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Colourimetric and fluorometric substrates for measurement of pullulanase activity

Barry V. McCleary*, David Mangan*, Vincent McKie, Claudio Cornaggia and Edward Rooney

Megazyme International Ireland, Bray Business Park, Southern Cross Road, Bray, County Wicklow, Ireland.

Corresponding authors. Tel.: +353 12861220.

E-mail addresses: david@megazyme.com (David Mangan)

barry@megazyme.com (Barry McCleary)

Abstract

Specific and highly sensitive colourimetric and fluorometric substrate mixtures have been prepared for the measurement of pullulanase and limit-dextrinase activity and assays employing these substrates have been developed. These mixtures comprise thermostable α- and β-glucosidases and either 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-maltotriosyl (1-6) α-maltotrioside ($BzCNPG_3G_3$, **1**; Figure 1) as a colourimetric substrate or 4,6-*O*-benzylidene-4-methylumbelliferyl-β-maltotriosyl (1-6) α-maltotrioside ($BzMUG_3G_3$, **2**) as a fluorometric substrate. Hydrolysis of substrates **1** and **2** by *exo*-acting enzymes such as amyloglucosidase, β-amylase and α-glucosidase is prevented by the presence of the 4,6-*O*-benzylidene group on the non-reducing end D-glucosyl residue. The substrates are not hydrolysed by any α-amylases studied, (including those from *A. niger* and porcine pancreas) and are resistant to hydrolysis by *Pseudomonas sp.* isoamylase. On hydrolysis by pullulanase (Figure 1), the 2-chloro-4-nitrophenyl-β-maltotrioside (**3**) or 4-methylumbelliferyl-β-maltotrioside (**4**) liberated is immediately hydrolysed to D-glucose and 2-chloro-4-nitrophenol or 4-methylumbelliferone. The reaction is terminated by the addition of a weak alkaline solution leading to the formation of phenolate ions in solution whose concentration can be determined using either

spectrophotometric or fluorometric analysis. The assay procedure is simple to use, specific, accurate, robust and readily adapted to automation.

Keywords: pullulanase, limit dextrinase, assay procedure, 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-maltotriosyl (1-6)-α-D-maltotrioside, $BzCNPG_3G_3$, 4,6-*O*-benzylidene-methylumbelliferyl-β-maltotriosyl (1-6)-α-D-maltotrioside, $BzMUG_3G_3$.

1. Introduction

Pullulanase (α-dextrin glucanohydrolase, pullulan 6-glucanohydrolase, limit dextrinase, amylopectin 6-glucanohydrolase)[1,2] (EC 3.2.1.4) cleaves the α-1,6-linkages in pullulan, amylopectin and α- and β-limit dextrins of starch. Hydrolysis of α-1,6-linkages in 1,4:1,6-α-gluco-oligosaccharides by pullulanase requires the presence of at least one α-1,4-linked glucosyl residue on each side of the α-1,6-linkage. Thus the smallest substrate for pullulanase is the tetrasaccharide 6^2-α-D-maltosyl maltose (**7,** Figure 2).[3] Hydrolysis of glycogen is restricted by the highly branched, short chain length and dense nature of the molecule.[2,3] Several groups of enzymes catalysing *endo*-hydrolysis of pullulan have been described including those that hydrolyse the α-1,6-glucosidic bonds in pullulan and branched α-1,4;1,6 glucans (*e.g.* amylopectin)[1,4] (Type I) and those referred to as amylopullulanases[5] (Type II) which can hydrolyse both α-1,4- and α-1,6-glucosidic linkages. Other *endo*-acting enzymes active on pullulan include pullulan hydrolase type I (neopullulanase) [6] and type II (isopullulanase);[7] which only cleave (α-1,4) linkages in pullulan releasing panose (**8**) or isopanose (**9**), respectively (Figure 2). A fifth type of *endo*-hydrolase active on pullulan is referred to as pullulan hydrolase type III. This enzyme has the ability to hydrolyse both (α-1,6) and (α-1,4) glucosidic linkages in pullulan leading to the formation of panose (**8**) as well as maltose, maltotriose and glucose. [8]

Pullulanase finds widespread application in the starch processing industry. In combination with amyloglucosidase (AMG) it gives more efficient conversion of starch to glucose, reducing the formation of isomaltose (**10**) and giving up to 1% greater yield of

glucose.[9,10] High maltose syrups are produced from starch using combinations of pullulanase and β-amylase.[9,11]

The industrial application of pullulanase has led to significant interest in this enzyme and considerable screening efforts to locate new and interesting forms with different optimal conditions of pH and temperature for activity.

Pullulanase activity in industrial enzyme preparations can conveniently be assayed with reduced pullulan using an appropriate reducing sugar method such as that of Nelson[12] and Somogyi.[13] In crude enzyme preparations, the activity can be more specifically assayed using soluble dyed substrates (Red Pullulan) or dyed and cross-linked pullulan either in powder (AZCL-Pullulan) or tablet (Limit-Dextrazyme) form.[14] While the latter two substrates are very useful and widely used, they do not readily lend themselves to automated analysis procedures.

The aim of this work was to develop simple assay procedures for pullulanase using the well-defined oligosaccharide, 6^3-α-D-maltotriosyl maltotriose (**11**, Figure 2) based on similar procedures previously developed and published for the measurement of α-amylase[15,16] and *endo*-cellulase.[17] The two substrates developed were 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-6^3-α-D-maltotriosyl maltotrioside ($BzCNPG_3G_3$; **1**) and 4,6-*O*-benzylidene-4-methylumbelliferyl-β-6^3-α-D-maltotriosyl maltotrioside ($BzMUG_3G_3$; **2**). In this paper, the synthesis of these oligosaccharide substrates and their use in enzyme-linked assays for pullulanase will be described. During the course of this work the synthesis and use of the 2-chloro-4-nitrophenyl 6^3-α-D-glucosyl 6^3-α-D-maltotriosyl maltotrioside (**12**) lacking a 4,6-*O*-benzylidene group for the assay of pullulanase and limit dextrinase was described.[18]

2. Results and Discussion

6^3-α-D-Maltotriosyl maltotriose (**11**) was obtained in a highly pure form from Megazyme International. **11** is derived from pullulan which is composed mainly of repeating maltotriosyl units (~ 93%),[19] but also contains approximately 7% maltotetraosyl units. It is important that there is no contamination present in the 6^3-α-D-maltotriosyl maltotriose used for the synthesis of these substrates as the most common contaminant (heptasaccharide containing a maltotetraosyl

unit) would be susceptible to hydrolysis by fungal α-amylases and thus would make the substrate less specific for the assay of pullulananse activity in enzyme mixtures.

The 2-chloro-4-nitrophenyl- and 4-methylumbelliferyl- derivatives of 6^3-α-D-maltotriosyl maltotriose (**11**) were prepared from oligosaccharide **11** following the procedure reported by Planas *et al.*[20] The 4,6-*O*-benzylidene protecting group was then introduced to prepare substrates **1** and **2** respectively as previously described for cello-oligosaccharides.[17] The principle of the assay procedures for pullulanase using **1** and **2** is shown in Figure 1. On hydrolysis of the oligosaccharide substrates **1** or **2** at the 1,6-α-linkage, the released 2-chloro-4-nitrophenyl-β-maltotrioside or 4-methylumbelliferyl-β-maltotrioside respectively are immediately hydrolysed to glucose and either 2-chloro-4-nitrophenol or 4-methylumbelliferone by the concerted action of the α-glucosidase and β-glucosidase enzymes in the reagent mixture. Reaction is terminated and phenolate ions are developed by addition of dilute alkali.

Unlike the substrate described by Bøjstrup *et al.*[18] these 4,6-*O*-benzylidine end-blocked substrates are absolutely resistant to all known *exo*-acting α-glucosidases and α-glucanases, such as AMG and β-amylase. Incubation of **1** under standard assay conditions with 200 Units of *Bacillus stearothermophilis* α-glucosidase, *Rhizopus* sp. AMG or barley β-amylase for up to 4 h gave no hydrolysis. In contrast, substrate **12** as used in the pullulanase assay procedure described by Bøjstrup *et al.*[18] is rapidly hydrolysed by AMG, and is even hydrolysed (*albeit* slowly) by the *B. stearothermophilis* α-glucosidase which is used in the reagent mixture. At a concentration of 1 mM and a pH of 5.5, the terminal non-reducing end 1,6-α-linked D-glucosyl residue in 6^3-α-D-glucosyl 6^3-α-D-maltotriosyl-maltotriose (**13**) is hydrolysed by *Bacillus stearothermophilis* α-glucosidase at 2% of the rate of hydrolysis of the non-reducing end 1,4-α-linked D-glucosyl residue in maltoheptaose. This observation introduces an instability issue into the substrate mixture used by Bøjstrup *et al.*,[18] meaning that the ancillary enzyme α-glucosidase must be added to the reagent mixture immediately at the time of performing the assay. Since β-glucosidase can only hydrolyse the substrate after removal of α-linked D-glucosyl residues, substrate **12** is stable in the presence of this enzyme. A lag phase was reported in the release of 2-chloro-4-nitrophenol from **12** by Bojstrup *et al.*[18] This was attributed to inadequate levels of the ancillary enzymes in the reagent mixture. With an unblocked substrate such as **12** the level of α-

glucosidase added to the reagent mixture must be balanced by the rate of hydrolysis of **12** by this enzyme.

In enzyme-linked assays, the optimal assay conditions for the enzyme of interest (pullulanase) must be matched by the properties of the ancillary enzymes, with particular reference to the pH activity profiles of these enzymes. The pH activity profiles for *B. stearothermophilis* α-glucosidase and *T. maritima* β-glucosidase are shown in Figure 3 and those for pullulanase from *K. planticola* and *B. licheniformis* are shown in Figures 4a and 4b. *K. planticola* pullulanase has optimal pH for activity on reduced pullulan at 5.0-5.5, while *B. licheniformis* pullulanase shows optimal activity (on reduced pullulan) at pH 4.5-5.0. *T. maritima* β-glucosidase shows over 65% of maximal activity over the pH range 4.5-7.0, but *B. stearothermophilis* α-glucosidase has just 20% of maximal activity at pH 4.5 and just 42% at pH 5.0. Clearly, this mixture of α-glucosidase and β-glucosidase cannot be used in assays at pH values below 5.0, and even at pH 5.0, high levels of α-glucosidase are required to ensure instant hydrolysis of the 2-chloro-4-nitrophenyl β-maltotrioside released by pullulanase hydrolysis of **1** or **2**. Since many of the pullulanases described in literature have a pH optimum at 5.0, we decided to perform the assays at pH 5.0.

