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Bioconversion of *Jatropha curcas* seed cake to hydrogen by a strain of *Enterobacter aerogenes*

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HIGHLIGHTS

- *Jatropha curcas* seed cake biomass (JSC) was used as feedstock for bioH_2 production.
- A pure culture of the bacteria *Enterobacter aerogenes* fermented JSC.
- *E. aerogenes* was efficient for 2.5–10 gVS_{JSC}/L_{FM} with a maximum of 238.2 mL H_2/L_{FM}.
- A new pathway to enhance by-products valorization in biodiesel production chain.

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ABSTRACT

Hydrogen (H_2) gas is considered the future energy carrier as a clean fuel. Biological processes to produce hydrogen are very attractive due to less energy expenditures and the possibility to use organic wastes as substrate. In this work, *Jatropha curcas* L. seed cake (JSC), a solid residue remaining after oil extraction from *J. curcas* seeds for biodiesel production, was used as substrate in a dark fermentation process by a pure strain of the bacteria *Enterobacter aerogenes*. Batch assays were performed using the substrate (2.5 gVolatile Solid/$L_{Fermentation\ Medium}$) submitted to thermal pretreatment in an autoclave for two different exposure times (15 and 30 min) and the results were compared with the ones obtained when the JSC was used without pretreatment. The best specific biohydrogen production (68.2 mL H_2/gVS_{iJSC}) was attained for the conditions of no substrate pretreatment, which is an advantage from the viewpoint of energy saving. In the best conditions, the increase of the initial JSC concentration from 2.5 to 10 gVS/L_{FM} led to the increase of the cumulative hydrogen production and to higher bioH_2 production rates. However a decrease on the specific H_2 production from 68.2 to 23.5 mL H_2/gVS_{iJSC} was observed.

1. Introduction

The energy sector is a major contributor to man-made greenhouse gas emissions, therefore, to reach a projected target of 80% reduction in gas emissions by 2050 will put pressure on existing energy systems [1].

There is a need therefore to explore sources of renewable energy that are viable and able to fulfill human energy demands and contribute towards a decarbonised economy. Hydrogen is considered the "future energy carrier" due to its proprieties as a clean fuel with no CO_2 emissions. It is the most promising fuel with most technical, socio-economic and environmental benefits [2]. H_2 has the highest energy content per unit weight of any known fuel (142 kJ/g) [2–4]. Among the many hydrogen production processes (conventional and non-conventional), biological methods are known to be the less energy intensive, since most of them can operate at ambient temperature and pressure [5,6]. Another advantage is the use of complex organic waste as substrate on bio-hydrogen production fermentative processes, degrading organic compounds and producing clean energy at the same time. In addition, the abundant availability and low cost of the feedstock makes it a very attractive option from both the environmental and economic viewpoint. In this context, the process of dark fermentation to produce bioH_2 requires carbohydrate-rich substrates and fermentative bacteria [7,8]. In recent years, bioH_2 production through dark fermentation has received increased attention due to its many advantages, such as the high hydrogen production rates, the potential to convert biomass or bio-wastes into hydrogen, and the feasibility of the effective process design and control [9].

During dark fermentation, bioH_2 can be produced by microbial consortia, where the prevailing feedstock are unsterilized waste

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materials, or through more controlled fermentation conditions by specific and highly H_2-producing strains. Good H_2-producers include mesophiles, such as species of clostridia and *Enterobacter*, and thermophiles such as *Thermotoga neapolitana* [10]. *Enterobacter aerogenes*, an anaerobic facultative bacterium, has been described as a good H_2 producer in fermentations with the most diverse substrates, such as organic urban solid wastes [11], biodiesel residues containing glycerol [12] and cyanobacterial and microalgal biomass, such as *Anabaena* [7], *Scenedesmus obliquus* [13] and *Nannochloropsis* [14] biomass.

Carbohydrates are the main source for bacteria during fermentative processes, for that reason, some types of waste can be suitable to use as substrate during those processes [3].

