

STARCH HYDROLYSIS USING (α -AMYLASE: A LABORATORY EVALUATION USING RESPONSE SURFACE METHODOLOGY

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Abstract

The South African cane sugar mills use a thermostable amylase enzyme (produced by genetically modified strains of *Bacillus licheniformis*) to control seasonally high starch levels in juice. The recommended point of application of the enzyme is the third or fourth evaporator effect since the temperatures in these effects are high enough for the starch to remain solubilised, but low enough to prevent deactivation of the enzyme. The extended residence time and lower Brix in these effects are also believed to favour enzymatic action compared to conditions in the syrup tank.

While considerable effort has been made to characterize the effect of factory parameters on the previous generation of commercially available α -amylases when they were first introduced to the cane sugar industry, not much work has been done on the thermostable enzymes, thus spurring this investigation. The effects and interaction effects of temperature, Brix, pH, starch concentration and enzyme concentration on the amount of starch hydrolysed by the enzyme in a sucrose solution were determined using statistical experimental design techniques. Experiments were conducted as batch processes, while the stirring rate, calcium content and the volume of each run were kept constant.

A model was derived and corresponding response surface diagrams were used to identify trends in terms of the evaluated parameters. Some experiments were done to lend support to the model, which was shown to be a good predictive tool within the experimental domain investigated.

Keywords: sugar, starch, enzyme, α -amylase, laboratory, statistics, design

Introduction

Sugarcane starch is a naturally occurring polysaccharide in sugar cane and consists of a mixture of linear (amylose) and branched (amylopectin) molecules, in a ratio of *ca.* 20 to 80, although this ratio varies according to the source and maturity of the plant. Starch is the primary product from photosynthesis and is temporarily stored in cane leaves as a reserve food until it can be converted to sugar. Starch content in cane varies widely with variety and growing conditions although the occurrence of high or low levels of starch in certain regions is still unexplained (Boyes, 1958; Bruijn and Vanis, 1970; Madsen, 1974; Meade and Chen, 1977). The presence of excessive starch in a cane sugar factory is known to cause, amongst other things, boiling and viscosity related problems and filterability impairment, particularly in carbonatation refineries (Meade and Chen, 1977).

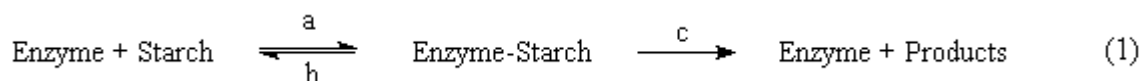
Since starch, as a factor of cane quality, is introduced into the sugar mill together with the cane the sugar miller has little choice but to deal with high levels of starch in the milling

process itself. Cane juice contains natural amylase enzyme which will hydrolyse starch once it is gelatinised (above 68°C), the use of which was first described by Nicholson and Horsley (1958). The natural enzymes are optimally active at pH 5.5-5.7 and 65°C but will be inactivated at temperatures above 75°C (Alexander, 1974; Sayed *et al.*, 1974; Wood, 1962; Madsen, 1974). In order to make full use of the natural enzymes, measures have to be taken to give the enzyme enough time at the appropriate conditions. However, since cane juice also contains natural invertase enzyme which is most active at pH 3.0-5.5 and 55°C and is only inactivated outside this pH range and above 65°C (albeit not instantaneously) (Anon, 2004), such conditions will cause severe sucrose inversion. In addition, the amount and type of natural enzymes cannot be controlled or predicted. With the advent of diffusers it has been shown on more than one occasion that conditions inside a diffuser can sustain the hydrolysis of starch by natural amylase enzyme and that diffuser juice typically has 25% less starch compared to milling juice (Koster, 1995; Mkhize, 2003; Rein, 1995).

The application of α -amylase enzymes to juice in one of the evaporators has been practised in South Africa for the last 20-30 years, but mainly by factories operating milling tandems as opposed to diffusers. Starch levels are also primarily monitored in resulting raw sugars while only one mill currently analyses juice for starch content (Bruijn and Jennings, 1968; Bruijn, 1970; Smith, 1970). A penalty is applied to local raw sugar arriving at the South African Sugar Terminal with a starch content of above 150 ppm, relating roughly to cane juice starch levels of about 400 ppm (based on Brix). In the 2003/04 season about R84 000.00 was paid in starch penalties (80 000 tons of sugar). The penalty threshold will most likely be reduced in the future according to international demands and trends; the limit for sugars accepted at the Hulett Refineries was recently lowered to 100 ppm (Mohabir and Kheswa, 2003).

