Appl Biochem Biotechnol (2017) 183:1209–1223
DOI 10.1007/s12010-017-2493-5

Development of Microtiter Plate Culture Method for Rapid Screening of ε-Poly-L-Lysine-Producing Strains

Yong-Juan Liu[1] · Xu-Sheng Chen[1] · Jun-Jie Zhao[1] · Long Pan[1] · Zhong-Gui Mao[1]

Received: 16 February 2017 / Accepted: 24 April 2017 /
Published online: 24 May 2017

Abstract ε-Poly-L-lysine (ε-PL) produced by *Streptomyces albulus* possesses a broad spectrum of antimicrobial activity and is widely used as a food preservative. To extensively screen ε-PL-overproducing strain, we developed an integrated high-throughput screening assay using ribosome engineering technology. The production protocol was scaled down to 24- and 48-deep-well microtiter plates (MTPs). The microplate reader assay was used to monitor ε-PL production. A good correlation was observed between the fermentation results obtained in both 24-(48)-deep-well MTPs and conventional Erlenmeyer flasks. Using this protocol, the production of ε-PL in an entire MTP was determined in <5 min without compromising on accuracy. The high-yielding strain selected through this protocol was also tested in Erlenmeyer flasks. The result showed that the ε-PL production of the high-yielding mutants was nearly 45% higher than that of the parent stain. Thus, development of this protocol is expected to accelerate the selection of ε-PL-overproducing strains.

Keywords ε-Poly-L-lysine · High-throughput screening · Ribosome engineering · Strain improvement · Miniaturized production

Introduction

ε-Poly-L-lysine (ε-PL) is a basic homo-poly-amino acid characterized by a peptide bond between ε-amino and α-carboxyl groups of L-lysine. It is a secondary metabolite, mainly produced by bacteria belonging to the family *Streptomycetaceae* [1–3]. The major advantages

✉ Xu-Sheng Chen
chenxs@jiangnan.edu.cn

✉ Zhong-Gui Mao
maozg@jiangnan.edu.cn

[1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

of ε-PL over synthetic homo-poly-lysine (α-PL) include its broad spectrum of antimicrobial activity, non-toxicity, and biodegradability [4–7]. Currently, ε-PL is used as a natural food preservative in Japan, South Korea, the USA, and other countries. Besides its use in the food industry, ε-PL has numerous applications in the pharmaceutical industry as a drug carrier, nanoparticle, gene carrier, liposome, interferon inducer, lipase inhibitor, hydrogel, coating material, etc. [8], and its applications are expected to broaden in the future [6, 9].

Among the various strain-improvement strategies developed to increase ε-PL productivity, the most successful one was screening for mutants with *S*-(2-aminoethyl)-L-cysteine plus glycine resistance after nitrosoguanidine (NTG) treatment and ultraviolet (UV) mutagenesis [5]. Recently, atmospheric and room temperature plasma mutagenesis combined with *S*-(2-aminoethyl)-L-cysteine and glycine resistance screening as well as genome shuffling were reported to improve the production of ε-PL [10, 11]. Traditionally, most of the screening methods have been performed in Erlenmeyer flasks [12], in which only a small proportion of randomly selected mutants could be screened and most of the mutagenic spore suspension is lost owing to the low throughput of Erlenmeyer flasks. Besides, these flasks require a large amount of material and the cultivation process is time-consuming and laborious, thus limiting the wide applications of such strain improvement strategies [13–15]. Furthermore, UV spectrophotometry, which has been extensively used to determine the concentration of ε-PL [3, 16], has limited screening throughput and allows screening about 100 samples/month [17]. Therefore, it is imperative to develop a high-throughput screening method to evaluate a large number of ε-PL-overproducing mutants with a good degree of accuracy and reproducibility.

In recent years, many successful high-throughput screening strategies have been reported, including screening of rapamycin in *Streptomyces hygroscopicus* [18], avermectins in *Streptomyces avermitilis* [19], 1,3-propanediol in *Clostridium butyricum* [20], poly(γ-glutamic acid) in poly(γ-glutamic acid)-producing bacteria [21], cephalosporin C in *Acremonium chrysogenum* [22], ethanol in *Saccharomyces cerevisiae* [23], gluconate in *Aspergillus niger* [24], and α-ketoglutaric acid in *Yarrowia lipolytica* [25]. In the present study, we report on a high-throughput procedure for screening ε-PL super-producers based on the microplate reader assay using 24- and 48-deep-well microtiter plates (MTPs) for cultivation. The accuracy of the method was compared with a UV assay, and its reproducibility was also examined.

Materials and Methods

Microorganisms and Medium

Streptomyces sp. M-Z18, preserved in our lab, was isolated from the soil as described by Nishikawa and Ogawa [26], subjected to UV and NTG mutagenesis as described by Hiraki et al. [27]. UV mutagenesis is one of the most commonly used physical mutagenic factors and NTG is a chemical mutagen; their mutagenic effect is mainly caused by the change of DNA structure. The solid medium was BTN agar medium and consisted of the following (per L): glucose, 10 g; yeast extract, 1 g; peptone, 2 g; and agar, 20 g. The pH of the medium was adjusted to 7.5 using 2 M NaOH before sterilization [10].

YH medium was used as the seed and product fermentation medium in this study. It contained the following (per L): glycerol, 60 g; yeast extract, 8 g; $(NH_4)_2SO_4$, 5 g; K_2HPO_4, 2 g; $MgSO_4$, 0.5 g; $ZnSO_4$, 0.04 g; and $FeSO_4$, 0.03 g. The medium pH was adjusted to 7.5 using 2 M NaOH before autoclaving [28].

Cultivation of the Microorganism

Cultivation in Erlenmeyer Flasks

The microbial cultivations were performed in two stages: spore germination and production. After spore germination, three loops of aerial spores were transferred into a 250-mL Erlenmeyer flask containing 40 mL of fermentation medium and incubated at 30 °C and 200 rpm on a rotating shaker (HYL-C, Qiang Le Laboratory Equipment Co., Taicang, China) for 24 h. Then, 8% of the seeds were transferred to fresh medium and incubated at 30 °C and 200 rpm on a rotating shaker for 72 h.