The concentration of β-glucosidase required to optimise the sensitivity of hydrolysis of **1** by pullulanase was determined by using *K. planticola* pullulanase and assay solutions containing substrate **1** (2 mM), 24 U/mL of *B. stearothermophilis* α-glucosidase and 0.7-6.5 U/mL of *T. maritima* β-glucosidase (Figure 5). The concentration of α-glucosidase required to optimise the sensitivity of hydrolysis of **1** by pullulanase was determined using assay solutions containing **1** (2 mM), 6.5 U/mL of *T. maritima* β-glucosidase and 2.4-24 U/mL of *B. stearothermophilis* α-glucosidase (Figure 6). An aliquot of substrate solutions (0.1 mL) was incubated with pullulanase (13.6 mU on this substrate) at 40 ^{o}C and the reaction terminated after 0-9 min by the addition of 1.5 mL of 2% Tris solution (pH 10.0). β-Glucosidase at a concentration of 0.39 U/assay (3.9 U/mL) was sufficient to give maximal colour-release on hydrolysis of the substrate (Figure 5), but a level of 0.65 U/assay was routinely used to allow for possible loss of enzyme activity on storage of the substrate over longer times. For α-glucosidase, a higher concentration of 2.4 U/assay (24 U/mL) was required to give maximal colour-release on hydrolysis of substrate **1** (Figure 6) because pH 5.0 is a sub-optimal pH for this particular enzyme. The activities of the

α-glucosidase and β-glucosidase stated here are those measured at the optimal pH for these enzymes (pH 6.5 for both).

Under the assay conditions employed, the effect of substrate concentration was determined by incubating 0.1 mL of substrate mixture containing **1** (0.2 to 4.0 mM), β-glucosidase (at 0.65 U/assay) and α-glucosidase (at 2.4 U/assay) with 0.1 mL of buffered solution containing either *K. planticola* (7.1 mU/assay on this substrate; 40 mU/assay on reduced pullulan) or *B. licheniformis* pullulanase (14.5 mU/assay on this substrate; 74 mU/assay on reduced pullulan). The reaction was terminated after fixed time intervals by the addition of 1.5 mL of 2 % Tris solution (pH 9.0) (Figure 7a and 7b). The maximum rate of hydrolysis for *K. planticola* pullulananse was obtained at a concentration of substrate **1** of approximately 0.5 mM in the assay mixture. Above this value there was slight substrate inhibition. For *B. licheniformis* pullulanase maximal rate was achieved at a concentration of substrate **1** of 1.2 mM, but there was no apparent inhibition at concentrations up to 2.0 mM. In the final assay mixture, a concentration of 2.0 mM substrate **1** was chosen to ensure a linear reaction range for the assay up to 1.0 absorbance units. The Michaelis-Menten curves for action of these two pullulanases on **1** are shown in Figure 8a and 8b. *K. planticola* pullulanase had a lower K_m value (0.032 mM) than *B. licheniformis* pullulanase (0.124 mM). The value obtained for *K. planticola* pullulanase (0.032 mM) is much lower than the value of 0.146 mM found by Bøjstrup *et al.*[18] using substrate **12**.

Standard curves relating absorbance increase at 400 nm to hydrolysis of **1** by *K. planticola* and *B. licheniformis* pullulanases standardised on borohydride-reduced pullulan (Nelson/Somogyi assay) are shown in Figure 9. It is evident that the slopes of the curves are dissimilar, indicating some difference in the ability of the two enzymes in hydrolysing the modified hexasaccharide relative to the pullulan polysaccharide. The reason for this difference is not clear, but may relate to interference of the chromogen or end-blocking group in the ability of the enzyme to accommodate substrate **1** in the active site. Equally, it could be due to differences in transglycosylation activities between the two enzymes. Indeed, a related enzyme, barley malt limit-dextrinase, has been shown to have strong transglycosylation properties.[21]

In some applications, highly sensitive substrates and assay procedures are required. It is well known that in assays such as those described here, fluorometric substrates give much higher sensitivity than colourimetric ones based on nitrophenyl- or chloronitrophenyl- chromogen groups. We have thus synthesised and evaluated compound **2** as a substrate for pullulanase. The

levels of α-glucosidase and β-glucosidase to ensure maximum sensitivity of the assay were, as expected, the same as those required in the assay employing compound **1**.

Under the assay conditions employed, the effect of substrate concentration was determined by incubating 0.1 mL of substrate mixture containing substrate **2** (0.1 to 1.0 mM), β-glucosidase (0.65 U/assay, *i.e.* per 0.1 mL) and α-glucosidase (2.4 U/assay, *i.e.* per 0.1 mL) with 0.1 mL of buffered solution containing *K. planticola* (1.4 mU/assay on this substrate, 6.6 mU/assay on reduced pullulan) or *B. licheniformis* pullulanase (1.8 mU/assay on this substrate, 9.1 mU/assay on reduced pullulan) and the reaction was terminated after fixed time intervals by the addition of 3.0 mL of 2% Tris solution (pH 10.0) (Figure 10a and 10b). Michaelis-Menten plots (Figure 11a and 11b) revealed Km values of 0.057 mM and 0.063 mM for *K. planticola* and *B. licheniformis* pullulanases respectively. At substrate concentrations above 0.2 mM in the assay mixture, *K. planticola* pullulanase showed some substrate inhibition. Fluorescence values were determined with a portable fluorimeter, Quantifluor® ST, from Promega which gave very stable and reproducible fluorescence readings in final sample volumes as low as 2.0 mL in the fluorimeter tube. A standard curve for 4-methylumbelliferone in 2% Tris solution (pH 10.0) is shown in Figure 12. The curve is linear up to a meter value of 1000 and allows direct conversion of fluorescence values to micromoles of 4-methylumbelliferone. Standard curves relating fluorescence increase to the level of either *K. planticola* or *B. licheniformis* pullulanases are shown in Figure 13.

The stability of the reagent mixture containing **1**, thermostable β–glucosidase (6.5 U/mL) and thermostable α–glucosidase (24 U/mL) at -20°C, 4°C and 20°C is shown in Figure S1 (Supplementary Information). Similar stability studies were performed with reagent mixtures containing substrate **2**. On storage of the reagent mixtures for up to 66 days, 0.1 mL aliquots were removed and the blank absorbance was determined as well as the absorbance on incubation of the substrate with a set amount of *K. planticola* pullulanase (13.8 mU on substrate **1**) at 40°C for 10 min. With reagent mixtures stored at 20°C there was a significant increase in the blank absorbance value, *albeit* only after 65 days. The analogous studies on substrate **2** using *K. planticola* pullulanase (7.4 mU on reduced pullulan) showed a similar stability of the substrate (results not shown). The increase in reaction absorbance value parallels the increase in the blank value, such that on subtracting the blank value from the reaction value, the determined absorbance value was very similar to those obtained on storage at -20°C and 4°C. This

demonstrates that the α-glucosidase and β-glucosidase in the reagent mixture are stable even on storage at 4°C for 50 days. For much longer storage times, a storage temperature of -20°C is recommended. These studies would indicate that the prepared substrate should be stable for > 2 years at -20°C.

For screening purposes in microplate format, the volumes of reagent mixture containing **2** and of the solution being assayed for pullulanase activity is reduced to 20 μL and the sensitivity is increased by reducing the volume of Tris solution used to terminate the reaction and to adjust the final pH, to 50 μL.

In this paper, the synthesis of two new substrates for the specific assay of pullulanase, namely $BzCNPG_3G_3$ (**1**) and $BzMUG_3G_3$ (**2**), has been described and assay formats have been suggested. Traditionally, pullulanase has been assayed with pullulan and more recently with borohydride-reduced pullulan using a reducing sugar method such as the Nelson/Somogyi procedure.[12,13] Dyed pullulan-based substrates were introduced to simplify the assay of pullulanase (and barley malt limit-dextrinase) in crude microbial fermentation broths and in grain and malted grain extracts where high levels of sugars preclude the use of reducing sugar methods. The most commonly used dyed substrates are Red Pullulan[14] (a soluble, dyed pullulan) and AZCL-Pullulan (a dyed and crosslinked, insoluble pullulan substrate). These dyed substrates have found widespread application since their introduction approximately 20 years ago. AZCL-Pullulan is most conveniently used in a tablet form (Limit Dextrizyme tablets).[14] For comparison purposes, the two pullulanases used in this study have been standardised on Red Pullulan and Limit Dextrizyme tablets and the results are shown in Figures 14 and 15. The new substrates described here ($BzCNPG_3G_3$, **1** and $BzMUG_3G_3$, **2**) are completely specific for the assay of pullulanase in the presence of all other hydrolytic enzymes studied. They are simple to use, extremely stable and are readily incorporated into automated formats. The major limitation in the introduction of these are the difficulties associated with the production of pure oligosaccharide **11** and the low overall yields for the synthesis of the colourimetric and fluorometric derivatives **1** and **2**.

3. Experimental

3.1 Materials

Thermostable β-glucosidase (*Thermotoga maritima*; Accession number Q08638, Megazyme Cat. No. E-BGOSTM), thermostable α-Glucosidase (*Bacillus stearothermophilus*; Accession number P94451, Megazyme Cat. No. E-TSAGS), Pullulanase M1 (*Klebsiella planticola*; Megazyme Cat. No. E-PULKP) and Pullulanase M2 (*Bacillus licheniformis*; Megazyme Cat. No. E-PULBL), Barley β-amylase (Megazyme Cat. No. E-BARBL100), Amyloglucosidase (*Rhizopus sp.*; Megazyme Catalogue No. E-AMGPU), 6^3-α-D-maltotriosyl maltotriose (Megazyme Cat. No. O-MTMT) and Glucose Test Kit (Megazyme Cat. No. K-GLUC) were obtained from Megazyme International Ireland. All other chemicals used in organic synthesis were purchased from Sigma Aldrich, Lennox Laboratory Supplies, Fischer Scientific Ireland or Apollo Scientific and were analytical reagent grade. A Bruker Avance 400 was employed for ^{1}H (400.13 MHz) and ^{13}C (100.61 MHz) NMR spectra. Resonances δ, are in ppm units downfield from an internal reference in C_2D_6SO (δ_H = 2.50). Mass spectrometry analysis was performed with a Q-Tof Premier Waters Maldi-quadrupole time-of-flight (Q-Tof) mass spectrometer equipped with Z-spray electrospray ionization (ESI) and matrix assisted laser desorption ionisation (MALDI) sources. Silica gel Florisil (200 mesh; Aldrich) was used for column chromatography. Analytical thin-layer chromatography was performed using Merck 60 F_{254} silica gel (pre-coated sheets, 0.2 mm thick, 20 cm x 20 cm) and visualised by UV irradiation or 5% H_2SO_4/EtOH staining.

3.2 Colourimetric substrate synthesis

3.2.1 2-Chloro-4-nitrophenyl-β-maltotriosyl (1-6) α-maltotrioside and 4-methylumbelliferyl-β-maltotriosyl (1-6) α-maltotrioside synthesis

The 2-chloro-4-nitrophenyl and 4-methylumbelliferyl derivatives of **11** were synthesised according to the general methodology employed by Planas *et al*.[20] These compounds were not purified following global deacetylation but were instead carried forward into the benzylidene acetal formation reaction as crude isolates (~75% *w*/*w* purity).

