Novel methods and tools for lactonases, acylases and proteases

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Content

Abbreviations

ADEPT	Antibody-directed enzyme prodrug therapy
AH	*N*-acyl-L-homoserine
AHL	*N*-acyl-L-homoserine lactone
AiiA	Quorum-quenching lactonase from *Bacillus* sp.
AidH	Quorum-quenching lactonase from *Ochrobactrum* sp.
E.C.	Enzyme Commission
GC	Gas chromatography
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography
HS	L-homoserine
HSL	L-homoserine lactone
MTP	Microtiter plate
NBM	*N*-benzoyl-L-methionine
pAcy1	Porcine aminoacylase I
PMPP	Protease-mediated protein purification
QS	Quorum sensing / quorum-sensing
QQ	Quorum quenching / quorum-quenching

Moreover, SI units and the usual codes for amino acids were used

Scope and Outline

This thesis is about the establishment and the application of novel methods and tools that are related to the most widely used enzyme class: hydrolases. It covers all fields from the identification to the application of these valuable enzymes with particular focus on lactonases, acylases and proteases. The activity assay introduced in **Article I** substantially extends the method toolbox for studies on lactonases and acylases that interfere with the bacterial cell-cell communication system. **Article II** describes a fully automatized robotic platform that represents the next-level tool for the high-throughput enzyme screening in the microtiter plate format. It was used, for instance, for the screening for improved porcine aminoacylase I variants. Diverse aspects of the protease-mediated hydrolysis of non-resistant proteins for the purification of resistant target proteins are highlighted in **Article III**.

Article I **Fast, continuous and high-throughput (bio)chemical activity assay for *N*-acyl-L-homoserine lactone quorum-quenching enzymes**

Last D.; Krüger G. H. E.; Dörr M.; Bornscheuer U. T., *Appl. Environm. Microbiol.* **2016**, *82* (14). doi:10.1128/AEM.00830-16

(Selected as spotlight article of this journal's issue)

The bacterial cell-cell communication system, termed quorum sensing (QS), is based on small molecules and controls several important processes such as biofilm formation and infection. Quorum-quenching (QQ) enzymes interfere with QS system by the conversion of QS-active to QS-inactive molecules. *N*-acyl-L-homoserine lactones (AHLs) are the most common QS molecules among Gram-negative proteobacteria. Despite the numerous prospective therapeutic and technical applications of AHL-converting QQ enzymes, only very few fundamentally different methods are reported for their activity measurements. Here, we describe a novel (bio)chemical activity assay that ideally complements the other methods in this field. In contrast to previous publications, our results indicate that porcine aminoacylase I (pAcy1) is not active towards AHLs, but towards their autohydrolysis products.

Article II **Fully automatized high-throughput enzyme library screening using a robotic platform**

Dörr, M.; Fibinger, M. P.; Last, D.; Schmidt, S.; Santos-Aberturas, J.; Böttcher, D.; Hummel, A.; Vickers, C.; Voss, M.; Bornscheuer, U. T., *Biotechnol. Bioeng.* **2016,** *113* (7), 1421-1432. doi:10.1002/bit.25925

(Selected as spotlight article of this journal's issue)

As soon as a protocol is established, the screening for new or improved enzymes is a routine, but time-intense and laborious task. In this article, the establishment of a robotic platform for the fully automatized high-throughput screening of enzyme libraries is presented. It was proven useful by four different projects. Besides other enzymes, a pAcy1 library was screened for variants with increased activity towards *N*-benzoyl-L-methionine (NBM). Although not mentioned in the publication itself for focus and simplification reasons, NBM is a simplified precursor of prodrugs that are tested for a novel cancer treatment strategy, the antibody-directed enzyme prodrug therapy (ADEPT). The results indicate that it is difficult to obtain pAcy1 variants that are active enough for ADEPT.

Article III **Highly efficient and easy protease-mediated protein purification**

Last, D.; Müller, J.; Dawood, A. W.; Moldenhauer, E. J.; Pavlidis, I. V.; Bornscheuer, U. T., *Appl. Microbiol. Biotechnol.* **2016,** *100* (4), 1945-1953. doi:10.1007/s00253-015-7206-9

The intended application of a protein determines to which degree it has to be purified from its natural or recombinant source. This downstream process is still one of the most cost-driving factors of the overall protein production, in particular for therapeutic proteins. In **Article III**, diverse aspects of the protease-mediated protein purification are addressed on the basis of five proteins including the QQ lactonase from *Bacillus* sp. (AiiA). It makes use of the known correlation between thermostability and protease resistance. Thus, non-resistant proteins are hydrolyzed by a low-specific protease while the resistant target protein remains unaffected. This study includes, for instance, a comparison with the traditional heat-mediated purification as well as the site-specific removal of an affinity tag by the low-specific trypsin.

Background

1. Hydrolases – Classifications and biological functions

Hydrolases are biocatalysts that use water molecules to cleave chemical bonds and are classified by the Enzyme Commission (E.C.) as group 3.[1] These enzymes are further divided into subclasses according to the chemical bonds they hydrolyze, for instance those acting on ester bonds (E.C. 3.1), glycosidic bonds (E.C. 3.2), peptide bonds (E.C. 3.4), carbon-nitrogen bonds other than peptide bonds (E.C. 3.5), acid anhydrides (E.C. 3.6) and carbon-halide bonds (E.C. 3.8).[2] Hydrolases mediate an enormous variety of biological functions including, but not limited to, i) the digestion of polymeric nutrients that range from natural ones such as proteins by proteases to the man-made poly(ethylene terephthalate) by two esterases[3], ii) the detoxification of epoxides by epoxide hydrolases[4] and haloalkanes by haloalkane dehalogenases[5], iii) the control of physiological processes such as the rapid termination of the acetylcholine-mediated neurotransmission by an esterase[6], iv) the establishment and the maintenance of infections by hydrolase virulence factors such as proteases secreted by the human pathogen *Pseudomonas aeruginosa*[7], v) the bacterial adaptive immune system that fights invading virus genes by a RNA-guided DNase[8] and vi) the release of progeny phages from host cells by murein hydrolases[9]. Due to this broad variety of activities, hydrolases constantly attract a very high level of interest in academia and industry.

2. Activity assays

For the identification, characterization, optimization and application of enzymes it is obligatory to determine their catalytic activities in a reliable manner. In this regard, analyses tend to be more accurate and less error-prone when product formation, but not substrate depletion, is monitored. This is due to the fact that the analyte is formed during the biocatalysis reaction in the first case, but is already present at a high background level in the second case.

Widespread analytical tools like gas chromatography (GC) and high-performance liquid chromatography (HPLC) that can be equipped with several detectors addressing diverse physicochemical properties, enable to follow an incredible variety of enzymatic reactions. Their scope is even further broadened by functional group derivatizations.[10] Nevertheless, GC and HPLC applications are limited by sample processing, the quite long analysis times per sample and the measurement of one sample after another, thus resulting in an overall low throughput of usually a few dozen samples per day.

For these reasons, methods that allow for direct sample readouts without any upstream compound separations via columns, are strongly preferred. GC and HPLC are often used only as independent methods to confirm the results obtained by other activity assays.

Generally, enzyme activity assays can be classified according to characteristics such as direct and indirect as well as continuous and discontinuous. Direct measurements are based on differences between the substrate(s) and the product(s) with regard to their physicochemical properties. This includes, in particular, the formation or depletion of a chromophore or fluorophore that can be monitored by photometers or fluorimeters. As most hydrolase reactions do not lead to such easily detectable changes, the substrate might be engineered accordingly. For instance, the hydrolysis of a non-fluorescent resorufin ester to the corresponding carboxylic acid and the fluorescent resorufin can be used for studies on esterases and lipases.[11] However, the results obtained with these modified substrates cannot always be transferred to the actually intended ones, as "you only get what you screen for".[12] That is why such surrogates are rather used as reference substrates and for screening purposes.

Indirect assays work either on the basis of a sensor/reporter, the derivatization of a reaction compound or an enzyme cascade. For instance, the fluorescence of the copper (II) calcein sensor increases in parallel to the enzymatically released α-amino acids.[13] Another example are the so-called pH shift assays that can be used if an acid is formed, which is in most cases a carboxylic acid obtained by ester or amide hydrolysis.[14] The minor modification of the acetylcholine ester to the acetylthiocholine ester enables the derivatization of the reaction product thiocholine with dithiobisnitrobenzoate, and thereby the formation of the yellow chromophore 5-thio-2-nitro-benzoic acid.[15] If none of these options is suitable for the intended reaction, it might be coupled with one or more other enzymes in a cascade until a detectable compound is released or consumed. In order to measure only the enzyme-catalyzed reaction of interest, the assisting enzymes have to be provided in excess. One example for this approach is the acetic acid assay that can be used for all hydrolase reactions that release acetic acid.[16] It comprises three assisting enzymes to finally monitor the formation of reduced nicotinamide adenine dinucleotide (NADH) by absorption measurement.

Continuous methods allow to run and monitor the reaction progress constantly until the desired information is obtained. In contrast to this, discontinuous methods require several, successively stopped samples to follow the reaction course. This approach is used when the analyte detection requires a derivatization or too strong changes of the reaction conditions that are incompatible with the enzyme-catalyzed reaction. This applies for instance to *ortho*-phthalaldehyde (OPA) that is often used to measure the amine formation during amide and peptide hydrolysis. It can only be used in an endpoint manner as it also reacts with the enzyme's amine functionalities.

Despite the high number of already established activity assays, new substrates and enzyme applications require the adaptation of existing methods or the development of new ones. In the following a new activity assay is described, compared to the commonly used methods in this field and classified according to the above mentioned characteristics.

2.1. Novel activity assay for *N*-acyl-L-homoserine lactone quorum-quenching enzymes (Article I)

A wide range of bacteria coordinate their protein expression patterns among local populations by quorum sensing (QS), a cell-cell communication system that is based on small molecules. Widespread processes such as infection and biofilm formation are controlled by QS. Consequently, mechanisms have evolved for the interference with the QS systems of other bacteria, termed quorum quenching (QQ), that are competing for the same habitat. QQ can be, for instance, mediated by enzymes that convert QS-active to QS-inactive molecules.[17] Besides their inherent environmental relevance, QQ enzymes have perspective therapeutic applications in humans, animals and plants as well as several technical applications like the reduction of biofilm formation on medical devices.

The most common QS molecules among Gram-negative proteobacteria such as *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* are *N*-acyl-L-homoserine lactones (AHLs). These compounds consist of the strictly conserved L-homoserine lactone and a variable acyl moiety, which is most often an unbranched fatty acid. Almost all AHL-converting enzymes are hydrolases and belong to the groups of QQ lactonases or QQ acylases.[17] Hence, they cleave the lactone or the amide bond of AHLs (all reactions are schematically shown in Fig. 1). Also abiotic factors like temperature and pH value have a strong influence on the lactone autohydrolysis rates of AHLs.[18]

When I started to work on AHL-converting QQ enzymes, I have realized that only a few fundamentally different methods with their respective strengths and weaknesses are available to measure their activities. These are either based on chromatography (HPLC, GC) or on biosensors.[19] The chemical structures of the substrates and the products of the enzyme catalysis do not enable direct activity measurements. There are also no obvious AHL-modifications that lead to signal formation upon conversion and, at the same time, are compatible with the enzyme specificities.

The crucial point of this project was to realize that AHLs strictly contain the α-amino acid L-homoserine (HS), which could be detected in its free form by the previously published calcein assay[13]. Hence, the reaction products of QQ lactonases (*N*-acyl-L-homoserines, AHs) and QQ acylases (HSL) have to be converted subsequently to HS. This was achieved by coupling of the pAcy1 to QQ lactonases or the exploitation of the quite fast autohydrolysis of HSL for QQ acylases as shown in Fig. 1.

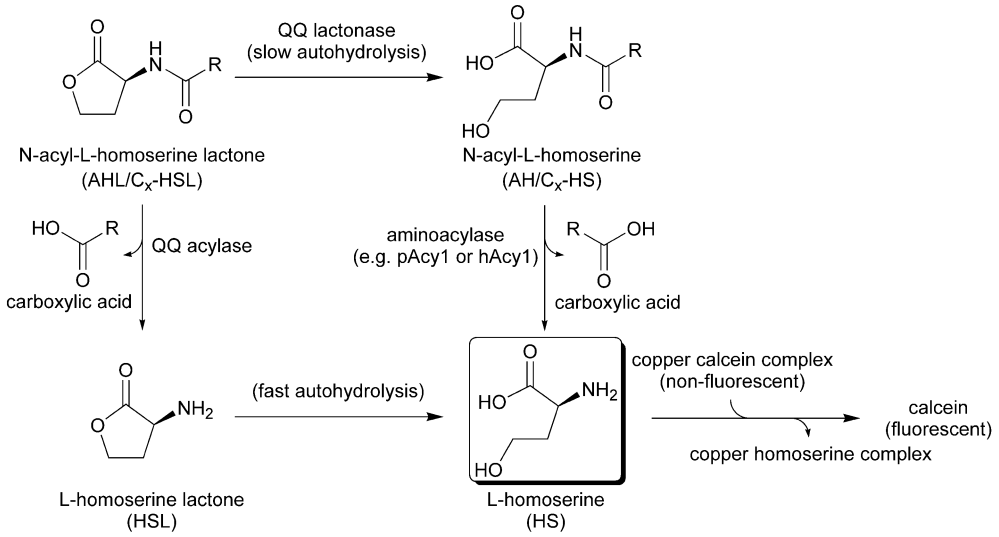

Fig. 1: Established activity assay for QQ lactonases and QQ acylases (identical with Fig. 1 in the related publication).

In order to show and prove the applicability of the established method, two examples were chosen. First, three previously undescribed QQ lactonases, which were identified by their homologies to the known QQ lactonase from *Ochrobactrum* sp. (AidH), were characterized. The importance of the amino acid position 160 for catalysis that was emphasized for AidH, could not be confirmed by the calcein assay for our three homologous enzymes. Second, *Escherichia coli* cells that express the QQ acylase QqaR were successfully identified in an artificial library, where they had been significantly underrepresented in a ratio of 1:125 colony forming units.

Our publication also indicates that pAcy1 does not convert AHLs, but their autohydrolysis products AHs. This contradicts four previous publications, which described pAcy1 to be active towards these QS molecules.[20] From our point of view, the authors of the first description[20a] did not consider the autohydrolysis of AHLs appropriately.[18] All four publications have in common that pAcy1 was used as crude, commercial preparation. The very low purity of such pAcy1 preparations is exemplarily shown in Fig. S4 of the **Article I**. Hence, the detected QQ activity might have been caused by other potential ingredients, for instance the mammalian paraoxonase 2 that is ubiquitously expressed.[21] Therefore, we would like to suggest to the responsible researchers, to repeat their activity measurements with recombinantly expressed and purified pAcy1, in order to provide indisputable results.

A comparison between the commonly used methods and the novel activity assay is provided in the following Tab. 1:

Tab. 1: Comparison of methods that can be used for studies on AHL-converting QQ enzymes. The color code indicates an advantage (green), disadvantage (red) or an intermediate level (yellow).

Aspect	Biosensor	HPLC / GC	Novel activity assay
Mode	Endpoint; analysis subsequent to biocatalysis	Endpoint; analysis subsequent to biocatalysis	Continuous; biocatalysis and analysis in parallel
Duration	Variable; e.g., overnight growth and color formation of microorganism	Depends on sample number and sample analysis time; e.g. 30 min for our GC method	Several minutes
High-throughput	Yes	No	Yes
Enzyme	Crude or purified	Crude or purified	Crude or purified
Flexibility	Biosensors most often accept only certain AHLs	All AHLs, but protocols have to be adapted	Unlimited for QQ acylases; high for QQ lactonases depending on the coupled aminoacylase
Spontaneity	Pre-culture has to be prepared on the day before	Depends on sample number and sample analysis time	Spontaneous measurements are possible
Quantification	Yes/no answer to semi-quantitative	Quantitative	So far established as semi-quantitative method
Equipment	Variable; from visible inspection to luminometer	HPLC or GC	Microtiter plate (MTP) fluorescence measurement device
Biosafety	Mainly S1, partially S2 (e.g., *Chromobacterium violaceum*)	Not applicable	Not applicable
Differentiation QQ lactonase / QQ acylase	Yes, acidification of QQ lactonase products leads to ring closure of AHs and biosensor signal formation	Yes, different retention times of the products	QQ lactonases only give signal after the addition of the assisting aminoacylase
Prize	Low amounts of AHLs	High amounts of AHLs are quite expensive; own synthesis cheap and easy	High amounts of AHLs are quite expensive; own synthesis cheap and easy

The finally established method can be classified as continuous, indirect, and involves a cascade with an additional enzymatic or an autohydrolysis step. All in all, this method significantly extends the scope of methods for the identification, characterization, optimization and application of QQ enzymes.

3. Optimization of nature's hydrolases to meet human's demands

During the evolution, enzymes were adapted to their natural environment and to their biological function. Besides other intrinsic properties, this applies in particular to their stability, activity and specificity. As the demands of analytical, industrial, therapeutic and other man-made applications usually differ in these regards, biocatalysts most often have to be optimized by *protein engineering*.[22] Basically, the opposing strategies of *rational design*[23] and *directed evolution*[24] with all degrees of combination[25] are commonly used for this purpose. Rational design is based on the detailed knowledge of the sequence, structure and mechanism of the particular or homologous enzymes. This facilitates the manual or computational identification of amino acid positions that have a significant influence on the property of interest. In turn, this enables the targeted generation of a few enzyme variants that can be studied in detail even with low-throughput methods such as GC and HPLC. In contrast to this, directed evolution can be applied without any in-depth understanding of the biocatalyst. It makes use of random mutagenesis for the generation of a gene library and subsequent testing of the encoded enzyme variants for the desired or a closely related property. For instance, one could screen for protease resistance when searching for a more thermostable enzyme variant.[26] Nowadays, several methods are available for the generation of huge mutant libraries.[27] Therefore, the reliable and high-throughput identification of the desired enzyme variants is often the bottleneck and determines the size of the library that is practically feasible to be screened.[28] In the following a brief overview of suitable techniques is presented along with a fully automated robotic enzyme screening platform that is described in **Article II**.

3.1. High-throughput selection and screening techniques

High-throughput techniques for the identification of new or improved enzyme variants require a stringent linkage between the genotype and the phenotype. In the case of selections, the enzymes and their encoding genes are usually entrapped by or associated with the same cell wall or capsid. In the course of a selection on solid or in liquid media only those clones (most often bacteria or bacteriophages) are further studied, that have a growth or replication advantage. For this, an artificial evolutionary pressure has to be established in such a way that the desired enzymatic activity mediates fitness, survival or replication. Hence, either the substrate or the product has to be of physiological relevance. For instance, the desired enzyme has to i) complement an auxotrophy of an essential metabolic pathway[29], ii) release a nutrient as the sole carbon or nitrogen source[30], iii) detoxify a growth inhibiting or lethal compound[5, 31], iv) maintain the infectivity or mediate capsid protein formation[26, 32]. All these methods enable a throughput of up to 10^{10} clones per day or even higher.[27b]

Screening methods are based on the spatial separation and individual measurement of each single clone. For instance, one can screen colonies on solid media with a throughput of about 10^5-10^6 clones per day, if the enzymatic activity can be converted to a detectable output.[27b] In particular, the formation or disappearance of color, fluorescence or turbidity are suitable in this regard.[28] Besides other enzymes, amylases, xylanases and cellulases[33] as well as lipases[34] were screened in this way. Another powerful technique is the fluorescence-activated cell sorting (FACS) with a throughput of about 10^6-10^9 clones per day.[27b, 35] Cells individually pass a fluorescence detector in a liquid stream and are separated into populations according to their fluorescence. Hence, this approach is limited to those enzymatic activities that can be converted to cell-associated fluorescence. This can be achieved, for instance, by a pH-sensitive variant of the green fluorescent protein (GFP) to detect hydrolase-induced pH shifts[36] or by dyes in the context of a live/dead-screening[37]. *In vitro* compartmentalization combined with *in vitro* transcription-translation systems further broadened the scope of high-throughput approaches and were, for instance, used for a cellulase screening.[38]

Despite all the versatile techniques briefly outlined above, screening in the MTP format is still the most frequently applied one although its throughput is limited to about 10^3 to 10^4 clones per day.[27b, 39] In contrast to the other methods, it allows for kinetic activity measurements and diverse measurement modes (absorbance, fluorescence, luminescence, etc.). Furthermore, it enables quite complex protocols that, for instance, involve an enzyme production and a target strain.[40] Prospectively, the trend towards "small, but smart libraries"[41] also favors this approach. Nevertheless, MTP-based screenings comprise a high number of simple, but most often manual steps. This obstacle was addressed in the following **Article II**.

3.2. A fully automated robotic platform for enzyme screening (Article II)

After the establishment of a protocol, the screening for new or improved enzymes in the MTP format is a routine, but time-intense and laborious procedure. It is usually performed manually or with partial robotic support. This particularly includes pipetting robots to add, mix and remove liquids in a fast and reliable manner. Nevertheless, enzyme screenings bind valuable capacities that are no more available for intellectually more challenging tasks. In **Article II**, a fully automated robotic platform is described that covers all screening steps from the enzyme production to the processing and visualization of the obtained biochemical data.

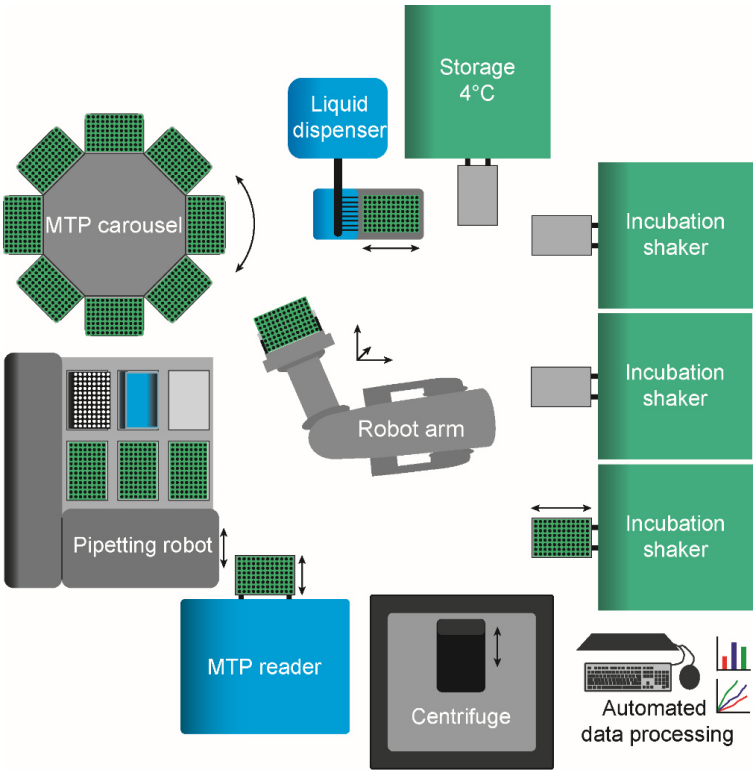

Fig. 2: Set-up of the robotic enzyme screening platform (identical with Fig. 1 in the related publication).

Compared to the (semi-)manual performance, not only the researchers are disburdened significantly, but the processes are also less error-prone and have a two- to threefold increased throughput. In contrast to robotic screening platforms in industry, which are typically designed to continuously perform the same process over and over again, our set-up is much more flexible. It can easily switch between or even interweave different screening protocols without any construction work.

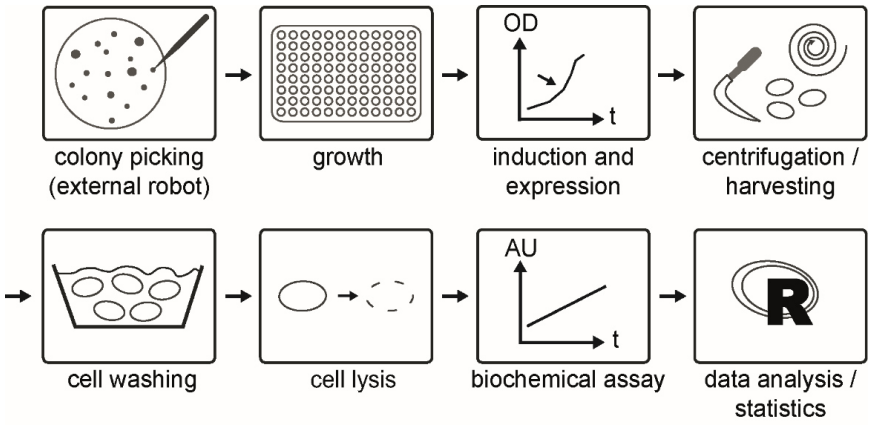

Fig. 3: Common process running on the robotic platform (identical with Fig. 5 in the related publication).

Besides other enzymes, the robot was tested for the screening for pAcy1 variants with improved activities. Although not mentioned in the publication for focus and simplification reasons, the initial intention was to evaluate the applicability of pAcy1 variants in the so-called antibody-directed enzyme prodrug therapy (ADEPT), an innovative cancer treatment strategy.[42] Here, in the first step, an antibody-enzyme conjugate is injected systemically, which accumulates at the tumor site due to the specific binding of the antibody to the cancer antigen.[43] In the following, a nontoxic prodrug is administered that is selectively converted to its toxic form by the tumor-localized enzyme. Compared to the conventional small-molecule anti-cancer drugs, this approach leads to less side effects. It also allows to use prodrugs, whose corresponding drugs have very short half-lives and are too toxic for systemic applications. In contrast to antibodies alone, these drugs can also kill adjacent antigen-free cancer cells and tumor growth supporting cells (bystander effect), and penetrate more deeply into the tumor tissue.

Most often bacterial enzymes without human counterparts were used for ADEPT studies to avoid non-selective prodrug activation. However, this resulted in immunogenicity issues that can be, for instance, circumvented by the elimination of immunogenic epitopes[44]. Alternatively, human or human-like enzymes can be engineered to convert prodrugs that are not accepted by the wild type enzyme.[45]

In this context, we focused on pAcy1 instead of the already tested human aminoacylase 1 (hAcy1),[46] because the pAcy1 wild type is already about 100-fold more active towards NBM.[47] Here, NBM was considered a suitable surrogate for prodrugs that were already used in combination with carboxypeptidase G2.[48] More precisely, our study was focused on the evolvability of the acyl-binding pocket of pAcy1 that is limiting the conversion of substrates with bulky acyl groups.[47] The pAcy1 library was already prepared and screened during my diploma thesis,[49] but now screened again with the help of the robotic platform. A few new variants were found, but these did not outcompete the ones identified during the diploma thesis. In the course of the PhD thesis, a HPLC protocol was also established to determine the specific activities of the wild type (0.13±0.0 U/mg), the basic library mutant T345G (1.57±019 U/mg) and one of the improved variants (T345G/L370I; 4.44±0.46 U/mg).

The specific activities of ADEPT enzymes towards their prodrugs are often much higher than the activity of pAcy1 towards the simplified prodrug precursor NBM.[43b, 50] Hence, it seems to be ambitious to obtain pAcy1 variants that are suitable for ADEPT, and therefore more promising to develop specific prodrugs for the pAcy1 variants.

4. Hydrolase applications

Hydrolases represent the most widely used enzyme class with a market share of 78 % in 2014.[51] Not only the high diversity of commercial applications but also their scales contribute to this remarkable value. In the following a brief overview of commercial hydrolase applications is given: Proteases, cellulases, lipases and amylases are added to laundry detergents to improve the washing performance by the enzymatic removal of the respective stains. In the food industry numerous hydrolytic enzymes are indispensable to "modify and improve the functional, nutritional and sensoric properties of ingredients and products".[52] For instance, proteases like chymosin mediate the coagulation of milk in the cheese production, pectinases are used to clarify fruit juices and lactases eliminate lactose from dairy products for those customers intolerant to lactose.[53] Moreover, the acrylamide formation during food processing, can be reduced by treatment with asparaginase.[52, 54] Phytases, fibre-degrading enzymes and other common additives in farm animal feed release otherwise inaccessible nutrients or break down digestion inhibiting compounds.[55]

In the production of fine, bulk and pharmaceutical chemicals, hydrolases are still of high value, "although hydrolase reactions are hardly novel or exciting chemistry".[56] This is due to their widespread availability, broad substrate specificity, high chemo-, regio- and stereoselectivity as well as independence from cofactors and their high stability.[1b] For instance, the lipase-based route for the production of pregabalin has a remarkably low E factor (ratio of the mass of waste per mass of product) of 12 compared to 86 for the chemical process, accompanied by a 80 % reduction in energy consumption.[57] In non- or low-aqueous media, hydrolases can even catalyze transfer or bond formation reactions.[1b, 58] At the same time, oxidoreductases like alcohol dehydrogenases[59] and transferases like transaminases[60] are of steadily increasing importance in the chemical and pharmaceutical industries.[61]

Also most therapeutic enzymes that are approved for the treatment of human diseases are hydrolases, certainly because of their cofactor independence. Besides other indications, these enzyme drugs address i) the depletion of amino acids that are essential for some forms of cancer, for instance asparagine by the asparaginase, ii) the intoxication with drugs, for instance methotrexate by the carboxypeptidase G2,[62] iii) the compensation of metabolic deficiencies, for instance glucocerebrosidase prevents the accumulation of glucocerebroside in Gaucher's disease[63] and iv) the blood coagulation, for instance the removal of life-threatening blood clots by tissue plasminogen activator[64]. Prospectively, also QQ and ADEPT enzymes will be approved for application to human diseases in the near future.

