Enzymatic Degradation of Poly(butylenesuccinate)/ Thermoplastic Starch Blend

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ABSTRACT: The degradation of thermoplastic starch blend in the presence of commercial α-amylase and unpurified amylase of microbial origin was investigated. The blends consisting of thermoplastic starch and poly(butylene succinate) have potential use in packaging applications thus, it is essential to establish susceptibility to degradation. Molar mass loss, gravimetric weight loss, and molecular structure were evaluated. The changes in the surface were observed with scanning electron microscopy. It was confirmed that there was a significant difference in gravimetric weight loss between the blends degraded in two different solutions. Unpurified enzymes of microbial origin, produced by *Rhizopus oryzae* cultures decomposed analyzed materials more efficiently than purified commercial ones. Moreover, it was proved that in applied conditions, the molar mass of PBS fraction did not change significantly.

KEYWORDS: Poly(butylene succinate), starch, α-amylase, *Rhizopus oryzae*, urea.

1 INTRODUCTION

Starch is a biodegradable, renewable and low cost alternative for synthetic polymers for short-term applications which require rapid degradation [1]. However, it has two main disadvantages: due to its disintegration in the water, starch loses its properties when exposed to moisture. Moreover, when heated, pyrolysis occurs before crystalline melting point of starch is reached [2]. Therefore, starch cannot be processed via conventional processing techniques. To improve its processability, elasticity and mechanical properties, a wide variety of plasticizers, such as glycerol, formamid, urea, acetamide, sugar, polyols amino acid, lipid and phosphate sorbates have been used [3]. Another route of starch modification is blending with aliphatic polyesters such as poly (ε–caprolactone) [4], polylactide [5] and poly (butylene succinate) (PBS). The significant advantage of using PBS is its relatively high melting point (higher than poly (ε–caprolactone)), good processability at temperatures up to 200°C [6], higher flexibility and thermal resistance than polylactide [7].

The preparation and characterization of starch/PBS blends have been reported several times in the literature [3, 8, 9, 10], however, such systems have not been tested for the degradation in the presence of commercial α-amylase and enzymes produced by the cells during culture into the medium.

Starch degradation by α-amylases is caused by the hydrolysis of α-1,4-glycosidic linkages of starch to maltose and dextrins [11]. The main degradation products are lower molar mass starch chains, fructose and maltose [2]. There are several microorganisms possessing the starch breaking ability such as bacteria [12] and fungi. The fungal source used mainly for commercial production of α-amylase are the strains of *Aspergillus* and *Rhizopus* [13, 14]. PBS exhibits a low biodegradation rate due to its high crystallization rate and crystallinity [15]. The main enzyme involved in the degradation of PBS is lipase [16]. It was proved, that the weight loss of PBS was around 9% after 60 days degradation in the presence of *Rhizopus delemar* lipase [17] and 100 % using *R. oryzae* lipase [18] at pH=5 and 40°C.

In the presented work, degradation of starch-polyester blend by unpurified microbial enzymes and commercially available α-amylase were investigated. The analyzed blend, containing 50 wt.% of thermoplastic starch (TPS) plasticized by urea and 50 wt.% of poly(butylene succinate) was studied in terms of degradation products, degradation time and weight loss. Fourier-transform IR spectroscopy (FTIR), NMR spectroscopy and differential scanning calorimetry (DSC) were used to determine the effect of degradation on chemical and molecular structures, as

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DOI: 10.32604/JRM.2018.00134

well as thermal properties of the blend. The studies were performed to gain insight into how these materials will behave as biodegradable thermoplastic blends under the presented conditions.

2 EXPERIMENTAL

2.1 Materials

α-amylase from *Aspergillus oryzae* (powder, ~30 U/mg), sodium azide, trichloroacetic acid (TCA), dinitrosalicylic acid (DNS) were obtained from Sigma-Aldrich. All medium ingredients (agar–agar, yeast extract, peptone) were purchased from BTL Łódź, Poland. Sodium chloride and soluble starch (cat. nr 789820118) were obtained from POCH, Poland, a division of Avantor Performance Products (Center Valley, PA, USA). All reagents were applied without further purification. The PBS homopolymer (Bionolle 1001MD) was supplied by Showa Denko, Tokyo, Japan. Native potato starch was purchased from Trzemeszno Sp. z o. o. Potato Industry (Poland). Urea was obtained from Grupa Azoty Puławy.

