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SCIENTIFIC PAPER

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BIOETHANOL FROM CORN MEAL HYDROLYZATES

The two–step enzymatic hydrolysis of corn meal by commercially available α–amylase and amyloglycosidase and the subsequent or simultaneous ethanol fermentation of the hydrolyzates by Saccharomyces cerevisiae yeast were studied. The conditions of starch hydrolysis, such as substrate and enzyme concentration and the time required for enzymatic action, were optimized taking into account both the effects of hydrolysis and ethanol fermentation. The corn meal hydrolyzates obtained were good substrates for ethanol fermentation by Saccharomyces cerevisiae. A yield of ethanol of more than 80% of the theoretical one was achieved with a satisfactory product to substrate yield $Y_{P/S}$ (g/g) and good ethanol volumetric productivity P (g/l·h). No shortage of fermentable sugars was observed during simultaneous hydrolysis and fermentation. Savings in time and energy could be realized by such a process.

Key words: Enzyme hydrolysis, Starch, Corn, Ethanol fermentation.

In recent years, research and development efforts directed the toward the commercial production of ethanol as the most most promising biofuel from renewable resources have increased. In many developed countries in Europe and in the USA the use of bioethanol as an alternative fuel or a gasoline supplement in amounts up to 15% is highly recommended [1–3] or even required as an ecologically favorable fuel oxygenate [4]. Concerning the EU, a new directive was accepted in November, 2001 that requires member states to establish legislation about the utilisation of fuels from renewable resources. In 2005 this utilisation should cover 2% of the total fuel consumption. This quota is expected to increase to 5.75% in 2010. Some member states such as Finland, Sweden or Austria have already fulfilled this quota [5].

Word ethanol production in 2003 was 32 Mm^3 [6]. The major world producers are Brazil and the US, which together account for about 80% of the world production. Around 60% of the ethanol is produced by fermentation [7]. The main feedstocks for bioethanol production are sugar cane (in Brazil) and corn grain (USA), while many other agricultural raw materials rich in fermentable carbohydrates, or those locally available that could be converted to yield fermentable sugars, are used worldwide. Feedstocks based on corn, potato, sorghum, Jerusalem artichoke and lignocellulosic biomass are of the greatest interest for ethanol production in Serbia [8].

An important issue regarding bioethanol production is whether the process is economical [9–11]. Research efforts are focused to design and improve a process which would produce a sustainable transportation fuel. A low cost of feedstock is a very important factor in establishing a cost effective technology [12,13]. In order to analyze the cost effectiveness of bioethanol derived from corn grain, estimates of the net energy balance of ethanol production were made. In a study which was based on data from processing technologies used in the early 1980s, Pimentel [13] concluded that the energy requirement for producing ethanol derived from corn grain was higher than the energy content of ethanol. However recently, Shapouri et al. [14] and Kim and Dale [1] estimated that the energy content of ethanol derived from corn grain was higher than the energy required to produce ethanol. It is believed that the improvement in the last decade was primarily due to increasing corn yields, lower agricultural chemical use, and improved corn processing technologies.

The hydrolysis of corn starch may be considered as the first and key step in corn processing for bioethanol production. The main role of this step is to effectively provide the conversion of two major starch polymer components: amylose, a mostly linear α–D–(1–4)–glucan and branched amylopectin, and α–D–(1–4)–glucan, which has α–D–(1–6) linkages at the branch points, to fermentable sugars that could subsequently be converted to ethanol by yeasts or bacteria. Recent advances in the development of thermostable α–amylases, starch liquefying enzymes which catalyze the hydrolysis of internal α–D–(1–4)–glycosidic linkages in starch in a random manner [15–18] and effective glucoamylases [19,20], starch saccharifying enzymes which catalyze the hydrolysis of the α–D–(1–4) and α–D–(1–6)–glycosidic bonds of starch from the non–reducing ends giving glucose as the final product, have led to commercial establishment of the so called "two enzyme cold process" [7]. The main advantages of this process are lower energy consumption and a lower content of non–glucosidic impurities, and thus much better suitability for ethanol production.

