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Scale-down of *Cephalosporium acremonium* Cultivations for High Throughput Screening of High-yield Cephalosporin C Strains.

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ABSTRACT

A high throughput screening (HTS) strategy was investigated for screening high-yielding cephalosporin C (CPC) using mutated *Cephalosporium acremonium.* The strategy was based on microtiter plates (MTPs) to culture the microorganisms and measure its products. The feasibility of MTPs was to replace the cultivation in shake flasks, and CPC production will be determined using turbidimetric assay by ELISA reader to replace HPLC assay. The HTS systems were verified by screening high-producing CPC mutants. By using this HTS strategy, two mutants, i.e. 5-A1 and 8-A2 were selected from the secondary mutagenesis. The volumetric productivity of Cephalosporin C from *Cephalosporium acremonium* was 7914.43 U/ml; 8639.24 U/ml in shake flasks. CPC production was improved about 246% and 269% compared with that of original strain.

Keywords: *Cephalosporium acremonium,* cephalosporin C, high throughput screening, scale-down, strain improvement.

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INTRODUCTION

Cephalosporin C (CPC) produced by *Cephalosporium acremonium* is a naturally occurring β-lactam antibiotic [1]. It is currently produced industrially as a precursor for more potent Cephalosporins for parenteral use in treating human infection caused by penicillanase-producing bacteria. The production of cephalosporin C has increased dramatically by process optimization and strain improvement [2]. The current industrial high-producing strains were derived mainly from multiple rounds of mutagenesis, screening, and selection. Industrial strain improvement can be done by mutagenesis, enrichment techniques, and genetic engineering [3]. Mutagenesis is the oldest method and is still adapted because of its advantages over other methods. Several mutagens such as nitrosoguanidine (NTG), 4-nitroquinolone-1-oxide, methylmethane sulfonate (MMS), X-rays, ultraviolet rays (UV), ethylmethane sulfonate (EMS), and hydroxylamine (HA) have successfully been applied for this purpose [4].

High throughput screening (HTS) is a method for scientific experimentation especially used in drug discovery and relevant to the fields of biology and chemistry. HTS allows a researcher to quickly conduct millions of biochemical, genetic or pharmacological tests by using robotics, data processing and control software, liquid handling devices, and sensitive detectors [5]. One of the most important advantages for HTS is miniaturization, which reduces the cost of reagents and also the amount of chemical waste. In addition, automation of screening processes have significantly reduced system variability and increased screening throughput. In the recent years, some major types of HTS approaches have been developed for different systems [6, 7]. The first approach focuses on the measurements of specific absorbance peaks of either the target product or its derived compound [8, 9], the second approach focuses on the morphology change of mutant colonies [10], and the another approach involves a bioactivity assay, which is generally used in the selection of antibiotic-producing strains [11, 12].

Traditionally, most of the screening methods are performed in Erlemeneyer flasks [13]. However, there are some disadvantages of traditional flask screening method. The main drawbacks were low screening throughput and poor parallelity. These limit the wide application of strain improvement strategies. Microtiter plates (MTPs) recently have been considered as alternative shaken fermentation vessels, because these arrays of micro-scale reactors are easily handled by laboratory automation equipment [14]. The use of MTPs for screening hundreds of thousands of individual mutants for improved strains has many advantages, including higher throughput, smaller footprint, inexpensive vessel, and lower operating cost [3]. Therefore, it is necessary to establish a high throughput method for screening Cephalosporin C producing strains. However, there are some challenges when MTPs were used for cultivation of microorganisms, especially for fungi cultivation and aerobic fermentation. One of the major concerns is limited maximum oxygen transfer capacity in MTPs, wherea strain could be a suitable candidate for the desired production process might fail during an oxygen-limited screening procedure [13]. Another concern with MTP for fermentation is medium evaporation because some fermentation screening processes require an incubation time of several days on a shaker. With that, significant loss of volume might occurred, especially when the small working volume is applied [3]. So it is essential to study the capabilities of MTP in micro-culture by determine the oxygen transfer coefficient and evaporative process. This study was designed to investigate a high throughput screening strategy for the efficient identification of Cephalosporin C high-yield strains.

MATERIALS AND METHODS

Microorganism strains and cultivation condition

Microorganism strains: *Cephalosporium acremonium* and *Alcaligenes faecalis* (ATCC8750) strains were obtained from National Engineering Research Center for Biotechnology (Shanghai) - East China University of Science and Technology. The cultures was grown on agar medium (per liter: 10 g polypepton, 3 g beef extract, 5 g NaCl, and 18 g agar) and preserved at 4^{o}C.

Culture media for Cephalosporium acremonium

Slant medium: The slant medium per liter contained 10 g polypepton, 12 g maltose extract, 40 g maltose monohydrate and 18 g agar. The pH of the medium was adjusted to 7.0 with 30% of NaOH before autoclaving. Culture on slants was grown at 28^{o}C for 10 days.

Seed medium: The seed medium contained (per liter): 5 g glucose hydrate, 35 g sucrose, 30 g corn steep liquor (CSL), 0.5 g methionine, 8 g $(NH_4)_2SO_4$, 5 g $CaCO_3$, and 5 ml soybean oil. Sucrose was aseptically added to the culture media before inoculation. Glucose hydrate was autoclaved at 115°C for 10 min. All other components were autoclaved at 121°C for 25 min. The pH of the medium was adjusted to 6.5 with 30% of NaOH before addition of $CaCO_3$ and soybean oil. Cultivation was done at 28°C for 68 h at 220 rpm in a rotary shaking incubator.

