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Cultivation Process Optimization Of Recombinant *Bacillus Subtilis* Harbouring Apoptin Gene

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The success of recombinan apoptin production in native form in the previous results open the way to develop this anticancer protein production to the larger scale. We optimized cultivation process of recombinant *Bacillus subtilis* 168 harbouring pOXGW12His8Arg with apoptin gene in a stirred tank fermentor and shake flasks. The parameters to optimize cultivation process are xylose-inducer concentration, agitation speed, and aeration rate. The xylose-inducer concentration variations are carried out in a shake flasks with 100 mL volume broth, while the agitation speed and aeration rate variation is carried out in a stirred tank fermentor with 3 L volume broth. The xylose concentration is varied between 0-5% w/v, while agitation speed and aeration rate are varied between 150-250 rpm and 0.5-1.5 NL min^{-1} respectively. The best condition in this cultivation is 1% w/v of xylose, 250 rpm of agitation speed and 1,5 NL min^{-1} of aeration rate giving the specific growth rate value for each parameter of 0.628 h^{-1}; 0.630 h^{-1}; and 0.747 h^{-1} respectively. The recombinant apoptin were purified by Ni-NTA column using AKTA system. However, the results showed that the optimum condition of producing recombinant apoptin was 1% w/v of xylose, 250 rpm of agitation speed, and 0.5 NL min^{-1} of aeration.

Key words: aeration, agitation, apoptin, optimization, xylose

Keberhasilan produksi apoptin rekombinan dalam bentuk *native* pada penelitian sebelumnya membuka jalan untuk mengembangkan produksi protein antikanker ini ke skala yang lebih besar. Di dalam studi ini, kami melakukan optimasi kultivasi bakteri rekombinan apoptin dalam *stirred tank fermentor* dengan bakteri *Bacillus subtilis* 168 rekombinan apoptin. Parameter yang dioptimasi adalah konsentrasi induksi *xylose,* kecepatan agitasi dan laju aerasi. Variasi konsentrasi induksi *xylose* dilakukan dalam *shake flasks* dengan volume kultur 100 mL dengan konsentrasi 0-5% b/v sedangkan variasi kecepatan agitasi dan laju aerasi dilakukan dalam *stirred tank fermentor* dengan volume kultur 3 L dengan kecepatan dan laju masing-masing adalah 150-250 rpm dan 0,5-1,5 NL min^{-1}. Hasil yang didapat adalah pertumbuhan bakteri optimum dicapai pada konsentrasi *xylose* 1% b/v, kecepatan agitasi 250 rpm, dan laju aerasi 1,5 NL min^{-1} dengan nilai laju pertumbuhan spesifik bakteri untuk masing-masing variasi adalah 0,628 h^{-1}; 0,630 h^{-1}; dan 0,747 h^{-1}. Apoptin rekombinan dimurnikan dengan menggunakan kolom Ni-NTA pada sistem AKTA. Namun, hasilnya menunjukkan bahwa kondisi optimum untuk memproduksi apoptin rekombinan dicapai pada konsentrasi *xylose* 1% b/v, kecepatan agitasi 250 rpm, dan laju aerasi 0,5 NL min^{-1}.

Kata Kunci: aerasi, agitasi, apoptin, optimasi, *xylose*

Apoptin is chicken anemia virus (CAV) protein which contains 121 kinds of amino acid and molecular weight about 14 kDa (Noteborn *et al.* 1991; Maddika *et al.* 2006). Apoptin was reported can induces apoptosis in human transformed or tumor cells but not in normal cells (Dannen *et al.* 1997). This specificity and selectivity properties may be the reason to develop apoptin production in larger scale.

