Design and development of *Pichia pastoris* based dengue specific virus-like particles concerted with insights into stress responses during expression using a proteomic approach

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften

Dr. rer. nat

genehmigte Dissertation

von

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geboren am 06.08.1985 in Tuni, India

2015

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Tag der Promotion	09.02.2015

DECLARATION

The work described here in the current thesis was carried out at Institute of Technical Chemistry, Leibniz University of Hannover under the guidance of Prof. Dr. Ursula Rinas. I hereby declare that the present work is my own and to the best of my knowledge and belief, it contains no material previously neither published or written by another person, nor submitted by another person for the award of any other university degree, except where acknowledgement has been made in the text.

Hannover, October 2014

Nemani Satish Kumar

ACKNOWLEDGEMENTS

During my Ph.D, I really feel grateful and indebted to every person who has provided me his/her kind support and invaluable time. The thesis would not have been possible without the help, support and encouragement from following people.

I owe my sincere thanks to my Supervisor Prof. Dr. Ursula Rinas to pursue my PhD under her guidance at the Institute of Technical Chemistry (TCI), Leibniz University of Hannover. She has been a constant source of motivation and support. I also thank you for providing all the necessary resources to complete my thesis. Your significant contribution to the content of this thesis is very much acknowledged.

I also express my sincere gratitude towards my co-supervisor Prof. Dr. Thomas Scheper, who has always been approachable, helped me by providing an opportunity to work at the institute and funding during my stay.

Also, I would like to owe a huge thanks to Dr. Navin Khanna, who helped me to gain my first research experience at International Centre for Genetic Engineering and Biotechnology (ICGEB), India. He provided me an opportunity to come to Germany and start my Ph.D here. I would be always indebted for that. I would also like to thank Dr. Satyamanglam Swaminathan, who was always supportive personally and professionally.

I am very grateful to Dr. Manfred Nimtz, Dr. Heinrich Lünsdorf and Dr. Wolfgang Kessler at Helmholtz Centre for Infection Research, Braunschweig for their support and friendly atmosphere while working with them.

I would like to express my heartfelt thanks to colleagues of TCI. I would like to express my heartfelt gratitude to Bernd Tscheschke and Dr. Michael Meyer for being always helpful, supportive emotionally and professionally. Without you both my stay in Germany would have been quite hard. I would also like to thank master's students Sompon Virojandra and Juri Böttcher. I would also like to thank Matthias Krätzig, Annette, Kristain Lüder, Emilia Schax, Bin, Daniel, Marcel, Carola, Michael, Dr. Ana Vanz, Dr. Zhaopeng Li and Gustavo Roth and all other colleagues of the institute. I would like to thank specially Martina Weiss for helping me in ordering, indenting and technical assistance. I would also take the opportunity to owe a special thanks to Dr. Fareed Ahmad, Iffat, Chodisetti Giriprakash, Dr. Shashi Gupta, Piyush Kapopara, Amita, Dr.Amrita Rai, Dr. Anurag Singh and Dr. Vijayalakshmi for their constant help and support.

I would like to express my heartfelt gratitude to Dr. Gurramkonda Chandrasekhar at ICGEB for helping in starting my research career and helping me in every time of need. I would also like to thank Dr. Gaurav Batra, Dr. Talha Mohammad for providing me basic training in the laboratory. I would like to thank to Dr. Krishan Kumar for helping to learn to be optimistic always. I am indebted to Dr. Upasana Arora (Dimpy) who has been always with me with patience correcting my thesis and supporting me personally. I would also like to thank to Dr. Krishnamurthy for carefully reviewing and correcting my thesis. I wish to thank Dr. Lav Tripathi (Dushman) for a splendid time during a stay in ICGEB. I would also like to thank Sushil, Rajen, Poornima, Meena, Shailendra Mani, Dr. Mallikarjun, Radha, Shwetha, K. Chandrasekhar and Dr. Mahedhar reddy.

I would like to thank the Department of Biotechnology, Government of India and Bundesministerium für Bildung und Forschung (BMBF) for funding during my initial stay in Germany.

I dedicate this thesis to my late father and my uncle who taught me to be strong and independent during all my phases of life. I wish to show respect and gratitude towards my uncle A. N. Murthy and A. Nagalakshmi for being a great means of support emotionally and financially throughout. Also, I would like to thank my brother, mother and my other family members A.K. Lakshmi, A.Srinivas Rao, Dr. A.Sridevi, Bharath, Monu and Jaji for constant motivation, love and support.

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SYNOPSIS

Currently, dengue represents highly spread arboviral disease for which there is no available vaccine. The infection can be caused by any of the four dengue virus serotypes DENV-1, -2, -3 and -4 [1]. Though live attenuated vaccines have reached the advanced phase of clinical trials, problems associated with live attenuated vaccines over safety issues have spurred interest toward subunit vaccine candidates [2]. Dengue virus envelope domain III (EDIII) is endowed with serotype specificity, host receptor recognition and capacity to elicit neutralizing antibodies [1, 3]. One way to exploit the vaccine potential of EDIII is to array-in multiple copies on the surface of a carrier moiety. Virus-like particles (VLPs) offer a versatile platform for display of epitopes on surface with potential therapeutic applications. Hepatitis B surface antigen (HBsAg) VLPs have been used as a successful carrier in case of malarial vaccine candidate which reached phase III clinical trials [4, 5].

The most affected dengue populations reside in economically poor and developing nations: therefore a vaccine needs to be economically feasible [6]. *Pichia pastoris* offers great advantages in vaccine production owing to its cost effectiveness, high expression, and importantly proteins produced are free of toxins and safe for human use [7]. Often, expression of viral surface proteins is not as efficient as in mammalian cells resulting in the formation of insoluble aggregates [8].

Based on the above information the current doctoral thesis was aimed to explore the feasibility and potential of EDIII presented on the surface of the virus-like particles as a vaccine candidate using eukaryotic expression system, *P. pastoris.* The first part of the work was to generate *P. pastoris* based DENV-2 EDIII based chimeric VLPs using HBsAg as modular display system and immunogenic evaluation. The second part of the work was focused on possible bottlenecks during expression of fusion protein in *P. pastoris* with insights into the unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD) using proteomic tools.

In the present work DENV-2 EDIII (E) was fused to the amino terminus of HBsAg (S), and expressed either individually (resulting protein termed as ES_1:S_0) or co-expressed along with 1 and 4 copies of HBsAg denoted as ES_1:S_1 and ES_1:S_4 in *P. pastoris* strain GS115. Also, only unfused 8 copies of HBsAg (ES_0:S_8) were expressed. The chimeric proteins were

purified to near homogeneity using conventional chromatographic techniques. All the purified proteins were characterized by electron microscopy to evaluate their ability to assemble into VLPs. ES_1:S_4 was best among other chimeric constructs based on solubility and yield and thus was evaluated for its stability and immunogenicity. Proteome analysis of GS115 cells expressing ES_1:S_0, ES_1:S_4, ES_0:S_8 and wild-type GS115 strain before and after induction was carried out by two dimensional gel electrophoresis combined with MALDI-TOF to gain deeper insights into host cell physiological response during expression of these proteins

ES and unfused S antigen in all chimeric constructs were expressed successfully to high yields. In the presence of non ionic detergent Tween-20 S antigen fraction was soluble whereas ES antigen fraction was hardly extractable. A significant fraction of the ES antigen in all the chimeric constructs was degraded whereas S antigen was very stable during expression. The chimeric proteins ES_1:S_0, ES_1:S_1 and ES_1:S_4 were purified to near homogeneity. Both ES_1:S_1 and ES_1:S_4 were able to assemble into VLPs whereas ES_1:S_0, represented only aggregates as revealed by electron microscopy. Purified ES_1:S_4 was evaluated for stability and it was found that both ES and S antigen fractions were stable on storage at 4°C for a period of two weeks without any additives. However, a small fraction of ES antigen in purified ES_1:S_4 resulted in the formation of insoluble aggregates. Antisera from mice immunized with ES_1:S_4 resulted in a modest neutralization titer against DENV-2. In order to assess the effect of expression of the fusion protein in host cell physiology, proteome analysis of cells expressing the fusion protein was carried out. Results revealed significant upregulation of chaperones involved in unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD).

Stable and optimal presentation of EDIII on surface of carrier needs to be optimized to make it a viable option for vaccine candidate, trials of which are underway. The present study highlights the possible bottlenecks during heterologous expression of viral proteins with deeper insights into the host cell response and perturbances caused by expression of these proteins.

Keywords: Dengue, Envelope domain III, Virus-like particles, Hepatitis B surface antigen, *Pichia pastoris*, Transmission electron microscopy, Two-dimensional gel electrophoresis, Unfolded protein response and Endoplasmic reticulum associated degradation.

ZUSAMMENFASSUNG

Denguefieber ist derzeit eine der am weitesten verbreiteten arboviralen Krankheiten für die noch kein Impfstoff verfügbar ist. Die Infektion kann von einem der vier Denguefieber-Serotypen DENV-1, DENV-2, DENV-3 oder DENV-4 ausgelöst werden. Obwohl einige Lebendimpfstoffe gegen das Denguefieber schon fortgeschrittene Stufen von klinischen Studien erreicht haben, treten immer wieder Sicherheitsbedenken gegenüber dieser Art Impfstoffe auf. Deshalb fokussiert sich die derzeitige Forschung vermehrt auf die ungefährlicheren Impfstoffe aus Proteinuntereinheiten. Als vielversprechenden Kandidaten für einen Proteinuntereinheiten-Impfstoff kann das Denguefieber Hüllprotein (EDIII) genannt werden, da es sowohl serotypenspezifisch ist, als auch eine Wirt-Rezeptor-Erkennung besitzt und die Fähigkeit hat neutralisierende Antikörper zu induzieren. Eine Möglichkeit das Potenzial von EDIII als Impfstoff zu nutzen, ist mehrere Kopien des EDIII-Proteins an die Oberfläche eines Trägerproteinkonstruktes zu koppeln. Als vielseitig einsetzbares Trägerproteinkonstrukt, um Epitope von Impfstoffen zu präsentieren, können Virus ähnliche Partikel (VLPs) dienen. So konnte ein Impfstoff mit VLPs bestehend aus dem Hepatits-B-Oberflächen-Antigen (HBsAg) als Trägerproteinkonstrukt kürzlich erfolgreich die klinische Phase III erreichen.

Die am stärksten vom Denguefieber betroffenen Gebiete befinden sich in wirtschaftlich schwachen Regionen und in Entwicklungsländern, sodass ein Impfstoff gegen Denguefieber kostengünstig produziert werden muss. *Pichia pastoris* bietet hier große Vorteile, da mit diesem Expressionsystem kostengünstig, mit hohen Expressionsraten und vor allem toxinfrei und somit sicher produziert werden kann. Jedoch ist die Expression von viralen Proteinen in Hefen häufig nicht so effizient wie in Säugerzelllinien, da hier unlösliche Proteinaggregate entstehen können.

Basierend auf diesen Informationen war das Ziel dieser Doktorarbeit die Expression von EDIII in dem eukaryotischen Organismus *P. pastoris* und dessen Kopplung und Präsentation auf VLPs im Hinblick auf einen Impfstoff gegen das Dengue Virus zu evaluieren. Der erste Teil der Arbeit beschäftigt sich mit der Entwicklung eines chimären VLPs bestehend aus HBsAg und DENV-2 EDIII, dessen Expression in *P. pastoris* und einer Immunogenitätsuntersuchung des entstehenden Konstrukts. Der zweite Teil der Arbeit

fokussiert sich auf mögliche Engpässe bei der Expression in *P. pastoris* unter besonderer Berücksichtigung der ungefalteten Proteinantwort (UPR) und der endoplasmatischen Retikulum assoziierten Degradation (ERAD) mithilfe von proteomischen Methoden.

In der vorliegenden Arbeit wurden DENV-2 EDIII (E) an den Amino-Terminus von HBsAg (S) fusioniert und entweder eigenständig (ES_1:S_0) oder mit 1 bzw. 4 Kopien von HBsAg (ES_1:S_1 bzw. ES_1:S_4) in *P. pastoris* GS115 co-exprimiert. Außerdem wurde auch unfusioniertes HBsAg (ES_0:S_8) produziert. Die chimären Proteine wurden bis zu einer hohen Reinheitsstufe mit konventionellen chromatographischen Methoden aufgereinigt. Alle aufgereinigten Proteine wurden mittels Elektronenmikroskopie charakterisiert, um den Aufbau von VLPs zu überprüfen. ES_1:S_4 konnte aufgrund der Löslichkeit und Ausbeute als bestes chimäres Konstrukt identifiziert werden und wurde daher hinsichtlich seiner Stabilität und Immunogenität untersucht. Ein Vergleich der Proteomanalyse von *P. pastoris* GS115 Zellen, die ES_1:S_0, ES_1:S_4 und ES_0:S_8 exprimieren mit dem Wildtyp GS115 vor und nach der Induktion wurde mittels 2D- Gelelektrophorese kombiniert mit MALDI-TOF durchgeführt.

ES- und unfusioniertes S-Antigen wurden in allen chimären Konstrukten mit hohen Ausbeuten exprimiert. In Gegenwart des nicht ionischen Detergenz Tween-20 S war die Antigenfraktion löslich, wogegen die ES-Antigenfraktion kaum zu extrahieren war. Ein signifikanter Anteil des ES-Antigens war in allen chimären Konstrukten degradiert, wobei das S-Antigen sehr stabil exprimiert werden konnte. Die chimären Proteine ES_1:S_0, ES_1:S_1 und ES_1:S_4 konnten stabile VLPs bilden. Von ES_1:S_0 dagegen konnten nur Aggregate auf den elektronenmikroskopischen Aufnahmen beobachtet werden. Aufgereinigtes ES_1:S_4 wurde hinsichtlich seiner Stabilität evaluiert. Es zeigte sich, dass sowohl die ES- als auch die S-Antigenfraktion über 2 Wochen bei 4°C ohne Additive stabil waren. Jedoch resultierte ein kleiner Teil des ES-Antigens im aufgereinigtem ES_1:S_4 in unlöslichen Proteinaggregaten. Ein Antiserum einer Maus, die mit ES_1:S_4 immunisiert wurde zeigte eine moderate Neutralisierung gegen DENV-2. Um den Effekt der Expression von unlöslichen Proteinaggregaten des Fusionsproteins auf die Wirtszellphysiologie zu untersuchen, wurde eine Proteomanalyse der Zellen durchgeführt. Die Ergebnisse zeigen eine signifikante Hochregulierung der Chaperone, die in der ungefalteten Proteinantwort (UPR) und der endoplasmatischen Retikulum assoziierten Degradation (ERAD) eine Rolle spielen.

Die stabile Expression und optimale Präsentation von EDIII auf der Oberfläche des Trägerkonstrukts werden derzeit weiter verbessert, um einen praktikablen

Impfstoffkandidaten zu erhalten. Die vorliegende Arbeit zeigt mögliche Engpässe in der heterologen Expression von viralen Proteinen in *P. pastoris*, die zu einer gestörten Wirtszellantwort führen und durch die Expression dieser Proteine hervorgerufen werden.

Schlagwörter: Dengue, Hüllprotein Domäne III, Virusartike Partikel, Hepatitis B Oberflächenantigen, *Pichia pastoris*, Transmissionselektronenmikroskopie**,** Zweidimensionale Gelelektrophorese, Ungefaltete Proteinantwort, Endoplasmatisches Retikulum assoziierte Degradierung.

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ABBREVIATIONS

µg	Microgram
µL	Microliter
Aa	amino acid
Ab	Antibody
ADE	Antibody dependent enhancement
AGL	Antigenic loop
AOX1	Alcohol oxidase 1
AOX2	Alcohol oxidase 2
APS	Ammonium persulphate
BMGY	Buffered complex medium containing glycerol
BMMY	Buffered complex medium containing methanol
CHAPS	3-[3-cholamidopropyl (dimethylammonio]-1-propanesulfonate
CVD	ChimeriVax-dengue virus
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DSS	Dengue shock syndrome
EDIII-	Envelope domain III
ELISA	Enzyme linked immunosorbent assay
ERAD	Endoplasmic recticulum associated degradation
h	Hour
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HEPES	2 [4-2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
His	Histidine
HSP	Heat shock protein
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kDa	Kilo Dalton
L	Litre
LAV	Live attenuated virus
M	Molar
mAb	Monoclonal antibody
MALDI TOF	Matrix associated laser desorption ionization time of flight
mAU	Milli absorbance units
MES	2-(N-morpholino) ethanesulfonic acid
mg	Milligram

min	Minutes
ml	Milliliter
mM	Millimolar
Mut^-	Methanol utilizing minus
Mut^+	Methanol utilizing plus
Mut^s	Methanol utilizing slow
NCR	Non coding region
ng	Nanogram
NGC	New Guinea C
NL	Non-linear
nm	Nanometer
NS	Non-structural
NTR	Non translating regions
OD/OD_{600}	Optical density/Optical density at 600 nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer (sodium salt)
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PDI	Protein disulphide isomerase
PVDF	Polvinylidene fluoride
PVP	Polyvinylpyrrolidone
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
STAT	Signal transducers and activation of transcription
TEMED	N, N, N', N'-Tetramethylethylenediamine
TMB	3, 3', 5, 5'- Tetramethylbenzidene
TNF	Tumor necrosis factor
TT	Transcription termination sequences
UPR	Unfolded protein response
VLP	Virus like particle
WHO	World health organization
wt	Wild-type
YF17D	Yellow fever virus vaccine
YPD	Yeast extract, peptone dextrose medium

1. THEORETICAL BACKGROUND

1.1 Dengue

Dengue, a mosquito borne viral disease caused by any of the four closely related antigenically distinct serotypes DENV-1, -2,-3,-4 has become a major concern during the recent decades [1, 9]. Dengue fever is caused by virus belonging to genus Flaviviridae transmitted by mosquito bite *Aedes aegypti and Aedes albopictus*. Infection with DENV results in varying degree of pathological conditions ranging from a mild febrile illness dengue fever to severe and fatal dengue hemorrhagic fever and dengue shock syndrome [9]. The world health organization (WHO) estimates about 3.6 billion people are at the risk of infection over 100 countries with about 100 million infections in its mild form and 500,000 cases of a severe form, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) getting infected every year [6]. There is no licensed vaccine yet. The steady expansion of disease has led to urgent needs in the development of vaccines. Low understanding of pathogenesis of disease, contribution of immune response in protection or progression of disease and lack of animal models are major barriers in development of vaccine [10].

1.1.1 History

Dengue like symptoms caused by flying insects in human dates back in Chin, Tang and Northern sung dynasties in common era 265-420, 610 and 990 AD respectively. The dengue endemics were later seen in 17 century onwards and rapid spread in disease was due to trade, shipping and transportation [11]. The troops movement during the second world war along with the human settlements and deforestation resulted in viral and mosquito vector spread all over the Western Pacific and South East Asia which remained endemic from then on [12]. Dengue virus serotype 1 (DENV-1) Hawaii strain and Dengue virus serotype 2 (DENV-2) New Guinea C were identified in 1944 by Sabin and Dengue virus serotype 3 and Dengue virus serotype 4 by Halstead in 1956 [13].

1.1.2 Epidemiology

During the recent decades dengue incidence has been increased to 30 fold with rise in geographic expansion to new countries (Figure 1-1) [14]. The world health organization (WHO) estimates about 3.6 billion people are in dengue endemics areas. Annually, 50-270 million cases of dengue fever, 500,000 cases of DHF/DSS and 21,000 cases of death are reported. The countries majorly endemic to dengue are shown in Figure 1-2. The major reasons for the rapid spread of dengue are demographic changes like population explosion, uncontrolled urbanization, deforestation, lack of proper sewage management and vector control measures. The dengue outbreaks in Hawaii caused by DENV1 serotype was first observed during the Second World War in 1944. Similar outbreaks as a result of infection by same virus strain in Cuba were observed in 1977 and1981 followed by 1997 outbreaks where severe form of disease DHF/DSS observed was associated with DENV2 strain leading to the deaths of thousands. Similar endemics were observed in 2006 in Singapore. The spread of epidemics in both Cuba and Singapore was reduced by effective mosquito control. But there was a resurgence of disease as a result of travelers from endemic countries [12].

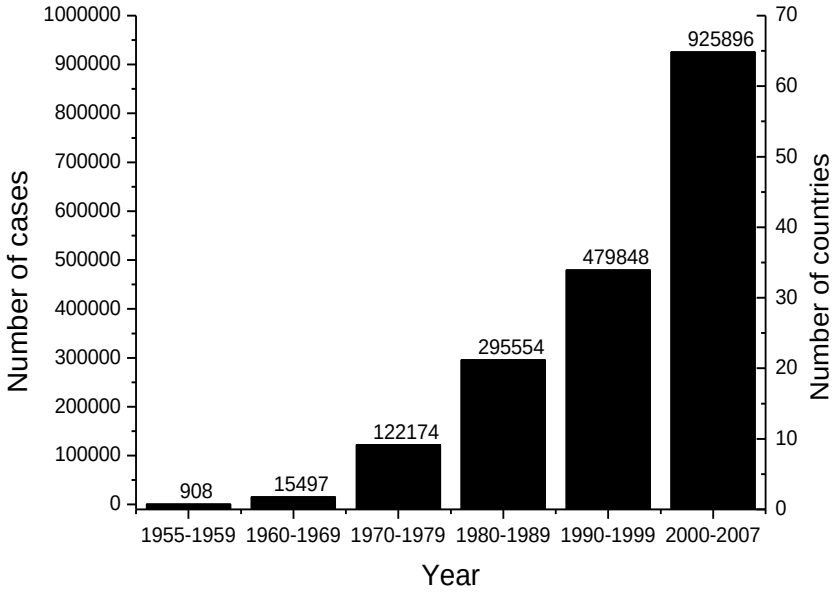


Figure 1-1: Rise in number of dengue cases in reported countries (1955-2007). Plot of annual average number of dengue cases, including dengue fever and dengue hemorrhagic fever reported against time in years.(WHO dengue guidelines, available in http://whqlibdoc.who.int/publications/ 2009/9789241547871_eng.pdf)

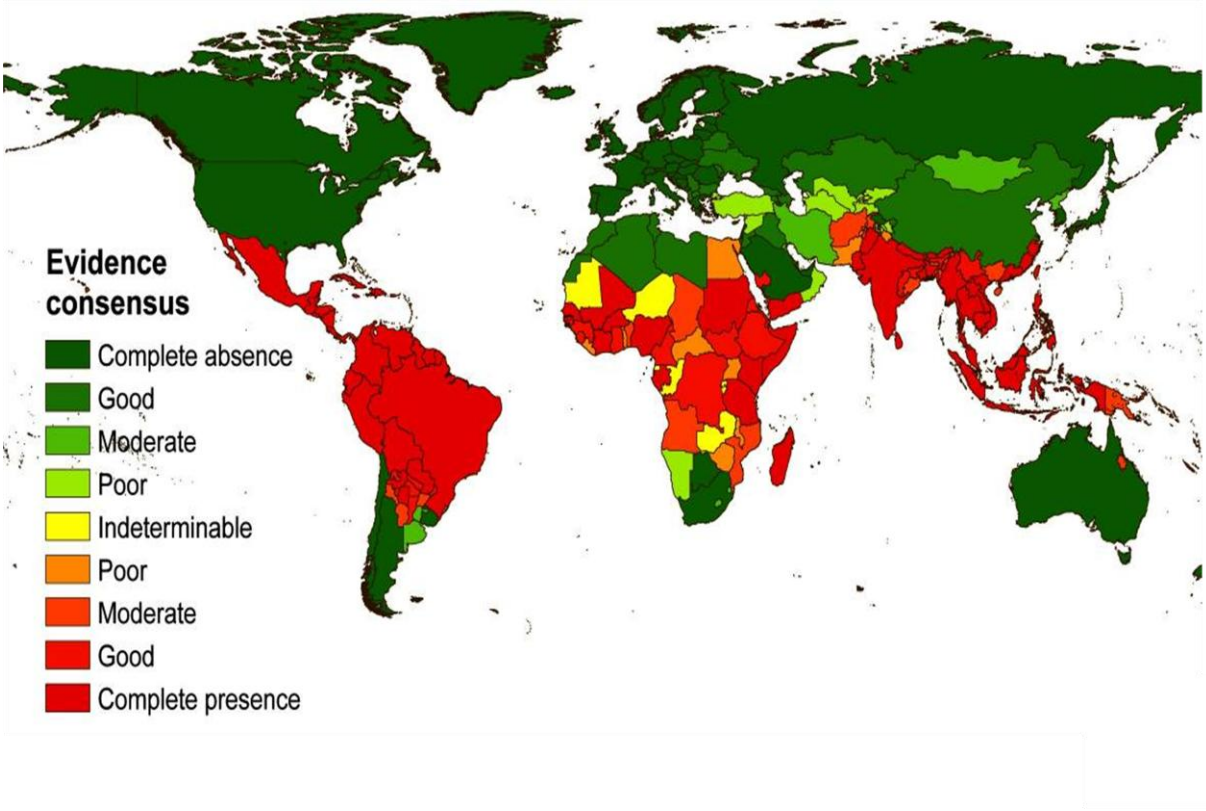


Figure 1-2: Global evidence, consensus, risk and burden of dengue in different parts of world. Adapted with permission from authors of reference [6].

1.1.3 Entomology

Dengue fever is an arthropod born disease transmitted by female *Aedes* species. These include *Aedes aegypti, which* is an African origin principal dengue virus vector and *Aedes albopictus*, a secondary vector for dengue transmission in Southeast Asia. The female Aedes is haematophagus and anthropophillic [11, 15].

1.1.4 Dengue transmission

The dengue serotypes transmission occurs in two ecologically and evolutionary distinct sylvatic and human cycles. The sylvatic transmissions involving arboreal *Aedes* and non human primates found in sylvatic environments of Southeast Asia, Western Africa and Peninsular Malaysia [11]. The principal *Aedes* vectors in Africa include *Aedes luteocephalus, A. furcifer* and *A.taylori*. The hosts include *Erythrocebus patas, Chlorocebus sabaeus and Papio cynocephalus*. The principal Aedes vectors in Asia includes *Aedes niveus s.l complex* and reservoir host *Macaca fasicularis, Macaca nemestrina and Presbytis cristata*. The human

cycle includes humans as amplifying hosts and pre-domestic and domestic *Aedes* species. [11, 15]

1.1.4.1 Dengue virus transmission cycle in humans

The human cycle of dengue virus transmission is shown in Figure 1-3. The transmission of dengue virus occurs by the bite of *Aedes mosquito* to an infected individual during the viremic phase which last between 2-7 days. The mosquito feeds on the blood of an infected individual and once the virus is ingested in the vector it migrates to posterior midgut, the first site of viral replication in mosquito from there it moves to haemocoel then to fat body and finally to salivary glands. The virus replicates in tissue tropism dependent manner in mosquito i.e. it can replicate in foregut, midgut, haemocel, haemolymph, fat body nervous system and salivary glands but not in hindgut, malphigian tubules and diverticula [16]. A glycoprotein 45 kDa receptor in *Aedes* species is said to be essential for viral entry and is found in all developmental stages and in tissues infected with virus [17]. The virus ingested in mosquito can persist for long time in mosquito and is transmitted to its progeny by transovarial transmission. [16]. The transmission to other humans occurs by the bite of an infected mosquito, which remains infective throughout its life.

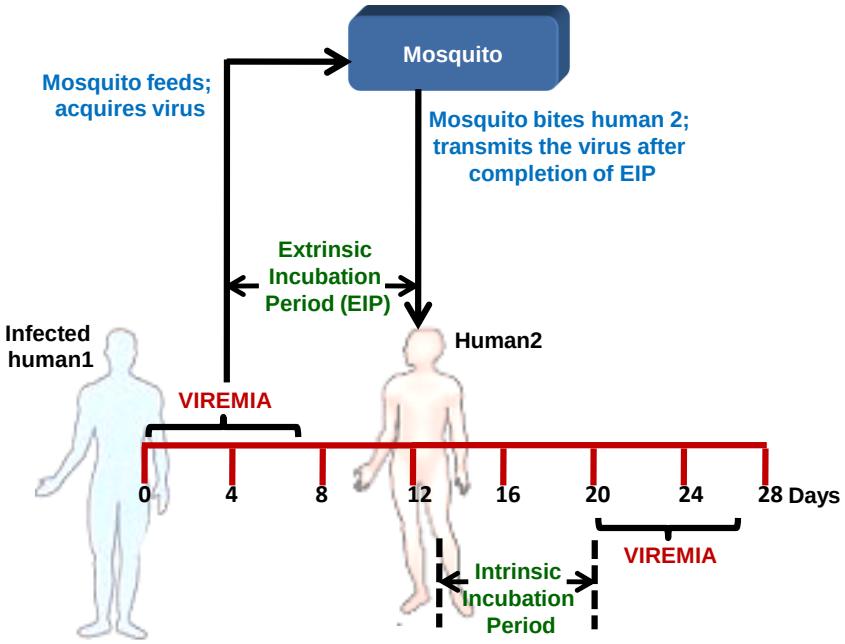


Figure 1-3: Dengue virus transmission cycle in humans. Adapted from (http://en.citizendium.org/wiki/Dengue_Virus).

1.1.5 Syndrome

1.1.5.1 Dengue disease classification

The clinical spectrum of the infection ranges from mild benign fever with febrile illness termed as dengue fever to fatal dengue hemorrhagic fever or dengue shock syndrome. Most of the dengue infected cases (about 50-90 %) are asymptomatic so remain unreported. There exists a very minor percentage (about 2-3 %) attaining fatal DHF/DSS apparently positioned at the apex of pyramid in Figure 1-4 A. The major clinical manifestations associated with the dengue fever and dengue haemorrhagic fever (DHF)/ dengue shock syndrome (DSS) are shown in Figure 1-4 B [18].

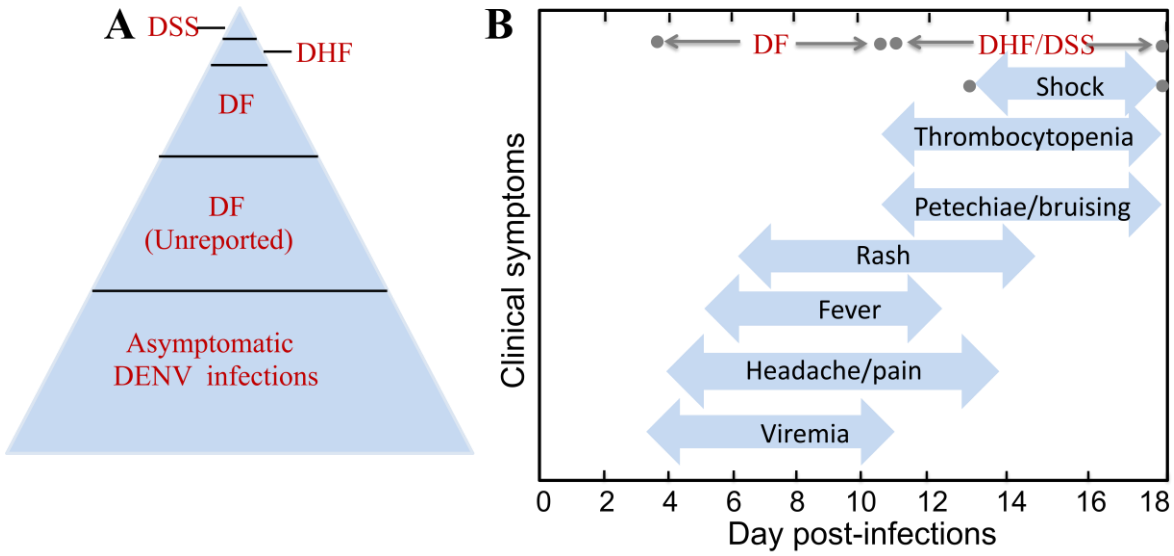


Figure 1-4: (A) Pyramid of dengue disease [12]. **(B)** Time course of clinical manifestations of dengue diseases. Adapted with permission from authors of reference [18].

