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Optimisation of ethanol fermentation of Jerusalem artichoke tuber juice using simple technology for a decentralised and sustainable ethanol production



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ABSTRACT

Ethanol production from Jerusalem artichoke was optimised using simple technology according to tuber harvest date. The optimal treatment for winter juice was the addition of 0.25 mL L⁻¹ of a commercial inulinase (17 U g⁻¹) and a juice heating at 52.5 °C for 60 min before the beginning of the fermentation. For autumn juice, the optimal treatment was a previous heating at 80 °C for 15 min followed by the addition of 0.75 mL L⁻¹ of the inulinase at 60 °C kept for 120 min, prior to the fermentation. Ethanol yields of 0.458 and 0.454 g g ⁻¹ were obtained with autumn juice and winter ones, respectively. Fermentation was conducted at 30 °C by *Saccharomyces cerevisiae*. These results could be useful for a staggered and decentralised ethanol production from a low-requirement crop which does not interfere with the food chain.

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Introduction

Nowadays, governments encourage the development and use of biofuels with the aim of reducing greenhouse gas (GHG) emissions and because of the need to find alternative sources of energy other than fossil fuels to increase security of supply. In this direction, the European Directive on biofuels (EU-Directive 2009/28/EC, 2009) established the goal of reaching a 10% share of renewable energy in the transport sector in Community by 2020 and introduced mandatory sustainability criteria which biofuels must meet under this Directive. Biofuels must deliver greenhouse gas savings of at least 35% compared to fossil fuels, rising to 50% in 2017 and to 60%, for biofuels from new plants, in 2018. According to this premise and taking into account the default values for GHG emission savings of some biofuels included in the annex V of this Directive, new sustainable crops and process are required to be investigated in order to achieve the European renewable targets.

Therefore, the use of biomass for energy is a great opportunity for agriculture in the twenty-first century, but it is required to choose the right crops, in the right place with the right techniques, with a different approach from traditional agri-food products (Fernández, 2006).

Sustainability of biofuels is increasingly taken into account. However, sustainable technologies to produce biofuels from different kinds of biomass resources are required (Coppola et al., 2009). Thus, an optimisation of the production process is needed to improve the sustainability of biofuels (Matías et al., 2011).

Ethanol is the most produced biofuel worldwide. The United States is the largest ethanol world producer, followed by Brazil. Corn accounts for more than half of global ethanol production, and sugar cane for more than one third (REN 21, 2010).

At present, most of the main raw materials for ethanol production are food and starchy grains (Li and Chan-Halbrendt, 2009). The conventional enzymatic saccharification of starch by amylases has many disadvantages and the process is complicated (Chi et al., 2009). In the process to convert starchy grains into ethanol, starch must be gelatinized and liquefied at high temperature before the saccharification and fermentation so, compared to inulin hydrolysis using inulinases, the process for hydrolysis of starch is more complex (Zhang et al., 2010). Ethanol can also be obtained from lignocellulosic biomass, but the development of cost-effective and sustainable technologies is required (Chi et al., 2011).

Decentralised bioenergy systems are receiving increasing attention due to the potential ability to support local development and to create local employment (Mangoyana and Smith, 2011). The decentralised production of biofuels has been proposed for several reasons, such as the possibility of small scale production, the fact that there is no need to use high technology or make large investments, and because small

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plants do not need highly specialised technical staff (Iglesias et al., 2012).

Jerusalem artichoke (Helianthus tuberosus L.) is a low-requirement crop which has been reported to have one of the highest carbohydrate yields. Nowadays it does not interfere with food chain. It is, then, a promising energy crop for sustainable ethanol production (Matías et al., 2011). The clone of JA called "Nahodka" has been reported to provide high sugar and biomass yield (Matías et al., 2013; Curt et al., 2006; González et al., 2004; Conde et al., 1991). The main storage carbohydrate of Jerusalem artichoke (JA) is inulin, which is a polydisperse β (2-1) fructan, mainly a mixture of two linear fructan oligosaccharides (FOS), one with a terminal sucrose (GFn) and the other with a fructopyranose (Fm) (Bruggink et al., 2005). In JA, inulin has an average degree of polymerisation (DP) of 8 to 10 (Vijn and Smeekens, 1999), although depolymerisation of inulin during ageing of tubers has been observed (Schorr-Galindo and Guiraud, 1997). This phenomenon should be taken into account for ethanol production from JA tubers, especially if harvest is staggered in order to optimise the production costs.

