Application Technology – US Fuel Research
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From: RMF/JPlm

Simultaneous liquefaction and protein degradation at 65, 67.5, & 70°C for SSF to Fuel ethanol

Study # RMF050118

Summary & Conclusions

- Simultaneously starch liquefaction and protein degradation has been carried out at 65, 67.5 and 70°C in a mini scale liquefaction set up using 15 ml tubes.
- Termamyl SC and Alcalase (Family S8), Novoren (Family A1) and ProMix have been tested and evaluated for their effect on standard fermentation parameters.
- Background conditions for the liquefaction was pH=5.8 and DS=34.10% and 50 NU/g DS Termamyl SC and for SSF Spirizyme Fuel was added @ 0.5 AGU/g DS. No urea was added.
- The effect of increasing the temperature contributed positively on the ethanol yield, as higher temperatures alone gave higher ethanol yields due to a more efficient liquefaction of corn starch.
- Alcalase was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalase was increased by increasing temperature. @ 70°C Alcalase enhances the ethanol yield by 6% compared to not having at protease added during liquefaction.
- Protease and temperature had significant impact on glycerol. Novoren and Alcalase lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.
- The glycerol:ethanol ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.
- The pH is higher at liquefaction conditions, and there could be a factor of substrate stabilization in corn flour as opposed to corn mash (post liquefaction studies)

Purpose

To study the effect of adding an α-amylase (Termamyl SC), and a peptidase (Alcalase, Novoren, ProMix) at the same time during liquefaction carried out @ 65, 67.5, and 70°C. Starch liquefaction and protein degradation is thereby run simultaneously for SSF.

Background

Post liquefaction trials have been carried out with Alcalase, where the effect has been very temperature dependent. Alcalase showed best performance at 50°C, and the performance decreased significantly with increasing temperature. In this experiment peptidases are added to the raw mash, where starch, protein and other corn sources are intact and liquefaction and protein degradation is carried out in 15 ml test tubes. Having a simultaneous step of starch liquefaction and protein degradation may be interest to industry, as temperature pH could be beneficial to the peptidases we have available and thereby secure better protein hydrolysis and possible starch release from protein bound starch sources, thus obtain higher yeast efficiency during fermentation.

The factors to be tested in each of the factorial experiments are as follows:
Temperature: 65, 67.5, 70°C
Enzyme: Alcalase, Novoren, and ProMix and no Protease.

Background conditions similar for all treatments during SSF are pH=5, and Spirizyme fuel @ 0.5 AGU/g DS. During liquefaction Alcalase and Novoren was added at 0.02 mg EP/g DS, and ProMix at 0.03 mg Product/g DS (corresponding to the dosage in SSF).

Experimental

The experiment was carried out in 15 ml test tubes using approximately 5 g of corn mash (34.10 % DS) (exact weigh and dry matter content is known) and 5 replicates. Corn flour from Broin (EXOL) was used for the experiments. Subsequently the impact from the liquefaction was accessed by conducting a standard SSF fermentation.

Table 1. HPLC ethanol results after 24 and 48 H of fermentation

Treatment	AGU/g DS	NU/g DS	mg EP/g DS	mg EP/g DS	mg product/g DS
Spirizyme fuel	0.5	50			
Termamyl SC	0.5	50			
Alcalase 0.02	0.5	50	0.02		
Novoren	0.5	50		0.02	
Promix	0.5	50			0.03

Liquefaction Treatment
Corn flour was added water to obtain a DS fraction of 34.10% and pH was adjusted to 5.6 and samples were taken for DS determination. 5g of this mash was transferred to 15ml test tubes, which were liquefied for 1H after the corn mash reached the designated liquefaction temperature, which were 65, 67.5, and 70°C.

SSF Fermentation

Spirizyme fuel was added to all the tubes after liquefaction. Fermentations were carried out as SSF at 32°C, 70 hours using Red Star yeast. Yeast addition was aimed at $1*10^7$ cells/ml. All treatments were each run in 8 replicates and non-liquefied controls were included in the fermentation. The fermentations were monitored by weighing the individual tubes and recording the time & date of the measurement. At the end of fermentation tubes were sampled for HPLC analysis of sugars and fermentation products, primary parameters evaluated were ethanol and glycerol.

Results:

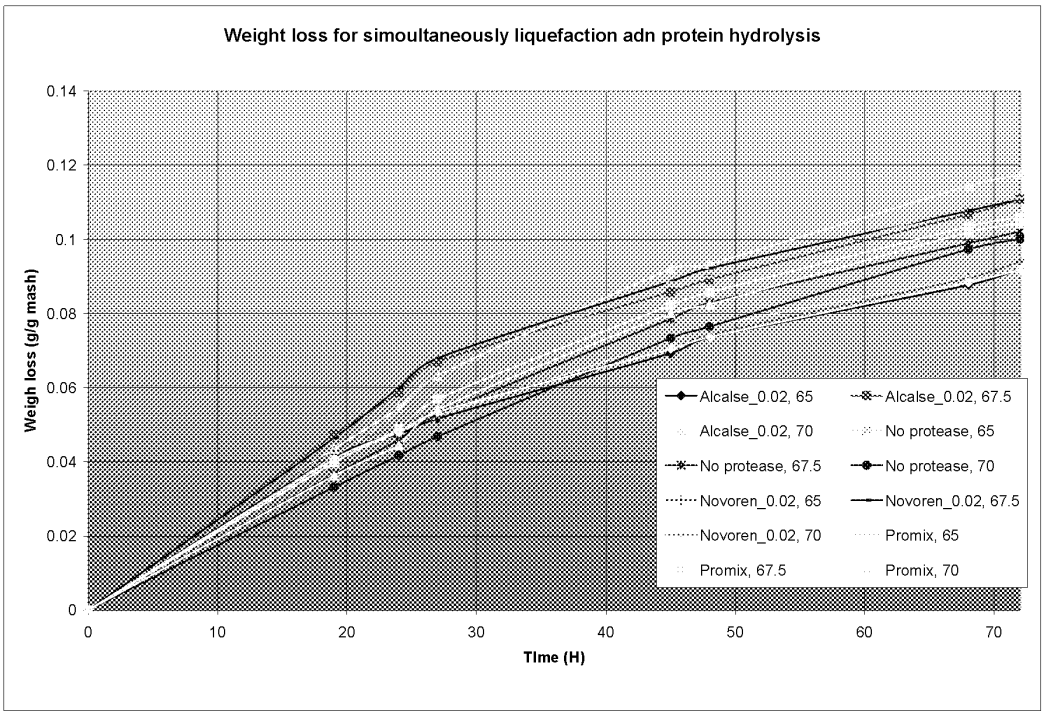
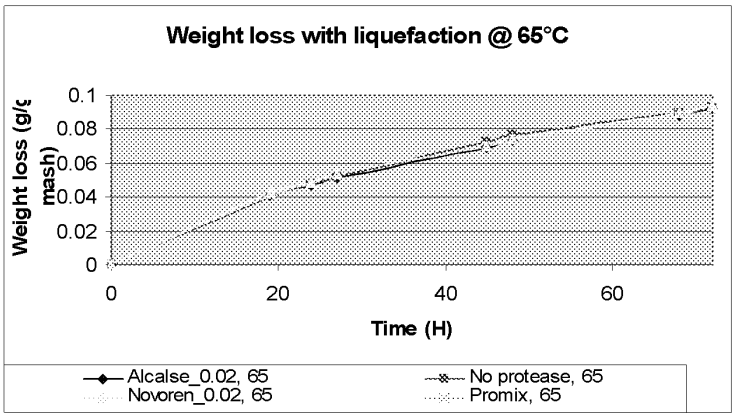
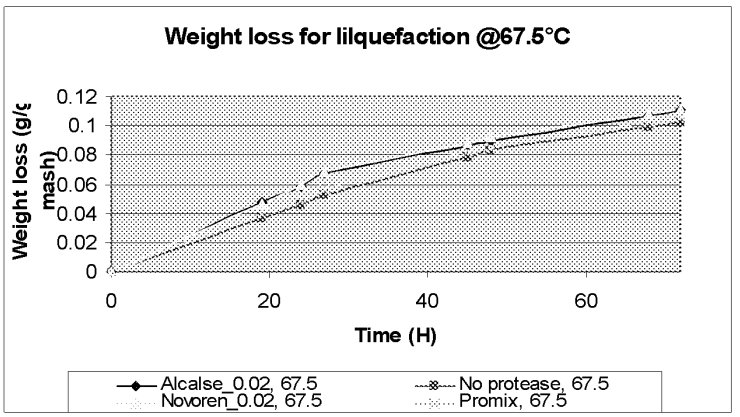


Figure 1. Weight loss for all samples as a function of fermentation time

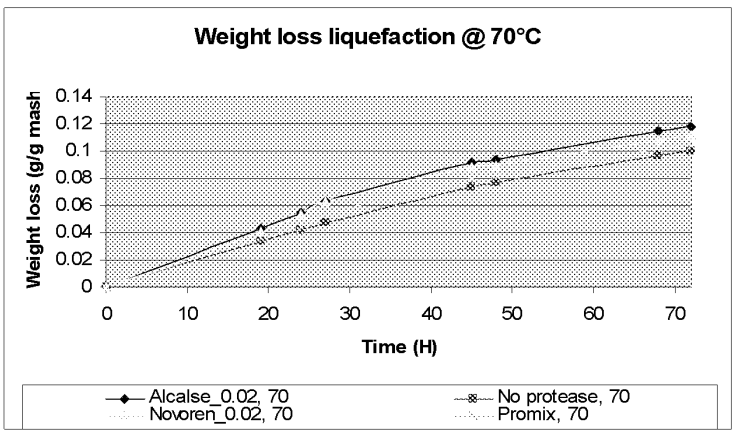
The weight loss curves are affected by the liquefaction temperatures as higher yeast growths and more ethanol is being produced in the high temperature liquefied samples. At the end of fermentation samples liquefied @ 70°C treated with Alcalase has considerably higher weight losses compared to the remaining treatments. The individual liquefaction temperatures are depicted below.



Very little difference between treatments at 65°



Differences in weight loss between Novoren and Alcalase to no Protease



Differences in weight loss pronounced particularly between Alcalase to no protease

Statically analysis of results:
Start Model for all response variables.
$Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Reduced models after response variable:
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model: **Y(glycerol)=**$I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model**: Y(gly/etoh)=** $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Where
α=(temp)= 65, 67.5 & 70°C
β=(enzyme) = Alcalase (Alc), Novoren (Novo) , ProMix (PM) and No protease (NoP)
γ=(enzyme*temp)=(Alc*65, 67.5, 70°), (Novo*65, 67.5, 70°), (PM*65, 67.5, 70°), NoP*65, 67.5, 70°),

Ethanol:

Ethanol has been analyzed after 24 & 48H, and furthermore at the end of fermentation. Only one sample was used for 24 & 48 H analysis, whereas 6 replicates were used at the end of fermentation, thus statistical analysis has been performed on the end HPLC data.

Table 2. HPLC ethanol results after 24 and 48 H of fermentation

	Ethanol	
Treatment	**24 H**	**48H**
NoP, 65	0.147	0.188
Alc 0.02, 65	**0.151**	**0.197**
No 0.02, 65	**0.151**	**0.192**
PM 65	0.147	0.188
NoP, 67.5	0.142	0.222
Alc 0.02, 67.5	**0.178**	**0.228**
No 0.02, 67.5	**0.179**	**0.230**
PM 67.5	0.146	0.226
NoP, 70	0.138	0.217
Alc 0.02, 70	**0.178**	**0.250**
No 0.02, 70	0.168	0.243
PM 70	0.139	0.224

The HPLC results after 24 and 48H show that Alcalase performs very well in respect to ethanol yield. Increasing the liquefaction temperature result in higher ethanol yield, due to higher degree of gelatinization and liquefaction thereby starch accessibility, thus higher ethanol yields. Protease addition results in adding a little extra ethanol yield on top of the temperature effect, which is a result of protein degradation either by releasing more starch and or hydrolysis of protein to amino acids to enhance fermentation. Alcalase @ 0.02 mg EP/g DS liquefied at 70°C gives the over all highest ethanol yield.

The reduced model for ethanol is listed below. Factor analysis showed
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$

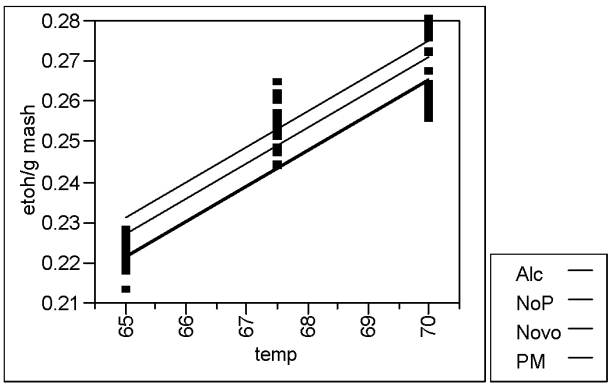
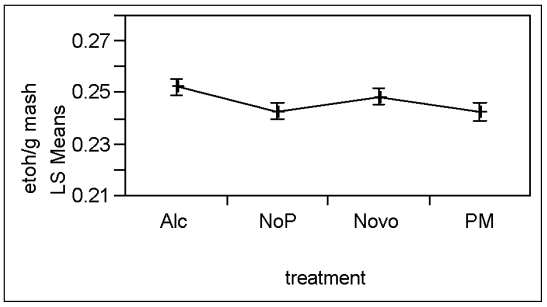


Figure 2. Response etoh/g mash, Whole Model. Regression Plot.

Ethanol increases by increasing liquefaction temperature, and fermentations with Alcalase reaches the highest level of ethanol. No Protease and ProMix performs equally poor. The thermo-stability of Flavourzyme in ProMix is not very high, as optimum is around 50°C, and the liquefaction temperatures in this trial is starting at 65°C.



Treatment			Least Sq Mean of ethanol (g/g mash)
Alc	A		0.252
Novoren	A	B	0.248
NoP		B	0.243
PM		B	0.243

Figure 3.LS Means Plot for ethanol and levels of significance.

The LS means numbers show Alcalase has the highest impact on improving ethanol yield and below it is clear that Alcalase performs better for all tested temperatures.

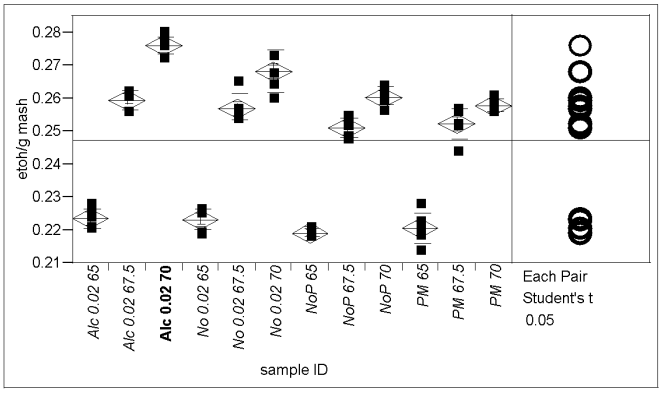


Figure 4: One-way Analysis of Ethanol Yield (g/g DS) by all treatments after 70H of fermentation.

At the end of fermentation Alcalase (0.02 mg EP/g DS at 70°C) demonstrate the best performance compared over temperature and treatment. The enzyme protein amount added from Novoren in ProMix is however considerably lower, as the total weight dose is only 0.03 mg Product/g DS, which is equivalent to ((0.03 mg/g DS*73%)/1000)*(0.15AU(RH)/g /22 AU(RH)g EP)=1.5*10-4 mg EP/g DS. This explains the poor performance of ProMix, but the dosage was included at this is the recommended start dose of ProMix added during SSF. ProMix is not as thermo-stable as Alcalase as the effect of increasing temperature is the same as for no protease, which is due to the lower thermo-stability of Flavourzyme in the product.

The table above confirms the observations and lists the significance levels, very clear effect of liquefaction temperature and Alcalase and Novoren. Below the leverage effect and prediction profiles are depicted. As can be seen there is linear increase in ethanol yield with increasing temperature, and that Alcalase gives higher ethanol yields compared to the other treatments.

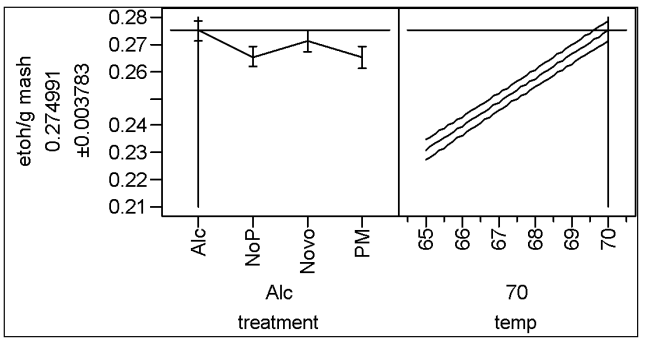


Figure 5. Prediction Profiles for treatment and temperature.

For any given temperature Alcalase will give the best ethanol yield, and increasing the temperature will ensure higher ethanol yields.

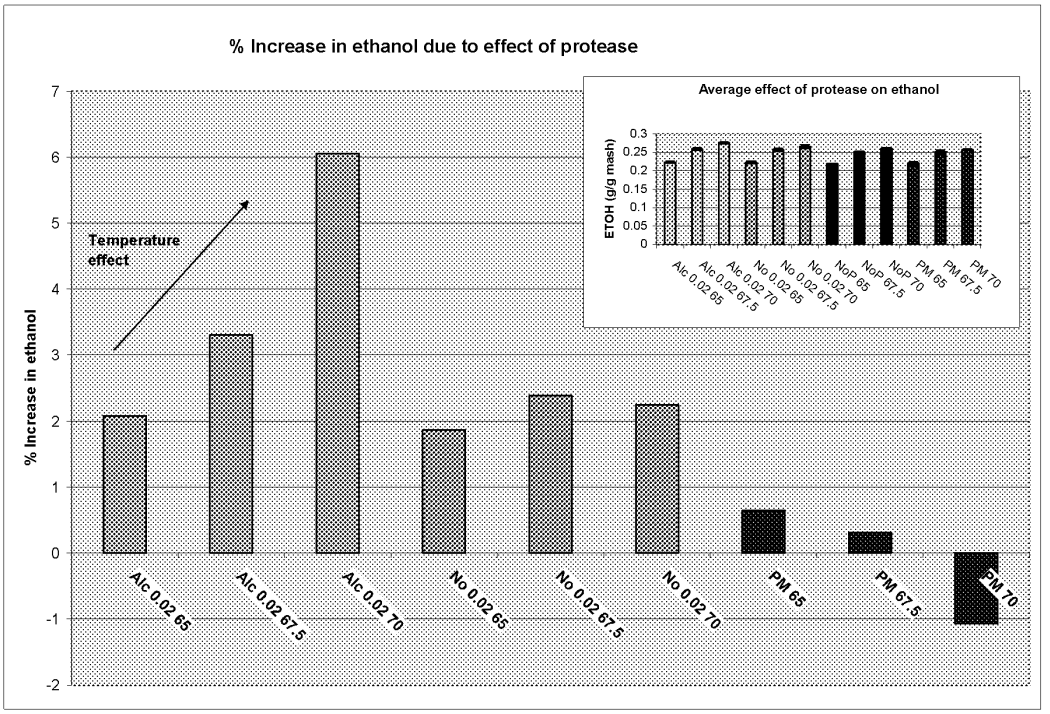


Figure 6. % increase in ethanol by treatment (enzyme and temperature) compared to no protease for every temperature.

In Figure 6 it is clear that Alcalase increases the ethanol yield up to 6% compared to not having a protease added (compared for every liquefaction temperature). Novoren also enhances the ethanol formation during SSF, but not to the same effect as Alcalase, and at 67.5°C Novoren reaches its maximum performance. ProMix on the other hand only increases the ethanol yield by 0.5% compared to n ot having a protease added, and there is absolutely no effect at 70°C. The poor performance of ProMix is very likely due to the low dosage in combination with low thermo-stability.

Sub conclusion:
Liquefaction time has significant impact on the ethanol yield, higher liquefaction temperature results in higher ethanol yields. The addition of protease improves the ethanol yield significantly at 67.5 and 70°C compared to not having a protease added. Alcalse was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalse was increased by increasing temperature. @ 60°C Alcalse enhances the ethanol yield by 6% compared to not having at protease added during liquefaction. This has not been seen at the post liquefaction studies, where increasing temperature above 50°C has shown decreasing performance of Alcalse.
The pH is higher at liquefaction conditions, and also there could be a factor of substrate stabilization in corn flour as opposed to corn mash.

Glycerol Yield

Again glycerol was measured after 24 & 48 H of fermentation, and more thoroughly analyzed with 6 replicates at the end of fermentation

Table 3. HPLC glycerol results after 24 and 48 H of fermentation

	Glycerol	
Treatment	**24 H**	**48 H**
NoP, 65	0.027	0.010
Alc 0.02, 65	0.026	0.017
No 0.02, 65	0.024	0.012
PM 65	0.026	0.013
NoP, 67.5	0.026	0.015
Alc 0.02, 67.5	0.027	0.013
No 0.02, 67.5	0.027	0.015
PM 67.5	0.028	0.014
NoP, 70	0.027	0.013
Alc 0.02, 70	0.027	0.015
No 0.02, 70	0.026	0.014
PM 70	0.026	0.013

Formation of glycerol was in general very similar for the protease treatments, in fact similar to not having a protease added during liquefaction.

After reduction of insignificant effects in the start model, the model describing ethanol yield at the end of fermentation looks as follows:

Model: **Y(glycerol)**=I+α(temp)+β(enzyme) +ε

No significant effect of the interaction (temp:enzyme) was found, and the regression plot below illustrate this very well, as the enzyme have similar pattern in glycerol formation for very all temperatures.

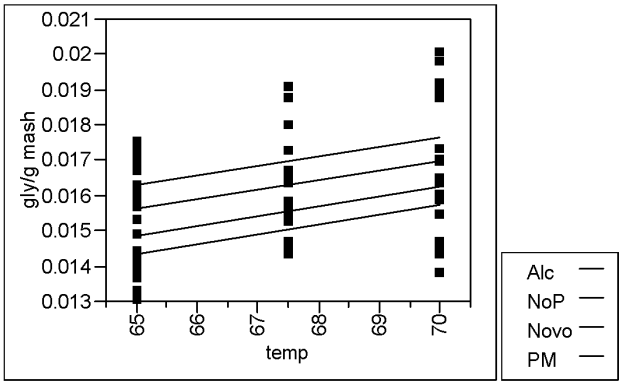
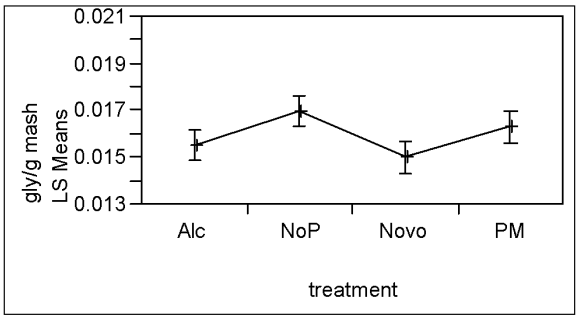


Figure 7. Response gly/g mash, Whole Model. Regression Plot.



Treatment		Least Sq Mean of glycerol (g/g mash)
NoP	A	0.0170
PM	A B	0.0163
Alc	B	0.0155
Novo	B	0.0150

Figure 8.LS Means Plot for glycerol and levels of significance.

Not having a protease added during liquefaction result in the highest formation of glycerol, and again Alcalase and Novoren show the best performance by lowering the glycerol the most.

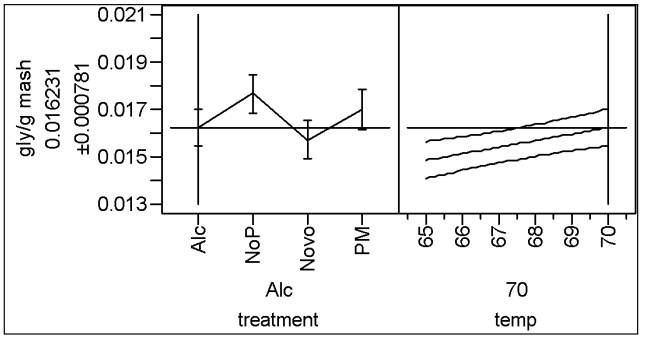


Figure 9: Prediction Profiles for response variable glycerol, for both treatment and temperature.

Temperature has the reverse effect on glycerol compared to ethanol, as increasing glycerol is being formed at higher temperature, indicating high yeast growth and more stress during SSF. Novoren secures the lowest glycerol yield however, not significantly different form Alcalase.

Sub conclusion:
Protease and temperature had significant impact on glycerol. Protease (particular Novoren) lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Glycerol/Ethanol – Rato

The reduced model for glycerol/ethanol relationship ended up being:
Model**: Y(gly/etoh)**= $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

The model varied from the previous response variables, as gly/etoh showed significant effect of the interaction between temperature:enzyme.

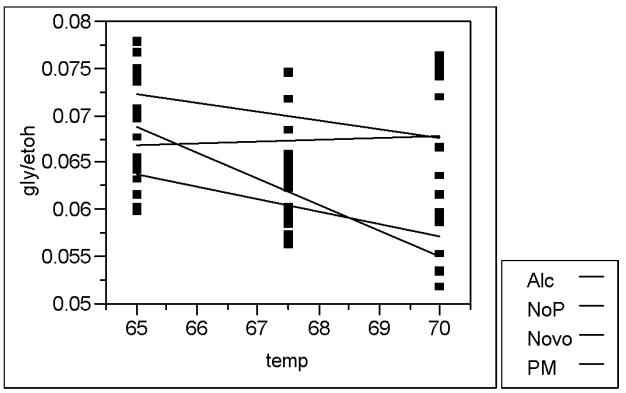
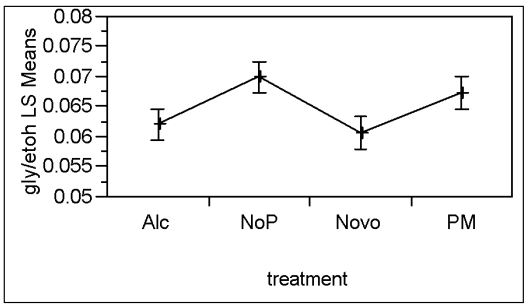


Figure 10. Response gly/etoh, Whole Model. Regression Plot.

The interaction between temperature and enzyme is very clear in the regression plot above, as the lines are not parallel, indicating different behaviors for each temperature. The different pattern is primary due to ProMix not showing increased ethanol at increasing temperature and decreasing glycerol, which is the picture for Alcalase and Novoren. The gly/etoh ratio with ProMix is the same at the different liquefaction temperatures, which is different from Alcalase and Novoren.



Treatment		Least Sq Mean of gly/etoh (g/g mash)
NoP	A	0.070
PM	A	0.067
Alc	B	0.062
Novo	B	0.061

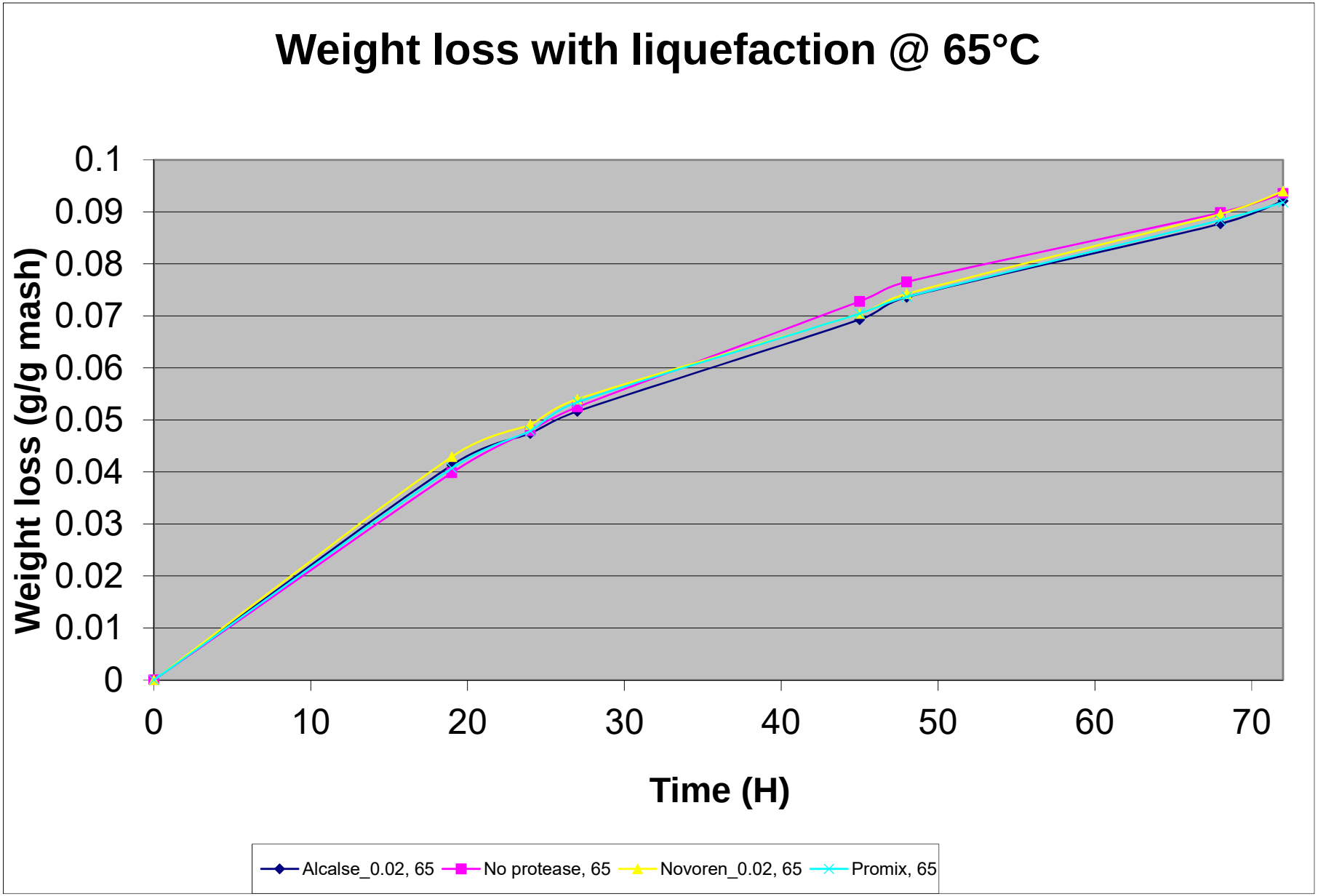
Figure 11. LS Means Plot for glycerol and levels of significance.

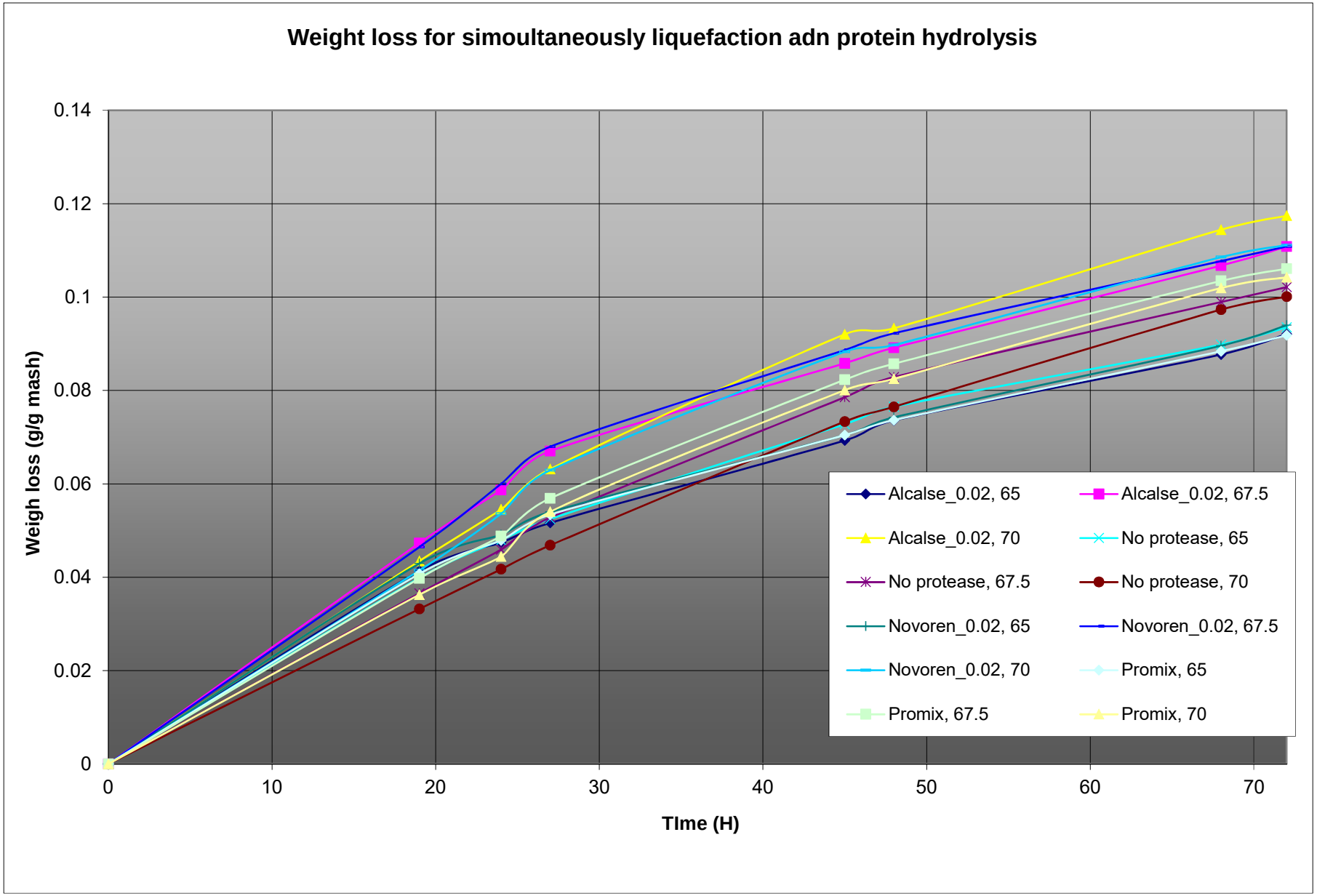
The highest ratio of glycerol being produced per g of ethanol is in the control samples, with no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.

