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Investigation of the Cellulose Pulp Modification Process with the Use of Cellulolytic Enzymes from the Aspergillus wentii ŁOCK 0459 Strain

Abstract

The use of cellulolytic enzymes in the process of cellulose biomodification is one of the current applications of these enzymes which allows us to join the process of controlled partial cellulose degradation with changes to the molecular, supermolecular, and morphological cellulose structure. The application of biotechnological methods in the chemical fibre industry allows us to solve problems connected with protecting the natural environment. This paper presents the results of investigations concerning the estimation of the usability of cellulolytic enzymes created by the Aspergillus wentii ŁOCK 0459 strain used for modifying cellulose pulps. The tests were carried out with the use of an Aspergillaceae strain from the Polish Culture Collection (Kolekcja Czystych Kultur) at the Technical University of Łódź. Biomodified cellulose pulps, characterised by a solubility of 98% in aqueous solutions of sodium hydroxide, were used for the preparation of alkaline cellulose solutions. The solutions obtained were applied as spinning solutions for the process of cellulose fibre and film formation.

Key words: *enzymes, cellulolytic enzymes, Aspergillus wentii, cellulose, biomodification, cellulose structure, spinning solutions.*

Introduction

The investigations concerning the production of cellulolytic enzymes which have so far been carried out on a great scale, are principally aimed at maximising the biodegradation process (down to simple sugars) of cellulose or of the wastes containing this polymer. The final goal was the application of the enzyme in practice [1-3].

The use of cellulolytic enzymes in the biomodification process of cellulose is one of the current applications of these enzymes which allows us to join the process of a controlled partial cellulose degradation with changes to the molecular, supermolecular, and morphological structure. The application of biotechnological methods in the chemical fibre industry allows us to solve problems connected with protecting the natural environment.

A cycle of investigations into the application of cellulolytic enzymes for cellulose pulp transformation has been carried out over many years at the Institute of Chemical Fibres [2-4]. A cellulose biomodification method which led to the obtaining of high reactive cellulose pulps has been developed into the basis for these investigations. The process of cellulose pulp biomodification was conducted with the use of high-specialised (selected) enzymes from the cellulase and xylanase groups which originated from the deep-seated fermentation of *Aspergillaceae* fungi from the *Aspergillus niger* and *Trichoderma reesei* types. Applying enzymatic processes for cellulose modification allows us to link the process of partial cellulose depolymerisation to changes to its molecular, supermolecular, and morphological structure, which in turn results in obtaining kinds of cellulose which can be dissolved to a degree of 98-99.5% in a 9% aqueous solution of sodium hydroxide.

The process of enzymatic cellulose processing has some advantages, such as soft proceeding conditions, controlled progress, the possibility of carrying out continuous processes, high specificity of action, the possibility of turning back the enzymes' solutions, and the lack of ecological threat. This process is a modern solution for cellulose processing and an approach to developing a method of manufacturing cellulose fibres and films without the use of toxic carbon disulphide, and should also be an alternative to the viscose manufacturing method.

The type of enzyme plays an essential part in the cellulose biomodification process. We use the expression 'type' here to refer to the quantitative and qualitative composition of the cellulolytic complex used, i.e. the micro-organism type which creates this complex. This importance is the reason why the search for new special cellulolytic enzymes has been continually conducted world-wide. What is more, the search is for such enzymes which should be directed to a significant degree at cellulose structure modification and at the same time securing a minimised process of decomposition of this polymer to simple sugars.

Smaller participation in the cellulolytic complex of those enzymes which splinter cellobiose and glucose from the cellulose chain such as 1,4-β-celobiohydrolase and exo-1,4-β-glucosidase and of the β-glucosidase which decompose the celobiose down to glucose could have the effect of improving the biomodification process' economy as the result of the decrease in the cellulose mass loss.

The aim of this paper is to present the results of investigations concerned with the biosynthesis process of cellulolytic enzymes by means of the *Aspergillus wentii* ŁOCK 0459 strain, and with the practical application of enzymes obtained from the process of biomodification cellulose pulps. Selected physico-chemical properties of the biomodified cellulose, together with an estimation of the possibilities of applying this cellulose to obtain alkaline spinning solutions, are presented in this paper. The preliminary results of the investigations into obtaining fibres and films from the alkaline cellulose solutions prepared during the tests carried out are also described.

