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BIOTECHNOLOGY METHODS

A high-throughput screening strategy for accurate quantification of menaquinone based on fluorescence-activated cell sorting

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Abstract To enhance the screening efficiency and accuracy of a high-yield menaquinone (vitamin K_2, MK) bacterial strain, a novel, quantitative method by fluorescence-activated cell sorting (FACS) was developed. The staining technique was optimized to maximize the differences in fluorescence signals between spontaneous and MK-accumulating cells. The fluorescence carrier rhodamine 123 (Rh123), with its ability to reflect membrane potential, proved to be an appropriate fluorescent dye to connect the MK content with fluorescence signal quantitatively. To promote adequate access of the fluorescent molecule to the target and maintain higher cell survival rates, staining and incubation conditions were optimized. The results showed that 10 % sucrose facilitated uptake of Rh123, while maintaining a certain level of cell viability. The pre-treatment of cells with $MgCl_2$ before staining with Rh123 also improved cell viability. Using FACS, 50 thousands cells can easily be assayed in less than 1 h. The optimized staining protocol yielded a linear response for the mean fluorescence against high performance liquid chromatography-measured MK content. We have developed a novel and useful staining protocol in the high-throughput evaluation of *Flavobacterium* sp. mutant libraries, using FACS to identify mutants with increased MK-accumulating properties. This study also provides reference for the screening of other industrial microbial strains.

Keywords High-throughput · Menaquinone · Quantification · Fluorescence-activated cell sorting

Introduction

A high-yield strain is the lifeblood in biopharmaceutical industries because its performance directly determines profit levels. Consequently, the improvement of strains has become an important issue for researchers, with random mutagenesis and rational modification mainly applied. Random mutagenesis consists of physical mutagens (e.g. N^+ ion beam implantation, He–Ne irradiation, UV irradiation) and chemical mutagens (e.g. nitrosoguanidine, $LiCl_3$). Generally, the yield of mutants screened by random mutagenesis is very stable, but limited by its lower screening efficiency from larger mutant libraries. Rational modification consists of gene knockout and overexpression based on transposons, plasmid overexpression, gene shuffling, and other methods of mutagenic manipulation. Both random mutagenesis and rational modification have been widely used for the reconstruction of microbial strains. However, the millions of isolates created after mutagenic treatment should be measured. Therefore, establishing a simple and accurate high-throughput screening technique for obtaining positive strains from mutant libraries is quite important.

So far, several rapid screening techniques have been reported such as microplate bioassays with 96-, 384-, 1536-, and even 3456-well plate formats [4, 5, 29, 33]. These screening methods can be easily established if positive strains have phenotype diversity or a bacteriostatic effect [2, 29]. However, they cannot be widely used if positive strains have no phenotypic diversity or bacteriostatic effect. Other

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screening techniques commonly used are time-consuming and laborious because they require the preparation of plates, the spreading of diluted bacteria suspensions, the cultivation of every single colony, and the target must be manually measured. Thus, it is particularly urgent to find other screening techniques. Fluorescence-activated cell sorting (FACS) is a specialized application of flow cytometry [11, 27] that has been used for the high-throughput sorting of a number of biomolecule interactions (e.g. protein–protein, protein–cells) and biomolecule formations (e.g. lipid, inclusion body), as well as for high-throughput screening of engineered proteins and host cells [20]. In a FACS instrument (Fig. 1), multiple fluorescent parameters (size, structure, and fluorescent signals) of individual sample particles (cells, microbeads, or emulsions) can be analyzed by a focused laser beam at rates of approximately 10^7 cells/h [34], for this reason, the efficiency of FACS is rather high. Furthermore, quantification of some intracellular biomolecules requires breaking the cells and separating the crude extractions, a procedure in which the cells would die. As a result, FACS, with its unique capability of in situ measurements, has been brought into focus. So far, the application of FACS in high-throughput screening of menaquinone (MK)-accumulating microorganisms has not been reported.

MK, an indispensable fat-soluble vitamin in human metabolism, has vital roles in blood coagulation, bone metabolism [3, 13], mitochondrial repairing and amyotrophic lateral sclerosis treatment [1, 21, 32]. These physiological functions have been discovered in the past 3 years and published in the top journals, indicating that MK has a bright application prospect and potential economic value. However, the low production capacity of strains has been one of the main obstacles to commercialization on a large scale. Many rational approaches such as mutation and genetic modifications have been implemented to enhance the performance of the host microorganism [12, 35–37]. Additionally, structural analogs of intermediates of MK biosynthesis such as vitamin K_3 [23], 1-hydroxy-2-naphthoate (HNA) [36], were used as primary screening methods, but these were still based on a time- and labor-intensive screening of colonies.

Rhodamine 123 (Rh123), a cationic lipophilic fluorescent dye, had been used to estimate the electrical potential across the inner mitochondrial membrane because it can be accumulated in the mitochondrial matrix for its charge and solubility in both the inner mitochondrial membrane and matrix space [24]. On the other hand, the mitochondrial transmembrane potential was affected by menaquinone [8, 25]. It had been reported that mitochondrial transmembrane potential reduced from −48 to −200 mV after inactivation of the menaquinone by exposure of the membranes to UV irradiation [8]. Therefore, the fluorescence signal of Rh123 maybe reflect MK content.

This work aims to examine the feasibility of using FACS as a high-throughput straightforward screening method for menaquinone-accumulating strains that were mutated by ion beam implantation. Rh123 was employed to selectively label target molecules with a high sensitivity. Subsequently, the labeling system (i.e., preparation method of competent cells, sucrose content, buffer type, and dye content) was optimized to further improve fluorescence signal. Furthermore, the optimized method was verified for its accuracy and precision using high performance liquid chromatography (HPLC). This research attempts to construct an efficient, quantitative, and high-throughput screen of MK production in industrial strains.

