J Ind Microbiol Biotechnol (2007) 34:807–811
DOI 10.1007/s10295-007-0257-x

SHORT COMMUNICATION

A semi-quantitative high-throughput screening method for microbial L-tyrosine production in microtiter plates

Tina Lütke-Eversloh · Gregory Stephanopoulos

Received: 17 May 2007 / Accepted: 11 September 2007 / Published online: 10 October 2007

Abstract In this study, we developed a fluorescence-based assay for quantifying the aromatic amino acid L-tyrosine in small sample volumes employing 96-well microtiter plates. The method was based on the specific derivatisation of L-tyrosine with 1-nitroso-2-naphthol and the formation of a stable complex for the fluorescent determination of L-tyrosine concentrations. The original procedure for L-tyrosine measurements in blood or tissue samples (Waalkes and Udenfriend in J Lab Clin Med 50:733–736, 1957) was modified to a simple assay suitable for high-throughput screening of L-tyrosine producing microorganisms such as engineered *Escherichia coli*.

Introduction

Metabolic engineering of microorganisms has employed in the past various approaches to achieve the goal of developing or improving a specific phenotype by genetic manipulation. Systematic methods follow a targeted approach whereby specific genetic modifications are used to alter metabolism and generate microbes with desired properties such as the production of valuable compounds like vitamins, antibiotics, solvents or amino acids. These rationally designed strategies include well-defined gene inactivation, native or heterologous gene overexpression and deregulation of targeted enzymes or multiple biochemical reactions [5, 21, 22]. Despite vastly increasing knowledge of microbial physiology and metabolic networks, many biomolecular interactions and regulatory circuits are still poorly understood and new methodologies are required to probe unknown factors in the largest possible space for phenotype improvement. Employing random gene knockout or gene overexpression libraries, combinatorial metabolic engineering approaches have been demonstrated as useful tool for strain improvement in recombinant lycopene producing *Escherichia coli* [2, 11]. These methods actually are contributing to the development of *inverse metabolic engineering*, that holds great promise in exploring the large unknown genomic space in contrast to directed, rationally designed manipulations of the product-forming biochemical pathway [6, 10]. Prerequisite for using such random approaches, is the ability to identify cells with superior phenotype out of a large pool of candidates. In recombinant lycopene production, for example, strains of interest could be easily identified by visual inspection of agar plates, because lycopene accumulation resulted in red-colored *E. coli* colonies. The increasing coloring of the cells was proportional to the lycopene content which allowed a simple, semi-quantitative high-throughput screening method to be used. Another promising combinatorial approach for phenotype improvement was applied recently by engineering transcription factors to alter the metabolic network in yeast and *E. coli* on a global level [3, 4]. Similarly, the success of this novel approach was so far only possible in systems with established high-throughput screening procedures like tolerance towards solvents or other toxic chemicals that allowed interesting mutants to be selected by survival. In general, however, there are many interesting primary or secondary metabolites of industrial impact that do not offer simple methods for quantification.

Aromatic amino acids represent valuable natural products for their use as pharmaceuticals, food and feed

T. Lütke-Eversloh · G. Stephanopoulos (✉)
Department of Chemical Engineering,
Massachusetts Institute of Technology,
77 Massachusetts Ave., Room 56-469,
Cambridge, MA 02139, USA
e-mail: gregstep@mit.edu

additives, dietary supplements and neutraceuticals [7]. Various systematic metabolic engineering strategies have been employed to generate production strains, primarily strains of *E. coli* and *Corynebacterium glutamicum* [8, 9, 13, 16], but combinatorial approaches have never been reported thus far due to the lack of suitable high-throughput screening methods. Recently, we have generated deregulated strains of *E. coli* overproducing L-tyrosine by means of rational metabolic engineering design [15, 17]. The genetic simplicity of these L-tyrosine producers offers an excellent basic platform to explore the metabolic space for improving aromatic amino acid production in *E. coli*. However, the fact that L-tyrosine is a colorless compound excreted into the medium makes it difficult to apply combinatorial methods to identify so far unknown gene targets for phenotype improvement. Therefore, we developed a screening method capable to analyze thousands of clones in a high-throughput manner, such as gene knockout or overexpression libraries, for altered L-tyrosine production profiles. The method described in this paper represents a simple chemical derivatisation assay for fluorescent L-tyrosine detection in microtiter plates.

