

Short Communication

A high-throughput method for screening of L-tyrosine high-yield strains by *Saccharomyces cerevisiae*

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A biosensor screening assay based on the synthesis of betaxanthin was applied to relatively high throughput screening of the L-tyrosine mutant library. In the assays, fluorescence output showed a linear relationship between extracellular L-tyrosine content and yellow pigment formation. In addition, the yellow pigment accumulation of the L-tyrosine high-yield strain can be easily distinguished with the naked eye compared with the wild-type strain. As a result, numerous mutants that exhibited significantly increased coloration, were screened out after random mutagenesis, and *p*-coumaric acid production in mutants NK-A3 and NK-B4, were remarkably improved by 4-fold more than that of the wild-type strain. In general, this study provides a novel strategy for screening mutant libraries in the search for highly L-tyrosine-producing strains.

Key Words: betaxanthin; high-throughput screening; L-tyrosine; *p*-coumaric acid; *Saccharomyces cerevisiae*

L-tyrosine as a precursor plays an important role in the biosynthesis of various plant flavonoids (Koopman et al., 2012; Li et al., 2015). A variety of strain improvement strategies have been developed to increase L-tyrosine production. Currently, classical strain engineering based on rational design approaches mainly focus on alleviating the feedback regulation of the L-tyrosine biosynthesis pathway (Helmstaedt et al., 2001; Luttkik et al., 2008), reducing by-product formation, and altering central carbon metabolism, in order to increase the supply of two main precursors, erythrose-4-phosphate and phosphoenolpyruvate (Hazelwood et al., 2008). Although these methods

have demonstrated a significant increase in the production of L-tyrosine, many biosynthesis interactions and regulatory circuits that are indirectly involved in the L-tyrosine biosynthetic pathway are still unclear. Therefore, random mutagenesis followed by an efficient screening strategy provides a feasible way to identify obscure targets or unknown mechanisms.

High Performance Liquid Chromatography (HPLC) is the most common method for the analysis of small molecule production, such as amino acids, organic acids and polymer precursors. However, it is a challenge to prepare and analyze, one by one, massive samples generated by random mutagenesis. Therefore, a high-throughput method for screening mutants having a phenotype of interest becomes imperative. Recently, a range of biosensors have demonstrated a great potential for converting the intracellular product formation into a screenable optical output (Mahr et al., 2015; Pflieger et al., 2007; Santos and Stephanopoulos, 2008; Shi et al., 2015). These systems enable a simple method for analyzing clones in a high-throughput manner according to colorimetric assays and fluorescence readout. Here, we describe a rapid and simple high-throughput method for the screening of L-tyrosine high-yield strains. The genes encoding tyrosine hydroxylation and DOPA dioxygenase were introduced into wild-type *S. cerevisiae* strain, which catalyze the conversion of L-tyrosine to betaxanthin (DeLoache et al., 2015). Since betaxanthin can be easily detected by both visual and fluorescence measurements, the synthesis of betaxanthin was employed for fast identifying the high L-tyrosine producer after random mutagenesis.

S. cerevisiae BY4741 was used as the parent strain (Liu et al., 2014), and the NK-T20 strain (unpublished data) was used as a positive L-tyrosine high-yield strain (Table S1). Expression plasmid pSP-G1 was donated by J. Nielsen (Partow et al., 2010). Synthetic complete (SC) medium,