Sub Conclusion:
The gly/etoh ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease.

Reference: RMF050118

Document Produced Natively





				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

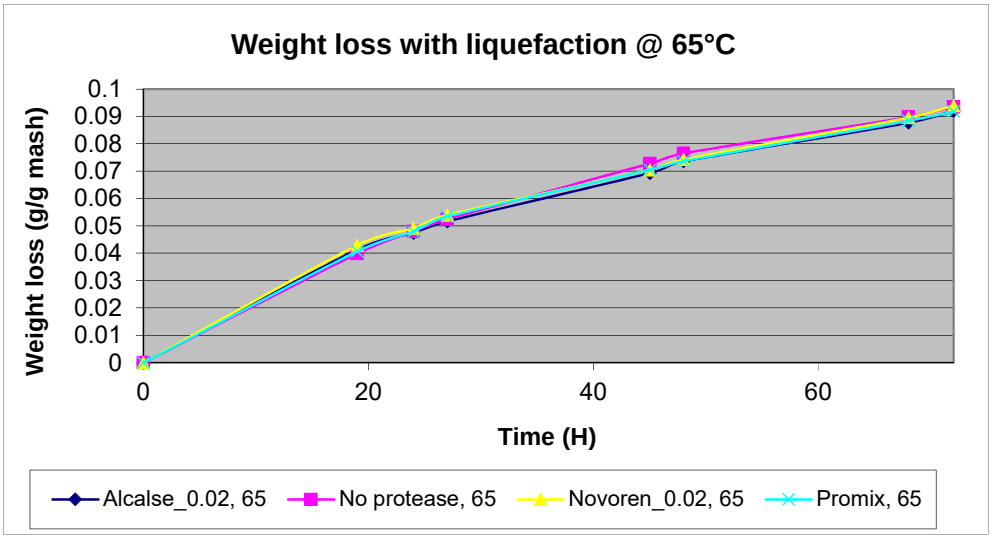
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842

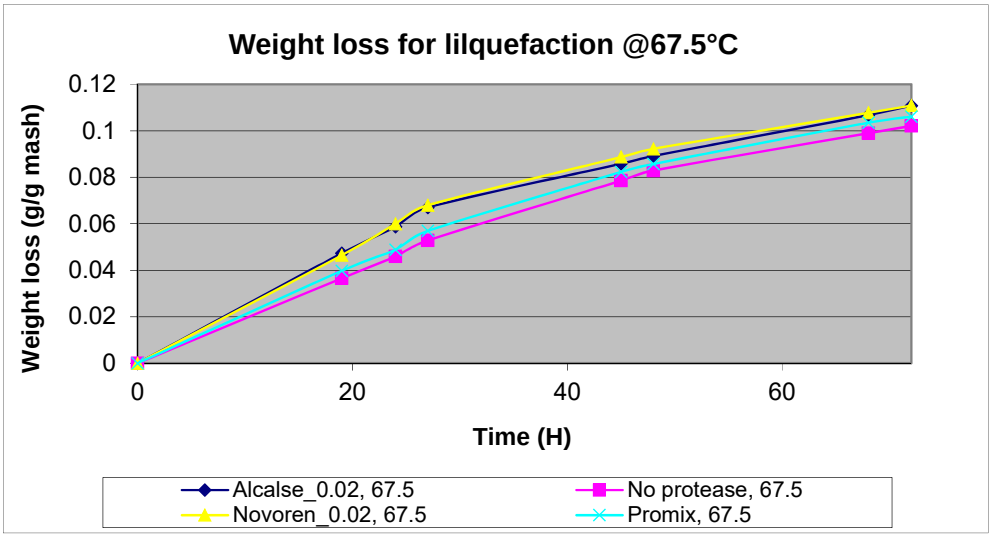
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

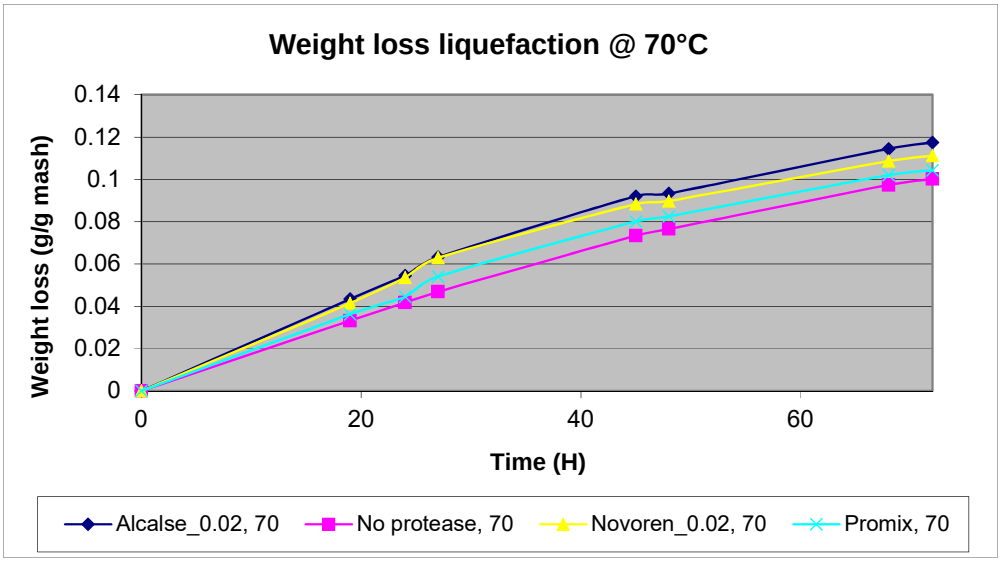
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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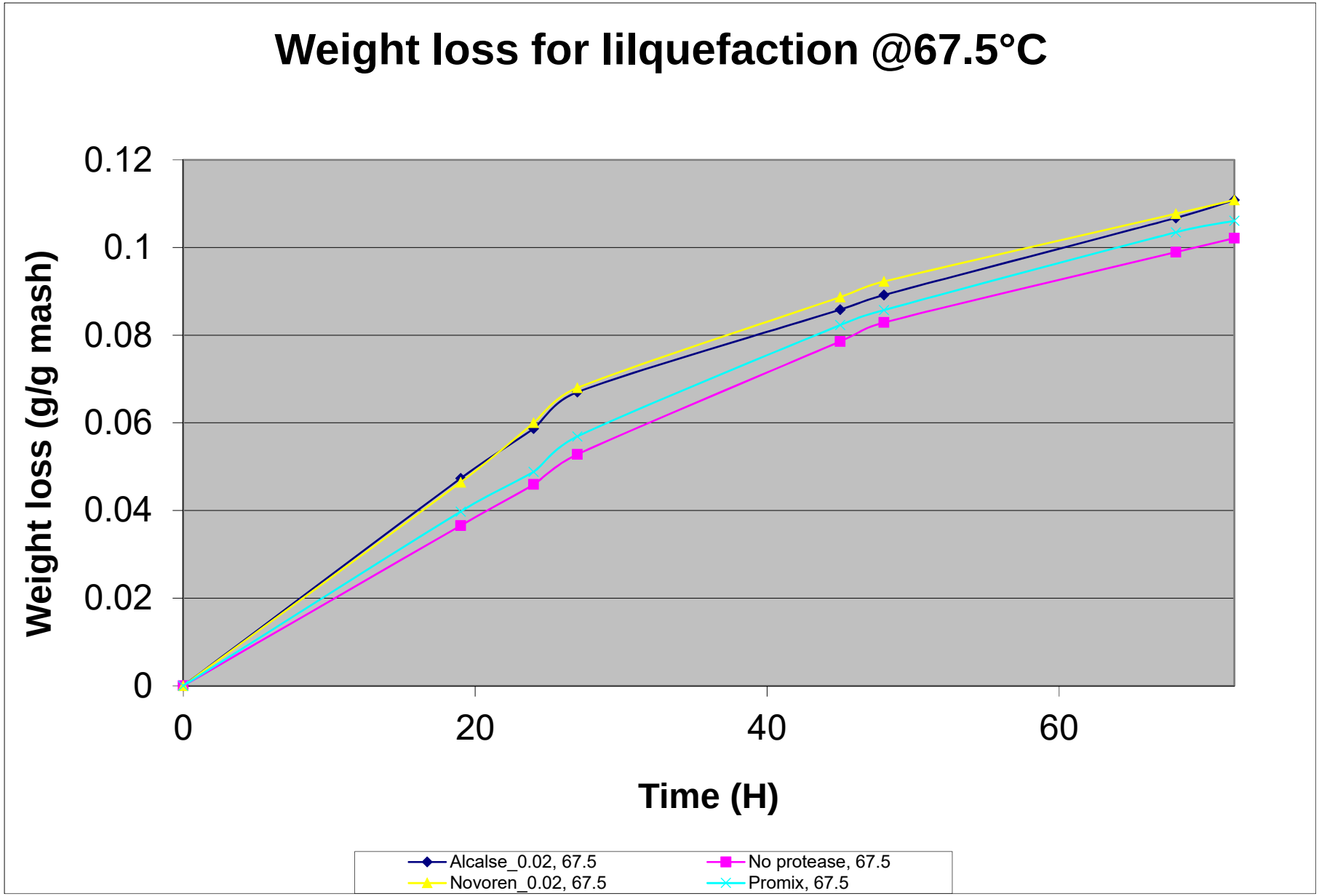

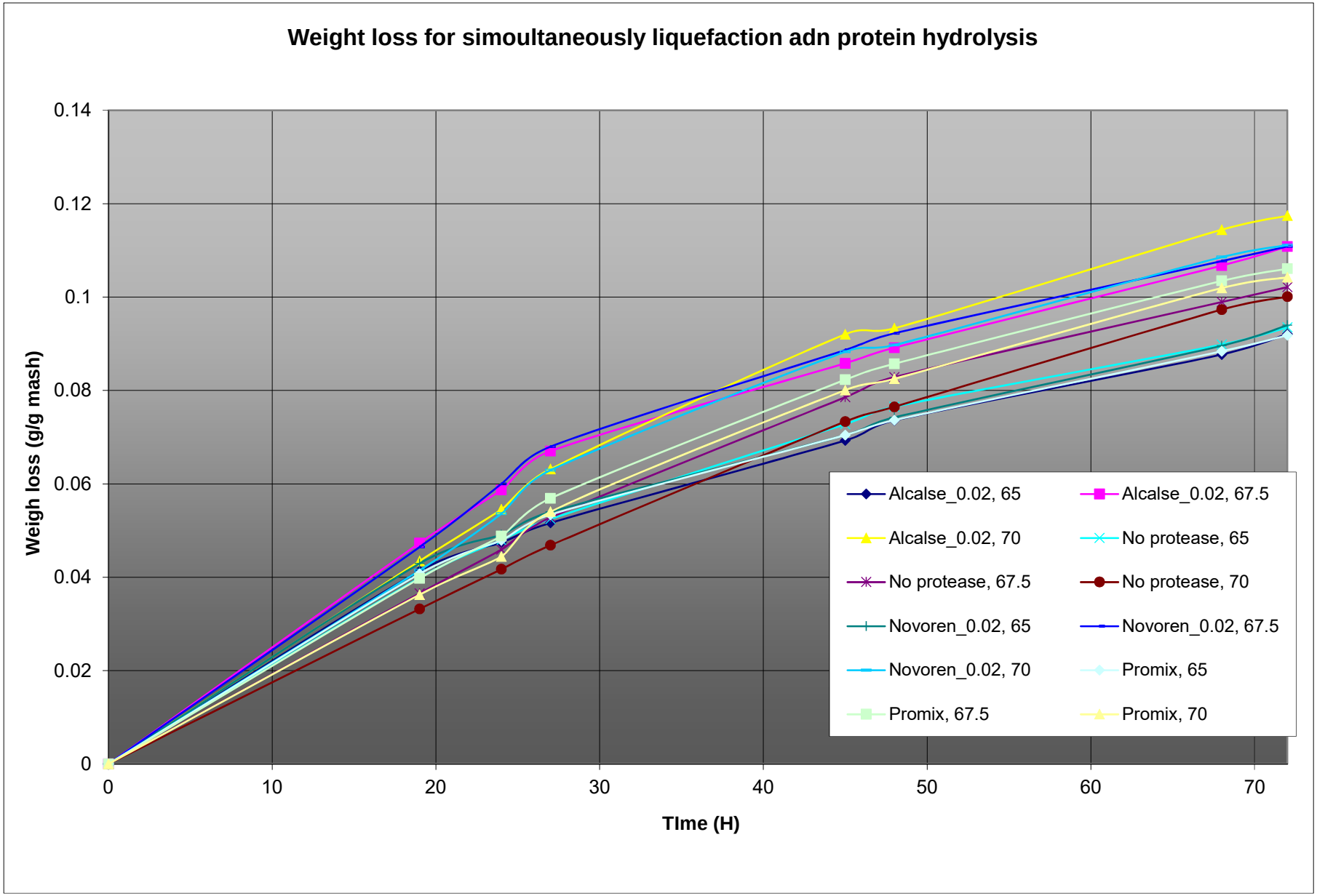

				mean	stdev	cv
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Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

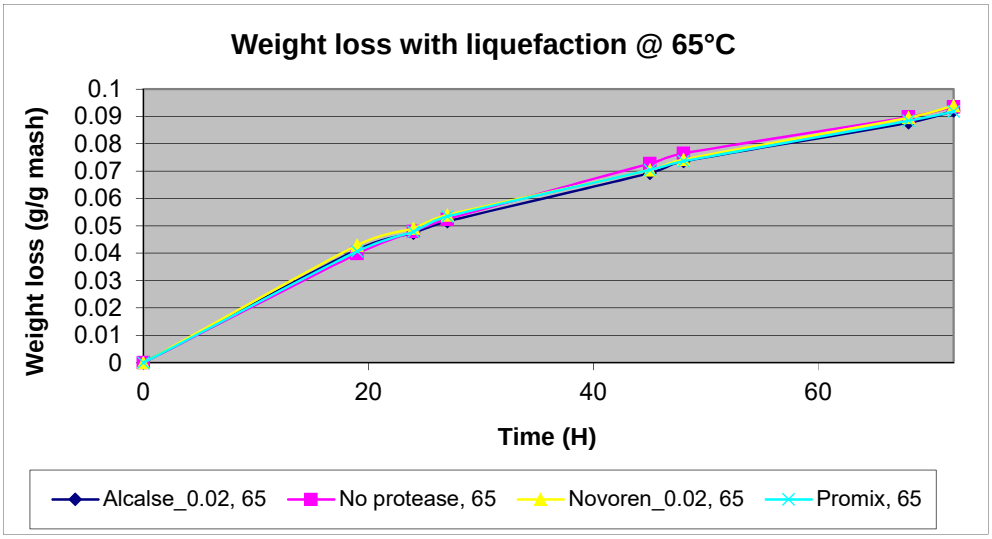
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

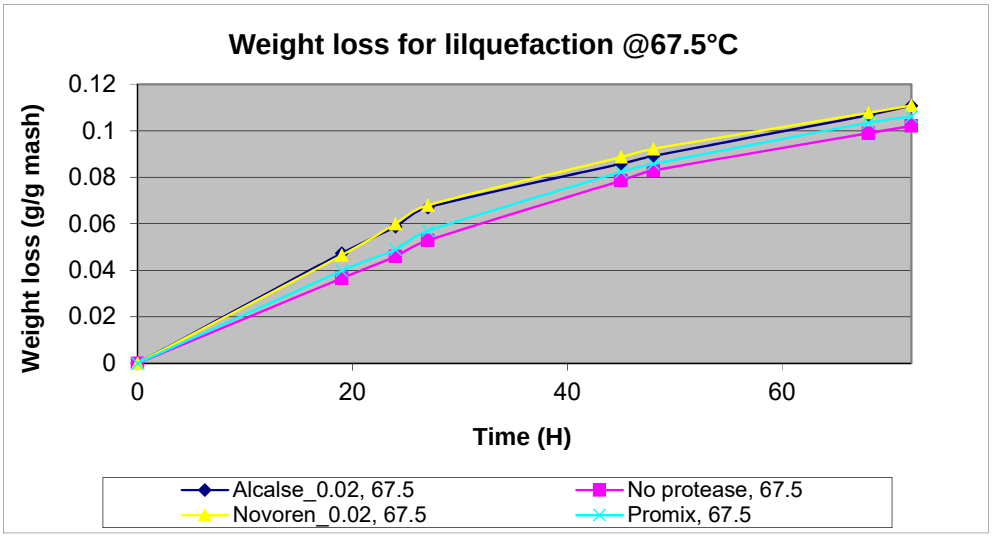
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

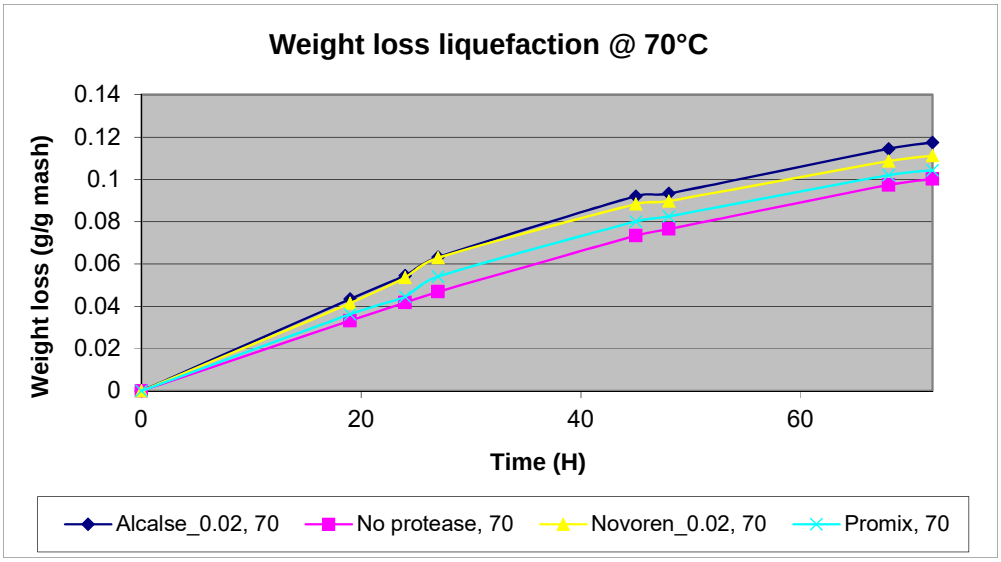
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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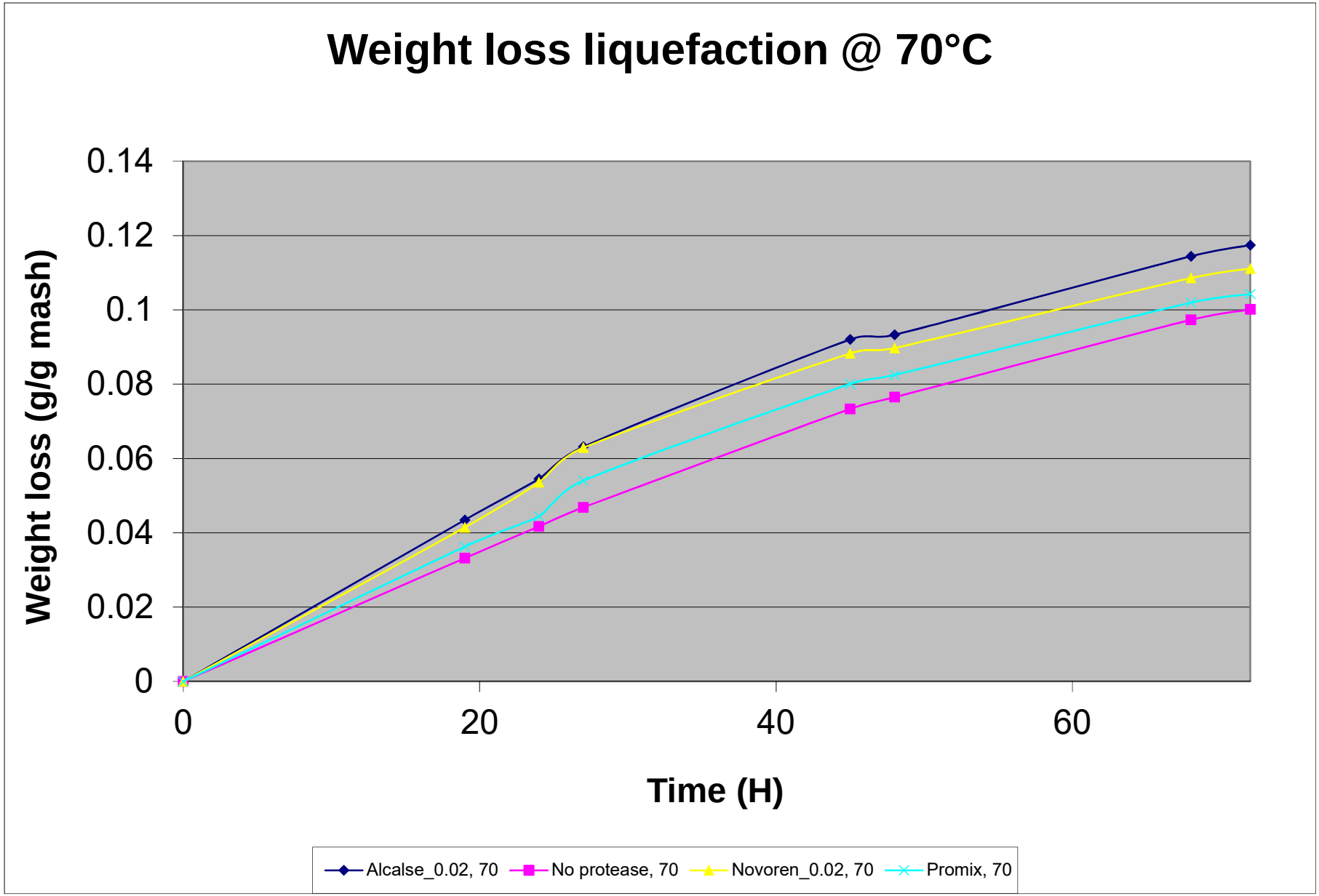

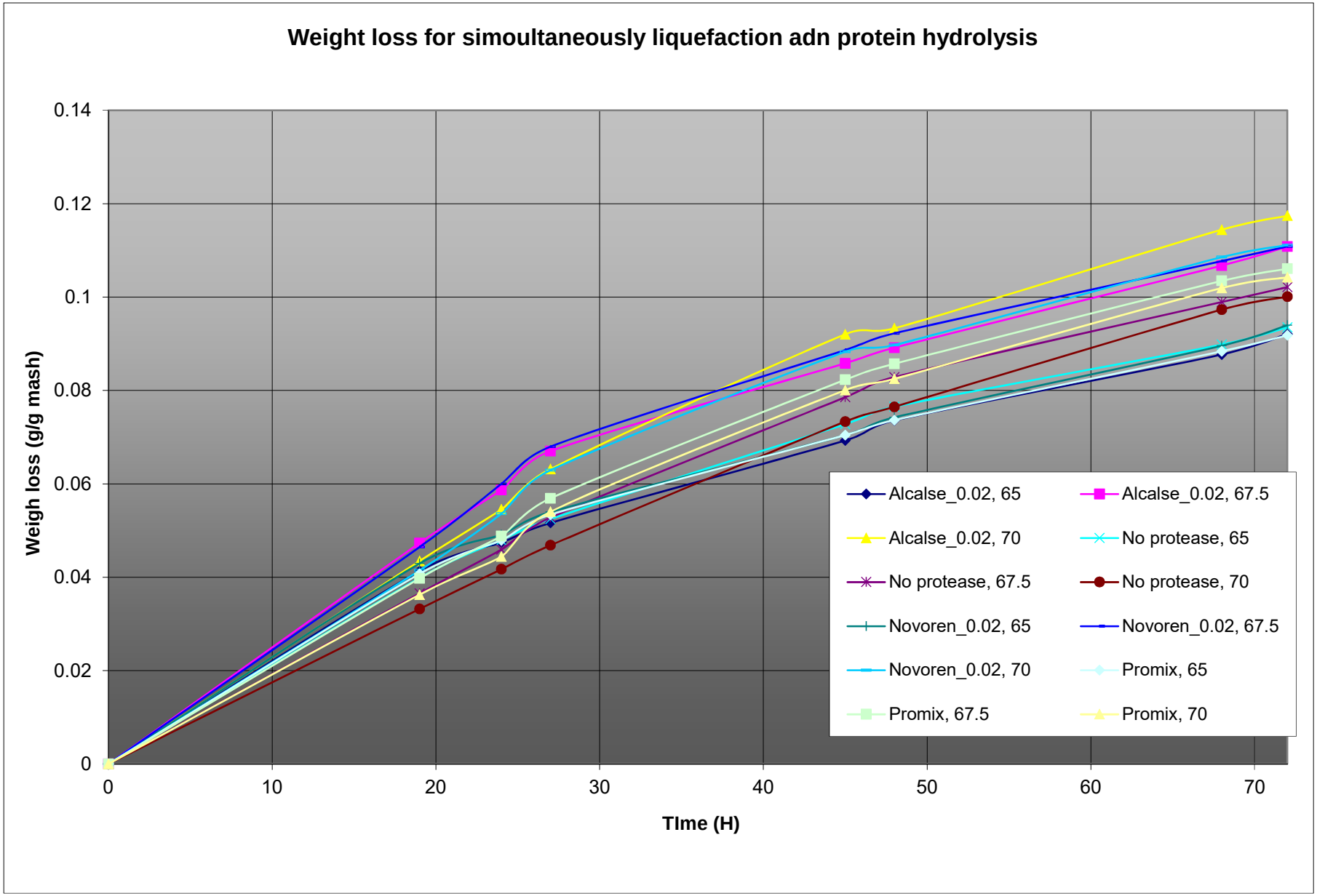

				mean	stdev	cv
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Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

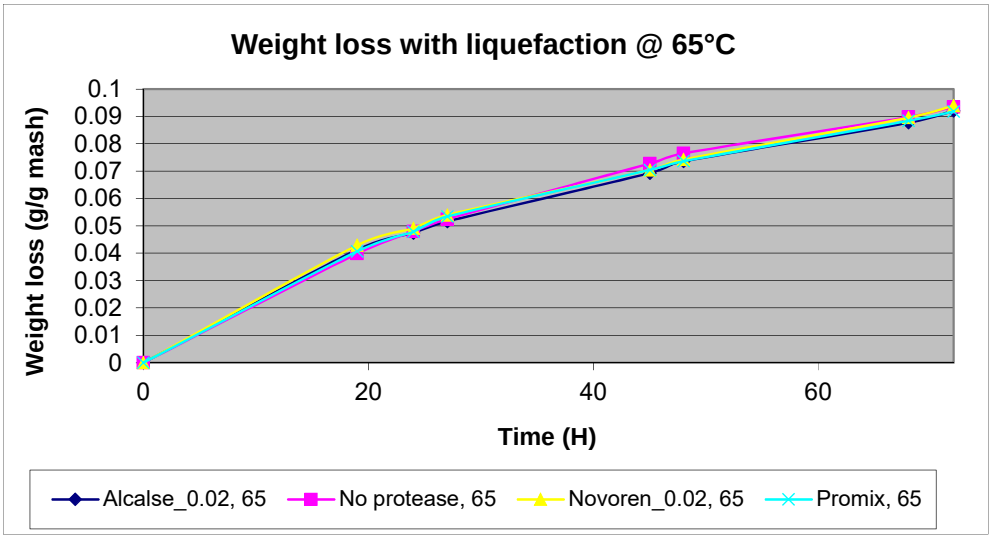
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

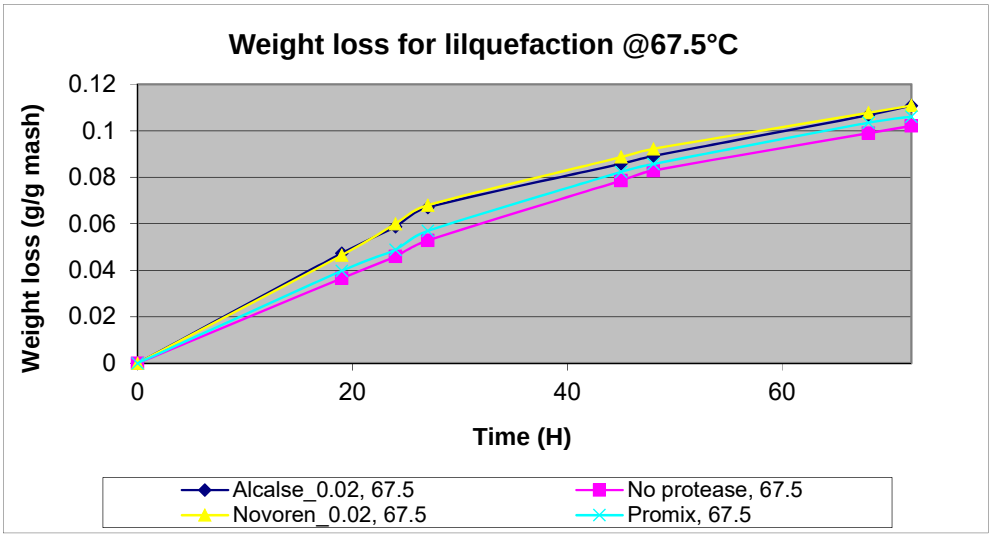
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

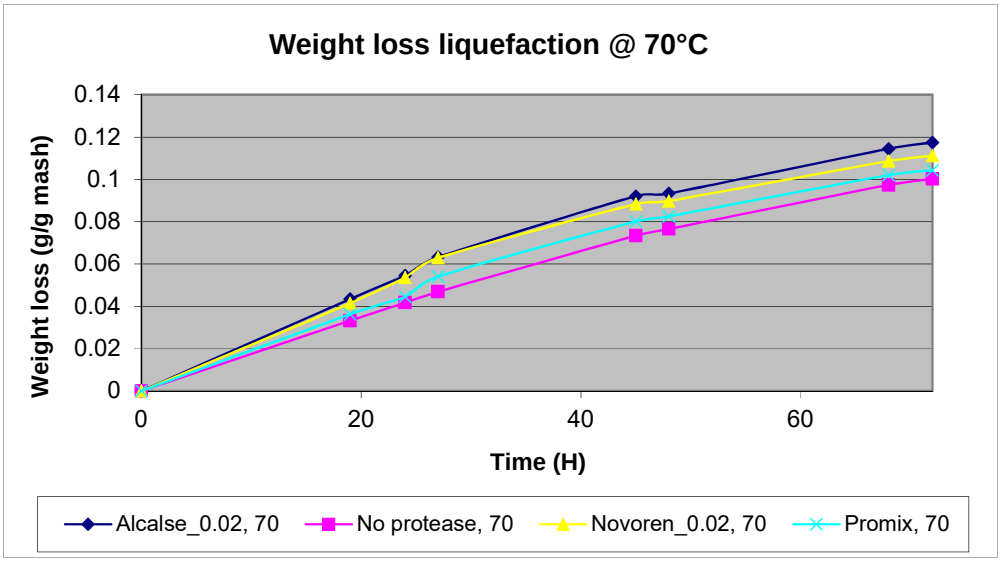
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







Application Technology – US Fuel Research
NL200589
LUNA: 2005-49534-03

To: HSO, KSW, RHeu, BJes, LWP, RDe,

From: RMF/JPlm

Simultaneous liquefaction and protein degradation at 65, 67.5, & 70°C for SSF to Fuel ethanol

Study # RMF050118

Experimental design:

- Simultaneously starch liquefaction and protein degradation has been carried out at 65, 67.5 and 70°C in a mini scale liquefaction set up using 15 ml tubes.
- Termamyl SC and Alcalase (Family S8), Novoren (Family A1) and ProMix (Novoren & Flavourzyme) have been evaluated for their effect when added during liquefaction.
- Background conditions for the liquefaction was pH=5.8 and DS=34.10% and 50 NU/g DS Termamyl SC and for SSF Spirizyme Fuel was added @ 0.5 AGU/g DS. No urea was added.

