

Biotransformations from and to methylated flavonoids

Subtitle

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Preface

1 Abstracts

1.1 English Abstract

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1.2 Deutsche Zusammenfassung

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Thesis

2 Introduction

Some introductory text

2.1 Natural products and secondary metabolites

2.1.1 General

2.1.2 Classes of natural products

Terpenoids and Steroids

... here is some text

Polyketides and non-ribosomal peptides

... here is some text

Alkaloids

... here is some text

Phenylpropanoids

... here is some text

2.2 Alkylating reactions in nature

2.2.1 Methylation

2.2.2 Prenylation

2.2.3 Glycosylation

2.3 Usage and expansion of nature's reaction toolbox

2.3.1 Terpene synthases and elongases

2.3.2 Methyl transferases

2.3.3 Glycosyl transferases

2.3.4 Other important enzymes in biotech research

BMVOs

Esterases/Lipases

Oxidases

Lyases

Transaminases

2.4 Conclusion

3 Material And Methods

3.1 Materials

3.1.1 Chemicals

Enzymes and buffers used for molecular cloning were obtained from Thermo Scientific (Darmstadt, Germany), unless otherwise noted. Flavonoid HPLC standards were purchased from Extrasynthese (Genay, France). Deuterated solvents were acquired from Deutero GmbH (Kastellaun, Germany). Solvents, purchased from VWR (Poole, England), were distilled in-house before use.

All other chemicals were obtained from either Sigma-Aldrich (Steinheim, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

3.1.2 Instruments

circular dichroism (CD)-spectrometer	Jasco J-815 (Eaton, USA)
electrophoresis (horizontal)	Biometra Compact XS/S (Göttingen, Germany)
electrophoresis (vertical)	Biometra Compact M (Göttingen, Germany) Biometra Minigel-Twin (Göttingen, Germany)
fast protein liquid chromatography (FPLC)	ÄKTA purifier (GE Healthcare, Freiburg, Germany)
gas chromatography coupled mass-spectrometry (GC/MS)	GC-MS-QP2010 Ultra (Shimadzu, Duisburg, Germany)
high-performance liquid chromatography (HPLC)	VWR-Hitachi LaChrom Elite (VWR, Darmstadt, Germany)
Isothermal Titration Calorimetry (ITC)	MicroCal iTC200 (Malvern, Worcestershire, UK)
micro-titer plate (MTP)-reader	SpectraMax M5 (Molecular Devices, Biberach, Germany)
nuclear magnetic resonance (NMR)-spectrometer	Varian Unity 400 (Agilent, Böblingen, Germany) Varian VNMRs 600 (Agilent, Böblingen, Germany)

photospectrometer	Eppendorf Biophotometer Plus (Hamburg, Germany) JASCO V-560 (Eaton, USA) Colibri Microvolume Spectrometer (Biozym, Hess. Oldendorf, Germany)
centrifuges	Eppendorf 5424 (Hamburg, Germany) Hettich Mikro 120 (Kirchlengern, Germany) Beckman Avanti J-E, Beckman Allegra X-30R (Krefeld, Germany)
centrifuge rotors	Beckman JA-10, JA-16.250, JS-4.3 (Krefeld, Germany)

