Anal Bioanal Chem (2002) 374 : 1069–1073
DOI 10.1007/s00216-002-1579-1

TECHNICAL NOTE

Michael Breuer · Martina Pohl · Bernhard Hauer
Bettina Lingen

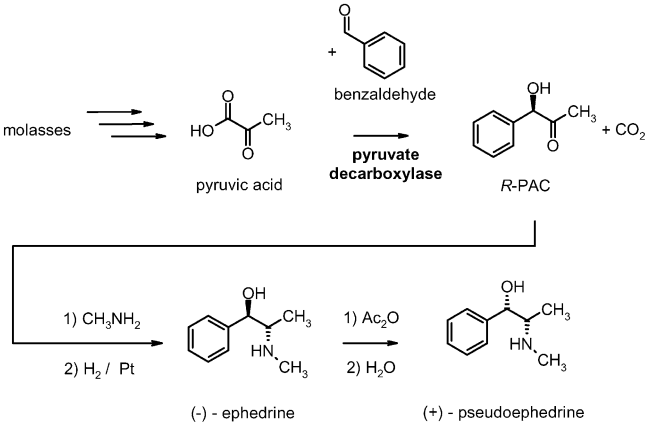
High-throughput assay of (*R*)-phenylacetylcarbinol synthesized by pyruvate decarboxylase

Received: 27 March 2002 / Revised: 8 August 2002 / Accepted: 20 August 2002 / Published online: 19 October 2002

Abstract A novel assay has been developed for the detection of (*R*)-phenylacetylcarbinol, (*R*)-PAC, a chiral intermediate in the industrial synthesis of ephedrine. It is the product of a biotransformation of benzaldehyde catalysed by the enzyme pyruvate decarboxylase. The assay, using 2,3,5-triphenyltetrazolium chloride, enables high-throughput photometric analysis of the activity of the enzyme thus avoiding time-consuming chromatographic procedures.

Keywords High-throughput assay · (*R*)-Phenylacetylcarbinol · Pyruvate decarboxylase · Tetrazolium red

Abbreviations *(R)-PAC* (*R*)-Phenylacetylcarbinol · *PDC* Pyruvate decarboxylase · *IPTG* Isopropyl-β-D-thiogalactoside · *MES* 2-[*N*-Morpholino]ethanesulfonic acid · OD_{510nm} Optical density at 510 nm · *ThDP* Thiamine diphosphate



Scheme 1 Industrial production of pseudoephedrine. Yeast cells synthesise pyruvic acid from sugar and finally convert it with benzaldehyde to (*R*)-PAC. This step is catalysed by pyruvate decarboxylase. Isolated (*R*)-PAC is further converted chemically; reductive methylamination of (*R*)-PAC yields ephedrine, which can be isomerised into pseudoephedrine

Introduction

One of the earliest examples of industrial biocatalysis is the production of pseudoephedrine and ephedrine, both of which are widely used for their decongestant and antiallergic properties. The process starts with the biotransformation of benzaldehyde to (*R*)-PAC, the first chiral intermediate of the overall synthesis (Scheme 1).

M. Breuer (✉) · B. Hauer
BASF AG, GVF/E-A030, 67056 Ludwigshafen, Germany
e-mail: Michael.Breuer@basf-ag.de

M. Pohl
MBP Cologne GmbH, Neurather Ring 1, 51063 Köln, Germany

B. Lingen
Institut für Enzymtechnologie
der Heinrich-Heine-Universität Düsseldorf,
im Forschungszentrum Jülich, 52426 Jülich, Germany

Since 1921 it has been known that yeast (*Saccharomyces cerevisiae*) can catalyse the formation of (*R*)-PAC [1, 2]. Further synthetic steps from (*R*)-PAC to pseudoephedrine are performed by classical chemical synthesis. The process for pseudoephedrine production was patented as early as 1932 [3].

