CHAPTER 4

DISCUSSION

Human Lymphatic filariasis is a major debilitating disease. World Health Organization has initiated the Global Program to Eliminate Lymphatic Filariasis by the year 2020 through mass drug administration and alleviation of disability (Ottesen 2000). A regional programme to combat lymphatic filariasis in the Pacific Islands is showing great promise as it reaches its halfway point. The Pacific Programme to Eliminate Lymphatic Filariasis (PacELF), established in 1999, aims to eliminate the disease from the Pacific by 2010, ten years ahead of the global target. Certainly much emphasis is given to easy, rapid and early diagnosis of the filariasis especially in individuals having new exposure to infections. WHO recommends baseline surveys using kits to assess prevalence of infection, elimination activities in countries that show prevalence of 1% by Mass Drug Administration and mosquito control (WHO 2005).

There has been an extensive research on diagnostic development and evaluation by several researchers. A simple and sensitive rapid immunodiagnostic method for detection of antigen and antibody for the ‘differential diagnosis’ of filarial infections is an imminent need. An unmistakable method of diagnosis of lymphatic filariasis has been the direct confirmation of microfilariae in blood by geimsa stained smears by microscopy (Khamboonruang et al 1987, Sabry 1992), knot’s concentration and membrane filtration techniques. However such techniques may become
less sensitive since microfilariae can be present in very low numbers and inaccessible, absent as in prepatent cases or show nocturnal periodicity.

The advent of recombinant DNA technology has paved way for construction of gene libraries or cDNA of parasite DNA. Such libraries were screened with either *B. malayi* or *W. bancrofti* infected patient’s sera or specific DNA probes to pick up antigen which are of diagnostic importance.

Several important contributions have been made in this field of research and some are herewith highlighted. An initial work of immunoscreening of λgt11 cDNA library of *B. malayi* adult parasite with MF sera resulted in the identification of clone expressing an antigen (SXP-1) which has been used in diagnosing individuals with patent infection (Dissanayake et al 1992). Another recombinant clone derived from *Brugia malayi* BmM14 fused to GST-tag was developed to measure the IgG4 levels in the different clinical groups (Ramzy et al 1995). However it was shown that the IgG4 levels do not correlate with the level of microfilariae or mf-antigen measured in bancroftian filariasis (Ramzy et al 1995).


Studies have clearly shown that filarial specific antibodies are the direct index of the degree and duration of filarial exposure in endemic populations (Mahanty et al 1994). Maizels et al (1995) found that the level of IgG4 antibody correlates with microfilariae counts. The recombinant antigen based rapid IgG4 antibody ELISA (Rahmah et al 2001) and Brugia rapid-dipstick test (Rahmah et al 2003) has been developed for the detection of
antibodies in sera of patients with brugian infection. However, this test showed only 54.5% positive reaction in *W. bancrofti*-infected individuals.

The antibody assays can be used extensively for diagnosis of clinical cases in travelers and expatriates, primary surveillance in areas of unknown endemicity, detection of early infection in childhood and monitoring of chempotherapeutic programs. However, the antibody tests have limitations in being unable to discriminate between past exposure and current infection.

4.1 DEVELOPMENT OF *rWbSXP-1* RAPID ANTIBODY TESTS FOR THE DIAGNOSIS OF BANCROFTIAN AND BRUGIAN FILARIASIS

The available commercial kits for filarial diagnosis are expensive and may not suit for developing and underdeveloped countries endemic to the disease. Hence, there is an immediate need to develop a cost effective rapid, simple and sensitive immunodiagnostic method for the detection of antigen and antibody for the differential diagnosis of filarial infections in individuals having an exposure to infection. An important factor to be considered in developing diagnostic assay is that the assay should be suitable to field conditions and simple to perform even by unskilled field workers.

An orthologue of *BmSXP-1* was identified from *W. bancrofti* L3 cDNA library using *BmSXP-1* specific DNA primers as a probe (Rao et al 2000). The identified *WbSXP-1* gene (Accession no. AF098861) was cloned in *EcoRI* site of *pRSETB* vector and was expressed in *E.coli* BL21 to derive the diagnostic antigen *rWbSXP-1* (Rao et al 2000). The identification of *WbSXP-1* set a platform for the development of a specific diagnostic method to detect both Brugian and Bancroftian filariasis. In a previous study, development of *rWbSXP-1* specific ELISA with predominantly IgG4 antibodies and a detection method for circulating filarial antigen in
bancroftian and brugian filariasis using monospecific polyclonal antibodies were demonstrated (Lalitha et al 1998, 2002).

