

ISSN 1682-296X (Print)

ISSN 1682-2978 (Online)



Bio Technology



ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Characterization of a Mutant Strain of *Streptomyces albulus* and Optimization of ϵ -polylysine Production

¹Tie-Dan Zhang, ¹Bo Yang, ^{1,2}Zhi-Wei Ye, ^{1,2}Jun-Fang Lin and ^{1,2}Li-Qiong Guo

¹Department of Bioengineering, College of Food Science, South China Agricultural University, 483 Wushan Road, Tianhe District, 510640, Guangzhou, China

²Institute of Biomass Research, South China Agricultural University, 510640, Guangzhou, China

Abstract: A novel method was developed for characterizing mutant strains with enhanced ϵ -PL production by adding 7 mg mL⁻¹ S-(2-aminoethyl)-l-cysteine hydrochloride and 0.002% methylene blue as selection markers on agar medium. With proposed method, a mutant strain YB13-2 of *Streptomyces albulus* with 13.03% higher ϵ -polylysine (ϵ -PL) yield than original strain was obtained through mutagenesis with diethyl sulfate. In addition, optimization of ϵ -PL production with YB13-2 was carried out by enhancing the culture media and culture conditions of the mutant strain through a combination of Plackett-Burman design, response surface methodology and traditional one-factor-at-a-time method. Through medium optimization a high ϵ -PL yield of 148.16 mg mL⁻¹ was achieved by using 42.23 g L⁻¹ glycerol, 8.15 g L⁻¹ yeast extract and 10.74 g L⁻¹ ammonium nitrate. The optimum culture conditions were determined to be at 30°C temperature, 220 rpm rotational speed, 100 mL medium volume, 7.0 initial pH value and 8% inoculum amount. Based on this optimization, a further higher yield of 168.91 mg mL⁻¹ ϵ -PL was obtained.

Key words: *Streptomyces albulus*, ϵ -polylysine, mutagenesis, response surface methodology, placket-burman

INTRODUCTION

ϵ -polylysine (ϵ -PL) is a naturally occurring homo-polypeptide which consists of 25 to 30 l-lysines and is characterized by a unique structure linking the ϵ -amino and carboxyl groups of L-lysine. ϵ -PL is usually biosynthesized extra cellularly by *Streptomyces albulus* and *Kitasatospora* (Wang *et al.*, 2013). Compared with the conventional chemical preservatives, ϵ -PL shows a broad spectrum of antibacterial activity against most Gram-positive and Gram-negative bacteria, fungi and some viruses in neutral and faintly acidic environment (Chang *et al.*, 2014; Hamano, 2011; Nani *et al.*, 2013). The attractive properties of ϵ -PL contain biodegradability, edibility, non-toxicity, good biocompatibility, flexible molecular backbone, excellent solubility, low immunogenicity and relative safety (Chen *et al.*, 2011; Groman *et al.*, 2009; Zhu *et al.*, 2012). These features make ϵ -PL interesting for applications in fields as diverse as medicine, pesticides, electronic material design and so on Rao *et al.* (2011).

Currently, ϵ -PL is generated commercially via fermentation process using a mutant strain of *S. albulus* (Wang *et al.*, 2013; Hamano, 2011). Strain mutation leads to a significant increase in the production of

ϵ -PL and most reports on strain mutation used S-(2-aminoethyl)-l-cysteine (AEC) as selection marker (Hamano *et al.*, 2007; Hiraki *et al.*, 1998). AEC-resistant mutant strains may have the ability to synthesize more ϵ -PL than the non-AEC-resistant ones. The present study aims to develop a simple method for strain enhancement by adding AEC and methylene blue as selection markers in agar medium.

Aside from strain improvements, designing and optimizing for an appropriate culture medium and culture conditions in fermentation process are also crucial for efficient and high-yield production of ϵ -PL. Traditional optimization of fermentation factors is generally a time consuming and labor-intensive process. Recently, statistically-based experimental designs have been widely used for optimizing culture media and culture conditions which are being gradually accepted in biotechnology (Nombona *et al.*, 2012; Martinez *et al.*, 2011; Sontakke and Yadav, 2011). To the author's knowledge, however, little literary information regarding statistical optimization of culture media for ϵ -PL production by *S. albulus* is available.

