Screening for Polysaccharide *endo*-Hydrolases using Insoluble Dyed Polysaccharide

04/17

BACKGROUND

Insoluble dyed substrates can be used to detect enzymatic activities in both agar plate and microtiter plate assays. These methods allow for high throughput screening of multiple samples and are accurate, cost effective and easily performed.

The substrates as traditionally supplied (e.g. **AZCL-Amylose**) are granular and quickly settle from substrate suspensions, making routine pipetting non-reproducible. Also, when added to agar plates, they rapidly settle to the bottom of the plate before the agar sets. Consequently, the substrates are not ideal in their current form for their intended uses. To resolve this limitation, Megazyme has investigated alternative ways to dry and mill these substrates to produce materials of a finer particle size which resolve many of the identified limitations. In the preparation of agar plates, the molten agar takes some minutes to solidify and during this time, settling of the insoluble substrate occurs, even with the finer particle size. In order to prevent this, we have evaluated various additives to increase the viscosity of the agar preparations and thus reduce "settling" problems. It was found that xanthan gum (0.5% w/v) effectively prevents settling (Figure 1). As a result, the substrate particles remain closer to the gel surface, resulting in an increased sensitivity of the assay (Figure 2). Xanthan gum is available from Megazyme (cat. no. **P-XANTH**).

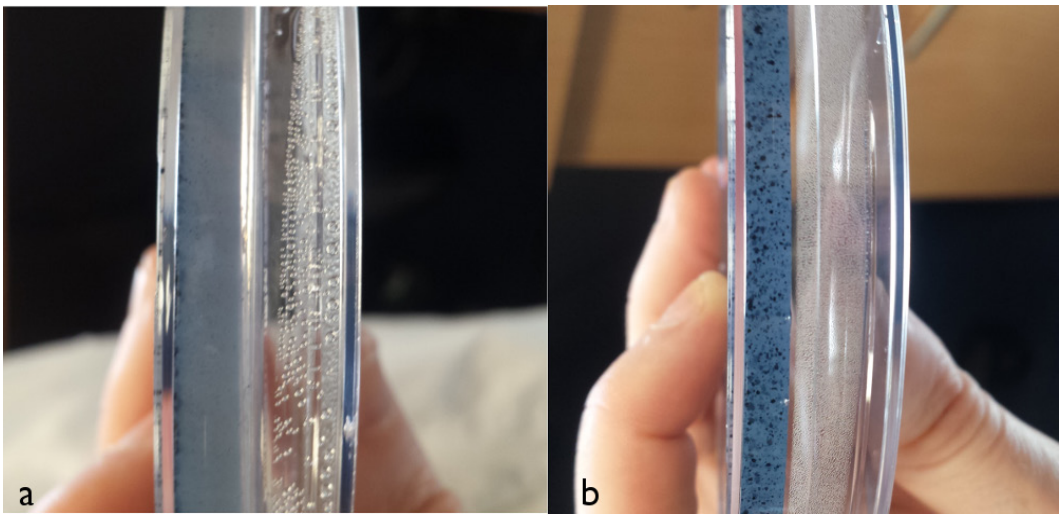


Figure 1. Settling of AZCL-Amylose (**I-AZAMY**) in agar/xanthan gum preparations.
a) AZCL-Amylose (0.1% w/v) in agar (2% w/v) with no added xanthan gum. b) same as (a) but with xanthan gum (**P-XANTH**) at a concentration of 0.5% w/v. The settling of substrate particles during the gelling process in plate (a) is clearly observable, while there is no detectable settling in plate (b), which contains xanthan gum in the agar mix.

Test-tube based assays are also facilitated by the addition of xanthan gum into the substrate suspension. In this case, xanthan gum at a concentration of 0.1% w/v dramatically reduces the rate of settling of the substrate (Figure 3) and thus allows the insoluble substrates to be pipetted to a high degree of accuracy. In the use of microtitre plate assays, xanthan gum should not be used to facilitate the suspension of the AZCL-polysaccharide substrate. The xanthan gum, even at a concentration of just 0.1% w/v, severely reduces the rate of filtration of the reaction solution through the filter assembly on centrifugation. In this case, accurate pipetting of the insoluble substrate is facilitated simply by the finer size of the substrate particles which remain in suspension with constant agitation of the suspension during pipetting (as shown in Table 1, page 7).