The rWbSXP-1 antigen based simple and rapid format for antibody flow through assay of human lymphatic filariasis was developed successfully developed by Basker et al (2004). The kit underwent extensive evaluation in India (Basker et al 2004) and global WHO surveillance (Lammie et al 2004). The kit showed with 91% sensitivity and 100% specificity in both these evaluation. The rapid kit was suitable to finger prick blood samples collected on filter paper (Basker et al 2004). However, Lammie et al (2004) have also emphasized on making antibody flow through kits to detect even low levels of antibody reactivity. Thus, the potential of the protein made it imminent to investigate the strategies to suit industrial or commercial production in large scale.

The technology transfer for production, quality control and application of recombinant antigen rWbSXP-1 was done as per the MoU between CBT, Anna University and Span Diagnostics Ltd. The ‘MF-Signal: rapid kit format’ was originally developed under the memorandum of understanding between Centre for Biotechnology Anna University, Chennai and SPAN Diagnostics Ltd., Surat, Gujarat, India.

In the present study, an attempt was made to optimize the conditions and development of methodology for large-scale production and purification of rWbSXP-1 protein to suit commercial application. Attempt was made to optimize and enhance the sensitivity and effectiveness of diagnostic kit developed for field application. The purified rWbSXP-1 was also used for development of a monoclonal antibodies for the detection of circulating SXP antigen in both bancroftian and brugian filariasis.
4.2 CHOICE OF HOST AND MEDIA OPTIMIZATION FOR PRODUCTION OF rWbSXp-1 PROTEIN

For economic production of this protein in large scale, a novel osmotically inducible *Escherichia coli* GJ1158 with NaCl as inducer was attempted. Initial expression studies with pRSETB: *Wb* SXP-1 in *E.coli* BL21 and GJ1158 showed some difference in the basal level expression. The salt inducible *proUp* promoter integrated in GJ1158 (Bhandari et al 1997) originally was sought to minimize uninduced T7 polymerase expression and to enhance protein yield. However, the expression was studied in LB/ON medium and the study mentions non-occurrence of basal expression and with apparently no requirement for pLysS (Studier 1990), during recombinant protein expression in GJ1158. However, in the GJ1158 cultivation there occurred basal level expression of protein even without induction at slightly higher cell density. Though all salts or ionic species could increase the osmolarity of the medium, only some media salts, e.g. Phosphates, are utilized during growth. Continuous cultivation of *E. coli* in basal M9 media was studied under dilution rates of 0.3 and 0.5 D. At steady states different media components were pulsed and media shifted using method adopted by Fiester et al (1980). Several works have been done in media and growth kinetics in batch or fed batch and they are often observed to be recombinant host organism specific (Friehs et al 1993, Flaschel and Ferenci 1999). Use of Glucose, yeast extract, trace metals were observed to be positive in *E.coli* growth. However to ascertain the concentration of glucose, yeast extract, the modified M9 with different C:N ratio in the form of glucose and yeast extract were batch cultivated in fermenter. The growth kinetics showed that the use of yeast extract reduced the oscillation in growth rates. Good expression results were obtained during batch operation with modified M9 media supporting low cell densities, which was comparable with *E.coli* BL21.
4.3 HIGH CELL DENSITY CULTIVATION OF *E.coli* GJ1158 FOR EXPRESSION OF rWbSXP-1

Since most proteins are intracellular in the recombinant *E.coli*, productivity is proportional to the cell density and the specific productivity (Fuchs et al 2002). In fed batches the substrate concentration is maintained by optimal feeding, for total consumption by the cells. *E.coli* can also adapt to low growth rate at low glucose concentration (Natarajan et al 1999, Riesenberg et al 1999, Lin et al 2000). Also, growth rate maintenance is very much essential for controlling the recombinant *E.coli* cultivation, which is helpful in maintaining the plasmid copy number and stability (Graberr 2002).

However, fed batch cultivation with osmotically inducible recombinant *E.coli* GJ1158 did not increase productivity in spite of optimized feeding which was expected to maintain the growth rate. In repeated fed batch cultivations it was found that the recombinant protein was expressed in the cultivation due to high basal level expression at higher cell densities, induced by the possible accumulation of metabolites produced during the growth. Over expression of recombinant protein in *E.coli* causes growth retardation due to metabolic imbalance and burden (Bentley et al 1990, 1991, OhMinkyu et al 2000, Sanchez et al 2002).