Conclusions:

- Alcalase was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalase was increased by increasing temperature. @ 70°C Alcalase enhances the ethanol yield by 6% compared to not having at protease added during liquefaction.
- Protease and temperature had significant impact on glycerol. Novoren and Alcalase lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Discussion:

- Alcalase had optimum performance @ 50°C when added at a post-liquefaction step, using this procedure liquefaction temperature can preferably be increased to 70°C.
- The pH is higher at liquefaction conditions compared to fermentation, and the protein matrix in corn flour could be stabilizing the peptidases as opposed to cooked corn mash where proteins are precipitated.

Purpose

To study the effect of adding an α-amylase (Termamyl SC), and a peptidase (Alcalase, Novoren, ProMix) at the same time during liquefaction carried out @ 65, 67.5, and 70°C. Starch liquefaction and protein degradation is thereby run simultaneously for SSF.

Background

Post liquefaction trials have been carried out with Alcalase, where the effect has been very temperature dependent. Alcalase showed best performance at 50°C, and the performance decreased significantly with increasing temperature. In this experiment peptidases are added to the raw mash, where starch, protein and other corn sources are intact and liquefaction and protein degradation is carried out in 15 ml test tubes. Having a simultaneous step of starch liquefaction and protein degradation may be interest to industry, as temperature pH could be beneficial to the peptidases we have available and thereby secure better protein hydrolysis and possible starch release from protein bound starch sources, thus obtain higher yeast efficiency during fermentation.

The factors to be tested in each of the factorial experiments are as follows:
Temperature: 65, 67.5, 70°C
Enzyme: Alcalase, Novoren, and ProMix and no Protease.

Background conditions similar for all treatments during SSF are pH=5, and Spirizyme fuel @ 0.5 AGU/g DS. During liquefaction Alcalase and Novoren was added at 0.02 mg EP/g DS, and ProMix at 0.03 mg Product/g DS (corresponding to the dosage in SSF).

Experimental

The experiment was carried out in 15 ml test tubes using approximately 5 g of corn mash (34.10 % DS) (exact weigh and dry matter content is known) and 5 replicates. Corn flour from Broin (EXOL) was used for the experiments. Subsequently the impact from the liquefaction was accessed by conducting a standard SSF fermentation.

Table 1. HPLC ethanol results after 24 and 48 H of fermentation

Treatment	AGU/g DS	NU/g DS	mg EP/g DS	mg EP/g DS	mg product/g DS
Spirizyme fuel	0.5	50			
Termamyl SC	0.5	50			
Alcalase 0.02	0.5	50	0.02		
Novoren	0.5	50		0.02	
Promix	0.5	50			0.03

Liquefaction Treatment
Corn flour was added water to obtain a DS fraction of 34.10% and pH was adjusted to 5.6 and samples were taken for DS determination. 5g of this mash was transferred to 15ml test tubes, which were liquefied for 1H after the corn mash reached the designated liquefaction temperature, which were 65, 67.5, and 70°C.

SSF Fermentation

Spirizyme fuel was added to all the tubes after liquefaction. Fermentations were carried out as SSF at 32°C, 70 hours using Red Star yeast. Yeast addition was aimed at $1*10^7$ cells/ml. All treatments were each run in 8 replicates and non-liquefied controls were included in the fermentation. The fermentations were monitored by weighing the individual tubes and recording the time & date of the measurement. At the end of fermentation tubes were sampled for HPLC analysis of sugars and fermentation products, primary parameters evaluated were ethanol and glycerol.

Results:

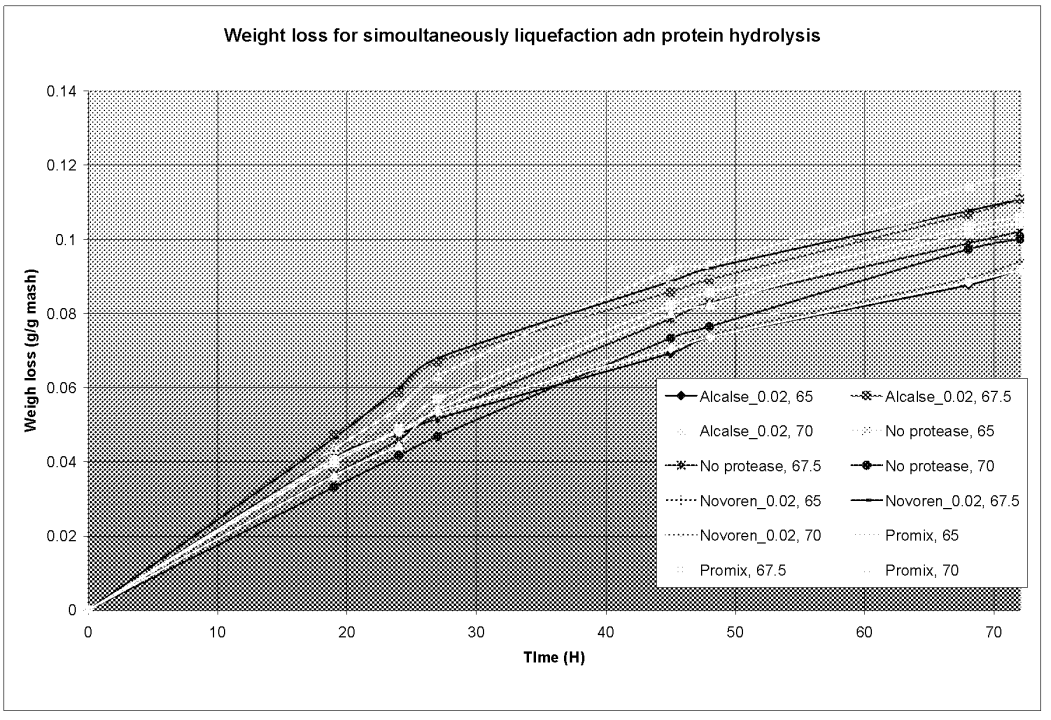
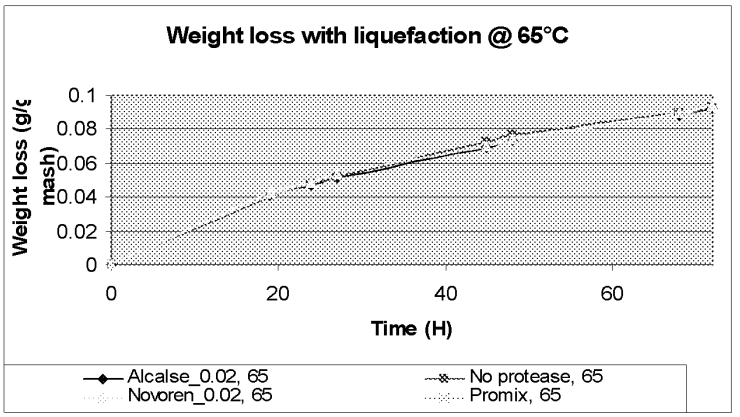
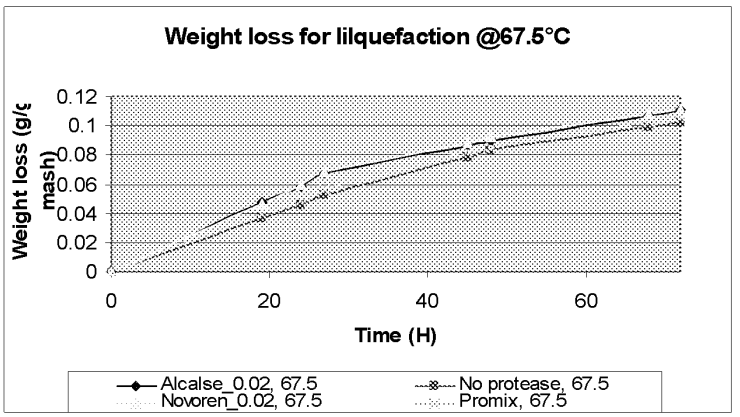


Figure 1. Weight loss for all samples as a function of fermentation time

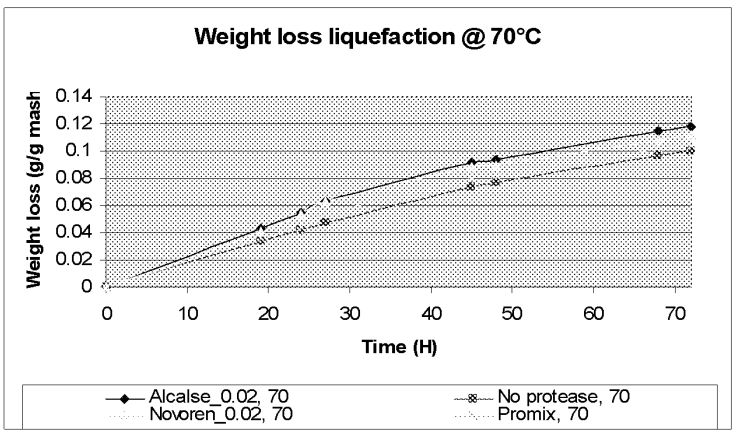
The weight loss curves are affected by the liquefaction temperatures as higher yeast growths and more ethanol is being produced in the high temperature liquefied samples. At the end of fermentation samples liquefied @ 70°C treated with Alcalase has considerably higher weight losses compared to the remaining treatments. The individual liquefaction temperatures are depicted below.



Very little difference between treatments at 65°



Differences in weight loss between Novoren and Alcalase to no Protease



Differences in weight loss pronounced particularly between Alcalase to no protease

Statically analysis of results:
Start Model for all response variables.
$Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Reduced models after response variable:
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model: **Y(glycerol)=**$I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model**: Y(gly/etoh)=** $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Where
α=(temp)= 65, 67.5 & 70°C
β=(enzyme) = Alcalase (Alc), Novoren (Novo) , ProMix (PM) and No protease (NoP)
γ=(enzyme*temp)=(Alc*65, 67.5, 70°), (Novo*65, 67.5, 70°), (PM*65, 67.5, 70°), NoP*65, 67.5, 70°),

Ethanol:

Ethanol has been analyzed after 24 & 48H, and furthermore at the end of fermentation. Only one sample was used for 24 & 48 H analysis, whereas 6 replicates were used at the end of fermentation, thus statistical analysis has been performed on the end HPLC data.

Table 2. HPLC ethanol results after 24 and 48 H of fermentation

	Ethanol	
Treatment	**24 H**	**48H**
NoP, 65	0.147	0.188
Alc 0.02, 65	**0.151**	**0.197**
No 0.02, 65	**0.151**	**0.192**
PM 65	0.147	0.188
NoP, 67.5	0.142	0.222
Alc 0.02, 67.5	**0.178**	**0.228**
No 0.02, 67.5	**0.179**	**0.230**
PM 67.5	0.146	0.226
NoP, 70	0.138	0.217
Alc 0.02, 70	**0.178**	**0.250**
No 0.02, 70	0.168	0.243
PM 70	0.139	0.224

The HPLC results after 24 and 48H show that Alcalase performs very well in respect to ethanol yield. Increasing the liquefaction temperature result in higher ethanol yield, due to higher degree of gelatinization and liquefaction thereby starch accessibility, thus higher ethanol yields. Protease addition results in adding a little extra ethanol yield on top of the temperature effect, which is a result of protein degradation either by releasing more starch and or hydrolysis of protein to amino acids to enhance fermentation. Alcalase @ 0.02 mg EP/g DS liquefied at 70°C gives the over all highest ethanol yield.

The reduced model for ethanol is listed below. Factor analysis showed
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$

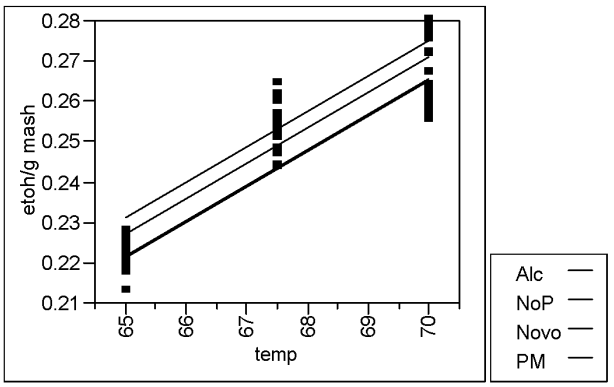
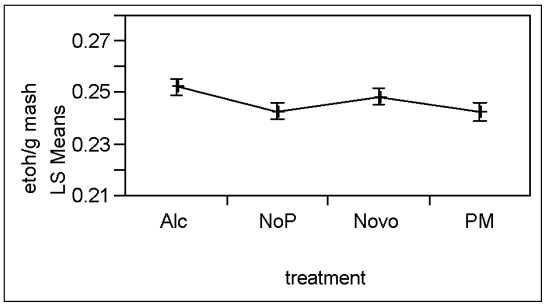


Figure 2. Response etoh/g mash, Whole Model. Regression Plot.

Ethanol increases by increasing liquefaction temperature, and fermentations with Alcalase reaches the highest level of ethanol. No Protease and ProMix performs equally poor. The thermo-stability of Flavourzyme in ProMix is not very high, as optimum is around 50°C, and the liquefaction temperatures in this trial is starting at 65°C.



Treatment			Least Sq Mean of ethanol (g/g mash)
Alc	A		0.252
Novoren	A	B	0.248
NoP		B	0.243
PM		B	0.243

Figure 3.LS Means Plot for ethanol and levels of significance.

The LS means numbers show Alcalase has the highest impact on improving ethanol yield and below it is clear that Alcalase performs better for all tested temperatures.

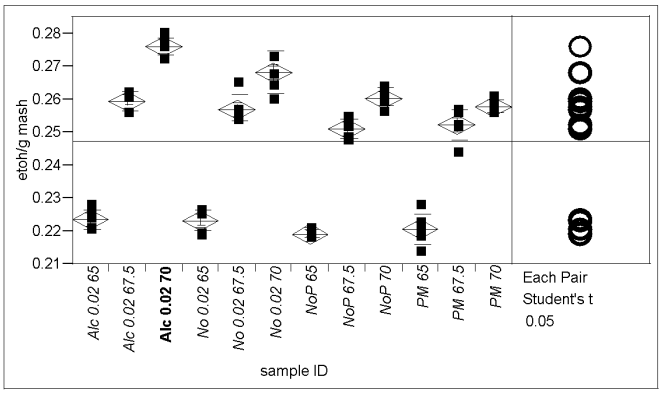


Figure 4: One-way Analysis of Ethanol Yield (g/g DS) by all treatments after 70H of fermentation.

At the end of fermentation Alcalase (0.02 mg EP/g DS at 70°C) demonstrate the best performance compared over temperature and treatment. The enzyme protein amount added from Novoren in ProMix is however considerably lower, as the total weight dose is only 0.03 mg Product/g DS, which is equivalent to ((0.03 mg/g DS*73%)/1000)*(0.15AU(RH)/g /22 AU(RH)g EP)=1.5*10-4 mg EP/g DS. This explains the poor performance of ProMix, but the dosage was included at this is the recommended start dose of ProMix added during SSF. ProMix is not as thermo-stable as Alcalase as the effect of increasing temperature is the same as for no protease, which is due to the lower thermo-stability of Flavourzyme in the product.

The table above confirms the observations and lists the significance levels, very clear effect of liquefaction temperature and Alcalase and Novoren. Below the leverage effect and prediction profiles are depicted. As can be seen there is linear increase in ethanol yield with increasing temperature, and that Alcalase gives higher ethanol yields compared to the other treatments.

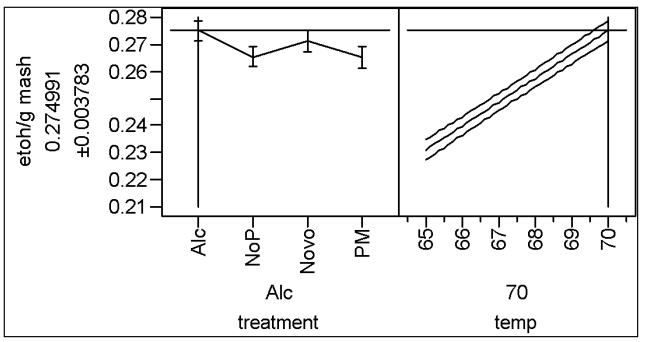


Figure 5. Prediction Profiles for treatment and temperature.

For any given temperature Alcalase will give the best ethanol yield, and increasing the temperature will ensure higher ethanol yields.

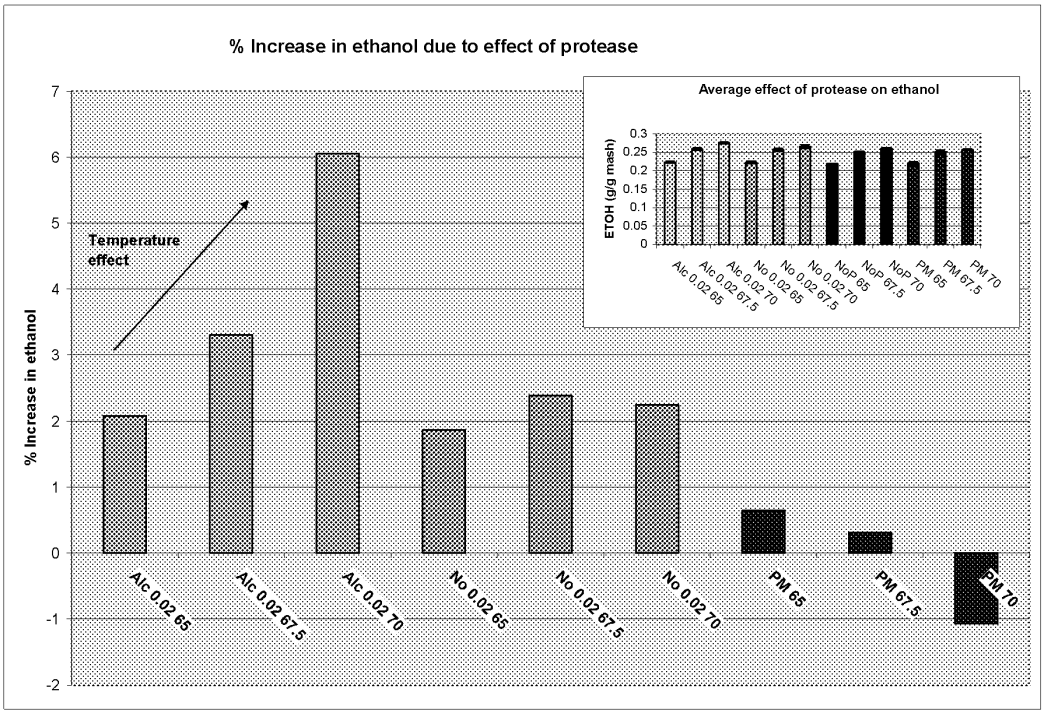


Figure 6. % increase in ethanol by treatment (enzyme and temperature) compared to no protease for every temperature.

In Figure 6 it is clear that Alcalase increases the ethanol yield up to 6% compared to not having a protease added (compared for every liquefaction temperature). Novoren also enhances the ethanol formation during SSF, but not to the same effect as Alcalase, and at 67.5°C Novoren reaches its maximum performance. ProMix on the other hand only increases the ethanol yield by 0.5% compared to n ot having a protease added, and there is absolutely no effect at 70°C. The poor performance of ProMix is very likely due to the low dosage in combination with low thermo-stability.

Sub conclusion:
Liquefaction time has significant impact on the ethanol yield, higher liquefaction temperature results in higher ethanol yields. The addition of protease improves the ethanol yield significantly at 67.5 and 70°C compared to not having a protease added. Alcalse was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalse was increased by increasing temperature. @ 60°C Alcalse enhances the ethanol yield by 6% compared to not having at protease added during liquefaction. This has not been seen at the post liquefaction studies, where increasing temperature above 50°C has shown decreasing performance of Alcalse.
The pH is higher at liquefaction conditions, and also there could be a factor of substrate stabilization in corn flour as opposed to corn mash.

Glycerol Yield

Again glycerol was measured after 24 & 48 H of fermentation, and more thoroughly analyzed with 6 replicates at the end of fermentation

Table 3. HPLC glycerol results after 24 and 48 H of fermentation

	Glycerol	
Treatment	**24 H**	**48 H**
NoP, 65	0.027	0.010
Alc 0.02, 65	0.026	0.017
No 0.02, 65	0.024	0.012
PM 65	0.026	0.013
NoP, 67.5	0.026	0.015
Alc 0.02, 67.5	0.027	0.013
No 0.02, 67.5	0.027	0.015
PM 67.5	0.028	0.014
NoP, 70	0.027	0.013
Alc 0.02, 70	0.027	0.015
No 0.02, 70	0.026	0.014
PM 70	0.026	0.013

Formation of glycerol was in general very similar for the protease treatments, in fact similar to not having a protease added during liquefaction.

After reduction of insignificant effects in the start model, the model describing ethanol yield at the end of fermentation looks as follows:

Model: **Y(glycerol)**=I+α(temp)+β(enzyme) +ε

No significant effect of the interaction (temp:enzyme) was found, and the regression plot below illustrate this very well, as the enzyme have similar pattern in glycerol formation for very all temperatures.

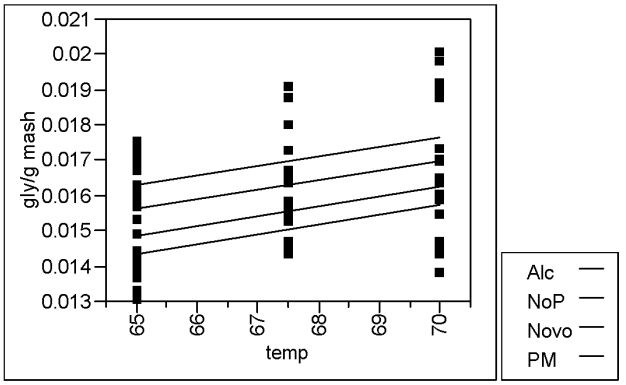
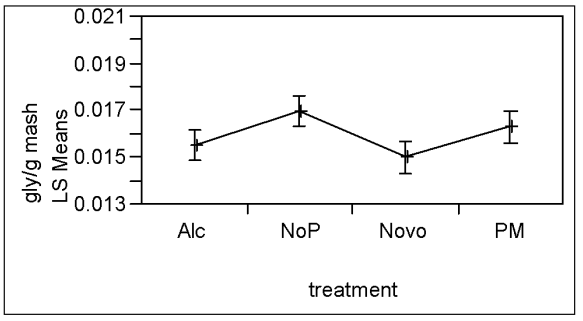


Figure 7. Response gly/g mash, Whole Model. Regression Plot.



Treatment		Least Sq Mean of glycerol (g/g mash)
NoP	A	0.0170
PM	A B	0.0163
Alc	B	0.0155
Novo	B	0.0150

Figure 8.LS Means Plot for glycerol and levels of significance.

Not having a protease added during liquefaction result in the highest formation of glycerol, and again Alcalase and Novoren show the best performance by lowering the glycerol the most.

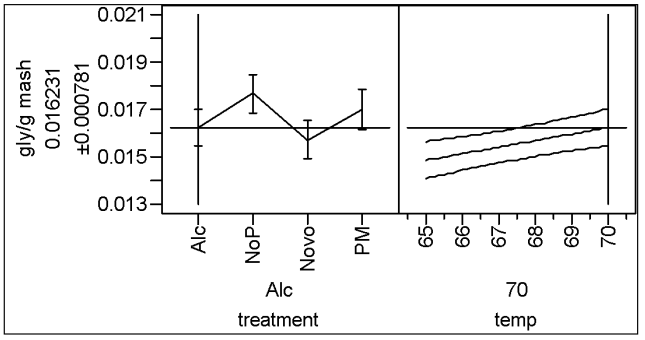


Figure 9: Prediction Profiles for response variable glycerol, for both treatment and temperature.

Temperature has the reverse effect on glycerol compared to ethanol, as increasing glycerol is being formed at higher temperature, indicating high yeast growth and more stress during SSF. Novoren secures the lowest glycerol yield however, not significantly different form Alcalase.

Sub conclusion:
Protease and temperature had significant impact on glycerol. Protease (particular Novoren) lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Glycerol/Ethanol – Rato

The reduced model for glycerol/ethanol relationship ended up being:
Model**: Y(gly/etoh)**= $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

The model varied from the previous response variables, as gly/etoh showed significant effect of the interaction between temperature:enzyme.

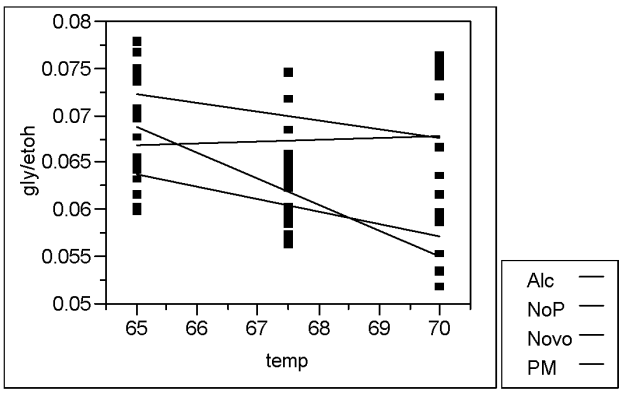
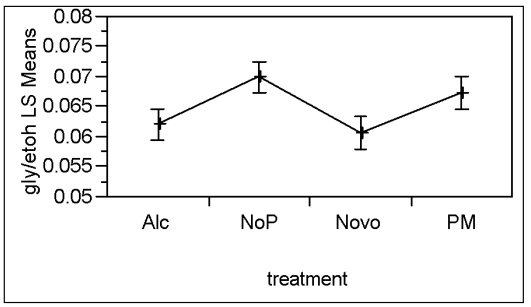


Figure 10. Response gly/etoh, Whole Model. Regression Plot.

The interaction between temperature and enzyme is very clear in the regression plot above, as the lines are not parallel, indicating different behaviors for each temperature. The different pattern is primary due to ProMix not showing increased ethanol at increasing temperature and decreasing glycerol, which is the picture for Alcalase and Novoren. The gly/etoh ratio with ProMix is the same at the different liquefaction temperatures, which is different from Alcalase and Novoren.



Treatment		Least Sq Mean of gly/etoh (g/g mash)
NoP	A	0.070
PM	A	0.067
Alc	B	0.062
Novo	B	0.061

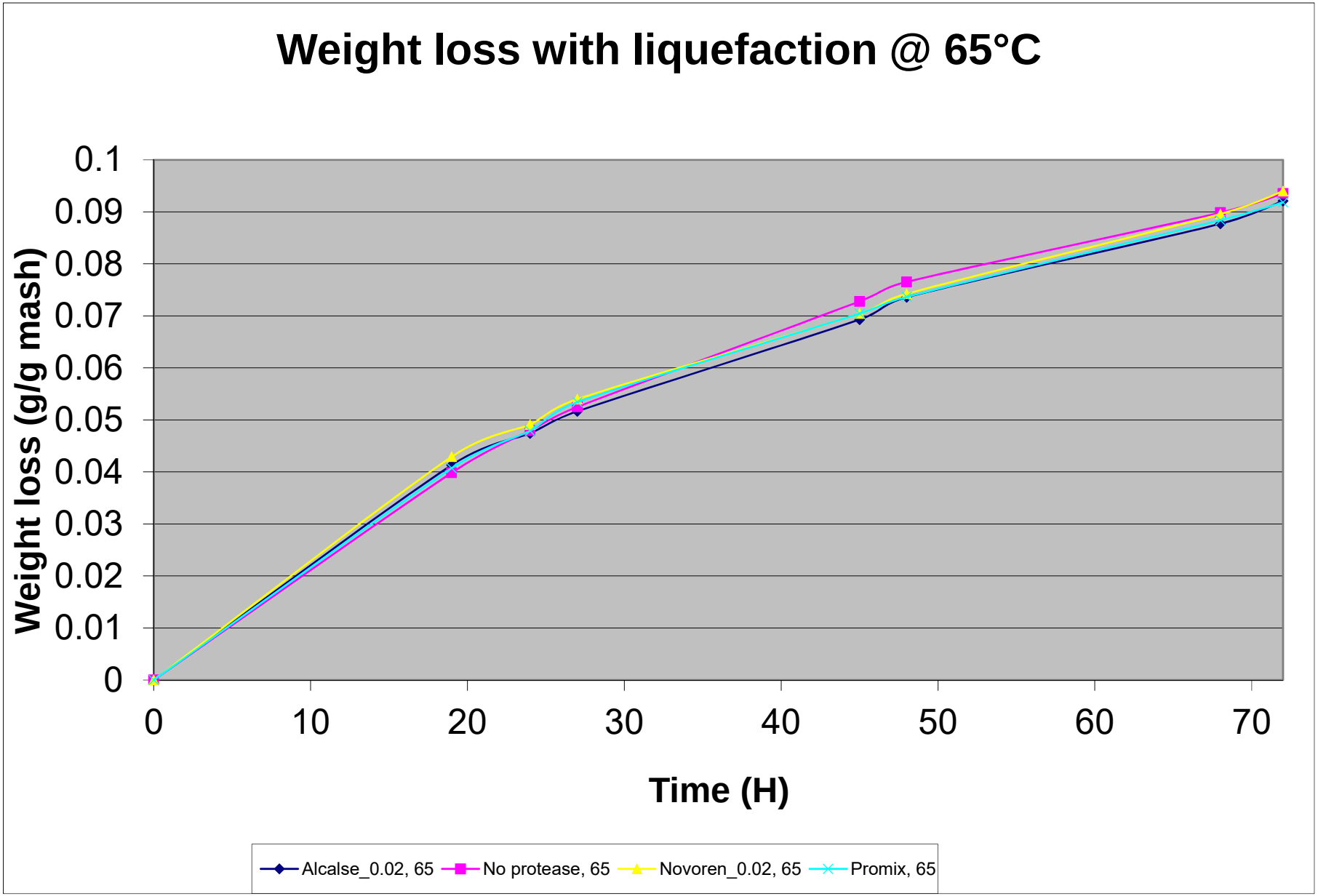
Figure 11. LS Means Plot for glycerol and levels of significance.

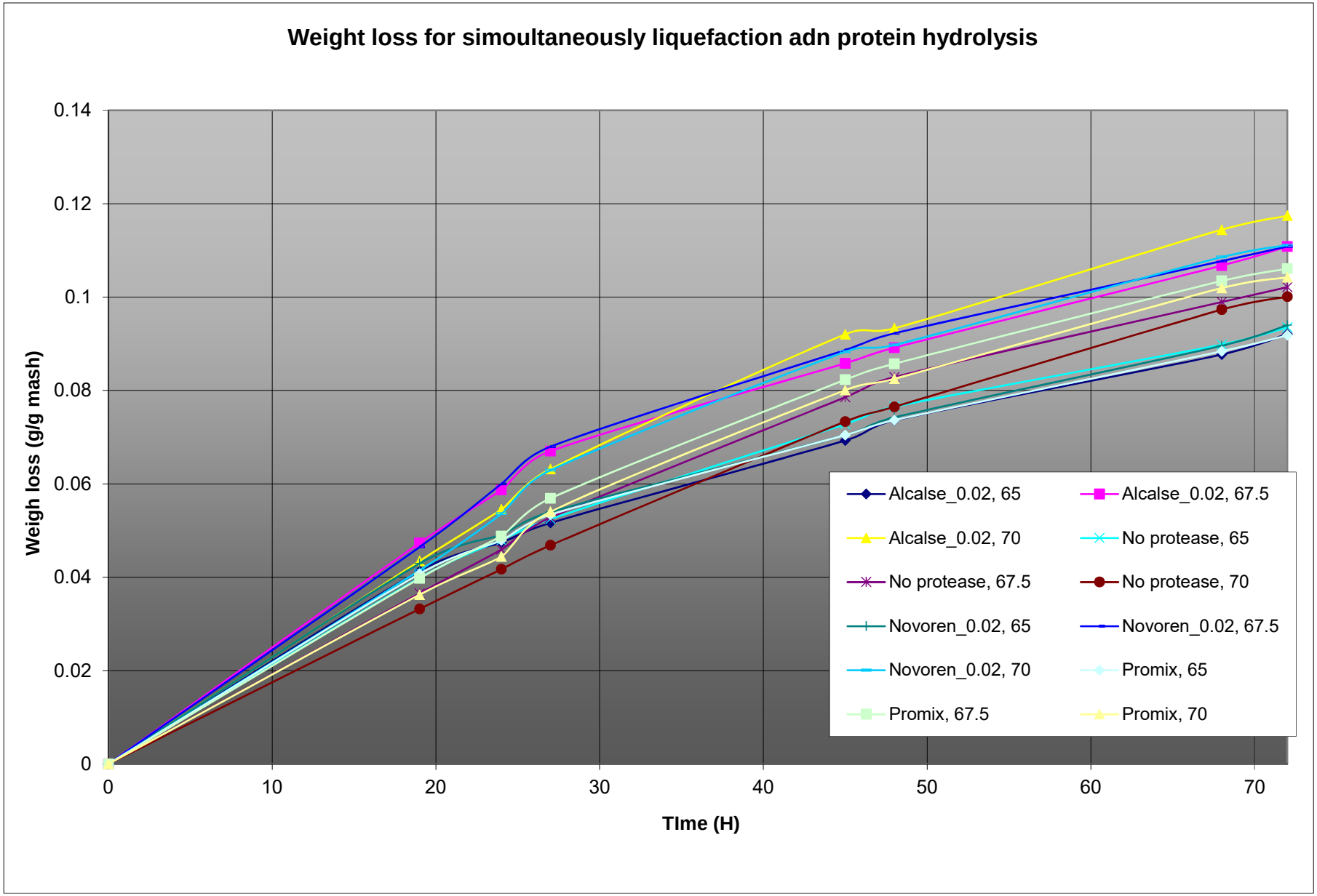
The highest ratio of glycerol being produced per g of ethanol is in the control samples, with no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.