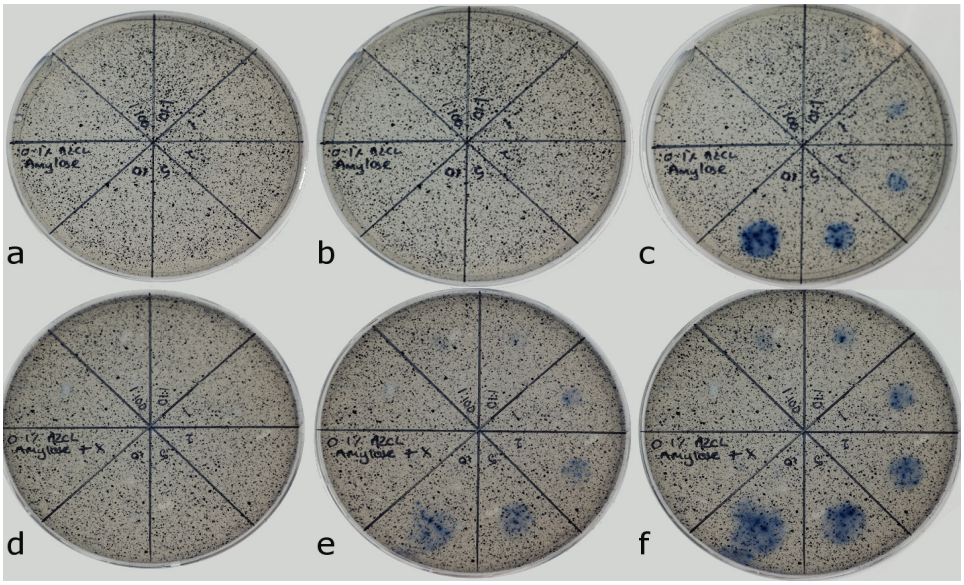


Figure 2. Comparison of plate activity screens with and without xanthan gum in the agar/substrate mixture. Plates a-c contain no xanthan gum, plates d-f contain 0.5% w/v xanthan gum (**P-XANTH**). All plates contain 0.1% w/v AZCL-Amylose (**I-AZAMY**) in 2% w/v agar and are spotted with varying concentrations (approx. 0.01 to 10 U/mL) of fungal α-amylase (**E-ANAAM**). Plates a and d are time zero plates, plates b and e after incubation for 1 h and plates c and f after incubation for 2 h. It can be seen that the plates containing xanthan gum show enzymatic activity more rapidly and with lower concentrations of enzyme.

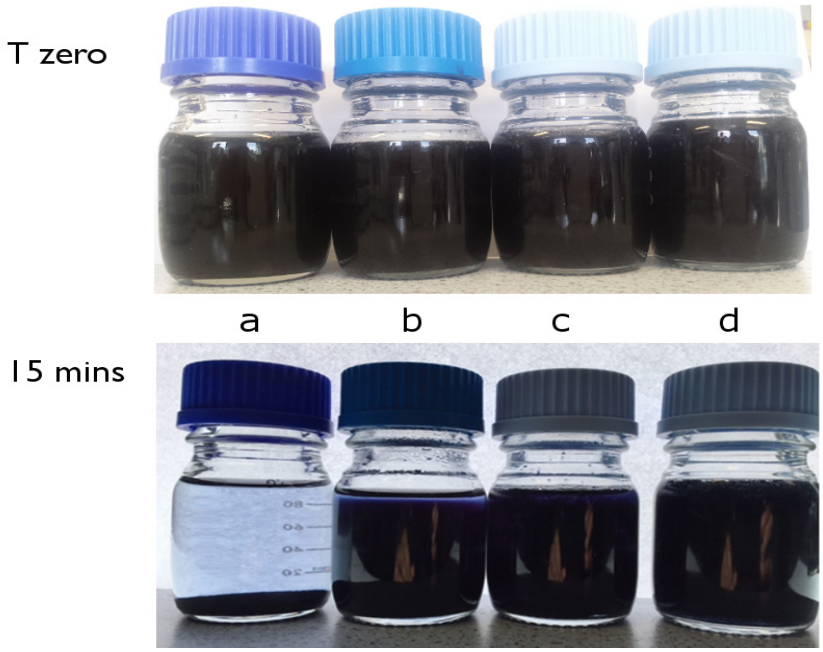


Figure 3. The effect of xanthan gum on the rate of settling of insoluble dyed substrate; time zero and after 15 min. Bottles a-d contain 0.2% w/v insoluble substrate with varying concentrations of xanthan gum.
a) 0% w/v xanthan gum, b) 0.05% w/v xanthan gum, c) 0.1% w/v xanthan gum and d) 0.5% w/v xanthan gum.

