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Hydrolysates from malt spent grain with potential application in the bioethanol production

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ABSTRACT

Spent malted grain from local brewery - a byproduct of beer brewing consisting of the residue of malt and grain which remains after the mashing and lautering process, was partially hydrolyzed using chemical and enzymatic methods. Chemical analysis of spent grain components was done. It consists primarily of grain husks, pericarp, and fragments of endosperm, and contains mainly pentosans – approx. 47% of dry mater, approx. 17% cellulose, and traces of starch – about 2.5% of dry matter. The nitrogen content is two times lower than the literature data. Hydrolysis was done with cellulase complex from *Trichoderma longibrachiatum* and xylanase complex from *Aspergillus niger* B03 after pre-treatment with hot water, hot diluted 0.1M alkali or diluted 0.05M acid. Enzyme hydrolysis of β-glucans was partially optimized. From 12% to 30% of the spent grain was hydrolyzed to mono- and disaccharides – mainly glucose, xylose, cellobiose. Hydrolysates containing predominantly (more than 95%) hexoses were obtained applying pre-treatment with hot water (115°C) and hydrolysis with the cellulase complex of *Trichoderma longibrachiatum*.

Key words: malt spent grain, hydrolysis, cellulase, xylanase, bioethanol

Introduction

Ethanol is produced by the fermentation of carbohydrate materials and is used for production of alcoholic beverages, for industrial purposes (as a solvent, disinfectant, or chemical feedstock) and, in recent years, as a blending agent with gasoline to increase octane and reduce carbon monoxide and other smog-causing emissions (Hahn-Hagerdal et al., 2006; Lin & Tanaka, 2006).

Bioethanol is the most widely used liquid renewable biofuel and suitable alternative to replace fossil fuels. The largest producers in the world are the United States, Brazil, and China. Ethanol is currently produced from sugar (Brazil) or grain (starch, USA). However, this raw material base will not be sufficient because the increasing demand for fuel ethanol and the lower than expected reduction of greenhouse gases.

Producing biofuels in the "second generation biofuel plants" out of feedstocks that cannot be used directly for food production or do not reduce the amount of land that can be used to produce food can be accomplished by capture of biomass that is currently treated as either waste or that is a co-product of existing production processes with very low or negative current economic value (Cardona & Sanchez, 2007; Sanchez & Cardona, 2008). Examples of waste streams that could potentially be converted into biofuels include perennial grasses, a portion of municipal trash and garbage (e.g., waste paper, waste food scrapes, used cooking oils), wood pulp residues, macroalgae, and forest residues (e.g., wood pieces leftover after timber extraction) (Hahn-Hagerdal et al., 2006). Currently these streams often generate negative value in that consumers and firms must pay for disposal. An alternative is the production of bioethanol from agroindustrial wastes containing abundant cellulosic fibers and carbohydrates (Table 1) such as grape pomace, sugar beet pomace, crop residues (in particular corn (maize) stover, barley, wheat and rice straw), corncobs, sunflower stalks and heads, cotton waste, brewer's spent grain, etc. (Pandey, 2010).

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Table 1. *The contents of cellulose, hemicellulose, and lignin in common agricultural residues (adapted from the literature (*Sun & Cheng, 2002; 19. Serena & Knudsen, 2007*).*

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Wheat sraw	30	50	15
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0

Bioethanol from wood or straw by enzyme hydrolysis and fermentation

The process of producing ethanol from wood or straw requires the production of ethanol from both C5 and C6 sugars - unlike the only the C6 sugars in conventional ethanol production from sugarcane (Table 2).This process is technically feasible but is complex and expensive and there are few industrial examples (Anish & Rao, 2008).

Lignocellulose-based production of ethanol is mixed-sugar fermentation in the presence of inhibiting compounds – low molecular weight organic acids, furan derivatives, phenolics and inorganic compounds – released and formed during pretreatment and/or hydrolysis of the raw material (Larsson et al., 2000). Cellulose monomers are principally glucose and cellobiose, whereas hemicellulose monomers are a mixture of hexoses (principally glucose, with some mannose and galactose) and substantial amounts of pentoses (principally xylose, with some arabinose) (Mussatto et al., 2008). Lignocellulosic raw materials, in particular hardwood and agricultural raw material, can contain 5–20% (or more up to 40%) of the pentose sugars xylose and arabinose. Xylose is by far the most abundant pentose sugar, whereas arabinose can constitute as much as 14–15% in corncob hulls and wheat bran, respectively (Sun & Cheng, 2002).The five sugars of interest for fermentation are glucose, mannose, galactose, xylose, and arabinose (Table 2).

