Ann Microbiol (2015) 65:733–743
DOI 10.1007/s13213-014-0913-0

ORIGINAL ARTICLE

Efficient production of ε-poly-L-lysine from agro-industrial by-products by *Streptomyces* sp. M-Z18

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Received: 4 March 2014 / Accepted: 5 May 2014 / Published online: 10 June 2014

Abstract ε-Poly-L-lysine (ε-PL)—a natural food preservative with wide antimicrobial activity and high food safety—is increasingly attracting widespread attention. However, the high cost of raw materials severely impairs its economy and utilization. In this study, agro-industrial by-products, i.e., fish meal coupled with corn steep liquor, were employed as alternative organic nitrogen sources for industrial ε-PL production by *Streptomyces* sp. M-Z18. An economical medium was then developed by using an artificial neural network. Amino acids analyses showed that the improved medium was rich in glutamate, arginine, lysine and aspartate, which not only elevated the acid tolerance capability of the mycelia but also enhanced cell growth and ε-PL production. Subsequently, a cost-effective and efficient strategy for ε-PL production was established on fermenter scale, based on the improved medium and two-stage pH control. Notably, ε-PL production and productivity reached 35.24 g/L and 4.85 g/L day in fed-batch fermentation. Further profit assessment at the 10 m^3 scale indicated that application of this strategy resulted in a net profit increase of 9,057 USD. Therefore, the proposed strategy has great potential for industrial production of ε-PL.

Keywords *Streptomyces* · ε-Poly-L-lysine · Artificial neural network · Agro-industrial by-products · Amino acid · Profit assessment

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Introduction

ε-Poly-L-lysine (ε-PL)—an L-lysine homopolymer with amide linkage between ε-amino and α-carboxyl groups—is produced mainly by the genus *Streptomyces* (Shima and Sakai 1977, 1981a, b). It is biodegradable, water-soluble, heat-stable and exhibits a wide antimicrobial spectrum, including yeast, fungi, Gram-positive and Gram-negative bacteria (Shih et al. 2006), as well as antiphage activity (Shima et al. 1982). Moreover, ε-PL also shows excellent behavior in high safety (Hiraki et al. 2003). Therefore, ε-PL has been used widely for many years as a natural food preservative in many countries, including Japan and the United States as well as Korea (Yoshida and Nagasawa 2003). Besides, it could also be used as hydrogel, dietary agent, interferon inducer and biochip coatings in the fields of agriculture, food, medicine and electronics. Consequently, the global demand for ε-PL is increasing tremendously. As a result, it is necessary to develop a cost-effective strategy, including exploration of economical raw materials, to reduce the cost of industrial ε-PL production.

The nutritional raw materials required in aerobic fermentation generally include a carbon source, organic and/or inorganic nitrogen source(s) and inorganic salts. The organic nitrogen source can be converted into different intermediate metabolites and significantly affect nitrogen-containing secondary metabolites biosynthesis. In addition, the organic nitrogen source is usually much more expensive than the carbon source. Even at relatively lower concentrations, it still accounts for a high share of the raw materials cost. In past decades, restricted organic nitrogen sources have been used for ε-PL production, and only yeast extract met the demand for high ε-PL production (Kahar et al. 2001, 2002; Jia et al. 2009; Zhang et al. 2010a; Shukla and Mishra 2013). Recently, beef extract was identified as an excellent organic nitrogen source for ε-PL production by *Streptomyces* sp. M-Z18 (Chen et al. 2011b). Moreover, the production of ε-PL was enhanced

markedly under proper process optimization through fed-batch fermentation (Chen et al. 2011a, 2012, 2013). However, the high cost of yeast or beef extracts would severely impair the economy of ε-PL production in industry. Therefore, it is essential to find cheaper substitutes while maintaining high ε-PL production and productivity. In fact, agro-industrial by-products, such as soybean meal, corn steep liquor and fish meal, have been used successfully as cheap organic nitrogen sources for the fermentation of diverse biological products, e.g., clavulanic acid (Hamedi et al. 2012), bacterial cellulose (Rani and Appaiah 2011) and delta-endotoxin (Zouari et al. 2002). Nevertheless, the consideration of employing these low-cost raw materials for ε-PL production has been scarcely reported to date.

In the present study, agro-industrial by-products from different bioresources were considered to replace the expensive beef extract in the production of ε-PL by *Streptomyces* sp. M-Z18. Following a systematic analysis of cost-reduction as well as enhancement of ε-PL production and cell growth, fish meal coupled with corn steep liquor were chosen as alternative organic nitrogen sources. A matched medium was then developed by an artificial neural network (ANN) to exploit the full potential for ε-PL production. Finally, a cost-effective and efficient strategy based on the improved medium and two-stage pH control was established. Further profit assessment demonstrated that the proposed strategy has an economic advantage over the original procedure. The information obtained in this work should be useful for the efficient reduction of raw materials cost and improvement of ε-PL production in industry.

Materials and methods

Materials

Industrial-glycerol was purchased from Kerry Oleochemical Industrial Co. (Shanghai, China). Soybean meal (nitrogen content 78.64 mg/g) and corn steep liquor (nitrogen content 35.43 mg/g) were kind gifts from Creat Medium Co. (Qingdao, China) and Linghua Group Co. (Jining, China), respectively. Fish meal (nitrogen content 97.00 mg/g) was obtained from Xinggang Co. (Binzhou, China). Beef (nitrogen content 88.89 mg/g) and yeast (nitrogen content 112.50 mg/g) extracts were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China) and Oxoid (Hampshire, England), respectively.

