

Kinetic modeling of simultaneous saccharification and fermentation of corn starch for ethanol production*

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Fuel ethanol production, using a simultaneous saccharification and fermentation process (SSF) of native starch from corn flour, has been performed using *Saccharomyces cerevisiae* and a granular starch hydrolyzing enzyme. The quantitative effects of mash concentration, enzyme dose and pH were investigated with the use of a Box-Wilson central composite design protocol. Proceeding from results obtained in optimal fermentation conditions, a kinetics model relating the utilization rates of starch and glucose as well as the production rates of ethanol and biomass was tested. Moreover, scanning electron microscopy (SEM) was applied to investigate corn starch granule surface after the SSF process. A maximum ethanol concentration of 110.36 g/l was obtained for native corn starch using a mash concentration of 25%, which resulted in ethanol yield of 85.71%. The optimal conditions for the above yield were found with an enzyme dose of 2.05 ml/kg and pH of 5.0. These results indicate that by using a central composite design, it is possible to determine optimal values of the fermentation parameters for maximum ethanol production. The investigated kinetics model can be used to describe SSF process conducted with granular starch hydrolyzing enzymes. The SEM micrographs reveal randomly distributed holes on the surface of granules.

Key words: native starch; optimization, modeling, kinetics, simultaneous saccharification and fermentation

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INTRODUCTION

Ethanol, a chemical alternative to petroleum-derived fuel, is produced from fermentation of carbohydrates such as starches, which are present in plants. Ethanol provides a renewable energy source that produces a number of additional benefits, including limited greenhouse gas emissions and creation of new employment opportunities by enhancing economic benefits in rural areas (Wyman, 2003).

It is well known that fermentation of starch is more complex than fermentation of sugars such as glucose or sucrose, because starch must first be converted into fermentable sugars and then into ethanol. To reach an almost total degradation of starch, two main groups of amylolytic enzymes are required: one comprised of liquefying thermostable α -amylases, and the other of saccharifying glucoamylases (Senn & Pieper, 2001). In conventional ethanol technology, a starchy feedstock such as corn flour, is first slurried with water containing a heat-

stable α -amylase. The preliminary addition of α -amylase is required in order to decrease the viscosity of the mash and to facilitate the cooking operation. This slurry is then cooked at high temperatures (90–95°C) to gelatinize and liquefy the starch. During the mashing process, starch polymer is hydrolyzed by α -amylase to produce shorter chains, namely dextrans, maltose and maltotriose. The resulting mash is then cooled to 60°C and a glucoamylase is added which converts dextrans to fermentable sugars, these being mainly glucose and maltose (Power, 2003). In the final step of the process, yeast are added and incubated at temperatures below 35°C for 48–72 h to ferment the sugars to ethanol. Due to the high energy consumption during the mashing process, the current production cost of traditional starch substrate processing for ethanol are high. According to Lim *et al.* (2003) the energy demand of the conventional cooking process is equivalent to 30–40% of the fuel value of ethanol produced. Hence to compete with the existing fossil fuel industry and become commercially viable, the cost must be reduced.

There are several possible ways to approach this problem. The most straightforward is to design new amylases capable of raw starch digestion (GSHE — the granular starch hydrolyzing enzymes). It is well known that amylases from cereals are capable of hydrolyzing raw starch, which is essential in the breakdown of stored carbohydrates. In addition to green plant sources, enzymes reported to have the ability to digest raw starch were also found in microbial sources. Recently, seven highly active enzyme systems with raw starch hydrolytic capabilities were created by using biological combinatorial processes, using naturally occurring amylases. According to Robertson *et al.* (2006) these systems have the potential to overcome limitations of a natural set of enzymes utilized currently. Application of tailored GSH enzymes eliminates the cost-intensive liquefaction cooking step, and thus reduces the energy input per unit of ethanol produced. In addition to the advantages already mentioned, another attractive feature of this enzymatic complex is that it can be used to break down starch molecules in very concentrated mashes, in which the dry solid content

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Abbreviations: St, starch concentration (kg/m³); Glu, glucose concentration (kg/m³); X, biomass concentration (kg/m³); Et, ethanol concentration (kg/m³); Enz, enzyme concentration (U/m³); k_{st} , rate constant (kg/Uh); K, inhibition and saturation constants (kg/m³); K_m , Michaelis' constant (kg/m³); Q_p , specific ethanol production rate (1/h); R, starch utilization rate (kg/m³/h); R_{glu} , formation, glucose formation rate (kg/m³/h); R_{glu} , utilization, glucose utilization rate (kg/m³/h); $Y_{p/s}$, yield coefficient of product (kg/kg); $Y_{x/s}$, yield coefficient of cell growth (kg/kg); T, time (h); μ , specific growth rate (1/h). **Subscripts:** 0, initial; max, maximum

oscillates in the range of 12–38% wt. Possible drawbacks of this technology include the cost and the amount of enzyme required for the process, and the increased chance of microbial contamination and corresponding loss of yield because partial pasteurization of the mash during the heating process does not occur. The heat applied during standard ethanol process also helps to release the starch that is bound to a fiber or proteins, and inactivates some toxins that may be present in the grain (Nichols *et al.*, 2006).

Great attention is being paid to optimization of the fermentation processes. It is well known that when starch is used as a raw material, amylases are strongly inhibited by hydrolysis products, such as glucose. This inconvenience can be overcome by a simultaneous saccharification and fermentation process (SSF), which combines enzymatic hydrolysis with fermentation. Furthermore, the SSF process also reduces osmotic stress since the yeast cells are exposed to a relatively lower sugar concentration, thereby offering a possibility of higher production rates. An additional advantage of SSF is that a multistage process for the conversion of starch into ethanol is carried out in one bioreactor, which provides not only a reduction in the overall fermentation time but also a reduction in the investment and operational costs (Kobayashi *et al.*, 1998). However, in spite of these advantages, there are some drawbacks of using SSF, such as different optimal temperatures for the action of α - and glucoamylase and the growth of yeast, which means that conditions used in SSF cannot be optimal for both, the enzymes and the yeast (Öhgren *et al.*, 2007). Thus, the SSF temperature, which is usually around 35°C, is a compromise between optimal temperatures for hydrolysis and fermentation. According to Philippidis & Smith (1995), hydrolysis is the main rate-limiting process in SSF, hence in recent years thermotolerant yeast and bacteria have been used to raise the process temperature close to the optimal hydrolysis temperature. Ward *et al.* (1995) found that a thermotolerant yeast *Kluveromyces marxianus* in a mixed culture with thermophilic filamentous fungi *Taloromyces emersonii*, produced ethanol with a high yield at 45°C, using a starch-containing media as a substrate.