Conventional production of ethanol from cellulose via fermentation involves a complex process of pretreatment in attempt to recover a maximum amount of sugars from the hydrolysis of cellulose and hemicellulose, and to ferment them into ethanol.

Lignocellulose needs to undergo treatments that release its monomeric sugars, which then can be converted by a microorganism. The two main steps are: (i) a pretreatment (by physical or chemical procedures) that releases hexoses and pentoses from hemicellulose, and (ii) an enzymatic treatment (or, alternatively, hydrolysis by chemical procedures) that generates glucose from cellulose.

Table 2. *Sources of sugars for ethanol production (*Petrova & Ivanova, 2010*).*

Sources	Carbohydrates	Hydrolysis products
1. Crops	A. Sucrose	Fructose + Glucose
	B. Starch	Glucose
2. Lignocellulose	C. Cellulose	Glucose
	D. Hemicellulose	Glucose + Galactose + Mannose + Xylose + Arabinose + Other (L-Rhamnose, L-Fucose, Uronic acids)
	E. Lignin	Lignols (coniferyl, sinapyl, coumaryl)

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The cellulose hydrolysis is the main bottleneck in cellulosic fuel production (Anish & Rao, 2008). There is no microorganism currently available that can utilize lignin monomers for ethanol production (Walker, 1998).

Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic structural and chemical composition and to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars. In liquid hot water pretreatment (LHW), pressure is utilized to maintain water in the liquid state at elevated temperatures. It has been shown to remove up to 80% of the hemicellulose and to enhance the enzymatic digestibility of pretreated biomass materials such as corn fiber (Hendriks & Zeeman, 2009).

Acid-catalyzed pretreatment primarily solubilizes the hemicellulose fraction into the liquid phase. Alkaline pretreatment is basically a delignification process, in which a significant amount of hemicellulose is solubilized as well (Laxman & Lachke, 2009).

A consortium of enzymes - endoglucanase, exoglucanase and β-glucosidase (cellobiase), collectively known as cellulase, are needed to break down cellulose into its constituent glucose monomers (Laxman & Lachke, 2009).

The aim of the present study was to obtain additional information about fermentation of substrates, obtained by hydrolysis of spent malted grain from brewery - a byproduct of beer brewing, by ethanol producing distillery yeasts *Saccharomyces cerevisiae* and evaluate the influence of substrate preparation method on fermentation parameters.

Materials and Methods

Spent malt grain

Spent barley grain which remains after the mashing and lautering process, was obtained from local brewery in 2011. It was frozen to -20°C and dried at 40°C for 4-5 days before analysis.

Enzymes and yeast strain

The yeast strain *Saccharomyces cervisiae* AL100 (Institute of Microbiology, Bulgarian Academy of Sciences) was maintained on YPG agar (pH 4.5), containing yeast extract (3.0 g/l), peptone (5.0 g/l), glucose (10.0 g/l), agar (20.0 g/l). Further propagation, prior to ethanol fermentation, was performed in 200 ml flasks statically at 30°C for 24 h in the YPG medium.

A thermostable α–amylase from *Bacillus licheniformis* – Termamyl 120L (Novozymes, Denmark), cellulase from *Trichoderma longibrachiatum* (Biovet, Peshtera, Bulgaria, specific activity 4500 U/g, optimal temperature 40°C and pH 4.5) and xylanase complex from *Aspergillus niger* B03 (specific activity 5800 U/g, optimal pH and temperature 5.0 and 50°C, respectively) were used for carbohydrate hydrolysis.

Pretreatment

Spent malted grain was treated with hot water at 115°C. Dry mass, reducing sugars, mono- and oligosaccharides were analysed in the extracts.

Two additional treatments were also applied - with 0.1M NaOH and 0.05 M H_2SO_4. After acid or alkali addition the samples were autoclaved at 115°C (0.7 atm) for 20 min. Then they were neutralized with 20% NaOH or 1.0 M HCl to pH 4.5-5.0. Reducing sugars and saccharides were analysed before and after these treatments.