The aim of this study was to investigate the two–step enzymatic hydrolysis of corn meal by

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commercially available α–amylase and amyloglycosidase and ethanol fermentation of the obtained hydrolyzates by various inoculum sizes of *Saccharomyces cerevisiae* yeasts. The conditions of starch hydrolysis such as the substrate and enzyme concentration and the time required for the enzymatic action were optimized taking into account both the effects of hydrolysis and the ethanol fermentation.

MATERIALS AND METHODS

Starch

Corn meal obtained by the dry milling process was a gift form the starch industry "Jabuka–Pančevo". The corn meal consisted of 90% of the particles with an average size between 0.5 to 1 mm and 10% of the particles with a size smaller than 0.5 mm.

Enzymes and microorganisms

Termamyl 120 L, a heat–stable α–amylase from *Bacillus licheniformis* was used for corn meal liquefaction. The enzyme activity was 120 KNU/g (KNU=Kilo Novo Units α–amylases – the amount of enzyme which breaks down 5.26 g of starch per hour according to Novozyme's standard method for the determination of α–amylase). Supersan 240L *Aspergillus niger* glucoamylase, activity 240 AGU/g (AGU is the amount of enzyme which hydrolyses 1 μmol of maltose per minute under specified conditions) was used for corn meal saccharification. The enzymes were a gift from Novozymes, Denmark.

Saccharomyces cerevisiae was used for the fermentation of hydrolyzed corn meal. The culture originated from the collection of BIB–TMF, Belgrade, and was maintained on a malt agar slant. The agar slant consisted of malt extract (3 g/l), yeast extract (3 g/l), peptone (5 g/l), agar (20 g/l) and distilled water (up to 1 l). Before use as an inoculum for the fermentation, the culture was aerobically propagated in 500 ml flasks in a shaking bath at 32°C for 48 hours and then separated by centrifugation. The liquid media consisted of yeast extract (3 g/l), peptone (3.5 g/l), KH_2PO_4 (2.0 g/l), $MgSO_4$ x $7H_2O$ (1.0 g/l), $(NH_2)_2SO_4$ (1.0 g/l), glucose (10 g/l) and distilled water. Various amounts of inoculum (1%, 1.35% and 2% w/w) were used for the fermentation of corn meal hydrolyzates.

Hydrolysis experiments

Corn meal, 100 g was mixed with water in various weight ratios (1:1.25, 1:3, 1:4, 1:5) and 60 ppm of Ca^{2+} (as $CaCl_2$) ions were added. The mixture was treated with enzymes in two steps. The first step, liquefaction, was performed at 85°C and pH=6.0 with various concentrations of Termamyl 120 L, and the second step, saccharification, was performed at 55°C and pH=5.0 with various concentrations of Supersan 240L. The hydrolysis was performed in flasks in a thermostated water bath with shaking (ν=150 rpm).

Ethanol fermentation of starch hydrolyzates

Starch hydrolyzates obtained by the two–step hydrolysis of corn starch meal were subjected to ethanol fermentation by Saccharomyces cerevisiae under anaerobic conditions (pH=5.0, t=32°C, mixing rate ν=100 rpm). The mashes were fermented up to 48 hours with various initial yeast concentrations. During the fermentation the consumption of the substrate was followed as well as ethanol formation.

Analytical methods

The starch content was determined by the Ewers polarimetric method [21]. The water content in the corn meal was determined by the standard drying method in an oven at 105°C to constant mass. The lipid concentration was determined according to the Soxlet method. The protein content was estimated as the total nitrogen by the Kjeldahl method multiplied by 6.25, and the ash content was determined by slow combustion of the sample at 650°C for 2 hours [22]. During the corn meal hydrolysis and fermentation, the content of reducing sugars was determined by 3, 5–dinitrosalicylic acid [23]. A standard curve was drawn by measuring the absorbance of known concentrations of glucose solutions at 570 nm. The ethanol concentration was determined based on the density of the alcohol distillate at 20°C and expressed in weight %. At least three measurements were made for each condition and the data given were averages.

