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Beverage waste derived biomaterials for tissue engineering

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* In memoriam

The waste material from the beverage manufacturing industry was valorized as a renewable source for the manufacture of biomaterials capable of acting as scaffolds for tissue engineering. Environmentally friendly design of both structural and textural properties, produced biomaterials whose *in vivo* properties were similar to those of a commercial biomaterial.

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Introduction

Currently, one of the major challenges in this century is to develop sustainable manufacturing models in both the industrial and agricultural fields. The use of agricultural wastes as a valuable source of renewable raw materials (RRM) and commodities within the bio-refinery concept is being studied as a way to both decrease pollution and avoid the depletion of non-renewable resources. This has important environmental benefits linked to the reduction in Greenhouse gas emissions derived from their production, leading towards a cyclic “zero waste” economy, where the derived materials can compete with conventional ones, to support a sustainable development for an ever-increasing world population.^{1,2}

As a consequence of gas emissions from industry and agriculture, the valorisation of waste biomass is recognised, by different sectors such as academy, industry and governmental agencies, as an essential first step to make industrial development compatible with the protection of the environment. In addition, it is noteworthy the urgent need to decrease the use of non-renewable natural resources whilst seeking renewable sources both of which are important strategies to control and reduce climate change.

A bio-refinery approach was used in this study to valorise agricultural industrial wastes in the search for value added materials and chemicals.³ The multidisciplinary nature of the authors, with a wide expertise for the modification of chemical processes, leading to greener methods that produce both a reduction in the amounts of contaminating residues from the food industry in addition to the production of value added products has been shown. Mediterranean agricultural wastes valorisation (mainly fruits, cereals and oleaginous) has been carried out since the early 2000' for cleaner production of hydrogen and fine chemical intermediates, catalysts designed from residues to valorise a company's own residues, supports for enzymes or optimisation of structured materials for decontamination of effluents.⁴⁻¹⁰

Bone tissue engineering is a promising approach for more than 60 million people that are expected to suffer from bone and dental defects by 2020. Bone implants should address specific requirements to achieve a final clinical success such as their biocompatible, biodegradability and non-toxic character. Furthermore, bone scaffolds should have suitable mechanical properties and reproduce the bone environment in both compositional and structural characteristics.¹¹ In fact, the research in bone biomaterials development try to pursue by surface modification treatments, an increase in porosity or bioactive components released to enhance the osteointegration process. Currently, the standard goal of the treatment is for autologous bone grafts which consist in inserting a piece of bone tissue from the same

patient, taken from another part of their skeleton. This method does not suffer from any immune risk and has led to successful osteointegration. However, the use of autografts present several drawbacks such as limited tissue applied or morbid area of the donor site decreasing the possibility to obtain enough biocompatible bone tissue, able to colonize and differentiate cells and with optimum mechanical properties in the same patient.¹² Other options, less attractive in the biomedical field, such as allografts (bone tissue from different donors but of the same species) or xenografts (tissue from other species) have shown higher risks of infection and implant rejection.

For these reasons, biomaterials have become interesting for use as supports to solve the disadvantages of other approaches since they can be produced in large quantities and with similar physicochemical and mechanical properties to bone tissue. The majority of the production of biomaterials is carried out using non-renewable raw materials that are sold at more than 100 euros per gram, increasing the cost and thus the accessibility for medical treatments. As a direct consequence, several alternative sources of materials for bone regeneration have been sought for decades *i.e.* using natural sources such as coral, but although of low cost, its extraction causes a negative environmental impact and cannot be considered as sustainable.

