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"Gheorghe Asachi" Technical University of Iasi, Romania

EFFECT OF NICKEL CONTAMINATION ON THE GROWTH OF OLEAGINOUS YEASTS IN HYDROLISATES OF *Arundo donax*

Domenico Pirozzi[1*], Giuseppe Toscano[1], Gerardo Caputo[1], Ciro Florio[1], Felicia Rugari[1], Giuseppe Travaglini[1], Abu Yousuf[2], Gaetano Zuccaro[1]

[1]Università Federico II, Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale (DICMAPI), 80 P.le Tecchio, 80125 Napoli, Italy
[2]University Malaysia Pahang, Faculty of Engineering Technology (Program of Energy and Environment), 26300 Gambang, Malaysia

Abstract

Hydrolysates of *Arundo donax*, a crop offering high productivity in contaminated or salinized soils with no inputs of irrigation and agrochemicals, were used in a discontinuous fermenter to grow the oleaginous yeast *Lipomyces starkey*, to obtain microbial oils potentially useful for the production of 2nd-generation biodiesel.
A mixture of fermentable sugars was obtained by steam-explosion and subsequent enzymatic hydrolysis of the lignocellulosic materials. The concentration of Ni^{2+} ions and of inhibitors of the microbial growth significantly affected both the biomass and the triglyceride yields. The microbial lipids produced were compatible with the synthesis of an automotive-grade biodiesel.
A physico-mathematical model, developed to describe the biomass growth, demonstrated that the concentration of heavy metals affected the maximum biomass concentration, though its influence on the specific growth rate of the yeasts was not significant.

Key words: Arundo donax, heavy metals, microbial oils, oleaginous yeasts.

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1. Introduction

The application of the first-generation biodiesel, mainly obtained 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 (Huang et al., 2009; Yu et al., 2011), and by the insufficient availability of fertile land. In addition, the use of edible oils to produce biodiesel is threatening food supplies and biodiversity, causing social and environmental problems in developing countries.

New perspectives are offered by the recent development of new preparations of cellulolytic enzymes, that are able to efficiently hydrolyze lignocellulosic materials (Hendriks and Zeeman, 2009; Jorgensen et al., 2007), such as non-food parts of crops, forest products, and industry wastes, that can be recycled to obtain fermentable sugars. Though so far the hydrolysates of lignocellulose have been mainly used for the production of bioethanol, they can be alternatively employed for culturing oleaginous microorganisms, that are able to produce more than 20% of their weight in the form of lipids (Huang et al., 2012a; Pirozzi et al., 2013; Yu et al., 2011; Zhao et al., 2012). These lipids are mainly triglycerides, potentially exploitable as feedstock for the synthesis of the so-called *2nd generation biodiesel*. Among oleaginous microorganisms, oleaginous yeasts are particularly attractive due to their simpler cultural requirements (aerobic condition and a C/N ratio > 50), as well as to their ability to metabolize low-cost industrial wastes (Angerbauer et al., 2008; Fakas et al., 2008; Papanikolau et al.,

* Author to whom all correspondence should be addressed: email: dpirozzi@unina.it

2009). They are able to metabolize a wide range of wastes (Huang et al., 2012a; Liang et al., 2012; Meng et al., 2009; Papanikolaou et al., 2009; Pirozzi et al., 2013; Wang et al., 2012; Yu et al., 2011; Zhao et al., 2012), and have very simple cultural requirements, as the lipid accumulation occurs under nitrogen limiting conditions and excess of the carbon sources (Papanikolaou et al., 2011a, 2011b). 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).

The *Arundo donax* is 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., 2013) and salinized, and guarantee efficient protection to soils subjected to accelerated erosion (Fagnano et al., 2015).

In order to exploit the plant biomasses obtained from the polluted soils for the production of 2nd-generation biofuels, it is of crucial importance to analyse the effect of the pollutants in each stage of the process.

Heavy metals, apart from affecting the plant growth (Jamali et al., 2014; Malschi et al., 2013; Rashid et al., 2014), may significantly affect the performance of cellulolytic enzymes. In some cases, heavy metals at low concentrations may act as co-factors and activators to enhance the enzyme activity (Nanda and Abraham, 2013). Yet, in many cases they cause the inhibition of the cellular enzymes (Liu et al., 2010), forming complexes with the substrates, or interacting with specific functional groups, with enzyme-substrate complexes (Mikanova, 2006) and with sulphydryl groups (Shaw and Raval, 1961). They can also displace essential metal ions in metalloenzymes (Tamás et al., 2014), and affect the enzyme transcription (Baldiran, 2003). Obviously, different metals have a different effect on the enzyme performance.

