CHAPTER I

INTRODUCTION

Plasmids are extrachromosomal DNA elements of bacteria that represent a reasonable stable, but dispensable, gene pool in bacteria. Certain plasmid elements also are capable of reversible integrating with the host chromosome as an alternative to their extrachromosomal state. Plasmids with this dual properties have been termed episomes [1]. Plasmids may be conveniently classified into two major types : Conjugative and non-conjugative. A plasmid is classed as conjugative if it is self-transmissible from one cell to another. Non-conjugative plasmid does not have the inherent ability to initiate self-transfer but they nevertheless can be transferred from cell to cell by conjugative plasmids by a process called mobilization. Moreover both conjugative and non-conjugative plasmids can be transmitted by a virus vector (transduction) or by the direct uptake of DNA (transformation) [2].

Bacterial plasmids carry a variety of determinants which may permit their bacterial hosts to better survive in an adverse environment or permit their bacterial hosts to more successfully compete with other microorganisms of the same or different species. The most obvious medical significant plasmids are the resistance factors (R factor) which confer on cells harbouring them resistance to various antibiotics [3]. The R factors were first observed in Japan in 1957 by Watanabe during an outbreak of dysentery, when strains of

Shigella sonnei which were resistant to more than one antibiotic were isolated [4]. R factors may determine resistance to one antibiotic or many, e.g. ten or more distinct antimicrobial agents. The resistance genes are often incorporated into discrete genetic units called transposon, which have the capacity to transpose from one DNA molecule to another. This has undoubtedly contributed to the rapid dissemination of antibiotic resistance by providing an efficient mechanism for incorporating resistant determinants into the new vectors which can transfer to and stably replicate in diverse hosts. It was readily apparent from early studies that R factors are widespread both geographically and in wide variety of pathogenic and non-pathogenic bacteria. More recent studies have emphasized the increased incidence of plasmid determined multiple antibiotic resistance concomitant with the increased employment of antibiotics and the importance of R factors in human and veterinary medicine.

β-lactamase enzyme is the common mechanism by which many aerobes resist to β-lactam antibiotic. The enzyme may be either controled by chromosomal gene or plasmid. The work on the bacterial production of β-lactamase enzymes are mainly concerned with aerobes. The report on β-lactamase producing anaerobes are seldom seen in Thailand. Therefore, the study of β-lactamase in the anaerobes is of considerable importance.

Bacteroides fragilis is an anaerobic Gram negative bacilli that normally resides in the human intestinal tract, where it constitutes a large part of the fecal flora (Fig. 1) [5]. As an opportunistic pathogen, B. fragilis is the anaerobe most frequently

isolated from clinical specimens [6-8]. Most of the B. fragilis produces β-lactamase enzyme of cephalosporinase type that hydrolyse cephalosporins [9-15] while Bacteroides species mainly produce penicillinase [16-19]. The bacterial resistance to β-lactam antitiotics may be plasmid mediated or chromosomal mediated [12]. They may carry specific β-lactamase plasmids which enable them to resist β-lactam antibiotics. They may also transfer the plasmids to other bacteria and delivered rapid increasing in number of β-lactamase producing bacteria [20-21].

The purposes of this project are firstly to study the prevalence of Bacteroides species which produce β-lactamase and their MIC to ampicillin, penicillin G and cefoxitin. Secondly, to study whether the β-lactamase production in B. fragilis group is plasmid mediated and whether the plasmid is transferable to susceptible strain or chromosomal mediated. Finally, the isoelectric point of the β-lactamase enzyme from these organisms will be determined by isoelectric focusing technique.

LITERATURES REVIEW

The important mechanism that Gram positive and Gram negative bacteria resist to β-lactam antibiotics is the production of inactivating enzymes. In Gram positive bacteria, e.g. Staphylococcus aureus, resistance to penicillin is due to destruction of β-lactam ring, the active site of β-lactam by β-lactamases which are usually plasmid encoded [20]. The enzymes are expressed at high level only after induction, with most of the activity being extracellular. On the contrary, Gram negative bacteria are intrinsically resistant to many β-lactam antibiotics. This was partly due to the inability of the drugs to diffuse easily through pores in the outer membrane [22], and partly to the low levels of chromosomally encoded β-lactamase [23]. However, high level of resistance to broad spectrum β-lactam antibiotics in Gram negative bacteria is due to β-lactamase activity specified by plasmids [20].

