PRODUCTION OF MBP-*TAQ* I RESTRICTION ENDONUCLEASE FUSION PROTEIN BY RECOMBINANT *ESCHERICHIA COLI* HARBORING PETMET

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ABSTRACT

PRODUCTION OF MBP-*TAQ* I RESTRICTION ENDONUCLEASE FUSION PROTEIN BY RECOMBINANT *ESCHERICHIA COLI* HARBORING PETMET

The gene encoding the *Taq* I restriction endonuclease was cloned into pMAL-c2 plasmid to construct p22 expressing the MBP-*Taq* I fusion protein. The affinity of MBP to amylose was exploited for the single–step chromatographic purification. To protect the *Taq* I endonuclease gene from the cleavage by its own product, the co-expression of *Taq* I methylase was recommended. Therefore, the gene encoding the *Taq* I methylase was PCR amplified and a recombinant plasmid (pETMET) expressing this enzyme was constructed.

In this study *E. coli* cells were transformed by p22 and pETMET. The optimum host strain, induction time and period using this system was determined by shake-flask experiments. The highest productivity (5.10^5 U/L *Taq* I endonuclease activity) was obtained when the recombinant *E. coli* TB1 (p22, pETMET) cells were grown in LB medium and induced at the early exponential phase of the growth for 10 hours. The growth, plasmid stability, and *Taq* I endonuclease activity were investigated under fully controlled operating conditions using a fermentor. The maximum total *Taq* I endonuclease activity ($7{,}5.10^5$ U/L) could be reached when the recombinant cells were induced for 12 hours in SB Medium.

In conclusion, the presence of pETMET has slightly improved the yield of MBP-*Taq* I endonuclease production.

ÖZET

MBP-TAQ I RESTRİKSİYON ENDONÜKLEAZ FÜZYON PROTEİNİNİN PETMET PLAZMİDİ İÇEREN REKOMBİNANT *ESCHERICHIA COLI* İLE ÜRETİMİ

Taq I restriksiyon endonükleaz enzimini kodlayan gen, MBP-*Taq* I füzyon proteininin ekspresyonu için pMAL-c2 plazmidine klonlanmıştır (p22). Füzyon proteininin tek aşamalı afinite kromatografisi ile saflaştırılması için MBP'nin amiloz'a afinitesi kullanıldı. *Taq* I endonükleaz geninin kendi ürettiği enzim tarafından kesilmesini önlemek için *Taq* I metilaz enziminin birlikte ekspresyonu tavsiye edilmiştir. Bu nedenle *Taq* I metilaz enzimini kodlayan gen PCR ile çoğaltılmış ve pET sistemi içine klonlanarak bu enzimin ekspresyonunu sağlayan pETMET adlı rekombinant plasmid yapılmıştır.

Bu çalışmada *E. coli* hücreleri p22 ve pETMET ile bu iki genin birlikte ekspresyonunu sağlamak için dönüştürülmüştür. Yüksek verimli *Taq* I endonükleaz üretimi için konakçı hücre, indükleme zamanı ve süresinin optimizasyonu farklı rekombinant *E. coli* suşlarının kullanıldığı shake flask deneyleri ile yapılmıştır. En yüksek üretim (5.10^5 U/L *Taq* I endonükleaz aktivitesi) rekombinant *E. coli* TB1 (p22, pETMET) hücrelerinin LB besi ortamında büyütülmesi ve erken büyüme evresinde 10 saat boyunca indüklenmesi ile elde edilmiştir. Büyüme hızı, biyokütle üretimi, plazmid kararlılığı, protein derişimi ve *Taq* I endonükleaz aktivitesi tam kontrollü fermentör ortamında incelenmiştir. En fazla toplam *Taq* I endonükleaz aktivitesine ($7{,}5.10^5$ Ünite/L) rekombinant *E. coli* TB1 (p22, pETMET) hücreleri SB besi ortamında 12 saat boyunca indüklenerek ulaşılmıştır.

