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Single Cell Oil Production from Undetoxified *Arundo donax* L. hydrolysate by *Cutaneotrichosporon curvatus*

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The use of low-cost substrates represents one key issue to make single cell oil production sustainable. Among low-input crops, *Arundo donax* L. is a perennial herbaceous rhizomatous grass containing both C5 and C6 carbohydrates. The scope of the present work was to investigate and optimize the production of lipids by the oleaginous yeast *Cutaneotrichosporon curvatus* from undetoxified lignocellulosic hydrolysates of steam-pretreated *A. donax*. The growth of *C. curvatus* was first optimized in synthetic media, similar in terms of sugar concentration to hydrolysates, by applying the response surface methodology (RSM) analysis. Then the bioconversion of undetoxified hydrolysates was investigated. A fed-batch process for the fermentation of *A. donax* hydrolysates was finally implemented in a 2-L bioreactor. Under optimized conditions, the total lipid content was 64% of the dry cell weight and the lipid yield was 63% of the theoretical. The fatty acid profile of *C. curvatus* triglycerides contained 27% palmitic acid, 33% oleic acid and 32% linoleic acid. These results proved the potential of lipid production from A. donax, which is particularly important for their consideration as substitutes for vegetable oils in many applications such as biodiesel or bioplastics.

Keywords: *Arundo donax* L. hydrolysates, single cell oil, microbial lipids, *Cutaneotrichosporon curvatus*, second-generation sugars

Introduction

The use of biomass to produce not only bioenergy but a number of biobased products is the key strategy to accelerate the global transition from a fossil-based economy toward the bioeconomy. In particular, biodiesel can be produced from different sources such as vegetable oils, animal fats, and cooking oil waste [1]. The recent demand for biodiesel worldwide has turned triacylglycerol (TAG) into an ever-growing and substantial consumption resource [2]. As a consequence, one main concern regards the use of oil crops for non-food applications as it is expected to increase the price of these commodities.

Microbial lipids, also known as single cell oils (SCOs), can be produced from a number of biobased intermediates or waste streams, such as, for instance, low-cost sugars and biodiesel-derived glycerol. The fatty acids profile is similar to most plant oils and animal fats and this could make them an alternative for replacing some conventional resources [3]. Besides biofuels, their potential use concerns additional production sectors such as bioplastics and food additives [4, 5].

Oleaginous microorganisms can convert biomass sugars to lipids up to more than 20% of their dry mass. The lipogenesis process is triggered by specific culture conditions, namely nitrogen [6], phosphate or sulphate limitations [7]. Lipids are stored as TAGs contained inside vesicles defined as lipid bodies. Moreover, some species, such as *Cutaneotrichosporon curvatus*, can utilize a number of carbon sources including hexose and pentose sugars and their oligomers [8]. The overall economics of this bioprocess becomes favorable when zero or low-cost substrates are utilized as carbon or nitrogen sources [9, 10].

Lignocellulosic biomass is a renewable feedstock for the

production of biofuels and bio based products. It is an abundant resource [11] with no direct competition with the food production chain. In particular, second-generation sugars obtained from the lignocellulose polysaccharides appear to be a renewable raw material even more sustainable with respect to the first-generation sugars obtained from food crops [12]. In recent years, some high-yield perennial crops have been investigated as feedstocks for the production of second-generation carbohydrates. Among them, giant reed (*Arundo donax* L.), a perennial herbaceous crop, could be a feedstock for lignocellulose-based biorefineries since it can adapt to a wide range of climatic habitats and produces high biomass yields [13].

The production of TAGs from lignocellulose-derived sugars depends on the composition and quality of the hydrolysates [14]. The main constituents of lignocellulosic materials are cellulose, hemicellulose and lignin. Cellulose is a linear polymer of glucose, hemicellulose is a heteropolysaccharide composed mainly of pentoses (xylose, arabinose, etc.) and a few hexoses (glucose, galactose, mannose, etc.), and lignin is an aromatic polymer mainly composed of phenylpropane subunits. Pretreatment processes are necessary to disrupt the plant cell wall complex, before the enzymatic hydrolysis of the polysaccharides. Depending on the severity of the pretreatment, some by-products, such as furfural and 5-hydroxymethyl-furfural (HMF), can be generated in this step from the degradation of pentoses and hexoses respectively. These molecules have a known inhibitory action against the metabolic activity of many microorganisms [15]. The pretreatment typically generates also short chain organic acids, namely acetic, formic and levulinic acids [16, 17]. Furthermore, phenolic compounds, such as syringaldehyde, p-hydroxybenzaldehyde (PHB), vanillin, etc., could be generated from lignin [18]. All these compounds are usually inhibitory to microorganisms in the down-stream fermentation step [19]. Tolerance to inhibitors appears to be strain dependent even if optimized process strategies could contribute to alleviate the overall toxic effect.

The aim of the present work was to optimize the microbial co-fermentation of glucose and xylose to lipids by *C. curvatus* grown on un-detoxified *Arundo donax* L. hydrolysates at high concentrations.

Materials and Methods

Culture Preparation

Yeast strain. The yeast strain used in the present study was *Cutaneotrichosporon curvatus* CA-3802 (DSM 70022, DSMZ, Germany). It was stored at 4°C and propagated every month on yeast peptone dextrose (YPD) agar slants (glucose 20 g/l, peptone 10 g/l, yeast extract 10 g/l, agar 20 g/l, pH 6.0).

Inoculum preparation. Before inoculation, all media were autoclaved at 121°C for 20 min. The pre-inoculum was prepared by using a rotary shaker with the agitation speed set at 150 rpm. The medium was inoculated with four-to-five colonies picked out from the agar plate and incubated for 72 h at 30°C. The inoculum for the batch tests was prepared by transferring 50 ml of the pre-culture medium into 250-ml Erlenmeyer flasks. For the fed-batch inocula, 1 L of pre-culture medium (YPD broth 50 g/l, Sigma-Aldrich) was transferred into 2-L Erlenmeyer flasks. After incubation, wet cells were recovered by centrifugation at 10,000 ×*g* for 10 min and resuspended in a few mL of 0.9% NaCl solution. 500 µl of resuspended cells were oven-dried at 70°C to constant weight to obtain the dry cell weight (DCW) concentration of the cell suspension. The average dry weight of NaCl was measured and subtracted from the overall dry weight. A precise volume of the cell suspension was inoculated to achieve the desired initial cell concentration of 1.7 g/l DCW for the batch tests and 6 g/l for the fed-batch cultures.

