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Production of Single-cell oils from Lignocellulosic Biomass from *Arundo donax* L.

Judith Cano-Ruiz[ab], Teresa Nunziata[a], Gaetano Zuccaro[a], Pedro Vicente Mauri[b], M. Carmen Lobo[b], Domenico Pirozzi[a]

[a]DICMaPI - Università Federico II, P.le Tecchio, 80, 80125 Napoli, Italia
[b]Dep. Agroenvironmental Research , IMIDRA, Finca "El Encin" A-2, Km 38,2 Alcalá de Henares (Madrid), Spain
dpirozzi@unina.it

Single-cell oils (SCOs) raise a growing interest due to their potential for the production of biofuels, bioplastics and more recently of food additives. The production of SCOs by exploitation of residual lignocellulosic biomass has become a realistic target, due to the recent advances in enzyme hydrolysis technology. The fermentation of yeasts offers a useful alternative to microalgae, due to their ability to use several agroforestry wastes as feedstock and their simple cultural requirements. In addition, the microbial oils obtained from yeasts have a composition quite similar to that of vegetable oils.
This study is focused on the exploitation of Giant reed (*Arundo donax*), a widely diffused perennial crop, well known for its capability to grow in marginal lands ensuring high biomass productivity. Different types of biomass, from crops cultivated under different agronomic management were tested. The effect of two types of treated sewage sludges as organic matter sources and inorganic nitrogen fertigation was evaluated to increase soil fertility. These practices were tested in order to improve crop composition and productivity.
The hydrolysates from *A. donax* biomass have been evaluated as a growth medium for oleaginous yeasts, for the production of microbial oils. The production kinetics and the yields in microbial oils have been analyzed taking into account the composition of the different biomasses used, in order to contribute to a complete optimization of the productive cycle. In general, the treatment acid hydrolysis with HCl followed by enzymatic hydrolysis led to the best results in order to obtain glucose production for the development of biofuels.

1. Introduction

In the last years single-cell oils (SCOs) have experiment a growing interest. They have been used for the production of biofuels, bioplastics and, more recently, food additives (Bellou et al., 2016). Its production by exploitation of residual lignocellulosic biomasses has become a realistic target, due to the recent advances of enzyme hydrolysis technology.
Oleaginous yeasts offer a useful alternative to microalgae, due to their ability to use several agroforestry wastes as feedstock and their simple cultural requirements (Li et al., 2013). In addition, the microbial oils obtained from yeasts have a composition quite similar to vegetable oils (Papanikolaou et al., 2011). These yeasts convert glucose into oils, and lignocellulosic material have been result a suitable source of these compounds.
Giant reed (*Arundo donax*) is a lignocellulosic perenial grass widely diffused as perennial crop. It is well known for its rapid spread, capability to grow in marginal lands, ensuring high biomass productivity with low agronomic inputs (Pirozzi et al., 2013; Pilu et al. 2012). It has been used as source of biomass in different Mediterranean countries (Angelini et al. 2005). The different soil management has resulted to be determinant in *Arundo* biomass production (Nasi o di Naso et al. 2010; Pari et al. 2016).
Previous studies have shown the increase in the *Arundo* production in soils treated with organic amendments. The same positive effect has been observed using inorganic nitrogen fertigation in field experiments (Plaza et al. 2015; Cano-Ruiz et al. 2016).
The base of the explotation of lignocellulosic *Arundo* residues for the production of single-cell oils consists in the use of hydrolysates of lignocellulosic materials from *Arundo donax* as a source of fermentable sugars, for

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culturing the oleaginous yeasts (Pirozzi et al. 2012, 2013). The hydrolysates from *A. donax* biomass have been evaluated as a growth medium for oleaginous yeasts, for the production of microbial oils. It is common that lignocellulosic biomass need a pretreatment in order to increase its digestability. The main effect of these pretreatments is to break the lignocellulose into monomers, in order to facilitate the access to bacteria to fermentable compounds. Also enzymes are capable of breaking down lignocellulosic materials to sugars (Yu et al 2011; Sun et al. 2002; Kumar et al. 2009). In occasions, during the pretreatments, reactions products that inhibit bacterial multiplication are formed (Ask et al 2012) and condition an optimal fermentation.
The objective of this work is to analyze the production kinetics and the yields in microbial oils, from different *Arundo donax* biomasses, in order to contribute to a complete optimization of the productive cycle.