Sonuç olarak pETMET plazmidinin varlığının MBP-*Taq* I endonükleaz üretimini geliştirdiği saptanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

μ_{max}	Specific Growth Rate
bp	Base pairs
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraaceticacid
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
MBP	Maltose Binding Protein
OD_{600}	Optical Density at 600 nm wavelength
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
T. aquaticus	*Thermus aquaticus*
U	Unit
UV	Ultraviolet

1. INTRODUCTION

Restriction endonucleases are important tools in biotechnology both in the cloning of the genes for the high level production of commercially important recombinant proteins and in the diagnosis of genetic disorders. Therefore purification and production of these enzymes are considered to be very important. *Taq* I restriction endonuclease is a thermostable type II endonuclease and has a large number of applications in the identification of point mutations and polymorphisms in the DNA diagnosis. Because of its high specificity, it also serves as a model enzyme to study DNA - protein interactions.

In bacteria, methylases are found and implicated in the protection of DNA from their own restriction endonucleases and in mismatch repair (Noyer-Weidner & Trautner, 1993). Methylation provides resistance to cleavage for the methylated gene by altering the specific recognition sequences. *Taq* I restriction endonuclease cleaves the palindromic 5' TCGA 3' DNA sequence between the T and C residues, whereas *Taq* I methylase puts a methyl group at the A residue. In order to protect the *Taq* I endonuclease gene from cleavage by its own product, the *Taq* I methylase gene should be expressed.

The low yield and high cost of the isolation of the enzyme from its host, *Thermus aquaticus,* made it necessary to clone and express *Taq* I restriction endonuclease in *Escherichia coli* and to develop purification techniques. The genes encoding both the *Taq* I endonuclease and methylase have been cloned from *Thermus aquaticus* YT-1 and sequenced. The expression of these genes together or separately under the control of various promoters were investigated (Barany, *et al.*1987, 1992).

In the Biochemical Engineering Laboratory, the gene encoding the *Taq* I restriction endonuclease was PCR amplified using *Thermus aquaticus* genomic DNA as a template and then inserted into pMAL-c2 plasmid. The recombinant *E. coli* strains were constructed using the pMAL-c2 plasmid, which expresses the MBP-*Taq* I fusion protein. The recombinant plasmid was transferred into *E. coli* TB1 cells. The recombinant cells containing this vector conferred resistance to ampicillin. Expression of the MBP-*Taq* I fusion protein by this construct in different strains of *E. coli* was investigated (Özkırımlı, 1998).

The affinity of MBP to amylose was exploited for the single – step chromatographic purification of the fusion protein by affinity chromatography. The MBP tail could be removed by cleaving with Factor Xa. The cleavage of the MBP tail of the purified fusion protein resulted in the production of a protein with a molecular weight corresponding to that of *Taq* I endonuclease (Özkırımlı, 1998).

In order to express *Taq* I methylase, its gene was PCR amplified and a recombinant plasmid (pETMET) expressing this enzyme and conferring resistance to kanamycin was constructed by E. Toksoy (unpublished results) and K. Çavuşoğlu (1999).

In this study, the coexpression of *Taq* I methylase and the *Taq* I restriction endonuclease genes was performed to increase the production of recombinant restriction endonuclease by protecting the *Taq* I restriction endonuclease gene from cleavage by its own product. Therefore, *E. coli* cells were transformed by pMAL-c2 expressing *Taq* I restriction endonuclease as an MBP-fusion protein (p22) and pETMET containing a *Taq* I methylase gene, so that these two genes would be coexpressed.

Shake flask experiments using different recombinant strains of *E. coli* was conducted to determine the optimum host strain for the high-yield production of *Taq* I endonuclease using this system. Plasmid stability and cell viability was also determined.

The effect of the presence of pETMET on the production of *Taq* I restriction endonuclease by genetically engineered cells was investigated under fully controlled operating conditions using a 2.5L BioFlo 3000 Fermentor. The growth rate, biomass yield, plasmid stability, protein concentration, and *Taq* I endonuclease activity were investigated.

2. THEORETICAL BACKGROUND

Currently one of the main aims of genetic engineering is to force a host cell to produce an optimal level of one or more desirable high value proteins. For the production of large amounts of a recombinant protein or intermediate enzymes that facilitate the synthesis of an end product in the host cell, genetically engineered cells are used. In the latter case, genetic engineering techniques are employed, for example, to produce an enzyme that speeds up the limiting reaction in the production of a desired product (Georgiou, 1988).