Fermentation Tests

Lipid production on synthetic media containing glucose and xylose in batch culture. *C. curvatus* was grown on autoclaved synthetic media (121°C for 20 min) containing glucose and xylose at three different concentrations of 40, 60, and 90 g/l. The initial pH was 5.5 (not monitored during the fermentation) and contained optimized amounts of KH_2PO_4 and yeast extract (obtained from DOE optimization), and trace elements, namely, $MgSO_4 \bullet 7H_2O$ 0.4 g/l, $MnSO_4 \bullet H_2O$ 0.003 g/l, $CuSO_4 \bullet 5H_2O$ 0.0001 g/l. A concentrated solution of yeast extract was separately sterilized through a 0.2-µm filter and then added to media in order to obtain the desired concentration. All experiments were conducted in triplicate.

Comparison of different nitrogen sources. Batch experiments were performed in shake flasks containing different nitrogen sources, namely, yeast extract, $(NH_4)_2SO_4$ and $(NH_4)_2SO_4$ plus vitamins, with the same C/N ratio (obtained from DOE optimization). The growth medium was prepared as follows: $MgSO_4 \bullet 7H_2O$ 0.4 g/l, KH_2PO_4 2 g/l, $MnSO_4 \bullet H_2O$ 0.003 g/l, $CuSO_4 \bullet 5H_2O$ 0.0001 g/l, $ZnSO_4 \bullet 7H_2O$ 0.04 g/l at initial pH 5.5. All media were sterilized through a 0.2-µm filter. After 5 days' incubation in a rotary shaker at 150 rpm at 30°C, the yeast cells were centrifuged, washed twice with distilled water and the dry weight was determined. All experiments were conducted in triplicate.

Design of Experiments (DoE) for improvement of medium composition. Inhibition by biomass degradation products is often the main challenge in the production of biochemicals through fermentation of second-generation sugars. Bio-detoxification of toxic products at high cell concentrations could represent a viable option to achieve high process yields. For this reason, the response surface methodology (RSM) was used to optimize the

Table 1. Factors and levels of the Box-Behnken design. All samples consist of glucose 90 g/l, $MgSO_4 \bullet 7H_2O$ 0.4 g/l, $MnSO_4 \bullet H_2O$ 0.003 g/l, and $CuSO_4 \bullet 5H_2O$ 0.0001 g/l.

Variable	Level		
	Low (-1)	Medium (0)	High (+1)
Yeast extract (X_1) (g/l)	0.50	2.75	5.00
KH_2PO_4 (X_2) (g/l)	2.00	4.50	7.00
Citric acid (X_3) (g/l)	0.00	0.50	1.00

biomass production in function of the nutrient concentrations, namely, yeast extract (X_1) as nitrogen source, phosphate (KH_2PO_4) (X_2) and citric acid ($C_6H_8O_7$) (X_3) by using a glucose concentration of 90 g/l which is close to lignocellulosic hydrolysates.

In particular, the Box-Behnken factorial design was applied to define a mathematical model describing the single and synergistic effects of the three independent variables in terms of biomass production. The selected algorithm was implemented with three factors (X_1, X_2, X_3) and three levels (-1, 0, +1), including three replicates at the center point. The factor concentration ranges are reported in Table 1. On the whole, according to previous experimental designs, 15 tests were selected to achieve statistically significant surface responses [20–22].

The dry weight biomass production was selected as model response (Y) and correlated to the independent variables through a second-degree polynomial equation whose general formula is:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (1)$$

where β_0 represents the intercept, β_i the linear coefficient, β_{ij} the quadratic coefficient, β_{ii} is the linear-by-linear interaction between x_i and x_j and x_i, x_j are input variables that influence the response variable Y. The robustness of the mathematical model (Eq. (1)) was evaluated by the coefficient of determination R^2. The model was tested by carrying out experiments in shaking flasks with the predicted medium compositions at the same culture conditions. The experiments were carried out in 150-ml Erlenmeyer flasks containing 25 ml of culture medium incubated at 30°C and pH 5.5.

Feedstock and Pretreatment

Giant reed (*Arundo donax* L.) was grown and harvested in Northern Italy (province of Vercelli) and delivered as a 200–300 kg milled residues. The residues were divided into stocks of roughly 30 kg and stored indoors. The dry-matter (DM) content of the raw material was 94%. Air-dried biomass was analyzed for carbohydrates, lignin and ash content by the following standard methods: carbohydrates and lignin by the Klason procedure (modified TAPPI T-13 m-54 and ASTM D1106) and ash by ASTM D1102. The raw material contained (%): 37.2 ± 0.9 glucan, 20.9 ± 1.2 xylan, 1.55 ± 0.11 arabinan, 0.48 ± 0.05 galactan, 21.8 ± 1.3 lignin, 7.84 ± 0.21 total ash, 3.5 ± 0.4 acetyl groups).

Biomass pretreatment was carried out by using the steam explosion (SE) batch technology from Stake Tech Ltd. (Canada) (10 L). *Arundo donax* was pretreated at 200°C for 5 min according to process conditions previously optimized [23]. Prior to the pretreatment, biomass was crushed to particles in the range of 1.7–5.6 mm. Acid catalyzed steam explosion pretreatment was carried out by impregnating the *Arundo donax* with H_2SO_4 before feeding the batch digester. *Arundo donax* was soaked in a dilute H_2SO_4 solution (0.07 M). After 10 min, impregnated biomass was pressed to separate the solids from the solution. The resulting acid load was 1.5% (w, w) and the final DM level of impregnated biomass was 36.0 ± 1.7%. The pretreated product had a DM content of 13.4 ± 0.9% and contained (% respect to DM): 42.7 ± 1.0 glucan, 17.8 ± 1.0 xylan, 1.23 ± 0.08 arabinan, 0.55 ± 0.07 galactan, 26.9 ± 1.8 lignin, 2.48 ± 0.32 acetyl groups, 0.23 ± 0.02 5-HMF, 0.51 ± 0.03 2-FUR. All data are reported with standard deviation achieved on three replicates.

