

## Article

# Production of Biomodified Bleached Kraft Pulp by Catalytic Conversion Using *Penicillium verruculosum* Enzymes: Composition, Properties, Structure and Application

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**Abstract:** The global development of bioeconomy is impossible without technologies for comprehensive processing of plant renewable resources. The use of proven pretreatment technologies raises the possibility of industrial implementation of enzymatic conversion of polysaccharides from lignocellulose considering the process complexity. For instance, a well-tuned kraft pulping produces a substrate easily degraded by cellulases and hemicellulases. Enzymatic hydrolysis of bleached hardwood kraft pulp was carried out using an enzyme complex of endoglucanases, cellobiohydrolases,  $\beta$ -glucosidases and xylanases produced by recombinant strains of *Penicillium verruculosum* at a 10 FPU/g mixture rate and a 10 % substrate concentration. As a result of biocatalysis the following products are obtained: sugar solution, mainly glucose, xylobiose, xylose, as well as other minor reducing sugars; modified complex based on cellulose and xylan. The composition of biomodified kraft pulp was determined involving the use of HPLC. The method for determining the degree of crystallinity on an X-ray diffractometer was used to characterize the properties. The article shows the possibility of producing modified cellulose cryogels by amorphization with concentrated 85 %  $H_3PO_4$  followed by precipitation with water and supercritical drying. Analysis of the enzymatic hydrolysate composition reveals the predominance of glucose (55...67 %) in reducing sugars with a maximum content of up to 6 % in the solution after 72 hours. The properties and structure of modified kraft pulp were shown to change during biocatalysis, in particular, the degree of crystallinity increased by 5 % after 3 hours of enzymatic hydrolysis. We obtained cryogels based on the initial and biomodified kraft pulp with conversion rates of 35, 50 and 70 %. The properties of these cryogels are not inferior to those based on industrial microcrystalline cellulose, as confirmed by the specific surface area, degree of swelling, porosity and SEM images. Thus, kraft pulp enzymatic hydrolysis offers prospects not only for producing sugar-rich hydrolysates for microbiological synthesis, but also for producing cellulose powders and cryogels with specified properties.

**Keywords:** enzymatic hydrolysis; biomodified kraft pulp; cellulose; xylan; cryogel; crystallinity

## 1. Introduction

Cellulose-based wood resources are the most widespread renewable resources in the world. The necessity of including them into the circular bioeconomy model has been growing over the last years [1-3]. The crucial biotechnological process of this model is

enzymatic hydrolysis, which involves deep polysaccharide degradation: cellulose and hemicelluloses to simple sugars [4-6]. The hydrolysate is possible to use in further microbiological conversion with such biosynthesis products as bioethanol, organic or amino acids.

The issue of lignin-containing wood raw materials pretreatment to enzymatic conversion is still thrown into sharp relief in terms of all the possibilities of their full use [7,8]. Raw materials pretreatment aims at increasing the fiber accessibility for the enzyme action [9]. The indispensable stages are raw materials grinding, pulp crystalline structure destruction and complete or partial delignification [4,10]. There are several technologies of wood chemical pretreatment. Among these, for example, are acid treatment, alkali cooking and kraft pulping. Kraft pulping is a cost-efficient and effective method of preparing wood raw materials for the production of pulp with good reactivity for enzymatic conversion [11-16]. Nowadays, the current high level of technology allows the integration of biorefineries into existing pulp and paper industrial enterprises [5,6,17-20].

The choice of effective enzymes acting on carbohydrates, which include a complex of glycosidases with various degradation pathways of macromolecules, is a decisive factor for deep and controlled bioconversion [8,21,22]. Enzymatic technologies based on the use of isolated hydrolytic enzymes (endoxylanases and endoglucanases) have been extensively introduced in the pulp and paper industry starting from the late 1990s [23]. Currently there is a wide variety of multienzyme commercial complexes for polysaccharide degradation of plant raw materials. Novozymes (Danmark), Genencor and Danisco Division (USA) are the international companies producing cellulolytic enzymes of the fungus *Trichoderma*. About 80% of multienzyme complexes for the production of biofuels and organic acids from lignin-containing raw materials are produced when using the fungus [4,24]. Their market prevalence is due to intense development of microbial platforms in recent decades. Enzyme complex based on the producer strains of *Penicillium verruculosum* have been proposed as their alternative [25]. Their effectiveness in the saccharification of kraft pulp has been confirmed by the number of studies [26,27].