3.1.3 Bacterial strains

E.coli

BL21(DE3)	$F^- ompT hsdSB(r_B^-, m_B^-) gal dcm \lambda(DE3)$ Invitrogen, Karlsruhe (Germany)
C41(DE3)	$F^- ompT hsdSB(r_B^-, m_B^-) gal dcm \lambda(DE3)$ Lucigen, Wisconsin (USA)
C43(DE3)	$F^- ompT hsdSB(r_B^-, m_B^-) gal dcm \lambda(DE3)$ Lucigen, Wisconsin (USA)
DH5 α	$F^- \Phi80lacZ\Delta M15 \Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(r_K^-, m_K^+) phoA supE44 \lambda^- thi-1 gyrA96 relA1$ Invitrogen, Karlsruhe (Germany)
JM110	$rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44 \Delta(lac-proAB) e14- [F' traD36 proAB^+ lacI^q lacZ\Delta M15] hsdR17(r_K^-, m_K^+)$ Martin-Luther-University Halle-Wittenberg
JW1593 (BW25113 derivative)	$rrnB \Delta lacZ4787 HsdR514 \Delta(araBAD)568 rph-1 \Delta ydgG$ (Kan ^R) Keio Collection, National Institute of Genetics (Japan)
MG1655	$F^- \lambda^- ilvG^- rfb-50 rph-1$ DSMZ, Hamburg (Germany)
One Shot TOP10	$F^- \Phi80lacZ\Delta M15 \Delta(mrr-hsdRMS-mcrBC) recA1 endA1 mcrA \Delta lacX74 araD139 \Delta(ara-leu)7697 galU galK rpsL$ (Str ^R) $\lambda^- nupG$ Invitrogen, Karlsruhe (Germany)
Origami(DE3)	$\Delta(ara-leu)7697 \Delta lacX74 \Delta phoA Pvull phoR araD139 ahpC galE galK rpsL F'[lac + lacI q pro] (DE3) gor522::Tn10 trxB$ (Kan ^R , Str ^R , Tet ^R) Novagen, Wisconsin (USA)

Rosetta(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm λ(DE3) pRARE (Cam^R)</i> Novagen, Wisconsin (USA)
Rosetta(DE3) pLysS	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm λ(DE3) pLysSRARE</i> (Cam ^R) Novagen, Wisconsin (USA)
T7 Express	<i>fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10-Tet^S)2 [dcm] R(zgb-210::Tn10-Tet^S) endA1 Δ(mcrC-mrr)114::IS10</i> NEB, Massachusetts (USA)

Agrobacterium tumefaciens

GV3101	chromosomal background: C58, marker gene: <i>rif</i> , tumor inducing plasmid (Ti-plasmid): cured, opine: nopaline Sylvestre Marillonet, IPB
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3.1.4 Plasmids

Table 3.3.: Plasmids used in this work.

name	description
pACYC Duet-1	..
pCDF Duet-1	..
pET20b(+)	...
pET28a(+)	..
pET32a(+)	..
pET41a(+)	..
pQE30	..
pUC19	..

3.1.5 Oligonucleotides

Oligonucleotides and primers were ordered from Eurofins Genomics (Ebersberg, Germany). The purity grade was *high purity salt free* (HPSF).

Table 3.4.: Primers used in this work. Recognition sites for endonucleases are underlined.

name	sequence (5'→3')	cloning site
somt1	TTGAAGACAAAATGGCTTCTTCATTAAACAATGGCCG	BpI
somt2	TTGAAGACAAGGACACCCCAAATACTGTGAGATCTTCC	BpI
somt3	TTGAAGACAAGTCCTTAGGAACACCTTTCTGGGAC	BpI
somt4	TTGAAGACAAAAGCTCAAGGATAGATCTCAATAAGAGAC	BpI

3.1.6 Software

All mathematical and statistical computations and graphics were done with the R software (versions 3.1.X, <http://cran.r-project.org/>). Visualizations of macromolecules were arranged using the PyMol Molecular Graphics System, version 1.7.0.0 (Schrödinger, New York (USA)).

Physicochemical calculations and calculations of different molecular descriptors were performed using Marvin Beans 15.4.13.0 (ChemAxon, Budapest (Hungary)) and Molecular Operating Environment 2008.10 (Chemical Computing Group, Montreal (Canada)).

3.2 Microbiology

3.3 Molecular Biology

Basic molecular biology methods like polymerase chain reaction (PCR), DNA restriction/ligation, DNA gel electrophoresis, preparation of competent cells and transformation were performed based on the protocols summarized by Sambrook and Russell [9].

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden (Germany)) according to the manufacturer's instructions.

In vitro site-directed mutagenesis was set-up according to the protocol of the *QuikChange Site-Directed Mutagenesis* kit [11] offered by Agilent Technologies (Santa Clara, USA).

Nucleotide fragments obtained by PCR, restriction/ligation procedures or excision from electrophoresis gels were purified and concentrated using the *Nucleospin Gel and PCR Clean-up* kit provided by Machery-Nagel (Düren, Germany) according to the instructions provided by the manufacturer.