Enzymatic Hydrolysis

The enzymatic hydrolysates of steam-pretreated *Arundo donax* were prepared in a 2-L stirred bioreactor (Braun Biotech International) equipped with a homemade helical impeller. The biomass consistency (insoluble fraction to liquid fraction ratio) used to produce the enzymatic hydrolysate was 25%. The enzymatic blend, Cellic Ctec2, was kindly provided by Novozymes. The process was run at pH 5 and 45°C in a fed-batch mode for both the biomass and the enzyme feeding. The overall enzyme dosage was 190 mg/g of insoluble glucan corresponding to 25 FPU/g of insoluble glucan. The process was interrupted after 90 h. The hydrolysate contained 90.1 g/l and 20.8 g/l glucose and xylose, respectively, corresponding to a glucose yield of 70%. The hydrolysates were then filtered to separate the residual fiber. The liquid fraction was added with nutrients and inoculated for fermentation.

Fermentation of *Arundo donax* Hydrolysates in Batch Mode

Arundo donax hydrolysate was supplemented with nutrients and then sterilized by membrane filtration (0.22 μm). The final fermentation medium had the following composition: glucose 90.1 g/l, xylose 20.8 g/l, $MgSO_4 \bullet 7H_2O$ 0.4 g/l, KH_2PO_4 2 g/l, $MnSO_4 \bullet H_2O$ 0.003 g/l, $CuSO_4 \bullet 5H_2O$ 0.0001 g/l, $ZnSO_4 \bullet 7H_2O$ 0.04 g/l, yeast extract 4.2 g/l. The tests were carried out at both pH 5.5 and pH 6.5. For each test, a working volume of 50 ml was used.

Fermentation of *Arundo donax* Hydrolysates in Fed-Batch Mode

Hydrolysates fermentation was carried in fed-batch mode to facilitate the adjustment of the C/N ratio in the course of the process, namely, 24 g/g at the beginning and 300 g/g upon consuming the sugars in the previous batch. The fermentation tests were carried out in a 2-L bioreactor. The medium contained: $MgSO_4 \bullet 7H_2O$ 1.5 g/l, KH_2PO_4 2 g/l, NaH_2PO_4 0.3 g/l, $MnSO_4 \bullet H_2O$ 0.004 g/l, $CuSO_4 \bullet 5H_2O$ 0.001 g/l, $ZnSO_4 \bullet 7H_2O$ 0.04 g/l, and 0.8 ml of a solution containing vitamin B6 hydrochloride 1 g/l,

Table 2. Composition of the *Arundo donax* hydrolysate before and after concentration by evaporation in a rotavapor.

	Glucose conc. (g/l)	Xylose conc. (g/l)	Acetic acid conc. (g/l)	5-HMF conc. (g/l)	2-FUR conc. (g/l)
A. donax hydrolysate (a)	90.1	20.8	3.2	0.32	0.68
A. donax hydrolysate after concentration (b)	342.4	77.0	4.3	1.98	0.13
Concentration ratio b/a	3.8	3.7	1.3	6.20	0.20

myoinositol 25 g/l, nicotinic acid 1 g/l, vitamin B1 hydrochloride 1 g/l. 7 g/l $(NH_4)_2SO_4$ were supplemented to achieve the target initial C/N ratio. 6 g/l DCW of *C. curvatus* were inoculated, and the temperature was held at 30 ± 0.5°C, while the pH was maintained at 5.5 ± 0.2 by automatic addition of 4 N NaOH solution. The dissolved oxygen concentration was maintained at least above 40% of air saturation by automatically changing the agitation speed and by adjusting the inlet air flow rate. The initial culture contained 0.8 L medium. In order to obtain a feed suitable for the fed-batch tests, the feed was concentrated in a laboratory rotavapor vacuum system at 8 kPa, 200 rpm and 35°C for 2 h. This step increased the concentration of the compounds in the hydrolysate as detailed in Table 2.

The nutrient composition of the concentrated feed was the same as the initial medium with the only difference being in the nitrogen source concentration ($(NH_4)_2SO_4$), which was kept at 0.52 g/l, corresponding to a C/N ratio in the bioreactor equal to roughly 300 g/g suitable to increase the lipid production [24].

Analytical Methods

Determination of monosaccharide concentrations. The carbohydrate analysis was performed with a HPIC (High Performance Ionic Chromatography) Dionex ICS-2500 System equipped with a Nucleogel Ion 300 OA operating at 40°C with 10 mM H_2SO_4 solution as mobile-phase (0.4 ml/min). The detector was a Shodex RI-101 Refractive Index. Each analysis was carried out in duplicate.

Determination of microbial growth inhibitors. The analysis of the degradation by-products was performed using the HP1100 system equipped with a Dionex AS1 column operating at 30°C with Milli-Q Water/Acetonitrile as mobile-phase (0.7 ml/min) and a diode array detector. Quantification was carried out at two different wavelengths, 205 and 280 nm.

Lipid extraction. The yeast cells were harvested, washed twice with distilled water and oven-dried at 70°C to constant weight. Extraction of total cellular lipids was performed by a modification of the original Bligh & Dyer [25] extraction procedure. About 500 mg of dried cells were suspended in chloroform/methanol/water 2:1:0.8 v/v/v in a separating funnel obtaining a monophasic system. In order to promote cell lysis, a sonication was carried out at 40°C for 30 min. Then, chloroform and water were added to obtain volumetric ratios of chloroform/methanol/water equal to 3:1:1.8 and the consequent formation of a biphasic system. A further sonication was then carried out at 40°C for 15 min and the extraction was completed overnight at room temperature with gentle shaking. The organic phase was evaporated in a rotavapor under a vacuum at 40°C for roughly one hour, until constant weight. Finally, the total lipids were gravimetrically quantified [26]. The extraction method was verified by using the certified reference material BCR 163 (beef-pork fat blend). Recovery yields were around 98%.

Fatty acids profile. The microbial lipids were directly trans-methylated according to the method of Morrison and Smith [27]. 2 ml of BCl_3/MeOH (12% v/v) and 1 ml of 2,2-dimethoxypropane were added to total dry lipids and put in a water bath for 30 min at 60°C. Then, 1 ml of distilled water was added to stop the reaction and 2 ml of hexane were added to extract the fatty acid methyl esters (FAMEs).