Jatropha curcas (*J. curcas*) is considered as a very promising feedstock crop for the production of biodiesel. In fact, there are studies that demonstrate that its low cost and high conversion yield of oil to biodiesel makes this crop a very competitive option [14]. In addition, *J. curcas* oil is non-edible because of the presence of toxic compounds. Several studies have shown that the major factor responsible for its toxicity is the high concentration of phorbolesters in the seeds with known tumour promoting activity [15,16].

J. curcas production has increased immensely in the past years due to the demand for biodiesel as an alternative fuel. It is estimated that by 2015 the global cultivated area of *J. curcas* will be around 12.8 million hectares [17].

J. curcas seed cake (JSC) is a by-product from the oil extraction process [17]. Authors have reported that a biodiesel plant using *J. curcas* produces 2.5–3.0 tons of solid waste per one ton of biodiesel [18]. The need for *J. curcas* waste management strategies should be addressed in order to avoid potential environmental hazards [18,19].

After oil mechanical extraction, JSC may hold 25 MJ/kg of energy, as well as 3.8–6.4% nitrogen and 0.9–2.8% phosphorus [20]. Therefore, energetic valorization of this waste has been pointed out as the most favorable choice from an environmental and energy point of view. However, most of complex organic wastes are lignocellulosic materials; therefore, pretreatment is commonly used to release sugars and other compounds from their complex structures, making these compounds more available to bacteria degradation and consequently leading to higher yields. Many authors have studied various types of pretreatment [21–26] such as physicochemical, biological and chemical treatments.

Recent studies using de-oiled *Jatropha* waste (DJW) as a feedstock in an anaerobic digestion process by a sewage sludge inoculum shows that the application of different pretreatments and hydrolysis methods to the DJW leads to the release of reducing sugars with consequent enhancement of the fermentative process yields [25].

The aim of this work was to evaluate the bioH_2 production by a strain of the bacteria *E. aerogenes* in batch dark fermentation using JSC as source of carbon feedstock. The effects of thermal pretreatment and concentration were studied. Specific hydrogen productions were compared. The results obtained represent an important contribution to increase the know-how on bioH_2 production from this agro-industrial solid waste, as carbon source feedstock, using a robust hydrogen producing bacteria (*E. aerogenes*). In fact, to our knowledge very few studies have been conducted to produce hydrogen from JSC as substrate [25,27].

2. Materials and methods

2.1. Fermentative bacteria

The fermentations were carried out by *E. aerogenes* ATCC 13048 *Sputum* (American Type Culture Collection, Manassas, USA), which was harvested from exponentially grown cultures. The original culture was kept at 4 °C in solid CASO Agar (from MERCK: 15 g/L peptone from casein, 5 g/L peptone from soymeal, 5 g/L sodium chloride and 15 g/L agar–agar). The bacterial synthetic growth medium used was a 20 g/L peptone and 5 g/L NaCl solution, without an additional carbon source. A preculture grown overnight at 37 °C and 150 rpm was inoculated at 1% (v/v) and the fermentation was conducted at 30 °C for 6 h, 220 rpm. The fermentation media for the bioH_2 production assays (basal fermentation medium) contained K_2HPO_4 (7.0 g/L), KH_2PO_4 (5.5 g/L), tryptone (5 g/), yeast extract (5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.25 g/L), $CaCl_2 \cdot 2H_2O$ (0.021 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.12 g/L), nicotinic acid (0.02 g/L), Na_2SeO_3 (0.172 mg/L), $NiCl_2$ (0.02 mg/L), with a pH of around 6.8.

2.2. J. curcas seed cake (JSC) feedstock

The JSC used in this study as feedstock was obtained from a biodiesel industry in Santiago Island (Cape Verde). Such JSC correspond to the solid waste remaining after mechanical oil extraction from *Jatropha* seeds.

The provided JSC was previously milled in a coffee grinder, before its use as a substrate and the powdered JSC was analyzed in terms of its content in volatile solids (VS, 867.7 gVS/Kg_{JSC}) [28] and in residual oil content (13.7% (w/w)) [29]. All analyses were performed in duplicate.

To characterization of JSC biomass in terms of volatile solids (VS) content, firstly, moisture was determined by drying the sample in an oven at 105 °C until constant weight; following, total ash was determined by incineration at 550 °C in a muffle furnace. The VS (% w/w) was calculated by the difference between dry and ash weights [28].

