ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Increase in production of baculovirus occlusion bodies through cold incubation in silkworms

Dayanand B Sajjan[1], Shivayogeppa B Hinchigeri[1,2*]

1. Department of Biochemistry, Karnataka University, Dharwad-580 003, Karnataka

2. Administrative block, REVA University, Bangalore-560064, Karnataka

Manuscript Info

Manuscript History:

Received: 12 February 2015
Final Accepted: 25 March 2015
Published Online: April 2015

Keywords:

Nucleopolyhedrovirus; *Bm*NPV; Occlusion bodies; Polyhedrin; Haemolymph; viral DNA.

Corresponding Author

Shivayogeppa B Hinchigeri

hinchigerisb@gmail.com

Abstract

Baculovirus is lethal to insects as they infect midgut, fat bodies and haemolymph leading to the liquefaction of host. In the final stage of disease, baculovirus enters the lytic phase and releases the occlusion bodies (OB) to the surrounding environment. OB encapsulates the one or more pathogenic occluded virions (ODV) within. Overcoming the difficulties in the isolation of pure viral OB from diseased wild and field insects, here we are reported an efficient method for recovery of baculovirus OB. By cold incubation of moribund larvae, it was possible to enrich the *Bm*NPV OB yield per larva to 1.86 fold (n=90). The present method of cold incubation of diseased worms had no effect on the genetic properties of OB as evident by DNA melting temperature (T_m) and restriction endonuclease analysis. Further insect bioassay revealed that the virulence potential of OB obtained from cold incubated worms was compatible with OB of naive control worms. This is a simple and most effective method to isolate pure homogenous baculovirus OB from the scarce samples such as single insect larva or its cadaver.

INTRODUCTION

Baculoviruses are insect pathogens that affect the most speciose orders among the class insecta i.e. *Lepidoptera*, *Hymenoptera* and *Diptera* (Rohrmann G F, 2011). *Bm*NPV belongs to the genus *alphabaculovirus* that infects the commercial sericulture insect *Bombyx mori* and causes a lethal disease called "grasserie".

Most of the earlier investigations related to baculovirus were due to the threat they posed to the sericulture. Baculoviruses also play a significant role in the control of natural insect populations of forest pests such as gypsy, douglas fir tussock moths and agricultural pests of food crops (Rohrmann G F, 2011). The high host specificity and the confinement of the baculoviruses to the members of the arthropod have paved way for the use of baculovirus as a biopesticide against forest and crop insect pests (Franklin H R and Julius J M, 1999) such as velvet bean caterpillar, cotton bollworm (Rohrmann G F, 2011) and gypsy moth (Reardon R, *et al*., 1996). Recombinant DNA technology and advances in insect cell culture have resulted in the use of baculovirus as expression vector for the production of recombinant proteins (Summer M D and Smith G E, 1987, Kost T A, *et al*., 2005), subunit vaccines and as surface display system (Jin R *et al*., 2008), gene delivery in mammalian cells and in cancer therapy (Wang C Y, *et al*., 2006; Mäkelä A R, *et al*., 2006; 2008). Further recombinant protein production in insect cells is appreciated due to high cell density, ease, safety and lower costs associated with it (Elias C B, *et al*., 2000). Most recent application being the development in the field of biomedical engineering where, nanohybridised baculovirus stents are used to promote wound healing by efficient delivery of vascular endothelial growth factor (*Vegf*) genes (Paul A, *et al*., 2013).

Globally, over 50 baculovirus products have been used against different insect pests (Moscardi F, *et al*., 2011). Isolation of the baculovirus is one of the tedious tasks especially from the forest and agricultural pest insects. A study by Fuller E, *et al*., (2012) on baculovirus of the gypsy moth *Lymantria dispar* have shown that viral epidemics are terminated by pupation and epidemic burnout thus minimising the chance of getting good number of OB. The viral sample isolation further affected by the characters induced by the infection of baculovirus like liquefaction of the host tissue (Hawtin R E, *et al*., 1997), enhanced wandering behavior of host insects (Kamita S G, *et al*., 2005), low host density and predation of worms by birds. Even though the soil is found to be a good source of baculovirus (Murillo R, *et al*., 2006a), the viral propagation requires an insect larva to be fed with the virus contaminated soil in artificial diet (Richards A R and Christian P D, 1999) or to add soil/ duff from forest floor to distilled water (Podgwaite J D, *et al*. 1979; Thompson C D, *et al*. 1981) and feed the solution to larva. Although such methods are utilised in the commercial production of the *Anticarsia gemmatalis* alphabaculovirus (*Ag*MNPV) in Brazil, for some insects there are no available artificial diet. Therefore, commercial production of baculoviruses of these insects is too difficult or impossible under laboratory conditions due to the dependency of host plant leaves for viral inoculation preparations. On the other hand, field production of baculovirus agents is viable, resulting in lower cost pesticide products (Moscardi F, 1999). However, field production is challenging when liquefaction of the insect body is very severe, as in baculovirus-infected *spodoptera* species, making it almost impossible to collect dead larvae. In such cases, live moribund larvae must be collected when the body has not yet ruptured. These larvae may, however, contain fewer viruses than would dead larvae (Moscardi F, *et al*., 2011). Also the method of initial viral propagation by feeding virus contaminated diet may be impractical for insect species as it may result in other soil born microbial infections to the larva. In such case, the larva may die due to unexpected microbial disease before it gets infected with baculovirus. It may also result in a genetic mix-up of different viral strain *in vivo* and lack of optimal concentration of OB in the soil to cause efficient viral propagation (Murillo R, *et al*., 2006b).

