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A versatile colony assay based on NADH fluorescence

Christoph Reisinger [a], Friso van Assema [b], Martin Schürmann [b], Zahid Hussain [c], Peter Remler [a], Helmut Schwab [a,c,*]

[a] *Applied Biocatalysis Research Centre, Petersgasse 14, 8010 Graz, Austria*
[b] *DSM Research B.V., ASC&D, P.O. Box 18, 6160 MD Geleen, The Netherlands*
[c] *Institute of Molecular Biotechnology, University of Technology Graz, Petersgasse 14, 8010 Graz, Austria*

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Abstract

Direct visualization of the activity of enzymes expressed by bacterial colonies attached to a solid support, often referred to as "filter assay", is a powerful strategy for the identification of new or improved biocatalysts. In this work we demonstrate the usefulness of NAD^+/NADH coupled enzymatic reactions as visualization tool in such experimental setups. Dehydrogenases, capable of oxidizing or reducing the reaction product released from the bacterial colony were supplemented to the screening solution, together with the screening substrate and a sufficient amount of NAD^+ or NADH, respectively. We also examined the screening of directly NAD^+/NADH coupled reactions. The release or consumption of NADH in the area of colonies was monitored on behalf of its fluorescence at 450 nm. Excitation was achieved by standard "black-light" UV tubes (340–360 nm). The visible fluorescence signal was recorded using a CCD-camera. We got excellent results for the screening of threonine aldolases and esterases and were able to show the principle utility for amidase, nitrilase, nitrile hydratase, hydroxynitrile lyase and benzaldehyde dehydrogenase active colonies.

Keywords: Colony screening; NADH fluorescence; Aldolase; Esterase; Nitrilase

1. Introduction

The screening of enzyme activity at colony level comprises a powerful tool for the finding, improvement or alteration of proteins by mutagenesis when high numbers of variants have to be evaluated [1–3]. Uniform cell growth and expression levels are more easily achieved in colonies than by other large scale cultivation approaches which require a higher degree of optimization and instrumentation, as well. On the other hand, the evaluation of activity within colony populations asks for more sophisticated assaying methods since standard spectroscopic lab-equipment cannot be employed with colonies. A successful colony assay needs an elaborate screening chemistry that – uninfluenced by cell background and growth media – delivers a continuous signal related to the enzymatic reaction. The availability of such techniques is very much dependent on the enzyme to be investigated. Often pH-indicators are used to observe pH shifts [4,5] caused by hydrolytic enzymes. Tailored (pro-)chromogenic substrates [6,7] allow visualizing substrate turn-over. Sometimes the specificity of a coupled enzyme reaction is an elegant technique to transform the investigated activity into a visible signal around a colony and has been used, e.g. for the screening of hydrogen peroxide producing enzymes [8]. Fluorescence signals have tremendous advantages over colorimetric ones when image documentation, analysis and quantification are demanded [9]. It is generally accepted that real time assays are more flexible and easier to handle compared to those based on endpoint detection. The additional time axis increases the analytical window and robustness as the kinetic behaviors of the reaction can be monitored.

As a general demand assay conditions (e.g. pH, substrate concentration or reaction additives) have to match the targeted operation conditions as close as possible in order to avoid a perturbation of the selection range in an unwanted manner. The application of a coupled enzymatic reaction represents a well-suited way to avoid harsh chemical staining methods. NAD(P)H coupled enzyme assays have been reported to be useful for the analysis of many different enzymatic reactions [10,11]. A broad range of $NAD(P)^+$/NAD(P)H linked enzymes exists for a wide variety of substrates. The reduced cofactor is easily monitored in a UV/VIS spectrophotometer by its absorbance at 340 nm or in a fluorescence spectrometer on behalf of its low quantum

* Corresponding author. Tel.: +43 316 873 4070; fax: +43 316 873 4071.
E-mail address: helmut.schwab@tugraz.at (H. Schwab).

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yield emission at 450 nm. While the fluorescence signal has been extensively used for the monitoring of intracellular reactions or the cell redox status [12] the staining of colonies for released NAD(P)H is usually accomplished by blue dye formation with nitrazolium blue in the presence of phenylmethane sulfonic acid [13,14].

In this report we present a useful fluorescence based colony detection method based on the application of supplemented NAD^+ or NADH together with co-acting dehydrogenases as detection tool for several enzymatic reactions at bacterial colony level. The presented technology does not require cost-intensive equipment and can be applied easily in any standard lab.

2. Materials and methods

2.1. Chemicals and enzymes for genetic manipulations

All chemicals were obtained from Roth, Sigma–Aldrich or Fluka in analytical quality. β-NAD^+ and β-NADH were purchased in crystalline form and stored as 200 mM stock solutions at −20 °C. Genetic manipulations were performed according to Ausubel et al. [15]. Restriction enzymes and T4-DNA ligase were obtained from Fermentas, *Phusion* DNA polymerase was purchased from Finnzymes and Hotstart-*Taq* DNA polymerase is a product of Qiagen.

