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Enzymatic Production of Reducing Sugars from Broomcorn Seed (*Sorghum vulgare*): Process Optimization and Kinetic Studies

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Abstract: This research aimed to add value to broomcorn seed, locally available as lignocellulosic biomass waste. Enzymatic hydrolysis was conducted to produce fermentable sugars from broomcorn seed. The enzymatic liquefaction and saccharification processes performed by α-amylase and amyloglucosidase were optimized to improve the release of reducing sugars. The optimization was carried out through a set of experiments designed by central composite design using response surface methodology (RSM). The concentrations of broomcorn seed flour (15-75 g/l), α-amylase (0.0-1.2 g/l) and amyloglucosidase (0.0-0.8 g/l) were selected as three independent process variables. It was found that the developed statistical model was suitable to predict optimum yield of reducing sugars. The optimum conditions were flour concentration of 48.13 g/l, α-amylase concentration of 0.76 g/l and amyloglucosidase concentration of 0.48 g/l to produce 42 g/l of reducing sugars. The kinetics of enzymatic hydrolysis was described by Michaelis-Menten equation and the kinetic constants were obtained using the Lineweaver-Burk plot. The kinetic parameters of V_{max} and K_m were determined as 24.57 g hydrolyzed flour/l. h and 49.15 g/l in the presence of dual enzymes.

Key words: Broomcorn seed · α-amylase · Amyloglucosidase · Reducing sugar · Enzymatic hydrolysis

INTRODUCTION

Sorghum vulgare, commonly known as broomcorn, is a type of sorghum with coarse, fibrous seed head which is used for the fabrication of various brooms and wooden decorative items [1]. The fibrous panicle of this plant is used for broom making purposes, whereas the leftover stalks are of little forage value. The majority of broomcorn seed is fed to chickens and other livestock. The feed value of broomcorn seed is estimated from nothing to almost as high as grain sorghum and corn, depending on the degree of ripening of the seed during the plant harvest time [2]. The literature suggests that the mature seeds have reasonable feed value, roughly comparable to oat. The broomcorn seed contains starch, protein, moisture, fiber, lipid and ash with weight percentage of 59.62,13.37,10.06, 9.04, 3.64 and 4.27%, respectively [3]. The relatively high content of starch in the broomcorn seed makes it a suitable precursor for the production of value-added bio-products.

Regardless of the botanical source of starch, it consists of two types of glucans, namely amylopectin and amylose [4, 5]. Amylose is a linear polymer of glucose with a molecular weight of around 10^6 Da, whereas amylopectine is a larger branched polymer of glucose with molecular weight of around 10^8 Da [6, 7]. The digestion of these polymers might take place in a two step process; the onset of the starch degradation is carried out by α-amylase. The α-amylase performs the starch liquefaction, where the amylopectin and amylose are broken into disaccharide, trisaccharide and α-dextrin units. The next process involves the starch saccharification in presence of amyloglucosidase, where the produced maltose, maltotriose and α-dextrin units are hydrolyzed to glucose units [8, 9].

A survey of literature explores that number of researches have been carried out on promotion of starch digestion from grain sorghum (*Sorghum bicolor* (L.) MOENCH). Ezeogu *et al.* [4] examined the effect of cooking condition on starch digestibility of grain sorghum. It was reported that cooking of sorghum with 2-mercaptoethanol improved starch digestion. Pressure-cooking of grain markedly increased the starch digestibility. It was inferred that such improvement was attributed to physical disruption of the proteins surrounding the starch granules. In another investigation

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carried out by Shewale and Pandit [10], the possibility of enhancing the yield of enzymatic glucose production from three types of grain sorghum was assessed through application of ultrasound pretreatment. It was observed that ultrasound processing of the sorghum slurry prior to liquefaction increased the starch saccharification to glucose from 74 to 90%. It was elucidated that such enhancement was due to the disruption of hydrophobic protein matrix enveloping the starch granules and amylase-lipid complex. The extent of starch digestibility could be also improved through the optimization of enzymatic liquefaction and saccharification processes. Barcelos *et al.* [11] evaluated the enzymatic hydrolysis of grain sorghum in presence of commercial α-amylase and glucoamylase. The concentrations of 73 U of α-amylase/ g grain and 1150 U glucoamylase/ g grain were found as optimum condition to yield almost 250 g/l glucose in batch bioreactors. Although production of value-added products from grain sorghum has been thoroughly studied, little data are found in the scientific literatures regarding the applications of broomcorn. To the best of authors' knowledge, there is no report available on the production of valuable bio-products from broomcorn seed. This agricultural waste can be considered as a low cost raw material for the production of ethanol through hydrolysis and fermentation processes.

