

Article

Arundo donax Refining to Second Generation Bioethanol and Furfural

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Abstract: Biomass-derived sugars are platform molecules that can be converted into a variety of final products. Non-food, lignocellulosic feedstocks, such as agroforest residues and low inputs, high yield crops, are attractive bioresources for the production of second-generation sugars. Biorefining schemes based on the use of versatile technologies that operate at mild conditions contribute to the sustainability of the bio-based products. The present work describes the conversion of giant reed (*Arundo donax*), a non-food crop, to ethanol and furfural (FA). A sulphuric-acid-catalyzed steam explosion was used for the biomass pretreatment and fractionation. A hybrid process was optimized for the hydrolysis and fermentation (HSSF) of C6 sugars at high gravity conditions consisting of a biomass pre-liquefaction followed by simultaneous saccharification and fermentation with a step-wise temperature program and multiple inoculations. Hemicellulose derived xylose was dehydrated to furfural on the solid acid catalyst in biphasic media irradiated by microwave energy. The results indicate that the optimized HSSF process produced ethanol titers in the range 43–51 g/L depending on the enzymatic dosage, about 13–21 g/L higher than unoptimized conditions. An optimal liquefaction time before saccharification and fermentation tests (SSF) was 10 h by using 34 filter paper unit (FPU)/g glucan of Cellic[®] CTec3. C5 streams yielded 33.5% FA of the theoretical value after 10 min of microwave heating at 157 °C and a catalyst concentration of 14 meq per g of xylose.

Keywords: lignocellulosic; bioethanol; high gravity; hybrid SSF; xylose dehydration

1. Introduction

Several initiatives are active in Europe to foster the bio-based economy. Pillars of this transition are the sustainable production of bioenergy, advanced biofuels and bio-based chemicals and materials. The use of lignocellulosic feedstocks is encouraged because it does not compete with the use of commodities destined for food production. Different biorefining schemes for the conversion of lignocellulosic feedstocks have been implemented so far which, in general, consist of converting the biomass to intermediate platform (macro) molecules or streams. Conversion processes can be based on both biochemical or thermochemical routes. Generally, thermochemical processes are widely used for biomass conversion and energy recovery since they use well-developed technologies [1–4]. On the other side, biomass-derived carbohydrates represent a versatile platform for the production of a variety of final products [5]. In particular, for the biotechnological route, the initial step consists of biomass pretreatment followed by enzymatic hydrolysis to fermentable sugars. Several leading pretreatment technologies have been developed for biomass deconstruction and fractionation [6–8]. Among these, steam explosion is a widely used physicochemical pretreatment suitable for several feedstocks. It uses saturated steam to produce high degrees of biomass deconstruction and facilitate the subsequent fractionation in its macro-components: cellulose, hemicellulose and lignin. For these reasons, it can

be considered a versatile and green technology for the exploitation of the biomass barrel [9,10]. Depending on the biomass composition, the use of catalytic amounts of acidic additives (i.e., SO_2 , H_2SO_4), can reduce the process temperatures and enhance the recovery yields of the hemicellulose carbohydrates [11–13]. Furthermore, acid-catalyzed steam explosion pretreatment (ACSEP) increases the hydrolysis extent of the water-soluble hemicellulose and facilitates the subsequent enzymatic digestion of the cellulose fraction. On the other side, acid catalysts can generate degradation by-products, typically small chain organic acids and furan derivatives, which typically lower the microbial conversion efficiency [14,15]. Important progress has been made in recent years concerning the fermentation of pentose (C5) sugars to ethanol [16,17], butanol [18] and xylitol [19,20]. In most cases, the process's efficiency depends on the capability of these microorganisms to withstand industrially relevant conditions (i.e., high concentration of solutes, inhibitors, etc.). On the other side, chemically catalyzed processes are in general less sensitive than microbial processes to side-chain products. In this regard, the biorefining scheme using ACSEP has the advantage of producing a depolymerized hemicellulose stream that can be more easily dehydrated to the final product, i.e., to furans derivatives. Coproduction of biofuels and chemicals can increase the biorefinery profitability. In particular, bio-based chemicals and intermediates from hemicelluloses could provide alternatives to many fossil products. Among these, levulinic acid, formic acid and furfural are the most promising. In many biomass fractionation processes, the hemicellulose soluble fraction already contains acids and furans that are often inhibitory toward the following fermentation process. Chemically catalyzed conversion of hemicellulose could be a process layout more easily integrated with the separate valorization of C6 sugars. The global market for furfural is expected to grow at a compound annual growth rate (CAGR) of 11.6%. Furfural can be easily converted to 2-methylfuran, a gasoline octane booster, which, in turn, can be the precursor of various condensation reactions, producing non-polar alkanes, suitable for high-quality diesel fuel. Furfural is also used for the production of furfuryl alcohol, tetrahydro-furfuryl alcohol, acetyl furane, furoic acid and tetrahydrofuran (THF). These furfural derivatives can be used for the production of lubricants, adhesives and polymers [21,22]. A new stimulus to the research on this chemical derives from the need to develop green processes, i.e., processes that are less energy-intensive than the current process and using recoverable catalysts. The use of a biphasic system employing heterogeneous catalysts could offer many advantages concerning homogeneous catalysts, although performances can depend on the hemicellulose composition [23].

Dehydration is typically carried out in autoclave apparatus at a temperature higher than 150 °C by mineral acids. The use of microwave energy combined with the use of solid acid catalysts, such as strong cation-exchange resins, could be a valuable set-up [24]. The controlled microwave dehydration decreases reaction times and energy and could reduce, in principle, the formation of secondary by-products. Furthermore, solid resins can be used in continuous operation, easily separated from the reaction mixture and reused and do not cause the corrosion problems typically associated with homogeneous acid catalysts [25]. Finally, a higher dehydration selectivity is typically obtained in biphasic water/organic solvent systems, which allows for the extraction of the dehydration product from the aqueous phase, thus preventing site-chain degradations into undesired byproducts such as humins [26,27]. Several conversion processes of *A. donax* to high-value products have been reported recently [28,29]. Concerning ethanol production, the recent commercial enzyme mixtures can withstand higher temperatures compared to the previous products. One major limit in the fermentation of lignocellulose carbohydrates is the final ethanol concentration before distillation. Techno-economic evaluations indicated that an optimal threshold concentration is 39 g/L [30]. Reaching this target implies the processing of concentrated biomass slurries, over 20% biomass consistency, which raises several process issues due to the more complex system rheology and to by-product inhibition of enzymes [13]. For this reason, a hybrid process scheme consisting of a biomass pre-liquefaction at high temperature followed by simultaneous saccharification and fermentation (HSSF) at lower temperature were more recently investigated and optimized. The combination of the effect of the hybrid process and pH for improved sugar conversion in high solid ethanol production from *A. Donax* was already studied

