BIOPOLYMER COMPOSITES BASED ON GALACTOGLUCOMANNANS (GGMS) AND MICRYSTALINE CHITOSAN (MCCH)

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Abstracts

The article presents a method of extracting galactoglucomannas (GGMs) from softwood (spruce). GGMs were extracted using thermal and enzymatic treatment in an aqueous environment. The extracted GGMs, depending on the extraction method, were characterized by different composition of simple carbohydrates i.e. glucose, galactose and mannose, as well as by the average molecular weight. Evaluation of the composition of GGMs obtained was performed using GC/MS and SEC.

Biopolymer composites were obtained by combining GGMs and microcrystalline chitosan (MCCh), which can be used as preparations for plant protection and growth stimulation. The studies were performed in order to evaluate biological activity of composites based on Petri dish test in which their ability to stimulate seed germination of selected plants was estimated. The effect of plant growth stimulation depended on GGMs composition of simple carbohydrates. GC/MS and SEC chromatographic tests and ^{13}C NMR analysis enabled to establish the composition and structural changes of the obtained GGMs and biocomposites.

Keywords: *spruce, galactoglucomannans, microcrystalline chitosan, thermal treatment, enzymatic treatment, biopolymer composites*

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1. Introduction

At present, there is a tendency to explore new active biomaterials based on natural polymers. These polymers are the most available for human, reproducible sources of raw materials. Their properties, like biodegradability, allow to use them to produce new ecological materials.

Chitin and its derivatives (chitosan) are the best known natural polymers, which can create totally new connections with other natural polymers such as wood hemicellulose.

Galactoglucomannas, belonging to hemicelluloses, are important to numerous branches of industry. Their usefulness is mainly the result of the ratio of galactose, glucose and mannose residues amounts, the average degree of polymerisation, and their ability to deposition on the surface of cellulose materials [1,2,3,4].

At present, synthetic pesticides demonstrate toxicity to people and pose a threat to the environment. The disadvantages of commercial pesticides, which lead to environmental degradation and genetic changes in living organisms, encourage growing interest in natural plant protection (e.g. biopreparations), by the means of plant growth regulators or biostimulators. A new generation of biopreparations are so-called elicitors. The elicitors activate defense mechanisms which induce resistance of plants to a number of diseases caused by fungi and bacteria [5,6]. The natural elicitors are components of cell membranes of phytopathogenic fungi and plants, like pectin, chitin, chitosan and glucan. Chitosan is a polysaccharide of specific biological properties. The biological activity of chitosan depends on its susceptibility to hydrolytic and enzymatic degradation, its impact on the membrane of a living cell, and its natural stimulation of organism's immunity [7, 8, 9,10]. Chitosan has a positive charge, therefore given to the plant from the outside it reacts with negatively charged molecules on the surface of fungal cell and enters into reactions with chemically compatible active sites, causing significant changes in the composition of membrane function [11].

Galactoglucomannans can create new natural chitosan composites. Galactoglucomannans and chitosan have the same characteristics, like biocompatibility and biodegradability, and can be linked to chitosan via hydrogen bonds leads to the formation"biological activity" complex [12, 13, 14, 15].

The aim was to formation of useful composite materials with controlled parameters of the structure. The test compositions were prepared on the base of chitosan and softwood extracted GGMs. The preliminary assessment of biological activity of prepared compositions was based on Petri dish test in which their ability to stimulate seed germination of selected plants was estimated. It has been found that the compositions containing GGMs stimulate germination of plant seeds.

2. Material and Methods

2.1. Materials

Raw material

Spruce (*Picea abies*) sawdust was utilised for the purposes of this research.

Chitosan

Chitosan from Primex ehf. was used in the study. The characteristics of chitosan are: average molar mass (Mv) = 373,0 kDa, deacetylation degree (SD) = 81,0 %, ash content - 0,31%, WRV=156,0%.

Microcrystalline chitosan (MCCh)

For the preparation of biopolymer composites microcrystalline chitosan (MCCh) of the following physico-chemical parameters was used: average molar mass (Mv) = 310,0 kDa, deacetylation degree (SD) = 81,0 %, polymer content – 2,68-3,89 %, WRV=650,0%.

Galactoglucomannans (GGMs)

GGMs extracted from sawdust of spruce wood were used in this study. Two main processing methods were used to extract GGMs: the thermal method and the thermal/enzymatic method involving selected enzymes from groups of cellulases and hemicellulases according to the methodology described in literature [16]. Characteristic of isolated GGMs are showed in Table 1.

