Research Article
Volume 14:4, 2020
DOI: 10.37421/jmgm.2020.14.463

Journal of Molecular and Genetic Medicine

ISSN: 1747-0862 **Open Access**

Expression of Human Basic Fibroblast Growth Factor Mediated by Mini Intein in *Bacillus subtilis*

Wu KC, Kwong WY*, Joey CY Chau, Choi MC and Christopher SK Chung

DreamTec Research Limited, Kowloon, Hong Kong, P.R. China

Abstract

Bacillus subtilis (*B. subtilis)* is an ideal host system in production of homologous proteins. However, the production of heterologous proteins in *B. subtilis* is rare due to low expression levels encountered in most cases. Inteins, also known as 'protein intron', which is capable of excised itself from its fusion partners, have been employed for the expression of recombinant proteins in various host systems especially in *Escherichia coli* (*E. coli)* but yet, only few paucity of employment of inteins for protein expression in *B. subtilis* has been found. In this communication, we demonstrated that *B. subtilis* was able to facilitate auto-cleavages between intein and C-extein. The construct, pECBS1-H6-DnaE-bFGF, in which a 6x His-tag (H6) and basic fibroblast growth factor (bFGF) were fused at the N- and C-terminus of *Asp* DnaE (intein) respectively, was shown to be capable of processing intracellular expression and auto-cleavages of bFGF with same primary sequence as *Homo Sapiens*. Moreover, switching shake of flask cultivation to small fermentative scale yielded 113 mg L^{-1} of biologically active bFGF. This approach of using intein *Asp* DnaE for the production of heterologous proteins is highly productive and should be explored further for industrial application.

Keywords: Basic Fibroblast Growth Factor (Bfgf) • FGF2 • Inteins • *Bacillus subtilis*

Introduction

Human basic fibroblast growth factor (bFGF, also known as FGF2), a member of the fibroblast growth factor family, has multiple therapeutic applications in neurodegenerative diseases, heart disease and in hard-to-heal wounds [1-3]. Moreover, bFGF plays an important role in tissue development by triggering the proliferation of fibroblasts and stem cells [4-8]. It also contributes vastly to the large-scale production of stem cells. However, low productivity of bFGF is encountered in current expensive production method which has hindered its commercial application in medical industry [9,10]. Unstable bFGF proteins are easy to be degraded in stem cell cultivation condition. Routine replacement of fresh culture media supplemented with commercial bFGF dramatically increases the research cost. In order to promote the stem cell research development, improvement of up-stream production of recombinant human bFGF in a cost-effective manner is crucial.

Escherichia coli (*E. coli)* is a widely utilized bacterial host for the expression of recombinant proteins without post-translational modifications. *E. coli* performs fast growth rate, cost effectiveness and ease of use for wide application in biotechnology Since *E. coli* is a gram-negative bacterium with an LPS outer membrane, the purified recombinant proteins always accompanied with substantial amount of endotoxins. These endotoxins might result in undesirable toxic effects when used for the purpose of treating tissue culture samples or on animal subjects. The endotoxins are difficult to isolate by downstream purification process, unless endotoxin-free water and endotoxin removal kit are employed. In this way, these kits scale up the production cost of protein interested.

Bacillus subtilis (B. subtilis) is a gram-positive bacterium which is regarded as Generally Recognized As Safe (GRAS) by FDA, as it is free of endotoxins [11]. Despite the expression levels of recombinant heterologous proteins are lower in comparison to *E. coli* [12], *B. subtilis* leads to relatively stable expression and the final products are safe to use. In this study, we aimed to exploit intein from DNA polymerase III unit of *Anabaena species* (*Asp* DnaE), with the 6x His tag on N-terminus in order to facilitate the intracellular expression of bFGF in *B. subtilis*. Surprisingly, bFGF was shown to be auto-cleavable during expression. Plasmid stability and cell viability remain maintained in high levels during shake flask cultivation and when the expression is scale-up to 4 L fermentation, no significant changed was found in both parameters while giving a great improvement of yield of bFGF, 113 mgL^{-1}. The study may provide a new option in production of bFGF and for others heterologous proteins by utilizing intracellular intein-mediated approach in *Bacillus subtilis*.

