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Production of Cellulases and Xylanases by *Trichoderma viride* and Biological Processing of Lignocellulose and Recycled Paper Fibers

U. VIESTURS*,[1,2], M. LEITE,[2] A. TREIMANIS,[2]
T. EREMEEVA,[2] A. APSÍTE,[2] M. EISIMONTE,[2] AND P. JANSONS[1]

[1]Latvian Biogas Association; and [2]Latvian State Institute of Wood Chemistry, 27 Dzérbenes Str., LV-1006 Riga, Latvia

ABSTRACT

The production of cellulases and xylanases by *Trichoderma viride* L-333 was studied. Significant amounts of extracellular enzymes were obtained when submerged fermentation was performed in the fed-batch regime on cellulose-and xylan-based media with addition of sophorose and Tween-80. The culture filtrate of *T. viride* was used for enzymatic treatment of recycling newspaper (TMP) fibers. To elucidate the mechanism of the enzymatic hydrolysis, the fractionation and separate processing of tree different fiber size fraction were performed. The biological processing of recycling fiber caused an increase in brightness and dewatering properties. The most significant improvement was observed with unfractionated and long fiber fractions at a ratio of cellulase to endoglucanase to xylanase of 1:10:100.

Index Entries: Cellulase; hemicellulase; fed-batch fermentation; enzyme activities; enzyme adsorption; recycled waste paper; freeness; drainage; brightness.

INTRODUCTION

Cellulases and xylanases are the main enzymes used for recycling fiber modification. *Trichoderma* spp. are well known as producers of cellulolytic enzymes. Several fermentation techniques have been applied to enhance mycelial growth and extracellular enzyme production *(1–6)*.

Recycled fibers have lower freeness and strength compared to virgin fibers. The treatment of the secondary fibers with cellulases and hemicellulases can result in the improvement of the fiber quality. It has been reported that the modification of the secondary fiber with a mixture of cellulases and hemicellulases can reverse the decrease in drainage that occurs when the secondary fiber is recycled *(7–9)*. Improved dewatering of pulp was presumed to derive from the "peeling" effect, which removes small fiber fibrils that have high affinity for water *(8–10)*. It has been found that the increase in drainage occurs owing to the hydrolysis of the

*Author to whom all correspondence and reprint requests should be addressed.

fines and an increase in the long fiber content *(7,11)*. Jackson and coauthors *(12)* found that the fines were subjected to both flocculation and hydrolysis by enzymes, and fibrils were removed or fragmented from the surface of large fibers. Visualization of enzyme binding to the secondary fibers by the silver-enhanced colloidal gold affinity labeling technique confirmed the previous finding reported by the authors *(12)* that fines are the preferred fiber fraction for cellulase attack *(13)*. Prasad and coauthors *(11)* found that xylanase was essential to increase brightness of recycled fiber and to reduce the ink area. In a joint effort, a European research consortium of microbiologists and biochemists, as well as wood and fiber technologists *(14)*, found that the action of endoglucanase (EG) was necessary for an improvement in recycled paper drainage. The effect did not appear to be owing to a selective hydrolysis of fines, but was a consequence of the hydrolysis of amorphous cellulose on the surface of the fibers *(14)*. In spite of the fact that this study included various fractions of virgin and recycled fibers and all possible enzymes of the cellulase and hemicellulase system, some unresolved questions on the mechanisms of the enzymatic treatment still remain, because of the large variation in the enzyme sources, activities and dosages, secondary fiber origin and composition, and so forth. The purpose of the present study was to test the culture filtrate of *Trichoderma viride* produced in the fed-batch fermentation regime, and containing a mixture of cellulolytic and hemicellulolytic enzymes. To elucidate the mechanism of the enzymatic hydrolysis of the recycled paper fibers, the study included the fractionation and separate processing of the three fiber-size fractions of the recycled newspaper made of spruce thermomechanical pulp (TMP).

