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Evaluation of Pre-treatments of Brewery's Spent Grain for Growing Bacteria in the Production of Polyhydroxyalkanoates

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A suitable solution to solve the problems related to the conventional plastics is their replacement by biodegradable materials with similar properties such as polyhydroxyalkanoates (PHAs). PHAs can be produced by microorganisms using abundant low-cost substrates such as brewery's spent grain (BSG). In this work, different pre-treatments were tested on BSG to improve the growth and production of biomass using three different strains highly recognized for their ability to produce PHA. A solid-state fermentation (SSF) was carried out to evaluate PHA production. An evaluation of biomass growth kinetics of the three selected bacteria was made after different hydrolytic pre-treatments of BSG. Results showed that bacteria did not grow in control BSG and the pre-treatments were necessary to release the principal substrate metabolised by bacteria. The best result was obtained with alkali pre-treatment although microwave pre-treatment showed good results too. The strains *P. putida* and *C. necator* showed productions of 1.95 and 1.45 mg biomass/g BSG and viable cell counts of 1.5 E+16 and 1.3 E+08 CFU/g BSG after 72 hours of SSF, respectively. On the other hand, *Bacillus cereus* was not able to grow in the pre-treated substrates. BSG is a promising substrate for bacterial growth and PHA production for its nutrient content.

1. Introduction

Lignocellulosic agroindustrial by-products are renewable and represent an inexpensive source of biomass. Brewery's spent grain (BSG) is a disposal residue generated throughout the year in large amounts, being an 85% of total generated by-products from beer production (Vitanza, 2016). Average annual global production is estimated to be approximately 39 million tonnes, from which about 3.4 million tonnes are produced in the European Union (Mussatto, 2014; Becker, 2015; Vitanza, 2016). BSG is a by-product of brewing readily available with a high potential for industrial explotation due its high-volume production, low cost and nutritional content (Robertson et al., 2010). However, BSG has a high proteolytic activity, humidity and carbohydrate content which can affect its composition after industrial processing due bacteria affinity with the substrate (Ikurior, 1995).

So far, BSG has been used as animal feed, for production of value-added compounds such as enzymes, xylitol, lactic acid, to cultivate microorganisms, to extract compounds such as sugars, proteins, acids and antioxidants or as adsorbent for removing organic materials from effluents and immobilization of various substances (Oliveira, Freire and Castilho, 2004; Mussatto, 2009; Sharma and Kumar Bajaj, 2016).

The indiscriminate dumping of plastic materials into the environment has led to excessive accumulation of such materials in landfills. A suitable solution could be to replace these conventional plastics by biodegradable materials with similar properties such as PHAs (Mendez et al., 2016; Viloria et al., 2017). PHAs are a group of biodegradable polymers synthesized by several bacterial strains as energy reserve granules, under limitation of essential nutrients such as nitrogen or phosphorus and excess of carbon (Amache et al., 2013). Due to their biodegradability, compatibility and piezoelectricity PHAs have many applications in several fields, such as

packaging, food services, bio-medical, and agriculture industries (Rai et al., 2011; Nicolò et al., 2014). However, the production of PHAs is limited mainly by their high cost that depends directly on the substrate used as raw material, the PHA/substrate yield factor and the quality of the product required for the downstream processes (Yang, Huang and Ni, 2006).

Oliveira and co-authors investigated the use of SSF from agro-industrial wastes for the production of PHAs (Oliveira et al., 2004). Until that moment, most of the PHA production is based on submerged fermentation and, to our knowledge, there are very few reports are about the PHA production under solid-state fermentation (SSF). Since PHA is an intracellular product, the main difficulty associated to SSF is the separation of bacterial cells from solid substrate. However, SSF provides the possibility of using solid agro-industrial wastes as raw materials, thus contributing to minimize environmental problems related to waste management and reduce production costs (Sathiyanarayanan et al., 2013). In the case of BSG, the intake of hemicellulose and cellulose compounds (41.9 and 25.3% w/w dry matter) is difficult for some bacteria which can produce PHA but not the enzymes to degrade the lignocellulosic complex to obtain simple carbon (Lynch, Steffen and Arendt, 2016). Therefore, BSG needs previous treatments to break the lignocellulosic complex and produce simple sugars to improve the intake for the bacteria. In this work, different pre-treatments of BSG were tested to study the growth of biomass and the production and PHA by three different strains highly recognized for their ability in the production of PHA such as *Pseudomonas putida*, *Bacillus cereus* and *Cupriavidus necator.*

