Megazyme

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ASSAY OF

CELLULASE
(*endo*-1,4-β-glucanase)

using

4,6-*O*-Benzylidene-4-methylumbelliferyl-β-cellotrioside

plus

Thermostable β-glucosidase

R-CELLFLR 10/14

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INTRODUCTION:

Cellulase [*endo*-1,4-β-glucanase (EC 3.2.1.4)] plays a key role in the hydrolysis of cellulosic biomass to fermentable sugars. This enzyme also finds widespread industrial applications in the modification of cellulosic materials and in the degradation of mixed linkage 1,3;1,4-β-glucans. Numerous methods are available for the measurement of cellulase, including those based on increase in reducing sugar levels on hydrolysis of CM-cellulose or 1,3:1,4-β-glucan. *endo*-Cellulase can be specifically assayed using viscometric methods with soluble CM-cellulose 7M as substrate or by employing soluble or insoluble (crosslinked) dyed cellulose or mixed-linkage β-glucan.[1] In general, assays based on the use of dyed polysaccharides (Megazyme Catalogue No. **T-CCZ**, **T-CTZ**, etc.) are standardised against a reducing sugar method that employs either CM-cellulose or β-glucan as substrate. However, these substrates are not suitable for automation.

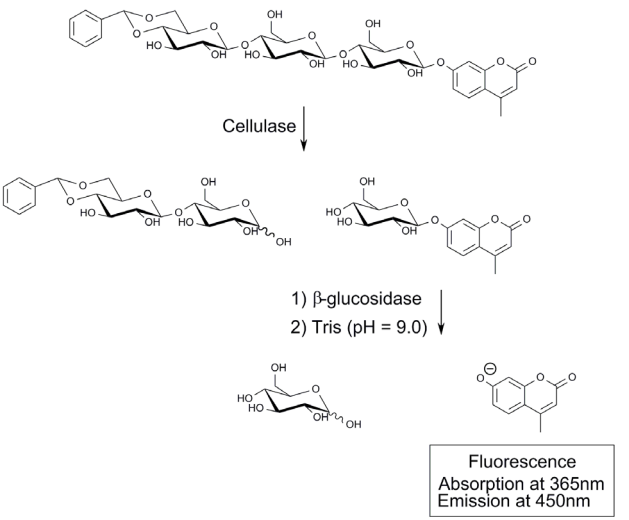

Figure 1. Principle of cellulase assay using Cellafluor.

The Megazyme Cellafluor test reagent[2], employs high purity β-glucosidase and 4,6-*O*-benzylidene-4-methylumbelliferyl-β-D-cellotrioside (BzMUG3). The level of β-glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of BzMUG3 to 4,6-*O*-benzylidene-cellobiose and 4-methylumbelliferyl-β-D-glucose by cellulase, the 4-methylumbelliferyl-β-D-glucose is immediately cleaved to D-glucose and free 4-methylumbelliferyl (4MU) by the β-glucosidase present in the substrate mixture (Figure 1).

Thus, the rate of release of 4MU relates directly to the rate of hydrolysis of BzMUG3 by cellulase.

The reaction is stopped and the phenolate colour is developed, on addition of Trizma base solution (pH 9). A standard curve relating enzyme activity to increase in absorbance at 450 nm following irradiation at 365 nm on hydrolysis of BzMUG3 by Trichoderma cellulase is shown in Figure 2. Cellulase enzymes from different sources vary in their ability to hydrolyse BzMUG3, so it is necessary to establish a specific standard curve for each cellulase to allow accurate quantitation. The assay can be used at temperatures up to 60°C and from pH 4.5 to 8. For cellulase enzymes with activity at high pH values, it is necessary to terminate the reaction with 2% tri-sodium phosphate (pH 11). BzMUG3 substrate solution as supplied in 50% dimethyl sulphoxide DMSO/H_2O is completely stable for at least 4 weeks at 20°C and for > 4 years at -20°C. An analogous UV absorption based assay kit is also available from Megazyme (**K-CELLG3**).[3]

ACCURACY:

Standard errors of less than 3% are readily achieved.

SPECIFICITY:

The assay is specific for cellulase. The substrate is not hydrolysed by β-glucosidase, cellobiohydrolase or any other enzymes tested.

CONTENTS:

Bottle 1: 4,6-*O*-benzylidene-4-methylumbelliferyl-β-D-cellotrioside (BzMUG3) in 50% (v/v) DMSO/H_2O (10 mL, 0.51 mM).
Stable for 4 weeks at 20°C/> 4 years at -20°C.

Bottle 2: Thermostable β-glucosidase suspension (0.4 mL).
Stable for 4 weeks at 20°C/> 4 years at 4°C.

PREPARATION OF REAGENT SOLUTION (Cellafluor):

NOTE: The Cellafluor reagent should be made up in relatively small quantities as required in order to maximise long term reagent stability. A typical preparation of 3 mL Cellafluor Reagent is described.

Before use, transfer 3 mL of the contents of Bottle 1 to a new container and to this add 50 µL of the contents of bottle 2 (after gentle swirling of contents). Mix well. This is termed Cellafluor reagent solution. Store at 4°C during use. Stable for approximately 1 week at 4°C. Stable for at least 4 weeks at -20°C.