EXPERIMENT

Materials and Methods

Enzyme source

The enzyme source was an unpurified filtrate of *T. viride* L-333 (Institute of Microbiology and Biotechnology, University of Latvia). The medium composition was similar to that used by Mandels et al. *(1)*, with the following alterations: instead of urea, the concentration of $(NH_4)_2SO_4$ was increased from 1.4 g/L to 3.0 g/L and the concentration of KH_2PO_4 from 1.0 g/L to 2.0 g/L (to ensure the buffering capacity). Proteose peptone was replaced by 0.1% yeast extract. The carbon sources were carboxymethylcellulase sodium salt (Sigma, St. Louis, MO) and pretreated or untreated lignocellulosic (LC) substrate (wheat straw, wheat bran, waste newspaper). EG formation was induced by sophorose (500 μg/mL) *(2)*. The fermentations were carried out in a laboratory fermentor FU-8 *(4)*. The use of a special stirring system for mycelial cultures designed by the authors allowed the increase in the LC substrate concentration in the initial nutrient medium from 2–8% dry matter. Tween-80 (0.02%) and the physiological regulation of the metabolic activity of the cell by acetate pulses in *T. viride* fed-batch culture were used to increase the production and excretion of extracellular cellulases and xylanases.

Enzyme Assays

1. Cellulase activities: Filter paper (FPA) and EG (EC 3.2.14) activities were measured against Whatman No. 1 filter paper and carboxymethylcellulase (CMC), respectively, according to Mandels and coauthors *(1)*, and expressed as international units (IU/mL).

2. Xylanase (1.4-β-xylanase, EC 3.2.1.8) was assayed by the method of Bailey and coauthors *(15)*.
3. Reducing sugars were determined by the 3.5-dinitrosalicylic acid (DNS) method *(16)*.
4. Substrate: Unprinted and 6-mo-old printed samples of the Latvian newspaper "Diena," manufactured from spruce TMP was used as a raw material in this study. The newsprint waste paper was torn into small pieces by hand and blended until a uniformity of the raw material and 76° Schopper Riegler (SR) were reached. Three types of fiber fractions were prepared using 17-, 240-, and 250-mesh screens. Type I (17-mesh screen) contained long fibers only. Type II (240-mesh screen) contained both long fibers and fines. Type III (250-mesh screen) consisted only of fines. All the fractions were used for the enzyme treatment.

Enzyme Treatment

Enzyme reactions were performed at pH 4.8, 50°C, and 3.0% consistency for 120 min. After the incubation, the residual enzyme activity adsorbed onto the fibers was destroyed by boiling the pulp for 5 min to prevent further enzyme action. The enzyme dosage was calculated according to FPA, EG, and xylanase activities based on the oven dry fiber.

Fiber Characterization

The influence of the enzyme treatment on brightness of the recycling paper (ISO 3688), freeness (°SR, ISO standard 5 267/1-1979), and dewatering properties (WRV) *(21)* was determined. Lignin was assayed as Klasson lignin according to the standard procedure (Tappi T222m).

High-Performance Size-Exclusion Chromatography (HP SEC) Conditions

HP SEC was used for the analyses of enzymatic hydrolysates and the low-molecular part of the fibers soluble in 5% NaOH. The HP SEC analyses were performed on a size-exclusion chromatograph (Laboratory Instruments, Prague, Czech Republic), equipped with refractometric and spectrophotometric detectors and a Rheodyne Model 7125 fixed-loop (100 μL) injector. Prepacked stainless-steel columns (250 × 8 mm ID) containing Separon S HEMA 1000 E10 100 (for the analysis of enzymatic hydrolysates) and Separon S HEMA 1000 (for the analysis of 5% NaOH soluble part of fiber) were used. Water was used as the eluent in both cases at a flow rate of 0.5 mL/min at ambient temperature.

RESULTS

Production of Enzymes

Technological parameters of the cultivation were studied with *T. viride* to elucidate the most effective way to promote cellulase and xylanase production. Significant amounts of cellulase (2.06–3.1 FPA IU/mL) and EG (20.6–33.0 IU/mL) were obtained on cellulose-based media, when submerged fermentation was performed in the fed-batch regime. Xylanase was produced efficiently on both cellulose- and xylan-based media, although the best yield (up to 116 IU/mL) was observed with birch xylan (1% dry wt) as inducer. The EG was slightly promoted from 19.3 to 23.0 IU/mL by sophorose. Tween-80 was used to favor extracellular enzyme excretion from the cell.