Microorganism, culture media, inoculum preparation and culture conditions

Streptomyces sp. M-Z18 was used throughout this study. This strain was isolated from soil as described by Nishikawa and Ogawa (2002) and subjected to ultraviolet and nitrosoguanidine mutagenesis as described by Hiraki et al. (1998). Agar slant medium comprised (g/L): glucose, 10; yeast extract, 5; beef extract, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $K_2HPO_4 \cdot 3H_2O$, 1; and agar 20. The pH was adjusted to 7.0 before sterilization. Seed culture medium (M3G) contained (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. The original fermentation medium consisted of (g/L) (Chen et al. 2011b): industrial-glycerol, 60; $(NH_4)_2SO_4$, 5; beef extract, 10; KH_2PO_4, 4; $MgSO_4 \cdot 7H_2O$ 0.8; $FeSO_4 \cdot 7H_2O$, 0.05. The improved medium composed of (g/L): industrial-glycerol, 83; $(NH_4)_2SO_4$, 8; fish meal, 15; corn steep liquor, 5; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 2; $FeSO_4 \cdot 7H_2O$, 0.1. Initial pH values of the above media were adjusted to 6.8 with 2 M NaOH and/or 1 M H_2SO_4. The media were sterilized in an autoclave for 20 min at 121 °C. In agar slant and M3G media, glucose was autoclaved separately.

The inoculated slants were incubated at 30 °C for 7 days to obtain a heavy sporulated growth. After that time, spores were used for seed culture inoculation (at a concentration of about 2×10^5 spores/L). The seed culture was grown in a 500 mL Erlenmeyer flask containing 80 mL liquid medium and incubated at 30 °C on a rotary shaker with 200 rpm for 24 h. Shake-flask fermentation was carried out in a 250 mL Erlenmeyer flask with 40 mL liquid fermentation medium. The fermentation medium was inoculated with 3 mL 24-h *Streptomyces* sp. M-Z18 seed culture containing approximately 3 g/L dry cells and then cultured at 30 °C with a rotary velocity of 200 rpm for 72 h. All experiments were performed at least in triplicate.

Batch and fed-batch fermentations were performed in a 5-L fermenter (Baoxing Corp., Shanghai, China) with a 3.5-L working volume and two Rushton turbines. Seed culture (300 mL) was inoculated into 3.2 L sterilized fermentation medium with an initial pH of 6.8. Dissolved oxygen (DO) was set at about 30 % of air saturation and controlled by adjusting agitation speed from 200 to 800 rpm and aeration rate with a range of 0.5–2.5 vvm during fermentation. The pH change was detected by a pH electrode (Mettler Toledo, Greifensee, Switzerland) and controlled by ammonia water (12.5 %, w/v) during fermentation. The fermentation temperature was maintained using a recirculating water bath at 30 °C. Samples were taken at appropriate time intervals for the analyses of glycerol, NH_3-N, dry cell weight (DCW) and ε-PL production.

Central composite rotatable design

Based on the screening experiment of different organic nitrogen sources (Fig. 1) and our previous work (Chen et al. 2011b), glycerol, $(NH_4)_2SO_4$ and corn steep liquor were identified as significant medium components for ε-PL production by Plackett-Burman design (data not shown). A central

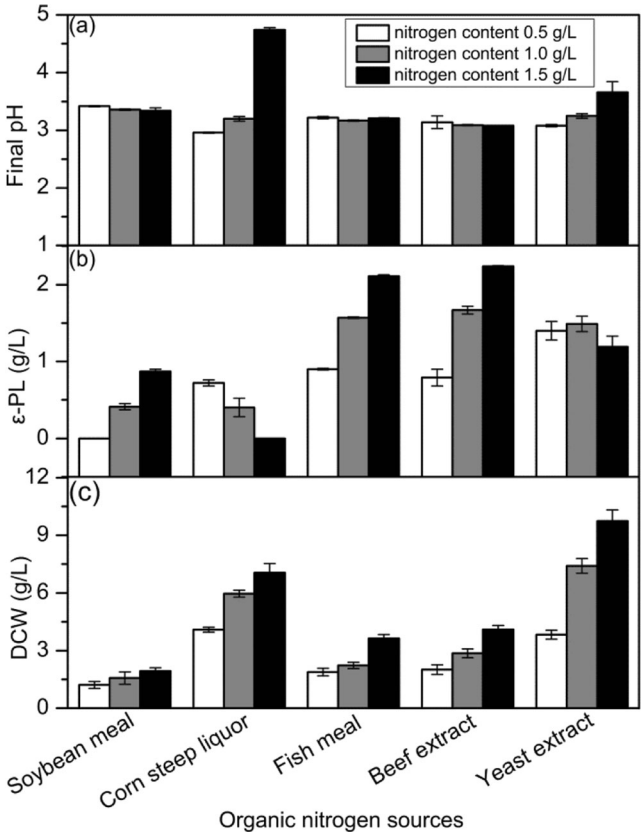

Fig. 1 Cell growth and ε-poly-L-lysine (ε-PL) production on various concentrations of organic nitrogen sources by *Streptomyces* sp. M-Z18. Data points represent means (n=3)±standard deviation (SD). Nitrogen content (g/L): *white* 0.5, *gray* 1, *black* 1.5

composite rotatable design (CCRD) was used to explore the influences of these experimental factors and their interactions on ε-PL production, and moreover to make predictions for different input values. The experiments (with 20 trials) consisted of eight factorial (2^3), six axial points (α=1.68) and six center points (Table 1). The combined effect of the three significant components [glycerol, $(NH_4)_2SO_4$ and corn steep liquor] was evaluated by artificial neural network (ANN) according to the experimental results of the CCRD.

