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Bioethanol Production via the Fermentation of *Phalaris aquatica* L. Hydrolysate

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The investigated integrated bioprocess for the production of bioethanol from lignocellulosic biomass, derived from the perennial herbaceous species *Phalaris aquatica* L., consists of three main stages, which were studied and optimised separately. The pretreatment process was investigated through a Taguchi statistical design where mild conditions were selected for the optimization of the hemicellulose hydrolysis. The solid residue, where cellulose lies, was enzymatically hydrolysed with the implementation of a Box-Denken statistical design and glucose was recovered at a concentration equal to 13.5 g/L. Glucose concentration increased to 45 g/L after the application of a semi-batch policy. The fermentation of the enzymatic hydrolysate with the yeast *Saccharomyces cerevisiae* was initially conducted under batch conditions in flask scale and the optimal regime was selected to be transferred to the bioreactor scale. Inoculum size, aeration, agitation speed, fermentation medium composition and type of strain were the parameters of study. The fermentation of the hydrolysate provided 47 g/L ethanol concentration under fed-batch conditions, corresponding to 90 % yield. The specific productivity was equal to 4.8 g/(L·h), while the overall productivity was maximized, through different feeding policies, to 2.3 g/(L·h).

1. Introduction

The modern demand for alternative energy resources, resulting from the fossil fuels deficiency, has driven the worldwide interest towards energy crops that can serve as raw materials for the production of biofuels and biochemicals of high-added value (Hamelinck et al., 2005). Perennial grasses are suitable as energy plants due to the high lignocellulosic biomass productivity of their plant tissues. *Phalaris aquatica* L. is a perennial herbaceous plant, native to the Mediterranean area, that can be cultured to non-food oriented agricultural areas (Pappas et al., 2009). The specific species display high structural polysaccharides percentage (>650 g/kg DM) and low lignin content (<10 g/kg DM) (Pappas, 2010). Fast growth, high biomass yield, low input cost, region-specificity, annual or less-frequent harvests, non-prime agricultural land need, non competitiveness for prime agricultural land required by commodity crops and high convertibility through biochemical processes to monomer sugars, are the key characteristics that appoint *P. aquatica* as a potential biomass feedstock for the production of second generation bioproducts.
The integrated biochemical production of bioethanol mainly involves three stages: i) the pretreatment of the dried and milled biomass, ii) the enzymatic hydrolysis of the solid residue, and iii) the fermentation of the cellulose sugar monomers to ethanol. Concerning the pretreatment stage, there is in general a great variety of employed methods. Mild acid hydrolysis is considered as suitable for lignocellulosic biomass like *P. aquatica*, due to its low lignin content, which allows dilute acid hydrolysis to break-up hemicellulose polysaccharides bonds and dissolve sugar monomers (Alvira, 2010). The remaining solid residue contains the undiluted cellulose of the plant tissues. A suitable mixture of enzymes is able to hydrolyze the β-glycosidic bonds and provide the respective glucose monomers. More specifically, cellulase and β-glucosidase are usually used in order to degrade the cellulose structure and release the fermentable

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sugars to the hydrolysate solution. Key-factors that serve important role to the conversion of cellulose into glucose are: the solid loading of the pretreated biomass, the quantity and the activity of the enzymes, the reaction conditions, the porosity of the cellulose surface, the crystallinity index and the lignin content. The role of surfactants is known to enhance the enzymes selectivity towards the cellulose surface (Kristensen, 2007). The surfactants, due to their hydrophobic nature, interact with the lignin content of the solid residue, allowing the enzymes to absorb more efficiently on the cellulose surface.

The fermentation stage involves an ordinary yeast metabolic pathway for energy production, where hydrocarbons are converted to ethanol. According to the stichiometric equation, the theoretical yield of the fermentation stage is estimated to 0.51 g ethanol/g glucose. The microorganism *Saccharomyces cerevisiae* is the most widely studied yeast, known also for its high ethanol productivities. There are plenty of wild type strains used for the production of bioethanol, leading to the demand for further research on the strain which yields to higher ethanol productivities. The investigation of several strains and the selection of DSM 70449 and Sigma Type II is thoroughly described by Yu et al. (2010) and Biener et al. (2012). For batch mode fermentations, ethanol productivities are measured between 0.3-1.1 g/(L·h), while for fed-batch fermentations the productivities are increased to values up to 0.9-1.6 g/(L·h) (Taherzadeh, 1999, Dehkhoda, 2009).

