Energy-saving and by-products-free production of ethanol from granular corn starch

DARIA SZYMANOWSKA*, WŁODZIMIERZ GRAJEK

Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, Poznań, Poland

*Corresponding author: darszy@up.poznan.pl

Abstract

Intense development of the transport sector and a rise in the prices of fossil fuels boost the demand for fuels from alternative sources of energy, including biofuels. Thus, new energy-efficient technologies of fuel production from renewable resources are being developed. Accordingly, we attempted to create a technology of bioethanol production by simultaneous enzymatic hydrolysis and fermentation of native corn starch. In our work we used commercially available STARGEN 001 enzymatic preparation for native corn starch hydrolyzis, and yeast *Saccharomyces cerevisiae* strain Red Star Ethanol Red for it's fermentation. A major part of the research was on developing a fermentation technology with repeated full recirculation of stillage liquid fraction. It has been shown that a completely effluentless ethanol production with separation of stillage solid fraction and using the liquid fraction to prepare the next portion of corn mash is possible. Thus the process can proceed for several months without any effluent.

Key words: ethanol, simultaneous saccharification and fermentation, stillage, recycling

Introduction

The technologies of biological ethanol production have been known for a long time. So far they have been based on the process of initial starch processing which requires liquefaction of starch at high temperature to obtain a starch solution. This generates high energy consumption, usually amounting to about 30-40% of all energy needed for ethanol production (Lim et al., 2003). In order to reduce the costs, there is constant pursuit of energy-efficient technologies that ensure high productivity. A possible solution is one-step method of ethanol production by simultaneous hydrolysis and fermentation in one reactor – SSF, Simultaneous Saccharification and Fermentation (Bednarski and Reps, 2003; Öhgren et al., 2007) using the enzymes that can hydrolyze native starch and prevent its gelatinization In this method, the products of hydrolysis are immediately used by microorganisms. Consequently, the enzymes inhibition is limited and ethanol productivity is higher by 25-40% in comparison to the traditional two-step method. Additionally, a one-step method eliminates the need of using two separate tanks (cost reduction) and theoretically reduces the length of the process by excluding the time of preliminary fermentation that occurs already during hydrolysis (Kobayashi et al., 1998). Since the process of hydrolysis occurs in higher temperatures than fermentation numerous projects are focused on finding the enzymes which are active at lower temperature or thermotolerant microorganisms that have the enzymes which can both ferment and hydrolyze a given substrate. The optimal temperature of glucoamylase and α-amylase activity is about 60°C, while most of distiller's yeast strains have the growth optimum of 30-32°C. In the process of simultaneous saccharification and fermentation, the optimal temperature of a medium is the highest that distiller's yeast can withstand. Thus, the temperature of this process usually fluctuates around 35°C, which is actually a compromise between the optimum temperature of enzyme activity and the yeast growth.

In addition, using native corn starch in the SSF process will enable considerable reduction of the costs of heating starch mashes. Generally it is assumed that native starch (in the form of non-gelatinized granules) is resistant to hydrolysis by amylolytic enzymes. However, owing to the progress in molecular biology in the last few years, a commercial enzymatic preparation has come

on the market, that exhibits amylolytic activity to native starch. It is STARGEN 001 developed by Genencor International. STARGEN 001 preparation is used for hydrolysis of native starch to fermenting sugars. It catalyzes the depolymerization of polysaccharides to glucose, hence it can be applied in the process of simultaneous hydrolysis of native starch and ethanol fermentation. STARGEN 001 allows to eliminate double pH regulation (liquefaction step – optimum pH = 5.0-6.0, saccharification step – optimum pH = 4.8-5.0). Furthermore, in the use of STARGEN 001 there are no limitations related to the viscosity of mashes, so mediums of high density can be used in the process. Employing STARGEN 001 results in higher ethanol productivity obtained by avoiding the loss of fermenting sugars, which may occur during heating (the Maillard reaction), and ensures energy and water saving. Additionally, it does not require using an activator, such as calcium ions for most of amylases.