3.3.1 Golden Gate Cloning

The Golden Gate cloning procedure is a one-pot method, meaning the restriction digestion and ligation are carried out in the same reaction vessel at the same time [5, 3]. Consequently PCR-fragments, destination vector, restriction endonuclease and ligase are added together in this reaction. The methodology employs type II restriction enzymes, which together with proper design of the fragments allow for a ligation product lacking the original restriction sites.

For digestion/ligation reactions of fragments containing BpI sites, 20 fmol of each fragment or vector, together with 5 U of BpI and 5 U of T4 ligase were combined in a total volume of 15 μ l 1 \times ligase buffer. For fragments to be cloned via BsaI sites, BpI in the above reaction was substituted by 5 U BsaI.

The reaction mixture was placed in a thermocycler and incubated at 37 °C for 2 min and 16 °C for 5 min. These two first steps were repeated 50 times over. Finally, the temperature was raised to 50 °C (5 min) and 80 °C (10 min) to inactivate the enzymes.

3.4 Protein biochemistry

Stock solutions of antibiotics, IPTG or sugars were generally prepared according to the pET System Manual by Novagen [8].

3.4.1 Protein production test (expression test)

The heterologous production of proteins in *E. coli* was assessed in a small scale protein production test, henceforth called expression test. Single colonies of *E. coli* transformed with the constructs to be studied were used to inoculate a 2 ml starter culture in lysogeny broth (LB)-medium containing the appropriate antibiotics. The concentrations of antibiotics used was as follows: 200 μ g ml⁻¹ ampicillin, 150 μ g ml⁻¹ kanamycin, 50 μ g ml⁻¹ chloramphenicol, 20 μ g ml⁻¹ tetracycline.

The starter culture was allowed to grow at 37 °C and 200 rpm over night. A 5 ml sampling culture of the medium to be studied containing the appropriate antibiotics was prepared. The media tested included LB, terrific broth (TB) and auto-induction media like ZYP-5052. The sampling culture was inoculated to an OD⁶⁰⁰ of 0.075 using the starter culture and incubated at different temperatures and 200 rpm in a shaking incubator. 1 mM isopropyl-D-thiogalactopyranosid (IPTG) was added when the OD⁶⁰⁰ reached 0.6-0.8, if appropriate for the studied construct. 1 ml samples were removed after different times of incubation (e.g. 4, 8, 12 hours), subfractionated (3.4.2) and analyzed via sodium dodecylsulfate (SDS)-polyacrylamid gel electrophoresis (PAGE) (3.4.5).

3.4.2 Protein subfractionation

The protein subfractionation procedure described herein was adapted from the protocol described in the pET Manual [8]. Overall 5 protein subfractions can be obtained, including *total cell protein*, *culture supernatant (medium) protein*, *periplasmic protein*, *soluble cytoplasmic protein* and *insoluble protein*.

The OD⁶⁰⁰ of the culture sample was measured and the cells harvested by centrifugation at $10000 \times g$, 4 °C for 5 minutes. The protein in the supernatant medium was concentrated by precipitation with trichloro acetic acid (TCA) (3.4.3) for SDS-PAGE analysis. The periplasmic protein was prepared (3.4.4) and also concentrated by TCA precipitation for SDS-PAGE. Cells were lysed by resuspending the cell pellet in $(OD^{600} \times V \times 50) \mu\text{l}$ of bacterial protein extraction reagent (B-PER) and vortexing vigorously for 30 s. The suspension was incubated at room temperature (RT) for 30 min to assure complete lysis. To separate insoluble protein and cell debris from the soluble cytosolic protein, the suspension was centrifuged at $10000 \times g$ and 4 °C for 10 min. Soluble cytoplasmic protein was contained in the supernatant, whereas insoluble protein remained in the pellet. For SDS-PAGE analysis of the insoluble protein, the pellet was resuspended in the same volume of B-PER. To obtain only the total cell protein fraction, the preparation of periplasmic and soluble cytosolic protein was omitted. Sample volumes of 10 μl of each fraction were used for SDS-PAGE analysis.

