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# Depolymerization of carboxymethylcellulose in a hydro-alcoholic medium by a mono-component endocellulase

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ABSTRACT

Carboxymethylcellulose (CMC) is a cellulose ether with a wide range of applications and markets. In order to tailor the molecular weight and the rheological profile of CMC, the industrial production process often implies a difficult to control depolymerization step with high concentrations of hydrogen peroxide or other oxidizing agents at alkaline pH.

Because CMC dissolved in an aqueous buffer at concentrations above 3–4% (w/w) generates extremely high and unmanageable viscosities, we have developed an alternative process of enzymatic depolymerization with a selected, recombinant mono-component endocellulase for the preparation of a highly concentrated aqueous solution of low molecular weight CMC, from a 240–300 kDa CMC dispersed as a slurry in a hydro-alcoholic reaction medium, followed by solvent recovery.

Wood cellulose from different tree sources and a few commercially available cellulases, either of natural origin or recombinant, were screened. The recombinant, mono-component endoglucanase Cel12A from *T. reesei* was selected for CMC hydrolysis carried out in isopropanol. At the end of the reaction, the enzyme is thermally inactivated and the solvent recovered by distillation. This novel industrial process yields a stable aqueous solution of a low viscosity, ready-to-use 35% (w/w) CMC, which is finding increasing new practical applications.



## 1. Introduction

Cellulose is the primary product of photosynthesis in plants and is the most abundant renewable polymer produced on the biosphere. It is a linear polymer composed of units of D-glucopyranose linked by β-(1,4) glycosidic bonds [1], in which the anhydrous cellobiose represents the repeating unit [2].

The degree of polymerization (DP) of cellulose represents the number of units of monomer (i.e., glucose) that, on the average, make up a cellulose filament. The DP varies considerably according to the plant of origin of cellulose and to the industrial process applied, with values ranging from a few hundreds to almost 20,000 [1]. For industrial applications as a versatile rheology modifier, cellulose undergoes chemical reactions, such as insertion on hydroxyl groups of various substituents in order to modulate its solubility and rheological properties. Two main classes of derivatives are produced: ethers and esters of cellulose [3]. Carboxymethylcellulose (CMC) is the cellulose ether with the greatest range of applications and markets, first synthesized by Jansen in 1918 [4]. CMC is obtained by carboxymethylation of alkali-swollen cellulose with monochloroacetic acid. Reaction and process conditions determine the degree of substitution (DS) of CMC, which corresponds to the average number of carboxymethyl groups bound to a glucose unit. This value can range from 0 to 3, which is the actual number of free hydroxyl groups available for carboxymethylation on a single monomer of the cellulose polymer [5]. The introduction of carboxymethyl substituents transforms the water-insoluble cellulose in water-soluble CMC.

In solution, the molecular weight of CMC correlates to viscosity according to Mark Houwink's law:

$$\eta_0 = kM^a$$

where $\eta_0$ is the intrinsic viscosity (i.e., the viscosity at zero concentration), $M$ is the molecular weight, "$k$" and "$a$" are constants determined by temperature, solvent and the type of polysaccharide itself [6].

For few applications (e.g., as pigment dispersing agent, in paper tissue production and in textile printing), the required rheological properties of CMC solutions are obtained only after controlled depolymerization of CMC. Traditionally, hydrolysis of CMC is performed by chemical oxidation with high concentrations of hydrogen peroxide or other oxidizing agents at alkaline pH.

*Abbreviations:* CMC, carboxymethylcellulose; CBM, cellulose binding module; EtOH, ethanol; IPA, isopropyl alcohol; BCA, bicinchoninic acid; DP, degree of polymerization; DS, degree of substitution.

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**Table 1**
Commercial cellulases used.

| Commercial name | Composition | Protein content (mg protein/g product) | Cellulase activity by Megazyme method (U/g product) |
|---|---|---|---|
| Primafast 200 (Genencor-Danisco) | Natural complex from *T. reesei* | 240 ± 14 | 4345 ± 77 |
| Indiage Max L (Genencor-Danisco) | Complex from *T. reesei* deleted in Cel7A (CBH I), endo-enriched in Cel7B (EG I) and Cel5A (EGII) | 205 ± 1 | 9635 ± 133 |
| Indiage Super L (Genencor-Danisco) | Mono-component r-EG III from *T. reesei* (Tr Cel12A) | 31 ± 2 | 956 ± 35 |
| Indiage Neutra L (Genencor-Danisco) | Mono-component r-EG III from *Streptomyces* (Ssp Cel12A) | 77 ± 6 | 3138 ± 99 |
| Cellulase 2000 L (Genencor-Danisco) | Natural complex from *P. funiculosum* | 53 ± 3 | 1270 ± 32 |
| β-Glucanase 750 L (Genencor-Danisco) | β-glucanase from *G. emersonii* | 18 ± 1 | 107 ± 2.9 |
| Novozym 613 (Novozymes) | EG I from *H. insolens* (Hi Cel7B) | 27 ± 2 | 350 ± 20 |

This process can be hazardous, difficult to control and might not always yield the desired low molecular weight CMC. An alternative approach implies the use of cellulases and many reports in the literature describe the enzymatic depolymerization of chemically modified forms of cellulose, even though all those reactions are performed in water-based media [7,8]. CMC is soluble in an aqueous environment, but has to be kept at relatively low concentrations in order not to develop extremely high viscosities, thus becoming hard to handle, stir and pump in an industrial setting.