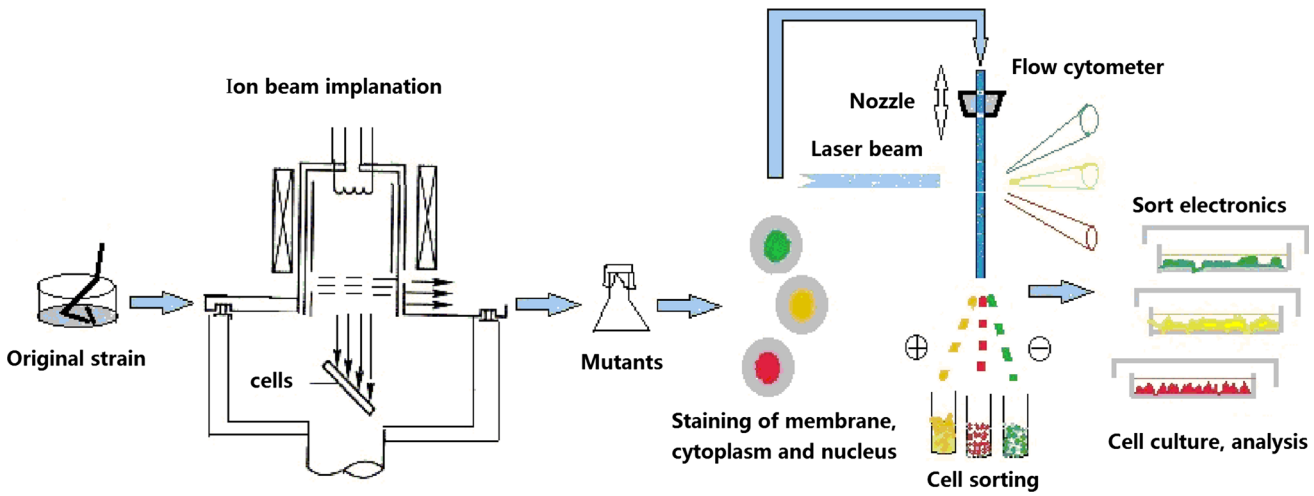

Fig. 1 Flow chart of screening cells with FACS. *Red drops* substance contained in cells with an emission wavelength above 650 nm, *Orange drops* substance contained in cells with an emission wavelength between 564 and 606 nm, *Green drops* substance contained in cells with an emission wavelength between 515 and 545 nm

Materials and methods

Bacterial strains and growth medium

The MK-producing strain *Flavobacterium* sp. FM2-6 was transferred from *Flavobacterium* sp. CICC 10970,which was purchased from China Center of Industrial Culture Collection (CICC). The mutants (130 strains) used for verifying the correlation between fluorescent signal intensity and MK content and mutants (30 strains) used for verifying whether FACS can be applicable in screening high-yield MK strains, were both obtained from mutant library. All strains were cultured at 37 °C in glycerol–peptone medium contained (g/L): glycerol 10, peptone 10, yeast extract 1.5, K_2HPO_4 4.5, NaCl 3, $MgSO_4{\cdot}7H_2O$ 0.3. The initial pH value was adjusted to 7.0.

Mutant library establishment

A *Flavobacterium* sp. mutant library was established by ion beam implantation. The strain *Flavobacterium* sp. FM2-6 was inoculated into fresh seed medium. When the seed culture reached the end of the exponential growth phase, 100 μL of appropriately diluted broth was spread on sterilized plates. A thin layer of cells was spread evenly on each plate. The plates were air-dried in an axenic workbench and then irradiated using the ion implantation facility at ASIPP (Chinese Academy of Sciences, Institute of Plasma Physics). The dose of nitrogen ions and energy were 2.6×10^{14} ions/cm^2 and 10 keV, respectively. Two dishes were placed sequentially in the target chamber. The upper one was exposed to ion beam bombardment, while the lower one was an unexposed control. Every sample was kept inside the target chamber for about 1.5–2 min to complete the treatment process, including vacuum pump-down, nitrogen ion beam implantation and normal pressure recovery. After ion bombardment, each plate was washed with 1 mL of sterile distilled water, and samples (50 or 100 μL) were placed onto the analog-resistant screening plate [18]. The plates were incubated at 37 °C for 96 h. The colonies that survived were selected and analyzed.

Screening process

The mutants were transferred from plate to test-tube containing 10 mL fermentation medium and cultured for 120 h. Cells were stained by fluorescent dye and sorted by flow cytometer. The flow chart of screening cells with FACS was as illustrated in Fig. 1. The strains were rendered differentially fluorescent and incorporated into a small liquid stream illuminated by a laser beam. The cells passed sequentially through the beam and fluorescent light from the cells gave rise to electrical signals. The stream was broken into a series of uniform size drops downstream of the laser. Cell signals were used to give appropriate electrostatic charges to drops containing cells. The drops then passed between two charged plates and were deflected to appropriate containers.

Staining and FACS

A Rh123 (Eugene, OR, USA) stock solution was made by dissolving the dye to 1 mg/mL in dimethyl sulfoxide (DMSO) unless otherwise stated. The mutants from a mutant library were incubated at 37 °C on a rotary shaker incubator at 200 rpm for 96 h. After cultivation, the cultures were cooled on ice for 10 min, and the cells were harvested by centrifugation (10 min, 8000*g*, 4 °C). Competent cells were prepared and bacterial suspension was centrifuged (10 min, 8000*g*, 4 °C). The cells were resuspended in 1 mL of ice-cold buffer solution [distilled deionized water (DDW), 0.9 % NaCl or 1 mM $MgCl_2$] and Rh123 was added. After being mixed vigorously by vortex, both mixtures were incubated in the dark for different lengths of time at room temperature. Finally, the cells were centrifuged (10 min, 8000*g*, 4 °C) and washed twice with ice-cold DDW. The sample was stored in the dark for different lengths of time at 25 °C and analyzed by flow cytometry.

FACS was carried out on a FACS can flow cytometer (Becton-Dickinson, Mountain View, USA) using the following settings: forward scatter = E00, side scatter = 420, FL-1 = 546 nm. Cells were excited with an air-cooled argon ion laser (485 nm), and FL-1 was used to detect Rh123 fluorescence.