THE STUDY OF β-LACTAMASE

Criteria Used in the Characterization of β-lactamases

β-lactamase could be characterized by the combination information from the following parameters.

1. Substrate profile

Substrate profile referred to the hydrolytic activity of β-lactamase against β-lactam substrates. The concept had been evolved strictly for comparative purposes to avoid the wide differences in the level of β-lactamase expression in bacterial cells. Profiles were often expressed as ratios related to value of 100 for a chosen substrate (usually benzylpenicillin). The substrate profile alone was not a very good parameter for classification of β-lactamases because a number of different enzymes had similar substrate profiles [24,25].

2. Enzyme inhibitor

Many antibiotics include β-lactam itself and other chemicals may inhibit β-lactamase. Their inhibitory effect on the β-lactamase enzyme is currently used for characterization of β-lactamase enzyme.

2.1. β-lactam antibiotic. Methicillin, isoxazoyl penicillins (oxacillin, cloxacillin, dicloxacillin), clavulanic acid and sulbactam are not only stable to β-lactamases but are also inhibitors of many types of β-lactamase. The degree of inhibition had been used in the classification of β-lactamases [26].

2.2. Non β-lactam. Chemical substances are also capable of inhibiting the β-lactamase enzyme. Among the known substances commonly used in characterization and classification of β-lactamase are chloride ions and para-chloromercuribenzoate (pCMB).

3. Analytical isoelectric focusing

A direct visual comparison of β-lactamases could be made by examination of the patterns of the enzymes in analytical isoelectric focusing [27]. This was a method of separation proteins at their isoelectric points in a pH gradient. A high degree of resolution is obtained because focusing is caused by forces that acted against diffusion and the β-lactamases are therefore concentrated during the separation.

Further detailed on isoelectric focusing technique was described in page 18.

4. Immunological studies

Antisera had been raised to β-lactamases from organisms of several genera and used to compare the identities of enzymes obtained from a variety of strains.

5. Molecular weight

The molecular weight of purified β-lactamases had been determined by various methods e.g. gel filtration, sodium dodecyl sulphate polyacrylamide gel electrophoresis, ultracentrifugation.

Classification of β-lactamases

There are long controversy concerning an optimal system for

classification of β-lactamase. The traditional system are based on the hydrolytic activity of the enzymes against a range of β-lactam substrates and on their inhibition by various inhibitors.

The classification of β-lactamase enzyme widely known are those of Richmond and Sykes [26] and Matthew [28].

Richmond and Sykes Classification There are five major classes.

Class I The enzymes are predominately active against cephalosporins and susceptible to isoxazoyl penicillins and carbenicillin but resistant to clavulanic acid.

Class II The enzymes are predominately active against penicillins. They are susceptible to isoxazoyl penicillins and clavulanic acid.

Class III The enzymes are approximately equal activity against penicillins and cephalosporins. They are susceptible to cloxacillin but resistant to pCMB.

Class IV The enzymes of this class have similar substrate profile to the enzymes in Class III the difference are the resistance to cloxacillin but susceptible to pCMB.

Class V The enzymes in this class have a penicillinase profile including activity against cloxacillin and resistant to pCMB.

In the scheme of β-lactamases classification by Sykes and Matthew, the enzymes are grouped on the basis of substrate profile and genetic location of the β-lactamase genes [25].

1. Chromosomally Mediated β-lactamases Most of the β-lactamase enzymes which are controled by chromosomal gene are mainly produced in the nature without stimulation by the inducer. Based on the hydrolytic activity the enzymes may be devided into :

1.1. Penicillinase The enzymes hydrolyse penicillin at a much greater rate than the cephalosporins and are inhibited by cloxacillin.

1.2. Cephalosporinases The enzymes hydrolyse cephalosporins at least 5-10 times as rapidly as benzylpenicillin and they are inhibited by cloxacillin and carbenicillin.

1.3. Broad Spectrum β-lactamases In addition to their broad spectrum activity the enzymes are not inhibited by cloxacillin but are inhibited by pCMB.

2. Plasmid Mediated β-lactamases. The plasmid mediated enzymes were most commonly found in all of the members of the Enterobacteriaceae. Many other Gram negative fastidious and non-fastidious organisms and Gram positive cocci were also known to have plasmid mediated β-lactamase enzyme. Based on hydrolytic activity the enzymes are divided into :

2.1. Enzymes that did not hydrolyse isoxazoyl penicillins.

2.2. Enzymes that hydrolysed isoxazoyl penicillins and methicillin.

2.3. Other β-lactamases specified by R-plasmids.

Further detailed on plasmid - mediated β-lactamases was described in page 10.