Artificial neural network

In this study, the most popular feed-forward ANN architecture, i.e., multi-layer perceptron (MLP) was used to predict the dependence of ε-PL production on the above three significant medium components. The main advantages of MLP over other ANN models are that it is easy to implement and can approximate almost all kinds of non-line maps. It consists of (1) an input layer with neurons representing input variables to the issue, (2) an output layer with neuron(s) representing the dependent variable(s), and (3) one or more hidden layers containing neuron(s) to help capture the nonlinearity in the problem (Fathi et al. 2011). A real number quantity, viz. weight, is responsible for the connection of these three layers (Desai et al. 2008). The complexity of the MLP network depends on the number of layers and the number of neurons in each layer. Since one hidden layer neural network could manage almost all kinds of the simulation tasks (Desai et al. 2008; Fathi et al. 2011), an ANN model with three layers was used in this research (Fig. 2).

All data (input and output) used in Table 1 were normalized between 0 and 1. Inputs are normalized to avoid numerical overflows caused by very large or very small weights. The use of the sigmoid transfer function maps outputs in a range between 0 and 1; therefore, the outputs should also be normalized to the same range (Bowen et al. 1998). The linear normalization equation used was as follows:

$$Z^{*}=\frac{Z\text{-}Z_{min}}{Z_{max}\text{-}Z_{min}} \quad (1)$$

Where, Z^{*} is the normalized value of Z. $Z_{\min}$ and $Z_{\max}$ are the minimum and maximum values of Z, respectively.

The neurons in the input layer receive the normalized data and directly pass them on to the hidden layer. After collecting the data from input layer, the neurons in the hidden layer multiply them by their corresponding weights, sum the values, and finally add a constant value called bias, mathematically:

$$y_i = \sum_{i=1}^{n} w_{ij}x_i + b_j \quad (2)$$

Where w_{ij} is the weight of the ith input neuron that is connected to the jth hidden neuron; n is number of inputs to the neuron; b_j is the bias associated with the jth neuron, which adds a constant term in the weighted sum to improve convergence. The sum is then passed through a transfer function. Generally, the transfer functions are sigmoidal function, hyperbolic tangent and linear function. In this study, a sigmoidal transfer function $f_1(x)$ used in the hidden layer is shown in Eq. 3.

$$f_1(y_i) = \frac{1}{1+e^{-yi}} \quad (3)$$

The output thus produced by the hidden layer becomes an input to the output layer. The neurons in the output layer produce the output identically to the hidden layer, with the only difference being that a linear transfer function $f_2(h)$ is used instead of the sigmoidal one. The normalized experimental data in Table 1 were partitioned into training and test sets. A back-propagation algorithm was employed to supervise the training process of the network. Back propagation is based on searching the weights produced from the inputs that fit the predicted outputs best over the entire training data set (Razavi et al. 2003). Each iteration in back-propagation constitutes two sweeps: forward activation to produce a solution, and

Table 1 Central composite rotatable design (CCRD) matrix of independent variables and their corresponding experimental and predicted production of ε-poly-L-lysine (ε-PL). *ANN* Artificial neural network

Run order	Glycerol (g/L)	$(NH_4)_2SO_4$ (g/L)	Corn steep liquor (g/L)	ε-PL (g/L)	
				Experimental	ANN predicted
1	70.00	5.00	10.00	2.24±0.10	2.26
2	100.00	7.00	5.00	2.67±0.01	2.71
3	70.00	5.00	10.00	2.23±0.16	2.26
4	100.00	3.00	15.00	0.12±0.05	0.08
5	70.00	1.64	10.00	0±0.00	0.00
6	70.00	8.36	10.00	2.41±0.04	2.48
7	70.00	5.00	10.00	2.25±0.01	2.26
8	40.00	3.00	5.00	1.41±0.18	1.61
9	120.45	5.00	10.00	1.96±0.08	1.86
10	19.55	5.00	10.00	1.68±0.07	1.50
11	40.00	7.00	5.00	2.58±0.03	2.62
12	70.00	5.00	1.59	2.56±0.06	2.37
13	70.00	5.00	10.00	2.31±0.03	2.26
14	100.00	7.00	15.00	1.17±0.28	1.23
15	40.00	7.00	15.00	2.03±0.11	2.00
16	70.00	5.00	10.00	2.32±0.03	2.26
17	100.00	3.00	5.00	1.22±0.06	1.34
18	40.00	3.00	15.00	0.52±0.07	0.55
19	70.00	5.00	10.00	2.23±0.03	2.26
20	70.00	5.00	18.41	1.11±0.04	1.07

the backwards propagation of the computed error to modify the neurons' weights (Fathi et al. 2011). The training cycle was performed by varying the number of neurons in the hidden layer (the number of hidden neurons varied from 3 to 15 in this study), and also by changing combinations of ANN-specific parameters such as learning rate and momentum value. The training process was carried out for 5,000 epochs or until the mean-squared error (MSE) in Eq. 4 reached 1e-4, and then the corresponding ANN was built. Figure 2 shows the schematic diagram of the training process using the back propagation algorithm for the three-layer feed-forward network used in this study. Testing was carried out with the best weights stored during the training step. The root mean square error (RMSE) in Eq. 5 and coefficient of determination (R^2) in Eq. 6 between the results of the predicted and actual outputs were used as measure of closeness of the model to the actual system. The generalization capacity of the model was ensured by selecting weights with the least test set RMSE and largest R^2 value. Once the ANN model was developed to the desired level of precision, a genetic algorithm (GA) was used to optimize ε-PL production.

$$MSE = \frac{\sum_{i=1}^{n} \left(y_{i,exp} - y_{i,pre}\right)^2}{n} \quad (4)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} \left(y_{i,exp} - y_{i,pre}\right)^2}{n}} \quad (5)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n} \left(y_{i,pre} - y_{i,exp}\right)^2}{\sum_{i=1}^{n} \left(y_{i,exp} - y_m\right)^2} \quad (6)$$

Where, $y_{i,exp}$ is the experimental value and $y_{i,pre}$ is the predicted value, n is the number of experiments and y_m is the average of experimental values.