1.1.5.2 Dengue fever

Primary infection with any of the four serotypes would result in mild febrile illness termed as dengue fever which lasts for 4- 7 days. Most of the cases being asymptomatic remain un-noticed. The symptoms include fever, headache rashes (maculopapular, petechial and scarlainform), muscle pain, joint pain and nausea. Sometimes dengue fever can be associated with abnormalities like hemorrhage, leucopenia and elevated levels of serum transaminases [19].

1.1.5.3 Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS)

The major fatal form of dengue disease is caused by infection with a serotype heterologous to that of primary infection. The disease is serotype specific. It has been reported that secondary infection by serotype 2 to an individual previously infected with serotype 1 or serotype 3 would result in this DHF/DSS [12]. The symptoms of DHF are identical to dengue fever but with hemorrhagic manifestations, thrombocytopenia, increased vascular permeability and organ impairment. The increased vascular permeability is of major concern which results in ascites, pleural effusion, hepatomegaly and hypovolemia. DSS is a severe form of DHF where patient condition deteriorates due to narrowing down of pulse pressure and decrease in blood volume. This shock is life threatening and patient either recovers within 24 hours and if fails to resist, dies [18].

1.1.5.4 Primary dengue infection

Initial infection with any of the four serotypes would result in symptomatic/asymptomatic disease. Severe disease during primary infection is associated with serotype 1 and 3 [12]. The first formed antibodies within 3-5 days (50 %) or 6-10 days (90-93 %) are transient IgM, which reaches a maximum by the end of two weeks then slowly diminishes to an undetectable level. The IgG antibodies can be seen in very low amount at the end of first week, then slowly expand and persist throughout the life. These antibodies provide lifelong immunity for infection with homologous serotype, but not for heterologous serotypes. People living in dengue endemic areas can be infected with all the four serotypes. It is said that secondary infection with a serotype heterologous to previous one increases the manifestation of getting DHF/DSS [10].

1.1.5.5 Secondary dengue infection

A secondary infection with heterologous serotype results in classical dengue fever with 2-3 % gaining life threatening DHF/DSS [10]. Secondary infection with serotype 2 to higher extent is associated with severe form of disease DHF/DSS [20]. Severity of disease associated with tertiary or quaternary infections is rare. DHF/DSS is found to be associated with high viremia and cytokines load. In literature there are many hypothesis explaining the pathogenesis of DHF/DSS in which both cellular immune response and humoral immune response are responsible for severity of disease. According to original antigenic sin theory by Halstead, primary infection with a serotype generates antibodies which can provide immunity for a long time, but the pre-existing cross reactive neutralizing or non neutralizing antibodies are of major concern. During heterologous infection with a serotype different from previous infecting one, the cross reactive antibodies binds to virion with low affinity and assist in gaining access to monocytes via Fcγ receptors and enhance viral load causing antibody dependent enhancement (ADE) [2]. ADE is a biological mechanism which results in increased uptake of virus by infected cell thereby increasing viral replication rather than decreasing as a result of humoral immune response of previous serotype. Experimental evidences for ADE have been shown by passive transfer of sub neutralizing antibodies for DENV envelope into non human primates and AG129 which led to enhancement of replication and viremic load from 10 -100 fold causing fatal disease [21]. The mechanism of ADE is shown in Figure 1-5. The other hypothesis suggest that secondary heterologous infection causes activation of memory T cells which binds virus with low avidity but are not

able to kill resulting in increased production of pro-inflammatory cytokines, TNFα/β and interferon γ leading to immunopathologic progression of disease [2]. ADE although associated with disease severity due to high viremia and increased production of pro-inflammatory cytokines but are not only sufficient to explain DHF/DSS in terms of ADE [18]

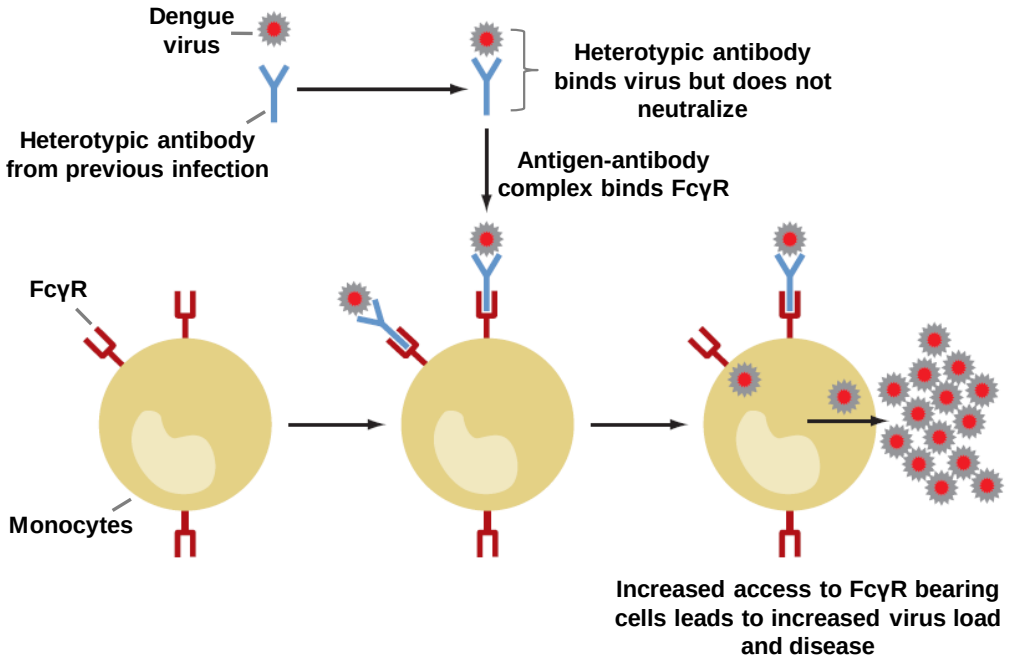


Figure 1-5: Illustration of antibody dependent enhancement (ADE). Binding of dengue virus with heterotypic semi-neutralizing antibodies results in formation of virus antibody complex and gets internalized enhancing viral replication and viremia. (Used with permission from authors of reference) [18].

1.1.6 Dengue virus structure

The structure of dengue virus type 2 strain PR159 –S1 was determined by fitting the known structure of tick born encephalitis virus (TBEV) envelope into the cryo-electron density map of DENV2. The dengue virus was revealed to be an enveloped virus of approximately 50 nm diameter. The surface of the virion is a condensed structure with 180 copies of envelope glycoprotein followed by membrane protein beneath it (Figure 1-6 B) [18]. The nucleocapsid and viral genome are surrounded by bilayer lipid envelope underneath the membrane protein. The dengue viral genome is composed of single stranded RNA of positive polarity about 11 kb size with 5'and 3' untranslated regions (UTRs) of 100 and 450 bp size. 5' UTR's has a methylated cap with S-adenosyl methionine activity and stem loop (SL) structure. 3' UTR lacks poly-A tail but contains conserved stem loop structure. The presence of upstream AUG regions (UAR) and cyclizable sequences in both 5' and 3' UTRs has an important role in viral RNA translation and replication. RNA is synthesized as a single open reading frame encoding a polyprotein which is cleaved by host proteases and viral NS3 protease into three structural proteins (envelope, capsid and pre-membrane) and non-structural proteins (NS1, NS2a NS2b, NS3, NS4a, NS4b andNS5) (Figure 1-6 A, Table 1-1).

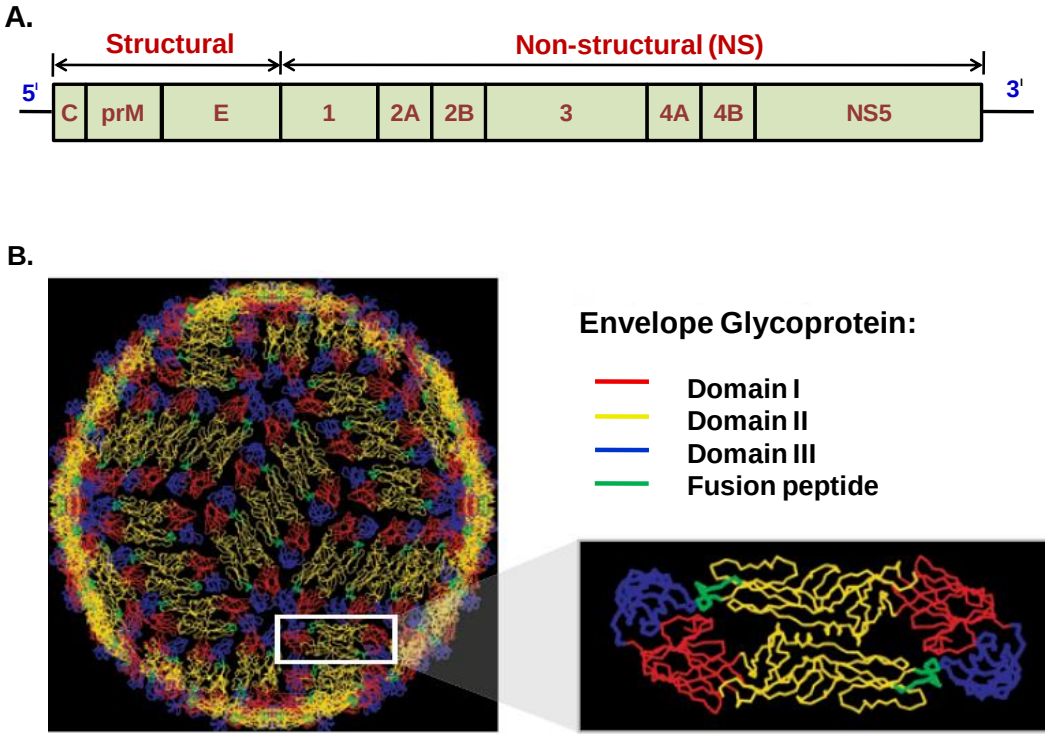


Figure 1-6: (A) Dengue virus genome organization encoding structural and non-structural proteins. **(B)** Dengue virus structure, with an enlarged view of envelope protein where domain I, II, III and fusion peptide are represented by red, yellow, blue and green color, respectively. (Used with permission from authors of reference) [18]. Abbreivations of structural and non-structural proteins are shown in Table 1-1.

The envelope protein is a major immunogenic glycoprotein playing an essential role in viral biology assisting in receptor recognition, viral attachment and virion morphogenesis. The envelope protein is said to be highly conserved among all the flaviviruses. There exists 60-67 % sequence similarity among all the four serotypes and 90-96 % homology among genotypes of same serotype [18]. The E protein has three distinct domains. Domain I is a flexible hinge with 8 ß barrels strands contains type specific epitopes and antibodies formed against this domain are non-neutralizing. Domain I also contributes in the conformational change during low pH. Domain II contains a highly conserved fusion loop playing a pivotal role in fusion with the endosomal membrane at acidic pH and dimerization. The domain contains type and subtype specific epitopes resulting in cross-reactivity. The antibodies to this domain act as neutralizing at a threshold concentration and below this concentration as enhancing. Domain III is an immunoglobulin like domain, easily accessible on virion surface playing an essential role in receptor recognition and antibodies formed against this domain are neutralizing, highly

immunogenic and non cross-reactive. It has been shown the antibodies against this domain could block the infectivity of the virus. These attributes together manifest the prominent role of domain III as subunit vaccine candidate [10, 22].

Table 1-1: Dengue virus proteins and their predicted functions [19, 23, 24].

	Protein	Function
Structural	Capsid (C)	Binds and stabilizes viral RNA
	Pre-membrane/ Membrane (prM/M)	pr peptide of prM prevents premature fusion and membrane protein forms ion channels
	Envelope (E)	Receptor recognition, viral attachment and viral morphogenesis
Nonstructural	NS1	Viral replication and provides a defense to virus by inhibiting complement activation
	NS2a	Part of viral replication complex
	NS2b	Cofactor for NS3
	NS3	Helicase and protease
	NS4a	Membrane alteration for viral replication
	NS4b	Also plays a role in viral replication by interacting with NS3 and also plays a role in blocking IFNα/β signaling.
	NS5	Methyl transferase and polymerase

1.1.7 Cellular targets and receptors for DENV

The dengue virus replicates in cell and tissue tropism specific manner [25]. The dengue virus when transmitted to humans by infected mosquito bite first reaches to Langerhans cells and dendritic cells where it replicates and disseminates into blood stream through lymph nodes reaching to monocytes and tissue macrophages. The viral replication is observed majorly in liver, endothelial cells, splenic macrophages and bone marrow. The entry of virus into these specific cells is facilitated by expression of specific receptors on cell surface recognized by virus. The receptors in human include majorly carbohydrates (sulfated glycans, glycosphingolipid) and carbohydrates binding proteins (lectin, mannose binding receptor). Carbohydrate acts as a co-receptor which associates with other molecules forming a complex which enhances viral entry. In case of mosquito majorly proteins act as receptors for viral entry. Some receptors are serotype specific, and virus uses combination of receptors to gain entry into different cell types using different receptors [25]. Table 1-2 lists the receptors found to be involved in viral entry in humans.

Table 1-2: Cell receptors of dengue virus. Adapted from reference [25].

Receptor	Properties	Cell/Expression system	Dengue serotypes
Heparin sulfate	Sulfated glycosaminoglycan	Vero cells, BHK-21 cells, SW-13 cells	DENV 1-4
Neolactotetraosylceramide (nLc_4 Cer)	Glycosphingolipid	Vero cells, BHK-21 cells, K562 cells	DENV 1-4
DC SIGN	Dendritic cell specific receptor with lectin activity	Dendritic cells, Macrophages	DENV 1-4
Mannose receptor	Lectin like activity	Macrophages	DENV 1-4
HSP70/HSP90	Heat shock proteins expressed on plasma membrane	Macrophages HepG2 cells, SK-SY-5Y cells	DENV-2
GRP78	Chaperone expressed on plasma membrane	HepG2 cells	DENV-2
Laminin receptor	High affinity laminin receptor	PS clone D cells, HepG2 cells	DENV 1-3
CD 14 associated protein	Lipopolysaccharide receptor	Monocyte, Macrophages	DENV-2

1.1.8 DENV life cycle

The virus enters the host cells by receptor mediated endocytosis in clathrin dependent manner. The low pH in the endosomal compartment causes conformational changes within the E protein which exist as spiky projections in heteromeric trimeric forms. This leads to the exposure of fusion peptide of E protein which causes the fusion of viral membrane with endosomal membrane, allowing uncoating and release of viral RNA [26]. The viral RNA has positive polarity and is translated as single ORF encoding a polyprotein precursor at rough endoplasmic reticulum (RER) which is cleaved into structural and nonstructural proteins by host and viral proteases. The viral RNA replicates in cytosol.

The viral RNA replicase components NS3 and NS5 bind to 3' UTR to initiate the replication of (+) strand forming a (-) strand which further acts as template forming (+) strand. In the later stages the RNA fuses with capsid to form RNA-capsid complex. The nucleocapsid with encapsulated genome buds into the ER and acquires envelope decorated with prM-E heterodimers from ER membrane and forms immature particles. The maturation of virions occurs with a conformational change induced by pH transition. At acidic pH during migration of immature virions the pr peptide gets dissociated from M protein by the furin protease causing maturation of viruses. Nevertheless, the pr peptide still exists associated with E protein, which gets separated when everyone is released into the extracellular milieu by exocytosis (Figure 1-7).[23, 26].

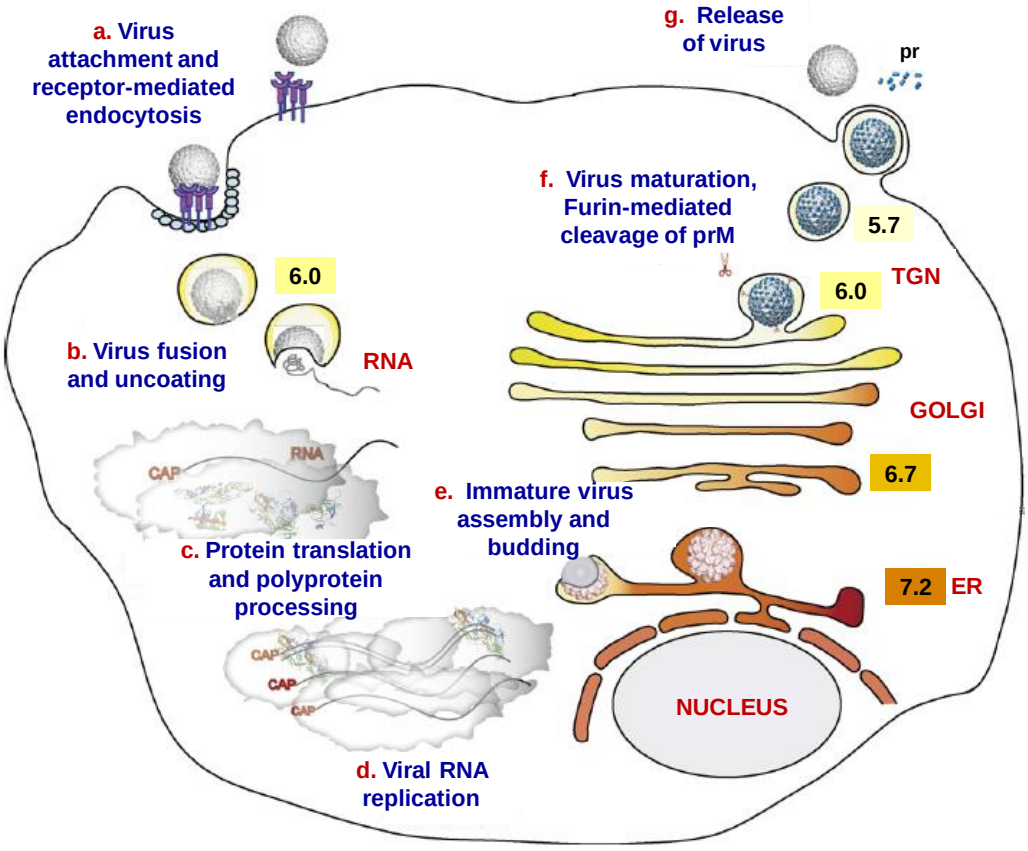


Figure 1-7: Dengue virus life cycle. **(A)** Viral attachment by receptor mediated endocytosis, **(B)** virus membrane fusion and uncoating causing release of viral RNA (+ strand) into cytosol, **(C)** translation of viral RNA into polyprotein and processing into individual units **(D)** viral RNA replication **(E)** association of viral RNA with structural proteins to form immature virion, **(F)** transport of immature virions through trans golgi network and cleavage of pr peptide from prM causing maturation of virions **(G)** final release of virion. Used with permission from authors of reference [26].

1.1.9 Pathogenesis

The mechanism underlying the pathogenesis of dengue virus is not completely known. Much of literature was focused on descriptive mechanisms and challenge is related to identification of immunopathological mechanisms [27]. The dengue pathogenesis is multifactorial and is affected by age, gender and genetic determinants in host, and also by virulence of virus genotype variants within serotype and comorbidities. Infants to age 4-12 months are more susceptible to infection than newborn babies and children of more than 1 year of age. Similarly children are more susceptible to DSS/DHF than adults. Gender based susceptibility is also seen in severe form of disease. Female children are more susceptible than males [27, 28].

Dengue pathogenesis is thought to be immune mediated due to its severity and higher incidence during secondary heterologous infection. Pathological features in case of severe DENV infection, i.e. DHF or DSS include majorly hemorrhagic manifestation in the skin, gastrointestinal tract and heart combined with fluid accumulation in body cavities. However, the mechanism for vascular leakage and hemorrhage are not well understood. Elevated concentration of cytokines including IL-2, IL-6, IL-8, IL-10, IL-13 and TNFα are found associated with immunopathogenesis of severe disease [19]. These elevated cytokines are associated with increased capillary permeability, thrombocytopenia and hematocrit. Understanding of dengue pathogenesis is hampered due to unavailability of infallible immunological markers for protection and pathological response and lack of suitable animal models [10].

1.1.10 Dengue vaccine candidates in clinical trails

With several goals which includes universal availability at affordable price, lifelong protection against all four serotypes, safe and well tolerable in immune compromised individuals different vaccine candidates have been developed [29] some of which reached clinical trials (Table 1-3). Due to unavailability of dengue therapeutics and vector control measures being the sole measure of disease control, development of vaccine would be a major breakthrough in disease control [30].

Table 1-3: Vaccine candidates under clinical trials. Adapted from [30].

Vaccine candidate	Vaccine approach	Developer	Clinical Phase
ChimericVax	Based on YF17D backbone and replacement of prM E gene with DENV genes	Sanofi Pasteur	Phase III
Live attenuated	Attenuation by serial passaging in PDK cell line	WRAIR and GSK	Phase II (Development on hold)
Tetravalent vaccine formulation	Defined deletion mutations and intertypic chimerisation	NIAID,Butantan and Biological E	Phase I
DenVax	Replacement of DEN1, DEN3 DEN4 prM E genes with DEN2 PDK 53 virus gene	Inviragen	Phase II
DEN1 80E	Truncated envelope protein expressed in insect cells	Hawaii Biotech/Merck	Phase I
D1ME-VR-P	DNA vaccine comprising prM and E genes	NMRC	Phase I

Abbreviations: YF17D: Live Attenuated Yellow Fever Vaccine; PDK: Primary Dog Kidney; WRAIR: Walter Reed Army Institute of Research, USA; GSK: GalaxoSmithKline Biologicals; UK NIAID: National Institute of Allergy and Infectious Diseases, USA; NMRC: Naval Medical Research Center, USA.

1.1.11 Envelope domain III based subunit vaccine candidate

Although, live attenuated vaccine (LAV) Chimeric Vax has reached to advanced clinical phase concerns, about safety still exist [10]. To overcome problems associated with live attenuated vaccines like viral interference, attenuation and reversion to virulent form, efforts were made to establish protein based subunit vaccine candidates. The envelope domain EDIII is considered to be a promising candidate. Domain III is highly immunogenic, possesses multiple type and subtype specific neutralizing epitopes, lacks cross-reactive and enhancing epitopes [22]. This would avoid the risk of antibody dependent enhancement associated with other domains. EDIII domains individually and in association with fusion partners were evaluated as monovalent and tetravalent vaccine candidates. Table 1-4 lists homologous/heterologous EDIII based chimeras as potential subunit vaccine candidates.

Table 1-4: List of EDIII based subunit vaccine candidates

Serotype	Fusion partner	Expression host	Developmental stage	Reference
DENV-2	MBP	*E. coli*	Monovalent evaluated in mice	[31]
DENV1-4	P64K of *Niesserria meningitides*	*E. coli*	Monovalent evaluated in NHPs	[32]
DENV-2	DENV4	*E. coli*	Bivalent evaluated in mice	[33]
DENV 1-4	Glycine linker	*P. pastoris*	Tetravalent evaluated in mice	[34]
DENV-2	HBsAg	*P. pastoris*	Monovalent evaluated in mice	[3]
DENV-2	HBcAg	*P. pastoris*	Monovalent evaluated in mice	[35]

Abbreviations: MBP: Maltose binding protein; NHPs: Non human primates; HBcAg: Hepatitis B core antigen.

1.2 Virus-like particles (VLPs)

Virus-like particles (VLPs) are supramolecular clusters with repetitive surface, particulate structures and pathogenic recognition patterns formed by self assembly of structural proteins including capsid and envelope [4]. VLPs usually exist as icosahedral or rod shaped structures in range of 20-150 nm. VLPs have structural and antigenic features identical to that of virus and induce early, rapid and sustained B and T cell responses. Identical with the viruses, VLPs also exist as non enveloped and enveloped classes. Non enveloped VLPs include single or multiple antigen of the pathogen with self assembly characteristic without any host cell components. HBsAg is exceptional since it is a non-enveloped VLP but still possesses host cell lipids, which are needed for its structural integrity and antigenicity [4]. Some of the other examples for non-enveloped VLPs include human papillomavirus L1 capsid, rotavirus (VP2, VP4, VP6 and VP7). Envelope VLPs include single or multiple antigens arranged on surface of host cell membrane. Envelope VLPs are more complex and also provide more flexibility in insertion of antigens. Some of the envelope VLPs include influenza virus (HA-hemagglutinin, NA: neuraminidase, M1: matrix protein, M2: ion channel), human immunodeficiency virus (HIV envelope), simian immunodeficiency virus (SIV) gag protein, hepatitis C virus (HCV)

(C: core protein, E1,E2 and P7: envelope proteins [4]. VLPs have three major functions: VLPs act as subunit vaccines (SUVs), serve as carrier for display of epitopes of foreign origin or epitopes of other proteins of the same strain or same epitopes of different strains, and as therapeutics by targeting self molecules [5, 36].

1.2.1 Subunit vaccine candidates (SUVs)

SUVs exploit the self assembly properties of an antigen component of the parental virus for immunization against cognate viral infection. VLPs offer a safe and effective vaccine platform owing to its immunological attributes like size, particulate nature, surface area, hydrophobic/hydrophilic interface and absence of genetic material [36]. A number of VLP vaccines have been subject of extensive research during the last two decades, some of which reached preclinical and clinical trials. Two VLP based vaccine candidates reached the market which include Hepatitis B surface antigen (HBsAg) VLP and human papilloma virus capsid (HPV L1) VLP. VLPs can function as homologous as well as heterologous vaccine candidates [37].

1.2.2 Platform for foreign antigen delivery

VLPs serve as a platform for immune presentation of foreign antigens with low immunogenic potential or which are not able to assemble into VLPs [38]. Incorporation of heterologous epitopes of the same virus or epitopes of a completely unrelated pathogen by genetic fusion or chemical linkage results in formation of chimeric VLPs and so fused antigens are exposed on surface of VLPs. HBsAg, HBcAg, HPV L1 capsid and parvovirus VP2 were used as efficient carriers of foreign antigens by compromising some features without disrupting VLP assembly [36]. Of many chimeric VLPs only two had shown promising results in clinical phase. These include homologous chimeric VLPs Sci-B-Vac and BioHepB against hepatitis B virus which includes L and M protein of HBV along with HBsAg [38]. RTS,S formed by fusion of circumsporozite protein from *Plasmodium falciparum* at amino terminus of HBsAg co-expressed along with 4 copies of HBsAg also successfully reached Phase III trials [37].

1.2.3 Targeting self molecules

The application of VLPs in presentation of self antigens which mediates disease on surface exposure, results in generation of autoantibodies. These induced autoantibodies can be used to overcome immunetolerance and can thus be used for treatment of chronic diseases like cancer, allergy, arthritis and cardiovascular diseases [36]. The self molecules include β-amyloid, angiotensin II, TNFα and ghrelin. The therapeutic efficacy by presentation of self molecules have been demonstrated in rheumatoid arthritis, atherosclerosis, osteoporosis, hypertension and Alzheimer disease and has shown positive results in preclinical trials [36].

1.2.4 Immunogenicity of VLPs

Physical attributes of VLPs are associated with eliciting potent B and T cell responses. This response can be further enhanced by removing the immunosuppressive epitopes in VLPs. The optimal number (20-25) and spacing (5-10 nm) between epitopes in VLPs has been associated with B cell activation [36]. Repetitive epitopes exposed on the surface of the VLPs cross link with B cell receptors of B lymphocytes and causes activation and proliferation of B-lymphocytes to produce IgM antibodies. Repetitive structures are also known to bind with complement proteins and causing further B cell activation. Additionally, VLPs can also induce T cell dependent IgG response, germinal center formation, hyper-mutation, class switching and memory cell formation. The small size of VLPs allows it to drain from the site of injection to lymph node and then spread to secondary lymphoid organs where they interact directly with B cells thereby stimulating activation [36].

The particulate nature and size of the VLPs help in efficient uptake and delivery by antigen presenting cells (dendritic cells). Dendritic cells can uptake antigen with dimensions of (10 nm-3 μm) [36]. Once internalized by the dendritic cells the antigens are processed into fragments and presented to class II MHC. Dendritic cells can also cause cross presentation to class I MHC, triggering maturation of dendritic cells resulting in production of cytotoxic T cell lymphocytes and cytokines for clearance of pathogen [39] (Figure 1-8). HBsAg VLPs are known to induce significant humoral immune response [40]. HPV VLPs are known to stimulate directly the maturation and activation of dendritic cells leading to expression of costimulatory cytokines and T-cell responses [36].

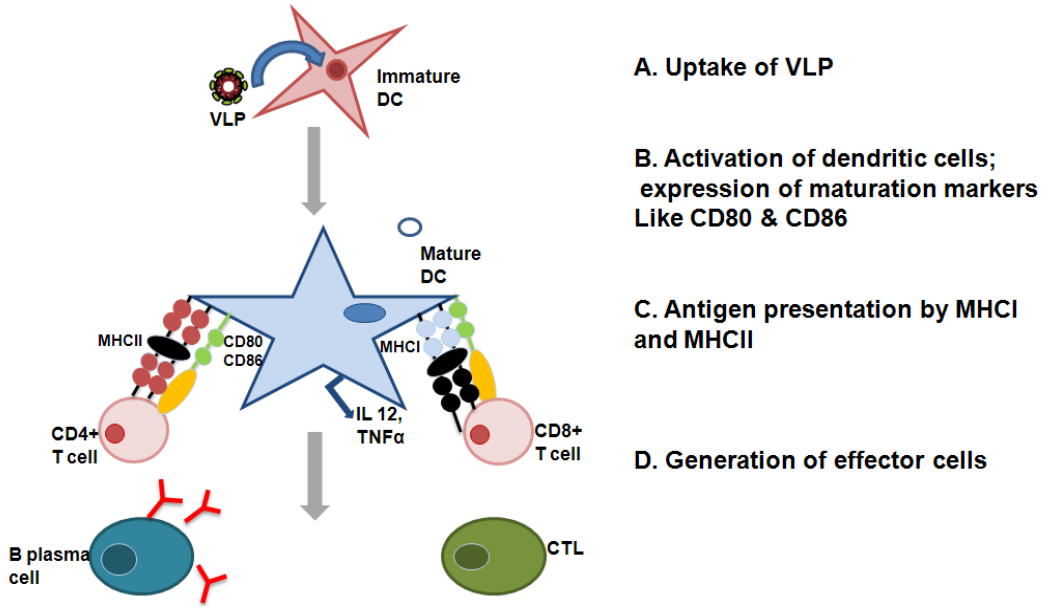


Figure 1-8: VLPs uptake, presentation, processing and maturation of dendritic cells. Adapted from [39].

1.3 Hepatitis B surface antigen

The hepatitis B surface antigen (HBsAg) was first discovered 50 years ago in Australian aborigines named as AuAg by Baruch Blumberg. HBsAg is a major marker of Hepatitis B virus (HBV) infection [41]. Hepatitis B surface antigen (HBsAg) is the major envelope protein found abundantly in individuals infected with HBV infection, belonging to Hepadnaviridae family. It exists in the form of 22 nm spherical or filamentous lipoprotein particles. HBsAg is synthesized in excess amount and act as bait to the immune system and helps in evasion of HBV [41]. HBsAg particles are composed of surface viral proteins (60 %) and host derived lipids (40 %) [42]. The particles have a molecular weight of 2.4 MDa and density of 1.2-1.21g/ml. Each particle is composed of about 100 monomers [43]. The other envelope protein includes L and M proteins. The envelope proteins L, M and S are encoded by same ORF with difference in phase translation initiation codons and same termination codon. The three proteins differ at amino terminus but are same at carboxy terminus. The S protein is found in higher proportions when compared to L and M proteins. L and S protein have role in virus maturation whereas M protein has no role in maturation [44].