***Address for Correspondence:** Dr. WY Kwong, DreamTec Research Limited, Kowloon, Hong Kong, P.R. China, E-mail: hkdreamtec@gmail.com*

Received 05 August 2020; **Accepted** 07 September 2020; **Published** 15September 2020

Materials and Methods

Bacterial strains and chemicals

E. coli strain, DH5α was purchased from New England Biolabs (Ipswich, MA). *B. subtilis* strain WB800 was described in earlier report [13]. The synthetic DNA fragments, restriction enzymes and antibody against bFGF were purchased from Thermo Fisher Scientific (Ipswich, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Engineering of *E. coli/ B. subtilis* expression shuttle vector

pRB374 and pBR322 were employed as templates and the *E. coli/B. subtilis* expression shuttle vectors, respectively [14]. Further, pECBS1 was constructed by the following modification. Firstly, pBR374 (5.9 kb) was digested with SalI and BglII. The fragments released were replaced by SalI and BglII digested shotgun PCR fragment containing the following components (5' to 3'): T7 RNA Polymerase gene, Lac promoter, LacI gene, LacIq promoter, bleomycin resistance gene and part of neomycin resistance gene (5.3 kb), leading to the formation of pECBSi vector. pECBSi was finally digested by EcoRI and BglI, replaced by EcoRI and BglI digested fragment from pBR322 (4.3 kb) to form pECBS1 shuttle vector (9.7 kb).

Construction of bFGF expression constructs

The *E. coli/B. subtilis* expression shuttle vector (pECBS1-H6-DnaE-bFGF), was constructed in the following way. A synthetic DNA fragment, encoding the sequence of EcoRI-T7 promoter-Lac operator (LacO)- ribosomal binding site (RBS)- 6x His tag (H6)-Asp DnaE int-c (DnaE)-bFGF-T7 terminator-XbaI (0.7 kb) from 5' to 3' end, was synthesized by Thermo Fisher Scientific. The

synthetic DNA fragment was digested with EcoRI and XbaI and this was followed by ligation with a *E. coli/B. subtilis* shuttle vector, pECBS1. The two same restriction enzymes were utilized again for further digestion. This resulted in the formation of pECBS1-H6-DnaE-bFGF construct.

Shake flask cultivation

B. subtilis transformants were grown at 37°C, (with rotations at 250 rpm) in 200 ml 2x LB medium supplemented with 25 µg ml^{-1} of kanamycin. A final concentration of 0.2 mM IPTG was added subsequently, when the A_{600} value reached 1.0, 1 ml of culture samples were collected at 3 h intervals for the analysis of bFGF expression. The cell pellet was resuspended in 200 µl of resuspension buffer (50 mM Tris-Cl, 200 mM EDTA, pH 8.0), followed by incubation on ice for 5 min. The mixture was then treated with 120 µl of lysozyme solution (10 mg mL^{-1}) at 37 °C for 20 min. 80 µl of lysis buffer (10 mM EDTA, 10% Triton X-100, and 50 mM Tris-Cl, pH 8.0) was then added. The tube with solutions was inverted gently, followed by centrifugation at 14,800 rpm for 5 min. Cell lysate samples were analyzed for bFGF expression by Western blotting.