Materials and methods

Bacterial strain and cultivation conditions The generation of the L-tyrosine producing strain *E. coli* T1 has been described in detail previously [17]. The strain was grown in Luria Bertani (LB) or MOPS-buffered minimal medium [18] comprising 5 g/l glucose and 2 g/l NH_4Cl. For plasmid maintenance, 50 mg/l spectinomycin and for the induction of the P_{lac} promoter, 0.5 mM isopropyl-β-D-thiogalctopyranoside (IPTG) were added [20]. L-Tyrosine production experiments were performed at 37°C as 50 ml cultures in 250 ml Erlenmeyer flasks or as 200 µl cultures in 96-well microtiter plates.

Fluorescence measurements Fluorescence was measured in fluorescence cuvettes (PMMA, Sarstedt) with a Hitachi F-2500 fluorescence spectrometer with an excitation wavelength of 502 nm and an emission wavelength of 532 nm. Samples in 96-well microtiter plates were analyzed in a Packard Fusion microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 590 nm.

HPLC analyses For quantification of L-tyrosine, cell-free culture supernatant samples were filtered through 0.2 µm PVDF membrane filters (Acrodisc LC 13 mm syringe filters, PALL Life Sciences, East Hills, NY, USA) and used for HPLC analysis with a Waters 2690 Separations module connected with a Waters 996 Photodiode Array (PDA) detector (Waters Corp., Milford, MA, USA), set to a wavelength of 278 nm. The samples were separated on a Waters Resolve C18 column with 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) as mobile phase. The following gradient was used at a flow rate of 1 ml/min: 0 min, 95% solvent A + 5% solvent B; 8 min, 20% solvent A + 80% solvent B; 10 min, 80% solvent A + 20% solvent B; 11 min, 95% solvent A + 5% solvent B. The L-tyrosine concentrations in the samples were determined according to a standard, the free acid of L-tyrosine (Sigma-Aldrich, St. Louis, MO, USA).

Results and discussion

Following derivatisation with 1-nitroso-2-naphthol and formation of a stable complex, L-tyrosine can be quantified colorimetrically or fluorometrically [23, 24]. The chemical reaction of nitrosonaphthol in the presence of nitrite and nitric acid at elevated temperature is specific for hydroxyphenol compounds such as L-tyrosine [12]. The original procedure was developed for plasma and tissue samples and included a protein removal step by trichloroacetic acid precipitation and a final extraction step with ethylene dichloride (Table 1). To adapt the method for quantitative analysis of L-tyrosine in microbial cultures, experiments were conducted with L-tyrosine producing *E. coli* cultures. For the generation of the L-tyrosine excreting strain *E. coli* T1, feedback-inhibition resistant derivatives of the *aroG* and *tyrA* genes, encoding a 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and the chorsimate mutase/prephenate dehydrogenase, respectively, were overexpressed in an *E. coli* K12 Δ*tyrR* strain, which lacked the TyrR-mediated transcriptional control of genes involved in the aromatic amino acid metabolism. *Escherichia coli* T1 produced 350 mg/l L-tyrosine in 50 ml batch cultures and up to 3.8 g/l in 2-l fed-batch cultivations [17].

In order to obtain culture samples with various L-tyrosine concentrations, *E. coli* strain T1 was cultivated in 50 ml MOPS-buffered minimal medium comprising 5 g/l glucose and 2 g/l NH_4Cl, and samples were drawn during the exponential growth phase. This provided samples with a broad range of L-tyrosine concentrations from 0.02 to 0.35 g/l under conditions permissive for future high-throughput screening approaches. In addition, L-tyrosine production was monitored in 96-well microtiter plate cultures, where the optical density at 600 nm, and thus the growth-associated product formation [17], was approximately fivefold lower with an average OD_{600} of 0.88 ± 0.43, as compared to Erlenmeyer flask experiments.

The major practical burden of the original method was the step of solvent extraction, which would technically be impossible to conduct at a large scale in microtiter plates. Therefore, *E. coli* culture samples were tested for L-tyrosine measurements with and without ethylene dichloride extraction, and did not show significant differences, leading to the

Table 1 Overview on nitrosonaphthol assay protocols for L-tyrosine measurements

Step	Original protocol for cuvettes[a]	Modified protocol for microtiter plates
1	1 ml sample + 3 ml H_2O	100 µl sample + 100 µl NN assay solution[b]
2	+1 ml 30% trichloroacetic acid	Incubation for 45 min at 55°C
3	Centrifugation	Incubation for ≥180 min at 25°C
4	2 ml of supernatant + 1 ml 0.1% 1-nitroso-2-naphthol (in ethanol)	Measurement of fluorescence
5	+1 ml 0.5 g/l $NaNO_2$ in 20% nitric acid	
6	Incubation for 30 min at 55°C	
7	+10 ml ethylene dichloride	
8	Centrifugation	
9	Transfer aqueous supernatant to cuvette	
10	Measurement (colorimetric or fluorescent)	

[a] Reference [24]

[b] NN assay solution: 1:1 mixture of 0.1% 1-nitroso-2-naphthol in ethanol and 20% nitric acid containing 0.5 g/l $NaNO_2$

conclusion that the extraction step could be omitted under the given conditions (Fig. 1a).

