

1 Article

2 **A novel method of creating thermoplastic chitosan blends to produce cell**
3 **scaffolds by FDM additive manufacturing**

4
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12 **Abstract:**

13 Due to its remarkable and promising biological and structural properties, chitosan has been widely
14 studied in several potential applications in the biomedical sector. Attempts are being made to use this
15 polymer and its properties in thermoplastics dedicated to 3D printing in FDM technology. However,
16 chitosan can be processed only from acid solution, which limits its applications. The paper presents a
17 new path for the production of filaments based on unstable chitosan hydrogels obtained by carbon
18 dioxide saturation, as well as synthetic polymers such as polyvinyl alcohol and polycaprolactone. The
19 results confirm that the absence of acid allows formation of thermally stable and printable filaments
20 containing from 5% to 15% of chitosan, capable of reducing *S. aureus* and *E. coli* bacteria by 0.41-
21 1.43 in logarithmic scale (56 – 94%) and 0.28-0.94 in logarithmic scale (36 – 89%), respectively.

22 **Keywords:** 3D printing; chitosan; polycaprolactone; filament; antimicrobial properties

24 **1. Introduction**

25 Creating polymer compositions by physical mixing or chemical coupling is one of the most
26 popular ways of producing materials with novel properties. These properties may correlate with the
27 concentration of components present in the created composites or give them completely new and
28 non-obvious features (Rajeswari, Sreerag Gopi, Jackina Stobel Christy Jayaraj, & Pius, 2020).
29 Polymers from renewable sources deserve particular attention because of their low cost, high
30 availability, non-toxicity and ability to be used in products from sustainable resources. Chitosan
31 (CS) is an example of such a resource. Chitosan is the product of chitin deacetylation, composed
32 of randomly distributed $\beta(1\rightarrow4)$ -linked D-glucosamine and N-acetyl-D-glucosamine units in a linear
33 polymer structure. The sources of chitin are exoskeletons of shellfish and insects, as well as the cell
34 walls of fungi (Muzzarelli, 2009). Due to its biocompatibility, biodegradability, antimicrobial
35 properties, non-toxicity, and film-forming properties, chitosan offers potential in a range of
36 applications (Rinaudo, 2006). The major challenge in the efficient production of renewable polymers
37 is the compatibility of the raw material with common industrial processing methods such as
38 extrusion, injection moulding and extrusion blow moulding. Over the last few years, the meltability of
39 thermoplastic polymers has been strongly focused on the versatility of production by 3D printing

63 # These authors contributed equally to this paper.

40 methods with a particular focus on the fused deposition method (FDM), which is based on creating
41 three-dimensional products by melting the raw material and depositing it on previously produced
42 layers of solidified material (Mania, Banach, & Tylingo, 2020). Despite a number of advantages, the
43 use of chitosan in the above methods is very limited as it does not melt. Therefore, obtaining
44 composites with biological properties resulting from the characteristics of intempersible
45 biopolymers, such as antimicrobial activity, is still very restricted.

46 In the scientific literature, there are examples of studies that attempted to overcome this
47 limitation. The simplest methods are the use of additives of chitosan or other biopolymers as a solid
48 material in the form of particles in the polymer matrix, which guarantees the meltability of the
49 material (Bonilla, Fortunati, Vargas, Chiralt, & Kenny 2013; Correló et al., 2009). This solution is
50 effective when chitosan, as a filler, is used in concentrations of 0.5 - 1.5% (Rojas-Martínez et al.,
51 2020). Our previous work has shown that direct co-extrusion of chitosan with a poly(lactic acid)
52 (PLA) matrix hinders the achievement of homogeneous and smooth 3D printing filaments. The
53 filaments containing more than 3% of chitosan in PLA matrix were not suitable for FDM printing and
54 had weak antimicrobial properties (Mania et al., 2019).

55 Epure et al. have modified the physicochemical properties of chitosan to make it thermoplastic.
56 Chitosan in powder form was mixed with glycerol and 2% acetic acid was added to form a plastic
57 paste which was pressed hot. The addition of glycerol made the product extendable and therefore it
58 did not crumble (Epure, Griffon, Pollet, & Avérous, 2011). However, it was not possible to
59 completely melt the chitosan and the final colour of the mixture was darkened in the presence of
60 acetic acid.

61 Grande, Pessan and Carvalho (2015) proposed a method of obtaining a plastic chitosan
62 composite with PLA. It consisted of mixing 1% chitosan solution in acetic acid with 1% poly(vinyl
63 alcohol) (PVA) solution as a compatibility enhancer and using glycerol as a plasticizer (Grande,
64 Pessam, & Carvalho, 2015). The mixture was gently dried, ground and re-dried and then mixed with
65 PLA granules. The method developed made it possible to obtain thermoplastic filaments but did not
66 solve the problem of polymer degradation, which results from the residual acid used to dissolve the
67 chitosan. The authors indicated severe degradation of chitosan and of PLA leading to a decrease in
68 the mechanical properties and strong darkening during processing, even when the PVA-chitosan
69 blend was dried at mild temperatures (<50°C). Thus, the extraction of acid residues proved to be an
70 obstacle to overcome.

71 Other research groups have also worked on obtaining thermoplastic chitosan using polyols,
72 water and acetic acid as an alternative to the thermomechanical method (Dang & Yoksan, 2015;
73 Mendes et al., 2016). Although this approach reduced the size of the dispersed chitosan phase to a
74 few microns, the irregular geometry of the dispersed phase indicated that chitosan was not
75 effectively melted during thermo-mechanical mixing. Unfortunately, the problem of the darkening of
76 the resulting mixtures has still not been solved and has intensified with the increase in chitosan
77 concentration in composites (Matet, Heuzey, Pollet, Aji, & Avérous, 2013).

78 Grande, Pessan and Carvalho (2018) suggested a way to obtain thermoplastic PLA, PVA and
79 chitosan blends. The first stage consisted of mixing a 1% solution of PVA and a 1% solution of
80 chitosan in 1% acetic acid, which was then spray-dried, while the volatile acetic acid at the drying

1 81 temperature was driven away together with water. Another way was to freeze the mixture of
2 82 solutions and subject it to freeze-drying. However, it must be taken into account that the
3 83 evaporation of acetic acid from the material during sublimation may not always be efficient and may
4 84 result in increased exploitation of the apparatus on an industrial scale (Grande, Pessan, &
5 85 Carvalho, 2018). Target composites presented by the authors contain from 2.5 to 6.25% chitosan
6 86 and the studies do not present the effect of long-term processing of the composite at extrusion
7 87 temperature.

8 88 In in our work, we tried to confirm the hypothesis that the elimination of acid during chitosan
9 89 dissolution will allow obtaining thermoplastic and thermostable filaments in a PCL matrix with
10 90 antimicrobial activity. This effect was attempted by using an innovative method of saturation with
11 91 gaseous carbon dioxide for the processing of chitosan in the form of a solution. To date, such an
12 92 approach to obtain thermoplastic chitosan composites has not been proposed in the scientific
13 93 literature.