Since 1970 the SSF process has been used and mentioned in general reports (Verma *et al.*, 2000; Öhgren *et al.*, 2006) but little has been cited on the SSF optimization of raw corn flour with a combination of GSHE amyloglucosidase and yeast *Saccharomyces cerevisiae*. Therefore the present work was carried out to identify optimal process conditions for the simultaneous saccharification and fermentation of granular corn starch using GSHE amyloglucosidase and *Saccharomyces cerevisiae* Ethanol Red yeast through a response surface methodology. Additionally, an unstructured kinetic model describing the rate of change in the starch, glucose and ethanol, as well as the biomass concentration, has also been tested.

MATERIALS AND METHODS

Raw material. Commercially available corn flour (BIO CORN, Ziebice, Poland) was used as a raw material for fermentation. It had a median diameter of 250 μ m, and contained 12.8% water, 90.05% starch, and 0.1% ash.

Yeast. A freeze dried distiller's yeast, Ethanol Red (*Saccharomyces cerevisiae*), obtained from Lesaffre Company (Marcq en Baroeul, France) was used in this study for production of ethanol from the corn mashes. The num-

ber of living cells at packing was above 2.0×10^{10} /g, as defined by the manufacturer.

Enzymes. A mixture of granular starch hydrolyzing enzymes, containing *Aspergillus kawachi* α -amylase expressed in *Trichoderma reesei*, and a glucoamylase from *Aspergillus niger* were employed in this study (STARGEN 001, Genecore International, USA). The enzymatic activity of this set of enzymes was ≥ 456 GSHU/g (Granular Starch Hydrolyzing Units), as defined by Genecore International. In addition, fungal acid protease GC 106 (*Aspergillus niger*), also obtained from Genecore International, was added to the mashes. The enzymatic activity of GC 106 was ≥ 1000 SAPU/g (Spectrophotometric Acid Protease Units), as defined by the manufacturer.

Other chemicals. All other chemicals used in this study, purchased from various commercial suppliers, were of analytical grade purity.

Experimental design and statistics. The SSF optimization, by the classical method of a single-dimensional search involving changing of one variable while fixing the others at a certain level, is laborious and time-consuming, especially when the number of variables is large. Furthermore, the other significant disadvantage of the one-factor-at-a-time strategy is that it fails to consider any possible interactions between factors. In fact, if interactions are present and the factorial experimental strategy is not used, incorrect or misleading results may be obtained (Montgomery, 2001). An alternative and more efficient approach to the investigation of a fermentation systems is the use of statistical methods. Many statistical experimental design methods are currently being employed in bioprocess optimization. Among these, response surface methodology (RSM), which employs a combination of mathematical and statistical techniques, is the one most suitable for identifying the effect of individual independent variables and their interactions, and for seeking optimal conditions for a multivariable system efficiently (Montgomery & Runger, 2003). A three-factor ($k = 3$), five-level central composite design (CCD) was used in this study. The effects of corn flour concentration in the mash (X_1), the dose of GSHE (X_2) and the mash pH (X_3), were selected as process (independent) variables. Ethanol concentration (Y_1 , g/l), and the overall process yield (Y_2 , % of theoretical ethanol yield) were the responses (dependent variables). As shown in Table 1 the variable levels X_i were coded as x_i according to the following equation (1):

$$x_i = \frac{2X_i - X_{(\min)i} - X_{(\max)i}}{X_{(\max)i} - X_{(\min)i}}$$

where x_i is the dimensionless value of an independent variable, X_i the real value of an independent variable and $X_{(\min)i}$, $X_{(\max)i}$ are the lower and the upper limit of the independent variable, respectively. The set points were selected according to the results obtained during a preliminary study. This design was chosen for its ability to estimate second-order effects, to detect the interrelationship between the factors, and to identify the response optima according to the experimenter formulated criteria. This experimental plan was divided into three blocks, comprised of 20 actual experiments and including 6 replicates. Replication allowed determination of experimental error and increased precision of the estimates. The order in which experiments were performed was randomised, according to the requirement for observations to be distributed independently and randomly which, in addition, helps to avoid the influence of unknown nuisance vari-

Table 1. Factor variation intervals

Factors	Variation levels				
	-1.4	-1.0	0	+1.0	+1.4
X ₁ — mash concentration [% wt.]	23.0	25.0	30.0	35.0	37.0
X ₂ — dose of GSHE [ml/kg flour dry matter]	0.30	0.75	1.875	3.00	3.45
X ₃ — mash pH	3.20	3.50	4.25	5.00	5.30

ables. The design matrix of the central composite design (CCD) for k = 3 is shown in Table 2.

A multiple regression analysis of the data was carried out in order to obtain empirical models that define the response (Y) in terms of the independent variables:

$$\begin{aligned} \bar{Y}_i = & b_0 + b_1 \cdot X_1 + \dots + b_k \cdot X_k + b_{12} \cdot X_1 \cdot X_2 + \dots \\ & + b_{k-1,k} \cdot X_{k-1} \cdot X_k + b_{11} \cdot X_1^2 + \dots + b_{kk} \cdot X_k^2 \end{aligned}$$

Coefficients of the polynomial, represented by b₀ (constant term), b_k (linear effects), b_{kk} (quadratic effects), and b_{k-1,k} (interaction effects) were correlated to the responses (Y_i). Statistical analysis was carried out in coded data sets in order to simplify interpretation of the results. The accuracy and general ability of the above polynomial model was evaluated by an adjusted coefficient of determination Adj-R², the significance of total regress F-value and the non-significance of lack of fit F-value. The commercial software STATISTICA, version 6.0 PL from StatSoft, Inc., was used for regression and graphical analyses of the data obtained.

