Polish Journal of Microbiology
2008, Vol. 57, No 2, 141–148

ORIGINAL PAPER

Isoglucose Production from Raw Starchy Materials Based on a Two-stage Enzymatic System

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Received 4 December 2007, revised 22 February 2008, accepted 25 February 2008

Abstract

A new low-cost glucoamylase preparation for liquefaction and saccharification of starchy raw materials in a one-stage system was developed and characterized. A non-purified biocatalyst with a glucoamylase activity of 3.11 U/mg, an α-amylase activity of 0.12 WU/mg and a protein content of 0.04 mg protein/mg was obtained from a shaken-flask culture of the strain *Aspergillus niger* C-IV-4. Factors influencing the enzymatic hydrolysis of starchy materials such as reaction time, temperature and enzyme and substrate concentration were standardized to maximize the yield of glucose syrup. Thus, a 90% conversion of 5% starch, a 67.5% conversion of 5% potato flour and a 55% conversion of 5% wheat flour to sweet syrups containing up to 87% glucose was reached in 3 h using 1.24 glucoamylase U/mg hydrolyzed substrate. The application of such glucoamylase preparation and a commercially immobilized glucose isomerase for the production of glucose-fructose syrup in a two-stage system resulted in high production of stable glucose/fructose blends with a fructose content of 50%. A high concentration of fructose in obtained sweet syrups was achieved when isomerization was performed both in a batch and repeated batch process.

Key words: *Aspergillus niger*, glucoamylase, glucose-fructose syrup, isomerization

Introduction

Increasing demands for refined sugar, coupled with its rising price and the adverse effects of sacharose consumption on human health, have necessitated the search for acceptable sucrose substitutes. With the development of glucoamylase it becomes a straightforward matter to produce high glucose syrups from raw starchy materials (Kearsley and Dziedzic, 1995; Crabb and Shetty, 1999; Mishra and Debnath, 2002). However, these sweeteners have shortcomings as objects of commerce. D-glucose has only about 70% of the sweetness of sucrose and is comparatively insoluble.

One of the successes of enzyme technology so far has been the development of glucose isomerase that isomerizes α-D-glucose to α-D-fructose (Pedersen, 1993; Bhosale *et al*., 1996). This isomerization process can be used to produce a starch-based glucose/fructose blends (so-called isoglucose) containing up to 55% fructose. When the fructose content is 42%, the glucose-fructose syrup is equivalent in sweetness to sugar. Fructose is up to 1.7 times sweeter than sucrose and twice as soluble as glucose at low temperatures, so a 50% conversion of glucose to fructose gives a stable syrup that is as sweet as a sucrose solution of the same concentration (Bray *et al*., 2004). The high-fructose syrups are preferred by the food industry since they do not cause the problem of crystallization as in the case with sucrose. Moreover, D-fructose plays an important role as a diabetic sweetener because it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood. The major uses of fructose-containing glucose syrups are in the beverage, baking, canning, dairy, and confectionery industries as well as in the beekeeping for the feeding of bees (Vuilleumier, 1993; Uusitupa, 1994; Mishra and Debnath, 2002).

The enzymatic production of glucose-fructose syrups from starch, where soluble and immobilized biocatalysts are used, involves three major processes: (i) liquefaction of starch by α-amylase, (ii) saccharification of starch to glucose by means of a glucoamylase, and (iii) isomerization, where part of the glucose is converted to fructose by means a glucose isomerase. Combination of saccharification of starch with isomerization will result in shortening of reaction time and

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lead to a major saving in terms of equipment cost. However, the major drawback in the development of the uni-pH process is that the wide difference in optimum reaction conditions for the respective biocatalysts tends to lower the efficiency of a simultaneous system (Pedersen, 1993; Bhosale *et al.*, 1996; Ge *et al.*, 1999).

Intensive studies have been carried out to increase the efficiency of glucose-fructose syrups production by the way of detailed optimization of the particular stages of this process. Research is focused on a few major aspects: (i) enhancement of amylases and glucose isomerase production, (ii) improvement of the yields of enzymatic hydrolysis of starchy materials, and (iii) optimization of glucose isomerization by glucose isomerase (Fiedurek *et al.*, 1989; Prabhakar and Raju, 1993; Bazaraa and Hassan, 1996; Zhang *et al*., 2004).