The objective of this research was to optimize enzymatic hydrolysis of broomcorn seed flour to reducing sugars. The optimum concentrations of α-amylase and amyloglucosidase to carry out liquefaction and saccharification processes were determined. The kinetics of starch digestion to the reducing sugars in presence of both enzymes was investigated.

MATERIALS AND METHODS

Raw Material: Broomcorn seeds were kindly supplied by the Agricultural Organization of Mazandaran, Sari, Iran. The seeds were finely ground to flour. The flour was washed several times to remove the seed shells. Then, the flour was recovered by filtration (Whatman), dried in oven at 105°C and stored in dessicator at room temperature for further experiments.

Alkali pretreatment: It is well known that the protein barrier surrounding the starch granules can reduce the starch digestibility and gelatinization due to the low availability of starch for enzymatic hydrolysis [7]. Thus, alkali pretreatment was used as an effective method for protein disruption and improving the abundance of starch granules for further action of the enzymes. For the alkali treatment of the broomcorn seed flour, a defined amount of the flour was steeped in 450 ml of NaOH solution (0.1 M) and stirred at 60°C for 1h.

Starch Digestion: The pretreated flour sample was mixed with 550 ml of potassium hydrogen phthalate buffer (0.1 M); the final pH was 6.1. Then, the mixture was boiled at 105°C, where the gelatinization initiated and the viscosity of slurry increased. After cooling the slurry to 95°C, a defined concentration of heat-resistant α-amylase (Serva, 30 Unit/mg) was added to the solution to carry out liquefaction; the solution was kept at this temperature for 2 h.

The liquefied starch was saccharified in presence of amyloglucosidase. After the completion of the liquefaction, the pH of the solution was reduced to 4.5 using phosphoric acid solution and a defined amount of amyloglucosidase (Serva, 120 Unit/mg) was added to the liquefied starch at 60°C and stirred at 120 rpm for 72 h. Samples were periodically withdrawn to analyze the produced reducing sugar concentration.

Analytical Procedure: Decrease in starch content of the solution during liquefaction and saccharification was monitored by means of resorcinol reagent. The concentration of reducing sugars was measured using dinitrosalicylic acid (DNS) method [12].

Optimization: Design-Expert 7.0 (Stat-ease Inc., USA) was used to design the experiments and to perform the regression and statistical and graphical analysis. Three independent variables including broomcorn seed flour (substrate) concentration (A), α-amylase concentration (B) and amyloglucosidase concentration (C) were selected as experimental parameters. The effect of these parameters on concentration of reducingsugars as the only major response was studied. The range of the independent variables and the experimental design levels are tabulated in Table 1.

The Central Composite Design (CCD) was used as a standard RSM model to optimumize the experimental parameters. In current study, CCD designed a set of 20 runs based on the implemented independent variables. The results were then fitted to the following second order polynomial equation to predict the optimum condition:

Table 1: Experimental range and levels of the independent variables

		Coded levels of variables				
Factors	Symbol	-2	-1	0	+1	+2
Flour concentration (g/l)	(A)	15	30	45	60	75
α-amylase concentration (g/l)	(B)	0	0.3	0.6	0.9	1.2
Amyloglucosidase concentration (g/l)	(C)	0	0.2	0.4	0.6	0.8

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ii} X_i X_j + \varepsilon$$

Where Y represents the response variable, β_0 is the intercept, β_i and β_{ii} are the first and second order quadratic model coefficients for the variable, respectively. Also, β_{ij} is the linear model coefficient for the interactions between i and j; while X_i and X_j are decoded independent process variables and $å$ is the atandard error.

