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Methods for the selection of hydrolases by applying *E. coli* uridine auxotrophic strain and synthetic nucleosides

SUMMARY OF DOCTORAL DISSERTATION

Natural sciences,
Biochemistry (N 004)

VILNIUS 2020

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This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:
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The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 10 AM on 22th of September 2020, in R401 auditorium of the Life Sciences Center, Vilnius University, Saulėtekio av. 7, LT- 10257 Vilnius, Lithuania. Phone: +370 5 223 4420, +370 5 223 4449, e-mail:info@gmc.vu.lt
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VILNIAUS UNIVERSITETAS

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Hidrolazių atrankos metodai, panaudojant *E. coli* uracilo auksotrofo kamieną ir sintetinius nukleozidų darinius

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Gamtos mokslai,
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INTRODUCTION

Every process in the biosphere is touched by the capacity of microbes to transform the world around them. The chemical cycles that convert the key elements of life - carbon, nitrogen, oxygen, and sulphur - into biologically accessible forms are largely directed by and dependent on microbes. All plants and animals have closely associated microbial communities that make necessary nutrients, metals, and vitamins available to their hosts. Through fermentation and other natural processes, microbes create or add value to many foods. At present, most industrial enzymes are also hydrolases of microbial origin, catalysing breakdown of natural polymeric substrates like proteins, starches, lipids, fibers, and complex cell walls into simpler molecules. The most frequently used industrial enzymes fall in the broad categories of proteases, amylases and cellulases. Pectinases, xylanases, phytases, lipases, lactases, invertases, isomerases, oxidases, and catalases also are widely used. In practice, the great majority of microbial enzymes come from *Aspergillus*, *Trichoderma*, *Bacillus*, *Streptomyces* and *Kluyveromyces* species [1] [2]. But Earth is home to as many as 1 trillion (10^{12}) microbial species and only about 10^4 species have been grown in a laboratory [3]. Thus, uncultivable microorganisms encouraged metagenomic studies. Metagenomics is the study of genetic material recovered directly from environmental samples. Metagenomics analysis includes 16S rRNA sequencing test that shows microbial biodiversity and metagenomics libraries – large fragments of metagenomic DNA cloned into appropriate vectors, that are used for selection of natural biocatalysts.

The revolution in the high throughput DNA sequencing technologies has resulted in a dramatic expansion of the databases, but numbers of microbial enzymes predicted *in silico* and those experimentally characterized in the lab, remain very low. Metagenomics, the improvement of high-throughput screening methods and the development of host expression systems for metagenome-derived genes, systems biology and gene synthesis may

together open the gateway to the useful information that hides in unexplored genetic resources, e.g., new enzymes with unique properties and novel scaffolds applicable for evolution *in vitro*.

Based on the abovementioned reasons, the objective of this study was to investigate and evaluate the opportunities of the application of *E. coli* uridine auxotrophic strain and synthetic nucleosides for the selection of hydrolases from metagenomic libraries. Towards this aim the following tasks had been formulated:

- to develop and to optimize methods for the selection of hydrolases from metagenomic libraries by using uridine esters and N^4-benzoyl-(deoxy)cytidine amides;
- to evaluate the diversity and catalytic properties of selected enzymes;
- to identify and to characterize metagenomic fucosidase;
- to synthesize substrates for the selection of glycosidases and to develop a model system for the selection of these enzymes.

Scientific novelty:

At present, all described selection methods of esterases have been applied only for the screening of the appropriate variants from libraries containing mutant sequences, but not from metagenomes, and only a single selection method has been developed for the selection of amidohydrolases by using leucine auxotrophic *E. coli* TOP10 strain and leucine amides as leucine source. Moreover, no selection method of fucosidases from metagenomes has been published in the scientific literature.

In this study, methods for the selection of metagenomic esterases and amidohydrolases have been developed and the principal selection system for glycosidases has been shown. The developed methods are based on the *E. coli* uracil auxotroph DH10B *ΔpyrFEC* strain and synthetic nucleoside derivatives adapted to esterases,

amidohydrolases and glycosidases. It is shown that a combination of uridine esters or amides and the uridine auxotroph *E. coli* DH10B*pyrFEC* are applicable for discovery of novel hydrolases with different structural and catalytic characteristics.

Also metagenomic 62Fuc1 fucosidase has been described and fucosyldeoxyuridine synthesis has been performed by using this novel enzyme. The prepared substrate has been used to check the expression of fucosidase in a selective medium and thus, for the first time, demonstrates a principal method of glycosidase selection, that can be applied for the selection of glycosidases from metagenomes.

The developed selection methods allow to find unique metagenomic enzymes. The 11 nearest homologues of selected hydrolases have been identified as hypothetical in the NCBI database. Also two unique amidohydrolases D8_R1 and YqfB have been found. The *E. coli* YqfB protein has been known for a long time, but its functional activity has only been determined recently. DUF998 family protein has been also determined as esterase for the first time.

Thesis statements:

1. The *E. coli* uracil auxotrophic strain and synthetic uridine esters or cytidine amides can be used for selection of esterases and amidohydrolases from metagenomic libraries.
2. The developed esterase selection method is more sensitive compared to the classical tributyrin method.
3. Developed selection methods of esterases and amidohydrolases allow to select enzymes with unknown or undetermined activity.
4. Chemical origin of substrates themselves and radical groups of substrates determine the specificity of selected hydrolases.
5. Metagenomic 62Fuc1 fucosidase is an α-L-fucosidase and catalyzes transglycosylation reactions.
6. A model glycosidase selection method based on the uracil auxotrophic *E. coli* strain and substrate glycosyl (deoxy) uridine can be applied for the selection of glycosidases.

Contents of the doctoral thesis

The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, List of References (173 positions), Tables (11), Figures (51). Total 123 pages.

MATERIALS AND METHODS

Materials

Restriction endonucleases, HindIII, BamHI, PstI, Phusion DNA polymerase, aLICator™ LIC Cloning and Expression System Kit 3, PageRulerTM Prestained Protein Ladder were purchased from Thermo Fisher Scientific, Lithuania, Pierce™ Coomassie Plus (Bradford) Assay Reagent and HisPur™ Ni-NTA spin column were purchased from Thermo Fisher Scientific, Rockford. Nutrient medium were purchased from Roth, Germany. pNP-acyl esters, tributyrin, β-D-glucose pentaacetate, β-D-galactose pentaacetate, uridine were purchased from Sigma-Aldrich. "ZR Soil Microbe DNA MidiPrep™", was purchased from Zymo Research, Germany. Nitrocefin was purchased from Oxoid, UK. 3'-*O*-benzoyl-2'-deoxyuridine, α-L-*p*-nitrophenylfucopyranoside, were purchased from Carbosynth, UK. 3'-*O*-acetyl-2'-deoxyuridine, 3'-*O*-acetyl-N^4-benzoyl-2'-deoxycytidine, 3'-*O*-levulinyl-N^4-benzoyl-2'-deoxycytidine, 5'-O-levulinyl-N^4-benzoyl-2'-deoxycytidine were purchased from Jena Bioscience, Germany, N^4-acetylcytidine, N^4-acetyl-2'-deoxycytidine, N^4-benzoyl-2'-deoxycytidine, N^4-isobutyryl-2'-deoxycytidine and isocytosine were purchased from Combi-Blocks, San Diego, USA. pNP-acyl esters, uridine, 2'-deoxycytidine, N^4-benzoylcytidine, N^4-acetylcytosine, *p*-nitroacetanilide and capecitabine were obtained from Sigma-Aldrich, Germany. N^4-hexanoyl-2'-deoxycytidine, N^4-nicotinoyl-2'-deoxycytidine, N^4-(2-acetyl-benzoyl)-2'-deoxycytidine, N^4-(3-acetyl-benzoyl)-2'-deoxycytidine, N^4-(4-acetyl-benzoyl)-2'-deoxycytidine, N^4-(2-benzoyl-benzoyl)-2'-deoxycytidine, N^4-(3-benzoyl-benzoyl)-2'-deoxycytidine, N^4-(4-benzoyl-benzoyl)-2'-deoxycytidine were synthesised as described previously [4]. N^2-acetylisocytosine and N^4-acetyl-5-fluorocytosine were prepared as published previously [5]. LActazymeB™ of *Bacillus circulans* was purchased from GenoFocus.