In the previous study, a fungal strain of *Aspergillus niger* C-IV-4 was selected, mutagenized, and immobilized in our laboratory as a new effective producer of extracellular glucoamylase, and an active enzyme preparation from this source was isolated and purified (Ilczuk *et al*., 1983; Fiedurek, *et al*., 1987; Fiedurek and Szczodrak, 1995). The objectives of this study were to optimize the medium composition for efficient production of glucoamylase; to determine of some important operating conditions for efficient hydrolysis of raw starchy materials by non-purified glucoamylase preparation from *A. niger* and isomerization of obtained glucose to fructose by commercially immobilized glucose isomerase; to use these enzymes for the production of glucose-fructose syrup in a two-stage system; and to test the stability of syrup production in a semi-continuous operation with a reusable glucose isomerase.

Experimental

Materials and Methods

Substrates and chemicals. Wheat flour (500 type) containing 69% starch and wheat bran (22% starch) were purchased from the Lubella Co. (Lublin, Poland), while potato flour (potato starch, superior standard type, 78% starch) was provided by the Potato Industry Concern (Trzemeszno, Poland). High purified starch (Analar) and casein were obtained from BDH Chemicals Ltd. Glucose, fructose, glucose oxidase, peroxidase, o-dianisidine, 3,5-dinitrosalicylic acid, 2-thiobarbituric acid and bovine serum albumin were supplied by Sigma Chemical Co., while yeast extract and bactopeptone were from Difco. All other products used were of reagent or analytical grade and purchased locally.

Sources of enzymes and assays. In the experiments *Aspergillus niger* C-IV-4 mutant from our laboratory collection was used. This strain is characterized by a high activity of extracellular α-amylase and glucoamylase. Conidia for the inoculations were produced by one week cultivation at 30°C on potato dextrose agar slants. The composition of the liquid basal medium for the production of both amylases in shaken flask cultures was given earlier (Paszczyński *et al*., 1985). This medium was optimized during experiments with respect to the initial pH, different sources and concentrations of carbon (starch, wheat flour) and nitrogen (mineral and organic). Shake cultures were conducted in 500 ml conical flasks containing 100 ml of sterile medium. The flasks were seeded with conidia to a final concentration of about 2×10^5 conidia/ml, and placed on an orbital rotary shaker at 220 rpm and 30°C for 4 days. The mycelium was separated by centrifugation (15 min at 7500 rpm) and the clarified supernatant was used as an enzyme solution for various tests, or it was lyophilized in a freeze-dryer (Labconco, Kansas City, MO, USA). Dry powder with an α-amylase activity of about 0.12 WU/mg and a glucoamylase activity of 3.11 U/mg as well as protein content of 0.04 mg/mg lyophilizate was used as a non-purified amylolytic preparation for liquefaction and saccharification of starch to glucose. A commercially immobilized glucose isomerase from *Streptomyces murinus* (350 U/g preparation) was obtained from Sigma Chemical Co. and used for isomerization of glucose to fructose. One unit (U) of enzyme converts glucose to fructose at an initial rate of 1 μmole per min at 60°C and pH 7.5.

The activity of α-amylase was measured with iodine according to the method described by Heinkel (1956). One unit of the enzyme activity (Wohlgemuth's unit, WU) was defined as the amount of α-amylase hydrolyzing 1 mg of starch into non-colored reaction products per minute at 60°C and pH 4.7. The standard glucoamylase assay mixture contained 3 ml of 2% Analar starch in 0.1 M phosphate-citrate (McIlvaine) buffer (pH 4.5) and 0.5 ml of suitably diluted enzyme solution. After 10 min incubation at 60°C, the released glucose was quantified by the glucose oxidase method (Lloyd and Whelan, 1969). Appropriate substrate and enzyme blanks were included to correct for any free glucose not emanating from starch. One unit of glucoamylase activity (U) was defined as the amount of enzyme that catalyzed the liberation of 1 mmole glucose from the soluble Analar starch per min under the described conditions. The activity was expressed as units per ml of culture (U/ml).

