| METHOD OF ANALYSIS | | No: 61903.<br>Version 3 |
|---|---|---|
| Department:<br><br>DFS/R&D/Analysis | Title: Meltamase activity determination using maltotriose as substrate, relative method (two steps, manual and Selectra analyzer). | |
| Date of issue: | Product: N.a. | Product code: N.a. |
| Compiled by: A. Vriend<br><br>Date: | | Validated method<br><br>YES |
| Checked by<br>Expert:<br><br>R. Busink<br><br>Date: | Approved by<br>Q-functionary:<br><br>A.F. Arendsen<br><br>Date: | Approved by<br>QA/QC-officer:<br><br>M.M. Immerzeel<br><br>Date: |

## 1 SAFETY AND ENVIRONMENT

Restrictions for working with chemicals and ML-I samples are mentioned in the work instructions concerning management, storage and use of chemicals, the handling of dangerous substances and standard rules for ML-I laboratories. These restrictions are also applicable for material that has been in contact with ML-I samples.

When working with strong acids, bases, carcinogenic matters and toxic matters etc. take all necessary precautions.

When working with highly concentrated enzyme preparations take all necessary precautions. Avoid inhalation of dust add/or prolonged contact with unprotected skin.

## 2 PRINCIPLE

### 2.1 Application

This method is applicable for the determination of meltamase (= amylomaltase) from Bacillus amyloliquefaciens (GMO, amylomaltase gene of Thermus thermophilus) in fermentation samples (broth, supernatant, filtrate), down stream processing samples and end products.

### 2.2 Description of the method

Meltamase is incubated with maltotriose at pH 6.50 and 70 °C, releasing glucose from the substrate. The incubation is stopped by adding hydrochloric acid. The amount of released glucose is a measure for the meltamase activity and is examined using a glucose test assay (NADH formation) on a Selectra analyzer at a wavelength of 340 nm.

## 2.3 Unit definition

One **A**mylo Mal**t**ase **u**nit (ATU) is defined as the amount of enzyme which produces 1 µmol of glucose per minute under the assay conditions of the test.

## 2.4 Measuring range

The measuring range of this method is 0.5 – 2.0 ATU / ml

## 2.5 Summary validation report

Validation report 61903, version 1.0, Meltamase activity determination using maltotriose as substrate, relative method (two steps, manual and Selectra analyzer).

*Table 1* Validation parameters, acceptance criteria and summarized results for Meltamase.

| **Validation parameters** | **Acceptance criteria** | **Result** | **Accepted** |
|---|---|---|---|
| Accuracy (confidence limits) | After standard addition:<br>110%>Recovery>90% | 93.6% - 102.6% | Yes |
| Repeatability<br>*Relative $SD_{within\ days}$*<br>Fermentation broth (FB)<br>Filtrate, ccUF, end-product | <br><br><br>< 5%<br>< 3% | *Relative $SD_{within\ days}$*<br>Control C329 0.4%<br>Sonificated FB 2.1%<br>Lysozyme/MPP FB 2.5%<br>Filtrate (non-conc) 1.0%<br>ccUF 1.2%<br>End-product 2.4% | <br>Yes<br>Yes<br>Yes<br>Yes<br>Yes<br>Yes |
| Intermediate Precision<br>*Relative $SD_{between\ days}$*<br>Fermentation broth (FB)<br>Filtrate, ccUF, end-product | <br><br><br>< 5%<br>< 3% | *Relative $SD_{between\ days}$*<br>Control C329 2.7%<br>Sonificated FB 3.8%<br>Lysozyme/MPP FB 0.4%<br>Filtrate (non-conc) 2.0%<br>ccUF 2.4%<br>End-product 2.3%<br><br>$RSD_{Single}$ $RSD_{Duplicate}$<br>Control C329 2.7% 2.7%<br>Sonificated FB 4.4% 4.1%<br>Lysozyme/MPP FB 2.6% 1.9%<br>Filtrate (non-conc) 2.3% 2.2%<br>ccUF 2.7% 2.5%<br>End-product 3.4% 2.9% | <br>Yes<br>Yes<br>Yes<br>Yes<br>Yes<br>Yes |
| Linearity ("Best Fit Model") | Range 0.50 – 2.0 $^{ATU}/_{ml}$ | Polynomial regression (2nd order) | Yes |
| Robustness pH and Temperature | Record | Satisfying | Yes |
| System Precision | Record | 0.52% | Yes |
| Limit of detection | [1]NI | [1]NI | [1]NI |
| Limit of quantitation | [1]NI | [1]NI | [1]NI |
| Selectivity / Specificity | [1]NI | [1]NI | [1]NI |