Heavy metals can also affect the growth of the yeasts. Some heavy metals (e.g. Cu, Fe, Mn, Ni and Zn) may act as micronutrients contributing to the yeast's growth, whereas others (e.g., Cd and Pb) are not necessary for biological functions and produce only toxic effects (Bayoumi Hamuda and Toth, 2012). The toxic effect of heavy metals is often due to their affinity for cellular ligands (phosphates, purines, pteridines, porphyrins, or cysteinyl and histidyl side chains of proteins), that may cause the formation of complexes and clusters (Gadd, 1993). As a consequence, heavy metals can bind on the cell surface and alter the net charge of the cell, leading to the alteration of different physiological functions of the yeasts (Collins and Stotzky, 1992). They can also disrupt the integrity of the yeast's membranes (Avery et al., 1996).

Another potential problem for the production of the 2nd generation biofuels stems from the phenomenon of biosorption, i.e. the ability of the yeasts to adsorb or bind heavy metals. Obviously, biosorption may increase the chance of contamination of the final product (i.e. the biofuel).

In this study, we have characterized the effect of a heavy metal, namely the Ni^{2+} ions, on the growth of the oleaginous yeast *Lipomyces starkeyi* in the presence of hydrolysates of *Arundo donax*, and on the lipid accumulation within their cells. We have analysed the implications of the sorption treatment of the polluted hydrolysate, by evaluating the changes in the concentrations of both the Ni^{2+} ions and the inhibitors of the microbial growth.

Though the detoxification of the heavy-metal-contaminated wastes has been widely studied, very few studies have been so far focused on the removal of heavy metals from lignocellulosic hydrolysates (Lee et al., 2011; Mussatto et al., 2010; Silveira Chaud et al., 2012). In particular, no attempts have been made to evaluate separately the effects of the heavy metals and of other inhibitors on the oleaginous yeast's growth.

Lipomyces starkeyi have been so far grown using different waste materials as culture media, such as sewage sludge (Angerbauer et al., 2008), olive mill wastewaters (Yousuf et al., 2010), *A. donax* hydrolysates (Pirozzi et al., 2012), potato starch (Wild et al., 2010).

2. Materials and methods

2.1. Lignocellulosic biomass and microorganisms

A. donax was grown in open field conditions in hilly areas in Piana di Monte Verna (Campania, Italy). The transplanting of *A. donax* was made from rhizomes with a plant density of 1 x 1 m, and nitrogen fertilization was made at the end of rainy period (April) using a low dose (100 kg N ha^{-1} from urea). A strain of the oleaginous yeast *Lipomyces Starkeyi DBVPG 6193* (Culture Collection of the Dipartimento di Biologia Vegetale of the Perugia University, Italy) was used in the experiments. The yeast was maintained at 5 °C on YPD agar plate containing 20 g/L agar (Sigma), 20 g/L D-glucose (Sigma), 10 g/L yeast extract (Sigma) and 20 g/L peptone (Fluka). Prior to fermentation, 100 mL preculture in YPD medium containing 20 g/L D-glucose, 20 g/L peptone and 10 g/L yeast extract in 250 mL flask was carried out at 161 rpm and 30 °C for 24 h (Minitron, Infors HT, Switzerland).

2.2. Hydrolysis of lignocellulosic biomasses

The hydrolysis of the *A. donax* biomass was carried out following a two-step protocol, based on a steam-explosion followed by enzymatic hydrolysis. The steam explosion was carried out loading a 100 g batch of biomass in a pressurized vessel (5 L), and

supplying saturated steam, heating the material at 205°C for 10 min.

Subsequently, the enzymatic hydrolysis was carried out using cellulase from *Trichoderma reseei ATCC 26921* (Sigma-Aldrich) and cellobiase from *Aspergillus niger* (Sigma-Aldrich). The activity of cellulase was determined as 161 filter paper units (FPU)/mL and 20.3 cellobiase units (CBU)/mL (Adney and Baker, 1996; Ghose, 1987).