JSC residual oil content was assessed by Soxhlet extraction, using 12 g of grounded raw material and hexane as extracting solvent, for 6 h. The amount of oil was determined gravimetrically after solvent evaporating and drying [29].

2.3. Experimental set up

2.3.1. Batch experiments

Batch fermentation assays were performed in around 160.0 mL serum bottles sealed with butyl rubber stoppers and crimped with aluminum seals, containing 26.0 mL of fermentation medium and treated (Section 2.3.2) and untreated JSC.

The sterilized serum bottles (and fermentation medium) were aseptically purged with bubbling N_2 to eliminate O_2, before inoculation with exponentially grown *E. aerogenes* at 1% (v/v). The fermentation was carried out under magnetic stirring, for 6 h (equilibrium time) at 30 °C in a P-SELECTA Hotcold-GL 2101507 heater, according to the operational conditions optimized in previous studies [12].

All the experiments were performed in triplicate and the results are expressed as average ± standard deviation. Control fermentation assays, without *J. curcas* seed cake, were also prepared for comparison.

2.3.2. Effect of thermal pretreatment and JSC concentration

The purpose of this study was to study the effect of thermal pretreatment and JSC (substrate) concentration on specific bioH_2 production by dark fermentation. Regarding the effect of thermal pretreatment, in this work it was associated with the fermentation medium sterilization, for energetic reasons. So, experiments were performed in which the powdered JSC was added to the fermentation medium and were submitted to thermal conditions of 121 °C for 15 and 30 min (2 atm) in an autoclave. This allowed for both thermally treating the substrate (substrate with pretreatment)

and sterilizing the fermentation medium before starting the fermentative process.

For comparison purpose, experiments were also conducted wherein the powdered JSC was added to the fermentation medium after its sterilization, under aseptic conditions and before inoculation of the serum bottles (substrate without thermal pretreatment). In all experiments, the JSC concentration was 2.5 gVS/L_{FM}.

To evaluate the effect of initial powdered JSC (substrate) concentration on the performance of the bacteria, experiments were carried out using increasing concentrations of JSC ranging from 2.5 to 10 gVS/L_{FM}, without thermal pretreatment.

2.4. Analytical methods

2.4.1. Analysis of the gaseous phase

The gaseous phase samples were collected directly from the headspace of the serum bottles by using a gas-tight syringe. The content of H_2 and CO_2 was analyzed by gas chromatography, at atmospheric pressure, in a Varian 430-GC equipped with TCD (Thermal Conductivity Detector) and a fused silica column (Select Permanent Gases/CO_2-Molsieve 5A/Borabound Q Tandem #CP 7430). Injector and column were operated at 80 °C and the detector at 120 °C. Helium was the carrier gas.

Specific hydrogen productions – mL H_2/gVS$_{JSC}$ and mL H_2/L_{FM} – were calculated by dividing the total volume of hydrogen produced by the initial amount (in terms of VS) of JSC used as fermentation substrate (according to the initial concentration of substrate), in terms of its volatile solids content and by the fermentation medium volume in the serum bottle, at 6 h of the process.

2.4.2. Analysis of the liquid phase

In order to evaluate the efficiency of the thermal pretreatment during 15 and 30 min on the release of fermentable compounds, into fermentation medium, samples were taken and the supernatants obtained by centrifugation (21,500 g; 2 min) and filtration (0.2 μm) were analyzed by HPLC using a Merck Hitachi HPLC system (Darmstadt, Germany) equipped with an Aminex HPX-87H column (BioRad) and a refractive index detector. The temperature of the column was set to 50 °C, and the eluent consisted of H_2SO_4 5 mM at 0.5 mL/min flow rate.

3. Results and discussion

3.1. Effect of thermal pretreatment

The effect of JSC thermal pretreatment on bioH_2 production was studied using as substrate the powdered JSC with and without pretreatment (Section 2.3.2.), for comparison (Table 1).