Cultivation in 24- and 48-Deep-Well MTPs

A high-throughput screening method was established to rapidly screen high-yielding mutants. The screening plate medium containing antibiotics was transferred to a thermostatic incubator and incubated for 7–8 days at 30 °C. Colonies were randomly picked, transferred to 24-deep-well MTPs containing 2 mL of fermentation medium or 48-deep-well MTPs containing 0.5 mL of fermentation medium, and incubated at 30 °C and 200 rpm on a rotating shaker for 96 h. Meanwhile, each well of an aseptic 96-well MTP was filled with 1 mL of sterile BNT agar medium and inoculated with spores from one colony randomly picked from the screening plate.

Ribosome Engineering Mutagenesis Procedure

The strain of *Streptomyces* sp. M-Z18 was subjected to mutagenesis using paromomycin. The spores were spread on BTN agar plates containing paromomycin, which belongs to the 2-deoxystreptamine-containing groups, is an aminoglycoside antibiotic that causes miscoding. It binds to the 30S subunit, which causes the ribosome to select incorrect aminoacyl-tRNAs and which leads to misreading during translation [29], and incubated at 30 °C to obtain single drug-resistant mutants. After cultivating for 7–10 days, mutants with abundant spores were picked out for fermentation assays, and high-producing mutants were utilized as the second screening strains.

Analytical Methods

The culture broth was centrifuged and the supernatant was subjected to the following assays for the measurement of ε-PL concentration.

Microplate Reader Assay

The absorbance of the supernatant was determined at 465 nm to measure the concentration of ε-PL. The 24-well MTP was directly centrifuged at 1600×*g* for 20 min, and the resulting supernatants (50 μL) in the wells were transferred into another 96-deep-well MTP containing 950 μL of phosphoric acid buffer (0.130 g/L $Na_2HPO_4{\cdot}12H_2O$ and 0.0546 g/L $Na_2HPO_4{\cdot}12H_2O$ and 0.0546 g/L $NaH_2PO_4{\cdot}2H_2O$; pH 6.9–7.1) using an eight-channel pipette (Eppendorf Xplorer plus, Hamburg, Germany). Subsequently, 500 μL of the diluted supernatant was transferred to another 48-deep-well MTP, and

500 μL of methyl orange (MeO) was added and vortexed. After incubation for 30 min at 30 °C and 200 rpm on a rotating shaker, the plate was centrifuged at 1600×*g* for 25 min, and the resulting supernatants (10 μL) in the wells were transferred into 96-shallow-well MTP containing 190 μL of phosphoric acid buffer. The absorbance of the supernatants was measured at 465 nm using a Multiskan FC microplate reader (Thermo Fisher, Waltham, MA, USA). A standard curve was generated using 500 μL of 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, and 0.16 g/L commercially available ε-PL sample (Bainafo, Zhengzhou, Henan Province, China). Owing to the sensitivity of the microplate reader, at least five replicates were employed.

Spectrophotometric Assay

The concentration of ε-PL in the samples was measured according to the method of Itzhaki [16] with modifications. In brief, 0.5 mL of the sample was mixed with 2 mL MeO solution (1 mM MeO and 50 mM sodium phosphate, pH 7.0) and vortexed. After incubation for 30 min at 30 °C, the mixture was centrifuged at 45,000×*g* for 15 min, and the supernatant was diluted tenfold and its absorbance was measured at 465 nm [30].

Analysis of Dry Cell Weight (DCW) and Glycerol Concentration

The samples (10 and 1.5 mL, respectively) were collected from Erlenmeyer flasks and MTPs and centrifuged (4500×*g* for 10 min and 13,000×*g* for 10 min, respectively). The precipitate obtained was washed twice with distilled water and dried (at 105 °C and vacuum-dried at 65 °C, respectively) to a constant weight to determine the dry cell weight of the culture. The concentration of glycerol was determined by using a HPLC system (Dionex, U-3000, USA) with a refractive index detector (Shodex RI-101, Japan) and an ion exchange column (Aminex HPX-87H, 300 × 7.8 mm, Hercules, USA). The column was eluted with 5 mM H_2SO_4 at 60 °C and a flow rate of 0.6 mL/min.

Statistical Analysis

The experimental data obtained were analyzed using Origin 8.5 professional software with Minitab15 software, and the results are the average of standard error difference.

Pearson correlation factors were calculated using IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Evaluation of Cell Growth and ε-PL Production in MTPs

While solid culture is advantageous over liquid culture for rapid screening, the microenvironment of liquid cultures, especially MTP cultures, is not necessarily the same as that of conventional Erlenmeyer flask culture systems. Accordingly, in the present study, cell growth and ε-PL production in MTPs were evaluated. Furthermore, the reproducibility of ε-PL production at different scales (MTP and Erlenmeyer flask) was investigated to determine whether microenvironment culture can be adopted for screening ε-PL-overproducing strains.

The maximum loading capacity of the 24-deep-well MTPs was 10 mL, and the maximum amount of ε-PL production in the most suitable liquid volume was optimized using spectrophotometric assay. As shown in Fig. 1a, the culture medium volume (1–5 mL) was optimized, and the ε-PL concentration was the highest when the culture medium volume was 2 mL. Furthermore, the ε-PL yield in 3 mL of the culture medium was lower than that obtained in 1 mL of the culture medium, and almost no ε-PL was produced when the culture medium volume was 4 and 5 mL (0 g/L). To increase the flux and improve the efficiency of screening of bacteria, the culture medium volume for the 48 (and 96)-deep-well MTPs was optimized. As shown in Fig. 1b, the ε-PL production presented a declining trend with the increasing volume of the culture medium from 0.5 to 2 mL. The yield of ε-PL was the highest in 0.5 mL of the culture medium. Therefore, 0.5 mL was considered to be the optimal culture medium volume. However, as the loading of 96-deep-well MTPs was very low and the fermentation time was quite long, only little fermentation medium was left after fermentation; therefore, the 96-deep-well MTPs may not be suitable for screening of strain. Furthermore, the mycelial morphology in different volumes of the culture medium was examined (Fig. 1c). The shape and amount of pellets were the most suitable when the culture medium volume was 2 and 0.5 mL, respectively.

