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Abstract:	In previous studies Bacillus subtilis has been used to control mould growth during red sorghum malting. The use of this biocontrol in steeping liquor has been optimized with some success and the combined use of 0.2% NaOH steeping followed by resteeping in a Bacillus subtilis-based biocontrol has been proposed. The sharpness and variability of the β-amylase peak and the higher levels of β-glucanase obtained in the presence of B. subtilis cells were highlighted. In this work the suitability of the Weibull 4 Parameters Model to predict sorghum malt α-amylase activity during the enzyme induction stage of red sorghum germination has been compared with those of 2nd Order Polynomial Model and General Linear Model. Results obtained show that the Weibull 4 Parameters Model can be used to predict α-amylase synthesis with significant goodness of fit when compared to the 2nd Order Polynomial Model and General Linear Model. The effect of steeping treatment (combined use of 0.2% NaOH and Bacillus subtilis S499 starters) and the germination temperature is highlighted. In fact, when the Bacillus subtilis culture used as starters is diluted, the treatment efficacy is lost. This study also shows that the germination temperature affects the α-amylase synthesis rate during the induction phase.

Suitability of the Weibull 4-parameters model to predict the induction phase of α-amylase production during red sorghum malting when steeping in dilute NaOH is used prior to resteeping in *Bacillus subtilis*-S499 based treatment

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Abstract

In previous studies *Bacillus subtilis* has been used to control mould growth during red sorghum malting. The use of this biocontrol in steeping liquor has been optimized with some success and the combined use of 0.2% NaOH steeping followed by resteeping in a *Bacillus subtilis*-based biocontrol has been proposed. The sharpness and variability of the β-amylase peak and the higher levels of β-glucanase obtained in the presence of *B. subtilis* cells were highlighted. In this work the suitability of the Weibull 4 Parameters Model to predict sorghum malt *α*-amylase activity during the enzyme induction stage of red sorghum germination has been compared with those of 2nd Order Polynomial Model and General Linear Model. Results obtained show that the Weibull 4 Parameters Model can be used to predict *α*-amylase activity with significant goodness of fit when compared to the 2nd Order Polynomial Model and General Linear Model. The effect of steeping treatment (combined use of 0.2% NaOH and *Bacillus subtilis* S499 starters) and the germination temperature is highlighted. In fact, when the *Bacillus subtilis* culture used as starters is diluted, the treatment efficacy is lost. This study also shows that the germination temperature affects the α-amylase activity increase rate during the induction phase.

Keywords: *Bacillus subtilis* biocontrol, *α*-amylase activity, Weibull 4-parameter model, red sorghum malting

Introduction

Sorghum is often malted for use in industrial food processes such as the production of various beverages or weaning foods. Sorghum malt is notably used in brewing. The main purpose of the malting step is to favor the production of enzymes which will render the grain constituents (starch, proteins) more digestible. The effect of the grain microbial ecosystem on barley malt quality has been clearly discussed by Laitila et al. *(1)*. Biocontrol treatments are often used during malting to control mould growth and to improve malt quality *(2-5)*. In fact, plant-bacteria interactions have

been widely studied in recent years and it is known that the grain microbial ecosystem affects the germination process. So, when harmless microbes are used as a biocontrol of pathogens and spoilage microbes, these interactions are complex (plant-microbes interactions and microbes-microbes interactions) and difficult to describe accurately.

Diastatic power (DP) is often used as a measure of malt quality. It is an expression of the collective activity of the following enzymes: α-amylase, β-amylase, α-glucosidase, and limit dextrinase *(6)*.

When *Bacillus subtilis* is used during the steeping step, malt α-amylase is significantly improved compared to steeping in distilled water *(4)*. As the predominant starch-hydrolyzing activity in sorghum is usually α-amylase activity (although in some varieties the β-amylase activity is higher *(7)*), it can be useful, when one wants to optimize the malting process, to have a good model of its development in the course of germination. During the germination step of the malting process, malt α-amylase activity, reaches a maximum, and then finally drops *(8)*. A key question to be addressed is: how long should germination be allowed to proceed for taking the maximum advantage of the underlying phenomenon *(8)*?

In the present study we have focused specifically on red sorghum malt α-amylase activity in the context of a combined steeping treatment (steeping in dilute alkaline followed by resteeping in biocontrol treatment). Our aim was to model the time course of the development of this enzyme activity during red sorghum germination, as affected by the steeping and germination conditions. For this, we have examined the performance of three models: the 2nd order polynomial model (2nd OPM) (the only model proposed to date for α-amylase activity during sorghum malting by Egwim and Adenomon *(9)*), the Weibull 4-parameter model (W-4-PM) chosen according to the individual distribution of experimental data using Minitab 16 software, and the general linear model (GLM) constructed using stepwise regression.

Materials and methods

Choice of variables

Malt enzymes activities during the sorghum malting process may be affected by several factors including: the nature of the steeping liquor *(10-12)*, the steeping temperature and time, aeration during steeping *(13,4)*, the final warm water steep and air rest cycles *(14)*, the steep-out moisture *(13)*, the use of microbial starters: lactic acid bacteria and yeasts *(3)*, *Bacillus subtilis (4)*, germination temperature *(15,16)* and germination time *(8,17)*.

Therefore in this study, we set the following steeping conditions: aeration, temperature, time, nature of the steeping solution for the initial 8 h steeping. The difference between steeping treatments has been made by varying the dilution of the *Bacillus subtilis* S499 culture (ln *BSP*: natural logarithm of the *Bacillus subtilis* S499 population) used as the biocontrol during the last 8 h steeping. For the germination conditions, two factors were manipulated; germination temperature (*GT*) and germination time (*GD*).

Red sorghum malting

Bacillus subtilis strain S499 was obtained from the Walloon Center of Industrial Biology (CWBI) and grown on Luria broth agar at 37°C for 24 h. An inoculating loopful was transferred to 100 ml Landy broth optimized for *B. subtilis* S499 lipopeptide production and incubated for 16 h. Finally, 10 ml was transferred to 350 ml optimized Landy broth and incubated at 30°C (with rotary shaking at 130 rpm) for 72 h. The culture (containing approximately 10^{11} cells/ml) was diluted with distilled water to 10^{8} and 10^{4} cells/ml used to obtain the steep liquors employed during the biocontrol step of the steeping process *(5)*.

The red sorghum cultivar used was obtained from the D.R. Congo and has been described previously *(4,5)*. Sorghum malts were obtained as described by Bwanganga et al. *(4)* and Bwanganga et al. *(5)* by manual sorting, steeping at 30°C for 8 h in 0.2% NaOH and then for 8 h in the biocontrol steep liquor (B1 and B2 refer, respectively, to the treatments containing 10^{8} and 10^{4}

cells/ml and C refers to the treatment with distilled water alone in the second phase of steeping), germination in the dark at 25°C (T1), 30°C (T2), or 35°C (T3) for 6, 12, 24, 36, 48, 60, 72, 84, 96, or 108 h, and kilning for 48 h at 40°C.

Alpha-amylase assays were performed on extracts of sorghum malt flour obtained by grinding kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). The activity was extracted and assayed using Megazyme methods (Ceralpha Method K-CERA 08/05): sodium maleate (100 mM, pH 6.0) plus $CaCl_2$ (5 mM) and sodium azide (0.02%) as the extraction buffer, azurine cross-linked amylose as the substrate, incubation at 40°C for exactly 10 min and 2% (w/v) Trizma base as the stopping solution. The absorbance was read at 590 nm against the reaction blank.

Modelling the *α*-amylase increase during germination

The first function used to represent the *α*-amylase activity data collected for the above-mentioned steeping and germination conditions was the 2nd order polynomial model as proposed by Egwim and Adenomon *(9)*.

$$AA = a + b(GD) + c(GD)^2 \quad (1)$$

where *AA* is the *α*-amylase activity. Parameters a, b, and c were fitted by stepwise regression performed with Minitab 16 software.