The enzyme in yeast catalysing the synthesis of (*R*)-PAC is pyruvate decarboxylase (PDC; EC 4.1.1.1). In vivo this enzyme converts pyruvate to acetaldehyde. 2-α-Hydroxyethyl-ThDP ("activated acetaldehyde") is an intermediate of the catalytic cycle of this enzyme. Its α-carbanion reacts with several aldehydes in a nucleophilic attack to form the respective acyloins [4]. In this manner benzaldehyde and pyruvic acid form (*R*)-PAC and CO_2. It has recently been shown that it is possible to replace pyruvate by acetaldehyde in the synthesis of (*R*)-PAC. This avoids accumulation of CO_2 and a pH-shift of the reaction mixture [5]. Pyruvate decarboxylase has been isolated from several different sources [6]. In contrast with the en-

zymes from different yeast and fungi, pyruvate decarboxylase from the bacterium *Zymomonas mobilis* catalyses the formation of substantial amounts of (*R*)-PAC from acetaldehyde and benzaldehyde [4]. Because acetaldehyde is considerably cheaper than pyruvate, this approach might have new development potential in the industrial synthesis of ephedrine.

PDC from *Z. mobilis* was optimised by directed evolution to improve the performance of the catalyst. In essence, directed evolution relies on generating several thousand different variants of the enzyme by genetic engineering, each of which must be tested individually for its catalytic activity. A detailed description of this methodology is given elsewhere [7]. Directed evolution requires a powerful and specific high-throughput assay system to handle large libraries of enzyme variants.

Such a test system should be capable of analysing samples in parallel. Very often high-throughput assays rely on spectroscopy for measuring the activity of the biocatalyst. Chromatographic methods are rarely used, although there are promising recent developments using, e.g., capillary electrophoresis [8].

Chromogenic substrates are especially useful for assaying enzymes, because they form a coloured dye on reaction with the enzyme. 5-Bromo-4-chloro-3-indolylderivates or *p*-nitrophenyl substrates are well established examples [9, 10]. Another useful means of analysis of many samples in parallel is IR spectroscopy, which has been used to analyse a biotransformation with lipase, with high throughput [11].

Occasionally the biocatalyst itself has spectroscopic properties enabling direct determination in an assay. This principle has been employed in the development of a green-fluorescent protein with a shifted maximum of absorption [12].

We found the determination of (*R*)-PAC formed by pyruvate decarboxylase was not manageable with either approach. An easy and specific assay based on reduction of a tetrazolium salt by (*R*)-PAC has therefore been developed. The test system enables rapid detection of (*R*)-PAC-forming enzyme variants. It is the first assay system for high-throughput analysis of aromatic acyloins.

Experimental

Expression of pyruvate decarboxylase

Escherichia coli harbouring a plasmid pPDC-His_6 with pyruvate decarboxylase from *Zymomonas mobilis* was prepared as described elsewhere [13]. Cells were grown at 37 °C in 96-well plates using Luria–Bertani medium [14] supplemented with 100 μg mL^{-1} ampicillin and 40 μg mL^{-1} kanamycin. Three hours after inoculating with an overnight culture protein expression was induced by adding IPTG (1 mmol L^{-1} final conc.). After growing the cultures for an additional 16 h cells were harvested by centrifugation (5 min, 3850 g).

Enzyme incubation

Cell pellets were suspended in 200 μL substrate solution containing acetaldehyde (30 mmol L^{-1}), benzaldehyde (40 mmol L^{-1}), $MgSO_4$ (20 mmol L^{-1}), ThDP (2 mmol L^{-1}) in 50 mmol L^{-1} MES-buffer, pH 7.0. Agitating the microplates for 10 s increases the efficiency of cell lysis. After incubation of the mixture for 45 min at 25 °C insoluble matter was removed by centrifugation (5 min, 3850 g). The supernatant was assayed for (*R*)-phenylacetylcarbinol.

