

# Optimization of Cultivation Conditions for *Azotobacter vinelandii* D-08, Producer of the Polysaccharide Levan, for Obtaining Biocomposite Materials

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A prospective binder composed of a microbial polysaccharide levan present in the culture fluid was obtained. The synthesis of levan was carried out by an *Azotobacter vinelandii* bacteria strain using molasses, distillery stillage, and milk whey as the nutrient medium. The maximum amount of levan produced in these experiments was 14.5 g/L. Composite materials were obtained based on wood waste and biological binder. Depending on the pressing behaviour, materials were obtained within a density range of 1083 to 1443 kg/m<sup>3</sup> and a tensile strength of 7.2 to 32.4 MPa. Water absorption and thickness swelling were 7.2% and 14.9%, respectively. During hot pressing, the resulting materials changed in their attenuated total reflection-frustrated total reflection (ATR-FTR) spectra at frequencies of 930, 1000, and 1750 cm<sup>-1</sup>, indicating the occurrence of chemical and structural changes in individual components of the lignocellulosic raw materials and changes in the composition of biological binding agent. Analysis of the physico-mechanical properties and other results of the composite materials using scanning electron microscopy (SEM) and X-ray microtomography suggested that composite materials based on the microbial polysaccharide levan-containing binder are advanced, new, and eco-friendly substances.

*Keywords:* *Azotobacter vinelandii* D – 08; Levan; Polysaccharide binders; Particleboard; Biocomposite material

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## INTRODUCTION

Significant progress has been made recently in the development of the industry dealing with production of microbial extracellular polysaccharides (exopolysaccharides, EPS) that possess new and unique industrial properties (Nicolaus *et al.* 2010; Freitas *et al.* 2011), in particular, Levan is a biopolymer with enormous potential used in various industries (Donot *et al.* 2012; Oner *et al.* 2016). However, compared to synthetic polymers, biopolymers of natural origin still represent a small fraction of the current polymer market, mostly due to expensive manufacturing processes. Therefore, many efforts have been devoted to the development of efficient and environmentally friendly manufacturing processes, searching for cheaper ways to obtain them.

Levan (fructan) – a fructan polysaccharide with remains of fructose in its structure – is a neutral branched polysaccharide constructed from the remnants of D-fructofuranose, which are connected by  $\beta$ -2  $\rightarrow$  6 bonds to the main chain, and by  $\alpha$ -2  $\rightarrow$

1 in branches. The molecular weight, depending on the producer and cultivation conditions, ranges from 5 to 1000 kDa (Dos Santos *et al.* 2013; Abou-Taleb *et al.* 2015).

Levan is formed due to the presence of the bacterial extracellular enzyme levansucrose, which converts sucrose to  $\beta$ -2,6-bonded fructooligosaccharides with different chain length and high molecular weight (Yanase *et al.* 2002; Abdel-Fattash *et al.* 2005).

Polyfructosans are formed by many bacteria, if the medium contains sucrose. The process of levans formation can be represented by the reaction:  $n \text{ Sucrose} = \text{Levan} + n \text{ Glucose}$ . The reaction is catalyzed by extracellular levansucrose. On media containing sucrose, such formation of levan with participation of exoenzyme is visible due to the appearance of small drops of levan near colonies (Schlegel 1989).

The technology used in the production of chipboards is one of the most dynamically evolving sectors of the wood industry (Thoemen *et al.* 2010; Burton *et al.* 2011). Today, the dominant materials on the market are wood fibre materials (chipboards, particleboards, MDF, OSB) containing a binder with various highly toxic resins. These materials emit phenol and formaldehyde into the environment.

Different methods are currently used to reduce the toxicity level of these materials (Haag *et al.* 2004; Pan *et al.* 2006; Kadimaliev *et al.* 2012), but they do not fundamentally solve the problem. Therefore, an intensive search is underway for new natural compounds that can replace resins with a biological binder.

Prospective binders include polysaccharides of microbial origin (Smith and Callow 2006; Revin and Vedyashkina 2009; Revin *et al.* 2010; Shutova *et al.* 2010; Revin and Novokuptsev 2014; Revin *et al.* 2016a; Revin *et al.* 2016b), such as the exopolysaccharide levan. Levan has high adhesion properties and can be used to glue woodparticles. Despite the high branching and force of cohesion, exopolysaccharide levan has a large number of hydroxyl groups, which helps it to adhere (to form bonds) with various substrates. Safe for humans and environment, levan is a so-called "green" and environment friendly adhesive to be used for special purposes. Levan has also been used to produce eco-friendly glue (Combie 2003; Combie *et al.* 2004; Kang *et al.* 2009). Due to the high tensile strength of levan when gluing aluminium and the excellent shear strength of levan in binding some plastics, compared with many other synthetic glues, levan is one of the most technically appropriate polysaccharides for use as an eco-friendly glue (Combie and Yavorsky 2005).

