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XYLANASE-/CELLULASE COMPLEXES FOR THE PRODUCTION OF GLUE-FREE MEDIUM DENSITY FIBRE BOARDS

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Taking the example of lignocellulosic fibres from pinewood, we could substitute the addition of resin by employing various hydrolases, especially xylanase plus cellulase from *Trichoderma reesei*. Differently to the use of oxidases, the polysaccharides of the wood in this case are activated for fibre-to-fibre bonding. Already after a few minutes incubation time and less than 3% of enzyme dosage, there is a substantial improvement in strength and hygroscopic properties of glue-free MDF. The material properties meet approximately the standard of EN 622. We received different results with different xylanase-cellulase-complexes. According to the present results, high activity in xylanase plus high activity in endo-glucanase are necessary for the successful modification of the lignocellulose fibres.

The utilisation of lignocellulosic materials from annual and perennial plants is gaining in importance in connection with the increasing utilisation of renewable raw materials. Medium Density Fibre Boards (MDF) are increasingly being used in the furniture industry and more and more recently in the construction industry as well as in the interior design sector because of their good mechanical and processing properties. However, according to the current technology it is necessary to insert about 8 to 12% synthetic resins to ensure the required physicomechanical

properties for the production of fibre boards. These binding agents cause about 50% of the material costs in direct relation to the respective prices for crude oil and other specific market factors. Moreover, they also cause environmental problems on a large scale both in the production and processing of the MDF as well as the use and the waste disposal.

Thus, the task is to minimize or substitute the synthetic resins which have been a major part up to now. The enzymatic modification of raw materials to activate inherent bonds seems to be a suitable approach to achieve this.

According to their ability and mechanism for the modification of lignocellulosic material, enzymes of white-rot fungi have been primarily investigated up to now. For this the extracellular enzymes lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase have been described so far. Above all the enzyme system of the *Phanaerochaete chrysosporium* fungi which is responsible for the degradation of lignin has been investigated [1, 2]. Above all the importance of laccase for the activation of the wood fibre material is being reported in many papers [3, 4].

Up to now only a few articles have been published in journals on the functioning of brown-rot fungi. There is a commonly held believe that these fungi preferably degrade cellulose and thereby enrich lignin. In previous investigations [5] small laccase activities have been found in certain liquid cultures. Milstein also verified laccase-like enzyme activities of brown-rot fungi [6]. The increase of strength properties of produced wood fibre boards was proved following a nine-day solid state fermentation [7], the reasons, however, for this property improvements have not been examined yet. The current paper deals with the discovering and the characterisation of enzymes for the modification of lignocellulosic fibre materials with the goal to produce materials which are free from synthetic resins and biodegradable.

MATERIAL AND METHODS

Fibre material. Fibre material from pine (*Pinus sylvestris*) has been used for the investigation because it shows more favourable requirements in comparison to fibre material from colza, cannabis, wheat or barley for the processing into new fibre materials. The production of the fibre material was carried out in a lab defibrator with hydrothermical pretreatment (5 minutes at 160°C, refiner disk gap of 0,25 mm, feed screw 20 RPM, 50 l/h water flow rate). Directly following, the fibre material was dried in an current dryer at 100°C to a humidity of 12%.

Enzymes. Enzymes of white-rot fungi *Phanerochaete chrysosporium*, *Trametes versicolor*, *Lentinus lepideus*, brown-rot fungi *Gloeophyllum trabeum, Coniophora puteana* as well as fungi imperfecti *Trichoderma reesei* and *Penicillium verruculosum* have been tested in terms of their suitability for the modification of the fibre material. The enzyme fermentation was carried out in discontinuous manner (80 liter NBS fermentors). Through the variation of the medium the composition of the enzyme complexes was changed; in the case of the white-rot fungi the induction of lignin-degrading enzymes was to be achieved through defining the level of nitrogen limitation. The downstream processing of the fermentation mediums was carried out through separation, cross-flow-microfiltration and subsequent concentration through cross-flow-ultra-filtration (rotary piston machine to ensure low sher stress, polysulfon membrane, molecular mass cut-off 10 kD). The ultra-concentrate served as the starting point for further investigations. Furthermore, some laccase, cellulase and xylanase commercial compounds have been included in the investigations.

