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Efficient production of ε-poly-L-lysine from glucose by two-stage fermentation using pH shock strategy

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ABSTRACT

In this study, a pH shock strategy was employed to enhance ε-poly-L-lysine (ε-PL) production from glucose. In the conventional fermentation, in the early stage, only 13% of total ε-PL production is achieved in 25% of the entire fermentation period, which severely affected ε-PL productivity. To improve the efficiency of ε-PL production during fermentation, a novel two-stage fermentation, namely culture and fermentation stages, was proposed on the basis of the analysis of conventional pH shock fermentation. After optimization of parameters such as inoculum growth conditions, initial fermentation pH, and inoculum volume, the ε-PL production and productivity achieved using the novel fermentation process in a 5-L fermenter reached 32.22 g/L and 5.86 g/L/day, which were 32.3% and 36.6% higher, respectively, when compared with those obtained in conventional fermentation. Furthermore, evaluation of acid tolerance of mycelia collected from the pH shock fermentation showed that pH shock enhanced ε-PL production, which might be related to the acid tolerance of *Streptomyces albulus* and pH stress (pH 3.0). The results obtained could be useful for large-scale ε-PL production and to provide new information on ε-PL biosynthesis mechanism.

1. Introduction

ε-Poly-L-lysine (ε-PL), which is a naturally occurring homopolymer (amino acid), consists of 25–35 L-lysine residues that are polymerized by ε-amide linkages [1]. Because of the presence of many free amino groups in the main chain, ε-PL displays multi-cation characteristics in acidic to slightly alkaline environments. Moreover, ε-PL has strong antibacterial activity against a wide spectrum of microorganisms, including most gram-positive and gram-negative bacteria, fungi, yeasts, and some viruses. In addition, this polymer is biodegradable and non-toxic. As a result, ε-PL has been routinely used as a natural and safe food preservative for many years in the food industry in Japan, Korea, the USA, and China. Concurrently, there has been significant interest in the applications of ε-PL and its derivatives in medical and other industries [2]. To meet the growing demand for ε-PL in various fields, numerous studies aimed at improving the production of ε-PL have been conducted.

ε-PL is predominantly produced by *Streptomyces albulus* by submerged fermentation[3]. In the past four decades, breeding of high-yield strains and development of efficient bioprocesses have become the core areas of research. In particular, many strategies with some clear objectives have been adopted to enhance ε-PL production. For example, to overcome potential end-product feedback inhibition or avoid product toxicity during ε-PL production, an *in situ* product removal method based on resin was used to increase the production of ε-PL to 23.4 g/L in 192 h in a 5-L fermenter during fed-batch fermentation, which was 6.2-fold higher than that obtained in the control [4]. Furthermore, to reduce energy consumption during ε-PL production, airlift bioreactor [5], immobilized cells with repeated fed-batch cultures [6], and solid-state fermentation [7] have been employed instead of the popular approach of using free cells in a jar fermenter. In addition, for providing optimal and cheap nutrition and a suitable environment for ε-PL-producing strains and increasing ε-PL production, carbon/nitrogen source selection and dissolved oxygen (DO) regulation have been systematically studied [8–13].

When compared with the above-mentioned strategies, pH control is the most efficient method for enhancing ε-PL production (Table 1). It has been reported that the control of pH in the range of 3.5-4.5 could inhibit the activity of ε-PL-degrading enzyme and accumulate intracellular ATP for ε-PL synthetase activity[14], which are the prerequisites for achieving ε-PL accumulation in strains producing this polymer. On the contrary, at pH above 5.0, no ε-PL production was noted during fermentation in the presence of high ε-PL-degrading enzyme activity; however, this pH was beneficial for mycelium growth. Accordingly, a two-stage pH control strategy was developed by Kahar et al. [3], which enhanced ε-PL production to 48.3 g/L, thus helping

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Table 1
Comparison of parameters using different fermentation strategies.

Strain	pH controlling strategy	ε-PL (g/L)	DCW (g/L)	Productivity (g/L day)	Fermentation Time (h)	Yield (%)	Reference
S. albulus S410	pH 6.8 → 5 → 4	48.3	27	6.04	192	18.6	Kahar et al. [3]
S. albulus IFO14147	pH 6.8 → 4	5.20	34.2	0.52	240	/	Shih et al. [24]
S. albulus TUST2	pH 6.8 → 4	20.0	18	4.00	120	/	Jia et al. [12]
Streptomyces sp. GIM8	pH 6.8 → 3.8	23.4	7.3	2.86	196	/	Liu et al. [4]
S. noursei NRRL 5126	pH 6.8 → 4 → 3	1.99	20.7	0.33	144	/	Bankar et al. [7]
S. albulus PD-1	pH 6.8 → 5 → 4	34.20	33.4	4.88	168	/	Xu et al. [11]
Kitasatospora sp. MY 5–36	pH 6.8 → 4	34.11	23.0	9.34	87.6	/	Zhang et al. [13]
Streptomyces sp. M-Z18	pH 6.8 → 3.0 → 3.8	54.7	76.4	6.84	192	/	Ren et al. [18]
Streptomyces sp. M-Z18	pH 6.8 → 3.0 → 3.85	38.0	60.5	4.76	192	5.7	This study
Streptomyces sp. M-Z18	pH 5.0 → 3.85	32.22	47.9	5.86	132	8.2	This study

achieve a ε-PL production capacity of 1000 t/a in Japan. In our previous study, glycerol was used as a carbon source for ε-PL production for the first time [15]. Moreover, a novel two-stage pH control strategy was proposed on the basis of kinetic analysis, and a ε-PL production of 30.11 g/L was achieved [16]. In our recent study, a pH shock strategy, in which the pH was spontaneously decreased from 4.0 to 3.0 in 12 h, was developed. This method significantly improved the production of ε-PL from glycerol (54.70 g/L ε-PL in 192 h of fed-batch fermentation), which is the highest ε-PL production reported to date. After pH shock, mycelial respiration and key enzymes in the central metabolic and ε-PL biosynthetic pathways are generally strengthened until the end of the fed-batch fermentation [17]. However, this strategy is not without limitations. First, a high amount of mycelium, with a dry cell weight (DCW) of 76.35 g/L, significantly affects ε-PL yield. Second, the high biomass results in high viscosity of the fermentation broth, decreasing the oxygen transfer rate and increasing power consumption. Finally, the use of glycerol as the carbon source for ε-PL biosynthesis by *S. albulus* reduced the polymerization by 2–3 degrees, when compared with that achieved using glucose as the carbon source; thus, this could decrease the antibacterial activity of ε-PL.

