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METHODS

Identification of avermectin-high-producing strains by high-throughput screening methods

Hong Gao · Mei Liu · Xianlong Zhou · Jintao Liu · Ying Zhuo · Zhongxuan Gou · Bing Xu · Wenquan Zhang · Xiangyang Liu · Aiqun Luo · Chuansen Zheng · Xiaoping Chen · Lixin Zhang

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Abstract Avermectins produced by *Streptomyces avermitilis* are potent against a broad spectrum of nematode and arthropod parasites with low-level side effects on the host organisms. This study was designed to investigate a high-throughput screening strategy for the efficient identification of avermectin high-yield strains. The production protocol was miniaturized in 96 deep-well microplates. UV absorbance at 245 nm was used to monitor avermectin production. A good correlation between fermentation results in both 96 deep-well microplates and conventional Erlenmeyer flasks was observed. With this protocol, the production of avermectins was determined in less than 10 min for a full plate without compromising accuracy. The high-yield strain selected through this protocol was also tested in 360 m^3 batch fermentation with 1.6-fold improved outcome. Thus, the development of this protocol is expected to accelerate the selection of superior avermectin-producing strains.

Keywords Avermectin · High-throughput screening · Strain improvement · Miniaturized production

Hong Gao, Mei Liu, and Xianlong Zhou contributed equally to this work.

H. Gao · M. Liu · X. Zhou · J. Liu · Y. Zhuo · Z. Gou · W. Zhang · X. Liu · A. Luo · C. Zheng · L. Zhang (✉)
Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing 100190, People's Republic of China
e-mail: Zhanglixin@im.ac.cn

L. Zhang
South China Sea Institute of Oceanology, CAS, Guangzhou 510301, China

X. Chen · L. Zhang
Guangzhou Institutes of Biomedicine and Health, CAS, Guangzhou 510663, China

J. Liu · Y. Zhuo
Graduate University of Chinese Academy of Sciences, Beijing 100049, China

B. Xu
Hebei Veyong Bio-Chemical Co., LTD, Shijiazhuang, Hebei 050091, China

Introduction

Avermectins, produced by *Streptomyces avermitilis*, are widely used as anathematic and insecticidal agents (Burg et al. 1979; Ikeda and Omura 1995). They featured a 16-membered pent acyclic with a disaccharide of methylated deoxysugar L-oleandrose polyketides (Zimmermann et al. 2003). The structural differences between eight major avermectin compounds are mainly at the C5, C22–C23, and C26. B1a is the most efficient component (Ikeda and Omura 1997), and there is an increasing interest in improving the production of avermectin B1a; however, the yield achieved for avermectins is still low despite the labor-intensive work invested in traditional strain improvements.

A variety of strain improvement strategies have been developed to increase avermectin productivity (Hwang et al. 2003; Lee et al. 2000). All of these approaches yield a population of mutants with a diverse range of properties. Traditionally, most of the screening methods are performed in Erlenmeyer flasks (Buchs 2001). However, these flasks require a large amount of material, and the cultivation process is time-consuming and laborious, limiting the wide applications of such strain improvement strategies (Duetz et al. 2000). Furthermore, it is also difficult to quantify the target compounds. High-performance liquid chromatography (HPLC), extensively used to determine the concentration of avermectins, has limited screening throughput and

allows for about 100 samples per day. Therefore, it is imperative to develop a high-throughput screening method to evaluate a large number of mutants with a good degree of accuracy and reproducibility.

Three major types of high-throughput screening (HTS) approaches have been developed for different systems over the recent years (Du et al. 2007; Tyo et al. 2006; Zhang et al. 2007; del Cardayré 2005). The first approach focuses on the measurements of specific absorbance peaks of either the target product or its derived compound (Askal et al. 2008; Yu et al. 2008; Zhang 2005a, b; Zhang et al. 2005); the second tracks the morphology change of mutant colonies (Dobson et al. 2008; Yu et al. 2008), and the third approach involves a bioactivity assay, which is generally used in the selection of antibiotic-producing strains (du Toit and Rautenbach 2000; Kumar et al. 2000; Xu et al. 2005). Here, we report a high-throughput procedure for screening avermectin super-producers based on the UV absorbance using 96 deep-well microtiter plates (MTP) for cultivation. The accuracy was compared with an HPLC assay, and the reproducibility of the method was also examined.