The common method that used by any researchers to develop production of this protein is by recombination. One of the major obstacles in apoptin recombinant production is to produce protein in native form. Recombinant protein which is expressed in *Escherichia coli* is often in an inclusion body (insoluble aggregate) which cause the protein can not form a proper tertiary structure and will be inactive (Ahsan *et al.* 2005). Besides, the use of isopropyl β-D-1 thiogalacto pyranoside (IPTG) as inducer in *E. coli* expression system would also inhibits the development of apoptin production in larger scale because it is not economical. Recently, we reported that he succeded to produce recombinant apoptin in native form with *Bacillus subtilis* 168 as cell host which has been transformed by Gateway system

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(Japan) with pOXGW12His8Arg plasmid and use xylose as the inducer. This method is more realistic to be applied in the larger scale because it needs a cheaper inducer compared to IPTG (Sahlan *et al.* 2013).

Before doing the apoptin production in large scale, optimization studies are needed to determine the optimum operating conditions. In this study several parameters of recombinant bacteria cultivation are considered, which are agitation speed, aeration rate, and xylose concentration. Optimization of agitation speed and aeration rate are carried out in a stirred tank fermentor (Eyela) with 3 L volume broth while the optimization of xlose concentration is carried out in shake flasks with 100 mL volume broth. This study will show how these parameters affect the growth profile, the specific growth rate value of recombinant bacteria and production of the protein.

MATERIAL AND METHODS

Microorganisms. Bacteria used in this study is *Bacillus subtilis* 168 has been transformed by Gateway system (funded by JSPS Young Researcher Invitation Program, NAIST, Japan) with pOXGW12His8Arg plasmid for apoptin expression.

Cultivation Medium. Cultivation mediun used in this study is Luria Bertani (LB) which contains (L^{-1}) 10 g of bacto tryptone (Becton, Dickinson & Company), 10 g of NaCl (Merck), and 5 g of yeast extract (Becton, Dickinson & Company). These three components are dissolved in aquadest and pH was adjusted to 7. This solution was then autoclaved at 121 °C for 15 min. The medium was then cooled down to ambient temperature and tetracycline (Darya Varia) is added as antibiotics until its concentration reach 10 µg mL^{-1} in the medium.

Cultivation in Stirred Tank Fermentor for Agitation Seed and Aeration Rate Optimization. Optimization of agitation speed and aeration rate starts with inculating 10% v/v of preculture to 3 L of sterilized medium that has contained 5 µg mL^{-1} of tetracycline at normal pH and 37°C. Agitation speeds are varied between 150-250 rpm and aeration rates are varied between 0.5-1.5 NL min^{-1}. When OD_{600} reaches 0.4, tetracycline was added again until its concentration was 10 µg ml^{-1} in medium. Cultivation was then continued until OD_{600} value reaches 0.6-0.8, xylose (Sigma-Aldrich) is added in this step with 1% w/v concentration. The culture was cultivated again until stationary phase is reached.

Cultivation in Shake Flasks for Xylose Concentration Optimization. LB medium which has contained 10 µg mL^{-1} of tetracycline is prepared in 18 flasks and volume of medium in each flask is adjusted so the xylose concentration in medium reach 0%, 1%, 2%, 3%, 4%, and 5% in 100 mL medium (each concentration is done in 3 repetitions). One mililitre of inoculums that has been dissolved in sterilized aquadest is added to each flasks. These flasks was then incubated in shaker incubator at 37 °C and 120 rpm until OD_{600} value reach 0.6-0.8. next step is xylose addition with the varied concentration and the culture was incubated again for 2 h and the growth profile will be evaluated.

Purification of Recombinant Apoptin. The recombinants apoptin were purified by AKTA system using Ni-NTA column (HisTrap FF) with buffer A (100 mM Tris HCl pH 7.4, 100 mM NaCl, and 20 mM imidazole) as binding and washing buffer and buffer B (100 mM Tris HCl pH 7.4, 100 mM NaCl, and 500 mM imidazole) as elution buffer. The purified recombinant apoptin was confirmed by 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration proteis analyzed by Bradford method with Bovine Serum Albumin (BSA) as standard.