The FAME profiles were determined by GC analyses carried out on an Agilent GC7890A gas chromatograph, equipped with a HP-INNOWax 19091N-213 capillary column (30 m × 0.32 mm × 0.50 µm) and a flame ionization detector. The oven temperature was programmed at 80°C for 11 min, from 80°C to 180°C at a rate of 20°C/min and held at 180°C for 22 min. Helium was the carrier gas at 80 kPa and the split ratio was 1:19 (v/v). Identification of methyl esters was performed comparing the peak retention times to FAME standards (Sigma-Aldrich).

Results and Discussion

Cell Biomass and Lipid Production on Glucose and Xylose by *C. curvatus*

Microbial conversion of sugars to lipids by means of *C. curvatus* has been already investigated [28–30]. Critical parameters are the concentration and composition of the available carbon sources [28, 31], the C/N ratio [32] and the nitrogen sources [24]. Despite the number of achievements documented through the literature regarding fermentation tests in synthetic media, the process performance in biomass hydrolysates is often scarce due to the low tolerance of most microorganisms to the common degradation by-products. The present paper aims at optimizing a process for the conversion of *Arundo donax*-derived carbohydrates to lipids without an mid-term detoxification step.

The ability of the oleaginous yeasts to produce high concentrations of intracellular lipids is due to their capacity of producing significant amounts of acetyl-CoA, the basic

Table 3. Sugar consumption, cell biomass and lipid production by *C. curvatus* separately cultured on glucose and xylose. All data are reported with standard deviation achieved on three replicates.

Carbon source	Sugar concentration (g/l)	C/N ratio (g/g)	DCW (g/l)	DCW yield[a] (g/g, %)	Lipid production (g/l)	Lipid content[b] (w, w %)	Lipid yield[c] (g/g, %)	Average sugar consumption rate[d] (g/l/h)
Glucose	40	29	12.4 ± 0.8	41.2 ± 1.5	6.4 ± 0.7	51.8 ± 2.0	21.3 ± 0.8	0.21 ± 0.01
	60	43	16.0 ± 0.4	51.9 ± 2.0	8.2 ± 0.4	51.2 ± 2.2	26.6 ± 0.7	0.21 ± 0.02
	90	64	16.5 ± 0.7	53.4 ± 1.6	10.2 ± 0.6	65.2 ± 1.8	31.0 ± 2.0	0.22 ± 0.01
Xylose	40	29	8.5 ± 0.3	29.4 ± 1.1	5.3 ± 0.9	62.4 ± 3.8	18.3 ± 0.7	0.20 ± 0.02
	60	43	9.8 ± 0.6	28.8 ± 2.1	5.9 ± 0.4	60.2 ± 3.0	17.4 ± 0.9	0.24 ± 0.03
	90	64	13.9 ± 0.7	44.4 ± 1.6	8.9 ± 0.5	64.0 ± 1.1	28.4 ± 0.9	0.22 ± 0.02

[a]Gram of dry cell biomass/gram of sugar consumed × 100%.

[b]Gram of lipids/gram of dry cell biomass × 100%.

[c]Gram of lipids/gram of sugar consumed × 100%.

[d]Sugar consumption rate (6 days).

unit in fatty acid biosynthesis. Acetyl-CoA derives from the breakdown of the citric acid that, under nitrogen-limitation, has been previously accumulated inside the mitochondria and then transported into the cytosol. The stoichiometry of the glucose metabolism indicates that the maximum theoretical yield of SCO produced per glucose consumed is around 0.32 g/g [33]. In order to optimize the set-up in terms of carbon sources and nutrient composition, some preliminary tests were performed in synthetic media by using ranges of carbohydrate concentrations comparable to the biomass hydrolysates.

Table 3 shows the cellular biomass production and the sugar consumption by *C. curvatus* at three initial glucose and xylose concentrations. In all experiments, sugar was not completely consumed, and fermentations were stopped at 216 h. After 144 h the yeast growth reached the stationary phase and after that the fermentation was continued for 72 h for further lipogenesis. The highest DCW concentration and yield, namely 16.5 g/l and 53.4% (g/g) respectively, were obtained at 90 g/l glucose. Similarly, 13.9 g/l and 44.4% (g/g) were obtained during the fermentation of 90 g/l xylose. The time-course curves of the xylose consumption were comparable to those of glucose even if the lipid yield was slightly lower. The highest lipid content of *C. curvatus* grown on glucose and xylose reached roughly 65% (w/w) of the DCW. This corresponded to 37% glucose consumption in the feed, with a final metabolic yield of about 0.31 ± 0.02 g lipids/g of consumed sugars, close to the maximum theoretical yield of 0.32 [33]. Nutrient intake from yeast extract can contribute to slightly increase the lipid yield calculated on initial glucose. Furthermore, dry lipid extract could also include minor non-triglyceride components.

Fig. 1 indicates that, in the explored range of glucose concentrations (corresponding to C/N ratios reported in Table 3), the lipid yield linearly depended on sugar amount and, as a consequence, on the C/N ratio. No higher concentrations of sugars were considered because in the implemented process the maximum concentration obtainable of glucose was about 90 g/l. This finding agrees with the results reported by Zhang *et al*. [30]. A non-linear relation was found by Béligon *et al*. who tested the C/N ratio between 300 and 900 g/g [24]. This could be due to competitive metabolic pathways, including for instance the conversion of monosaccharides to exopolysaccharides, which are activated by certain process conditions [34, 35].

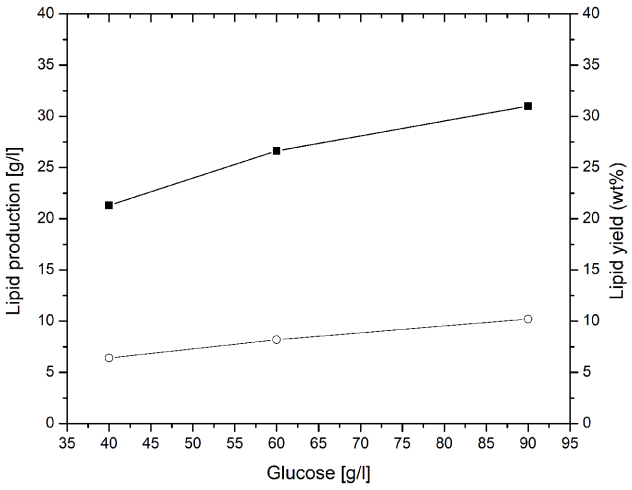

Fig. 1. Lipid production (white dot) and lipid yield (black square) at different initial glucose concentrations (process time: 9 days).