Competent cell preparation

Cells were grown to stationary phase and centrifuged. The effect of creating competent cells by isopropanol shock, DMSO shock, polyoxyethylene oleyl ether (POE) treatment, or heat shock methods on mean fluorescence were compared, respectively. Centrifuged cells were resuspended in either: 1 mL ice-cold TSE buffer (10 mM Tris–HCl [pH 8.0], 10 % sucrose, 0.5 mM EDTA); 1 mL 70 % (wt/vol) isopropanol; 1 mL 1 % (wt/vol) DMSO; or 1 mL 1 % (wt/vol) POE. Then the mixture was incubated on ice for 10 min.

Heat shock was performed as previously described for preparation of competent cells [22]. After centrifugation, cells were resuspended in 1 mL of ice-cold 1 mM $MgCl_2$ buffer solution and incubated on ice for 10 min. The sample was centrifuged (10 min, 8000*g*, 4 °C) and resuspended in

1 mL 1 mM $MgCl_2$ with 5 μL stock Rh123 solution. After vigorous mixing by vortex, mixtures were heat shocked at 42 °C for 90 s, incubated for 10 min in the dark, and analyzed on the FACS can flow cytometer.

Cell viability analysis

During flow cytometric analysis of the stained cells, the cells with higher fluorescence were sorted by "single cell" mode. Each cell was collected in a 96 deep-well plate (Bioneer, Daejeon, Republic of Korea) containing 1 mL glycerol-peptone medium. After 48 cells (half plate for each sample) were sorted, the plates were incubated at 37 °C with vigorous shaking (200 rpm). After 48 h incubation, the number of wells with viable cells was counted.

Menaquinone analysis by HPLC

Cells and culture fluid were separated by centrifuging the culture broth at 8000*g* for 5 min. The cells collected were freeze-dried and dissolved with 3 mL methanol by shaking at 55–60 °C for 10 min. The amounts of MK in the upper organic phase were assayed by HPLC (Waters 600, Waters, USA) equipped with a Phenomenex C18 column with 250 × 4.60 mm, 4 μm at 35 °C. Methanol-dichloromethane (8:2, v/v) was used as mobile phase with a flow rate of 1.0 mL/min. A wavelength of 248 nm was selected for calibration and analysis [28]. Commercially available MK (Sigma-Aldrich, St. Louis, MO, USA), processed in parallel with the samples, was used as a standard.

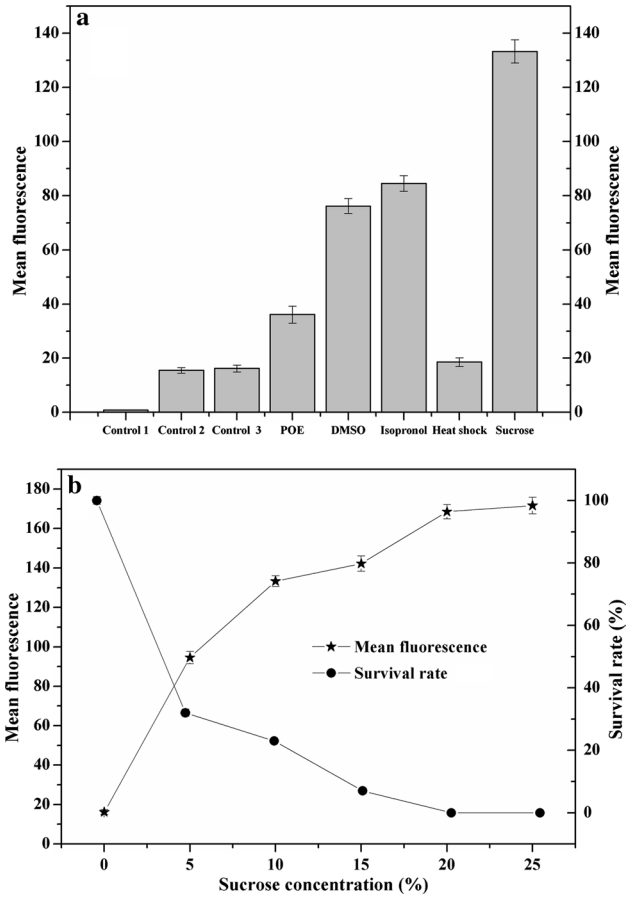

Fig. 2 Effect of different preparation methods of competent cells (**a**) and different concentrations of sucrose (**b**) on mean fluorescence. *Control 1* dye without cells, *control 2* cells not stained by Rh123, *control 3* non-competent cells stained by Rh123. Competent cells were resuspended in 1 mL of ice-cold 1 mM $MgCl_2$ solution

Results

Optimization of competent-cell preparation conditions

Previous studies had shown that the internalization of dyes passing into host cells may require a carrier such as acetone [7], glycerol [9], DMSO [6, 9], ethanol [14] or sucrose [31] to make the cells competent. To improve the transportation of fluorescent dye across the *Flavobacterium* sp. cell membrane, competent-cell preparing protocols and other permeabilization methods were attempted. Figure 2a shows the mean fluorescence, which was achieved using different competence-inducing factors such as POE, DMSO, isopropanol, heat, sucrose shock on stationary-phase *Flavobacterium* sp. FM2-6. As can be seen from the histogram, the fluorescence value of Rh123 can hardly be detected (0.82 ± 0.08, control 1). The fluorescence value of non-competent cells (16.18 ± 1.23, control 3), which were stained by Rh123 but without pretreatment in stationary-phase to acquire competence, was exactly equal to spontaneous fluorescence value of untreated cells without staining (15.46 ± 1.08, control 2). The fluorescence value increased obviously in cells prepared for competence. Of the competence-inducing methods, sucrose shock permeabilization gave the highest mean fluorescence 133.29 ± 4.23, albeit with the same incubation and detecting conditions as other preparation methods. These results suggest that sucrose treatment might help fluorescent dyes permeate the cell membrane and allow cells to be stained more effectively. Therefore, sucrose shock was selected for the proceeding experiments.