The most update regrouping of the β-lactamases had been carried out using data obtained from sequence homology studies. Based on the molecular weight, substrate profile and sequence homology, Sykes classified β-lactamases into three classes [29,30].

Class A Comprised the enzymes of molecular weight about 30,000 daltons that are preferentially active against penicillins and shared considerable homology with one another.

Class B The enzymes in this class have the molecular weight of about 23,000 daltons and have activity mainly against cephalosporins.

Class C The enzymes in the last class are the enzymes which have large molecular weight of about 39,000 daltons and showed no amino acid sequence homology with the other classes. This class included the chromosomal cephalosporinases of Gram negative bacteria.

Plasmid Mediated β-lactamases

The first report of R plasmid specified β-lactamase that could be transferred between bacteria was isolated from Escherichia coli strain of a young girl in Athens whose first name was Temoniera in 1965 [21,31] and the enzyme was named after her initial as TEM enzyme. Since then many investigators described plasmid mediated β-lactamase enzymes from various sources of aerobic and anaerobic bacteria using different combination of substrate profile, enzyme inhibitor, molecular weight and isoelectric point. From the extensive reviews of Foster [20] and Medeiros [32,33] together with the report of other investigators, the various types of β-lactamase can be summarized as shown in Table 1.

The plasmid mediated β-lactamase were classified by their substrate profile into 4 groups named; broad spectrum penicillinases, oxacillinases, carbenicillinases, and unique substrate profile.

The broad spectrum penicillinases were the enzymes that hydrolysed penicillin as well as cephalosporin. The enzyme in this group was known as TEM type β-lactamase which composed of TEM-1, TEM-2, SHV-1, HMS-1, TLE-1, ROB-1, and LCR-1 as shown in Table 1. The TEM-1 β-lactamase was the most common β-lactamase found in clinical isolated [28,31,34,35]. The SHV-1 (sulphydryl variable) and HMS-1 (Hedges, Matthew, and Smith) were included in the TEM type β-lactamase due to the similarity of their substrate profiles [36].

Recently new types of TEM β-lactamase group were detected in ampicillin resistant isolate of E. coli named TLE-1 (TEM-like enzyme). TLE-1 resembled TEM-1 in substrate profiles and reaction with inhibitors but differed in isoelectric point.

Another β-lactamase, ROB-1, had been found in Haemophilus influenzae type b which isolated from a child with meningitis who failed to respond to ampicillin therapy [37]. Further studied about this enzyme was done by Medeiros et al. [38]. The enzyme had broad spectrum activity like TEM-1 but with an isoelectric point of 8.1 which was quite different from the TEM-1 pI of 5.4.

LCR-1 was another TEM type β-lactamase, identified by Simpson in a carbenicillin resistant isolate of Pseudomonas aeruginosa from a burn patient [39]. Isoelectric focusing study showed a single band nearly equidistant between those of PSE-1 and PSE-2, clearly different from the TEM-1 banding pattern [39] (Table 1).

Oxacillinases were other β-lactamases of plasmid origin found in E. coli that hydrolyzed isoxazoyl β-lactam substrates and methicillin. There were seven distinct OXA enzymes on the basis of isoelectric focusing and substrate studies (Table 1). The OXA-1 enzyme hydrolyzed methicillin about ten times more rapidly than the OXA-2 and OXA-3 enzymes. OXA-4, OXA-5, OXA-6, and OXA-7 hydrolyzed oxacillin, methicillin and cloxacillin readily but differed from OXA-1, OXA-2 and OXA-3 in substrate profiles, inhibitor reactions and isoelectric points (Table 1). OXA-4 and OXA-6 were unusual for members of the OXA group in their susceptibility to inhibition by

cloxacillin. OXA-5 and OXA-7 had isoelectric points close to SHV-1 [33]. It was postulated that the rarity of the OXA β-lactamases was a result of the limited transfer capacity of plasmids [31].