The laccase activity, the cellulase activity (filter paper activity, CMC activity) as well as the xylanase activity served as criterion for the choice and characterisation of the enzymes.

Incubation of the fibre material and enzyme activity assay:

As a criterion for the enzyme activity served the change of the fibre sheets' strength in comparison to a control measurement without enzyme activity. At first the fibre material was incubated at a temperature of 20 to 60°C with an incubation time of 0,5 and 1 hour with the favoured enzyme composition. 5g of the fibre material was mixed with 100ml enzyme composition and incubated in basin marin stirring all the time. The production of the fibre sheets was carried out in a hand sheet maker following the Rapid-Kothen process. After climatisation the breaking length was calculated in a Zwick-universal test machine according to DIN 53112 with a test speed of 0,8 to 1 mm/min. The resulting breaking length describes the power which is necessary to tear apart a test sample. It is dependent on the fibre to fibre bonding.

RESULTS

The results of the change in the fibre sheets' breaking length in correlation to the incubation of the pine fibre material with culture filtrates from brown- and white-rot fungi is shown in Table 1. Due to the fact that enzyme compositions of the brown- and white-rot fungi did not lead to the expected degree of binding strength, enzyme compositions were produced with an increased level of xylanase and then included in the investigations. These considerations are based on the fact that xylanase and mannanase enzymes play a role in the pulp industry (e.g. the so called *biobleaching*). Enzyme complexes of *Trichoderma reesei* (SIAB-01/SIAB-03) and *Penicillium verruculosum* (SIAB-02) were purposely produced on a small scale. The results of the investigation of the tensile strength are shown in Fig. 1 and the characteristics of these enzyme samples are presented in Table 2. The enzymes described were tested at different temperatures and duration of effec-

Table 1

Breaking length (in kilometres) of fibre sheets

Carbon source / inducer in the fermentation medium	Water	Medium (control)	*Trametes versicolor*	*Gloeophyllum trabeum*	*Coniophora puteana*
Wheat bran, cellulose	0,12	0,08	0,09	0,05	0,02
Wheat bran, cellulose, (nitrogen limitation)	0,12	0,1	0,08	0,08	0,05
Cellulose, fibre material	0,12	0,04	0,06	0,06	0,06
Cellulose, fibre material, (nitrogen limitation)	0,12	0,03	0,07	0,07	0,06

Table 2

Composition of the enzyme complexes

Strain	*T. reesei* „SIAB-01“	*P. verruculosum* „SIAB-02“	*T. reesei* „SIAB-03“
	Lactose	Cellulose Wheat bran	Lactose Cellulose
Carbon source/Inducer	Cellulose Wheat bran		Distillers spent grain
Endoglucanase CMC-Activity	13 IU/mg	3,0 IU/mg	23,6 IU/mg
Cellulase [FPU]	0,35 IU/mg	0,28 IU/mg	0,31 IU/mg
Xylanase	0,72 IU/mg	14 IU/mg	18 IU/mg
Mannanase	4,74 IU/mg	4,46 IU/mg	5,29 IU/mg
Ratio of Xylanase to Cellulase [XA / FPU]	1 : 2,1	1 : 48	1 : 58
Activity	IU/mg Protein in the liquid concentrate		

tiveness on the pine fibre material. For this the ultra-concentrates were diluted with distilled water at a ratio of 1:5 and then incubated with the pine fibre material for 15 minutes, for 60 minutes as well as for 5 hours at 20°C or 40°C. The highest tensile strength was achieved with the enzyme composition SIAB-03 (*T. reesei*-18.2-03) at 40°C and with an incubation time of one hour. This supernatant also exhibits the highest activity in xylanase.

The thorough empirical tests of enzymatic pretreatment show that it is always possible to cause a modification of the examined pine fibre material which leads you to expect reductions in the use of synthetic resins. From various series of tests with different culture filtrates (different enzyme complexes) it becomes obvious that the composition of the fermentation medium for the cultivation of the enzyme forming strains (*basidiomyceten*, *deuteromyceten*) has a significant influence on the strength properties of the resulting fibre sheets. In this manner, a doubling of the breaking length of produced fibre sheets was observed, for example, with enzyme complexes of similar fungi but from different mediums (Fig. 1).

