Journal of Engineering and Natural Sciences
Mühendislik ve Fen Bilimleri Dergisi

Sigma
2004/2

CORN, RICE AND WHEAT STARCH HYDROLYSIS BY USING VARIOUS ALPHA-AMYLASE ENZYMES AT TEMPERATURE 40^{O}C

Dilek KILIÇ APAR, Belma ÖZBEK*

Yıldız Technical University, Department of Chemical Engineering, Davutpaşa-/ISTANBUL

Geliş/Received: 27.06.2003 Kabul/Accepted: 14.04.2004

40^{O}C SICAKLIKTA MISIR, PİRİNÇ VE BUĞDAY NİŞASTALARININ ÇEŞİTLİ ALFA-AMİLAZ ENZİMLERİ KULLANILARAK HİDROLİZİ

ÖZET

Bu çalışmada; 40^{O}C sıcaklıkta mısır, pirinç ve buğday nişastalarının çeşitli alfa-amilaz enzimleri kullanılarak hidrolizleri incelenmiştir. Nişasta hidroliz deneyleri için kullanılan üç ticari alfa-amilaz enzimleri sırası ile *Bacillus species, Aspergillus oryzae* and *Bacillus licheniformis* mikroorganizmalarından üretilmiş olup, Sigma şirketinden temin edilmiştir (Ürün Kodları sırası ile: A6814, A6211 and A3403). Herbir nişasta hidroliz prosesi için, karıştırmalı kesikli bir reaktörde, kalan nişasta konsantrasyonu ve alfa-amilaz enzim aktivitesi (%) zamanın bir fonksiyonu olarak incelenmiştir. Daha sonra, herbir nişasta için kalan nişasta konsantrasyon değerlerine uyan matematiksel ifadeler geliştirilmiştir. Ayrıca, nişasta hidrolizi prosesinde kullanılan herbir enzim için, enzim stabilitesi ve proses zamanı arasındaki bağıntıyı incelemek üzere literatürde var olan bazı inaktivasyon modelleri test edilmiştir.
Anahtar Sözcükler: Mısır, pirinç ve buğday nişastası hidrolizi, alfa-amilaz enzimleri, inaktivasyon, proses zamanı, matematiksel modelleme, karıştırmalı kesikli reaktör

ABSTRACT

The work reported here investigates the effects of various alpha-amylase enzymes on the hydrolysis of corn, rice and wheat starch at temperature 40^{O}C. Three commercial alpha-amylase enzymes which are produced from *Bacillus species, Aspergillus oryzae* and *Bacillus licheniformis* (obtained from Sigma Company, Product Code: A6814, A6211 and A3403, respectively) were used for the starch hydrolysis experiments. For each starch hydrolysis process, the residual starch concentration and the residual alpha-amylase enzyme activity (%) were investigated depending on the processing time in a stirred batch reactor. Then, the mathematical models are derived by using the experimental data of residual concentration for each starch used. Some inactivation models were tested for understanding the relation between the temperature and enzyme stability during hydrolysis process for each enzyme used.
Keywords: Corn, rice and wheat starch hydrolysis, alpha-amylase enzymes, inactivation, processing time, mathematical modelling, stirred batch bioreactor

1. INTRODUCTION

The rapid enhancement of many biotechnology processes has been severely constrained by enzyme deactivation. An improved knowledge of enzyme deactivation would significantly

* Sorumlu Yazar/Corresponding Autor: e-mail: bozbek@yildiz.edu.tr ; tel: (0212) 449 1731

enhance the feasibility of quite a few biotechnological processes. Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. A large scale processing industry has emerged in the last century. In the past decades, it was seen a shift from the acid hydrolysis of starch to the use of starch converting enzymes in the production of maltodextrin modified starches, or glucose and fructose syrups. Currently, these enzymes comprise about 30% of the world's enzyme production. Besides the use in starch hydrolysis, starch-converting enzymes are also used in a number of other industrial applications, such as laundry and porcelain detergents or as anti-staling agents in baking. A number of these starch-converting enzymes belong to a single family: the alpha-amylase family or family 13 glycosyl hydrolases [1-3]. So, alpha-amylase enzyme is known to effectively attack both insoluble starch and starch granules held in aqueous suspension. Before designing a successful hydrolysis system, information is required describing phenomena which affect the kinetics of starch hydrolysis such as temperature, pH, enzyme concentration and etc. [1-9].

In the present study, the effect of processing time on maximal efficiency of active alpha-amylase enzyme is investigated using a stirred batch reactor system. Alpha-amylase enzymes used are obtained from Sigma Company, Product Code: A6814, A6211 and A3403), which are produced from *Bacillus species, Aspergillus oryzae and Bacillus licheniformis,* respectively. The effect of processing time for alpha-amylase hydrolysing wheat, rice and corn starch at temperature value of $40^{O}C$ are presented by investigating the residual starch concentration and residual alpha-amylase enzyme activity. Then, the mathematical models, which represent the residual starch concentration and residual alpha-amylase enzyme activity as a function of the processing time, were developed according to the data obtained from the experiments.