Although sucrose shock was found to be more beneficial to the dye accessing the target, it was harmful to the viability of strains [31]. To balance higher viability of cells with better staining effect, the sucrose concentration was studied. Figure 2b showed that sucrose concentrations

ranging from 0 % to 40 % resulted in a significant decrease ($p < 0.05$) in the survival rate of cells and an increase in fluorescent intensities. Overall, a 10 % (wt/vol) sucrose concentration was found to maintain a suitable viability of 23 %, meanwhile mean fluorescence (133.29 ± 4.23) was similar to those obtained by a higher sucrose concentration. Thus, in the present study, the concentration of 10 % (wt/vol) was selected for further investigation.

Effect of different buffer

To realize high throughput FACS in *Flavobacterium* sp., important factors were as follows: (1) promoting specific fluorescent molecule binding to the target and (2) maintaining a high cell survival rate after fluorescence staining. Consequently, staining buffer was optimized to increase specific staining while minimizing nonspecific staining and to enhance the survival rate.

DDW, 0.9 % NaCl and 1 mM $MgCl_2$ were examined as staining buffers to test the effect of solution ionic strength on Rh123 staining and fluorescence. DDW and $MgCl_2$ showed a relative higher fluorescence in cell staining than the NaCl solution (Fig. 3a). However, the survival rate reached the highest when using $MgCl_2$ solution.

Effect of dye concentration

The concentration of dye had a significant effect on the detection results. It has been reported that higher dye concentration led to a certain extent of fluorescence self-quenching [15]. However, the target could not be fully stained when the dye concentration was low. Therefore, Rh123 concentration was varied to optimize fluorescence signal.

The increase in fluorescent intensity was observed when Rh123 concentration ranged from 0.5 to 15 μg/mL. Fluorescent intensity was kept stable when Rh123 concentration was above 5 μg/mL. In addition, cell viability was not affected when the dye concentration was changed from 0.5 to 15 μg/mL (Fig. 3b). To determine the optimum incubation time required for cell staining, *Flavobacterium* sp. cells were stained with Rh123 for five different times (0, 5, 10, 15 or 20 min). The results showed that the mean fluorescence increased abruptly when the time

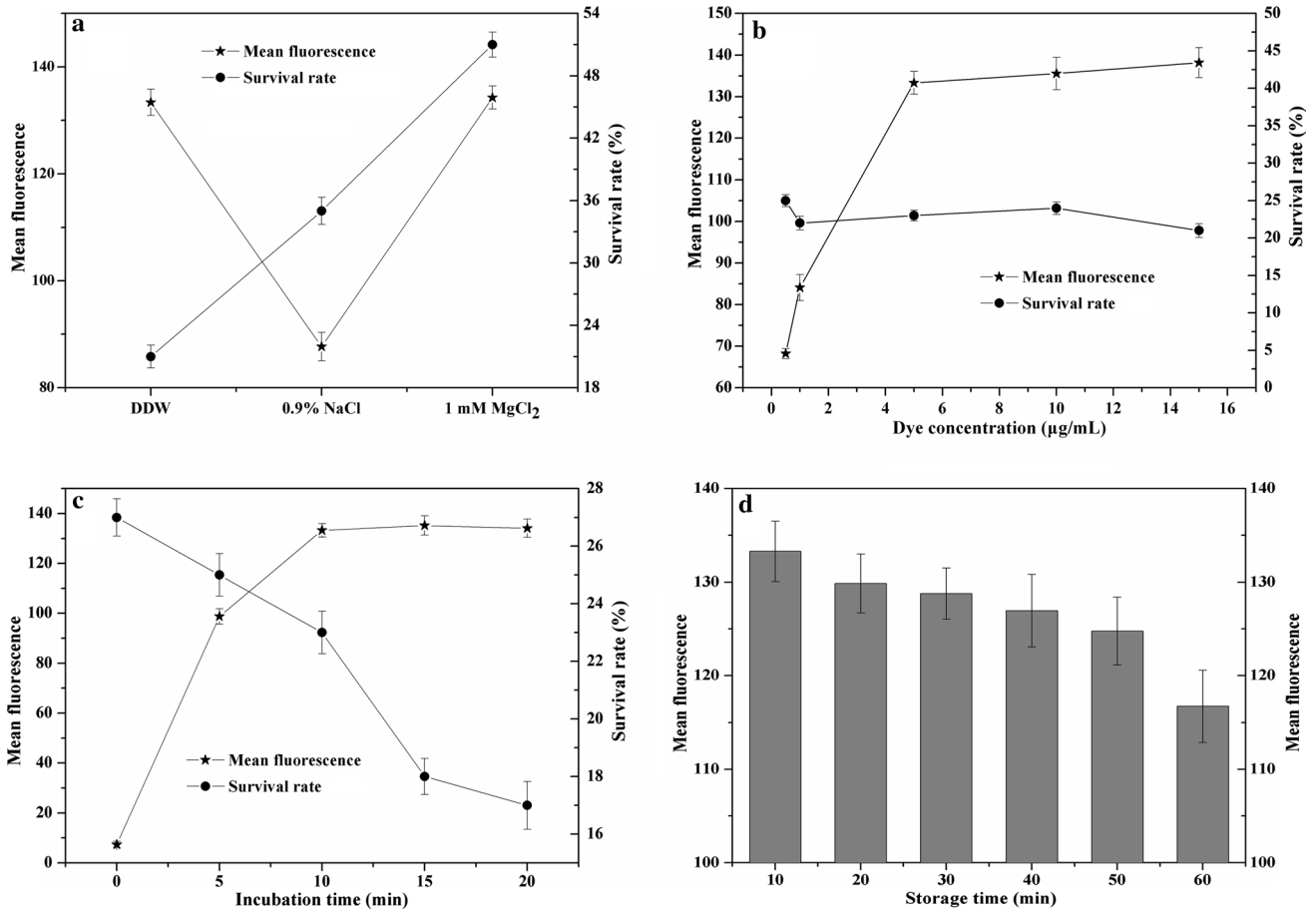

Fig. 3 Effect of different buffers (**a**), Rh123 concentrations (**b**), incubation times (**c**), storage time (**d**) on mean fluorescence and survival rate

ranged from 0 to 10 min, and grew slowly after 10 min. Whereas, survival rate declined with the increase of time (Fig. 3c).