Inulin cannot be directly fermented by classic fermentation yeasts, so either a hydrolysis followed by fermentation with classical yeasts or the use of yeasts with inulinase activity is required to produce ethanol from inulin sources. In the case of JA, acid hydrolysis was the main method used at first (Kays and Nottingham, 2007). Nevertheless, for a sustainable and decentralised production, this method has several disadvantages because it uses large amounts of both acid (toxic reagents) and energy (Guiraud et al., 1982). Enzymatic hydrolysis of inulin has also been used later (Ricca et al., 2009; Rocha et al., 2006; Szambelan and Nowak, 2006; Szambelan et al., 2004, 2005; Nakamura et al., 1996), but the aim of most of these researches was the syrup production. Szambelan and Nowak (2006), studied the enzymatic hydrolysis of JA tubers for further ethanol production, but only two doses of inulinases from Aspergillus niger were studied and the average degree of polymerisation of inulin was not taken into account. Inulinases can be produced by a series of microorganisms, including fungi, yeasts, and bacteria. There are two different subclasses of inulinase, endoand exo-inulinase: exoinulinase (EC 3.2.1.80) hydrolyses the terminal fructose from the inulin chain, whereas endoinulinase (EC 3.2.1.7) reduces the long chain of inulin into smaller oligosaccharides (Basso et al., 2010). Ethanol from Jerusalem artichoke can also be produced by simultaneous enzymatic saccharification and fermentation (SSF) (Zhang et al., 2010; Szambelan and Nowak, 2006; Xiang-Yang and Wei-Guo, 2005; Nakamura et al., 1996; Ohta et al., 1993). However, in previous works, high enzyme concentrations and non-commercial microorganisms were required to obtain relatively high ethanol yields. On the other hand, yeasts with inulinase activity, like Kluyveromyces marxianus, had also been studied. These kinds of microorganisms can produce both active inulinase and ethanol. Nevertheless, compared to Saccharomyces cerevisiae, K. marxianus cannot tolerate a high concentration of ethanol in the medium and produces less ethanol, so that it has not been used for ethanol production from inulin-containing materials in the fermentation industry so far (Zhang et al., 2010). Furthermore, K. marxianus requires more stringent anaerobic conditions than S. cerevisiae, which means a more complex industrial installation (Guiraud et al., 1982).

The aim of this work has been to optimise ethanol fermentation of juice from JA tubers using commercial reagents and simple technology in order to favour decentralised industrial production.

Material and methods

Jerusalem artichoke tubers

Tubers used in the experiments were harvested from field trials conducted in experimental plots of Agricultural Research Centre of Extremadura, located in the Guadiana River Basin. The Nahodka clone of Jerusalem artichoke was used. Two harvest dates were carried out during the same season: one in autumn (December 2, 2010) and one late in winter (March 1, 2011).

Extraction of the juice of Jerusalem artichoke tubers

Just after harvest, juice was extracted from tubers by liquefying, using a blender (Model F2000, Frucosol). Previously, tubers were washed with water. Then, juice was immediately stored at -25 °C for further use.

Microorganism

S. cerevisiae was used in the fermentation trials. It was purchased from Laffort (Actiflore Cerevisiae), which contains about 20,000 million of live yeast cells per gram of dry yeast.

Experiments

Different experiments were carried out in order to optimise the ethanol fermentation yield of juice of Jerusalem artichoke tubers by a juice treatment followed by a simultaneous saccharification and ethanol fermentation. Three variables (heat treatment of juice prior to fermentation, dose of inulinase and moment of enzyme addition) were adjusted successively through different series of experiments.

Juice treatment

Juice heat treatment and partial enzymatic hydrolysis of the inulin were performed prior to the beginning of the fermentation. 500 mL Erlenmeyer flasks filled with 200 mL of JA juice were employed. Water baths were used to maintain the temperature. Different heat treatments and doses of enzyme were studied. The enzyme used was a commercial (Sigma-Aldrich) liquid mixture (density = 1.12 g mL^{-1}) of exo- and endo-inulinases, obtained from *A. niger*; the declared activity was 17 U g⁻¹.

