OPTIMIZATION OF L-ASPARAGINASE PRODUCTION FROM ESCHERICHIA COLI USING RESPONSE SURFACE METHODOLOGY

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Received: 07.11.2017
Accepted: 20.12.2018

SUMMARY

Among the antitumor drugs, bacterial enzyme L-asparaginase has been employed as the most effective chemotherapeutic agent in pediatric oncotherapy especially for acute lymphoblastic leukemia. In previous study, the L-asparaginase from Erwinia chrysanthermy was expressed in Escherichia coli BL21(DE3). The recombinant L-asparaginase was produced from recombinant E.coli BL21(DE3) under different cultivation conditions (inducer concentration, inoculum concentration and KH₂PO₄ concentration). The optimized conditions by response surface methodology using face centered central composite design. The analysis of variance coupled with larger value of R² (0.9) showed that the quadratic model used for the prediction was highly significant (p < 0.05). Under the optimized conditions, the model produced L-asparaginase activity of 123.74 U/ml at 1.03 mM IPTG, 3% (v/v) inoculum and 0.5% (w/v) KH₂PO₄. Recombinant protein was purified by two step using gel filtration and DEAE chromatography. The purified L-asparaginase had a molecular mass of 37 kDa with specific activity of 462 U/mg and identified by MALDI-TOF mass spectrometry. Results of MALDI-TOF analysis confirmed that recombinant protein was L-asparaginase II. Recombinant L-asparaginase has antiproliferative activity with K562 cell line. In conclusion, this study has innovatively developed cultivation conditions for better production of recombinant L-asparaginase in shake flask culture.

Keywords: Escherichia coli BL21(DE3), K562, L-asparaginase, MALDI-TOF, response surface

INTRODUCTION

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) which catalyses the hydrolysis of the amide group of asparagine to yield aspartate and ammonia is an important enzyme as therapeutic agents used in combination therapy with other drugs in the treatment of acute lymphoblastic leukemia in children, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma, and melanosarcoma (Stecher et al. 1999; Verma et al. 2007). The drug depletes the blood of asparagine, nonessential amino acid on which many cells depend for normal metabolic processes. Whereas normal cells compensate by synthesizing L-asparagine from aspartic acid and glutamine via the enzyme, asparagine synthetase, selected malignant lymphoid cells have low levels of the synthetic enzyme and depend on intracellular pools of L-asparagine for protein synthesis and cell functioning (Broome 1981; El-Bessoumy et al. 2004)This deprives the leukemic cell of circulating asparagine, which leads to cell death. The L-asparaginasases of Erwinia chrysanthemi (Erw. chrysanthemi) and Escherichia coli (E. coli) have been employed for many years as effective drugs in the treatment of acute lymphoblastic leukemia and leukemia lymphosarcoma (Graham 2003). L-asparaginase has an antioxidant property (Maysa et al. 2010). It is also used in food industry as a food processing aid; it can effectively reduce the level of acrylamide up to 90% in a range of starchy fried foods without changing the taste and appearance of the end product (Hendriksen et al. 2009).

Production of L-asparaginase is greatly influenced by fermentation medium composition and culture conditions such as temperature, pH, inoculum size, agitation rate, and incubation time.
(Hymavathi et al. 2009). Production of recombinant L-asparaginase from E. coli, optimization of culture medium composition and expression condition are important strategies to enhance the yield of biological active L-asparaginase. Response surface methodology (RSM) have been used for many decades by several researchers in biotechnology for an optimization strategy (El-Naggar et al. 2015; Erva et al. 2017; Kumara et al. 2013) and can be adopted on several steps, the first step is to screen the important parameters and the second step is to optimize those parameters (Nawani & Kapadnis 2004). These have several advantages that included less experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable conditions and forecast response (Chang et al. 2006). Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system can be determined. In the present study, strain recombinant E. coli BL21(DE3) harboring gene L-asparaginase was optimized expression condition using design of experiments and response surface methodology to enhance the production of the active form of recombinant L-asparaginase. The biological activity of recombinant L-asparaginase was also tested on human blood cancer cell line.

MATERIALS AND METHODS

Bacterial Strains

Recombinant E.coli BL21(DE3) harboring gene L-asparaginase (E-ASPG) was obtained from Department of Enzyme Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology, Vietnam.

Strain E-ASPG was grown in Lysogeny broth (LB) (pH 7.0) which comprised peptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L).

Chemicals

L-asparagine, Nessler’s reagent were from Sigma (Louis, USA). IPTG, trichloroacetic acid, bactotryptone and yeast extract were from Bio Basic Inc (New York, USA).... All other reagents were of analytical grade unless otherwise stated.