METHODS OF SCREENING:

Method A is used to screen for enzymatic activity using liquid enzyme samples. This is an agar plate based method and is most appropriate when small numbers of samples are to be screened. Relative activity is detected by the release of soluble dyed fragments from the insoluble AZCL or RedCL-polysaccharide substrates.

Method B is suitable for use when screening for activity in isopropyl-β-D-1-thiogalactopyranoside (IPTG) based promoter systems, e.g. pET system vectors in recombinant *Escherichia coli* cells etc. for a particular protein expression. The method should be modified as appropriate, including a suitable selection and induction agent for the expression system/vector used in the application.

Method C is used to screen multiple liquid enzyme samples. This method is useful for screening clarified lysed cell supernatants from expression studies etc. Relative activity levels can be detected using this method. It is important to note however that although the spectrophotometric values obtained in this method are quantitative in nature they are not intended to replace traditional methods which are capable of measuring absolute activity levels. The values obtained are most useful in identifying activity, and also to compare relative activity levels between multiple samples.

Method D may be used to quantify enzymatic activity in test tube formats. This method is most suitable in applications where sample volumes are plentiful and the user wishes to measure relative activity of a number of enzymatic samples. As with method C, the values are most useful in identifying activity and also to compare relative activity levels between multiple samples and are not designed to replace traditional methods of activity determination.

PROCEDURES:

Method A: Agar plate format with enzyme preparations.

Components: 0.1% insoluble substrate, 0.5% xanthan gum, 2% agar and 100 mM buffer.

This method will prepare 100 mL of solution which is sufficient for approx. three 10 cm plates.

0.1 g Insoluble substrate of interest
0.5 g Xanthan gum (**P-XANTH**)
2 g Agar
~ 5 mL Industrial methylated spirits (IMS) or ethanol
100 mL of suitable buffer, e.g. 100 mM sodium acetate buffer, pH 5.0 (dependent on optimal pH of enzyme being screened)

1. Accurately weigh all components into a 250 mL beaker.
2. Pipette approx. 5 mL IMS into the beaker and mix until a smooth paste is formed.
3. Add ~ 80 mL of buffer and stir to combine.
4. Adjust the final volume to 100 mL with buffer using a graduated cylinder. Transfer the solution into a suitable autoclavable container.
5. Sterilise by autoclaving for 20 min at 121°C and 15 psi (if non-sterile plates are suitable for the application heating the solution until the xanthan gum and agar dissolve is sufficient).
6. Allow to cool to ~ 60°C.
7. Pour ~ 30 mL into sterile plates while **swirling the solution**. Ensure the surface is flat to avoid sloping of the plates.
8. For screening: pipette the required volume (~ 1-10 µL) of diluted (if required) enzyme onto the plates and incubate at the required temperature for varying amounts of time.

Enzymatic activity is observed by the release of soluble dyed fragments. An example of this method is shown in Figure 4. The larger the halo of activity observed the higher the activity of the sample.

