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1 **Single cell oil production from undetoxified *Arundo donax* L. hydrolysate**
2 **by *Cutaneotrichosporon curvatus***

3

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17 **Single cell oil from undetoxified hydrolysates**

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18 **ABSTRACT**
19 The use of low-cost substrates represents one key issue to make single cell oil production
20 sustainable. Among low-input crops, *Arundo donax* is a perennial herbaceous rhizomatous
21 grass containing both C5 and C6 carbohydrates. The scope of the present work was to
22 investigate and optimize the production of lipids by the oleaginous yeast
23 *Cutaneotrichosporon curvatus* from undetoxified lignocellulosic hydrolysates of steam
24 pretreated *A. donax.* The growth of *C. curvatus* was firstly optimized in synthetic media,
25 similar in terms of sugar concentration to hydrolysates, by applying the response surface
26 methodology (RSM) analysis. Then the bioconversion of undetoxified hydrolysates was
27 investigated. A fed-batch process for the fermentation of *A. donax* hydrolysates was finally
28 implemented in a 2 L bioreactor. Under optimized conditions, the total lipid content was 64%
29 of the dry cell weight and the lipid yield was 63% of the theoretical. The fatty acids profile of
30 *C. curvatus* triglycerides contained 27% palmitic acid, 33% oleic acid and 32% linoleic acid.
31 These results proved the potential of lipids production from A. donax, which is particularly
32 important for their consideration as substitutes of vegetable oils in many applications such as
33 biodiesel or bioplastics.
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35
36 **Keywords:** *Arundo donax* L. hydrolysates; Single cell oil; Microbial lipids;
37 *Cutaneotrichosporon curvatus*; Second generation sugars
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40 **1. Introduction**
41
42 The use of biomass to produce not only bioenergy but a number of biobased products is the
43 key strategy to accelerate the global transition from a fossil-based economy toward the
44 bioeconomy. In particular, biodiesel can be produced from different sources such as vegetable
45 oils, animal fats, and wastes of cooking oils [1]. The recent demand for biodiesel worldwide
46 has turned triacylglycerol (TAG) into an ever-growing and substantial consumption resource
47 [2]. As a consequence, one main concern regards the use of oil crops for non-food
48 applications as it is expected to increase the price of these commodities.
49 Microbial lipids, also known as single cell oils (SCOs), can be produced from a number of
50 biobased intermediates or waste streams, such as, for instance, low cost sugars and biodiesel
51 derived glycerol. The fatty acids profile is similar to most plant oils and animal fats and this
52 could make them an alternative for replacing some conventional resources [3]. Besides
53 biofuels, their potential use concerns additional production sectors such as bioplastics and
54 food additives [4, 5].
55 Oleaginous microorganisms can convert biomass sugars to lipids up to more than 20% of
56 their dry mass. The lipogenesis process is triggered by specific culture conditions, namely
57 nitrogen [6], phosphate or sulphate limitations [7]. Lipids are stored as TAGs contained inside
58 vesicles defined as lipid bodies. Moreover, some species, such as *Cutaneotrichosporon*
59 *curvatus*, can utilize a number of carbon sources including hexoses and pentoses sugars and
60 their oligomers [8]. The overall economics of this bioprocess becomes favourable when zero
61 or low-cost substrates are utilized as carbon or nitrogen sources [9, 10].
62 Lignocellulosic biomass is a renewable feedstock for the production of biofuels and bio-
63 based products. It is an abundant resource [11] with no direct competition with the food
64 production chain. In particular, second generation sugars obtained from the lignocellulose
65 polysaccharides appear as a renewable raw material more sustainable with respect to the first-
66 generation sugars obtained from food crops [12]. In recent years, some high yields perennial
67 crops have been investigated as feedstocks for the production of second generation
68 carbohydrates. Among them, giant reed (*Arundo donax* L.), a perennial herbaceous crop,
69 could be a feedstock for lignocellulose-based biorefineries since it can adapt to a wide range
70 of climatic habitats producing high biomass yields [13].
71 The production of TAGs from lignocellulose-derived sugars depends on the composition
72 and quality of the hydrolysates [14]. The main constituents of lignocellulosic materials are
73 cellulose, hemicellulose and lignin. Cellulose is a linear polymer of glucose, hemicellulose is
74 a heteropolysaccharide composed mainly of pentoses (xylose, arabinose, etc.) and a few
75 hexoses (glucose, galactose, mannose, etc.), and lignin is an aromatic polymer mainly
76 composed of phenylpropane subunits. Pretreatment processes are necessary to disrupt the
77 plant cell wall complex, before the enzymatic hydrolysis of the polysaccharides. Depending
78 on the severity of the pretreatment, some by-products, such as furfural and 5-hydroxymethyl-
79 furfural (HMF), can be generated in this step from the degradation of pentoses and hexoses
80 respectively. These molecules have a known inhibitory action against the metabolic activity
81 of many microorganisms [15]. The pretreatment typically generates also short chain organic
82 acids, namely acetic, formic and levulinic acids [16, 17]. Furthermore, phenolic compounds,
83 such as syringaldehyde, p-hydroxybenzaldehyde (PHB), vanillin, etc. could be generated from
84 lignin [18]. All these compounds are usually inhibitory to microorganisms in the down-stream
85 fermentation step [19]. Tolerance to inhibitors appears to be strain dependent even if
86 optimized process strategies could contribute to alleviate the overall toxic effect.
87 The aim of the present work was to optimize the microbial co-fermentation of glucose and
88 xylose to lipids by *C. curvatus* grown on un-detoxified *Arundo donax* L. hydrolysates at high
89 concentrations.

