

# Expression, purification and evaluation of recombinant L-asparaginase in methylophilic yeast *Pichia pastoris*

*Biểu hiện, tinh sạch và đánh giá hoạt tính của L-asparaginase tái tổ hợp trong nấm men Pichia pastoris*

Research article

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L-asparaginase (EC 3.5.1.1), a therapeutic enzyme used in the treatment of childhood acute lymphoblastic leukemia (ALL). Hence, the goal of this work is study the expression and evaluation of hydrolysis activity of native sequence (X12746) encoding for L-asparaginase from *Erwinia chrysanthemi* NCPBB1125 in the popular expression system *Pichia pastoris*. The sequence of *asn* encoded for mature protein was expressed in *P. pastoris* SMD1168 and X33. SDS-PAGE analysis showed recombinant L-asparaginase was secreted efficiently. Stable and high hydrolysis activity of extracellular L-asparaginase in *P. pastoris* SMD1168 making it a potential candidate to produce recombinant protein. After purification, a specific band whose appearance approximately 45 kDa indicating the glycosylated protein with specific activity by 6.251 U $\text{mg}^{-1}$  and about 3 folds purifications.

*L-asparaginase (EC 3.5.1.1), một loại enzyme được sử dụng trong điều trị bệnh ung thư bạch cầu mãn tính ở trẻ em. Mục tiêu của nghiên cứu này là biểu hiện và đánh giá hoạt tính thủy phân của L-asparaginase mã hóa bởi đoạn gene (X12746) tương ứng từ Erwinia chrysanthemi NCPBB1125 được biểu hiện trong nấm men Pichia pastoris. Gene đã được cắt signal peptide và biểu hiện trong P. pastoris SMD1168 and X33. Qua phân tích kết quả điện di SDS-PAGE của môi trường sau lên men, L-asparaginase tái tổ hợp được tìm thấy trong dịch ngoại bào của P. pastoris. Với khả năng sản xuất protein có hoạt tính cao hơn so với chủng P. pastoris X33, SMD1168 được lựa chọn để biểu hiện L-asparaginase tái tổ hợp. Sau khi tinh sạch, sự xuất hiện của một băng có kích thước phân tử xấp xỉ 45 kDa trên điện di SDS-PAGE cho thấy protein tái tổ hợp đã bị glycosyl hóa với hoạt tính riêng 6.251 U $\text{mg}^{-1}$  và đạt độ sạch 3.471 lần.*

**Keywords:** L-asparaginase, *Pichia pastoris*, recombinant protein

## 1. Introduction

L-asparaginase (EC 3.5.1.1) was used as a chemotherapy regimen with other chemotherapy drugs including prednisolone, dexamethasone or vincristine in the treatment of childhood acute lymphoblastic leukemia (ALL). The molecular weight of L-asparaginase ~140kDa composed by four identical sub units. Structural studies showed *E. coli* L-asparaginase (EcA) existed in two types: a low affinity enzyme EcAI in the cytoplasm and a high affinity periplasmic enzyme EcAII (Campbell et al., 1967; Sanches et al., 2007). As administrated in the body, the drug hydrolyze exogenous L-asparagine leading to the

starvation of cancer cells which is the main reason of its apoptosis state. Besides, the toxic acrylamide in industrialized foods formed during browning process by Maillard reaction between the  $\alpha$ -amino group of free amino acid L-asparagines and carbonyl group of reducing sugar (Becalski et al., 2003) can be detected by of L-asparaginase reaction (Mashburn and Wriston, 1964) towards its substrates. Since the hydrolysis activity of L-asparaginase was first discovered by Lang in several beef tissues (Lang, 1904), the variable sources of L-asparaginase have been found including bacteria, yeast, fungi, plant, animal and even in soil (Kuldeep Kumar, 2012). Among of those, gene encoded for L-asparaginase (*asn*) from *Escherichia coli* and *Erwinia* has been used

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frequently for the production of recombinant L-asparaginase on the pharmaceutical market, but *Erwinia* L-asparaginase is more favour for patients with high sensitive toward EcA because of its low efficacy related to low toxicity (Mashburn and Landin, 1970; Adamson, 1971; Shifrin et al., 1973; Ashworth and MacLennan, 1974; Duval et al., 2002).

