

# Investigating the catalytic properties of a cupin-like protein from Thermotoga maritima (TM1010).

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Maarten Smits

# Investigating the catalytic properties of a cupin-like protein from *Thermotoga maritima* (TM1010).

by

Maarten Smits

Student Name	Student Number
Maarten Smits	5609313

Instructor: U. Hanefeld  
Teaching Assistants: N. Karakitsou, L. Koekkoek & M. Strampraad  
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Faculty: Faculty of Applied Sciences, Delft

Cover: *GtHNL* (green) and *Tm1010* (blue). Visual created using PyMol  
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# Preface

This study represents the work I have done over the last three months researching the catalytic activity and metal complex formation of *Thermotoga maritima* 1010 (*Tm1010*) at the Delft University of Technology. These months have allowed me to gain essential skills and knowledge while working on the fascinating topic of enzyme catalysis. I would like to thank Prof. Ulf Hanefeld for the opportunity, the support as well as the guidance he has given me. I would also like to thank the technical assistants, Laura Koekkoek, Marc Strampraad, Natalia Karakitsou, and Remco van Oosten, who guided and helped me throughout the project. Lastly, I would like to thank the amazing people of the BOC research group, who made it a joy to work on this project.

*Maarten Smits  
Delft, July 2024*

# Summary

*Granulicella tundricola* hydroxynitrile lyase (*GtHNL*) is an enzyme that interestingly has both hydroxynitrile lyase activity, catalysing reversible cyanohydrin cleavage, and oxidative alkene cleavage activity. This study aimed to investigate the hydroxynitrile lyase activity, the oxidative cleavage activity, and the metal co-factor properties of a structurally similar enzyme, *Thermotoga maritima* 1010 (*Tm1010*). Structurally, *Tm1010* is similar to *GtHNL*. However, *Tm1010* has an extra beta-strand at the C-terminus and no metal co-factor in the crystal structure. *GtHNL* and *Tm1010* were produced using pET-28a(+) plasmids, transformed into competent gold BL21(DE3) *Escherichia coli* cells. The specific activity of the hydroxynitrile lyase activity of *GtHNL* was found to be up to  $0.076 \pm 0.014$  U / mg, compared to  $0.011 \pm 0.002$  U / mg of *Tm1010*. Oxidative cleavage assays showed *GtHNL* and *Tm1010* activities of up to  $10.29\% \pm 0.07\%$  and  $21.40\% \pm 1.53\%$  respectively. *Tm1010* was found to have a Mn co-factor, in contrast to the crystal structure. The ratio of Mn to enzyme in purified *Tm1010* and *GtHNL* was found to be  $\sim 0.1$  and  $0.7-0.9$  respectively, suggesting *Tm1010* binding Mn less strongly than *GtHNL*. Mn saturation increased the oxidative cleavage activity. The removal of Mn reduced hydroxynitrile lyase activity marginally, while the almost completely removing oxidative cleavage activity, indicating Mn to be crucial for the oxidative cleavage activity. In conclusion, *Tm1010* is more efficient at catalysis of the oxidative cleavage reaction while *GtHNL* favours the hydroxynitrile lyase activity. In addition, *Tm1010* binds Mn less strongly than *GtHNL*. Lastly, Mn was found to be crucial for the oxidative cleavage activity of *GtHNL* and *Tm1010*.

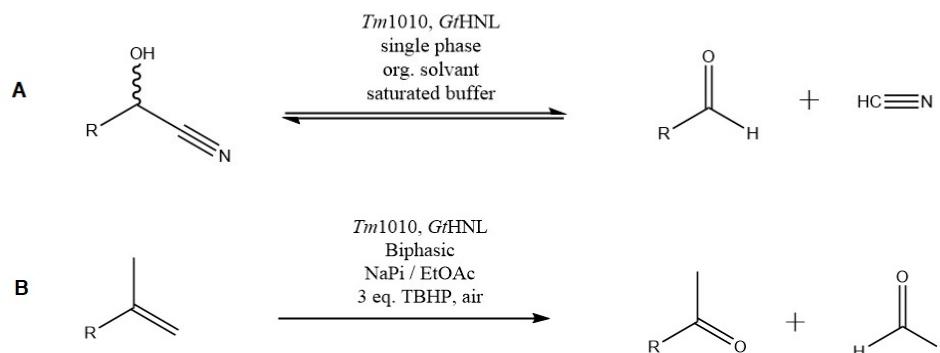
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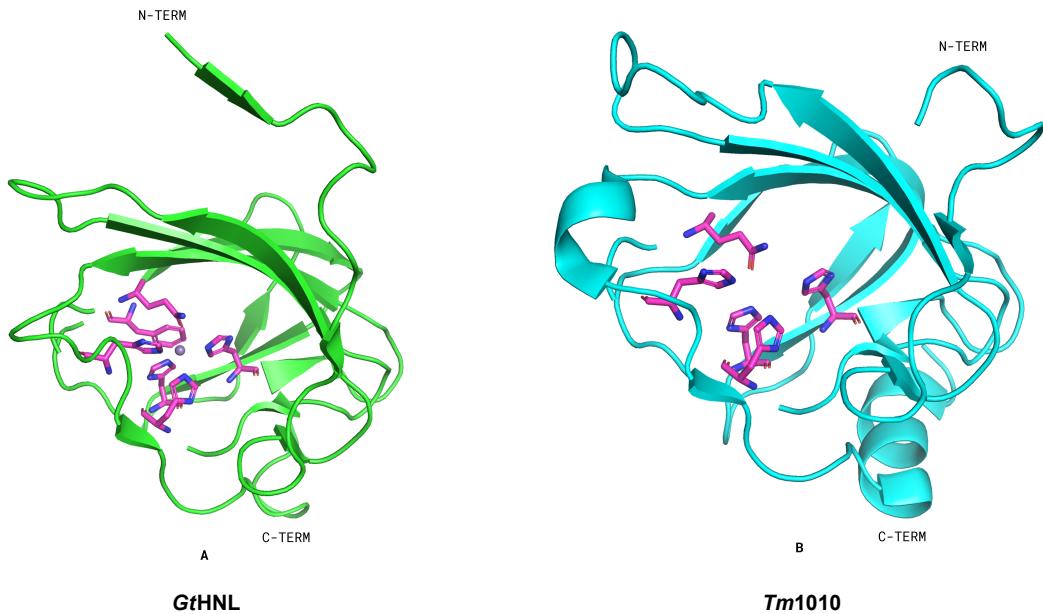
# Introduction

The Mn dependent *Granulicella tundricola* hydroxynitrile lyase (GtHNL) of the cupin superfamily has been investigated for its hydroxynitrile lyase activity, used to synthesise enantiopure cyanohydrins (figure 1.1A) [5]. Similarly, *Thermotoga maritima* 1459 (*Tm1459*) has been investigated for its ability to catalyse the oxidative alkene cleavage reaction (figure 1.1B) [4]. A reaction which is primarily achieved with the rather unsafe method of ozonolysis [3], to which enzymes can provide a safer alternative. Both GtHNL and *Tm1459* show structural similarity in the active site as well as forming Mn complexes. Hence, in recent years, the enzymes have been examined for multipotent activity of both the reversible cyanohydrin synthesis and the oxidative alkene cleavage reaction [2].



**Figure 1.1: Oxidative cleavage and hydroxynitrile lyase assay reaction mechanisms.** A: Hydroxynitrile lyase mechanism catalyzed by GtHNL and *Tm1010*. B: Oxidative cleavage reaction mechanism catalyzed by GtHNL and *Tm1010*.

The enzyme *Thermotoga maritima* 1010 (*Tm1010*) shows a similar active site to GtHNL (figure 1.2) and *Tm1459*. A key difference in the crystal structures of *Tm1010* and GtHNL is that the crystal structure of *Tm1010* was found not to have a metal ion in the active site (figure 1.2B) [2] [11]. Moreover, the C-terminus of *Tm1010* has an extra beta-strand compared to GtHNL [2]. Further details about the properties of this enzyme are limited. This study aims to identify the catalytic oxidative alkene cleavage and hydroxynitrile lyase (cyanohydrin cleavage) properties of *Tm1010*. Moreover, the Mn content of *Tm1010* was investigated to identify if the enzyme does form enzyme-Mn complexes. Activity assays were performed both on *Tm1010* and GtHNL. This enables a comparison of *Tm1010* with GtHNL. In addition, GtHNL data can be compared with previous research.



**Figure 1.2: Crystal structures of GtHNL and Tm1010.** The crystal structures of GtHNL [5] and Tm1010 [11] shown in green and blue respectively. The active site is highlighted purple. The side-chains of the amino acids in the active site are shown and the C-terminus and N-terminus of both enzymes are labelled. A purple dot in the active site marks the presence of a Mn ion.

The enzymes were produced using pET-28a(+) plasmids containing the kanamycin and Lac operon genes. Kanamycin serves as a selectable marker and the Lac operon enables isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) induction. The plasmids were transformed into gold BL21(DE3) *Escherichia coli* cells to overexpress GtHNL and Tm1010. Purification was done using heat treatment to denature all proteins except for GtHNL and Tm1010. The purified enzymes were supplemented with a tenfold excess of Mn to saturate Mn in the active sites. Excess Mn was removed using a PD-10 desalting column. The hydroxynitrile lyase (cyanohydrin cleavage) assay utilises the racemic mandelonitrile to benzaldehyde reaction (figure 2.2). Benzaldehyde absorbs at 280nm ( $A_{280}$ ) and can thus be analysed using a spectrophotometer. The oxidative cleavage assay utilises the reaction of  $\alpha$ -methylstyrene to acetophenone with tert butyl hydroperoxide (TBHP) as oxidising agent (figure 2.1). Concentrations of  $\alpha$ -methylstyrene and acetophenone were obtained using gaschromatography (GC). Mn contents of Tm1010 and GtHNL were investigated using inductively coupled plasma mass spectrometry (ICP-MS). In addition, apoprotein of GtHNL and Tm1010 was made and analysed by ICP-MS.