In a commercial process, the overall objective is to maximize the yield of a desired product and to increase the rate of production if possible. However, the overproduction of a recombinant protein usually disrupts the coordination of metabolic processes and may ultimately result in cell death, and is, hence, unfavorable. Therefore, the maximization problem of the chemical engineer becomes an optimization problem in which the physiology of the genetically engineered cells must be taken into account (Georgiou, 1988, Cserjan-Puschmann, 1999).

Recombinant proteins can be produced in high levels in various expression systems, and the choice depends on; (a) Cell growth characteristics; (b) Expression levels; (c) Intracellular and extracellular expression; (d) Post – translational modifications such as the proteolytic degradation, and formation of inclusion bodies; (e) Biological activity of the protein when expressed with that specific expression system; (f) Degree of authenticity required and regulatory issues in the production of therapeutic proteins (Makrides, 1996).

2.1. *Escherichia coli* as a Host

The choice of the host for the expression of a recombinant protein is the most important step in the selection of the expression system. For large-scale productions, the host must propagate fast with simple nutritional requirements. It must not be pathogenic and must not produce any toxins that may contaminate the product. The cells should be able to grow to high cell densities to maximize protein production. *Escherichia coli* is by far the most widely used host for the high level production of recombinant proteins, because it grows fast on simple nutrients and there is extensive knowledge on the genetics and microbiology of *Escherichia coli,* which makes it easier to model, predict and use in large scale productions (Georgiou, 1988, Baneyx, 1999).

Escherichia coli is also versatile because there are many high expression vectors available for it. However, there are some drawbacks to this host, too. It is unable to perform post-translational modifications required for eukaryotic proteins, it cannot facilitate disulfide bond formation necessary for the three dimensional conformation of some proteins. Some proteins cannot assume their correct three - dimensional structure that is essential for biological activity and instead precipitate inside the cell forming inactive protein aggregates called inclusion bodies. It cannot secrete the protein into the culture medium, which would be beneficial from a biochemical engineering point for the simple purification of the target protein. The stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, differences of codon usage between the foreign gene and *Escherichia coli,* and the toxicity of the protein to the host determine the efficiency of expression of a protein in *Escherichia coli* (Makrides, 1996).

2.1.1. Strain Selection

The choice of the strain for the expression of recombinant protein depends on the target protein, the expression vector, the fermentation conditions and the particular goal of overproduction. For instance, for proteins that are highly susceptible to proteolysis, it may be necessary to use protease deficient strains.

TB1 has been reported to be the most widely used strain with pMAL expression vector. It is an α-complementing host, allowing selection of recombinant plasmids harboring an insert.

XL1-Blue is often used for high quality plasmid DNA purification. It carries the gene endAl, preventing nonspecific activity of endonuclease 1 on DNA. It also carries the recA gene and hence homologous recombination is abolished increasing plasmid stability. This is particularly desirable when working with sequences containing direct repeats larger than 50 bp. Moreover, it is an α - complementing host. (Maniatis *et al.*, 1989)

ER2508 is a protease deficient strain. The *lon* protease is not expressed and hence this strain is most frequently used when proteolysis is a problem. The malB gene deletion includes the malE gene, so this strain does not produce any chromosomal MBP. This is beneficial when MBP is expressed as a fusion partner to a target protein. All the MBP produced is part of the fusion partner, increasing the efficiency of amylose column chromatographic purification. The proteins secreted to the periplasm can be released into the culture medium when expressed in a periplasmic leaky *Escherichia coli* host such as E609L.

2.1.2. Transcriptional Regulation

The gene coding for a desired protein acts as a template for the production of that protein. The gene may be inserted into the host chromosomal DNA for expression, however, the amount of mRNA determines the rate of expression of a protein. Therefore, if the number of copies of the target gene in the host can be increased, the rate of product formation will be increased. To achieve this aim, the gene is inserted into high copy number plasmids that are propagated from one generation to the next and exist in several copies per cell. Plasmids containing a desired foreign gene and designed specifically to effect a high level of product synthesis are called expression vectors (Georgiou, 1988). An ideal plasmid should have a low molecular weight for easier handling and higher copy number, and should have single sites for a large number of restriction endonucleases. The expression vector should also harbor a gene that confers a phenotypic trait to simplify selection and propagation of the recombinant colonies. This is usually an antibiotic resistance gene. Hence, only those cells harboring the plasmid, i.e. recombinant cells, can grow in the presence of antibiotic supplemented to the medium (Primrose, *et al.* 1983). The essential components of an expression vector are the promoter, the ribosome binding site, the transcription terminator and the origin of replication.