Analytical methods

Dry cell weights were measured as follows: 10 mL culture broth was subjected to centrifugation at 4,500 *g* for 10 min, and then the precipitate was collected and washed twice with distilled water. The washed mycelia was filtered through a pre-weighed filter paper and dried at 105 °C to a constant weight prior to measuring the biomass of the culture. The supernatant was used to determine ε-PL concentration according to the procedure described by Kahar et al. (2001), and also

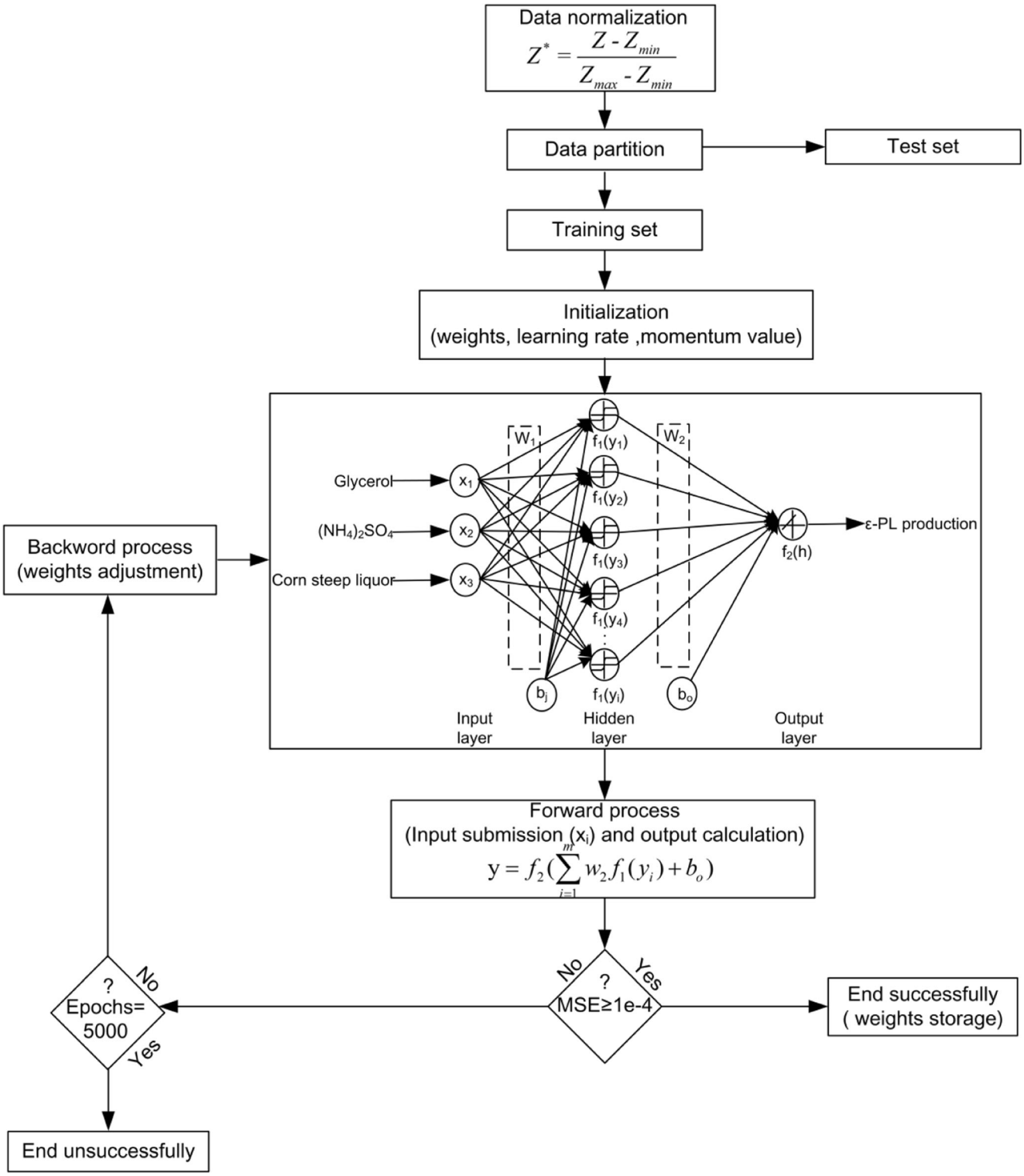

Fig. 2 Schematic representation of the neural network and training process using back propagation algorithm. W_1 is a weight matrix including all of the weights between input and hidden neurons. W_2 represents the weight matrix that connects the neurons between hidden and output layers. Transfer functions f_1 in the hidden layer and f_2 in the output layer are sigmoid and linear functions, respectively

employed to detect the concentration of glycerol using an HPLC system (U-3000, Dionex, Sunnyvale, CA) with a refractive index detector (Shodex RI-101, Tokyo, Japan) and an ion exchange column (Aminex HPX-87H, 300×7.8 mm, Hercules, CA). The column was eluted with 5 mM H_2SO_4 at a temperature of 60 °C and a flow rate of 0.6 mL/min. NH_3-N

was analyzed by means of a colorimetric method using Nessler reagent (AOAC International 1995). For the determination of amino acid contents in beef extract, fish meal and corn steep liquor, 100 mg aliquot samples were first hydrolyzed using 10 mL 6 M HCl for 24 h at 110 °C in vacuo (Blackburn 1978). The hydrolysates were then used to determine the individual free amino acids with amino acid analyser (Agilent Technologies, Santa Clara, CA) as detailed in Feng et al. (2013).