Currently, the introduction of new classes of compounds of biological origin is deterred by their cost; furthermore, materials made from these compounds fail to comply with the physical-mechanical requirements of existing standards. Therefore, the aim of this study was to optimize the culture conditions of the *Azotobacter vinelandii* D-08 strain in order to increase the biosynthesis of levan, which is used as a biological binder in biocomposite materials. With this goal in mind, the following goals were set: 1) identify the optimal conditions for synthesis of polysaccharide levan using recycled industry waste; 2) produce biocomposite materials using a levan-containing binder as the culture fluid; and 3) study the physical and mechanical properties of the binder itself and obtained materials.

## EXPERIMENTAL

### Materials

#### *Preparation of nutrient media*

The bacteria *Azotobacter vinelandii* strain D-08 was cultivated in test tubes at 28 °C using an agar medium composed of the following in g/L: KH<sub>2</sub>PO<sub>4</sub>, 0.2; K<sub>2</sub>HPO<sub>4</sub>, 0.8; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.2; CaSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1; FeCl<sub>3</sub>, 0.005; Na<sub>2</sub>MnO<sub>4</sub>, 0.005; yeast extract, 0.5; sucrose, 20.0; and agar-agar, 15.0 to 20.0. The inoculum was obtained in a sucrose-based medium with the following composition in g/L: KH<sub>2</sub>PO<sub>4</sub>, 0.2; K<sub>2</sub>HPO<sub>4</sub>, 0.8; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.2; CaSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1; FeCl<sub>3</sub>, 0.01; Na<sub>2</sub>MnO<sub>4</sub>, 0.01; yeast extract, 0.5; and sucrose, 20.0. The media was sterilized at 120 °C for 20 min, and the pH range was 6.8 to 7.2 (Schlegel 1989).

#### *Cultivation conditions for Azotobacter vinelandii D-08*

The cultivation of bacteria in liquid medium was carried out using 250-mL conical flasks containing 100 mL of medium. The starting inoculum was the slant culture agar medium, which was washed off using 10 mL of the prepared medium. Flasks with 100 mL of medium were seeded *via* the suspension of microorganisms in 10 mL of inoculum. The cultivation of *A. vinelandii* was performed using a temperature controlled shaker (Inkubator ES-20/60, "BioSan", Riga, Latvia) for 24 h at 250 rpm and 28 °C.

The resulting inoculum was seeded in a nutrient medium containing food industry wastes including molasses, distillery dregs, and milk whey in a 3:2:5, 5:2:3, 3:5:2, or 3:3:3 ratio; 10% of the seed material was then added. Direct seeding was carried out from a slant (slant culture on agar medium) onto a medium consisting of molasses, distillery dregs, and milk whey wastes in a ratio of 3:2:5, which was washed off using 10 mL of prepared-on-waste medium. Static cultures of *A. vinelandii* D-08 strain were obtained using a thermostatic oven at 28 °C for 72 h.

### Methods

#### *Determination of physical and mechanical properties of the obtained culture fluid containing levan*

The viscosity of the culture fluid was measured with a Viscotester VT-04F viscometer (Rion, Tokyo, Japan). Measurement of the pH of the *A. vinelandii* culture fluid was performed using the potentiometric method with an HI 98129Combo pH metre (Hanna Instruments, Sande, Germany). The content was determined *via* the precipitation of the native levan polysaccharide from the culture medium using 96% ethanol in a 1:2 ratio, and then drying the precipitate at 105 ± 2.5 °C to a constant weight (Sutherland 1990). Biomass was determined using the weight method. This method consists of three sequential steps: bringing the filtered mass to a constant value, separating the microbial cells from the culture fluid, and determination of their mass. To bring the filtered mass to a consistent value, the filtrate was put in an open Petri dish and then oven-dried for 1 h at 105 °C. The cells were separated from the medium by centrifugation (CL-16, Polikom, Moscow, Russia). A total of 5 mL from a stirred culture was centrifuged for 45 min at 7000 rpm (5000 × g). The supernatant was decanted, and the precipitate was washed with distilled water. The sucrose content was determined using an AP-300A automatic polarimeter (Atago Co., Ltd., Tokyo, Japan). To determine the elemental composition (C, H, N, S) of the precipitated exopolysaccharide obtained after bacterial culture, CHNOS was performed using a Vario MICRO elemental analyser (Elementar, Hanau, Germany).

The protein content was determined using the biuret and Bradford methods (Bradford 1976; Dawson *et al.* 1991; Janairo *et al.* 2011). The infrared spectra of the culture liquid were collected using a Shimadzu IR Prestige-21 Fourier infrared spectrophotometer (Tokyo, Japan).