Subsequently, the growth curves of *Streptomyces* sp. M-Z18 in Erlenmeyer flasks and 24-deep-well MTPs were compared. As shown in Fig. 2a, b, pH showed a declining trend in the fermentation process. The decrease in the pH in the 24-deep-well MTP was slower than that in the Erlenmeyer flask, and as a result, the final pH in the 24-deep-well MTP was higher than that in the Erlenmeyer flask. Meanwhile, the concentration of glycerol in the fermentation medium in the Erlenmeyer flask was lower than that in the 24-deep-well MTP, which may be related to the fermentation system. The dry weight of mycelium and ε-PL production showed an upward trend during the fermentation process in both the flask and MTP, and the final output was about the same. As illustrated in Fig. 2c, the amount of mycelial pellets was higher in the 24-deep-well MTP, when compared with that in the Erlenmeyer flask, with most of the pellets remaining intact. These results indicated that the 24-deep-well MTPs were ideal for screening of strain.

The microtiter-based cultivation system has been tested for use with streptomycetes before [31]. The medium, namely the sources of carbon and/or nitrogen and phosphate [32–34], but also the size and type of inoculum or type of vessel and agitation will all greatly affect growth of *Streptomyces* strains in liquid culture and the production of secondary metabolites [35]. In this study, it was notable that the *Streptomyces* strains tested did grow very well in the MTPs.

Comparison of ε-PL Production in MTPs and Erlenmeyer Flasks

Ideally, fermentation in Erlenmeyer flasks can be replicated in MTPs [36]. To verify the feasibility of using 24-(48)-deep-well MTPs instead of Erlenmeyer flask fermentation, the correlations between fermentation in the Erlenmeyer flasks and 24-(48)-deep-well MTPs were investigated. A total of 24 strains screened from the antibiotic plate were simultaneously inoculated into Erlenmeyer flasks and 24-deep-well MTPs, and the final amount of ε-PL produced was determined by UV spectrophotometry. As shown in Fig. 3a, ε-PL production in the Erlenmeyer flasks was well correlated with that in the MTP cultures, and the correlation efficiency was high for ε-PL production (Pearson factor: $r = 0.812$, $p < 0.01$). Subsequently, the correlations between ε-PL production in the Erlenmeyer flask tested by UV spectrophotometry and the microplate reader were investigated. As illustrated in Fig. 3b, regression

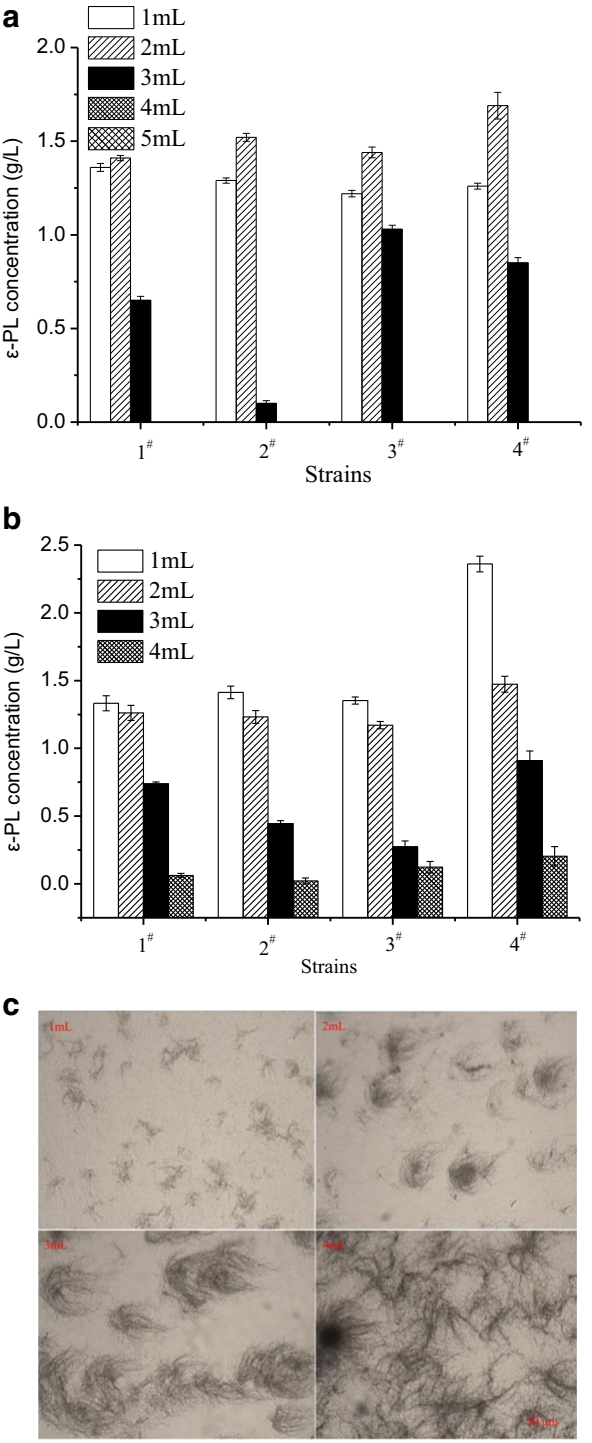

Fig. 1 Optimization of the culture medium for 24-deep-well MTPs (**a**) and 48-deep-well MTPs (**b**). Comparison of the mycelial pellet in different volumes of the culture medium (**c**)