## 2. Materials and methods

In this study, gene encoded for L-asparaginase from *Er. chrysanthemi* NCPPB1125 was obtained from GenBank (X12746) was removed the signal sequence and expressed in *P. pastoris* SMD1168 and X33 (Invitrogen, USA). Other chemicals were purchased from Sigma-Aldrich and Merk (Germany).

### 2.1 Construction of recombinant vector pPICZ $\alpha$ A and *asn* gene namely pPICZ $\alpha$ Aasn

The signal peptide was removed using Signal IP 4.1 Server (at <http://www.cbs.dtu.dk/services/SignalIP/>) with two restriction sites of *Pml*I and *Xba*II were designated at the two ends of sequence to ligate into vector pPICZ $\alpha$ A. Forward primer 5' – ATA TAT CAC GTG GGC CGA TAA ACT GCC GAA T – 3' and reverse primer 5' – ATA AAG TCT AGA GCG TAG GTA TGG AAG TAT TCT T – 3' have the restriction site underlined respectively. The total volume of PCR reaction is 25 $\mu$ l containing: 15.5  $\mu$ l H<sub>2</sub>O; 2.5  $\mu$ l 10X *Pfu* buffer (without MgSO<sub>4</sub>); 2  $\mu$ l dNTP (2.5 mM); 1.5  $\mu$ l MgSO<sub>4</sub> (25 mM); 1  $\mu$ l forward primer (10 pM $\mu$ l<sup>-1</sup>); 1  $\mu$ l reverse primer (10 pM $\mu$ l<sup>-1</sup>); 0.5  $\mu$ l *Pfu* DNA polymerase (5 U $\mu$ l<sup>-1</sup>); 1  $\mu$ l DNA template (50-100 ng). Selected mode: 95C/5"; 30 cycles (95C/45"; 54C/45"; 72C/1'30"); 72C/10'.

PCR product was purified by GeneJET PCR Purification Kit (Thermo, USA). The DNA copies was ligated to cloning vector pJET1.2/blunt (2974 bp) by the following mixture: 5.5  $\mu$ l H<sub>2</sub>O; 1  $\mu$ l buffer 10X T4 DNA ligase; 1  $\mu$ l T4 DNA ligase; 0.5  $\mu$ l vector pJET1.2/blunt; 1.5  $\mu$ l PCR product of *asn* gene. The mixture was incubated at 22C for 16 hours.

The recombinant plasmid was transformed into *E. coli* DH10B competent cells using heat shock methods and screened on Luria Agar (LA) dish with additional ampicilin of 100  $\mu$ gml<sup>-1</sup>. DNA was extracted from recombinant vector using Accuprep<sup>®</sup> Gel Extraction Kit (Bioneer, USA) and ligated to expression vector pPICZ $\alpha$ A.

The resulting recombinant vector namely pPICZ $\alpha$ Aasn was transformed into competent cells of *P. pastoris* SMD1168 and X33 by the procedure described in *Pichia* Expression Kit (Invitrogen, Catalog no. K1710-01). The recombinant colonies was screened on YPD agar dish including yeast extract 1% (w/v), peptone-A 2% (w/v), D-sorbitol 2% (w/v) with additional 25 $\mu$ gml<sup>-1</sup> Zeocine<sup>®</sup>. To check the insertion of *asn* gene into *P. pastoris*, *AOX* forward primer 5' – GAC TGG TTC CAA TTG ACA AGC – 3' and reverse primer 5' – GCA AAT GGC ATT CTG ACA TCC – 3' was used in PCR experiment. DNA

template of recombinant *P. pastoris* was prepared by method described by Looke (Looke et al., 2011). The PCR selected mode was similar as that using *asn* gene.

### 2.2 Expression of recombinant L-asparaginase in methyl tropholic yeast *P. pastoris*

One colony of recombinant *P. pastoris* strain harbouring plasmid pPICZ $\alpha$ Aasn was cultured in 5 ml YP with glycerol 1% (v/v) in 16-18 hours. The inoculated media was added to 250 ml media in a shake flask 1 L by 1% (v/v) for induction with 0.5% absolute methanol. This carbon source was added to the media each 24 hours.