Simultaneous saccharification and fermentation.

The SSF experiments were performed in 250 ml Erlenmeyer flasks, each containing 200 ml of fermentation medium formulated according to the experimental design (Table 1). A weighed amount of corn flour was suspended in sterilized water. The pH of the fermentation broth was measured at each sampling and

adjusted by addition of either 10% wt. H₂SO₄ or 20% wt. NaOH. In all cases, the medium was supplemented with acid protease GC 106 (40 μl/kg corn flour dry matter) and chloramphenicol (50 μg/L of the fermentation medium). Fermentation was initiated with addition of freeze-dried distiller's yeast Ethanol Red (0.5 g/L of fermentation medium). The number of living cells in the medium was 1.0 × 10⁷ cells/ml after inoculation. Flasks were incubated in a rotary shaker (200 rpm) at 35°C for 72 h. Samples were taken and analyzed for yeast cell viability as well as for the starch, glucose and ethanol concentration after fermentation. Average results of triplicate experiments are shown.

Model validation. In order to validate the regression model, a new series of fermentation trials was carried out in triplicate. Using the point prediction capability of the software, values of dependent variables were predicted, together with the 95% confidence intervals, and then compared with the experimental results.

Kinetics studies. The SSF kinetics of mashes containing native corn starch were described in a modified unstructured kinetic model developed by Kroumov *et al.* (2006). Kinetics experiments were carried out in a 5 L

Table 2. Central composite design: 2³+(2 × 3)+6 = 20. X₁ — corn flour concentration (% wt.), X₂ — GSHE dose (ml/kg of dry matter), X₃ — mash pH

Run	Block	Actual values X _i			Coded values x _i		
		X ₁	X ₂	X ₃	x ₁	x ₂	x ₃
1	1	25.00	3.000	5.00	-1.0	1.0	1.0
2	1	30.00	1.875	4.25	0.0	0.0	0.0
3	1	35.00	3.000	3.50	1.0	1.0	-1.0
4	1	30.00	1.875	4.25	0.0	0.0	0.0
5	1	25.00	0.750	3.50	-1.0	-1.0	-1.0
6	1	35.00	0.750	5.00	1.0	-1.0	1.0
7	2	35.00	3.000	5.00	1.0	1.0	1.0
8	2	35.00	0.750	3.50	1.0	-1.0	-1.0
9	2	25.00	3.000	3.50	-1.0	1.0	-1.0
10	2	25.00	0.750	5.00	-1.0	-1.0	1.0
11	2	30.00	1.875	4.25	0.0	0.0	0.0
12	2	30.00	1.875	4.25	0.0	0.0	0.0
13	3	30.00	0.300	4.25	0.0	-1.4	0.0
14	3	37.00	1.875	4.25	1.4	0.0	0.0
15	3	30.00	1.875	4.25	0.0	0.0	0.0
16	3	23.00	1.875	4.25	-1.4	0.0	0.0
17	3	30.00	1.875	4.25	0.0	0.0	0.0
18	3	30.00	1.875	3.20	0.0	0.0	-1.4
19	3	30.00	1.875	5.30	0.0	0.0	1.4
20	3	30.00	3.450	4.25	0.0	1.4	0.0

Table 3. Results of central composite design: $2^3+(2 \times 3)+6 = 20$

Run	Ethanol (Y_1) [g/l]		Overall process yield (Y_2) [%]	
	Actual	Predicted	Actual	Predicted
1	112.43	108.25	62.426	59.263
2	113.30	116.20	73.394	74.708
3	116.78	117.85	64.841	65.667
4	117.19	116.20	75.908	74.708
5	94.58	96.49	73.519	76.184
6	106.82	106.10	83.031	82.589
7	119.43	121.20	77.364	78.187
8	115.81	113.25	64.299	62.742
9	118.47	121.20	76.744	78.187
10	120.02	122.85	66.636	69.147
11	107.55	101.49	83.602	79.664
12	109.80	111.10	85.351	86.068
13	79.09	86.22	51.233	55.273
14	103.53	99.67	67.065	64.240
15	102.86	102.40	86.910	87.381
16	115.39	110.63	74.745	71.345
17	112.77	110.63	73.046	71.345
18	119.85	118.86	62.946	63.691
19	106.47	110.63	68.967	71.345
20	109.69	110.63	71.052	71.345

batch bioreactor (BIOFLO III, New Brunswick Scientific, New Brunswick, NJ) with 3.0 L working volume, at 35°C, 100 rpm and an initial biomass concentration of 1.0×10^7 cells/ml. All computations were performed using the Matlab and Simulink Release 2013 (The MathWorks, Inc., Natick, Massachusetts, United States).

Analysis. Samples for the analysis of glucose and ethanol concentrations were first centrifuged at $4000 \times g$ for 10 min at 4°C (Multifuge 3SR, Germany), filtered through a 0.22 μm membrane filter (Millex-GS, Millipore, USA), and then analyzed with an HPLC system (Merck Hitachi, Germany). Glucose and ethanol were separated on Aminex HPX-87P (Bio-Rad, USA) at 30°C using 5 mM H_2SO_4 solution as the mobile phase at a flow rate of 0.6 ml/min, and then detected with a refractive index detector (Model L-7490, Merck Hitachi, Germany). Starch content was analyzed according to the enzymatic method developed by Holm *et al.* (1986). Yeast cell populations were determined by a direct microscopic count in a counting chamber after staining with methylene blue. Yeast cell number was recalculated to express the biomass concentration as kg/m^3 , according to the data presented by Haddad & Lindegren (1953).