Smith (1970) derived a set of curves using variables suitable to South African conditions which was to be used with two commercial α -amylases, both derived from *Bacillus subtilis*. The effects of reaction time, Brix and temperature combinations, viscosity, starch concentration and enzyme dosage were evaluated. These enzymes were stable up to about 95°C, which rendered the third or fourth evaporator effects most suitable for their application. In order to ensure longer reaction times, a holding tank between the third and fourth or fourth and fifth evaporators was sometimes utilised (Madsen, 1974).

The kinetics of starch hydrolysis by α -amylase can be represented as follows:



According to this equation α -amylase and starch form an enzyme-starch complex in an equilibrium reaction. This complex then yields the original enzyme and oligosaccharide products in an irreversible step. It is believed that initial reaction kinetics are typically first order and that the reaction therefore has a fixed half-life. The reaction rate is then dependent only on the starch concentration while the active enzyme sites are being filled. Once all the sites are occupied the kinetics essentially become zero order and the rate is dependent only on the enzyme concentration (Dixon and Web, 1964). Typically, the initial rate of reaction is limited by the dissolution of starch which is aided by temperature; thermostability in the enzyme is therefore highly desirable (Bruijn and Jennings, 1968).

With the introduction of a heat stable α -amylase (produced by genetically modified strains of *Bacillus licheniformis*) to the South African sugar industry, Bruijn (1973) conducted an initial investigation and concluded that the enzymes showed potential to be used successfully at

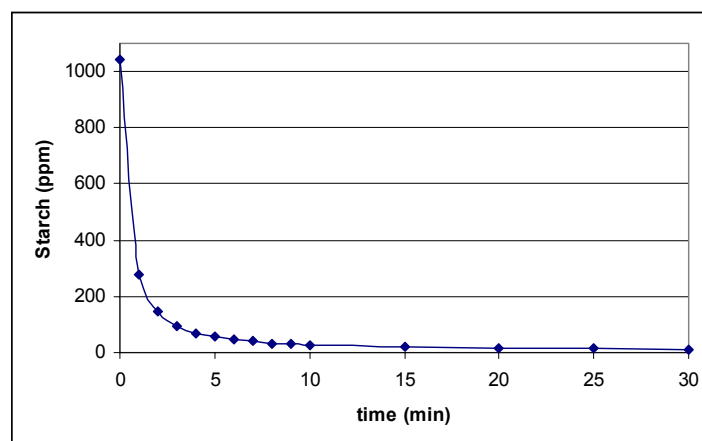
higher temperatures. However, he reported that the enzymatic action in mixed juice was somehow hindered and that application would therefore only be viable after the clarifier. Subsequent trials were conducted at Umfolozi mill by Madsen (1974) at the end of the season to determine the best-suited conditions for this enzyme. The effect of pH was determined at 37, 60 and 95°C and the effect of temperature at pH 5.7. The supplier's current recommended conditions for use with this enzyme are based on results of these trials. The third or fourth evaporator effects are still indicated as the best point of entry into the factory although the enzymes can withstand higher temperatures (up to 105°C).

Sporadic problems with high starch levels in sugar despite the use of enzymes under the recommended conditions suggested that a more in-depth investigation into the commercial application of thermostable α -amylase to a cane sugar factory was needed. Past studies have also mostly been done prior to the use of diffusers becoming widespread in the South African industry. This paper discusses the findings of a laboratory investigation into parameters affecting the enzymatic hydrolysis of starch in a sucrose solution.

Reaction conditions

Figure 1 depicts a typical enzyme progress curve over time.

Because enzyme kinetics invariably slow down as the reaction progresses due to the changing environment, it is customary in enzyme studies to use the initial velocity as a primary response factor (Dixon and Webb, 1964). With the reaction indicated in Figure 1 the biggest change on the y-axis occurs between zero and one minutes. One minute was therefore deemed to be a suitable sampling time to evaluate the effect of various reaction conditions on the amount of starch hydrolysed by the enzyme since this would cause relatively large variations. Although a single sampling point would be greatly affected by experimental error, sampling at one minute was found to be reproducible within a tolerance of 50 ppm.



42.5° Brix, 90°C, 1000 ppm maize starch on Brix,
60 ppm thermostable α -amylase on Brix and pH 6.25.

Figure 1. Typical α -amylase progress curve.

A number of reaction variables were identified as potentially having significant effects on the enzymatic performance of thermostable α -amylase. Investigating the effects of reaction variables may become quite involved and time-consuming when there is more than one variable affecting the outcome of an experimental procedure. Various techniques have been developed to assist experimenters in studying the effects of reaction variables. Of these techniques, the best insight into a system is most frequently gained by employing statistical

design methods. (Box and Draper, 1987; Davies, 1993; Montgomery, 1991; Lionnet and Baker, 1977).

