

# Storage of the Recombinant Protein hPDGF-BB in the Culture of *Pichia pastoris*

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Received: 15 Feb 2018/ Revised: 01 April 2018/ Accepted: 22 June 2018

## ABSTRACT

Human platelet-derived growth factor-BB (hPDGF-BB), a proliferation factor, has been successfully manufactured and approved by FDA as the treatment for diabetic foot ulcer and self bone grafting. There have been no reports on the storage of the recombinant hPDGF-BB (rhPDGF-BB) in the *Pichia pastoris* fermentation broth although during the research on process development and the production manufacture it needs to be stored at low temperature. The concentration of rhPDGF-BB protein in the fed-batch fermentation broth of *P. pastoris* was stable during 3-week storage at -20°C but its bioactivity was reduced by 20%. The addition of a mixture of 50% glycerol with either 1 mM EDTA or 1 mM PMSF into the fermentation broth could fully preserve the bioactivity of rhPDGF-BB until 3 weeks at -20°C. The addition of 50% glycerol with either 1 mM EDTA or 1 mM PMSF was found no affection in the protein purification process.

**Key-words:** Bioactivity, Fed-batch fermentation, *Pichia pastoris*, Protein stability, rhPDGF-BB, Storage

## INTRODUCTION

The human platelet-derived growth factor-BB (hPDGF-BB) is a proliferation factor and a potent recruiter for mesenchymal stem cells, osteogenic cells and tenocytes [1]. The rhPDGF-BB has been approved by FDA as the treatment for diabetic foot ulcer and self-bone grafting [1]. It has been produced in a variety of heterologous systems including *Escherichia coli* [2-4], Chinese hamster ovary cells [5], *Saccharomyces cerevisiae* [6,7], baculovirus [8], vaccinia viruses [9], mushroom [10], plant [11] and *Pichia pastoris* [12]. Babavalian *et al.* [12] reported a high efficiency of 30 mg/L in *Pichia pink*, a *P. pastoris* mutant cell, without optimization.

*P. pastoris* has attracted considerable interest in recent years, surpassing *S. cerevisiae* as the preferred yeast recombinant expression system [13], because of its high volumetric productivity, resulting in cell densities up to 130 gL<sup>-1</sup> with a minimal amount of native proteins expressed [14], and a more favorable glycosylation pattern with N-linked oligosaccharides chains of no more than 20 links [15]. However, there have been several reports of proteolytic degradation of recombinant proteins produced in *P. pastoris* [16-20]. Sinha *et al.* [20] reported that phenyl methyl sulfonyl fluoride (PMSF) (1 mM) reduced the proteolytic degradation by 78%, while 1 mM EDTA reduced the activity by 45%, and a combination of 1 mM EDTA and 1 mM PMSF reduced protease activity by 94.2%.

There has been no report on the storage of the recombinant protein in the *P. pastoris* fermentation broth, although during the research of process development and the production manufacture it needs to be stored for a while. In this study, the quantity and the quality of rhPDGF-BB protein after 3-week storage of the fermentation broth at -20°C were evaluated. The treatment of the fermentation medium with a

### How to cite this article

Pham VM, Quoc HHH, Le HXM, Nguyen TN. Storage of the Recombinant Protein hPDGF-BB in the Culture of *Pichia pastoris*. Int. J. Life Sci. Scienti. Res., 2018; 4(4): 1934-1939.



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cryoprotectant {glycerol, 50% (w/v)} and the protease inhibitor (PMSF, 1 mM and EDTA, 1 mM) for the storage of rhPDGF-BB at -20°C was also further examined.

## MATERIALS AND METHODS

This present study proceeded in the duration of November 2017. The recombinant *Pichia pastoris* X-33 strain in this study transformed with the *pdgf-b* gene integrated to the *P. pastoris* chromosome and expressing rhPDGF-BB protein under the control of the AOX1 promoter was obtained from the Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University Ho Chi Minh City (Vietnam).