RESULTS AND DISCUSSION

Enzymatic hydrolysis

The corn meal used for the experiments was dry milled without previous separation of the germ. The content of the main components was determined by chemical analysis and presented in Table 1.

The degree of hydrolysis of native starch from the corn meal depends on factors such as the substrate concentration, type and concentration of the enzyme used, and on the applied process conditions such as pH, temperature and the mixing rate. The first set of experiments was conducted in order to determine the

Table 1. Chemical composition of the corn meal

Component	(%)
Starch	70.82
Proteins	11.80
Lipids	7.89
Ash	5.58
Water	3.91

concentration of α–amylase (Termamyl) and the time needed for an appropriate liquefaction of the corn meal. The substrate concentration represented by the ratio of substrate to water (hidromodulus) was fixed at 1:3, which corresponded to an initial concentration of starch of 17.5%.

The effect of the concentration of Termamyl (represented as activity units) on the dextrose equivalent of corn meal is presented in Figure 1. The dextrose equivalent (DE) of the corn meal hydrolyzate was directly proportional to the added activity of the enzyme in a given time of enzyme action. It may be seen from Figure 1 that DE value of 16 is reached after one hour of action of 12 KNU of Termamyl, while a DE value of 19 could be obtained with 14 KNU. As shown in Figure 2, by extending the enzyme reaction in the first step, higher DE values could be attained, indicating that the same conversions could be achieved with lower enzyme concentrations in a longer period of time. However, in our opinion, extension of the enzyme reaction in the first step is not economically justified because of the high temperature which is employed. In addition, the longer exposure of enzyme to high temperatures, which is needed for gelation of the starch granules required for its good susceptibility to enzyme action, could lead to slight enzyme deactivation.

A further objective was to determine the optimal degree of liquefaction of the corn meal needed for adequate saccharification in the subsequent step. For this purpose liquefied corn meal with various degrees of liquefaction (eg. with different DE values) was subjected to the action of the sacharifying enzyme Supersan glycoamylase (24 AGU). The results are presented in Figure 3. The highest DE value after treatment with both liquefying and sacharifying enzymes was obtained when the DE after the treatment with Termamyl was around 16 (Figure 3). A similar optimal DE after liquefaction was reported earlier [7,24].

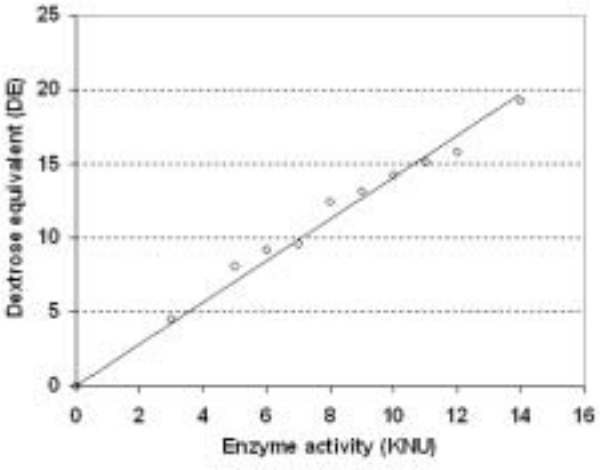

Figure 1. Effect of the added activity of α–amylase (Termamyl) on the dextrose equivalent of corn meal. Process conditions: Hidromodulus: 1:3; t=85°C; pH=6.0; τ=1h.