The second model was a classical general linear model obtained after stepwise regression of *α*-amylase experimental values using ln *BSP*, *GT* and *GD* with their 1st and 2nd order interaction.

The third model tested was the W-4 PM

$$AA = AA_0 + (AA\omega - AA_0) \exp(-\alpha \times GD^{\beta}) \quad (2)$$

where AA_0 is the *α*-amylase activity at the start of germination and $AA\omega$ is the value towards which the activity tends during the induction phase of the *α*-amylase activity.

Equation (1) can be written as:

$$\ln[\ln((AA_{\omega}-AA_0)/(AA-AA_0))] = \ln\alpha+\beta\ln GD \quad (3)$$

This is the equation of a straight line with slope (β) and x-intercept -(ln α)/β. The parameters α and β were obtained by plotting experimental data according to equation (3) and for each case the smallest and the highest value were first considered respectively as AA_0 and $AA\omega$. The true values of AA_0 and $AA\omega$ were obtained using the Gauss-Newton algorithm and a convergence tolerance of 0.00001, after fixing the values of α and β equal to those obtained with experimental data (straight line of Eq. 3) using Minitab software. $AA_{\#} = [((AA_{\omega}-AA_0)/(AA-AA_0))]$ is dimensionless and tends to one at the maximum activity. β is the expression of the speed at which the maximum activity is achieved [$\beta = \partial\ln(\ln AA_{\#})/\partial\ln GD$], and -(ln α)/β is the starting point, i.e., the advantage offered by the steeping treatment or the expression of the capacity of the treatment to improve *α*-amylase activity (Bwanganga et al., 2013). Minitab 16 software was used for statistical analyses: analysis of variance, goodness of fit, stepwise regression, general linear model, fitted line plot and scatterplot.

Results

1. Modelling *α*-amylase activity during germination

Three-way ANOVA was applied to the experimental data obtained with different steeping treatments after different germination times; temperatures and results are presented in Table 1. All the main effects and their 1st and 2nd order interactions were significant ($p<0.05$).

The kinetic parameters of the 2nd OPM and W-4-PM obtained with experimental data are presented in Table 2. The equation of the GLM obtained using Minitab software after stepwise regression was:

$$AA = -9.97 + 0.107\ GT*GD + 0.174\ \ln BSP*GD - 0.00455\ GT*\ln BSP*GD - 0.00967\ GD^2 \quad (4)$$

The regression analysis of the 3 models is presented as supplementary data in Tables S2, S3 and S4.

Predicted and experimental data scaterplots are presented in Fig. 1.

2. Goodness of fit

The goodness of fit of all 3 models obtained using the decomposition of the residual error is presented as supplemental data Tables S2, S3 and S4 respectively for the 2nd OPM, W-4-PM and GLM. The mean square error [$MSE = n^{-1}(SSE)$] and its root (RMSE) are presented in Table 3. SSE (sum of square error) $= \sum(\text{experimental data-predicted data})^2$. When we consider only the values of R-sq (adj) obtained: 86.3 - 96.2% for the 2nd OPM and 82.8% for the GLM one may be tempted to consider these two models as good fits. However, the decomposition of the residual error associated to the fits (Tables 2, S2, S3 and S4) and the RMSE (Table 3) clearly show that neither the 2nd OPM nor the GLM give good fits (the lack of fit being significant for these two models). From this point of view, the W-4-PM can be considered as a good fit: RMSE = 0.95 and for all fits obtained, except for the steeping in control and germination at 35°C, where the lack of fit wasn't significant.

3. The effect of germination temperature and steeping treatment on *α*-amylase activity

The question to be asked is: how does each of the malting factors affect *α*-amylase activity?

To answer this question, two approximations were made according to the results obtained with the experimental data (Table 2):

1. Parameter β varies very little with steeping treatments for a given temperature so that it can be considered as a constant regardless of the steeping treatment. This parameter is a function of germination temperature and the effect of germination temperature on β is shown in Fig. 2a.
2. *ln α* varies minimally for a given treatment regardless of the temperature so that it can be considered as a constant in this study. The effect of steeping treatment on this parameter is presented in Fig. 2b.

So, from equation (3) to be combined with the regression equations of Fig. 2 (a and b) we obtained

the following model:

$$\ln(\ln AA_{\#}) = 14.20 - 0.08306 \ln(BSP) - (0.02775\, GT + 2.677) \ln GD \quad (5)$$

Discussion

Seed germination is well documented. It is known that sorghum germination is under hormonal control *(18)*. In addition, when germination is well advanced, the seed in contact with the external environment can synthesize ABA, known to be involved in stress responses to changing environmental conditions *(19)*. The role of carbohydrates in the regulation of plant hormone action has been extensively discussed *(20-23)*. The steeping and germination conditions can improve, to a greater or lesser extent, the release of absorbable simple sugars and induce ABA synthesis and/or activation. Excess glucose during seedling development, for example, induces growth arrest and differentiation, which some authors attribute to the biosynthesis of the ABA and ABA signaling *(19)*. The ABA is important in the blockage of germination by reducing the permeability of membranes and its action is highly modulated by the concentration of glucose *(19)*. So, it is clear that when using a model to predict enzyme activity, steeping and germination conditions have to be taken into account. The effect of steeping conditions on α-amylase activity during sorghum malting is well known. Steeping conditions are known to be able to affect grain moisture *(4,13)*, phenolic content *(12)*, cell walls degradation *(11)*, protein matrix hydrolysis *(24)* and α-amylase activity *(4,11-13)*. It is also known that during germination, α-amylase activity rises, reaches a maximum, and then finally drops *(9,25)*. Everything that happens after the peak (maximum α-amylase activity) - despite the effect of germination conditions - is strongly related to the underlying phenomenon, the growth of the seedling. The 2nd OPM or the GLM can be used in modelling α-amylase activity during the red sorghum germination step. When such models are used, R-square, Chi-square, F-test and/or the root mean square error (RMSE) are often used to evaluate the goodness of fit. The basis of these statistics is the sum of square total (SST) (deviation from the average) and the sum of square error (deviation from the model's predicted values). Egwim and Adenomon *(9)* obtained an

R-sq value between 67 to 90% using the 2nd OPM to model α-amylase activity during sorghum malting and suggested that the model can be used to predict future values. It is known that when a lack of fit exists, standard deviations for regression coefficients are overestimated, and this gives rise to confidence intervals that are too large *(26)*. As it can be seen in Table 3, Suppl. Tables S2 and S4, in such a situation the acceptable values of R-sq and the R-sq (adj) do not guarantee the goodness of the fit. The advantage of such models is to cover the whole process and therefore can afford to give an idea of the germination time corresponding to the maximum for this enzyme activity. We obtain an R-sq (adj) = 86.3 - 96.0% using the 2nd OPM but the p-value for the lack of fit was less than 0.05 (Table 2). The same observation is made with the GLM which gave an R-sq (adj) = 82.82% but the lack of fit was not significant. From this point of view, W-4-P M presented significant goodness, the lack of all fits was not significant except for the model obtained from the steeping treatment C followed by germination at 35°C (see Table 2).

During the malting process the effect of conditions created by the maltster must be evaluated correctly to be sure whether or not malting conditions have to be improved because during germination, on the one hand there are a series of reactions that take place in the non-living part of the grain (endosperm), which can be controlled by the maltster and on the other hand all reactions taking place in the living part of the grain are highly regulated *(27)*. The sensitivity of enzyme synthesizing cells varies, so that the aleurone layer cells are not affected by the level of sugars while in the embryo; the repression of the enzymes synthesis is effective *(28)*. In sorghum, it has been reported that the synthesis of enzymes during germination is mainly achieved in the scutellum *(29,30)* and therefore more sensitive to repression by sugars. The conditions affecting the occurrence of these two phases are strongly influenced by the conditions of malting (effect of malting conditions on the hydrolysis of the endosperm reserve, etc.). From this point of view the W-4-PM clearly highlights the effect of steeping and germination conditions on α-amylase activity during malting. The ST affects the grain capacity ($\partial \ln\alpha / \partial \ln BSP = 0.08306$) and the germination temperature affects the rate of the α-amylase increase ($\partial\beta/\partial GT = -0.02775$) as shown in Fig. 2.