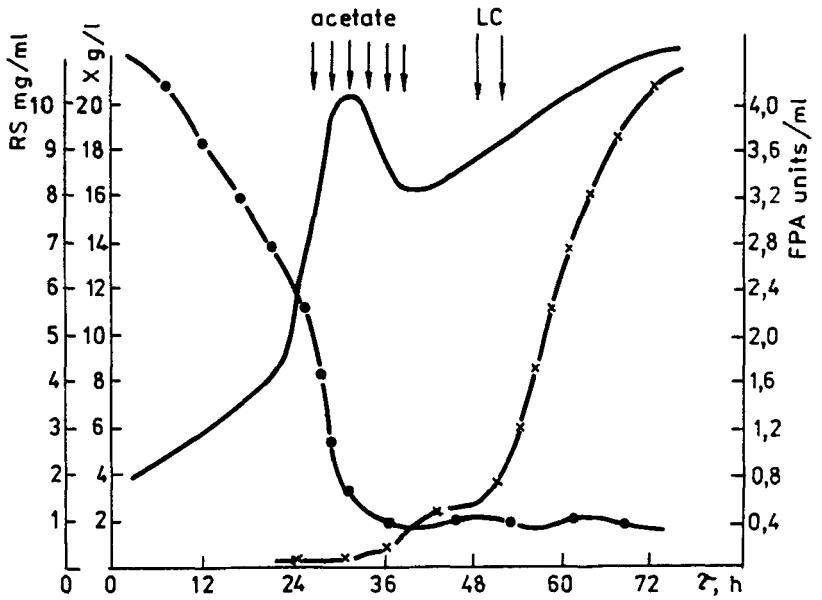

Fig. 1. The growth of *Trichoderma* L-333 biomass (—), cellulase (FPA) synthesis (–x–), and reducing substances (RS) consumption (–●–) on alkali-treated wheat straw (LC). Arrows indicate acetate and LC substrate feeding. The LC substrate feeding allowed to increase the LC substrate concentration from 2–8% dry wt.

The main technological advantage used in this study follows from our previous investigations. It was found that it is possible to promote the exoenzyme synthesis by affecting the general metabolic activity of the cell with energy-rich, cell-energy supplying substrates, such as ethanol, lactose, acetate, or glucose *(17–19)*. The data obtained on the cultivation of *T. viride* L-333 on alkali-treated wheat straw, unprinted newspaper, and so forth, confirmed our previous findings. The acetate pulses at the moment when the biomass and enzyme synthesis begins to decrease and restores both the synthesis of cellulase and cell growth (Fig. 1). Acetate and ethanol are preferable auxiliary sources with weakened carbon limitations, probably because these nonfermentable substrates enter more easily into the Krebs cycle *(18)*. A gradual addition of the LC substrate has a double effect: the intensification of the general metabolism by the soluble carbohydrate pulse and the intensification of the cellulase synthesis at the expense of an increased substrate concentration in the culture media. Owing to the media rheology and mass-transfer problems, it is not advisable to add high LC substrate concentrations at the beginning of the process. The gradual increase in the cellulose concentration and the feeding by small pulses of energy-rich carbon source (acetate) gave the best synthesis of extracellular xylanases and cellulases. The influence of the addition of sorphose, Tween-80, variations of carbon sources, pH profiling, and so on, was comparatively low. Comparative data characterizing enzyme activities in the various fed-batch cultivation processes of *T. viride* L-333 are presented in Table 1.

Treatment of Recycled Fibers

As can be seen from Table 1, the concentration of extracellular enzymes in the *T. viride* culture filtrate was rather different for various fermentation processes. In

Table 1
Effect of Various Carbon Sources and Energy-Rich Carbon Substrate Pulses on the Enzyme Activities During the Fed-Batch Cultivation of *T. viride* L-333

		Enzyme activities, IU/mL			Protein
Substrate	Addition	FPA	EG	Xylanase	% dry wt
CMC	Lactose	2.10	28.4	34.2	22.2
LC untreated	Glucose	1.12	28.3	33.1	19.9
LC alkali Treated, birch xylon, sophorose	LC alkali Treated + acetate	3.40	36.8	116.1	22.1
Unprinted newspaper (TMP), Tween-80	Acetate	1.6	20.1	86.5	20.04

[a]LC, lignocellulosic (wheat straw); CMC, carboxymethylcellulose.

