UTILIZATION OF FERMENTATION AND PURIFICATION STRATEGIES TO ENHANCE THE YIELD OF *Bm*R1 ANTIGEN

by

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	Description	Abbreviations
1	Ampicillin resistance (β-lactamase) gene	Amp^r
2	Base pair(s)	bp
3	Bovine serum albumin	BSA
4	Cell concentration	X
5	Celsius	C
6	Centimetre(s)	cm
7	Dalton	Da
8	Deoxyribonuclease	DNase
9	Deoxyribonucleic acid	DNA
10	*E.coli* TOP10 host (Invitrogen, USA)	TOP10
11	Ethylenediaminetetraacetic acid	EDTA
12	Fermentation time	t
13	Global Programme for the Elimination of Lymphatic Filariasis	GPELF
14	Growth rate	μ_m
15	Horseradish peroxidase	HRP
16	Hour(s)	hr
17	Initial cell concentration	X_i
18	Initial substrate concentration	S_i
19	Isopropyl-β-D-thiogalactoside	IPTG
20	Kilobase(s)	kb
21	Kilodalton(s)	kDa
22	Kilogram(s)	kg
23	Luria Bertani	LB
24	Maximum concentration of cell	X_m
25	Molar	M
26	Molecular weight	MW
27	Microgram(s)	µg
28	Microlitre(s)	µm
29	Micrometre(s)	µm
30	Milliampere(s)	mA
31	Milligram(s)	mg
32	Milliliter(s)	ml
33	Millimetre(s)	mm
34	Millimolar(s)	mM
35	Minute(s)	min.
36	Nanogram(s)	ng
37	Nanomolar(s)	nM
38	Nickel-nitrilotriacetic acid	Ni-NTA
39	Phosphate-buffered saline	PBS
40	Revolutions per minute	rpm
41	Ribonuclease	RNase
42	Ribonucleic acid	RNA
43	Second(s)	sec.
44	Substrate concentration at Xm	S_m
45	Ultra violet	UV

46	Volume	vol.
47	Volt(s)	V
48	Watt(s)	W
49	World Health Organization	WHO

PENGGUNAAN STRATEGI FERMENTASI DAN PENULENAN BAGI PENINGKATAN HASIL ANTIGEN *Bm*R1

ABSTRAK

Brugia Rapid merupakan ujian diagnostik komersial yang digunakan untuk mengesan jangkitan terhadap *Brugia malayi* dan *Brugia timori* (filariasis brugia). Ujian ini merupakan format ujian batang pencelup aliran sehala yang menggunakan antigen rekombinan *Bm*R1 yang diekspreskan daripada klon *Bm17DIII*/pPROEX^{TM}HT/TOP 10. Oleh kerana penggunaan ujian ini mendapat permintaan yang tinggi di pasaran, maka peningkatan penghasilan ke skala besar dan peningkatan efisiensi penulenan adalah perlu untuk mengurangkan kos penghasilan ujian tersebut.

Pengkulturan ke atas bakteria rekombinan ini dijalankan melalui kaedah fermentasi kultur sekelompok, di mana sel ditumbuhkan di dalam media kaldu "Terrific" terubahsuai dan glukosa disuapkan secara eksponen pada kadar yang terkawal menggunakan "Multifermenter Control Software" (MFCS) untuk suapan secara automatik semasa sebelum dan selepas aruhan. Kepelbagaian ke atas kadar pertumbuhan spesifik menunjukkan kepekatan akhir sel sebanyak 24.3 g/l diperolehi pada kadar pertumbuhan spefisik ditetapkan pada 0.15 h^{-1}, diikuti dengan suapan pada kadar yang tinggi selepas aruhan. Walau bagaimanapun, didapati peningkatan yang lebih tinggi ke atas kadar pertumbuhan spesifik tidak meningkatkan hasil sel, sebaliknya mengurangkan penghasilan protein rekombinan *Bm*R1. Didapati

penghasilan tertinggi protein rekombinan *Bm*R1 sebanyak 9.82 g/l diperolehi apabila suapan dilakukan pada kadar tetap yang rendah (1.9 ml/min), digabungkan dengan kadar pertumbuhan spesifik yang dikawal pada 0.075 h^{-1}. Selain itu juga, dapat diperhatikan bahawa pada kadar ini, produktiviti spesifik keseluruhan, hasil koefisien fermentasi [hasil biomas (Yx/s) dan hasil produk (Yp/s)] adalah yang tertinggi dibandingkan dengan proses lain. Strategi ini telah berjaya mengawal pengumpulan bahan-rencatan asid asetik di bawah tahap rencatan iaitu 1.26 g/l.

Ekspresi terhadap antigen rekombinan *Bm*R1 adalah optimum dengan dua kali aruhan IPTG berkepekatan 1 mM. Aruhan yang dilakukan pada fasa log pertengahan telah menghasilkan protein terlarut dalam jumlah yang signifikan. Oleh itu, penulenan antigen rekombinan *Bm*R1 dilakukan melalui penulenan tidak ternyahasli (non-denaturing) kromatografi afiniti logam tidak bergerak ("immobilized metal affinity chromatography").

Kajian mempelbagaikan isipadu penimbal pembasuh, kepekatan imidazol dan garam telah dijalankan untuk meningkatkan efisiensi proses penulenan protein. Kepekatan imidazol pada 20 mM adalah yang terbaik di mana ia memberikan kepekatan antigen rekombinan *Bm*R1 yang tertinggi dengan ketulenan yang baik. Apabila penimbal basuhan sebanyak lima isipadu kolum digunakan semasa proses penulenan, hasil kepekatan antigen rekombinan yang diperolehi adalah sebanyak 5.14 mg/l, dibandingkan dengan hasil yang diperolehi iaitu sebanyak 2.84 g/l apabila 10 isipadu kolum penimbal basuhan digunakan. Hasil keseluruhan protein sasaran meningkat sebanyak tujuh kali ganda dibandingkan dengan hasil protein yang diperolehi melalui kaedah konvensional pengkulturan di dalam kelalang.