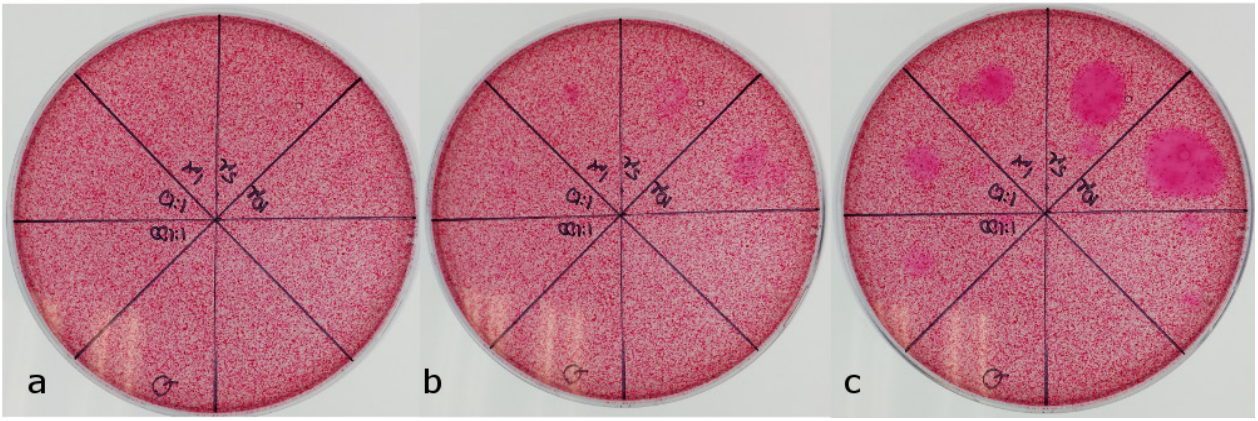


Figure 4. Plate activity screens containing 0.1% w/v RedCL-Galctomannan (Carob) (fine) (**I-RCLGMAF**), 0.5% w/v xanthan gum (**P-XANTH**) and 2% w/v agar in 100 mM sodium acetate pH 5.0 spotted with varying concentrations (approx. 5 µL of 0.05 to 5 U/mL) of *Aspergillus niger* β-mannanase (**E-BMANN**) and incubated at 37°C. Images are arranged as follows: a) time zero, b) 1h incubation and c) 2 h incubation.

Method B: Agar plate format with microbial preparations.

Components: 0.1% insoluble dyed substrate, 0.5% xanthan gum, LB media (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) and 2% agar. After autoclaving and cooling to ~ 60°C add antibiotic and inducing agent to the required concentration for the application.

This method will prepare 100 mL of solution which is sufficient for approx. three 10 cm plates.

0.1 g Insoluble dyed substrate
0.5 g Xanthan gum (**P-XANTH**)
2 g Agar
~ 5 mL Industrial methylated spirits or ethanol
100 mL of LB media pH 7.0 (100 mL water; 1 g tryptone; 1 g NaCl; 0.5 g yeast extract; pH 7.0)

NOTE: Depending on the application, an inducing agent and an antibiotic may be required. Check the plasmid literature for more information.

1. Accurately weigh all components into a 250 mL beaker.
2. Pipette approx. 5 mL IMS into the beaker and mix until a smooth paste is formed.
3. Add ~ 80 mL of buffer and stir to combine.
4. Adjust the final volume to 100 mL with buffer using a graduated cylinder. Transfer the solution into a suitable autoclavable container.
5. Sterilise by autoclaving for 20 min at 121°C and 15 psi (if non-sterile plates are suitable for the application heating the solution until the xanthan gum and agar dissolve is sufficient).
6. Allow to cool to ~ 60°C.
7. If required for the application, add an inducing agent and an appropriate antibiotic to the manufacturers required concentration. For example, if using pET22b, add IPTG to a final concentration of 1 mM and ampicillin to 100 µg/mL. Mix well.
8. Pour ~ 30 mL into sterile plates while **swirling the solution**. Ensure the surface is flat to avoid sloping of the plates.
9. It is recommended that the plates are left at room temperature (protected from light) overnight in order to lose excess moisture.
10. For screening: Dot bacterial cultures (~ 1-10 µL) onto the plates and incubate at 37°C until a signal is detected. In order to prevent excessive drying of the plates seal with parafilm after the bacterial culture sample has been absorbed.

    For detection of lower levels of activity incubate overnight at 37°C and continue to incubate at room temperature for a number of days.

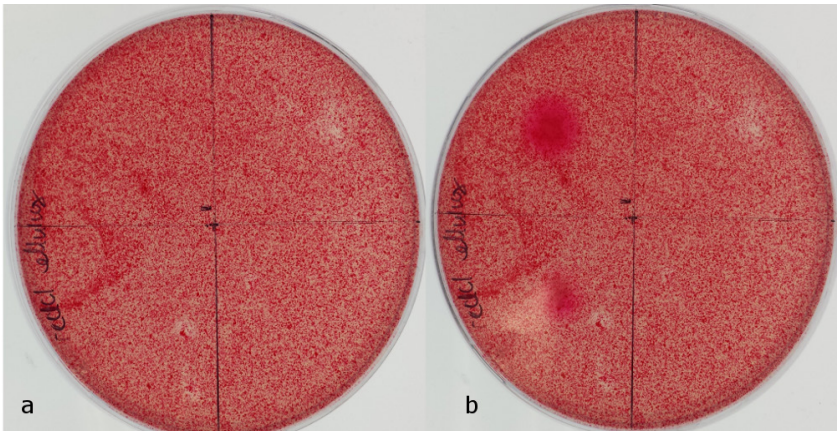


Figure 5. Plate activity screen containing 0.1% w/v RedCL-HE-Cellulose (**I-RCLCELF**), 0.5% w/v xanthan gum (**P-XANTH**) and 2% w/v LB agar. A cellulase producing *E. coli* strain was inoculated onto the plate and incubated at 37°C. a) is the plate at time of inoculation and b) is the plate after overnight incubation.