In the present study, silkworm-*Bm*NPV was employed as model insect-baculovirus system to isolate enriched OB without compromising quality, morphology, genetic content and virulence of baculovirus.

2. Material and methods

2.1. Viral inoculation

All the experiments were carried in triplicates with 30 larvae, in each replicate. The eggs of the multivoltine Pure Mysore silkworms were procured from the Central Silkworm Germplasm Resource Centre, Hosur, Tamil Nadu, India. Hatched larvae were reared at a mean temperature of 25.75 ± 1^{o}C and a relative humidity of 67.4 ± 2%. The healthy 5th instar 2nd-day larvae were employed for inoculation of *Bm*NPV using *per os* method developed by Lakkappan V J, *et al*., (2007). Larvae were individually weighed before proceeding to next step. A suspension of 6 ×10^{5} OB (LD_{100}) in sterile water was applied to fresh and clean mulberry leaves. The virus contaminated leaves were air dried and fed to the individual larva of both control and test sets simultaneously. The control and test sets of larvae were allowed to feed on the fresh mulberry leaves. 10 h after post infection, individual larvae of the test set were transferred to airtight 30 ml amber plastic bottles (Tarsons, Kolkata, India). They were incubated at different temperatures of 0, 5, 10, 15, 20, 25, 30 and 35^{o}C in incubation chamber (LCI-030EL, Daihan labtech, Korea) until the collection of viral occlusion bodies. Larvae and insect cadaver were retrieved at the time interval of 6 h after viral infection for the isolation of OB. Viral samples were designated as COB (control OB) and cold incubation at 5 ^{o}C as IOB (incubated OB) respectively throughout the text.

2.2. Isolation and purification of OB

Insects and cadavers were dissected individually in sterile water to collect the OB rich haemolymph and fat body tissue. Carcasses were further flushed with sterile water using 1 ml micropipette to get the maximum OB yield. The solution was homogenised by vortexing, which was followed by filtration through muslin cloth to remove tissue debris. To collect the crude OB pellet, filtered solution was centrifuged at 5,000 × *g* for 10 min at 4 ^{o}C in C-24BL refrigerated cold centrifuge (Remi, Kolkata, India). The walls of centrifuge tubes were cleaned with tissue paper wick to remove adhered fat body cells, haemocytes and other cell debris before the suspension of the pellet. The pellet was suspended in sterile water and made homogenous with the help of micropipette and suspension was filtered through a muslin cloth. This step of centrifugation was repeated ten times to clear off odour and microscopic cell debris. The pellet was suspended in ten volumes of 2.0% SDS. The solution was made homogenous with the

help of a micropipette and stirred on an orbital shaker for 30 min at 110 rpm followed by centrifugation at 6,400 × *g* for 10 min at 4°C. The resultant pellet was collected and re-suspended in distilled water. The solution was filtered once again through a muslin cloth and centrifuged at 6,400 × *g* for 10 min at 4°C. Residual cell debris adhered to the wall of centrifuge tubes were removed with tissue paper wick, and the pellet was resuspended in sterile water and made homogenous with the help of a micropipette. This step was repeated thrice to remove residual SDS present in the solution. The pellet was suspended in 1 ml sterile water to check integrity and purity of OB by observing aliquots under the compound microscope (100×).

For the field experiment, the individual baculovirus-infected 5th instar 2nd day silkworm larvae were collected from two different geographical locations in Karnataka state of India, from rearing farms based on the "grasserie" disease characters i.e. milky white body, swollen internodes and fragile skin. Further steps of incubation and retrieval of the viral OB sample were carried out in the same way as above. Finally, OB pellets were lyophilised, and the OB concentration per insect cadaver was calculated using the hemocytometer.

2.3. Density gradient purification of OB

500 µl crude COB suspension was applied to sucrose gradient of 40% to 65% and centrifuged for 60 min at 2,86,000 × *g* in Optima LE-80K ultracentrifuge (Beckman Coulter, California, USA) swing-out rotor to get a single purified band of OB.

2.4. Insect bioassay

To check the viability of the IOB isolated from this method, insect bioassay was performed by *per os* method of Lakkappan V J, *et al*., (2007) with a mean temperature of 29.05 ± 1°C and a relative humidity of 53.2 ± 2%. Experiments were carried in 3 replicates of 30 healthy 5th instar 2nd- day Pure Mysore larvae. The LD_{50} concentration of 4.8×10^4 OB/larva was taken which was determined for the earlier experiment (section 2.1).

2.5. Scanning Electron Microscopy (SEM)

SEM analysis was carried by McDougall L A, *et al*., (1994) method with some modifications. The lyophilised OB samples were directly fixed on a gold plate, and the specimen coated with a thin layer of gold using the Polaron SEM coating system. Specimen was subjected to scanning electron microscopy (Leo 435-VP, England, UK) with a magnification of 10000 ×.