1.3.1 Structural features

HBsAg monomer is a typical transmembrane protein composed of 226 amino acids with a molecular weight of 24 kDa. HBsAg is composed of four transmembrane domains. The first transmembrane domain (TM1) is in an amino acid position 4-24 which assists in co-translational translocation of the amino terminus of the protein into the ER lumen. Downstream to TM I is cytosolic loop followed by TM II at amino acid position 80-100 which acts as a signal/anchor domain followed by antigenic loop. The third and fourth TM domains are located within third hydrophobic domain at carboxy terminus of HBsAg which helps in translocation of C-terminal end of protein into lumen [44]. The central hydrophilic region (110-156) contains (a) antigenic determinants [45]. The lipid components of HBsAg are arranged in an ordered interface and are essential for antigenicity [4]. The lipid composition of HBsAg particles includes phosphatidyl choline (60 %), cholesteryl ester (14 %), cholesterol (15 %) and triglycerides (3 %) [43].

1.3.2 Significance of HBsAg

HBsAg is a major marker of viral hepatitis. Antibodies against HBsAg can neutralize the virus infectivity [46]. Presence of HBsAg antibodies in acute hepatitis indicates the suppression of viral replication, clearance of infected hepatocytes and prevention of viral dissemination in new cells. The acute hepatitis is marked by presence of HBsAg which starts developing after few weeks of infection, reaches to the peak and decreases after onset of infection [41]. Protection is achieved by decrease in HBsAg, presence of anti HBcAg antibodies and development of anti HBsAg antibodies. The chronic infection is characterized by the presence of HBsAg even after 6 months of infection with HBV. Occult infections are characterized by the presence of anti HBcAg antibodies in HBsAg and HBV DNA negative patients in serum. The chronic and occult infection can lead to fatal hepatitis or hepatocellular carcinoma only in case of immunodeficiency. Hotspots of variability are also present in HBsAg in antigenic loop which would result in generation of HBV mutants which when attain ability to transmit lead to fatal hepatitis. The recombinant HBsAg vaccine is the first recombinant virus-like particle vaccine in the market [41].

1.3.3 HBsAg as carrier

The potential use of HBsAg as carrier by expression in systems like yeast, baculovirus and mammalian systems has resulted in success to a significant extent. Insertions in amino terminus to that of S protein in preS region and antigenic determinant loop in regions (110-156) resulted in exposure of epitopes on surface of the particle without disruption in particle assembly [47, 48]. The feasibility of HBsAg as carrier was first tested by fusion of herpes simplex virus (HSV) glycoprotein fragment (300 aa) to amino terminus of HBsAg in preS region which resulted in exposure on surface of particle [49]. The second malaria vaccine candidate formed by the fusion of repetitive T cell epitope circumsporozite protein (189 aa) to amino terminus of HBsAg and co-expression of HBsAg in yeast resulted in the formation of RTS, S vaccine candidate which successfully reached phase III trials [47]. HBsAg based chimeras are listed in Table 1-5

Table 1-.5: List of HBsAg based chimeras.

Epitope (AAs)	Site	VLPs	Host	Developmental stage	Reference
Glycoprotein (gD) fragment of HSV (300)	N	+	Yeast	ND	[49]
Capsid protein VP1 of poliovirus type 1 (10)	AGL	+	Mammalian cells	Evaluated in mice	[48]
Receptive epitope of circumsporozite protein in *P. facliparum* (64)	N	+	Yeast	Evaluated in mice and rabbit	[50]
gp 120 V3 domain of HIV/BRU isolate (24)	N	+	Mammalian cells	Evaluated in monkeys	[51]
C-terminal CSP in *P. facliparum* (189)	N	+	Yeast	Evaluated in humans	[47]
HVR E2 immunodominant eptiope of HCV1a/1b strains	AGL	+	Mammalian cells	Evaluated in mice	[52]
E protein of dengue virus serotype 2 (395)	N	+	Yeast	Evalauted in mice	[53]
C-terminal sequence of Kat A protein in *Helicobacter.pylori* (130)	AGL	+	Mammalian cells	Evalauted in mice	[54]
EDIII of dengue virus serotype 2 (104)	N	+	Yeast	Evaluated in mice	[3]

Abbreviations: N: Amino terminus; AGL: Antigenic loop; HSV: Herpes simplex virus; HCV:Hepatitis C virus; HIV: Human immunodeficiency virus.

1.4 *Pichia pastoris* as an expression host

Industries keep on looking for improved efficiency of bioprocess or alternative host system for recombinant expression of heterologous proteins in a cost effective manner [7]. Methylotropic, *Pichia pastoris* is one such host which offers a magnitude of advantages over both prokaryotic (*Escherichia coli*) and eukaryotic (yeast (*Saccharomyces cerevisiae*), baculovirus and mammalian cells) expression system [55]. Some of which includes:

- Growth to high cell densities in simple defined medium.
- Proper polypeptide folding by presence of chaperones.
- Post translational modifications, including glycosylation, disulphide bond formation and phosphorylation.
- Easy genetic manipulation and stably expressing cell lines.

The advantages of *P. pastoris* over *S. cerevisiae* includes a preference for respiratory rather than fermentative growth and presence of strongly regulated promoter (alcohol oxidase) [56]. There are ample *Pichia* strains and vectors used for heterologous protein expression. The choice of strain and vector depends on protein to be expressed. The strains vary genotypically and phenotypically. Genotypical variation includes protease deficient strains (*pep4*) and auxotrophic mutants *(his4, arg4*) [55]. Phenotypical difference depends on the ability of strains to use methanol efficiently, which depends on the presence of alcohol oxidase gene (*AOX*). There are two *AOX1* and *AOX2* genes which have homology of about 96 %. *AOX1* accounts for about 85 % of methanol utilization with high expression level of about 30 % of total cell protein [57]. *AOX2* has low expression levels and are not able to metabolize methanol efficiently. The presence of *AOX1* and *AOX2* genes results in efficient methanol utilizing phenotype (MUT^+) whereas the presence of defective *aox1* and *AOX2* results in methanol slowly utilizing phenotypes (MUT^S). AOX catalyses the first step in the methanol metabolism causing oxidation of methanol to formaldehyde and generating hydrogen peroxide in peroxisomes. The hydrogen peroxide is detoxified by catalase. Formaldehyde is involved in an assimilation pathway for generation of biomass and dissimilation pathway for generation of energy. Numerous cytosolic and secretory proteins were expressed under AOX promoter. Alternatively, GAP YPT1, FLD1 and DHAS were also employed for protein expression [58].

The ability of *P. pastoris* to produce proteins with authentic conformations resulted in its extensive use as a heterologous expression system. *P. pastoris* allows expression of high disulphide bonded proteins, oligomeric forms and proteins with its cognate partners in very high quantities by multicopy integration [59].

P. pastoris can be used for the production of intracellular or secretory proteins with structural, diagnostic and therapeutic potential [60]. Intracellular or extracellular targeted expression depends on the protein. If the protein is secreted in its native system, then protein can be fused to its native signal or with an alpha mating factor from *S. cerevisiae*. The alpha mating factor is in upstream of the multiple cloning site (MCS) and at the amino terminus to that of recombinant protein. The α-mating factor is composed of a pre sequence (19 amino acids) and pro sequence (60 amino acids) with cleavage site for Kex2 endopeptidase and dipeptide repeats (EA) to enhance the proteolytic cleavage necessary for protein maturation. Nevertheless, if protein is integral membrane protein or has problem with secretion in such cases, they can be expressed intracellulary which can result in high expression levels. Some of high expressed cytosolic proteins include hydroxynitrile lyase (22 g/l), tetanus toxin C (12 g/l) and HBsAg (6 g/l) [58, 61].

Despite extensive use of *P. pastoris* as a platform for heterologous protein expression, there are several bottlenecks in protein production [62]. During recent times with the *Pichia's* available genome sequence in literature and studies on the physiological response to secretory proteins or intracellular protein expressed revealed obstacles during protein production. Along with the protein intrinsic properties (hydrophobicity, number of disulphide bonds and PEST sequences), the environmental factors such as temperature, oxygenation, medium, composition and pH also affect the recombinant protein expression [58, 60]. The protein function depends on three dimensional conformations which it acquires during and after synthesis from ribosomes. After synthesis at ribosome proteins are translocated to the ER where they undergo folding and posttranslational modifications. The properly folded proteins are translocated to specific functional site and terminally misfolded proteins or unfolded proteins are retained in the ER and targeted to degradation in the ubiquitin mediated pathway termed as endoplasmic reticulum associated degradation (ERAD). Accumulation of these misfolded proteins results in activation of ER-Nucleus signaling pathway called as unfolded protein response (UPR). The cascade of folding, translocation and degradation are aided by both cytosolic and ER resident chaperones [63].

1.4.1 Molecular chaperones and their role in protein biogenesis

The major classes of chaperones, helper proteins HSP70, HSP40 (co-chaperones) nucleotide exchange factors (NEF), HSP110 and HSP90 belong to class of heat shock protein (HSP) family. HSPs are found in endoplasmic reticulum (ER), cytosol and mitochondria and play a vital role in protein biogenesis, translocation and degradation (Table 1-6) [64]. Chaperones work as a part of dynamic and complex network in cooperation with other factors. The same chaperones, which aid in protein folding in ER, when not able to attain native conformation, target the protein to degradation [65]. The major classes of chaperones and co-chaperones that acts on the substrate are described below

- Members of Heat shock proteins 70 (HSP70s) are composed of three domains 44 kDa amino (N)-terminal ATPase domain, 15 kDa substrate binding domain and carboxy (C)-terminal lid of unknown function. Chaperone activity of HSP70 is ATP dependent and includes cycle of ATP hydrolysis and protein binding. HSP70s also called as stress induced proteins, play a vital role in protein folding, translocation, disassembly of aggregates and targeting to ERAD [64].
- HSP40s act as cochaperones and enhance the ATPase activity of HSP70. Three classes of HSP40s exist. All three are composed of 70 amino acids conserved J domain which contains a HPD motif necessary for binding to HSP70 and enhancement of ATPase activity. Type III is composed of only J domain whereas Type I is composed of J domain, a glycine/phenyl alanine (G/F) and cysteine rich region (CR). The G/F rich region acts as spacer needed for stimulation and CR rich regions are needed for peptide binding. HSP40s aid in folding and translocation along with HSP70s [64].
- NEFs help in release of ADP from ATPase binding domain, a rate limiting step in HSP70 cycle. Three classes of NEFs were identified as GrpE, BAG1(Bcl2 associated athanogene which binds to anti apoptotic Bcl2 and other proteins including HSP70) and HSP BP (HSP binding protein). NEFs along with HSP70s assist in folding [64].
- HSP110s are distantly related to HSP70s and are composed of similar N-terminal ATPase domain and distinct C-terminal domain and have extended regions between N-terminal and C-terminal domains. HSP110s can act as NEF stimulating ATPase activity and also act as holdase preventing protein aggregation [64].

- HSP90s contain an N-terminal ATP binding domain, a middle region substrate binding domain and a C-terminal dimerization domain. HSP90s have weak ATPase activity and requires HSP70s and HSP40s. HSP90 promotes folding and degradation [66]
- PDI like proteins are characterized by presence of catalytic C-xx-C active site motif. PDI has a twisted U shape with two inactive catalytic sites between two thioredoxin domains that face each other. Members of PDI play a role in disulphide bond formation, folding and ERAD [66].
- Lectin like chaperones majorly recognize glycoproteins and facilitates protein folding and ERAD [66].

Table 1-6: Chaperones, co-chaperones and NEFs found in yeast cytosol and ER. Adapted from [64].

Factors	ER	Cytosol
HSP70	KAR2	SSA1,SSAB, SSZ1
HSP40	JEM1, SCJ1, SEC63	HLJ1, YDJ1, SIS1
NEFs	LHS1,SSE	FES1, SNL1
HSP90		HSP90, CLPB
HSP110	LHS1	SSE1, SSE2
Thioloxidoreductases	PDI, EPS1, MPD1, MPD2	
Lectin like	MNS1/HTM1,YOS9	

1.4.2 Unfolded protein response (UPR)

The unfolded protein response (UPR) is an intracellular signaling pathway to counteract and relieve from accumulated misfolded proteins and maintain homeostasis in ER by increasing the transcription of genes expressing ER resident chaperones [63]. Three genes *HAC1*, *IRE1* and *RLG1* were identified in *S. cerevisiae* which act as major UPR transcription factors. A single 22 bp element unfolded protein response (UPRE) element which is conserved among UPR targets is necessary for activation of ER resident proteins upon accumulation of misfolded proteins. *HAC1* mRNA is constitutively expressed, but HAC1 protein is expressed in abundance only under the accumulation of misfolded proteins. A prerequisite for translation of HAC1p involves mRNA splicing. The *IRE1* gene is required for inositol prototrophy and encodes for IRE1 protein with amino-terminal signal sequence and single transmembrane domain and serine/threonine kinase and ribonuclease activity at carboxy terminus. Kinase activity is needed for activation by trans-autophosphorylation and on

activation IRE1p cleaves the *HAC1* mRNA at both splice junctions and released exons are joined by tRNA ligase encoded by RLG1. Once HAC1p is produced in abundance, it causes transcriptional upregulation of UPR targets genes (KAR2, PDI) to cope up with accumulated misfolded intermediates [63].

1.4.3 Endoplasmic reticulum associated degradation (ERAD)

The mechanism of ERAD involves majorly aberrant proteins recognition (by chaperones), translocation to cytosol (SEC61), ubiquitinlyation (ubiquitin activation (E1), conjugation (E2) and ligation (E3 andE4) and degradation via proteasome (CDC48 and 19s subunit) [67] (Figure 1-9). This has been well defined in *S. cerevisiae* and homologues of proteins involved in ERAD were found in *P. pastoris* so we expect an identical role of degradation of misfolded proteins [68, 69].Two major pathways of ERAD are identified based on the defects in domains of misfolded proteins ERAD-C (cytosolic domain) and ERAD-L (lumen domain). Both pathways include different subsets of chaperones and different ubiquitin ligase complexes. The ERAD-L pathway includes chaperones KAR2, PDI, SEC63p, LHS1, calnexin, HTM1 and ubiquitin ligase HRD1 complex composed of Yos9 and Hrd3p. The ERAD-C pathway includes chaperones SSA, YDJ1, SSE, HSP104 and HSP90 and ubiquitin ligase complex DOA10. In both pathways the ligases are conjugated with ubiquitin conjugate enzymes and recruited to membrane by CUE1p and converge at CDC48 ATPase [67].

After translation polypeptide gets associated with KAR2 along with its HSP40 cofactors JEM1 and SCJ1 are translocated to the ER where it is glycosylated at amino-terminus Asp/X/Ser/Thr with sequon $Glucose_3$-$Mannose_9$-N acetylglcuosamine$_2$ (Glc_3-Man_9-$GlcNAc_2$) and disulphide bonds are formed. The two outermost glucose moieties are removed by α-glucosidases present in ER. Calnexin and calrecticulin interact with sequon Glc_1-Man_9-$GlcNAc_2$ and cause protein maturation. On release from the glycan, α-glucosidase removes the terminal glucose moiety. The mature forms are translocated to Golgi for further delivery to their final destination. Nevertheless, immature proteins are recognized by folding sensor UDP-glucose/glucosyl transferase (UGGT) which reglucosylates the glycan and initiates folding cycle by association with calnexin and calreticulin. The calnexin/calreticulin cycle serves as first quality control mechanism in ER. If the protein doesn't get folded by repeated calnexin/calreticulin cycles then the protein is targeted by α-mannosidase (MNS1) and HTM1 which removes two mannose residues from Man_9-$GlcNAc_2$. HTM1 is known to interact with

PDI reduced protein substrate. In case of lumenal misfolded domains the formed Man_7-$GlcNAc_2$ is recruited by KAR2 to HRD ligases HRD1 composed of Yos9 and Hrd3. Yos9 scans for α1,6 terminal linkages and interacts with Hrd3, which binds at misguided domains on the substrate. HRD1 forms a complex with substrate bound Hrd3 and together with ubiquitin conjugate enzyme E2 (UBC7) gets attached to the ER membrane and causes ubiquitinlyation of substrate. In case of cytosolic defects the misfolded domains are recruited to DOA10 together with UBC7 and CUE1 which ubiquitinylate the substrate. Both HRD1 and DOA10 ubiquitinlyated substrates are converged at CDC48. UFD2, CDC48, 19s subunit proteins RPT5 and RPN10 and proteins associated with 19s RP RAD23, DSK2 associate with ubiquitinlyated proteins and target them to the proteasomal core where the proteins are degraded into peptides [67, 70].

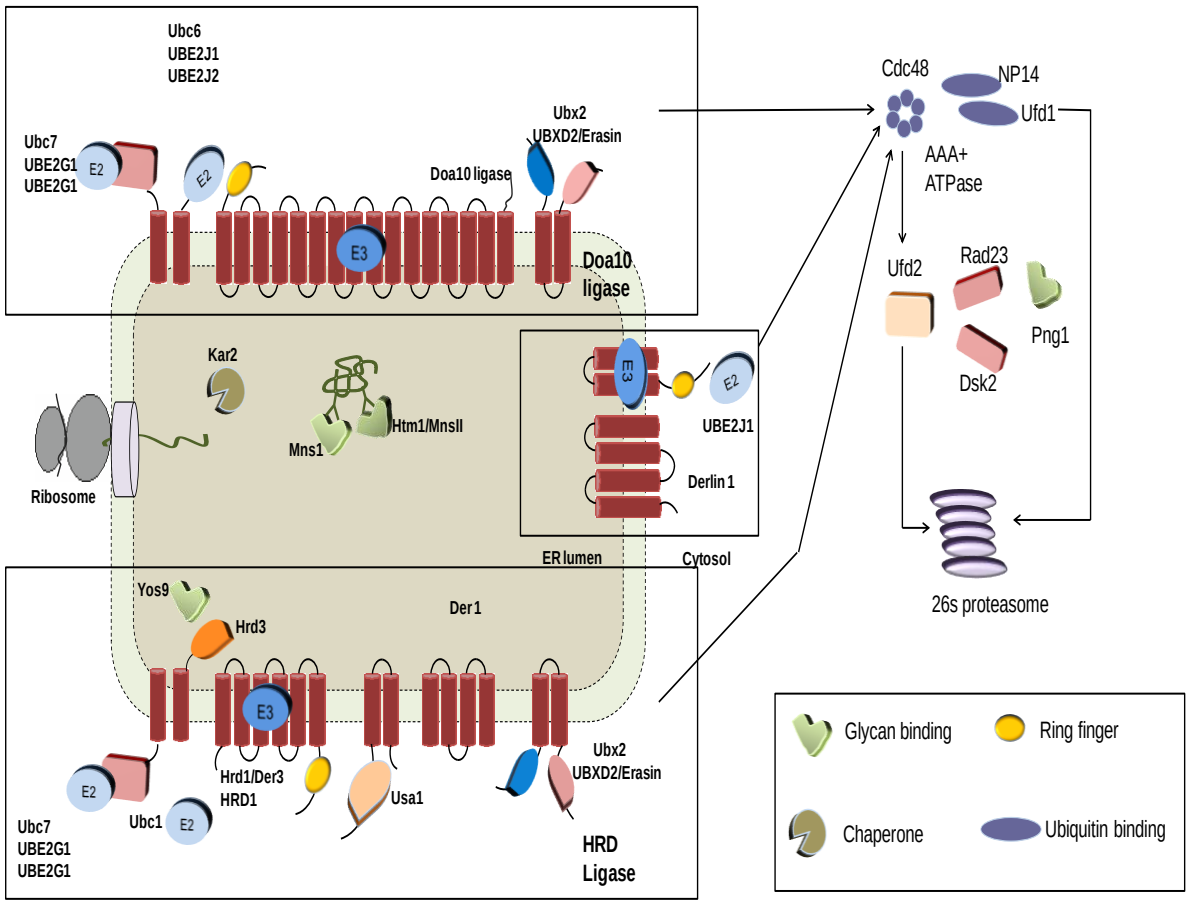


Figure 1-9: Endoplasmic reticulum associated protein degradation mechanism in yeast. Adapted with permission from authors of reference [70].

Studies on UPR targets suggest intimate harmony between ERAD and UPR. On accumulation of misfolded proteins in ER, cells would exhibit fix/ clear mechanism i.e. enhancement of misfolded proteins in ER causes activation of UPR which results in activation and increased synthesis of ER resident chaperones and cytosolic chaperones which aid in folding and retrotranslocation of authentic conformation attained molecules to target sites and in case the chaperones fail to fold, proteins are retrotranslocated to cytosol and eliminated via ERAD [63].

2. AIM AND OBJECTIVES

Dengue is a globally spread arthropod borne infection caused by any of the four serotypes DENV-1,-2,-3 and -4 [3]. Currently there are no licensed vaccine or therapeutics available. Although, live attenuated vaccines have reached advanced clinical trials concerns over safety issues has drifted interest towards subunit vaccines. As a result envelope domain III (EDIII) has come into focus as potential subunit vaccine candidate. EDIII is endowed with host cell receptor recognition, serotype specificity and capacity to elicit neutralizing antibodies [1]. The present work is focused on the development of the dengue EDIII based vaccine candidate.

In order to further annex the immunogenic potential of EDIII domain, it can be presented on surface of virus-like particle. The success of Hepatitis B surface antigen (HBsAg) VLPs as a carrier for presentation of malarial epitopes reaching advanced clinical phase [4] has impelled interest on application of HBsAg as carrier for display of foreign epitopes. In this study HBsAg is used as a modular display system to present DENV-2EDIII on its surface. Dengue being endemics of mostly developing or economically poor countries vaccine needs to be produced at affordable prices. *Pichia pastoris* is one such system which offers great advantages in vaccine production owing to its growth in relatively inexpensive media, high expression levels and proteins produced are free of toxins and considered to be safe for human use [7]. Often, viral protein production in yeast is not as efficient as in mammalian cells, resulting in the formation of insoluble aggregates.

The major objective of the work presented here was aimed to explore the feasibility and potential of dengue EDIII on the surface of VLPs as a vaccine candidate using the eukaryotic expression system *Pichia pastoris.* The first part of the work was to generate *P. pastoris* based DENV-2 EDIII based chimeric VLPs using HBsAg as modular display system and immunogenic evaluation. The second part of the work was focused on possible bottlenecks during expression of the fusion protein in *P. pastoris* with insights into the unfolded protein response (UPR) and endoplasmic reticulum associated degradation (ERAD) using a proteomic approach.

The specific aims of this study were as follows

- Heterologous co-expression of 0,1 and 4 copies of unfused HBsAg (S) and EDIII-HBsAg (ES) denoted as $ES_1:S_0$, $ES_1:S_1$ and $ES_1:S_4$ in *P. pastoris* by methanol based-induction.
- Heterologous expression of 8 copies only unfused HBsAg denoted as $ES_0:S_8$ in *P. pastoris* as control.
- Purification of $ES_1:S_0$, $ES_1:S_1$ and $ES_1:S_4$ from *P. pastoris*
- Electron microscopic evaluation of purified $ES_1:S_0$, $ES_1:S_1$ and $ES_1:S_4$
- Stability evaluation of $ES_1:S_4$
- Immunological evaluation of $ES_1:S_4$ in mice.
- Proteome analysis of cell samples from before and after induction with methanol of non producing strain and cells expressing $ES_1:S_0$, $ES_1:S_4$ and $ES_0:S_8$ using 2D gel electrophoresis combined with MALDI TOF

3. MATERIALS

3.1 Chemicals, media components, kits and other consumables

Chemicals used in the present work and their sources are listed in Table 3-1. Particle specific quantitative Hepanostika micro ELISA kit was obtained from Biomerieux, France. Pierce BCA (bicinchoninic acid) protein assay kit was obtained from Thermo Fisher Scientific, Germany. Unstained and pre-stained protein ladders were obtained from Thermo Fisher scientific, Germany. A butyl sepharose column for hydrophobic interaction chromatography was procured from Sigma-Aldrich GmbH, Germany. DEAE Sepharose (anion exchange columns) and Hi-prep^{TM} Sephacryl S-300 (26/60) and Sephacryl S-300 (16/60) (size exclusion columns) were obtained from GE Healthcare, Sweden. 3µM SUPELCOSIl^{TM} LC-DB-18 column (3.3 cm x 4.6 mm) for reverse phase high performance liquid chromatography (RP-HPLC) was purchased from Sigma-Aldrich GmbH, Germany. Glass beads (0.45-0.6 mm) were obtained from Sigma-Aldrich, Germany. Immobiline dry strip gels (IPG) pH 3-10 NL, 18 cm length and Immobilized pH gradient (IPG) buffer were procured from GE Healthcare, United Kingdom. Dialysis membrane with molecular weight cut off 6-8 kDa was purchased from Sigma-Aldrich, Germany.

3.2 Strains

Wild-type (wt) GS115 cells (Invitrogen, USA) and engineered GS115 cells harboring chimeric constructs EDIII-HBsAg$_1$: HBsAg$_0$ (ES$_1$:S$_0$), EDIII-HBsAg$_1$: HBsAg$_1$ (ES$_1$:S1), EDIII-HBsAg$_1$: HBsAg$_4$ (ES$_1$:S$_4$) and EDIII-HBsAg$_0$: HBsAg$_8$ (ES$_0$:S$_8$) used in the present work were a kind gift from Dr. Navin Khanna, ICGEB, India The subscript denotes copy number of genes in the chimeric construct. The cloning details have been described in [3].

3.3 Antibodies and immunochemicals

Anti-dengue envelope domain III (24A12) specific [3] and anti hepatitis B surface antigen (5S mAb) specific mouse monoclonals [71] were a kind gift from Dr. Navin Khanna, ICGEB India. Anti HDEL (2E7) and anti ubiquitin antibody (P4D1) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology, USA. Goat anti-mouse IgG specific peroxidase conjugate was obtained from Calbiochem, Germany. Immune module polystyrene strips for development of in house ELISA were procured from Thermo Fisher Scientific, Germany. Soluble TMB substrate for ELISA and insoluble TMB substrate for Western blot detection was obtained from Sigma-Aldrich GmbH, Germany. PVDF membrane for Western blotting was obtained from Bio-Rad GmbH, Germany.

Table 3-1: List of reagents used in the present work

Chemical	Manufacturer
Agarose (DNA grade)	Biozym Scientific GmbH, Germany
Acetic acid	Carl Roth GmbH + Co.KG, Germany
Acetonitrile	Carl Roth GmbH + Co.KG, Germany
Acrylamide and bisacrylamide stock solution [Rotiphorese Gel 30 (37:5:1)]	Carl Roth GmbH + Co.KG, Germany
Ammonium hydrogen carbonate	Merck KgaA, Germany
Ammonium persulfate	Sigma-Aldrich GmbH, Germany
Ammonium sulfate	Carl Roth GmbH + Co.KG, Germany
Bromophenol blue	Sigma-Aldrich GmbH, Germany
Bacto Agar	Becton, Dickinson and Company, USA
Bacto Peptone	Becton, Dickinson and Company, USA
D-Biotin	Sigma-Aldrich GmbH, Germany
Bovine serum albumin	Bio-Rad, Germany
D-Biotin	Sigma-Aldrich GmbH, Germany
CHAPS 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate	Biomol GmbH, Germany
Coomassie brilliant blue G-250	Merck KGaA, Germany
Calcium chloride dihydrate	Merck KGaA, Germany
Dithiothreitol (DTT)	Sigma-Aldrich GmbH, Germany
Dextrose	Merck KGaA, Germany
Ethanol	Merck KGaA, Germany
Ethylenedimainetetracetate disodium salt	Carl Roth GmbH + Co.KG, Germany
Formic acid	J.T.Baker Chemical company, USA
Formaldehyde	Sigma-Aldrich GmbH, Germany
Glycerol (86 %)	Carl Roth GmbH + Co.KG, Germany

Glycine	Carl Roth GmbH + Co.KG., Germany
Histidine	Sigma-Aldrich GmbH, Germany
Iodoacetamide	GE Healthcare, UK
Luria Bertani (LB) medium	Difco, Becton, Dickinson Company, USA
2-Mercaptoethanol	Sigma-Aldrich GmbH, Germany
Methanol	Carl Roth GmbH + Co.KG, Germany
2-Propanol	Carl Roth GmbH + Co.KG, Germany
Polvinylpyrrolidone	Sigma-Aldrich GmbH, Germany
Dipotassium hydrogen phosphate	Carl Roth GmbH + Co.KG, Germany
Potassium dihydrogen phosphate	Carl Roth GmbH + Co.KG, Germany
Polyethylene glycol (PEG-6000)	Sigma-Aldrich GmbH, Germany
Potassium chloride	Sigma-Aldrich GmbH, Germany
Potassium thiocyanate	Sigma-Aldrich GmbH, Germany
Sodium bicarbonate	Sigma-Aldrich GmbH, Germany
Sodium carbonate	Sigma-Aldrich GmbH, Germany
Sodium chloride	Sigma-Aldrich GmbH, Germany
Sodium dihydrogen phosphate	Sigma-Aldrich GmbH, Germany
Disodium hydrogen phosphate	Sigma-Aldrich GmbH, Germany
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories GmbH, Germany
Silicone M 100	Carl Roth GmbH + Co.KG, Germany
Sulfuric acid	Carl Roth GmbH + Co.KG, Germany
Sodium hydroxide	Sigma-Aldrich GmbH, Germany
TEMED N,N,N’,N’ tetramethylethylenediamine	Sigma-Aldrich GmbH, Germany
Trifluoroacetic acid (TFA)	Sigma-Aldrich GmbH, Germany
Thiourea	Sigma-Aldrich GmbH, Germany
Tris base	Carl Roth GmbH + Co.KG, Germany
Tween 20	Sigma-Aldrich GmbH, Germany
Trypsin	Promega Corporation, USA
Yeast extract	Bio-Rad Laboratories GmbH, Germany
Yeast nitrogen base without amino acids and ammonium sulfate	Becton, Dickinson and Company, USA
Urea	Sigma-Aldrich GmbH, Germany

4. METHODS

4.1 Shake flask cultivation

A starter culture was set up by inoculating YPD (100 ml) with glycerol stock cultures (100 µl) and grown at 28°C, under constant shaking in an orbital shaker at 250 rpm for 18 h to an OD_{600} 10. About 1 % of starter culture was used to inoculate 500 ml of buffered glycerol complex medium (BMGY) in a 2 L baffled shake flask. The culture was incubated at 28°C, under constant shaking in an orbital shaker at 250 rpm until an OD_{600} ~20 is reached. Subsequently, cells were pelleted by centrifugation (3347 × *g*), washed with sterile PBS, re-centrifuged and re-suspended in buffered methanol complex medium (BMMY) to an OD_{600} ~100 and incubated at 28°C under shaking at 250 rpm. Recombinant protein production was induced through the addition of 1 % (v/v) methanol twice a day at 12 h intervals for a total period of 72 h. Finally, cells were harvested by centrifugation, washed with PBS, and the cells were stored at -80°C until use.

4.2 Preparation of total cell extracts

Samples analogous to OD_{600} ~100 (1 ml) were transferred to fresh 1.5 ml microcentrifuge tubes (Eppendorf, Germany) and pelleted by centrifugation at 16,000 x *g* for 10 min and washed with PBS pH 7.2. The washed pellet was mixed with 0.5 ml of lysis buffer (25 mM phosphate buffer pH 8.0, 5 mM EDTA, 8 % (v/v) glycerol and 500 mM NaCl) and 0.6 g glass beads. The cells were disrupted using glass beads in thermomixer (Eppendorf, Germany) at 1400 rpm and 4°C for 12 h. After lysis, lysate was transferred to fresh tubes and beads were washed with 0.5 ml of lysis buffer and pooled with early lysates.

4.3 Preparation of soluble and insoluble fractions

100 µL of lysates were solubilized by adding 1 % (v/v) Tween 20 at final concentration and kept for 12 h at 4°C in a thermomixer at 1400 rpm. The solubilized lysates were centrifuged at 16,000 x *g* and resultant supernatant and pellet fractions were separated. The pellet fraction was further solubilized with 100 µL buffer containing urea (25 mm PB pH 8.0, 135 mm NaCl and 8 M urea) for 4 h in thermomixer at room temperature.