Implementation of a comprehensive strategy in renewable resources pretreatment presumes a propensity to maximize the use of all resource components. Kraft pulp production has an advantage towards to utilization of lignin, the most part of which is used in the chemical and energy recovery cycle. However, studying the processes of further enzymatic conversion of kraft pulp polysaccharides as a rule was carried out towards soluble products (glucose and reducing sugars). Papers devoted to the insoluble residue after hydrolysis exist in limited number [28]. Along with this, it is known that the structure of plant fibers is modified during substrate enzymatic conversion; kraft pulp acquires new characteristics of crystallinity, degree of polymerization, etc. [29]. Thus, we can speak about the possibility of producing a new product – biomodified kraft pulp, the properties of which are not fully disclosed. According to the waste-free bioeconomy concept, it is necessary for the economic dimension to carry out follow-up studies of this product in order to use it in nanotechnology, pharmaceuticals, energy and other industries.

Production of porous, lightweight materials with high specific surface area known as cryogels is a possible way to use biomodified kraft pulp. These polysaccharide gels are produced by amorphization in a solvent, freezing and subsequent supercritical drying, which makes their production simple and cost-efficient [30]. Polysaccharide cryogels have the potential to be used in regenerative biomedicine as cell scaffolds, and tissue engineering, with high fluid absorption parameters [31,32].

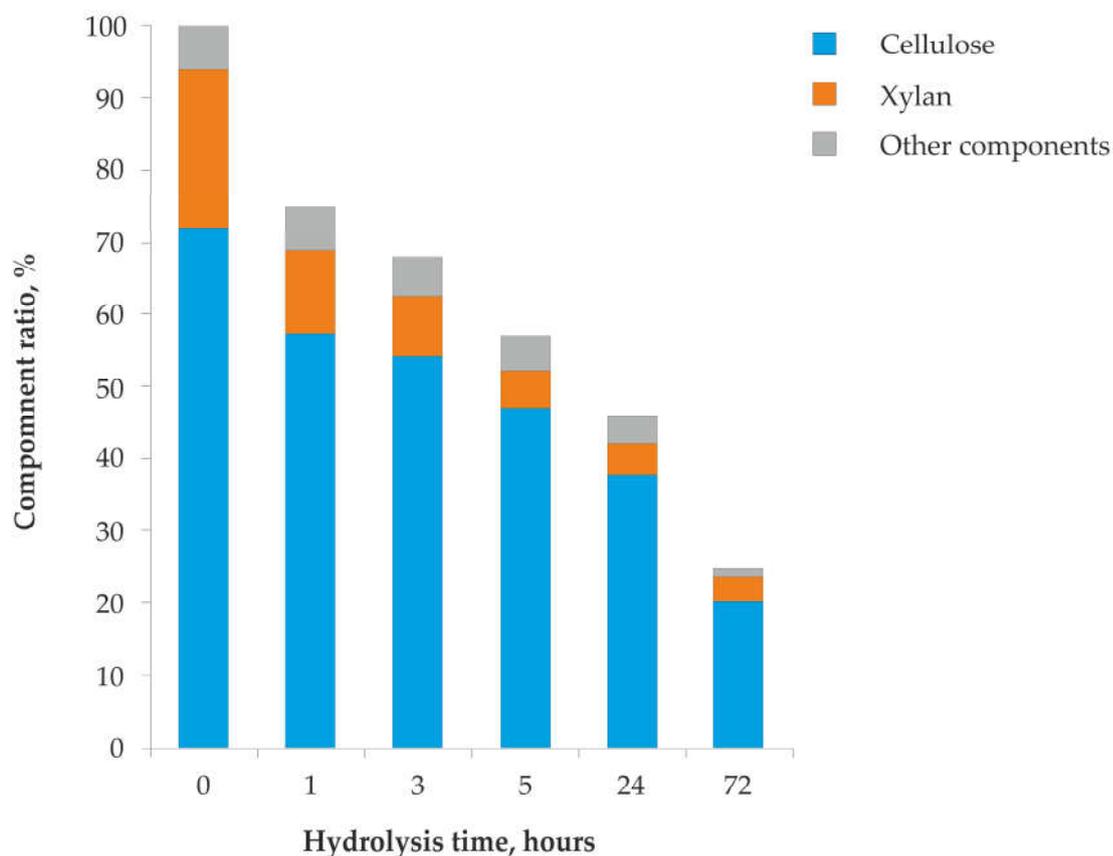
The present work is a continuation of the previous works [9,11,30], which aimed at obtaining hydrolysates with high content of cellulosic sugars after kraft method of wood raw materials pretreatment by means of conversion with enzyme complexes based on the producer strains *P. verruculosum*. This study deals with modified kraft pulp produced by such biocatalysis. Its composition, properties and structure can be regulated during deep hydrolysis to meet various purposes, including high-quality cryogels production.