Effect of different storage time

A loss in cellular fluorescence or photobleaching is of concern when undertaking fluorescent studies, particularly when the screening is performed over a prolonged period of time. Therefore, the fluorescent intensities were measured by FACS after cells were stained for 10 min and left for various durations (up to 60 min). Cells stained by Rh123 showed a gradual loss in fluorescent intensity with prolonged time (Fig. 3d). On the whole, a relatively high intensity (87.6 % of initial intensity) was retained for 60 min. These results indicated that the method was still useful for long-term screening processes.

Correlation of fluorescence intensity with menaquinone content

Figure 4 indicated a good correlation between fluorescent signal intensity and MK content. To verify this correlation, an additional 130 mutants with variable amounts of MK were examined. Each cell was stained by Rh123 under optimum conditions, and the fluorescent intensities were analyzed by flow cytometer. MK content of each cell was analyzed by HPLC. As illustrated in Fig. 4, a high significance of the geometric mean tested by flow cytometer correlated ($R^2 = 0.9869$) well with the MK content measured by HPLC. It can be concluded that FACS analysis data for Rh123-stained cells are highly reliable. In other words, Rh123 staining in combination with FACS analysis can be a useful tool for MK analysis.

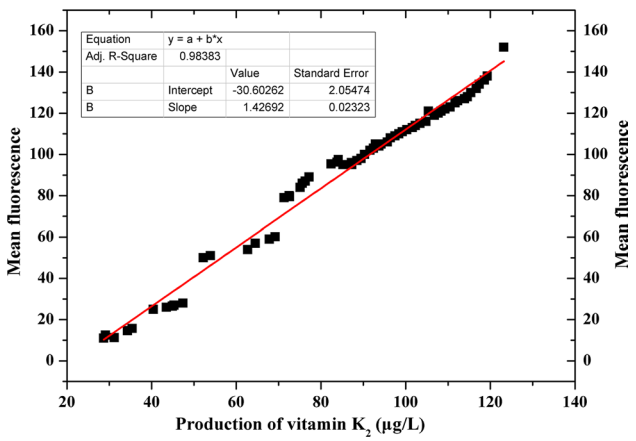

Fig. 4 Correlation of detection value by HPLC and mean fluorescence

Comparison of the pre-optimized and optimized protocol by flow cytometry (FCM)

The optimized protocol was as follows. Cool cell culture to 4 °C and harvest cells by centrifugation (10 min, 8000*g*, 4 °C). Cells were resuspended in ice-cold TSE buffer (10 mM Tris–HCl [pH 8.0], 10 % sucrose, 0.5 mM EDTA). The mixture was incubated on ice for 10 min and centrifuged (10 min, 8000*g*, 4 °C). The cells were resuspended in 1 mL of ice-cold 1 mM $MgCl_2$ solution and 5 μL of Rh123 solution was added to the cell suspension. After vigorous mixing by vortex, both mixtures were incubated in the dark for 10 min at room temperature. Finally, the cells were centrifuged (10 min, 8000*g*, 4 °C), washed twice by ice-cold DDW and analyzed by flow cytometer immediately.

To demonstrate the dramatic improvement of fluorescent intensity after staining in the optimal protocol, a higher-yield mutant FM2-6 was stained under three different conditions. Figure 5a was the negative control, which was not stained by Rh123 in the optimal protocol; Fig. 5b was the pre-optimized result, which was stained by Rh123 but without the step of sucrose shock; Fig. 5c was the optimized result, which was manipulated completely according to the optimized protocol. As indicated in Fig. 5a, a large portion of the population was able to form fluorescence spontaneously. These biological noises were most likely to contribute false positives in the screening procession. Figure 5a, b showed that there was a little shifting of the peak to higher fluorescence, which indicated that the autofluorescence and fluorescence formed from a MK-producing strain cannot be effectively distinguished. When comparison histograms between the fluorescence in the pre-optimized (Fig. 5b) and the optimized (Fig. 5c) protocols, the latter demonstrated a greater reduction of negative staining and a significant improvement in positive staining. Strains selected from peak 1 and peak 2 were analyzed by HPLC, respectively. The results show that the latter contained MK while the former did not, which further confirmed the feasibility of this high-throughput screening strategy.

Application of the high-throughput screening method

To verify that FACS can be applicable in screening high-yield MK strains, mutants treated by ion beam were cultured for 120 h and sorted by flow cytometry (FCM) using an optimized protocol. The whole process is illustrated in Fig. 1. As shown in Fig. 6a, three fluorescent intensity regions were observed, which ranged from 10^0 to 10^2, 10^2 to 10^3 and 10^3 to 10^4, respectively. There were 4, 10 and 16 mutants were sorted from region 1, 2 and 3, respectively. The sorted mutants were cultivated in fermentation medium for 120 h, MK content was measured by HPLC (Fig. 6b). As shown in Fig. 6b, MK content measured by HPLC had