*([1])NI = not included.*

Validation results for examined meltamase samples: fermentation broth (sonificated and lysozyme/MPP treated), non-concentrated filtrate, ccUF and (50% glycerol) formulated end product did meet all set acceptance criteria as shown in *table 1*.

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## 3 APPARATUS AND CONDITIONS

### 3.1 Apparatus

- Clinical analyzer : Selectra, Vital
- Selectra sample tubes : Contents 5 ml
- Reagent bottle : 25 ml
- Balance, accuracy at 0.0001 g : Mettler AE240
- Balance, accuracy at 0.001 g : Mettler PM 400
- Balance, accuracy at 0.01 g : Sartorius model 2004 MP
- PH meter : Radiometer PHM82
- Diluter, provided with 0.5 and 5.0 ml cylinders : Hamilton Microlabs
- Magnetic stirrer : Variomag, Multipoint HP15
- Magnetic stirrers : IKA labortechnik, MINI MR
- Vortex : Genie-2 Scientific Industries
- Disposible culture tubes (glass 16 x 150 mm) : Corning
- Pipettor 1000 adjusted to 1.00 ml : Eppendorf
- Pipettor 5000 adjusted to 4.00 ml : Eppendorf
- Centrifuge, 3000 rpm : Megafuge 1.0 Heraeus, provided with inserts for centrifuge tubes of 15 ml each
- Ultrasonic disintegrator provided with process timer: MSE, Soniprep 150
- Waterbath adjusted to 70.0°C +/- 0.2°C : Grant W28
- Thermometer 50°C – 100°C : Testo, 110

Or equivalent apparatus

### 3.2 Conditions

Not applicable

## 4 MATERIALS

### 4.1 Chemicals

- di-Sodiumhydrogenphosphate 2aq ($Na_2HPO_4.2H_2O$) : Merck, 1.06580.1000
- Hydrochloric acid 0.5 mol/l : Merck, 1.09058.1000
- Triton X-100 : Merck, 1.12298.0101
- Maltotriose : Fluka Biochemica, 63430
- Glucose Hexokinase test kit (Ecoline $S^+$) : DiaSys,1.2511.99.90.314
- Protease inhibitor cocktail tablets : Roche, Complete 1 836 145
- System liquid : Merck, 1.07906.1000
- Sputofluol : Merck, 1.08000.1000
- Hydrochloric acid 0.100 mol/l : Merck, 1.09060.1000

Or equivalent quality

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4.2 References, standards and controls

Meltamase standard preparation with an official assigned activity. The activity is expressed in ATU/ml. Store the stock of the standard amylomaltase preparation in the freezer and amounts for daily use in the refrigerator.
Meltamase control preparation with an officially assigned activity. The activity is expressed in ATU/ml. Store the stock of the amylomaltase control preparation and amounts for daily use in the refrigerator.

4.3 Reagents

Water:
Ultra High Quality water, conductivity $\leq$ 0.10 µS.cm

Phosphate buffer pH 6.50
Dissolve 9.0 g di-sodiumhydrogenphosphate 2aq in approximately 800 ml water. Adjust the pH at 6.50 +/- 0.05 with hydrochloric acid 0.5 mol/l. Make up to 1000 ml with water and mix. This solution may be kept for 3 month in the refrigerator.

Phosphate / Triton X-100 buffer solution:
Add and dissolve 1.0 g Triton to 1 l phosphate buffer solution. This solution may be kept for 3 month in the refrigerator.