some experiments, the culture filtrate was supplemented with a commercial xylanase preparation, Pulpzyme HB (Novo Nordisk A/S, Bagsvaerd, Denmark). In the range of the enzyme dosages under study, the most significant improvement in WRV and brightness values of the recycled fibers was observed at a comparatively low cellulase content (FPA 0.3 IU/g cellulose dry wt). The optimal ratio of FPA to EG to xylanase was estimated as 1:10:100. The treatment of the printed newspaper "Diena" with such a culture filtrate gave an increase in brightness from 46–52% ISO, and an increase in the WRV values from 109.3–123.0% during a 2-h treatment at 50°C with mixing. It was observed that most of the enzymes were adsorbed rapidly onto fibers within the first 10 min of the contact at 50°C (Fig. 2), which resulted in a rapid increase in the WRV values. The increase in the contact time during a 2-h treatment caused a slight loss in the WRV and °SR values.

Fiber Fractions

To elucidate the mechanism of the enzyme action, the recycled fibers were fractionated into three fractions according to the fiber length and used to separate enzyme treatments. The fractions were subjected to pentosan and Klason lignin analysis. A remarkable difference was established in the carbohydrate and lignin content of the different fractions. As compared to the other two fractions—Type I and Type II—the fines fraction (Type III) probably consisted of more hemicellulose, from the primary cell wall and the outer layer of the secondary cell wall. This was confirmed by the Klason lignin content, which was higher in the Type III fraction (37.2%) than in the longer fiber fractions (24.0 and 24.7% for Type II, respectively). The adsorption of enzymes (Fig. 2) and the hydrolysis rate, according to the release of reducing sugar (Fig. 3), were two times higher for the fines fraction (Type III). At the same time, only the long fiber fraction (Type I) showed an increase in WRV and °SR values (Fig.4), while a rapid decrease in WRV and °SR values for the fines fraction and a slight decrease in those of the Type II fraction were observed (Fig. 5). The HP SEC data confirmed a remarkable difference between the fiber fractions (Table 2). The solubility in 5% alkali and absorbance at 313 and 405 nm for the fines fraction was twice as high compared to the same characteristics for Type I and Type II fibers. The changes in absorbance during the course of experiment, estimated