Analysis of (*R*)-phenylacetylcarbinol

The determination of (*R*)-phenylacetylcarbinol was performed in 96-well plates. Addition of 20 μL tetrazolium red solution (0.2% 2,3,5-triphenyltetrazolium chloride dissolved in methanol) and 10 μL NaOH (3 mol L^{-1}) gives a red dye. This reaction was measured photometrically at 510 nm by use of a Molecular Devices (Sunnyvale, CA, USA) Spectra Max 190 micro plate reader.

On each micro plate unmutated clones are assayed as standards. Colour development at 510 nm is compared with that for these standards, for 2 min, to identify improved enzyme variants. When scaled up appropriately the reaction can also be monitored in a conventional spectrophotometer (e.g. Hewlett–Packard 8452A diode-array spectrophotometer).

Interaction of tetrazolium red with other compounds of the enzyme incubation is not desirable. To examine the specificity of the assay we checked for false positive reaction of tetrazolium red in the presence of substrates (benzaldehyde and acetaldehyde) or by-products (acetoin) of the reaction. Substrate solution was incubated without pyruvate decarboxylase as described above. To estimate the effect of acetoin a 3 mmol L^{-1} solution in incubation buffer (20 mmol L^{-1} $MgSO_4$, 2 mmol L^{-1} ThDP, 50 mmol L^{-1} MES, pH 7.0) was incubated for 45 min at 25 °C.

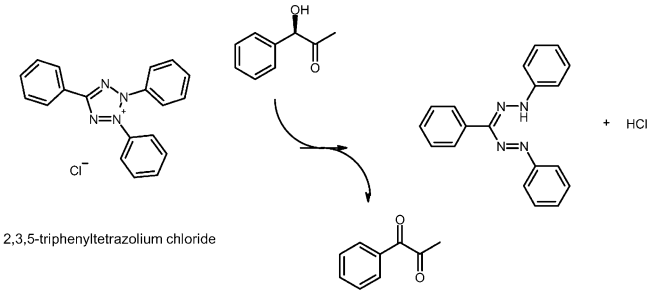
2,3,5-Triphenyltetrazolium and acetoin were obtained from Sigma Chemicals (Deisenhofen, Germany); authentic (*R*)-phenylacetylcarbinol was a gift from BASF PharmaChemikalien (Minden, Germany).

Results and discussion

Several methods for detecting (*R*)-PAC and similar acyloins are described in literature [15]. Those which are based on chromatographic procedures (e.g. Ref. [13]) are too slow to be employed in a high-throughput screening process. A colorimetric method using the Voges–Proskauer reaction has been described [15] and subsequently modified [16]. This method is not sufficiently specific, because it also detects acetoin (3-hydroxy-2-butanone), a by-product of the PDC-reaction formed by addition of acetaldehyde to 2-α-hydroxyethyl-ThDP. Interference by acetoin should be avoided in an assay for (*R*)-PAC.

Reagents based on tetrazolium blue chloride have been described for detection of acyloins after paper chromatography [17]. Tetrazolium blue (3,3′- [3,3′-Dimethoxy-(1,1′-biphenyl)-4,4′-diyl]-bis [2,5-diphenyl-2*H*-tetrazolium] dichloride) is reduced by acyloins and other compounds with a similar reduction potential. When used for photometric detection of (*R*)-PAC, however, tetrazolium blue turned out to be inappropriate, because a precipitate was observed concomitant with the formation of the respective formazane. This precipitation dramatically affected photometric quantification.

The reactivity of 2,3,5-triphenyltetrazolium (tetrazolium red) is similar to that of tetrazolium blue (Scheme 2). When tetrazolium red was used for detection of (*R*)-phenylacetylcarbinol no precipitate was detected during the



Scheme 2 (*R*)-PAC reduces 2,3,5-triphenyltetrazolium chloride to the respective formazane which has an intense red colour