To overcome these limitations, in the present study, we used glucose as the carbon source and optimized the process of pH shock fermentation. From the results of fed-batch pH shock fermentation using glucose as the carbon source, we divided the conventional pH shock fermentation into two stages and developed a novel two-stage fermentation strategy for ε-PL production from glucose after optimizing some parameters, such as inoculum growth conditions, initial fermentation pH, and inoculum volume. Finally, the bioprocess parameters and advantages of the optimized pH shock fermentation process were compared with those of conventional fermentation, and the reasons for enhanced ε-PL biosynthesis following pH shock are also discussed here.

2. Materials and methods

2.1. Microorganism

S. albulus M-Z18 was used in this study, which is a mutant of *S. albulus* Z-18 (CGMCC 10479). The bacterial culture was stored in broth containing 20% (v/v) glycerol at −80 °C for long-term preservation.

2.2. Inoculum preparation

For the production of spores from surface cultures, a stock culture was plated on BNT agar and incubated for 7–8 days at 30 °C. The spores were harvested using a NaCl solution (0.85% w/v), and the liquid was agitated using a vortex mixer for breaking the spore chains. Then the suspension was filtered through sterilized non-absorbent cotton wool to remove mycelial fragments and pieces of agar medium. Finally, the concentration of the spore suspension was maintained at 10^6 spores/mL, which was determined using a hemocytometer, by centrifugation and dilution.

A 1-mL spore suspension was used to inoculate a 500-mL shake flask containing 80 mL seed medium, and the culture was cultivated at 30 °C on a rotary shaker (HYL-C, Qiangle Experimental Co., Ltd, Taicang, China) at 200 rpm for 24 h. These cultures were used for all fermentations in this study.

2.3. Culture and fermentation media composition

BNT agar [17] was used for spore preparation; BNT agar composition was as follows (g/L): glucose, 10; peptone, 2; yeast extract, 1; and agar, 20, with an initial pH of 7.5 adjusted by 1 M NaOH and/or 1 M H_2SO_4 solution.

M3G medium [3] as seed medium was used for seed cultivation in this study; its composition was as follows (g/L): glucose, 50; yeast extract, 5; $(NH_4)_2SO_4$, 10; KH_2PO_4, 1.36; $K_2HPO_4{\cdot}3H_2O$, 0.8; $MgSO_4{\cdot}7H_2O$, 0.5; $ZnSO_4{\cdot}7H_2O$, 0.04; $FeSO_4{\cdot}7H_2O$, 0.03, with an initial pH of 6.8 adjusted by 1 M NaOH and/or 1 M H_2SO_4 solution.

The fermentation medium [18] was the same as that used in our previous study but with minor modifications (g/L): glucose, 60; $(NH_4)_2SO_4$, 10; beef extract, 10; KH_2PO_4, 4; $MgSO_4{\cdot}7H_2O$, 0.8; and $FeSO_4{\cdot}7H_2O$, 0.05, with an initial pH of 6.8 adjusted by 1 M NaOH and/or 1 M H_2SO_4 solution.

All media components were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and were of analytical and biochemical grades. All the media were sterilized in an autoclave at 121 °C for 20 min. In each case, glucose was autoclaved separately.

2.4. Fermentation in a 5-L stirred tank bioreactor

Fermentation in a 5-L glass stirred tank bioreactor (Biotech-5BG, Baoxing Bio-Engineering Equipment Co., Ltd., Shanghai, China) was performed by filling it 3.5 L of media. Before the inoculation, the sterilized bioreactor's temperature, aeration rate, and agitation speed were maintained at 30 °C, 0.5 vvm, and 200 rpm, respectively, and the initial pH was controlled at 6.8 by the manual addition of ammonia water (12.5%, w/v). When the system was stable, 300 mL of 24-h-old seed culture was inoculated into the bioreactor. During the fermentation, pH and DO were monitored online using pH and DO electrodes (K8S-225 and InPro6800-12-220, Mettler Toledo, Zurich, Switzerland), respectively. The DO was maintained above 30% of air saturation before pH declined to 4.0; afterward, the DO was maintained above 20% of air saturation until the end of fermentation, which was controlled by adjusting agitation speed from 200 to 950 rpm. When agitation speed reached 800 rpm, aeration rate was then manually increased stepwise with steps of 0.5 vvm and a range of 0.5–2.5 vvm. In addition, when the glucose concentration in the fermentation broth was below 10 g/L, sterilized glucose (90%, w/v) was automatically added and maintained at about 10 g/L for preventing carbon source limitation. Moreover, when the ammonia nitrogen (NH_4^+-N) concentration decreased below 0.5 g/L, sterilized $(NH_4)_2SO_4$ solution (40%, w/v) was automatically added and maintained at about 0.5 g/L for preventing nitrogen source

limitation. Those conditions were used throughout this study when fermentations were performed in the 5-L bioreactor. However, different fermentation strategies were used for the regulation of pH.

In the pH shock fermentation from glucose experiment (Section 3.1), the pH was controlled according to our previously developed method by Ren et al. [16] with some modifications: pH was spontaneously decreased from initial pH 6.80 to pH 5.00, then maintained constant at pH 5.00 by the automatic addition of ammonia water (12.5%, v/v) for 8 h. After that, the pH was uncontrolled for 12 h, then recovered to 3.85, and maintained until DO recovery to below 50% air saturation; finally, the pH was adjusted to 3.85, and it remained stable until the end of fermentation at 192 h.

In the two-stage fermentation validation experiment (Section 3.3), the initial pH of the fermentation medium was 5.00, which was inoculated for the first of stage cultivation with 15% (v/v) inoculation volume at a DO recovery to 50% air saturation. The pH was maintained constant at 3.85 until the end of fermentation at 132 h.

2.5. Fermentation in a 1-L multi-bioreactor system

The fermentations were performed in a 1-L glass bioreactor system (T & J Bio-Engineering Co. Ltd., Shanghai, China) consisting of four identical stirred tank reactors with a filling volume of 0.6 L. The broth was agitated at 300 rpm with a mechanical stirrer at 30 °C with a 1 vvm aeration rate. The initial pH was adjusted to 6.80 by adding ammonia water (12.5%, v/v), and then the broth was inoculated with 50 mL of 24-h-old seed culture. During the fermentation, pH and DO were also monitored online using pH and DO electrodes, respectively (K8S-120 and InPro6800-12-120, Mettler Toledo, Zurich, Switzerland). The control of DO was the same as that in the abovementioned 5-L bioreactor. However, the agitation speed was varied from 300 to 1500 rpm, and aeration rate was manually increased stepwise with steps of 0.5 vvm and a range of 1–3 vvm. Moreover, the controls of carbon and nitrogen sources were also the same as that in the 5-L bioreactor.