Investigations on the optimisation and production of xylanase-/cellulase complexes

The culture filtrates of *Trichoderma reesei* and *Penicillium verruculosum* contain components which cause the activation of the fibre material and improve the breaking length of the fibre sheets. The best results were achieved with the culture filtrate that had the highest xylanase activity (enzyme complex SIAB-03), wherefore in the following investigations the extraction and characterisation of this enzyme was more intensively examined with the goal to optimise the enzyme complexes and the fermentation in terms of commercial as-

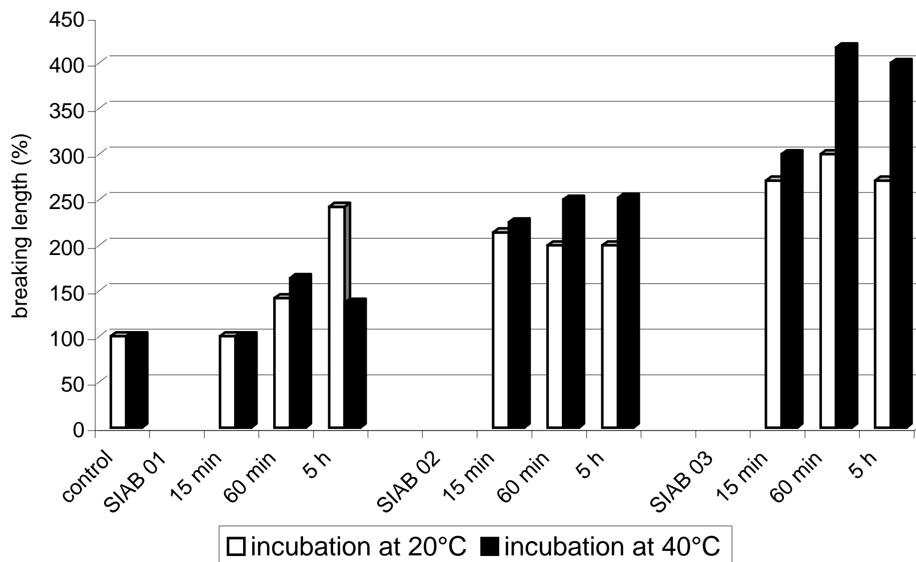
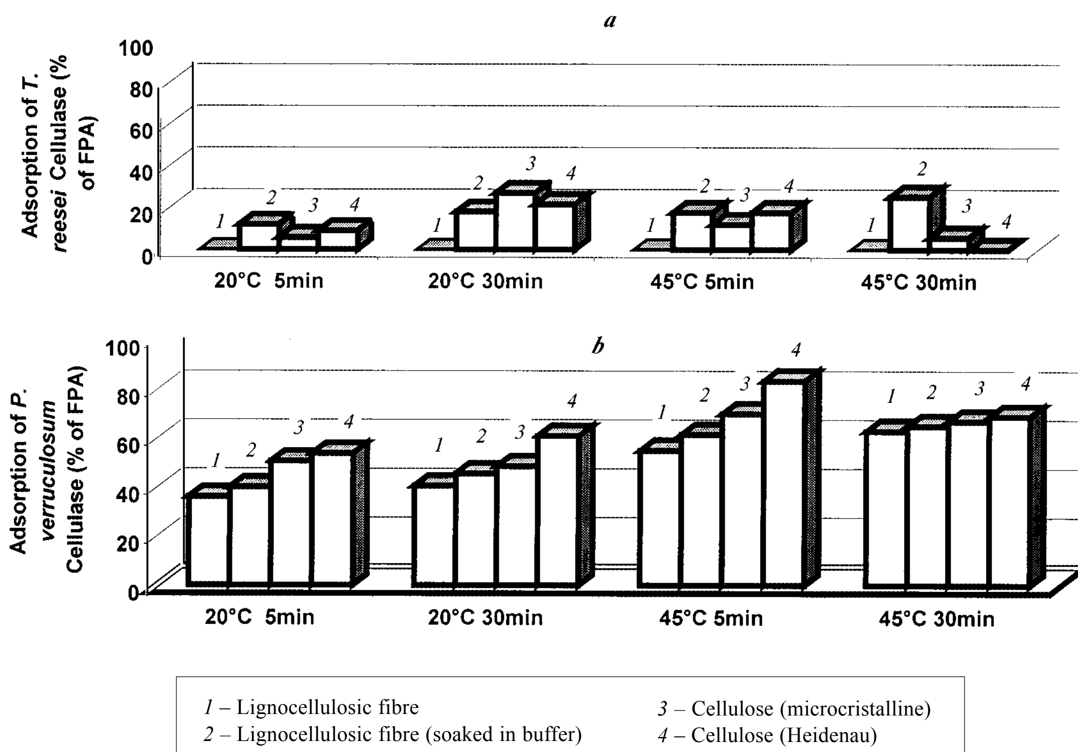