2.2 Preparation of PBS/TPS Blend

The PBS/TPS (50 wt.% /50 wt.%) blend in the film form used to biodegradation tests, was prepared by blown film method using a single screw extruder (Labtech Engineering, Thailand, L/D=30:1, screw diameter 20 mm). The temperatures profile ranging from 130°C to 140°C and the temperature of die was set at 140°C. Starch was plasticized by urea in amount 30 wt.% on dry weight of starch. The film sample from neat PBS was prepared by the same method but at the different temperature-extruder was operated at 135-150°C. The PBS film was characterized by M_w =78.9 kg mol^{-1} and $Đ_M$ =7.9.

2.3 Microorganism

Rhizopus oryzae DSM 2031 (classified as a GRAS filamentous fungus [19]) was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Spores were grown on a potato dextrose agar (PDA) slant at 30°C for 5 days. They were collected with a loop and suspended in saline solution. The initial spore concentration was 4.45×10^5 spores/mL.

2.4 Culture Media and Batch Processes

All culture media contained 10 g/L yeast extract, 20 g/L peptone and 20 g/L starch and pH of the medium was adjusted to 6.0. The yeast cells were cultivated on a rotary shaker (200 rpm) in 500 mL Erlenmeyer flasks containing 200 mL of medium at 30°C for 180 h. After the experiment, the biomass was separated by centrifugation at 10°C for 10 min (8000 rpm). The biomass was characterized by dry cell mass (d.m.) measured by the thermogravimetric method at 105°C. Each data point is the average result of three replica experiments.

2.5 Degradation Test

The film used in the biodegradation test was cut into 2.0×2.0 cm strips with thickness 0.1 mm. Each film was placed in separate vial containing 10 mL of acetate buffer (pH 6.0), 6 mg of α-amylase and 60 mg of sodium azide. In case of degradation in the presence of *R. oryzae* enzymes present in supernatant, 10 mL of supernatant and 30 mg of sodium azide were used to perform the test. The film-enzyme incubations were carried out at 37°C in a rotary shaker (150 rpm). At the specific time points, respective films were removed from the shaker incubator, rinsed thoroughly with distilled water, and then dried under reduced pressure (0.5 mbar) at the room temperature for 48 h. Experimental weight loss values represent averages of determinations from the three replicate films.

2.6 Measurement

Reducing sugars in the degradation solutions was detected by the DNS method [20]. **Amylase activity** of supernatant was determined in culture filtrates by measuring the amount of starch hydrolyzed in the reaction mixture by the iodine method [21]. Analyzed reactions were initiated by adding 50 μL of soluble starch solution (10 g/L) and 50 μL of clear supernatant. After 30 min of incubation at 50°C, when the analyzed enzymes were the most active, 50 μL of 10% TCA was added to stop the enzymatic reaction, followed by the addition of 150 μL of iodine reagent and 5 mL of distilled water. The absorbance of the solution was determined at a wavelength of 580 nm. One unit (U) of enzyme activity for the starch-iodine assay is defined as the disappearance of an average of 1 mg of iodine binding starch material per min in the analyzed reaction [21]. U/mL was calculated using the formula: $U/ml=(A_{580control}-A_{580sample})\div A_{580}/mg\ starch\div 30\ min\div 0.05\ ml$, where $A_{580\ control}$ is the absorbance obtained from the starch without the addition of enzyme, $A_{580sample}$ is the absorbance for the starch digested with enzyme, A_{580}/mg starch is the absorbance for 1 mg of starch as derived from the standard curve, 30 min is the assay incubation time, and 50 μL is the volume of the enzyme used in the assay. The **starch amount** in the degradation solutions was determined by a colorimetric method: 100 μL of sulphuric acid 2 M and 0.5 mL of KI-I_2 were added to a 5 mL of sample. The absorbance of the solution was determined using Helios Gamma UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) at a wavelength of 580 nm. The amount of starch released was obtained through the comparison with