In the present study, a *S. albulus* mutant strain (YB13-2) with ϵ -PL hyperproduction from *S. albulus*

YB12-32 was obtained by diethyl sulfate (DES) treatment. On this basis, an attempt was made to optimize the medium components and culture conditions for improved ϵ -PL production from the YB13-2 mutant strain via RSM, PB design and traditional one-factor-at-a-time method.

MATERIALS AND METHODS

Microorganism and culture conditions: The ϵ -PL-producing strain *S. albulus* YB12-32 was isolated from soil samples collected in southern China, identified and then cultured in the laboratory. The strain was maintained on Bennett's agar slant at 30°C. Batch fermentation was carried out with 220 rpm orbital agitation at 28°C in 250 mL flasks containing 50 mL of the fermentation medium inoculated with 6% (v/v) seed culture.

Preliminary characterization for high ϵ -PL producing mutant strains: A stock spore suspension was prepared via cultivation of the strain YB12-32 on fresh Bennett's slants at 30°C for 5 to 7 days. Then collected the spores and adjusted the concentration to 1×10^5 spore mL⁻¹. AEC was added to each tube containing 1 mL spore suspension with the final concentration of 0 to 8 mg mL⁻¹, respectively. The spore was spread onto the basal agar medium and incubated at 30°C for 5 days. The MIC of AEC to cells was observed by strain growth on gradient plates.

Analytical methods: An aliquot of 100 μ L ϵ -PL standard solution with concentrations of 0.5, 1.0, 2.0 and 4.0 g L⁻¹, respectively added to each hole of the basal medium containing 0.002% methylene blue which was then incubated at 30°C for 24 h. The concentration of methylene blue was confirmed by a previous study of Nishikawa and Ogawa (2002). The ratio between the diameters of the transparent halo (H) and colony (C), namely the H/C value, was calculated. The concentration of ϵ -PL was measured as described Itzhaki in (1972). Biomass accumulation was estimated using dry cell weight analysis.

The spores of the YB12-32 strain were suspended in phosphate buffer (0.1 mol L⁻¹, pH 7.2) and the concentration was adjusted to 10^6 mL⁻¹. The spore suspension was treated with DES (final concentration 1%) and various exposure times (10 to 60 min) were examined. Treatment was terminated by adding equal volumes of 25% Na₂S₂O₃. The treated spores were harvested by centrifugation, washed twice with phosphate buffer and resuspended in the phosphate buffer. Afterwards, the spore suspension was mutated for optimum exposure time and the survivors were screened for resistance to the MIC of AEC and 0.002% methylene blue on basal agar plates incubated at 30°C for 5-7 days.

Table 1: Variables and their levels in the Plackett-Burman design.

Variable	Component	Levels of variables (g L ⁻¹)	
		-1 (Low)	+1 (High)
A	Glycerol	20.00	40.00
B	Yeast extract	05.00	10.00
C	Ammonium nitrate	05.00	10.00
D	Na ₂ HPO ₄	00.80	01.20
E	KH ₂ PO ₄	01.00	01.50
F	MgSO ₄ ·7H ₂ O	00.25	00.50
G	ZnSO ₄	00.05	00.10
H	FeSO ₄	00.01	00.02

Potential mutant strains were cultured in 50 mL fermentation medium by shaking at 220 rpm and stored at 28°C for 3 days. The concentration of ϵ -PL was determined according to the above analytical method. Finally, YB13-2 mutant strain with genetic stability and ϵ -PL yield up to 132.72 mg mL⁻¹ was obtained.

