Reduction of Fines in Recycled Paper White Water *via* Cellulase Enzymes

Đorđe Jevtović,[a,*] Predrag Zivković,[a] Ana Milivojević,[b] Dejan Bezbradica,[b] and Luc Van Der Auwera [c]

Due to the high wastepaper recyclability and water-loop system closure, packaging paper mills struggle with increased fines, causing runnability issues. Cellulase enzymes are a preferred treatment choice for the improvement of the pulp refining in stock preparation area but are not widely used or easy to introduce in the production process. Different cellulase enzymes were tested, and those with the highest activity were introduced to the white-water (WW) samples with the aim to reduce fines content as potentially new enzyme applications on the paper machine. The first portion of the study involved the development of an experiment model to find and confirm the optimal enzyme process parameters (40 °C, pH 5.7, reaction time 3 h, and 0.18% v/v enzyme addition) for laboratory made white-water. The second portion of the study included turbidity, colloidal charge, flow cytometry (FCM), and chemical oxygen demand (COD) analysis on industrial and laboratory made white-water samples at optimized process parameters. Obtained results corresponded to reduced fines content in white-water samples, which justified commercial usage of cellulase enzymes on recycled paper machine short loop and potentially increased machine runnability without negative influence on wastewater treatment plant.

DOI: 10.15376/biores.19.1.635-655

Keywords: Cellulase enzymes; Fiber fines; Recycled paper; Flow cytometry; RSM optimization; Sustainability

Contact information: a: Department of Material Science, University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11120 Belgrade, Republic of Serbia; b: Department of Biochemical Engineering and Biotechnology, University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11120 Belgrade, Republic of Serbia; c: Solenis Technologies Germany GmbH, Paper Application Laboratory, 41066 Mönchengladbach, Germany;
** Corresponding author: 20194009@estudent.tmf.bg.ac.rs*

INTRODUCTION

Fines in a paper machine process are the fraction of the fiber furnish passing through a 200-mesh (76 μm) screen (Britt and Unbehend 1976; Sjostrom and Alen 1998). They are often rod-shaped, semicrystalline, fibrillar particles having widths of 0.1 to 0.5 μm and lengths up to several tens of micrometers (William 1996). Marton and Marton (1976) reported that the specific surface area of the fines is 5 to 8 times that of the fibers. This leads to a higher water absorption on the fines and potential undesired reactions with other interfering substances in the paper process water. Due to their small size, large specific surface area, and higher swelling, fines affect the paper sheet structure and properties in several ways (Seth 2003; Lee *et al*. 2011; Odabas *et al.* 2016; Gulsoy and Uysal 2020). A high fines concentration in water short loop could lead to poor water

clarification efficiency and in the paper forming section with reduced dewatering properties or low retention on the forming wire. Therefore, fines retention is a very important parameter relative to paper machine operations, and much work has been completed to select adequate retention polymers for efficient control (Van de Ven 2005). If the wire retention is not high enough, the fines and stickies will circulate in water short loop, generating an "unhealthy" situation with respect to potential deposit agglomeration. Fines, like stickies, often carry negative charge from the hydroxyl and carboxyl groups and together with cellulose fiber in water environment affect the overall system charge (William 1996; Smook 1999). Agglomeration of fines might possibly have adverse effects on end-use properties, such as dusting, printing, curl, *etc*. (Hubbe 2002). Fibers and fines management dictates the addition of majority additives in water short-loop and directly influences the total chemical oxygen demand (COD) values in the wastewater treatment plant. With reduced freshwater usage per ton of paper, it is becoming challenging for paper producers to maintain the wastewater COD levels and the desired paper quality. Papermaking system closure increases the number of fines and organic and inorganic substances in process water. This can significantly reduce the product quality and the process efficiency (Bajpai 2012).

Since the 1980s, the use of enzymes in pulp and paper industry has grown rapidly (Bajpai 1999). Bleaching with hemicellulase and xylanase, pitch control with lipase, viscosity control of size/film press, and pigment coating starches with amylase enzymes are standard commercial treatments in the paper industry nowadays. Bajpai (2011) summarized most enzymes treatments and their potential in pulp and paper industry. By adding enzymes directly in the pulp stock, the adsorption on fibers is fast and almost complete, so enzymes cannot be reused as reaction catalysators (Fischer and Messner 1992). Therefore, constant addition is needed to keep the desired activity in process.

Some authors decided to test enzymes effects, mainly lipase and esterase, in wastewater or process water of thermomechanical pulp (TMP) or recycled furnish (Xiao *et al.* 2000; Zhang *et al.* 2000; Dube *et al.* 2008; Liu *et al.* 2012b) with promising colloidal phase cleaning results in terms of reduced colloidal charge. Sundberg *et al.* (1998) showed that pectinase treatment substantially decreased anionic colloidal charge in a peroxide-beached mechanical pulp furnish. Others reported attempts to reuse the enzyme catalytic properties by immobilization on fiber surfaces (Zhang *et al.* 2017; Wu *et al.* 2019). Polymers with cationic charge were found to enhance cellulase hydrolysis on recycled fibers (Yu *et al.* 2018).

Cellulase enzymes hydrolyze 1,4-β glucoside bonds in cellulose fibers, leading to mono, di, and oligosaccharides (*e.g*., reducing sugars). Cellulases appear as multicomplex or mono component forms, and they react with different parts of cellulose chain (Couturier *et al.* 2016). Multicomplex cellulase products can contain up to three forms: endoglucanase (EG), cellobiohydrolases (CBH), and β-glucosidases (BG). Only multicomplex cellulase containing all three mentioned activities can effectively degrade cellulose fiber down to glucose monomer (Knezevic-Jugovic 2008). Glucose does not ionize in water, and therefore it does not contribute to the system charge (Williams 1996). This means that creation of glucose and reducing sugars leads to reduced colloidal charge. With increased concentration of soluble reducing sugars in system, theoretically, the number of nephelometric turbidity units (NTU) should be reduced. All mentioned parameters lead to a cleaner colloidal water phase, creating a healthier environment for improved paper production efficiency.

Cellulase enzymes have mainly been used directly in pulp for achieving higher drainage with potentially improved paper mechanical properties (Maximino *et al.* 2011; Kim *et al.* 2017). Endoglucanase activity can effectively catalyze hydrolysis of crystalline and amorphous structure of cellulose and reduce the refining energy (Nagl *et al.* 2022). Kamaya (1996) showed that commercial cellulase from *Trichoderma reesei* with dominant endoglucanase activity can boost dewatering properties and reduce the refining energy in bleached kraft pulp. Verma *et al.* (2015) reached the same conclusion by comparing different cellulase enzyme activities on wood-free bleached recycled pulp and the enzyme's influence on dewatering and final paper mechanical properties such as tensile strength and smoothness. Rashimi and Nishi (2010) summarized one decade of laboratory results from other authors, with cellulase and hemicellulase usage in recycled pulp, and concluded that mainly in acidic conditions and temperatures higher than 50 °C with 30 min contact time, cellulase enzymes usually contribute to paper process with higher dewatering and drainage properties. Later, response surface methodology (RSM) on three levels with three factors was performed by Liu *et al.* (2012a) on recycled pulp with the aim to optimize the enzyme dosage for the highest possible drainage and lowest required beating energy consumption with improved fiber flexibility. Min *et al.* (2015) used cellulase enzymes to convert fines from recycled paper waste rejects into sugars for biofuels or bioplastic. Calcium carbonate was noted as a potential hydrolysis reaction inhibitor.

The goal of this study was to test cellulase enzymes in white-water (WW) (instead of traditional pulp slurry addition) generated from recycled paper, with the aim to reduce the fines content as potentially new enzyme application area. Paper forming wire filtered water or WW was selected for analysis as representative short loop process water from paper production.

EXPERIMENTAL

Materials

The wastepaper for lab WW sample was supplied from the raw material stockyard of a local paper producer. To simulate the industrial pulp conditions, the chemical additives supplied by Solenis Technologies Germany were added in the formulation: dry strength cationic polymer (Xelorex RS1200), sizing aid (Aquapel J220), wet-strength polymer (Kymene 720), and polyvinyl acetate-based glue sample from Mitol Company (Mekol 1413/G).

