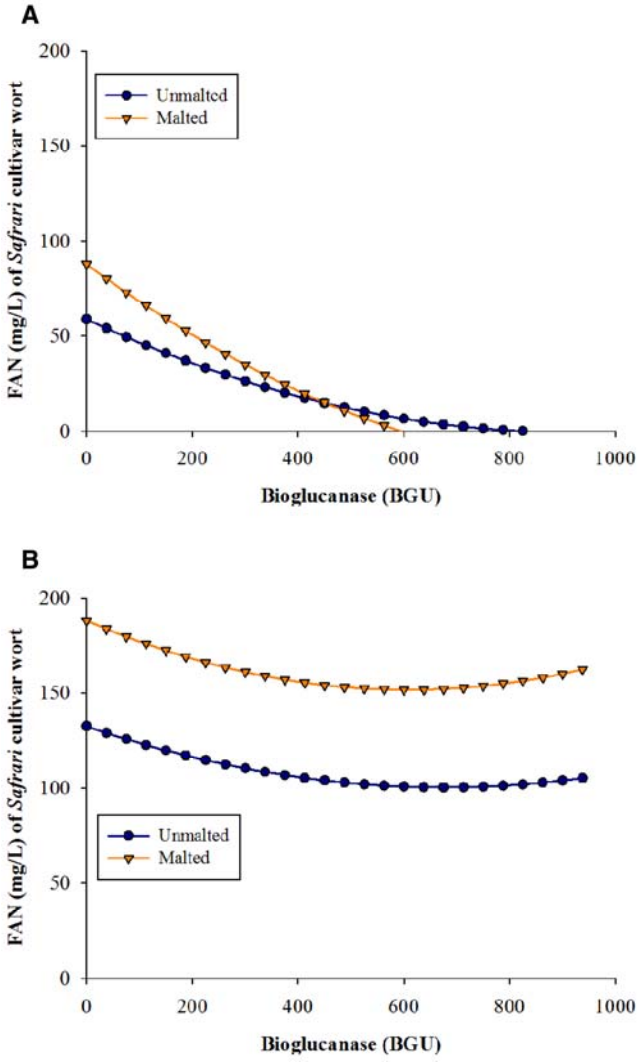

Figure 7. A, Effect of concentration of Bioglucanase (β-glucanase) as sole mashing enzyme (concentrations of Hitempase and Brewer's Protease set at 0) on yield of wort free amino nitrogen of sorghum cultivar *Safrari*. **B,** Effect of concentration of Bioglucanase (β-glucanase) in the presence of fixed concentrations of Hitempase (2000 U) and Brewers protease (60 mg) on yield of wort free amino nitrogen of sorghum cultivar *Safrari*.