The optimal C/N ratio of the hydrolysate concentrations achieved in the present work was 85.7 g/g, corresponding to 100 mol/mol. This was obtained by fixing the glucose concentration at 90 g/l and varying the nitrogen source in a range of C/N ratios wider than the previous one. It's worth noting that the optimal ratio for cell biomass production follows within the optimal range for the lipid production, namely 80–350 mol/mol [31]. This indicates that the optimized conditions for biomass growth are close to the optimal range for lipid production.

Optimization of the Medium Composition

In general, the C/N ratio that maximizes biomass or lipid production is strain dependent, and it is also correlated to the nature of the carbon and nitrogen sources. Therefore, in the present work, the medium composition and the specific C/N ratio for *C. curvatus* were optimized accordingly. Nitrogen and phosphorus limitation play an important role since they can limit biomass growth and induce the intracellular accumulation of lipids. Similarly, citrate in the cytosol plays an important role since it is the precursor of acetyl-CoA which, in turn, represents the immediate starting point of fatty acid synthesis [33]. Citrate was used as a nutrient by several authors such as Luo *et al*. [36], Liang *et al*. [37], Gong *et al*. [38] and Wu *et al*. [39]. In the experimental design, yeast extract was initially selected as a nitrogen source as it is also one of the most complete sources of auxiliary nutrients for fermentation such as vitamins and other important co-factors. This enabled the evaluation of the effect of the C/N ratio without introducing additional stress factors to the cells.

As already mentioned, lipid biosynthesis is favored by cell stress conditions, namely the depletion of some key nutrients. If this stress is further intensified by the action of biomass degradation products, the most likely result is the yeast's inability to grow on biomass hydrolysates. Therefore, in the present paper, the nutrient concentration was first optimized to maximize cell growth. Table 4 reports the experimental design including the response (Y) values. The biomass production was taken as the dependent variable or response. The mathematical model returned a coefficient of determination (R^2) of 0.9586, and the adjusted R^2 of 0.8841. Both the coefficients confirmed the good capacity of the obtained model to fit 95.86% of the response and to predict values of biomass production. Table 5 shows the main statistical data relevant to the Box-Behnken design. The

Table 4. Experimental design matrix.

Run	X_1 (g/l)	X_2 (g/l)	X_3 (g/l)	Y (g/l)
1	2.75	2.0	1.0	14.30
2	2.75	4.5	0.5	12.60
3	0.50	4.5	1.0	9.35
4	0.50	7.0	0.5	10.10
5	2.75	7.0	0.0	12.45
6	5.00	2.0	0.5	14.75
7	5.00	7.0	0.5	12.75
8	5.00	4.5	1.0	11.60
9	2.75	4.5	0.5	13.50
10	0.50	4.5	0.0	8.25
11	5.00	4.5	0.0	12.90
12	0.50	2.0	0.5	8.70
13	2.75	2.0	0.0	16.40
14	2.75	4.5	0.5	13.60
15	2.75	7.0	1.0	13.60

Table 5. Analysis of variance for the obtained mathematical model.

ANOVA										
Coefficients		*P* value	Standard error		*df*	*SS*	*MS*	*F*	*Prob > F*	*LF*
b_0	10.260	0.002820	1.88100	Model	9	72.56	8.062	12.87	0.00584	2.78
b_1	4.704	0.000497	0.58900	Error	2	0.607	0.303			
b_2	-1.615	0.056400	0.65300	Total	14	75.69				
b_3	-1.554	0.553000	2.44800							
b_{12}	-0.151	0.084470	0.07035							
b_{13}	-0.533	0.190000	0.35200							
b_{23}	0.650	0.09527	0.31660							
b_{11}	-0.526	0.001320	0.08136							
b_{22}	0.160	0.059180	0.06590							
b_{33}	-0.192	0.912000	1.64800							

df, degree of freedom; *SS*, Sum of Squares; *MS*, Mean Square; *F*, Fisher coefficient; *LF*, Lack of Fit.