Materials and Methods

The cellulose pulp designated V-67 (Californian pine, USA) was used for investigations of the biomodification process. This cellulose pulp was characterised by an α-cellulose content of 97%, an average polymerisation degree DPv=641, and a coefficient of the secondary water retention value WRV=151%.

Biosynthesis of enzymes
The culture medium of the composition shown in Table 1 was used for the synthesis of cellulolytic enzymes by the *Aspergillus wentii* ŁOCK 0459 strain. The culture medium was elaborated according to the work described in [5].

The biosynthesis of the cellulolytic enzymes was carried out by means of the dynamic method with the use of a Labfors bioreactor, produced by Infors A.G., Switzerland, with a 7.5 dm^3 capacity, using stirring and aeration. The process was conducted for 7-8 days, at a temperature of 35-40°C. Microcrystalline cellulose of the 402 2b type produced by Mikro-Technik, Germany was added to the culture medium. A suspension of *Aspergillaceae* spores with a concentration of 10^6 cells/cm^3 in a 0.85% NaCl solution was used as the inoculum for the culture inoculation. After the completion of biosynthesis, the culture medium containing enzymes was separated from the bio-mass, and the cellulose remains were introduced into the medium by means of filtration in vacuum by means of a Büchner funnel with a blotting-paper filter.

Biomodification of cellulose pulps
After preliminary mechanical processing performed with the use of a Werner-Pfleiderer shredder, the cellulose sample was processed by means of cellulases of the *Aspergillus wentii* ŁOCK 0459 strain. The enzymatic reaction was conducted by two methods: with the use of an Erlenmeyer flask of 250 cm^3 capacity immersed in an Elpan 357 type water bath and shaken for mixing, and with the use of a glass reactor of 6 dm^3 capacity equipped with a mechanical stirrer.

The following parameter ranges were used for the processes of cellulose biomodification:
- cellulose content in the solution: 5% by weight,
- modulus enzyme/substratum (E/S): 12.6-22.8 U CMC,
- time: 6-8 hours,
- temperature: 50°C, and
- pH 4.8.

After biomodification, the cellulose was drained in vacuum with the use of a Blüchner funnel, then water at a temperature of 95°C was poured on it and left for a period of 10 minutes to allow deactivation of the enzyme remains; next, the cellulose was once more drained with the use of the Blüchner funnel and washed out three times with distilled water.

Preparation of alkaline solutions from biomodified cellulose
Biomodified cellulose with 71% humidity and an amount of 5% by weight was dissolved in a 7.7-8.3% aqueous sodium hydroxide solution containing 0.86% by weight of zinc oxide and 4.2% by weight of urea. The dissolution process was performed in a Treiber-type mixer equipped with a high-velocity stirrer at a temperature of 8°C over a period of 60 minutes. The alkaline cellulose solutions obtained were analysed, and the viscosity, filtration factors, the cellulose content of the solution and the total alkalinity of the solution were determined. The solution prepared was used for tests consisting of formation of cellulose fibres and films [6].

Cellulose film manufacturing on laboratory scale
The cellulose films were obtained by uniform distribution of the alkaline cellulose solution on the surface of glass plates, and next by immersing them in a coagulation solution composed of 150g/l of H_2SO_4, and of 80 g/l of Na_2SO_4. The bath temperature was 20°C. After the coagulation process, the films were washed out by distilled water and dried at small tension at a temperature of 50°C [6].

Cellulose fibre formation
The spinning solution after filtration and aeration was poured into a pressure container of 1 dm^3 capacity at a pressure of 0.5 MPa. The tests of cellulose fibre formation were carried out with the use of an experimental spinning machine. The spinning solution was pressed into the coagulation bath with the use of a toothpump at a yield of 1.2 cm^3/rev. through a PtRh spinning die with 300 orifices of 0.08 mm hole diameter.

The following baths were used:
- as the coagulation bath: 110 g/l of sulphuric acid at a temperature of 20°C, and
- as the washing bath: water at a temperature of 75°C.

The lengths of both baths (the coagulation and the washing bath) were 80 cm. The velocity of formation was set at 20 cm/minute. The fibres were also drawn in the washing bath at a drawing ratio of R_{max}=20%. The fibres obtained were washed with water, and a preparation containing surface active compounds was deposited on the fibres, which were then dried at a temperature of 20°C [6].