The primary objective of the present work is the maximisation of ethanol productivity by the implementation of a simple feeding strategy for fed-batch fermentation. Different feeding policies are investigated, resulting in high productivities, along with high ethanol concentrations. Moreover, the need for high glucose concentration in the hydrolysate/fermentation medium is addressed via statistical experimental design and optimisation of the pretreatment and enzymatic hydrolysis processes.

2. Materials and methods

2.1 Lignocellulosic biomass pretreatment

Lignocellulosic biomass was harvested from a 5 y old *Phalaris aquatica* crop, cultivated in an experimental field. The naturally dried biomass was milled in a rotor mill (Retsch Cutting Mill SM 100) with a resulting particle size <1 mm. Initially, biomass was treated with dilute sulphuric acid (1, 1.5, 2 % w/v) at 110, 120, 130 ^{o}C for 30, 45, 60 min reaction time. These three factors were investigated in three different levels. The constant parameter was the biomass loading (10 % w/v). The statistical analysis response was the xylose concentration, following the hemicelluloses hydrolysis. A combined severity factor (CSF) was used in order to evaluate the intensity of the pretreatment method. The CSF was defined based on the following equation:

$$CSF = \log_{10}R_o - pH, \quad R_o = \log[t\cdot\exp((T_r - T_b)/\omega)] \tag{1}$$

where: T_r is the reaction temperature, T_b is the reference temperature (100 ^{o}C) and ω is an empirical coefficient, equal to 14.75 for the method of dilute acid hydrolysis (Lloyd, 2005).

2.2 Enzymatic hydrolysis

The enzymes used were commercially available enzyme solutions: cellulase from *Trichoderma reesei* (Celluclast 1.5L) and β-glucosidase from *Aspergillus niger* (Novozym 188). The reaction mixture consisted of 2, 3, 4 % w/v solid substrate, 10, 15, 20 FPU/g substrate cellulase concentration, while the ratio of cellulase to β-glucosidase was equal to 1:1.75, and the ratio of surfactant to substrate loading was 0.02, 0.04, 0.06 g/g; the reaction time was equal to 24, 48, 72 h, and the reaction temperature was kept constant at 50 ^{o}C. PEG 4000 was used as the surfactant. The hydrolysate, after centrifugation, was detoxified with NaOH treatment up to pH 11 and then it was neutralized with H_2SO_4 to pH 6.

2.3 Microorganisms and inoculum preparations

The two *Saccharomyces cerevisiae* strains, Sigma Type II and DSMZ 70449, were purchased by Sigma Aldrich and The German Collection of Microorganisms and Cell Cultures, respectively. The strains were maintained in agar plates containing solid YPD rich medium (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L and agar 20 g/L) at 4 ^{o}C. The seed culture was carried out in Erlenmeyer flasks containing the preculture medium, consisted of: glucose 30 g/L, yeast extract 5 g/L, $(NH_4)_2SO_4$ 10 g/L, KH_2PO_4 4.5 g/L, $MgSO_4\cdot 7H_2O$ 1 g/L and $ZnSO_4\cdot 7H_2O$ 0.65 g/L. The inoculum was originated from the agar plates, as a single colony. The flasks were incubated in a rotary shaker incubator (GFL 3033) at 150 rpm, 30 ^{o}C. pH was initially controlled at 6 with the use of sterilized 1M HCl and 1M NaOH solutions.