According to the results, tests carried out using the powdered JSC (substrate) without thermal pretreatment led to higher biohydrogen production (68.2 ± 5.3 mL H_2/gSV$_{iJSC}$/169.0 ± 6.3 mL H_2/L_{FM}) than the ones performed with the substrate previously submitted to 121 °C by 15 for 30 min (60.1 ± 1.3 mL H_2/gSV$_{iJSC}$/148.9 ± 1.1 mL H_2/L_{FM}) (Table 1). In fact, the thermal pretreatment of the powdered JSC did not result in a significant bacteria performance improvement.

This behavior may be related to the release of toxic compounds to the liquid phase (fermentation medium) when JCS was submitted to the thermal conditions (121 °C), which might have caused inhibitory effects on bacteria performance. Studies conducted by the authors demonstrated that this toxicity is not related with chemical reactions between hydrolyzed compounds from JSC and nutrients in the fermentation medium during sterilization. In fact, it was found that very similar specific H_2 productions were obtained when the powdered JSC was added to the fermentation medium before sterilisation in autoclave (121 °C for 15 min) – 60.9 ± 0.3 mL H_2/gVS$_{iJSC}$ (Table 1) or when the powdered JSC was

Table 1
Biohydrogen production performance using powdered JSC (substrate) with and without thermal treatment in an autoclave for 15 and 30 min. Control: in the absence of JSC; fermentation operational conditions: JSC concentration = 2.5 gVS/L_{FM}; $T_{Process}$ = 30 °C; magnetic stirrer ~200 rpm.

JSC pretreatment	Specific H_2 productions	
	(mL H_2/gVS$_{iJSC}$)	(mL H_2/L_{FM})
Control	0.0	0.0
Without	68.2 ± 5.3	169.0 ± 6.3
121 °C/15 min	60.9 ± 0.3	154.0 ± 0.8
121 °C/30 min	60.1 ± 1.3	148.9 ± 1.1

FM – Fermentation Medium.

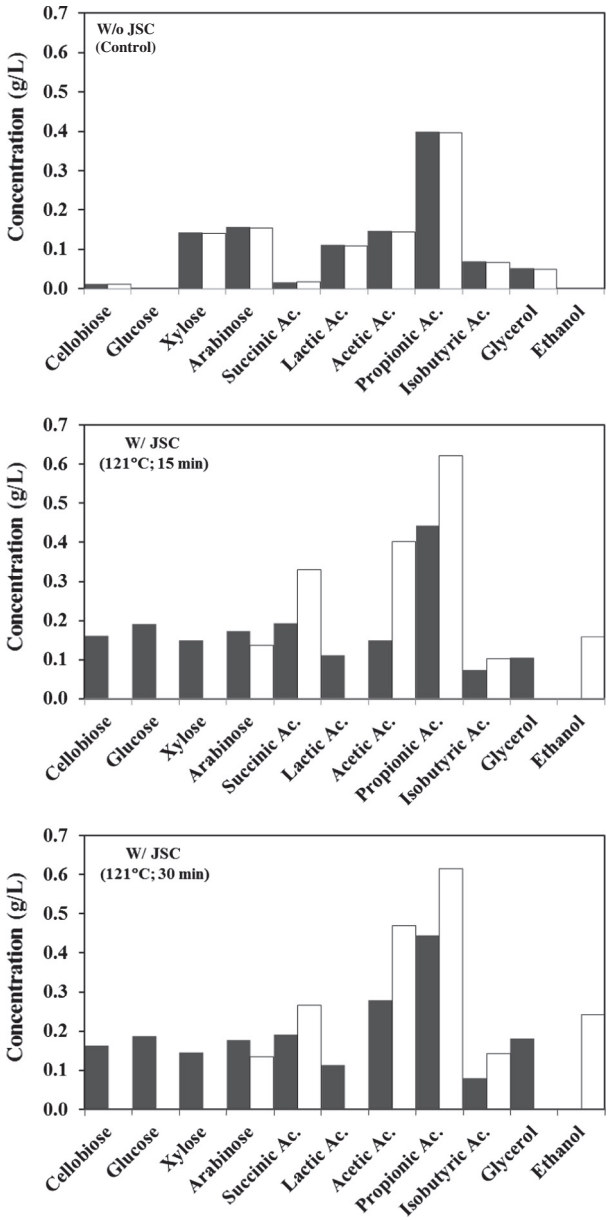

Fig. 1. Initial (■) values of sugars, organic acids, glycerol and ethanol contents in the fermentation medium, after thermal treatment in an autoclave (121 °C) for 15 and 30 min, in the absence and in the presence of powdered JSC (2.5 gSV/L_{FM}), and final (□) values in the liquid phase after fermentation process.