Submerged cultures were performed in three replicate experiments, and analyses of amylases activities were carried out at least in duplicate. The values reported here are mean values with standard deviations being less than 5% in all cases.

Hydrolysis of starchy materials. The standard hydrolysis reaction was conducted in plugged conical

flasks (100 ml). The reaction mixture (0.1 M McIlvaine buffer, pH 4.5; 50 ml) contained 5% of starchy material and 566 U/g substrate of crude *A. niger* glucoamylase preparation containing a small amount of α-amylase as an accompanying hydrolytic enzyme. Unless otherwise stated, the flasks were incubated for 3 h at 45°C in a water bath shaker, agitated at 100 rpm. Samples were withdrawn periodically, heated at about 100°C for 15 min to stop the reaction and analyzed for reducing sugars and glucose. Some of the important factors specified in Tables I and II influencing the efficiency of hydrolysis of starchy materials were optimized during the experiments. The detailed experimental conditions are described in the subsequent tables.

The percentage of hydrolysis of starchy materials was calculated using the equation: saccharification (%) = reducing sugars formed (mg) × 0.9 × 100/starchy material (mg). Saccharifications were performed in three replicate experiments, and analyses were carried out in duplicate. The mean standard error of the saccharification of starchy materials was ±1.45% and ranged from ±0.02 to 2.75.

Isomerization of glucose in batch and repeated batch procedure. Pure solutions of 5% glucose or solutions of 3.6–4.3% starch-base glucose obtained after enzymatic hydrolysis of different starchy materials, were transferred (50 ml each) into plugged conical flasks (100 ml) where their pH-value was adjusted to that of 7.5. Then, an immobilized glucose isomerase preparation was added to the reaction mixture at a dose of 297–405 U/g glucose. Batch isomerization of glucose was carried out for 1 h at 65°C (if not otherwise stated) in a water bath shaker, agitated at 100 rpm. Repeated batch isomerization was done under the same conditions for 4 h, but the reaction mixture was replaced every 1 h and the carrier-bound enzyme was washed off with water before being transferred into the fresh medium. Samples were withdrawn periodically and analyzed for glucose and fructose. Some of the essential factors specified in Tables III and IV influencing the efficiency of glucose isomerization were optimized during the experiments. The detailed experimental conditions are described in the subsequent tables.

Isomerizations were performed in three replicate experiments, and analyses were carried out in duplicate. The mean standard error of the isomerization of glucose to fructose was ±1.35% and ranged from ±0.04 to 2.52.

Table I
Selection of suitable reaction time and amounts of *A. niger* glucoamylase preparation for efficient hydrolysis of starch[a]

Time (h)	Glucoamylase concentration (U/mg starch)									
	0.31		0.62		1.24		1.86		2.48	
	Saccharification (%)	Reaction with iodine	Saccharification (%)	Reaction with iodine	Saccharification (%)	Reaction with iodine	Saccharification (%)	Reaction with iodine	Saccharification (%)	Reaction with iodine
0.5	+	10.03	+	15.05	+	32.62	+	57.70	+	65.24
1.0	+	25.10	+	42.66	+	70.30	–	57.70	–	90.33
2.0	+	45.20	–	70.26	–	82.80	–	100.30	–	90.30
3.0	+	75.30	–	87.82	–	87.80	–	100.30	–	100.00
4.0	–	85.30	–	85.31	–	95.35	–	100.30	–	100.00

[a] Hydrolysis conditions: Analar starch, 1%; 45°C; pH, 4.5.

Table II
Effect of temperature on enzymatic hydrolysis of starchy raw materials[a]