by Palmqvist and Liden (2014) [31]. More recent investigations were carried out on the conversion of wheat straw. In particular, Bondesson and Galbe (2016) developed a process for the simultaneous saccharification and co-fermentation (SSCF) of acid-catalyzed steam-pretreated wheat straw [30]. The SSCF of SO₂-pretreated wheat straw was assessed by Cassels et al. (2017) [32]. Nielsen et al. (2020) investigated the conversion of mixed agricultural feedstocks by SSCF [33]. Lignocellulosic biomasses are generally waste from the agri-food industry or derived from dedicated crops in marginal soils with low rainfall and requiring low amounts of nutrients; in the latter category, the *Arundo donax* is very promising as a source of carbohydrates [13,34]. The goal of this work was to enhance all the carbohydrates present in the plant as required by the biorefinery approach by using green technologies and mild process conditions. Bioethanol was obtained from cellulose through the optimization of the enzymatic hydrolysis and fermentation process, while the hemicellulose was processed in microwaves with regenerable catalysts to obtain 2-furaldehyde, a bio-based molecule with high added value for the industry. In particular, after acid-catalyzed steam explosion pretreatment, C6 was converted to bioethanol by means of high gravity hydrolysis and fermentation while the hemicellulose-derived xylose was converted to furfural in biphasic media H₂O-butanol heated up by microwave irradiation. Several process conditions were tested to achieve the highest ethanol concentration and the highest xylose conversion at mild conditions. Before the hydrolysis and fermentation process, a detailed characterization of Cellic[®] CTec3 product inhibition (glucose and ethanol) is provided in this work.

For the first time, we tested the modulation of the temperature during the C6 bioconversion steps coupled with multiple inoculations.

2. Materials and Methods

2.1. Raw Material and Pretreatment

Biomass was grown in Northern Italy. It had an initial dry matter (DM) content of 94.5%. Prior to the pretreatment, biomass was grounded in the range 1.7–5.6 mm. The optimal pretreatment for the giant reed was assessed in a previous investigation [13]. In particular, acid-catalyzed steam explosion pretreatment (ACSEP) was carried out by impregnating the biomass with homogenous H₂SO₄ catalyst before feeding the steam explosion batch digester located in ENEA Trisaia (Italy). For the impregnation, 1 kg of raw *Arundo donax* was soaked in a cold dilute H₂SO₄ solution (30 L; 0.07 M). After 10 min of soaking, biomass was squeezed to separate the solids from the solution. The DM after the impregnation and squeezing was 27%. The acid loading was 1.4% wt. It was estimated either based on the volume and molarity of the retained acid solution and by titration of the residual acid after the impregnation. Steaming temperature and process time were 200 °C and 5 min, respectively.

After the pretreatment, the material was pressed in order to separate the solid from the liquid fraction. The solid fraction containing cellulose was used for the hydrolysis and fermentation process in order to obtain ethanol while the pentose rich-liquid fraction (hemicellulose) was treated with a heterogeneous catalyst (Amberlyst-15) in order to convert xylose into furfuraldehyde.

2.2. Hydrolysis Tests

Cellic[®] CTec3 (Novozymes, Bagsvaerd, Denmark) was used for the hydrolysis and fermentation tests. The filter paper activity of the enzymatic preparation was 190 filter paper unit (FPU)/g, determined according to the Ghose method (1987) [35]. The enzyme dosage and the potential inhibition by glucose and ethanol were assessed in 500 mL Schott Duran laboratory glass bottles closed with screw caps, which were agitated in an orbital shaker incubator at 150 rpm. Preliminary product-inhibition study tests were assessed to determine the effect of glucose and ethanol on enzyme performances. Unless otherwise specified, the temperature was kept at 50 °C and pH 5.

A hybrid process consisting of a pre-liquefaction time followed by a saccharification and fermentation tests (SSF) process (HSSF), which was optimized by studying various variables, including the enzymatic dosage, the pH and the fermentation temperature. Liquefaction tests at high DM content

were carried out in a custom high-solids bioreactor built at ENEA. This bioreactor is a stainless-steel reactor consisting of a 5 L horizontally vessel with a helicoidal impeller mounted on a horizontal rotating shaft. Temperature control was achieved by putting the reactor vessel in an external oven.

In the HSSF process, biomass liquefaction was carried out in the fed-batch mode. This is a common process strategy to reduce the excessive viscosities of concentrated biomass suspensions. The overall biomass feed was divided into four batches regularly loaded in the maximum time selected for the biomass partial liquefaction. After all feedings, the final water-insoluble content (WIS) was 28%.

2.3. Fermentation Tests

The simultaneous saccharification and fermentation tests (SSF) were carried out in 500 mL Erlenmeyer flasks closed by silicon stoppers with inserts for the vent gas. The flasks were shaken at 150 rpm. The nutrients composition was (g/L): yeast extract 2; KH_2PO_4 5; MgSO_4 0.4; $(\text{NH}_4)_2\text{SO}_4$ 2. The pH was continuously adjusted through the process by adding NaOH 4 M. The medium was supplemented with antibiotics to avoid bacterial contaminations and consequent formation of lactic acid in the long run. *Saccharomyces cerevisiae* (SIGMA Type II) was used for the fermentation of glucose. The technical sheet indicated that it could tolerate temperatures up to 37 °C when the cell lyses start. Cultures were maintained on Yeast Extract–Peptone–Dextrose (YPD) agar plate at 4 °C. For the pre-inoculum preparation, one colony of the yeast in the maintenance medium was transferred to 100 mL Erlenmeyer flasks containing 20 mL of the liquid medium YPD and incubated at 26 °C and 150 rpm. After 24 h, the pre-inoculum was transferred to 1000 mL Erlenmeyer flasks containing 500 mL of liquid YPD. Inoculated flasks were incubated under the same conditions as pre-inoculum for 72 h. After this time, the cells were recovered by centrifugation (6000 rpm for 10 min) and re-suspended in the saline solution (0.85% NaCl). The obtained suspensions were added to the fermentation medium to obtain the desired cell concentration in the range 2–7 g/L dry cells. After each sampling, the fermentation broth was centrifuged at 4000 rpm for 10 min. The supernatant was filtered through 0.22 µm filters and stored at –20 °C.

