Stoja Milovanovic, Gesa Hollermann, Cornelia Errenst, Jelena Pajnik, Sulamith Frerich, Stephen Kroll, Kurosch Rezwan, Jasna Ivanovic

Supercritical CO2 impregnation of PLA/PCL films with natural substances for bacterial growth control in food packaging

Supercritical CO_2 impregnation of PLA/PCL films with natural substances for bacterial growth control in food packaging

Stoja Milovanovic[a], Gesa Hollermann[b], Cornelia Errenst[c], Jelena Pajnik[a], Sulamith Frerich[c], Stephen Kroll[b,d], Kurosch Rezwan[b,e], Jasna Ivanovic[a,*]

[a] Department of Organic Chemical Technology, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia
[b] Advanced Ceramics, University of Bremen, Am Biologischen Garten 2, 28359 Bremen, Germany
[c] Institute of Thermo and Fluid Dynamics, Ruhr-University Bochum, Universitätsstraße 150, 44801 Bochum, Germany
[d] IfBB - Institute for Bioplastics and Biocomposites, Hochschule Hannover - University of Applied Sciences and Arts, Heisterbergallee 10A, 30453 Hannover, Germany
[e] Centre for Materials and Processes (MAPEX), University of Bremen, Bibliothekstraße 1, 28359 Bremen, Germany

Keywords:
Antibacterial food packaging
Poly(lactic acid)
Poly(ε-caprolactone)
Supercritical CO_2 impregnation
Thyme extract
Thymol

A B S T R A C T

Biodegradable polymers with antibacterial properties are highly desirable materials for active food packaging applications. Thymol, a dietary monoterpene phenol with a strong antibacterial activity is abundant in plants belonging to the genus *Thymus*. This study presents two approaches for supercritical CO_2 impregnation of poly (lactic acid)(PLA)/poly(ε-caprolactone)(PCL) blended films to induce antibacterial properties of the material: (i) a batch impregnation process for loading pure thymol, and (ii) an integrated supercritical extraction-impregnation process for isolation of thyme extract and its incorporation into the films, operated in both batch or semi-continuous modes with supercritical solution circulation. The PCL content in films, impregnation time and CO_2 flow regime were varied to maximize loading of the films with thymol or thyme extract with preserving films' structure and thermal stability. Representative film samples impregnated with thymol and thyme extract were tested against Gram (−) (*Escherichia coli*) and Gram(+) (*Bacillus subtilis*) model strains, by measuring their metabolic activity and re-cultivation after exposure to the films. The film containing thymol (35.8 wt%) showed a strong antibacterial activity leading to a total reduction of bacterial cell viability. Proposed processes enable fast, controlled and organic solvent-free fabrication of the PLA/PCL films containing natural antibacterial substances at moderately low temperature, with a compact structure and a good thermal stability, for potential use as active food packaging materials.

1. Introduction

Environmental and economic concerns associated with the limitation of fossil resources and the accumulation of non-degradable waste materials have led to the global interest for replacing petroleum-based polymers in food packaging and disposable product manufacture sector with biodegradable polymers derived from renewable resources (Tawakkal, Cran, Miltz, & Bigger, 2014; Vieira, Da Silva, Dos Santos, & Beppu, 2011; Vink et al., 2004).

As a synthetic biodegradable polyester whose monomers are derived from renewable, agricultural feedstock, poly(lactic acid)(PLA) represents the most promising and a commercially viable alternative towards petroleum-based polymers in the domain of packaging (Murariu & Dubois, 2016). Besides being thermoplastic and biodegradable, poly (lactic acid)(PLA) has a good processability, a reasonably good barrier and mechanical properties for a wide spectrum of applications (Lim, Auras, & Rubino, 2008). However, inherently low impact strength and elongation at break, slow crystallization, poor thermal resistance, sensitivity to moisture and degradation by hydrolysis of the neat PLA need to be altered for its use in the packaging and engineering sectors (Lim et al., 2008; Murariu & Dubois, 2016). Blending of PLA with poly(ε-caprolactone)(PCL) has been, therefore, suggested for creation of new biomaterials with tailored mechanical, thermal and viscoelastic and biodegradation properties (Goriparthi, Suman, & Nalluri, 2012; Wu, Zhang, Yuan, Zhang, & Zhou, 2010; Yeh et al., 2009). PCL is a biodegradable and biocompatible aliphatic polyester with lower tensile strength and slower degradation rate mainly due to hydrophobicity but better toughness than PLA (Wu et al., 2010). Both PLA and PCL are

Abbreviations: PLA, Poly(lactic acid); PCL, Poly(ε-caprolactone); PLA, PLA-1, PLA-5, PLA-10, PLA without, and with 1 wt%, 5 wt% and 10 wt% of PCL, respectively; SFE-SSI, Coupled supercritical fluid (CO_2) extraction and impregnation; SSI, Supercritical solvent (CO_2) impregnation; T, Thymol; T, Supercritical extract of thyme

* Corresponding author.
E-mail address: jasnai@tmf.bg.ac.rs (J. Ivanovic).

approved by the United States Food and Drug Administration (FDA) for contact with food.

A growing global concern about foodborne diseases associated with an outbreak of microorganisms in food products has led to an increased interest in active food packaging as an emerging technology for controlling microorganism growth in the food systems and extending of food shelf life (Krepker et al., 2017; Raouche, Mauricio-Iglesias, Peyron, Guillard, & Gontard, 2011). Typical antimicrobial agents in food packaging systems include benzoic acid, sorbates, lauric acid, lysozyme, nisin, EDTA, glucose oxidase, chlorine dioxide, ethanol, sulphur dioxide, silver, triclosan, synthetic antibiotics (Malhotra, Keshwani, & Kharkwal, 2015; Sung et al., 2013). Health-related issues regarding metal (oxide) nanoparticles as antimicrobial agents (Jeng & Swason, 2006; Martirosyan & Schneider, 2014) and the increase in microorganism resistances towards conventional antimicrobial substances (Malhotra et al., 2015; Nostro et al., 2004) arose interest in natural antimicrobial substances (Ivanovic et al., 2013; Ivanovic, Misic, Zizovic, & Ristic, 2012; Raouche et al., 2011; Villegas et al., 2017).

Volatile fraction of the supercritical thyme extract isolated at 10–15 MPa and 35–40 °C is mainly composed of thymol (39.7–80.3%), carvacrol (3.13–5.96), and caryophyllene (1–6.06%) (Ivanovic et al., 2014, 2012). Thymol content in the supercritical extract of cultivated thyme used in this study is 4.14 wt% and it was determined by GC/MS using calibration-curve method. Thyme extracts and thymol have proven antimicrobial and antioxidant activity (Babovic et al., 2010; Ivanovic et al., 2012; Milovanovic et al., 2016) and are generally recognized as safe (GRAS) substances by FDA (Rivas et al., 2010). Thymol has already been incorporated in different synthetic and biopolymer films based on PLA (Boonruang, Chinsirikul, Hararak, Kerddonfag, & Chonhenchob, 2016; Milovanovic et al., 2016; Torres et al., 2017), polypropylene (Ramos, Jiménez, Peltzer, & Garrigós, 2012), low-density polyethylene (Krepker et al., 2017; Torres, Romero, Macan, Guarda, & Galotto, 2014), zein and gelatine (Del Nobile, Conte, Incoronato, & Panza, 2008; Kavoosi, Mohammad, Dadfar, & Purfard, 2013). The PLA/PCL, gelatin and zein films containing 8–35 wt% of thymol have been reported for antibacterial activity against Gram(+) (*B. subtilis*, *S. aureus*, *L. monocytogenes*) and Gram(−) (*P. aeruginosa*, *E. coli*) bacteria strains (Del Nobile et al., 2008; Kavoosi et al., 2013). Soy edible films loaded with commercial essential oil of thyme were proven for significant inhibition of *E. coli* and *S. aureus* (Emiroğlu, Yemiş, Coşkun, & Cando, 2010).

Traditional processes such as solvent casting, melt blending or compression moulding are used to load thymol and/or thymol-rich essential oils into PLA films (Emiroğlu et al., 2010; Liu et al., 2016; Tawakkal, Cran, & Bigger, 2016; Wu et al., 2014). However, these processes suffer from certain drawbacks. Solutions containing volatiles such as thymol for polymer impregnation using the solvent casting method can result in a low penetration into the polymer and simultaneous evaporation of the active substance with the solvent (Wu et al., 2014). This can have an adverse effect on the thymol loading and structure of the film (Wu et al., 2014). Melt blending implies high processing temperatures (145–190 °C) which can result in hydrolytic degradation of PLA (Karst & Yang, 2006) as well as thymol degradation (Tawakkal et al., 2016; Trivedi, Patil, Mishra, & Jana, 2015), partial vaporization and uneven distribution when thymol is added in the last minutes of the compounding process for preventing its evaporation (Ramos et al., 2014).