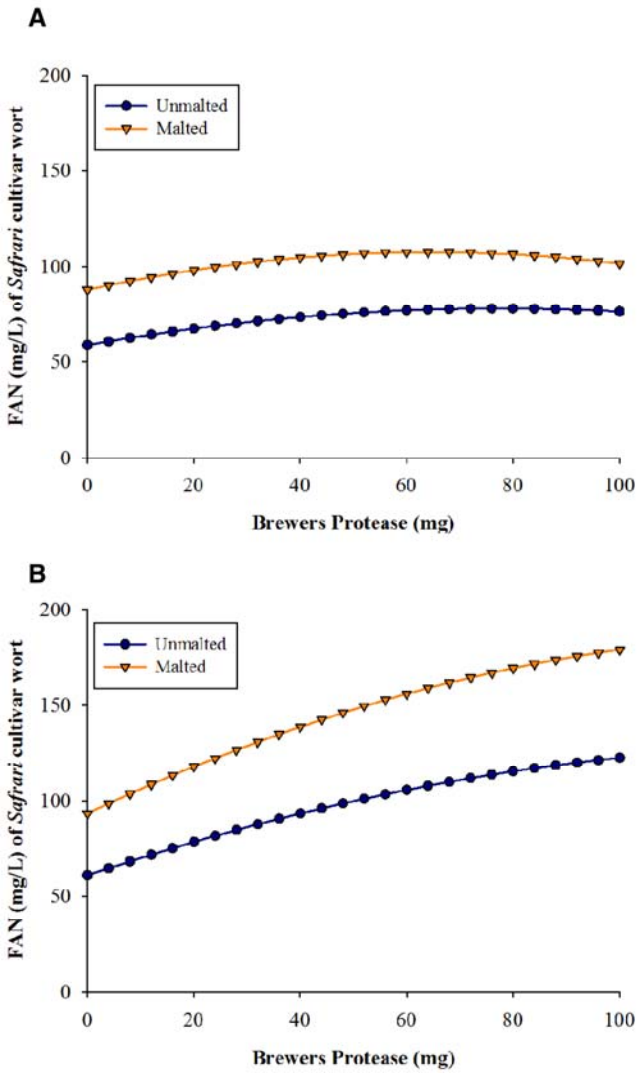

Figure 8. A, Effect of concentration of Brewers protease as sole mashing enzyme (concentrations of Bioglucanase and Hitempase set at 0) on yield of wort free amino nitrogen of sorghum cultivar *Safrari*. **B,** Effect of concentration of Brewers protease in the presence of fixed concentrations of Hitempase (2000 U) and Bioglucanase (400 BGU) on yield of wort free amino nitrogen of sorghum cultivar *Safrari*.

that the decrease of FAN levels to zero could be stopped and controlled (Fig. 7B). This confirms the need to have all mashing enzymes present in appropriate proportions during mashing to permit obtain sustainable amounts of FAN contents. According to the models, Bioglucanase (X_2), in its linear form, contributed 11% of the FAN content of the unmalted *Safrari* mash and 9% of the malted *Safrari* mash (Figures 6A and B). This contribution was statistically significant for the two mash types (P = 0.006 and 0.007, respectively) (Table 6). Similarly, in its quadratic form (X_2^2), the effect of the enzyme remained significant for the two mash types (P = 0.038 and 0.029, respectively) (Table 6). Its contribution in this form was 12% and 11%, respectively (Figures 6A and B).

The effect of Brewers protease as sole mashing enzyme on FAN content for unmalted and malted *Safrari* is presented in Figure 8A. Free amino nitrogen content increased very slightly from 60 mg/L to 75 mg/L for unmalted *Safrari* mash and from 90 mg/L to 110 mg/L for malted *Safrari* mash with an increase in enzyme concentration. This level was maintained constant from about 60 mg of enzyme input thereof. The model predicted that coupling Hitempase (at 2,000 U) and Bioglucanase (at 400 BGU) to the action of Brewers protease induced a steady increase in FAN content (Fig. 8B). This once more indicates the need of all mashing enzymes in order to obtain higher FAN yields. The additional FAN content observed for malted *Safrari* mash as compared to unmalted *Safrari* mash could once more be attributed to the natural and additional virtues of the malting process. The mathematical models statistically showed that the action of Brewers protease was in its linear form significant for both mash types (P = 0.000 for both) (Table 6). Its contribution to FAN content in this form (X_3) was 21% and 19% for both the unmalted and malted *Safrari* mashes, respectively (Figs. 6A and B). The effect of the enzyme in its quadratic form (X_3^2) is however not significant for the two mash types (P = 0.175 and 0.129, respectively) (Table 6). Its contribution decreased to as low as 7% for both

Table 7. ANOVA for free amino nitrogen of umalted and malted *Safrari.*

		Sum of squares		Mean of squares		F-value		P-value	
Source	**DF**	**Unmalted**	**Malted**	**Unmalted**	**Malted**	**Unmalted**	**Malted**	**Unmalted**	**Malted**
Regression	9	11,814.795	25,319.50	1,312.755	2,813.278	13.032	16.671	0.001	0.001
Linear	3	9,794.014	20,169.46	3,264.671	6,723.156	32.408	39.841	0.000	0.000
Quadratic	3	1,577.067	3,427.379	525.689	1,142.46	5.218	6.77	0.033	0.018
Interactions	3	443.714	1,722.655	147.905	574.218	1.468	3.403	0.303	0.083
Residual error	7	705.153	1,181.253	100.736	168.75				
Total error	16	12,519.94	26,500.75						

Table 8. ANOVA for comparing free amino nitrogen of unmalted and malted *Safrari* worts.