2. MATERIALS AND METHODS

2.1. Bioreactor

The Gallenkamp Modular Bioreactor system (Model No: FER-195-010, manufactured by Sanyo Gallenkamp PLC, Loughborough) was used for the starch hydrolysis experiments. The controls of various parameters such as impeller speed, pH and temperature were performed by its modules. The 1.0 L vessel (round bottom design) was constructed of glass and stainless steel with an aspect ratio (height/diameter) of 1.545. The important design details were as follows: operating volume, 0.5 L; internal diameter, 11 cm; height, 17 cm; number of baffles, 4; baffle height, 13.5 cm; baffle width, 1.5 cm, number of impellers, 1; location of impeller from top plate, 14 cm; location of impeller from bottom plate, 3 cm; type of impeller, Rushton disc turbine; impeller diameter of disc, 4.8 cm; impeller blade width, 1.4 cm; impeller blade length, 1.9 cm; number of blades, 6. The proportionality of diameter of impeller to diameter of tank (D/T) is 0.436.

2.2. Determination of the Residual Starch Concentration

For determination of the residual starch concentration in the reaction solution [10], the samples were taken at certain time intervals. 5 ml of Iodine solution (0.5% KI and 0.15% I_2) and known volumes of the samples were mixed. The final volume was completed to 15 ml by addition of distilled water. The absorbency was measured at 550 nm against blank containing 5 ml of iodine solution and 10 ml of distilled water. Absorbencies were converted to starch concentration using the calibration chart prepared. At least 5 measurements were made for each condition and the data given is an average of these results.

2.3. Determination of the Alpha-amylase Enzyme Activity

The method used to determine the alpha-amylase activity of the samples was described by De Moraes et al. [11]. According to this method; 0.2 g of soluble starch was dissolved in 100 ml boiling 50 mM sodium acetate buffer (pH 5.9). The solution was cooled to 40^{O}C. 200 µI of the appropriately diluted enzyme solution was added to 1 ml of starch solution and the mixture was incubated at 40^{0}C in a water bath for 10 minutes. Iodine reagent was prepared by addition of 1 ml stock solution (0.5% I_2 in 5% KI) and 5 ml 5 M HCl to 0.5 litre distilled water. 200 µI incubated reaction mixture was added to 5 ml of iodine solution to stop the reaction. The degradation of starch by the enzyme was measured at 620 nm against 200 µI water in 5 ml of iodine solution as blank. At least 5 measurements were made for each condition and the data given is an average of these results. An original sample was kept without processed for a control activity measurement.

3. COMPUTATIONAL WORK

The software package MATLAB 5.0 was used in the numerical calculations. The parameters were evaluated by the nonlinear least squares method of Marquardt-Levenberg until minimal error was achieved between experimental and calculated values. The residual (SSR) is defined as the sum of the squares of the differences between experimental and calculated data and is given by

$$SSR= \sum_{m=1}^{Nd}(C^{obs}{}_m-C^{cal}{}_m)^2$$

where m is observation number and N_d is total number of observations. The estimated variance of the error (=population variance) is calculated by the SSR at its minimum divided by its degrees of freedom:

$$\sigma^2 \approx s^2 = (SSR)_{min}/ (m-p)$$

where p is the number of parameters and s^2 is the variance. The standard error σ (= the estimated standard deviation) is calculated by taking the square root of the estimated variance of the error.

4. RESULTS AND DISCUSSION

The experiments were performed at temperature value 40^{0}C for processing time of 90 minutes at impeller rate 300 rpm and pH 6.5 ∓ 0.05. The reactions were carried out in 0.5 litre of aqueous solutions containing 10 g/l starch. These reaction solutions contained approximately 90000 units enzyme per litre for each experiment. The degree of corn, rice and wheat starch hydrolysis (%) and residual alpha-amylase enzyme activity (%) were investigated. Enzyme activities prior to hydrolysis process was also determined and used as the control. In calculations, A_{max} was denoted as the alpha-amylase enzyme activity prior to hydrolysis process. This value was then considered as 100% activity. Activity at any operational condition (A) was then given in terms of percentage values of the control.

4.1. The Effect of Alpha-Amylase Enzymes Obtained from Different Sources on Corn Starch Hydrolysis

The effect of alpha-amylase enzymes produced by *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae* was investigated on the hydrolysis process of corn starch. Figures 1 and 2 show the residual alpha-amylase enzyme activities and residual corn starch concentrations depending on the processing time, respectively. The hydrolysis degrees of corn starch, by using alpha-amylase enzymes produced by *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae* at temperature value of 40^{0}C after 90 minutes of processing time, were obtained as 5.5%,

40.4% and 0%, respectively. The residual enzyme activity values were obtained as 88.3%, 80.7% and 98.5%, respectively.

