

BATCH AND HIGH CELL DENSITY FED-BATCH CULTURE PRODUCTIONS OF AN ORGANOPHOSPHORUS HYDROLASE

Michael H. Kim, Vipin K. Rastogi
Geo-Centers, Inc., Gunpowder Branch, P. O. Box 68, APG, MD 21010

Tu-chen Cheng, and Joseph J. DeFrank
U. S. Army Edgewood Chemical Biological Center, APG, MD 21010

ABSTRACT

Organophosphorus hydrolases (OPH) are of great interest to the U. S. Army for their potential use in the non-toxic, non-corrosive decontamination against VX. OPH genes had been cloned and over expressed in *Escherichia coli* by other researchers. However, a common problem has been that of a low expression level. In this study, batch cultures in both complex (e.g., Lauria Broth) and minimal (succinate) media as well as high cell density cultures in the succinate medium were performed to obtain a yield information for a cobolt-requiring OPH.

High cell density fed-batch cultures of *E. coli* carrying a cloned OPH gene were achieved by a pH control with an acidic 200 g/L succinate (pH = 4 to 4.3) medium. The uptake of carbon source, succinic acid instead of succinate, by the cell cultures raised the pH of broth culture which triggered an addition of the acidic succinate medium. This also provided an additional amount of carbon source for high cell density cultures. Up to 300 mg OPH/L of fermentation broth was achieved using the high cell density fed batch cultures.

INTRODUCTION

Current decontamination of areas and equipment potentially exposed to the various chemical agents requires the use of a caustic solution DS2. However, the use of DS2 in field uses is limited due to its toxic and corrosive properties, in addition to being environmentally hazardous. Alternative technology to DS2 decontamination, organophosphate (OP) degrading enzymes could be potentially used in detoxification of chemical agents. To this end, a number of such enzymes responsible for degradation of nerve agents have been identified¹. Particularly, organophosphorus hydrolase (OPH), which degrades VX, has been cloned into *E. coli*, produced, and purified. However, two common problems for cloned OPHs have been their low activities² toward VX and low levels of expressed protein in the host *E. coli*³.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 00 JAN 2002		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Batch And High Cell Density Fed-Batch Culture Productions Of An Organophosphorus Hydrolase Stem				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Geo-Centers, Inc., Gunpowder Branch, P. O. Box 68, APG, MD 21010; U. S. Army Edgewood Chemical Biological Center, APG, MD 21010				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES This article is from ADA409494 Proceedings of the 2001 ECBC Scientific Conference on Chemical and Biological Defense Research, 6-8 March , Marriott's Hunt Valley Inn, Hunt Valley, MD., The original document contains color images.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 5	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

Effective enzyme decontamination technology requires research on three areas of development: manipulating the OPH gene for a higher activity toward VX, optimizing fermentation for higher cell mass and expressed protein level, and establishing a purification protocol that maximizes purity of the enzyme while minimizing loss of yield that also requires a minimum number of downstream process steps.

MATERIALS AND METHODS

ORGANISM

The strain employed for this study was *E. coli* XL1 that carries a plasmid pVSEOP7. The plasmid contain the ampicillin resistance marker and the gene for *Flavobacterium* organophosphorus hydrolase. In the pVSEOP7 plasmid, the gene is under regulation of the *trc* promotor, which is inducible by IPTG.

FERMENTATION

Batch cultures fermentations were carried in a Bio-Flow 3000 (New Brunswick) unit fitted with 5 L working volume vessel. Both complex Lauria Broth (LB: 10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH adjusted to 7.0 with NaOH) and succinate-minimal medium consisting (per L) of 9.2 g KH_2PO_4 , 1.2 g $(\text{NH}_4)_2\text{SO}_4$, 10 ml of modified Wolin salts and 25 g succinic acid were used. In both media, 100 mg/L each of ampicillin and thiamine was added to maintain plasmid stability and for thiamine auxotroph of the host strain *E. coli* XL1. The modified Wolin salts contained the following ingredient (per L): 3.0 g nitrilotriacetic acid, 6.0 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NaCl, 1.0 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g H_3BO_3 , 0.01 g $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.01 g CuSO_4 .

Fed-Batch Fermentations were carried out in the same Bio-Flow 3000 unit fitted with 10 L working volume vessel. The bioreactor was initially filled to 5 L with 50 g KH_2PO_4 , 50 g $(\text{NH}_4)_2\text{SO}_4$, 50 g succinic acid, 50 mL of modified Wolin salt, and 500 mg each of ampicillin and thiamine, and the pH was adjusted to 6.5 with NaOH. As growth will increase pH of the bioreactor, an acidic succinate-minimal medium (pH adjusted to 4 to 4.3 by adding NaOH) containing (per L) 200 g succinic acid, 40 mL modified Wolin salts, 40 mL NH_4OH , and 100 mg each of ampicillin and thiamine was used to control the pH at 6.5. Induction was started by adding 3 mL of 200 mM IPTG per L into both the bioreactor and the acidic succinate-minimal pH control medium.