## 2. Results

### 2.1 Yield and component composition of hydrolysate and biomodified kraft pulp during biocatalysis

The process of biocatalysis of kraft pulp by *P. verruculosum* enzymes resulted in the formation of a mixture of mono-, di-, and oligosaccharides of the wood origin in the liquid phase. The predominant products after 72 hours of enzymatic hydrolysis were glucose with a concentration of 6.0 % and the xylan hydrolysis products, such as xylose (1.0 %) and xylobiose (0.7 %); in addition there were minor amounts of mannose (0.2 %), galactose (0.2 %) and arabinose (less than 0.1 %) (Table S1).

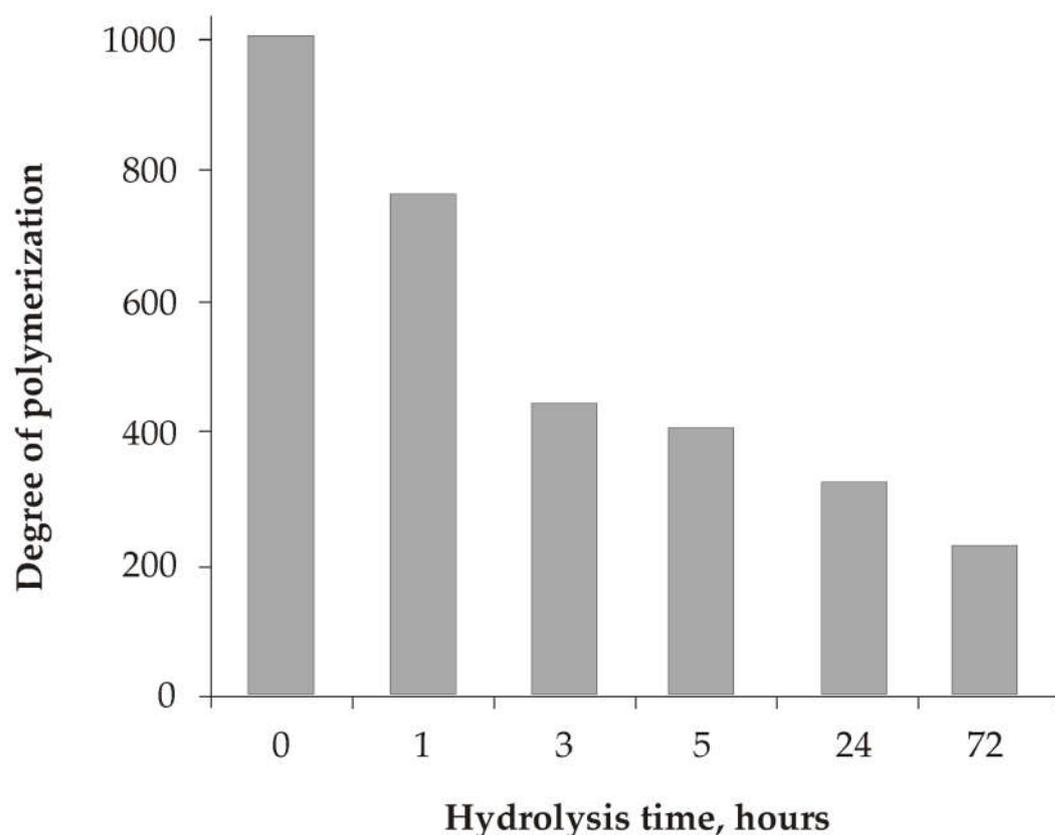
The simultaneously flowing enzymatic modification of kraft pulp significantly reduced its yield, from 75 % after 1 hour of biocatalysis to 25 % after 72 hours of hydrolysis (Figure 1). There were changes in the amounts of the main components of biomodified kraft pulp: cellulose and xylan. Their initial amounts were 72 % and 22 %, respectively. The maximum amount of cellulose in biomodified kraft pulp ranged from the initial value to 82.7 %, the maximum value was achieved during treatment for 24 hours with the *P. verruculosum* enzyme complex. The xylan content decreased sharply as a result of the action of xylanases after the first hour of the process to 15.3 % and reached a minimum of 9 % after 24 hours of enzymatic modification.



**Figure 1.** Changes in yield and component composition of biomodified kraft pulp during enzymatic hydrolysis by the *P. verruculosum* complex.

### 2.2 Degree of polymerization of biomodified kraft pulp

After hardwood pulping and several bleaching stages, the degree of polymerization (DP) of kraft pulp used in the experiments was within 1000. As a result of the action of *P. verruculosum* cellulases, a sharp change in DP of biomodified kraft pulp was observed: up to 761 units in the first hour, then up to 400 units in 3 hours and gradually decreasing to 240-250 units by 72 hours, which corresponds to the level of commercial microcrystalline cellulose (MCC) (Figure 2).