Materials and methods

Microorganism and media

S. avermitilis 3-115, an industrial strain (average avermectin CGMCC No. 3229), and the mutants derived from 3-115, were grown on YMG agar medium (per liter: 4 g of yeast extract, 10 g of malt extract, 4 g of glucose, 18 g of agar; pH 7.2).

The seed medium contained (per liter) 30 g of corn starch, 8 g of soy flour, 10 g of peanut meal, 4 g of yeast extract, 0.03 g of $CoCl_2$, and 0.04 g of α-amylase. The pH of the seed medium was adjusted to 7.0 with NaOH before autoclaving. The fermentation medium contained (per liter) 140 g of corn starch, 0.1 g of α-amylase, 28 g of soy flour, 10 g of yeast extract, 0.022 g of $Na_2MoO_4{\cdot}2H_2O$, 0.0023 g of $MnSO_4{\cdot}H_2O$, 0.25 g of $(NH_4)_2SO_4$, 0.02 g of $CoCl_2$, and 0.8 g of $CaCO_3$. The medium pH was adjusted to 7.5 with NaOH before autoclaving (15 g/L agar was added for the solid fermentation medium).

Culture of microorganism

Culture in Erlenmeyer flasks

Cultivations were performed in two stages: spore germination and the production. During the spore germination stage, mycelium from a YMG agar medium plate were transferred into 250-ml flasks containing 40 ml seed medium and incubated at 28 °C on a rotary shaker at 220 rpm (5 cm shaking diameter, 50/300, Long March, China) for 40 h. Fermentation media were then inoculated with 5% seed cultures and incubated on a rotary shaker at 220 rpm at 28 °C for 10 days.

Solid-state micro-culture in 96-well format

Solid-state micro-cultures in a 300-μl scale were carried out in deep MTP covered with a lid. Single colonies were inoculated in MTP and cultivated at 28 °C for 10 days.

Fermentor studies

The production of avermectin was carried out in a 360-m^3 fermentor (TIAN GONG JI TUAN, Hebei, China) containing 235 m^3 fermentation medium as a fed-batch process. Initially, all the medium components were added into the fermentor; the pH was adjusted to 6.5, and the medium was sterilized in situ. Seventeen cubic meters of seed culture was inoculated aseptically, and the impeller speed was maintained at 90 rpm and temperature at 28 °C. The pH was maintained between 6.1 and 6.5 using 1 N HCl and NaOH solution. When the concentration of reducing sugar dropped below 0.7%, saccharified starch was fed until the concentration of reducing sugar was 1.0%. The avermectin synthesis was monitored for every 24 h.

Analytical methods

The following procedure was carried out to prepare samples for quantification of avermectins. One milliliter of culture broth was extracted with 9 ml of methanol for Erlenmeyer flask culture (Gao et al. 2009), and 1 ml of methanol was added to the wells for MTP cultures. The supernatants were then analyzed with the following assays.

Spectrophotomic assay (UV assay)

The optical densities of the supernatants at 245 nm were measured for the concentration of all major avermectin components with the following procedure. For MTP cultures, supernatants (40 μl) in MTP wells were transferred into another MTP (UVstar, Greiner, Germany) using a 12-channel pipette. Two hundred microliters of methanol was added to dilute the samples into the linear range of detection (Multilabel Plate Reader, Perkin–Elmer, USA). A standard curve was generated using 200 μl of 750, 375, 187.5, and 93.75 mg/L commercially available avermectins (96% B1a and 4% B1b, DR, Germany). At least three replicate experiments were carried out.

HPLC assay

The concentrations of the major avermectin component B1a were determined by reverse phase HPLC (Agilent 1200, USA). The samples were separated on a Waters C18 column and eluted by methanol/water (85:15, *v*/*v*) at a flow rate of 1 ml/min (Curdova et al. 1989) with a UV absorbance at 245 nm.

Statistical analysis

Pearson correlations factors were calculated by SPSS for Windows 11.5 software (SPSS Inc. USA).