3.4.3 Protein sample concentration by TCA precipitation

Diluted protein samples were concentrated by TCA precipitation in microcentrifuge tubes. Therefore 0.1 volume (V) of 100 % (w/v) TCA in water was added to the clarified sample, which was then vortexed for 15 s and placed on ice for a minimum of 15 min. The sample was centrifuged at $14000 \times g$, 4 °C for 15 min. The supernatant was discarded and the pellet was washed twice with 0.2 V ice-cold acetone. The acetone was removed and the pellet set to air-dry in an open tube. After drying, the protein pellet was resuspended in 0.1 V phosphate buffered saline (PBS) containing $1 \times$ SDS-sample buffer by heating to 85 °C and vigorous vortexing, to achieve a $10 \times$ concentration. After resuspension the sample was analyzed by SDS-PAGE or stored at -20 °C until use.

3.4.4 Preparation of periplasmic protein

Target proteins may be directed to the periplasmic space by N-terminal signal sequences like *pelB* or *DsbA/C* [7]. The periplasma is, other than the cytosol, an oxidizing environment and often used for the production of proteins containing

dilsulfide linkages. The preparation of periplasmic protein was accomplished by an osmotic shock protocol modified from Current Protocols in Molecular Biology [1]. The cell pellet was resuspended in the same volume as the culture sample of 30 mM tris-HCl, 20 % (w/v) sucrose, pH 8 and 1 mM ethylenediaminetetraacetic acid (EDTA) was added. The suspension was stirred for 10 min at RT and the cells were collected by centrifugation at $10000 \times g$, 4 °C for 10 min. The supernatant was discarded and the cell pellet was resuspended in the same volume of ice-cold 5 mM MgSO_4 . The suspension was stirred for 10 min on ice, while the periplasmic proteins were released into the solution. The cells were collected by centrifugation as before. Periplasmic proteins were contained in the supernatant.

3.4.5 Discontinuous SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

3.4.6 Production of recombinant protein

Heterologous production of PFOMT

3.5 Analytics

3.5.1 In vitro determination of glucose

The glucose concentration in clarified, aqueous samples was determined by a modified version of the glucose assay kit procedure provided by Sigma-Aldrich [10]. Glucose oxidase (GOD) oxidizes D-glucose to gluconic acid, whereby hydrogen peroxide is produced. The hydrogen peroxide can be detected and quantified by horseradish peroxidase (HRP), which reduces the produced H_2O_2 and thereby oxidizes its chromogenic substrate *o*-dianisidine. The oxidized form of *o*-dianisidine can then be measured photospectrometrically [2].

The methodology employs a coupled photospectrometric assay using GOD and HRP with *o*-dianisidine as reporter substrate. The assay was prepared in MTP-format. A reaction solution containing 12.5 U/ml GOD, 2.5 U/ml HRP and 0.125 mg ml^{-1} *o*-dianisidine dihydrochloride in 50 mM sodium acetate pH 5.1 was prepared.

Sample solutions from culture supernatants were typically diluted in 9 volumes of water. The reaction was started, by adding 50 μl reaction solution to 25 μl of sample and was incubated at 37 °C and 200 rpm for 30 min in a shaking incubator. 50 μl 6 M sulfuric acid was added to stop the reaction and achieve maximum color development (full oxidation of any *o*-dianisidine charge transfer complexes).

The developed pink color was measured at 540 nm in a MTP-reader. A calibration

curve of a standard D-glucose solutions (0 to $100\text{ }\mu\text{g ml}^{-1}$), that was always part of the experiments, was used to quantify the sample measurements.

