

**ANALYSIS OF THE DEGREE OF MYCOLOGICAL
BIODEGRADATION OF LIGNO-CELLULOSE MATERIAL
WITH ORGANIC FILLING THROUGH SELF-BREAKING
ESTIMATION AND WITH THE USAGE OF ELECTRONIC
MICROSCOPE**

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ABSTRACT

In the given work, one compared biodegradation rate of paper products without filling and with admixture of wheat and rye bran, with and without 0.125 % fungicide (mixture: 4, 5-dichloro-2-octylo-2H-izotiazol-3-on and 3-iodoprop-2-ynyl-N-butylcarbamate). As a biotic factor, one used *Ch. globosum* axenic culture and mixture of micro fungi (consisting of *A. niger*, *T. viride*, *P. funiculosum*). Ligno-cellulose products with different percentage filling were obtained from recycled cardboard pulp. As a criterion of biodegradation, resistance to overgrowing of sample surface by test fungi was taken into consideration. Moreover, self-breaking was estimated according to PN-74/P-50133 (2012) after mycological test. In the end, research material was subjected to microscopic analysis with the help of scanning microscope.

The lowest resistance to mycelium development on the sample surface and breaking, showed waste paper with admixture of wheat bran of 3 % and rye of 5 %. The analyzed products with 5 % of wheat bran were resistant to biodegradation caused by all test fungi. Addition of fungicide of 0.125 % into paper products caused complete resistance to test microfungi. Microscopic analysis supported results that were obtained while implementing endurance and standard method (using indexation in order to describe the degree of overgrowth of a product by mycelium).

KEYWORDS: Paper samples, microfungi, biodegradation, breaking strength, microscopic analysis .

INTRODUCTION

Biodegradable materials are an interesting alternative for traditional plastic since packaging manufactured from them may undergo regeneration thanks to composting. Composting is a much cheaper method than recycling of materials, and ecologically accepted. The basic idea of biodegradable polymer production is acceptance of nature conditions (Czaja 2004). Biodegradable products and packaging arouse bigger and more frequent interest from the producers of food packaging and industrial articles. On the other hand, they make a perfect argument in the hands of politicians in the fight against growing number of waste. They also gain support of consumers whose eco-awareness constantly increases, especially because of mass media. Attempts to implement organic materials for the production of i.a., packaging result from the huge need for waste disposal which is being produced in frightening amounts in the contemporary world without the possibility of its management. All these factors influence spreading of bio-packaging products, which become more attractive than traditional plastic and slowly supersede artificial materials from all fields of economy. Application of waste paper in paper production is connected with usage of waste materials (Przybysz et al. 2001, Raczyńska 2003). The same concerns the attempt to use rye bran as a filling. Not only we find application for waste but also gain decrease in costs of production.

In the present article, there are examination results of one of the stages of the research on the broad issue, i.e. the possibility of mill waste disposal (rye bran), in paper industry and its processing (Modzelewska and Pietrzak 2010). In the pretest stage, which confirmed plausibility of further 2 attempts, one has analyzed its impact, as an additive, on the selected properties of paper products, and foremost on biodegradation of ligno-cellulose materials, depending on their intended use. Wheat bran which is extracted from modern technology is devoid of all nutrients such as fiber, and consequently becomes useless in the food industry as well as for the production of provender for farmed animals. Therefore, there is a problem how to manage such great amounts of bran. Currently, there are three disposal possibilities of organic waste available: as eco-fuel, as a filling at the production of chipboards, and at the production of eatable table-ware (Mościcki 2000). The currently ongoing research will enable estimation of usefulness of cereal bran as a filling in production of paper products, extrusions and pots of ecological ligno-cellulose pulp, and estimate their biodegradation. Conducted preliminary research has shown that only 3 % of the aforementioned content in substances has an impact on the progress of biodegradation. The main aim of the research on biodegradation of paper products with different organic content (in the form of cereal bran: rye and wheat) was to determine dynamics of the process and degree of biodegradation under the influence of microfungi activity, using in this case three methods: standard method, self-breaking indication of paper products and microscopic analysis. Since standard method, using indexation (Tab. 1) for the description of overgrowth degree of a sample by microfungi is a subjective one - in order to make results more credible, one decided to apply 2 other methods as more appropriate ones.

