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Development of polycaprolactone scaffold with antibacterial activity by an integrated supercritical extraction and impregnation process

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ABSTRACT

The present study is aimed to develop a process for production of functionalized scaffolds impregnated with natural compounds extracted from Patagonian *Usnea* lichen. A setup for an integrated supercritical CO_2 extraction of natural compounds with posterior impregnation on solid matrices (polycaprolactone, PCL) is developed and presented here. In order to establish optimized operating conditions, supercritical extraction of *Usnea* as well as sorption kinetics and resulting material properties have been studied separately first. *Usnea* extracts isolated by supercritical carbon dioxide at 30 MPa and 40 °C have shown strong antibacterial activity with values of the minimum inhibitory concentration (MIC) ranging from less than 1.25 μg/mL to 320 μg/mL against *Listeria innocua* and Methicillin-resistant *Staphylococcus* (MRS) strains. Useful scaffolds of PCL for tissue engineering containing a porous structure with pore diameters between 150 and 340 μm can be obtained when PCL is exposed to carbon dioxide at 35 °C and 15 MPa. The degree of crystallinity of functionalized PCL was shown to be influenced by the incorporated antibacterial agent. The presented results showed that the impregnated PCL samples are promising bactericidal compounds against *L. innocua*. Screening of antibacterial activity of functionalized PCL against a Methicillin-resistant *Staphylococcus aureus* (MRSA) strain showed a higher activity when a low bacterial inoculum level (2×10^4 Colony-forming Units/mL [CFU/mL]) was assayed.

1. Introduction

An ideal scaffold for bone tissue engineering should be biocompatible, biodegradable, osteoconductive and it should provide structural support to the new formed bone [1]. These materials, obtained from natural or synthetic compounds, must have a proper degradation rate and some required characteristics related with the morphological feature for good performance. A scaffold should have an interconnected porous structure, sufficient mechanical strength and good cell–scaffold interaction. Pore size, pore morphology and degree of porosity are very important parameters in tissue engineering. An interpenetrating network of pores in the range of 100–500 μm is required to allow vascularization and tissue in-growth [2].

Polycaprolactone (PCL) is a semicrystalline and biodegradable polyester widely used for scaffold assembly [3]. A number of varied techniques has been developed to fabricate porous scaffolds of PCL, including fiber bonding [4], particulate leaching [5], solvent casting [6], selective laser sintering [7], supercritical fluid technology [8,9] and melt molding. Among these techniques, foaming with supercritical carbon dioxide ($scCO_2$) is superior to other methods for producing solvent-free porous structures. Hence, the use of $scCO_2$ is of high interest as an alternative "green" solvent for processing biodegradable and biocompatible polymers in pharmaceutical and medical applications [10–12].

On the other hand, fatal bacterial infections in humans and animals caused by multiresistant pathogenic bacteria have led to increased interest in research on isolation of natural antibacterial agents as well as production of new biomaterials with antibacterial properties. In this respect, lichens have been used in traditional medicine as a source of antibacterial substances [13]. Their efficiency in elimination of multiresistant pathogenic bacteria is addressed to the synthesis of unique secondary metabolites. Reportedly, lichens produce over 800 secondary metabolites comprising many classes of compounds [14,15]. Usnic acid, common to *Usnea* species, is proved to exhibit antibacterial, antiviral, antiprotozoal, antiproliferative, anti-inflammatory, analgesic and antipyretic activities [16]. Lichen extracts from *Usnea* species are

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used as natural drugs in topical preparations, mouthwashes, etc. with excelent results. However, due to the low concentration of the metabolites in the lichen, it is necessary to employ methods to achieve high purities and yields when extracting the active substances from lichens [17]. Additionally, functionality of scaffolds used in human medicine can be further improved by adsorption of active substances [3,18,19].

Since supercritical carbon dioxide is not only known as a possible solvent for valuable compounds but also for its high diffusion ability in organic matter, the latter property is used for impregnating solid matrices with natural antibacterial agents. In literature there are few studies that combine the above mentioned processes synergistically [20–22].

The aim of the present study is to design a process for production of functionalized PCL scaffolds with natural compounds extracted from Patagonian lichen and to assess its antibacterial properties. In this study, setup for supercritical extraction of natural compounds with posterior impregnation on a solid matrix was developed and presented. To our knowledge, this paper is the first to report the functionalization of PCL scaffolds with a natural antibacterial agent by using an integrated process at high pressure. The resulting materials show high potential as antibacterial devices against Methicillin-resistant *Staphylococcus aureus* (MRSA) strain.

2. Materials and methods

2.1. Materials

Usnea lichen was used as raw material for supercritical extraction of antibacterial substances. Patagonian lichen *Usnea lethariiformis* (Voucher specimens No. 16644, Herbarium of Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade) was collected in Ushuaia, Tierra del Fuego (Argentinian Republic) in 2011 (GPS-54.544221-67.202307). (+)-Usnic acid (UA) (Carl Roth, Germany) CAS 9012-76-4, was used for antibacterial analysis and as a standard substance for High Performance Liquid Chromatography (HPLC) analysis.

Granules of polycaprolactone (PCL), with a molecular weight of 80,000 purchased from Sigma Aldrich, Germany, were used as material for scaffold production. Commercial carbon dioxide (99% purity) supplied by Gamasol (Argentine) was used for all supercritical processes.

2.2. Supercritical fluid extraction (SFE)

Extractions with supercritical carbon dioxide from lichen of *U. lethariiformis* were performed at a pressure of 30 MPa and temperature of 40 °C. These conditions have been recently suggested for the isolation of *Usnea* extracts with strong antibacterial activity [17]. Sufficient effort had been taken to find the best operating conditions while there is still work to be done on optimizing pretreatment of the source material. In this work, the lichen was cut and subsequently subjected to milling either in a planetary mill (PM) (Fritsch, Germany) or a blade mill (BM) (IKA, Germany), using stainless steel recipients, during 15 min.

In each experiment, a known mass of lichen (of about 40–50 g) was introduced into the extractor vessel of a High Pressure Extraction Unit (HPE500, Eurotechnica, Germany) trapped by filter paper to prevent any carry over of particles. The $scCO_2$ flow rate used in the SFE experiments was 0.6 kg/h.

The effect of pretreatment of the lichen on the yield of the extraction was determined. The extracts were characterized by Fourier Transform Infrared spectroscopy, using a Nicolet spectrometer to identify the presence of functional groups of the active ingredients of the extracts.

2.3. Behavior of polycaprolactone in supercritical CO_2

The investigations on swelling and the sorption of CO_2 into polycaprolactone were performed in a high-pressure view chamber (P_{max} = 50 MPa, T_{max} = 120° C, Eurotechnica, Germany) provided with a digital camera on spherical polycaprolactone samples (diameter = 6 mm). Data of change in volume (dV %) and mean pore size after depressurization were collected as preliminary studies on impregnation of polycaprolactone with usnea extracts dissolved in supercritical CO_2. The parameters being investigated were pressure (10, 13, 15, 17 and 18 MPa), temperature (30, 35 and 40 °C) and depressurization rate (0.1, 0.5, 1 and 2 MPa/min).

Also, the shape and size of rectangular polycaprolactone samples were recorded as a function of time at constant pressure and temperature by means of a digital camera as mentioned above.

2.4. Integrated extraction-impregnation process

The proposed process design for inline impregnation of solids with antibacterial lichen extracts after supercritical fluid extraction thereof is shown in Fig. 1. This laboratory scale unit has been designed for an integrated extraction-impregnation process and was extended by closing the solvent (supercritical CO_2) cycle in a way that the extractor (E, 500 mL) and the adsorption column (A, 100 mL) can be operated at different temperatures and pressures independently from each other. The extractor vessel is filled with the raw material from which a target substance is to be extracted. The adsorption column is filled with solid to be impregnated by the extract from the prior extraction step. The maximum allowable working pressure of the extractor and adsorption column is 50 MPa. Electric heating enables heating of the extractor and adsorption vessel up to temperatures of T_{max} = 100 °C. The CO_2 is pumped into the extractor until the required pressure is obtained by a double acting pneumatically driven piston pump. In case of supercritical extraction of the *Usnea* lichen, the conditions are set to 30 MPa and 40 °C. Additionally, a view cell (T_{max} = 120 °C, P_{max} = 50 MPa, window diameter 18 mm) is connected to the extractor outlet and equipped with a microspectrophotometer (Ocean Optics, model USB4000 Miniature Fiber Optic Spectrometer) for inline detection of changes in solute concentration. The temperature inside the adsorption column containing PCL is set to 35 °C. A manual back pressure valve TESCOM (BPR) is used for pressure regulation within the CO_2-cycle between the extractor and adsorption vessels. A CO_2 flow meter (Ritter, Germany) is provided to indicate the consumption of CO_2 passing through the system.