Additionally, studies in which the effectiveness of the processes of saccharification and fermentation in one (SSF) and in two tanks (SHF – Separate Hydrolisis and Fermentation) were compared on a bench scale with the use of steam-pretreated corn stover, proved the advantage of the SSF process due to the reduction of enzyme inhibition (Öhgren et al., 2007). It should be emphasized that the SSF process is described in the literature also with respect to the second-generation bioethanol production (Thomsen et al., 2008; Erdei et al., 2010). Thomsen et al. (2008) assessed the process of ethanol production from corn silage by the SSF method preceded by high-temperature pretreatment of the raw material. The highest yield obtained by these authors was 37 g of ethanol /l of corn silage. Other authors (Shanavas et al., 2011), who examined the process of simultaneous hydrolysis of cassava starch (using STARGEN 001 preparation) and ethanol fermentation, obtained ethanol efficiency of 295.20 g/kg of starch.It should also be mentioned that most published studies that describe research on the SSF process, do not concern native starch. The stage of hydrolysis and fermentation is often preceded with high-temperature treatment of the raw material (Wang et al., 2005; Nikolic et al., 2009; Shanavas et al., 2011).

Modern microbial biosynthesis technologies should ensure not only a high product yield, but also take into account the problem of environmental pollution. In ethanol fermentation using yeast, the main waste product is stillage (Decloux et al., 2002). The volumetric proportion of stillage to the amount of ethanol obtained is about 13:1 (Madejon et al., 2001). A medium-sized distillery manufacturing 50 m^3 of alcohol per day produces stillage in quantities of about 650 m^3 daily. Stillage is considered to be a highly polluting waste and its utilization is difficult and costly. Stillage has a high water content (91-95%) and high values of COD (chemical oxygen demand) and BOD (biochemical oxygen demand) (60-100 g O_2/dm^3) (Fitzgibbon et al., 1995). Therefore a major part of the research was on developing a fermentation technology with repeated full recirculation of stillage liquid fraction.

The aim of the present study was to develop a modern technology of ethanol production from corn flour with omitting gelatinization and liquefaction by using enzymes digesting native corn starch. This technology should involve the simultaneous saccharification of native starch and ethanol fermentation combined with recirculation of stillage liquid fraction.

Materials and methods

Raw material

Commercially available corn flour (BIO CORN, Ziębice, Poland) was used as the raw material for fermentation. It had a median diameter of 250 μm, and contained 12.8% of water, 84.7% of starch, and 0.1% of ash.

Yeast

Freeze-dried distiller's yeast, Red Star Ethanol Red (*Saccharomyces cerevisiae*), obtained from Lesaffre Company (Marcq en Baroeul, France) was used in this study for the production of ethanol from corn mashes. The number of the living cells at packing was >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 glucoamylase from *Aspergillus niger* were employed in this study (STARGEN 001, Genencore International, USA). The enzymatic activity of this set of enzymes was ≥456 GSHU/g (Granular Starch Hydrolyzing Units), as defined by Genencore International. In addition, the fungal acid protease GC 106 (*Aspergillus niger*), also obtained from Genencore 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.

Simultaneous saccharification and fermentation

The SSF experiments were performed in 5 l bioreactor BioFlo III (New Brunswick, USA) containing 4 l of the fermentation medium. A weighed amount of corn flour was suspended in unsterilized water. The pH of the fermentation broth was measured and adjusted to 5.0 by the addition of either 10% H_2SO_4 or 20% NaOH. In all cases, the medium was supplemented with the acid protease GC 106 (40 μl/kg corn flour dry matter) and STARGEN 001 (2.05 ml/kg corn flour dry matter). The fermentation was started with the addition of freeze-dried distiller's yeast Red Star Ethanol Red (0.5 g/l of the fermentation medium). After the inoculation the number of the living cells in the medium was 1.0×10^7 cells/ml. The repeated batch SSF fermentations were performed in anaerobic non-sterile conditions at 35 °C with medium agitation rate of 200 rpm for 72 h. The samples were taken and analyzed for yeast cell viability as well as for starch, glucose and ethanol concentrations after the fermentation. The bacterial contamination in the mash was avoided by the addition of 50 μg/ml chloramphenicol. All the experiments were performed in triplicate.
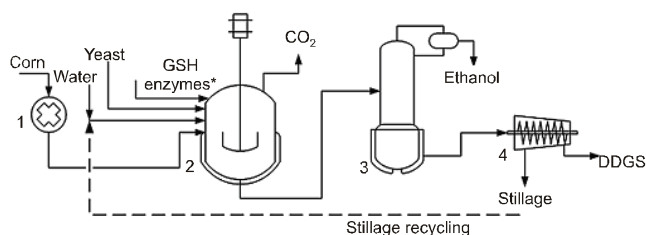
Fig. 1. Simultaneous saccharification and fermentation process of raw starch using GSH enzymes: 1) hammer mill, 2) fermentor, 3) distillation column, 4) decanter, DDGS – Dried Distillers Grains with solubles