2.3. Fermentation of yeasts in hydrolysates of A. donax

The growth tests were carried out using a fixed volume (150 mL) of hydrolysate without external organic supplement, contained in conical flask of 500 mL. The liquid medium was inoculated by 2 mL of microorganism suspension (optical density = 0.12 at 600 nm, which corresponds to ca. $2 \cdot 10^6$ CFU/mL). 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 effect of Ni^{2+} ions on the growth of oleaginous yeasts was analyzed by addition of $NiSO_4$ in three different concentrations (0, 50, 100 μM).

2.4. Removal of heavy metals

Ni^{2+} ions were removed by adsorption. The hydrolysate was first neutralized with NaOH to pH 6.5, and then treated with activated charcoal at different weight ratios. The hydrolysates were then incubated 8 h at 30°C and 160 rpm, then filtrated to remove the adsorbent. Finally, pH was adjusted to 6.5 with $Ca(OH)_2$ or 5 M H_2SO_4.

2.5. Lipid extraction and measurement

Methanol (5.0 mL) and chloroform (2.5 mL) were added to 200mg 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 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). After the extraction, the lipid amount was determined by weighting the extracted lipids after drying at 105°C for 1 h (Fontanille et al., 2012; Gong et al., 2013; Huang et al., 2012a; Liang et al., 2012; Tapia et al., 2012; Wang et al., 2012; Zhao et al., 2012).

2.6. Biomass and chemical analyses

The biomass growth was evaluated in terms of dry biomass weight. To this scope, the biomass was collected by centrifugation at 6000 rpm for 10 min and dried at 105°C until constant mass was achieved.

The concentration of Ni was measured following a modified method of Demirci et al. (1999) to prepare the samples. Briefly, the yeasts were precipitated by centrifugation (10,000 rpm and 4ºC for 15 min), collected, washed three times (with 0.9% saline water) to remove the compounds adsorbed on the cell surface, and dried. Concentrated nitric acid (2.5 ml) was added to dried yeasts (0.05 g) in a kjeldahl flask, and heated at 160ºC. Concentrated sulfuric acid (1 ml) was then added under constant heating.

Small amounts of concentrated nitric acid were added until the solution was colorless, to keep oxidizing conditions. The sample was heated until a fume of sulfuric acid was observed, then cooled, diluted with distilled water (10 ml), filtered and diluted again with distilled water. The concentration of Ni was measured by an atomic absorption spectrophotometer at 213.9 nm.

Potential inhibitor compounds were analyzed by HPLC (LC2010c), equipped with a refractive index detector (RID-10A, Shimadzu, Japan), following the method adopted by Chen and co-workers (2009). The reducing sugars were measured by the Nelson-Somogyi method (Sadarivam et al., 1996).

2.7. Statistical analysis

All experiments were carried out adopting a minimum of three replicate tests. All values are means of three separate experiments. Differences were considered statistically significant for $P < 0.05$.

3. Results and discussion

3.1. Hydrolysis of A. donax biomass

In a first series of experiments, samples of *A. donax* were hydrolyzed by a two-step procedure, based on a steam-explosion followed by enzymatic hydrolysis, obtaining a final concentration of reducing sugars of 15.6 g/L in the hydrolysate. The pH of the hydrolysate was 5.2. The hydrolysate was characterized as regards the inhibitors of the microbial growth produced in the course of the hydrolysis (Papanikolaou et al., 2009; Yousuf et al., 2010) due to the degradation of sugars (e.g. furfural from xylose and 5-hydroxymethylfurfural), of lignin (e.g. vanillin, syringaldehyde, and 4-hydroxybenzaldehyde) and of other components of the lignocelluloses (e.g. acetic acid from acetyl groups, formic acid from xylose oxidation, and levulinic acid from glucose oxidation) (Palmqvist and Hahn-Hägerdal, 2000).

The concentrations of some potential inhibitors in the hydrolysate of *A.donax* are reported in the Table 1.

Table 1. Concentrations (g/L) of the main inhibitors of the microbial growth in the raw hydrolysate of *A. donax*

Inhibitors	*Concentrations (g/L)*
Acetic acid	5.6
Levulinic acid	1.6
Formic acid	1.8
Furfural	0.091
5-HMF	0.82

3.2. Effect of Ni^{2+} ions on the growth of L- starkeyi and growth model

The subsequent tests were aimed at analysing the growth of *L. starkeyi* in the hydrolysates of *A. donax*. A typical growth profile is reported in Figure 1 (square symbols).