Experimental design and data analysis: The evaluation and optimization of culture medium components through the YB13-2 mutant strain were carried out by a combination of traditional one-factor-at-a-time method and statistical experimental designs which could be divided into two phases. In the first phase, one-factor-at-a-time method and PB design were used to screen the major variables affecting the performance of the culture in terms of ϵ -PL yields. A total of eight PB design variables were studied to screen the most significant nutrient components and each variable was represented by two levels, high and low, denoted by (+1) and (-1), respectively (Table 1). The effect of each variable ($E_{(x)}$) on each response was determined by subtracting the average response of the low level from that of the high level. The effects of the dummy variables were used to calculate the Standard Error (SE) and the variables with confidence levels above 95% significantly influence ϵ -PL production.

In the second phase, based on the results obtained from the PB design, RSM based on Box-Behnken design was conducted in an optimum vicinity to locate the true optimum variable concentrations at three levels (-1, 0, 1) for the ϵ -PL production response. This design could be explained by applying the following quadratic equation:

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} X_i^2 + \sum a_{ij} X_i X_j$$

where, (Y = predicted response; a_0 = offset term; a_i = linear effect; a_{ii} = squared effect; a_{ij} = interaction effect; X_i and X_j =variables).

In this study, all experiments were performed in triplicate. SPSS software (Version 17.0) was used for analysis of variance (ANOVA) and statistical significance of the data. Design-Expert software (Version 7.1.5) was used for experimental design and analysis of the results.

RESULTS AND DISCUSSION

Characterization for high ϵ -PL-producing mutants:

According to the “Materials and methods” section, the growth of the YB12-32 strain was inhibited gradually with the addition of increasing concentration of AEC. Strain growth was completely inhibited when the agar plates contained 7 mg mL^{-1} of AEC. Therefore, 7 mg mL^{-1} AEC was chosen as the MIC of AEC in the agar medium to preliminarily characterize the AEC-resistant mutants.

The results in Fig. 1 a-e demonstrated that the gradient concentrations of ϵ -PL gave rise to the production of the increasing size of transparent zones with different H/C values on agar medium. The standard curve was found to better describe a positive linear relationship above because a higher linear correlation coefficient, R^2 , of 0.9928 was obtained by regression analysis. In other words, increasing the ϵ -PL concentration within the range of 0 to 4 g L^{-1} correspondingly resulted in a linear increase in the H/C value of the transparent zone. Hence, the above assumption is feasible.

The spore suspension was treated for 30 min with 1% DES, with a loss of viability exceeding 85%. Approximately 130 mutants with increased H/C values were independently derived, compared with that of the parent strain YB12-32 ($\text{H/C} = 2.65$). The ϵ -PL productions of the 107 mutants were improved by the determination of ϵ -PL concentration in the fermentation broth, indicating that the method was effective and useful to preliminarily characterize the mutants using AEC and methylene blue. The traditional method for mutant characterization had the disadvantage of transferring the mutants randomly to slants for sporulation to examine their ϵ -PL production through fermentation without knowing whether their ϵ -PL yields were elevated (Hiraki *et al.*, 1998; Chen *et al.*, 2007). Thus, application of the simple, effective and intuitive method presented in the current study could reduce the screening procedure and effectively minimize the workload.

Optimization of medium components by RSM: Out of the 107 selected mutants, 10 were found to improve ϵ -PL production by more than 10%. After verifying the ϵ -PL productions of these 10 mutants through secondary