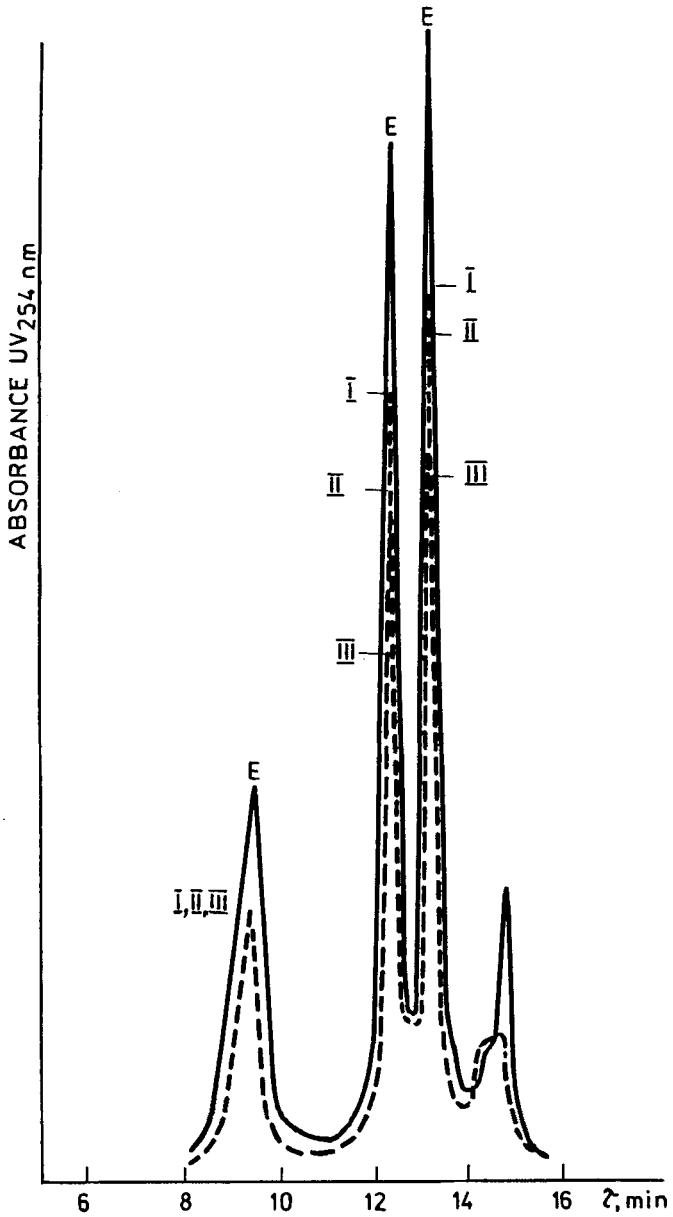

Fig. 2. SEC-chromatograms of the initial enzyme solution, containing *T. viride* culture filtrate, enriched with commercial xylanases (– E) and enzymatic hydrolysates after 10 min of processing of the fiber fractions (– –). I, maxima of the absorption peak for the long fiber fraction (Type I); II, absorption peak for a fraction consisting of both long and short fibers (Type II); and III, absorption peak for fines fraction (Type III).

according to the alkali soluble part of fibers, indicated a dependence of the changes on the type of fiber fraction (Table 2). The absorbance at different wavelengths remained practically unchanged for the long fiber fraction (Type I), but sharply increased for fines (Type III).

An increase in absorption at 313 nm (particularly for the fines fraction) indicated that the carbonyl group content calculated on the mass unit was increased.

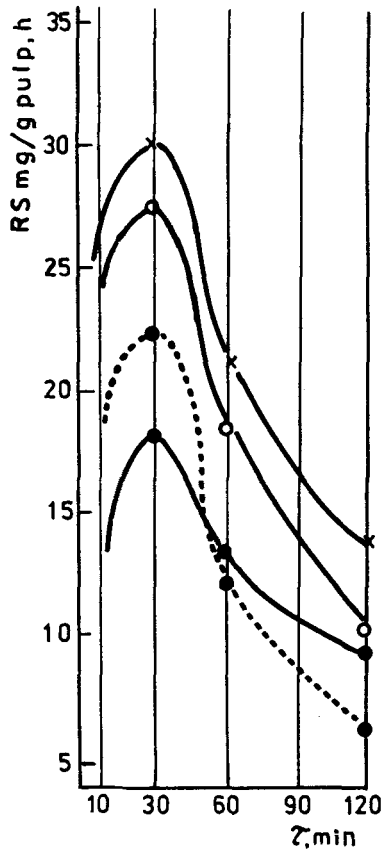

Fig. 3. Rate of hydrolysis as a function of the treatment time for different types of fiber fractions. (-●-) Type I, (-○-) Type II, (-x-) Type III, (—) unfractionated.

An increase at 405 nm, which usually refers to quinone-type substances, confirmed that an oxidation process probably took place in the fines fraction of fibers.

DISCUSSION

The cellulase complex in *T. viride* consists mainly of three enzymes. In processes of submerged cultivation in the Mandels-Andreotti media, these enzyme concentrations are usually as follows: 1.0 IU/mL FPA, 10 IU/mL endoglucanase, and 1.0 IU/mL β-glucosidase *(1)*. A doubling of the cellulase yield is possible either by increasing the cellulose *(20,21)* and nitrogen concentration or controlling pH during the growth *(22)* (or both). The addition of different concentrations of Tween-80 has a similar effect on FPA activities *(3)*. There are reports on the possibility of increasing the cellulase yield by the induction of cellulase synthesis by several soluble inducers, such as sophorose, cellobiose, and lactose *(5,23)*, and of reducing the adsorption action using soluble-substituted derivatives of cellulose, such as carboxymethylcellulose *(24)*. The synthesis of cellulolytic enzymes as regulated by the substrate metabolism has received less attention. The literature reports on the possibility of inducing the cellulase synthesis by affecting the general metabolic activity of the cells by lactose *(5)* or glucose *(25)*. Various organisms, includ-