Table 1. Wastepaper and Chemical Additive Formulation for Lab WW Sample

Wastepaper Type	Weight (g)	Chemical Additive	Chemical Addition (g)
Old corrugated container (OCC)	50	Xelorex RS1200	1.0
Printed coated paper	10	Aquapel J220	0.3
Monoglazed paper (MG paper)	10	Kymene 720	0.5
Coated OCC	5	Mekol 1413/G	0.8
Office paper or copy paper	5		
Heavy coated copy paper	5		
Newspaper	4		
Polyethylene coated OCC	10		
Mass paper with adhesive coating	1		

Industrial WW sample was collected from the local recycled paper mill production in Germany with 0.23% consistency and 6.25 pH value. The buffers for water samples were made with citric acid (Lachema, Czech Republic), trisodium citrate dihydrate (NRK engineering, Serbia), disodium hydrogen phosphate (Centrohem d.o.o. Serbia), sodium dihydrogen phosphate (Centrohem d.o.o. Serbia), sodium hydroxide (Centrohem d.o.o. Serbia), and hydrochloric acid (Lach-Ner, Czech Republic).

Carboxymethyl cellulose (CMC) was supplied by Sigma Aldrich, USA, and 3,5-dinitrosalicylic acid (DNS) by Acros Organic, UK. Three cellulase enzymes, supplied by Solenis Technologies Germany were used for experiments. The enzymes are referred to as *Hercobond A, Hercobond B,* and *Hercobond C*. The supplier defined the first two enzymes as multi complex cellulase. *Hercobond B* has addition of cationic charge in its formulation for improved fiber bonding, and *Hercobond C* consists mainly of enzymes having endoglucanase activity.

Methods

Experimental design

Three commercial cellulase enzymes supplied by Solenis Technologies Germany GmbH were tested, namely: *Hercobond A, Hercobond B,* and *Hercobond C.* After determining the cellulase activity, the enzyme with the highest activity value was selected for further analysis. The enzyme was introduced in specially formulated laboratory (lab) WW, which contained the fines and "dead fibers" as the main cellulosic material. The term "dead fibers" refers to recycled fiber fractionations caused by high shear forces in the course of their redispersion in a disintegrator. The presence of cellulosic material was established with Fourier-Transform Infrared spectroscopy (FTIR) analysis on the dry lab WW residue. As an outcome, changes in the concentration of reducing sugars (CORS) were followed as a function of different process variables: temperature, enzyme concentration, reaction time, and pH value. The ranges selected corresponded to industry averages, and they were in line with ranges used in previously mentioned articles. Generated experiment data were used for the RSM experiment model creation with four variables on five value levels, precisely predicting CORS on given process parameters.

The obtained parameters for the highest CORS generation were used as an optimum experiment set up for future experiments including: (1) WW turbidity, (2) charge demand or colloidal charge, (3) flow cytometry (FCM) as a potentially new method for fines content monitoring, and (4) COD to investigate influence on wastewater treatment. The mentioned methods were applied on two different samples for comparison: lab made and industrial (ind.) WW, from the local recycled paper mill production as a real-life sample. To assess the enzyme selectivity and the CORS change, High Performance Liquid Chromatography (HPLC) was used on selected lab-generated WW samples.

Lab WW sample preparation

The paper and chemical mixture were mixed in a lab TAPPI disintegrator (FRANK-PTI, Germany) for 25 min at 3000 rpm, and then dispersed in 2 L of lab tap water to reach 5% consistency. The obtained pulp was filtered through a steel 200-mesh and the collected filtrate with pH values of 7.56 and 0.019% consistency was used for the tests as lab WW.

Enzyme addition in WW samples

Two WW samples (lab, ind.) were placed in 600 mL beakers on defined experiment parameters with a magnetic stirrer consistently mixing at 170 rpm. The selected enzyme

was added in both samples based on substrate concentration to reach the same activity per g of substrate. At defined intervals of reaction time, samples were taken for the turbidity, PCD, FCM, and COD measurements.

Enzyme selection method

For enzyme selection, a preliminary experiment was performed. Enzymes were added (0.1% v/v) to 50 mL of lab prepared WW and left for 24 h on 40 °C, after which the turbidity was measured. The temperature was selected based on the most common temperature of the wet end area on the packaging paper machine.

FTIR

Lab-generated WW sample was filtered through a 2 µm glass microfiber filter supplied by Fitlers Fioroni, and the substrate was dried at 105 °C for 2 h. The dry substrate was measured on an FTIR Nicolet iS10, USA, with the ATR mode.

Cellulase enzyme activity test method

The cellulase activity was determined spectrophotometrically by monitoring the CORS in reaction of the CMC hydrolysis using the DNS method (Maximino *et al.* 2011). Properly diluted 0.5 mL sample was mixed with 0.5 mL of the DNS reagent. After 5 min of incubation in boiling water, 4 mL of distilled water was added, and the samples were vortexed. The absorbances against the blank were measured at 540 nm using a Ultrospec ™ 3300, Germany spectrophotometer. The blank was prepared in the same manner using water or buffer instead of a sample.

The CORS was determined using a glucose standard curve, and the results were expressed as mM glucose equivalents.

The substrate used in the reaction was 2% (v/v) solution of CMC in 50 mM citrate buffer pH 4.8. The reaction was performed in a thermostatic water bath at 50 °C. The reaction was initiated by the addition of 0.1% (v/v) enzyme to the substrate solution and samples were taken (250 µl) in previously determined time and analyzed using the DNS method.

The activity was calculated using Eq. 1,

$$Act\ \left(\frac{IU}{ml}\right) = R * \frac{\frac{dA}{dt} * V_{rs}}{k * V_e} \quad (1)$$

where *Act* is enzyme activity (IU/ml), *R* is dilution factor, *A* is absorbance, *t* is time (s), V_{rs} is volume of reaction (mL), *k* is slope of the standard curve**,** and V_e is enzyme volume (mL).

RSM analysis

Statistical experiment planning and RSM were used to optimize the reaction parameters (Milivojevic *et al.* 2019). It is possible to investigate the influence of several parameters using the process with a relatively small number of experiments, which can significantly reduce the time and budget of the research. Four variables were analyzed with a central composite rotatable design experiment plan (CCRD) on five value levels. The base characteristic is that the dispersion of the model obtained with the plan is constant in all points from the hypersphere radius $\sqrt{\sum {X_i}^2}$, and it is independent of the direction in the multifactor experimental space, but only from the radius of the factor space. This means

that all points equidistant from the central point of the experimental plan have the same amount of information contained in the resulting model.

The obtained experimental results were modeled using a second-order equation (2) which includes variables and interactions between different factors,

$$Y = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} X_i + \sum_{i=1}^{n} \beta_{kii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} X_i X_j \quad (2)$$

where Y is the response, β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant regression coefficients, X_i and X_j are independent variables, k is number of single factors, and n is the number of factors.

The values of the coefficients and their statistical significance evaluation were determined by the least-squares method using Design-Expert program version 13 (Stat-Ease Inc. Minneapolis). The adequacy of the obtained model was tested using the Fisher test, while the Student distribution was used to estimate the significance of the coefficients. Only the significant coefficients ($p \leq 0.05$) were considered for the final reduced models. This experiment plan included 30 experimental points: 16 factorial, 8 axial, and 6 centrals. The influence of four factors was examined in the following ranges: reaction time (1 to 3 hours), temperature (20 to 40 °C), enzyme concentration (0.02 to 0.18% (v/v)), and pH value (4 to 8). The selected range corresponds to paper industry average values, and all out of this range are considered not to be realistic for research.

20 mL of lab WW sample was introduced in a 100 mL Erlenmeyer flask. The pH of the samples was adjusted with buffers in accordance with the experimental plan. The enzyme was added in every Erlenmeyer flask in the previously mentioned concentration, depending on experiment number. The Erlenmeyer flasks were introduced inside a thermostatic vibrating shaker (IKA KS 4000i), which operated at 170 rpm, and the temperature was set also in accordance with the experimental plan. After the defined reaction period, the samples were taken from reaction mixture for the total reducing sugars measurements. Supernatant, for selected experiments, was taken for HPLC analysis. The reference samples (without enzyme addition) with different pH values had the same reaction time and temperatures as the samples with the enzymes. The measured CORS value is the difference between the reference and the experiment concentration, so it characterizes the enzyme contribution in the substrate hydrolysis.

HPLC analysis

Composition mixture and concentration change of mono- and oligosaccharides in selected samples were analyzed using Dionex Ultimate 3000 high performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Valtam, SAD) and carbohydrate column (Hi-plex Ca^{2+}, 300 mm × 7.7 mm, 8µm) at 80 °C. As a mobile phase, HPLC grade water was used with flow rate of 0.6 mL/min. The detection was performed with an RI detector (RefractoMax 520) at 40 °C. The data collection and analysis were run through the Chromeleon 7.2 Data System program (Thermo Scientific).