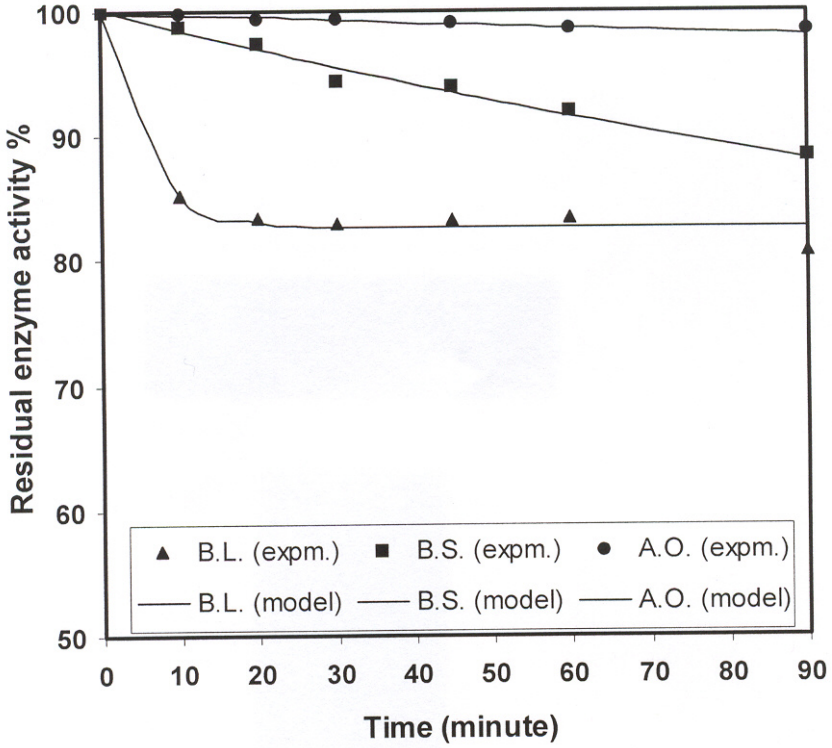

Figure 1. The residual activities of alpha-amylase enzymes produced from different sources versus processing time during corn starch hydrolysis

To predict the effect of the processing time on the enzyme stability, the data of residual α-amylase enzyme activity for each enzyme used were evaluated for corn starch hydrolysis process. Some inactivation models were tested to understand the relationship between the residual enzyme activity and the processing time during the starch hydrolysis process [12, 13]. For this purpose, following residual α-amylase enzyme activity-processing time expressions were used:

***A single step non first order inactivation model*:** for some enzymes, it is reported by Sadana and Henley [14] that the single step inactivation leads to a final state that does exhibit some residual activity.

$$E \xrightarrow{k_D} E_1^{\alpha 1}$$

In above reaction, E and E_1 are enzyme states of different specific activities and they are homogeneous α_i is the ratio of the specific activity of the final state to the initial state. k_D is the degradation coefficient (min^{-1}). The activity-processing time relationship is then as follows:

$$A = (100-\alpha_1) \exp(-k_D [t]) + \alpha_1 \quad (1)$$

where A is residual enzyme activity (percentage values after hydrolysis);

***Quadratic fit for inactivation model*:**

$$A = A_{max} + k_{D1}*t + k_{D2}*t^2 \quad (2)$$

where A_{max} is enzyme activity (percentage value prior hydrolysis); k_{D1}, k_{D2} are the coefficients dependent on the processing time ($(min)^{-1}$ and $(min)^{-2}$), respectively.