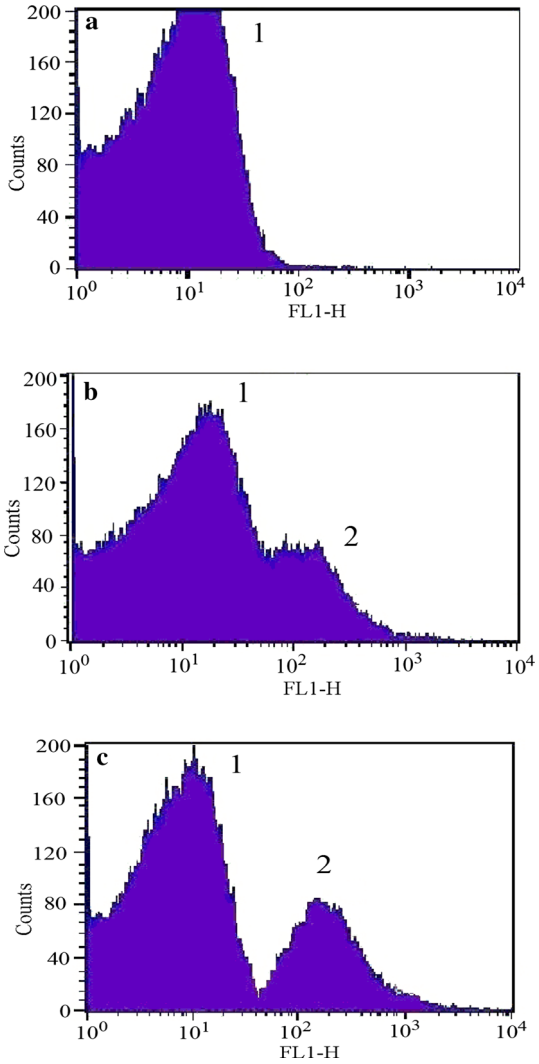

Fig. 5 FCM histogram obtained by negative control (**a**), pre-optimized (**b**) and optimized conditions (**c**). FACS was used by the following settings: forward scatter = E00, amp gain = 2.56, side scatter = 420, FL-1 = 546 nm

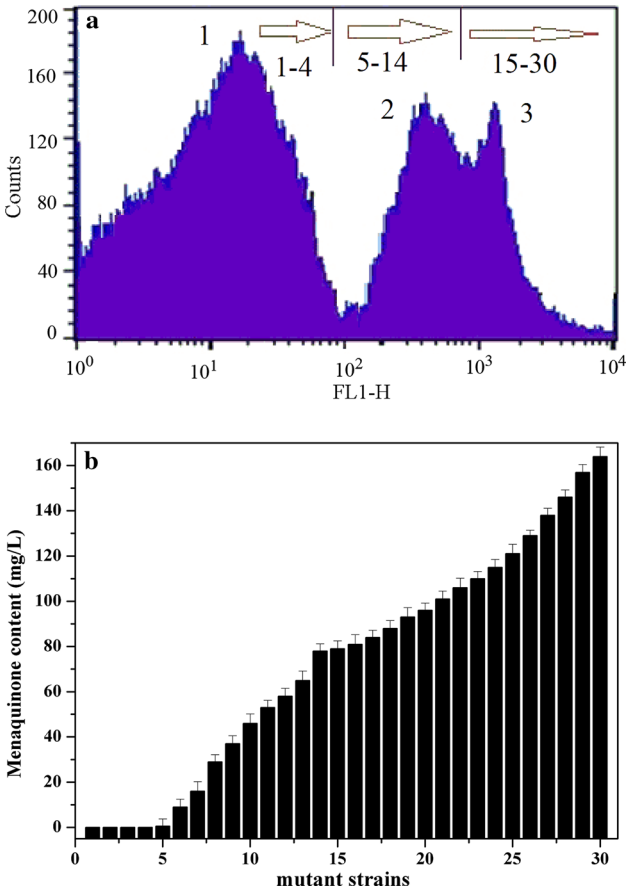

Fig. 6 High-throughput screening of positive strains. **a** FCM histogram of mutants treated by ion beam. Mutants were cultured for 120 h and sorted by FCM using optimized protocol. **b** MK contents of 30 mutants sorted by FCM and measured by HPLC. FACS was used by the following settings: forward scatter = E00, amp gain = 8.41, side scatter = 420, FL-1 = 546 nm

the same tendency as the fluorescent intensity measured by FCM. The data suggested that the level of MK granules in individual cells can be analyzed by flow cytometer which distinguish the different light-scattering properties caused by accumulation of MK granules in whole cells.

Discussion

Generally, screening methods depended on the phenotypic change, antibacterial activity of the strains, or characteristics of target metabolites. However, MK-containing strains had no obvious phenotypic change. Furthermore, it also has no antibacterial effect. Therefore, to realize high-throughput screening of high-yield MK strains there is a need to consider the nature of the target metabolites. In some prokaryotic cells, MK could transfer two electrons in a process of respiration which occurs in the cell membrane [16]. The reason maybe that it consists of a polar head group (quinone ring) and a hydrophobic side chain (polyisoprenoid). The former group has a crucial activity in a two-step reversible reduction reaction to form a quinol structure. There are three oxidized states of the quinone ring. The transformation between three forms of the quinone ring accompany the transfer of a proton and an electron (Fig. 7) [10, 17]. Such chemical features of MK make them contribute to the formation of transmembrane potential via electron accepting or donating reactions with other respiratory components. Therefore, there was a certain relationship between membrane potential and MK content. What was more important, an additional 130 mutants with variable amounts of MK were examined by flow cytometer and HPLC, respectively. There was a high significance of the geometric mean between flow cytometer

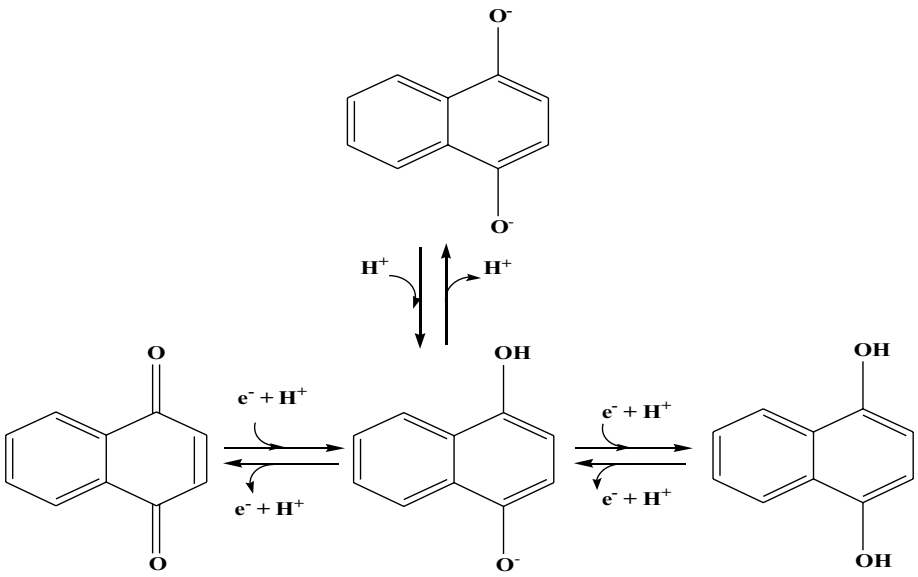

Fig. 7 Mechanism of membrane potential changes in quinone ring. *Upper* semiquinone radical ion, *left* oxidized form, *middle* semiquinone intermediate, *right* reduced form