Contrary to α-amylase activity, it had been shown that *GT* affects negatively the rate of the β-amylase increase *(5,15)*.

It is therefore understandable that the value of $AA\omega$ estimated in this study is only a potential value. This model is cut by the curve of the repression phase of the α-amylase activity (see Fig. 1c. experimental values). The true maximum is found at the intersection of the two models with two different bases: that is to say the junction of the induction phase and that of the repression phase. It explains the peak obtained early (around 72 h germination) with a germination temperature of 35°C (Fig. 1c.). Knowledge of this phenomenon is crucial. Indeed, when comparing two steeping treatments one tends to fix conditions for germination. However, the steeping treatment and germination temperature have an effect over time after which this maximum is reached. Thus we perceive that two treatments should not be compared on this basis. This is one of the advantages of the W-4-PM; taking into account the effect of steeping conditions on the one hand and that of germination conditions on the other hand.

Conclusion

The α-amylase activity time course during the first germination phase, characterized by the induction of the α-amylase activity, can be suitably modelled using the W-4-PM with significant goodness of fit (all models obtained haven't presented significant lack of fit except the fit obtained with the control (C) when the germination temperature was 35°C). The advantage of such a model is to highlight the effect of steeping and germination conditions. The W-4-PM hasn't been used to model the entire germination step as the second phase of this process is highly regulated and should be approached differently. This limitation of the model isn't a problem when the objective is the monitoring of the malting process. In fact, despite the importance of α-amylase during malting, what is sought is not always the maximum of this activity, but a compromise between a range of characteristics: the other enzymatic activity levels (β-amylase, α-glucosidase, limit dextrinase, β-glucanase, endo- and exo-peptidases, etc.), the reduction of the Total Malting Loss, the achievement

of good grain modification level, reduced phenolic compounds content, etc.

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Fig. 1a.1.

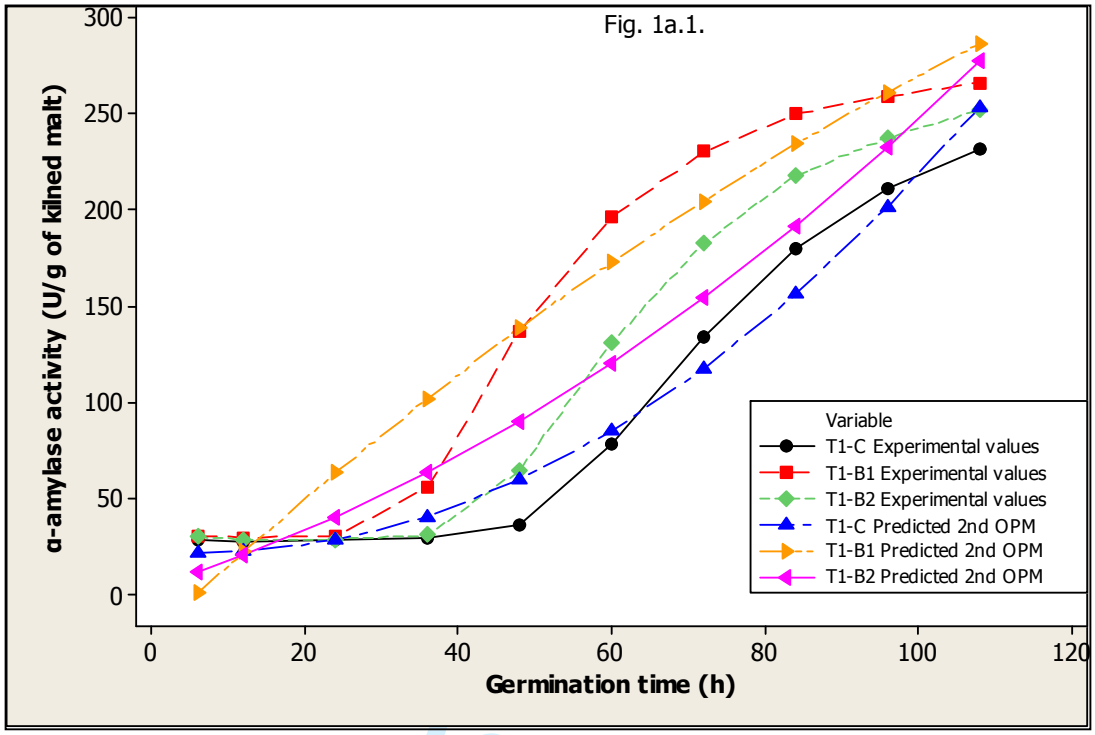
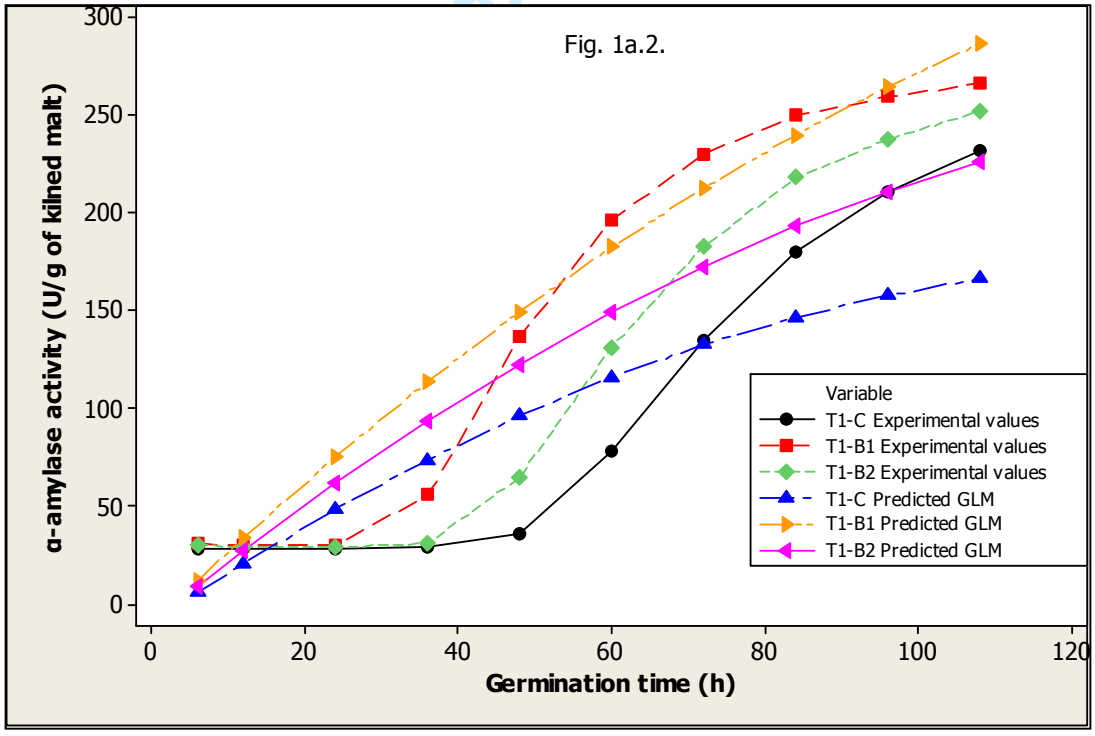


Fig. 1a.2.