4.4 COMPARISON OF PROTEIN PRODUCTION BETWEEN *E.coli* GJ1158 AND BL21

In the present work, the over expression of *Wb*-SXP1 in osmotically inducible *E.coli* GJ1158 in modified M9 medium did not produce inclusion bodies. The non-formation of inclusion bodies for a recombinant protein in GJ1158 was earlier reported by Bhandari et al (1997) using NaCl induction protocol. In our work, the rWbSXP-1 expression was optimized the protein was predominantly available in soluble form and was easily purified using
‘Immobilized Metal Affinity Chromatography’ under native conditions (Janardhan et al 2007).

# 4.5 STUDY ON BASAL LEVEL OR LEAKY EXPRESSION DURING GJ1158 CULTIVATION

The T7 expression system is known for leaky expression, which could be prevented by the co-transformation with pLys(s) (Studier et al 1990). The use of T7 lysozyme coded in plasmid pLys(S) is to inhibit T7 RNA polymerase transcription in the cells. However, the act of T7 lysozyme may reduce the expression level of cloned genes, and the use of these plasmids is also restricted in some aspects (Studier et al 1990). In our study we have tried both with osmotically favorable environment and the use of pLys(S). pLys(S) uses chloramphenicol as antibiotic marker which is known for its influences in plasmid amplification.

However, in this study ineffectiveness of pLys(S) in osmotically inducible *E.coli* GJ1158 made it evident that the leaky expression was mainly due to secreted metabolites. The results were further substantiated by the conductivity data obtained during batch cultivations of *E.coli* GJ1158. The conductivity (or in other words osmolarity) increased steadily with increase in biomass.

In the over expression of recombinant protein using *E.coli* GJ1158 in LB/ON medium no basal level expression was observed (induced at 0.6 OD) with out co-transformation of pLysS (Bhandari et al 1997). Similar results were obtained with Wb-SXP-1 protein over expression in LB/ON at same cell density. The inclusion body formation was observed to minimum during protein expression. However, basal level expression was observed in the uninduced cultures at higher cell densities, at 2.0 OD. The rWbSXP-1 results followed the same trend in modified basal M9 medium without NaCl.
Also the metabolic burden in dual plasmid systems has profound effect on \textit{E.coli} growth.

There has been a report that, the of basal level expression in \textit{E.coli} BL21(SI) and \textit{E.coli} BL21(DE3) is very high when grown in LBON and LB medium respectively (Ana 2002) contrary to Bhandari et al (1997).

Additionally, to understand the plasmid copy increase during cultivation, there are works reported in ColE1-derived, \textit{tac} promoter based plasmids containing different recombinant genes, amplification occurred following induction with IPTG, but no amplification occurred if product formation was not induced.

The plasmid in recombinant \textit{E. coli} were shown to have increased plasmid copies three- to six fold within a short period in glucose-limited batch fed-batch cultivations. The amplification was not caused by the toxic effect of IPTG, but was related to a strong inhibition of translation and chromosomal replication after the induction of heterologous gene expression. Similar to the amplification after chloramphenicol addition, ColE1 derived plasmid replication proceeded even if \textit{oriC} replication and translation were inhibited following strong induction of a recombinant gene. In accordance with the effect of chloramphenicol, the level of ppGpp, which is a negative regulator of ColE1 derived plasmid replication, decreased after induction. Plasmid amplification is sometimes used to increase the formation of recombinant product by a higher gene copy number or gene dosage (e.g. run away systems). Moreover, ColE1-related plasmids are amplified during the production of recombinant proteins as long as product synthesis leads to growth inhibition. Plasmid amplification was observed for different products and was found to be independent of the strain and the inductor, but dependent on the replication mode of the plasmid origin (Dong 1995, Rinas 1996, Teich 1998).
4.6 CONTINUOUS CULTIVATION OF *E.coli* GJ1158 AND CULTURE STABILITY

Generally, in continuous culture of microbial cells, the cell concentration in a fermentor is directly related to the product concentration, and the productivity of the system (Silva et al 1995, Moueddeb et al 1996, Becker et al 1997). It had been well shown that during cultivation, productivity in *E.coli* cells is plasmid linked (Demain 2000, Sanchez et al 2002, Graberr et al 2002).

In the present work, during the continuous cultivation operation with modified minimal (M9) medium, the recombinant cell behavior was observed with respect to various dilution rates. In continuous cultivation with osmotically inducible recombinant *E.coli*, the recombinant protein expression decreased with increasing dilution rates, leading to better plasmid stability at higher dilution rate. This was mainly related to the dilution and removal of metabolites produced during the growth phase.

The effect of plasmid mediated burden on plasmid instability or maintenance have been reported in chemostat culture of recombinant *E. coli*. The chemostat population became rapidly heterogeneous and the competition, in terms of burden and selection among evolved strains was found to be crucial for kinetics of plasmid loss (Chew et al 1988, Kysilk et al 1992, Ronald et al 1990).