Table 1. Characteristic of GGMs isolated from spruce wood shavings after thermal and enzymatic treatment (enzyme Hemicellulase)

Symbol	Average Mw, Da	Carbohydrate content – mass ratio		
		Glucose	Glucose	Glucose
	After thermal treatment			
GGM-T	39 253	1	13	2
	After enzymatic treatment			
GGM-E	113 316	206	1	1

Seeds test

”Lidka” radish seeds, PNOS, Ożarów Mazowiecki, were used to test germination stimulation.

2.2. Preparation of microcrystalline chitosan (MCCh)

Microcrystalline chitosan (MCCh) was prepared by agglomeration from solution, according to continuous method developed in Institute of Biopolymers and Chemical Fibres (IBWCh) [17], with the use of a continuous reactor Dispax Reactor Labor-Pilot 2000/4 by IKA.

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2.3. Preparation of biopolymer composites to plant growth stimulation

In order to prepare biopolymer composites, GGMs were added to a suspension of microcrystalline chitosan in the form of aqueous solutions in a proportion of 10, 15, 20% wt. with respect to chitosan (Table. 2). The mixture was homogenized for 10 to 15 min using a high speed stirrer type T 50, IKA, at 2.000-3.000 r.p.m.

Table 2. The quantitative composition obtained biopolymer composites

Symbol of sample	Quantitative composition
MCCh + 10% GGM-T	Microcrystaline chitosan – 90% wt. Galactoglucomannnans – 10% wt.
MCCh + 15% GGM-T	Microcrystaline chitosan – 85% wt. Galaktoglukomannany – 15% wt.
MCCh + 20% GGM-T	Microcrystaline chitosan – 80% wt. Galactoglucomannnans – 20% wt.

2.4. Study of radish seeds germination

Testing of biological activity of prepared compositions was based on Petri dish test in which their ability to stimulate seed germination of selected plants was estimated. Filter paper disks and 20 seeds were placed on 9 cm diameter Petri dishes, then 10 ml of aqueous solution of tested biopolymers was added at concentrations: 0.1%, 0.01% and 0.005%. The effect of preparations on germination of radish seeds was evaluated after 72 hours by the number of germinated seeds, sprouts green mass and length, in comparison to the control test (water, pH = 7.00). Evaluated preparations were: microcrystalline chitosan, galactoglucomannans and MCCh/GGM biocomposites.

2.5. Determination of average molecular weight of chitosan

Determination of average molecular weight of chitosan was made according to the viscosimetric method according to SPR/BPB/5 IBWCh procedure [18].

2.6. Determination of chitosan deacetylation degree

Determination of the chitosan deacetylation degree (SD) was made at the UV spectrum by the first derivative method according to SPR/BLF/21 IWBCh procedure[19].

2.7. Determination of ash content

The ash content of chitosan was determined by weight, based on the residue after ashing the sample at 800°C according to SPR/BLF/6 IBWCh procedure [20].

2.8. Determination of chitosan content in the preparation

The polymer content of microcrystalline chitosan was determined according to SPR/BPB/11 IBWCh procedure [21].

2.9. Determination of the water retention value (WRV)

The water retention value of chitosan was determined gravimetrically according to SPR/BPB/14 IBWCh procedure[22].

2.10. Determination of qualitative and quantitative composition of GGMs – GC/MS chromatography

The quantitative and qualitative composition of the obtained GGMs was tested using GC/MS chromatography, according to the method developed by Pszonka and Stupińska [23]. Hewlett Packard 5890, II/5972 series GC-MS equipment was utilised. A full carbohydrate separation was performed on a capillary column DB-5 (60 m x 0,25 mm x 0.25 μm) in the following conditions of device operation: gas chromatography device: T_{dos}-275°C, T_{det}-280°C $T_{furnace}$= 45(1 min) 20°C/min to 170°C (2°/min) to 230°C (20 min). Carrier gas flow: He=0,9 cm^3/min (in T_p=45°C). A quadruple mass spectrometer in SCAN (qualitative assaying) and SIM (quantitative assaying) mode was used to identify and determine the amount of separated analytes. Quantitative assaying was performed using the internal standard method. Myo-inositol was used as the internal standard. The method's detection threshold is 5 μg/g for each simple carbohydrate, and the standard relative deviation is up to +/- 10%.