Sub Conclusion:
The gly/etoh ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease.

Reference: RMF050118

Document Produced Natively





				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

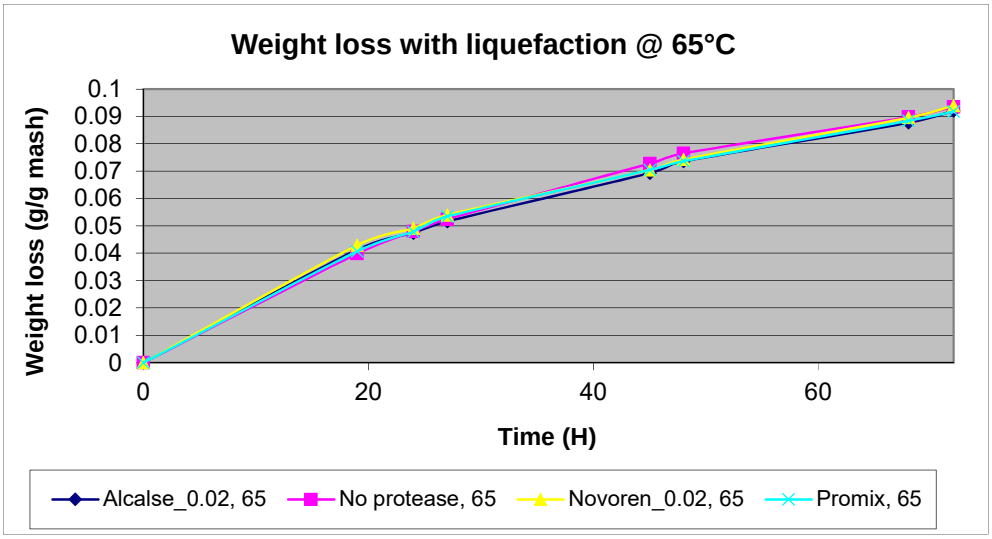
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842

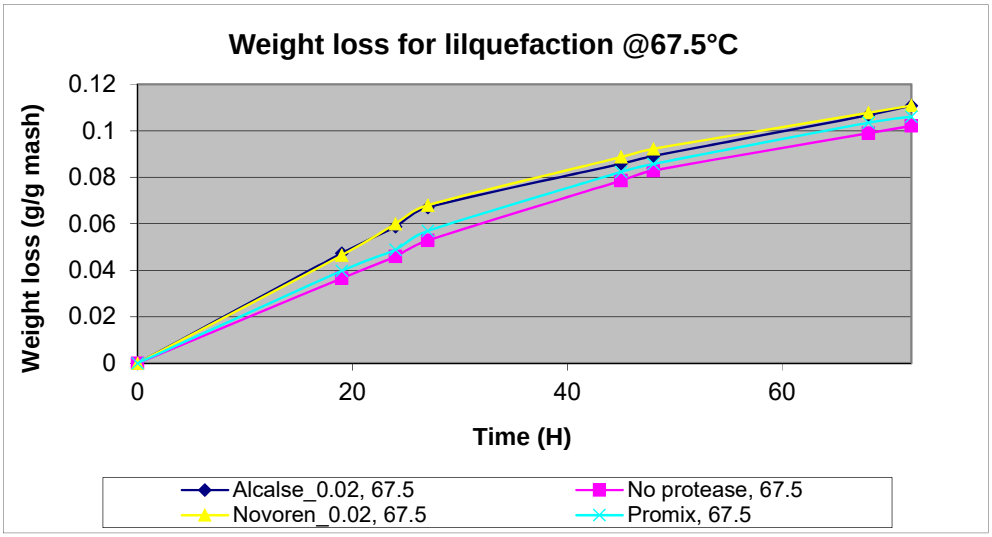
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

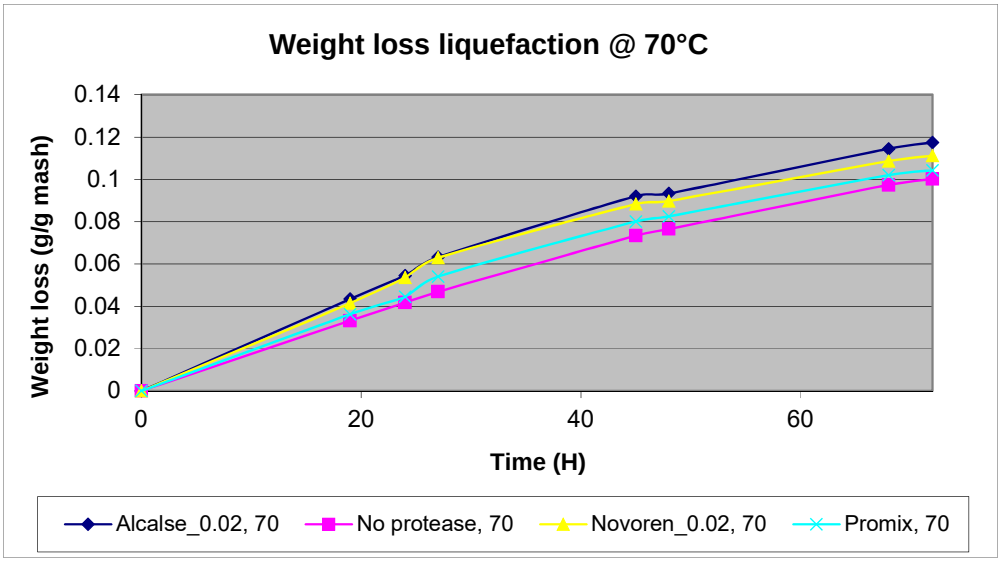
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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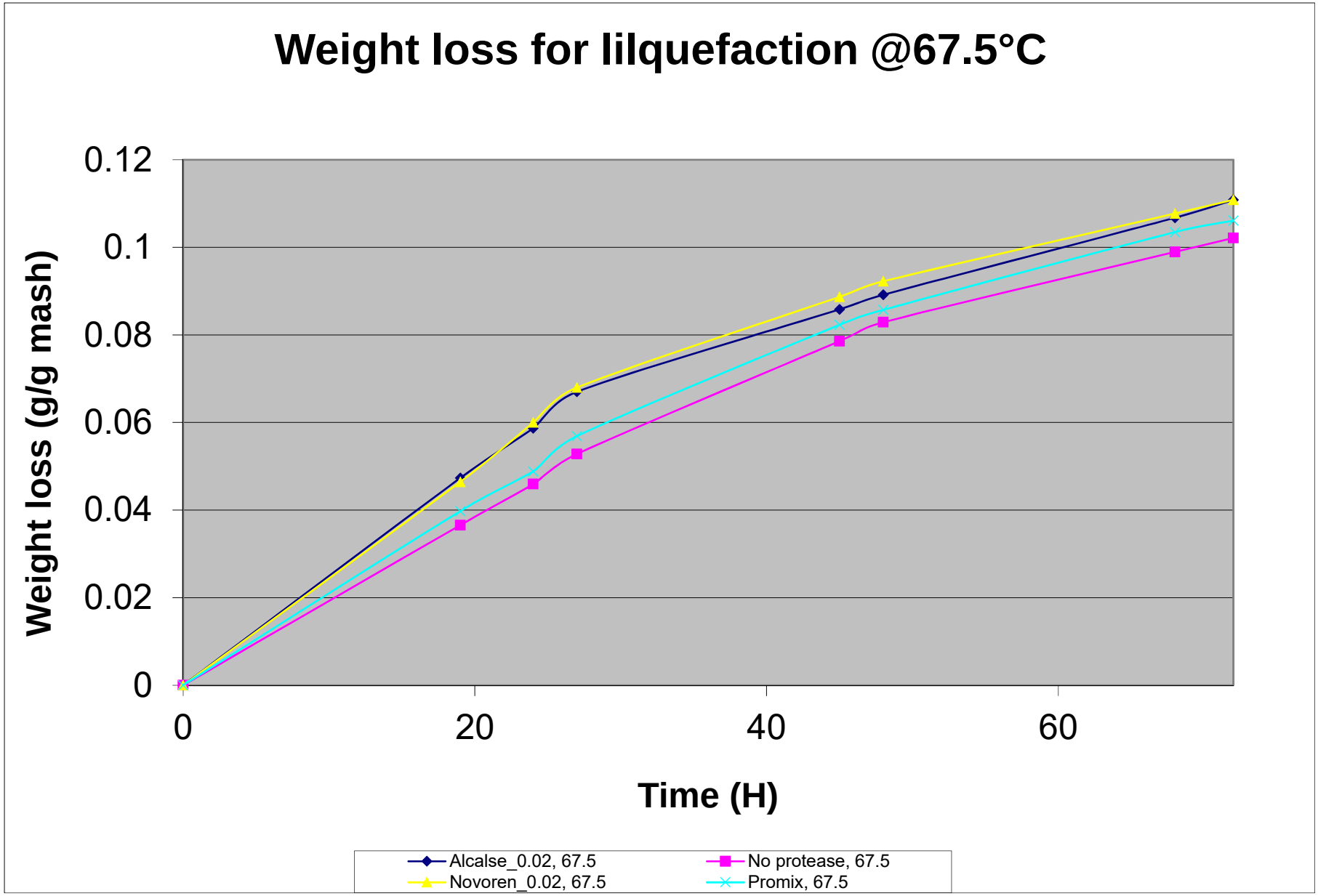
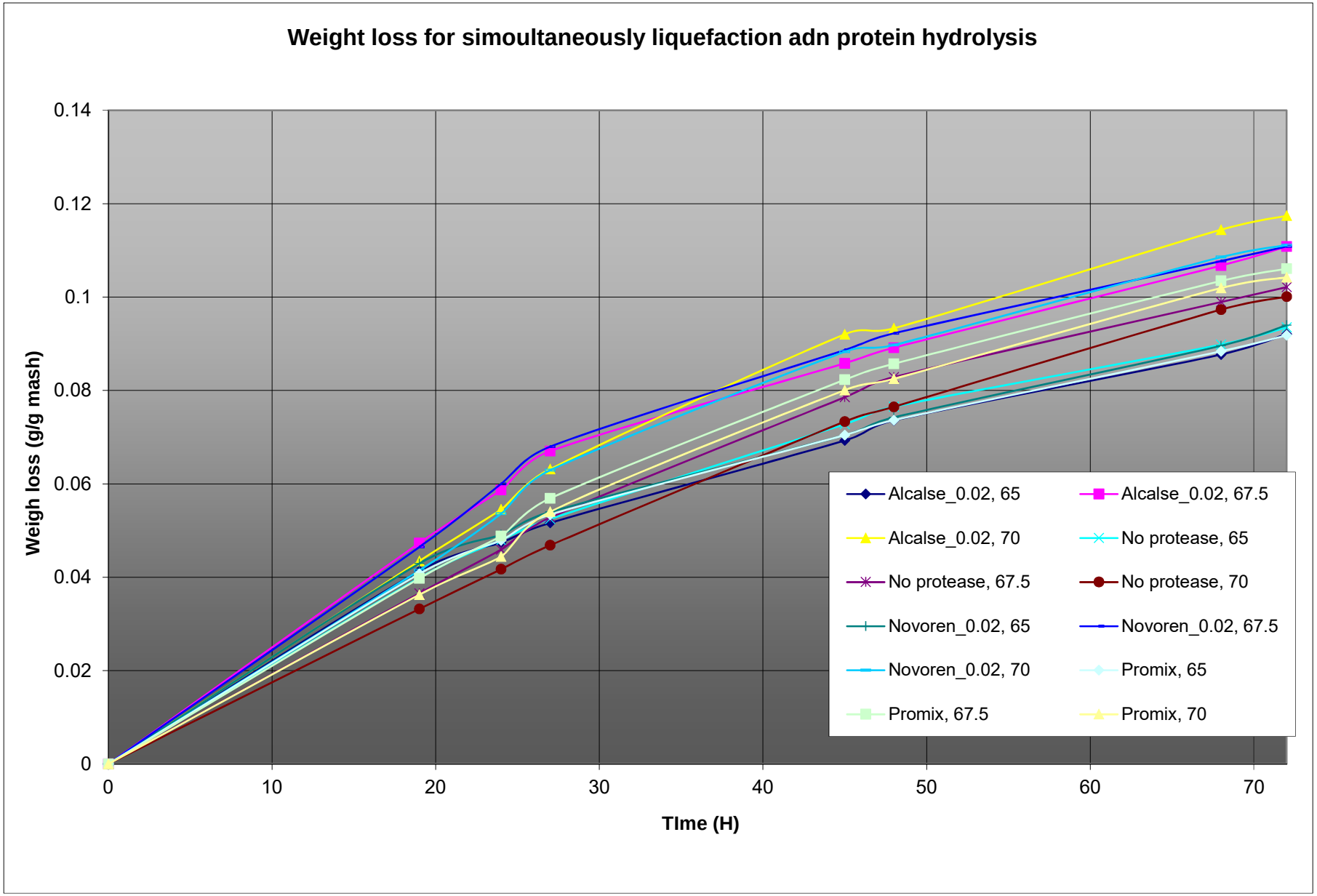




				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

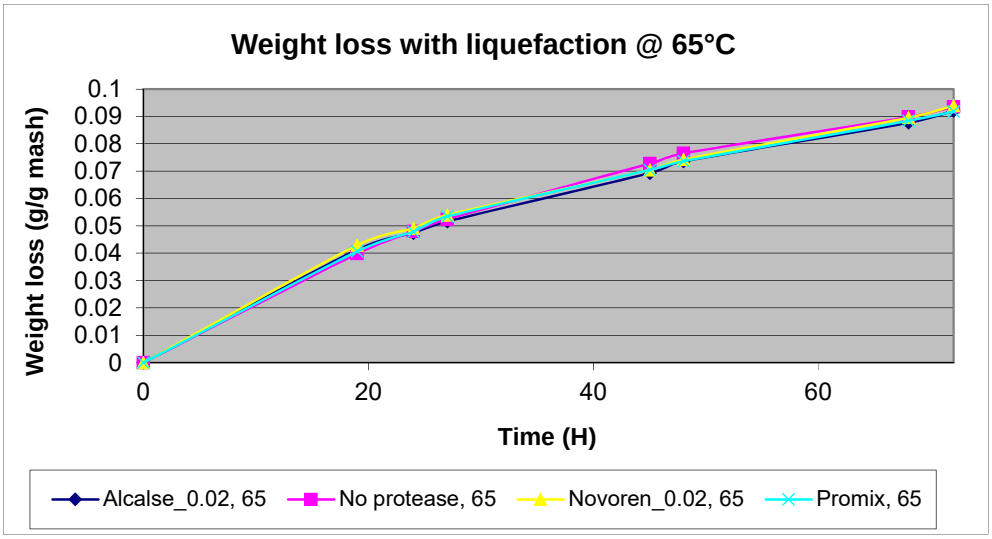
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

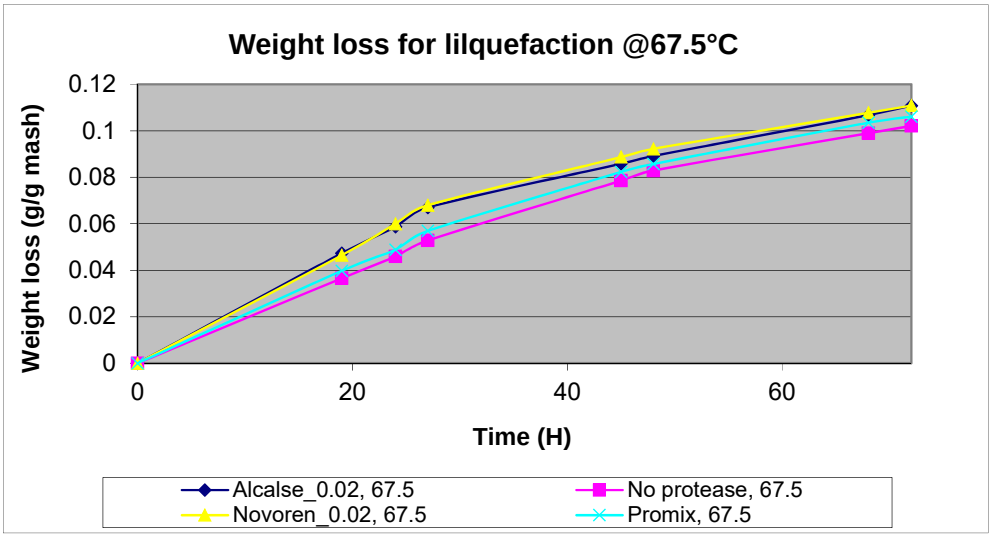
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

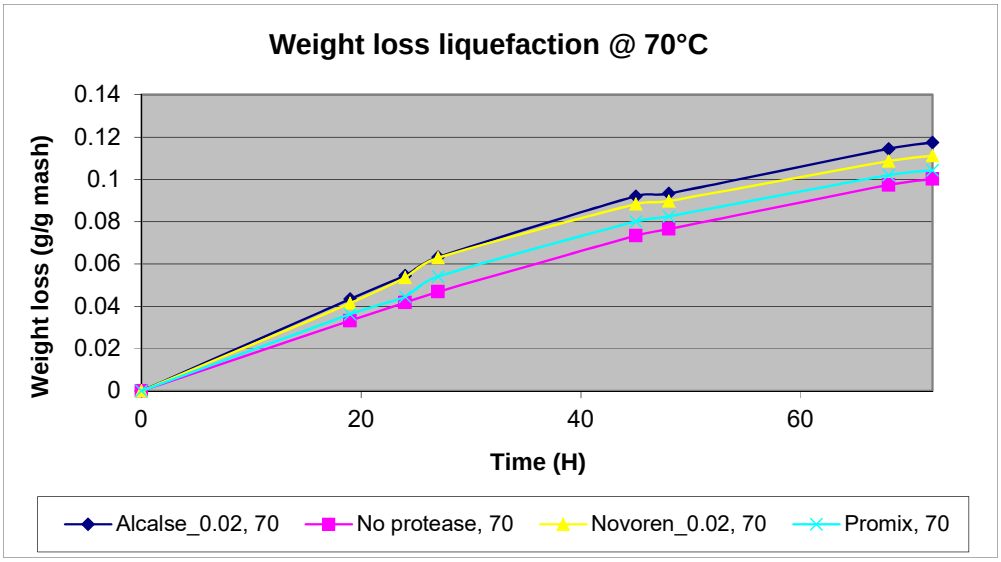
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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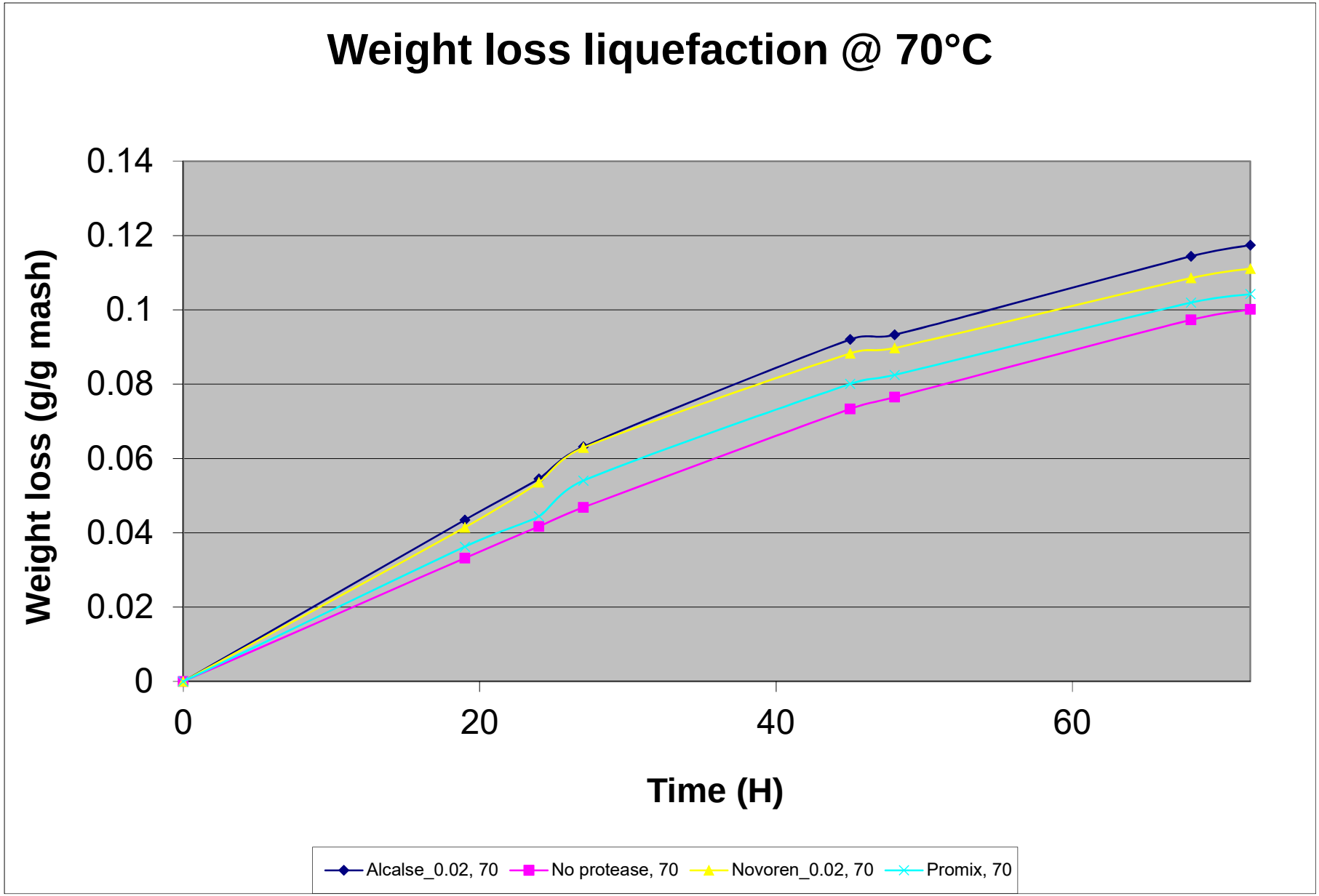

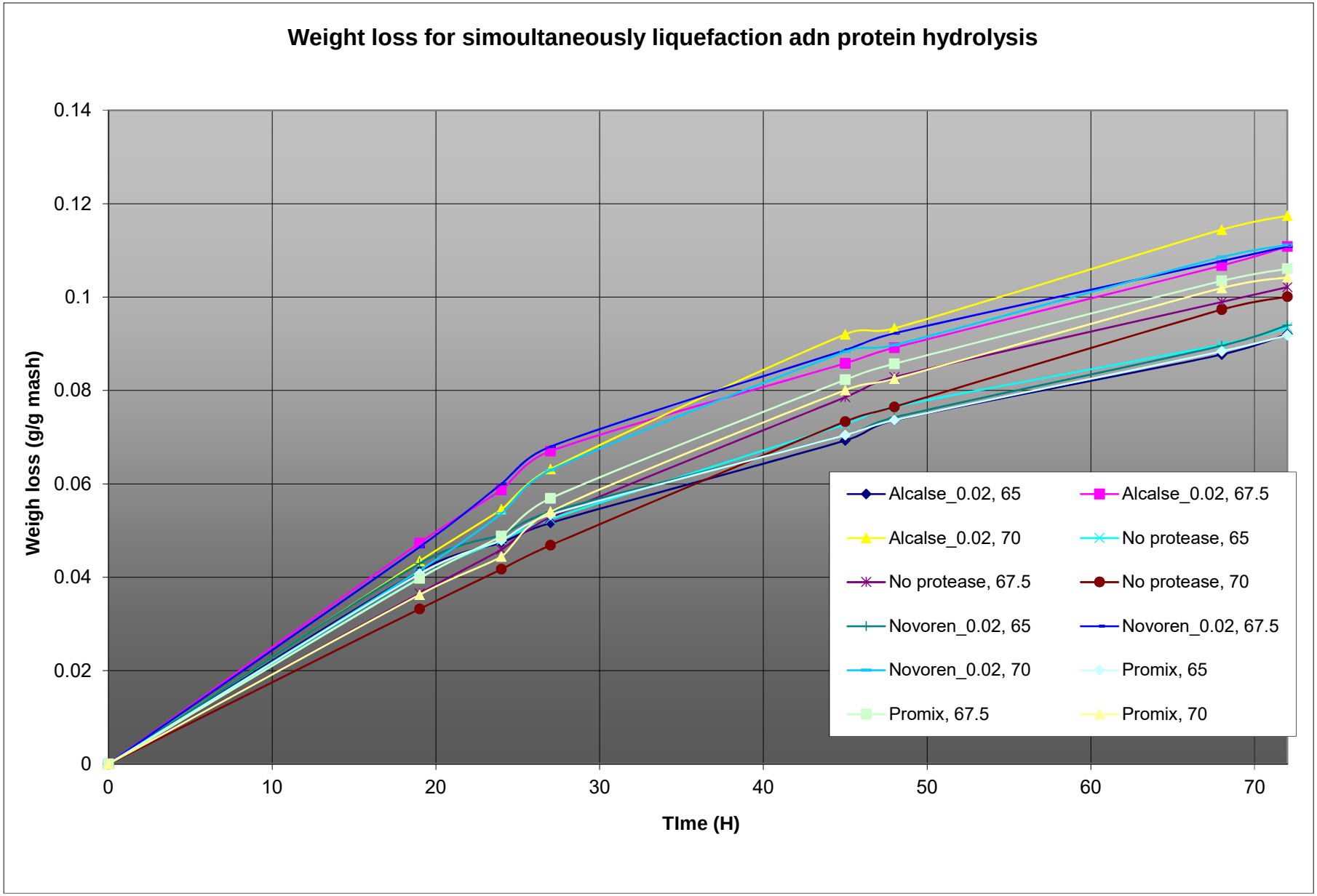

				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
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No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
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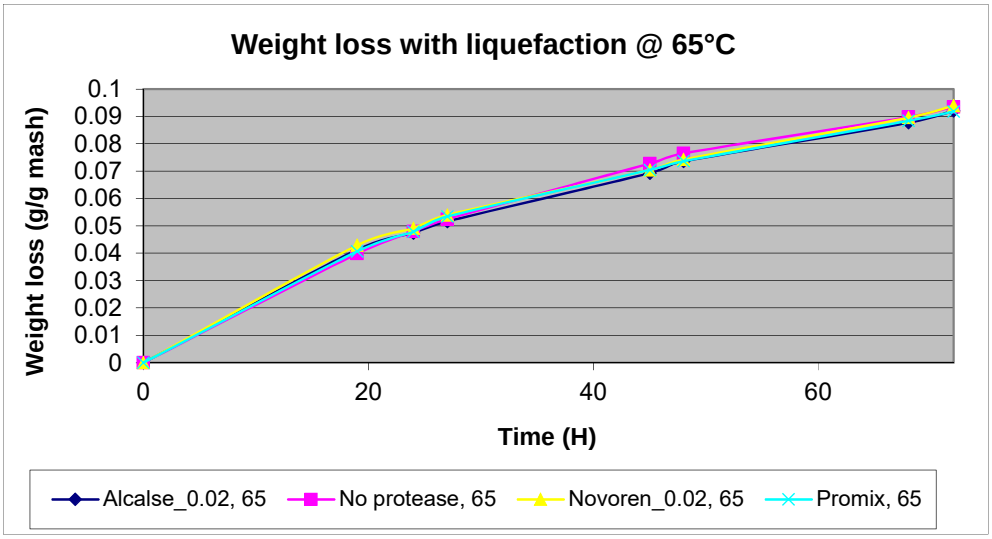
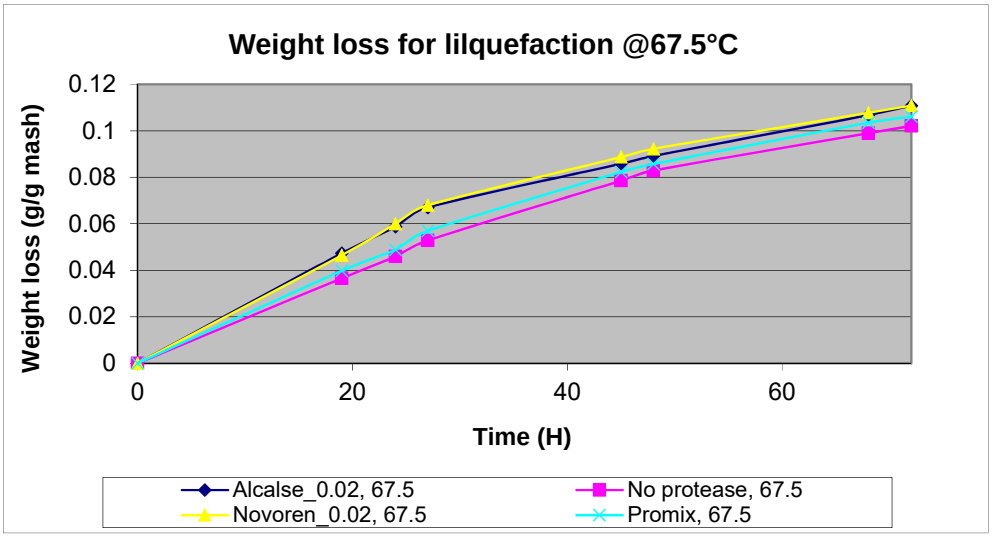
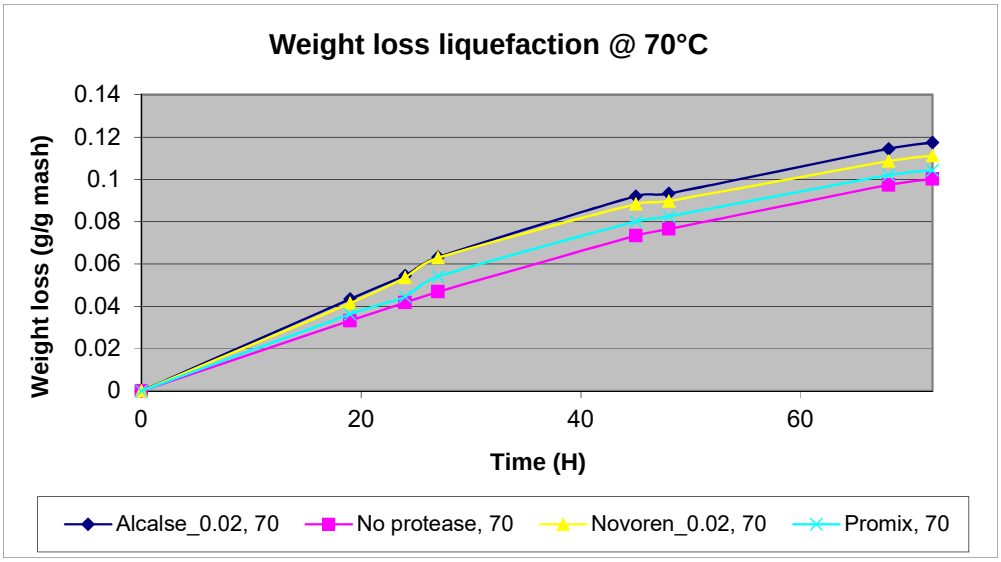
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







Application Technology – US Fuel Research
NL200589
LUNA: 2005-49534-01

To: HSO, KSW, BJes, LWP, RDe,

From: RMF/JPlm

Simultaneous liquefaction and protein degradation at 65, 67.5, & 70°C for SSF to Fuel ethanol

Study # RMF050118

Summary & Conclusions

- Simultaneously starch liquefaction and protein degradation has been carried out at 65, 67.5 and 70°C in a mini scale liquefaction set up using 15 ml tubes.
- Termamyl SC and Alcalase (Family S8), Novoren (Family A1) and ProMix have been tested and evaluated for their effect on standard fermentation parameters.
- Background conditions for the liquefaction was pH=5.8 and DS=34.10% and 50 NU/g DS Termamyl SC and for SSF Spirizyme Fuel was added @ 0.5 AGU/g DS. No urea was added.
- The effect of increasing the temperature contributed positively on the ethanol yield, as higher temperatures alone gave higher ethanol yields due to a more efficient liquefaction of corn starch.
- Alcalase was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalase was increased by increasing temperature. @ 70°C Alcalase enhances the ethanol yield by 6% compared to not having at protease added during liquefaction.
- Protease and temperature had significant impact on glycerol. Novoren and Alcalase lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.
- The glycerol:ethanol ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.
- The pH is higher at liquefaction conditions, and there could be a factor of substrate stabilization in corn flour as opposed to corn mash (post liquefaction studies)

Purpose

To study the effect of adding an α-amylase (Termamyl SC), and a peptidase (Alcalase, Novoren, ProMix) at the same time during liquefaction carried out @ 65, 67.5, and 70°C. Starch liquefaction and protein degradation is thereby run simultaneously for SSF.