19 94 **2. Experimental methods**

20 95 **2.1. Materials**

21 96 Medium molecular weight chitosan (MMW) with deacetylation degree of 81-96% was
22 97 purchased from Primex (Iceland). PVA with molecular weight of 9-10 kDa and a degree of
23 98 hydrolysis of 80% was purchased from Merck (Germany). Medium molecular weight PCL with a
24 99 melting index equal to 11 g/10 min at 100°C was purchased from Esun Industrial Co., Ltd.
25 100 (Shenzhen, China). The acetic acid, hydrochloric acid and sodium hydroxide were produced by
26 101 Avantor Performance Materials Poland S.A (Gliwice, Poland). The carbon dioxide used to saturate
27 102 the chitosan preceipitate was derived from Linde Gaz Polska Sp. z o. o. (Gdańsk, Poland). For
28 103 microbiological tests, the following bacterial strains were used: Gram (-) *Escherichia coli* (ATCC
29 104 25922) and Gram (+) *Staphylococcus aureus* (ATCC 29213) from the Polish Collection of
30 105 Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish
31 106 Academy of Sciences (Wrocław, Poland). Phosphate Buffered Saline (PBS), Tryptic Soy Agar
32 107 (TSA), and Tryptic Soy Broth (TSB) media were purchased from Merck (Germany).

33 108 **2.2. Preparation of CS/PVA composites**

34 109 Chitosan/poly(vinyl alcohol) (CS/PVA) composites were made in three variants: 1% CS solution
35 110 in 0.1M acetic acid, 1% CS solution in 0.1M hydrochloric acid, and 1% CS solution in carbonic acid.

36 111 The 1% CS solution in 0.1M acetic acid and 0.1M hydrochloric acid was obtained by indirect
37 112 dissolving of polymer in acid solution under mechanical stirring at a speed of 300 RPM (RA 2020,
38 113 Heidolph Instruments GmbH & Co. KG, Kelheim, Germany) until a homogenous and clear solution
39 114 was obtained (3 hours). The 1% chitosan solution in carbonic acid (CS-CO₂) was prepared
40 115 according to the methodology proposed by Gorczyca and colleagues with slight modifications
41 116 (Gorczyca et al., 2014).

42 117 In the first step, 1.5% CS solution in 0.1M acetic acid was obtained by indirect dissolution of
43 118 polymer in proper acid solution during mechanical stirring at a speed of 300 RPM (RA 2020,
44 119 Heidolph Instruments GmbH & Co. KG, Kelheim, Germany). Then, during mixing, 0.5M solution of
45 120 sodium hydroxide was added until a pH value in the range of 9-10 was reached. This was
46 121 equivalent to complete precipitation of chitosan in the microcrystalline form. The precipitated

122 chitosan was filtered with the use of a seepage kit under reduced pressure and was washed several
123 times with distilled water until the pH of the rinsing water reached a value equal to 7.0. Finally, the
124 precipitated chitosan was weighed and suspended in such an amount of distilled water to obtain a
125 solution of 1 % in relation to the dry matter of the polymer. The CS suspension was homogenized at
126 10000 RPM for 3 minutes (Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Kelheim,
127 Germany) and then saturated with CO₂ with simultaneous mechanical mixing using a hollow shaft
128 stirrer for gas saturation (BIOMIXBMX-10, Gdańsk, Poland) until completely dissolved.

129 The PVA solution was made by heating distilled water to 60°C and adding to it small portions of
130 the appropriate amount of PVA to obtain a concentration of 1%. The mixture was strongly mixed
131 with simultaneous heating to 50-60°C (MR Hei-Standard, Heidolph Instruments GmbH & Co. KG,
132 Kelheim, Germany). After obtaining a homogeneous solution, it was cooled to room temperature.
133 The finished PVA solution and chitosan solution dissolved in hydrochloric or acetic or carbonic acid
134 were mixed at a 1:1 mass ratio, frozen at -24°C and then freeze-dried for 72 h (p = 0.94 mbar,
135 sample temperature 20°C, condenser temperature -80°C; Christ Alpha 12-4 LD Plus, Osterode am
136 Harz, Germany).

137 **2.3. Evaluation of the effect of extrusion time on the colour of CS/PVA composite**

138 Freeze-dried PVA/chitosan composites were ground in a laboratory mill (ChemLand, FW 135,
139 Stargard, Poland) to obtain fine chips (approx. 5 s). The crushed material was placed in glass petri
140 dishes with lids, which were then placed in a laboratory oven heated to 150°C (BINDER FDL 115,
141 GmbH, Tuttlingen, Germany). The process of heating the composites was carried out for 4
142 hours. The results were presented in the form of photos of the composites taken before heating and
143 after 1, 2, 3 and 4 hours of heating (Nikon D7200 camera (Kumagaya, Japan).

144 **2.4. Filament preparation**

145 To produce thermoplastic filaments based on chitosan, poly(vinyl alcohol) and polycaprolactone, in
146 the first stage CS-CO₂/PVA composites were prepared by mixing 1% CS-CO₂ solution and 1% PVA
147 solution in 3:1, 1:1, 1:3 mass ratios and freeze-drying for 72 hours (p = 0.94 mbar, sample
148 temperature 20°C, condenser temperature -80°C; Christ Alpha 12-4 LD Plus, Osterode am Harz,
149 Germany). The dry CS-CO₂/PVA composites were ground in a laboratory mill (ChemLand, FW 135,
150 Stargard, Poland), until a fine powder was obtained. Prior to the extrusion process, an extruder was
151 started (Wellzoom C, Zhenzen, China), setting a temperature of 150°C in the dosing and extrusion
152 zone. When the temperature was reached, the milled material was placed in the dispenser of the
153 extruder and the dosing auger drive was started. The extrusion process was carried out at a speed
154 of 2000 mm/min. The molding nozzle diameter was equal to 1.75 mm. The resulting extrudate was
155 cut by hand into sections not exceeding 3 mm in length. The CS-CO₂/PVA/PCL filaments were
156 produced by mixing 80g of PCL granules and 20g of selected CS-CO₂/PVA composite granules and
157 extrusion. The thoroughly mixed combination was then re-extruded using the same extruder and the
158 same extrusion conditions as were used to produce granules of the CS-CO₂/PVA composites.

159 **2.5. 3D Print Preparation**

160 The CAD model in the form of a 12.5 mm diameter and 20 mm high cylinder was created with
161 AutoCAD 2019 software and exported in "stl" format. The file was converted to "gcode" using Z-

162 Suite V 2.12.0.0 software. The object was printed using the FDM technique with a Zortrax M200
163 Plus printer (Olsztyn, Poland) with working space dimensions 200 x 200 x 300 mm. Printing
164 parameters for control (PCL) and CS-CO₂/PVA/PCL filaments were as follows: extruder nozzle size
165 0.4mm, layer height 0.1mm, print filling: 30%, print temperature: 150°C, working field temperature:
166 30°C.

167 **2.6 Material characterization**

168 **2.6.1 Morphological evaluation (SEM)**

169 The topography of the filaments was evaluated using a VP-SEM S3400-N scanning electron
170 microscope (Hitachi, Hyogo, Japan). The acceleration voltage was 25 kV. Compensation of the
171 charge was ensured by recording the images at a vacuum of 120 Pa and using a back-scattering
172 electron detector (BSE). The use of the BSE detector allowed increase of the contrast of local areas
173 differing in mass and/or density of the material. Visual recording of the samples was obtained with
174 Nikon D7200 (Warsaw, Poland).

175 **2.6.2. Chemical structure (FTIR)**

176 FT-IR spectra have been recorded using a spectrometer (Nicolet 8700; Thermo Electron Corp.,
177 Waltham, MA, USA) equipped with GoldenGate (Specac Corp., Oprington, UK) with a single-
178 reflection diamond. The temperature of the crystal was maintained at $25.0 \pm 0.1^\circ\text{C}$ during
179 measurement. 64 scans were obtained with a resolution of 4 cm^{-1} within the wavelength range from
180 4000 to 550 cm^{-1} . The spectrum of each material was measured, averaged and corrected by
181 background removal. Spectragryph V software was used to process the obtained spectra. 1.2.10
182 (Oberstdorf, Germany).

