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Exploitation of Agricultural Biomasses to Produce II-Generation Biodiesel

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Hydrolysates of lignocellulosic materials from *Arundo donax* (Giant reed, GR) and *Sorghum vulgaris* (SV) were used as a source of fermentable sugars, employed for culturing the oleaginous yeasts *Lipomyces starkey*. A significant fraction of the *L. starkey* biomass was made of triglycerides, potentially exploitable for the synthesis of biodiesel. The conditions of the hydrolysis of the AD and SV, as well 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. In order to reduce the effect of the microbial growth inhibitors produced in the course of the lignocellulose hydrolysis, *L. starkeyi* were adapted to fermentation media containing progressively higher concentrations of hydrolysates. The fatty acids distribution of the microbial lipids obtained was compatible with the production of a biodiesel offering excellent performances as automotive fuel.

1. Introduction

A full industrial application of biodiesel is still limited, as the relatively high costs of the traditional feedstock materials (i.e. vegetable oils and animal fats) make 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.
In order to overcome these problems, alternative sources of triglycerides are consequently sought, requiring only a limited use of fertile soil. Hydolysates of lignocellulosic materials can offer an alternative source of fermentable sugars, to be used as a feedstock for the production of the so-called II generation biofuels. As a matter of facts, an efficient technology for processing lignocellulosic biomasses could enable the recycle of a large range of agroforestal waste biomasses, using the non-food parts of crops (stems, leaves and husks), as well as industry wastes (woodchips, skin and pulp from fruit pressing, etc.). In this view, suitable non-food crops (switchgrass, jatropha, miscantus, etc.) could be cultivated in partially-fertile soils, to obtain lignocellulosic biomasses for biofuel production. 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).
Though so far lignocellulosic biomasses have been mainly exploited for the production of bioethanol, in the last years a growing interest has been devoted to growth of oleaginous microorganisms in lignocellulose hydrolysates (Li et al., 2008). Oleaginous microorganisms are able to produce more than 20% of their biomass as triglycerides, potentially exploitable for the synthesis of biodiesel. In particular, oleaginous yeasts offer very simple cultural requirements, as the lipid accumulation occurs under nitrogen limiting conditions and excess of the carbon sources (Angerbauer et al., 2008; Huang et al., 2009; Li et al., 2007; Meng et al., 2009; Papanikolaou and Aggelis, 2011a and 2011b; Yu et al., 2011).
In this study, the oleaginous yeast *Lipomyces starkeyi* were cultured in the presence of raw hydrolysates of biomasses AD and SV, with no addition of organic supplements. Both plants are known for their adaptability to different climatic and soil conditions. In particular, AD is able to grow in soils polluted, salinized or subjected to erosion (Fiorentino et al., 2010). The experimental activity was aimed at the

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achievement of satisfactory yields in terms of biomass concentration and triglyceride yields Specific attention was devoted to the effect of inhibitors of the cellular growth generated in the course of lignocellulose hydrolysis. In this view, a progressive adaptation of the *L. Starkey* was carried out culturing the yeasts in the presence of progressively more concentrated solutions of hydrolysates.

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

AD and SV were collected from S. Angelo dei Lombardi (Campania, Italy) agro-land, 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). In a typical test, H_2SO_4 at 10%, 5%, 2.5% (w/v) was used to oven-dried biomass at a solid to liquid ratio of 1:10 with 3g of samples in 30 ml of acid solution in a 100ml glass bottle. Then they were autoclaved at 121°C for 20 min. After vacuum filtration the filtrate was neutralized to pH 6.5 with saturated KOH solution. Hydrolysates were sterilized in autoclave before inoculation.

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 by sequential dilution and insemination in plate count agar medium (Difco Laboratories, USA). The colonies were counted after 48 h of culture on agar medium.

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

Potential inhibitor compounds were analyzed following the method adopted by Chen and co-workers (Chen et al., 2009).

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 Hydrolysis of cellulosic biomass

The composition of the biomass samples of AD and SV is reported in the Table 1, in terms of cellulose, lignocelluloses and lignin content.

Table 1: Composition of the lignocellulosic biomass

Biomass	Cellulose, %	Hemicellulose, %	Lignin, %
Arundo donax (AD)	43,9	23,9	18,9
Sorghum vulgaris (SV)	45,2	27,3	17,5

The preliminary hydrolysis of the biomass samples was carried out with dilute H_2SO_4. In a preliminary experiment, we observed the effect of the H_2SO_4 concentration on the concentration of reducing sugars in the hydrolysate, as shown in the Figure 1. A maximum in the sugar yield, for both the *Arundo donax* hydrolysate (ADH) and the *Sorghum vulgaris* hydrolysate (SVH), was observed treating the biomass in the presence of 5% H_2SO_4. This result can be explained considering that higher concentrations of acid, though increasing the biomass conversion, promote a further sugar degradation towards undesired products (Saha et al., 2005). Consequently the biomass hydrolysis for the following tests was carried out in the presence of 5% H_2SO_4.