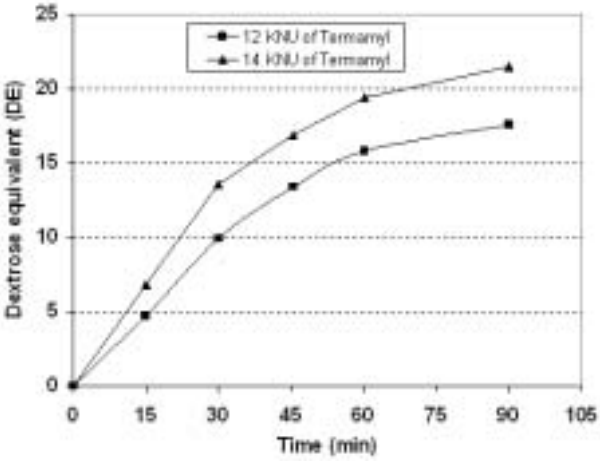

Figure 2. Kinetics of corn meal hydrolysis by α–amylase (Termamyl). Process conditions: Hidromodulus: 1:3; t=85°C; pH=6.0.

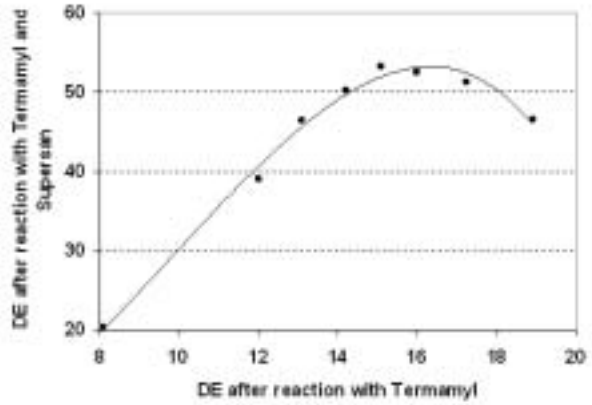

Figure 3. Effect of the DE value of the hydrolyzate liquefied by Termamyl on the DE value of the hydrolyzate obtained by Supersan. Process conditions for Termamyl: Hidromodulus 1:3; t=85°C; pH=6.0; τ=1h. Process conditions for Supersan: t=55°C; pH=5.0, A=24 AGU, τ=4h.

In order to determine the optimal concentration of glycoamylase, the saccharifying enzyme which is necessary for the complete conversion of starch to glucose, the corn meal hydrolyzate treated for an hour with 12 KNU of Termamyl (up to DE=16) was subsequently treated with various amounts of Supersan glycoamylase. Figure 4 presents the DE values obtained. It may be seen that the highest conversion of corn meal starch (DE=93.8%) was achieved with the combined action of 12 KNU of Termamyl and 48 AGU of Supersan after 48 hours. However, a similar and rather high conversion (DE=92.1%) was obtained in a two–step enzymatic hydrolysis with 36 AGU of Supersan, thus indicating that the lower amount of enzyme is sufficient for the effective conversion of the substrate. In all experiments within 24 hours more than 90% of the total conversion performed within 48 hours was attained.

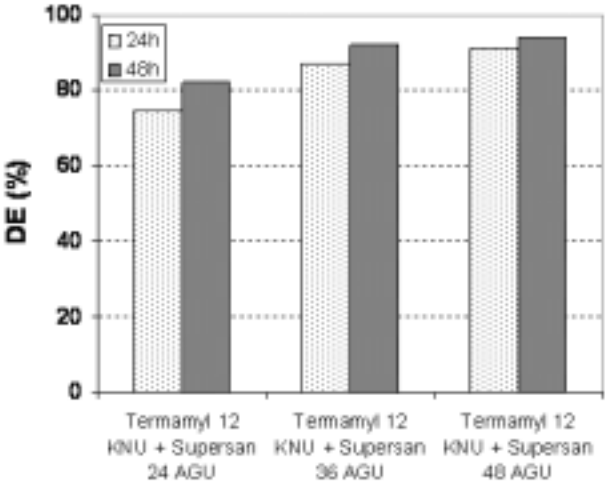

Figure 4. Influence of the amount of Supersan on the DE values of the hydrolyzates. Process conditions for Termamyl: Hidromodulus: 1:3; t= 85°C; pH=6.0; τ=1h; A=12 KNU. Process conditions for Supersan: t=55°C; pH=5.0, A=24, 36 or 48 AGU, τ = 24 and 48 h.