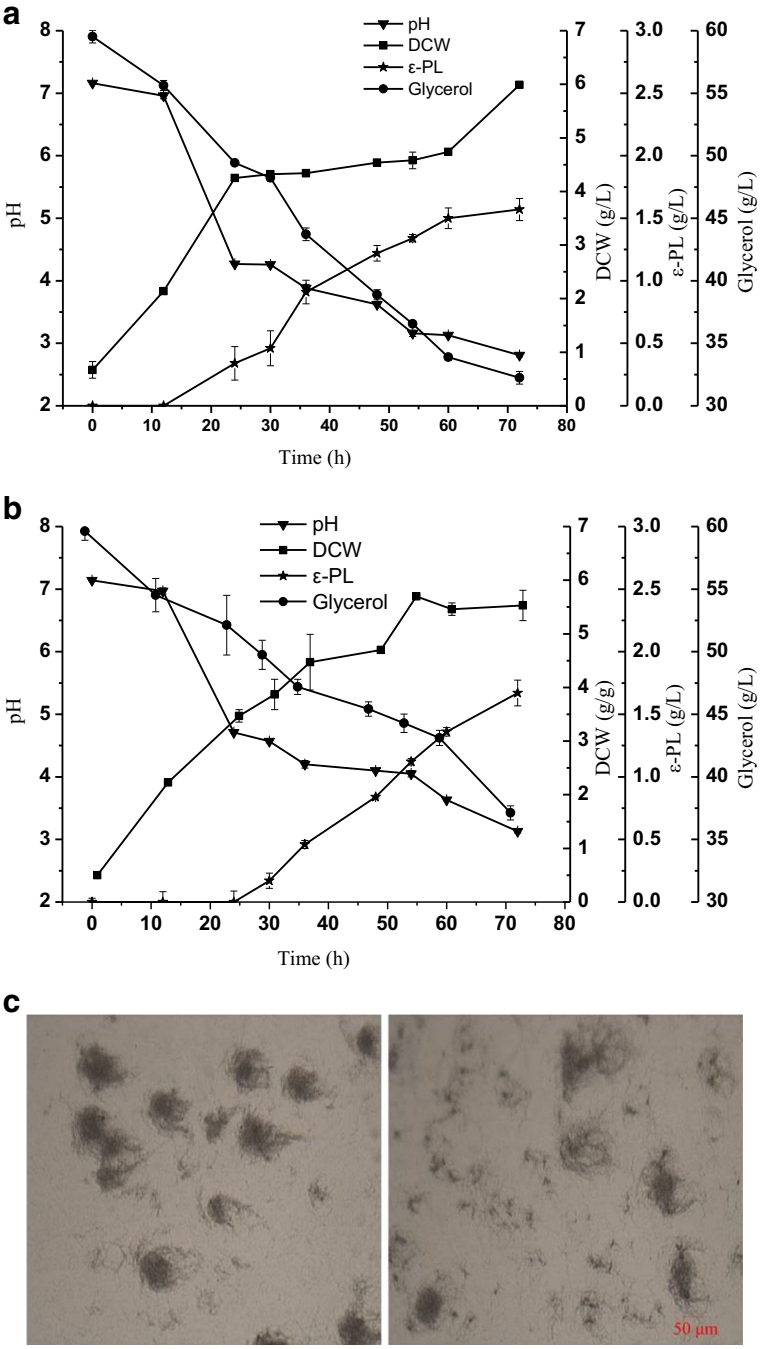

Fig. 2 Growth curve of *Streptomyces* sp. M-Z18 in Erlenmeyer flask (**a**) and 24-deep-well MTP (**b**). Cell dry weight (*pentagram*), pH (*inverse triangle*), glycerol concentration (*circle*), and ε-PL production (*square*) were measured at different time points. The morphology of mycelium in Erlenmeyer flask (*right*) and 24-deep-well MTP (*left*) (c)

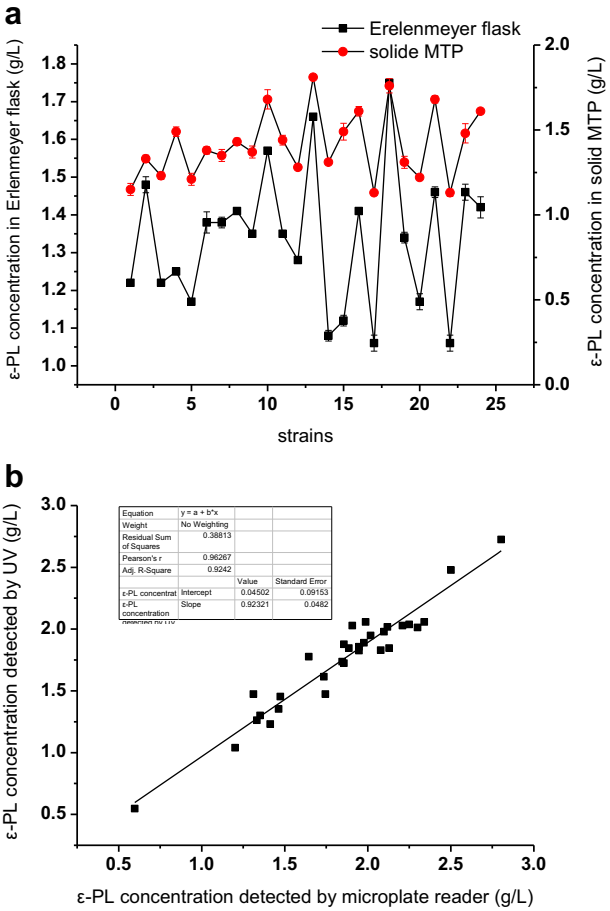

Fig. 3 Correlation between fermentation in Erlenmeyer flask and solid-state MTP cultures. A total of 24 strains were studied (**a**). The correlation between ε-PL production in 48-deep-well MTPs determined by microplate reader assay and that in Erlenmeyer flasks detected by UV spectrophotometry. A total of 31 randomly selected strains were studied (**b**)

analysis revealed that the correlation coefficient between UV spectrophotometry and microplate reader assay was 0.9242, suggesting that fermentation in Erlenmeyer flasks could be replicated in 24-(48)-deep-well MTPs and that MTP cultures could be used for predicting ε-PL production. Thus, Erlenmeyer flask culture can be replaced with microenvironment culture for the evaluation of ε-PL production.

In this study, mutants were cultured in 24-(48)-deep-well MTPs. Researchers have studied liquid MTP cultures and present some methods to overcome the main obstacles in liquid MTP cultures—low aeration rates and the effect of surface tension [14, 37, 38]. Results showed the feasibility of the MTP culture for screening high-ε-PL-producing strains as there was a good correlation between scales of the MTP and Erlenmeyer flask cultures. The ε-PL production by UV and microplate reader assay in both the Erlenmeyer flask cultures and MTP cultures had good correlation suggesting that the microplate reader was reliable.