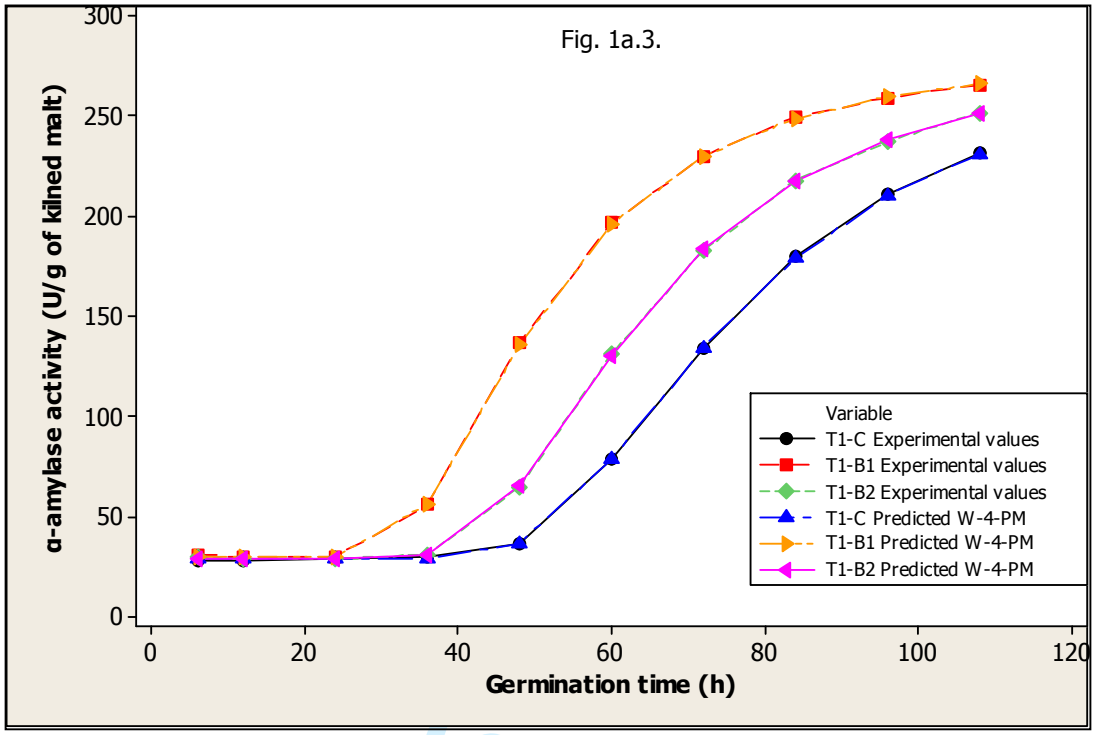


Fig. 1a.3.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T1-C Experimental values
T1-B1 Experimental values
T1-B2 Experimental values
T1-C Predicted W-4-PM
T1-B1 Predicted W-4-PM
T1-B2 Predicted W-4-PM

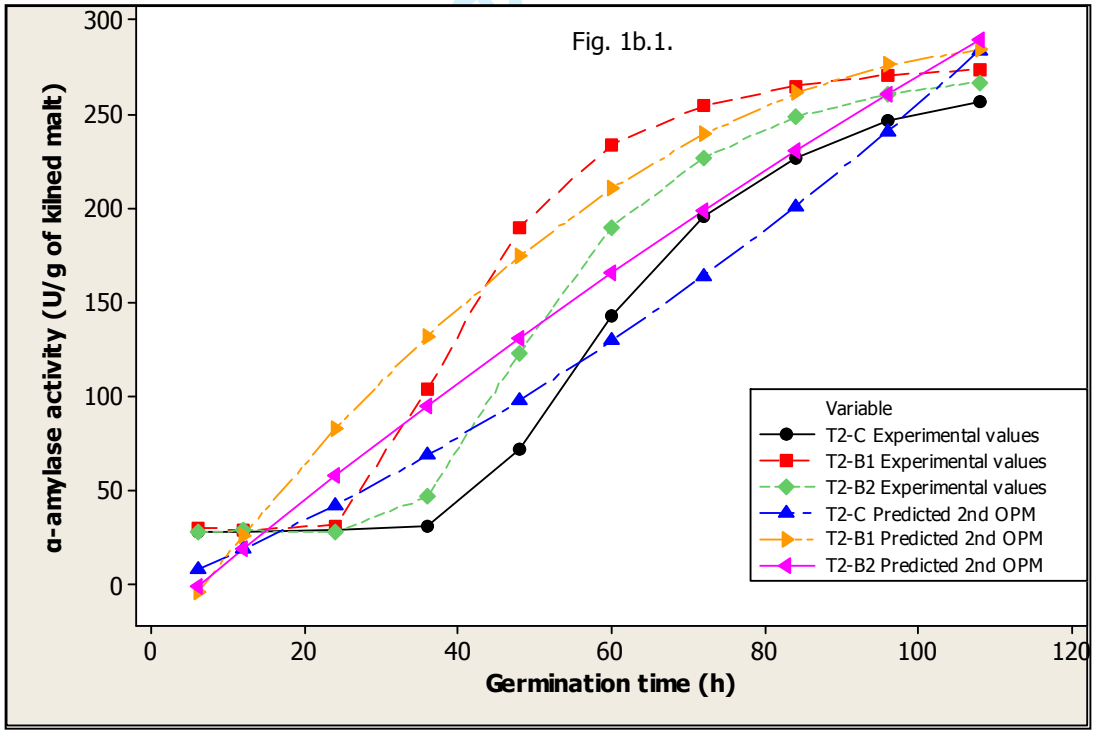


Fig. 1b.1.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted 2nd OPM
T2-B1 Predicted 2nd OPM
T2-B2 Predicted 2nd OPM

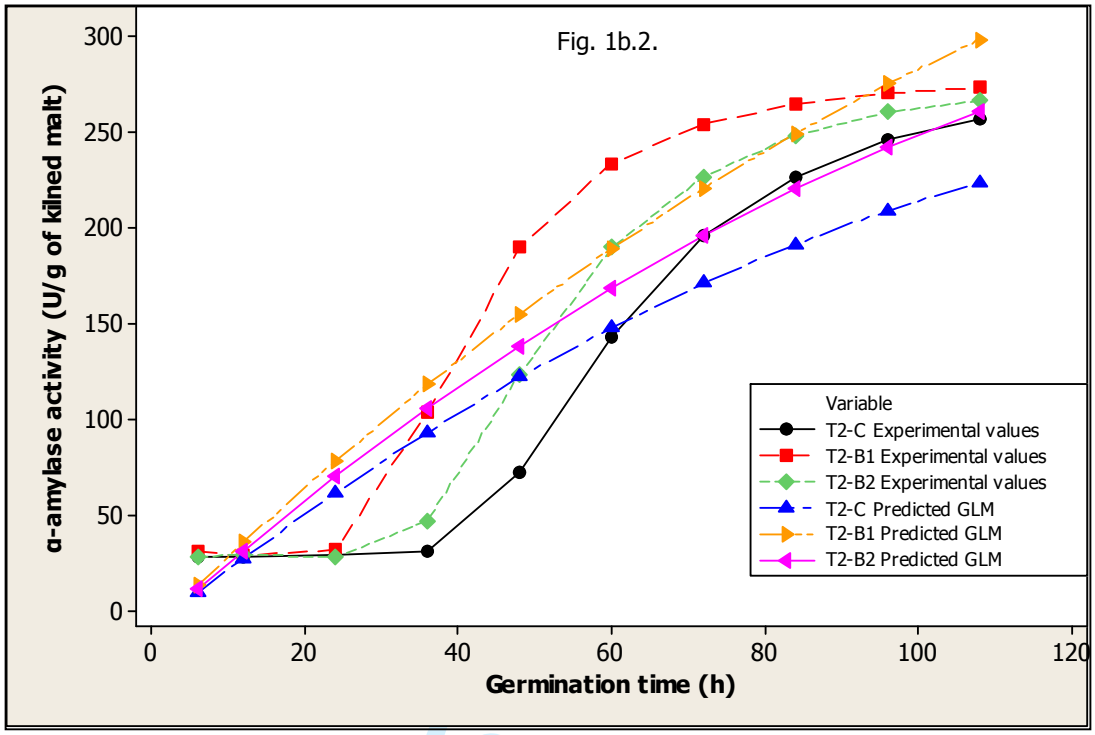


Fig. 1b.2.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted GLM
T2-B1 Predicted GLM
T2-B2 Predicted GLM