2.11. Determination of molecular weight – Size Exclusion Chromatography (SEC)

The GGMs molecular weight was determined by Size Exclusion Chromatography using Agilent chromatography device with triple detection: Refractive Index (RI), Right Angle Light Scattering (RALS) and Viscometer (DP), equipped with three TSK GEL columns. A water/NaN_3 system was used as the solvent.

2.12. Determination of structural changes of obtained GGMs and MCCh/GGMs biocomposites – Nuclear Magnetic Resonance ^{13}C NMR

Evaluation of structural changes in the obtained GGMs and MCCh/GGMs composites was performed by ^{13}C NMR. The analysis was performed on a Bruker Avance III 400 MHz Spectrometer. The spectra for ^{13}C nuclei were obtained at 100.61 MHz frequency in a MAS BB DVT wide-band probe.

3. Results and discussion

3.1. Evaluation of biological activity used GGMs and composites MCCh/GGMs

Today most of synthetic plant protection agents available on the market have significant toxicity, which poses a threat to the environment and people,

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and these preparations do not have the ability to simultaneously combat a number of fungal and bacterial strains.

The use of synthetic pesticides leads in short time to resistance against pathogens, and reported defects of commercial pesticides induce growing interest in natural plant protection products (e.g. bioformulations), eligible as plant growth regulators or biostimulators [6, 7] [6,7]. Therefore, one of the goals of this project was to develop and produce plant protection preparation, ecological and safe for humans and environment. The preparation is based on properly selected bioactive polymers from the polysaccharide group, which will be used not only to treat the plants by spraying, but also for the so-called "activation of the soil".

The project aim was to evaluate the biological activity of GGMs and MCCh/GGMs composites (based on Petri dish test). The effectiveness of tested preparations was determined on the basis of the quantity of germinated seeds and the sprout green mass and length, in relation to controls consisting of water at pH = 7.0. Structural changes in GGMs and MCCh/GGMs composites were also evaluated.

GGMs (characteristics showed in Tab.1.) and MCCh/GGMs composites produced with the participation of microcrystalline chitosan and selected galactoglucomannans (GGMs) isolated from spruce wood sawdust after thermal treatment (sample GGM-T) and after enzymatic treatment with Hemicellulase commercial enzyme (sample GGM-E) have a significant effect on the germination of radish seeds, as shown in Tab. 3.

Galactoglucomannans preferentially bind to the chitosan via hydrogen bonds and via the amino group of chitosan and the carboxyl group of GGMs. The structural changes of MCCh/GGMs composites were examined using ^{13}C NMR chromatography (Fig. 1-4). In the NMR spectrum (sample GGM-T, Fig. 1), there are visible peaks mainly deriving from carbon C1 at 104.29 ppm and C4 at 81.47 ppm, as well as a sharp peak from carbon C3 at 71.34 ppm and C5 at 74.8 ppm. In the case of sample GGM-E (Fig. 2), additionally appeared peaks at 26.34 and 176.49 ppm. These peaks are characteristic to the methyl group CH_3 and carbonyl group C=O. These spectra show structural differences between GGMs obtained after thermal and enzymatic treatments.

In studied MCCh/GGMs composites structural changes in the ^{13}C NMR spectrum were observed (Fig. 3, 4). Overlapping peaks were visible at 63 ppm (carbon C6) and 57 ppm (C2), showing how MCCh and GGMs connect via the amino group at the C2 carbon. The difficulty in the interpretation of the results is associated with overlapping peaks of carbons C6 and C2.