Bacterial strains and plasmids

- *Escherichia coli* DH10B: F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara, leu*)7697 *araD139 galU galK nupG rpsL* λ⁻ (Thermo Fisher Scientific),
- *Escherichia coli* DH10B Δ*pyrFEC* ([6],
- *Escherichia coli* HMS174 Δ*pyrFEC* (constructed in MMB department, Bchi,VU),
- *Escherichia coli* BL21 (DE3): *hsdD gal (λcIts857 indl Sam7 nin5 lacUV-T7* genas *1* (Novagen),
- *Escherichia coli* HMS174: F⁻ *recA1 hsdR* (rK12⁻ mK12+) (DE3) (Rif R) (Novagen),
- *Escherichia coli* Arctic express (DE3): B F⁻ *ompT hsdS*(rB⁻ mB⁻) *dcm*⁺ Tetrgalλ(DE3) *endA* Hte [cpn10 cpn60 Gent^r] (Agilent).

Primers

Table 1. Primers used for the amplification of the genes of the selected esterases, amidohydrolases and 62Fuc1 fucosidase. The 5' end of the primer (highlighted in the underlined italic font) includes the pLATE31 vector-specific sequences for the ligation-independent cloning.

Clone	Forward primer (5'->3')	Reverse primer (5'->3')
24T5	TACATATGCTGAGAAAA TGGCTG	CTAAGCTTGTGCGCTTC GATGAAG
24T1	*AGAAGGAGATATAACTATG* *G*ATCAAACACTTCTCGC	*GTGGTGGTGATGGTGAT* *GGCC*CTCTGCGAGGTAA
24T3	*AGAAGGAGATATAACTATG* *G*AAAGCCAGACGTTTG	*GTGGTGGTGATGGTGAT* *GGCC*CTTCAGGCTCTCC
33T1	TACATATGAAAGCCAGA CGTTTG	TACTCGAGTCTTCAGGC TCTCAGCAAAG
30T1	*AGAAGGAGATATAACTATG* CGTCGTCCATCCTTG	*GTGGTGGTGATGGTGAT* *GGCC*CTGGGCCAGATGC
30T2	*AGAAGGAGATATAACTATG* AAAGTTAAAATTTTAAT	*GTGGTGGTGATGGTGAT* *GGCC*TTGAGTAATTTTAT

36T2	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	ACCCTGAGACTTGCG	*GGCC*CAGGTTGTCGCGG
3T	ATGCATATGGCGTTTTTC	ATGCTCGAGATCCAGCA
	GATTTGCC	GATCATGCAG
GRU1	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CCGGTTATCGACATG	*GGCC*CAAACCAAGCATA
BD1	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	ATCAGAAACATCTTTGG	*GGCC*CTGCAGACCACG
12T	TACATATGCTTCATCGTC	TAGTCGACGAGTTTGGT
	ATTGC	CGCGGGATC
33T3	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AATAAATCAAAACGTAC	*GGCC*TTTGAAAATGGAC
BD9	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AAGCCCACTCGCTG	*GGCC*GAACAGCGCAGC
36T1	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	ATAAAACTCAATCCTGT	*GGCC*GAAGAACAGCCG
45T3	GGCGAGTTGCATATGAC	GGAAGCTTGCCCGTGG
	CC	GAAGG
C233	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	ACGAACCCCAAGTCG	*GGCC*GACCTCCGTGCTG
PLA1	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CGCCGCCGC	*GGCC*CGGCTTTGCCATC
MO101T	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AACATCGTATTGATTCA	*GGCC*TAAAGGCACTGA
SVG1	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AGTCTCCACCTCAAGTG	*GGCC*CGGTTTGGCGTAG
SVG3	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CGCAAGGGG	*GGCC*GTTAAAGACAGA
B11	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CGCGATGCCTC	*GGCC*GTGCGCTTCGATG
4H1T	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AGAATCATTTTATCCCT	*GGCC*TACTTTAGAAAAG
RIEB	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CGCACATCTTTGC	*GGCC*GAGGTGCGCCGT
MO4B	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	AAAAGTCCATCCAAG	*GGCC*ACGTAAGGCCGTA
EN1H	*AGAAGGAGATATAACTATG*	*GTGGTGGTGATGGTGAT*
	CAATCAACCAA	*GGCC*CGAATAGATTTTT

EN3H	*AGAAGGAGATATAACTATG* CCCAACAACATCG	*GTGGTGGTGATGGTGAT GGCC*GGACACATCTTCC
BD2H	*AGAAGGAGATATAACTATG* AGTTCGCTCCGCC	*GTGGTGGTGATGGTGAT GGCC*AAGCGTCCTCTCA
CAP3H	*AGAAGGAGATATAACTATG* CATCGAGCTGTTTC	*GTGGTGGTGATGGTGAT GGCC*CGGCGTACACGG
1315H	*AGAAGGAGATATAACTATG* TGGGCCACCAGC	*GTGGTGGTGATGGTGAT GGCC*CGAGACGGACTT
SVGPA-2T	*AGAAGGAGATATAACTATG* CCGGCGCTTGACGGC	*GTGGTGGTGATGGTGAT GGCC*CGGCCGTCATCCG
K3H2	*AGAAGGAGATATAACTATG* TCCGACCCGCAA	*GTGGTGGTGATGGTGAT GGCC*GCCGGCGAGCGC
Tb_10-7T	*AGAAGGAGATATAACTATG* AATCATAACGTATCCGC	*GTGGTGGTGATGGTGAT GGCC*ACGGTCGAGAAA
Tb_7_1T	*AGAAGGAGATATAACTATG* ACACATGGATTCGACA	*GTGGTGGTGATGGTGAT GGCC*GTCATCAATCGCG
MO13_Est631	TCCATATGAGCTCACTTT TTATTG	GCTCGAGTTTAATCAGT TTGATAT
MO13_Est537	TCCATATGAGCTCACTTT TTATTG	ACTCGAGTGCTTTTAAG CATG
BRM_Am	*AGAAGGAGATATAACTATG* GACATCCAGAACCTG	*GTGGTGGTGATGGTGAT GGCC*TAACCGACAGGC
K3_Am	*AGAAGGAGATATAACTATG* ACGAACCAACTCTGGC	*GTGGTGGTGATGGTGAT GGCC*GACCGGGCACCG
Mo10_Am	*AGAAGGAGATATAACTATG* CGCCAGACACCCG	*GTGGTGGTGATGGTGAT GGCC*CGAGAAGGCCTG
CIAN4	*AGAAGGAGATATAACTATG* AAGAGACATATTCAGAT	*GTGGTGGTGATGGTGAT GGCC*ATGCACGGCGCA
P4FUM M07_Ac	*AGAAGGAGATATAACTATG* AGCTTAGAACAACAGG	*GTGGTGGTGATGGTGAT GGCC*GACTGCAACTTGA
P4FUM M07_Am	*AGAAGGAGATATAACTATG* ACCCAGGTTCAGGAAA	*GTGGTGGTGATGGTGAT GGCCG*TGGACGCGGCG
D8_RL	*AGAAGGAGATATAACTATG* GAACAATTAAAATTTCA	*GTGGTGGTGATGGTGAT GGCC*ATCGTGTGCTGGT
62Fuc1	*AGAAGGAGATATAACTATG* GACAAGATGTGGGGGG	*GTGGTGGTGATGGTGAT GGCC*GCGAATGAGTGTC
62Fuc1_422	CGCGCTTGTGAGTTGGA GTTC	GGCCATCACCATCACCA CCAC

Methods

Screening of esterases, amidohydrolases and glycosidases on agar plates by using acylated uridine esters, cytidine amides or glycosyluridines

The clones exhibiting hydrolase activity were identified on a MD medium (33.9 g/L Na2HPO4 15 g/L KH2PO4, 5 g/L NH4Cl, 2.5 g/L NaCl, 0.2% (w/v) glucose, 0.2% Casamino acid, 1 mM IPTG) containing 100 μg/mL ampicillin and 40 μg/mL kanamycin, 0.02 mg/mL 2',3',5'-*O*-acetyluridine, 2',3',5'-*O*-hexanoyluridine, N^4-benzoyl-2'-deoxycytidine, β-D-galactosyl-2'-deoxyuridine or α-L-fucosyl-2'-deoxyuridine as the sole source of uridine, allowing only the growth of recombinants that can complement the uridine auxotrophy of the *E. coli* DH10B *ΔpyrFEC*::Km strain by hydrolysing the uridine esters, amides or glycosides.