Results and discussion

Effect of organic nitrogen sources on ε-PL production by *Streptomyces* sp. M-Z18

Organic nitrogen sources have profound effects on ε-PL-producing strains, and without them ε-PL production and cell growth will be seriously suppressed (Shima and Sakai 1981a). The aim of this experiment was to investigate the effect of different organic nitrogen sources on cell growth and ε-PL production by *Streptomyces* sp. M-Z18, and further study the possibility for substitution of the expensive beef extract in the original fermentation medium with low-cost organic nitrogen sources, viz. agro-industrial by-products. Therefore, organic nitrogen sources derived from different origins, including plants (soybean meal and corn steep liquor), animals (fish meal and beef extract) and microbial material (yeast extract) were employed for ε-PL production in shake-flask culture (Fig. 1). Due to the distinct nitrogen contents of different organic nitrogen sources, the experiment was performed according to the principle of equivalent amounts of nitrogen, which was determined by the Kjeldahl method using a Kjeldahl Apparatus SKD-100 (Peiou Analytical Instruments Co. Shanghai, China) following the manufacturer's instructions.

As shown in Fig. 1, cell growth and ε-PL production was enhanced with the increase of nitrogen-source concentration, except for ε-PL production on yeast extract and corn steep liquor. Amongst the selected organic nitrogen sources, those derived from animal materials seemed more suitable for ε-PL production. The maximum volumetric ε-PL production of 2.24±0.01 g/L was obtained in the medium containing beef extract. Meanwhile, fish meal yielded ε-PL production of 2.11 ±0.02 g/L, and both of them were much higher than that of traditional yeast extract (1.49±0.10 g/L). In contrast, the performance of plant organic nitrogen sources (soybean meal and corn steep liquor) on ε-PL production was rather poor. However, biomass increased substantially on corn steep liquor. Bankar and Singhal (2010) reported that proteose peptone is the best organic nitrogen source for ε-PL production by *Streptomyces noursei* NRRL 5126, superior to soya peptone, yeast extract, beef extract, corn steep liquor, peptone bacteriological and meat peptone. However, yeast extract was deemed to be a suitable organic nitrogen source for ε-PL production in many previous reports (Kahar et al. 2001, 2002; Jia et al. 2009). These results confirmed that the choice of organic nitrogen source could severely affect ε-PL production, and is strain dependent.

Considering the higher price of beef extract and its poorer improvement of ε-PL production as compared with fish meal, fish meal was reasonably selected as a low-cost alternative organic nitrogen source in this study. Moreover, since production of ε-PL by *Streptomyces* sp. M-Z18 was dependent on cell growth, corn steep liquor was also supplemented to promote cell growth. Therefore, a mixed organic nitrogen source containing both fish meal and corn steep liquor was used for further optimization of ε-PL production.

Optimization by ANN of medium components for ε-PL production

Response surface methodology (RSM) is a powerful tool commonly used for media optimization. There are also a few reported successful cases of the production of ε-PL using RSM (Shih and Shen 2006a; Chen et al. 2011b). However, RSM is useful only for quadratic approximations. All systems containing curvature, such as symmetrical or non-symmetrical bell-shaped curves, may not be well explained by the second-order polynomial of RSM (Nelofer et al. 2012). In last two decades, ANN has emerged as an attractive tool for system optimization that has been proven to be superior and more accurate than RSM in its ability to approximate almost all kinds of non-linear functions (Desai et al. 2008; Nelofer et al. 2012). Moreover, like RSM, ANN can also work well with relatively little data, if these data are statistically well distributed in the input domain, which is the case with CCRD (Desai et al. 2008). A few case studies are available in the literature where models were developed by RSM and ANN using the same CCRD; ANN models have consistently worked better than RSM (Zhang et al. 2010b; Bingöl et al. 2012).

The topology of the network selected in this study consisted of three layers (3:10:1): an input layer consisting of three medium components [glycerol, $(NH_4)_2SO_4$ and corn steep liquor], a middle hidden layer with ten neurons and one output layer for ε-PL production (Fig. 2). The experimental and predicted ε-PL production by ANN is shown in Table 1. It was noted that the ANN-predicted values were very close to the experimental values both towards the center points, such as experiments 1, 3, 7, 13, 16, 19, and edge points, e.g., experiments 4, 6, 9 and 14. The selected ANN network with ten nodes in the hidden layer had the lowest value for the test set RMSE ($RSME_{test}=0.035$) and the corresponding $RSME_{train}$ was 0.093. The R^2 value between the experimental and ANN predicted ε-PL production for training and test sets

data were 0.986 and 0.999, respectively. The high R^2 value and low RMSE for both the training and test sets proved the good simulation and generalization characteristics of the selected ANN. Using genetic algorithm (GA), the optimal concentrations of the significant medium components for ε-PL production predicted by ANN were 83 g/L glycerol, 8 g/L $(NH_4)_2SO_4$ and 5 g/L corn steep liquor with 3.12 g/L ε-PL production. Interestingly, the optima predicted by ANN were located at the edges and even outside of the topology. The same phenomena were also reported in previous studies (Desai et al. 2008; Nelofer et al. 2012). Three independent verification experiments were carried out and ε-PL production of 3.07±0.02 g/L was obtained, which was coincident with the predicted result of ANN.