Isolation and scanning electron microscopy of starch granules from fermented mash. A sample (5 g) of the fermented mash was first centrifuged at $4000 \times g$ for 10 min at 25°C (Multifuge 3SR, Germany) and then suspended with 45 ml of sterilized water and shaken for 5 minutes. Then, the sample was centrifuged, and the

supernatant was discarded. The sediment was mixed with 50 ml of 0.02 M sodium hydroxide and stirred for 15 min at room temperature. The mixture was centrifuged at $4000 \times g$ for 10 min at 25°C. The supernatant was discarded along with the first layer on top of the starch, which was carefully removed by scraping. The starch was washed with water (6×50 ml), collected by centrifugation and dried in lab-scale freeze-dryer (Christ, Germany). The granules were placed on specimen stubs covered with double sided adhesive tapes and sprayed with gold particles (99.9%) in sputter coater Bal-Tec SCD050 (Balzers, Lichtenstein). The granules were observed and photographed using Scanning Electron Microscope (SEM) Zeiss EVO40 (Carl Zeiss AG, Oberkochen, Germany) with accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

The response surface model developed for the ethanol concentration (Y_1)

New strategy to minimize ethanol production cost is to run simultaneous saccharification and fermentation (SSF) by using the GSH enzymes. In SSF, due to the fact that yeast consume glucose, an inhibition of α - and glucoamylase is almost completely eliminated which ensures that all reactions proceed at a high rate. However, because of the system's complexity and interactions between the system components, several process parameters must be optimized: the substrate concentration, the dose of active components (α -amylase and glucoamylase) in the enzymatic mixture, and the pH of the fermentation slurry.

As shown in Table 3 the maximum predicted ethanol concentration reached approximately 121.8 g/L. Analysis of the variance indicated that the response surface model developed for ethanol concentration (Y_1) was statistically significant, with the probability of the F test being at a level below 0.0001. Furthermore, the probability value of the lack-of-fit test was 0.117, indicating that the regression model is a good predictor of the experimental results. In addition, as was mentioned above, the precision of a model can be checked by the adjusted coefficient of determination Adj-R^2 . In the case of response Y_1 , the value of Adj-R^2 was equal to 0.841, indicating good agreement between the experimental results and the theoretical values predicted by the model equation presented in Table 4. According to this polynomial equation, the ethanol concentration (Y_1) was linearly related to the corn flour concentration in the mash (X_1), while the quadratic term was not found to be significant, resulting in a linear increase in Y_1 with X_1 for all doses of GSHE (X_2) (Fig. 1). The pH effect was regarded as not significant ($p > 0.05$), hence it was removed from the model by the ANOVA backward elimination procedure. Furthermore, it was found that the dose of GSHE (X_2) was highly significant ($p < 0.0001$) in linear and quadratic terms, giving an overall curvilinear effect (Figure 1). The optimal concentrations for the two factors, as obtained from the maximum point of the model, were calculated by the Statistica software to be 34.8% and 2.08 ml/kg for the corn flour concentration (X_1) and the dose of GSHE (X_2), respectively. Similar results were presented by Wang *et al.* (2007), who compared the amount of ethanol and the profile of fermentable sugars during fermentation conducted according to traditional and novel technology, based on the granular starch hydrolyzing enzymes. They found that the final ethanol yield with GSH

Table 4. Estimated coefficients of polynomial equations for investigated dependent variables using coded values. X_1 — corn flour concentration (% wt.), X_2 — GSHE dose (ml/kg of dry matter)

Dependent variable	Term	Coefficient	Std. Err.	F-value	p-value
Ethanol (Y_1) [g/l]	b_0 (interc.)	116.10	1.19		
	$b_1(X_1)$	5.88	1.12	27.69	0.0001
	$b_2(X_2)$	4.81	1.12	18.49	0.0007
	$b_2^2(X_2^2)$	-9.02	1.33	45.99	<0.0001
	$Y_1 = 116.1 + 5.88 b_1 + 4.81 b_2 - 9.02 b_2^2$ (Adj. R^2 0.841; Lack of Fit 0.017)				
Process yield (Y_2) [%]	b_0 (interc.)	74.74	0.97		
	$b_1(X_1)$	-8.46	0.78	118.07	<0.0001
	$b_2(X_2)$	3.20	0.78	16.91	0.0012
	$b_1^2(X_1^2)$	2.13	0.93	5.29	0.0387
	$b_2^2(X_2^2)$	-5.91	0.93	40.36	<0.0001
$Y_2 = 74.74 - 8.46 b_1 + 3.2 b_2 + 2.13 b_1^2 - 5.91 b_2^2$ (Adj. R^2 0.913; Lack of Fit 0.092)					

enzymes was comparable to the conventional enzymes, while the glucose, maltose and maltotriose concentrations had consistently lower GSH enzymes throughout fermentation. The authors concluded that GSH enzymes allow for simultaneous liquefaction, saccharification and fermentation. As mentioned above, high substrate concentration increased ethanol concentration in the SSF, but our results also indicated that ethanol concentration was not highly correlated with the second investigated variable — the overall process yield (Y_2), as shown by the value of Pearson correlation coefficient r , equal to 0.22, with $p = 0.35$. For example, when fermentation was conducted under optimal conditions to reach maximum ethanol concentration, the predicted overall yield was only 69.4%. If we assume that the yields attained in traditional technology, based on fermentation of gelatinized starch, do not usually exceed 90 to 95% of the theoretical value, our results are definitely lower. This clearly indicates that besides ethanol concentration, the

overall process yield — expressed as the theoretical yield of ethanol/starch — is also an important factor affecting the ethanol production cost from starchy feedstock. Consequently, the overall process yield (Y_2) was taken into consideration in the optimization of simultaneous saccharification and fermentation of corn flour mashes.