183 **2.6.3. Thermal stability (TGA)**

184 A thermogravimetric analyser (TA Instruments SDT 600, Warsaw, Poland) was used to measure
185 the change in weight of the sample due to decomposition. During the measurement, the samples
186 were heated at a constant rate of $10^\circ\text{C}/\text{min}$ between 30°C and 800°C . The tests were conducted in
187 a nitrogen atmosphere.

188 **2.6.4. Mechanical properties and printability**

189 The mechanical properties were tested using a universal testing machine (Instron model 5543
190 with "Merlin" V 4.42. software, Warsaw, Poland). Filaments were tested in accordance with ISO
191 527: 2012. The filaments were cut into 10 cm lengths and conditioned in an air climatic chamber at
192 25°C for 24 hours under relative humidity equal to 50%. In order to perform the measurement, each
193 of the filaments was mounted in the jaws of the apparatus so that the length of the measurement
194 section was 50 mm. The speed of the head movement causing the filament stretching was 100
195 mm/min. Each test was performed in 10 replicates. Based on the diagram of the relationship
196 between breaking stress and deformation, test parameters such as tensile strength, strain at break
197 and Young's modulus were determined. The printability of the filaments was assessed by the
198 toughness parameter measurement in the indirect instrumental stiffness test proposed by Xu et
199 others: $\text{kg}/\text{mm}^2\%$, pressure unit expressed as a percentage and used to describe the mechanical
200 parameters of tests using texture analysis methods (Xu et al., 2020). The universal testing machine

201 was equipped with a three-point-bending rig with thin blades (3 mm thickness). The filament was cut
202 into 6 cm in length and placed on top of the machine platform. Trigger force was set to 50 g, and the
203 blade was set to cut the filament until 1 mm in distance (57% strain) at a speed of 2 mm/s. The
204 maximum force and fracture distance were recorded by Merlin software. Based on the area under
205 the curve and maximum stress, the toughness parameter was calculated. The mechanical
206 properties of the cylindrically shaped 3D prints were characterized according to the method
207 described by Bi and Hoyer with co workers with slight modifications (Bi et al., 2011; Hoyer et al.,
208 2012). Five cylindrical samples (\varnothing 12.5 mm h=20 mm) were compressed up to 10% deformation in
209 two cycles at a test speed of 0.5 mm/s. The hardness, flexibility, and cohesiveness were measured.
210 Hardness was determined as the ratio of the maximum force in the first compression cycle to the
211 compressed surface. Elasticity is a dimensionless quantity expressed as the ratio of the distance of
212 the pin compressing the sample from the beginning of compression to the achievement of the
213 maximum force in the second cycle with the same distance determined in the first cycle.
214 Cohesiveness, also a dimensionless value, was determined as the ratio of the area under the curve
215 in the second compression cycle to the area under the curve in the first compression cycle.

216 **2.6.5. Antimicrobial activity**

217 The evaluation of antimicrobial properties of the materials was performed according to the ASTM
218 E2149 method with slight modification using *E. coli* and *S. aureus* strains. Colonies of bacteria were
219 first subcultured to triptic soya broth and incubated for 24 h at 37°C. Then microbial suspensions
220 were diluted in phosphate buffered saline to obtain a number of bacterial cells between 1.5×10^7 to
221 5×10^7 CFU/mL. Dillutions were made with a spectrophotometer by measuring the absorbance at
222 600 nm wavelengths (optical density $0.08 \div 0.1$). Antimicrobial activity tests were conducted for CS-
223 CO₂/PVA composites, CS-CO₂/PVA/PCL filaments, and 3D prints.

224 **To determine the antimicrobial activity of CS-CO₂/PVA composites**, squares with 5 cm edge
225 length were cut out of the composites and PE film (negative control without the antimicrobial
226 properties). Then square samples were sterilised under UV radiation (30 minutes on each side).
227 Afterwards, 0.4 mL of inoculum containing 1.5×10^7 to 5×10^7 CFU/mL of tested bacterial strain
228 was applied to each of the square. The inoculated samples were covered with sterile square-
229 shaped PE foils with an edge length of 4 cm to ensure proper contact of the bacterial suspension
230 with the tested material (16 cm²). The samples prepared in this way were incubated 24 hours at
231 37°C (Heidolph Incubator 1000, Merck Sp. z o.o., Warsaw, Poland). After incubation each sample
232 was transferred to 10 mL PBS solution and shaken 5 times for 5 seconds each using a vortex. Ten-
233 fold dilutions of PBS extracts in peptone water were made ($10^0 \div 10^8$) and inoculations on TSA
234 medium by flooding. The plates were incubated at 37°C for 24 hours. After the incubation, bacterial
235 colonies that grew on Petri plates were counted.

236 **For determination of antimicrobial activity of filaments**, cell suspensions containing 1.5×10^7
237 to 5×10^7 CFU/mL of tested bacterial strain, were placed in separate bottles of 45 mL, followed by
238 addition of 5 g of PCL filament (control sample) or proper CS-CO₂/PVA/PCL filament (tested
239 sample). Then samples were incubated at 37°C for 24 hours in an incubator with constant shaking
240 at 200 rpm (Heidolph Unimax 1010, Merck Sp. z o.o., Warsaw, Poland). After incubation, ten-fold
241 dilutions in peptone water were made ($10^0 \div 10^8$) and inoculations on TSA medium by flooding. The

242 plates were incubated at 37°C for 24 hours. After the incubation, bacterial colonies that grew on
243 Petri plates were counted.

244 **The antimicrobial activity of the 3D prints** was evaluated analogously to the procedure used
245 for CS-CO₂/PVA composites. The test samples were prepared by printing the tiles in a square
246 shape, with a side length of 5 cm and a thickness of 0.5 cm. The negative control was a plaque of
247 the same dimensions printed from PCL.

248 To calculate the number of cells per mL of the initial suspension, the results of the colony counts
249 were used, the number of which, after incubation on the plates, ranged from 30 ÷ 300 units,
250 according to the equation:

$$V_c = N \times D,$$

251 where V_c is the bacteria concentration, in colony forming units per ml (CFU/mL), N is the average
252 value, in colony forming units (CFU), from Petri dishes, D is the dilution factor from the plates
253 counted. Antimicrobial activity was calculated according to the formula:

$$R = \log \frac{B}{C},$$

254 where B is the average of the number of viable cells on the tested sample after 24 h incubation at
255 37°C (CFU/mL), C is the average of the number of viable cells on the control sample after 24 h
256 incubation at 37°C (CFU/mL). A percentage reduction of bacteria/fungi on a logarithmic scale (R)
257 equal to 1, 2, and 3 corresponds to a reduction of 90%, 99%, and 99.9%, respectively.