Hydrochloric acid 0.05 mol/l:
Dilute 50 ml hydrochloric acid 0.5 mol/l with 450 ml water and mix. This solution may be kept for 3 month at room temperature.

Substrate solution:
Dissolve 5.00 g maltotriose in approximately 40 ml phosphate buffer pH 6.50 solution in a 50 ml volumetric flask. Make up to volume with the same and mix. Always use a freshly prepared solution.

Glucose Hexokinase reagent:
Mix 20 ml (one bottle) reagent R1 with 5 ml reagent R2. This reagent mixture (mono reagent) is stable for 3 months in the refrigerator. The reagent must be protected from light. **The reagents contain 0.95 g/l sodium azide as preservative. Avoid contact with skin.**

Protease inhibitor solution:
Dissolve 1 tablet protease inhibitor Complete in 1 ml phosphate buffer solution pH 6.50. Always use a freshly prepared solution.

Diluted system liquid solution (wash water analyzer):
Add to 10 l water 25 ml system liquid and mix. This solution is stable for 1 months.

Sputofluol solution 10% (washing solution analyzer):
Mix 1 part Sputofluol solution with 9 parts water.

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## 5 PROCEDURE

### 5.1 Preparation

Not applicable.

### 5.2 Pretreatment reference

Not applicable.

### 5.3 Pretreatment standard

Before use allow the standard to attain room temperature. With the diluter dilute from the standard 0.500 ml with 4.500 ml phosphate / triton buffer solution and mix. Next with the diluter prepare dilutions as indicated in the table below in culture tubes and mix. Prepare the final dilutions in duplicate (sample and sample blank).

| Activity to be incubated [ATU/ml] | Total dilution | Standard sample | Standard solution [ml] | Phosphate triton buffer solution to be added [ml] | Standard solution [ml] | Phosphate triton buffer solution to be added [ml] |
|---|---|---|---|---|---|---|
| 2.0 | 1250 | S1 | 0.400 | 4.600 | 0.100 | 0.900 |
| 1.7 | 1538 | S2 | 0.325 | 4.675 | 0.100 | 0.900 |
| 1.3 | 2000 | S3 | 0.250 | 4.750 | 0.100 | 0.900 |
| 1.0 | 2500 | S4 | 0.200 | 4.800 | 0.100 | 0.900 |
| 0.8 | 3333 | S5 | 0.150 | 4.850 | 0.100 | 0.900 |
| 0.5 | 5000 | S6 | 0.100 | 4.900 | 0.100 | 0.900 |

### 5.4 Pretreatment control

Weigh in duplicate the control sample accurately to within 0.001 g, in volumetric flasks. Dilute the control sample with phosphate/triton buffer solution to an activity of approximately 1.3 ATU per 1 ml solution to be incubated.

### 5.5 Pretreatment samples

#### 5.5.1 Broth

Pipette from the sample 1.5 ml in a 2 ml plastic sample cup. Add with a pipettor 60 µl protease inhibitor solution and mix (dilution factor 1.04). Place the sample cup in melting ice. Using a ultrasonic disintegrator adjusted to 10 cycles, 10 sec on and 5 sec off, for sonification of the broth sample. During sonification cool the sample mixture in melting ice. Check the amplitude value, this must be 10 microns. After sonification replace the sample cup in melting ice. Next dilute in culture tubes the samples with phosphate / triton buffer to a final concentration of approximately 1.3 ATU per 1 ml to be incubated. Prepare the final dilutions in duplicate (sample and sample blank). Store all diluted solutions in a bath with melting ice until starting the

incubation.

5.5.2 Downstream processing samples

Activity per g:
Weigh samples accurately to within 0.001 g, in volumetric flasks. Make up to volume with phosphate / triton buffer and mix. Next dilute in culture tubes the sample solution with phosphate / triton buffer to a final concentration of approximately 1.3 ATU per 1 ml to be incubated. Prepare the final solutions in duplicate (sample and sample blank). Store all diluted solutions in a bath with melting ice until starting the incubation.

Activity per ml:
Dilute the sample in culture tubes with phosphate / triton buffer to a final concentration of approximately 1.3 ATU per 1 ml to be incubated. Prepare the final solutions in duplicate (sample and sample blank). Store all diluted solutions in a bath with melting ice until starting the incubation.