RESULTS

Optimization of Agitation Speed in Stirred Tank Fermentor. Optimization of agitation speed was carried out in stirred tank fermentor with 3L of volume broth at constant temperature and aeration rate which are 37 °C and 0,5 NL min^{-1} respectively and three variations of agitation speed (150, 200, and 250 rpm). The growth profile on Fig 1 shows that bacteria grows faster in higher agitation speed. It can be seen on the highest speed, the lag phase is shorter and the specific growth rate value is the highest (Fig 2).

Optimization of Aeration Rate in Stirred Tank Fermentor. Optimization of aeration rate was also carried out in stirred tank fermentor with 3L volume broth at constant temperature and agitation speed which are 37 °C and 250 rpm respectively, with 3 variation of aeration rates (0.5, 1, and 1.5 NL min^{-1}). Based on specific growth rate value (µ) for each aeration rates (Fig 4), the highest rates gives the highest µ which means the bacteria grows faster in higher aeration rate. The aeration rate that gives the best bacterial growth in this study is 1.5 NL min^{-1}.

Optimation of Xylose Concentration as Inducer. Optimization of xylose concentration is aimed to

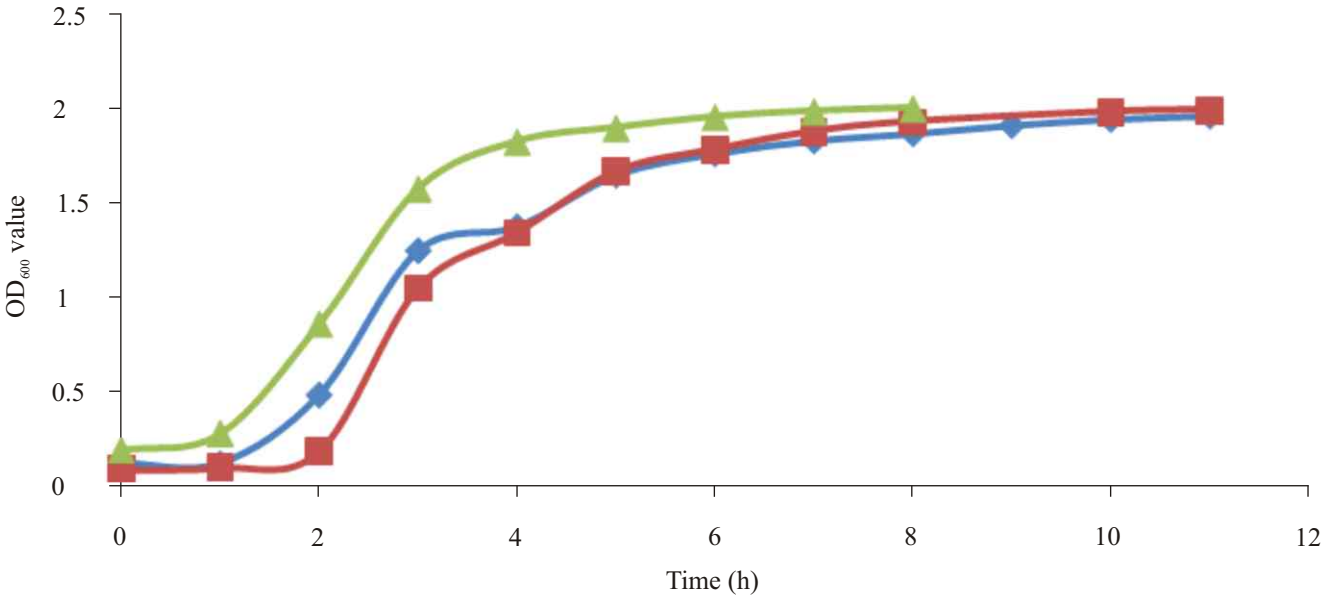

Fig 1 Bacterial growth profile in each agitation speed. : 150 rpm, : 200 rpm, and : 250 rpm.