#### *Conditions for obtaining biocomposite materials*

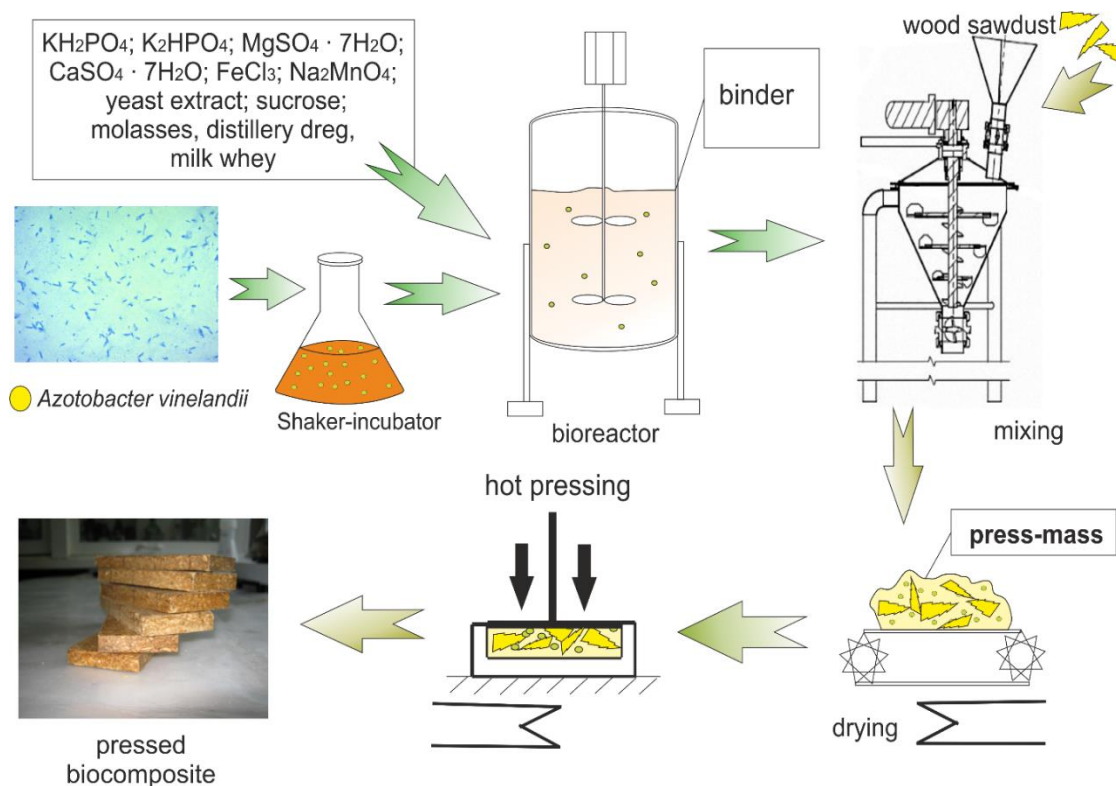
A total of 120 mL of culture fluid with a viscosity value of 0.41 to 0.44 dPa·s was added to 100 g of pine chips (*Pinus sylvestris*), with sizes of 4 to 7 × 1 to 2 × 0.5 to 1.5 mm. To ensure uniform wetting, the sawdust was thoroughly mixed. The resulting press-mass was treated *via* hot-pressing on a GT-7014-H hydraulic press mould (Gotech Testing Machines Inc., USA) at a pressure equal to 26.1MPa and 39.2 MPa for 10 minutes at temperatures of 100, 120, and 140 °C.

The general scheme for obtaining the biocomposite material is presented in Fig. 1.

#### *Determination of physical and mechanical properties and structure of biocomposites*

The principle objective of the second portion of this study was to obtain lignocellulosic materials where the main binder was present using culture fluid containing levan. Biocomposite materials were obtained *via* hot pressing in a GT-7014-H hydraulic molding press with a cooling system. The densities of the biocomposites were determined using an automatic high resolution H-300S densitometer (Hildebrand, Oberboihingen, Germany) that was used for calculating the specific weight with an extremely high resolution of 0.001 g/cm<sup>3</sup>. The tensile strength was determined using a GT-AI-7000M device (Gotech Testing Machines Inc.) in accordance with State Standards 28840-90 (2004) and 10635-88 (2006). The determination of water absorption and thickness swelling was carried out in accordance with State Standard 10634-88 (1991). The structure of the obtained materials was studied using a Skyscan 1172 X-ray microtomograph (Bruker, Brussels, Belgium). The infrared spectra of the biocomposite materials was examined using a Shimadzu IR Prestige-21 Fourier infrared spectrophotometer.

Physical and mechanical properties of obtained bio-composite materials were compared with their corresponding values from the standards for particleboards (EN 312 (2003) and State Standard 10632 (2007)).