The procedure for every test is characterized by two variables of time, t_1 and t_2, where t_1 is the time of continuous extraction–adsorption in a single passing mode at given conditions (extraction: 30 MPa/40 °C and adsorption: 15–17 MPa/35 °C) and t_2 represents the time of recycling of the solution at 15–17 MPa/35 °C through both. This process is referred to in the following as E-A procedure.

The obtained materials from this E-A procedure were compared to samples processed by an impregnation method without recycling, named E-VC procedure. For this semicontinuous extraction-impregnation process, polycaprolactone samples were placed in the view cell adjusting the pressure and temperature to 15–17 MPa and 35 °C respectively. The extractor vessel is filled with the raw material and operated at the same conditions as the E-A process. A valve between the extractor and the view cell was used for adjusting the pressure in both recipients. In this method the supercritical solvent – extract solution was not recycled but conducted in a one-way manner through the depressurization valve, where the residual extract remaining after the sorption step precipitates.

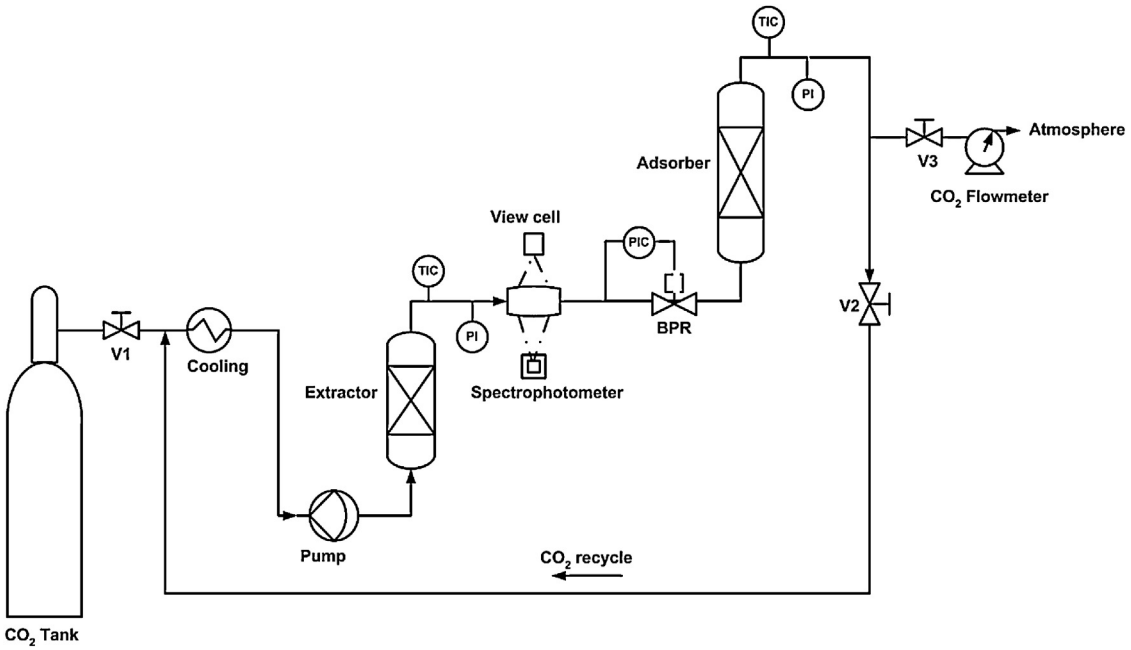

Fig. 1. Setup for a proposed process that integrates extraction, impregnation and foaming steps.

The name of the impregnated samples was assigned according to the process parameters: PCLt_1/t_2. Thus, the obtained samples were named: PCL3/0; PCL1/1; PCL2/1 and PCL2/4. For example, PCL2/1 means that CO_2 was passed through the equipment without recycling during $t_1 = 2$ h and afterwards recycling was carried out over $t_2 = 1$ h at homogeneous pressure throughout the equipment.

2.5. Characterization techniques

Morphologies of the used lichen and all polycaprolactone samples were investigated by scanning electron microscopy (SEM), employing a Jeol JXA-8600 microscope after coating the samples with a thin gold layer. The obtained images were analyzed by means of the free excess imaging software tool, ImageJ, in order to describe polymer morphology and pore diameter.

Thin Layer Chromatography (TLC) [23] was used for chemical analysis of metabolites in Patagonian lichen. Acetone and solvent C (toluene and acetic acid) were used as solvents.

Infrared spectra of the obtained extracts were recorded by using a FTIR spectrometer Nicolet 6700 (Thermo Scientific) over the wavenumber range 400–4000 cm^{-1} employing reflectance mode.

Differential Scanning Calorimetry (DSC) was performed on polycaprolactone samples at atmospheric pressure with a Shimadzu DSC-50 thermal analyzer under nitrogen atmosphere at a heating rate of 10 °C min^{-1}, from room temperature to 120 °C. Also, high pressure DSC analysis on polycaprolactone samples was undertaken in CO_2 atmosphere with a Calvet-Calorimeter BT 2.15 (SETARAM, France).

Thermogravimetric analysis was carried out with a thermobalance model TGA 50 (Shimadzu) in the temperature range of 25–500 °C, using alumina cruciales, under dynamic air atmosphere (30 mL min^{-1}) and heating rate of 10 °C min^{-1}. Impregnated extract content % (w/w), was determined from the obtained curves using a $scCO_2$ processed polycaprolactone as reference.

Density values of the polycaprolactone scaffolds were determined by using a Sartorius Balance (YDK01) with a density kit. The porosity (ε) represents the "void space" of the scaffold and was calculated from the density of the scaffold δS = (scaffold weight/scaffold volume) and the density of untreated polycaprolactone, $\delta_{PCL} = 1.14\ g/cm^3$: $\varepsilon = (1 - \delta S/\delta_{PCL}) \times 100$.

2.6. Antibacterial activity of lichen extracts

The antimicrobial activity of extracts of lichen *U. lethariiformis* was evaluated against gram positive bacterium, *Listeria innocua* CIP 8011 (provided by the University of Buenos Aires, Argentina) and a number of 31 Methicillin-Resistant *Staphylococcus* (MRS) strains isolated from clinical samples obtained from dogs and cats (provided by the Faculty of Veterinary Medicine, University of Belgrade). *L. innocua*, a non-pathogenic species, may be used as a biological indicator for the food borne pathogen *Listeria monocytogenes* because of its similar response to physical, chemical or thermal treatments [24]. For the isolation of staphylococci, conventional microbiological methods were applied with use of Columbia sheep blood agar (bioMerieux) and nutrient broth (BioLab). For the purpose of identification of isolated strains, commercial systems ID32 Staph (bioMeriux) and a BBL Crystal Gram positive ID kit (Becton Dickinson) were used. Presumptive categorisation of MRS strains was done with MRSA Chrom ID agar (bioMerieux). All suspected MRS strains were confirmed by detection of *mec*A gene using Polymerase Chain Reaction assay previously described by Murakami and Minamide [25]. Extraction of bacterial DNA was performed with commercial bacterial genomic DNA extraction kit (Metabion). Primers were purchased from Invitrogen, deoxyribonucleoside triphosphates were purchased from Fermentas, and final visualization of Polymerase Chain Reaction products was conducted on 1.5% agarose gels (Serva) in 1× Tris/Borate/EDTA buffer (Serva) by agar-gel electrophoresis. Positive and negative controls were MRSA American Type Culture Collection 43300 (MRSA ATCC 43300), and *S. aureus* American Type Culture Collection 25923 (*S. aureus* ATCC 25923).

