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Micro-colony array based high throughput platform for enzyme library screening

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Abstract

Enzymes are becoming increasingly important tools for synthesizing and modifying fine and bulk chemicals. The availability of biocatalysts which fulfil the requirements of industrial processes is often limited. Recruiting suited enzymes from natural (e.g. metagenomes) and artificial (e.g. directed evolution) biodiversity is based on screening libraries of microbial clones expressing enzyme variants. However, exploring the complex diversity of such libraries needs efficient screening methods. Overcoming the "screening bottleneck" requires rapid high throughput technology allowing the analysis of a large diversity of different enzymes and applying different screening conditions.

Facing these facts an efficient and cost effective method for high throughput screening of large enzyme libraries at the colony level was developed. Therefore, ordered high density micro-colony arrays were combined with optical sensor technology and automated image analysis. The system generally allows the simultaneous monitoring of enzyme activities reflected by up to 7000 micro-colonies spotted on a filter in the size of a micro-titer plate. A developed replica option also allows the analysis of clones under varying external conditions. The method was verified by a model screening using esterases and was proved to provide reliable enzyme activity measurements within single micro-colonies allowing the discrimination of activity differences in the range of 10–20%.

Keywords: Esterases; High throughput screening; Enzyme variant libraries; Image analysis; High density array

1. Introduction

Enzymes are versatile biocatalysts becoming increasingly employed in many applications. The

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major advantages of using enzymes in biocatalytic transformations include their chemo-, regio- and stereo-specificity as well as the mild reaction conditions that can be used (Bornscheuer, 2003). The performance of an enzyme, though showing activity for a desired reaction, is in many cases insufficient for its application in industrial biocatalytic processes. Wild type enzymes often need to be optimised to fulfil the needs with respect to various parameters such as, e.g. high stability and activity under process conditions, desired substrate selectivity or high enantioselectivity (Bornscheuer and Pohl, 2001; Bornscheuer et al., 2002). One important strategy enabling the adaptation of enzymes towards such needs is directed evolution.

Recruiting suited enzymes from artificial biodiversity generated by evolutionary enzyme engineering strategies is based on screening libraries of genes encoding enzyme variants. These are usually established in *Escherichia coli* but due to limitations in functional expression, with increasing demand also in other microbial hosts. Ultimately, the success of finding useful enzyme variants hinges on the throughput capacity as well as the accuracy and discriminatory capacity of the screening methods.

Although in recent years a number of promising strategies for high throughput in vitro screening of protein variants have emerged, there is still a lack of powerful methods for enzyme library screening (Olsen et al., 2000). Most current strategies use microtiter plate based assays and there is a strong incentive in the chemical and pharmaceutical industry to miniaturize these micro plate assays in order to reduce costs and waste, and to speed up timelines. The option to change from the 96-well format to higher well densities such as 384- and 1536-well formats however often come to borders of limitation with respect to, e.g. reproducible liquid handling, signal detection instrumentation or assay technology. In addition, typical difficulties encountered when using microtiter plate based screening assays include high variance in growth and/or gene expression behaviour of identical clones in separate wells of the microtiter plate and inhomogeneity in exposure to environmental parameters (e.g. temperature, humidity, oxygen supply) across the plate (Blok et al., 2003). Only few attempts to overcome such limitations with working in microtiter plates have yet been described. For example, Kairos Scientific Inc., patented a system for automated and time resolved spectroscopic enzyme activity determination of a large number of cellular colonies expressing variants of a selected enzyme (MicroColonyImager; Bylina et al., 1999, 2002). Other approaches include the application of optical imaging fibre arrays, which may find application in the environmental sensing area (Walt, 2002), and cell immobilization on adsorbed polyacrylamide beads (Freeman et al., 2004). However, these methods either need expensive equipment or are based on specifically labelled substrates.