Background

Post liquefaction trials have been carried out with Alcalase, where the effect has been very temperature dependent. Alcalase showed best performance at 50°C, and the performance decreased significantly with increasing temperature. In this experiment peptidases are added to the raw mash, where starch, protein and other corn sources are intact and liquefaction and protein degradation is carried out in 15 ml test tubes. Having a simultaneous step of starch liquefaction and protein degradation may be interest to industry, as temperature pH could be beneficial to the peptidases we have available and thereby secure better protein hydrolysis and possible starch release from protein bound starch sources, thus obtain higher yeast efficiency during fermentation.

The factors to be tested in each of the factorial experiments are as follows:
Temperature: 65, 67.5, 70°C
Enzyme: Alcalase, Novoren, and ProMix and no Protease.

Background conditions similar for all treatments during SSF are pH=5, and Spirizyme fuel @ 0.5 AGU/g DS. During liquefaction Alcalase and Novoren was added at 0.02 mg EP/g DS, and ProMix at 0.03 mg Product/g DS (corresponding to the dosage in SSF).

Experimental

The experiment was carried out in 15 ml test tubes using approximately 5 g of corn mash (34.10 % DS) (exact weigh and dry matter content is known) and 5 replicates. Corn flour from Broin (EXOL) was used for the experiments. Subsequently the impact from the liquefaction was accessed by conducting a standard SSF fermentation.

Table 1. HPLC ethanol results after 24 and 48 H of fermentation

Treatment	AGU/g DS	NU/g DS	mg EP/g DS	mg EP/g DS	mg product/g DS
Spirizyme fuel	0.5	50			
Termamyl SC	0.5	50			
Alcalase 0.02	0.5	50	0.02		
Novoren	0.5	50		0.02	
Promix	0.5	50			0.03

Liquefaction Treatment
Corn flour was added water to obtain a DS fraction of XX and pH was adjusted to 5.8 and samples were taken for DS determination. 5g of this mash was transferred to 15ml test tubes, which were liquefied for 1H after the corn mash reached the designated liquefaction temperature, which were 65, 67.5, and 70°C.

SSF Fermentation

Spirizyme fuel was added to all the tubes after liquefaction. Fermentations were carried out as SSF at 32°C, 70 hours using Red Star yeast. Yeast addition was aimed at 1*107 cells/ml. All treatments were each run in 8 replicates and non-liquefied controls were included in the fermentation. The fermentations were monitored by weighing the individual tubes and recording the time & date of the measurement. At the end of fermentation tubes were sampled for HPLC analysis of sugars and fermentation products, primary parameters evaluated were ethanol and glycerol.

Results:

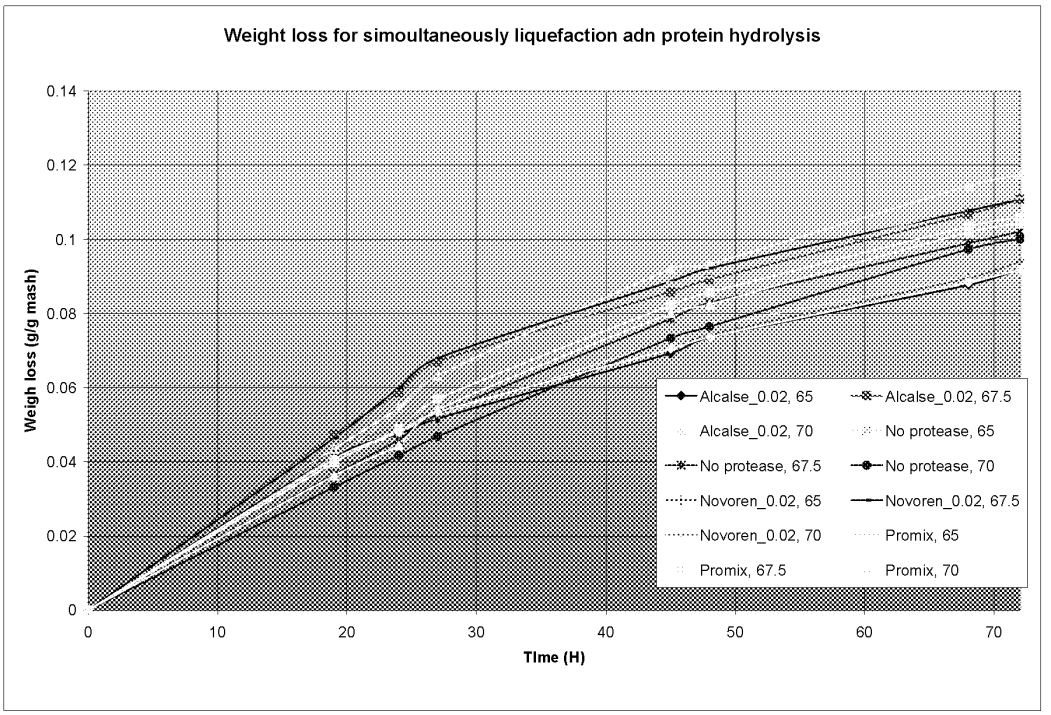
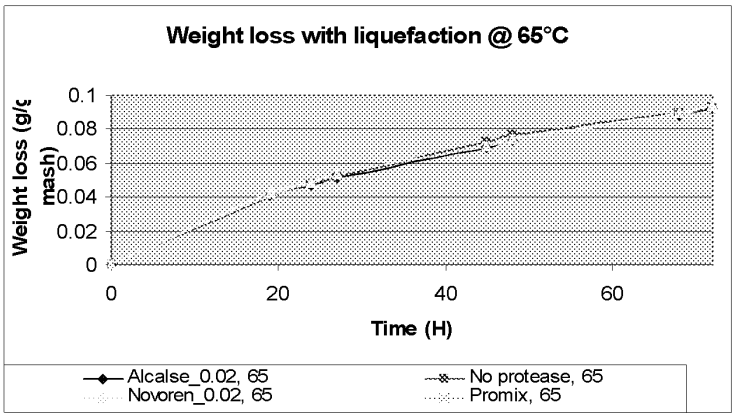
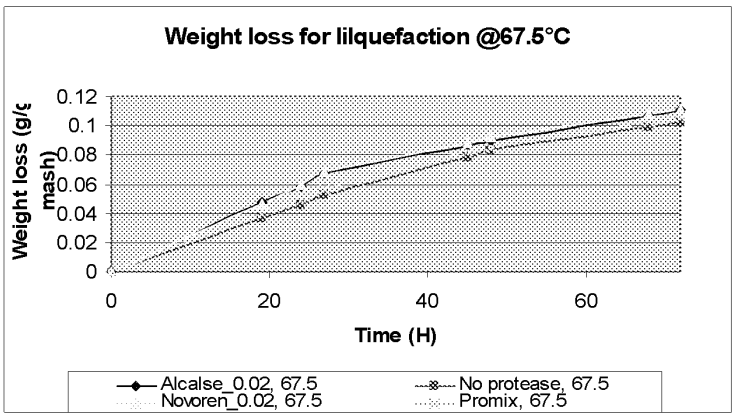


Figure 1. Weight loss for all samples as a function of fermentation time

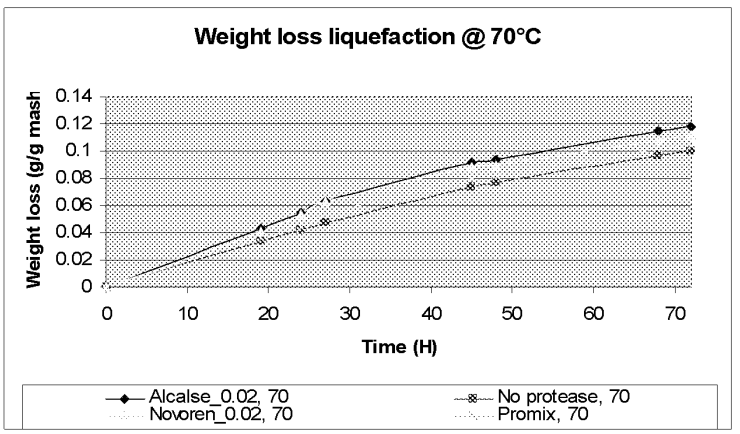
The weight loss curves are affected by the liquefaction temperatures as higher yeast growths and more ethanol is being produced in the high temperature liquefied samples. At the end of fermentation samples liquefied @ 70°C treated with Alcalase has considerably higher weight losses compared to the remaining treatments. The individual liquefaction temperatures are depicted below.



Very little difference between treatments at 65°



Differences in weight loss between Novoren and Alcalase to no Protease



Differences in weight loss pronounced particularly between Alcalase to no protease

Statically analysis of results:
Start Model for all response variables.
$Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Reduced models after response variable:
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model: **Y(glycerol)=**$I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model**: Y(gly/etoh)=** $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Where
α=(temp)= 65, 67.5 & 70°C
β=(enzyme) = Alcalase (Alc), Novoren (Novo) , ProMix (PM) and No protease (NoP)
γ=(enzyme*temp)=(Alc*65, 67.5, 70°), (Novo*65, 67.5, 70°), (PM*65, 67.5, 70°), NoP*65, 67.5, 70°),

Ethanol:

Ethanol has been analyzed after 24 & 48H, and furthermore at the end of fermentation. Only one sample was used for 24 & 48 H analysis, whereas 6 replicates were used at the end of fermentation, thus statistical analysis has been performed on the end HPLC data.

Table 2. HPLC ethanol results after 24 and 48 H of fermentation

	Ethanol	
Treatment	**24 H**	**48H**
NoP, 65	0.147	0.188
Alc 0.02, 65	**0.151**	**0.197**
No 0.02, 65	**0.151**	**0.192**
PM 65	0.147	0.188
NoP, 67.5	0.142	0.222
Alc 0.02, 67.5	**0.178**	**0.228**
No 0.02, 67.5	**0.179**	**0.230**
PM 67.5	0.146	0.226
NoP, 70	0.138	0.217
Alc 0.02, 70	**0.178**	**0.250**
No 0.02, 70	0.168	0.243
PM 70	0.139	0.224

The HPLC results after 24 and 48H show that Alcalase performs very well in respect to ethanol yield. Increasing the liquefaction temperature result in higher ethanol yield, due to higher degree of gelatinization and liquefaction thereby starch accessibility, thus higher ethanol yields. Protease addition results in adding a little extra ethanol yield on top of the temperature effect, which is a result of protein degradation either by releasing more starch and or hydrolysis of protein to amino acids to enhance fermentation. Alcalase @ 0.02 mg EP/g DS liquefied at 70°C gives the over all highest ethanol yield.

The reduced model for ethanol is listed below. Factor analysis showed
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$

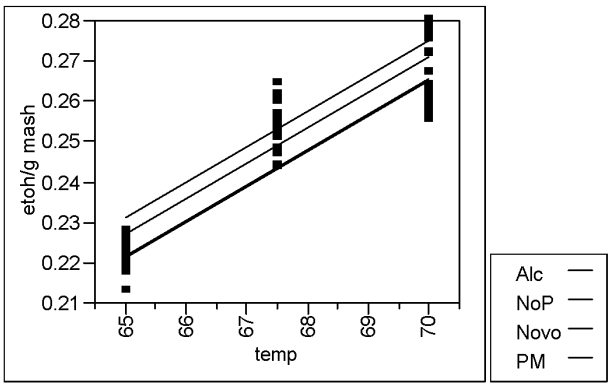
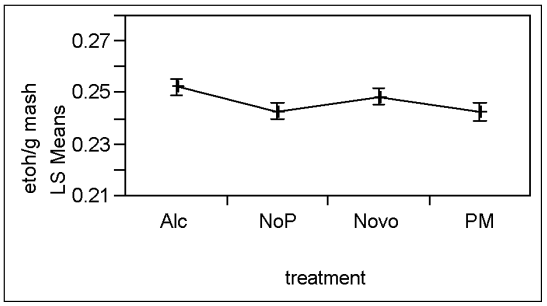


Figure 2. Response etoh/g mash, Whole Model. Regression Plot.

Ethanol increases by increasing liquefaction temperature, and fermentations with Alcalase reaches the highest level of ethanol. No Protease and ProMix performs equally poor. The thermo-stability of Flavourzyme in ProMix is not very high, as optimum is around 50°C, and the liquefaction temperatures in this trial is starting at 65°C.



Treatment			Least Sq Mean of ethanol (g/g mash)
Alc	A		0.252
Novoren	A	B	0.248
NoP		B	0.243
PM		B	0.243

Figure 3.LS Means Plot for ethanol and levels of significance.

The LS means numbers show Alcalase has the highest impact on improving ethanol yield and below it is clear that Alcalase performs better for all tested temperatures.

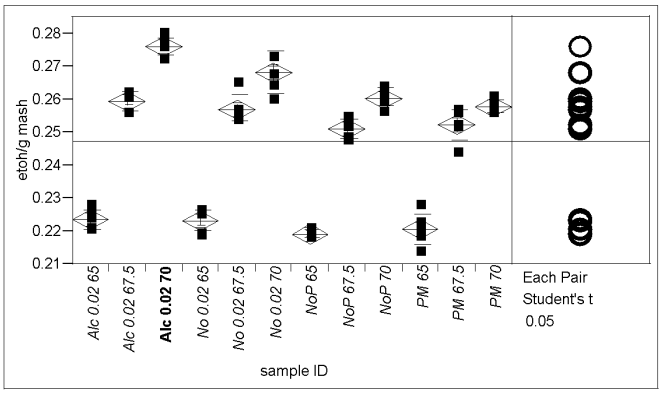


Figure 4: One-way Analysis of Ethanol Yield (g/g DS) by all treatments after 70H of fermentation.

At the end of fermentation Alcalase (0.02 mg EP/g DS at 70°C) demonstrate the best performance compared over temperature and treatment. The enzyme protein amount added from Novoren in ProMix is however considerably lower, as the total weight dose is only 0.03 mgPrdouct/g DS, which is equivalent to ((0.03 mg/g DS*73%)/1000)*(0.15AU(RH)/g /22 AU(RH)g EP)=1.5*10-4 mg EP/g DS. This explains the poor performance of ProMix, but the dosage was included at this is the recommended start dose of ProMix added during SSF. ProMix is not as thermo-stable as Alcalase as the effect of increasing temperature is the same as for no protease, which is due to the lower thermo-stability of Flavourzyme in the product.

The table above confirms the observations and lists the significance levels, very clear effect of liquefaction temperature and Alcalase and Novoren. Below the leverage effect and prediction profiles are depicted. As can be seen there is linear increase in ethanol yield with increasing temperature, and that Alcalase gives higher ethanol yields compared to the other treatments.

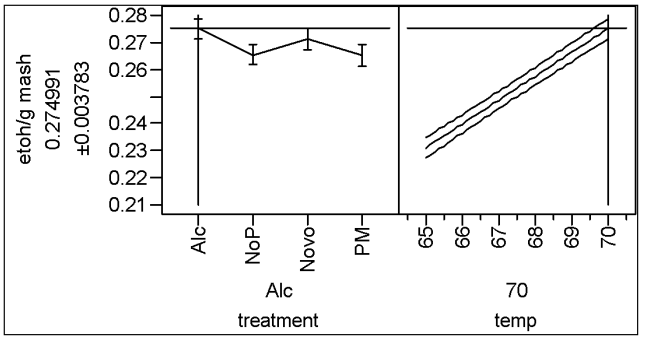


Figure 5. Prediction Profiles for treatment and temperature.

For any given temperature Alcalase will give the best ethanol yield, and increasing the temperature will ensure higher ethanol yields.

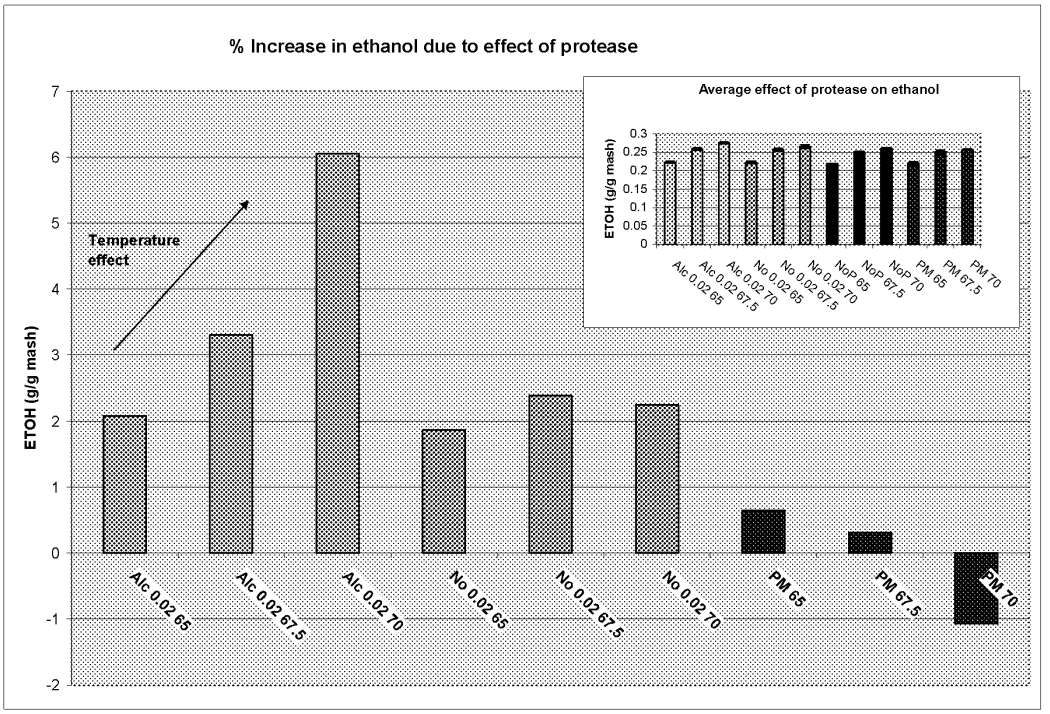


Figure 6. % increase in ethanol by treatment (enzyme and temperature) compared to no protease for every temperature.

In Figure 6 it is clear that Alcalase increases the ethanol yield up to 6% compared to not having a protease added (compared for every liquefaction temperature). Novoren also enhances the ethanol formation during SSF, but not to the same effect as Alcalase, and at 67.5°C Novoren reaches its maximum performance. ProMix on the other hand only increases the ethanol yield by 0.5% compared to n ot having a protease added, and there is absolutely no effect at 70°C. The poor performance of ProMix is very likely due to the low dosage in combination with low thermo-stability.

Sub conclusion:
Liquefaction time has significant impact on the ethanol yield, higher liquefaction temperature results in higher ethanol yields. The addition of protease improves the ethanol yield significantly at 67.5 and 70°C compared to not having a protease added. Alcalse was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalse was increased by increasing temperature. @ 60°C Alcalse enhances the ethanol yield by 6% compared to not having at protease added during liquefaction. This has not been seen at the post liquefaction studies, where increasing temperature above 50°C has shown decreasing performance of Alcalse.
The pH is higher at liquefaction conditions, and also there could be a factor of substrate stabilization in corn flour as opposed to corn mash.

Glycerol Yield

Again glycerol was measured after 24 & 48 H of fermentation, and more thoroughly analyzed with 6 replicates at the end of fermentation

Table 3. HPLC glycerol results after 24 and 48 H of fermentation

	Glycerol	
Treatment	**24 H**	**48 H**
NoP, 65	0.027	0.010
Alc 0.02, 65	0.026	0.017
No 0.02, 65	0.024	0.012
PM 65	0.026	0.013
NoP, 67.5	0.026	0.015
Alc 0.02, 67.5	0.027	0.013
No 0.02, 67.5	0.027	0.015
PM 67.5	0.028	0.014
NoP, 70	0.027	0.013
Alc 0.02, 70	0.027	0.015
No 0.02, 70	0.026	0.014
PM 70	0.026	0.013

Formation of glycerol was in general very similar for the protease treatments, in fact similar to not having a protease added during liquefaction.

After reduction of insignificant effects in the start model, the model describing ethanol yield at the end of fermentation looks as follows:

Model: **Y(glycerol)**=I+α(temp)+β(enzyme) +ε

No significant effect of the interaction (temp:enzyme) was found, and the regression plot below illustrate this very well, as the enzyme have similar pattern in glycerol formation for very all temperatures.

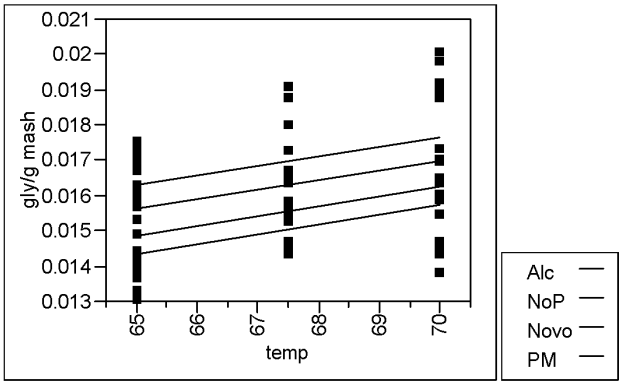
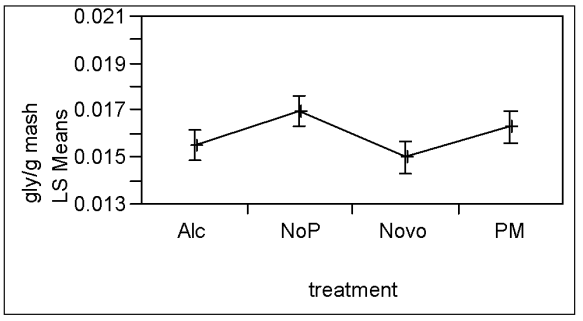


Figure 7. Response gly/g mash, Whole Model. Regression Plot.



Treatment		Least Sq Mean of glycerol (g/g mash)
NoP	A	0.0170
PM	A B	0.0163
Alc	B	0.0155
Novo	B	0.0150

Figure 8.LS Means Plot for glycerol and levels of significance.

Not having a protease added during liquefaction result in the highest formation of glycerol, and again Alcalase and Novoren show the best performance by lowering the glycerol the most.

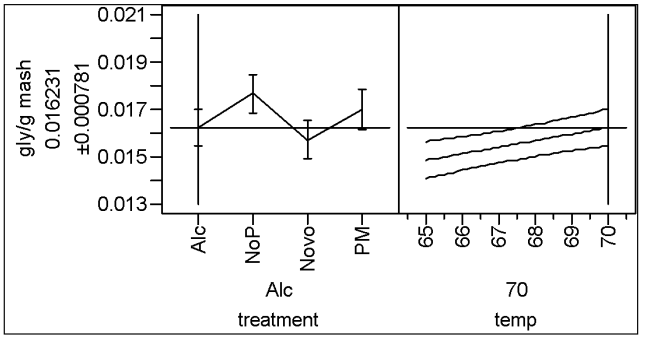


Figure 9: Prediction Profiles for response variable glycerol, for both treatment and temperature.

Temperature has the reverse effect on glycerol compared to ethanol, as increasing glycerol is being formed at higher temperature, indicating high yeast growth and more stress during SSF. Novoren secures the lowest glycerol yield however, not significantly different form Alcalase.

Sub conclusion:
Protease and temperature had significant impact on glycerol. Protease (particular Novoren) lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Glycerol/Ethanol – Rato

The reduced model for glycerol/ethanol relationship ended up being:
Model**: Y(gly/etoh)**= $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

The model varied from the previous response variables, as gly/etoh showed significant effect of the interaction between temperature:enzyme.

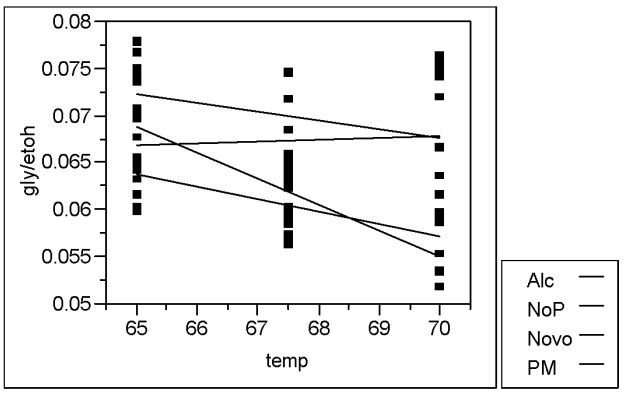
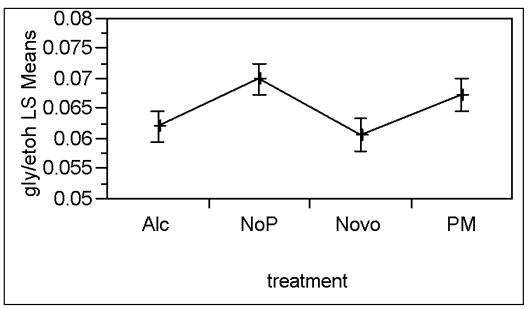


Figure 10. Response gly/etoh, Whole Model. Regression Plot.

The interaction between temperature and enzyme is very clear in the regression plot above, as the lines are not parallel, indicating different behaviors for each temperature. The different pattern is primary due to ProMix not showing increased ethanol at increasing temperature and decreasing glycerol, which is the picture for Alcalase and Novoren. The gly/etoh ratio with ProMix is the same at the different liquefaction temperatures, which is different from Alcalase and Novoren.



Treatment		Least Sq Mean of gly/etoh (g/g mash)
NoP	A	0.070
PM	A	0.067
Alc	B	0.062
Novo	B	0.061

Figure 11. LS Means Plot for glycerol and levels of significance.

The highest ratio of glycerol being produced per g of ethanol is in the control samples, with no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.

Sub Conclusion:
The gly/etoh ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease.

Reference: RMF050118

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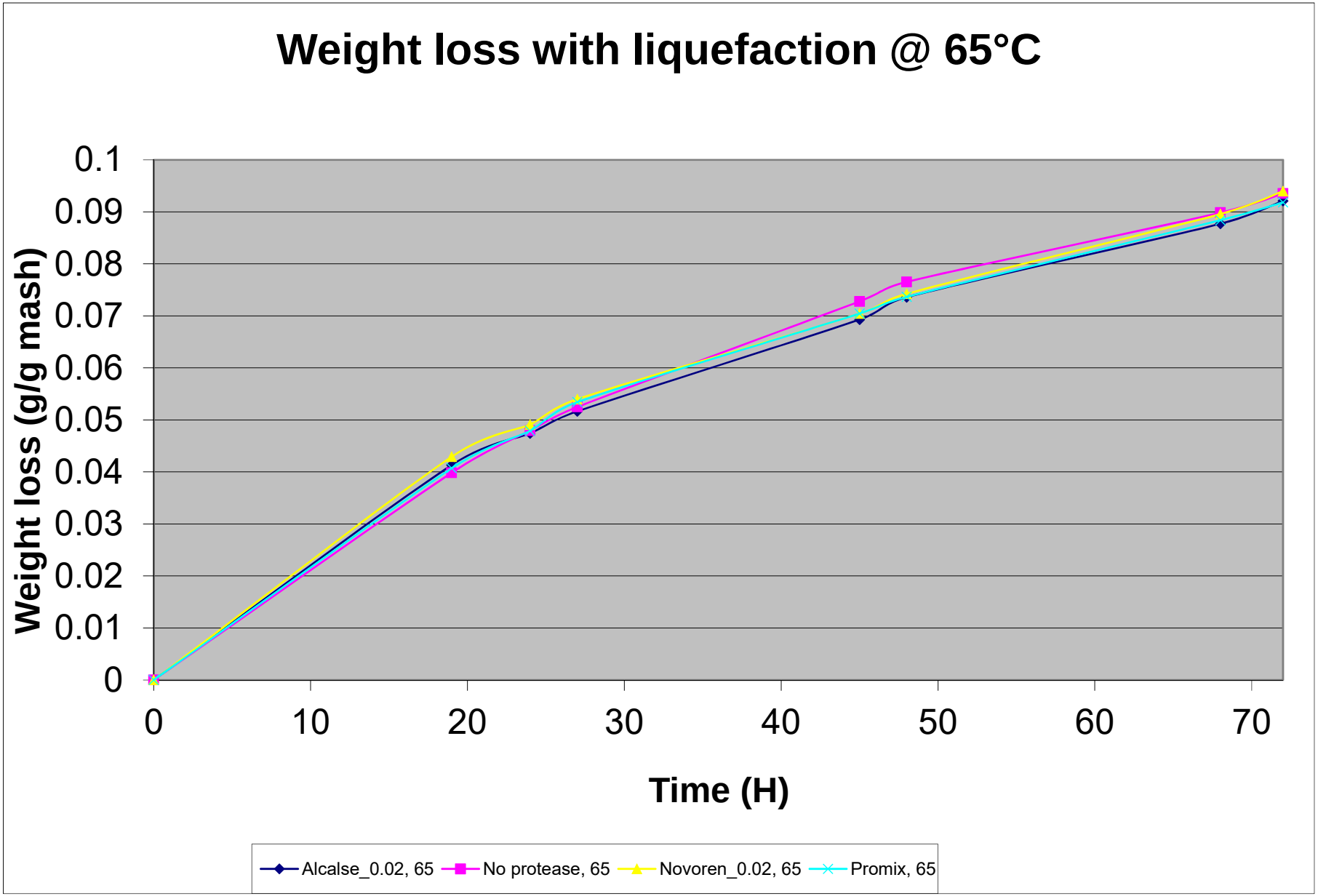
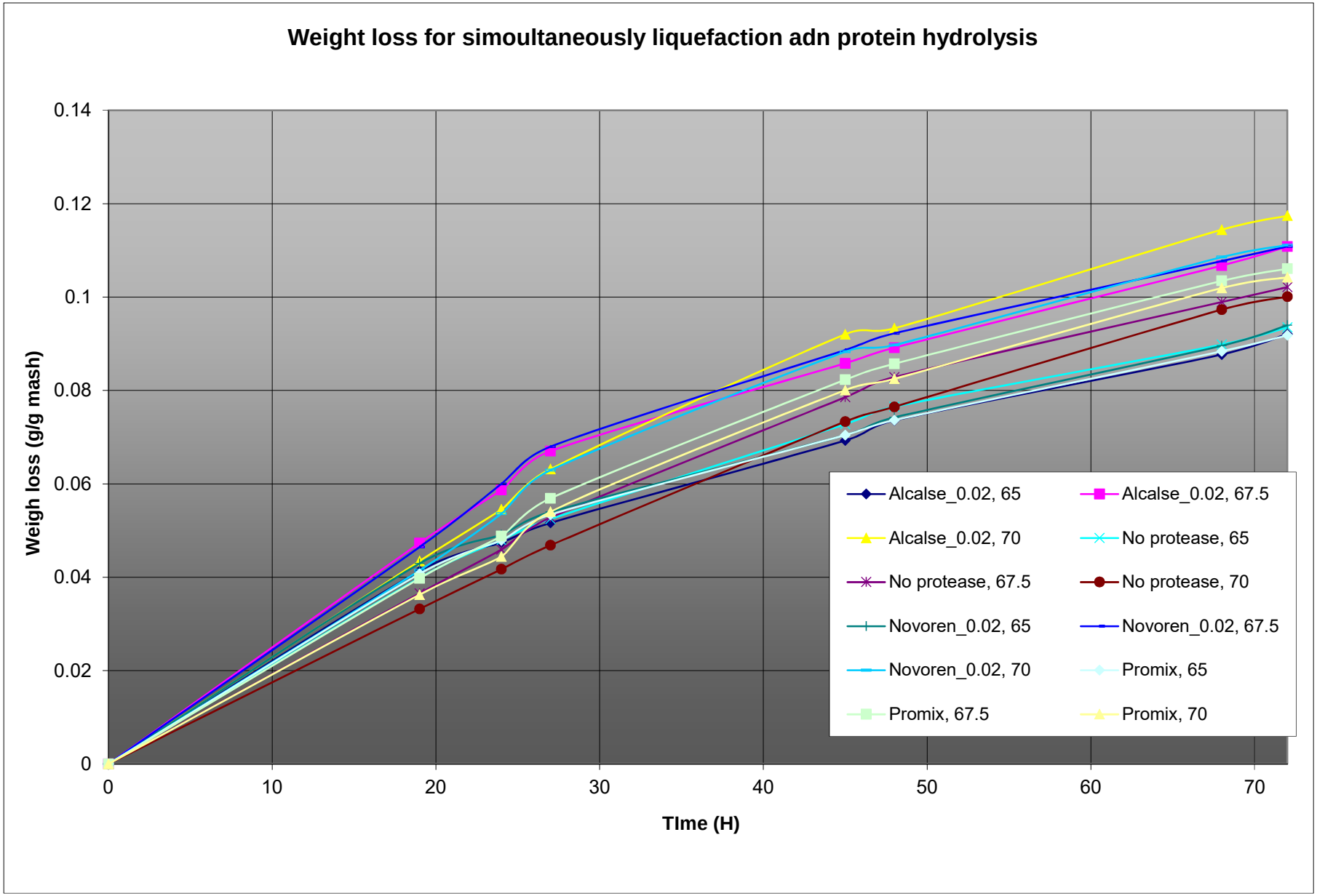