2.4 Fermentation

Fermentation processes were initially conducted in flasks for batch screening experiments, under different conditions. The optimal combination was transported to a 3 L bioreactor (BioFlo 110 Bioreactor/Fermentor, New Brunswick Scientific Co. Inc.). The investigated parameters were: glucose loading (5, 10, 20, 100 g/L), nitrogen source loading (total nitrogen source 8, 16 g/L, consisted of 3, 5, 6, 10 g/L of $(NH_4)_2SO_4$ and 5, 3, 10, 6 g/L of yeast extract, respectively), pH (5.5, 6, 6.5, 7), aeration (agitation speed and working to flask volume ratio) and different inoculum size (initial optical density 0.5 and 4). The rest of the fermentation medium contained: KH_2PO_4 3 g/L, Na_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L and 2 mL of trace elements solution. The latter contained: $ZnSO_4 \cdot 7H_2O$ 0.9 g/L, $FeSO_4 \cdot 7H_2O$ 0.6 g/L, H_3BO_3 2 g/L, $MnCl_2 \cdot 4H_2O$ 1.5 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.8 g/L, $CoCl_2 \cdot 6H_2O$ 0.8 g/L and $CuSO_4 \cdot 5H_2O$ 0.5 g/L. The bioreactor had initial working volume equal to 800 mL. Glucose concentration was set to 20 g/L and 100 g/L for low and high loading cultivations, respectively. pH was controlled by the addition of 3M NaOH and 2M HCl solutions. The dissolved oxygen concentration was also controlled through the impeller agitation speed. The aeration of the fermentation broth was manually controlled at 1 vvm. The exhaust gases composition, in terms of O_2 and CO_2, were measured by a gas analyzer (5200 Multi Purpose, Servomex Ltd). All cultivations were performed at 30 ^oC. The three fed-batch fermentation policies were: constant feeding, pulses feeding and exponential feeding. The feeding medium consisted of 300 g/L glucose, while the total nitrogen source was 7.5 times concentrated. For the high loading experiments, the initial medium was also 5 times concentrated. The ethanol yield was calculated as the ratio of grams of produced ethanol to grams of consumed glucose, multiplied by 0.51, which is the maximum theoretical yield for the ethanol production from glucose.

2.5 Analytical methods

Cell growth was monitored via the measurement of the culture optical density, with the use of a Spectrophotometer (Hitachi U-18000) at 600 nm. Glucose, ethanol, glycerol, acetic and formic acid concentrations were analyzed by high-performance liquid chromatography (HPLC), coupled with a refractive index detector (RI). A Hi-Plex H+ Analysis column (300×7.7 mm) was used. Xylose, glucose, arabinose, galactose and cellobiose were analyzed by a Zorbax Carbohydrate Analysis column (4.6×150 mm).

3. Results and discussion

The experimental design of the pretreatment stage was used to estimate the combined severity factor (Eq. 1). The highest values for hemicellulose conversion are reported for values exceeding 1, as it is shown in Figure 1. These coefficient values are correlated to the middle levels of the experimental design (i.e., 1.5 % w/v H_2SO_4, 120 ^oC and 45 min reaction time). Those results are in agreement with the results of Lloyd et al. (2005), who demonstrated that higher hemicellulose conversion values are in correspondence to CSF between the limits 1 and 1.5. Under these pretreatment conditions, xylose and total sugars concentrations, recovered from this stage, were 20 g/L and 29 g/L, respectively.

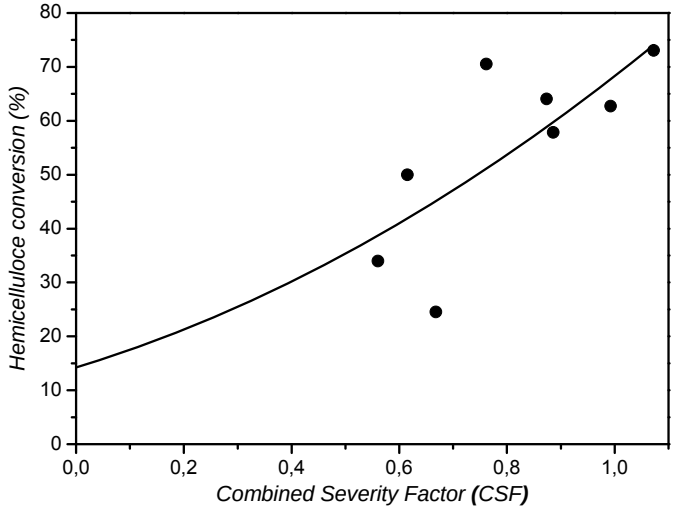

Figure 1: The effect of pretreatment conditions, as a combined factor, on the hemicellulose conversion