Results and discussion

Erlenmeyer flask culture can be replaced by the MTP solid-state cultures for the evaluation of avermectin production

While solid culture is advantageous over liquid culture for rapid screening, the micro-environment for solid cultures, especially solid-state MTP cultures, is not necessarily the same as more traditional Erlenmeyer flask culture systems. Therefore, avermectins production in MTP was studied, and the reproducibility of avermectin production between scales (MTP and Erlenmeyer flask) was investigated to determine whether solid culture can be adopted for screening high avermectin-producing strains.

Ideally, the solid-state MTP fermentations would replicate Erlenmeyer flask culture (Isett et al. 2007). As shown in Fig. 1, avermectin production from Erlenmeyer flask is well correlated with that from solid-state MTP cultures. The correlation efficiency was high for avermectin B1a production (Pearson factor: r=0.727, p<0.01). This suggested that solid-state MTP cultures could predict avermectin titer.

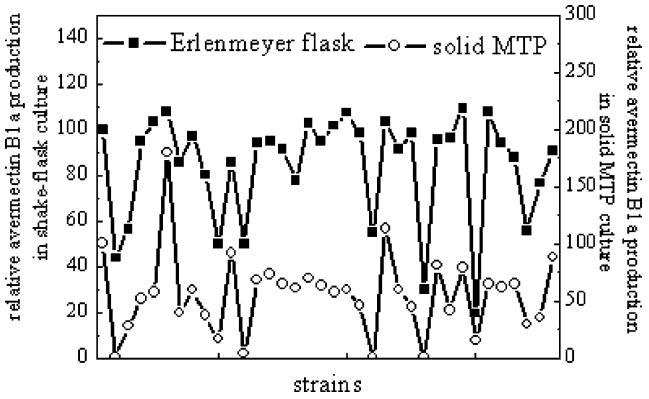

Fig. 1 The correlation of Erlenmeyer flask and solid-state MTP cultures. Thirty-six strains were studied in this experiment

The MTP assay is reproducible for the growth of strains and their respective avermectin production

The reproducibility of solid-state MTP was studied to further validate the assay. Eight hundred fifty-three mutants were studied. In all experiments, each clone was inoculated into two adjacent wells with mock controls. Subsequently, the microplates were covered with lids and cultured at 28 °C. After 10 days of incubation, no cross-contamination was found.

The productivity of those colonies was also studied. For all strains tested, the mean standard error in the duplicates was 13.35%, with variability of less than 5% for 25.3% of the strains, 5–10% for 21.6% of the strains, and more than 35% for only 4.8% of the strains. The results were reproducible, indicating that the solid-state MTP assay is reliable.

UV assay is comparable to HPLC assay for rapid quantification of avermectins

HPLC assay is not suitable for high-throughput quantitative analysis. Therefore, we have developed an UV assay to predict avermectin titer.

The avermectin complexes have an ultraviolet absorbance maximum at 245 nm (Sams 1993). A standard curve was generated with concentrations of commercial avermectin standards at 750, 375, 187.5, and 93.75 mg/L. As shown in Fig. 2, a second-order polynomial formula with R^2= 0.99999 was obtained that fits avermectin–OD_{245} curve in the concentration range of 100–750 mg/L. Furthermore, there is a high correlation between total avermectins and avermectin B1a production (Erlenmeyer flask, r=0.941, p<0.01; solid-state MTP, r=0.899, p<0.01; n=12).

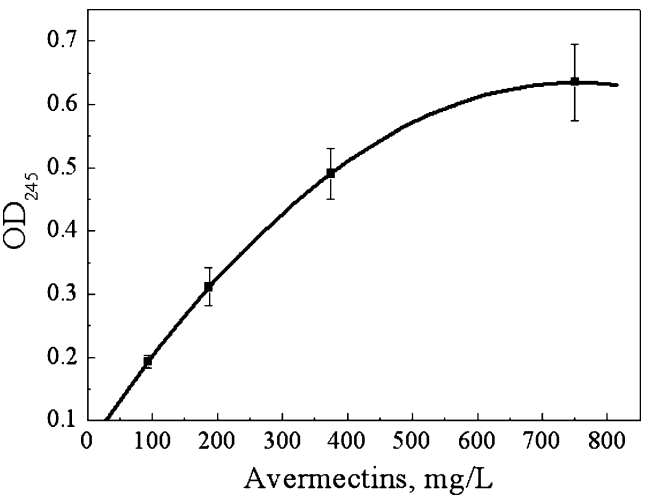

Fig. 2 The standard curve of avermectins. A second-order polynomial fit was used. Avermectin (mg/L)=205.02−1101.60×OD_{245}+ 3062.79×OD_{245}^2. R^2=0.99999