Culture condition

Strain E-ASPG was grown in Lysogeny broth. Inoculum of overnight cultures (1%) grown in LB medium was made in 25 mL LB medium in 100 mL Erlenmeyer conical flasks and grown to an optical density at 600 nm (OD$_{500	ext{ nm}}$) 0.4 - 0.6 at 37°C with shaking at 220 rpm. IPTG was then added to 1 mM final concentration, the culture was continuously incubated at 28°C with agitation of 220 rpm for 6 h of induction. Cells were harvested by centrifugation 8000 rpm/5 min.

Enzyme assay

Activity analysis of L-asparaginase II was performed according to Chung et al (Chung et al. 2010) using Nessler’s reagent to measure the released ammonia after L-asparagine hydrolysis. The enzyme activity of recombinant protein was determined using an ammonium sulphate calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μM of ammonia per minute.

Response surface methodology

The parameters namely induction concentration, inoculum concentration and KH$_2$PO$_4$ were optimized. These values were used in the RSM design and are as shown in Table 1.

Table1. Experimental range and level of the process variables for L-asparaginase production.

| Component                  | Unit | Variables | Range          | Level     |
|----------------------------|------|-----------|----------------|-----------|
|                            |      |           | -1,316 (-α)    | -1, 0, +1, +1,316 (+α) |
| IPTG concentration         | mM   | A         | 0.04-1.36      | 0.04, 0.2, 0.7, 1.2, 1.36 |
| Inoculum concentration     | % (v/v) | B     | 0.68-3.32     | 0.68, 1, 2, 3, 3.32 |
| KH$_2$PO$_4$ concentration | % (w/v) | C      | 0.26-2.24     | 0.26, 0.5, 1.25, 2, 2.24 |

For each run triplicate study was carried out. The 20 set of batch experiments designed by software are as given in Table 2. All the experiments were carried out in triplicates and the average of L-
asparaginase activity (U/ml) was considered as the response (Y). The following second-order polynomial equation explains the relationship between dependent and independent variables:

\[ Y = b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{23}BC + b_{13}AC \]

where Y is the dependent variable (L-asparaginase production); A, B and C are independent variables (inducer concentration, inoculum concentration and KH₂PO₄ concentration, respectively); b₀ is an intercept term; b₁, b₂ and b₃ are linear coefficients; b₁₁, b₂₂ and b₃₃ are the quadratic coefficients; and b₁₂, b₁₃ and b₂₃ are the interaction coefficients. The evaluation of the analysis of variance (ANOVA) was determined by conducting the statistical analysis of the model. In order to depict the relationship between the responses and the experimental levels of each of the variables under study, the fitted polynomial equation was expressed in the form of contour and response surface plots.

Table 2. RSM design for L-asparaginase production with experimental and predicted L-asparaginase activity

| Trails | A  | B  | C   | L-asparaginase activity (U/mL) |
|--------|----|----|-----|--------------------------------|
|        |    |    |     | Experimental                  | Predicted    |
| 1      | 1  | -1 | -1  | 105.248 ± 0.912                | 107.600      |
| 2      | -1 | -1 | 1   | 51.488 ± 0.101                 | 47.336       |
| 3      | -1 | -1 | -1  | 108.76 ± 0.405                 | 102.722      |
| 4      | 1.316 | 0  | 0   | 98.581 ± 0.101                 | 88.559       |
| 5      | 1  | 1  | 1   | 92.883 ± 0.456                 | 91.392       |
| 6      | -1 | 1  | -1  | 106.072 ± 0.963                | 95.523       |
| 7      | 0  | 0  | 0   | 100.983 ± 0.558                | 95.605       |
| 8      | 0  | 0  | 0   | 94.762 ± 0.811                 | 95.605       |
| 9      | 1  | 1  | -1  | 114.064 ± 1.419                | 122.622      |
| 10     | 1  | -1 | 1   | 49.194 ± 0.811                 | 52.214       |
| 11     | -1 | 1  | 1   | 62.24 ± 1.318                  | 64.293       |
| 12     | 0  | 0  | -1.316 | 120.659 ± 0.405             | 124.104      |
| 13     | 0  | 0  | 0   | 97.327 ± 2.788                 | 95.605       |
| 14     | 0  | 0  | 0   | 98.223 ± 0.101                 | 95.605       |
| 15     | 0  | 1.316 | 0  | 102.882 ± 0.608                | 106.127      |
| 16     | 0  | 0  | 1.316 | 67.544 ± 0.203                | 67.107       |
| 17     | 0  | -1.316 | 0  | 79.264 ± 0.152                 | 85.084       |
| 18     | -1.316 | 0  | 0   | 53.889 ± 0.152                 | 67.517       |
| 19     | 0  | 0  | 0   | 101.305 ± 0.101                | 95.605       |
| 20     | 0  | 0  | 0   | 90.446 ± 0.152                 | 95.605       |