Mainly depending on the intended applications, enzymes have to be purified to certain degrees from their natural or recombinant sources. Obviously, therapeutic enzymes have to be of the highest purity possible as impurities and misfolded forms of these drugs cause immune responses and other severe side effects. In contrast to this, large scale technical applications usually require less or even no enzyme purification at all.

Therefore, new protein purification methods have to be, first and foremost, easy and inexpensive. In **Article III**, the protease-mediated protein purification is presented, which meets these two and some other beneficial criteria. Hence, this method further extends the already very broad scope of hydrolase applications.

4.1. Protease-mediated protein purification (Article III)

Proteases hydrolyze the peptide bonds of proteins in terms of processing, inactivation or digestion. The latter one is conducted by proteases with low specificities like trypsin and proteinase K. Both enzymes were also used to establish and assess the method of protease-mediated protein purification (PMPP) that is presented in **Article III**. The underlying idea of this approach is that non-resistant proteins are degraded by low-specific proteases while the protease-resistant target protein remains unaffected. Based on the correlation between thermostability and protease resistance,[65] five thermostable proteins were included in this study.

GFP was used as a proof of principle for this rather unconventional approach. Indeed, both proteases degraded most host proteins. While GFP remained unaffected by trypsin, it was terminally truncated by the even less specific proteinase K. Both, trypsin and proteinase K treatment did not affect the biological function (fluorescence) of GFP.

Subsequently, a commercial preparation of the *Candida antarctica* lipase A (CAL-A) was chosen to compare PMPP with the obviously competing thermal denaturation of protein impurities. In this case, PMPP was advantageous with regard to both purification factor and recovery. Hence, it is as a valuable alternative to the traditional heat-mediated protein purification. Most likely it has to be assessed case-by-case, which method works better.

The protease resistance of the (*R*)-specific transaminase from *Aspergillus fumigatus* (AspFum), which was initially susceptible to terminal truncation by trypsin, was improved by a rationally designed amino acid substitution. As expected, the critical position was located on the enzyme's surface and the substitution did not affect the catalytic activity. Hence, the application scope of PMPP can be extended to proteins that are not completely protease-resistant.

Inspired by these results, I reasoned that it should be possible to remove an affinity tag in a site-specific manner by a low-specific protease if the rest of the protein is resistant to it. Indeed, this was shown by trypsin treatment of an accordingly modified AiiA, a QQ lactonase that was already described to be protease-resistant.[66] Compared to the highly specific proteases that are commonly used for this purpose,[67] trypsin is much cheaper and more active. Additionally, misfolded AiiA was degraded as it exposes many cleavage sites in contrast to its correctly-folded form. As shortly outlined above, this aspect is of particular interest for therapeutic enzymes.

Finally, it was conceived that the proteolytic digestion of interfering impurities would be a straightforward approach for the application of cell extracts containing the protease-resistant target protein. This was exemplified by the activity assay of an alanine dehydrogenase (AlaDH), which was previously impaired by the host's enzymes. In this regard, it was sufficient to eliminate the background activity by proteolysis without the removal of the resulting protein fragments. Thereby, the amount of AlaDH that was required to exceed the limit of detection and the limit of quantification was significantly decreased.

All in all, this multi-facetted study shows that the purification of protease-resistant proteins by proteolysis of the non-resistant impurities is an easy and inexpensive approach. The efficiency of PMPP is comparable to other unspecific protein purification methods such as ion exchange chromatography.[68] Further, as a non-chromatographic method it is theoretically easily scalable. Hence, the only limitation of this method is that the target protein has to be protease-resistant. As many technical and therapeutic proteins are very stable, for instance with regard to high temperatures, PMPP holds a great potential. Hence, it is another promising application for hydrolases and more precisely for low-specific proteases.

5. Conclusion

Hydrolases represent the most widely used enzyme class.[51] As many new applications are still emerging for these valuable biocatalysts, including QQ, ADEPT and PMPP, they will prospectively retain their leading position also in the future. In this thesis, the identification, characterization, optimization and application of hydrolases are addressed with focus on lactonases, acylases and proteases. The novel methods and tools that were developed, established and applied in the course of this thesis, will lead to significant progresses in their respective fields.

An activity assay for AHL-converting QQ lactonases and QQ acylases was described in **Article I** that ideally complements the currently available methods. Its usefulness was proven by the characterization of three previously undescribed QQ lactonases and a screening for clones expressing a QQ acylase. Finally, our results indicate that pAcy1, the first mammalian enzyme described to be active towards AHL, is not active towards AHLs but their autohydrolysis products AHs.

The lessons we have learned in the establishment and application of a fully automatized robotic screening platform are presented in the **Article II**. These information will facilitate the establishment of fully or at least partially automated robot variants in other research groups or companies. It could also be shown that pAcy1 and its variants might not be active enough for ADEPT applications.

The method that is described in **Article III** enables to purify protease-resistant target proteins by hydrolysis of the non-resistant host proteins in an easy and inexpensive manner, which is most likely of interest for industrial applications. Thus, it will compete with the heat-mediated protein purification as well as the site-specific cleavage by highly-specific proteases.

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7. Author contributions

Article I **Fast, continuous and high-throughput (bio)chemical activity assay for *N*-acyl-L-homoserine lactone quorum-quenching enzymes**

Last D.; Krüger G. H. E.; Dörr M.; Bornscheuer U. T., *Appl. Environm. Microbiol.* **2016**, *82* (14). doi:10.1128/AEM.00830-16

DL initiated the project, designed the experiments and performed them with the help of GHEK and MD. DL wrote the manuscript. All authors read and edited the manuscript.

Article II **Fully automatized high-throughput enzyme library screening using a robotic platform**

Dörr, M.; Fibinger, M. P. C.; Last, D.; Schmidt, S.; Santos-Aberturas, J.; Böttcher, D.; Hummel, A.; Vickers, C.; Voss, M.; Bornscheuer, U. T., *Biotechnol. Bioeng.* **2016,** *113* (7), 1421-1432. doi:10.1002/bit.25925

UTB, MD, DB and AH initiated the project. MD, MPCF, DL, SS, JSA, CV and MV designed and performed the experiments. DL was mainly responsible for the studies on porcine aminoacylase 1. UTB and MD wrote, MPCF, DL, SS, JSA, CV read and edited the manuscript.

Article III **Highly efficient and easy protease-mediated protein purification**

Last, D.; Müller, J.; Dawood, A. W.; Moldenhauer, E. J.; Pavlidis, I. V.; Bornscheuer, U. T., *Appl. Microbiol. Biotechnol.* **2016,** *100* (4), 1945-1953. doi:10.1007/s00253-015-7206-9

DL initiated the project, designed the experiments and performed them with the help of JM, AWHD, EJM and IVP. DL wrote the manuscript. All authors read and edited the manuscript.

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Daniel Last Prof. Uwe. T. Bornscheuer

8. Articles

8.1. Article I

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Fast, Continuous, and High-Throughput (Bio)Chemical Activity Assay for *N*-Acyl-L-Homoserine Lactone Quorum-Quenching Enzymes

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ABSTRACT

Quorum sensing, the bacterial cell-cell communication by small molecules, controls important processes such as infection and biofilm formation. Therefore, it is a promising target with several therapeutic and technical applications besides its significant ecological relevance. Enzymes inactivating *N*-acyl-L-homoserine lactones, the most common class of communication molecules among Gram-negative proteobacteria, mainly belong to the groups of quorum-quenching lactonases or quorum-quenching acylases. However, identification, characterization, and optimization of these valuable biocatalysts are based on a very limited number of fundamentally different methods with their respective strengths and weaknesses. Here, a (bio)chemical activity assay is described, which perfectly complements the other methods in this field. It enables continuous and high-throughput activity measurements of purified and unpurified quorum-quenching enzymes within several minutes. For this, the reaction products released by quorum-quenching lactonases and quorum-quenching acylases are converted either by a secondary enzyme or by autohydrolysis to L-homoserine. In turn, L-homoserine is detected by the previously described calcein assay, which is sensitive to α-amino acids with free N and C termini. Besides its establishment, the method was applied to the characterization of three previously undescribed quorum-quenching lactonases and variants thereof and to the identification of quorum-quenching acylase-expressing *Escherichia coli* clones in an artificial library. Furthermore, this study indicates that porcine aminoacylase 1 is not active toward *N*-acyl-L-homoserine lactones as published previously but instead converts the autohydrolysis product *N*-acyl-L-homoserine.

IMPORTANCE

In this study, a novel method is presented for the identification, characterization, and optimization of quorum-quenching enzymes that are active toward *N*-acyl-L-homoserine lactones. These are the most common communication molecules among Gram-negative proteobacteria. The activity assay is a highly valuable complement to the available analytical tools in this field. It will facilitate studies on the environmental impact of quorum-quenching enzymes and contribute to the development of therapeutic and technical applications of this promising enzyme class.

The bacterial cell-cell communication by small molecules known as quorum sensing (QS) enables these single-celled organisms to coordinate their gene expression among a local population, depending on environmental conditions (1–4). Important processes such as infection and biofilm formation are controlled by QS. Hence, QS is a highly relevant target for naturally occurring and human-made interference termed quorum quenching (QQ), and it is mediated in particular by small-molecule inhibitors and QQ enzymes (5). From an ecological point of view, QQ has a significant influence on the interactions between bacteria (6, 7) and also bacteria and eukaryotes (8). Prospective therapeutic applications of QQ include the prevention and treatment of bacterial infections in humans, animals, and plants (5, 9–11). Technical applications are, for instance, the reduction of biofilm formation on medical devices and technical membranes and the reduction of food spoilage (12–14).

N-Acyl-L-homoserine lactones (AHL) are the most common type of communication molecules among Gram-negative proteobacteria (5). They consist of a strictly conserved L-homoserine lactone (HSL) moiety linked to a variable acyl side chain via an amide bond (all chemical structures relevant to this study and the overall scheme of the established activity assay are shown in Fig. 1). The acyl side chains are usually unbranched fatty acids, which mainly differ in length and substitution at the third carbon atom. Nevertheless, AHL with more unusual acyl side chains like branched-chain or unsaturated fatty acids and *p*-coumaric acid are also present in nature (15, 16).

AHL-converting enzymes mainly belong to the groups of QQ lactonases and QQ acylases besides the rarely occurring oxidoreductases (17). QQ lactonases hydrolyze the HSL ring of AHL with low acyl side chain specificity, thereby forming *N*-acyl-L-homoserine (AH) (Fig. 1) (18). QQ acylases preferentially act on the amide bond of AHL with medium to long acyl side chains, thereby releasing HSL and the respective carboxylic acid (Fig. 1). However, for the identification, characterization, and optimiza-

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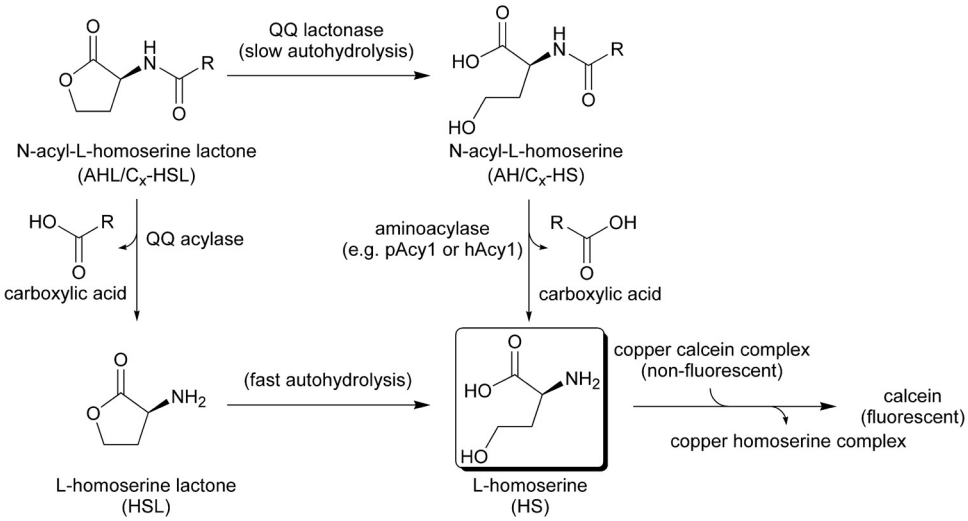

FIG 1 Reaction scheme of the established assay for the measurement of QQ lactonase and QQ acylase activities. The autohydrolysis rates of AHL and HSL strongly depend on the reaction conditions, in particular an increase in the pH or the temperature strongly accelerates the autohydrolysis (28, 29). Due to the stabilization effect of the variable acyl side chain, AHL are much more stable than HSL (28, 29).

tion of these valuable biocatalysts, only a very limited number of fundamentally different methods are described in the literature with their respective strengths and weaknesses. Unfortunately, the combination of AHL structure and specificity profiles of the QQ enzymes prevents any obvious chromogenic or fluorogenic AHL derivatization; this approach would otherwise enable easy monitoring of the reaction progress (19). Hence, QQ activity measurements have to be performed indirectly, preferentially by the detection of a reaction product or, alternatively, the remaining AHL.

Biosensors are by far the most widely used AHL detection systems, work in the physiological concentration range (low nanomolar to low micromolar in planktonic cell cultures), and are cost-effective and suitable for high-throughput screening (20). In turn, these biological systems often require several hours to form a detectable signal, are usually restricted to certain types of AHL, and monitor the remaining AHL in an endpoint manner. For instance, one of the most frequently used biosensors, *Chromobacterium violaceum* strain CV026 (biosafety level 2 organism), produces the pigment violacein upon exposure to AHL with short acyl side chains during overnight incubation (21). At least for some biosensors, including CV026, technical tricks have to be applied in order to obtain semiquantitative results instead of simple yes or no answers concerning the absence or presence of QQ enzymes (22). In addition to this, more laborious but application-oriented methods are available, for instance, the measurement of the AHL-dependent formation of pathogenicity factors by *Pseudomonas aeruginosa* (23–25).

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are much less frequently used than biosensor-based methods (15, 25, 26). Although providing quantitative data, both systems are working in the nonphysiological range (high micromolar to low millimolar) unless samples are concentrated or derivatized. Furthermore, they are restricted to endpoint measurements and low throughput.

Here, we present an alternative method for the fast and continuous high-throughput measurement of QQ enzyme activities, perfectly complementing the assays described above. Our method was verified for the characterization of three previously undescribed QQ lactonases as well as for a QQ acylase screening in an artificial library. Moreover, in contrast to previous publications, our results indicate that porcine aminoacylase 1 (pAcy1) is not active toward AHL but rather toward the corresponding autohydrolysis product AH.

MATERIALS AND METHODS

Consumables. All chemicals and the commercial pAcy1 preparation (grade II, 500 to 1,500 U/mg protein) were purchased from Sigma-Aldrich (Steinheim, Germany). The BC assay quantitation kit obtained from Interchim (Montlucon, France) was used to determine the protein contents; bovine serum albumin served as the standard.

Syntheses and heat-accelerated autohydrolyses of *N*-acyl-L-homoserine lactones. Syntheses of *N*-hexanoyl-L-homoserine lactone (C_6-HSL) and *N*-octanoyl-L-homoserine lactone (C_8-HSL) were performed according to the method of Hodgkinson et al. (27). Stock solutions of C_6-HSL and C_8-HSL (400 mM) were prepared in anhydrous dimethyl sulfoxide (DMSO) containing 0.2% glacial acetic acid and stored in aliquots at −20°C.

N-Hexanoyl-L-homoserine (C_6-HS) was obtained by heat-accelerated autohydrolysis of C_6-HSL (95°C, 500 rpm, 20 min) in sodium phosphate buffer (SPB) (50 mM, pH 7.5); C_6-HSL and related compounds were previously described to be sensitive to higher temperatures (28, 29). The conversion was monitored with a LaChrom HPLC (Merck, Darmstadt, Germany) at a wavelength of 205 nm. It was equipped with a LiChrospher 100 RP-18e column (5 μm, 250 mm by 4 mm; Merck). The solvent (74.75% MilliQ water, 25% acetonitrile, 0.25% glacial acetic acid) was used in an isocratic mode with a flow rate of 1 ml/min. Elution times were 3.4 min (C_6-HS) and 7.0 min (C_6-HSL).

Expression vectors. The expression plasmids for pAcy1 and for the QQ acylase QqaR originated from previous studies (23, 30). The synthetic and codon-optimized gene of hAcy1 (GenBank accession no. KU922759) was provided already cloned into pET28b(+) by GenScript (Piscataway, NJ, USA). The synthetic and codon-optimized genes *aiiM* (KU922758), *qqlB* (KU922760), *qqlG* (KU922761), and *qqlM* (KU922762) were ordered from Life Technologies (Regensburg, Germany) and cloned into pET28b(+). The complete coding DNA sequences were deposited in GenBank as indicated. All constructs were confirmed by sequencing (Eu-

TABLE 1 Enzymes relevant for this study

Enzyme	Activity	Natural origin	Source	Reference or source
pAcy1	Aminoacylase	*Sus scrofa*	Recombinant from *E. coli* or commercial	30
hAcy1	Aminoacylase	*Homo sapiens*	Recombinant from *E. coli*	This study
AiiM	QQ lactonase	*Microbacterium testaceum*	Recombinant from *E. coli*	38
AidH	QQ lactonase	*Ochrobactrum* sp. T63	Not practically used	40
QqlB	QQ lactonase	*Paraburkholderia glathei*	Recombinant from *E. coli*	This study
QqlG	QQ lactonase	*Geminicoccus roseus*	Recombinant from *E. coli*	This study
QqlM	QQ lactonase	*Mesorhizobium ciceri*	Recombinant from *E. coli*	This study
QqaR	QQ acylase	*Deinococcus radiodurans* R1	Recombinant from *E. coli*	23

rofins, Ebersberg, Germany). An overview of all of the enzymes relevant for this study is provided in Table 1.

Transformation, protein expression, cell lysis, and protein purification. Chemocompetent *E. coli* BL21(DE3) cells were transformed by the heat shock method (31). Cells were grown in lysogenic broth (LB) medium (10 g/liter tryptone, 10 g/liter sodium chloride, 5 g/liter yeast extract; supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin after autoclaving) at 37°C and 180 rpm until the optical density at 600 nm (OD_{600}) reached 0.6 ± 0.1. Then protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG); cultivation was continued at 20°C and 180 rpm for 18 h. The aminoacylase expression cultures were additionally supplemented with chloramphenicol (25 μg/ml) and L-arabinose (2 mg/ml) for chaperone coexpression (30). Cell cultures were centrifuged (15 min, 4,000 × *g*, 4°C), washed with washing/lysis buffer (pAcy1, hAcy1, and AiiM: 100 mM Tris-HCl, 150 mM NaCl [pH 8.0]; all other enzymes: SPB, 250 mM NaCl [pH 7.25]), centrifuged again, and stored as cell pellets at −20°C. These were resuspended in washing/lysis buffer followed by two French press passages (each 1,500× psi) and cell debris removal (45 min, 10,000 × *g*, 4°C). Proteins were purified with gravity flow columns of Strep-Tactin Sepharose (pAcy1, hAcy1, and AiiM; IBA, Göttingen, Germany) or Co-IDA resin (all other enzymes; Carl-Roth, Karlsruhe, Germany). Elution was mediated by the respective washing/lysis buffer additionally containing 2.5 mM desthiobiotin (pAcy1, hAcy1, and AiiM) or 300 mM imidazole (all other enzymes). Imidazole was removed from His tag elution fractions by dialysis overnight (12,000 molecular weight cutoff [MWCO]; >1,000-fold dilution) against SPB.

Purification of pAcy1 from the crude commercial preparation was achieved by size exclusion chromatography with a Superdex 75 column (150 ml) on an ÄKTApurifier (GE Healthcare Europe, Freiburg, Germany), which was run with SPB as the buffer solution.

The crude *E. coli* cell lysates as used for the CV026 assay, for instance, were prepared from the respective cultivations as follows. The number of harvested cells per aliquot was normalized by the formula 5 absorption units per OD_{600} in milliliters. Cells were centrifuged (15 min, 4,000 × *g*, 4°C), washed with SPB, centrifuged again, and stored at −20°C. The cell pellets were resuspended in 500 μl SPB and sonicated on ice (Bandelin Sonopuls HD 2070; 1 min, 50% pulse, 50% power), followed by cell debris removal by centrifugation (5 min, 17,000 × *g*, 4°C).

Biocatalyses and GC-MS analyses. Biocatalysis reactions (0.75 ml in SPB) contained 2 mM C_6-HSL or C_6-HS as the substrate and 100 μg/ml of the specified aminoacylase. AiiM was added in purified form (10 μg/ml) or as a crude cell lysate (15 μl) if applicable. Reactions were carried out at 37°C and 750 rpm for the indicated periods of time and stopped by the addition of 50 μl concentrated hydrochloric acid. Each sample was extracted two times with 0.3 ml hexane containing 5 mM external standard methyl benzoate. The combined organic phases were dried with anhydrous sodium sulfate. For the derivatization of the reaction product hexanoic acid, 10 μl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide was added to 90 μl of the organic phase followed by incubation at 60°C and 500 rpm for 20 min. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu GC2010 coupled to a QP2010S MS device) equipped with a BPX-5 column (25 m, 0.25 mm), and the following program parameters: injector, 250°C; pressure, 38.3 kPa; column flow rate, 0.93 ml/min; MS ion source temperature, 200°C; MS interface temperature, 220°C; and column oven temperature profile, 0 min at 60°C, 5 min at 60°C, 15 min at 220°C, and at 20 min at 220°C.

Calcein assay. The calcein assay was performed according to the method of Dean et al. (32). Briefly, 5- to 20-μl samples were filled up to 200 μl with preincubated substrate/sensor solution [SPB, 1 to 2 mM substrate, 1 μM calcein, 5 μM copper(II) chloride]. For the fluorescence measurements (excitation, 450 nm; emission, 530 nm; incubation temperature, 37°C), an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland) was used.

Biosensor assay with *Chromobacterium violaceum* strain 026. In order to gain more information than a simple yes or no answer concerning the absence or presence of QQ enzyme activity, the CV026 assay was performed in a modified and technically easier way than described elsewhere (21, 22). For this, samples were mixed in equal amounts with serial C_6-HSL dilutions (0 to 800 μM in LB medium) in the wells of a microtiter plate. Following the biocatalysis reactions (4 h, 750 rpm, 37°C), 20 μl of each sample was transferred to a new microtiter plate filled with 80 μl of a CV026 culture per well (1:50 dilution of an overnight culture in LB medium; stored at 4°C until use). After overnight incubation at 750 rpm and 30°C, the violacein color formation was determined.

Screening for the QqaR expression strain in an artificial library. The challenge of this experiment was to identify the *E. coli* expression strain of the QQ acylase QqaR in an artificial library with an excess of negative-control clones (visualized in Fig. S1 in the supplemental material). The *E. coli* expression strain of the QQ lactonase AiiM served as a negative control as it possess the same plasmid backbone and therefore the same antibiotic resistance and burden after the induction by IPTG as the QqaR expression strain. In detail, separated 4-h-old cultures of the uninduced QqaR and AiiM expression strains were separately diluted with LB medium to an OD_{600} of 0.5, mixed in a volumetric ratio of 3:93 (aiming for a high excess of the negative control over the positive control), and plated on agar plates. On control agar plates prepared from nonmixed cultures, the AiiM expression strain showed about 4 times higher CFU than the QqaR expression strain. Hence, the overall probability to find the QqaR expression strain within the library was estimated to be about 1:125. Clones were transferred with a 96-pin head microbial colony picker QPix 420 (Molecular Devices, Sunnyvale, CA, USA) from agar plates into the wells of microtiter plates (MTPs). One positive (QqaR expression strain) and one negative control (AiiM expression strain) were positioned manually in the middle of each MTP. The resulting master MTPs (150 μl LB medium per well) were incubated for 14 h at 37°C and 700 rpm, supplemented with glycerol (90%, 50 μl per well) and stored at −80°C. Preexpression MTPs (200 μl LB medium per well) were inoculated and incubated for 5 h at 37°C and 700 rpm. The subsequently inoculated expression MTPs filled with 200 μl of Zyp-5052 autoinduction medium per well (33) were loaded onto an automated robotic platform (34), which carried out all of the following screening steps. The expression MTPs were incubated for 6 h at 37°C and 700 rpm, followed by another 16 h at 20°C and 700 rpm. Cells were centrifuged (20 min, 5,000 × *g*, 4°C), washed with

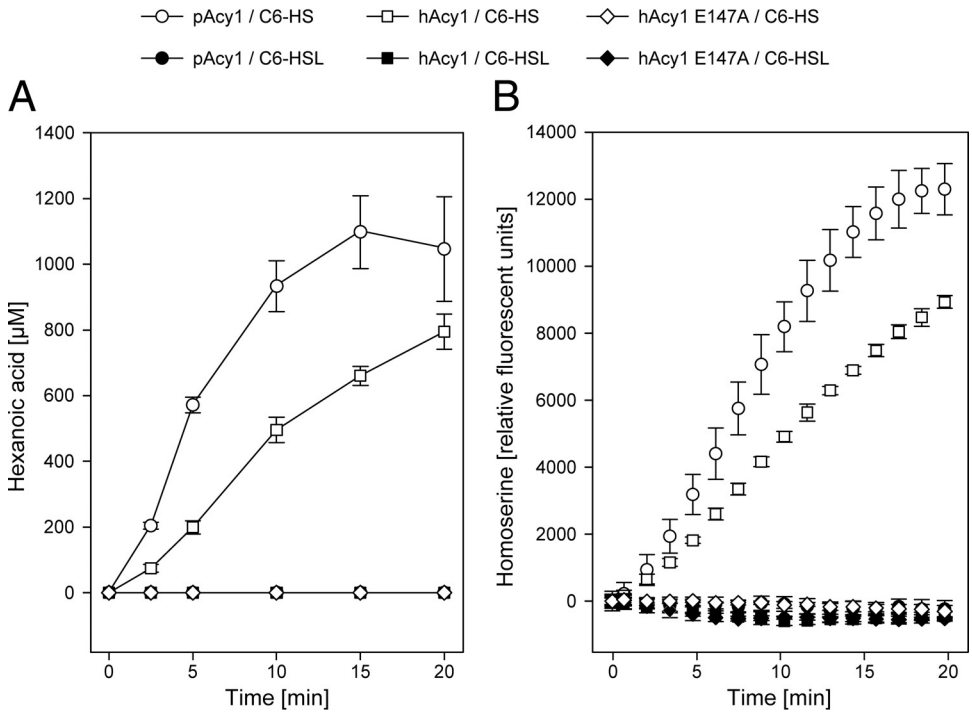

FIG 2 Investigations on the conversion of C_6-HS and C_6-HSL (each 2 mM) by aminoacylases (each 100 μg/ml). (A) Quantification of hexanoic acid formation by GC-MS analysis. The three measurements involving C_6-HSL are hidden by the curve of hAcy1 E147A/C_6-HS as no hexanoic acid formation was found for all of them. (B) Continuous monitoring of HS formation by the calcein assay normalized to the respective starting fluorescence values. The error bars indicate the standard deviations ($n = 3$).

SPB, and centrifuged for a second time. Cell lysis was mediated by resuspension in a 100-fold dilution of BugBuster 10× protein extraction reagent (Merck, Darmstadt, Germany) in SPB and incubation for 2 h at 30°C and 700 rpm, followed by a final centrifugation step. The calcein assay was performed as described above with C_8-HSL (1 mM) as the substrate but using a Varioskan microplate reader (Thermo Scientific, Waltham, MA, USA). It was assumed that the signal formation by clones expressing QqaR would take too long for individual and continuous activity measurements of one MTP after another. Therefore, all six MTPs were screened in parallel with a single point fluorescence measurement after 4 h of biocatalysis at 37°C and 700 rpm.

Nucleotide sequence accession numbers. The complete coding DNA sequences for the synthetic and codon-optimized genes were deposited in GenBank under accession no. KU922758 to KU922762.

RESULTS

Overall concept of the activity assay for quorum-quenching enzymes. The method presented here is based on L-homoserine (HS), which is present in the strictly conserved HSL moiety of each AHL (Fig. 1). The respective biocatalysis products of QQ lactonases and QQ acylases are converted either by a secondary enzyme or by autohydrolysis to HS. This compound is finally detected with the previously described calcein assay, which is sensitive to α-amino acids with free N and C termini (32).

At first, the sensitivity of the calcein assay toward HS was determined by a standard curve mimicking a biocatalysis reaction with *N*-hexanoyl-L-homoserine lactone (C_6-HSL) as the model AHL. Thus, the concentrations of the final cascade reaction products HS and hexanoic acid were increased, while the concentration of C_6-HSL was decreased proportionally. Under the conditions tested, the calcein assay was sensitive in the micromolar range and linear up to 2 mM HS (see Fig. S2 in the supplemental material). At this point, it should be mentioned that exact quantifications based on relative fluorescence units should be handled with care due to the strong impact of the sample composition and reaction conditions and the inner-filter effects and photo bleaching. Here, the calcein assay was established and used as a semiquantitative method only. For this reason, the data obtained were not converted to concentrations or activity units.