2. Material and methods

2.1 Biomass

Biomass samples were collected from *Arundo* plants grown in experimental plots in El Encin (Alcala de Henares, Madrid, Spain). Plants were grown under different agricultural management: sewage sludge composted with pruning wastes at 50 t/ha as organic amendment (CP); sewage sludge thermically dried at 50 t/ha as nitrogen source(ST); inorganic nitrogen applied by fertigation at dose 120kgN/ha (N). Plants aerial parts were harvested two years after crop establishment. Samples were dried in an oven at 60ºC during 72 hours; milled; and stored in a dry place before analysis.

2.2 Microorganism and Culture media

Culture media was realizing according to Pirozzi et al (2015). *Lipomyces Starkeyi DBVPG 6193* was used as oleaginous yeast, purchased from the Culture Collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The strains were maintained at 5 °C on a YPD solid medium with the composition (g/L): yeast extract (10), peptone (20), D-glucose (20), agar (20). Before fermentation, yeast was grown in a 100 mL Erlenmeyer flasks with an initial volume of 50 mL of preculture which contained (g/L): KH_2PO_4 (3.0), Na_2HPO_4 (1.0), yeast extract (5.0), glucose (10.0), peptone (5.0). The pH of media was adjusted to 6 and prior to inoculation, the pre-culture broth was sterilized at 121 °C for 21 min. Cultures for lipid production were inoculated with 5% v/v of the pre-culture media. The incubation of the pre-culture was carried out at 30 °C, 160 rpm for 48 hours (Minitron HT Infors, Switzerland).

2.3 Hydrolysis of cellulosic biomass

Different hydrolysis experiments were developed in order to get higher values of glucose production. All hydrolysis were done using 100 mL suspensions of *Arundo donax* biomass 5%, (w/v) in phosphate buffer (50 mM, pH 6).
Two different acid hydrolysis with different inorganic acid were tested. One of them, using 5% sulfuric acid, according to Zuccaro et al. (2014) and the other one with HCl (0.2M) according to Meinita et al (2012). Both hydrolysis were done in autoclave at 121ºC for 30 minutes.
The enzymatic hydrolysis was carried out at 50°C and 150 rpm during 48 hours. 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.
Acid hydrolysis followed for enzymatic hydrolysis, was carried out following consecutively the two methods described above. In this case, the pH was corrected before start enzymatic hydrolysis, in order to avoid the inactivity of enzymes due to the low pH produced for acid hydrolysis process.
Glucose was measured using an enzymatic kit (Sigma Aldrich) in order to know which of the treatments has more fermentable compounds. Also fermentation inhibitors were measured using colorimetric methods: phenols concentration was calculated according to Folin- Ciocalteau method (Singleton et al., 1999); and the furans according to the methodology proposed by Martinez et al. (2000).

2.4 Lignin composition

Lignin of each biomass was evaluated using Klason method modified by Moreira-Vilar et al. (2014) with H_2SO_4 at 72 % and using autoclave at 105 °C during 60 minutes.

2.5 Fermentation of hydrolysates

The fermentation tests were carried out only using hydrolysis products of the procedure with the best glucose production results. They were developed in conical flask of 500 ml. Before starting fermentation the pH of hydrolysis product was correct to achieve values of 5-5.5. 80 ml of this medium were inoculated using 2 ml of

microorganism suspension from preculture medium, mentioned in paragraph 2.2 of this work. The flasks were incubated in a rotary shaker at an agitation rate of 160 rpm and an incubation temperature of 30°C. At the end of fermentation pH value of the medium was 5-7.5, that varies with the composition of medium. After each fermentation, biomass and glucose concentration were determined following a modified Nelsol-Somogyi method (Nelson, 1944). Estimation of biomass was done measuring the obtained extract at 600nm with a Shimadzu UV6100 spectrophotometer (Japan).

2.6 Statistical analysis

All data were collected by triplicate. Average and graphs were done using Microsoft Excell 2010. Lignin results were analyzed using IBM SPSS 21 in order to evaluate the possible correlation with hydrolysates results, using Pearson and Spearman correlation analysis

3. Results and discussion

Hydrolysates concentration obtained in different hydrolysis assayed is shown in Figures 1 and 2.