In the fermentation condition optimization experiments (Section 3.2), one bioreactor was used for the first stage of cultivation, and the other three bioreactors were used for the second stage fermentation. All experiments were divided into three groups, shown in Table 2: (i) inoculum growth condition optimization: when the DO of first stage of cultivation recovered to 95%, 50%, and 20%, cultures with 15% inoculum volume were separately transferred into the other three reactors with an initial pH of 5.00, and the second cultivation was started; (ii) initial pH of fermentation optimization: when the DO of first stage of cultivation recovered to 50%, cultures with 15% inoculum volume were separately transferred into the other three reactors with an initial pH of 5.00, 4.00, and 3.85, and the second fermentation was started; and (iii) inoculation volume optimization: when the DO of first stage of cultivation recovered to 50–60%, cultures with 10%, 15%, and 20% inoculum volume were separately transferred into the other three reactors at an initial pH of 5.00, and the second fermentation was started. All fermentations were fed-batch fermentations performed for 132 h.

2.6. Acid tolerance and genetic stability assays

To evaluate the acid tolerance of *S. albulus* M-Z18 during the pH shock fermentation, biomass was drawn from the bioreactors with and without pH shock at 60 h (without pH shock as control experiment), then diluted to the same multiple (10^{-2}) with sterile water on a super-clean bench, immediately plated on BTN agar with pH 3.85 (10% agar content), and incubated at 30 °C for 8 days to observe the growth of mycelia and production of spores. Subsequently, the spore pretreatment and seed culture were conducted as in Section 2.2.

To further evaluate the genetic stability of *S. albulus* M-Z18 after pH shock strategy (without pH shock as control), the biomass was drawn from the bioreactors with pH shock at 60 h (without pH shock as control experiment), then diluted to the same multiple (10^{-1}) with sterile water on a super-clean bench, immediately plated on BTN agar with pH 6.8 (2% agar content), and incubated at 30 °C for 8 days to obtain mycelia and production of spores. The spores were used for seed cultivation and inoculated into a 1-L multi-bioreactor system for testing their fermentation ability. The batch fermentation conditions were as following: when the pH spontaneously dropped to 3.85 from the initial pH of 6.80, pH of 3.85 was maintained by automatically adding ammonia water (12.5%, v/v) until the carbon source depleted. The DO was maintained above 30% of air saturation before the pH declined to 3.85; then, the DO was maintained above 20% of air saturation until the end of fermentation, which was controlled by adjusting the agitation speed from 300 to 1500 rpm. The other conditions were the same as in Section 2.5.

Table 2
Levels of selected fermentation conditions for inoculation.

Parameters	Levels		
	1	2	3
Inoculum growth conditions[a]	95%	50%	20%
Initial pH	5.0	4.0	3.85
Inoculums volume (%)	10	15	20

[a] The levels of inoculation time indicate the DO value in seed culture after recovery.

2.7. Analytical methods

All samples were centrifuged at 4500*g* for 10 min at 20 °C. For the determination of the DCW, the precipitate was collected and washed twice with deionized water. Then the washed mycelia were filtered through a pre-weighed filter paper and dried at 105 °C to a constant weight. The supernatant was used for further analysis. The ε-PL concentration was determined using the method described by Itzhaki [19]. Briefly, the supernatant was diluted at a suitable multiple, and then 2.0 mL diluent was mixed with 2.0 mL 1 mM MeO solution (pH 6.90). After vortex mixing, the precipitation reaction was incubated at 30 °C for 30 min in a shaker at 200 rpm. Subsequently, the mixture was centrifuged at 4500*g* for 15 min. The supernatant was diluted 20-fold, and its absorbance was measured at 465 nm. The concentration of glucose was determined using a biosensor (SBA-40C, Biology Institute of Shandong Academy of Sciences, Shandong, China), and NH_4^+-N was analyzed colorimetrically using Nessler's reagent [20].

2.8. Statistical analysis

The values reported are the means of three replications. The results are expressed as mean ± standard deviation (mean ± S.D.). The difference between experimental group and control group in this study was distinguished by statistical analysis using the GraphPad Prism 6.0 and p-value < 0.05 was considered significant.

3. Results

3.1. Production of ε-PL from glucose using pH shock strategy in 5-L fermenter

In addition to being an efficient strategy for enhancing secondary metabolite production in *Streptomyces* spp., pH shock has also been proved to be effective in increasing ε-PL production in *S. albulus* M-Z18 from glycerol [16]. This strategy was adopted to improve glucose-based ε-PL production, and the results of fed-batch fermentation are shown in Fig. 1. The ε-PL production and DCW reached 38.06 and 60.6 g/L after 192 h of fed-batch fermentation, which were 55.2% (24.54 g/L) and 146.9% (24.53 g/L) higher than those achieved using the two-stage pH control strategy, respectively [22], indicating that the pH shock strategy is also beneficial for increasing ε-PL production using glucose

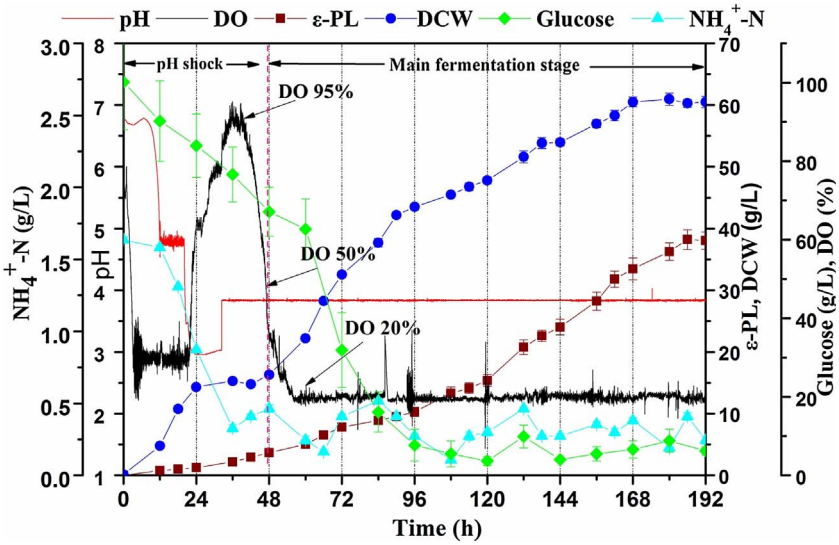

Fig. 1. Time profile of ε-PL production from glucose using pH shock strategy by fed-batch fermentations in a 5-L fermenter.