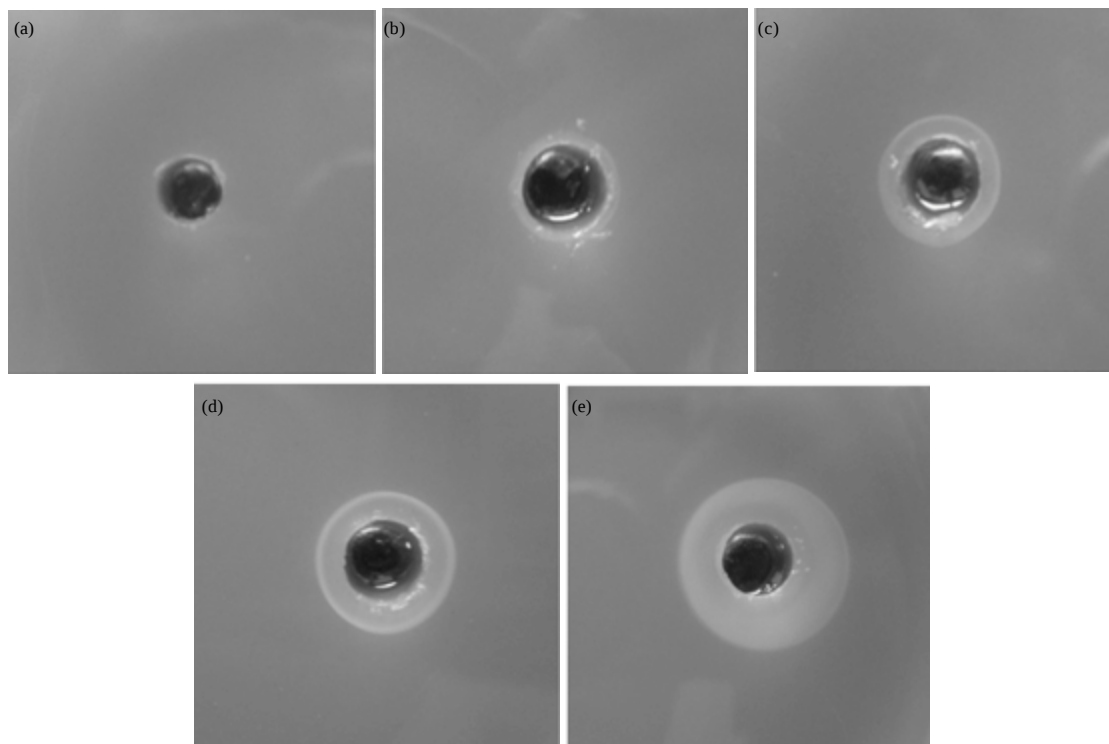


Fig. 1(a-e): Photographs of transparent zones of in agar medium. Increasing size of transparent zones occurred with gradient concentrations of ϵ -PL in agar medium containing 0.002% methylene blue after incubation at 30°C for 24 h. The concentrations of ϵ -PL of Fig a-e were 0, 0.5, 1.0, 2.0 and 4.0 g L^{-1} , respectively. Correspondingly, the H/C values of the resulting transparent zones were 0, 0.57, 1.03, 2.01 and 3.60

fermentation, only two mutant strains, YB13-2 and YB47-3, maintained stable yields. The two mutant strains yielded ϵ -PL productions of 132.72 and 129.83 mg mL⁻¹ (13.03 and 10.90% increase), respectively, compared with that of the parent strain (117.45 mg mL⁻¹). Such mutagenesis subjected to DES treatment gave rise to an obvious problem, wherein most of the mutants derived from *S. albulus* YB12-32 with enhanced ϵ -PL productions decreased in production when sub-cultured for generations. Thus, obtaining high ϵ -PL-producing mutants with good genetic stabilities by DES mutagenesis is difficult. This disparate finding might be explained by assuming that degeneration probably occurs with a spontaneous reverse mutation and the chemical mutagen of DES has certain limitations to *Streptomyces* mutagenicity. Moreover, low enhancement levels of ϵ -PL productions by the obtained mutants which might be attributed to the structure of the spore walls that would probably disturb the effect of DES mutagen, were observed.

A PB design was used to screen the most significant nutrient components on the production of ϵ -PL by YB13-2 mutant strain. First, pre-experiments were conducted to investigate the influence of various carbon and nitrogen sources on the production of ϵ -PL by one-factor-at-a-time method. Among the existing components of the culture medium, glycerol was determined the best carbon source and yeast extract and ammonium nitrate to be the best nitrogen sources for ϵ -PL production which provided a basis for the following PB design.