# 2

## Materials & Methods

Parts of the methods used in this study were inspired by or use the same method as was done in previous literature [13] [2].

### 2.1. Transformation of GtHNL & Tm1010 into Gold E. coli BL21(DE3)

The plasmids pET-28a(+)GtHNL and pET-28a(+)Tm1010 (Appendix B) were transformed into competent Gold BL21(DE3) cells. BL21(DE3) gold cells allow for GtHNL and Tm1010 induction with IPTG. First, the competent cells were thawed on ice, after which 1  $\mu$ L of 100 ng /  $\mu$ L plasmid-DNA was added to the cells. The cells were placed on ice a second time for 30 minutes. Hereafter, the samples were heat shocked in a water bath of 42 °C for 30 seconds and placed on ice for 2 minutes. 500  $\mu$ L of LB medium (Appendix C.1) was added after which the cells were incubated for 1 hour at 37 °C & 200 rpm. 150  $\mu$ L culture was plated on LB agar (Appendix C.2) containing 50  $\mu$ g/mL kanamycin and incubated overnight at 37 °C.

### 2.2. Cell cultivation and protein expression using IPTG and MnCl<sub>2</sub>

Single *E. coli* colonies containing one of the plasmids were inoculated into 20 mL LB-medium + 50  $\mu$ g/mL kanamycin to obtain a pre-culture. The pre-cultures were incubated overnight at 37 °C, 120 rpm. A blank, needed for optical density (OD) measurements, was taken from TB medium (Appendix C.4) with 50  $\mu$ g/mL kanamycin . 1 L TB medium was inoculated with the pre-culture and cultivated at 37 °C, 180 rpm. The OD at 600nm (OD600) was measured during cultivation. When the OD600 reached 0.6-0.8, a sample was taken and spun down after which the pellet was stored at -20 °C. In addition, 0.1 mM IPTG and 1 mM MnCl<sub>2</sub> were added to the culture. The cultivation was continued at 25 °C, 180 rpm overnight (20 h). Again, the OD600 was measured, this time to obtain a diluted sample of 0.5 mL with an OD600 of around 0.500. After spinning down the cultivation broth and the sample, the supernatant was discarded. The pellet of cells obtained from the fermentation was washed with 50 mL of 50 mM sodium phosphate buffer, pH 7.0 (Appendix C.5). The washed pellet and the sample were stored at -20 °C.

### 2.3. Purification of GtHNL & Tm1010

The pellets obtained from cultivating the *E. coli* cells were resuspended in  $\pm$  15 mL lysis buffer (Appendix C.6) after which the cells were lysed by cell disruption, 1.37 bar at 4 °C. During the cell disruption, additional lysis buffer was added resulting in obtaining a solution of  $\pm$  50-100 mL. The lysed cells were centrifuged at 4500 g, 4 °C for 30 minutes and the pellet was removed. The remaining supernatant, the cell free extract (CFE), was heated to 65 °C for 30 minutes to denature all proteins except for GtHNL and Tm1010. An additional shortened 65 °C purification of 15 minutes was performed for GtHNL. The heated CFE was then centrifuged at 4500 g, 4 °C again for 15 minutes. Half of the protein was stored at -20 °C, the other half was stored on ice to be supplemented with MnCl<sub>2</sub>.

## 2.4. Mn saturation of enzyme active sites with MnCl<sub>2</sub>

1 mM of MnCl<sub>2</sub> was added to 0.1 mM of both enzymes in 5mL of 50 mM sodium phosphate buffer, pH 7.0. The solution was incubated for 4 hours at room temperature. After incubation, excess Mn was removed by a PD-10 desalting column (Cytiva, G-25 resin). Each column was equilibrated with 25 mL of water, after which 2.5 mL of sample was added, discarding the flow-through. The samples were eluted with 3.5 mL water. The columns were washed again with 25 mL water and stored in a 0.05% NaN<sub>3</sub> solution.

## 2.5. BCA protein concentration analysis

A bicinchoninic acid (BCA) assay was used to determine the concentration of protein. Calibrations were created using standards of different known bovine serum albumin (BSA) concentrations (Table 2.1). The samples were diluted with water. A 50:1 (A:B) BCA reagent mix was made, of which 2 mL was added to 0.1 mL of the standards and protein dilutions (Table 2.2). The mixtures were incubated for 30 minutes at 37 °C. After cooling to room temperature, the OD562 levels of the calibration and dilution samples were measured using a spectrophotometer (Agilent Cary 60). The calibration tangent was used to obtain the protein concentration of the samples. All measurements were taken in duplo.

**Table 2.1:** Calibration standards for BCA assay.

Standard	BSA Stock (2 mg/mL)	Water	Final Concentration
A	300 µL	0 µL	2 mg/mL
B	300 µL	300 µL	1 mg/mL
C	420 µL	700 µL	750 µg/mL
D	100 µL	300 µL	500 µg/mL
E	100 µL	700 µL	250 µg/mL
F	200 µL of standard E	300 µL	100 µg/mL
G	100 µL of standard E	400 µL	20 µg/mL
Blank	0 µL	700 µL	0 µg/mL

**Table 2.2:** Preparation of different protein dilutions.

Dilution	Protein	Water
1x	200 µL	0 µL
1/10x	20 µL	180 µL
1/5x	40 µL	160 µL
1/2.5x	80 µL	120 µL
1/2x	100 µL	100 µL

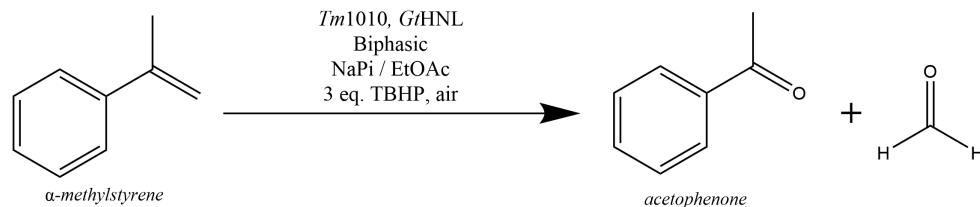
## 2.6. Protein purity analysis using SDS-PAGE

At different stages during protein expression and purification, 1 mL samples were taken. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the protein purity of the samples by separation based on protein size. The samples were prepared with DTT and Laemmli buffer and heated for 2 minutes at 95 °C. The samples were loaded on an Any kD™ Criterion™ TGX Stain-Free™ Protein Gel, 18 well, 30 µl. TGS buffer was used as running buffer. The SDS-PAGE ran at a constant 200 V. The unstained gels were imaged on a Bio-Rad Chemidoc MP.

## 2.7. Oxidative cleavage activity assay

### 2.7.1. Reaction mechanism

The oxidative cleavage assay utilises the reaction of  $\alpha$ -methylstyrene to acetophenone (figure 2.1).



**Figure 2.1: Oxidative cleavage reaction mechanism.** The oxidative cleavage reaction of  $\alpha$ -methylstyrene to acetophenone catalysed by GtHNL and Tm1010.

### 2.7.2. TBHP quantification with an iodine - sodium thiosulfate titration

The concentration of TBHP in decane needed to be identified to be able to achieve a desired TBHP concentration in the assay. 100  $\mu\text{L}$  TBHP in decane was added to 10 mL of a 3:2 acetic acid / chloroform mixture in an Erlenmeyer flask. The oxygen in the mixture was removed by flushing the solution with nitrogen for 2 minutes. Furthermore, 0.01 M sodium thiosulfate solution and a saturated sodium iodide solution were flushed with nitrogen. 500  $\mu\text{L}$  of the sodium iodide solution was added to the TBHP Erlenmeyer. The headspace of the flask was flushed with nitrogen for 1 minute. To the flask, 15 mL of distilled water and four drops of a 1% starch solution, both oxygen-free, were added. A dark purple colour should appear. The sodium thiosulfate solution was added dropwise until the TBHP mixture turned clear. The concentration of TBHP was calculated using the specific amount of sodium thiosulfate that was needed to turn the solution clear..

### 2.7.3. Tm1010 and GtHNL activity assay

100 mM  $\alpha$ -methylstyrene and 150 mM TBHP in ethyl acetate (EtOAc) with 1 mg of protein in 50 mM sodium phosphate buffer, pH 7.0, (NaPi) were mixed into a reaction mixture with a 1:9 ratio of organic / aqueous phase (Table 2.3). To achieve 150 mM TBHP with 100 mM  $\alpha$ -methylstyrene in 100  $\mu\text{L}$  organic phase, a stock of 0.5 M  $\alpha$ -methylstyrene and 1.5 M TBHP was made. A 0.1 mM  $\text{MnCl}_2$  blank is used to assess the background reaction.