**Protein purification**

The supernatant cell free extract containing the crude L-asparaginase was loaded into sephacyr S-200 column (2.6 × 6 cm) equilibrated with 50 mM potassium phosphate (pH 8) and eluted with the same buffer at the flow rate of 0.5 ml per minute. Fractions showing L-asparaginase activity were pooled and concentrated with bench top protein concentrator at 4°C. The homogeneity of the protein was checked by SDS-PAGE. The concentrated enzyme solution was added on the top of diethylaminoethyl sepharose ion exchange column (DEAE - sepharose) (2.6 × 6 cm) equilibrated with 50 mM Tris HCL (pH 8.6). The column was washed with 2 volumes of starting buffer and the protein was...
eluted with linear gradient of NaCl (0 - 1 M) prepared in 50 mM Tris HCL (pH 8.6) at the rate of 30 ml per hour. The eluate was collected with 1.5 ml per fractions. The fractions showing L-asparaginase activity were stored at 4°C.

**Molecular weight determination and quantitative protein determination**

The molecular weight (MW) of the purified protein was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), according to the method of Laemmli (Laemmli 1970).

Protein concentrations were estimated using the Bradford method, with BSA as the standard (Bradford 1976).

**Protein identification**

The purified protein was identified by MALDI-TOF mass spectrometry. The predicted protein was trypsin-digested and peptides were extracted according to standard techniques (Bringans et al. 2008). Peptides were analyzed by MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer (AB Sciex)(Applied Biosystems, USA). Spectra were analyzed to identify the protein of interest using Mascot sequence matching software (Matrix Science (Matrix Science Ltd, UK) with the MSPrn100 Database. Peptide fragments showing ion scores of >59 were identified as unique or highly similar (P < 0.01).

**Antiproliferative activity of L-asparaginase**

The human leukemia cell line K562 (chronic myelogenous leukemia) were used in this study. The antiproliferative activity of recombinant L-asparaginase was evaluated by the MTT reduction assay (Shanmugaparakash et al. 2015)

**RESULTS AND DISCUSSION**

**Optimization of recombinant L-asparaginase using response surface methodology**

The effect of medium components and condition expression (KH$_2$PO$_4$ concentration, inducer concentration, and inoculum concentration) on the L-asparaginase production was investigated. Table 2 shows the CCD design and the levels of each variable, L-asparaginase activity as the responses. The wide range of L-asparaginase activity from 49.2 to 120.7 U/ml was observed under these investigated expression condition. Correlation of L-asparaginase activity and the investigated variables was determined using the Design Expert sofware and was represented by the following equation:

$$Y = 95.61 + 7.99A + 7.9B - 21.65C + 5.56A*B + 6.04B*C - 10.14A$$

Where the response (Y) is the L-asparaginase activity, while A, B and C are the inducer concentration, inoculum concentration and KH$_2$PO$_4$ concentration, respectively.

The analysis of variance (ANOVA) tested using Fisher’s statistical analysis, was used to verify the adequacy of the model. The closer $R^2$ is to the 1, the stronger the model is and the better it predicts the response (Kaushik et al. 2006). In this case, the value of the determination coincident ($R^2 = 0.921$) indicates that 92.1% of the variability in the response was attributed to the given independent variables and only 6.9% of the total variations are not explained by the independent variables. In addition, the value of the adjusted determination coefficient (Adj $R^2 = 0.884$) is also very high which indicates a high significance of the model. In this model, a lower value of 8.17 for the coefficient of variation (CV), suggested a good precision and reliability of the experiment. As lack of fit is not significant, it clearly implies that the obtained experimental responses adequately fit with the model.

In order to understand the interactions of induction expression and to find the optimum conditions required for maximum L-asparaginase production, the 3-D response surface curves were plotted. Figure 1 shows the interaction between inoculum concentration and inducer concentration by keeping KH$_2$PO$_4$ concentration at optimum value. It showed that increase of IPTG concentration and inoculum concentration result in higher L-asparaginase activity; the highest value of L-asparaginase activity was obtained with high level of IPTG and inoculum concentration. It can be seen that maximum L-asparaginase production was attained at inducer concentration of 1.03 mM and inoculum concentration at optimum value. The analysis of the plots also demonstrated that the highest asparaginase activity was achieved when the concentrations of KH$_2$PO$_4$ were 0.5% (w/v). Further increase in KH$_2$PO$_4$ concentration decreases the activity.