2.2. Bacterial strains and expression constructs

Escherichia coli strains Top10F′ DH5α and BL21AI were obtained from Invitrogen and used as hosts for cloning and colony assay experiments, and for the over-expression of dehydrogenases and amidase by pET26b(+) (Stratagene) and pMS470Δ8 [16] derived expression constructs, respectively. An *Acinetobacter* sp. ADP1 strain for subsequent cloning of dehydrogenases had been isolated from river water and was confirmed by PCR amplification of the 16S-rDNA region [17] followed by sequencing.

Clones used for the assay evaluation expressed L- and D-threonine aldolase (*Pseudomonas putida* NCIMB 12565 and *Alcaligenes xylosoxidans* IFO 12669, respectively, contributed by DSM Research B.V., ASC&D, The Netherlands), amidase, nitrile hydratase (*Rhodococcus erythropolis* NCIMB11540) and nitrilase (*Rhodococcus rhodochrous* BT1821 [20]) from a *tac*-promoter of the pMS470Δ8 plasmid. The structural genes were cloned by PCR from the genomic DNA with primers according to Table 1. An esterase (*Exiguobacterium* sp. A19 isolate [18]) was expressed from a pZero2 plasmid carrying a 3.5 kb chromosomal fragment from a *Bsp*143I partial fragment library. It had been identified from there by pH-shift screening using a saturated solution of ethyl acetate in 0.02 M sodium phosphate buffer (pH 8) with 3 g/l phenol red as indicator. The *Hevea brasiliensis* hydroxynitrile lyase (HNL) expression plasmid pHNL-200 was described by Hasslacher et al. [21]. Benzaldehyde dehydrogenase for the evaluation of the direct dehydratase assay was expressed from the same construct used for the production of the sensing enzyme preparation.

For the purpose of sensing enzymes benzaldehyde dehydrogenase and arylalcohol dehydrogenase [22], glutamate dehydrogenase and aldehyde dehydrogenase genes were cloned from *Acinetobacter* sp. ADP1 by PCR using primer pairs designed

Table 1
Names of expression plasmids, their construction primers and their purpose in this study

Construct name	Construction primers or origin	Purpose
pMS470-33/3/1/11	Reisinger et al. [19]	Amidase expression, amidase acivity assay evaluation
pK470-33/3/1/11	Same as pMS470-33/3/1/11 but Amp^R replaced by Kan^R cassette	Negative control for esterase assay evaluation
pBbvR-LTA	5′-GCCATatgacagacaagagccaacaattc-3′ 5′-GCAAgcttcagccaccaatgatcgtgcgg-3′	L-Threonine aldolase assay evaluation
pBbvR-DTA	5′-GCCATatgtcccaggaagtcatacgc-3′ 5′- GCAAGCTtcagcgcgagaagccgcgc-3′	D-Threonine aldolase assay evaluation
pHNL-200	Hasslacher et al. [21]	HNL assay evaluation
pZ-Exba-est1	*Exiguobacter* sp. A19 7 kb chromosomal fragment	Esterase assay evaluation
pMSNhaserep7 pMSNhaseSDSrep9	Reisinger et al. [19]	Nitrile hydratase assay evaluation
pMS470-37/4/1/4	5′-CTCATatggtcgaatacacaaacac-3′ 5′-GGATCCGCATGCagtcagatggaggctgtcgc-3′	Nitrilase assay evaluation
pET-BALD	5′-GATTCAtatggttttaaacaatcgtaagaac-3′ 5′-GATTaagcttaaaatggatagtgtggggcct-3′	Benzaldehyde dehydrogenase expression
pET-AADH	5′-GATTcatatgacaaagtttaccgaaatcac-3′ 5′-GATTaagcttaaccaatttttaaaatgggtttg-3′	Arylalcohol dehydogenase expression
pET-GLUT	5′-GATTCATatgtcactttcatatcaaatagaaaataat-3′ 5′-GATTAAGCttaaccaggaaagataccacgttc-3′	Glutamate dehydrogenase expression
pET-DH1	5′-GCTCATatgcgttatatcgatcctaatcaac-3′ 5′-GCAAGCttagaagaagcccattggttttg-3′	Aldehyde dehydrogenase II expression, dehydrogenase activity assay evaluation

according to the database sequence [23] and summarized in Table 1. PCR fragments amplified with *Phusion* DNA polymerase were subcloned into pET26b(+) plasmids using *Nde*I and *Not*I or *Hin*dIII restriction sites. Amidase needed for the assaying of nitrile hydratases was expressed from the pMS470Δ8 construct (pMS470–33/3/1/11) mentioned before.