				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

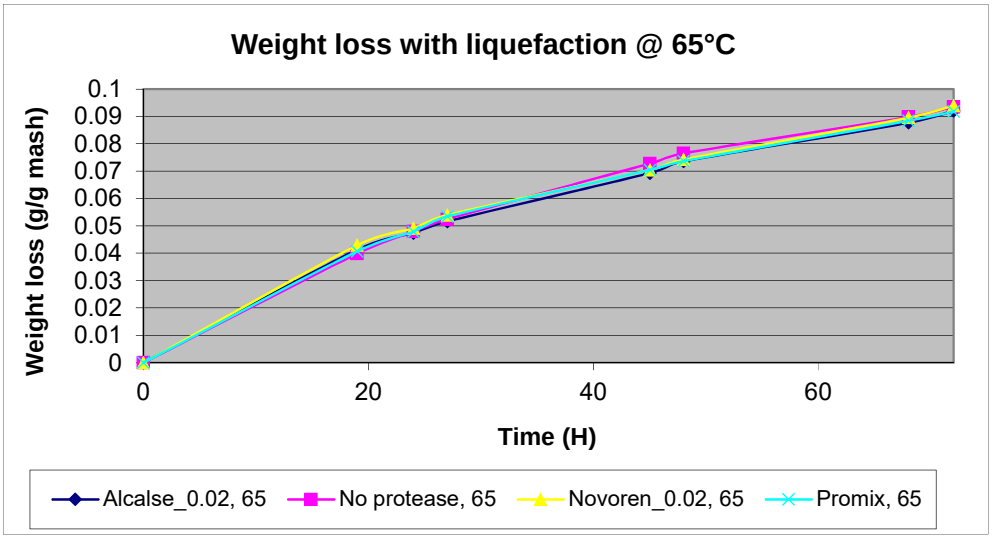
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

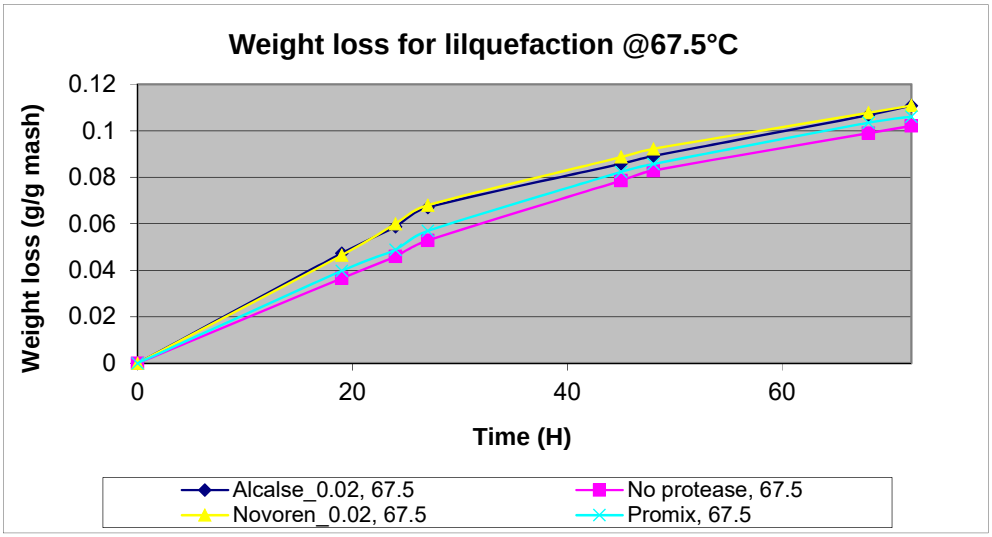
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

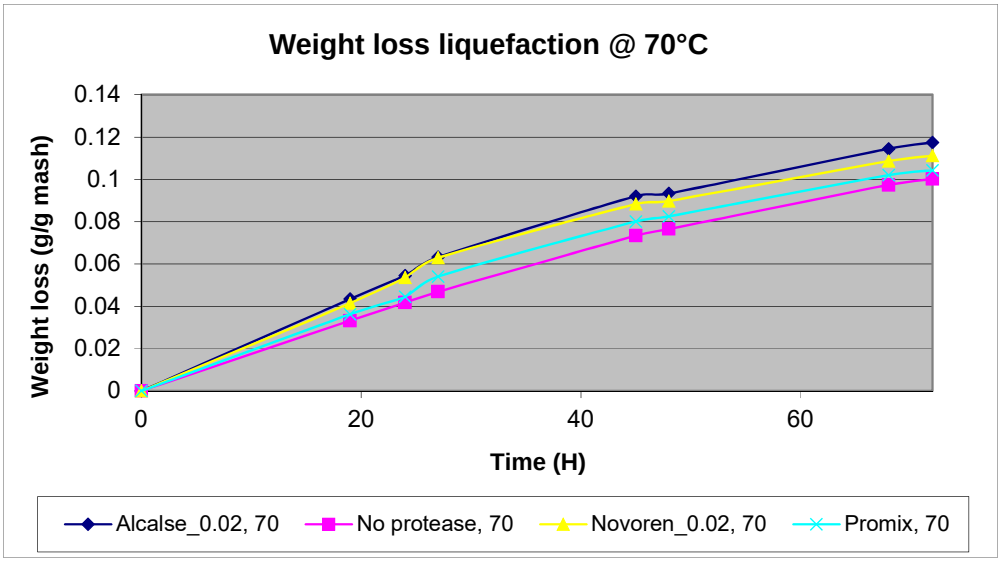
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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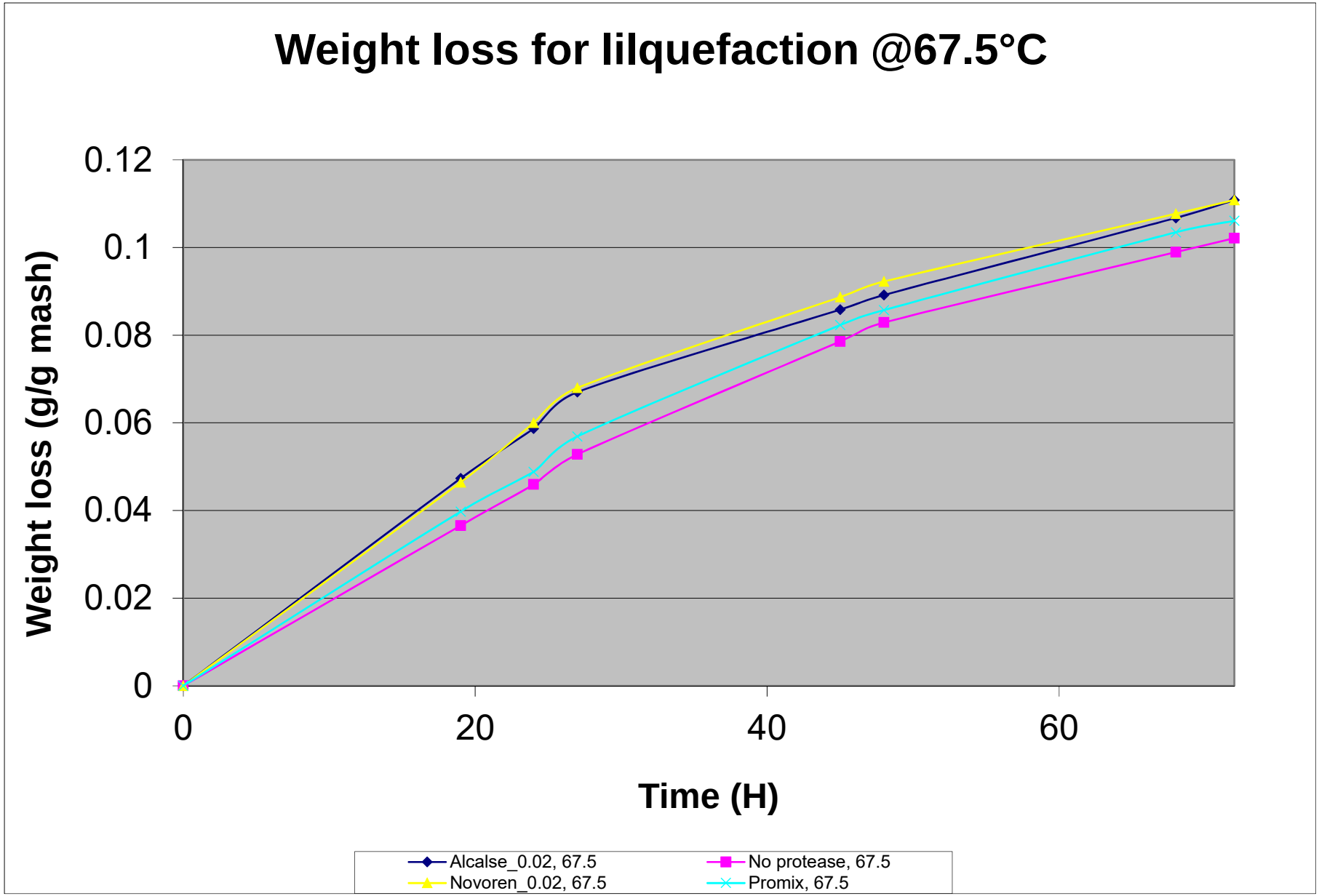
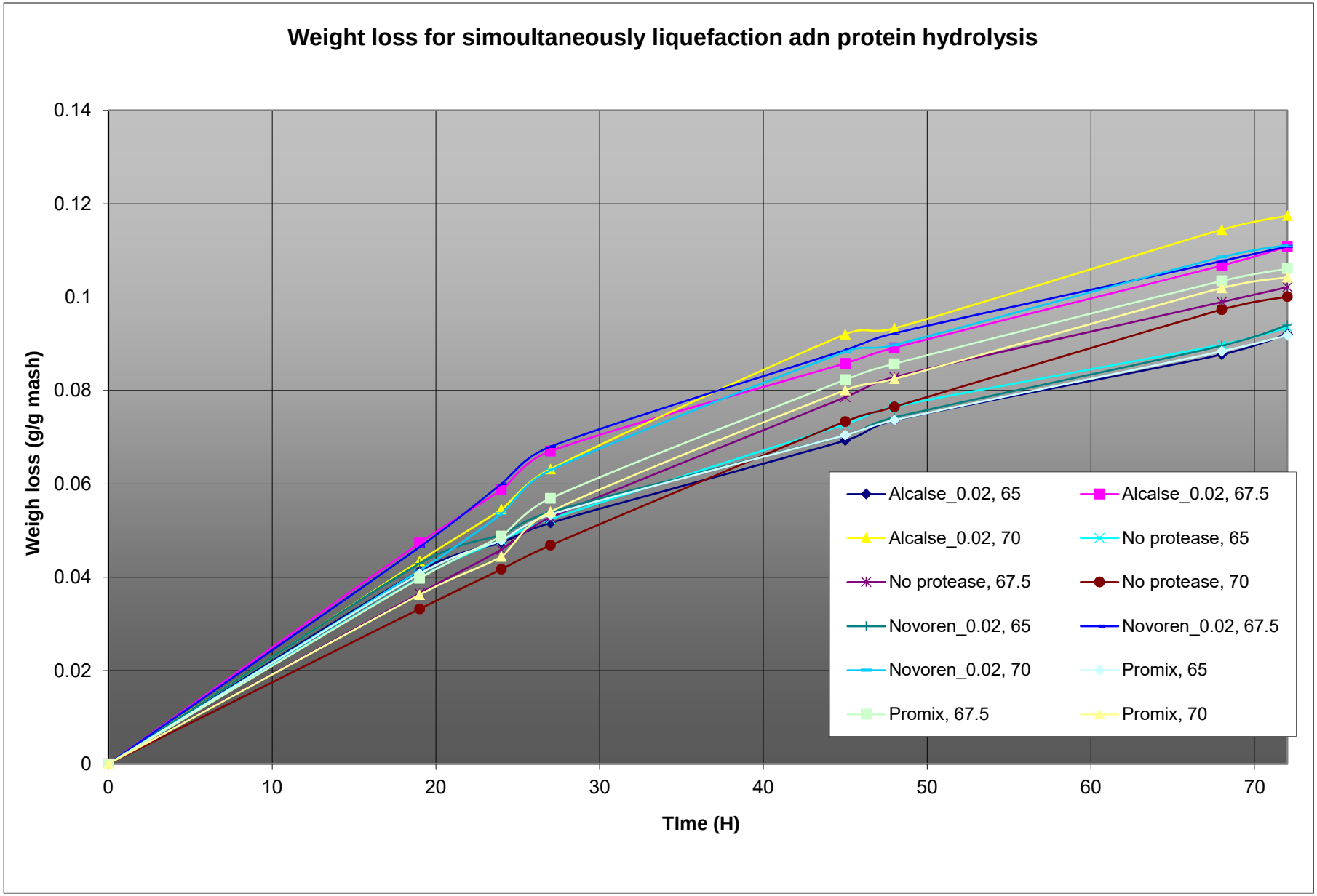




				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

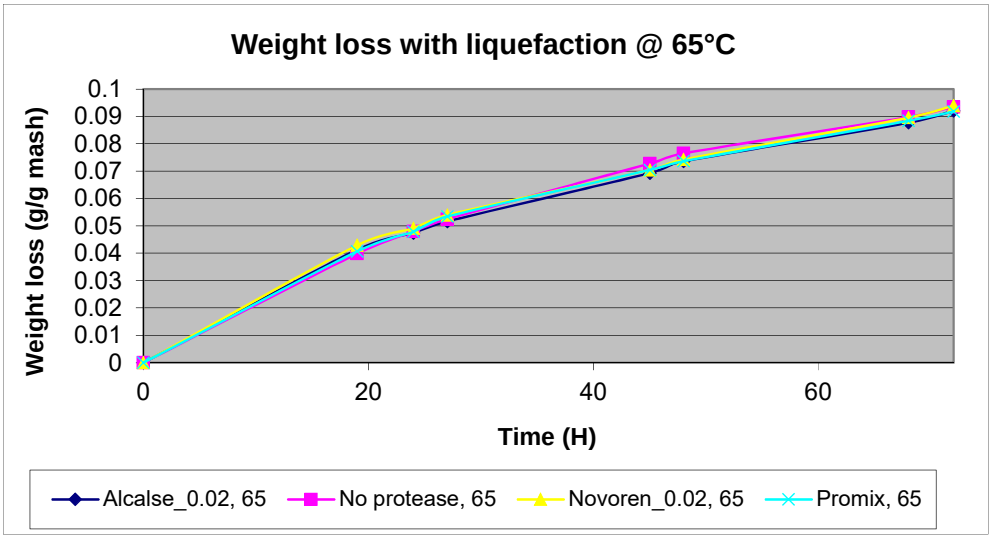
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

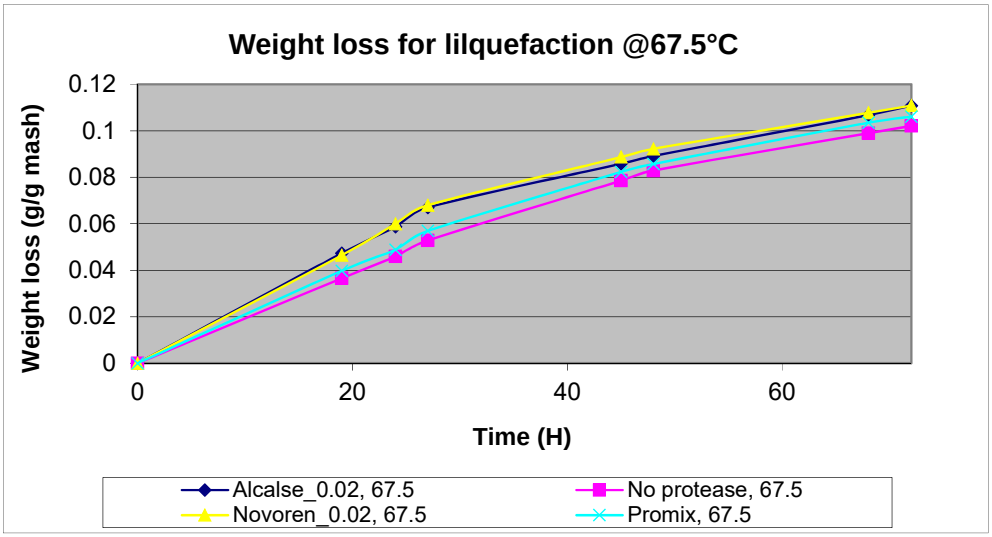
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

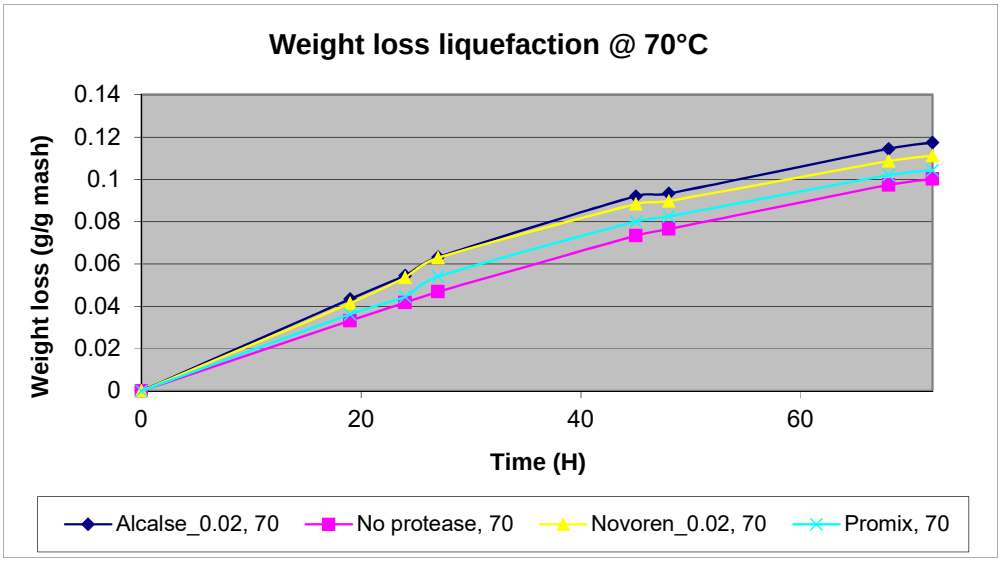
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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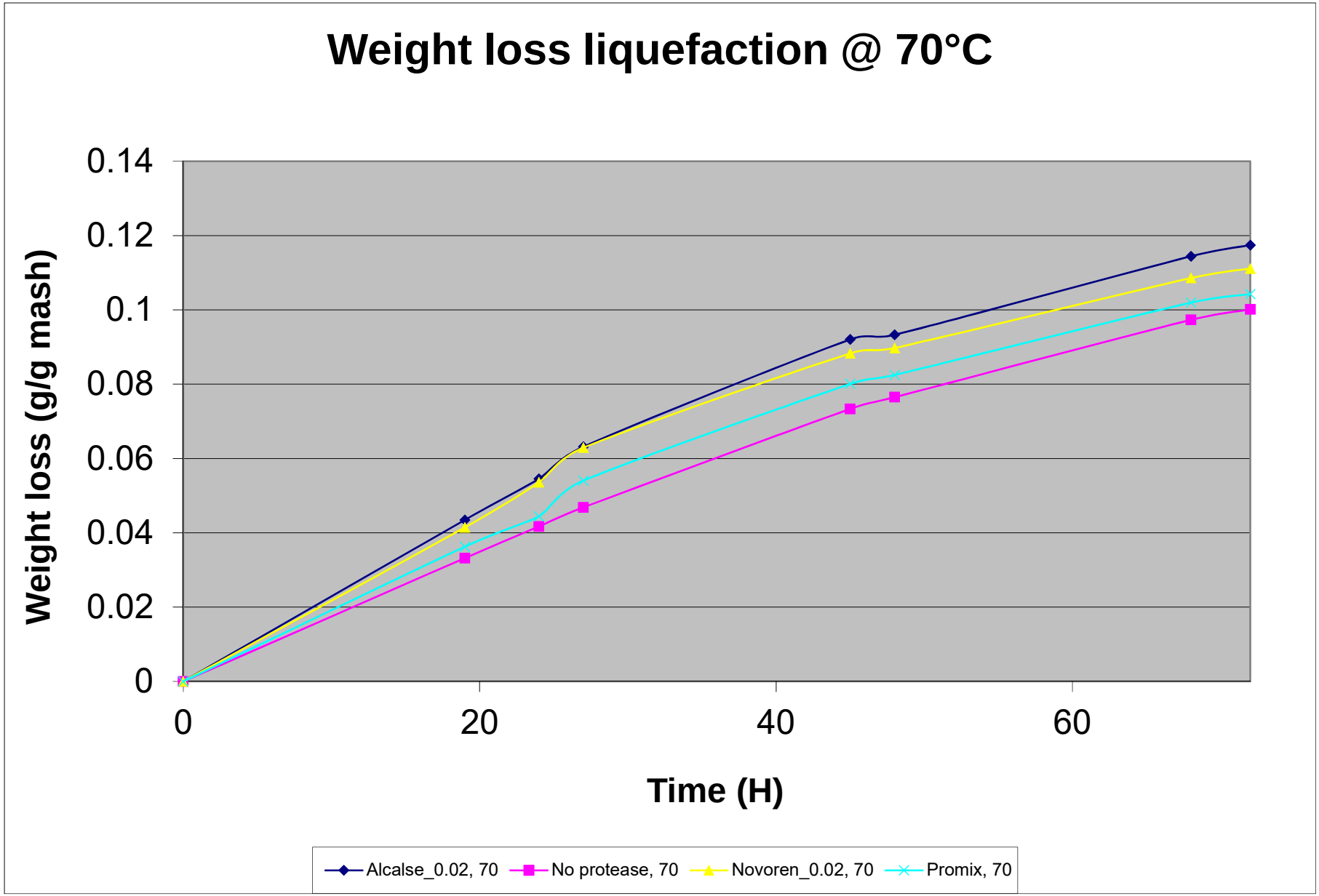
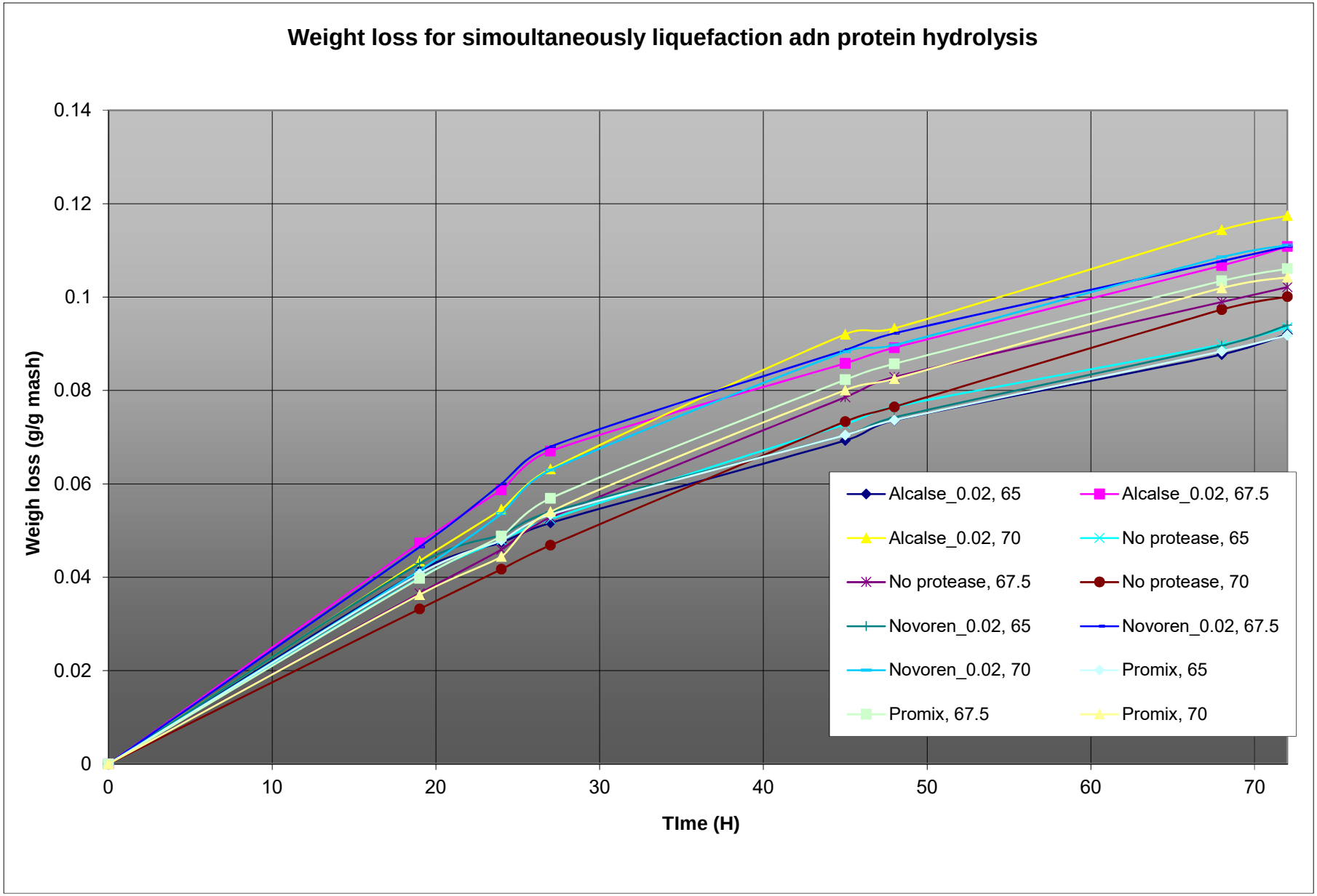




				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

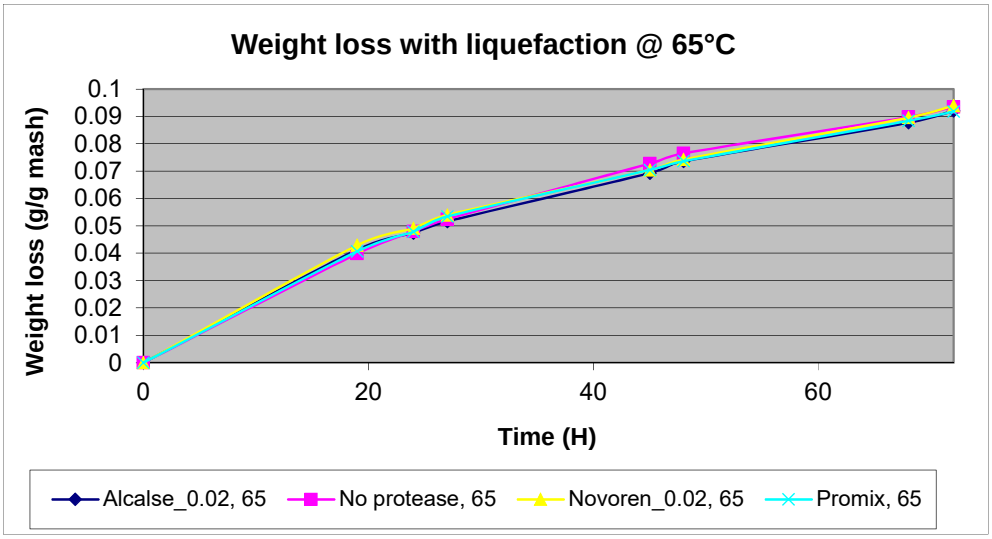
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

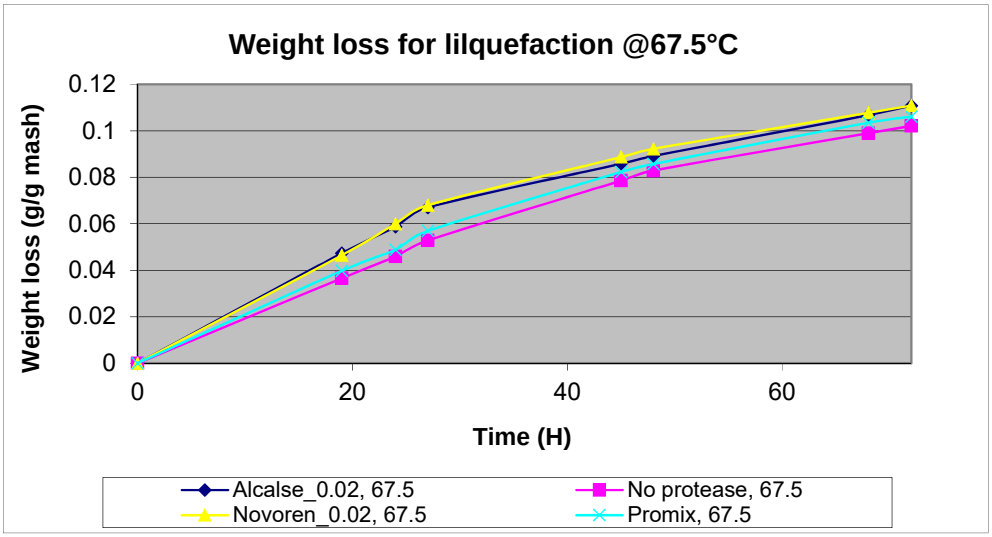
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

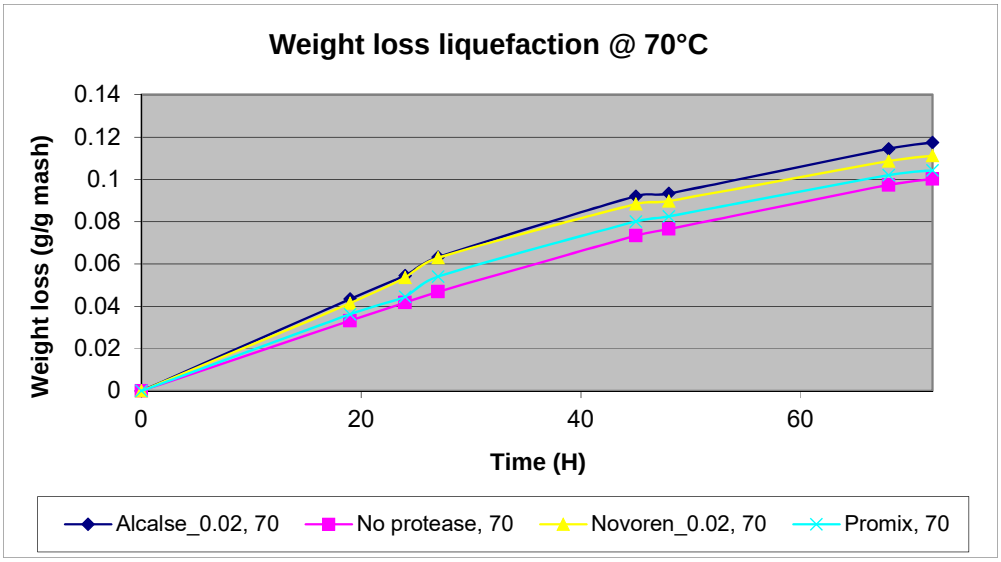
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







Application Technology – US Fuel Research
NL200589
LUNA: 2005-49534-03

To: HSO, KSW, RHeu, BJes, LWP, RDe,

From: RMF/JPlm

Simultaneous liquefaction and protein degradation at 65, 67.5, & 70°C for SSF to Fuel ethanol

Study # RMF050118

Experimental design:

- Simultaneously starch liquefaction and protein degradation has been carried out at 65, 67.5 and 70°C in a mini scale liquefaction set up using 15 ml tubes.
- Termamyl SC and Alcalase (Family S8), Novoren (Family A1) and ProMix (Novoren & Flavourzyme) have been evaluated for their effect when added during liquefaction.
- Background conditions for the liquefaction was pH=5.8 and DS=34.10% and 50 NU/g DS Termamyl SC and for SSF Spirizyme Fuel was added @ 0.5 AGU/g DS. No urea was added.

Conclusions:

- Alcalase was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalase was increased by increasing temperature. @ 70°C Alcalase enhances the ethanol yield by 6% compared to not having at protease added during liquefaction.
- Protease and temperature had significant impact on glycerol. Novoren and Alcalase lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Discussion:

- Alcalase had optimum performance @ 50°C when added at a post-liquefaction step, using this procedure liquefaction temperature can preferably be increased to 70°C.
- The pH is higher at liquefaction conditions compared to fermentation, and the protein matrix in corn flour could be stabilizing the peptidases as opposed to cooked corn mash where proteins are precipitated.

Purpose

To study the effect of adding an α-amylase (Termamyl SC), and a peptidase (Alcalase, Novoren, ProMix) at the same time during liquefaction carried out @ 65, 67.5, and 70°C. Starch liquefaction and protein degradation is thereby run simultaneously for SSF.

Background

Post liquefaction trials have been carried out with Alcalase, where the effect has been very temperature dependent. Alcalase showed best performance at 50°C, and the performance decreased significantly with increasing temperature. In this experiment peptidases are added to the raw mash, where starch, protein and other corn sources are intact and liquefaction and protein degradation is carried out in 15 ml test tubes. Having a simultaneous step of starch liquefaction and protein degradation may be interest to industry, as temperature pH could be beneficial to the peptidases we have available and thereby secure better protein hydrolysis and possible starch release from protein bound starch sources, thus obtain higher yeast efficiency during fermentation.