Recycling of stillage

After the fermentation period was completed, mash containing ethanol was pumped to a continuous distillation column (UOP3CC, Armfield, UK). At the top of this column, operating at 78.5°C and reflux ratio of 4:1, ethanol concentrated to 93-95% (volume based) is collected in a side stream. The liquid fraction collected at the bottom of the distillation column, was first cooled down to 30°C and then centrifuged at 4000g for 20 min. Supernatant was used instead of water for dilution of native corn starch for next SSF run. The recirculation of stillage was performed at constant recirculation degree, 75% of fresh water was replaced by stillage. The proce-

dure was repeated twenty times over a period of 60 days, using a fresh yeast culture for inoculation on each run.

Analysis

Samples for the analysis of glucose and ethanol concentrations were firstly centrifuged at 4000 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 on an HPLC system (Merck Hitachi, Germany). Glucose and ethanol were separated on an Aminex HPX-87P (Bio-Rad, USA) at 30°C using 5 mM H_2SO_4 solution as the mobile phase at the flow rate of 0.6 ml/min, and detected with a refractive index detector (Model L-7490, Merck Hitachi, Germany). The starch content was analyzed according to the enzymatic method developed by Holm et al. (1986). The yeast cell populations were determined by a direct microscopic count in a counting chamber after staining with methylene blue.

Results and Discussion

The process of fed-batch simultaneous saccharification of native corn starch and ethanol fermentation (SSF)

In the conducted research, the previously experimentally determined optimal conditions of the SSF process were applied: the dose of STARGEN 001 enzymatic preparation of 2.05 ml per kilogram of flour dry matter, environmental pH 5.0 and 25% (w/v) concentration of starch mash (Białas et al., 2009).

In order to verify the optimal conditions of SSF and assess its kinetic parameters, the process of simultaneous saccharification of native starch and ethanol fermentation in 5 l bioreactors was performed. The results presented in Figure 2 show that the SSF process ran correctly. Together with the formation of starch hydrolysis products, the mash ethanol concentration was increasing. After 72 hours, the efficiency amounting to 85.7% of the fermentation theoretical efficiency and ethanol concentration of about 14% v/v (110 g/l) were obtained. The starch utilization was 96.7%. Figure 2 presents changes of ethanol, starch and glucose concentrations during the SSF process. The data indicate that the starch hydrolysis was effective and ran parallel to the fermentation process.

According to the data by Genencor International Inc., the application of STARGEN 001 preparation and corn mash in the concentration of 33% of dry mass enables obtaining final ethanol concentrations of 16-18%.
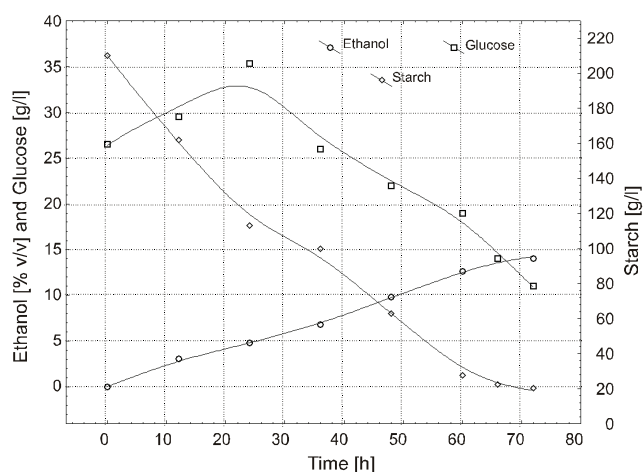
Fig. 2. Changes in starch, glucose and ethanol concentrations
during the process of simultaneous starch hydrolysis
and ethanol fermentation