The two enzymes used in our study exhibited a high efficiency in corn starch conversion, which was comparable with the results of many other researchers. Arasaratnam et al. [25] reported a lower glucose yield of 76.0% on corn flour by using a similar combination of amylase and glucosidase. Some differences in the results may originate from different compositions of the initial substrate, the substrate concentration and different experimental conditions applied. Dettori–Campus et al. [26] hydrolyzed starch granules from various cereals by amylase from *Bacillus stearothermophilus* and obtained up to 80% conversion for barley, corn and rice starches, while the conversion of potato starch was less efficient. Similar yields, lower than 80%, were obtained in one step enzymatic hydrolysis with glucoamylase performed by Kimura and Robyt [27]. Leach and Schoch [28] reported that raw corn starch is more susceptible to α–amylase action than high amylase corn starch. A very efficient conversion of corn starch of more than 96% after 24 h was reported by Karakastsanis and Liakopoulus–Kyriakidis [29] using the simultaneous action of α–amylase and glucoamylase. Generally considered, the two step enzymatic process was shown to be more efficient than one–step enzymatic action.

ETHANOL FERMENTATION OF THE CORN MEAL HYDROLYZATES

Effect of the initial substrate concentration

In the first set of experiments the effect of the initial corn starch concentrations on ethanol fermentation was assessed. Mixtures of various concentrations of corn meal and water, which corresponded to the initial starch concentrations C_0=11.5%; 14%; 17.5% and 20%, were liquefied by treatment with Termamyl (τ=1h, t=85°C, pH=6.0, 12 KNU per 100 g of corn meal), then cooled to 55°C, adjusted to pH=5, and treated with Supersan (36 AGU per 100 g of corn meal) for 4 hours. After that the temperature was decreased to 32°C and the mixture was inoculated with 1% of *Saccharomyces cerevisiae* and subjected to ethanol fermentation. The results are presented in Table 2.

Table 2. Results of the batch fermentation of corn meal hydrolyzates with different initial starch content

Hidro-modulus (weight ratio of corn meal to water)	Initial substrate concentration (% of starch)	DE after two–step enzymatic action[1]	Maximum % of ethanol[2]	% of the theoretical yield of ethanol	$Y_{P/S}$ (g/g)	P (g/l·h)
1:5	11.5	75.5	5.7	89.2	0.50	1.21
1:4	14.0	72.2	6.8	87.5	0.48	1.41
1:3	17.5	68.1	7.7	78.5	0.44	1.60
1:2.5	20.0	58.4	6.9	62.4	0.34	1.43

[1]Enzymatic treatment: 12 KNU of Termamyl at t=85°C; pH=6.0; τ=1h; 36 AGU of Supersan at t=55°C; pH=5.0; τ=4h.

[2]Ethanol fermentation: t=32°C; pH=5.0; τ=48h; 1% of Saccharomyces cerevisiae.

The initial substrate concentration had a pronounced effect on both the starch hydrolysis and the ethanol fermentation. Regarding the yields, lower substrate concentrations are more suitable since substrate inhibition can be avoided. Arasaratnam et al. [25] reported lower effects of the hydrolysis of corn flour starch for higher initial starch concentrations, e.g., when a 16% suspension of corn flour was hydrolyzed, the glucose yield was 76%, but when a 40% suspension was hydrolyzed the yield was only 50.2%. Similar substrate inhibition was also noticed for ethanol fermentation [9,30]. The inhibition of fermentation becomes significant with increase of the initial substrate concentration to higher than 15% [24]. According to the results presented in Table 2, the maximum ethanol concentration (%) and the maximum value of the product yield on the substrate ($Y_{P/S}$) were achieved for the lowest initial corn starch concentration in the mixture (e.g., 11.5%).

However, from the economic viewpoint, it is desired to attain as high ethanol concentrations as possible, in order to decrease the costs of ethanol distillation, which are considerable in the economical evaluation of the overall process [2,30]. In addition, the use of higher initial substrate concentrations is economically favorable, since it could decrease the reactor volumes [7]. Taking into account the ethanol concentrations achieved, the yields of products per substrate ($Y_{P/S}$) and the volumetric productivities of ethanol (P) presented in Table 2, and related economic points, we can suggest using an initial concentration of 17.5% which corresponds to a hidromodulus of 1:3.