Tahap sensitiviti dan spesifisiti antigen rekombinan *Bm*R1 yang dihasilkan dalam kajian ini ditentukan melalui ujian Pemblotan Western dan dua lagi ujikaji imunoasai iaitu ELISA dan ujian pantas imunokromatografi. Melalui kaedah ELISA, didapati antigen rekombinan *Bm*R1 yang dihasilkan dalam kajian ini mengekalkan sifat antigenik apabila dikesan oleh serum pesakit filariasis (100% sensitif) dan tidak bertindakbalas apabila diuji menggunakan serum dari jangkitan lain dan individu yang sihat (100% spesifik). Apabila ia diaplikasikan pada ujian pantas, antigen rekombinan *Bm*R1 bertindakbalas terhadap antibodi anti-filaria di dalam serum pesakit yang dijangkiti dengan *Brugia malayi* dan menunjukkan tahap sensitiviti 100%. Oleh itu, antigen rekombinan *Bm*R1 yang dihasilkan ini adalah sesuai untuk digunakan dalam penghasilan kit Brugia Rapid™.

Secara keseluruhannya, kajian ini mencadangkan penambahbaikan, kaedah yang lebih efisien, dan lebih jimat ke atas proses penghasilan berskala besar dan proses hiliran antigen *Bm*R1, seterusnya sesuai untuk aplikasi industri.

UTILIZATION OF FERMENTATION AND PURIFICATION STRATEGIES TO ENHANCE THE YIELD OF *Bm*R1 ANTIGEN

ABSTRACT

Brugia Rapid ™ is an established diagnostic test that is commercially available to detect infection to both *B. malayi* and *B.timori* infections (brugian filariasis). The test is a dipstick lateral flow test format that utilizes *Bm*R1 recombinant antigen expressed by the clone *Bm17DIII*/pPROEX™HT/TOP 10. Due to a significant demand for the test in the market; there is a need to scale up the production of the recombinant antigen and increase the purification efficiency to reduce the production cost of the test.

The cultivation of the recombinant bacteria was performed using fed-batch fermentation where cells were grown in a modified Terrific broth medium and glucose was fed exponentially at a controlled rate using Multifermenter Control Software (MFCS) for automated feeding during pre-induction and post-induction feeding. Varying the specific growth rate (μ) prior to induction showed that a final cell concentration of 24.3 g/L was obtained at specific growth rate of 0.15 h^{-1} with feeding at high rate during post-induction. However, further increase of the specific growth rate during pre-induction feeding does not produce a higher cell mass, in fact it decreased the *Bm*R1 recombinant protein production. It was found that the highest *Bm*R1 production of 9.82 g/L was obtained when the feeding was carried out at low constant rate (1.9 ml/min), combined with the specific growth rate controlled at

0.075 h^{-1}. It was also observed that at this feeding rate, the overall specific productivity, the fermentation yield coefficients [biomass yield ($Y_{x/s}$) and product yield ($Y_{p/s}$)] was the highest amongst all the runs tested. This strategy has successfully controlled the accumulation of acetic acid by-inhibitory product below the reported growth inhibitory level of 1.26 g/L. .

The expression of *Bm*R1 recombinant antigen was found to be optimal with twice induction of 1 mM of IPTG concentration. Initiation of expression at mid log had generated significant amounts of the soluble protein. Therefore, the *Bm*R1 recombinant antigen was then purified under non-denaturing conditions using immobilized metal affinity chromatography.

In order to increase the efficiency of purification process, various volumes of wash buffer, imidazole and salt concentration were performed. Imidazole at 20 mM was found to be the best concentration that gave the best yield of *Bm*R1 recombinant antigen while achieving sufficient purity. When 5 column volumes (CV) of washing buffer was used in the purification step, the production of *Bm*R1 recombinant antigen was 5.14 mg/l; as compared to 2.84 mg/l when 10 CV of washing buffer was employed. The overall production of the target protein was improved seven-fold compared to the conventional flask cultivation method.

The sensitivity and specificity of the purified *Bm*R1 recombinant antigen produced in this study was confirmed by Western blot and two other immunoassays techniques namely ELISA and immunochromatographic rapid test. By the ELISA technique, it was found that the *Bm*R1 recombinant antigen produced in this study retained its

CHAPTER ONE

INTRODUCTION

1.1 Introduction to lymphatic filariasis

1.1.1 Lymphatic filariasis

Lymphatic filariasis (LF), or more commonly known as elephantiasis, is a parasitic disease caused by thread-like worms that live in the human lymphatic system. This disease is widespread and occurs throughout the tropical and sub-tropical regions of the world, such as Asia, Africa, Central and South America (Figure 1.1). The World Health Organization (WHO) has identified lymphatic filariasis to be one of the six infectious diseases that has the potential to be eliminated as a public health problem (WHO, 1998). Consequently a global programme for elimination of the disease known as the 'Global Programme to Eliminate Lymphatic Filariasis' (GPELF) was initiated in year 2000. In 2006, WHO has reported that more than 20% of the world's populations are at risk of acquiring LF. Meanwhile, it is estimated that over 120 million of people in at least 83 countries in the world have already been infected and more than 40 million people are incapacitated by the disease (The Global Alliance to Eliminate Lymphatic Filariasis (GAELF), *http://www.filariasis.org*). Statistics have shown that over 25 million men suffered from the genital form of the disease, while more than 15 million of people suffered from lymphoedema or elephantiasis of the leg. Out of this, 90% of the cases are caused by *Wuchereria bancrofti* whereas *Brugia malayi* accounts for about 10% (or 13 million) of the infected people especially in South and Southeast Asia, South Korea, and parts of China. However, *Brugia timori* is only restricted to Timor Leste and a few islands in Indonesia (Michael,2000)