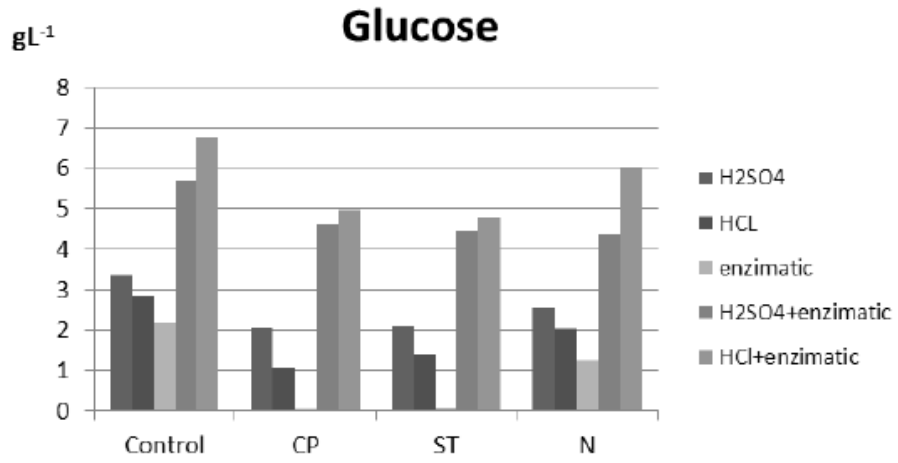

Figure 1: Glucose concentration after hydrolysis of Arundo donax *biomass. The experimental conditions adopted for each hydrolysis test are described in Paragraph 2.*

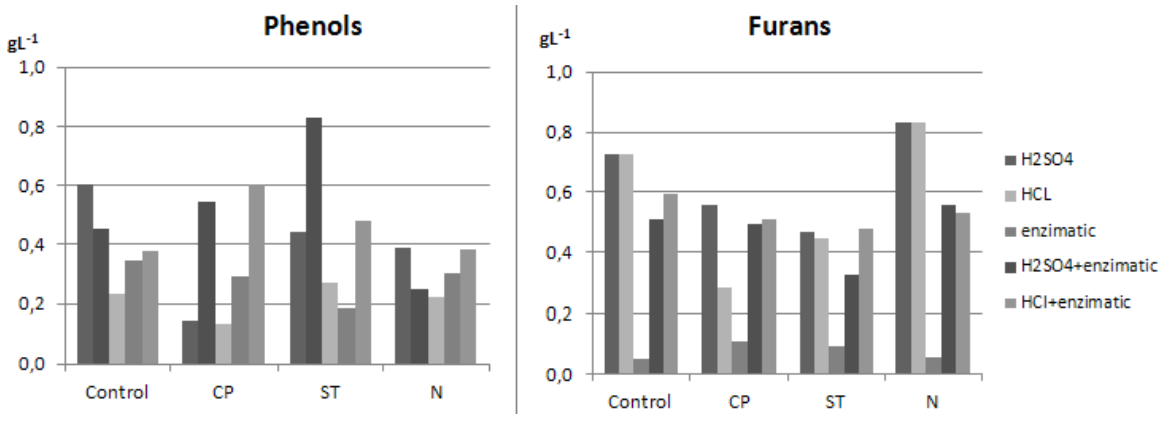

Figure 2: Phenols and furans concentration after hydrolysis of Arundo donax *biomass. The experimental conditions adopted for each hydrolysis test are described in Paragraph 2.*

In general, high differences between hydrolysates concentration in relation to the assayed hydrolysis treatments are observed. Phenols and furans present differences in the cases that only acid hydrolysis was done. These differences are not shown when acid hydrolysis and enzymatic hydrolysis consecutively were done. Both compounds present lower values in enzymatic hydrolysis, but the lack of glucose produced after enzymatic hydrolysis shows that doing only enzymatic process, the hydrolysis is not successfully developed.

The glucose concentration (Figure 1) is higher in cases where both kind of hydrolysis were done consecutively, being slightly higher when acid hydrolysis is done with HCl. In this case, the efficiency of hydrolysis, i.e. the ratio glucose concentration/theoretical glucose concentration, is close to 60% (data not shown), similar to most data reported in the Literature. Higher values of glucose are shown in samples which pre-acid hydrolysis, that could be similar to other pretreatments done previously to the hydrolysis. Steam exploded material was used by Zuccaro et al. (2014). Also You et al. (2016) used an acid reaction combined with heating in order to break cell walls. Scordia et al. (2011), use oxalic acid and high temperatures for improving the production of hydrolysates of *Arundo donax*.
Also in relation to glucose values, in most of hydrolysis, the control samples present slightly higher glucose concentration than plants from treatments with organic or inorganic fertilization.
Lignin values are shown in figure 3.
It is shown that plants grown in plots treated with inorganic nitrogen have lower lignin values than plants from plots treated with sewage sludges. This fact could explain the higher glucose production obtained after hydrolysis in samples from these plots. Correlation analysis (Table 1) shows the strong and significant negative correlation between lignin values and glucose concentration with HCl hydrolysis followed by enzymatic hydrolysis. These results agree with those obtained by Dien et al. (2009) that explains that better sugar conversion has been obtained with lower values of lignin. A correlation at 0.1 in Pearson method in acid hydrolysis using HCl is observed.

