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Microalgae *Chlorella sp.* as an Alternative Source of Fermentable Sugars

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The aim of this work was to optimize the enzymatic hydrolysis of starch originating from microalgae (34.0 ± 1.2% wt. starch in the dry algal biomass) grown on flue gas from waste incineration plant and to study the influence cell disintegration method on the yield of hydrolysis. Apart from the starch, the possibility of cellulose usage from microalgae cell wall as a further source of fermentable sugars was studied and preliminary results are presented.

1. Introduction

The threat of global warming, due to the growth of carbon dioxide in the atmosphere, along with thinning of global oil reserves and increasing cost of raw materials are the main motivations of an intensive search for alternatives to conventional fuels. Attention has been focused on biofuels (bioethanol, biodiesel) (Scharlemann, 2008; Hill, 2006; Farrell et al, 2006) from raw plant material (Novák, 2009; Doucha et al, 2008), mainly crops (Petr, 2008a; Petr, 2008b; Geussová, 2009; Karpenko, 2008). One of the key steps of development and implementation of an alternative method of biofuel production is an effective conversion of starch from unicellular algae to ethanol. These photoautotrophic microorganisms have the ability to enjoy faster growth than higher plants as well as lower water and fertilizer consumption. Furthermore, the produced biomass can be consumed completely, without causing significant waste and ultimately the photobioreactors may be located in areas that are not suitable for agricultural use (Patil and Giselrod, 2008; Doucha and Livansky, 2006; Livansky and Doucha, 2000; Huntley and Redalje, 2007). Different kinds of microscopic algae has been mentioned as a potential source of lipids for biodiesel production (Huntley and Redalje, 2007; Kheshgi etal, 2000), but the technology still faces fundamental problems. Algae with high lipid content in biomass are characterized by low growth rates and sensitivity to shear forces. However, there are also resistant unicellular green algae (*Chlorella*), whose main intracellular storage material is starch. In cells exposed to sulphur limitation, the starch content increased up to 65 % dry weight. A significant reduction of cultivation

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costs (up 50 %) can be achieved by using carbon dioxide (Douskova et al, 2009; Doucha et al, 2005). Bioconversion of CO_2 from flue gas to fermentable sugars could thus in the near future contribute to the direct reduction of carbon dioxide in the atmosphere (Doucha and Livansky, 2006; Doucha et al, 2005; Benemann, 1997; Brown, 1996). The algal biomass, with 34.0 ± 1.2% wt. of starch, used in the experiments was grown in large outdoor open thin-layer photobioreactor (Doucha and Livansky, 2006).

The two-step enzymatic hydrolysis of algal starch by commercially available α-amylase and glucoamylase was studied. The conditions of starch hydrolysis such as substrate and enzyme concentration and the time required for the enzymatic action were taken as recommended by the producer of thermostable enzymes and were further optimized. The impact of the mechanical disintegration of cells on the yield of starch conversion was studied (Doucha and Lívanský, 2008). The maximum degree of cell disintegration (95 % mechanically disrupted cells) led after 14 h of enzymatic treatment to a 96 % conversion of starch into glucose. The achieved maximum yield of the algal starch hydrolysis is comparable with the enzymatic corn starch breakdown.

2. Experimental

Materials and methods. The algal biomass used throughout the experiments was produced in outdoor open thin-layer batch cultures (Doucha and Livansky, 2006; Doucha and Lívanský, 2009) under sulphur limitation (Doušková et al, 2008) carried out at the Institute of Microbiology of the Academy of Sciences of the Czech Republic in Třeboň. In order to facilitate the enzymatic starch hydrolysis the rigid cellulosic cell wall disruption was accomplished by a bead mill Dyno-Mill KDL-Pilot A (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland). The optimum glass beads diameter was 0.3–0.5 mm. In this homogenizer approximately 95 % of algal cells were disrupted (Doucha and Lívanský, 2009). Amylose/Amylopectin ratio of the algal starch was determined by an assay kit (K-AMYL/04/06 Kit, Megazyme, Ireland). Starches containing algal biomass samples were completely dispersed by heating in dimethyl sulphoxide (DMSO) (Yun and Matheson, 1990; <secure.megazyme.com/downloads/en/data/K-AMYL.pdf>, 2009).Conversion of algal starch into fermentable sugars**.** For starch hydrolysis thermostable amylases developed by Genencor (www.genencor.com) were used. A summary of their optimal conditions is presented in the Tab. 1.