Three factors, glycerol (A), yeast extract (B) and ammonium nitrate (C), functioning as the carbon and nitrogen sources, were found to play significant roles in the production of ϵ -PL because of their low P values (<0.0500). Therefore, these three factors were used as the variables for further optimization by RSM based on Box-Behnken design.

The experimental design and results of the Box-Behnken design are presented in Table 2. By applying multiple regression analysis on the experimental data, the second-order polynomial equation was given as:

$$Y = 2.6373 + 3.8158X_1 + 8.0458X_2 + 5.9863X_3 - 0.0442X_1^2 - 0.0313X_1X_3 + 0.0733X_2X_3 - 0.0369X_1^2 - 0.4274X_2^2 - 0.2448X_3^2$$

(Y = ϵ -PL yield; X_1 = glycerol; X_2 = yeast extract; X_3 = ammonium nitrate). The ANOVA of the quadratic regression model demonstrated that this equation was a highly significant model because of the low F- and P-probability values (F-value = 115.48, p-value < 0.0001). In the current case, the model was statistically significant at 0.05 level of significance with $R^2 = 0.9933$ and adjusted $R^2 = 0.9847$, indicating that only 1.53% of variability in the response could not be explained by the second-order polynomial prediction equation. A relatively lower value

Table 2: Experimental design and results of the box-behnken design

Run	Glycerol (g L ⁻¹ , X ₁)	Yeast extract (g L ⁻¹ , X ₂)	Ammonium nitrate (g L ⁻¹ , X ₃)	ϵ -PL (mg mL ⁻¹)
1	30.0	7.5	8	138.93 ^a
2	37.5	9.0	8	145.12 ^b
3	45.0	6.0	10	146.15 ^c
4	37.5	6.0	12	144.17 ^b
5	30.0	6.0	10	139.44 ^b
6	45.0	9.0	10	147.10 ^c
7	37.5	7.5	10	146.86 ^c
8	37.5	9.0	12	146.92 ^b
9	37.5	7.5	10	146.66 ^c
10	30.0	9.0	10	142.38 ^c
11	45.0	7.5	8	146.21 ^c
12	45.0	7.5	12	147.63 ^c
13	37.5	6.0	8	143.25 ^b
14	37.5	7.5	10	146.75 ^c
15	37.5	7.5	10	146.92 ^c
16	37.5	7.5	10	146.84 ^c
17	30.0	7.5	12	142.23 ^b

^aProduction increase <5% level, ^bProduction increase between 5 and 10% level, ^cProduction increase >10% level

of the coefficient of variation (CV = 0.23%) implied a better precision and reliability of the current experiments. Furthermore, the distribution from the normal plot of residuals for ϵ -PL production presented a valid assumption of the error of normality.

Among the variables tested, X_1 , X_2 and X_3 , were found to play significant roles in ϵ -PL production ($p < 0.05$). Likewise, significant interactions were found between X_1X_2 , X_1X_3 , X_1^2 , X_2^2 and X_3^2 ($p < 0.05$). However, no direct relationship between X_2 (yeast extract) and X_3 (ammonium nitrate) was found ($p > 0.05$).

The fitted response for the above regression model was plotted in Fig. 2a-c. The 3D graph was generated for the pair-wise combination of the three variables while keeping the other one at its optimum level for ϵ -PL production by the YB13-2 mutant. Fig. 2a-c displays the response surface curves for the interaction between X_1 and X_2 , X_1 and X_3 and X_2 and X_3 , respectively. Based on the response surface plot and RSM regression, the predicted maximum ϵ -PL production (148.16 mg mL⁻¹) was obtained when the optimal values of the variables in the coded units were $X_1 = 42.23$ g L⁻¹, $X_2 = 8.15$ g L⁻¹ and $X_3 = 10.74$ g L⁻¹. Hence, the optimal fermentation medium for ϵ -PL predicted by the model was 42.23 g L⁻¹ glycerol, 8.15 g L⁻¹ yeast extract, 10.74 g L⁻¹ ammonium nitrate, 0.8 g L⁻¹ Na₂HPO₄, 1 g L⁻¹ KH₂PO₄, 0.25 g L⁻¹ MgSO₄•7H₂O, 0.05 g L⁻¹ ZnSO₄•7H₂O and 0.01 g L⁻¹ FeSO₄•7H₂O. Three independent fermentations were carried out to verify the predicted optimum yield and a ϵ -PL yield of 147.53±1.37 mg mL⁻¹ was obtained with the predicted medium which was consistent with the predicted yield by the quadratic polynomial.