Fed-batch fermentation

B. subtilis transformants were grown in 200 ml 2x LB medium supplemented with 25 µg ml^{-1} of kanamycin at 37 °C, (with rotation at 250 rpm) until the A_{600} = 1.0. 50 ml of the culture was then transferred to a 2 L flask containing 450 ml of 2x LB medium supplemented with 25 µg ml^{-1} of kanamycin at 37 °C, (with rotation at 250 rpm) until the A_{600} value reached 1.0. The entire culture was inoculated into a 5 L fermenter containing 3.5 L of 2x LB medium supplemented with 25 µg ml^{-1} of kanamycin. The addition of 1 M NaOH maintained the pH of the culture at 7.0. pO_2 in the culture was set at 1.5 vvm. Further, a 50% glucose feeding solution was added to maintain the pH of the culture at 7.0, when the pH began to rise. This was followed by induction of culture with a final concentration of 0.2 mM IPTG as we got A_{600} = 8. The pH adjustment was maintained by 1 M H_2SO_4. Culture samples were collected at 2 h intervals for the analysis of bFGF expression.

Small scale purification of bFGF

Cation exchange (CE) chromatography and heparin-agarose (HA) chromatography were used for the purification of bFGF. The protein concentration of eluted fraction was first measured by Nanodrop Microvolume Spectrophotometer. Further, the eluted fractions with significant readings (≥ 1 mg/ml) were pooled and dialyzed by 0.1x PB. Purified bFGF was visualized on 10% SDS-PAGE stained with Coomassie Brilliant Blue R-250. A band containing bFGF was retrieved from the SDS-PAGE gel and was subjected to analysis by LC-MS.

Biological assays of bFGF

The mitogenic effects of purified bFGF on the proliferation of NIH/3T3 fibroblast cells were analysed by the MTT assay. NIH/3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin until 80% confluency. NIH/3T3 cells were trypsinized, resuspended in DMEM medium supplemented with 1% FBS and seeded into 48-well plates at a density of 2×10^4 cells. Cells were continued to culture at 37°C, 5% CO_2 for 24 h. All the wells were then treated with various concentration of bFGF and continued to culture for 3 days. A final concentration of 0.5 mg ml^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and one incubation at 37°C, 5% CO_2 for 4 h was given. All solutions were aspirated from plate wells and 150 µl DMSO (MTT solvent) was added for dissolving the purple crystal. The plates shook continuously in dark for 10 mins and the absorbance was read at 570 nm in a microplate reader.

Results

Engineering of plasmid construct expressing intracellular bFGF in *B. subtilis*

The plasmid, pECBS1 *E. coli/B. subtilis* shuttle vector was transformed into *B. subtilis* WB800N (Figure 1). This vector was designed for high intracellular expression of the protein of interest. T7 promotor and lac operator were employed to direct the expression. Since, 6x His was a common affinity tag for purification of fusion protein. So, it was selected for fusion at the N-terminus of DnaE intein to enhance the expression of C-extein (bFGF). pECBS1 *E. coli/B. subtilis* shuttle vector was employed in this study for simplifying the DNA manipulation work and providing a T7 cassette expression backbone for expressing the protein of interest.