### 2.3 Purification of recombinant L-asparaginase

Ni-NTA column was used to purify recombinant L-asparaginase which has a high affinity for recombinant proteins according to the instruction of ProBond<sup>™</sup> Nickel-Chelating Resin (Invitrogen, USA).

For recombinant protein in methyltropholic yeast, the culture supernatant was collected 48 hours after induction by centrifuging the cell at 4000 rpm for 15 minutes at 4C and this was used for purification of recombinant L-asparaginase by Ni-NTA affinity chromatography. A column was added 2 ml Ni-NTA resin and equilibrated with 8 ml binding buffer (NaH<sub>2</sub>PO<sub>4</sub> 250 mM; NaCl 2.5 mM; imidazole 10 mM; pH 8.0). 8 ml of culture supernatant was loaded on to the Ni-NTA column. The column was washed with 24 ml of washing buffer (NaH<sub>2</sub>PO<sub>4</sub> 250 mM; NaCl 2.5 mM; imidazole 20 mM; pH 8.0) and protein was eluted with elution buffer (NaH<sub>2</sub>PO<sub>4</sub> 250 mM; NaCl 2.5 mM; imidazole 250 mM; pH 8.0). Fractions of 1 ml were collected and analyzed by SDS-PAGE.

### 2.4. Enzyme assay

#### 2.4.1 Protein concentration

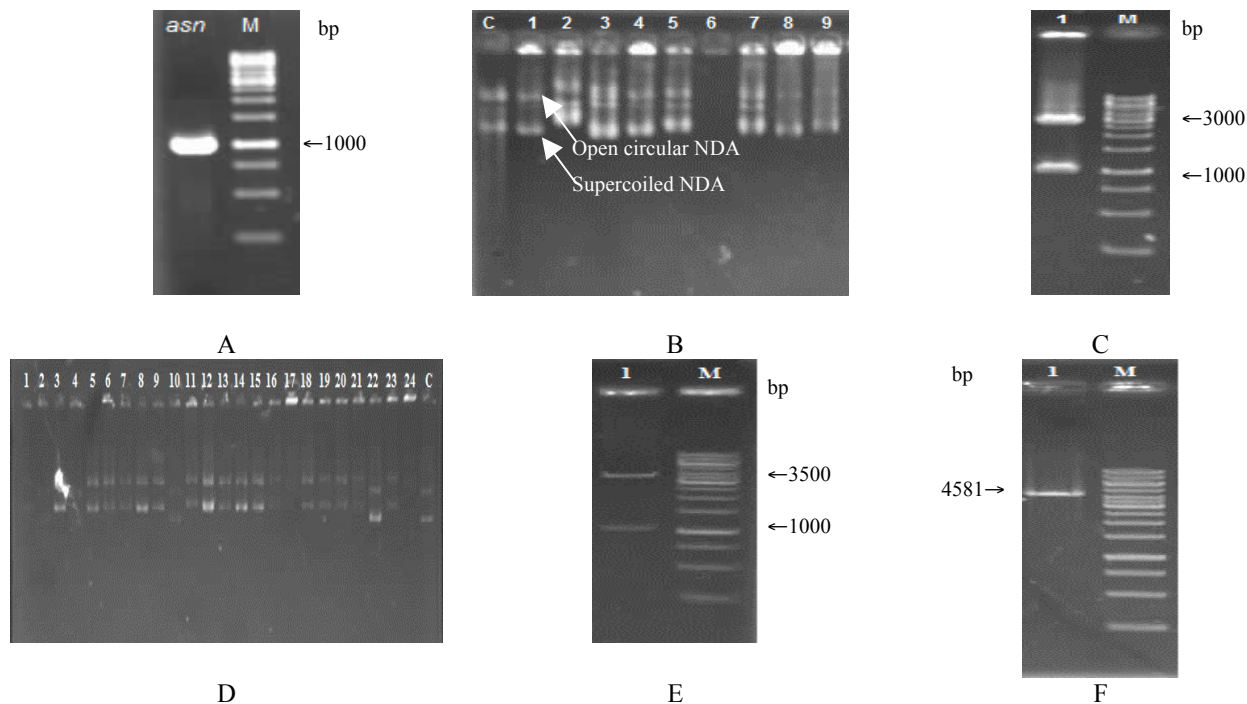
The concentrations of protein L-asparaginase were determined using the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard protein.