Overcoming the "screening bottleneck" for developing industrial biocatalysts requires cost effective and rapid high throughput techniques that include widely applicable activity assays and can be applied with a wide variety of different enzymes and screening conditions. Facing these facts we developed a broadly useable, cost effective, flexible and efficient method for high throughput screening of large enzyme libraries at the colony level. The study focuses on finding proper setups for generation of ordered micro-colony arrays and on applying suitable chemo-sensor-based detection principles for time-resolved monitoring enzyme activities within single micro-colonies. Here, we report on the use of the system in screening for esterase activity based on pH shifts, but the general setup is transmittable to many other enzymes.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli BL21 (ATCC BAA-1025) was used as host for *tac* promoter based recombinant expression plasmids pKN27EX and pKNJ70EX (Valinger et al., 2007) encoding for solvent resistant variants of the bacterial esterase EstB from *Burkholderia gladioli* (Petersen et al., 2001) showing esterase activity in 35% (v/v) formamide.

2.2. Chemicals and lab materials

Fluorescein sodium salt was purchased from Merck Chemical Co., Rotiphorese NF Acrylamid/BIS, dichlorodimethylsilane and methacrylic acid-[3-(trimethoxysilyl)-propyl-ester] were purchased from Roth, ammonium persulfate, TEMED and methyl(*S*)-(+)-3-hydroxy-2-methylpropionate were from Sigma–

Aldrich Chemical Co. and Biodyne A nylon membrane (45 μm) was obtained from Pall.

2.3. Media

E. coli strains were cultivated in LB media (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} NaCl); for agar plates 15 g L^{-1} agar agar were added. For selection of plasmids media were supplemented with 40 g L^{-1} kanamycin (LB-Kan). For induction of esterase expression 0.2 mM isopropyl-(beta)-D-thiogalactopyranoside (IPTG) were added.

2.4. Cultivation

Over night cultures (ONC) of *E. coli* BL21(pKN27EX) and *E. coli* BL21(pKN70JEX) were made by inoculation of one colony from a fresh agar plate into 20 ml LB-Kan (100 ml Erlenmeyer flask) and incubation at 37 °C and 180 rpm for 12–14 h. Cultures for spotting cells were run in a standardised manner to ensure comparable conditions: ONCs were diluted to an OD_{600} of 0.5 (SPECTRAmaxPLUS, Molecular Devices) and cultivated at 30 °C and 180 rpm to an OD_{600} of 1. This preculture was again diluted to an OD_{600} of 0.1 into fresh LB-Kan and cultivated at 30 °C and 180 rpm to an OD_{600} of 0.5.

For cultivating mutant libraries cells were harvested from agar plates by suspending in 1 ml LB-Kan. Ten microlitres of this suspension were used to inoculate ONCs (30 ml LB-Kan) and cultivation was performed as described above for the standardised cultivation scheme.

2.5. Generation of high density micro-colony arrays

Cell cultures grown under standardised conditions were diluted to an OD_{600} of 0.01 in 0.9% NaCl. In the range of 5000–7000 spots (1 nL each) were transferred by a MicroGridII spotter (Biorobotics) on a nylon membrane sheet (Biodyne A, 45 μm pore size, 12 cm × 8 cm filter size), which was placed on an LB-Kan agar plate (in Omnitrays, Nunc). The selected dilution produced in the average about 35–45% single cell generated micro-colonies. Thereby, 35–45% of the spot positions were empty and 10–15% of the colonies were generated out of two or more cells.

The membranes on the Omnitray agar plates were then incubated at 30 °C for about 15 h (till micro-colonies appear on the membranes). For induction of gene expression from *tac* promoter based constructs the membranes were transferred on induction plates (LB-Kan supplemented with 0.2 mM IPTG) and incubated for 2 h at 30 °C.

For testing various parameters for the desired screening reactions, colony arrays were alternatively produced in a larger format by stamping using the 384 Pin Replicator from Nunc. In this case due to the much larger transferred volume the optimal OD_{600} for single cell generation was 5×10^{-5}.

2.6. Replication of the micro grid arrays

Master filters of the micro-colony arrays were replicated (up to five times) by first placing the wet master membrane with the micro-colony array on a soft silicone mate sterilised with ethanol. Then a sterile pre-wetted membrane was pressed very carefully on the micro-colony array master, subsequently placed on a LB-Kan plate and incubated for 15 h at room temperature (RT) for 5–7 h at 37 °C.