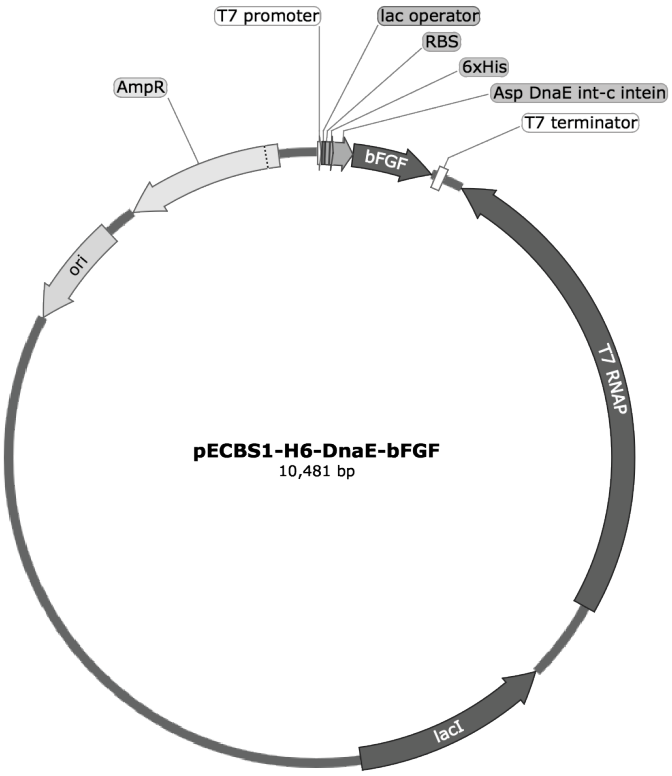
Expression of bFGF in shake flask scale

Different combinations of inteins and C-exteins were examined for successful expression of soluble bFGF. bFGF was selected to fuse with *Asp* DnaE intein on its C-terminus since *in vitro* cleavage at C-terminal of DnaE was controllable by pH shift or by treatment with reducing agents. GST, Chitin binding domain (CBD) and H6 affinity tag were tested to serve as N-exteins for the expression of bFGF. However, only insoluble form of precursor resulted for the first two constructs (data not shown). While for H6, with relatively smaller in size, gave a positive expression result of mature and bioidentical bFGF (Table 1). Results from time course experiments in shake flask (Figure 2) showed that construct pECBS1-H6-DnaE-bFGF (Figure 1) expressed satisfactory levels of bFGF as final product under induction, whereas the precursor form did not detect from the western blot.

Table 1. Analysis of purified bFGF by liquid chromatography tandem mass spectrometry.

Constructs	Peptide[a]	Mr(Calc)[b]	Mr(Expt)[c]	Ion Score
H6-DnaE-bFGF	^{NH2}-PALPEDGGSGAFPPGHFKD	1779	1781.5	46
	KAILFLPMSAKS-^{COOH}	1105	1106	52

Note: [a]subsequent to partial trypsin digestion of purified bFGF, the N-terminal and C-terminal sequences were identified by the Mascot search engine
[b]Theoretical mass-to-charge ratio of the peptide
[c]Experimental mass-to-charge ratio of the peptide



Note: This figure shows plasmid constructs vector (10.4kb) expressing H6-DnaE-bFGF insert cassettes. Symbols for the genetic components shown are: *ori*=Origin of replication in *B. subtilis*; *AmpR*=Ampicillin resistance gene; *lacI*=lacI gene; *T7 RNAP*=T7 RNA polymerase gene; *bFGF=bFGF* gene; *DnaE=Asp* DnaE intein; *H6*=6x His tag; RBS=ribosomal binding site. Arrows indicate directions of gene expression.

Figure 1. Schematic representation of DNA constructs expressing H6-DnaE-bFGF.

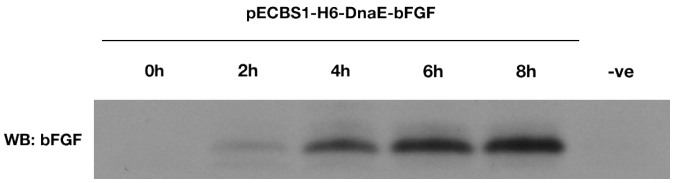
Fermentative scale expression of bFGF

Since *B. subtilis* is an aerobic bacterium, levels of dissolved oxygen were expected to be an important parameter for the enhancement of cell growth and also, the overall expression levels of bFGF. However, though only compressed air was supplied during the whole fermentative process, no remarkable difference was found when a mixture of pure oxygen and compressed air was provided for the growth of *B. subtilis*. Luria-Bertani (LB) broth is a widely used nutrient source for bacteria growth. By previous methodology, 2x LB medium worked well for expression of recombinant proteins in *B. subtilis* [15] and same medium was used in this fermentative study.

The results showed that both bFGF expression and cell mass of the *B. subtilis* were significantly increased. The overall yield of bFGF of the expression constructs increased approximately 2 folds from 64 mg L-1 to 113 mg L-1 and 6 folds, from shake flask cultivation (Figure 3) to small fermentative scale (Figure 4) respectively. Although cells entered stationary phase after induction, plasmid curing was not traceable throughout the post induction.