The factors to be tested in each of the factorial experiments are as follows:
Temperature: 65, 67.5, 70°C
Enzyme: Alcalase, Novoren, and ProMix and no Protease.

Background conditions similar for all treatments during SSF are pH=5, and Spirizyme fuel @ 0.5 AGU/g DS. During liquefaction Alcalase and Novoren was added at 0.02 mg EP/g DS, and ProMix at 0.03 mg Product/g DS (corresponding to the dosage in SSF).

Experimental

The experiment was carried out in 15 ml test tubes using approximately 5 g of corn mash (34.10 % DS) (exact weigh and dry matter content is known) and 5 replicates. Corn flour from Broin (EXOL) was used for the experiments. Subsequently the impact from the liquefaction was accessed by conducting a standard SSF fermentation.

Table 1. HPLC ethanol results after 24 and 48 H of fermentation

Treatment	AGU/g DS	NU/g DS	mg EP/g DS	mg EP/g DS	mg product/g DS
Spirizyme fuel	0.5	50			
Termamyl SC	0.5	50			
Alcalase 0.02	0.5	50	0.02		
Novoren	0.5	50		0.02	
Promix	0.5	50			0.03

Liquefaction Treatment
Corn flour was added water to obtain a DS fraction of 34.10% and pH was adjusted to 5.6 and samples were taken for DS determination. 5g of this mash was transferred to 15ml test tubes, which were liquefied for 1H after the corn mash reached the designated liquefaction temperature, which were 65, 67.5, and 70°C.

SSF Fermentation

Spirizyme fuel was added to all the tubes after liquefaction. Fermentations were carried out as SSF at 32°C, 70 hours using Red Star yeast. Yeast addition was aimed at $1*10^7$ cells/ml. All treatments were each run in 8 replicates and non-liquefied controls were included in the fermentation. The fermentations were monitored by weighing the individual tubes and recording the time & date of the measurement. At the end of fermentation tubes were sampled for HPLC analysis of sugars and fermentation products, primary parameters evaluated were ethanol and glycerol.

Results:

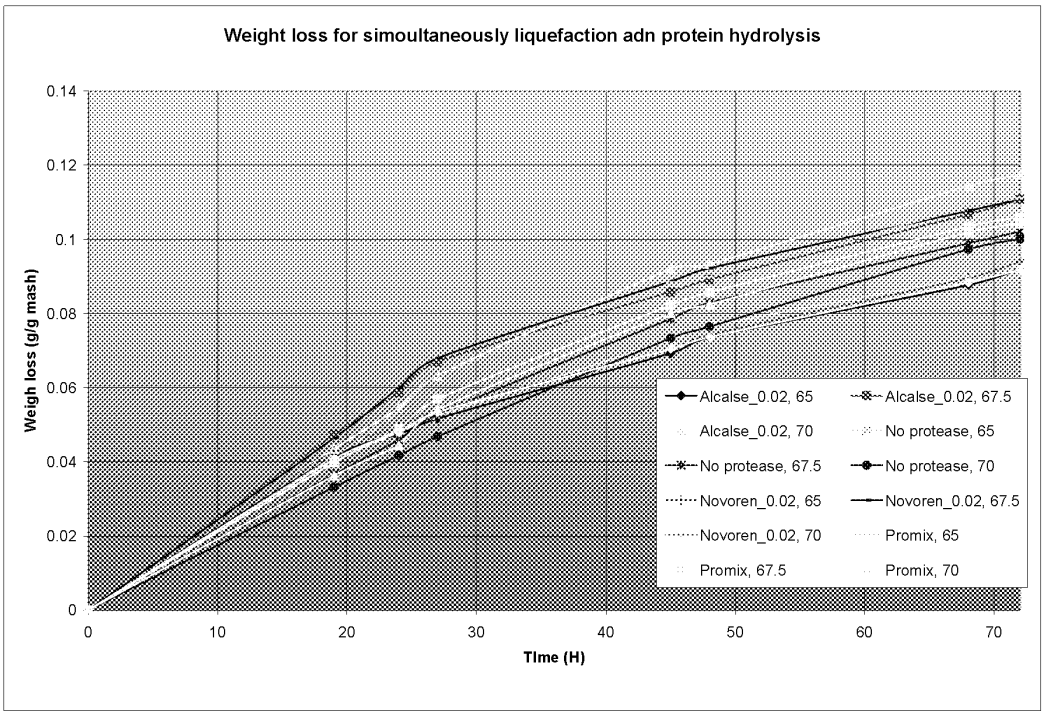
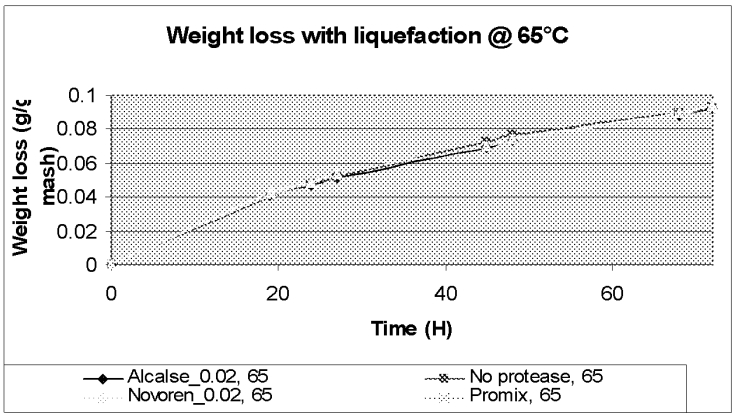
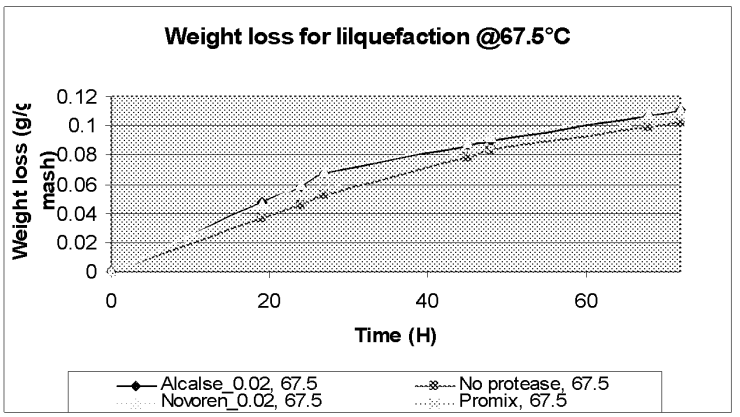


Figure 1. Weight loss for all samples as a function of fermentation time

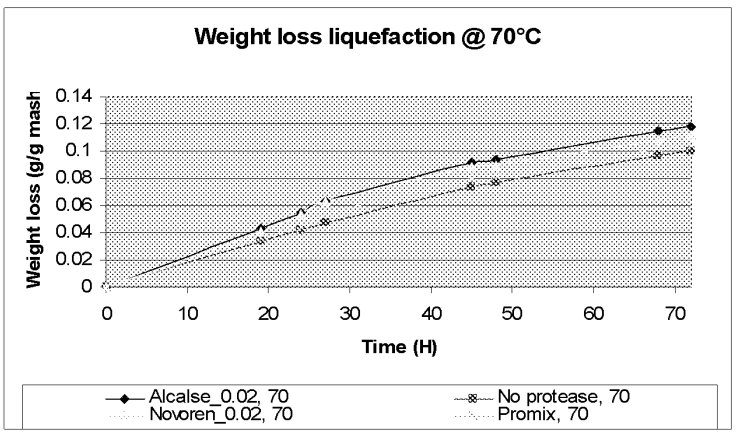
The weight loss curves are affected by the liquefaction temperatures as higher yeast growths and more ethanol is being produced in the high temperature liquefied samples. At the end of fermentation samples liquefied @ 70°C treated with Alcalase has considerably higher weight losses compared to the remaining treatments. The individual liquefaction temperatures are depicted below.



Very little difference between treatments at 65°



Differences in weight loss between Novoren and Alcalase to no Protease



Differences in weight loss pronounced particularly between Alcalase to no protease

Statically analysis of results:
Start Model for all response variables.
$Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Reduced models after response variable:
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model: **Y(glycerol)=**$I+\alpha(temp)+\beta(enzyme)+\varepsilon$
Model**: Y(gly/etoh)=** $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

Where
α=(temp)= 65, 67.5 & 70°C
β=(enzyme) = Alcalase (Alc), Novoren (Novo) , ProMix (PM) and No protease (NoP)
γ=(enzyme*temp)=(Alc*65, 67.5, 70°), (Novo*65, 67.5, 70°), (PM*65, 67.5, 70°), NoP*65, 67.5, 70°),

Ethanol:

Ethanol has been analyzed after 24 & 48H, and furthermore at the end of fermentation. Only one sample was used for 24 & 48 H analysis, whereas 6 replicates were used at the end of fermentation, thus statistical analysis has been performed on the end HPLC data.

Table 2. HPLC ethanol results after 24 and 48 H of fermentation

	Ethanol	
Treatment	**24 H**	**48H**
NoP, 65	0.147	0.188
Alc 0.02, 65	**0.151**	**0.197**
No 0.02, 65	**0.151**	**0.192**
PM 65	0.147	0.188
NoP, 67.5	0.142	0.222
Alc 0.02, 67.5	**0.178**	**0.228**
No 0.02, 67.5	**0.179**	**0.230**
PM 67.5	0.146	0.226
NoP, 70	0.138	0.217
Alc 0.02, 70	**0.178**	**0.250**
No 0.02, 70	0.168	0.243
PM 70	0.139	0.224

The HPLC results after 24 and 48H show that Alcalase performs very well in respect to ethanol yield. Increasing the liquefaction temperature result in higher ethanol yield, due to higher degree of gelatinization and liquefaction thereby starch accessibility, thus higher ethanol yields. Protease addition results in adding a little extra ethanol yield on top of the temperature effect, which is a result of protein degradation either by releasing more starch and or hydrolysis of protein to amino acids to enhance fermentation. Alcalase @ 0.02 mg EP/g DS liquefied at 70°C gives the over all highest ethanol yield.

The reduced model for ethanol is listed below. Factor analysis showed
Model: **Y(etoh)=** $I+\alpha(temp)+\beta(enzyme)+\varepsilon$

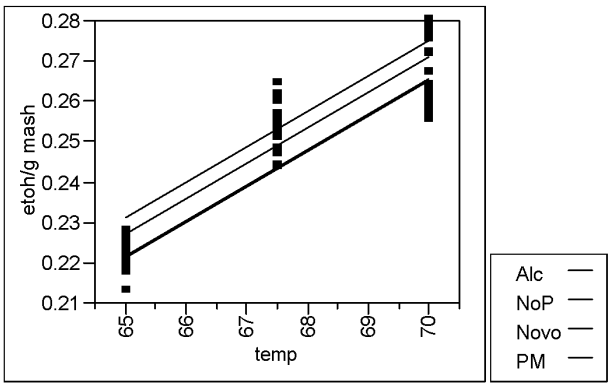
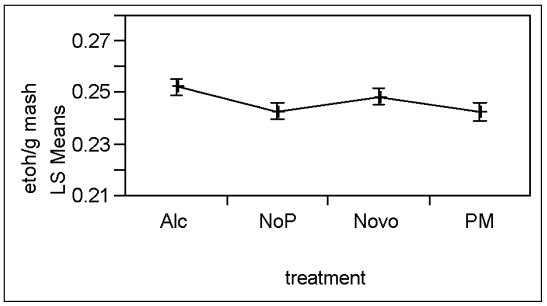


Figure 2. Response etoh/g mash, Whole Model. Regression Plot.

Ethanol increases by increasing liquefaction temperature, and fermentations with Alcalase reaches the highest level of ethanol. No Protease and ProMix performs equally poor. The thermo-stability of Flavourzyme in ProMix is not very high, as optimum is around 50°C, and the liquefaction temperatures in this trial is starting at 65°C.



Treatment		Least Sq Mean of ethanol (g/g mash)
Alc	A	0.252
Novoren	A B	0.248
NoP	B	0.243
PM	B	0.243

Figure 3.LS Means Plot for ethanol and levels of significance.

The LS means numbers show Alcalase has the highest impact on improving ethanol yield and below it is clear that Alcalase performs better for all tested temperatures.

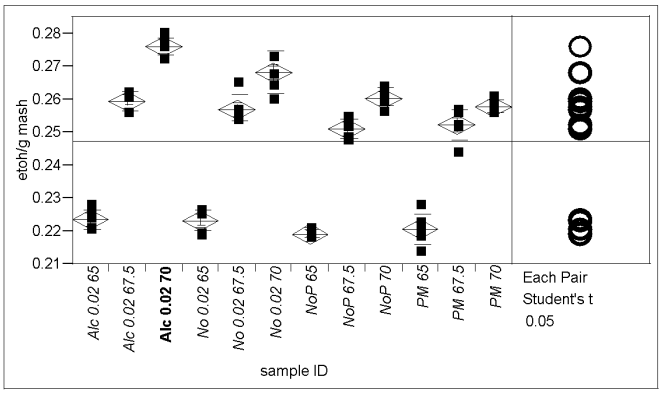


Figure 4: One-way Analysis of Ethanol Yield (g/g DS) by all treatments after 70H of fermentation.

At the end of fermentation Alcalase (0.02 mg EP/g DS at 70°C) demonstrate the best performance compared over temperature and treatment. The enzyme protein amount added from Novoren in ProMix is however considerably lower, as the total weight dose is only 0.03 mg Product/g DS, which is equivalent to ((0.03 mg/g DS*73%)/1000)*(0.15AU(RH)/g /22 AU(RH)g EP)=1.5*10-4 mg EP/g DS. This explains the poor performance of ProMix, but the dosage was included at this is the recommended start dose of ProMix added during SSF. ProMix is not as thermo-stable as Alcalase as the effect of increasing temperature is the same as for no protease, which is due to the lower thermo-stability of Flavourzyme in the product.

The table above confirms the observations and lists the significance levels, very clear effect of liquefaction temperature and Alcalase and Novoren. Below the leverage effect and prediction profiles are depicted. As can be seen there is linear increase in ethanol yield with increasing temperature, and that Alcalase gives higher ethanol yields compared to the other treatments.

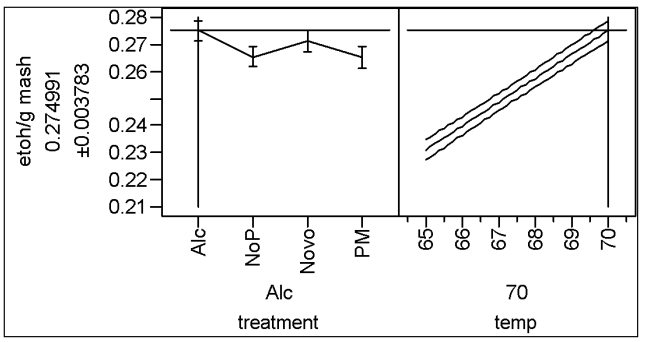


Figure 5. Prediction Profiles for treatment and temperature.

For any given temperature Alcalase will give the best ethanol yield, and increasing the temperature will ensure higher ethanol yields.

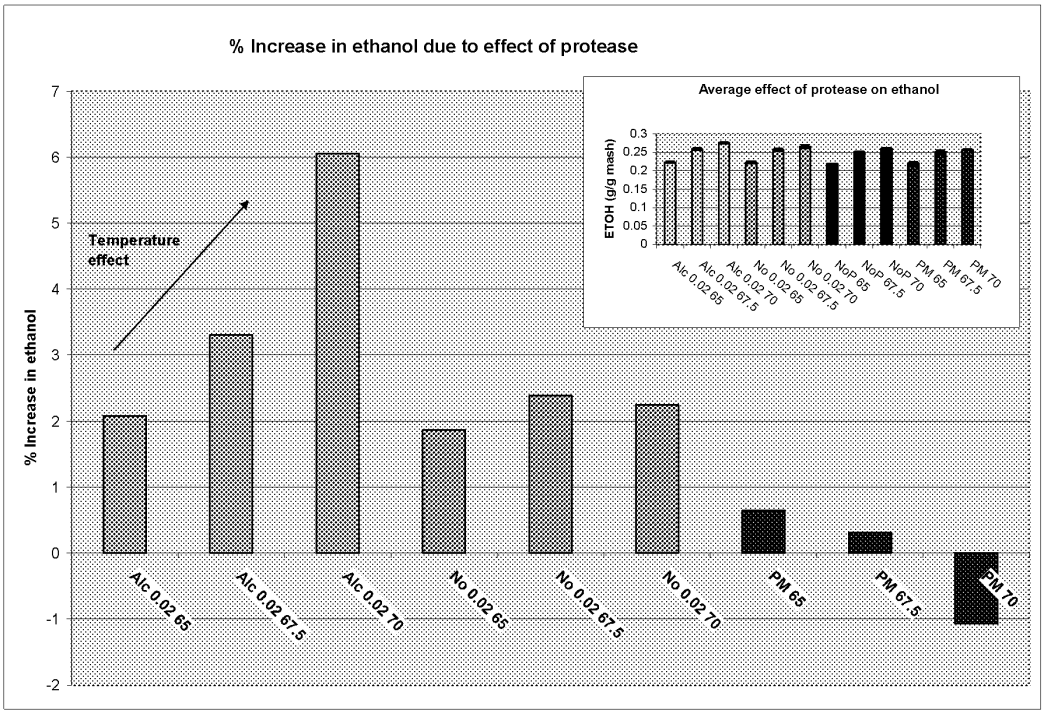


Figure 6. % increase in ethanol by treatment (enzyme and temperature) compared to no protease for every temperature.

In Figure 6 it is clear that Alcalase increases the ethanol yield up to 6% compared to not having a protease added (compared for every liquefaction temperature). Novoren also enhances the ethanol formation during SSF, but not to the same effect as Alcalase, and at 67.5°C Novoren reaches its maximum performance. ProMix on the other hand only increases the ethanol yield by 0.5% compared to n ot having a protease added, and there is absolutely no effect at 70°C. The poor performance of ProMix is very likely due to the low dosage in combination with low thermo-stability.

Sub conclusion:
Liquefaction time has significant impact on the ethanol yield, higher liquefaction temperature results in higher ethanol yields. The addition of protease improves the ethanol yield significantly at 67.5 and 70°C compared to not having a protease added. Alcalse was the best performing protease compared to Novoren and ProMix, and very interestingly the effect of Alcalse was increased by increasing temperature. @ 60°C Alcalse enhances the ethanol yield by 6% compared to not having at protease added during liquefaction. This has not been seen at the post liquefaction studies, where increasing temperature above 50°C has shown decreasing performance of Alcalse.
The pH is higher at liquefaction conditions, and also there could be a factor of substrate stabilization in corn flour as opposed to corn mash.

Glycerol Yield

Again glycerol was measured after 24 & 48 H of fermentation, and more thoroughly analyzed with 6 replicates at the end of fermentation

Table 3. HPLC glycerol results after 24 and 48 H of fermentation

	Glycerol	
Treatment	**24 H**	**48 H**
NoP, 65	0.027	0.010
Alc 0.02, 65	0.026	0.017
No 0.02, 65	0.024	0.012
PM 65	0.026	0.013
NoP, 67.5	0.026	0.015
Alc 0.02, 67.5	0.027	0.013
No 0.02, 67.5	0.027	0.015
PM 67.5	0.028	0.014
NoP, 70	0.027	0.013
Alc 0.02, 70	0.027	0.015
No 0.02, 70	0.026	0.014
PM 70	0.026	0.013

Formation of glycerol was in general very similar for the protease treatments, in fact similar to not having a protease added during liquefaction.

After reduction of insignificant effects in the start model, the model describing ethanol yield at the end of fermentation looks as follows:

Model: **Y(glycerol)**=I+α(temp)+β(enzyme) +ε

No significant effect of the interaction (temp:enzyme) was found, and the regression plot below illustrate this very well, as the enzyme have similar pattern in glycerol formation for very all temperatures.

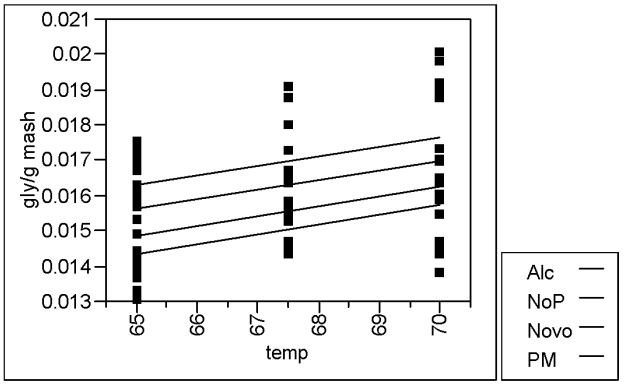
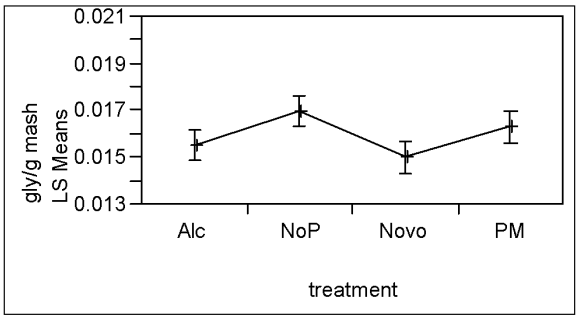


Figure 7. Response gly/g mash, Whole Model. Regression Plot.



Treatment		Least Sq Mean of glycerol (g/g mash)
NoP	A	0.0170
PM	A B	0.0163
Alc	B	0.0155
Novo	B	0.0150

Figure 8.LS Means Plot for glycerol and levels of significance.

Not having a protease added during liquefaction result in the highest formation of glycerol, and again Alcalase and Novoren show the best performance by lowering the glycerol the most.

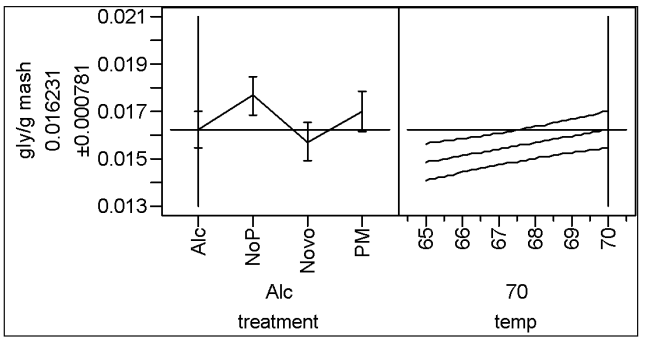


Figure 9: Prediction Profiles for response variable glycerol, for both treatment and temperature.

Temperature has the reverse effect on glycerol compared to ethanol, as increasing glycerol is being formed at higher temperature, indicating high yeast growth and more stress during SSF. Novoren secures the lowest glycerol yield however, not significantly different form Alcalase.

Sub conclusion:
Protease and temperature had significant impact on glycerol. Protease (particular Novoren) lowered the glycerol formation, whereas increasing liquefaction temperature resulted in higher glycerol formation.

Glycerol/Ethanol – Rato

The reduced model for glycerol/ethanol relationship ended up being:
Model**: Y(gly/etoh)**= $Y=I+\alpha(temp)+\beta(enzyme)+\delta(enzyme*temp)+\varepsilon$

The model varied from the previous response variables, as gly/etoh showed significant effect of the interaction between temperature:enzyme.

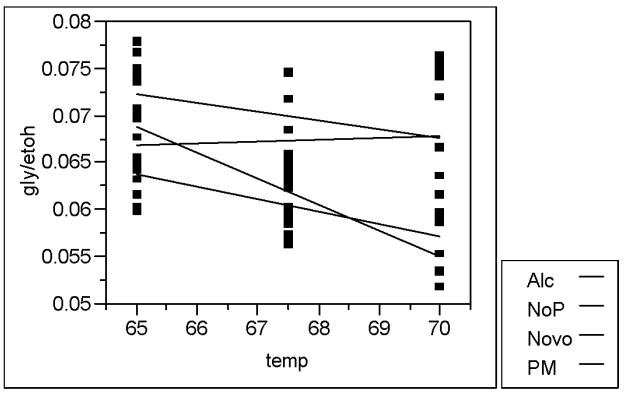
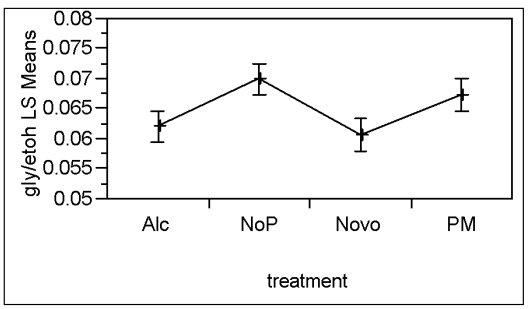


Figure 10. Response gly/etoh, Whole Model. Regression Plot.

The interaction between temperature and enzyme is very clear in the regression plot above, as the lines are not parallel, indicating different behaviors for each temperature. The different pattern is primary due to ProMix not showing increased ethanol at increasing temperature and decreasing glycerol, which is the picture for Alcalase and Novoren. The gly/etoh ratio with ProMix is the same at the different liquefaction temperatures, which is different from Alcalase and Novoren.



Treatment		Least Sq Mean of gly/etoh (g/g mash)
NoP	A	0.070
PM	A	0.067
Alc	B	0.062
Novo	B	0.061

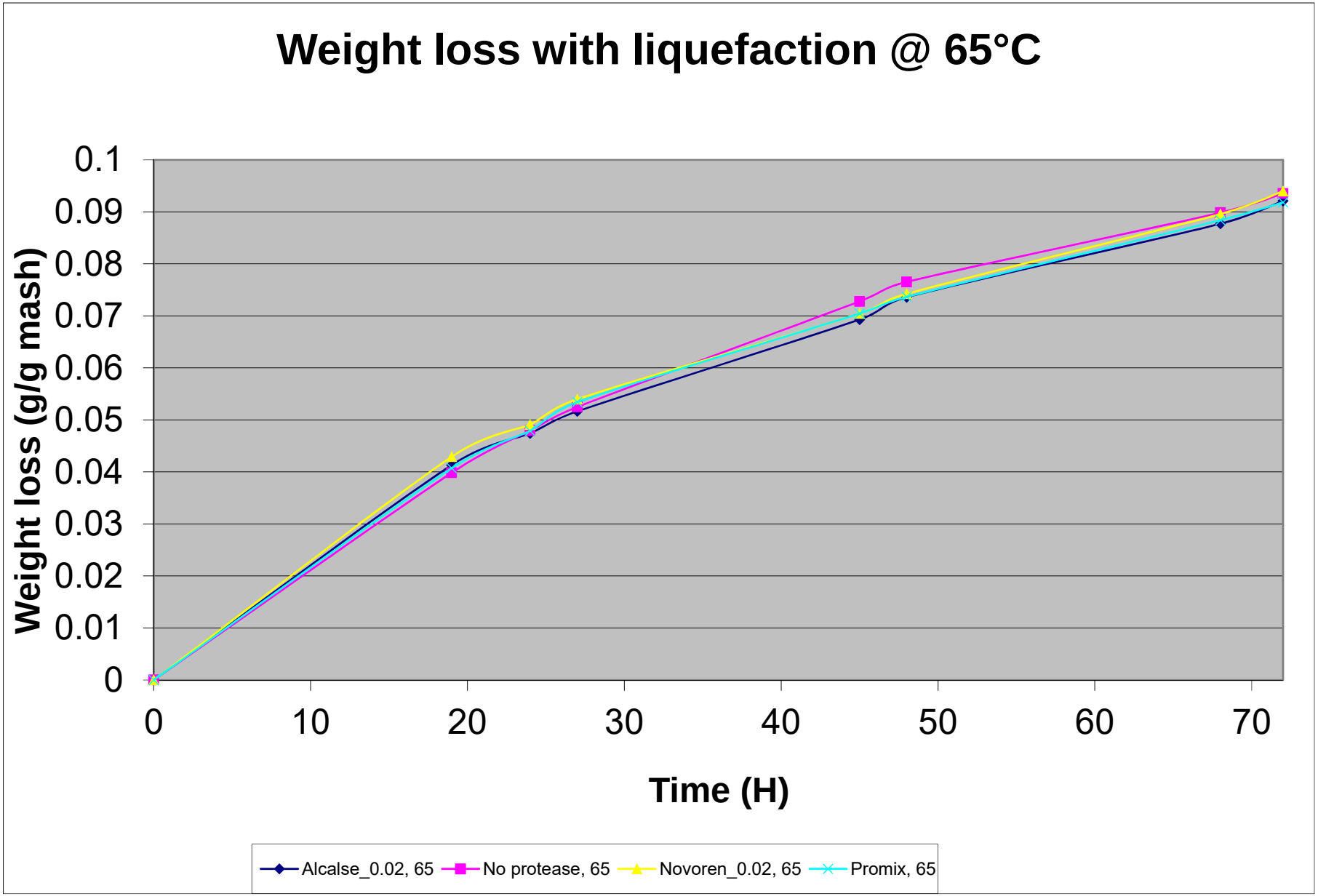
Figure 11. LS Means Plot for glycerol and levels of significance.

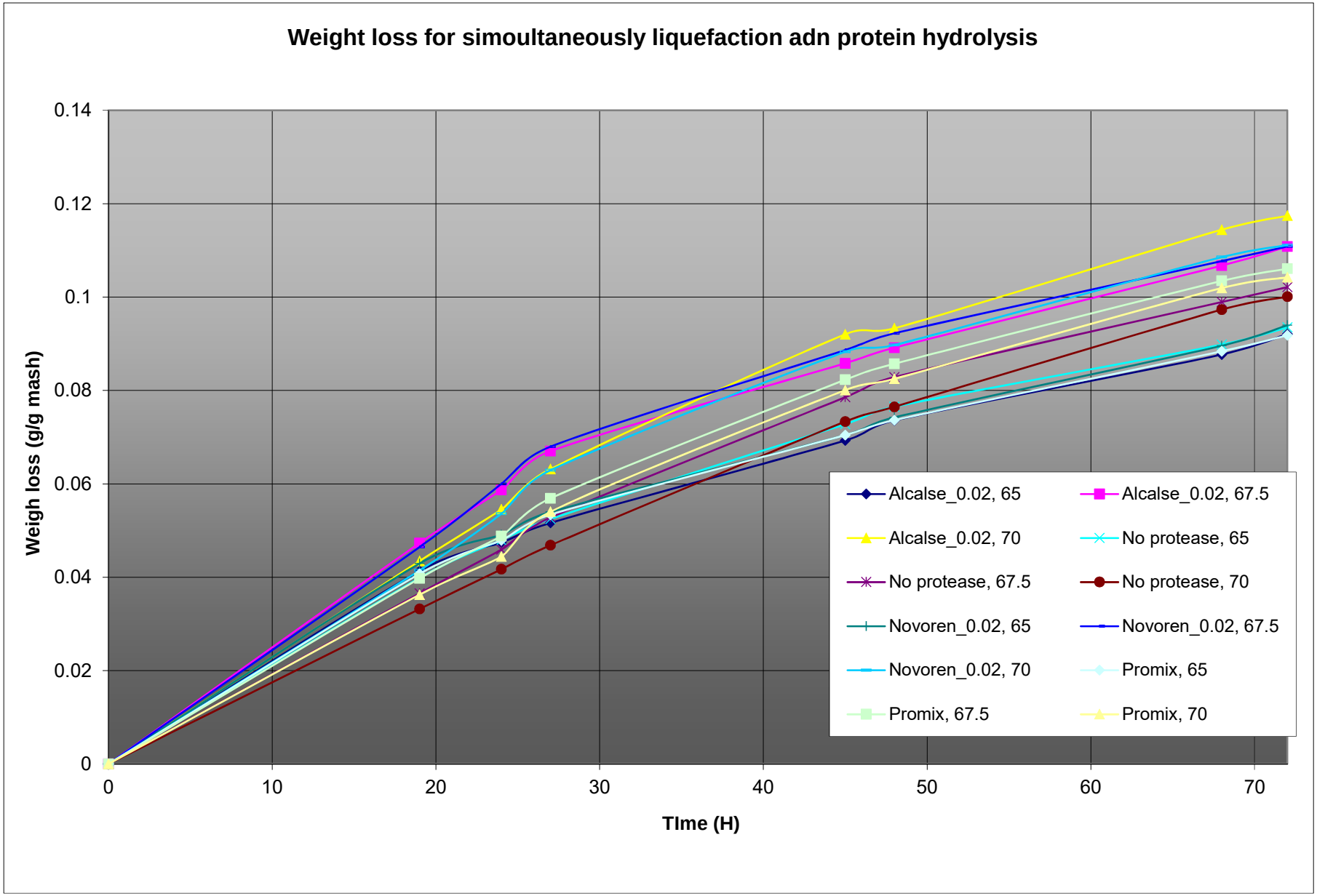
The highest ratio of glycerol being produced per g of ethanol is in the control samples, with no protease. The enzymes behave different at increasing temperatures due to thermo-stability, and due to the impact they have on ethanol yield.

Sub Conclusion:
The gly/etoh ratio was lowest for Alcalase and Novoren treated samples, and these were significantly lower than fermentations with ProMix or no protease.