Fermentation medium: The fermentation medium contained (per liter): 70 g starch, 30 g dextrin, 0.15 g amylase, 50 g CSL, 6 g methionine, 3 g CH_4N_2O, 13 g $(NH_4)_2SO_4$, 3 g $MgSO_4 \cdot 7H_2O$, 9 g K_2PO_4, 5 g yeast extract, 10 g $CaCO_3$, 20 ml soybean oil, and 10 ml trace element solution. The trace element solution contained (per liter): 8 g $FeSO_4 \cdot 7H_2O$, 2 g $CuSO_4 \cdot 5H_2O$, 2 g $ZnSO_4 \cdot 7H_2O$, and 2 g $MnSO_4 \cdot H_2O$. The pH of the trace element solution was adjusted to 1.0 by 1N sulfuric acid. The pH of the medium was adjusted to 6.2 with 30% of NaOH before addition of $CaCO_3$ and soybean oil. Fermentation was carried out at 28°C for 6 days at 220 rpm in the rotary shaking incubator.

Culture media for Alcaligenes faecalis (ATCC8750)

Nutrient agar medium was used for slants and plates which contained (per liter): 10 g polypepton, 3 g beef extract, 5 g NaCl, and 18 g agar. For liquid medium, agar was not added into the medium. The pH of the medium was adjusted to 7.0 – 7.2 with 30% of NaOH. *A. faecalis* was grown at 37°C for 24 hours.

Analytical methods

Volumetiric oxygen transfer coefficient K_La (h^{-1}) measurement

Volumetric oxygen transfer coefficient K_La (h^{-1}) was determined using the optical sulfite oxidation method (Hermann R et al., 2003) for the cultivation in 96-wells MTP, 48-wells MTP, and 500 mL shake flasks. A sulfite solution system (0.5 M sodium sulfite, 10^{-7} M cobalt sulfate, 0.012 M Na_2HPO_4/NaH_2PO_4 phosphate buffer (pH 8.0)) was mixed with bromothymol blue solution ($2.4x10^{-5}$ mol) that serve as indicator as color changes from blue to yellow. The initial pH of the solution mixture was adjusted to 8.0 with 2M sulfuric acid. The pH remains almost constant at 8.0 and then sharply drops upto 5.0 at the end of the reaction. The catalyst $CoSO_4$ (10^{-4} mol) was added and time was recorded until the color changed from blue to yellow due to reaction.

The maximum filling volumes of 48-well and 96-well MTPs were 5000 µl and 2000 µL for each well, respectively. The oxygen transfer coefficient K_La (h^{-1}) and evaporative process were studied at different working volumes i.e. 300, 400, 500, 600, 700 µL for 96-well MTP, and 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500µL for 48-well MTP. To prevent liquid spillage, maximum working volume for 96-well MTP and 48-well MTP was 700 and 1500 µL, respectively. In this experiment, all the data represent mean values of three MTPs and six shake flasks.

Determination of water evaporation capacity

Water evaporation was determined by the loss of water volume at every 24 h for 6 consecutive days. The loss of water was determined by weighing the MTP after every 24 h. All wells of each MTP were filled with an equal volume of water and loss of water volume was determined after 24 h. The MTPs were put in similar fermentation conditions (28°C at 220 rpm). Evaporation rates and ratios were calculated according to the following formulae:

Evaporation rate = Loss water weight after 6 days/144 h

Evaporation percentage (%) = (Evaporative water weight after 6 days × 100)/Initial water weight

Determination of cephalosporin C by turbidimetric assay

The turbidimetric assay was used for the estimation of CPC content in HTS by microbiological assay using *A. faecalis* (ATCC8750) as indicator organism in a 48-well MTP, as CPC effectively inhibited the growth of *A. faecalis* during this study. A 5% suspension of *A. faecalis* (ATCC8750) was prepared by growing in a slants agar for 24 h. Then, the concentration of cell suspension was diluted with nutrient broth to obtain an optical

density (OD) of 1.5 at 600 nm. Each well of 48-well MTP was inoculated with 450 µL of 5% *A. faecalis* suspension and 50 µL test sample (CPC fermenting broth supernatant). Test sample liquid was diluted in linear range with0.1M phosphate buffer before use. The standard CPC zinc salt (Sigma Chemicals, USA) in the range of 10-80 $UmL^{-1}$was used to construct a standard curve (R^2=0.99). Bioassay was conducted at 37°C for 4 h and 40 min (220 rpm). In the end OD was determined by microplate reader (Thermo, Shanghai) Instruments Co., Ltd) at 600 nm.

Determination of cephalosporin C by HPLC assay

An Agilent 1100 series HPLC system equipped with a TSKgel ODS-100S reversed-phase analytical column (250 mm × 4.6 mm) was used to quantify CPC. The mobile phase was prepared by mixing 850 mL of H_2O, 40 mL of acetonitrile, 140 mL of methanol, 0.8 g of $NaH_2PO_4 \cdot 2H_2O$. The pH of the mobile phase was adjusted to 7.3 with 1N NaOH. The flow rate for the mobile phase was 1.0 mL min^{-1} and the eluate was detected at 254 nm. CPC zinc salt (Sigma, USA) was used as standard.