5.6 Preparation measurement

Incubation:
Samples:
Starting at time = 0 minutes (stopwatch), in order of the series and with regular time intervals place one of the tubes to be incubated into the waterbath at 70.0°C +/- 0.2°C. Starting at time = 5 minutes, in the same order of the series and at the same regular time intervals add 1 ml maltotriose substrate solution at 70.0°C +/- 0.2°C with the dispenser and mix. Place the tubes into the waterbath. At time = 35 minutes, in the same order and with the same time intervals terminate the incubation by adding 4.00 ml hydrochloric acid 0.05 mol/l with a dispenser and mix.

Blanks:
Add respectively 4 ml hydrochloric acid 0.05 mol/l and 1 ml maltotriose substrate to the blank tubes.
Centrifuge the sample and sample blank tubes for 10 minutes at 3000 rpm (1550 x g).
Introduce approximately 2 to 4 ml of the reaction mixtures into the Selectra sample tubes.

5.7 Measurement

*Starting the Selectra analyzer*
- Fill the external wash container with diluted system liquid solution.
- Empty the external diluted waste– and concentrated waste container.
- Place a tube filled with 10% sputofluol in position W of the sample tray.
- Place a tube filled with water in position B of the sample tray.
- Place a 25 ml bottle filled with 0.1 mol/l HCl solution in position 24 of the reagent tray.
- Place a 25 ml bottle filled with Glucose Hexokinase reagent at position 4 of the reagent tray.
- Once a day an automatically cuvette rotor blank is measured and printed.

Check for each filter (wavelength) the *sd* value of the measured cuvette rotor blank. The *sd* must be lower than 0.020.

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**In case the analyser was not in the stand-by modus and no rotor cuvette blank was determined and printed, run this test as follows;**

- *Press "F5", (Special Functions).*
- *Press "F1", (Rotor / System).*
- *Select in the menu, "Reset System".*
- *Press "Enter".*
- *Press "F1", (Reset System). System initialising is running now.*
- *Press "F10", (return), after finishing "system initialising"*
- *Select in the menu, "blank rotor".*
- *Press "Enter".*
- *Press "F2", (blank), the rotor cuvette blank test is started now.*
- *After finishing of the rotor cuvette blank test, the results are automatically printed.*
- *Check for each filter the sd value (< 0.020 !).*

Continue the starting procedure as follows:

- Press "F10", (return).
- Select in the menu "fill / empty System".
- Press "Enter".
- Press "F1", (System Fill). Flushing is started now.
- During flushing check the syringes and the tubing of the reagent and sample probe for the absence of air bubbles and leakage.
- After flushing press "F10", (return).
- Press again "F10", (return).
- Press "F2", (install).
- Select in the menu "change reagent disk".
- Check if the Beta reagent disk is selected. In case another disk is selected, press "Enter".
- Select "Beta reagent disk".
- Press "Enter", the Beta reagent disk is selected now.
- Press "F10", (Main Menu).

The analyser is ready for use now.

*Sample request:*

- Press in the Main Menu "F8", (request samples).
- Type the run number in "sample number".
- Link method 61903 with the first sample number.
- Press "F4" (repeat mode).
- Press "F8", (new sample). Run number two and method 61903 are automatically requested now. Press "F8" for each following sample.
- Press "F9" (load samples).
- Type the first sample run and press "Enter".
- The position of the first sample run is programmed on the sample disk.
- Place the curse at the second position (under run number 1) and press "Enter".
- Press "Enter" for each following sample run.
- Press "F3" (confirm load). The analyser start the series now.

*After finishing of the series follow the next procedure:*

Clear the result buffer:

- Press "F7", (Eval. Samples).
- Press "F2", (Clr. Res. Buffer).
- Answer the question "Res. Buffer" with "Yes".

Unload the sample disk:

- Press “F9”, (load samples).
- Press “F4”, (confirm unload).
- Press “F10”, (Main Menu).

