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Study of a Discontinuous Fed-Batch Fermentor for the Exploitation of Agricultural Biomasses to Produce II-Generation Biodiesel

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A discontinuous fed-batch fermenter was used to grow the oleaginous yeast *Lipomyces starkey* in the presence of hydrolysates of lignocellulosic wastes from *Arundo donax* (AD). The lignocellulosic materials were first steam-exploded and subsequently treated with commercial preparations of cellulases and β-glucosidases, to obtain a mixture of fermentable sugar.
The discontinuous fed-batch fermenter was adopted to maintain a lower concentration of the nitrogen sources, and consequently an higher C/N ratio, thus promoting the accumulation of triglycerides within the yeasts' cells. Its use increased significantly the final concentration of biomass in comparison to that observed when using a batch fermenter.
The composition of the biodiesel produced was compatible with a satisfactory performance as automotive fuel, in terms of both the resistance to oxidation and the cold performance.

1. Introduction

Oleaginous microorganisms, that are able to produce more than 20% of their biomass as triglycerides (Hu et al., 2011; Yu et al., 2011; Huang et al., 2012; Zhao et al., 2012), are attracting increasing interest. As a matter of facts, the microbial oils that can be produced by these microoganisms offer a renewable and cheap feedstock for the production of bioplastics, biodiesel and other products (Yousuf et al., 2010; Pirozzi et al., 2014). Among the oleaginous microorganisms, yeasts offer very simple cultural requirements, as they grow under aerobic conditions, and only require a carbon-to-nitrogen ratio C/N > 30 to enable the triglyceride accumulation within their cells (Li et al, 2007; Papanikolaou and Aggelis, 2011a; Papanikolaou and Aggelis, 2011b). In addition, the microbial oils obtained from yeasts have a composition quite similar to that of vegetable oils (Papanikolaou and Aggelis, 2011a).
In this study, Lipomyces starkeyi were selected for the subsequent tests, as they have been proved to store large amounts of lipids, showing only a minimal reutilisation of the stored lipids (Holdsworth et al., 1988). A discontinuous fed-batch fermenter was used to control the concentration of the nitrogen sources, thus carrying out the growth of the oleaginous yeasts at an higher C/N ratio, and consequently promoting the accumulation of triglycerides within the yeasts' cells.
Hydrolysates of *Arundo donax* (AD) were used as a source of fermentable sugars to grow the oleaginous yeast *Lipomyces starkeyi* with no addition of organic supplements. The development of an efficient process to obtain II-generation biofuels from lignocellulosic biomasses is of great interest (Zhang et al., 2011; Pirozzi et al., 2013; Toscano et al., 2013), as 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

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biofuel production. In addition, the yield of feedstock biomasses per unit area is increased until 40 t d.m. ha^{-1} (Angelini et al., 2009), as cellulose and hemicelluloses, that are the main component of plants, can be both hydrolysed to obtain mixtures of fermentable sugars.
The composition of microbial oils produced was characterized, to ensure the production of a biodiesel offering excellent performances as automotive fuel.

2. Materials and methods

2.1 Microorganisms and culture in synthetic medium

The oleaginous yeasts *Lipomyces starkeyi* were obtained from 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). The desired values of C/N ratio were achieved by addition of $(NH_4)_3SO_4$ salt.

2.2 Hydrolysates of lignocellulosic biomass

Arundo donax (AD) was 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. The AD biomass was processed by steam explosion , followed by 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 hours.
The activities of cellulase and β-glucosidase were determined as 60 FPU/mL and 360 CBU/mL, respectively, according to a standard procedure (Adney et Baker, 1996; Ghose, 1987).

2.3 Fermentation of oleaginous yeasts

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 hours.

2.4 Analytical methods

The biomass concentration in the culture medium was measured by OD determination at 600 nm.
Dried samples containing oleaginous biomass were subject to lipids extraction. Methanol (5.0mL) and chloroform (2.5mL) 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 Nitrogen multiple additions tests

A first series of experimental tests was carried out growing *Lipomyces starkeyi* in a synthetic medium (described in the Method paragraph). As oleaginous yeasts accumulate lipids as storage materials under N-limiting conditions, a C/N ratio = 58 was adopted.