Antibacterial activity of *Usnea* extracts was investigated by a broth microdilution method recommended by the Clinical and Laboratory Standards Institute [26]. The only modification of the method was application of *Usnea* extracts instead of antibiotics. Investigated concentrations of extracts were in the range of 2560–1.25 expressed in μg/mL. The extracts were dissolved

in dimethyl sulfoxide at 25.6 mg/mL and afterwards diluted at 1:10 with Cation Adjusted Mueller Hinton Broth (CAMHB, Becton Dickinson). Titration was performed in microplate wells as previously described. The final bacterial innoculum density of 5×10^5 Colony-forming Units/mL (CFU/mL) was achieved by adding 5 μL of 1–2 × 10^7 Colony-forming Units/mL (CFU/mL) suspension of investigated strain in microplate wells with 100 μL of previously added Cation Adjusted Mueller Hinton Broth (Becton Dickinson). Microplates were incubated 18–24 h at 37 °C. The minimum inhibitory concentrations (MIC) were defined as the lowest concentration (highest dilution) of extracts that inhibited the visible growth (no turbidity), when compared to the control. Afterwards, the minimum bactericidal concentration (MBC) was determined for which 10 μL of each well were transferred to Mueller Hinton agar plates and incubated for 24 h at 37 °C. The minimum bactericidal concentration (MBC) value is the lowest concentration where 99.9% or more of the initial inoculum was killed. Determinations were performed in triplicate. Commercial usnic acid powder (Aldrich) and commercial extracts of *Usnea barbata* (Flavex 1 and 2) were used as positive controls.

2.7. Determination of the antibacterial activity of impregnated PCL scaffolds

The modified broth macrodilution method was used for the investigation of antibacterial activity of impregnated polycaprolactone samples and Cation Adjusted Mueller Hinton Broth (Becton Dickinson) was used as a standard culture medium. The assays were performed in sterile 13 mm × 100 mm glass tubes. For the tests of each strain the concentration of the polycaprolactone samples was kept constant (200 mg/mL in *L. innocua* assays and 100 mg/mL in MRSA assays). The assays were performed by triplicate, during incubation time of 24 h, at 37 °C.

A standardized inoculum of investigated strains (*L. innocua* and MRSA ATCC 43300) was prepared by suspending colonies directly in Cation Adjusted Mueller Hinton Broth (Becton Dickinson) to the same density as 0.5 McFarland turbidity standard (approximately 1–3 × 10^8 CFU/mL). Serial 1:10 dilutions of this suspension were made and inoculum sizes of 3–4 × 10^4 CFU/mL and 2 × 10^5 CFU/mL were used as initial CFU numbers in these tests. Controls without polycaprolactone and with non-impregnated polycaprolactone were also analyzed. The total number of bacteria after incubation (CFU/mL) was determined by plate counts for each microorganism. The mean value for log (CFU/mL) was registered for each sample ($P < 0.05$).

3. Results

3.1. Characterization of patagonian lichen

Morphological features of the Patagonian lichen (*U. lethariiformis*) are shown in Fig. 2a. The lichen is anatomically composed of a thin cortex, a lax medulla (containing the algal layer) and a central axis. Samples of Patagonian lichen were analyzed by Thin Layer Chromatography (TCL) for chemical identification of their components. According to the TCL, the cortex of *U. lethariiformis* contained usnic acid and the medulla contained difractaic acid as major secondary metabolites. Fig. 2b and c shows the morphological changes produced by the pretreatment of the lichen after being milled with the planetary mill. The morphology of blade milled lichen was similar to planetary milled lichen. An important reduction in length of the hyphae of lichen with respect to the length observed in the untreated lichen becomes evident.

Table 1
Antibacterial activity of the extract of *Usnea lethariiformis* against *Listeria Innocua*. Commercial usnic acid was used as positive control. MIC and MBC values were determined by triplicate.

	Obtaining process	MIC (μg/mL)	MBC (μg/mL)
Usnic acid (UA)	Control, commercially available	2.5	320
EA-extract	Obtained after depressurization during E-A procedure	2.5	160
PM-extract	SFE/pretreatment of lichen with Planetary Mill	10	40
BM-extract	SFE/pretreatment of lichen with Blade Mill	5	40

3.2. Yield and characterization of Usnea extracts

The kinetics of the extraction process are shown in Fig. 3, where the extraction yield is presented as a function of the specific CO_2 consumption. The yields of lichen extracts isolated at 30 MPa and 40 °C are clearly improved by pretreatment although not much difference becomes evidente among both used milling methods. After a consumption of supercritical CO_2 of 12–14 $kgCO_2/kg_{solid}$ a similar yield of around 1% was obtained based on the original mass of the source material.

The content of usnic acid within the isolated extracts of *Usnea* lichen was 50.0% (w/w) in both cases.

Fig. 4 shows the infrared spectra of the two extracts obtained with the pretreated lichens, BM-extract and PM-extract. The characteristic bands at 2930, 2854, 1691, 1630, 1612, 1541, 1458, 1423, 1375, 1357, 1334, 1317, 1290, 1221, 1189, 1145, 1118, 1070, 1041, 991, 962, 931, 840, 819, 599 cm^{-1} were assigned to the functional groups of the usnic acid structure. The band at 1741 cm^{-1} that appears only in the BM-extract can be assigned to a C=O acid group present in the acid difractaic structure [27]. Otherwise, no substantial differences were detected for the different methods of pretreatment. For this reason pretreatment with a planetary mill was chosen for being faster and easier to be handled for future extractions.

Supercritical extracts from *Usnea* lichen have been tested against *L. innocua* and Methicillin-resistant *Staphylococcus* (MRS) strains. Results of comparative analysis of antibacterial activities of lichen extracts and controls are presented in Tables 1 and 2 [28].

The extracts obtained from *Usnea* (obtained at 30 MPa and 40 °C) showed strong antibacterial activity against the microorganisms tested. In trials using *L. innocua*, the obtained MCB values for the extracts were lower than those obtained for the usnic acid used as control sample, being 40 and 320 (μg/mL) respectively. This indicates that for eliminating 99.9% of the inoculated cells a lower concentration is required when the extracts are used instead of pure usnic acid.

In antibacterial tests against MRS strains, MIC values for PM-extract were obtained in a range of ≤1.25–320 μg/mL. As can be seen in Table 2, among 31 strains presented, 8 strains were more susceptible to *Usnea* extracts than against pure usnic acid with MIC values from ≤1.25 to 320 μg/mL which is also illustrated in Fig. 5. However, the concentration of usnic acid in the tested samples needs to be considered. Taking into account an inversal relationship between MIC value and usnic acid content in the sample, MIC values for lichen extract and commercial usnic acid against each strain were compared. As a result, 22 strains showed similar