Turbidity measurements

The turbidity was measured using a portable turbidity device WTW 320 with a measuring range up to 1100 NTU at 280 nm. At the end of the reaction, 5 mL of sample was poured into 15 mL of distilled water, which was in the cuvette and measured in the turbidity device. The reading was multiplied by the dilution factor.

Charge demand analysis

A BTG, Mütek PCD 05 device was used for the colloidal charge analysis. A 0.001 M polydiallyldimethylammonium chloride (polyDADMAC) cationic polymer solution was used as the titration medium. Before the reaction, 10 mL of sample was introduced in the PCD device chamber. After the titration reached an endpoint of zero, the value provided on device was multiplied by 100 and marked in μeq/L units, presenting the amount of titrant needed for charge neutralization.

FCM analysis

The Sysmex Cube 6 V2m device was used for the flow cytometry measurements. During the enzyme reaction, 2 mL of samples were taken and filtrated through Celtrix 50 μm mesh filter. The filtered samples were mixed *via* a vortex mixer, and 10 μL were pipetted into the sample tube with addition of 7.5 μL Nile red reagent supplied by Sysmex. After 5 min exposure time to the Nile red dye, the mixture was diluted by 1 mL of distilled water, mixed with a vortex mixer, and then measured.

COD analysis

Hach LCK114 and LCK014 kits were used for COD analysis. A two mL of sample was mixed with reagent in a cuvette and digested at 148 °C for 2 hours in the heating block (HACH HT200S). After cooling down to 20 to 25 °C, the COD measurements were completed on HACH DR6000 spectrophotometric device at 420 nm wavelength.

RESULTS AND DISCUSSION

Enzyme Selection

The lab WW dry content was analyzed with the FTIR device, which indicated the cellulose structure presence in the sample (Fig. 1), confirming cellulase enzymes as suitable treatment for the investigated substrate.

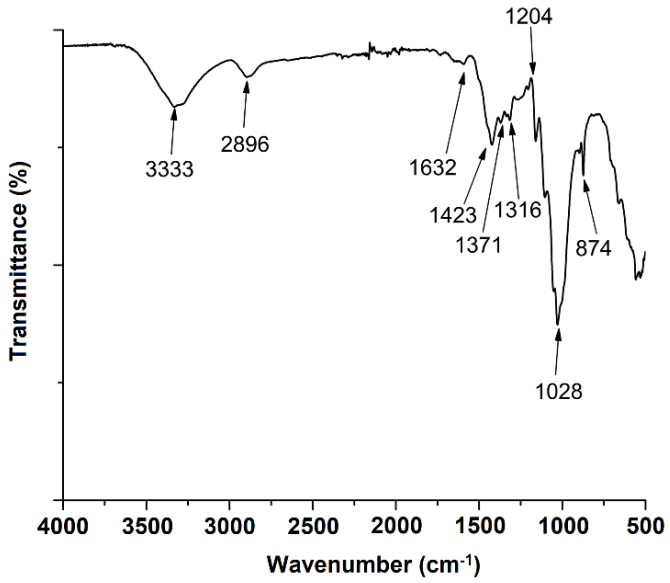

Fig. 1. Fourier-Transform Infrared spectroscopy diagram of filtered and dried lab WW sample

The stretching vibration of a hydroxyl group (O-H) was observed at 3333 cm^{-1} (Choe *et al.* 2019; Chung *et al.* 2004). The symmetric and asymmetric C-H bond present in the polysaccharide structure appeared in the 2916 to 2850 cm^{-1} region. The small peak at 1632 cm^{-1} was assigned to the deformation vibrations of the O-H groups. Other bands related to C-H bending and wagging vibration of the methylene and methyl groups, O-H in-plane bending, as well as asymmetric and symmetric vibration of the C-O-C and C-O groups, appeared at 1423, 1371, 1316, and in the 1204 to 1028 cm^{-1} region, respectively. The vibration at 874 cm^{-1} originates from the β-glucosidic bond (Chung *et al.* 2004).

With the aim to select the most suitable enzyme for research, the activity of enzymes was measured as a preliminary experiment at two different pH values, as shown in Fig. 2. The cellulase enzymes are known to have the highest activity at acidic pH conditions (Pardo and Forchiassin 1999). Recycled paper production pH is close to neutral; therefore, the activity was also performed at pH 7.

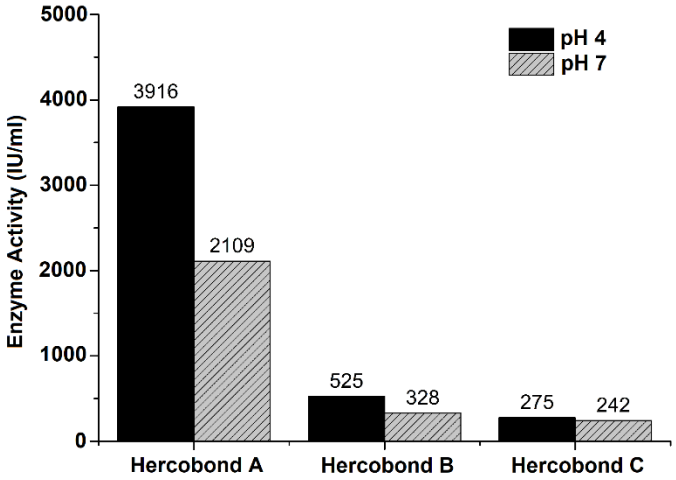

Fig. 2. Activity of cellulase enzymes on different substrate pH values

Hercobond A exhibited the highest activity at both pH values. *Hercobond C* revealed the lowest activity and relatively the most stable activity at both pH values.

Lower turbidity values are indicative of a cleaner water phase in the paper industry. Higher turbidity values are indirectly connected with lower machine runnability, issues with dewatering on forming wire and dusting effects in the paper drying section. Table 2 shows the turbidity results from a preliminary experiment after 24 h of enzyme reaction. The turbidity values were lower under acidic pH conditions, in line with the results obtained in other studies (Sarja *et al.* 2004).

Table 2. Turbidity of Lab WW Samples after 24 h on 40 °C

pH	Sample	Turbidity (NTU)
4.5	Referent sample	452
	Hercobond A	251
	Hercobond B	302
	Hercobond C	303
7.0	Referent sample	882
	Hercobond A	436
	Hercobond B	569
	Hercobond C	763

The turbidity results followed the enzyme activity values and confirmed the high interaction of used substrate and *Hercobond A*. This enzyme was selected for the further investigation of influence on the different process parameters by using RSM.

Optimization of experimental factors of process water enzyme treatment

RSM methodology was performed in the lab-prepared WW sample. All factors and process variables range are presented in Table 3, and the obtained CORS results are shown in Table 4.

Table 3. Four Variated Process Parameters Used for RSM Modeling

Factors	Coordinated Values				
	-2	-1	0	1	2
Temperature, X_1 (°C)	20	25	30	35	40
pH, X_2	4	5	6	7	8
Enzyme Concentration, X_3 (%, v/v)	0.02	0.06	0.10	0.14	0.18
Reaction Time, X_4 (h)	1	1.5	2	2.5	3