The idea behind statistical experimental design techniques is to arrange a set of experiments in such a way as to obtain significant variations in the experimental response with different settings of the experimental variables, whilst varying the experimental variables simultaneously instead of one by one. The variations in the experimental response must then be traceable to the variations in the experimental variables. Such an arrangement will allow for the determination of individual as well as combined influences of each experimental variable on the experimental response in the presence of a simultaneous variation of all other variables. A response surface experimental design was deemed to be the most suitable technique for the current investigation.

Five experimental variables, namely Brix, temperature, starch concentration, enzyme concentration and pH, were selected for statistical evaluation using a sucrose solution. A constant amount of calcium (based on sample) was added since α -amylase is a metalloenzyme and requires the presence of calcium cations in order to stabilize the transition states *via* electrostatic interactions (Dixon and Webb, 1964). Maize starch (Merck) was used due to the problematic isolation of cane starch (Alexander, 1974) and since maize starch is deemed to closely resemble cane sugar starch in terms of its amylose to amylopectin ratio (Vignes, 1974) as well as its response to α -amylase (personal communication with enzyme supplier). The starch was gelatinised in water prior to use.

The adjustment of the solution pH was done after mixing of all the ingredients but before heating of the solution and thus before the enzyme was added. A 0.05M hydrochloric acid solution was used for pH adjustment in accordance with recommendations from the enzyme supplier. For the purpose of this paper, pH of the samples is therefore defined according to this procedure and does not relate directly to a typical solution pH that is measured in a cane sugar factory.

A constant stirring rate was used to aid swift interaction between the substrate and the enzyme, although Smith (1970) indicated that the stirring rate did not appear to make a difference. The enzyme was diluted (1:20) prior to use and the activity of the stock enzyme monitored on a weekly basis using the SASTA α -amylase activity method (Anon, 1985).

Experimental

Materials

The following materials were used during the enzyme reactions:

- sucrose H-18 refinery first boiling sugar
- maize starch Merck (uniLAB), analytical grade, 20-30% amylose
- α -amylase Novo Enzymes, Termamyl 120 LS.

Enzyme reactions

A three-armed 500 cm³ round-bottom flask equipped with a water-cooled condenser was immersed in a heated oil bath with magnetic stirring. 106.25 g of sucrose (42.5°Bx), 21.26 g solubilized maize starch (1000 ppm on Brix), 4 g *ca.* 10% lime (*ca.* 150 ppm on sample) and distilled water were mixed to a final mass of 250 g and the pH of the solution adjusted to 6.25 using 0.05M hydrochloric acid. The solution was heated to 90°C and the reaction started by the addition of 0.09 g (60 ppm on Brix) diluted (1:20) enzyme. A sample (3 cm³) was taken after 1 minute and quenched with 1.2 cm³ 0.5M acetic acid.

The above amounts were adjusted according to the levels indicated in Table 1 and the experiments indicated in Appendix A.

Starch analysis

Samples were analysed for starch by the method supplied by the Sugar Processing Research Institute (SPRI) in Louisiana, which is a modified version of the SASTA simplified starch method (Anon, 1985; SPRI, 2002). The centrifuge step in the SPRI method was omitted since there were no insoluble solids present in the samples. Although no tolerance was given, the SPRI data and subsequent evaluation of the method at the SMRI laboratory indicated that a tolerance of ± 50 ppm starch on Brix would be appropriate.

Enzyme activity

α -Amylase activity of the stock enzyme was determined using the SASTA method (Anon, 1985). An activity of 96% starch hydrolysis in 15 minutes was recorded.

Table 1 lists the full experiment domain selected for the response surface experiment design.

Table 1. Experiment domain.

Code	Dependent Variable	Unit	Variable level		
			Low (-1)	Centre (0)	High (+1)
x ₁	Brix	%	25.0	42.5	60.0
x ₂	Temperature	°C	80	90	100
x ₃	Enzyme concentration	ppm on Brix	20	40	60
x ₄	Starch concentration	ppm on Brix	400	700	1000
x ₅	pH	(measured at 25°C)	5.50	6.25	7.00
Fixed Variables					
Variable		Unit	Level		
Calcium concentration (lime)		ppm on sample	ca. 150		
Stirring rate (magnetic stirrer bar)		rpm	ca. 400		
Enzyme dilution with distilled water		ratio	1:20		
Total sample size		g	250		
Time		minutes	1		

A reduced central composite design was used for the evaluation of the five experimental variables selected. Although such a design does not necessarily have the same predictive power as a full, rotatable central composite design, its use results in a significant saving in experimental runs (Davies, 1993). This design considered the effect of each of the variables as well as interaction effects between two or more of the variables (Lionnet and Barker, 1977). The design also included axial or star points which fall outside the experimental design to lend it some rotatability and orthogonality (Box and Draper, 1987). The response (or independent) variable selected was the amount of starch hydrolysed after one minute.