High Throughput Screening Method

Mutagenesis:

Combination ofUV irradiation and LiCl were employed for mutagenizing the original strain of *C. acremonium* (named 1-D1) which was grown on slant medium at 28°C for 10 days to form spores. Agar slant containing populated fungi were washed with 10 mL sterile water to harvest the mutant spore suspension. And the spore suspension was gently homogenized with glass beads (3 mm in diameter), and resuspended with sterilized water at approximately 1×10^8 spores mL^{-1}. This liquid containing spores was exposed to UV irradiation at a distance of 30 cm for 2 min. Then 5 ml liquid containing mutant spores transferred into 5 ml LiCl liquid for 20 min (12 g LiCl L^{-1}) which results in the 98% kill rate.

High throughput screening

Mutagenized liquid (10µL) was transferred into each of the well in the 48-well MTP for spore germination using eight-multichannel pipette (Biohit Biotech SuZhou, Co., Ltd). After 5 days of incubation at 28°C, 220 rpm, 10% (v/v) of seed culture was transferred into another 48-well MTP for fermentation. Each deep well (total volume 5000 µL) was added with 1200 µL seed or fermentation media. The original 48-well MTP were kept at 4°C in the refrigerator as seed plates. After 6 days of fermentation, the 48-well MTPs contents were centrifuged (Shanghai Luxiangyi centrifuge instrument Co., Ltd) at 3,000 g for 10 min and the supernatant was analyzed by turbidimetric assay at 600 nm to quantify the CPC concentration. The well with the highest concentration of CPC was identified and confirmed after each screening cycle. And the well with the highest CPC concentration was inoculated on slant medium from 48-well MTP of seed. After that it was used as new parent strain in a new cycle formutation. The process was repeated until a strain was confirmed to be statistically superior in its performance as compared to the wild/parent strain used prior to the mutagenic treatment.

Single colony selection:

The wells that produced highest CPC concentration were selected by transferring well contents on agar medium using pour plate method and then single colonies were obtained. After getting single colonies, sample high throughput method was applied. Mutants with higher CPC concentration were selected for future evaluation in shake flask and fermentor. The screening strategy is summarized in Fig 1 for the identification of mutants with increased CPC production from the original strain.

RESULTS AND DISCUSSION

Volumetric oxygen transfer coefficient ($K_La\ h^{-1}$) in 48- and 96-well microtiter plates

For the 48-well MTPs, the volumetric oxygen transfer coefficient (K_La) was 141.62±2.72, 124.80±2.50, 119.55±2.48, 112.64±2.25, 102.64±2.93, 95.63±2.79, 86.64±2.92, 83.24±6.28, 79.07±6.00, and 76.55±7.20 h^{-1} for 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, and 1500 µL filling volumes, respectively. Whereas, for

96-well MTPs, the K_La obtained was 79.35±1.02, 69.32±1.09, 58.77±2.26, 52.42±3.52, and 46.72±3.37 K_La h^{-1} for 300, 400, 500, 600, and 700 µL, respectively. Shake flasks (500 mL) were also incubated for comparing the K_La with the MTPs. The obtained K_La in the 500 mL shake flasks (162.73 ± 2.02 h^{-1}) was higher than 48- and 96-well MTPs at all working volumes. Based on the results obtained, t by decreasing the filling volumes in the 48- and 96-well MTPs could increase the K_La values. The K_La values for 48-well MTPs were higher than 96-well MTPs.

Evaporation percentage and evaporation rate

In the 96-well MTPs, the evaporation percentages were 66.96, 51.05, 43.46, 36.39, and 32.25 % for 300, 400, 500, 600, and 700 µL of filling volumes, respectively. As shown in Fig 2 the evaporation percentages were obtained in the range of 135.00 to 148.82 mg h^{-1} with the range of filling volume 300 µL. The 48-well MTPs were less evaporative as compare to the 96-well MTPs with 45.11, 40.83, 34.69, 31.07, 28.44, 26.45, 24.23, 22.71, 22.52, and 21.26% of evaporation percentage; and evaporation rate of 89.65, 94.65, 91.84, 92.50, 94.06, 96.11, 95.97, 97.53, 104.23, and 105.41 mg h^{-1} for 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, and 1500 µL filling volumes, respectively. Shake flasks (500 mL) were moderately evaporative with 25.24% evaporation percentage and 52.78 mg h^{-1} evaporation rate. The results for 48-well MTPs and shake flask are represented in Fig 3.

The amount of evaporative water substantially changed every day whether in the 96- or 48-well MTPs during the whole fermentation course. The water evaporation rate was least affected by the filling volumes. In 96-well MTPs, when the filling volumes doubled from 300 to 600 µL/well, the evaporation rate only increased a bit which was from 135 to 143.96 mg h^{-1}. Standard deviation of the evaporation rate was ± 0.03%. When the water volume was increased double in the 48-well MTPs, e.g. increased from 600 µL/well to 1200 µL/well, the evaporation rate was slightly increased from 89.65 to 95.97 mg h^{-1} with a standard deviation of ± 0.03%.

Since the evaporation rate was seldom influenced by the water volume for the microplate format (96-well or 48-well), hence more the initial liquid quantities, the little the evaporation ratio. With a small evaporation percentage, the stability of the media concentration could be maintained. When the filling volume in the 48-well MTPs reached its upper limit (1500 µL/well), the evaporation percentage (21.26%) was lower as compare with 30 mL in the shake flask (25.24%). However，in the 96-well MTPs, the evaporation percentage for the upper limit (700 µL/well) was 32.25% that was higher than in the shake flask.