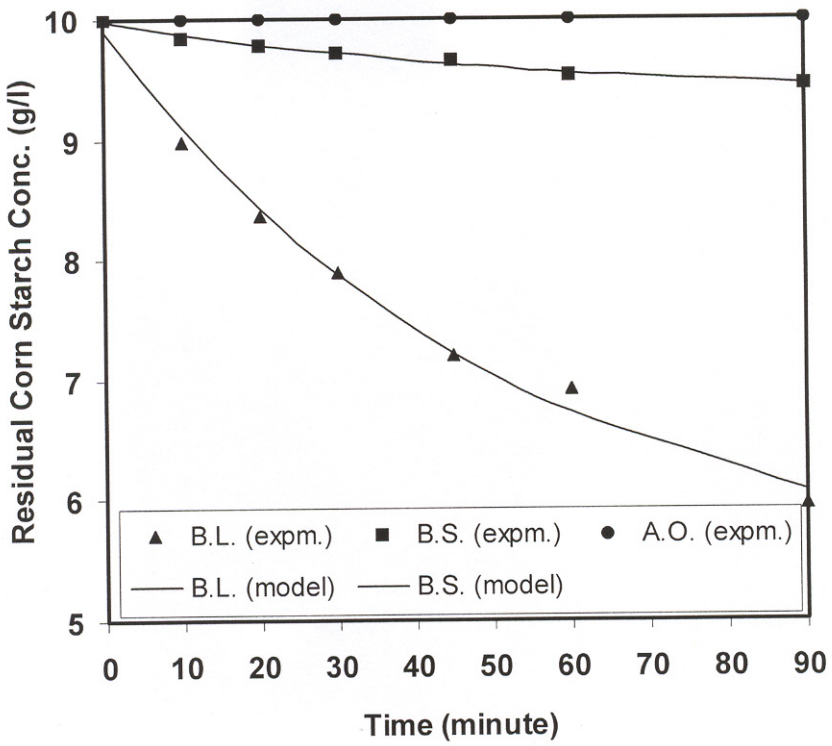

Figure 2. The residual corn starch concentrations versus processing time during starch hydrolysis process by using alpha-amylase enzymes produced from different sources

***Zero order inactivation model*:**

$$A = A_{max} - k_D * t \quad (3)$$

where A_{max} is enzyme activity (percentage value prior hydrolysis); k_D is the zero order inactivation coefficient dependent on the processing time (min^{-1}).

First order inactivation model:

$$A = A_{max} * \exp(-k_D * t) \quad (4)$$

where A_{max} is enzyme activity (percentage value prior hydrolysis); k_D is the first order inactivation coefficient dependent on the processing time (min^{-1}).

After evaluation, the data of alpha-amylase enzyme obtained from *Bacillus species*, *Bacillus licheniformis* and *Aspergillus oryzae* were produced quadratic fit (equation (2)), a single-step non-first-order inactivation model (equation (1)) and a single step non first order inactivation model (equation (1)), respectively, depending on the processing time (from 0 to 90 min) (see Table 1a).

For corn starch hydrolysis process, the parameters, k_D, k_{D_1} and k_{D2} were estimated using the Marquardt-Levenberg optimisation routine of the MATLAB 5.0 software and are presented in Table 1a for each equation. The values of standard error (σ) and R^2 statistical were also given in Table 1a for each experimental set.

Starch hydrolysis:

To predict the effect of the processing time on the starch hydrolysis, the data of residual starch concentration versus the processing time were evaluated. The following residual starch concentration-processing time expression (an empirical first-order model) given by Komolprasert and Ofoli [3] is used:

$S_1 = a * \exp(-b*t) + c$ (5)

where S_1 is the residual starch concentration (g/L) and t is processing time (min). The parameters in equation (5), a (g starch/l), b (min^{-1}) and c (g starch/l) were estimated using the Marquardt-Levenberg optimisation routine of MATLAB 5.0 software. The estimated parameters for corn starch depending on the processing time for enzymes produced by *Bacillus species* and *Bacillus liceniformis* were given in Table 1b. In the same table, the residual (SSR), standard error (σ) and R^2 statistic values for each experimental set were also given.

Table 1a. Estimated parameters of residual alpha-amylase enzyme activity (A)

Enzyme produced by *Bacillus species*

Model	A_{max}	k_{D1} (min^{-1})	k_{D2} (min^{-2})	Standard error (σ)	R^2 Statistic
Eq. (2)	100.10	-0.1609	0.0003	0.6966	0.9903

Enzyme produced by *Bacillus licheniformis*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	82.60	0.1874	0.0146	0.9835	0.9904

Enzyme produced by *Aspergillus oryzae*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	97.65	0.0119	0.0146	0.0919	0.9883

Table 1b. Estimated parameters of residual corn starch concentration (S_1)

Enzyme produced by *Bacillus species*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	0.6774	0.0168	9.3019	0.0030	0.0274	0.9928

Enzyme produced by *Bacillus licheniformis*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	4.7158	0.0186	5.1821	0.0793	0.1408	0.9964

4.2. The Effect of Alpha-Amylase Enzymes Obtained from Different Sources on Rice Starch Hydrolysis

On the hydrolysis process of rice starch, the effect of alpha-amylase enzymes, obtained from by *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae*, was investigated. Figures 3 and 4 show the residual alpha-amylase enzyme activities and residual rice starch concentrations depending on the processing time, respectively. The hydrolysis degrees of rice starch at

temperature of 40^{O}C after 90 minutes of processing time were obtained as 19.1%, 48.1% and 0%; and the residual enzyme activity values were obtained as 74.8%, 82.7% and 93.1%, for alpha-amylase enzymes produced by *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae*, respectively.