#### 2.4.2 L-asparaginase activity

Enzyme activity of recombinant L-asparaginase was measured by method introducing in previous study (Mashburn and Wriston, 1964) in which the rate of ammonia formation will be detected by Nessler's reagent at 37C. One unit of L-asparaginase activity was determined as the amount of enzyme that liberates 1  $\mu$ mol of ammonia from L-asparaginase per minute at 37C.

## 3. Results and discussions

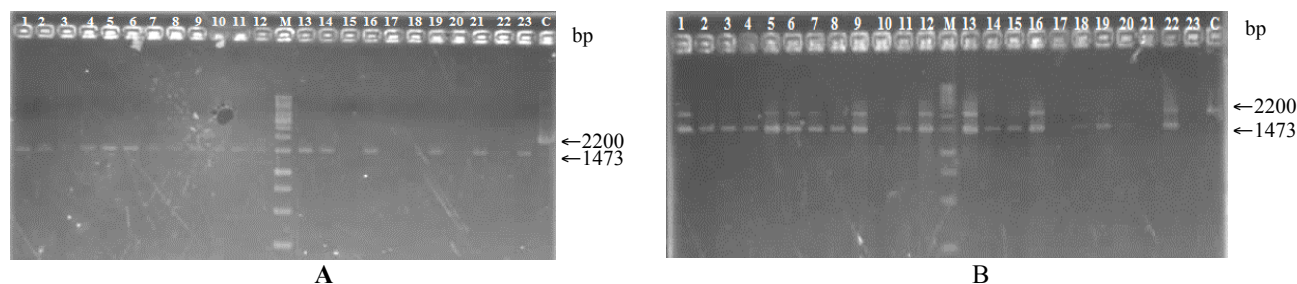
### 3.1 Construction of recombinant vector



**Figure 1. Construction of recombinant vector pPICαZAasn.** DNA electrophoresis (A) PCR product of gene encoded L-asparaginase from *Er. chrysanthemi* NCPPB1125 with 2 restriction sites of *PmlI* and *XbaI*; (B) Recombinant cloning vector, C - pJET1.2/blunt; (C) Digestion of recombinant cloning plasmid; (D) Recombinant expression vector, C- pPICZαA ; (E) Digestion of recombinant expression plasmid; (F) Linearized pPICZαAasn. M: DNA ladder 10kpbs

The PCR product on electrophoresis gel showed a band near the position of 1000 kb ladder indicating the *asn* gene with the molecular weight of 981 bp (Fig. 1A). An insertion event of *asn* gene into cloning vector pJET1.2/blunt was showed on electrophoresis gel resulting a higher molecular weight of recombinant cloning vector in comparison with “empty” vector which represented by lane 2 (Fig. 1B). The expected results should be one band of interested plasmid but in every lane, two visible bands could be seen clearly, it is important to realize there are five types of plasmid in order of electrophoresis mobility including nicked open-circular DNA, relaxed circular DNA, linear DNA, supercoiled DNA and supercoiled denatured DNA. On the electrophoresis gel, there are usually two types of plasmid such as supercoiled DNA and open-circular DNA, and other bands corre-

sponding to different form of plasmids. The rate of migration of plasmid on the gel is proportional with applied voltage so open-circular DNA migrates slower than supercoiled DNA because of its stretched conformation. The insertion of *asn* gene into cloning vector was confirmed again by the digestion of two restriction enzymes *PmlI* and *XbaI* at the two ends of sequence (Fig. 1C). Next, foreign gene was inserted into vector pPICZαA, the heavier molecular weight of recombinant vector corresponds to lane 1-21 and lane 23-24 indicating the insertion of *asn* gene (Fig. 1D). This was confirmed again by using two restriction enzymes resulting one band of interested gene and one band of pPICZαA (3600 bp) on the electrophoresis gel (Fig. 1E). For transformation into *P. pastoris* cells, the recombinant vector was linear (4581 bp) for integration into yeast genome (Fig. 1F).