2.7. Preparation of the gel immobilized sensor

The preparation of the sensing layer consisting of a gel matrix fixed on a glass plate and carrying the substrate and the pH-sensitive fluorescence sensor was done as described elsewhere (Gerlach et al., 2006). For esterase screening the sensing layer consisted of a 12% polyacrylamide gel supplemented with 140 mM methyl(*S*)-(+)-3-hydroxy-2-methylpropionate, 0.17 mM fluoresceine and 50 mM Tris–HCl (pH 7.5).

2.8. Activity measurements

After induction, the micro-colony replicas were transferred to the sensing layer fixed on a glass plate. The glass plate with the sensing layer and the micro-colony replica on the surface was immediately inserted in a moistured metering chamber and the online fluorescence signal detection was performed essentially as described by Gerlach et al. (2006).

2.9. Calibration of the micro-colony based high throughput system

For calibration of the system we generated micro-colony arrays by spotting different mixtures of two esterase mutants exhibiting different enzyme activities. In this case the spotting was performed with dense cell suspensions in order to generate colonies derived from a large number of cells. In such multi-cell generated micro-colonies the overall activity will be determined by the ratio of the two differently active enzyme variants. The strains expressing esterase mutants, BL21(pKN27EX) and BL21(pKNJ70EX) were grown to an $OD_{600} = 1$ (1 L culture each) under the expression conditions as described above. Mixtures of the differently active clones were prepared by mixing aliquots in ratios of (%) 0/100, 20/80, 40/60, 60/40, 80/20 and 100/0. The mixtures (150 ml final volume each) were centrifuged for 10 min at 4 °C and 4000 rpm. Cell pellets were resuspended in 30 ml 0.9% NaCl ($OD_{600} = 5$). One nanolitre of these mixtures were spotted as described above on nylon membranes. After incubation of the calibration filters at 30 °C for approximately 10 h the membranes were transferred on induction plates and incubated for 2 h at 30 °C prior to measuring esterase activity as described above.

2.10. Determination of esterase activity and protein expression

For checking protein expression crude lysates of the respective cell suspensions were analyzed by standard SDS-PAGE and Coomassie blue staining. Esterase activity of the two mutants on the substrate methyl(*S*)-(+)-3-hydroxy-2-methylpropionate was determined by autotitration (Mettler Toledo autotitrator DL50). Activity was determined with cell suspensions grown and induced under standardised conditions as described above, centrifuged 20 min at 4000 rpm at 4 °C and resuspended in 0.9% NaCl. For comparability of the systems the concentrations of all components and the pH were chosen as used in the micro-colony array assay described above. Alternatively, a microtiter plate assay based on measuring the fluorescence decrease of fluoresceine (Fluostar optima, BMG labtech, Germany) run under the same conditions as used with the micro-colony assay described above was applied.

3. Results and discussion

3.1. Optimal density of colonies in spotted high density arrays

One main concept of this developed micro-colony array platform is based on the idea of directly spotting cells in an ordered array onto membrane supports out from cell suspensions. We wanted to avoid the time and material consuming strategy of picking single colonies and bring them into an ordered array in microplates. The idea was to have an optimum of single cells in a defined volume spotted onto a membrane which is then placed on an agar-solidified medium and incubated to grow colonies. From statistical calculations one can define a concentration at which a defined volume containing just one cell is in an optimal range. This calculation reveals an optimal range of around 40% empty positions where about 40% single cell generated colonies and 20% colonies out of two or more cells are present. The number of volume units not containing a cell can be taken as a measure for the ratio of single cell containing units.

In order to experimentally prove this concept, different dilutions of a cell suspension of *E. coli* BL21[pKNJ27EX] were transferred to membranes using the standard 384 pin stamp from Nunc. The stamp transfers 0.1 μl cell suspension per pin and the spot area is sufficiently large, that after growth to micro-colonies one can distinguish the outgrowth of one or more colonies at one spot position. Thereby, the empty positions, single cell generated colonies and colonies generated out of two or more cells were counted (72 stamped membranes). The results obtained clearly proved the calculated correlation between empty positions and single cell generated colonies. Fig. 1 depicts the correlation between empty positions and single cell generated colonies determined for densities around the calculated optimal point of operation.