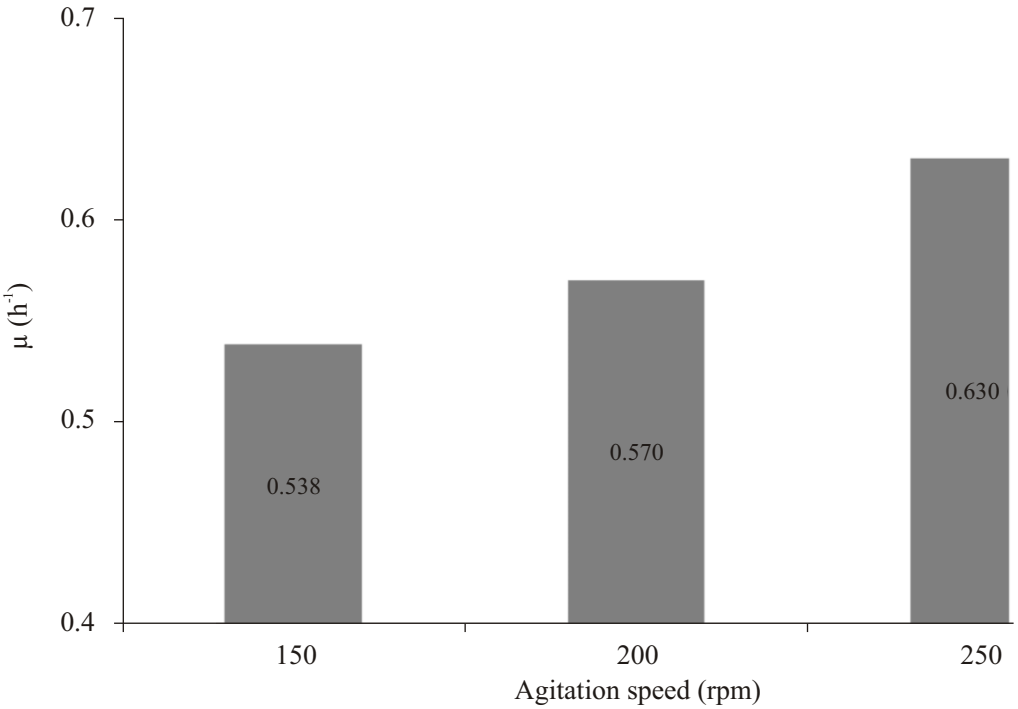

Fig 2 Specific growth rate value on each agitation speed.

determine if increasing xylose concentration will affect the cell growth or not since xylose is not the secondary nutrition but as inducer to apoptin expression. This optimization was carried out in shake flasks with 100 ml of volume broth and the xylose concentrations are varied between 0-5% w/v. The growth profile on Fig 5 shows that there is no significant effect in increasing xylose concentration to the bacterial growth. Based on specific growth rate calculation (Fig 6), the highest μ value is reached at 1% xylose concentration and decrease as the xylose concentration increase.

The apoptin recombinan expression. The cells were collected and purified by liquid chromatography AKTA system using HisTrap FF column. The cell was lysed by sonication process, centrifugation and applied to the column. Unbound protein was washed by binding buffer, after unbound protein was removed, the recombinant apoptin was eluted gradiently by the elution buffer. The apoptin was eluted in conductivity about 17-22 mS cm^{-1} (Fig. 7). The fraction of protein signal peak was confirmed by SDS-PAGE. The results