The limitations of the conventional methods can be avoided by using supercritical solvent impregnation (SSI), and namely impregnation with supercritical carbon dioxide ($scCO_2$). $ScCO_2$ is good solvent for a wide range of bioactive phytocompounds, including thymol (Milovanovic, Stamenic, Markovic, Radetic, & Zizovic, 2013; Reverchon, 1997). Thymol solubility in $scCO_2$ at pressure 7.8–25 MPa and temperature 35–50 °C expressed as mole fraction of thymol in $scCO_2$ ranges from $0.8{\cdot}10^{-3}$ to $14.7{\cdot}10^{-3}$ (Milovanovic et al., 2013). $ScCO_2$ establishes low energy interactions with the polymer matrix, which leads to its plasticization and swelling (Champeau, Thomassin, Jérôme, & Tassaing, 2014; Kazarian et al., 1996; Nalawade et al., 2006b; Nalawade, Picchioni, Marsman, & Janssen, 2006a). Consequently, more space between macromolecular chains and their increased mobility favour sorption of CO_2 (Aionicesei, Škerget, & Knez, 2009; Champeau et al., 2014) and substances soluble in $scCO_2$ (Ivanovic et al., 2016; Von Schnitzler & Eggers, 1999). High diffusivity, low viscosity and near zero surface tension of $scCO_2$ enable rapid penetration of thymol or thyme extract into a high variety of matrices including PLA (Alvarado et al., 2018; Ivanovic et al., 2014, 2016; Ivanovic, Zizovic, Ristic, Stamenic, & Skala, 2010; Milovanovic, Jankovic-Castvan, Ivanovic, & Zizovic, 2015; Torres et al., 2017) which can be easy controlled by changing CO_2 pressure, temperature and contact time. Therefore, in the impregnation process, $scCO_2$ is employed as a solvent for the given impregnating substance and the medium for its transport to polymer matrix. Additionally, low critical parameters of CO_2 ($p_c = 7.4$ MPa and $T_c = 31.1$ °C) allow material processing at relatively low temperatures.

Impregnation of an active substances into a polymer using $scCO_2$ can be designed either as a batch (Ivanovic et al., 2016; Torres et al., 2017; Villegas et al., 2017) or semi-continuous process (Fanovich et al., 2013; Ivanovic et al., 2014). In the batch process, the impregnating substance and the polymer are placed into the same vessel with or without provided circulation of the supercritical fluid solution. Semi-continuous impregnation involves subsequent introduction of fresh CO_2, dissolution of an active substance in $scCO_2$ in a separate vessel and flow of supercritical solution through a polymer placed in an adsorption column (Manna et al., 2007). If there is a demand to load the plant extract (thyme extract) rather than the pure substance (thymol) into the polymer, a coupling of the supercritical extraction and impregnation processes is reasonable regarding energy and time savings. In addition, a coupling of supercritical fluid extraction and impregnation processes (SFE-SSI) minimizes extract's loss in the tubes, vessels and exchangers of the equipment (Fanovich et al., 2013; Fanovich, Ivanovic, Zizovic, Misic, & Jaeger, 2016). Thyme extraction and extract's incorporation into cotton gauze, polypropylene, cellulose acetate, PCL and starch using SFE-SSI was recently reported (Ivanovic et al., 2014).

This study was aimed to test feasibility of two approaches in the eco-friendly production of biodegradable films containing natural compounds for potential use as new antibacterial food packaging materials. PLA and PCL were chosen as model polymers for the film production. A batch impregnation process was used to load pure thymol into the films. An integrated supercritical extraction-impregnation process, operated in batch or semi-continuous mode with constant circulation of the supercritical solution was used to isolate thyme extract and incorporate it into the films. To testify feasibility of the aforementioned processes for production of the functional thymol and thyme extract containing PLA/PCL films antibacterial activity, structural and thermal properties of the obtained films were analysed.

2. Materials and methods

2.1. Materials

PLA/PCL blended films were prepared by solvent casting method using two semi-crystalline aliphatic polyesters, poly(lactic acid) (PLA, Ingeo™ Biopolymer 3052D, NatureWorks LLC, USA) with $Tm = 160$ °C (Kuska et al., 2016) and poly (ε-caprolactone) (PCL, Aldrich, Mn = 80,000) with $Tm = 62.5$ °C (Ivanovic et al., 2016) with chloroform (98.3%, Lachema, Neratovice, Czech Republic) as a solvent. PCL (0.067 g/mL) and PLA (0.067 g/mL) were dissolved in chloroform and left at room temperature in separate glasses overnight. PLA and PCL solutions were mixed together using a magnetic stirrer at 700 rpm for 8 h under ambient conditions. Thereafter, the mixture was poured in a Petri dish to dry in air for a week. Prior to experiments the samples were vacuum dried at 40 °C for 24 h. PLA films without and with 1 wt%,

5 wt% and 10 wt% of PCL denoted as PLA, PLA-1, PLA-5 and PLA-10, respectively, were used for further impregnation tests. Solid, crystalline thymol with melting point of 48–51 °C and boiling point of 232 °C (purity < 99%, Sigma Aldrich, Germany), and dried aerial parts of the cultivated thyme (*Thymus vulgaris* L) donated by a local producer (BIOSS-Petrović Slobodan i ostali, Beograd, Serbia) were used for the impregnation tests. Carbon dioxide (purity of 99.9%, Messer-Technogas AD, Belgrade, Serbia) was used for thyme supercritical extraction and the films' supercritical impregnation.

2.2. Impregnation of PLA/PCL films

2.2.1. Processing of PLA/PCL films containing thymol

Impregnation of PLA/PCL films with thymol was performed by batch SSI in a high-pressure view cell (Eurotechnica GmbH, Bargteheide, Germany) described elsewhere (Milovanovic et al., 2015, 2013). A schematic presentation of the high-pressure view cell and the setup inside it is given in Fig. S1. Thymol (0.23 ± 0.02 g) was placed in the glass container at the bottom of the vessel, below a porous basket containing a PLA/PCL film (30 mm × 17 mm × 0.3 mm).

Polytetrafluoroethylene (PTFE) coated fiberglass fabrics below and above the film prevent possible splashing of thymol onto the film surface during CO_2 introduction into the vessel and its venting of the system (1.4 MPa/min). A film to thymol mass ratio of 1:1 was used in all the experiments. The system was heated to the desired temperature (40 °C). Thereafter, CO_2 was introduced and compressed to 10 MPa. The impregnation time was varied between 1 h and 15 h, while the system was kept at the constant pressure and temperature conditions. A slow depressurization of the system (1.4 MPa/min) was chosen as it favours thymol impregnation into PLA films (Torres et al., 2017).

2.2.2. Processing of PLA/PCL film containing thyme extract

A coupled process for supercritical fluid extraction (SFE) of thyme and isolated thyme extract's loading into representative PLA/PCL film (PLA-5) was performed in a laboratory scale unit (HPEA 500, Eurotechnica, Germany) shown schematically in Fig. S2. An extractor vessel (280 mL) was filled with the dried aerial parts of thyme (23 ± 2 g), while PLA-5 film (0.23 ± 0.02 g) was placed into the porous basket in the adsorption column (100 mL). A mass ratio of 100:1 of thyme to PLA-5 was used. Once the desired temperature was reached (40 °C), CO_2 was pumped into the system until the operating pressure (10 MPa) was reached in both extraction and adsorption vessel. Afterwards, a gear pump was used to provide circulation of the supercritical solution ($scCO_2$ + extract) through both extractor and adsorber, for desired time intervals (2–6 h). Three operation regimes were applied as given by Table 1.

SFE-SSI experiments in Regime 1 were operated in a batch mode. This implied only one filling of both vessels with CO_2 followed by subsequent circulation of the solution ($scCO_2$ + extract) through both extractor and adsorber over different time (2, 4 and 6 h) denoted as t_1 (Table 1, runs 1–3). On the other hand, SFE-SSI experiments operated in a semi-continuous mode involved cyclic introduction of a new quantity of $scCO_2$, after t_1 (Regime 2, runs 4 and 5) or after t_1 and t_2 (Regime 3, run 6) (Table 1). After each introduction of fresh $scCO_2$, supercritical solution was circulated through both extractor and adsorber over 2 h (runs 4–6). After every cycle of t_1 and t_2, before CO_2 introduction in the system, the extractor was kept closed while the adsorption vessel was being depressurized to atmospheric conditions by opening valve V-9 at a decompression rate of 1.4 MPa/min controlled by back pressure regulator (BPR) (Fig. S2). Film was taken out and weighed after each cycle and at the end of process. After each cycle in runs 4–6, the PLA/PCL film was taken from the adsorber, weighed using an analytical balance (accuracy ± 0.01 mg) and put back in the adsorber. After sealing the adsorber, valve V-3 was opened and the system was pressurized by introducing fresh CO_2 for the next cycle. Once the operating pressure in the adsorption vessel was reached, the valves on the extractor were opened and the $scCO_2$ + extract solution continued to circulate through the whole system. The thymol or thymol extract loadings in the films were calculated using Eq. (1):

$$Loading\ (wt\%) = \frac{m_L}{m_{L+F}} \cdot 100 \quad (1)$$

where m_L is mass of thymol or thyme extract loading and m_{L+F} mass of film loaded with pure thymol or thyme extract. m_L represents differential mass of the film before and after impregnation which is determined gravimetrically.

2.3. Determination of thymol content in the films

The representative samples impregnated with thymol and thyme extract were analysed with respect to thymol content using a gas chromatography/mass spectrometry (GC/MS) (GC, Agilent technologies, 7890A GC) (MS, Agilent technologies, 5975C MSD) and a capillary column (ZB-WAXplus, 30 m × 0.32 mm I.D., df = 0.25 μm). The impregnated films (0.05 g) were dissolved in 10 mL of chloroform (≥ 99.8%, CAS No 67-66-3, Carl Roth, Karlsruhe Germany) and afterwards diluted in a ratio of 1:100 (*v*/v) with chloroform. The GC/MS analysis was performed using a GC (Agilent technologies, 7890A GC) coupled with a MS (Agilent technologies, 5975C MSD) and a capillary column (ZB-WAXplus, 30 m × 0.32 mm I.D., d_f = 0.25 μm). The flow rate of the carrier gas (Helium) was 1 mL/min. The initial oven temperature was set to 120 °C, increased to 240 °C at a rate of 20 °C/min and maintained at 240 °C for 1 min. Quantification of thymol was based on an internal standard and calibration curve (thymol concentration range between 0.8 and 100 mg/L).