**Table 2.3:** Preparation table of oxidative cleavage gaschromatography reaction samples.

	GtHNL	Tm1010	Control	Blank
NaPi ( $\mu\text{L}$ )	900 – tbd.	900 – tbd.	900	800
$\alpha$ -methylstyrene + TBHP stock ( $\mu\text{L}$ )	100	100	100	100
Enzyme in buffer ( $\mu\text{L}$ )	tbd.	tbd.	-	-
1 mM $\text{MnCl}_2$	-	-	-	100
Total volume ( $\mu\text{L}$ )	1000	1000	1000	1000

The reaction samples were shaken at 1000 rpm, 30 ° C for 20 hours in an Eppendorf Thermomixer C + ThermoTop. After the reactions had taken place, 400  $\mu\text{L}$  10 mM dodecane in EtOAc was added as an internal standard. The organic phase containing the  $\alpha$ -methylstyrene and acetophenone were extracted, stopping the reaction. The samples were dried using  $\text{MgSO}_4$  and analysed with gaschromatography

(Shimadzu GC-2010 Pro). A split injector with a temperature of 340 °C injected the samples into the column. Nitrogen gas carried the samples with a linear velocity of 30 cm / s. A cp-sil 8 CB column with a length of 25 m, an internal diameter of 0.025 mm and a film thickness of 1.25 µm was used. The GC run plan utilized two temperature plateaus (Table 2.4). The FID detector was set to a temperature of 360 °C with a hydrogen flow of 40 ml / min, an airflow of 400 ml / min and a makeup flow of 30 ml / min. Using Labsolutions, the chromatographs were obtained and analysed. The background reaction was subtracted from the enzymatic reactions. The reactions were performed and analysed in duplo.

**Table 2.4:** Oxidative cleavage gaschromatography method.

<i>Gradient</i>	<i>Temperature</i>	<i>Time maintained</i>
-	150°C	2 min
30 °C/min	190°C	2 min
30 °C/min	200°C	1 min
30 °C/min	345°C	1 min

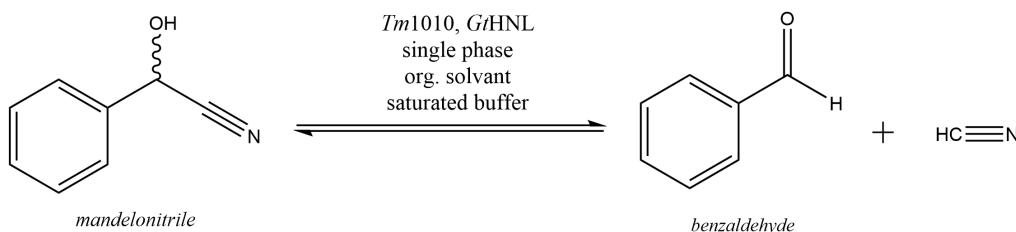
To quantify the substrate and product after the reactions, eight different calibration samples were tested alongside the protein samples (Table 2.5). The substrate and product were diluted with EtOAc to achieve 1 M and 0.1 M S/P stocks. Again, dodecane was used as an internal standard. The substrate and product peak areas were obtained, normalised using the internal standard, and used to create a calibration curve.

**Table 2.5:** Preparation table of oxidative cleavage gaschromatography calibration samples.

## 2.8. Hydroxynitrile lyase activity assay

### 2.8.1. Reaction mechanism

The hydroxynitrile lyase assay uses the reaction of mandelonitrile to benzaldehyde and hydrogen cyanide (figure 2.2).



**Figure 2.2: Hydroxynitrile lyase reaction mechanism.** The hydroxynitrile lyase reaction of mandelonitrile to benzaldehyde catalysed by GtHNL and Tm1010.

### 2.8.2. Racemic mandelonitrile purification

The racemic (rac-)mandelonitrile, purified using flash chromatography, 9:1 petroleum ether (PE) / methyl-tert-butyl- ether (MTBE) followed by 3:7 PE / MTBE, was obtained from previous research [13] [7]. The pure mandelonitrile was a yellow liquid, solidifying at freezing temperatures (-20 °C).

### 2.8.3. Tm1010 and GtHNL activity assay

50 mM sodium citrate buffer, pH 5.0 (Appendix C.7), enzyme and mandelonitrile were added to a quartz cuvette (Table 2.6). The mixture was kept at 25 °C, 1200 rpm while measuring absorbance.

1.4 mL sodium citrate buffer was added to 0.2 mL of enzyme solution. The enzyme solution was diluted to ±1 mg / mL enzyme for absorption levels to be between 0,2 and 2. 50 mM phosphate buffer, pH 5.0, was used as blank (Appendix C.3). After mixing at 25 °C, 0.4 mL 6 mM rac-mandelonitrile in sodium citrate buffer was added to the buffer and enzyme mixture to achieve a final volume of 2 mL.

The absorbance at 280nm was measured for 5 minutes. The optical path length was 1 cm. Based on the ΔA280/min obtained reaction rate was used to calculate the specific activity of the enzymes.

**Table 2.6:** Preparation table of spectroscopy HNL activity samples.

	<i>GtHNL</i>	<i>Tm1010</i>	<i>Background</i>	<i>Blank</i>
50 mM Sodium Citrate buffer, pH 5.0 (mL)	1.4	1.4	1.4	-
50 mM Phosphate buffer, pH 5.0 (mL)	-	-	-	2.0
Enzyme in buffer (mL)	0.2	0.2	-	-
6 mM rac-mandelonitrile (mL)	0.4	0.4	0.4	-
1 mM MnCl <sub>2</sub> (mL)	-	-	0.2	-
Total volume (mL)	2.0	2.0	2.0	2.0

## 2.9. GtHNL and Tm1010 apo-enzyme

This removal of Mn from *GtHNL* and *Tm1010* was based on protocols from previous research [6] [12]. The enzymes were diluted in 50 mM ice-cold potassium phosphate buffer, pH 7 (table 2.7). Dialysis tubes, MWCO of 3.5 kDa, were prepared by cutting the desired length and boiling the tubes for 20 minutes in 2% sodium bicarbonate / 1 mM EDTA dissolved in water. After the tubes were rinsed with water, 3 mL of the enzyme samples were added. The tubes were placed in 1.5 L of 20 mM 2,4-pyridinedicarboxylic acid (PDCA) in water. The dialysis was run for 64 hours, stirring slowly at 4 °C. Purified, Mn saturated and apo-enzyme samples of 15HT *GtHNL*, 30HT *GtHNL* and *Tm1010* ( $\pm$  0.5 - 1 mL) were sent to the Reactor Institute Delft (RID) for Mn content analysis with ICP-MS.

**Table 2.7:** *GtHNL* and *Tm1010* apoprotein preparation table. The concentrations of the samples were equalised to 3 mL of 1.58 mg/mL enzyme.

	15HT <i>GtHNL</i>	30HT <i>GtHNL</i>	<i>Tm1010</i>
Purified enzyme (mL)	2.873	3.0	0.394
50 mM potassium phosphate buffer, pH 7 (mL)	0.127	-	2.606
Total volume (mL)	3.0	3.0	3.0

# 3

## Results & Discussion

### 3.1. Protein concentration and purity

Before *GtHNL* and *Tm1010* could be analysed, the enzymes had to be produced and purified. The enzymes were over-expressed and purified using a 65 °C 30-minute heat treatment. *Granulicella tunericola* thrives at 21-24 °C [10], in contrast to *Thermotoga maritima*, which thrives at 80 °C [1]. Therefore, in addition to a 65 °C 30-minute heat treatment purification (30HT), a shortened 15 minutes purification (15HT) was performed for *GtHNL* to possibly improve the purification efficiency. The purity and concentration of the purified and MnCl<sub>2</sub> supplemented enzymes were assessed with a bicinchoninic acid (BCA) assay and a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, samples were taken during fermentation and purification to assess the enzyme production process.

#### 3.1.1. BCA assay concentration analysis

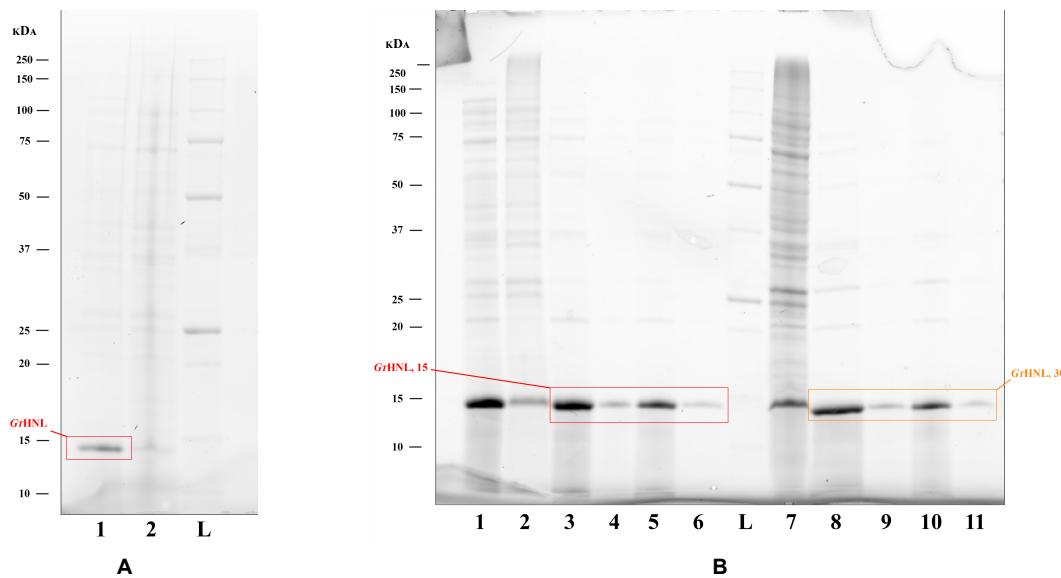
Overall, there is a loss of protein after the enzymes have been saturated with Mn (table 3.1). Due to less buffer used during cell lysis, the concentration of purified *Tm1010* is significantly higher than the *GtHNL* solutions. 15HT increased the concentration of purified *GtHNL* only slightly.

