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Exploitation of Oleaginous Yeasts for the Production of Microbial Oils from Agricultural Biomass

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Lignocellulosic wastes from *Arundo donax* (AR) and *Sorghum bicolor* (SB) were used as a source of fermentable sugars for culturing the oleaginous yeasts *Lipomyces starkey*. In order to minimize the generation of inhibitors of the microbial growth, that is a serious bottleneck still limiting the industrial production of microbial oils, the lignocellulosic materials were first steam-exploded and subsequently treated with commercial preparations of cellulases and β-glucosidases.
This treatment made possible the growth of yeasts in the presence of raw hydrolysates, thus improving the production of microbial oils from oleaginous yeasts, potentially allowing a sustainable production of II-generation biodiesel.
The conditions of the hydrolysis of the AD and SB, as well as the conditions of the fermentation of the hydrolysates, were optimized in order to maximize both the microbial biomass yield and the lipid fraction of the biomass. The composition of the triglycerides obtained adopting such conditions were compatible with the production of a good-quality automotive biodiesel.

1. Introduction

Microbial oils are attracting increasing interest, as they offer a renewable and cheap feedstock for the production of bioplastics, biodiesel and other products. They can be obtained from oleaginous microorganisms, that are able to produce more than 20 % of their biomass as triglycerides (Li et al, 2008).Among the oleaginous microorganisms, yeasts are considered for their very simple cultural requirements, as they grow under aerobic conditions, only needing a higher C/N ratio (>30) to enable the triglyceride accumulation within their cells (Papanikolaou and Aggelis, 2011a-b). Several oleaginous yeasts can metabolize different carbon sources (Amaretti et al., 2012), under different operating conditions (Amaretti et al., 2010). They be grown in the presence of hydrolysates of lignocellulosic materials, as they are able to metabolize both hexose and pentose sugars (Hu et al, 2011). A possible bottleneck for this process is due to inhibitors of the microbial growth produced during the lignocelluloses hydrolysis (Yu et al., 2011; Huang et al., 2012), that may produce synergical effects (Zhao et al., 2012). These effect can be reduced by adopting fed-batch reactors (Li et al, 2007; Zhang et al., 2011).
In this study, hydrolysates of AD and SB were used as a source of fermentable sugars to grow, with no addition of organic supplements, the oleaginous yeast *Lipomyces starkeyi*. An efficient technology for processing lignocellulosic biomasses could open new perspectives as regards the biofuel production. As a matter of facts, a large range of waste biomasses can be recycled, such as non-food parts of crops (stems, leaves and husks), forest products, and also industry wastes (woodchips, skin and pulp from fruit pressing, etc.). In addition, suitable non-food crops (switchgrass, jatropha, miscantus, etc.) can be cultivated in partially-fertile soils, not used for agriculture, to obtain both vegetable oil (to produce biodiesel according to the traditional method) and lignocellulosic biomasses for biofuel production. Also, since

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cellulose and hemicelluloses are the main component of plants, the yield of feedstock biomasses per unit area is significantly increased until 40 t d.m. ha^{-1} (Angelini et al., 2009).
The experimental activity was aimed at overcoming the problems still limiting the industrial application of the process. In this view, the hydrolysis of AD and SB was carried out by steam explosion and subsequent enzymatic treatment, based on the simultaneous action of cellulases and cellobiases, in order to achieve a lower concentration of inhibitors of the cellular growth (furfural, acetic acid, etc.), that are usually produced by the degradation of sugars.
The composition of microbial oils was characterized as a function of the conditions adopted in the whole process. In all instances, the oils were prevalently made of triglycerides, containing a fraction of oleic oil residuals close to 50 %. This composition allows the production of bioplastics, as well as a biodiesel offering excellent oxidation stability and cold performance.

2. Materials and methods

2.1 Microorganisms and culture media

The oleaginous yeasts *Lipomyces starkeyi* were obtained by the collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The microorganisms were kept on potato dextrose agar (Sigma) at T = 5 °C and cultivated in a synthetic N-limiting medium, containing (g/L): KH_2PO_4 , 1.0; $MgSO_4$ $7H_2O$, 0.5; $(NH_4)_2SO_4$, 2.0; yeast extract, 0.5; glucose, 70.0. The growth was carried out under aerobic conditions at 30 °C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland).