Establishment of Microplate Reader Assay for ε-PL Detection

Although UV spectrophotometry is one of the methods used for the detection of ε-PL, it is time-consuming, laborious, and not suitable for high-throughput screening. Therefore, in the present study, we established the fast and efficient microplate reader assay to detect ε-PL. It is known that the maximum absorbance of ε-PL is at 465 nm, and a standard curve of the microplate reader must be prepared to reduce the reaction system. In the present study, a standard curve was generated using 500 μL of 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, and 0.16 g/L commercially available ε-PL. The experiments were performed at least five times because of the sensitivity of the microplate reader. The standard curve of ε-PL was obtained. The data fitted the experimental findings, and the linearity was up to 0.99871, which indicated the feasibility and validity of this method.

The reproducibility of MTP cultures and the use of the microplate reader assay for the measurement of ε-PL production were further studied to validate the assay. A total of 55 mutants were examined. In all the experiments, each colony was inoculated into two adjacent wells of the MTPs with controls. Subsequently, the MTPs were covered with lids and cultured at 30 °C and 200 rpm on a rotating shaker. After 4 days of incubation, no cross-contamination was detected. The productivity of the colonies formed in the MTPs was studied using the established measurement methods. For majority of the strains tested, the mean standard error in duplicates was 19.38%; however, six strains exhibited a mean standard error in duplicates of >35%. The results were reproducible, indicating that the microplate reader assay is a reliable method for the measurement of ε-PL production.

Correlations Between Fermentation in Erlenmeyer Flasks Tested by UV Spectrophotometry and that in MTPs Determined by Microplate Reader Assay

Following the confirmation of the feasibility of the MTP assay using established measurement methods, it was necessary to determine whether the microplate reader assay results represented those of the same strains in Erlenmeyer flasks. Therefore, the correlations between fermentations in Erlenmeyer flasks tested by UV spectrophotometry and those in 24-deep-well MTP cultures determined by the microplate reader assay were investigated.

A total of 50 randomly selected mutant strains derived from parent strain *Streptomyces* sp. M-Z18 were cultured in Erlenmeyer flasks and 24-deep-well MTPs. As shown in Fig. 4, although the detection rates of the microplate reader assay were slightly higher than those of the UV spectrophotometry, both the assays presented the same trend. The results obtained using UV spectrophotometry were in close agreement with those obtained by the microplate reader assay, and the correlation coefficient between both the assays was 0.86755. Thus, high-throughput screening using the microplate reader assay could be an effective method for the determination of ε-PL production.

As mentioned above, two major issues were addressed: fermentation reproducibility between the MTP system and conventional flasks and detection of reproducibility between analytical assays. The assay is based on the UV absorbance of ε-PL at 245 nm, and it proved that the strain screened is trustworthy.

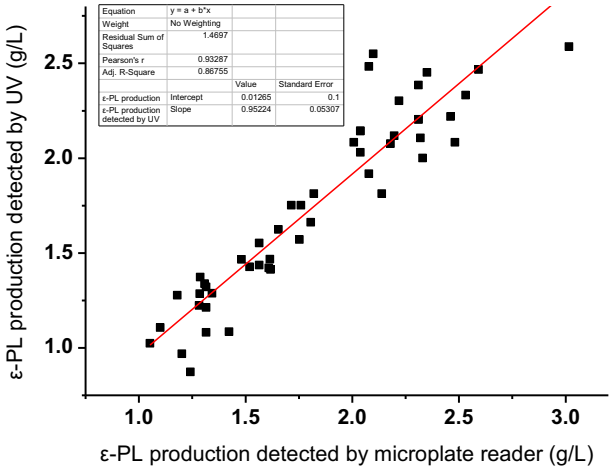

Fig. 4 Correlation between ε-PL concentrations in 24-deep-well MTPs determined by microplate reader assay and those in Erlenmeyer flasks determined by UV spectrophotometry. A total of 50 randomly selected strains were examined

Efficient Screening of ε-PL-Overproducing Strain Based on High-Throughput Assay Using Ribosome Engineering Technology

The ribosome engineering technique is a powerful method to activate the potential ability of bacteria to produce various secondary metabolites involved in the effects of antibiotics [5, 39, 40]. It has been reported that antibiotic-resistant bacteria show not only improvement in enzymatic activity but also organic chemical tolerance [41, 42]. In the present study, a high-throughput assay using ribosome engineering technology was applied for the overproduction of ε-PL. A screening strategy was developed (Fig. 5), combining micro-cultivation in 24-(48)-deep-well MTPs and high-throughput assay using the microplate reader. The single spore suspension was spread on a screening plate containing paromomycin and incubated at 30 °C in a thermostatic incubator for 7–10 days. Based on the growth rates, colonies were randomly selected and transferred to 24-deep-well MTPs containing 2 mL of fermentation medium using sterile toothpicks for preliminary screening by the microplate reader. Simultaneously, the colonies were also inoculated into 96-deep-well MTPs containing 1 mL of BTN medium and stored as a backup at 4 °C. Only strains in the top 10% of the medium with low OD_{465} values were further evaluated. Actually, there are two stages in the process for screening overproducing strains: the first stage is primary screening performed in 24-deep-well MTPs; the higher-producing strains were chosen and entered into the second screening performed in Erlenmeyer flasks. Finally, the highest-producing strain in the second screening was selected.