2.6. Viral DNA isolation and amplification of viral gene

The viral DNA was isolated according to PCR-REN based method developed by Christian P D, *et al*., (2001) with modifications, such as, addition of equal volume of CI (chloroform:isoamyl alcohol, 24:1) to upper aqueous layer of PCI (phenol/chloroform/isoamyl alcohol) extract. CI treated solution was mixed gently and centrifuged at 9,500 × *g* for 5 min. Five volumes of ice-cold absolute ethanol was added to the upper aqueous phase and incubated overnight at -20 °C. The DNA pellet was collected by centrifuging at 7,000 × *g* for 10 min. The DNA pellet was washed twice with 70% ethanol. The air dried DNA pellet was suspended in sterile TE and stored in -20 °C until use. The DNA yield was quantitatively measured using a Nano-Drop 1000 spectrophotometer (Thermo, Massachusetts, USA). For the amplification of the viral major gene polyhedrin, the primers were designed spanning the whole length of the gene using Primer-3 software (v. 0.4.0). The primer, forward *polh* F 5' GCAAGGGCCTCAATCCTATT 3' and reverse *polh* R 3' CAACAACGCACAGAATCTAACG 5' (Sigma–Aldrich, Bangalore, India) were used to amplify the gene using C-1000 thermocycler (Biorad, Massachusetts, USA) with initial denaturation at 95 °C for 5 min and 34 cycles of 1 min at 95 °C with annealing step of 58 °C for 45 sec, extension step of 1 min at 72 °C, final extension of 10 min at 72 °C and 4 °C for infinity.

2.7. Restriction endonuclease analysis of viral DNA

Fresh viral DNAs were isolated as mentioned in section 2.6. For restriction analysis, DNA was dissolved in sterile nano-pure water instead of TE buffer. The viral DNA from both COB and OB were subjected to restriction profiling according to the method mentioned elsewhere (Graham R I, *et al.,* 2004). Appropriate quantities of DNA

were digested with restriction enzymes *Hind* III and *Eco* RI and their combination (New England Biolabs). Digestion was carried out for 2.5 hour at 37 ^{0}C and products were visualised on 1 % agarose gel.

Restriction profile was detected using Bio-Rad Tm XR$^+$ gel image system, and analysis was carried out using Image Lab software version 4.0 (Biorad, Massachusetts, USA) against 1kb molecular weight marker (New England Biolabs).

2.8. Determination of T_m value and GC content of viral DNA

T_m values of the viral DNAs were calculated according to the method of Allen M W, (2007). Melting of the DNA was carried in the temperature controlled cell holder of the spectrophotometer U-3310 (Hitachi, Tokyo, Japan). The absorbance was noted at 260 nm at temperatures starting from 60 ^{o}C up to 95 ^{o}C with the incremental rate of approximately 0.5 ^{o}C/min and all other conditions were kept constant. T_m was determined from the graph as the midpoint at which half of the DNA exists in single-stranded conformation. Additionally GC content was determined according to the following equation (Marmur J and Doty P, 1962).

$$\%GC=T_m- 69.3/0.41$$

3. Results

3.1. Viral OB production efficiency

The viral OB produced per larvae was determined by using hemocytometer and differences in the number of OB was reported as fold increase/decrease. The optimal condition for the recovery of the OB was found to be 54 h after post infection at which maximum number of OB produced. The optimum temperature for the increased yield in OB was found to be at 5 ^{O}C. Almost two-fold (1.86 ± 0.05, n=90) increase in IOB production was observed at 54 h when compared to COB (Graph 1A). At 54 h after post infection the total COB produced per insect in control set (continued on mulberry feeding) was ranging from 7.1×10^8 to 7.4×10^8 OB with an average of 7.2×10^8 ± 0.1 OB (*SD*=9.9 $\times10^6$, n=90). For cold incubated larval set (insect cadavers), 12.5×10^8 to 13.9×10^8 IOB were observed with an average of 13.5×10^8 ± 0.4 OB (*SD*=3.8 $\times10^7$, n=90). At 54 h after post infection the ratio of number of OB produced to respective initial body weight was significantly higher (2.1 ± 0.3 fold, $p<0.001$) in experimental sets compared to the control sets of larvae (Graph 1B). Similar results were obtained for the field isolates of silkworm larvae picked from two different geographical locations within Karnataka state, India (data not shown).

3.2. Determination of lethality of OB

Virulence potential of the IOB was determined by performing the insect bioassay. The lethality of IOB at LD_{50} was 48.89% with an insignificant difference ($p>0.05$) with COB LD_{50} of lethality of 47.78 % (Table 1). The deviation in LD_{50} mortality from standard was insignificant in both COB, $\chi^2(1, N=90) =0.77$, $p=0.38$ and IOB, χ^2 (1, $N=90$) =0.82, $p=0.37$.

3.3. SEM analysis

The SEM imaging was carried out to study the effect of cold condition on the assembly of the polyhedron and associated proteins during OB production. Morphological and qualitative characteristics of COB and IOB are depicted in Figure1A and 1B respectively. Both COB and IOB demonstrated the size of 2-3µm.

3.4. PCR amplification of viral polyhedrin gene

The viral DNA was isolated from IOB, COB and OB obtained from infected larvae picked from two different geographical locations within Karnataka state. Structural gene polyhedrin is the most conserved gene in baculovirus genome. The amplification of which is the most conserved gene in baculovirus has yielded the product of 955 base pairs in all DNA samples when observed in 1.0% agarose gel (Figure 2).