Temperature (°C)	Starchy raw materials											
	Analar starch				Potato flour				Wheat flour			
	Reducing sugars (mg/ml)	Saccharification (%)	Glucose		Reducing sugars (mg/ml)	Saccharification (%)	Glucose		Reducing sugars (mg/ml)	Saccharification (%)	Glucose	
			(mg/ml)	(%)			(mg/ml)	(%)			(mg/ml)	(%)
30	18.61	33.50	15.42	82.85	10.40	18.72	7.70	74.00	6.25	11.25	4.68	74.88
45	27.88	50.18	25.00	89.67	22.90	41.22	18.10	79.03	20.80	37.44	16.70	80.28
55	43.05	77.50	38.44	89.29	29.16	52.50	23.90	81.96	26.38	47.50	23.00	87.18
60	50.00	90.00	42.90	85.80	37.50	67.50	32.22	85.92	30.55	55.00	26.60	87.00
70	38.19	68.74	23.41	87.48	20.83	37.50	17.27	82.90	13.88	24.50	11.80	85.00
80	7.20	12.96	5.84	81.11	4.16	7.50	3.35	80.52	3.47	6.25	2.90	83.57

[a] Hydrolysis conditions: starchy material, 5%; *A. niger* glucoamylase, 1.2 U/mg substrate; time, 3 h; pH 4.5.

Table III
Selection of suitable temperature for efficient batch isomerization of glucose[a]

Temperature (°C)	Glucose		Fructose	
	(mg/ml)	%	(mg/ml)	%
30	43.90	87.80	4.42	8.85
45	37.90	75.80	11.43	22.86
50	33.17	66.34	15.24	30.48
60	31.75	63.50	16.76	33.52
65	25.60	51.20	26.67	53.30
70	25.00	50.00	25.17	50.34
80	21.32	42.64	24.23	48.46

[a] Isomerization conditions: glucose, 5%; immobilized glucose isomerase, 0.21 U/mg substrate; pH, 7.5; time, 1 h.

Table IV
Time course of glucose isomerization by immobilized glucose isomerase[a]

Time (min)	Glucose		Fructose	
	(mg/ml)	%	(mg/ml)	%
15	35.07	70.14	12.34	24.68
30	30.80	60.16	20.12	40.24
60	25.60	51.20	26.67	53.30
120	18.95	37.90	30.48	60.10
180	18.95	37.90	29.72	59.44

[a] Isomerization conditions: glucose, 5%; glucose isomerase, 0.21 U/mg substrate; temp. 65°C; pH, 7.5.

Other analyses. Reducing sugars were analyzed by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Glucose was determined with a glucose oxidase-peroxidase reagent (Lloyd and Whelan, 1969) and fructose content was measured after a reaction with 2,5 thiobarbituric acid (Percheron, 1962). Soluble protein was estimated using the Schacterle and Pollack (1973) method. All analyses were done at least in duplicate and the data given are the averages of all the measurements.

Results and Discussion

Production of glucoamylase and characteristics of non-purified preparation. It had been reported by other authors that secretion of amylolytic enzymes by different fungi was strictly dependent upon growth conditions, in particular, on carbon and nitrogen sources (Pandey *et al*., 2000; Gupta *et al*., 2003). Given this, different kinds and concentrations of carbon and nitrogen in the culture medium and its initial pH were evaluated to improve the yields of glucoamylase production by *A. niger* C-IV-4 in shaken flasks. Different variants of a basal culture medium were prepared where the concentration of carbon (added as pure starch or wheat flour) varied from 1 to 15% while that of nitrogen (added as different mineral salts or organic compounds) ranged from 0.5 to 0.7%. Glucoamylase activity was also assayed at initial medium pH values between 4.0 and 6.0. In all cases, titers of enzyme were measured after 4 days cultivation, and results of these shaken-flask experiments are depicted in Figures 1–3. The data clearly indicated that the fungus cultivated on the medium with wheat flour (3%) and $(NH_4)_2HPO_4$ (0.7%) as well as with a initial pH of 5.0 gave the maximum glucoamylase activity (from 150 to 175 U/ml) after 4 days of submerged culture. Taking into consideration these results, the optimized medium composition was used to produce active glucoamylase preparation.