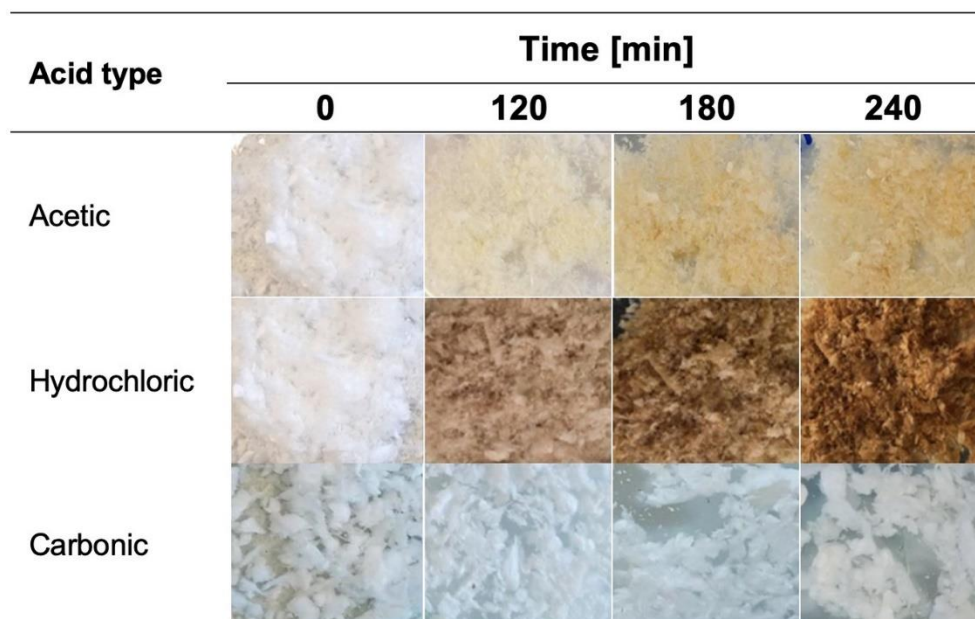
261 **2.7. Statistical Analysis of the Data**

262 The STATISTICA software (StatSoft, Inc., Tulsa, OK, USA) was used for analyses. The statistical
263 significance was determined at $p < 0.05$. All data reported were based on the means of three
264 replicates ($n = 3$) or five ($n=5$) replicates in the case of mechanical tests. Experimental results were
265 expressed as mean \pm standard deviation (SD). Student's t-test and one-way analysis of variance
266 (ANOVA) were applied. The differences were considered to be statistically significant at $p < 0.05$.

267 **3. Results and discussion**

268 **3.1 Assessment of the effect of temperature treatment on the stability of CS-CO₂/PVA** 269 **composites**

270 During visual evaluation of the influence of temperature treatment on the stability of CS-CO₂/PVA
271 composites, significant differences in their colour were observed. They resulted from the type of
272 acid used during composites preparation (Fig.1).



273

274 Fig.1. Comparison of color changes of CS-CO₂/PVA composites at the ratio of 1:1 m/m heated at 150°C for 4
275 hours.

276 The change in the colour of samples indicated the degradation of polymers present in the
277 composite. It is an undesirable phenomenon as it leads to structural changes in polymer chains
278 (e.g. loss of molecular weight) and as a result, changes in the physicochemical and biological
279 properties of the material. In the context of the use of chitosan in various biomaterials, the greatest
280 threats are biodegradation, degradation by ultrasounds, thermal degradation, and
281 photodegradation. The presence of acid in chitosan materials mainly causes the hydrolysis of the
282 polymer to a lower molecular weight without significantly affecting the deacetylation degree and
283 polydispersity (Sikorski, Gzyra-Jagiela, & Draczyński, 2021). The strongest darkening of the
284 composites was observed while using hydrochloric acid. During four hours of heating at 150°C, the
285 colour changed from white to brown. A less intense colour change during heating was shown in the
286 material for which a solution of chitosan in acetic acid was used. After heating this material under
287 the same conditions a change in colour from white to yellow was observed. In the case of
288 composites prepared with chitosan solution in carbonic acid, no effect of temperature on the change
289 of their colour was observed. Grande and co-workers found that the color change of CS/PVA blends
290 is dependent on the drying method, and best results can be obtained by using freeze-drying and
291 spray-drying (an only slightly yellow tone) (Grande et al., 2018). However, subjecting thus dried
292 composites containing at least 25% (m/m) of chitosan to hot-pressing (150°C), still causes their
293 darkening, even if volatile acetic acid was used to dissolve chitosan. Hot-pressed composites did
294 not change their color due to the temperature treatment, when the chitosan content in their
295 composition did not exceed 10% (m/m). The results we presented confirmed that the innovative
296 method of dissolving chitosan with the use of carbon dioxide allowed the creation of thermostable
297 CS-CO₂/PVA composites, even if chitosan constitutes as much as 50% of their mass.

298 3.2. CS-CO₂/PVA chemical structure

299 The infrared spectroscopy was used to identify individual chemical groups in pure chitosan and
300 PVA as well as to register structural changes in composites of both polymers (Fig. 2). Figure 2.A
301 shows the chitosan spectrum. Intense signals at wavelengths of 1150 cm^{-1} , 1058 cm^{-1} , 1026 cm^{-1}
302 and 893 cm^{-1} indicate the presence of a saccharide structure. Another characteristic band at
303 3349/3301 cm^{-1} is the signal coming from the hydroxyl group of the chitosan molecule. A series of
304 signals at 1652 cm^{-1} , 1557 cm^{-1} and 1315 cm^{-1} correspond to amides I, II and III. The remaining
305 marked signals come from groups $-\text{CH}_2-$ (Mania et al., 2019; Staroszczyk, Sztuka, Wolska, Wojtasz-
306 Pająk, & Kołodziejska, 2014).

307 The PVA spectrum is shown in Figure 2.B. Both PVA and chitosan spectra have several analog
308 signals. The signal at about 3342 cm^{-1} corresponds to tensile vibrations of the hydroxyl group, at
309 2917 cm^{-1} and 1426 cm^{-1} to tensile and deformation vibrations of alkyl groups. Other signals
310 characteristic for PVA are in the range 1750-1000 cm^{-1} . For example, the band at 1726 cm^{-1} is a
311 signal of C-O and C=O tensile vibrations of the acetate group. In addition, several signals have
312 been observed which are the result of interactions between PVA mers such as at 1371 cm^{-1} ((OH)-
313 C-OH), 1238 cm^{-1} (=C-O-C), 1088 cm^{-1} (C-O-C) and 1020 cm^{-1} ((C-O)-C-OH) (Alhosseini et al.,
314 2012; Mansur, Sadahira, Souza & Mansur, 2008).

315 Analyzing the spectrum of PVA/CS composites (Figure 2.C), characteristic peaks were detected
316 as in the spectra of individual components of this composite. Chitosan and PVA do not show strong
317 interactions between each other as evidenced by the lack of new signals and significant shifts of
318 existing peaks. The only shift observed was found in the case of signals corresponding to tensile
319 vibrations of hydroxyl and alkyl groups. Moreover, on the basis of the obtained spectrum it was
320 confirmed that the intensity of its signals increases with the increase of the share in the composition
321 of a given polymer. These results are consistent with the work of Grande and co-workers (Grande
322 et al., 2018).