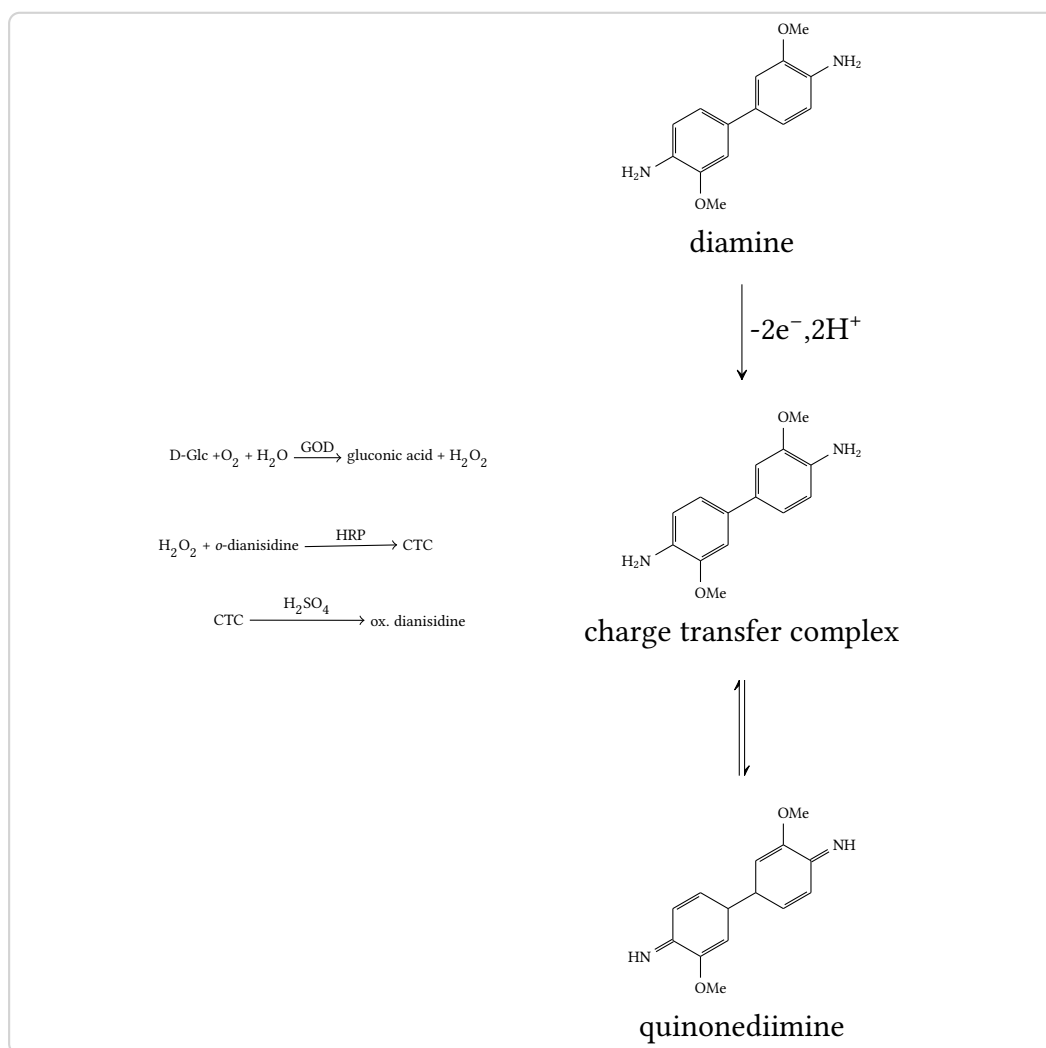


Figure 3.1.: GOD assay

3.5.2 HPLC measurements

4 Evaluation of PFOMT towards the acceptance of long-chain SAM analogues

4.1 Introduction

4.2 Substrate binding studies using ITC

4.3 Determination of the structure of *apo*-PFOMT

4.3.1 PFOMT activity in deep eutectic solvent (DES) / Solubility-enhancing effects of DES

vielleicht eigenes kapitel DES?

4.4 Study of variants for long-chain alkylations

4.4.1 PFOMT-Paper (DIM)

4.4.2 Dockings???

4.5 Colclusion/Discussion

5 Enzymatic methylation of Non-catechols

5.1 Introduction

Non-catechols in nature (biosynthesis, mode of action?), chemical methylation???

5.2 SOMT-2

5.2.1 In vivo methylation studies using *N. benthamiana*

5.2.2 In vivo studies in *E. coli*

5.2.3 In vitro studies using recombinantly produced SOMT-2

5.3 PFOMT

5.3.1 Acidity and Nucleophilicity of phenolic hydroxyl-groups

5.3.2 pH-Profiles of PFOMT-catalysis

5.3.3 Influence of Mg^{2+} on PFOMT activity

5.4 Consensus or Bioinformatic points-of-view (COMT)???

5.5 Conclusion/Discussion

6 Development of an whole cell methyl transferase screening system

6.1 Introduction

6.2 Theoretical considerations / design of system

6.3 Detectability of *S*-adenosyl-L-homocysteine (SAH)

S-adenosyl-L-methionine (SAM)