Simultaneous saccharification and ethanol fermentation of Jerusalem artichoke juice

Later, the saccharification and ethanol fermentation of the JA juice from previous step were performed simultaneously in batch mode at 30 °C in partially anaerobic conditions, in the same Erlenmeyer flask of 500 mL Inoculation was done with 3 g dry wt of the yeast described above per litre of juice. Yeast had been previously activated in a liquid medium (13 mL distilled water and 0.5 g sucrose per gram dry wt.) in a shake flask at 40 °C for 20 min. During the fermentation, samples (3 mL) were periodically withdrawn, cooled for 10 min at 3 °C and centrifuged at 4000 rpm. The supernatants were filtered through a 0.2 μ m polyethersulfone syringe filter (Albet LabScience) and analysed for ethanol and sugars. The ethanol yield was determined as grams of ethanol produced per gram of total sugars. During the fermentation the medium pH was regularly measured using a digital pH metre (Model Basic 20, Crison). Juice was not previously sterilised due to the high cost of this operation.

Table 1

Sugar content and composition in the juice of Jerusalem artichoke tubers according to harvest date.

Harvest date	Free sugars $(g L^{-1})$	Total sugars $(g L^{-1})$	Inulin (% s/total sugars)	DP
Autumn	14.2	212.3	84.5	10.8
Winter	22.7	215.2	82.1	4.6

--- 60 °C 1 h --- 45 °C 1 h ---- Without heating



Fig. 1. Influence of the heat treatment and the inulinase concentration (mLL^{-1}) on the ethanol yield in winter juice. Bars mean the standard deviation (n = 2).

Analytical methods

Ethanol assay

Ethanol concentration in the medium, expressed as % (v/v), was determined by gas chromatography using a Perkin Elmer Clarus 500 apparatus, equipped with an auto-sampler and fitted with a split/splitless injector and a flame ionisation detector. The column was a capillary column (VF-624 ms, 30 m × 0.25 mm i.d., 1.4 µm film thickness) packed with 6% cyanopropylphenyl–94% PDMS. Analyses were carried out at a programmed temperature: initial temperature 40 °C (kept for 10 min), then increasing at a rate of 25 °C min⁻¹ and final temperature of 250 °C (kept for 3 min.). The detector and injector temperature was 150 °C. Helium was employed as carrier gas at a pressure of 12 psi. The injection volume was 1.0 µL. 4 methyl-2 pentanol was used as internal standard.

Determination of sugar content and average degree of polymerisation (DP) of inulin in juice

Total and reducing sugars and the average DP of inulin in the juice were determined by liquid chromatography–mass spectrometry (LC– MS) following the method of Matías et al. (2011). The chromatographic system consisted of a binary gradient LC–MS pump and a Prostar 410 HPLC Autosampler from Varian and a column heater from Chrom Tech. The atmospheric pressure (AP)-electrospray ionisation (ESI) mass spectrometer was a triple quadrupole from Varian (310-MS TQ).



Fig. 2. Reducing sugars in winter juice after three different heat treatments and five concentrations of inulinase (added before the heat treatment). Total sugar of winter juice = 215.2 g L^{-1} .

Results and discussion

Juice characteristics

Juice sugar composition was different according to tuber harvest date, as it is shown in Table 1. The inulin DP was much higher in the autumn juice (10.8) than in the winter ones (4.6). It is well known that there is a gradual decrease in the average DP during the ageing of tubers due to a natural depolymerisation of inulin (Matías et al., 2013; Schorr-Galindo and Guiraud, 1997; Soja et al., 1990; Chabbert et al., 1983). On the contrary, total sugar content was similar in both types of juice (Table 1). Higher viscosity was observed in autumn juice than in winter one, which can be explained due to the higher inulin DP. Accordingly, process optimisation was performed according to tuber harvest date, which has not been taking into account in previous works.

Process optimisation using juice of tubers from winter harvest (winter juice)

In a first series of experiments, five concentrations of inulinase and three heat treatments of the juice prior to fermentation were investigated. In Fig. 1 it can be clearly observed that the highest ethanol yield was obtained by the heat treatment at 60 °C for 1 h. Similar ethanol yields were obtained at 45 °C for 1 h and without heating, with higher standard deviations in comparison with the treatment at 60 °C. These results can be explained because the risk of development of undesirable microorganisms is lower at higher temperature, but also because of the higher inulinase activity at 60 °C (Fig. 2). Reducing sugar concentrations after the treatment were directly proportional to the inulinase dose for treatments at 60 °C and 45 °C, while practically remained constant



Fig. 3. pH evolution during fermentation with respect to inulinase concentration in winter juice heated at 60 $^{\circ}$ C.