### Fed-batch cultivation (According to the Invitrogen protocol <sup>[21]</sup>)-

Inoculum for bioreactor cultures was prepared as follows: 50 mL of inoculation medium was inoculated with a single colony of *P. pastoris* X33::pdgf-b strain grown on YPD agar plate supplemented with 100 µg L<sup>-1</sup> of zeocin. The flask was incubated at 30°C with 250 rpm shaking for 24 h until the cell density reached an OD<sub>600</sub> of 2-6. Inoculation medium of 50 mL volume used in 100 ml shake flask consisted of 0.5 g yeast extract, 1 g meat peptone, 100 mM potassium phosphate buffer pH 6.0, 0.67 g YNB, 20 µg biotin, and 0.5 g of pure glycerol. 450 mL of BSM medium for bioreactor fermentation was prepared in a final volume of 500 mL as follows: 13.35 g H<sub>3</sub>PO<sub>4</sub> 85%, 0.47 g CaSO<sub>4</sub>, 9.1 g K<sub>2</sub>SO<sub>4</sub>, 7.45 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 2.58 g KOH, 40 g pure glycerol, and 2.18 mL of PTM<sub>1</sub> salt supplement solution (Invitrogen). Feed medium consisted of 500 mL<sup>-1</sup> L<sup>-1</sup> of glycerol or 500 mL<sup>-1</sup> L<sup>-1</sup> of methanol and supplemented with 12 mL<sup>-1</sup> PTM<sub>1</sub> solution.

Fed-batch cultivation was carried out in a 1 L bioreactor (Biotron LiFlus GX, Korea) with 450 mL initial volume of modified BSM medium. After sterilization by using the autoclave, and cooling down, the medium was supplemented with a PTM<sub>1</sub> solution. The fermentation conditions were controlled and kept on the following values: initial mixing 300 rpm, temperature 30°C, pH adjusted to 6.5 with the addition of 28% (v/v) ammonium hydroxide, and dissolved oxygen (DO) was kept above 5 to 50% by manual regulation of airflow (up to 10 vvm) and mixing agitation control (up to 1100 rpm). Fermentation was initially carried out in standard batch phase at the beginning with defined amount supplementation of glycerol and lasted until carbon

source in the BSM medium was utilized. The second phase was started as the glycerol feed medium being fed into the cultivation for 4 more hours then followed by the methanol-fed phase when methanol feed medium being introduced to the cultivation gradually from 3.6 mL L<sup>-1</sup> h<sup>-1</sup> to 10.9 mL L<sup>-1</sup> h<sup>-1</sup> rate for total 72 hours as described by Invitrogen®. The BSM-based culture medium was harvested after 72 hours of methanol induction, centrifuged at 6000 rpm to obtain a cell-free supernatant. The supernatant was then instantly aliquoted and stored at -20°C, fridge with or without the addition of additives for later examines. All the samples in this study were collected from 3 individual fermentation batches. The concentration of rhPDGF-BB in the fermentation broth was approximately 686.7 µg/mL.

**Purification of rhPDGF-BB-** The protocol was optimized for the study of Wang *et al.* <sup>[7]</sup>. Cells were separated from the biomass by centrifugation at 6000 rpm, 4°C. The medium was kept cool and pH adjusted to 4.0 with glacial acetic acid then filtered through a 0.2 µm Sartorius filter. 70mg of protein rhPDGF-BB from the purified-ready medium was applied to a 5 mL SP FF Sepharose column (GE Healthcare) using AKTA START protein purification system (GE Healthcare) at a constant flow rate of 2 mL/min. The column was then washed with 50% elution buffer for 40 mL before rhPDGF being eluted with 90% elution buffer for 20 mL. The eluted fractions of rhPDGF-BB were pooled and dialyzed overnight against PBS buffer pH 7.5 then stored at 4°C for further examinations and bioactivity evaluation.