2. Materials and methods

2.1 Culture preparation

2.1.1 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).

2.1.2 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-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 cells concentration, namely 1.7 g/L DCW for the batch tests and 6 g/L for the fed-batch cultures.

2.2 Fermentation tests

2.2.1 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, namely 40 g/L, 60 g/L 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 $MgSO_4 \cdot 7H_2O$ 0.4 g/L, $MnSO_4 \cdot H_2O$ 0.003 g/L, $CuSO_4 \cdot 5H_2O$ 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.

2.2.2 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: $MgSO_4 \cdot 7H_2O$ 0.4 g/L, KH_2PO_4 2 g/L, $MnSO_4 \cdot H_2O$ 0.003 g/L, $CuSO_4 \cdot 5H_2O$ 0.0001 g/L, $ZnSO_4 \cdot 7H_2O$ 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,

140 washed twice with distilled water and the dry weight was determined. All experiments were
141 conducted in triplicate.
142
143 *2.2.3 Design of Experiments (DOE) for improvement of medium composition*
144
145 Inhibition by biomass degradation products is often the main challenge in the production of
146 biochemicals through fermentation of second generations sugars. Bio-detoxification of toxic
147 products at high cells concentration could represent a viable option to achieve high process
148 yields. For this reason, the response surface methodology (RSM) was used to optimize the
149 biomass production in function of the nutrients concentration, namely yeast extract (X_1) as
150 nitrogen source, phosphate (KH_2PO_4) (X_2) and citric acid ($C_6H_8O_7$) (X_3) by using a glucose
151 concentration of 90 g/L which is close to lignocellulosic hydrolysates.
152 In particular, the Box-Behnken factorial design was applied to define a mathematical
153 model describing the single and synergic effects of the three independent variables in terms of
154 biomass production. The selected algorithm was implemented with three factors (X_1, X_2, X_3)
155 and three levels (-1, 0, +1), including three replicates at the center point. The factors
156 concentration ranges are reported in Table 1. On the whole, according to previous
157 experimental designs, 15 tests were selected to achieve statistically significant surface
158 responses [20-22].
159 The dry weight biomass production was selected as model response (Y) and correlated to the
160 independent variables through a second-degree polynomial equation whose general formula is:
161 $$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad \text{(Eq. 1)}$$
162 where β_0 represents the intercept, β_i the linear coefficient, β_{ij} the quadratic coefficient, β_{ii} is
163 the linear-by-linear interaction between x_i and x_j and x_i, x_j are input variables that influence
164 the response variable Y[17]. The robustness of the mathematical model (Eq. 1) was evaluated by
165 the coefficient of determination R^2. The model was tested by carrying out experiments in
166 shaking flasks with the predicted medium compositions at the same culture conditions. The
167 experiments were carried out in 150 mL-Erlenmeyer flasks containing 25 mL of culture
168 medium incubated at 30°C and pH 5.5.
169
170 *2.3 Feedstock and pretreatment*
171