2.4. Dehydration Tests

The acid-catalyzed conversion of hemicellulose into furfural (FA) by the microwave-assisted reaction was carried in a microwave apparatus Mars-6 (CEM corporation, Charlotte, USA) equipped with a 16 position carousel with Teflon closed vessels (CEM corporation, Charlotte, USA). The temperature was controlled through an optical fiber sensor (CEM corporation, Charlotte, USA). The conversion tests were carried out in 75 mL vessels containing 45 mL 1-butanol (Sigma-Aldrich, St. Louis, MO, USA) and 15 mL hemicellulose stream for ten minutes. The water phase was saturated with NaCl (35%) to maintain the biphasic reaction conditions even at a high butanol-to-water ratio [36]. A strongly acidic cation exchange resin, Amberlyst-15 (Sigma-Aldrich, St. Louis, MO, USA, product code 216380), was selected as a solid acid catalyst. The specific set-ups, namely temperature and catalyst dosage, were detailed in the following for the various tests as they are part of the set-up optimization. After processing, the extraction vessels were allowed to cool down to room temperature before filtration with a Whatman GF/A (Sigma-Aldrich, St. Louis, MO, USA) and separation of the two phases in separator funnels. Each phase was then analytically characterized. Carbohydrates and fermentation metabolites were analyzed with an HPIC DX 300 system (Dionex, Sunnyvale, CA, USA) equipped with a Nucleogel® Ion 300 OA (Macherey–Nagel, Düren, Germany). The detector was a Shodex RI101 refractive index (Showa Denko, Japan). The analysis of the FA was performed using Agilent HP 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Dionex AS1 column (Dionex, Sunnyvale, CA) and a diode array detector set at 280 nm (Agilent Technologies, Palo Alto, CA, USA).

3. Result and Discussion

3.1. Enzymatic Hydrolysis of Giant Reed

Raising the solids loading is, in principle, a direct way to achieve concentrated hydrolysates [37]. The enzymatic digestion of biomass suspensions at high DM content is called high gravity hydrolysis and has the advantage of producing higher concentrations of the final product, thus reducing distillation costs, bioreactors capacity and amounts of wastewaters. The challenges of high gravity hydrolysis are the high viscosities that limit the mass transfer and results in poor mixing [13]. Furthermore, high concentrations of products, namely cellobiose and glucose, could have an inhibitory effect on cellulases [38,39], whereas high solids concentrations could enhance the unspecific enzyme adsorption by interaction with lignin or lignin-carbohydrate complex [40,41]. In the simultaneous saccharification and fermentation (SSF), high gravity conditions could generate osmotic stress and increase the inhibition by degradation products in the pretreated biomass. Finally, previous studies showed that the ethanol produced could affect cellulase activity [42]. Leading enzyme manufacturers are investigating strategies to reduce enzyme production costs and increase specific activities. As a result, the latest commercial mixtures have been remarkably improved with respect to the previous blends. In particular, Novozymes developed Cellic[®] CTec3 containing high concentrations of cellobiose hydrolases, which ensures a low concentration of cellobiose. For this reason, only the effect of glucose and ethanol on the cellulases activities was investigated in the present study.

Initial indications on the enzyme dosage at high DM content were obtained in the batch mode (Figure 1).

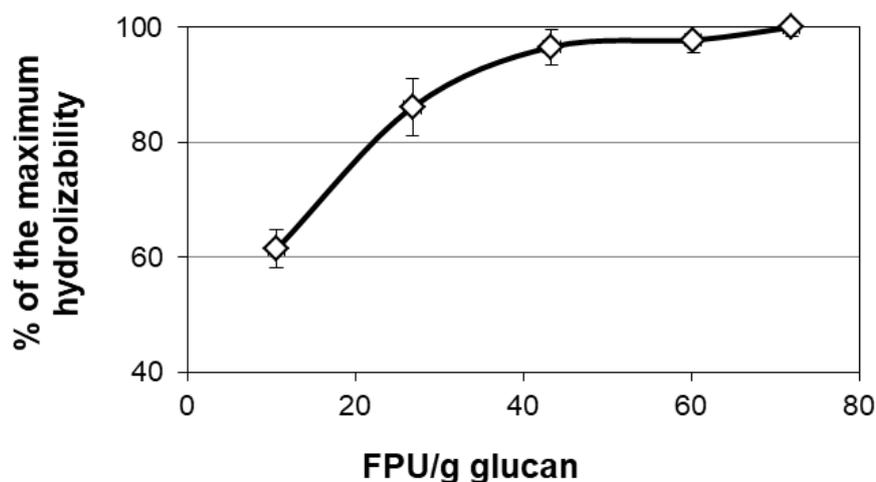


Figure 1. Determination of enzymatic dosage in the high-solids hydrolysis system. This investigation was assessed in batch mode.

The maximum hydrolyzability was observed above 38–45 FPU of Cellic[®] CTec3 per g of glucan. This dosage was considered as an upper limit and the process tests were intentionally carried out at roughly half the dosage with the attempt of reducing the enzyme load by optimizing the process.

Tests were then carried out to assess any specific thresholds for glucose and ethanol inhibition of Cellic[®] CTec3 in order to optimize the HSSF process settings. To do so, the solid fiber obtained after pressing the pretreated slurry was hydrolyzed in simulated hydrolysates already containing glucose at increasing concentration (3; 41; 80 g/L) and by using an enzymatic dosage of 19 FPU/g glucan. Figure 2A describes the time course of the three hydrolysis processes and Figure 2B shows the final process yields.