The effect of the addition of $NiSO_4$ on the yeast's growth is also described in the Fig. 1. Significant decreases in the final biomass concentration were observed in the presence of $NiSO_4$: 22% with 50 μM $NiSO_4$, 30% with 100 μM $NiSO_4$. The addition of $NiSO_4$ also reduced the growth rate of *L. starkeyi* (Table 2).

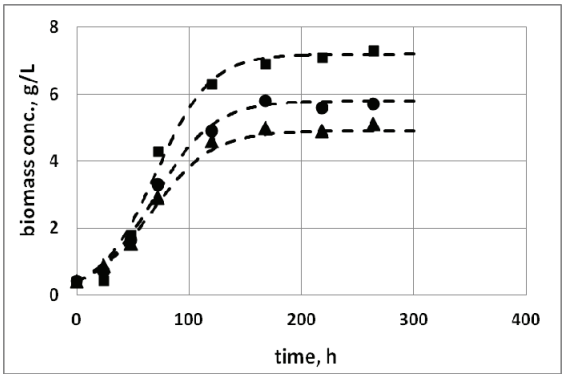

Fig. 1. Effect of the $NiSO_4$ concentration on the growth profiles. L. starkeyi cultured in batch reactors, in the presence of *A. donax* hydrolysate, T = 30°C. The dashed curves represent the theoretical data obtained with the logistic model. $NiSO_4$ concentrations: 0 μM (■), 50 μM (●), 100 μM (▲)

These results can be explained taking into account that, in the presence of 100 μM nickel, the absorption of Ni^{2+} ions in the yeast's cells may occur (Mariano da Silva et al., 2007). The nickelous ions absorbed may inhibit both the growth and the cellular metabolism of the yeasts (Joho et al., 1995), due to their interaction with enzymes (Hausinger, 1987), or other cellular components (Martin, 1988; Van Steveninck, 1966).

The pH value may affect the toxicity of the nickel. At lower values of the pH, nickel free ions are more available for interaction with the yeast, as their tendency to form nickel oxide and hydroxide is reduced (Hahne and Kroontje, 1973). As a matter of facts, the toxicity of nickel at pH 6.0 has been found very low (Soares et al., 2003), whereas the growth rate of yeasts is significantly reduced at pH 3,5 (Mariano da Silva et al., 2007). In our experiments, during the oleaginous fermentations, the pH dropped from an initial value of 5.2 to final values in the range 3.5-3.8. This may explain the observed reduction in the growth rate and in the final biomass concentration of *L. starkeyi*.

The profiles of biomass concentration obtained under different experimental conditions were described by a logistic growth model. The model includes a biomass balance (Eq. 1), where μ is the specific growth rate, defined by the logistic Eq. (2).

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

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

In order to obtain the *X(t)* profiles, Eqs. (1-2) 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 = 0.95). In the Fig. 1 a graphic comparison is made between the model predictions (dashed curves) and the experimental results obtained at different concentrations of $NiSO_4$.

The results reported in Table 2 demonstrate a good correspondence between the experimental and theoretical data. The experimental data indicate that the final biomass concentration (X_{max}) decreases as higher concentrations of $NiSO_4$ are adopted, confirming the results shown in the Fig. 1. On the contrary, the specific growth rate (μ_{max}) appears not to be significantly affected by the $NiSO_4$ concentration.

3.3. Effect of the preliminary sorption of Ni^{2+} ions

The Fig. 2 summarizes the results obtained in the tests for the removal of Ni^{2+} ions from the hydrolysate at pH 6.5. The removal fraction increased with the amount of charcoal added.