4.7 SECRETED METABOLITES AND INCREASE IN MEDIA OSMOLARITY

It has been commonly observed that gratuitous overexpression of proteins in *E. coli* causes growth retardation. However, the molecular events
involved in the metabolic response to the over expression of proteins is largely unclear.

The protein productivity is also affected by the metabolic properties of *E.coli* strains, especially the acetate metabolizing capacity (Shiloach 1996, Michele 1998, Sanchez et al 2002). Phue et al (2004), has shown using northern blot analysis that the glyoxalate shunt and acetyl-Co-A synthethase are constitutively expressed in *E.coli* BL21 while same functions are repressed in *E.coli* JM109 when cultivated in both low and high glucose regimes. Glyoxalate shunt was repressed in JM109 even when acetate concentration was greater than 5 g/L while it was expressed in BL21 when acetate level was only 1 g/L.

In our investigation, the ionic metabolites secreted during GJ1158 cultivation, predominantly acetate increased medium osmolarity and influenced the osmo-responsive proUp promoter causing basal expression and burden. Thus, GJ1158 could not effectively metabolize acetate and the basal expression due to increase in osmolarity owing to the accumulation of secreted metabolites was equivalent to induction with 100 mM NaCl. Although, the original report (Bhandari et al 1997) suggests a final 300 mM concentration of NaCl for a strong induction, the expression was apparent at 100 mM NaCl concentration and the rate of expression varied with salts concentration.

The major metabolic acids in the fermentation broth, formed during the fermentation, namely, pyruvic, succinic, acetic, propionic and lactic acids were identified and analyzed by HPLC (Yang et al 2005). DNA micro- array technologies have been used to characterize the changes in transcriptional patterns of selected host genes during protein overexpression (Oh et al 2000). About 132 E. coli genes, including those in the central metabolism, key biosynthetic pathways, and selected regulatory functions, were used as probes.
for detecting the level of mRNA transcripts in *E. coli* strains JM109, MC4100, and VJS676A during protein overexpression. Upon induction, these strains shared several common responses, such as the upregulation of *glk* and the heat shock genes as well as the downregulation of *fba, ppc, atpA*, and *gnd*. In addition, the biosynthesis genes *glnA, glyA*, and *leuA* were down-regulated in all three strains.

Importantly media-dependent responses were also observed and studied. For example, many respiratory genes that were upregulated in defined media showed an opposite effect in complex media under protein-overproducing conditions. These results demonstrate that gratuitous overexpression of proteins triggers a complex global response that involves several metabolic and regulatory systems. Explanations based on either existing knowledge of global regulations such as the heat shock response and the stringent response or stoichiometric analysis without regulatory considerations cannot account for the response induced by protein overexpression (MinOh et al 2000). In another *E.coli* strain MG1655, functional genomic expression pattern for about 4290 coding genes has been studied in late log phase culture grown in minimal glucose and LB with glucose medium. The hallmark of the work when *E. coli* is grown in minimal glucose medium, where the biosynthetic pathway genes the switched on, including amino acid biosynthesis and formation and excretion of acetate (Tao et al 1999, Phue et al 2004). Thus, variations may need to be extensively studied and understood on basis of *E.coli* strains and the media used.

### 4.8 NEED FOR MODIFICATION OF PROCESS AND FED BATCH CULTIVATION FOR GJ1158

The main problems arising from high cell density cultivation are high oxygen and substrates uptake rates and accumulation of low molecular, growth-inhibiting metabolites in the cell suspension during the cultivation.
Metabolite accumulation such as acetate, pyruvate etc is a common problem in HCDC of recombinant *E.coli* but varies from strain to strain (Rocha et al 2002). Even though reports with other *E.coli* strains show that growth rate was reduced with accumulation of metabolites like acetate and pyruvate in medium with excess glucose and under oxygen limiting conditions (Natarajan et al 1999, Akesson et al 2001, Rocha et al 2002, Phue et al 2004). In salt inducing *E.coli* GJ 1158 a different problem was encountered in batch cultivation signifying that, the metabolic overflow created osmotic induction and expression of recombinant *Wb*-SXP1 with decrease in growth rate. A number of fermentation processes have been developed to remove metabolites from fermentation broth using membrane recycle (Crespo et al 1991, Sarad et al 1994, Won et al 1997, McIntyre et al 1999, Miyano et al 2000, Fuchs et al 2001). To increase the cell density in a fermentor, filters or type of separating equipment is needed in the effluent stream to filter and return the cells to the fermentor. Recycling facilities of this type, such as microfilters have some limitations in terms of larger industrial scale applications. Membrane fouling during long term operation results in reducing membrane capacity, and requires replacement membranes, especially in the fermentation industry (Hawkes 1996, Simon 2000).