In one screening cycle, 818 viable single colonies were found on the plates. Based on their sporulation ability, 588 isolates were selected for subsequent culture in MTPs. Figure 6 shows the screening results. The red dot represents the starting strains' ε-PL production. Out of 588 viable single colonies in 24-deep-well MTPs, 57 mutants were selected through preliminary screening, and their corresponding strains preserved at 4 °C in 96-well MTPs were inoculated onto Erlenmeyer flasks containing fresh fermentation medium for the second screening. The production of ε-PL in Erlenmeyer flasks was measured by UV spectrophotometry, and the results are shown in Fig. 7. It can be observed that the ε-PL production of the 57 mutants was comparable with that of the parent strain, and 29 mutants exhibited at least 11% higher ε-PL production than the parent strain M-Z18. Among these mutants, the ε-PL production of the

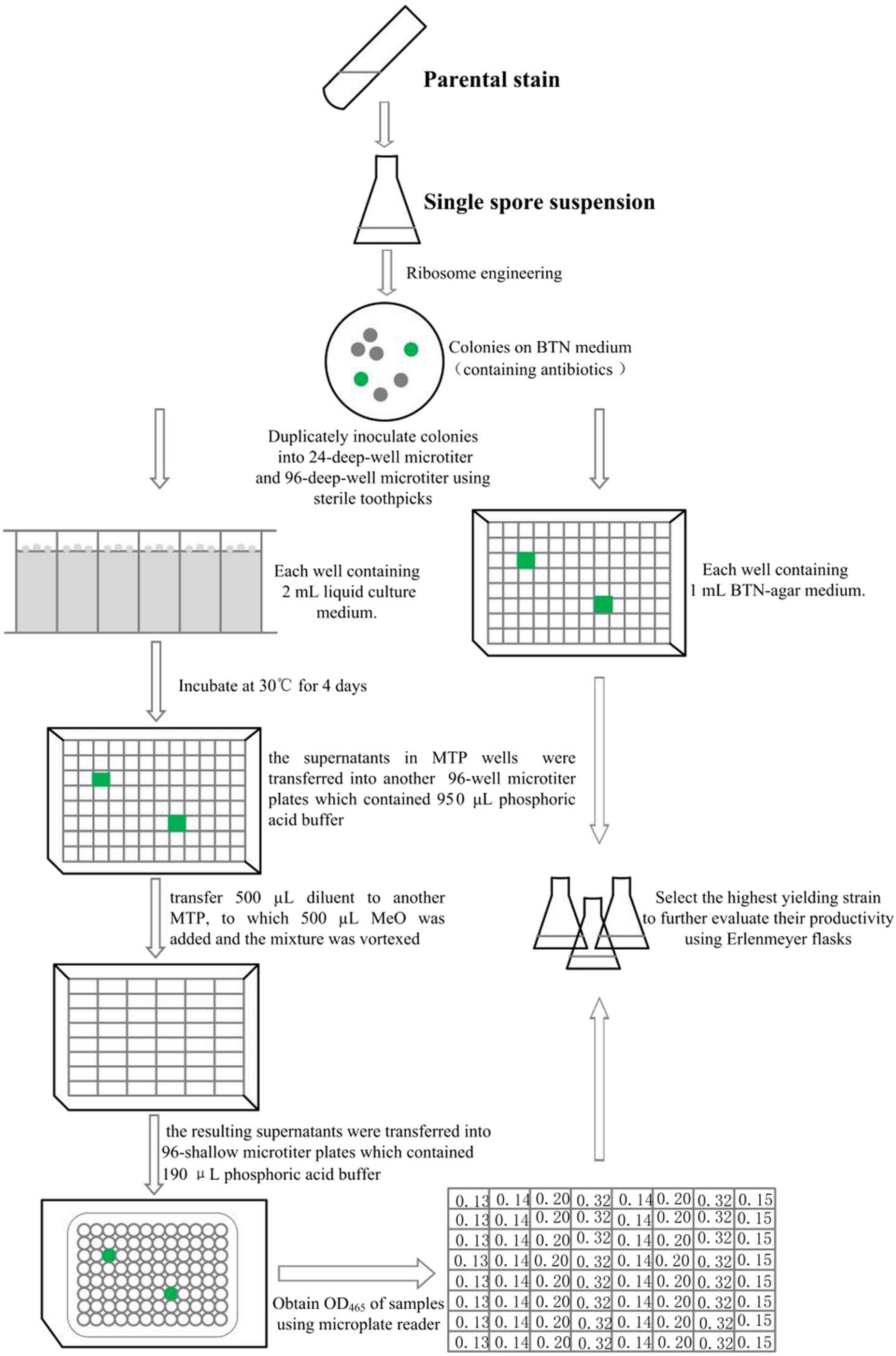

Fig. 5 An integrated procedure with micro-cultivation in 24-(48)-deep-well MTPs and high-throughput assay using a microplate reader

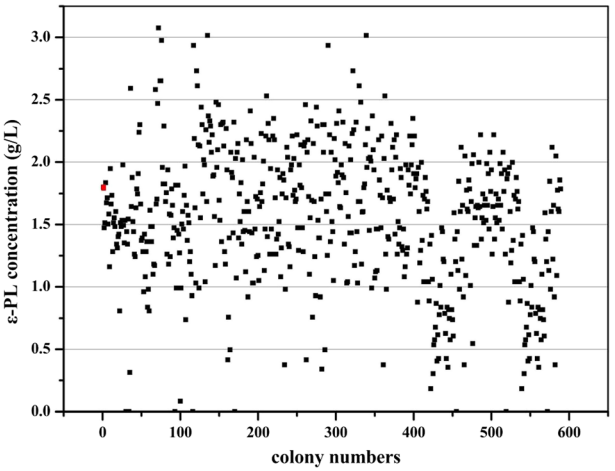

Fig. 6 High-throughput screening of ε-PL-overproducing mutants

high-yielding mutant was 2.59 g/L, which was nearly 45% higher than that of the parent stain. Among the mutants isolated, only a few were low-yielding ones, suggesting that the developed high-throughput screening method is more efficient, when compared with the traditional Erlenmeyer-flask screening.

The screening procedure is usually divided into two processes, preliminary screening and secondary screening. A reasonable screening strategy could play a multiplier effect for the random mutagenesis [22]. This study established a new preliminary screening process by using 24-(48)-deep-well MTPs. It disposes of the key problem of low probability because it allows screening of a massive number of samples within a short time. To the best of our knowledge, this study is the first to develop a high-throughput screening strategy using ribosome engineering technology for ε-PL production.