These values, however, were not confirmed by any publications or descriptions of really performed production processes. The discrepancies between the obtained results and the values provided by the enzyme manufacturer may result from several reasons. Course of this digestion/fermentation process strongly depends on the quality of the raw material – its origin, species and variety, affecting the shares of particular starch fractions, and also on the flour granulation that determines starch availability to the enzymes.

The data presented by Wang et al. (2000 and 2005) indicate that to liquefy starch by STARGEN 001 preparation, the process of fermentation must run at the temperature of 33 °C and be preceded by one-hour mash incubation at the temperature of 48 °C at pH 4.2. Thus, there were significant differences between the conditions applied in Wang's and our experiments. In our experiment STARGEN 001 was added directly together with yeast to the mash of the temperature of 35 °C. In addition, Wang used yeast *Saccharomyces cerevisiae* from different supplier (Fleischmann's Yeast, Fenton, MO, USA). However, the analysis of the publications that were based on the research using STARGEN 001 preparation reveals that the results of the present study are very similar to those published by Wang et al. (2005). The authors compared the process performed according to STARGEN 001 technology with the traditional process of ethanol production with liquefaction at the temperature of 90-120 °C, then saccharification with the use of two different combinations of liquefying and sac-

charifying enzymes (DG1 and DG2; α-amylase and glucoamylase), and fermentation. In both methods the authors obtained similar final ethanol concentrations of 14.1-14.2% (v/v) with significantly different sugar profiles of the analyzed systems.

Sharma et al. (2007) compared the catalytic efficiency of STARGEN 001 preparation with the activity of traditional amylolytic enzymes. The raw material included starch with various ratios of amylose and amylopectin. The best results were obtained when starch without amylose was used. In this case, the final ethanol concentration (8.2% v/v) was the same regardless STARGEN 001 preparation or traditional enzymes were used. However, for starch with a high content of amylase, higher concentrations of ethanol were obtained with STARGEN 001. Lately Sharma et al. (2010) reported that one can further increase the efficacy of bioethanol production by applying new liquefying enzymes. The obtained ethanol concentrations were 0.54% higher (v/v) after 72-hour SSF process than in experiment involving STARGEN 001 only. However, it should be emphasized that each time SSF was preceded with a high-temperature stage (48-90 °C). STARGEN 001 was also used for bioethanol production from barley starch (Gibreel et al. 2009) and the efficiency of this process was similar to the efficiency of corn starch processing.

Simultaneous saccharification of native corn starch and ethanol fermentation (SSF) with stillage recycling

One of the key factors determining the effectiveness of the conversion of plant biomass to bioethanol is the total costs of this process, including the cost of technological water. In this context, it is worth emphasizing that the water shortage has began a global issue, which leads to the increasing cost of each water-consuming process. This situation affects also Poland, hence the efforts aiming at the reduction of technological water consumption are of great importance. Another factor that is intrinsic to water management is effluent production. Distillery stillage is ranked among especially burdensome industrial effluents particularly at large biorefineries, and its utilization is expensive. One of the methods of lowering the costs of water used by distilleries is re-utilization of stillage in the production process. Scientific literature provides a range of suggestions concerning the reutilization of distillery stillage for technological purposes (Kishimoto et al., 1997). However, there are

Table 1. Time course of SSF processes with stillage recycling (SR) and without stillage recycling (S)

Time fermentation [h]	Ethanol [g/l]		Starch [g/l]		Glucose + Maltose [g/l]		Yeast density [1×10^8 CFU ml^{-1}]	
	SR	S	SR	S	SR	S	SR	S
0	0	0	218.4	208.3	23.53	23.87	1.82	1.93
24	35.7	39.6	123.9	167.3	10.94	18.00	1.81	1.93
48	77.6	85.0	70.3	78.0	8.95	7.50	1.73	1.93
72	91.9	95.5	37.7	32.2	8.59	3.44	1.69	1.93

only a few described processes where the whole stillage or its liquid fraction are repeatedly turned back and re-utilized to prepare a new batch of medium. So far, the possibility of stillage turning back in the SSF process involving native starch hydrolysis has not been investigated.