Results and discussion

Beer bagasse (BB), obtained from the spent grain waste produced during the beer brewing process, has been used to prepare value added materials. It is a low cost, abundant by-product, produced in *ca.* 0.2 kg per L of beer that at present is mainly used in animal feed.¹³ The BB derived materials have been shown to be rich in calcium, phosphorous, silicon and magnesium (Table 1), as major inorganic components, which are at the same time the main components present in bone.¹⁴ Furthermore, previous work has shown that from a ton of BB, marketed at less than 50 euros, approximately 10 Kg of biomaterial can be produced. The difference in prices of commercial BB and the derived biomaterial is of several orders of magnitude and the latter has the added advantage of being renewable and environmentally benign.¹⁵

Silica is important in bio-mineralisation of living organisms, with osteogenic potential as the major component of bio-glasses, as well as being a promoter of resistance to dissolution of biocompatible calcium phosphates in acid environments such as those encountered during the first weeks in injured tissues. Amorphous silica is more effective to induce hydroxyapatite

(HA) formation than crystallized silica onto materials.¹⁶ Magnesium (Mg) is associated with the metabolism of calcified tissues, such as enamel, bone or dentin and it has been demonstrated that its content increases the mechanical properties of bio-glasses and stimulates osteoblast proliferation (amounts of magnesium oxide up to 13 wt.% are related to an optimal/favourable behaviour of biomaterials) and its depletion is related to lack of bone growth.^{17, 18}

In this study the BB was first dried at 150 °C, then milled to below 2.5 mm before heat treatment at 600 °C to drive off the organic fraction and leave just the inorganic constituents. This heat treated material was then milled to below 120 µm and pelletized in a die press at 4 tons/cm². The pellets were then heat treated during four hours at 700 °C (P7), 900 °C (P9), 1100 °C (P11) or 1200 °C (P12), respectively.¹⁹ The chemical compositions of P7, P9, P11 and P12 were similar within experimental error. The results obtained with P7 are chosen as an example in Table 1.

The XRD spectra of the BB-derived materials indicated the presence of calcium-magnesium phosphate (31.5 ° (100 %), 29.7 ° (85 %) and 29.3 ° (75 %)) and cristobalite (21.9 ° (100 %, (111)), 36 ° (12 %, (220)), 31.3 ° (10 %, (102)) and 28.4 ° (8 %, (111)) (Figure 1A), which increased in intensity with heat treatment temperature, with silica particle sizes of 70, 115, 250 and >300 nm and for the phosphate 124, 259, >300 and > 300 nm for P7, P9, P11 and P12, respectively.

Mercury intrusion porosimetry (MIP) of the pelleted materials indicated that with the increase in heat treatment temperature the total pore volume and porosities were reduced but the through pore diameter or threshold diameter, shown by the sudden upswing in the intrusion curve, shifted to wider pores (Figure 1B and Table 2). The pellets produced from the commercial HA had much narrower pore widths and a lower total pore volume due to the much smaller particle size of the starting material.

Literature search results indicated that osteoblasts MC3T3 cell growth is inhibited on crystalline silica containing biomaterials.^{20,21} The XPS analyses for the BB-derived materials (Figure 2 and Table 3) indicate an enrichment of silicon on the surface of the materials, by comparison of peaks for Si 2p (103eV, silica), P 2p (133.4 eV, phosphate), Ca 2p_{3/2} (347.5 eV, Ca phosphate) and Mg 2p (51 eV, magnesium phosphate). Low angle XRD analyses also confirmed that the silica-rich surface layer was formed mainly by cristobalite, especially as the heat treatment temperature was increased. Thus, although higher heat treatment temperatures improved the mechanical strengths of the pellets (an important property for the

biomaterials behaviour in bone tissue engineering applications), the formation of cristobalite, especially with heat treatments greater than 700 °C was detrimental for the behaviour of the biomaterials.¹⁶⁻¹⁸

In vitro tests of the cytocompatibility of these materials were performed in MC3T3E1 cells growing on pellets of P7, P9, P11 and P12 by the lactate dehydrogenase (LDH) assay. The obtained results indicated that cells cultured using both different cell culture media, basal and osteogenic, on BB-derived materials showed no obvious cytotoxicity effect when compared to cells grown on hydroxyapatite (HA) and on normal plastic (polystyrene) plates, used as control (Figure 3A). Furthermore, the cell death rate was similar in all BB samples. In addition, osteoblastic MC3T3E1 cells were able to grow over the BB samples which were analysed up to seven days in cell culture (Figure 3B). The number of cells growing on HA significantly increased after three and seven days in culture, unlike those growing on BB-derived materials that remained almost constant, most likely due to the presence of crystalline silica on their surface.¹⁸