The experimental design of the enzymatic hydrolysis, according to Box-Denken, was analyzed with the aid of the statistical package Minitab 14.0. The results are shown in Figure 2. The factor with the most significant effect on the glucose production is the interaction between the reaction time and the enzyme loading. The optimal factors combination was found to be: 4 % w/v solid substrate quantity, 20 FPU/g substrate cellulase concentration, 0.04 g/g substrate surfactant concentration and 72 h reaction time. The presence of surfactants did not enhance the glucose liberation, probably due to the lower lignin content of *Phalaris aquatica* in comparison to other plants. The final glucose concentration was 13.5 g/L. An improvement of this concentration was succeeded with the implementation of a semi-batch policy: six feeding pulses every 6 hours, containing 1 % w/v substrate loading (up to 12 % total) and 30 FPU/g cellulase loading, which resulted in 45 g/L glucose concentration. The results are in agreement with Zheng et al. (2009) who performed a similar process, increasing by 18 % the final glucose concentration, in comparison to the batch process. The achieved concentration is in general suitable for further utilization of the hydrolysate for the fermentation process, without any prior condensation. The amount of the derived sugars from pretreatment and enzymatic hydrolysis stage is estimated to 720 g/kg DM, which corresponds to yield value 90,7 %. These results are similar to those found at Caetano's research for brewer's spent grains (Caetano et al., 2013).

The results of the screening experiments based on different conditions for the fermentation stage are presented in Table 1. In the optimal case, the medium contains 20 g/L glucose and 8 g/L total nitrogen source (sulphuric ammonium 3 g/L and yeast extract 5 g/L). The aeration effect was estimated via the study of the medium to flask volume ratio (optimal value 1:2.5). Moreover, the additional optimal parameter values that maximised the ethanol production were: agitation speed 150 rpm, pH 6.0 and inoculum size corresponding to an initial optical density 0.5. The strain selected for further investigation was Sigma Type II, since it demonstrated significantly shorter lag phase and increased ethanol concentration (9.98 g/L). The above optimal conditions were implemented in flask fed-batch experiments, where ethanol productivity and concentration were increased to 0.82 g/(L·h) and 18.84 g/L, respectively. Furthermore, fed-batch cultivations, with different feeding policies, were conducted in the bioreactor, with primary objective to maximize the final ethanol concentration. The highest ethanol productivity was fulfilled with the exponential feeding regime, taking into account the cells' growth rate (Laopaiboon, 2007). The overall productivity was equal to 2.3 g/(L·h) and the maximum ethanol concentration was equal to 37.8 g/L. The specific productivity displayed the significantly increased value of 4.8 g/(L·h). The sugars consumption resulting in biomass growth and ethanol production are shown in Figure 3. Finally, the glucose initial loading was increased to 100 g/L and combined with the exponential feeding, resulted in 47 g/L ethanol concentration.

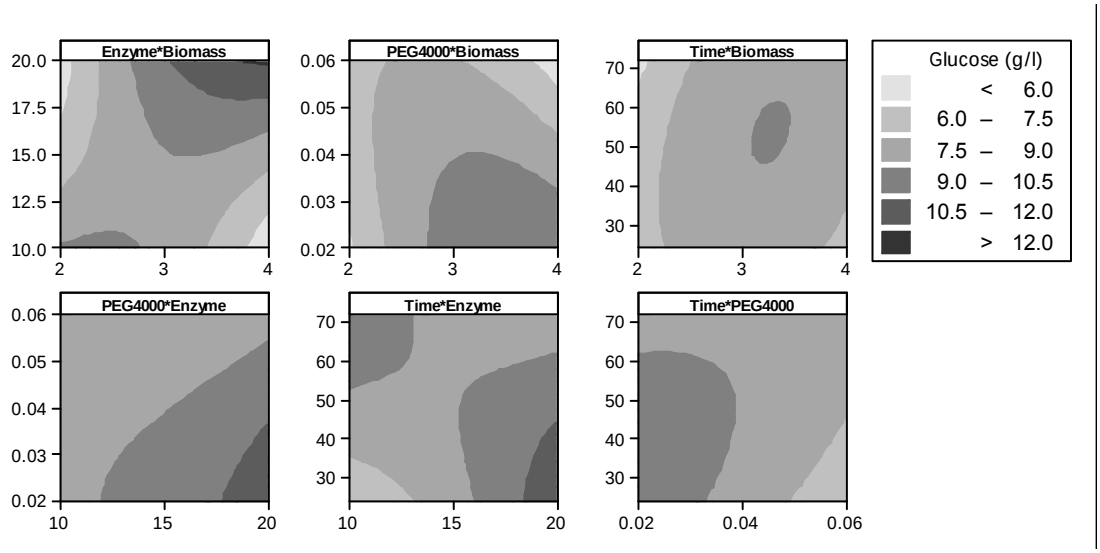

Figure 2: Contour plots showing the interactions between the enzymatic hydrolysis parameters after Box-Denken statistical analysis design

Among other studies on Mediterranean lignocellulosic biomass sources that have been reviewed in Faraco's (2011) paper, the treatment on olives' wastes, residues of a two-step olives centrifugation process, is presented. The sugars derived from the olive wastes (OP) biomass were fermented with baker's yeast and 11.2 g/l ethanol is produced with overall productivity 2.1 g/(L·h).