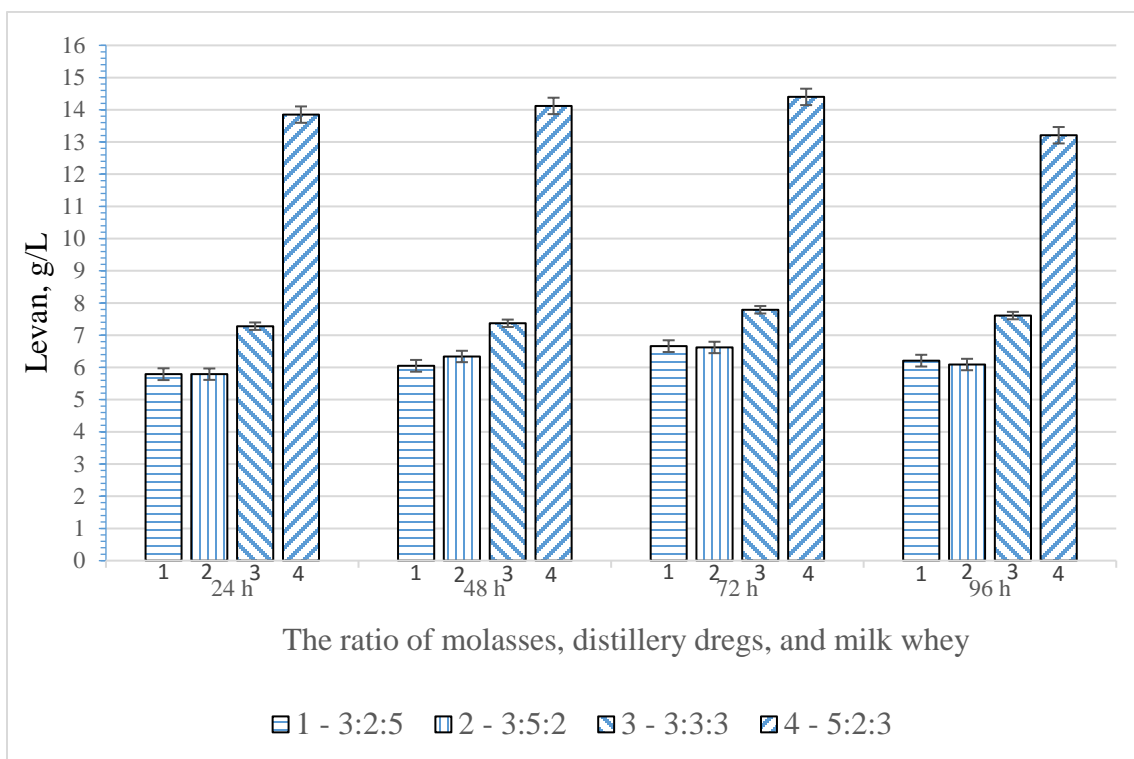
**Fig. 1.** Scheme for obtaining composites from wood particles and levan-containing culture fluid

### Scanning electron microscopy

To determine and analyze the structure of biocomposite materials using a scanning electron microscope Hitachi Tabletop SEM TM, 3000 (Hitachi High - Technology Corp., Tokyo, Japan) with an increase in only 800. For photos of a scanning electron microscope dimensions of the samples were  $20 \times 20 \times 10$  mm. All samples were treated with ethanol to remove grease on the entire surface of the samples in order to obtain the highest quality images.

## RESULTS AND DISCUSSION

To produce composite materials with adhesive properties, it is important to have the precise amount of binder required for the largest possible interaction between the functional groups of components of the raw wood. Therefore, there was an initial concern over the optimization of the synthesis conditions of the levan polysaccharide from the molasses, distillery dregs, and milk whey industry wastes. Accumulation of levan was observed at 96 h and 28 °C. The experiments covered four variations that differed from each other in the proportion of molasses, distillery dregs, and milk whey present in the culture medium (Fig. 2).

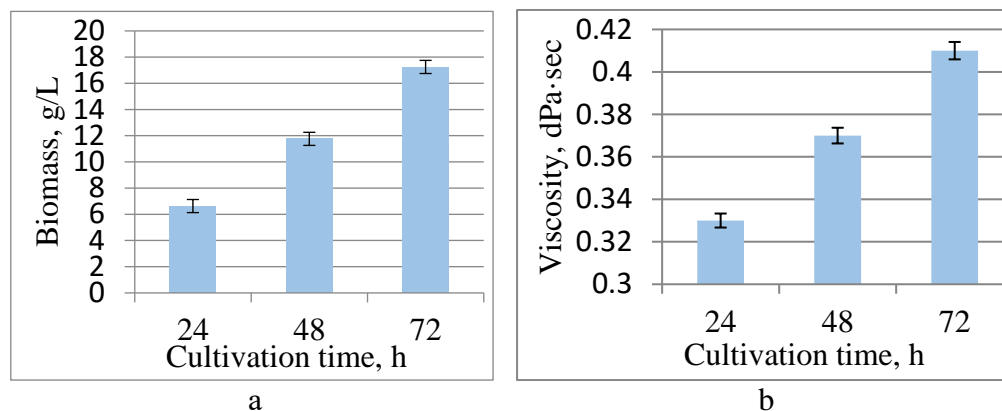


**Fig. 2.** The dynamics of levan accumulation by *Azotobacter vinelandii* strain D - 08 in a liquid culture medium consisting of molasses, distillery dregs, and milk whey ( $V = 250$  rpm,  $T = 28$  °C)