Table 2. Comparison of experimental measurements of growth parameters with the theoretical data obtained with the logistic model, at different concentrations $NiSO_4$. Values are significantly different (P < 0.05, n = 3)

Concentration of $NiSO_4$, μM	*Xo*	*μ_{max}, h^{-1} (exp)*	*μ_{max}, h^{-1} (pred)*	*X_{max}, g/L (exp)*	*X_{max}, g/L (pred)*
0	0.4	$4.33 \cdot 10^{-2}$	$4.12 \cdot 10^{-2}$	7.12	7.21
50	0.4	$3.70 \cdot 10^{-2}$	$3.81 \cdot 10^{-2}$	5.88	5.86
100	0.4	$3.66 \cdot 10^{-2}$	$3.70 \cdot 10^{-2}$	5.01	4.96

A maximum removal fraction of 82% was obtained adopting charcoal concentrations above 0.06 g/(100 mL). Subsequently, the effect of the sorption treatment on the oleaginous fermentation was evaluated. To this scope, we carried out a growth test of *L. starkeyi* in a medium obtained from an *A. donax* hydrolysate with an initial Ni^{2+} ion concentration of 100 μM, after sorption treatment with 0.08 g/(100 mL) of charcoal (indicated as **sample A** in the Fig. 2). The residual concentration of Ni^{2+} ion concentration in the sample A was 25 μM. The sorption treatment did not cause appreciable changes in the concentration of reducing sugars (15.3 g/L in the sample A).

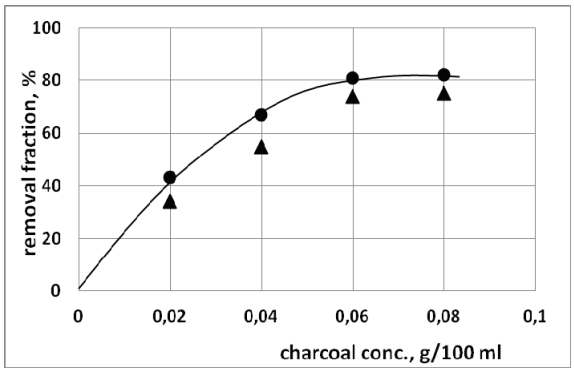

Fig. 2. Removal fraction of Ni^{2+} ions from the *A. donax* hydrolysate as a function of the charcoal concentration adopted. T = 30°C, pH 6.5, incubation time: 8 h. Initial concentration of Ni^{2+} ions: 50 μM (●), 100 μM (▲)

In order to achieve a clearer evaluation of the effect of the sorption treatment, we prepared a reference growth medium (**sample B**) from the raw hydrolysate of *A. donax*, adding a similar concentration of Ni^{2+} ion as in the sample A (25 μM). The Fig. 3 describes the effect of the Ni^{2+} ion concentration and the sorption treatment on the growth profiles of *L. Starkeyi*.

The experimental results showed a significant difference between the maximum biomass concentrations obtained using samples A and B as growth media, though the Ni^{2+} ion concentrations in these media was the same. The higher biomass production observed in the presence of sample A could be explained taking into account that the sorption treatment of the hydrolysate removes not only the Ni^{2+} ion, but also the inhibitors of the microbial growth contained in the raw hydrolysate (see Table 1).

Therefore, the growth of *L. Starkeyi* in the presence of sample B was slower as compared to that in the presence of sample A, due to the lower concentration of inhibitors in the sample A caused by the sorption treatment. In order to confirm this hypothesis, we measured the concentration of some inhibitors in the sample A. The data shown in Table 3 demonstrate that the content of all the measured inhibitors in the sample A is lower as compared to their content in the raw hydrolysate of *A. donax*. The pH values of samples A and B were very similar to the pH of the *A. donax* hydrolysate (i.e. 5.2). Therefore, these results were not affected by changes in the availability of nickel free ions for interaction with the yeast, as discussed in Paragraph 3.2.

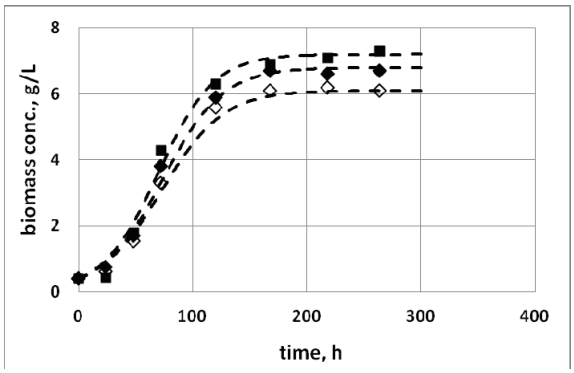

Fig. 3. Effect of Ni^{2+} ions and sorption treatment on the growth profiles. L. starkeyi cultured in batch reactors, in the presence of *A. donax* hydrolysate, T = 30°C. The dashed curves represent the theoretical data obtained with the logistic model. Growth media used:- raw hydrolysate of *A. donax* (■);- sample A (◆): raw hydrolysate of *A. donax,* after addition of $NiSO_4$ (100 μM) and sorption treatment with 0.06 g/(100 mL) of charcoal, to obtain a final Ni^{2+} ion concentration of 25 μM;- sample B (◇): raw hydrolysate of *A. donax,* after addition of $NiSO_4$ (25 μM)