Another time-consuming step in the nitrosonaphthol assay was the removal of cells from the culture broth samples to obtain cell-free culture supernatant samples. Although necessary for the colorimetric determination of L-tyrosine [23], fluorescent measurements did not require centrifugation for L-tyrosine measurements in the nitrosonaphthol assay (Fig. 1b).

Finally, the nitrosonaphthol assay was simplified for the application in microtiter plates: to 100 µl of sample, i.e. bacterial culture or L-tyrosine standard solution, 100 µl of the reaction solution consisting of a 1:1 (v/v) mixture of 0.1% (w/v) 1-nitroso-2-naphthol in ethanol and 20% (v/v) nitric acid containing 0.5 g/l (w/v) $NaNO_2$, was added (Table 1).

The derivatisation step was conducted at 55°C for 45 min, whereas extended incubation at 55°C for up to 60 min did not show a significant effect on the subsequent measurements. Reaction times shorter than 30 min resulted in incomplete derivatisation, particularly in samples with high L-tyrosine concentrations (Fig. 2). After the reaction, the samples were incubated for approximately 180 min at room temperature before the fluorescence was measured. The post-reaction time at room temperature had to be at least 120 min for samples with ≤100 mg/l L-tyrosine and at least 180 min for samples with ≥100 mg/l L-tyrosine to allow a stable fluorescence measurement (Fig. 2). The effect of the slowly adjusted equilibrium between the non-reacted nitrosonaphthol and its L-tyrosine derivative was most likely due to the omission of the ethylene dichloride extraction [24]. However, the time flexibility of the assay represents an advantageous property if respective controls were included on each microtiter plate to allow comparable results, considering the handling of large numbers of microtiter plates for high-throughput screening approaches. Moreover, the nitrosonaphthol assay solution did not exhibit light-sensitivity and was usable for up to 24 h without changes in the measurements. No significant differences in L-tyrosine measurements were observed if samples were mixed with the assay solution and stored at room temperature up to 8 h prior the initiation of the derivatisation reaction at 55°C. Recapitulatory, the modified nitrosonaphthol assay revealed sufficient flexibility in order to handle large

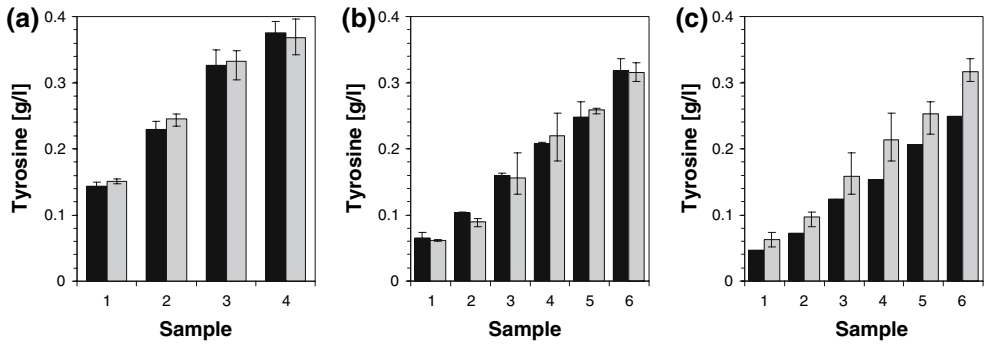

Fig. 1 Development and accuracy of the nitrosonaphthol assay. Different samples from *Escherichia coli* T1 flask cultures were used to analyze the influence **a** of ethylene dichloride extraction (*black* without extraction, *grey* with extraction) and **b** the centrifugation step (*black* culture broth, *grey* culture supernatant). **c** The L-tyrosine concentrations of various samples were measured by HPLC (*black*) and by the nitrosonaphthol assay (*grey*). At least four replicates per sample were assayed and the *error bars* represent the data range