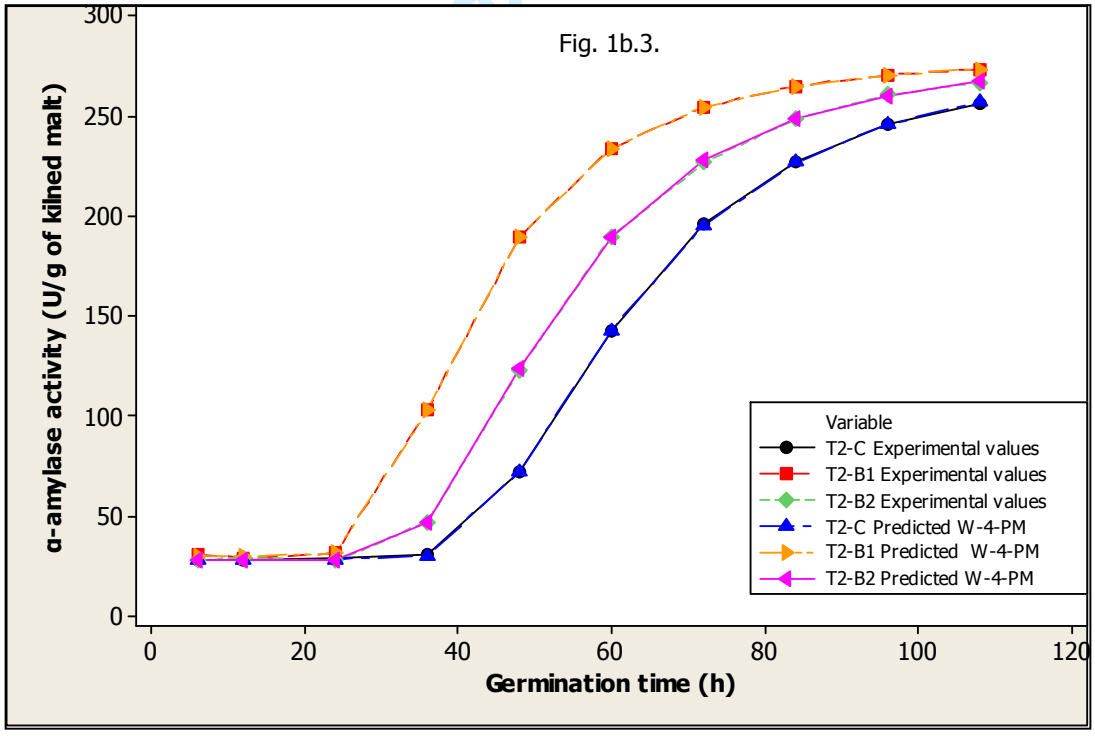


Fig. 1b.3.
α-amylase activity (U/g of kilned malt)
Germination time (h)
Variable
T2-C Experimental values
T2-B1 Experimental values
T2-B2 Experimental values
T2-C Predicted W-4-PM
T2-B1 Predicted W-4-PM
T2-B2 Predicted W-4-PM

Fig. 1c.1.

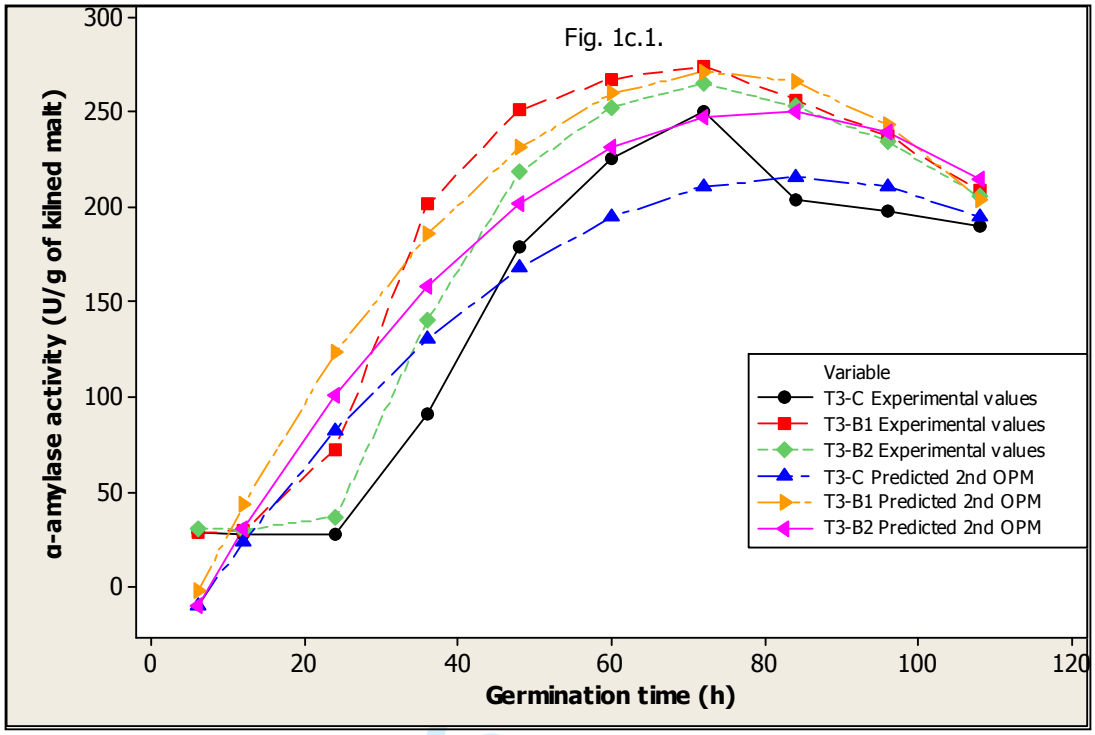


Fig. 1c.2.