Screening of esterases by using the tributyrin-supplemented agar plates

LB agar medium supplemented with 1% tributyrin was used to screen for lipolytic/esterolytic activity. The clones showing a halo around the individual colonies, which indicated hydrolysis of the tributyrin, were selected on the emulsified tributyrin medium after growth at 37 °C for 1–2 days [7] [8] [9].

DNA sequencing and gene annotation

Nucleotide sequences were determined at Macrogen Europe (the Netherlands) using the following sequencing primers: M13F-pUC (5'-GTTTTCCCAGTCACGAC-3'), M13R-pUC (5'-CAGGAAACAGCTATGAC-3'), T7 Promoter (5´-TAATACGACTCACTATAGGG-3´), T7 terminator (5'-TAATACGACTCACTATAGGG-3') or LIC Reverse Sequencing primer, 24-mer (5'-GAGCGGATAACAATTTCACACAGG-3'). Some individual clones contained more than one ORF in each DNA fragment. ORFs were analysed by using the Unipro UGENE program, and homology search was conducted using the Blast server

(http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was conducted using the Maximum Likelihood Tree algorithm of MEGA 10 software. The alignment was performed using ClustalW in MEGA 10 [10].

Overexpression and purification of esterases

The recombinant proteins were overexpressed in *E. coli* strain BL21 (DE3) or HMS 174 (DE3). *E. coli* cells were grown in BHI (Brain-Heart-Infusion Broth) medium containing ampicillin (100 μg/mL) at 37°C with aeration. Protein expression was induced by adding IPTG to a final concentration of 0.1–0.5 mM at 0.6–1 OD_{600}, and cells were grown at 16–30 °C after induction for 4–18 h. Wet cell biomass from culture broth was suspended in the buffer A (50 mM potassium phosphate, pH 7.5), and disrupted by sonication for 2.5 min. A lysate was cleared by centrifugation at 15,000×*g* for 4 min. Cleared lysate was applied to Ni-NTA column (equilibrated with the buffer A). The column was washed with the buffer A, and the proteins were eluted with buffer A containing 300 mM imidazole. The active fractions were combined and dialyzed against the buffer B (50 mM potassium phosphate, pH 7.5), at 25 ºC. All the purification procedures were performed at room temperature.

Determination of protein concentration and purity

The concentration of protein was determined using Pierce™ Coomassie Plus (Bradford) Assay Reagent by Standard Microplate Protocol. Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE, 14% separating and 4.0% stacking) according to Laemmli. Gels were developed in Coomassie Brilliant Blue G-250 dye, scanned in 16 bit format and quantified by GelAnalyser program. Each sample contained 2 μg of total protein. Quantities of impurities and target proteins were estimated using calibration curve generated from known amounts of BSA: 0.125, 0.25 and 0.5 μg per band. The purity of analysed protein

was calculated as the ratio between quantity of target protein and quantity of all proteins.

Enzymatic activity of enzymes

Hydrolysis of pNP-esters. The activities of esterases were assayed by incubating the enzyme with 1 mM pNP-substrate (from 10 mM stock in DMSO) in 50 mM potassium phosphate, pH 7.5, buffer at a 37 ºC for 10 min. 50–3000 ng/1 ml of protein, depending on the enzyme specificity for the substrate, were added into the reaction mixture. The absorption of the reaction mixture at 405 nm was measured against enzyme-free blank to compensate for the substrate auto-hydrolysis [11] [12]. One unit is defined as the amount of enzyme that catalyzes the formation of 1 µmol of 4-nitrophenol (molar extinction coefficient $\varepsilon = 12.3\ M^{-1}cm^{-1}$) per minute. Enzyme activity was tested against different pNP-acyl esters (acetate, butyrate, valerate, decanoate, palmitate, and stearate).

Hydrolysis of nitrocefin. Nitrocefin is a chromogenic cephalosporin substrate routinely used to detect the presence of beta-lactamase enzymes [13]. Once hydrolysed, the degraded nitrocefin compound rapidly changes colour from yellow to red. A hydrolytic activity was assayed in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM nitrocefin at 37 ºC for 2 h. The change of the colour was evaluated visually.

pH-indicator-based assay. A hydrolytic activity was assayed in reaction mixture containing 5 mM potassium phosphate buffer, pH 7.5, 0.5 mM Phenol Red (from 10 mM stock in water), 1–5 µg/ml enzyme and 10 mM substrate: *R*/*S*-1-phenylethyl acetate/hexanoate/benzoate (from 100 mM stock in acetone), pentaacetylglucose or pentaacetylgalactose (from 100 mM stock in acetone), 3'-*O*-acetyl-2'-deoxyuridine, 3'-*O*-acetyl-N4-benzoyl-2'-deoxycytidine, 3'-*O*-levulinyl-N^4-benzoyl-2'-deocytidine, 5'-*O*-levulinyl-N^4-benzoyl-2'-deocytidine (from 100 mM stock in DMSO). Reaction mixtures were incubated at room temperature for up to 5 h.

A change of colour from red to yellow indicated the hydrolysis of esters [14] [15] [16].

Hydrolysis products test by thin-layer chromatography (TLC) method. A hydrolytic activity was assayed in reaction mixture containing 45 mM potassium phosphate buffer, pH 7.5, 1–4.6 μg/ml enzyme and 10 mM substrate: β-D-glucose pentaacetate, β-D- galactose pentaacetate (from 100 mM stock in acetone), N^4-acylcytidines/cytosines, 3'-*O*-levulinyl-N^4-benzoyl-2'deoxycytidine and 5'-*O*-levulinyl-N^4-benzoyl-2'-deoxycytidine (from 100 mM stock in DMSO). Reaction mixtures were incubated at 30 ºC temperature for up to 3 h. Thin-layer chromatography (TLC) was conducted on the Merck silica gel 60 F254 plates, using the cloroform and methanol (5:1) mixture of solvents. β-D-glucose pentaacetate, β-D-galactose pentaacetate were visualized by anisaldehide stain (50 mL ethanol, 1.9 mL of concentrated sulfuric acid, 0.54 mL of glacial acetic acid and 0.14 mL of p-anisaldehyde). The plate was developed by heating on a hot plate. Synthetic nucleosides were visualised by exposing to UV light.

HPLC-MS analyses

HPLC-MS analyses were performed using a high performance liquid chromatography system (Shimadzu, Japan) equipped with a photo diode array (PDA) detector (SPD-M20A Prominence diode array detector; Shimadzu, Japan) and a mass spectrometer (LCMS-2020, Shimadzu, Japan) equipped with an ESI source. The chromatographic separation was conducted using a Hydrosphere C18 column, 4 × 150 mm (YMC, Japan), or YMC Pack Pro column, 3× 150 mm (YMC, Japan) at 40 °C and a mobile phase that consisted of 0.1 % formic acid water solution (solvent A), and acetonitrile (solvent B) delivered in the gradient elution mode. Mass scans were measured from m/z 10 up to m/z 500, at 350 °C interface temperature, 250 °C DL temperature, ±4,500 V interface voltage, neutral DL/Qarray, using N 2 as nebulizing and drying gas. Mass spectrometry data was

acquired in both the positive and negative ionization mode. The data was analyzed using the LabSolutions LCMS software.

Enzymatic synthesis of glycosylated nucleosides

Enzymatic reaction of 2´-deoxyuridine galactosylation was prepared as follows: 0.1 M 2´-deoxyuridine 0.3 M *o*-nitrophenyl-β-D-galactopyranoside (oNP-Gal), 5 mg/ml Lactazyme-B™, 30 mM potassium phosphate buffer, pH 6.0 in total of 1000 µl reaction volume. Reactions were incubated with shaking at 50 ºC for 2 hours.

Fucosylation enzymatic reaction was prepared in 1000 µl reaction volume mix consisting of 120 mM 2´-deoxyuridine, 40 mM *p*-nitrophenyl-α-L-fucopyranoside, 30 mM potassium phosphate buffer, pH 7.5 and 0.35 mg/ml 62Fuc1 fucosidase. Reactions were incubated with shaking at 37 ºC for 2 hours.

Products of reactions were analyzed by TLC (mobile phase ethylacetate:methanol:water in proportion 7:3:1) and HPLC-MC. After incubation, reaction products were purified by chromatography. The chromatographic separation was conducted using a C18 column, and a mobile phase that consisted of water (solvent A) and 50% methanol solution (solvent B) delivered in the gradient elution mode.