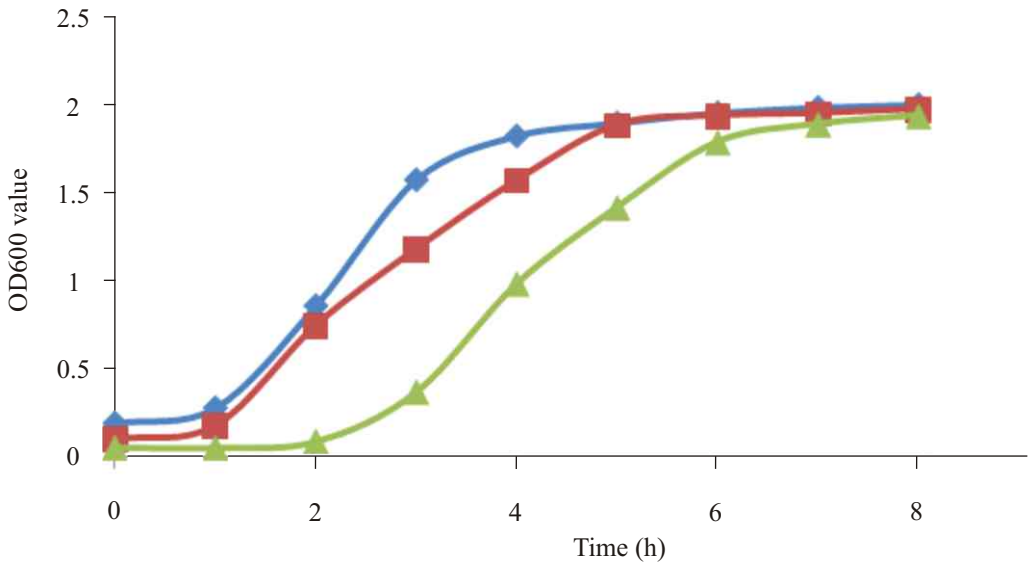

Fig 3 Bacterial growth profile in each aeration rate. : 0.5 NL min^{-1}, : 1 NL min^{-1}, and : 1.5 NL min^{-1}.

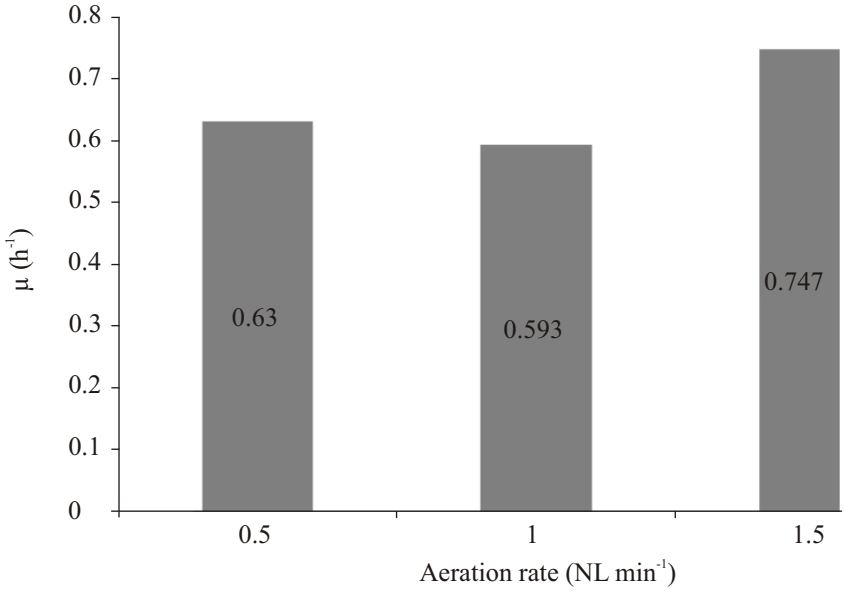

Fig 4 Specific growth rate value on each aeration rate.

showed that the molecular weight of apoptin about 15 kDa (Fig 8). The amount of protein produced in each cultivation process was analysed by Bradford method. The results showed in Table 1. The results showed that the optimum cultivation process for production the recombinant apoptin for agitation speed, aeration rate, and xylose concentration for induction which are 250 rpm, 0.5 NL min^{-1}, and 1% w/v respectively.