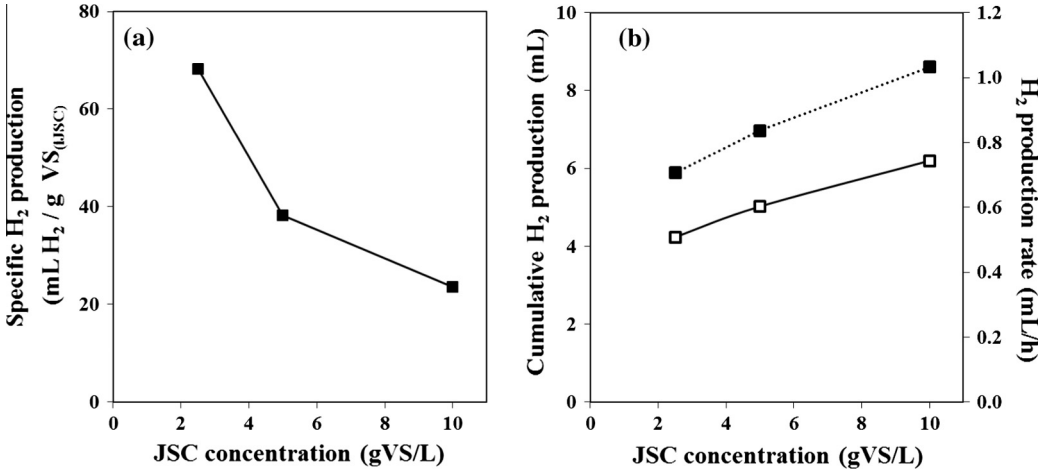

Fig. 2. Effect of JSC concentration on (a) the fermentative hydrogen yield and (b) cumulative H_2 production (open symbols) and rate (filled symbols) by *Enterobacter aerogenes*.

firstly autoclaved (121 °C for 15 min) in deionized water and the hydrolyzed posteriorly added to the fermentation medium – 57.1 ± 0.2 mL H_2/gVS_{iJSC}.

An important aspect to point out in this work is that although thermal pretreatment did not result in the improvement of dark fermentation, the conversion of JSC into hydrogen was occurred with specific bioH_2 production values well above those reported by other authors using as feedstock (substrate) de-oiled *Jatropha* waste collected from a biodiesel industry in Taiwan [27]. In this case, only 10.6 ± 0.2 mL H_2/gVS was obtained by a consortium (sewage sludge collected from a municipal wastewater treatment plant) in the optimal process conditions, corresponding to a substrate concentration of 200 $g/L_{nutrient\ medium}$, a process temperature of 55 °C and an initial pH solution of 6.5. Addition to low biogas (containing H_2 and CO_2) production the energy costs involved in this thermophilic process (55 °C) should also be emphasized in comparison with the one presented in the present study (mesophilic process, 30 °C) [27].

Fig. 1 presents the initial values of sugars, organic acids, glycerol and ethanol contents in the fermentation medium after thermal treatment in an autoclave (121 °C) for 15 and 30 min, in the absence and in the presence of powdered JSC (2.5 gSV/L_{FM}) for comparison with its final concentration values in the liquid phase after fermentation process.

As can be verified the initial concentration ranges of the compounds are quite similar being observed only a small increase in the concentrations of cellobiose, glucose and arabinose, after thermal treatment in the presence of powdered JSC. Already, after dark fermentation process a total consumed of sugars and glycerol and an organic acids and ethanol production can be observed proving the bioconversion of the substrate into biogas (containing H_2 and CO_2). However, relating results presented in the Table 1 with the one presented in the Fig. 1 it can be concluded that the JSC delivered to the fermentation medium other compounds in addition to the organic acids, sugars and glycerol, which acted as a carbon source for the specific and highly H_2-producing strain used in this work (*E. aerogenes*), since in the absence of substrate production of hydrogen did not occur (control fermentation assays).