DIRECTIONS FOR USE:

NOTE: It is anticipated that this reagent will find extensive use in the area of metagenomics as a solution screening tool. The procedure for use can be adapted to suit most automated instruments. In our hands, fluorescent measurements were made using a single tube Promega Quantifluor® ST fluorometer (available from www.promega.com) following the procedure described here. Cellafluor is not pH buffered and so the assay is effectively run in the pH buffer of the cellulase solution to be assayed. The only limitation in pH range comes from the ancilliary β-glucosidase employed in Cellafluor which can be used from pH 4.5 to 8.

ASSAY OF CELLULASE:

1. Dispense 0.10 mL aliquots of Cellafluor solution directly to the bottom of 13 mL glass test tubes and pre-incubate the tubes at 40°C for 3 minutes.
2. Pre-incubate the cellulase solution to be assayed, suitably diluted in an appropriate buffer, at 40°C for 3 min.
3. To each tube containing Cellafluor solution, add 0.1 mL of the cellulase solution to the bottom of the tube, stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of 2% Tris solution (pH 9) (Stopping Reagent) and stir the tube contents.
5. Read the fluorescent absorbance of the reaction solutions and the reagent blank using a Quantifluor® ST fluorometer or similar. 4MU exhibits absorption at 365 nm and emission at 450 nm.
6. The reagent blank is prepared by adding 3.0 mL of Trizma base solution (2%) to 0.1 mL of reagent mixture with vigorous stirring, followed by the enzyme solution (0.1 mL) with stirring.

Quantitation of fluorescent output is dependent on a large number of variables. For this reason, calibration standards need to be run on the fluorometer to be used in order to relate fluorescent absorbance to concentration of 4-methylumbelliferone liberated. In our hands, fluorescent measurements were made using a single tube Promega Quantifluor® ST fluorometer calibrated using a standard of 50 µg/L 4-methylumbelliferone in 2% Tris (pH 9.0).

Figures 2, 3 and 4 below may be helpful to the user.

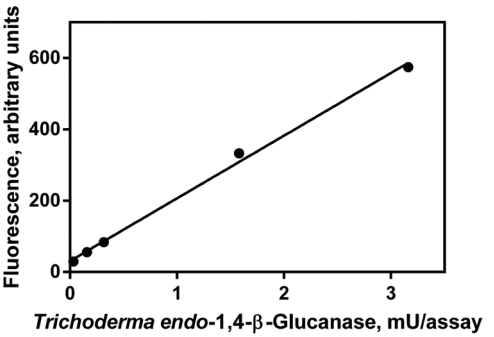

Figure 2. Standard curve relating fluorescence absorbance to *Trichoderma longibrachiatum* cellulase (Megazyme Catalogue Number: **E-CELTR**) activity in 100 mM sodium acetate buffer (pH 4.5). Incubations were stopped after 10 minutes with the addition of 3 mL 2% Tris (pH 9).

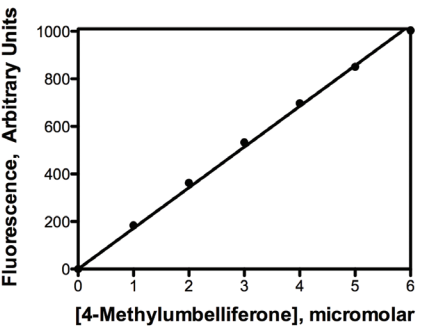

Figure 3. Standard curve relating fluorescent response to 4-methylumbelliferyl concentration in 2% Tris (pH 9) using a Quantifluor® ST fluorometer.

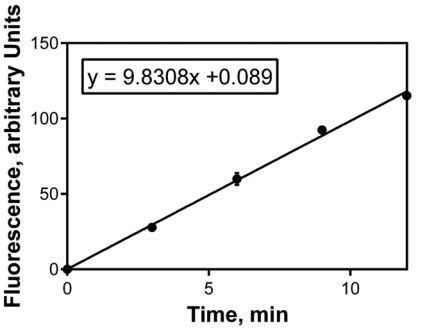

Figure 4. Fluorescent response observed over 12 min at 40°C with Cellafluor reagent (0.1 mL) incubated in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (0.1 mL, pH 4.5 100 mM sodium acetate, 3.16 mU) (Megazyme Catalogue Number: **E-CELTR**). Reactions were terminated at 0, 3, 6, 9 and 12 minutes by addition of 3 mL 2% Tris (pH 9.0).

REFERENCES:

1. McCleary, B.V., McKie, V. and Draga, A. (2012). Measurement of *endo*-1,4-β-glucanase. In "Methods in Enzymology", Volume 510, (H. Gilbert, Ed.), Elsevier Inc., pp. 1-17.
   Link to article

2. Mangan, D., McCleary, B.V., Liadova, A., Ivory, R. & McCormack, N. (2014). Quantitative fluorometric assay for the measurement of *endo*-1,4-β-glucanase. Carbohydrate Research, 395, 47-51.
   Link to article

3. McCleary, B.V., Mangan, D., Daly, R., Fort, S., Ivory, R. & McCormack, N. (2014). Novel substrates for the measurement of *endo*-1,4-β-glucanase (*endo*-cellulase). Carbohydrate Res., 385, 9-17.
   Link to article

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