Cascade reaction to monitor quorum-quenching lactonase activity. The conversion of AHL to AH by QQ lactonases does not lead to signal formation in the calcein assay due to the blocked N terminus of HS (Fig. 1). Therefore, a cascade reaction was conceived, additionally involving an aminoacylase that cleaves the amide bond of AH but not that of AHL. Thereby, the detectable HS and the respective carboxylic acid are released.

For this purpose, the activities of recombinant porcine aminoacylase 1 (pAcy1) and recombinant human aminoacylase 1 (hAcy1) toward the model AH C_6-HS were tested. This AH was anticipated to be at least an acceptable substrate for both aminoacylases with regard to its amino acid and acyl moieties (35, 36) and was obtained by heat-accelerated autohydrolysis of C_6-HSL (see Fig. S3 in the supplemental material). On the other hand, no detectable conversion of C_6-HSL was expected due to its unusual amino acid structure. The inactive hAcy1 variant E147A served as a negative control (37).

Indeed, pAcy1 and hAcy1 selectively converted C_6-HS but not C_6-HSL, which was determined by GC-MS analysis of hexanoic acid formation (Fig. 2A). The results for the calcein assay were similar to those for the GC-MS analysis (Fig. 2B).

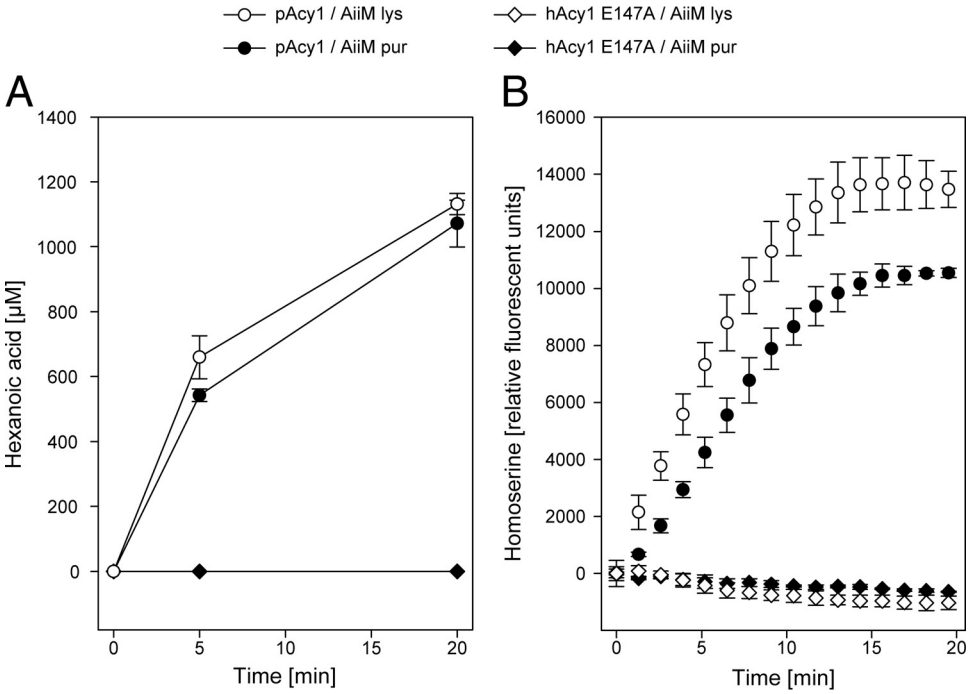

FIG 3 Cascade reactions involving AiiM (as purified enzyme or as crude cell lysate) combined with the most active aminoacylase pAcy1 or the negative-control hAcy1 E147A (each 100 μg/ml) with C_6-HSL (2 mM) as the substrate. (A) Quantification of hexanoic acid formation by GC-MS analysis. (B) Continuous monitoring of HS formation by the calcein assay normalized to the respective starting fluorescence values. The error bars indicate the standard deviations ($n = 3$).

In order to perform the cascade reaction with the physiologically relevant substrate C_6-HSL, the most active aminoacylase, pAcy1, was combined with the model QQ lactonase AiiM (9, 38). Both forms of AiiM, purified and crude cell lysate, led to the expected increase in calcein fluorescence within a few minutes (Fig. 3). Here, the secondary enzyme pAcy1 was the limiting factor of the two-step cascade reaction as 1:20 dilutions of purified AiiM gave similar results in the calcein assay (data not shown). All of these results show that the new method enables fast activity measurements of purified and unpurified QQ lactonases when combined with a suitable aminoacylase.

Finally, a commercial and inexpensive pAcy1 preparation derived from porcine kidney was tested as a potential substitute for the recombinant and purified one. However, also without the addition of the substrate, a strong increase in fluorescence was observed. This background activity was removed completely by size exclusion chromatography (see Fig. S4 to S6 in the supplemental material) but only partially by dialysis (12,000 MWCO; data not shown). The chromatographically purified pAcy1 was used for the subsequent experiment.

Application to the characterization of three previously undescribed quorum-quenching lactonases. AidH is one of the three known QQ lactonases with an α/β-hydrolase fold (17, 39, 40). Based on its amino acid sequence (GenBank accession no. GQ849010) and active-site residues (39), three homologous open reading frames were identified by a NCBI BLAST search. These belong to the two soil- and plant-associated bacteria, *Paraburkholderia glathei* (QqlB) (KDR41687.1; 49% amino acid identity; annotated as epoxide hydrolase) and *Mesorhizobium ciceri* (QqlM) (WP_013530214.1; 68% amino acid identity; annotated as α/β-hydrolase), and to the salt water-associated bacterium *Geminicoccus roseus* (QqlG) (WP_027134646.1; 66% amino acid identity; annotated as α/β-hydrolase) (41–45). Recombinantly expressed QqlB, QqlG, and QqlM showed QQ activity in the modified CV026 assay with crude cell lysates (see Fig. S7 and S8 in the supplemental material).

All 13 active-site residues described for AidH were also found in QqlG and QqlM. QqlB was selected as the third enzyme as it differed in 7 of these 13 positions. From biochemical and structural studies on AidH, Gao et al. (39) concluded that Y160 plays a key role in biocatalysis by hydrogen bond formation via its hydroxyl group. However, QqlB possesses a phenylalanine at the homologous position and therefore no such hydroxyl group. In order to investigate the importance of the position and especially of the hydroxyl group, all variants of QqlB, QqlG, and QqlM with tyrosine and phenylalanine at the homologous positions (QqlB, QqlB F160Y, QqlG, QqlG Y160F, QqlM, QqlM Y160F) were generated, recombinantly expressed (see Fig. S7 in the supplemental material), purified via His tag (see Fig. S9), and dialyzed. In order to unequivocally measure the activities of the QQ lactonases, the secondary enzyme of this cascade reaction, pAcy1, was necessarily provided in excess; dilutions of the QQ lactonases showed lower slopes but the same tendencies (data not shown). The calcein assay revealed no differences between the respective phenylalanine and the tyrosine variants of QqlG and QqlM (Fig. 4). QqlB F160Y was even less active than the natural phenylalanine variant. It has to be mentioned that both QqlB variants were less well expressed (see Fig. S7) and less stable than those of QqlG and QqlM. QqlB and QqlB F160Y partially formed precipitates during dialysis, which were removed by centrifugation.

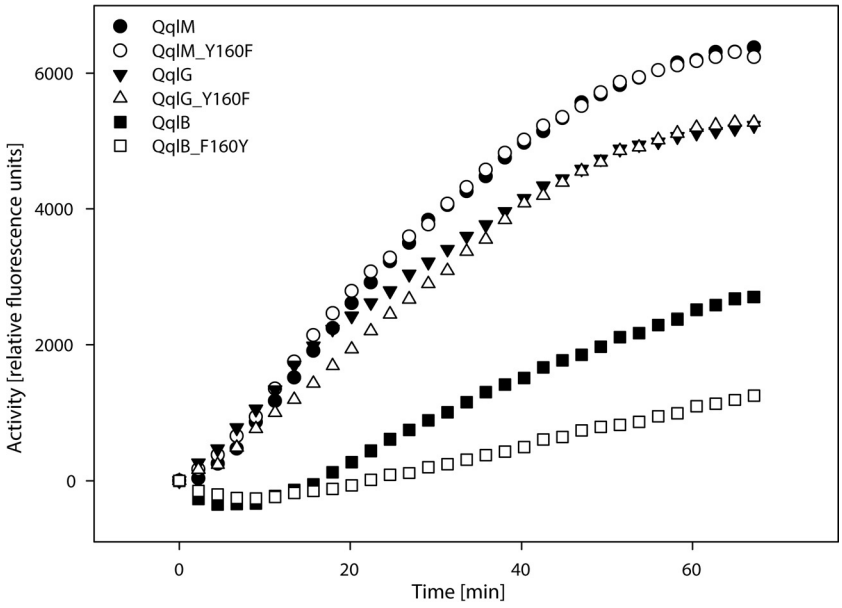

FIG 4 Calcein assay for the comparison of phenyl and tyrosine variants of QqlB, QqlG, and QqlM at the homologous position of AidH Y160 (each 30 μg/ml) normalized to the respective starting fluorescence values. C_6-HSL (2 mM) served as the substrate. Standard deviations (average: 3%; individual maximum: 8%) are not indicated for a better clarity.

Cascade reaction to monitor quorum-quenching acylase activity. In order to monitor QQ acylase activity with the calcein assay, the biocatalysis product HSL has to be converted to HS in a second step (Fig. 1). For this purpose, one might simply exploit the autohydrolysis of the HSL ring, which increases disproportionally with decreasing lengths of the acyl side chains of AHL (29). Thus, HSL should be much more susceptible to autohydrolysis than AHL as it lacks such a stabilization effect. In particular, AHL with medium to long acyl side chains would be suitable as these are also strongly preferred by QQ acylases (23, 25, 26). *N*-Octanoyl L-homoserine lactone (C_8-HSL) served as the model AHL, and QqaR was used as the model QQ acylase (23).

Indeed, the calcein assay showed an increase in fluorescence, depending on the concentration of QqaR (Fig. 5). It is also obvious that imidazole, derived from the elution buffer of the His tag pu-

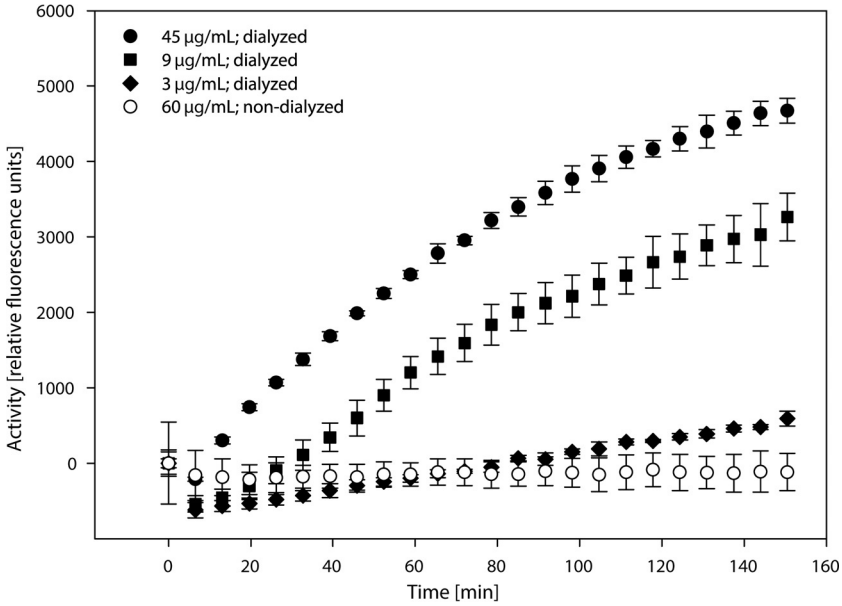

FIG 5 Continuous calcein assay monitoring the activities of the indicated concentrations of purified and dialyzed QQ acylase QqaR normalized to the respective starting fluorescence values. Nondialyzed QqaR directly obtained from His tag purification was also tested, showing no increase in fluorescence despite a high enzyme concentration. This indicates a strong quenching (so-called inner filter) effect by imidazole. C_8-HSL (1 mM) served as the substrate. The error bars indicate the standard deviations ($n = 3$).

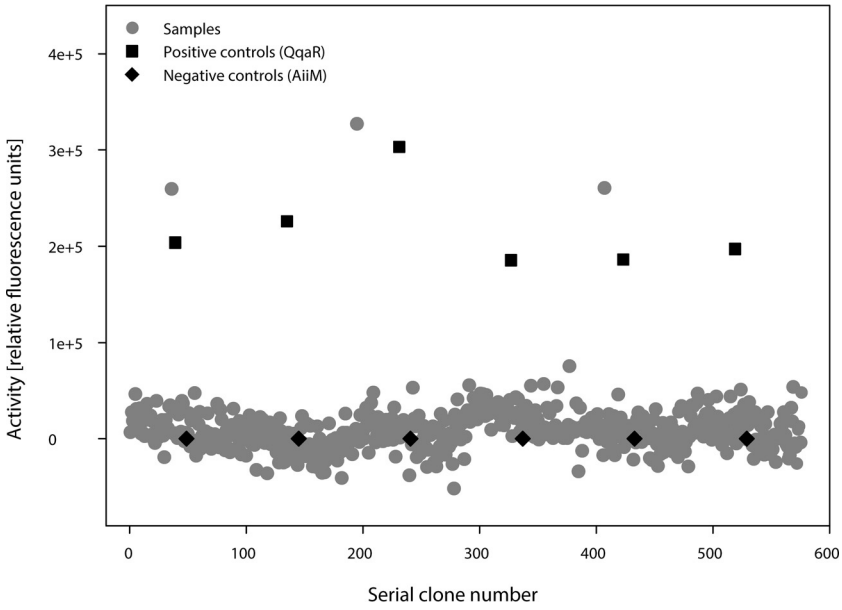

FIG 6 Identification of three *E. coli* clones with QQ acylase activity in an artificial library presented as an assembly of all clones of the six MTPs and normalized to the respective negative control. C_8-HSL (1 mM) was used as the substrate.

rification (300 mM; final assay concentration, ≤30 mM), strongly quenches the fluorescence of calcein.

Application to the screening for quorum-quenching acylases in an artificial library. The method described might be used, for instance, to identify QQ acylases in a metagenome-derived *E. coli* library, which would be a classical application for biosensors (24). In order to obtain an artificial metagenome library, the *E. coli* expression strains for QqaR and AiiM were mixed in a ratio of about 1:125 CFU (the entire screening procedure is visualized in Fig. S1 in the supplemental material). In total, 576 clones, including 6 clones of the positive (QqaR) and 6 clones of the negative (AiiM) controls were screened in the MTP format on a fully automated robotic platform (34). Indeed, 3 active clones were found with the calcein assay (Fig. 6), which is a statistically plausible number with regard to the Gaussian distribution (theoretically, four to five hits were expected). Their sequencing revealed that all three belonged to the QqaR expression strain. Hence, the proposed method was working in a reliable manner and is a useful alternative to biosensor-based screening.

DISCUSSION

In the present study, a (bio)chemical activity assay for the identification, characterization, and optimization of QQ lactonases and QQ acylases, which is based on the strictly conserved HSL moiety of AHL, was established. The respective biocatalysis products are converted either by a secondary enzyme or by autohydrolysis to HS. This compound is detected by the previously described calcein assay, which is sensitive only to α-amino acids with free N and C termini (32).

As QQ lactonases convert AHL to AH, a secondary enzyme that specifically cleaves the amide bond of AH and releases HS is required. For this purpose, hAcy1 and pAcy1 were tested, and the latter enzyme showed the higher activity (Fig. 2A and B). pAcy1 is especially suitable as a secondary enzyme as it can be stored at 4°C for several days to few weeks (data not shown) or in solution with 50% glycerol at −20°C for several months (according to commercial suppliers) with acceptable or even no loss in activity. Unfortunately, commercial preparations of recombinantly expressed and purified pAcy1 are too expensive for the intended purpose. In order to obtain pAcy1 in large amounts and without possessing the recombinant expression strain, an inexpensive commercial preparation derived from porcine kidney was tested. However, this preparation led to a fast increase in the calcein assay even without the addition of a substrate (see Fig. S6 in the supplemental material). Hence, commercial pAcy1 preparations should be tested properly as amino acids, metal chelators, and fluorescence-quenching compounds like imidazole (Fig. 5) might interfere with the calcein assay. Here, pAcy1 was purified to an appropriate degree in only one step by size exclusion chromatography (see Fig. S4 to S6). Probably other unspecific protein purification methods like hydrophobic interaction or ion-exchange chromatography would be suitable as well. If the specific activity of pAcy1 is too low toward a certain type of AH, most likely those with very bulky or branched acyl side chains (35), and this cannot be overcome by higher enzyme concentrations, one should consider the use of pAcy1 variants (35) or even other aminoacylases instead. Moreover, cells that should be examined for QQ enzymes have to be washed with a buffer solution prior to cell disruption. This step removes amino acids and other interfering compounds that might be present in the growth medium.

In order to obtain the GC-MS data shown in Fig. 2A and 3A, it took about 4 days of lab work to perform the biocatalysis reactions, to extract and derivatize the samples, and to analyze them. In contrast to this, the calcein assay measurements visualized in Fig. 2B and 3B were performed in less than 2 h. Hence, our method enables much faster and less expensive activity measurements than the GC-MS method. Biosensor-based methods require several additional hours of signal formation as well as forward planning as a suitable preculture is needed. The continuous and high-throughput character of the method presented also

facilitates the ability to find a suitable combination of enzyme concentration and biocatalysis time; several enzyme dilutions can be tested in parallel, and the measurements can be stopped once the signal formation is sufficient. In contrast to this, the GC-MS method is more or less a trial-and-error approach in this regard due to the measurement of one endpoint sample after another.

Another aspect that has to be discussed here in detail is that pAcy1 was previously described to be active toward AHL (46). For this, Xu et al. (46) monitored the formation of an amine by *o*-phthaldialdehyde (OPA) derivatization. However, the highest activities toward C_4-HSL (the least stable AHL) were found at high pH values (optimum, 10) and at high temperatures (optimum, 76°C). Both conditions are known to accelerate the autohydrolysis of AHL to AH (28, 29). An increase in pH from 7.85 to 8.5 reduces the half-life of the much more stable C_8-HSL from 7 h to only 30 min (28). As shown in this study, 10 min of incubation at 95°C led to almost full autohydrolysis of C_6-HSL (see Fig. S3 in the supplemental material). Xu et al. (46) performed their biocatalysis reactions over 2 h. In addition to this, pAcy1 was found here to be highly active toward C_6-HS, while no conversion was found for C_6-HSL (Fig. 2). Moreover, the supposed high temperature optimum of 76°C contradicts the relation of thermostability and activity of pAcy1 (47). While 80% of pAcy1 is inactivated after only 2.5 min at 70°C, it retains its activity almost completely after 10 min at 50°C. As the activities of pAcy1 are quite similar at both temperatures according to Wang et al. (47), one should expect that the QQ performance of pAcy1 over the 2 h of biocatalysis should have been much better at 50°C than at 70°C. Taken together, these results indicate that Xu et al. (46) have measured the activity of pAcy1 toward AH but not toward AHL as claimed. Not only in the course of this study but also in the literature, doubts concerning the QQ activity of pAcy1 have been mentioned (5) or no QQ activity was found at all (48). Perhaps the antibiofilm activity of the pAcy1 preparation, which was linked by Xu et al. (46) to its supposed QQ activity, was (i) due to the activity of pAcy1 toward another biofilm component, (ii) due to another enzyme like paraoxonase (49, 50), or (iii) due to low-molecular-weight compounds. The very low purity of such crude commercial pAcy1 preparations is exemplarily shown in Fig. S4. Nevertheless, pAcy1 did not show any measurable QQ activity under the tested conditions and thus can be used as a secondary enzyme for the method presented.

Following its establishment, our method was used to study the three previously undescribed QQ lactonases QqlB, QqlG, and QqlM originating from *Paraburkholderia glathei* (QqlB), *Geminicoccus roseus* (QqlG), and *Mesorhizobium ciceri* (QqlM). The open reading frames were identified on the basis of their homologies to the amino acid sequence and active-site residues of AidH, one of the three known QQ lactonases with an α/β-hydrolase fold (39, 40). The QQ activities of the recombinantly expressed proteins were confirmed by a modified CV026 assay (see Fig. S8 in the supplemental material).

Based on AidH crystal structures, Gao et al. (39) highlighted that the hydroxyl group of the amino acid Y160 is crucial for biocatalysis. Interestingly, QqlB possesses phenylalanine at the homologous position and therefore no such hydroxyl group. For this reason, the activities of the respective phenylalanine and tyrosine variants of QqlB, QqlG, and QqlM were compared by means of the calcein assay (Fig. 4). Indeed, the amino acid substitutions did not affect the activities of QqlG and QqlM, whereas the mutation F160Y further decreased the activity of QqlB. These results at least reveal that the importance of the hydroxyl group of Y160 in AidH cannot be generalized for homologous QQ lactonases. With biosensors, it would have been difficult or even impossible to obtain activity measurements with such a high resolution as shown here with the modified calcein assay.

In addition to QQ lactonases, the calcein assay was used for studies on a QQ acylase, which belongs to the other important class of AHL QQ enzymes. Here, the fast autohydrolysis of the biocatalysis product HSL to HS was exploited as the second step of the cascade reaction. This approach offers maximum flexibility concerning the acyl side chain of AHL, which is of particular interest for AHL with unusual acyl side chains, thereby circumventing the laborious development of specific biosensor strains (16, 18). The activity measurements took only several minutes and in the case of the screening a few hours (Fig. 5 and 6). Nevertheless, the autohydrolysis can be the rate-limiting step, depending on the concentration and the activity of the QQ acylase. For this reason, one might search for a lactonase as a secondary enzyme that hydrolyzes HSL, but not AHL (such HSL-hydrolyzing enzymes were not investigated here). Alternatively, one might perform the calcein assay in an endpoint manner with temporary incubation at a high temperature. This should strongly accelerate the autohydrolysis of HSL as shown here for the much more stable C_6-HSL (see Fig. S3 in the supplemental material).

In general, our method is also suitable for screening applications with crude cell lysate as shown by the reliable identification of clones expressing the QQ acylase QqaR in an artificial library (Fig. 6). It took only 4 h to distinguish between active and inactive clones. While the calcein assay enables the direct monitoring of the biocatalysis reactions, several additional hours of signal formation would have been required with biosensors subsequent to the biocatalysis reactions. On the other hand, the calcein assay requires much higher AHL concentrations than biosensor methods, which is of particular relevance for large-scale screening applications. Due to the high commercial price, one should consider that AHL synthesis is quite easy, robust, and inexpensive (27).

All in all, our method enables fast, continuous, and high-throughput activity measurements of QQ lactonases and QQ acylases. Therefore, it perfectly complements the few and fundamentally different methods described so far in this field.

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Electronic Supplementary Material

Article title: Fast, continuous and high-throughput (bio)chemical activity assay for *N*-acyl-L-homoserine lactone quorum quenching enzymes

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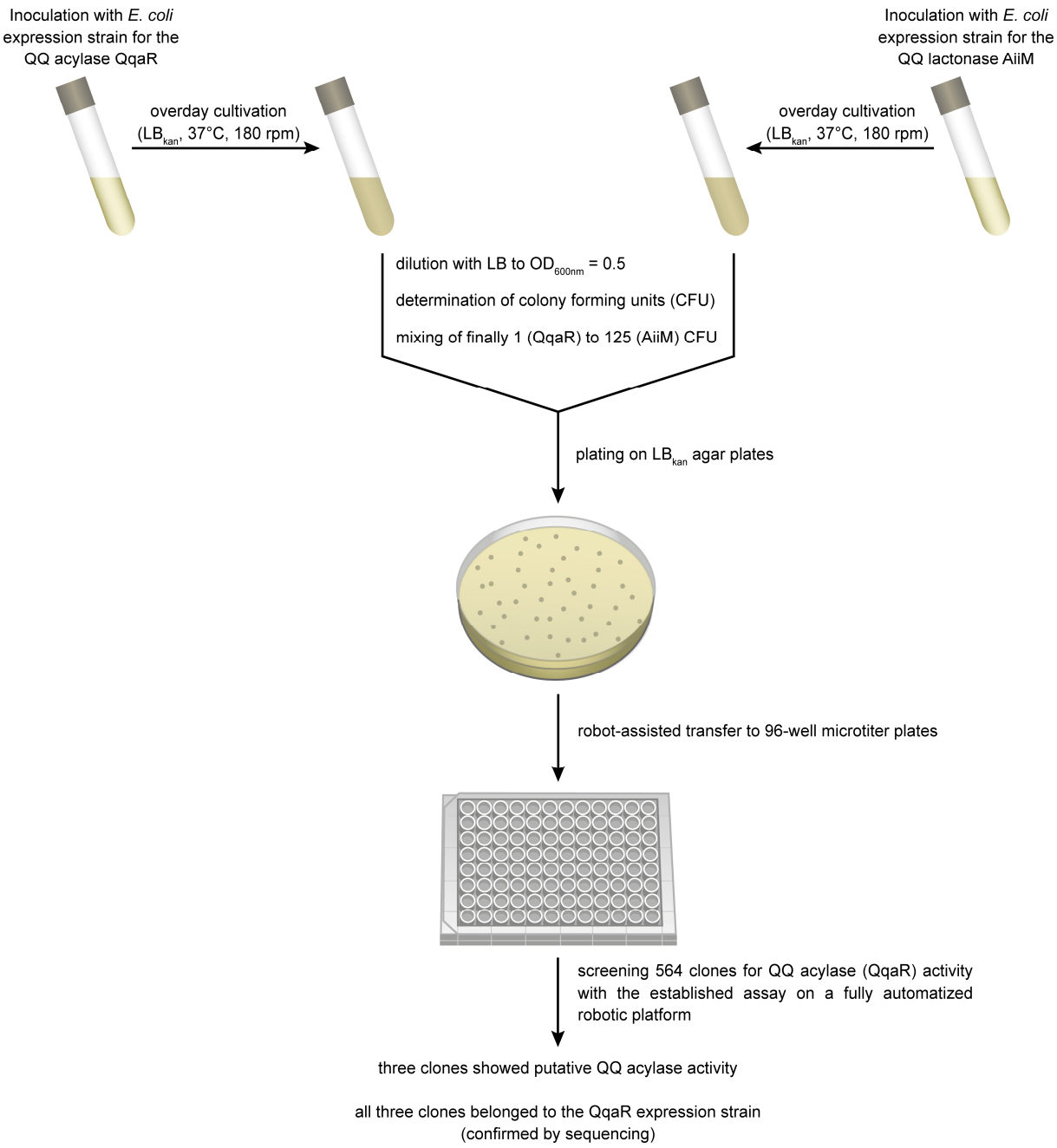

FIG S1: Screening procedure used for the identification of *E. coli* clones with QQ acylase activity in an artificial library. The results confirm, that the established activity assay works in a reliable manner.

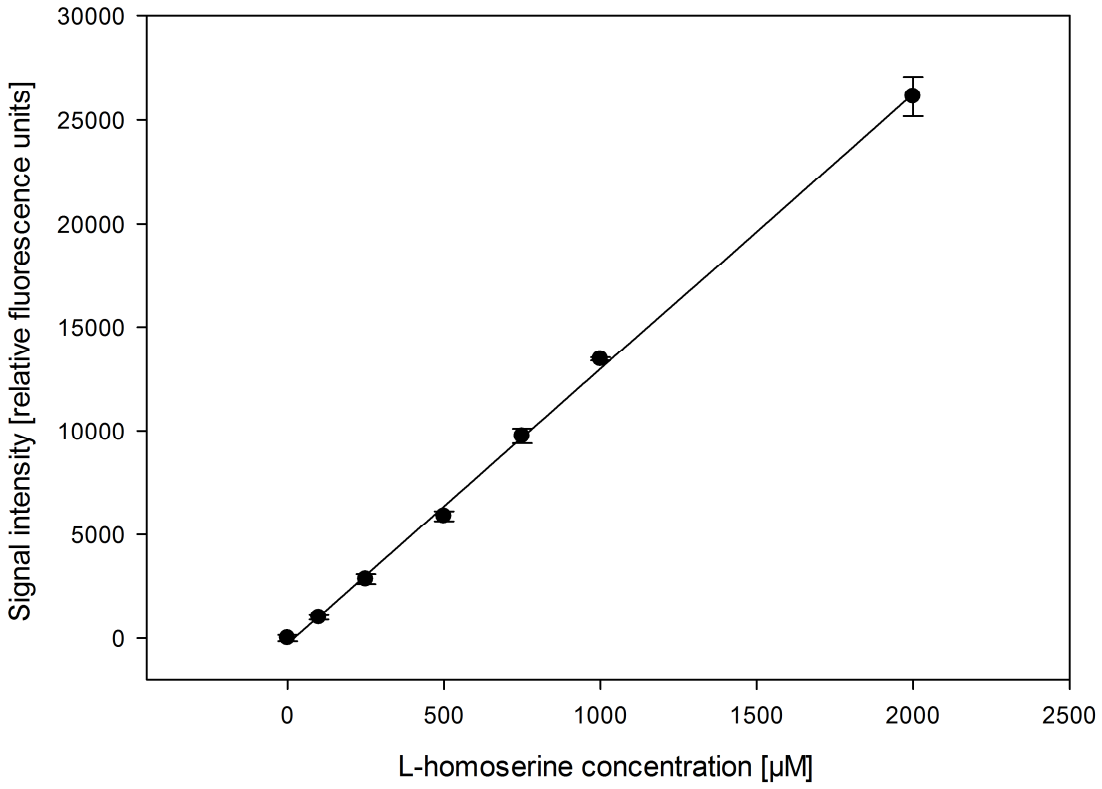

FIG S2: Sensitivity of the calcein assay towards HS was determined by a standard curve mimicking a biocatalysis reaction. Thus, the concentrations of HS and hexanoic acid were increased while the concentration of C_6-HSL was decreased proportionally.