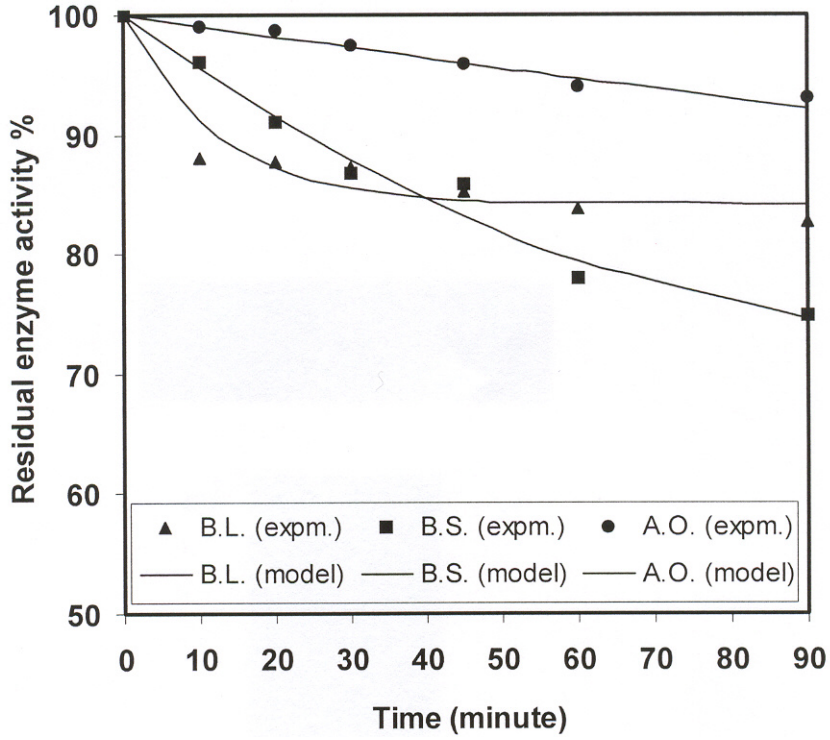

Figure 3. The residual activities of alpha-amylase enzymes produced from different sources versus processing time during rice starch hydrolysis

After evaluation of the data of alpha-amylase enzyme from *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae*, a single step non first order inactivation model (equation (1)) simulated very effectively the inactivation data of alpha-amylase enzyme. The parameters $\square_1$ and k_D estimated for rice starch hydrolysis and the values of standard error (σ) and R^2 statistical were given in Table 2a.

To predict the effect of the processing time on the starch hydrolysis, the residual starch concentration-processing time expression (an empirical first-order model, equation (5)) given by Komolprasert and Ofoli [3] is used. The estimated parameters of rice starch depending on the processing time for enzymes produced by *Bacillus species* and *Bacillus liceniformis* were given in Table 2b. In the same table for each experimental set, the residual (SSR), standard error (σ) and R^2 statistic values were also given.

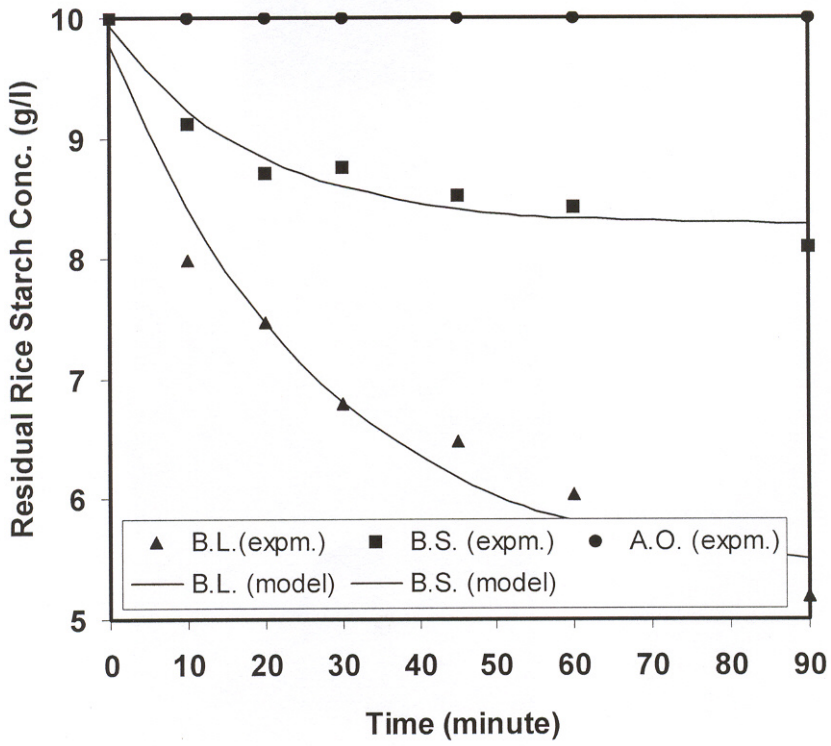

Figure 4. The residual rice starch concentrations versus processing time during starch hydrolysis process by using alpha-amylase enzymes produced from different sources