2. Methodology

2.1 Pre-treatments and BSG

BSG was kindly provided by *Companyia Cervesera del Montseny* (Catalunya, Spain) and stored at -20 ºC until its use. BSG dried at 60 ˚C for 48 hours was submitted to four different pre-treatments: (a) alkaline pre-treatment was carried out immersing BSG in 10 g/L NaOH to obtain a liquid-solid ratio of 8% (w/w) and autoclaved at 121˚C for 60 min. Then, the autoclaved BSG was washed using tap water until it reached to pH 10. The BSG was then immersed in 3% (v/v) hydrogen peroxide at 50 ˚C for 24 h and washed to adjust at pH 9 (Zhang *et al.*, 2013). (b) In the acid pre-treatment BSG was mixed with 3% (w/w) H_2SO_4 to obtain a liquid-solid ratio of 8% (w/w) and this mixture was autoclaved at 130 ˚C for 15 min. The pH was then adjusted to 5.5 with NaOH (Akhtar, Goyal and Goyal, 2017). (c) Thermal hydrolysis consisted in mixing the BSG with water in order to obtain a liquid-to-solid ratio of 8% (w/w). The mixture was autoclaved at 120 ˚C for 30 min (Carvalheiro, 2004). (d) In the microwave-alkaline pre-treatment, 5 g of BSG was placed in sealed vessels with 5 mL of 1% (w/v) NaOH, then it was heated in a Microwave Digestion System (MDS-2000 ®, CEM, Buckingham, UK) for 3 min at 850 W. After that, BSG was neutralized using distilled water and then dried at room temperature. Dried BSG was immersed in 1% (v/v) H_2SO_4 for 1h to achieve a liquid-solid ratio of 10:1 (v/w). At the end, BSG was washed thoroughly using distilled water until neutral pH (Akhtar, Goyal and Goyal, 2017). All of the pre-treated samples were dried overnight in an oven (60 ˚C) and then stored in moisture free container for further studies.

2.2 Solid State Fermentation

SSF was carried out in flasks (250 ml) adding 10 g of dry mass of different pre-treated and not pre-treated BSG moistened at 70 % w/w with Ringer solution in sterile conditions. *Pseudomonas putida* DSM 6125, *Bacillus cereus* DSM 31 and *Cupriavidus necator* DSM 428, obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) were inoculated individually to pre-treated BSG after a preadaptation steps in LB medium (24 h, 30 ºC). The flasks were sealed, and the fermentation was carried out at 30 ºC with agitation at 120 rpm, during 72 h. After 72h, the PHA production was evaluated qualitatively thought microscopy observation. After this first trial, a second assay of SSF was carried out only using alkaline pre-treated BSG as it showed the best results in the previous experiment. In this case, 30 g of the alkaline pre-treated BSG moistened at 70 % w/w with ringer solution were added in flasks of 500 ml in sterile conditions. These flasks were inoculated with the selected three strains. The fermentation parameters were the same mentioned above with an air flow of 50 ml/min. Bacterial growth, dry biomass and PHA accumulation were evaluated at 24, 48 and 72h in triplicate during the fermentation.

2.1 PHA observation

Heat-fixed smears of bacterial cells obtained washing with sterile water the solid medium was stained with the Nile blue solution at 55°C for 10 min in a coplin staining jar. After being stained, the slides were washed with tap water to remove excess stain and with 8% aqueous acetic acid for 1 min. (Ostle and Holt, 1982). PHA accumulation was qualitatively evaluated through microscopy observation (Zeiss Axioskop, Oberkochen, Germany)

2.2 Biomass recovery

After SSF assays, a sample of 5 g was taken from every flask, mixed with 15 ml of water and vigorously agitated for 20 min. After that, the mixture was filtered through AP25 paper (Millipore, Billerica, MA, USA) and the cake residues were washed with 10 ml water and filtered again. The filtrates were centrifuged for 30 min at 3400g and washed twice with water. The final bacterial pellet was dried at 60 ºC and weighted to determinate the quantity of biomass. Biomass data were expressed as dry weight (DW) of biomass per weight of BSG (Oliveira, Freire and Castilho, 2004).

2.3 Cell growth measurements

Growth of the three strains was monitored by plating out the cultures in agar LB media (Panreac) after serial dilutions 1:10. Agar cultures were incubated for 24–48 h at 30 ºC. Colonies were enumerated in terms of colony-forming unit per g of BSG (CFU/g) (Dahman and Ugwu, 2014).