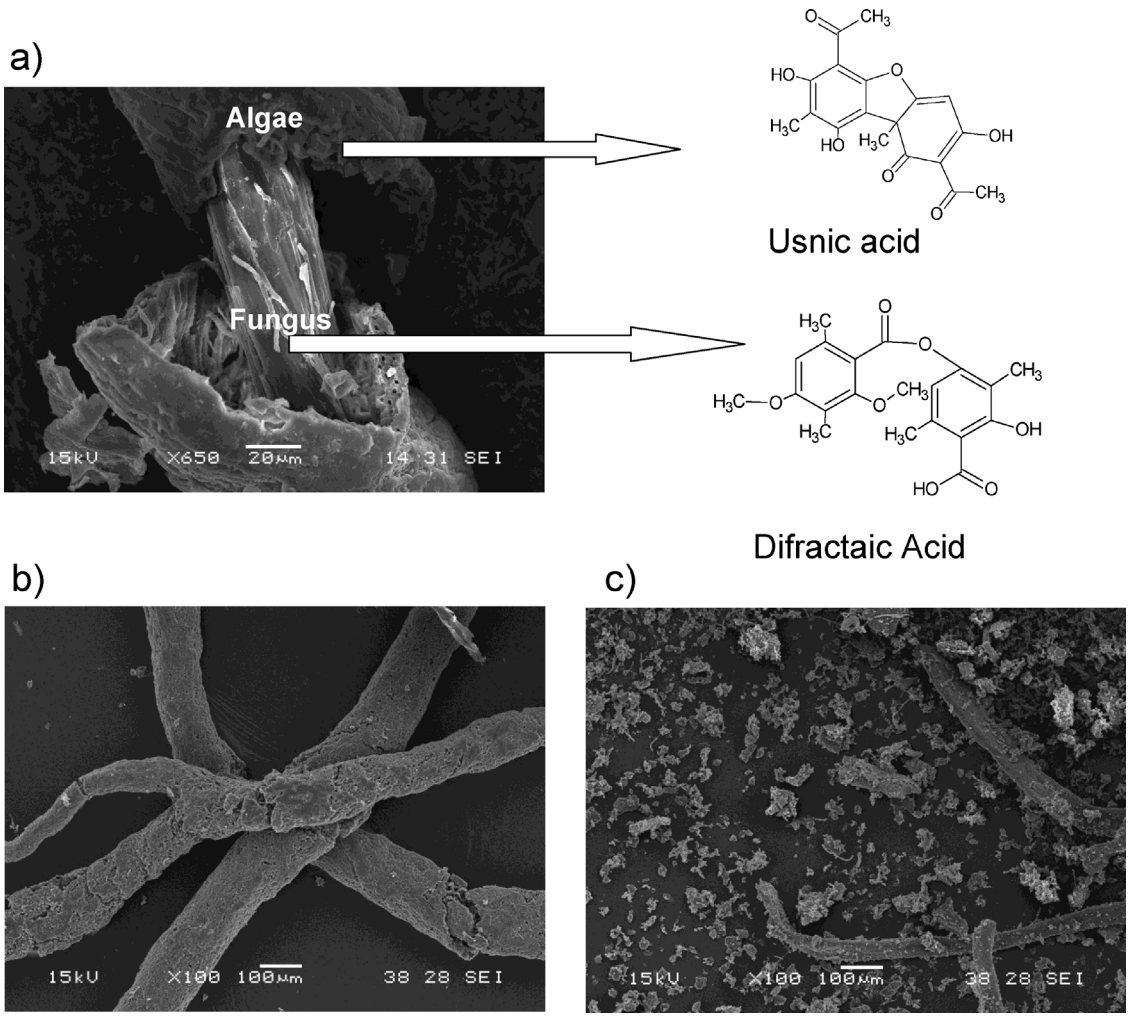

Fig. 2. (a) Structure of *Usnea* lichen and its main metabolites, (b) hyphae of lichen and (c) hyphae of lichen milled in planetary mill.

or higher susceptibility to natural usnic acid than commercial one.

MIC values for Flavex 1 were in the range from 80 μg/mL to >2560 μg/mL, while values for Flavex 2 were in the range from ≤1.25 μg/mL to 320 μg/mL. These results show that the reference sample "Flavex 2" has a similar behavior as the commercial usnic acid. It is in accordance with the amount of usnic acid in each sample, 830 g/kg and 1000 g/kg respectively. "Flavex 1" has a very low usnic acid concentration (40 g/kg) and consequently showed low efficiency as antibacterial substance.

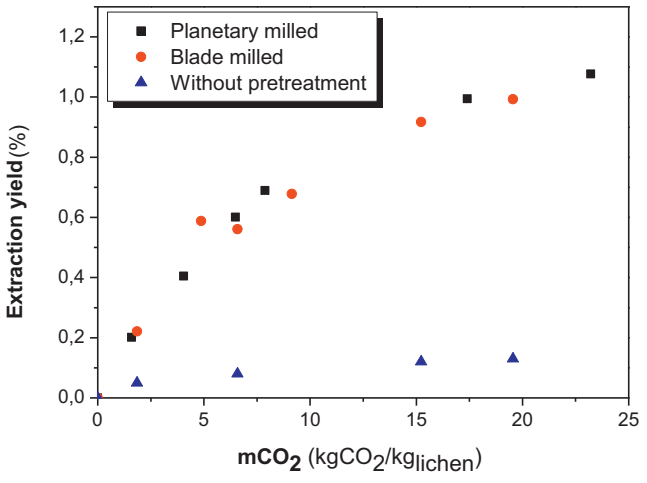

Fig. 3. Extraction yields (%) by using supercritical CO_2 at $P = 30$ MPa, $T = 40$ °C from non-pretreated lichen and milled with blade mill (BM) and planetary mill (PM).

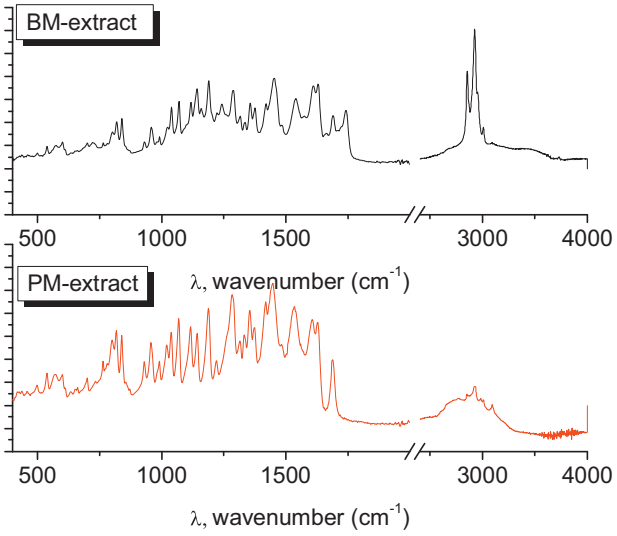

Fig. 4. Infrared spectra of the extracts isolated from pretreated Patagonian lichen *Usnea lethariiformis:* blade milled (BM-extract) (a) and planeraty milled (PM-extract) (b).

Table 2
Antibacterial activity of the PM-extract of *Usnea lethariiformis* against MRS strains. Commercial usnic acid and extracts (Flavex 1 and 2) were used as positive controls. Usnic acid content of each extract was included. MIC values were determined by triplicate.

Exp#	PM-extract *U. lethariiformis*	Flavex 1	Flavex 2	Usnic acid (UA)
UA content (g/kg) [28]	500	40	830	1000
MRS strain #		MIC (μg/mL)		
1	40	2560	20	20
2	40	2560	20	20
3	20	2560	10	10
4	20	2560	160	160
5	2.5	1280	10	20
6	40	2560	160	160
7	20	2560	5	5
8	40	2560	5	5
9	10	>2560	2.5	2.5
10	10	2560	5	5
11	20	2560	20	40
12	2.5	2560	40	20
13	20	2560	5	10
14	20	2560	5	20
15	20	2560	20	10
16	10	640	40	20
17	20	640	20	10
18	320	1280	20	10
19	20	2560	20	10
20	10	80	2,5	≤1.25
21	≤1.25	1280	≤1.25	≤1.25
22	≤1.25	80	10	≤1.25
23	20	2560	5	5
24	10	1280	5	10
25	40	1280	5	2.5
26	40	2560	10	10
27	40	1280	320	320
28	≤1.25	2560	80	80
29	40	640	20	10
30	20	2560	20	20
31	40	2560	20	20

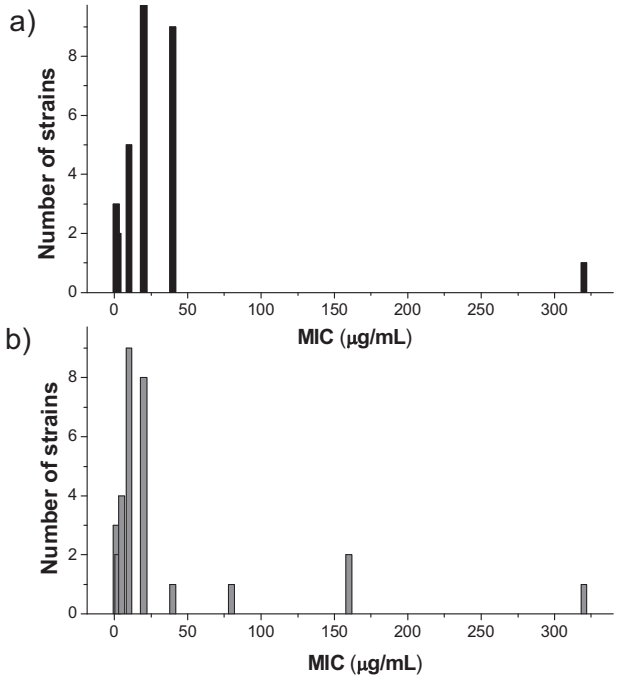

Fig. 5. Distribution of MIC values for *Usnea* extract (a) and usnic acid (b) against MRS strains.