Table 2a. Estimated parameters of residual alpha-amylase enzyme activity (A)

Enzyme produced by *Bacillus species*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	63.93	0.0137	0.0146	1.4748	0.9891

Enzyme produced by *Bacillus licheniformis*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	84.09	0.0817	0.0146	1.3389	0.9774

Enzyme produced by *Aspergillus oryzae*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	80.97	0.0053	0.0146	0.4937	0.9852

Table 2b. Estimated parameters of residual rice starch concentration (S_1)

Enzyme produced by *Bacillus species*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	1.6671	0.0546	8.2696	0.1142	0.1690	0.9744

Enzyme produced by *Bacillus licheniformis*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	4.4375	0.0369	5.3395	0.4597	0.3390	0.9841

To predict the effect of the processing time on the starch hydrolysis, the residual starch concentration-processing time expression (an empirical first-order model, equation (5)) given by Komolprasert and Ofoli [3] is used. The estimated parameters of rice starch depending on the processing time for enzymes produced by *Bacillus species* and *Bacillus liceniformis* were given in Table 2b. In the same table for each experimental set, the residual (SSR), standard error (σ) and R^2 statistic values were also given.

4.3. The Effect of Alpha-Amylase Enzymes Obtained from Different Sources on Wheat Starch Hydrolysis

Figures 5 and 6 show the residual alpha-amylase enzyme activities (obtained from *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae*) and residual wheat starch concentrations depending on the processing time, respectively. The hydrolysis degrees of wheat starch, by using alpha-amylase enzymes produced by *Bacillus species, Bacillus licheniformis* and *Aspergillus oryzae* at temperature value of 40^0C after 90 minutes of processing time, were obtained as 29.1%, 58.1% and 17.5%, respectively. The residual enzyme activity values were obtained as 92.6%, 82.1% and 94.5%, respectively.

After evaluation of the data of alpha-amylase enzyme from *Bacillus species* and *Bacillus licheniformis*, a single step non first order inactivation model (equation (1)) simulated very effectively the inactivation data. But, a quadratic model (equation (2)) simulated very effectively the data of alpha-amylase enzyme from *Aspergillus oryzae*. The parameters α_1 and k_D estimated for wheat starch hydrolysis and the values of standard error (σ) and R^2 statistical were given in Table 3a.

To predict the effect of the processing time on the starch hydrolysis, the residual starch concentration-processing time expression (an empirical first-order model, equation (5)) given by Komolprasert and Ofoli [3] is used. The estimated parameters for wheat starch depending on the processing time for each enzyme used were given in Table 3b. In the same table, the residual (SSR), standard error (σ) and R^2 statistic values for each experimental set were also given.

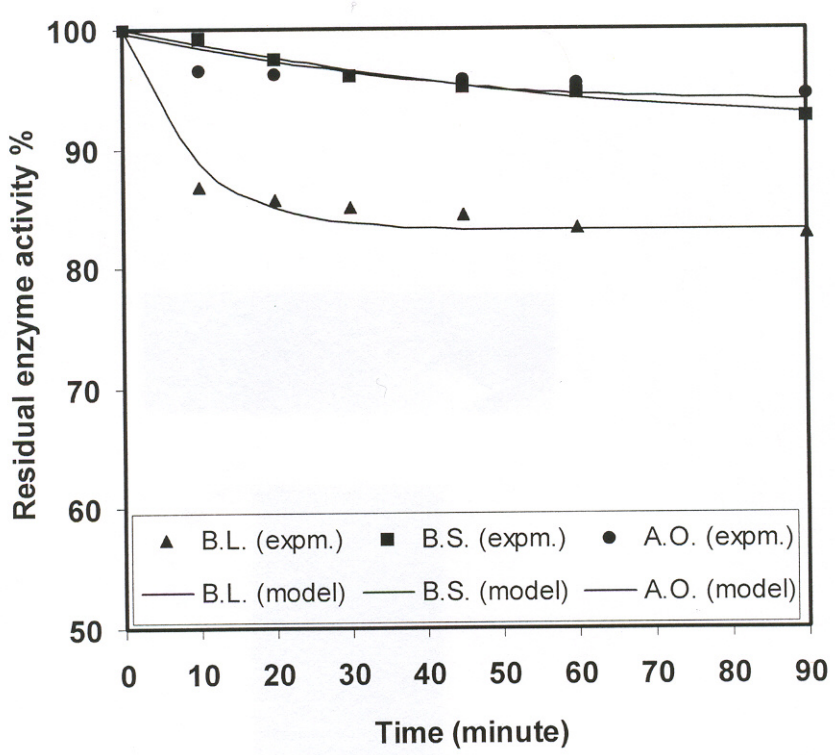

Figure 5. The residual activities of alpha-amylase enzymes produced from different sources versus processing time during wheat starch hydrolysis