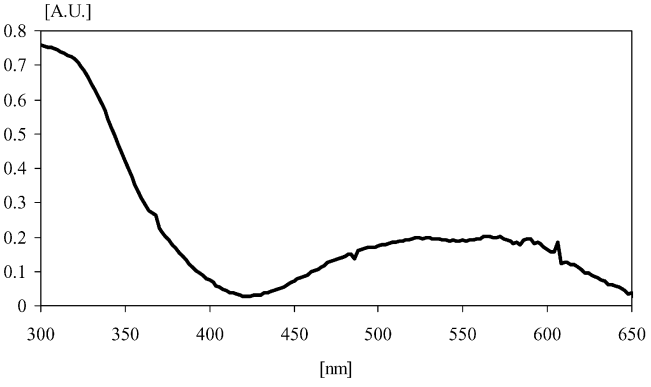


Fig. 1 UV–visible spectrum of the formazane dye produced by reduction of tetrazolium red with 0.2 mmol L^{-1} (*R*)-phenylacetylcarbinol

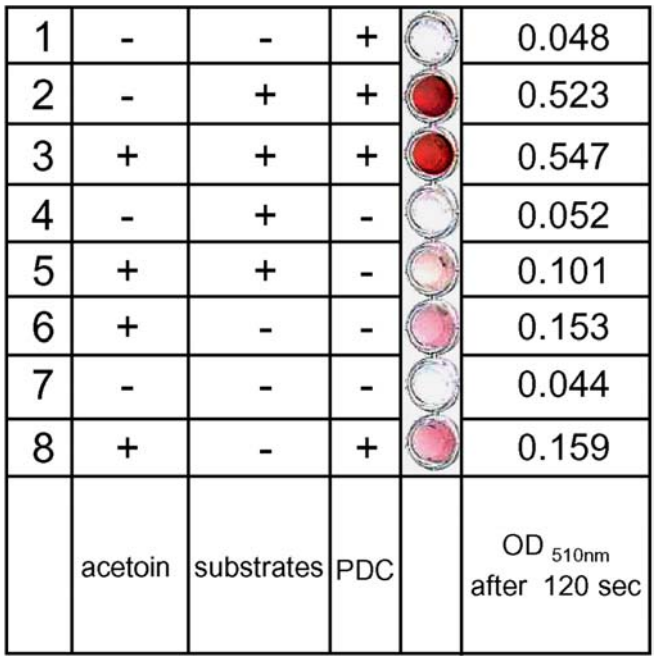


Fig. 2 Detection of (*R*)-PAC with tetrazolium red. Formation of the red formazane dye can be observed only in the presence of active PDC and its substrates (samples *2* and *3*). In sample *2* 0.2 mmol L^{-1} R-PAC are formed No colour is observed when either substrates or catalyst, or both are omitted from the reaction mixture (samples *1*, *4*, and *7*). Acetoin (3 mmol L^{-1}) gives rise to much less colour (samples *5*, *6*, and *8*) and does not affect (*R*)-PAC-detection (sample *3*)

reaction. Thus the assay can easily be performed in a spectrophotometer. Figure 1 shows the UV–visible spectrum of the red formazane dye after reduction of tetrazolium red by (*R*)-PAC.

Figure 2 shows the specificity of the microplate-assay for (*R*)-PAC. It is known that pyruvate decarboxylase also catalyses the synthesis of acetoin from two acetaldehyde molecules. To estimate any interference by this by-product the response of the assay to acetoin was tested. Cells harbouring the PDC-plasmid were grown and the crude extract containing pyruvate decarboxylase was incubated as described above. Significant formation of the red formazane is observed only when both active enzyme and the respective substrates are present in the reaction mixture (samples 2 and 3). The positive control (sample 2) gives an intense red colour (OD_{510nm}=0.523). This is equivalent to 0.2 mmol L^{-1} (*R*)-PAC. Both acetaldehyde and benzaldehyde alone do not react with tetrazolium red (sample 4). When assaying 3 mmol L^{-1} acetoin only a very faint reddish hue (OD_{510nm}=0.15, sample 6) was observed.