To improve the cell proliferation the powdered BB materials were optimised by reducing the amounts of silica through a pre-treatment of the materials heated at 500 °C with NaOH solutions of different concentrations (0.5, 1 or 3N). The surface and bulk compositions of the alkaline treated BB-derived materials are included in Tables 1 and 3, designated P70.5N, P71N and P73N for 0.5, 1 and 3N NaOH treatments, respectively. The amount of silicon progressively decreased with increasing strength of the alkaline treatment (see Figure 4A) and consequently the amounts of the other main cations (P, Ca and Mg) increased. For the 3N NaOH treated material the contents of the main cations were close to those indicated in the literature as optimum for osteoblast growth.²⁰ Furthermore, heat treatments at only 700 °C helped to avoid formation of cristobalite.^{21,22}

The XRD analysis of alkaline treated materials indicated that there was no cristobalite (peak at 22 °) and the main crystalline components were calcium and magnesium phosphates (Figure 4A). This peak was still visible for the untreated and least concentrated base treated sample P7 and P70.5N respectively (see red arrows).

The porosities of the 700 °C pelleted materials after alkaline treatment (P70.5N, P71N and P73N) are shown in Table 2. From these results it may be observed that with increasing the alkali concentration there was a reduction in the total pore volume and porosity of the material, with a significant reduction in the through pore diameter. In addition, there was an increase in bulk density in the P73N for which the textural data was the most similar to that

found with the commercial HA, in terms of pore size, volume and surface area (see Figure 4 B and Table 2).

XPS results (Table 3 and Figure 4A) indicated that the amount of silica on the surface was greatly diminished. After 3N NaOH pre-treatment there was no surface enrichment of this element, while the other three main inorganic cations were in amounts similar to their bulk values. Mechanical testing experiments were undertaken, given the importance of this parameter on the *in vivo* behaviour of biomaterials related to bone performance, indicating (Table 2) that the BB-derived material after 3N NaOH treatment (P73N) was much harder than the other materials. In order to evaluate the potential use of these materials in the biomedical field, powdered BB bagasse of the untreated P7 and its alkali treated derivatives were developed as pellets in order to test them in *in vitro* experiments^{23, 24}.

Pellets of both BB-derived materials and HA were submerged in simulated body fluid (SBF) to promote the formation of bone-like apatite for osteoblastic cell proliferation.²⁵ Proliferation rates of MC3T3-E1 cells growing on P7, P73N and HA treated with SBF were analysed up to seven days (Figure 5A) indicating that osteoblastic proliferation rates were higher on P73N biomaterial compared to untreated P7 and more importantly to the commercial HA. To analyse the effect of the alkali treatment on cell differentiation, the expression of alkaline phosphatase, an early marker of osteoblastic cell differentiation, in MC3T3E1 cells was analysed. From Figure 5, alkaline treatment, apart from having an optimal effect on cell proliferation (Figure 5A), also improved significantly the differentiation of cells growing on BB-derived materials as shown by the increase in ALP enzyme activity (Figure 5B). Furthermore, osteoblast cells growing on the P73N biomaterial reached a differentiation degree twice that of cells growing on the reference material, HA. In addition, the cytoskeletal organization was determined by actin labelling using phalloidin-TRITC and nuclei staining with Hoechst. The stress fibres of actin were highly apparent in all cells on all material surfaces tested (Figure 5C) after three days of cell incubation. On P7 material cells did not spread out as much as on the other surfaces, showing shorter stress fibres. On P73N biomaterial, cells were well spread out, even compared with reference HA. On the HA materials, the stress fibres were also highly apparent but not as long and well organized as those found in cells growing on P73N biomaterial.