As shown in Fig. 2, the highest accumulation of levan occurred when using molasses, distillery dregs, and milk whey in a ratio of 5:2:3. In this case, the content of levan reached almost 14 g/L after 24 h of cultivation. Increases in the duration of cultivation to either 48 or 72 h had no effect on the content of levan. However, at longer times, there was a slight decrease in the amount of levan in all versions of the experiments. The maximum accumulation of levan, which occurred when using molasses (where it accounted for 50% of the total nutrient medium), may be explained by the necessity for high levels of sucrose during polysaccharide synthesis. Evidence that sucrose is one of the most important components in the culture medium, ensuring a high yield of levan (up to 14.50 g/L), is provided by data gathered using a polarimetric analysis of the culture medium. Carbohydrate content was reduced by 45% within 24 h of culturing. Evidence of active sucrose consumption for the synthesis of levan can also be found in the literature (Shih *et al.* 2005; Dos Santos *et al.* 2013).

The maximum accumulation of levan took place in the medium where molasses content was half of the nutrient medium. Therefore, in later experiments a 5:2:3 proportion of molasses, distillery dregs, and milk whey (by weight) was used.

The physical and chemical properties of the culture liquid used as a binder depend not only on the polysaccharide content, but also on the biomass amount, pH, and viscosity of the medium. Culturing *Azotobacter vinelandii* D-08 for 24 h resulted in an increase in biomass up to 6.63 g/L. When the duration of growth was increased to 48 and 72 h, there were additional increases in biomass to 11.76 g/L and 17.25 g/L, respectively (Fig. 3a).



**Fig. 3.** The accumulation of biomass (a) and viscosity changes (b) in the liquid culture obtained by culturing *A. vinelandii* in dynamic conditions at 250 rpm and 28 °C

Regarding the accumulation of levan and biomass growth, while the polysaccharide biosynthesis was mostly complete after 24 h of cultivation, biomass growth continued up to 72 h. It can be assumed that the cultivation of the *Azotobacter vinelandii* D-08 strain increased the shortage of the main nutritive (sucrose), which resulted in a reduced rate of levan synthesis, while biomass growth continued at the expense of other nutrients present in the culture medium (*i.e.*, molasses, distillery dregs, and milk whey).

Another important property of the culture medium is the viscosity of the binder. The dynamic viscosity increased from 0.33 (24 h of incubation) to 0.41 dPa·sec (72 h of incubation) (Fig. 3b). The increase in the viscosity of the medium after 72 h of incubation at the termination of levan synthesis indicated that viscosity is not only modified by levan, but also by the biomass component of the culture. In addition, the proteins present in the nutrient medium and culture fluid (Sutherland 2007) formed during the biosynthesis of the polysaccharide may have also modified the viscosity of the medium. The fact that the culture fluid contained proteins apart from polysaccharides was demonstrated using an elemental analysis of the data (Table 1). In particular, the presence of nitrogen and sulphur atoms unambiguously indicated the presence of proteins in the sample fluid.

**Table 1.** Elemental Analysis of Synthesized Levan

No	Name	C%	H%	N%	S%
1	Levan	40.82	6.513	0.35	0.093
2		40.57	6.432	0.36	0.159
3		40.12	6.335	0.32	0.073
Mean Value		40.51	6.426	0.34	0.108
Deviation, abs.		0.35	0.089	0.02	0.045

The viscosity of the medium, as previously emphasized, depends mostly on the polysaccharides content and other bioorganic components in the liquid medium (Sutherland 1990). However, the pH of the medium also affects the viscometric properties of biopolymer solutions. Therefore, in the next series of experiments, changes in pH during the cultivation of *A. vinelandii* D-08 were studied.

The pH was 7.0 at the initial stage of cultivation. During biosynthesis and biomass growth, gradual acidification of the environment occurred and reached a pH of 6.08 after

72 h. Thus, medium acidification during cultivation of the *A. vinelandii* D-08 strain may cause the formation of additional functional groups capable of participating in adhesion processes (Loginov 2011).

The resulting culture fluid, which consisted of the levan polysaccharide, bacterial cells, and other metabolites, was used as a biological binder to obtain biocomposite materials (Table 2).