A colony from either LB or succinate-minimal (10 g/L) medium plate was transferred to 5 mL (10 x 18 mm) tube of either LB or succinate-minimal (10 g/L) suspension medium and incubated in a rotary shaker (New Brunswick) overnight at 25°C and 200 rpm, after which 4 mL was transferred to 100 mL of LB or succinate-minimal (10 g/L) medium in a 250-mL Erlenmeyer flask and incubated in a rotary shaker at 25°C and 200 rpm. The overnight-grown cultures were used to inoculate a New Brunswick 5- or 10-liter bioreactor with pH control and dissolved oxygen (DO) monitoring. A strip chart (LKG) was used to record DO with a range of 0 to 100 saturation, and pH of the bioreactor was controlled at 7.0 for batch fermentations with 6 N HCl and 6.5 for fed-batch fermentations. Temperature was maintained at 25°C (otherwise noted), agitation at 700 rpm, air pressure at 50 psi. Periodic samples were taken for monitoring optical density and OPH activities at different times of growth. Protein expression was induced with 0.6 mM IPTG at optical density of 0.5 to 1 for an early induction and at optical density of 3 to 8 for a late induction. For fed-batch fermentations, 0.6 mM IPTG was separately added to both the

bioreactor and the acidic succinate-minimal (e.g., 200 g/L) medium used for pH control.

Cells were harvested by centrifugation (10,000 rpm for 15 minutes) at 4°C, resuspended in 4 mL of 10 mM BTP buffer (pH = 7.2) containing 1 mM CoCl₂ to wet gram cell pastes, and broken with a French press at 1000 psi. The resulting crude lysate mixture was centrifuged (17500 rpm for 20 minutes) at 4 °C, decanted, and the supernatant stored at -40 °C.

ANALYTICAL

Optical Density (OD) at 600 nm was measured with a Spectronic 20D spectrometer. Chemical oxygen demand (COD) of culture media without cells was colorimetrically measured using HACH COD kit. Protein determination was carried out using the Pierce protein assay kit. OPH enzyme activity was quantitatively assayed via colorimetric assay using a chromogenic substrate, paraoxon, at a concentration of 100 iM, in 1 mL of 50 mM CHES buffer (pH = 9.1).

RESULTS

BATCH FERMENTATION

The clone pVSEOP7 expressed the enzyme at a relatively similar level in crude lysates for both LB and succinate-minimal (25 g/L) batch fermentations (Table 1). However, the amount of cells harvested was much higher from the succinate-minimal batch than the LB fermentation, resulting a higher volumetric yield (approximately 50.3 mg OPH/L), as compared to only 18.1 mg OPH/L. Specific growth rate of the host *E. coli* XL1 was higher in LB than the succinate-minimal medium (Figure 1), but it appears that not all the soluble substrates in the LB medium was used for cell growth, resulting in a less amount of cells. The final ODs achieved in the succinate-minimal media, i.e., the host *E. coli* XL1 without pVSEOP7, XL1 with pVSEOP7-induced and uninduced control were similar to 4.5 to 5.0.

FED-BATCH FERMENTATION

Typical time-dependent data from a high cell density fed batch fermentation are shown in Figures 2-a, 2-b, 2-c. Cell growth was exponential until the DO became a limiting factor around the culture time of 50 hrs (Figure 2-c). The decreases in OD values could also be attributed to the increases in the total fed-batch culture volume due to pH control and the high succinate concentration. The COD concentration eventually reached 15000 mg/L (Figure 2-a), and, by noting the low extracellular protein concentration of 250 mg/L (Figure 2-a), the high COD is mostly consisted of succinate added for pH control. In fact, high cell density fed batch cultures with 200 g/L succinate resulted in a higher COD of 30000 mg/L. The volume activity exponentially increased upon the IPTG induction (Figure 2-b).

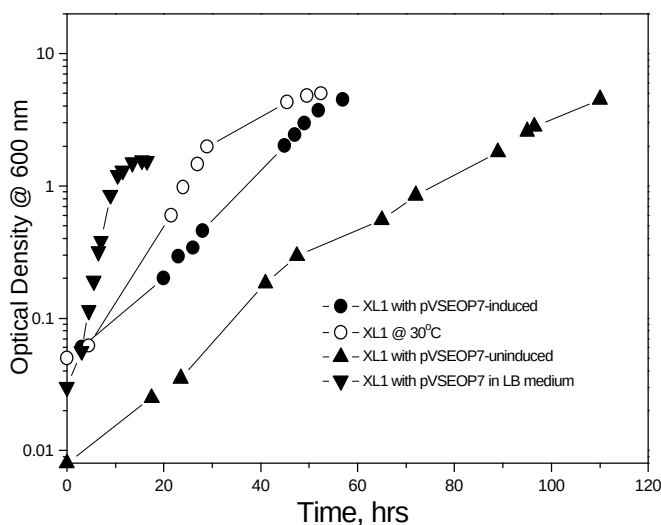


Figure 1. Batch culture growth data .

density fed batch cultures with 200 g/L succinate resulted in a higher COD of 30000 mg/L. The volume activity exponentially increased upon the IPTG induction (Figure 2-b).