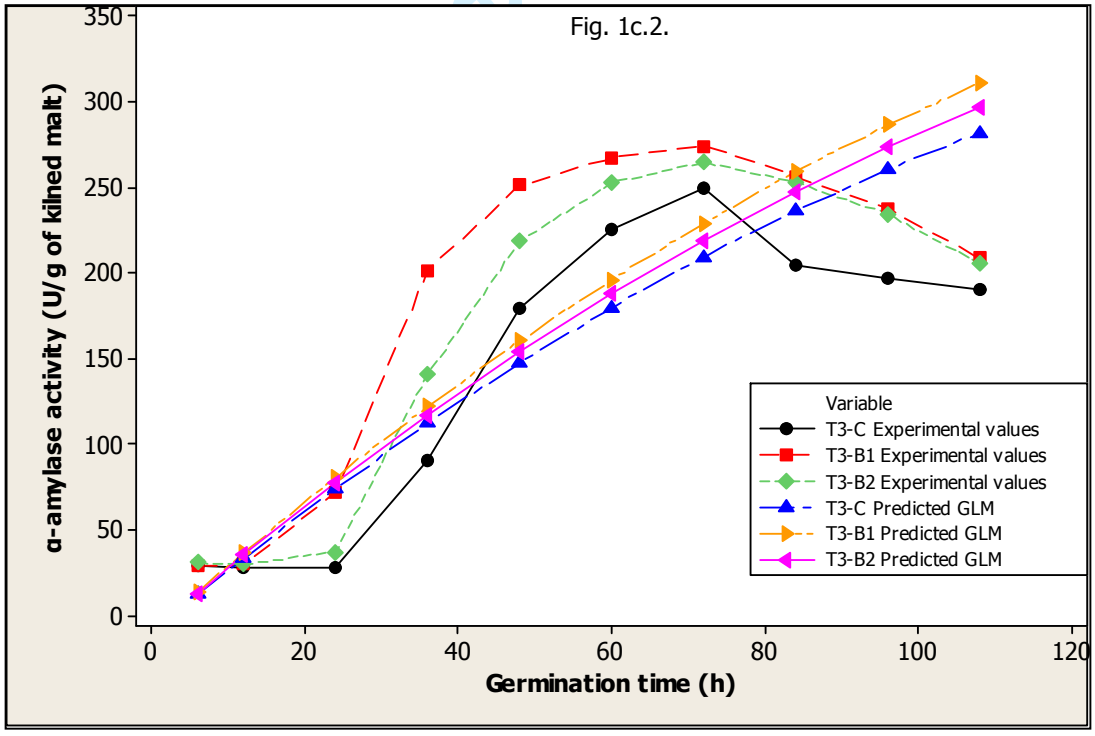
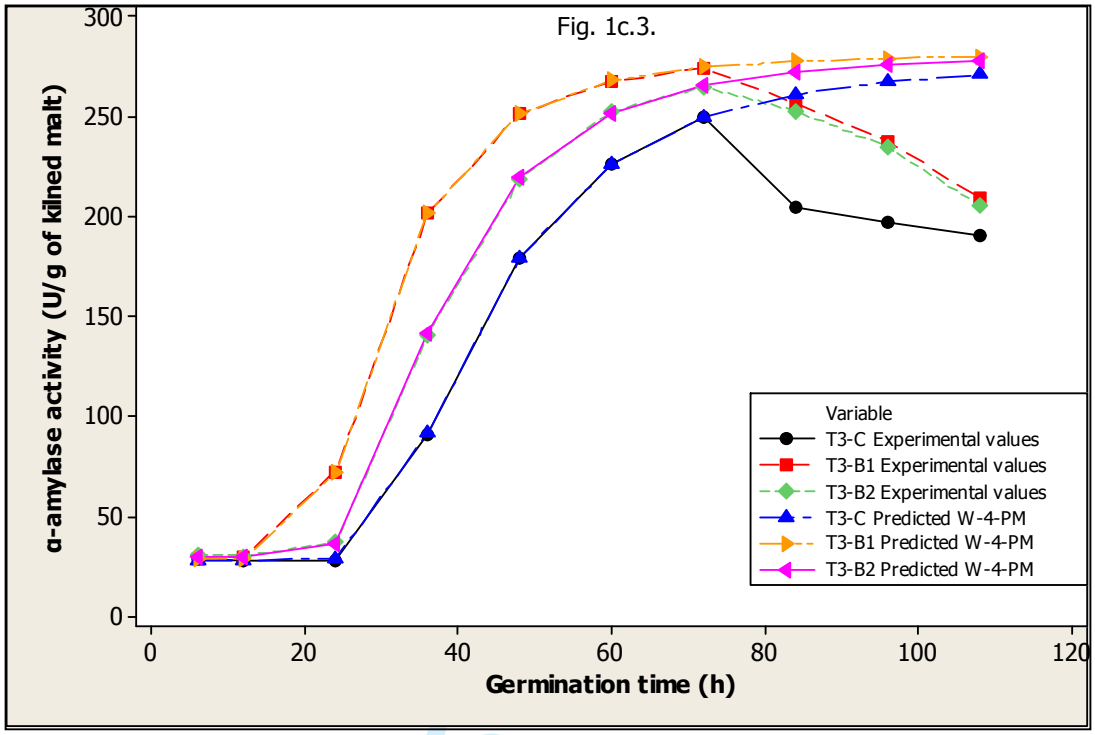


Fig. 1c.3.



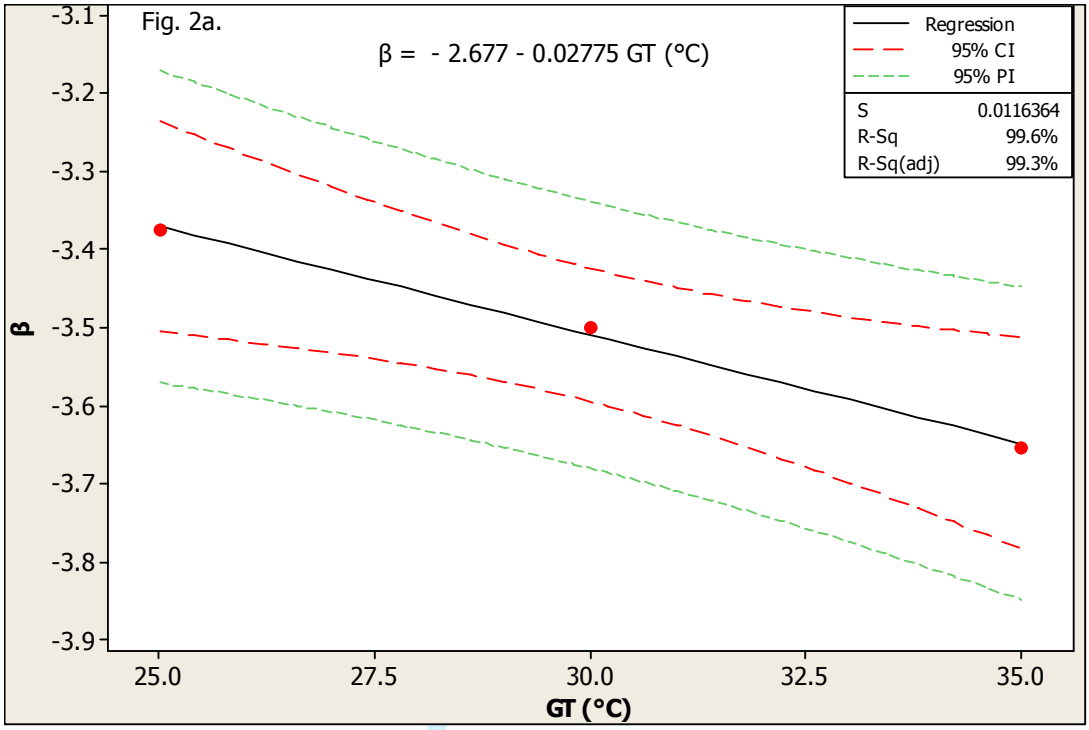


Fig. 2a.
β = - 2.677 - 0.02775 GT (°C)
Regression
95% CI
95% PI
S 0.0116364
R-Sq 99.6%
R-Sq(adj) 99.3%
β
-3.1
-3.2
-3.3
-3.4
-3.5
-3.6
-3.7
-3.8
-3.9
25.0
27.5
30.0
32.5
35.0
GT (°C)