MATERIAL AND METHODS

Experimental material

For the needs of research, one applied sheets of paper that were produced in laboratory conditions using Rapid-Köthen apparatus, with g.s.m. of $100 \pm 5 \text{g.m}^{-2}$. Composition of paper pulp was as following: strong waste paper-type D (cardboard), water, 2 % starch in s.d.m., organic

filling (cereal bran). One produced sheets of paper without or with 3-, 5- and 10 % filling content, in the form of wheat and rye bran.

Moreover, during indication process, one used agaric culture medium and test microfungi: *Chaetomium globosum* and mixture: *Aspergillus niger*, *Trichoderma viride*, *Penicillium funiculosum*.

Additionally, in the case of research on retardation of biodegradation process of selected paper products, where the most important issue is the longest possible durability of a product, one has added into the samples (in proportion to dry fibrous pulp): 0.125 % commercial anti-mould preparation, containing two fungicides (mixture: 4, 5-dichloro-2-octylo-2H-izotiazol-3-on and 3-iodoprop-2-ynyl-N-butylcarbamate: 5 % - 7 %).

Due to the composition of paper pulp, produced sheets of paper may be described using symbols in the following way:

- paper of waste paper pulp without filling **WP**
- paper of waste paper pulp with 3 % additive of wheat bran **WP +3 % w**
- paper of waste paper pulp with 5 % additive of wheat bran **WP +5 % w**
- paper of waste paper pulp with 10 % additive of wheat bran **WP +10 % w**
- paper of waste paper pulp with 3 % additive of rye bran **WP +3 % r**
- paper of waste paper pulp with 5 % additive of rye bran **WP +5 % r**
- paper of waste paper pulp with 10 % additive **WP +10 % r**

Research methodology

Forming of paper sheets was conducted using Rapid-Köthen apparatus. Fibrous pulp was previously ground in the PFI mill, and before sample forming – defibrated with the help of separator and defiberetor (Modrzejewski et al. 1985).

Production of sheets of paper and examination of their resistance to biodegradation was conducted with accordance to methodology proposed by Cofta et al. (2006).

From each configuration of paper products, 24 samples were cut, each having dimension of 15 x 95 mm. Simultaneously, one prepared also control samples of Whatman blotting paper no.3, of the same dimension as the ones mentioned above.

Samples underwent sterilization in order to eliminate the possibility of infection. On the earlier prepared and sterilized Petri dishes with agaric culture medium and Czapek-Dox salt additive, in sterile conditions, one has spread a sample of waste paper and blotting paper. The prepared samples underwent sterilization in order to eliminate possibility of infection.

Examination of fungicides must be conducted with the use of microorganisms, specific for a given product, which we want to protect. In order to obtain utilitarian results – examination with the presence of fungi which cause the most damage of a given material, is indispensable. According to laboratory practice, it follows that it is good to work with microorganisms of different susceptibility to fungicide and those that do not show much deviation in case of repeated tests. Due to this reason, one applied *Ch. globosum*, which develops very well on clean cellulose and is often isolated in various archives and libraries (Zyska 1997, 2000). Fungi that were used in the mixture include: *A. niger*, *P. funiculosum*, *T. viride*. Dishes were placed in the thermostatic chamber, maintaining constant temperature of $25 \pm 1^\circ\text{C}$ and air humidity of $95 \pm 3\%$.