In several works, continuous or cell retention cultures have been started as batch culture for few hours followed by continuous culture. The medium was pumped to the fermentor at a constant dilution rate of 0.1 to 0.07 h\(^{-1}\) for several hours till a steady state was reached. The continuous fermentation with cell recycling was carried out for days. The recycle rate of the liquid flow back to the fermentor was often set at different rates i.e, 1.5 to 2 times of the medium feed rate of the liquid flow to the fermentor. The important parameters of continuous fermentation, namely, the yield coefficient and productivity were obtained after the cell concentration, and
glucose and concentrations after the steady state had been reached (Nataraj et al 1999, Won et al,1997 Hewitt et al 2000).

4.9 HCDC OF RECOMBINANT GJ1158 BY TOTAL CELL RETENTION CULTURE

To minimize the formation of inhibitors, providing optimal growth conditions is essential. Many reports suggest the removal of inhibitors, toxins and metabolites were achieved using fermentation with cell recycle which resulted in an increased productivity because of dilution or removal of many unidentified growth inhibiting components in the process (Miyano et al 2000, Fuchs et al 2001). Therefore, an attempt was made to raise high cell densities with total cell retention culture with membrane recycle where the metabolites are removed continuously while feeding the nutrients simultaneously.

Broth circulation flow rate, dilution rate and cell bleed rate are identified as the most important process variables that affect process performance (Crespo et al 1991, Sarad et al 1994, Won et al 1997, McIntyre et al 1999, Miyano et al 2000, Fuchs et al 2001). The other important process related factors are addition of antifoaming agent i.e, for instance use of polypropylene glycol higher than 300 ppm have a slight negative impact on growth rate (Koch et al 1995). Most importantly is the maintenance of dissolved oxygen levels during TCRC. The selection criteria for these process variables depend upon the process, organism and the product on case-by-case basis. In GJ1158 TCRC system, cell bleed was not done, instead total recycle mode was preferred as the objective of experiment to remove metabolites secreted and maintain the osmolarity of the medium at low level. The circulation rate was selected based on the continuous cultivation experiments where the metabolite induced protein expression was alleviated. The medium conductivity was monitored continuously and was maintained at a possible lowest level as at the start of the cultivation. As a consequence, during the
TCRC the recombinant *E. coli* system was not self-inducing which was clearly indicated by the protein profile during time course. The *Wb*-SXP1 basal level expression was controlled and well regulated in TCRC, resulting in significant increase in biomass.

This investigation suggests increased biomass productivity with acceptable recombinant protein expression can be achieved using osmotically inducible *E. coli* by elimination of unidentified growth inhibiting small ionic metabolites or molecules from the cell culture during the fermentation. This work demonstrated a successful high cell density fermentation process with an external membrane module in laboratory scale for an osmotically induced *E. coli* system.

The use of NaCl induction protocol makes this system economical with expression levels on par to *E. coli* BL21. Moreover, on over production, the protein did not form inclusion bodies and remained in soluble form, making the purification by immobilized metal affinity chromatography easy. The Further investigation of the regulatory mechanism in physiological pathways in osmotically inducing *E. coli* host would greatly contribute to strain improvement and protein overproduction.

The advantage with protein over-expression in osmotically inducible GJ1158 was that inclusion body formation was three times less (Janardhan et al 2007).

4.10 **EXTENSION OF BATCH CULTIVATION FROM 3.0 L TO 30 L BIOREACTOR**

Moreover, in this work modified airlift bioreactors (ALB) were opted. ALB’s are economical and energy efficient compared to CSTR’s (Tiejun et al 2006). In glucose based media oscillations in growth rate affects
productivity, thus it is important to design media depending on the *E.coli* strain for the avoidance of acetate accumulation (Bentley 1990, Akesson 2001, Rocha 2001). The recombinant rWbSXP-1 protein expression in GJ1158 batch cultivation with modified M9 medium in 30 L ALB was successful and consistent.

Interestingly production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), by fed-batch culture of recombinant *Escherichia coli* harboring a plasmid containing the polyhydroxyalkanoate (PHA) biosynthesis genes in a pilot-scale fermentors with air supply only has been reported. The 30 L fermentor had a KLa value of 0.11 s⁻¹, showed a productivity of 1.37 g P(3HB/V)/L-h when compared to 300 L fermentor having a KLa of 0.03 s⁻¹, and a productivity of 1.06 g P(3HB/V)/L-h. These results suggest that economical production of P(3HB/V) is possible by fed-batch culture of recombinant *E. coli* in a large-scale fermentor having low KLa value (Jong-il 2002).