We have developed an alternative and innovative industrial process for the production of aqueous solutions of an extensively depolymerized, highly concentrated and low viscosity CMC by hydrolysis of powder 240–300 kDa CMC, dispersed as a slurry in a hydro-alcoholic medium to prevent its dissolution, with a selected recombinant mono-component endocellulase, followed by inactivation of the enzyme and recovery of the solvent by distillation [9].

Cellulases are glycosyl hydrolases that split the β-1,4 glycosidic bond of cellulose and its derivatives. Cellulases are conventionally divided in two groups:

- endoglucanases (endo-β-1,4-D-glucano 4-glucanohydrolases, E.C.3.2.1.4)
- exoglucanases or cellobiohydrolases (exo-β-1,4-D-glucano 4-cellobiohydrolases, E.C.3.2.1.74)

Endoglucanases (EG) randomly hydrolyze the β-(1,4) glycosidic bonds within the polymer, while cellobiohydrolases (CBH) generate cellobiose from the reducing and non-reducing ends of the polymer, thus generating the disaccharide cellobiose [10]. A wide body of literature is available on the biochemistry, molecular biology and structure of microbial cellulases and reference is made only to some relevant publications on the subject [11–17].

Three delignified celluloses of different plant origin were studied and seven different industrial celullases were tested, either natural enzyme complexes or recombinant. Two approaches were pursued: (a) cellulose hydrolysis by cellulase, followed by carboxymethylation (which could eventually be carried out in the same reaction mixture and reactor); (b) carboxymethylation of cellulose followed by enzymatic depolymerization in isopropanol. The latter turned out to be the most efficient reaction and was further developed.

Because the carboxymethylation process of cellulose takes place in high alcohol concentration (ethanol or isopropanol) and CMC is insoluble in 40% alcohol concentration and above, the activity and stability of the cellulases were evaluated at increasing concentrations of EtOH or IPA, using cellulose or CMC as substrate. Having selected the best cellulase in terms of hydrolytic efficiency under process conditions, it was found that enzymatic hydrolysis of cellulose in alcohol, followed by carboxymethylation, reaches only limited depolymerization as estimated by the viscosity of the resulting CMC's. On the other hand, the molecular weight of the 240 kDa CMC hydrolyzed by endocellulase in 40% IPA decreases by almost one order of magnitude, yielding a stable aqueous solution of a highly concentrated (35% w/w), easy-to-handle CMC, with a molecular weight of around 30 kDa and with low viscosity.

## 2. Materials and methods

### 2.1. Materials

The celluloses used were from commonly available commercial sources, from the following trees: *Eucalyptus* spp, *Pinus pinaster*, *Picea abies* + *Pinus nigra*.

Reagent grade CMC was from Fluka, with the following characteristics:

- Brookfield viscosity of a 4% solution = 500–2500 mPa s (25 °C, 20 rpm)
- DS = 0.60–0.95

Industrial grade CMC produced by Lamberti spa was used, with a DS of 0.60–0.95, referred to as CMC/MV, where MV stands for "medium viscosity" (i.e., viscosity of a 4% solution = 200–500 mPa s, 60 rpm, 20 °C). DS was established by a potentiometric method described in ASTM D 1439-2003 [18].

All cellulases used in this work are in liquid form and commercially available (see Table 1) [19–22].

### 2.2. Characterization of celluloses

Cellulose fibers were observed and characterized by optical microscopy following the procedures described by the Tappi 401 om-08 method [23]. DP of celluloses was determined by a viscosimetric method [24] based on the elution time of a cellulose solution (dissolved in cuproethylendiamine as solvent) from a capillary viscosimeter at 25 °C. The intrinsic viscosity was extrapolated by Martin's equation, while DP was calculated according to Immergut [25,26].

Initial characterization of three types of celluloses was performed by depolymerization with a natural whole cellulase complex from *T. reesei*, to establish which cellulose is more extensively hydrolyzed. Enzymatic depolymerization of cellulose was performed at 50 °C as follows: 2 g of cellulose are dispersed in 98 g of a sodium acetate 0.05 M buffer solution at pH 4.8 or in 80% hydro-alcoholic mixture, 11 units of enzyme are added, the mixture is stirred for 1 h and then filtered on a 0.45 μ nitrocellulose filter. The pH of the filtered liquid is adjusted to pH 12 with 4 N NaOH to inactivate the enzyme, while the insoluble portion is extensively washed with water, oven dried at 100 °C for 1 h and finally resuspended in a 0.05 M trisodium phosphate buffer at pH 12. Reducing sugars were determined on both the soluble and insoluble portions by the bicinchoninic acid (BCA) method [27]. In order to estimate the possible contribution of proteins to color development in the BCA assay, controls were always run in parallel in the absence of reducing sugars and in the presence of an equivalent amount of enzyme protein. With the highest amount of cellulase complex used in the above depolymerization reaction, absorbance values were on the average 0.0178. They were even lower in all other cases.