Comparison of glucoamylase productivity obtained by *A. niger* C-IV-4 with those reported by other

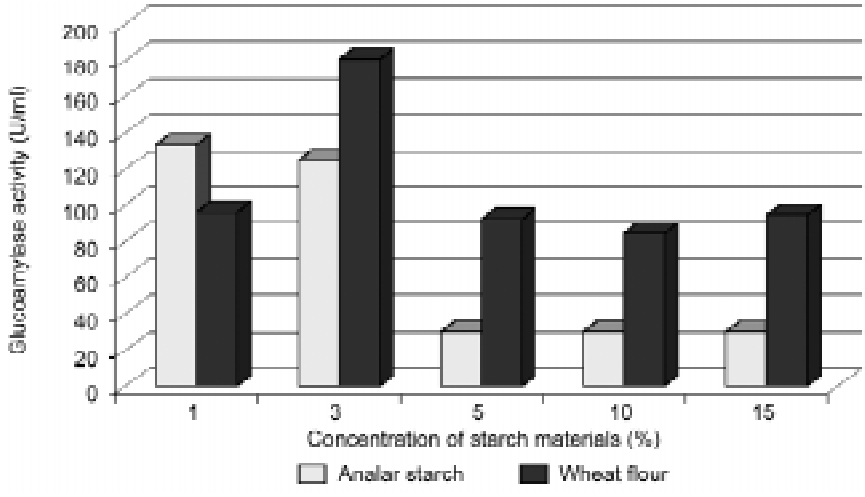

Fig. 1. Extracellular glucoamylase production by *Aspergillus niger* C-IV-4 in shaken flask cultures on basal medium containing starchy materials at different concentrations.

The basal medium (pH 4.5) was used, except that starch or wheat flour were applied at different concentrations. After 4 days of cultivation in shaken flask, the glucoamylase activity of the culture supernate was assayed by the standard method.

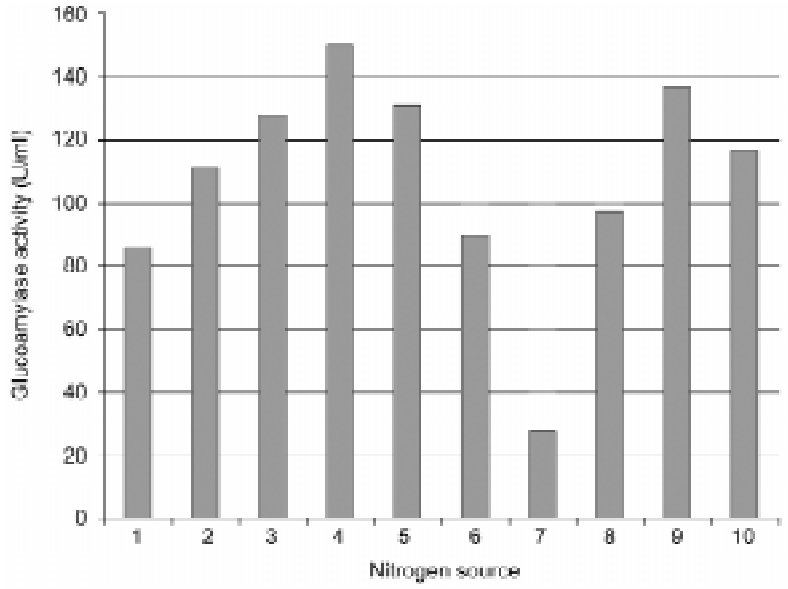

Fig. 2. Effect of nitrogen source on glucoamylase production by *Aspergillus niger* C-IV-4.
The basal medium (pH 4.5) with 3% wheat flour was used, except that different nitrogen sources, mineral (0.7%) and organic (0.5%), were applied as indicated. The nitrogen sources applied were: 1 – NH_4Cl; 2 – NH_4NO_3 ; 3 – $(NH_4)_2 SO_4$; 4 – $(NH_4)_2 HPO_4$; 5 – $NH_4H_2 PO_4$; 6 – $NaNO_3$; 7 – urea; 8 – casein; 9 – yeast extract; 10 – peptone. After 4 days of cultivation in shaken flasks, the glucoamylase activity of the culture supernate was assayed by the standard method.