In order to increase the biomass yield obtained under N-limiting conditions, *Lipomyces starkeyi* were grown under multiple additions of the nitrogen source, restoring the initial concentration of $(NH_4)_2SO_4$ once a stationary phase was established. The Fig. 1 shows the growth profile of *Lipomyces starkeyi* under multiple additions of the nitrogen source.

The data in Fig. 1 show that, after each addition of $(NH_4)_2SO_4$, a new exponential phase started. This result demonstrates that the stationary phases are reached due to the nitrogen depletion. Also, this shows that the addition of a reduced amount of $(NH_4)_2SO_4$ can significantly increase the yield in oleaginous biomass.

After a first stationary phase was observed, corresponding to a biomass concentration of about 5.1 g/L (sample A in Fig. 1 and Tab. 1), a new addition of the salt produced a second growth-cycle, increasing the yeasts concentration to about 7.2 g/L (sample B in Fig. 1 and Tab. 1). Subsequently, a second addition of salt yielded a new growth cycle, until a biomass concentration of 8.2 g/L was reached (sample C in Fig. 1 and Tab. 1). Then, a third addition of $(NH_4)_3SO_4$ increased the biomass concentration to 8.8 g/L (sample D in Fig. 1 and Tab. 1).

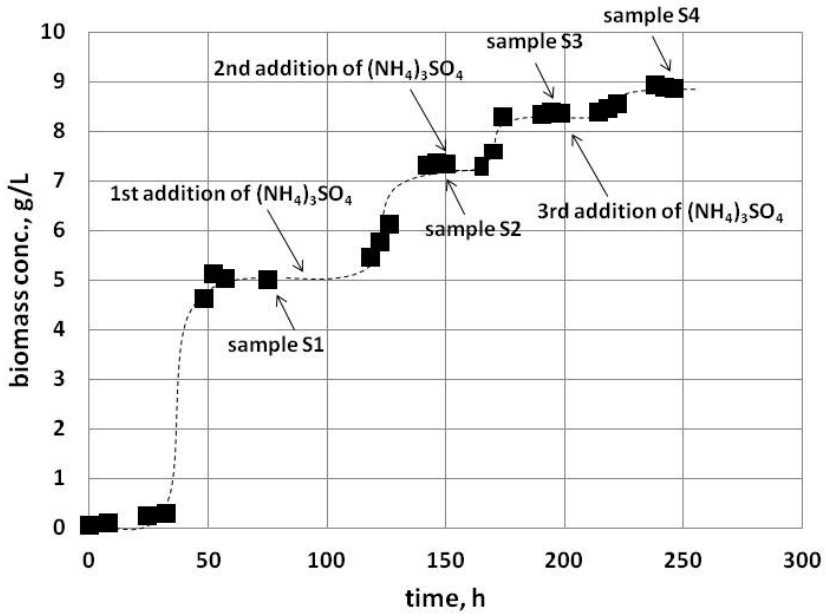

Figure 1: Growth of Lipomyces starkeyi in the presence of a N-limiting synthetic medium in batch reactors, under multiple additions of $(NH_4)_2SO_4$. Operating conditions: T = 30°C, 160 rpm. Medium composition as in the Method paragraph.

The samples (S1-S2-S3-S4) collected after each growth cycle were evaluated as regards the concentration of biomass and triglycerides, as reported in the Tab. 1. The results indicate that the lipid fraction within the oleaginous biomass increased as new growth cycles were carried out. This result confirms that nitrogen-limiting conditions enabled the accumulation of triglycerides in the biomass of the oleaginous yeasts.

On the other hand, the data in Fig. and Tab. 1 show that, when only nitrogen addition were carried out with no carbon supplement, the increases in the biomass concentration observed after each growth cycle were progressively reduced.