Method C: Plate reader with microtiter plates.

Components: 0.2% insoluble dyed polysaccharide in 100 mM of suitable pH buffer, assay buffer (e.g. sodium acetate buffer used to re-suspend the insoluble substrate) and enzyme (for example supernatants from cell lysis or cell culture, diluted with assay buffer as required).

Required materials

- 0.45 μM filtered 96-well microtiter 'reaction' plate available from EMD Millipore #MSHVN4510
- 'Catch' plate #MSCPNUV40 available from EMD Millipore (or similar 96 well plate suitable for UV analysis)
- Centrifuge capable of centrifuging microtiter plates at 2,000 x *g*
- Incubator
- Plate reader (capable of reading at appropriate wavelength*)
- Multichannel pipette (not required but recommended)
- Pipetting reservoir (available from VWR International # 89094-662 [or similar])

This method will prepare 100 mL of solution which is sufficient for approx. 10 microtiter plates.

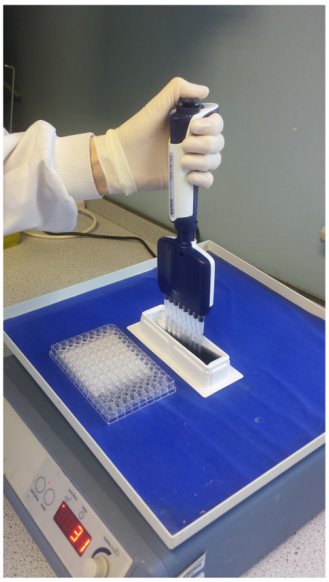
0.2 g Insoluble dyed polysaccharide
100 mL of suitable buffer, e.g. 100 mM sodium acetate pH 5.0 (dependent on optimal pH of enzyme being screened)

1. Accurately weigh the insoluble dyed polysaccharide into a 250 mL beaker.
2. Add ~ 80 mL of buffer.
3. Transfer the solution into a graduated cylinder and adjust the final volume to 100 mL using buffer.
4. Pre-warm the prepared insoluble substrate solution to assay temperature.
5. Pour the suspension into a pipetting reservoir.
6. **Constantly agitate** the substrate suspension (on a see-saw rocker or plate shaker as shown in Figure 6a) to ensure no settling of the substrate, pipette 100 μL of the suspension into the 'reaction' microtiter plate.
7. Equilibrate the reaction plate to assay temperature for ~ 5 min.
8. Pipette a suitable volume of the enzyme solution (10-100 μL) into the reaction plate. It may be necessary to dilute the samples with assay buffer if the relative activities of the samples are to be compared.
9. Include at least one reaction blank where an equal volume of assay buffer only is pipetted into a well.
10. Incubate for a suitable time period at assay temperature in order to detect a signal (10 min to > 1 h).
11. Stop the reaction by placing the 'catch' plate directly underneath the reaction plate (as in Figure 6c-d) and centrifuge at 2,000 x *g* for 10 min.
12. Measure the absorbance of the reaction plate at the appropriate wavelength as described below:

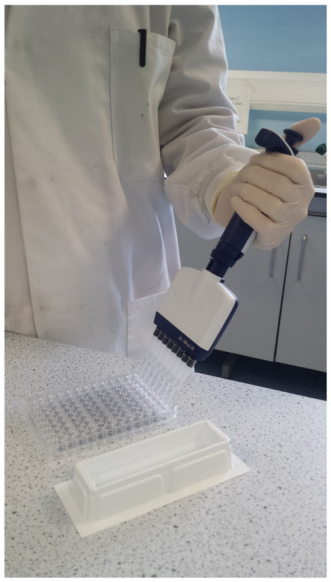
*For the AZCL substrates read at ~ 590 nm
For RedCL substrates read at ~ 510 nm

The values obtained are used to identify a particular activity and to compare relative activities between multiple samples. Units of activity cannot be obtained from these values as these substrates have not been standardised against known substrates.