Table 4. RSM Experiment Results

Number	Temperature (°C)	pH	Enzyme Concentration (%, v/v)	Time (h)	CORS (mM)
1.	-1 (25)	-1 (5)	-1 (0.06)	-1 (1.5)	1.14
2.	1 (35)	-1 (5)	-1 (0.06)	-1 (1.5)	1.29
3.	-1 (25)	1 (7)	-1 (0.06)	-1 (1.5)	1.66
4.	1 (35)	1 (7)	-1 (0.06)	-1 (1.5)	0.95
5.	-1 (25)	-1 (5)	1 (0.14)	-1 (1.5)	1.90
6.	1 (35)	-1 (5)	1 (0.14)	-1 (1.5)	2.35
7.	-1 (25)	1 (7)	1 (0.14)	-1 (1.5)	1.66
8.	1 (35)	1 (7)	1 (0.14)	-1 (1.5)	2.28
9.	-1 (25)	-1 (5)	-1 (0.06)	1 (2.5)	1.82
10.	1 (35)	-1 (5)	-1 (0.06)	1 (2.5)	2.57
11.	-1 (25)	1 (7)	-1 (0.06)	1 (2.5)	1.02
12.	1 (35)	1 (7)	-1 (0.06)	1 (2.5)	1.90
13.	-1 (25)	-1 (5)	1 (0.14)	1 (2.5)	2.02
14.	1 (35)	-1 (5)	1 (0.14)	1 (2.5)	3.16
15.	-1 (25)	1 (7)	1 (0.14)	1 (2.5)	1.70
16.	1 (35)	1 (7)	1 (0.14)	1 (2.5)	3.25
17.	-2 (20)	0 (6)	0 (0.10)	0 (2.0)	1.70
18.	2 (40)	0 (6)	0 (0.10)	0 (2.0)	2.32
19.	0 (30)	-2 (4)	0 (0.10)	0 (2.0)	1.14
20.	0 (30)	2 (8)	0 (0.10)	0 (2.0)	0.90
21.	0 (30)	0 (6)	-2 (0.02)	0 (2.0)	0.74
22.	0 (30)	0 (6)	2 (0.18)	0 (2.0)	2.98
23.	0 (30)	0 (6)	0 (0.10)	-2 (1.0)	1.12
24.	0 (30)	0 (6)	0 (0.10)	2 (3.0)	2.25
25.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	1.80
26.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	1.59
27.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	1.46
28.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	1.89

Number	Temperature (°C)	pH	Enzyme Concentration (%, v/v)	Time (h)	CORS (mM)
29.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	1.73
30.	0 (30)	0 (6)	0 (0.10)	0 (2.0)	2.00

After the elimination of terms that were not statistically significant, the final second-order regression model that described the influence of reaction factors on the CORS, Y (equation 3) was as follows:

$$Y = 1.94 + 0.2529X_1 - 0.0963X_2 + 0.4354X_3 + 0.2696X_4 + 0.1681X_1X_3 + + 0.2381X_1X_4 - 0.1598{X_2}^2 \quad (3)$$

Therefore, the results of the experiment showed that all linear coefficients in the model were significant and that the square regression coefficient β_{22} was also significant. The quadratic coefficient had a negative value, so its influence on the CORS can be described by a quadratic function with a maximum. Liu *et al.* (2012a) had a quadratic function with a minimum but a different substrate and cellulase enzyme were used.

In addition, the presented model showed a positive interaction between temperature (X_1) and enzyme concentration (X_3) as well as between temperature (X_1) and reaction time (X_4).

The highest change in CORS in comparison with the reference sample (3.25 mM) was obtained in experiment number 16 at 35 °C and pH 7 with 0.14% (v/v) enzyme addition and 2.5 h reaction time. The lowest change in CORS (0.74 mM) was noted in experiment number 21 on 30 °C, pH 6, 0.02% volumetric enzyme addition and 2 h reaction time.

The effects of the most interesting pairs of factors on the CORS are illustrated by the three-dimensional diagrams of response surfaces, as well as by the contour diagram (Figs. 3 to 6). The effect of pH (X_2) illustrated a bell-shaped curve with maximum at pH 5.7 (Fig. 5 and 6), independent of values of other factors because X_2 did not show any statistically significant interaction with the other factors (Eq. 3). The effect of temperature (X_1) is defined only by positive linear coefficient (0.2529); hence it can be seen (Fig. 4 and 6) that the CORS increased linearly with the increase of temperature throughout the examined temperature range.

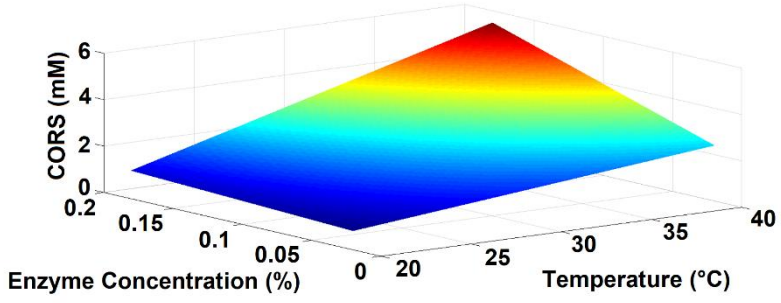

Fig. 3. Influence of enzyme concentration and temperature on CORS

Also, due to positive interactions with enzyme concentration (β_{13}=0.1681) and reaction time (β_{14}=0.2381), this increase became steeper with the increase of these two factors. Similarly, the concentration of reducing sugar increased along the increase of

enzyme concentration (Fig. 3) and the reaction time (Fig. 4) due to positive linear coefficients β_3=0.4354 and β_4=0.2696, respectively. Also, due to the already mentioned positive interaction, the increase became steeper at higher temperatures.

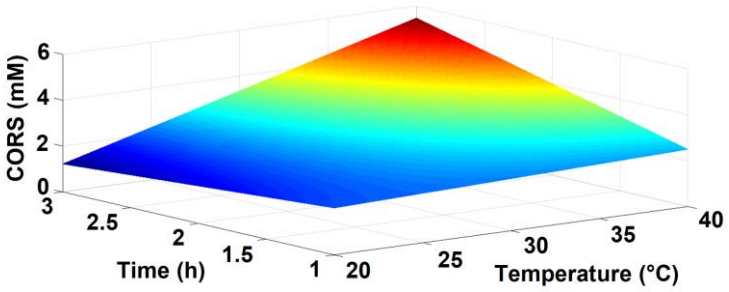

Fig. 4. Influence of time and temperature on CORS

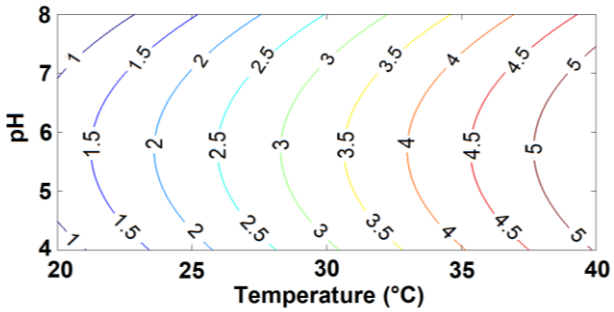

Fig. 5. Contour diagram; Influence of pH and temperature on CORS

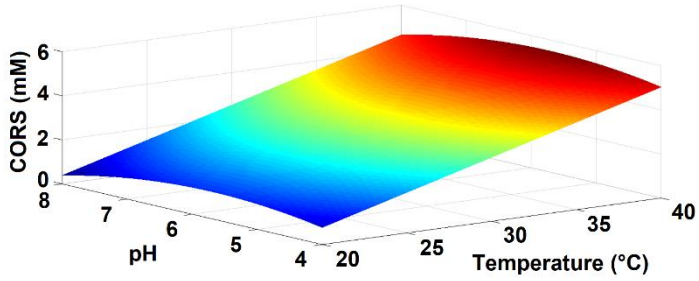

Fig. 6. Influence of Temperature and pH on CORS

Based on the generated model, it is possible to predict that concentration of the reducing sugars, at the optimum reaction parameters (temperature 40 °C, pH 5.7, 0.18% (v/v) enzyme and 3 h reaction time), would be 5.50 mM.

RSM model evaluation

To evaluate the adequacy and validity of the obtained model for the influence of the experimental factors on the CORS, additional experiments were performed under the optimal conditions (at a temperature of 40 °C, at a pH of 5.7, and enzyme concentration of 0.18% v/v and at a reaction time of 3 h). A representative sample was prepared in the lab, with CORS of 5.23 mM, which represents a relative error of the model of 4.9%.

The results achieved in these experiments indicated that the model (Eq. 3) provided a good prediction and that the application of statistical planning in the optimization of reaction parameters was fully justified.

Hercobond A in 0.18% volumetric addition represented 25000 to 27000 IU/g calculated on the substrate concentration. This activity, with other process parameters from RSM optimization, was used in further work.

Results of HPLC analysis

The change in the concentration of mono- and oligosaccharides in the selected samples was also monitored by high-performance liquid chromatography.

Figure 7 presents the superimposed chromatograms from the analysis of samples of the enzymatically treated lab WW. Filtrate samples obtained after 1, 2, and 3 h of the enzymatic reaction at a temperature of 30 °C, at a pH of 6 and an enzyme concentration of 0.1% v/v were analyzed together with reference sample on given parameters (0 h). WW samples correspond to experiment number 23 (1 h), 24 (3 h), and 25 (2 h) from the Table 4. The mentioned experiment numbers had the middle value of investigated RSM variables range, and as such gave clear insight into CORS distribution.