2.3. Production of the sensing enzyme preparations

Plasmids were transformed into competent *E. coli* BL21AI cells using the SEM-protocol [24]. Three millilitres of overnight cultures (ONCs) in 2xTY media, supplemented with ampicillin (100 mg/l, for pMS470 clones) or kanamycin (40 mg/l, for pET26 clones) were used to inoculate 330 ml of the same media in 1 l baffled flasks followed by incubation at 37 °C. At OD_{600} 1.2 (approximately) the incubation temperature was then reduced to 25 °C and for induction L-arabinose (0.2%, for pET-plasmids) or IPTG (0.3 mM for pMS470 plasmids) was added. Cells were harvested after 16 h of induction and disrupted in 0.1 M sodium phosphate buffer (pH 7) using an ultrasonic homogenizer. Cell fragments were removed by centrifugation at 30,000 × *g* for 1 h at 4 °C. Protein expression was examined by SDS-PAGE followed by Coomassie blue staining. Enzyme preparations were employed as cell free extracts (CFE) except for benzaldehyde dehydrogenase which was further purified by ammonium sulfate precipitation (at 1.2 M) and dialysis of the supernatant against a 40-fold volume of 0.1 M phosphate buffer (pH 7). Protein concentration was determined using the Biorad assay referring to a BSA standard. All enzymes were stored frozen at −20 °C and portions were thawed directly before use. Aldehyde dehydrogenase, benzaldehyde dehydrogenase and glutamate dehydrogenase activity were determined in HEPES (0.1 M, pH 8) containing 600 μM β-NAD^+ and 3 μl/ml acetaldehyde, 3 μl/ml benzaldehyde or 40 mM glutamic acid as substrates by following the increase of absorbance at 340 nm using a Beckman Coulter DU800 spectrophotometer. For aryl alcohol dehydrogenase activity determination β-NAD^+ was replaced by 200 μM β-NADH and the decrease of absorbance was recorded. Amidase activity was determined using the conductivity assay [18] and acetamide as substrate. The absorbance coefficient of NADH at 340 nm was taken as 6200 M^{-1} cm^{-1}. Parameters of the enzyme preparations used as "sensing enzymes" are given in Table 2.

Table 2
Activities and protein content of cell free extracts (CFE) used in this study

Enzyme preparation	Total protein [mg/ml]	Activity [U/ml]	Substrate
Benzaldehyde dehydrogenase*	24	19	Benzaldehyde
Arylalcohol dehydrogenase	7	6.5	Benzaldehyde
Aldehyde dehydrogenase	13	1.2	Acetaldehyde
Glutamate dehydrogenase	11	5.2	Glutamic acid
Amidase	20	70	Acetamide

* Partially purified by ammonium sulfate precipitation.

2.4. Measurement setup and image analysis

The experimental setup is shown in Fig. 1. Standard 18 W UV "black-light" tubes (365 nm) manufactured by Omnilux were used for fluorescence excitation. A 45 mm Ø × 3 mm Schott color glass detection filter GG435 together with a Sony DSC-V1 digital still camera were used for recording of the images. Filter papers or membranes were placed on the bottom of a black polypropylene storage box with inner dimensions of 40 cm × 30 cm × 23 cm. Four UV fluorescent tubes were situated pair wise on the very left and right of the opening and the camera with the screwed up emission filter was fixed above the centre of the box. Images were saved in TIFF format to avoid errors caused by compression/decompression cycles. Image analysis was done using the National Institute of Health (NIH) public domain tool ImageJ. The green image channel was extracted from the color image and used for the calculation of the fluorescence intensity by measuring the average pixel intensities of a region of interest (ROI). The ROI size was kept constant within datasets in order to allow relative comparison of the average values. Subtraction of the average ROI intensities at time point zero was used for normalization of the values. Areas not containing spots or colonies were taken for background calculations. For quantification of the fluorescence signal the background was subtracted from the corresponding intensity signal. Care was taken that no pixels inside the ROI got saturated (overexposure).

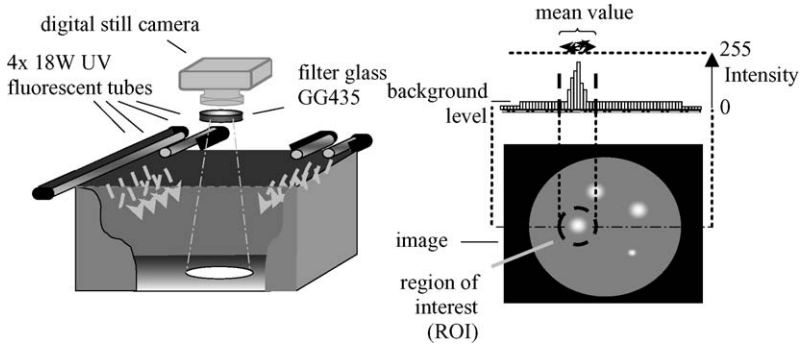


Fig. 1. Schematic drawing of the measurement setup and data processing. "Black-light" tubes were used for excitation, images were recorded with a Sony DSC-V1 camera and image evaluation was done with ImageJ.