Enzyme hydrolysis

Starch hydrolysis was done at 80°C and pH 7.0 for 30 min at 140 rpm using enzyme activity 5.0 U/g substrate. Cellulose was hydrolysed with cellulose complex in concentration 40.5-162 U/g spent grain at 40°C, pH 4.5., 3-24 h at 140 rpm. Xylanase was applied in concentration 35-140 U/g spent grain at pH 4.5 and 40°C, for 3-24 h at 140 rpm.

Fermentation

Alcoholic fermentations were carried out at pH 4.5 without additives at 30°C in 300-ml bottles with final volume of 50-100 ml of the filtered hydrolysates (extracts after hydrolysis step). The bottles were fitted with rubber bungs and fermentation valves and incubated statically. The loss of weight by CO_2 liberation during the fermentation and the ethanol concentration were monitored daily until fermentation ceased.

Analytical methods

Ethanol concentration was measured by ethanol-oxidase test (Gonchar et al., 2001). Dry mass was measured using the UltraX apparatus (Germany). Protein (as nitrogen) in the spent grain was determined by the Kjeldahl method using UDK 152 apparatus (Marver). Reducing sugars were examined by the method of Miller (1969). Ethanol was analysed by the ethanol-oxidase test or on the base of CO_2 liberation.

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The mono- and disaccharides in the extracts were determined by HPLC (Waters model 2695 equipped with a column Macherey Nagel and diffraction refractometer 2414; temperature of detection 40°C, elution with acetonitrile:water (80:20) and rate of 1.5 ml/min) or by TLC on Silicagel-60 (Merck, Germany). Xylose, arabinose, glucose, mannose, galactose, cellobiose, rhamnose were used as detection standards in concentration 2.5 mg/ml.

Starch was measured by the polarimetric method of Ewers (ISO 10520) (FAO, 1997) using the P3001RS (Krüss, Germany) apparatus after treatment of the assay mixture with hot diluted HCl and filtration.

The cellulose content was determined by the method of Updegraff (1969), pentosans were determined by the method of Douglas (1981).

Results and Discussion

Chemical characteristics of malt spent grain

The results for the dry mass, reducing sugar concentration, reducing sugars, starch, pentosans and nitrogen compounds are summarized in Tables 3 and 4. Data were calculated as % from the dry mass of the spent grain. The basic components of the grain are the pentosans – approx. 47% of the dry mass, and the cellulose – 17% of DM. It contains traces of starch – about 2.5% of dry matter. The nitrogen content is two times lower than the literature data (Table 2). Spent grain consists primarily of grain husks, pericarp, and fragments of endosperm, and is used as additive in animal feed.

Spent grain from maize or wheat contain low amount of cellulose (about 7% of DM), about 7-9% starch, 6-11% lipids and 38-45% proteins (Mussatto et al., 2008; White et al., 2008) and are used in animal feed. Spent malt grain differ from the maize or wheat spent grain and contain approx. 55% total saccharides, approx. 35% pentosans and about 4-5% lignin. The weight ratio cellulose/hemicellulose/lignin in this barley spent grain the ratio was 9:3.5:1 (Table 3). The results differ significantly from those from plant material - normally 4:3:3, or for other malt spent grains - 6:3:1 (Table 4). It could be concluded that this agroindustrial waste could not be used as animal feed because of the low protein content.

Table 3. *Chemical characteristics of spent malt grain.*

Water content %		**Starch**	**Pentosans**	**Cellulose**	**Reducing sugars**	**Nitrogen compounds**
Wet malt spent grain	Dry malt spent grain	% from dry mass (DM)	% from DM	% from DM	% from DM	% from DM
69.49 ± 1.1	2.61 ± 0.53	2.57	46.77	17.56	2.40	12.26

Table 4. *Chemical composition of malt spent grain (literature data).*

Chemical composition	**Quéméré et al., 1983**		**www.bonda.fr Bonda nutrition animale**	**Archives of FAO www.fao.org/docrep/field/003/ AB607F/AB607F02.htm**
	Dry spent grain	**Wet spent grain**		
Water content, %	9.0	76.0-80.0	78.0	50.0
Starch, % from DM	-	-	7.0	-
Pentosans, % from DM	35.3	-	-	-
Cellulose, % from DM	15.0	14.0-20.0	15.0	18.8
Total saccharides, % from DM	55.1	-	-	46.4
Lignin, % from DM	4.8	-	-	-
Nitrogen compounds, % from DM	28.5	21.0-38.0	30.0	22.8
Lipids, % from DM	8.2	6.0-11.0	10.0	7.8

