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Innovative Methods for the Production of II Generation Biodiesel by Exploitation of Agricultural Biomasses Through the Use of Oleaginous Yeasts

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A discontinuous fermenter was used to grow the oleaginous yeast *Lipomyces starkeyi* in the presence of hydrolysates of lignocellulosic wastes from *Arundo donax*. 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. A physico-mathematical model was developed to find the optimal condition of production. Specific attention was devoted to the effect of temperature on the growth of *L. Starkeyi* and on the intracellular accumulation of lipids. 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

The second-generation biodiesel, based on the exploitation of agro-industrial wastes through the use of oleaginous microorganisms, can potentially ensure significant environmental benefits and improve the energy security of the developed countries, overcoming the problems associated with the production of the first-generation biodiesel. As a matter of facts, the application of the first-generation biodiesel (obtained mainly from vegetable oils or animal fats) is critically limited by the relatively high costs of the feedstocks, that make the biodiesel still more expensive than mineral diesel (You et al., 2008). In addition, the use of edible oils to produce biodiesel is threatening food supplies and biodiversity, causing social and environmental problems in different developing countries.
Currently, the most promising methods for the production of second-generation biodiesel are based on the exploitation of the oleaginous microorganisms, that are able to produce a significant fraction of their biomass as triglycerides, potentially exploitable for the synthesis of biodiesel (Li et al., 2008). So far, the most studied oleaginous microorganisms are the microalgae, that are offering promising results, though their industrial performance is critically affected by the light availability and by the need to control CO_2 concentration.
Oleaginous yeasts (Papanikolaou and Aggelis, 2009) and fungi (D'Urso et al., 2008) offer an useful alternative to microalgae. They are able to metabolize a wide range of wastes (Papanikolaou and Aggelis, 2009; Yu et al., 2011; Huang et al., 2012; Zhao et al., 2012; Pirozzi et al., 2013a), and have very simple cultural requirements, as the lipid accumulation occurs under nitrogen limiting conditions and excess of the carbon sources (Papanikolaou and Aggelis, 2011a-b). In addition, the microbial oils obtained from yeasts have a composition quite similar to that of vegetable oils (Angerbauer et al., 2008), and are consequently suitable for the production of a biodiesel offering satisfactory performances as automotive fuel (Pirozzi et al., 2012). However, further studies are needed to improve the efficiency of the microbial oils production, and to improve the economical balance of the process. As a matter of facts, the cost of the biodiesel obtained by oleaginous

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yeasts still exceeds that of fossil diesel (Ratledge, 2004; Ratledge and Cohen, 2008; Wynn and Ratledge, 2005).

So far, different residual materials have been tested as possible nutrients for the oleaginous yeasts, such as sewage sludge (Angerbauer et al., 2008), primary effluent wastewaters (Hall et al., 2011), sugar cane bagasse (Huang et al., 2012), tomato waste (Pirozzi et al., 2013b), glycerol (Papanikolaou and Aggelis, 2009), olive-mill wastewater (Yousuf et al., 2010) or other souces (Li et al., 2008). However, lignocellulosic biomasses represent the more promising feedstock. As a matter of facts, a large range of residual 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. As cellulose and hemicelluloses are the main component of plants, the yield of feedstock biomasses per unit area could be as high as 40 t d.m. ha^{-1} (Mantineo et al., 2009).

In this study, the oleaginous yeast *Lipomyces starkeyi* was cultured in the presence of hydrolysates of biomasses from Arundo donax, a perennial crop known for its adaptability to different climatic and soil conditions, offering good yields in marginal lands and with low input cropping systems. A. donax is able to grow in soils polluted (Fiorentino et al., 2010; 2013) and salinized (Impagliazzo et al., 2011), and guarantee efficient protection to soils subjected to accelerated erosion (Fagnano et al., 2012 and 2014) , This is of particular importance, due to the recent increase of the rain erosivity promoted by the climate changes (Diodato et al., 2009).

Aim of the experimental activity was the achievement of satisfactory yields in terms of biomass concentration and triglyceride yields A physico-mathematical model was developed to find the optimal condition of production. Specific attention was devoted to the effect of temperature on the growth of *L. Starkeyi and* on the intracellular accumulation of lipids. The analysis of the microbial oils was carried out to evaluate their potential for the production of a biodiesel suitable as automotive fuel.