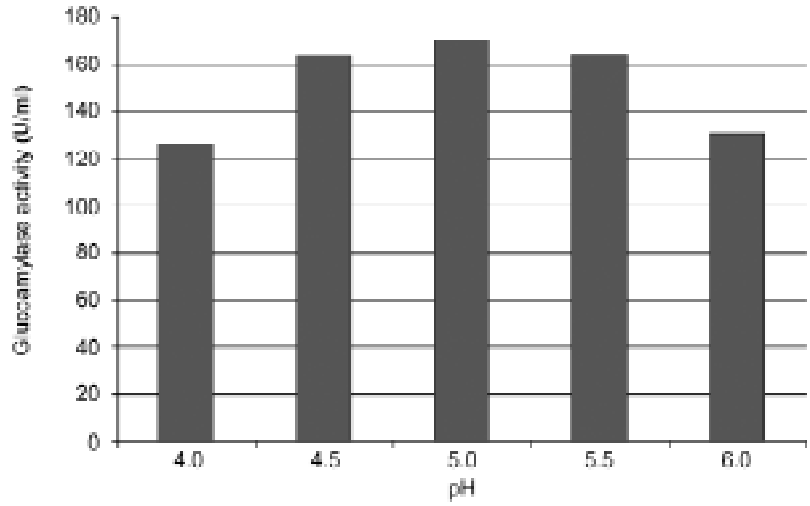

Fig. 3. Effect of initial medium pH on glucoamylase production by *Aspergillus niger* C-IV-4.
The optimized basal medium with 3% wheat flour and 0.7% $(NH_4)_2 HPO_4$ was used. After 4 days of cultivation in shaken flasks, the glucoamylase activity of the culture supernate was assayed by the standard method.

authors for different fungal species (Dhawale and Ingledew, 1983; Abouzied and Reddy, 1986; Milosavljevic *et al.*, 2001) revealed that the selected *A. niger* strain, constitutes, at this stage of our studies, a culture of relatively high biosynthetic activity, that gives reasonable glucoamylase yields on a starch-based medium in submerged liquid cultures.

Post-culture supernatant of *A. niger* C-IV-4, obtained after 4 days of shaken flask cultures in optimized medium with wheat flour, was than lyophilized and used as non-purified glucoamylase preparation in the production of isoglucose from starchy materials. Besides high glucoamylase activity, the crude enzyme also contained small amounts (0.12 WU/mg preparation) of α-amylase. It is significant that both amylases were most active under the same reaction conditions, *i.e.*, at pH 4.5–4.7 and temperature 60°C. The presence of α-amylase in *A. niger* C-IV-4 culture supernatant is very advantageous because it allows the use of such a preparation in simultaneous liquefaction and saccharification of starch. Normally, the both enzymatic processes are completely separated. The application of one-stage enzymatic system for liquefaction and saccharification of starch results in shortening of

reaction time and leads to a major saving in terms of equipment cost.

Enzymatic hydrolysis of starchy raw materials to glucose. In the enzymatic hydrolysis of starch it is essential to obtain a high syrup yield per enzyme unit. Many factors affect this yield, including the kind of hydrolyzed starchy material, inhibition of enzyme action by heat or degradation products, enzyme and substrate concentration, rate of enzyme action, and degree of agitation. Thus, the optimization of hydrolysis conditions plays a significant role in the economy of the saccharification process.

To maximize the sugar yield from starchy raw materials, the basic hydrolytic variables, *i.e.*, reaction time, enzyme concentration, kind of substrate and its concentration, and temperature were optimized. In the first step of the studies pure starch (1%) was exposed for 4 h at 45°C to enzymatic hydrolysis using various concentrations of crude *A. niger* glucoamylase preparation. The dynamics of saccharification of this material is presented in Table I. A rate of glucoamylase action on starch rose gradually with increasing biocatalyst dose. At enzyme concentration of 1.86 U/mg substrate no iodine-colored products of starch degradation were found after 1 h of hydrolysis. A high degree of starch conversion to sweet syrup (about 88% of soluble carbohydrate fraction) was obtained after 3 h of hydrolysis when a glucoamylase preparation of 1.24 U/mg starch was used. It follows clearly from the data in Table I that a 1.5-fold increase in the enzyme amount from 1.24 to 1.86 U/mg starch increased after 2 h of reaction the hydrolysis efficiency by only 12%. An extension of the hydrolysis time to 3 or 4 h did not cause substantial changes in hydrolysis yield or had no effect on starch saccharification. Accordingly, in further studies a 3-h period and 1.24 glucoamylase U/mg starch were finally decided as a optimum reaction time and enzyme dose for liquefaction and saccharification of starch.