3.2. Development of the screening setup

The aim of this work was to provide a flexible and adaptable tool for investigating in parallel a high number of enzyme variants with respect to desired features relevant for establishing efficient industrial biocatalytic processes. To meet this goal with a broad

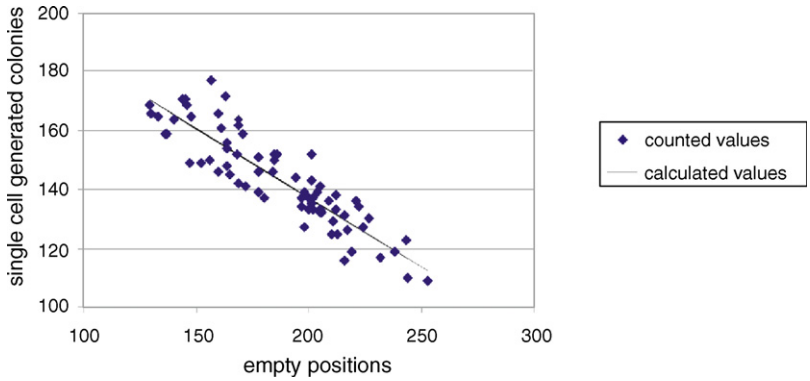

Fig. 1. Evaluation of colonies generated by direct spotting from cell suspensions. Data from 72 stamped membranes shows the linear correlation between empty positions and single cell generated colonies.

range of reactions and conditions the experimental setup should be as flexible and easy to handle as possible.

Based on the micro-colony array described above the following basic setup was designed (Fig. 2):

(i) A proper chemo-sensor allowing the online-monitoring of the enzyme reaction is established by embedding a reporter molecule (chemo-sensor) in a suitable polymer (gel) matrix fixed on a glass carrier plate in a thin layer (Gerlach et al., 2006).

(ii) The micro-colony array established on a membrane is brought into tight contact with this chemo-sensor layer. Either options, direct contact of the colonies to the sensor layer or contact of the porous membrane to the sensor (colonies on the opposite side as shown in Fig. 2), are possible. In addition, the cells of colonies may be lysed or permeabilized prior to activity analysis

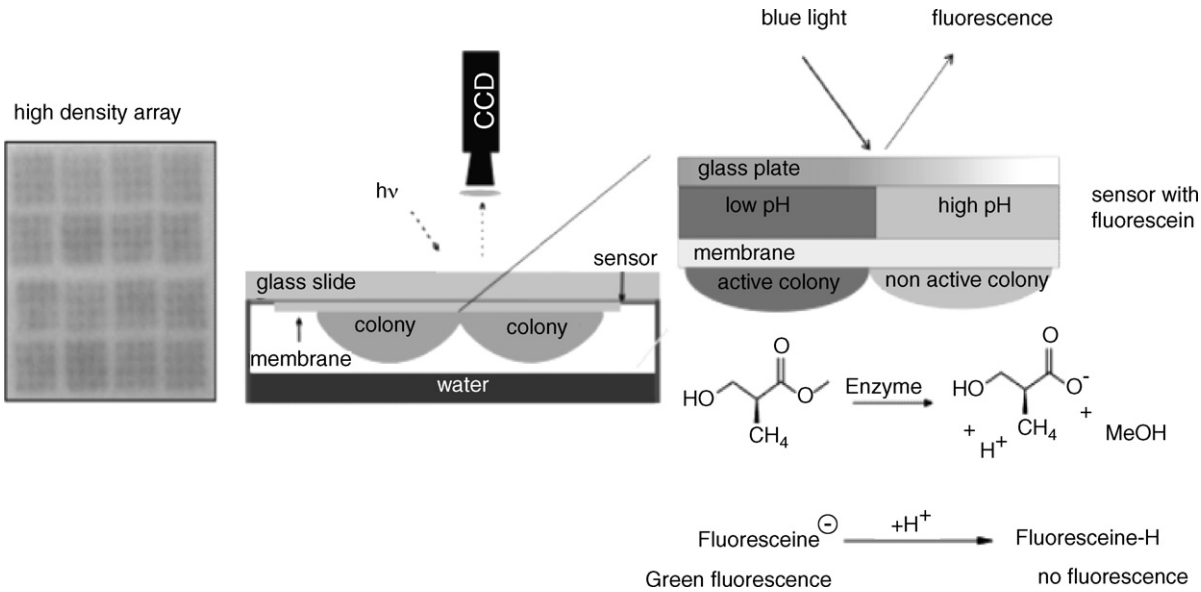

Fig. 2. The experimental setup and detection principle. For detailed explanations see text.

by, e.g. chloroform or polymyxin B treatment, respectively.

