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Biotechnological Synthesis of Succinic Acid By *Actinobacillus succinogenes* by Exploitation of Lignocellulosic Biomass

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Succinic acid is increasingly used in pharmaceutical industries, for the production of additives in food industries, in agriculture and in refinery processes as a precursor of many chemical compounds among which the most important is the succinate salt. It is also used as an ion chelator and surfactant, and for the biochemicals production. Currently, succinic acid is mainly produced through chemical petroleum-based processes, usually from n-butane using maleic anhydride. However, the use of petrochemical feedstocks raises serious environmental problems, due to the higher values of temperature and pressure required. The biotechnological production of succinic acid by microbial conversion of lignocellulosic biomass is attracting growing interest due to the environmental and economic advantages offered.
This research is focused on the exploitation of Arundo donax (Giant reed) as a source of lignocellulosic biomass. Arundo donax is a perennial crop particularly suitable for energy production, as it offers high yields per hectare, even in partially fertile or polluted soils, not used for agriculture. Hydrolyzate of Arundo donax will be used as growth media for the *Actinobacillus succinogenes 130Z*, a bacterium typically found in the bovine rumen, that is recognized as one of the most promising for the biotechnological production of succinic acid, as it is able to produce higher concentrations of succinic acid. The experimental analysis is carried out to optimize the production of succinic acid taking into account the effect of the most critical parameters of the process (microbial biomass, pH, reducing sugars, volatile fatty acids, and succinic acid). Tests have shown that in 48h the sugars are completely biodegraded with a total production of bio-succinic acid of 5.9 g for 9.1 g of reducing sugars, an hourly production 0.12 g h^{-1} with a yield equal to 65%.

1. Introduction

Succinic acid (SA) is currently used as ion chelator, surfactant and as additive in the food and pharmaceutical industries (Zeikus et al., 1999). Recently SA has also generated interest for the preparation of biopolymers such as polyamides and polybutylene succinate (McKinlay et al., 2007; Song and Lee, 2006). The market of SA is projected to witness a CAGR (Compound Annual Growth Rate) of 22.6% by volume between 2014 and 2019, and is expected to generate a global market value of $486.7 million by 2019 (Research and Markets, 2014). Currently SA is mainly produced with chemical-based refinery process through maleic anhydride from *n*-butane and this kind of production is expansive and could causes serious pollution problems (Chen et al., 2012). The biotechnological production of succinic acid (Bio-SA) from lignocellulosic biomass (LB) could contribute to alleviate the dependence on oil for the production of these platform and derivates in the future (Delhomme et al., 2009), so the microbial conversion of LB into Bio-SA is attracting great and increasing interest as energy-saving and environmental-friendly process (Li et al, 2010).
In this perspective, in recent years many efforts have been directed to the development of efficient technologies to obtain renewable energy and biochemicals from LB, by recycling a large range of agricultural wastes and industry wastes (Toscano et al., 2013 and 2014). In this research paper Arundo donax (Giant reed), resulting from a treatment of steam explosion and subjected to enzymatic hydrolysis (EHY), was

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chosen as a source of LB because it is a perennial crop particularly suitable for energy production owing to biomass yields as high as 37.7 t of dry matter per ha and per year (Angelini et al., 2009). Being a non-food crop, it can be cultivated in partially fertile or polluted soils, not used for agriculture (Toscano et al., 2013). *Actinobacillus succinogenes 130Z* was chosen bacterial inoculum. As a matter of facts, this microorganism produces large quantities of SA compared to other bacteria and is recognized as one of the most promising bacteria for the optimization of the Bio-SA production on an industrial scale.