The gene of interest must be placed under the control of an *Escherichia coli* promoter. Two types of promoters exist that initiate transcription; constitutive and regulated. Constitutive promoters are always turned on and the rate of mRNA synthesis is not modulated. On the other hand, a regulated (controllable) promoter controls the level of mRNA synthesis and hence production of the protein under its control. Repressible or inducible promoters may be employed to control gene expression. The inducible promoters are usually preferred because they are induced by the addition of a substrate or by temperature shifts; however, repressible promoters require the dilution of the repressor from the growth medium by changing the culture medium, which is not practical (Georgiou, 1988).

There are basically four desirable features of a regulated promoter. First, it should be strong. Thus, the yield of the target protein can be as high as 30 per cent of the total cellular protein. It must have a low basal expression level, meaning it must be tightly regulated and not "leaky". Leaky protein expression before induction of the cells may result in the accumulation of the usually toxic recombinant protein, causing cell death due to excess toxicity or metabolic burden. Plasmids also lose stability as a result of expression of the foreign protein, and hence a decrease in growth rate occurs and recombinant protein production may drop significantly. The promoter must be easily transferable to other *Escherichia coli* strains to study expression in different strains. Finally, its induction must be simple and cost effective. For laboratory research, strong promoters such as *tac* or *trc* are usually employed that may be induced by the addition of IPTG. However, IPTG is both toxic and very expensive, making it unsuitable for large-scale use. For large-scale production, promoters induced by thermal induction or chemical inducers are used (Table 2.1).

Table 2.1. A few promoters used for the high level expression of genes in *Escherichia coli* with their advantages and disadvantages (Weickert *et al.,* 1996)

Promoter	**Induction**	**Characteristics**
lac	IPTG, high temperature	Low level, leaky expression
phoA	Phosphate starvation	Limited media options
recA	Nalidixic acid	High level expression
T7	IPTG, high temperature	Very high level, leaky expression
tac	IPTG, high temperature	High level, leaky expression Induction causes cell death
trp	*Tryptophan starvation*	*Leaky expression*

Improvements have been made in promoter control that increase efficiency of transcription and provide tighter control at a lower cost some of which are listed in Table 2.1 (Weickert *et al.* 1996). A temperature sensitive mutant *lacI* gene that encodes a thermosensitive *lac* repressor is a recent development with which *lac* based promoters can be induced thermally with a tight regulation. However, although thermal induction is cost effective and simple, it may result in the increased production of proteases, which degrade the product. Some expression vectors use the moderately strong arabinose inducible araB promoter and the araC repressor. The araB promoter can both be tightly repressed and modulated over a wide range of inducer conditions which is advantageous for the control of gene expression. The araB expression systems could be used with other promoters to independently regulate expression of two or more recombinant proteins in Escherichia coli. The regulatory region of the cadA gene was used to construct a pH inducible expression system. This is advantageous since bulk chemical inducers such as acids and bases can be employed. Another advantage of pH induction over temperature induction is that high temperatures induce formation of inclusion bodies and heat shock proteins including lon protease, which result in degradation.

To achieve a high yield of protein it is common practice to use strong expression systems, which are often too strong and lead to rapid loss of host-cell metabolic activity or even to cell death after induction, because the recombinant protein production imposes a high metabolic load on the host-cell. Therefore the recombinant protein expression rate should be adapted for optimal exploitation of the host-cell's synthetic capacity. The reduction of the promoter strength of strong expression systems, e.g. by feeding limiting quantities of inducer, is an effective way of tuning the expression rate (Cserjan-Puschmann, 1999).