Table 1
Effect of $scCO_2$ flow regime, number of cycles and impregnation time on the thyme extract loading (TE) measured by mass change and on thymol content (T) analysed by GC/MS in PLA-5 films processed *via* coupled SFE-SSI process.

Run	Flow regime (No. of cycles)	Time of impregnation (h)[£]			m_{scCO2}:m_{thyme}[*]	TE (wt%)[¥]	T (wt%)[§]
		t_1	t_2	t_3			
1	1	2	–	–	17	4.95 ± 0.48	–
2	1	4	–	–	17	4.33 ± 0.42	–
3	1	6	–	–	17	5.08 ± 0.50	0.064 ± 0.006
4	2	2	2	–	22	6.06 ± 0.59	–
5	2	4	2	–	22	4.44 ± 0.43	0.067 ± 0.006
6	3	2	2	2	26	5.09 ± 0.50	0.027 ± 0.002

[*] m_{thyme}:m_{film} = 100 = const.
[¥] Gravimetric method.
[§] GC analysis.
[£] With provided constant circulation of supercritical solvent through extractor and adsorber.

2.4. Spectroscopic analysis of the films

Attenuated total reflectance Fourier transforms infrared (ATR-FTIR) spectroscopy was used to study the presence of thymol and thyme extract in the films and its interaction with the polymer. FTIR spectra of the samples were recorded in absorbance mode using a Nicolet™ iS™ 10 FT-IR Spectrometer (Thermo Fisher Scientific) with Smart iTR™ ATR Sampling accessories equipped with a diamond crystal, within a range of 400–4000 cm^{-1}, at a resolution of 4 cm^{-1} and in 20 scan mode.

2.5. Scanning electron microscopy

The effect of impregnated thymol and thyme extract on the film morphology was investigated by the field emission scanning electron microscopy (FE-SEM, Mira3 XMU TESCAN a.s., Brno, Czech Republic) operated at 15 kV. The film samples were coated with a thin layer of Au/Pd (85/15), using a sputter coater (POLARON SC502, Fisons Instruments, Ipswich, UK) prior to the analysis.

2.6. Thermogravimetric and differential thermal analysis

Thermogravimetric and Differential Thermal Analysis (TGA/DTA) of the representative samples of thymol and thyme extract containing films was performed using a simultaneous thermal analyser STA 503 (BAEHR Thermo-Analyse GmbH, Germany). TGA tests were conducted to monitor corresponding weight loss in the temperature range of 25 to 1000 °C under dry N_2 atmosphere (2 L/h). For the degradation kinetic analysis, each sample was heated at a rate of 10 °C/min, following a holding time of one hour at 1000 °C and cooling back to 25 °C at a rate of 10 °C/min.

2.7. Evaluation of antibacterial activities of impregnated films

For determining the antibacterial properties of selected PLA/PCL films, *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) were purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ, No. 1077 and No. 1088), Braunschweig, Germany. The cells were grown in overnight culture (37 °C, 150 rpm) in lysogenic broth (LB) media, collected by centrifugation and re-suspended in phosphate-buffered saline (PBS) to a final concentration of approximately 8×10^8 cells/mL of *E. coli* and 2×10^9 cells/mL of *B. subtilis* according to McFarland standards (Cat. No. R20421, Remel, Thermo Fisher Scientific). All materials were used in sterile conditions. Non-impregnated and impregnated PLA-5 films were sterilized in a UV-chamber overnight before the experiments. Pieces of films with the same size (5 × 10 mm) were placed in a 96-well plate, each filled with 250 µL of bacterial suspension, followed by incubation at 37 °C shaking for 3.5 h. For controls, the bacterial suspensions were incubated without material. All tests were performed in triplicates. After incubation, a part of the sample suspension was used to determine the adenosine triphosphate (ATP) level using a luminescence BacTiter-Glo™ assay (Promega No. G8231, Germany). The remaining suspension of each sample was diluted by a factor of 1:1.000 in PBS. 50 µL of the dilution was added to a 96-well plate filled with 250 µL LB medium each, sealed with a breathable membrane (Sigma Aldrich, No. **Z763624**) and followed by incubation at 37 °C under constant shaking. As blank controls, pure LB medium and PBS in LB medium were incubated in the same plate. The bacterial growth was observed by measuring the optical density (OD) at 590 nm for 18 h, using the UV/VIS spectrophotometer Multiskan® GO (Thermo Fisher Scientific) under kinetic mode.

2.8. Statistical analysis

Quantitative data were reported as mean ± standard deviation. A one-way ANOVA (analysis of variance) method followed by post hoc Tukey's HSD test was used to evaluate the significant difference among various treatments. Different letters were used to indicate that the means difference is significant at level $p < 0.05$.

3. Results and discussion

3.1. Effect of PCL content and impregnation time on the thymol loading using batch SSI

Batch impregnation experiments in the high-pressure view cell were performed to optimize impregnation time regarding the thymol loading in the PLA/PCL films with various PCL content (0, 1, 5 and 10 wt%) at constant CO_2 pressure and temperature (10 MPa, 40 °C). These pressure and temperature conditions were selected for two reasons: moderately high thymol solubility at 10 MPa and 40 °C of $4.5 \cdot 10^{-3}$ expressed as mole fraction in $scCO_2$ (Milovanovic et al., 2013) and $scCO_2$ plasticizing effect on PLA and PCL (Ivanovic et al., 2016; Kuska et al., 2016). At higher pressures (30 MPa, $T = 40$ °C), $scCO_2$ density and its solvent power increase. This can result in an increased affinity of thymol to supercritical fluid phase rather than polymer phase, and consequently, to a lower thymol loading of the polymeric carrier (Ivanovic et al., 2016). Plasticizing effect of $scCO_2$ on both PCL and PLA, which is desired for incorporation of thymol, is the most pronounced at moderately high pressures in the range of 10–20 MPa (Ivanovic et al., 2016; Kuska et al., 2016). Temperatures above 40 °C were excluded due to the previously studied melting behaviour of PCL under elevated CO_2 pressures (Ivanovic et al., 2016) to prevent structural changes of films.

By using GC/MS, the influence of the PCL content on the thymol loading (Fig. 1a) at constant time (2 h) and the effect of the impregnation time on the thymol loading in PLA-5 films (Fig. 1b) were given. Within the two-hour impregnation, an increased PCL content from 1 wt % to 10 wt% had positive but not significant effect on the thymol loading (11.3–13.3 wt%) and the thickness of films (10–15%). Torres

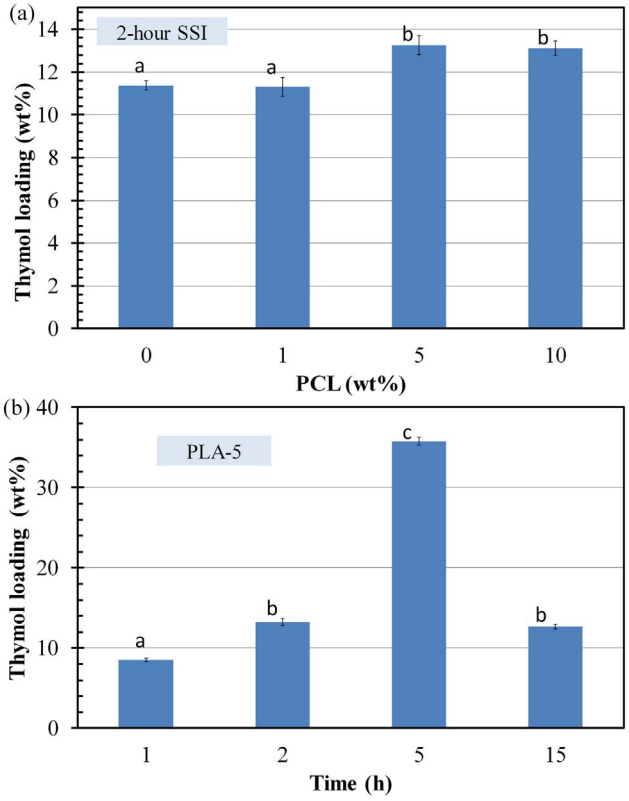

Fig. 1. Effect of: (a) PCL content in PLA films for 2-hour SSI, and (b) impregnation time for SSI of the PLA-5 on thymol loading at 10 MPa and 40 °C.

et al. (2017) achieved similar loading of thymol (13.5–20.5 wt%) in PLA films using the SSI process at similar pressure (9–12 MPa) and temperature conditions (40 °C) after 3 h by applying depressurization rates of 0.1–10 MPa/min. A slight increase of the thymol loading and film swelling with adding 5–10 wt% of PCL could be due to plasticizing effect of the PCL on the polymer matrix (Semba, Kitagawa, Ishiaku, & Hamada, 2006) which favours sorption of CO_2 (Aionicesei et al., 2009; Champeau et al., 2014) and of the soluble substances in it (Ivanovic et al., 2016; Von Schnitzler & Eggers, 1999).The highest and similar thymol loading (13.1–13.3%) was observed for the film containing 5 wt % and 10 wt% of PCL (PLA-5) (Fig. 1a). The PLA-5 was, however, used for further SSI tests to identify the optimal impregnation time for achieving the maximum thymol loading (Fig. 1b). In our recent study (Ivanovic, Rezwan, & Kroll, 2018), PLA blend with 10 wt% of PCL showed double decomposition peak after $scCO_2$ treatment indicating an increasing effect of PLA and PCL immiscibility which could have an adverse effects on structural changes of film during processing with $scCO_2$.