Source	**DF**	**Sum of squares**	**Mean of squares**	**F-value**	**P-value**
Inter-groups	1	13,916.3	13,916.3	11.41	0.001
Intra-groups	32	39,020.7	1,219.4		
Total	33	52,937			

mash types (Figs. 6A and B). Excess doses of this enzyme during mashing therefore appear to be of no technological importance.

The global action of these enzymes in their interaction or coupled forms (X_1X_2, X_1X_3 and X_2X_3) on the FAN content was statistically not significant (P = 0.303 for unmalted *Safrari* and P = 0.083 for malted *Safrari*) (Table 7). Their contributions of FAN content are shown in Figures 6A and B. The effect of the X_1X_2 (Hitempase/Bioglucanase) interaction was not significant for both mash types (P = 0.801 for unmalted *Safrari* and P = 0.420 for malted *Safrari*) (Table 6). Its contribution of FAN content in both mash types was 2% and 4%, respectively. Similarly, the action of the couple X_1X_3 (Hitempase/Brewers protease) was also not significant for both mash types (P = 0.353 for unmalted *Safrari* and P = 0.354 for malted *Safrari*) (Table 6). Its contribution of FAN content in both mash types was 7% and 6%, respectively (Figs. 6A and B). Finally, for the couple X_2X_3 (Bioglucanase/Brewers protease) (P = 0.253 for unmalted *Safrari* and P = 0.081 for malted *Safrari*) (Table 6), the contribution of FAN content in both mash types was 9% and 12%, respectively (Figs. 6A and B). Once more, Table 8 statistically confirmed the observation that malted *Safrari* sorghum type samples were more potential mashing materials than unmalted *Safrari* sorghum adjuncts to which commercial enzymes were supplemented for production of worts of higher FAN content (P = 0.001).

The results obtained for the action of the enzymes on extract and FAN yields after mashing on the basis of the models were optimised to define satisfactory domains of compromise for the mashing enzymes. These domains were obtained for the two key brewing parameters by fixing the wort conditions at extract ≥12°P and FAN ≥ 80 mg/L. The theoretical optimal combination of enzyme action for unmalted *Safrari* gave the following triplet of coded variables for extract: 0.399, 0.866 and –0.816 (2,098.5 U, 937.5 BGU and 0 mg real variables) for Hitempase, Bioglucanase and Brewers protease, respectively. This triplet allowed for a maximal extract of 18°P. The theoretical optimal enzyme combination for maximal contribution of FAN for unmalted *Safrari* mash gave as triplet of coded variables: 0.623, –0.866 and 0.816 (2,434.5 U, 0 BGU and 100 mg real variables). This triplet allowed for maximal free amino nitrogen of 144.77 mg/L (Fig. 9A). This triplet for maximal extract yields for malted *Safrari* was –1, –0.813 and –0.816 (0 U, 28.68 BGU and 0 mg real variables) for Hitempase, Bioglucanase and Brewers protease, respectively. It allowed for a maximal extract of 18.82°P. The optimal enzyme combinations were thus different for the two mash types, but both gave comparable yields of extracts. The triplet for maximal FAN content for malted *Safrari* was 0.461, –0.866 and 0.816 (2,191.5 U, 0 BGU and 100 mg real variables). It allowed for maximal FAN of 196.73 mg/L (Fig. 9B). These results once more confirmed