Fig. 1. Breaking length of fibre sheets incubated with culture filtrates of *Trichoderma reesei* and *Penicillium verruculosum*



1 – Lignocellulosic fibre
2 – Lignocellulosic fibre (soaked in buffer)
3 – Cellulose (microcristalline)
4 – Cellulose (Heidenau)

Fig. 2. Absorption of Cellulase from *T. reesei* (*a*) and *P. verruculosum* (*b*) to different lignocellulotic fibres

pects. The following investigations were carried out to prove this.

Investigations on enzyme adsorption. The enzymes' ability to bind itself to the substrate (in the case of insoluble substrates) is a significant criterion of the enzymatic decomposition process. In correlation to the properties of xylanase-/cellulase enzyme complexes with the ability to modify lignocellulosic fibre materials, investigations on adsorption were carried out. After the incubation, the remaining activity in the supernatant was investigated with a surplus of insoluble substrates. Through the measurement of the differences one could then determine the amount of the adsorbed enzymes.

The adsorbability of the *T. reesei* enzyme and the *P. verruculosum* enzyme (Fig. 2–4) was examined by means of the original substrate (lignocellulosic fibre material, native and washed) and to control the cellulose. After the separation of the solids from the adsorbed enzyme the remaining activities in the medium were measured. The

Table 3

Analytical characterisation of the commercial enzyme preparations

Activity Sample/Enzyme	Xylanase [IU/ml]	FPA [IU/ml]	AZO–CMC [IU/ml]	Protein [mg/ml]
Dyad 2 [1)]	456 45,6 IU/mg	1,10 0,11 IU/mg	7,92 0,792 U/mg	–
Dyad 3	438	34,7	304,8	162,1
Dyad 1	10.545	18,5	370,9	89,9
Biopr	1.332	14,2	292,2	145,8
Neutral Cellulase NC-40	308	9,2	98,7	28,7

Dry sample: 10 mg /ml in citric acid buffer.

sorption from cellulase (as FPA), endo-β-D-glucanase (AZO-CMC) and xylanase was also measured.

Binding Substrates:

lignocellulosic fibre material (from IHPT), unwashed

lignocellulosic fibre material, washed with 0,025 M citric acid buffer (after 1 hour leaving in the buffer to soak)

microcristalline cellulose (Serva)

cellulose of the *Papiertechnische Stiftung Heidenau*.

The comparison between the adsorption of both enzyme complexes shows that in each series of tests, cellulase and endoglucanase of *P. verruculosum* are more strongly bound to the unwashed fibre material than the respective enzymes of *T. reesei* (Fig. 2, 3). Only the xylanase of *T. reesei* is bound more strongly to the unwashed fibre material at higher temperatures and following a longer incubation (Fig. 4). A faster binding ability of those enzymes which degrade cellulose to the substrate is possibly unfavourable for a positive modification of the lignocellulosic fibre or speeds up the decomposition of the cellulosic structure. However, it is not obvious which enzymes have the described activating effect.

Table 4

Xylanase formation with different substrates, inducers (shake flask experiments)

Medium (Inducer/carbon source)	Cellulase [FPU/ml]	Xylanase [IU/ml]
Wheat bran + Cellulose	0,456	5,55
Wheat bran + DHEA-Cellulose	0,98	6,98
Distiller?s spent grain + Cellulose	3,08	438
DHEA-Cellulose	0,881	3,75
Cellulose (from *PTS Heidenau*)	n.m.	10,75

Analytical characterisation of cellulase-/xylanase preparations. The investigations also included the commercial enzyme preparations which are particularly used in the paper industry for the improvement of the pulp properties (degree of whiteness, degree of drainage). After the solid (strength) investigations, some of the used industrial enzymes proved well suited for the production of MDF which are free from synthetic resins.