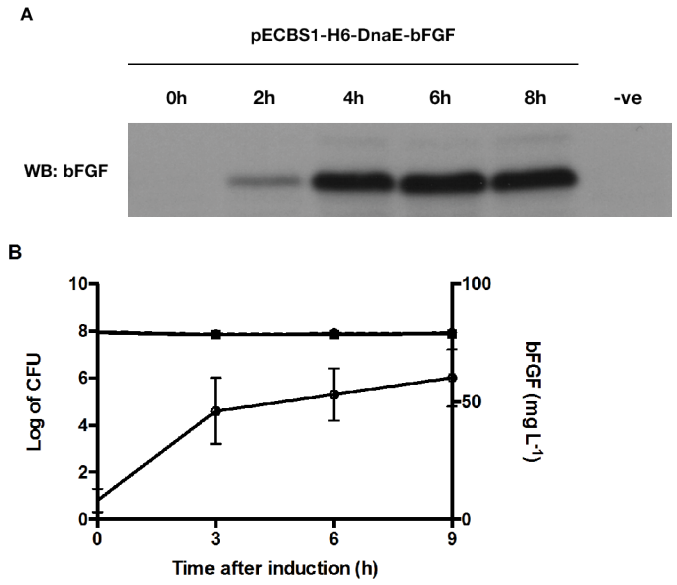
Bio-identicality of bFGF expressed in *B. subtilis*

Soluble bFGF retrieved from the lysate was observed to have same molecular weight as of bFGF purchased from Thermo Fisher Scientific in Western blot analysis (Figure 4). Purified bFGF sample was subjected to LC-MS and mass determination by MALDI-TOF. The results confirmed that



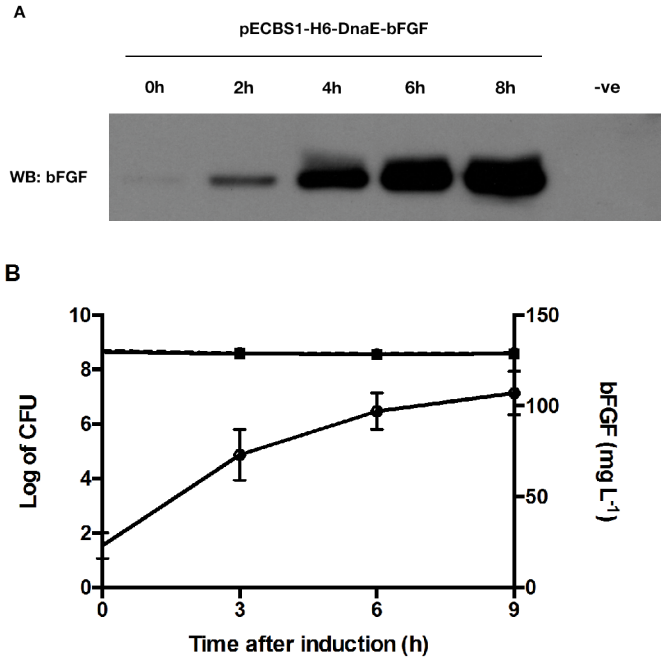
Note: pECBS1-H6-DnaE-bFGF *B. subtilis* transformants post IPTG induction samples collected from different time intervals were analyzed. Lanes 0 h, 2 h, 4 h, 6 h and 8 h: samples collected from cultures induced for 0 h, 2 h, 4 h, 6 h and 8 h respectively, each lane loaded with 5 µl of cell lysate; Lanes -ve: 5 µl of cell lysate from pECBS1 vector cultures induced for 8 h.

Figure 2. Western blot analysis of bFGF present in cell lysate samples of *B. subtilis*.