4.4 Purification of $ES_1:S_4$

$ES_1:S_4$ was purified by the approach employed for purification of HBsAg in [72] with some modifications. 100 g wet cell weight (WCW) from 1L culture broth OD_{600} ~100 was used for purification. The cells were suspended in 500 ml of lysis buffer (25 mM phosphate buffer pH 8.0, 5 mM EDTA and1 % (v/v) Tween 20) and made into a uniform suspension and disrupted with microfluidizer (Microfludics/ USA) connected to the chiller unit at 12,000 PSI and 4°C by passing for 15 cycles. NaCl was added slowly to a final concentration of 0.5 M to the lysate, followed by addition of PEG 6000 at a final concentration of 5 % (v/v) under constant stirring at 4°C. Both NaCl and PEG 6000 were added in three intervals within a period of thirty minutes. After addition of PEG 6000, lysate was stirred for 2 h and left overnight for precipitation. Next morning, PEG lysate was centrifuged at 3347 × *g* for 1 h and the supernatant was collected and equilibrated for 4 h at 4°C with Aerosil-380 (Evonik Corporation, Germany), pre-washed and pre-equilibrated with 25 mM PB pH 7.2, 500 mM NaCl, (0.13 g of dry Aerosil 380 per 1 g of wet biomass) for binding. The protein adsorbed Aerosil was washed thrice with 300 ml of 25 mM PB pH 7.2 to remove unbound or weakly bound proteins. The desorption of the fusion protein was done by suspending the Aerosil pellet in 100 ml of desorption buffer (50 mM Na_2CO_3/$NaHCO_3$ pH 10.6, 1.2 M urea) and incubated at 37°C under shaking at 100 rpm for 12 h followed by centrifugation at 16,000 × *g* for 1 h. The eluant was collected and filtered with 0.2 µM filter and dialyzed against 1L of 50 mM Tris-HCl pH 8.5. The binding of dialyzed retentate to 30 ml of DEAE Sepharose Fast flow (GE healthcare, Sweden) pre equilibrated with 50 mm Tris HCl, pH 8.5 was carried out as a batch process for 3h at 4°C. The bound resin was then packed into a 50 ml Bio-Rad column, connected to a FPLC instrument and program based washing and elution were carried out. The resin was then washed with 250 ml of 50 mM Tris-HCl, pH 8.5 and eluted

with 100 ml of 50 mM Tris-HCl pH 8.5, 500 mM NaCl. The peak fractions were collected using a fraction collector. The DEAE elutes were concentrated to 6 ml with 100 kDa cut off membrane and subjected to gel filtration with Sephacryl S-300 (26/60 Hi- prep^{TM} GE Health care, Sweden) pre-packed column with a column volume of 320 ml and void volume ~110 ml. About 6 ml of sample was loaded onto the column and the elution was carried out using PBS (10 mM KH_2PO_4, 2 mM Na_2HPO_4 pH 7.2, 2.7 mM KCl and 135mM NaCl) at a flow rate of 1ml min^{-1}. The peak fraction within the void volume were collected and analyzed by SDS PAGE and Western blotting. Positive peak fractions were pooled and treated with 1.5 M KSCN at 4°C for 16-18 h. KSCN was removed by dialysis against PBS. Only unfused HBsAg (ES_0:S_8) was purified as described in [72] (Appendix II Figure 10-1). The purification of ES_1:S_1 was carried out by the same way as described above (Appendix II Figure 10-2). However, ES_1:S_0 was purified from membrane fraction using chaotropic agents.

4.5 Purification of ES_1:S_0

Owing to the lower solubility of ES_1:S_0 as confirmed from ELISA and Western blotting, purification was carried out following a membrane extraction protocol using chaotropic agents, as described by [71] with modifications. 50 g wet cell weight from 500 ml culture broth OD_{600} ~100 was dissolved in 300 ml of lysis buffer containing (25 mM PB pH 8.0, 5 mM EDTA, 5 % (v/v) glycerol and 500 mM NaCl), made into a uniform suspension and lysed by passing through the microfluidizer (Microfludics/ USA) connected to the chiller unit at 12,000 psi and 4°C for 15 cycles. Next, the lysate was centrifuged at 16,000 × *g* for 30 min and the supernatant was discarded. The pellet was washed twice with lysis buffer and dissolved in 100 ml of 25 mM PB pH 7.5, 5 mM EDTA, 150 mM NaCl, 4 M urea and 2 % (v/v) Tween 20 and solubilized at RT for 4 h. Later, the urea solubilzed pellet was centrifuged at 16,000 × *g* for 30 min and the supernatant was mixed with equal volume of 25 mM PB pH 8.0, 5 mM EDTA, 150 mM NaCl, followed by the slow addition of PEG 6000 at a concentration of 5 % (v/v) under constant stirring at 4°C within 30 min. The suspension was stirred for 2 h and then left overnight for PEG precipitation. The precipitate was removed by centrifugation and the supernatant was collected and diafiltered using a 30 kDa MWCO membrane (Sartorius, Germany) against 25 mM PB pH 7.5, 5 mM EDTA and 150 mM NaCl. 5L of this buffer was used to diafilter 200 ml of PEG precipitated supernatant. The concentration of NaCl in the diafiltered retentate was increased to 2.5 M. The retentate was

bound to 25 ml pre-packed butyl sepharose column pre-equilibrated with 25 mM PB pH 7.5, 2.5 M NaCl interfaced to FPLC. The binding was performed by passing the retentate twice through the column connected to FPLC. After binding, the column was washed with 250 ml of 25 mM PB, pH 7.5 followed by washing with 150 ml of 25 mM sodium carbonate/bicarbonate buffer, pH 9.6 and elution with 75 ml of 25 mM sodium carbonate/bicarbonate buffer pH 9.6, 8 M urea. All binding, washing, and elution steps were essentially carried out using FPLC. Butyl Sepharose elute fractions were pooled and dialyzed against 250 ml of 25 mM sodium carbonate/bicarbonate buffer, pH 9.6 to remove urea and then dialyzed against 25 mM PB, pH 8.0. Dialysis was carried out at 4°C with three changes. The dialyzed protein was concentrated with 30 kDa membrane and subjected to gel filtration with Sephacryl S-300 (16/60 Hi- prep^{TM} GE Healthcare, Sweden) pre-packed column with a column volume of 120 ml and void volume of ~40 ml. About 2.5 ml of sample was loaded into the column and the elution was carried out using PBS, pH 8.0 at a flow rate of 1ml min^{-1}. The peak fraction within the void volume were collected and analyzed by SDS-PAGE and Western blotting. Purified peak fractions containing protein were pooled and treated with1.5 M KSCN at 4°C for 16 h. The KSCN was removed by dialysis against PBS, pH 8.0.

4.6 Analytical methods

4.6.1 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were casted and run in the presence of the SDS (SDS-PAGE). The formulation of stacking and separation gels mixture for 12 % SDS-PAGE is given in Table 4-1. The 2X stringent loading dye was used for solubilzing the fractions to be analyzed. The composition of the stringent loading dye (Laemmli buffer) includes 125 mM Tris HCl pH 6.8, 30 % (v/v) glycerol, 500 mM DTT, 7.24 M β-mercaptoethanol, 8 % (w/v) SDS and 0.005 % bromophenol blue. Equal volumes of loading dye were added to the protein samples, kept at 95°C for 15 min, centrifuged and the supernatant was loaded onto the gels. Gels were run at constant voltage of 100V and were stained with Coomasssie brilliant blue/Silver staining or subjected to Western blotting.

Table 4-1: Composition of resolving (12 % acrylamide) and stacking (6 % acrylamide) gels.

Components	Resolving gel (ml)	Stacking gel (ml)
30 % Acrylamide	4	0.75
Resolving buffer 1.5M Tris HCl pH 8.8	2.8	-
Stacking buffer 1.5M Tris HCl pH 6.8	-	0.63
1 % SDS	1	1
Water	2.2	3.77
25 % APS	0.02	0.01
TEMED	0.02	0.01
Total	10	5.5

4.6.2 Silver staining

Gels were washed with Milli-Q water thrice for 5 min each and immersed in a fixative solution (30 % (v/v) ethanol, 10 % (v/v) acetic acid) for 30 min, followed by addition of sensitizer solution, (Farmer's reducer: 15 mM potassium ferricyanide and 50 mM sodium thiosulfate) and incubated for 3 min with gentle shaking. Later gels were washed thrice (5 min each) with water and stained with silver nitrate solution at a concentration of 1 mg ml^{-1} for 30 min. Gels were then rinsed with 235 mM sodium carbonate solution for a short time and developed by adding 50 ml developer solution (235 mM sodium carbonate and 0.4 % (v/v) formaldehyde) to the gels. As soon as bands were visualized, developer solution was drained out and 5 % (v/v) acetic acid was added to arrest development and the gels gently shaken for 10 min. Finally, gels were washed with water and scanned. All steps were essentially carried out in an orbital shaker at a speed of 50 rpm.

4.6.3 Western blotting

The EDIII-HBsAg was detected by Western blotting using anti-Hepatitis B surface antigen specific (5S mAb) and anti-dengue envelope domain III (24A12) specific mouse monoclonal antibodies while HBsAg was detected only with 5S mAb. Proteins separated on SDS-PAGE were electrotransferred using Bio-Rad Semi-dry Western blot apparatus onto polyvinylidene difluoride (PVDF) membrane. Prior to transfer PVDF membrane was soaked in methanol for a short time and then soaked in transfer buffer (25 mM Tris base, 200 mM glycine and 20 % (v/v) methanol along with thick filter pads for 5 min. The transfer was carried out at constant voltage of 15 V for 50 min at room temperature. After transfer, the membrane was removed and incubated in blocking solution (5 % (w/v) skimmed milk, 2 % (w/v) polyvinylpyrrolidone, 1 % (v/v) Tween 20 in PBS pH 7.2) for 2 h on a shaker at room temperature. Subsequently, the membrane was washed thrice with PBS-T (PBS containing 1 % (v/v) Tween 20) and incubated with mouse monoclonal primary antibody (1 µg ml^{-1}) for 1h on a shaker at room temperature. After incubation blots were washed thrice with PBS-T and incubated with secondary antibody (0.1µg ml^{-1} goat anti-mouse IgG H and L chain specific peroxidase conjugate) for 1 h in a shaker at room temperature. Thereafter blots were again washed thrice with PBS-T and developed with TMB substrate until the bands were clearly

visualized. All steps in immunoblotting were carried out on an orbital shaker. Each wash step was carried out for 5 minutes.

4.6.4 Protein quantification

Lysate and soluble fraction were prepared as described previously. The total protein in lysate and soluble fraction in all the chimeric constructs was measured by Pierce^{TM} BCA (bicinchoninic acid) protein assay kit with bovine serum albumin (BSA) as standard (BCA Standard curve, Appendix III Figure 11-3). The total protein was quantified identically as described in protocol provided with kit.
(https://www.piercenet.com/instructions/2161296.pdf).

4.6.5 Quantification by RP-HPLC

The quantification by RP-HPLC was performed as mentioned in [61]. The ES and S antigen in the total lysate were quantified by RP-HPLC using 3 µM SUPELCOSIL TM LC-DB-18 column (3.3 cm x 4.6 mm). For RP-HPLC, a 100 µL aliquot of lysate was suspended in an equal volume of solubilization reagent (8 % (w/v) SDS; 50 % (v/v) β- mercaptoethanol and 1 M DTT) boiled for 15 min and clarified by centrifugation and filtration. A 50 µL of clarified sample was injected into the column maintained at 70°C in HPLC column oven, at a flow rate of 1ml min^{-1} using degassed buffer A [0.15 % (v/v) trifluoroacetic acid (TFA) in Milli-Q water] and buffer B [0.12 % (v/v) TFA, 80 % (v/v) 2-propanol: 20 % (v/v) acetonitrile] to impel a gradient in which buffer B percentage changes with time as follows: 0-5 min, 45 %; 5-20 min, 45-95 %; 20-25 min, 95 %; 25-30 min, 95-45 %; 30-35 min 45 %. The entire process was carried out with VWR Hitachi Chromaster equipped with autosampler, column oven, diode array detector and four isocratic pumps. All samples were analyzed at 214 nm. In-house purified HBsAg ranging in a concentration range of 1-4 mg ml^{-1} was processed in parallel (HPLC calibration curve, Appendix III Figure 11-1). The concentration of standard HBsAg was measured using a BCA protein assay kit.

4.6.6 Hepanostika HBsAg ELISA

Immunoassay was performed in accordance with manufacturer instructions (http://www.biomerieux.fr/upload/Hepanostika_HBsAg_Ultra_Poster_2005_ISBT_BKK.pdf) .The Tween 20 solubilzed fractions were diluted appropriately in an assay diluent containing 0.1 % (w/v) BSA in PBS (10 mM KH_2PO_4, 2 mM Na_2HPO_4 pH 7.2, 2.7 mM KCl and 135mM NaCl). 25 µL of sera diluent provided in kit were added to the wells pre-coated with anti-HBsAg antibody, followed by the addition of 100 µL of diluted sample and incubated at 37°C for 1h. 50 µL of anti-HBsAg conjugate were then added and further incubated at 37°C for 1h. Subsequent steps include five washes with wash buffer provided with the kit and the addition of 100 µL of substrate composed of urea peroxide (UP) and tetramethylbenzidine (TMB) in a ratio of 1:1 to the wells and incubated at RT in the dark for 30 min. The reaction was stopped by addition of 0.5 M H_2SO_4 and absorbance measured at 450 nm. In-house purified HBsAg quantified by BCA protein assay kit in the range of 1-100 ng ml^{-1} was used as standard (ELISA calibration curve, Appendix III Figure 11-2). Induction interval samples from GS115 wild-type strain and uninduced sample from chimeric strains were used as negative controls.

4.7 Electron microscopy

The purified recombinant proteins were visualized by electron microscopy. Electron microscopy examination was performed as described previously [61]. Purified proteins were diluted in PBS, pH 7.2 at a concentration of 50 µg ml^{-1}, adsorbed for 2 min onto a glow-discharged C-Formvar foil and negatively stained with 2 % (w/v) uranyl acetate. An energy filtered transmission electron microscope Libra 120 (Zeiss, Oberkochen, Germany) was used and zero-loss images were acquired with a 2048 x 2048 CCD camera (Troendle, Moorenweis, Germany) using an energy-width of 15eV and an objective aperture of 90 µm.

4.8 Stability evaluation

The stability of purified ES_1S_4 was evaluated by incubating the protein under various conditions of pH, temperature, with and without excipients for 2 weeks. The concentration of the protein was maintained at 0.2 mg mL^{-1} in all the conditions as evaluated by the BCA

protein assay kit. Two weeks later at 37°C and 25°C precipitation was visualized in the samples. The samples were centrifuged and the pellet was solubilized in $1/10^{th}$ volume of the supernatant in PBS, pH 7.2. Day zero and day fourteen supernatant and pellet samples were analyzed by Western blotting with anti hepatitis B surface antigen (5S mAb) and anti dengue envelope domain III (24A12 mAb) specific mouse monoclonals.

4.9 Identification of KAR2 and PDI (UPR markers)

5 µL of whole cell lysate from before and after 72 h of methanol induction from chimeric constructs and wild-type GS115 were applied to reducing SDS PAGE and electroblotted onto PVDF membrane at 15 V for 50 min. The UPR response in all the chimeric constructs and wild-type strains at different induction intervals was assessed by presence of UPR markers which contains HDEL ER resident sequence at carboxy terminus with anti-HDEL (2E7) mouse monoclonal antibody at a concentration of 0.2 µg ml^{-1} in conjunction with 0.1µg ml^{-1}goat anti-mouse IgG HRP conjugate using TMB substrate for detection.

4.10 Detection of global change in endogenous ubiquitylation

5 µL of whole cell lysate from before and after 72 h of methanol induction from chimeric constructs and wild-type GS115 were applied to reducing SDS PAGE and electroblotted onto PVDF membrane at 15 V for 50 min. Endogenous ubiquitylation in all the chimeric constructs and wild-type strains at different induction intervals were identified with anti ubiquitin (P4D1) mouse monoclonal antibody at a concentration of 0.2 µg ml^{-1}in conjunction with 0.1 µg ml^{-1} goat anti-mouse IgG HRP conjugate using TMB substrate for detection.

4.11 Two-dimensional gel electrophoresis

A 2D gel electrophoresis of GS115 strain expressing ES_1:S_0, ES_1:S_4 and ES_0:S_8 and non producing strain GS115 were carried out with the aim to identify a regulation pattern of chaperones involved in stress response, folding and degradation during expression of these proteins. Samples from supernatant fraction of centrifuged lysate before and after 72 h of methanol induction from wild-type GS115, recombinant GS115 producing ES_1: S_0, ES_1: S_4 and ES_0:S_8 were analyzed by two-dimensional gel electrophoresis. All samples were run in

triplicates. All steps involved in two-dimensional gel electrophoresis were carried out as described previously [69].

4.11.1 Sample preparation

Cell pellets corresponding to OD_{600} ~100 (1 ml) stored in -80°C from shake flask cultivation of all chimeric constructs (ES_1:S_0, ES_1:ES_4, ES_0:S_8 and wild-type GS115) were thawed and washed by suspending the pellet in 1ml of PBS pH, 7.2 and centrifuged at 16,000 x *g* for 10 min. The supernatant was discarded and the pellet was dissolved in 500 µL of lysis buffer (25 mM PB pH 8.0, 5 mM EDTA, 8 % (v/v) glycerol and 500 mM NaCl). To the above suspension 0.6 g of glass beads were added and cells were disrupted in thermomixer overnight at 4°C. The lysate was transferred to a fresh vial and glass beads were washed with 0.5 ml of lysis buffer, pooled with initial lysate. The lysate was centrifuged at 16,000 x *g* for 15 min and supernatant and pellet fraction were separated. The supernatant from all the constructs was analyzed by two-dimensional gel electrophoresis.

4.11.2 Precipitations with chloroform/methanol

The protein concentration in the supernatant from the lysate was quantified by BCA assay. Approximately 300 µg of protein was precipitated with chloroform/methanol to get rid of salts and nucleic acids. All samples were analyzed in triplicates and for each sample one water control was used. An appropriate volume of sample corresponding to 300 µg (or 100 µL waters as a control) was taken in 2 ml microcentrifuge tube and made up to 100 µL with Milli-Q water. 800 µL methanol was added and vortexed for 10 s. Then 200 µL of chloroform was added to the above mix and vortexed for 10 s. Followed by the addition of 600 µL of Milli-Q water, vortexed for 10 s and centrifuging at 16,000 x *g* for 7 min at 4°C. After centrifugation supernatant from the lysate containing tubes was discarded. The pellet was washed with supernatant from water control tubes by gentle shaking at 300 rpm for 1 min. without disturbing the pellet, supernatant was completely drained out and dried under vacufuge (Eppendorf, Germany) for 10 min to remove methanol and chloroform.

4.11.3 Solubilization of pellet

The dried pellet from 300 µg of protein was made into solution by dissolving in 500 µL solubilzation buffer containing (9 M urea; 2 M thiourea, 4 % (w/v) CHAPS; 0.2 % (w/v) SDS; 0.002 % bromophenol blue and 17 mM Tris pH 8.5). To the above solution, 7.5 µL of IPG buffer and 7.5 µL of 1 M dithiothreitol were added and incubated for 4 h at 25°C and 100 rpm in thermomixer to facilitate solubilzation. Amidst 1h intervals, samples were sonicated in a sonicator bath (Sigma-Aldrich, Germany) for 15 min for better solubilzation. The solubilized fractions were stored at -80°C for later use.

4.11.4 First dimension isoelectric focusing (IEF)

Prior to isoelectric focusing, the Ettan IPGphor strip holder (GE Healthcare, UK) was cleaned with 10 % SDS using a toothbrush, rinsed well with Milli-Q water and air dried. Samples were removed from -80°C and thawed in thermomixer at 25°C with constant stirring for 2h at 1000 rpm. DTT was further added to the vials at a final concentration of 30 mM during last 10 min of stirring. Later, samples were centrifuged at 16,000 x *g* and the supernatant was transferred to a fresh tube. Two IEF sample application pieces (GE Healthcare UK) were placed in lateral wells at both ends of immobilized pH gradient (IPG) strip holder and 20 µL of rehydration buffer (9 M urea; 2 M thiourea, 4 % (w/v) CHAPS, 17 mM Tris pH 8.5), (GE Healthcare, UK) were added. 360 µL of sample were added slowly, avoiding bubbles in the center of the strip holder two electrodes. Immobilized pH gradient (IPG) strips pH 3-10 NL (GE Healthcare, UK) were taken out from -20°C ten minutes prior to use and strip number and corresponding sample to be loaded was noted. The protective cover of the IPG strips was removed using forceps by holding at positive end (+) and IPG strip was slided slowly over the sample holder with gel side facing down in bubble free manner and making sure that IPG gels were in contact with the strip holder electrodes. About 3 ml of silicone oil was added on top of the strip, ceramic holder was closed with lid and placed in IPGphor in an orientation where + end of strip faces + end of machine. The IEF program (Table 4-2) was then started.

Table 4-2: Steps entailed in isoelectric focusing

Procedure	Program steps	Voltage (V)	Step duration (h)	Gradient type	Theoretical voltage hours (Vhr)
Rehydration		0	35	Step-n-hold	0
Isoelectric focussing	1	50	4	Step-n-hold	200
	2	100	4	Gradient	400
	3	300	3	Gradient	900
	4	1000	4	Gradient	2600
	5	3500	3	Gradient	6750
	6	5000	3	Gradient	12750
	7	5000	3	Step-n-hold	15000
	8	8000	3	Gradient	19500
End	9	8000	10	Step-n-hold	80000
Temperature: 20°C Current limit for each strip: 30 µA (3-10 NL strip)					

4.11.5 Second dimension SDS –PAGE

Subsequent to IEF, silicone oil in strip holder was drained completely using a pipette and IPG strips were placed with gel side facing upwards individually in channels of Plexiglas tray. The IPG strips were equilibrated by gentle agitation with 4 ml of SDS equilibration buffer (50 mM Tris-HCl pH 8.8; 6 M urea; 30 % (v/v) glycerol and 2 % (w/v) SDS) containing 1 % (w/v) DTT and then with 4 ml of equilibration buffer comprising 5 % (w/v) iodoacetamide for 15 min. Thereafter, the strips were incubated in SDS running buffer before transfer to polyacrylamide gels.

20 L of SDS running buffer (24 mM Tris base, 200 mM glycine, pH 8.3 and 0.1 % (w/v) SDS) was prepared and transferred to electrophoresis tank about 2 h prior to strip transfer to polyacrylamide gels. The pre-casted SDS-PAGE slab gel cassettes stored at 4°C were taken out and washed with Milli-Q water and placed in multi channel SDS-PAGE gel holder. The SDS-PAGE cassettes were filled with SDS running buffer to facilitate easy strip transfer. IPG strips were transferred to the gel cassettes with plastic side of strip against the glass plate with the help of a clean forceps and 5 ml of agarose sealing solution (SDS equilibration buffer; 1 % (w/v) agarose and 0.002 % (v/v) bromophenol blue) was overlaid over the strip in bubble free manner and left for 10 min for solidification. After solidificaiton cassettes were assembled in electrophoresis tank and gels were run at 40 V for 2 h and then increased to 100 V for an overnight run.

4.11.6 Colloidal Coomassie staining

After the run, gels were removed from the plates and rinsed well with Milli-Q water. Soon after gels were fixed for 2 h with fixative solution and stained with Colloidal Coomassie stain overnight. The gels were destained with water till spots were clearly visualized and background was clear. The composition of fixative solution is 10 % (v/v) acetic acid; 30 % (v/v) ethanol) and of Colloidal Coomassie stain includes 10 % (w/v) ammonium sulfate; 8.5 % (v/v) orthophosphoric acid; 0.12 % (w/v) Coomassie brilliant blue G-250 and 20 % (v/v) methanol.

4.11.7 Scanning, image analysis and data surveillance

Destained gels were scanned with (Epson perfection V 750 pro, EPSON, Germany) at 300 dpi resolution in 16 bit grayscale format. The images were analyzed, matched and quantified by Proteomeweaver 3.0 software and data was exported to Microsoft excel. The data analysis was performed as described previously in [73]. The spots were normalized by dividing each spot intensity with total spots intensity and converted to percent. The spot intensity corresponding to each protein from wild type strain and producing strain after 72 h of methanol induction was divided with intensity before methanol induction. Then log2 ratio of induced and uninduced samples of wild type and producing strain were carried out. To, see the difference in regulation pattern of proteins in producing strain in comparison with non-producing strain, log2 ratios from producing strain was substraced with value from wild-type strain. Log2 fold change above 0.6 was considered significant [73].

4.11.8 Identification of spots by MALDI TOF

The spot corresponds to proteins with difference in regulation when compared with wild-type were excised from the gels, washed thrice with Milli-Q water and additional two wash steps alternatively with Milli-Q water and acetonitrile by keeping in thermomixer for 5 mins at 300 rpm. Followed by an additional wash step with water and drying the samples at 30°C. The spots were processed as follows. Firstly proteins were reduced by adding 50 µL of 100 mM ammonium bicarbonate containing 20 mM DTT at 56°C for 30 min and then carbamidomethylated with 50 µL of 100 mM ammonium bicarbonate containing 55 mM iodoacetamide in the dark. Proteins were dehydrated with 200 µL acetonitrile. Later, 200 µL of 100 mM ammonium bicarbonate was added to the spots and incubated for 15 min at RT. The incubation solution was removed, proteins were dehydrated with 200 µL acetonitrile and dried under vacufuge at 30°C for 10 min. Spots containing protein were then digested by adding 30 µL trypsin at the concentration of 20 µg ml^{-1} in 50 mM ammonium bicarbonate for each spot and incubating at 37°C for 14h. The trypsin digested peptides were then eluted with desalting buffer (10 mM ammonium phosphate, monobasic in 0.1 % TFA) and loaded onto the PAC (Prespotted Anchor Chip, Bruker Daltonics GmbH, Germany) target and analyzed using Bruker ultra flex time of flight mass spectrometer MALDI TOF.

Protein identification was performed identically as described in [69]. Briefly, peptide mass fingerprints obtained by the MALDI-TOF MS were processed using FlexAnalysis 2.0 (Bruker Daltonics GmbH, Germany) and used to search the NCBInr database by using Mascot 2-.10 software (http://www.matrixscience.com). The parameters used for searching were as follows: taxonomy: other Fungi, tryptic digestion, modifications were allowed for carbamidomethylation of cysteine (fixed modification) and methionine oxidation. All proteins with MASCOT score greater than 76 are considered as significant with P value < 0.05.

5. RESULTS

5.1 HBsAg as carrier

The ability of HBsAg to self assemble into virus-like particles (VLPs) and to retain this property even on fusion with heterologous domain and exposing them on surface is well documented hence has been used as a tool for the display of epitopes on its surface [4, 36]. Bacteria, although prominently used expression system for recombinant proteins, have not been preferred for the production of HBsAg due to the absence of the post translational modifications as found in eukaryotes [4]. Expression systems such as mammalian cells and insect cell lines can ensure proper folding of proteins, however are very expensive and thus not economically feasible. Methylotropic yeast *P. pastoris* which enables cost effective and large scale production of heterologous proteins has been well documented in production of HBsAg [61].

The major objective of the work presented here has been to evaluate feasibility and potential of DENV-2EDIII exposed on the surface of the HBsAg carrier as a vaccine candidate. In this work EDIII was fused with the amino terminus of HBsAg and co-expressed along with 1 and 4 copies of unfused HBsAg in GS115 *P. pastoris*. Recombinant proteins were purified by conventional chromatographic techniques and evaluated for VLP formation by transmission electron microscopy (TEM).

5.2 Co-expression of DENV-2 EDIII-HBsAg (ES) with 0, 1, 4 copies of HBsAg (S) antigens and only 8 copies of HBsAg (S)

Positive co-transformants for ES (DENV-2 EDIII-HBsAg) and S (HBsAg) genes were analyzed for co-expression upon methanol induction. The induced cells were lysed with glass beads and total lysate was treated with 1 % (v/v) Tween 20 and fractionated into supernatant and pellet fractions by centrifuging at 16,000 x *g* for 10 mins. The presence of the 37 kDa ES and 24 kDa S antigen in soluble and pellet fractions were detected and confirmed by Western blotting with an in house anti HBsAg (5S) specific mouse monoclonal [71] in $ES_1:S_1$, $ES_1:S_4$. Only 24 kDa band was seen in $ES_0:S_8$ and only 37 kDa band was visualized in $ES_1:S_0$ (Figure 5-1 A, B). The presence of the 37 kDa ES was further assured by an in house anti dengue EDIII (24A12) specific mouse monoclonal [3] in $ES_1:S_0$, $ES_1:S_1$ $ES_1:S_4$ (Figure 5-1 C, D). RP-HPLC was performed to quantify the ES and S antigens in lysate of positive transformants. The RP-HPLC data show a linear fold increase in HBsAg protein as in accordance with multimerization of the HBsAg gene (Table 5-1). RP-HPLC profile shows peaks with retention time of 14.8 for HBsAg and 14.4 for ES antigen (Figure 5-1 E). In house purified HBsAg standard was processed in parallel at the concentration ranging from 0-4 mg ml^{-1}. HBsAg peak was seen in $ES_1:S_1$, $ES_1:S_4$ and $ES_0:S_8$ and not in $ES_1:S_0$, whereas ES antigen peak was seen in $ES_1:S_0$, $ES_1:S_1$ and $ES_1:S_4$ and not in $ES_0:S_8$. Hepanostika HBsAg ELISA of the soluble fraction in positive transformants also confirmed HBsAg and ES antigen (Table 4-1). RP-HPLC and ELISA results suggest $ES_1:S_4$ to be the best clone among all the chimeric constructs based on their yield and solubility.