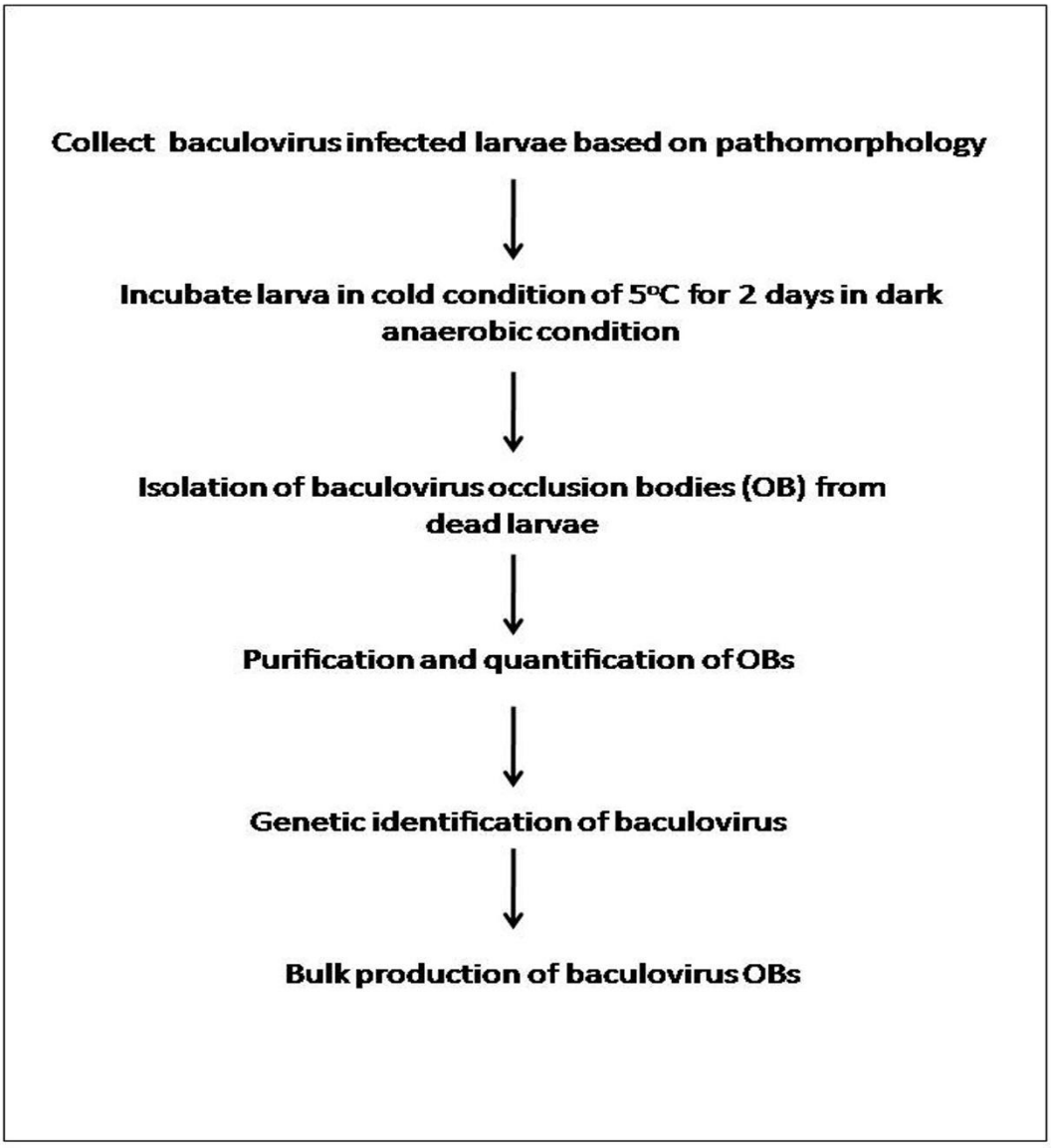
3.5. Viral DNA restriction profile

The restriction endonuclease analysis of viral DNAs with enzymes *Eco*RI, *Hind*III and their combination yielded the same length restriction fragments of 8, 9 & 18 respectively for both control and cold incubated sets. Cumulative molecular weight of all restriction fragments were found to be 83270.2 bp, 87299.8 bp and 87354.6 bp for restriction with *Eco* RI, *Hind* III and their combination, respectively, for DNA of both COB and IOB (Figure 3). The length of individual DNA fragments is reported in table 2.