Starchy raw materials (pure starch as well as wheat and potato flours), used at a concentration of 5%, were then hydrolyzed for 3 h by fixed dose of glucoamylase preparation at temperatures ranging from 30–80°C. The optimum temperature for hydrolysis of all tested materials was 60°C, at which maximum degrees of hydrolysis (90%, 67.5%, and 55%) were recorded for starch, potato flour and wheat flour, respectively (Table II). Moreover, the obtained starch syrups showed in their compositions a high percentage content of glucose, *i.e.*, 85.8% for starch, 87.0% for wheat flour and 85.92% for potato flour. It is very important for further experiments where starch hydrolysates containing glucose will be used in effective production of glucose-fructose syrup. It should be stressed that at a temperature of 70°C only the little decrease in glucose yield was observed. However, a temperature rise to 80°C caused a significant decrease in the hydrolysis efficiency.

For comparison, a saccharification efficiency of cooked wheat-starch of up to 89% after 24 h hydrolysis at 40°C was obtained by Dhawale and Ingledew (1983) for amylolytic complex from a derepressed yeast mutant of *Schwanniomyces castellii*. A high degree of liquefaction and saccharification of potato starch (over 80%) by α-amylase and glucoamylase from *A. niger* S was also reported by Bolach *et. al*. (1985). In the case of corn syrup applied in the production of glucose the enzyme/enzyme process was used. The yield of glucose in enzyme hydrolyzed syrups of a 96 DE was 90% while it was 70% with an acid-hydrolyzed syrups containing 86% glucose. The higher dextrose content of the enzyme-hydrolyzed syrup also permits faster crystallization (Macalister *et al*., 1975).

Saccharification of starchy materials can be carried out as a batch process or as continuous method with immobilized enzymes. For instance, Hausser *et al.* (1983) have immobilized both fungal α-amylase and glucoamylase on microporous plastic sheets. A maltose syrups (30% maltose and 35% glucose as % of solids) could be produced from 42 DE feedstock by first processing continuously through an glucoamylase reactor followed by continuous processing through an α-amylase reactor.

A pullulanase from *Bacillus acidopullulyticus* capable of hydrolyzing α-1-6-linkages, with a pH optimum very close to that of the fungal glucoamylase, was also described (Jensen and Norman, 1984). This would facilitate combined use of the two enzymes. Janse and Prestorius (1995) showed that using a recombinant strain of *Saccharomyces cerevisiae* producing α-amylase, glucoamylase and pullulanase allows the hydrolysis of starch in a single step.

Production of isoglucose by enzymatic isomerization of glucose and starch-based glucose to fructose. Isomerization of glucose was performed in batch or repeated batch system, *i.e.*, in successive cycles of starch hydrolysis and isomerization of obtained glucose. At first, some of the most substantial parameters affecting the efficiency of glucose isomerization (*i.e.*, reaction time and temperature) by immobilized glucose isomerase were standardized. A pure glucose (5%) was used as a substrate in these experiments. As Tables III and IV show a reaction time of 1 h and temperature of 65°C were the best factors for efficient isomerization of glucose to fructose. Under these conditions, a 53% concentration of fructose was attained. This kind of biotransformation gave stable syrups that were as sweet as sucrose solution of the same yield. In other cases, the tested glucose-fructose syrups contained higher or lower concentrations of fructose which are not preferred by the food industry. Therefore, the fixed conditions were then also applied for efficient isomer-

Table V
Repeated batch isomerization of starchy raw materials using immobilised glucose isomerase[a]

Starch raw materials	Cycles							
	I		II		III		IV	
	glucose (%)	fructose (%)	glucose (%)	fructose (%)	glucose (%)	fructose (%)	glucose (%)	fructose (%)
Analar starch	49.05	50.94	52.67	47.33	61.49	37.75	73.62	26.38
Potato flour	56.72	43.27	53.03	46.96	62.24	31.08	71.61	28.38
Wheat flour	57.96	42.04	60.99	39.00	68.90	47.33	79.48	20.51

[a] The isomerization was performed is shaken flasks in 1-h cycles

ization of starch-based glucose (*i.e.*, glucose coming from pure starch as well as from wheat and potato flours) in a repeated batch isomerization procedure.