RESULTS AND DISCUSSION

Empirical Model Equation and Analysis of Variance (ANOVA): The obtained results based on the experimental design for the enzymatic hydrolysis of broomcorn seed flour are summarized in Table 2. The concentration of reducing sugar was in the range of 5.0 to 41.2 g/l depending on the experimental condition. A quadratic regression model was developed in terms of the coded factors (effective parameters) by the software. After exclusion of the insignificant terms, the model is presented as follows:

$$\text{Reducing sugar (g/l)} = -61.17 + 3.42\,A + 36.02\,B + 28.36\,C - 0.036\,A^2 - 25.24\,B^2 - 38.63\,C^2 + 0.11\,AC + 4.81\,BC \quad (2)$$

The model consists of single, quadratic and interaction terms and their corresponding coefficients. The synergistic and antagonistic effects are indicated by positive and negative signs in front of the model terms, respectively.

The adequacy of the developed quadratic model was justified through analysis of variance (ANOVA). The ANOVA for the concentration of reducing sugar model after enzymatic hydrolysis as a function of flour, α-amylase and amyloglucosidase concentration is illustrated in Table 3. The terms with *"Prob > F"* less than 0.05 are considered significant. Thus, the terms AC and BC are not also significant and could be omitted from the model. The coefficient of variation of 6.71% shows a high degree of agreement between experimental and simulated results.

Interaction Between Process Parameters: Figure 1 depicts the response surface 3D plot illustrating the interactive effect of broomcorn seed flour and α-amylase concentration on production of reducing sugar. As observed in this plot, increase in the flour concentration from 15 to 50 g/l, increased the concentration of reducing sugar produced in the hydrolysis process. However, increasing flour concentration to beyond 50 g/l in the

Table 2: Experimental design and results for enzymatic hydrolysis of broomcorn seed flour to reducing sugars

Run	A Flour (g/l)	B α-amylase (g/l)	C Amyloglucosidase (g/l)	Response Reducing sugar (g/l)
1	60.0	0.9	0.6	35.9
2	45.0	0.6	0.4	41.2
3	60.0	0.3	0.2	30.0
4	45.0	0.6	0.4	41.2
5	60.0	0.9	0.2	33.2
6	45.0	0.6	0.8	38.1
7	45.0	0.0	0.4	27.0
8	45.0	0.6	0.4	41.2
9	45.0	0.6	0.0	33.2
10	60.0	0.3	0.6	32.1
11	30.0	0.9	0.2	24.0
12	30.0	0.3	0.2	21.4
13	45.0	0.6	0.4	41.2
14	45.0	1.2	0.4	38.5
15	45.0	0.6	0.4	41.2
16	30.0	0.3	0.6	21.6
17	30.0	0.9	0.6	26.0
18	45.0	0.6	0.4	41.2
19	75.0	0.6	0.4	14.1
20	15.0	0.6	0.4	5.0

Table 3: Results of ANOVA for the developed quadratic model

Source	Degree of freedom	Sum of square	Coefficient estimate	Mean square	F-value	Prob > F
Model	9	1951.67	3.53	216.35	48.95	< 0.0001
A	1	199.3	2.31	199.3	44.99	< 0.0001
B	1	85.42	1.05	85.42	19.28	0.0014
C	1	17.58	0.00375	17.58	3.97	0.071
A^2	1	1837.98	-8.07	1637	369.76	< 0.0001
B^2	1	129.7	-2.27	129.7	29.28	0.0003
C^2	1	60.03	1.55	60.03	13.55	0.0042
AB	1	0.00011	0.00375	0.00	0.00	0.9
AC	1	0.85	0.33	0.85	0.19	0.6
BC	1	0.67	0.29	0.67	0.15	0.7
R^2=0.9778			Std. Dev.[a]=2.10		CV[b]=6.71	