**Figure 2.** Changes in the degree of polymerization of biomodified kraft pulp during biocatalysis.

### 2.3 Characteristics of cryogels produced from original and biomodified kraft pulps

The following samples were chosen as test samples for obtaining polysaccharide cryogels: original bleached hardwood pulp (P) and biomodified (BP) with different conversion rates: 5 h (BP1), 24 h (BP2) and 72 h (BP3). The BP1 sample after amorphisation in phosphoric acid, precipitation, freezing and freeze-drying had the highest yield - 70.4 %. Samples of original P, BP2 and BP3 had lower yield of 2.0...6.8 % (Table 1). The lowest cryogel volume is typical for BP2, this may be due to the high shrinkage (70 %) and the lowest porosity (91.6 %). The remaining samples had a volume of about 3.0 cm<sup>3</sup>, a shrinkage rate of 34-40 % and an average porosity of 96 %. All cryogels from original and biomodified kraft pulp had low density of 0.049-0.115.

**Table 1.** Properties of cryogel samples from biomodified kraft pulp with different conversion rates.

Sample	V, cm <sup>3</sup>	Cryogel yield, %	Q max, %	ρ, g/cm <sup>3</sup>	Porosity, %	Δ V, %
P	3.30	64.4	4316	0.049	96.5	40
BP1	2.96	70.4	4201	0.062	95.6	52
BP2	1.48	68.4	3908	0.115	91.6	36
BP3	3.14	63.6	4168	0.051	96.4	38

Figure 3 shows SEM-images of cryogel samples from original bleached and biomodified kraft pulp.