After the evaluation of the *in vitro* cytocompatibility of the BB derived materials, their *in vivo* behaviour in both powder and pellet form was determined. The biocompatibility character of BB samples were achieved by inserting them in direct contact with muscle tissue of Wistar rats (Figure 6A). The powder materials, a possible filling material in bone defects, were

surgically inserted into small pockets of muscle tissue and left for 21 days. Subsequently, macroscopic visualisation of removed samples and adjacent tissue, showed no signs of infection, oedema, exudates or inflammation of the muscle tissue around the implanted area. In addition, this absence of toxicity was confirmed also through histological images of Haematoxylin-Eosin stained (H/E) (Figure 6B) which was in accordance with the cytotoxic effect demonstrated under *in vitro* conditions. These materials were well integrated into the animal's tissue after 21 days where they were in direct contact with muscle fibres where some particles could still be discerned (see black asterisk). The same procedure was performed using pellets inserted into muscle tissue (see Figure 6C). The haematoxylin-eosin images showed the BB-derived materials (marked by a black asterisk) surrounded by muscle tissue and in some cases a soft and thinner fibrous capsule that covered the material was detected also. The latter effect was related to the recognition of the BB as a foreign body by the hosts' immune system, something frequently observed in biomaterial implantation. However, there were no signs of infection or rejection after 21 days, becoming accepted by the organism. It is noteworthy that in some areas the BB materials were in direct contact with the living tissue (without fibrotic capsule) highlighting their biocompatible and non-toxic behaviour. Furthermore, it was possible to detect higher amounts of connective tissue inside the damaged area, due to the porous structure of the samples which could allow connective tissue ingrowth. This interesting fact is a pre-requisite parameter to address in bone scaffolds because it has been shown that osteointegration is improved in biomaterials with a porous structure where bone tissue and new vessels can grow and penetrate through the scaffold regenerating the damaged area. The higher porosity found in P7 and P9 revealed an increase of integration compared to P11 and P12. Additionally, the presence of adipose tissue near the implantation site in P7 and P9 was remarkable as shown in their respective image inserts (A1 and B1), while P11 and P12 showed a highly compact structure that prevented tissue ingrowth. From these results, the calcination temperature of 700 °C was chosen for further experimentation.

After the encouraging *in vitro* and *in vivo* results, the best biomaterial P73N from the *in vitro* results was structured by inverse templating with polyurethane sponges (see Supplementary information for details) to achieve a 3D bone scaffold. These scaffolds present suitable pore diameters to allow the correct vascularization when implanted (*ca.* several hundred microns)^{26,27} by changing the preparation conditions of the structured materials, mainly using different template densities and ratios P73N/template. The structuration conditions were chosen to give materials with mechanical strengths of *ca.* 1700 Kg/cm² (> 95 % that of the pelletized P73N (Table 2) (see Supplementary information). The textural properties of material P73N could not be studied by MIP, since the through pores were above the maximum limit for the MIP

measurements (*ca.* 150 μm), however, microscopy studies confirmed that its pores were several hundred microns, as required to ensure cell viability (Figure 7, structure size $1\times 1\times 1\text{ cm}^3$).

With these 3D scaffolds we intend to evaluate under *in vitro* conditions with osteoblastic cell lines in order to analyse the effect of the micro and macroporosity on cell behaviour. In addition, further research is still needed in order to confirm the *in vivo* results in a bone defect model. The best samples will be evaluated alongside the commercial HA as a reference for further study of these scaffolds surgically located within pigs jaws as a model of bone tissue engineering.

Conclusions

To the best of our knowledge, this is the first time that beer production wastes have been successfully used as scaffolds for tissue engineering *in vivo*, after their transformation into tailored biocompatible, non-toxic materials.

These 3D scaffolds are several orders of magnitude cheaper than commercial ones and the methodology used for their optimisation is simple and environmentally friendly. This waste valorisation has a great potential market as well as important environmental benefits, allowing the preparation of value added materials.

Currently ways of conformation (inverse templating and 3D printing) are being studied in order to use these new waste derived biomaterials at a pilot scale.

Conflicts of interest

There are no conflicts to declare.