In the Erlenmeyer flask and solid-state MTP cultures, correlation between UV and HPLC assay was investigated. The productivity of 40 random selected strains was determined by UV and HPLC assay, respectively. Pearson factor was calculated to determine the correlation between the two assays. It was shown that the correlation efficiency between UV and HPLC assays Erlenmeyer flask was high (r=0.948, p<0.01, n=40) as was the correlation efficiency between UV and HPLC assays for solid-state MTP (r=0.875, p<0.01, n=40). So the UV assay could be applied to determine avermectins production in solid-state MTP culture.

It is necessary to determine whether UV assay results represent the avermectin productivity of the same strains in Erlenmeyer flask. Therefore, the correlations were investigated between experiments in Erlenmeyer flask tested by HPLC and those in solid-state MTP cultures tested by UV assay (Fig. 3).

Figure 3 shows the avermectin B1a productivity of 56 mutants derived from parent strain 3-115, and the correlation between experiments in Erlenmeyer flask tested by HPLC and those in solid-state MTP cultures tested by UV assay was divided into four areas. When strains were cultured in Erlenmeyer flasks and productivity were determined by HPLC (avermectin B1a) for the spots in areas of A and B, avermectin production in these two areas was 10% higher than the control strain, 3-115, and therefore identified as overproduction mutants. Therefore, the spots in areas of A and B were real "hits". However, when strains were cultured in solid-state MTP and their respective productivity was determined by UV assay (total avermectins), the spots in areas B and D were identified as overproduction mutants. Thus, area D was a "false-positive" as strains represented by the spots in D area were not the real hits in conventional screening, and area A served as "false-negative" in the assay. The high degree of correlation between HPLC and UV data was observed (Pearson factor of r=0.629, p<0.01, Table 1). Thus, the UV assay could be applied to screen avermectin-overproducing strains.

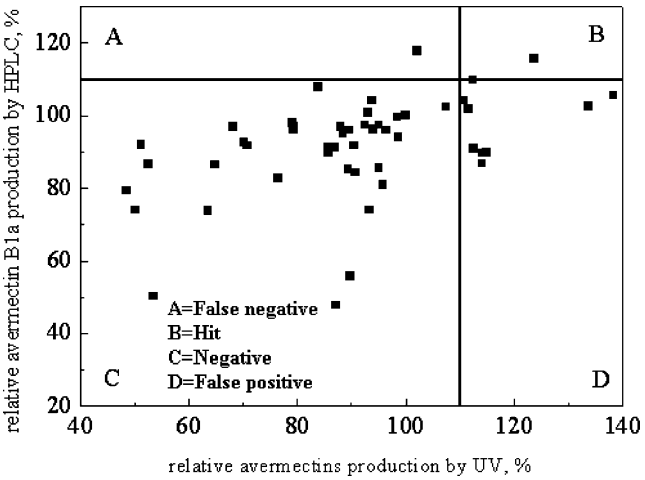

Fig. 3 Screening avermectin-overproducing strains by HPLC in Erlenmeyer flask vs. by UV in solid-state MTP

As shown in Table 1 and Fig. 3, the percentage of false-negatives was 3.57%, and the percentage of false-positives was 12.5%. The results suggested that the UV assay is suitable to be used in high-throughput screening of avermectins/avermectin B1a overproduction mutants.

High-throughput screening of avermectin-overproducing strains

Utilizing the solid-state MTP cultures and the UV assay, we have developed a screening strategy (Fig. 4) to identify mutants with increased avermectin production from strain 3-115. There are three steps in the preliminary screen. Step 1: Plate the spores derived from 3-115 on agar and incubate at 28 °C until single colonies were observed clearly (about 240 h). Select the colonies randomly. Step 2: Inoculate the selected colonies on solid-state MTPs. Incubate the MTPs for 240 h. Step 3: Extract with methanol for 6 h and analyze the supernatant by multilabel reader to quantify the avermectin concentration. There are two more steps in the second screen. Step 4: The strains with 10% higher absorption than 3-115 were selected to the second round of validation in shaking flasks. Step 5: The cell-free culture supernatant is collected and analyzed by HPLC to quantify the B1a content.