Table 3
Representative parameters that describe the sorption of CO_2 within polycaprolactone at P = 15 MPa and two different temperature conditions.

P [MPa]	T [°C]	dV [%]	S [wt%]	D [10^{-9} m^2/s]	Shape of sample
15	35	9.2	20.0	4.0	Non deformed
15	40	8.0	40.0	10.0	Deformed

dV, swelling; S, solubility of CO_2; D, diffusivity of CO_2 within polycaprolactone.

3.3. Behavior of polycaprolactone in supercritical CO_2

Fig. 6 shows the effect of different process parameters on the change in volume during CO_2 sorption under pressure and the mean pore diameter in the polycaprolactone samples obtained after depressurization. While the change in volume during exposure to carbon dioxide does not show a clear tendency as a function of pressure (Fig. 6a-1), it is clearly shown that at lower temperature swelling is more pronounced (Fig. 6a-2).

A slightly increasing pore size can be observed as the pressure prior to depressurization is increased (Fig. 6b-1): a higher amount of gas is dissolved in the polymer which of course results in a larger void space during foaming. Bubble growth is enhanced by including the increased amount of CO_2 present in their vicinity. Anyway, the pore size is mainly influenced by temperature and depressurization rate (Fig. 6b-2 and b-3). A faster depressurization results in an enhanced nucleation rate because supersaturation is higher at onset of nucleation. Hence a higher number of bubbles are formed with less time for growing and coalescing. In order to interprete the effect of temperature on the pore size the plasticizing behavior needs to be accounted for. During the step where the bubbles grow, the polymer needs to remain in a fluid state, which is obviously rather the case at higher temperatures, where the viscosity is reduced. Anyway, even at temperatures below the melting temperature at atmsopheric pressure, bubbles are formed giving evidence of a change in melting behavior during exposure to compressed carbon dioxide.

When polycaprolactone is contacted by compressed CO_2 at 35 °C (Fig. 7), a profile of plasticization propagates throughout the sample. As can be seen in Fig. 7a, polycaprolactone is not completely plasticized at 35 °C and 15 MPa after 30 min of treatment. However, when the pressure is increased to 17 MPa the sample is completely plasticized after 30 min. If the pressure is increased to 20 MPa, the shape is totally changed within only 20 min.

Properties of the samples of polycaprolactone at 15 MPa and temperatures of 35 °C and 40 °C are presented in Table 3. Results show that changes in morphology occur as a consequence of the pressure treatment. Compared to the melting temperature at atmospheric pressure there is a dramatic reduction observed under pressure where melting occurs already at a temperature of 33.3 °C at 10 MPa, as is shown in Fig. 8.

3.4. Characterization of impregnated polycaprolactone scaffolds

In Table 4 the characteristics of sc CO_2 processed polycaprolactone samples in terms of porosity (ε), mean pore size (dp), wt% of impregnation, melting temperature (T_m), melting enthalpy (ΔH_m) and crystallinity (χ_c) are listed. For comparison, scCO_2-treated polycaprolactone samples without impregnation are also shown. The crystallinity of polycaprolactone samples was determined using a heat of fusion value (ΔH_{100}) of 135.31 J/g [29] for 100% crystalline polycaprolactone according to Eq. (1)

$$\chi_c\,(\%) = \frac{\Delta H_m}{\Delta H_m^o} \times 100 \qquad (1)$$

Fig. 9 illustrates the microstructure of the scCO_2-processed samples in which the mean pore diameter was determined.

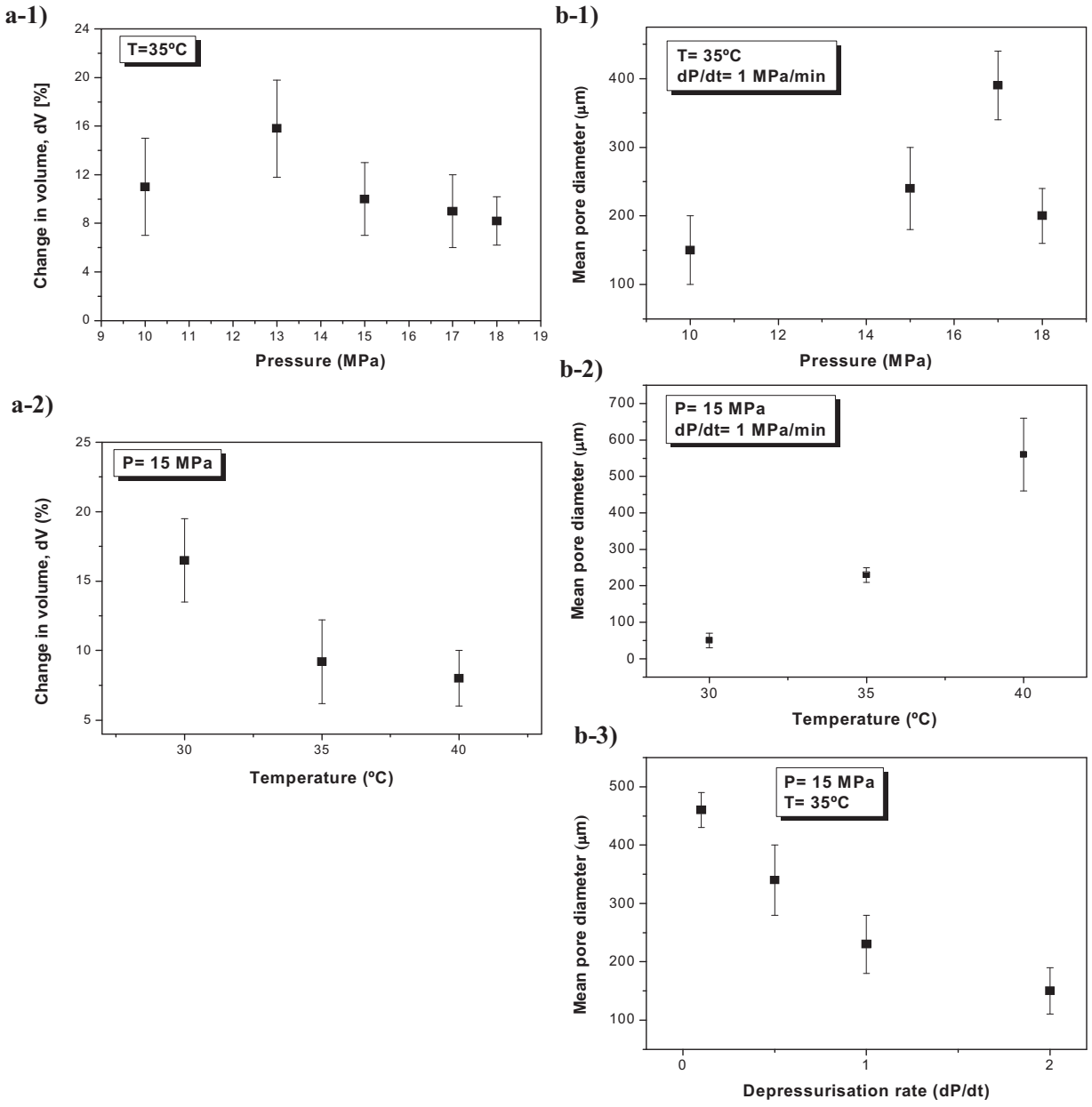

Fig. 6. (a-1) Effect of pressure on change in volume (%, swelling) of polycaprolactone samples at $T = 35\,^{\circ}C$; (a-2) effect of temperature on change in volume (%, swelling) of polycaprolactone samples at $P = 15$ MPa; (b-1) effect of pressure (MPa) on mean pore diameter (μm) of polycaprolactone samples after depressuration rate of 1 MPa/min at constant temperature of 35 °C; (b-2) effect of temperature (°C) on mean pore diameter (μm) of polycaprolactone samples after depressuration rate of 1 MPa/min at constant pressure of 15 MPa; (b-3) effect of depressurisation rate (MPa/min) on mean pore diameter (μm) of polycaprolactone samples at 15 MPa and 35 °C.