### 4.11 EFFECTIVE DOWNSTREAM PROCESSING FOR PURIFICATION OF rWbSXP-1 PROTEIN

To cover large endemic areas for surveillance, developing an economical production of rWbSXP-1 based diagnostic kits was critical for extending the technology to industrial application. Despite the fact that large-scale cultivation was successful with GJ1158, there were other critical aspects like application of protein for diagnostics, testing and evaluation. Though the kit developed by Basker (2004) was a great success, Lammie et al (2004) has recommended that, the antibody kits in general should be capable of detecting even low levels of antibody at initial level of infection or exposure to disease. Thus, the sensitivity was needed to be improved further to detect weakly reactive serum antibodies. It was very important that purity of the protein was of highest quality for effective results.
Further it was important to work on all aspects of downstream processing and protein purification. Many cell disruption methods such as sonication, french pressure and enzyme treatment have been reported for *E.coli* (Asenjo 1990, Ana 2002, Wolfgang 2007). The purification of any protein by chromatography and number of steps required varies and depends on the nature of protein itself (Liesiene et al 1997, Lammotte 2005). Multi-step purification strategies reduce the recovery of the target protein though the purity may improve tremendously (Sulkowski 1996).

In an work with temperature-inducible expression *E. coli* system recombinant human bone morphogenetic protein-2 (rhBMP-2) produced as a non-active aggregated form using a high concentrations of both biomass and inactive rhBMP-2 (8.6 gL$^{-1}$) during high-cell-density cultivation. However after washing and solubilizing the inclusion bodies, rhBMP-2 was refolded and dimerized at by means of a simple dilution method with yields only around 50% and the purification procedure based on affinity chromatography gave yields of 10 mg rhBMP-2 dimer per gram cell dry weight (Luis 2002).

In an interesting work in *E.coli* BL21(SI) the level of purified recombinant protein recovered was several fold higher than from *E.coli* BL21(DE3), when grown in LBON and LB medium respectively. However, the recovery of protein during urea solubilization, purification and refolding has been reported to be around 10% of the total expressed protein (Ana 2002). Shin et al (2002) working in *E.coli* BL21 had reported 20-30% recovery of a recombinant transglutaminase enzyme though a 100-fold increase in activity was seen after purification compared to bacterial lysate. Thus much importance is on type of *E.coli* and the optimal downstream processing conditions for protein purification.

The purity and quality of rWbSXP-1 was observed to be very critical in performance of the diagnostic kit. The purification of rWbSXP-1 protein,
although was successful in 2.5 mL bed volume, posed problem in achieving purity at higher bed volumes. In addition during IMAC, the recovery of rWbSXP-1 from soluble fraction was significantly high compared to urea solubilization.

Thus, in our study the rWbSXP-1 largely available in soluble fraction was considered for further purification experiments in this work. The inclusion of anion exchange chromatography step after the IMAC considerably enhanced the purity of the rWbSXP-1, but an increased loss was also observed. Remarkably, the purity and recovery of the rWbSXP-1 greatly improved with the inclusion of gel filtration after IMAC. The estimated loss of rWbSXP-1 recovered after IMAC followed by gel filtration was 25% as compared to 31% in anion exchange. Considering the maximum rWbSXP-1 released into soluble fraction, the loss after IMAC and gel filtration was 5%. The quality of purified rWbSXP-1 protein significantly improved to meet the manufacturing requirements in diagnostic industry.

4.12 ENHANCEMENT OF SENSITIVITY IN rWbSXP-1 BASED RAPID KIT

Further, the sensitivity of rapid diagnostic kit was considerably improved to detect even weak exposure to lymphatic filarial infections. The enhanced rapid assay was particularly sensitive in identifying the degree of exposure by virtue of the antibody reactivity and titre related spot intensity. The enhancement of spot size (0.5 μg/0.7 μl) and sensitivity based spot intensity was a key development in assessing the antibody reactivity and exposure in individuals or MF positive cases, compared to a less sufficient results of mere positive or negative tests as described previously (Basker et al 2004). Thus, the rapid test will be useful in providing status of endemic population for MDA programs and its withdrawal for early or newly infected individuals were the antibody level or reactivity is low. During evaluation
studies the immuno-characteristics and response of purified rWbSXP-1 in detecting anti-SXP antibodies in specific cases of microfilareamic positive serum and from healthy test groups were satisfactory as shown in results.

