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REVIEW ARTICLE

FUEL ETHANOL BIOPRODUCTION FROM INULIN RICH FEEDSTOCK

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Abstract

Due to its important environmental benefits, bioethanol promise to be a good biofuel substitute for gasoline. To make it competitive with other fuels, the production costs should be reduced by using alternative raw materials. Inulin rich feedstock, like dahlia (*Dahlia hortensis*) and Jerusalem artichoke (*Helianthus tuberosus*) tubers or chicory (*Cichorus intybus*) roots would be a cheap and convenient source for fermentable sugars for bioethanol production. These are suitable crops for European countries and in low-requirements environmental conditions.

Two processes were studied for conversion of inulin rich feedstock to fermentable sugars:"acid-based" and "enzyme-based" hydrolysis. The fermentable sugars (mainly fructose and low amounts of glucose) are then fermented using different alcohol-tolerant *Saccharomyces cerevisiae* yeast strains. Direct fermentation of inulin to ethanol was also performed, as some *Kluyveromyces* spp. yeast strains were found to have the ability to ferment inulin.

This paper highlights the on-going developments in fuel ethanol bioproduction from inulin rich feedstock, with focus on inulin hydrolysis which is the major problem of the overall process.

Keywords: fuel ethanol, inulin, inulinase, biotechnology

Introduction

Biofuels have recently received attention as alternative fuels, as known fossil fuels reserves are estimated to last between 41 and 700 years (Marica, 2009). Nowadays, ethanol is the most employed liquid biofuel, next to methanol, esters and biodiesel (Marica, 2009, Taherzadeh and Karimi, 2007, Niga, 2009). Bioethanol is considered a good gasoline alternative as the energy crops can be grown in most climates around the world (RESTMAC Project).

The bioethanol market was continuous increasing during the last decades. According to Renewable Fuels Association (RFA), in 2011 North and Central America was the main bioethanol producer, with 54513 million litres, followed by South America and Brazil with over 20820 million litres and Europe with 4420 million litres (http://ethanolrfa.org/pages/World-Fuel-Ethanol-Production, http://cta.ornl.gov/bedb/biofuels.shtml). In Europe, Sweden is the highest producer and consumer of bioethanol, having almost 800 E85 fuel stations in 2007. It is followed by Germany, France and United Kingdom (RESTMAC Project).

Although Romania is not one of the largest users of bioethanol in Europe, some bioethanol plants were constructed in the last years, one of them in Zimnicea, having the capacity of 100 000 tons of bioethanol per year (http://www.wall-street.ro/articol/Companii/74837/Cum-arata-investitia-de-90-mil-euro-a-lui-Ioan-Niculae-in-bioetanol.html). Moreover, the Dacia automobile plant produces Dacia Sandero Stepway on bioethanol (http://www.ziare.com/dacia/sandero/dacia-lanseaza-sandero-stepway-pe-bioetanol-1161997).

The cost of fuel ethanol production is still too high to compete with gasoline, but the ecological impact is of primary importance in some countries for the development of its obtaining technology. The environmental and infrastructure advantages offered by the use of biofuels are more appreciated nowadays (Wyman, 2001). The fuel ethanol does

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not contribute to the greenhouse effect; the CO_2 resulted from fermentation is usually used by the plants in their metabolism. Bioethanol use as fuels can reduce CO, hydrocarbons (HC) and volatile organic compounds (VOC) emissions, contributing to the reduced ozone layer in the urban zones. Bioethanol does not contain aromatics and sulphur derivate compounds and its use can reduce nitrogen oxides emissions and particulate air pollution (Roehr, 2001, Wyman, 2001).

Bioethanol can be obtained from energy crops such are starchy crops (barley, wheat, potatoes, maize etc.), sugar crops (sugar beet, sugar cane, Jerusalem artichoke, dahlia, chicory etc.), cellulose crops (straw, wood etc.) and solid energy crops (cardoon, sorghum whole crop maize etc.) (Smith, 2006). The primary feedstock for bioethanol obtaining worldwide remains sugar and starch from agricultural crops (Smith, 2006).

Inulin rich feedstock as Jerusalem artichoke, dahlia and chicory are considered one of the promising energy crops because of its high yield in bioethanol and fermentable sugars, and relatively low input requirements.

Alcohol production from Jerusalem artichoke was studied since the beginning of the 19th century and the obtained bioethanol was used in the World Wars (Guiraud *et al.*, 1982, Matias *et al.*, 2011).

In the biotechnological process of bioethanol obtaining from inulin rich feedstock, the raw material is firstly hydrolysed in order to obtain the fermentescible sugars for yeasts metabolism. Despite the numerous hydrolysis processes available, the use of enzymatic saccharification is presented as advantageous as it requires less energy and uses mild environmental conditions. Some new fermentation techniques were developed to increase bioethanol yield, including separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Ferreira *et al.*, 2011).