RESULTS AND DISCUSSION

Selection of esterases from metagenomic libraries

The principle of the developed method for the selection of ester-hydrolysing enzymes is shown in Figure 1 on the left colum. For comparison, a standard tributyrin agar plate method is represented on the right.

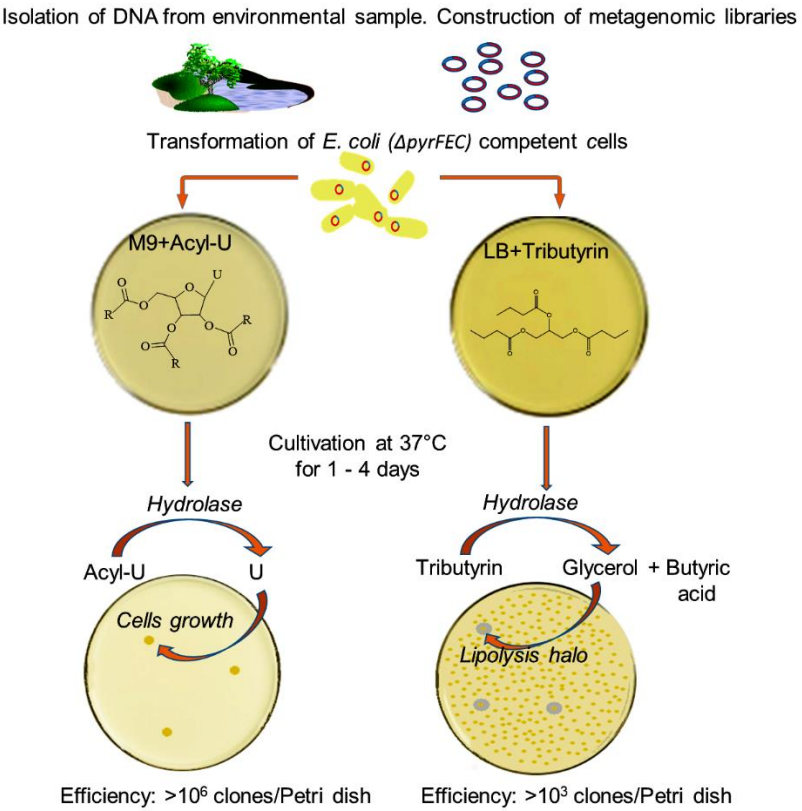

Figure 1. The principle of the developed selection method.

In short, metagenomic DNA isolated from the environmental samples is fragmented, inserted into an appropriate vector and used to transform competent cells of *E. coli* DH10B *ΔpyrFEC*::Km. A mineral medium containing 2',3',5'-tri-*O*-acetyluridine or 2',3',5'-tri-*O*-hexanoyluridine as the sole source of uridine is used to select the clones exhibiting acyl-esterase activity. The positive hits complement the uridine auxotrophy of the *E. coli* DH10B *ΔpyrFEC*::Km strain by hydrolysis of the substrate to uridine allowing colony formation.

In this study metagenomic libraries constructed from different soil-originated metagenomic DNA, fragmented and ligated in pUC19 vector were used. In total, 19 libraries were tested and 87 positive clones were selected. All clones were re-streaked on MD medium without uridine or uridine derivative. Four of the selected hits capable of forming colonies on MD medium without the uridine esters were considered as false positives (approximately 5%) and were omitted from the further analysis. The plasmid DNA from the remaining clones was isolated, and the fragments obtained after restriction digestion were analysed by sequencing to exclude the redundancy. Hence, 30 clones were chosen for further analysis.

Bioinformatics analysis showed that the selected clones exhibiting esterase activity contained ORFs with medium (31%) to high (100%) sequence identity to proteins found in the databases (Table 2). Eleven of the closest homologs were annotated as hypothetical proteins or with unknown esterase activity.

Table 2 The list of selected ester hydrolases. The clones selected using hexanoyluridine as the uridine source are marked by asterisk.

Clone, GenBank accession No.	**The nearest homolog, GenBank accession No.**	**Identities %**
24T5, MH423251	*Kaistia soli*, WP_073056985.1, alpha/beta hydrolase	67
24T1, MH423255	*Devosia epidermidihirudinis*, WP_046138431.1, hypothetical protein	99

Clone, GenBank accession No.	The nearest homolog, GenBank accession No.	Identities %
24T3, MH423254	*Devosia riboflavin*, WP_035086921.1, hypothetical protein	95
33T1, MH423252	*Devosia* sp. Root685, WP_082561207.1, hypothetical protein	95
30T1, MH423263	*Sinorhizobium* sp. GL2, KSV78272.1, hypothetical protein N182_21555	61
30T2, MH423266	*Acidobacteria* bacterium, OLB17 KXK06970.1, Alpha/beta hydrolase	74
36T2, MH423275	*Paracoccus aminophilus*, WP_020950583.1, alpha/beta hydrolase	74
BD1, MH423279	*Rhizobium tropici*, WP_015341790.1, alpha/beta hydrolase fold-3 catalytic domain- containing protein	51
EN3H*, MH423261	*Pseudomonas lini*, WP_048393178.1, alpha/beta hydrolase	95
MO101T, MH423257	*Lysinibacillus* sp. AR18-8, WP_066036519.1, alpha/beta hydrolase	99
CAP3H*, MH423259	MULTISPECIES: *Cupriavidus*, WP_092295063.1, alpha/beta hydrolase	96
BD2H*, MH423277	MULTISPECIES: *Duganella*, WP_090189706.1, alpha/beta hydrolase	70
3T, MH423256	*Roseiflexus* sp. RS-1, WP_011955564.1, acetylxylan esterase	61
GRU1, MH423265	*Paenibacillus phocaensis*, WP_068787184.1, acetylesterase	100
SVG3, MH423258	*Rheinheimera* sp. KL1, WP_053423444.1, alpha/beta hydrolase	93
K3H2*, MH423271	*Dehalococcoidia* bacterium, PWB48329.1, hypothetical protein	67
4H1T, MH423260	*Sphingobacterium mizutaii*, WP_093095847.1, esterase	87
RIEB, MH423273	Rhizobium sp. P44RR-XXIV, WP_077472732.1, alpha/beta hydrolase	68
SVGPA2T, MH423270	*Sphingopyxis* sp. C-1, WP_062186324.1, alpha/beta hydrolase	93

Clone, GenBank accession No.	The nearest homolog, GenBank accession No.	Identities %
C233, MH423278	*Paracoccus* sp. TRP, WP_010397925.1, glycoside hydrolase	74
SVG1, MH423269	*Ensifer* sp. LC163, WP_083222508.1, serine hydrolase (Beta-lactamase)	92
12T, MH423253	*Acidobacteria* bacterium, OFW37874.1, hypothetical protein A3J29_14090	65
45T3, MH423262	*Brevundimonas* sp. Leaf363, WP_056098529.1, ribosomal-protein-alanine N-acetyltransferase	67
PLA1, MH392251	*Gemmata* sp. SH-PL17, AMV27246.1, GDSL-like Lipase/Acylhydrolase	58
33T3, MH423272	*Pseudohongiella acticola*, WP_047492018.1, hypothetical protein	61
BD9, MH423268	*Mesorhizobium temperatum*, WP_095491896.1, SGNH/GDSL hydrolase family protein	50
36T1, MH423267	*Firmicutes* bacterium CAG:272, CDC74944.1, sialate O-acetylesterase	31
MO4B, MH423274	*Microvirga ossetica*, WP_099513428.1, peptidase	92
EN1H*, MH423276	*Bacillus* sp. J33, WP_026581439.1, hypothetical protein	100
1315H*, MH423264	*Microbacterium gorillae*, WP_094770426.1, DUF998 domain-containing protein	57

The phylogenetic analysis of the selected hydrolases showed that the enzymes represent very diverse groups of proteins. Most of the hits were representatives of ABhydrolase (α/β hydrolase) superfamily (SSF53474) (19 hits) and SGNH hydrolases (SSF52266) (4 hits) followed with proteins belonging to β-lactamases (SSF56601) (2 hits), α/β hydrolases/galactose-binding domain-like (2 hits), glycosyl hydrolases (SSF51445) (1 hit), *N*-acyltransferase superfamily (SSF55729) (1 hit) and one DUF998 family protein.