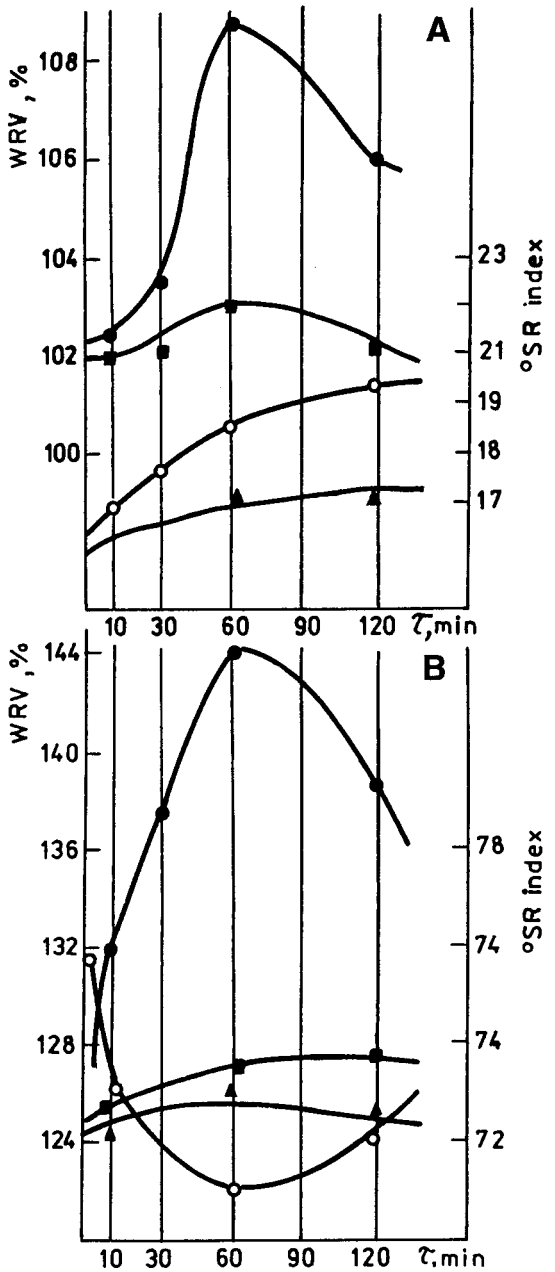

Fig. 4. Effect of enzymetic treatment on the long fiber (Type I) fraction **(A)** and unfractionated fiber **(B)** characteristics. WRV values: (-●-) enzyme treated, (-■-) control. Fiber freeness in °SR: (-○-) enzyme treated, (-▲-) control.

ing, *T. viride*, have been reported as sources for high cellulase and xylanase activities on cellulose and xylan-based media *(3)*. In our previous investigations, a relationship among the cell-energy-supplying substrate, the ATP intracellular concentration, and the exoenzyme activity was observed *(19)*. It was established that within the pulse of the additional carbon substrate, the respiration oscillation was followed by a rapid increase in the ATP intracellular concentration and a slightly slower

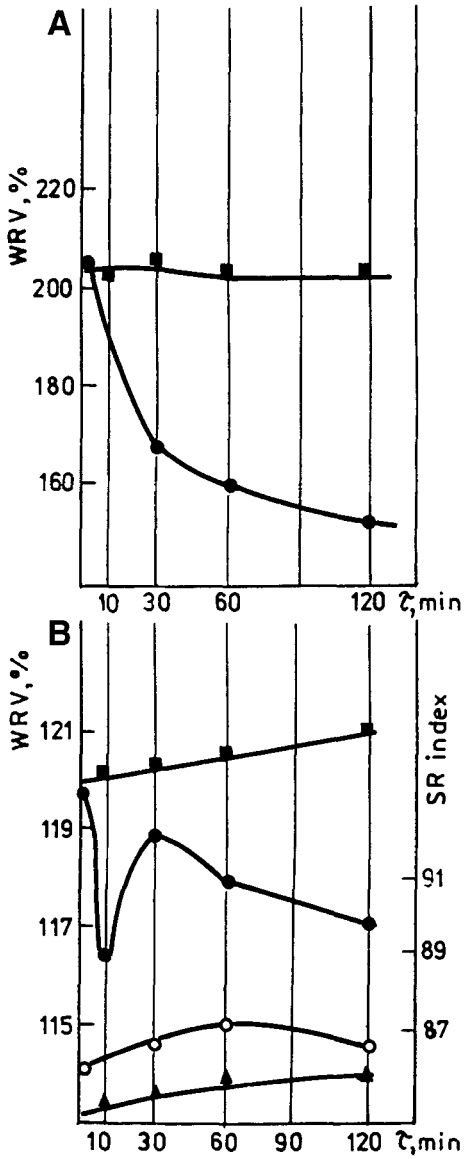

Fig. 5. Effect of enzymatic treatment on the fines (Type III) fraction **(A)** and fraction consisting of both long and short fibers (Type II) **(B)** WRV values: (–●–) enzyme treated, (–■–) control, fiber freeness in °SR: (–○–) enzyme treated, (–▲–) control.