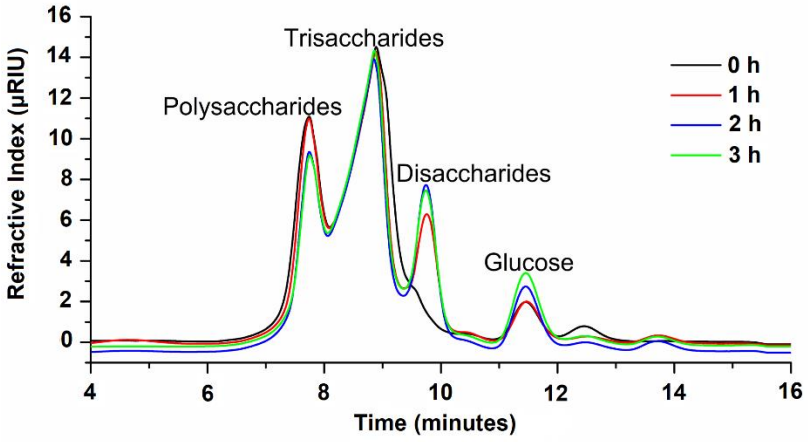

Fig. 7. Changes in the concentration of mono- and oligosaccharides over time in selected samples of treated lab WW

The attached chromatogram revealed the concentration of monosaccharides, most prominently glucose, to increase steadily throughout the hydrolysis. The disaccharide concentration increased steeply in the first hour of hydrolysis, then it slowed down due to the simultaneous formation by hydrolysis of longer oligosaccharide and degradation by their hydrolysis to monosaccharides. This result may give a hint regarding enzyme activity structure. The increase in glucose concentration indicated the presence of BG, and the increase in disaccharides concentration indicated the presence of CBH component in *Hercobond A*. The concentration of the other oligosaccharides (tri-, tetra- and higher) changed insignificantly, which was probably due to the equilibrium between their formation by hydrolysis catalyzed by EG within preparation and degradation by CBH.

Results of turbidity testing

Lower turbidity values in WW samples are indicative for a cleaner water phase and less impurities. Such impurities can harm the paper production operation, and potentially

there will be a need to increase the retention polymers dosage, thereby increasing production costs.

The trend of turbidity values as a function of reaction time and dependent on RSM optimized parameters (at a temperature of 40 °C, at a pH of 5.7, and enzyme concentration of 0.18% v/v and during a reaction time of 3 h) are shown in Fig. 8. Values represent two experiment's average results with standard deviation.

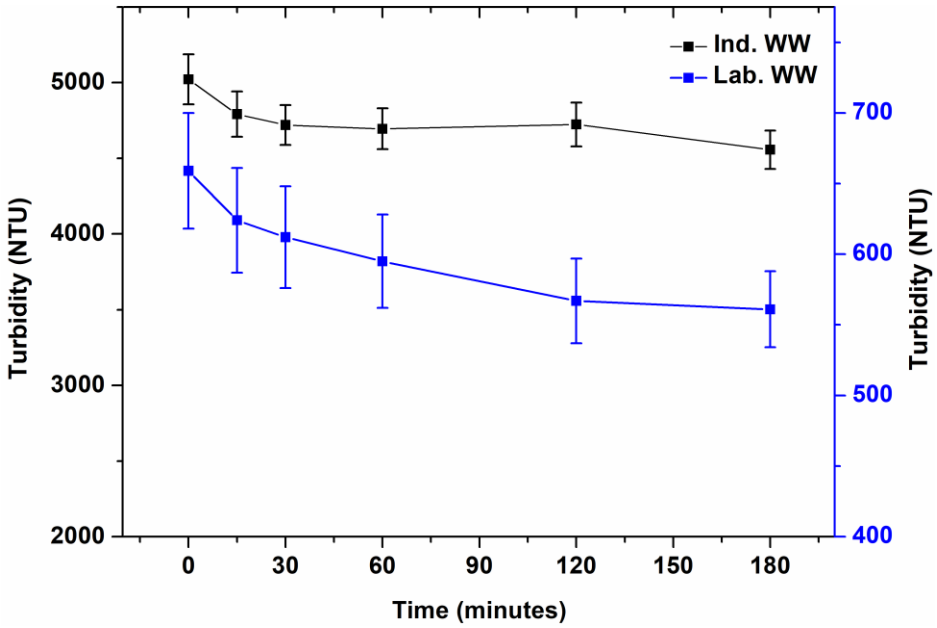

Fig. 8. Turbidity in function of reaction time

The turbidity in the ind. WW sample was far higher compared to the lab generated WW sample due to higher load in the short-loop water of the paper machine circulation and the higher WW concentration. Still, in both cases, a stable downwards trend was noticeable.

After 180 min, the turbidity was reduced by 9.26% on ind. and 14.87% on lab WW compared to reference value (0 min), with a constant slope. The highest turbidity reduction on both samples was noted within the first 15 min of reaction indicating a fast enzyme-substrate interaction.

With increased sugar concentration in the sample, the water-soluble materials content increased, leaving less particles to cause turbidity in the water phase.

Charge titration measurement results

Lower colloidal charge values are indicative for a cleaner water phase and less anionic trash substances (fines included), which usually have negative influence on the machine retention and wire dewatering properties. Low colloidal charge leads to lower cationic additive usage on paper machines, therefore lower costs, and less COD to wastewater plants.

The colloidal charge value dependency on reaction time with standard deviation on RSM optimized parameters (at a temperature of 40 °C, at a pH of 5.7, and enzyme concentration of 0.18% v/v and during a reaction time of 3 h) is depicted in Fig. 9. The values represent an average from two experiments. In both curves, one could notice a decreasing trend. The reference values (0 min) for WW samples were different. The ind.

WW sample had more anionic charge due to more impurities in the real-life system, and this corresponds to the higher turbidity values, as noted earlier.

Hercobond A carries a negative charge. Therefore, during the first 15 min of reaction the charge was more negative in the ind. WW sample compared to reference value. This sample had approximately 12 times higher concentration in comparison to the lab sample, so more enzyme was added volumetrically with the same end activity per g of substrate.

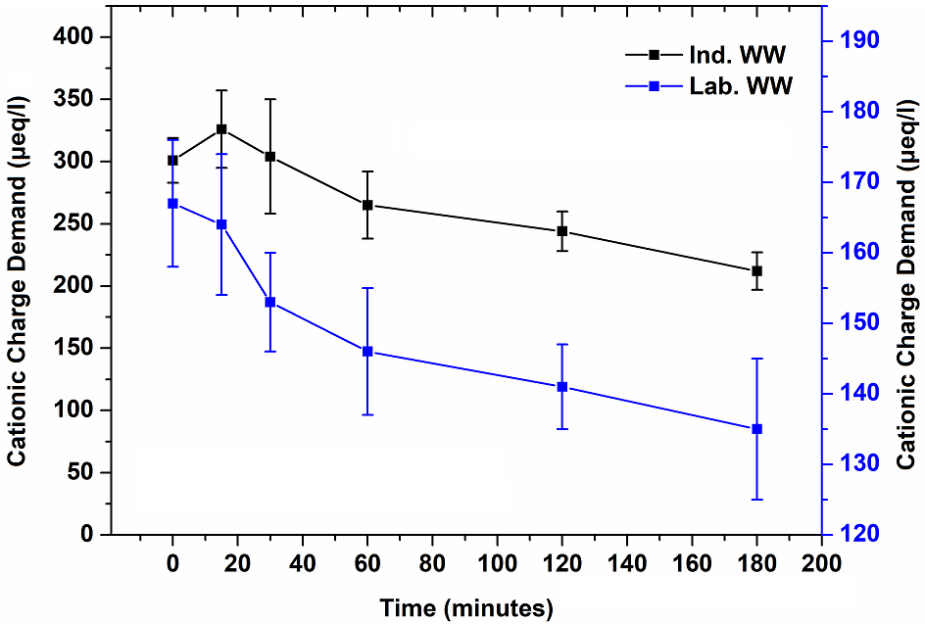

Fig. 9. Colloidal charge as a function of reaction time

The ind. WW sample had a charge reduction of 29.6% and the lab WW sample had a charge reduction of 19.2% after 180 min of reaction compared to referent values (0 min).