4.13 ACCELERATED STABILITY TESTING OF THE RAPID KIT

Stability of rWbSXP-1 protein based rapid flow through antibody assay kit was incompliance with European pharmacopoeia and WHO-cGMP regulations for manufacturing. The kit was stable if stored at 4°C for a period of one year. We successfully optimized protein purification in technical scale considering the future applications. The performance of improved rapid flow through antibody assay for diagnosis of human lymphatic filariasis was more sensitive and satisfactory. This work is expected to suit the conditions in developing and under developed endemic countries where the diagnosis, surveillance and eradication of the disease is an important concern.

4.14 DEVELOPMENT OF MONOCLONAL ANTIBODY AGAINST rWbSXP-1 ANTIGEN

The rapid diagnostic procedure of human lymphatic filariasis is very essential and emphasized by WHO for proper surveillance and eradication of the disease. Out of 1.2 billion cases Wb accounts for greater 90% of total filarial infections. Parasite adults lodges in the lymphatic system, which is crucial for maintaining the tissue fluid balance of the body and has a major role in the immune system. Infection is usually acquired in childhood and although infected many individuals never show outward symptoms (WHO 2005). Assays should be easy-to-use, rapid tests to identify adult filarial worm antigen within a few minutes using a finger-prick blood sample. Thus, these can be used to help assess prevalence, map the disease and measure the impact of MDA programmes.
At present, there is commercial assay currently available to detect *brugian* infections and two other diagnostic tests, Og4C3 ELISA (More and Copeman 1990, Weil et al 1997) and an immunochromatographic card test (ICT) (Weil et al 1997) are available to detect circulating antigens of *W.bancrofti* in serum/whole blood.

Sensitivity assessment of the ICT card test in detecting microfilaria carriers was studied in comparison with Og4C3 ELISA and conventional microscopic techniques (Pani et al 2004). Though, the sensitivity of the ICT test was good among microfilaria carriers, the format also reported 25% of microfilaria-negative individuals as being positive for circulating filarial antigens and the diagnostic lines were not stable beyond few minutes (particularly in the case of amicrofilaraemic persons). The ICT showed lower sensitivity (71.2%) compared to the filtration technique and requires further improvement (Pani et al 2000) The sensitivity of the Og4C3 test was lower than that of ICT test which is comparatively more sensitive in detecting microfilaria carriers in endemic communities. Moreover, the main drawback is both are not effective in detection of *B.malayi* antigens. Thus, improvement factor in the formats to provide stable diagnostic lines, specificity of the formats to detect *brugian* and *bancroftian* parasites and costs of the test kit and ELISA formats are to be considered before its large-scale use (Pani et al 2004). Hence, it may be argued that neither Og4C3 nor ICT can be considered ideal and standard tests and costly for economic situation existing in developing countries endemic to disease.

In this situation, we identified recombinant *Wuchereria bancrofti* SXP-1 (rWbSXP-1) protein antigen, an orthologue of *BmSXP-1* and developed an immunoassay for diagnosis. The assay could detect circulating antibodies in both bancroftian and brugian infections (Rao et al 2000, Lalitha et al 2002). An improved rWbSXP-1 antigen based immunoassay was highly
specific and rapid field applicable format for diagnosis (Basker et al 2004) and surveillance or elimination programmes (Lammie et al 2004). However, to identify active filarial infection in endemic population, it is important to detect the circulating filarial antigen in blood.

4.15 SELECTION OF MAb FOR ANTIGEN DETECTION AND ITS CHARACTERIZATION

Though a previous work on MAb raised against rWbSXP-1 was successful, the affinities of such antibodies were very less (Basker 2004b).

Sasisekar et al (2004) have shown using confocal microscopy, that polyclonal antibodies raised against rWbSXP-1 could show localized SXP as a partial-surface protein in Sertaria digitata adult worms. Also WbSXP-1 protein has been proved to be expressed in all the parasite stages is a right candidate for diagnostic application.

We explored the scope of antigen detection circulating microfilaria by developing MAb against WbSXP-1 protein considering its highly immunogenic nature and its presence in all the stages of the Wb parasite life cycle. Our work aimed for the detection of both circulating Wb and Bm microfilaria.

The raising of MAb was successful against rWbSXP-1 protein. MAbs Screening was done by combining ELISA, western blot and sandwich ELISA methods.

The Western blot protocol for screening monoclonal antibodies has significant advantages over other screening approaches. A critical step in monoclonal antibody production procedure is the primary screening of hybridoma supernatants. The primary screening protocol utilizing highly
sensitive Western blot analysis was reported using fusion of a human separase fragment with different purification tags, (Anton et al 2003). The approach was reported to have significantly reduced number of false positives during the primary screening.