Out of 738 viable single colonies on YMG plates, 116 mutants were selected through the preliminary screen. The mutants were subjected to the second round of validation in shaking flasks, and the avermectin B1a production was measured by HPLC. The results were shown in Fig. 5. Of these selected strains, 93 mutants were comparable to the control strain, 3-115, producing more than 90% of avermectin B1a produced by 3-115. Thirty-eight mutants were found to possess B1a titers at least 10% higher than that of the parental strain, 3-115.

Table 1 Numbers of spots in each area for Fig. 3

Area	Description	Numbers of spots
A	False-negative	2
B	Hit	2
C	Negative	45
D	False-positive	7
Total		56
A/total	Percentage of false-negatives	3.57%
D/total	Percentage of false-positives	12.5%

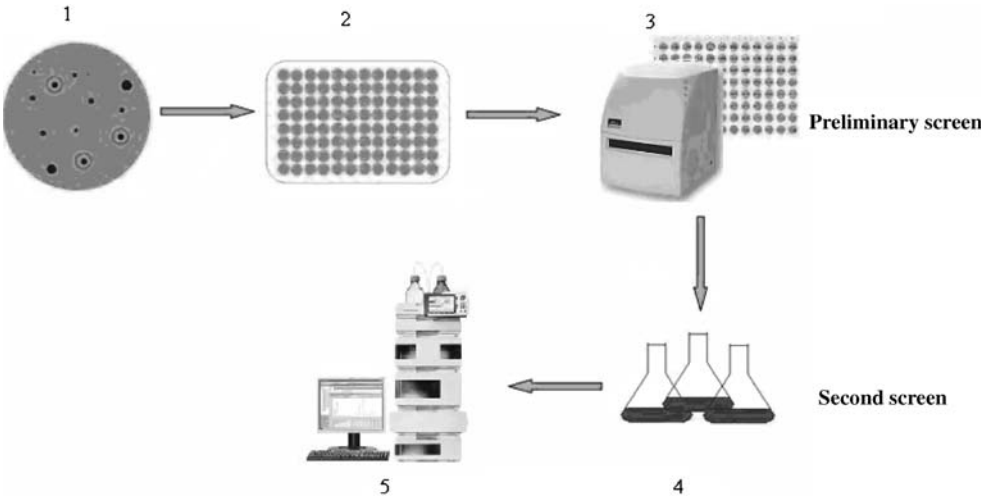

Fig. 4 Strategy for libraries screening on solid-state MTP cultures

Scale-up fermentation of selected strain

The mutants with higher avermectin B1a production in Fig. 5 were selected to be cultivated in a 360-m^3 fermentor, and PE1 exhibited the highest productivity. As shown in Fig. 6, PE1 (CGMCC No. 2712) exhibited remarkable avermectin B1a titer and productivity increase of 60% more than the parent strain (3-115). This indicated that the high-throughput method successfully selected the most potent avermectin B1a-producing strains, and the selected strains could be well adapted to industrial-scale production. The selected strain possessed the potential to greatly improve the industrial productivity of avermectins/avermectin B1a by fermentation.

Discussion

Although the strain improvement for avermectin production has been extensively studied and reviewed in recent years, the absence of high-throughput screening for overproducers limited the application of new tools such as genome shuffling, etc. In this paper, we developed a simple high-throughput culturing and screening strategy for avermectin-overproducing strains. The evaluation was performed on a 96-well MTP system. 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 avermectins at 245 nm. An industrial-scale test proved that the strain screened holds great potential for fermentation industry.

In such a screening strategy, mutants were cultured in 96 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 (Duetz et al. 2000; Hermann et al. 2003; Zimmermann et al. 2003).