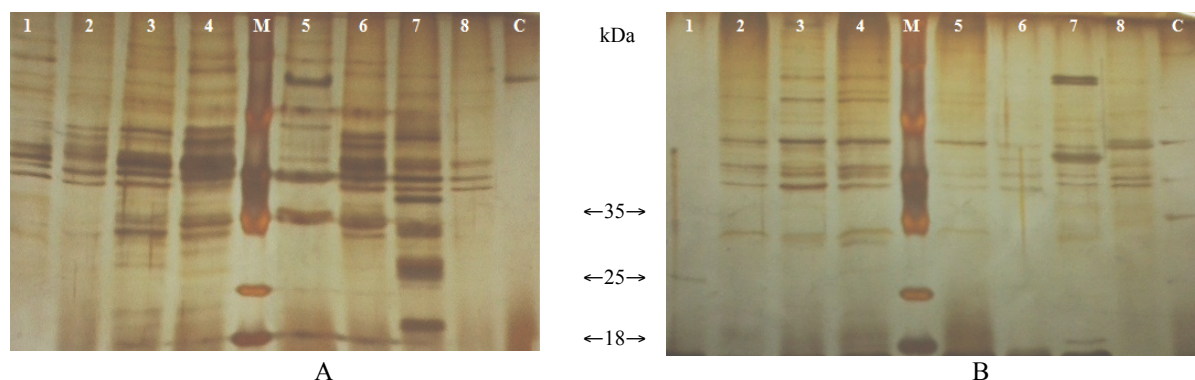
**Figure 2. DNA electrophoresis of PCR product of DNA extracted from recombinant *P. pastoris* SMD1168 (A) and X33 (B) using *AOX* primers with C (control) are those of *P. pastoris* SMD1168 and X33 without the inserted gene respectively. M: DNA ladder 10 kpbs**

As can be seen, the result showed there is one band of wild-type (2200 bp) and one band near the position of

1500 bp ladder which equivalents to the inserted gene (981 bp) plus *AOX* primer (492 bp) (Fig. 2). This con-

firms the foreign gene had been integrated into *P. pastoris* SMD1168 and X33 genome.

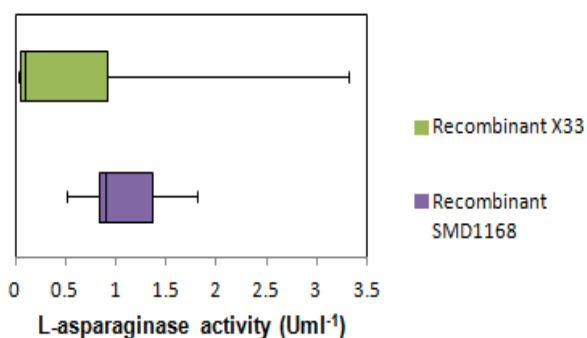
### 3.2 Extracellular proteins in *P.pastoris*



**Figure 3.** SDS-PAGE of crude supernatant of recombinant *P. pastoris* SMD1168 (A) and X33 (B); C (control) are that of *P. pastoris* SMD1168 and X33 without *asn* gene respectively. M: Protein marker.

The appearance of several bands on electrophoresis gel indicated the recombinant L-asparaginase was secreted efficiently into the broth. In contrast, the heterologous L-asparaginase encoded by *ASP3* gene of *Saccharomyces cerevisiae* was expressed in *P. pastoris* GS115 was intracellular proteins (Ferrara et al., 2006). Thus, the supernatant was used to evaluate the L-asparaginase activity.