2. Material and Methods

2.1 Arundo donax enzymatic hydrolysis

Arundo donax (GR) was collected from Torre Lama (Campania, Italy) agro-land. Leaves were separated from stems, washed, dried overnight at 80 °C and minced with a chopper. The powder was pre-treated in the steam explosion plant of ENEA Research Center of Trisaia (Matera, Italy) at 210 °C for 6 min. The steam exploded LB was then subjected to EHY by the action of cellulase (Celluclast 1.5L, from Novozymes) and cellobiase (Novozyme 188, from Novozymes). Following Gong et al. (2013), the optimal ratios of 15 filter paper units of Celluclast and 30 cellobiose units of Novozyme 188 per gram of LB have been used. Hydrolysis of GR has been performed at 50 °C for 72h with 10% (w/v) of dry steam-exploded biomass in water. The hydrolyzate has been filtered (with filter paper) and it was subsequently centrifuged for 20 min at 2200 RPM; the supernatant was collected and was again filtered (with filter paper). Finally, the pH was adjusted to 6.8 with addition of a basic solution of Na_2CO_3 at pH 11.5 before use in fermentation process.

2.2 Actinobacillus succinogenes (As) 130Z

Actinobacillus succinogenes (As) 130Z was used in all experiments and was obtained from DSMZ. Cells have been activated in 50 mL sealed anaerobic bottles containing 10 mL of medium BHI (from Sigma-Aldrich). During the exponential growth phase, 10 mL were collected and inserted into in 125 mL sealed anaerobic bottles containing 90 mL of medium BHI, to get the final working volume of 100 mL (II-adaptation). Before inoculation medium BHI was heat sterilized at 121 °C for 20 min and were created the conditions of anaerobiosis (even after the bacterial inoculum) by bubbling nitrogen for 20 min. The pH of the medium was maintained at 6.8 with addition of a basic solution of Na_2CO_3 at pH 11.5. Anaerobic bottles were incubated at 37 °C and 150 RPM; at 24h of II-adaptation it was made the withdrawal then used as bacterial inoculum in fermentation of GR.

2.3 Arundo donax (GR) fermentations

Fermentation medium contained GR hydrolyzate as sole carbon source; resazurin (0.025 %v/v) was also added as anaerobiosis indicator. As bioreactor was used 125 mL sealed anaerobic bottles containing 90 mL of GR hydrolyzate and 10 mL of the bacterial inoculum (taken from the growth medium of *As 130Z* II-adaptation at 24h) were placed inside the bioreactor to get the final working volume of 100 mL.
Inside the bioreactor were created the conditions of anaerobiosis (even after the bacterial inoculum) by bubbling nitrogen for 20 min. The pH inside the bioreactor was maintained at 6.8 with addition of a basic solution of $NaCO_3$ at pH 11.5 every 24h. Anaerobic bottles were incubated for 48h at 37 °C and 150 RPM.

2.4 Analytical methods

Sampling of liquid and gaseous phases from crimped vials was performed according to standard anaerobic techniques (Strobel, 2009). The pH was analyzed by pH meter WTW Series Terminal 740.
The microbial biomass concentration was monitored by measuring optical density of liquid samples at 600 nm (OD_{600}). After centrifugation and filtration with 0.2 µm cut-off filters, the liquid sample was analyzed for reducing sugars and soluble fermentation products as organic acids; the concentration of reducing sugar was measured following a modified Nelson-Somogyi method (Nelson, 1944) while concentration of organic acids (acetic acid, butyric acid, propionic acid) was determined by GC analysis, using a Shimadzu GC-17A equipped with a FID detector and a capillary column with a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 µm film thickness, from SGE). Bio-SA was determinated wit commercial KIT of Megazyme (K-SUCC 01/14).

3. Results and Discussion

3.1 Enzymatic Hydrolysis of Arundo donax

The composition of the LB samples (Arundo donax) is reports in the Table 1, in terms of cellulose, hemicelluloses and lignin content (Pirozzi et al., 2013).