Results and discussion

Refer to Appendix A for a full experimental design and the resulting experiment responses. A tolerance of ± 50 ppm is associated with the starch analysis results.

The analysis of variance (ANOVA) of the model (refer to Appendix B, Table 2) shows that there is no significant lack-of-fit (small F value and probability > 0.1). This is also confirmed by the normal probability plot of the residuals (Figure 2) and the plot of the residuals versus predicted response (Figure 3), both of which show none of the deviations expected for poor

models. (Normal probability plots should give a straight line in view of the fact that random experimental error is always normally distributed, while a plot of residuals versus predicted response should show a random scatter of points equally distributed about the zero line.) Trends from this model may therefore be used to predict the effect of these factors on the action of thermostable α -amylase on maize starch in laboratory solutions.

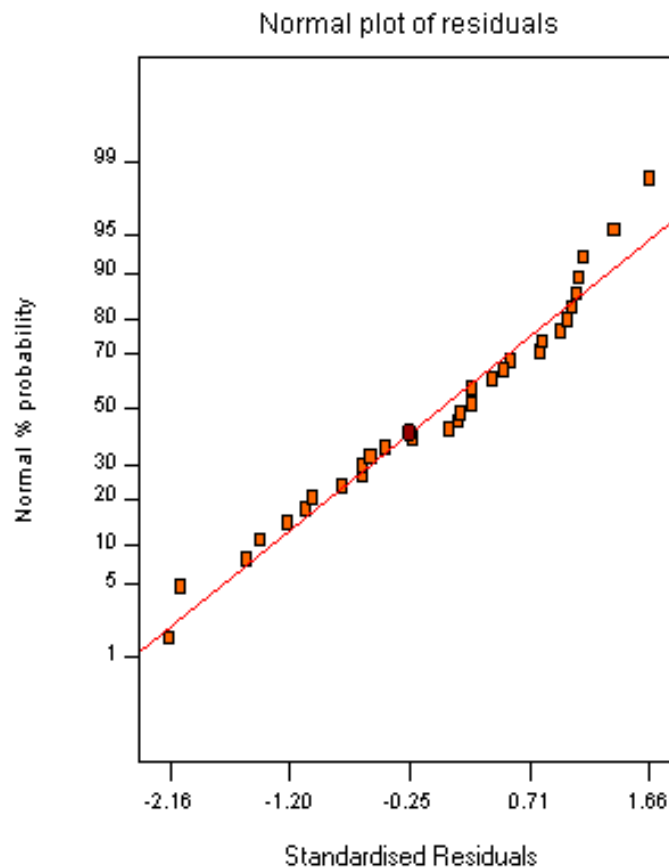


Figure 2. Normal probability plot of residuals.

Preliminary inspection of the model indicated that a high initial starch level and high enzyme dosage would result in high starch removal and that this was also favoured by a low Brix and possibly by a low pH, although this effect may not be significant. From experience it is known that the removal of starch is promoted by a high enzyme dosage and a low Brix (Smith, 1970). The temperature did not have a significant effect. In addition, the two-factor interaction effects between Brix, starch content and pH, respectively were significant. The effect of temperature and enzyme dosage did not appear to be much affected by the other factors and temperature was therefore omitted from the model.

Since the model derived (Appendix B, Equation 1) was shown to be valid within the experiment domain investigated, it could be used to move closer to optimum experiment conditions. Two approaches may be followed to establish the direction of the experiment optimum, namely canonical analysis of the two response surface models, or a graphical interpretation. The latter approach will be followed here as it gives a clear picture and some additional insight into the different trends within the experiment domain investigated.