the standard curve. **Water sorption** was evaluated by measuring the percentage weight increase of a dry polymer sample after immersion for 24 h in distilled water at 37°C. ^{1}H NMR measurement was performed on Varian Mercury 400 MHz spectrometer using $CDCl_3$ as solvent. FTIR spectra were obtained with a Nicolet iS5 FTIR (Thermo Fisher Scientific) in ATR mode. The DSC measurement was performed using a TA Instruments DSC Q20 TA. The first heating run from 24°C to 200°C was performed at heating rate of 10 °C/min in order to study crystallinity, then the process underwent cooling at the rate of 10 °C/min. The molar mass and polydispersity were determined by GPC on a Viscotek system comprising GPC_{max} and TDA 305 triple detection unit (RI, IV, LS) equipped with one guard and two DVB Jordi gel columns (102-107, linear, mix bed) in CH_2Cl_2 as eluent at 35°C at a flow rate of 1.0 mL/min. SEM images were obtained on a Zeiss LEO 1530.

3 RESULTS AND DISCUSSION

3.1 Degradation in the Presence of Commercial α-amylase

Degradation of PBS/TPS blend was performed using commercial α-amylase at 37°C in the acetate buffer solution. The collected data: Weight loss of the samples, water sorption and mass of glucose and starch released to solution are presented in the Table 1.

The weight loss of the samples after 120 h reached around 18%. Surprisingly, the weight loss of the control samples was only 4% lower, even if in the degradation solution, amount of glucose and starch did not exceed 1 wt.% and 0.16 wt.% respectively. These results can be explained by the high percentage of urea, which was around 30 wt.% in the samples. During incubation, urea and water used as additives were easily leached from the samples, reducing the mass of the polymer film.

The mass of the glucose and starch in the degradation solution was measured in all batches. As can be seen from the data presented in the Table 1 (Column 4 and 5), during first 48 h degradation, the amount of glucose released was rapidly increased, which corresponds to the fast hydrolysis phase. For a longer period, the increase was rather minor. On the other hand, the starch concentration in the solution gradually rises during the entire experiment, even though the quantity released amounting to 0.23% was rather small.

Table 1 The effect of degradation process on weight loss and water sorption of the PBS/TPS blend, and the mass of starch and glucose released to the degradation solution.

Time (h)	Weight loss[a] (%)		Water sorption[b] (%)		Mass of glucose[c] g glucose/g specimen (%)		Mass of starch[d] g starch/g specimen (%)	
	enzyme [e]	control [f]	enzyme	control	enzyme	control	enzyme	control
6	13.6 ±0.8	9.7 ±0.9	19 ±0.5	21 ±1.3	4.0 ±0.05	0.89 ±0.14	0.19 ±0.01	0.16 ±0.03
24	15.1 ±0.9	8.5 ±0.8	19 ±0.74	17 ±0.3	5.6 ±0.62	0.78 ± 0.01	0.18 ±0.01	0.10 ±0.05
48	16.7 ±0.2	10.0 ±0.6	17 ±0.08	19 ±1.6	8.0 ±0.26	0.76 ±0.01	0.21 ±0.01	0 ±0.01
120	17.6 ±0.5	13.2 ±0.6	18 ±0.1	19 ±1.1	8.8 ±0.45	1.2 ±0.06	0.23 ±0.01	0 ±0.01

[a]Weight loss percentage was calculated from the equation $[(m_0-m_1)/m_0] \times 100$, where m_1 is the sample weight at the specific time point and m_0 is the initial weight of the sample before immersion. [b]Water sorption percentage was calculated from the equation $[(m_0-m_1)/m_0] \times 100$, where m_0 is the weight of the sample after immersion in water for 24 h at 37°C, and m_1 is the weight of a dry sample. [c]Mass of starch released to the solution per initial specimen mass. [d]Mass of glucose released to the solution per initial specimen mass. [e]Data obtained for the experiment performed in the presence of 0.6 mg/mL α–amylase in the acetate buffer solution. [f]Data obtained for the experiment performed without enzyme in the acetate buffer solution.