### 3.3 Determination L-asparaginase activity from fermented supernatant



**Figure 4.** L-asparaginase activity of crude supernatant of recombinant *P. pastoris* SMD1168 and X33

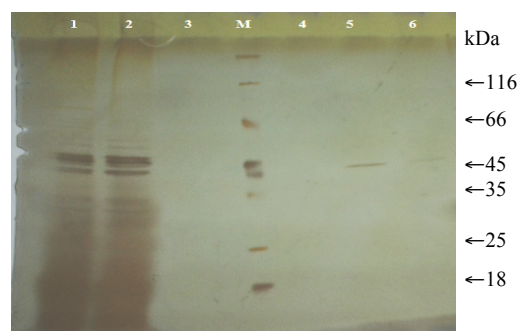
The distribution of data (Fig. 4) of hydrolysis activity of two recombinant strains revealed the recombinant L-asparaginase in *P. pastoris* SMD1168 is more favored than that in *P. pastoris* X33. One factor contributes for this difference can be explained by the efficiency of transformation (Fig. 2A) in which the number of homozygous double transformants are minority in *P. pastoris* X33. The highest activity of recombinant L-asparaginase in crude extract recorded of *P. pastoris* X33 was 3.3 Uml<sup>-1</sup> while in *P. pastoris* SMD1168 was 1.8 Uml<sup>-1</sup>.

### 3.4 Purification and evaluation of recombinant L-asparaginase

The recombinant L-asparaginase was fused with 6xHis tag has a high affinity with Ni-NTA column so the hetero-

ologous protein accumulated in elution fragment corresponding to desired proteins

The appearance of a single band at lane 5 and lane 6 (Fig. 5) ~ 45 kDa of ladder protein indicated the recombinant L-asparaginase. The unexpected higher molecular mass can be explained by the glycosylation in *P. pastoris*. The purified protein was used to evaluate the hydrolysis activity of recombinant L-asparaginase.



**Figure 5.** Purification of supernatant of a recombinant *P. pastoris* SMD1168 (Lane 1: Crude supernatant; Lane 2: Flow through; Lane 3: Wash; Lane 4 – 6: Elution fragments)

The appearance of a single band obtained after the supernatant had flowed through the affinity chromatography column was ~45 kDa marker (Fig. 5) indicate the expected extracellular L-asparaginase. In fact, the molecular weight was higher than predicted value (~35 kDa). This difference can be explained by the contribution of glycosylations to the molecular mass of protein molecule because of the adjacent *O*-linked (hydroxyl group of serine, threonine or hydroxylisine) and *N*-linked (group of asparagines carbohydrate chains) to heterologous proteins in *P. pastoris* (Lin-Cereghino et al., 2002). This was also observed when recombinant L-asparaginase was produced in *P. pastoris* GS115 possessing a molecule mass of ~45 kDa (Ferrara et al., 2010) higher than expected of ~35 kDa (Ferrara et al., 2006), the difference of the L-asparaginase band on electrophoresis gel can be explained by different glycosylation degrees. Accordingly, the ma-

ture protein was predicted to have 4 potential O-linked sites by NetOGly 4.0 (available at <http://www.cbs.dtu.dk/services/NetOGlyc/>) and 4 potential N-linked sites by NetNGly 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>). The specific activity of recombinant L-asparaginase in *P. pastoris* SMD1168 after affinity step obtained by  $6.251 \text{ U mg}^{-1}$  with a recovery rate of 16.667% and 3.471 fold purification. The values obtained from this result are higher than that of intracellular proteins in *P. pastoris* GS115 of  $800 \text{ U g}^{-1}$  (Ferrara et al., 2006) but lower when the extraction procedure of heterologous protein was selected respectively  $\sim 15000 \text{ U mg}^{-1}$  (Ferrara et al., 2010).

## 4. Conclusions

We have cloned the *asn* gene from *Er. chrysanthemi* NCPPB1125 and expressed successfully in *P. pastoris* SMD1168 and X33. The recombinant L-asparaginase was secreted efficiently into the media. After purification, a specific band  $\sim 45 \text{ kDa}$  implied the desired protein with the specific activity by  $6.251 \text{ U mg}^{-1}$ , the recovery rate by 16.67% and  $\sim 3$  fold purification. Characterization and optimization of this protein can be accessed in the next works.

## 5. References

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