2.2 Lignocellulosic biomasses

Arundo donax (AD) and *Sorghum bicolor* (SB) were grown in open field condition in the Torre Lama experimental station, University of Napoli, (40°37'N, 14°58'E, 30 m a.s.l.). The soil texture was Silty-Clay and poor in organic matter, with higher carbonate content.

2.3 Enzymatic hydrolysis

The enzymatic hydrolysis was carried out using 100 mL of 2.5 %, 5 %, or 10 % (w/v) suspensions of pre-treated *Arundo donax* biomass in phosphate buffer (50 mM, pH 5) at 50 °C and 150 rpm. The treatment was conducted using commercial preparations of cellulase (Celluclast 1.5L, Novozymes, Bagsvaerd, Denmark), and β-glucosidase (Novozymes 188, Bagsvaerd, Denmark). The enzyme loading per gram of cellulose were 15 FPU and 30 CBU, respectively. A typical hydrolysis time was 72 h.
The activities of Cellulase and β-glucosidase were determined as 60 FPU/mL and 360 CBU/mL, respectively, according to a standard procedure (Ghose, 1987).

2.4 Fermentation with steam-exploded Arundo donax

The fermentation tests were carried out using 150 mL of the sugar mixture obtained by enzymatic hydrolysis. in conical flask of 500 mL. The liquid medium was inoculated by 2 % v/v of pre-adapted yeasts. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and T = 30 ± 1 °C for 96 h.

2.5 Analytical methods

The biomass concentration in the culture medium was measured by OD determination at 600 nm. Glucose was measured in using an enzymatic kit (Sigma Aldrich). Reducing sugars and xilose were measured with Nelson-Somogyi (Sadavarim et al., 1996) and fluoroglucinol (Douglas, 1981) colorimetric assays, respectively. UV spectra were recorded on a SHIMAZU-UV1601 spectrophotometer using 1 cm cells.
The total organic carbon (TOC) was measured by a TOC-$V_{CSH/CSN}$ (Shimadzu Japan) upon suitable diluition of a culture medium sample. The TOC was obtained by adding the NPOC (Not Purgable Organic Carbon) to POC (Purgable Organic Carbon) value.
Dried samples containing oleaginous biomass were subject to lipids extraction. Methanol (5.0mL) and chloroform (2.5 mL) were added to 200 mg of dry biomass and vortexed 5 sec. Subsequently the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, USA) at 50 % power and 90 % pulser. The cells were then filtered off with Whatman no.1 filter paper and the solvent-lipid mixture was placed in a 50 mL centrifuge tube. The layers were separated by centrifugation for 10 min at 2000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich, USA) at 20 °C. The lower layer was then transferred to a pear-shape flask with Pasteur pipette. Again, 10mL of 10 % (v/v) methanol in chloroform were added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pear-shape flask was evaporated to dryness (BÜCHI Rotavapor R-200, Switzerland) and the extracted weight was finally recorded after drying at 105 °C for 1 h.
The concentration of volatile acids (acetic acid, butyric acid) and ethanol was determined by GC analysis, using a Shimadzu GC-17A equipped with a FID detector and a capillary column with a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 μm film thickness, from SGE). 1 μL samples were injected with a

split-ratio of 1:10. Helium was fed as carrier gas with a flow rate of 6.5 mL/min. Injector and detector temperatures have been set to 320 C and 250 C, respectively. Initial column temperature has been set to 30 C, kept for 3 min, followed by a ramp of 10 °C/min till 140 C, kept for 1 min.

3. Results and discussion

3.1 Hydrolysis of lignocellulosic biomass

The data shown in the Figure 1 describe the enzymatic hydrolysis of AD biomass preliminary treated by steam explosion. All the profiles grows with the treatment time, though the slopes of all the experimental curves are decreasing, suggesting the achievement of an horizontal asymptote. In all cases, the final concentrations of sugars increase as higher values of the steam-exploded biomass concentration are adopted.