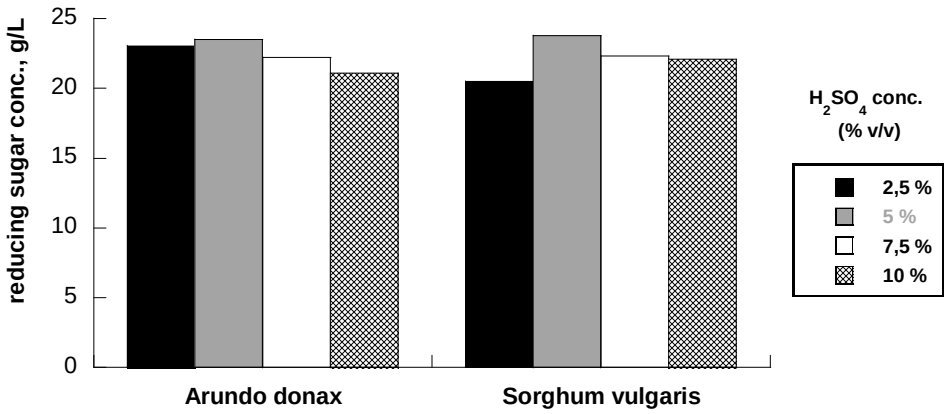

Figure 1. Effect of the H_2SO_4 concentration on the reducing sugars concentration in the hydrolysate

3.2 Growth of the oleaginous yeasts

An important bottleneck for the synthesis of II-generation biofuels from lignocellulose hydrolysates stems from the generation of microbial growth inhibitors in the course of the hydrolysis treatment. Acetic acid was the most abundant inhibitor contained in ADH (5,5 g/L) and SVH (5,1 g/L). The concentrations of other potential inhibitors (furfural, formic acid, hydroxybenzaldeyde, etc.) were in all cases lower than 1 g/L.

When culturing *L. starkeyi* in the raw ADH without any external organic supplement, no appreciable growth was observed, due to the higher concentration of inhibitors. For this reason, the microorganisms were cultured in fermentation media containing 75%, 50% and 25% fractions of ADH, with addition of water. The growth profiles, reported in the Figure 2, demonstrate that the maximum initial growth rate was observed in the presence of the most diluted hydrolysate (25%), due to the lower concentration of inhibitors. However, the yeasts grown in the presence of the most concentrated hydrolysate (75%) reached the highest concentration within 7 days (150-200 hrs). For longer times than 200 hrs, a progressive reduction of the yeasts concentration was observed whatever the hydrolysate concentration used.

Qualitatively similar results were obtained when culturing *L. starkeyi* in the presence of the same concentrations of SVH (data not shown). In this case, the maximum concentration obtained with 75% hydrolysate was slightly lower (8,9 10^7 CFU/ml with SV, 1,2 10^8 CFU/ml with AD), and the onset of the final decrease of the concentration was anticipated at about 150 hrs.

The biomass and lipid production for both ADH and SVH, obtained in the presence of 75% hydrolysate, are described in the Table 2. The results show that, though the lipid content with ADH is lower, its biomass yield is significantly higher, and consequently the overall lipid yield with ADH is more satisfactory.

3.3 Adaptation of yeasts to inhibitors

In order to overcome the effect of the inhibitors, consecutive *L. Starkey* growth cycles (250 hrs each) were carried out in the presence of progressively more concentrated hydrolysates, using the biomass obtained from each cycle as inoculum for the subsequent cycle.

The experimental results shown in the Figure 3 indicate that, after two growth cycles carried out in the presence of 50% ADH and 75% ADH, respectively, the pre-adapted *L. Starkeyi* were able to grow in the presence of 100% ADH (i.e. undiluted raw hydrolysates), overcoming the negative effect of the inhibitors. The biomass concentration obtained after the third growth cycle was about 9,7 g/L Similar results were obtained when using SVH as growth medium, though the concentration of biomass collected was slightly lower.