With increasing impregnation time from 1 h to 5 h, PLA-5 films were loaded with up to four times higher amount of thymol (35.8 wt%) (Fig. 1b). Certainly, a longer processing time (15 h) led to significant decrease of thymol loading of 12.6 wt% (Fig. 1b). The aforementioned phenomenon can be attributed to the increased crystallinity of the polyester matrix saturated with $scCO_2$ over time (Mauricio et al., 2011). Both $scCO_2$ and thymol act as plasticizers enhancing mobility and a better organization of the polymer chains which can result in increased polymer crystallinity. Lowering of the amorphous phase and free volume of the polymer matrix consequently result in a lower sorption of $scCO_2$ and an impregnating substance (thymol) in the polymer (Ivanovic et al., 2016; Kuska et al., 2016). Therefore, the PLA-5 film impregnated with 35.8 wt% of thymol after 5 h of SSI was used as a representative for further characterization. A good dispersion of thymol is observed for all the films impregnated with thymol. This is primarily attributed to interaction of the thymol and the polymer matrix through the hydrogen bonding between the carbonyl groups of both polyester components and the hydroxyl groups of thymol (Boonruang et al., 2016).

3.2. Effect of CO_2 flow regime in the SFE-SSI process on the loading of thyme extract

The optimization of the coupled SFE-SSI process is based on harmonization of the dynamics of the process of thyme extraction and the process of thyme extract's impregnation into the films since the latter is being much slower. In this regard, the mass ratios of the $scCO_2$ to thyme ($m_{scCO2:}$ m_{thyme}) and thyme to film (m_{thyme}: m_{film}), processing time and the CO_2 flow regime need to be synchronized in order to maximize the extraction and loading of the thyme extract, achieve an even distribution of the extract into the film and prevent the desorption of already impregnated extract from the film (Ivanovic et al., 2014). The coupled SFE-SSI process was carried out at the same operating pressure and temperature as SSI of thymol (10 MPa, 40 °C). Experiments were carried out in a batch (Regime 1) and semi-continuous mode (Regimes 2 and 3) with constant circulation of supercritical solution through both extractor and adsorber. Impregnation time of up to 6 h was chosen in order to minimize crystallization of the film due to saturation with $scCO_2$ and/or the thyme extract whose components (i.e. thymol) can act as plasticizers as well as to prevent desorption of the impregnated extract from the film (Ivanovic et al., 2014). The effect of impregnation time and introduction of fresh CO_2 into the system (which increases m_{scCO2}:m_{thyme} ratio) on the thyme extract loading is given in Table 1. The colour of the PLA-5 films changed from whitish (non-impregnated) and pale yellow (shorter processing time) to dark yellowish with increasing of impregnation time and/or number of cycles. This indicated a successful incorporation of the thyme extract into the films (Fig. 2).

Running of the process in the Regime 1 prevents loss of the thyme extract that occurs during intermediate step(s) of adsorber decompression when the Regimes 2 and 3 are applied. The impregnation of the PLA-5 with the thyme extract at constant pressure and temperature conditions is supposed to be favoured by prolongation of contact time from 2 to 6 h (runs 1–3) due to the fact that this process is much slower than the process of thyme extraction. However, prolongation of impregnation time did not have significant effect on the thyme extract loading in the film, which was between 4.33 wt% and 5.08 wt% TE (Table 1). Yet it had improved the distribution of the extract inside the carrier film which was reflected by darker yellow colour of the film (run 3, Fig. 2).

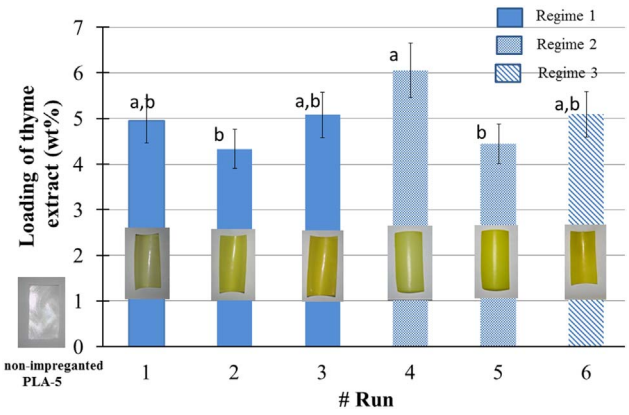

Fig. 2. Influence of $scCO_2$ flow regime in SFE-SSI process on loading and distribution of thyme extracts into PLA-5 film.

A two-hour impregnation followed by another cycle of introducing of fresh CO_2 was the most effective regime for increasing the loading up to 6.06 wt% TE (run 2). On the other hand, running the process in three cycles (2 h + 2 h + 2 h times of impregnation), with two intermediate steps of decompression of the adsorber led to the loss of thyme extract from the vessels, which in turn resulted in a lower thyme extract loading (5.09 wt% TE). This was due to elutriate of thyme extract from the impregnated film with multiple introducing of fresh CO_2 which also resulted in a partial loss of the volatiles (thymol) (Table 1, run 6). Longer time (6 h) of the film impregnation either by using Regime 1 (run 3) or Regime 3 (run 6) led to the most even distribution of the impregnated thyme extract (Fig. 2). Sample obtained from run 3 (Regime 1, one cycle process) was used for further characterization due to the relatively high thyme extract loading (5.08 wt%), thymol content in the thyme extract-loaded film (0.064 wt%) and a lower CO_2 consumption for its production compared to run 4 (2 h + 2 h) using SFE-SSI process. (Table 1) as well as even distribution of the extract (Fig. 2).

3.3. Morphological analysis of PLA/PCL films impregnated with thymol and thyme extract

The SEM images of non-impregnated (PLA-5) and representative PLA-5 films impregnated with thymol (35.8 wt%, PLA-5 + T) and thyme extract (5.08 wt%, PLA-5 + TE) are shown in Fig. 3. Submicron voids on the surface and pits of the non-treated PLA-5 film is due to a partial miscibility of PLA and PCL in the solvent casting process (Fig. 3a) (Wu et al., 2014). In this regard, an increased amount of PCL (i.e., 30 wt%) is expected to evoke the appearance of larger voids and pits on and in the PLA/PCL blend films which might have a negative effect on mechanical properties of the film (Wu et al., 2014). The presence of 35.8 wt% of thymol in the PLA-5 blend favoured formation of submicron-sized voids on the surface (Fig. 3b). However, the density and size of the voids (< 1 μm) on the surface of the PLA-5 + T were notably smaller compared to the previously reported for the PLA film blended with 30 wt% of PCL with three times lower thymol load (12 wt %) produced by solvent casting method (Wu et al., 2014). This was due

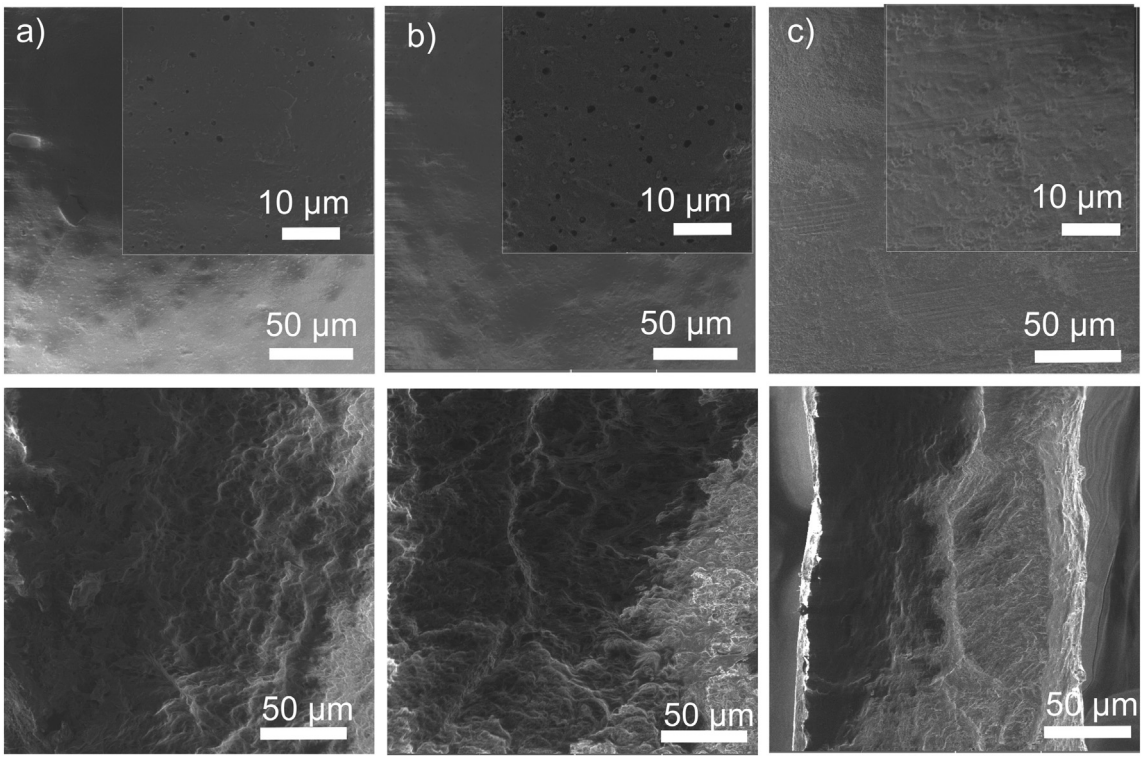

Fig. 3. SEM images of (a) PLA-5, (b) PLA-5 + T, and (c) PLA-5 + TE surface (upper row) and cross-section of corresponding samples (lower row).

to thymol evaporation with chloroform used for the PLA-thymol composite film preparation in the aforementioned study. A more compact structure of the PLA-5 + TE film containing thyme extract could be due to the lower amount of isoprenoids (including thymol) due to the co-extraction of higher molecular weight compounds such as waxes (Fig. 3c). Co-extraction of waxes with volatile compounds present in aromatic plants during single-step SFE process is common even at moderately low $scCO_2$ pressures and temperatures (9–10 MPa, 40–50 °C) (Ormeño, Goldstein, & Niinements, 2011; Reverchon, 1997). However, co-extracted waxes can stabilize the essential oil leading to a delay of evaporating volatile components (Ormeño et al., 2011). A more compact structure of the PLA-5 containing 5.08 wt% of the thyme extract compared to the PLA-5 loaded with 35.8 wt% of pure thymol (Fig. 3b) might be due to a lower plasticizing effect of the thyme extract (contains only 0.064 wt% of the thymol).