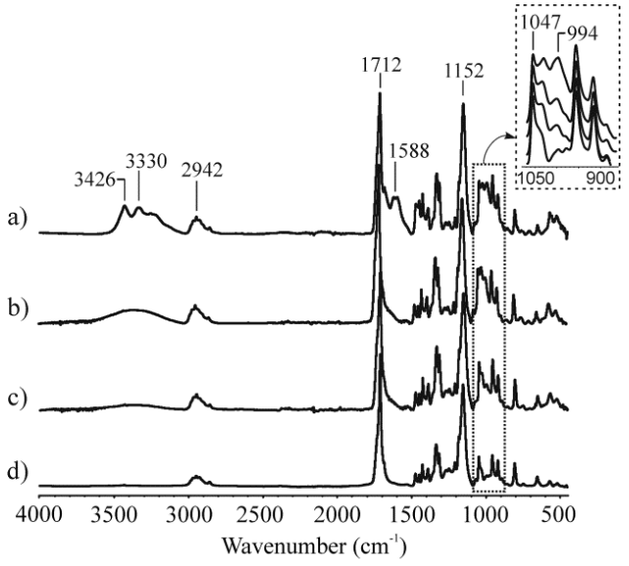

Figure 1 FTIR spectra of PBS/TPS blend before (a) and after 24 h (b), 120 h (c) degradation as well as neat PBS (d).

Figure 1 shows the FTIR spectra of PBS/TPS blend before and after degradation as well as the neat PBS. The effect of α-amylase can be observed by a decrease in the intensity of the peak at 3600-3000 cm^{-1} and 1015-970 cm^{-1} assigned to O-H and C-O stretching bonds in the starch, respectively [22]. The first region (3427-3255 cm^{-1}) can also be attributed to N-H stretching bond of urea, however, the most exposed peak in the spectrum, characterizing the presence of urea in the blend is located at 1675 cm^{-1} and 1588 cm^{-1}, assigned to the C=O stretching bond and N–H bending bond, respectively [23]. The intensity of the urea characteristic bonds rapidly decrease during the degradation, even for the samples incubated without enzyme (control). Moreover, based on thse FTIR analysis, it was observed that the intensity of PBS characteristic peak at 1712 cm^{-1} after degradation did not change significantly. This indicates that probably α–amylase did not accelerate the PBS degradation. To confirm these results, we separated the PBS fraction of the sample by filtration. After 120 h degradation the molar mass was determined by GPC analysis. It was found that the polymer was characterized by slightly higher M_w in comparison with the neat PBS. The GPC chromatogram was monomodal with narrower distribution Đ=6.49.

In the next step, we conducted additional experiments, where neat PBS was incubated under the same conditions of temperature and enzyme concentration as the blend. It was found that after 24 h, the weight loss of the samples was around 1.4%. Longer incubation time up to 30 days, did not cause further changes in the weight loss of the sample. The molar mass of PBS after 24 h of degradation increased by 2.4% (M_w=80.4 kg mol^{-1}) which indicate that the hydrolysis of soluble PBS oligomers occurred and some of the polymer additives could be removed from the sample. As a consequence, the polymer after degradation was characterized by lower polydispersity. After 120 h of degradation, the M_w and Đ decreased to 77.6 kg mol^{-1} and 3.72 kg mol^{-1}, respectively.

The changes in M_n of PBS during degradation were also followed by ^{1}H NMR analysis. The obtained results showed that the molar mass of neat PBS was almost twice higher than the one calculated from GPC. Moreover, after 120 h of degradation the $M_{n,HNMR}$ of PBS was almost twice lower than the initial sample. The difference between data achieved from GPC and ^{1}H NMR analysis can be explained by the presence of some macrocyclic products which could overestimate the M_n of PBS calculated from the ^{1}H NMR spectrum. Moreover, it should be also noticed that the integral ratio of the signal of ~C$\underline{H_2}$-OH in the end-groups in PBS structure at 3.60 ppm to signal assigned to methylene protons in ester group at 4.1 ppm was too high to calculate the M_n of the PBS precisely.

The thermal properties of PBS/TPS blend before and after degradation in the presence of α-amylase were investigated using DSC analysis (Table 2). The melting point of the PBS fraction in the PBS/TPS blend was approximately 110°C. After incubation, the T_m did not change significantly. The crystallinity of the PBS fraction was calculated based on the theoretical ΔH values of PBS (110.5 J g^{-1}) [24]. Along with the degradation time, the crystallinity of PBS fraction increased from 24.6% to 28.8%. Such an increased tendency can be explained by the degradation in the amorphous phase of PBS which results in higher crystallinity.

Table 2 Thermal parameters of PBS/TPS blend before and after 120 h degradation in the presence of α-amylase.