Analytic methods
The contents of reducing sugars in the enzyme solutions were assessed by a method using dinitrosalicyl acid (DNS) [7]. The activity of endo-1,4-β-glucanase (CMC) was estimated by the colorimetric method with the use of carboxymethylocellulose [8]. The filter-paper activity (FPA) was estimated by the colorimetric method with the use of Whatman filter-paper No. 1 [8], whereas the β-glucosidase activity (CB) by the colorimetric method with the use of salicin [8]. The activity of endo-1,4-β-xylannase was also estimated by the colorimetric method but with the use of xylose [9]. The protein content in the post-culture filtrates was assessed by means of the Lowry method [10]. The average polymerisation degree ($\overline{DP}v$) in the cellulose samples tested was estimated by the viscometric method with the use of an alkali complex of sodium-ferric-tartrate solution. The secondary swelling index - the water retention value (WRV) - was determined by the gravimetric method [12]. The α-cellulose content was estimated in a sodium hydroxide solution according to the method described in standard [13]. The dissolution degree of cellulose in the solutions was determined according to [14]. The following quantities and coefficients were determined within the frame of property estimation of the biomodified cellulose solutions: the filtration index Kw [15], the corrected filtration index Kw*, the dynamic viscosity, and the total alkalinity of the solution. The mechanical properties of fibres and films were estimated according to Polish standards [16].

Photos of the alkaline cellulose solutions were taken with the use of an BIOLAR optical polarisation microscope and a computerised image analyser manufactured by the IMAL company.

Photos of the cross-sections and surfaces of the cellulose fibres were taken with the use of a JSM-35C electron-scanning microscope (SEM) manufactured by Jeol, Japan; magnifications of 1300 were applied.

Investigation Results

Investigations of the process of the biosynthesis of cellulolytic enzymes
Investigations into cellulase biosynthesis were conducted for the *Aspergillus wentii* ŁOCK 0459 strain, which was selected for the preliminary research period from

Table 1. *Culture medium composition; pH within the pH range of 4.5-8.0.*

Culture composition	Content in g/1000cm ^{3}
Sodium nitrate	2.0
Alkaline potassium phosphate (I)	0.7
Alkaline potassium phosphate (II)	0.3
Potassium chloride	0.5
Hydrated manganese sulphate	0.5
Hydrated ferric (II) sulphate	0.01
Malt extract	5 - 10
Cellulose	10 - 20
pH	4.5 - 8.0

Table 2. *Conditions of the cellulolytic enzymes' biosynthesis with the use of the Aspergillus wentii ŁOCK 0459 strain.*

Enzyme designation	Cellulose content in medium	Malt extract content in medium	pH of medium		Temperature	Time of culture	Stirring velocity
			before culture	after culture			
	%	%			°C	days	r.pm./min
B-4	1.0	1.0	4.5	4.9	35	8	50
B-5	1.0	1.0	4.5	6.5	35	8	75
B-6	1.0	1.0	8.0	4.6	35	7	70
B-8	2.0	1.0	8.0	4.8	40	7	70
B-10	2.0	0.5	8.0	4.9	35	7	70

among many strains with cellulolytic abilities. During the optimisation tests, the influence of the following parameters on the quality and quantity content of the cellulolytic enzyme complex were estimated: the cellulose and malt extract content in the culture medium, and the conditions of culture proceeding, such as temperature and the rotational velocity of the stirrer. On the basis of the results obtained by means of the above-mentioned tests, a culture medium was selected, for which the strain used produced cellulases of the highest activity, with the aim of being used for further investigations (Table 1) aimed at scale increasing of the biosynthesis process. This medium containing cellulose of an amount of 1-2% by weight was used for the enzyme culture carried out with the use of the bioreactor. The parameters of the biosynthesis process are listed in Table 2.

The activity of cellulolytic enzymes and of endo-1,4-β-xylanase was determined in the post-culture liquids after draining off the fungi spawn and cellulose residues. The protein contents determined and the enzymes' activity measured in CMC units per 1 mg of proteins (the specific activity) are shown in Table 3 which presents a characteristic of the enzymes obtained.

It was determined that the *Aspergillus wentii* ŁOCK 0459 strain on a medium containing 2% of cellulose and 1% or 0.5% of malt extract yields cellulases with the highest activity (the enzymes designated B-8 and B-10). The highest activity of endo-1,4-β-glucanase of 1.14 U CMC/cm^3 was stated for a medium with a cellulose content of 2% and a decrease down to 0.5% content of malt extract, and for a culture conducted on this medium at a temperature of 35°C over 7 days, at pH 8 of the medium and with intensive mixing at 70 r.p.m. (Table 2).