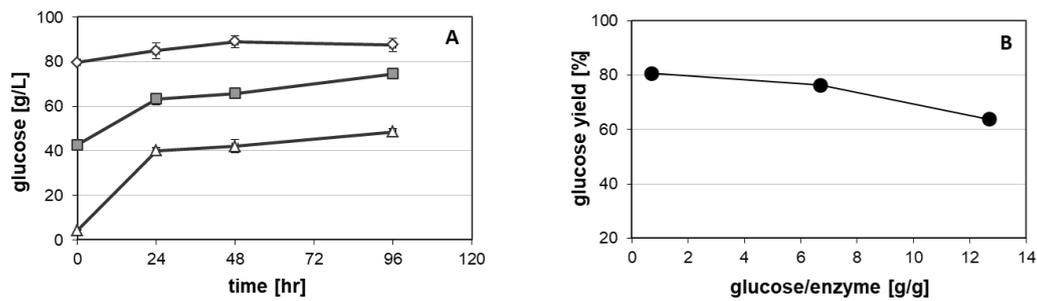


Figure 2. Effect of glucose inhibition on enzymatic hydrolysis process at different initial concentrations of glucose ((A): white triangle 3 g/L; grey square 41 g/L; white rhombus 80 g/L); relative final process yields (B).

The data indicated that the inhibition due to glucose was negligible up to glucose-to-enzymes-ratio of 7. Higher glucose concentrations reduced the hydrolysis yield of the fresh substrate up to 18%. Thus, in the fed-batch process, 10–12% was chosen as the initial solids loading and SSF was started before 80 g/L glucose to reduce enzyme inhibition.

The first commercial enzyme blends were strongly inhibited by ethanol [42,43]. For this purpose, the effect of ethanol added before the hydrolysis of the biomass by Cellic[®] CTec3 was investigated at fermentation temperature of 37 °C (Figure 3A) and hydrolysis temperature process of 50 °C (Figure 3B). The data displayed in Figure 3 indicate that concentrations of ethanol in the range 30–50 g/L did not significantly affect the Cellic[®] CTec3 activity. This result emphasizes that Cellic[®] CTec3 has improved process performances with respect to other old enzymatic mixtures [44] and can be effectively used for high gravity fermentation.

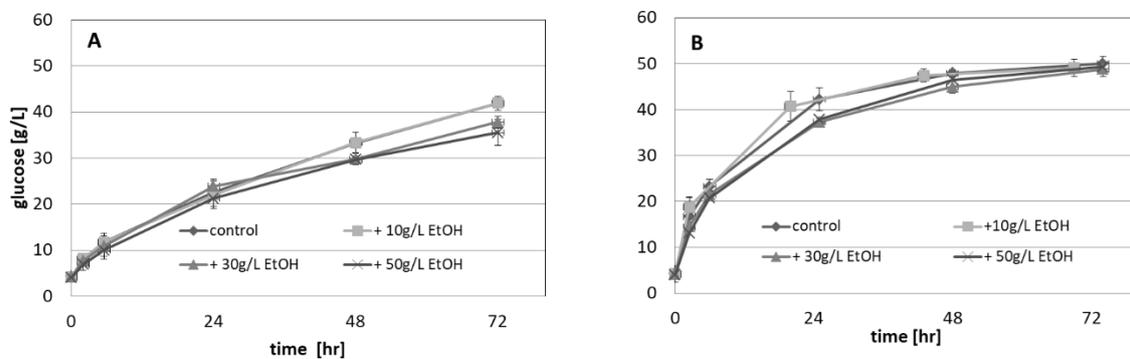


Figure 3. Effect of ethanol inhibition on enzymatic hydrolysis process. Comparison between enzymatic kinetics carried out at increasing ethanol concentrations in hydrolysis processes at 37 °C (A) and 50 °C (B).

3.2. Hybrid SSF of *Arundo Donax*

SSF processes consume the sugars in the hydrolysates, thus, reducing the inhibition of the enzyme and promoting the further hydrolysis of the residual cellulose. Finding the compromise between the optimal temperature and pH of hydrolysis and fermentation has been the object of several investigations. Process temperature is a critical factor, especially at high DM content. High temperatures reduce the medium viscosity and improve mixing efficiency. Furthermore, as reported in the product datasheet, the enzyme activity is higher at temperatures close to 50 °C. On the other hand, temperatures higher than 35 °C typically reduce the cell viability of most microorganisms. SSF with intermittent increasing ramps of temperature was investigated to increase the process yields [45]. In particular, the set-up consisted of rising the temperature twice at 50 °C during the process: at the beginning of the process, before the yeast inoculation, to produce partial liquefaction of the biomass, and when approaching the ethanol plateau level. Short biomass liquefaction at the optimal enzyme temperature before SSF is the

compromise needed between the high temperatures that better assists the liquefaction at high DM and the shift effect of the SSF processes. In fact, at the liquefaction stage, part of the cellulose is still in the polymeric or oligomeric form and the SSF process could produce the desired shift effect on the hydrolysis of residual biomass. This hybrid SSF process could offer the advantage of a step-wise acceleration of the hydrolysis, which avoids sugar accumulation as in separated hydrolysis and fermentation (SHF) or sugar starvation due to unfavorable hydrolysis conditions as in pure SSF. The duration of the initial pre-liquefaction step results in this process being closer to a pure SSF (short liquefaction time) or, on the contrary, to a separate hydrolysis and fermentation step, SHF, (prolonged liquefaction time). The liquefaction times (10 and 24 h) were chosen so that the glucose concentration at the beginning of the SSF was in the range 40–80 g/L. The effect of raising the temperature after reaching the ethanol plateau level was compared to control experiments in which the temperature was kept constant after the initial pre-liquefaction step. The combination of various process variables, namely pH, temperature and yeast dosage, was then explored. The detailed time course of the experiments obtained after a liquefaction time of 10 h is displayed in Figure 4.