2.5. Evaluation of the analytical window

Different quantities of NADH in 100 mM HEPES buffer (pH 8) were dropped on Whatman (grade 1) filter disks. The spots were either left for drying before or photographed immediately. Images were taken with different exposure times and analyzed for the intensity of the fluorescent spots.

2.6. Colony assays

In order to obtain separated single colonies deriving from two different strains that should further on be discriminated in the assay, serial dilutions of mixed ONCs were spread on LB media that contained the required antibiotic. The colonies were lifted onto Whatman (grade #1) filter disks which were afterwards dried for 5 min in the air stream of the laminar flow bench. Dry filters with the colonies on top were soaked with the corresponding screening solutions and the development or disappearance of the fluorescence signal was followed over time. Several colonies categorized as "positive" or "negative" were subsequently streaked on plates for over night cultivation followed by plasmid isolation and restriction analysis in order to confirm their identity. The following conditions were chosen in detail.

2.6.1. Threonine aldolase

E. coli DH5α cells containing pBbvR-LTA and -DTA plasmids or pMS470-33/3/1/11 (amidase expression, employed as negative control) were grown at 30 °C on LB-ampicillin plates for 36 h to generate single colonies. L- and D-threonine (40 mM in 100 mM HEPES, pH 8) together with 5 mM β-NAD^+ and 30 μl/ml aldehyde dehydrogenase CFE were taken as screening reagent. Alternatively, the substrate DL-syn-phenylserine was applied the same way using the benzaldehyde dehydrogenase preparation, instead.

2.6.2. Esterase

E. coli Top10F′ carrying pZ-Exba-est1 or pK470-33/3/1/11 (negative control) were used for the esterase assay. Colonies were grown at 30 °C for 36 h on LB-kanamycin plates. Benzyl acetate saturated HEPES (100 mM pH 8) containing 5 mM β-NAD^+ and a supplement of 30 μl/ml benzaldehyde dehydrogenase together with 30 μl/ml aryl alcohol dehydrogenase were used as screening solution for the detection of ester hydrolyzing activity.

2.6.3. Nitrilase and amidase

E. coli Top10F′ cells carrying pMS470-37/4/1/14 (Nitrilase) and pMS470-33/3/1/11 (amidase) plasmids were grown to colonies on LB-ampicillin-IPTG plates (0.1 mM IPTG) at 30 °C for 36 h. After lifting up the colonies to filters and drying, the filter disks were pre-incubated with 100 mM HEPES (pH 8) containing 5 mM NADH and 50 mg/ml 2-ketoglutarate for 20 min at 30 °C. Excess liquid was removed with a dry filter paper and the filters were soaked in the screening solution containing 5 mM NADH and 10 mg/ml 2-ketogluterate in HEPES (pH 8) and 60 μl/ml glutamate dehydrogenase CFE saturated with benzonitrile (nitrilase) or supplemented with 100 mM acetamide (amidase), respectively. In this case the decrease of fluorescence around colonies was monitored.

2.6.4. Nitrile hydratase

pMSNhaserep7 and pMSNhaseSDSrep9 in *E. coli* Top10F′ were used as positive strains while the amidase expressing pMS470-33/3/1/11 strain was taken as negative control. Colonies were obtained by streaking cells with sterile toothpicks on LB-ampicillin plates. Incubation for 16 h at 30 °C followed by another 24 h period at 25 °C was done in order to express active nitrile hydratase. After transfer of the cells to filters by lifting, a pre-incubation step with 5 mM NADH was carried out as described above. The screening solution contained 120 mM methacrylonitrile, 5 mM NADH, 10 mg/ml α-ketoglutarate in 100 mM HEPES, pH 8. Sixty microlitres per millilitres glutamate dehydrogenase and 30 μl/ml amidase (pMS470-33/3/1/11, CFE) were supplemented to the screening mixture and the disappearance of fluorescence was observed.

2.6.5. Hydroxynitrile lyase (HNL)

E. coli TOP10F′ containing pHNL-200 or pMS470-33/3/1/11 (negative control) were grown to colonies and induced as described for the nitrile hydratase experiment, however, in this case the expression temperature was reduced to 21 °C. Due to the instability of mandelonitrile the pH of the screening solution had to be lowered to pH 5.4 (0.1 M sodium phosphate buffer). Ten microlitres per millilitres mandelonitrile were used together with 5 mM NAD^+ and 30 μl/ml of benzaldehyde dehydrogenase. The appearance of fluorescence was monitored over a range of 5 min.