172 Giant reed (*Arundo donax)* was grown and harvested in Northern Italy (province of
173 Vercelli) and delivered as a 200-300kg milled residues. The residues were divided in stocks
174 of roughly 30 kg and stored indoor. The dry-matter (DM) content of the raw material was
175 94%. Air dried biomass was analyzed for carbohydrates, lignin and ash content by the
176 following standard methods: carbohydrates and lignin by the Klason procedure (modified
177 TAPPI T-13 m-54 and ASTM D1106) and ash by ASTM D1102. The raw material contained
178 (%): 37.2 ± 0.9 glucan, 20.9 ± 1.2 xylan, 1.55 ± 0.11 arabinan, 0.48 ± 0.05 galactan, 21.8 ±
179 1.3 lignin, 7.84 ± 0.21 total ash, 3.5 ± 0.4 acetyl groups).
180 Biomass pretreatment was carried out by using the steam explosion (SE) batch technology
181 from Staketech (10 L). *Arundo donax* was pretreated at 200°C for 5 min according to process
182 conditions previously optimized [23]. Prior to the pretreatment, biomass was crumbled to
183 particles size in the range 1.7-5.6 mm. Acid catalyzed steam explosion pretreatment was
184 carried out by impregnating the *Arundo donax* with H_2SO_4 before feeding the batch digester.
185 *Arundo donax* was soaked in a dilute H_2SO_4 solution (0.07 M). After 10 min, impregnated
186 biomass was pressed to separate the solids from the solution. The resulting acid load was 1.5 %
187 (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.

2.4 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 enzymes feeding. The overall enzymes 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.

2.5 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, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, KH_2PO_4 2 g/L, $MnSO_4 \cdot H_2O$ 0.003 g/L, $CuSO_4 \cdot 5H_2O$ 0.0001 g/L, $ZnSO_4 \cdot 7H_2O$ 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.

2.6 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: $MgSO_4 \cdot 7H_2O$ 1.5 g/L, KH_2PO_4 2 g/L, NaH_2PO_4 0.3 g/L, $MnSO_4 \cdot H_2O$ 0.004 g/L, $CuSO_4 \cdot 5H_2O$ 0.001 g/L, $ZnSO_4 \cdot 7H_2O$ 0.04 g/L, and 0.8 mL of a solution containing vitamin B6 hydrochloride 1 g/L, 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 nutrients composition of the concentrated feed was the same as the initial medium with the only difference 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].

2.7 Analytical methods

2.7.1 Determination of monosaccharide concentrations

The carbohydrates analysis was performed with a HPIC (High Performance Ionic Chromatography) DIONEX ICS2500 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 RI101 refractive index. Each analysis was carried out in duplicate.

2.7.2 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.

2.7.3 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 the cells 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 vacuum at 40°C for roughly one hour, till constant weight. Finally, the total lipids were gravimetrically quantified [26]. The extraction method was verified by using certified reference material BCR® 163 - “Beef Pork fat blend”. Recovery yields were around 98%.

2.7.4 Fatty acids profile

The microbial lipids were directly transmethylated 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 acids methyl esters (FAMEs).