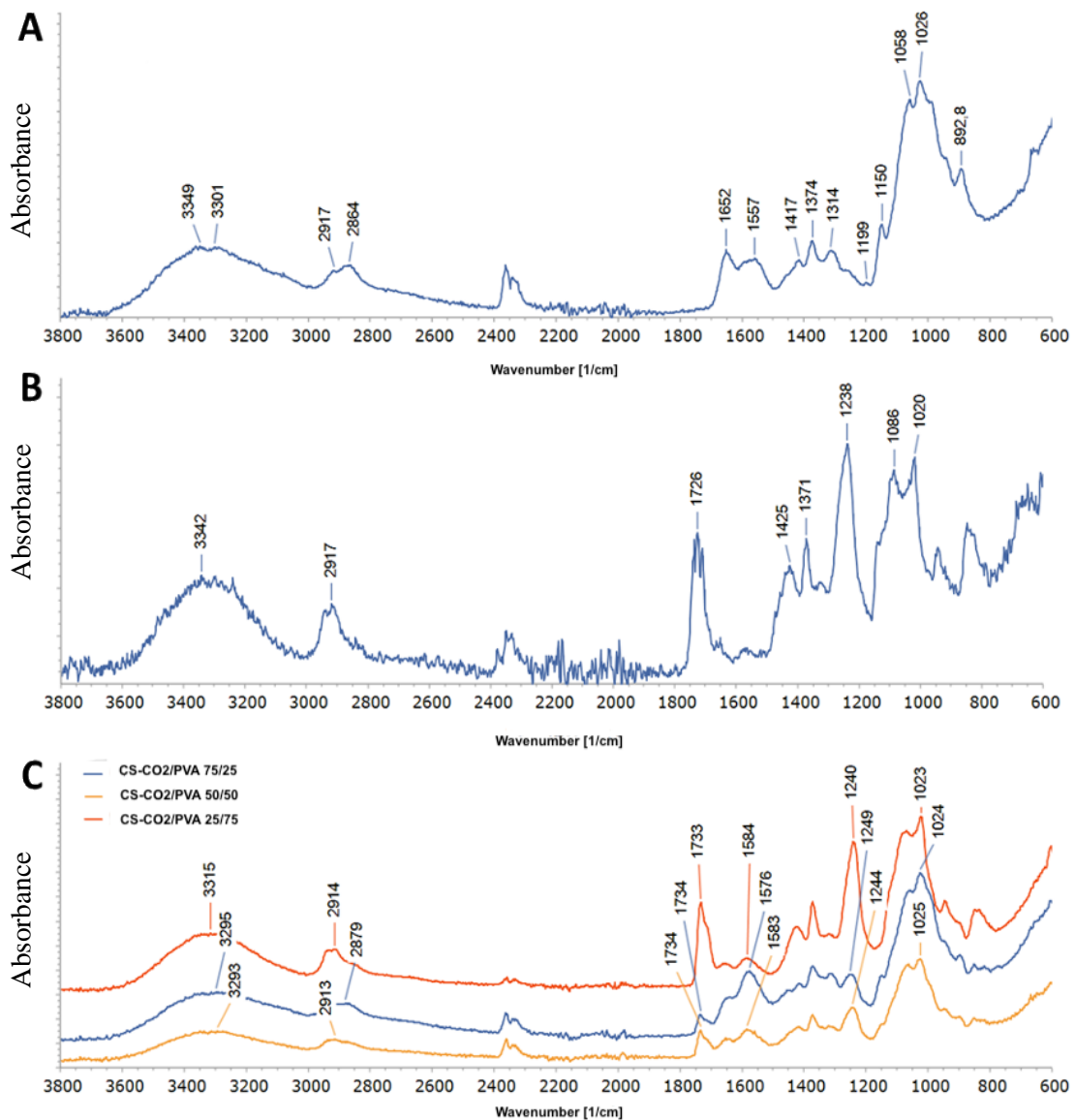


Fig.2. FTIR spectra obtained for CS (A), PVA (B) and PVA/CS (C) composites.

3.3. CS-CO₂/PVA/PCL filaments production

PCL was used as the matrix for the production of filaments and doped with one of the three CS-CO₂/PVA obtained composite. The 1% solutions of CS-CO₂ and PVA were combined to obtain CS-CO₂/PVA composites with mass fractions of 75/25, 50/50 and 25/75. The freeze-dried composites subsequently subjected to extrusion are shown in Figure 3.



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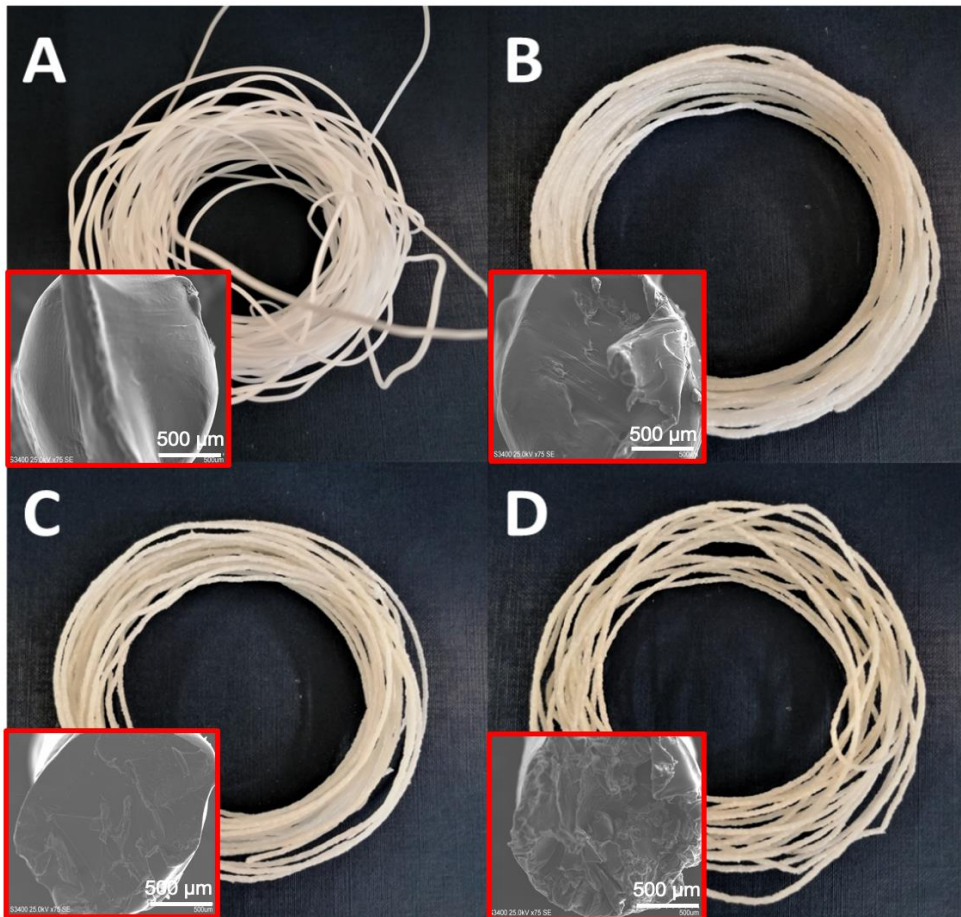
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Fig. 3. Granules obtained by combining CS-CO₂ and PVA solutions, freeze drying and extrusion (150°C) at mass ratios 25/75 (A), 50/50 (B) and 75/25 (C).

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334

Filaments obtained by extrusion PCL with 20% addition of CS-CO₂ composite are shown in Figure 4.



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Fig. 4. Photographs and SEM micrographs of CS-CO₂/PVA/PCL filaments at mass ratios: 0/0/100 (A), 5/15/80 (B), 10/10/80 (C), 15/5/80 (D).