To confirm that the hits encoded the enzymes with esterolytic activity, the selected genes were PCR-amplified and the resulting fragments were ligated into pET21 or pLATE31 expression vectors. *E. coli* strain BL21 (DE3) was transformed with the recombinant plasmids and used for the expression of the recombinant proteins. In total, 27 recombinant proteins were purified by Ni-NTA chromatography and 23 of them showed purity higher than 90% (Table 3). Due to hydrophobic nature, the protein encoded by the clone 1315H was not purified, and an insoluble fraction of the cells was used for the determination of activity. The proteins encoded by clones RIEB and 4H1T, SVGPA2T were not purified due to a poor expression. The purity of the protein MO4B was not analysed because the concentration of the purified enzyme was very low due to its poor solubility. During overexpression, sufficient amounts of five proteins 24T1, 24T3, 33T1, SVG1 and SVG3 were released into the extracellular space, and the enzymes were purified from the medium, most likely, without the signal peptides.

The hydrolytic activity of the purified proteins was analysed with various *p*-nitrophenyl (pNP) esters: acetate, butyrate, valerate, decanoate, palmitate and stearate (Table 3). All hydrolases were active towards the short-chain esters pNP-acetate and pNP-butyrate, most of them used pNP-valerate, but only a few of them hydrolysed pNP-decanoate (Table 3). Neither pNP-palmitate nor stearate was recognized as the substrate (data not show).

Table 3. The activity of the recombinant esterases towards p-nitrophenyl esters.

Clone	Purity, %	Activity, U/μg of protein			
		pNP-acetate	pNP-butyrate	pNP-valerate	pNP-decanoate
24T5	96	38±3.8	26.4±4.8	38.2±3.8	0.3±0.01
24T1	94	66.6±10.7	10±20.8	84±8.7	0.9±0.01
24T3	85	45±11	46±8.1	60.5±6.2	-
33T1	96	49±0.8	80±2.4	27.5±6.5	0.9±0.01
30T1	93	55±2.3	54.4±5.8	20±2.1	-

Clone	Purity, %	Activity, U/μg of protein			
		pNP-acetate	pNP-butyrate	pNP-valerate	pNP-decanoate
30T2	93	52±4.7	8±0.1	2.6±0.8	-
36T2	77	32.7±2.9	4.8±0.3	2.2±0.25	-
BD1	93	12.9±0.3	5.7±0.8	4.3±0.5	-
EN3H	93	283±15	336±15	196±18	8.6±0.6
MO101T	96	159±19	0.2±0.1	-	-
CAP3H	74	29.1±6.2	75±10	34.3±6.5	10.3±0.34
BD2H	93	1.9±0.6	18±0.5	24.3±1.1	3.1±0.5
3T	93	23±4	0.9±0.2	-	-
Gru1	98	177±0.3	2.7±0.8	1.3±0.5	-
SVG3	99	346±7.5	35±9.6	33.6±3.6	-
K3H2	98	349±35	425±21	49.3±2.5	16.9±2.7
4H1T#	n.a.	n.a.	n.a.	n.a.	n.a.
RIEB#	n.a.	n.a.	n.a.	n.a.	n.a.
SVGPA2T#	n.a.	n.a.	n.a.	n.a.	n.a.
Tb107T*	94	122±4.7	133±8.8	7.4±0.6	2.6±1.4
C233	90	3.7±0.5	5.8±1.4	4.7±1.6	-
SVG1	95	343±15	367±15.8	189±52.8	2.1±0.34
12T	77	4.1±0.5	0.3±0.2	0.3±0.2	-
45T3	86	104±12	78±.5.4	49.5±6.1	-
PLA1	93	30±0.8	2.4±0.02	-	-
33T3	82	80±6.7	7.6±0.7	0.9±0.4	-
BD9	69	3.2±1.4	3±2	3.2±2.6	-
36T1	95	4.5±0.5	6±0.7	6.7±3	-
MO4B**	-	1.3±0.1	0.12±0.1	-	-
EN1H	95	14.4±2	47±17	74.4±8.9	2.0±0.2
1315H***	-	2.2±1.3	17.1±10	35.6±17.4	1.±0.6

– the proteins were not purified due to a poor expression. *Tb10_7T was screened on the tributyrin agar plate. ** a concentration of the protein was too low for a purity evaluation. ***the protein was expressed but not purified. The activity calculated based on proteins of crude extract. n.a. – not analyzed, "-" – no activity.

The recombinant enzymes selected using acetyluridine were able to hydrolyse short-chain esters of pNP with a high efficiency, but displayed a very weak activity against pNP-decanoate. The enzymes selected on hexanoyluridine demonstrated the activity towards the

longer-chain esters. These esterases hydrolysed pNP-decanoate with a high efficiency. Further analysis of the substrate specificity of the selected enzymes showed that approximately half of the tested esterases could accept the bulky peracetylated carbohydrates (Fig. 2), and fourteen esterases could hydrolyse tributyrin (tested on standard tributyrin agar plate method).

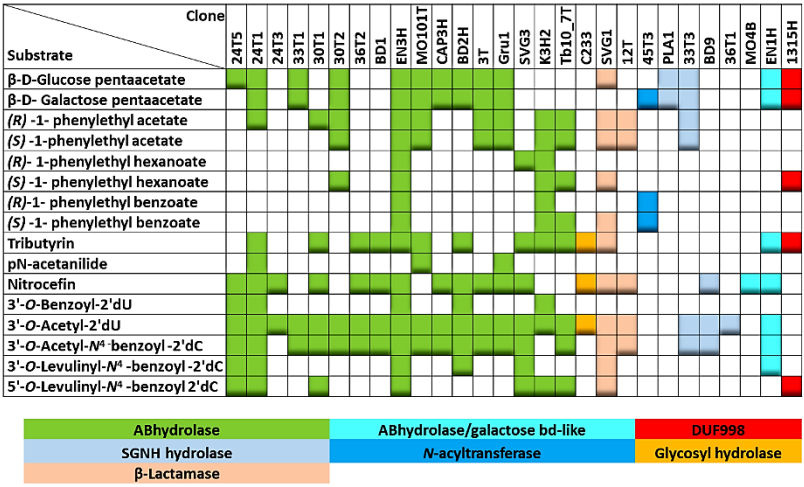

Figure 2. The substrate specificity of the recombinant esterases.

To test if the standard selection on agar plates with tributyrin would result in clones exhibiting activity towards 2',3',5'-tri-*O*-acetyluridine or 2',3',5'-tri-*O*-hexanoyluridine, several metagenomics libraries were evaluated and two hits (Tb7_1T and Tb10_7T) forming the halos indicative of the hydrolysis were screened. Tb7_1T (MH423281) encoded the group β-lactamases protein, and Tb10_7T (MH423280) was the most similar to ABhydrolases. Neither of the two clones were capable of growing on acetyl- or hexanoyluridine as a uridine source.

Selection of amidohydrolases from metagenomic libraries

After successfully developing the method for functional selection of esterases, the same principle was applied to the selection of amidohydrolases from metagenome. To develop this selection method, N^4-benzoyl-2'-deoxycytidine (**4**) (Fig. 3 A), as a sole source of uridine in the minimal M9 medium, the *E. coli* DH10B *pyrFEC*::Km strain and the metagenomic libraries were used. The amidohydrolases, supposedly encoded in the metagenomic libraries, convert N^4-benzoyl-2'-deoxycytidine to 2'-deoxycytidine, and the native *E. coli* cytidine deaminase converts 2'-deoxycytidine to 2'-deoxyuridine, upon which the growth phenotype of uridine auxotrophic cells is restored. Also, N^4-acetylcytidine was tested as a uridine source. However, it was found that *E. coli* metabolizes this substrate and it was demonstrated that the 103-amino acid *Escherichia coli* protein YqfB, previously identified as hypothetical, is a unique ASCH domain-containing amidohydrolase responsible for the catabolism of N^4-acetylcytidine. The data on the YqfB protein have been published [5] and are not discussed in this study in more detail.

The sequence analysis showed that 6 of the 7 selected clones contain ORFs with medium (40%) to high (99%) sequence identity to hydrolases found in the NCBI GenBank database [17] (Table 4). The clone P4FUMM07 contains two ORFs (80–84% identity) encoding potential amidohydrolases, which are therefore named P4FUMM07_AcAm and P4FUMM07_AmH.

Table 4. The list of selected amidohydrolases and their nearest homologues.