As *Hercobond A* consumes fines, producing water-soluble sugars, the total number of $RCOO^-$ (sugars carboxylate groups) is reduced in the colloidal phase, leading to reduced cationic charge demand values.

A similar colloidal charge trend was noted by Liu *et al.* (2010), where immobilized pectinase enzymes were used on bleached softwood thermo-mechanical pulp.

Flow cytometry

Flow cytometry analysis measures the number of particles, along with their size, in size range from 0.1 to 50 µm, and indirectly via staining the particle's hydrophobicity. In the paper industry, this analysis is mainly used to track micro-stickies size and number in the colloidal water phase (Grubb *et al.* 2009). Considering the average fines size, this method can give insight into the enzyme reaction with the fines. The main flow cytometry parameters were on particle number (Forward scatter, *FSC*) and particle hydrophobicity (Intensity of fluoresce at > 630 nm, *FL3*). The results are presented in Tables 5 and 6 on RSM optimized process parameters. The trends were similar in both cases. There was an overall increase in particle number after the enzyme addition in both samples.

The fiber classification in terms of response combinations of particle count and "hydrophobicity" in a matrix of 4 squares were defined as: Q1 (low FSC, high FL3); Q2 (high FSC, high FL3); Q3 (low FSC, low FL3); Q4 (high FSC and low FL3); and RN1, the

sum of Q1 + Q2, line of positive particle population or number of particles with high dye fluorescence intensity. Again, the difference between the lab and the ind. WW sample was visible. The industrial sample had approximately 12 times higher load of particles in the measured range, following the WW concentration difference.

Table 5. FCM Results for Lab WW sample; Q1 (low FSC, high FL3); Q2 (high FSC, high FL3); Q3 (low FSC, low FL3); Q4 (high FSC and low FL3); and RN1, the sum of Q1 + Q2

No	Reaction Time (min)	Particle number	RN1	Q1	Q2	Q3	Q4
1.	0	101860	34991	28071	6920	62936	3933
2.	15	138687	65424	48111	17313	68591	4672
3.	30	143176	69826	50586	19240	68285	5065
4.	60	156301	73792	53055	20737	77177	5332
5.	120	197202	101618	71158	30460	89972	5612
6.	180	189228	84823	57305	27518	98321	6084

Table 6. FCM Results for Ind. WW Sample; Q1 (low FSC, high FL3); Q2 (high FSC, high FL3); Q3 (low FSC, low FL3); Q4 (high FSC and low FL3); and RN1, the sum of Q1 + Q2

No	Reaction time (min)	Particle number	RN1	Q1	Q2	Q3	Q4
1.	0	1718325	55648	35805	19843	1652014	10663
2.	15	1729932	236559	171322	65237	1478821	14552
3.	30	1634520	196920	155373	41547	1431054	6546
4.	60	1722313	263024	211355	51669	1452358	6931
5.	120	1993594	213691	143233	70458	1765046	14857
6.	180	1713731	173545	96746	76799	1524517	15669

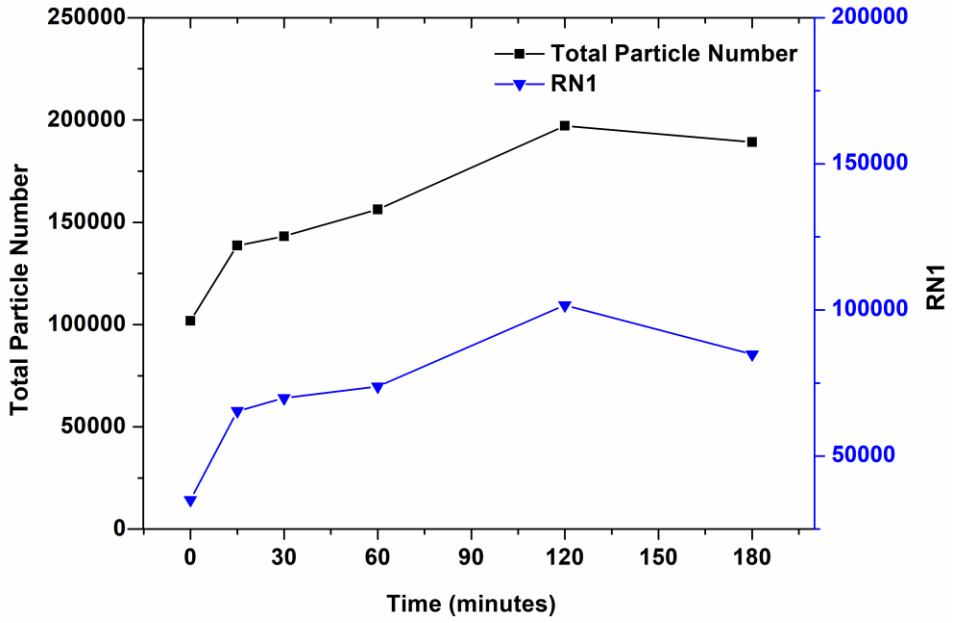

Fig. 10. FCM lab WW sample; Total particle number and RN1 value in function of reaction time

On both samples, the total number of particles peaked at 120 min of reaction time, after which it started to decline. This trend was in agreement with the previous results of HPLC analysis (Fig. 7), where after 120 min of reaction a strong increase in disaccharide and glucose concentration was recorded.

Both disaccharide and glucose are water soluble and originate from the consumed fines, so the total particle number was reduced after a certain time as particles (fines) were becoming shorter.

An increased number of particles was visible in all squares. However, in the first 60 min of reaction for ind. WW and 120 min for the lab WW, the dominant increase was in Q1 and Q2 (RN1), indicating an increased particle hydrophobicity. *Hercobond A* actively consumed hydrophilic parts of substrate, so during the reaction, the total hydrophobicity share was increasing. Later during the reaction, the particles number was increasing in Q3, indicating a shorter fines length.

The lab WW sample showed a strong Q1 and Q2 (RN1) increase followed by a noticeable increase in Q3 and Q4 corresponding exactly to total particle number (Fig. 10).

The industrial sample had a steadier total particle trend for the first 60 min of reaction with a strong increase in Q1 and Q2 (RN1) values (Fig. 11).

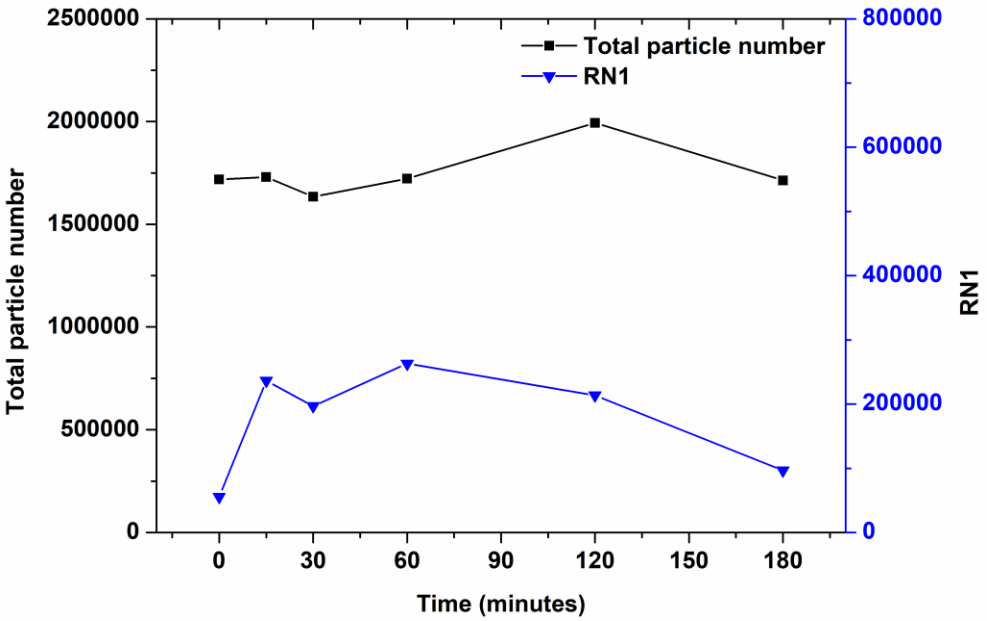

Fig. 11. FCM ind. WW sample; Total particle number and RN1 value in function of reaction time

As shown in Fig. 12, the particle distribution (%) of the WW samples in four quadrants before reaction start and at 120 min of reaction had peaks in total particle number. The percentage share in Q1 and Q2 increased in both cases, compared to the total share.