In the present study, various initial pH, inoculum amounts, medium volumes, rotation speeds and temperatures were examined for biomass and ϵ -PL production by the YB13-2 mutant, as shown in Fig. 3a-e.

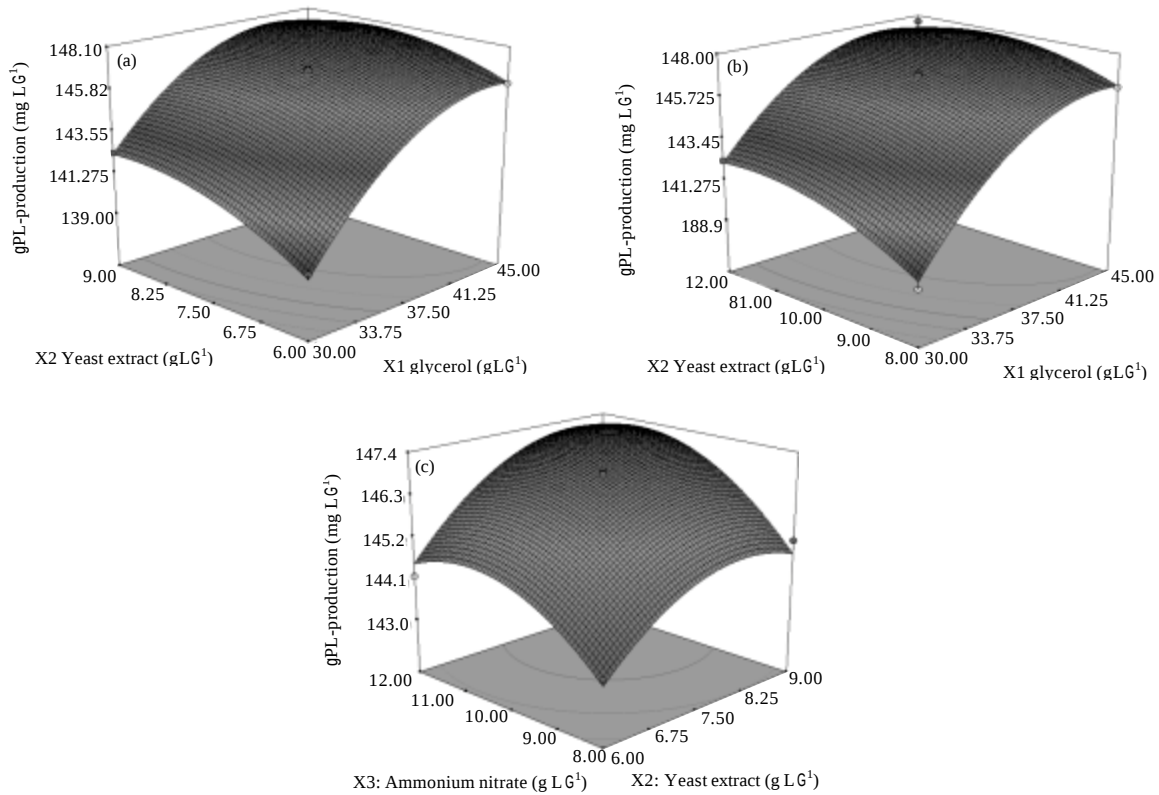


Fig. 2(a-c): Response surface plot of the Box-Behnken model. (a) Glycerol-yeast extract; (b) Glycerol-ammonium nitrate; (c). yeast extract-ammonium nitrate