Effect of inoculum size

The time course of the ethanol fermentation of corn starch hydrolyzate was performed with various inoculum sizes of *Saccharomyces cerevisiae* and the results are presented in Figure 5. The increase of inoculum size did not have a pronounced effect on the final ethanol concentration. The final ethanol yields obtained by fermentation with 1%, 1.35% and 2% of inoculum were 78.5, 80.1 and 81.6% of the theoretical yield, respectively. However, the duration of fermentation decreased with increasing inoculum size. Thus, fermentation with 1% of yeast lasted 48 hours, with 1.35% of yeast 36 hours, while with 2% of yeast the fermentation was accomplished in 32 hours (Figure 5).

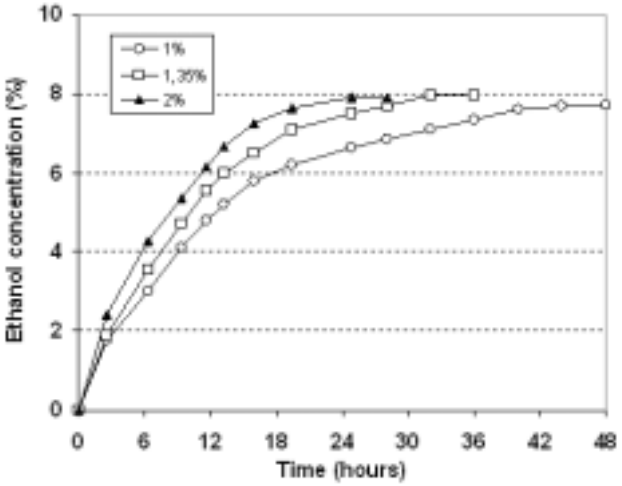

Figure 5. Effect of the inoculum size of Saccharomyces cerevisiae on the fermentation of starch hydrolyzate. Enzyme treatment: Hidromodulus: 1:3; 12 KNU of Termamyl at t=85°C; pH=6.0; τ=1h; 36 AGU of Supersan at t=55°C; pH=5.0; τ=4h. Ethanol fermentation was performed at pH=5.0 t=32°C, mixing rate ν=100 rpm with various inoculum sizes of Saccharomyces cerevisiae

Simultaneous hydrolysis and fermentation

In order to reduce the time of the complete process and make beneficial energy savings, we performed a simultaneous process of the second hydrolysis step and fermentation of corn meal. For that purpose, a mixture of corn meal with water (17.5% of starch) was liquefied (12 KNU of Termamyl per 100 g of corn meal τ=85°C, pH=6). The mixture was then cooled to 32°C, the pH adjusted to 5.0, and simultaneous fermentation by *Saccharomyces cerevisiae* and hydrolysis by Supersan glucosidase were performed. The kinetics of the process are presented in Figure 6.

The time course of the ethanol fermentation and the final ethanol concentration achieved (Figure 6) were very similar to those obtained by fermentation which followed two step enzymatic actions. These data suggested that the ethanol produced during the fermentation did not have an inhibitory effect on the enzymatic hydrolysis. In the literature, there are very few recent reports on the stimulation of enzyme action by ethanol [31]. Apar and Ozbeck, 2005 [31] found that the