Table 1: Biomass and lipid concentrations obtained during the fermentation in the presence of synthetic medium under multiple additions of $(NH_4)_2SO_4$. Operating conditions: T = 30°C, 160 rpm. Medium composition as in the Method paragraph.

Sample	Oleaginous biomass concentration, g/L	Lipid fraction, %	Lipid conc., g/L
S1	5.1	16.9	86.2
S2	7.2	17.3	125
S3	8.2	19.2	157
S4	8.8	19.4	171

3.2 Multiple addition tests with hydrolysates of lignocellulosic biomass

Multiple additions tests were carried out also with yeasts growing in hydrolysates of *Arundo Donax* (AD), obtained following the methods described in the Methods paragraph. Againg, as soon as a stationary phase was reached, $(NH_4)_2SO_4$ was added to restore the initial concentration of nitrogen. The Fig. 2 shows the growth profile of Lipomyces starkeyi in the presence of the hydrolysate, under multiple additions of the nitrogen source.

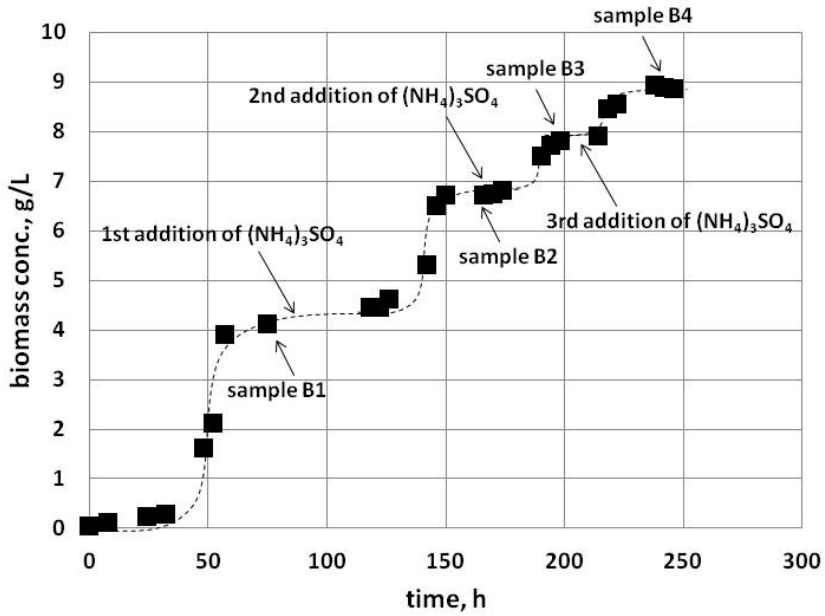

Figure 2: Growth of Lipomyces starkeyi in the presence of hydrolysate of Arundo donax in batch reactors, under multiple additions of $(NH_4)_2SO_4$. Operating conditions: T = 30°C, 160 rpm. The procedure followed to obtain the hydrolysate is described in the Method paragraph.

Again, after each addition of $(NH_4)_2SO_4$, a new exponential phase started, leading to higher values of the final biomass concentration in comparison to those obtained with a batch fermenter. The increases in biomass concentration were progressively reduced as subsequent growth cycles were carried out. A comparison between Figs. 1 and 2 show that, when using the hydrolysate of AD, the lag phase of each growth cycle was longer in comparison to that observed in the tests carried out with synthetic medium.

The Tab. 2 reports the concentration of biomass and triglycerides of the four samples (B1-B2-B3-B4) collected after each growth cycle. A comparison between Tabs. 1 and 2 demonstrates that the yeasts

grown in the presence of AD hydrolysate reach a slightly lower concentration of oleaginous biomass, though the biomass content in lipids is higher.

Table 2: Biomass and lipid concentrations obtained during the fermentation in the presence of hydrolysate of Arundo donax under multiple additions of $(NH_4)_2SO_4$. Operating conditions: T = 30°C, 160 rpm. The hydrolysis procedure is described in the Method paragraph.