Table 3a. Estimated parameters of residual alpha-amylase enzyme activity (A)

Enzyme produced by *Bacillus species*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	89.88	0.0141	0.0146	0.3939	0.9905

Enzyme produced by *Bacillus licheniformis*

Model	α_1	k_D (min^{-1})	SSR	Standard error (σ)	R^2 Statistic
Eq. (1)	83.12	0.1084	0.0146	1.0784	0.9873

Enzyme produced by *Aspergillus oryzae*

Model	A_{max}	k_{D1} (min^{-1})	k_{D2} (min^{-2})	Standard error (σ)	R^2 Statistic
Eq. (2)	99.62	0.1334	0.0008	0.5603	0.9713

Table 3b. Estimated parameters of residual wheat starch concentration (S_1)

Enzyme produced by *Bacillus species*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	3.8352	0.0163	6.2164	0.0299	0.0866	0.9976

Enzyme produced by *Bacillus licheniformis*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	6.2169	0.0227	3.5472	0.2817	0.2654	0.9937

Enzyme produced by *Aspergillus oryzae*

Model	a (g starch/l)	b (min^{-1})	c (g starch/l)	SSR	Standard error (σ)	R^2 Statistic
Eq. (5)	1.7213	0.2037	8.2772	0.0134	0.0578	0.9972

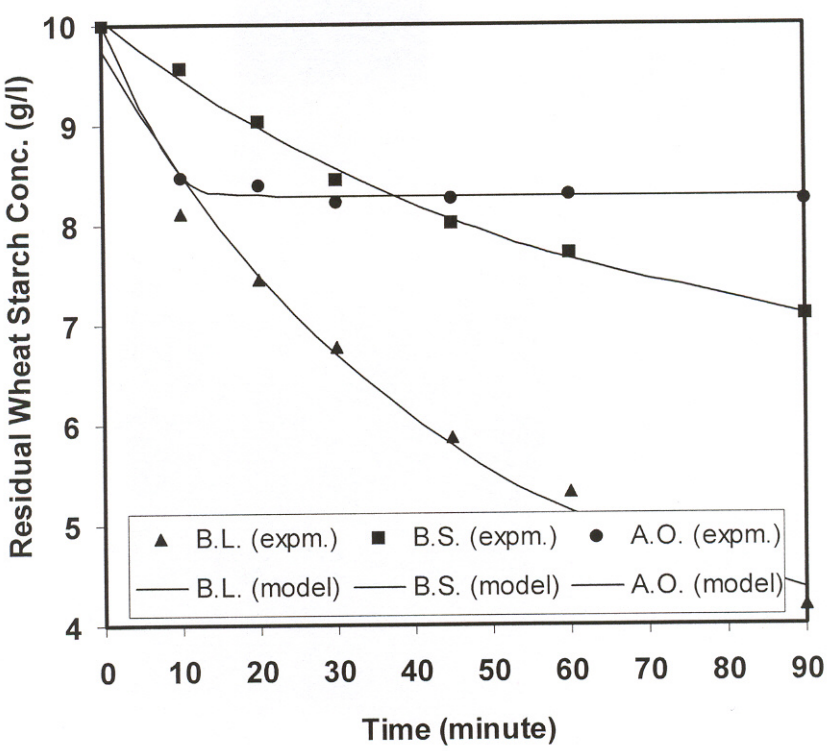

Figure 6. The residual wheat starch concentrations versus processing time during starch hydrolysis process by using alpha-amylase enzymes produced from different sources

5. CONCLUSIONS

An evaluation of the experimental data showed that the processing time is involved in the inactivation of alpha-amylase enzymes (produced from different sources) after starch hydrolysis process.

By using enzyme produced by *Bacillus species,* the hydrolysis degrees for corn, rice and wheat starch were obtained as 5.5%, 19.1% and 29.1%, respectively. The residual enzyme activities were obtained as 88.3%, 74.8% and 92.6%, respectively. By using enzyme produced by *Bacillus licheniformis,* the hydrolysis degrees for corn, rice and wheat starch were obtained as 40.4%, 48.1% and 58.1%, respectively. The residual enzyme activities were obtained as 80.7%, 82.7% and 82.1%, respectively. On the other hand, using enzymes produced by *Aspergillus oryzae,* the hydrolysis degrees for corn, rice and wheat starch were obtained as 0%, 0% and 17.5%, respectively. The residual enzyme activities were obtained as 98.5%, 93.1% and 94.5%, respectively.