The analytical characterisation of the commercial enzyme preparations is presented in Table 3.

Due to the fact that the enzyme complexes consist of different components, the definition of the single activities carries less weight regarding their suitability for the described application. It became evident that the endoglucanase in addition to the xylanase gains more and more importance for the modification of the fibre material, which was why the AZO-CMC activity was included in the investigations. At least the comparison between *T. reesei* enzyme complexes (SIAB-03) and *P. verruculosum* enzyme complexes (SIAB-02) in the previous investigations has shown that the cellulase and xylanase activities do not serve as a sufficient criterion for the suitability of application.

Fermentation of the xylanase-/cellulase complexes. Basic criterion for the technical realisation of the enzymatic modification of lignocellulosic fibre materials are the composition of the enzyme complexes and the efforts for their production. Investigations on the procedure and the induction were carried out to optimise the fermentation of *T. reesei* xylanase-/cellulase complexes SIAB-03. For the induction technically relevant substrates and waste products were investigated and as a result distiller's spent grain proved to be most suitable (Table 4). In the investigations on fermentation, the feeding medium and the time regime for the fed-batch-technique was investigated with the goal to obtain a linear increase in xylanase formation over the period of fermentation similar to the cellulase formation with the same mutant on the basis of lactose, cellulose and

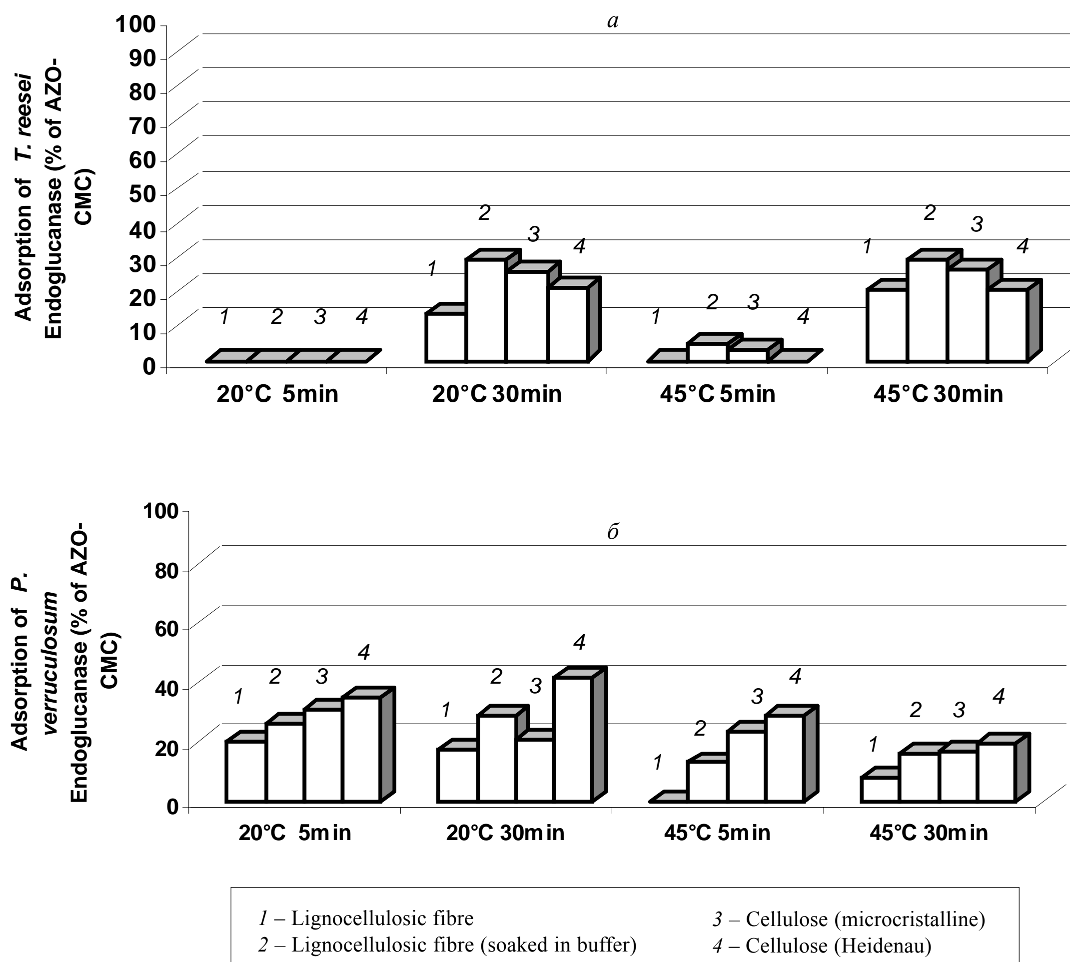


Fig. 3. Absorption of Endoglucanase from *T. reesei* (*a*) and *P. verruculosum* (*b*) to different lignocellulotic fibres

Table 5