Determination took place 10 times (10 series), and the obtained results constitute average of all the conducted marking. Because of the time for incubation, examination for the first series lasted 8 weeks, for the second 4 weeks, other series were kept in thermostatic chamber during 3 weeks (since after 14th day, samples without fungicide additive reached index 0 and further monitoring of the experiment was unnecessary), where regular reading of the degree of samples' overgrowth took place: on the 4th, 7th, 10th, 14th and 21st day, all according to the 4-step scale (Tab. 1).

Tab. 1: Scale used for evaluation of the susceptibility of the experimental samples to fungal infestation (Cofta et al. 2006)

Degree of sample colonisation	Index
no sign of mycelium growth on a sample, there is a zone of inhibition on the medium between the sample and mycelium	3
no sign of mycelium growth on a sample, there is no zone of inhibition on the medium between the sample and mycelium	2
less than 1/3 of the sample surface colonised by the test fungus mycelium	1
more than 1/3 of the sample surface colonised by the test fungus mycelium	0

Determination of self-breaking resistance of ligno-cellulose samples was conducted according to the ISO 1924-2/PN-74-50133 norm, and preparation of the samples for the durability examination: PN-EN ISO 5269-2: 2007. All mechanical tests were performed for the papers subjected to impact microfungi for a period of 21 days. Relative humidity of the analyzed products, before determination of self-breaking endurance, estimated 7±1 %. Before placing samples in the handles of a breaking machine, sheets were taken out of Petri dish, cleared and dried. Paper products from waste paper were later subjected to drying, series without filling and with 3-, 5- and 10 % additive of rye bran were dried over 7 days, the rest during 10 days.

Photographs of the surface and structure of paper with different content of organic filling, at different stages of biodegradation, were taken with the help of electron scanning microscope (SEM) S-3400N Hitachi, using the mode of high-vacuum (detector of secondary electrons SE).

Paper products from lingo-cellulose pulp, as a non-conducting material, underwent earlier gold sputtering in order to gain appropriate SE mode picture. The following enlargements were chosen:

- series 1: x50, x100, table height: 65 mm and accelerating voltage: 15 kV,
 - series 2: x200, x500, x1000, x1500 (x3000, x4500), table height; 35 mm and accelerating voltage;
- 15 kV. Due to huge amount of taken pictures, in the work only selected ones were presented.

RESULTS AND DISCUSSION

At this stage of work, one compared the rate of biodegradation of paper products with admixture of wheat bran, rye bran and without filling. As a biotic factor axenic culture *Ch. globosum* and mixture of microfungi (*A. niger*, *T. viride*, *P. funiculosum*) were applied. As a criterion of biodegradation one established resistance to overgrowing of samples' surface by test mycelium (called here as standard method due to its common usage in different modifications, depending on infected material) and estimated self-breaking after mycological test (Cofta et al. 2006).

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In the conducted examination, mycelium *Ch. globosum* on the 7th day almost completely covered the surface of paper samples. Sheets of paper, made of waste paper pulp without additive of any fungicide, were very susceptible to biodegradation. Earlier research showed that addition of rye bran to paper pulp causes even more susceptibility to degradation caused by *Ch. globosum* (Cofta et al. 2006), due to huge amount of food substances for the occupying microorganisms (Perkowski et al. 2008). Currently presented research partially supports this hypothesis.

However, sheets produced from waste paper pulp with additive of 5 % wheat bran were completely resistant to *Ch. globosum* mycelium development until 7th day of mycological test. After 3 weeks, samples were slightly covered by test microfungus. It is probably caused by the synergism phenomenon occurring among substances inhibiting growth of microorganisms, found in waste paper pulp (printing ink, fillers etc.), used for the samples production, and compounds occurring in rye bran (Tab. 2). Research on the material (Modzelewska et al. 2009) in question proved that the presence of inks had an impact on the content of heavy metals in the tested ligno-cellulose materials, which also may have contributed to the limited increase of the applied microfungi (Schmitz et al. 1993, Baldrian 2003).

Tab. 2: Fungal infestation of the examined paper materials by test microfungi (*Ch. globosum* and mixture *A. niger*, *T. viride*, *P. funiculosum*).