3. Results and discussions

3.1 PHA production

Microscopy observation showed that non-treated BSG had the lowest production of PHA. On the other side, the best pre-treatment with high presence of PHA observed was alkaline followed by microwave pre-treatment using the three strains (Fig 1). The other pre-treatments were not successful, might be due to the lack of washing steps after the acid, alkaline or thermal process, then the presence of inhibitory compounds or toxic acids in the BSG reduce the biomass growth and subsequent PHA production (Carvalheiro, 2004). (microscopy pictures of other pre-treatments were not showed for its low visibility). Alkaline pre-treatment was selected to further evaluate the biomass kinetic growth of *P. putida* and *C. necator* during SSF of pre-treated BSG.

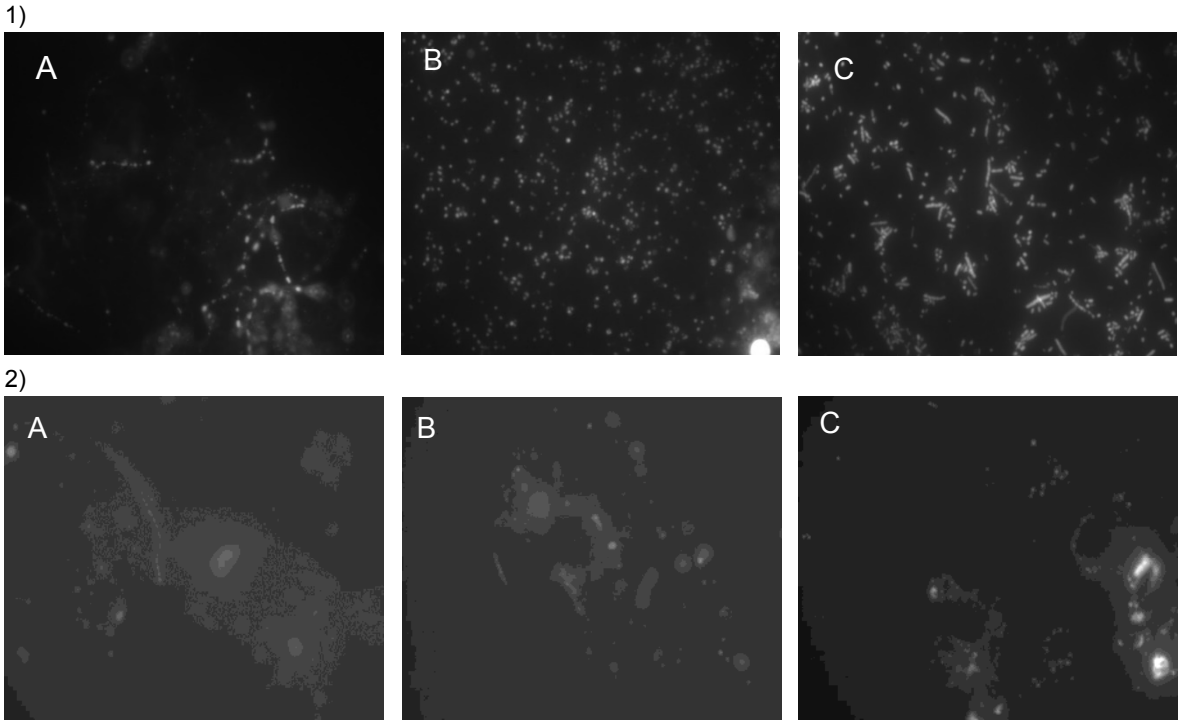

Figure 1. Microscopy observations with Nile blue technique for the PHA production in Bacillus cereus (A), Pseudomonas putida (B) and Cupriavidus necator (C) using BSG alkaline (1) and microwave (2) pre-treatment.

3.2 Kinetic growth of strains in alkaline pre-treated BSG

The growth of *C. necator*, *B. cereus* and *P. putida* in alkaline pre-treated BSG was studied. In case of *C. necator*, the population decreased at 4.0 E+01 CFU/g BSG in the first 24h and then it grew until 6.6 E+07 CFU/g at 48 h

maintaining a slow growing rate reaching values of 1.3 E+08 CFU/gBSG at the end of the experiments (Fig. 2A). The behaviour of this strain during the first 24 h could be due to an adaptation process of the bacteria to the substrate. In the case of *P. putida*, any adaptation step was observed, and it grew from the beginning of the fermentation until reaching the highest value of 1.5 E+16 CFU/gBSG at 72h. These results demonstrated that the *P. putida,* contrarily to *C. necator,* probably has the enzymes needed to metabolise sugars obtained after alkaline pre-tretament of BSG, and therefore, it could use this substrate to grow (Fig 2B). On the other hand, *Bacillus cereus* was not able to grow in the pre-treated BSG. Some authors reported a *B. cereus* biomass production of 14.4 mg/g of malt (Sharma and Bajaj, 2016), which means that the selected strain and the differences in nutritional value of the substrate could have high repercussions in bacterial growth.