3.2.2 4,6-*O*-Benzylidene-2-chloro-4-nitrophenyl-β-maltotriosyl-α-(1,6)-maltotrioside (BzCNPG$_3$G$_3$, 1)

To a solution of 2-chloro-4-nitrophenyl-β-maltotriosyl-α-(1,6)-maltotrioside (1 g, 0.87 mmol) and *p*-toluenesulfonic acid monohydrate (43 mg, 0.22 mmol) in anhydrous dimethylformamide (10 mL) containing activated 4Å molecular sieves (200 mg), was added benzaldehyde dimethylacetal (1.05 mL, 6.98 mmol) over 1 hour *via* syringe. The reaction was stirred for 14 hours under an argon atmosphere at 50ºC. After this time, triethylamine (43 µL, 0.31 mmol) was added and the reaction cooled to room temperature. The crude reaction mixture was absorbed onto silica gel and purified by flash chromatography to afford $BzCNPG_3G_3$ (**1**) as a pale yellow solid (215 mg, 0.17 mmol) in 6% overall yield from maltotriosyl-maltotriose.

mp (H_2O) 196-199ºC (dec.); ^{1}H NMR (400MHz, C_2D_6SO) δ 3.03-3.16 (m, 1H), 3.21-3.82 (m, 33H), 3.87-3.99 (m, 1H), 4.08-4.19 (m, 1H), 4.52 (t, J = 5.52 Hz, 1H), 4.64 (t, J = 5.27 Hz, 1H), 4.67-4.73 (m, 2H), 4.81 (t, J = 5.52 Hz, 1H), 5.00 (d, J = 3.77 Hz, 1H (anom)), 5.02-5.13 (m, 5H), 5.14 (d, J = 3.77 Hz, 1H (anom)), 5.32 (d, J = 7.78 Hz, 1H (anom)), 5.35 (d, J = 5.02 Hz, 1H), 5.43 (d, J = 3.02 Hz, 1H), 5.47 (d, J = 2.51 Hz, 1H), 5.51 (d, J = 3.26 Hz, 1H), 5.59 (s, 1H), 5.60-5.75 (m, 6H), 7.35-7.43 (m, 3H), 7.43-7.51 (m, 3H), 8.23 (dd, J = 9.29, 2.76 Hz, 1H), 8.35 (d, J = 2.76 Hz, 1H); ^{13}C NMR (100MHz, C_2D_6SO) δ 60.5, 60.8, 60.8, 61.3, 63.8, 67.5, 68.5, 70.5, 70.6, 71.2, 72.1, 72.2, 72.2, 72.3, 72.6, 72.9, 72.9, 73.1, 73.5, 73.5, 73.7, 73.8, 74.0, 76.0, 76.5, 79.4, 79.8, 80.6, 81.4, 81.4, 99.2, 100.1, 100.9, 101.2, 101.3, 101.7, 101.9, 116.1, 122.7, 124.7, 126.1, 126.9, 126.9, 128.5, 128.5, 129.4, 138.2, 142.1, 158.1; HRMS MALDI$^+$ $[M+Na]^+$ Calc. 1256.3260, Found 1256.3219.

3.2.3 4,6-*O*-Benzylidene-4-methylumbelliferyl-β-maltotriosyl-α-(1,6)-maltotrioside ($BzMUG_3G_3$, 2)

To a solution of 4-methylumbelliferyl-β-maltotriosyl-α-(1,6)-maltotrioside (1 g, 0.87 mmol) and *p*-toluenesulfonic acid monohydrate (43 mg, 0.22 mmol) in anhydrous dimethylformamide (10 mL) containing activated 4Å molecular sieves (200 mg), was added benzaldehyde dimethylacetal (1.05 mL, 6.98 mmol) over 1 hour *via* syringe. The reaction was stirred for 14 hours under an argon atmosphere at 50ºC. After this time, triethylamine (43 µL, 0.31 mmol) was added and the reaction cooled to room temperature. The crude reaction mixture was absorbed onto silica gel and purified by flash chromatography to afford $BzMUG_3G_3$ (**2**) as a pale yellow solid (167 mg, 0.14 mmol) in 5% overall yield from maltotriosyl-maltotriose.

mp (H_2O) 230-235 ºC (dec.); ^{1}H NMR (400MHz, C_2D_6SO) δ 2.39 (d, J = 1.25 Hz, 3H), 3.01-3.11 (m, 1H), 3.17-3.77 (m, 34H), 3.84-3.94 (m, 1H), 4.06-4.16 (m, 1H), 4.49 (t, J = 6.02 Hz, 1H), 4.63, (t, J = 5.02 Hz, 1H), 4.66 (d, J = 3.51 Hz, 1H (anom)), 4.68 (t, J = 5.27 Hz, 1H), 4.98 (d, J = 3.76 Hz, 1H (anom)), 5.02 (d, J = 3.77 Hz, 1H (anom)), 5.03-5.20 (m, 6H)), 5.30-5.38 (m, 1H), 5.41-5.79 (m, 10H), 6.24 (d, J = 1.00 Hz, 1H), 6.99-7.06 (m, 2H), 7.32-7.40 (m, 3H), 7.40-7.47 (m, 2H), 7.66-7.73 (m, 1H); ^{13}C NMR (100MHz, C_2D_6SO) δ 18.7, 60.7, 60.79, 60.82, 61.4, 63.8, 67.5, 68.5, 70.49, 70.54, 71.2, 72.09, 72.12, 72.2, 72.3, 72.6, 72.8, 72.9, 73.2, 73.5, 73.6, 73.7, 73.8, 74.0, 75.8, 76.5, 79.7, 79.8, 80.5, 81.4, 81.4, 99.1, 100.1, 100.8, 101.1, 101.3, 101.6, 101.8, 103.6, 112.2, 113.7, 114.6, 126.8, 126.8, 126.9, 128.5, 128.5, 129.3, 138.3, 153.9, 154.9, 160.5, 160.6; HRMS ESI$^+$ $[M+Na]^+$ Calc. 1259.3854, Found 1259.3854.

3.3 Dissolution of colourimetric and fluorometric oligosaccharide substrates $BzCNPG_3G_3$ (1) and $BzMUG_3G_3$ (2)

Compound **1** was dissolved at a concentration of 12.4 mM in dimethylsulfoxide and stored at -20ºC between use. In this form **1** is stable for > 4 years. For use as a substrate, 1.0 mL of this solution was added to 2.0 mL of distilled water and mixed thoroughly. Thermostable β-glucosidase (50 µL, 3.2 M $(NH_4)_2SO_4$, 400 U/mL) and α-glucosidase (50 µL, 3.2 M $(NH_4)_2SO_4$, 1500 U/mL) was then added, mixed thoroughly and stored at -20ºC between use (final concentration of substrate **1** (4 mM). In this form, the substrate is stable for > 2 years.

Compound **2** was dissolved at a concentration of 3.1 mM in dimethylsulfoxide and stored at -20ºC between use. In this form **2** is stable for > 4 years. For use as a substrate, 1.0 mL of this solution was added to 2.0 mL of distilled water and mixed thoroughly. Thermostable β-glucosidase (50 µL, 3.2 M $(NH_4)_2SO_4$, 400 U/mL) and α-glucosidase (50 µL, 3.2 M $(NH_4)_2SO_4$, 1500 U/mL) was then added, mixed thoroughly and stored at -20ºC between use (final concentration of substrate **2** (1.0 mM). In this form, the substrate is stable for > 2 years.

3.4 Standard assay of pullulanase using $BzCNPG_3G_3$ (substrate 1) (4 mM) in the presence of β-glucosidase (6.5 U/mL) and α-glucosidase (24 U/mL)

A pre-equilibrated aliquot of pullulanase (0-40 mU on reduced pullulan) in 200 mM sodium acetate buffer, pH 5.0 containing 0.5 mg/mL of BSA (0.1 mL) was added to a pre-equilibrated aliquot of substrate **1** solution (0.1 mL, 4 mM) containing thermostable β-glucosidase (0.65 U) and thermostable α-glucosidase (2.4 U) and the mixture was incubated at 40°C for exactly 10 min. The reaction was terminated by adding 1.5 mL of 2% Tris (pH 10.0). The tube contents were well mixed and the absorbance measured at 400 nm against a reagent blank. One Unit of activity is the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenolate per minute at 40°C and pH 5.0.

3.5 Standard assay of pullulanase using $BzMUG_3G_3$ (substrate 2) (1 mM) in the presence of β-glucosidase (6.5 U/mL) and α-glucosidase (24 U/mL)

A pre-equilibrated aliquot of pullulanase (0-6 mU on borohydride-reduced pullulan) in 200 mM sodium acetate buffer, pH 5.0 containing 0.5 mg/mL of BSA (0.1 mL) was added to a pre-equilibrated aliquot of substrate **2** solution (0.1 mL, 1 mM) containing thermostable β-glucosidase (0.65 U) and thermostable α-glucosidase (2.4 U) and the mixture was incubated at 40°C for exactly 10 min. The reaction was terminated by adding 3.0 mL of 2% Tris (pH 10.0). The tube contents were well mixed and the fluorescence measured with a portable fluorimeter, Quantifluor ST, from Promega, U.K.

3.6 Standard assay of pullulanase using borohydride reduced pullulan (10 mg/mL) by reducing sugar determination.

A pre-equilibrated aliquot of pullulanase (0.2 mL; 0-100 mU on borohydride-reduced pullulan) in 100 mM sodium acetate buffer, pH 5.0 containing 0.5 mg/mL of BSA (0.1 mL) was added to a pre-equilibrated aliquot of borohydride-reduced pullulan (Lot 100902, 0.5 mL, 10 mg/mL) in 100 mM sodium acetate buffer (pH 5.0). The resulting mixture was agitated on a vortex stirrer and incubated at 40°C. Incubations were terminated by the addition of Nelson–Somogyi solution C[12,13] (0.5 mL) after 0, 3, 6, 9 and 12 min. The colour was developed according to the Nelson–Somogyi procedure.[12,13] The contents of tubes were stirred vigorously

and the absorbance was measured at 520 nm against a substrate/reagent blank and a glucose standard solution (50 μg) was included. One Unit of activity is the amount of enzyme required to release one micromole of glucose reducing sugar equivalents per minute at 40°C and pH 5.0.

3.7 Determination of the pH activity profiles of α-glucosidase, β-glucosidase and pullulanases.

To determine the effect of pH on the activity of α-glucosidase and β-glucosidase on 4-nitrophenyl α-glucoside or 4-nitrophenyl β-glucoside respectively, the substrates were prepared at a concentration of 10 mM in 100 mM buffer solutions over a range of pH values; citrate-phosphate (pH 4.0-7.0), phosphate (pH 7.0-9.0), acetate (pH 4.5-5.0) or maleate (pH 5.5-6.5). A pre-equilibrated aliquot of the enzyme (0.1 mL, 140 mU) in 100 mM KCl containing 0.5 mg/mL BSA was added to a pre-equilibrated aliquot of substrate solution (0.1 mL, 10 mM) at the various pH values and the mixtures were incubated at 40°C for exactly 10 min. The reactions were terminated by adding 3.0 mL of 2% Tris (pH 10.0). To determine the effect of pH on the activity of pullulanase on borohydride-reduced pullulan, a pre-equilibrated aliquot of pullulanase (0.2 mL; 60 mU on borohydride-reduced pullulan) in 100 mM KCl containing 0.5 mg/mL of BSA was added to a pre-equilibrated aliquot of borohydride-reduced pullulan (Lot 100902, 0.5 mL,10 mg/mL) in 100 mM sodium acetate buffer (pH 4.5 or 5.0) or sodium maleate buffer (pH 5.0, 5.5, 6.0 or 6.5). The resulting mixture was agitated on a vortex stirrer and incubated at 40°C. Incubations were terminated by the addition of Nelson–Somogyi solution C[12,13] (0.5 mL) after 0, 3, 6, 9 and 12 min. The colour was developed according to the Nelson–Somogyi procedure.[12,13] The contents of tubes were stirred vigorously and the absorbance was measured at 520 nm against a substrate/reagent blank and a glucose standard solution (50 μg) was included. One Unit of activity is the amount of enzyme required to release one micromole of glucose reducing sugar equivalents per minute at 40°C and pH 5.0.