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Table 1. Chemical composition determined by ICP (main cations, wt. %) of BB derived biomaterials

Samples	P	Ca	Mg	Si
P7	13.0	7.5	6.4	17.0
P7 0.5N	17.7	11.4	8.1	11.3
P7 1N	19.5	13.1	9.2	10.7
P7 3N	21.9	14.4	10.3	6.8

Table 2. Textural data from MIP and Mechanical Strength of BB derived biomaterials

Samples	Pore Volume (cm³g⁻¹)	Porosity (%)	Surface Area (m²g⁻¹)	Bulk Density (gcm⁻³)	Skeletal Density (gcm⁻³)	Mechanical Strength (Kg/cm²)
P7	0.315	43.6	2.1	1.35	2.12	250
P9	0.267	40.3	0.9	1.39	2.46	421
P11	0.251	39.7	0.4	1.56	2.62	637
P12	0.258	36.1	0.3	1.59	2.63	880
P70.5N	0.236	38.4	7.0	1.47	2.25	538
P71N	0.210	37.9	7.0	1.83	2.96	808
P73N	0.144	34.7	12.5	2.63	4.24	1843
HA	0.106	20.2	13.2	1.90	2.38	1951

Table 3. Bulk (ICP) and Surface (XPS) compositions of BB derived biomaterials

Samples	Ca/P _{ICP}	Ca/P _{XPS}	Si/P _{ICP}	Si/P _{XPS}	Mg/P _{ICP}	Mg/P _{XPS}
P7	0.45	0.36	1.45	1.78	0.64	0.49
P9	0.56	0.22	1.44	1.96	0.59	0.31
P11	0.59	0.17	1.42	2.14	0.61	0.25
P12	0.60	0.14	1.43	2.32	0.62	0.20
P70.5N	0.50	0.43	0.71	1.06	0.59	0.47
P71N	0.52	0.50	0.61	0.88	0.61	0.54
P73N	0.52	0.51	0.34	0.34	0.61	0.63

Figure 1. A. X-ray diffraction of P7, P9, P11 and P12. B. Mercury intrusion porosimetry of P7, P9, P11, P12 and commercial hydroxyapatite (HA)

Figure 2. X-ray photoelectron spectroscopy analysis (Si 2p and P 2p regions) of P7, P9, P11 and P12

Figure 3. Cell viability on BB-derived materials. Cell death (A) and cell proliferation (B) rates of MC3T3E1 cells cultured on P7, P9, P11, P12 and HA materials were evaluated by the LDH and XTT assays, respectively. Values are shown as mean $\pm$ SEM ($n = 4$ per each group). * $p < 0.05$ vs. their respective 1 day data.

A

Figure 4. A. X-ray diffraction of P7, P73N, P71N, P70.5N. B. Mercury intrusion porosimetry of P73N, P71N, P70.5N and HA

Figure 5. Proliferation (A), differentiation (B) and morphology (C) of MC3T3-E1 cells cultured on modified BB derived materials. Cell proliferation (A) and differentiation (B) of MC3T3-E1 cells growing on HA and BB derived materials was evaluated by XTT and ALP activity, respectively. Values are shown as mean $\pm$ SEM (n = 4 per each group). * p < 0.05 vs. their respective 1 day data. § p < 0.05 vs. each other (A); * p < 0.05 vs. HA (B). Cell morphology was evaluated in C by phalloidin-TRITC staining for cytoskeleton visualization (red). Nuclei were counterstained with Hoechst (blue).

Figure 6. *In vivo* biocompatibility evaluation of BB derived materials applied in powders and in pellets in muscle tissue of Wistar rats. Surgery process (A) of the BB implantation, (B) the histological results of powder materials in muscle tissue by hematoxylin eosin staining and (C) the histological results of pellets in muscle tissue by hematoxylin eosin staining 21 days after surgery.

Figure 7. Photographs of powdered, pelletized and inverse templated BB derived material

For the first time waste from beverage manufacturing industry has been valorised as *in vivo* tissue engineering scaffold.