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Based on SEM micrographs, it was observed that the filaments became less homogeneous with increasing chitosan concentration. They have a more coarser structure compared to the filament obtained exclusively from PCL granules. The increase in filament heterogeneity with the increase in chitosan concentration was also observed in our previous work, where PLA was used as the base

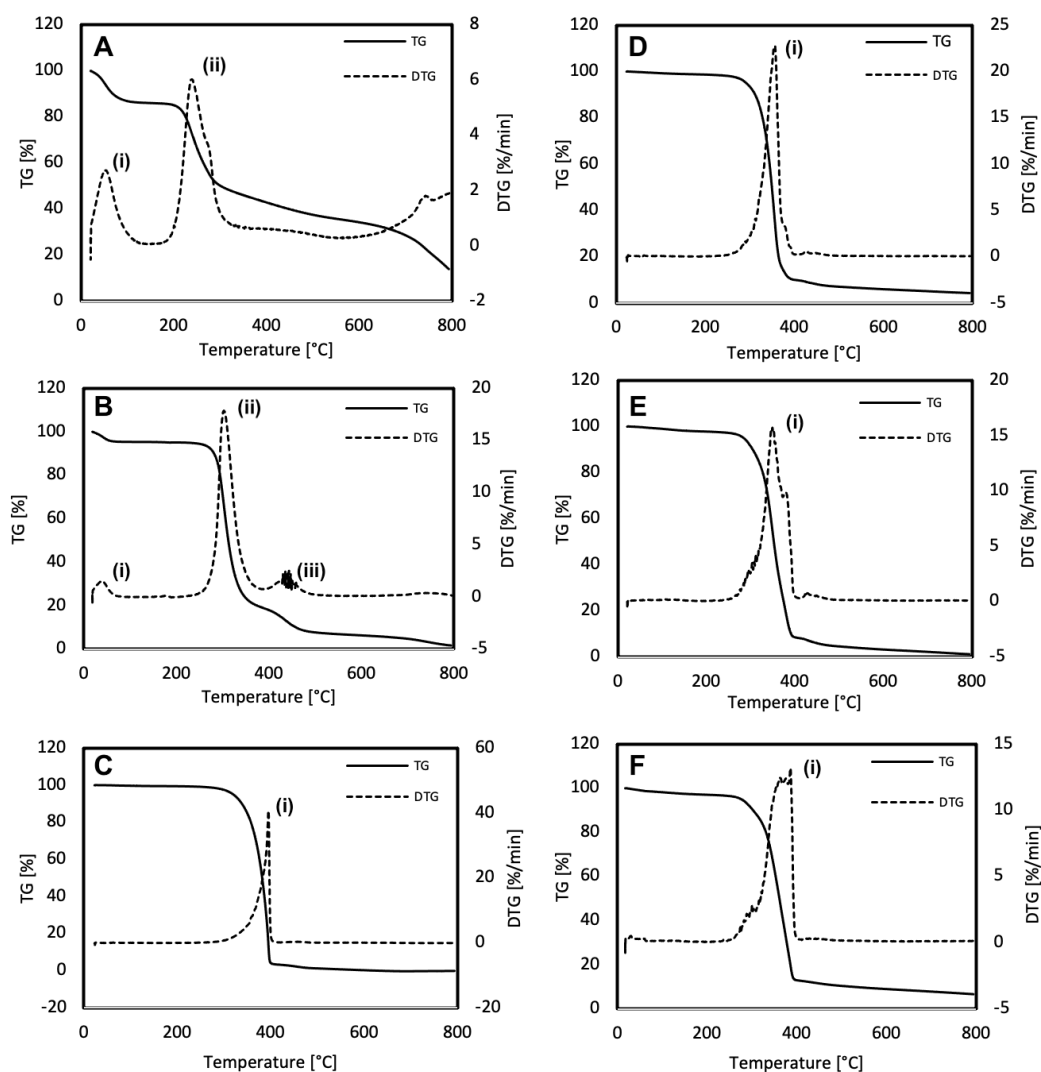
342 thermoplastic material. Due to the lack of the compatibilizer in the form of PVA, the deposition of
343 chitosan in the PLA matrix resulted in the formation of large pores in the filament and air pockets,
344 when the chitosan concentration was 10% (Mania et al., 2019). The use of PVA prevents such
345 problems, even when chitosan constitutes 15% of the filament's weight. Grande et al (2015)
346 demonstrated that the use of freeze-drying in the formation of PVA/chitosan composites significantly
347 improves the dispersion of these composites in PLA. The evidence is the observed particle size
348 distribution and a reduction in the dispersed particle reaching 0.5–5 μm during the
349 thermomechanical mixing process, as demonstrated by the SEM analysis (Grande et al., 2015). In
350 our investigation we observed that increase of the chitosan content in CS-CO₂/PVA composite
351 caused the worst distribution and the worst homogeneity of the material. For CS-CO₂/ PVA
352 composite containing 25%, 50%, and 75% of chitosan (m/m), the particle distribution was in the
353 range 5 -10 μm , 25 - 40 μm , and 50 - 60 μm , respectively (*Supplementary materials*). Chen and
354 colleagues suggested that particle distribution is associated with hydrogen bonds between the
355 hydroxyl groups of PVA and the amino groups of chitosan. Increasing the chitosan content in the
356 material increases the the level of ionic cross-linking tended to form the bigger CS/PVA particle
357 agglomerates (Chen et al., 2017). This understanding matches the results obtained for filaments
358 using the SEM technique (Fig. 4).

359 **3.4. Thermal stability**

360 The thermograms, derivative weight plots of CS-CO₂/PVA/PCL and their components are
361 presented in figure 5. The TGA curve of chitosan exhibited an endothermic effect attributable to water
362 release at the range 25-145°C (Fig. 5A) with 13.1% weight loss. The second decomposition stage is
363 attributed to a complex process including dehydration of saccharide rings, depolymerization and
364 decomposition of the acetylated units of the polymer (42.0% weight loss) (Lewandowska, 2009;
365 Moussout, Ahlafi, Aazza & Bourakhouadar, 2016). According to Eulalio and others, the third
366 decomposition stage (measured for chitosan powder) is a thermo-oxidative process that starts at
367 approx. 500°C, with maximum velocity at approx. 600°C and a weight loss of 40-45%, leaving no
368 residue behind (Eulalio, Rodrigues, Santos, Peniche, & LiaFook, 2019) . In our tests, there was also
369 a loss in weight that occurred above 400°C continuously with a small offset at 740°C (Fig.5A). The
370 PVA curve consisted of three stages (Fig. 5B). The first weight loss at 20-120°C is related to moisture
371 vaporization (4% weight loss). The second occurs at 305°C with 75% weight loss due to dehydration
372 of the chain with the release of volatile compounds. The last stage of weight loss above 400°C is
373 caused by degradation of polyene residues with formation of carbon and hydrocarbon (Lewandowska,
374 2009; Raju, Rao, Reddy, & Veera Brahmam, 2007). The TGA curve of PCL shows only one stage of
375 mass loss due to the lack of water absorption capacity of this polymer. In the temperature range from
376 350°C to 420°C almost complete weight loss due to depolymerization with the formation of ϵ -
377 caprolactone, which is subject to further decomposition is observed (Aoyagi, Yamashita, & Doi, 2002).
378 In turn, the TGA curves of CS-CO₂/PVA/PCL filaments were characterized by only one stage of
379 decomposition, during which almost complete loss of mass occurred (Fig. 5.D-F). Both the curve
380 course and the temperature value range for the stage of greatest mass loss coincide with the TGA
381 PCL curve. The results of the thermal analysis confirm the results of the visual assessment of the CS-
382 CO₂/PVA composites (Fig. 1). The PCL degradation starts above 350°C, so any loss of filament

383 weight until the printing temperature is reached may result from changes in chitosan and PVA.
 384 However, the weight loss of all produced filaments up to 150°C did not exceed 2% which suggests
 385 that the composites do not absorb nearly as much water as the CS and PVA materials. (Fig. 5 D-F).
 386 The weight loss measured up to 150°C for chitosan, PVA and CS-CO₂/PVA composites containing
 387 25%, 50% and 75% chitosan, was 18%, 5%, 5%, 7%, 10%, respectively. Thermograms for CS-
 388 CO₂/PVA composites are found in the *Supplementary materials* file. The decomposition temperature
 389 of them appears to shift from 275°C towards 225°C with increasing CS content. This is above the
 390 extrusion and printing temperature of 150°C, but is consistent with decomposition temperatures of
 391 275°C for PVA and 225°C for CS.