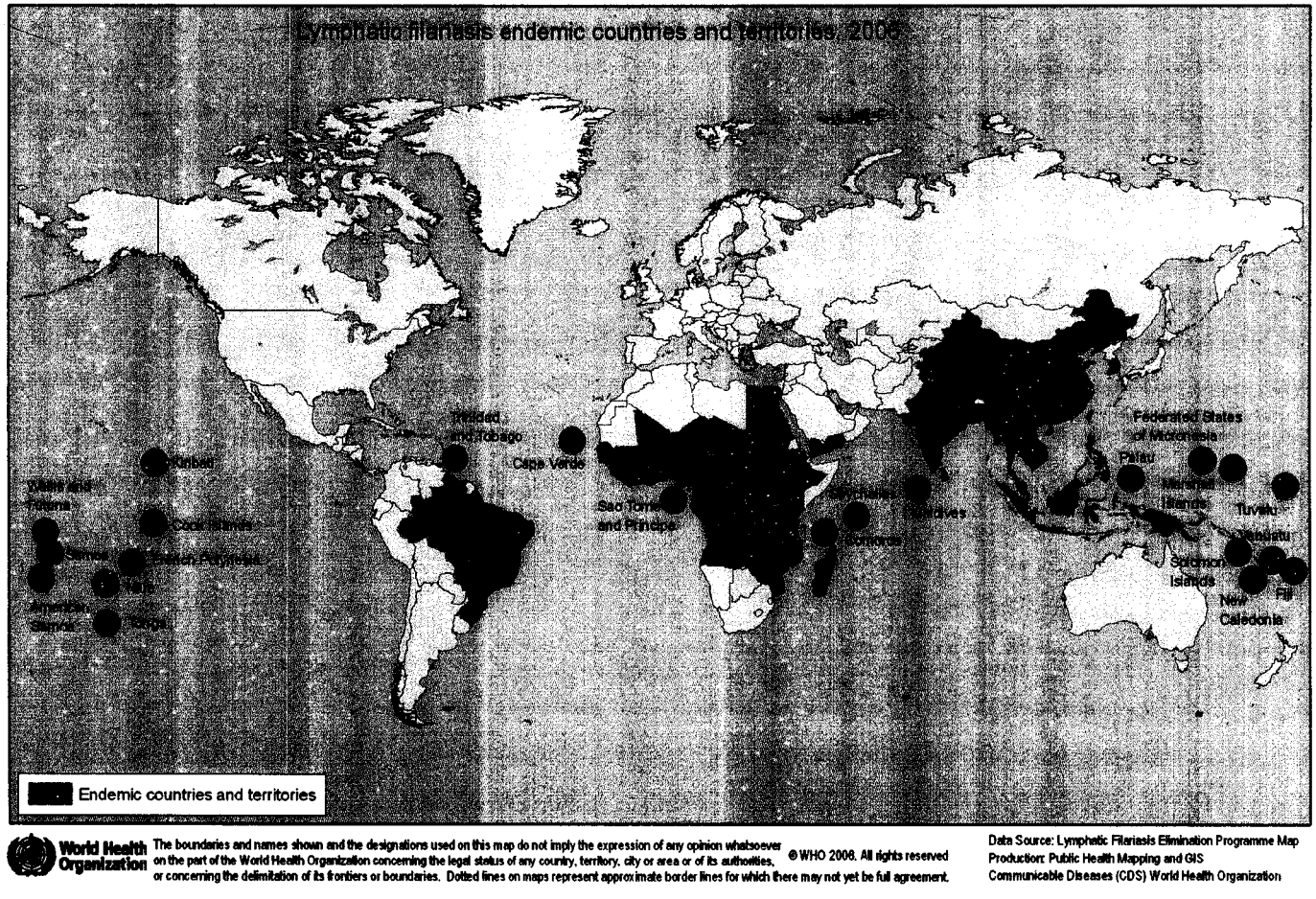

Figure 1.1 World endemic countries of lymphatic filariasis in 2006

Source: http://gamapserver.who.int/mapLibrary/app/searchResults.aspx

Lymphatic filariasis is a major cause of permanent disability and disfigurement in the endemic areas. The areas endemic for this disease are underdeveloped and poor, hence it is known as the disease of poverty. Most often, young children get infected before the age five and it takes years before the infection can be clinically detected. Besides, the infection also has serious clinical consequences for pregnant women and infants. Once infected, the disease will slowly develop and the patient may suffer from disfiguration with swelling of the limbs and breasts (lymphoedema) and genitals (hydrocele), or swollen limbs with dramatically thickened, hard, rough and fissured skin (elephantiasis) This scenario will prevent the patients from experiencing normal working and social life because of physiological consequences and obvious physical handicap. As a result, this will lead to economic instability due to loss of manpower and decreased productivity which will aggravate the cycle of poverty (*http://www.filariasis.org*).

1.1.2 Transmission and life cycle

There are three lymphatic filaria that infect human namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. The disease is being transmitted to human by the bite of more than 70 species and subspecies of mosquitoes mainly *Anopheles, Aedes*, *Culex* and *Mansonia*; hence it is referred to as a vector-borne disease (Stone *et al.*, 1959; Nanduri and Kazura, 1989). The three lymphatic filariae have similar biphasic life cycle where larval development takes place in mosquito (intermediate host) and adult development takes place in the human (definitive host). Basically, there are four different stages of the life cycle of lymphatic filaria as depicted in Figure 1.2.