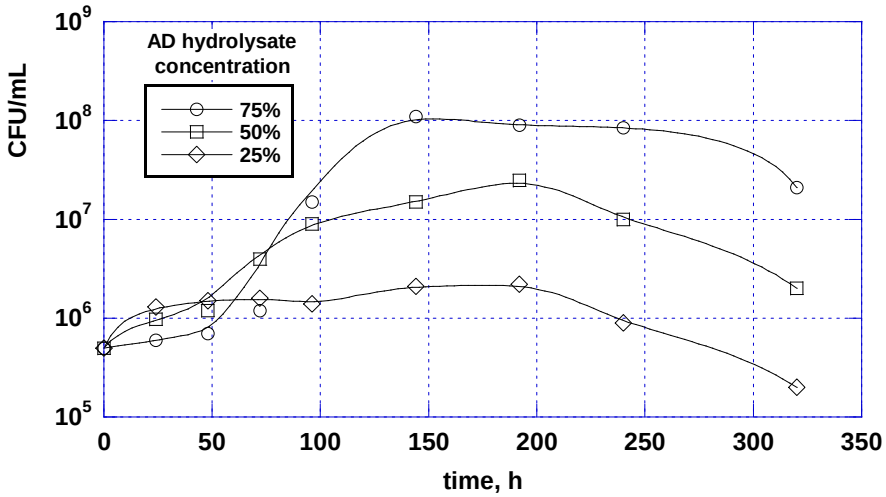

Figure 2. Growth profiles of Lipomyces starkeyi *in the presence of Arundo donax (AD) hydrolysates at different concentrations. C*

Table 2: Biomass and lipid production of Arundo donax *(AD) and* Sorghum vulgaris *(SV), obtained in the presence of 75% hydrolysate.*

Biomass	Dry biomass yield, g/L	Lipid content, %	Lipid yield, g/L
Arundo donax (AD)	6,9	15,9	110
Sorghum vulgaris (SV)	5,1	18,5	94,4

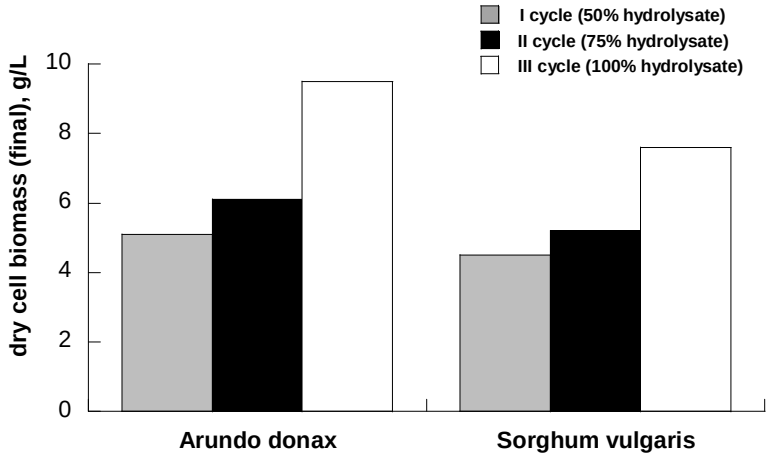

Figure 3. Effect of consecutive adaptation cycles (250 hrs each) on the biomass yield of L. starkeyi*, in the presence of hydrolysates of AD and SV.*

3.4 Distribution of fatty acids

The lipids obtained from *L. Starkey* grown in the presence of 75% ADH and 75% SVH were characterized as regards the distribution of fatty acids, in order to evaluate the characteristics of the II-generation biodiesel that can be potentially obtained. The concentrations of the most abundant fatty acids found (i.e. palmitic, stearic, oleic and linoleic) are shown in the Table 3. The results obtained using 75% ADH indicate a balanced distribution between unsaturated fatty acids (improving the low-temperature performance of biodiesel) and saturated fatty acids (increasing the oxidative stability). Consequently, an excellent quality biodiesel can be obtained. The fatty acid distribution in the lipids obtained using 75% SVH was similar, though slightly increased fractions of unsaturated fatty acids were observed.

Table 3: Distribution (%) of the most abundant fatty acids in the lipids obtained from Arundo donax *(AD) and* Sorghum vulgaris *(SV), cultured in the presence of 75% hydrolysate.*

Biomass	*Arundo donax* (AD)	*Sorghum vulgaris* (SV)
Palmitic acid (C16:0)	22,1	19,4
Stearic acid (C18:0)	16,4	16,1
Oleic acid (C18:1)	46,0	48,2
Linoleic acid (C18:2)	6,2	6,5

4. Conclusions

In conclusion, the results obtained demonstrate that *Arundo donax* and *Sorghum vulgaris* biomasses can be used for the II-generation biodiesel synthesis, allowing a sustainable production of renewable energy, and reducing the competition with food crops for fertile lands.

A main bottleneck for this process was caused by the cellular growth inhibitors produced in the course of the preliminary hydrolysis of the lignocellulosic biomasses. This bottleneck was overcome using diluted hydrolysates as fermentation medium for the oleaginous yeasts. Alternatively, *L. Starkeyi* were preliminary adapted to inhibitors and eventually used in the presence of raw non-diluted hydrolysates, obtaining a satisfactory biomass yield.

The fatty acids distribution of the lipids accumulated in the *Lipomyces starkeyi* was in all cases compatible with the production of a biodiesel offering excellent performances as automotive fuel.

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