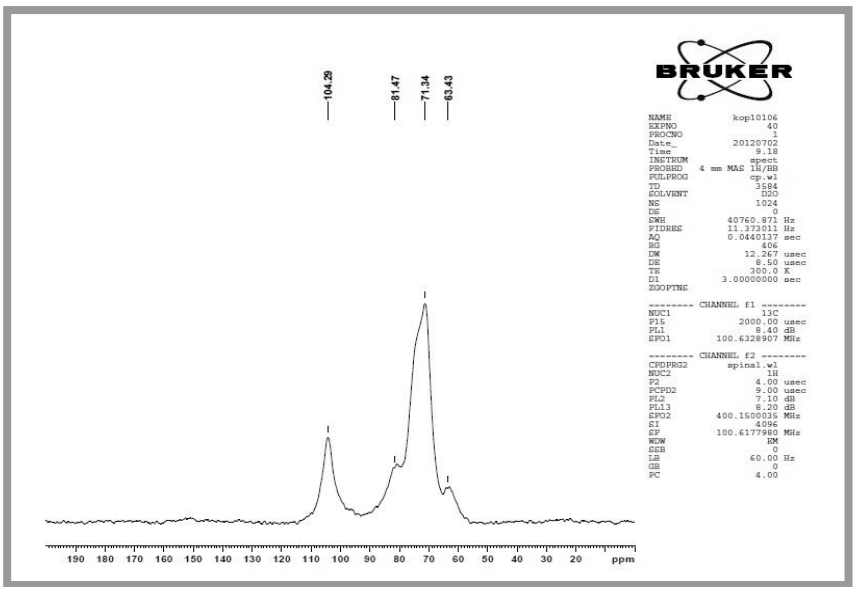

Figure 1. Spectrum of ^{13}C NMR – GGMs after thermal treatment (sample GGM-T)

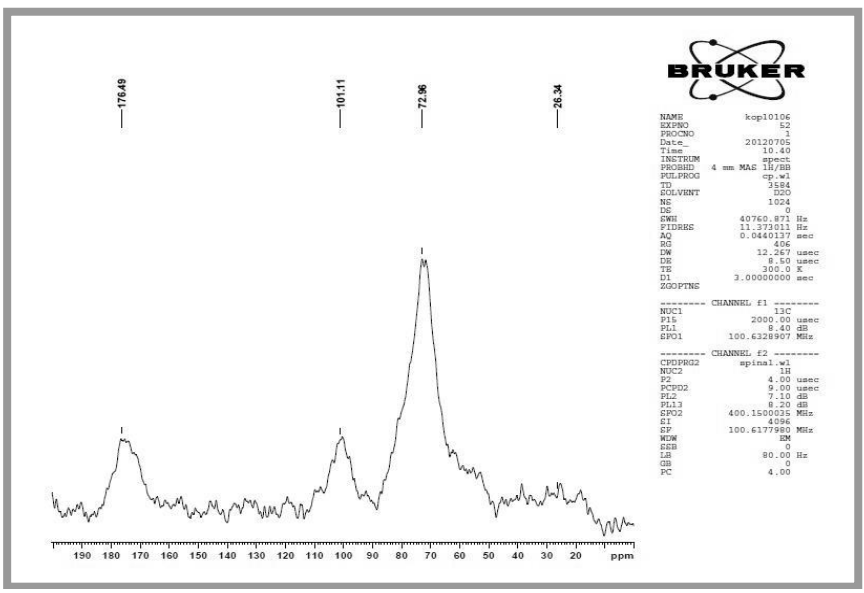

Figure 2. Spectrum of ^{13}C NMR – GGMs after enzymatic treatment (sample GGM-T)

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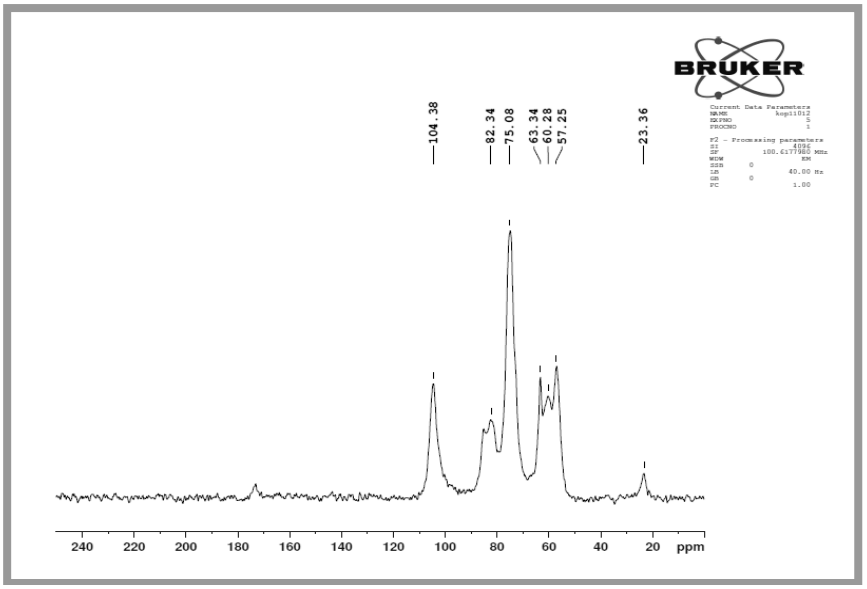