The response surface model developed for the overall process yield (Y_2)

According to Lee (2007) the principal advantage of ethanol production through the fermentation of starch, especially gelatinized starch, is in its technological simplicity and efficiency. As was mentioned earlier, the process yield in traditional technology falls into the range of 90–95%, thereby providing high economic profitability. From the data

presented in Table 3, it can be seen that the yield was within the limits of 51.2–86.9%, which corresponds to the residual starch range being between 3.4 and 11.5 g/l (not shown). Not surprisingly, these two dependent variables were highly correlated. The calculated Pearson correlation coefficient r was equal to — 0.95 ($p < 0.0001$). Experimental results of the CCD design were fitted into the second-order polynomial (Table 4). The fit of the model was checked by the adjusted coefficient of determination Adj- R^2 , which was calculated to be 0.913, indicating that 91.3% of the variability in the response could be explained by the model. Statistical significance of the second-order model equation was evaluated by the F-test analysis of variance, which revealed that this regression is statistically significant, showing $p < 0.0001$ at 95% of confidence level. In addition, the p value of lack of fit test was 0.092 which exceeds the critical value of 0.05. This means that the model adequately fits the response data.

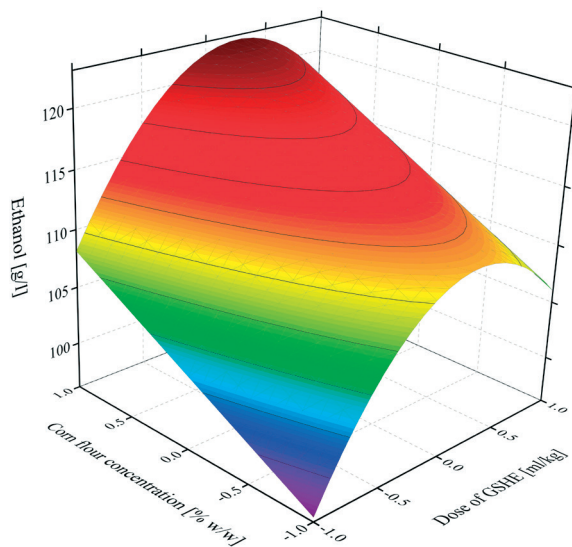


Figure 1. Three dimensional plot showing the effect of corn flour concentration and dose of GSHE on the ethanol concentration after fermentation (Y_1). The pH was fixed at zero coded level. GSHE — granular starch hydrolyzing enzymes

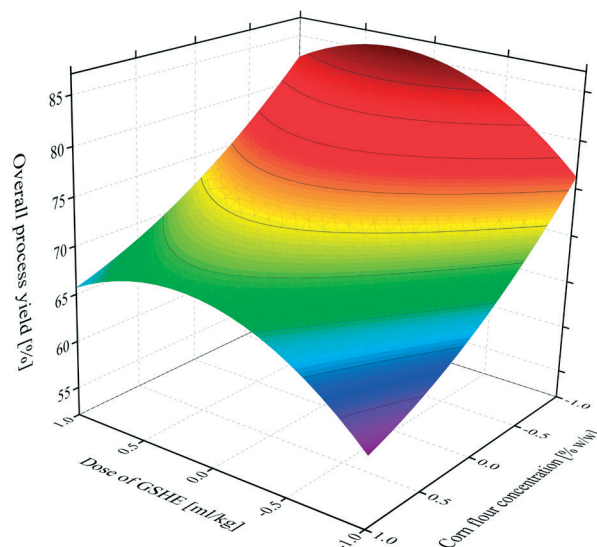


Figure 2. Three dimensional plot showing the effect of corn flour concentration and dose of GSHE on the overall process yield (Y_2). The pH was fixed at zero coded level. GSHE — granular starch hydrolyzing enzymes

Regression analysis of Y_2 showed that when the independent variables X_1 and X_2 were investigated, they were significant at the probability levels of 95%, and proved to be the two most influential factors on the process yield. The pH (X_3) was found to be insignificant at the probability level of 95% and consequently was removed from the model by the ANOVA backward elimination procedure. Moreover, a comparison of the regression coefficient (Table 4) indicates that the value of Y_2 was much more influenced by the value of X_1 than by the value of X_2 . It can further be seen that the main effects for the independent variables X_1 have a negative sign, which means that the investigated value progressively decreased when the corn flour concentration in the mash increased (Fig. 2). The data show that this dependence is different to that obtained during the analysis of variable Y_1 (ethanol concentration). Additionally, the second-order effects for independent variables X_2 also have a negative sign, meaning that the investigated value increased to reach a maximum, and then sharply decreased (Fig. 2). The following conclusions can be drawn from these relationships: to achieve the maximum ethanol concentration Y_1 a high hydrolysis rate is crucial to provide a sufficient level of reducing sugars. At the same time, to obtain the highest possible process yield Y_2 , the utilization of mashes with a lower substrate concentration is recommended. Thus, starch losses may have occurred to a relatively reduced extent. Furthermore, the optimal concentrations for the two factors, as obtained from the maximum point of the model, were calculated by the Statistica software to be 25.0% and 2.18 ml/kg for the corn flour concentration in the mash (X_1) and for the dose of GSHE (X_2), respectively. It is worth noting that the results obtained in our investigation are in excellent agreement with an earlier study of Suresh *et al.* (1999). They also reported that the maximum ethanol yield was obtained in a 250 g/l starch medium. Similar observations concerning relationship between starch concentration and ethanol yield have been made by Abouzied & Reddy (1987). It must be taken into account that contrary to the previous optimization step concerning ethanol concentrations, the indicated value of the mash concentration (X_1) is considerably lower. Therefore, in order to find the best compromise between ethanol concentration and the process yield, a multicriteria decision making approach has to be used.

