novozymes
Rethink Tomorrow

Analytical Method

Biomass Hydrolysis Activity by FCD, BHU(2)

Purpose
To describe the procedure for determination of BHU(2) activity

Scope
Novozymes Enzyme QC laboratories involved in analysis of samples from production and R&D

Principle
The method measures the decay of cellulose in a substrate consisting of **g**rinded and **s**ieved – **p**retreated **c**orn **s**tover (GS-PCS) mixed with a fluorescence enhancer (Calcoflour White, FB28). The cellulose hydrolysis results in a decrease in fluorescence (excitation/emission: 360nm/460nm). This is monitored relative to a Biomass enzyme standard. The method thus measures hydrolysis activity by Fluorescence Cellulose Decay (FCD).

In the method the incubation is terminated and monitored after 24 hours at 50°C.

Unit definition
The Biomass Hydrolysis activity is measured in BHU(2) units relative to an enzyme standard of declared activity

Sample types
The method can be used for analysis of finished goods samples as well as for process samples and samples from development.

Reaction conditions

Parameter	Reaction conditions
Temperature	50°C ± 1°C
pH	5.0 (Room temperature)
Substrate conc. (% Insoluble Solids)	3.02 %
FB28 conc.	150 µM
Na-Acetate	50 mM
$MnSO_4$	1 mM
Enzyme conc.	1.631 - 6.524 BHU(2) / mL
Reaction time	24 hours
Wave length	Excitation 360 nm / Emission 460 nm

Method parameters

The following parameters were determined by validation:

Intermediate precision

$CV_{\text{single determination}}$ = 4.8 %

Range

The range is 1.63 – 6.53 BHU(2)/mL

Limit of determination

The limit of determination is 40.8 BHU(2)/g for a minimum preparation of 1 g sample dissolved in 25 mL

Equipment

Equipment	
Multi-detection Micro plate Reader	Synergy 2 from Bio Tec (critical equipment)
Micro plate Heat Sealer	e.g Thermo Scientific ALPS 3000 or Thermo Scientific ALPS 50V (manual type)
Pipettes	Eppendorf multi pipette Xstream (art. No.#613-0618) with combitip 25 mL and e.g. Biohit 8-canal e-line pipette (not larger than e300)
Incubator	e.g. Binder (BF115)
Diluter	e.g. Hamilton Microlab
Analytical balance	e.g. Sartorius, Mettler
pH meter	e.g. Radiometer, Metrohm
Table shaker or Teleshaker	e.g. Ika Minishaker or Teleshaker
Roller mixer	e.g. Stuart SRT2/SRT6 at 33 rpm, 16 mm amplitude
Magnetic stirrer plates	-

Materials

Reagents/materials	Distributer
Sealer tape for Heat Sealer	e.g. Thermo Scientific cat#AB3739 (for Heat Sealer model 3000) or Thermo Scientific cat#AB0745 (Easy Peel for manual Heat Sealer model 50V)
Costar 96 well micro plate	Costar 96 well micro plate cl. Flat bottom, N-ST, Costar 734-1551
Ultra Pure water	Resistivity: ≥18.2 M Ω·cm at 25°C
Substrate	GS-PCS is available upon request

Substrate

The substrate is prepared and adjusted with the necessary reagents and no additional reagents are needed. The standard, QC and samples are therefore dissolved and diluted in Ultra Pure water. The substrate has been autoclaved and is stored refrigerated

Reagents

As the substrate is provided as "ready to use" and the samples are dissolved and diluted in Ultra Pure water no additional reagents has to be prepared

Handling of enzymes and chemicals

- Valid procedures for handling enzymes and GMOs apply.
- Enzymes and enzyme solutions should be handled in a fume hood or in closed containers.
- Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation.
- Avoid inhalation of dust aerosols and contact with skin and eyes.
- Handling of chemicals and disposal of waste must be performed according to valid procedures.

Standard

The standard is available upon request.

Step	Action
1	Prepare a stock solution of the standard of a concentration of 65.24 BHU(2)/mL (±0.1%) in Ultra Pure water Storability of the stock solution: 2 hours at room temperature
2	Prepare a working solution. Place the dilutions as described in *Assay plate layout* here below

No.	Obtain dilution ratio by dilution as: (Example)		Dilution ratio	Concentration (BHU(2) / mL)
	Stock solution µl	Diluent µl		
1	30	1170	40	1.632
2	40	1160	30	2.176
3	50	1150	24	2.719
4	60	1140	20	3.263
5	80	1120	15	4.351
6	100	1100	12	5.439
7	120	1080	10	6.527

QC sample

The QC sample is available upon request.