2. Materials and methods

2.1 Microorganisms and culture media

Lipomyces starkeyi were kept on potato dextrose agar (Sigma) at T = 5°C. The microorganisms were cultivated under N-limiting medium, containing (g l-1): KH_2PO_4 (Serva), 1.0; $MgSO_4 \cdot 7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka) 0.5, glucose 70.0. The microorganisms were grown under aerobic conditions at 30°C on a rotary shaker 160 rpm (Minitron, Infors HT, Switzerland).

2.2 Hydrolysis of cellulosic biomass

Arundo donax biomass was collected from an experimental field in S. Angelo dei Lombardi (Campania, Italy) used for studying the impact of cropping systems on soil erosion (Fagnano et al., 2014), washed and dried over night at 80°C and grind with a chopper. The powdered biomasses were stored in desiccators. Cellulose, hemicellulose and lignin were measured following a standard method (Ververis et al., 2007). The lignocellulosic materials were first steam-exploded and subsequently treated with commercial preparations of enzymes. 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 concentrations of glucose and xylose in the hydrolysates were 36-40 g/L and 12-16 g/L, respectively.

2.3 Fermentation of hydrolysates

The fermentation tests were carried out in conical flask of 500 ml. The liquid medium was inoculated by 2 ml of microorganism suspension, obtained dissolving 10 loops of solid culture in 8 ml of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature T = 30 ± 1 °C. The pH value of the medium was 6.5- 6.68 before sterilization. One hundred fifty ml of the medium was transferred in a 500-ml shaking flask. At the end of fermentation pH value of the medium was 7.5-9.0, that varies with the composition of medium. After each fermentation ordeal, the biomass was recovered by centrifugation (4000 rpm for 10 min) and lyophilized to enable the determination of the dry biomass and the lipid content. The total count of microorganisms was carried out in plate count agar medium (Difco Laboratories, USA) after 48 h of culture.

The concentration of reducing sugars was measured according to the Nelson-Somogyi method (Somogyi, 1952). Optical density was measured at 620nm.

2.4 Extraction and characterization of microbial lipids

Total lipids were extracted according to a standard method (Bligh and Dyer, 1957) with little modification. In a typical test, 5 ml of methanol and 2.5 ml of chloroform were added to 200 mg of dry biomass and vortexed 5 seconds. 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 tube fitting with centrifuge racks. 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, 10 ml 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 extracted weight was finally recorded after.
The fatty acids composition was determined by GC analysis on a Shimadzu GC 17/3 gas-chromatograph equipped with a flame ionisation detector, following the method adopted by Li and co-workers (Li et al., 2007).

3. Results and discussion

3.1 Effect of the temperature on the growth kinetics

A first series of experiments was carried out growing the oleaginous yeasts at different temperatures (Figure 1). The growth rate in the exponential phase was higher as the temperature increased. However, the maximum concentration of biomass was observed at 35°C.