**Table 2.** Effects of Compression and Temperature on Density and Tensile Strength (Pressing Time 10 Min)

Compression Pressure (MPa)	Compression Temperature (°C)	Density (kg/m <sup>3</sup> )		Tensile Strength (MPa)	
		No binder (Control)	With binder	No binder (Control)	With binder
26.1	100	1058.9 ± 3.8	1083.9 ± 8.2	5.0 ± 0.4	7.2 ± 0.3
	120	1151.4 ± 1.2	1215.1 ± 3.8	10.0 ± 0.1	11.2 ± 0.1
	140	1257.4 ± 2.7	1303.8 ± 25.9	16.8 ± 0.0	21.2 ± 0.5
39.2	100	1270.1 ± 0.4	1352.2 ± 18.3	16.8 ± 0.0	22.5 ± 0.5
	120	1286.3 ± 4.6	1404.9 ± 8.8	17.4 ± 0.1	28.9 ± 6.5
	140	1311.2 ± 2.1	1443.5 ± 30.5	18.2 ± 0.1	32.4 ± 8.5

The densities and tensile strength limits for the obtained biocomposite materials are presented in Table 2. The maximum cross-breaking strength of samples without culture fluid was 18.2 MPa when the thickness of the boards ranged from 7 to 13 mm (depending on pressing conditions). Biocomposite materials had a higher limit when experiments were carried out at pressing temperatures of 100 and 120 °C, and culture fluid containing levan was added (Table 1). These results suggest that the tensile strength and density of the obtained biocomposites depends on both temperature and pressure values. Thus, the greatest density and tensile strength was observed at 140 °C and 39.2 MPa.

**Table 3.** Water Absorption and Thickness Swelling of a Biocomposite Made from Sawdust and Levan Biological Binder

Compression Pressure (MPa)	Compression Temperature (°C)	Water Absorption (%)	Thickness Swelling (%)
26.1	100	Did not pass the test	Did not pass the test
	120	Did not pass the test	Did not pass the test
	140	53.7 ± 8.9	Did not pass the test
39.2	100	23.9 ± 4.1	71.8 ± 0.6
	120	16.6 ± 2.9	41.6 ± 10.7
	140	7.2 ± 0.9	14.9 ± 1.7

One of the most important requirements for biocomposite materials is water resistance, which is the resistance to swelling in a humid environment. A typical disadvantage of wood and composite wood materials is their water resistance. This disadvantage is inherent to solid wood and to other materials manufactured using wood (including chipboard), with the only difference lying in the boards' inability to be restored to their former thickness after drying. This is due to the destruction of the adhesive bonds of chipboard wood particles during the swelling process, a phenomenon

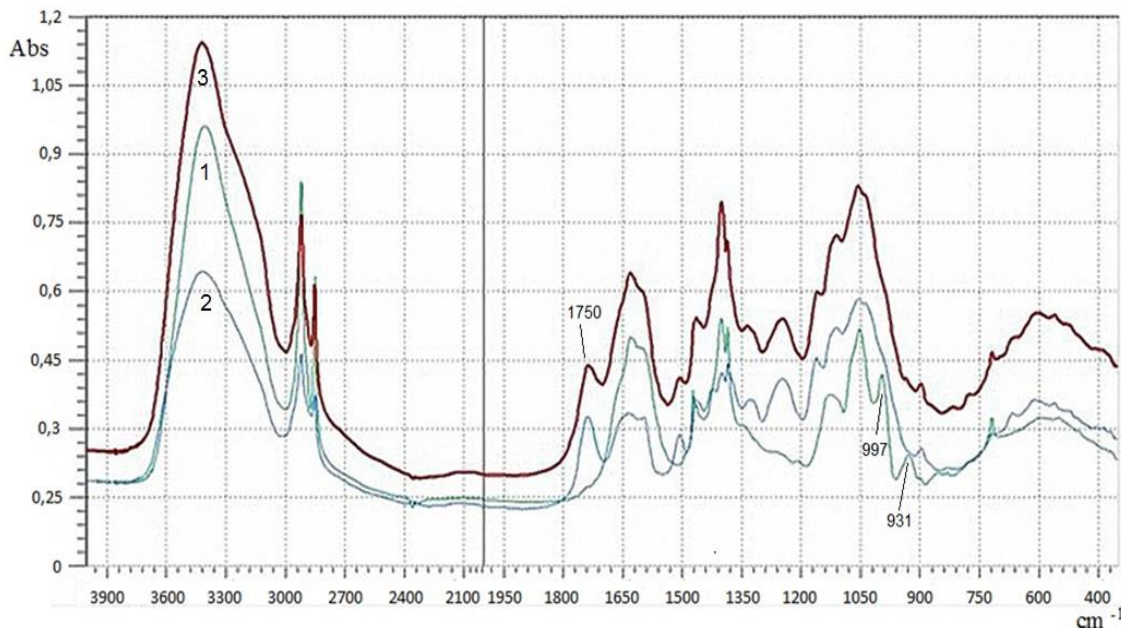


known as decompression (Doronin and Ivanov 1993). Data on water absorption and swelling for the wood sawdust samples are presented in Table 3.

The samples obtained at a pressure of 26.1 MPa failed the test for water absorption and thickness swelling. In an experiment performed at 39.2 MPa and 140 °C (Table 3), water absorption and thickness swelling met the domestic European (EN 312 2003) and (State Standard 10632 2007) requirements for lignocellulosic materials. It is possible that during high pressure and temperature, condensation occurred for the levan, lignin, and cellulose components of the sawdust, which was instrumental in the formation of water-resistant materials (Ye *et al.* 2007; Kang *et al.* 2009). In previous studies, considerable attention has been given to the description of the chemical interaction and structural changes that occur during hot pressing of a press-mass. These studies indicate that functional groups of polysaccharides and other wood components are involved in the crosslinking process (Sivonen *et al.* 2002; Hennecke and Roffael 2006; Müller *et al.* 2009).