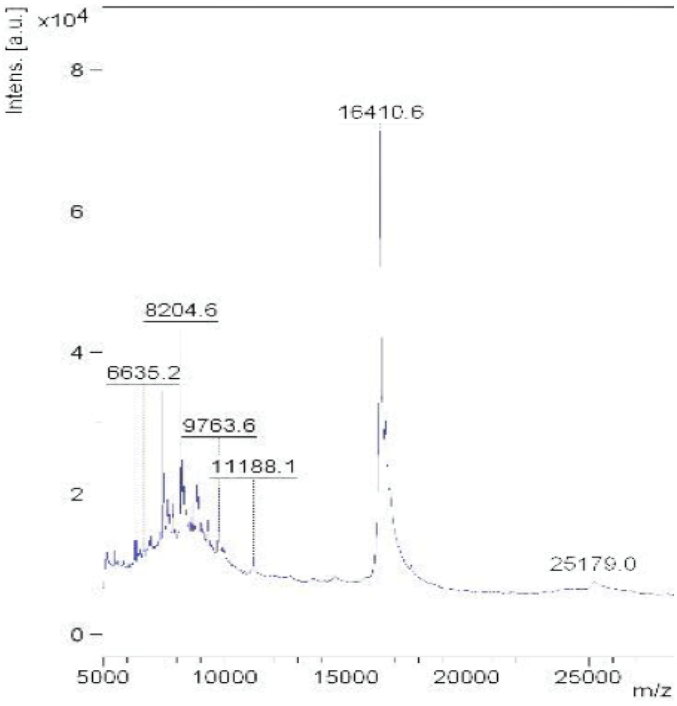
Note: Culture samples were obtained at different time points before and after IPTG induction. (A) Western blotting of bFGF present in cell lysate (CL) samples. All lanes were loaded with 5 µl of cell lysates. (B) Cell viabilities and quantification of bFGF. Levels of bFGF detected in the cell lysates ( ) are presented. CFU refers to colony-forming units. Viable cell counts were determined on plain agar plates ( ) and plates supplemented with kanamycin ( ), respectively. Growth experiment of the transformant was repeated three times and standard error bars are shown.

Figure 3. Time course study of bFGF in *B. subtilis* in shake flask cultivation.



Note: Culture samples were obtained at different time points before and after IPTG induction. (A) Western blotting of bFGF present in cell lysate (CL) samples. All lanes were loaded with 5 µl of cell lysates. (B) Cell viabilities and quantification of bFGF. Levels of bFGF detected in the cell lysates ( ) are presented. CFU refers to colony-forming units. Viable cell counts were determined on plain agar plates ( ) and plates supplemented with kanamycin ( ), respectively. Growth experiment of the transformant was repeated three times and standard error bars are shown.

Figure 4. Time course study of bFGF in *Bacillus subtilis* in fed-batch fermentative scale.



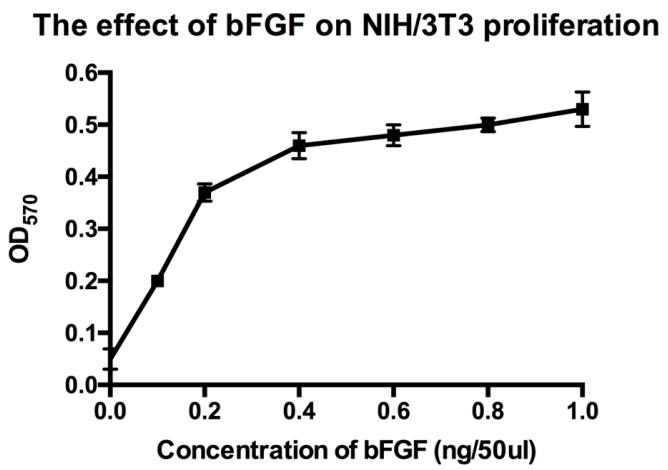
Note: Molecular size of purified bFGF sample from pECBS1-H6-DnaE-bFGF is shown.

Figure 5. Mass determination of bFGF.

the final bFGF product obtained from H6-DnaE-bFGF expression construct, possessing the 146 amino acid bioidentical structure (Table 1) and with 16.4 kDa in size (Figure 5).