The FAMEs profiles were determined by GC analyses carried out on Agilent GC7890A gas chromatograph, equipped with HP-INNOVAX 19091N-213 capillary column (30 m x 0.32 mm x 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. Split ratio was 1:19 (*v/v*). Identification of methyl esters was performed comparing the peaks retention times to FAMEs standards (Sigma-Aldrich).

3. Results and discussion

3.1 Cell biomass and lipid production on glucose and xylose by C. curvatus

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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 any intermediated 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 unit in the 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 nutrients composition, some preliminary tests were performed in synthetic media by using ranges of carbohydrates 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 hours. After 144 hours the yeast growth reached the stationary phase and after that the fermentation was continued for 72 hours 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 lipids yield was slightly lower. The highest lipids 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]. Nutrients intake from yeast extract can contribute to slightly increase the lipid yield calculated on initial glucose. Furthermore, dry lipids extract could also include minor non-triglycerides 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 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, that are activated by certain process conditions [34, 35]. The optimal C/N ratio at the hydrolysate concentrations achieved in the present work was 85.7 g/g, corresponding to 100 mol/mol. It 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 the cell biomass production follows within the optimal range for the lipids production, namely 80-350 mol/mol [31]. This indicates that the optimized conditions for the biomass growth are close to the optimal range for the lipids production.

3.2 Optimization of the medium composition

In general, the C/N ratio that maximizes the biomass or lipids 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 the 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 acids synthesis [33]. Citrate was used as 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 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, the lipids biosynthesis is favoured 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 inability to grow on biomass hydrolysates. Therefore, in the present paper, the nutrients concentration was firstly optimized to maximize the 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 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. 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:

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

This response equation was used to generate the response surface plots (Fig. 2) and to optimize the biomass production. The predicted optimal conditions to maximize the biomass production of *C. curvatus* DSM 70022 up to17 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 the of 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.

3.3 Effects of the nitrogen source

Nutrients 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 the biomass growth and lipid accumulation [24]. It has been reported that the organic nitrogen improves both cell growth and lipid accumulation of *Cutaneotrichosporon curvatus*, while inorganic nitrogen enhances the biomass accumulation [30].