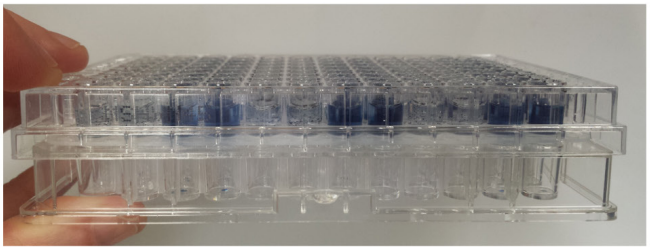
An example of the procedure for microtiter plate screens is shown in Figure 6.



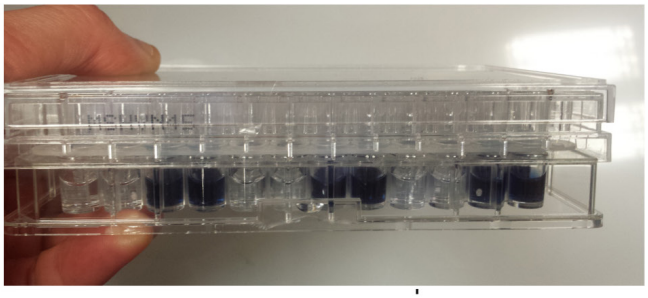
a. Pipette the insoluble substrate into the reaction plate ensuring that the solution is agitated at all times. This will prevent the settling of the insoluble substrate.



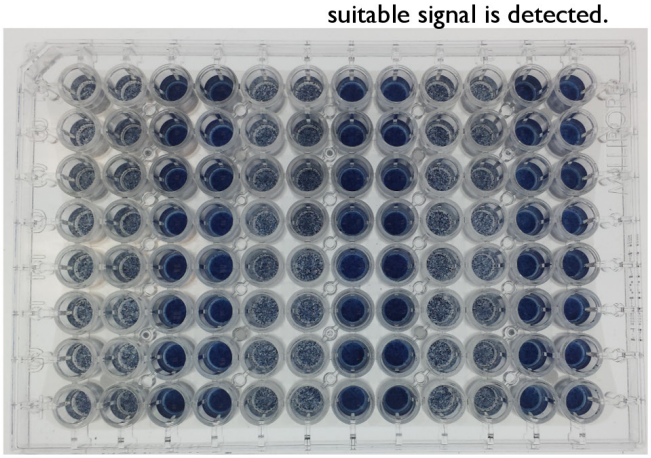
b. Pipette the enzyme solution into the reaction plate. Ensure that the plate and enzyme solution has been equilibrated to assay temperature. Include a reaction blank (assay buffer only). Incubate the plate at assay temperature until a suitable signal is detected.



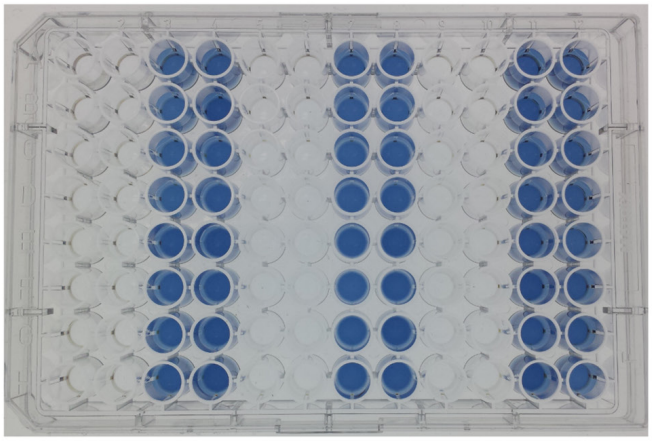
d. Sandwich the plates together as above, ensuring to align the plates in the same order to avoid confusion. Centrifuge at 2,000 x *g* for 10 min.



e. Reaction and catch plate after centrifugation. All the liquid should have transferred into the catch plate.



c. The reaction plate after incubation at assay temperature and for an appropriate time (e.g. 40°C for 10 min).



f. The catch plate after centrifugation. The plate is now ready to be read spectrophotometrically at the appropriate wavelength for the insoluble substrate (e.g. 590 nm for AZCL substrates as here).