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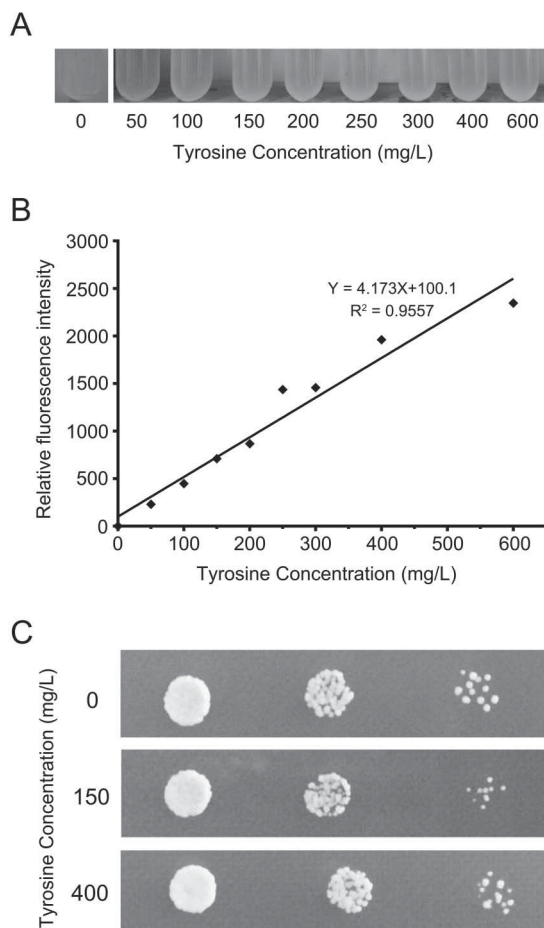


Fig. 1. Biosensor detection of L-tyrosine on liquid and solid media.

The color (A) and yellow fluorescence intensity (B) of the NK-WT1 strain cultured in SC-Ura-Tyr liquid medium with L-tyrosine supplementation. C. The NK-WT1 strain was spotted onto agar plates without or with L-tyrosine supplementation.

as well as drop-out media (SC-Ura, SC-Ura-Tyr), and agar plates were used in transformation and complementation experiments (Gietz and Schiestl, 2007; Mao et al., 2017). When required, different concentrations of L-tyrosine were added in liquid or solid media.

To demonstrate the feasibility of probing L-tyrosine concentration through this method, the sensor plasmid pLC84 (Fig. S1) was first transformed into *S. cerevisiae* BY4741 wild type strain, yielded an NK-WT1 strain. The NK-WT1 strain was cultured in SC-Ura-Tyr liquid and solid medium supplemented with various concentrations of L-tyrosine. As expected, an elevated extracellular L-tyrosine level resulted in a deeper color of the medium through the biosensor cells, as shown by fluorescence spectroscopy (Figs. 1A, B and C). These results suggested that the sensor strain could be distinguished by visual inspection in varying L-tyrosine-supplemented medium.

Subsequently, we selected an engineered strain NK-T20 (unpublished data, overexpressing mutants of Aro4p and Aro7p to avoid feedback inhibition, and deletion of *ARO10* and *PDC5* genes to decrease byproduct formation) as the positive control. Similarly, the deeper coloration of the NK-T21 strain was observed in SC-Ura-Tyr liquid medium or agar plates (Fig. 2A). For further analysis, a confocal

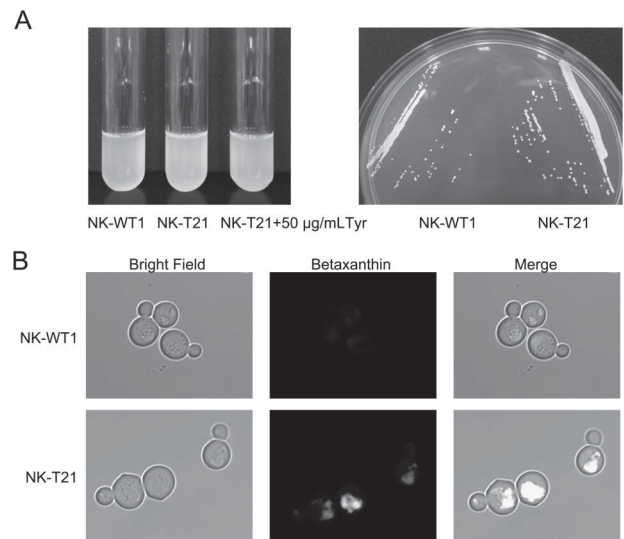


Fig. 2. Color comparison between wild-type and L-tyrosine high-yield strains.