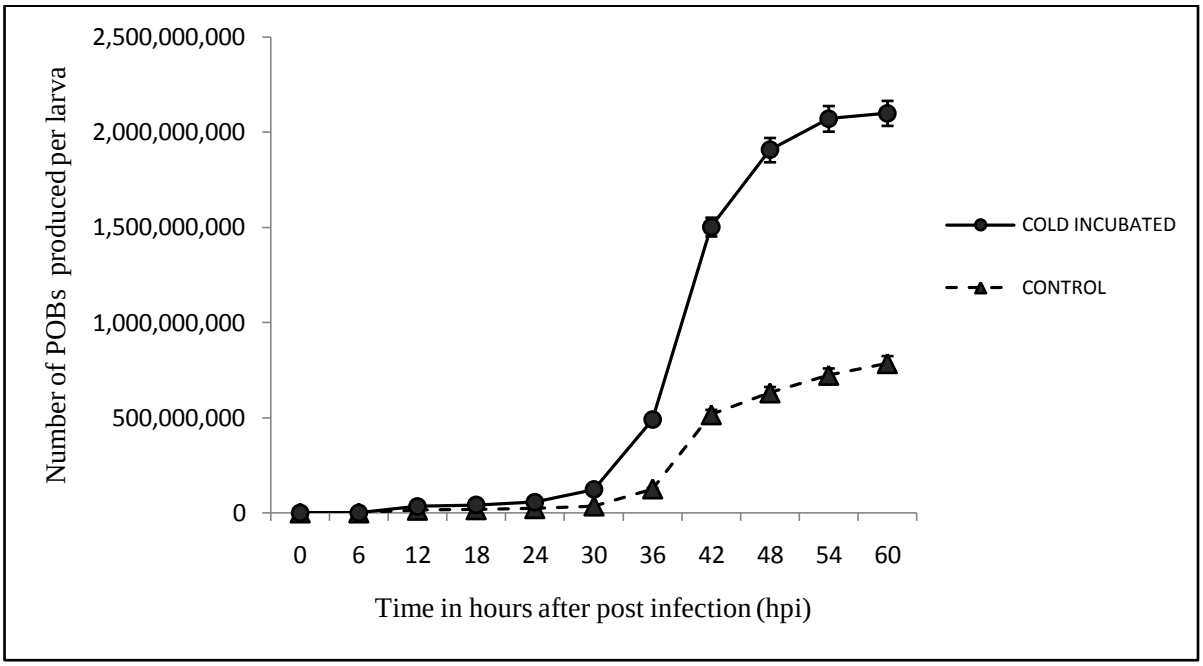
3.6. T_m value and GC content

Melting curve of viral DNAs obtained from COB and IOB were determined by plotting a graph of absorbance (260 nm) versus temperature (^oC). The T_m was defined by the midpoint of sloping intercept to melting curve. The T_m value of 84.45 ^oC & 84.13 ^oC were obtained for DNA of COB and IOB respectively. GC content of 36.95 % & 36.17 % were derived from DNA of COB and IOB, respectively (Table.3).

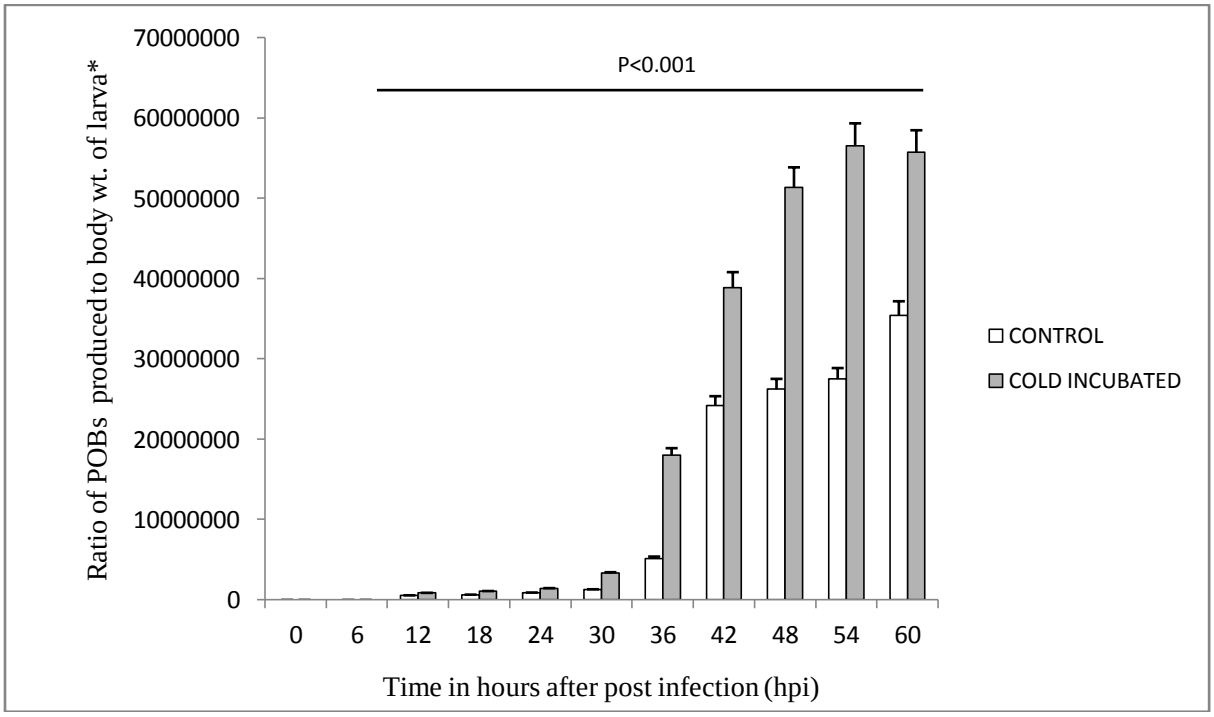
Flow chart



A



B



Graph.1: (A) Number of OBs produced per larva after post infection. Cold incubation (5^0C) for the experimental sets of larvae was carried out from 10 h post infection (**B)** Ratio of OBs produced per larva to body weight after post infection. Cold incubation for experimental sets of larvae was carried out from 10 h post infection, *initial body weight of 5^{th} instar 2^{nd}-day larvae at 0^{th} h post infection.