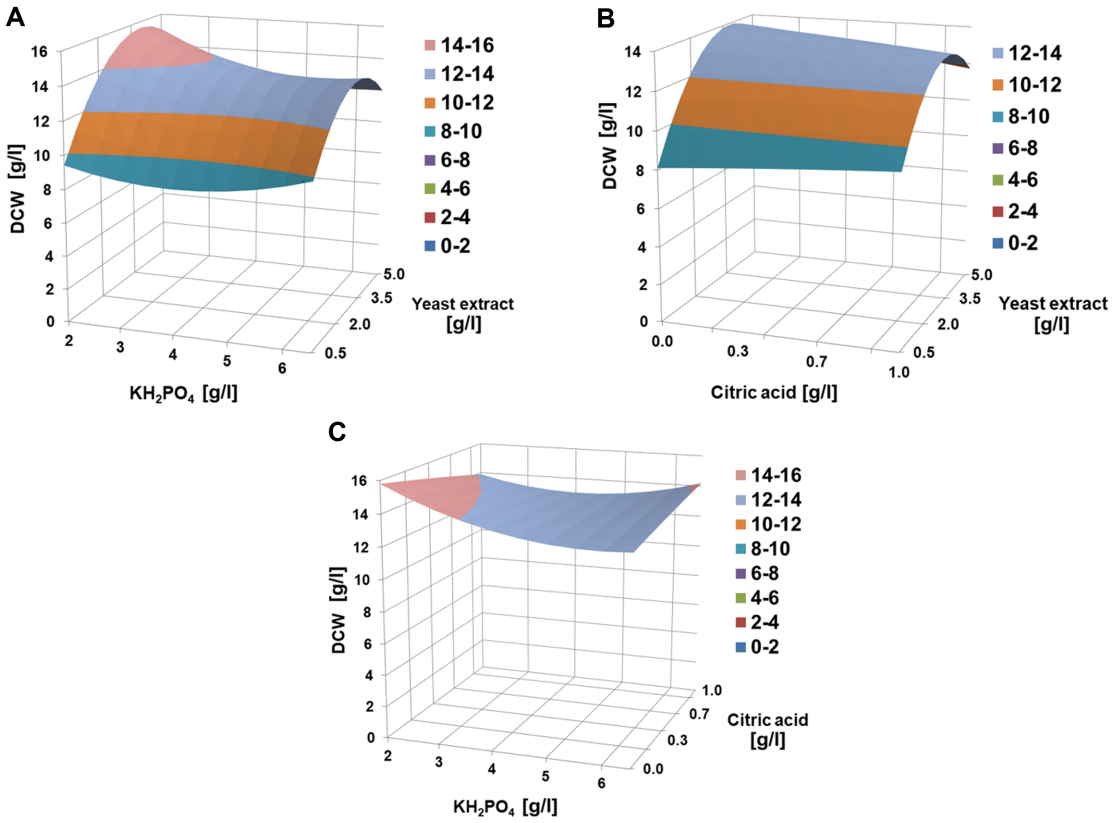

Fig. 2. (**A**) Response surface of biomass production *versus* KH_2PO_4 and yeast extract; (**B**) Response surface of the biomass production *versus* citric acid and yeast extract; (**C**) Response surface of the biomass production *versus* KH_2PO_4 and citric acid.

model F-value of 12.87 implies the model is significant. There is only a 0.58% chance that an F-value this large could occur due to noise. A value of "Prob > F" less than 0.0500 indicates model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit of 2.78 implies the Lack of Fit is not significant relative to the pure error.

The model equation obtained from the Box-Behnken design based on Eq. 1 is given below:

$$Y = b_0 + b_1{*}X_1 + b_2{*}X_2 + b_3{*}X_3 + b_{12}{*}X_1{*}X_2 + b_{13}{*}X_1{*}X_3 + b_{23}{*}X_2{*}X_3 + b_{11}{*}X_1^2 + b_{22}{*}X_2^2 + b_{33}{*}X_3^2 \quad (2)$$

This response equation was used to generate the response surface plots (Fig. 2) and to optimize biomass production. The predicted optimal conditions to maximize the biomass production of *C. curvatus* DSM 70022 up to 17 g/l were 4.2 g/l of yeast extract and 2 g/l KH_2PO_4. Citric acid did not significantly affect biomass production. In order to validate the model, a control experimental test was carried out in triplicate and the measured response value equal to 16.8 g/l resulted very close to the predicted value, thus confirming the accuracy of the model. The experimental design was aimed at finding set-up values of the C/N ratio and phosphorus concentration for the specific yeast strain used in the present paper, which were then applied in the tests with biomass hydrolysates.

Effects of the Nitrogen Source

Nutrient composition is an important requirement to drive the fermentation process toward a preferential product [31]. Inorganic nitrogen (*i.e.*, NH_3, $(NH_4)_2SO_4$, NH_4Cl, KNO_3) and organic nitrogen (*i.e.*, yeast extract, peptone, urea and free amino acids) play different roles in biomass growth and lipid accumulation [24]. It has been reported that organic nitrogen improves both the cell growth and lipid accumulation of *Cutaneotrichosporon curvatus*, while inorganic nitrogen enhances the biomass accumulation [30].

Besides the composition, the medium cost is an important constraint for the economic feasibility of the processes. Thus, considering that the cost per kg of the yeast extract is roughly 1.6-fold higher than the cost of $(NH_4)_2SO_4$, a

Table 6. Growth of *Cutaneotrichosporon curvatus* in media containing different nitrogen sources and the same C:N ratio. Dry weight biomass data are reported with standard deviation achieved on three replicates.

Nitrogen source	Glucose concentration (g/l)	Nitrogen source concentration (g/l)	C:N ratio (g/g)	Dry weight biomass (g/l)
Yeast extract	90	4.20	85.7	9.39 ± 0.6
$(NH_4)_2SO_4$	90	1.98	85.7	5.54 ± 0.5
$(NH_4)_2SO_4$ + vitamins*	90	1.98	85.7	7.40 ± 0.3

*1% of the vitamin solution containing B1 hydrochloride 1 g/l, B6 hydrochloride 1 g/l, nicotinic acid 1 g/l and myoinositol 25 g/l was added to the media.

potential alternative is the use of a medium containing inorganic nitrogen sources along with trace concentrations of the most effective vitamins. In the present investigation, three nutrient compositions, namely yeast extract, ammonium sulphate and ammonium sulphate, added with vitamin B1 hydrochloride, vitamin B6 hydrochloride, nicotinic acid and myoinositol, were evaluated. For all the tests, glucose was used as the sole carbon source at the same C/N ratio. In these conditions, the fermentation process with yeast extract achieved a biomass concentration 1.6-fold higher than the sole ammonium sulphate as nitrogen source (Table 6). The addition of vitamins increased the biomass production by about 35% with respect to the sole $(NH_4)_2SO_4$ (Table 6). On the other hand, ammonium sulphate, added with the four vitamins, has an overall cost 3-fold lower with respect to the cost of yeast extract. Vitamins have, in fact, a negligible cost and typically account for 0.05% of total costs [40]. Based on these results, $(NH_4)_2SO_4$ plus vitamins was selected as the favorite nutrient composition for subsequent hydrolysate fermentation.

Microbial Conversion of *Arundo donax* Hydrolysates

The microbial production of lipids by using *C. curvatus* strains was mostly investigated by using synthetic media [24, 26, 28, 30]. However, both the nature and the level of the main biomass degradation products determine different process efficiencies.

In the present paper, *Arundo donax* was used to produce second-generation sugars. *Arundo donax* is a biomass belonging to perennial rhizomatous grasses and recently received much attention thanks to an easy soil management and low demand for nutrients [41, 42]. Despite the low production costs, the conversion of the *Arundo donax* biomass to sugars presents some difficulties mainly due to the recalcitrant nature of lignin. This could require severe pretreatment conditions with the effect of generating degradation by-products with antimicrobial action. *Arundo donax* hydrolysates have been tested for the production of second-generation bioethanol [13, 43], methane [44, 45] and a number of bio-based products [46] including single cell oils (SCOs) [47]. To the best of our knowledge, there are no studies on the bioconversion of *Arundo donax* carbohydrates into lipids by the oleaginous yeast *C. curvatus*.