3.8 Assay of pullulanase on $BzCNPG_3G_3$ (1) and $BzMUG_3G_3$ (2).

To determine the effect of substrate concentration (Figure 8) on the rate of hydrolysis of **1** by

Klebsiella planticola and *Bacillus licheniformis* pullulanase, 0.1 mL of **1** (0.2–4.0 mM) in the presence of thermostable β-glucosidase (0.65 U) and thermostable α-glucosidase (2.4 U), was incubated with either 0.1 mL of *K. planticola* pullulanase (7.1 mU on this substrate; 40 mU on reduced pullulan) or *B. licheniformis* pullulanase (14.5 mU on this substrate; 74 mU on reduced pullulan) in 100 mM sodium acetate buffer (pH 5.0) at 40°C. The reaction was terminated after fixed time intervals by adding 1.5 mL of 2% Tris solution (pH 9.0) and absorbance measured at 400 nm. To determine the effect of substrate concentration (Figure 10) on the rate of hydrolysis of **2** by *K. planticola* and *B. licheniformis* pullulanase, 0.1 mL of **2** (0.05–1 mM) in the presence of thermostable β-glucosidase (0.65 U) and thermostable α-glucosidase (2.4 U), was incubated with 0.1 mL of *K. planticola* pullulanase (1.39 mU on this substrate, 6.6 mU on reduced pullulan) or *B. licheniformis* pullulanase (1.8 mU on this substrate, 9.1 mU on reduced pullulan) in 100 mM sodium acetate buffer (pH 5.0) at 40°C. Reaction was terminated after fixed time intervals by adding 3.0 mL of 2% Tris solution (pH 9.0) and the fluorescence measured with a portable fluorimeter, Quantifluor® ST, from Promega, U.K. The effect of β-glucosidase concentration on the rate of increase in absorbance at 400 nm was determined by preparing solutions containing **1** (4 mM), α-glucosidase (24 U/mL) and β-glucosidase (0.7-6.5 U/mL) and was determined as follows (Figure 6): aliquots (0.1 mL) of the solutions were incubated with *K. planticola* pullulanase (13.6 mU on this substrate) at 40°C and reaction terminated after 0, 3, 6 and 9 min by adding 1.5 mL of 2% Tris solution (pH 9.0). The effect of α-glucosidase concentration on the rate of increase in absorbance at 400 nm was determined by preparing solutions containing **1** (4 mM), β-glucosidase (6.5 U/mL) and α-glucosidase (2.4 - 24 U/mL) and was determined as follows (Figure 7): aliquots (0.1 mL) of the solutions were incubated with *K. planticola* pullulanase (13.6 mU on this substrate) at 40°C and reaction terminated after 0, 3, 6 and 9 min by adding 1.5 mL of 2% Tris solution (pH 9.0). The effect of α-glucosidase and β-glucosidase concentrations on the rate of increase in fluorescence on hydrolysis of **2** was determined by preparing solutions containing **2** (1 mM) , β-glucosidase and α-glucosidase mixtures as described for studies on substrate **1**. The fluorescence was measured with a Quantifluor® ST portable fluorimeter (Promega, U.K).

Standard curves for the action of *K. planticola* and *B. licheniformis* pullulanases on **1** (Figure 10) were determined by incubating **1** (4 mM, containing β-glucosidase at 6.5 U/mL and

α-glucosidase at 24 U/mL) with 0.1 mL of *K. planticola* and *B. licheniformis* pullulanases (0-40 mU/assay on borohydride-reduced pullulan in pH 5.0, 100 mM sodium acetate buffer containing 0.5 mg/mL BSA) at 40°C for 10 min. The reaction was terminated by the addition of 1.5 mL of 2% Tris solution (pH 10.0). The tube contents were well mixed and the absorbance measured at 400 nm against a reagent blank. Standard curves for the action of *K. planticola* and *B. licheniformis* pullulanases on **2** (Figure 14) were determined by incubating **2** (1 mM, containing β-glucosidase at 6.5 U/mL and α-glucosidase at 24 U/mL) with 0.1 mL of *K. planticola* and *B. licheniformis* pullulanases (0-6 mU/assay on borohydride-reduced pullulan in pH 5.0, 100 mM sodium acetate buffer containing 0.5 mg/mL BSA) at 40°C for 10 min. The reaction was terminated by the addition of 3.0 mL of 2% Tris solution (pH 10.0). The tube contents were well mixed and the fluorescence was measured with a Quantifluor® ST portable fluorimeter (Promega, U.K).

Long term stability of compound **1** in the presence of β-glucosidase at 6.5 U/mL and α-glucosidase at 24 U/mL) was determined by storing the substrate mixture at -20°C, 4°C and 20°C for up to 66 days. After the various time intervals, stability was determined by running a standard assay by mixing 0.1 mL of the substrate mixture with 0.1 mL of *K. planticola* pullulanase (6.8 mU on substrate **1** mixture) for 10 min at 40 °C and stopping the reaction with 1.5 mL of 2% Tris solution (pH 10.0) (Figure S1 - Supplementary Information). Changes in blank absorbance values were determined by adding 1.5 mL of 2 % Tris solution (pH 10.0) to 0.1 mL of the substrate solution and 0.1 mL of the buffer solution, mixing well and measuring the absorbance at 400 nm. Long term stability of compound **2** (in the presence of β-glucosidase at 6.5 U/mL and α-glucosidase at 24 U/mL) was determined by storing the substrate mixture at -20°C, 4°C and 20°C for up to 66 days. After the various time intervals, stability was determined by running a standard assay by mixing 0.1 mL of the substrate mixture with 0.1 mL of *K. planticola* pullulanase (28 mU on reduced pullulan) for 10 min at 40 °C, stopping the reaction with 3.0 mL of 2% Tris solution (pH 10.0) and measuring the fluorescence with a Quantifluor® ST portable fluorimeter. Changes in blank absorbance values were determined by adding 3.0 mL of 2% Tris solution (pH 10.0) to 0.1 mL of the substrate solution and 0.1 mL of the buffer solution and mixing well. It appears clearly from the results shown in Figure S1 (Supplementary Information) that mixtures containing **1** are very stable at 4°C and -20°C, but less stable at 20°C. On storage at

20°C, the absorbance of the blank solution of the substrate mixture containing **2** in the assay (containing no enzyme) increased from 0.025 to 0.104 over 66 days (*i.e.*, four-fold), but the assay absorbance increased by the same amount such that the determined enzyme activity remained unchanged. This increase in absorbance value represents a degradation of approximately 3% of the substrate in the assay mixture. At -20°C and 4°C, the absorbance of the blank solution in the assay increased by just 0.000 and 0.015 respectively, over the same period. In the assay, the substrate behaved exactly as in the freshly prepared reagent solution. Similar stability was observed with the substrate mixture containing compound **2**.

3.9 Hydrolysis of 6^1-α-D-glucosyl 6^3-α-D-maltotriosyl maltotriose (13) and maltoheptaose by α-glucosidase and amyloglucosidase

Pre-equilibrated aliquots of *B. stearothermophilus* α-glucosidase (0.1 mL; 1.0 U) or *A. niger* amyloglucosidase (0.1 mL, 1.0 U) in 100 mM sodium maleate buffer, pH 5.0 containing 0.5 mg/mL of BSA was added to a pre-equilibrated aliquots of 6^1-α-D-glucosyl 6^3-α-D-maltotriosyl maltotriose (**13**) or maltoheptaose in 100 mM sodium maleate buffer, pH 5.5 and the mixture was incubated at 40°C. The reaction was terminated after 0, 5, 10, 20, 40 and 90 min by incubating the tubes in a boiling water bath for 2 min. Free glucose was measured using glucose oxidase/peroxidase reagent from a Glucose (GOPOD) test kit (Megazyme Cat. No. K-GLUC). The relative rates of hydrolysis of these substrates by α-glucosidase was calculated from these values. Under these conditions, AMG gave complete hydrolysis of both substrates within 20 min.

3.10 Hydrolysis of $BzCNPG_3G_3$ (1) and $BzMUG_3G_3$ (2) by α-glucosidase, β-amylase and *Rhizopus* sp. amyloglucosidase

Hydrolysis of substrates **1** and **2** by *exo*-acting enzymes was determined using substrate solutions containing **1** (4 mM), 24 U/mL of *B. stearothermophilis* α-glucosidase and 6.5 U/mL of *T. maritima* β-glucosidase. Aliquots of substrate solution were incubated with *B. stearothermophilus* α-glucosidase (0.1 mL; 20 U), *A. niger* amyloglucosidase (0.1 mL, 20 U) or barley β-amylase (0.1 mL, 20 U) in 100 mM sodium maleate buffer, pH 5.0 containing 0.5

mg/mL of BSA. The tubes were incubated at 40°C and the reaction was terminated after 0-4 h by the addition of either 1.5 mL (substrate 1) or 3.0 mL (substrate 2) of 2% Tris solution (pH 10.0).

3.11 Standard assay of pullulanase using Red Pullulan

A pre-equilibrated aliquot of pullulanase (1.0 mL; 0-400 mU on reduced pullulan) in 200 mM sodium acetate buffer, pH 5.0 containing 0.5 mg/mL of BSA was added to a pre-equilibrated aliquot of Red Pullulan (Lot 100906, 0.5 mL, 20 mg/mL) in 500 mM KCl. The resulting mixture was agitated on a vortex stirrer and incubated at 40°C. The incubations were terminated after 10 min by the addition of 95/5 *v*/*v* ethanol/H_2O (2.5 mL) which precipitated non-hydrolysed, high-molecular weight Red Pullulan. The tube contents were stirred vigorously on a vortex mixer and the tubes were allowed to equilibrate at room temperature for 10 min. The tubes were again stirred and then centrifuged at 1,000 *g* for 10 min. The supernatant solutions were then poured directly from the tubes into spectrophotometer cuvettes and the absorbance of the blank and of the reaction solutions were measured at 510 nm against distilled water (Figure 14). Activity was determined by reference to a standard curve. A reagent blank was prepared by replacing the enzyme preparation with 100 mM sodium acetate buffer (pH 5.0, containing 0.5 mg/mL BSA).