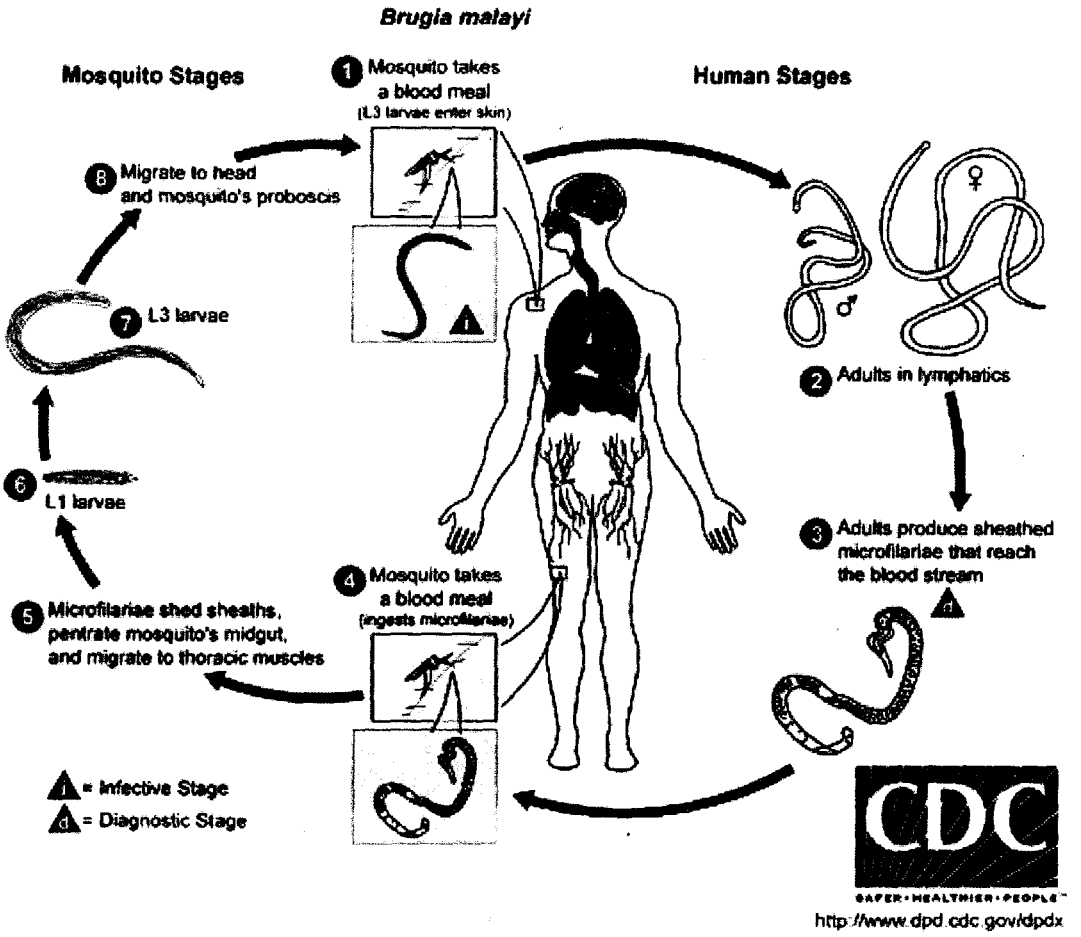

Figure 1.2 The life cycle of *B. malayi*

Source: www.dpd.cdc.gov/dpdx

Mosquito ingests microfilariae as part of its blood meal. Within a few hours, the microfilaria penetrates the mosquito gut wall, migrates to the flight muscles and molts to the second larval stage (L_2). The L_1 and L_2 molt takes place between days 6 to 10 depending on the environmental conditions. After several days, the parasite undergoes an additional molt to the L_3 parasite. The L_3 then migrates from the flight muscles to the mouth parts of the vector, where they are positioned to be passed on to the vertebrate host during a subsequent blood meal (Scott, 2000).

Infection is initiated by the deposition of the third stage larvae (L_3) on the skin of human host following a bite by an infective mosquito. In the definitive host, the larval undergo an additional molt to the fourth larval stage (L_4) between days 9 and 14 post-infection as they mature into the lymphatic-dwelling adult male and female worms to complete the life cycle. Adult female parasites can remain reproductively active for more than 5 years. The lymphatic-dwelling filariae are diecious and undergo ovoviviparous reproduction resulting in the release of fully formed, sheathed first stage larvae (L_1 of microfilariae) from the female. Then, the microfilariae enter the peripheral circulation of the human host where they are available to be ingested by the vector during a blood meal (Scott, 2000).

The unique characteristic of the microfilaria (mf) stage of the parasites is that they have "periodicity" that restricts their appearance in the blood to only certain periods of the day. In general, the microfilariae can be classified into two main groups based on their circadian rhythms; i) the noctural periodic strain, ii) the nocturnaly subperiodic strain. In the nocturnally periodic strain, they virtually disappear from the peripheral circulation and are found predominantly in the blood vessels of lungs

and deep tissues during the daytime. Conversely, during the night time (especially between 10 pm until 2 am), the microfilariae can be found in the peripheral blood. Meanwhile, for the nocturnally subperiodic strain, the mf is present in the blood both day and night but the density is much more higher during the night and tend lose their sheath in the process of dying on microscope slides (Bowman *et al.,* 2002). In addition to this basic strain, there is also nonperiodic or diurnally subperiodic strain of mf found in the South Pacific where it is transmitted mainly by day-biting mosquitoes of genus *Aedes* (Nanduri and Kazura, 1989).

In Malaysia, the predominant strain is the nocturnal subperiodic *B. malayi.* Due to its predominant appearance in blood at night, conventional diagnostic method is by microscopic examination of night blood. Studies of *Wuchereria* and *Brugia* species suggested that their periodicity is due to the difference in the oxygen tension between the arterial and venous blood in the lungs (Hawking and Gammage, 1968; Burren, 1972; Nanduri and Kazura, 1989). However, Wang and Saz (1974) suggested that these parasites have adapted their periodicity to the vector feeding behavior, possibly to facilitate their transmission (Nanduri and Kazura, 1989).

1.1.3 *Brugia malayi*

Brugian filariasis is caused by *Brugia malayi* infection and it is endemic in parts of India, China, Indonesia, Philippines, Thailand, Vietnam and Malaysia (WHO, 1998). This species was first identified by Lichtenstein and named by Brug in 1927. The *B. malayi* species is classified under the family of Filariidae and the genus of Brugia. The distribution of *B. malayi* is very similar to that of *W. bancrofti.* However, the

differences between *W. bancrofti, B. malayi* and *B. timori* reside in their epidemiology, vectors, reservoirs, symptoms, but mainly the species morphology.

The brugia female worm measures 43 to 55 mm in length by 130 to 170 μm in width, and male worm measures 13 to 23 mm in length by 70 to 80 μm in width. Adult worms produce microfilariae, measuring 177 to 230 μm in length and 5 to 7 μm in width (Figure 1.3), which are sheathed and have nocturnal periodicity (*http://www.dpd.cdc.gov*). The adult male and female worms of *B. malayi* inhabit primarily the lumen of lymphatics whereby the microfilariae usually migrate from the lymphatics into the blood stream (Nanduri and Kazura, 1989).