Based on results of oxygen transfer coefficient and evaporative rates of MTPs obtained, we can concluded that 48-well MTPs have higher filling volumes capacity, lower evaporation rate and higher oxygen transfer coefficient than 96-well MTPs. The values for these parameters were much closed to the values obtained in the shake flask. With that, the 48-well MTPs were selected for the further experiments.

In 48-well MTPs with filling volumes of 1100 and 1200 µL, the evaporative percentage (26.45 and 24.23%) was close to shake flasks (25.24%). Hence, it was chosen for further experiments with 1200 µL filling volume.

High throughput screening for selecting high yielding strains

The mutants obtained after preliminary mutagenesis by UV and LiCl combination were transferred into 480 wells (10 MTPs) to check for its growth. As depicted in Fig 4, only 75 wells were positive for growth with a total mortality rate of 84.37%. The highest CPC producing mutant No. 33 was selected among the 75 wells and renamed as 7-B1. This mutant strain (7-B1) was used for the secondary screening.

For secondary screening, 960 wells (20 MTPs) were used to screen for the mutants. As shown in Fig 5, positive growth was observed in 371 wells and mutant No. 165 which was renamed as 12-E6 was selected for further screening. The strain was grown on slants agar to obtained single colony. Further, 472 viable single colonies of strain 12-E6 were randomly picked to check for their ability to produce the metabolite of interest (10 MTPs), and production of CPC from these 472 strains were compare with 7-B1, and parental strains 1-D1 as shown in Fig 6. From these mutants, top twenty high yielding mutants were inoculated on slant agar for future work.

Comparison of parent strain with mutant strains

Finally, after four more steps of secondary screening, two mutated *Cephalosporium acremonium* i.e. 5-A1 and 8-A2 with higher CPC production (Fig 6) were identified and their single colony was compared with original strain. The results were reported in Table 3 and Fig 7. The results showed that CPC production of mutant strains selected by high throughput screening, was higher than the original strain. The volumetric productivity of CPC for strains 5-A1 and 8-A2 in shake flasks was found to be 7914 and 8639 U/ml which was 246 and 269% increase compared to parent strain, respectively. This indicated that the high throughput method successfully selected the most potent CPC producing strains. The selected strains possessed the potential to greatly improve the industrial productivity of CPC through fermentation. The results indicated that the high throughput method was more promising for selecting the high producing CPC strains.

Ellaiah et al. (2002) reported that the yield of CPC was improved when the spore suspension of *Cephalosporium acremonium* ATCC 48272 was treated with UV rays and N-methyl-N-nitro-N-nitrosoguanidine. Their results showed that antibiotic yield was improved from 630 to 1995 μgmL^{-1} of the broth that resulting in a high yielding mutant. In another study, Ellaiah et al. (2003) reported the productivity was 2.4 times higher in mutant strains as compared to parent strain. In addition, in order to improve CPC production by recombinant DNA integration in *Acremonium chrysogenum*, Liu et al. (2010) found a 116.3% increase in CPC production [15]. Tan et al. (2013) reported an increase of 50% in CPC production after high throughput screening of *A. chrysogenum* [1]. The improvement of CPC production obtained in current study is comparable with those reported earlier.

Table 1: Effect of different filling volumes on volumetric oxygen transfer coefficient (K_La,h^{-1}) in 96-well MTPs and 500 mL shake flask.

Filling volumes (µL)	96-well MTP		500 ml shake flask (30 ml of solution volume)	
	K_La (h^{-1})	Relative standard deviation RSD (%)	K_La (h^{-1})	RSD (%)
300	79.35	1.02	162.73	2.02
400	69.32	1.09		
500	58.77	2.26		
600	52.42	3.52		
700	46.72	3.37		

Table 2: Effect of different filling volumes on volumetric oxygen transfer coefficient (K_La,h^{-1}) in 48-well MTPs.

Filling volumes (µL)	48-well MTP	
	K_La (h^{-1})	RSD (%)
600	141.62	2.72
700	124.80	2.50
800	119.55	2.48
900	112.64	2.25
1000	102.64	2.93
1100	95.63	2.79
1200	86.64	2.92
1300	83.24	6.28
1400	79.07	6.00
1500	76.55	7.20

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Table 3: Comparison of cephalosporin C of different strains after using high throughput screening method (HPLC assay).

Strains	Cephalosporin C (U mL^{-1})
Strain 1-D1 (Original strain)	3211.10
Strain 7-B1 (Mutant strain in the first preliminary screening)	5961.61
Strain 12-E6 (Mutant strain in the second preliminary screening)	6891.57
Strain 5-A1	7914.43
Strain 8-A2	8639.24