Reference: RMF050118

Document Produced Natively





				mean	stdev	cv
.	.	4140	.		.	.
Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
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Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

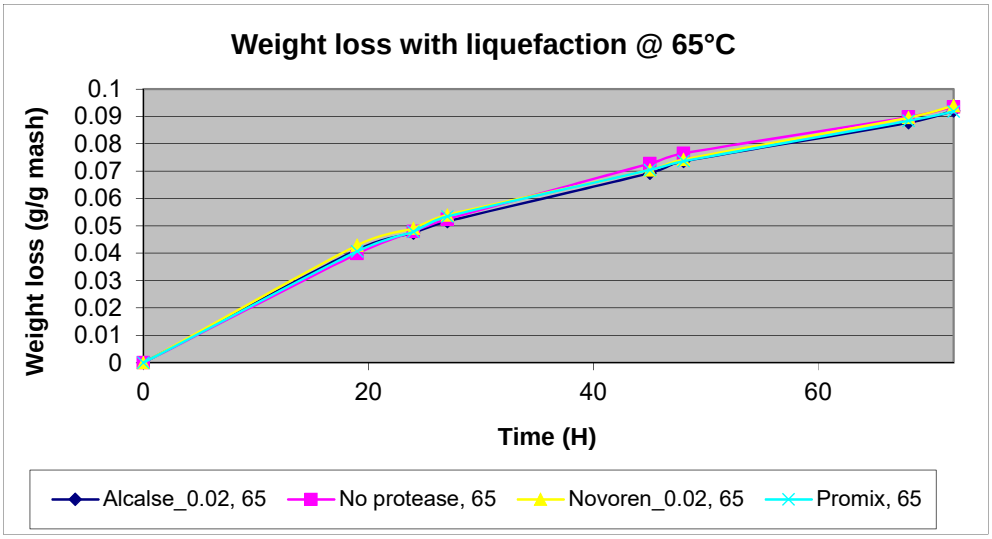
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842

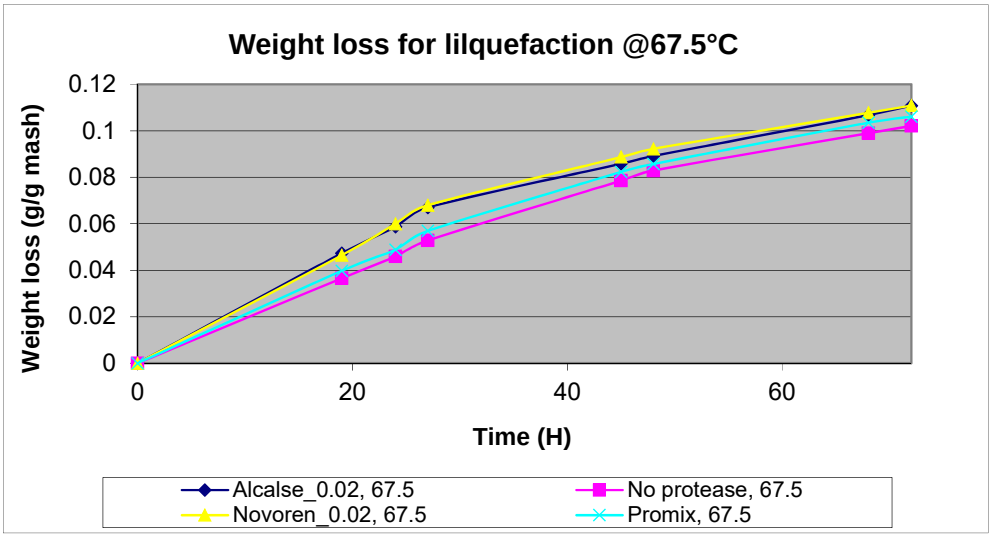
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

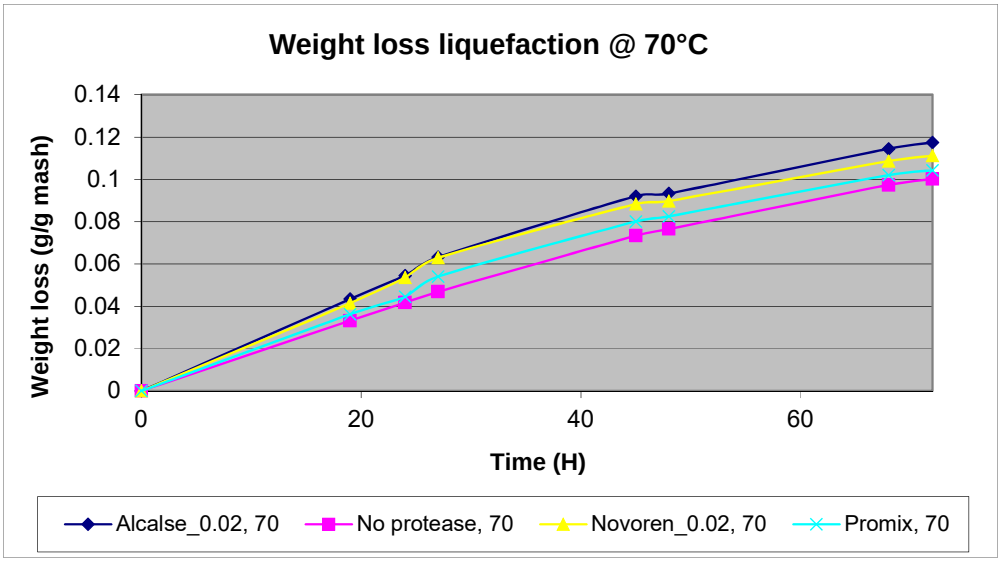
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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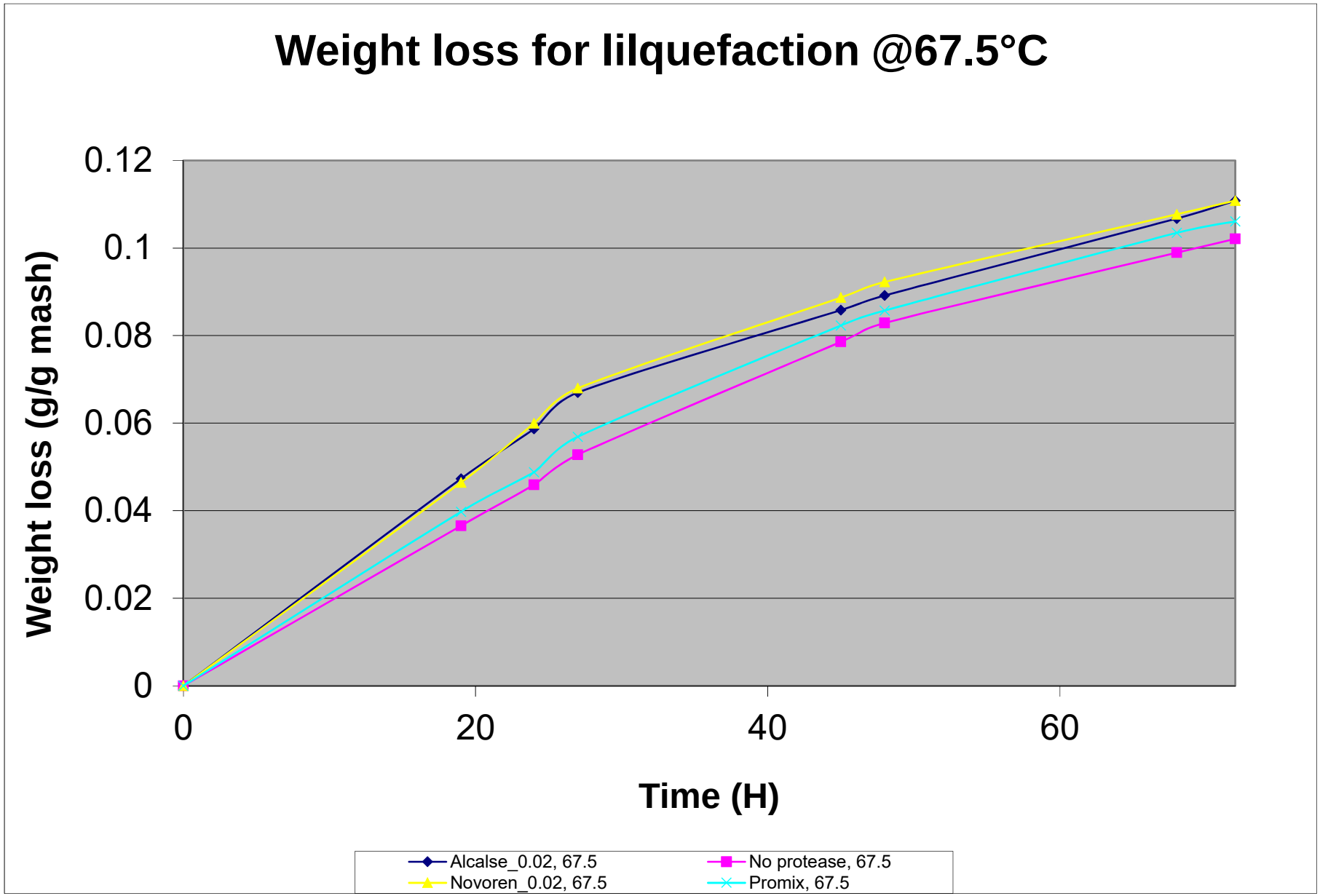

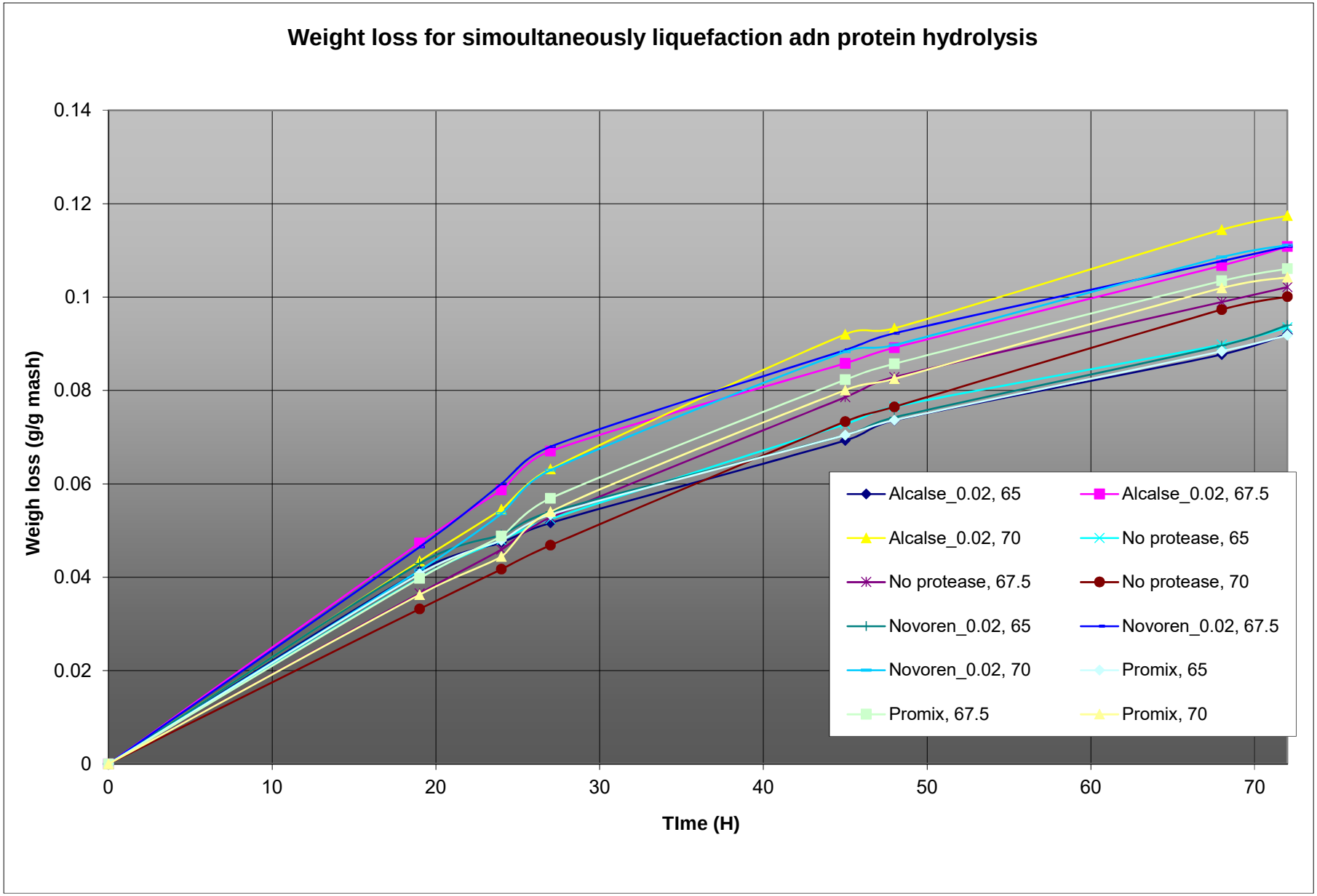

				mean	stdev	cv
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Alcalse_0.02	65	19	8	0.041331	0.005818	14.1
Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

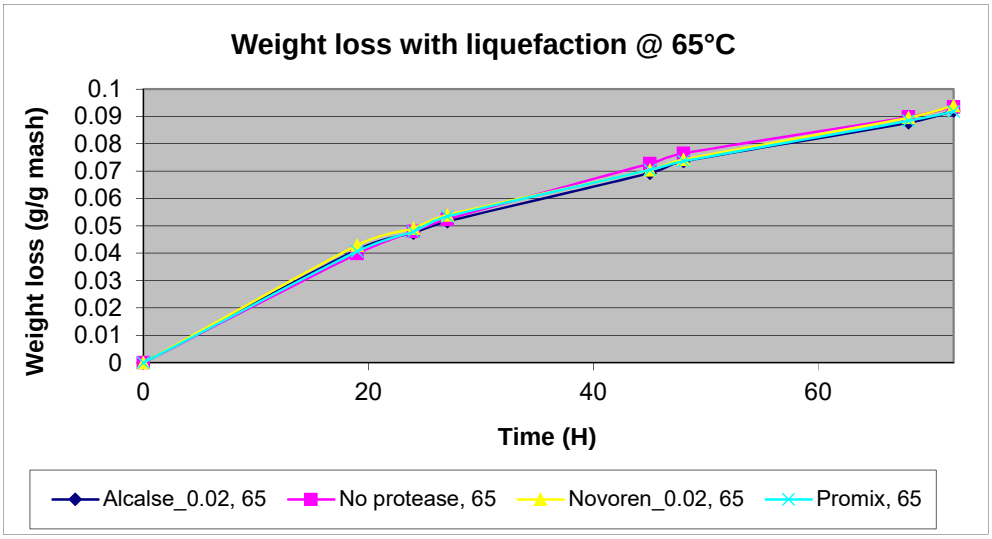
				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

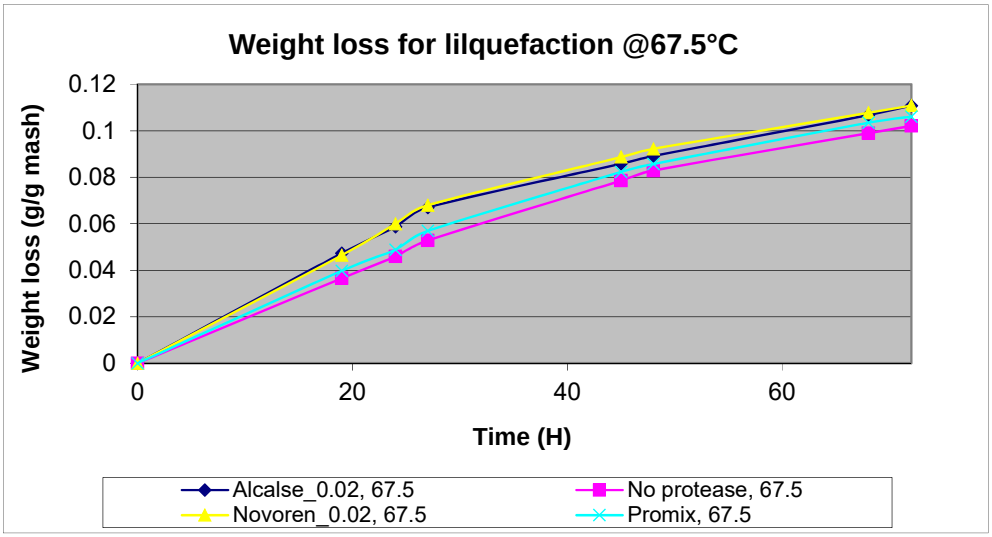
	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

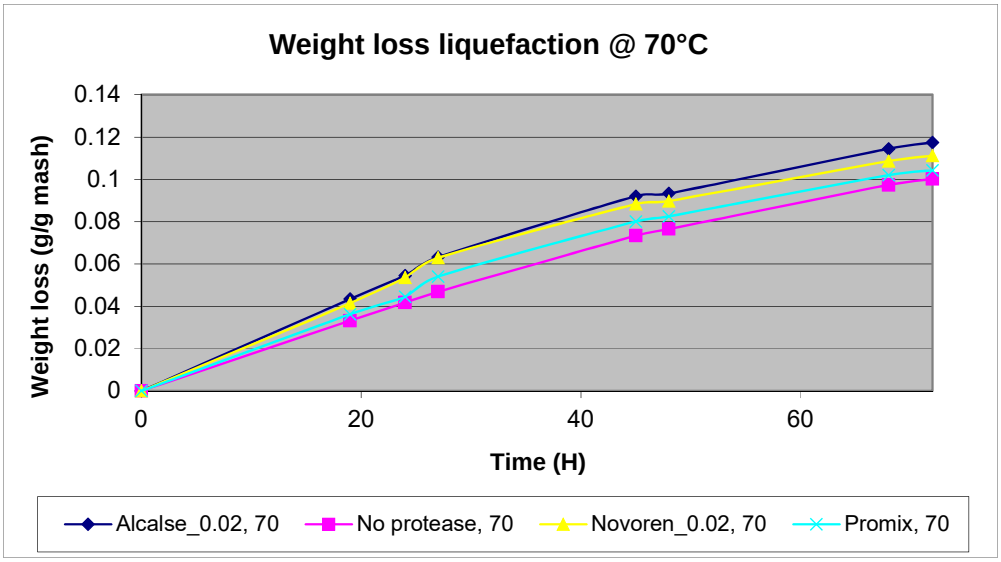
65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236







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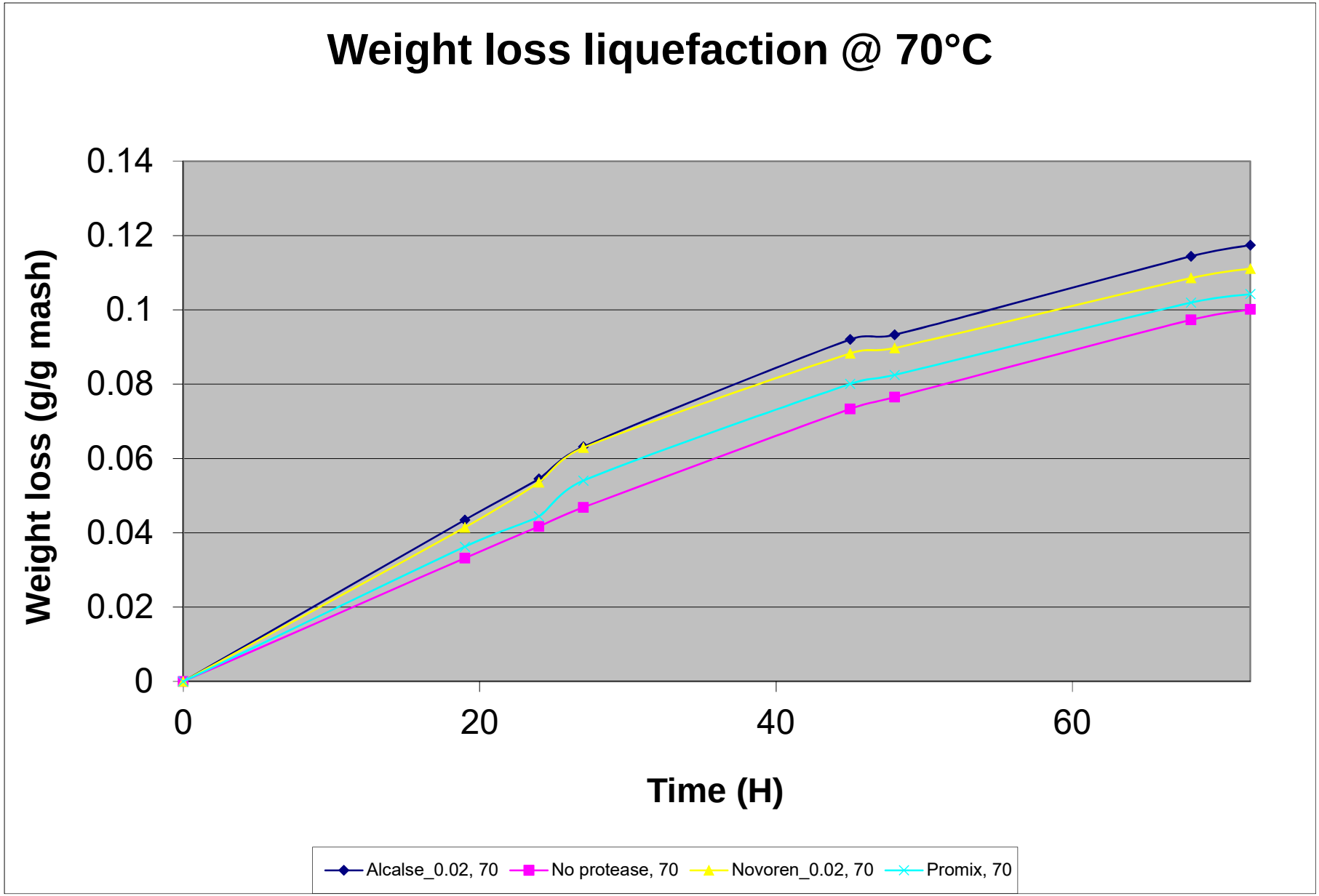

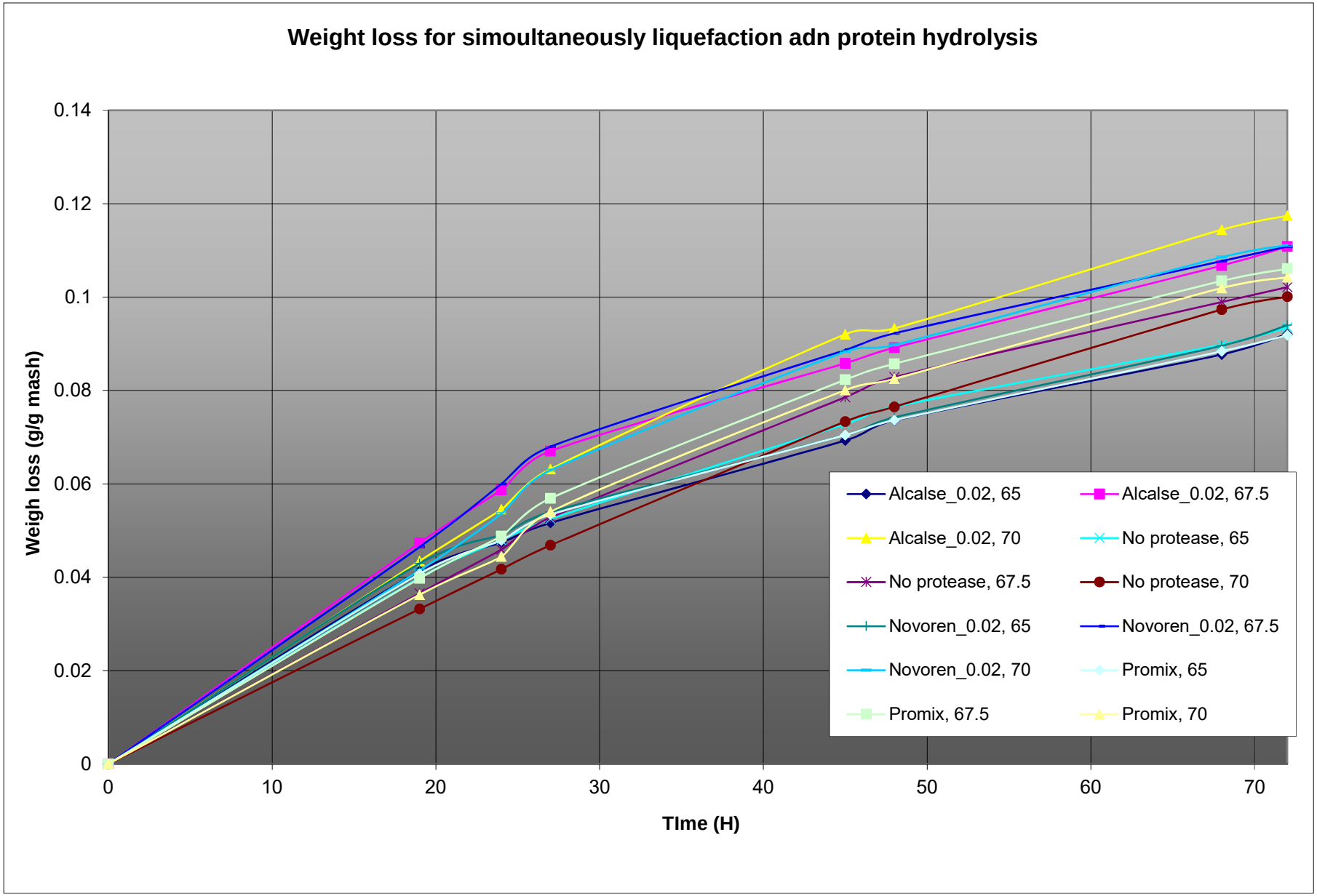

				mean	stdev	cv
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Alcalse_0.02	65	24	8	0.0474	0.002299	4.9
Alcalse_0.02	67.5	19	8	0.047312	0.003802	8.0
Alcalse_0.02	67.5	24	8	0.058668	0.002064	3.5
Alcalse_0.02	70	19	8	0.043466	0.005268	12.1
Alcalse_0.02	70	24	8	0.054523	0.001935	3.5
No protease	65	19	8	0.039852	0.005608	14.1
No protease	65	24	8	0.047938	0.00188	3.9
No protease	67.5	19	8	0.036551	0.003076	8.4
No protease	67.5	24	8	0.045922	0.001178	2.6
No protease	70	19	8	0.033205	0.005652	17.0
No protease	70	24	8	0.041713	0.003324	8.0
Novoren_0.02	65	19	8	0.04293	0.005345	12.5
Novoren_0.02	65	24	8	0.049104	0.001826	3.7
Novoren_0.02	67.5	19	8	0.04645	0.003795	8.2
Novoren_0.02	67.5	24	8	0.059991	0.003533	5.9
Novoren_0.02	70	19	8	0.04146	0.006098	14.7
Novoren_0.02	70	24	8	0.053584	0.003201	6.0
Promix	65	19	8	0.040715	0.003742	9.2
Promix	65	24	8	0.047895	0.002224	4.6
Promix	67.5	19	8	0.03975	0.006964	17.5
Promix	67.5	24	8	0.048816	0.003673	7.5
Promix	70	19	8	0.03626	0.006008	16.6
Promix	70	24	8	0.044433	0.001893	4.3

				mean	stdev	cv
Alcalse_0.02	65	27	7	0.051647	0.002476	4.794277
Alcalse_0.02	65	45	7	0.069285	0.003603	5.200367
Alcalse_0.02	67.5	27	7	0.067016	0.002517	3.756257
Alcalse_0.02	67.5	45	7	0.085824	0.003544	4.1299
Alcalse_0.02	70	27	7	0.063182	0.002218	3.510299
Alcalse_0.02	70	45	7	0.09204	0.003676	3.994156
No protease	65	27	7	0.052478	0.00369	7.031789
No protease	65	45	7	0.072782	0.006553	9.003686
No protease	67.5	27	7	0.05279	0.001329	2.51664
No protease	67.5	45	7	0.078564	0.001967	2.503658
No protease	70	27	7	0.046848	0.002494	5.323616
No protease	70	45	7	0.073353	0.004362	5.946012
Novoren_0.02	65	27	7	0.053994	0.002872	5.318509
Novoren_0.02	65	45	7	0.070437	0.00302	4.28756
Novoren_0.02	67.5	27	7	0.067976	0.004863	7.153422
Novoren_0.02	67.5	45	7	0.088684	0.005937	6.694886
Novoren_0.02	70	27	7	0.062928	0.004155	6.603506
Novoren_0.02	70	45	7	0.088294	0.005227	5.920368
Promix	65	27	7	0.053422	0.002331	4.363806
Promix	65	45	7	0.070452	0.003502	4.970663
Promix	67.5	27	7	0.0569	0.004674	8.214751
Promix	67.5	45	7	0.082306	0.006375	7.745133
Promix	70	27	7	0.054059	0.002326	4.302213

Promix	70	45	7	0.080073	0.003723	4.649016

				mean	stdev	cv
Alcalse_0.02	65	48	7	0.073589	0.004403	5.983606
Alcalse_0.02	65	68	6	0.087711	0.006618	7.545554
Alcalse_0.02	67.5	48	7	0.089162	0.00408	4.576296
Alcalse_0.02	67.5	68	6	0.106753	0.005245	4.913334
Alcalse_0.02	70	48	7	0.093307	0.00427	4.576362
Alcalse_0.02	70	68	6	0.114439	0.004853	4.241027
No protease	65	48	7	0.076531	0.006759	8.831576
No protease	65	68	6	0.089835	0.004277	4.760926
No protease	67.5	48	7	0.082879	0.001862	2.247051
No protease	67.5	68	6	0.098947	0.002312	2.336248
No protease	70	48	7	0.076499	0.003985	5.20946
No protease	70	68	6	0.09733	0.004682	4.81029
Novoren_0.02	65	48	7	0.074236	0.003143	4.233917
Novoren_0.02	65	68	6	0.089577	0.004218	4.709259
Novoren_0.02	67.5	48	7	0.092244	0.006219	6.74211
Novoren_0.02	67.5	68	6	0.107715	0.00615	5.709601
Novoren_0.02	70	48	7	0.08974	0.005798	6.461027
Novoren_0.02	70	68	6	0.108571	0.007454	6.865462
Promix	65	48	7	0.073649	0.004058	5.510476
Promix	65	68	6	0.088399	0.003837	4.340601
Promix	67.5	48	7	0.085717	0.006662	7.771797
Promix	67.5	68	6	0.103474	0.00819	7.915296
Promix	70	48	7	0.082492	0.003638	4.41003
Promix	70	68	6	0.101955	0.00279	2.736027

				mean	stdev	cv
Alcalse_0.02	65	72	6	0.092094	0.006514	7.073188
Alcalse_0.02	67.5	72	6	0.110813	0.005519	4.980348
Alcalse_0.02	70	72	6	0.117437	0.00505	4.299858
No protease	65	72	6	0.093508	0.004256	4.551979
No protease	67.5	72	6	0.102129	0.002668	2.612625
No protease	70	72	6	0.100103	0.004718	4.71299
Novoren_0.02	65	72	6	0.093967	0.005156	5.486863
Novoren_0.02	67.5	72	6	0.11085	0.006205	5.597967
Novoren_0.02	70	72	6	0.111125	0.007995	7.19469
Promix	65	72	6	0.091818	0.003952	4.304201
Promix	67.5	72	6	0.106083	0.008317	7.839842
Promix	70	72	6	0.104236	0.002801	2.687611

	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236

65	0	19	24	27	45	48	68	72
Alcalse_0.02, 65	0	0.041331	0.0474	0.051647	0.069285	0.073589	0.087711	0.092094
No protease, 65	0	0.039852	0.047938	0.052478	0.072782	0.076531	0.089835	0.093508
Novoren_0.02, 65	0	0.04293	0.049104	0.053994	0.070437	0.074236	0.089577	0.093967
Promix, 65	0	0.040715	0.047895	0.053422	0.070452	0.073649	0.088399	0.091818

67.5	0	19	24	27	45	48	68	72
Alcalse_0.02, 67.5	0	0.047312	0.058668	0.067016	0.085824	0.089162	0.106753	0.110813
No protease, 67.5	0	0.036551	0.045922	0.05279	0.078564	0.082879	0.098947	0.102129
Novoren_0.02, 67.5	0	0.04645	0.059991	0.067976	0.088684	0.092244	0.107715	0.11085
Promix, 67.5	0	0.03975	0.048816	0.0569	0.082306	0.085717	0.103474	0.106083

70	0	19	24	27	45	48	68	72
Alcalse_0.02, 70	0	0.043466	0.054523	0.063182	0.09204	0.093307	0.114439	0.117437
No protease, 70	0	0.033205	0.041713	0.046848	0.073353	0.076499	0.09733	0.100103
Novoren_0.02, 70	0	0.04146	0.053584	0.062928	0.088294	0.08974	0.108571	0.111125
Promix, 70	0	0.03626	0.044433	0.054059	0.080073	0.082492	0.101955	0.104236