However, it was also determined that the reduced content of malt extract in the culture medium influences the increase in β-glucosidase activity of the cellulases produced. This activity was noted as 0.91 U CMC/cm^3. The lowest activity of β-glucosidase was observed for the B-8 enzyme test with a medium containing 2% of cellulose and 1% of malt extract after 7 days of culture at a temperature of 40°C. This activity was on the level of 0.05 U CMC/dm^3.

Investigation of the process of biomodification of cellulose pulps

Investigations into estimating the applicability of enzymes to the biomodification of V-67 cellulose were carried out at this stage. The enzymes designated as B-4, B-5, B-6, B-8, and B-10 obtained from the *Aspergillus wentii* ŁOCK 0459 strain were tested. The properties of the V-67 cellulose biomodified for 6 and 8 hours at a temperature of 50°C and cellulose content in the enzyme solution of 5% by weight are presented in Table 4. The E/S modulus oscillated within the range of 12.6 to 22.8 U CMC/g over the whole biomodification period. The following quantities and coefficients were determined: the percentage of cellulose saccharisation during the reaction, the average polymerisation degree DPv of cellulose, the α-cellulose content, the secondary swelling index (i.e. the water retention value (WRV)), and the dissolution degree (Sa) of cellulose in the alkaline solutions.

On the basis of the results obtained, it was stated that for all enzymes from the *Aspergillus wentii* ŁOCK 0459 strain tested, the cellulose obtained was characterised by a dissolution degree of over 97% in a 9% aqueous solution of sodium hydroxide. At the same time, the polymer mass loss did not exceed 5% with the exception of the V-67/6/B-10 and V-67/8/B-10 tests.

Table 3. *Characteristic of enzymes used for cellulose biomodification.*

Enzyme designation	Protein content	Sugar content	Enzyme activity							
			Endo-1,4-β-glucanase		Filter-paper (FPA)		β-glucosidase		Endo-1,4-β-xylanase	
	mg/cm^3	mg/cm^3	U CMC/cm^3	Specific U CMC/mg	U CMC/cm^3	Specific U CMC/mg	U CMC/cm^3	Specific U CMC/mg	U CMC/cm^3	Specific U CMC/mg
B-4	0.46	0.28	0.83	1.80	0.16	1.00	0.50	1.09		14.28
B-5	0.52	0.19	0.63	1.21	0.13	0.25	0.74	1.42	27.80	53.46
B-6	0.85	0.61	0.68	0.80	0.21	0.25	0.31	0.37	31.13	36.62
B-8	0.58	0.72	1.00	1.70	0.18	0.31	0.05	0.09	34.31	59.16
B-10	0.92	0.69	1.14	1.24	0.25	0.27	0.90	0.98	40.25	43.75

On the basis of the tests carried out, the enzymes B-4, B-5, and B-8 were selected for investigation of the biomodification process with use of the bioreactor. The enzymatic processing of the V-67 cellulose pulp was carried out at a temperature of 50°C for 6 hours, and at a cellulose content in the enzyme solution of 5% by weight. The investigation results are presented in Table 5.

All biomodified cellulose pulps obtained were characterised by a dissolution degree (Sa) of cellulose in the aqueous solution of sodium hydroxide of over 98%. From the samples whose parameters are shown in Table 5, alkali solutions were prepared according to the procedures described previously. The properties of these solutions are presented in Table 6.

The alkaline cellulose solutions were characterised by an α-cellulose content of 5% by weight, total alkalinity within the range of 7.73-8.33% by weight, the filtration index Kw within the range from 5165 to 19425, the corrected filtration index Kw* within the range of 373 to 1273, and the dynamic viscosity assessed with the use of the Brookfield viscometer at a temperature of 6°C ranging from 7400 to 12500 cP. The alkaline cellulose solutions with an α-cellulose content of 5% were characterised by very high viscosity, which results from the high (over 450) polymerisation degree (DP) of the biomodified cellulose. Photos of unfiltered alkaline solutions of the biomodified V-67 cellulose are shown in Figure 1.