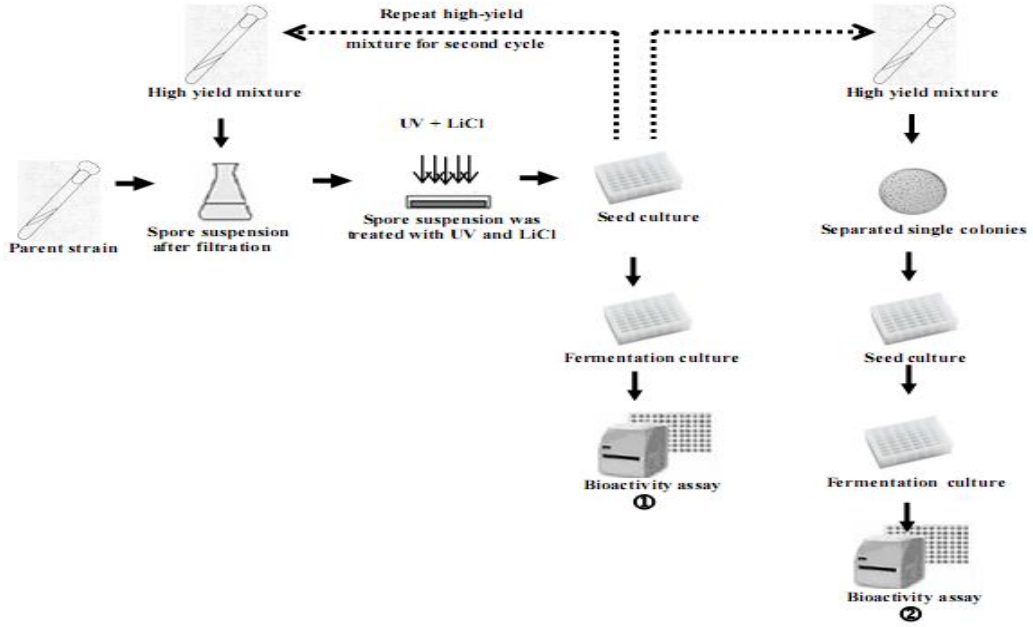

Figure 1: Strategy for libraries screening on 48-well microtiter plate cultures. ①Screening for high yield mixture.②Screening for high yield strain.

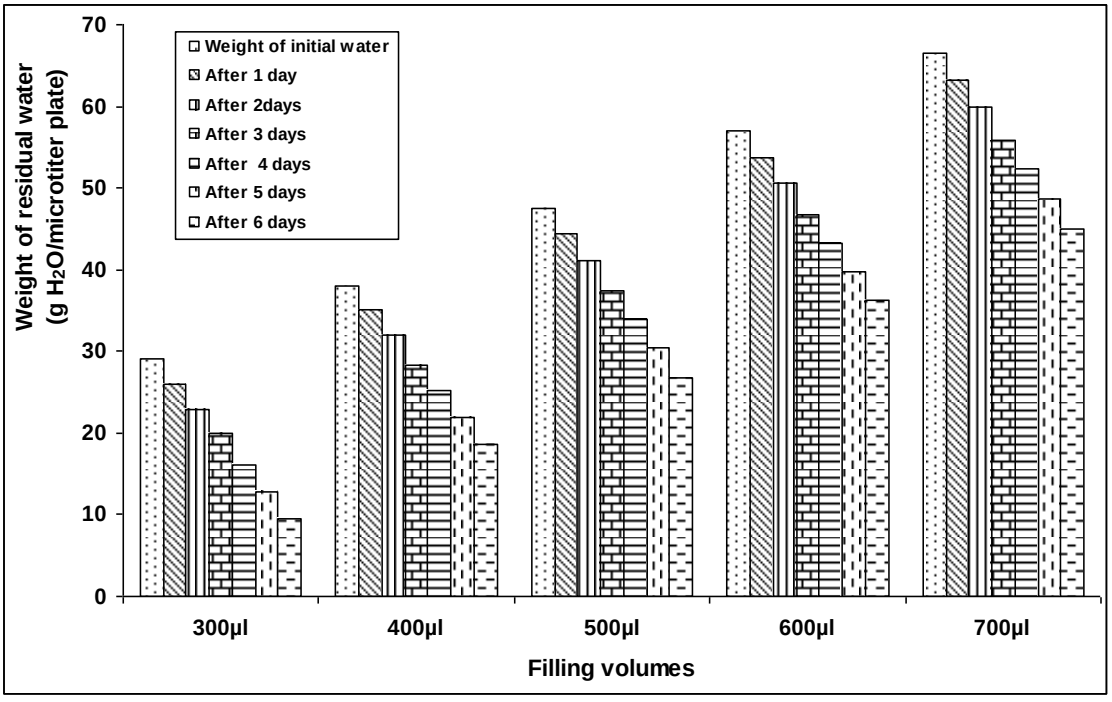

Figure 2: Effect of different filling volumes on evaporative process in 96-well microtiter plate.