382 Besides the composition, the medium cost is an important constraint for the economic
383 feasibility of the processes. Thus, considering that the cost per kg of the yeast extract is
384 roughly 1.6-folds higher than the cost of $(NH_4)_2SO_4$, a potential alternative is the use of a
385 medium containing inorganic nitrogen sources along with trace concentrations of the most
386 effective vitamins. In the present investigation, three nutrients compositions, namely yeast
387 extract, ammonium sulphate and ammonium sulphate, added with vitamin B1 hydrochloride,
388 vitamin B6 hydrochloride, nicotinic acid and myoinositol, were evaluated. For all the tests,
389 glucose was used as the sole carbon source at the same C/N ratio. In these conditions, the
390 fermentation process with yeast extract achieved a biomass concentration 1.6-fold higher than
391 the sole ammonium sulphate as nitrogen source (Table 6). The addition of vitamins increased
392 the biomass production by about 35% with respect to the sole $(NH_4)_2SO_4$ (Table 6). On the
393 other side, ammonium sulphate, added with the four vitamins, has an overall cost 3-fold lower
394 respect to the cost of yeast extract. Vitamins have, in fact, a negligible cost and typically
395 account for 0.05% of total costs [40]. Based on these results, $(NH_4)_2SO_4$ plus vitamins was
396 selected as the favourite nutrients composition for the subsequent hydrolysates fermentation.
397
398 *3.4 Microbial conversion of Arundo donax hydrolysates*
399
400 The microbial production of lipids by using *C. curvatus* strains was mostly investigated by
401 using synthetic media [24, 26, 28, 30]. However, both the nature and the level of the main
402 biomass degradation products determine different process efficiencies.
403 In the present paper, *Arundo donax* was used to produce second generation sugars. *Arundo*
404 *donax* is a biomass belonging to perennial rhizomatous grasses and recently received much
405 attention thanks to an easy soil management and low demands of nutrients [41, 42]. Despite
406 the low production costs, the conversion of the *Arundo donax* biomass to sugars presents
407 some difficulties mainly due to the recalcitrant nature of lignin. This could require severe
408 pretreatment conditions with the effect of generating degradation by-products with
409 antimicrobial action. *Arundo donax* hydrolysates have been tested for the production of
410 second generation bioethanol [13, 43], methane [44, 45] and a number of bio-based products
411 [46] including single cell oils (SCOs) [47]. To the best of our knowledge, there are not studies
412 on the bioconversion of *Arundo donax* carbohydrates into lipids by the oleaginous yeast *C.*
413 *curvatus*.
414 As reported in Table 2 the most abundant degradation product from acid-catalysed steam
415 pretreatment of the biomass was acetic acid. In general, the microbial toxicity of the short
416 chain organic acids at pH lower than 5 is due to the permeation of the undissociated form
417 across the phospholipid bilayer, followed by its dissociation and acidification of the cytosol.
418 This influences the normal biochemical processes in the cell [15]. Previous research
419 investigations reported that potassium acetate could even be a carbon source for *C. curvatus*
420 at pH 7 in batch mode with the production of 0.63g/L DCW at 72nd h [48]. In the present
421 investigation, the acetic acid concentration remained constant through the process (detailed
422 data not shown). This could be due to the presence of relatively higher concentrations of
423 glucose and xylose that represented the preferred carbon sources. In order to explore the effect
424 of the pH on the biomass growth rate, two different initial pH set-ups, namely 5.5 and 6.5,
425 were tested. Fig. 3 shows the results of these tests. The data indicated that growth stopped at
426 the lower pH after 24 h, but especially in hydrolysate the DCW was on the same level after 24
427 h, meaning that the specific growth rate was not different, but that the entry into stationary
428 phase happened earlier. This indicate that changed in cultures grown in non-regulated pH
429 occurred for instance because of the secretion of acids like citrate, N-transport into the cell
430 (proton antiport) or assimilation of additional acids different from acetic acid, with an overall
431 effect of interrupting the yeast growth.

The investigation of the yeast growth in synthetic media enabled the selection of the 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 performances. Besides acetic acid, *Arundo donax* hydrolysates contained both 5-HMF and 2-furaldehyde reaching a total concentration 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 lipids 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 lipids 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 hours was comparable to the synthetic media at both the investigated pH. On the other side, *C. curvatus* in the biomass hydrolysates entered the stationary growth rate earlier than in the synthetic media. This also coincided with the stop 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 hydrolysates toxicity as 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, 6 g/L, was used accordingly. An additional advantage of the fed-batch mode consists 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 nutrients 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. The biomass growth was very rapid already in the early process hours. Glucose and xylose were almost completely consumed in 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 hours 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 1:1 to 1:4 and in all the tests *C. curvatus* preferentially consumed glucose. Nevertheless, in the sugars mixture containing glucose and xylose, xylose consumption greatly increased when glucose 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 hours of process, corresponding to a decrease of glucose concentration from 12 g/L 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 lipids production. The fresh carbohydrates were exhausted after additional 119 h. Table 7 summarizes the process performances. 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 lipids 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 inhibitors concentration, 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 if have still margins for improvements, are close to the benchmark (0.25 g/g, 60%) and 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 the step of hydrolysates clarification included in the conversion pathway simulated by Biddy and co-workers. After transesterification, the lipids profile was analysed 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 lipids profile on *Arundo donax* hydrolysate appears quite similar to those obtained by *C. curvatus* on other kinds of lignocellulosic hydrolysate [29, 14]. These results indicate that the hydrolysates composition does not affect significantly the fatty acids 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 bioreactor which enabled the control of oxygen and pH. A fermentation strategy ensuring a high cell density was successfully implemented in 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 hours 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 was 63.6 g lipids/100 g dry cells biomass corresponding to a lipid yield of about 20 g lipids/100 g monosaccharides.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

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

Fig. 2. **A** Response surface of the 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.