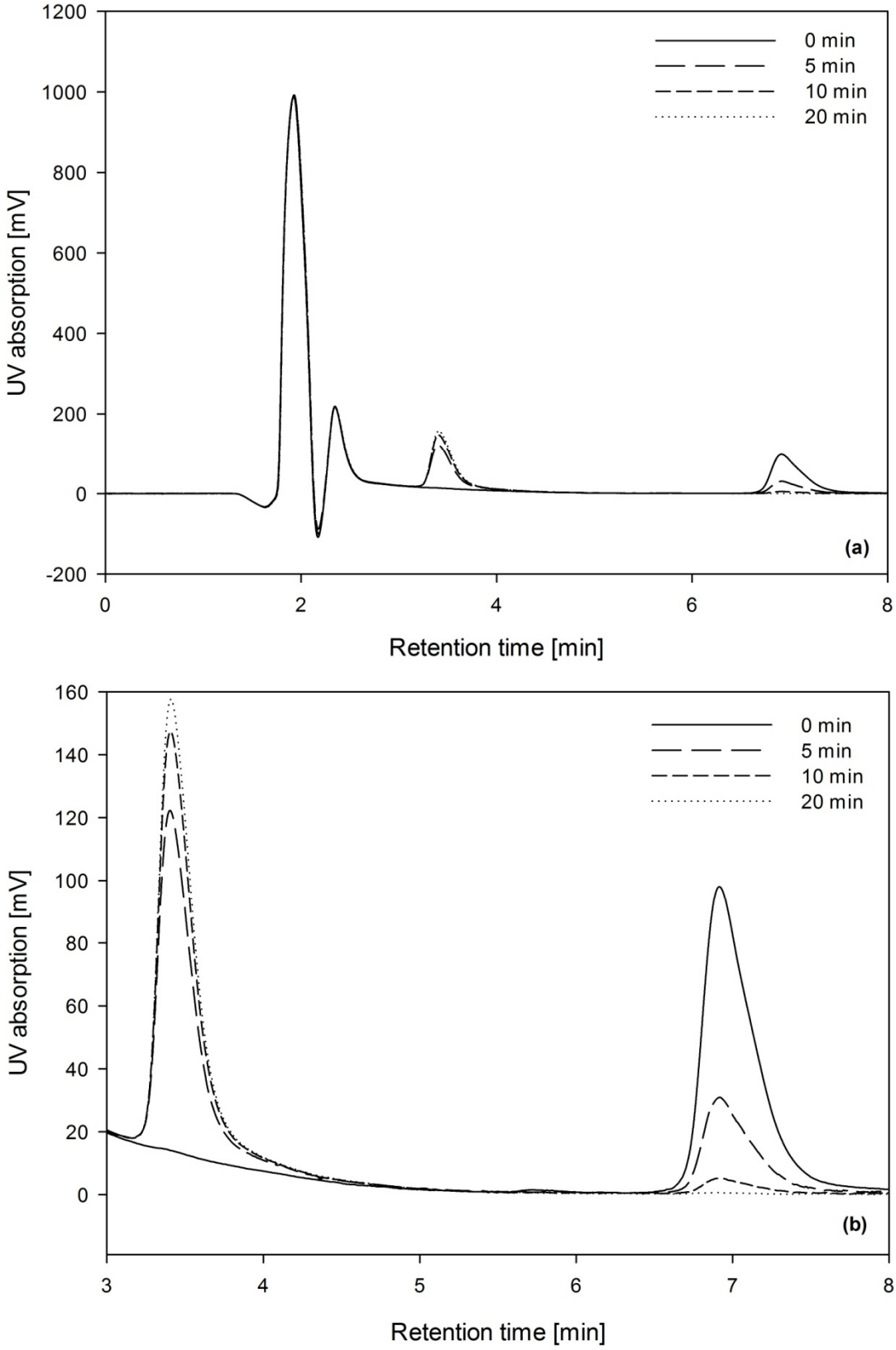

FIG S3: HPLC chromatograms of the heat-accelerated autohydrolysis of C_6-HSL (retention time 7.0 min) to C_6-HS (retention time 3.4 min) by incubation at 95°C for the indicated periods of time (samples were immediately transferred to ice and analyzed). Biocatalysis reactions with QQ lactonase AiiM were used to prove the identity of C_6-HS (data not shown). **(a)** Overview of the entire runs. **(b)** Detailed view on the peaks of C_6-HSL and C_6-HS.

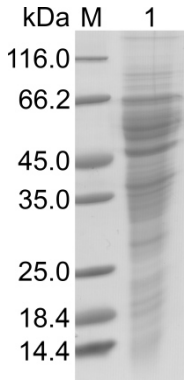

FIG S4: SDS-PAGE analysis of the crude commercial pAcy1 preparation derived from porcine kidney. The pAcy1 has a molecular weight of ~45 kDa. *M*, protein molecular weight marker; *lane 1*, pAcy1 preparation.

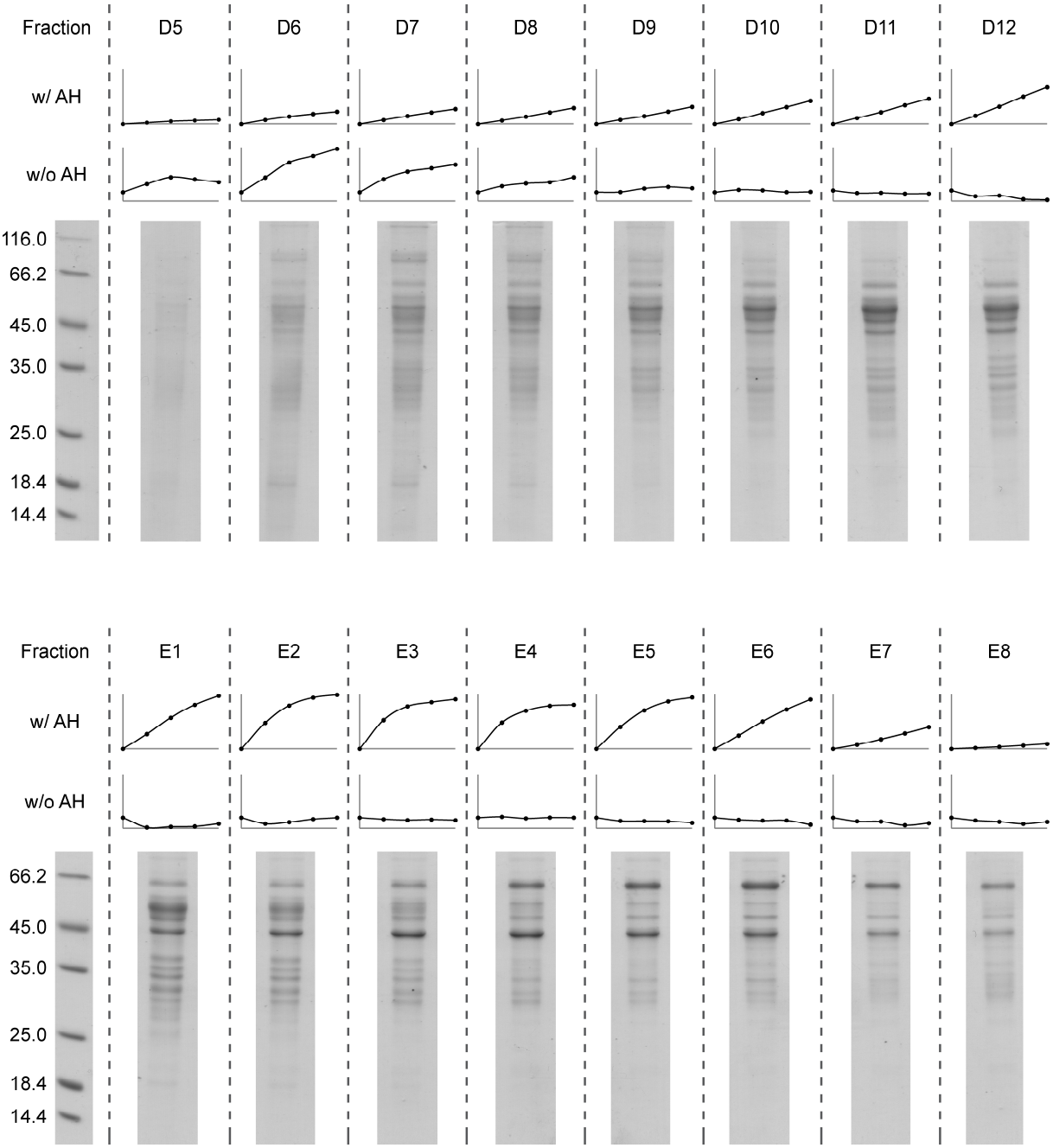

FIG S5: The fractions, obtained by size exclusion chromatography of the commercial pAcy1 (45 kDa) preparation, were subjected to calcein assay measurements with and without C_6-HS (AH) as well as to SDS-PAGE analyses. The highest activities of pAcy1 were found in the fractions E2 to E4, while the highest background activities were found in the fractions D5 to D8. Also low-molecular weight compound(s) seem to be involved as ultrafiltration (12 kDa MWCO) led to a strong but incomplete reduction of the background activity in the calcein assay even in the absence of a substrate (data not shown).

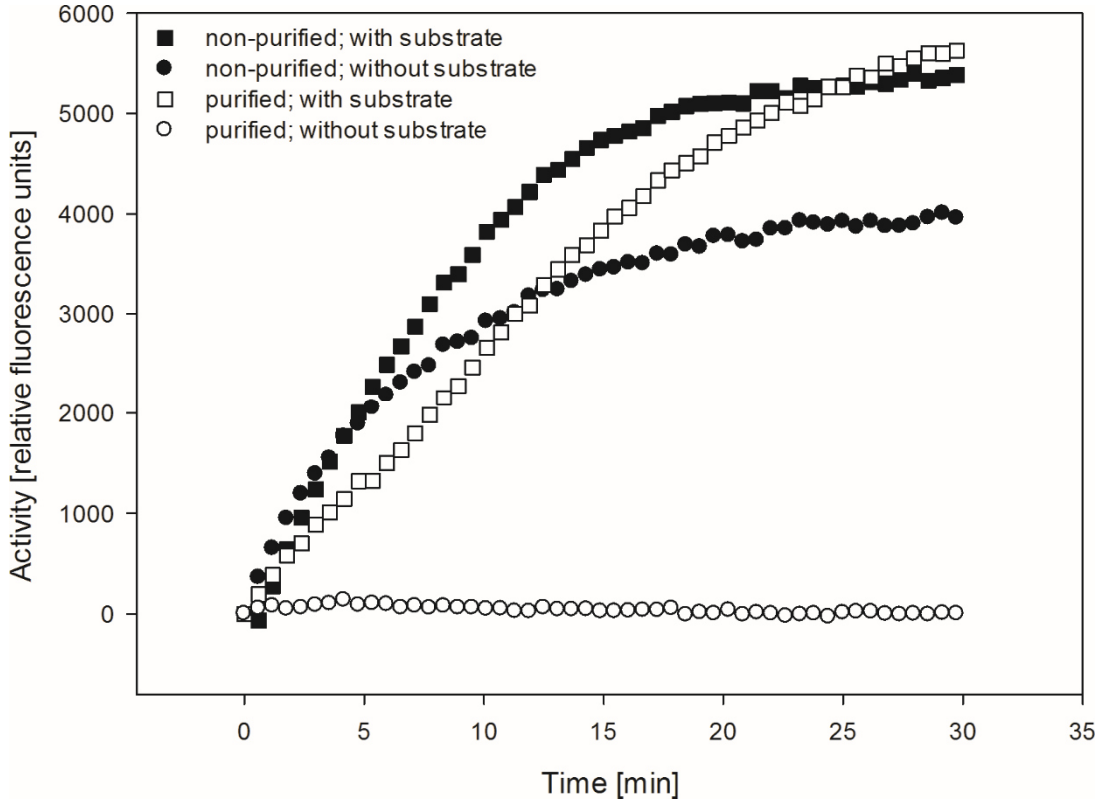

FIG S6: Calcein assay with commercial pAcy1 preparation derived from porcine kidney with (fraction E3) and without previous purification by size exclusion chromatography. C_6-HS (2 mM) was used as substrate. In contrast to the purified pAcy1, the non-purified form showed strong activity in the calcein assay even without any substrate. This observation might be due to amino acids or metal chelators present or released in the commercial pAcy1 preparation. Standard deviations (average: 7.3 %; individual maximum: 14.5 %) are not indicated for a better clarity.

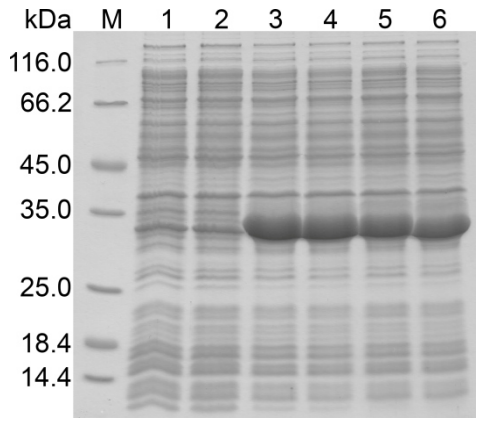

FIG S7: SDS-PAGE analysis of the expressions of QqlB, QqlG, QqlM and variants thereof in *E. coli*. *M*, protein molecular weight marker; *lane 1*, QqlB; *lane 2*, QqlB F160Y; *lane 3*, QqlG; *lane 4*, QqlG Y160F; *lane 5*, QqlM; *lane 6*, QqlM Y160F.

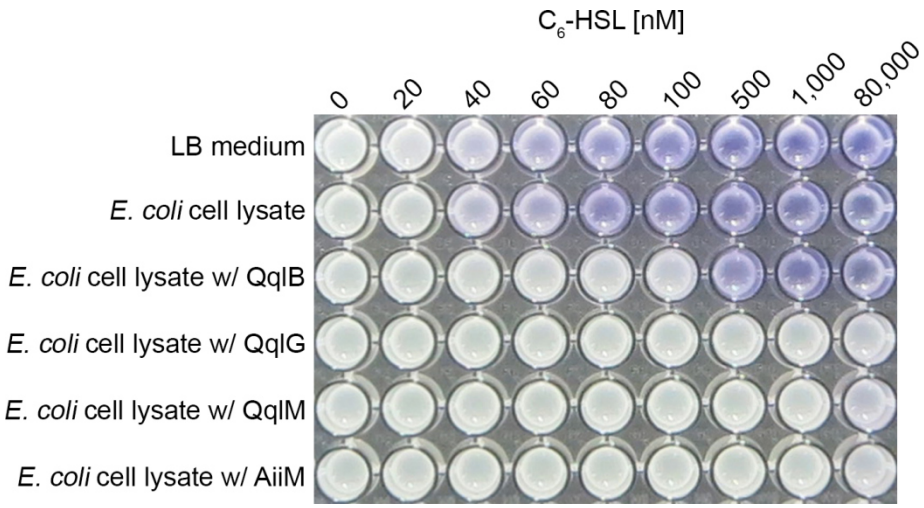

FIG S8: Modified CV026 assay to measure the QQ activities of *E. coli* cell lysates containing QqlB, QqlG and QqlM. LB medium and *E. coli* cell lysate containing the empty expression vector served as negative controls while *E. coli* cell lysate containing the known QQ lactonase AiiM served as positive control. The lower QQ activity of QqlB compared to QqlG, QqlM and AiiM might be due to the much lower expression level as shown in Fig. S6.

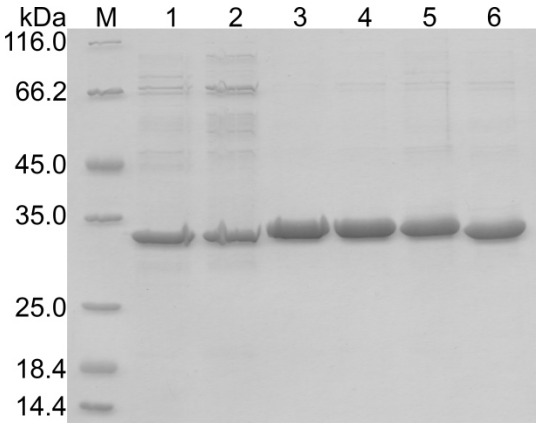

FIG S9: SDS-PAGE analysis of the His-tag purified QqlB, QqlG, QqlM and variants thereof. *M*, protein molecular weight marker; *lane 1*, QqlB; *lane 2*, QqlB F160Y; *lane 3*, QqlG; *lane 4*, QqlG Y160F; *lane 5*, QqlM; *lane 6*, QqlM Y160F. Interestingly, QqlM Y160F shows a slightly different electrophoretic mobility than QqlM.

8.2. Article II

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Fully Automatized High-Throughput Enzyme Library Screening Using a Robotic Platform

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ABSTRACT: A fully automatized robotic platform has been established to facilitate high-throughput screening for protein engineering purposes. This platform enables proper monitoring and control of growth conditions in the microtiter plate format to ensure precise enzyme production for the interrogation of enzyme mutant libraries, protein stability tests and multiple assay screenings. The performance of this system has been exemplified for four enzyme classes important for biocatalysis such as Baeyer–Villiger monooxygenase, transaminase, dehalogenase and acylase in the high-throughput screening of various mutant libraries. This allowed the identification of novel enzyme variants in a sophisticated and highly reliable manner. Furthermore, the detailed optimization protocols should enable other researchers to adapt and improve their methods.

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KEYWORDS: activity assay; enzymes; growth conditions; high-throughput screening; protein engineering; robotic platform

Introduction

We currently experience an almost exponential increase in the number of DNA sequence data (e.g., currently approx. 38 million non-redundant (nr) sequences available at NCBI-nr). The Environmental Genome Shotgun Sequencing Project of the Sargasso Sea and the Global Ocean Survey alone discovered 7.3 million previously unknown gene sequences comprising 1,700 new protein families (Venter et al., 2004). These new, yet uncharacterised proteins, can be considered a richly filled cornucopia pouring out unexplored enzymes applicable as biocatalysts. These are important for environmentally friendly "green" production of pharmaceutical building blocks or in synthetic biology approaches for the production of bulk chemicals and new materials like biopolymers and nano-structured materials (Bershtein and Tawfik, 2008; Bornscheuer et al., 2012; Woodley, 2008). Furthermore, protein engineering to adapt enzymes to new substrates, to alter their stereoselectivity or to make them fit to meet demands for their use in industrial processes (Bornscheuer et al., 2012) also needs sophisticated tools to identify desired improved biocatalysts. This requires firstly, the transformation of the genes into a working expression system and functional activity screening assays for the characterization of potential enzymes. Unfortunately, the challenges to explore novel enzymes from sequencing projects as well as within mutant libraries currently cannot be addressed in a satisfactory manner using ultra-high-throughput techniques. Even though methods such as micro-compartmentalization, microfluidics, or fluorescence activated cell sorting (FACS) (Colin et al., 2015; Fernandez-Alvaro et al., 2011; Griffiths and Tawfik, 2000) offer very high throughput useful for initial pre-evaluation of large enzyme libraries, the required limitation of substrates to fluorescent molecules with conditions usually far away from properties required for their industrial applications, will only enable a prefiltering. Although here the assay can lead to a sensitive and easily measurable signal in the form of a fluorescent compound, the resulting 'hits' commonly do not show the expected performance when exposed to the real substrate and conditions ["*you get what you screen for*" (Schmidt-Dannert and Arnold, 1999)]. Furthermore, enzyme activity and especially reliable kinetic data can often not be determined using these micro/nano techniques. Hence, a reliable and automated screening system is ultimately required to: (i) follow-up on putative hits identified by the ultra-high-throughput techniques mentioned above and (ii) directly

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interrogate "*small, but smart*" mutant libraries, which are currently at the forefront of protein engineering (Floor et al., 2014; Jochens and Bornscheuer, 2010; Kuipers et al., 2010; Wijma et al., 2014, 2015).

In this article, we present a widely applicable setup for in-depth high-throughput screening of enzymes for biocatalytic applications in a fully automatized, robotic platform. To the best of our knowledge such advanced automation systems with high flexibility for protein engineering tasks have not yet been described in literature. At the time of this publication only examples of automated systems for microtiter plate cultivation (Huber et al., 2009), the production of monoclonal antibodies (Yoshioka et al., 2011), or protein crystallization (Ferrer et al., 2013; Li et al., 2012) had been reported. Although these systems are useful to, for example, identify optimized conditions for microbial growth or to yield sufficient amounts of pure protein for structure elucidation, they do not enable automated characterization of enzymes for biocatalytic applications and/or the screening of mutant libraries in directed evolution approaches. The system described here covers controlled cell growth cycles, induction of the protein expression at defined cell density, controlled protein expression cycles, harvesting and lysis of the cells adapted to the host cell and enzyme of interest with optional purification of the desired protein, characterization of the enzymes by suitable activity assays and very importantly, a final statistical data evaluation of the measurements enabling visualization and documentation of the results (Figs. 1 and 2). The suitability and general applicability of this platform is exemplified for four different enzymes with relevance for biocatalysis: a (*S*)-selective amine transaminase [VFL–ATA, from *Vibrio fluvialis* (Genz et al., 2015)], a Baeyer–Villiger monooxygenase [CHMO, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (Donoghue et al., 1976)], two haloalkane dehalogenases [(HLD, DhaA from *Rhodococcus rhodochrous* (Newman et al., 1999), and DhlA from *Xanthobacter autotrophicus* (Janssen et al., 1989)] and porcine acylase I [(pAcyl, from *Sus scrofa* (Lindner et al., 2008; for details see Table SI, Supporting Information)]. In all four cases, mutant libraries were created and screened for variants with altered properties using the automated robotic platform (Figs. 1 and 2).

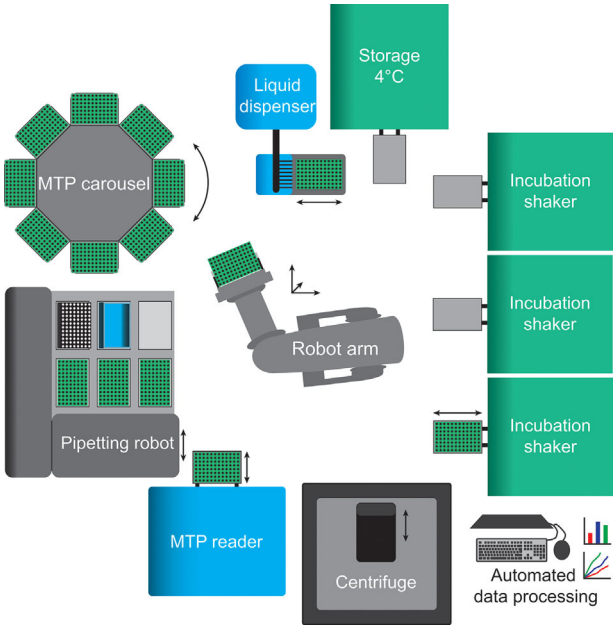


Figure 1. Set-up of the robotic platform. Microtiter plates (MTP) are handled by the central robot arm. The pipetting robot as well as the liquid dispenser allow fast and precise addition or removal of liquids, two stacked MTP readers enable determination of protein content and enzymatic activities, three incubation shakers are used to cultivate MTPs before and after induction; plates can be stored in a cooled device at 4°C or on the MTP carousel. The centrifuge allows harvesting the cells and removal of cell debris.

Figure 2. Picture of the robotic platform.

Materials and Methods

This section only covers the setup of the robotic platform and the description of the enzymatic assays used. All other experimental details are given in the Supporting Information.

Devices of the Robotic Platform

The robotic platform was custom designed by Thermo Fisher (Burlington, Canada) and later optimized and extended by us. It consists of a central robotic arm (Fanuc/Thermo Fisher F5), a dispenser (Nanodrop, Thermo Fisher), four incubators (2× Cytomat 1550, 1× Cytomat 470; all three equipped with linear tower shaker units for gyratory shaking <1,000 rpm, 1× Cytomat 2, Thermo Fisher), a liquid handling robot equipped with a 96 tips head (Bravo, Agilent Inc.), a centrifuge (Rotanta 460-Robotic, Hettich GmbH), two microtiter plate UV-vis and fluorescence spectrophotometer/reader (Varioskan, Thermo Scientific and Omega, BMG, respectively), a bar-code reader (M3, Microscan), a MTP turn table (Regrip, Thermo Scientific)—removed in later stages of the platform optimization—4 uninterruptable power sources (400 kW each), and a 96 pin head microbial colony picker (QPix 420, Molecular Devices). The central process management software is Momentum (Ver. 3.2.5, Thermo Fisher), the control software for the pipetting robot is Agilent VWorks 9. For the feedback cycles, data evaluation, visualisation and presentation a linux server (Ubuntu 14.04LTS) with the statistical analysis package R (R-cran-project.org, Ver. 2.14) and python (python.org, Ver. 2.7)

as scripting language was used. To complement limitations of Momentum and VWorks, the LARA software package was developed to plan, program, archive and evaluate all complex robotic processes. LARA is an open source software suite and can be freely downloaded for academia at lara.uni-greifswald.de. Some details of the LARA software package are described in the SI and a complete description will be published elsewhere.

Standard Process Using the Robotic Platform

If not stated otherwise the following standard process was running on the robotic platform: The bacteria colonies of mutant libraries, cultivated in standard petri dishes (ø 90 mm), were picked with the colony picker into a 96 well microtiter plate (MTP) filled with initial growth medium [in most cases Lysogenic Broth (LB)]. These master plates were incubated for 5 h at 37°C and 700 rpm until the cells are in the maximal exponential growth phase. Freshly filled screening plates (Zym 5042 autoinduction medium) were inoculated with a tiny fraction of the master plate culture transfered by the small needles of the 96 pin picking robot head. The remaining culture of the masterplate was stored at 4°C and kept for hit-picking and sequencing of interesting mutants. This procedure ensured a very even growth (Fig. 3C), a precondition for reduction of false positive and false negative samples. The screening plates were incubated for 6 h at 37°C and 700 rpm and then moved into the 20°C incubator for expression for further 12 h. For induction of the gene expression, the liquid handling robot added 20 μL of the inducer (IPTG, 0.1 mM final concentration) if no autoinduction medium is used. Growth and expression were monitored by measuring the optical density of all wells at 600 and 660 nm at defined intervals (usually every 2 h, Fig. 3). After 18 h total time for growth and expression, the cells were harvested with the robotic centrifuge at 5000*g* for 20 min at 4°C and the supernatant was removed by the liquid handling robot. Washing steps with the same buffer as used for cell lysis (not including the lysis agent) removed residual growth medium. The remaining cell pellet was lysed by the addition of lysis buffer—the nature of which depends on the enzyme expressed (see examples below)—and consecutively incubated at 30°C and 700 rpm for 2 h. Cell debris was removed by centrifugation (5000*g* for 20 min at 4°C) and the lysate was transfered into a new lysate plate. This lysate plate was kept at 4°C for the enzymatic assays. The identity of the microtiter plates was tracked by their barcodes. For the non-optimized growth (Fig. 3B) we cultivated a manually picked masterplate to the stationary growth phase overnight and inoculated a freshly prepared growth and expression plate with 10 μL of the masterplated cell suspension with the help of the pipetting robot.

Platform Optimization

During the course of the screenings several rounds of optimization of the platform setup and container flow were necessary since we faced many delays when running several processes simultaneously and screening results were not reliable. These optimization steps required the reorientation of devices (dispenser, barcode reader, incubators and plate rotation station, which was finally removed), to allow a single plate orientation (in our case "landscape" only) for most of the devices. With an additional re-teaching of the robotic arm to enable a smoother and faster movement, the transfer time of each plate could be drastically reduced. Furthermore, the following process steps underwent many cycles of optimization: initial cell growth to achieve the desired homogeneity (Fig. 3B and C), harvesting conditions (centrifugation time), pelleting, cell lysis (various cell lysis strategies had been tested), assay pipetting (to cope with fast reactions and slow transport speeds and device delays). These improvements are described in more detail below and in the Supporting Information. The normalisation of the protein content by the split-GFP system has just been reported by us (Santos-Aberturas et al., 2015).