Figure 6. Microtiter plate activity screen containing 0.2% w/v AZCL-Amylose (fine) (**I-AZAMYF**). For this screen a 1:1000 dilution of fungal α-amylase (**E-ANAAM**) was pipetted into rows 3-4, 7-8 and 11-12. Assay buffer only was pipetted into rows 1-2, 5-6 and 9-10. Activity is observed in (f) where the release of soluble dyed fragments in the enzyme reaction rows is clearly visible. The absorbance values and %CV values of the plate shown in f are given in Table 1 of the appendix.

Method D: Test tube procedure.

Components: 0.2% Insoluble dyed polysaccharide in 100 mM of suitable pH assay buffer, 0.1% xanthan gum and sample enzyme (for example supernatants from cell lysis or cell culture) diluted with assay buffer as required.

This method will prepare 100 mL of solution.

0.2 g Insoluble dyed polysaccharide
0.1 g Xanthan gum (**P-XANTH**)
~ 5 mL Industrial methylated spirits (IMS) or ethanol
100 mL of suitable buffer

To stop the reaction; prepare 2% w/v Tris base (Megazyme Cat. no. **B-TRIS500**).

1. Accurately weigh all components into a 250 mL beaker.
2. Pipette approx. 5 mL IMS into the beaker and mix until a smooth paste is formed.
3. Add ~ 80 mL of buffer and stir to combine.
4. Adjust the final volume to 100 mL with buffer using a graduated cylinder.
5. Bring the solution to the boil with constant stirring in order to dissolve the xanthan gum.
6 Using a pipette, or a positive displacement pipette, pipette 0.5 mL of substrate solution into a 16 x 100 mm test tube.
7. Incubate to assay temperature for ~ 5 min.
8. Pipette 0.5 mL of sample into the test tube and incubate for a suitable time period (e.g. 10 min - 1 h depending on the activity of the sample). Prepare a reaction blank containing only assay buffer as a control.
9. Add 5 mL of 2% Tris base (pH 10) and mix the tube contents.
10. Centrifuge at 2,000 x *g* for 10 min.
11. Activity may be observed visually or by decanting into 1 mL cuvettes and reading the absorbance against the reaction blank at the appropriate wavelength as described below.

    For the AZCL substrates read at ~ 590 nm
    For RedCL substrates read at ~ 510 nm

APPENDIX:

Absorbance values obtained for experiment shown in Figure 6.

a. Absorbance values for plate shown in Figure 6f

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
A	0.051	0.050	0.788	0.873	0.051	0.050	0.822	0.847	0.051	0.049	0.936	0.870
B	0.050	0.050	0.750	0.991	0.053	0.056	0.769	0.788	0.051	0.053	0.937	0.829
C	0.049	0.058	0.768	0.839	0.050	0.050	0.755	0.796	0.053	0.050	0.866	0.853
D	0.049	0.050	0.780	0.843	0.050	0.053	0.784	0.821	0.052	0.052	0.858	0.835
E	0.049	0.050	0.905	0.950	0.051	0.051	0.840	0.915	0.051	0.053	0.910	0.779
F	0.049	0.049	0.850	0.898	0.051	0.051	0.787	0.879	0.052	0.050	0.967	0.868
G	0.050	0.050	0.878	0.962	0.053	0.052	0.854	0.954	0.052	0.051	0.920	0.891
H	0.050	0.051	0.916	1.029	0.050	0.052	0.886	0.914	0.053	0.051	1.031	0.811

b. Statistical analysis (by column) of the absorbance values obtained above

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
Sample Description	**Reaction Blank**	**Reaction Blank**	**Enzyme dilution**	**Enzyme dilution**	**Reaction Blank**	**Reaction Blank**	**Enzyme dilution**	**Enzyme dilution**	**Reaction Blank**	**Reaction Blank**	**Enzyme dilution**	**Enzyme dilution**
Average	0.050	0.051	0.829	0.923	0.051	0.052	0.812	0.864	0.052	0.051	0.928	0.842
Standard deviation	0.001	0.003	0.066	0.070	0.001	0.002	0.046	0.061	0.001	0.001	0.055	0.036
% coefficient of variation	0.015	0.056	0.079	0.076	0.024	0.038	0.056	0.070	0.016	0.029	0.060	0.043

c. Statistical analysis of the combined reaction blank and enzyme dilution values shown above

Overall values		
	Reaction Blank	**Enzyme dilution**
Average	0.051	0.867
Standard deviation	0.001	0.013
% coefficient of variation	0.016	0.015

Table 1. Analysis of microtiter plate screen shown in Figure 6f. The % coefficient of variation (%CV) values obtained for both the enzyme dilution and the reaction blank show that when using the outlined method the values obtained from well to well are replicable. Therefore, this method is suitable for the screening of enzymatic activity in a high throughput fashion.