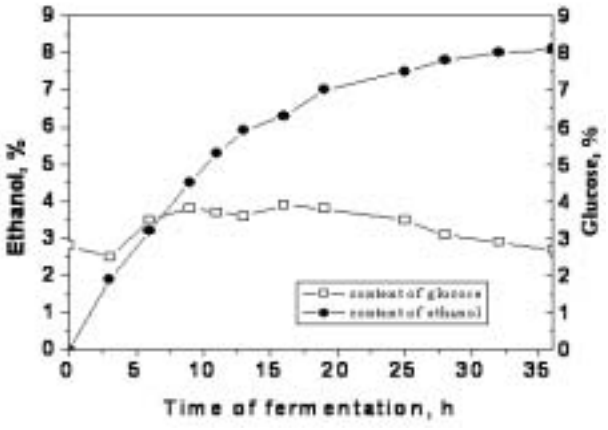

Figure 6. Time course of simultaneous hydrolysis and fermentation. Enzyme treatment first step: Hidromodulus: 1:3;12 KNU of Termamyl at t=85°C; pH=6.0; τ=1h. Ethanol fermentation: pH=5.0; t=32°C; ν=100 rpm; 1.35% of Saccharomyces cerevisiae. The second step enzyme treatment with 36 AGU of Supersan was performed concurrently with fermentation.

addition of ethanol in the range of 5–15% could increase the degree of hydrolysis by α–amylase. This incentive effect was exhibited in the presence of starch as a substrate, while in the absence of starch the residual enzyme activity was decreased. As presented in Figure 6, the concentration of glucose was constantly maintained low during the fermentation, since the produced glucose was simultaneously consumed by the yeast and converted to ethanol. The time course of the glucose concentration during the fermentation (Figure 6) suggested that the sugar production from starch was in accordance with its consumption by yeasts. The progress of ethanol production showed that there was not a shortage of fermentable sugars during the process.

CONCLUSION

The two–step enzymatic hydrolysis of corn meal by commercially available α–amylase and amyloglycosidase and the subsequent or simultaneous ethanol fermentation of the hydrolyzates by *Saccharomyces cerevisiae* was studied. The conditions of starch hydrolysis such as substrate and enzyme concentration and the time required for the enzymatic action were optimized taking into account both the effects of hydrolysis and the ethanol fermentation.

The optimal DE which should be reached in the first enzymatic liquefaction step with 12 KNU of Termamyl per hundred grams of corn meal was observed to be around 16. Further saccharification and fermentation steps were effectivly performed by using 36 AGU of Supersan glucoamylase per hundred grams of corn meal and choosing an initial substrate concentration of 17.5% of starch (1:3 hidromodulus). In this way, an ethanol yield of more than 80% of the theoretical one was attained.

By increasing the inoculum size from 1% to 2% of *Saccharomyces cerevisiae* the fermentation time could be reduced from 48 to 32 hours, respectively.

Fermentation and hydrolysis can be concurrently performed, and no shortage of fermentable sugars was observed, suggesting that these two processes were in accordance. In this way, the time of the overall process may be reduced by at least for four hours (the time required for the second hydrolysis step) and certain energy savings could be obtained.

Acknowledgement

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IZVOD

PROIZVODNJA BIOETANOLA NA HIDROLIZOVANOM KUKURUZNOM BRAŠNU

(Naučni rad)

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U radu je ispitivan postupak dvojno enzimske hidrolize kukuruznog brašna pomoću komercijalnih enzima α–amilaze i amiloglukozidaze i naknadna ili uporedna fermentacija hidrolizata pomoću kvasca Saccharomyces cerevisiae. Uslovi hidrolize kao što su koncentracija supstrata i enzima i vreme dejstva pojedinih enzima su optimizovani sa aspekta oba procesa (i fermentacije i hidrolize). Dobijeni hidrolizati kukuruznog brašna su se pokazali kao pogodni supstrati za etanolnu fermentaciju pomoću kvasca *Saccharomyces cerevisiae* pri čemu je ostvaren prinos etanola od više od 80% od teorijskog prinosa i sa zadovoljavajućim vrednostima prinosa produkta po substratu $Y_{P/S}$ (g/g) i volumetrijske produktivnoosti P (g/l·h). U toku postupka sa uporednim odvijanjem hidrolize i fermentacije nije primećen nedostatak fermentabilnih šećera. Osim toga ovim postupkom moguće je skratiti ukupno vreme procesa i ostvariti odredjene energetske uštede.

Ključne reči: Enzimska hidroliza, Skrob, Kukuruz, Alkoholna fermentacija.