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Table 5. *Effect of the pretreatment on the reducing sugar content in the extract*

	Type of pretreatment (115°C, 0.7 atm., 20 min.)		
	Water	**Diluted 0.1M NaOH**	**Diluted 0.05 M H_2SO_4**
Reducing sugars (% from DM)	2.57	1.85	9.85

Table 6. *Effect of the pretreatment and the type of hydrolysis on the degree of hydrolysis and the process duration (data for the highest enzyme concentrations studied - 162 U/g substrate cellulase u 140 U/g substrate xylanase).*

Type of pretreatment, 115 °C	**Hydrolysis**	**Degree of hydrolysis, % from DM**	**Duration, hours**
Water			
	Simultaneous	**12.1**	**8**
	Consecutive		
	1. cellulase →xylanase	11.2	48
	2. xylanase → cellulase	12.8	48
0.1M NaOH (0.4%)			
	Simultaneous	**13.5**	**24**
	Consecutive		
	1. cellulase →xylanase	14.1	48
	2. xylanase → cellulase	13.8	32-48
0.05M H_2SO_4 (0.49%)			
	Simultaneous	**30.8**	**32**
	Consecutive		
	1. cellulase →xylanase	20.7	48
	2. xylanase → cellulase	24.1	48

Effects of pre-treatment on fermenting sugar composition

Hot water physical pretreatment at 115°C breaks down the shield formed by lignin and hemicellulose, disrupts the crystalline structure and reduces the degree of polymerization of cellulose and releases hexoses and pentoses from hemicellulose. Combining the physical hot water pretreatment with a chemical pretreatment with diluted alkali (0.1 M NaOH) an uronic acids esterification and saponification of intermolecular ester bonds. It is a delignification process (Laxman & Lachke, 2009; Zheng et al., 2009). As a result spent malt barley grain was transformed in dense mash of fine particles, increased in volume and could not be filtered. The thermal pretreatment with diluted acid favoured the hydrolysis of hemicellulose to monosaccharides – the reducing sugar content in the extract increase 5 times in comparison with the hot water and alkali treatment (Table 5). A high xylose yield could be expected but also sugar degradation products.

Starch hydrolysis

Non-pretreated and treated with hot water barley spent grain was subjected to starch hydrolysis with thermostable amylase. The reducing sugars increase with 28.33%, which corresponds to hydrolysis of 26.5% of the starch (without pretreatment). After thermal pretreatment, 51.6% of the starch could be hydrolysed within 30 minutes. Nevertheless, starch hydrolysis could be applied only when the substrate concentration is higher.

Effect of pretreatment on cellulose and hemicellulose hydrolysis

Enzyme reactions were carried out with 3 different cellulose and xylanase concentrations and two types of hydrolysis process: simultaneous with both enzymes and consecutive with alteration of the enzymes. The results are summarized on Table 6.

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Hydrolysis was weakly influenced by the enzyme concentration. Processing with cellulase complex from *Trichoderma longibrachiatum* of treated with hot water barley spent grain could be achieved with enzyme quantity 81-162 U/g substrate. The net hydrolyzed dry mass is 4.5-5.5%. The optimal time for hydrolysis with xylanase from *Aspergillus niger* B03 was 8 h and the degree of hydrolysis 5% of DM at the lower enzyme concentration of 35 U/g and 6.5% of DM at the highest enzyme quantity tested – 140 U/g substrate.

Both enzymes hydrolyse simultaneously lower part of the spent grain cellulose and hemicellulose than the sum of their independent action on the substrate. At higher enzyme concentrations the degree of hydrolysis reached approx. 12% and the net increase of reducing sugars was 9.5%. Simultaneous hydrolysis by both enzymes ended after 8 hours, but the consecutive process is slower and needed more than 24 hours for each enzyme action. Practically similar degree of hydrolysis (13.5%) could be achieved when alkali pretreated spent malt grain was subjected to simultaneous cellulose/xylanase hydrolysis but for 24 hours. Consecutive hydrolysis reached similar yields after 48 hours. The net reducing sugar increase was 9.5%.