Types of the examined paper	day 4		day 7		day 10		day 14		day 21	
	Ch*	M**	Ch	M	Ch	M	Ch	M	Ch	M
WP	1.85	1.75	0.25	1.62	0	1.62	0	1.5	0	1.37
WP+ 3 %r	2.75	2.37	1	2.37	0	2.37	0	2.37	0	2.37
WP+ 5 %r	0.12	2.25	0	2.25	0	1.87	0	1.75	0	1.75
WP+10 %r	0.12	0.12	0	0	0	0	0	0	0	0
WP+ 3 %w	0	0.12	0	0	0	0	0	0	0	0
WP+ 5 %w	3	3	3	2.87	2.12	2.75	1.5	2.75	1.5	2.75
WP+10 %w	2.25	2.5	1	1,5	0	0	0	0	0	0
WP 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 3 %r 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 5 %r 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 10 %r 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 3 %w 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 5 %w 0,125 % f	3	3	3	3	3	3	3	3	3	3
WP+ 10 %w 0,125 % f	3	3	3	3	3	3	3	3	3	3

Ch* - *Ch. globosum*

M** - mixture (*A. niger*, *T. viride*, *P. funiculosum*)

f- fungicide

Additional examination was carried out, the aim of which was to check if fungicide additive would cause any changes in the case of rate of covering of samples' surface by test fungi. Diversity

of variants, together with results has been presented in Tab. 2. The most interesting variant was 0.12 % additive of waste paper containing 3 % of wheat bran. The obtained results confirmed big effectiveness of the applied fungicide (Tab. 2, Fig. 2). In this case, as well as in other ones, overgrowth indices estimated 3.

Due to huge susceptibility of products with rye bran additive to the attacks of various microorganisms, one may find some mycotoxins inside them. Very frequent kind of fungi attacking cereal in the Central-European climate *Fusaria* spp. (Fassatiova 1983; Chełkowski et al. 2001). It is a common species producing big amount of mycotoxins which may inhibit growth of other microorganisms (Bottalico 1999).

Examination results, obtained for variants without fungicide additives and varying in biotic factors, are different for the mixture of microfungi and *Ch. globosum*. Exception to the rule is the test in which samples were created from waste paper pulp and with 5 % additive of wheat bran. Results indicate great susceptibility to biodegradation, both by the mixture: *A. niger*, *T. viride*, *P. funiculosum* and axenic *Ch. globosum*. Differences occur in the case of biological activity of 5 fungi mixture and *Ch. globosum*, for other test variants. Microfungi: *A. niger*, *T. viride*, *P. funiculosum* on the 4th day of testing gain comparable indices of overgrowing. It means that they overgrow the surface of samples with similar rate. However, in the next days of research, one observed bigger susceptibility to overgrowing of samples by *Ch. globosum*, in comparison to the mixture of test microfungi (Cofta et al. 2008), what has been also supported by the microscopic analysis (Figs. 3-5).

Mycelium *Ch. globosum* was evenly developing on most of the samples, firstly from white and yellow moldy spots and single spores, to black and brown spore areas. For the absolute development of this species of fungus indicated yellowish gold drops of spores, appearing on the surface of mycelium. As mycelium progressed, resistance of samples decreased. During the last days of observation, samples' overgrowing was very intense, except from paper without mass additives, in which mycelium development was very slow, and occurred only during the last day of observation. *Ch. globosum* colonies develop even in conditions with slight amount of oxygen and high content of CO₂ and extreme amounts of water (Zyska 1993).