Figure 3. Spectrum of ^{13}C NMR – MCCh/GGMs composite (sample MCCh + 10% GGM-T)

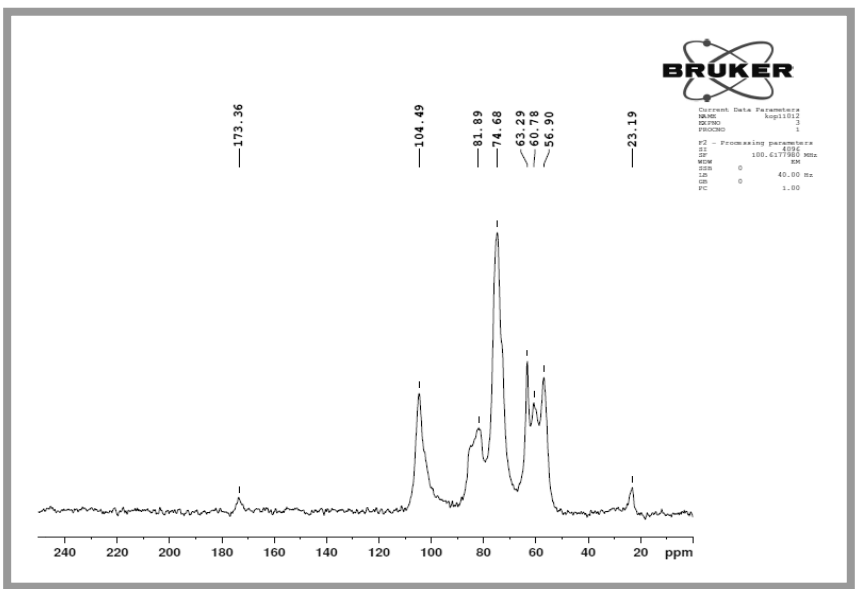

Figure 4. Spectrum of ^{13}C NMR – (sample MCCh + 15% GGM-E)

Table 3. Influence of MCCh/GGMs composites on radish seed germination rate

Symbol of sample	Conc. of biocomposite %	pH	Amount of sprouts		Weight of sprouts		Length of sprouts cm	
Control			The average of two reps	% relative to controls	The average of two reps	% relative to controls	The average of two reps	% relative to controls
	-	7.0	19.5	100.0	1.1280	100.0	5.41	100.0
MCCh/HQG	0.1	7.44	19.5	100.0	1.0823	95.9	4.76	88.0
	0.01	7.28	20.0	102.6	1.1239	99.6	5.93	109.5
	0.005	7.21	19.5	100.0	1.1241	99.7	5.45	100.6
Control	-	7.00	17.0	100.0	0.6989	100.0	3.33	100.0
GGM-E	0.1	6.69	15.5	91.2	0.8499	121.6	3.47	104.1
	0.01	6.64	18.5	108.8	0.9172	131.2	3.12	93.7
	0.005	6.73	18.0	105.9	0.9911	141.8	4.20	126.0
GGM-T	0.1	6.74	16.5	97.1	0.8622	123.4	4.86	145.9
	0.01	6.77	18.5	108.8	1.0687	152.9	3.51	105.4
	0.005	6.82	16.5	97.1	0.9456	135.3	4.75	142.6
Control	-	7.00	17.5	100.0	0.8027	100.0	3.46	100.0
MCCh + 10% GGM-T	0.1	6.93	18.5	105.7	0.8842	110.1	4.70	135.7
	0.01	6.99	18.0	102.9	1.0254	127.7	4.18	120.8
	0.005	6.93	18.0	102.9	0.9232	115.0	3.92	113.2
MCCh + 15% GGM-T	0.1	6.93	19.0	108.6	0.9105	113.4	3.24	93.5
	0.01	6.99	19.0	108.6	0.9397	117.1	4.46	128.9
	0.005	6.93	15.5	88.6	0.8279	103.1	4.44	128.2
MCCh + 20% GGM-T	0.1	6.87	18.0	102.9	0.9171	114.2	3.75	108.2
	0.01	6.92	18.5	105.7	0.8602	107.2	3.48	100.6
	0.005	6.91	18.5	105.7	0.9579	119.3	4.01	115.8
Control	-	7.00	18.5	100.0	0.8234	100.0	3.11	100.0
MCCh + 10% GGM-E	0.1	7.09	18.5	100.0	0.8747	106.2	2.75	88.6
	0.01	7.09	16.5	89.2	0.7912	96.1	3.29	106.0
	0.005	7.13	17.5	94.6	1.0048	122.0	3.50	112.7
MCCh + 15% GGM-E	0.1	7.17	19.5	105.4	1.1527	140.0	3.71	119.5
	0.01	7.17	16.5	89.2	1.0740	130.4	3.68	118.4
	0.005	7.19	19.0	102.7	1.0733	130.4	3.75	120.8
MCCh + 20% GGM-E	0.1	7.12	16.5	89.2	0.8095	98.3	3.44	110.6
	0.01	7.13	16.0	86.5	0.9297	112.9	4.08	131.2
	0.005	7.16	16.5	89.2	0.9775	118.7	4.33	139.5