[a] Standard deviation

[b] Coefficient of variation

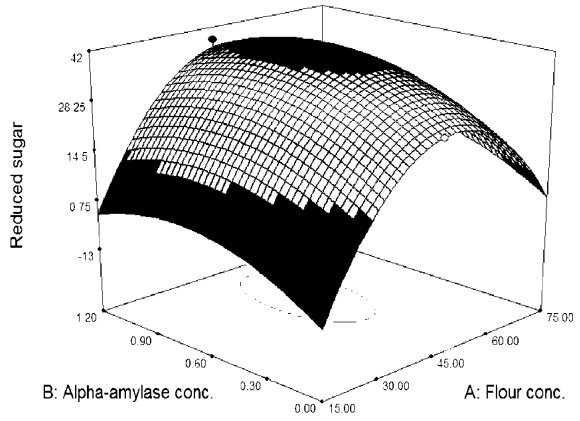


Fig. 1: Response surface 3D plot indicating the interaction between flour (A) and α-amylase (B) concentrations on the concentration of reducing sugars

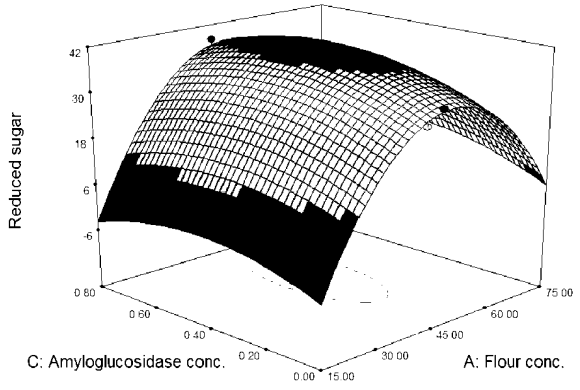


Fig. 2: Response surface 3D plot indicating the interaction between flour (A) and amyloglucosidase (C) concentrations on the concentration of reducing sugars

solution adversely affected the production of reducing sugars. Such reduction in the concentration of product was confidently attributed to the substrate inhibition effects imposed at flour concentrations above 50 g/l. From Figure 1, it could be observed that at any designated flour concentration, the release of reducing sugar during the enzymatic hydrolysis increased proportionally with increase of the α-amylase concentration to about 0.8 g/l. Increase in enzyme concentration to above 0.8 g/l did not significantly enhance the starch hydrolysis.

The simultaneous effect of flour and amyloglucosidase concentrations on the release of reducing sugars during the hydrolysis process is demonstrated in Figure 2. The concentration of reducing sugars augmented with increase of amyloglucosidase concentration to 0.5 g/l; further increase in enzyme concentration was not effective in promoting the concentration of reducing sugars.

Figure 3 shows the 3D plot of the α-amylase and amyloglucosidase concentrations on production of reducing sugars during the starch hydrolysis. As illustrated in this plot, the concentrations of 0.8 and 0.5 g/l of α-amylase and amyloglucosidase were found as the optimum concentrations to improve the yield of enzymatic hydrolysis of starch to reducing sugars during the starch liquefaction and saccharification, respectively.

Figure 4 presents the actual results obtained from the experiments against the predicted values given by the proposed model. The actual data were in full agreement with the predicted values as observed in this plot. In cases where a complete agreement is found between the experimental and predicted values, the R^2 values are very close to unity. The high correlation coefficient

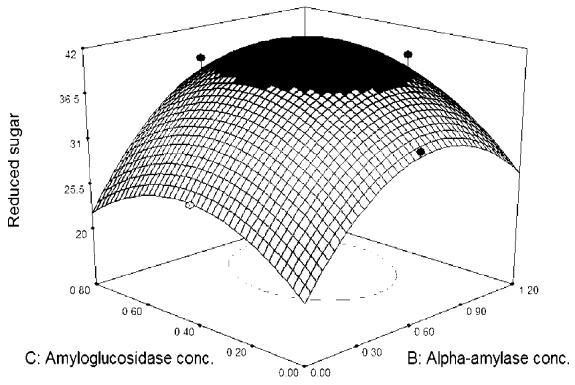


Fig. 3: Response surface 3D plot indicating the interaction between α-amylase (B) and amyloglucosidase (C) concentrations on the concentration of reducing sugars