3.4. Structural analysis of the PLA/PCL films impregnated with thymol and thyme extract

The presence of thymol and thyme extract in the films was analysed by ATR-FTIR. The corresponding spectra of (i) thymol, (ii) non-impregnated and (iii) thymol containing PLA-5 films are given in Fig. 4a. ATR-FTIR spectra of (i) thyme extract, (ii) non-impregnated and (iii) thyme extract containing PLA-5 films are presented in Fig. 4b. Observed shifts in the following ATR-FTIR absorption bands showed that the thymol interacts with polymer matrix through intermolecular hydrogen bonds between thymol terminal hydroxyl group and carbonyl groups of the ester moieties of both PLA and PCL. A shift of the broad band at 3196 cm^{-1} in the spectra of thymol (Fig. 4a(i)) corresponding to the stretching of phenolic –OH, to 3503 cm^{-1} was observed after thymol impregnation into the film (Fig. 4a(iii)). Shift and broadening of the band related to -OH stretching was also observed for PLA/kenaf-fiber composite films containing 20 wt% of thymol (Tawakkal et al., 2016). The aforementioned shift was not evidenced for PLA-5 loaded with the 5.08wt of thyme extract. Adsorption bend of ester COC group (overlapping with CH) is shifted from 2867 to 2873 cm^{-1} after thymol impregnation (Fig. 4a(i) and Fig. 4a(iii)).

The band at 805 cm^{-1} (Fig. 4a(i)) attributed to the out-of-plane CH wagging vibrations (Topala & Tataru, 2016) from isoprenoids is also shifted to 808 cm^{-1} after thymol impregnation into the film (Fig. 4a (iii)). PLA/PCL film containing thyme extract showed the same band in the FTIR spectra although at somewhat lower wave number, 811 cm^{-1}, which arises from the overlapping of thymol and carvacrol bands (Topala & Tataru, 2016) (Fig. 4b(iii)). A shoulder at 1724 cm^{-1} (Fig. 4a (ii)), on the peak observed in the PLA carbonyl group absorption band at 1748 cm^{-1}, is attributed to the carbonyl group stretching vibrations (Wisam, Mansor, Emad, Jaffar, & Nor Azowa Bt, 2010). Its disappearance after thymol impregnation supports the notion of an intermolecular interaction between hydroxyl group of thymol and carbonyl groups present in both polyester components of PLA-5 film. This was not the case for thyme extract impregnated into the PLA-5 film where the shoulder at 1724 cm^{-1} was still observed (Fig. 4b(ii)) thus indicating insufficient amount of thymol (OH) groups to interact with carbonyl groups present in both polyester components, PLA and PCL.

Increased intensity of the adsorption peak attributed to C═O bending at 1754 cm^{-1} (Wisam et al., 2010) is observed only for the PLA-5 film containing thymol (Fig. 4a(iii)) which also indicates carbonyl group partition in intermolecular interactions. Slight shift of peaks at 1082 cm^{-1} and 1182 cm^{-1} attributed to C—O bending to the higher wave numbers was observed for the thymol containing PLA-5 film (Fig. 4a(iii)). The presence of thymol and thyme extract in the PLA-5 films was also evidenced by appearance of the four peaks ranging from 1620 to 1457 cm^{-1} specific to C═C stretching vibrations in phenolic ring (Topala & Tataru, 2016), OH and = CH out-of-plane bending adsorption band at 738 cm^{-1} and 945 cm^{-1} (Fig. 4a(iii) and Fig. 4b(iii)). Two bands related to the crystalline and amorphous phases of PLA were found at 870 cm^{-1} and 754 cm^{-1}, respectively (Fig. 4a(ii), Fig. 4a(iii), Fig. 4b(ii) and Fig. 4b(iii)). Weak peaks at ~590 cm^{-1} originate from the out-of plane bending of hydroxyl group of thymol (Fig. 4a(iii)).

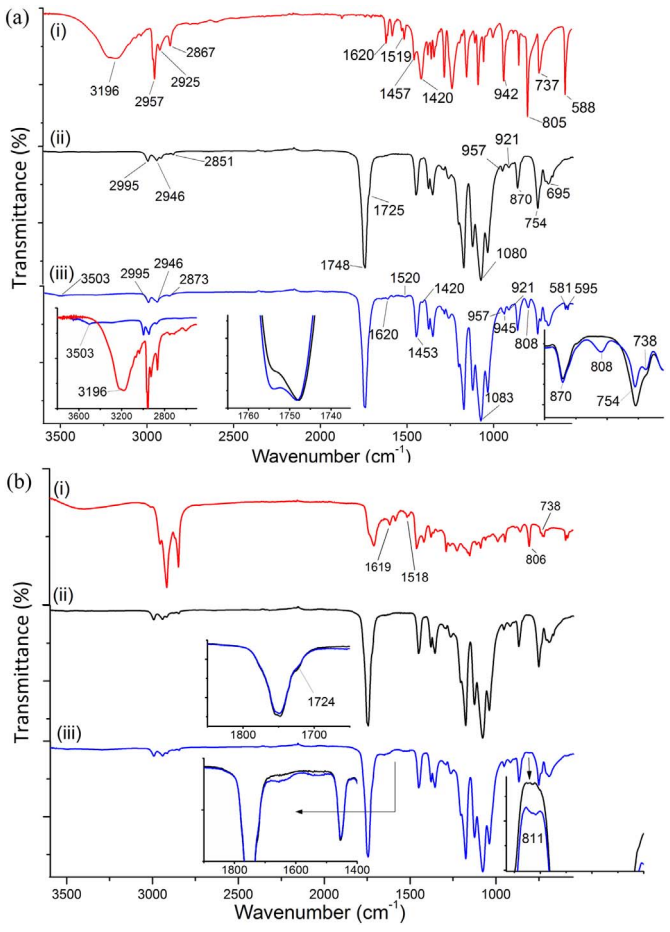

Fig. 4. ATR-FTIR spectra of (a) thymol (i), PLA-5 film (ii), and PLA-5 impregnated with thymol (iii) and (b) thyme extract obtained by extraction with $scCO_2$ (i), PLA-5 film (ii), and PLA-5 impregnated with supercritical extract of thyme (iii).

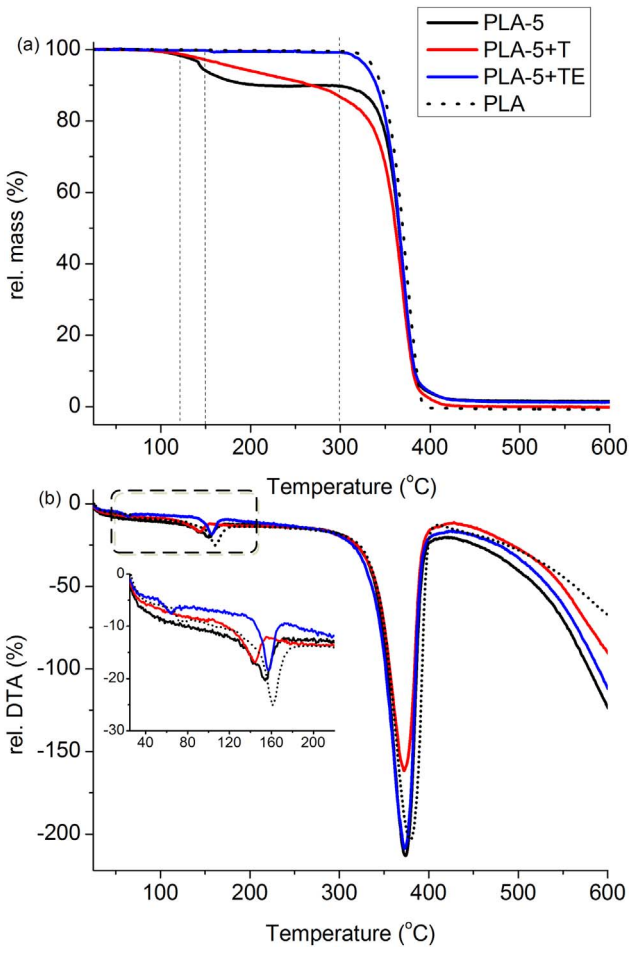

Fig. 5. (a) TGA and (b) DTA thermograms obtained for the PLA, non-treated PLA-5, PLA-5 impregnated with thymol and thyme extract under nitrogen.