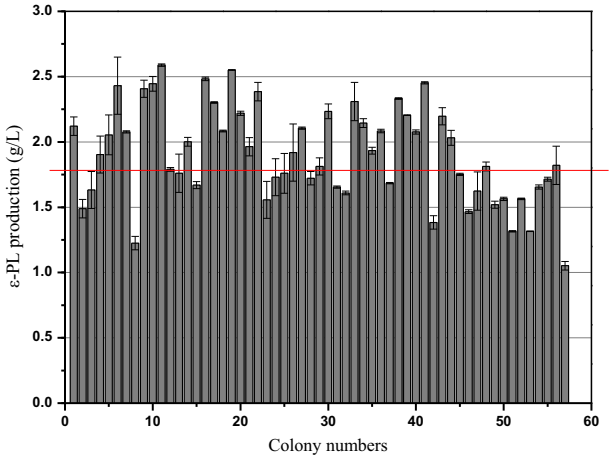

Fig. 7 Shake-flask cultivation of ε-PL-overproducing strains screened in 24-deep-well MTPs (The *red line* represents the average yield of the starting strain)

Conclusions

Although strain improvement for ε-PL production has been extensively studied and reviewed in recent years, the absence of high-throughput screening for overproducers has limited the application of new tools such as ribosome engineering technology. In this study, a novel and integrated high-throughput screening assay using ribosome engineering technology was established for screening the entire mutant library of *Streptomyces* sp. M-Z18 after mutagenesis. The mutants were cultured in 24-(48)-deep-well MTPs, and the results suggested that the antibiotic productivity of industrial *Streptomyces* was enhanced by inducing combined drug resistance-producing mutations. Currently, we are investigating the possibility of further development of high-yielding strains in a similar way to obtain better industrial strains.

Acknowledgments This work was supported by the Cooperation Project of Jiangsu Province among Industries, Universities and Institutes (BY2016022-25), the Program of the National Natural Science Foundation of China (31671846,31301556), and the Jiangsu Province Collaborative Innovation Center for Advanced Industrial Fermentation Industry Development Program.

References

1. Shima, S., & Sakai, H. (1977). Polylysine produced by *Streptomyces*. *Agricultural and Biological Chemistry, 41*, 1807–1809.
2. Shima, S., & Sakai, H. (1981). Poly-L-lysine produced by *Streptomyces*. Part II. Taxonomy and fermentation studies. *Agricultural and Biological Chemistry, 45*, 2497–2502.
3. Shima, S., & Sakai, H. (1981). Poly-L-lysine produced by *Streptomyces*. Part III. Chemical studies. *Agricultural and Biological Chemistry, 45*, 2503–2508.
4. Hiraki, J., Ichikawa, T., Ninomiya, S.-I., Seki, H., Uohama, K., Seki, H., Kimura, S., Yanagimoto, Y., & Barnett, J. W. (2003). Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. *Regulatory Toxicology and Pharmacology, 37*, 328–340.
5. Hosoya, Y., Okamoto, S., Muramatsu, H., & Ochi, K. (1998). Acquisition of certain streptomycin-resistant (str) mutations enhances antibiotic production in bacteria. *Antimicrobial Agents and Chemotherapy, 42*, 2041–2047.
6. Shih, L., Shen, M. H., & Van, Y. T. (2006). Microbial synthesis of poly (ε-lysine) and its various applications. *Bioresource Technology, 97*, 1148–1159.
7. Shima, S., MATSUOKA, H., IWAMOTO, T., & SAKAI, H. (1984). Antimicrobial action of ε-poly-L-lysine. *The Journal of Antibiotics, 37*, 1449–1455.
8. Bankar, S. B., & Singhal, R. S. (2013). Panorama of poly-ε-lysine. *RSC Advances, 3*, 8586–8603.
9. Ren, X. D., Chen, X. S., Tang, L., Sun, Q. X., Zeng, X., & Mao, Z. G. (2015). Efficient production of ε-poly-l-lysine from agro-industrial by-products by *Streptomyces* sp. M-Z18. *Annals of Microbiology, 65*, 733–743.
10. Li, S., Li, F., Chen, X. S., Wang, L., Xu, J., Tang, L., & Mao, Z. G. (2012). Genome shuffling enhanced ε-poly-l-lysine production by improving glucose tolerance of *Streptomyces graminearus*. *Applied Biochemistry and Biotechnology, 166*, 414–423.
11. Zong, H., Zhan, Y., Li, X., Peng, L., Feng, F., & Li, D. (2012). A new mutation breeding method for *Streptomyces albulus* by an atmospheric and room temperature plasma. *African Journal of Microbiology Research, 6*, 3154–3158.
12. Büchs, J. (2001). Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal, 7*, 91–98.
13. Du Toit, E., & Rautenbach, M. (2000). A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. *Journal of Microbiological Methods, 42*, 159–165.
14. Duetz, W. A., Rüedi, L., Hermann, R., O'Connor, K., Büchs, J., & Witholt, B. (2000). Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Applied and Environmental Microbiology, 66*, 2641–2646.
15. Kumar, M. S., Kumar, P. M., Sarnaik, H. M., & Sadhukhan, A. (2000). A rapid technique for screening of lovastatin-producing strains of *Aspergillus terreus* by agar plug and Neurospora crassa bioassay. *Journal of Microbiological Methods, 40*, 99–104.