Process optimization

A typical problem in a simultaneous saccharification and fermentation processes is to find a set of conditions for the input variables that ensure the highest concentration of ethanol and overall process yield. The procedures used to solve this problem involve several steps: responses on the dependent variable Y_i are predicted by

fitting the observed response, using an equation based on the levels of the independent variables, and by finding the levels of independent variables which produce the most desirable predicted response. This relationship between predicted response Y_i on one or more dependent variables and the desirability of the response is called the desirability function (Derringer & Suich, 1980). Presented results clearly indicate that the concentration of ethanol as well as the overall process yield can be affected mainly by altering the investigated independent variables X_1 and X_2 . For that reason, the following conditions were imposed: the corn flour concentration in the mash, the dose of GSHE and the pH had coded values from -1.0 to +1.0. At the same time, the ethanol concentration Y_1 and the overall process yield Y_2 were maintained at the highest possible level, respectively. Before optimization, the response variables Y_i were converted into the desirability function D that varies from 0 to 1 where, if the response is at its goal or target, then $D = 1$, and if the response is outside an acceptable region, $D = 0$. Applying desirability function methodology, the best combination of variables X_1 , X_2 and X_3 for maximizing both Y_1 and Y_2 responses simultaneously has been found to be $X_1 = 25\%$, $X_2 = 2.05$ ml/kg and $X_3 = 5.0$, respectively.

Validation of the model and optimal conditions

In order to verify the adequacy of the model and the optimal conditions developed, four confirmation run experiments were performed (Table 5). The test conditions for the first three confirmation run experiments were taken from the cutting conditions performed previously, whilst the last confirmation run experiment was performed within the range of levels defined at the optimization stage. The predicted values and the actual experimental values were compared, and it can be observed that most values determined experimentally were similar to those determined by the developed models, which definitely proved the validity of the experiments.

Kinetics study

In order to increase starch conversion efficiency, several kinetics models have been developed. Important contributions in this field were made by Polaković & Bryjak (2004), as well as by Kroumov *et al.* (2006). The essential feature of the model developed by Polaković & Bryjak (2004) was that starch was structured into susceptible and resistant fractions that differed in the rate constants of hydrolysis. This assumption gave a good approximation of the substrate and product concentration profile during hydrolysis. A mathematical description of a simultaneous saccharification and fermentation of starch by recombinant strain of *Saccharomyces cerevisiae* YPB-G

Table 5. Validation results. Act. — actual, Pred. — predicted, X_1 — corn flour concentration (% wt.), X_2 — GSHE dose (ml/kg of dry matter), X_3 — mash pH

X_1	X_2	X_3	Y_1 — ethanol concentration				Y_2 — overall process yield			
			Act.	Pred.	Conf. Interval		Act.	Pred.	Conf. Interval	
					-95%	95%			-95%	95%
26.04	0.87	4.0	101.1	99.9	96.5	103.4	75.7	75.2	72.8	77.6
32.92	2.79	5.0	115.0	117.5	114.5	120.5	67.9	69.2	67.0	71.4
33.66	1.95	3.6	115.2	120.7	117.6	123.8	66.5	69.9	67.7	72.1
25.00	2.05	5.0	110.4	110.8	107.3	114.3	85.7	85.7	83.1	88.3

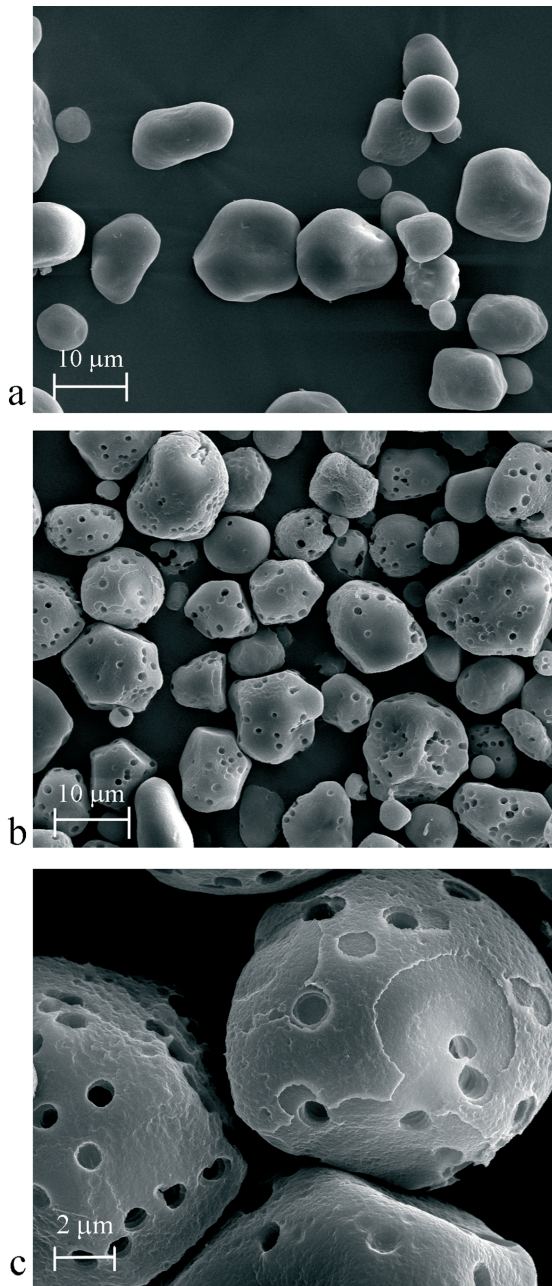


Figure 3. Scanning electron micrograph showing untreated raw corn starch granules (a) and ruptured raw corn starch granules after SSF with granular starch hydrolyzing enzymes (b, c). SSF — simultaneous saccharification and fermentation

was developed and described in detail by Kroumov *et al.* (2006). In this work, the concept of starch being structured into susceptible and resistant fractions was also applied. Furthermore, the authors assumed that the specific growth rate of the recombinant strain was influenced by glucose, and by initial and total starch concentrations. The specific ethanol production rate was described as a function of glucose, initial starch and ethanol concentrations. According to the data presented by Kroumov *et al.* (2006) the proposed model showed excellent flexibility for different operational conditions of the SSF process, and could be used successfully to describe microbial physiology of the genetically modified yeast *Saccharomyces cerevisiae*, secreting enzymes for starch degradation. In the present work, however, an attempt has been made to apply this knowledge to the modeling of saccharifica-