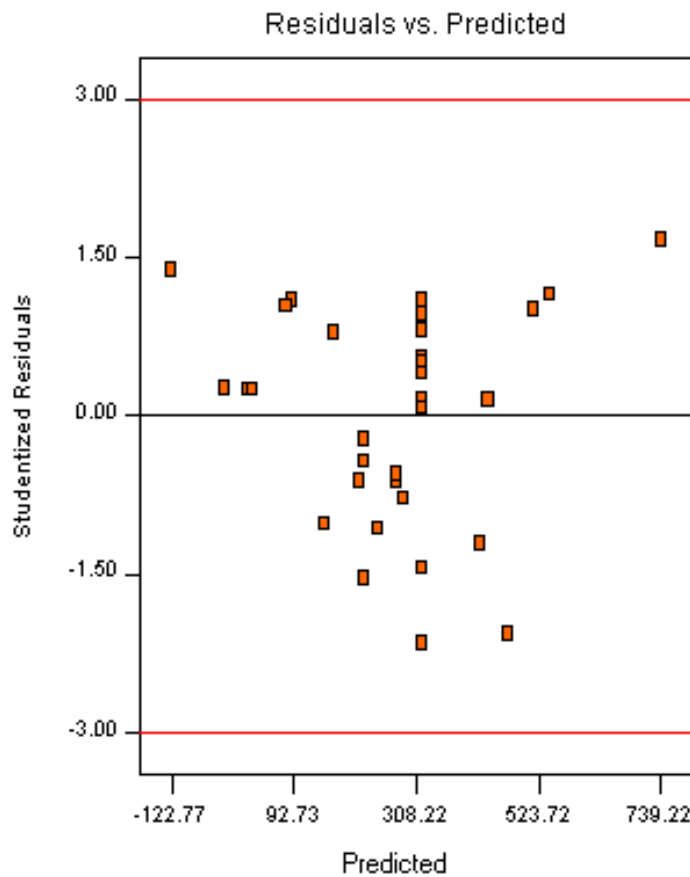


Figure 3. Residuals versus predicted response.

Some contour diagrams for the variation in experimental response as a function of two variables at a time (the other two variables being constant at these respective midpoint values) are illustrated in Figures 4 to 6.

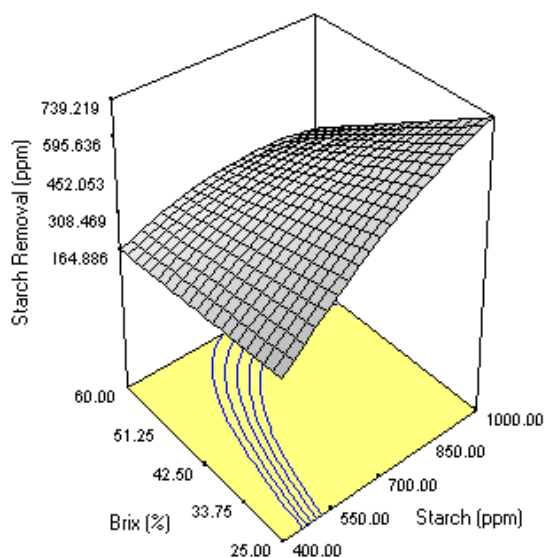


Figure 4a. Interaction between Brix and starch content (pH 5.5, 60 ppm enzyme).

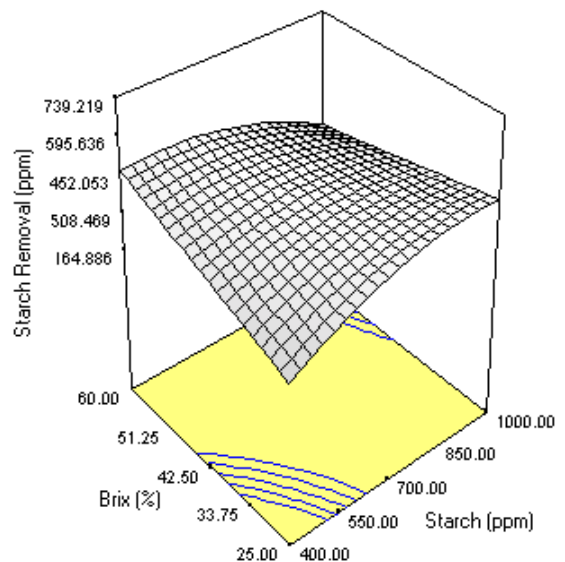


Figure 4b. Interaction between Brix and starch content (pH 7.0, 60 ppm enzyme).