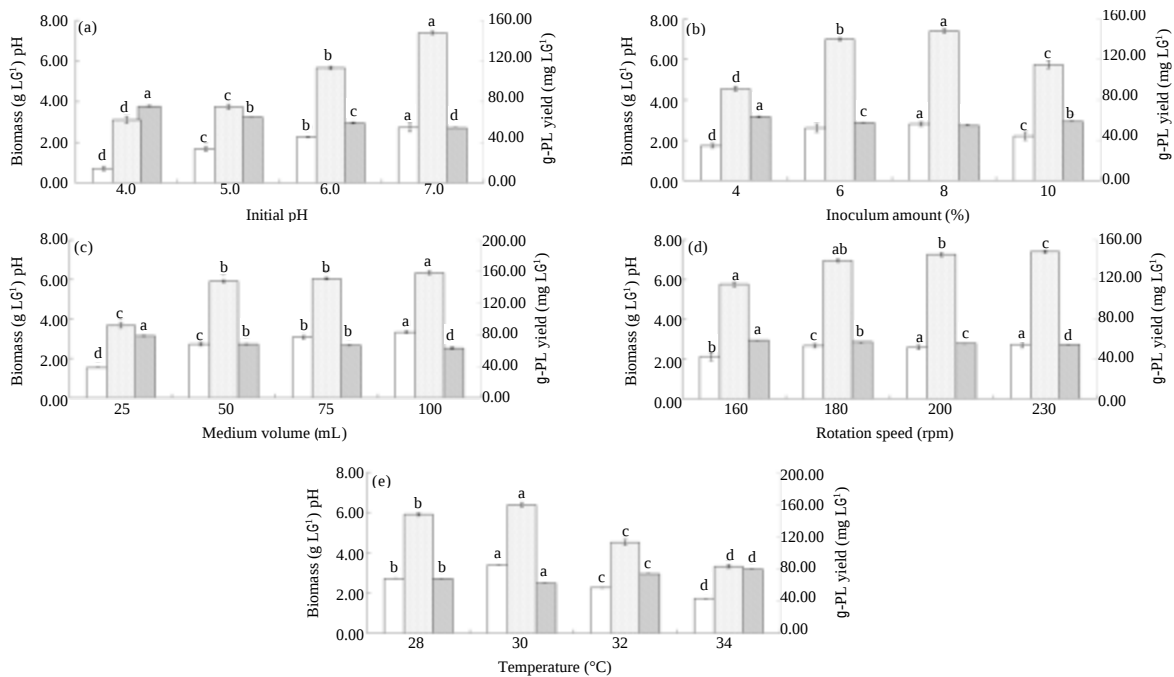


Fig. 3(a-e): Effects of various factors on the production of ε-PL by the mutant strain YB13-2. (a) Initial pH, (b) Inoculum amounts, (c) Medium volumes, (d) Rotation speeds and (e) Temperatures

The optimum culture conditions were observed at a fermentation temperature of 30°C, rotational speed of 220 rpm, medium volume of 100 mL, initial pH value of 7.0 and inoculum amount of 8%.

Based on the above optimizations, a ϵ -PL yield of 168.91 mg mL⁻¹ was finally attained under the optimum medium components and culture conditions by the YB13-2 mutant strain. The yield was higher by 27.28% than that of the previous optimization. The results of this study are helpful for further large-scale industrial production of ϵ -PL by *S. albulus*.

ACKNOWLEDGMENT

We would like to thank the National Natural Science Foundation of China (Grant No. 31071768 and 3107137), for financial assistance.