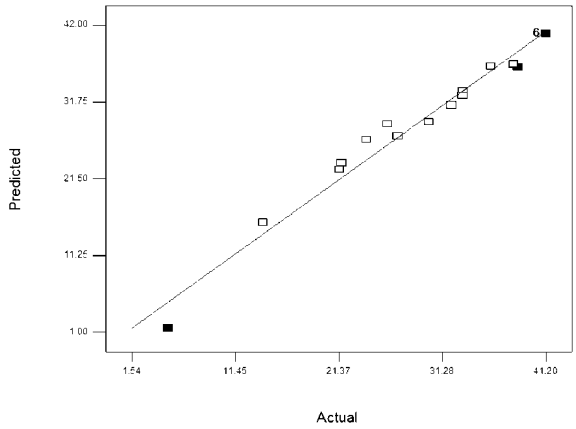


Fig. 4: Predicted versus actual results for the enzymatic hydrolysis process

(R^2= 0.9778) of the model indicate that 97.78% of the variations in the result were attributed to the three investigated variables. The relatively high R^2 value obtained for this model proves the reliability of the developed model to interpret the experimental results.

Process Optimization: In order to find the optimum conditions for the independent variables which results in improved production of reducing sugar from starch hydrolysis, numerical optimization was carried out. The objective of the optimization process was to obtain the maximum response that simultaneously satisfies all the variables. In order to determine the optimum condition, a set of solutions were generated by the Design Expert Software. The optimum condition was calculated based on the process ranges which were adjusted using Eq. (2). The optimum condition of the experiment, predicted and experimental concentration of reducing sugars are summarized in Table 4. The experimental value of reducing sugar concentration (41.95 g/l) was in a very good agreement with the predicted one (42.28 g/l). This implies the adequacy of the developed model to predict the release of reducing sugars during the starch hydrolysis process.

Kinetics of Enzymatic Starch Hydrolysis: During the course of enzymatic hydrolysis, starch is converted to glucose and other reducing sugars. Studying the kinetics of this process provides useful information for the better understanding of the pathways of the reaction. The kinetics of many regular enzymatic reactions is described by Michaelis-Menten equation stated as follows [13]:

$$\frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]} \quad (3)$$

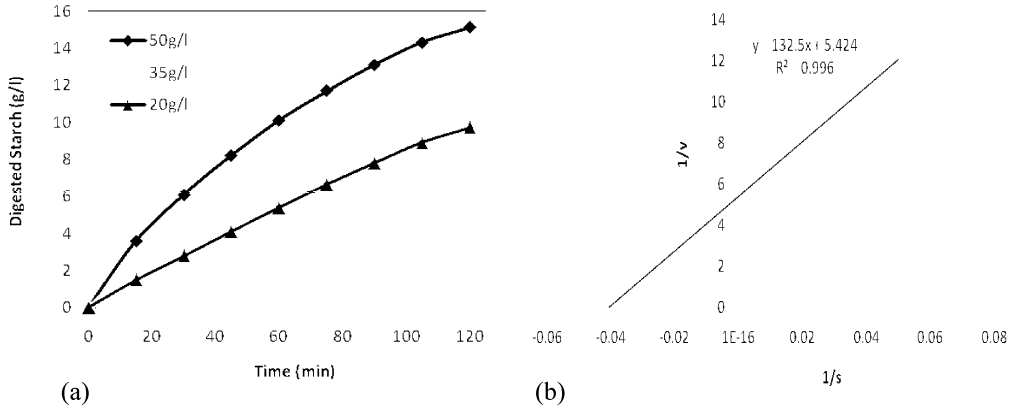
Where V_{max} represents the maximum velocity (g/l.h), K_m is the Michaelis constant (g/l), [P] and [S] are respectively the concentrations of product and substrate (g/l) and t shows the time of the enzymatic process (h). The constant K_m shows the substrate concentration at which the rate of reaction reaches half of V_{max}. The data obtained during the course of starch digestion could be graphically illustrated using the Lineweaver-Burk plot:

$$\frac{1}{V} = \frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}} \quad (4)$$