**Table 3.1: Total protein concentrations after purification and metal saturation.** Concentrations of protein in *GtHNL* samples after 15-minute heat treatment and *GtHNL* and *Tm1010* samples after 30-minute heat treatment have been measured with a BCA assay. The concentrations of the samples after MnCl<sub>2</sub> supplementation were measured as well. The concentrations are denoted by mean ± variance (n = 2).

Protein	Purified (mg/mL)	Mn saturated (mg/mL)	Purified (μM)	Mn saturated (μM)
15HT <i>GtHNL</i>	1.65 ± 0.02	0.70 ± 0.03	115 ± 1.39	49.0 ± 2.10
30HT <i>GtHNL</i>	1.58 ± 0.02	0.65 ± 0.04	110 ± 1.39	45.5 ± 2.80
<i>Tm1010</i>	12.03 ± 0.01	1.67 ± 0.05	805 ± 0.67	112 ± 3.35

### 3.1.2. SDS-PAGE GtHNL purity analysis

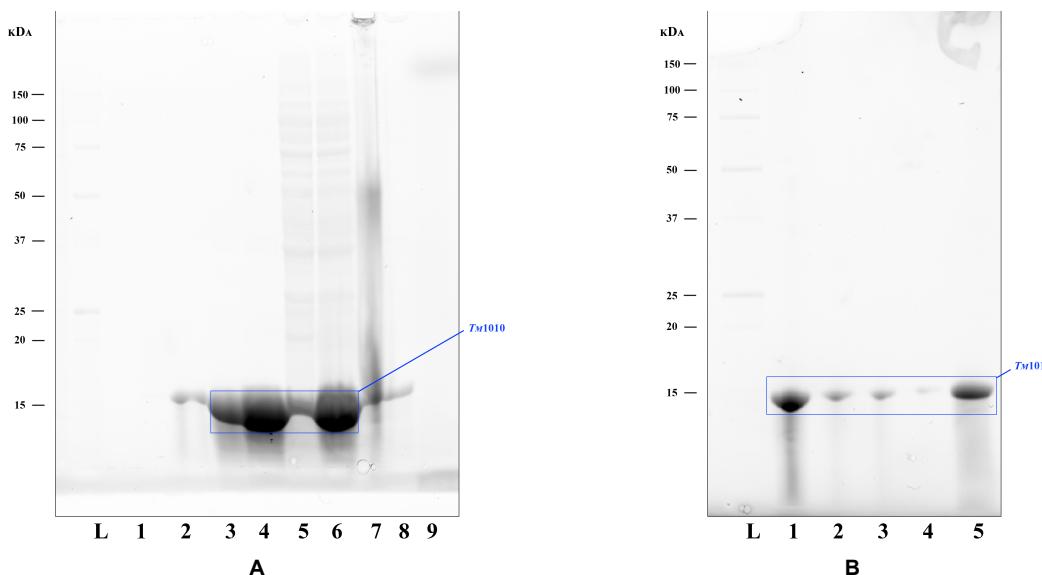
The molecular weights of GtHNL and *Tm*1010 are 14.29 kDa and 14.93 kDa respectively. Figure 3.1A shows a band corresponding to GtHNL only after induction of IPTG (lane 1-2). Figure 3.1B shows that purification utilizing 15HT and 30HT both resulted in the same amount of separation of GtHNL from other proteins (lanes 2, 3-4 & 7, 8-9). The GtHNL band remains prominent after Mn saturation (lanes 5-6 & 10-11). GtHNL was thus produced after induction with IPTG and purified effectively with both 15HT and 30HT. 15HT purification increased the concentration of purified GtHNL by a small amount, while not decreasing the purity. Overall, 15-minute heat treatment at 65 °C thus increases purification efficiency only slightly. Mn saturation did not affect the purity of the GtHNL solutions.



**Figure 3.1: SDS-PAGE analysis of fermentation broth and heat treated GtHNL samples.** Unstained SDS-PAGE was done to identify production of GtHNL and purity in the purified and MnCl<sub>2</sub> supplemented samples. A: (1) fermentation broth sample after GtHNL induction. (2) fermentation broth before induction. (L) Bio-Rad Precision Plus Protein Unstained ladder. B: (1) CFE. (2) Denatured protein after 15-minute heat treatment (15HT). (3) 15HT 1x diluted purified GtHNL. (4) 15HT 10x diluted purified GtHNL. (5) 15HT 1x diluted MnCl<sub>2</sub> supplemented GtHNL. (6) 15HT 10x diluted MnCl<sub>2</sub> supplemented GtHNL. (L) Bio-Rad Precision Plus Protein Unstained ladder. (7) 30-minute heat treated (30HT) denatured proteins. (8) 30HT 1x diluted purified GtHNL. (9) 30HT 10x diluted purified GtHNL. (10) 30HT 1x diluted MnCl<sub>2</sub> supplemented GtHNL. (11) 30HT 10x diluted MnCl<sub>2</sub> supplemented GtHNL. Of all samples, 10 µL was loaded onto the gels. Bands of notable wells at the height of 15HT GtHNL and 30HT GtHNL is shown in red and orange respectively.

### 3.1.3. SDS-PAGE *Tm*1010 purity analysis

Figure 3.2A shows a fermentation & purification gel with *Tm*1010 bands at around 15 kDa. The gel shows that after IPTG induction, a *Tm*1010 band appears (lanes 8-9) and that bands of other proteins disappear after purification (lanes 3-6). A 30-minute heat treatment thus resulted in pure *Tm*1010. A first Mn saturation of *Tm*1010 was performed with miscalculated concentrations (lanes 1-2), therefore, a second Mn saturation of *Tm*1010 was done (figure 3.2B). Similar to GtHNL, the *Tm*1010 Mn saturation lowered yield but did not affect purity (lanes 1-5).



**Figure 3.2: SDS-PAGE analysis of *Tm1010* fermentation and heat treatment purification.** Unstained SDS-PAGE was performed to identify production and purity of *Tm1010*. 10  $\mu$ L of sample was loaded into each well. A: (L) Bio-Rad Precision Plus Protein Unstained ladder. (1 & 2) failed Mn saturation of *Tm1010*. (3) 2x diluted purified *Tm1010*. (4) 1x diluted purified *Tm1010*. (5) Denatured proteins after 30-minute heat treatment. (6) CFE. (7) Pellet after disruption, smeared. (8) Fermentation broth after *Tm1010* induction with IPTG. (9) Fermentation broth before induction. B: (L) Bio-Rad Precision Plus Protein Unstained ladder. (1) 10x diluted purified *Tm1010*. (2) 100x diluted purified *Tm1010*. (3) 10x diluted  $MnCl_2$  supplemented *Tm1010*. (4) 100x diluted  $MnCl_2$  supplemented *Tm1010*. (5) 1x diluted  $MnCl_2$  supplemented *Tm1010*. Bands of notable wells at the height of *Tm1010* are labelled in blue.

## 3.2. Enzyme activity assessment

As *GtHNL* and *Tm1010* were now produced and purified, the activities of both enzymes could be investigated. Using the  $\alpha$ -methylstyrene to acetophenone and mandelonitrile to benzaldehyde reaction mechanisms (figure 2.1 & 2.2), both the oxidative cleavage and hydroxynitrile lyase activities could be analysed.

### 3.2.1. Oxidative cleavage analysis with gaschromatography

The reaction of  $\alpha$ -methylstyrene to acetophenone was catalysed by *GtHNL* and *Tm1010* with tert-butyl hydroperoxide (TBHP) as oxidising agent for 20 hours. The concentrations of  $\alpha$ -methylstyrene and acetophenone in the reaction mixtures after 20 hours were analysed with gaschromatography. The peaks of the substrate, product, internal standard and decane (TBHP solv.) were identified and the peak areas were converted to concentrations using a calibration line (appendix E). The total *GtHNL* and *Tm1010* catalysed reaction was quantified by subtracting the 0.1 mM  $MnCl_2$  background conversion from the total  $\alpha$ -methylstyrene to acetophenone conversion.