3.12 Standard assay of pullulanase using Limit Dextrizyme tablets

A Limit Dextrizyme tablet (Lot 121101) was added to a pre-equilibrated aliquot of pullulanase (0.5 mL; 0-25 mU on reduced pullulan) in 100 mM sodium acetate buffer (pH 5.0 containing 0.5 mg/mL BSA). The tablet was allowed to swell without agitation. The suspension was incubated at 40°C for 10 min and the reaction was terminated by the addition of 10 mL of 2% Tris solution (pH 10.0) with vigorous agitation on a vortex mixer and the tubes were left at room temperature. After approximately 10 min the tube contents were agitated again on a vortex mixer and the contents were filtered through Whatman No. 1 (9 cm) filter circles. The filtrates were poured into spectrophotometer cuvettes and the absorbance of the blank and of the reaction solutions were measured at 590 nm against distilled water (Figure 15). Activity was determined

by reference to a standard curve. A reagent blank was prepared by replacing the enzyme preparation with 100 mM sodium acetate buffer (pH 5.0 containing 0.5 mg/mL BSA).

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References

1. Bender, H.; Wallenfels, K. *Meth. Enzymol.* **1966**, *8*, 555–559.

2. Lee, E. Y. C.; Whelan, W. J. *The Enzymes* (*3rd ed.*). New York: Academic Press. **1972**, vol. 5, 191–234.

3. Banks, W.; Greenwood, C. T.; *Starch and its components.* Aberdeen University Press, **1975**, 233-234.

4. Kim, C. H.; Nashiru, O.; Ko, J. H. *FEMS Microbiol. Lett.* **1996**, *138*, 147-152.

5. Roy, A.; Messaoud, E. B.; Bejar, S. *Enzyme Microbiol. Technol.* **2003**, *33*, 720-724.

6. Imanaka, T.; Kuriki, T. *J. Bacteriol.* **1989**, *171*, 369–374.

7. Sakano, Y.; Masuda, N.; Kobayashi, T. *Agric. Biol. Chem.* **1971**, *35*, 971–973.

8. Niehaus, F.; Peters, A.; Groudieva, T.; Antranikian, G. *FEMS Microbiol. Lett.* **2000**, *190*, 223-229.

9. Norman, B. E. *Starch* **1982**, *34*, 340-346.

10. Crabb, W. D.; Mitchinson, C. *Trends Biotechnol.* **1997**, *15*, 349-352.

11. Shaw, J. F.; Sheu, J. R. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1071-1073.

12. Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375-380.

13. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19-23.

14. McCleary, B. V. *Carbohydr. Res.* **1992**, *227*, 257-268.

15. Blair, H. E. U.S. Patent 4 794 078, 1988.

16. McCleary, B. V.; Sheehan, H. *J. Cereal Sci.*, **1987**, *6*, 237-251.

17. McCleary, B. V.; Mangan, D.; Daly, R.; Fort, S.; Ivory, R.; McCormack, N. *Carbohydr. Res.* **2014**, *385*, 9-17.

18. Bøjstrup, M.; Christensen, C. O.; Windahl, M. W.; Henriksen, A.; Hindsgaul, O. *Anal. Biochem.* **2014**, *449*, 45-51.

19. Catley, B. J. *Carbohydr. Res.* **1978**, *61*, 419-424.

20. Planas, A.; Abel, M.; Millet, Ó.; Palasí, J.; Pallarés, C.; Viladot, J.-L. *Carbohydr. Res.*, **1998**, *310*, 53-64.

21. McDougall, G. J.; Ross, H. A.; Swanston, J. S.; Davies, H. V. *Planta*, **2004**, *218*, 542-551.

Figures

Figure 1. Overview of pullulanase assay procedure using $BzCNPG_3G_3$ (**1**) and $BzMUG_3G_3$ (**2**).

Figure 2. Pullulan-derived structures of interest.

Figure 3. pH activity profiles for α-glucosidase and β-glucosidase.

Figure 4. pH activity curves for (a) *K. planticola* and (b) *B. licheniformis* pullulanases on borohydride-reduced pullulan (Lot 100901) at 40°C.

Figure 5. Effect of β-glucosidase concentration on reaction kinetics over 9 min in the presence of *K. planticola* pullulanase (13.6 mU/assay) and $BzCNPG_3G_3$ (**1**) (2 mM) in pH 5.0 100 mM sodium acetate at 40°C.

Figure 6. Effect of α-glucosidase concentration on reaction kinetics over 9 min in the presence of *K. planticola* pullulanase (13.6 mU /assay) and $BzCNPG_3G_3$ (**1**) (2 mM) in pH 5.0 100 mM sodium acetate at 40°C.

Figure 7. Effect of $BzCNPG_3G_3$ (**1**) concentration on reaction kinetics over 6 min in the presence of (a) *K. planticola* pullulanase (7.1 mU/assay on this substrate, 40 mU/assay on reduced pullulan) and (b) *B. licheniformis* pullulanase (14.5 mU/assay on this substrate, 74 mU/assay on reduced pullulan) plus β-glucosidase (0.65 mU/assay) and α-glucosidase (2.4 mU/assay) in pH 5.0 100 mM sodium acetate at 40°C.

Figure 8. Determination of the Michaelis-Menten K_m constant for (a) *K. planticola* and (b) *B. licheniformis* pullulanases on $BzCNPG_3G_3$ (**1**). Initial rates of reaction were determined as described in Figure 7 and in the Experimental Section.

Figure 9. Standard curves relating absorbance increase at 400 nm observed with $BzCNPG_3G_3$ (**1**) to level of *K. planticola* and *B. licheniformis* pullulanase activity on borohydride reduced pullulan at 40°C in pH 5.0 100 mM sodium acetate.

Figure 10. Effect of $BzMUG_3G_3$ (**2**) concentration on reaction kinetics over 6 min in the presence of (a) *K. planticola* pullulanase (1.39 mU/assay on this substrate, 6.6 mU/assay on reduced pullulan) and (b) *B. licheniformis* pullulanase (1.8 mU/assay on this substrate, 9.1 mU/assay on reduced pullulan) in the presence of β-glucosidase (0.65 mU/assay) and α-glucosidase (2.4 mU/assay) in pH 5.0 100 mM sodium acetate at 40°C.

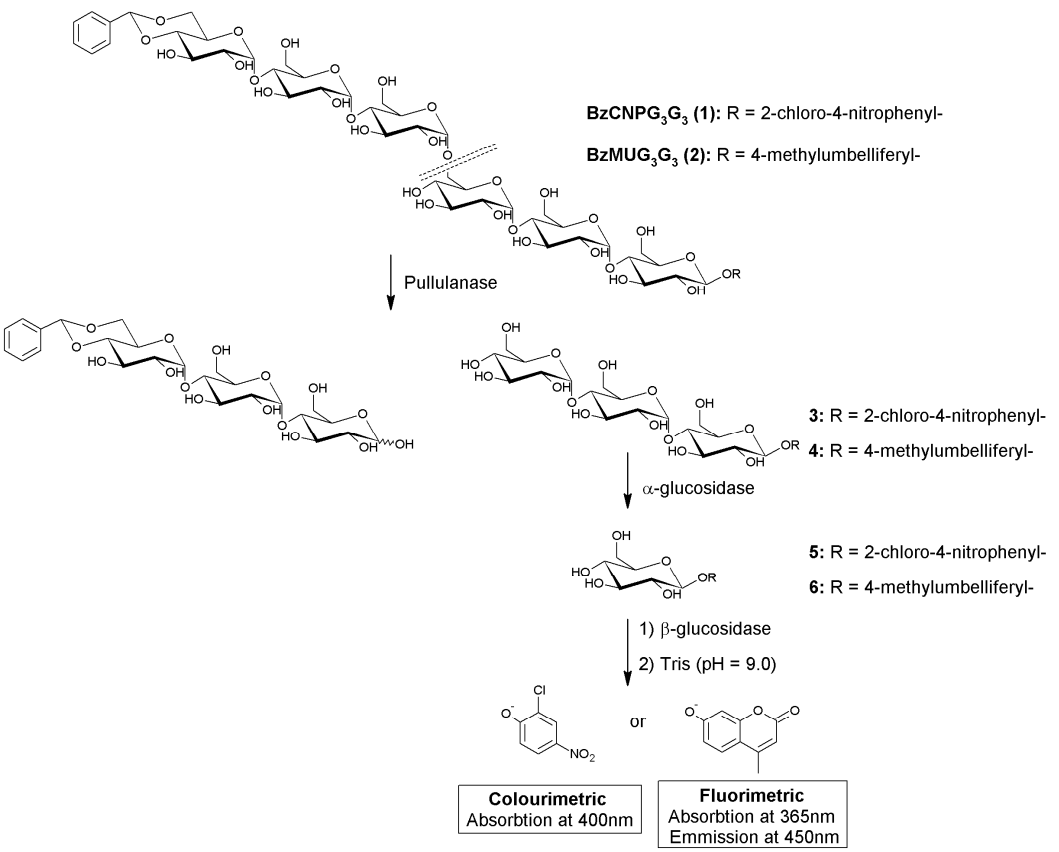
Figure 11. Determination of the Michaelis-Menten K_m constant for (a) *K. planticola* and (b) *B. licheniformis* pullulanases on $BzMUG_3G_3$ (**2**). Initial rates of reaction were determined as described in Figure 10 and in Experimental Section.

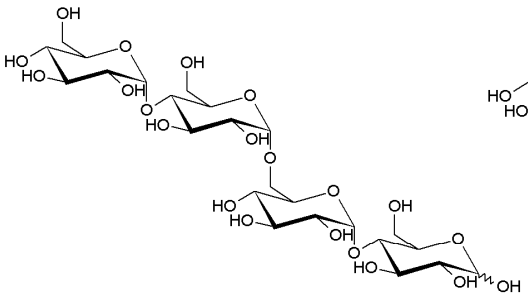
Figure 12. Standard curve relating fluorescence (arbitrary units) against concentration (micromolar) of 4-methylumbelliferone in 2% Tris (pH 10.0).

Figure 13. Standard curves relating increase in fluorescence observed with $BzMUG_3G_3$ (**2**) to level of *K. planticola* and *B. licheniformis* pullulanase activity on borohydride-reduced pullulan (Lot 100901) at 40°C in pH 5.0 100 mM sodium acetate.

Figure 14. Standard curve relating the activity of pullulanase (mU/assay, *i.e.* /1.0 mL) to increase of absorbance at 510 nm on hydrolysis of Red-Pullulan (Lot 100906). Incubations were performed under standard conditions at pH 5.0 for 10 min at 40°C. Pullulanase activity (mU/assay) was determined on borohydride-reduced pullulan (Lot 100901) using the Nelson-Somogyi reducing-sugar assay.

Figure 15. Standard curve relating the activity of pullulanase (mU/assay, *i.e.* /0.5 mL) to increase of absorbance at 590 nm on hydrolysis of Azurine-CL-Pullulan (in Limit-Dextrizyme tablets, Lot 121101). Incubations were performed under standard conditions at pH 5.0 for 10 min at 40°C. Pullulanase (U/mL) was determined on borohydride reduced pullulan (Lot 100901) using the Nelson-Somogyi reducing-sugar assay.