The MAb IgG1k (1A6C2) was more stable and was able to clearly detect microfilaria isolated from MF positive cases. Significantly, the monoclonal 1A6C2 showed no reactivity in the endemic normal and chronic pathology sera groups in ELISA. The MAb-1A6C2 showed greater specificity in detecting Wb and Bm microfilaria and no reactivity with endemic normal and chronic pathology cases. Moreover, it did not cross react with other protein of filarial origin such as rALT-2, rThioredoxin, rThioredoxin-peroxidase and common non-filarial protein like E.coli cells lysate and BSA. Biotinylazation of IgG1k was stable and did not show to lose its activity.

In a similar type of work involving Fasciola parasite, monoclonal antibody (MAb) against the 28.5 kDa tegumental antigen of was produced by the hybridoma technique. The MAb was found to be of the isotype IgG1, k-light chain and shown by immunoblotting to specifically react with the 28.5 kDa antigen present in the whole-body extracts of adult parasites. It did not cross-react with antigens from other trematode parasites, including Schistosoma, Eurytrema and Paramphistomum spp. Immunolocalization of this antigen showed it was expressed in all developmental stages of the parasite, and it could be a strong candidate for immunodiagnosis and vaccine development (Kulathida 2002).

Thus the work results show that a successful detection of circulating microfilaria using MAb’s raised against rWbSXP-1. Further improvement for a sensitive and field applicable rapid strip format for antigen detection in MF in patient’s blood or serum will pave way for an economic, single step method for active brugian or bancroftian infections in human lymphatic filariasis in
endemic population in developing countries. Monoclonal based diagnostics are extensively used in a wide array of different applications and are moving from immunoassays towards protein chips.

**Expected Outcome:** The present work on ‘optimization of high cell density cultivation for the production of recombinant filarial protein wbsxp-1 and diagnostic application in human lymphatic filariasis’ has certainly made the protein expression in *E.coli* GJ1158 feasible. The high cell densities and protein expression was achieved in TCRC mode of operation. The TCRC setup and design of operation is hitherto unreported for *E.coli* GJ1158 cultivation. The batch bioreactor scale up was successful from 3 L to 30 L. The purification of rWbSXP-1(6xHis) protein from *E.coli* GJ1158 was successful using immobilized metal affinity chromatography and followed gel filtration as polishing step. The purification strategy resulted in highly pure protein for diagnostic application. The rWbSXP-1 based ‘MF-Signal’ rapid format was optimized for high sensitivity and effectiveness for field application. The technology transfer to M/s SPAN Diagnostics Ltd was successfully completed. Successful development of MAb against rWbSXP antigen and specificity on ELISA based antigen assay has shown promising result to proceed further towards rapid antigen detection kit format.
CHAPTER 5

CONCLUSIONS

- \textit{Wuchereria bancrofti} protein \textit{Wb-SXP1} was identified as potential candidate for immunodiagnosis of Human Lymphatic filariasis. For economic production of this protein in large scale, a novel osmotically inducible \textit{Escherichia coli} GJ1158 host with NaCl as inducer was attempted.

- The work suggests increased biomass productivity with acceptable recombinant protein expression can be achieved using osmotically inducible \textit{E.coli} by elimination of unidentified growth inhibiting small ionic metabolites or molecules from the cell culture during the fermentation.

- The work demonstrated a successful high cell density fermentation process with an external membrane module in laboratory scale for \textit{E.coli} GJ1158. The biomass increase in cell retention culture was significantly higher than in batch mode. A successful downstream processing and purification of r\textit{WbSXP-1} using a combination of IMAC and gel filtration in technical scale was developed. The performance of improved rapid flow through antibody assay for diagnosis of human lymphatic filariasis was increased. The concentration of r\textit{WbSXP-1} protein per spot was optimized at 0.5 µg/ 0.7 µl. The kit is more sensitive to low levels of antibodies in exposed individuals.
The raising of MAb was successful against rWbSXP-1 protein. The MAb IgG1k (1A6C2) was stable and clearly detected Wb and Bm microfilariae isolated from MF positive cases. MAb 1A6C2 showed no reactivity to the endemic normal, chronic pathology sera and did not cross react with other recombinant protein of filarial origin.

The enhanced antibody kit and MAb’s is expected to suit the conditions in developing and under developed endemic countries where the diagnosis, surveillance and eradication of the disease is an important concern.

Future scope: Characterization of rWbSXP-1 in eukaryotic expression system would produce a better post-translationally modified protein which would be similar to the native filarial protein for better diagnostic applications. To synthesize antigenic peptide epitopes based on polymorphic variants of WbSXP. Study the diagnostic responses to these peptides as single and multiple epitope combinations. These approaches would enhance better diagnosis of the disease.