A. Pictures of the color accumulation of the NK-WT1 and NK-T21 strains cultured in the SC-Ura-Tyr liquid medium or agar plate. B. Fluorescence microscopy images of individual cells of the NK-WT1 and NK-T21 strains. Fluorescence images were taken using a confocal laser scanning microscope (CLSM, Zeiss.LSM710.Germany), with excitation at 514 nm for betaxanthin.

laser scanning microscope was used to visualize the fluorescence of single cells (Fig. 2B). The images also showed that the higher L-tyrosine producer exhibited stronger fluorescence compared with the wild type strain. These results indicated that the system could be effectively applied to distinguish the L-tyrosine high-yield strain and to qualitatively detect the level of intracellular tyrosine.

Taken together, as shown in Fig. 3, we developed a new strategy for screening large mutant libraries. The overall procedure consists of two steps: (I) Isolation of the analogue resistant mutants; and (II) Rapid quantification of intracellular tyrosine levels by HPLC assay.

(I) The original NK-WT1 was chemically mutagenized by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and plated on the agar medium containing 2 mg/L of L-tyrosine ethylester or D-tyrosine (Fig. S2). The plates were incubated at 30°C for 3~5 days. Then, the resistant colonies were selected by visual inspection based on the pigmentation intensity and size of the colony (Fig. S3). Subsequently, 10 mutants that exhibited significantly increased coloration were selected from the analogue plates. The original mutant strains were further cultured in the same screening medium to determine the stability of mutations (Fig. 4A).

(II) The selected strains were repeatedly cultured in SC medium to the loss of the sensor plasmid. The plasmid was easily lost in the absence of auxotrophic selective pressure after three rounds, with each round lasting 12 h (Fig. 4B). Eventually, the tyrosine ammonia lyase (*TAL*) gene from *Rhodobacter capsulatus* was over-expressed in the mutants to convert L-tyrosine to *p*-coumaric acid (Fig. S1) (Kyndt et al., 2002; Mao et al., 2017). Unlike the precursor L-tyrosine, *p*-coumaric acid is cell permeable and cannot be further metabolized by yeast, making it better

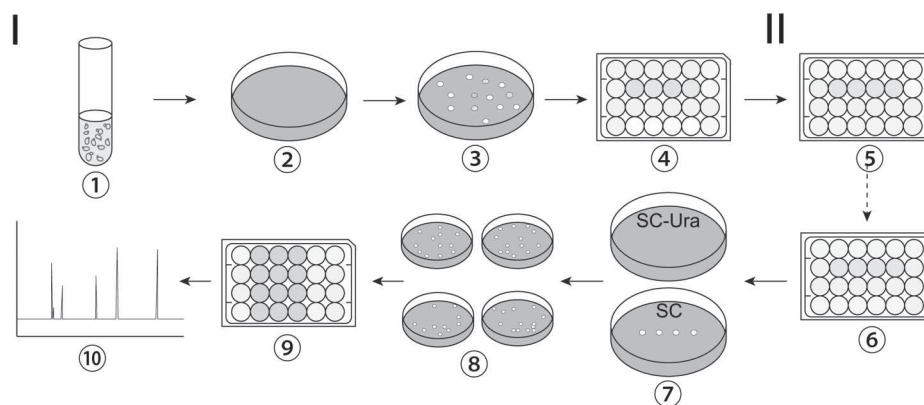


Fig. 3. Biosensor-based two-step high-throughput screening.