	T_c[a] (°C)	H_c[a] (J g^{-1})	T_m[b] (°C)	H_m[b] (J g^{-1})	x_c[c] (%)
TPS/PBS	69.0	31.4	110	27.1	24.6
TPS/PBS 120 h	73.0	37.0	108	31.8	28.8

[a]Determined by the cooling scan from melt at 10 °C/min. [b]Determined by the 1st heating scan at 10 °C/min. [c]The crystallinity degree was calculated according to equation $x_c = \Delta H_m / \Delta H_m^o$, where ΔH_m^o=110.5 J g^{-1}.

3.2 Degradation in the Presence of Amylase of *R.oryzae* Origin

Microbial amylases production was performed using *R. oryzae* species at 30°C and pH=6.0 on a laboratory scale in 200 mL shake culture flasks. Soluble starch and peptone were used as carbon and nitrogen source, respectively. *R. oryzae* was cultivated for 24 h, 48 h, 72 h, 96 h, 144 h and 166 h, in order to find the highest amylolytic activity of the applied strain. As can be seen from the Figure 2, the maximum amylolytic activity occurred after 48 h of cultivation. The growth phase of the cells was observed up to 96 h, and after that moment, biomass yield decreased to 38%.

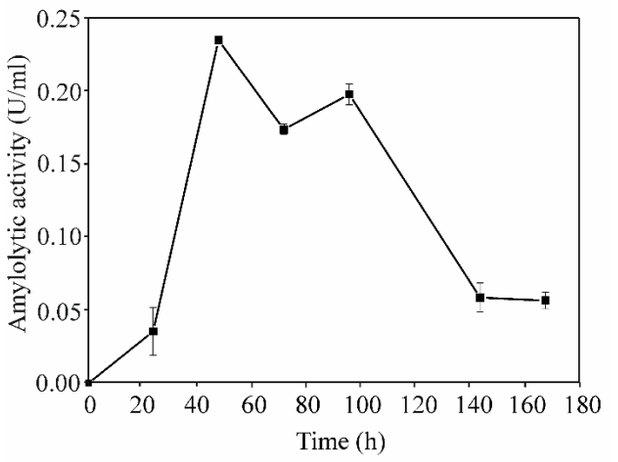

Figure 2 α-amylase production by *R.oryzae* at 30°C.

Afterwards, the obtained supernatant with the highest amylolytic activity of 0.235 U/mL was used for the degradation of PBS/TPS blend at 37°C for 144 h. The results of weight loss and amylolytic activity are shown in Figure 3.

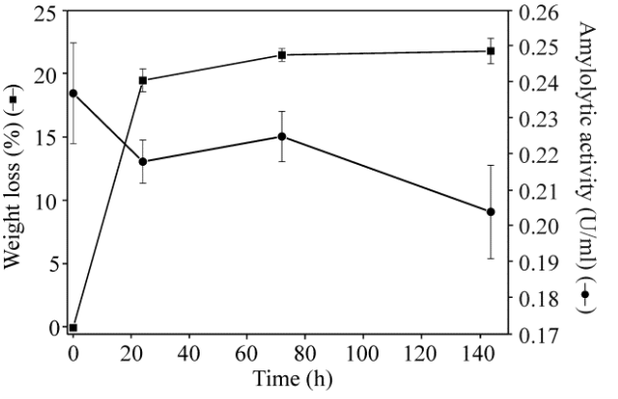

Figure 3 Weight loss and amylolytic activity of supernatant during biodegradation of PBS/TPS blend.

Surprisingly, the weight loss of the samples after 5 days of degradation was higher by approximately 5%, in comparison with the experiment performed in the presence of commercial enzyme. During incubation, amylolytic activity decreased by 14%. The presented results indicated that even if the supernatant generally exhibits lower enzymatic activity than commercial products, due to the presence of different types of amylase (not only α–amylase), it is able to degrade the starch fraction more efficiently than the commercial enzyme. Thermal properties of the samples degraded with *R. oryzae* enzymes (Table 3) are similar to those described above where α-amylase was used for degradation blends. Crystallization temperature did not change significantly during degradation, as well as the melting temperature which for the PBS fraction was equal to 110°C. After degradation in supernatant, the crystallinity of PBS fraction increased and was higher than in the samples treated by commercial α–αmylase.

Table 3 Thermal parameters of PBS/TPS blend before and after 24 h, 72 h and 144 h degradation in the presence of supernatant.