From the mixture of used fungi, most active were: *A. niger* and *T. viride*. Samples without fungicide participation underwent overgrowing by the mixture of test fungi in very diverse way, changing in successive days of observation. There were samples covered only by *A. niger* or only by *T. viride*. Fungus *T. viride* developed completely during 14 days of observation, regardless from the type of cellulose paper. At the beginning, there was white cotton coating of loose structure, which in the following days changed its color into yellowish green. While analyzing mycelium *T. viride* visually, it appeared extensively on the whole surface of scale, however finally taking into account only the surface of samples – it was covered by this species only on the edges. During observation, a characteristic smell of coconut, specific for the species *T. viride*, was perceptible (Fig. 1). This fungus, similarly to *Penicillium occurs* broadly i.a., in soil that is why also this species of microfungus was recognized on the surface of samples taken out of black or forest soil.

Fungus *A. niger* on the 4th day after infection produced single black spores, placed on a delicate white sheath, during successive days one could observe clusters of spores forming black and brown mycelium, exactly at the place of infestation of samples. Lower side of colony was colorless or light yellow. Conidiophores grew directly from the substratum.

Fungus *P. funiculosum* appeared on single scales in the form of rusty-orange spots on the bottom side of the culture medium. However, it did not become active directly on the surface of paper straps (Fig. 1). The species: *Penicillium occurs* mainly in soil. During analysis of biodegradation degree of the examined products in soil, one also observed it on the surface of

samples that were taken out of black or forest soil. It produces toxic compounds, such as oxalic acid and kojic acid, which cause rotting and fasten degradation.

After mycological test, one has checked the rate of biodegradation of examined paper products through determination of their resistance to breaking under their own weight. In Tab. 3 and in the Fig. 2, average values of the obtained results, concerning self-breaking, were presented.

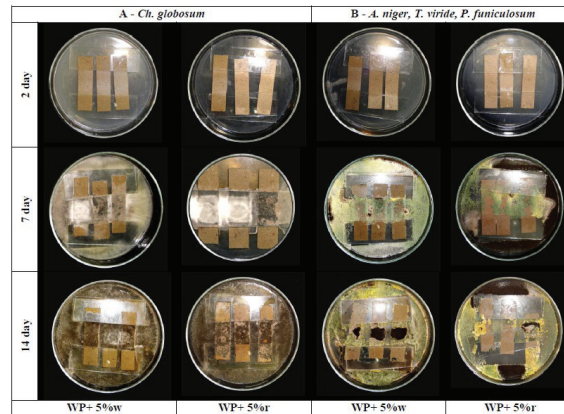


Fig. 1: Examples of photographs showing different degrees of colonisation of samples during 14 days after infection: A – test fungus *Ch. globosum*, B – mixture.

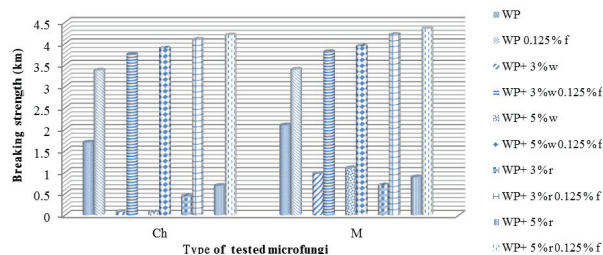


Fig. 2: Diagram illustrating and comparing breaking strength of paper samples manufactured from waste paper stock with or without addition of fillers and fungicide after biodegradation by microfungi (km).

The lowest resistance to breaking showed samples prepared without filling and with 3 and 5 % additives of rye bran, which in turn were characterized by fragility and brittleness. All the samples were strongly twisted and difficult to be put in the handles of breaking machine. Despite surface of the tested papers being totally covered, additives of 10 % aforementioned bran had a positive effect on the endurance tests, which is also confirmed by Modzelewska (2011) research. Samples with fungicide additives were less fragile and deformed which probably allowed gaining in higher values,

Analyzing results concerning resistance to self-breaking of the infected samples in the successive days of reading, most resistant one turned to be waste paper without organic filling. Paper with rye filling had higher values of self-breaking than the one with wheat filling. Also the type of applied fungus was of great importance: weaker turned to be samples infected with fungus *Ch. globosum*, e.g.: on the 7th day of observation of self-breaking WP+5 %w estimated 1.91 km in the case of *Ch. globosum*., and for the mixture 1.95 km. Even though this difference is slight

(0.04 km), the tendency in all the examined variants is the same (Tab. 3).