392



393

394 Fig. 5. TGA and TGA derivative curves of chitosan (A) CS, (B) PVA, (C) PCL, (D) CS-CO₂/PVA/PCL filament
 395 with 5/15/80 components ratio, (E) CS-CO₂/PVA/PCL filament with 10/10/80 components ratio, (F), CS-
 396 CO₂/PVA/PCL filament with 15/5/80 components ratio.

397 3.5. Mechanical properties and printability

398 The comparison of the filaments printability, mechanical properties of filaments and 3D prints obtained
 399 from them are presented in Table 1. The obtained CS-CO₂/PVA/PCL filaments were subjected to

mechanical tensile tests to determine the following parameters: tensile strength, elongation at break and Young's modulus. Based on the obtained results, significant differences between pure PCL and subsequent CS-CO₂/PVA/PCL samples with different chitosan content were found. The addition of the filler in the form of the CS-CO₂/PVA composite decreased the tensile strength by 73.6%, 67.8% and 56.6%, in relation to the PCL value, when the chitosan content in the filament was 5%, 10% and 15%, respectively. Filaments containing chitosan had 50-64% lower extension at break value and 50-75% lower Young modulus than the PCL filament. Deterioration of the mechanical properties of filaments with an increase in chitosan concentration was also observed in our previous work (Mania et al., 2019). Reduction in tensile strength by about 60% in PLA filaments with addition of 10% chitosan corresponded to the same reduction in tensile strength of CS-CO₂/PVA/PCL filaments with a 15/5/80 ratio. The equal reduction in tensile strength even at 5% higher concentration of chitosan in thermoplastic filaments may be due to the use of PVA as a compatibilizer in the physical mixture of raw materials. Filaments containing a filler in the form of a composite of chitosan with PVA, in relation to filaments only with the addition of chitosan, but at a similar concentration of this polymer, are characterized by lower elongation ability (Mania et al., 2019). Based on Young modulus-tensile strength chart, all obtained CS-CO₂/PVA/PCL filaments are classified as natural materials (Shah, 2014). Properties closest to thermoplastic materials were demonstrated by a filament with addition of 15% chitosan. To be included in this group, the same filament should have approx. 2.7 times greater tensile strength with recent Young modulus.

Despite the significant deterioration of the mechanical parameters of the filaments resulting from the use of the CS-CO₂/PVA composite filler in the PCL matrix, all filaments obtained can be described as printable. They fall within the printability window described in the experiment of Xu and colleagues: > 80 kg/mm²*% (Xu et al., 2020). The printability of the filaments demonstrated in the instrumental test was confirmed in a printout using a 3D FDM printer (Fig.6).

Table 1. Comparison of the filaments' printability, their mechanical properties and 3D prints obtained from them (n=10, p<0.05).

Material type	PCL	CS-CO ₂ /PVA/PCL 5/15/80	CS-CO ₂ /PVA/PCL 10/10/80	CS-CO ₂ /PVA/PCL 15/5/80
Filament				
Tensile strength [MPa]	21.49 ± 1.02 ^a	5.68 ± 0.15 ^b	6.91 ± 0.21 ^c	9.32 ± 0.43 ^d
Extension at break [%]	21.96 ± 0.79 ^a	10.89 ± 0.30 ^b	10.34 ± 0.44 ^b	7.83 ± 0.19 ^c
Young modulus [MPa]	15.50 ± 0.48 ^a	8.03 ± 0.50 ^b	6.22 ± 0.18 ^c	3.98 ± 0.09 ^d
Printability [kg/mm²*%]	187.9 ± 3.2 ^a	131 ± 2.0 ^b	103.1 ± 1.4 ^c	95.3 ± 1.1 ^d
3D print				
Hardness [kPa]	3.42 ± 0.08 ^a	3.95 ± 0.06 ^b	4.35 ± 0.09 ^c	4.67 ± 0.04 ^d
Flexibility [-]	0.88 ± 0.03 ^a	0.72 ± 0.05 ^b	0.69 ± 0.03 ^b	0.57 ± 0.02 ^c
Cohesiveness [-]	0.73 ± 0.05 ^a	0.70 ± 0.04 ^a	0.62 ± 0.06 ^a	0.65 ± 0.02 ^a

Values with different letters marked from a to d differ significantly from the control sample in each row.

Compression tests have shown that increasing the chitosan concentration in the printout increases its hardness and decreases its flexibility. The samples with addition of 5%, 10% and 15% chitosan were 15.5%, 27.2% and 36.5% harder and 18.2%, 21.6% and 35.2% less flexible compared to PCL, respectively. The addition of chitosan did not significantly affect the cohesiveness of the 3D prints, which was comparable to that of the prints made of polycaprolactone (Table 1). Visually, there was no difference between the samples.



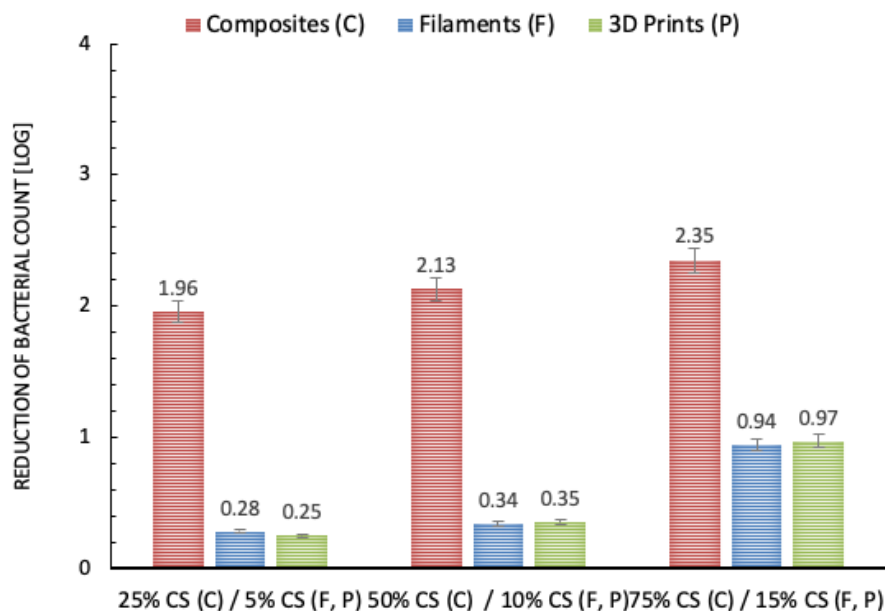
Fig.6. The photography of 3D objects in the form of cylinders printed with the FDM method from the obtained composite filaments (from the left 3D prints containing 5%, 10% and 15% of chitosan).