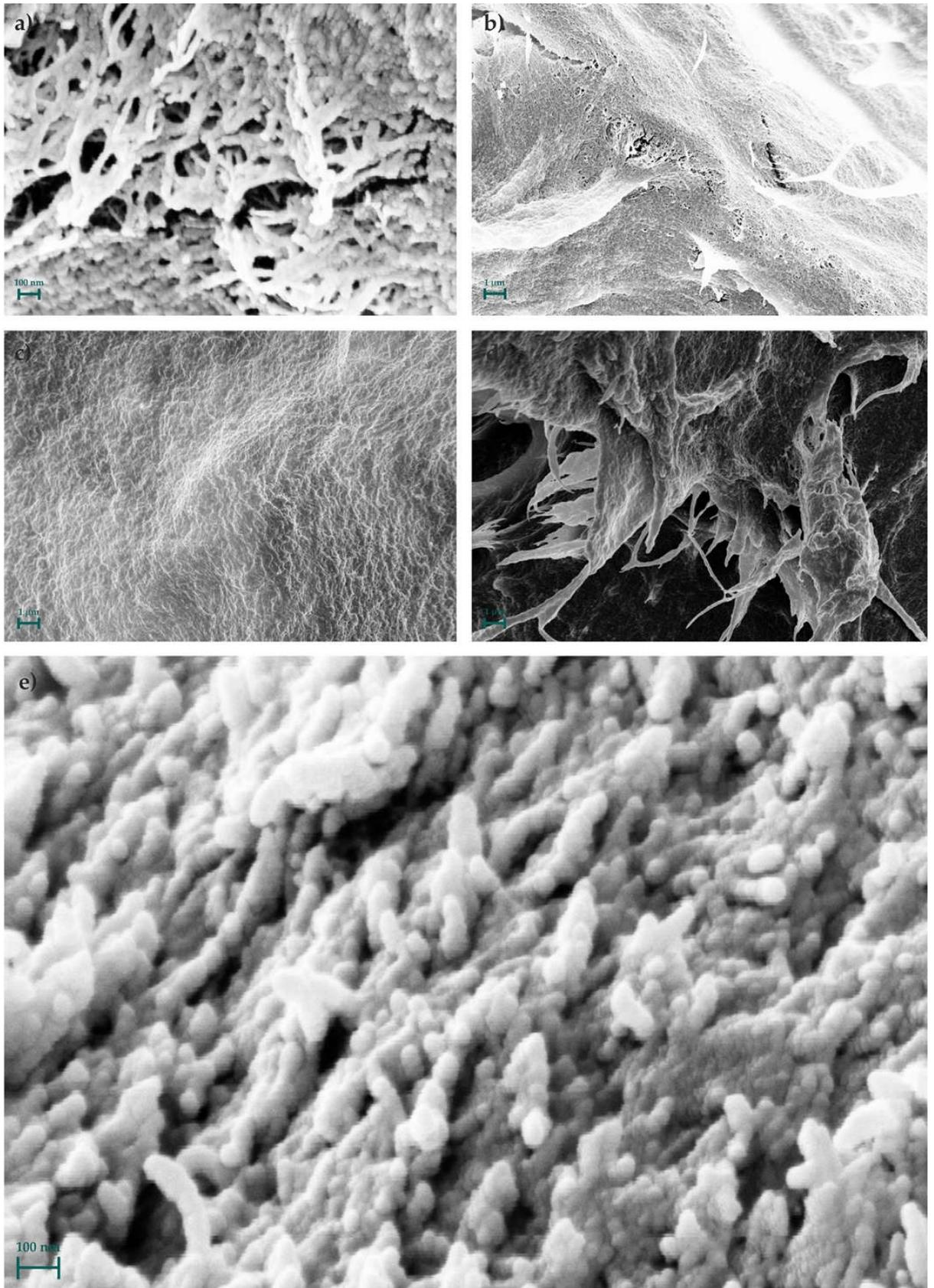


Figure 3. SEM-images of cryogel samples from original and biomodified kraft pulp.

### 3. Discussion

#### 3.1 Composition of biomodified kraft pulp

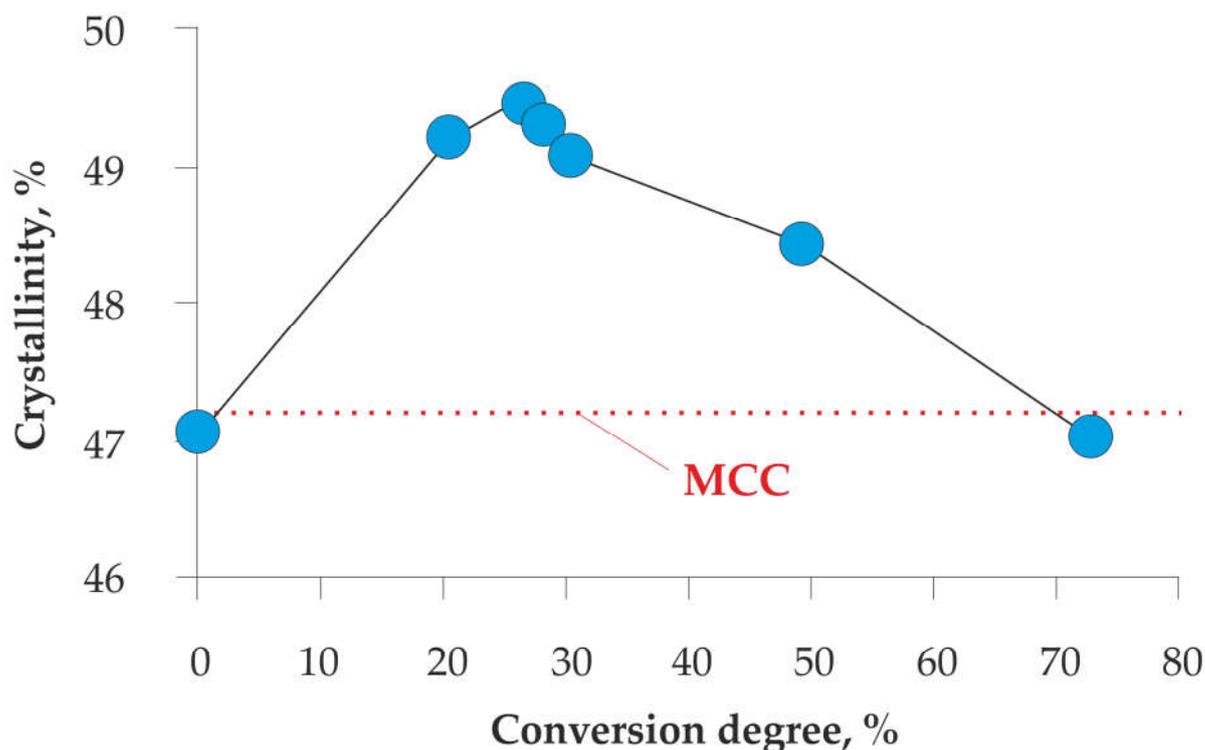
Previous studies on kraft pulp enzymatic hydrolysis with specially designed enzymes focused mostly on determination of monosaccharide composition and content [33-35], as well as cellulose hydrolysis efficiency [36]. A well selected enzyme complex and the absence of inhibitors are crucial for production of glucose-rich hydrolysates [9,33]. In this work we used bleached cellulosic pulps to eliminate hydrolysis limiting factors, such as high lignin content [9,38]. Furthermore, the enzyme complex of *P. verruculosum* has been shown previously to be highly efficient towards kraft pulps [9]. We monitored compositional and structural changes of cellulosic pulp during enzymatic digestion. The substrate contained more than 25 % of hemicelluloses, which were also hydrolyzed by hemicellulases of *P. verruculosum* into mono-, di- and oligosaccharides (Table S1). The pulp composition was changing continuously as hydrolysis proceeded (Fig. 1). The composition at particular time point depended on hydrolysis rates of *P. verruculosum* individual enzymes, accessibility of pulp polysaccharides, as well as synergistic action of cellulases and xylanases. The initial cellulose : xylan ratio of 3.2:1 changed to 5:1 during the first hour, then to 6:1 during the second hour, and by 24 h it reached the maximum value of 9:1. Further hydrolysis up to 72 h resulted in the final ratio of 6.5:1 in biomodified kraft pulp. Hemicelluloses other than xylan were less affected by *P. verruculosum* enzymes, however, with increasing hydrolysis time more mannose, galactose and arabinose was found in the solution (Table S1). Maximum amount of hemicelluloses dissolved after 5...24 h of hydrolysis, and the total amount of non-cellulosic components decreased from 30 to 17...19 %.