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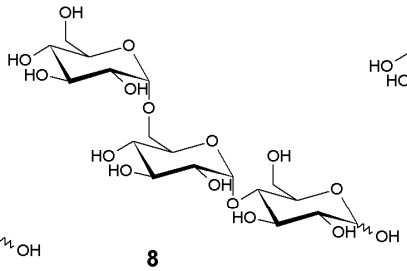

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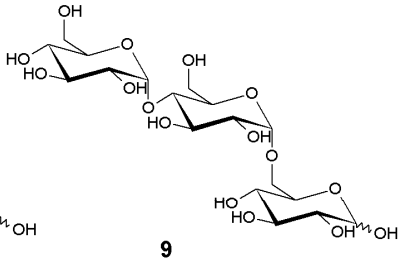

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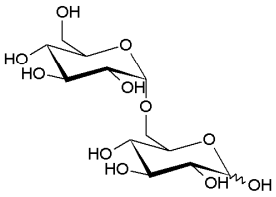

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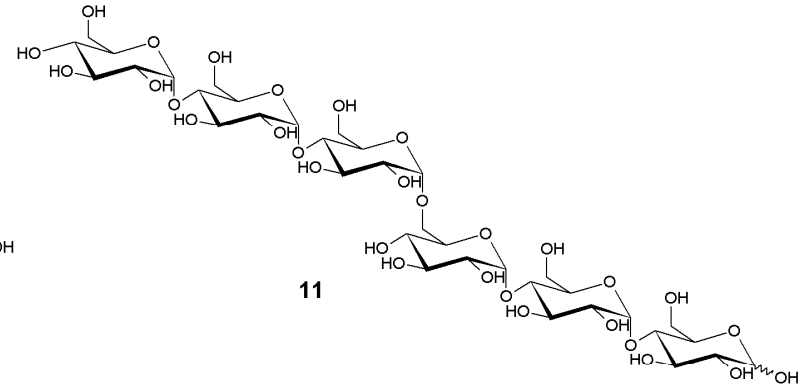

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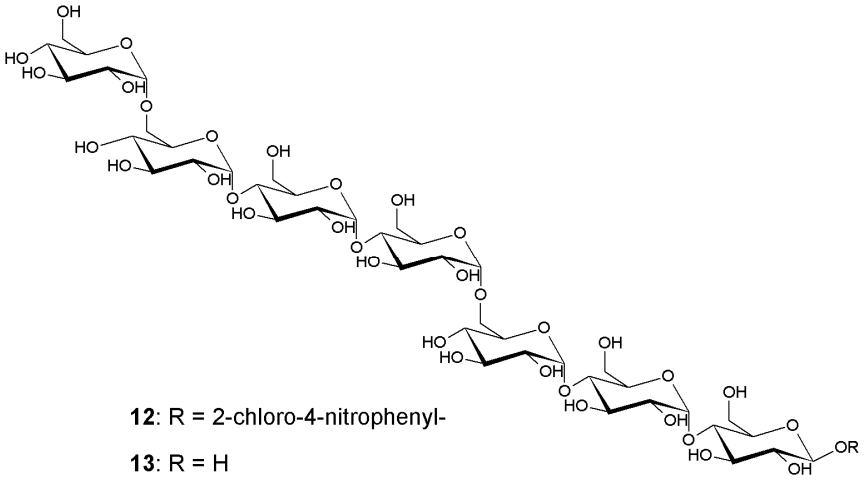
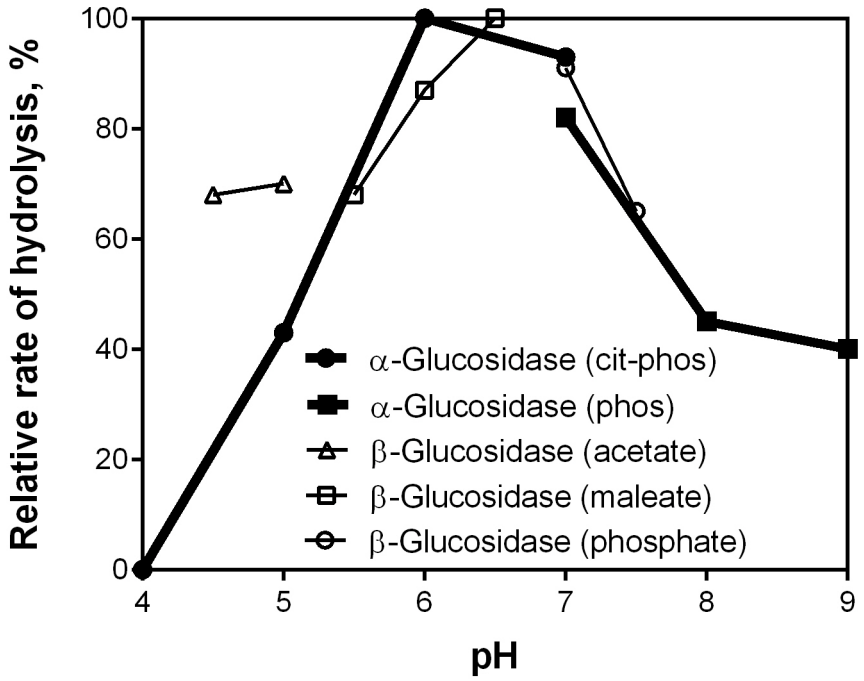
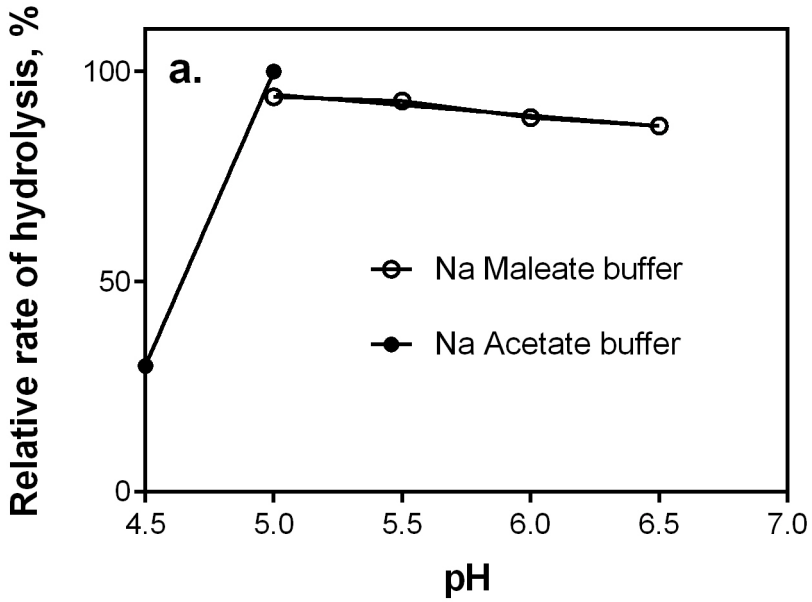
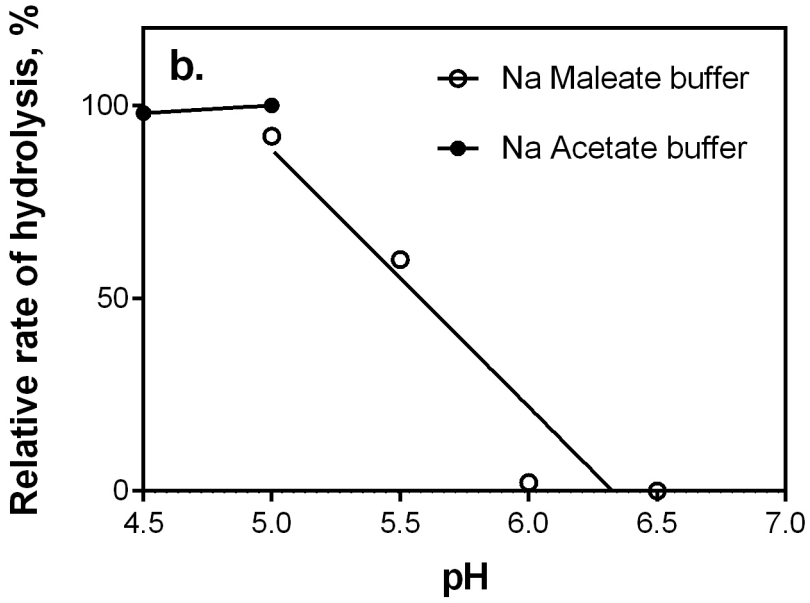