	T_c^a (°C)	H_c^a (J g^{-1})	T_m^b (°C)	H_m^b (J g^{-1})	x_c^c (%)
PBS/TPS	69.0	31.4	110	27.1	24.6
PBS/TPS-24 h	71.9	37.6	110	32.2	29.1
PBS/TPS-72 h	69.3	40.6	110	34.7	31.4
PBS/TPS-144 h	69.5	39.5	110	34.3	31.0

[a]Determined by the cooling scan from melt at 10 °C/min. [b]Determined by the 1st heating scan at 10 °C/min. [c]The crystallinity degree were calculated according to equation
$x_c = \Delta H_m / \Delta H_m^o$, where $\Delta H_m^o = 110.5$ J g^{-1}.

To analyze the degradation behavior of neat PBS in the microbial unpurified amylase solution, the additional experiments were performed in supernatant and the molar mass, weight loss and thermal properties of samples were studied. The collected data is presented in Table 4. The molar mass of the PBS and polydispersity increased during incubation as a result of oligomers degradation. The weight loss was in the same range as PBS treated by α-amylase, reported above. Degree of crystallinity slightly decreased from 53% to 48% with a prolonged degradation time which indicated that the degradation occurred in the amorphous and crystalline regions of PBS, causing some damage to the crystalline structure.

Table 4 Weight loss, molecular weight and thermal properties of PBS before and after 24 h, 72 h and 144 h degradation in the supernatant.

	Weight loss (%)	M_w[a] (kg mol^{-1})	$Đ_M$[b]	T_c[c] (°C)	H_c[c] (J g^{-1})	T_m[d] (°C)	H_m[d] (J g^{-1})	x_c[e] (%)
PBS	-	78.9	7.9	83.3	68.3	114	58.9	53
PBS-24 h	1.7±0.3	76.3	6.8	80.2	65.5	115	52.8	48
PBS-72 h	1.4±0.5	80.8	5.9	85.3	61.0	115	56.7	51
PBS-144 h	1.1±0.2	81.0	5.8	76.8	65.0	114	52.9	48

[a]Results obtained from RI detector based on PS narrow standards calibration. [b]$Đ_M = M_w/M_n$. [c]Determined by the cooling scan from melt at 5 °C/min. [d]Determined by the 1st heating scan at 10 °C/min. [e]The crystallinity degree were calculated according to equation $x_c=\Delta H_m / \Delta H_m^o$, where ΔH_m^o=110.5 J g^{-1}