The present work focuses on the bioethanol production technologies from inulin rich feedstock and developments made in biofuel obtaining in the last decades.

Inulin and inulin producing plants

Inulin is a polyfructan, a molecule consisting mostly of β-fructo-furanoside residues, with a glucose molecule at the terminating end (Fig. 1). Until recently, inulin was considered a linear molecule, but it was demonstrated by permethylation analysis, that even native inulin has a small degree of branching (Vandamme *et al.*, 2004, Roberfroid, 2005).

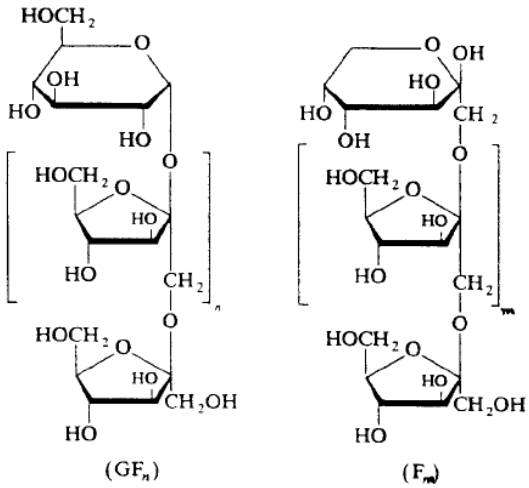

Figure 1. *Chemical structure of inulin (GFn – number of fructose units linked to glucose; Fm – number of fructose units) (Vandamme et al., 2004)*

Inulins are reserve carbohydrates in plants such are Jerusalem artichoke, chicory, dahlia, garlic, onion and salsify. They are also present in fungi and bacteria, and only brief summaries of the occurrence of inulins in algae have been provided (Roberfroid, 2005) (Table 1).

Jerusalem artichoke and chicory seem to be the richest sources of plant inulin (Bekers *et al.*, 2008). The advantages of these crops consist in high inulin content, increased rate of growth in poor soils and high yields productivity, minimal or no fertilizers requirements, overwintering is not expensive, high tolerance to frost and diseases (Bajpai and Margaritis, 1982, Gibbons, 1989, Danilcenko *et al.*, 2008, Matias *et al.*, 2011).

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Table 1. *Occurrence of inulins in plants and microorganisms (Vandamme et al., 2004, Roberfroid, 2005)*

Occurrence of inulin	Inulin content, g/100g
Plants:	
- Oat (*Avena sativa*)	NA*
- Barley (*Hordeum vulgare*)	0.5-1.5
- Rye (*Secale sativa*)	0.5-1
- Wheat (*Triticum durum*, *Triticum aestivum*)	1-4
- Leek (*Allium ampeloprasum*)	3-10
- Onion (*Allium cepa*)	1-7.5
- Garlic (*Allium sativum*)	9-16
- Asparagus (*Asparagus officinalis*, *A. racemosus*)	NA
- Palm lily (*Cordyline terminalis*)	NA
- Chicory (*Cichorium intybus*)	15-20
- Dandelion (*Taraxacum officinale*)	12-15
- Jerusalem artichoke (*Helianthus tuberosus*)	17-20.5
- Salsify (*Tragopogon porrifolius*)	4-11
- Yacon (*Polymnia sonchifolia*)	3-19
Algae:	
- *Acetabularia mediterranea*	NA
- *Cladophora* spp.	
- *Rhizoclonium* spp.	
Fungi:	NA
- *Aspergillus sydowi*	
Bacteria:	NA
- *Streptococcus mutans*	
- *Lactobacillus reuteri*	

*NA – data not available

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Inulins have different characteristics, according to their provenience. Inulins from plants have a lower degree of polymerization (DP_{max}–200) than inulins of bacterial origin (DP_{max}–10000÷100000). Also, the bacterial inulins are highly branched (15%), while inulins from plants have a small degree of branching (1-2%) (Vandamme *et al.*, 2004, Roberfroid, 2005). Composition depends mainly on the plant source, harvesting conditions and extraction process (Ronkart *et al.*, 2007).

Since the price of feedstock contributes substantially to the final price of bioethanol, inexpensive feedstock is considered to make the bioethanol competitive on the market (del Campo *et al.*, 2006).

Inulin hydrolysis

Inulin rich feedstock like Jerusalem artichoke and dahlia and chicory tubers are formed from carbohydrate polymers that can be hydrolysed to single sugars and fermented to ethanol. Inulin can be hydrolysed using enzymes or directly inulinase producing microorganisms, but also by acid hydrolysis.