As shown in Table 1 minor differences were observed in ethanol concentration when the conventional SSF process (S) and the process with stillage recycling (SR) were compared.

Approximately 3.2% higher final ethanol concentration was achieved for S process. However, this difference can be regarded as insignificant due to their *p* value being greater than 0.05. The same was observed for ethanol productivity. The calculated average value for this parameter for SR process was 1.28 g l^{-1} h^{-1} and 1.32 g l^{-1} h^{-1} for S process, respectively. However, ethanol yields, $Y_{p/s}$ for SR and S process were 0.49 and 0.53 g/g respectively. This means that the efficiency of starch conversion to ethanol for SR and S system were 87.5% and 94.6% of theoretical value, respectively. The F-test analysis of the variance revealed that they were significantly different (*p* = 0.021). Arguably, such differences can be related to the lower count of yeast in the recycled system resulting from the environmental stress which can cause disruption of cellular ionic homeostasis, leading to a reduction of metabolic activity and, eventually, the death of a cell (Table 1).

Effect of stillage recycling on the final ethanol concentration

In the first place, attention was paid to the changes in the ethanol concentration in the medium, depending on the number of recirculations. The results of the chemical and statistical analyses prove that the process was stable through all the cycles and could have been con-

tinued longer (the fermentations were continued in 5 l bioreactors with no interruptions until the 30th cycle).

The final ethanol concentration in the process with stillage recycling on average amounted to 91.9 g/l (Fig. 3). Each subsequent recirculation entailed a slight reduction of the final ethanol concentration on average by 0.01%, however, the regression analysis demonstrated that the changes were statistically insignificant (*p* > 0.05).
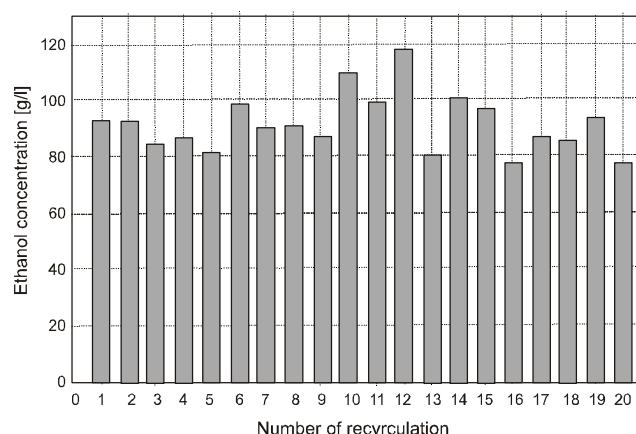
Fig. 3. Effect of stillage recycling on the final ethanol concentration

Effect of stillage recycling on the efficiency of ethanol fermentation

Changes in the fermentation efficiency were analysed with regard to the number of recirculations. The efficiency of the fermentation process in 5 l bioreactors maintained on the constant mean level of 83.74% of the theoretical fermentation efficiency (Fig. 4). In subsequent recirculations some fluctuations in fermentation efficiency were observed, but they can be assumed as statistically insignificant (0.31%; *p* > 0.05).
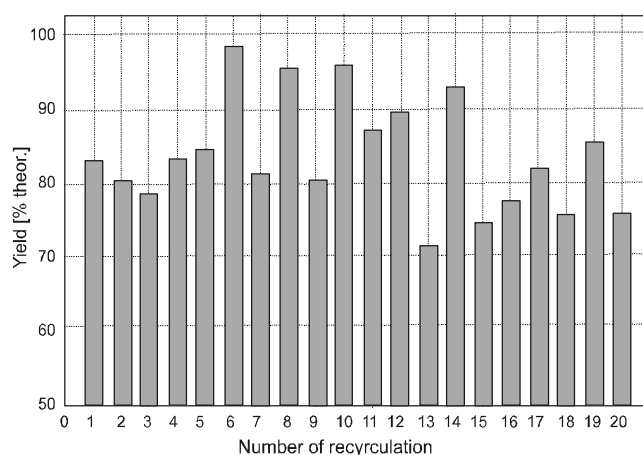
Fig. 4. Effect of stillage recycling
on the yield of ethanol fermentation