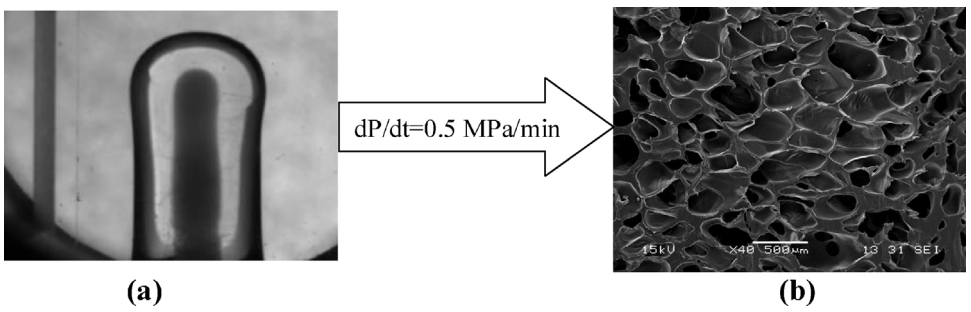

Fig. 7. (a) Image of polycaprolactone sample at 15 MPa and 35 °C and (b) microstructure of obtained polycaprolactone scaffold with average pore diameter of 340 μm.

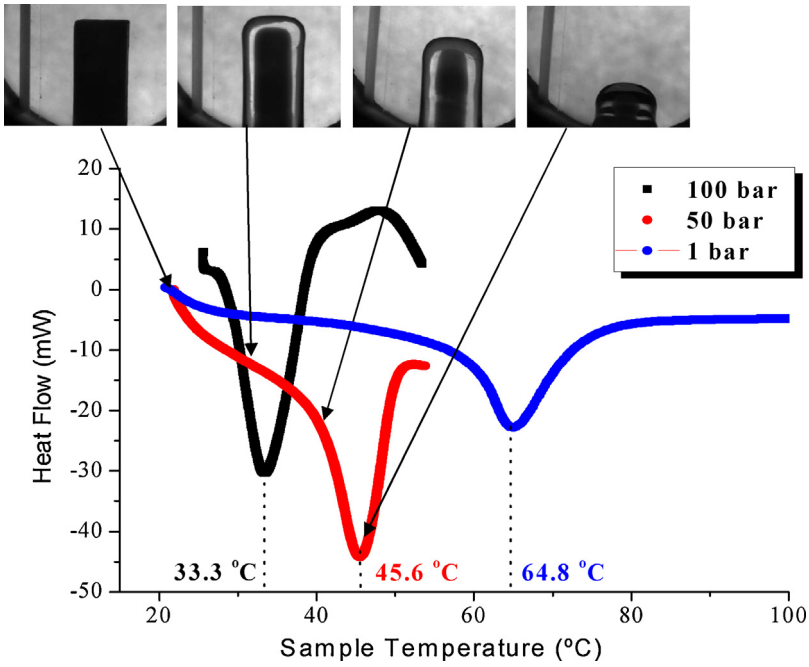

Fig. 8. Differential Scanning Calorimetry (DSC) of polycaprolactone at pressures of 0.1, 5 and 10 MPa of CO_2.

Homogeneous macropores were observed in all samples except for sample PCL2/4, in which different pore sizes were detected.

3.5. Antibacterial activity of impregnated polycaprolactone scaffolds

The results of the antibacterial screening of the tested functionalized polycaprolactone scaffolds are presented in Fig. 10. The impregnated polycaprolactone samples (PCL3/0, PCL1/1, PCL2/1, PCL2/4) showed a bactericidal effect on *L. innocua*, reducing viability of inoculated cells by more than 99%. Growth of *L. innocua* was reduced by logarithmic orders of 7.11, 6.15, 7.84 and 6.76 using the PCL3/0, PCL1/1, PCL2/1, PCL2/4, respectively (Fig. 10a) relative to the reference samples Control (without PCL) and PCL (non-impregnated sample). The most effective sample (PCL2/1) was the one with highest percentage of impregnation (2.8%). However, the bactericidal effect was not directly proportional to the percent of impregnation as was observed for PCL3/0, PCL1/1.

Fig. 10b shows the effect of percent of impregnation and the inoculum size on the antibacterial activity against MRSA strain. The tested samples showed a higher antimicrobial activity against MRSA ATCC 43300 when a low bacterial inoculum level (2×10^4 CFU/mL) was assayed. With a low inoculum size, growth reductions were more pronounced for most samples. In this case, a minimum amount of 1.5% of impregnation was needed for producing a bacteriostatic effect (PCL1/1, PCL2/1, PCL2/4). PCL3/0 with the lowest content of impregnated extract, allowed the development of the strain, although the growth was reduced by more than one logarithmic order with respect to the references Control and PCL. When a higher inoculum size (2×10^5 CFU/mL) was assayed, only PCL2/4 showed a bacteriostatic effect since counts after 24 h were not significantly different to the initial inoculum. However, PCL1/1, PCL2/1, PCL2/4 showed growth reductions of more than two logarithmic orders when compared to the PCL without impregnation (PCL reference).

4. Discussion

A conceptual design of an integrated process is proposed by integrating three steps for producing scaffolds of polycaprolactone with antibacterial properties. The strategy followed is based on minimizing the loss of extract mass in the tubes, vessels and exchangers of the equipment by directly using the $scCO_2$-extract solution for

Table 4
Parameters of processing (t_1, t_2); porosity (ε, [%]), mean pore size (μm), impregnation (%, w/w), melting temperature (T_m), melting enthalpy (ΔH_m) and crystallinity (χ_c [%]) of $scCO_2$ processed polycaprolactone samples.

Sample	Procedure	ε, Porosity [%]	Mean pore size [μm]	Impregnation [%, w/w]	T_m [°C]	ΔH_m [J/g]	χ_c [%] Crystallinity
PCL	Ref. [9]	70	340	–	63.6	85.7	63.3
	E-VC	64	342	0.7	59.8	77.3	57.1
PCL3/0	t_1: 3 h t_2: 0						
	E-A	72	290	1.5	59.6	76.1	56.3
PCL1/1	t_1: 1 h t_2: 1 h						
	E-A	40	270	2.8	58.4	64.7	47.8
PCL2/1	t_1: 2 h t_2: 1 h						
	E-A	72	365	2.2	59.6	69.0	51.0
PCL2/4	t_1: 2 h t_2: 4 h						