(iii) Positioning of the chemo-sensor–micro-colony array setup in a measuring chamber ensuring constant vapour pressure for water and volatile substrates.

(iv) Monitoring the enzyme reaction based on a LED light source for fluorescence excitation, a proper filter and a sensitive CCD camera for fluorescence emission detection (Gerlach et al., 2006).

In this study, the screening setup was established for monitoring hydrolase (esterase) activity based on assaying pH-shift. The carrier material consisted of a standard 12% polyacrylamide gel. For other screening tasks different gel matrices such as agarose or polyvinylchloride can also be applied as carrier material (Gerlach et al., 2006). As chemo-sensor, fluoresceine which shows intense green fluorescence at alkaline pH and shifts to non-fluorescence between pH 7 and 5 was employed. Methyl(*S*)-(+)-3-hydroxy-2-methylpropionate was used as chiral model substrate.

For testing the setup, high density micro-colony arrays containing about 5000 colonies on one standard filter were produced as described above using nylon membrane filters reflecting a low intrinsic fluorescence background and high resistance to chemicals, e.g. organic solvents (see Section 2). Two recombinant *E. coli* strains expressing two differently active muteins of *B. gladioli* esterase EstB from (N27 and NJ70, Valinger et al., 2007) were used as model systems to test the screening setup.

3.3. Image analysis and hit detection

Membranes with the micro-colony arrays were contacted with the chemo-sensor containing the substrate. The measurement time was between 10 and 30 min.

In order to achieve high throughput analysis of the micro-colony array based screening reactions, software based on matlab was developed allowing automated finding the centre of the spots. The only manual intervention is to roughly delineate the entire array of spots with a rectangle. The approach is based on the work of Bischof et al. (2003). A normalized cross-correlation using an ideal spot mask was employed to enhance the spot signals. The radon transform estimates the rotation and starting point of the spot grid resulting in a rough

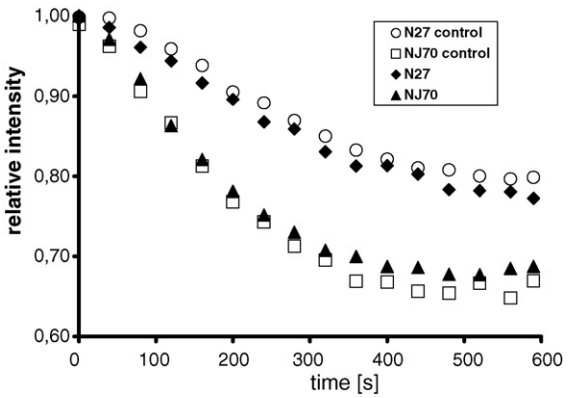

Fig. 3. Example of a test screening setup using two esterase variants. A micro-colony array was generated in a sub-divided manner. Sixteen sub-arrays (16 colonies each) contained either only mutant N27 (left upper sub-array), only mutant NJ70 (left lower sub-array) or a mixture (1:1) of both clones (right upper and lower sub-arrays). The substrate used was methyl(*S*)-(+)-3-hydroxy-2-methylpropionate. The presented values show the mean values of five randomly selected clones of each type. The open symbols represent the values for the known control clones; the filled symbols represent the values for the screened clones. The mean deviation was below 20% with all values.

grid alignment. For detection of the exact spot centres a mean shift algorithm was used (Comaniciu and Meer, 2002). To speed up execution time the fast mean shift algorithm was applied (Beleznai et al., 2004).