The results demonstrate that the concentration of hexose sugars is much higher as compared to pentoses. This can be explained considering that, during the steam explosion, the hemicelluloses is partially hydrolysed and lost. As a matter of facts, the initial value of the concentration-time profiles of pentoses is significantly higher than zero, whereas the initial concentration of glucose is zero.

Preliminarily steam-exploded SB was treated by enzymatic hydrolysis, as well, adopting the same treatnebt conditions. In this case, qualitatively similar results were observed, as shown in the Figure 2. When using SB, the concentration-time profiles of total reducing sugars and glucose indicated the achievement of lower values of the final concentrations. On the contrary the yield in pentose sugars was higher.

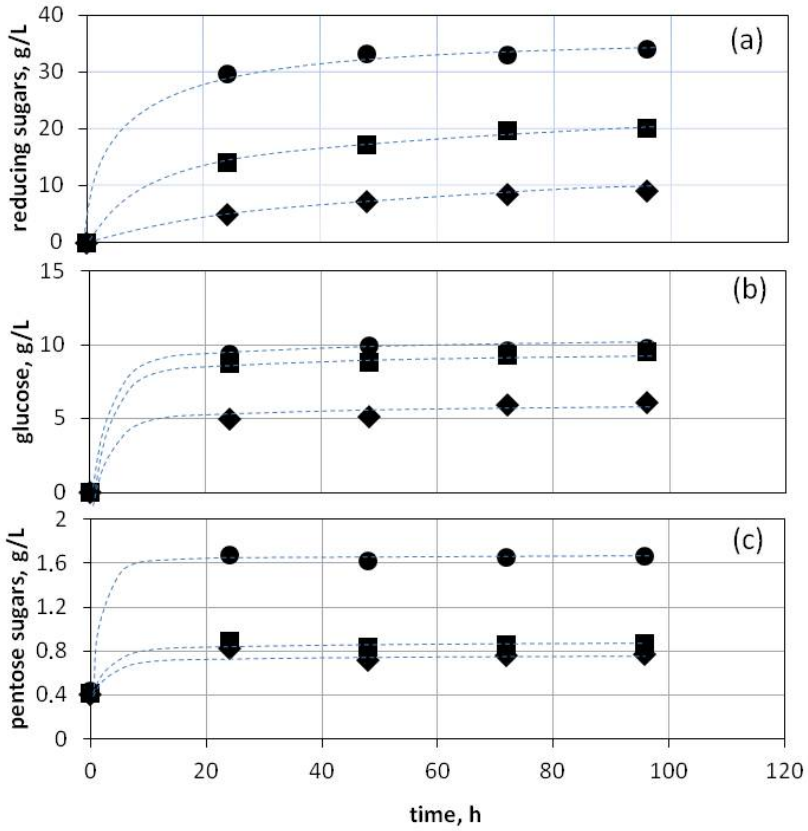

Figure 1: Enzymatic hydrolysis of A. donax (AD) biomass pretreated by steam explosion. T=50 °C, pH=5.0, 150 rpm. Concentration-time profiles of reducing sugars (a), glucose (b) and pentoses (c). Concentrations of the biomass: 2.5 % (♦), 5 % (■), 10 % (●)