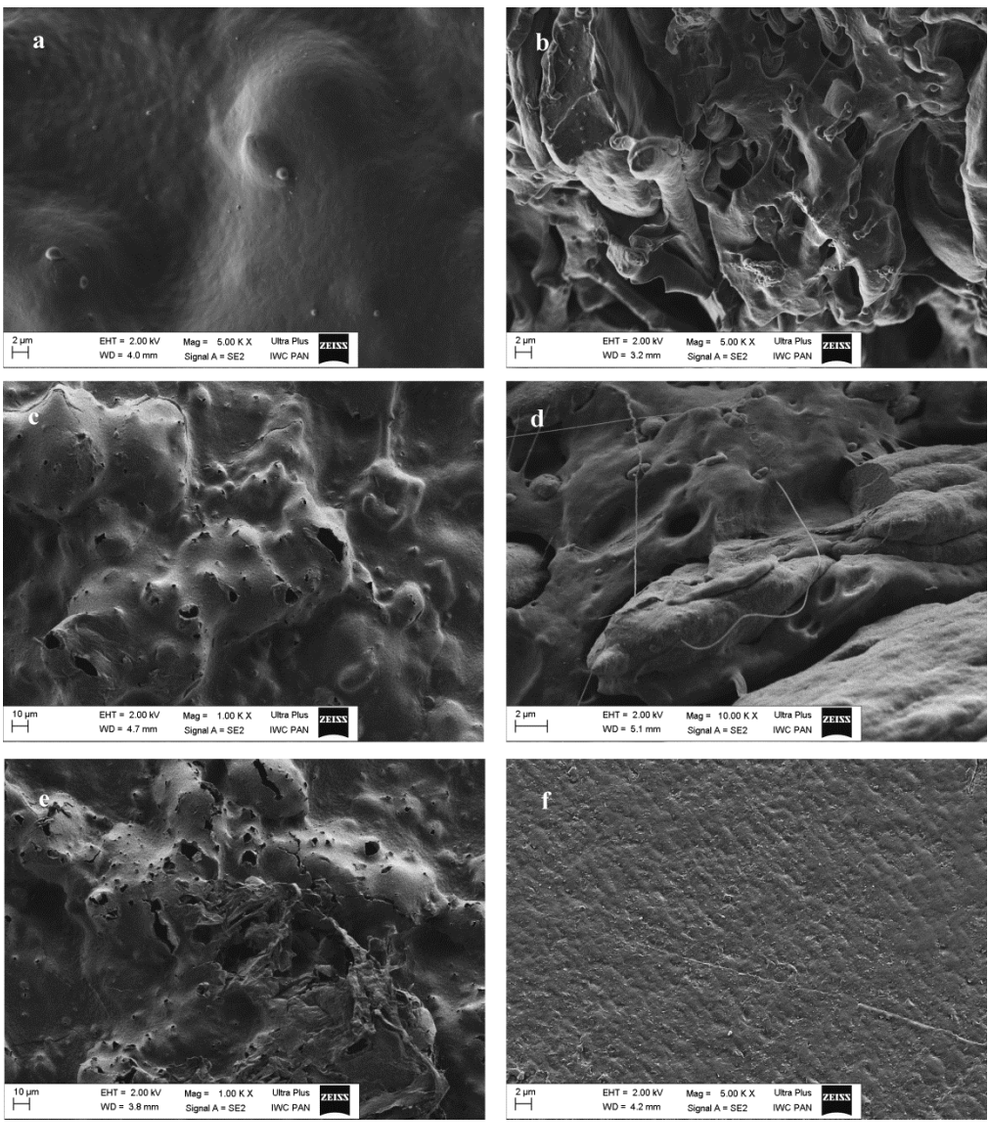

Figure 4 Scanning electron micrographs of: (a) and (b) PBS/TPS blend, (c) and (d) PBS/TPS blend after 120 h degradation in the presence of commercial α-amylase, (e) PBS/TPS blend after 144 h degradation in the presence of *R. oryzae* supernatant solution, (f) neat PBS after 144 h degradation in the presence of *R. oryzae* supernatant solution.

Finally, we used SEM images to observe the morphology of blend surface before and after degradation. As shown in Figures 4a and 4b, the starch particles are dispersed throughout the PBS matrix and exhibit a tendency to form a separate phase. The sample incubated with commercial α-amylase showed small holes on the surface (Figure 4c) in all of analyzed pictures, which are probably related to the degradation of starch. From the Figure 4d it can be seen that partial degradation occurred also inside the sample where groove/channels in polymer matrix associated with starch degradation are clearly visible. The most pronounced changes were observed after degradation in supernatant (Figure 4e) where the number of holes on the film surface was higher in comparison with the samples analyzed above. Moreover, as can be observed in Figure 4f, incubation with amylase produced by *R.oryzae* did not cause cracks/holes on the surface of neat PBS which proves the limited degradation of polyester.

CONCLUSIONS

In this study we examined the degradation profile of thermoplastic starch/poly(butylene succinate) blend in the presence of commercial α-amylase and unpurified solution of fungal amylases. It turned out that starch exhibited a limited ability to be degraded by α–amylase, probably due to the difficulties in reaching the interior domains. Moreover, the increase of crystallinity during degradation process, impeded the penetration of water into the sample which resulted in only partial decomposition of the starch. Higher degree of degradation was achieved when the polymer blend was exposed to the *R. oryzae* supernatant solution. In this case, we used enzyme preparation, which can hydrolyze starch bonds in different locations, contrary to the commercial α-amylase. It should also be pointed out that the use of the clear supernatant as enzymatic source is highly advantageous because it decreases the cost of the hydrolysis. Furthermore, we proved that none of the two degradation agents caused significant damage on the PBS matrix. Nevertheless, an extensive knowledge of the degradation mechanisms and their effect on the performance of presented materials will be the subject matter of further studies.

ACKNOWLEDGMENT

This work was financially supported by National Science Centre of Poland (MINIATURA-1, registration number 2017/01/X/ST8/00883 entitled: "Effect of selected sterilization methods on structural, mechanical and microbiological properties of starch thermoplastic blends used in the food and medicine packaging industry.").

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