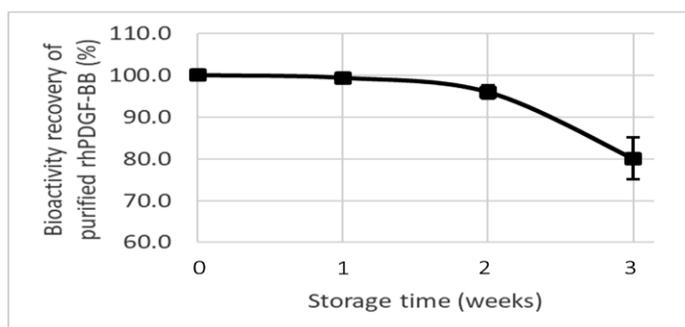
**Protein quantification-** The total protein concentration was determined by the Bradford protein assay using BSA as standard <sup>[22]</sup>. The concentration of rhPDGF-BB was calculated based on the total protein concentration and the ratio of rhPDGF-BB protein to total protein, which was determined by calculating the target band intensity after SDS-15% PAGE gel and silver staining analysis using GelAnalyzer (<http://www.gelanalyzer.com/>).

**Bioactivity assay-** Biological activity of rhPDGF-BB was evaluated by a modified method based on the report of Karumuri *et al.* <sup>[4]</sup> using the NIH-3T3 fibroblast cell line (American Type Culture Collection) and the Sigma-Aldrich® MTT Cell Proliferation Assay Kit. The cells were cultured in Dulbecco's Modified Eagle Medium/

Nutrient Mixture F-12 Ham (DMEM/ F12, 1:1 mixture; Himedia) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 1X Antibiotic Antimycotic Solution (100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin and 0.25 µg ml<sup>-1</sup> amphotericin B; Sigma-Aldrich) at 37°C in 5% CO<sub>2</sub>. For bioassay, cells were seeded in 96-well plates at a density of 5×10<sup>3</sup> cell/well in DMEM/F12 containing 10% FBS and incubated for 24 h. The cultured cells were then subjected to the treatment of rhPDGF-BB at the concentration of 40 ng ml<sup>-1</sup>. MTT was then added into each well of the 96-well assay plate, and the plate was incubated for another 3 h. OD550 was measured and mean values were calculated for each well. The rhPDGF-BB (BioLegend) was used as the reference standard in the experiments.

## RESULTS

**Content and bioactivity of rhPDGF-BB after 3-week storage of the fermentation broth at -20°C-** The fermentation broth was stored at -20°C for 3 weeks. The content and bioactivity of rhPDGF-BB were determined every week. Surprisingly, there was no significant change in the concentration of rhPDGF-BB among samples at the end of each week. However, although the content of rhPDGF-BB has remained, the bioactivity of rhPDGF-BB was reduced by approximately 4% in the second week and 20% in the third week. In other word, the bioactivity recovery of rhPDGF-BB after 3-week storage at -20°C was about 80% (Fig. 1).



**Fig. 1:** Time course of the bioactivity recovery of purified rhPDGF-BB during the storage of *P. pastoris* in 3 weeks.

The data are expressed as means±SD of three independent measurements

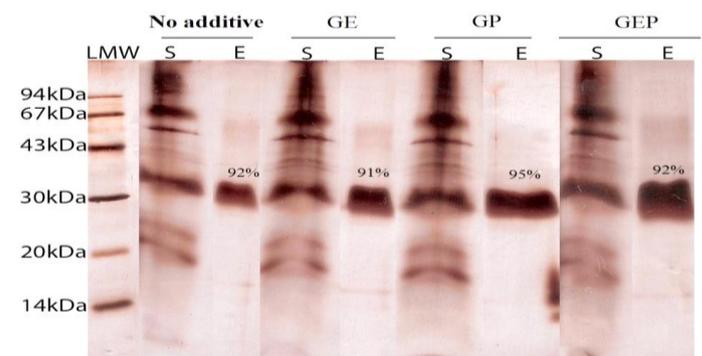
It was reported that freezing and thawing of protein without using the cryoprotectant might affect the activity of the protein [23]. Moreover, although the concentration of rhPDGF-BB was unchanged, the limited proteolysis by the protease in the fermentation broth