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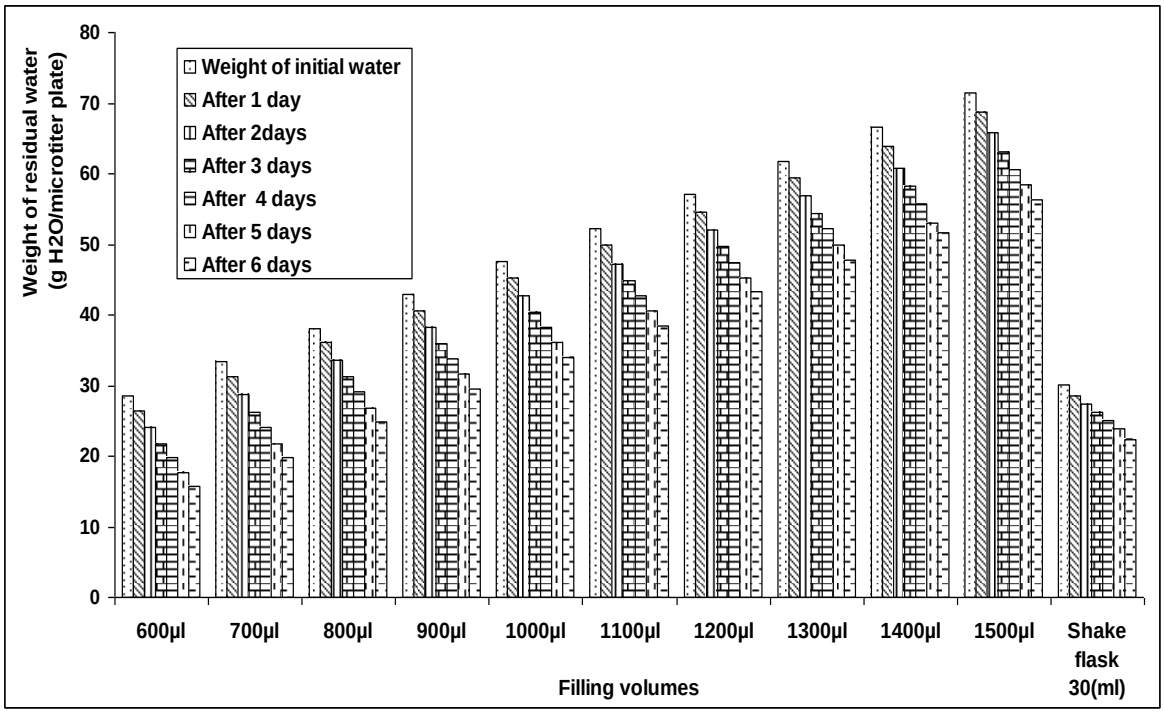

Figure 3: Effect of different filling volumes on evaporative process in 48-well microtiter plate.

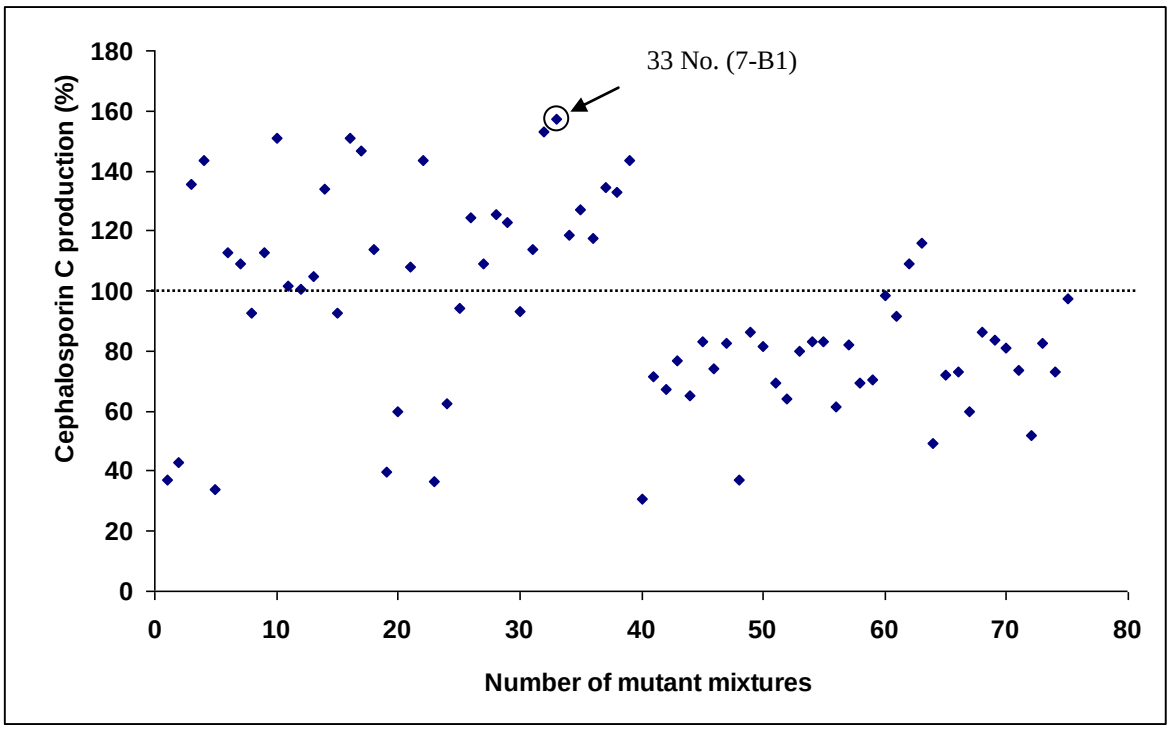

Figure 4: The CPC concentration of wells after the four steps of preliminary.