In Malaysia, the main species of parasite causing lymphatic filariasis are the subperiodic strains of *B.malayi*. This parasite is principally transmitted by mosquitoes belonging to the species *Mansonia bonneae, M. dives, M. uniforms* and *M. Indiana* (Chang *et al.*, 1991; Kwa, 2008). Generally, brugian filariasis leads to symptoms and manifestations that may include recurrent fever, lymphatic damage, renal damage, adenolymphangitis, lymphoedema, elephantiasis and pulmonary disease. However, the infection is usually limited to legs below the knee and upper limbs distal to elbow.

Figure 1.3 Microfilaria of *Brugia malayi*

Source : http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

1.1.4 Clinical manifestation

In areas where LF is endemic, majority of the infected individuals fall into the following categories: asymptomatic amicrofilaraemia, symptomatic microfilaraemia, asymptomatic microfilaraemia, acute infection with filarial fever and adenolymphangitis (inflammation of the lymphatics and lymph nodes), chronic elephantiasis and occult filariasis. Asymptomatic amicrofilaraemia individuals are the infected persons who do not exhibit any physicals symptom and has no circulating microfilaria. Microfilaraemic individuals are those with microfilaria circulating in their blood, and they may have symptoms (symptomatic) or have no physical symptom (asymptomatic). Individual with acute symptoms may experience fever, chill, malaise, headache and vomiting. However, lymphoedema is the worst clinical manifestation of lymphatic filariasis and the chronic stage of lymphoedema is elephantiasis.

The clinical manifestations of LF vary from one endemic area to another and also differ to some extent on the species of the parasite. For example, in parts of Africa where bancroftian filariasis is prevalent, the most common clinical form of the disease is hydrocoele while tropical pulmonary eosinophilia has been reported most commonly from the India subcontinent and Brazil. Meanwhile, it is recognized that inguinal lymphadenitis and lymphangitis are more common in brugian filariasis with the exceptions of genital disease (hydrocoele and filarial scrotum) (Kumaraswami, 2000).

1.1.5 Diagnosis of lymphatic filariasis

The diagnosis of brugian LF caused by *B. malayi* and *B. timori* can be performed using conventional laboratory methods such as thick blood smear technique, DNA-based laboratory assay using the PCR technique, ultrasonography and immunoassays. For thick blood smear technique, a drop of blood is spread onto the slide, dried, stained and examined under the microscope for the presence of microfilariae. This method is insensitive for active infections; it misses people with low mf counts and those with amicrofilaremic infections; these individuals have the potential to contribute to future transmission. In addition, night blood collection is troublesome to both the staff and villagers and impractical in some endemic areas (Weil and Ramzy, 2006). The sensitivity of this technique can be improved by membrane filtration of 1-5 ml of blood prior to microscopy.

Molecular diagnostics by PCR is available for LF diagnosis. The main obstacle with the PCR method is that it requires sophisticated laboratory equipment and trained personnel to perform the analysis; and requires several hours to obtain the results. Thus, it is not practical to be used with large number of samples and for field screening. In addition, PCR generally do not detect people with amicrofilaraemic infection and still requires night blood smear for maximum sensitivity.

Ultrasonography, the detection is based on 'filarial dance sign' which refers to live adult worm inside the lymphatic vessels. Other than not suitable for large scale studies, it is not very useful for brugian filariasis in which the adult worms are not found in the peripheral lymphatics.

Immunoassays detect the presence of specific antigens or antibodies in the blood of individuals. Antigen detection assay is not available for brugian filariasis. With regard to detection of specific antibodies, it was recognized that the elevated levels of IgG4 is a marker of active infection rather than past exposure and it does not cross-react with antigens from non-filarial helminthes (Lal and Ottesen, 1988; Kwan-Lim *et al.,* 1990; Turner *et al.,* 1993; Haarbrink *et al.,* 1995; Chanteau *et al.,* 1995; Terhell *et al.,* 1996; Rahmah *et al.,* 1998). To date, there are two commercialized rapid tests for diagnosis of brugian lymphatic filariasis which is based on detection of anti-filarial IgG4 antibody test namely Brugia Rapid™ and PanLF Rapid™. The latter is for detection of both brugian and bancroftian filariasis and also employs the *Bm*R1 recombinant antigen.

1.1.6 Elimination of lymphatic filariasis

In 1997, the WHO advocated the global elimination of LF as a public health problem and targeted to eliminate the disease by the year 2020. In conjunction with this, three years later (year 2000), the Global Alliance to Eliminate Lymphatic Filariasis (GAELF) was formed with the sole purpose of supporting the Global Programme to Eliminate Lymphatic Filariasis (GPELF). This programme aims to eliminate LF by interrupting the transmission of infection and to alleviate and prevent both suffering as well as disability caused by the disease. *(http://www.filariasis.org/resources/globalalliancehistory.htm).*

In order to interrupt the transmission of the infection, the entire 'at risk' population must be treated for a period long enough to make sure that level of microfilariae in the blood remain below that necessary to sustain transmission. In this case, a single

dose of two drugs regimens has being advocated (albendazole 400 mg plus diethylcarbamazine (DEC) 6 mg/kg, or albendazole 400 mg plus ivermectin 200 μg/kg) for a period of 4-6 years corresponding to the reproductive life span of the parasite (Ottesen, 2000).

In addition to alleviate suffering and decrease the disability caused by LF disease, a principal strategy focuses on decreasing the secondary bacterial and fungal infection of limbs and genitals whose lymphatic function has already been compromised by filarial infection, since secondary infection is the primary pathogenetic determinant of worsening lymphoedema and elephantiasis (Ottesen, 2000).

The importance of mapping and surveillance studies is to identify endemic areas so that repeated cycles of mass drug administration (MDA) can be performed in order to reduce both infection prevalence and transmission rates to levels below those required for sustained transmission (Ottesen *et al.*, 1997; Molyneux, 2001; Ottesen, 2006; Weil and Ramzy, 2006). To this end, sensitive and specific field-applicable diagnostic tools are required for mapping the distribution of the disease and monitoring various phases of the program. Many endemic areas for LF are remote and have poor access to well-equipped laboratories, thus a rapid and field-applicable diagnostic test is important to ensure that it can be performed easily by field workers while at the same time gives reliable and reproducible results. Furthermore, an important technical consideration to ascertain the success of this programme is the need for sensitive tests which obviates night blood sampling for proper mapping and surveillance exercises after the cessation of the mass-treatment (Ottesen *et al.*, 1997).