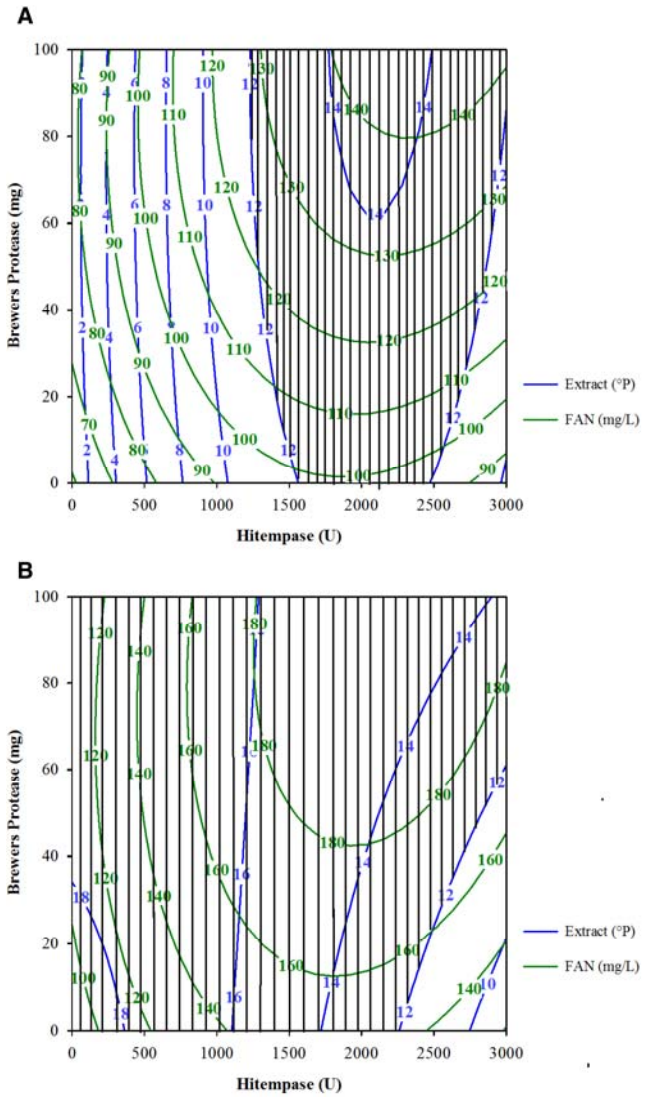

Figure 9. A, Response surface curves for the enzyme combinations providing for optimal yields in extract and free amino nitrogen for unmalted sorghum cultivar *Safrari.* **B,** Response surface curves for the enzyme combinations providing for optimal yields in extract and free amino nitrogen for malted sorghum cultivar *Safrari.*

that commercial enzyme supplements for mashing malted *Safrari* are not indispensable for obtaining maximal yields of extract as compared to unmalted *Safrari*. The enzymes supplements however proved useful for obtaining maximal FAN.

Conclusions

The effects of three commercial mashing enzymes (Hitempase 2XL, Bioglucanase TX and Brewers protease) on yields of extract and FAN were studied during the mashing of unmalted and malted *Safrari* grist. Hitempase 2XL was principally responsible for extract yields in unmalted *Safrari* mash but had no impact on the malted *Safrari* mash type. Bioglucanase TX barely played a supporting role in these yields, while Brewers protease showed no significant role. Hitempase 2XL and Brewers protease individually contributed to yields in FAN in both unmalted and malted *Safrari* mashes, though the milling operation contributed to FAN yields for more than 50% in both mashes. This study shows that proper malting and mashing of this sorghum cultivar could lead to satisfactory wort properties in terms of extract and FAN for brewing purposes. Supplements of commercial mashing enzymes to boost their yields of extract in particular are thus not indispensable when mashing with malted *Safrari*. Optimisation of mashing properties through models clearly describing the actions of individual commercial mashing enzymes, as displayed in this study using the response surface methodology, is however of interest, particularly when mashing with high amounts of sorghum adjuncts. Further studies on the fermentability of worts obtained after such studies would be of importance in order to assess the exploitability of the results for improved brewing practices with this sorghum cultivar.

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