COD results

Hercobond A had no effect on COD values during the reaction, as shown on Fig. 13, in which experiment parameters were set to RSM optimized values. The COD values for the lab WW samples were in the range from 3700 to 3900 ppm without any noticeable trend in function of time.

The COD for the ind. WW sample were in the range from 10250 up to 10803 ppm, also without a noticeable trend, meaning that the cellulase enzymes could be used commercially for the fines control, without possibility to disturb the COD range in wastewater. Enzyme immobilization could be a way to reduce COD values while keeping all positive enzyme attributes.

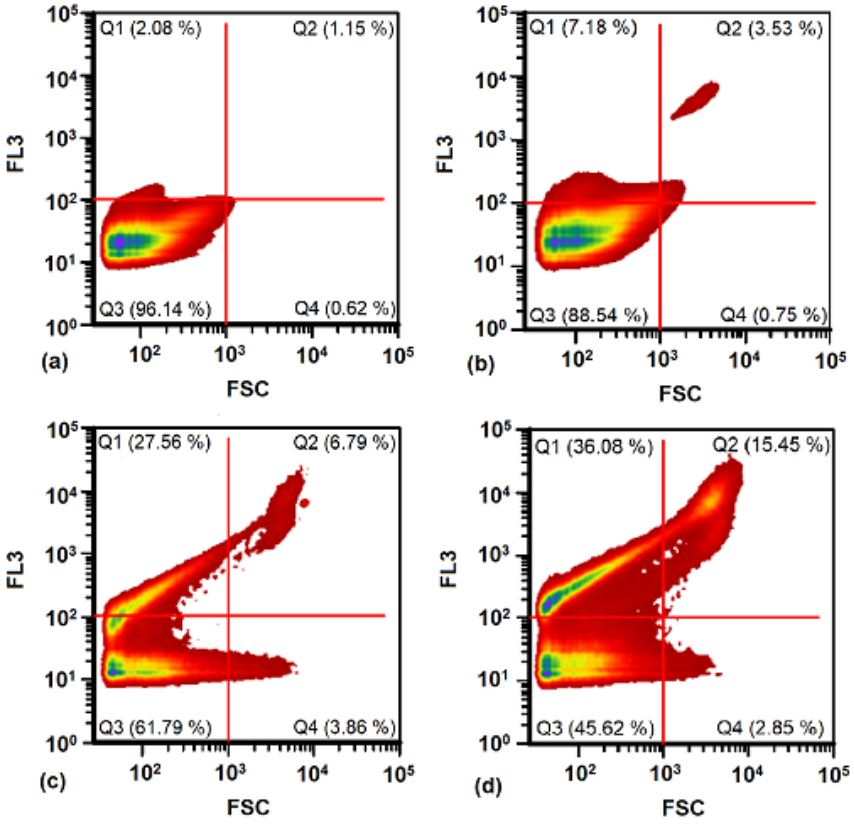

Fig. 12. Flow cytometry results: (a) lab WW 0 min; (b) lab WW 120 min of reaction; (c) ind. WW 0 min; (d) ind. WW 120 min of reaction.

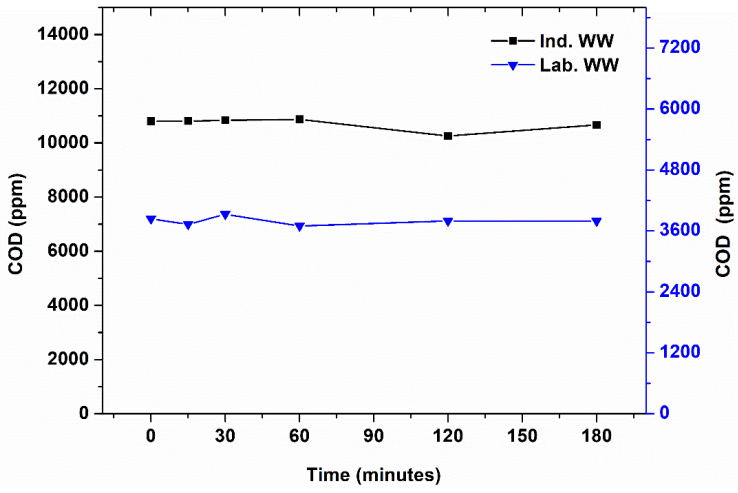

Fig. 13. COD values in function of reaction time

CONCLUSIONS

1. In the specially formulated lab whitewater (WW) system, cellulase *Hercobond A* revealed the highest activity and the lowest turbidity after 24 h reaction at two pH values. Therefore, *Hercobond A* was selected for further testing and response surface methodology (RSM) optimization.
2. RSM is an efficient and suitable method for experiment parameters optimization with low experimental error, where lab generated WW from recycled paper was used as cellulase substrate. The resulting RSM model showed a bell-shaped curve with a maximum at pH 5.7 and a linear response for the temperature, the reaction time, and the enzyme concentration. The optimum process parameters for temperature (40 °C), enzyme concentration (0.18% v/v), and reaction time (3 h) were identified with the equation model (Eq. 3).
3. The mono and disaccharides concentration in the WW increased considerably after 2 h reaction time (HPLC), suggesting that *Hercobond A's* main activities were β-glucosidase (BG) and cellobiohydrolase (CBH) and indicating *Hercobond A* as an appropriate enzyme selection for the WW fines management and degradation.
4. The second series of experiments compared the enzyme effects in the lab and ind. WW samples under the RSM model parameters. The trends noted on the two WW systems were alike. A downward trend for turbidity and colloidal charge (14.9 and 9.3%; 19.2 and 29.6% retrospectively) was confirmed with the lower anionic charge, presumably corresponding with less available $RCOO^-$ groups and more water-soluble sugars generated from the fines. The curve trend in both cases was the steepest at the first 15 min of reaction, indicating rapid interaction of *Hercobond A* with the fines.
5. The enzyme treatment increased the particle number in the range from 0.1 to 50 μm and led to higher hydrophobic share, indicating the interaction of *Hercobond A* with the hydrophilic particles, showing that FCM can be effectively used as a new method for fines distribution and content monitoring.
6. The chemical oxygen demand (COD) values were unchanged in both samples, suggesting the WW enzyme treatment will not negatively affect the wastewater treatment plant, and confirming cellulase usage as "green" and sustainable chemistry.
7. *Hercobond A* under demonstrated and optimized process parameters can reduce the fines content in recycled paper process WW with all potential benefits for the paper machine to improve overall runnability, without negative influence on wastewater treatment plant. This novel approach uses enzyme application in the short loop process water instead of traditional addition directly into the pulp slurry.

ACKNOWLEDGMENTS

The authors would like to acknowledge support from Solenis Technologies Germany GmbH for enzymes, chemical additive samples, and for the use of the laboratory with all needed equipment. This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No. 451-03-47/2023-01/200135).

REFERENCES CITED

Bajpai, P. (1999). "Application of enzymes in the pulp and paper industry," *Biotechnology Progress* 15(2), 147-157. DOI: 10.1021/bp990013k

Bajpai, P. (2011). "Enzymatic modification of starch for surface sizing," in: *Biotechnology for Pulp and Paper Processing*, 1st Ed., Springer, New York, NY, pp 317-326. DOI: 10.1007/978-981-10-7853-8

Bajpai, P. K. (2012). "Effects of increased closure water-loop systems in pulp and paper industry," *IPPTA: Quarterly Journal of Indian Pulp and Paper Technical Association* 24(2), 91-95.

Britt, K. W., and Unbehend, J. E. (1976). "New methods for monitoring retention," *TAPPI Journal* 59, 67-70.

Choe, E. K., Lee, M., Park, K. S., and Chung, C. (2019). "Characterization of cotton fabric scouring by Fourier transform-infrared attenuated total reflectance spectroscopy, gas chromatography-mass spectrometry and water absorption measurements," *Textile Research Journal* 89(12), 2305-2315. DOI: 10.1177/0040517518790976

Chung, C., Lee, M., and Choe, E. (2004). "Characterization of cotton fabric scouring by FT-IR ATR spectroscopy," *Carbohydrate Polymers* 58(4), 417-420. DOI: 10.1016/j.carbpol.2004.08.005

Couturier, M., Bennati-Granier, C., Urio, M. B., and Ramos, L. P., and Berrin, J. G. (2016). "Fungal enzymatic degradation of cellulose," in: *Green Energy and Technology*, C. Soccol, S. Brar, C. Faulds, and L. Ramos (eds.), Springer, Cham. DOI: 10.1007/978-3-319-30205-8_6

Dube, E., Shareck, F., Hurtubise, Y., Beauregard, M., and Daneault, C. (2008). "Enzyme-based approaches for pitch control in thermomechanical pulping of softwood and pitch removal in process water," *Journal of Chemical Technology and Biotechnology* 83, 1261-1266. DOI: 10.1002/jctb.1939

Fischer, K., and Messner, K. (1992). "Adsorption of lipase on pulp fibers during biological pitch control in paper industry," *Enzyme and Microbial Technol*ogy 14(6), 470-473. DOI: 10.1016/0141-0229(92)90139-F

Grubb, M., Wray, H., and Richardson, D. (2009). "The use of flow cytometry for managing extractives associated with pitch deposits," *Appita Annual Conference*, 347-352.