Remove the sample tubes from the sample disk.
Remove the bottle with Glucose hexokinase reagent from the reagent disk.
Place the covers on the reagent and sample disk.

## 6 CALCULATION

Carry out the calculation with the aid of the computer program available for this analysis. If this is not possible carry out the calculations as follows:

Prepare a calibration curve by plotting the absorbance versus the exactly calculated activity of the standards S1 – S6. This calibration curve must be fitted according second polynomial regression, in which y = absorbance and x = activity in ATU/ml incubation solution. This curve is used to determine the activity in the unknown samples.

Calculate the activity in the samples as follows:

ATU / ml sample = ATU obtained from the curve x Df

ATU / g sample = ATU obtained from the curve x Df / W

Where:
Df = Dilution factor
W = weight of sample [g]

See appendix 2, example shape of standard calibration line.

## 7 ASSESSMENT

### 7.1 Requirements

- A (diluted) sample solution must have an activity fitted within the measuring-range.
- The level of each control value must fit in the range : $C_{assigned} \pm 3 \times SD_{overall}$ ($C_{assigned}$ = Assigned control value; $SD_{overall}$ = overall standard deviation of the average control value calculated from past series).
- The relative *(absolute)* difference in level between (duplicate) control values within a daily series is not allowed to exceed a value of 2.8 x $RSD_{within\ day}$. (Relative absolute difference in control values = $(^{|\text{control value 1} - \text{control value 2}|}/_{\text{Average control value}}) \times 100\%$ ; $RSD_{within\ day}$ = relative overall standard deviation “within a day” calculated from past series using control values e.g. as determined in validation of the method).
- The relative *(absolute)* difference in level between (duplicate) sample values is not allowed to exceed a value of 2.8 x $RSD_{within\ day}$. (Relative absolute difference in sample values = $(^{|\text{sample value 1} - \text{sample value 2}|}/_{\text{Average sample value}}) \times 100\%$ ; $RSD_{within\ day}$ = relative overall standard deviation “within a day” calculated from past

sample series with a comparable type of matrix e.g. as determined in validation of the method).
- A standard curve point exceeding 5% deviation (after fitting) should be discarded and should be considered non-valid (= outlier). Refitting of the standard curve is required.
- The fitted standard curve should consist of at least 80% of the standard curve points.

The results of the control sample must be expressed as percentage of the assigned value. The results of the control samples must be imported into the control charts available for this method of analysis. All results have to be evaluated.

### 7.2 Actions

- Repeat the analysis with an adjusted dilution when the outcome is outside the measuring range.
- Repeat the analysis whenever the calibration curve does not comply with the requirements.
- Repeat the analysis whenever the controls do not comply with the requirements.
- Repeat the analysis whenever the difference between duplicate do not comply with the requirements.

### 7.3 Authorisation

The standards and selected samples meet the criteria as stated in section 7.1. After a training period by a for this method authorized technician, a technician will be authorized for this method when she/he succeeds in performing the test single-handed whereby

## 8 REFERENCES

Not applicable.

## 9 REMARKS

Appendix 1, Selectra program Meltamase glucose assay

Appendix 2, Example meltamase standard calibration line

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Appendix 1: Selectra program Meltamase glucose assay

Test parameters

| | |
|---|---|
| Name | : 61903 |
| Abbr. Naam | : melt |
| Mode | : Endpoint |
| Wavelenght | : 340 nm |
| Units | : Abs. |
| Decimals | : 3 |
| Low Conc. | : 0.000 Abs |
| High Conc. | : 0.000 Abs |
| Calibrator name | : none |
| Prozone Check | : No |
| Ref. male low | : 0.000 Abs |
| Ref. male high | : 0.000 Abs |
| Ref. female low | : 0.000 Abs |
| Ref. female high | : 0.000 Abs |
| Ref. ped. low | : 0.000 Abs |
| Ref. ped. high | : 0.000 Abs |
| Ref. panic low | : 0.000 Abs |
| Ref. panic high | : 0.000 Abs |
| Control 1 | : none |
| Control 2 | : none |
| Control 3 | : none |
| Correlat. faktor | : 1.000 |
| Correlat. offset | : 0.000 Abs |