TABLE 1*. Comparison of Batch OPH Yields in Different Media

Medium	Cell conc., Wet g/L	Volume of crude lysate, mL	OPH activity, U**/mL	Volume activity, U/L	Eq. OPH, mg OPH/L
LB	7.1	120	3767	90370	18.1
Succinate (25 g/l)	21.6	385	3333	256640	51.3

* These values are based on an average of two batch fermentations in each medium.

** The OPH enzyme unit activity (U) is defined as μ mole p-NP produced/min

The high cell density fed-batch fermentation produced a much higher amount of cells per liter as well as higher unit activities in the crude lysates (Table 2), resulting in as much 300 mg OPH/L of fermentation broth. The increased amount of OPH production is largely due to a higher cell mass (up to 63.5 wet g cells/L) obtained through high cell density fed-batch technique.

However, a higher unit activity in the crude lysates (up to 6500 U/mL) was also contributing factor to the overall increases in the OPH yield. The final volumes achieved in these fed batch fermentations varied greatly and could be an important factor for optimization of OPH production.

CONCLUSIONS

High cell density fed batch cultures were successfully used to achieve up 300 mg OPH/L of fermentation broth. The high concentration of recombinant enzyme allows multi-gram quantitie of enzyme to be produced on the bench scale (10-liter). The overall OPH yield can be further improved by optimizing the fermentation and/or carrying out the fermentation in a larger pilot scale (100-liter).

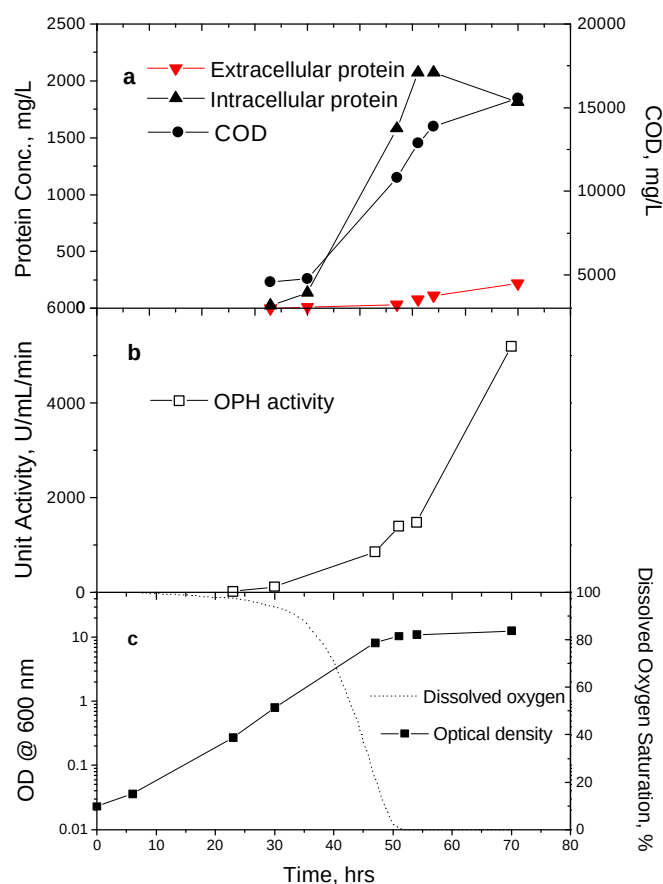


Figure 2. Time-dependent data from fed-batch cultures.

TABLE 2. Data from High Cell Density Fed Batch Cultures.

Final volume, L	Cell conc., wet g/L	Volume of crude lysates, mL	OPH activity, U/mL	Volume activity, U/L	Eq, OPH, mg OPH/L	Succinate conc., g/L
6.0	44.6	1050	4738	829100	166	200
8.5	44.7	1534	5786	1044151	209	200
11.0	50.8	2036	6516	1206052	241	150
10.5	61.3	2728	6000	1558857	312	150
10.2	63.5	2500	5301	1262143	252	200
9.4	60.0	2139	5028	1024275	205	200

REFERENCES

1. Grimsley, J. K., Rastogi, V. K. and Wild, J. R. (1998) ABiological Detoxification of Organophosphorus Neurotoxins..@ in S. K. Sikdar and R. L. Irvine (eds), Bioremediation: Principle and Practice, Vol. II: Biodegradation Technology Developments, Technomic Publishing Inc., 577-613.
2. Sriram, G., Rastogi, V. K., Ashman, W., and Mulbry, W. (2000) AMutagenesis of Organophosphorus Hydrolase to Enhance Hydrolysis of the Nerve Agent VX.@ *Biochem. Biophys. Res. Commun.* **279**(2), 516-519.
3. Lai, K., Dave, K. I., and Wild, J. R. (1994) ABimetallic Binding Motifs in Organophosphorus Hydrolase are Important for Catalysis and Structural Organization.@ *J. Biol. Chem.*, **269**, 16579-16584.