3.6. Antimicrobial properties

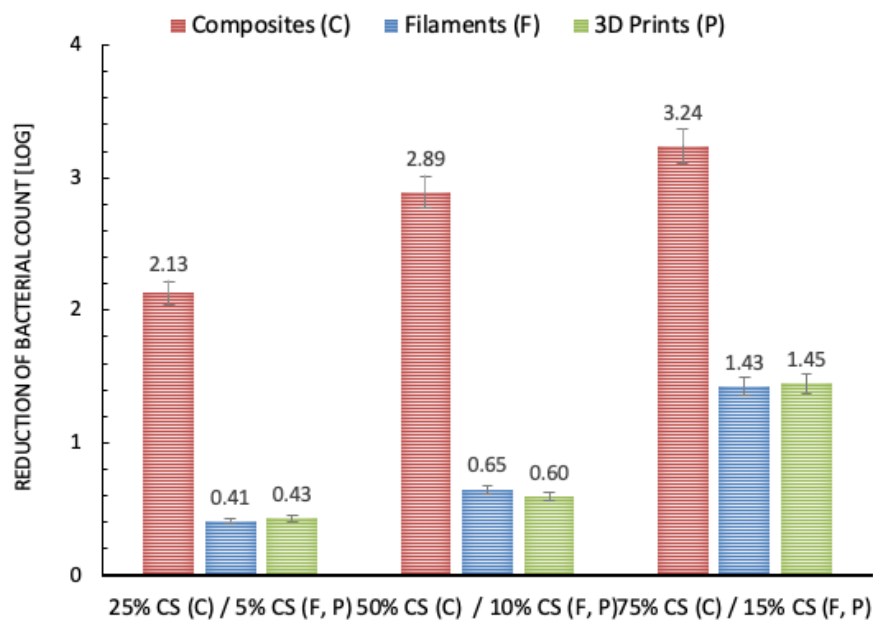
To evaluate the antimicrobial properties of the filaments the ASTM: E2149 method with slight modifications was used, which is designed to measure the antimicrobial activity of non-leaching (non-water soluble) antimicrobials surfaces made of plastic, rubber, silicone, and treated fabric material and is one method to test an irregularly shaped antimicrobial object, such as a thread, powder or 3D molded plastic. The results of the antimicrobial activity tests are presented in the form of the degree of reduction obtained after 24-hour contact of *S. aureus* and *E.coli* inoculum with the tested materials. PCL and PVA have no antimicrobial properties (Aslam, Kalyar, & Raza, 2018; Balcucho, Narváez, & Castro-Mayorga, 2020). It was found that all CS-CO₂/PVA composites had the strongest antimicrobial effect against *S. aureus* and *E. coli* (at least 2 log orders which corresponds to 99% reduction), compared to PCL filaments and prints. This activity also increased for both bacterial strains along with an increase in chitosan concentration in the material (Fig.7.) The highest value was recorded for CS-CO₂/PVA composites containing 75% of chitosan against a *S. aureus* strain. The same composite also caused the strongest antimicrobial effect after being used to produce filaments and for 3D printing. The greater antimicrobial effect of the CS-CO₂/PVA composite, CS-CO₂/PVA/PCL filaments and 3D prints was obtained with respect to the Gram-positive strain. The 3D prints showed virtually the same antimicrobial properties as the filaments from which they were printed. It means that re-melting the CS-CO₂/PVA/PCL material has no significant effect on the inhibition of the material's antimicrobial activity. The antimicrobial activity was due to the presence of chitosan and depends on the degree of its deacetylation, molecular weight, concentration in solution, pH, and ionic strength of the solution (Rinaudo, 2006). According to Goy and colleagues, there is a lack of conclusive data on whether chitosan has higher activity on Gram-positive or on Gram-negative bacteria (Goy, de Britto, & Assis, 2009). On both strains, chitosan seems to act differently, though in both cases satisfactorily. The main reason for the antimicrobial activity of chitosan is the interaction of its positively charged chains with anionic components of microorganisms—lipopolysaccharides (Gram-negative bacteria) and teichoic acids

462 (Gram-positive bacteria) that cause bacterial cell lysis. However, this mechanism takes place when
463 the amino groups of the polymer are protonated: $\text{pH} < 6$ (Kong, Chen, Xing, & Park, 2010).

A



B



464

465 Fig. 7. The antimicrobial activity against (A) *E. coli* and (B) *S. aureus* of CS-CO₂/PVA composites, and CS-
466 CO₂/PVA/PCL filaments and prints estimated according to Standard ASTM: E2149.

467 It has been concluded that for neutral or alkaline media, the cationic nature of chitosan can no
468 longer explain its antibacterial activity. In this case, the strong coordination capability of -NH₂

469 groups in the chitosan chain might be one possible mechanism (Mania et al., 2018; Xie, Wang, &
470 Liu, 2002).
471 In the scientific literature, it has been confirmed that the antimicrobial activity of chitosan does not
472 imply a cytotoxic effect. Da Silva and co-workers used chitosan from the same producer and with
473 the same parameters to obtain scaffolds for cartilage tissue engineering. They confirmed that the
474 viability of the chondrocytes from pig stifles estimated by the live / dead assay did not differ between
475 the four groups of chitosan scaffolds and was above 80% (Da Silva et al., 2016). Moreover, in the
476 earlier work of our team it was shown that chitosan hydrogels obtained by the CO₂ saturation
477 method are characterized by higher biocompatibility towards mouse NIH 3T3 fibroblasts,
478 than their counterparts in solutions of organic or mineral acids (Gorczyca et al., 2014). Also no
479 cytotoxic effect was noted for polycaprolactone and polyvinyl alcohol (Ragetyl et al., 2010; Zhang et
480 al., 2019)

481 **4. Conclusion**

482 In our work we present an innovative method for processing chitosan in a thermoplastic matrix. The
483 use of the CO₂ saturation technique in the preparation of CS-CO₂/PVA composites allows
484 generation of a polar filler for thermoplastics containing up to 75% chitosan. So far, such a solution
485 has not been presented by any research team and it overcame the limitation previously described in
486 the literature regarding the presence of residual solvents. The use of a 20% filler addition in a
487 thermoplastic material allows it to achieve a chitosan concentration in the range of 5-15% m / m,
488 and to produce a filament suitable for use in 3D printing with the FDM technology. Both, CS-
489 CO₂/PVA/PCL filaments and prints are characterized by antimicrobial activity against gram negative
490 and gram positive indicator bacteria. This activity depends on the concentration of chitosan and
491 ensure reduction of bacterial growth even above 0.94 in logarithmic scale (89%) in the case of
492 materials containing 15% of chitosan. The filler decreases the mechanical properties of the
493 filaments, but it does not significantly affect their printability. On the other hand, 3D prints become
494 harder, less flexible and consistent with increasing chitosan concentration. The absence of acid in
495 the materials allows the thermal stability of the filaments to be ensured, which is extremely
496 important in its further processing, including in 3D printing conditions. The presented results may
497 have great potential in biological applications as cell growth scaffolds, including in regenerative
498 medicine.

499 **Author Contributions:**

500 Conceptualization: R.T, S.M., Data curation, S.M., P.K., A.B-K., and R.T.; Formal analysis, R.T. and S.M.;
501 Funding acquisition, R.T. and S.M. Investigation, R.T, P.K. and S.M.; Methodology, R.T., P.K. and S.M.; Project
502 administration, R.T. and S.M.; Resources, R.T., P.K., A.B-K. and S.M.; Software, R.T., P.K., A.B-K. and S.M.
503 Supervision, R.T. and S.M.; Validation, R.T., A.B-K. and S.M.; Visualization, R.T., P.K., A.B-K., and S.M.;
504 writing—original draft preparation, R.T., P.K., A.B-K., and S.M.; writing—review and editing, R.T., P.K., A.B-K.,
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