as the carbon source. As described in Fig. 1, both cell growth and ε-PL production slowly increased before 48 h (culture stage) and then were simultaneously enhanced rapidly until the end of fermentation (192 h, fermentation stage). It must be noted that in the culture stage, cell growth occupied almost one-fourth of the entire fermentation with limited ε-PL production (5 g/L), which could severely affect ε-PL productivity and waste the energy input during fermentation. Therefore, this fermentation was divided into two stages (Fig. 1): with the first stage of culture, including pH shock and mycelia recovery, was performed in seed culture fermenter and the second stage comprised cell culture inoculation into the fermenter and initiation of main fermentation. To conduct the novel two-stage fermentation, some key parameters, such as inoculum growth conditions, inoculum volume, and initial fermentation pH, were first optimized.

3.2. Optimization of parameters of two-stage fermentation in a 1-L multi-bioreactor system

3.2.1. Effect of inoculum growth conditions on ε-PL production

It is well known that seed culture age represents the physiological state of the seed culture; therefore, inoculation time could significantly influence ε-PL production. In the present study, inoculation time was determined according to the DO level in the first culture stage after pH recovery to 4.0. To investigate the effect of inoculation time on ε-PL production from glucose by *S. albulus* M-Z18, the fed-batch fermentation systems were inoculated at three different DO levels: 95%, 50%, and 20% (indicated by arrows in Fig. 1). As shown in Fig. 2, irrespective of the inoculation time, cell growth did not show a lag phase. The mycelia exhibited exponential growth in 48 h in the three fermentation systems and subsequently reached stationary phase with maximum DCW of 42.5, 38.72, and 38.05 g/L until the end of fermentation (132 h). Meanwhile, the production of ε-PL in the three fermentation systems steadily increased from 24 h of fermentation and finally reached 12.09, 15.56, and 12.75 g/L, respectively. In addition, ε-PL productivity and yield in the system inoculated at 50% DO (2.83 g/L/day, 6.22%) were higher than those achieved in systems inoculated at 95% DO (2.20 g/L/day, 5.49%) and 20% DO (2.32 g/L/day, 5.83%) (Table 3). These findings indicated that seed cultures inoculated at 50% DO might possess mycelia with better environmental adaptability and ε-PL biosynthesis ability, which is similar to the findings observed in our previous study [23]. As the fermentation system inoculated at 50% DO level showed the highest ε-PL concentration, productivity, and yield, seed inoculation at 50% DO level was selected as the seed transfer criterion in subsequent analyses.

3.2.2. Effect of initial pH on ε-PL production

The initial pH might influence mycelial growth and ε-PL production following inoculation of first-stage culture into second-stage fermentation. Thus, the effects of the initial pH on cell growth and ε-PL production were evaluated, and the results are shown in Fig. 3. The changes in pH exhibited the following trend: the initial pH (5.0, 4.0, and 3.85) decreased to pH 3.85 by 12 h after a slight increase. The mycelia grew rapidly at the initial pH of 5.0 before 12 h and maintained this trend until the end of fermentation with a maximum DCW of 48.52 g/L, when compared with that noted at the initial pH of 4.0 (41.58 g/L) and 3.85 (39.85 g/L). Moreover, the highest ε-PL production (17.75 g/L) was achieved at the initial pH of 5.0, which was 10.04% and 39.22% higher than that obtained at the initial pH of 4.0 (16.13 g/L) and pH 3.85 (12.75 g/L), respectively. It is also worth mentioning that there was a significant difference in ε-PL production between pH 4.0 and pH 3.85, indicating that the initial fermentation pH plays an important role in ε-PL production. Moreover, the ε-PL productivity and yield at the initial pH of 5.0 (3.23 g/L/day, 5.87%) were also higher than those achieved at pH 4.0 (2.93 g/L/day, 5.51%) and pH 3.85 (2.32 g/L/day, 5.23%) (Table 3). Thus, considering ε-PL production, productivity, and yield, an initial pH of 5.0 was employed in subsequent analyses.

3.2.3. Effect of inoculum volume on ε-PL production

Inoculum volume plays an important role in fermentation. While small inoculum volume can cause slow cell growth in the early stage of fermentation and decrease the productivity, larger inoculum volumes can result in rapid cell growth, leading to lack of nutritional substrates or DO deficiency and decreased productivity [23]. To determine optimal inoculum volume in the second stage of fermentation, the first-stage culture was inoculated at three inoculum volumes, i.e., 10%, 15%, and 20% (v/v) (Fig. 4). The results indicated that all the three inoculum volumes produced similar trends in mycelial growth and a DCW of 41–43 g/L. However, when the inoculum volume was increased from 10% to 15%, ε-PL production also increased and reached a maximum of 18.29 g/L. However, a further increase in the inoculum volume to 20% did not achieve significant improvement in the final ε-PL production (18.61 g/L),when compared with that obtained with 15% inoculum volume ($p = 0.7151 > 0.05$). Furthermore, the ε-PL productivity and yield achieved with 15% inoculum volume reached 3.33 g/L/day and 6.13%, which were similar to those obtained with 10% and 20% inoculum volume (Table 3). Taken together, to achieve the best DCW, ε-PL concentration, ε-PL productivity, and ε-PL yield, the second stage of fermentation should be performed at an initial pH of 5.0 with first-stage