and HPLC measurements (Fig. 4). Therefore, Rh123 as a dye that reflects the value of membrane potential, could be used to quantitatively connect the MK content with mean fluorescence.

To validate the use of Rh123 as a carrier that can bind fluorescence signal with MK content accurately, the geometric mean of the fluorescence distribution was compared to a chemical measurement value of MK in the culture. The geometric mean of the flow cytometer measurements correlated very well ($R^2 = 0.98$) with the analytical MK measurements over a wide dynamic range (from 28 to 125 mg/L) of MK content. From this, it can be inferred that the MK levels at the Rh123 fluorescence level can be estimated accurately based on the FACS. Furthermore, 30 mutants located in different fluorescent intensity regions were sorted. Their MK contents measured by HPLC had the same tendency as the fluorescent intensity measured by FCM. Accordingly, FACS could be used for high-throughput screening of high-yield MK strains using Rh123 staining.

Consistent access of the fluorescent molecule to the target was necessary for determining a quantitative fluorescence level. Gram-negative bacteria do not accumulate Rh123—nor other dyes such as hexamethylindodicarbocyanine [26] or 1-*N*-phenylnaphthylamine [30]—because these dyes did not rapidly cross the outer membrane. From Fig. 2a, it can be concluded that there was little fluorescent signal intensity with the dye itself (0.82 ± 0.08) and little change in the fluorescence intensity when cells were added to dyes (16.18 ± 1.23) or not (15.46 ± 1.08). Therefore, a large portion of the *Flavobacterium* sp. cells were not stained for membrane potential without cell permeabilization. To improve the dye transporting across the *Flavobacterium* sp. cell membrane, cell permeabilization methods had been developed to obtain competent-cells. In the previous study, we found that nonionic surfactant POE had a great leakage effect on the cell [19]. However, compared with sucrose shock, it was not the optimal. The result was consistent with the reports that demonstrated sucrose shock was the most effective method for staining *E. coli* cells with Nile red [31], the plausible reason was that extracellular hypotonic treatment formed by adding sucrose made the cells swell, then cell membrane permeability was enhanced.

The concentration of Rh123 used for cell staining varied considerably (i.e., 0.5–15 μg mL^{-1}) when the fluorescence was detected by FCM [24]. However, for quantitative analysis of MK using a spectrofluorometer, the background fluorescence associated with Rh123 concentration should be taken into consideration. In some cases, it was necessary to wash stained cells by centrifugation or filtration to remove residual Rh123 and thus the background fluorescence level was lowered. In this study, 5 μg mL^{-1} of Rh123 was found to be the optimal concentration at cell densities of 5×10^4 cells mL^{-1}.

Rh123 fluorescence was also influenced by the environment of staining such as buffer category, incubation time and storage time. When the incubation environment was changed from a NaCl solution to deionized water or $MgCl_2$, the Rh123 stained the membrane potential more specifically (Fig. 3a). Previously, Tyo et al. [31] reported that the pre-treatment of cells with $MgCl_2$ prior to staining with Nile red improved cell viability. However, the effect of Rh123 on the cell viability had not been reported. Therefore, buffer category was optimized in the study. Using 1 mM $MgCl_2$ solution improved the viability to 23 %, which provide an adequate efficiency for screening mutant libraries by FACS.

The application of FACS to screen high-yield MK strains had many advantages. First, this protocol allowed single-cell measurements of MK levels in *Flavobacterium* sp. to such a level of precision that mutants with incrementally increased MK accumulation could be sorted from the library and characterized. Second, the efficiency of FACS was more than 50 thousands cells per hour. While there would be a loss due to nonviable cells in the *Flavobacterium* sp. system, which did not prohibit the assay from screening genome scale libraries. Third, this method was an in situ measurement of the MK content, because quantification of intracellular biomolecule did not require breaking cells and separating the crude extractions. The cells were still alive through this procedure. As a result, the application of FACS was generalizable to other intracellular biomolecules whose high-throughput screens do not currently exist.