I. Isolation of the analogue resistant mutants: (1) Mutagenesis. (2), (3) Screening approach, The mutants were firstly plated on the agar medium containing 2 mg/L of L-tyrosine ethylester or D-tyrosine, then incubated at 30°C until noticeably different pigmentation intensities were detected. (4) Selected mutants were re-cultivated in the liquid medium containing 2 mg/L of L-tyrosine ethylester or D-tyrosine to maintain the stability of the mutations. II. Rapid detection of L-tyrosine levels by *p*-coumaric acid production in the mutants. (5), (6) Proceed to the plasmid-curing step. The selected mutants were cultured in liquid medium without auxotrophic selective pressure and repeated three times. (7) To further verify the loss of the sensor plasmid, the cultures were spotted on SC-Ura and SC plates. (8) Transformation, the *TAL* gene from *Rhodobacter capsulatus* was transformed into the selected mutants to convert L-tyrosine to *p*-coumaric acid. (9) Mutants that contain pLC-*TAL* plasmid are cultured in microtiter plates for *p*-coumaric acid production. (10) HPLC analysis of supernatant.

to analyse than L-tyrosine in the culture (Jakociunas et al., 2015; Rodriguez et al., 2015). As shown in Fig. 4C, the *p*-coumaric acid production of most mutants was higher than that of the wild type strain, except for one mutant that remained at the same level. Among these 10 mutants, the yield of *p*-coumaric acid in the NK-A3 and NK-B4 mutants was markedly improved by a factor of four greater than the wild type strain. Therefore, this system could be effectively used as a means of preliminary screening tyrosine mutant libraries.

Laboratory mutagenesis is an extremely important technique for improving L-tyrosine production in *S. cerevisiae*. However, owing to the absence of high-throughput screening for assessing the phenotype of interest, it may be difficult to screen through the number of mutants generated using highly mutagenic strategies. In the present study, we describe a high-throughput method for screening L-tyrosine high-yield strains based on the synthesis of yellow fluorescent pigment. This screening strategy is simple, and does not require expensive equipment. The L-tyrosine-producing strains could be isolated sensitively by the naked eye or microplate reader. In theory, the strategy in this study could also be applied to the analysis of L-tyrosine production using an L-tyrosine auxotrophic strain as a biosensor. Mutants from the mutation library are first individually cultured in 96-well microtiter plates. Subsequently, the cell lysis supernatants are used as a growth medium for the L-tyrosine auxotrophic strain with yellow pigment synthesis. The growth and fluorescence of the auxotrophic report strain are monitored to screen the L-tyrosine high-producing mutants. Additional modifications of this approach will be further investigated in future studies. This approach could also be applied to other microorganism, and might find new gene targets for improved L-tyrosine production by means of random mutagenesis.

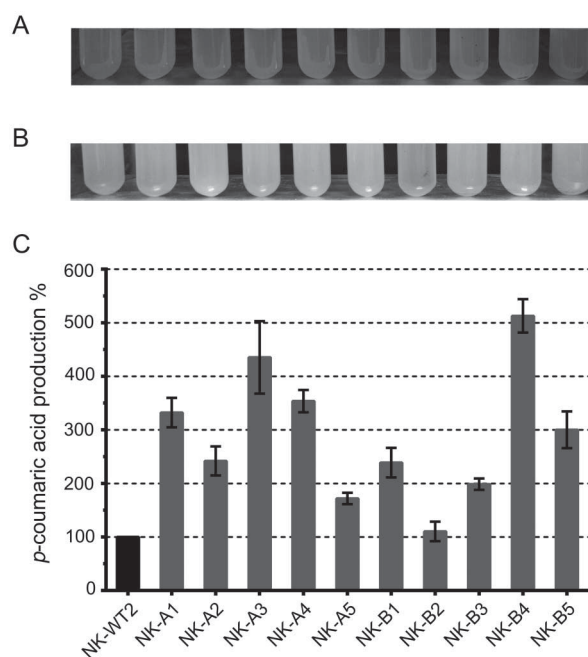


Fig. 4. The verification of L-tyrosine high-yield mutants by *p*-coumaric acid production.

A and B. Identification of the loss of the sensor plasmid in the selected mutants. C. The *p*-coumaric acid production of the L-tyrosine high-yield mutants. Average $\pm$ standard deviations were calculated from three biological replicates.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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