tion and fermentation of mashes containing native corn starch, using enzymes of hydrolyzing granular starch and the distiller's yeast Ethanol Red (*Saccharomyces cerevisiae*). It is well known that most starches are triphasic with alternating concentric growth rings or lamella of amorphous and semi-crystalline character, as well as lipid amylase inclusion complexes (Oostergetel & van Bruggen, 1993; Morgan *et al.*, 1995). Moreover, scanning electron microscopy has shown that there are randomly distributed depressions on the surface of wet starch granules. These depressions are suspected architecturally of being enzyme-susceptible regions (Robertson *et al.*, 2006). This suggests that the biphasic kinetic model developed by Polaković & Bryjak (2004) could also be useful for describing the digestion of raw starch by α - and glucoamylase. However, according to Robertson *et al.* (2006), an uncritical extrapolation of the laboratory observations to technical processes has to consider that starch preparation for the enzymatic studies may, for instance, include a solvent extraction in order to eliminate lipid effects. Sample preparation may introduce artefacts, through dehydration and/or physical abrasion. Thus, contrary to the data presented by Polaković & Bryjak (2004) as well as that of Kroumov *et al.* (2006), granular starch in the corn flour was considered to be a homogenous material containing only one fraction. In our opinion, based on limited available information concerning the proportion of potentially susceptible and resistant fractions in raw starch, it is difficult or impossible to formulate correct estimate of the rate constants. Consequently, if it is assumed that only a single substrate containing starch with some percentage of glucose is added, then the model equations are written as follows:

Enzymatic rate of starch degradation:

$$R_{St} = \frac{k_{St} \cdot Enz \cdot St(t)}{K_m \cdot \left(1 + \frac{Glu(t)}{K_{Glu}}\right) + \frac{St(t)^2}{K_{St}} + St(t)}$$

Mass balance of starch degradation:

$$\frac{dSt(t)}{dt} = -R_{St}$$

Balance of glucose produced during starch degradation:

$$\frac{dGlu(t)}{dt} = 1.111 \cdot (R_{St})$$

Biomass balance:

$$\frac{dX(t)}{dt} = \mu \cdot X(t)$$

and the specific growth rate model:

$$\mu = \frac{\mu_{max} \cdot Glu(t) \cdot \left(\frac{St(t)}{St_0}\right)}{K_s + Glu(t)}$$

Product balance:

$$\frac{dEt(t)}{dt} = q_p \cdot X(t)$$

and the production rate model:

$$q_p = \frac{q_{pmax} \cdot Glu(t) \cdot Et(t) \cdot \left(1 - \frac{Et(t)}{Et_{max}}\right)}{(K_{s1} + Glu(t)) \cdot \left(K_{ps1} + Et(t) + \frac{Et(t)^2}{K_{pi}}\right)}$$

Glucose balance:

$$\frac{dGlu(t)}{dt} = R_{Glu}(\text{formation}) - R_{Glu}(\text{utilization})$$

where:

$$R_{Glu}(\text{formation}) = 1.111 \cdot (R_{St})$$

$$R_{Glu}(\text{utilization}) = \frac{1}{Y_{X/S}} \cdot \frac{dX(t)}{dt} + \frac{1}{Y_{P/S}} \cdot \frac{dEt(t)}{dt}$$

The reaction rate for hydrolysis of the starch is a Michaelis-Menten kinetic model, which considers the substrate inhibition of starch K_{St} as well as the competitive product inhibition of glucose K_{Glu} . It should be noted that the model considers only one enzyme activity involved in starch degradation to glucose by the synergistic action of α - and glucoamylases. In the present study, similar to the works of Kroumov *et al.* (2006) and Davis (2008), we have assumed that this two-enzyme action can be simplified and represented as a sum of individual enzyme activities. As shown in Table 6, estimated values of the kinetic parameters concerning starch hydrolysis indicate that the enzyme complex investigated is greatly inhibited by glucose ($K_{Glu} = 0.0857$), whereas the substrate inhibition of starch should be regarded as an insignificant factor ($K_{St} = 999.98$). Similar conclusions were drawn by Polaković & Bryjak (2004) as well as by Kroumov *et al.* (2006). However, a striking discrepancy between their results was observed concerning K_{St} and K_{Glu} and the present data. Respectively, the inhibition constant K_{St} value exceeded 60 kg/m³, whereas the K_{Glu} value was 1.1 kg/m³. It might therefore contribute to the use of the raw starch as a substrate and a different enzyme system. In addition, the estimated value of the rate constant k_{St} was almost 100 times lower than the rate constant obtained by Kroumov *et al.* (2006). It is probable that the rate of soluble starch digestion was much faster, because α -1,4-bonds for α -amylase and α -1,4-bonds at nonreducing ends for glucoamylases were readily susceptible to an enzyme attack, whereas in the case of the digestion of native starch granules, polysaccharide backbones were concentrated in the solid-phase portion

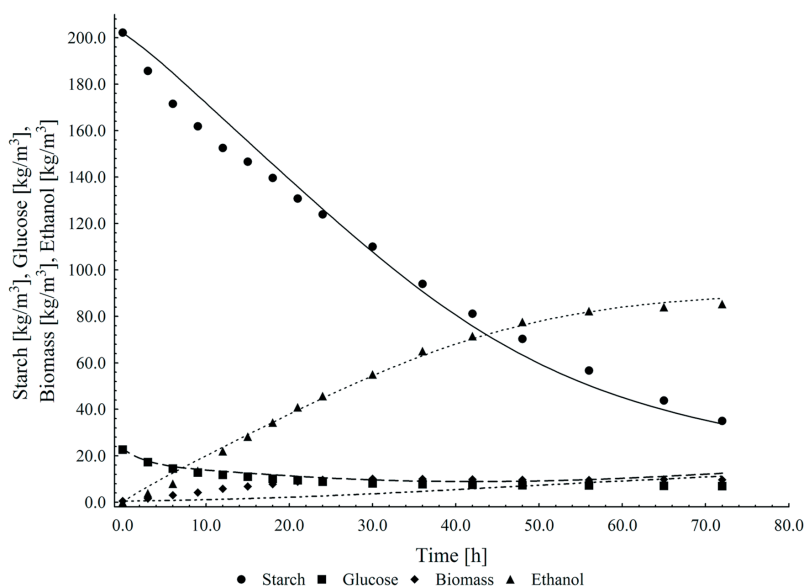


Figure 4. Kinetics of simultaneous saccharification and fermentation of corn flour mash conducted in a 5 L volume bioreactor