Selected amidase, GenBank accession No.	The nearest homologue genus/species, GenBank accession No., protein name	Identity (%)
BRM_Am, MN734430	*Pseudomonas* sp. 1 R 17, WP_065947988.1, amidase	99
K3_Am, MN734429	*Achromobacter xylosoxidans*, WP_013394140.1, amidase	97
MO10_Am, MN734431	*Rhodococcus erythropolis*, WP_084324709.1, amidase	99
P4FUMM07_AcAm, MN734432	*Agromyces* sp. Root80, Wp056655740.1, amidase	84
P4FUMM07_ AmH, MN734432	*Agromyces* sp. Root81, Wp056655731.1, amidase	80
CIAN4, MN734434	*Pusillimonas noertemannii*, PVY61383.1, feruloyl esterase	73
MO13_Est631, MN734432	*Bacillus asahii*, WP_119118064.1, tannase/feruloyl esterase	56
D8_RL, MN734435	*Runella limosa*, WP_028525627.1, hypothetical protein	40

The phylogenetic analysis of the selected hydrolases showed that these enzymes belong to varying groups of proteins. Four of these enzymes (BRM_Am, MO10_Am, K3_Am and P4FUMM07_AcAm) belong to the Amidase signature (AS) enzyme superfamily SSF75304. P4FUMM07_AmH is a representative of metal dependent amidohydrolase superfamilies SSF51338 and SSF51556. MO13 and CIAN4 are representatives of ABhydrolase superfamily SSF53474 and belong to the group C of the tannase/feruloyl_esterase family. Out of all 8 of the selected amidohydrolases, the most intriguing is the D8_RL enzyme: it is a 128 amino acid uncharacterized protein without any predictable hydrolytic function. The amino acid sequence analysis using SMART [18] revealed that D8_RL contains the ASCH (Human

activating signal cointegrator homology) [19] and GAS2 (Growth-Arrest-Specific Protein 2) domains [20]. ASCH domain-containing proteins are widespread and diverse but, at present, the vast majority of those proteins have no function assigned to them [19], except for an ASCH domain-containing ribonuclease from *Zymomonas mobilis* [21] and the enzyme YqfB from *Escherichia coli*, which is active towards N^4-acylcytidines [5].

To confirm that the enzymes encoded in the selected fragments of the metagenomic DNA have the amidohydrolytic activity, their genes were PCR-amplified and the resulting fragments were ligated into pLATE31 or pET21a expression vectors. BL21 (DE3) strain of *E. coli* was transformed with these plasmids and used for the synthesis of the recombinant amidohydrolase proteins. In total, seven recombinant proteins were successfully purified by Ni-NTA chromatography. Due to its hydrophobic nature, the P4FUMM07_AmH protein was not purified to homogeneity, and a partially purified protein fraction was used for the activity assays.

The hydrolytic activity of the recombinant enzymes was assayed qualitatively by testing 29 different substrates (Figure 3). All tested hydrolases were not very strictly specific to 2'-hydroxylation of ribose; however, they preferred the nucleosides as substrates and most of the enzymes did not hydrolyse cytosine (**15**–**16**) or isocytosine (**17**) derivatives. The bulkiness of the acyl group played a significant role, since the selected hydrolases were specific towards longer acyls in the case of aliphatic radicals (**1, 2, 3, 11, 13**). Seven of the selected hydrolases recognized esters as substrates, only the P4FUMM07_AmH was a "true" amidohydrolase without any detected esterase activity. Five hydrolases (MO13_Est537, CIAN4, K3_Am, D8_RL and P4FUMM07_AcAm) showed a beta-lactamase activity in the presence of nitrocefin (**29**). The enzymes were active towards the short-chain esters (**20**–**22**), most of them used compounds **24** and **25**, but only three amidases – BRM_Am, MO13Est537 and D8_RL – hydrolysed the pNP-decanoate (**23**).

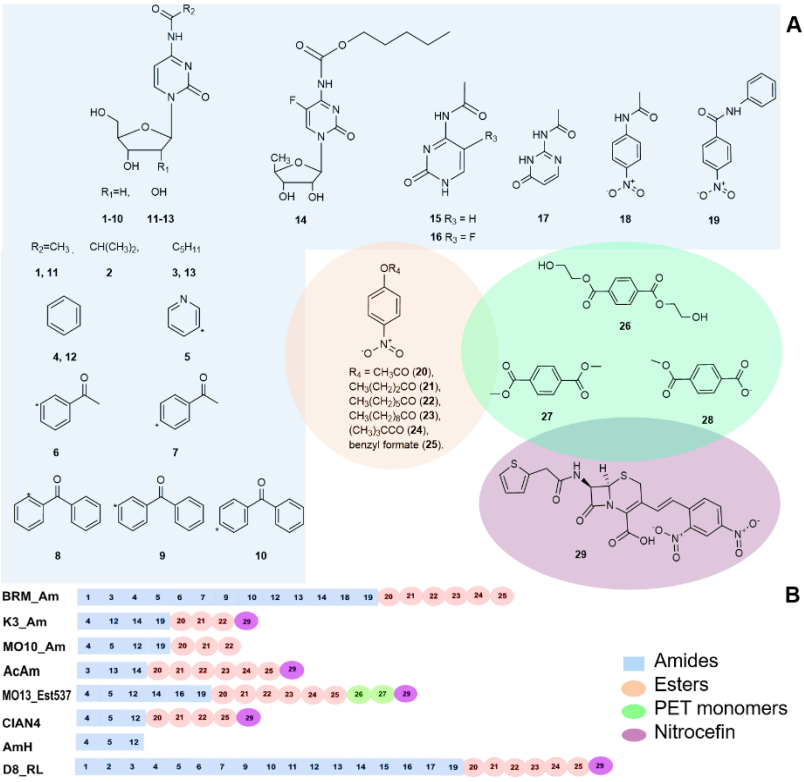

Figure 3. A Compounds tested as substrates for hydrolases. An asterisk marks the carbon atom through which the group was linked. **B**. Substrate spectra of the individual hydrolases identified in this study; AcAm – P4FUMM07_AcAm, AmH – P4FUMM07_AmH.

Thus, the selected hydrolases were the most active towards short-chain esters, in contrast to the larger amides. Among the identified enzymes, the MO13_Est537 showed distinguished catalytic properties: in addition to cytidine-based substrates, the enzyme hydrolysed dimethyl terephthalate (**27**) and *bis*(2-hydroxyethyl) terephthalate (**26**).

Model method for selection of glycosidases

The aim of this part of work was to test the suitability of the method by using *E. coli* uridine auxotrophic strains DH10B Δ*pyrFEC* or HMS174 Δ*pyrF* and substrates glycosyluridine derivatives for the selection of glycosidases. Substrate β-D-galactosyl-2'-deoxyuridine and α-L-fucosyl-2'-deoxyuridine were prepared by adapting and modifying methods reported in the literature [22] [23] [24]. Lactazyme B™ β-D-galactosidase from *Bacillus circulans* and metagenomic 62Fuc1 fucosidase from Curonian lagoon water sample (received from Faculty of Marine Technology and Natural Sciences, Klaipeda University) were used for the enzymatic reaction. Metagenomic 62Fuc1 fucosidase has been used regarding poor commercial availability of fucosidases.

62Fuc1 α-L-fucosidase gene was amplified from the above-mentioned metagenomic DNA, protein was overexpressed in *E. coli* BL21 (DE3) cells, purified and its activity was tested. HHPred structure analysis showed, that the nearest homologue of 62Fuc1 fucosidase is *Bacteroides thetaiotaomicron* (PDB 4J27) α-L-fucosidase (identity 27%). 62Fuc1 fucosidase is 64 kDa polypeptide with hydrolase and transfucosidase activity. The trasnsfucosylation activity of the 62Fuc1 enzymes was assayed qualitatively by testing different acceptors: uridine, 2´-deoxyuridine, 2´-deoxycytidine, 2´,3´-dideoxyuridine, 2´,5´-dideoxyuridine, X-Gal, IPTG, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-L-fucopyranoside and metyl-α-L-fucopyranoside. In all reactions *p*-nitrophenyl-α-L-fucopyranoside was used as a donor of the fucosyl group. Fuc621 fucosidase recognized 2´-deoxyuridine, uridine, X-Gal, 2´,5´-dideoxyuridine and *p*-nitrophenyl-α-L-fucopyranoside (reaction product – *p*-nitrophenyl-difucopyranoside) as an acceptor. After evaluation these results, fucosyl-dU and galactosyl-dU was chosen as a substrate for selection of fucosidases and galactosidases in M9 medium, respectively. Selection of glycosidases was tested on the selective M9 media agar plate supplemented with the purified β-D-galactosyldeoxyuridine or

α-L-fucosyl-2'-deoxyuridine as a sole source of uridine and the strains producing β-D-galactosidase (*E. coli* DH10B Δ*pyr*FEC/pUC19) and 62Fuc1 fucosidase (HMS174 Δ*pyr*F/pLATE31-62Fuc1), respectively. *E. coli* DH10B Δ*pyr*FEC/pUC19 colonies were observed after 2 days and HMS174 Δ*pyr*F/pLATE31-62Fuc1 after 4 days (a very weak growth) of incubation (Figure 4). Most likely, a poor expression of the 62Fuc1 gene or a difficult transportation of the substrate into the cells from the culture medium determines the weak growth of clone of fucosidase.