Gülsoy, S. K., and Uysal, S. (2020). "Effects of recycled fiber fines on handsheet properties of different unbeaten and beaten pulps," *Wood Industry/Drvna Industrija*, 71(4), 327-337. DOI:10.5552/drvind.2020.1904

Hubbe, M. A. (2002). "Fines management for increased paper machine productivity," in: *Proc. Sci. Tech. Advan. Wet End Chemistry*, Pira, Barcelona.

Kamaya, Y. (1996). "Role of endoglucanase in enzymatic modification of bleached kraft pulp," *Journal of Fermentation and Bioengineering* 82(6), 549-553. DOI: 10.1016/S0922-338X(97)81250-0

Kim, K. J., Lee, J. M., Ahn, E. B., and Eom, T. J. (2017). "Effect of enzyme beating on grinding method for microfibrillated cellulose preparation as a paper strength enhancer," *Cellulose* 24, 3503-3511. DOI: 10.1007/s10570-017-1368-9

Knežević-Jugović, Z. (2008). in: *Enzimsko Inženjerstvo*, Tehnološko-metalurški fakultet Univerziteta u Beogradu, Belgrade, pp. 399-404.

Lee, H., Nam, W. S., Sohn, S. D., and Paik, K. H. (2011). "Effect of different types of fines on the properties of recycled chemical pulp," *Journal of Industrial and Engineering Chemistry* 17(1), 100-104. DOI:10.1016/j.jiec.2010.12.004

Liu, K., Li, X. F., Li, X. M., He, B. H., and Zhao, G. L. (2010). "Lowering the cationic demand caused by PGA in papermaking by solute adsorption and immobilized pectinase on chitosan beads," *Carbohydrate Polymers* 82(3), 648-652. DOI: 10.1016/J.CARBPOL.2010.05.028

Liu, J., Hu, H., Xu, J., and Wen, Y. (2012a). "Optimizing enzymatic pretreatment of recycled fiber to improve its draining ability using response surface methodology," *BioResources* 7(2), 121-2140. DOI: 10.15376/biores.7.2.2121-2140

Liu, T. Z., Wang, D. F., Zhao, H. Y., and Zheng, K. Y. (2012b). "Stickies treatments of recycled fiber pulping wastewater by lipases," *Advanced Materials Research* 534, 225-229. DOI: 10.4028/www.scientific.net/AMR.534.225

Marton, J., and Marton, T. (1976). "Wet end starch: Adsorption of starch on cellulosic fibers," *TAPPI* 59, 121-124.

Maximino, M. G., Taleb, M. C., Adell, A. M., and Formento, J. C. (2011). "Application of hydrolytic enzymes and refining on recycled fibers," *Cellulose Chemistry and Technology* 45, 397-403.

Milivojević, A. D., Corović, M. M., Simović, M. B., Banjanac, K. M., Blagojević, S. N., Pjanović, R. V., and Bezbradica, D. I. (2019). "Novel approach for flavonoid esters production: statistically optimized enzymatic synthesis using natural oils and application in cosmetics," *Industrial & Engineering Chemistry Research* 58(9), 3640-3649. DOI: 10.1021/acs.iecr.8b06113

Min, B. C., Bhayani, B. V., Jampana, V. S., and Ramarao, B. V. (2015). "Enhancement of the enzymatic hydrolysis of fines from recycled paper mill waste rejects," *Bioresources and Bioprocessing* 2, 1-10. DOI: 10.1186/s40643-015-0068-2

Nagl, M., Haske-Cornelius, O., Skopek, L., Bausch, F., Pellis, A., Bauer, W., and Guebitz, G. M. (2022). "Mechanistic investigation of the effect of endoglucanases related to pulp refining," *Cellulose* 29, 2579-2598 DOI: 10.1007/s10570-021-04386-5

Odabas, N., Henniges, U., Potthast, A., and Rosenau, T. (2016). "Cellulosic fines: Properties and effects," *Progress in Materials Science* 83, 574-594. DOI: 10.1016/j.pmatsci.2016.07.006

Pardo, A. G., and Forchiassin, F. (1999). "Influence of temperature and pH on cellulase activity and stability in *Nectria catalinensis*," *Revista Argentina Microbiologia* 31(1), 31-35.

Rashmi, S., and Nishi, B. (2010). "Enzymatic treatment of secondary fibers for improving drainage: An overview," *IPPTA: Quarterly Journal of Indian Pulp and Paper Technical Association* 23(2), 121-126.

Sarja, T., Zabihian, M., Kourunen, P., and Niinimäki, J. (2004). "New method for measuring potential secondary stickies in deinked pulp filtrates," *Water Science and Technology* 50, 207-215. DOI: 10.2166/wst.2004.0197

Seth, R. S. (2003). "The measurement and significance of fines," *Pulp & Paper Canada* 104(2), 41-44.

Sjostrom, E., and Alen, R. (eds.) (1998). *Analytical Methods in Wood Chemistry, Pulping, and Papermaking*, Springer Berlin, Heidelberg, pp. 20-26. DOI: 10.1007/978-3-662-03898-7

Smook, G. A. (1999). *Handbook for Pulp & Paper Technologies*, Angus Wilde Publications, Vancouver, Canada.

Sundberg, K. E., Sundberg, A. C., Thornton, J. W., and Holmbom, B. R. (1998). "Pectic acids in the production of wood-containing paper," *TAPPI Journal* 81(7), 131-136.

Van de Ven, T. G. M. (2005). "Filler and fines retention in papermaking," in: *Advances in Paper Science and Technology*, S. J. I'Anson (ed.), Robinson College, Cambridge, pp. 1193-1224. DOI: 10.15376/frc.2005.2.1193

Verma, P. K., Bhardwaj, N. K., and Singh, S. P. (2015). "Selective hydrolysis of amorphous cellulosic fines for improvement in drainage of recycled pulp based on ratios of cellulase components," *Journal of Industrial and Engineering Chemistry* 22, 229-239. DOI: 10.1016/j.jiec.2014.07.015

William, E. S. (1996). *Principles of Wet End Chemistry*, TAPPI Press, Atlanta, pp 10-17.

Wu, R., Wang, Q., and Wang, G. (2019). "Immobilized enzyme on pulp fiber through layer-by-layer technique using cationic polyacrylamide for whitewater treatment from papermaking," *Bioprocess and Biosystems Engineering* 42, 1583-1589. DOI: 10.1007/s00449-019-02155-0

Yu, H., Xu, Y., Ni, Y., Wu, Q., Liu, S., Li, L., Yu, S., and Ji, Z. (2018). "Enhanced enzymatic hydrolysis of cellulose from wastepaper fibers by cationic polymers addition," *Carbohydrate Polymers* 200, 248-254. DOI: 10.1016/j.carbpol.2018.07.079

Zhang, A., Wang, G., Gong, G., and Shen, J. (2017). "Immobilization of white rot fungi to carbohydrate-rich corn cob as a basis for tertiary treatment of secondarily treated pulp and paper mill wastewater," *Industrial Crops and Products* 109, 538-541. DOI: 10.1016/j.indcrop.2017.09.006

Zhang, X., Stebbing, D. W., Saddler, J. N., Beatson, R. P., and Kruus, K. (2000). "Enzyme treatments of the dissolved and colloidal substances present in mill white water and the effects on the resulting paper properties," *Journal of Wood Chemistry and Technology* 20(3), 321-335. DOI: 10.1080/02773810009349639

Article submitted: October 9, 2023; Peer review completed: November 4, 2023; Revised version received and accepted: November 15, 2023; Published: December 1, 2023.
DOI: 10.15376/biores.19.1.635-655