Table 1. The thermostable amylolytic enzymes used in the experiments and some of their optimal conditions

Product	Enzymes	Optimum temp. (°C)	Optimum pH	Recommended amount ($kg_{enzyme}\ t^{-1}_{dry\ starch}$)
Spezyme XTRA	α-amylase	85	5.0-6.7	0.4-0.8
Distillase L-400	amyloglucosidase	58-65	3.0-5.0	0.6-0.8
Optimash™ BG	glucanase/xylanase	60-70	4.0-5.0	0.025-0.05

The starch hydrolysis was carried out as follows. At the beginning four mashes were prepared. The concentration of the suspension of dry microalgae material (DB-disintegrated biomass, NDB-non-disintegrated biomass) was 22 g dry biomass weight

L^{-1} with the pH adjusted to 6.0. Furthermore, the suspension was heated up in a thermostated water bath to 85 °C under constant mixing with a magnetic stirrer bar (4.5 cm in diameter, 250 rpm). Subsequently 8.80/17.60 µL of thermostable starch liquefying and high performance alpha-amylase was added (Spezyme® XTRA, Genencor, Denmark). The temperature was kept at 85 °C for 30 min. The enzymatic saccharification process continued with cooling the suspension down to temperature 65 °C and the pH of the suspension was adjusted to 4.0 using hydrochloric acid. Then 8.80/17.60 µL of saccharifying glucoamylase (Distillase® L-400, Genencor, Denmark) and 0.55/1.10 µL of beta-glucanase/xylanase complex (Optimash™ BG, Genencor, Denmark) was added. This step was performed for 24 hours at 65 °C with permanent agitation of the suspension. The summary of added amount of individual enzymes is shown in Tab. 2.

Table 2. Dosage of enzymes in different mashes

Mash	Material	Amount of Spezyme® XTRA (µL)	Amount of Distillase® L-400 (µL)	Amount of Optimash™ BG (µL)
NDB-A	non-disintegrated	8.80	8.80	0.55
NDB-B	non-disintegrated	17.60	17.60	1.10
DB-A	disintegrated	8.80	8.80	0.55
DB-B	disintegrated	17.60	17.60	1.10

(A- basic amount of enzyme; B- increased amount of enzyme)

Analysis of fermentable sugar content. The samples taken from the suspension were cooled to room temperature, filtered through cellulose acetate microfilters (pore size 0.2 µm) and were analysed by HPLC (Agilent 1100) using a ionex column with a little pre-column Ag+ (Phenomenex Rezex RSO Oligosaccharides 200x10 mm) and detected by a refraction index detector (Agilent 1100 RID). The mobile phase was degassed demineralized water operating at flow rate of 0.4 $mL.min^{-1}$, the column temperature was 80 ºC. Before injection, the samples were filtered (through 0.2 µm filter), degassed and pH was adjusted to 7.0. All the data was obtained using Agilent Chemstation software.

3. Results and discussion

Many of the properties of starches that determine their suitability for particular end-uses are dependent upon their amylose/amylopectin ratios. These properties include gelatinisation characteristics, solubility, and the formation of resistant starch. Thus, the measurement of the amylose content of starches is an important quality parameter for the selection of applicable amylolytic enzymes (especially thermostable amylases) (Buléon et al, 1998; Gupta et al, 2003). The level of amylose determined in starch samples by the modified Con A procedure is presented in the Tab. 3. From the results it can be seen that amylose content in algal starch is comparable with main cereal starch sources, thus it can be assumed that the starch rich algal biomass will not require any special treatment compared to cereals. The gelatinization temperature of algal starch (ca. 65°C), as determined by viscosity measurements, also suggests to a structural

similarity between algal and cereal starches (data not shown).For an optimization of the yield of enzymatic hydrolysis of the real algal starch substrate, the influence of hydrolysis conditions (temperature, duration of enzymatic degradation) were studied. The substeps of the hydrolysis (gelation, liquefaction, saccharification) (Buléon et al, 1998; Gupta et al, 2003) were carried out during experiments in batch arrangement under controlled conditions (enzyme addition, temperature profile, agitation). For the complete conversion into high glucose syrup, the first step is the liquefaction into soluble, short-chain dextrins and oligosaccharides. The use of enzymes in starch liquefaction is well established and has been extensively reviewed. This process requires the use of highly thermostable α-amylases. The next step is the saccharification of the starch–hydrolysate syrup to high concentration glucose syrup. This is done by using a glucoamylase that hydrolyzes α-1-4 glycosidic bonds from the non-reducing end of the chain (Marc et al, 2002).