Fourier IR spectroscopy was used to evaluate the chemical and structural changes that occurred during hot pressing (Fengel and Wegener 2003; Fabo 2004; Müller *et al.* 2009; Kadimaliev *et al.* 2015). This method also allowed for an analysis of the formation of bonds between wood particles and levan or other components in the binder. Figure 4 shows the IR spectra of the culture fluid and the obtained biocomposite materials.

Evaluation of IR spectra for the biocomposite materials (curves 2 and 3) revealed a correspondence to lignocellulosic materials (Garcia *et al.* 2011). The peak at 980  $\text{cm}^{-1}$  in the culture fluid spectrum (curve 1) corresponds to the absorption of fructose (Grube *et al.* 2002), which is the basic building material of the polysaccharide levan (Abdel-Fattash *et al.* 2005). A change in the obtained biocomposite material that occurred around 997 and 931  $\text{cm}^{-1}$  (curve 1) indicates rupture of the furanose ring in levan, and the release of -OH and -CH<sub>2</sub>OH groups. Because of the heat treatment, the main components of the wood underwent significant changes. In hemicellulose, end bonds were destroyed, resulting in the formation of uronic acids, which is indicative of the increase in the amount of -C=O groups around 1750  $\text{cm}^{-1}$ . Cellulose is also subject to degradation, which encourages the binding of the released -OH groups with free groupings of -OH and -CH<sub>2</sub>OH on the furanose ring (Yang *et al.* 2007).



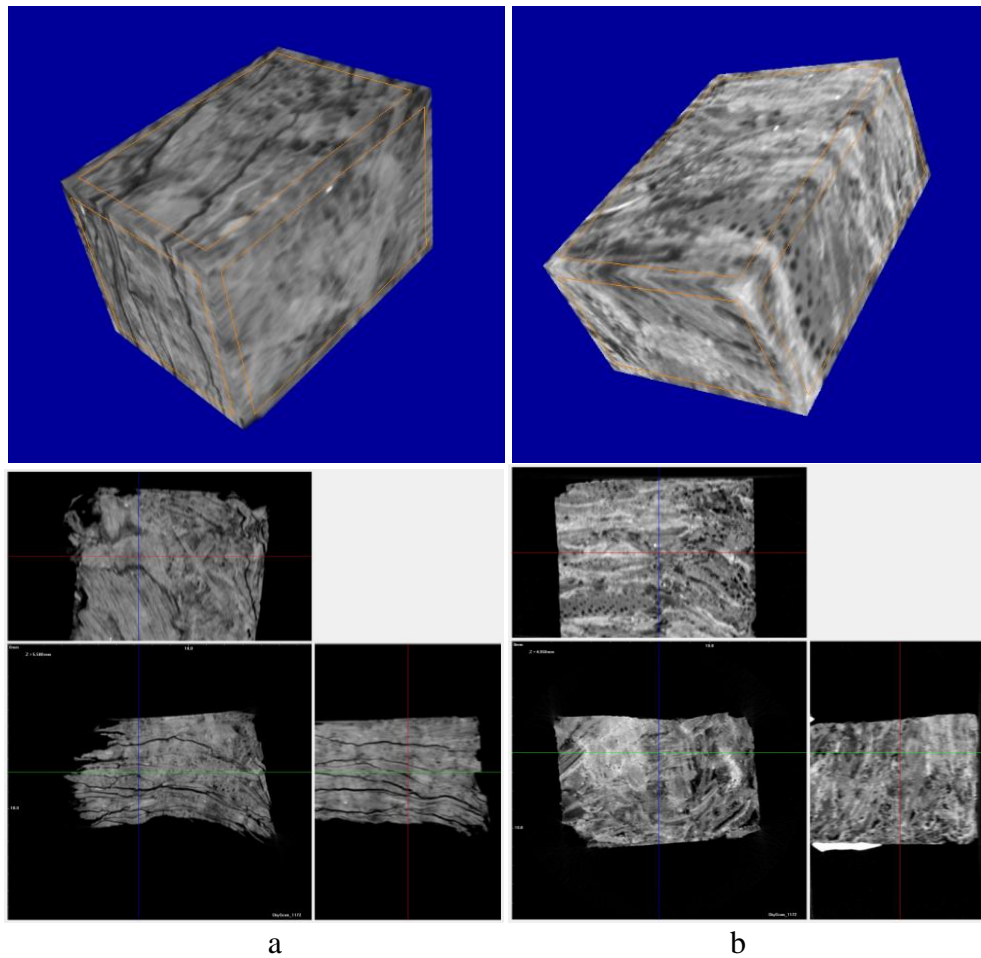
**Fig. 4.** IR-spectra for the culture fluid obtained after cultivation of the *A. vinelandii* D-08 strain (1 - green); the control material obtained without the addition of culture media (2 - blue); and the experimental biocomposite material supplemented with culture liquid (3 - red) (pressing at 180 °C and 39.2 MPa for a 5 min duration)

Scanning X-ray microtomography was used for a morphological analysis of the composites made with biological binders and commercially available chipboards containing phenol-formaldehyde resins (Wieland *et al.* 2013; Charwat-Pessler *et al.* 2014). A large number of micro-cracks were discovered in the samples where the binder was not used in the construction of the material (Fig. 5a).