Table 1: Composition of Arundo donax

Lignocellulosic Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Arundo donax	43.9	23.9	18.9

Figure 1 shows the trend of reducing sugars in the treatment of EHY of GR.
The increasing trend of the sugars in enzymatic hydrolysis process is regular with a slight increase in the rate of production in the initial stages of the process (up to 24h), arriving in the final step at a concentration of about 9.1 g/L of total reducing sugars; if is considered the composition of the lignocellulosic biomass (Table 1) this results showed a conversion of approximately 67% and the data are consistent with the tests previously optimized by Zuccaro et al. (2014).

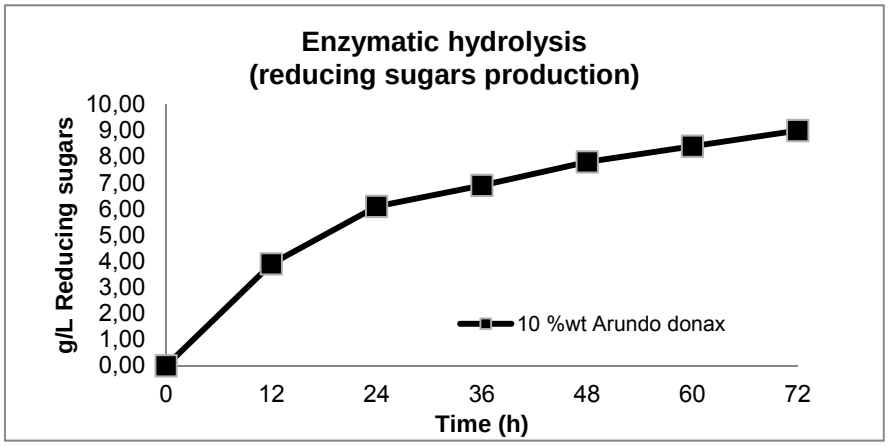

Figure 1: Enzymatic hydrolysis with 10% (w/v) Arundo donax for 72h at 150 RPM and 50 °C

3.2 Actinobacillus succinogenes 130Z

Figure 2 shows the trend of the microbial biomass during the reactivation process of *As 130Z* on BHI medium (from Sigma-Aldrich). The activation process was continued for 192h for understanding the behaviour of the microbial biomass on a time scale greater than that of the fermentation of GR.
Microbial biomass has an increasing trend constant and more rapid in the first 72h (in particular in the first 24h) of the process. After the growth is slower then to reach an absorbance peak at 168h; occurs after an endogenous phase. A T_{168} was taken then the sample that is used for the second adaptation in 100 mL of BHI medium.

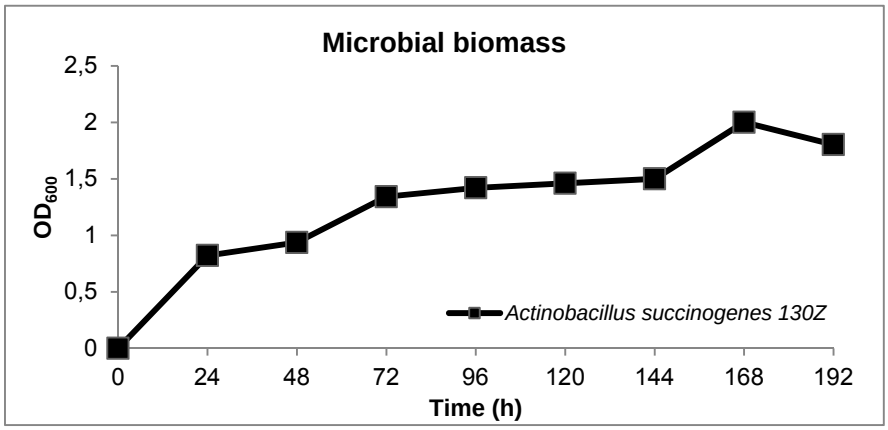

Figure 2: Reactivation of Actinobacillus succinogenes 130Z on BHI medium (Sigma-Aldrich) at 37 ° C and 150 RPM.