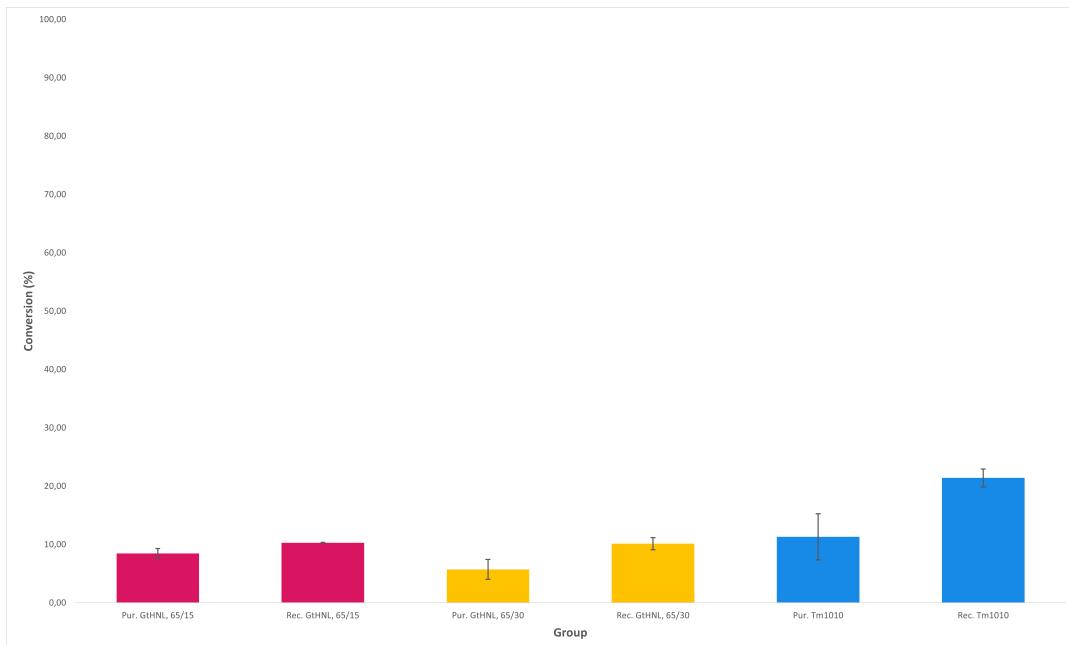
Gaschromatography analysis of the oxidative cleavage reactions shows that *GtHNL* converted  $\sim$ 5-8% of the  $\alpha$ -methylstyrene and *Tm1010*  $11.30\% \pm 3.96\%$  (table 3.2). Saturation of the enzymes with excess Mn significantly increases conversion percentages overall. Purified 15HT *GtHNL* has slightly more conversion than purified 30HT *GtHNL*. After Mn saturation, this difference almost disappears. Visualising the conversion percentages, a significant difference can be seen between the activities of *Tm1010* and *GtHNL* (figure 3.3). *Tm1010* catalyses the oxidative cleavage reaction better than *GtHNL*, especially after Mn saturation,  $10.29\% \pm 0.07\%$  compared to  $21.40\% \pm 1.53\%$ .

The conversion of  $\alpha$ -methylstyrene by *GtHNL* has been assessed in previous literature. This literature shows more than double the amount of conversion for *GtHNL* (23.6%) [2]. This difference might be explained by this experiment using double the amount of  $\alpha$ -methylstyrene (100 mM - 50 mM) and half the amount of enzyme (1 mg - 2 mg). To conclude, Mn saturation of the enzymes has a significant

positive effect on the oxidative cleavage activity of both enzymes. Moreover, the difference in *GtHNL* purification method does not have a significant effect on the oxidative cleavage activity when *GtHNL* is saturated with Mn. Lastly, *Tm1010* is better at catalysing the oxidative cleavage reaction than *GtHNL*.

**Table 3.2: Oxidative cleavage conversion efficiency for *Tm1010* and *GtHNL*.** 100 mM  $\alpha$ -methylstyrene in ethyl acetate with 150 mM TBHP converted by 1 mg 15-minute heat treated *GtHNL*, 30-minute heat treated *GtHNL* and *Tm1010* in 50 mM sodium phosphate buffer, pH 7.0. The reaction took place for 20 h at 30 °C, 1000 rpm. Both purified and MnCl<sub>2</sub> supplemented enzymes were measured. The conversions have been denoted mean  $\pm$  variance (n = 2).

Conversion %	15HT <i>GtHNL</i>	30HT <i>GtHNL</i>	<i>Tm1010</i>
Purified	8.46 $\pm$ 0.83	5.73 $\pm$ 1.70	11.30 $\pm$ 3.96
Mn saturated	10.29 $\pm$ 0.07	10.12 $\pm$ 1.04	21.40 $\pm$ 1.53



**Figure 3.3: Enzymatic oxidative cleavage conversion efficiency by *GtHNL* and *Tm1010*.** The bar height shows the percentage of 100 mM  $\alpha$ -methylstyrene in ethyl acetate converted by 1 mg of *GtHNL*, 15 and 30 minutes heat treated at 65 °C (red and yellow respectively), and *Tm1010* (blue) in 50 mM sodium phosphate buffer, pH 7.0.. Conversion by purified enzyme as well as MnCl<sub>2</sub> supplemented enzyme were investigated. The reaction took place for 20 h at 30 °C, 1000 rpm. The 0.1 mM MnCl<sub>2</sub> background reaction was subtracted from the enzymatic reactions. Error bars show the variability of the data (n = 2).

### 3.2.2. Hydroxynitrile lyase activity analysis

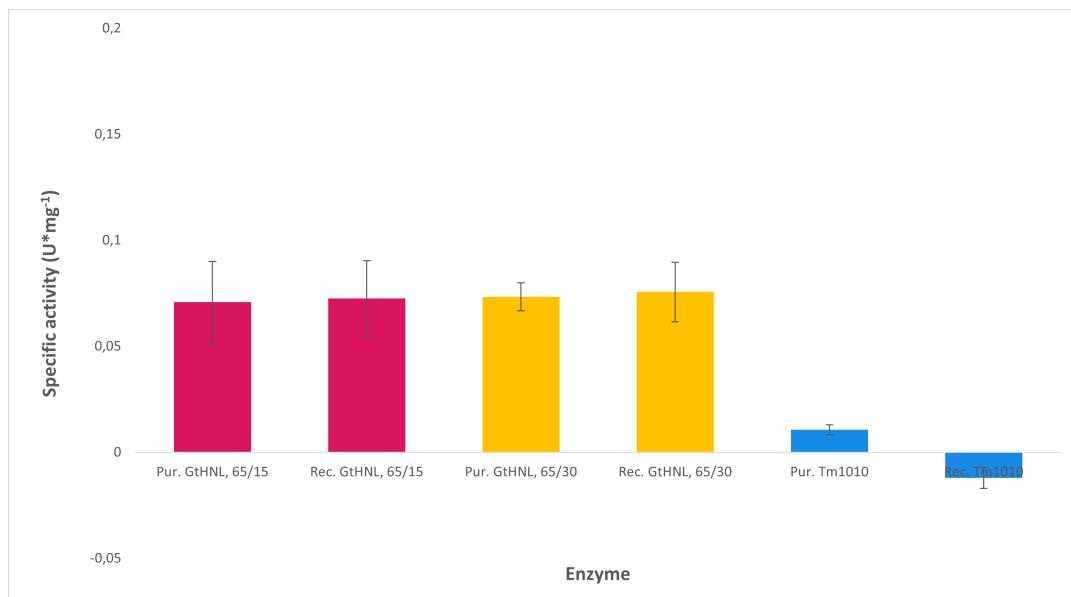
The hydroxynitrile lyase (HNL) activity was assayed using the racemic (rac-)mandelonitrile to benzaldehyde reaction. The benzaldehyde formation was analysed by measuring A<sub>280</sub> with a spectrophotometer. The absorbances were converted into activities using the Lambert-Beer law and the benzaldehyde molar absorption coefficient of 1147,9 M<sup>-1</sup> · cm<sup>-1</sup> [8]. 0.1 mM MnCl<sub>2</sub> background activity (appendix F) was subtracted from the activities of *GtHNL* and *Tm1010*. Specific activities of both purified and Mn supplemented *GtHNL* and *Tm1010* were obtained.

The specific activities obtained from the assay show *GtHNL* outperforming *Tm1010* in the lyase of rac-

mandelonitrile (figure 3.4). Table 3.3 shows that purified 15HT GtHNL has a similar specific activity as 30HT GtHNL,  $0.071 \pm 0.019$  U / mg compared to  $0.073 \pm 0.007$  U / mg. After Mn saturation the activities are similar as well,  $0.073 \pm 0.018$  U / mg compared to  $0.076 \pm 0.014$  U / mg. Literature shows a significantly higher specific activity for the lyase of rac-mandelonitrile, 1.74 U / mg [5]. An explanation for this can be that a buffer with a pH of 5 was used instead of a pH of 6, resulting in lower activity [5]. Visualised by figure 3.4, Mn saturation of GtHNL does not increase activity significantly, it even decreases the activity for Tm1010. To conclude, GtHNL has a greater HNL activity than Tm1010. In addition, a 15-minute heat treatment reduction does not affect the activity. Lastly, in contrast to the oxidative cleavage activity, Mn saturation of the enzymes with Mn does not seem to affect the HNL activity.

**Table 3.3: Tm1010 and GtHNL hydroxynitrile lyase activity.** Specific activities of 15HT GtHNL, 30HT GtHNL and Tm1010 in 50 mM sodium citrate buffer, pH 5.0. Catalysed 6 mM mandelonitrile conversion to benzaldehyde denoted in U / mg. Activities of both purified and MnCl<sub>2</sub> supplemented GtHNL and Tm1010 have been measured. The activities have been denoted by mean  $\pm$  variance (n = 2).

Specific activity (U/mg)	15HT GtHNL	30HT GtHNL	Tm1010
Purified	$0.071 \pm 0.019$	$0.073 \pm 0.007$	$0.011 \pm 0.002$
Mn saturated	$0.073 \pm 0.018$	$0.076 \pm 0.014$	$-0.012 \pm 0.005$



**Figure 3.4: Hydroxynitrile lyase specific activity of GtHNL and Tm1010.** 6 mM rac-mandelonitrile reaction catalysed by purified and Mn supplemented enzymes in 50 mM sodium citrate buffer, pH 5.0. Initial 5-minute rac-mandelonitrile reaction rate quantised by spectrophotometer at 280nm. Specific activities of 15HT GtHNL (red), 30HT GtHNL (yellow) and Tm1010 (blue) after subtracting the 0.1 mM MnCl<sub>2</sub> background reaction are visualised by the bar height. Error bars in black show the variance in the specific activities.