The results show that the applied galactoglucomannans (samples GGM-E and GGM-T) stimulate germination of radish seeds contributing to an increase in sprout weight and a bit weaker increase in sprout length (Table 3). The GGMs in the concentrations of 0.005 - 0.1% resulted in an increase in spouts green mass weight of about 23,0- 52.9% and an increase in sprouts length of about 4.1 - 45.9%, comparing to the control. It should be noted, however, that the most beneficial effect on the plant growth stimulation was obtained by GGMs after the heat treatment (sample GGM-T).

The addition of microcrystalline chitosan had no significant influence on the growth of sprouts when compared to GGMs (Table 3).

Presented results (Table 3) show that the addition of microcrystalline chitosan to GGMs cause the growth of plants. It depends on the dilution of

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composite as well as the method of isolating GGMs from spruce sawdust and the percentage share of GGMs in the MCCh/GGMs composite.

The greatest effect inducing the growth of sprouts was presented by biocomposites prepared with addition of 0.01% and 0.005% MCCh and 15% GGM-T (based on the dry weight of chitosan). On the other hand, the greatest stimulating effect of radish green mass growth had MCCh/GGM-E composite in an amount of 15% by weight. (Tab. 3, Fig. 5).

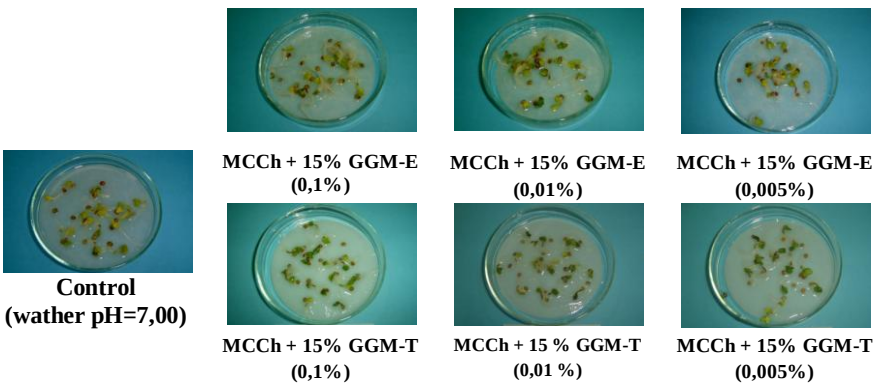

Figure 5. Influence of selected chitosan compositions with GGMs obtained by thermal treatment of spruce – sample (MCCh+15% GGM/T), and GGMs obtained by enzymatic treatment of spruce – sample (MCCh+15% GGM/E) on radish germination rate

The presented GGMs differ in quantitative composition of monosaccharides. In the case of GGMs obtained by thermal treatment dominant monosaccharide was galactose, while in the case of GGMs prepared by the enzymatic treatment – glucose. It may be concluded that the decisive component of MCCh/GGM biocomposite stimulating the growth of plants is GGM. Moreover, the effect of the stimulation of plants (increase in length and weight) depends on monosaccharide content of GGMs.

4. Conclusions

As a result of thermal and enzymatic treatment of spruce sawdust, galactoglucomannans of varied compositions of monosaccharides and molecular mass were obtained.

The research confirmed the usefulness of GGMs for the production of biocomposites with MCCh intended for the formation of preparations to stimulate plant growth.

5. List of abbreviations:

GGM – galactoglucomannans

MCCh – microcrystalline chitosan
GGM-T – galactoglucomannans after thermal treatment
GGM-E – galactoglucomannans after enzymatic treatment

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