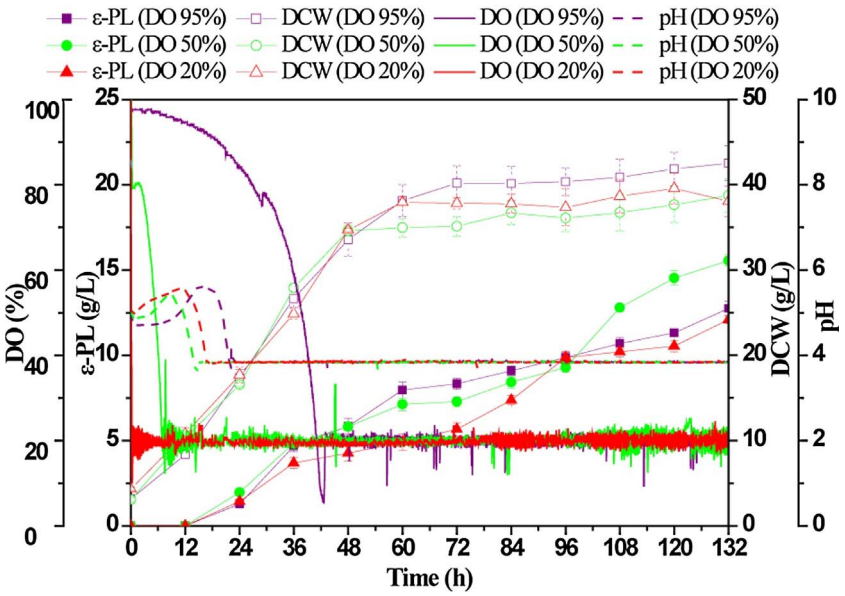

Fig. 2. Time profiles of ε-PL production at different inoculum growth conditions in fed-batch fermentation.

culture recovered from pH shock and inoculated at 50% DO level and 15% inoculum volume.

3.3. Production of ε-PL from glucose using two-stage fermentation strategy in a 5-L fermenter

Following optimization of the fermentation parameters, a novel two-stage fermentation strategy based on the modified conventional pH shock fermentation was developed and performed in a 5-L jar fermenter. As shown in Fig. 5, the DO level decreased to 20% and the pH was reduced to 3.85 in 5 h after a brief increase. The mycelia grew rapidly in the initial 48 h and subsequently reached the stationary stage with the highest DCW of 47.94 g/L. Meanwhile, ε-PL steadily accumulated in the culture broth from the start of fed-batch fermentation and finally reached 32.22 g/L by 132 h. Although the final ε-PL concentration was lower than that achieved in the pH shock fermentation, the productivity and yield of ε-PL reached 5.86 g/L/day and 8.2%, which were 23.1% and 43.9% higher than that obtained in the pH shock fermentation, respectively (Table 3).

3.4. Evaluation of acid tolerance and genetic trait of S. albulus M-Z18 following pH shock

When compared with the control, *S. albulus* M-Z18 cells subjected to pH shock (acidic pH stress [pH 3.0] for 12 h) showed a significant increase in ε-PL production. To evaluate the effect of acidic pH stress on *S. albulus* M-Z18, the acid tolerance of these cells was investigated at pH 3.85. As shown in Fig. 6a, mycelia subjected to pH shock could form numerous colonies on the agar plate, whereas those not subjected to pH shock formed only a few colonies, thus indicating that *S. albulus* M-Z18 developed strong acid tolerance after pH shock, which might be responsible for its high ε-PL production.

Subsequently, we examined whether pH shock could endow *S. albulus* M-Z18 with high ε-PL production genetic traits. For this purpose, *S. albulus* M-Z18 was cultivated on BNT agar plates. After 8 days of incubation, the spores were used as seed, cultured in a 250-ml flask for 24 h, and then inoculated into a 1-L jar fermenter for batch production. The batch fermentation results showed that the time profiles of *S. albulus* M-Z18 with and without pH shock were the same, implying that pH shock did not endow *S. albulus* M-Z18 with high ε-PL production genetic traits (Fig. 6b). Therefore, it can be concluded that high ε-PL productivity might be the result of signal transduction produced by low pH-induced environmental stress and not caused by genetic changes.

4. Discussion

When compared with the original pH shock fermentation, the novel two-stage fermentation process exhibited two advantages. First, the two-stage fermentation process decreased the fermentation time by 60 h. Fed-batch fermentation is a common approach to produce ε-PL, which is aimed at maximum ε-PL accumulation in the fermentation broth to reduce production cost, including the cost of the fermentation and extraction processes. As a result, 168–192 h has become the standard duration for ε-PL fermentation (Table 1). However, such a long fermentation time period could increase the risk of contamination, increase power consumption [24] (aseptic air preparation and mixing), and produce other disadvantages. Therefore, reduction of the fermentation duration is the appropriate method to decrease the cost of ε-PL production while maintaining a relatively high production level. The novel two-stage fermentation process developed in this study presented

Table 3
Results of seed transferring condition optimization.

Parameters	Inoculum growth conditions			Initial pH			Inoculation volume		
	95%	50%	20%	5.0	4.0	3.85	10%	15%	20%
Fermentation time (h)	132	**132**	132	**132**	132	132	132	**132**	132
ε-PL production (g/L)	12.09	**15.56**	12.75	**17.75**	16.13	12.75	16.28	**18.29**	18.61
DCW (g/L)	42.5	**38.72**	38.05	**48.52**	41.58	39.85	41.10	**41.47**	43.18
Productivity (g/L/day)	2.20	**2.83**	2.32	**3.23**	2.93	2.32	2.96	**3.33**	3.38
Yield (g/g)	5.49	**6.22**	5.83	**5.87**	5.51	5.23	5.94	**6.13**	6.07

Bold values signifies the optimal results in each condition.

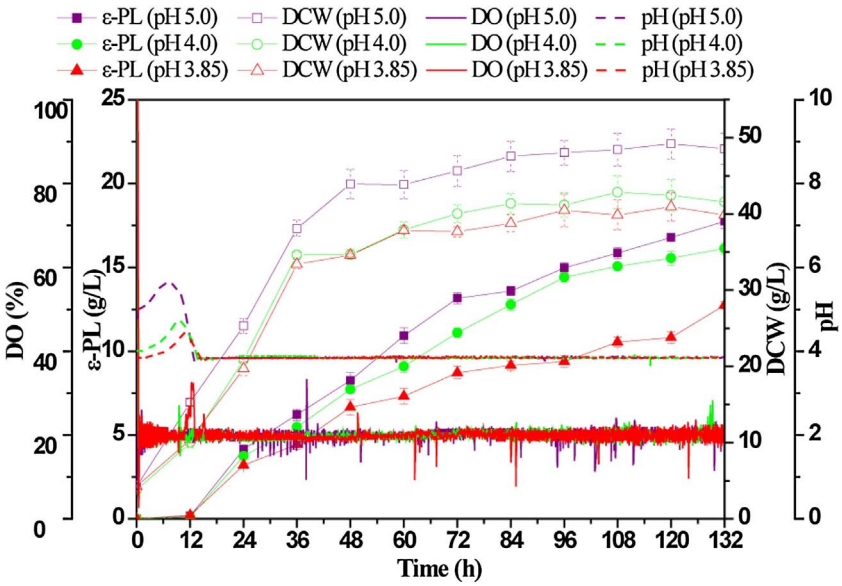

Fig. 3. Time profiles of ε-PL production at different initial pH values in fed-batch fermentation.