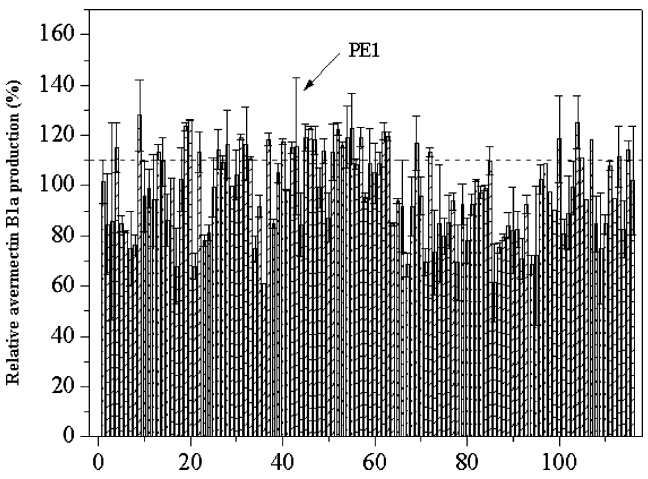

Fig. 5 Screening of avermectin B1a overproduction mutants. *Dashed line*, 110% production of the control strain

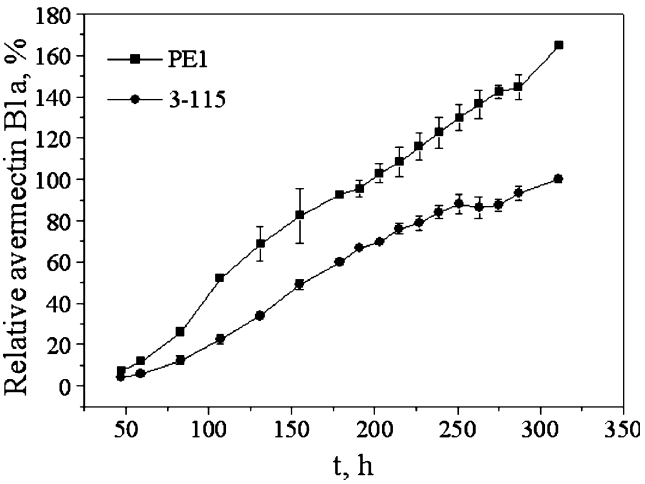

Fig. 6 The comparison of avermectin B1a production of parent strain 3-115 and mutant PE1 in 360-m^3 fermentor. The final titer of 3-115 strain was set as 100%, and the *error bars* represent standard deviations. For each strain, three fermentor studies were carried out

However, by considering cross-contamination, convenience of extraction, and other application factors, solid-state MTP culture was used in this paper. Results showed that feasibility of solid culture for screening high avermectin-producing strains as there was a good correlation between scales of MTP and Erlenmeyer flask cultures. The relative avermectins production by UV and by HPLC assay in both the Erlenmeyer flask cultures and solid-state MTP cultures had good correlation suggesting that the UV absorbance at 245 nm was a reliable indicator of avermectin production. Finally, we validated the high-throughput screening assay. These results suggested that this assay corresponds well to conventional Erlenmeyer flask culture and HPLC determination of avermectins. Based on the above results, we developed a selection strategy for utilizing the assay to screen large libraries of mutant strains. The strain identified in this paper was cultured in a fermentor, and the yield implied that the improved strain screened by the HTS method could be successfully applied and well-adapted to industrial fermentation processes.

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References

Askal HF, Refaat IH, Darwish IA, Marzouq MA (2008) A selective spectrophotometric method for determination of rosoxacin antibiotic using sodium nitroprusside as a chromogenic reagent. Spectrochim Acta A Mol Biomol Spectrosc 69:1287–1291

Buchs J (2001) Introduction to advantages and problems of shaken cultures. Biochem Eng J 7:91–98

Burg RW, Miller BM, Baker EE, Birnbaum J, Currie SA, Hartman R, Kong YL, Monaghan RL, Olson G, Putter I, Tunac JB, Wallick H, Stapley EO, Oiwa R, Omura S (1979) Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother 15:361–367

Curdova E, Jechova V, Zima J, Vanek Z (1989) The effect of inorganic phosphate on the production of avermectin in *Streptomyces avermitilis*. J Basic Microbiol 29:341–346

del Cardayré SB (2005) Developments in strain improvement technology: evolutionary engineering of industrial microorganisms through gene, pathway, and genome shuffling. In: Zhang L, Demain AL (eds) Natural products: drug discovery and therapeutics medicines. Humana, Totowa, NJ, pp 33–56