In order to determine the kinetic parameters, 0.75 g α-amylase (as the optimum concentration) was incubated with various concentrations of broomcorn seed flour solution (20, 30 and 50 g/l) as substrate. The starch digestion profile during the course of enzymatic

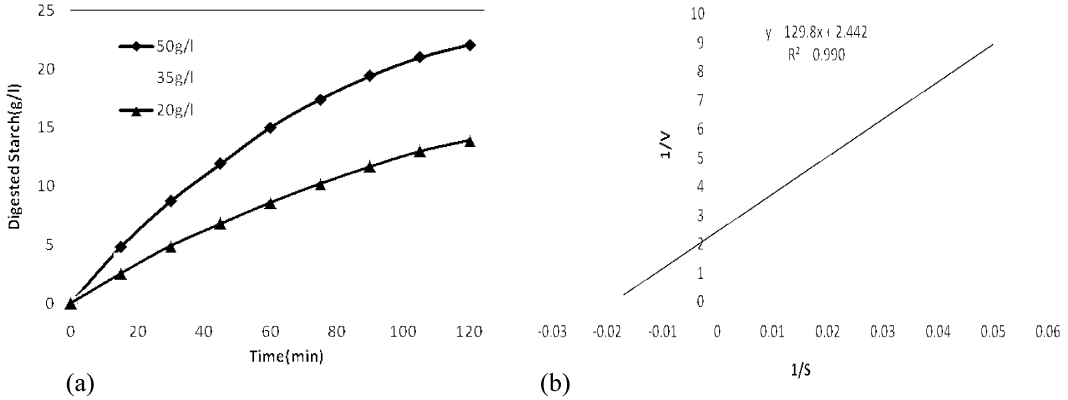
Table 4: Optimum condition for the enzymatic hydrolysis of starch to reducing sugars

				Reducing sugars (g/l)	
Run	Flour (g/l)	α-amylase (g/l)	Amiloglucosidase (g/l)	Obtained	Predicted
1	48.13	0.76	0.48	41.95	42.28



(a) (b)

Fig. 5: (a) Time-dependent profile of digested flour and (b) Lineweaver-Burk plot for the enzymatic hydrolysis in the presence of α-amylase



(a) (b)

Fig. 6: (a) Time-dependent profile of digested flour and (b) Lineweaver-Burk plot for the enzymatic hydrolysis in the presence of α-amylase and amyloglucosidase

hydrolysis is depicted in Figure 5 (a). The kinetic constants of V_{max} and K_m were obtained from the intercept and slope of the Lineweaver-Burke plot as illustrated in Figure 5 (b). The values of V_{max} and K_m were found as 11.06 g hydrolyzed flour/l. h and 24.42 g/l.

For the purpose of improving the rate of starch digestion and enhancing the kinetic parameters, the enzymatic hydrolysis was performed in presence of α-amylase and amyloglucosidase. The concentrations of the two enzymes were kept constant at optimum values i.e. 0.75 and 0.48 g/l for the α-amylase and amyloglucosidase, respectively, while the concentration of starch was varied in the solution (20, 30 and 50 g/l). The digested enzyme profile in presence of both enzymes and the corresponding Lineweaver-Burke plot are shown in Figure 6 (a) and (b). As expected, the values of V_{max} (24.57 g hydrolyzed flour/l. h) and K_m (49.15 g/l) were improved compared to the condition where α-amylase was lonely used to carry out the enzymatic hydrolysis.

CONCLUSION

Enzymatic hydrolysis of broomcorn seed flour to reducing sugars was studied in a series of batch experiments. The main objective of present work was to find out an optimum condition to improve the release of reducing sugars from the broomcorn seed starch. RSM was successfully applied to predict the effect of broomcorn seed flour, α-amylase and amyloglucosidase concentrations on production of reducing sugars. It was concluded that with use of RSM, the optimum conditions for enzymatic hydrolysis were flour concentration of 48.13 g/l, α-amylase concentration of 0.76 g/l and amyloglucosidase concentration of 0.48 g/l to produce 42 g/l of reducing sugars. The kinetics of the enzymatic hydrolysis process was also studied and the kinetic parameters were determined.

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