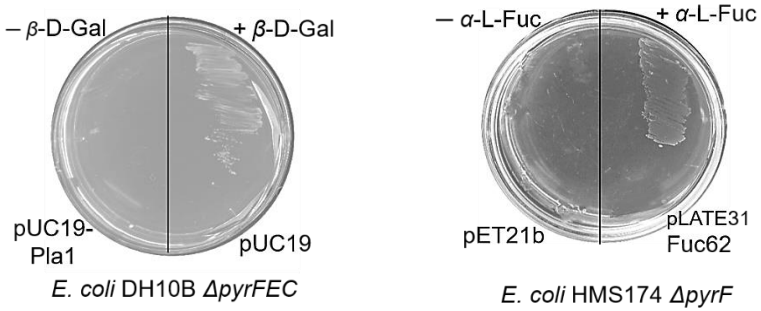

Figure 4. Selection of LacZ galactosidase and metagenomic 62Fuc1 fucosidase on M9 media with β-D-galactosyl-2'-deoxyuridine or α-L-fucosyl-2'-deoxyuridine.

Although the method of selection of glycosidase still requires improvement, this proof-of-principle test showed that the appropriate combination of the *E. coli* uridine auxotrophic strain and glycosylated deoxyuridine a sole source of uridine allowed the selection of the desired glycosidases.

RESUMÉ

In this study, two selection methods for the screening of esterases and amidohydrolases from metagenomic libraries were developed and a model method for the selection of glycosidases was presented.

To develop the selection method for esterases, 2',3',5'-tri-*O*-acetyluridine and 2',3',5'-tri-*O*-hexanoyluridine were chosen as the sole source of uridine, supporting growth only of those recombinant clones, which encode ester hydrolases that complement the uridine auxotrophy of the *E. coli* DH10B *ΔpyrFEC*::Km [6] strain by hydrolysis of uridine esters. Regarding the application of two structurally different substrates used for the selection, it was supposed that the uridine derivatives with a varied acyl length could predetermine the properties of the selected hydrolases, and the enzymes with a different preference to acyl size would be identified.

The developed esterase selection method is fast, of high performance and easy to use. Only 14 clones from 30 clones selected using uridine esters as substrates, formed tributyrin hydrolysis zones and clones selected on tributyrin (Tb7_1T and Tb10_7T) did not hydrolyze modified uridine esters. The amount of substrate required for selective selection (1–2 mg/100 ml medium) is a thousand fold lower than the amount of tributyrin (1 g/100 ml of medium). This indicates that the developed selective method is more sensitive, requires significantly less substrate, moreover the number of clones tested per process is at least 10^3 higher.

The esterases selected by this method are very diverse in their sequences and catalytic properties. Analysis of the protein sequences and phylogenetic analysis revealed that the selected esterases belong to as many as eight enzyme superfamilies. The closest homologues of the ten esterase sequences belong to hypothetical proteins, three of the selected enzymes belonging to the glycoside hydrolases, *N*-acetyltransferases, or DUF998 families. There is no evidence in the literature that classical screening methods for esterases or lipases

reveal these family members. This proves that the method is also suitable for the search for enzymes with previously uncharacterized unknown esterase activity.

In total, 27 selected esterase genes were expressed in *E. coli* cells, active enzymes were purified, and their activities and specificities for substrates were determined. Also, it has been found that some of the enzymes have not only esterase but also lactamase and amidase activities, moreover, they showed regio- and stereo-specificity. Using acetyluridine in the selection of esterases, enzymes that more efficiently hydrolyse p-NP short-chain esters and hexanoyluridine were used to find enzymes capable of hydrolysing longer-chain esters. These results showed that the ester group of the substrate used for selection determined, at least partly, the specificity of the enzymes selected.

A combination of a synthetic cytidine derivative and *E. coli* uridine auxotrophic DH10B *ΔpyrFEC* strain allows for the functional selection of amidohydrolases from metagenomic libraries. The expression of eight selected amidohydrolase genes in *E. coli* cells was performed, and seven enzymes were purified. The selected amidohydrolases in the hydrolysis reactions of aliphatic amide radicals were more specific for longer acyls, and the opposite effect was observed in the hydrolysis reactions of ester linkages – more efficient catalysis of hydrolysis of short-chain esters. Several of the selected hydrolases recognized nitrocefin as a substrate. The spectrum of selected amidohydrolase substrates suggests that both the type of substrate (nucleoside) and the size of the amide side group (aromatic group) used for selection, at least partially, determine the biocatalytic properties of the selected enzymes. Hence, the unique metagenomic amidohydrolases D8_RL, BRM_Am and MO13_Est631, which hydrolyse a wide spectrum of substrates, have been discovered.

Results of the model method for selection of glycosidases showed that the basic principle of the method can be applied to glycosidase screening, too. The appropriate substrates such as β-D-galactosyl-2'-deoxyuridine and α-L-fucosyl-2'-deoxyuridine were

prepared by using Lactazyme B^{TM} β-D-galactosidase from *Bacillus circulans* and metagenomic 62Fuc1 fucosidase, respectively. Metagenomic 62Fuc1 was screened from Curonian lagoon water sample by genomic DNA sequence analysis.

A major advantage of nucleosides as substrates used for selection is that desired enzymes can be chosen by altering the acyl groups of substrates. The principle of this method could be applied to the selection of other hydrolases, such as nucleoside/nucleotide hydrolases. The method has the potential expansion by using auxotrophic host of other metabolic pathways and different modified nucleosides, nucleotides or heterocyclic bases derivatives. Also it is possible variant based on the synthesis of compounds, supporting growth.

CONCLUSIONS

1. The developed selection methods based on the uracil auxotroph *E. coli* strain and synthetic uridine esters or cytidine amides can be applied to the selection of esterases and amidohydrolases from metagenomic libraries.
2. The developed selection method for esterases is more sensitive compared to the classical tributyrin method. The amount of substrate used for selection is 1000-fold lower and the amount of selected hits is 2-fold higher.
3. The developed selection approach allow identification of very different hydrolases. Hence, the selected esterases belong to eight different enzyme families and amidohydrolases are from four different families of enzymes. The selected enzymes showed different catalytic profiles towards miscellaneous substrates.
4. The developed selection methods of esterases and amidohydrolases allow to select enzymes with unknown or undetermined activity. In total, 50% of homologues of the selected enzymes are hypothetical proteins or with unknown ester or amide hydrolase function.
5. Substrates determine the specificity of selected hydrolases.
6. Metagenomic 62Fuc1 fucosidase is an α-L-fucosidase and catalyzes transglycosylation reactions.
7. A model glycosidase selection method based on the uracil auxotrophic *E. coli* strain and substrate glycosyl(deoxy)uridine can be applied for selection of glycosidases.

LIST OF PUBLICATIONS

Articles

1. **Urbelienė, N.**; Kutanovas, S.; Meškienė, R.; Gasparavičiūtė, R.; Tauraitė, D.; Koplūnaitė, M.; Meškys, R. Application of the uridine auxotrophic host and synthetic nucleosides for a rapid selection of hydrolases from metagenomic libraries. Microbial Biotechnology, 2019, 12, 148–160, doi:10.1111/1751-7915.13316.
2. Stanislauskienė, R.; Laurynėnas, A.; Rutkienė, R.; Aučynaitė, A.; Tauraitė, D.; Meškienė, R.; **Urbelienė, N.**; Kaupinis, A.; Valius, M.; Kaliniene, L.; R. Meškys. YqfB protein from *Escherichia coli*: an atypical amidohydrolase active towards N^4-acylcytosine derivatives. Scientific Reports, 2020, 10, 788, doi:10.1038/s41598-020-57664-w.
3. **Urbelienė, N.**; Meškienė, R.; Tiškus, M.; Stanislauskienė, R.; Aučynaitė, A.; Laurynėnas, A.; Meškys, R. A rapid method for the selection of amidohydrolases from metagenomic libraries by applying synthetic nucleosides and a uridine auxotrophic host. Catalysts, 2020, 10, 445, doi:10.3390/catal10040445.