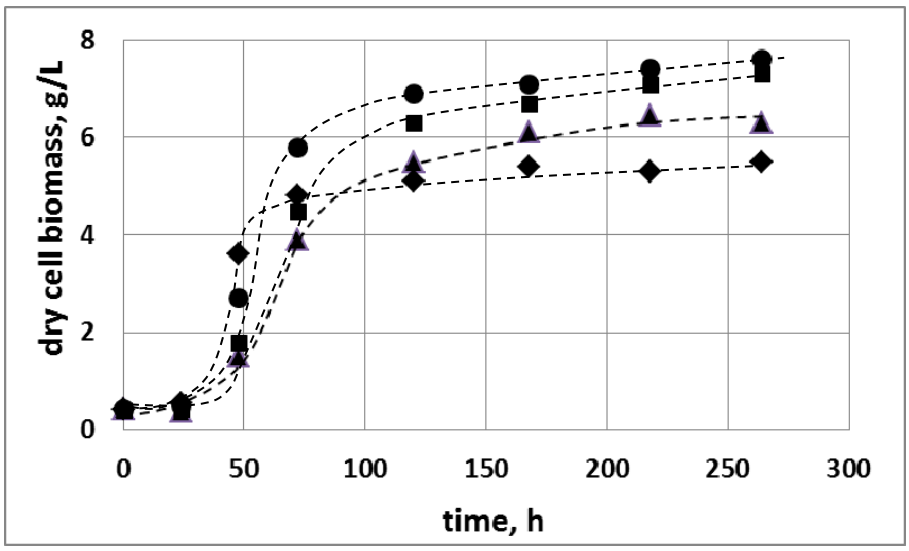

Figure 1 - Effect of the temperature on the growth kinetics of L. starkeyi grown in the presence of the hydrolysate ADH 50%. T = 25°C (▲),30°C (■), 35°C (●), 40°C (◆).

Table 1 – Effect of the temperature on the biomass and the triglyceride fraction of L. starkeyi grown in the presence of the hydrolysate ADH 50%

Temperature, °C	Biomass conc., g/L	Lipid fraction, %	Lipid conc., g/L
25	6.21	20.4	1.27
30	7.61	20.1	1.53
35	7.88	18.6	1.47
40	5.60	17.4	0.97

After each growth test, the lipids were extracted from the yeasts, following the procedure described in the Methods paragraph. The results reported in the Table 1 show that the lipid fraction measured was a decreasing function of the temperature.
On the basis of these results, the optimal temperature as regards the lipid concentration per liquid volume is 30°C, as shown in Table 1.

The TOC levels were measured in the course of the each tests. The values of biomass concentration and TOC obtained at 30°C are reported in the Figure 2 as a function of the time. In all the tests carried out, the TOC reduction was mostly achieved in the first 4-5 days, that is in the period of the biomass growth, showing the use of the organic carbon as a primary carbon source. In any case, the TOC removal was never complete, demonstrating that the break in the biomass growth was not due to the exhaustion of organic compounds.

3.2 Growth model

The profiles of biomass concentration and TOC obtained under different experimental conditions were described by a logistic growth model. The model includes a biomass balance:

$$\frac{dX}{dt} = \mu X \tag{1}$$

where μ is the specific growth rate, defined by the logistic equation:

$$\mu = \mu_{\max}\left(1 - \frac{X}{X_{\max}}\right) \tag{2}$$

In order the obtain the TOC profiles, the hypothesis of proportionality between TOC reduction rate and biomass growth rate was adopted:

$$\frac{d[TOC]}{dt} = \mu X \frac{1}{Y_{X/TOC}} \tag{3}$$

where $Y_{X/TOC}$ is a yield factor representing the ratio of the amount of biomass produced to the amount of TOC consumed (g biomass/g TOC).

In order to obtain the X(t) and TOC(t) profiles, the equations (1) (2) (3) were integrated using a fourth-order Runge-Kutta integration method. The least-square method was used to obtain the parameter estimates. The model fitted the experimental data with a satisfactory R-squared value (R^2 = 0,93). In the Figure 2 a graphic comparison between the model predictions (dashed line) and the experimental results obtained at T=30°C is reported.