3.5. Thermal stability of the PLA/PCL films impregnated with thymol and thyme extract

The results of thermal analysis of the non-impregnated PLA and PLA-5 films impregnated with 35.8 wt% of thymol (PLA-5 + T) and 5.08 wt% of thyme extract (PLA-5 + TE) extract are given in Fig. 5 and Table 2. Both non-impregnated films (PLA and PLA-5) and the PLA-5 impregnated with thymol (PLA-5 + T) showed two inflections (Fig. 5b). The first one centred 144–161 °C can be associated with melting of the PLA – Ingeo™ Biopolymer 3052D (Kuska et al., 2016), whereas the second one centred around 373–379 °C (Table 2) corresponds to the polymer thermal degradation (Alvarado et al., 2018; Torres et al., 2017). The low temperature inflection due to thymol (or thymol-rich oil) dehydration and melting at 40–61.6 °C (Alvarado et al., 2018; Kumar Trivedi et al., 2015; Mourtzinos et al., 2008; Torres et al., 2017) is visible only on the DTA curve for the film loaded with 5.08 wt% of the thyme extract which contains only 0.064 wt% of thymol. At DTA curve for PLA-5 + T sample that contains 35.8 wt% of thymol (600 times higher amount than the PLA-5 + TE sample) this peak is, however, not evidenced. Following explanation can be given. Alvarado et al. (2018) produced neat PLA films and PLA nanocomposite films containing natural nanofibers impregnated with thymol using supercritical fluid impregnation. Operating conditions applied for supercritical impregnation of PLA-based films in the recent studies (Alvarado et al., 2018; Torres et al., 2017) (9–12 MPa and 40 °C) enabled their loading with 18.0–23.8 wt% of thymol. Despite poor stability of the pure thymol at low temperatures (~75 °C), PLA films loaded with thymol were stable at 110–120 °C (Alvarado et al., 2018; Torres et al., 2017). What is even more interesting, the same authors (Alvarado et al., 2018) confirmed that the corresponding weight loss at the 110 °C did not affect the thymol content determined by HPLC. The PLA-5 containing 35.8 wt% obtained in the present study was also stable at 110–120 °C and showed a relatively good thermal stability at 150 °C (corresponding weight loss of ~3%) (Fig. 5a). At the same temperature (150 °C), weight loss of only 0.2% was evidenced for the PLA-5 loaded with thyme extract. An increased thermal stability of thymol loaded in the PLA-based films was recently explained by interactions between the

Table 2
Results of TGA/DTA analysis referring to degradation and melting temperature under nitrogen atmosphere.

Sample	Degradation	Melting
	T_{max} (°C)	T_m (°C)
Thymol (this study)	232**	48–51
Thymol (literature)*	268	40–61.6
PLA	379.3	161
PLA-1	373	153
PLA-5	374.1	154
PLA-10	373	154
PLA-5 + T	372.5	144
PLA-5 + TE	372.7	157

* Literature data (Kumar Trivedi, Patil, Mishra, & Jana, 2015; Mourtzinos, Kalogeropoulos, Papadakis, Konstantinou, & Karathanos, 2008; Tao, Hill, Peng, & Gomes, 2014; Zhang et al., 2014).
** Boiling point.

PLA chains and the molecules of thymol, mainly by hydrogen bonding (Alvarado et al., 2018). This was confirmed by ATR-FTIR in this study for the PLA-5 loaded with pure thymol (35.8 wt%). Accordingly, evidenced low-temperature inflection for the same film loaded with 5.08 wt% of thyme extract could be the due to a weaker interaction of the extract with the polymer matrix which was also indicated by ATR-FTIR analysis (Fig. 4b). The thermal degradation profile the PLA-5 film loaded with 35.8 wt% and its weight loss between 120 °C and 300 °C (~86%) is in accordance to recently reported for the PLA film loaded with 19.1 wt% of thymol by SSI process at similar operating conditions (12 MPa, 40 °C, decompression rate of 1 MPa/min) (Torres et al., 2017). Corresponding weight loss centred at 200 °C is associated with thymol evaporation (Fig. 5a) (Torres et al., 2017).

Incorporation of thymol resulted in 10 °C lower melting point compared to the non-impregnated PLA-5 (Table 2, Fig. 5b) which confirms plasticization effect of thymol. Similar observation was reported for SSI of thymol in the pure PCL (Ivanovic et al., 2016) and PLA (Kuska et al., 2016; Torres et al., 2017).

3.6. Antibacterial activity of representative thymol and thyme extract containing films

The antibacterial activity of PLA-5 film impregnated with 35.8 wt% thymol (PLA-5 + T) and 5.08 wt% thyme extract (PLA-5 + TE) (0.064 wt% of thymol) were investigated against the bacterial model organisms *E. coli* (Gram$^-$) and *B. subtilis* (Gram$^+$). To determine the viability of the bacterial cells after incubation with the PLA-5 films in buffer, an ATP-dependent enzyme was used to quantify the ATP level by generating a luminescence signal. The ATP level is an indicator for a vital metabolism and is directly proportional to the number of living cells. To ensure the comparability samples treated without PLA-5 films at all were set as 100% (control) viable as shown in Fig. 6.

The results showed that after incubation with PLA-5 + T the viability of *E. coli* was almost completely vanished, reduced down to 20% of the values found for untreated PLA-5 (Fig. 6). Similar bactericidal properties were shown against *B. subtilis* cells, whereby PLA-5 + T led to a nearly total reduction of the ATP level. *B. subtilis* growth was reduced in contact with the materials in general, most probably due to its sensitivity to environmental changes rather than any antibacterial effect pure PLA. On the other hand, the films loaded with thyme extract (PLA-5 + TE) had no significant antibacterial effect on the tested bacterial strains (Fig. 6).

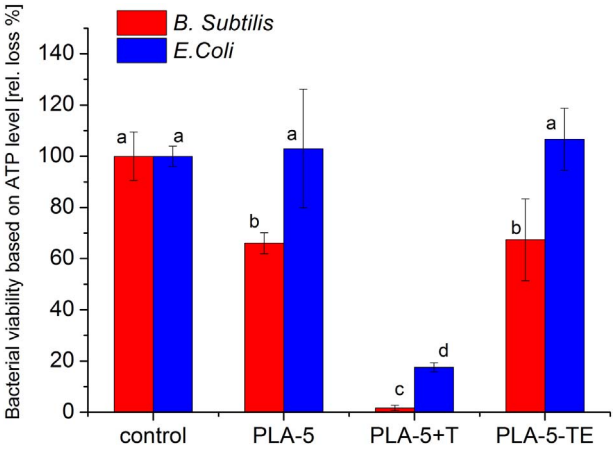

Fig. 6. The effect of PLA-5 films impregnated with thymol (35.8 wt%) and thyme-extract (5.08 wt%) on the viability of and *E. coli* after 3.5 h of incubation based on *B. subtilis* the metabolic ATP level. According to Tukey's HSD test ($p < 0.05$) *B. subtilis* PLA-5 + T (c) and *E. coli* PLA-5 + T (d) show significant differences towards the control, PLA-5 and PLA-5-TE, *B. subtilis* PLA-5 and PLA-5-TE (b) show significant difference towards the control, thus not related to antibacterial effects.

Previously determined minimum inhibitory concentrations (MIC) of supercritical thyme extract isolated at 10 MPa and 40 °C against *B. subtilis* and *E. coli* using macrodilution method were ≤40 and 640 μg/mL, respectively (Ivanovic et al., 2012). In the same study, MIC values for thymol were 160 μg/mL and 160–320 μg/mL, respectively. Despite stronger antibacterial activity of the supercritical extract of thyme against the tested Gram(+) bacterial strain (*B. subtilis*), the above mentioned study (Ivanovic et al., 2012) showed that the thymol is mainly responsible for the antibacterial effect, in particular against Gram(−) bacterial strains (*E. coli*) (Ivanovic et al., 2012). According to our present study, the PLA-5 loaded with 5.08 wt% thyme extract contained only 0.064 wt% of thymol (Table 1) which is two orders of magnitude (~600 times) lower compared the PLA-5 film containing 35.8 wt% of pure thymol and thus insufficient to show comparable antibacterial effect on the tested bacterial strains.

To substantiate these findings of bactericidal properties of the thymol impregnated films, the ability of the bacterial cells to reproduce in fresh growth medium after being exposed to the non-impregnated and impregnated PLA-5 films was analysed. This was done by measuring the increase of optical density at 595 nm (turbidity) as a function of time (Fig. 7). The increase in turbidity over time reflects the amount of bacterial cell growth in the medium. Neat PLA-5 or PLA-5 + TE films did not show any inhibition effect on the bacterial density. The anticipated bactericidal effect of PLA-5 + T was clearly shown. It was even stronger than expected, as the turbidity did not increase at all. Results from both tests suggest that PLA-5 film impregnated with

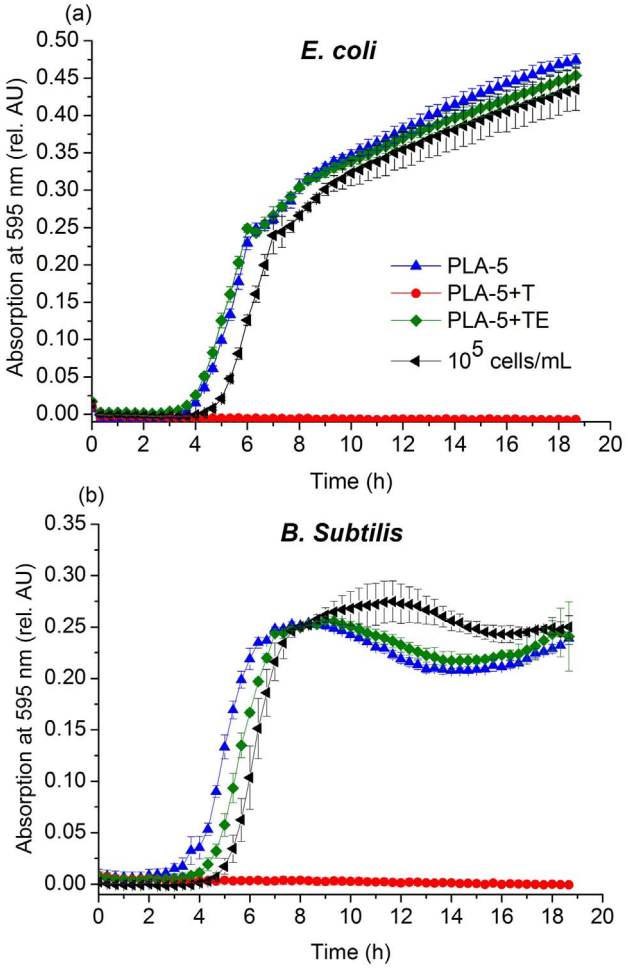

Fig. 7. The effect of PLA-5 films impregnated with thymol (T) and thyme-extract (TE) on the growth of (a) *E. coli* and (b) *B. subtilis* in batch culture over 18 h at 37 °C.