Evaluation of ε-PL production in shake-flask fermentation

Time profiles of ε-PL production in the original and improved media are illustrated in Fig. 3. The pH changes of both media showed the same trend, decreasing sharply to below 3.5 from an initial 6.8 within 24 h and maintaining stable afterwards (Fig. 3a). The differences among the experimental results of DCW and ε-PL production were distinguished by statistical analysis (Fisher's least significant difference, LSD). The results showed that the differences in DCW from 18 to 36 h were not significant ($P>0.05$) in the original medium, while those in the improved medium were not significant from 30 to 36 h. Besides, there were no significant differences in ε-PL production in the original medium from 30 to 72 h, while that period in the improved medium was from 48 to 72 h. Otherwise, the differences were significant ($P<0.05$). Figure 3b shows that DCW in the original medium ceased increasing at 18 h while cell growth in the improved medium did not stop until 30 h. Moreover, the biosynthesis rate of ε-PL was faster throughout fermentation and did not stop until 48 h of fermentation in the improved medium, with 2.96±0.10 g/L of maximum ε-PL accumulation, which was 55.8 % higher than that in the original medium with production of ε-PL ceasing at 30 h (Fig. 3c). In conclusion, although cell lysis happened at the same time point of 36 h in both media, the mycelia in the improved medium still seemed better adapted to the low pH value than those in the original medium. Obviously, the remaining glycerol concentration were quite high until the end of shake-flask fermentation in both media,

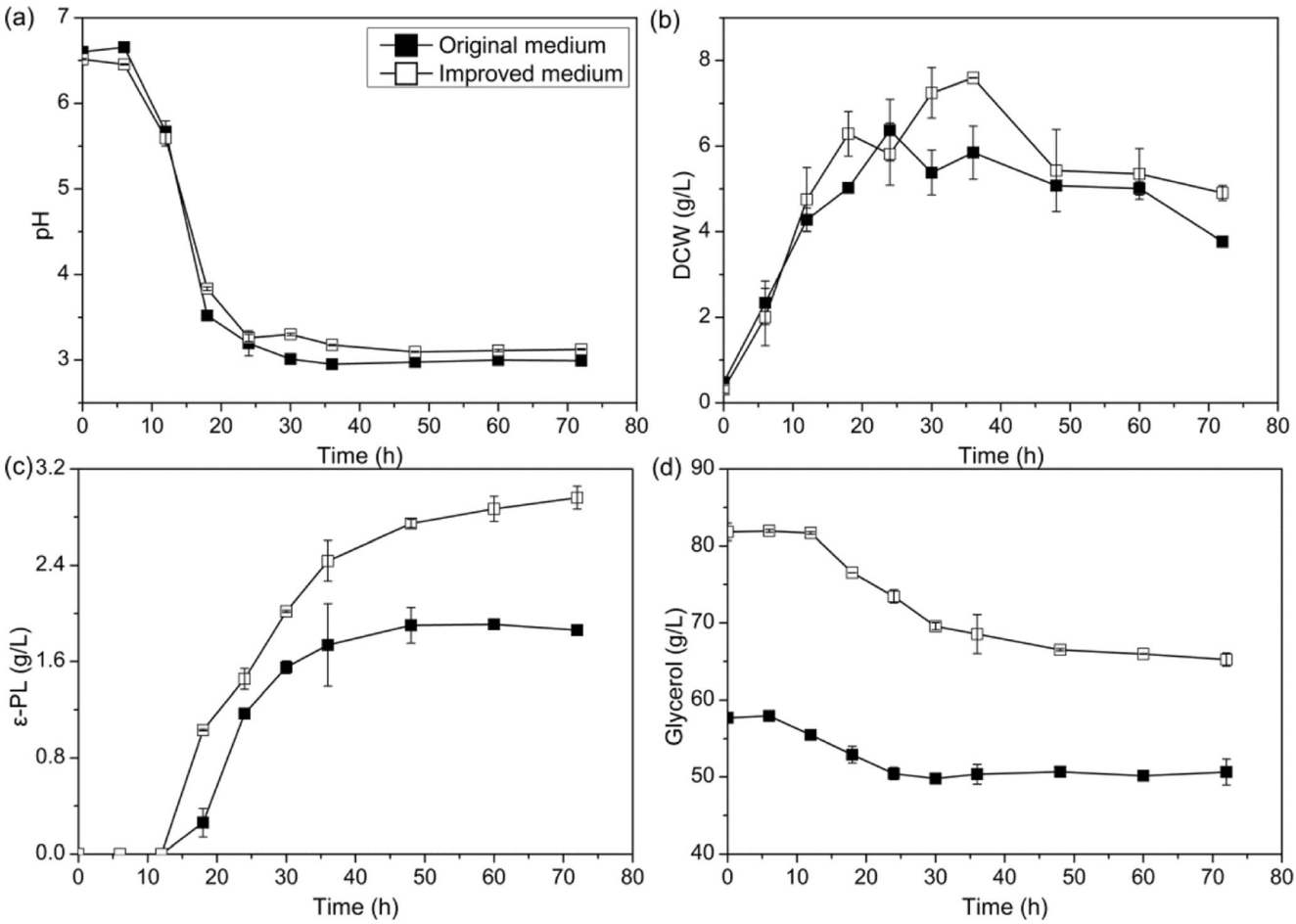

Fig. 3 Time profiles of ε-PL production in original and improved media in shake-flask fermentation without pH control. *Filled squares* Original medium, *open squares* improved medium

attributed to the suppression of metabolism by the low pH value (Fig. 3d). However, the pH could be controlled automatically at an optimal level in the fermenter, which would lead to the continuous consumption of glycerol. Therefore, the remaining glycerol could be negligible at the end of fermentation, which would not obstruct product recovery.