a 31.25% reduction in fermentation duration, when compared with pH shock fermentation, and achieved 32.2 g/L ε-PL production in 132 h. To the best of our knowledge, this study is the first to report a ε-PL production of more than 30 g/L in 132 h in a jar fermenter. Furthermore, ε-PL productivity was also improved, reaching 5.86 g/L/day, which was 23.1% higher than that achieved using glucose in the original pH shock fermentation. In addition, the ε-PL yield attained was 8.2%, which was 43.9% higher than that noted in the pH shock fermentation, indicating that the utilization of raw materials had increased. Interestingly, reduction of the fermentation duration could also increase batch production and improve the production capacity in industries. Thus, the novel fermentation strategy could be significantly important for the rapid and efficient production of ε-PL.

Moreover, the novel two-stage fermentation strategy could considerably decrease power consumption. In the novel two-stage fermentation process, the first stage comprised pH shock and mycelia viability recovery, while the second stage included ε-PL production at a constant pH of 3.85. As the first stage could be combined with seed cultivation, the fermenter volume could be as low as 1/5–1/10 the volume of the second-stage fermenter, indicating that the consumption of aseptic air could be reduced by 80%–90% at the same aeration rate (vvm). In fact, ε-PL fermentation is a high oxygen consumption bioprocess, with the power consumption for aseptic air preparation covering over 50% of the cost of ε-PL fermentation. Thus, the novel two-stage fermentation strategy could reduce the consumption of aseptic air and decrease the cost of fermentation. Moreover, this process produced a DCW of 47.9 g/L, which was 20.8% lower than that produced in the original fermentation process. It has been reported that lower DCW could result in decreased viscosity of the fermentation broth and increased oxygen transfer coefficients [25]. These changes in turn could reduce the consumption of aseptic air and power for stirring during fermentation and ultimately decrease the total power consumption. Thus, the advantages of shortened fermentation duration and reduced power consumption exhibited by the novel two-stage fermentation strategy developed in this study could decrease ε-PL production costs.

It must be noted that ε-PL production by *S. albulus* M-Z18 following pH shock fermentation was similar irrespective of the use of glucose or glycerol as the carbon source. Therefore, we evaluated the acid tolerance of *S. albulus* M-Z18 after pH shock and found that the acid tolerance of *S. albulus* M-Z18 after pH shock was significantly higher than

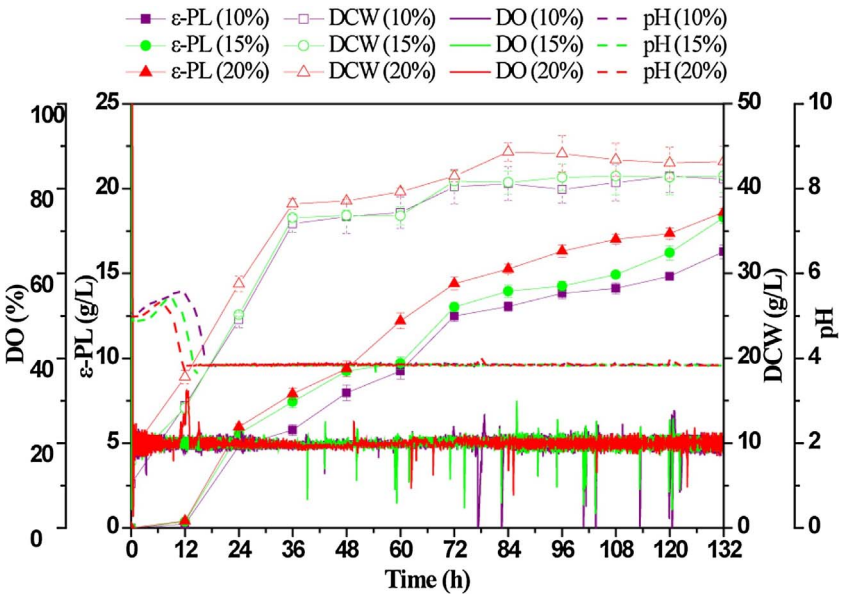

Fig. 4. Time profiles of ε-PL production at different inoculums volumes in fed-batch fermentation.

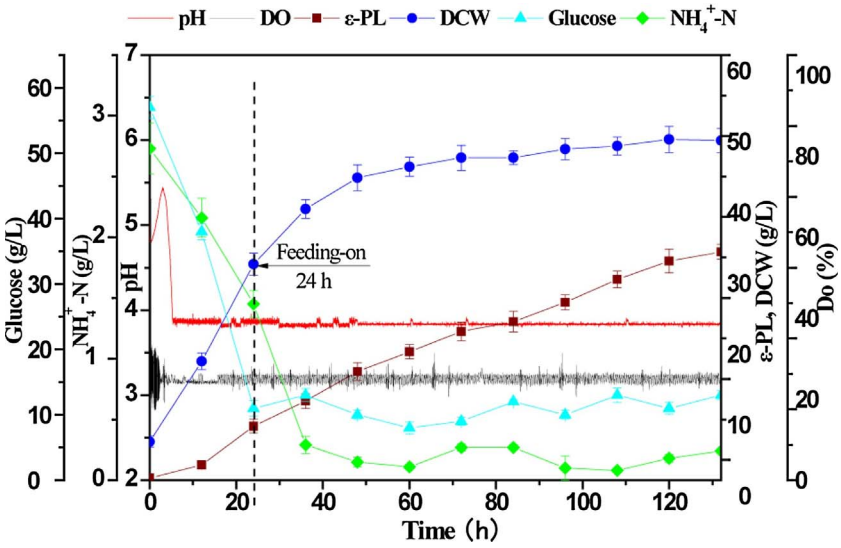

Fig. 5. Fed-batch fermentations for ε-PL production with optimal conditions in a 5-L fermenter.