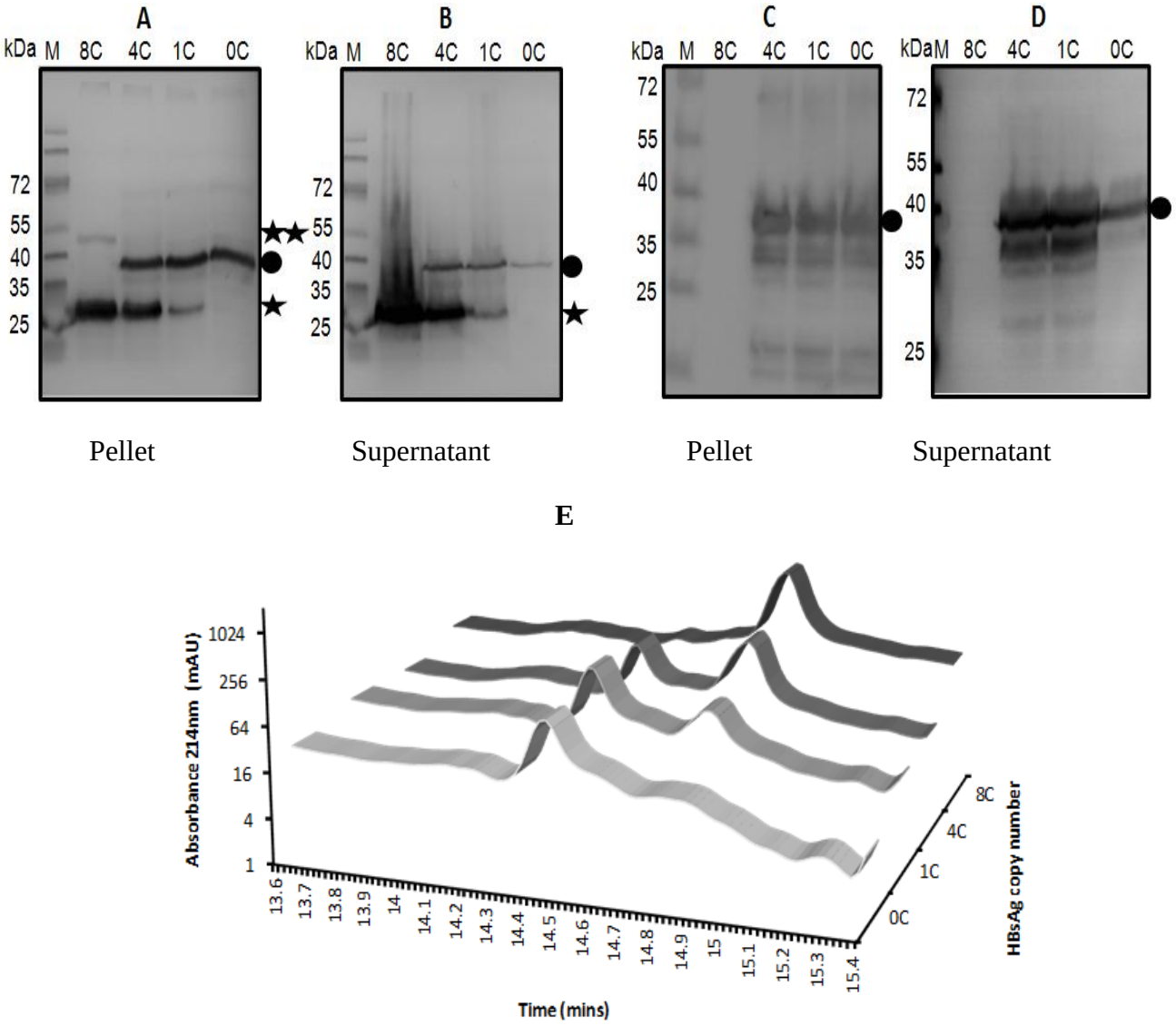


Figure 5-1: Co-expression of fusion protein DENV-2 EDIII-HBsAg with 0, 1 and 4 copies of unfused HBsAg ($ES_1:S_0$, $ES_1:S_1$,$ES_1:S_4$) and only 8 copies of unfused HBsAg ($ES_0:S_8$). The chimeric proteins are denoted in the figure as $ES_0:S_8$ (8C), $ES_1:S_4$ (4C), $ES_1:S_1$ (1C) and $ES_1:S_0$ (0C). Subscript denotes the copy number. Induced cells equivalent to 100 OD (1 ml) from all the chimeric constructs were lysed with glass beads and treated with 1 % (v/v) Tween 20 and fractionated into supernatant and pellet The pellet was solubilized in equal volume of urea buffer. Equal volume of the supernatant and pellet fractions from all the chimeric constructs were loaded. 10 µL of samples corresponding to supernatant or pellet fractions from different chimeric constructs were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied in the gel and electroblotted on PVDF membrane **(A)** Western blot of pellet fraction revealed by anti hepatitis B surface antigen specific mouse monoclonal antibody. **(B)** Western blot of supernatant revealed by anti hepatitis B surface antigen specific mouse monoclonal antibody. **(C)** Western blot of pellet fraction revealed by anti dengue EDIII specific mouse monoclonal antibody. **(D)** Western blot of supernatant revealed by anti dengue EDIII specific mouse monoclonal antibody. Single and double asterisks represent HBsAg monomer and dimer respectively. Single filled circle represents fusion protein (ES). **(E)** RP HPLC

profile of ES_0:S_8 (8C), ES_1:S_4 (4C), ES_1:S_1 (1C) and ES_1:S_0 (0C) induced lysate. Retention time of ES antigen is 14.4 min for and S antigen is 14.8 min.

Table 5-1: Co-expression of fusion protein EDIII-HBsAg (ES) with 0, 1 and 4 copies of unfused HBsAg (S) and expression of only 8 copies of unfused HBsAg (S). The chimeric constructs harboring the S and ES antigens were grown in BMGY media for 18 h and then induced in BMMY media with 1 % (v/v) methanol for every 12h

ES:S	VLP method	Total S antigen (mg L^{-1})[a]	Total ES antigen (mg L^{-1})[a]	S antigen in soluble fraction (mg L^{-1})[b]
1:0	+(EM)	-	750	1
1:1	+(EM)	330	1000	15
1:4	+(EM)	1522	810	165
0:8	ND [72]	3063	-	815

+ denotes detection of virus-like particles by electron microscopy (EM); ND indicates not determined;

[a] indicates quantification of total lysate by RP-HPLC; [b] refers to the quantification of the soluble fraction by Hepanostika HBsAg micro ELISA.

5.3 Purification of DENV-2 EDIII-$HBsAg_1$:$HBsAg_4$ (ES_1:S_4)

The protein being hydrophobic mostly gets associated as insoluble fraction. To solubilize the protein, Tween 20 was used at a concentration of 1 % (v/v). The protein was purified from the soluble fraction under native conditions. The conventional chromatography techniques applied for purification includes adsorption and desorption using Aerosil 380, followed by anion exchange chromatography with DEAE sepharose in conjunction with size exclusion chromatography (Sephacryl S-300 26/60). The size exclusion peak fractions within the void volume were analyzed by silver stained SDS PAGE. Silver stained gels revealed presence of ES and S antigens within the void volume of size exclusion peak fractions, which were further confirmed by Western blotting with mouse monoclonal anti hepatitis B surface antigen specific (5S) mouse monoclonal antibody (Figure 5-2). The ES_1:S_4 positive fractions after size exclusion chromatography were pooled and treated with KSCN. The mixture was dialyzed with PBS, pH 7.2 to remove KSCN. The protein recovery during subsequent steps of purification quantified by Hepanostika HBsAg ELISA is shown in (Table 5-2). From about 100 g of biomass (wet cell weight) 4 mg of purified protein was recovered. As can be seen in

gel picture there are some other bands at higher molecular weight along with desired one, these are the higher order structures of the proteins as confirmed by Western blotting with 5S mAb.

Table 5-2: ES_1:S_4 recovery during subsequent purification steps quantified by Hepanostika HBsAg microELISA from the 100 g biomass wet cell weight.

Step	Total S antigen Fraction (mg)[a]	Purification recovery (%)[a]
PEG precipitated supernatant	40	100
Aerosil flowthrough	5.6	14.1
Aerosil wash	0.1	0.3
Aerosil eluate	8	20.
DEAE eluate	5.3	13.3
Size exclusion eluate	4.6	11.6
After KSCN treatment	4.0	10.00

[a] refers to quantification by Hepanostika HBsAg microELISA

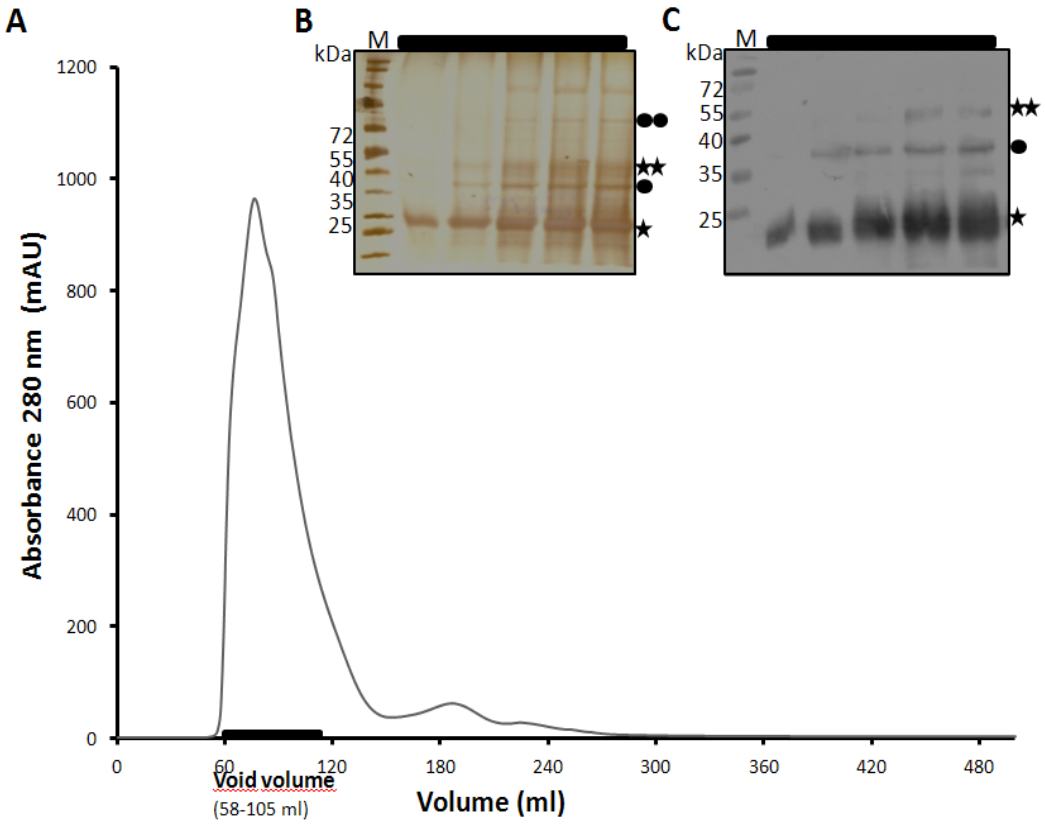


Figure 5-2: Size exclusion chromatography (Sephacryl S-300 26/60) of ES_1:S_4. **(A)** Size exclusion chromatogram resulted from passing pooled and concentrated ion exchange fractions of ES_1:S_4 onto sephacryl S-300 column. Protein elution was measured at 280 nm. **(B)** Size exclusion peak fractions within void volume (58-105 ml) analyzed by silver staining. **(C)** Size exclusion peak fractions within void volume (58-105 ml) analyzed by Western blotting with 5S mAb. 10 µL of peak fractions containing protein within void volume were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied corresponding to gels for Silver staining and Western blotting. Single and double asterisks represent HBsAg monomer and dimer respectively. Single and double filled circles denotes fusion protein (ES) monomer and dimer respectively. Bar denotes void volume

5.3.1 Stability evaluation of $ES_1:S_4$

The purified $ES_1:S_4$ at a concentration of 0.2 mg ml^{-1} was tested for stability for a period of two weeks under different conditions (Table 5-3). After two weeks precipitation was observed with protein aliquots in 25mM PB pH 7.2, 135 mM NaCl (PBS) without any additive kept at 37°C and 25°C, protein aliquots in 25 mM PB pH 7.2, 135 mM NaCl with DTT, 20 % (w/v) glycerol and protein aliquot in 25 mM MES pH 5.5, 135 mM NaCl at 37°C. The protein aliquots in all conditions were centrifuged at 16,000 x *g* for 30 mins fractionated into supernatant and pellet. The pellet was dissolved in $1/10^{th}$ volume of 25 mM PB pH 7.2, 135 mM NaCl, 8 M urea corresponding to the supernatant. The stability of ES and S antigen in day 0 and day 14 supernatant and pellet fractions was evaluated by Western blotting with a mixture of anti hepatitis B surface antigen (5S) specific and anti dengue EDIII specific (24A12) mouse monoclonals at a concentration of 1 µg ml^{-1} (Figure 5-3). Western blotting results revealed ES and S antigen were highly stable in PBS pH 7.2 at 4°C, PBS pH 7.2 with 5 mM EDTA, 2 mM PMSF and in 25mM HEPES pH 7.2 at 37°C. Nevertheless, a small fraction of the ES antigen under the above conditions formed insoluble particulates. Purified recombinant protein containing ES and S antigen in PBS pH 7.2 at 25°C, PBS pH 7.2 with 20 % (w/v) sucrose, 0.1 mM pepstatin and in 25mM Tris HCl pH -8.5, 135 mM NaCl at 37°C were moderately stable with a significant fraction of ES antigen forming insoluble particulates and S antigen was a stable The S antigen fraction of the $ES_1:S_4$ in PBS pH 7.2 with 20 % (w/v) glycerol and 25 mM MES pH 5.5, 135 mM NaCl at 37°C was stable whereas ES antigen fraction formed insoluble aggregates. S antigen fraction of the chimeric protein in PBS, pH 7.2 with DTT at 37°C seems to be degraded completely and ES fraction was unstable with respect to the formation of degraded products and adducts of these degraded products as confirmed by Western blotting.

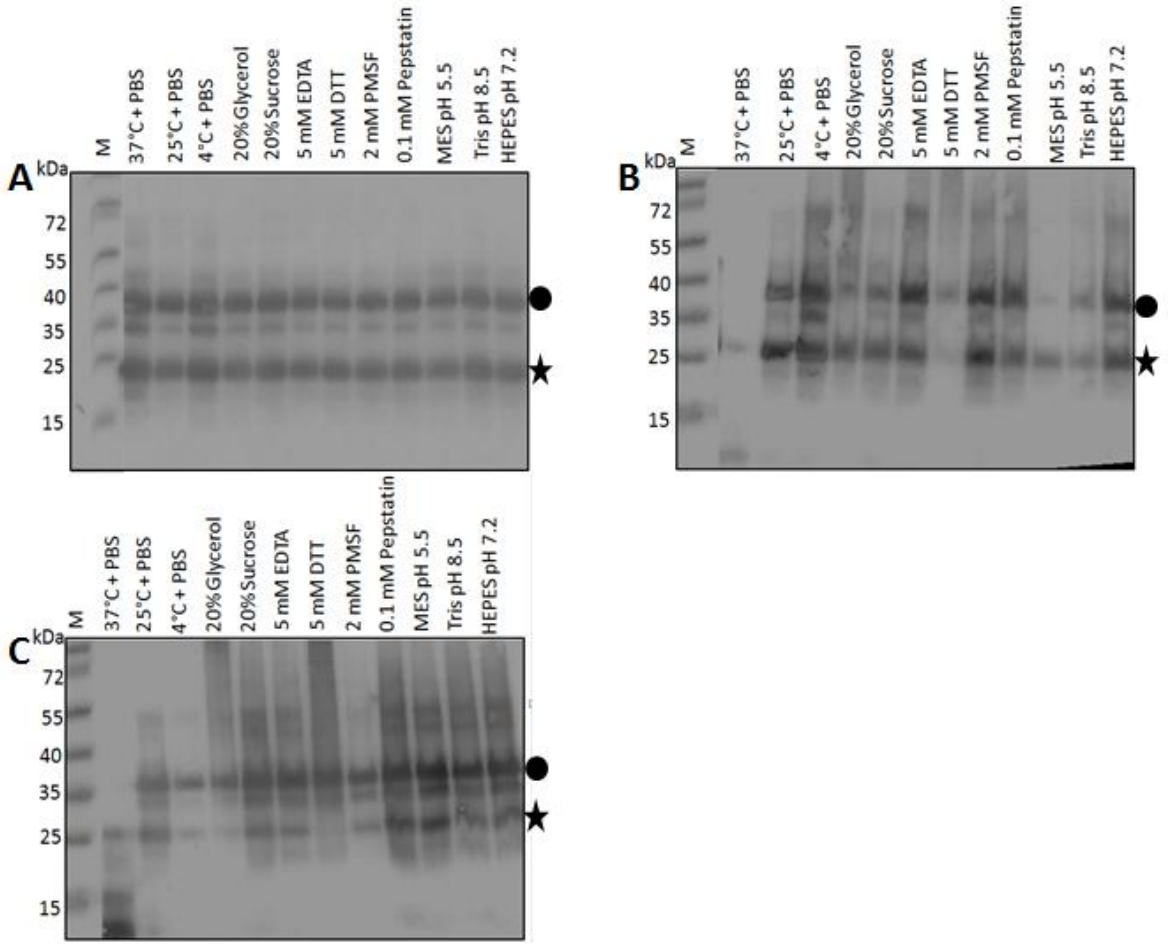


Figure 5-3: Stability evaluation of purified $ES_1:S_4$ by Western blotting **(A)** Western blot of day zero purified protein with and without excipients revealed by a mixture of 5S mAb and 24A12. After two weeks 250 µL of protein samples in different conditions described in table 4-3 were centrifuged and fractionated into supernatant and pellet fraction. Pellet fraction was dissolved in 25 µL of PBS with 8M urea. **(B)** Western blot of day 14 supernatant fractions revealed by 5S mAb and 24A12 mAb. **(C)** Western blot of day 14 pellet fractions revealed by 5S mAb and 24A12 mAb. 10 µL of purified protein incubated under different conditions were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied in the gel and electroblotted on PVDF membrane Single asterisk represents HBsAg (S) monomer and filled circle represents fusion protein (ES) respectively.

Table 5-3: Stability evaluation of $ES_1:S_4$ at a concentration of 0.2 mg ml^{-1} for 2 weeks. The Western blotting in Figure 5-3 was used to estimate the percentage of stability of purified $ES_1:S_4$ in respective lanes under various conditions of pH and temperature with and without excipents. The calculations were made by the use of ImageJ software densitometric scan of the blot (http://www.lukemiller.org/ ImageJ_gel_analysis.pdf).

Temperature	Buffer	pH	Excipients	Concentration	Stability
37°C	Phosphate	7.2	-	-	-
			Sucrose	20 %	++
			Glycerol	20 %	+
			EDTA	5 mM	++
			DTT	5 mM	-
			PMSF	2 mM	++
			Pepstatin	0.1 mM	++
37°C	HEPES	7.2	-	-	++
	MES	5.5	-	-	+
	TRIS	8.5	-	-	+
25°C	Phosphate	7.2	-	-	++
4°C	Phosphate	7.2	-	-	+++

-Indicates unstable (<20 %); + mildly stable (20-35 %); ++ moderately stable (35-70 %); +++ highly stable (70-90 %) in terms of solubility determined by Western blotting. Concentration of phosphate, HEPES, MES and Tris buffers used in this study was 25mM.

5.3.2 Immunological evaluation of $ES_1:S_4$

Purified protein was sent to our collaborator Dr. Navin Khanna, ICGEB, New Delhi and India for immunological characterization in 4-6 weeks old Balb/c mouse.The immunological evaluation was performed by Rajendra Raut, ICGEB, New Delhi, India The immunological acitivity was essentially carried out as described in [35]. Briefly, the purified recombinant proteins ($ES_1:S_4$) was incubated with allohydrogel (500 µg of alum was used for 20 µg of ES antigen fraction in $ES_1:S_4$ as quantified densitometrically by ImageJ) for 36 h at 4°C under filp-flop condition. The suspension was centrifuged at 5000 rpm for 5 mins and protein bound alum was resuspended in sterile PBS in such a way that 100 µL of suspension contained 20 µg of protein captured on 500 µg of alum. In-house EDIII-2 protein was also similarly coated on alum. Four to six weeks odl Balb/c mice were immunized with intra-peritoneal injections on days 0, 30 and 90 with 100 µL of protein-alum suspension. An additional group of Balb/c

was inoculated with 100 µL PBS-alum mix as negative control. Immunized mice were bled 1 week after the booster dose by retro-orbital puncture. The ability of antisera against ES_1:S_4 to reduce virus infectivity was evaluated by using plaque reduction neutralization test as reported [35]. Antibodies specific to ES_1:S_4 were found to recognize DENV-2 virus and reduce its infectivity, however neutralizing titers were low.

5.4 Purification of DENV-2 EDIII-HBsAg (ES_1:S_0)

Prelude experiment on the solubility of ES antigen by Hepanostika HBsAg ELISA has revealed antigen to be highly insoluble and gets associated with membrane (Table 5-1). Thus the pellet fraction from the lysate was solubilized with detergent and urea, followed by polyethylene glycol (PEG 6000) precipitation, diafiltration with 30 kDa cut off membrane and hydrophobic interaction chromatography with butyl sepharose. The peak fractions of butyl sepharose elutes were pooled, dialyzed and passed finally onto size exclusion sephacryl S-300 (16/60) column. The size exclusion peak fractions within the void volume were analyzed by silver stained SDS PAGE. Silver stained gels reveals the presence of ES antigen within size exclusion peak fractions, which was further confirmed by Western blotting with 5S mAb (Figure 5-4). The ES_1:S_0 positive fractions after size exclusion chromatography were pooled and treated with KSCN.The mixture was dialyzed with PBS to remove KSCN. From 50 g induced biomass wet cell weight only 1 mg of purified protein was recovered.

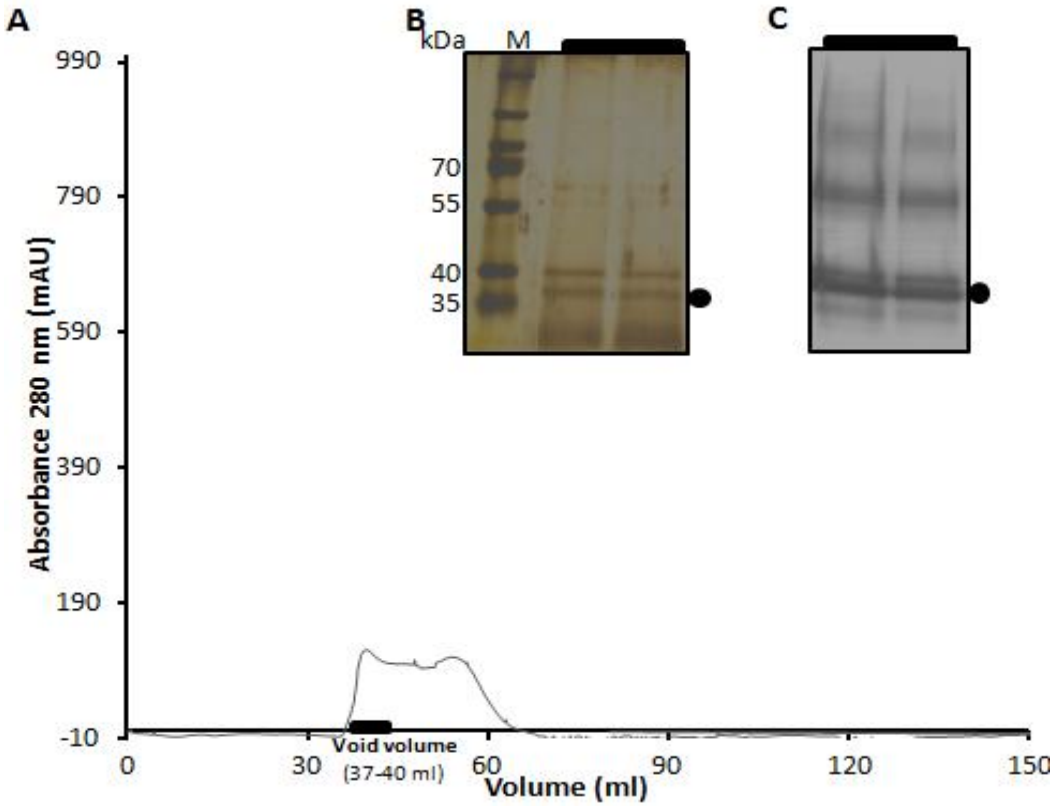


Figure 5-4: Size exclusion chromatography (Sephacryl S-300 16/60) of ES_1:S_0. **(A)** Size exclusion chromatogram resulted from passing pooled and concentrated ion exchange fractions of ES_1:S_0 onto sephacryl S-300 column. Protein elution was measured at 280 nm. **(B)** Size exclusion peak fractions within void volume (37-40 ml) analyzed by silver staining. **(C)** Size exclusion peak fractions within the void volume (37-40 ml) analyzed by Western blotting developed with 5SmAb. 10 µL of peak fractions containing protein within void volume were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied corresponding to gels for Silver staining and Western blotting. Filled circle represents fusion protein (ES). Bar represents void volume.

5.5 Electron-microscopic evaluation of purified proteins for formation of VLPs

Purified proteins, ES_1:S_4, ES_1:S_1 and ES_1:S_0, were evaluated for the formation of VLPs by electron microscopy. Purified proteins were coated on grids and negatively stained, using uranyl acetate. ES_1:S_1 and ES_1:S_4 containing increasing proportions of HBsAg were able to assemble into VLPs whereas only ES_1:S_0 containing only ES antigen was not able to assemble into VLPs and represented only aggregates (Figure 5-5). The presence of aggregates in ES_1:S_0 were characterized by size exclusion chromatography (Figure 5-4).

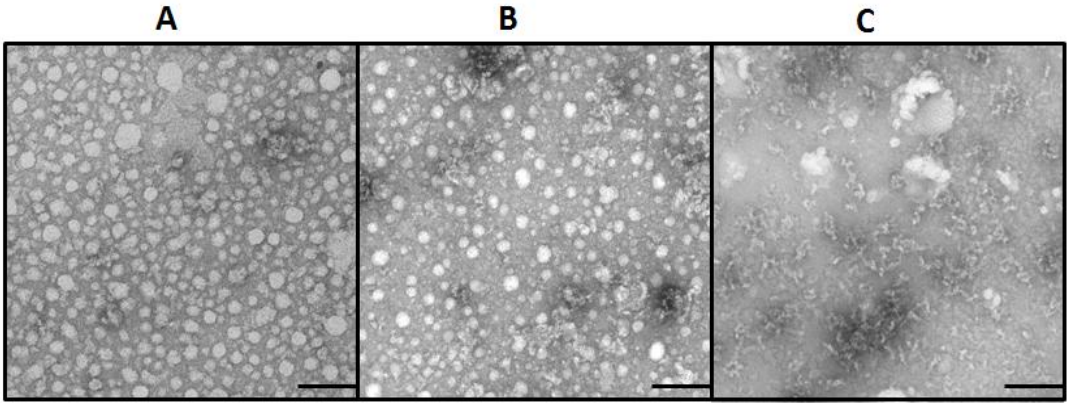


Figure 5-5: Electron microscopy visualization of purified proteins after size exclusion chromatography, KSCN treatment and dialysis. **(A)** Purified ES_1:S_4. **(B)** Purified ES_1:S_1. **(C)** Purified ES_1:S_0. The bar corresponds to 200 nm.

5.6 Conclusions

- Expression of ES_1:S_0, ES_1:S_1:ES_1:S_4 and ES_0:S_8 under alcohol oxidase promoter was achieved.
- Both ES and S antigen were highly insoluble with a significant fraction retained in insoluble fraction
- Co-expression of unfused S antigen in higher copy number assisted in enhancing solubility of ES antigen.
- A significant fraction of ES antigen was degraded during expression. This was inferred from the presence of low molecular weight products. The chances of low molecular weight products being non specific interactions as a result of anti dengue EDIII specific mouse monoclonal antibody are eliminated by using constructs expressing only 8 copies S antigen as a negative control where no such bands are visualized. Time course analysis of ES_1:S_4 induction intervals by Western blotting revealed a significant fraction of ES antigen is degraded. (Appendix I Figure 9-2)
- ES_1:S_4 was best among chimeric constructs in terms of solubility and yield so was purified under native conditions to an apparent homogeneity.
- ES_1:S_4 was able to form VLPs .

- $ES_1:S_4$ was able to elicit virus neutralizing antibodies against DENV-2 however, titer was not very high. $ES_1:S_4$ was sent to ICGEB, India for immunological evaluation. To, obviate the loss of protein activity during transport, $ES_1:S_4$ purified under identical conditions in ICGEB was processed in parallel which revealed an identical result.
- $ES_{1:}S_1$ purified under the same conditions employed for $ES_{1:}S_4$ was also able to form VLPs.
- $ES_1:S_0$ being relatively insoluble was purified to an apparent homogeneity from the insoluble fraction under denaturing conditions followed by removal of chaotropic agents by dialysis.
- $ES_1:S_0$ was not able to assemble into VLPs and represented only aggregate forms. This would indicate that fusion of EDIII domain to the S antigen hindered the formation of VLPs. However, the ability to form VLPs was augmented by co expression of S antigen at higher copy numbers [3] (Figure 5-5).

5.7 Induction of unfolded protein response (UPR) in wild-type (wt) GS115 and GS115 recombinant strains expressing $ES_1:S_0$, $ES_1:ES_4$, $ES_0:ES_8$

The solubility of ES antigen was quite lower compared to the S antigen as evidenced by Western blotting So, we presumed that ES antigen might not be able to attain native conformation rendering them insoluble. The accumulation of improperly folded proteins provokes unfolded protein response (UPR) [74]. ER resident chaperones KAR2 and PDI which are considered to be most prominent mediators of UPR contains an ER resident signal sequence HDEL at carboxy-terminus [75]. The UPR induction in the wild-type and recombinant strains expressing $ES_1:S_0$, $ES_1:S_4$, $ES_0:ES_8$ were evaluated by mouse monoclonal anti HDEL antibody. Immunoblotting data revealed significant down regulation of KAR2 and PDI in wild-type GS115, upregulation in strains producing $ES_1:S_0$, $ES_1:ES_4$ and downregulation in strains producing $ES_0:S_8$ on comparing before and after induction with methanol (Figure 5-6). The low molecular weight products could be degraded products of KAR2.

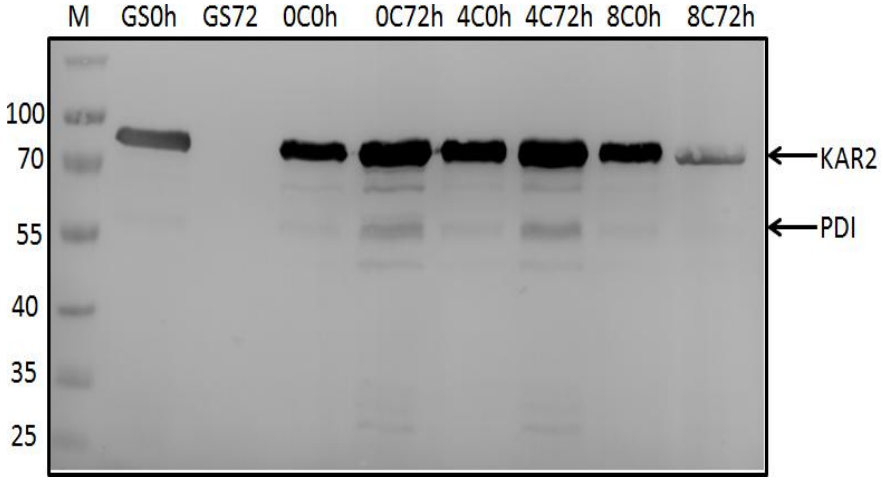


Figure 5-6: UPR induction in wild-type GS115 (GS) and GS115 recombinant strains expressing $ES_1:S_0$ (0C), $ES_1:ES_4$ (4C) and $ES_0:ES_8$ (8C).Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol and resuspended in complex medium (BMMY) containing methanol. Samples were taken before (0) and after 72 h of induction with 1 % (v/v) methanol. 10 µL of samples corresponding to total cell lysate before and after 72h of induction with methanol corresponding to GS, 0C, 4C and 8C were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied in the gel and electroblotted on PVDF.

5.8 Global change in endogenous ubiquitylation in wild-type GS115 and GS115 recombinant strains expressing ES_1:S_0, ES_1:ES_4, ES_0:ES_8

Cells to get relieved from misfolded proteins which cause UPR are retrotranslocated to cytosol and degraded by the ubiquitin proteasomal system, a major component of ERAD [66, 67]. Immunoblot data revealed increased levels of endogenous ubiquitylation in ES_1:S_0, ES_1:ES_4, decreased levels in wild-type (GS115) and no change in strains producing ES_0:S_8 (Figure 5-7).