Table.1. Determination of lethality of OBs by insect bioassay

5th instar 2nd-day larvae	**Replicates (n=30)**	**Mean weight (mg ± SE)**	**Mortality of worms at LD_{50} of 4.8×10^4 OBs/larva**	**Mortality (%) mean**
Control(COB)	1	2.46 ± 0.24	15	52.22 %
	2	2.56± 0.24	13	
	3	2.50± 0.27	15	
Cold incubated (IOB)	1	2.36± 0.20	16	51.11%
	2	2.43± 0.27	14	
	3	2.49± 0.20	14	
Difference				2.17 %

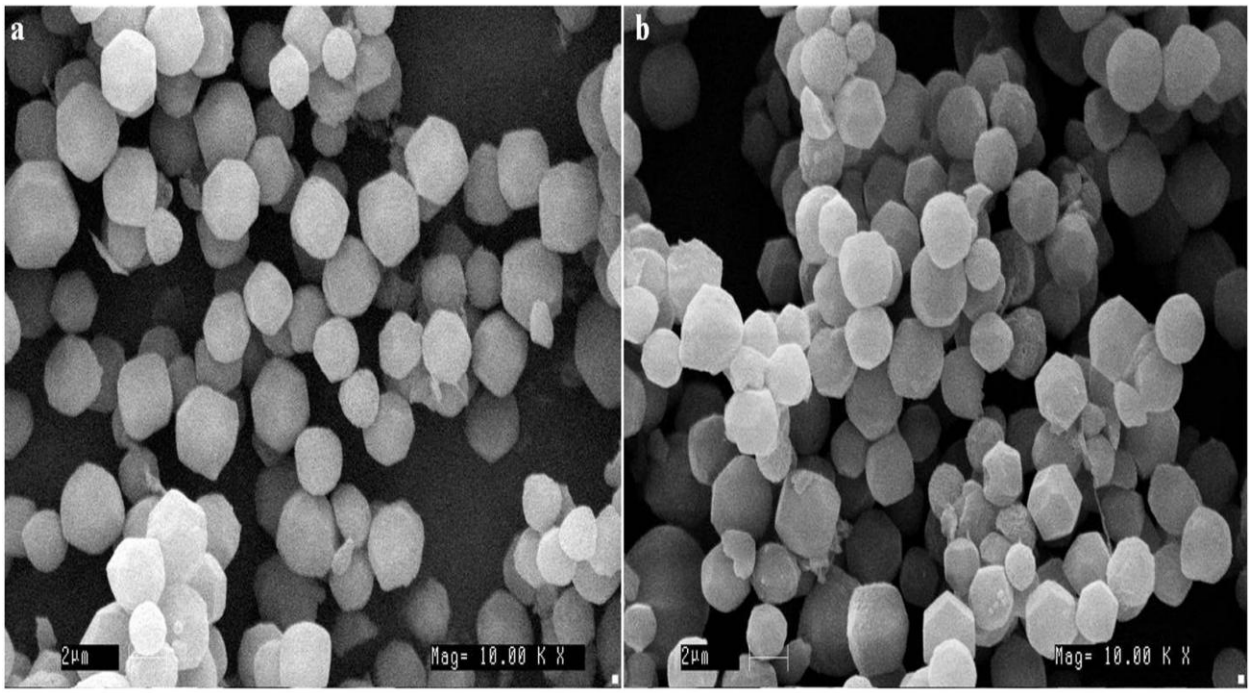


Figure.1 Electron micrograph of the baculovirus OB. (A) OB obtained from control larvae (COB) and purified by density gradient ultracentrifugation, (B) OB from larval cadaver incubated at 5^0C and purified by normal centrifugation(IOB), bar=2µM.