DISCUSSIONS

The process of agitation in the aerobic cell culture is important to capture oxygen and disperse it into medium. The results are appropriate to the theory that state when the impeller speed increase, air containing oxygen more captured in the behind of agitator blades and is dispersed into medium. The oxygen is mixture with medium and contact with cell surface. The agitation also acts to homogenous pH and temperature in the medium. The cell growth fast when the agitation speed is increased (Ducros *et al*. 2009). The agitation speed that gives the best bacterial growth in this study is 250 rpm. The interesting part that seen on Fig 1 is there are tendency to form diauxic growth pattern at 150 and 200 rpm growth profile but not at 250 rpm. It shows that the agitation speed may be affect the bacterial growth pattern on substrate consumption because there is more than one carbon source in this cultivation (LB and xylose) so this phenomenon may be happened, but still need further study to prove this

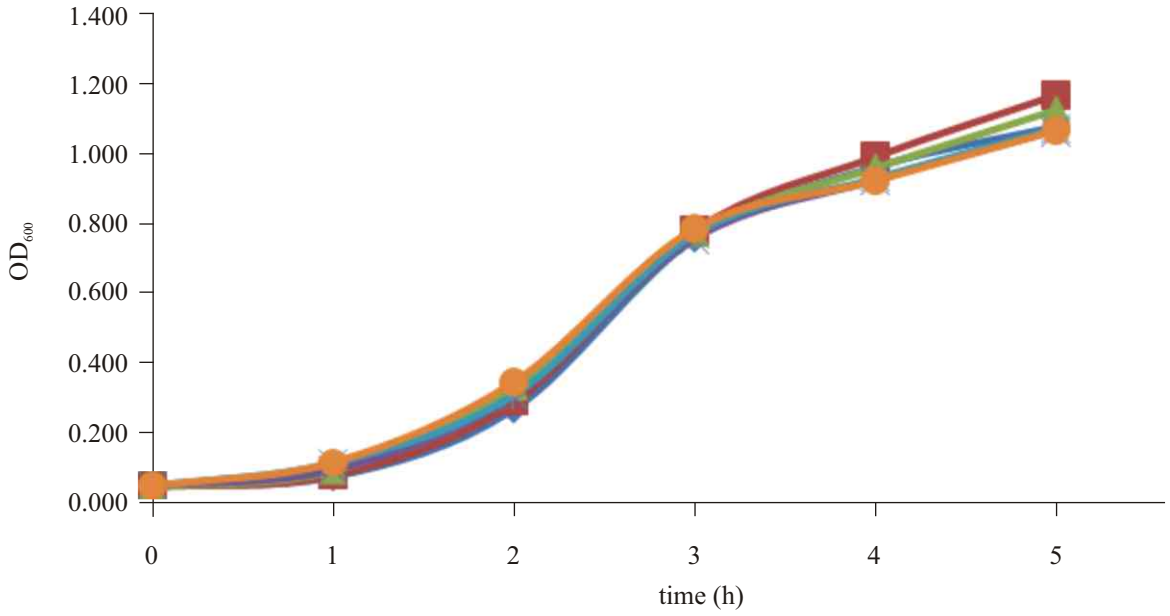

Fig 5 Bacterial growth profile in each xylose concentration. : 0%, : 1%, : 2%, : 3%, : 4%, and : 5% xylose concentration.