Fig. 3. Biomass growth in batch fermentation at two different initial pH. Black square: pH 5.5 in biomass hydrolysate. White dot: pH 6.5 in biomass hydrolysate. Black triangle: pH 5.5 in synthetic medium. White diamond: pH 6.5 in synthetic medium. The concentration of glucose and xylose in the hydrolysate were 90.1 g/L and 20.8 g/L, respectively.

Fig. 4. Time course of glucose (black square), xylose (grey triangle) and dry cells weight (DCW, white dot) during *Arundo donax* hydrolysate fermentation. Hydrolysate was supplied in fed-batch mode.

Tables

Table 1
Factors and levels of the Box-Behnken design. All samples composition is 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

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

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

698 [a] Gram of dry cell biomass/gram of sugar consumed × 100%.
699 [b] Gram of lipids/gram of dry cell biomass × 100%.
700 [c] Gram of lipids/gram of sugar consumed × 100%.
701 [d] Sugar consumption rate (6 days).
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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

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719 **Table 5**
720 Analysis of variance for the obtained mathematical model.
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Coefficients		*P value*	Standard error	ANOVA	*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							

722 *df*, degree of freedom; *SS*, Sum of Squares; *MS*, Mean Square; *F*, Fisher coefficient; *LF*, Lack of Fit.
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Table 6
739 Growth of *Cutaneotrichosporon curvatus* in media containing different nitrogen sources and the same C:N ratio. Dry weight biomass data are
740 reported with standard deviation achieved on three replicates.
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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

742 *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
743 to the media.
744
745
Table 7
746 Sugar consumption, cell biomass and lipid production by *C. curvatus* cultured on *Arundo donax* hydrolysate in fed-batch mode. All data are
747 reported with standard deviation achieved on three replicates.
748

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

749 [a] Gram of dry cell biomass/gram of sugar consumed x 100%.
750 [b] Gram of lipids/gram of dry cell biomass x 100%.
751 [c] Gram of lipids/gram of sugar consumed x 100%.
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758 **Table 8**
759 Fatty acid profile of lipid extracted from *C. curvatus* after the culture in bioreactor compared with the most similar vegetable oil, namely Cocoa-
760 butter [30].
761