Mitogenicity of purified bFGF

The purified bFGF expressed in *B. subtilis* was observed to be biologically



Note: Mitogenic effects exhibited by different concentrations of purified bFGF samples (—▪—) from pECBS1-H6-DnaE-bFGF are shown.

Figure 6. Mitogenicity of bFGF.

active in triggering the cell proliferation of NIH/3T3 cells (Figure 6) and expansion of human mesenchymal stem cell (data not shown).

Discussion

The gram-positive, GRAS bacterium, *B. subtilis*, is a well characterized common host employed for valuable proteins production because it does not consist of endotoxins. Thus, it is an attractive host system for the production of proteins for commercial and medical applications. *B. subtilis* has been engineered to express both secretory homologous and heterologous proteins [16]. However, attaining high levels of intracellular expression of soluble heterologous proteins has been shown difficult. Despite many groups studied inteins and its applications on protein expression, the mechanism of action of inteins yet still not fully understood in different host systems. Our group attempt to express endotoxin-free recombinant proteins in bacterial host systems for both research and commercial purposes. As compared to other approaches, protein expression by employing intein was the simplest and cheapest way in producing recombinant proteins with bioidentical structure, which ensured high biological activity and prevented any immunological response when treated on animal subjects. Expression of heterologous proteins intracellularly by intein-mediated approaches in *B. subtilis* is also not well-studied, which encouraged us to engineering an intein-mediated plasmid constructs expressed in *B. subtilis*. bFGF was chosen to be expressed due to its multiples potential therapeutic value in many areas.

To simplify the DNA manipulation and allow the expression of bFGF in both *E. coli* and *B. subtilis*, a shuttle vector was engineered by combining the elements from two different expression vectors. The terminator regions of pRB374 and parts of the multiple cloning sites were deleted, followed by replacement of terminator regions from pBR322 to result pECBS1 shuttle vector. The bFGF encoding sequence was first designed to fuse on the C-terminal end of intein and an affinity tag was acted as an anchor for the purification of precursor after expression, if bFGF cannot be self-excised from its fusion partner. From the result, fusing GST or CBD tag on the N-terminus of DnaE gave only aggregation form of precursor, while changing the N-terminal affinity tag to H6 gave a surprising overcome. The bFGF expressed not only in soluble form, but also in mature state with reasonable yield (Figure 2, and Figure 3A). Changing the fusion partner of N-terminal end of DnaE to a relatively smaller H6 might alter the overall conformation of whole fusion protein, which favoured the segregation of C-extein and avoided the formation of inclusion body. When the expression was scaled up to 4L fermentation, the overall yield of bFGF increased 2 fold as compared to shake flask cultivation (Figure 4A) while cell viability and plasmid stability also remained stable throughout the whole post induction period (Figure 4B).

Conclusion

The bFGF expressed was subjected to purification in examination of its biological activity and bio-identicality. Our previously trials showed that heparin-agarose chromatography alone was not efficient enough for elimination of all intracellular host proteins, thus the retrieved bFGF was not exceedingly pure. A cation exchange chromatography was decided to be employed prior to heparin-agarose chromatography for get rid of majority of the housekeeping proteins first, followed by removing high concentration of salt through dialysis. Purified bFGF was sent for amino acid determination and tested for the ability in triggering the proliferation of mouse fibroblast cells and human mesenchymal stem cell. The results shown that the purified bFGF not only processed the same primary sequence of 146 aa, but also with high biological activity in triggering NIH/3T3 cell proliferation. Our findings provide a new intein option on expression of bFGF and the shuttle expression vector may also available for high intracellular expression of other proteins in *B. subtilis*.

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How to cite this article: Chau, Wu Kam, Kwong Wai Yeung, Joey Cho Yi Chau and Choi Man Chung, et al. "Expression of Human Basic Fibroblast Growth Factor Mediated by Mini Intein in Bacillus Subtilis." *J Mol Genet Med* 14 (2020): 463 doi: 10.37421/jmgm.2020.14.463