Step	Action
1	The QC sample is weighed out. From this weighing dilutions are prepared and used to load four wells (A9-A12) in the microtiter plate. *Note:* There is thus four replicates of the QC sample.
2	Prepare a stock solution of a concentration of 20.6 BHU(2)/mL with an accuracy of ±1.0% in Ultra Pure water
3	Stir for minimum 15 min. and maximum 30 min. Storability of the stock solution: 2 hours at room temperature
5	Prepare a working solution by diluting the stock solution 5 times with Ultra Pure water. Place the dilution in four wells as described in *Assay plate layout* here below.

Samples

Step	Action
1	Weigh out the samples with an accuracy of ± 10% and transfer quantitatively to a measuring flask
2	Dissolve the sample in the measuring flask with Ultra Pure water
3	Stir the solutions for between 15 and 30 minutes. Storability: 2 hours at room temperature
4	Stir the stock solution prior to dilution
5	Dilute the samples further with Ultra pure water. *Note!* The activity of the final dilution should if possible be approx. 4.10 BHU(2)/mL
6	Place the dilutions as described in the *Assay plate layout* described here below where the samples are numbered 1 to 42.
7	Each weighing of sample is placed in 2 wells as shown in the *Assay plate layout*

Blank

Use Ultra Pure water as blank. Place the blank in the Assay plates just prior to the standard curve in well A1 as described in *Assay plate layout* here below

Procedure

Substrate loading

Step	Action
1	The substrate (GS-FCD-PCS) is stored refrigerated in Nalgene bottles of approximately 250 mL. Storability: 3 month refrigerated after opened.
2	Make sure that the substrate is thoroughly mixed before proceeding to the next step.
3	<table><tr><th>If…</th><th>Then…</th></tr><tr><td>An aliquot of the substrate is poured out into a beaker</td><td>It must be stirred without foaming for approximately 15 minutes before continuing to the next step</td></tr><tr><td>The Nalgene bottle is placed on a roller mixer for maximum 90 minutes</td><td>An aliquot must be poured out into a beaker and stirred without foaming before continuing to the next step</td></tr></table>
4	Prepare one Costar plate for each maximum of 42 samples. *NOTE:* Mark the plate with plate ID, date and initials.
5	While the substrate stirs aliquots are withdrawn using the Eppendorf multi pipette Xstream and 250 µL substrate are loaded pr. well
6	Only part of the microtiter plate has to be loaded with substrate when fewer than 42 samples are to be analyzed. *NOTE:* The remaining wells in the row started upon, plus the following row has to be filled with substrate
6	The plates are now ready to load the enzyme samples.

Sample loading

Step	Action
1	Dilute the standard, QC and samples as described above
2	Load 50 µl of the diluted samples carefully on top of the substrate in the Costar substrate plate with an 8 channel pipette *Note!* Change pipette tips between every load (e.g. between A1-H1 and A2-H2)
3	Load 50 µL water in the wells filled with substrate and no sample
4	The plate are immediately sealed and read as described below

Assay plate layout

Row A is loaded with:

- Blank (A1), Standard (A2 to A8) and QC sample (A9 to A12)

Rows B to H are loaded with:

- Sample 1 (S1) is placed in wells B1 and B7,
- Sample 2 (S2) is placed in wells B2 and B8, and so on.
- Row C is loaded with samples no. 7 to no. 12,
  Row D is loaded with sample no. 13 to no. 18, etc.
- Continue loading row E to H with samples no. 19 to 42

The 96 well microtiter assay plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	St1	St2	St3	St4	St5	St6	St7	QC	QC	QC	QC
B	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4	S5	S6
C	S7	S8	S9	S10	S11	S12	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S13	S14	S15	S16	S17	S18
E	S19	S20	S21	S22	S23	S24	S19	S20	S21	S22	S23	S24
F	S25	S26	S27	S28	S29	S30	S25	S26	S27	S28	S29	S30
G	S31	S32	S33	S34	S35	S36	S31	S32	S33	S34	S35	S36
H	S37	S38	S39	S40	S41	S42	S37	S38	S39	S40	S41	S42

Microtiter plate sealing and mixing

Step	Action
1	Turn on the micro plate heat sealer. *NOTE:* The heat sealer should be turned on at least 15 minutes before use to reach the desired 168°C.
2	The plate is sealed 2.0 sec. at 168°C.
3	Check that the seal is intact.
4	Mix the enzyme with substrate by shaking upside-down thoroughly or by using a table shaker/teleshaker with a horizontal rotation for approximately 15 seconds at 1200 rpm.