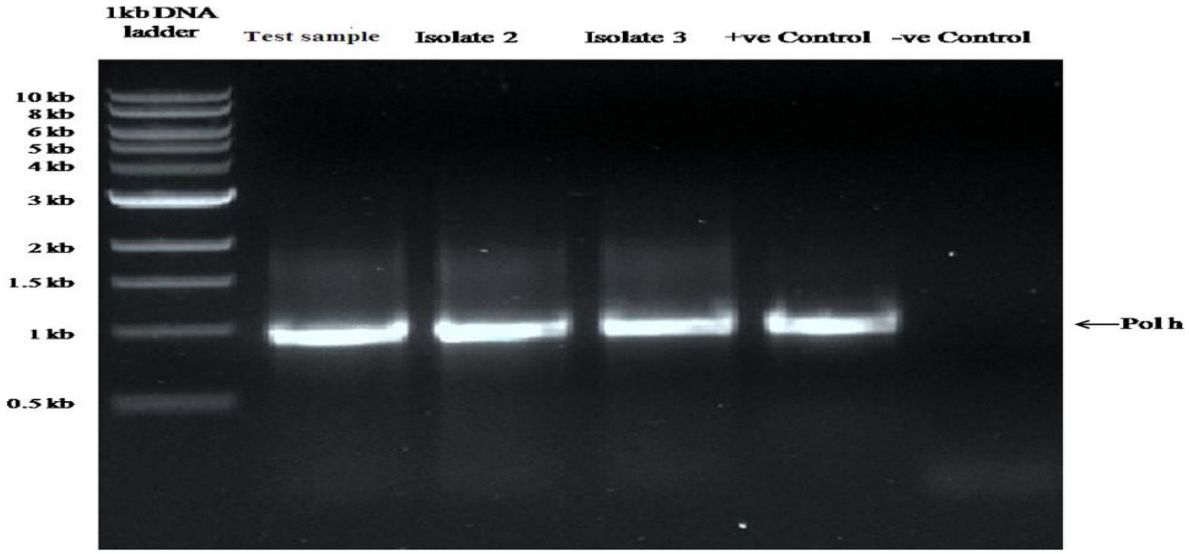


Figure.2 Amplification of viral gene *polyhedrin* (*polh*) and visualized on 1.0% agarose gel.

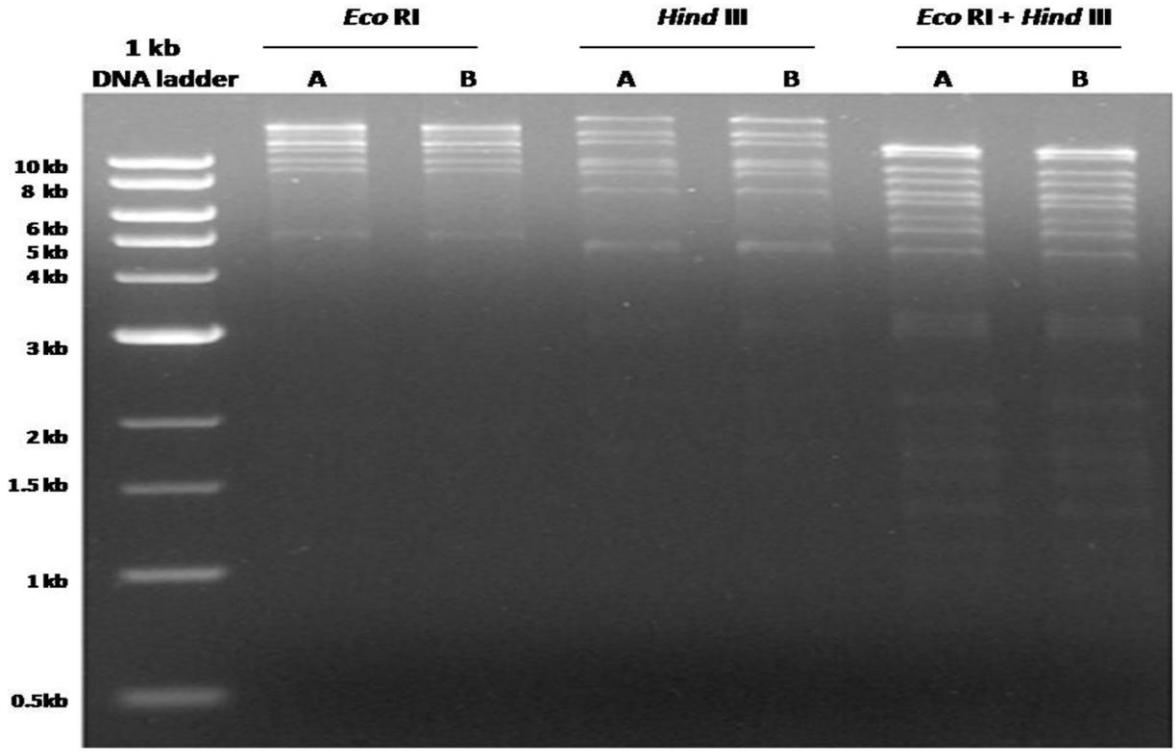


Figure.3 *Bm*NPV DNA digested with *Eco* RI, *Hind* III and double digest and electrophoresed on 1% agarose gel at 30 V for 9 h. *Bm*NPV DNA from (A) COB (B) IOB. New England Biolabs 1kb ladder used as a standard.

Table.2. Frgment lengths of *Bm*NPV DNAs after treatment with restriction enzymes.

New England Biolabs 1kb ladder		*Eco*RI				*Hind*III				*Eco*RI + *Hind*III			
		Control		Cold incubated		Control		Cold incubated		Control		Cold incubated	
Lane 1		**Lane 2**		**Lane 3**		**Lane 4**		**Lane 5**		**Lane6**		**Lane7**	
Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)	Band No.	Base Pairs (bp)
1	10000.0	1	18000.0	1	18000.0	1	19000.0	1	19000.0	1	11000.0	1	11000.0
2	8000.0	2	14500.0	2	14500.0	2	15000.0	2	15000.0	2	10400.0	2	10400.0
3	6000.0	3	12000.0	3	12000.0	3	12000.0	3	12000.0	3	9064.8	3	9064.8
4	5000.0	4	11700.0	4	11700.0	4	9911.1	4	9911.1	4	8000.0	4	8000.0
5	4000.0	5	11200.0	5	11200.0	5	8984.3	5	8984.3	5	7162.0	5	7162.0
6	3000.0	6	9394.3	6	9394.3	6	7541.6	6	7541.6	6	6603.9	6	6603.9
7	2000.0	7	5334.2	7	5334.2	7	6751.6	7	6751.6	7	5826.1	7	5826.1
8	1500.0	8	1141.7	8	1141.7	8	4897.3	8	4897.3	8	5334.2	8	5334.2
9	1000.0					9	3213.9	9	3213.9	9	4673.8	9	4673.8
10	500.0									10	3253.2	10	3253.2
										11	3136.7	11	3136.7
										12	3024.4	12	3024.4
										13	2198.2	13	2198.2
										14	1914.5	14	1914.5
										15	1728.9	15	1728.9
										16	1572.7	16	1572.7
										17	1350.1	17	1350.1
										18	1111.0	18	1111.0
	Cumulative bp =		83270.2		83270.2		87299.8		87299.8		87354.6		87354.6