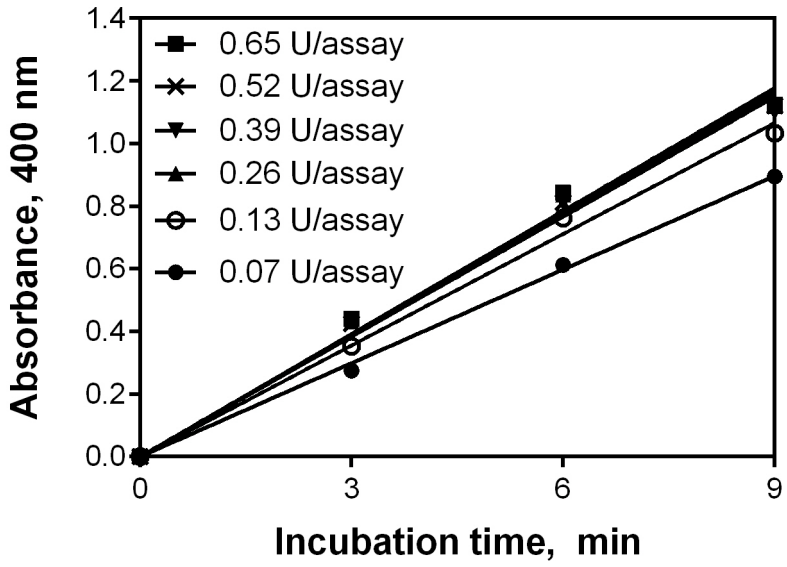
12: R = 2-chloro-4-nitrophenyl-

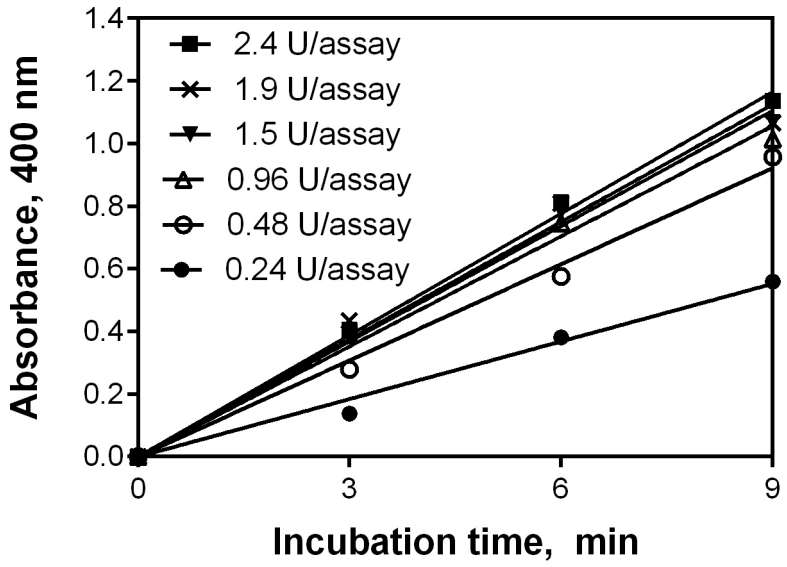
13: R = H

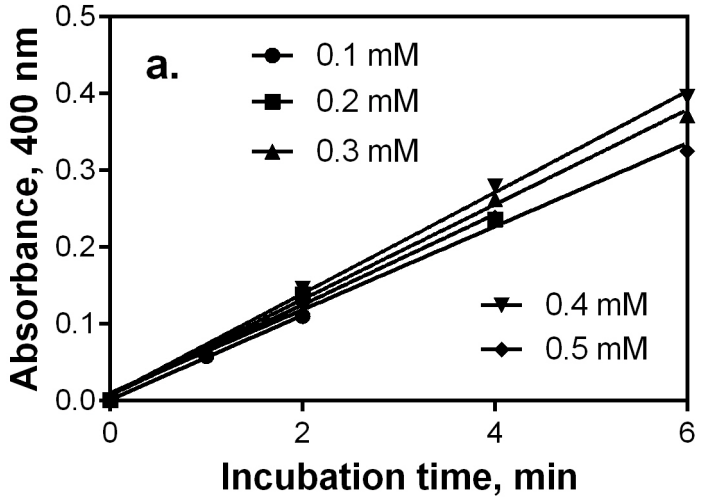


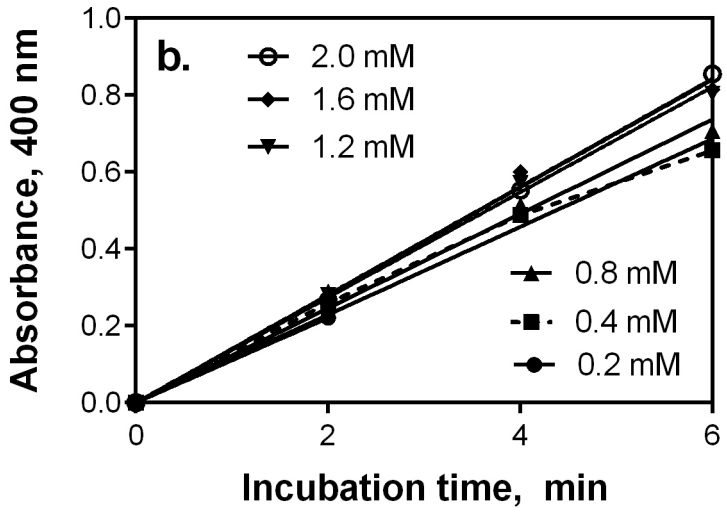

a.
Relative rate of hydrolysis, %
100
50
0
4.5
5.0
5.5
6.0
6.5
7.0
pH
Na Maleate buffer
Na Acetate buffer

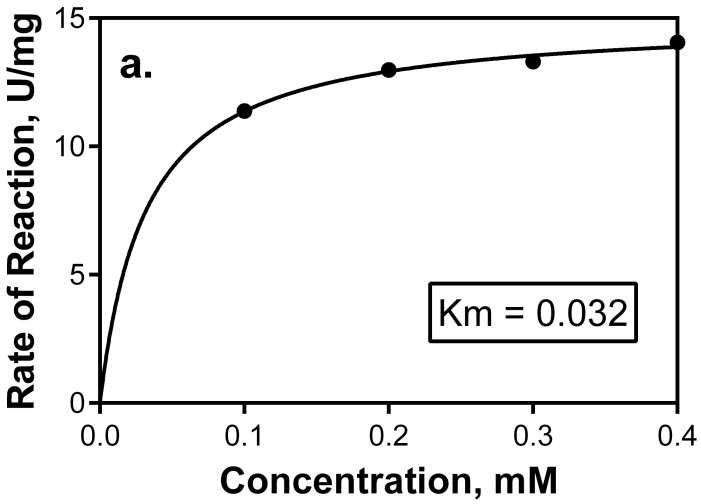

b.
Relative rate of hydrolysis, %
100
50
0
4.5
5.0
5.5
6.0
6.5
7.0
pH
Na Maleate buffer
Na Acetate buffer