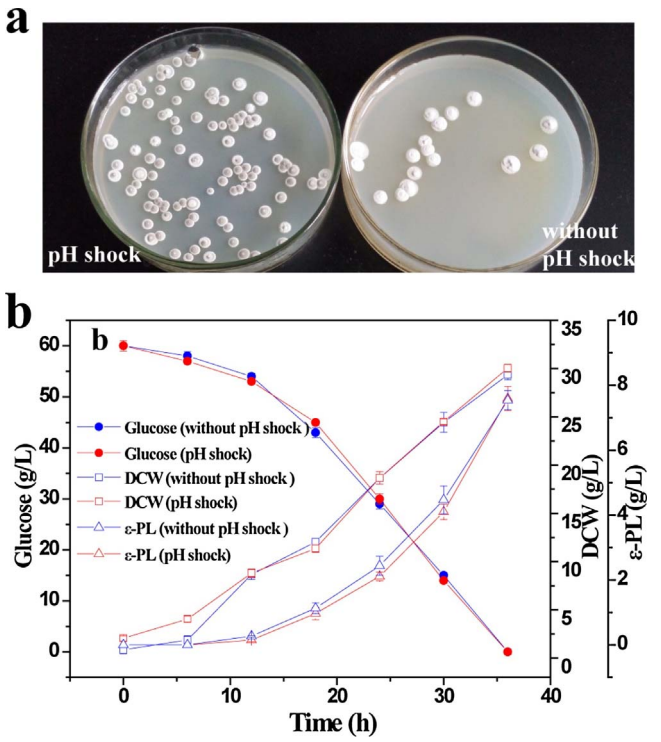

Fig. 6. Growth (a) and batch fermentation (b) of *S. albulus* M-Z18 with experimental (with pH shock) and control (without pH shock) group.

that of cells not subjected to pH shock (Fig. 6a), suggesting that enhanced acid tolerance may be related to the increased ε-PL production. To further investigate the genetic traits of *S. albulus* M-Z18 with high ε-PL production, spores of *S. albulus* M-Z18 derived from mycelia subjected to pH shock were inoculated in batch fermentation. The results showed that the high ε-PL production trait of *S. albulus* M-Z18 was not inherited, indicating that this trait might result from environmental stress (acidic pH of 3.0) during pH shock. It must be noted that *S. albulus* M-Z18 can spontaneously adapt to an environmental pH of 3.0, whereas most of the *Streptomyces* spp. cannot thrive in environments with pH lower than 4.5 [26,27]. Therefore, acidic pH stress might have changed the physiological state of *S. albulus* M-Z18 and subsequently triggered rapid mycelial growth and overproduction of ε-PL.

It has been reported that the production of actinorhodin and kasugamycin could also be markedly enhanced by the application of pH shock in the fermentation process [28,29]. It was confirmed that pH shock upregulated the expression of a wide range of stress-response genes, including those of the biosynthesis pathway regulator, secretion system, two-component systems, various sigma factors, and shock-related proteins. These changes synergistically contributed to enhanced actinorhodin productivity by mediating the pH shock signal to regulators or regulating the biosynthesis genes for actinorhodin production [30–32]. Thus, it can be reasonably assumed that the signal regulatory system is involved in ε-PL biosynthesis during pH shock fermentation.

5. Conclusion

In conclusion, a novel two-stage fermentation strategy was developed by dividing the conventional pH shock fermentation process into two stages after optimizing the parameters, including inoculum growth conditions, initial fermentation pH, and inoculum volume. Under the optimal conditions, ε-PL production and productivity reached 32.22 g/L and 5.86 g/L/day after 132 h of fed-batch fermentation in a 5-L fermenter, which were 32.3% and 36.6% higher, respectively, when compared with those achieved in conventional pH shock fermentation. As a result, the proposed strategy might significantly reduce the cost of fermentation and increase the profit gained from industrial production of ε-PL. Moreover, enhancement of ε-PL production following pH shock might be related to the acid tolerance of *S. albulus* M-Z18 and acidic pH stress (pH 3.0). The results obtained in this study could be useful for large-scale ε-PL production and to provide new information on ε-PL biosynthesis mechanism.

Acknowledgements

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References

[1] S. Shima, H. Sakai, Polylysine produced by *Streptomyces*, Agric. Biol. Chem. 41 (9) (1977) 1807–1809.
[2] M. Saimura, S. Takehara, K. Mizukami, Biosynthesis of nearly monodispersed poly (ε-l-lysine) in *Streptomyces species*, Biotechnol. Lett. 30 (3) (2008) 377–385.
[3] P. Kahar, T. Iwata, J. Hiraki, E.Y. Park, M. Okabe, Enhancement of ε-polylysine production by *Streptomyces albulus* strain 410 using pH control, J. Biosci. Bioeng. 91 (2) (2001) 190–194.