Table.3.T_m and % GC content determined from melting curve of *Bm*NPV DNA using sloping intercept model.

***Bm*NPV DNA**	**T_m**	**% GC**
COB	84.45	36.95
IOB	84.13	36.17
% difference	0.38%	0.78%

Analysis was carried in temperatures ranging from 60^0C to 95^0C.

4. Discussion

In most developing countries, starting a baculovirus culture by obtaining insects from non- specific disease-free insect colonies may not be possible. The only other option is to initiate the colony from wild populations (Grzywacz D, *et al*., 2004). The larval skin is composed of mainly chitin and is periodically replaced during molting. Baculoviruses have a strategy to degrade the insect chitin by encoding viral *chitinase* and viral–encoded *cathepsin* (*V-CATH*) at later stage of infection cycle causing the host to liquefy (Slack J and Arif B M, 2007). It was supported

by the observation that when the activities of these viral enzymes were lowered by incubating the insect samples in cold condition (5 ^{o}C) it resulted in the intact insect body loaded with OB even after 54 h post infection.

Baculoviruses are vulnerable to the harmful effects of UV radiation when they get exposed to the environment (Griego V M, *et al*., 1985). Viability and persistence of OB on the leaf surface is negatively correlated with irradiation by solar ultraviolet light, high pH and temperature (Jones K A, *et al*., 1993; Young S, 2001). After such exposures, OB may lose their insecticidal properties within a few hours (Murillo R, *et al*., 2007). The samples were incubated in dark using amber bottles to minimise the physiochemical effects of light and temperature on the integrity of viral DNA. Restriction profile together with T_m analysis confirmed that the genetic integrity of viral DNA was intact even after incubation in the cold for long hours. The negligible difference in T_m (0.38 ^{o}C) and GC content (0.78 %) between COB and IOB may be attributed to the experimental and instrumental errors.

The *phenol-oxidase* (*PO*) is widely distributed in living organisms, and the insects are no exception. When OB egress out of the haemocytes in lytic phase, the cytosolic *serine protease* and *PO* are released leading to browning of haemolymph (Clem R J, 2001). In this study, the incubation of insects in anaerobic cold (5^{o}C) condition resulted in the lowered PO activity as the oxygen was deprived to the enzyme. The remarkable power of OB assembly at this low temperature may be attributed to the high density of 1.29 g/cm^3 and lowest solvent content of 22% of polyhedrin subunit along with the predominant hydrophobic domain of polyhedrin trimeric subunit (Coulibaly F, *et al*., 2009). However, further reduction of temperature might result in little OB production as evident by the experiments by Zwart M P, *et al*. (2009) on baculovirus infected *Spodoptera exigua* at a low temperature of -20^{o}C. At the ultra-low temperatures, polyhedrin subunits are incapable to assemble into OB due to solvent constrain operating upon the mobility of subunits to assemble.

These results support the observation by Kang K, *et al*., (2011) that the starvation only in early stages of viral infection causes reduction in virus replication and pathogenicity suggesting that there is a critical viral load beyond which starvation has little impact on larval mortality. It is evident from electron microscopic results and insect bioassay that neither starvation nor anaerobic incubation has an effect on the OB morphology, size and virulence. *Bm*NPV is reported to be present as single nucleocapsid (SNPV) in OB (Hukuhara T, 1967). It is clear from insect bioassay results that the virulence of baculovirus of IOB and COB yield the comparable number of OB inferring that both of them possess the same bio infectivity features (section 3.2).

As this method allows the isolation of baculovirus from as low as single insect larva, there is less scope for the incidence of loss of baculovirus under interest. Such a method can be useful for the labs that are involved in the production of homogenous baculovirus for novel insecticidal development, genomic sequencing, biochemical and genetic engineering, of baculoviruses from single insect cadaver.

Competing interest

The authors of this article declare that they have no financial and non-financial competing interests.

Author's contribution

DBS carried out the collection of samples and performed experiments. SBH designed the experiment, interpreted the data and drafted the manuscript. Both the authors read and approved the final manuscript.

Acknowledgement

The authors highly appreciate and gratefully acknowledge the CSGRC, Hosur, Tamil Nadu, India for providing silkworm layings. One of the authors Mr. Dayanand B. Sajjan is the awardee of UGC-RFSMS (No.11-03/2008) from the University Grant Commission, New Delhi, Government of India. Dr. S.B.Hichigeri is thankful to REVA University for providing the necessary facility to complete this manuscript.

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