Carbenicillinases were the plasmid mediated enzymes in Pseudomonas described by Hedges and Matthew in 1970 [40]. These enzymes, PSE-1, PSE-2, PSE-3, and PSE-4 were originally thought to be restricted to Pseudomonas aeruginosa, but they had recently been found in the enteric species as well [41,42]. These PSE β-lactamases were able to hydrolyze carbenicillin as rapidly as benzylpenicillin. They had been divided into four types on the basis of molecular weight, isoelectric point and inhibition by β-lactamase inhibitors. PSE-3 and PSE-4 were generally not self-transmissible, even among Pseudomonas strains. The PSE-4 enzyme which also known as the Dalgleish β-lactamase was a carbenicillin hydrolyzing enzyme that would also hydrolyze ticarcillin, piperacillin, cefoperazone and cefsulodin. Recently, a new plasmid mediated β-lactamase from Pseudomonas aeruginosa had been characterized as NPS-1 [43]. This enzyme focused between PSE-2 and PSE-3 enzymes, at a pI of about 6.5. Another new carbenicillin hydrolyzing β-lactamase, designated AER-1 was found in Aeromonas hydrophila isolated from the blood culture from India. The enzyme had a pI of 5.9 and focused slightly higher than the LCR-1 β-lactamase in polyacrylamide gel [28].

The unique substrate profile β-lactamase was another plasmid determined β-lactamase discover in Achromobacter species by Levesque et al. [44]. It composed of CEP-1 and CEP-2. Cephaloridine was the best substrates for this enzyme. The enzyme, CEP-2 had a pI of 8.1

and preferentially hydrolyzed the cephalosporins like CEP-1. The CEP-1 determinant was transferred from Proteus mirabilis to E. coli, where it specified a β-lactamase with properties indistinguishable from those of the E. coli chromosomal enzyme. It had not been demonstrated unequivocally that the CEP-1 structural gene resides on the plasmid [45].

Another new plasmid determined β-lactamase, SAR-1, was identified from Vibrio cholerae biotype El Tor [46]. The enzyme was able to hydrolyze carbenicillin as well as benzylpenicillin and quite distinct from all other plasmid determined β-lactamases by virtue of it unusually low isoelectric point at 4.9 and a combination of its size, substrate profile and inhibition properties.

An increasing number of these β-lactamases were now known to be encoded by transposons, genetic elements capable of transfer among a wide variety of plasmids and between plasmids and chromosomes [47]. Three of the TEM-like β-lactamases were encoded by transposons (Tn). TEM-1 was determined by Tn3 and TEM-2 by Tn1 which shared much homology with Tn3 [48,49]. SHV-1, on the other hand, was encoded by a transposon of 15 kilobase pairs unrelated to Tn1 [50]. Several of the OXA-like β-lactamases and PSE-type enzymes also were encoded by transposons [51,52]

β-lactamases of Bacteroides fragilis Group

The Bacteroides fragilis in this study comprised the following species: B. fragilis, B. distasonis, B. ovatus, B. vulgatus,

B. thetaiotaomicron and B. uniformis. These were formerly regarded as subspecies of B. fragilis except B. uniformis which was a new species now included in B. fragilis group [53].

Penicillin-hydrolyzing activity of B. fragilis was first noted by Pinkus et al. in 5 out of the 18 strains tested [54]. Since that report, several studies had shown 50-90% of B. fragilis strains possess constitutive, membrane-bound β-lactamases [12,55-57]. However, an occational strain of B. fragilis produce extracellular β-lactamase under the certain conditions [57,58]. The β-lactamases of B. fragilis were predominantly active against cephalosporins and were only moderately active against penicillin [55,57-60]. Relatively low levels of β-lactamase were found in intermedietely resistant strains. Highly resistant strains possessed a ten fold higher concentration of enzyme [12,57]. A few investigators had isolated a number of B. fragilis strains that were highly resistant to β-lactam antibiotics, included cefoxitin [61,62]. However, cefoxitin was seldom degraded, and it seemed not to be a substrate for the β-lactamase enzymes produced by these strains. These organisms seemed to possess a barrier to the target sites of β-lactam antibiotics [63, 64].