Theoretical maximum enzyme activity (123.74 U/ml) was obtained at the optimal values of IPTG concentration at 1.03 mM, inoculum concentration at
3% (v/v) and KH$_2$PO$_4$ at 0.5% (w/v). Validation of model was carried out with the optimum values predicted by the software. Results showed that experimental value of enzyme activity (120 U/ml) was very closer to the predicted response and the predicted model fitted well (Figure 3).

Figure 2. Response surface plot of asparaginase production by recombinant E. coli showing the effect of inoculum concentration and IPTG concentration

Figure 3A. SDS-PAGE analysis of L-asparaginase expression at optimum condition. Lane 1: EASPG with IPTG induction, Lane 2: EASPG without induction, M: protein marker

Figure 3B. SDS-PAGE of the overexpressed and purified of rASPG in E. coli BL21 (DE3). (Lane M: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA)
Theoretical maximum enzyme activity (123.74 U/ml) was obtained at the optimal values of IPTG concentration at 1.03 mM, inoculum concentration at 3% (v/v) and KH₂PO₄ at 0.5% (w/v). Validation of model was carried out with the optimum values predicted by the software. Results showed that experimental value of enzyme activity (120 U/ml) was very closer to the predicted response and the predicted model fitted well (Figure 3A).

According to the results of our study the most important factors affecting protein expression is inducer concentration low inducer concentration may result in an inefficient induction and consequently, low recombinant protein yields. On the other hand, inducers added in excess can result in toxic effects including reduced cell growth or resulting in high protein expression, but it was inclusion bodies which were inactive forms of the recombinant proteins. Inoculum concentration also affects the recombinant L-asparaginase yield, higher levels of inoculum increases recombinant L-asparaginase yield but inoculum level depends on the inducer concentration. Another aspect of expression of recombinant L-asparaginase is KH₂PO₄ concentration, higher levels of KH₂PO₄ decreases recombinant L-asparaginase expression and its level depends on the inoculum concentration. In study of Bahreini et al (2014) high cell densities can be obtained associated with improving the productivity of recombinant L-asparaginase per cell but optimal IPTG concentration was very low (Bahreini et al. 2014).

Identification recombinant enzyme

The recombinant EASPG strain was expressed at optimum condition to harvest recombinant enzymes. The rASPG was purified from the cell lysis of EASPG by filter chromatography sephacryl S-200 and DEAE sepharose showed only one protein band about 37 kDa on SDS-PAGE (Fig.3B).

Table 4. The specific activity of recombinant L-asparaginase II from E. coli K-12 express in E. coli BLR(DE3) was 190 U/mg, recombinant L-asparaginase II from Erw. chrysanthemii 3937 express in E. coli BL21(DE3)pLysS was 118.7 U/mg (Kotzia & Labrou 2007), L-asparaginase II from B. subtilis express in E. coli JM109 (DE3) was 45.5 U/mg L-asparaginase from Rhizomucor miehei express in E. coli was 1.985 U/mg and activity of purified L-asparaginase from B. licheniformis was 697.09 U/mg.

| Purification steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification factor |
|-------------------|-----------------|-------------------|------------------------|-----------|-------------------|
| Cell lysis        | 360             | 2                 | 45                     | 100       | 1                 |
| Sephacryl S-200   | 318             | 0.52              | 101.6                  | 88.5      | 2.26              |
| DEAE-Sepharose    | 143             | 0.1               | 462.5                  | 44.9      | 4.55              |

Figure 5. Alignment of three neutral identified peptides (3 peptides) with L-asparaginase from (WP-039108651).
Identification of recombinant ASPG the single protein on SDS-PAGE (Fig.4) was cut out from the gel and used for MALDI-TOF analysis. There peptide fragments of the purified enzyme identified by MALDI-TOF mass spectrometry agreed with those of the L-asparaginase found in GenBank WP-039108651 GVMVVLNDR (position 171-179), GRGVMVVLNDR (position 169–179), TNATSLDTFR (position 189–198) (Figure 6). Whereas the peptide fragments showing ion scores above 44 were identified uniquely or highly similarly to p < 0.05. These peptides of the recombinant enzyme expressed by EASPG was matched to L-asparaginase resulting in a sequence coverage of 7% (relative RMS error = 90 ppm), mascot PMF score was 147, mass was 36777 Da.