REFERENCES

- Chang, Y., L. McLandsborough and D.J. McClements, 2014. Antimicrobial delivery systems based on electrostatic complexes of cationic ϵ -polylysine and anionic gum arabic. *Food Hydrocoll.*, 3: 137-143.
- Chen, W.W., H.Y. Zhu and H. Xu, 2007. Breeding of mass-producing ϵ -polylysine mutant and its batch fermentation. *Ind. Microbiol.*, 37: 28-30.
- Chen, X., Z. Wang, Y.Z. Wang, F.T. Jiang, D.S. Li and J.H. Wang, 2011. Effects of yeast extract on poly- ϵ -lysine production in batch culture of *Streptomyces Albulus*. *Adv. Mat. Res.*, 343-344: 1023-1028.
- Groman, E.V., M. Yang, C.P. Reinhardt, J.S. Weinberg and D.E. Vaccaro, 2009. Polycationic nanoparticles: (1) synthesis of a polylysine-MION conjugate and its application in labeling fibroblasts. *J. Cardiovasc. Transl. Res.*, 2: 30-38.
- Hamano, Y., I. Nicchu, T. Shimizu, Y. Onji, J. Hiraki and H. Takagi, 2007. ϵ -poly-L-lysine producer, *Streptomyces albulus*, has feedback-inhibition resistant aspartokinase. *Appl. Microbiol. Biotechnol.*, 76: 873-882.
- Hamano, Y., 2011. Occurrence, biosynthesis, biodegradation and industrial and medical applications of a naturally occurring ϵ -poly-L-lysine. *Biosci. Biotechnol. Biochem.*, 75: 1226-1233.
- Hiraki, J., M. Hatakeyama, H. Morita and Y. Izumi, 1998. Improved ϵ -poly-L-lysine production of an S-(2-aminoethyl)-L-cysteine resistant mutant of *Streptomyces albulus*. *Seibutsu-Kogaku Kaishi*, 76: 487-493.
- Itzhaki, R.F., 1972. Colorimetric method for estimating polylysine and polyarginine. *Anal. Biochem.*, 50: 569-574.
- Martinez, L., V. Redondas, A. Garcia and A. Moran, 2011. Optimization of growth operational conditions for CO₂ biofixation by native *Synechocystis* sp. *J. Chem. Technol. Biotechnol.*, 86: 681-690.
- Nani, W., P.F. Yang and C.K. Lee, 2013. Epsilon-polylysine fermentation and its recovery using Carboxymethyl Cellulose (CMC)-conjugated magnetite. *Sep. Sci. Technol.*, 48: 1086-1092.
- Nishikawa, M. and K. Ogawa, 2002. Distribution of microbes producing antimicrobial ϵ -poly-L-lysine polymers in soil microflora determined by a novel method. *Applied Environ. Microbiol.*, 68: 3575-3581.
- Nombona, N., E. Antunes, W. Chidawanyika, P. Kleyi, Z. Tshentu and T. Nyokong, 2012. Synthesis, photophysics and photochemistry of phthalocyanine- ϵ -polylysine conjugates in the presence of metal nanoparticles against *Staphylococcus aureus*. *J. Photochem. Photobiol. A: Chem.*, 233: 24-33.
- Rao, S.S., N. Han and J.O. Winter, 2011. Polylysine-modified PEG-based hydrogels to enhance the neuro-electrode interface. *J. Biomater. Sci. Polym. Edn.*, 22: 611-625.
- Sontakke, J.B. and G.D. Yadav, 2011. Optimization and kinetic modeling of lipase catalyzed enantioselective *N*-acetylation of ($\pm$)-1-phenylethylamine under microwave irradiation. *J. Chem. Technol. Biotechnol.*, 86: 739-748.
- Wang, L., S. Li, Z.G. Mao and F.L. Zhao, 2013. Optimization of fermentation medium for ϵ -polylysine production by recombinant *Streptomyces* sp. FEEL-1. *J. Agric. Sci. Technol.*, 15: 76-83.
- Zhu, M.Q., L. Xiang, K. Yang, L.J. Shen and F. Long *et al.*, 2012. Synthesis and characterization of biodegradable amphiphilic triblock copolymers methoxy-poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-lactic acid). *J. Polym. Res.*, Vol. 19. 10.1007/s10965-011-9808-y