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References

1. Bhalerao S, Clandinin TR (2012) Vitamin K_2 takes charge. Science 336:1241–1242
2. Bochner BR, Gadzinski P, Panomitros E (2001) Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res 11:1246–1255
3. Boukaert HJ, Said AH (1960) Fracture healing by vitamin K. Nature 19:849
4. Bradley J, Gill J, Bertelli F, Letafat S, Corbau R, Hayter P, Harrison P, Tee A, Keighley W, Perros M, Ciaramella G, Sewing A, Williams C (2004) Development and automation of a 384-well cell fusion assay to identify inhibitors of CCR5/CD4-mediated HIV virus entry. J Biomol Screen 9:516–524
5. Brandish PE, Chiu CS, Schneeweis J, Brandon N, Leech CL, Kornienko O, Scolnick EM, Strulovici B, Zheng W (2006) A cell based ultra-high-throughput screening assay for identifying inhibitors of D-amino acid oxidase. J Biomol Screen 11:481–487
6. Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Meth 77:41–47
7. Cooksey KE, Guckert JB, Williams S, Callis PR (1987) Fluorometric determination of the neutral lipid content of microalgal cells using Nile red. J Microbiol Meth 6:333–345
8. Das A, Hugenholtz J, Halbeek HV, Ljungdahl LG (1989) Structure and function of a menaquinone involved in electron transport in membranes of *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. J Bacteriol 171:5823–5829
9. Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock. Biomass Bioenergy 35:2534–2544
10. Fiorini R, Ragni L, Ambrosi S, Littarru GP, Gratton E, Hazlett T (2008) Fluorescence studies of the interactions of ubiquinol-10 with liposomes. Photochem Photobiol 84(1):209–214
11. Givan AL (2011) Flow cytometry: an introduction. Methods Mol Biol 699:1–29
12. Hisataka T, Takashi S, Chiranun D, Tani Y (1989) Menaquinone-4 production by a sulfonamide-resistant mutant of *flavobacterium* sp. 238-7. Agric Biol Chem 53:3017–3023
13. Ishida Y (2008) Vitamin K_2. Clin Calcium 18:1476–1482
14. Kacmar J, Carlson R, Balogh SJ, Srienc F (2005) Staining and quantification of poly-3-hydroxybutyrate in *Saccharomyces cerevisiae* and *Cupriavidus necator* cell populations using automated flow cytometry. Cytometry 69:27–35
15. Kaprelyants AS, Kell DB (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J Appl Bacteriol 72:410–422
16. Kurosu M, Begari E (2010) Vitamin K_2 in electron transport system: are enzymes involved in vitamin K_2 biosynthesis promising drug targets? Molecules 15:1531–1553
17. Lenaz G, Fato R, Formiggini G, Genova ML (2007) The role of Coenzyme Q in mitochondrial electron transport. Agric Biol Chem 7:8–33
18. Liu Y, Wang L, Zheng ZM, Qiu HW, Wang P, Zhao GH, Gong GH, Song JY, Dai J (2015) Vitamin K_2 production using *Escherichia*. sp mutant obtained by ion beam implantation induction. Plasma Sci Technol 17:159–166
19. Liu Y, Zheng ZM, Qiu HW, Zhao GH, Wang P, Liu H, Wang L, Li ZM, Wu HF, Liu HX, Tan M (2014) Surfactant supplementation to enhance the production of Vitamin K_2 metabolites in shake flask cultures using *Escherichia* sp. mutant FM3-1709. Food Technol Biotech 52:269–275
20. Mattanovich D, Borth N (2006) Applications of cell sorting in biotechnology. Microb Cell Fact 5:12
21. Nakagawa K, Hirota Y, Sawada N, Yuge N, Watanabe M, Uchino Y, Okuda N, Shimomura Y, Suhara Y, Okano T (2010) Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme. Nature 468:117–122
22. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, NY
23. Sato T, Yamada Y, Ohtani Y, Mitsui N, Murasawa H, Araki S (2001) Efficient production of menaquinone (vitamin K_2) by a menadione-resistant mutant of *Bacillus subtilis*. J Ind Microbiol Biotechnol 26:115–120
24. Scaduto RC, Grotyohann LW (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 76:469–477
25. Shibayama-Imazu T, Sonoda I, Sakairi S, Aiuchi T, Ann WW, Nakajo S, Itabe H, Kazuyasu Nakaya (2006) Production of superoxide and dissipation of mitochondrial transmembrane potential by vitamin K_2 trigger apoptosis in human ovarian cancer TYK-nu cells. Apoptosis 11:1535–1543
26. Shapiro HM (1990) Flow cytometry in laboratory microbiology: new directions. Am Soc Microb News 56:584–588
27. Shapiro HM (2004) Practical flow cytometry, 4th edn. Wiley-Liss, NY
28. Tani Y, Asahi S, Yamada H (1984) Vitamin K_2 (menaquinone): screening of producing microorganism and production by *Flavobacterium meningosepticum*. J Ferment Technol 62:321–327
29. Tan J, Chu J, Hao YY, Wang YH, Yao SC, Zhuang YP, Zhang SL (2013) A high-throughput screening strategy for accurate quantification of erythromycin. J Taiwan Inst Chem 44:538–544
30. Tsuchido T, Aoki I, Takano M (1989) Interaction of the fluorescent dye-*N*-Phenylnaphthylamine with *Escherichia coli* cells during heat stress and recovery from heat stress. J Gen Microbiol 135:1941–1947
31. Tyo KE, Zhou H, Stephanopoulos GN (2006) High-Throughput screen for poly-3-hydroxybutyrate in *Escherichia coli*

and *Synechocystis* sp. strain PCC6803. Appl Environ Microb 72:3412–3417
32. Vos M, Esposito G, Edirisinghe JN, Vilain S, Haddad DM, Slabbaert JR, Meensel SV, Schaap O, Strooper BD, Meganathan R, Morais VA, Verstreken P (2012) Vitamin K_2 is a mitochondrial electron carrier that rescues pink1 deficiency. Science 336:1306–1310
33. Wunder F, Stasch JP, Hutter J, Alonso-Alija C, Hüser J, Lohrmann E (2005) A cell-based cGMP assay useful for ultra-high-throughput screening and identification of modulators of the nitric oxide/cGMP pathway. Anal Biochem 339:104–112
34. Yang G, Rich JR, Gilbert M, Wakarchuk WW, Feng Y, Withers SG (2010) Fluorescence activated cell sorting as a general ultra-high-throughput screening method for directed evolution of glycosyltransferases. J Am Chem Soc 132:10570–10577
35. Yoshiki T, Naoki S (1987) Menaquinone-4 production by a mutant of *flavobacterium* sp. 238-7. Agric Biol Chem 51:2409–2415
36. Youshiki T, Satoru A, Hideaki Y (1985) Production of menaquinone (vitamin K_2)-5 by a hydroxynaphthoate-resistant mutant derived from *flavobacterium meningosepticum*, a menaquinone-6 producer. Agric Biol Chem 49:111–115
37. Yoshiki T, Satoru A, Hideaki Y (1986) Menaquinone (vitamin K_2)-6 production by mutant of *flavobacterium meningosepticum*. J Nutr Sci Vitaminol 32:137–145