Tab. 3: The breaking strength in examined paper samples after biodegradation by microfungi (km).

Type of the examined paper	The mean results of breaking strength in examined paper samples (km)										Sample of paper product after taking it out of a dish and after degradation	
WP	2.44	2.45	2.1	2.32	1.87	1.9	1.8	2.33	1.7	2.1		
WP+ 3 %w	2.6	2.62	2.3	2.4	1.9	2.1	1	1.10	0.08	0.97		
WP+ 5 %w	2.87	2.87	2.45	2.5	2.2	2.2	1.2	1.29	0.07	1.1		
WP+ 10 %w	2.78	2.79	2.34	2.36	1.99	2.08	0.8	1	0.70	1.23		
WP+ 3 %r	2.34	2.35	2	1.9	1.78	1.82	0.67	0.89	0.45	0.7		
WP+ 5 %r	2.43	2.43	2.11	2.23	1.91	1.95	0.85	1.02	0.69	0.89		
WP+ 10 %r	2.39	2.41	1.9	1.98	1.82	1.87	0.56	1.51	0.28	1.23		
kind of micro-fungus	Ch	M	Ch	M	Ch	M	Ch	M	Ch	M		
day	day 2		day 4		day 7		day 10		day 14			day 21

Analyzing results concerning samples with fungicide, one may conclude that particularly resistant were series with 0.125 % fungicide additive and wheat bran of 3 and 5 % (Fig. 3). For instance, in the case of samples, filled with wheat bran and fungicide, values were as follows: 3 % of filling and 0.125 % of fungicide for the sample infected by *Ch. globosum*: 4.1 km, and infected by the mixture of fungi 4.2 km; 5 % of filling and 0.125 % of fungicide for the sample infected by *Ch. globosum*: 4.2 km, and by the mixture of fungi 4.35 km.

Having conducted selection of microscopic pictures (due to their huge number), in the work only a few were presented, which reflect general tendency of the obtained results. On the basis of microscopic observation of material before biodegradation, after the attack of test microfungi – one may conclude that there were changes in the structure of cellulose fibers, brought about by degradation factors.

In the case of samples of ligno-cellulose products subjected to biodegradation caused by test fungi, having analyzed photographs (Figs. 3, 5), it may be concluded without any ambiguity that regardless of composition of sheets of paper, much bigger destruction underwent materials infected by microfungus *Ch. globosum* than by the mixture consisting of *A. niger*, *T. viride*, *P. funiculosum*. Differences are significant both on the surface and in the structure of fibers. Sprawling hyphae and the reaction of enzymes in the tested mycelium not only reshaped but also contributed to numerous cellulose fiber cracks which significantly reduced the mechanical properties and changed the paper structure (Strzelczyk and Karbowska-Berent 2004, Rojas et al. 2009). Even in the case of small enlargement, slime molds of *Ch. globosum*. mycelium are noticeable and are much longer than other test fungi occurring in the mixture. In the pictures, before biodegradation, one may observe single fibers and a lot of space among them. After mycological attack, examined material significantly uniformed its surface but only visually (it is visible in the case of x 100 enlargement SE). Spores and slime molds of fungi filled the space among fibers and (what is visible at big enlargement) damaged also fibers, making them shorter and gradually colonizing in them.