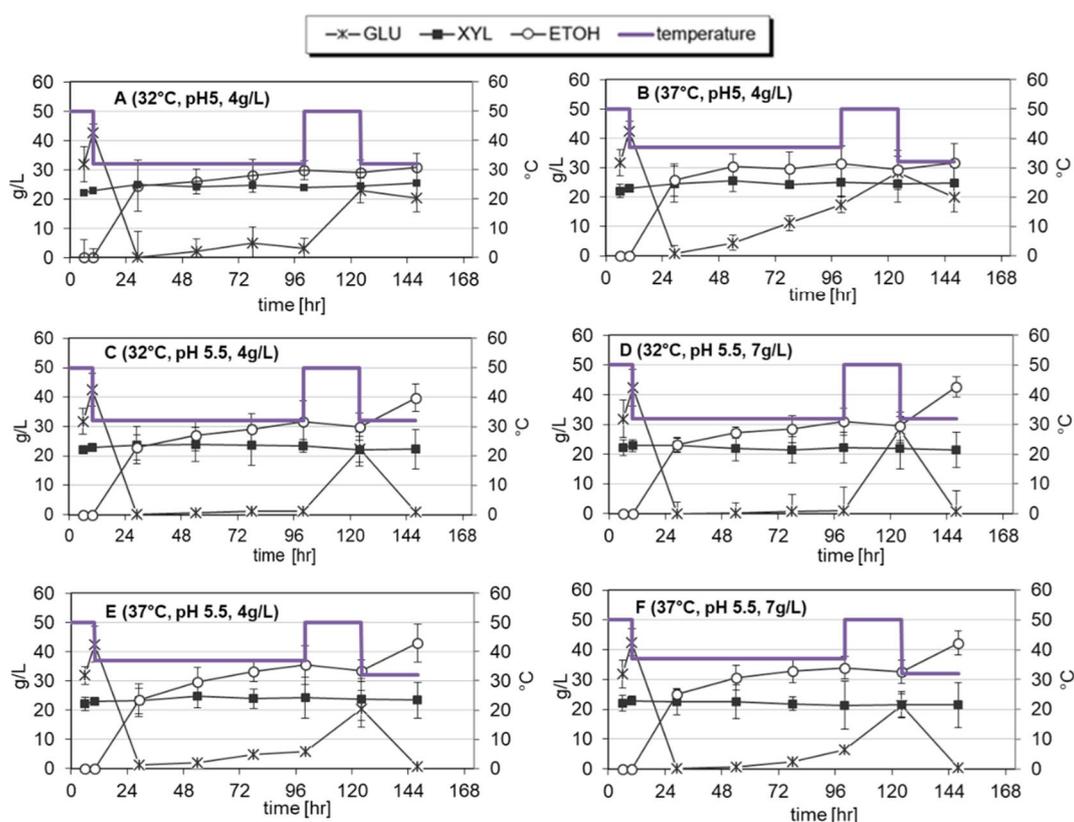


Figure 4. Hybrid simultaneous saccharification and fermentation tests (SSF) of *Arundo donax* at different conditions of temperature, pH and yeast dosage (g/L). Process set-ups: (A) (32 °C; pH 5; 4 g/L), (B) (37 °C; pH 5; 4 g/L), (C) (32 °C; pH 5.5; 4 g/L), (D) (32 °C; pH 5.5; 7 g/L), (E) (37 °C; pH 5.5; 4 g/L), and (F) (37 °C; pH 5.5; 7 g/L). White circle: ethanol g/L; Black square: xylose g/L; black x: glucose g/L. After the temperature step at 50 °C, the flasks were all re-inoculated with 3 g/L yeast. After the second hydrolysis step (124 h of processes), the selected temperature was 32 °C in all tests in order to favor the yeast activity.

In all the experiments, the xylose concentrations remained constant. At pH 5 in both experiments (Figure 4A,B), after 50 h of process, cellulose hydrolysis was more rapid than glucose fermentation and, as result, glucose accumulated. This effect was more evident at 37 °C. At 32 °C, the ethanol concentration approached the plateau after 72 h while a similar ethanol concentration was achieved at 37 °C already

after 50 h. Measurements of the viability of the cells during the process steps (Figure 5) indicated that at 50 h, the number of viable cells was similar in the two assays.