2.6.6. Benzaldehyde dehydrogenase

pET-BALD1 and pET-GLUT1 (negative control) in *E. coli* BL21AI were grown on LB-kanamycine plates, supplemented with 0.02% L-arabinose at 30 °C for 18 h. The dried filters were soaked in HEPES (pH 8, 100 mM) containing 5 mM NAD^+ and 10 mM benzaldehyde and fluorescence increase was monitored.

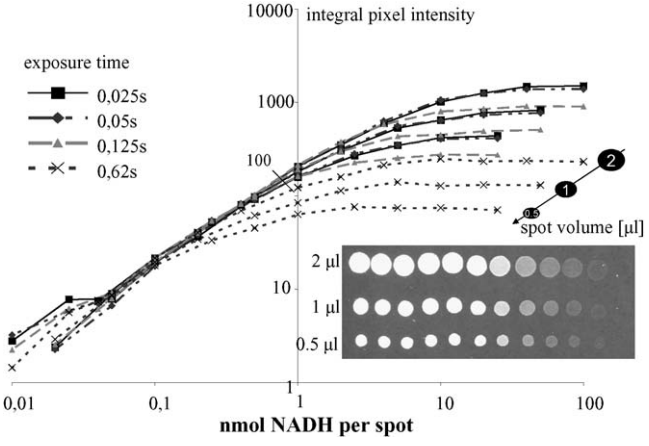


Fig. 2. Correlation of the NADH quantity with pixel intensities. Different exposure times and spot sizes (i.e. local concentrations) show different ranges where a precise quantification is possible. When saturated or noisy images are avoided the integral intensity of a spot correlates to the contained amount of NADH.

3. Results and discussion

3.1. Calibration of the NADH fluorescence imaging

To figure out in how far the amount of NADH on a filter paper could be quantified, images of spots resulting from varying dilutions and drop volumes of an NADH solution were examined upon UV-excitation (Fig. 2). The useful signal range has a span over 2 orders of magnitude and the signal recovery rate is fairly independent from the concentration and thus distribution of the NADH, as long as no saturated pixels appear. A 50 μM concentration of NADH is still recognized as a faint glow by the unaided eye. Drying of the filters caused only minor perturbations of the NADH spots but resulted in a strong increase of fluorescence yield (Fig. 3). This finding is especially useful when trace activities shall be localized in an endpoint manner.

3.2. Intrinsic fluorescence of colonies

E. coli colonies showed a variable intrinsic fluorescence when excited at 360 nm, assumingly as a result of their own NADH level. The strength of this signal depends on the history of the culture. While actively growing colonies show weak fluorescence only, prolonged incubation periods and induction phases can result in very strong light emission. The interference of this observation with the presented assay is limited, however, since continued monitoring allows a differential analysis. Moreover, the glow of the colonies on the filter paper prior to the addition of screening solution can be useful for automated image analysis, e.g. when the coordinates of all colonies shall be acquired.

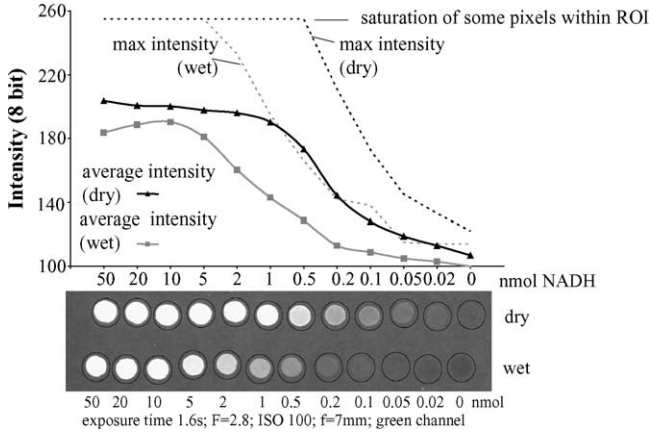
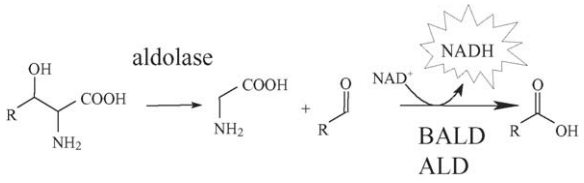
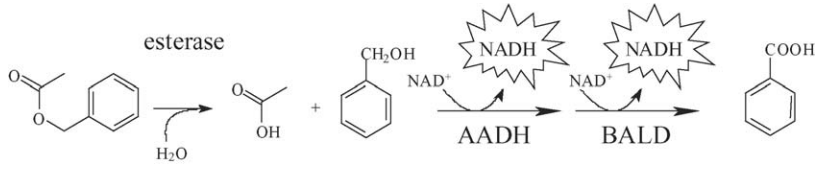


Fig. 3. Different fluorescence intensities are emitted by dry and wet spots of NADH. For continuous assaying the "wet" graph is of relevance but endpoint assay for finding of trace activities can take advantage of the "dry" series. "Bright" areas contain saturated pixels (maximum intensity) and quantification becomes imprecise.