Thus, this work has demonstrated for the first time that biomodified kraft pulp of different composition could be obtained together with glucose-rich hydrolysates using enzyme complex of *P. verruculosum*. Resulting biomodified kraft pulp together with hydrolysates becomes essential for lignocellulose biorefinery. Similar technologies have been recommended for implementation at pulp and paper mills for several years [16,39], and implementation results can integrate well as new elements in the forest industry. For instance, 100 tons (dry weight) of bleached kraft pulp after 24 h of hydrolysis with enzyme dosage of 10 FPU/g can give 60 tons of sugars (approximately 40 tons of glucose) and 46 tons of biomodified kraft pulp (cellulose content more than 80 %).

#### 3.2 Structure and properties of biomodified kraft pulp

The degree of polymerization (DP) of commercial cellulosic pulps is essential for their application. Biocatalytic treatment of lignocelluloses with cellyolytic complexes is known to decrease DP depending on substrate type and cooperative action of cellulases and xylanases [40,41]. Kaffle et al. reported a decrease in DP of bleached softwood kraft pulp from 1700 to 250 after 96 h of hydrolysis by *Trichoderma* cellulases [28]. The DP of 250 corresponds to Avicel celluloses intensively applied in various industries. We observed decrease in DP of hardwood kraft pulp during hydrolysis with *P. verruculosum* enzymes. The DP decreased from 1000 to 322 during the first 24 h; further hydrolysis resulted in further smooth decrease of DP up to 240 after 72 h. Thus, to obtain pulp samples with higher DP (1.5...2 times higher than commercial Avicel) hydrolysis should be stopped at no longer than 24 h. However, the further microbiological treatment of hydrolysates, glucose and reducing sugar concentration should be considered as well.

Crystallinity is another important characteristic of lignocelluloses [28,40]. We used a new original method for calculating the crystallinity of kraft pulp and biomodified cellulosic product based on XRD data and NMR calibration [42]. The destruction of cellulose and hemicelluloses of kraft pulp by *P. verruculosum* enzymes led to wave-like changes in pulp crystallinity (Fig.4). At the first stage of hydrolysis up to 30 % conversion crystallinity increased by almost 2.5 % comparing to initial sample. Further conversion to 70 % resulted in decrease in crystallinity to the initial value of bleached kraft pulp. Interestingly, the crystallinity of 5...65 % converted kraft pulp was higher than that for commercial MCC.



**Figure 4.** Changes in crystallinity of biomodified kraft pulp during enzymatic hydrolysis by *P. verrucosum* cellulases.

The observed composition, DP and crystallinity of the pulps together with high sugar concentration of hydrolysates confirmed high catalytic performance of *P. verrucosum* enzymes. After achieving the required quality of hydrolysate one can vary the composition and properties of the new product, biomodified kraft pulp: high purity, DP of 250...500 and crystallinity higher than for commercial MCC.

### 3.3 Application of biomodified kraft pulp as polysaccharide matrix for cryogel formation

All cryogels obtained from kraft pulps before and after enzymatic hydrolysis were stable in water. Stability was measured at room temperature ( $20 \pm 3^\circ\text{C}$ ); the samples were weighted after soaking in water for 24 h. The swelling ratios (SR) are collected in the table 2. The less dense samples (P, BP1, BP3) gave the biggest SR. The dense structure of the BP2 sample hindered efficient water penetration; therefore, BP2 had the smallest SR. Similar values of 10...15 g/g were obtained earlier for cryogels from MCC [32].