Acid hydrolysis

The acidic treatment of inulin has been investigated using mainly sulphuric and hydrochloric acids, at pH between 1.0 and 4.0 and temperatures of 60…100°C, hydrolysis time of 5 minutes to several hours (Szambelan and Nowak, 2006, Glibowski and Bukowska, 2011). Nasab *et al.* (2009) also found that pH, temperature and time are significant variables for acid hydrolysis of inulin, while at alkaline pH, these variables are insignificant. During the acid treatment of sugars some degradation compounds like furfural and 5-hydroxymethylfurfural (5-HMF) are formed, toxic compounds that inhibit further enzymatic and fermentation processes (del Campo *et al.*, 2006).

The acid pretreatment of the feedstock improves the release of sugars (del Campo *et al.*, 2006). Also, as del Campo *et al.* (2006) observed, a direct relation between acid concentration and the released fermentescible sugars. Also, it has been shown that acid hydrolysis is faster than enzymatic hydrolysis (Szambelan and Nowak, 2006). On the other hand, the high concentration of the acid conducts to the formation of higher amounts of inhibitory compounds (Bekers *et al.*, 2008).

Szambelan and Nowak (2006) showed that sulphuric acid hydrolysis of Jerusalem artichoke tubers at pH 2.0 yielded the highest amounts of fermentable sugars, more than phosphoric and hydrochloric acids, using hydrolysis pH between 2.0 and 3.0, at 100°C, after 60 minutes.

In their study, Thuesombat *et al.* (2007) showed that acid hydrolysis of Jerusalem artichoke mash, with concentrated sulphuric acid at 80°C, for 40 minutes gave the highest reducing sugar content and ethanol yield respectively. The maximum ethanol concentration obtained was 88.1 g/l, after 72 hours of batch fermentation (Thuesombat *et al.*, 2007).

Harsh acidic treatment conditions should be avoided for inulin hydrolysis. Also, in the case of the high content of inulin in the feedstock, dilute acid hydrolysis does not seem to be efficient, therefore alternative treatment with enzymes should be considered for higher yields.

Enzymatic hydrolysis

Several enzymes able to hydrolyse inulin have been described in the literature. The inulinases (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7), classified among hydrolases, can be classified as exo-inulinases and endo-inulinases. Inulinases act on the β-2,1 linkage of inulin and hydrolyse it into fructose and glucose (Chi *et al.*, 2009). These enzymes are originating from plants or microorganisms. Microorganisms are the best producers for inulinases, as they are easy to cultivate and of their high productivity (Chi *et al.*, 2009). The main inulinase producers described in the literature are *Aspergillus niger* (Gern *et al.*, 2001, Ge and Zhag, 2005, Kango, 2008, Kumar *et al.*, 2005), *Kluyveromyces marxianus* (Rosa *et al.*, 1986, Kalil *et al.*, 2010, Santisteban *et al.*, 2005, Golunski *et al.*, 2011, Treichel *et al.*, 2009, Kushi *et al.*, 2000, Singh and Bhermi, 2008, Mazutti *et al.*, 2010, Sguarezi *et al.*, 2009) and *Pichia guilliermondii* (Gao *et al.*, 2007, Chi *et al.*, 2009, Yu *et al.*, 2009, Gong *et al.*, 2007). Also, *Rhizoctonia* spp. strains were used as inulinase producers (Ertan *et al.*, 2003, Neagu Bonciu *et al.*, 2011, Neagu Bonciu *et al.*, 2012). The inulinases have, in general, molecular weight between 50 and 300 kDa, optimum pH of 3.5-7.5, optimum temperature 30...60°C, according to their provenience (Gern *et al.*, 2001, Ge and Zhag, 2005, Kango, 2008, Kumar *et al.*, 2005, Kalil *et al.*, 2010, Santisteban *et al.*, 2005, Golunski *et al.*, 2011, Treichel *et al.*, 2009, Kushi *et al.*, 2000, Singh and Bhermi, 2008, Mazutti *et al.*, 2010, Sguarezi *et al.*, 2009).

The exo-inulinases remove the terminal fructose residues of inulin, while the endo-inulinases act on the internal linkages of the inulin, resulting highly soluble and sweet oligosaccharides with prebiotic properties (Chi *et al.*, 2009; Ertan *et al.*, 2003).

Although enzymatic hydrolysis has slow rates of inulin decomposition, this allows to fermentation yeasts to better ferment available sugars, without catabolic repression to interfere.

Also, some catalysts (acidic zeolite LZ-M-8) were developed and used for inulin hydrolysis with commercial inulin. This catalyst was found to be very selective and hydroxymethylfurfural was not detected in the medium (Abasaeed and Lee, 1995).