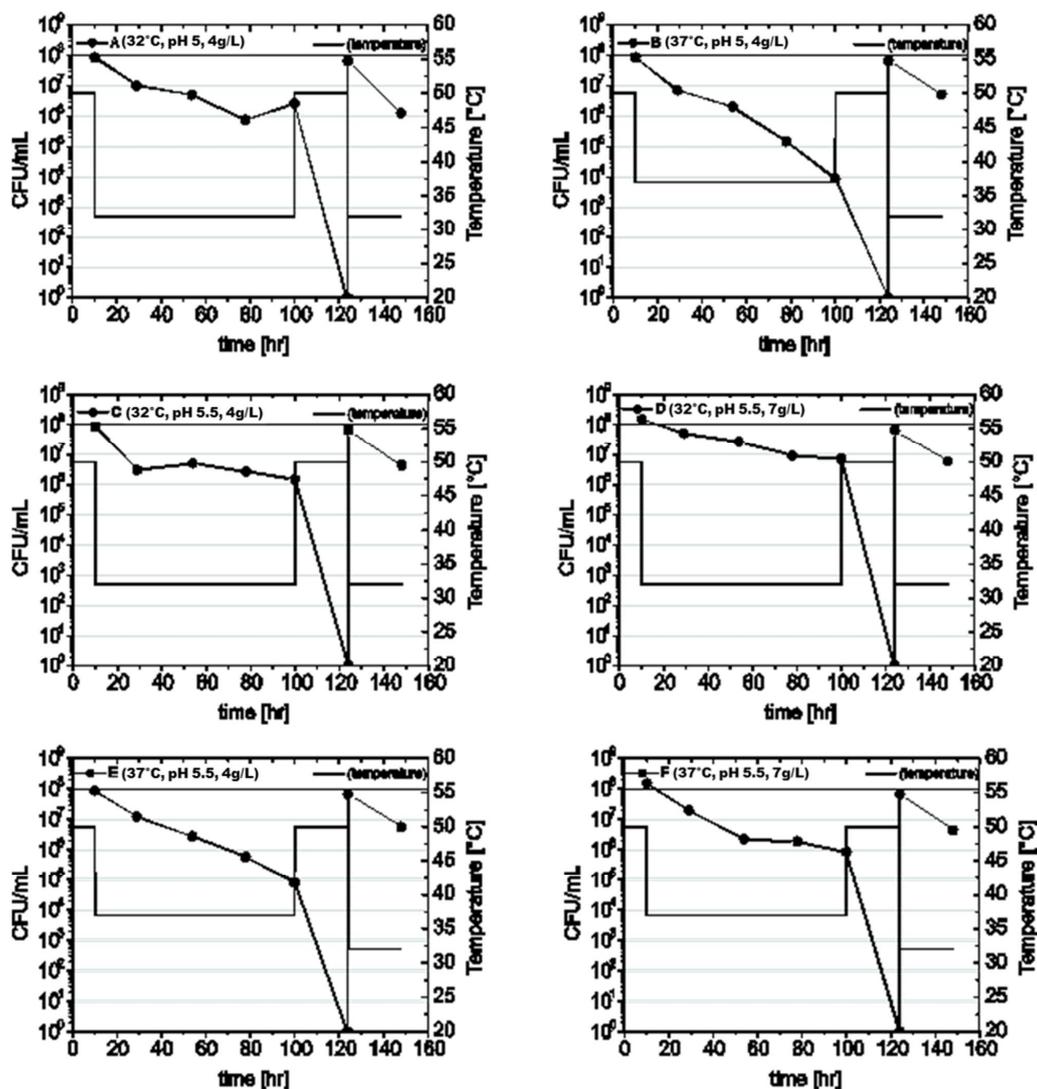


Figure 5. Cells viability (full circles) versus the temperature program. Fermentation process set-ups: (A) (32 °C; pH 5; 4 g/L), (B) (37 °C; pH 5; 4 g/L), (C) (32 °C; pH 5.5; 4 g/L), (D) (32 °C; pH 5.5; 7 g/L), (E) (37 °C; pH 5.5; 4 g/L), and (F) (37 °C; pH 5.5; 7 g/L).

Panels C and D in Figure 4 show the fermentation at pH 5.5, 32 °C and two different yeast dosages. Unlike the previous tests, in this case, fermentation restarted after the temperature step. This indicates that pH 5.5 represented a more favorable condition to the yeast. No meaningful improvements were obtained by doubling the yeast dosage.

Finally, panels E and F in Figure 4 displays the fermentation at pH 5.5, 37 °C and two different yeast dosages. Ethanol concentrations similar to that obtained at pH 5.5 and 32 °C were achieved. On the whole, the data indicate that pH was the most determining factor. At pH 5.5 and 37 °C, the cell viability (Figure 4E) was one order of magnitude higher than at pH 5 (Figure 4B) and this indicates that working at pH 5.5 enables the setting of a higher SSF temperature.

Figure 5 shows cell viability during the process steps.

In the following 50 h, in the test at 32 °C (Figure 5A), the viability of the cells diminished by less than one order of magnitude, whereas at 37 °C it diminished by two orders of magnitude. This indicates that at 37 °C, the cells did not undergo significant thermal stress for at least 40 h; however, in both tests,

fermentation did not restart after the second hydrolysis step at 50 °C, even though the medium was re-inoculated with fresh cells. Furthermore, it is worth noting that the second hydrolysis step after the ethanol plateau produced, in assay A, an increment of the glucose concentration 1.8 higher than in assay B and that the maximum glucose concentration achieved after the temperature increase was similar in both tests. This indicates that there was an intrinsic limit in the cellulose hydrolysability probably due to the structure of the fiber after the pretreatment more than the optimal hydrolysis temperature.

Table 1 sorts the final results by the increasing concentration of ethanol. The data indicated that the cellulose conversion was mostly in the range 78–88%, significantly higher than the control tests at 32 °C. This was due to the use of temperatures higher than 32 °C and the double step of hydrolysis.

Table 1. Final ethanol concentration and overall cellulose conversion corresponding to different hydrolysis and fermentation (HSSF) process conditions. All flasks were inoculated with an additional 3 g/L yeast after the temperature step at 50 °C. ^a: Yeast dosage during the first SSF step; ^b: fermentation temperature; ^c: estimated as residual glucose + ethanol/0.51. Letters in the brackets correspond to the panel label in Figure 4.

Test	Yeast Dosage [g/L] ^a	T (°C) ^b	Fermentation Mode	pH	EtOH [g/L]	Overall Converted Cellulose ^c
1	4	37	control	5	29.8 ± 1.0	88 ± 2
2	4	32	control	5	30.1 ± 1.2	62 ± 2
3	7	32	control	5.5	30.8 ± 1.4	62 ± 3
4 (A)	4	32	T step	5	30.9 ± 1.7	81 ± 3
5	4	32	control	5.5	31.6 ± 1.8	63 ± 4
6 (B)	4	37	T step	5	31.7 ± 1.0	82 ± 2
7	7	37	control	5.5	33.9 ± 0.8	82.0 ± 1.8
8	4	37	control	5.5	35.4 ± 1.5	78 ± 3
9 (C)	4	32	T step	5.5	39.8 ± 1.8	79 ± 4
10 (F)	7	37	T step	5.5	42.3 ± 0.9	83.0 ± 1.8
11 (D)	7	32	T step	5.5	42.7 ± 1.4	85 ± 3
12 (E)	4	37	T step	5.5	42.9 ± 1.7	85 ± 3

The data in the table indicate that at pH 5 and constant temperature after the liquefaction step, similar ethanol concentrations were achieved at 37 and 32 °C (tests 1 and 2). Conversely, at pH 5.5, SSF at 37 °C achieved 10% more ethanol than at 32 °C (tests 3 and 7) and a sharp difference was evident in the overall converted cellulose through the fibers hydrolysis and glucose fermentation. Raising the process temperature once the ethanol plateau level was reached produced roughly 20% more ethanol (tests 5 and 9, 8 and 12) when compared to the control tests.

Overall, the maximum concentration of ethanol was 43 g/L. The same setups were investigated after a liquefaction time of 24 h. In particular, the comparison between the twin assays is summarized in the histogram of Figure 6. In this case, the ethanol plateau level was reached earlier than in the previous case and the entire process lasted 116 h (detailed data not shown).