might modify the conformation of the protein, therefore reducing its activity. In order to preserve the bioactivity of rhPDGF-BB in the fermentation broth, the cryoprotectant {glycerol, 50% (w/v)} and the protease inhibitor (PMSF, 1 mM and EDTA, 1 mM) were used for further investigation of rhPDGF-BB storage.

### Examine the effect of the mixture of glycerol with EDTA and PMSF on the purification and bioactivity of rhPDGF-BB-

In order to use glycerol as a cryoprotectant and EDTA, PMSF as protease inhibitors on the storage of rhPDGF-BB at -20°C, their effects on the purification process and the bioactivity of rhPDGF-BB was examined in advance. Three mixture combinations prepared for the protein storage were glycerol and EDTA (GE), glycerol and PMSF (GP), and glycerol, EDTA and PMSF (GEP). Of all the mixtures, the concentration of the components was glycerol, 50% (w/v) referred from a review of Simpson, [24], and EDTA, 1 mM and PMSF, 1 mM referred from the report of Sinha *et al.* [20].

The recovery yields of the purified rhPDGF-BB from the fermentation samples mixed with GE, GP and GEP were 42%, 54%, and 49%, respectively, which were comparable with that of the sample without additives, about 44%. The purity of rhPDGF-BB from all formulas was also very comparable for each other, which was higher than 90% (Fig. 2). The bioactivities of the purified rhPDGF-BB from all the samples of no additive, GE, GP, and GEP showed no significant difference to each other and to the reference rhPDGF-BB protein.



**Fig. 2:** Purification analysis of rhPDGF-BB protein from the fermentation broths adding with no additive, GE, GP and GEP by SDS-15% PAGE gel and silver staining. LMW: low weight molecule protein marker, Lane S: fermentation broth, Lane E: elution fraction with the purified protein

It could be concluded that the presence of the mixture of 50% glycerol (w/v) with 1 mM EDTA and 1 mM PMSF in

either individual or combination has no significant effect on the purification process and the bioactivity of rhPDGF-BB.

**Effect of the mixture of glycerol with EDTA and PMSF on the storage of rhPDGF-BB at -20°C-** Three samples of the fermentation broth were mixed with glycerol and EDTA (GE), glycerol and PMSF (GP), and glycerol, EDTA and PMSF (GEP) and a sample without any additive were stored at -20°C for 3 weeks. The content and bioactivity of rhPDGF-BB in each sample were determined every week.

As expected, the concentration of rhPDGF-BB in all of the samples with or without additives remained unchanged after 3-week storage on -20°C. Noticeably, the bioactivity recovery of rhPDGF-BB protein in the GE, GP and GEP samples at the end of the third week was 100%, 100%, and 89%, respectively. It means that the addition of 50% glycerol with either 1 mM EDTA or 1 mM PMSF could preserve completely the bioactivity of rhPDGF-BB protein in 3 weeks at -20°C. However, unexpectedly in the presence of both 1 mM EDTA and 1 mM PMSF together with 50% glycerol, approximately 11% of the bioactivity of rhPDGF-BB was still lost.