1.1.7 Brugia Rapid™

Brugia Rapid™ is a dipstick test format that utilized *Bm*R1 recombinant antigen for the detection of brugian filariasis. This rapid test is based on the significantly elevated level of IgG4 antibody in active infection (Rahmah *et al.*, 2001b, 2003b). In this test, the anti-filarial antibodies in patient sera will react with this antigen, followed by binding of this complex with monoclonal anti-human IgG4 conjugated to colloidal gold. Thus, samples containing anti-filarial IgG4 antibodies that react specifically to the antigen will result in the appearance of a purple-reddish colour at the test line (Rahmah *et al.*, 2003b).

Previously, this test was performed in an ELISA format known as *Brugia*-ELISA. Evaluation studies on this test demonstrated the specificity rates of 95.6-100%, the sensitivity rates of 96-100%, the positive predictive values of 75-100%, and the negative predictive values of 98.9-100% (Rahmah *et al.*, 2001a). However, since this test is more suitable for laboratory rather than field use, therefore the format of the test has been further developed into a rapid immunochromatographic dipstick test and cassette format, which later was named as Brugia Rapid™ test. This new format is easy to perform, rapid and robust. Most importantly, it does not require any night blood sampling and does not need any laboratory facilities; make it useful for field-work in remote areas.

Evaluation studies of this test have been performed to validate the sensitivity and specificity of the rapid test which involved both endemic and non-endemic areas of filariasis. The results showed 97% sensitivity, 99% specificity, 97% positive predictive value, and 99% negative predictive value (Rahmah *et al.*, 2001b; 2003a).

In the past, the mapping activities for LF endemicity has been hampered by the lack of data on the geographical distribution of levels of infectious or disease (Micheal and Bundy, 1997). With this regard, other than for patient diagnosis, this assay is very useful for mapping of endemic areas, and surveillance post- mass treatment towards helping to achieve the goals of the Global Elimination Program for Lymphatic Filariasis (GPELF).

1.2 *E.coli* fermentation

1.2.1 History

In the 19th century, interest in microbial fermentations has received great attention when people started to realize the potential use of microbes especially in the production of drinks and dairy products. *E.coli* has been the most widely used prokaryotic host because it is well characterized in terms of its molecular genetics, physiology, and expression system (Makrides, 1996; Choi and Lee, 2004), and thus was the pioneer organism for large scale fermentation studies. Since early 1970s, growing *E.coli* to high densities has been the subject of numerous studies where initially it was focused on high cell density growth of *E.coli* to investigate the limits of bacterial growth in liquid culture and to obtain large quantities of exponentially grown *E.coli* needed for biochemical studies (Choi *et al.,* 2006). Besides *E.coli*, the use of microorganisms such as *Pichia pastoris* (Stratton *et al.,* 1998), *Saccharomyces cerevisiae* (Lee *et al.,* 1994), *Candida brassicae* (Yano *et al.,* 1985) and *Bacillus subtilis* (Park *et al.,* 1992) are very commonly grown to high cell density using fermentation methods. However, growing *E.coli* to a high cell density culture has become the method of choice for the production of recombinant proteins mainly

because of the high volumetric productivities associated with this method (Choi *et al.*, 2006).

Large scale fermentation in the fermenter has the added advantage of increased cost-effectiveness, enhanced downstream processing, reduced wastewater, lower production cost and a reduced investment in equipment (Choi *et al.*, 2006). The development of this technique for *E.coli* has facilitated the production of recombinant proteins and non-protein biomolecular products such as amino acids, primary and secondary metabolites with high productivities (Jeong and Lee, 1999; Gerigk *et al.*, 2002; Mijts and Schmidt-Dannert, 2003; Jeong *et al* 2004; Choi *et al.*, 2006). Considering the commercial potential of a synthetic human IL-lra, Zanette and co-workers (1998) has highlighted the economical process for the production of this cytokine in a 50L scale fermentation process of *E.coli*. Such similar study also has been demonstrated by Yazdani and co-workers (2004) for the production of recombinant malarial antigen, PvRII in *E.coli* in 10L culture volume. In addition, Janardhan and co-workers (2007) has established the production of WbSXP-1 recombinant protein for diagnostic application in lymphatic filariasis.

The increasing industrial demand for robust fermentation control requires improved strategy to increase the production of the target product. In an effort to further improve the fermentation technique, the principal strategy focuses on the development of growth medium, culture conditions and nutrient feeding strategy.

1.2.2 Characteristics of *E.coli*

E.coli is a Gram negative bacterium which does not produce endospores in response to starvation conditions (Rinas *et al.,* 1995). It is generally grown under aerobic conditions because anaerobic growth provides less energy for metabolic processes such as protein synthesis (Xu *et al.* 1999; Manderson *et al.* 2006). There are four different phases in *E.coli*'s growth namely lag, log, stationary and death phase. At each phase, *E.coli* has different morphological state and the medium has different condition in terms of nutrient availability and levels of inhibitory by-product. When the bacteria first enter into a new culture medium, the cells will take time to adapt to the new environment. Consequently, the slow growth is observed during the lag phase as there is no or very little cell division. Conversely, in the log phase, there is a rapid exponential growth of the cells whereby nutrients are metabolized at its maximum speed until one of the nutrients is depleted and starts limiting the growth. The rate at which cells grow during this phase is known as the *growth rate* (k), and the time it takes for the cells to double is known as the *generation time* (d). However the cells start to reduce their metabolic activity as a consequence of depleted nutrients during the stationary phase, where the rate of cells dividing is equal to the rate of cell death. When the cells are unable to resume division and cannot survive the harsh conditions caused by few nutrients availability and too much toxins, the cells start to die which result in a decrease in the numbers of cells within the system.