Enzymatic Assays and Their Optimizations

Transaminase Activity Assay

Transaminase activity was measured using the 'acetophenone' assay (Scheme 1; Schätzle et al., 2009). The screening plates (Greiner UV-MTP 96-well plates) were charged with substrate solution (180 μL/well, composition: 10% DMSO, 1 mM amino donor [(*S*)-1-phenylethylamine, (*S*)-1-phenylpropylamine or (*S*)-1-phenylbutylamine], 2 mM amino acceptor (pyruvate or pentanal), in CHES buffer (50 mM, pH 9.0), and crude enzyme lysate was added (20 μL/well). Absorbance was then measured with a whole plate spectrophotometer at 245 nm (acetophenone) or 242 nm (1-phenylpropan-1-one and 1-phenylbutan-1-one) over 15 min with a sampling rate of 1/30 s.

CHMO Assays to Measure Activity, Long Term Stability, and Product Inhibition

For assaying the CHMO activity in the conversion of cyclohexanone **3** to ε-caprolactone **4** (Scheme 1), optimization experiments (i.e., amount of lysate, buffer type, pH, measurement intervals) revealed the following procedure, which was then kept as standard: The best kinetic measurements were obtained by adding 20 μL cell lysate to 160 μL sodium phosphate buffer (50 mM, pH 8.0). After mixing of the components by the pipetting robot, 20 μL of a buffered assay mix (500 μM NADPH, 10 mM cyclohexanone) were mixed into the MTP. The so prepared assay plates were moved as fast as possible into the plate reader (total duration approx. 60 s). The kinetics of the reaction were recorded by measuring the decrease of absorption at 340 nm every 30 s over a periode of 180 s. The optical pathlength was corrected according to the procedure described in the Supporting Information.

In some cases it was necessary to decrease the amount of cell lysate to obtain evaluable slopes. This was done by pre-dilution of the cell lysate, which was then subjected to the NAD(P)H assay using same amounts and concentrations of buffer, substrate and NAD(P)H. The total volume of each reaction per well was 200 μL. It was necessary to adjust the manual cuvette based NAD(P)H assay to the robotic platform. This was a special challenge due to the fast consumption of the NAD(P)H by the enzymatic reaction. A sensitive, but slow enough measurable decrease of absorption over an interval of 120 s was desired. After preparing the assay master mix, the robot arm needs approximately 30 s to add the cell lysate to

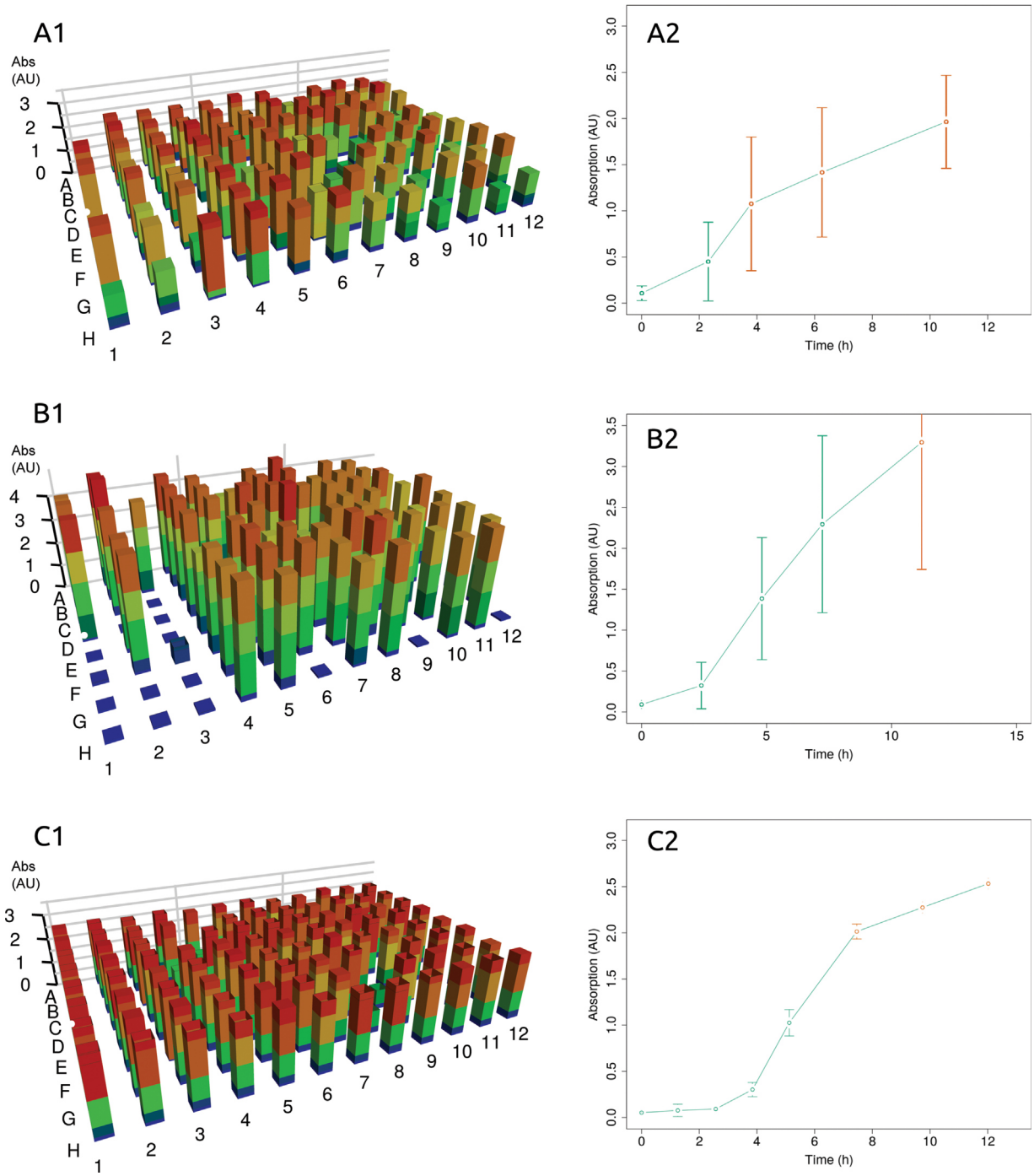
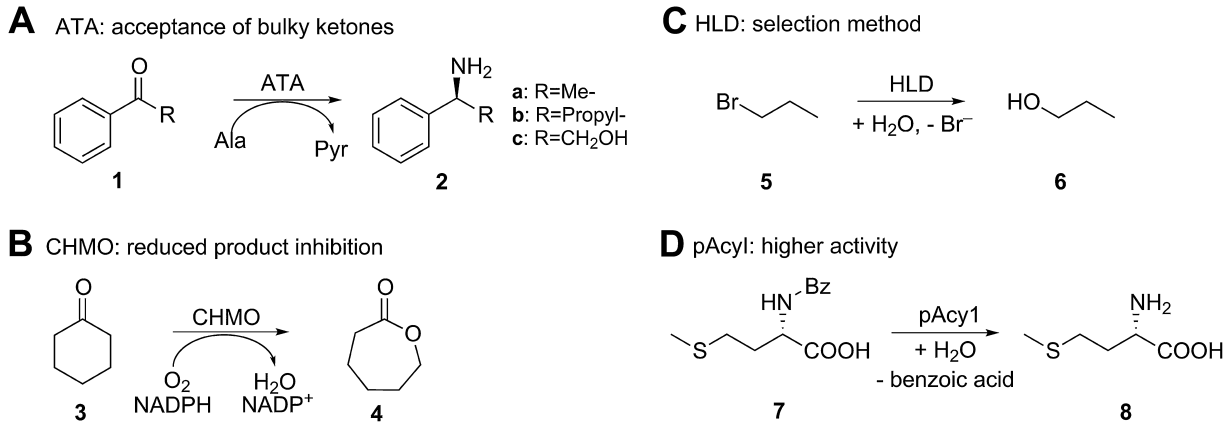


Figure 3. 3D barplot representations of per-well growth (1) and 2D growth plot averaged over the whole plate (2), measured by absorption at 600 nm. Each 3D-bar section represents the cellular growth during a constant time interval. (**A**) Inhomogeneous growth resulting from manual picking and direct inoculation of the expression plates with *E. coli* cells. (**B**) Inoculation of the growth media with 10 μL cell suspension by the pipetting robot. (**C**) Inoculation with freshly grown cells and colony picker replication. Here, the few empty wells refer to controls.

start the assay reaction and 30 s to transfer the MTP to the plate reader. Additional 30 s are elapsing until the enzyme activity is measured (due to instrument initialisation and a short additional shaking step). Therefore, suitable substrate and NAD(P)H concentrations needed to be investigated otherwise the reaction is too fast to obtain evaluable slopes. To find CHMO mutants with improved stability, the lysate was incubated for several days (24–68 h) at 25°C. After defined time intervals samples of 20 μL lysate have been taken and CHMO activity was measured according to the above described procedure. The decay of activity for each well was visualised with 3D plots (Fig. 4). The mutants displaying the highest remaining activity after 36 h were then selected for



Scheme 1. Screening objectives: (**A**) Assay for the (*S*)-ATA transaminase converting **1** to **2**. (**B**) Baeyer–Villiger oxidation of cyclohexanone **3** to ε-caprolactone **4**. (**C**) Dehalogenation of 3-bromopropane **5** to 3-hydroxypropane **6**. (**D**) Amidolysis of **7** to **8** by pAcyl.

sequencing and in-depth characterization. Mutants with higher tolerance against the reaction product ε-caprolactone, were assayed by several rounds of increasing amounts of ε-caprolactone (0–300 mM). The results are visualised by per well 3D plots and the best hits were further investigated by sequencing and manual characterization.

HLD Activity Assay

To determine the HLD activity (Scheme 1), a whole cell assay was applied, since the cellular membranes are permeable for the desired substrates. The harvested cells were washed twice with 200 μL HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM, pH 8.2) buffer to reduce the background signal. Fifty microliter of the whole cell suspension were added to 150 μL of the assay solution (140 μL HEPES, 1 mM, pH 8.2, 20 mg/mL phenol red supplemented with 10 μL of a mixture of 100 mM haloalkanes 1,2-dibromoethane, 1-iodopropane, 1-bromobutane, 1-iodobutane, and 4-bromobutyronitrile in acetonitrile). All five substrates are known to be accepted by the HLD and using a mixture increases the chance to find desired active variants (Fibinger et al., 2015). The absorption at 430 and 560 nm was measured at the beginning and after 24 h of incubation at 37°C. The phenol red indicator color change to yellow, indicated by a change in the ratio of both absorption values, was used for signaling the release of protons and subsequent catalytic conversion of the haloalkanes to the corresponding alcohols.

Acylase Activity Measurements

The activity of the acylase (Scheme 1) was measured using the calcein assay according to Dean et al. (2003). 20 μL crude cell lysate or purified enzyme solution (0.15 mg/mL) were supplemented with 180 sensor solution [10 mM *N*-benzoyl-L-methionine (NBM), 10 μM copper (II) chloride, 2 μM calcein] in sodium phosphate buffer (50 mM, pH 7.5). L-methionine formation was monitored by the increase in calcein fluorescence (450 nm excitation and 530 nm emission) after 0, 40, 80 and 120 min. Activity was also quantified by HPLC: biocatalysis reactions (in quadruplicate) were prepared by supplementation of 10 mM NBM with 0.01 mg/mL of purified enzyme in sodium phosphate buffer (50 mM, pH 7.5), and incubation in a thermoshaker (37°C, 750 rpm, 30 min). The addition of an equal volume of ice-cold methanol and transfer to −20°C terminated the reactions. The activities of the variants were analyzed by HPLC with a LiChrospher 100 RP-18e column (5 μM, 250 mM × 4 mm, Merck) at 271 nm. The conversion of NBM never exceeded 15% within the 30 min of biocatalysis. The solvents A (water; 0.1 % trifluoracetic acid) and B (acetonitrile; 0.1% trifluoracetic acid) were used in gradient mode: 0 min (80% A), 15 min (50% A), 17 min (10% A), 19 min (80% A), and 19–30 min (80% A) at a flow rate of 1 mL/min. The retention times for benzoic acid and NBM were 13.3 min and 14.8 min, respectively (Fig. S1).

Results

Set-Up of the Robotic Platform

The robotic platform (Fig. 1: schematic set-up, Fig. 2: picture of the device) was designed in a manner that all steps commonly required to produce and analyze enzyme mutant libraries are fully automated in a 96 well microtiter plate (MTP) format. This includes cultivation of the host strain, for example, *E. coli* containing the plasmids encoding the enzyme/variants of interest, induction of gene expression and hence protein production, cell lysis to release the enzyme into the supernatant, centrifugation for removal of cell debris followed by removal of cell lysate for the subsequent assays to determine protein content and enzyme activity. Figures 5 and 6 exemplify these steps and the work-flow, including time requirements for each step, is shown in Figure 5. To achieve a fully automated and reliable system with a throughput as high as possible, a large number of challenges needed to be met and detailed protocols for each step had to be designed and

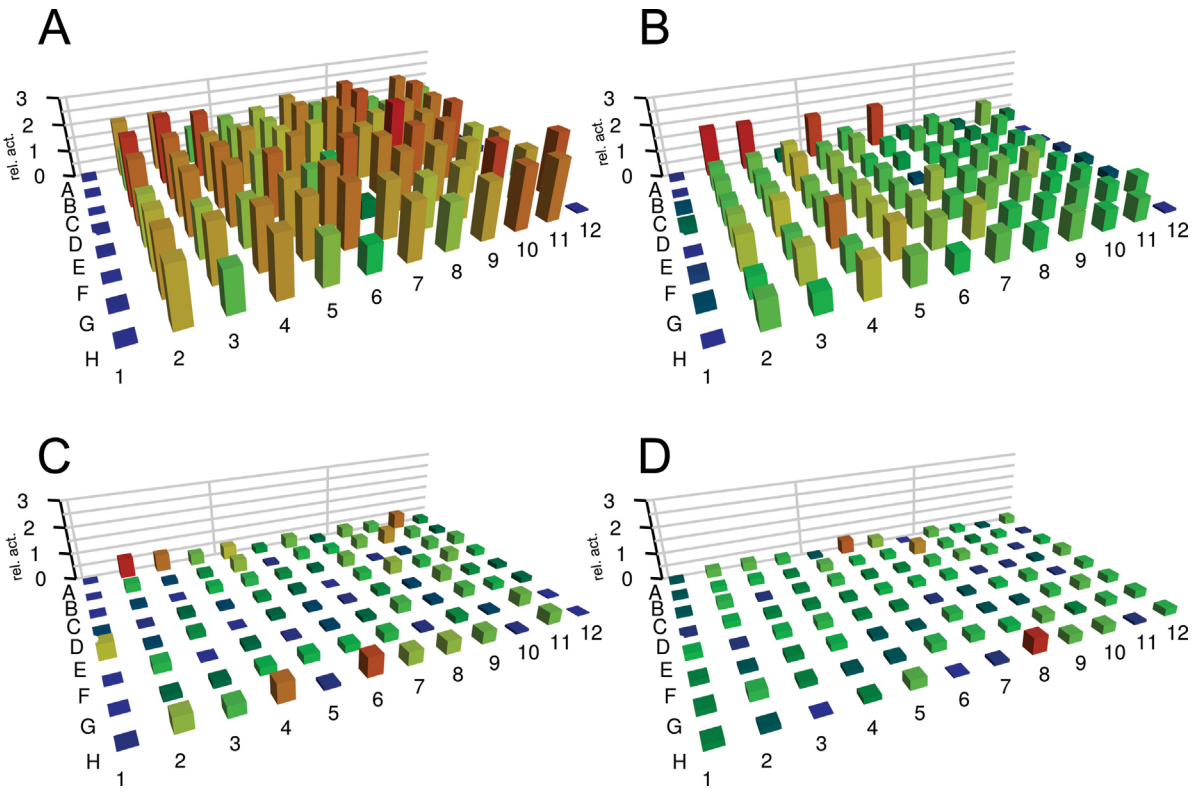


Figure 4. Decay of CHMO activity for each well visualized by 3D plots after screening an error-prone PCR-derived mutant library to identify variants of the CHMO with reduced product inhibition. With (**A**) 0 mM, (**B**) 75 mM, (**C**) 125 mM and (**D**) 300 mM ε-caprolactone. The highest NADPH consumption is displayed as a red bar showing improved and still active variants at high concentrations of product.

implemented (Fig. 6A). For instance, as cultivation of *E. coli* to an optical density suitable for induction usually requires 3–4 h, but a centrifugation step lasts only 20 min and the activity assay needs, for example, 2 min of continuous measurements in the case of CHMO, but two single point measurements within 24 h were required for HLD. The challenge to interweave concurrent processes with very different time constraints necessitates the software—see section "Devices of the Robotic Plattform" and supporting information for details—to be trained to ensure maximum use of the entire platform with minimal dead times (Fig. 6B). Most likely sources of errors also must be predicted and eliminated from the very beginning of the design process (Fig. 6A and E). Obviously the goal of this process is highest accuracy and reproducibility, process speed, user-friendliness and flexibility. Commercially available robotic software solutions focus on rigid process designs that are repeated several thousand times without alteration,

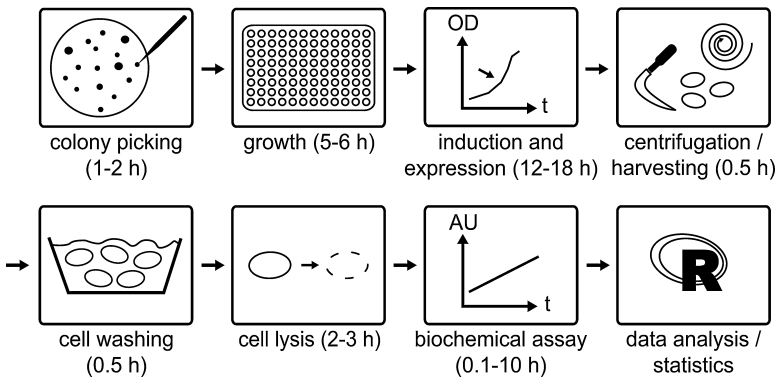


Figure 5. Schematic representation of the processes running on the robotic platform.

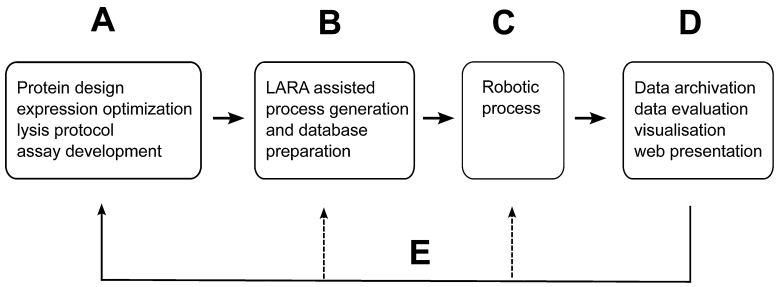


Figure 6. Total screening workflow. (**A**) Pre-screening phase. (**B**) Screening initiation. (**C**) Actual screening at the platform. (**D**) Post-processing. (**E**) Feedback cycle.

neglecting the planning and evaluation phase of the experiments. In our case every process was unique, also requiring individual evaluation. For this purpose the LARA software suite for process planning, program generation, data evaluation and visualisation needed to be developed (Fig. 6B and D; http://lara.uni-greifswald.de). The LARA software generated the individual process programs that can be executed on the platform and finally, as one outcome of the LARA data evaluation and visualisation module, all data are statistically evaluated and visualised. So were, for example, all figures showing experimental data in this publication algorithmically generated and similar figures are presented to the user in the web interface.

Commonly in the absence of such a sophisticated robotic platform, scientists performing enzyme engineering follow simple standard protocols for *E. coli* growth, induction, cell disruption and the assay itself. Although this obviously allows identification of improved enzyme variants from mutant libraries, every researcher is aware of improper conditions through all these steps leading to a considerable number of false positive and false negative hits. The robotic platform now provides the unique advantage that all steps are precisely controlled and detailed data for each clone (enzyme) per well of a microtiter plate can be obtained. This easily allows that the best variants found in a first survey screening can be feedbacked into the design process for confirmation and further enzyme improvement (Fig. 6E).

In the following paragraphs, these advantages are exemplified for the four different enzyme classes and the respective engineering targets.

Optimization of Conditions for *E. coli* Growth, Gene Expression, and Enzyme Isolation Exemplified for CHMO

The first often overlooked challenge is even growth of *E. coli* across the entire MTP and hence even production of the enzyme of interest to get comparable enzyme activities per well of the MTP. Figure 3A1/A2 exemplify this using a common protocol, in which colonies were picked manually and grown under standard conditions. Figure 3B1/B2 illustrates the non-optimized growth in the robotic plattform. Further optimization of this step resulted in very even growth and protein production as shown in Figure 3C1/C2. Key to success was the preparation of a master plate, picked automatically using a colony picker from single colonies plated on petri dishes.

Even though the growth of *E. coli* is equally high over the entire microtiter plate, the expression of the protein of interest is not necessarily on the same level as in normal shaking flasks. In case of the CHMO, nearly no enzyme activity was measurable when we started with the library screening. Although the cells were growing very well during the exponential growth phase as well as after induction, SDS–PAGE protein expression analysis of the lysed cells revealed hardly any band that corresponded to the CHMO (Fig. S2). Furthermore, no enzyme activity was measurable due to the low enzyme amount, especially when cultivating at 20°C. Thus, we performed an expression optimization for this particular enzyme on the robotic platform. The expression of CHMO in MTPs was substantially improved by changing from LB to terrific broth (TB) medium. Furthermore, applying smaller amounts of IPTG for induction of protein expression (0.1 mM instead of 1.0 mM) gave the desired success. Additionally, the durations of growing *E. coli* cells and protein expression of CHMO were prolonged to ensure a slow, but soluble enzyme expression. All optimisation steps were visualised by SDS–PAGE analysis (Fig. S3). Moreover, omitting the washing step of the cells further improved the measurable enzyme activity. We successfully verified the crucial improvement of protein expression by SDS–PAGE analysis as well as by the determination of the CHMO activity. We also demonstrated that even sensitive enzymes like the CHMO can be successfully produced and screened in a robotic system and that the established expression protocols are easily transferable to other enzyme classes under their particular conditions. Most importantly, the robotic platform allowed us a detailed monitoring of this improvement as shown in Figure 3.

Cell Lysis

Several chemical and enzymatic methods are used for the production of cell lysates during the screening of protein engineering libraries. These are based on the resuspension of the pelleted enzyme containing cells in a disrupting solution (which normally consists of the reaction buffer containing the appropriate disruption agent), followed by a certain incubation time in a shaker. In the case of chemical methods, these solution (home-made or commercial) contain one or more detergents. Enzymatic methods are normally based on the resuspension of the cells in a solution of

lysozyme (1 mg/mL that digests the cell walls) and DNAse (1 μg/mL) to reduce the viscosity of the resulting cell extracts, thus making their further pipetting easier. There is no ideal disruption method, and its selection depends completely on the stability of the studied enzymatic activity, which is in principle difficult to predict. The enzymatic disruption of the cells offers probably the most reliable option, as no negative interference with the activity of the enzyme studied could be observed. Thus, we recommend its use for cell disruption in first instance, in order to avoid unexpected negative effects derived from the employment of detergents. However, it must be taken into account that these solutions cannot be stored for long time, so it is recommended to prepare them freshly before their use. In principle, the simplest disruption solution could be based on the addition of 0.1% Triton X-100 to the reaction buffer for the enzymatic assay. This approach offers an efficient cell disruption in a very affordable way. In addition, the disruption solution can be stored for long times, even at room temperature. So, if the studied enzyme is able to tolerate these conditions, this preprepared solution can constitute an excellent option. However, in our experience many proteins (like VFL–ATA or CHMO) show very significant decreases or even complete abolishment of their enzymatic activities when 0.1 or 0.05% Triton X-100 is employed for disruption. Commercial chemical disruption solutions, such as BugBuster™, offer in principle good cell disruption rates and are designed to preserve enzymatic activities with higher efficiency. However, we have noticed that BugBuster™ also interferes at some level with certain enzymatic activities (such as VFL–ATA and CHMO, data not shown) and increases the cost of the overall process.

Protein Content Normalisation With the Split-GFP System

Another challenge in the screening of mutant libraries in microtiter plates is the different expression level and solubility of each individual enzyme variant. Hence, false positive hits suggesting, for example, higher specific activity are often simply wild-type enzyme expressed at a higher level. Purification in a 96-well format using immobilized metal affinity chromatography (IMAC) for His-tagged enzymes followed by common protein quantification methods is very expensive and time consuming (Scheich et al., 2003). We have recently developed a tool based on the application of the split-GFP principle (Cabantous et al., 2005) for the quantification of the target enzyme within mutant libraries directly in crude *E. coli* cell lysates. Briefly, a short tag (usually 16 amino acids) is cloned (either C- or N-terminal, depending on the enzyme investigated) to the gene encoding the enzyme of interest and after expression purified GFP (lacking this tag) is added resulting after a short incubation time in the formation of mature GFP enabling quantification of the target protein by measuring fluorescence. Quantification is performed in parallel to the enzymatic activity assay, employing a different 96-well plate for each of them. Together, these data allow the reliable determination of specific activities, as exemplified for the transaminase from *Vibrio fluvialis*. Further details of this procedures including the tolerance of different model enzymes to the GFP11 tagging, and the results obtained have been published already (Santos-Aberturas et al., 2015).

Improving the Acceptance of Bulky Substrates for Amine Transaminase

Amine transaminases (ATA) are an important enzyme class for the asymmetric synthesis of chiral amines (Höhne and Bornscheuer, 2009; Kohls et al., 2014). Unfortunately, natural ATA convert bulky ketones (such as 1b or 1c in Scheme 1) with very low activity or not all. For (*R*)-selective ATA, an intensive protein engineering study performed in a joint effort by researchers from Merck & Co. and Codexis (Redwood City, CA) created a variant of ATA-117 bearing 27 mutations allowing the production of the drug Sitagliptin on industrial scale and at the same time being able to accept isopropylamine as amine donor (Savile et al., 2010). As (*R*)- and (*S*)-ATA belong to different fold classes, the strategy successful for (*R*)-ATA cannot be transfered to identify (*S*)-ATA able to accept bulky ketones. To challenge our robotic screening platform, a first screening of approximately 1,400 (*S*)-ATA variants had been initiated. To reduce incubation times in the platform our standard lab protocol for producing the transaminases was revised: we investigated if the duration of both incubation steps, one for growth and one for expression, could be drastically reduced, but still ensure sufficient enzyme activity. It was further verified, if the complexity of the IPTG induction steps (with an additional pipetting step and movement of many containers) could be replaced by auto-induction of the VFL–ATA applying Zym 5052 medium. Since comparable cell densities were reached with auto-induction medium, this protein expression method was kept. As a result, the overall incubation and handling time were halved (from 40 to 20 h) while maintaining a good activity level of the enzyme using the platform. After these improvements we used the robotic device for mutant library sceening to find an altered amine donor/acceptor spectrum of a (*S*)-selective transaminase. For this two libraries based on the *Vibrio fluvialis* transaminase were created *via* a step wise QuikChange® (Stratagene, La Jolla, CA) mutagenesis approach. Library A covered three target sites: F19, F85, and F86, the sterically limiting residues located in the so-called small binding pocket. The primer pairs applied included degenerate codons, which encode for A, V, S, and F (see Table S2 for the primer sequences used in this study), creating a library with 81 mutants. Library B covered four target sites: L56, W57 and R415, L417, the sterically limiting residues located in the so-called large binding pocket. The degenerate codons at each target site encode for the following amino acids: A/L/P/V at residue 56, C/L/F/W at residue 57, R/C/L/F at residue 415 and A/L/P/V at residue 417, creating a library of 256 mutants. The robotic platform was then used to prepare and screen 280 colonies from library A and 1,120 colonies from library B for activity against the following donor:acceptor pairs: (*S*)-1-phenylbutylamine:pyruvate and (*S*)-1-phenylbutylamine:pentanal. Whilst no mutants from either library showed increased activity for (*S*)-1-phenylbutylamine:pyruvate, five mutants from library B showed higher than wild-type activity for (*S*)-1-phenylbutylamine:pentanal. These active mutants were sequenced and cultivated on a larger scale for purification and specific activity determination. The activities against the two acceptor:donor pairs are shown in Table I. Notably, two of these mutants showed >3-fold higher than WT activity towards (*S*)-1-phenylbutylamine:pentanal, M2 (W57F/R415L/L417V) and M3 (W57F/R415L). It appears that the

Table I. Specific activities of transaminase hits identified accepting pentanal instead of pyruvate.