### 3.3. Metal analysis & apoprotein

In addition to analysing the activities of *GtHNL* and *Tm1010*, the amount of Mn present in the enzymes before and after saturation was identified. Moreover, apoprotein was made by dialysis and investigated as well. The Mn contents were measured by ICP-MS.

The  $\mu\text{M}$  Mn per  $\mu\text{M}$  enzyme ratios of 0.913 and 0.757 of purified *GtHNL* show it is almost saturated with Mn, even before supplementing with  $\text{MnCl}_2$  (table 3.4). In contrast, supplementing Mn to *Tm1010* after purification increased Mn content tenfold, from 0.099  $\mu\text{M}$  Mn per  $\mu\text{M}$  enzyme to over 1 (saturated). Both *GtHNL* and *Tm1010* lose most of the metal after a 64-hour dialysis. The activity measurements of *GtHNL* and *Tm1010* apoprotein show a loss of activity in oxidative cleavage (table 3.4). In contrast, the hydroxynitrile lyase activity decreased only marginally after Mn removal. No duplo was assessed for the apoprotein activity measurements. In addition, the enzyme samples tested by ICP-MS were not clear, indicating impurity. These factors hamper the accuracy of the data.

The ratio of  $\mu\text{M}$  Mn per  $\mu\text{M}$  enzyme of *GtHNL* and *Tm1010* show Mn binding strongly to *GtHNL* and weak to *Tm1010*. Previous research supports *GtHNL* binding Mn strongly [12]. Oxidative cleavage activity is drastically decreased after removing Mn from the enzyme, suggesting metal complex formation is crucial for the oxidative cleavage activity. In contrast, *GtHNL* still has HNL activity after a 64-hour dialysis, suggesting Mn is not as crucial. Further research is needed to conclude these findings.

**Table 3.4: Mn content & activities of *GtHNL* and *Tm1010*.** Purified, Mn supplemented and apoprotein were analysed for metal content by ICP-MS. The apoprotein was obtained by 64-hour dialysis with 20 mM PDCA in water. The initial 5-minute reaction rate of 6 mM rac-mandelonitrile in 50 mM sodium citrate buffer, pH 5.0, to benzaldehyde was used to obtain the hydroxynitrile lyase activity (HNL), 25 °C, 1200 rpm. Moreover, oxidative cleavage conversions conditions were 100 mM  $\alpha$ -methylstyrene in ethyl acetate with 150 mM TBHP reacting for 20 hours, 30 °C, 1000 rpm. Activities were measured in duplo except for the apoprotein (n=1).

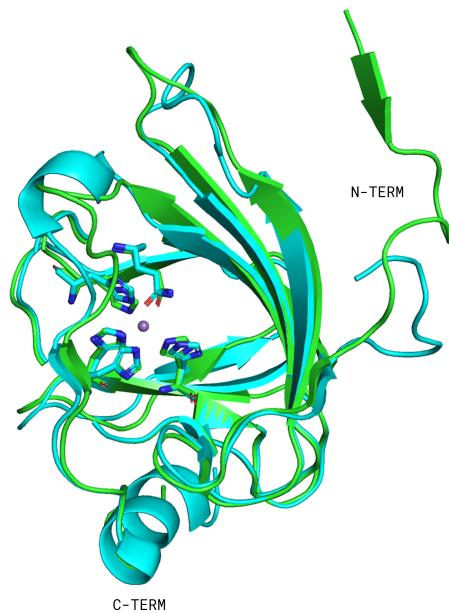
Protein	Mn (ppm)	Concentration ( $\mu\text{M}$ )	Mn ( $\mu\text{M}$ )	Ratio Mn/enzyme ( $\mu\text{M}/\mu\text{M}$ )	HNL (U/mg)	Ox. Cleavage (Conversion%)
15HT <i>GtHNL</i> pure	9.4	118.6	108.3	0.913	$0.084 \pm 0.019$	$8.46 \pm 0.83$
15HT <i>GtHNL</i> rec.	1.9	52.56	49.41	0.940	$0.073 \pm 0.018$	$10.29 \pm 0.07$
30HT <i>GtHNL</i> pure	8.5	123.9	93.77	0.757	$0.073 \pm 0.007$	$5.73 \pm 1.70$
30HT <i>GtHNL</i> rec.	3.7	48.80	103.6	2.123	$0.076 \pm 0.014$	$10.12 \pm 1.04$
<i>Tm1010</i> pure	59	903.2	89.27	0.099	$0.011 \pm 0.002$	$11.30 \pm 3.96$
<i>Tm1010</i> rec.	13.3	125.4	145.0	1.156	$-0.012 \pm 0.005$	$21.40 \pm 1.53$
15HT <i>GtHNL</i> apo	0.07	118.6	0.806	0.0068	0,002	3,65
30HT <i>GtHNL</i> apo	0.08	118.6	0.922	0.0078	-0,005	5,49
<i>Tm1010</i> apo	0.06	118.6	0.691	0.0058	-0,020	-1,84

### 3.4. *GtHNL* and *Tm1010* crystal structure

The favouritism of *GtHNL* and *Tm1010* for either oxidative cleavage or hydroxynitrile lyase activity can be explained by the differences in structure between the two enzymes. In previous research, a *GtHNL* variant, *GtHNL-H96A*, was analysed [2]. This variant was found to favour the oxidative cleavage reaction over the hydroxynitrile reaction, in contrast to wildtype *GtHNL*. Moreover, it was found to be structurally similar to *Tm1010* as well as having a beta-strand in the C-terminus. This shows that the key structural differences of *GtHNL* and *Tm1010*, e.g. the extra beta-strand, play a major role in the shifting the favoured reaction of the enzymes.

Superimposition of *GtHNL* and *Tm1010* shows the structural differences (figure 3.5). Due to these differences, in the active site and in the C-termini structure, the active cavity is greater for *Tm1010* than *GtHNL*. Research has shown that a greater active cavity accessibility increases oxidative cleavage

activity in *GtHNL* [2]. Therefore, a greater activity of oxidative cleavage for *Tm1010* can be explained by the increased active site accessibility. A greater active site accessibility leads to increased distance between the Mn ion and the residues which bind the metal. *Tm1010* having a larger active site accessibility thus supports *Tm1010* binding Mn less than *GtHNL*. A lesser Mn binding affinity of *Tm1010* compared to *GtHNL* might explain the Mn ion missing from the *Tm1010* crystal structure.



**Figure 3.5: Superimposition of the crystal structures of *GtHNL* and *Tm1010*.** The aligned crystal structures of *GtHNL* (green) and *Tm1010* (blue) shown superimposed. The side-chains of active site amino acids in both enzymes are shown as well the C- and N-terminus.

# 4

## Conclusion

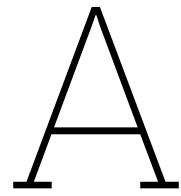
The catalytic oxidative cleavage, hydroxynitrile lyase, and metal complex formation properties of *Tm1010* were investigated. In addition, two different heat treatment purification methods of *GtHNL* were analysed. A heat treatment method shortened by half was found not to have a significant effect on the oxidative cleavage or hydroxynitrile lyase activity. However, a small increase in obtained purified *GtHNL* was observed. *GtHNL* favours the hydroxynitrile lyase reaction, while *Tm1010* favours the oxidative cleavage. Moreover, *Tm1010* had a higher oxidative cleavage activity than *GtHNL*, with an  $\alpha$ -methylstyrene conversion of up to  $21.40\% \pm 1.53\%$ . However, *Tm1010* showed little hydroxynitrile lyase activity.

In contrast to the crystal structure, *Tm1010* was found to form a Mn complex in the active site similar to *GtHNL* and *Tm1459*. Moreover, saturating the active sites of *Tm1010* and *GtHNL* with an excess of Mn has a significant positive effect on oxidative cleavage activity. In contrast, Mn saturation did not seem to affect the hydroxynitrile lyase activity. The Mn content of purified *GtHNL* was significantly higher than *Tm1010*. In addition, supplementing the enzymes with  $MnCl_2$  raised Mn contents only for *Tm1010* significantly, suggesting *GtHNL* binding Mn is much stronger than *Tm1010*. *Tm1010* and *GtHNL* were found to have structural differences, especially in the C-termini structure. Because of this, *Tm1010* has a greater active site accessibility than *GtHNL*, possibly causing higher oxidative cleavage activity. Moreover, *Tm1010* having greater active site accessibility supports *Tm1010* binding Mn less strongly. After removing Mn from *Tm1010* and *GtHNL*, oxidative cleavage activity was drastically lowered, indicating Mn is crucial for oxidative cleavage activity. The removal of Mn affected the hydroxynitrile lyase activity only marginally.

There is potential for optimisation of the *Tm1010* oxidative cleavage activity. Modifying the enzyme might lead to higher yields or a broader substrate scope. Moreover, assessing the binding affinity might help improve the Mn complex formation and activity.