16. Itzhaki, R. F. (1972). Colorimetric method for estimating polylysine and polyarginine. *Analytical Biochemistry, 50*, 569–574.
17. Wang, L., Chen, X., Wu, G., Zeng, X., Ren, X., Li, S., Tang, L., & Mao, Z. (2016). Genome shuffling and gentamicin-resistance to improve ε-poly-l-lysine productivity of Streptomyces albulus W-156. *Applied Biochemistry and Biotechnology, 180*, 1601–1617.
18. Xu, Z. N., Shen, W. H., Chen, X. Y., Lin, J. P., & Cen, P. L. (2005). A high-throughput method for screening of rapamycin-producing strains of *Streptomyces hygroscopicus* by cultivation in 96-well microtiter plates. *Biotechnology Letters, 27*, 1135–1140.
19. Gao, H., Liu, M., Zhou, X., Liu, J., Zhuo, Y., Gou, Z., Xu, B., Zhang, W., Liu, X., Luo, A., Zheng, C., Chen, X., & Zhang, L. (2010). Identification of avermectin-high-producing strains by high-throughput screening methods. *Applied Microbiology and Biotechnology, 85*, 1219–1225.
20. Ringel, A. K., Wilkens, E., Hortig, D., Willke, T., & Vorlop, K. D. (2012). An improved screening method for microorganisms able to convert crude glycerol to 1,3-propanediol and to tolerate high product concentrations. *Applied Microbiology and Biotechnology, 93*, 1049–1056.
21. Zeng, W., Lin, Y., Qi, Z., He, Y., Wang, D., Chen, G., & Liang, Z. (2013). An integrated high-throughput strategy for rapid screening of poly(gamma-glutamic acid)-producing bacteria. *Applied Microbiology and Biotechnology, 97*, 2163–2172.
22. Tan, J., Chu, J., Hao, Y., Guo, Y., Zhuang, Y., & Zhang, S. (2013). High-throughput system for screening of cephalosporin C high-yield strain by 48-deep-well microtiter plates. *Applied Biochemistry and Biotechnology, 169*, 1683–1695.
23. Wood, J. A., Orr, V. C. A., Luque, L., Nagendra, V., Berruti, F., & Rehmann, L. (2014). High-throughput screening of inhibitory compounds on growth and ethanol production of *Saccharomyces cerevisiae*. *Bioenergy Research, 8*, 423–430.
24. Shi, F., Tan, J., Chu, J., Wang, Y., Zhuang, Y., & Zhang, S. (2015). A qualitative and quantitative high-throughput assay for screening of gluconate high-yield strains by *Aspergillus niger*. *Journal of Microbiological Methods, 109*, 134–139.
25. Zeng, W., Du, G., Chen, J., Li, J., & Zhou, J. (2015). A high-throughput screening procedure for enhancing α-ketoglutaric acid production in *Yarrowia lipolytica* by random mutagenesis. *Process Biochemistry, 50*, 1516–1522.
26. Nishikawa, M., & Ogawa, K. i. (2002). Distribution of microbes producing antimicrobial ε-poly-L-lysine polymers in soil microflora determined by a novel method. *Applied and Environmental Microbiology, 68*, 3575–3581.
27. Hiraki, J., Hatakeyama, M., Morita, H., & Izumi, Y. (1998). Improved epsilon-poly-L-lysine production of an S-(2-aminoethyl)-L-cysteine resistant mutant of *Streptomyces albulus*. *Seibutsu-kogaku Kaishi, 76*, 487–493.
28. Wang, L., Chen, X., Wu, G., Li, S., Zeng, X., Ren, X., Tang, L., & Mao, Z. (2017). Enhanced ε-poly-L-lysine production by inducing double antibiotic-resistant mutations in Streptomyces albulus. *Bioprocess and Biosystems Engineering, 40*, 271–283.
29. Wang, G., Inaoka, T., Okamoto, S., & Ochi, K. (2009). A novel insertion mutation in Streptomyces coelicolor ribosomal S12 protein results in paromomycin resistance and antibiotic overproduction. *Antimicrobial Agents and Chemotherapy, 53*, 1019–1026.
30. Li, S., Chen, X., Dong, C., Zhao, F., Tang, L., & Mao, Z. (2013). Combining genome shuffling and interspecific hybridization among *Streptomyces* improved epsilon-poly-L-lysine production. *Applied Biochemistry and Biotechnology, 169*, 338–350.
31. Minas, W., Bailey, J. E., & Duetz, W. (2000). Streptomycetes in micro-cultures: growth, production of secondary metabolites, and storage and retrieval in the 96-well format. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology, 78*, 297–305.
32. Hobbs, G., Frazer, C. M., Gardner, D. C. J., Flett, F., & Oliver, S. G. (1990). Pigmented antibiotic production by Streptomyces coelicolor A3(2) kinetics and the influence of nutrients. *Journal of General and Applied Microbiology, 136*, 2291–2296.
33. Liao, X., Vining, L. C., & Doull, J. L. (1995). Physiological control of trophophase-idiophase separation in streptomycete cultures producing secondary metabolites. *Canadian Journal of Microbiology, 41*, 309–315.
34. Melzoch, K., Teixeira de Mattos, M. J., & Neijssel, O. M. (1997). Production of actinorhodin by Streptomyces coelicolor A3(2) grown in chemostat culture. *Biotechnology and Bioengineering, 54*, 577–582.
35. Whitaker, A. (1992). Actinomycetes in submerged culture. *Applied Biochemistry and Biotechnology, 32*, 23–35.
36. Isett, K., George, H., Herber, W., & Amanullah, A. (2007). Twenty-four-well plate miniature bioreactor high-throughput system: assessment for microbial cultivations. *Biotechnology and Bioengineering, 98*, 1017–1028.
37. Hermann, R., Lehmann, M., & Buchs, J. (2003). Characterization of gas–liquid mass transfer phenomena in microtiter plates. *Bioprocess and Biosystems Engineering, 81*, 178–186.

38. Zimmermann, H. F., John, G. T., Trauthwein, H., Dingerdissen, U., & Huthmacher, K. (2003). Rapid evaluation of oxygen and water permeation through microplate sealing tapes. *Biotechnology Progress, 19*, 1061–1063.
39. Kurosawa, K., Hosaka, T., Tamehiro, N., Inaoka, T., & Ochi, K. (2006). Improvement of alpha-amylase production by modulation of ribosomal component protein S12 in *Bacillus subtilis* 168. *Applied and Environmental Microbiology, 72*, 71–77.
40. Shima, J., Hesketh, A., Okamoto, S., Kawamoto, S., & Ochi, K. (1996). Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3 (2). *Journal of Bacteriology, 178*, 7276–7284.
41. Hosokawa, K., Park, N. H., Inaoka, T., Itoh, Y., & Ochi, K. (2002). Streptomycin-resistant (rpsL) or rifampicin-resistant (rpoB) mutation in *Pseudomonas putida* KH146-2 confers enhanced tolerance to organic chemicals. *Environmental Microbiology, 4*, 703–712.
42. Liu, Z., Zhao, X., & Bai, F. (2013). Production of xylanase by an alkaline-tolerant marine-derived *Streptomyces viridochromogenes* strain and improvement by ribosome engineering. *Applied Microbiology and Biotechnology, 97*, 4361–4368.