		U/mg[a] (fold increase relative to wild type)	
Mutant ID	Mutations	Pyruvate	Pentanal
WT	—	0.86	0.28
M1	L56V/R415C	0.00 (0.003)	0.43 (1.53)
M2	W57F/R415L/L417V	0.00 (0.003)	0.95 (3.39)
M3	W57F/R415L	0.00 (0.003)	0.86 (3.08)
M4	R415L	0.00 (0.005)	0.52 (1.86)
M5	L56V/W57F	0.03 (0.035)	0.31 (1.11)

[a]Reaction conditions: 1 mM amine, 2 mM acceptor, 10% DMSO, pH 9, 30°C, measured at 242 nm.

combined replacement of W57 and R415 [the flipping arginine (Steffen-Munsberg et al., 2013)] with hydrophobic residues unable to form the stabilizing interactions with pyruvate found in the wild-type enzyme, leads to specificity towards pentanal as the preferred amino acceptor over pyruvate. These findings not only confirm the importance of the flipping arginine in dual substrate recognition, but we also identified mutants that can convert an aldehyde. Further investigation of the substrate scope of these interesting mutants has been reported elsewhere (Genz et al., 2015).

Reduced Inhibition and Enhanced Long-Term Stability of CHMO

A completely different enzyme optimisation task for the robot was pursued for the CHMO. Instead of improvement of enzymatic activity, here the reduction of product inhibition and the enhancement of long term stability moved into focus. Furthermore, this is also an example how to handle more delicate enzymes in the screening platform: The major challenge for the application of Baeyer–Villiger monooxygenases such as the cyclohexanone monooxgenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 in organic synthesis is the instability of these enzymes and severe substrate and product inhibition. Especially for the synthesis of the bulk chemical ε-caprolactone from cyclohexanone (Scheme 1), such limitations are unfortunate due to the high application potential of this compound, for example, for polymer synthesis, for which we recently reported an enzyme cascade synthesis (Schmidt et al., 2015). In contrast to a rational protein design approach, which is hampered by the lack of a crystal structure of this particular CHMO, these limitations can be addressed by directed evolution—the combination of error-prone PCR (epPCR) with high-throughput screening. Thus, a mutant library of the CHMO was prepared by epPCR and screened for both long-term stability and reduced product inhibition (Fig. 4) using the robotic platform. These mutant libraries were generated at different mutation frequencies (>9 mutations per kb in one library and <9 mutations per kb in the two other libraries). Each library included ~1,100 clones in which the wild type was present at 4.1–10.6% depending on the mutation rate. Screening of the epPCR-mutant libraries identified a total of ten variants with improved long-term stability. Screening of the libraries for reduced product inhibition identified initially 56 putative hits. Subsequent analysis resulted in six variants with reduced product inhibition, which all contain besides other variations three mutations (2 × R60K, 3 × D264A, 2 × D267A) suggesting that these amino acid positions are crucial for the enzyme's stability and/or activity. After a deeper characterization of the putative hits, the best identified variant showed a 2-fold higher resistance against 600 mM product (ε-caprolactone) compared to the wild-type enzyme.

Implementing a Selection Assay for HLD

In a first step towards a continuous selection system for molecular evolution experiments (Fig. 7), the robotic platform was programmed to screen for dehalogenase activity. Haloalkane dehalogenases (HLD) are useful enzymes for industrial, environmental or fine chemical applications. Removing selectively halides from haloalkane compounds by cleaving the carbon-halogen bond leads to enantiopure chemicals. Additionally, haloalkanes are common pollutants that can be degraded to harmless alcohols resulting in the generation and/or release of growth substrates. To find appropriate enzyme variants for this purpose, a selection methodology was established for the selective accumulation of *E. coli* cells expressing active HLDs (Fibinger et al., 2015). In this study the activity of DhaA (Newman et al., 1999) and DhlA (Janssen et al., 1989) were destroyed by substituting catalytically necessary residues. Consequently, these positions were targeted by site-directed saturation mutagenesis. The resulting mutant library was applied to a selection methodology using toxic haloalkanes. The phenol red assay (Holloway et al., 1997) was chosen for finally analysing the surviving cells in a simple high-throughput assay to cover the whole libraries. The adaptation to the robotic platform

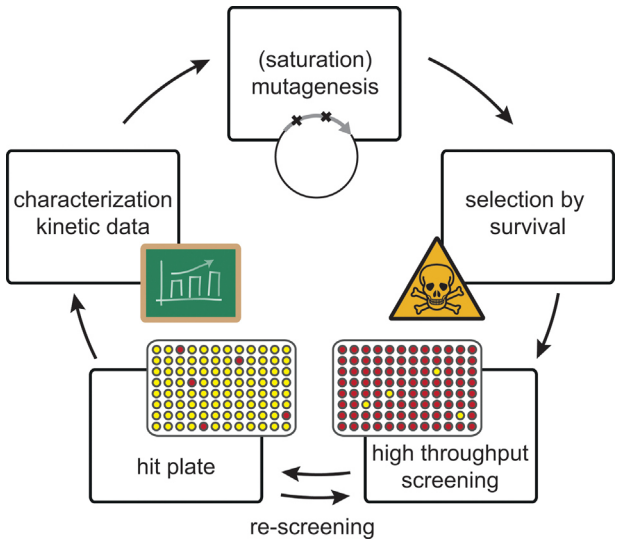


Figure 7. Engineering cycle for iterative selection and screening of haloalkane dehalogenases. The created mutant library undergoes selection by exposure to toxic haloalkane substrate. Surviving cells were screened with the high-throughput phenol red assay, hits were pooled and screened again to minimize the content of false-positive variants. Final hits can be further analysed separately to determine their characteristics and kinetic data.

required as described above optimization of the critical steps for cellular growth, protein expression, buffer composition, and also substrate and dye concentration. The successful accumulation of revertants in a continuous selection process (Fig. 7) was exemplified for a knock-out library (Fibinger et al., 2015). The statistical portion of wild-type revertants—containing the re-introduced residues in the active site—in the libraries was 0.26–3.125%. First, selection was performed under standard culture conditions followed by screening the libraries containing surviving cells in the platform in microtiter plates using the phenol red assay. Here, specific signals for wild-type revertants (yellow colored wells) and inactive clones (orange-red wells) were found. The genotype of cells in random wells was checked and only wild-type DhaA/DhlA was found to produce positive signals. No false positives were found, which could potentially be wrongly identified by medium acidification through metabolic changes. 1 mM of HEPES buffer seemed to be the best condition for the given substrate concentration, the application of a substrate mixture and the used whole cell assay. After screening 166 MTPs of the selection-based evolutionary process a strong accumulation of revertants was found by the automatized phenol red assay. Further details have been already reported elsewhere (Fibinger et al., 2015).

Improving Aminoacylase Activity

The application of combined mutations to improve enzyme activity and the applicability of the calcein assay was investigated with the help of the robotic platform within the aminoacylase project. For this, mutations in the acyl-binding pocket of porcine aminoacylase 1 (pAcy1) were combined to identify more active variants towards *N*-benzoyl-L-methionine (NBM). Lindner et al. (2008) predicted that the residues T345, L370 and to a minor degree L176 restrict the acyl-binding pocket of pAcy1 and therefore the conversion of *N*-acyl-L-amino acids with bulky acyl groups. Here, T345G served as basis for a mutant library (L176X, T345G, L370X) as the variant T347G in the highly homologous human aminoacylase 1 showed much higher activity towards NBM than the wild type (Lindner et al., 2008).

In the screening 34 out of 1,450 clones (2.3%) indicated higher activities than T345G; sequencing of the 18 most active ones revealed quite high variations at both randomized position (Table S2). These results were proven by a second calcein assay with five purified variants (Fig. 8). HPLC analysis of biocatalysis reactions with the most active variant T345G/L370I confirmed that its specific activity (4.44 ± 0.46 U/mg) is almost 3-fold higher than that of T345G (1.57 ± 0.19 U/mg) and about 34-fold higher than that of the wild type (0.13 ± 0.0 U/mg). All in all these results are also in agreement with the predictions made by Lindner et al. (2008). Thus, the robotic platform could be successfully used to identify several variants of pAcy1 in a mutant library having higher activities towards the bulky substrate NBM, while focusing the mutations on the spatially restricted acyl-binding pocket. It should be noted that the activities of pAcy1 and its variants towards NBM were too low for continuous measurements in the microtiter plate reader. Therefore multiple point measurements (0, 40, 80, 120 min) were made and in-between the assay, plates were stored in the incubation shaker at 37°C.

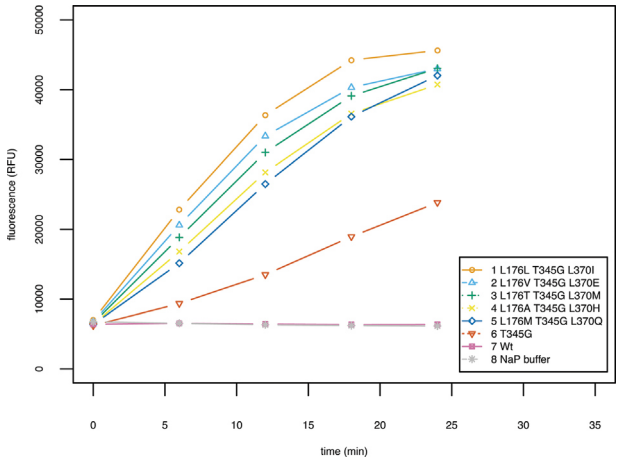


Figure 8. Calcein assay measurement of five pAcy1 variants with improved activities towards the bulky substrate NBM. Wild type (Wt) and T345G served as reference variants. NaP buffer was used to determine the background activity. Calcein assay measurements of five selected improved pAcyl variants.

Discussion

As already outlined in the introduction, microfluidic or cell sorting methods such as FACS can ensure ultrahigh-throughput of huge mutant libraries at high speed, but provide only limited information about the hits identified, for instance because only an artificial substrate can be used leading to a fluorescent product or because only endpoint measurements are possible. Therefore these methods are only useful for a prefiltering of novel enzymes derived from metagenome or mutant libraries. Furthermore, a major trend in protein engineering is to create "*small, but smart*" mutant libraries guided by bioinformatic tools and thus only a few thousand variants are usually investigated. Hence, screening of variants in the microtiter plate format is commonly the method of choice, especially as this can provide detailed information about the enzymes/variants, such as kinetic data, and allow the use of the "true" substrate and reaction conditions close to the real problem to be solved. Nevertheless, this involves many laborious steps required to express and screen a few thousand variants in MTPs. To address this challenge, we successfully set-up and implemented a robotic platform, which enables the coverage of all required steps in enzyme production and biochemical characterization necessary for the study of enzyme mutant libraries (or a series of novel enzymes identified by metagenome approaches). This wide applicability of the platform was demonstrated for four non-related enzyme classes covering a range of rather common protein engineering tasks: alteration of substrate scope, improved long term stability, reduced product inhibition, improved activity and also the implementation of a selection strategy. In all mutant libraries created to address these characteristics, we could identify a considerable number of improved variants (Table II).

The major benefit of the platform is the liberation of the researcher from routine work and at the same time providing a reproducible and precise handling of the clones/enzymes during all

Table II. Screening results for the featured projects—overview.

Enzyme class	Number of clones	Results
Transaminase	Library A: 280 Library B: 1,120	Five clones with improved substrate spectrum in library B
CHMO	4,200	10 mutants with improved long-term stability Six mutants with lowered product inhibition
HLD	14,600	Discrimination between active and inactive clones
Acylase	1,450	34 clones with higher activity

steps from expression to final characterization of the biocatalyst. We estimate that the throughput per week compared to manual handling of clones, microtiter plates and enzyme assays is at least increased 2–3 fold, whereas the human labor part is substantially reduced once the protocol has been implemented into the robotic platform. These savings in time and labor can also compensate in part for the higher investment costs required for the robotic platform and the process automatization. Furthermore, the documentation of all steps combined with the fast processing of the resulting data in a standardized manner allows the researcher to primarily focus on the creative work of the scientific process, like identifying the best suited experiments, designing optimal mutant libraries and finally interpreting the results of the experiments. The detailed analysis and the automated set-up of the robotic platform enabled us to address many critical steps such as even cell growth across the entire MTP, time point of induction, subsequent expression time and suitable methods for cell disruption to obtain the enzyme of interest for final assay measurements. The detailed protocols developed here are for sure also useful for researchers who do not have access to a robotic platform as they hopefully allow them to better identify critical steps and use our solutions to address issues related to cell growth, expression and enzyme isolation.

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Authors' Contribution

A.H., D.B., U.T.B., and M.D. planned the set-up of the robotic platform. M.D. and M.V. optimized the robotic platform. M.D. wrote the programs for process generation and evaluation and visualisation of the data. M.D., M.V., S.S., C.V., D.L., M.P.C.F., U.T.B., and J.S-A. planned and performed the experiments and analysed the data. M.D., S.S., C.V., D.L., M.P.C.F., J.S-A., and U.T.B. wrote the manuscript. U.T.B. supervised the work.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supporting information

Fully Automatized High-Throughput Enzyme Library Screening Using a Robotic Platform

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Materials and Methods

Chemicals, Strains and Plasmids

All chemicals were of analytical grade or higher quality and purchased from Sigma-Aldrich (Steinheim, Germany), VWR (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany) unless specified otherwise. Primers were ordered from Invitrogen/Life Technologies, microtiter plates (MTPs) from Greiner (96 round wells, flat bottom, BioOne 655101). The plasmid pET24b containing the gene encoding the (*S*)-ATA from *Vibrio fluvialis* (UniProt: F2XBU9) bearing an additional C-terminal His6-tag was kindly provided by Prof. Byung Gee Kim (Seoul National University, South-Korea).

Table S1: Expression strains used.

Enzyme class	Expression strain	Plasmid
Transaminase	BL21 (DE3)	pET24b
CHMO	BL21 (DE3)	pET28a(+)
HLD	BL21 (DE3)	pET11a, pET21b, pET28a
Acylase	BL21 (DE3)	pET52(b)

Library Generation

Amine Transaminase (ATA) Library

Preparation of Libraries A and B:

For the generation of the mutant libraries, degenerate primers containing the desired codons were used (Table S2). The library was created using site-directed mutagenesis of the VF-ATA gene using the standard QuikChange™ mutagenesis method. After PCR, the reaction mixtures were digested for 2 h at 37°C with *Dpn*I, followed by transformation into *E. coli* TOP10. After overnight growth on agar plates supplemented with kanamycin (50 µg/mL), all clones were washed off from the plate and used for 5 mL overnight cultivation (LB-media, supplemented with kanamycin 50 µg/mL). Plasmids were isolated from the 5 mL overnight culture (innuPREP Plasmid Isolation Kit, Analytik Jena, Germany) and the quality of the library was verified by sequencing.

Table S2: Degenerate primer sequences used for QuikChange™ mutagenesis of the amine transaminase from *Vibrio fluvialis* (VF-ATA), to create libraries A and B.

Mutations		**Primer sequence**
F19A/V/F/S	fw	CTATTCGCTCTATGGTKYCACCGACATGCCTTC
	rv	GGCATGTCGGTGRMACCATAGAGCGAATAGGTC
L56A/L/P/V and W57C/L/F/W	fw	CTCGGGCSYGTKSAACATGGTCGCGGGCTTTGACC
	rv	CCATGTTSMACRSGCCCGAGTTGGCGTCCAGATAACGCC
F85A/V/F/S and F86 A/V/F/S	fw	CCCGGTTATCACGCCKYTKYCGGCCGCATGTCCGATCAGACG GTAATGCTGTCGG
	rv	GGACATGCGGCCGRMARMGGCGTGATAACCGGGAAAACGCT CGTATTGGGC
R415R/C/L/F and L417A/L/P/V	fw	GATTTGCYKCCCGSYTGGTCAGTCCGTCGTCC
	rv	CTGACCARSCGGGMRGCAAATCAGCCCCAGATCGGTGC

The library of plasmids was then transferred into chemical competent *E. coli* BL21(DE3) by the heat shock method (Chung et al., 1989), and the transformed library was directly plated out on LB agar supplemented with Kanamycin (50 µg/mL).

Creation of the CHMO Error-Prone PCR Mutant Library

Error-prone PCR

For creation of the CHMO mutant libraries the GeneMorph® II Random Mutagenesis kit (Stratagene) was used. Three different approaches with varying DNA template concentrations from 50, 100 and 200 ng were used to generate libraries with different mutation frequencies. The following primers were used for amplification: 5´-CGCGGATCCAT GTCACAAAAAATGGATT-3` (CHMO_*Bam*HI_fw) and 5´-ATTCTTATGCGGCCGCTTAGGC ATTGGCAGGTTGC-3` (CHMO_*Not*I_rv). The composition of the PCRs is shown in Table S3.

Table S3: Composition of the error-prone PCRs.

Compound	**Volume** [µL]
Mutation buffer (10x)	5
dNTPs	1
pET28a_CHMO (101 ng/µL)	0.5-2.5
CHMO_*Bam*HI_fw (10 pmol)	2.5
CHMO_*Not*I_rv (10 pmol)	2.5
Polymerase	1
dH_2O	add to 50

Table S4 shows the temperature program used for the PCRs.

Table S4: Temperature program used for the error-prone PCRs.

Step	**Time** [s]	**Temperature** [°C]	**Cycles**
Denaturation	300	95	1
Denaturation	45	95	30
Annealing	45	58	
Extension	120	72	
Final extension	600	72	1

The epPCR products were analyzed on an agarose gel (1%). The total reaction volume (50 µL) was purified and concentrated to a final volume of 20 µL using the MiniElute® PCR Purification kit (Thermo Fisher).

MEGAWHOP

The MEGAWHOP PCR was composed as shown in Table S5. To 40 µL of the total reaction volume, 10 µL purified PCR product were added. The temperature program for the reaction is shown in Table S6.

Table S5: Composition of the MEGAWHOP PCR.

Compound	**Volume** [µL]
Buffer B (10x)	15
dNTPs (each 10 mM)	3
pET28a_CHMO (101 ng/µL)	2
Pfu Plus! Polymerase	3 (15 U)
dH_2O	add to 150

Table S6: Temperature program for the error-prone PCRs.

Step	**Time** [s]	**Temperature** [°C]	**Cycles**
Denaturation	300	95	1
Denaturation	45	95	18
Annealing	45	55	18
Extension	426	72	18

Preparation of the pAcy1 Mutant Library

The mutant library was based on the variant T345G, which was obtained by site-specific mutagenesis of the wild type plasmid (Wardenga et al. 2008). Consecutively, the positions L176 and L370 were randomized followed by *Dpn*I digestion of parental plasmids, electroporation of *E. coli* TOP10 (Dower et al. 1988) and plasmid isolation. Finally, *E. coli* BL21 DE3 pGro7 was transformed by electroporation. Transformants were selected on LB agar plates (100 µg/mL ampicillin; 25 µg/mL chloramphenicol).

Table S7: Primers used for library preparation.

Primer	Sequence
T345G_fw	5‘-CGGCGAGCGGCGATGCGCGTTATATTCGTGC-3‘
T345G_rv	5‘-GCGCATCGCCGCTCGCCGGGCAAATTTCC-3‘
L176X_fw	5‘-GGGCTTTGCGCTGGATGAAGGCNNKGCCAGCCCG-3‘
L176X_rv	5‘-CGGTAAACGCATCGGTCGGGCTGGCMNNGCCTTCATC-3‘
L370X_fw	5‘-GATGAACCATACCCCGGTGCTGNNKCATGATCATG-3’
L370X_rv	5’-GCAGACGTTCATCATGATCATGMNNCAGCACCG-3’

Table S8: Composition of the PCRs.

Compound	Volume [µL]
Polymerase reaction buffer B (10x)	5
dNTPs	1
Parental plasmid (ca. 50 ng/µL)	1
Primer_fw (10 pmol)	2.5
Primer_rv (10 pmol)	2.5
Pfu polymerase	1
Dimethyl sulfoxide	1.5
dH_2O	add to 50

Table S9: Temperature program used for the PCRs.

Step	Time [s]	Temperature [°C]	Cycles
Denaturation	180	95	1
Denaturation	60	95	18
Annealing	50	65	
Extension	386	72	
Final extension	300	72	1

HLD Saturation Library

A knockout library for DhaA and DhlA was created as described before (Fibinger et al. 2015). The plasmids obtained were pooled and enriched via *E. coli* TOP10 transformations and finally brought into the host strain *E. coli* BL21 (DE3). The random substitution of essential residues of the catalytic triad was confirmed by sequencing.

Enzyme Production

Expression of ATA

Master screening plates were prepared as follows: 16 sterile 96-well microtiter plates were charged with 200 µL/well of LB media (supplemented with Kanamycin 50 µg/mL). The wells were inoculated with the transformed library (1 colony/well) and incubated for 18 hours at 30 °C, 700 rpm as described above. Expression plates were prepared as follows: 16 sterile 96-

well microtiter plates were charged with 180 µL/well of TB media (supplemented with Kanamycin 50 µg/mL), the master plates were then duplicated onto the expression plates using the Colony Picker, the expression plates were then incubated on a gyratory shaker for 8 hours at 37°C, 600 rpm (the master plates were stored as a glycerol stock at –80°C). The expression was then induced upon addition of 20 µL/well of TB media (supplemented with IPTG, 0.1mM), and incubated on a gyratory shaker at 20°C, 600 rpm for 16 hours.

Expression, Purification and Analysis of pAcy1

Enzyme expression and purification of pAcy1 were performed according to Wardenga et al. (2008). For the expression in MTPs, the autoinduction medium ZYP-5052 was used (Studier 2005). Hence, the MTPs were only transferred from the 37°C to the 20°C incubation shaker at the beginning of the exponential phase for soluble and active expression. Protein contents of purified samples were determined with BC Assay Quantitation Kit (Interchim, Montlucon, France), bovine serum albumin served as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4 % stacking gel; 12.5 % resolving gel) was applied to visualize the protein expression and purity.

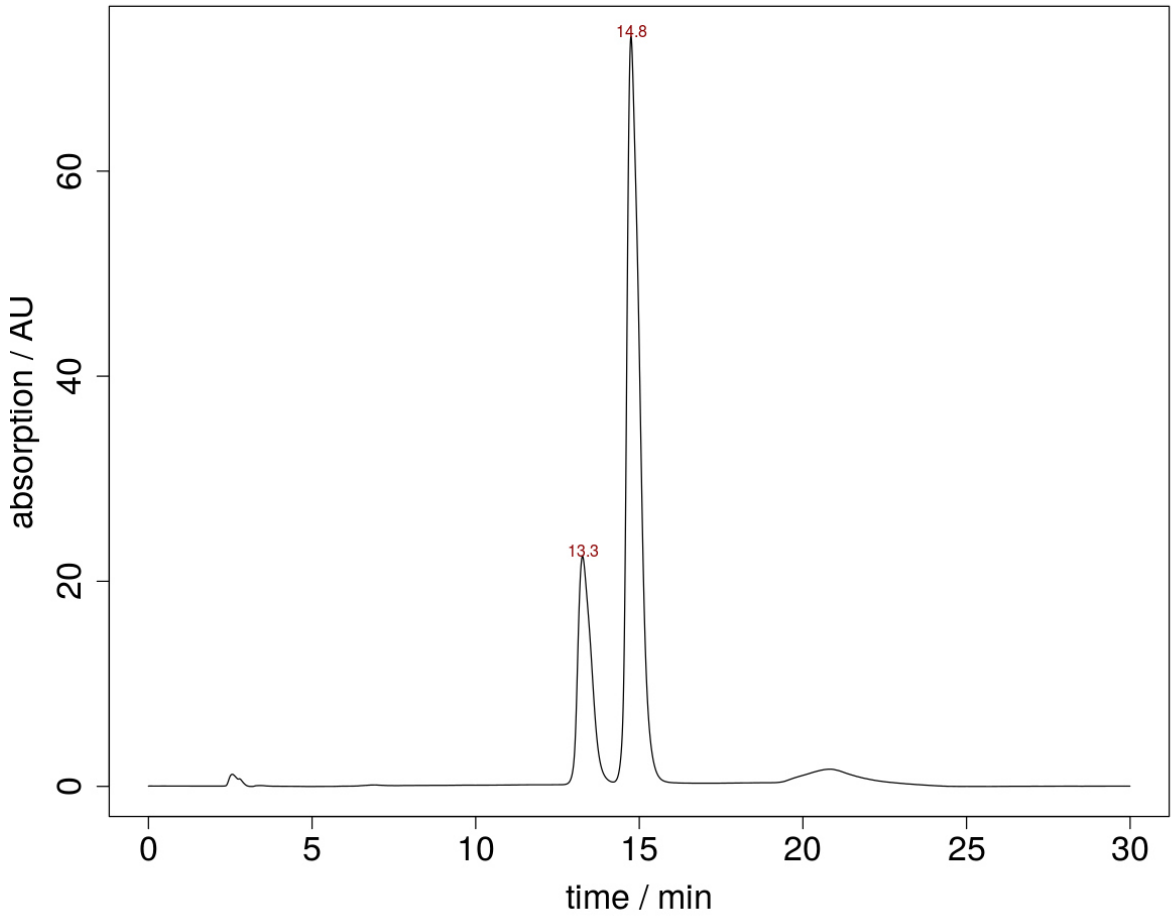

Figure S1: HPLC chromatogram for the analysis of the conversion of NBM (14.8 min) to benzoic acid (13.3 min) by pAcy1 variant T345G L370I after 30 min of biocatalysis.

Expression of CHMO variants

The master screening plates, transformed into BL21 (DE3), were prepared as follows: colonies from 16 sterile MTPs were picked and grown at 37°C and 500 rpm overnight in MTPs charged with 160 µL LB media (supplemented with Kanamycin 50 µg/mL). For protein expression, the transformed library (1 colony/well) and incubated MTP was replicated by cultivating the plate in the cytomat for 18 hours at 20°C and ca. 700 rpm. CHMO expression plates were prepared as follows: 16 sterile 96-well microtiter plates were charged with 180 µL/well of TB media (supplemented with Kanamycin 50 µg/mL), the master plates were then replicated into the expression plates using the Colony Picker, these expression plates were incubated in the cytomat for 9 h at 37°C and 700 rpm (the master plates were stored as a glycerol stock at –80 °C). The enzyme expression was induced by addition of 20 µL/well IPTG in TB media (final concentration of 0.1 mM), and incubated in the cytomat at 20°C and 700 rpm for 16 hours.

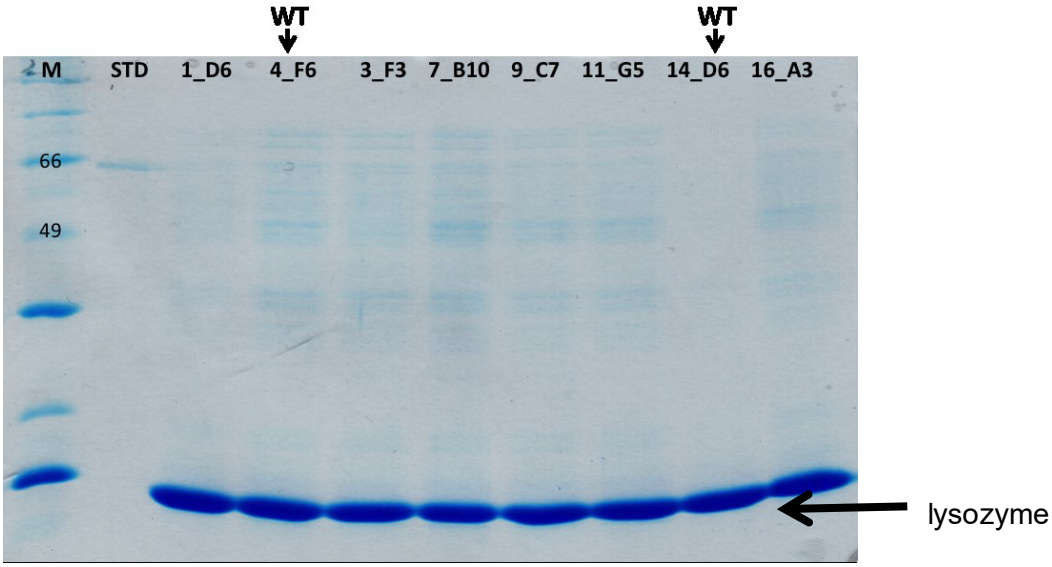

Figure S2: SDS-PAGE protein expression analysis of randomly picked variants from the mutant library (after cell lysis) prior to expression optimization. The expression was routinely performed in LB medium using 1.0 mM of IPTG for protein induction. For each variant and the wild type (WT) a band at 66 kDa, corresponding to the CHMO, should appear, but due to insufficient protein expression under these conditions it is not present. Protein-Marker: RotiMark™ Standard.