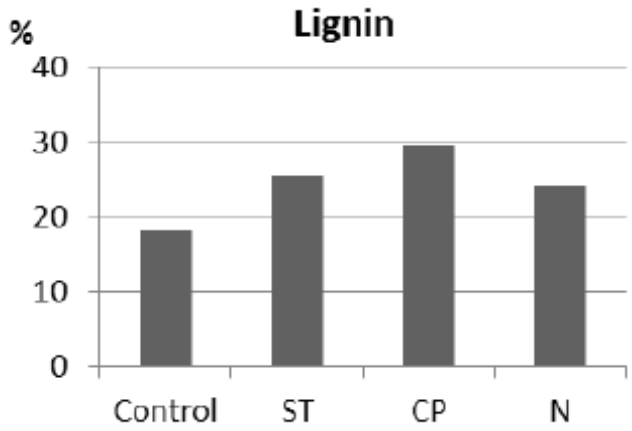

Figure 3: Lignin composition of biomass (Moreira-Vilar et al., 2014). Samples: sewage sludge composted with pruning wastes at 50 t/ha as organic amendment (CP); sewage sludge thermically dried at 50 t/ha as nitrogen source(ST); inorganic nitrogen applied by fertigation at dose 120kgN/ha (N)

*Table 1: Correlation between soluble lignin using Klason method and Glucose obtained using different treatments ** correlation of 0.05 * correlation of 0.1*

Soluble Lignin	Glu H_2SO_4	Glu HCL	Glu enzymatic	Glu H_2SO_4 +enzymatic	Glu HCl + enzymatic
Pearson	-0,755	-0,841*	-0,654	-0,547	-0,889**
Spearman	-0,400	-0,700	-0,400	0,000	-0,900**

Fermentation was developed in samples where higher glucose values were obtained. The results of biomass of SCO and glucose postfermentation are shown in figure 4.
Higher values of SCO biomass and lower of glucose are shown in hydrolysis in HCl, which means that an optimal fermentation has been produced. The lack of glucose consumption and bacterial formation shows that an unsuccessful fermentation occurs in the product of the double hydrolysis done with H_2SO_4. This could be due to the formation of fermentation inhibitors that are not measured in this study.

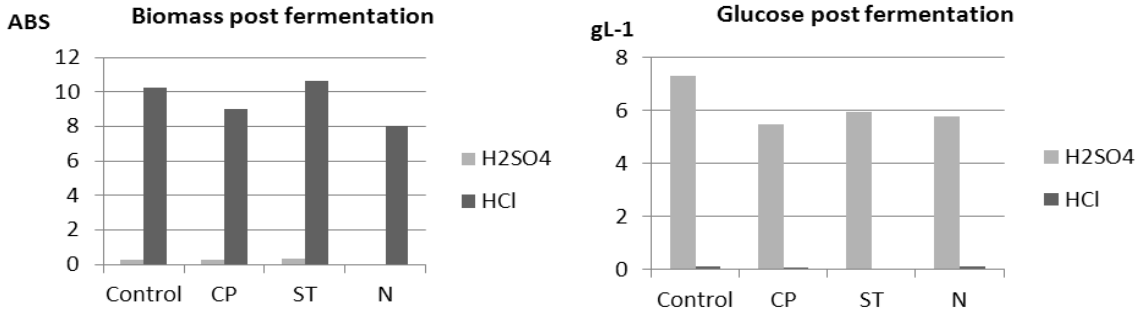

Figure 4: Biomass estimation (by absorbance at 600 nm) and glucose concentration (gL-1) after fermentation of Arundo donax hydrolysate. The experimental conditions adopted in are described in Paragraph 2.

4. Conclusions

The treatment acid hydrolysis with HCl followed by enzymatic hydrolysis led to the best results in order to obtain glucose production for the development of biofuels. Therefore, a better SCO formation after fermentation is obtained in the assayed conditions.
Lignin plant composition cause differences in glucose concentration obtained after hydrolysis. Agricultural management could cause differences in lignin composition, and the quantification of this compound is determinant in the hydrolysis feasibility.

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