Comparison of the results from samples 2 and 6 shows the specificity to be approximately 70 for (*R*)-PAC over acetoin. When (*R*)-PAC (0.2 mmol L^{-1}) is analysed in the presence of 3 mmol L^{-1} acetoin (sample 3) the absorption is increased by 2.9%only. Acetoin, the by-product of the biotransformation does not interfere substantially with

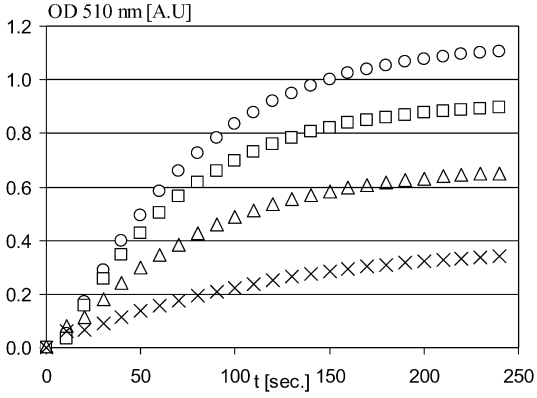


Fig. 3 Time course of colour development after use of different concentrations of (*R*)-phenylacetylcarbinol (*crosses*, 0.1 mmol L^{-1}; *triangles*, 0.2 mmol L^{-1}; *squares*, 0.3 mmol L^{-1}; *circles*, 0.4 mmol L^{-1})

colour formation resulting from the presence of (*R*)-PAC. A 15-fold excess of acetoin yields only a small increase of the assay response. Under standard assay conditions the concentration of acetoin formed by pyruvate decarboxylase is less than 15% of (*R*)-PAC. Thus when screening for PDC activity the additional formation of formazane by acetoin is negligible.

Different amounts of (*R*)-PAC were analysed to evaluate the correlation between (*R*)-PAC concentration and assay response. Figure 3 shows the increase of absorbance

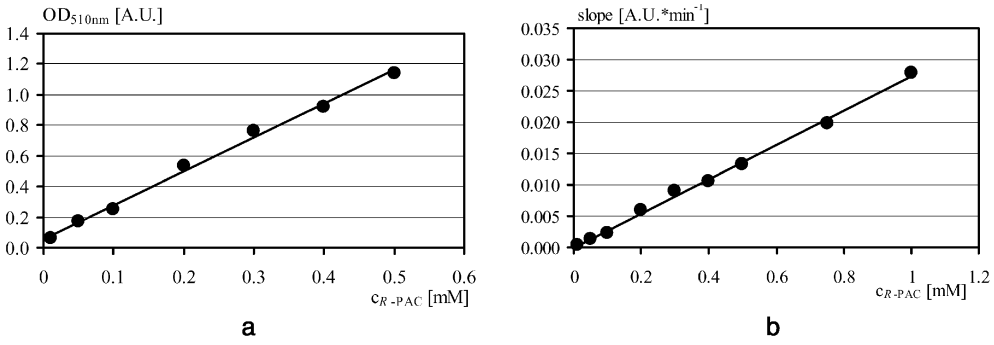


Fig. 4 Correlation between (*R*)-phenylacetylcarbinol concentration and formation of the red formazane dye. (**a**) OD_{510nm} measured 2 min after addition of 2,3,5-triphenyltetrazolium chloride to different amounts of (*R*)-PAC (r^2=99.5%); (**b**) correlation between the absorption increase at 510 nm and (*R*)-PAC concentration (r^2=99.7%)