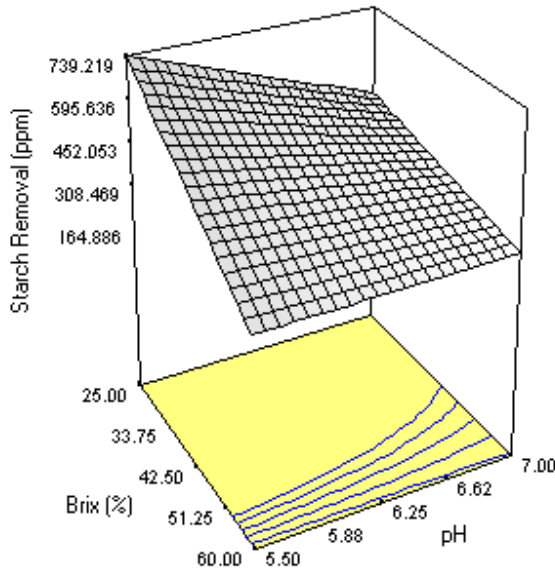


Figure 5a. Interaction between Brix and pH (1000 ppm starch, 60 ppm enzyme).

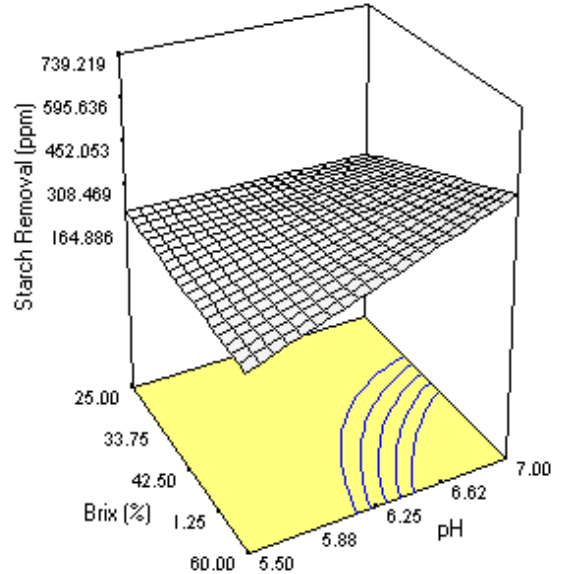


Figure 5b. Interaction between Brix and pH (400 ppm starch, 60 ppm enzyme).

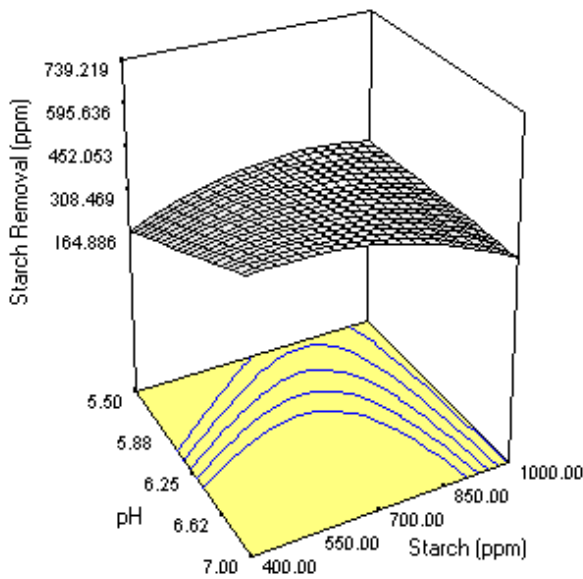


Figure 6a. Interaction between starch and pH (60°Bx, 60 ppm enzyme).

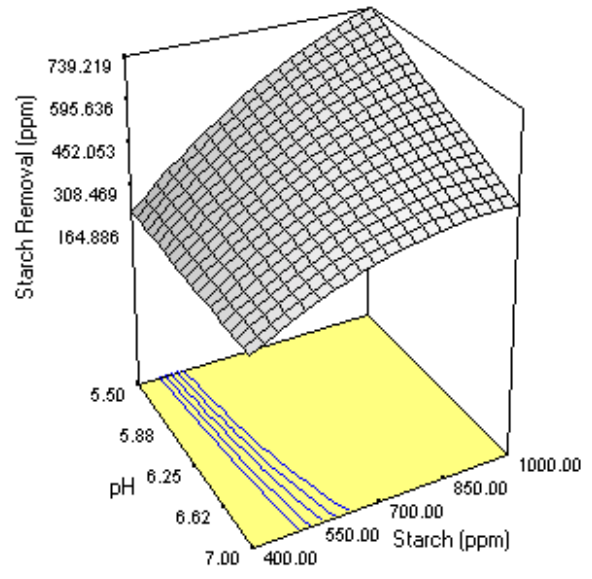


Figure 6b. Interaction between starch and pH (25°Bx, 60 ppm enzyme).

A number of aspects regarding these contour diagrams deserve further comment:

- At a high pH value (7.00) starch hydrolysis at high initial starch levels (>800 ppm) was favoured by low Brix while a higher Brix favoured starch hydrolysis at lower starch levels (between 400 and 700 ppm). At a low pH value (5.50) starch hydrolysis at high initial starch levels was favoured by low Brix while starch hydrolysis at lower initial starch levels was not much affected by the actual Brix, as indicated by the ridges in Figures 4a and b.