As reported in Table 2, the most abundant degradation product from acid-catalyzed steam pretreatment of the biomass was acetic acid. In general, the microbial toxicity of the short chain organic acids at pH lower than 5 is due to the permeation of the undissociated form across the phospholipid bilayer, followed by its dissociation and acidification of the cytosol. This influences the normal biochemical processes in the cell [15]. Previous research investigations reported that potassium acetate could even be a carbon source for *C. curvatus* at pH 7 in batch mode with the production of 0.63 g/l DCW at 72nd h [48]. In the present investigation, the acetic acid concentration remained constant through the process (detailed data not shown). This could be due to the presence of relatively higher concentrations of glucose and xylose that represented the preferred carbon sources. In order to explore the effect of the pH on the biomass growth rate, two different initial pH set-ups, namely 5.5 and 6.5, were tested. Fig. 3 shows the results of these tests. The data indicated that growth stopped at the lower pH after 24 h, but especially in hydrolysate the DCW was on the same level after 24 h, meaning that the specific growth rate was not different, but that the entry into stationary phase happened earlier. This indicates that changes in cultures grown in non-regulated pH occurred for instance because of the secretion of acids like citrate, N-transport into the cell (proton antiport), or assimilation of additional acids different from acetic acid, with an overall effect of interrupting yeast growth.

The investigation of yeast growth in synthetic media enabled the selection of initial set-up for fermentation. However, a fine tuning of the process conditions was necessary due to the presence of additional degradation by-products that could affect the process performance. Besides acetic acid, *Arundo donax* hydrolysates containe both 5-HMF and 2-furaldehyde reaching a total concentration

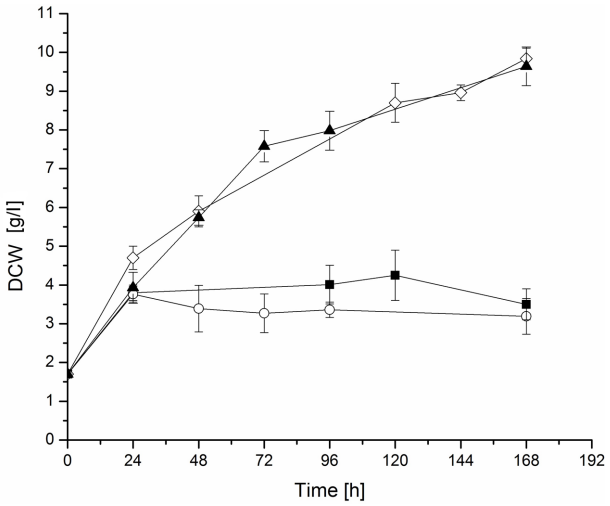

Fig. 3. Biomass growth in batch fermentation at two different initial pH levels.

Black square: biomass of hydrolysate at pH 5.5. White dot: biomass of hydrolysate at pH 6.6. Black triangle: biomass of hydrolysate at pH 5.5 in synthetic medium. White diamond: biomass of hydrolysate at pH 6.5 in synthetic medium. The concentrations of glucose and xylose in the hydrolysate were 90.1 g/l and 20.8 g/l, respectively. DCW:dry cell weight.

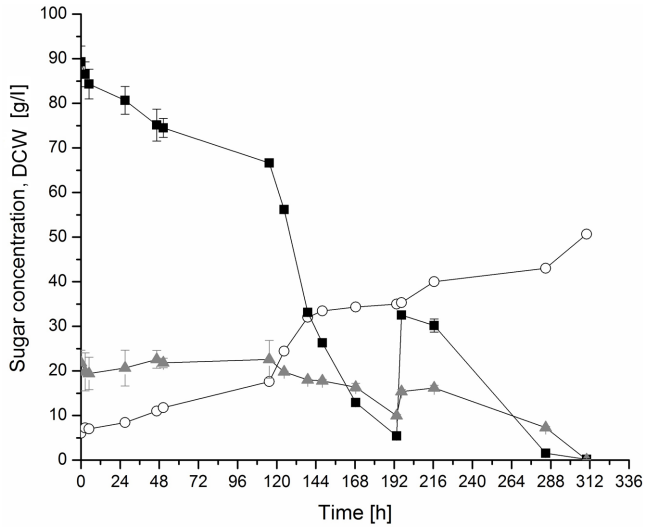

Fig. 4. Time course of glucose (black square), xylose (grey triangle) and dry cell weight (DCW, white dot) during *Arundo donax* hydrolysate fermentation.

Hydrolysate was supplied in fed-batch mode. DCW:dry cell weight.

in the fermentation medium of 1.0 g/l. Yu *et al*. investigated the effects of some biomass degradation by-products on the cell growth and lipid production by *C. curvatus* [49]. Low concentrations of furfural of around 1.0 g/l were found to have a strong inhibitory effect, reducing the dry cell weight and the lipid production respectively by 72.0% and 62.0%. At similar concentration of furfural (1.0 g/l), Chang et al. demonstrated that cell production and lipid yields were reduced by around 40.0% in corncob hydrolysates with respect to control systems [29]. Data indicate that, the specific growth rate of *C. curvatus* in the hydrolysates during the early 24 h was comparable to the synthetic media at both the investigated pH levels. On the other hand, *C. curvatus* in the biomass hydrolysates entered the stationary growth rate earlier than in the synthetic media. This also coincided with cessation of the glucose uptake (data not shown) implying an overall growth reduction of 70% with respect to the control trials. On the whole, these data indicated that the biomass degradation products in the hydrolysates did not affect the yeast growth in the pH range of 5.5-6.5 but that pH changes in these non-regulated trials could determine the late assimilation of biomass-derived compounds with an inhibitory effect on the process.