Sample	Oleaginous biomass concentration, g/L	Lipid fraction, %	Lipid conc., g/L
B1	4.3	19.4	83.4
B2	6.8	20.1	136
B3	7.9	20.3	160
B4	8.7	20.3	176

3.3 Composition of triglycerides

In order to evaluate the effect of the discontinuous fed-batch fermenter on the quality of the biodiesel produced, the triglycerides obtained from samples S1, S4 (produced using synthetic medium) and B1, B4 (produced using AD hydrolysate) were characterised as regards their fatty-acid distribution, as shown in the Tab. 3. No significant variations were observed between the compositions of the different samples collected, though the oleic acid fractions increase as subsequent growth cycles are carried out (i.e. between samples S1-S4 and between samples B1-B4).
In all cases, however, the fraction of insaturated fatty acids is about one half of the total. This ensures satisfactory performances of the biodiesel obtained both in terms of oxidative stability and low-temperature behaviour.

Table 3: Composition (%) of triglycerides obtained from samples S1, S4 (see Figure 1), B1, B4 (see Figure 2). The procedure followed to extract the triglycerides from the oleaginous yeasts is described in the Method paragraph.

Fatty acid	S1	S4	B1	B4
Palmitic acid (C16:0)	23.4	23.1	23.3	23.3
Stearic acid (C18:0)	15.2	15.0	15.3	15.3
Oleic acid (C18:1)	47.2	48.1	46.8	47.5
Linoleic acid (C18:2)	6.0	6.2	6.0	6.1

4. Conclusions

This study demonstrates that, growing the oleaginous yeasts *Lipomyces starkeyi* in a discontinuous fed-batch fermenter, it is possible to increase significantly the yield in oleaginous biomass. As a matter of facts, the use of a discontinuous fed-batch fermenter makes possible to carry out the process at reduced concentrations of the N sources, thus promoting the accumulation of triglycerides within the yeasts' cells.
The same result was achieved when using hydrolysates of lignocellulosic wastes from *Arundo donax* as a growth medium. The results obtained suggest that a continuous fed-batch reactor could be developed, with progressive addition of N-sources, to maximize triglyceride accumulation within the oleaginous biomass. In order to keep a lower concentration of N-.source, the glycerol co-produced during the biodiesel synthesis could be recycled as a nutrient for the oleaginous yeasts Amaretti et al., 2012). Studies are still ongoing to evaluate the effect of glycerol as a growth medium component.
The composition of microbial oils was characterized as a function of the conditions adopted in the whole process. In all instances, the oils were containing a fraction of oleic oil residuals close to 50%. This composition allows the production of bioplastics, as well as of a biodiesel offering excellent oxidation stability and good cold performance.

References

Adney, B., Baker, J., 1996. Measurement of cellulase activities. NREL LAP-006.
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.

Holdsworth J.E., Veenhuis M., Ratledge C., 1988, Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids, J. Gen. Microbiol. 134, 2907-2915.

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 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.

Pirozzi D., Ausiello A., Strazza R., Trofa M., Zuccaro G., Toscano G., 2013, Exploitation of Agricultural Biomasses to Produce II-Generation Biodiesel, Chemical Engineering Transactions, 32, 175-180.

Pirozzi D., Ausiello A., Yousuf, A., Zuccaro G., Toscano G., 2014, Exploitation of Oleaginous Yeasts for the Production of Microbial Oils from Agricultural Biomass , Chemical Engineering Transactions, 37, 469-474.

Sadarivam, S., Manickam, A., 1996. Biochemical Methods, New Age International (pt) Ltd , 5.

Toscano G., Ausiello A., Micoli, L., Zuccaro G., Pirozzi D., 2013, Anaerobic Digestion of Residual Lignocellulosic Materials to Biogas and Biohydrogen, Chemical Engineering Transactions, 32, 487-492.

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.

Yousuf, A., Sannino, F., Addorisio, V., Pirozzi, D., 2010, Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production, J. Agric. Food Chem., 58(15):8630-5.

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.