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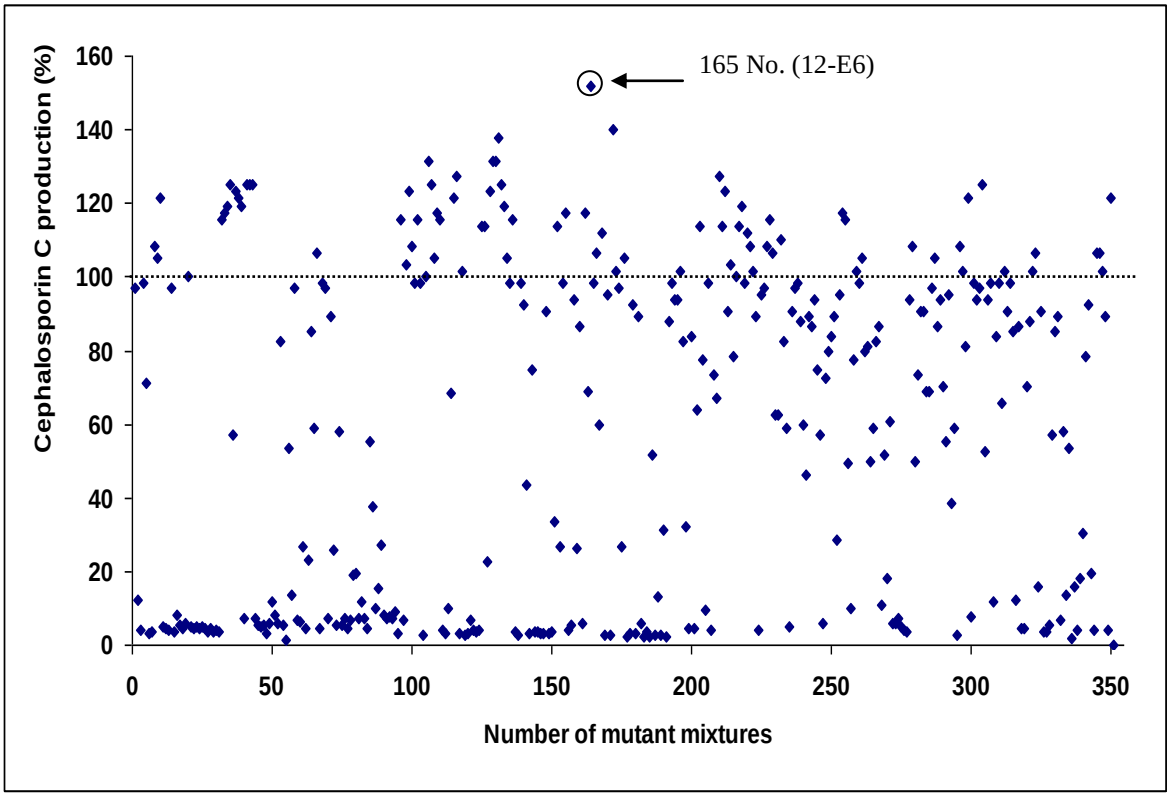

Figure 5: The CPC concentration of wells after the four steps of preliminary in second cycle (each hole).

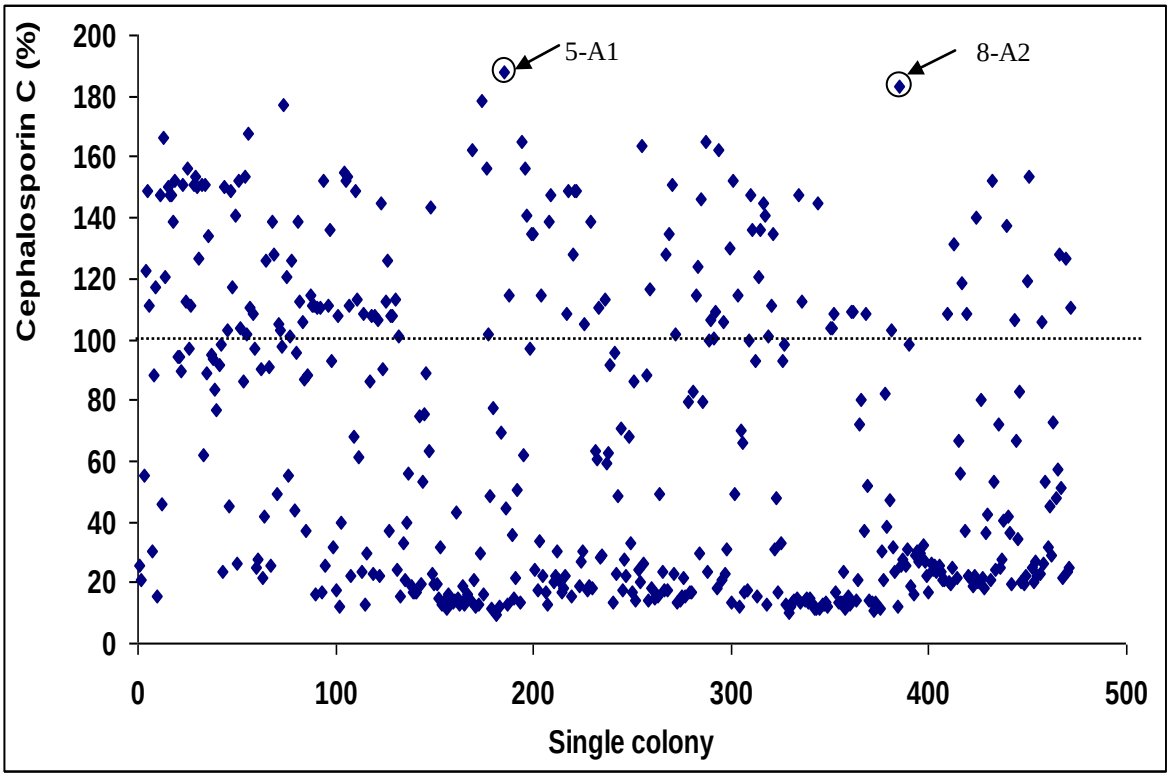

Figure 6: The CPC concentration of the strains after four more steps in the second screen of strategy.