3.3 Arundo donax fermentation

Figure 3 shows the trend of the microbial biomass, pH and reducing sugars during fermentation of GR.
The trend of the microbial biomass is regular and reflects the ideal growth curve of microorganisms with an initial lag phase (very reduced, testimony that GR could be considered a good starting substrate), the exponential growth phase intermediate (very quick especially in the first 24h of the process) and the final endogenous phase. The metabolic activity of the microorganisms also agrees with the performance of pH that starting from the correct value substrate of about 6.8 and then reach a final value of 5.75, despite during the fermentation the pH was controlled at intervals of 24h by the addition of a basic solution.
The decreasing trend of reducing sugars is regular and constant during the entire fermentation process and it is slightly more rapid in the first 24h of the process in accordance with the growth of the microbial biomass; to T_{48} reducing sugars are next to zero, in accordance with the start of the endogenous phase of Actinobacillus succinogenes (*As*) *130Z.*

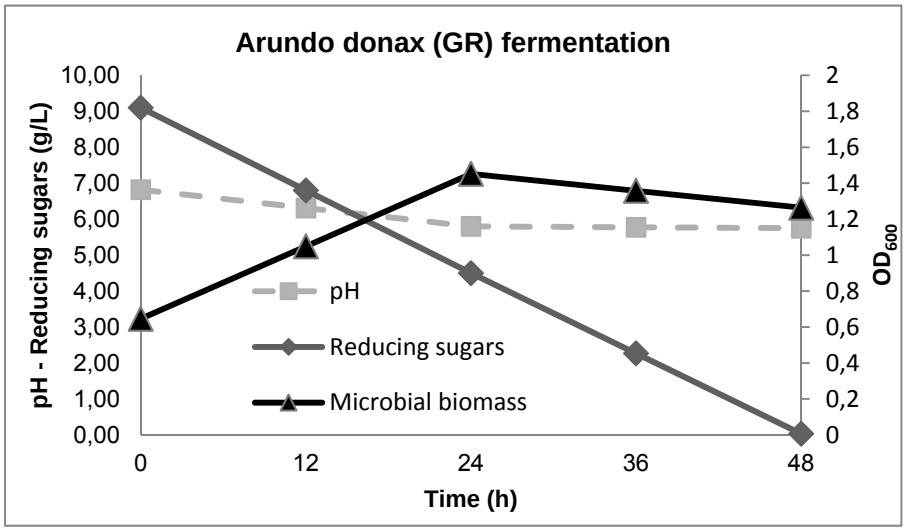

Figure 3: Fermentation of Arundo donax (GR) with Actinobacillus succinogenes (As) 130Z at 37 ° C and 150 RPM for 48h.

3.4 Volatile Fatty Acids (VFAs)

Figure 4 shows the trend of Volatile Fatty Acids (propionic, butyric and acetic) during fermentation of GR, that is in line with the data present in the literature (Gunnarsson et al., 2014). The presence of butyric acid could mean that inside the bioreactor the hydrolysis step continues performed by the bacteria; given the trend of the sugars, however, can be said that the rate of hydrolysis is slower than the rate of consumption of sugars.

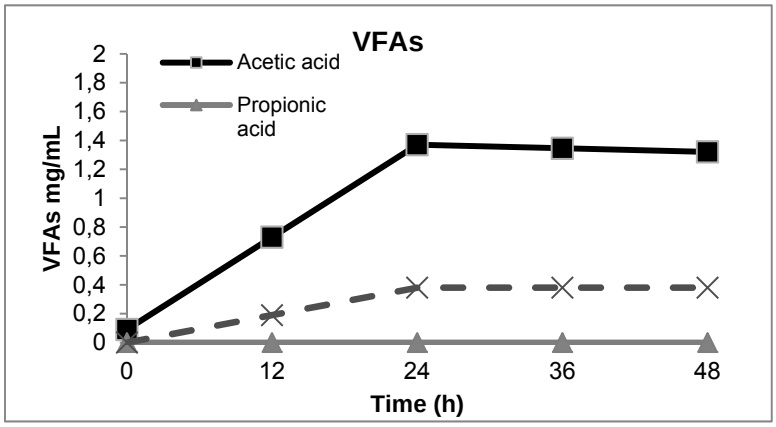

Figure 4: VFAs production during fermentation of Arundo donax (GR) with Actinobacillus succinogenes (As) 130Z at 37 °C and 150 RPM for 48h.