***fed batch*-Fermentation of *Trichoderma reesei* „SIAB-03"**

No.	Initial medium	Fed-batch	XA [IU/ml]	XA-Mex [IU/ml*h]
1	DS + Cell. + Lac	DS (50%) + Lac	340	9,2
2	DS + Cell. + Lac	Lac	83	n.d.
3	DS + Cell. + Gluc	DS (100%)	616	27,3
4	DS + Cell. + Gluc	DS (100%)	460	5,6
5	DS + Cell. + Gluc	DS (100%), Cell.	555	13,3
6	DS + Cell. + Gluc	DS (100%), Cell.	282	19,4
7	DS + Cell. + Gluc	DS (100%), Cell.	833	n.d.
8	DS + Cell. + Lac	DS (100%), Cell.	333	9,3

DS—Distiller's spent grain;Lac—Lactose; Gluc—Glucose; XA-Mex—maximum Xylanase-excretion rate XA—Xylanase

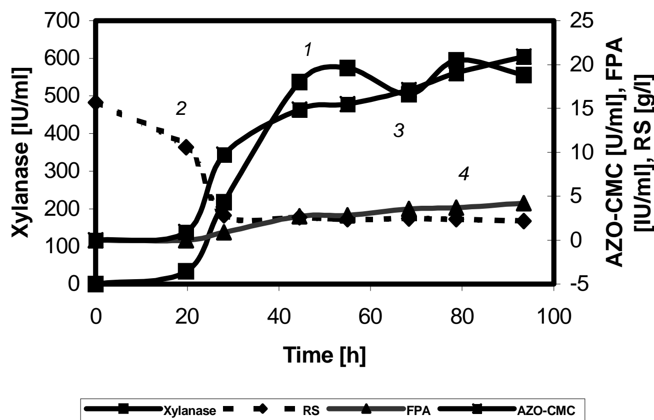


Fig. 5. Fermentation of Xylanase/Cellulase ("SIAB-03") with distiller's spent grain

wheat bran in the feedback-controlled-fed-batch-technique. The results are shown in Table 5 and in Fig. 5.

The feeding of distiller's spent grain in the fed-batch-technique lead to a high xylanase activity; lactose in the feeding substrate reduced the xylanase formation. While using the *T. reesei* mutant M 18.2-03 it became obvious that at least the xylanase formation was partially repressed by lactose in contrast to the cellulase formation, where lactose acted as a weak inducer. Sustained substrate limitations before and during the fed-batch-technique lead to a decrease of the xylanase formation which was greater than the cellulase formation. The following conditions are necessary and suitable for the fermentation of the SIAB-03 enzyme for the enzymatic fibre material modification:

Basic medium. lactose plus cellulose as carbon source for mycelium growth and induction of cellulase, 50% distiller's spent grain in basic medium for the induction of xylanase, Feeding medium for the fed-batch-technique:

distiller's spent grain (intermittent addition, e.g. controlled by the CO_2-content in the exit gas of the fermentation), plus cellulose for the substitution of the used cellulose which was added to the basic medium.