Conference posters

1. N. Urbelienė, S. Kutanovas, R. Meškienė, R. Gasparavičiūtė, D. Tauraitė, R. Meškys. The use of the auxotrophic host and synthetic nucleosides for a rapid selection of hydrolases from metagenomic libraries. BioCat, August 26–30, 2018, Hamburg, Germany.
2. N. Urbelienė; R. Meškienė; E. Gustaitė; R. Meškys. Application of *E. coli* auxotrophic host and synthetic nucleosides for a selection of hydrolases from metagenomic libraries. COINS 2019 – the 14th International Conference of Life Sciences, February 26–28, 2019, Vilnius, Lithuania.

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SANTRAUKA

Šio darbo metu sukurti ir patikrinti du selektyvios atrankos metodai, skirti esterazių ir amidohidrolazių atrankai iš metagenominių bibliotekų, bei parodytas modelinis glikozidazių atrankos metodas.

Sukurtas esterazių atrankos metodas yra greitas, didelio našumo ir patogus naudoti. Iš 30 klonų, atrinktų panaudojus acetil- ar heksanoil-uridinų substratus, tik 14 klonų formavo tributirino hidrolizės zonas, o atrinkti naudojant substratą tributiriną Tb7_1T ir Tb10_7T klonai nehidrolizavo modifikuotų uridino esterių. Selektyviai atrankai reikalingas substrato kiekis (1–2 mg/100 ml terpės) yra tūkstantį kartų mažesnis lyginant su naudojamu tributirino kiekiu (1 g/100 ml terpės), o selektyviai atrinktų klonų gauta dvigubai daugiau. Tai rodo, kad sukurtas selektyvus metodas yra jautresnis. Be to patikrinamų klonų kiekis viename procese yra bent trim eilėm didesnis.

Šiuo metodu atrenkamos esterazės yra labai įvairios savo sekomis ir katalizinėmis savybėmis. Išanalizavus baltymų sekas ir atlikus filogenetinę jų analizę, paaiškėjo, kad atrinktos esterazės priklauso net 8 fermentų superšeimoms. Dešimties esterazių sekų artimiausi homologai priskiriami hipotetiniams baltymams, trys iš atrinktų fermentų priklauso glikozidų hidrolazių, *N*-acetiltransferazių ar DUF998 šeimoms. Literatūroje nėra duomenų, kad klasikiniais atrankos metodais ieškant esterazių ar lipazių būtų rasta šių šeimos atstovų. Tai įrodo, kad metodas tinka ir fermentų su nežinomu esteraziniu aktyvumu paieškai.

Darbo metu optimizuota 27 atrinktų esterazių genų raiška *E. coli* ląstelėse, išgryninti aktyvūs fermentai bei patikrintas jų aktyvumas ir savitumas substratams, panaudojus pNP acetatą, butiratą, valeratą, dekanoatą, palmitatą ir stearatą. Nustatyta, kad dalis surastų fermentų pasižymi ne tik esteraziniu, bet taip pat laktamaziniu ir amidaziniu aktyvumais, bei pasižymi regio- ir stereo-savitaisiais aktyvumais. Esterazių atrankoje panaudojus acetiluridiną, atrinkti fermentai, efektyviau hidrolizuojantys pNP trumpų grandinių esterius,

o heksanoiluridiną, rasti fermentai, gebantys hidrolizuoti ilgesnių grandinių esterius. Šie rezultatai parodė, kad substrato esterio grupė, naudojama atrankai, bent iš dalies lėmė pasirinkto fermentų specifiškumą.

Sukurtas amidohidrolazių atrankos metodas leidžia greitai atrinkti unikalius fermentus tokius kaip D8_RL amidohidrolazę, paižyminčią labai plačiu substratų spektru, ar MO13_Est631 (MO13_Est537) katalizuojančią PET komponentų hidrolizę. Darbo metu optimizuota 8 atrinktų amidohidrolazių genų raiška *E. coli* ląstelėse, išgryninti 7 fermentai. Nustatyta, kad dalis surastų fermentų pasižymi amidohidrolaziniu, esteraziniu, o keletas iš jų ir laktamaziniu aktyvumais, kai kurie taip pat pasižymi regio-savitaisiais aktyvumais (BRM_Am, D8_Rl). Alifatinių amidų radikalų hidrolizės reakcijose atrinktos amidohidrolazės buvo labiau savitos ilgesnių acilų atžvilgiu, o esterinių jungčių hidrolizės reakcijose buvo stebimas atvirkščias efektas – efektyvesnė trumpų grandinių esterių hidrolizės katalizė. Atrinktų amidohidrolazių substratų spektras leidžia daryti išvadą, kad atrankai naudotas tiek substrato tipas (nukleozidas), tiek substrato šoninės amido grupės dydis (aromatinė grupė) bent iš dalies nulemia atrinkų fermentų biokatalizines savybes – atrinkti fermentai hidrolizavo nukleozidų amidus, bet ne heterociklinių bazių amidus, išskyrus D8_RL hidrolazę, ir rodė didesnį savitumą aromatinių grupių pakaitams.

Glikozidazių atrankos modelinio metodo rezultatai parodė, kad principinė metodo schema gali būti taikoma glikozidazių atrankai. Darbo eigoje susidurta su dviejų tipų problemomis – maža substrato sintezės išeiga ir nepakankama fukozidazės geno raiška. Esant gerai geno raiškai (LacZ galaktozidazė), uridino auksotrofo požymio kompensavimas *in vivo* yra stebimas, o kai geno raiška silpna (62Fuc1 fukozidazė), tai kolonijų augimas yra pernelyg lėtas. Siekiant padidinti glikozidazių atrankos efektyvumą reikia ieškoti geresnių fermento raiškos sąlygų, o efektyviai fukozilnukleozidų sintezei reakcijos pusiausvyrą paslinkti produkto sintezės, o ne hidrolizės kryptimi. Galimas sprendimo būdas yra 62Fuc1 mutagenezė (atsitiktinė arba

tikslinė) ir patobulintų variantų atranka. Taip pat literatūroje yra duomenų, kad pakeitus fukozidazės katalizinį nukleofilą, prarandamas hidrolizinis aktyvumas ir fukozidazė virsta fukosintaze.

Šio darbo metu atrinkti fermentai turi potencialų panaudojimą įvairiuose srityse – nukleozidų modifikacijos reakcijose, vykdant stereoselektyvias ar regioselektyvias sintezes. Atrinkta unikali amidohidrolazė D8_RL, kaip ir YqfB, kelia tiek industrinį, tiek mokslinį susidomėjimą, nes ASCH domenus turinčių fermentų funkcijos dar yra labai menkai ištirtos, be to abu fermentai pasižymi puikiomis biokatalizinėmis savybėmis. 1315H esterazė gali būti panaudota kaip inkarinis membraninis baltymas, o MO13_Est537 turi panaudojimo potencialą PET perdirbimo srityje. Mutagenezės pagalba galima būtų sukurti MO13_Est537 mutantų biblioteką ir ją patikrinti selektyvios atrankos metodu vietoj N^4-benzoil-2'-deoksicitidino panaudojant struktūriškai artimesnį PET monomerams substratą.

Didelį potencialą panaudojimui įvairiose chemofermentinėse sintezėse turi ir plataus substratų spektro esterazės ir amidohidrolazės SVG1, BRM_Am, D8_Rl, MO13Est537, bei enantioselektyvios esterazės 24T1, 30T1, 30T2, SVG3, SVG1, 1315H.

Didelis nukleozidų kaip substratų panaudojimo atrankoms privalumas yra tame, kad keičiant substrato acilo pakaitų grupes, galima atrinkti įvairius pageidaujamus fermentus. Šio metodo principą galima būtų pritaikyti ir kitų hidrolazių, pavyzdžiui nukleozidų/nukleotidų hidrolazių, atrankoms. Metodas turi potencialą plėstis, panaudojant kitų biosintezės kelių auksotrofų mutantus bei atitinkamus nukleozidus, nukleotidus ar heterociklinių bazių darinius, o taip pat yra galimi metodų variantai paremti išgyvenimą salygojančių junginių sintezės, o ne hidrolizės principu.

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2004-2009		Biochemist
2002-2004		Junior Biochemist

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