## DISCUSSION

In this study, the content of rhPDGF-BB was well preserved after 3-week storage at -20°C. Previous reports have demonstrated the loss of the recombinant protein during the fed-batch fermentation of *P. pastoris*, especially in the final hours of the fermentation process [16-19]. Kobayashi *et al.* reported that further cultivation after 96.5 h of fermentation resulted in the rapid disappearance of the recombinant human serum albumin (HSA) from the culture supernatant [17]. Furthermore, about 50% of the HSA was degraded after 20 h of incubation at 30°C with the culture supernatant of the fermentation [17]. The similar result was observed in the report of Sinha *et al.* [19] in which the level of the recombinant ovine interferon- $\tau$  (r-oIFN- $\tau$ ) produced during methanol induction of the Mut+ strain of *P. pastoris* X-33 drops typically after 50–55 h fermentation. More than 50% of the purified r-oIFN- $\tau$  was also degraded after 48 h of incubation with fermentation culture supernatant at 30°C [20]. Therefore, the preservation of rhPDGF-BB concentration after 3 weeks of incubation in the fermentation broth is likely due to

the inhibition of protease activity at the low temperature of -20°C.

The 20% reduction in the bioactivity of rhPDGF-BB after 3-week storage at -20°C was compensated by the addition of the mixture of 50% glycerol with either 1 mM EDTA or 1 mM PMSF. It is likely that the cryoprotection of glycerol [24], and the inhibition of EDTA or PMSF [20] to the limited proteolysis of rhPDGF-BB remain the bioactivity for rhPDGF-BB.

As the result, in the previous report of Sinha *et al.* [19] the addition of both EDTA and PMSF could reduce protease activity by 94.2%, which was more efficient than those of using either EDTA or PMSF individually. However, the presence of both 1 mM EDTA and 1 mM PMSF together with 50% glycerol could not preserve completely the bioactivity of rhPDGF-BB as the individual presence of either EDTA or PMSF could do. Therefore, the reduction in bioactivity of rhPDGF-BB in the sample of 50% glycerol, 1 mM EDTA and 1 mM PMSF could possibly not relate to the protease activity, but likely relate to the protein conformational change or aggregation under freezing condition. It might be implied that the cryoprotection effect of glycerol was impaired by the presence of 1 mM EDTA and 1 mM PMSF at once, but not affected by the presence of either EDTA or PMSF separately. However, this assumption by far still needs further investigation.

## CONCLUSIONS

The analysis of rhPDGF-BB protein content and bioactivity after 3-week storage at -20°C in the fermentation broth with or without additives was summarized in Table 1.

**Table 1:** Recovery of rhPDGF-BB's content and bioactivity at the end of the third-week storage at -20°C in different fermentation broth samples. No additive: fermentation broth without any additive, GE: adding 50% glycerol and 1 mM EDTA; GP: adding 50% glycerol and 1 mM PMSF; GEP: adding 50% glycerol, 1 mM EDTA and 1 mM PMSF

Sample	Content recovery (%)	Bioactivity recovery (%)
No additive	100	80
GE	100	100
GP	100	100
GEP	100	89

The rhPDGF-BB protein in the fed-batch fermentation broth of *P. pastoris* was stable during 3-week storage at -20°C in terms of protein concentration but not its bioactivity. The 20% loss of its bioactivity could be overcome by the addition of a mixture of 50% glycerol with either 1 mM EDTA or 1 mM PMSF. The reduction in bioactivity recovery of the combination of 1 mM EDTA and 1 mM PMSF with 50% glycerol was uncertain and needed to be clarified.

This study could be helpful for the storage of the fermentation medium during the production and quality control process in the manufacture of rhPDGF-BB for pharmaceutical applications.

#### ACKNOWLEDGMENTS

This research was partially funded by the Ho Chi Minh City Department of Science and Technology (Vietnam), according to the Scientific Research Contract No. 199/2017/HD-SKHCN, November 9, 2017 between the Ho Chi Minh City Department of Science and Technology and University of Science, Vietnam National University Ho Chi Minh City.

#### CONTRIBUTION OF AUTHORS

Research concept and design were framed by VMP, HHHQ, TNN and practical implementation, data collection, and analysis was carried out by VMP, HHHQ, HXLM. Final review of work was carried out by VMP and TNN.

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