- At high starch levels (1000 ppm) starch hydrolysis was favoured by a lower Brix and lower pH, while starch hydrolysis at lower initial starch levels (400 ppm) was favoured by a higher Brix and higher pH, as indicated by the ridges in Figures 5a and b.
- The interaction between initial starch and pH at high Brix (60 ppm) was minimal. However, at a low Brix starch hydrolysis is clearly favoured by a lower pH throughout the initial starch content range, as indicated by the ridges in Figures 6a and b.

From the above comments and according to the model, the best conditions for the thermostable α -amylase within the experimental domain with high starch levels are low Brix and low pH. These conditions incidentally also favours the action of natural invertase on sucrose although concurrent high temperatures may be used to inactivate the natural invertase enzymes.

Table 2 lists predicted starch removal using the model and actual starch removal from experiments at some typical conditions to test the above observations.

Table 2. Starch removal after one minute at various conditions using 60 ppm α -amylase and 1000 ppm initial starch.

	Brix (%)	Temperature (°C)*	pH	Predicted starch removal (ppm)	Actual starch removal (ppm)
Best suited conditions	25	100	5.5	739	664
Diffuser	13	85	5.5	**	782
Clarifier	13	100	7.0	**	641
Enzyme tank	13	65	5.5	**	787
1st evaporator	13	112	7.0	**	651
2nd evaporator	25	105	6.8	475	532
3rd evaporator	40	90	6.4	438	410
4th evaporator	50	87	6.1	375	342
5th evaporator	65	68	6.0	**	258

*Temperature has no effect on the predicted starch removal

**Conditions outside the experiment domain

A tolerance of ± 50 ppm is associated with starch analysis results

Good correlations were obtained between the predicted and actual starch removal as indicated in Table 2. Results suggest that conditions in the diffuser and enzyme tank would be the most suitable for thermostable α -amylase enzyme application. These values are, however, based on technical laboratory solutions and conditions in a cane sugar factory will most certainly be much more complex. In addition, inversion of sucrose by cane invertase under these conditions needs to be considered.

Conclusions

A model was derived to describe the effect of a thermostable α -amylase on maize starch in the presence of sucrose and calcium, using Brix, temperature, starch concentration, enzyme concentration and pH as dependent variables. The model was shown to be valid within the experiment domain and could be used to make predictions relating to these sucrose solutions.

Adjustment of the solution pH was done at room temperature using hydrochloric acid and does therefore not relate directly to a typical solution pH that is measured in a cane sugar factory.

Significant interaction effects were observed between the solution pH and starch concentration, solution pH and Brix, and between the starch concentration and Brix. The best-suited conditions for thermostable α -amylase according to the model are low Brix and low pH, while starch hydrolysis benefited from a high initial starch content and a high enzyme dosage.

Applicability of the model to actual cane sugar factory samples and conditions is currently being investigated.

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APPENDIX A

Experiment design and response values

To prevent bias, runs were conducted in a completely randomised order.

Table 1. Experiment design and response values.