35.8 wt% thymol is a highly potent antibacterial material that efficiently compromises survival of Gram(−) and Gram(+) bacteria.

4. Conclusions

Supercritical fluid technology was employed for the first time to impregnate PLA/PCL films with natural antibacterial agents such as thymol and thyme extract for potential use in antibacterial packaging. Supercritical CO_2-assisted batch and semi-continuous impregnation processes were successfully used to load thymol and thyme extract into PLA/PCL films. Impregnation time, PCL content and $scCO_2$ flow regime were varied to maximize the thyme extract or thymol loading and to retain a compact structure and a good thermal stability of the films. Proposed processes enable solvent-free and controlled film impregnation at mild temperature (40 °C) which minimizes vaporization of volatile impregnation substances and thus formation of micro voids within the film. All the obtained films had a good thermal stability at temperatures up to ~150 °C. The PLA blended film impregnated with thymol (35.8 wt%) displayed strong bactericidal properties against *B. subtilis* and *E. coli* indicating a great potential as a material for antibacterial food packaging. The same blend containing 5.08 wt% of thyme extract did not stop growth of above mention bacterial strains, possibly due to an insufficient amount of pure thymol (0.064 wt%). Therefore the coupled $scCO_2$ extraction-impregnation process needs to be further optimized towards increasing loading of thyme extract of the mentioned PLA/PCL blend film. Presented processing routes can be also applied for fast and controlled incorporation of antibacterial agents soluble in $scCO_2$ into other polymeric films. Low temperature and waterless processing of the films is particularly beneficial for loading volatile active compounds into polymers sensitive to heat and hydrolysis.

Acknowledgment

This research project was financially supported by Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No III 45017), a common initiative of the German Federal Government (Project ID: FKZ 01PL11082B) and German Academic Exchange Service (Project ID: 57140416). Financial support of DAAD Funding programme Research Stays for University Academics and Scientists (ID 57210259) is acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.02.065.

References

Aionicesei, E., Škerget, M., & Knez, Ž. (2009). Mathematical modelling of the solubility of supercritical CO_2 in poly(L-lactide) and poly(D,L-lactide-co-glycolide). *Journal of Supercritical Fluids, 50*, 320–326.

Alvarado, N., Romero, J., Torres, A., López de Dicastillo, C., Rojas, A., Galotto, M. J., & Guarda, A. (2018). Supercritical impregnation of thymol in poly(lactic acid) filled with electrospun poly(vinyl alcohol)-cellulose nanocrystals nanofibers: Development an active food packaging material. *Journal of Food Engineering, 217*, 1–10.

Babovic, N., Djilas, S., Jadranin, M., Vajs, V., Ivanovic, J., Petrovic, S., & Zizovic, I. (2010). Supercritical carbon dioxide extraction of antioxidant fractions from selected Lamiaceae herbs and their antioxidant capacity. *Innovative Food Science & Emerging Technologies, 11*, 98–107.

Boonruang, K., Chinsirikul, W., Hararak, B., Kerddonfag, N., & Chonhenchob, V. (2016). Antifungal poly(lactic acid) films containing thymol and carvone. *MATEC Web of Conferences, 67*, 6107.

Champeau, M., Thomassin, J. M., Jérôme, C., & Tassaing, T. (2014). In situ FTIR microspectroscopy to investigate polymeric fibers under supercritical carbon dioxide: CO_2 sorption and swelling measurements. *Journal of Supercritical Fluids, 90*, 44–52.

Del Nobile, M. A., Conte, A., Incoronato, A. L., & Panza, O. (2008). Antimicrobial efficacy and release kinetics of thymol from zein films. *Journal of Food Engineering, 89*, 57–63.

Emiroğlu, K. Z., Yemiş, P. G., Coşkun, K. B., & Cando, K. (2010). Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. *Meat Science, 86*, 283–288.

Fanovich, M. A., Ivanovic, J., Misic, D., Alvarez, M. V., Jaeger, P., Zizovic, I., & Eggers, R. (2013). Development of polycaprolactone scaffold with antibacterial activity by an integrated supercritical extraction and impregnation process. *Journal of Supercritical Fluids, 78*, 42–53.

Fanovich, M. A., Ivanovic, J., Zizovic, I., Misic, D., & Jaeger, P. (2016). Functionalization of polycaprolactone/hydroxyapatite scaffolds with *Usnea lethariiformis* extract by using supercritical CO_2. *Materials Science and Engineering: C, 58*, 204–212.

Goriparthi, B. K., Suman, K. N. S., & Nalluri, M. R. (2012). Processing and characterization of jute fiber reinforced hybrid biocomposites based on polylactide/polycaprolactone blends. *Polymer Composites, 33*, 237–244.

Ivanovic, J., Knauer, S., Fanovich, A., Milovanovic, S., Stamenic, M., Jaeger, P., Zizovic, I., & Eggers, R. (2016). Supercritical CO_2 sorption kinetics and thymol impregnation of PCL and PCL-HA. *The Journal of Supercritical Fluids, 107*, 486–498.

Ivanovic, J., Meyer, F., Misic, D., Asanin, J., Jaeger, P., Zizovic, I., & Eggers, R. (2013). Influence of different pre-treatment methods on isolation of extracts with strong antibacterial activity from lichen *Usnea barbata* using carbon dioxide as a solvent. *The Journal of Supercritical Fluids, 76*, 1–9.

Ivanovic, J., Milovanovic, S., Stamenic, M., Fanovich, M. A., Jaeger, P., & Zizovic, I. (2014). Application of an integrated supercritical extraction and impregnation process for incorporation of thyme extracts into different carriers. In J. Osborne (Ed.). *Handbook on supercritical fluids: Fundamentals, properties and applications* (pp. 257–280). Hauppauge, N.Y. USA: Nova Science Publishers, Inc.

Ivanovic, J., Misic, D., Zizovic, I., & Ristic, M. (2012). *In vitro* control of multiplication of some food-associated bacteria by thyme, rosemary and sage isolates. *Food Control, 25*, 110–116.

Ivanovic, J., Rezwan, K., & Kroll, S. (2018). Supercritical CO_2 deposition and foaming process for fabrication of biopolyester–ZnO bone scaffolds. *Journal of Applied Polymer Science, 135*, 1–11.

Ivanovic, J., Zizovic, I., Ristic, M., Stamenic, M., & Skala, D. (2010). The analysis of simultaneous clove/oregano and clove/thyme supercritical extraction. *The Journal of Supercritical Fluids, 55*, 1–11.

Jeng, H. A., & Swason, J. (2006). Toxicity of metal oxide nanoparticles in mammalian cells. *Journal of Environmental Science and Health, Part A, 41*, 2699–2711.

Karst, D., & Yang, Y. (2006). Molecular modeling study of the resistance of PLA to hydrolysis based on the blending of PLLA and PDLA. *Polymer, 47*, 4845–4850.

Kavoosi, G., Mohammad, S., Dadfar, M., & Purfard, A. M. (2013). Mechanical, physical, antioxidant, antimicrobial properties of gelatin films incorporated with thymol for potential use as nanowound dressing. *Journal of Food Science, 78*, E244–E250.

Kazarian, S. G., Vincent, M. F., Bright, F. V., Liotta, C. L., Eckert, C. A., & February, R. V. (1996). Specific intermolecular interaction of carbon dioxide with polymers. *Journal of the American Chemical Society, 118*, 1729–1736.

Krepker, M., Shemesh, R., Danin, Y., Kashi, Y., Vaxman, A., & Segal, E. (2017). Active food packaging films with synergistic antimicrobial activity. *Food Control, 76*, 117–126.

Kumar Trivedi, M., Patil, S., Mishra, R. K., & Jana, S. (2015). Structural and physical properties of biofield treated thymol and menthol. *Molecular pharmaceutics & organic process research. 3. Molecular pharmaceutics organic process research* (pp. 1000127–). .

Kuska, R., Milovanovic, S., Janowski, J., Frerich, S., Zizovic, I., & Ivanovic, J. (2016). Supercritical foaming and impregnation processes design based on thermodynamic measurements of pla under high pressure CO_2. *Proceedings of the 15th european meeting on supercritical fluids (EMSF)* (Essen, Germany, May 8–13).

Lim, L.-T., Auras, R., & Rubino, M. (2008). Processing technologies for poly(lactic acid). *Progress in Polymer Science, 33*, 820–852.

Liu, D., Li, H., Jiang, L., Chuan, Y., Yuan, M., & Chen, H. (2016). Characterization of active packaging films made from poly (lactic acid)/poly (trimethylene carbonate) incorporated with oregano essential oil. *Molecules, 21*, 695.

Malhotra, B., Keshwani, A., & Kharkwal, H. (2015). Antimicrobial food packaging: Potential and pitfalls. *Frontiers in Microbiology, 6*, 611.