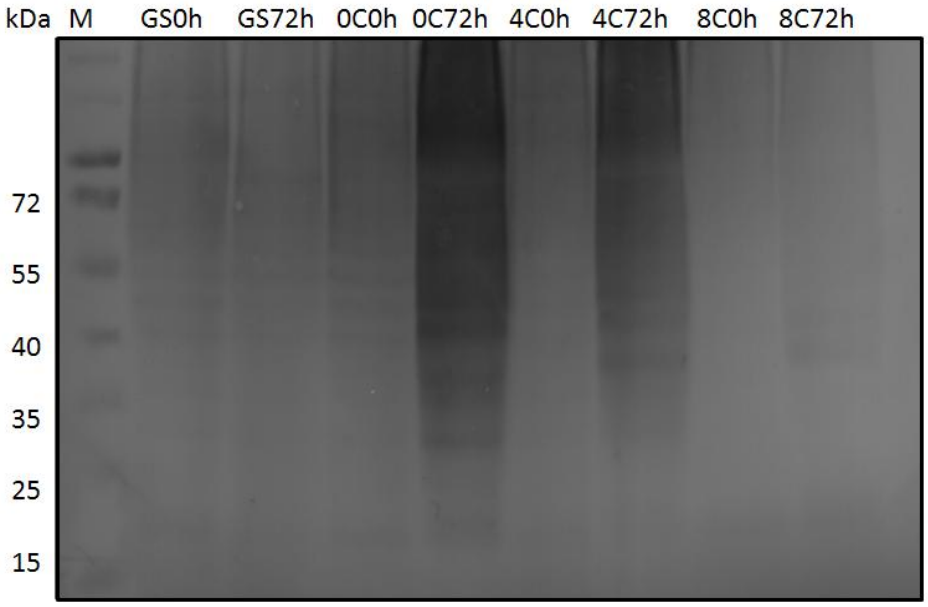
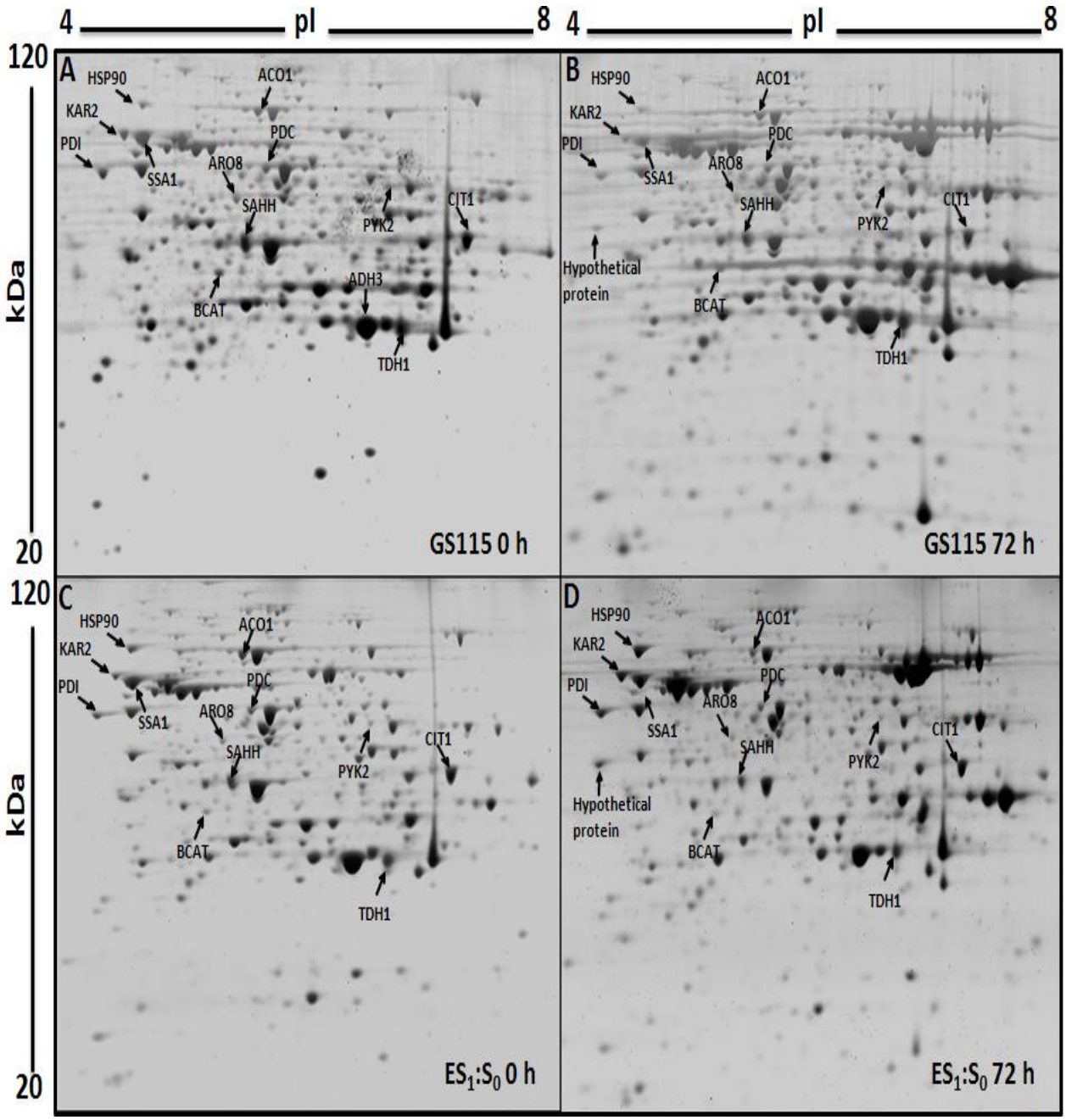


Figure 5-7: Change in endogenous ubiquitinylation before induction (0h) and after induction (72h) in wild-type GS115 (GS) and GS115 recombinant strains expressing ES_1:S_0 (0C), ES_1:ES_4 (4C) and ES_0:ES_8 (8C). Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol and resuspended in complex medium (BMMY) containing methanol. Samples were taken before (0) and after 72 h of induction with 1 % (v/v) methanol. 10 µL of samples corresponding to total cell lysate before and after 72h of induction with methanol corresponding to GS, 0C, 4C and 8C were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied in the gel and electroblotted on PVDF.

5.9 Evaluation of intracellular proteome

Two dimensional gel electrophoresis of before and after induction with methanol in wt GS115, ES_1:S_0, ES_1:S_4 and ES_0:S_8 resulted in a very reproducible pattern (Figure 5-8). The expression of ES_1:S_0 antigen and ES_1:S_4 on comparison with ES_0:S_8 antigen is relatively low (Table 5-1) but still generated higher UPR over ES_0:S_8 (Figure 5-6). From the above observations, we assumed that ES antigen is majorly causing UPR and could be targeted to ERAD. Proteome profiling revealed most of the identified proteins involved in UPR and ERAD was significantly upregulated in ES_1:S_0 and ES_1:S_4 on comparison with wt GS115 (Figure 5-9). An increase of UPR markers PDI and KAR2 identified in ES_1:S_0 and ES_1:S_4 was seen in comparison with wt GS115.There was a slight increase in expression of KAR2 and PDI in protein on comparison with ES_0:S_8, A significant increase in hypothetical protein which has thioredoxin like fold and homology with glutathione S transferase (GST) of *Rhizoctonia solani* in ES_1:S_0 and ES_1:S_4 on comparison with wild-type GS115 and ES_0:S_8 was observed. GST is known to be an antioxidant protein belonging to UPR [76]. An increase of cytosolic chaperones HSP70 (SSA1), HSP90 was observed in ES_1:S_0 and ES_1:S_4 in comparison with wild-type GS115 and ES_0:S_8. SSA1 and HSP90 are known to facilitate the degradation of apoprotein B [77]. The proteins related to pyruvate metabolism, glycolysis and amino acid synthesis were downregulated in ES_1:S_0, ES_1:S_4 and ES_1:S_8 on comparison with wt GS115 (Figure 5-10). Aconitase (ACO1) involved in TCA cycle was decreased, whereas citrate synthase (CIT1) did not show any significant change. A list of identified proteins classified by functional category is given in Appendix V Table 13-2.


4
pl
8
120
20
kDa
A
HSP90
ACO1
KAR2
PDC
PDI
SSA1
ARO8
SAHH
CIT1
PYK2
ADH3
BCAT
TDH1
GS115 0 h
B
HSP90
ACO1
KAR2
PDC
PDI
SSA1
ARO8
SAHH
CIT1
PYK2
Hypothetical protein
BCAT
TDH1
GS115 72 h
C
HSP90
ACO1
KAR2
PDC
PDI
SSA1
ARO8
SAHH
CIT1
PYK2
BCAT
TDH1
ES1:S0 0 h
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BCAT
TDH1
ES1:S0 72 h

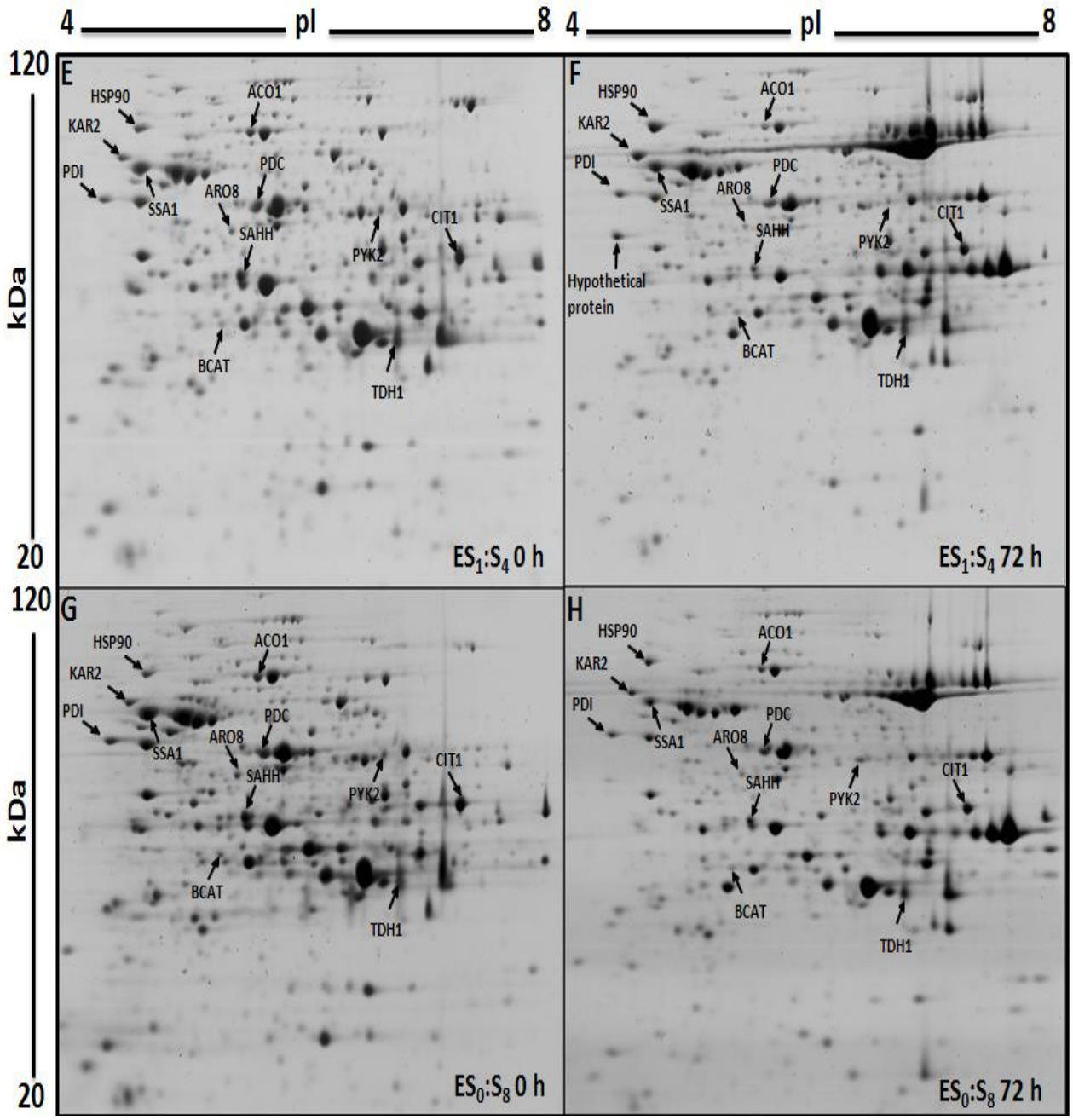

Figure 5-8: Intracellular changes in proteome of GS115 wild-type strain and GS115 strain producing ES_1:S_0, ES_1:S_4 and ES_0:S_8 before and after induction. **(A)** Wild-type strain 0h of induction. **(B)** 72h after induction with methanol in wild-type strain. **(C)** GS115 ES_1:S_0 0h of induction. **(D)** 72h after methanol induction of ES_1:S_0. **(E)** GS115 ES_1:S_4 0h of induction. **(F)** 72h after methanol induction of ES_1:S_4. **(G)** GS115 ES_0:S_8 0h of induction. **(H)** 72h after methanol induction of ES_0:S_8. Only section of gels which includes identified proteins from pI 4-8 are considered.

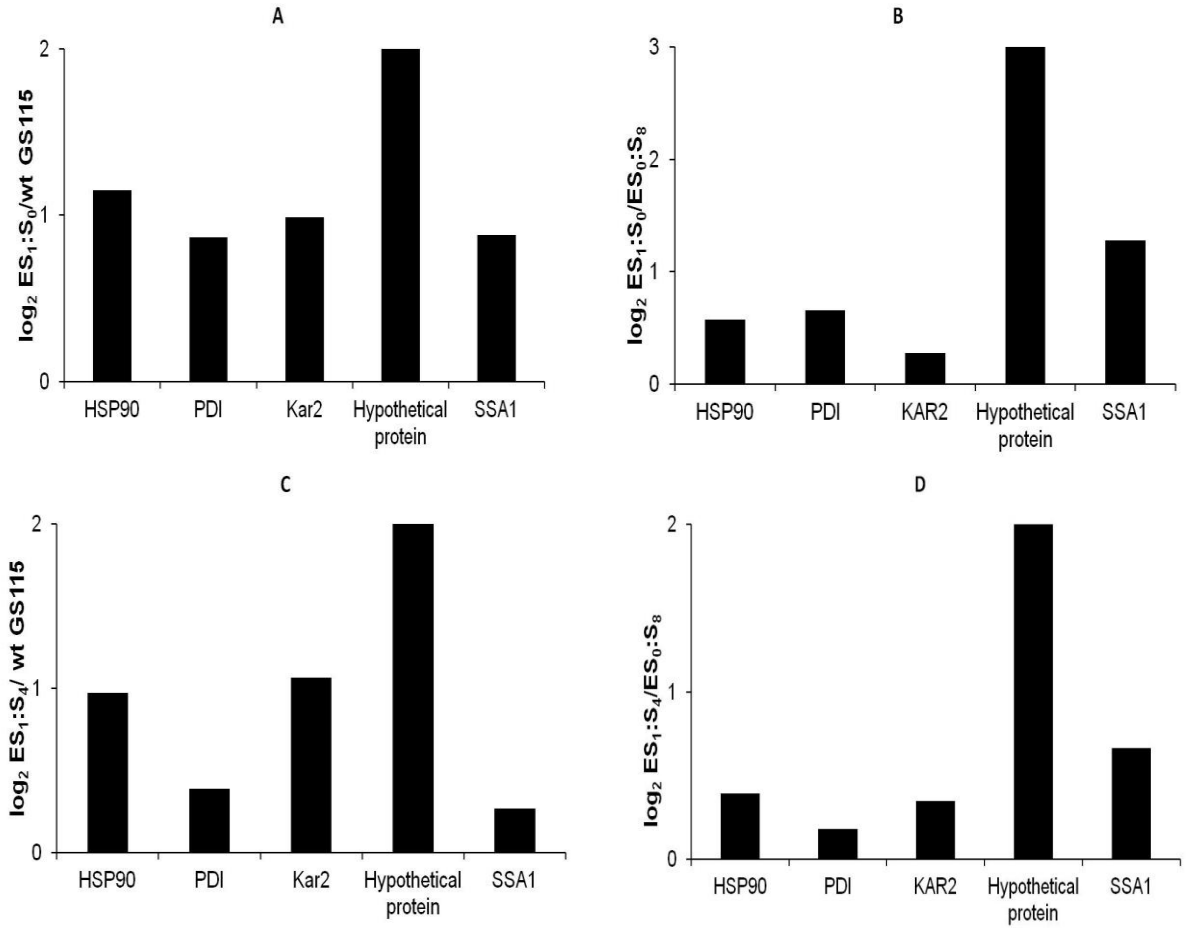

Figure 5-9: Changes of UPR and ERAD related proteins in GS115 strain producing chimeric proteins on comparison with non producing GS115 strain. **(A)** Log_2 ratio of cultures producing ES_1:S_0 compared to wild-type (wt) GS115. **(B)** Log_2 ratio of cultures producing ES_1:S_0 compared to ES_0:S_8. **(C)** Log_2 ratio of cultures producing ES_1:S_4 compared to wild-type (wt) GS115. **(D)** Log_2 ratio of cultures producing ES_1:S_4 compared to ES_0:S_8.

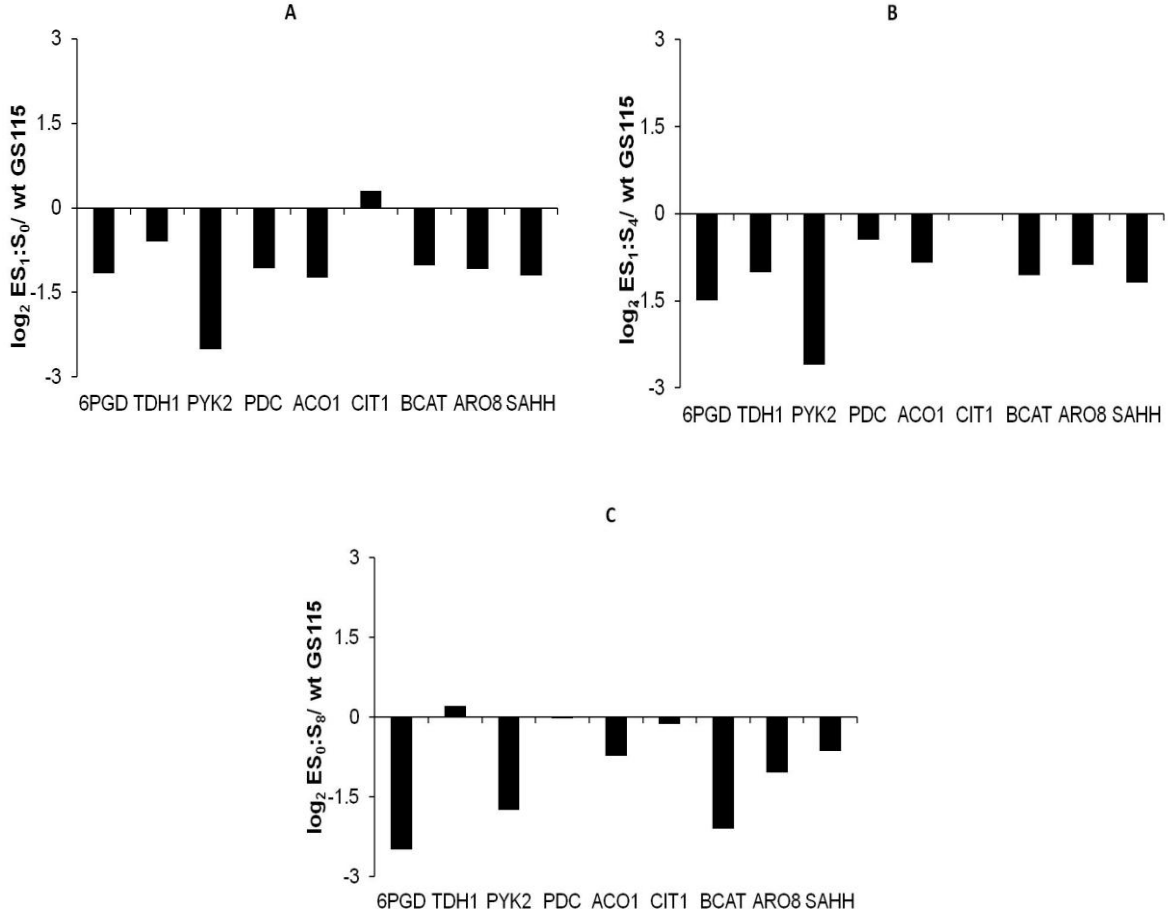

Figure 5-10: Changes of proteins related to central metabolism in GS115 strain producing chimeric proteins on comparison with non producing GS115 strain**. (A)** Log_2 ratio of cultures producing ES_1:S_0 compared to wild-type (wt) GS115. **(B)** Log_2 ratio of cultures producing ES_1:S_4 compared to wild-type (wt) GS115. **(C)** Log_2 ratio of cultures producing ES_0:S_8 compared to wild-type (wt) GS115.

5.10 Conclusions

- Expression of ES_1:S_0, ES_1:S_4 resulted in upregulation of UPR markers KAR2 and PDI after induction with methanol, indicating activation of unfolded protein response. UPR activation can be as a result of ES antigen expression as insoluble aggregates.
- Increased expression of endogenous ubiquitylation during expression of ES antigen in ES_1:S_0 and ES_1:S_4 after induction with methanol indicates activation of ERAD pathway and targeting of ES antigen to ERAD.
- During expression of 8 copies of only S antigen (ES_0:S_8) there is no significant change in expression of KAR2 and PDI after induction of methanol representing slightly detectable non prominent UPR response. There was no upregulation of endogenous ubiqutiylated proteins during expression of ES_0:S_8 .
- Expression of ES antigen caused increased expression of cytosolic chaperones SSA1 (HSP70), HSP90 on comparison with wt GS115 and also ES_0:S_8 .SSA1 and HSP90 are known to target improperly folded proteins to ERAD.
- An increased upregulation of hypothetical protein with homology to that of glutathione S transferase with antioxidant activity and probable role in UPR during ES_1:S_0 and ES_1:S_4 production.
- During expression of ES_1:S_0, ES_1:S_4 and ES_0:S_8 there was downregulation of proteins involved in central carbon metabolism.

6. DISCUSSION

Dengue, an arthropod borne febrile illness is rapidly spreading across the world with 2.5 billion people at risk of infection. Infection can be caused by any of the four dengue virus serotypes DENV-1, -2, -3 and -4 [9]. There are no licensed vaccine or specific therapeutics for this rapidly growing disease and vector control measures did not make a significant contribution to the prevention of the emergence and rapid spread [6]. A diverse number of vaccine candidates are in development using different strategies where the live attenuated vaccine (LAV) candidates are the front-runners. Live attenuated vaccine candidates, although, in advanced clinical phase concern over safety still remains due to reversion and interference. Therefore, subunit vaccine candidates are gaining importance because of being safer. One such subunit vaccine candidate, based on dengue envelope protein has reached clinical phase I [18]. A large number of diverse dengue vaccine candidates are in preclinical phase to ensure the continuous influx of innovation which is critical for maximizing chances of success in dengue vaccine development [29].

The first part of the study focuses on HBsAg as a carrier for surface presentation of domain III of DENV-2 envelope (EDIII-2). The study involves *P. pastoris* based co-expression of HBsAg based EDIII-2 chimera (ES) and 0, 1, 4 copies of HBsAg (S) denoted as $ES_1:S_0$, $ES_1:S_1$ and $ES_1:S_4$ purification and characterization by electron microscopy. Stability evaluation of $ES_1:S_4$ was performed.

The rationale for this work is based on four important factors. Firstly, in a field of dengue vaccine development research there has been an increasing awareness to explore non-replicating candidates as potential dengue vaccine candidates due to problems associated with live attenuated virus vaccine candidates. Domain III of DENV envelope (EDIII) has emerged as a promising vaccine candidate due to its potential attributes. These include receptor binding domain, exposure of domain on the surface of virus, dengue type and subtype specific neutralizing epitopes and anti EDIII antibodies are efficient virus blockers [10, 22].

Secondly, virus-like particles (VLPs) are supramolecular, non-infectious, non-replicative assemblies with repetitive surfaces, particulate structures and dimensions rendering them safe and immunogenic [4]. These attributes associated with VLPs contribute significantly in

generation of B and T-cell responses. Two VLP based vaccine candidates HBsAg and HPVL1 have successfully reached the market [5].

Thirdly, hepatitis B surface antigen (HBsAg) possesses an intrinsic quality of self assembly into VLPs and its ability to retain this potential on incorporation of foreign antigens. Thus, it has been widely used as carrier for display of vaccine antigens. With the success of HBsAg based malarial vaccine candidate RTS,S vaccine candidate has reached clinical phase III trials there is a tremendous interest in application of HBsAg as carrier [37].

Fourthly, dengue vaccine needs to be produced at affordable price since dengue is mainly found in developing and poor countries The yeast *P. pastoris* combines advantages of prokaryotic and eukaryotic hosts [78] and can be used as an effective platform for production of vaccine candidates in cost effective manner and hence is recognized as an ideal system. It has been used as an expression system for HBsAg which assembles into VLPs [72] and disulphide (S-S) linked viral antigens [9]. Thus we sought to integrate all this information to co-express EDIII-2-HBsAg (ES) with 0, 1 and 4 copies of HBsAg (S) to generate stable hybrid particles and evaluate immunogenicity of vaccine candidate.

A fusion of epitopes at amino terminus (circumsporozite protein from *P. falciparum*)or insertion at antigen determinant loop (VP1 capsid protein from polio virus) of HBsAg resulted in generation of hybrid VLPs which were protective [47, 48]. The chimeric EDIII-2-HBsAg (ES) antigen was designed by fusing of envelope domain III at the amino terminus of HBsAg and expressed individually as well as with unfused HBsAg (S) at different copy numbers (1 and 4) in *P. pastoris* under control of methanol inducible alcohol oxidase promoter (AOX1). An analysis of the relative proportion of chimeric and unfused S antigen revealed both proteins are insoluble in the absence of any detergents or chaotropic agents (Localization of ES and S antigen, Appendix I Figure 9-1). A high copy number of S antigen resulted in higher solubility in the presence of Tween 20 (1 % v/v). Co-expression of unfused S antigen of higher copy number also assisted in increasing the solubility of ES antigen (Figure 5-1 B, D). The expression of only unfused S antigen at high copy number (8 copies) has the highest solubility whereas ES antigen when expressed in the absence of the unfused S antigen was relatively insoluble with only a very minor fraction found in the soluble fraction in the presence of 1 % (v/v) Tween 20 (Table 5-1). Although expression of an only ES gene resulted in high yield (Table 5-1), most of ES antigen was associated with the insoluble fraction with significant fraction getting degraded into fragments. Degraded proudcts were

seen in Tween 20 soluble and insoluble fractions when revealed with anti dengue EDIII specific mouse monoclonal. (Figure 5-1 C,D). The Co-expression of S antigen at higher copy number (1, 4) aided in solubilzing ES protein, but had no effect on inhibiting degradation of ES antigen. Even cell lysis in the presence of PMSF and pepstatin had no effect on inhibiting degradation of ES antigen whereas unfused S antigen when expressed individually at high copy number (8 copies) or co-expressed as 1 and 4 copies along with ES antigen was not degraded. Expression of S antigen was proportional to the copy number (Table 5-1).

A recent report describes the purification of HBsAg VLPs from the soluble fraction of *P. pastoris* lysate [72]. $ES_1:S_4$ which was best soluble among other chimeric clones $ES_1:S_1$ and $ES_1:S_0$ was purified using the same strategy employed for HBsAg purification [71] with slight modifications as described in materials methods section.The purification of $ES_1:S_4$ which involved PEG precipitation of host cell contaminants, Aerosil-380 adsorption and desorption followed by ion-exchage with DEAE sepharose and size exclusion with sephacryl S-300. During the Aerosil adsorption and desorption step about 15 % of protein remained unbound to Aerosil and 20 % of protein was recovered by desorption with 25 mM sodium carbonate/bicarbonate buffer pH 10.6 , 1.2 M urea. Near to 60 % of protein prevailed adsorbed to Aerosil-380 even after desorption. The protein yield and recovery during subsequent steps of purification was carried out by Hepanostika HBsAg microELISA (Table 5-2) A similar tendency was also seen in case of purification of HBsAg where a prominent fraction of protein remained tightly bound to Aerosil-380 and could not be desorbed using buffer containing 25 mM Sodium carbonate/bicarbonate buffer pH 10.6, 1.2 M urea Increase in concentration of urea has helped in improving the recovery of HBsAg from Aerosil, however denatures the protein. A better desorption condtions for elution of protein from Aerosil needs to be optimized. Purified protein was able to assemble into VLPs confirmed by electron microscopy. However, the VLPs population were heterogenous indicating the presence of VLPs possibly corresponding to both S and ES antigens. $ES_1:S_1$was also purified from the soluble fraction in the same way as $ES_1:S_4$ and was also able to form VLPs seen by EM.

Purified $ES_1:S_4$ was evaluated for stability in the presence of different buffers, temperature and additives. Stability evaluation revealed that S antigen fraction in $ES_1:S_4$ was quite stable and soluble as determined by incubation at 37°C for 14 days in the presence of additives. Most of S antigen fraction remained soluble in the presence of polyols (sucrose, glycerol),

protease inhibitors (EDTA, PMSF and pepstatin) with a small fraction forming insoluble aggregates. Whereas a significant fraction of ES antigen formed insoluble aggregates on incubation at 25°C without additives and at 37°C in the presence of additives for 14 days. Addition of sucrose, protease inhibitors EDTA and PMSF to purified ES_1:S_4 assisted in keeping ES antigen in soluble form. At 4°C both ES and S antigen in ES_1:S_4 were stable. Nevertheless, a small fraction of ES antigen formed insoluble aggregates.

ES_1:S_0 being highly insoluble cannot be purified by same method employed for purification of ES_1:S_4, hence was purified from the insoluble fraction [71] under denaturing conditions, followed by removal of chaotropic agent by dialysis. Preliminary analysis by size exclusion chromatography with sephacryl S-300 revealed purified protein was able to form highly ordered structures, but was not able to assemble into VLPs as confirmed by EM. The yield of purified protein was quite low and conditions are to be optimized to increase the recovery.

In conclusion, this work demonstrates the fusion of DENV-2-EDIII to the amino terminus of HBsAg and co-expression with 1 and 4 copies of unfused HBsAg. HBsAg co-expression in higher copy number assisted in solubility of ES antigen. (Figure 5-1 B, D) Co-expression of HBsAg also aided in co-purification of ES antigen and formation of VLPs [3]. However, only ES antigen was not able to assemble into VLPs. Immunological evaluation in mice revealed ES_1:S_4 was able to generate modest neutralizing titers. However, in a recent work in our collaborating group at ICGEB, India fusion of EDIII domains from all the four stereotypes at the amino terminus of HBsAg and co-expression along with four copies of unfused HBsAg resulted in a very prominent neutralizing activity to all the four DENV serotypes (data not published). These interesting results lead us to hypothesize perhaps some of the antigenic determinants of DENV-2EDIII are hidden resulting in low neutralization. However on fusion with EDIII from other serotype resulted in better exposure of these epitopes and hence higher neutralization. This is in agreement with literature where generation of tetravalent soluble EDIII antigen resulted in improved presentation of hidden antigenic determinants in EDIII and enhanced immunogenicity than in monovalents [1]. Stable and optimal presentation of EDIII on surface of carrier needs to be optimized to make it a viable option for vaccine candidate, trials of which are ongoing.

Overexpression of only ES antigen or ES antigen along with unfused S antigen in different copy numbers (1, 4) using AOX1 promoter was achieved however, most of ES antigen remained insoluble. Expression of a virus surface glycoprotein or membrane proteins in yeast

often resulting in misfolding and aggregate formation are well documented, the exception is with HBsAg which on expression in yeast attains native conformation [8]. Yeast cell response to accumulation of intracellulary expressed aggregated proteins have not been well studied. The second part of the study was to gain insights into the yeast cell respone with special focus on UPR and ERAD during expression of *P. pastoris* GS115 ES_1:S_0, ES_1:S_4, ES_0:S_8 and wild-type (wt) GS115 as control with proteomic approach.