6.4 Usage of the *lsr*-promoter for true autoinduction

6.5 Conclusion/Discussion

7 DES in protein crystallography

7.1 Introduction

7.2 Solubility enhancement of hydrophobic substances by addition of DES

7.3 Enzymatic *O*-methylation in DES

7.4 DES as precipitants in protein crystallization

7.5 Conclusion/Discussion

8 Acknowledgements

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Appendix

A Figures

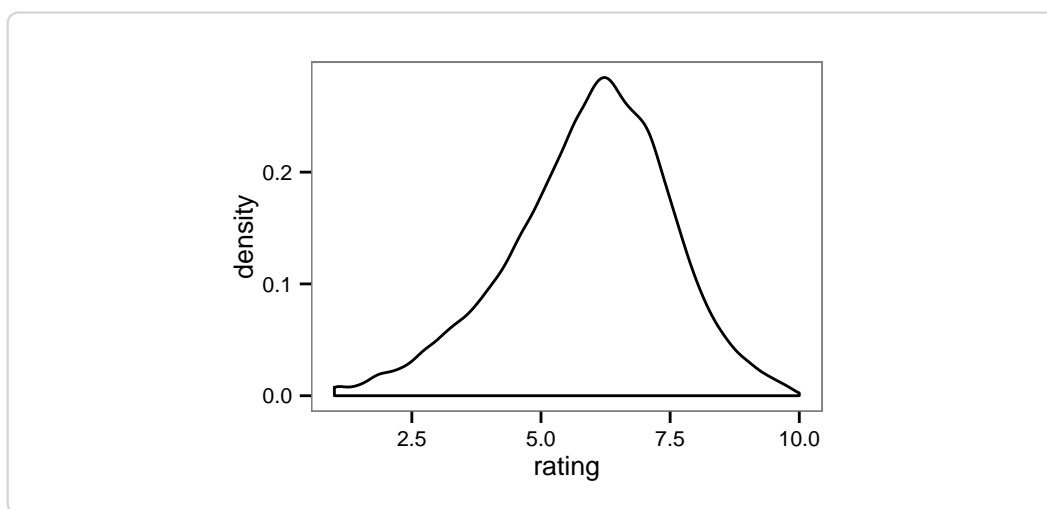


Figure A.1.: Lorem ipsum dolor sit amet, consectetur adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.

B Tables

Table B.1.: Lorem ipsum dolor sit amet, consectetur adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.

A	B	C	D	E	F	G	H	I
1	2	3	4	5	6	7	8	9
1	2	3	4	5	6	7	8	9
1	2	3	4	5	6	7	8	9

C Affidavit

I hereby declare that this document has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this document other than those indicated in the thesis itself.

Date:....., Location:....., Signature:.....

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Acronyms

B-PER	bacterial protein extraction reagent
CD	circular dichroism 9
EDTA	ethylenediaminetetraacetic acid 15
FPLC	fast protein liquid chromatography 9
GC/MS	gas chromatography coupled mass-spectrometry 9
GOD	glucose oxidase 15, 39
HPLC	high-performance liquid chromatography 9
HRP	horseradish peroxidase 15
IPTG	isopropyl-D-thiogalactopyranosid 13
ITC	Isothermal Titration Calorimetry 9, 39
LB	lysogeny broth 13
MTP	micro-titer plate 9, 15, 39
NMR	nuclear magnetic resonance 9
PAGE	polyacrylamid gel electrophoresis 13, 14
PBS	phosphate buffered saline 14
PCR	polymerase chain reaction 12, 13
RT	room temperature 14, 15
SAM	S-adenosyl-L-methionine 21
SDS	sodium dodecylsulfate 13, 14
TB	terrific broth 13
TCA	trichloro acetic acid 14
Ti-plasmid	tumor inducing plasmid 11, 39
V	volume

Glossary

GOD Glucose oxidase is an enzyme.... 37

Isothermal Titration Calorimetry (ITC) Fill in description here 37

MTP Micro-titer plate. Small format rectangular plastic plate containing wells to allow for storage of multiple small samples or the containment multiple simultaneous reactions. Typical sizes include 24, 96 and 384-wells 37

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence 37