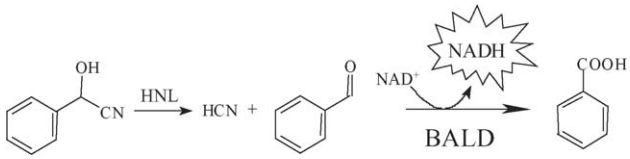
3.3. Discussion of the specific colony assays



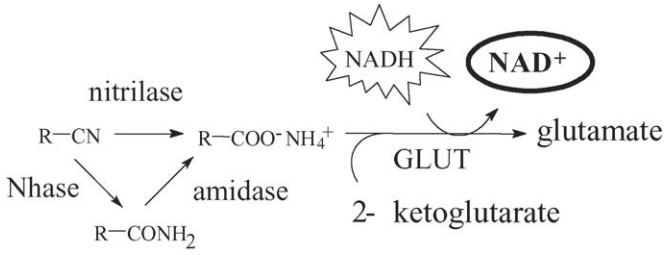
(1)



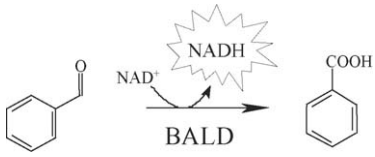
(2)



(3)



(4)



(5)

The Eqs. (1)–(5) describe the screening reactions investigated. A distinction can be made between configurations that produce NADH (Eqs. (1)–(3) and (5)), and others that consume it in the course of the assay (Eq. (4)). While both types are applicable the appearance of fluorescence proves to be much more efficient. Reasons for this include NADH oxidation by *E. coli* colonies or the crude protein preparations added as sensing enzyme and diffusion of the fluorescent NADH from the screening-solution pool towards a colony.

The analysis of D-threonine aldolase active colonies (according to Eq. (1)) is shown in Fig. 4. For the quantification well separated colonies were chosen. The benzaldehyde released from phenylserine by active colonies was readily converted by the benzaldehyde dehydrogenase, causing an increase in NADH derived fluorescence next to active colonies. Similar results were obtained for L-threonine aldolase active colonies with DL-phenylserine or L-threonine, then using aldehyde dehydrogenase (data not shown).

The release of benzyl alcohol from benzyl acetate by the esterase active colonies was investigated using the sequential action of aryl alcohol dehydrogenase and benzaldehyde dehy-

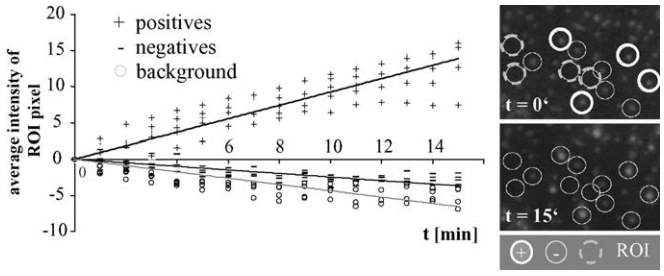


Fig. 4. Evaluation of DL-syn-phenylserine cleavage by D-threonine aldolase active colonies. Benzaldehyde dehydrogenase uses the liberated benzaldehyde around active colonies to reduce the supplemented NAD^+ to the fluorescent NADH. The graph shows the average intensity development of regions with active (+), inactive (−) and without (◯) colonies over time.

drogenase (Eq. (2)). In this case two fluorescent molecules are obtained from each alcohol released resulting in clearer signals (Fig. 5). Low background activity of *E. coli*'s own esterases on the chosen benzyl acetate allows the direct application of crude cell free extracts containing the two dehydrogenases. In contrast to classic esterase colony assays, which use pH-shift detection of the released acid, the presented method picks up the liberated alcohol as analyte. This fact can be advantageous when the release of very weak acids does not allow a pH-shift based visualization.

Among aldehyde coupled reactions the hydroxynitrile lyase (HNL) catalyzed addition of cyanide enjoys considerable interest. Since this type of reaction requires rather a low pH to omit unspecific chemical reaction the application of *Acinetobacter* benzaldehyde dehydrogenase (Eq. (3)) did not produce optimal results. A small pH window around 5.4 allowed a clear distinction between active and non-active colonies; however, for a more reliable screening of HNLs the availability of a low-pH stable dehydrogenase would be beneficial.