Table1: The effect of different process parameters on ethanol production under batch fermentation conditions

No.	Strain	Glucose (g)	Aeration (v:v)	Agitation speed (rpm)	Nitrogen source (g/L)	Inoculum (optical density)	pH	Ethanol (g/L)
1	DSM 70449	20	1:2.5	150	3 + 5	0.5	6.5	8.2
2	Type II	20	1:5	150	3 + 5	0.5	6.5	9.8
3	Type II	20	1:4	150	3 + 5	0.5	6.5	9.84
4	Type II	20	1:5	250	3 + 5	0.5	6.5	10.2
5	Type II	5	1:2.5	150	3 + 5	0.5	6.5	1.02
6	Type II	10	1:2.5	150	3 + 5	0.5	6.5	1.58
7	Type II	20	1:2.5	150	1.5 + 2.5	0.5	6.5	7.6
8	Type II	20	1:2.5	150	5 + 3	0.5	6.5	9.5
9	Type II	20	1:2.5	150	10 + 6	0.5	6.5	8
10	Type II	20	1:2.5	150	6 + 10	0.5	6.5	9.15
11	Type II	20	1:2.5	150	3 + 5	4	6.5	9.5
13	Type II	20	1:2.5	150	3 + 5	0.5	5.5	8.9
14	Type II	20	1:2.5	150	3 + 5	0.5	6	9.98

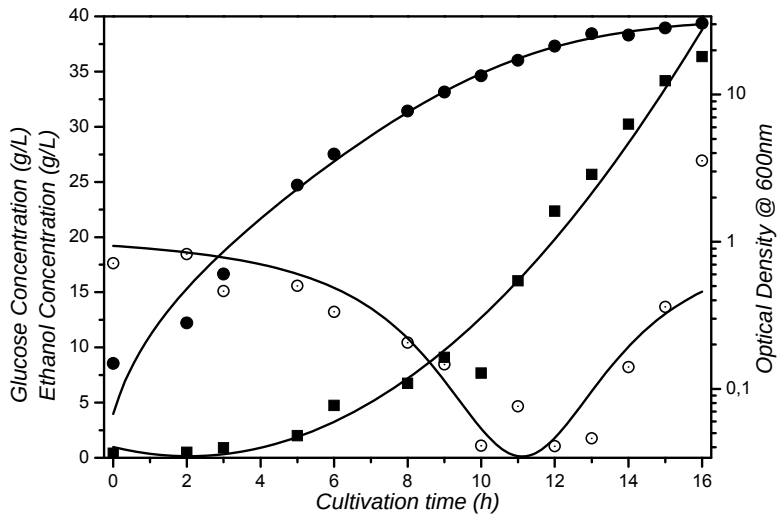

Figure 3: Ethanol production, cells' optical density and glucose consumption during the fed-batch fermentation with exponential feeding. Symbols standing for: (●) optical density, (■) ethanol concentration and (⊙) sugars consumption

4. Conclusions

The present work demonstrated a simple three stage process for the production of ethanol from lignocellulosic biomass with high concentration and productivity. *P. aquatica* biomass, which possesses reduced lignin content, allowed the efficient hemicellulose solubilisation under mild pretreatment conditions. The enzymatic hydrolysis stage was statistically optimized, employing also a semi-batch policy. The high glucose containing hydrolysate was utilized as the cultivation medium in the third sub-process. The fermentation was optimized under batch and fed-batch conditions, both on flask- and bioreactor- scale experiments. The high glucose loading coupled with the exponential fed-batch feeding policy resulted in significantly high ethanol productivities. The overall integrated bioprocess succeeded to obtain high ethanol concentration which is mandatory for the down-stream process, targeting at ethanol recovery. More specifically, distillation processes require a rich ethanol stream in order to be cost effective. The present technology could be part of an integrated biorefinery, targeting in the co-production of biofuels and high-added value chemicals in a cost-effective way.

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