For comparing the signals obtained from different colonies the background image was subtracted and images were normalized to the first image of the stack. The fluorescence intensity of the colonies was plotted for all images and the intensity gradient over time evaluated. Gerlach et al. (2006) could already demon-

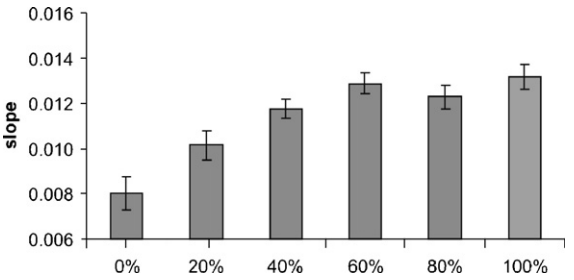

Fig. 4. Calibration of micro-colony arrays. Five sub grids (4×4 spots) of each of the mixtures from 0 to 100% proportion of both mutants were spotted and micro-colonies were analyzed for esterolytic activity on methyl(*S*)-(+)-3-hydroxy-2-methylpropionate using the chemo-sensor system.

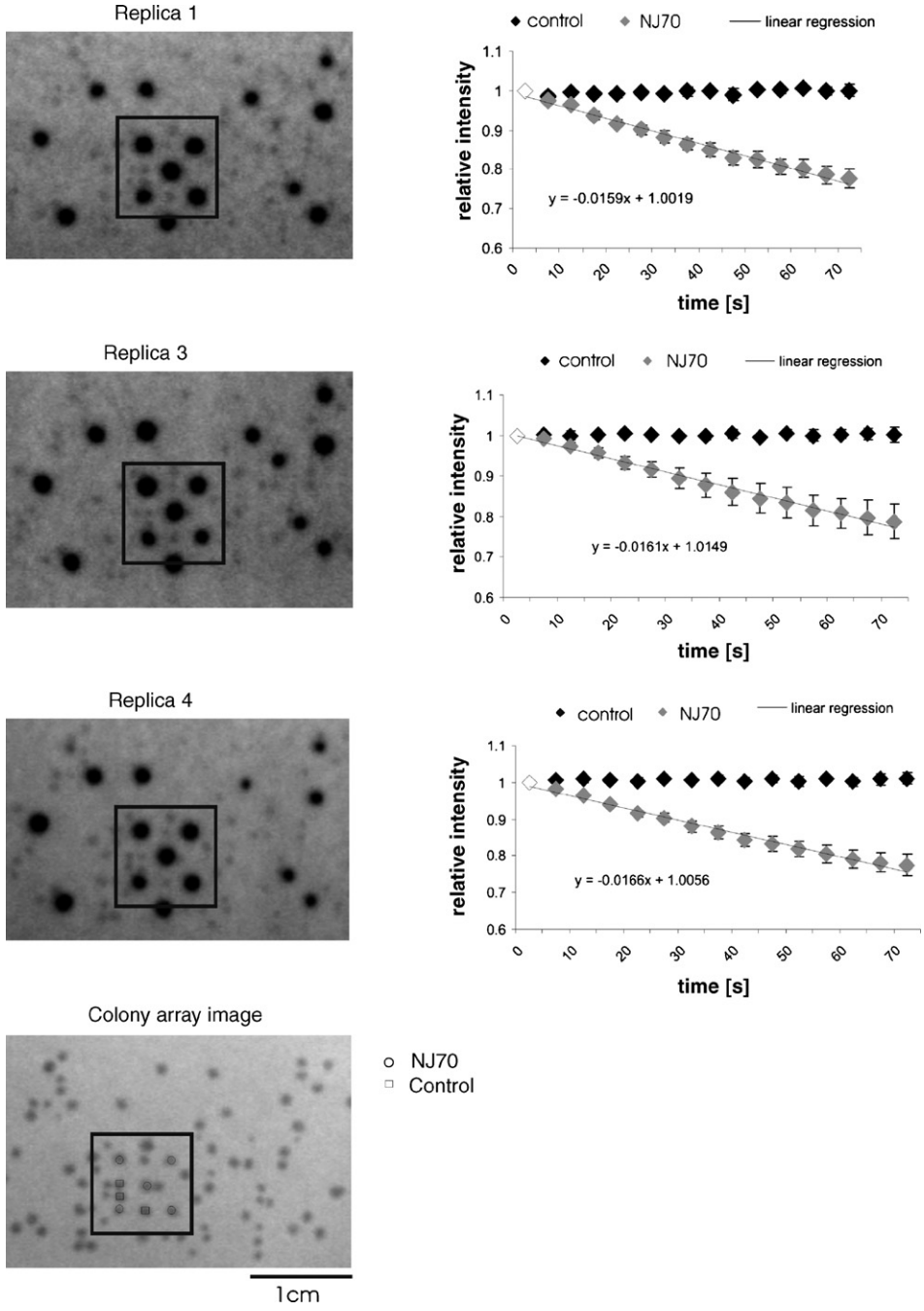

Fig. 5. Analysis of replicated micro-colony arrays. An array was spotted from a suspension of a mixture of mutant strain *E. coli* BL21[pKNJ70EX] and the host strain containing the insert-free vector (control) and subjected to activity analysis using methyl(*S*)-(+)-3-hydroxy-2-methylpropionate as substrate. The upper three images show the activity analysis of the replicates 1, 3 and 4. The lower image shows a picture of the colonies under standard illumination. The colonies within the enboxed area were analyzed with respect to activity and the results obtained are shown in the corresponding graphs at the right.

strate that the sensor technology allows to accurately following activity in each single colony. In this study clones showing high and low slopes were randomly selected. The data shown in Fig. 3 demonstrate that the randomly selected clones can be clearly assigned to the two different enzyme variants.

3.4. Validation of the screening system

For reasonable application and understanding the potentials of the screening method the *E. coli* strains BL21[pKN27EX] and BL21[pKNJ70EX] expressing differently active mutants of esterase EstB were used as references for the evaluation of the system. Determination of the specific activity using the autotitrator method as well as the microplate assay performed under comparable experimental conditions as used with the micro-colony array system resulted in differing activity values in the ratio of about 1:5 (data not shown). Substrate diffusion from the sensor layer into the colonies and inhomogeneity of expression of the enzyme within different parts of the colony may represent the main factors causing these differences. However, the relative signal differences of the two mutants are sufficiently high to allow accurate discrimination in the screening assay.