Table 3. Amylose content in major plant sources (Buléon et al, 1998) compared with amylose content in Chlorella vulgaris grown under sulphur limitation

Source	Amylose content (% of total starch)
Microalgae –*Chlorella sp.*	**34-38**
Barley*	21-24
Wheat*	25-29
Maize*	25-28
Potato*	18-21
Pea*	33-36

** (Buléon et al, 1998)*

Table 4. Concentration of sugars before and after enzymatic hydrolysis of 22.00 g L^{-1} algal biomass in water and the yields of glucose

Sugar	Concentration of sugar before hydrolysis				Concentration of sugar after hydrolysis				Yield of sugar			
	NDB (gL^{-1})		DB (gL^{-1})		NDB (gL^{-1})		DB (gL^{-1})		NDB (%)		DB (%)	
	A	B	A	B	A	B	A	B	A	B	A	B
Maltose	0.7	0.1	1.1	0.2	0.4	0.1	0.2	0.2	-	-	-	-
Glucose	0.7	0.1	1.0	0.2	6.0	7.1	8.6	8.9	56.8	70.2	73.4	96.9
Maltotriose	0.3	0.1	0.8	0.2	0.3	0.1	0.2	0.5	-	-	-	-

(NDB- non-disintegrated biomass; DB- disintegrated biomass; A- basic amount of enzyme; B- increased amount of enzyme)

The algal biomass used throughout the whole experimental work was grown in outdoor open thin-layer batch cultures under sulphur limitation and contained 34 ± 1.2 % wt. of starch. In Tab. 4 there are shown the concentration of extracted sugars after 60 min of biomass maceration (11 g) in 500 mL of distilled water. The 22.00 g of the dry biomass contained approximately 7.50 g of the starch at the beginning of the enzymatic breakdown experiments. Based on the analysis of saccharides in reaction mixture after the hydrolysis (Tab. 4) it was found that the starch, as well as smaller quantities of

maltose and maltotriose, was converted into glucose. The mechanical disintegration of cells of algal biomass significantly influenced the yield of the process. In the absence of mechanical milling of the algae cell walls, the glucose concentration in the reaction mixture increased to 6 g L^{-1}. The yield of converting starch into glucose was approximately 57 %. Mechanical disruption of solid cell walls and intracellular membrane structures of algae before the enzymatic hydrolysis resulted in an increase in the yield of starch hydrolysis to 73 % (Tab. 4). However, to increase the economic feasibility of the production process it was necessary to further optimize the process. This process required the regulation of the conditions (addition of enzymes, temperature profile, mixing, time of enzyme action). Due to increasing the quantities of enzymes and extending the reaction time, the yield of glucose in the non-disintegrated algal biomass increased by 13 % to 70 %. The yield of glucose in the disintegrated biomass increased by ca. 24 % to 97 %. This value is comparable to the enzymatic hydrolysis of corn starch.

4. Conclusion

In this work the two-step enzymatic hydrolysis of algal starch by commercially available α-amylase and glucoamylase was studied. The conditions of starch hydrolysis such as substrate and enzyme concentration and the time required for the enzymatic action were taken as recommended by the producer of thermostable enzymes. During the treatment following the recommended doses of enzymes the starch was hydrolysed by 73 % into fermentable sugars. Still, the yield of starch hydrolysis is slightly lower than in the case of hydrolysis of corn starch (ca. 90%). This was due to imperfect optimization of the process of hydrolysis (disintegration, enzyme dosage, duration of treatment). It was found that increasing the amount of enzymes, as compared with the manufacturer's instructions, the yield of glucose from starch of disintegrated biomass increased to 97 %. This value is comparable to the enzymatic hydrolysis of corn starch.

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