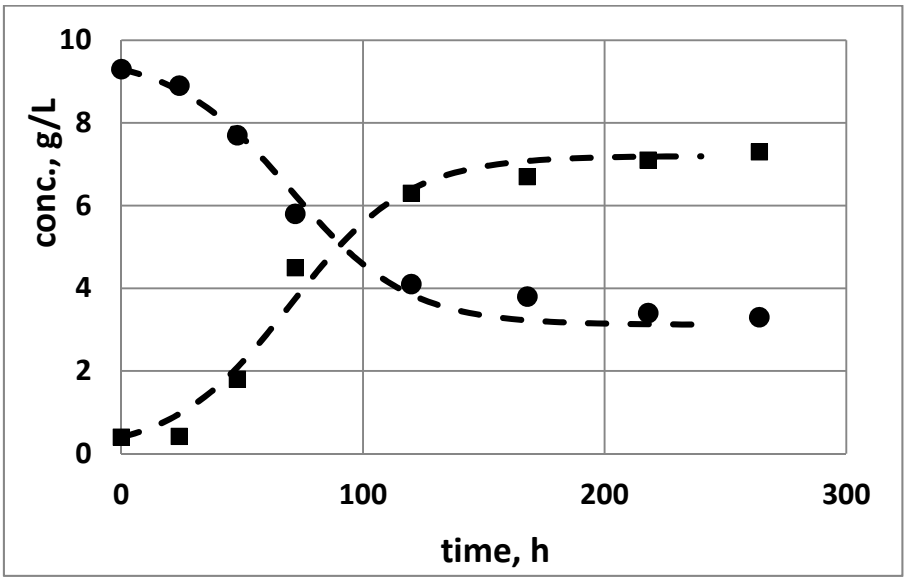

Figure 2 - Comparison of experimental measurements of biomass concentration (■, g/L) and TOC (●, g/L) with the theoretical data obtained with the logistic model, with reference to the culture of Lipomyces starkeyi in batch reactors, in the presence of the hydrolysate ADH 50%. T = 30°C.

A comparison between the experimental and theoretical data is given in the Table 2. The experimental value of the specific growth rate (μ_{max}) was determined as the slope of the regression line obtained by plotting the natural log of biomass versus time during the exponential phase. The specific growth rate (μ_{max}) increases

with the temperature, confirming the results shown in the Figure 1. The biomass yield based on TOC consumption ($Y_{X/TOC}$) is substantially constant, suggesting that changes in the temperature do not cause a significant increase in the maintenance requirements.

3.3 Effect of the temperature on the triglyceride composition

The concentrations of the most abundant fatty acids found in the microbial oils obtained from *L. Starkeyi* (palmitic, stearic, oleic and linoleic) is reported in the Table 3 as a function of the growth temperature. The composition of linolenic acid is not reported in Table 3 as its fraction was negligible. The percentages of the saturated fatty acids are slightly higher as the growth temperature increases. Whatever the temperature adopted, the results obtained indicate a balanced distribution between unsaturated fatty acids and saturated fatty acids. This indicates that an excellent quality biodiesel can be obtained, offering a good stability to the oxidation and a satisfactory cold performance.

4. Conclusions

The results obtained demonstrated that *Arundo donax* biomass can be used to produce II-generation biodiesel, allowing a sustainable production of renewable energy, and reducing the competition with food crops for fertile lands. A physico-mathematical model was developed to find the optimal temperature to maximize the growth of *L. Starkey* as well as the intracellular accumulation of lipids. The effect of the temperature was analysed also as regards the fatty acids distribution of the lipids accumulated in the *Lipomyces starkeyi*, The composition of the microbial oils was in all cases compatible with the production of a biodiesel offering excellent performances as automotive fuel, in terms of both the resistance to oxidation and the cold performance.

Table 2 –Comparison of experimental measurements of growth parameters with the theoretical data obtained with the logistic model, with reference to the culture of Lipomyces starkeyi in batch reactors, under different experimental conditions.

T	X_0	$[TOC]_0$	μ_{max} (exp)	μ_{max} (pred))	X_{max} (exp)	X_{max} (pred)	$Y_{X/TOC}$ (exp)	$Y_{X/TOC}$ (pred)
25	0.42	9.42	$3.91\ 10^{-2}$	$3.72\ 10^{-2}$	6.28	6.35	1.12	1.32
30	0.40	9.20	$4.12\ 10^{-2}$	$4.03\ 10^{-2}$	7.34	7.31	1.21	1.19
35	0.41	9.28	$4.17\ 10^{-2}$	$3.98\ 10^{-2}$	7.61	7.86	1.19	1.26
40	0.43	9.41	$4.33\ 10^{-2}$	$4.25\ 10^{-2}$	5.44	5.31	1.03	1.18

Table 3: Distribution (%) of the most abundant fatty acids in the microbial oils obtained from Lipomyces starkeyi in batch reactors, in the presence of the hydrolysate ADH 50% at different temperatures.

acid	**T=25°C**	**T=30°C**	**T=35°C**	**T=40°C**
Palmitic acid (C16:0)	22.0	22.3	22.3	22.5
Stearic acid (C18:0)	17.4	17.6	18.1	18.5
Oleic acid (C18:1)	45.1	44.9	44.8	44.6
Linoleic acid (C18:2)	6.0	5.9	6.1	5.9

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