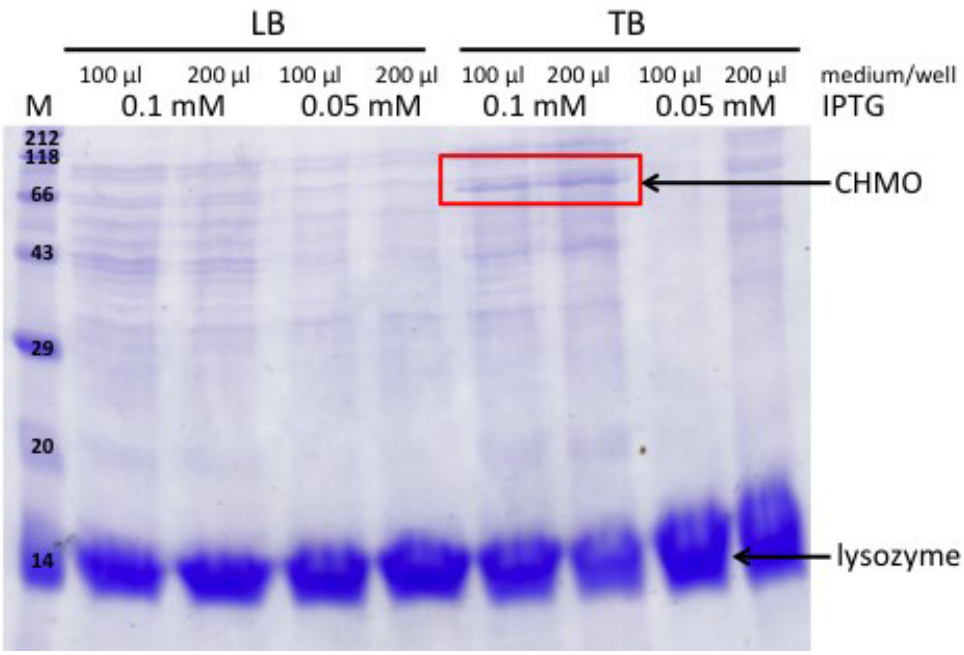

Figure S3: SDS-PAGE analysis after expression optimization. The best soluble expression was achieved with TB medium and 0.1 mM IPTG for induction of protein expression. Furthermore, a total volume of 200 µL per well resulted in a higher amount of soluble enzyme. The red box indicates the CHMO at 66 kDa. Protein-Marker: RotiMark™ Standard.

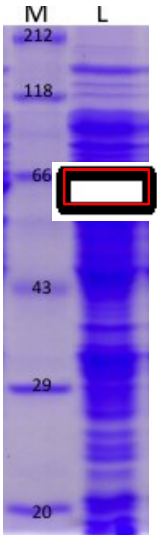

Figure S4: SDS-PAGE analysis of shaking flask expression of the CHMO. L = Lysate, Marker: RotiMark Standard.

Lysis of the ATA library

Lysate plates were prepared as follows: the expression plates were centrifuged for 45 min at 4500 rpm at 4°C and the supernatant removed. The pellets were then washed: the pellets were resuspended in 30 µL/well of CHES buffer (50 mM, pH 9, supplemented with pyridoxal-5'-phosphate (PLP), 8 µM), and then centrifuged for 45 min at 4500 rpm at 4°C and the supernatant removed. The pellets were then resuspended in 30 µL/well of lysis buffer (CHES buffer 50 mM, pH 9, supplemented with Lysozyme 1 mg/mL, DNAse 1µg/mL), and incubated for 3 h at 30°C. The plates were then screened immediately.

Lysis of the CHMO library

Lysate plates were prepared as follows: the expression plates were centrifuged for 10 min at 2200*g* at 4°C and the supernatant removed. The cell pellets were resuspended in 100-200 µL of the prepared lysis buffer (1 mg/mL lysozyme + 1 mL/mL DNase I stock solution in assay buffer). The cell pellets were lysed for 3 h at 30°C with agitation. After lysis, MTPs were centrifuged for 10-15 min at 2200*g* at 4°C to pellet the cell debris and clarify the lysate. The lysates were kept cooled and stored at 4°C until they were assayed.

Lysis of the pAcy1 library

The lysis of *E. coli* cells was mediated by resolubilization in 200 µL of a 100-fold dilution of BugBuster 10X Protein Extraction Reagent (Merck KGaA) in sodium phosphate buffer and subsequent incubation for 2 h at 30°C, respectively.

UV-vis absorption measurements

All UV-vis absorption measurements have been standardized to 10 mm optical path length d by using the equation **(1)** for the volume of an ideal cylinder V multiplied by a correction factor (f_{minisc}) that corrects for the meniscus as an approximation. f_{minisc} depends on the surface tension of the liquid and was set to 0.95 as a first order approximation for all experiments (the average well radius r was approximated to 0.34 cm).

$$d\ [cm] = V\ [\mu L]\ f_{minisc} / (1000\ \pi\ r^2\ [cm^2])\quad \textbf{(1)}$$

Cell growth with colony picker help

Cells in the master plate were grown for 4-5 hours and afterwards this master plate was replicated by the colony picker and served as backup plate. The small needle tips of the colony picker transferred a tiny fraction (sticking to the small needle tips) of seeding cells evenly into the fresh expression plate, which grows very evenly as illustrated in Fig. 3. This formed the basis for a comparable protein expression level of all wells.

LARA software

LARA is a software that was completely programmed at the Institute of Biochemistry in Greifswald. It is open source and for academia freely available at lara.uni-greifswald.de. It is written using the free and open source tools python with Qt4, SQLite (www.sqlite.org)/PostgresSQL(www.postgresql.org) and django (www.djangoproject.com) python modules, and the statistical software package R (R-cran-project.org, Ver. 2.14). It consists of three major components: firstly a process planning tool, secondly a program generator for the robots, and as a third component a data evaluation and visualisation pipeline. All parts are programmed in modules that can be included into the program core as plugins. This makes the core system very extensible. The planning tool is a stand-alone python/Qt4 application, which uses a graphical user interface with a bio-process paradigm. That means that the user formulates the process as he would plan it in the laboratory, like, e.g., incubate 16 plates at a certain temperature, induce protein expression on these plates with a special inducer, harvest the cells, lyse the cells and do a crude cell lysate assay. This "biochemist's natural language" formulation is translated by LARA into robot programs, applying a simple compiler that uses the visitor-pattern algorithm for code generation. Since

processes are represented in an abstract, system independent data structure, any code for a robotic device can in principle be generated if the target syntax is documented by the robot constructor. These generated programs can be loaded and executed by the commercial robot control software (e.g., Thermo Momentum, Agilent VWorks) that control actually the robots. The code generator was written to overcome many limitations of these commercial softwares and draw the connection to the database that holds the data of all measurements. The evaluation daemon is constantly supervising a dedicated directory for new files. When a device in the platform finishes a measurement, the raw data will be copied to this directory and be analysed. Data analysis plugin modules are triggered according to the content of the file and its association to a certain experiment using the UNIX notify mechanism. The plate barcodes, measurement output filenames and layouts help the system to decide what to evaluate. R scripts calculate, e.g. the best linear slopes for each well, compare the slopes to the mean of reference measurements and generate a graph with the best 15 slopes or an overview plot of the growth for each individual well of a microtiterplate. These evaluation modules and database entries can also be used to combine different measurements resulting from different devices or measurement types, like enzyme activity and fluorescence to evaluate the split-GFP normalization. These results are then presented to the user, using the powerful django database abstraction and dynamic web presentation framework in combination with the apache webserver (httpd.apache.org) or nginx webserver (nginx.org).

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8.3. Article III

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METHODS AND PROTOCOLS

Highly efficient and easy protease-mediated protein purification

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Abstract As both research on and application of proteins are rarely focused on the resistance towards nonspecific proteases, this property remained widely unnoticed, in particular in terms of protein purification and related fields. In the present study, diverse aspects of protease-mediated protein purification (PMPP) were explored on the basis of the complementary proteases trypsin and proteinase K as well as the model proteins green fluorescent protein (GFP) from *Aequorea victoria*, lipase A from *Candida antarctica* (CAL-A), a transaminase from *Aspergillus fumigatus* (AspFum), quorum quenching lactonase AiiA from *Bacillus* sp., and an alanine dehydrogenase from *Thermus thermophilus* (AlaDH). While GFP and AiiA were already known to be protease resistant, the thermostable enzymes CAL-A, AspFum, and AlaDH were selected due to the documented correlation between thermostability and protease resistance. As proof of principle for PMPP, recombinant GFP remained unaffected whereas most *Escherichia coli* (*E. coli*) host proteins were degraded by trypsin. PMPP was highly advantageous compared to the widely used heat-mediated purification of commercial CAL-A. The resistance of AspFum towards trypsin was improved by rational protein design introducing point mutation R20Q. Trypsin also served as economical and efficient substitute for site-specific endopeptidases for the removal of a His-tag fused to AiiA. Moreover, proteolysis of host enzymes with interfering properties led to a strongly improved sensitivity and accuracy of the NADH assay in *E. coli* cell lysate for AlaDH activity measurements. Thus, PMPP is an attractive alternative to common protein purification methods and facilitates also enzyme characterization in cell lysate.

Keywords Protein purification method · Protease resistance · Proteolysis · Thermostability · Site-specific cleavage · NADH activity assay

Introduction

Protein-based processes and products are of steadily increasing importance, for instance in biocatalysis and as therapeutical proteins. In order to meet the requirements of their characterization and application, proteins usually get purified to an appropriate degree. This downstream processing has become one of the main issues of the overall protein production process with regard to capacity and costs (Thommes and Etzel 2007; Straathof 2011).

Common protein purification methods depend either on general protein properties (like the ionic character of the surface) or on specific ones (like metal affinity tags for recombinant proteins) (Janson 2011; Gagnon 2012). Although affinity tags usually enable quite high purities in only one step, the additional amino acids can interfere with the expression, stability, biological function, and application-related regulations of the protein of interest (POI) (Pina et al. 2014). Moreover, purification methods which are based on general protein properties and preferentially do not involve column chromatography, are often favored at industrial scale due to substantially lower costs. Nevertheless, resistance towards nonspecific

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proteases remained widely unnoticed as an alternative protein property for purification and related purposes.

Under native conditions only a limited number of surface-exposed peptide bonds can get cleaved by proteases (Hubbard 1998). Not only the surrounding amino acid sequence has to match the specificity of the protease, but also the side chains of the amino acids (for recognition) and the peptide bond (for cleavage) have to be accessible by the protease. For instance, peptide bonds in flexible regions like loops and termini are strongly preferred over those that are located within secondary structures, like α-helices (Hubbard et al. 1994). Protease resistance also correlates with thermostability (Daniel et al. 1982; Ahmad et al. 2012), an important property for instance for many enzyme applications (Haki and Rakshit 2003; Elleuche et al. 2014). Besides inherent protease resistance, this feature can be engineered by rational design or directed evolution (Wyss et al. 1999; Harmsen et al. 2006; Hussack et al. 2011; Ahmad et al. 2012).

The present study proposes the method of protease-mediated protein purification (PMPP) employing cheap non-specific proteases. The process of PMPP consists of two steps: First, the addition of a nonspecific protease to a protein mixture (i.e., a crude cell extract) leads to the digestion of non-resistant proteins, while the protease-resistant POI remains unaffected. In the second step, the resulting peptides are removed, for instance by ultrafiltration or dialysis. Alternatively, the first step can be directly followed by other purification techniques such as size exclusion chromatography, in order to obtain even higher purity levels than with PMPP alone. An inactivation step of the protease by an irreversible inhibitor and/or protease removal can also be included.

Five model proteins were chosen to evaluate the applicability of PMPP, and to investigate diverse related aspects of this method. Green fluorescent protein (GFP) served as a model protein as it was already described to be resistant towards several proteases (Ward 2005), and due to the easy monitoring of its biological function. Quorum quenching lactonase AiiA variant AI96, a prospective therapeutical protein, was described to resist the protease-rich environment of the intestine (Cao et al. 2012). Three thermostable enzymes, namely lipase A from *Candida antarctica* (CAL-A) (Høegh et al. 1995; Dimitrijevic et al. 2012; Müller et al. 2015), a transaminase from *Aspergillus fumigatus* (AspFum) (Höhne et al. 2010; Thomsen et al. 2014; Skalden et al. 2015), and an alanine dehydrogenase from *Thermus thermophilus* (AlaDH) (Vali et al. 1980), were included due to the correlation between both properties. Trypsin and proteinase K were chosen as proteases due to their complementary specificities; trypsin cleaves peptide bonds at the C-termini of the basic amino acids arginine and lysine, whereas proteinase K prefers aromatic and aliphatic amino acids (Keil 1992; Pan et al. 2014).

Materials and methods

Consumables

All chemicals and enzymes were purchased from Sigma-Aldrich (Steinheim, Germany), New England Biolabs (Frankfurt am Main, Germany), Fermentas (St. Leon-Rot, Germany), Cayman Europe (Tallinn, Estonia), Thermo Fisher Scientific (Braunschweig, Germany), Sartorius (Göttingen, Germany), Carl Roth (Karlsruhe, Germany), or Fluka (Buchs, Switzerland) unless otherwise specified. Trypsin from porcine pancreas (1473 U/mg solid towards N_α-benzoyl-L-arginine ethyl ester hydrochloride; ≤0.1 % chymotrypsin) and proteinase K from *Tritirachium album* (40 U/mg towards urea-denatured hemoglobin) were supplied by Sigma-Aldrich. CAL-A lyophilizate was provided by Roche (Basel, Switzerland). Protein contents were determined with the BC Assay Quantitation Kit (Interchim, Montlucon, France) with bovine serum albumin as standard.

Plasmids and plasmid constructions

Constructs for the recombinant expression of AspFum [GenBank: XP_748821] (Höhne et al. 2010) and GFP variant sg11 [GenBank: 1EMC_A] (Palm et al. 1997) originated from previous studies. All primers were ordered from Life Technologies (Darmstadt, Germany) and their sequences are provided in the Supplementary Material (Table S1). Primers R20Q_fw and R20Q_rv were used to obtain AspFum variant R20Q by standard method of site-directed mutagenesis (Liu and Naismith 2008). The codon-optimized synthetic gene *aiiA* [GenBank: KT726963] was supplied by Life Technologies (Darmstadt, Germany). It was cloned into pET28b(+) by restriction enzymes *Nco*I and *Xho*I (both restriction sites framed *aiiA*). The N-terminal His-tag was introduced with primers M-His_6-EVAR-AiiA_fw and M-His_6-EVAR-AiiA_rv, also encoding a theoretically trypsin-sensitive linker. The resulting plasmid was further modified with the primer pairs M-His_6-EVAR-**A**-AiiA_fw and M-His_6-EVAR-**A**-AiiA_rv as well as M-His_6-EVAR-**AAA**-AiiA_fw and M-His_6-EVAR-**AAA**-AiiA_rv. The codon-optimized synthetic gene of AlaDH [GenBank: KT759160] with C-terminal His-tag was provided by GenScript (Piscataway, NJ, USA), readily cloned into pET28b(+). All constructs were confirmed by sequencing (Eurofins, Ebersberg, Germany).

Transformation, protein expression, and cell lysis

Chemocompetent *Escherichia coli* (*E. coli*) BL21 (DE3) cells transformed by the heat-shock method (Chung et al. 1989) were grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotic (100 μg/mL ampicillin or 50 μg/mL kanamycin) at 37 °C and with 180 rpm. Protein

expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside when the optical density at 600 nm (OD_{600}) reached 0.5; cultivation was continued for 16 to 18 h at 30 °C for GFP and at 20 °C for AspFum, AiiA, and AlaDH. For the measurement of background activity of *E. coli* cell lysate in the NADH assay, plain *E. coli* BL21 (DE3) was grown in LB medium for 4.5 h at 37 °C.

At analytical scale, the number of harvested cells per aliquot was normalized by the following formula: 7 absorption units per OD_{600} in milliliters. Cells were centrifuged (15 min, 4000×*g*, 4 °C), washed with proteolysis buffer (50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0), and stored at −20 °C. For cell disruption, the required number of cell pellets were individually resuspended in 500 μL proteolysis buffer and subjected to sonication on ice (Bandelin Sonopuls HD 2070; 1 min, 50 % pulse, 50 % power), followed by cell debris removal by centrifugation (5 min, 17,000×*g*, 4 °C). For protein purification, cultivation samples (50 mL for AspFum variants, 400 mL for AiiA variants, 100 mL for AlaDH) were centrifuged (15 min, 4000×*g*, 4 °C), washed, and stored as cell pellets at −20 °C. Resuspension in 20 mL lysis/washing buffer (AspFum: 50 mM HEPES, 300 mM NaCl, 0.1 mM pyridoxal-5'-phosphate, pH 7.5; AiiA and AlaDH: 50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, pH 7.5) was followed by two french press passages (each 1500×psi) and cell debris removal (45 min, 10,000×*g*, 4 °C).

Proteolysis and other protein purification methods

Proteolysis was initiated by the addition of either trypsin (147 U/mL; 100 μg/mL of commercial preparation) or proteinase K (2 U/mL; 50 μg/mL of commercial preparation) unless otherwise specified. Reactions were typically performed at 37 °C and 750 rpm in an Eppendorf shaker and were stopped at the specified time points by the addition of 5 mM phenylmethanesulfonyl fluoride (PMSF) (stock solution: 150 mM in dry ethanol). Controls were handled in parallel to protease-treated samples, but were supplemented with buffer instead of protease solution.

Thermal purification of CAL-A was achieved by incubation of 500 μL solution (5 mg/mL in 50 mM Tris–HCl, pH 8.0; Ca^{2+} ions caused strong precipitation) for 10 min at 70 °C and removal of denatured proteins by centrifugation (5 min, 16,000×*g*, 4 °C). For PMPP, 3 mL of CAL-A solution were treated with proteinase K (0.2 U/mL) for 30 min, and resulting protein fragments were removed by ultrafiltration (molecular weight cut off [MWCO] 10 kDa).

Metal affinity chromatography was used to purify AspFum variants with an Äkta purifier (5-mL HiTrap Fast Flow column, GE Healthcare, Freiburg, Germany) as well as AiiA variants and AlaDH with gravity flow columns (each 2-mL column volume of Co-IDA resin, Carl-Roth, Karlsruhe, Germany). Elution was mediated by the respective lysis/washing buffer additionally containing 300 mM imidazole. AspFum was desalted using three HiTrap desalting columns in line (each 5 mL; GE Healthcare, Freiburg, Germany), while ultrafiltration (MWCO 10 kDa) was used for the exchange to proteolysis buffer for AiiA and to amination buffer (100 mM NH_4Cl/NH_4OH, pH 8.5) for AlaDH.

Analytical procedures

Analysis of protein mixture composition was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 4 % stacking gel and 12.5 % resolving gel. Unstained protein molecular weight marker (Thermo Scientific, Waltham, MA, USA) was used as reference.

Fluorescence intensities of GFP-containing samples (100 μL) were measured in black microtiter plates with excitation at 395 nm and emission at 509 nm (Tecan Infinite M200 Pro, Crailsheim, Germany).

For CAL-A activity measurements, a pH stat assay was used (Titrino Plus 877, Metrohm, Filderstadt, Germany). Substrate solution (5 % *v*/*v* tributyrin, 2 % *w*/*w* gum arabic in deionized water) was emulsified by Ultraturrax T25 (IKA, Staufen, Germany) for 5 min at 22,000 rpm. Then 20 mL of substrate solution were pre-equilibrated in the reaction chamber (40 °C, pH 7.5). The reaction was initiated by the addition of 50 μL enzyme solution and was measured for 5 min; 10 mM sodium hydroxide solution served as titrant. One unit of lipase activity releases 1 μmol of butyric acid per min.

Transaminase activity of AspFum was determined by the acetophenone assay (Schätzle et al. 2009). Briefly, 10 μL of AspFum solution were mixed with 90 μL HEPES buffer (50 mM, pH 7.5). The reaction was initiated by the addition of 100 μL reaction solution containing 5 mM (*R*)-1-phenylethylamine, 5 mM pyruvate, and 0.5 % DMSO in HEPES buffer (50 mM, pH 7.5), and acetophenone formation was monitored at 245 nm (Tecan Infinite M200 Pro, Crailsheim, Germany) at 30 °C. One unit of transaminase activity corresponds to the formation of 1 μmol acetophenone per min (ε= 12 mM^{-1} cm^{-1}).

NADH consumption by *E. coli* BL21 (DE3) cell lysate was followed spectrophotometrically (Jasco V-550, Jasco, Gross-Umstadt, Germany) at 340 nm and 25 °C. To 800 μL proteolysis buffer, 200 μL cell lysate and 0.5 μL NADH stock solution (300 mM in deionized water) were added. Initial absorbance of every measurement was below 0.8, to ensure linearity of the response. Subsequently, AlaDH-related NADH assays were measured in microtiter plates (Tecan Infinite M200 Pro, Crailsheim, Germany). The assay mixtures contained 130 μL amination buffer, 10 μL sodium pyruvate stock solution (400 mM in amination buffer), and 40 μL of sample. Reactions were initiated by the addition of 20 μL NADH stock solution (2 mM in amination buffer) and were followed at 340 nm. One unit of ADH activity was defined as

the consumption of 1 μmol NADH per min (ε = 6.022 mM^{-1} cm^{-1}). The impact of trypsin treatment on the NADH assay was determined in quadruplicates as follows: *E. coli* BL21 (DE3) cell lysate as well as *E. coli* BL21 (DE3) cell lysate supplemented with different amounts of AlaDH were split up, respectively. Subsequently, one half of each sample was treated with trypsin while the other one remained untreated. The signal to noise ratios (S/N) of trypsin-treated samples and the ones of untreated samples were calculated separately as well as individually for each amount of AlaDH by the following equation:

$$S/N = \frac{A(\text{cell lysate} + \text{AlaDH}) - A(\text{cell lysate})}{A(\text{cell lysate})}$$

Results

GFP as proof of concept protein and investigation of the temperature dependency of proteolysis

GFP served as model protein for the proposed PMPP method. Therefore, GFP-containing cell lysate of *E. coli* was treated either with trypsin or proteinase K. As intended, both proteases degraded many host proteins (Fig. 1). GFP was not affected by trypsin, while proteinase K cleaved a small terminal fragment. This was visualized as double band after 3 min of incubation and later on as single band with slightly lower molecular weight. Despite this observation, it should be emphasized that the fluorescence as the inherent biological function of GFP was not affected by both proteases in this study (trypsin treatment: 100±2 %, proteinase K treatment: 101± 1 %), which is in agreement with the literature (Ward 2005).

Subsequently, temperature was selected as one of several parameters which are known to have an influence on proteolysis efficiency (Turapov et al. 2008) and prospectively on PMPP. *E. coli* cell lysate containing GFP was incubated with and without trypsin at temperatures from 20 to 50 °C (Fig. 2). As expected, the thermostable GFP remained unaffected over the whole range, while its purity increased in parallel to the temperature. Only at the highest temperature the purification seems to rely on both proteolysis and thermal denaturation of host proteins. This was concluded from the control sample, which was purer than the ones at lower temperatures. In order to study the effect of proteolysis in a selective manner, all further experiments were carried out at 37 °C.

Comparison of protease- and heat-mediated purification of CAL-A

Based on the correlation between thermostability and protease resistance, thermostable lipase CAL-A was used as second example for PMPP. A commercial lyophilizate of CAL-A with several impurities was treated either with proteinase K or trypsin. Indeed, both proteases led to the degradation of most impurities with a much better performance of proteinase K (Fig. S1 in the Supplementary Material). This result further necessitated a comparison between heat-mediated purification and PMPP. In the present case, the latter one was highly advantageous with regard to both purification factor and recovery (Fig. 3, Table 1). Heat treatment of CAL-A preparation even led to a negative purification factor due to a higher loss in activity than reduction in protein content. Interestingly, CAL-A was not inhibited by PMSF although it shares the

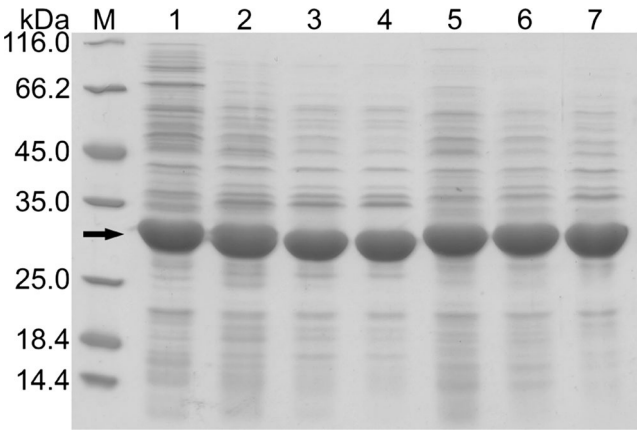

Fig. 1 SDS-PAGE analysis of the effects of protease treatment on crude cell lysate of *E. coli* containing GFP (27 kDa; indicated by an *arrow*). *M*, protein molecular weight marker; *lane 1*, control cell lysate; *lanes 2 to 4*, cell lysate treated with proteinase K for 3, 30, and 120 min; *lanes 5 to 7*, cell lysate treated with trypsin for 3, 30, and 120 min. When the standard concentration of 2 U/mL of proteinase K was applied for proteolysis, an additional band was always visible at about 36 kDa

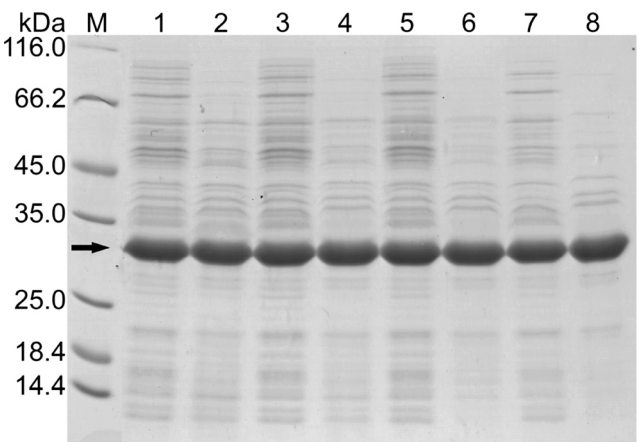

Fig. 2 SDS-PAGE analysis of the influence of temperature on the proteolysis of GFP-containing *E. coli* cell lysate after 30 min of incubation with trypsin. The band corresponding to GFP is indicated by an *arrow*. *M*, protein molecular weight marker; *lanes 1 to 8*, alternating control (*left*) and trypsin-treated sample (*right*) incubated at 20, 30, 40, and 50 °C, respectively

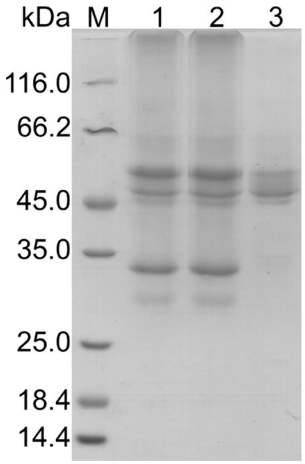

Fig. 3 SDS-PAGE analysis of CAL-A purification by heat and proteinase K treatment. *M*, protein molecular weight marker; *lane 1*, untreated CAL-A lyophilizate; *lane 2*, heat-treated sample (70 °C, 10 min); *lane 3*, proteinase K-treated sample (0.2 U/mL, 30 min). According to the amino acid sequence, CAL-A should have a molecular weight of approx. 45 kDa

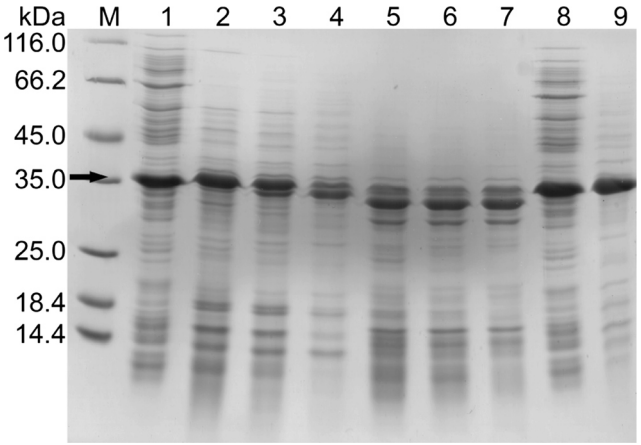

Fig. 4 SDS-PAGE analysis of the protease treatment of *E. coli* cell lysate containing AspFum (37 kDa; indicated by an *arrow*). *M*, protein molecular weight marker; *lane 1*, control cell lysate of AspFum; *lanes 2 to 4*, cell lysate treated with trypsin for 5, 30, and 120 min; *lanes 5 to 7*, cell lysate treated with proteinase K for 5, 30, and 120 min; *lane 8*, control cell lysate of AspFum R20Q; *lane 9*, cell lysate of AspFum R20Q treated with trypsin for 120 min

same catalytic triad with the PMSF-sensitive trypsin and proteinase K (Betzel et al. 2001; Polgar 2005; Ericsson et al. 2008).

Rational design of an AspFum variant resistant to trypsin

The transaminase AspFum was previously described in its biochemical properties and its crystal structure (Höhne et al. 2010; Thomsen et al. 2014; Skalden et al. 2015). The enzyme is stable for several hours at 60 °C (Fig. S2 in the Supplementary Material), correlating with its origin from a thermotolerant fungus (Bhabhra and Askew 2005), and designating it as a promising candidate for PMPP. Therefore, cell lysate of *E. coli* containing AspFum was treated either with trypsin or proteinase K. Host proteins were degraded to a large extent as shown by SDS-PAGE analysis (Fig. 4). Short terminal parts of AspFum were cleaved by both proteases, as indicated by slightly lower molecular weights. However, no loss in activity was observed for the truncated enzymes (data not shown).