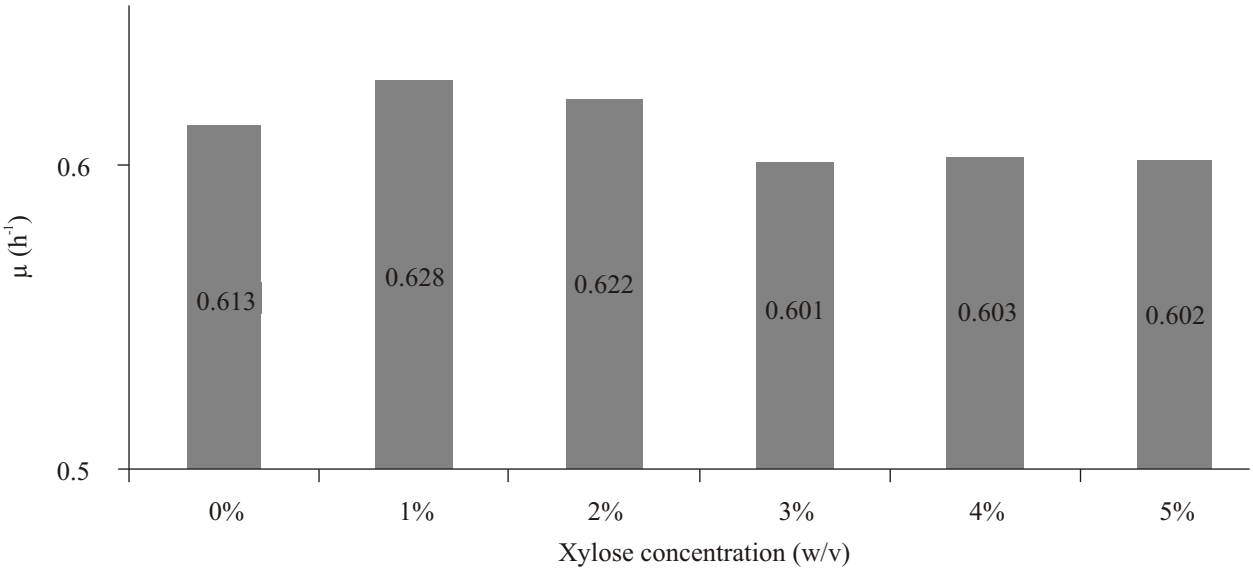

Fig 6 Specific growth rate value on each xylose concentration.

Tabel 1 The recombinant apoptin production

Cultivation condition			Weight of the apoptin recombinant (mg)
No.	Agitation (rpm)	Aeration (nl min^{-1})	
1.	150	0.5	0.025
2.	200	0.5	0.012
3.	250	0.5	0.152
4.	250	1.5	0.149