Dobson LF, O'Cleirigh CC, O'Shea DG (2008) The influence of morphology on geldanamycin production in submerged fermentations of *Streptomyces hygroscopicus* var. geldanus. Appl Microbiol Biotechnol 79:859–866

Du CY, Zhang YP, Li Y, Cao Z (2007) Novel redox potential-based screening strategy for rapid isolation of *Klebsiella pneumoniae* mutants with enhanced 1,3-propanediol-producing capability. Appl Environ Microbiol 73:4515–4521

du Toit EA, Rautenbach M (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J Microbiol Methods 42:159–165

Duetz WA, Ruedi L, Hermann R, O'Connor K, Buchs J, Witholt B (2000) Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Appl Environ Microbiol 66:2641–2646

Gao H, Liu M, Liu JT, Dai HQ, Zhou XL, Liu XY, Zhuo Y, Zhang WQ, Zhang L (2009) Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14–12A using response surface methodology. Bioresour Technol 100:4012–4016

Hermann R, Lehmann M, Buchs J (2003) Characterization of gas–liquid mass transfer phenomena in microtiter plates. Biotechnol Bioeng 81:178–186

Hwang YS, Kim ES, Biro S, Choi CY (2003) Cloning and analysis of a DNA fragment stimulating avermectin production in various *Streptomyces avermitilis* strains. Appl Environ Microbiol 69:1263–1269

Ikeda H, Omura S (1995) Control of avermectin biosynthesis in *Streptomyces avermitilis* for the selective production of a useful component. J Antibiot (Tokyo) 48:549–562

Ikeda H, Omura S (1997) Avermectin biosynthesis. Chem Rev 97:2591–2610

Isett K, George H, Herber W, Amanullah A (2007) Twenty-four-well plate miniature bioreactor high-throughput system: assessment for microbial cultivations. Biotechnol Bioeng 98:1017–1028

Kumar MS, Kumar PM, Sarnaik HM, Sadhukhan AK (2000) A rapid technique for screening of lovastatin-producing strains of *Aspergillus terreus* by agar plug and *Neurospora crassa* bioassay. J Microbiol Methods 40:99–104

Lee J, Hwang Y, Kim S, Kim E, Choi C (2000) Effect of a global regulatory gene, *afs*R2, from *Streptomyces lividans* on avermectin production in *Streptomyces avermitilis*. J Biosci Bioeng 89:606–608

Sams R (1993) Chemical assay of avermectins by high performance liquid chromatography with fluorescence detection. Vet Parasitol 48:59–66

Tyo KE, Zhou H, Stephanopoulos GN (2006) High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803. Appl Environ Microbiol 72:3412–3417

Xu ZN, Shen WH, Chen XY, Lin JP, Cen PL (2005) A high-throughput method for screening of rapamycin-producing strains of *Streptomyces hygroscopicus* by cultivation in 96-well microtiter plates. Biotechnol Lett 27:1135–1140

Yu H, Tyo K, Alper H, Klein-Marcuschamer D, Stephanopoulos G (2008) A high-throughput screen for hyaluronic acid accumulation in recombinant *Escherichia coli* transformed by libraries of engineered sigma factors. Biotechnol Bioeng 101:788–796

Zhang L (2005a) Integrated approaches for discovering novel drugs from microbial natural products. In: Zhang L, Demain AL (eds) Natural products: drug discovery and therapeutics medicines. Humana, New Jersey, pp 33–56

Zhang L (2005b) Screening for synergistic compounds. PCT Patent WO 2005/051303

Zhang L, An R, Wang J, Sun N, Zhang S, Hu J, Kuai J (2005) Exploring novel bioactive compounds from marine microbes. Curr Opin Microbiol 11:655–662

Zhang L, Yan K, Zhang Y, Bian J, Zheng C, Sun H, Chen Z, Sun N, Song Y, Zhuo Y, You J et al (2007) High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. PNAS 104:4606–4611

Zimmermann HF, John GT, Trauthwein H, Dingerdissen U, Huthmacher K (2003) Rapid evaluation of oxygen and water permeation through microplate sealing tapes. Biotechnol Prog 19:1061–1063