Subsequently, the combination of AspFum and trypsin was chosen to gain insights into the structural details of limited proteolysis and to probe the completion of protease resistance by rational design. According to SDS-PAGE analysis, the difference in molecular weights between untreated and trypsin-treated AspFum was about 2 kDa. Considering the amino acid sequence of AspFum, only cleavage at the N-terminal positions R14 (1.6 kDa), K16 (1.9 kDa), and R20 (2.4 kDa) and none at the C-terminus matched this estimation. In the crystal structure (Protein Data Bank [PDB] entry 4CHI), amino acids R14 and K16 are both located within the N-terminal α-helix and possess polar contacts involving side chains and C-termini, thus should be insusceptible to trypsin (Fig. S3 in the Supplementary Material). Amino acid R20 is situated next to the end of the α-helix and at the edge of the protein. Its side chain and its carboxy functionality are not involved in polar contacts and partially show high motional flexibilities (B-factors). Computational analysis by *Nickpred* (Hubbard et al. 1998) also indicated R20 to be more likely the cleavage site than R14 and K16 (Fig. S4 in the Supplementary Material).

Table 1 Comparison of heat- and proteinase K-mediated purification of CAL-A from commercial lyophilizate

Purification	Total activity (U)	Recovery (%)	Specific activity (U/$mg_{protein}$)	Purification factor
Untreated	67±3[a]/281±14[b]	100	79±4	1
Heat-mediated purification	49±1	73±1	64±1	0.81±0.01
Proteinase K-mediated purification	236±2[c]	84±1[c]	191±1	2.42±0.02

[a] Value for heat-mediated purification

[b] Value for proteinase K-mediated purification

[c] 274±1 U total activity and thus 97±1 % recovery remained after proteinase K treatment and before ultrafiltration

Indeed, the variant R20Q that was created was not degraded by trypsin (Fig. 4, lanes 8 and 9). As expected, His-tag purified wild type (4.7±0.3 U/mg) and R20Q (4.6±0.2 U/mg) did not differ in their specific activities (SDS-PAGE analysis: Fig. S5 in the Supplementary Material).

Trypsin as highly economical and very efficient alternative to site-specific endopeptidases for His-tagged AiiA

Recombinant proteins are frequently fused to a peptide or a protein, which enables affinity chromatography, soluble expression, or other features (Esposito and Chatterjee 2006; Young et al. 2012). For various reasons, the fusion partner sometimes has to be removed from the POI. For this purpose, usually a defined amino acid sequence is integrated between them, which can be cleaved by a site-specific endopeptidase (Waugh 2011). However, due to their very high commercial prizes, these enzymes are usually used only on a laboratory scale. In the context of this study, it was investigated if the nonspecific protease trypsin can be used instead when working on a trypsin-resistant POI as this would be extremely more economical and thus enable large-scale processes. As an application example, a His-tag was N-terminally fused to AiiA via a prospectively trypsin-sensitive linker (M-His_6-EVAR↓AiiA), theoretically enabling a traceless removal.

In agreement with the literature, AiiA was marginally expressed in soluble form in *E. coli* (Cao et al. 2012); the His-tag purified preparations contained several impurities, typical for low expression levels (Fig. S6 in the Supplementary Material) (Andersen et al. 2013). Unfortunately, trypsin treatment did not lead to the intended cleavage of the fusion construct (Fig. 5). Instead there was a reduction in band intensity of AiiA within the first 0.5 min, but no further degradation during the following 4.5 min. This observation was very likely due to a fraction of AiiA that was soluble, but partially misfolded and thus trypsin sensitive, similar to investigations on rhodopsin with correctly folded, trypsin-resistant fractions and partially misfolded, trypsin-sensitive fractions (Liu et al. 1996). Nevertheless, it is known that trypsin treatment does not affect the activity of AiiA preparations (Cao et al. 2012).

The linker (EVAR) in N-terminal direction of the cleavage site was supposed to be highly accessible, and its sequence was optimized for trypsin acceptance (Pan et al. 2014). In contrast to this, the unmodified AiiA in C-terminal direction was assumed to be acceptable with regard to the sequence, but questionable with regard to its accessibility (PDB entry 2A7M, highly homologous AiiA variant; Fig. S7 in the Supplementary Material). Consequently, variants with one (M-His_6-EVAR↓**A**-AiiA) and three additional alanines (M-His_6-EVAR↓**AAA**-AiiA) between cleavage site and AiiA were generated. Indeed, about half of M-His_6-EVAR↓**A**-AiiA was cleaved within 5 min, and M-His_6-EVAR↓**AAA**-AiiA was completely cleaved in only 0.5 min (Fig. 5).

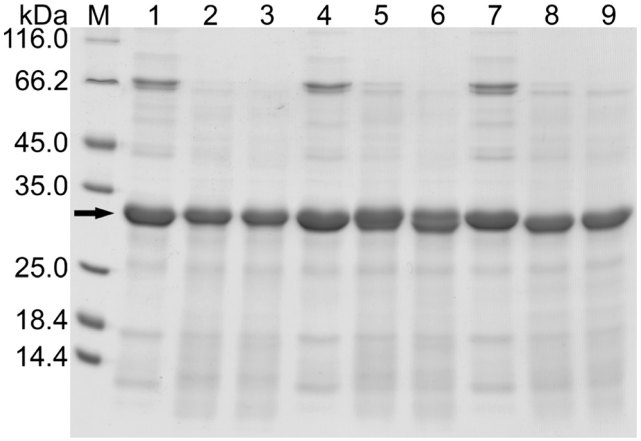

Fig. 5 SDS-PAGE analysis of the site-specific removal of the His-tag and linker from AiiA constructs (29 kDa; indicated by an *arrow*) by trypsin treatment for 0, 0.5, and 5 min, respectively. *M*, protein molecular weight marker; *lane 1 to 3*, M-His_6-EVAR↓AiiA; *lane 4 to 6*, M-His_6-EVAR↓**A**-AiiA; *lane 7 to 9*, M-His_6-EVAR↓**AAA**-AiiA

In order to provide further evidence for the site-specific cleavage, trypsin-treated samples were immediately subjected to another round of His-tag purification. In contrast to M-His_6-EVAR↓AiiA, the cleavage product of M-His_6-EVAR↓**AAA**-AiiA did not bind to the affinity column (Fig. S8 in the Supplementary Material).

Improved sensitivity and accuracy of the NADH assay in *E. coli* cell lysate by proteolysis of interfering host enzymes

In some cases, the main purpose of protein purification is the elimination of one or few proteins with interfering properties rather than the isolation of the POI from the protein mixture. When working on a protease-resistant POI, proteolysis of the responsible impurities without the removal of the degradation products would be a straightforward approach in this respect. As proof of principle, the high background activity of *E. coli* cell lysate towards the cofactor NADH was chosen. This often impedes or even prevents the application of the widely used NADH assay due to a low signal to noise ratio (*S*/*N*) (Fong et al. 2000).

In the context of this study, *E. coli* BL21 (DE3) cell lysate was pre-treated either with trypsin or proteinase K for 30 min. Indeed, NADH oxidation was reduced by 89±2 % for trypsin-treated samples and 92±3 % for proteinase K-treated samples. Thermostable AlaDH (Vali et al. 1980) was chosen as a model enzyme. Purified AlaDH was resistant towards trypsin with no measureable loss in activity (99±6 %; SDS-PAGE analysis: Fig. S9 in the Supplementary Material), but it was sensitive towards proteinase K (69±11 %; SDS-PAGE analysis: Fig. S9 in the Supplementary Material). This observation is in

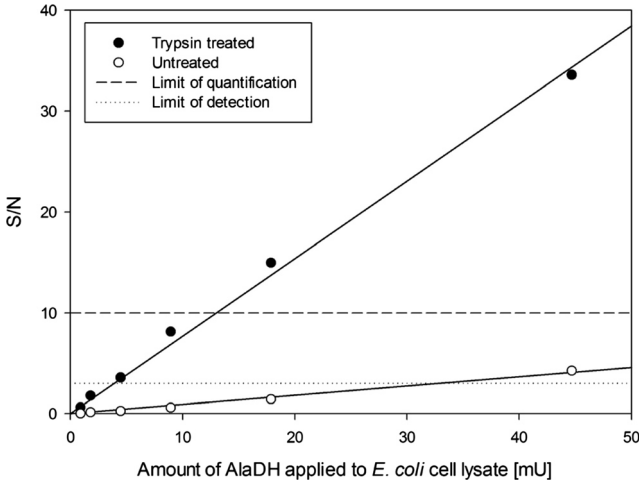

Fig. 6 Impact of trypsin treatment on the sensitivity and accuracy of the NADH assay. The conversion of pyruvate to alanine by AlaDH was used as a model system

agreement with the protease treatment of crude cell lysate containing AlaDH (Fig. S10).

Subsequently, the impact of trypsin treatment on the *S*/*N* was studied in detail by supplementation of *E. coli* BL21 (DE3) cell lysate with different amounts of purified AlaDH (Fig. 6). The limit of detection (*S*/*N*=3) for AlaDH activity was decreased from 33 to 4 mU by pre-incubation with trypsin. In turn, trypsin-treated samples containing 45 mU of AlaDH exceeded the limit of quantification (*S*/*N*=10) more than three times while the untreated samples were still far below the required *S*/*N* value and thus not quantifiable.

Discussion

In this study, the application of nonspecific proteases for protein purification was investigated on the basis of trypsin and proteinase K with five POIs.

First, GFP served as proof of concept protein for PMPP; treatment with both proteases led to the degradation of most host proteins. However, several proteins of the mesophilic expression host *E. coli* were also resistant, thereby reducing the overall efficiency of the method. In part, this might be also due to the inhibitory effect of proteolysis products (Kasper et al. 2014). Thus, proteins with a higher degree of protease resistance might have been protected by the gradually formed fragments derived from less resistant proteins. All in all, the efficiency of PMPP was similar to other unspecific protein purification methods like ion exchange chromatography (Imai and Okada 2008) or size exclusion chromatography (Tokunaga et al. 1997), but obviously PMPP is less expensive and does not require chromatography equipment.

Lipase CAL-A was used for the essential comparison between heat-mediated purification and PMPP. At least in the present case, the latter one was highly advantageous with regard to recovery as well as purification factor. However, it needs to be mentioned that we are not aware if the commercial CAL-A has been purified by heat treatment already, so that no further improvement was possible by another round of heat treatment. At this point, it should be also mentioned that some thermostable enzymes undergo activation by heat treatment for instance as reported by Lee et al. (1998) and for AspFum (Fig. S2 in the Supplementary Material), thus tending to outcompete PMPP.

The resistance of AspFum towards trypsin was successfully completed, facilitated by the formation of only one degradation product under standard PMPP conditions. Nevertheless, the approach of limited proteolysis can be also used for more complex issues like proteinase K-treated AlaDH (Fig. S9 in the Supplementary Material), especially when combined with techniques such as N-terminal sequencing and mass spectrometry (Fontana et al. 2004). Protease cleavage sites are generally characterized as highly surface exposed and weakly involved in structural interactions. Therefore, we reason that most of these positions can be mutated to similar, but non-cleavable, amino acids without a negative impact on protein properties as shown here for the specific activity of AspFum variant R20Q.

Site-specific endopeptidases are often used to remove peptides and proteins fused to recombinant POIs (Waugh 2011). However, their very high commercial prizes and low activities restrict their applications to the laboratory scale or to high-value products. For instance, it was estimated that the site-specific endopeptidase factor Xa would account for 88 % of the overall production costs of a recombinant protein purified via maltose-binding protein and with subsequent fusion tag removal (Banki and Wood 2005). On the basis of AiiA, it was shown here that trypsin could be a very efficient substitute if working on a trypsin-resistant POI. In comparison to site-specific endopeptidases, trypsin can be purchased at very low cost and is much more active. Hence, even large-scale applications are feasible and affordable. A further advantage of the presented approach was the additional polishing effect on the affinity-tag purified protein by degradation of nonresistant impurities and (very likely) partially misfolded AiiA. The latter aspect should be of particular interest for biotherapeutics as misfolding correlates with aggregation, one of the critical quality attributes affecting product safety and efficiency (Eon-Duval et al. 2012). Overall, this approach is also competitive to self-cleaving fusion tags; although these enable low cost separations by shifts in temperature, pH, or buffer composition, they usually possess low activities and add quite high molecular weights to the POI during its expression (Banki and Wood 2005; Li 2011).

In the case of AiiA, introduction of one additional amino acid between cleavage site and protein was enough to obtain a high proteolysis rate. Nevertheless, also with the frequently used TEV protease (EXXYXQ↓S/G) and other site-specific endopeptidases one or few amino acids remain attached to the POI (Waugh 2011). From our point of view, the traceless removal of a fused peptide or protein seems to be possible with trypsin, depending on both the sequence and the structure of the N-terminus of the POI.

The sensitivity and the accuracy of the NADH assay in *E. coli* cell lysate was improved significantly by proteolysis of NADH oxidizing host enzyme(s) (Fig. 6), thereby circumventing the laborious and expensive classical as well as the alternative heat-mediated purification. This could be of particular interest for NADH-based high-throughput screenings, for instance useful for the directed evolution of oxidoreductases (Fong et al. 2000), but also for other enzyme classes via coupled assays (Baumann et al. 2001). Thus, protease-resistant POIs do not necessarily have to be purified from protein mixtures if proteolysis of interfering proteins is sufficient for the intended application.

While GFP and AiiA were known to be thermostable and protease resistant, the results obtained with the thermostable enzymes CAL-A, AspFum, and AlaDH further confirmed the correlation between these properties (Daniel et al. 1982; Ahmad et al. 2012). Hence, the numerous thermostable proteins already known and in use in science and industry (Haki et al. 2003; Elleuche et al. 2014) are promising candidates for the here described PMPP.

The characteristics of the presented approaches (inexpensive, fast, freely scalable, easy to perform, low demands for equipment) render them attractive alternatives to common protein purification methods. Nevertheless, several aspects should be addressed in the future including the application of immobilized proteases instead of free ones (Xi et al. 2005) and the purification of protease-resistant proteins from natural sources.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Electronic Supplementary Material

Article title: Highly efficient and easy protease-mediated protein purification

Journal: Applied Microbiology and Biotechnology

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Table S1 Primer sequences

R20Q_fw	5’-GCTGGAACAAAGTGATAATCCGTTCAG-3’
R20Q_rv	5’-GATTATCACTTTGTTCCAGCAGTTTCTGG-3’
M-His_6-EVAR-AiiA_fw	5’-CACCATCACCATCACCATGAAGTGGCGCGTATGACCGTGAAAAA ACTGTATTTTC-3’
M-His_6-EVAR-AiiA_rv	5’-ATGGTGATGGTGATGGTGCATGGTATATCTCCTTCTTAAAG-3’
M-His_6-EVAR-**A**-AiiA_fw	5’-GTGGCGCGTGCCATGACCGTGAAAAAACTGTATTTTC-3’
M-His_6-EVAR-**A**-AiiA_rv	5’-CACGGTCATGGCACGCGCCACTTCATGGTG-3’
M-His_6-EVAR-**AAA**-AiiA_fw	5’-GCCGCAGCGATGACCGTGAAAAAACTGTATTTTCTGC-3’
M-His_6-EVAR-**AAA**-AiiA_rv	5’-CGCTGCGGCACGCGCCACTTCATGGTGATG-3’

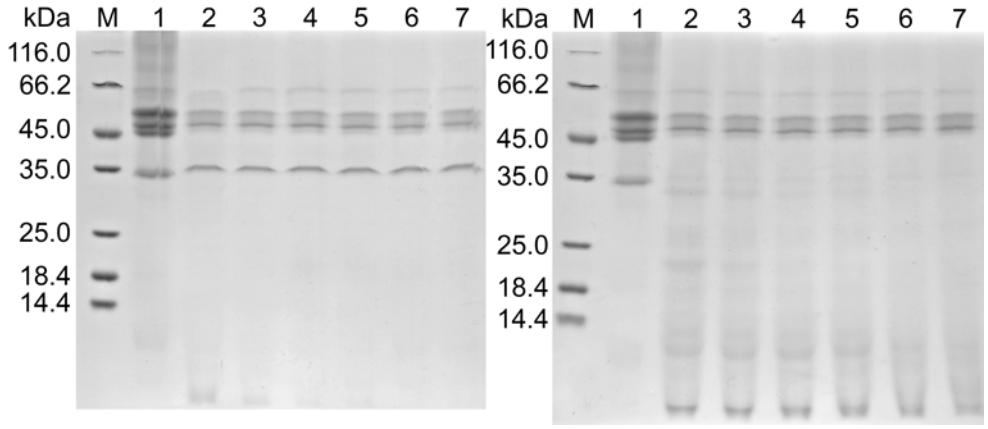

Fig. S1 SDS-PAGE analysis of the treatment of CAL-A solution with proteinase K *(left)* and trypsin *(right)*. *M*, protein molecular weight marker; *lane 1*, control; *lanes 2 to 7*, CAL-A preparation treated with protease for 2.5, 5, 7.5, 10, 20, and 30 min

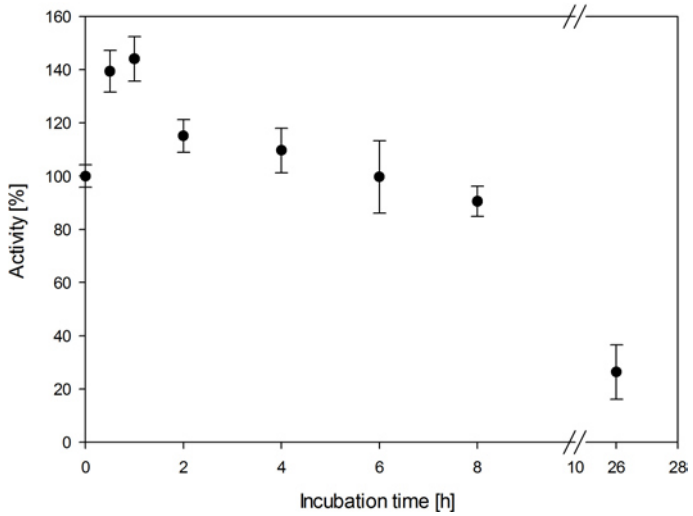

Fig. S2 Thermostability of AspFum determined in triplicate and incubated at 60°C for the indicated periods of time

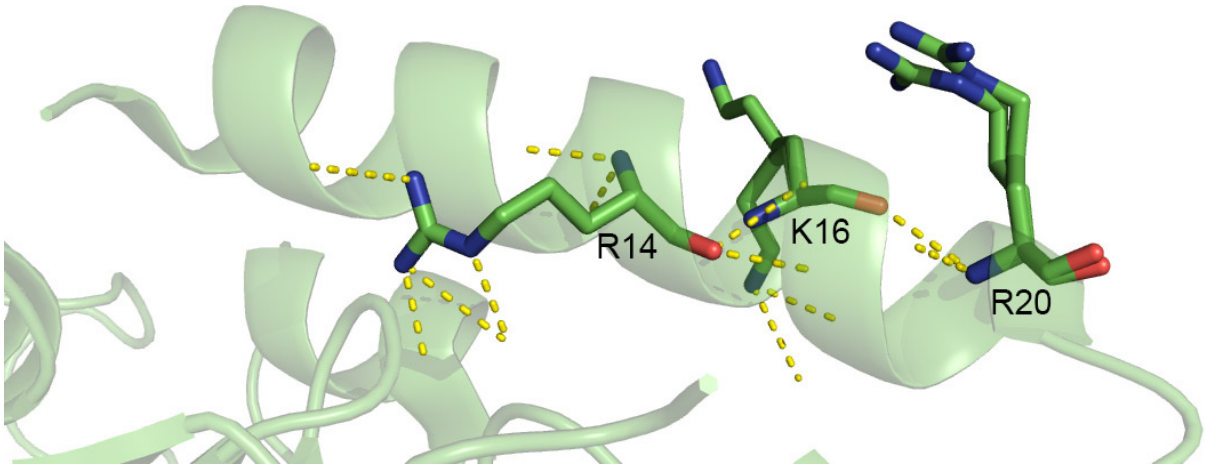

Fig. S3 Structural details of residues R14, K16 and R20 of AspFum (PDB code: 4CHI)

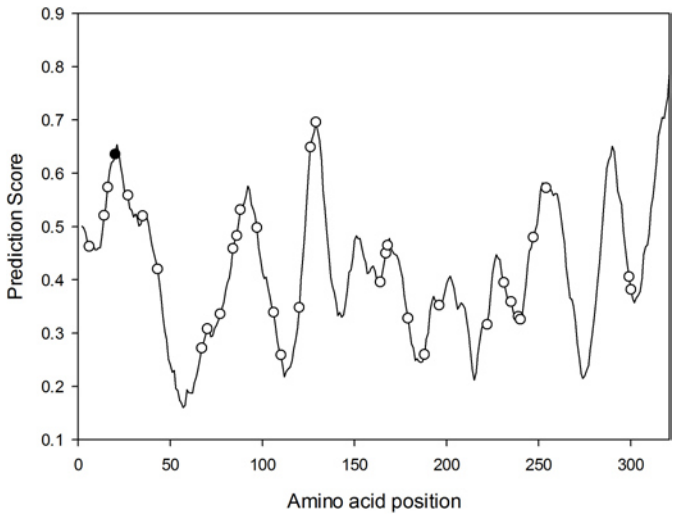

Fig. S4 Visualization of *Nickpred* calculation for AspFum. Open circles indicate arginine and lysine residues, and the closed circle indicates the experimentally confirmed trypsin cleavage site R20. Theoretically, the two active site residues R126 (flipping arginine) and K129 possess higher prediction scores than R20, but do not represent real trypsin cleavage sites. This might be due to the unfavored amino acid motif K129-P130-E131-D132 (Pan et al. 2014) and the fact that R126 is buried behind the highly mobile K129 (B-factors up to 88)

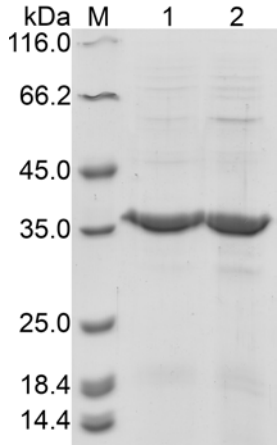

Fig. S5 SDS-PAGE analysis of His-tag purified AspFum wild type and R20Q. *M*, protein molecular weight marker; *lane 1*, wild type; *lane 2*, variant R20Q

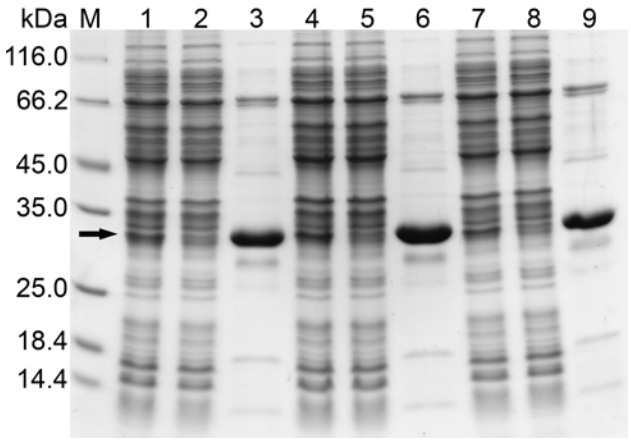

Fig. S6 SDS-PAGE analysis of the expression of AiiA constructs with crude cell lysate, flow through and purified enzyme adjacent to each other, respectively. *M*, protein molecular weight marker; *lanes 1 to 3*, M-His_6-EVAR↓AiiA; *lanes 4 to 6*, M-His_6-EVAR↓**A**-AiiA; *lanes 7 to 9*, M-His_6-EVAR↓**AAA**-AiiA

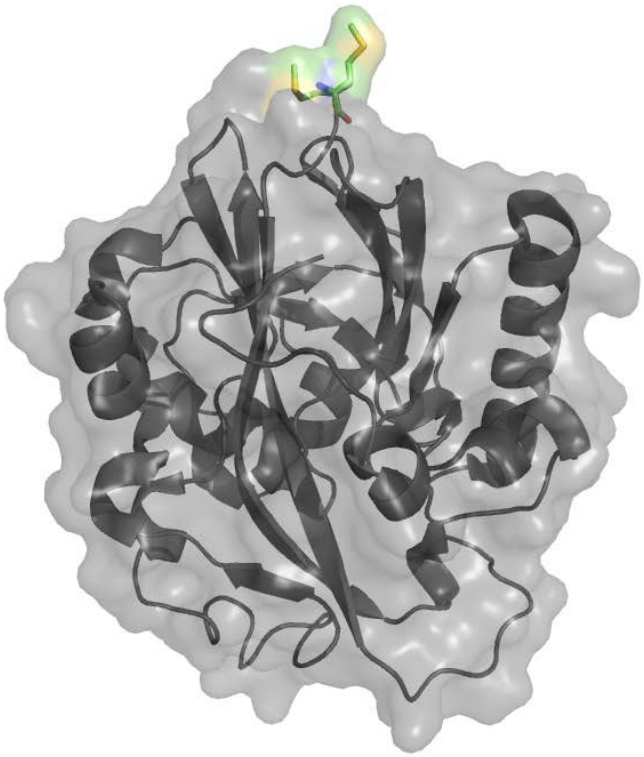

Fig. S7 Position of the N-terminal methionine in the overall structure of AiiA (PDB code: 2A7M). The following amino acids and especially their C-termini are much less exposed to the surface

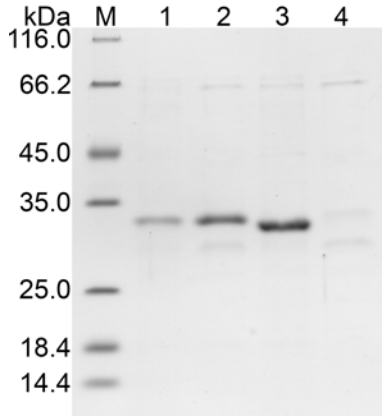

Fig. S8 SDS-PAGE analysis of the binding and the elution of trypsin treated AiiA variants to a metal affinity column. *M*, protein molecular weight marker; *lanes 1 and 2*, M-His_6-EVAR↓AiiA flow through and elution fraction; *lanes 3 and 4*, M-His_6-EVAR↓**AAA**-AiiA flow through and elution fraction

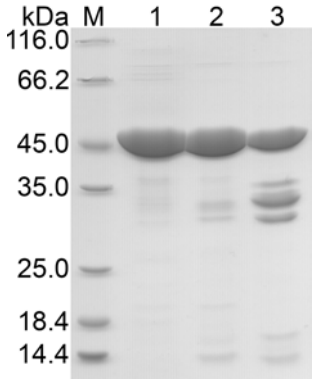

Fig. S9 SDS-PAGE analysis of the effect of 60 min protease treatment on purified AlaDH (theoretically 40.0 kDa). Obviously a gel shift of the AlaDH band occurs due to a lower mobility (Shi et al. 2012). *M*, protein molecular weight marker; *lane 1*, untreated AlaDH; *lane 2*, trypsin-treated AlaDH; *lane 3*, proteinase K-treated AlaDH

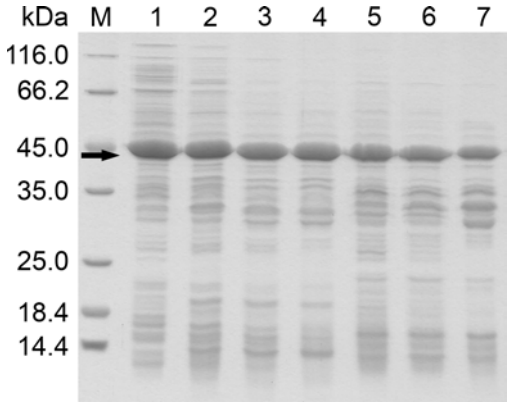

Fig. S10 SDS-PAGE analysis of the protease treatment of *E. coli* cell lysate containing AlaDH. *M*, protein molecular weight marker; *lane 1*, control cell lysate of AlaDH; *lanes 2 to 4*, cell lysate treated with trypsin for 5, 30, and 120 min; *lanes 5 to 7*, cell lysate treated with proteinase K for 5, 30, and 120 min

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9. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

……

Daniel Last

10. Publications

Last D.; Krüger G. H. E.; Dörr M.; Bornscheuer U. T., Fast, continuous and high-throughput (bio)chemical activity assay for *N*-acyl-L-homoserine lactone quorum-quenching enzymes, *Appl. Environm. Microbiol.* **2016**, *82* (14). doi:10.1128/AEM.00830-16

Dörr, M.; Fibinger, M. P. C.; Last, D.; Schmidt, S.; Santos-Aberturas, J.; Böttcher, D.; Hummel, A.; Vickers, C.; Voss, M.; Bornscheuer, U. T., Fully automatized high-throughput enzyme library screening using a robotic platform, *Biotechnol. Bioeng.* **2016,** *113* (7), 1421-1432. doi:10.1002/bit.25925

Last, D.; Müller, J.; Dawood, A. W.; Moldenhauer, E. J.; Pavlidis, I. V.; Bornscheuer, U. T., Highly efficient and easy protease-mediated protein purification, *Appl. Microbiol. Biotechnol.* **2016,** *100* (4), 1945-1953. doi:10.1007/s00253-015-7206-9

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