Microtiter plate reading and incubation

Step	Action
1	Start the Synergy reader
2	Choose the BHU(2) protocol for reading the plate
3	Read the plate , T=0
4	Save the T=0 data in the file corresponding to the working list *NOTE:* Name the file with a relevant name e.g. date_plate no_T0
5	Place the plate in an incubator at 50°C
6	Read the plate after 24 hours of incubation, T=24 after mixing the plates manually or by use of a table shaker/teleshaker with a horizontally for approximately 15 seconds at 1200 rpm. *NOTE:* Name the plate with the same name as for time zero but change the time from _T0 to _T24
7	Save the T=24 data in the file corresponding to the working list

Data processing and calculation

Step	Action
1	The Synergy readings T0 and T24 are copy-pasted into a spread sheet corresponding to the working list
2	The plate is sealed 2.0 sec. at 168°C.
3	Check that the seal is intact.
4	Mix the enzyme with substrate by shaking upside-down thoroughly or by using a table shaker/teleshaker with a horizontal rotation for approximately 15 seconds at 1200 rpm.

Calculations

Step	Action
1	The activity of the enzyme samples is determined relative to the standard curve. A validated and version controlled spreadsheet is used for calculations.
2	In all calculations the reader response (fluorescence emission intensity) is normalized using the equation: $Normalized\ reading\ (24h) = \frac{AU(0h) - AU(24h)}{AU(0h)}$
3	On the basis of the results in Signal for the 7 standards, a standard curve is drawn with the activities of the standards in BHU(2)/mL as the x-values and the Normalized Signal of the standards as the y-values

4	The enzyme activity of the diluted samples is read from the standard curve. The results are calculated automatically in the spreadsheet. Calculation of activity of a sample in BHU(2)/g is performed as stated in the formula: $Activity\ Unit/g = \frac{S \cdot V \cdot F}{W}$ S = Reading from the standard curve in BHU(2)/mL V = Volume of the measuring flask used in ml F = Dilution factor W = Weight of sample in g
5	0.569 g sample is dissolved in a 50 ml measuring flasks and further diluted 5 times. Two read signals (T0h and T24h) that are read on the Synergy reader are recalculated (Reading AU0h-Reading AU24h)/ReadingAU0h) to give a value of 0.413 that is the Normalized Response. From the standard curve an activity of 3.967 BHU(2)/mL is calculated. $Activity = \frac{3.967 \cdot 50 \cdot 5}{0.569} = 1743\ BHU(2)/g$

Approval

Standard curve

Approval of standard curve:

Parameter	Requirement
Quality of fit (lower r^2 limit)	r^2 should be $\geqslant 0.985$
Curve appearance	The standard curvature is linear and increasing

QC sample

Approval of QC sample (level control):

Parameter	Requirement
QC sample	The result of the QC sample must not deviate from the control limit, which is set to: Declared value ± 2 · SD

Samples

Approval of sample results:

Parameter	Requirement
CV of 6 results (= 2 replicates on 3 different standard curves i.e. corresponding to microtiter plates)	≤ 8.6%

Statement of analytical results

The results are reported with three significant digits.

If a result is below the lowest standard and the sample was diluted in the minimum dilution, the result is reported as < 40.8 BHU(2)/g

Synergy reader configuration

Protocol Definition Report: BHU(2)

Procedure

Plate Type	Costar 96 flat bottom
Set Temperature	ncubator off
Read	Fluorescence Endpoint
	Full Plate
	Filter Set 1
	Excitation: 360/40, Emission: 460/40
	Optics: Bottom, Sensitivity: 57
	Light Source: Tungsten, Standard Dynamic Range
	Read Speed: Normal, Delay: 100 msec, Measurements/Data Point: 10

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
Denmark

Tel. +45 4446 0000
Fax +45 4446 9999