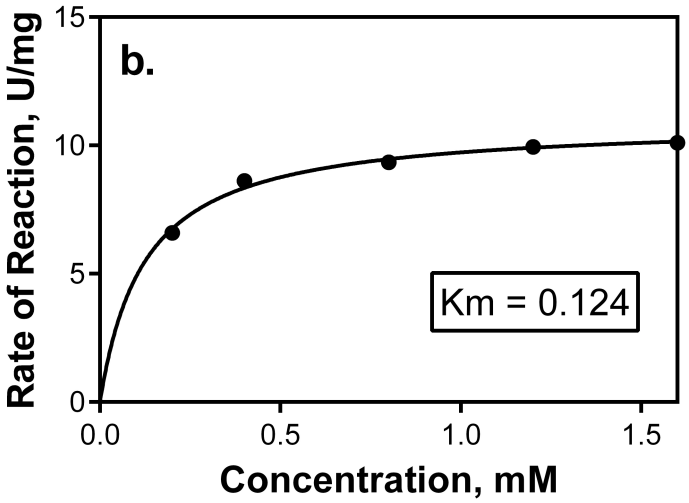


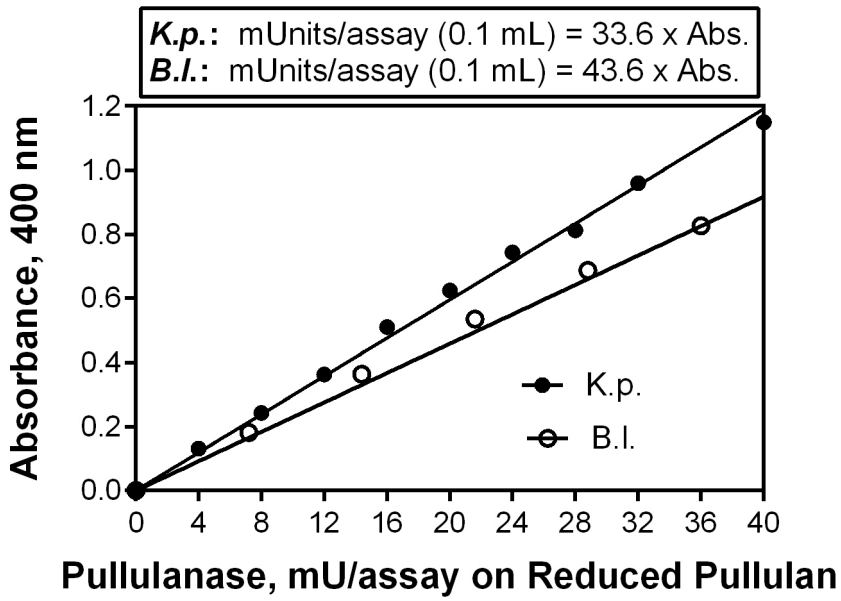


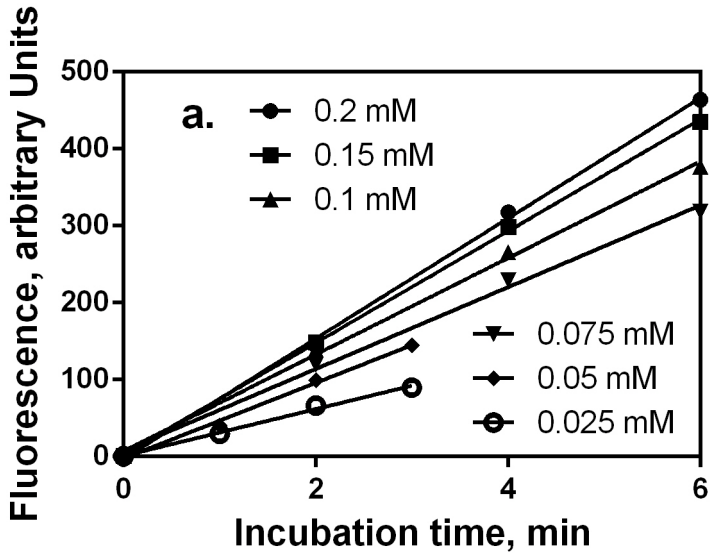


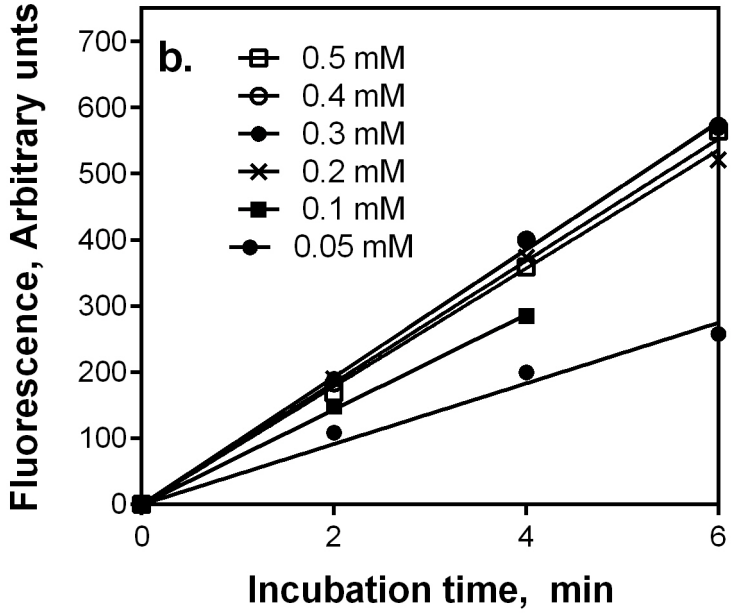


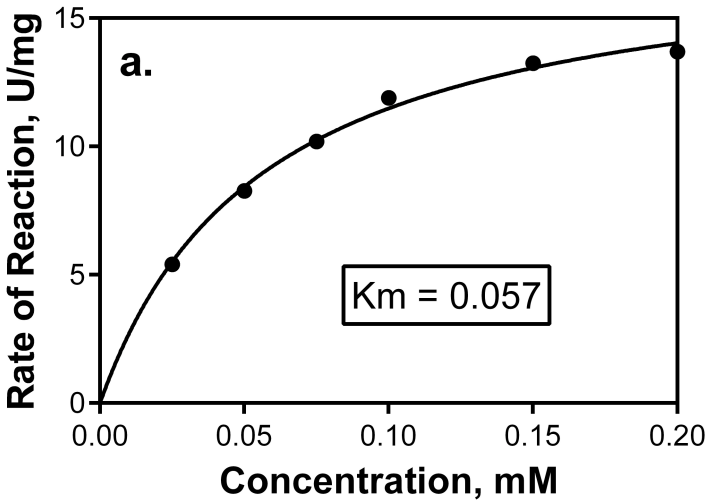


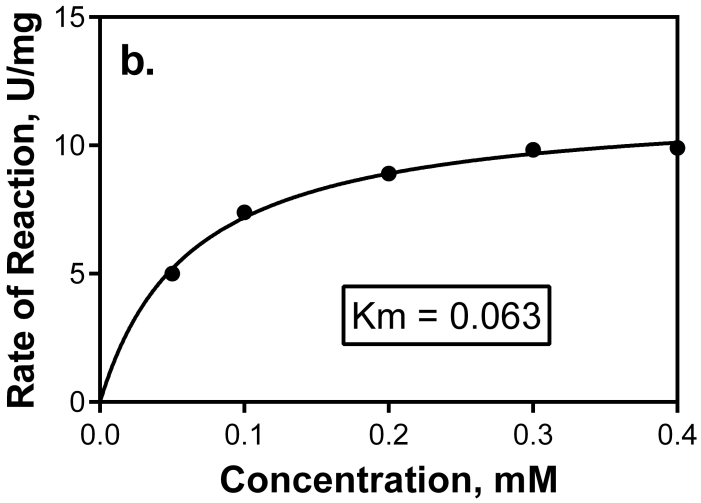


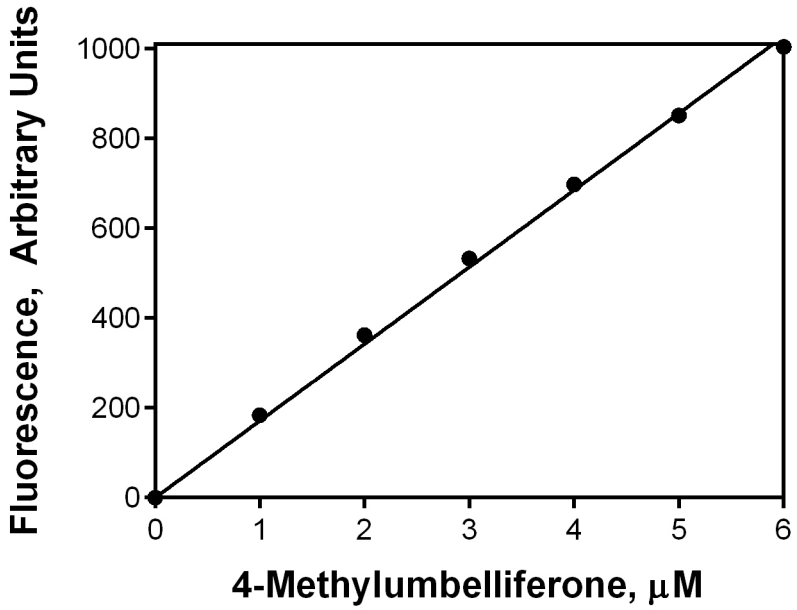


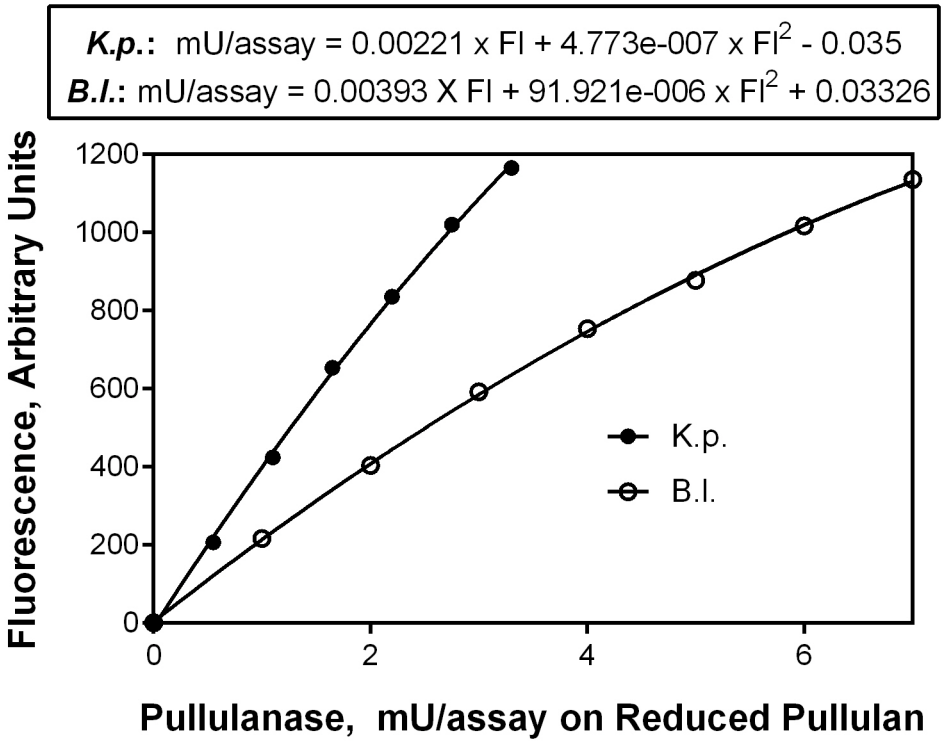

a.
0.2 mM
0.15 mM
0.1 mM
0.075 mM
0.05 mM
0.025 mM
Fluorescence, arbitrary Units
Incubation time, min
0
100
200
300
400
500
0
2
4
6

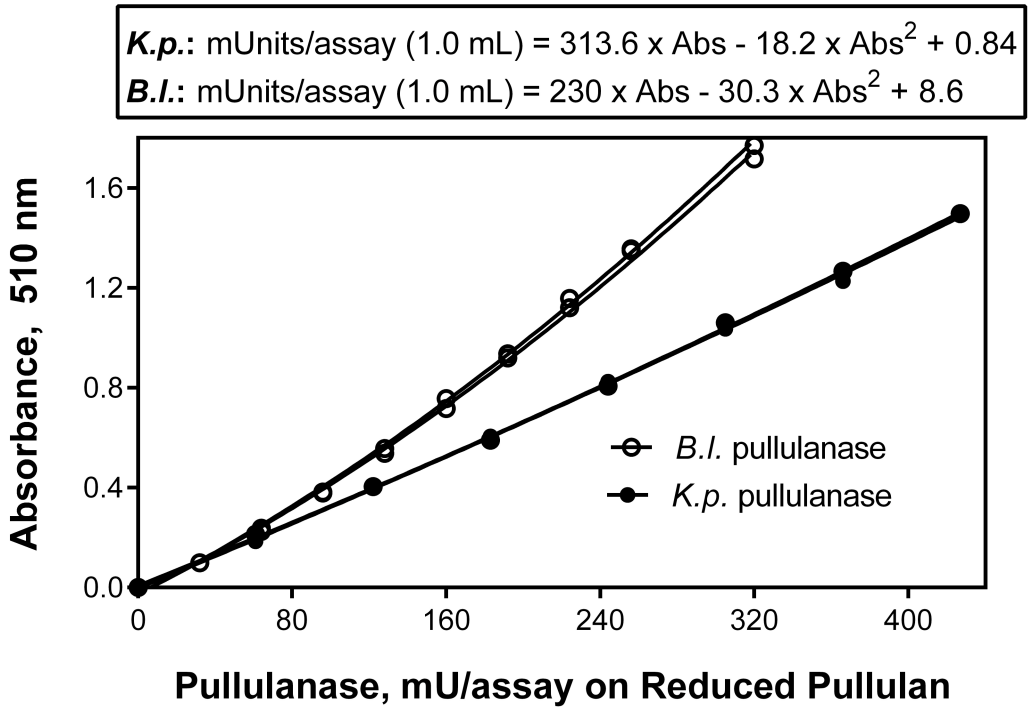

b.
0.5 mM
0.4 mM
0.3 mM
0.2 mM
0.1 mM
0.05 mM
Fluorescence, Arbitrary unts
Incubation time, min
0
100
200
300
400
500
600
700
0
2
4
6

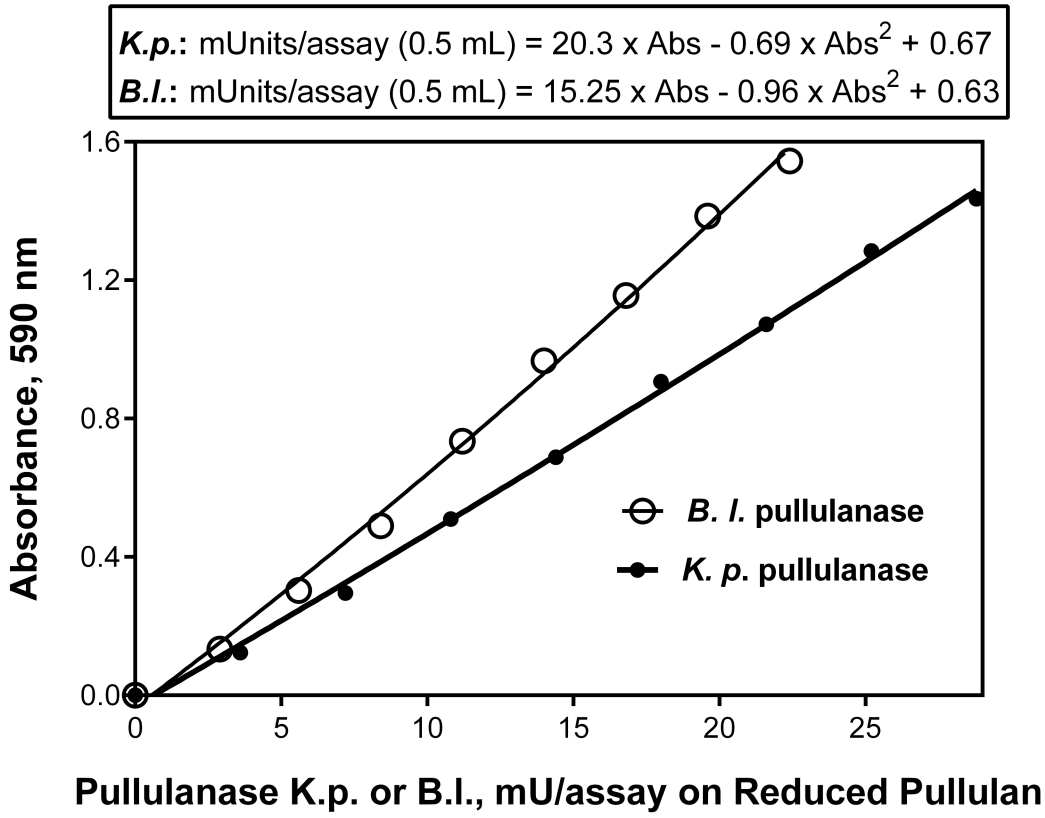












Highlights

- Novel, selective, enzyme-coupled assay for the measurement of pullulanase is reported
- The synthesis of a colourimetric and a fluorometric substrate is described
- Benzylidene acetal functionalization prevents action of the exo-glycosidases present
- Nitrophenyl functionalisation provides chromophore for simple quantitative detection
- Methylumbelliferyl functionalization provides fluorophore for increased sensitivity

Graphical abstract