**Antiproliferative activity of recombinant L-asparaginase**

The antiproliferative effects of L-asparaginase were evaluated on the human leukemia cell line K562 by using MTT cell viability assay. It was observed from Figure 6 that incubation of K562 with L-asparaginases resulted in decrease in the number of viable (metabolically active) cells as compared with control.

Recombinant L-asparaginase showed positive activity against leukemia cell line K562. The number of surviving cells decreases with increasing rASPG concentration. Recombinant L-asparaginase at concentration of 85 µg/ml inhibited 25% K562 cell (Fig 7).

**Figure 6.** The anticancer effect of the purified L-asparaginase on K562 cells after 72 of treatment A: Control cell, B: Treated cell

**Figure 7.** Recombiant L-asparaginase induces growth inhibition in K562 CML cells

![Figure 6](image)

![Figure 7](image)
In another study conducted by Guo et al., it was showed that the antitumor effects of L-asparaginase were observed in vitro with tumor cells K562, L1210, and P815 (P=0.01). As the concentration of recombinant L-asparaginas increased from 2.5-40 mg/L, the inhibitory rate with K562 increased from 20-50% (Guo et al. 2002). Song et al also showed that treatment of K562 and KU812 cells with different concentrations of asparaginase (0.02, 0.1, and 0.5 IU/mL) for 48 h, K562 cells increased the percentage of apoptotic cells (Song et al. 2015).

CONCLUSION

In conclusion, we were successful to optimize recombinant L-asparaginase expression and purification. The levels of the significant variables were optimized using response surface methodology with the following conditions: IPTG concentration 1 mM, inoculum concentration 3% (v/v) and KH2PO4 0.5% (w/v). Recombinant enzyme was purified and confirmed to be exactly L-asparaginase by MALDI−TOF. L-asparaginase has antiproliferative activity with human leukemia cell K562.

Acknowledgements: This study was supported by Vietnam Academy of Science and Technology (project VAST02.03/13-14: "Study on the production of recombinant L-asparaginase to inhibit cancer cell lines and treatment of acute lymphoblastic leukemia" project manager Prof. Quyen Dinh Thi]. We also extend our thanks to Dr. Hoang Thi My Nhung (Hanoi University of Science) for tumor cell line.

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**TÔI UỨ HÓA KHÀ NẴNG SỊNH TÔNG HỢP L-ASPARAGINASE TÀI TÔ HỢP TỪ CHỤNG *ESCHERICHA COLI* SỬ DỤNG PHƯƠNG PHÁP DẤP ÚNG BÈ MẤT**

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**TÔM TẮT**

Enzyme L. asparaginase từ vi khuẩn là một trong những thuốc sử dụng điều trị ung thư máu ở người, hiệu quả nhất trong điều trị ung thư bạch cầu cấp tính. Trong những nghiên cứu trước, L-asparaginase từ *Erwinia chrysanthemi* đã được chúng tôi biểu hiện thành công trong *Escherichia coli* BL21(DE3). Trong nghiên cứu này chúng tôi sử dụng phương pháp đáp ứng bể mặt để tối ưu kiến tạo các biến đổi đặc biệt cho sinh tổng hợp enzyme tài tổ hợp đạt hoạt tính cao. Các thông số lựa chọn để tối ưu bao gồm: nồng độ chất cắm ỨPTG, ti lệ giống và ti lệ KH₂PO₄. Bằng phương pháp quy hoạch thực nghiệm đã xây dựng được phương trình hồi quy mô tả mối quan hệ giữa hoạt tính enzyme và các biến đổi tự trị với hệ số hồi quy R² là 0,9, mức ý nghĩa cao với p < 0,05. Mô hình đã dự đoán hoạt tính L-asparaginase tài tổ hợp tối đa đạt được là 123,74 U/ml ở các giá trị yếu tố 1,03 mM ỨPTG, 3 % (v/v) ti lệ giống và 0,5 % (w/v) KH₂PO₄. Enzyme tài tổ hợp sau khi được tinh sạch đã được nhận dạng chính xác bằng phương pháp MALDI-TOF. L-asparaginase tinh sạch đạt hoạt tính riêng 462 U/mg và có hoạt tính ức chế sinh trưởng với dòng tế bào ung thư tự nhiên của người K562.

**Keywords:** *Escherichia coli* BL21(DE3), K562, L-asparaginase, MALDI-TOF, đáp ứng bể mặt