As an example of a "high to low"-fluorescence approach the amination of α-ketoglutarate by glutamate dehydrogenase was used to monitor nitrilase, amidase or (together with latter) nitrile hydratase reactions by their release of ammonium (Eq. (4)). However, the use of crude enzyme preparations caused a considerable background of non-specific NADH loss. Nevertheless, we were able to produce a discernible difference between active and inactive colonies for nitrilase (Fig. 5) as well as ami-

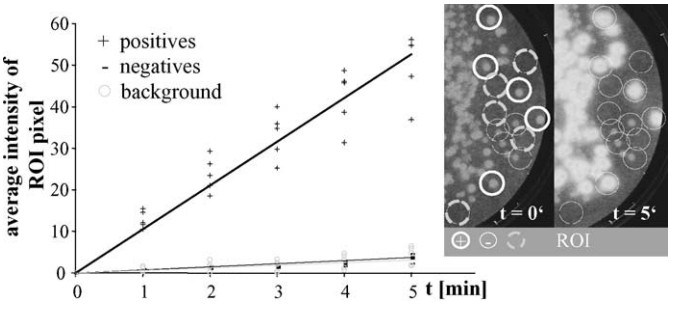


Fig. 5. Evaluation of the benzyl acetate cleavage by esterase active colonies. Aryl alcohol dehydrogenase and benzaldehyde dehydrogenase use the released benzyl alcohol around active colonies to subsequently reduce the supplemented NAD^+ to the fluorescent NADH. The graph shows the average intensity development of regions with active (+), inactive (−) and without (◯) colonies over time.

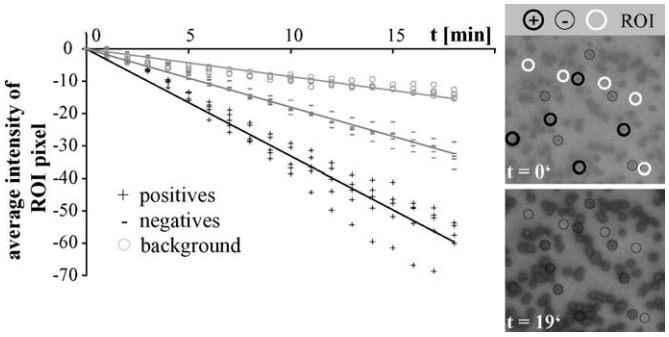


Fig. 6. Monitoring of nitrilase activity in colonies is done by tracing the disappearance of fluorescence over time. Ammonium released from benzonitrile is further on coupled to β-ketoglutarate by glutamate dehydrogenase causing parallel oxidation of NADH. This effect is recognized as an accelerated decrease of fluorescence intensity next to active colonies.

dase, and nitrile hydratase (data not shown) activity. Both, the consumption of NADH by "inactive" colonies as well as the NADH loss by background reactions in the screening solution are evident from the intensity versus time graph (Fig. 6).

The above presented screening approaches function on behalf of an enzymatic detection reaction, with both enzyme and coenzyme situated outside the intact cells forming the colony. We also examined the possibility to directly assay dehydrogenase activity on colony level using our setup. We found that externally supplied NAD^+, unable to pass cell membranes within a useful timeframe, does not contribute to the enzymatic reaction taking place inside of the intact cell. While induced cell lysis could break down the diffusion barrier and solve this problem we found an interesting alternative to this step. When testing for benzaldehyde dehydrogenase with benzaldehyde as substrate active colonies started to glow under UV light. The cell-internal NAD^+-pool was found to be sufficient to produce a detectable signal. With a substrate passing the cell membrane easily, direct

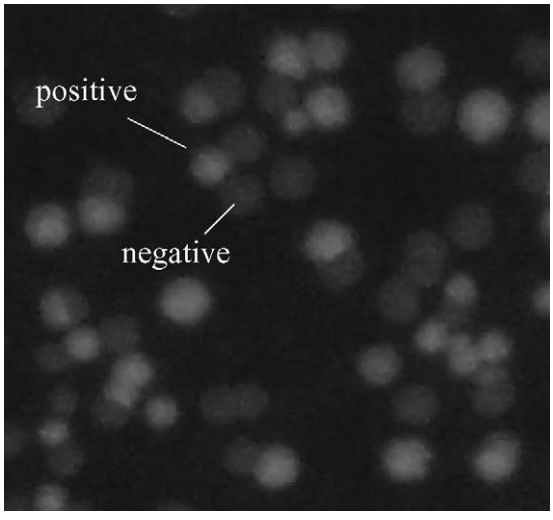


Fig. 7. Identification of benzaldehyde dehydrogenase expressing colonies can be achieved by exploiting the internal NAD^+ pool of the cells. By addition of benzaldehyde the strong fluorescence can be observed, which is independent from additionally added NAD^+.

monitoring of benzaldehyde dehydrogenase in intact cells was possible (Fig. 7).