increase in the exoenzyme activity *(19)*. A similar approach used in this study gave high activities of cellulase, EG, and xylanase during fed-batch cultivation of *T. viride* L-333. Our data on the cultivation of *T. viride* L-333 indicate that energy-rich carbon substrate feeding in the cellulose based fed-batch culture of *T. viride* could be an additional energy source and an inducer of extracellular enzyme synthesis to obtain high yields of both cellulase and xylanase.

The effects of enzymatic treatment on pulp characteristics has been studied by several authors. Stork and coauthors *(14)* attributed the shifting of the fibrous material composition from the long fiber fraction to the fines fraction to EG. It

Table 2
HP SEC Characteristics for Different Types of Fiber Fractions

Type of fibers	Time, min	Absorbance coefficient[a]							
		S^b5,%		290 nm		313 nm		405 nm	
		Buffer	Enzyme	Buffer	Enzyme	Buffer	Enzyme	Buffer	Enzyme
I	0	2.3	2.5	1.5	1.5	1.4	1.4	1.2	1.4
	30	2.3	2.3	1.5	1.6	1.4	1.6	1.3	1.5
	60	2.3	2.1	1.5	1.5	1.4	1.4	1.5	—
	120	2.3	2.1	1.8	1.5	1.7	1.5	1.7	—
II	0	2.4	2.6	1.5	2.0	1.3	1.4	1.2	1.7
	30	2.4	2.6	1.5	2.2	1.2	1.5	1.2	1.8
	60	2.4	2.6	1.5	2.1	1.1	1.5	1.2	1.9
	120	2.4	2.9	1.5	2.1	1.0	1.6	1.1	2.0
III	0	3.1	3.6	1.7	2.5	2.6	3.0	2.3	3.1
	30	3.1	3.2	1.8	2.7	2.6	3.3	2.7	3.2
	60	3.1	3.5	1.9	2.8	2.4	2.9	2.6	3.5
	120	3.1	4.1	2.0	3.1	2.2	3.8	2.8	3.7
Unfractionated	0	2.5	2.5	1.6	1.8	1.5	1.5	1.4	1.5
	30	2.6	2.7	1.6	0	1.6	1.6	1.4	1.7
	60	2.5	2.8	1.6	2.1	1.5	1.7	1.4	1.7
	120	2.4	2.7	2.7	2.4	1.4	1.7	1.2	1.8

[a]The ratio of absorbance to mass unit of soluble part.
[b]Solubility in 5% NaOH.

was found that the presence of the EG activity is a prerequisite for improvement of the WRV values of recycled fibers *(14)*. EG hydrolyzes amorphous cellulose randomly at its glucosid linkages into fragments. Celobiohydrolase (CBH) attacks the nonreducing end of both crystalline and amorphous cellulose macromolecula, releasing cellobiose *(26)*. When the activities of CBH and xylanase were presented, the EG effect was increased *(14)*.

The effect of EG and CBH on the cellulose microfibril structure has been investigated by transmission electron microscopy *(27)*. It was determinate that the EG action evokes the splitting of the microfibril structure into submicrofibrils. This leads to the appearance of whisker-like submicrofibrils at the cellulose microfibril periphery. According to Sprey and Bochem *(27)*, after the surface erosion initiated by EG, microfibrils were transformed into amorphous cellulose by CBH. This step occurred concomitant with an increase in the surface area in the submicrofibril part and an increase in water uptake into the intercrystalline structure *(27)*. Noe and coauthors *(28)* investigated the action of the crude xylanase complex on pulp, and concluded that the enzyme action can be summarized in terms of external and internal fibrillation. Our data confirmed the opinion that the fines fraction is preferable for the enzyme action, particularly when the enzymatic complex enriched in xylanases is used. The hydrolysis of the fines seems to be concomitant with an increase in the recycled newspaper brightness. The improvement of WRV and °SR values can be attributed to the hydrolysis of the long fiber fraction rather then the fines fraction, and it could be assumed that the improvement of drainage occurs owing to the EG activity, improved by its synergistical action with xylanase.

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