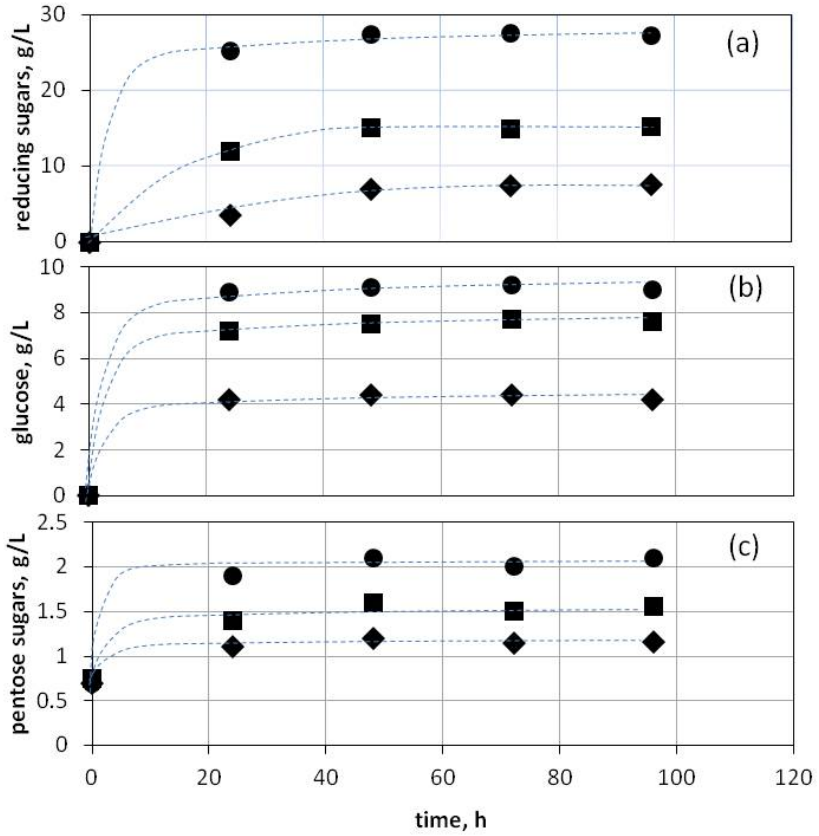

Figure 2: Enzymatic hydrolysis of S. bicolor (SB) biomass pretreated by steam explosion. T=50 °C, pH=5.0, 150 rpm. Concentration-time profiles of reducing sugars (a), glucose (b) and pentoses (c). Concentrations of the biomass: 2.5 % (♦), 5 % (■), 10 % (●)

3.2 Growth of oleaginous yeasts

After the enzymatic treatment, all the sugar mixtures were evaluated as regards the growth of oleaginous biomass and the fraction of triglycerides, adopting a growth period of 96 h. The results shown in the Table 1 indicate that, in all cases, the oleaginous yeasts were able to grow in the presence of raw hydrolysates. This is a satisfactory result, as it has been found (data not shown) that, when using raw hydrolysates obtained by other methods (i.e. acid hydrolysis), the growth is extremely slow or completely absent.

Table 1: Biomass and lipid concentrations obtained after a 96 h fermentation of hydrolysates. Conditions of enzymatic treatment: T= 30 °C, 150 rpm, pH = 5.0

Test	Conditions of enzymatic hydrolysis		Results of the oleaginous fermentation		
	Lignocellulosic waste	Conc. of heat-expl. biomass., %	Oleaginous biomass concentration, g/L	Lipid fraction. %	Lipid conc., g/L
AD1	Arundo donax	2.5	6.25	21.0	131
AD2	Arundo donax	5	7.44	20.2	150
AD3	Arundo donax	10	2.12	20.5	43.5
SB1	Sorghum bicolor	2.5	5.44	17.6	95.7
SB2	Sorghum bicolor	5	5.21	16.4	85.4
SB3	Sorghum bicolor	10	1.22	17.1	20.9

The improvement offered by the use of the steam explosion and the enzyme treatment is likely due to the reduced generation of inhibitors of the microbial growth. This is indirectly shown by the poorer results obtained when carrying out the enzymatic hydrolysis with 10 % heat-exploded biomass (tests AD3 and SB3 in Table 1). Obviously, these results can be explained observing that higher amounts of exploded

biomass lead to higher concentrations of inhibitors. The highest values of lipid concentration were obtained when carrying out the enzyme treatment in the presence of 5 % (test AD2 in Table 1) and 2.5 % (test SB1 in Table 1) steam-exploded biomass.

3.3 Composition of triglycerides

Consequently, the triglycerides obtained from tests AD2 and SB1 (Table 1) after a fermentation period of 96 h, were evaluated as regards their fatty-acid distribution. The results, reported in the Table 2, indicate that triglycerides from AD possess an higher fraction of oleic acid. It is worth noting that, if the triglycerides are to be used for the synthesis of automotive biodiesel, an higher fraction of insaturated fatty acids corresponds to a reduced oxidative stability, though a better cold-performance can be obtained.