2.1.3. Plasmid Stability

The vector contains the origin of replication, which determines the average plasmid molecules per cell, which is also called the plasmid copy number (Georgiou, 1988). However, the exact number of plasmids in individual cells may differ. Moreover, the fraction of colonies harboring the plasmid may change throughout the fermentation. This is called plasmid instability. Plasmid stability is the ability of plasmid harboring cells to maintain the structure of the plasmid with a high copy number. Batch culture studies show that the recombinant plasmid suffers from segregational and structural instabilities. Structural instability is the result of insertions or rearrangements within the plasmid or the loss of some plasmid material by deletions (Primrose, *et al.* 1983). Segregational instability is due to the defective partitioning of plasmids resulting in the loss of the entire plasmid. Plasmid free cells have a higher growth rate and can outgrow the plasmid containing cells. Plasmid free cells do not produce the desired protein and hence their domination results in a rapid decline in the efficiency of the culture. Plasmids with small DNA inserts are more stable. The production of a protein toxic to the host cell results in the death of the plasmid containing cells. Hence the plasmid free cells dominate the culture much faster. Dissolved oxygen concentration, growth temperature, presence of essential amino acids, dilution rate are some of the environmental factors affecting plasmid stability. The most common method of preventing plasmid free cells from dominating is using ampicillin selection. The plasmid harbors the gene for ampicillin resistance and when the cells are grown in ampicillin containing media, the loss of the plasmid results in cell death. However, in large-scale cultivations, the antibiotic in the medium can be inactivated by a small portion of the cells and hence the plasmids free cells can propagate. Moreover, the cost and waste disposal considerations make antibiotic selection undesirable in large scale productions. The stability of the plasmid can also be increased when a plasmid-encoded protein is essential for cellular metabolism. The plasmid stability problem can be solved definitely by the insertion of the target gene into the bacterial chromosome at the expense of having only one copy of the gene per cell which would limit the mRNA synthesis and hence protein production (Georgiou, 1988). Plasmids can also be maintained if they harbor the partitioning function, par, which ensures that the plasmid is efficiently segregated to the daughter cells at cell division (Primrose, *et al.* 1983).

2.1.4. Cytoplasmic Expression

The cytoplasmic expression usually yields high amounts of the desired protein. However, the drawbacks of the cytoplasm usually obliterate this advantage. First of all, the cytoplasm is crowded with all the other proteins of the cell, making the purification of a recombinant protein from this pool a very tedious task. Fusion protein strategies discussed below that use fusion partners or tags with affinity to certain substances may be used to purify the protein using affinity chromatography. Application of the heat to the total cell protein pool and then precipitation of the denatured proteins may easily be exploited for the purification of the thermostable proteins.

The fermentation conditions can be altered to directly express the protein as a soluble active protein in the cytoplasm of *Escherichia coli.* The rate of protein synthesis may be reduced so that intracellular concentration of aggregation prone intermediates is minimized. The bacterial cells can be grown at lower temperatures to slow down the metabolic processes of the host and to allow sufficient time for the protein to fold correctly. When the recombinant cells that express human interferon were grown at temperatures lower than 30°C, most of the protein was active and soluble while protein from cultures grown at 37°C was insoluble (Schein, 1989). Selection of different *Escherichia coli* strains such as strains deficient in thioredoxin reductase that maintain a favorable redox potential may also minimize inclusion body formation. Chaperones that interact with the nascent polypeptide chain preventing self-association may be co-expressed. It is also possible to co-express foldases that catalyze kinetic barriers associated with the folding reaction (Georgiou *et al.,* 1996). Thioredoxin may be used as a fusion partner or coproduced with the protein of interest. Other highly soluble fusion partners may also aid formation of the native three-dimensional structure. Growth and induction of the cells under osmotic stress in the presence of sorbitol or glycyl betain, addition of nonmetabolizable sugars to the medium may also prove successful (Makrides, 1996).

2.2. Fermentation Conditions

Protein production can be increased significantly through the use of high-cell density culture systems in batch, fed batch and continuous systems. The growth medium composition, oxygen availability, pH, temperature and induction conditions are the parameters that influence recombinant protein overexpression (Hannig and Makrides 1998).

The fermentation medium can be chemically defined (synthetic) or undefined (natural, complex). Although defined media are not frequently developed for industrial processes, they exhibit favorable characteristics at large scale that are not observed with undefined media composed of complex nitrogen and carbon sources. As a result, fermentation performance may vary due to variability in media, whereas performance consistency is expected when defined media are applied to fermentations (Zhang and Greasham, 1999).