The data indicate that at pH 5 the ethanol concentration achieved after 24 h liquefaction (conditions of test 6 in Table 1) was 31% higher than that obtained after a liquefaction time of 10 h. At pH 5.5 the two liquefaction times yielded almost the same results in all the process conditions. These findings could tentatively be explained by the effect of a higher amount of initial sugars, as obtained after 24 h of liquefaction, on the activity of the cells. In this regard, some investigations proved that while acetic acid causes a decrease of the intracellular NADH, higher glycolytic fluxes determine an increase of the NADH levels [46]. As a result, the inhibition by acetic acid at pH 5 could have been partly counterbalanced by higher glycolytic fluxes. A further test on the hybrid process optimized at pH 5.5 and 37 °C was investigated by increasing the enzymatic load from 19 to 34 FPU of Cellic[®] CTec3 per g of glucan. The results showed a further increase in ethanol obtained, from 43 to 51 g/L. The final ethanol concentration was higher with respect to the literature processes at high dry matter consistency. Bondesson et al. obtained 37.5 g/L ethanol from wheat straw using the acetic-acid-catalyzed steam pretreatment and co-fermentation approach [30]. About 39 g/L of ethanol was obtained by Palmqvist and Liden [31] for the conversion of *A. donax* in a hybrid process consisting of the pre-liquefaction of a slurry containing 21% solids for 48 h with an enzymatic dosage of 75 mg Cellic[®] CTec3 per g

of glucan. Cassels et al. (2017) achieved 35 g/L ethanol from wheat straw using an SO₂-catalyzed steam pretreatment and an SSF process with 10% solids [32]. Nielsen et al., 2020, obtained an ethanol concentration of about 50 g/L from mixed agricultural feedstocks at 14% solids by using a long liquefaction step of 48 h [33].

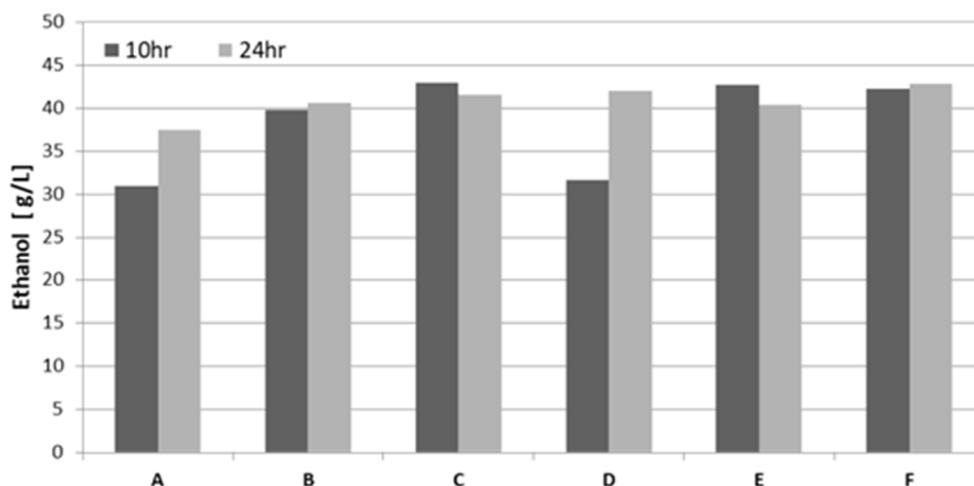


Figure 6. Ethanol concentrations achieved at different process conditions (yeast dosage/SSF temperature/pH + second hydrolysis step). Bars compare two preliquefaction times: 10 and 24 h. (A) (32 °C; pH 5; 4 g/L), (B) (37 °C; pH 5; 4 g/L), (C) (32 °C; pH 5.5; 4 g/L), (D) (32 °C; pH 5.5; 7 g/L), (E) (37 °C; pH 5.5; 4 g/L), and (F) (37 °C; pH 5.5; 7 g/L).

3.3. Conversion of Hemicellulose

Xylose concentration in the mixed hydrolysates was in the range 23–29 g/L depending on the length of the liquefaction time. Many efforts have been made to construct recombinant yeast strains for the xylose co-fermentation over the past few decades. However, this process task remains challenging due to the complexity of lignocellulosic hydrolysates. An alternative *Arundo donax* refining scheme could be the separation of cellulose and hemicellulose and the conversion of the hemicellulose derived xylose to FA (2-formaldehyde). Acid-catalyzed dehydration of C5 sugars to furfural is already an industrial process [47]. New challenges consist of developing more sustainable processes with reduced energy input and making use of catalysts suitable for continuous processes. The combined use of microwave energy and solid acid catalysts, such as resins, could be a valuable option. Strong cation-exchange resins offer the advantages of continuous processing of pretreatment liquid, reusability and short reaction times when compared to enzymes. One limitation is the thermal instability at temperatures of 150–170 °C, typically used for oligosaccharide hydrolysis [48]. Considering that the microwave-assisted processes typically last less than other thermal processes, the extent of the resin's deactivation is expected to be limited. Amberlyst-15 was selected for this process because it is a heterogeneous acid catalyst with good thermal and chemical resistance [49]. The microwave (MW) dehydration process was investigated by varying the reaction temperature and the catalyst concentration. The reaction time was 10 min for all the experiments. FA production yields, xylose conversion rate and process selectivity were then determined.

Table 2 displays the FA yields obtained by the microwave-assisted dehydration of hemicellulose derived xylose at increasing the process temperature. Three catalyst dosages were tested. The trend indicated that the yields increased at increasing the process temperature and the catalyst dosage up to a maximum of around 34% achieved at 157 °C with a selectivity of 57%. Comparable yields were obtained at 179 °C. There was no benefit of increasing the process temperature. The mineral acid gave yields of 37% at catalyst dosage over 10 meq/g_{xylose}, namely similar to solid catalyst under similar conditions. However, the process selectivity was higher than with the solid catalysts likely due to

a higher tar formation in the trials with the solid catalysts. The highest yield of 49% was obtained with the mineral acid at 179 °C. Matsagar et al. (2017) yielded 42% furfural by using Amberlyst-15 in water/toluene (1:5 v/v) biphasic system at 170 °C for 4 h [50]. After the process, the solid catalyst was recovered and reused in a subsequent batch without regeneration. However, the used catalyst did not show any activity. After regeneration, the catalysts recovered most of their original activity. Figure 7 displays the catalyst activity over three batches. The results indicated that even if the selected resin did not display the same catalytic activity as the mineral acid at the highest temperature, it offered the advantage of being used in continuous operations.