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## Amino acid sequences

The amino acid sequence of *Tm1010*:

>2F4P  
MGSDKIHHHHHHMVDDIFERGSKGSSDFFTGNVVVKMLVTDENGVFNTQVYDVVFEPGARTHWHSHPG  
GQILIVTRGKGFYQERGKPARILKKGDVVEIPPNVVWHGAAAPDEELVHIGISTQVHLGPAEWLGSVTEEE  
YRKATEGK

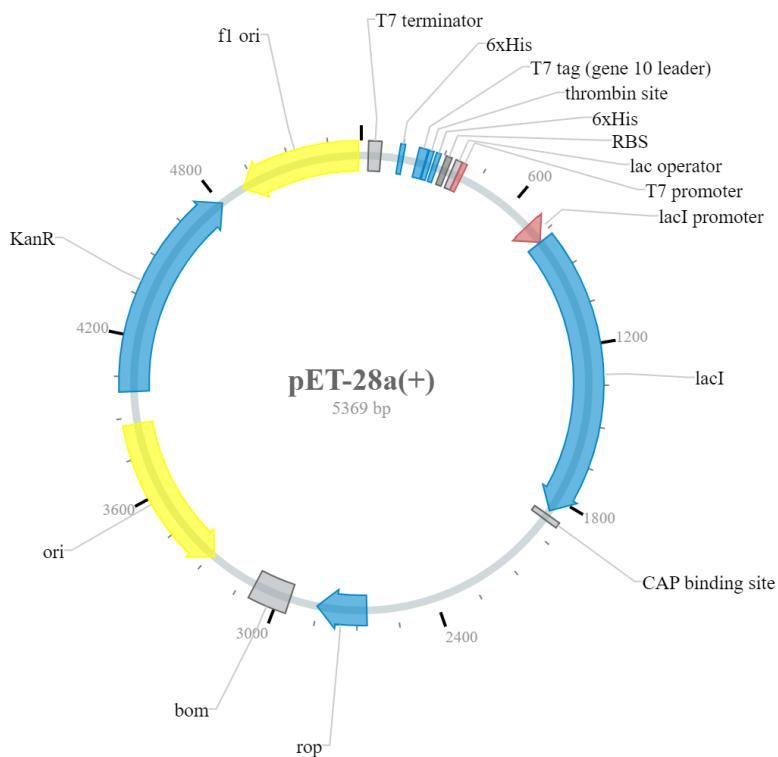
The amino acid sequence of *GtHNL*:

>4BIF  
MSYYHHHHHDYDIPTTENLYFQGAMEIKRVSQASGKGPADWFTGTVRIDPLFQAPDPALVAGASVTFEP  
GARTAWHTHPLGQLTIVAGCGWAQREGGAVEEIHPGDVVWFSPGEKHWGAAPTTAMTHLAIQERLDG  
KAVDWMEHVTDEQYRR

# B

## Plasmids

Production of *Tm1010* and *GtHNL* was realized by utilizing the properties of the pET-28a(+) plasmid (Figure B.1). Both protein sequences were inserted between the NcoI and HindIII sites. Induction of the Lac operon via IPTG enables transcription of the *Tm1010* and *GtHNL* genes. The KanR gene is used as a kanamycin selectable marker. The length of the plasmid is 5369 bp. The insert of *Tm1010* has a length of 147 bp and the insert of *GtHNL* has a length of 156 bp.



**Figure B.1:** Vector map of pET-28a(+) [9].

# C

## Media preparation

### C.1. Lysogeny broth (LB) medium

In a 1 L bottle, combine:

- 10 g of Bacto-tryptone
- 10 g of NaCl
- 5 g of Bacto-yeast extract
- 950 mL of deionized water

Shake or stir until everything is dissolved. Use 5 N NaOH to adjust the pH of the solution to 7.0. Add water to reach a final volume of 1 L. Autoclave the solution, liquid cycle (20 minutes at 15 psi). Allow the solution to cool before usage or storage.

### C.2. Lysogeny broth (LB) agar

In a 1 L bottle, combine:

- 7.5 g of agar
- 5 g of tryptone
- 5 g of NaCl
- 2.5 g of yeast extract
- 400 mL of deionized water

Shake until everything has dissolved. Use 1 N NaOH to adjust the pH of the solution to 7.0. Add water to reach a final volume of 500 mL. Autoclave the solution, liquid cycle (20 minutes at 15 psi). Allow the solution to cool to  $\pm 55^{\circ}\text{C}$ . Pour 10-20 mL of the solution into a Petri dish, avoid bubble formation, and make sure that the environment is sterile.

### C.3. Phosphate buffer, 50 mM & pH 5.0

Make 50 mM  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  concentrations using distilled water. Adjust mixture to achieve a final desired pH of 5.0, make sure the pH-meter is calibrated.

## C.4. Terrific broth (TB) medium

In a 1 L bottle, combine:

- 20 g of tryptone
- 24 g of yeast extract
- 900 mL of deionized water
- 4 mL of glycerol

Shake or stir until everything is dissolved. Autoclave the solution, liquid cycle (20 minutes at 15 psi). Allow the solution to cool to  $\pm 60^{\circ}\text{C}$  before use or storage. After autoclaving, add 100 mL of sterilized phosphate buffer. Add additional sterilized deionized water to achieve a final volume of 1 L.

## C.5. Sodium phosphate buffer, 50 mM & pH 7.0

Create Sodium Phosphate Dibasic Heptahydrate and Sodium Phosphate Monobasic Monohydrate concentrations of 50 mM using distilled water. Adjust the mixture of the two solutions to achieve a pH of 7.0 while using a calibrated pH-meter.

## C.6. Lysisbuffer

Combine and mix to achieve a desired quantity of lysisbuffer:

- 50 mM sodium phosphate buffer
- 50–70 mg DNase, pH 7.0
- 1 mM MgSO<sub>4</sub>
- 1 mM PMSF

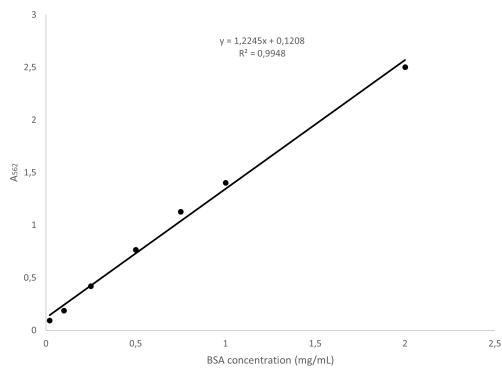
## C.7. Sodium citrate buffer, 50 mM & pH 5.0

Make 50 mM Sodium Citrate dihydrate and Citric Acid concentrations using distilled water. Adjust mixture to achieve a final desired pH of 5.0 (pH-meter calibrated for a pH of 5.0).