Acetate accumulation is a challenge in the production of proteins since acetate is a lipophilic agent that is detrimental to cell growth (Makrides, 1996). Uptake of glucose, when present in excess amounts normally exceeds the need for proper cell functions and subsequently leads to acetic acid formation. Monitoring glucose uptake rate can reduce acetate excretion. Methyl α-glucoside can be used as a nontoxic competitive inhibitor to modulate cellular glucose uptake rate, which delays the onset of acetate excretion. Initial concentration of glucose is important in acetate accumulation (Chou *et al.,* 1994). It has been reported that when the glucose concentration is higher than 5 g/L, protein production and cellular growth decreases (Narciandi *et al.,* 1996). Acetate accumulation is shown to be strain and growth medium specific. In BL2l, high glucose concentration results in a reduction in growth rate after induction, while low glucose concentration leads to exponential growth throughout induction. On the other hand, with JM109, high glucose concentration leads to liner growth throughout induction. It was reported that BL21 is a superior strain with regard to acetate accumulation suggesting that it probably possesses an acetate self control mechanism (Shiloach *et al.,* 1996). Lower acetate excretion was attained in fructose-supplemented growth media compared with those of glucose media (Aristidou, 1999).

The supply of yeast extract to the culture medium has been reported to decrease the rate of proteolysis of the recombinant protein. It was proposed that the reason for this is that the proteases select the smaller peptides supplied in the feed (Harrison *et al.*, 1997).

The oxygen supply to the feed is also important for protein production. Better oxygen supply to the culture provided by smaller working volumes and higher shaking speeds increases total and extracellular protein production. Moreover, glycerol increases secretion to the medium (Mikch, *et al.*, 1997).

The induction conditions affect recombinant protein production. The time and period of induction and amount of inducer are the important parameters that affect the recombinant protein expression (Andersson, *et al.*, 1996).

Highest expression of genes cloned under the control of the *tac* promoter was obtained when IPTG was added during the exponential growth phase. It was demonstrated that the time of induction rather than the cell concentration is associated with recombinant protein activity. It was verified that IPTG induction in early or mid-exponential phase of growth retards growth of cells (Yarzabal *et al.*, 1997).

It was shown that higher growth rates prior to induction makes the cells better equipped to adapt to stressful conditions. When the pre-induction growth rate is higher, post-induction growth rate, cell viability and cell mass concentration increase (Ryan *et al.*, 1996).

The amount of inducer can also be manipulated. The maximum activity of induced protein was found with one mM IPTG however, the activity was almost constant between IPTG concentrations 0.4 and one mM (Yarzabal *et al.*, 1997). It was also stated that different IPTG concentrations resulted in no difference in the yield of protein and final growth. In such cases, the IPTG concentration can be reduced to the minimum amount possible to economize (Narciandi *et al.*, 1996).

On the other hand, it has also been reported in many cases that change in IPTG concentration also affects the yields of recombinant protein. It was demonstrated that an increase from 0.01mM IPTG concentration to one mM resulted in 102 fold increase in activity while an increase from one mM to seven mM resulted in 23 per cent increase only. It has also been shown that an increase from 0.01mM IPTG to 7.5mM IPTG resulted in 42 fold increase in transcription while only 3.6 fold increase in mRNA level. These results prove that the mRNA stability is important and the rate of degradation of the cloned gene transcript increases as synthesis rate increases. They have also found that rRNA synthesis is highest when the culture is not induced. As the amount of IPTG increases, load increases and there is less priority to rRNA synthesis toward mRNA synthesis (Wood and Peretti, 1991).

It was reported that the activity increase is not proportional with mRNA synthesis increases as IPTG concentration increases. They have also shown that as the inducer level increases, plasmid retention decreases (Huang *et al.*, 1994).

Presence of glucose increases transcription rate and overall protein synthesis at exponential phase but specific recombinant protein level stays unaffected. This supports the hypothesis that translation rate and mRNA stability are significant factors in recombinant protein production (Li and Taylor, 1994).

2.3. Fusion Proteins

The use of gene fusions has greatly facilitated the expression and subsequent purification of heterologous proteins in *Escherichia coli.* Specific fusion peptides may confer advantages to the target protein during expression, such as increased solubility, protection from proteolysis, improved folding, increased yield and secretion. The presence of the additional amino acid sequence stabilizes the hybrid protein against degradation. Fusion of short peptide tags can be used to target the protein to different compartments of the cell or even to the culture medium, allowing accumulation of the protein in the most suitable environment. Furthermore, fusion protein tags can be removed by *in vitro* cleavage of the fusion protein, which makes the formation of the native amino terminus of the protein possible. The highly soluble fusion partner facilitates folding of the target protein. A problem of gene fusions is that the fusion proteins are sometimes cleaved *in vivo* at the junction between the fusion partner and the target protein, which creates problems and decreases yield of target protein if the fusion partner serves as an aid in purification (Baneyx, 1999).