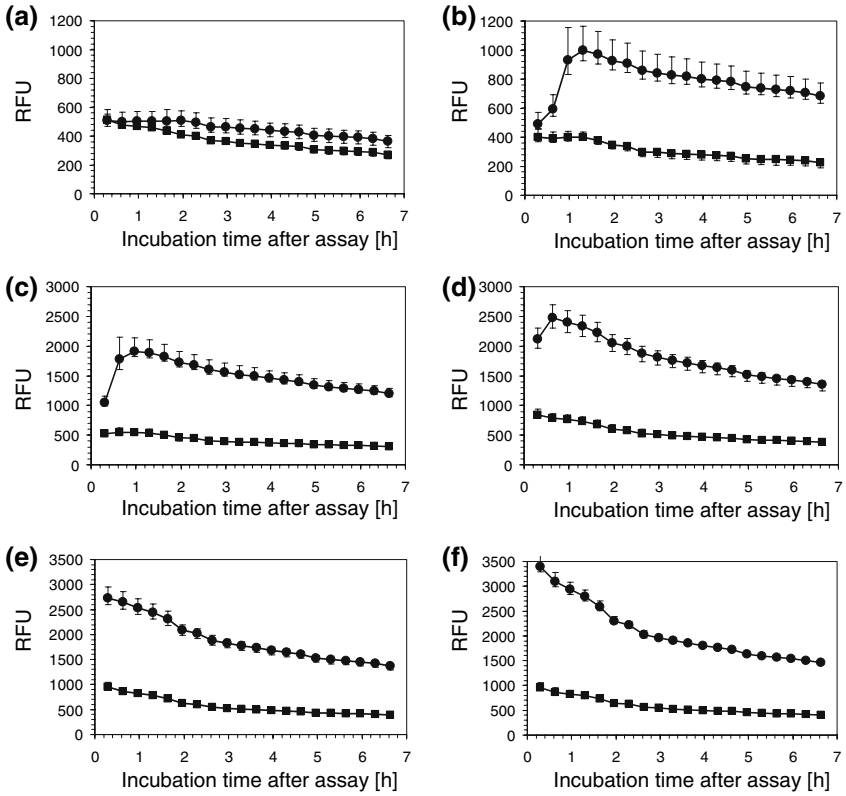

Fig. 2 Derivatisation time at 55°C and post-reaction incubation time at 25°C of the nitrosonaphthol assay. Two different samples from *E. coli* T1 flask cultures (*squares* 0.1 g/l L-tyrosine, *circles* 0.3 g/l L-tyrosine) were mixed with nitrosonaphthol assay solution and incubated for 10 (**a**), 20 (**b**), 30 (**c**), 40 (**d**), 50 (**e**) and 60 min (**f**) in a 55°C water bath. Afterwards, the samples were cooled on ice for 20 min and fluorescence (relative fluorescence units) was measured every 20 min. Twelve replicates per sample were assayed and the *error bars* represent the data range

sample numbers and the simplified protocol included only four steps (Table 1). In comparison to the original procedure, protein removal by trichloroacetic acid precipitation and centrifugation, separation of cells and culture supernatants by centrifugation and solvent extraction were not necessary, resulting in an easy and convenient method and a saving of time of approximately 30 min per sample (not considering incubation times).

The results of L-tyrosine measurements by the modified nitrosonaphthol assay were controlled by HPLC analyses (Fig. 1c). Although the L-tyrosine values obtained by the nitrosonaphthol method were usually up to 20–25% higher as compared to HPLC measurements of the same samples, the correlation among the samples, e.g. low versus high L-tyrosine concentrations, fit well. Therefore, this assay was confirmed to be suitable for screening large sample numbers to identify putative microbes with altered L-tyrosine production as compared to control samples. Such controls could be cultures of parental strains or L-tyrosine standard samples and should preferentially be included on each microtiter plate to allow comparability among different sets of microtiter plates. Depending on the amount of excreted L-tyrosine, samples may be used as they are or as dilutions since the optimal detection of L-tyrosine concentrations was determined to be in the range of 0.05–0.5 g/l L-tyrosine. Screening libraries in the order of 10^5–10^7 clones would eventually select a feasible number of mutants for subsequent HPLC analysis for more precise L-tyrosine quantification.

Screening of "invisible" microbial products generally hampers the utilization of novel promising combinatorial metabolic engineering strategies. Very recently, a sophisticated method for the quantitative detection of mevalonate was published: the authors combined a mevalonate auxotrophic *E. coli* strain with the expression of a green fluorescent protein gene, and this strain was used as a biosensor for growth-dependent fluorescence measurements to detect other mevalonate-overproducing strains [19]. In theory, this idea could also be applied for L-tyrosine production using an L-tyrosine auxotrophic strain to generate such a biosensor. Another possible approach could be the coupling of L-tyrosine overproduction with melanin biosynthesis, where the conversion of L-tyrosine by a tyrosinase leads to the formation of a dark pigment. The cultivation conditions of recombinant *E. coli* in mineral salts medium for optimum melanin production has been described recently [14] and the use of the *melC* operon from *Streptomyces glaucescens* as reporter for an inexpensive high-throughput screening method in corynebacteria has been suggested [1].