Table 3. Concentrations (g/L) of the main inhibitors of the microbial growth. Values are significantly different ($P < 0.05$, $n = 3$)

	raw hydrolysate of A. donax	***sample A[1]***
Acetic acid	5.6	4.4
Levulinic acid	1.6	1.4
Formic acid	1.8	1.3
Furfural	0.091	0.073
5-HMF	0.82	0.24

[1]*sample A has been obtained as described in the paragraph 3.3*

3.4. Production of the microbial triglycerides

Table 4 reports the final biomass concentration and the lipid fraction within the yeast's biomass (%) obtained under different experimental conditions. The experimental results indicate that, when using the raw hydrolysate of *A. Donax* as growth medium, the lipid fraction was not significantly affected by the Ni^{2+} ion concentration.

Yet, the lipid fraction obtained when growing the yeasts in the presence of sample A was significantly higher, showing that the accumulation of lipids is more significantly affected by the inhibitors contained in the growth medium than by Ni^{2+} ions. However, when using the sample A the biomass fraction was lower. Consequently, the maximum lipid concentration (g/L) was obtained when using the raw hydrolysate with no $NiSO_4$ addition.

Table 4. Biomass and lipid concentrations of *L. starkey* grown under different conditions. Values are significantly different ($P < 0.05$, $n = 3$), unless they have a common superscript

$NiSO_4$ conc., µM	*Biomass conc., g/L*	*Lipid fraction, %*	*Lipid conc., g/L*
0	7.2	19.9[a]	1.43
25	6.8	20.1[a]	1.37
50	5.8	19.9[a]	1.15
100	4.9	19.8[a]	0.97
sample A[1]	6.1	22.5	1.37

[1]*sample A: raw hydrolysate of A. donax, after addition of $NiSO_4$ (100 µM) and sorption treatment with 0.06 g/(100 mL) of charcoal, to obtain a final Ni^{2+} ion concentration of 25 µM.*

The increase in lipid accumulation at lower concentrations of the inhibitors produced in the course of the hydrolysis is in agreement with previous studies In particular, it has been shown (Huang et al., 2012b; Zhao et al., 2012), that furfural is a strong inhibitor of the lipid accumulation of the yeasts.

As regards the effect of the heavy metals on the lipid accumulation, some conflicting indications are given by the studies available on this topic. Redox-active metals, such as Nickel, can induce free-radical production and promote oxidative stress (Hosiner et al., 2014). As a consequence, lipid peroxidation can be induced, causing a reduction of the lipid yield in the oleaginous yeasts. On the other hand, recent studies on fungi have shown (Yilancioglu et al., 2014) that oxidative stress can promote the lipid accumulation. In any case, our results demonstrate that Ni^{2+} ions, under the conditions adopted, do not affect significantly these mechanisms.

4. Conclusions

In spite of the presence of inhibitors of the microbial growth and of Ni^{2+} ions, *A. donax* hydrolysates could be used to produce microbial triglycerides potentially useful for the synthesis of II-generation biodiesel.

In all instances, the growth profiles of L. starkeyi in the *A. donax* hydrolysates could be described adopting the logistic curve model, obtaining a satisfactory correspondence between the experimental and theoretical data. Increases in the $NiSO_4$ concentration resulted in the reduction of the biomass yield and of the specific growth rate of the yeasts.

We demonstrated that the treatment with charcoal of *A. donax* hydrolysates containing heavy metals can significantly increase the yields in biomass and in lipids, due to the simultaneous removal of Ni^{2+} ions and of inhibitors of the microbial growth.

The lipid fraction within the biomass was increased by the removal of inhibitors. On the contrary, the lipid fraction was not significantly influenced by the concentration of Ni^{2+} ions.

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