Only a few particles of insoluble, swelled cellulose fragments are visible in these photos. The solution designated as V-67/6/B-4, and characterised by a lower value of the filtration index Kw (5165), was used for investigating the process of fibre and film formation.

The cellulose films from biomodified cellulose were prepared according to the procedure described. The mechanical properties of the films obtained are presented in Table 7.

The film obtained from the V-67/2/B-4 solution was characterised by high elongation strength of 58.1 MPa and relative elongation of 4.88%. The mechanical parameters of the films obtained by tests confirm the usability of the biomodified cellulose alkali solutions for cellulose film manufacturing.

A part of the V-67/2/B-4 solution, which was characterised by a lower corrected filtration index Kw* (373) than that of the film solutions, was additionally filtered.

Table 4. *Characteristic of the biomodified V-67 cellulose pulps.*

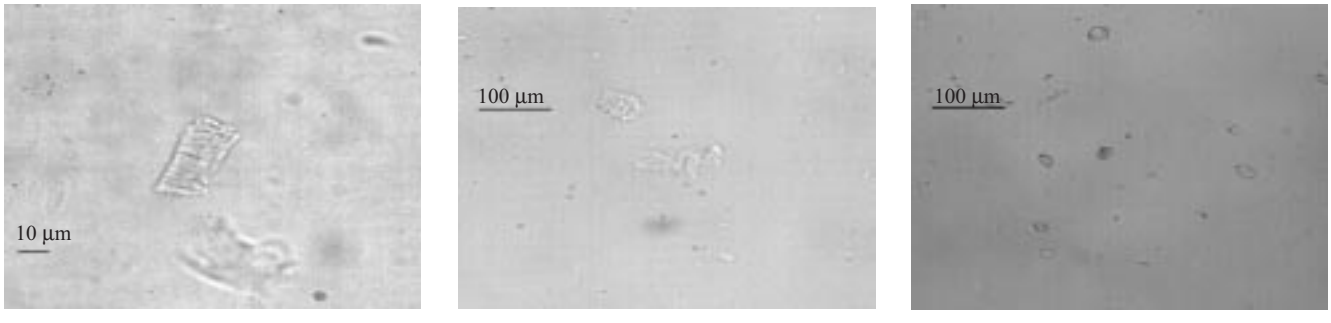
Test designation	Parameters of the biosynthesis process			Percent of saccharisation	Features of biomodified cellulose			
	Enzyme designation	Modulus E/S U CMC/g	Reaction time h	%	$\overline{DP}$v	α-cellulose content %	WRV %	Sa %
V-67 after mechanical treatment	-	-	-	-	543	98.0	90.5	52.4
V - 67/6/B-4	B-4	16.6	6	4.24	462	94.5	88.0	98.4
V - 67/8/B-4			8	4.66	460	94.3	82.3	98.0
V - 67/6/B-5	B-5	12.6	6	4.22	475	93.1	85.1	98.8
V - 67/8/B-5			8	4.57	470	93.5	82.5	99.0
V - 67/6/B-6	B-6	13.6	6	3.71	472	92.6	85.6	97.7
V - 67/8/B-6			8	5.98	465	91.7	80.0	98.9
V - 67/6/B-8	B-8	20.0	6	4.56	452	96.4	78.7	97.6
V - 67/8/B-8			8	4.56	430	95.8	78.6	99.0
V - 67/6/B-10	B-10	22.8	6	7.98	470	91.0	83.0	98.3
V - 67/8/B-10			8	8.59	448	91.6	81.2	98.7

Table 5. *Characteristic of cellulose pulps after biomodification with the use of a bioreactor.*

Test designation	Parameters of the biosynthesis process			Mass loss	Features of biomodified cellulose			
	Enzyme designation	Modulus E/S U CMC/g	Reaction time h	%	$\overline{DP}$v	α-cellulose content %	WRV %	Sa %
V - 67/B-4	B-4	16.6	6	3.5	462	95.2	80.3	99.3
V - 67/B-5	B-5	20.0		4.5	460	92.1	83.9	98.6
V - 67/B-8	B-8	12.6		5.1	453	94.3	78.8	98.0