Fermentation of inulin hydrolysates

Alcoholic fermentation is the conversion of sugars into ethanol. The fermentation of inulin hydrolysates was made using different yeast strains, selected after the following criteria: ethanol and temperature tolerance, resistance to the inhibitory products, fermentation rate, and ethanol yield (Ogbonna, 2004).

Different methods for inulin hydrolysis and fermentation processes were developed and optimized until now. The most researched hydrolysis and fermentation methods were separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Also, solid state fermentation was widely studied. Gibbons (1989) reported ethanol yield levels of 41-53% in his study, using solid-phase fermentation of Jerusalem artichoke tubers with *Kluyveromyces marxianus* strains. These low yields obtained can be explained by the low availability of inulin and the metabolic functionality of yeasts in the solid state fermentation process (Gibbons, 1989).

Separate hydrolysis and fermentation traditionally process consists in mixing the feedstock with the enzyme or inoculated with the microorganisms able to synthesize enzyme and incubated for the established time. After hydrolysis the enzymes or microorganisms are inactivated (usually by heating) and the fermentation yeasts are inoculated for fermentation (Ogbonna, 2004). The disadvantages of this method are the enzyme or

cells inhibition by the high concentration of fermentescible sugars released in the medium and consequently low yields in ethanol (Ogbonna, 2004).

The simultaneous saccharification and fermentation process consists in inoculating the inulinase producing microorganisms or the inulinases at the same time with the fermentation microorganisms, so the hydrolysis products are simultaneously bioconverted to ethanol. Therefore, the problem of feedback inhibition is avoided. Other advantages of this method are low contamination risk, higher productivities and yields compared to SHF process, less enzyme needed (Ogbonna, 2004).

Using the SSF process, Ge and Zhang (2005) obtained a maximum ethanol concentration of 19.6% and conversion efficiency of Jerusalem artichoke to ethanol of 90% of the theoretical yield after 48 hours, using *A. niger* SL-09 strain for inulin hydrolysis and *S. cerevisiae* Z-06 strain for fermentation. Their objective was to reduce the fermentation period of 5 days used by their predecessors. Similar results were obtained also by Nakamura *et al.* (1996) using the same substrates. Also, Ohta *et al.* (1993) obtained maximum concentrations of ethanol of 20.4 and 21% v/v from chicory and dahlia inulins respectively, using SSF process and *A. niger* and *S. cerevisiae* strains within 3 days at 30°C.

Use of yeasts with inulinase activity for inulin fermentation was also considered because it would eliminate preliminary acidic or enzymatic hydrolysis and thereby it would reduce the production costs (Gibbons, 1989). Numerous yeast strains posses the ability to ferment inulin and *Kluyveromyces marxianus* strains were particularly the most studied.

Kim *et al.* (1998) developed a recombinant *Saccharomyces cerevisiae* strain, secreting exo-inulinase that would allow the development of a SSF process of inulin to ethanol. They obtained 9 g/l ethanol from Jerusalem artichoke inulin (Kim *et al.*, 1998). Also, the recombinant strain *Saccharomyces* sp. W0/YCPlac33 PGK/CYC1-INU1, carrying the inulinase gene from *Pichia guilliermondii* strain, was used for ethanol obtaining from tuber meal of Jerusalem artichoke by Zhang *et al.* (2010). They obtained very high ethanol concentrations (12.05 ml ethanol in 100 ml of fermented medium in 144 hours) and over 98.9% of total sugar was utilized in one step process.

Lim *et al.* (2011) reported a strain of *S. cerevisiae* that utilizes inulin from Jerusalem artichoke without pretreatment for inulin hydrolysis. They obtained 36.2 g/l ethanol after 34 hours of fermentation, and a conversion efficiency of 70% of the theoretical yield (Lim *et al*, 2011). Also, Rosa *et al.* (1986) obtained high ethanol concentration (12.8% v/v) from the juice of Jerusalem artichoke tubers using *Kluyveromyces marxianus* yeast, with a theoretical yield of 77%.

Conclusions

From the data presented above it is clear that many advances have been made in bioethanol production from inulin rich feedstock. Many raw materials rich in inulin have been used for bioethanol production. Also, some significant advances in microbial inulinase bioproduction, characterization of the enzymes and inulinase gene cloning have been made.

Different hydrolysis and fermentation techniques have been used to increase the ethanol yield. However, the feasibility for fuel ethanol production from different feedstock depends on the availability of an efficient technology. Nowadays, the technologies needed for the inulin rich raw materials to bioethanol are relatively complex and expensive. To further decrease the costs of biomass conversion to ethanol it is essential to minimize the sugar losses and efficient conversion of inulin into fermentescible sugars has become an important task and the opportunity to reduce the bioethanol costs. In this context, lately, efforts have been made to isolate or to develop new strains of microorganisms able to ferment directly inulin.

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