Previous studies carried out by one of the authors [7,13,30], showed that the strain of the *E. aerogenes* used in this study was found to be more efficient in the degradation of JSC than of the other complex substrates (residual biomass) such as microalgal biomass of *S. obliquus* (57.6 mL $H_2/g\ VS_{alga}$ from 2.5 g_{alga}/L) [13], *Nannochloropsis* sp. (60.6 $mL/g_{dry\ biomass}$) [30] and biomass of the cyanobacteria *Anabaena* sp. after photoautotrophic H_2 production (15.2 $mL/g_{dry\ biomass}$) [7].

3.2. Effect of substrate concentration

The effect of the initial substrate (JSC) concentration on hydrogen production was evaluated in the fermentations of increasing JSC concentrations from 2.5 to 10.0 gVS/L_{FM} (Fig. 2). Following the previous results, all experiments were performed using the substrate without pretreatment.

Fig. 2 shows clearly that the specific bioH_2 production were influenced by the increase in substrate concentration, leading to a decrease on the specific H_2 production from 68.2 to 23.5 mL H_2/gVS_{iJSC}, after 6 h of the process. On the other hand, with the increased of the initial powdered JSC concentration from 2.5 to 10.0 gVS/L_{FM} (Fig. 2a) an increase of both the cumulative H_2 production (4.2–6.2 mL, Fig. 2b) and of the specific H_2 productions in terms of fermentation medium volume (168.95–238.19 mL $H_2/L_{fermentation\ medium}$, Table 2) were observed. In addition, a higher bioH_2 production rate (0.7–1.0 mL/h) was attained (Fig. 1b). However, these increases were not proportional with the increased of JSC concentration, suggesting inhibitory effects (by substrate (JSC) and/or products) with increasing initial substrate concentration. This behavior was also observed by Kumar and Lin [27].

Another effect which may have contributed to the behavior of the system is the increase the partial pressure of hydrogen in the headspace of the serum bottles with the increase of the initial substrate concentration, which may also lead to inhibitory effects on the performance of the fermentative bacteria [12].

Table 2
Biohydrogen production performance using different initial concentrations of powdered JSC (2.5, 5 and 10 gVS/L_{FM}). Control: in the absence of JSC; operational conditions: $T_{Process}$ = 30 °C; magnetic stirrer ~200 rpm.

Powdered JSC concentration (gVS_{iJSC}/L_{FM})	Specific H_2 productions	
	($mL\ H_2/gVS_{JSC}$)	($mL\ H_2/L_{FM}$)
Control	0.0	0.0
2.5	68.2 ± 5.3	169.0 ± 6.3
5.0	38.2 ± 2.4	193.0 ± 12.3
10.0	23.5 ± 0.3	238.2 ± 6.2

FM – Fermentation Medium.

4. Conclusions

The strain of the bacteria *E. aerogenes* used in this study proved to be efficient to convert *J. curcas* seed cake into hydrogen through a dark fermentation process (bioH_2).

The highest specific hydrogen production (68.2 mL H_2/gVS_{iJSC}) was obtained with a concentration of untreated JSC of 2.5 gVS/L_{FM} after 6 h of fermentation (equilibrium time), what can be considered as an advantage in terms of reducing energy expenditure.

The specific bioH_2 production were negatively influenced by the increase in substrate (JSC powdered) concentrations from 2.5 to 10 gVS/L_{FM}. Taking into account that the biological process presented in this study depends on the performance of the hydrogen producing microorganism (strain of the bacteria *E. aerogenes*) this behavior may be associated with inhibition factors caused by an increase of the substrate (JSC) concentration and/or the partial pressure at the *headspace* of the serum bottle, since an increase in the volume of H_2 produced was observed.

The process developed in this work, in mesophilic regime (25–35 °C), led to higher specific bioH_2 productions than those obtained by Kumar and Lin [27] in thermophilic conditions (55 °C), proving that the pure culture of bacteria (facultative anaerobic) used was effective for biologically converting JSC into H_2. This is advantageous both from the economic and environmental point of view, because it not only uses the waste as a carbon source but it also produces a clean energy (H_2) through a biological process that occurs at 30 °C and atmospheric pressure conditions.

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