Fatty acid	Lipids 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

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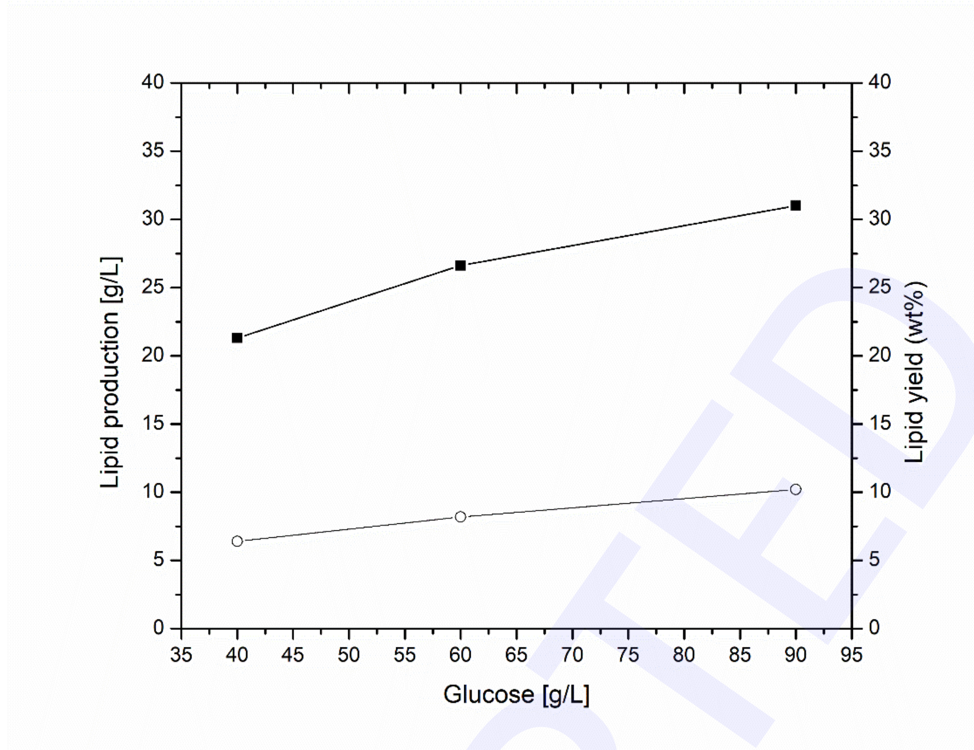
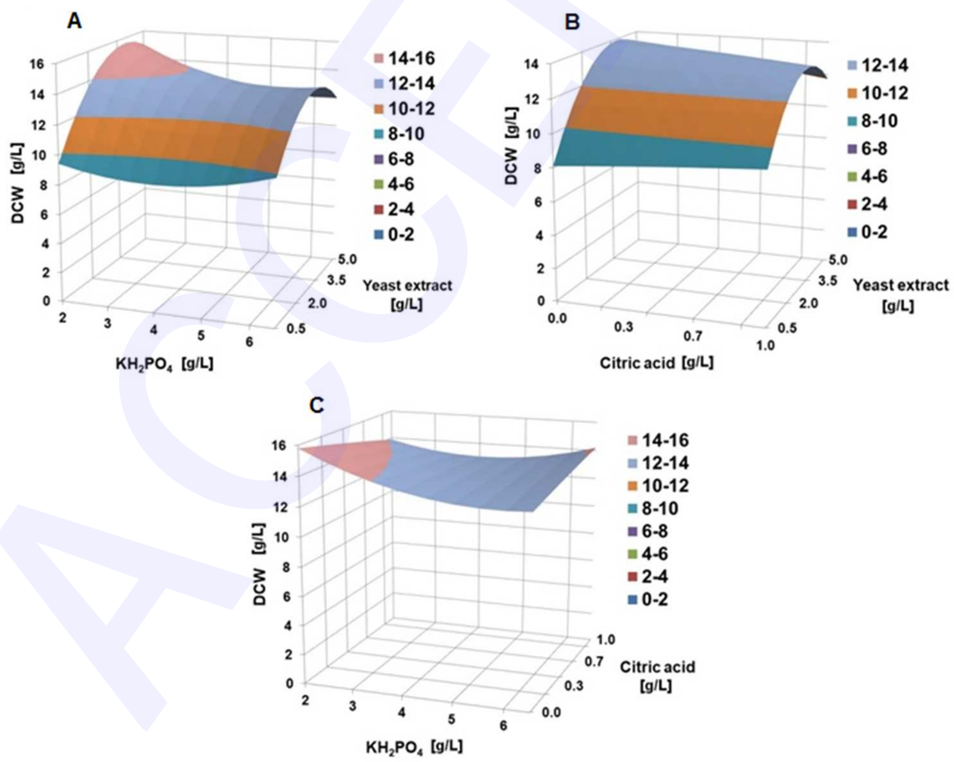
1 **Figures**