Expression studies on ES_1:S_0, ES_1:S_1, ES_1:S_4 and ES_0:S_8 revealed solubility of ES antigen is lower than S antigen; although the hydropathic index of ES antigen is lower than S antigen (i.e. ES antigen is less hydrophobic than S antigen). A grand average of hydropathicity (GRAVY) for ES antigen is 0.362 and for S antigen is 0.688 measured based on the number of hydrophobic residues in protein with protparam software (web.expasy.org/protparam). Also a significant fraction of ES antigen is getting degraded during expression. We speculated fusion of EDIII domain to HBsAg and *P. pastoris* based expression resulted in insoluble aggregate formation which would be the rationale for lower solubility of ES antigen

To further confirm the effect of the DEN-2-EDIII domain, transformants harboring only secretory EDIII was expressed and analyzed by Western blotting. Although, EDIII was fused with alpha mating factor only a minor fraction was secreted and most of the protein was retained intracelluarly, confirmed by immunoblotting with 24A12 mAb. Western blot results revealed the presence of aggregates in both secretory and intracellular fractions (Appendix IV Figure 12-1). Also the expression of only EDIII resulted in significant upregulation of the UPR (Appendix IV Figure 12-2).

The accumulation of improperly folded proteins causes considerable stress [8]. Cells respond to these proteins by inducing unfolded protein response (UPR) which results in increased synthesis of chaperones, which assist in folding and targeting proteins to their destined sites and when failed to fold retrotranslocate to cytosol and are cleared by endoplasmic reticulum associated degradation (ERAD) pathway mediated by ubiquitinlyated proteasomal complex [63].

UPR induction is indicated by increased expression of UPR markers. The major UPR markers are KAR2, PDI and LHS1 which contains HDEL ER resident signal at the carboxy terminus [75]. Western blotting of induction intervals (before and after induction) with anti HDEL monoclonal antibody revealed increased expression of KAR2 in ES_1:S_0.ES_1:S_4, and decreased

expression in ES_0:S_8 and wild-type GS115. These results would infer strong induction of UPR as a result of ES antigen expression in comparison with wild-type and ES_0:S_8 where there is downregulation of UPR markers during shake flask cultivation in BMGY media with glycerol as carbon source and induction with BMMY medium containing methanol.

Abberantly folded proteins targeted to ERAD are tagged with polyubiquitin which acts as degradation signal. Attachment of polyubiquitin to the protein involves coordinated action of E1, E2 and E3 enzymes [70]. Western blotting of induction intervals with anti ubiquitin antibody revealed increase in endogenous ubiquitylation in ES_1:S_0 and ES_1:S_4, no change in ES_0:S_8 and decreased levels in wt GS115. A significant upregulation of ubiquitylation in ES_1:S_0 in comparison with ES_0:S_8 would indicate targeting of ES_1:S_0 to ERAD. A clear degradation is evidenced by presence of ,low molecular weight products by Western blotting with anti dengue EDIII specific mouse monoclonal during ES antigen expression (Time course induction of ES_1:S_4, Appendix I Figure 9-2 B) However, there is no significant change in yield of fusion protein during the time of induction as quantified by RP-HPLC as described previously (Appendix I Figure 9-2 B, C). From this it can be inferred that most of ES antigen expressed is retained as insoluble deposits and is stable within cells by evading the targeting to ERAD.Nevertheless, a fraction of ES was degraded into fragments. We assume this degradation is mediated by chaperones KAR2 and PDI by solubilzing the aggregates and targeting them to ERAD. Such mechanism of solubilizing aggregates and targeting to degradation have been documented in literature [70].

Proteomic analysis of GS115 strains producing ES_1:S_0, ES_1:S_4 ES_0:S_8 and wild-type strain GS115 revealed a regulation pattern of UPR markers (KAR2 and PDI) identical with Western blotting. UPR response is known to be induced during accumulation of unfolded proteins [79]. There was an upregulation of KAR2 and PDI in ES_1:S_0 and ES_1:S_4 on comparison with wild-type GS115 and ES_0:S_8 indicating heretical conformation as a result ES antigen expression. A noteworthy upregulation of hypothetical protein was seen during production of ES_1:S_0, ES_1:S_4 on comparison with wild-type GS115 and ES_0:S_8. Hypothetical protein has thioredoxin like fold and has homology with glutathione S transferase of *Rhizoctonia solani*. Glutathione S transferase is an antioxidant protein belonging to UPR regulated pathway [76]. An increase in cytosolic chaperones SSA1, HSP90 was seen in ES_1:S_0 and ES_1:S_4 when compared with wild-type GS115 and ES_0:S_8. HSP90 in cooperation with SSA1 is known to target apoprotein B for ERAD [66, 77]. A similar role of SSA1 and HSP90 can be attributed

in degradation of $ES_1:S_0$ and $ES_1:S_4$. Expression of UPR markers, SSA1 and HSP90 was higher in $ES_1:S_0$ and $ES_1:S_4$ in comparison with $ES_0:S_8$. Expression of $ES_0:S_8$ although resulted in very high yield did not induce any prominent UPR and activation of SSA1. This further strengthens our hypothesis that on expression in yeast, ES antigen in $ES_1:S_0$ and $ES_1:S_4$ resulted in formation of insoluble deposits. The insoluble deposits induced a considerable stress indicated by an increase in expression of ER and cytosolic chaperones with a prominent role in UPR and ERAD.

Expression of ES antigen in yeast resulted in the formation of insoluble deposits. The evaluation of protein during ES antigen expression vindicated prominent upregulation of chaperones involved in UPR and ERAD to maintain cellular homeostasis. The present study illustrates consequences of intracellular protein expression and deeper understanding of stress responses and ERAD in *P. pastoris*. Collectively, these findings underline application of ES antigen as a model protein for gaining deeper insights into the stress responses.

7. FUTURE PROSPECTS

The present work demonstrates a fair trial of monovalent dengue subunit vaccine candidate using DENV-2EDIII expressed on the surface of HBsAg VLPs using *P. pastoris* as an expression host. We were able to successfully express the EDIII on the surface of HBsAg which resulted in modest immunogenicity. EDIII displayed on HBsAg can have potential vaccine and diagnostic applications. A stable and optimal presentation of antigenic epitopes on surface of carrier needs to be optimized for making this a viable option for vaccine candidate trials which are underway. Use of flexible glycine serine linker in antibody engineering for fusion with HBsAg probably may provide a stable and better exposure of antigenic epitopes. This approach could serve as the basis for platform for other infections as well. In a recent work in our collaborating group at ICGEB, India fusion of EDIII domain from all the four serotypes with glycine linker at the amino terminus of HBsAg and co-expression along with four copies of unfused HBsAg resulted in a very prominent neutralizing activity to all the four DENV serotypes paving the way for development of tetravalent subunit vaccine.

The fusion protein expression in *P. pastoris* resulted in formation of insoluble aggregates which were stable against proteolysis during induction intervals invivo evading the proteasomal degradation and causing a prominent stress in the host cells. Increased levels of KAR2 and PDI during expression of ES antigen presumably aids in solubilizing aggregates and targeting to ubiquitin mediated degradation. Most of the literature is focused on the degradation of glycoproteins and with less insight on degradation of non glycosylated proteins. Further work is underway to identify the endogenous ubiquitin proteins involved in mediating degradation of non glycosylated fusion protein by co-immunopreciptation with anti ubiquitin antibody.

8. REFERENCES

1. Zhao H, Jiang T, Zhou XZ, Deng YQ, Li XF, Chen SP, Zhu SY, Zhou X, Qin ED, Qin CF: **Induction of neutralizing antibodies against four serotypes of dengue viruses by MixBiEDIII, a tetravalent dengue vaccine.** *PLoS One* 2014, **9:**e86573.
2. Raviprakash K, Defang G, Burgess T, Porter K: **Advances in dengue vaccine development.** *Hum Vaccin* 2009, **5:**520-528.
3. Khetarpal N, Poddar A, Nemani SK, Dhar N, Patil A, Negi P, Perween A, Viswanathan R, Lunsdorf H, Tyagi P, et al: **Dengue-specific subviral nanoparticles: design, creation and characterization.** *J Nanobiotechnology* 2013, **11:**15.
4. Kushnir N, Streatfield SJ, Yusibov V: **Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development.** *Vaccine* 2012, **31:**58-83.
5. Tan M, Jiang X: **Subviral particle as vaccine and vaccine platform.** *Curr Opin Virol* 2014, **6:**24-33.
6. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, et al: **The global distribution and burden of dengue.** *Nature* 2013, **496:**504-507.
7. Rabert C, Weinacker D, Pessoa A, Jr., Farias JG: **Recombinants proteins for industrial uses: utilization of *Pichia pastoris* expression system.** *Braz J Microbiol* 2013, **44:**351-356.
8. Ciplys E, Samuel D, Juozapaitis M, Sasnauskas K, Slibinskas R: **Overexpression of human virus surface glycoprotein precursors induces cytosolic unfolded protein response in *Saccharomyces cerevisia*e.** *Microb Cell Fact* 2011, **10:**37.
9. Mani S, Tripathi L, Raut R, Tyagi P, Arora U, Barman T, Sood R, Galav A, Wahala W, de Silva A, et al: ***Pichia pastoris*-expressed dengue 2 envelope forms virus-like particles without pre-membrane protein and induces high titer neutralizing antibodies.** *PLoS One* 2013, **8:**64595.
10. Swaminathan S, Batra G, Khanna N: **Dengue vaccines: state of the art.** *Expert Opin Ther Pat* 2010, **20:**819-835.
11. Chen R, Vasilakis N: **Dengue-where are we and where are we going?** *Viruses* 2011, **3:**1562-1608.
12. Kyle JL, Harris E: **Global spread and persistence of dengue.** *Annu Rev Microbiol* 2008, **62:**71-92.
13. Halstead SB, Sukhavachana P, Nisalak A: **In vitro recovery of dengue viruses from naturally infected human beings and arthropods.** *Nature* 1964, **202:**931-932.
14. Halstead SB: **Dengue.** *Lancet* 2007, **370:**1644-1652.

15. Vasilakis N, Cardosa J, Hanley KA, Holmes EC, Weaver SC: **Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health.** *Nat Rev Microbiol* 2011, **9:**532-541.

16. Leake CJ: **Arbovirus-mosquito interactions and vector specificity.** *Parasitol Today* 1992, **8:**123-128.

17. Yazi Mendoza M, Salas-Benito JS, Lanz-Mendoza H, Hernandez-Martinez S, del Angel RM: **A putative receptor for dengue virus in mosquito tissues: localization of a 45-kDa glycoprotein.** *Am J Trop Med Hyg* 2002, **67:**76-84.

18. Murphy BR, Whitehead SS: **Immune response to dengue virus and prospects for a vaccine.** *Annu Rev Immunol* 2011, **29:**587-619.

19. Back AT, Lundkvist A: **Dengue viruses - an overview.** *Infect Ecol Epidemiol* 2013, **3**.

20. Murrell S, Wu SC, Butler M: **Review of dengue virus and the development of a vaccine.** *Biotechnol Adv* 2011, **29:**239-247.

21. Zellweger RM, Prestwood TR, Shresta S: **Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease.** *Cell Host Microbe* 2010, **7:**128-139.

22. Batra G, Gurramkonda C, Nemani SK, Jain SK, Swaminathan S, Khanna N: **Optimization of conditions for secretion of dengue virus type 2 envelope domain III using *Pichia pastoris*.** *J Biosci Bioeng* 2010, **110:**408-414.

23. Fischl W, Bartenschlager R: **Exploitation of cellular pathways by Dengue virus.** *Curr Opin Microbiol* 2011, **14:**470-475.

24. Perera R, Kuhn RJ: **Structural proteomics of dengue virus.** *Curr Opin Microbiol* 2008, **11:**369-377.

25. Hidari KI, Suzuki T: **Dengue virus receptor.** *Trop Med Health* 2011, **39:**37-43.

26. Perera R, Khaliq M, Kuhn RJ: **Closing the door on flaviviruses: entry as a target for antiviral drug design.** *Antiviral Res* 2008, **80:**11-22.

27. Whitehorn J, Simmons CP: **The pathogenesis of dengue.** *Vaccine* 2011, **29:**7221-7228.

28. Yacoub S, Mongkolsapaya J, Screaton G: **The pathogenesis of dengue.** *Curr Opin Infect Dis* 2013, **26:**284-289.

29. Schmitz J, Roehrig J, Barrett A, Hombach J: **Next generation dengue vaccines: a review of candidates in preclinical development.** *Vaccine* 2011, **29:**7276-7284.

30. Coller BA, Clements DE: **Dengue vaccines: progress and challenges.** *Curr Opin Immunol* 2011, **23:**391-398.

31. Simmons M, Nelson WM, Wu SJ, Hayes CG: **Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice.** *Am J Trop Med Hyg* 1998, **58:**655-662.

32. Hermida L, Bernardo L, Martin J, Alvarez M, Prado I, Lopez C, Sierra Bde L, Martinez R, Rodriguez R, Zulueta A, et al: **A recombinant fusion protein containing the domain III of**

the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. *Vaccine* 2006, **24:**3165-3171.

33. Khanam S, Etemad B, Khanna N, Swaminathan S: **Induction of neutralizing antibodies specific to dengue virus serotypes 2 and 4 by a bivalent antigen composed of linked envelope domains III of these two serotypes.** *Am J Trop Med Hyg* 2006, **74:**266-277.

34. Etemad B, Batra G, Raut R, Dahiya S, Khanam S, Swaminathan S, Khanna N: **An envelope domain III-based chimeric antigen produced in *Pichia pastoris* elicits neutralizing antibodies against all four dengue virus serotypes.** *Am J Trop Med Hyg* 2008, **79:**353-363.

35. Arora U, Tyagi P, Swaminathan S, Khanna N: **Virus-like particles displaying envelope domain III of dengue virus type 2 induce virus-specific antibody response in mice.** *Vaccine* 2013, **31:**873-878.

36. Jennings GT, Bachmann MF: **The coming of age of virus-like particle vaccines.** *Biol Chem* 2008, **389:**521-536.

37. Roldao A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM: **Virus-like particles in vaccine development.** *Expert Rev Vaccines* 2010, **9:**1149-1176.

38. Grgacic EV, Anderson DA: **Virus-like particles: passport to immune recognition.** *Methods* 2006, **40:**60-65.

39. Ludwig C, Wagner R: **Virus-like particles-universal molecular toolboxes.** *Curr Opin Biotechnol* 2007, **18:**537-545.

40. Hauser P, Voet P, Simoen E, Thomas HC, Petre J, De Wilde M, Stephenne J: **Immunological properties of recombinant HBsAg produced in yeast.** *Postgrad Med J* 1987, **63 Suppl 2:**83-91.

41. Gerlich WH: **Medical virology of hepatitis B: how it began and where we are now.** *Virol J* 2013, **10:**239.

42. Greiner VJ, Egele C, Oncul S, Ronzon F, Manin C, Klymchenko A, Mely Y: **Characterization of the lipid and protein organization in HBsAg viral particles by steady-state and time-resolved fluorescence spectroscopy.** *Biochimie* 2010, **92:**994-1002.

43. Gavilanes F, Gonzalez-Ros JM, Peterson DL: **Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins.** *J Biol Chem* 1982, **257:**7770-7777.

44. Patient R, Hourioux C, Roingeard P: **Morphogenesis of hepatitis B virus and its subviral envelope particles.** *Cell Microbiol* 2009, **11:**1561-1570.

45. Tiollais P, Charnay P, Vyas GN: **Biology of hepatitis B virus.** *Science* 1981, **213:**406-411.

46. Bocher WO, Herzog-Hauff S, Herr W, Heermann K, Gerken G, Meyer Zum Buschenfelde KH, Lohr HF: **Regulation of the neutralizing anti-hepatitis B surface (HBs) antibody response in vitro in HBs vaccine recipients and patients with acute or chronic hepatitis B virus (HBV) infection.** *Clin Exp Immunol* 1996, **105:**52-58.

47. Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, Heppner DG, Yuan G, Hollingdale M, Slaoui M, et al.: **Safety, immunogenicity, and efficacy of a**

recombinantly produced *Plasmodium falciparum* circumsporozoite protein-hepatitis B surface antigen subunit vaccine. *J Infect Dis* 1995, **171:**1576-1585.

48. Delpeyroux F, Chenciner N, Lim A, Malpiece Y, Blondel B, Crainic R, van der Werf S, Streeck RE: **A poliovirus neutralization epitope expressed on hybrid hepatitis B surface antigen particles.** *Science* 1986, **233:**472-475.

49. Valenzuela P, Coit D, Medina-Selby MA, Kuo CH, Van Nest G, Lyn Burke R, Bull P, Urdea MS, Graves PV: **Antigen engineering in yeast: synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1 gD particles.** *Nat Biotech* 1985, **3:**323-326.

50. Rutgers T, Gordon D, Gathoye AM, Hollingdale M, Hockmeyer W, Rosenberg M, Wilde MD: **Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of *Plasmodium falciparum*.** *Nat Biotech* 1988, **6:**1065-1070.

51. Schlienger K, Mancini M, Riviere Y, Dormont D, Tiollais P, Michel ML: **Human immunodeficiency virus type 1 major neutralizing determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates.** *J Virol* 1992, **66:**2570-2576.

52. Netter HJ, Macnaughton TB, Woo WP, Tindle R, Gowans EJ: **Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes.** *J Virol* 2001, **75:**2130-2141.

53. Bisht H, Chugh DA, Raje M, Swaminathan SS, Khanna N: **Recombinant dengue virus type 2 envelope/hepatitis B surface antigen hybrid protein expressed in *Pichia pastoris c*an function as a bivalent immunogen.** *J Biotechnol* 2002, **99:**97-110.

54. Kotiw M, Johnson M, Pandey M, Fry S, Hazell SL, Netter HJ, Good MF, Olive C: **Immunological response to parenteral vaccination with recombinant hepatitis B virus surface antigen virus-like particles expressing *Helicobacter pylori* KatA epitopes in a murine *H. pylori* challenge model.** *Clin Vaccine Immunol* 2012, **19:**268-276.

55. Higgins DR, Cregg JM: **Introduction to *Pichia pastoris*.** *Methods Mol Biol* 1998, **103:**1-15.

56. Darby RA, Cartwright SP, Dilworth MV, Bill RM: **Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review).** *Methods Mol Biol* 2012, **866:**11-23.

57. Koutz P, Davis GR, Stillman C, Barringer K, Cregg J, Thill G: **Structural comparison of the *Pichia pastoris* alcohol oxidase genes.** *Yeast* 1989, **5:**167-177.

58. Daly R, Hearn MT: **Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production.** *J Mol Recognit* 2005, **18:**119-138.

59. Vassileva A, Chugh DA, Swaminathan S, Khanna N: **Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the GAP promoter.** *J Biotechnol* 2001, **88:**21-35.

60. Porro D, Sauer M, Branduardi P, Mattanovich D: **Recombinant protein production in yeasts.** *Mol Biotechnol* 2005, **31:**245-259.

61. Gurramkonda C, Adnan A, Gabel T, Lunsdorf H, Ross A, Nemani SK, Swaminathan S, Khanna N, Rinas U: **Simple high-cell density fed-batch technique for high-level**

recombinant protein production with *Pichia pastoris*: Application to intracellular production of Hepatitis B surface antigen. *Microb Cell Fact* 2009, **8:**13.

62. Mattanovich D, Gasser B, Hohenblum H, Sauer M: **Stress in recombinant protein producing yeasts.** *J Biotechnol* 2004, **113:**121-135.

63. Chapman R, Sidrauski C, Walter P: **Intracellular signaling from the endoplasmic reticulum to the nucleus.** *Annu Rev Cell Dev Biol* 1998, **14:**459-485.

64. Buck TM, Wright CM, Brodsky JL: **The activities and function of molecular chaperones in the endoplasmic reticulum.** *Semin Cell Dev Biol* 2007, **18:**751-761.

65. Fewell SW, Travers KJ, Weissman JS, Brodsky JL: **The action of molecular chaperones in the early secretory pathway.** *Annu Rev Genet* 2001, **35:**149-191.

66. Stolz A, Wolf DH: **Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell.** *Biochim Biophys Acta* 2010, **1803:**694-705.

67. Romisch K: **Endoplasmic reticulum-associated degradation.** *Annu Rev Cell Dev Biol* 2005, **21:**435-456.

68. Gasser B, Maurer M, Rautio J, Sauer M, Bhattacharyya A, Saloheimo M, Penttila M, Mattanovich D: **Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions.** *BMC Genomics* 2007, **8:**179.

69. Vanz AL, Nimtz M, Rinas U: **Decrease of UPR- and ERAD-related proteins in *Pichia pastoris* during methanol-induced secretory insulin precursor production in controlled fed-batch cultures.** *Microb Cell Fact* 2014, **13:**23.

70. Hirsch C, Gauss R, Horn SC, Neuber O, Sommer T: **The ubiquitylation machinery of the endoplasmic reticulum.** *Nature* 2009, **458:**453-460.

71. Patil A, Khanna N: **Novel membrane extraction procedure for the purification of hepatitis B surface antigen from *Pichia pastoris*.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2012, **898:**7-14.

72. Gurramkonda C, Zahid M, Nemani SK, Adnan A, Gudi SK, Khanna N, Ebensen T, Lunsdorf H, Guzman CA, Rinas U: **Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and in vivo analysis of their immunogenic properties.** *J Chromatogr B Analyt Technol Biomed Life Sci* 2013, **940:**104-111.

73. Vanz AL, Lunsdorf H, Adnan A, Nimtz M, Gurramkonda C, Khanna N, Rinas U: **Physiological response of *Pichia pastoris* GS115 to methanol-induced high level production of the Hepatitis B surface antigen: catabolic adaptation, stress responses, and autophagic processes.** *Microb Cell Fact* 2012, **11:**103.

74. Geiler-Samerotte KA, Dion MF, Budnik BA, Wang SM, Hartl DL, Drummond DA: **Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast.** *Proc Natl Acad Sci U S A* 2011, **108:**680-685.

75. Pfeffer M, Maurer M, Kollensperger G, Hann S, Graf AB, Mattanovich D: **Modeling and measuring intracellular fluxes of secreted recombinant protein in *Pichia pastoris* with a novel 34S labeling procedure.** *Microb Cell Fact* 2011, **10:**47.

76. Boden G, Merali S: **Measurement of the increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals.** *Methods Enzymol* 2011, **489:**67-82.

77. Gusarova V, Caplan AJ, Brodsky JL, Fisher EA: **Apoprotein B degradation is promoted by the molecular chaperones Hsp90 and Hsp70.** *J Biol Chem* 2001, **276:**24891-24900.

78. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM: **Heterologous protein production using the *Pichia pastoris* expression system.** *Yeast* 2005, **22:**249-270.

79. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P: **Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.** *Cell* 2000, **101:**249-258.

80. Liu YY, Woo JH, Neville DM, Jr.: **Overexpression of an anti-CD3 immunotoxin increases expression and secretion of molecular chaperone BiP/Kar2p by *Pichia pastoris*.** *Appl Environ Microbiol* 2005, **71:**5332-5340.

9. APPENDIX I

9.1. Localization of ES and S antigen in ES_1:S_4

It was reported earlier S antigen is associated mostly with insoluble pellet fraction [71]. This would predict ES antigen may also evidence similar tendency.The distribution of ES and S antigen between soluble and insoluble fractions were analyzed by Western blotting with anti hepatitis B surface antigen specific mouse monoclonal. Cell pellets corresponding to 100 OD (1 ml) were lysed with glass beads as described previously and fractioned by centrifugation into supernatant and pellet fraction. The pellet fraction was solubilized for 4 h at RT by dissolving in 1 ml of PBS containing 8 M urea. After solubilization the supernatant from lysate and urea solubilized pellet fractiosn were analyzed by Western blotting with anti hepatitis B surface antigen specific mouse monoclonal (5S mAb). This revealed both ES and S antigen were completely insoluble in the absence of detergent or denaturants (Figure 9-1).

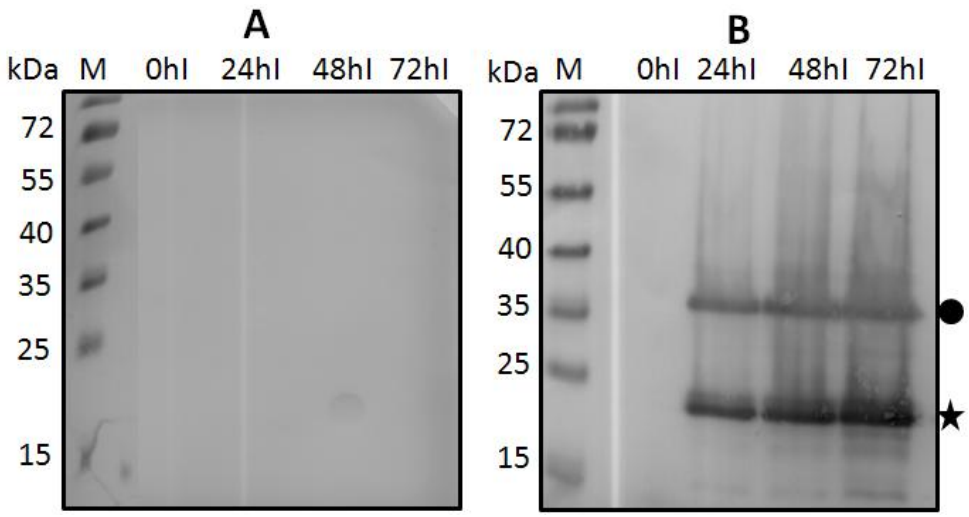

Figure 9-1: Localization of ES and S antigen in ES_1:S_4 revealed by anti heptatitis B surface antigen specific mouse monoclonal .Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol and induced at 100 OD by resuspending in complex medium (BMMY) containing 1 % (v/v) methanol at every 12 h intervals for a period of 72 h. Induced *P. pastoris* (100 OD) cell pellets during time course of induction were lysed with glass beads on thermomixer for 12 h by dissolving in 0.5 ml of lysis buffer containing 25 mM PB pH 7.2, 5mM EDTA, 500 mM NaCl and 8 % (v/v) glycerol. The total cell lysate (around 0.5 ml) was collected and beads were washed with 0.5 ml of lysis buffer and pooled with intial lysate collected earlier.The lysate was centrifuged at 16,000 x *g* and fractionated into supernatant and pellet **(A)** Immunoblot analysis of lysate fractionated supernatant taken before and after 24, 48 and 72h of methanol induction using anti hepatitis B surface antigen specific mouse monoclonal antibody. **(B)** Immunoblot analysis of lysate fractionated pellet taken before and after 24, 48 and 72h of methanol induction using anti hepatitis B surface antigen specific mouse monoclonal antibody. 10 µL of samples corresponding to supernatant or pellet fractions from different induction intervals were mixed with 10 µL of 2X Laemmli buffer and boiled for 15 mins and 10 µL were applied in the gel and electroblotted on PVDF membrane.

9.2. Time course analysis of ES_1:S_4

Recombinant cells expressing ES_1:S_4 were grown and induced as described previously in materials and methods section. Recombinant ES_1:S_4 was analyzed during induction phase with samples aliquoted at different intervals by Western blotting using anti hepatitis B surface antigen specific (5S) and anti dengue EDIII specific (24A12) mouse monoclonals. Sample before induction was used as negative control. Immunoblotting results with anti dengue EDIII specific mouse monoclonal antibody revealed ES antigen fraction in ES_1:S_4 was degraded (Figure 9-2 B). No prominent degradation can be seen in ES antigen when revealed by anti hepatitis B surface antigen specific mouse monoclonal antibody (Figure 9-2 A), we presumed this is as a result of degradation at the carboxy terminus which resulted in the loss of epitopes for detection by 5S mAb. Although there is a prominent degradation in ES antigen there is no significant change in the concentration of ES antigen during induction intervals quantified by RP-HPLC as described previously (Figure 9-2 B, C). This would infer that only a fraction of the protein is targeted for degradation and the rest gets accumulated as insoluble deposits evading degradation. The S antigen fraction expression is slightly increased during induction intervals as quantified by RP HPLC

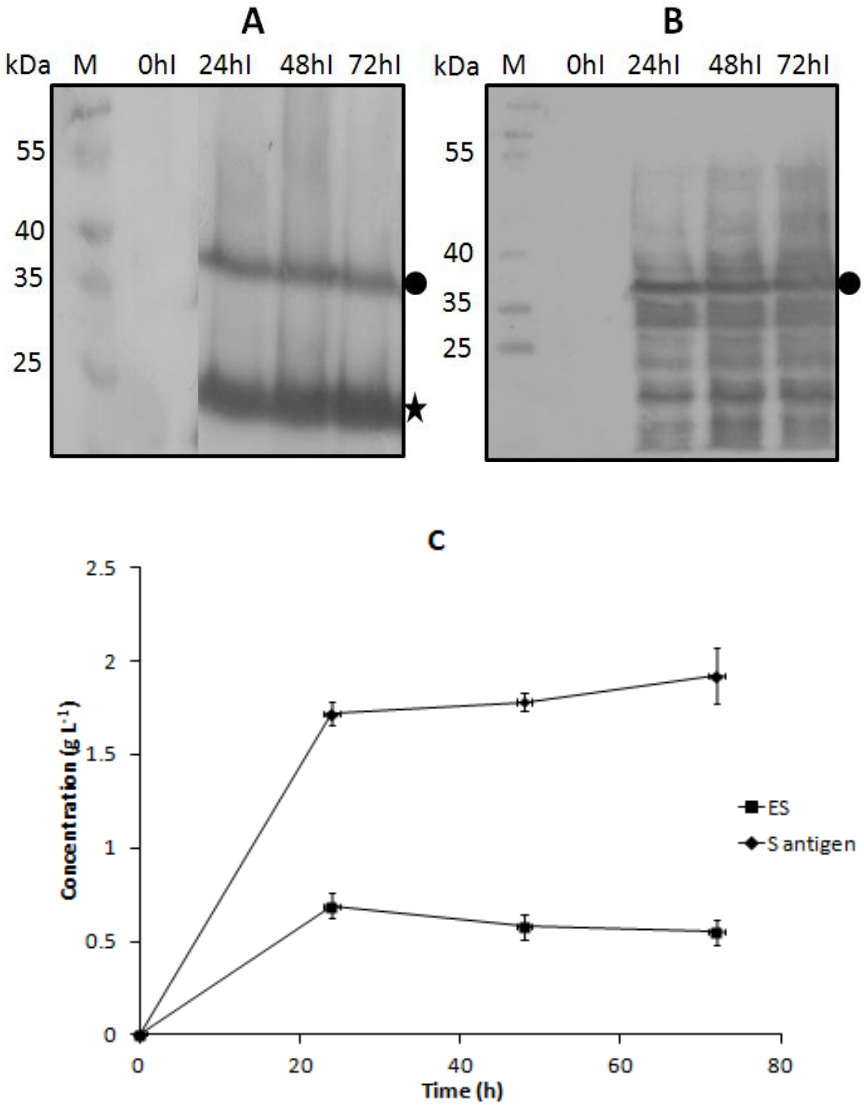

Figure 9-2: Time course analysis and quantification of ES_1:S_4 production. .Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol until OD_{600}~ 20 and resuspended in complex medium (BMMY) containing 1 % (v/v) methanol to final OD_{600}~100 and induced with 1 % (v/v) methanol at every 12 h intervals for a period of 72 h. Samples were taken before (0) and after 24, 48, 72 h of induction with 1 % (v/v) methanol. Induced *P. pastoris* 100 OD (1 ml) cell pellets during time course of induction were lysed with glass beads on thermomixer for 12 h by dissolving in 0.5 ml of lysis buffer containing 25 mM PB pH 7.2, 5mM EDTA, 500 mM NaCl and 8 % (v/v) glycerol. The total cell lysate (around 0.5 ml) was collected and beads were washed with 0.5 ml of lysis buffer and pooled with intial lysate collected earlier 10 µL of whole cell lysate was mixed with 10 µL of 2X Laemmli buffer, boiled for 15 mins and 10 µL was applied on SDS PAGE and then electroblotted on PVDF membrane. **(A)** Immunoblot analysis of ES_1:S_4 whole cell lysate taken before and after 24, 48 and 72h of methanol induction using anti hepatitis B surface antigen specific mouse monoclonal antibody.**(B)** Immunoblot analysis of ES_1:S_4 lysate taken before and after 24, 48 and 72h of methanol induction using anti dengue EDIII specific mouse monoclonal antibody. **(C)** quantification of ES and S antigen in ES_1:S_4 in whole cell lysate before and after 24, 48 and 72h of methanol induction by RP-HPLC using purified HBsAg as standard. For RP-HPLC 100 µL lysate was solubilized by adding 100 µL solubilization reagent (8 % (wv) SDS, 50 % (v/v) ß-mercaptoethanol, 1 mol L^{-1} DTT) and boiled for 15 min and clarified by centrifugation and 50 µL aliquot was loaded into C-18 column at flowrate of 1 ml min^{-1}

10. APPENDIX II

10.1. Purification of only HBsAg (ES_0:S_8)

HBsAg (ES_0:S_8) was purified from soluble fraction under native conditions as described in [72]. The chromatogram and purity profile further confirmed by Western blotting with anti hepatitis B surface antigen (5S) specific mouse monoclonal antibody is shown in Figure 10-1.