Under these conditions, the formation of xylanase is at least 800 IU/ml in activity. In correlation to the results in the IHPT the filtrates of such a fermentation are well suited without further concentration for the modification of the fibre material.

DISCUSSION AND CONCLUSIONS

The investigated *T. reesei* xylanase-/cellulase enzyme complexes as well as some of the commercial preparations are suited for the modification of lignocellulosic fibre material to produce materials which are free from synthetic resins (Fig. 6).

The exact effects have not been clarified up to now. The differences which have been found between the xylanase-/cellulase enzyme complexes of *T. reesei* and *P. verruculosum* show that alongside the enzyme activities (above all xylanase and endoglucanase) the sorption properties are also decisive for the outcome. Thus, a faster sorption of xylanase, in contrast to endoglucanase, seems to be most favourable.

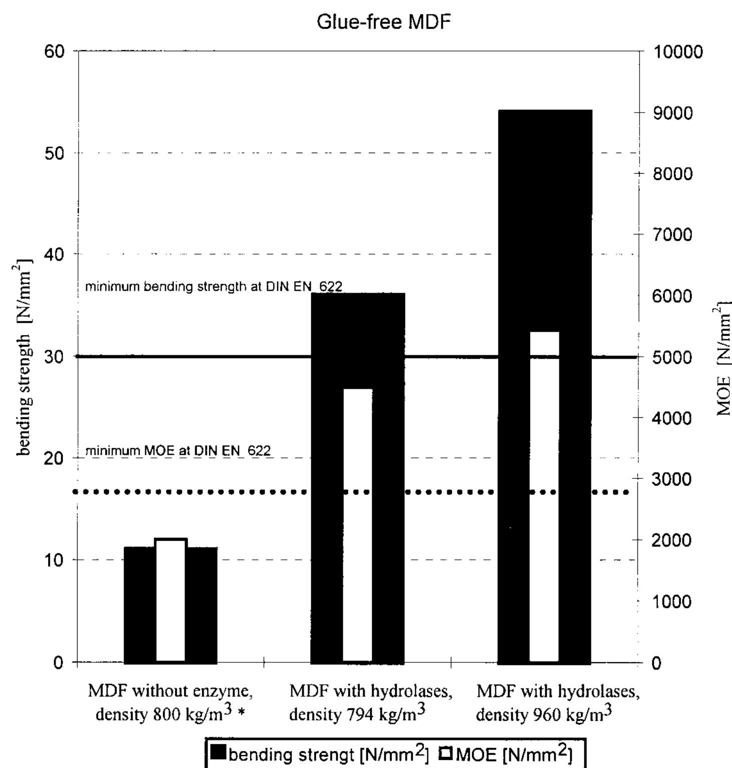


Fig. 6. Investigation of physicomechanical proporties of MDF: Bending strength, elasticity

The commercial realisation of a procedure for the enzymatic modification of lignocellulosic fibre material for the production of MDF is substantially determined by the enzyme costs. Hydrolases are also available as technically *cheap enzymes* [8], whereas the use of ligninases from white-rot fungi has not yet been taken into account. The culture filtrates have come from the distiller's spent grain *T. reesei-xylanase-/cellulase* complexes are, without any additional subsequent concentration, suitable for the modification of the fibre material, through which this process can be commercialised.

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КСИЛАНАЗНО-ЦЕЛЛЮЛАЗНЫЕ КОМПЛЕКСЫ ДЛЯ ПРОИЗВОДСТВА БЕСКЛЕЕВЫХ ВОЛОКОННЫХ ПЛИТ СРЕДНЕЙ ПЛОТНОСТИ

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На примере лигноцеллюлозных волокон из древесины сосны показана возможность замены смолы (клея) гидролазами (например, ксиланазой с целлюлазой из *Trichoderma reesei*). В отличие от оксидаз полисахариды древесины активируются для межволоконной связи. Через несколько минут инкубации при дозе фермента <3% происходит заметное улучшение прочности и гигроскопических свойств бесклеевых волоконных плит. Свойства материала приблизительно отвечают стандарту EN 622. При использовании разных ксиланазно-целлюлазных комплексов были получены разные результаты. Показано, что высокие значения ксиланазной и эндоглюканазной активности необходимы для успешной модификации лигноцеллюлозных волокон.