It was reported that amino acids can maintain intracellular pH (pH_i) stability by consuming hydrogen ions as part of the decarboxylation reaction when the bacteria are surrounded by a lower pH environment. Glutamate, arginine and lysine are specifically responsible for the pH_i homeostasis of Gram-positive bacteria (Senouci-Rezkallah et al. 2011). In that case, the pH_i of the bacteria cultured in media rich in these amino acids may not drop sharply, and the activity of bacterial cells will not be affected severely when the environmental pH decreases dramatically. As summarized in Table 2, the contents of glutamate, arginine and lysine were much higher in the improved medium with fish meal and corn steep liquor as mixed organic nitrogen sources as compared with the original medium using beef extract as sole organic nitrogen source, which might be responsible for the elevated acid resistance ability of *Streptomyces* sp. M-Z18, and result in prolonged cell growth and ε-PL production at low pH values (Fig. 3b,c). Besides, the glutamate decarboxylation system converts one molecule of extracellular glutamate to a less acidic extracellular γ-amino butyrate equivalently, which helps to alkalinize the environment (Waterman and Small 1998). This might be the reason for the final pH of the improved medium being slightly higher than that of the original medium (Fig. 3a). On the other hand, the abundant amino acid content in the improved medium could also be used as a precursor for mycelia biosynthesis, as reflected in the increased cell growth observed (Fig. 3b). Meanwhile, aspartate and glutamate are precursors for L-lysine biosynthesis (Takehara et al. 2010). In addition, our previous study proved that external L-lysine could be assimilated directly for ε-PL synthesis by *Streptomyces* sp. M-Z18 (Chen et al. 2013). Therefore, the higher concentrations of these amino acids in the improved medium not only elevated acid tolerance capability of the mycelia but also led to accelerated ε-PL production and cell growth.

Production of ε-PL in fed-batch fermentation

A novel fermentation-strategy based on the improved medium and two-stage pH control was established to further enhance the production of ε-PL on fermenter scale (Chen et al. 2011a). Fed-batch fermentation was then performed in a 5-L fermenter to investigate the efficiency of the proposed strategy. As shown in Fig. 4, when residual glycerol and NH_3-N were below 10 and 0.5 g/L, respectively, the sterilized pure glycerol and 600 g/L $(NH_4)_2SO_4$ were automatically fed to maintain their concentrations. After 176 h of cultivation, ε-PL production and productivity of 35.24 g/L and 4.81 g/L day were obtained. These results clearly demonstrate that *Streptomyces* sp. M-Z18 can efficiently use fish meal and corn steep liquor

Table 2 Amino acid contents of the organic nitrogen sources and media employed in this study

Amino acid	Beef extract (g/g N)[a]	Fish meal (g/g N)[b]	Corn steep liquor (g/g N)[c]	Original medium (g/L)	Improved medium (g/L)
Asp	0.36	0.80	0.27	0.32	1.22
Glu	0.78	1.37	0.53	0.69	2.10
Ser	0.21	0.39	0.17	0.19	0.60
His	0.06	0.27	0.13	0.05	0.42
Gly	1.33	0.66	0.18	1.18	1.00
Thr	0.10	0.37	0.14	0.09	0.57
Arg	0.40	0.56	0.21	0.36	0.86
Ala	0.56	0.63	0.24	0.50	0.96
Tyr	0.07	0.27	0.08	0.06	0.41
Cys	0.00	0.02	0.02	0.00	0.03
Val	0.18	0.44	0.18	0.16	0.67
Met	0.05	0.25	0.05	0.04	0.37
Phe	0.11	0.34	0.11	0.10	0.52
Ile	0.11	0.37	0.11	0.10	0.56
Leu	0.17	0.75	0.29	0.15	1.15
Lys	0.27	0.83	0.15	0.24	1.24
Pro	0.91	0.33	0.29	0.81	0.53

[a] Organic nitrogen source of the original medium with a total nitrogen content of 0.89 g N/L

[b, c] Organic nitrogen sources of the improved medium with a total nitrogen content of 1.46 and 0.18 g N/L, respectively

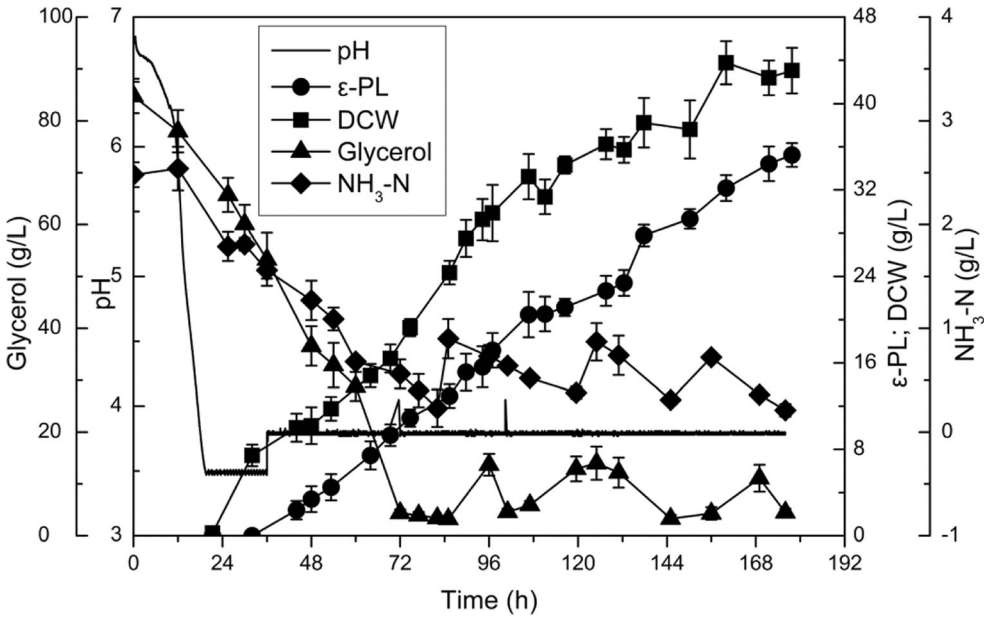

Fig. 4 Time profiles of ε-PL production using the improved strategy in fed-batch fermentation by *Streptomyces* sp. M-Z18. *Error bars* SD from three independent samples (some are too small to be distinguished). *Solid line* pH filled circles ε-PL, *filled squares* dry cell weight (DCW), *filled triangles* glycerol, *filled diamonds* NH_3-N

as mixed organic nitrogen sources for ε-PL production on fermenter scale.