Cellulosic gel can shrink in its volume by 40 % during cellulose regeneration [43]. The replacement of a solvent by a non-solvent results in closer arrangement of cellulose macromolecules. The  $\Delta V$  values above 0 corresponded to cellulose swelling after regeneration (Table 1). The sample with the highest density (BP2) had the lowest volume shrinkage (36%). The degree of swelling of hydrogel ( $Q_{\text{max}}$ ) corresponds to the volume shrinkage of cryogel. The more the density of cryogel was, the lower swelling had the corresponding hydrogel (Table 1).

Specific surface area is one of the most important parameters of porous materials [44]. The cryogel with the highest density and the cryogel from the initial kraft pulp had the highest values of the specific surface area (7.94 and 8.97  $\text{m}^2/\text{g}$ , respectively). The results were similar to those obtained previously for cryogels from MCC [45]. Other samples showed specific surface area similar to cryogels from pharmaceutical grade MCC [32].

Mechanical tests showed that the BP3 sample had the lowest strength (Table 2). It corresponded to the lowest DS of biomodified kraft pulp. The BP2 sample showed the highest compressive modulus (E) value. The irregular shape of the sample could contribute to the results, as well as its high volume shrinkage and low porosity and SR. Other

samples had regular cylindrical shape. Therefore, our cryogels demonstrated similar mechanical strength comparing to previously obtained cryogels from MCC (126-3675 κΠα) [32,45].

**Table 2.** The properties of cryogels obtained from biomodified pulp of different conversion level.

№, %, t	DP	Swelling ratio, g/g	E, kPa	Specific Surface
				Area, m <sup>2</sup> /g
P	1003	12,4	1120±508	8,97
BP1	405	13,9	792±157	0,63
BP2	323	5,8	2835±295	7,94
BP3	231	11,4	767±117	0,69

The morphology of the cryogel samples was similar to each other; it was represented by networks, flat (layered) or spherical structures, and clusters of spherical structures (Fig. 3). It was previously reported that regenerated cellulose can form 10-100 nm nanospheres and lamellar structures [46-48]. In our case, the diameter of the spheres was 20–100 nm. A similar morphology was previously observed for gels obtained after supercritical CO<sub>2</sub> drying [49,50] and aerogels obtained by the direct regeneration of dissolved cellulose [51]. This morphology of cellulose cryogels is suitable for biomedical applications, sorption, filtration, and others.

Thus, we investigated the ability of commercial hardwood bleached kraft pulp before and after profound enzymatic hydrolysis to form cryogels. Bleached pulps have suitable purity, and their non-hydrolysed residues are similar to commercial MCC. The cryogel properties and morphology were found to be similar to those observed previously for cryogels from microcrystalline cellulose [32,45]. Thus, biomodified kraft pulps can be promising materials for producing scaffolds in tissue engineering.

## 4. Materials and Methods

### 4.1. Kraft pulp

In the experiments we used samples of bleached hardwood pulp obtained from a mixture of aspen and birch at the Arkhangelsk Pulp and Paper Mill (Arkhangelsk region, Russia). Bleaching was carried out under industrial conditions using chlorine dioxide and sodium hydroxide in several stages. The polysaccharide composition of freeze-dried pulp was determined using acid hydrolysis (72 % H<sub>2</sub>SO<sub>4</sub>, 4 %, 1 h, 121 °C) [52] and further analysis of the monosaccharides formed by high-performance liquid chromatography using the LC-20 system.

Before use the substrate was additionally washed with distilled warm water for three cycles in order to remove residual chemical content and moisture to a humidity of 70...80 %. After that the pulp was refrigerated (+ 4 °C) for 24 hours till homogeneous humidity of all the substrate.

### 4.2. Enzymes

The research used enzyme complex (B1-221-151 and F10) produced by the recombinant strains of *P. verruculosum*. The enzyme complex B1 contained endoglucanases, cellobiohydrolases, and xylanases, whereas the enzyme complex F10 predominantly contained β-glucosidase. A balanced composition, comprising cellobiohydrolases 1 and 2 (40%), β-glucosidase (25%), endoglucanases 1, 2 and 3 (10%) and endoxylanase (2%), determined the effective use of enzyme complex for hydrolysis of industrial samples of cellulose-based raw materials. Approximately 23% of the enzyme complex consisted of ballast proteins [53]. Enzyme activity towards various substrates was determined using common

methods [54]. The enzyme dosage was adjusted so that the total activity was 10 FPU/g of dry pulp, as in previous studies [9,55].

#### 4.3. Enzymatic hydrolysis

Enzymatic hydrolysis involved the use of a Biostat A Plus laboratory bioreactor. The pulp concentration was 10%, the hydrolysis temperature was kept at about 50 °C with constant stirring (200-300 rpm) for 72 h, and the pH was kept at 5.0 with 0.05 M sodium acetate buffer. The mixture was centrifuged at 4200 rpm for 15 min for separating the biomodified kraft pulp and hydrolysate. The supernatant was sampled and analyzed for sugars. Samples were taken every hour during the first 6 hours, as well as after 24, 48 and 72 h.