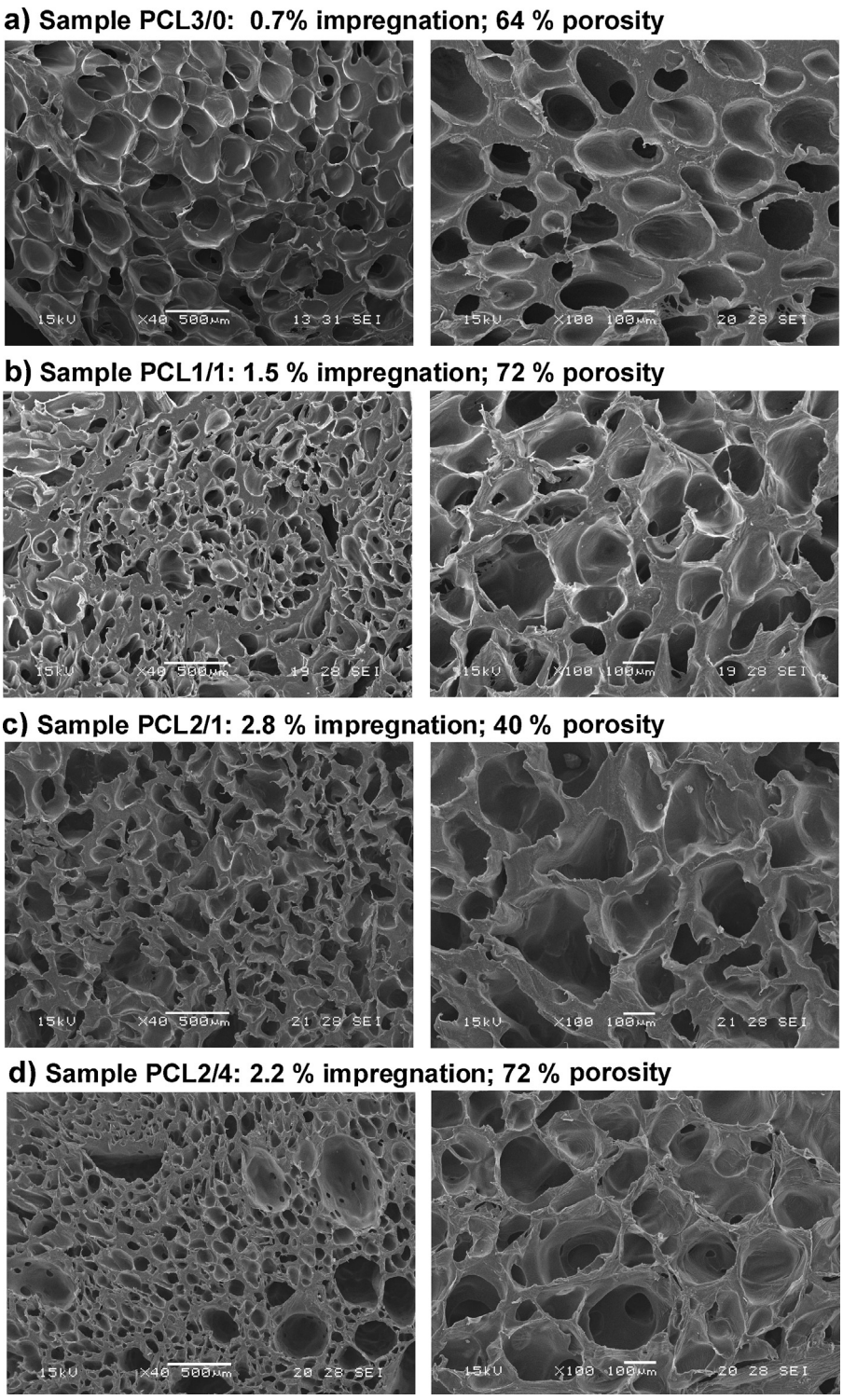

Fig. 9. Morphology of the polycaprolactone samples obtained after processing: (a) PCL3/0: extraction-impregnation during 3 h without recycling; (b) PCL1/1: extraction-impregnation (1 h) with recycling (1 h); (c) PCL2/1: extraction-impregnation (2 h) with recycling (1 h) and (d) PCL2/4: extraction-impregnation (2 h) with recycling (4 h).

impregnation, avoiding the low efficiency of extract recovery in a separation step by pressure reduction. The idea of the developed integrated process is presented in Fig. 11.

The recovery of the extract from *Usnea* lichen was quantified by separate extraction tests. These results showed that 1% (w/w) of extract containing 50% (w/w) of usnic acid can be isolated from the source material. Hence, an integrated process seems promising to improve the useful amount of extract incorporated into the PCL matrix.

It is important to note that the solubility of extract in $scCO_2$ changes according to the conditions in each vessel. The extraction variables are temperature, pressure, particle size and moisture content of the raw material, extraction time, CO_2 flow rate and solvent-to-source material ratio that all need to be optimized for an

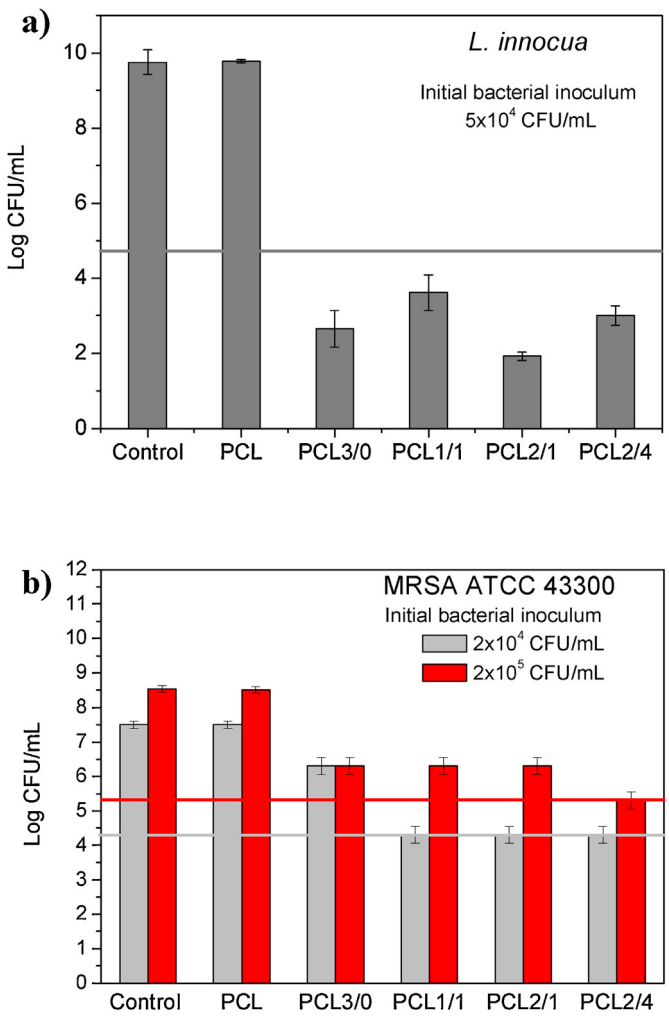

Fig. 10. Growth inhibition by functionalized polycaprolactone (PCL) scaffolds. (a) *L. innocua*; PCL concentration tested: 200 mg/mL and (b) MRSA; PCL concentration tested: 100 mg/mL. Vertical bars represent means of three replicates ± standard deviation. Control: culture medium without sample; PCL: culture medium + PCL without impregnation.

efficient process. In general, extraction yield increases with pressure due to an increase of the solubility in $scCO_2$ and decreases with an increase in temperature due to the decrease of CO_2 density. However, the antibacterial activities of extracts on specific bacteria may be adversely affected. This fact leads to a compromise situation to select the extraction conditions. In this study, pressure and temperature used for extraction were fixed to 40 °C and 30 MPa according to the previously published antibacterial activities of usnea extracts against MRSA [17].

On the other hand, according to the results on the CO_2 sorption in the polycaprolactone [9], useful scaffolds of polycaprolactone with a porous structure, with pore diameter of approximately 340 μm, can be obtained when the polycaprolactone samples are treated at 35 °C and 15–17 MPa (Fig. 9). The most homogeneous microstructure was obtained by expansion at moderate rates (0.5 MPa/min). Porous scaffolds of polycaprolactone with a density around 0.4 g/cm^3 can be achieved by using the mentioned conditions. Results show that changes in morphology occur as a consequence of the pressure treatment. As was mentioned above, compared to the melting temperature at atmospheric pressure, there is a dramatic reduction observed under pressure where melting occurs already at a temperature of 33.3 °C at 10 MPa (Fig. 8). This effect is used for improving the impregnation rate of polycaprolactone samples with active substances due to the increased diffusivity of the supercritical solution in the polymeric matrix [9].

In the proposed process, the variation in P and T in the adsorption vessel (A) taking into account the conditions established within the extractor vessel (E), is the main factor that ensures an enviroment supersaturated with solute in the neighborhood of the polymeric matrix. Table 4 shows that impregnation of *Usnea* extract achieved values are in the range of 0.7–2.8% (w/w) of the solid scaffold. PCL3/0 was found to be superficially impregnated, since the processing time was not sufficient for complete diffusion of the solute into the matrix. This superficial impregnation, can be the cause of greater bactericidal effect of sample PCL3/0 on *L. innocua* respect to the PCL1/1 sample.

Recycling of the $scCO_2$-extract solution was beneficial to increase the amount of extract within the matrix. Thus, an efficient procedure with 2 h for extraction-impregnation (t_1) ensures the maximun amount of extract in solution with $scCO_2$. Then a recycle time (t_2) of only 1 h was sufficient to achieve an acceptable impregnation value. A recycling time (t_2) greater than 2 h was not beneficial, probably due to the deposition of the extract on the walls and piping of the equipment when reducing temperature and pressure and due to dilution during the recycling process. Hence, recycling with a $scCO_2$-extract-solution with less concentration of extract can lead to "washing" the impregnated polymeric matrix.