| | |
|---|---|
| Dual Mode | |
| Sample Blank | : No |
| R1 bottle | : 25 ml |
| normal volume | : 240 µl |
| re-run volume | : 240 µl |
| Sample | |
| normal volume | : 5.0 µl |
| rerun volume | : 5.0 µl |
| R2 bottle | : 5 ml |
| normal volume | : 0 µl |
| rerun volume | : 0 µl |
| Pre-dilution | : No |
| Incubation time | : 11.5 min |
| Low Absorbance | : 0.000 Abs |
| High Absorbance | : 3.000 Abs |
| R. Ext. L. Limit | : 0.000 Abs |
| R. Ext. H. Limit | : 3.000 Abs |
| Reagent Blank | : No |
| Factor | : 1.0000 |

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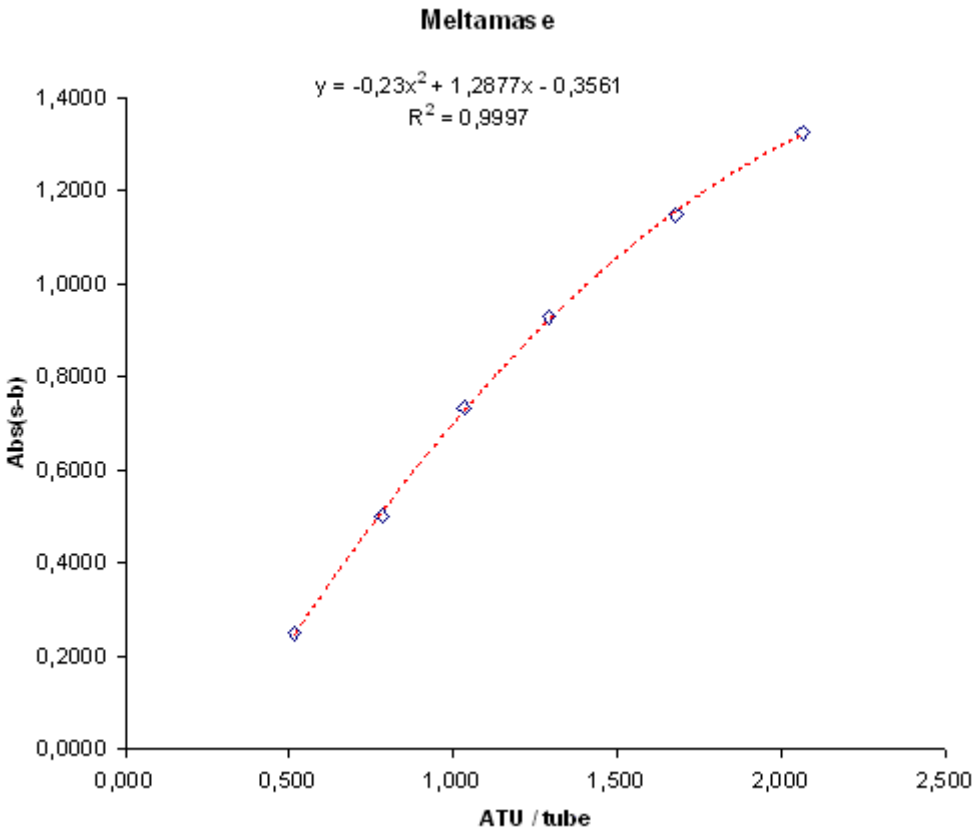
Appendix 2, Example meltamase standard calibration line



DSM

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| Department:<br><br>DFS/R&D/Analysis | Title:<br>Meltamase activity determination using maltotriose as substrate, relative method (two steps, manual and Selectra analyzer) |
| Product: not applicable<br><br>Product code: not applicable | |

HISTORY

| Version | Description of the modification |
|---|---|
| 1 | Draft. |
| 2 | Method adapted for introduction of new DSM standard preparation. |
| 3 | Activity expressed in new ATU units.<br>Granutest 250 replaced by glucose Hexokinase reagent.<br>Validation report summary added. |