Run No.	Variable settings*					Response
	Brix (°Bx)	Temperature (°C)	Enzyme (ppm on Brix)	Starch (ppm on Brix)	pH (pH units)	Starch removed (ppm on Brix)
1	-1 (25.0)	-1 (80)	-1 (20)	-1 (400)	+1 (7.00)	43
2	+1 (60.0)	-1 (80)	-1 (20)	-1 (400)	-1 (5.50)	1
3	-1 (25.0)	+1 (100)	-1 (20)	-1 (400)	-1 (5.50)	52
4	+1 (60.0)	+1 (100)	-1 (20)	-1 (400)	+1 (7.00)	198
5	-1 (25.0)	-1 (80)	+1 (60)	-1 (400)	-1 (5.50)	164
6	+1 (60.0)	-1 (80)	+1 (60)	-1 (400)	+1 (7.00)	216
7	-1 (25.0)	+1 (100)	+1 (60)	-1 (400)	+1 (7.00)	133
8	+1 (60.0)	+1 (100)	+1 (60)	-1 (400)	-1 (5.50)	262
9	-1 (25.0)	-1 (80)	-1 (20)	+1 (1000)	-1 (5.50)	684
10	+1 (60.0)	-1 (80)	-1 (20)	+1 (1000)	+1 (7.00)	222
11	-1 (25.0)	+1 (100)	-1 (20)	+1 (1000)	+1 (7.00)	109
12	+1 (60.0)	+1 (100)	-1 (20)	+1 (1000)	-1 (5.50)	207
13	-1 (25.0)	-1 (80)	+1 (60)	+1 (1000)	+1 (7.00)	453
14	+1 (60.0)	-1 (80)	+1 (60)	+1 (1000)	-1 (5.50)	207
15	-1 (25.0)	+1 (100)	+1 (60)	+1 (1000)	-1 (5.50)	943
16	+1 (60.0)	+1 (100)	+1 (60)	+1 (1000)	+1 (7.00)	189
17	- α (7.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	259
18	+ α (77.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	189
19	0 (42.5)	- α (70)	0 (40)	0 (700)	0 (6.25)	462
20	0 (42.5)	+ α (110)	0 (40)	0 (700)	0 (6.25)	344
21	0 (42.5)	0 (90)	- α (0)	0 (700)	0 (6.25)	9
22	0 (42.5)	0 (90)	+ α (80)	0 (700)	0 (6.25)	650
23	0 (42.5)	0 (90)	0 (40)	- α (100)	0 (6.25)	18
24	0 (42.5)	0 (90)	0 (40)	+ α (1300)	0 (6.25)	58
25	0 (42.5)	0 (90)	0 (40)	0 (700)	- α (4.75)	1
26	0 (42.5)	0 (90)	0 (40)	0 (700)	+ α (7.75)	331
27	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	440
28	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	482
29	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	380
30	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	402
21	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	395
22	0 (42.5)	0 (90)	0 (40)	0 (700)	0 (6.25)	400

*Natural values given in parenthesis

α indicates axial points which fall outside the experimental domain for rotatability, i.e. to ensure the precision of the estimated response is dependent only on the distance from the design centre

A tolerance of ± 50 ppm is associated with the starch analysis results

APPENDIX B

Analysis of variance for the model

Table 1. Response surface estimation.

Factor	Coeff Estimate	DF	Std Error	t for H ₀ , Coeff = 0
Intercept	350.88	1	70.90	-
x ₁	-50.79	1	36.53	-1.39*
x ₂	-5.54	1	36.53	-0.15
x ₃	96.91	1	37.47	2.59*
x ₄	84.38	1	36.53	2.31*
x ₅	-12.38	1	36.53	-0.34
x ₁ ²	-24.61	1	32.98	-0.75
x ₂ ²	20.14	1	32.98	0.61
x ₃ ²	4.77	1	36.36	0.13
x ₄ ²	-71.11	1	32.98	-2.16*
x ₅ ²	-39.11	1	32.98	-1.19
x ₁ × x ₂	19.81	1	44.74	0.44
x ₁ × x ₃	-34.94	1	44.74	-0.78
x ₁ × x ₄	-103.06	1	44.74	-2.30*
x ₁ × x ₅	78.31	1	44.74	1.75*
x ₂ × x ₃	54.44	1	44.74	1.22
x ₂ × x ₄	-21.19	1	44.74	-0.47
x ₂ × x ₅	-44.56	1	44.74	-1.00
x ₃ × x ₄	5.56	1	44.74	0.12
x ₃ × x ₅	-13.31	1	44.74	-0.30
x ₄ × x ₅	-73.69	1	44.74	-1.65*

*indicates significance at 5% confidence

Coeff = Coefficient, DF = degrees of freedom, Std Error = standard error, Prob = probability, t = Student's t-test and H₀ = the null hypothesis

x₁ ≡ Brix, x₂ ≡ Temperature, x₃ ≡ Enzyme concentration, x₄ ≡ Starch concentration, x₅ ≡ pH.

$$\text{Response} = 350.88 - 50.79x_1 + 96.91x_3 + 84.38x_4 - 71.77x_4^2 - 103.06x_1x_4 + 1.75x_1x_5 - 73.69x_4x_5 \quad (1)$$

Table 2. Analysis of variance (ANOVA).

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	951154	7	135879	5.9	0.0004
Residual	550574	24	22941		
<i>Lack of fit</i>	462245	19	24329	1.4	0.3877
<i>Pure error</i>	88329	5	17666		
Correlation Total	1501730	31			
Root MSE	151		R-Squared	0.63	
Dep Mean	269		Adj R-Squared	0.53	
CV	56		Pred R-Squared	0.28	
PRESS	1080480		Adeq Precision	11	Desire > 4

MSE = mean sum of errors, Dep = dependent, CV = correlation variation, PRESS = predicted residual sum of squares, R = residual, Adj = adjusted, Pred = predicted, Adeq = adequate and F = F-test value.