Lower isomerization degrees (from about 25% to 27%) of pure glucose solutions were published by Błaszków and Miśkiewicz (1980). The authors achieved those fructose yields after 23 h isomerization at 50°C and pHs 8.3–9.3 using a glucose isomerase immobilized on polyamide in a batch or continuous system.

Table V summarizes the isomerization activity of immobilized glucose isomerase towards the hydrolysates containing glucose obtained after enzymatic conversion of starchy materials (pure starch, wheat and potato flours) in a repeated batch isomerization process. The enzyme activity was expressed as the efficiency of glucose and fructose in glucose-fructose syrups. The carrier-bound glucose isomerase could be used repeatedly for two 1-h cycles without any appreciable loss in fructose yield which reached average values of 44.9% after every 1 h of enzymatic isomerization. A decrease in glucose isomerization (by only 7% in fructose efficiency in relation to the first cycle) was recorded in 3th cycle. Later, the glucose isomerase activity (measured as fructose yield) decreased distinctly and in the fourth cycle it achieved a value of about 25%. A relatively constant ratio (glucose/fructose yield) obtained in successive cycles of isomerization gives an indication that the immobilized enzyme was catalytically active over the whole isomerization period. This is encouraging and suggests that further applications might be practicable.

For example Błaszków and Miśkiewicz (1980) obtained a low isomerization degree (3.4%) of potato starch-based glucose which was converted to fructose by polyamide-bound glucose isomerase in a batch isomerization process. However, using a column with immobilized enzyme in a glucose recycling system for 24 h at optimized flow-rate resulted in a tenfold increase in isomerization of potato starch-based glucose.

Arthrobacter cells immobilized on K-carrageenan beads (NRRL-B-3728) were used for continuous isomerization of glucose to fructose in a bioreactor system. Maximal bioreactor isomerization of 44% was achieved when a buffered feedstock containing 40% of glucose was feed into the column (60ºC) at a flow rate of 0.2 ml/min. The biological half-life of glucose isomerase activities in the system was 400 h (Bazaraa and Hamdy, 1989).

The isomerization of D-glucose in mixed ethanol-water solutions was studied at various reaction temperatures (40–70°C), employing glucose isomerase from *Streptomyces phaeochromogenes* and *Clostridium thermohydrosulfuricum*, respectively. The thermophilic *Clostridium* enzyme was considerably more stable towards the combination of organic co-solvent and increased temperature and with this enzyme a 55% yield of fructose from glucose was obtained at relatively low concentration of ethanol (40%) (Nilson *et al*., 1991).

The glucose isomerase from the thermophile *Thermus thermophilus* seems to have potential for the development of new isomerization processes using high temperatures and slightly acidic pH. The isomerase has an optimum temperature at 95°C, and is also very stable at high temperatures. The optimum pH is around 7.0, close to that one where by-product formation is minimal. Since *Thermus* produces only a little of this useful isomerase, the production of the cloned gene in *Escherichia coli* and *Bacillus brevis* were compared. Especially *B. brevis* was able to produce the isomerase efficiently (more than 1 g/l) (Dekker *et al.*, 1992).

Conclusion. The data presented here imply that the non-purified amylolytic preparation from *A. niger* C-IV-4 represents a good source of indispensable enzymes for the highly efficient process of simultaneously liquefaction and saccharification of starch to glucose syrups. The use of such preparation and immobilized glucose isomerase for the production of isoglucose from different starchy raw materials in a two-stage enzymatic system resulted in a high production of stable syrups with a fructose content of 50%. Moreover, a high concentration of fructose in glucose/fructose blends was achieved when

isomerization was performed both in a batch and repeated batch process. Experiments are currently in progress to test the use of both enzymatic preparations (immobilized *A. niger* C-IV-4 glucoamylase and glucose isomerase) in column hydrolysis of starchy materials and column isomerization of starch-based glucose. This two-column continuous system could lead to a potential cost reduction in large scale glucose-fructose syrup production.

Acknowledgments

This work was financially supported by the Research Program BW/BS/BiNoZ/UMCS.

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