Table 2. Production of furfural (FA) by microwave-assisted C5 dehydration (a 4.7 meq/g_{RESIN}, * g_{CATALYST}/g_{XYLOSE}, b = HCl).

T [°C]	Catalyst [meq/g _{XYLOSE}]*	FA [%]	Xylose Conversion [%]	Selectivity [%]
Solid catalyst (a)				
132	9	9.9 ± 0.5	24 ± 3	65 ± 5
132	14	17.6 ± 0.9	54 ± 3	51 ± 3
132	17	19.9 ± 0.6	61 ± 3	51 ± 2
157	9	27.0 ± 1.1	86 ± 3	49 ± 3
157	14	33.5 ± 1.7	91 ± 3	57 ± 3
157	17	32.0 ± 0.6	92 ± 3	54 ± 3
179	9	35.4 ± 1.1	96 ± 3	58 ± 2
179	14	31.3 ± 1.3	99 ± 3	49 ± 2
179	17	31.0 ± 0.9	99 ± 3	49 ± 2
Mineral acid (b)				
157	1.6	3.7 ± 0.3	48.6 ± 1.5	11.7 ± 1.0
157	2.4	3.4 ± 0.2	41.9 ± 1.7	12.5 ± 0.7
157	3.0	4.7 ± 0.2	40.3 ± 1.1	17.9 ± 1.2
157	6.0	24.1 ± 0.7	44.6 ± 1.4	83 ± 4
157	10	27.7 ± 1.1	58.0 ± 1.5	74 ± 4
157	26	37.4 ± 1.0	79 ± 2	73 ± 3
179	6	49.4 ± 0.8	95 ± 3	80 ± 3

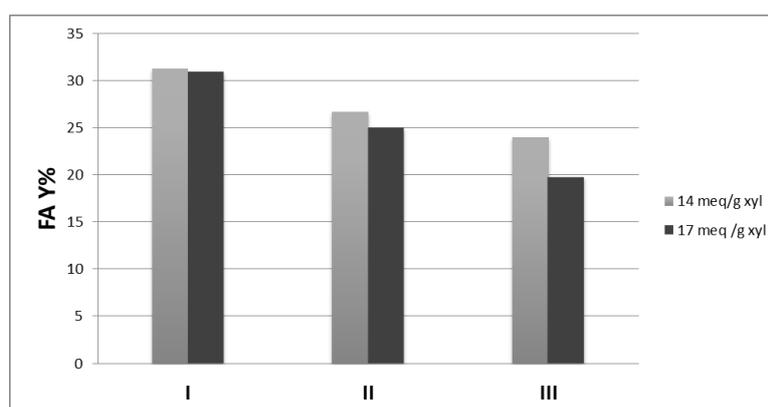


Figure 7. Effect of two cycles of catalyst regeneration on process carried out at 157 °C and 14 meq of catalyst per g of xylose. (I): FA yielded by using Amberlyst-15; (II): FA yielded by using Amberlyst-15 after first regeneration; (III): FA yielded by using Amberlyst-15 after second regeneration.

The highest FA yield achieved in the present investigation was 49% (g FA obtained per 100 g of xylose) corresponding to 5.3 g FA/100 g of *Arundo donax* by using HCl and 3.8 g FA/100 g of *Arundo donax* by using recyclable Amberlyst-15.

There are few works in the literature detailing the conversion of raw hemicellulose from *Arundo donax* to furfuraldehyde. A microwave catalyzed process was investigated by Raspolli Galletti et al. (2015) for the hydrolysis and dehydration of *Arundo donax* at 150 °C by using

HCl 37% [51]. The authors achieved a yield of 57%, higher than 49 % obtained in the present paper by using an inorganic acid catalyst. Differences in the set-up to dissolve and hydrolyze hemicellulose could explain the difference in the final yields. Jeon et al. (2016) investigated the conversion of sugars from microalgae by Amberlyst-15 catalyst achieving a yield of 19%, lower than that obtained in the present paper, by working at 180 °C for 30 min [52]. The yield obtained in the present paper, 34%, by using Amberlyst-15 at 157 °C for 10 min, resulted similarly to that of Matsagar et al. [50] also taking into account that one major difference is represented by the hemicellulose composition and in particular by the presence of secondary products in the *Arundo donax* hemicellulose that could have reduced the catalyst performances.

4. Conclusions

In this work *A. donax* pretreated by steam explosion was evaluated as a renewable feedstock to co-produce ethanol and 2-furaldehyde (FA). In order to exploit both the cellulose and hemicellulose streams, a biorefinery scheme was evaluated. The cellulose fraction was hydrolyzed by using Cellic® CTec3 and fermented with a novel hybrid process at high gravity conditions consisting of a biomass pre-liquefaction followed by simultaneous saccharification and fermentation with a step-wise temperature program and multiple inoculations. A list of specific findings and achievements is reported below:

- Cellic® CTec3 inhibition thresholds by glucose and ethanol were assessed under high gravity process conditions.
- pH 5.5 resulted in the optimal condition because at this pH, *S. cerevisiae* cells demonstrated higher viability at 37 °C.
- Ethanol concentration of 51 g/L was finally achieved by using an optimized hybrid process.
- The maximum yield of FA obtained by using a regenerable Amberlyst solid catalyst was 3.8 g per 100 g of original dry *A. donax*.
- Under the process conditions tested in the present paper, the catalyst maintained a significant residual activity after three batch processes.

On the whole, the biorefinery layout optimized in the present paper allowed us to obtain 51 g/L ethanol, well above the minimum concentration threshold required to make the industrial distillation phase economically convenient. Undetoxified hemicellulose was converted to furaldehyde through a microwave-assisted process using a regenerable solid catalyst with the advantage of converting xylose almost quantitatively; however, higher furfural selectivity is still required. The overall feasibility of the investigated process layout mainly depends on the scalability of the dehydration process. On the whole, the integrated exploitation of the lignin residue to energy and chemicals could significantly affect the techno-economic sustainability of the entire process.

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Abbreviations

ACSEP	acid-catalyzed steam explosion pretreatment
SHF	separated hydrolysis and fermentation
SSF	simultaneous saccharification and fermentation
SSCF	simultaneous saccharification and co-fermentation
HSSF	hybrid simultaneous saccharification and fermentation
FPU	filter paper unit

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