4. Conclusions

The reported principle of monitoring conversions of $NAD^+ \leftrightarrow NADH$ by direct fluorescence analysis provides a potential screening solution for a variety of enzymatic reactions at the colony level. By employing NAD^+/NADH coupled dehydrogenases, which function as a sensing enzyme, a huge palette of analytes can be covered in a universal assembly using simple and cheap standard components. Fluorescence signals provide high sensitivity and allow semi-quantification by image analysis. We were highly successful in adapting the procedure for the screening of aldolase and esterase activity on colony level. Furthermore were able to show the principle application for HNL, ammonium releasing enzymes and dehydrogenases. The ease of application and the simple required instrumentation coupled with the high throughput level of colony based screening systems represent a powerful tool whenever high clone numbers have to be checked for biocatalytic activities, especially in the context of enzyme engineering programs based on evolutionary strategies.

Acknowledgements

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References

[1] L. Wan, M.B. Twitchett, L.D. Eltis, A.G. Mauk, M. Smith, Proc. Natl. Acad. Sci. U.S.A. 95 (22) (1998) 12825.
[2] J.H. Zhang, G. Dawes, W.P. Stemmer, Proc. Natl. Acad. Sci. U.S.A. 94 (9) (1997) 4504.
[3] B. van Loo, J.H. Spelberg, J. Kingma, T. Sonke, M.G. Wubbolts, D.B. Janssen, Chem. Biol. 11 (7) (2004) 981.
[4] U.T. Bornscheuer, J. Altenbuchner, H.H. Meyer, Bioorg. Med. Chem. 7 (1999) 2169.
[5] U.T. Bornscheuer, J. Altenbuchner, H.H. Meyer, Biotechnol. Bioeng. 58 (5) (1998) 554.
[6] J.C. Moore, F.H. Arnold, Nat. Biotechnol. 14 (4) (1996) 458.
[7] P. Babiak, J.-L. Reymond, Anal. Chem. 77 (2) (2005) 373.
[8] S. Delagrave, D.J. Murphy, J.L. Pruss, A.M. Maffia, B.L. Marrs, E.J. Bylina, W.J. Coleman, C.L. Grek, M.R. Dilworth, M.M. Yang, D.C. Youvan, Protein Eng. 14 (4) (2001) 261.
[9] F. van Ommen Kloeke, A.M. Baty, C.C. Eastburn, Z. Diwu, G.G. Geesey, J. Microbiol. Methods 38 (1–2) (1999) 25.
[10] H. Beutler, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. VI, third ed., VCH Publishers (UK) Ltd., Cambridge, 1988, p. 598.
[11] E.I. Garvie, Microbiol. Rev. 44 (1) (1980) 106.
[12] S. Huang, A.A. Heikal, W.W. Webb, Biophys. J. 82 (5) (2002) 2811.
[13] K.M. Mayer, in: F.H. Arnold, G. Georgiou (Eds.), Directed Enzyme Evolution, Humana Press, Totowa, 2003, p. 183 (Chapter 19).
[14] A. Glieder, P. Meinhold, in: F.H. Arnold, G. Georgiou (Eds.), Directed Enzyme Evolution, Humana Press, Totowa, 2003, p. 157 (Chapter 16).
[15] F.M. Ausubel, et al. (Eds.), Current Protocols in Molecular Biology, Wiley-Interscience Editorial Board, 2002.
[16] D. Balzer, G. Ziegelin, W. Pansegrau, V. Kruft, E. Lanka, Nucleic Acids Res. 20 (8) (1992) 1851.
[17] S. Weidner, W. Arnold, A. Puhler, Appl. Environ. Microbiol. 62 (3) (1996) 766.
[18] B. Reiter, U. Pfeifer, H. Schwab, A. Sessitsch, Appl. Environ. Microbiol. 68 (5) (2002) 2261.
[19] C. Reisinger, I. Osprian, A. Glieder, H.E. Schoemaker, H. Griengl, H. Schwab, Biotechnol. Lett. 26 (21) (2004) 1675.
[20] M. Kobayashi, H. Komeda, N. Yanaka, T. Nagasawa, H. Yamada, J. Biol. Chem. 29 (1992) 20746.
[21] M. Hasslacher, M. Schall, M. Hayn, H. Griengl, S.D. Kohlwein, H. Schwab, J. Biol. Chem. 271 (10) (1996) 5884.
[22] R.M. Jones, L.S. Collier, E.L. Neidle, P.A. Williams, J. Bacteriol. 181 (15) (1999) 4568.
[23] V. Barbe, D. Vallenet, N. Fonknechten, A. Kreimeyer, S. Oztas, L. Labarre, S. Cruveiller, C. Robert, S. Duprat, P. Wincker, L.N. Ornston, J. Weissenbach, P. Marliere, G.N. Cohen, C. Medigue, Nucleic Acids Res. 32 (19) (2004) 5766.
[24] H. Inoue, H. Nojima, H. Okayama, Gene 96 (1990) 23.