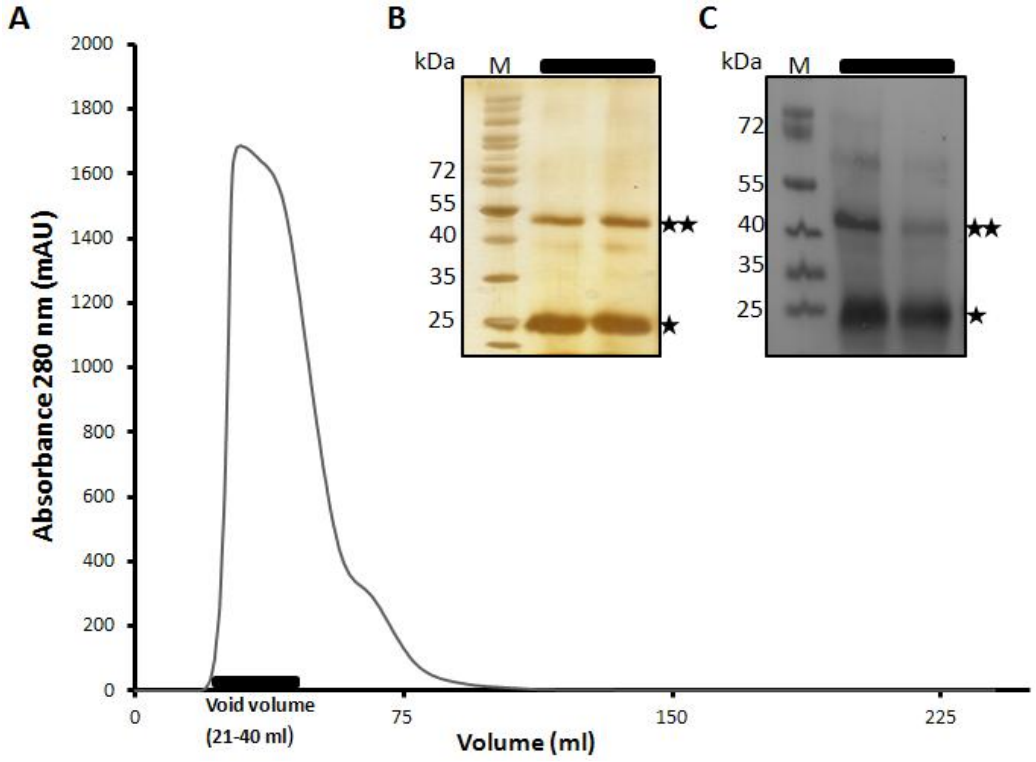

Figure 10-1: Size exclusion chromatography (Sephacryl S-300 16/60) of ES_0:S_8. **(A)** Size exclusion chromatogram resulted from passing pooled and concentrated ion exchange fractions of ES_0:S_8 onto sephacryl S-300 (16/60) column. Protein elution was measured at 280 nm. **(B)** Size exclusion peak fractions within void volume (21-40 ml) were pooled and analyzed by silver staining (15 µL of sample was loaded in each lane). **(C)** Size exclusion peak fractions within void volume (21-40 ml) were pooled and analyzed by Western blotting with 5SmAb (15 µL of pooled peak fractions containing protein was mixed with 15 µL of 2X Laemmli buffer and boiled for 15 mins and 15 µL was loaded in each lane for Silver staining and Western blotting). Single and double asterisks represent HBsAg monomer and dimer respectively. Bar corresponds to the void volume.

10.2 Purification of $ES_1:S_1$

$ES_1:S_1$ was purified precisely in the same way as $ES_1:S_4$ described previously. The chromatogram and purity profile is shown in Figure 10-2 Pure $ES_1:S_1$ was characterized by TEM and found to be forming particles (Figure 4-5 B).

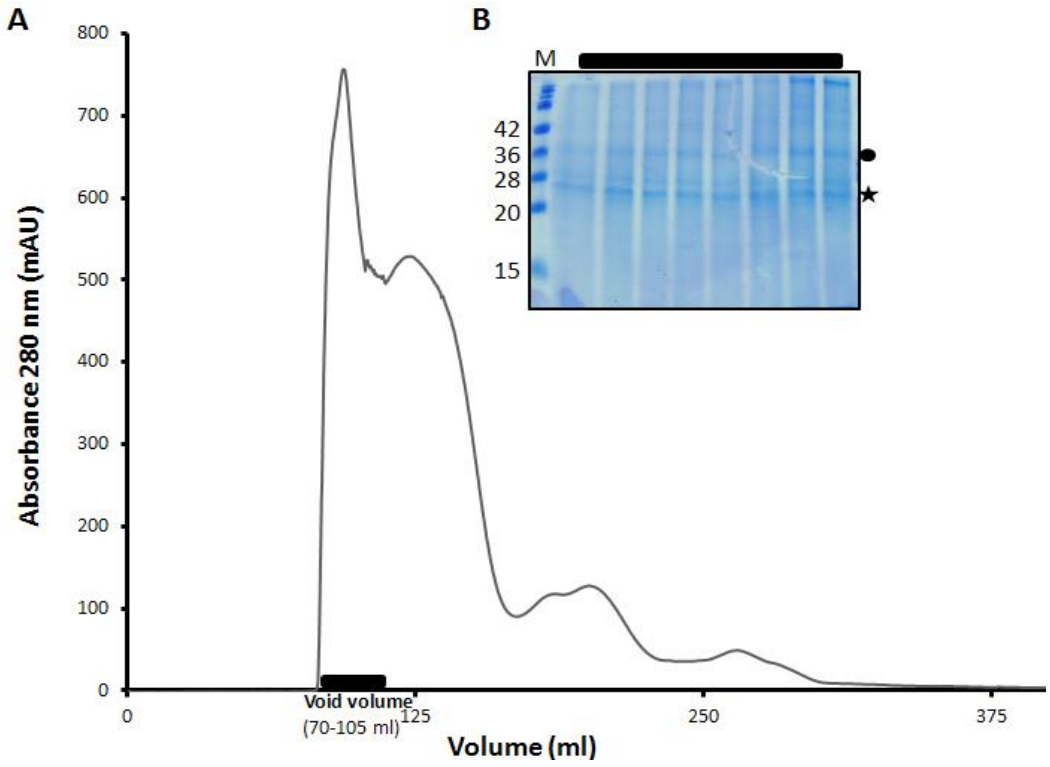

Figure 10-2: Size exclusion chromatography (Sephacryl S-300 26/60) of $ES_1:S_1$. **(A)** Size exclusion chromatogram resulted from passing pooled and concentrated ion exchange fractions of $ES_1:S_1$ onto sephacryl S-300 (26/60) column. Protein elution was measured at 280 nm. **(B)** Size exclusion peak fractions within the void volume (70-105 ml) were analyzed by Colloidal Coomassie staining. (15 µL of pooled peak fractions containing protein was mixed with 15 µL of 2X Laemmli buffer and boiled for 15 mins and 15 µL was loaded in each lane) Single asterisk represents HBsAg (S) monomer and single filled circle represents fusion protein (ES). Bar corresponds to void volume.

11. APPENDIX III

11.1 RP-HPLC Calibration curve

Calibration curve was constructed by plotting peak area as a function of protein amount injected (Figure 11-1). The obtained graph shows a good linear correlation. The known concentration of purified HBsAg (ES_0:S_8) to make external calibration curves were 0.5, 1, 2, 4 mg ml^{-1} as quantified by BCA assay. The concentration of HBsAg (S) and fusion protein were calculated by using the equation from the calibration curve.

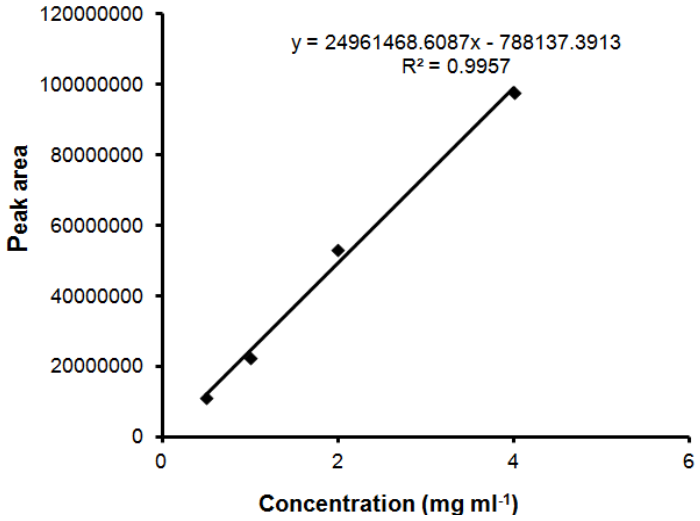

Figure 11-1: A plot of concentration (mg ml^{-1}) vs peak area of purified HBsAg (ES_0:S_8) with a linear correlation fitted using least square method.

11.2 ELISA Calibration curve

Calibration curve was constructed by plotting absorbance as a function of protein concentration (Figure 11-2). The obtained graph shows a good linear correlation. The known concentration of purified HBsAg (ES_0:S_8) to make external calibration curves were 0, 6.25, 12.5 and 25 ng ml^{-1}. The concentration of HBsAg fraction in soluble part of ES_1:S_0, ES_1:S_1, ES_1:S_4 and ES_0:S_8 were calculated by using the equation from the calibration curve.

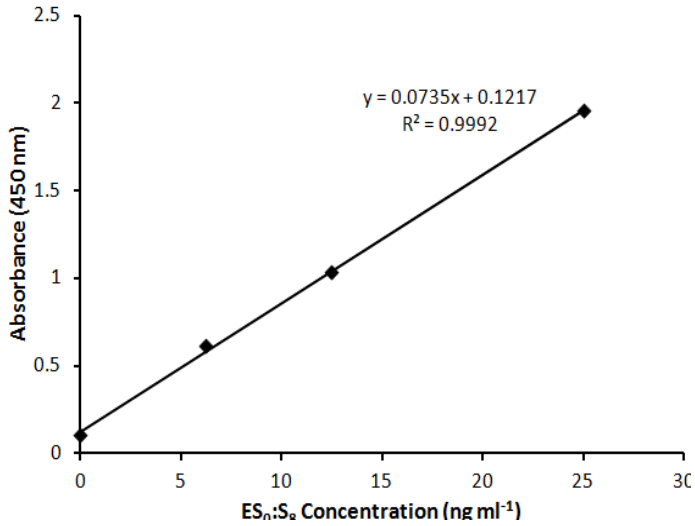

Figure 11-2: A plot of concentration (ng ml^{-1}) vs absorbance of purified HBsAg (ES_0:S_8) with a linear correlation fitted using least square method

11.3 BCA Calibration curve

Calibration curve was made by plotting absorbance as a function of protein concentration (Figure 11-3). The obtained graph shows a good linear correlation. The known concentration of pure BSA to make external calibration curves were 0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg ml^{-1}. Total protein concentration in soluble and whole lysate of ES_1:S_0, ES_1:S_4 and ES_0:S_8 in were calculated by using the equation from the calibration curve.

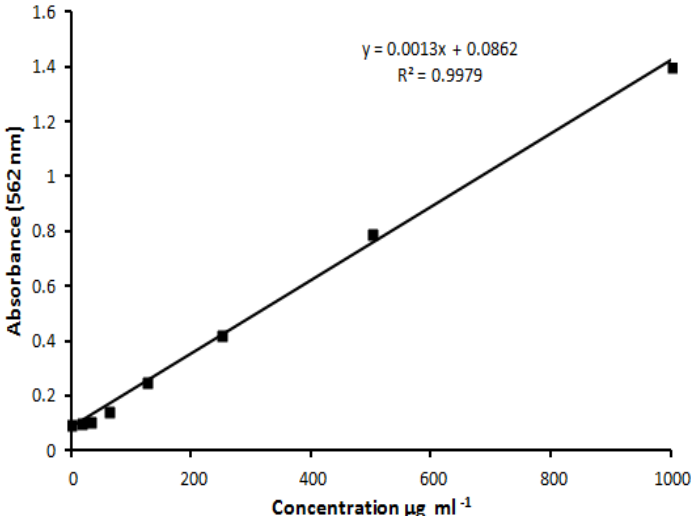

Figure 11-3: A plot of concentration (µg ml^{-1}) vs absorbance of pure BSAwith a linear correlation fitted using least square method.

12. APPENDIX IV

12.1 Expression of DENV-2-EDIII

12.1.1 Clone:

P. pastoris strain KM71H (Mut^S) harboring secretory EDIII gene used in the present work was a kind gift from Dr. Navin Khanna, ICGEB, New Delhi and India. The details of cloning of EDIII-2 can be found in [22].

12.2 Methods

12.2.1 Production of sEDIII-2 in shake flask

A starter culture was set up by inoculating YPD (100 ml) with glycerol stock cultures (100 µl) and grown at 28°C, under constant shaking in an orbiatal shaker at 250 rpm for 18 h. About 1 % of starter culture was used to inoculate 500 ml of buffered glycerol complex medium (BMGY, Invitrogen Life Technologies, Carlsbad, USA) in a 2 L baffled shake flask. The culture was incubated at 28°C, under constant shaking at 250 rpm until an OD_{600} ~20 was reached. Subsequently, cells were pelleted by centrifugation (3347 × *g*), washed with PBS, recentrifuged and resuspended in buffered methanol complex medium (BMMY, Invitrogen life technologies, Carlsbad, USA) to a final OD_{600} ~100 and incubated at 28°C under shaking at 250 rpm. Recombinant protein production was induced through the addition of 0.5 % (v/v) methanol twice a day at 12 h intervals for a total period of 72 h. At every 24 h intervals cells were fractionated by centrifugation at 16,000 x *g* and supernatant and pellet fraction were separated and stored at 80°C untill use. Both supernatant and cell pellet fractions were analyzed for expression by Western blotting. Cell pellets corresponding to 100 OD units (1 ml) from different induction intervals were lysed using glass beads as described previously in materials and methods section. Extracellular supernatant and whole cell lysate were analyzed by Western blotting with anti dengue EDIII specific mouse monoclonal

12.3 Results

12.3.1 Expression of sEDIII-2

Extracellular and lysate induction intervals were analyzed for expression of EDIII domain. Western blotting results revealed a small fraction of 12 kDa protein was secreted however prevailed in form of aggregates (Figure 12-1A). The molecular weight of EDIII retained intracellulary was about 23 kDa this could be due to presence of secretory signal (~10 kDa) along with EDIII domain (Figure 12-1B).

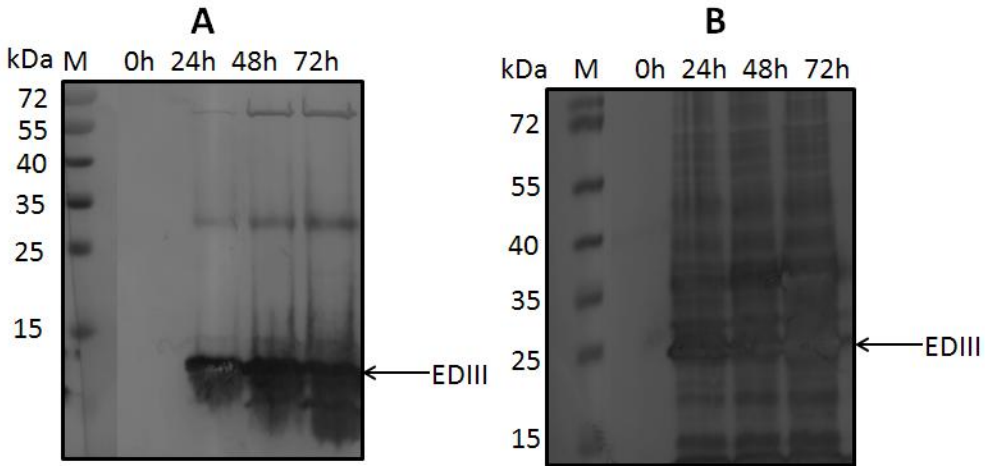

Figure 12-1: *P. pastoris* KM71H expressing sEDIII-2. Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol and .Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol until OD_{600} ~20 and resuspended in complex medium (BMMY) containing 0.5 % (v/v) methanol to a final OD_{600} ~100 and induced with 0.5 % (v/v) methanol at every 12 h intervals for a period of 72 h. Later cultures corresponding to 100 OD units (1 ml) from before (0 h) and after 24, 48 and 72 h of onset of methanol feeding were centrifuged to separate supernatant and cells. Induced *P. pastoris* 100 OD (1 ml) cell pellets during time course of induction were lysed with glass beads on thermomixer for 12 h by dissolving in 0.5 ml of lysis buffer containing 25 mM PB pH 7.2, 5mM EDTA, 500 mM NaCl and 8 % (v/v) glycerol. The total cell lysate (around 0.5 ml) was collected and beads were washed with 0.5 ml of lysis buffer and pooled with intial lysate collected earlier. 10 µL of extracellular supernatant and whole cell lysate were mixed with 10 µL of 2X Laemmli buffer, boiled for 15 mins and 10 µL was applied on SDS PAGE and then electroblotted on PVDF membrane **(A)** Western blotting of extracellular fraction from before (0 h) and fter 24, 48 and 72 h of methanol addition revealed by anti dengue EDIII specific mouse monoclonal. **(B)** Immunoblotting of intracellular whole cell lysate from before (0 h) and after 24, 48 and 72 h of methanol addition revealed by anti dengue EDIII specific mouse monoclonal. Arrow towards gel in A represent 12 kDa EDIII-2 protein and arrow towards B denotes 23 kDa EDIII with α-mating factor secretory signal.

12.3.2 Induction of UPR in *P. pastoris* KM71H sEDIII-2

The induction of UPR in cells producing sEDIII-2 was assessed by probing for UPR markers with mouse monoclonal anti HDEL antibody. These contain an ER resident signal peptide HDEL at carboxy terminus. Western blot results revealed presence of KAR2 both extracellularly and intracellularly (Figure 10-2). In case of medium there was no KAR2 found during growth in glycerol however there is increased expression of KAR2 correlated with time course production of EDIII. Intracellulary there was slight increase in KAR2 expression during induction.

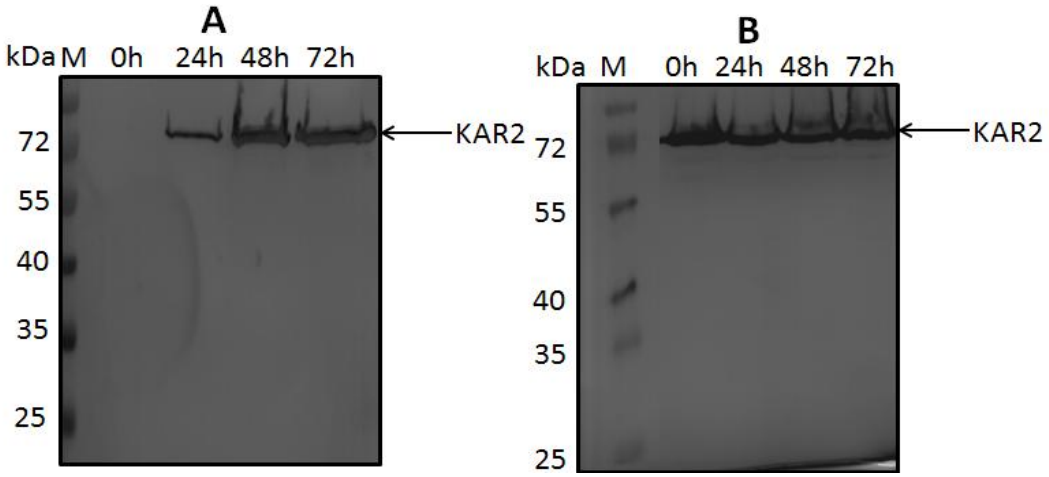

Figure 12-2: Induction of UPR response in *P. pastoris* KM71H expressing sEDIII-2. .Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol until OD_{600}~20 and resuspended in complex medium (BMMY) containing 0.5 % (v/v) methanol to a final OD_{600}~100 and induced with 0.5 % (v/v) methanol at every 12 h intervals for a period of 72 h. Later cultures corresponding to 100 OD units (1 ml) from before (0 h) and after 24, 48 and 72 h of onset of methanol feeding were centrifuged to separate supernatant and cells. Induced *P. pastoris* (100 OD) cell pellets during time course of induction were lysed with glass beads on thermomixer for 12 h by dissolving in 0.5 ml of lysis buffer containing 25 mM PB pH 7.2, 5mM EDTA, 500 mM NaCl and 8 % (v/v) glycerol. The total cell lysate (around 0.5 ml) was collected and beads were washed with 0.5 ml of lysis buffer and pooled with the initial lysate collected earlier. 10 µL of extracellular supernatant and whole cell lysate were mixed with 10 µL of 2X Laemmli buffer, boiled for 15 mins and 10 µL was applied on SDS PAGE and then electroblotted on PVDF membrane **(A)** Western blotting of extracellular fraction from before (0 h) and after 24, 48 and 72 h of methanol addition revealed by anti HDEL mouse monoclonal. **(B)** Immunoblotting of intracellular whole cell lysate from before (0 h) and after 24, 48 and 72 h of methanol addition revealed by anti HDEL mouse monoclonal. Arrow towards gel in A and B represents 74 kDa KAR2.

12.4 Discussion

Expression of EDIII-2 fused with α mating factor at the amino terminus resulted in a small fraction secreted in supernatant. Along with monomer, aggregates can also be seen in supernatant (Figure 12-1 A). Most of EDIII fraction was retained intracellular which was prevalent as aggregates. We assume that EDIII domain during expression was not able to attain native conformation, but due to the presence of secretory signal a small fraction was secreted but the most fraction retained intracellularly forming insoluble aggregates (Figure 12-1 B). This was further strengthened by leakage of KAR2 a major UPR marker indicating heretical conformations [75] in supernatant during EDIII expression. We hypothesize expression of EDIII resulted in a conformation which increased synthesis of KAR2 to such an extent that exceeded the cell's resuscitation capacity leading to secretion of KAR2 in the medium. This is in agreement with another report [80] on overexpression of anti-CD3 immunotoxin resulting in increasing expression and secretion of KAR2.

13. APPENDIX V

13.1 Growth of non producing *P. pastoris* GS115 strain and GS115 strain producing $ES_1:S_0$, $ES_1:S_4$ and $ES_0:S_8$

A growth curve during growth on glycerol as a carbon source for wild-type GS115 cells and GS115 cells producing recombinant proteins was plotted. Cells were grown in BMGY complex medium containing 1 % (v/v) glycerol until OD_{600} ~20 and resuspended in complex medium (BMMY) containing 1 % (v/v) methanol to a final OD_{600} ~100 and induced with 1 (v/v) methanol at every 12 h intervals for a period of 72 h.

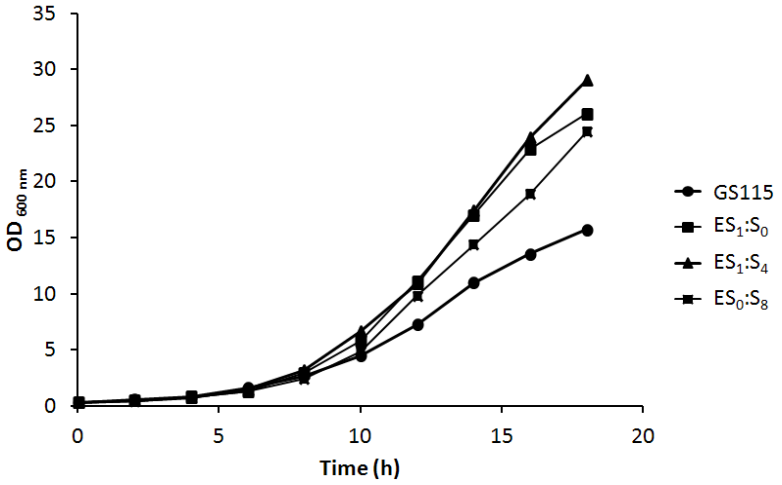

Figure 13-1: Growth curve during shake flask cultivation of non producing GS115 and GS115 strain producing recombinant proteins using glycerol as carbon source.

13.2 Quantification of total protein before and after 72 of induction in *P. pastoris* GS115 strain and GS115 strain producing $ES_1:S_0$, $ES_1:S_4$ and $ES_0:S_8$

Cell pellets corresponding to OD_{600} ~100 (1 ml) stored in -80°C from shake flask cultivation of all chimeric constructs ($ES_1:S_0$, $ES_1:ES_4$, $ES_0:S_8$ and wild-type GS115) were thawed and washed by suspending the pellet in 1ml of PBS pH, 7.2 and centrifuged at 16,000 x *g* for 10 min. The supernatant was discarded and the pellet was dissolved in 500 µL of lysis buffer (25 mM PB pH 8.0, 5 mM EDTA, 8 % (v/v) glycerol and 500 mM NaCl). To the above

suspension 0.6 g of glass beads were added and cells were disrupted in thermomixer overnight at 4°C. The lysate was transferred to a fresh vial and glass beads were washed with 0.5 ml of lysis buffer, pooled with initial lysate. The lysate was centrifuged at 16,000 x *g* for 15 min and supernatant and pellet fraction were separated. The total protein in supernatant before and after 72h of methanol induction in wild-type GS115 cells and GS115 cells producing recombinant proteins were quantified by BCA assay using BSA as standard (Table 13-1)

Table 13-1: Total protein in whole cell lysate fractionated supernatant before and after induction quantified by BCA assay with BSA as standard.

Clones	**0h (mg ml^{-1})**	**72h (mg ml^{-1})**
wt GS115	9.9	11.9
ES_1:S_0	11.9	13.3
ES_1:S_4	9.8	12.7
ES_0:S_8	9.3	11.9

Table 13-2: List of identified proteins classified by functional category

NCBI Accession-No[1]	**Gene Name[2]**	**Protein name[2]**	**MW kDa[3]**	**pI[3]**	**Mascot score**
Metabolism Carbohydrate metabolism					
Glycolysis					
XP_002491345	*TDH1*	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	35.6	6.2	172
XP_002490159	*PFK*	Beta subunit of heterooctameric phosphofructokinase involved in glycolysis [*Komagataella pastoris* GS115]	102	6.4	79
Ethanol metabolism					
XP_002491382	*ADH3*	Mitochondrial alcohol dehydrogenase isozyme III	37.3	5.8	174
Citric acid cycle					
XP_002489444	*ACO1*	Aconitase	85	5.6	215

XP_002489764	*CIT1*	Citrate synthase	51.9	7.8	200
Pyruate metabolism					
XP_002492397	*PDC*	Major of three pyruate decarboxylase	61.4	5.6	193
Amino acid metabolism					
XP_002490747	*ARO8*	Aromatic aminotransferase	54.1	5.3	184
XP_002493126	*SAHH*	S-adenosyl-L homocysteine hydrolase	49.2	5.4	149
XP_002493658	*BCAT*	Mitochondrial branched-chain amino acid aminotransferase, homolog of murine ECA39	44.9	5.3	
Sorting and degradation					
XP_002490239	*HSP90*	Heat shock protein (HSP90)	80.9	4.8	153
XP_002493991	*HSP70-HSF (SSA1)*	ATPase involved in protein folding and nuclear loca lization signal (NLS)-directed	69.7	4.1	89
XP_002489443	*ClpB*	HSP cooperates with Ydj1p (HSP40) and SSa1p (HSP70)	100.5	5.2	311
Folding and Stress response					
XP_002494292	*PDI*	Protein disulfide isomerase, multifunctional protein resident in the endoplasmic reticulum lumen [Komagataella pastoris GS115]	53	4.5	122
XP_002491027	*KAR2*	ATPase in protein import to ER and chaperone to mediate folding	74.2	4.7	322

[1] Accession numbers according to the NCBI reference standard (http://www.ncbi.nlm.nih.gov/RefSeq/). Functional classification is mostly according to KEGG pathway database (http://www.genome.jp/kegg/metabolism.html).

[2] Gene/protein names are according to the sequence genome of *P. pastoris* GS115 (http://www.uniprot.org/). If no name was found for *P. pastoris* GS115, a Blast search of the respective *P. pastoris* GS115 gene/protein against different *P. pastoris* strains was carried out in

following order: search against ATCC 76273/CBS 7435/CECT 11047/NRRL Y-11430/wegner 21-1 and *P. pastoris* (yeast). Gene and protein names were adopted from other *P. pastoris* strains only in case of 100 % sequence identity.

[3] Theoretical molecular mass and isoelectric point (pI)

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Publications

Khetarpal N[†], Poddar A[†], **Nemani SK**[†], Dhar N, Patil A, Negi P, Perween A, Viswanathan R, Lünsdorf H, Tyagi P, Raut R, Arora U, Jain SK, Rinas U, Swaminathan S, Khanna N ([†] Equal contributors). **Dengue-specific subviral nanoparticles: design, creation and characterization**. *J. Nanobiotechnol.* 2013 May 25; 11:15.

Gurramkonda C **,** Zahid M , **Nemani SK**, Adnan A , Gudi SK , Khanna N , Ebensen T **,** Lünsdorf H , CA Guzmán **,** U Rinas **. Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and in vivo analysis of their immunogenic properties.** *J Chromatogr B Analyt Technol Biomed Life Sci.* 2013 Dec 1; 940:104-11**.**

Batra G, **Nemani SK**, Tyagi P, Swaminathan S, Khanna N: **Evaluation of envelope domain III-based single chimeric tetravalent antigen and monovalent antigen mixtures for the detection of anti-dengue antibodies in human sera.** *BMC Infect Dis 2011,* 11(64):1471

2334.

Batra G, Talha SM, **Nemani SK**, Dhar N, Swaminathan S, Khanna N: **Expression, purification and characterization of in vivo biotinylated dengue virus envelope III based tetravalent antigen.** *Protein Expr Purif 2010*, 74(1):99-105.

Batra G, Gurramkonda C, **Nemani SK**, Jain SK, Swaminathan S, Khanna N: **Optimization of conditions for secretion of dengue virus type 2 envelope domain III using *Pichia pastoris*.** *J Biosci Bioeng 2010*, 110(4):408-414.

Gurramkonda C, Adnan A, Gabel T, Lunsdorf H, Ross A, **Nemani SK**, Swaminathan S, Khanna N, Rinas U: **Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: Application to intracellular production of Hepatitis B surface antigen.** *Microb Cell Fact 2009*, 8(1):13.