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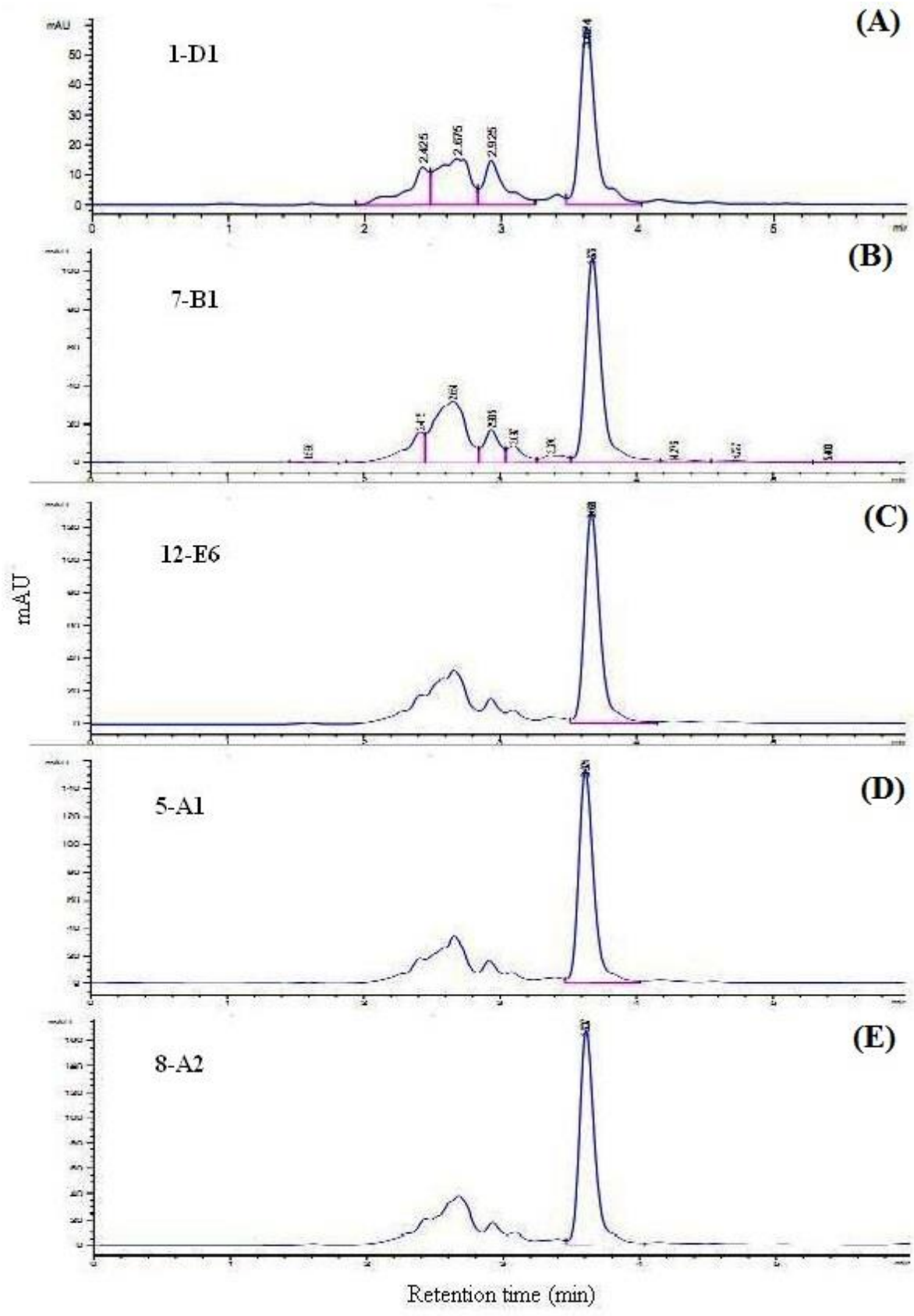

Figure 7: The chromatogram of cephalosporin C concentration produced from (A) 1-D1, (B) 7-B1, (C) 12-E6, (D) 5-A1, and (E) 8-A2.

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CONCLUSIONS

In this study, we had developed a simple high throughput culturing and screening strategy for CPC overproducing strains. The methods include two high throughput screening systems. One is a miniaturization culture system based on MTPs. Another is an analysis system based on ELISA plate format. Microtiter plate of 48-well was more suitable for cultivation of aerobic microorganism than the widely used 96-well microtiter plate. This is due to the oxygen transfer coefficient in the 48-well microtiter plate was higher than that in 96-well microtiter plates. Secondly, the working volume of 48-well MTP was larger than that of 96-well MTP, which ensured minimum evaporation percentage and stable cultivation after a long incubation period in the 48-well MTP.

Based on isolating single colony after mixed culture and evaluation of mutant population and mentioned-above high throughput screening strategy, two mutants of *Cephalosporium acremonium* (5-A1; 8-A2) were selected. The volumetric productivity of CPC was 7914 U mL^{-1} in 48-well MTP, 8639 U mL^{-1} in shake flasks, separately. It was about 246%, 269% improvement compared with that of original strain. The strain 8-A2 that showed highest CPC production (269%) is better. Thus, the development of this strategy is expected to accelerate the selection of superior CPC producing strains.

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