Fig. 4. Influence of the heat treatment and the moment of the inulinase addition [before (\blacksquare) or after (\blacktriangle) the heat treatment] on the ethanol yield in winter juice. Heat treatment time = 1 h. Inulinase concentration = 1.25 mL L⁻¹. Bars mean the standard deviation (n = 2).

without heating. But more reducing sugars were released at 60 °C (Fig. 2), which is in line with that reported by others (Rocha et al., 2006; Ricca et al., 2009). However, inulin was only partially hydrolysed during the heat treatments with the inulinases, reaching a maximum of 38% of total sugars with 1.25 mL L^{-1} of inulinases at 60 °C. The exoinulinases released only a portion of all potential reducing sugars in this step. However, relatively high ethanol yields were obtained, as it is shown in Fig. 1, which means that the hydrolysis or saccharification of inulin had to continue during the fermentation. This process would be facilitated by the action of the endo-inulinases, reducing the length of the inulin chains, during the partial hydrolysis step. Furthermore, a slow rate of carbohydrate decomposition allows yeast to better ferment available sugars (Szambelan and Nowak, 2006). The simultaneous saccharification and alcoholic fermentation (SSF) could be carried out because the inulinases were not inactivated after the juice heat treatments and could exhibit activity during fermentation. It should be noted that a rapid deactivation of these kinds of inulinases at temperatures higher than 60 °C was observed by Ricca et al. (2009). Although fermentation was conducted at a much lower temperature than the optimal for the inulinase activity, but better for thermostability, inulin could be successfully hydrolysed and converted into ethanol due to the relatively long fermentation times. On the other hand, an important pH drop of the juice was observed in the first hours of fermentation in all the experiments, from about 6.5 to around 5.5, which is within the



Fig. 5. Influence of the heat treatment and the inulinase concentration (mL L^{-1}) on the ethanol yield in autumn juice. Bars mean the standard deviation (n = 2).

optimal pH range for inulinases from A. *niger*, as reported by others (Rocha et al., 2006; Szambelan and Nowak, 2006). A pH of 5.5 was reached in less than 8 h in all cases (Fig. 3). Therefore, in order to reduce the cost of the process and make it easier with the aim to favour a decentralised production, the pH of the juice was not adjusted, unlike in other works reported up to date. Regarding the influence of the inulinase concentration on the ethanol yield, the highest yield $(0.424 \pm 0.016 \text{ g g}^{-1})$ was achieved with 0.25 mL of inulinase per litre of juice (Fig. 1), although more fermentable sugars were released during the juice treatment with 1.25 mL L⁻¹ (Fig. 2). Similar ethanol yields were obtained with the inulinase concentrations of 0.05 mL L⁻¹ (0.396 \pm 0.029 g g⁻¹) and 1.25 mL L⁻¹ (0.417 \pm 0.026 g g⁻¹). Nevertheless, lower time of fermentation was required with 1.25 mL L⁻¹ (68 h) than with the other doses (92 h). New experiments were carried out to adjust the inulinase dose, which will be explained later.

A new series of experiments was carried out in order to check if the ethanol yield continued to increase with a heat treatment at a higher temperature. The influence of the time of the inulinase addition (before and after the heat treatment) on the ethanol yield was also studied. A concentration of inulinases of 1.25 mL L⁻¹, was used in all experiments. Results are shown in Fig. 4. The influence of a juice heating at a temperature higher than 60 °C (75 °C) on the ethanol yield was different according to the time of the inulinase addition. When inulinases were added before the heat treatment the ethanol yield decreased at 75 °C, which is explained due to the decrease in the residual inulinase activity, as pointed out by Ricca et al. (2009). On the contrary, when inulinases were added after the heat treatment, the ethanol yield increased with the temperature and reached the maximum value at the highest temperature (75 °C). These results can be explained due to the better control of undesirable microorganisms at higher temperature, because inulinase activity was not affected by heating. On the other hand, higher ethanol yield was obtained when inulinases were added before the heat treatment because, in this case, a partial inulin hydrolysis could be carried out prior to the fermentation.

With the aim to adjust the inulinase rate, three inulinase concentrations (0.01, 0.05 and 0.25 mL L⁻¹) were studied. Enzymes were added before the heat treatment (60 °C for 1 h). Differences among the ethanol yield achieved with 0.25 mL L⁻¹ (0.439 ± 0.010 g g⁻¹) and that obtained with 0.05 mL L⁻¹ (0.393 ± 0.014 g g⁻¹) and 0.01 mL L⁻¹ (0.356 ± 0.013 g g⁻¹) were again significant, so it would be the optimal inulinase concentration for winter juice.