Table 2: Composition (%) of the samples of lipids AD2 and SB1 (see Table 1, obtained after a 96 h fermentation of hydrolysates. Conditions of enzymatic treatment: T= 30 °C, 150 rpm, pH = 5.0

Fatty acid	AD2	SB1
Palmitic acid (C16:0)	22.1	23.4
Stearic acid (C18:0)	16.4	14.1
Oleic acid (C18:1)	47.0	44.2
Linoleic acid (C18:2)	6.2	6.5

4. Conclusions

The treatment of AD and SB by steam-explosion and enzymatic hydrolysis improved the production of microbial oils from oleaginous yeasts, potentially allowing a sustainable synthesis of II-generation biodiesel. In particular, this treatment made possible the growth of yeasts in the presence of raw hydrolysates, partially overcoming the bottlenecks caused by the generation of cellular growth inhibitors in the course of the hydrolysis of the lignocellulosic biomasses.
The optimal process conditions were found, as regards the triglyceride production. Under these conditions, it was possible to produce a good-quality automotive biodiesel.

References

Amaretti A., Raimondi S., Sala M., Roncaglia L., De Lucia M., Leonardi A., Rossi, M., 2010, Production of single-cell oils by the cold-adapted oleaginous yeast Rhodotorula glacialis AS 4.7: effects of the growth temperature and the C:N ratio. 20, 109-114.

Amaretti A., Raimondi S., Leonardi A., Rossi, M., 2012, Candida Freyschussii: an Oleaginous Yeast Producing Lipids from Glycerol. Chemical Engineering Transactions, 27, 139-144.

Angelini L.G., Ceccarini L., Nassi O., Di Nasso N., Bonari E., 2009, Comparison of Arundo donax L. and Miscanthus x giganteus in a long-term field experiment in Central Italy: Analysis of productive characteristics and energy balance. Biomass and Bioenergy, 33, 635-643.

Douglas S.G., 1981. A rapid method for the determination of pentosans in wheat flour, Food Chemistry, 7, 139-145.

Ghose T.K., 1987. Measurement of cellulase activities. Pure Applied Chemist, 59(2):257–268.

Hu C., Wu S., Wang Q., Jin G., Shen H., Zhao Z.K., 2011, Simultaneous utilization of glucose and xylose for lipid production by Trichosporon cutaneum, Biotechnology for Biofuels, 4, 25.

Huang C., Wu H., Li R.F., Zong M.H., 2012, Improving lipid production from bagasse hydrolysate with Trichosporon fermentans by response surface methodology, New Biotechnology, 29(3), 372-278.

Li Q., Du W., Liu D., 2008, Perspectives of microbial oils for biodiesel production, Appl. Microbiol. Biotechnol, 80, 749–756.

Li Y.H., Zhao Z.B., Bai F.W., 2007, High-density cultivation of oleaginous yeasts Rhodosporidium toruloides Y4 in fed-batch culture. Enzyme Microb. Technol., 41, 312-317.

Papanikolaou S., Aggelis G., 2011a, Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production, Eur. J. Lipid Sci. Technol., 113, 1031-1051.

Papanikolaou S., Aggelis, G., 2011b Lipids of oleaginous yeasts. Part II: Technology and potential applications, Eur. J. Lipid Sci. Technol., 113, 1052–1073.

Sadasivam S., Manickam, A., 1996 Carbohydrates. In: Sadasivam S., Manickam A, eds., Methods in Biochemistry, New Age International Pvt. Ltd., 11-12..

Zhao X., Peng F., Du W., Liu C., Liu D., 2012, Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid, Bioprocess Biosyst. Eng., 35(6), 993-1004.

Zhang J., Fang X., Zhu X.L., Li Y., Xu H.P., Zhao B.F., Chen L., Zhang X.D., 2011, Microbial lipid production by the oleaginous yeast Cryptococcus curvatus O3 grown in fed-batch culture, J. Agric. Food Chem., 59, 4606–4613.

Yu X.C., Zheng Y.B., Dorgan K.M., Chen S., 2011, Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid, Bioresource Technology, 102, 6134–6140.