2

3 Figure 1

4



5

6 Figure 2

7

ACCEPTED

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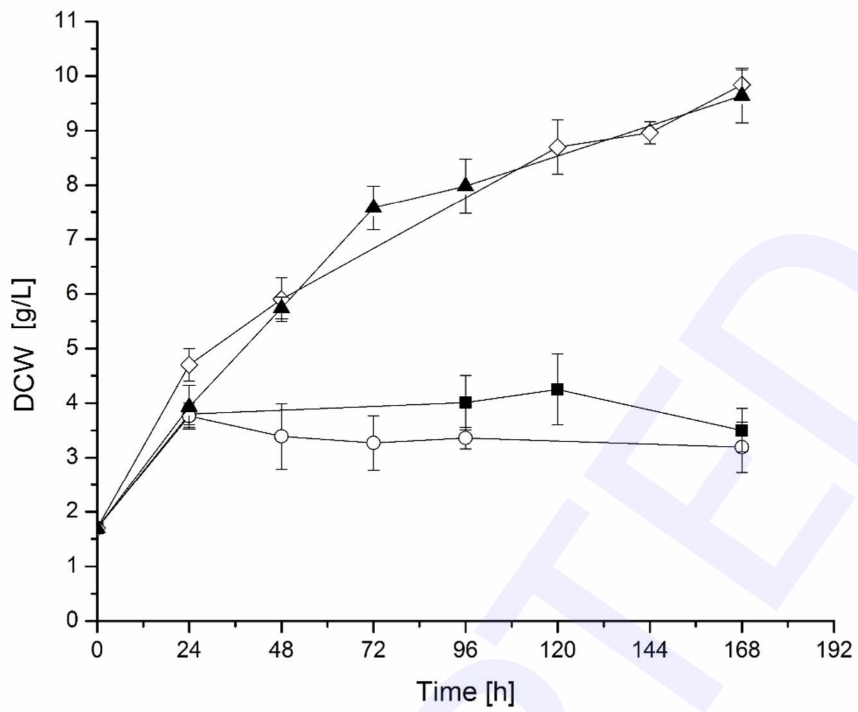
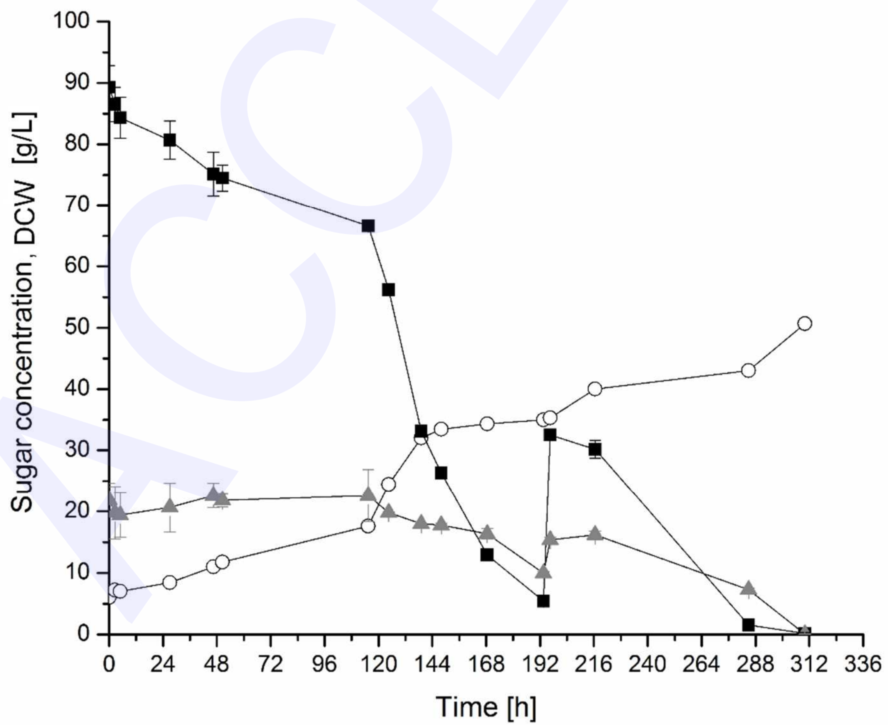


9 Figure 3

10



11

12 Figure 4