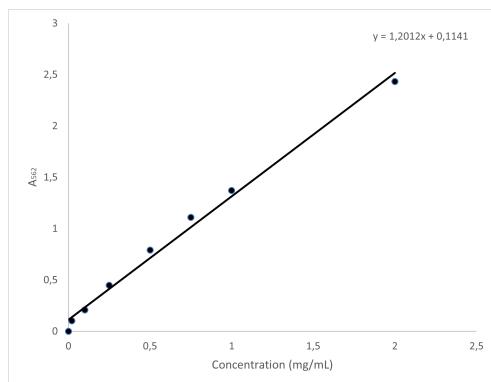
D

## BCA assay

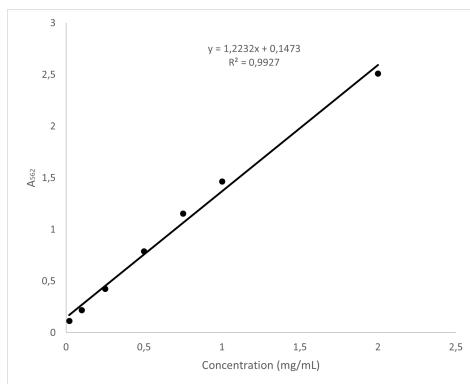
### D.1. BCA calibration



**Figure D.1:** BCA calibration curve of BSA dilutions for purified *GtHNL* measurements.



**Figure D.2:** BCA calibration curve of BSA dilutions for purified *Tm1010* measurements.

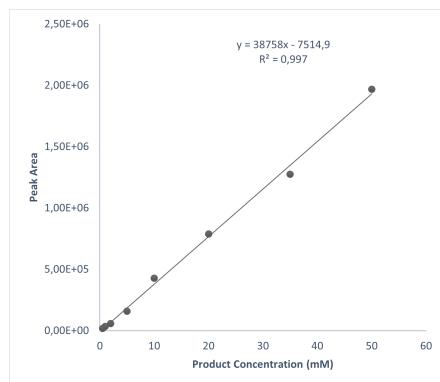


**Figure D.3:** BCA calibration curve of BSA dilutions for reconstituted enzyme measurements.

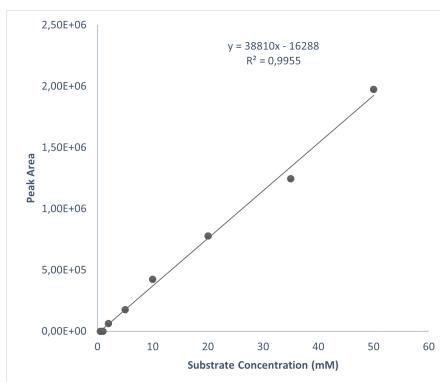
# E

## Gaschromatography

### E.1. Gaschromatography calibration and compound reference

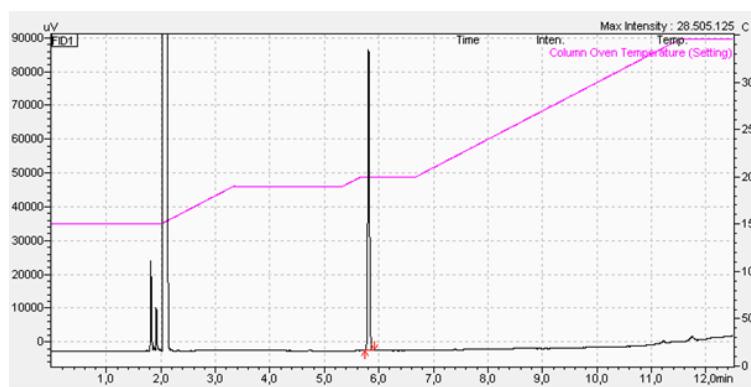


**Figure E.1:** Gaschromatography product calibration curve.

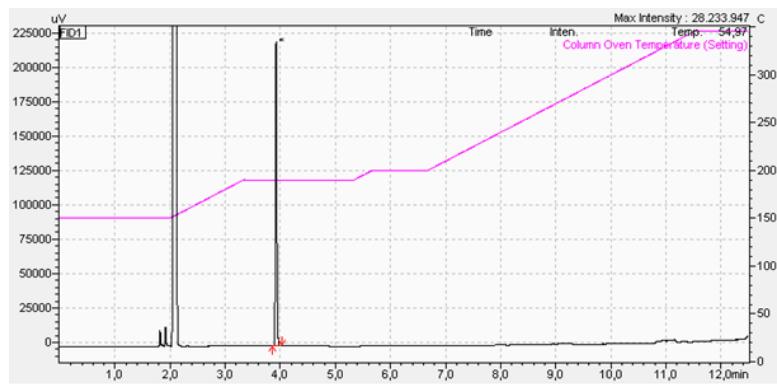


**Figure E.2:** Gaschromatography substrate calibration curve.

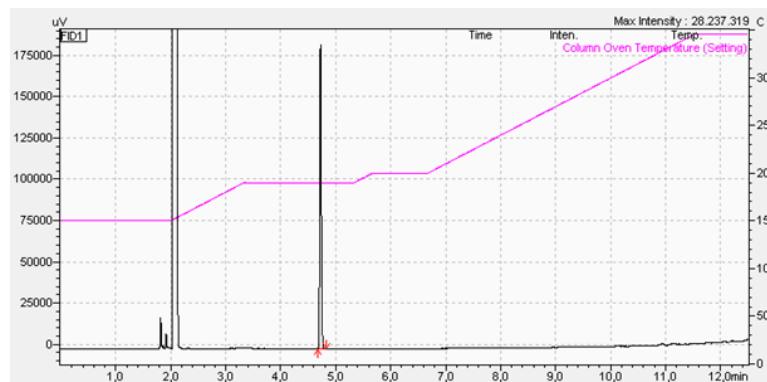
## E.2. $\alpha$ -methylstyrene & acetophenone identification



**Figure E.3:** Gaschromatography internal standard, dodecane, reference.



**Figure E.4:** Gaschromatography  $\alpha$ -methylstyrene reference.



**Figure E.5:** Gaschromatography acetophenone reference.

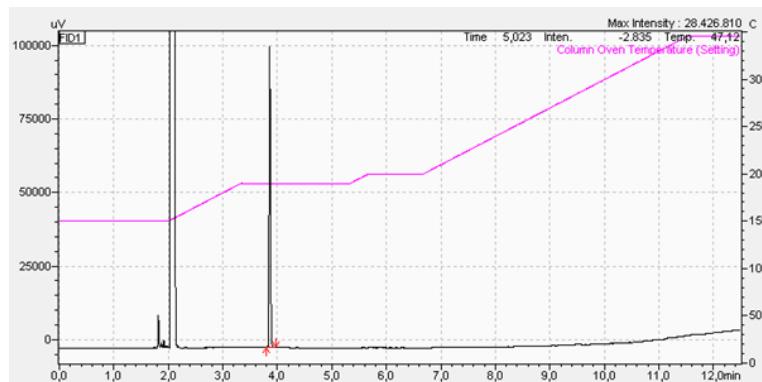


Figure E.6: Gaschromatography decane reference (TBHP).

### E.3. Background reaction chromatogram

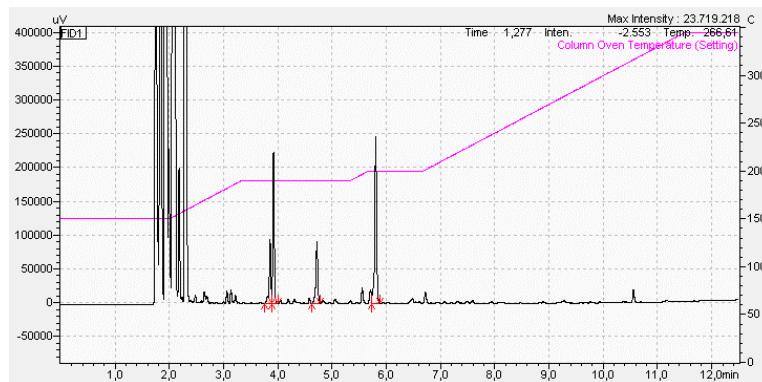


Figure E.7: Gaschromatogram of MnCl<sub>2</sub> background reaction.

### E.4. Conversion chromatogram example

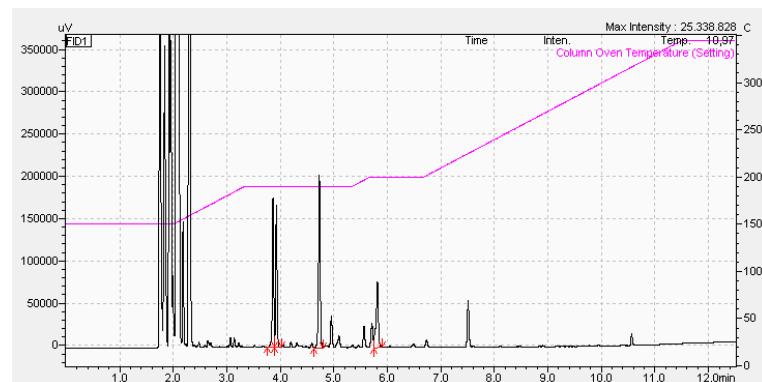
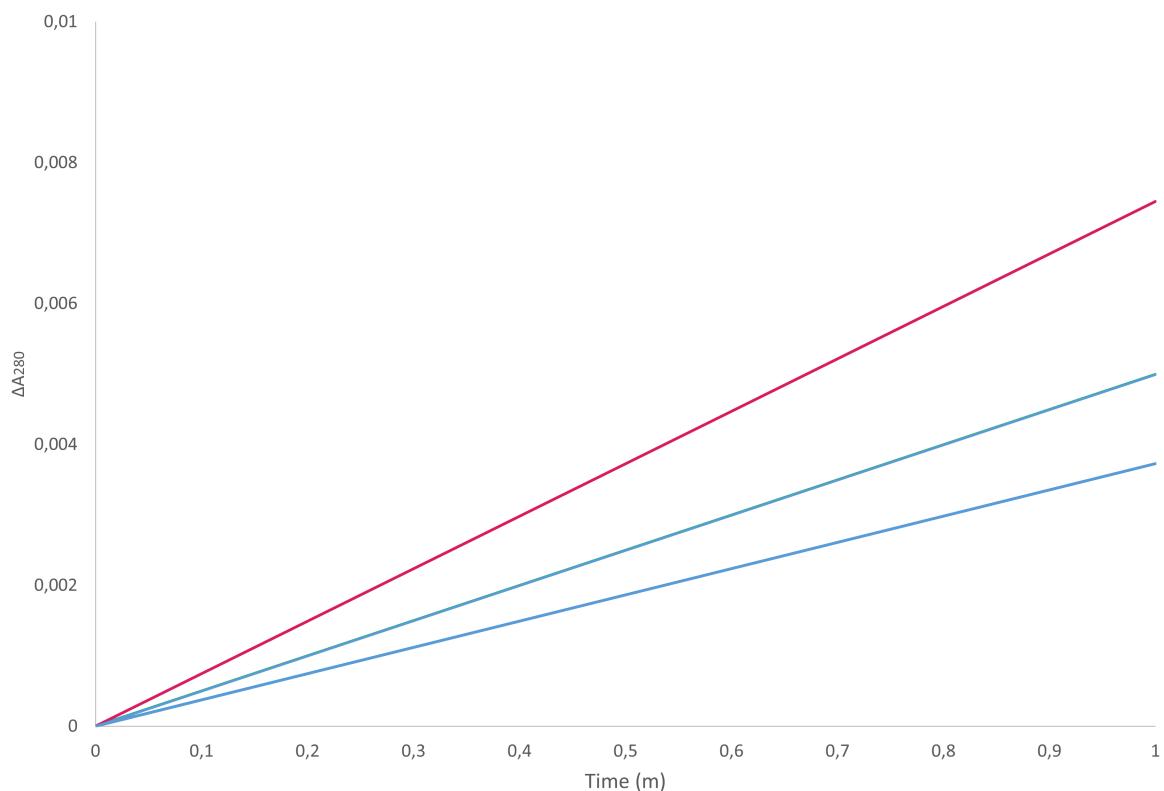


Figure E.8: Gaschromatogram of Tm1010 α-methylstyrene reaction after 20 h.

# F

## Hydroxynitrile lyase assay

### F.1. Background & reaction rate



**Figure F.1:** Normalised linear part of initial absorbance increase over time due to 30HT MnCl<sub>2</sub> supplemented GtINL mandelonitrile decomposition (red), mandelonitrile background decomposition with MnCl<sub>2</sub> (green), and in absence of MnCl<sub>2</sub> (blue).