Table 6. The SSF initial and estimated kinetic parameters

No.	Parameters	Value	Unit
Initial			
1	St_0	202.34	kg/m ³
2	Glu_0	23.03	kg/m ³
3	X_0	0.5	kg/m ³
4	Et_0	0.0	kg/m ³
5	Enz	262612.5	U/m ³
Estimated			
1	K_m	26.998	kg/m ³
2	k_{st}	0.000333	kg/U·h
3	K_{st}	999.98	kg/m ³
4	K_{Glu}	0.0857	kg/m ³
5	μ_{max}	0.6057	h ⁻¹
6	K_s	7.0237	kg/m ³
7	q_{pmax}	4.9951	h ⁻¹
8	Et_{max}	90.007	kg/m ³
9	K_{s1}	0.00633	kg/m ³
10	K_{ps1}	0.00046	kg/m ³
11	K_{pi}	14.687	kg/m ³
12	$Y_{p/s}$	0.4759	kg/kg
13	$Y_{x/s}$	0.8133	kg/kg

of the reaction mixture. Several different suggestions have been put forward concerning the routes of raw starch granules digestion. These include: local or distributed digestion at the surface pores, centripetal digestion along the starch polymer chains, digestion at the cracks, or diffusion through the starch structure to susceptible sites (Robertson *et al.*, 2006). However, the most probable explanation has been deduced by Matsubara *et al.* (2004), from the microscopic observation of corn starch granules hydrolyzed with a mixture of α - and glucoamylase. They claim that glucoamylase initially acted on the starch granule surface, forming small holes, whereas α -amylase was adsorbed around the hole and released oligosaccharides, which were further decomposed to glucose by the action of glucoamylase. Furthermore, when the above-mentioned enzymes were used together, they created more holes per unit area, and the size of the holes was expanded further. Thus, when the substrate was digested, additional sites for the reaction were exposed. Scanning electron microscopy of the starch granules isolated from fermented mash supports the efficient action of enzyme preparation used in this study towards raw corn starch. Figure 3a shows that the surface of untreated starch granules was smooth, whereas Figs 3b and 3c shows that the pores of the hydrolyzed starch were randomly distributed due to the breakdown of granules by the

GSH enzyme. It is worth pointing out that, according to Robertson *et al.* (2006) the representation of an initial substrate concentration is ambiguous, and probably has some effect on the estimate of the rate constant k_{st} . On the other hand, when the STARGEN 001 was used during the SSF of food waste containing starch, the inhibition coefficients K_{st} and K_{glu} also differed from those estimated in the present work, despite the fact that the estimated value of K_m equal to 26.998 kg/m³ agreed well with the value of 27 kg/m³ found by Davis (2008). According to Davis (2008) the values of K_{st} and K_{glu} were equal to 0.076 and 0.5 kg/m³, respectively. Thus, under these conditions, the rate of starch hydrolysis was highly dependent on the substrate as well as the product concentration. This definitely confirms that both, molecular and physical structure of the solid substrate, and molecular configuration of the enzyme used, may contribute to the hydrolysis as well as to the fermentation efficiency. Thus, a direct comparison with other studies becomes difficult, especially since most available correlations are made for gelatinized starch. However, our results for ethanol yield $Y_{p/s}$ agree well with the results obtained by Kroumov *et al.* (2006) and Davis (2008) as well as Montesinos & Navarro (2000), who studied the SSF process of alcohol production from gelatinized wheat flour using amyloglucosidase and the yeast *Saccharomyces cerevisiae*.

Finally, it could be said that, in spite of complexity, the model predicts experimental data reasonably well, as can be seen in Fig. 4. The discrepancies between simulation results and experimental data could be due to several reasons, which differ from those discussed earlier. There is some uncertainty in the method used to determine the concentration of yeast cells employed in these experiments. Because of the high concentration of corn flour particles, the yeast cell populations were determined by a direct microscopic count in a counting chamber after staining with methylene blue. The yeast cell number was recalculated to express the biomass concentration as kilogram per cube meter, according to the assumption that weight of an individual yeast cell is equal to 7.99×10^{-11} g, with a standard deviation 3.188×10^{-11} g (Haddad & Lindergren, 1953). Consequently, considering this standard deviation, if this approach is used for biomass determination, it can lead to inaccurate results. Unfortunately, besides the data presented by Haddad & Lindergren (1953), no relevant experimental framework exists for the determination of the weight of an individual yeast cell.

CONCLUSIONS

According to the optimization results, the maximum ethanol concentration of 110.36 g/l was obtained using mash concentration of 25%, which resulted in the predicted ethanol yield of 85.71%. The optimum conditions for the above yield were found for enzyme dose of 2.05 ml/kg and pH of 5.0. Nevertheless, it is possible to increase the concentration of starch in the stream feeding the fermentor to above 35%. A reasonable approach to the increase in the productivity of alcoholic fermentation of the concentrated mashes is the removal of the product, for instance by applying pervaporation or membrane distillation. Coupling of the SSF with the above mentioned separation systems reduces natural inhibitions of cell growth caused by high concentrations of ethyl alcohol, whereas the SSF process permits reduction of the enzyme inhibition by glucose during starch hydrolysis. In summary, the results reported in this work show that the

SSF process applied to native starch, using novel GSH enzymes, stands as an excellent alternative for traditional technology in the production of fuel ethanol. This may lead to more sustainable processing, characterized by less energy consumption, larger volumetric productivity, as well as a better utilization of substrates.

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