Manna, L., Banchero, M., Sola, D., Ferri, A., Ronchetti, S., & Sicardi, S. (2007). Impregnation of PVP microparticles with ketoprofen in the presence of supercritical CO_2. *Journal of Supercritical Fluids, 42*, 378–384.

Martirosyan, A., & Schneider, Y. (2014). Engineered nanomaterials in food: Implications for food safety and consumer health. *International Journal of Environmental Research and Public Health, 11*, 5720–5750.

Mauricio, M. R., Manso, F. C. G., Kunita, M. H., Velasco, D. S., Bento, A. C., Muniz, E. C., de Carvalho, G. M., & Rubira, A. F. (2011). Synthesis and characterization of ZnO/PET composite using supercritical carbon dioxide impregnation technology. *Composites Part A: Applied Science and Manufacturing, 42*, 757–761.

Milovanovic, S., Jankovic-Castvan, I., Ivanovic, J., & Zizovic, I. (2015). *Effect of starch xero- and aerogels preparation on the supercritical CO_2 impregnation of thymol. 67*, Starch - Stärke174–182.

Milovanovic, S., Kuska, R., Skoric-Lucic, M., Kalagasidis-Krusic, M., Frerich, S., Zizovic, I., & Ivanovic, J. (2016). Swelling kinetics and impregnation of PLA with thymol under supercritical CO_2 conditions. *Tehnika, 71*, 16–20.

Milovanovic, S., Markovic, D., Aksentijevic, K., Stojanovic, D. B., Ivanovic, J., & Zizovic, I. (2016). Application of cellulose acetate for controlled release of thymol. *Carbohydrate Polymers, 147*, 344–353.

Milovanovic, S., Stamenic, M., Markovic, D., Radetic, M., & Zizovic, I. (2013). Solubility of thymol in supercritical carbon dioxide and its impregnation on cotton gauze. *Journal of Supercritical Fluids, 84*, 173–181.

Mourtzinos, I., Kalogeropoulos, N., Papadakis, S. E., Konstantinou, K., & Karathanos, V. T. (2008). Encapsulation of nutraceutical monoterpenes in β-cyclodextrin and modified starch. *Journal of Food Science, 73*, S89–S94.

Murariu, M., & Dubois, P. (2016). PLA composites: From production to properties. *Advanced Drug Delivery Reviews, 107*, 17–46.

Nalawade, S. P., Picchioni, F., Marsman, J. H., & Janssen, L. P. B. M. (2006a). The FT-IR

studies of the interactions of CO_2 and polymers having different chain groups. *Journal of Supercritical Fluids, 36*, 236–244.
Nalawade, S. P., Picchioni, F. J., Marsman, H., Grijpma, D. W., Feijen, J., & Janssen, L. P. B. M. (2006b). Intermolecular interactions between carbon dioxide and the carbonyl groups of polylactides and poly(ε-caprolactone). *Journal of Controlled Release, 116*, e38–e40.
Nostro, A., Blanco, A. R., Cannatelli, M. A., Enea, V., Flamini, G., Morelli, I., Sudano, R. A., & Alonzo, V. (2004). Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiology Letters, 230*, 191–195.
Ormeño, E., Goldstein, A., & Niinements, Ü. (2011). Extracting and trapping biogenic volatile organic compounds stored in plant species. *Trends Trends in Analytical Chemistry, 30*, 978–989.
Ramos, M., Fortunati, E., Peltzer, M., Dominici, F., Jiménez, A., Garrigós, M.d. C., & Kenny, J. M. (2014). Influence of thymol and silver nanoparticles on the degradation of poly(lactic acid) based nanocomposites: Thermal and morphological properties. *Polymer Degradation and Stability, 108*, 158–165.
Ramos, M., Jiménez, A., Peltzer, M., & Garrigós, M. C. (2012). Characterization and antimicrobial activity studies of polypropylene films with carvacrol and thymol for active packaging. *Journal of Food Engineering, 109*, 513–519.
Raouche, S., Mauricio-Iglesias, M., Peyron, S., Guillard, V., & Gontard, N. (2011). Combined effect of high pressure treatment and anti-microbial bio-sourced materials on microorganisms' growth in model food during storage. *Innovative Food Science & Emerging Technologies, 12*, 426–434.
Reverchon, E. (1997). Supercritical fluid extraction and fractionation of essential oils and related products. *Journal of Supercritical Fluids, 10*, 1–37.
Rivas, L., McDonnell, M. J., Burgess, C. M., O'Brien, M., Navarro-Villa, A., Fanning, S., & Duffy, G. (2010). Inhibition of verocytotoxigenic *Escherichia coli* in model broth and rumen systems by carvacrol and thymol. *International Journal of Food Microbiology, 139*, 70–78.
Semba, T., Kitagawa, K., Ishiaku, U. S., & Hamada, H. (2006). The effect of crosslinking on the mechanical properties of polylactic acid/polycaprolactone blends. *Journal of Applied Polymer Science, 101*, 1816–1825.
Sung, S. Y., Sin, L. T., Tee, T. T., Bee, S. T., Rahmat, A. R., Rahman, W. A. W. A., ... Vikhraman, M. (2013). Antimicrobial agents for food packaging applications. *Trends in Food Science and Technology, 33*, 110–123.
Tao, F., Hill, L. E., Peng, Y., & Gomes, C. L. (2014). Synthesis and characterization of β-cyclodextrin inclusion complexes of thymol and thyme oil for antimicrobial delivery applications. *LWT - Food Science and Technology, 59*, 247–255.
Tawakkal, I. S. M. A., Cran, M. J., & Bigger, S. W. (2016). Interaction and quantification of thymol in active PLA-based materials containing natural fibers Intan. *Journal of Applied Polymer Science, 133*, 42160.
Tawakkal, I. S. M. A., Cran, M. J., Miltz, J., & Bigger, S. W. (2014). A review of poly(lactic acid)-based materials for antimicrobial packaging. *Journal of Food Science, 79*, R1477–R1490.
Topala, C. M., & Tataru, L. D. (2016). *ATR-FTIR study of thyme and rosemary oils extracted by supercritical carbon dioxide.* Revista de Chimie842–846.
Torres, A., Ilabaca, E., Rojas, A., Rodríguez, F., Galotto, M. J., Guarda, A., Villegas, C., & Romero, J. (2017). Effect of processing conditions on the physical, chemical and transport properties of polylactic acid films containing thymol incorporated by supercritical impregnation. *European Polymer Journal, 89*, 195–210.
Torres, A., Romero, J., Macan, A., Guarda, A., & Galotto, M. J. (2014). Near critical and supercritical impregnation and kinetic release of thymol in LLDPE films used for food packaging. *Journal of Supercritical Fluids, 85*, 41–48.
Trivedi, M. K., Patil, S., Mishra, R. K., & Jana, S. (2015). Structural and physical properties of biofield treated thymol and menthol. *Journal of Molecular Pharmaceutics & Organic Process Research, 3*, 1000127.
Vieira, M. G. A., Da Silva, M. A., Dos Santos, L. O., & Beppu, M. M. (2011). Natural-based plasticizers and biopolymer films: A review. *European Polymer Journal, 47*, 254–263.
Villegas, C., Torres, A., Rios, M., Rojas, A., Romero, J., de Dicastillo, C. L., Valenzuela, X., Galotto, M. J., & Guarda, A. (2017). Supercritical impregnation of cinnamaldehyde into polylactic acid as a route to develop antibacterial food packaging materials. *Food Research International, 99*, 650–659.
Vink, E. T. H., Rábago, K. R., Glassner, D. A., Springs, B., O'Connor, R. P., Kolstad, J., & Gruber, P. R. (2004). The sustainability of nature works™ polylactide polymers and ingeo™ polylactide fibers: An update of the future. *Initiated by the 1st international conference on bio-based polymers (ICBP 2003), November 12–14 2003, Saitama, Japan. Macromolecular Bioscience. 4. Initiated by the 1st international conference on bio-based polymers (ICBP 2003), November 12–14 2003, Saitama, Japan. Macromolecular Bioscience* (pp. 551–564).
Von Schnitzler, J., & Eggers, R. (1999). Mass transfer in polymers in a supercritical CO_2-atmosphere. *Journal of Supercritical Fluids, 16*, 81–92.
Wisam, H. H., Mansor, B. A., Emad, A., Jaffar, A.-M., & Nor Azowa Bt, I. (2010). Preparation and characterization of polylactic acid/polycaprolactone clay nanocomposite. *Journal of Applied Polymer Science, 10*, 97–106.
Wu, D., Zhang, Y., Yuan, L., Zhang, M., & Zhou, W. (2010). Viscoelastic interfacial properties of compatibilized poly(ε-caprolactone)/polylactide blend. *Journal of Polymer Science, Part B: Polymer Physics, 48*, 756–765.
Wu, Y., Qin, Y., Yuan, M., Li, L., Chen, H., & Cao, J. (2014). Characterization of an antimicrobial poly (lactic acid) film prepared with poly (ε-caprolactone) and thymol for active packaging. *Polymers for Advanced Technologies, 25*, 948–954.
Yeh, J.-T., Wu, C.-J., Tsou, C.-H., Chai, W.-L., Chow, J.-D., Huang, C.-Y., Chen, K.-N., & Wu, C.-S. (2009). Study on the crystallization, miscibility, morphology, properties of poly(lactic acid)/poly(ε-caprolactone) blends. *Polymer-Plastics Technology and Engineering, 48*, 571–578.
Zhang, Y., Niu, Y., Luo, Y., Ge, M., Yang, T., Yu, L., & Wang, Q. (2014). Fabrication, characterization and antimicrobial activities of thymolloaded zein nanoparticles stabilized by sodium caseinate-chitosan hydrochloride double layers. *Food Chemistry, 142*, 269–275.