#### 4.4. Biomodified kraft pulp analysis

After hydrolysis the biomodified kraft pulp was washed thoroughly with distilled water for removing the soluble hydrolysis products. The washing was performed by successive repeated cycles of mixing the biomodified kraft pulp with water and centrifugation at 4200 rpm for 15 min. After each cycle, the supernatant was carefully drained, tested for sugars, and a fresh batch of distilled water was added. This procedure was carried out before zero glucose values. The conversion rate and the biomodified kraft pulp yield were calculated as described in Aksenov et al. [9].

The crystallinity of initial and biomodified kraft pulp was analyzed relative to microcrystalline bacterial cellulose in accordance with [42,56] using XRD-7000S diffractometer (Shimadzu, Japan). For this purpose, samples were taken after 1, 3, 4, 5, 24, and 72 h of hydrolysis.

The viscometric method was used to determine the degree of polymerization, and cadmium ethylenediamine with pulp concentration of 0.1% (GOST 25438-82) was used as a solvent. The average degree of polymerization is determined based on the obtained results of intrinsic viscosity. For this purpose samples were taken in 2-5, 24, 48 and 72 h.

We used the inversion method to study the biomodified kraft pulp composition. The method involved adding H<sub>2</sub>SO<sub>4</sub> to the hydrolysates to a concentration of 4% by weight and incubating them for 20 min at 100 °C. The resulting mixture was cooled and neutralized using 25% NaOH. The concentrations of sugars (glucose, xylose, mannose, arabinose, galactose) and disaccharides (cellobiose, xylobiose) in hydrolysate before and after acidic inversion were determined using an LC-20 Prominence system (Shim-pack ISA-07/S2504 column, Shimadzu, Kyoto, Japan) with post-column derivatization and fluorometric detection. Then, the calculation of the polysaccharide composition of the biomodified kraft pulp was determined for each point by the difference between the initial pulp and the amount of monosaccharides after inversion in a particular enzymatic hydrolysate [52].

#### 4.5. Preparation and characterization of biomodified kraft pulp cryogels

Cryogels based on kraft pulp before and after 5, 24, 72 hours of enzymatic hydrolysis were obtained using phosphoric acid according to the method presented in [32]. For this, solutions containing 5% cellulose were prepared. The dissolution time was 25 hours at a temperature of 22-24 °C. Distilled water was used as a cellulose precipitant from phosphoric acid solutions. The regenerated cellulose was washed by distilled water until neutral with phenolphthalein, frozen at -18 °C, and freeze-dried. Parameters (yield of cryogel, volume variation, density, porosity, swelling and swelling ratio), specific surface area, mechanical characteristics of cryogels, and morphology were evaluated according to the study [32].

Scanning electron microscopy (SEM) of cryogels was used to evaluate the morphology. The images were obtained using a SIGMA VP instrument (Zeiss, Germany) operated at 10 kV accelerated voltage. Prior to SEM cryogel samples were sputter-coated with 5 nm Pt/Pd mixture while using a Q150TES sputtering system (Quorum, Laughton, UK).

## 5. Conclusion

The application of biocatalytic technologies based on conversion of polysaccharides to sugars in the plant raw materials processing requires procedures considering the degree of modification of cellulose-containing matrices preserved as a result of incomplete hydrolysis. Bleached kraft pulp can serve as a commercially available model substrate for hydrolysis by cellulases and hemicellulases. We have shown that the monitoring of the composition and characteristics of the non-hydrolyzed residue can be useful for the biocatalytic performance assessment using the interaction between *P. verruculosum* enzymes and kraft pulp as a case study. Targeted modification of a complex consisting of cellulose, xylan and other hemicelluloses changes the characteristics of kraft pulp for its use both as a finished product and as a raw material for cryogel production. These processes are highly scalable to existing pulp and paper mills and lay the groundwork for the bioeconomy development.

**Supplementary Materials:** Table S1: Chemical composition of peat interstitial water and ice.

**Author Contributions:** Conceptualization, A.R.S. and A.S.A.; methodology, A.M.R., D.G.C., I.V.G. and A.S.A.; resources, E.A.T., V.D.T.; data curation, A.V.M.; writing—original draft preparation, I.V.T., A.R.S. and A.S.A.; writing—review and editing, O.A.S.; visualization, D.G.C. and A.S.A.; cryogel production and characterization I.V.T.; project administration, I.V.T. and A.S.A. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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