The modelling studies showed that the inactivation data of the alpha-amylase enzyme produced from *Bacilius species* during hydrolysis of corn starch, represented by a quadratic fit inactivation model and during hydrolysis of rice and wheat starch represented by a single step non-first-order inactivation model. On the other hand, the data of alpha-amylase enzyme produced from *Bacillus licheniformis* were fitted to a single step non-first-order enzyme inactivation model during hydrolysis of each starch. The data of alpha-amylase enzyme produced from *Aspergillus oryzae* resulted in a single step non-first-order enzyme inactivation model for corn and rice starch hydrolysis and quadratic fit inactivation model for wheat starch hydrolysis.

For the modelling studies of the residual starch concentration, an empirical first-order model given by Komolprasert and Ofoli [3] accurately represented the data for each starch hydrolysed in the case of each enzyme used. But, for the alpha-amylase enzyme produced by *Aspergillus oryzae*, for corn and rice starch, no model was derived as no hydrolysis was achieved.

Finally, maximum hydrolysis degrees were obtained for the alpha-amylase enzyme produced by *Bacillus licheniformis* for each starch used. The stability behaviour observed was different for each alpha-amylase enzyme. These could be related to molecular weights or sources of the alpha-amylase enzymes and starches used. Thus, the operational parameters of the starch hydrolysis process should be optimised specifically for each enzyme and each starch used, as these process parameters (such as processing time) could cause degradation of the alpha-amylase enzyme during the starch hydrolysis process.

ACKNOWLEDGMENTS

This work was supported by the Yıldız Technical University Research Fund. Project Number: 20-A-07-01-02.

REFERENCES

[1] Colonna, P., Buleon, A. and Lemarie, F., (1988), ‘‘Action of *Bacillus Subtilis* Alpha-amylase on Native Wheat Starch’’, Biotechnol. Bioeng., 31:895-904.

[2] Textor, S. D., Hill, G. A., Macdonald, D. G. and Denis, E. S., (1998), ‘‘Cold Enzyme Hydrolysis of Wheat Starch Granules’’, The Canadian Journal of Chemical Engineering, 76:87-93.

[3] Komolprasert, V. and Ofoli, R. Y., (1991), ‘‘Starch Hydrolysis Kinetics of *Bacillus licheniformis* Alpha-amylase’’, J. Chem. Biotechnol., 51:209-223.

[4] Somers, W., Rozi, H., Bonte, A., Visser, J., Rombouts, K., Riet.,K., (1991), “On the Interaction of Alpha-amylase with Croslinked Starch: Evaluation of Process Conditions”, Enzyme Microb. Technol., 13:997-1006.

[5] Salieri, G., Vinci, G. and Antonelli, M. L., (1995), ‘‘Microcalorimetric Study of the Enzymatic Hydrolysis of Starch: An Alpha-amylase Catalyzed Reaction’’, Analytica Chemica Acta, 300:287-292.

[6] Bajpai, P., Gera, K., Bajpai, K., (1992), “Optimization Studies for The Production of Alpha-amylase Using Cheese Whey Medium”, Enzyme Microb. Technol.,14:679-683.

[7] Salieri, G., Vinci, G. and Antonelli, M. L., (1995), ‘‘Microcalorimetric Study of the Enzymatic Hydrolysis of Starch: An Alpha-amylase Catalyzed Reaction’’, Analytica Chemica Acta, 300:287-292.

[8] Somers, W., Rozi, H., Bonte, A., Visser, J., Rombouts, K., Riet.,K., (1991), “On the Interaction of Alpha-amylase with Croslinked Starch: Evaluation of Process Conditions”, Enzyme Microb. Technol., 13:997-1006.

[9] Hill, G. A., Macdonald, D. G. and Lang, X., (1997), ‘‘Alpha-amylase Inhibition and Inactivation in Barley Malt during Cold Starch Hydrolysis’’, Biotechnol. Lett., 19(11):1139-1141.

[10] Astolfi-Filfo, S., Galembeck, E. V., Faria, J. B. and Frascino, A. C. S., (1986), ‘‘Stable Yeast Transformants that Secrete Functional Alpha-amylase Encoded by Cloned Mouse Pancreatic cDNA’’, Biotechnology, 4:311-315.

[11] De Moraes, L. M. P., Astolfi-Filho, S. and Oliver, S. G., (1995), ‘‘Development of Yeast Strains for the Efficient Utilisation of Starch: Evaluation of Constructs that Express Alpha-amylase and Glucoamylase Separately or as Bifunctional Fusion Proteins’’, Appl. Microbial. Biotechnol., 43:1067-1076.

[12] Özbek, B., and Ülgen, K., (2000), "The Stability of Enzymes After Sonication", *Process Biochemistry*, **35(9):**1037-1043.

[13] Özbek, B., and Yüceer, S., (2001), "Alpha-amylase inactivation during wheat starch hydrolysis process", Process Biochemistry, 37(1): 87-95.

[14] Sadana, A., and Henley, J.M., Single step unimolecular non-first-order enzyme deactivation kinetics. *Biotechnology and Bioengineering*, 1987, **30**, 717-723.