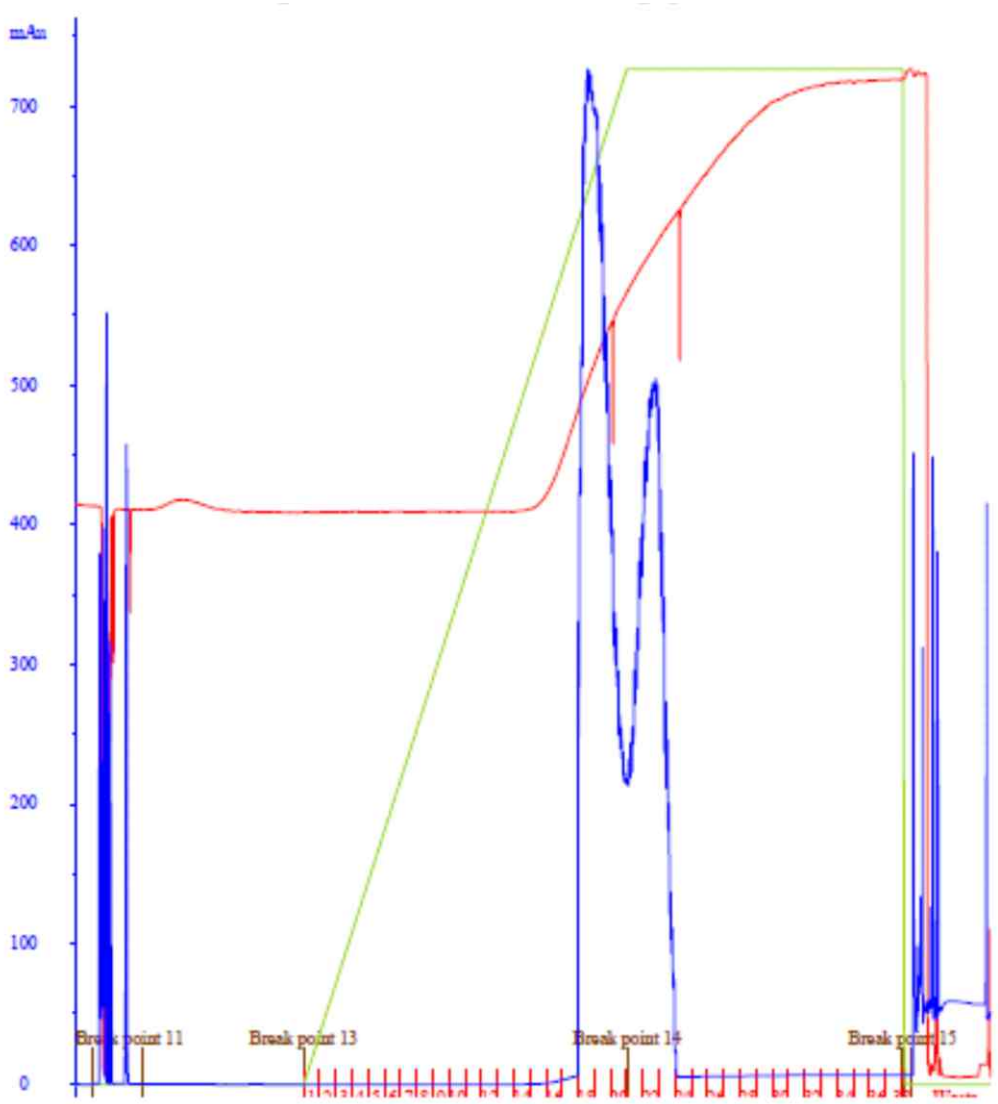

Fig 7 Chromatogram of the purification step the recombinant apoptin in Ni-NTA column. (blue) UV detection at 280 nm, (red) conductivity, and (green) gradient program.

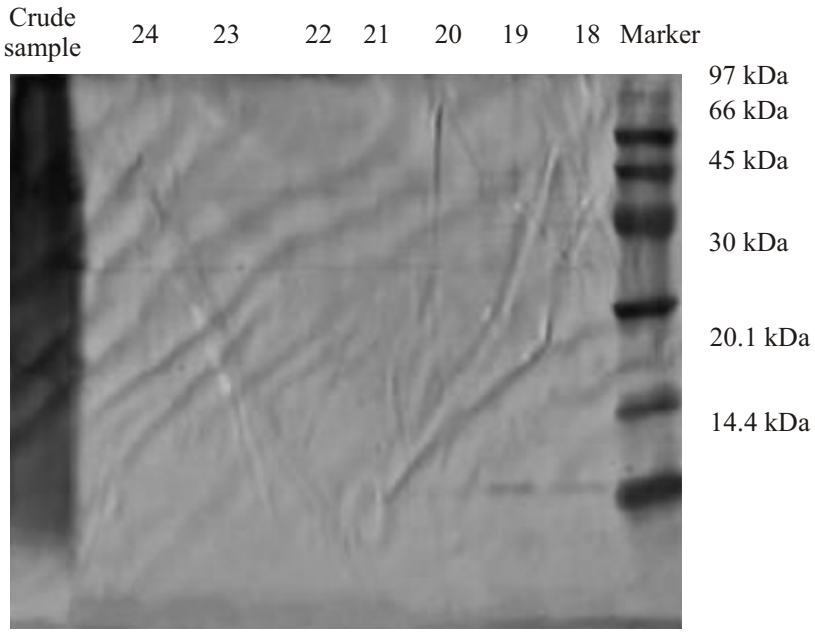

Fig 8 SDS PAGE of the recombinant apoptin. The molecular weight of recombinan apoptin was about 15 kDa.

possibility. Similar with agitation effect, the aeration rates also increased the dissolved oxygen level in the medium so the metabolism activity that also increased and the cell growth goes faster.

The xylose is not directly influence cell growth, it is an inducer molecules for protein expression and not affect cell growth. It shown that apoptin is not toxic protein for *B. subtillis*. The Fig 5 showed phenomenon of cell growth inhibition after supplemented by xylose. This inhibition phenomenon may be happened due to catabolite repression during cultivation. As for metabolites that may be formed are still being obstacle to be analyzed since the medium contains complex compounds. This phenomena is also related to the expression of the recombinant protein. In fast cell growth rate the recombinant protein is not well expressed. The results showed that the optimum cell growth rate was different with the recombinant protein production.

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