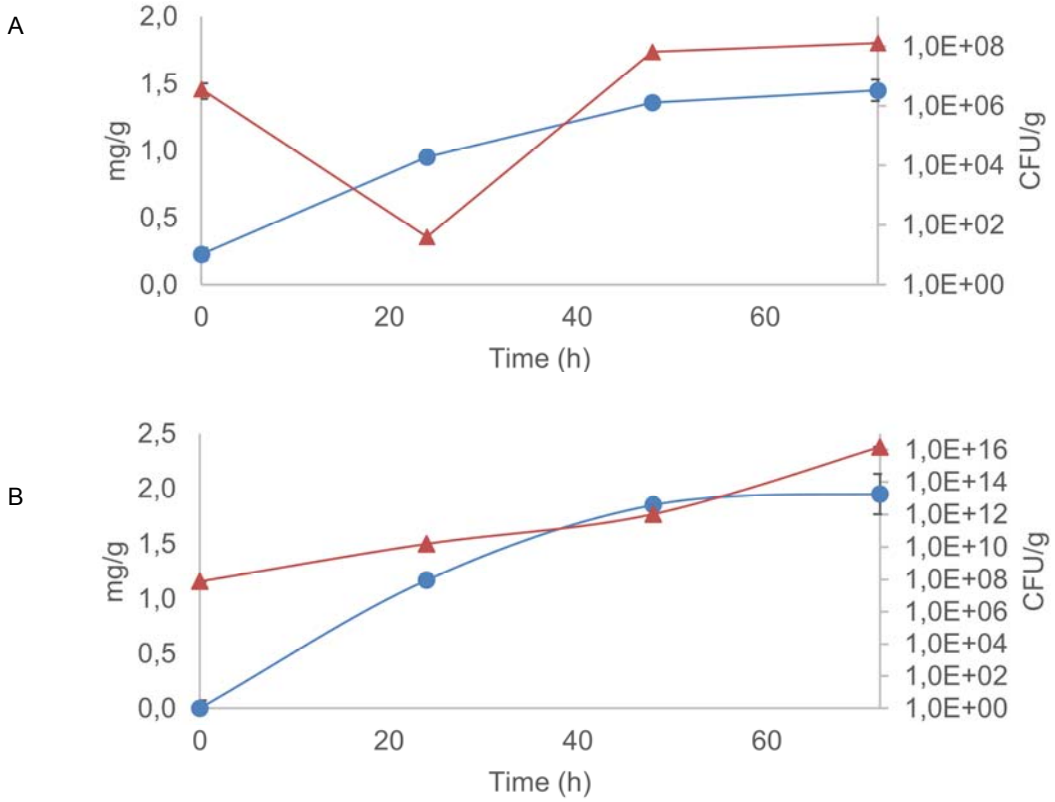

Figure 2. Kinetics of biomass production and bacterial growth of (A) *C. necator* and (B) *P. putida*. Biomass production (mg biomass/ g BSG) in the left axis (●) and bacterial growth (CFU/g BSG) in the right axis (▲)

The dry weight of produced biomass for *C. necator* and *P putida* at the end of the experiment (72 h) was 1.5 and 2 mg/g respectively lower values compared with other authors (Oliveira, Freire and Castilho, 2004; Ramadas *et al.*, 2013; Sathiyanarayanan *et al.*, 2013; Sharma and Kumar Bajaj, 2016). However, no additional nutrients or minerals were added to the pre-treated BSG in this work, testing the high nutrient value that the BSG has as substrate for bacteria growth. The results show a good kinetic growth behaviour for *P. putida* compared with *C. necator,* with a stationary state after 72 h for both strains, which probably indicate the lack of an important nutrient for the normal growth bacteria. However, as a first approach the success of the BSG used as a substrate for growing bacteria only with the pre-treatment, shows a high potential for further studies with the evaluated strains.

4. Conclusion

Production of biomass is an important step to improve the production of PHA. Furthermore, the use of agro-industrial residues as feedstock reduces the cost of the PHA production improving the economic feasibility of these bioplastics. This is essential to reach competitive prices and replace the petroleum-based plastics by biodegradable sources. The application of pre-treatments seems to be an important approach to obtain available substrates from industrial residues to the production of PHA. This investigation showed a significative release

of substrates from BSG, being the alkaline pre-treatment the most effective among the different pre-treatment methods evaluated. There was also observed the good behaviour of the different strains excluding the *B. cereus*. However, with this research it is possible conclude a good affinity of *P. Putida* for this type of substrate, allowing further investigations related with the medium optimization and better methods for biomass extraction.

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