After thermal acid pretreatment and simultaneous enzyme action the hydrolysis degree was 30.8% of the dry mass after 32 hours of enzyme action (net degree of hydrolysis 20%). The consecutive enzyme action hydrolyzed for 48 hours a lower part of the substrate probably due to inhibition by the reaction products (20-24% totally, 10-14% net action). The net degree of hydrolysis by the cellulase complex was approx. 8.5% of the substrate dry mass. The xylanase complex hydrolyzed approx. 12% of the hemicellulose in the barley malt spent grain.

Effect of pretreatment and enzyme hydrolysis on hydrolysis products

Glucose, cellobiose and cello-oligosaccharides were obtained as a result only of thermal water pretreatment. These saccharides and xylose were detected after diluted alkali pretreatment.

Xylanase hydrolysis of thermal water pretreated substrate leads to accumulation of xylose and traces of galactose. Accumulation of hexoses, cellobiose and cello-oligosaccharides was observed after cellulose hydrolysis. The simultaneous enzyme action yielded glucose, xylose, cellobiose and traces of galactose and cellodextrins. The hydrolysis scheme – simultaneous or consecutive did not affect the type of final hydrolysis products. Xylanase hydrolysis of alkali treated at 115°C spent grain yielded C5-xylose, traces of rhamnose, arabinose and galactose. Cellulose hydrolysis yielded glucose, cellobiose and cello-oligosaccharides.

The simultaneous hydrolysis with both enzymes of acid treated barley spent grain lead to fast accumulation of glucose, xylose, cellobiose and traces of rhamnose than after separate or consecutive enzyme action.

Effects of pretreatment on the fermentation with Saccharomyces cerevisiae AL100

Chemical composition of reducing sugars require the application of microorganism that could assimilate C5 and C6 sugars. The ethanologenic yeast *Saccharomyces cerevisiae* AL100 was used in this part of the investigation. The strain could ferment only C6 saccharides to ethanol and is not able to ferment the pentoses. The yielded ethanol was only 2.7 (NaOH treated barley spent grain) - 4.9 g/l (hot water treatment). From 58% (NaOH treatment) to 75% (acid treatment) of the sugars were non-fermentable.

Conclusions

Investigations on the hydrolysis of agricultural wastes were done earlier and from them is evident that the wheat straw, coco-nut coir, the sugarcane bagasse and the sawdust could be hydrolyzed from 34.5 % of dry mass (wheat straw) to 27.0% of DM (sawdust) to reducing sugars. Yoon & Kim (Yoon & Kim, 2005) reported the 43 h hydrolysis of 3.2% of DM to saccharides with avicel as a substrate; Lee et al. (Lee et al., 2008) hydrolyzed only 0.353% of the cellulose from *Pinus densiflora* to reducing sugars.

In our experiments, sugars as glucose, xylose, cellobiose were liberated from barley spent malt grain. From 12% up to 30% of the dry mass was hydrolyzed depending on the pretreatment – with hot water, hot diluted NaOH or acid. Hot water pretreatment leads to partial hydrolysis of cellulose content and affected less the hemicellulose hydrolysis.

The results confirm the importance of the advance pretreating on the degree of the cellulose and hemicellulose hydrolysis. However the quantity of hydrolysed cellulose and hemicellulose after hot water pretreatment reached no more than 8-10% of their amount in barley spent malt grain.

To increase the amount of hydrolysed cellulose spent grain was subjected to thermal pretreatment with diluted NaOH and H_2SO_4. Alkali pretreatment was proved to be

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unsuitable because the spent malt barley grain was transformed in dense mash of fine particles, increased in volume and could not be filtered. Glucose and traces of xylose, cellobiose and cello-oligosaccharides were detected in the hydrolysates. The pretreatment with H_2SO_4 affected significantly the hemicellulose hydrolysis. About 6% of the substrate dry mass were hydrolyzed within 8 hours by the xylanase compared to 2.5-4% (after hot water pretreatment) and 3.5-5.5% (diluted NaOH). Initial hydrolysis products inhibited the enzyme action. Pretreatment affected the degree of hydrolysis by both enzymes and the time necessary for maximal hydrolysis. Hydrolysis products had a negative effect on the fermentation process. Results indicate that acid treatment is unsuitable because it increases the content of non-fermentable sugars, for example the content of pentoses.

Because of the low reducing sugar concentration in the hydrolysates, the liquid fraction could be used as additive to another substrate (saccharose, glucose, fructose).

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