Synthesizing recombinant protein at the beginning of the culture may not be advantageous because many proteins are sensitive to proteases, thus an early protein production will increase to proteolytic degradation before the final harvest (Aucoin *et al.,* 2006) and can unnecessarily slow down the doubling time of the bacterial cells

(Manderson *et al.*, 2006). Conversely, induction at the stationary phase reduces culture viability and could lead to production of proteases which can breakdown the desired recombinant protein (Chisti, 1998; Corchero *et al.*, 2001; Manderson *et al.*, 2006). Therefore, the recombinant protein production is generally induced at late exponential growth phase, where the induction at this stage will promote exponentially grown cells to express the target protein.

E.coli cells consist of inner and outer membranes that divide the organism into three compartments; the cytoplasm, the periplasm and the extracellular space where the recombinant proteins can be targeted to one of these compartments (Choi *et al.*, 2006). However, many research group have focused on the secretory production system to target the recombinant protein production into the periplasmic space (Choi and Lee, 2004; Choi *et al.*, 2006) based on some exclusive characteristics. One of it is because periplasmic space is known to have less protease activity which in turn would maintain the stability of the protein by prevention of proteolytic degradation. In addition, the purification of the target protein could be more simplified as the periplasmic space contains less native host proteins (Choi *et al.*, 2006).

As a whole, the unique characteristic of recombinant *E.coli* as an economical host, coupled with the advent technology in recombinant DNA and large-scale culture processes has enabled high production of therapeutic proteins such as insulin, antibiotics, hormones and specialized proteins such as antibodies at industrial scale. In this case, Ouelette *et al.* (2003) reported that 10 mg/l of human rhIL-7 was successfully achieved in 1000 L batch culture of *E.coli* HMS174 (DE3). In the

production of TGF-α-PE40 (a candidate anti-tumor agent), Lee and co-workers (1997) had successfully obtained 950 mg/l of TGF-α-PE40 in 800 L fermenters.

1.2.3 Fermenter

The common types of fermenter used for industrial fermentation are the stirred-tank fermenter (STF) (Knorre *et al.,* 1990), air-lift fermenter (ALF) (Holst *et al.,* 1997) and dialysis reactor (Nakano *et al.*, 1997) (Riesenberg and Guthke, 1999). However, the most frequently used for industrial application, especially in the pharmaceutical industry, is the conventional mixing vessel STF.

Commonly, the STF (Figure 1.4) is fitted with baffles to prevent a large central vortex being formed and improve mixing by breaking the liquid flow. For an efficient mixing, high turbulence is required; hence this is created by the vortex field which forms behind the blades. The sparger located underneath the disk allows all the gas to flow through into the fermenter. Furthermore, the temperature in a vessel can be controlled by removing heat by means of water circulating through a jacket outside the vessel.

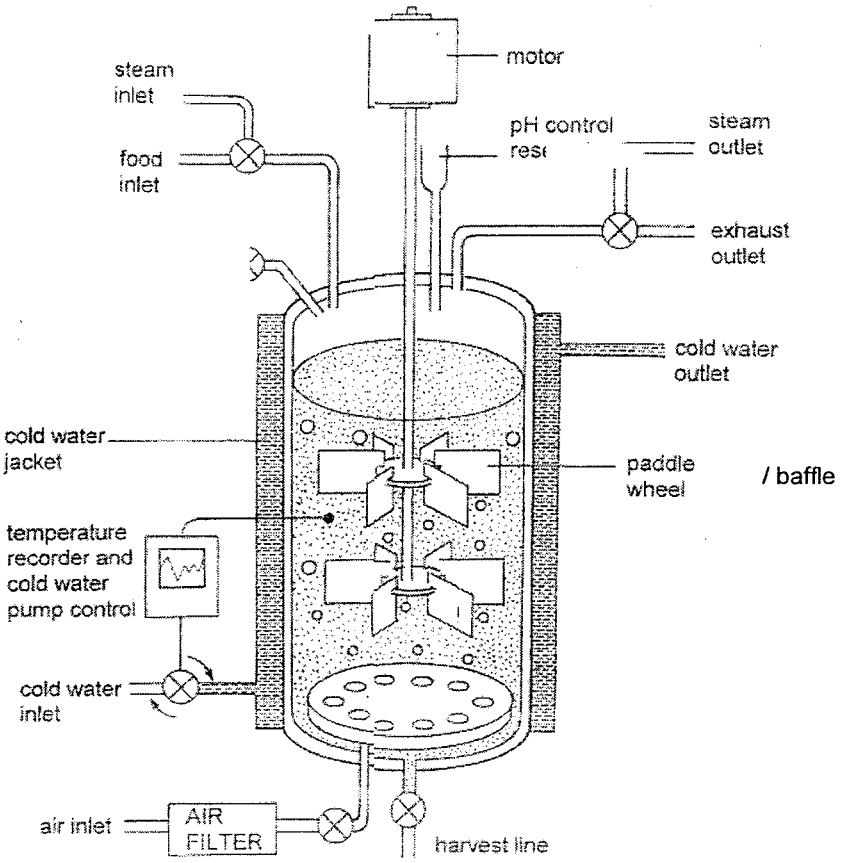

Figure 1.4 Schematic diagram of a stirred tank fermenter (STF)

Source: www.biotopics.co.uk/edexcel/penicl.html

In fed-batch fermentation, monitoring and controlling the running process can be assisted with the presence of software. In this study, a 5 L fermenter (Biostat B5, B. Braun International, Germany) was used to carry out the whole experiment. This stirred tank fermenter is equipped with data acquisition and automated feeding system controlled by the Multifermenter Control System Software (MFCS) supplied by B. Braun International, Germany, which allows monitoring and control of substrate feeding into the culture and other fermenter operating variables. The B. Braun's DCU was used as a front-end controller and a PC was connected to the MFCS-Win computer via a local network to enable data exchange via a software interface. The fermenter is also equipped with important control modules such as probes for temperature, pH, dissolved oxygen (DO) and pumps for feeding of the substrate to the culture. The probes provide direct measurements of temperature, pH and dissolved oxygen concentration during the cultivation process. Figure 1.5 shows the example of instrument set-up for a typical fermentation process.