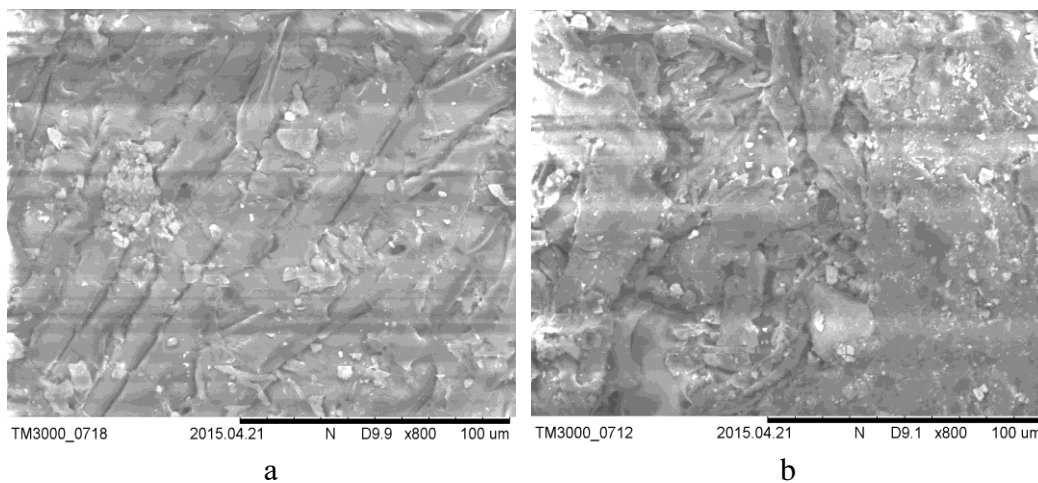
When scanning these samples, cracks were present throughout the volume of the material. This material will not meet the standards established for lignocellulosic materials, as suggested by results demonstrating the capacity of this material for high water absorption. Scanning results for samples with culture fluid binder containing levan showed that this material had the least number of defects in the entire volume (Fig. 5b).

Scanning electron microscopy (SEM) was used for additional comparative morphological analysis and to identify agglomerates of wood particles with biological and synthetic binders (Fig. 6). Figure 5a shows the samples prepared without using a binder. The adhesion process can hardly be observed, except for a small agglomeration of wood particles caused during heat treatment and pressure moulding, and partial destruction of the lignocellulosic filler. This is visually evident by the presence of multiple diagonal micro-cracks, which are present throughout virtually the entire surface of the material.

The microstructures of biocomposite materials derived from bio-binder containing the levan polysaccharide were homogeneous (Fig. 6b). Furthermore, micro-cavities and micro-cracks were almost absent from these materials (Fig. 6b), which can be confirmed by the adhesion properties of the polysaccharide and the biocomposite hydrophobic characteristics. Moreover, composite materials based on this bio-binder can better meet the standards for biodegradability and environmental safety in comparison with chipboards based on phenol-formaldehyde resins.



**Fig. 5.** X-ray microtomography of composite materials obtained at 180 °C and 39.2 MPa, for a duration of 5 min. (a) Composite material without binder; (b) composite material with the levan-based bio-binder



**Fig. 6.** SEM of biocomposite materials obtained without binder (a) and with bio-binder (b)

These results suggest that when hot pressing lignocellulosic materials with biological binder, a composite material was obtained with a structure that was moulded by a large number of functional groups located on the surface of the wood particles and the levan polysaccharide, as well as other components of the culture fluid. Water absorption and thickness swelling of the obtained material was in accordance with EN 312 (2003). However, the density of the obtained material exceeded the standard by more than 2-fold. One of the possible uses of the obtained materials lies in the production of large-sized wall panels. Changes in the physico-mechanical properties of the obtained materials according to furniture nomenclature can be reached by using other types of hot pressing.

## CONCLUSIONS

1. An eco-binder was made where the main component—the polysaccharide levan—was obtained by microbiological synthesis.
2. While optimising the conditions of cultivation of *Azotobacter vinelandii* D-08 on the media based on molasses, distillery dregs and milk whey the greatest accumulation of levan occurs in the proportion (ratio) 5:2:3. The amount of levan comprised 14 g/L during 24 h of cultivation.
3. Composite material having relatively high density was generated using wood raw material and the biological binder containing levan.

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