In conclusion, we presented a new high-throughput screening assay for the detection of L-tyrosine in small sample volumes. Based on the chemical derivatisation with nitrosonaphthol and subsequent fluorescence measurements, no additional biomarker or any genetic manipulation of the host are required and basically any type of microorganism capable for the cultivation in microtiter plates can be used for this application.

Acknowledgments We thank the Deutsche Forschungsgemeinschaft (DFG) for providing the research fellowship LU 893/2-1 and the DuPont-MIT Alliance for financial support.

References

1. Adham SAI, Rodríguez S, Ramos A, Santamaría RI, Gil JA (2003) Improved vectors for transcriptional/translational signal screening in corynebacteria using the *melC* operon from *Streptomyces glaucescens* as reporter. Arch Microbiol 180:53–59
2. Alper H, Miyaoku K, Stephanopoulos G (2005) Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. Nat Biotechnol 23:612–616
3. Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568
4. Alper H, Stephanopoulos G (2007) Global transcription machinery engineering: a new approach for improving cellular phenotype. Metab Eng. doi:10.1016/j.ymben.2006.12.002
5. Bailey JE (1991) Towards a science of metabolic engineering. Science 252:1668–1674
6. Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS (1996) Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol Bioeng 52:109–121
7. Bongaerts J, Krämer M, Müller U, Raeven L, Wubbolts M (2001) Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metab Eng 3:289–300
8. Ikeda M (2003) Amino acid production processes. In: Scheper T, Faurie R, Thommel J (eds) Advances in biochemical engineering/biotechnology, vol 79. Springer, Berlin, pp 1–35
9. Ikeda M (2006) Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. Appl Microbiol Biotechnol 69:615–626
10. Jin YS, Alper H, Yang YT, Stephanopoulos G (2005) Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through inverse metabolic engineering approach. Appl Environ Microbiol 71:8249–8256
11. Jin YS, Stephanopoulos G (2007). Multi-dimensional gene target search for improving lycopene biosynthesis in *Escherichia coli*. Metab Eng. doi:10.1016/j.ymben.2007.03.003
12. Knight JA, Robertson G, Wu JT (1983) The chemical basis and specificity of the nitrosonaphthol reaction. Clin Chem 29:1969–1971
13. Krämer M, Bongaerts J, Bovenberg R, Kremer S, Müller U, Orf S, Wubbolts M, Raeven L (2003) Metabolic engineering for microbial production of shikimic acid. Metab Eng 5:277–283
14. Lagunas-Muñoz VH, Cabrera-Valladares N, Bolívar F, Gosset G, Martínez A (2006) Optimum melanin production using recombinant *Escherichia coli*. J Appl Microbiol 101:1002–1008
15. Lütke-Eversloh T, Stephanopoulos G (2005) Feedback inhibition of chorismate mutase/prephenate dehydrogenase (TyrA) of *Escherichia coli*: generation and characterization of tyrosine-insensitive mutants. Appl Environ Microbiol 71:7224–7228
16. Lütke-Eversloh T, Santos CNS, Stephanopoulos G (2007) Perspectives of biotechnological production of L-tyrosine and its applications. Appl Microbiol Biotechnol (submitted)
17. Lütke-Eversloh T, Stephanopoulos G (2007) L-Tyrosine production by deregulated strains of *Escherichia coli*. Appl Microbiol Biotechnol 75:103–110
18. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. J Bacteriol 119:736–747
19. Pfleger BF, Pitera DJ, Newman JD, Martin VJJ, Keasling JD (2007) Microbial sensors for small molecules: development of a mevalonate biosensor. Metab Eng 9:30–38
20. Sambrock J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn Cold Spring Harbor Laboratory Press, Cold Spring Harbor
21. Stephanopoulos G, Sinskey AJ (1993) metabolic engineering: issues and methodologies. Trends Biotechnol 11:392–396
22. Stephanopoulos G (1999) Metabolic fluxes and metabolic engineering. Metab Eng 1:1–11
23. Udenfriend S, Cooper JR (1952) The chemical estimation of tyrosine and tyramine. J Biol Chem 196:227–233
24. Waalkes TP, Udenfriend S (1957) A fluorometric method for the estimation of tyrosine in plasma and tissues. J Lab Clin Med 50:733–736