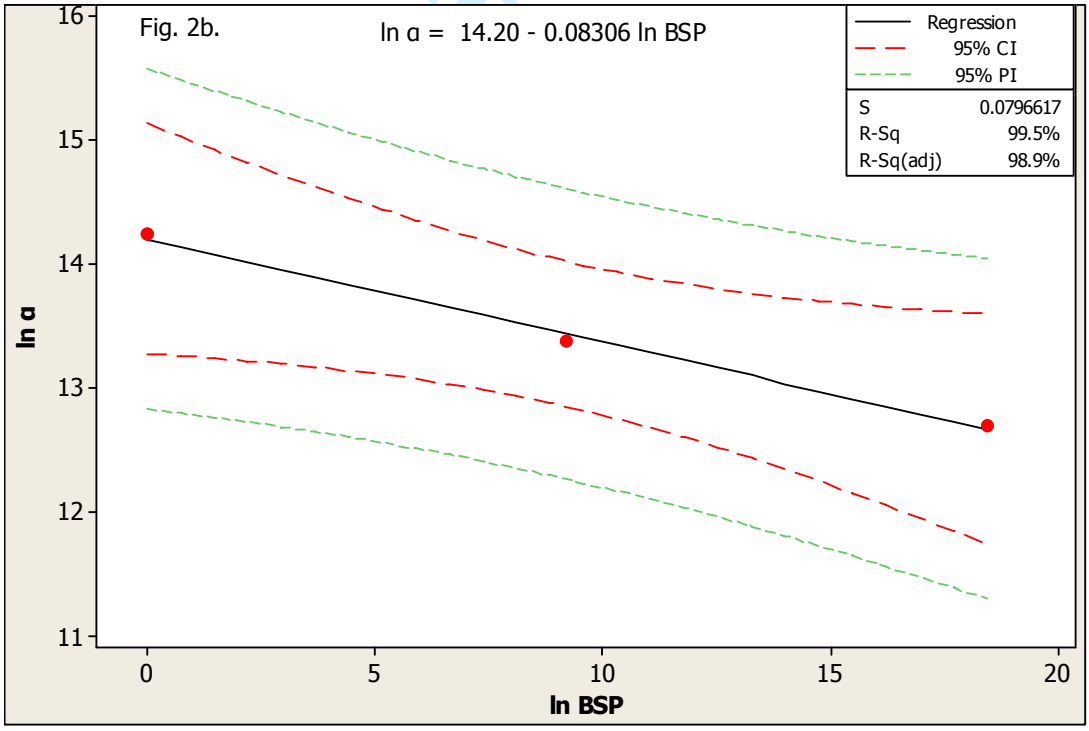


Fig. 2b.
ln α = 14.20 - 0.08306 ln BSP
Regression
95% CI
95% PI
S 0.0796617
R-Sq 99.5%
R-Sq(adj) 98.9%
ln α
16
15
14
13
12
11
0
5
10
15
20
ln BSP

Table 1. Three-way Analysis of Variance for α-amylase activity

Source	DF	SS	MS	F	P
GT	2	77733	38866	19755.61	0.000
ln BSP	2	88332	44166	22449.49	0.000
GD	9	2075955	230662	117244.42	0.000
GT*ln BSP	4	1359	340	172.75	0.000
GT*GD	18	183965	10220	5194.92	0.000
ln BSP*GD	18	48361	2687	1365.64	0.000
GT*ln BSP*GD	36	19158	532	270.49	0.000
Error	180	354	2		
Total	269	2495216			

S = 1.40263 R-Sq = 99.99% R-Sq(adj) = 99.98%

GT: germination temperature, BSP: Bacillus subtilis population, GD: germination time.

Table 2. Kinetic parameters of W-4-PM and 2nd OPM

Germination Temperature (°C)	Steeping Treatment	W-4-PM			2nd OPM					
		ln α	β	Lack of fit (p-value)	a	b	c	R-sq (%)	R-sq(adj)	Lack of fit (p-value)
25	C	14.4032972	-3.40443	0.696	23.3	-0.342	0.0229	96.4	96.2	0.000
	B1	12.7068479	-3.32415	0.507	-21.2	3.72	-0.00810	93.6	93.2	0.000
	B2	13.8054602	-3.39737	0.600	4.3	1.20	0.0123	94.4	94.0	0.000
30	C	14.2209757	-3.53351	0.423	-2.2	1.63	0.00939	93.8	93.4	0.000
	B1	12.7938593	-3.5137	0.713	-36.5	5.55	-0.0239	94.4	94.0	0.000
	B2	13.3374748	-3.45218	0.417	-20.9	3.40	-0.00485	93.5	93.0	0.000
35	C	14.0778748	-3.4764	0.000	-45.3	6.22	-0.0370	87.3	86.3	0.000
	B1	12.6115378	-3.79624	0.125	-52.6	8.74	-0.0589	94.8	94.4	0.000
	B2	12.9715405	-3.6857	0.591	-53.3	7.57	-0.0471	91.7	91.1	0.000

Table 3. Goodness of fit

	GLM	2ndOPM	W-4-PM
MSE	1568.49096	570.669896	0.90030675
RMSE	39.6041786	23.8886981	0.94884496

Figure captions

Figure 1. Experimental and 2nd OPM, GLM and W-4-PM predicted data (at different temperatures: T1=25°C, T2=30°C and T3=35°C for different steeping treatments: B1 and B2 refer, respectively, to the treatments containing 10^8 and 10^4 cells/ml and C refers to the treatment with distilled water alone in the second phase of steeping)

Figure 2a. Effect of germination temperature (GT) on the rate of α-amylase synthesis (α: a parameter of the W-4-PM).

Figure 2b. Effect of steeping treatment on the capacity of the α-amylase synthesis. BSP: *Bacillus subtilis* Population, β: a parameter of the W-4-PM.

Table S1. General Linear Model Stepwise regression

Step	1	2	3	4	5	6	7	8
Constant	11.028	9.802	7.672	-9.971	-22.705	-22.705	-202.3	-216.5
GT*GD	0.0813	0.0702	0.0720	0.1067	0.0592	0.0458	-0.034	-0.034
T-Value	30.29	24.43	24.71	12.95	4.14	5.06	-2.07	-2.09
P-Value	0.000	0.000	0.000	0.000	0.000	0.000	0.040	0.037
lnBSP*GD		0.0385	0.1055	0.1744	0.0799	0.0363	0.0363	0.0159
T-Value		7.38	4.25	6.11	2.19	7.39	7.82	1.81
P-Value		0.000	0.000	0.000	0.029	0.000	0.000	0.071
GT*lnBSP*GD			-0.00229	-0.00455	-0.00145			
T-Value			-2.75	-4.79	-1.21			
P-Value			0.006	0.000	0.229			
GD^2				-0.0097	-0.0150	-0.0150	-0.0150	-0.0150
T-Value				-4.47	-6.03	-6.03	-6.38	-6.46
P-Value				0.000	0.000	0.000	0.000	0.000
GD					2.08	2.48	4.87	5.05
T-Value					4.01	6.23	8.66	9.04
P-Value					0.000	0.000	0.000	0.000
GT							6.0	6.0
T-Value							5.72	5.79
P-Value							0.000	0.000
ln BSP								1.54
T-Value								2.74
P-Value								0.007
S	45.9	41.9	41.4	40.0	38.9	38.9	36.8	36.3
R-Sq	77.40	81.23	81.75	83.03	84.00	83.92	85.69	86.09
R-Sq(adj)	77.31	81.09	81.54	82.77	83.70	83.67	85.42	85.77
Mallows Cp	159.0	89.0	81.2	59.1	42.8	42.4	11.1	5.6

Table S2: 2nd OPM regression analysis

c) Germination temperature = 25°C

AA (for T1, C) = 23.3 - 0.342 GD + 0.0229 GD^2

Predictor	Coef	SE Coef	T	P
Constant	23.285	8.114	2.87	0.008
GD	-0.3424	0.3474	-0.99	0.333
GD2	0.022885	0.003018	7.58	0.000

S = 15.7074 R-Sq = 96.4% R-Sq(adj) = 96.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	179331	89665	363.43	0.000
Residual Error	27	6661	247		
Lack of Fit	7	6647	950	1319.73	0.000
Pure Error	20	14	1		
Total	29	185992			

AA (for T1, B1) = - 21.2 + 3.72 GD - 0.00810 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-21.19	13.43	-1.58	0.126
GD	3.7164	0.5752	6.46	0.000
GD2	-0.008097	0.004998	-1.62	0.117