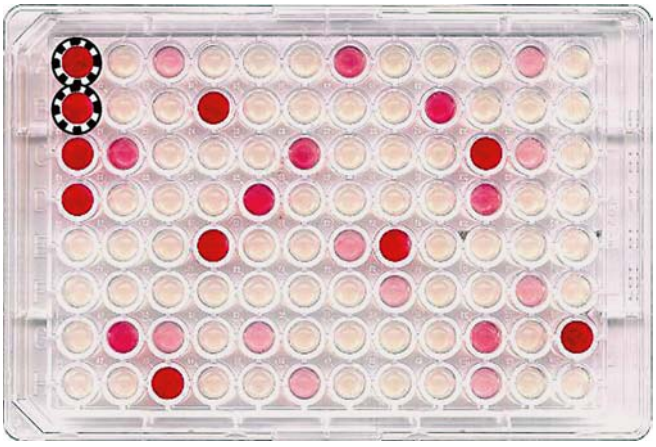
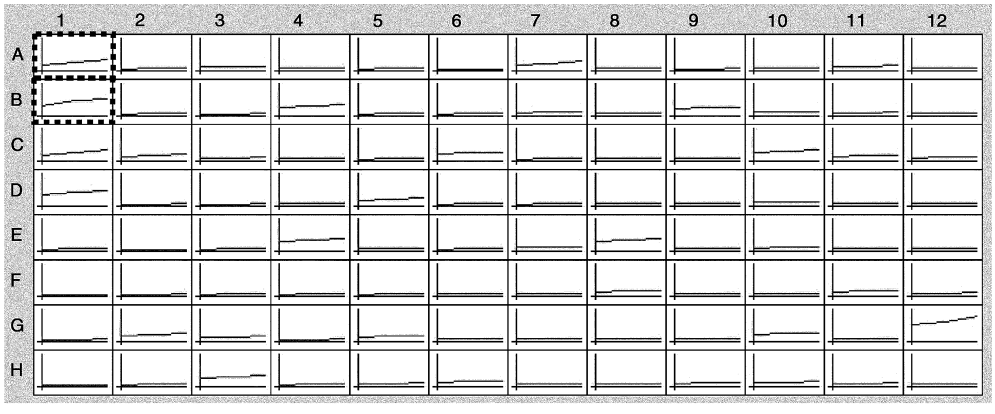


Fig. 5 Microplate with different variants of pyruvate decarboxylase. Cells were lysed and incubated for (*R*)-PAC synthesis. (*R*)-PAC was detected by use of tetrazolium red as described above. Standard clones are highlighted

at 510 nm as a function of time. Both, maximum absorption and slope of the respective curves correlate with the concentration of (*R*)-PAC (Fig. 4). We observed a linear correlation between (*R*)-PAC concentration and OD_{510nm}. (*R*)-PAC concentrations higher than 0.5 mmol L^{-1} cannot be assessed by measurement of OD_{510nm}. Two minutes after addition of 2,3,5-triphenyltetrazolium chloride absorption data are no longer within the linear range of the photometer (Fig. 4a), although plotting the slope of the linear range between 0 and 70 s against (*R*)-PAC concentration results in good correlation between these parameters in the range 0.05 to 1 mmol L^{-1} (Fig. 4b).

In addition, analysis of (*R*)-PAC produced by biotransformation of benzaldehyde can be quantified with this assay system with high throughput. Crude extracts of recombinant *E. coli* expressing the PDC gene were used to synthesise (*R*)-PAC. To achieve high reliability of high-throughput analysis plate-to-plate differences must be minimised. This was achieved by growing un-mutated

Fig. 6 Data analysis of microplate from Fig. 5. (**a**) Increase of OD_{510nm} as a function of time; (**b**) OD_{510nm} measured 2 min after addition of tetrazolium red. The position of standard clones is highlighted



a

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1.87**	0.33	0.61	0.13	0.33	0.29	1.58	0.35	0.34	0.36	0.71	0.35
B	**1.81**	0.37	0.37	1.44	0.26	0.29	0.37	0.40	1.18	0.37	0.34	0.39
C	1.81	1.25	0.28	0.29	0.30	1.18	0.32	0.30	0.32	1.44	0.63	0.32
D	1.68	0.31	0.33	0.33	1.24	0.31	0.38	0.30	0.37	0.71	0.41	0.35
E	0.35	0.34	0.26	1.53	0.32	0.38	0.61	1.21	0.37	0.35	0.30	0.33
F	0.26	0.29	0.29	0.36	0.22	0.35	0.38	0.66	0.44	0.23	0.72	0.35
G	0.27	1.24	0.64	0.33	0.65	0.40	0.25	0.33	0.35	1.25	0.35	1.67
H	0.28	0.26	1.30	0.25	0.37	0.71	0.36	0.32	0.32	0.67	0.31	0.34