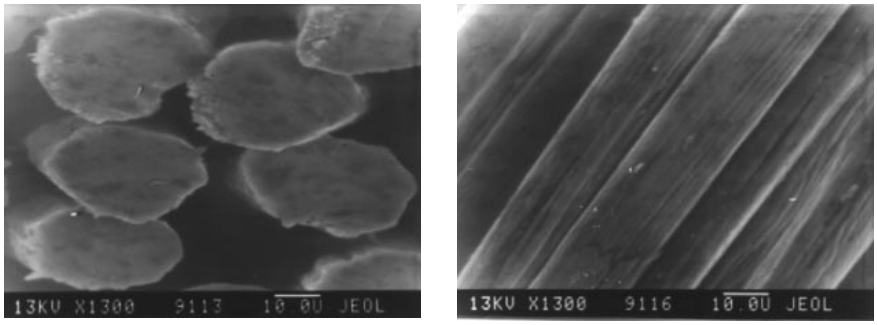
Table 6. *Properties of alkaline solutions from biomodified cellulose.*

Solution designation	α-cellulose content % wt.	Total solution alkalinity % wt.	Dynamic viscosity cP	Temperature of viscosity determination °C	Alkali ratio assessed	Kw	Kw*	Content of insoluble part %
V - 67/6/B-4	5.04	8.33		6.0	1.65	5165	373	0.20
V - 67/6/B-5	5.00	7.73	12 500	6.2	1.55	19425	1273	0.57
V - 67/6/B-8	5.03	8.17	12 100	6.3	1.62	6359	418	0.59



a) b) c)

Figure 1. *Microscope photos of the cellulose solutions: a) V-67/6/B-5, b) V-67/6/B-8, c) V-67/6/B-4.*



a) b)

Figure 2. *SEM photos of cellulose fibres: a) cross-section, b) surface.*

Table 7. *Mechanical properties of films from biomodified cellulose.*

Solution designation	Thickness	Tensile strength	Variability coefficient of the tensile strength	Elongation at maximum load
	mm	MPa	%	%
V - 67/6/B-4	0.014	58.1	15.5	4.85

Table 8. *Mechanical properties of fibres from biomodified cellulose.*

Test designation	Linear density	Tenacity at conditioned state	Variability coefficient of breaking force at conditioned state	Elongation at break at conditioned state	Variability coefficient of elongation at break at conditioned state
	dtex	cN/tex	%	%	%
V - 67/6/B-4	2.98	11.96	13.3	15	19.7

The filtration was performed with the use of filtration fabrics such as a PP/PET nonwoven and a cotton batiste. Then, the solutions were aerated at a temperature of 15°C for 8 hours. The spinning solution prepared in this way was used for fibre formation on laboratory scale.

The fibres were spun according to the conditions described previously, at a velocity of 20 m/minute. The fibre spinning was performed without any disturbances. The fibre samples, after deposition a standard preparation preserving the elementary fibres against sticking on its surface, were dried at ambient temperature, and then tested to estimate their mechanical properties. The test results are presented in Table 8. As can be seen from this Table, the fibres obtained with linear density of 9.98 dtex are characterised by tenacity of 11.96 cN/tex and elongation at break of 15% in a conditioned state. The SEM photos of the fibre's cross-section and of the longitudinal view of the fibres are shown in Figure 2. The fibres are characterised by an oval cross-section with little developed boundary line; they are regular and show no sticking.

Conclusions

- The enzymes obtained by means of the biosynthesis process with the use of a reactor characterised by endo-1,4-β-glucanase activity within the range of 0.63-1.14% U CMC/cm^3 were tested in the biomodification process of cellulose pulps. The biomodified cellulose pulps characterised by a dissolution degree within the range of 98-99% and a percent of cellulose saccharisation of 4.2-4.6, were used to prepare the cellulose alkaline solutions.
- The alkaline cellulose solution characterised by an α-cellulose content of 5.04% by weight, dynamic viscosity of 7,400 cP, value of the filtration index Kw of 5,165 and value of the corrected filtration index Kw* of 373, and with a content of indissoluble parts of 0.20%, was used for manufacturing cellulose films and fibres.
- The cellulose films obtained were characterised by a high elongation strength of 58.1 MPa and a relative elongation of 4.85%.
- The cellulose fibres manufactured were characterised by a linear density of 2.98 dtex, a tenacity of 11.96 cN/tex, and elongation at break of 15% in a conditioned state.
- The investigations carried out within the frame of the research project indicate the possibility of synthesising cellulolytic enzymes from the *Aspergillus wentii* ŁOCK 0459 strain which possess activities required for application in the process of cellulose biomodification.

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