There were controversy in the result of transferability of B. fragilis. Several laboratories failed to transfer penicillin and ampicillin resistance from B. fragilis to E. coli and between strains of B. fragilis by both broth mating and filter mating technique [55, 62,65-67], while many success were reported [68-77]. Furthermore, Mancini and his associates succeeded in transferring multiple

antibiotic resistance factor included ampicillin and penicillin resistance from a strain of B. fragilis to E. coli K12 by broth mating technique but the detail information of genetic control of the resistance was not reported [76]. Butler et al. was able to transfer the high level ampicillin resistance between strains of B. fragilis. The resistance element was however required mobilization by a tetracycline resistance transfer element [77-78]. After plasmid analysis, the plasmids were shown not to bear the resistance factor and the exact location of the ampicillin resistant determinant was not conclusive. In 1979 Tally et al. studied the β-lactamases produced by Bacteroides and suggested that gene coding for β-lactamase production in the B. fragilis group were likely located on chromosome [12]. In the year 1981, Rashtchian et al. could transform penicillin and tetracycline resistance from a strain of B. fragilis to E. coli and a 1.9 megadalton plasmid was supposed to associate with the resistance [79]. In the same report they could not transfer the resistance by filter mating technique. A year later, they were successful in transferring cefoxitin resistance from B. thetaiotaomicron to B. fragilis by a conjugation like process. The donor was harboured 4 plasmids with molecular masses of approximately 5.1, 2.9, 2.2 and 2.1 megadaltons. From 20 transconjugants, 8 were found to contain all 4 plasmids, 10 contained 1 to 3 plasmids, and 2 contained no plasmids. They suggested that the cefoxitin resistance genes either were not carried on any of the four plasmids or were carried on one or more plasmids which could integrate into the chromosome of the transconjugants, as an episome. Another possibility was that the resistance genes were located on conjugative transposons [80]. Recently, Cuchural et al. demonstrated a cefoxitin resistance

B. fragilis was able to transfer β-lactamase mediated cefoxitin resistance to a susceptible B. fragilis recipient by conjugation, but no plasmids were detected in the donor or transconjugants and the pI of the cefoxitin resistant β-lactamase was 8.1 [81].

Isoelectric focusing of the β-lactamases revealed that the B. fragilis group possessed enzymes which focused in the acid range (Table 2). An isoelectric point of 4.9, 4.7 and 4.8, 4.9 and 5.1 were found for the β-lactamases in the species B. fragilis [57,82-88]. Isoelectric point of β-lactamases from B. distasonis had been determined to be 4.8-5.6 [9,12,82,83]. Most of β-lactamases from B. ovatus focused at pI 4.0, 4.3 and 4.6 [12,82,85]. Most of B. thetaiotaomicron produced β-lactamases with isoelectric points of 4.3 [9,82,84,85]. β-lactamases from B. vulgatus had been found to have isoelectric points between pI 4.4 and 4.9 [9,12,82,85].

From many reports on substrate profile study, the β-lactamases so far described in B. fragilis group were of cephalosporinase character (Table 3) [11,57-60]. They hydrolyzed cephaloridine more rapidly than benzylpenicillin. Liljequist et al. studied β-lactamases from the two strains each of B. fragilis and B. uniformis [62]. Crude and purified β-lactamase preparations were used and given similar results. The β-lactamases from two strains of B. fragilis showed almost identical substrate profiles and the rates of hydrolysis were in the following order : cephaloridine > cefuroxime > penicillin. The pI of both strains were 4.9. β-lactamases from the two B. uniformis strains were different from each other and also from the B. fragilis β-lactamases. Their respective rates of hydrolysis of

different substrates were as follows : for one strain, cephaloridine > cefuroxime > penicillin, and for another strain, cefuroxime > cephaloridine > penicillin. The pI of the two strain were 5.6 and 5.3. The low hydrolysis rates of benzylpenicillin and the inability to hydrolyze cefoxitin were common features of all four enzymes (Table 3).

The studies of inhibition revealed that most β-lactamase enzymes from the B. fragilis group were susceptible to inhibition by cloxacillin, cefoxitin, para-chloromercuribenzoate, clavulanic acid and sulbactam [57,64,85].

The molecular weight between 30,000-40,000 daltons had been reported for β-lactamases from B. fragilis [57-59]. These value were unusually high compared with β-lactamases from aerobic Gram negative bacilli which had molecular weight between 20,000-30,000 daltons [26].

The studies on localization of β-lactamases from B. fragilis were also reported [57,59]. β-lactamase of one B. fragilis strain was found to be cell-bound and associated with the cell envelope based on the following observations :

(i) the enzyme was not released into the surrounding medium except under certain growth conditions. At these conditions other enzymes which were believed to be periplasmically located such as alkaline phosphatase, 3'-nucleotidase, and 5'-nucleotidase were also released to a great extent;

(ii) the cell-bound part of the β-lactamase activity was divided between the cytoplasmic fraction (40%) and the washed cell wall fraction (10%);

(iii) an osmotic shock procedure released 40% of the cell-bound β-lactamase (20% of the total B-lactamase) [57].