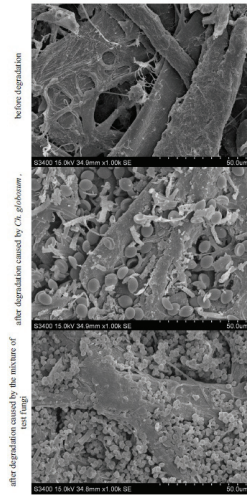


Fig. 3: Microscopic pictures showing the surface of the analyzed ligno-cellulose products without

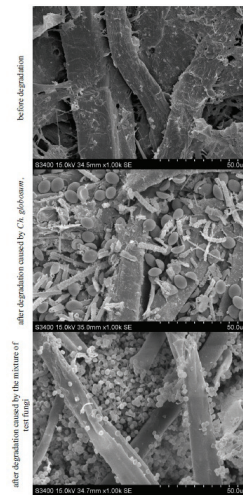


Fig. 4: Microscopic pictures showing the surface of the analyzed ligno-cellulose products with 3 % wheat filling before and after mycological degradation

Spores of fungus *Ch. globosum* are much bigger than others. However, spores of test microfungi: *A. niger*, *T. viride*, *P. funiculosum*, although tinier – occur in more numerous clusters, they colonize with more ease. Their slime molds develop more intensively on the surface of ligno-cellulose products, and only then cause degradation inside samples of paper. In the case of *Ch. globosum* it happens simultaneously and equally strongly.

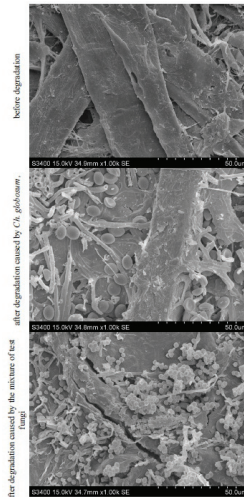


Fig. 5: Microscopic pictures showing the surface of the analyzed ligno-cellulose products with 3 % rye filling before and after mycological degradation

Generally, microscopic analysis confirmed previous research. Biodegradation caused by microfungi is intense and aggressive. Fungus *Ch. globosum* causes bigger changes in ligno-cellulose products than applied in the examination mixture of microfungi (*A. niger*, *T. viride*, *P. funiculosum*).

CONCLUSIONS

In the introduction, one has presented current trends of using rye bran, both in the world and Poland. The conducted at The Institute of Chemical Wood Technology, Poznań University of Life Sciences, research showed that application of cereal bran (wheat and rye as fillers in the production of selected paper products) is possible (Modzelewska et al. 2009, Modzelewska, Pietrzak 2010).

On the basis of gained results of this stage of research on the broad issue of biodegradation of paper products gained from ligno-cellulose materials, containing various mass and organic additives, one may arrive at the following conclusion:

1. Addition of wheat bran (3 %) and rye bran (3 % and 5 %) as a filling of pulp caused increase in susceptibility to overgrowing by test fungi, in comparison to samples devoid of bran. Waste paper was more susceptible to overgrowing by test mycelium *Ch. globosum*, which was evenly developing as opposed to test mycelium consisting of *A. niger*, *T. viride*, *P. funiculosum*, where, especially in the case of samples with fungicide additive, it developed longer.
2. Samples of waste paper with or without organic filling showed very low resistance to overgrowing, in relation to paper with 0.125 % fungicide additive; fungicide caused that examined ligno-cellulose products showed very high resistance to overgrowing by test fungi, none of the samples was covered with fungus, and inhibition area was maintained during the whole period of experiment (21 days).
3. By competent dosing of preparations into fungicide and application of various test fungi, one may shorten or lengthen time of mycological biodegradation of ligno-cellulose materials, depending on their final purpose.
4. Decrease in self-breaking resistance, as the research showed, before and after mycological biodegradation, most probable results from i.a., the loss of some chemical substances and changes in their properties in relation to original material. Enzymatic activity of microfungi caused shortening of cellulose chain, decrease in strength of bonds among fibrils in paper products, and finally decrease of its resistance properties (the accepted criterion was self-breaking resistance).
5. Usage of two or more methods simultaneously gives possibility for objective interpretation of results. The conducted analyses (standard method and microscopic analysis) mutually support the obtained research results.

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