The third step integrated within this process is the foaming of the samples. The foaming process can be divided into three steps: 1 – sorption of $scCO_2$ until saturation, 2 – nucleation of foam bubbles, 3 – growth of foam bubbles. Karimi et al. [30] reported a detailed study on the influence of the pressure, temperature, rate of quenching, etc. on the microstructure of PCL foams by using $scCO_2$. In their analysis foaming is described as a process that only occurs when the sample is melted. Also, when the foaming occurs at a lower rate of depressurization (slow quenching) the pressure decay rate can be idealized as an isothermal procedure. Thereby, the foaming temperature determines the width of the pore size distribution. The results of this work show that all processed samples have an adequate microstructure for use in tissue engineering. In Fig. 9 pores with appropriate diameters for vascularization and tissue regeneration can be observed. The mean size of the pores (150–340 μm) and the interconnectivity of these enable use as scaffolds for bone reconstruction.

The foaming process follows a complex mechanism, mainly three factors being involved in the process:

(i) release of CO_2 from PCL during nucleation and growth of bubbles
(ii) expansion of CO_2 inside the bubbles with decreasing pressure
(iii) increase of the matrix viscosity.

The balance between these factors determines the resulting pore morphology. Following [30], depressurization starts from a melted state of polycaprolactone and continues toward the melting line which is known from the DSC-experiments at elevated pressure (Fig. 12). The extent of supersaturation when arriving at the melting line, i.e. the pressure difference down to this point determines the foam structure significantly.

The degree of crystallinity of the obtained samples showed to be influenced by incorporation of the antibacterial agent. The lowest value of crystallinity was observed for the sample PCL2/1, indicating that the crystallization process (during depressurization and post-treatment) are affected by the amount of impregnated solute. This would indicate that the solute is retained within the amorphous phase of the semicrystalline polymer.

On the other hand, the present study shows the antibacterial activity of the obtained extracts against *L. innocua* and MRS strains

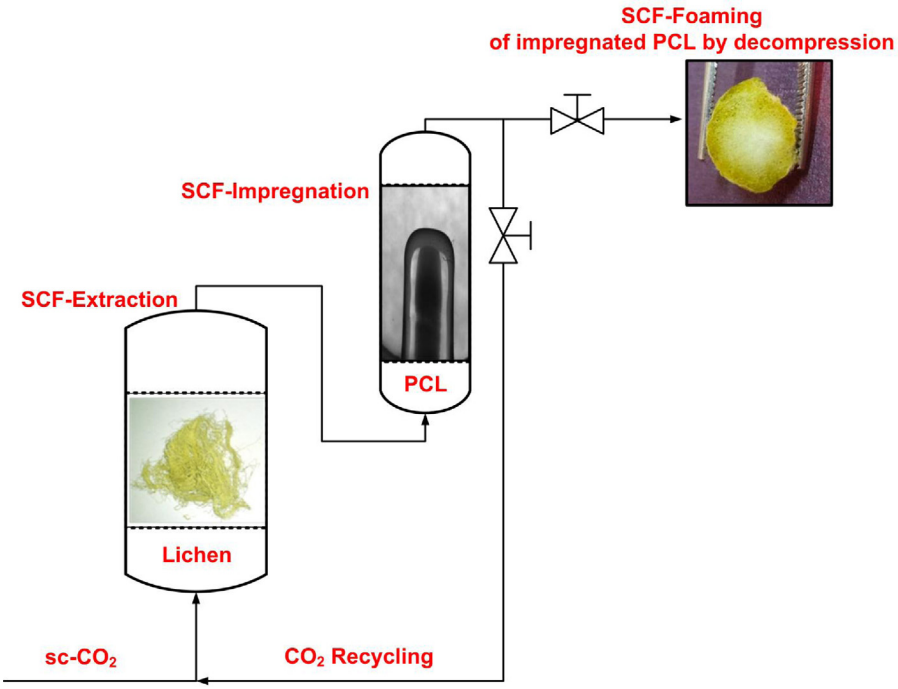

Fig. 11. Scheme of the developed integrated process.

(Tables 1 and 2), indicating that this capacity is conserved during the first step of the integrated process (E-A procedure). An antibacterial agent is defined as bactericidal when it exhibits the distinctive endpoint of causing a 99% reduction in bacterial inoculum within a 24-h period of exposure. Otherwise, it is considered bacteriostatic. The presented results showed that the samples PCL3/0, PCL1/1, PCL2/1, PCL2/4 are promising bactericidal compounds against *L. innocua*. Although these bacteria were used as models for testing the principle behavior, the presented results can be exploited for applying these functionalized samples as preservatives in food technology. Screening of antibacterial activity of functionalized polycaprolactone against MRSA ATCC 43300 strain showed higher activity when a low bacterial inoculum level (2×10^4 CFU/mL) was assayed.

Possible infective dose of MRSA was considered when determined total CFU/mL which will be tested in investigation of polycaprolactone antibacterial activity. Infectious dose is the amount of pathogenic organisms that will cause infection in susceptible hosts. The minimum infectious dose varies by organisms and is strain dependent so some pathogens appear to be able to infect at doses of 1–100 CFU, but others may not cause infection even at doses of 10^9 CFU [31]. The exact minimum infectious dose for individuals and populations is impossible to determine due to the route of transmission, content of food, gastric fluids, organic stuff, immune status of individuals and other factors. But for the most pathogenic microorganisms, the infectious dose is 500–1000 CFU. In this investigation numbers of 10,000 CFU and 100,000 CFU were chosen as indubitable infectious dose of MRSA strains no matter other factors are present or not.

According to results presented in Fig. 10b, impregnated polycaprolactone scaffolds at investigated concentration (100 mg/mL) slowed down multiplication of MRSA strains depending on the initial bacterial inoculum size. These results indicated that polycaprolactone scaffolds impregnated with *U. lethariiformis* extract could contribute to prevention of MRSA infections. The promising behavior of obtained polycaprolactone scaffolds could be explained by the presence of other secondary metabolites that have lichen synergistic action when are combined with natural usnic acid.

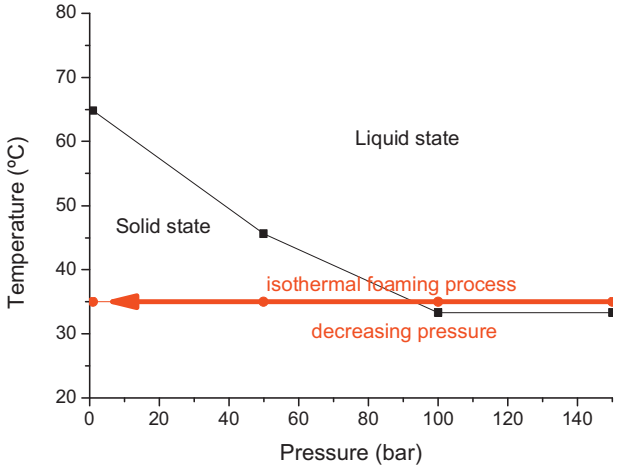

Fig. 12. Melting point curve determined from high pressure DSC data.

5. Conclusions

Design studies for an integrated extraction-impregnation process are presented. Various basic investigations have been performed systematically on extraction yield, sorption kinetics, scaffold structures, process performance and antibacterial effects in order to find the optimum process conditions. Optimum conditions for supercritical extraction of *U. lethariiformis* lichen with respect to yield and antibacterial activity were previously found to be 30 MPa and 40 °C. *Usnea* extracts isolated at these conditions showed strong antibacterial activity against MRS strains with values of MIC ranging from less than 1.25 μg/mL to 40 μg/mL.

On the other hand, this study demonstrates that by use of $scCO_2$ it is possible to obtain polycaprolactone of homogeneous morphologies containing porosities in the order of 70% and mean pore sizes from 150 to 340 μm at moderate pressures and temperatures. Further, the extraction of antibacterial substances like usnic acid

and its adsorption on polymeric base materials by an appropriate combination of extraction, impregnation and foaming conditions is feasible and results in impregnated products of considerable antibacterial effect.

As a consequence, a "three step in one" $scCO_2$ impregnation of biopolymers may provide an efficient method for tailoring the chemistry and morphology of the afore mentioned type of scaffolds by simultaneously obtaining the desired microstructure.

In future, further testing will be performed for optimizing process conditions and extending work to composite materials as base scaffolds for impregnation.

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