Finally, three heat treatments prior to fermentation (T1: 45 °C, 1 h; T2: 52.5 °C, 1 h; T3: 60 °C, 1 h) were studied in a new series of experiments with the aim to optimise energy requirements. Taking into account the results obtained previously, the optimal concentration of inulinases for winter juice (0.25 mL L⁻¹) was added before the heat treatment in all cases. The highest ethanol yield (0.454 \pm 0.010 g g⁻¹; 88.8% of the theoretical maximum value) was obtained at 52.5 °C in around 70 h, which was slightly higher than the ethanol yield reached with T3 (0.446 \pm 0.012 g g⁻¹), but considerably higher than the ethanol yield obtained with the heat treatment T1 (0.372 \pm 0.015 g g⁻¹). Fermentation time was similar in all cases. Accordingly, the optimal heat treatment for winter juice would be a heating at 52.5 °C for 1 h.

Process optimisation using juice of tubers from autumn harvest (autumn juice)

A low ethanol yield was achieved by the optimised process for winter juice when autumn juice was used. The enzymatic hydrolysis of jnulin was probably hampered by the high viscosity of the medium. Furthermore, due to the higher inulin DP, higher inulinase dose was probably required. For this reason, new experiments were carried out. A heat pre-treatment of juice was performed prior to the beginning of the inulin hydrolysis in order to reduce the viscosity. As in winter juice, a pH drop of juice was observed in the first hours of fermentation, so pH was not adjusted. In a first series of experiments the juice was heated at 70 °C for 15 min prior to the heat treatment (at 60 °C for 2 h). Four different concentrations of inulinase, ranging between 0.25 and 1.75 mL L^{-1} , were evaluated. Enzymes were added at 60 °C, after the heat pretreatment and just before the beginning of the heat treatment. However, the results suggested that higher reduction of viscosity was required. Thus, experiments were repeated but increasing the heat pretreatment temperature to 80 °C. As can be observed in Fig. 5, the highest ethanol yield $(0.480 \pm 0.012 \text{ g s}^{-1})$ was obtained using an inulinase concentration of 1.25 mL L^{-1} . Nevertheless, a similar ethanol yield $(0.458 \pm 0.014 \text{ g s}^{-1}; 89.6\% \text{ of the theoretical maximum value})$ was reached adding a significant lower enzyme dose (0.75 mL L^{-1}), although higher fermentation time was required (87 h instead of 63 h). Therefore, the optimal inulinase concentration would be 0.75 mL L^{-1} , taking into account sustainable criteria.

As can be noted, similar ethanol yields were achieved with both types of juices (autumn and winter), after having optimised the process, although higher inulinase dose and energy inputs were required for autumn juice. Almost 90% of the theoretical maximum value was achieved, which is higher than those obtained by others using S. cerevisiae as inoculum (Szambelan and Nowak, 2006; Szambelan et al., 2004, 2005). On the contraryXiang-Yang and Wei-Guo (2005) and Nakamura et al. (1996) obtained a yield slightly higher than 90%, but much higher inulinase doses were used. It should be noticed that the sugar yield of JA can decrease strongly in winter harvest in some conditions due to the rotting of tubers, as was observed by Matías et al. (2013). These authors obtained 14.4 t ha⁻¹ of sugars in autumn harvest of the same JA clone used in this study. Therefore, considering the results of the present work, a potential ethanol yield of 8200 L ethanol ha⁻¹ could be achieved from [A tubers, which is in line with that reported by Kays and Nottingham (2007).

Conclusions

Juice treatment followed by simultaneous enzymatic saccharification and fermentation were successfully employed to optimise ethanol fermentation of Jerusalem artichoke tuber juice using commercial reagents and simple technology. Optimal juice treatment varied according to tuber harvest date. The optimal treatment for juice extracted from tubers harvested in autumn requires higher inulinase dose and energy inputs than optimal treatment for juice extracted from tubers harvested in winter. Similar ethanol yield was obtained with both types of juice and the optimised process (almost 90% of the theoretical maximum value). These results could be beneficial to favour a staggered and decentralised ethanol production from a low-requirement crop which does not interfere with food chain.

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