3.5 Succinic acid production

Tests of fermentation of GR have shown that in 48h there is a total Bio-SA production of 5.9 g for 9.1 g of reducing sugars. As can be seen from the daily production (Figure 5), in accordance with the trend of the microbial biomass and with the reduction of the sugars (Figure 3) the production of Bio-SA is more rapid in the first 24h of the process with a peak of production precisely a T_{24}; the hourly production is 0.12 g h^{-1}. *As 130Z* in these fermentation tests of GR hydrolyzate showed yields of 65%; these data are consistent with data reported in the literature (Song and Lee, 2005; Gunnarsson et al., 2014).

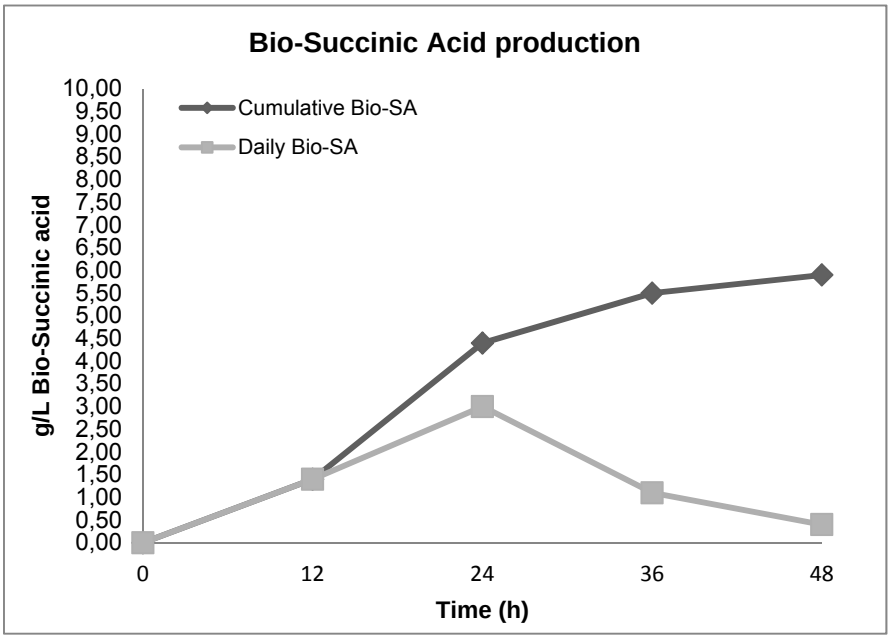

Figure 5: Bio-SA production during fermentation of Arundo donax (GR) with Actinobacillus succinogenes (As) 130Z at 37 °C and 150 RPM for 48h.

4. Conclusion

The results obtained in this experimental study identify the Arundo donax as a promising lignocellulosic biomass for the biotechnological production of succinic acid. The present investigation shows that Arundo donax can be easily hydrolyzed and fermented and *Actinobacillus succinogenes 130Z* presents good yields even in the absence of nitrogen and other nutrients source added inside the bioreactor. This study clearly shows that the use of Arundo donax is very attractive for the biotechnological production of succinic acid thanks to its high content of carbohydrates and related simple bioconversion, in addition to the fact that it grows even in adverse conditions of soil. In future tests it could be optimized process Simultaneous Saccharification and Fermentation (SSF) for further savings in economic and environmental costs.

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