A technology based on stillage recycling was successfully applied in the laboratory scale for three months. On the basis of material balance, it can be assumed that post-distillation drained liquid, replaced 75-80% of the technological water necessary for preparing starch mash. However, Navarro et al. (2000) observed the reverse correlation between final ethanol concentration in fermented wort and the number of recirculations. The former changed from 6.5 to 1.8% v/v. The authors assumed the accumulation of fermentation by-products (glycerol, lactic acid, propanol) hinders normal yeast metabolism. The results obtained in this study suggested that drastic changes in the ethanol concentration could not be associated with stillage recycling. Lalov et al. (2000) suggested that stillage dilution with water would solve this problem. Such efforts increased the ethanol concentration to 7% v/v (5.23% w/v) during 14 recirculations. The same authors described a method of side metabolite removal (salts, acids) with the use of electrodialysis. Chitosan, can also be used as an ion exchanger which eliminates all acids from the culture medium and increases pH. Kishimoto et al. (1997) presented a bioreactor integrated with the distillation column where stillage that left the column was recycled to the bioreactor. The experiment was conducted for 500 hours without any complications and included 56 distillations with the mean theoretical efficiency of the process amounting to 92%. After finishing the experiment, the effluent volume amounted to 6% of the volume of the effluent originating from the traditional technology. After 500 hours the process was halted since its effectiveness started to lower. This was most likely caused by the elevated accu-

mulation of glycerol and lactic acid in cells as well as by the formation and accumulation of Maillard reaction products (in the distillation column). Ohashi et al. (1998) increased the density of cells in the medium by using a ceramic filter that kept the cells in the bioreactor during stillage recirculation. As a result, both cell density and process productivity amounted up to 236 g/l and 13.1 g l^{-1} h^{-1}, respectively. However, it was the microfilter blocking that turned out to be a problem in that case. The impediment was overcome with yeast immobilization in calcium alginate, which resulted in the increase of volume productivity to 14.5 g l^{-1} h^{-1}, even though those solutions did not reach beyond the laboratory scale. For comparison, the Nicolic's team (2009) obtained volume productivity amounting to 1.96 g l^{-1} h^{-1} after 48 hours of the SSF process with the use of gelatinized corn starch (without stillage recirculation) and immobilized cells of *Saccharomyces cerevisiae* var. *ellipsoideus*.

Couallier and coworkers suggested that the application of nanofiltration or reverse osmosis can reduce the content of organic compounds in stillage by 99% (Couallier et al., 2008). The filtrate obtained in the process showed a very low chemical oxygen demand index, which allows to reuse it not only during the subsequent fermentation, but also to maintain the work of other technological devices, such as steam generators or CIP (cleaning-in-place) cleaning systems. However, the discussed system has some severe limitations. The use of membrane filtration techniques requires filtrated medium clarification before separation on NF (nanofiltration) or RO (reverse osmosis) membranes. Too high content of the insoluble fraction may lead to irreparable damages to the filtration modules and contribute, thereby, to increased costs.

The above-described methods of water recycling from the distillery stillage were used in the traditional technology of ethanol production, when starch was subjected to the process of gelatinization, followed by hydrolysis at about 60°C. However, there are no reports on the using of membrane techniques in the process of stillage recirculation after the simultaneous hydrolysis and fermentation (SSF) of native starch.

Conclusions

Here we demonstrated that the developed technology enables an effective bioethanol production. The final ethanol concentration was about 11% (v/v), practical

fermentation efficiency amounted to about 84% of the theoretical efficiency and starch utilization was over 90%.

Additionally, we showed that the application of the developed technology can: (i) reduce technological water consumption by more than 80%; (ii) drastically decrease the amount of post-production wastewater and (iii) partly reduce the energy consumption in the production process.

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