Parameters of fed-batch fermentations for ε-PL production from different organic nitrogen sources are listed in Table 3. It can be seen that the production and productivity of ε-PL obtained from this study were 17.0 % and 15.1 % higher than those of fed-batch fermentation using beef extract as organic nitrogen source (Chen et al. 2011a), which were similar to those of glucose and glycerol co-fermentation by the same *Streptomyces* sp. M-Z18 (Chen et al. 2012). Moreover, the production and productivity of 35.24 g/L and 4.81 g/L day obtained in this study were considerable as compared with other strategies, indicating the predominant potential of the proposed strategy (Table 3). To the best of our knowledge, this is the first report on ε-PL production using agro-industrial by-products, viz. fish meal and corn steep liquor, as mixed organic nitrogen sources with both higher production and productivity in fed-batch fermentation.

Table 3 Comparison of ε-PL production in fed-batch fermentation in this study and previous reports

Strain	Carbon/organic nitrogen sources	Fermentation strategy	ε-PL production (g/L)	DCW (g/L)	ε-PL productivity (g/L day)	Fermentation Time (h)	Reference
Streptomyces albulus S410	Glucose/yeast extract	pH 6.8→5→4[a]	48.3	~ 27	6.04	192	Kahar et al. 2001
	Glucose/yeast extract	pH 6.8→5→4[b]	30	~ 25	3.75	192	Kahar et al. 2002
S. albulus IFO 14147	Glucose/yeast extract	pH 6.8→4[a]	5.16	34.2	0.516	240	Shih and Shen 2006b
S. albulus TUST2	Glucose/yeast extract	pH 6.8→4[a]	20.2	~ 18	5.05	96	Jia et al. 2009
Kitasatospora sp. MY 5-36	Glucose/yeast extract	pH 6.8→4[c]	34.11	22.98	9.34	87.6	Zhang et al. 2010a
Streptomyces sp. GIM8	Glucose/yeast extract	pH 6.8→3.8[a,d]	23.4	~ 7.3	~ 2.86	~ 196	Liu et al. 2011
Streptomyces sp. M-Z18	Glycerol/beef extract	pH 6.8→3.5→3.8[a]	30.11	40.66	4.18	173	Chen et al. 2011a
	(Glycerol+Glucose)/beef extract	pH 6.8→3.8[a]	35.14	41.68	4.85	174	Chen et al. 2012
	Glycerol/(fish meal+corn steep liquor)	pH 6.8→3.5→3.8[a]	35.24	43.05	4.81	176	This study

[a] Free cells in stirred fermenter

[b] Free cells in airlift fermenter

[c] Immobilized cells in stirred fermenter

[d] Resin-based in situ adsorption

Table 4 Profit evaluation for the production of ε-PL in a 10 m^3 fermenter

Material	Price[d] (USD/metric ton)	Revenue and expenditure (USD)	
		Original strategy	Proposed strategy
Glycerol[a]	~900	−1,540	−1,802
Beef extract[b]	~2.6×10^4	−1,820	0
Fish meal[b]	~1.7×10^3	0	−178.5
Corn steep liquor[b]	~5.0×10^2	0	−17.5
ε-PL[c]	~2.0×10^5	+45,165	+52,860

[a] Carbon source

[b] Organic nitrogen source

[c] Target product

[d] The market prices of different materials based on http://www.alibaba.com released on 5 January 2014

Profit evaluation of ε-PL production on industrial scale

Profit evaluation was conducted for a 10 m^3 fermenter with a working volume of 7 m^3, and the final volume of fermentation broth was taken to be 7.5 m^3 after fed-batch fermentation. Compared to carbon and organic nitrogen sources, the cost of inorganic nitrogen sources and inorganic salts was negligible and was therefore not considered for profit evaluation in this study. The general prices of different materials and profit assessment are listed in Table 4. As mentioned in our previous study, the yield of ε-PL by *Streptomyces* sp. M-Z18 from glycerol using two-stage pH control strategy was 0.132 g/g (Chen et al. 2011a). Hence, raw materials costs of fed-batch fermentations in the previous study using beef extract as sole organic nitrogen source (Chen et al. 2011a) and the proposed strategy using fish meal and corn steep liquor as mixed organic nitrogen sources were 3,360 and 1,998 USD, respectively. As compared with the original strategy, the cost reduction of raw materials in the proposed strategy was 1,362 USD. Furthermore, the proposed strategy gave 17.0 % increase of ε-PL production over that of the previous study, which resulted in an extra profit of 7,695 USD (Table 4). Therefore, the net profit in the proposed strategy was improved by 9,057 USD. As a result, the proposed strategy can significantly reduce raw materials cost and increase ε-PL profit for industrial scale production.

Conclusions

In the present work, fish meal and corn steep liquor as mixed organic nitrogen sources exhibited excellent performance for ε-PL production. A cost-effective and efficient strategy was developed that simultaneously reduced raw materials cost and enhanced ε-PL production. Thus, this study demonstrates the potential of the proposed strategy for the economical ε-PL production in industry.

Acknowledgments This work was supported financially by the Jiangsu Key Project of Scientific and Technical Supporting Program (BE2012616), the Cooperation Project of Jiangsu Province among Industries, Universities and Institutes (BY2013015-11), the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (111-2-06), the Open Project Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (KLIB-KF201302) and National Natural Science Foundation of China (21376106).

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