[4] S. Liu, Q. Wu, J. Zhang, S. Mo, Production of ε-poly-L-lysine by *Streptomyces* sp. using resin-based, in situ product removal, Biotechnol. Lett. 33 (8) (2011) 1581–1585.
[5] P. Kahar, K. Kobayashi, T. Iwata, J. Hiraki, M. Kojima, M. Okabe, Production of ε-polylysine in an airlift bioreactor (ABR), J. Biosci. Bioeng. 93 (3) (2002) 274–280.
[6] Y. Zhang, X. Feng, H. Xu, Z. Yao, P. Ouyang, ε-Poly-L-lysine production by immobilized cells of *Kitasatospora* sp. MY 5–36 in repeated fed-batch cultures, Bioresour. Technol. 101 (14) (2010) 5523–5527.
[7] D. Xu, H. Yao, Z. Xu, R. Wang, Z. Xu, S. Li, X. Feng, Y. Liu, H. Xu, Production of ε-poly-lysine by *Streptomyces albulus* PD-1 via solid-state fermentation, Bioresour. Technol. 223 (2017) 149–156.
[8] S.B. Bankar, R.S. Singhal, Improved poly-ε-lysine biosynthesis using *Streptomyces* noursei NRRL 5126 by controlling dissolved oxygen during fermentation, J. Microbiol. Biotechnol. 21 (6) (2011) 652–658.
[9] J. Xia, Z. Xu, H. Xu, J. Liang, S. Li, X. Feng, Economical production of poly (ε-l-lysine) and poly (l-diaminopropionic acid) using cane molasses and hydrolysate of *streptomyces* cells by *Streptomyces albulus* PD-1, Bioresour. Technol. 164 (2014) 241–247.
[10] Z. Xu, X. Feng, Z. Sun, C. Cao, S. Li, Z. Xu, Z. Xu, F. Bo, H. Xu, Economic process to co-produce poly (ε-l-lysine) and poly (l-diaminopropionic acid) by a pH and dissolved oxygen control strategy, Bioresour. Technol. 187 (2015) 70–76.
[11] Z. Xu, F. Bo, J. Xia, Z. Sun, S. Li, X. Feng, H. Xu, Effects of oxygen-vectors on the synthesis of epsilon-poly-lysine and the metabolic characterization of *Streptomyces albulus* PD-1, Biochem. Eng. J. 94 (2015) 58–64.
[12] S. Jia, G. Wang, Y. Sun, Z. Tan, Improvement of epsilon-poly-L-lysine production by *Streptomyces albulus* TUST2 employing a feeding strategy, Bioinformatics and Biomedical Engineering, 2009. ICBBE 2009. 3rd International Conference On, IEEE (2009) 1–4.
[13] Z. Xu, C. Cao, Z. Sun, S. Li, Z. Xu, X. Feng, H. Xu, Construction of a genetic system for *Streptomyces albulus* PD-1 and improving poly (ε-L-lysine) production through expression of *Vitreoscilla* hemoglobin, J. Microbiol. Biotechnol. 25 (11) (2015) 1819–1826.
[14] K. Yamanaka, N. Kito, Y. Imokawa, C. Maruyama, T. Utagawa, Y. Hamano, Mechanism of ε-poly-l-lysine production and accumulation revealed by identification and analysis of an ε-poly-l-lysine-degrading enzyme, Appl. Environ. Microbiol. 76 (17) (2010) 5669–5675.
[15] X. Chen, L. Tang, S. Li, L.J. Liao, J.H. Zhang, Z.G. Mao, Optimization of medium for enhancement of ε-poly-L-lysine production by *Streptomyces* sp. M-Z18 with glycerol as carbon source, Bioresour. Technol. 102 (2) (2011) 1727–1732.
[16] X.S. Chen, S. Li, L.J. Liao, X.D. Ren, F. Li, L. Tang, J.H. Zhang, Z.G. Mao, Production of ε-poly-l-lysine using a novel two-stage pH control strategy by *Streptomyces* sp. M-Z18 from glycerol, Bioprocess Biosyst. Eng. 34 (5) (2011) 561–567.
[17] X.D. Ren, X.S. Chen, X. Zeng, L. Wang, L. Tang, Z.G. Mao, Acidic pH shock induced overproduction of ε-poly-L-lysine in fed-batch fermentation by *Streptomyces* sp. M-Z18 from agro-industrial by-products, Bioprocess Biosyst. Eng. 38 (6) (2015) 1113–1125.
[18] S. Li, F. Li, X.S. Chen, L. Wang, J. Xu, L. Tang, Z.G. Mao, Genome shuffling enhanced ε-poly-l-lysine production by improving glucose tolerance of *Streptomyces graminearus*, Appl. Biochem. Biotechnol. 166 (2) (2012) 414–423.
[19] X.D. Ren, X.S. Chen, L. Tang, Q.X. Sun, X. Zeng, Z.G. Mao, Efficient production of ε-poly-l-lysine from agro-industrial by-products by *Streptomyces* sp. M-Z18, Ann. Microbiol. 65 (2) (2015) 733–743.
[20] R.F. Itzhaki, Colorimetric method for estimating polylysine and polyarginine, Anal. Biochem. 50 (2) (1972) 569–574.
[22] X.S. Chen, X.D. Ren, N. Dong, S. Li, F. Li, F.L. Zhao, L. Tang, J.H. Zhang, Z.-G. Mao, Culture medium containing glucose and glycerol as a mixed carbon source improves ε-poly-l-lysine production by *Streptomyces* sp. M-Z18, Bioprocess Biosyst. Eng. 35 (3) (2012) 469–475.
[23] Q.X. Sun, X.S. Chen, X.D. Ren, Z.G. Mao, Improvement of ε-poly-L-lysine production through seed stage development based on in situ pH monitoring, Appl. Biochem. Biotechnol. 175 (2) (2015) 802–812.
[24] L. Shih, M.H. Shen, Optimization of cell growth and poly (ε-lysine) production in batch and fed-batch cultures by *Streptomyces albulus* IFO 14147, Process Biochem. 14 (7) (2006) 1644–1649.
[25] C. Bandaiphet, P. Prasertsan, Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, k_La in exopolysaccharide production from *Enterobacter cloacae* WD7, Carbohydr. Polym. 66 (2) (2006) 216–228.
[26] A. Hayes, G. Hobbs, C.P. Smith, S.G. Oliver, P.R. Butler, Environmental signals triggering methylenomycin production by *Streptomyces coelicolor* A3 (2), J. Bacteriol. 179 (17) (1997) 5511–5515.
[27] J.Y. Song, Y.J. Kim, Y.S. Hong, Y.K. Chang, Enhancement of geldanamycin production by pH shock in batch culture of *Streptomyces hygroscopicus* subsp. *duamyceticus*, J. Microbiol. Biotechnol. 18 (5) (2008) 897–900.
[28] C.J. Kim, Y.K. Chang, G.T. Chun, Enhancement of kasugamycin production by pH shock in batch cultures of *Streptomyces kasugaensis*, Biotechnol. Progr. 16 (4) (2000) 548–552.
[29] Y.J. Kim, J.Y. Song, M.H. Moon, C.P. Smith, S.K. Hong, Y.K. Chang, pH shock induces overexpression of regulatory and biosynthetic genes for actinorhodin production in *Streptomyces coelicolor* A3 (2), Appl. Microbiol. Biotechnol. 76 (5) (2007) 1119–1130.
[30] Y.J. Kim, M.H. Moon, J.Y. Song, C.P. Smith, S.-K. Hong, Y.K. Chang, Acidic pH shock induces the expressions of a wide range of stress-response genes, BMC Genom. 9 (1) (2008) 604.
[31] Y.J. Kim, J.Y. Song, S.-K. Hong, C.P. Smith, Y.K. Chang, Effects of pH shock on the secretion system in *Streptomyces coelicolor* A3 (2), J. Microbiol. Biotechnol. 18 (4) (2008) 658–662.
[32] Y.J. Kim, A.N. Moon, J.Y. Song, E.S. Kim, C.J. Kim, Y.K. Chang, Gene-expression analysis of acidic pH shock effects on two-component systems in *Streptomyces coelicolor*, Biotechnol. Bioproc. Eng. 14 (5) (2009) 584.