High inoculum concentrations could reduce the hydrolysate toxicity as an effect of the biological detoxification. A fed-batch fermentation was therefore set-up in order to increase the yeast-to-inhibitors ratio and reduce the toxic effect. A high inoculum concentration of 6 g/l was used accordingly. An additional advantage of the fed-batch mode exists in the possibility to adjust the C/N ratio by continuously replacing the consumed carbon source. The nitrogen and phosphorous concentrations as achieved through the experimental design were used to set up the nutrient levels for the fermentation of hydrolysates. The initial concentration of the $(NH_4)_2SO_4$ was set at 7 g/l taking into account the optimized nitrogen level calculated through the response surface equation (Eq. (2)), 0.42 g/l, proportionally increased to the current inoculum concentration of 6 g/l. This corresponded to an equivalent initial C/N ratio of 24 g/g. Fig. 4 reports the time-course of monosaccharides and cell biomass concentrations. Biomass growth was very rapid already in the early hours of the process. Glucose and xylose were almost completely consumed at 190 h of culture. Glucose was consumed first, and this agreed with the results reported by Yu *et al*. [28]. The xylose consumption started 120 h later in correspondence with an internal glucose-to-xylose ratio of around 3, suggesting a sequential utilization pattern, which was generally observed in most microorganisms. Yu and co-workers [28] tested several glucose : xylose mass ratios in the range of 1:1 to 1:4 and in all the tests *C. curvatus* preferentially consumed glucose. Nevertheless, in the sugar mixture containing glucose and xylose, xylose consumption greatly increased when glucose

Table 7. Sugar consumption, cell biomass and lipid production by *C. curvatus* cultured on *Arundo donax* hydrolysate in fed-batch mode. All data are reported with standard deviation achieved on three replicates.

Glucose consumption (g/l)	Xylose consumption (g/l)	DCW (g/l)	DCW yield[a] (g/g, %)	Lipid production (g/l)	Lipid content[b] (w, w %)	Lipid yield[c] (g/g, %)
116.2 ± 4.4	27.2 ± 3.6	44.7 ± 0.8	31.2 ± 4.4	28.4 ± 0.7	63.6 ± 2.8	19.8 ± 1.3

[a]Grams of dry cell biomass/gram of sugar consumed x 100%.

[b]Grams of lipids/gram of dry cell biomass × 100%.

[c]Grams of lipids/gram of sugar consumed × 100%.

remaining in the medium dropped to the concentrations ranging from 0.4 to 7.0 g/l corresponding to an internal ratio of 1:2. This agreed with the findings reported in the present paper. In fact, the major xylose consumption rate was observed between 168 and 190 h of the process, corresponding to a decrease of glucose concentration from 12 to 5 g/l with a glucose : xylose mass ratio of 1:2. New concentrated feed was added to the bioreactor at 190 h and the C/N ratio was adjusted at 300 g/g to maximize the lipid production. The fresh carbohydrates were exhausted after an additional 119 h. Table 7 summarizes the process performance. The overall biomass concentration and yield, and lipid yield were 31.2 g/l, 63.6% and 0.20 g/g of consumed sugars, respectively. In this process configuration, *C. curvatus* was able to achieve 63% of theoretical lipid yield. These values were slightly lower than those obtained in synthetic media (Table 3), namely 53.4 g/l, 65.2% and 0.348 g/g. The lipid yield (0.20 g/g) was two folds higher than that (0.12 g/g) obtained by Chang *et al*. in batch cultures on the corncob hydrolysates [29] and that (0.14 g/g) obtained by Liang *et al*. in batch cultures on the sweet sorghum bagasse hydrolysate [37]. Similar yield (0.17 g/g) but lower lipid production (5.8 g/l) were obtained by Yu et al. in batch cultures on wheat straw hydrolysate by using diluted feeds (24.3 g/l pentoses, 4.9 g/l hexoses) [14]. The lipid content obtained by *C. curvatus* on *A. donax* hydrolysate (63.6%) was comparable to the results (60%) obtained on corncob hydrolysate [29] and significantly higher than that (34%) obtained on wheat straw hydrolysate [14] and on sweet sorghum bagasse hydrolysate (34%) [37]. These differences are mainly due to the composition of lignocellulosic hydrolysates, in terms of inhibitor concentrations, but also to the strategy (batch/fed-batch) of the bioprocess. The selection of the fed-batch culture mode enabling a higher initial inoculum concentration, and the adjustment of C/N ratio during the fermentation yielded a higher process efficiency.

The process yield (0.20 g/g) and lipid content (63.6%) achieved in the present work from undetoxified biomass hydrolysates, even with margins for improvements, are close to the benchmark (0.25 g/g, 60%) and the improved case (0.27 g/g, 70%) recently used by Biddy *et al*. [2] for the techno-economic analysis of hydrocarbon fuels production from lignocellulosic biomass. The difference in the metabolic yield is reasonably due to the step of hydrolysates clarification included in the conversion pathway simulated by Biddy and co-workers. After transesterification, the lipid profile was analyzed by GC. The results are listed in Table 8.

The lipids synthesized by *C. curvatus* DSM 70022 were mainly composed of long-chain fatty acids with 16 and 18 carbon atoms with an internal distribution similar to vegetable fats [30]. The main fatty acids were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). On the whole, the lipid profile on *Arundo donax* hydrolysate appears quite similar to those obtained by *C. curvatus* on other kinds of lignocellulosic hydrolysate [14, 29]. These results indicate that hydrolysate composition does not affect significantly the fatty acid profile of *C. curvatus* while it could significantly affect the process yields and productivity.

In conclusion, this work demonstrated that *C. curvatus* DSM 70022 was able to produce lipids using undetoxified biomass hydrolysate from steam-pretreated *Arundo donax*.

After the process analysis in shaken flasks, fermentation of biomass hydrolysates was optimized in a bioreactor which enabled the control of oxygen and pH. A

Table 8. Fatty acid profile of lipid extracted from *C. curvatus* after the culture in bioreactor compared with the most similar vegetable oil, cocoa butter [30].

Fatty acid	Lipid composition by *C. curvatus* (w/w %)	Typical composition of vegetable oils (w/w %)
Palmitic acid (C16:0)	26.9	25.2
Stearic acid (C18:0)	8.2	35.5
Oleic acid (C18:1)	33.3	35.2
Linoleic acid (C18:2)	31.6	5.2
Linolenic acid (C18:3)	0.0	0.2

fermentation strategy ensuring a high cell density was successfully implemented in the bioreactor to reduce the hydrolysate toxicity. It enabled a low inhibitors to-yeast ratio without the need of diluting the substrate and consequently the product. Fresh medium was supplied in a fed-batch mode at 192 h to modulate the C/N ratio during the process, thus yielding a further increase of the biomass. On the whole, the process yielded a lipid content of 63.6 g lipids/100 g dry cell biomass corresponding to a lipid yield of about 20 g lipids/100 g monosaccharides.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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