b

standard clones on every plate. Possible variation between different plates can be neglected when enzyme variants are only compared with standards on the same plates. A typical plate from a high-throughput analysis of pyruvate decarboxylase variants is shown in Fig. 5. The (*R*)-PAC content of the wells in the plate was measured spectrophotometrically. These analyses can be evaluated in a kinetic representation showing the increase of OD_{510} as a function of time (Fig. 6a). Alternatively, it is also feasible to record slopes and maximum absorption of each well as numerical values (Fig. 6b). For every plate the results of all wells are finally compared with data from the respective standard samples to identify pyruvate decarboxylase muteins with altered catalytic properties.

Conclusions

Pyruvate decarboxylase is the catalyst for the synthesis of (*R*)-phenylacetylcarbinol, a chiral intermediate in the synthesis of pseudoephedrine. A spectrophotometric assay has been developed for the determination of (*R*)-PAC. (*R*)-PAC reduces 2,3,5-triphenyltetrazolium chloride to an intensely coloured formazane dye. Tetrazolium red is apparently a very useful indicator for detection of (*R*)-PAC. Acetoin, a by-product of the pyruvate decarboxylase does not interfere with the assay, because tetrazolium red is more specific for (*R*)-PAC. It was also found that it can also be used to detect an isomer of (*R*)-phenylacetylcarbinol, i.e. 2-hydroxypropiophenone [18]. This compound is not, however, formed by pyruvate decarboxylase, but by benzoylformate decarboxylase (EC 4.1.1.7). This assay is a prerequisite for the optimisation of pyruvate decarboxylase by directed evolution. This is the first assay system for high-throughput analysis of aromatic aclyoins. It is suitable for testing large libraries of enzyme variants. Depending on the laboratory automation 50,000 clones can easily be assayed per day.

By use of this analytical procedure, the entire process of enzyme preparation, incubation, and (*R*)-PAC-detection can be performed automatically in micro-plate format.

Acknowledgements The skilful technical assistance of Mr Jens Henninger is gratefully acknowledged. This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 380).

References

1. Neuberg C, Hirsch J (1921) Biochem Z 115:282–310
2. Neuberg C, Ohle H (1922) Biochem Z 128:610–618
3. Hildebrandt G, Klavehn W (1932) German Patent 548 459
4. Breuer M, Hauer B, Mesch K, Goetz G, Pohl M, Kula M-R (1997) German Patent 19 736 104 A1
5. Goetz G, Iwan P, Hauer B, Breuer M, Pohl M (2001) Biotechnol Bioeng 74:317–325
6. Pohl M (1997) Adv Biochem Eng Biotechnol 58:15–43
7. Arnold FH, Moore JC (1997) Adv Biochem Eng Biotechnol 58:1–14
8. Reetz MT, Kuhling KM, Deege A, Hinrichs H, Belder D (2000) Angew Chem Int Ed 39:3891–3893
9. Agban A, Ounanian M, Luu-Duc C, Monget D (1990) Eur J Med Chem 25:697–699
10. Lowry OH (1957) Methods Enzymol IV:3710
11. Reetz MT, Hermes M, Becker MH (2001) Appl Microbiol Biotechnol 55:531–536
12. Crameri A, Whitehorn EA, Tate E, Stemmer WPC (1996) Nature Biotechnol 14:315–319
13. Pohl M, Siegert P, Mesch K, Bruhn H, Grötzinger J (1998) Eur J Biochem 257:538–546
14. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. CSH Laboratory Press, Cold Spring Harbor, NY
15. Gröger D, Erge D (1965) Pharmazie 20:92–96
16. Nikolova P, Long A, Ward OP (1991) Biotechnol Tech 5:31–34
17. Rothrock JW, Watchung NJ (1967) US Patent 3 338 796
18. Lingen B, Grötzinger J, Kolter D, Müller M, Kula M-R, Pohl M (2001) in preparation