S = 26.0080 R-Sq = 93.6% R-Sq(adj) = 93.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	269210	134605	199.00	0.000
Residual Error	27	18263	676		
Lack of Fit	7	18248	2607	3343.61	0.000
Pure Error	20	16	1		
Total	29	287473			

AA (for T1, B2) = 4.3 + 1.20 GD + 0.0123 GD^2

Predictor	Coef	SE Coef	T	P
Constant	4.35	11.54	0.38	0.709
GD	1.2018	0.4939	2.43	0.022
GD2	0.012288	0.004291	2.86	0.008

S = 22.3315 R-Sq = 94.4% R-Sq(adj) = 94.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	227745	113872	228.34	0.000
Residual Error	27	13465	499		
Lack of Fit	7	13447	1921	2155.67	0.000
Pure Error	20	18	1		
Total	29	241210			

b) Germination temperature = 30°C

AA (for T2, C) = - 2.2 + 1.63 GD + 0.00939 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-2.19	12.62	-0.17	0.863
GD	1.6345	0.5404	3.02	0.005
GD2	0.009394	0.004695	2.00	0.056

S = 24.4324 R-Sq = 93.8% R-Sq(adj) = 93.4%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	245571	122786	205.69	0.000
Residual Error	27	16117	597		
Lack of Fit	7	16097	2300	2249.21	0.000
Pure Error	20	20	1		
Total	29	261689			

AA (for T2, B1) = - 36.5 + 5.55 GD - 0.0239 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-36.47	13.16	-2.77	0.010
GD	5.5480	0.5633	9.85	0.000
GD2	-0.023936	0.004894	-4.89	0.000

S = 25.4700 R-Sq = 94.4% R-Sq(adj) = 94.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	295351	147676	227.64	0.000
Residual Error	27	17515	649		
Lack of Fit	7	17489	2498	1867.98	0.000
Pure Error	20	27	1		
Total	29	312866			

AA (for T2, B2) = - 20.9 + 3.40 GD - 0.00485 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-20.93	13.82	-1.51	0.142
GD	3.3982	0.5918	5.74	0.000
GD2	-0.004849	0.005142	-0.94	0.354

S = 26.7602 R-Sq = 93.5% R-Sq(adj) = 93.0%

Analysis of Variance

Regression	2	276520	138260	193.07	0.000
Residual Error	27	19335	716		
Lack of Fit	7	19312	2759	2401.37	0.000
Pure Error	20	23	1		
Total	29	295855			

c) Germination temperature = 35°C

AA (for T3, C) = - 45.3 + 6.22 GD - 0.0370 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-45.34	16.34	-2.77	0.010
GD	6.2175	0.6998	8.89	0.000
GD2	-0.036977	0.006080	-6.08	0.000

S = 31.6398 R-Sq = 87.3% R-Sq(adj) = 86.3%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	184974	92487	92.39	0.000
Residual Error	27	27029	1001		
Lack of Fit	7	26947	3850	940.58	0.000
Pure Error	20	82	4		
Total	29	212003			

AA (for T3, B1) = - 52.6 + 8.74 GD - 0.0589 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-52.57	11.72	-4.49	0.000
GD	8.7384	0.5017	17.42	0.000
GD2	-0.058856	0.004359	-13.50	0.000

S = 22.6853 R-Sq = 94.8% R-Sq(adj) = 94.4%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	253300	126650	246.10	0.000
Residual Error	27	13895	515		
Lack of Fit	7	13838	1977	693.65	0.000
Pure Error	20	57	3		
Total	29	267194			

AA (for T3, B2) = - 53.3 + 7.57 GD - 0.0471 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-53.28	14.67	-3.63	0.001
GD	7.5719	0.6283	12.05	0.000
GD2	-0.047090	0.005459	-8.63	0.000

S = 28.4078 R-Sq = 91.7% R-Sq(adj) = 91.1%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	241721	120860	149.76	0.000
Residual Error	27	21789	807		
Lack of Fit	7	21692	3099	637.03	0.000
Pure Error	20	97	5		
Total	29	263510			

Table S3. W-4-PM regression analysis

a) Germination temperature = 25°C

AA (for T1, C) = 28.8 + 251.8 * exp (-1.831*10^{6} * GD$^{-3.40443}$)

Source	DF	SS	MS	F	P
Error	22	62.1085	2.82311		
Lack of fit	6	12.0526	2.00877	0.64	0.696
Pure Error	16	50.0559	3.12850		

AA (for T1, B1) = 29.8 + 250.5 * exp (-333540 * GD-3.32415)

Source	DF	SS	MS	F	P
Residual Error	22	84.0109	3.81868		
Lack of fit	6	21.5233	3.58722	0.92	0.507
Pure Error	16	62.4876	3.90548		

AA (for T1, B2) = 29.1 + 251.6 * exp (-997895 * GD-3.39737)

Source	DF	SS	MS	F	P
Residual Error	22	64.8472	2.94760		
Lack of fit	6	14.6137	2.43562	0.78	0.600
Pure Error	16	50.2335	3.13959		

b) Germination time = 30°C

AA (for T2, C) = 28.2 + 252.8 * exp (-1520390 * GD$^{-3.53351}$)

Source	DF	SS	MS	F	P
Residual Error	22	48.9772	2.22624		
Lack of fit	6	13.9662	2.32770	1.06	0.423
Pure Error	16	35.0110	2.18819		

AA (for T2, B1) = 29.8 + 250.0 * exp (-361059 * GD$^{-3.5137}$)

Source	DF	SS	MS	F	P
Residual Error	22	27.8758	1.26708		
Lack of fit	6	5.2512	0.87519	0.62	0.713
Pure Error	16	22.6246	1.41404		

AA (for T2, B2) = 28.1 + 253.3 * exp (-617336 * GD$^{-3.45218}$)

Source	DF	S	MS	F	P
Residual Error	22	187.990	8.54500		
Lack of fit	6	54.066	9.01096	1.08	0.417
Pure Error	16	133.924	8.37026		

c) Germination temperature = 35°C

AA (for T3, C) = 28.1 + 250.3 * exp (-353576 * GD$^{-3.4764}$)

Source	DF	SS	MS	F	P
Residual Error	13	748.140	57.549		
Lack of fit	3	719.937	239.979	85.09	0.000
Pure Error	10	28.203	2.820		

AA (for T3, B1) = 28.8 + 252.6 * exp (-307238 * GD$^{-3.79624}$)

Source	DF	SS	MS	F	P
Residual Error	13	55.5943	4.27648		
Lack of fit	3	23.4807	7.82691	2.44	0.125
Pure Error	10	32.1135	3.21135		

AA (for T3, B2) = 29.7 + 251.2 * exp (-446978 * GD$^{-3.68857}$)

Source	DF	SS	MS	F	P
Residual Error	13	48.1187	3.70144		
Lack of fit	3	8.0191	2.67303	0.67	0.591
Pure Error	10	40.0996	4.00996		

Table S4. GLM regression analysis

The regression equation is

AA = - 9.97 + 0.107 GT*GD + 0.174 lnBSP*GD - 0.00455 GT*lnBSP*GD - 0.00967 GD^2

Predictor	Coef	SE Coef	T	P
Constant	-9.971	6.060	-1.65	0.101
GT*GD	0.106678	0.008237	12.95	0.000
lnBSP*GD	0.17443	0.02853	6.11	0.000
GT*lnBSP*GD	-0.0045463	0.0009484	-4.79	0.000
GD^2	-0.009667	0.002161	-4.47	0.000

S = 39.9741 R-Sq = 83.0% R-Sq(adj) = 82.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	4	2071765	517941	324.13	0.000
Residual Error	265	423451	1598		
Lack of Fit	85	423097	4978	2530.10	0.000
Pure Error	180	354	2		
Total	269	2495216			