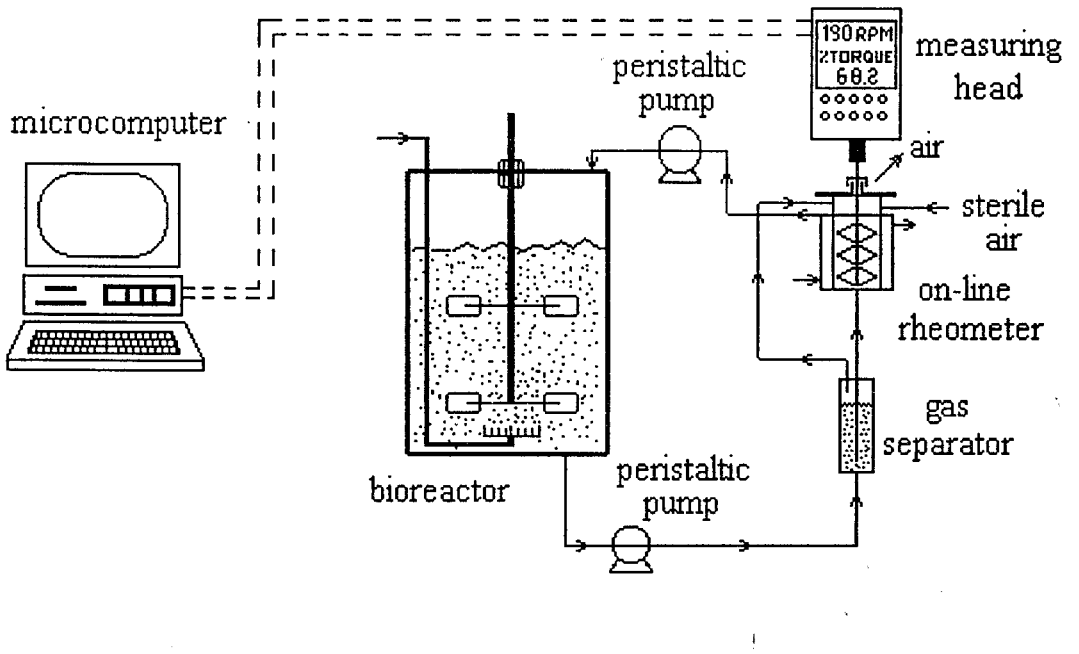

Figure 1.5 Instrument set-up for fed-batch fermentation

1.2.4 Mode of fermentation

Protein production in *E.coli* can be increased significantly through the use of large scale cell cultivation performed in fermenter. There are four main modes of fermenter operation namely batch, fed-batch, repeated batch and continuous fermentation.

1.2.4.1 Batch fermentation

In batch cultures, all substrate components are added initially, except for oxygen and pH-controlling agents. Typically, cells will grow exponentially until some substrate is exhausted or the concentration of by-products becomes inhibiting. The major disadvantage of batch culture is the exhaustion of nutrients and substrate that may cause the system to be retarded, thus affected the pattern of cell growth to reach death phase quickly in an old culture.

1.2.4.2 Continuous fermentation

In contrast to the batch culture, continuous cultures are mostly operated at steady state with an incoming substrate feed flow in balance with an outgoing flow. The substrate feed rate is normally growth limiting and this gives a possibility to control the cell growth at a constant physiological state and growth rate. Continuous cultures outperform batch culture economically by eliminating the inherent downtime that is lost for cleaning, sterilization, and the re-establishing of biomass within the fermenter. However, contaminations represent a major problem in continuous culture, a reason why this process is much less common than batch process in industrial microbial fermentation (El-Mansi *et al.*, 2006).

1.2.4.3 Fed-batch fermentation

In fed-batch cultures, nutrient feeding substrate is added at certain mode with no removal of the cultures from the reactor. By adding nutrient this way, cells are maintained under low substrate or nutrient conditions. Moreover, the production of by-inhibitory product can be prevented by controlling the nutrient supply appropriately. Thus, it allows growth to be prolonged where it is possible to overcome oxygen limitation and consequently, high biomass and product formation can be achieved (Manderson *et al.*, 2006). For the successful large scale bacterial cultivation *via* fed-batch fermentation allowing high target protein production, the selection of nutrient feeding strategy is critical because it affects the metabolic pathway fluxes; therefore it affects the maximum attainable cell concentration, the specific productivity of recombinant proteins and the formation of by-products (Choi *et al.,* 2006). In fed-batch operation, the initiation, processing and harvesting modes are utilized in a single sequence, contrary to cyclic operation (repeated batch) which involves the repetitive cycle of processing and harvesting modes.

1.2.5 Cultivation in fed-batch fermentation

In an effort to further improve the fermentation strategy, a number of studies on the effect of growth medium, nutrient feeding strategies and culture conditions on recombinant protein production have been carried out. Studies also have shown that the fermentation variables such as temperature, pH, and dissolved oxygen level can affect the transcription, translation, proteolytic activity, secretion, production levels, and stability of the protein (Choi *et al.,* 2006).

1.2.5.1 Growth medium

The most important factors that need to be considered when developing a medium are the concentrations and the ratios of the carbon/ energy sources, nitrogen sources, vitamins and trace elements (Riesenberg *et al.,* 1991; Kleman and Strohl, 1992). The composition of the cell growth medium may have significant metabolic effects on both the cells and protein production (Jana and Deb, 2005). In general, the available media for bacterial cultivation can be grouped into two different categories namely defined medium and complex medium.

Defined medium is a medium in which the exact chemical composition is known. A defined medium that provides only the exact nutrients (including any growth factor) needed by the organism for growth is known as minimal medium. This chemically-defined medium is of value in studying the minimal nutritional requirements of microorganisms, for enrichment of the cultures, and for a wide variety of physiological studies. Therefore, use of chemically defined medium in producing recombinant proteins is a common practice because these media attain more consistent titres, allowing easier process control, monitoring, and simplify the downstream recovery of the target protein (Lim and Jung, 1998; Zhang and Greasham, 1999; Cserjan-Puschmann *et al.*, 1999; Kweon *et al.*, 2001). However, it is noted that this medium is generally known to produce slower growth and lower protein production titres as compared to complex medium (Zanette *et al.,* 1998; Manderson *et al.*, 2006).

A complex medium contains some complex ingredients in which the exact constituent of the medium is not known, such as yeast extract, blood, or casein