In order to evaluate the potential of the micro-colony array system, mixtures of the two differently active mutant clones were prepared (proportions: 0, 20, 40, 60, 80 and 100%). These mixtures were used to generate arrays which were spotted in sub grids of 4×4 spots. In this case a low dilution of the suspensions was used for spotting, which provided a high number of cells (about 2000) applied to each spot. Thereby, colonies reflecting different activity levels should arise. The data obtained (Fig. 4) indicated that a relative activity difference of 20% could be easily differentiated up to 60%. At higher levels the system did not allow sufficient differentiation indicating a situation of saturation. Possible reasons therefore could be limitations in substrate supply or breakdown of the buffer system.

Generated micro-arrays were also replicated by contact printing onto fresh membranes and growing the clones again to micro-colonies. The analysis of replicated colony arrays generated from the two mutants N27 and NJ70 is summarized in Fig. 5. The results clearly demonstrate the reliability of this replication technology. Thereby, reactions of replicated arrays can be analyzed in parallel and also under different screening conditions.

4. Conclusions

We have developed a new high throughput screening platform based on micro-colony arrays which makes it feasible to screen about 5000–7000 enzyme variants per filter unit in the size of a micro-titer plate. In this study the feasibility of this micro-colony array system was demonstrated with a hydrolytic esterase-catalyzed reaction generating pH shifts. We could demonstrate the accurate discrimination of different enzyme activity levels in a model system employing two differently active esterase mutants.

The major benefits of this system are:

(I) Ordered colony arrays can be easily and rapidly generated on nylon membranes from large numbers of clones present in complex libraries.

(II) The developed method ensures the generation of a sufficient high number of single cell generated colonies. Such colonies show a very uniform growth behaviour which guaranties a high degree of accuracy in discrimination of enzyme activity levels.

(III) The developed array system allows easy production of replicas by simple contact transfer to new membranes.

(IV) The combination of the micro-colony array system with a chemo-sensor strategy based on fluorescent reporter molecules providing universal detection of specific reactions and automated image analysis allows simultaneous time-resolved monitoring of enzyme activity for any single colony. Besides pH-shift detection, suitable chemo-sensors could already be developed for monitoring concentration levels of molecular oxygen, hydrogen peroxide, primary and secondary amines and aliphatic and aromatic aldehydes (Gerlach et al., 2006).

In sum, the introduced system provides a cost and time saving versatile tool for high throughput screening of various enzymatic activities expressed in microbial hosts applicable to screening needs of directed evolution programs and of exploring metagenomes.

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