Insoluble Chromogenic Substrates available from Megazyme

Name	Catalog No.
AZCL-Amylose	I-AZAMY
AZCL-Amylose (fine)	I-AZAMYF
RedCL-Amylose (fine)	I-RCLAMYF
AZCL-Arabinan (Debranched)	I-AZDAR
AZCL-HE-Cellulose	I-AZCEL
AZCL-HE-Cellulose (fine)	I-AZCELF
RedCL-HE-Cellulose (fine)	I-RCLCELF
AZCL-Xyloglucan	I-AZXYG
AZCL-Xyloglucan (fine)	I-AZXYGF
RedCL-Xyloglucan (fine)	I-RCLXYGF
AZCL-Barley β-Glucan	I-AZBGL
AZCL-Barley β-Glucan (fine)	I-AZBGLF
RedCL-Barley β-Glucan (fine)	I-RCLBGLF
AZCL-Pachyman	I-AZPAC
AZCL-Curdlan	I-AZCUR
AZCL-Curdlan (fine)	I-AZCURF
RedCL-Curdlan (fine)	I-RCLCURF
AZCL-Chitosan	I-AZCHAN
AZCL-Chitosan (fine)	I-AZCHANF
AZCL-Galactan (potato)	I-AZGLP
AZCL-Galactan (potato) (fine)	I-AZGLPF
AZCL-Galactomannan	I-AZGMA
AZCL-Galactomannan (fine)	I-AZGMAF
RedCL-Galactomannan (fine)	I-RCLGMAF
AZCL-Casein	I-AZCAS
AZCL-Collagen	I-AZCOL
AZCL-Pullulan	I-AZPUL
AZCL-Pullulan (fine)	I-AZPULF
RedCL-Pullulan (fine)	I-RCLPULF
AZCL-Dextran	I-AZDEX
AZCL-Dextran (fine)	I-AZDEXF
AZL-Rhamnogalacturonan I	I-AZRHI
AZCL-Xylan (Birchwood)	I-AZXBW
AZCL-Xylan (Beechwood)	I-AZXBE
AZCL-Arabinoxylan (Wheat)	I-AZWAX
AZCL-Arabinoxylan (Wheat)(fine)	I-AZWAXF
RedCL-Arabinoxylan (Wheat)(fine)	I-RCLWAXF

Tablet tests for polysaccharide *endo*-hydrolases

Product name	Catalog No.	Enzyme assayed
Amylazyme	T-AMZ	α-Amylase
Amylazyme BG	T-AMZBG-200T	α-Amylase
Amylazyme HY	T-AMZHY-200T	α-Amylase
Amylazyme Red	T-AMZRD	α-Amylase
Arabinazyme	T-ARZ-200T	*endo*-1,5-α-L-Arabinanase
Cellazyme AF	T-CAF-1000T	*endo*-Cellulase
Cellazyme C	T-CCZ	*endo*-Cellulase
Cellazyme T	T-CTZ	*endo*-Cellulase
β-Glucazyme	T-BGZ	Malt-Glucanase/Cellulase
1,3-β-Glucazyme	T-PAZ-200T	*endo*-1,3-β-Glucanase
1,3-β-Glucazyme HS	T-CUR-200T	*endo*-1,3-β-Glucanase
Chitozyme	T-CHZ-200T	Chitosanase
Alpha-Dextrazyme	T-DEXT-200T	α-Dextranase
Limit-Dextrizyme	T-LDZ	Limit Dextrinase/Pullulanase
Galactazyme	T-GLZ	*endo*-1,4-β-Galactanase
Mannazyme	T-MNZ	*endo*-1,4-β-Mannanase
Protazyme AK	T-PRAK	*endo*-Protease
Protazyme OL	T-PROL	*endo*-Protease
Psyllazyme	T-PSYL-200T	*endo*-Hydrolases
Rhamnozyme	T-RHAM-200T	Rhamnogalacturonan hydrolase and Rhamnogalacturonan lyase
Xylazyme AF (40 mg)	T-XAF-100T	β-Xylanase
Xylazyme AX (60 mg)	T-XAX	β-Xylanase
Xylazyme (100 mg)	T-XYZ	β-Xylanase