In another study, release of 26% of the total amount of β-lactamase was achieved by a modified osmotic shock procedure [59].

Darland and Birnbaum investigated the intracellular distribution of β-lactamase in nine strains of the B. fragilis group by testing both the supernatant fraction and pellet after centrifugation of crude material obtained by sonication of bacterial suspensions. In six of the nine strains, they found that more than 97% of enzyme activity was in the supernatant fractions, whereas a considerable amount of enzyme activity of the three remaining isolates (33-51%) was found in the pellets [56]. These results indicated that the B. fragilis β-lactamase was most likely located in the periplasmic space [87].

Principle of Isoelectric Focusing Technique [88]

The technique of isoelectric focusing is an electrophoretic one, but instead of the separation being carried out at a constant pH, the separation is carried out in a pH gradient, which is established between two electrodes and is stabilized by carrier ampholytes (an ion that is charged both positively and negatively). In this technique proteins migrate until they align themselves (at their pI) at the point where a protein possesses no net overall charge and will therefore concentrate while migration ceases at the point called pI. It is therefore an equilibrium technique in which the effects of

diffusion are overcome and is the electrophoretic technique with the highest resolution, in the components that differ by 0.001 of a pH unit, or less, can be resolved [89]. The pI of a protein defines a physical parameter of the protein and has the great significance.

Polyacrylamide Gels [88]

Polyacrylamide gels are extensively used for isoelectric focusing technique. They have the advantage of possessing an exceptionally low number of charged groups and this means that exceptionally high field strengths can be used without ill effects. This technique gives rise to exceptionally high resolution and faster running times.

The Process of Polymerization

Polyacrylamide gels are formed by the polymerization of the monomer acrylamide together with a cross-linking agent, normally N,N'-methylene bisacrylamide, as shown in Fig. 2.

Polymerization of the acrylamide monomers only occurs in the presence of free radicals. These are normally produced chemically by the presence of persulphate ions. Tertiary amine groups are acquired as accelerators and frequently the base, N, N, N', N'-tetramethyl-ethylenediamine (TEMED) is added for this purpose. The polymerization reaction is strongly inhibited by high levels of oxygen. Therefore the gel solution must be thoroughly degassed under vacuum. Riboflavine is used as catalyst of the polymerization. Saccharose is

an additive used for stabilizing the pH gradient by increasing the viscosity of the system as well as osmolality.

The isoelectric focusing technique is an important technique for fractionation and characterization of proteins included enzymes. This technique has been used widely for β-lactamases studied. Moreover, the specific type of β-lactamase with the other current bacterial properties, make it possible for recognition of the species of β-lactamase producing bacteria [27,90-92].

THE STUDY OF PLASMID

Principle of Plasmid Isolation

The first step to study plasmids of bacteria is to isolate the plasmids. Many methods used for plasmids isolation usually based on the principle that the size of plasmids DNA is smaller than the size of the chromosomal DNA. With larger size, the bacterial chromosomes are easily broken into linear fragments and easily precipitated out whereas plasmids DNA usually remain intact and remain in supernatant because of the smaller sizes. The main difficulty is to ensure that plasmid DNA is free from contamination by chromosomal DNA, and to avoid the breakage and loss of plasmids.

There are usually three major steps of plasmid isolation.

1. Bacterial cells are broken in lysis solution which covalently closed circular plasmids are not broken unless chromosomal DNA are denatured. This method varies according to the species of

bacteria. Enterobacteria are usually treated with lysozyme to weaken the cell wall then lysed completely with sodium dodecyl sulfate.

2. Cell debris, protein and chromosomal DNA are removed from the cell lysate by salting out or phenol chloroform extraction [93,94].

3. The plasmid DNA is concentrated by precipitation with cold ethanol and can be separated from fragments of chromosome which remain in the supernatant by dye-bouyant density centrifugation such as cesium chloride ethidium bromide centrifugation.

The sizes of plasmid as expressed by its molecular weight are determined by the migration of DNA in agarose gel electrophoresis [95]. The rate of migration is proportional to the molecular weight of the DNA. After the gel electrophoresis, the position of plasmid DNA was determined by staining the gel with ethidium bromide solution which intercalates into the DNA and fluoresces when exposed the gel to an ultraviolet light. The size of plasmid can be determined relatively to the mobility of reference plasmids by plotting the logarithm of relative migration of reference plasmids versus the logarithm of plasmid molecular weight.