The protein of interest may be fused to itself to increase the stability of the protein against proteolytic degradation. With this method, inclusion body formation may increase. The advantages and disadvantages of inclusion bodies have been described previously and must be taken into account (Ford *et al.*, 1991).

The gene may be spliced directly after a signal sequence. With this strategy, if it is possible to remove the tag, a recombinant protein with a native amino terminus may be obtained. Moreover, the signal sequence can target the gene to different compartments of the cell; hence it may serve as a secretion tag (Makrides, 1996).

The selection of the fusion tail depends on the desired final destination and authenticity of the protein, the stability of the protein and the purification method suitable for the recovery of the protein. The advantages and disadvantages of cytoplasmic and periplasmic expression and secretion to the culture medium have been discussed. The end use of the protein determines the desired final authenticity of the protein. The affinity provides a simple purification for the proteins that are hard to purify using their own properties (Makrides, 1996).

Secretion-affinity fusions are used both for targeting the protein to the culture medium and for simplifying the purification of the protein by affinity chromatography. Secretion to the medium makes it possible to purify the product in a continuous system, an advantage from a chemical engineering point of view. Dual affinity fusions are especially used to express proteins that are highly susceptible to proteolysis. In this system, the gene of interest is fused to two fusion partners at each terminus, with specific affinity for two different ligands. This system requires two subsequent purification steps for complete recovery of the target protein. The solubility, stability, size, binding constant and subunit structure of both of the fusion partners must be considered in the construction of dual affinity fusions. It is also possible to fuse a secretion signal with a sequence that inserts the fusion product into the cell wall or one of the cellular membranes. This method is used to expose receptors or antigens on the outer surface of the bacteria or to assemble the protein into virus-like particles (Ford *et al.*, 1991).

The fusion partner may or may not be removed upon expression of the gene depending on the final desired authenticity of the protein and the biological interference of the fusion partner with the activity of the target protein. If the fusion partner interferes with the biological activity of the target protein, it is necessary to cleave the fusion protein and obtain the target in homogeneous form even in laboratory scale. If the target protein is to be used for therapeutic purposes, it is strictly necessary to obtain the target protein homogeneously in its authentic form not to cause immune response in the patient (Ford *et al.*, 1991; Uhlen and Moks, 1990).

It is possible to cleave the fusion protein enzymatically or chemically. The choice of method depends on the composition, sequence and characteristics of the particular protein. Chemical cleavage is efficient and the reagents are widely and cheaply available, making it possible to apply it on a large scale. However, it is nonspecific and harsh. Therefore, cleavage of the recombinant protein at additional cleavage sites occurs frequently. Moreover, the protein of interest may not always be able to withstand the harsh conditions that must be applied for chemical cleavage.

Enzymatic cleavage is desirable for relatively mild reaction conditions and most importantly, for the high degree of specificity exhibited. Exopeptidases and endopeptidases may be used for the cleavage of the fusion protein. However, enzymatic cleavage is affected by steric factors, therefore the cleavage site must be carefully engineered to be accessible by the enzyme. Among the useful enzymes are factor Xa, thrombin, enterokinase, renin and collagenase. All of these enzymes have extended substrate recognition sequences (up to seven amino acids in the case of renin), which reduces the likelihood of unwanted cleavages elsewhere in the protein. Factor Xa and enterokinase are the most advantageous because they cleave on the carboxy-terminal of their recognition sequences, allowing the release of the target protein with its authentic N-terminal (Ausubel *et al.*, 1992).

2.3.1. Maltose Binding Protein

Maltose Binding Protein (MBP) is the product of the MalE gene of *Escherichia coli* K12. MalE gene encodes for the pre-protein of 396 amino acid residues, 26 of which constitute the signal peptide. The expression of MBP is controlled by malEp, a strong promoter that is activated by protein MalT in the presence of inducing maltose or maltodextrins and repressed by glucose (Bedoulle and Duplay, 1988). It is a binding protein specific for maltose and maltodextrins. The binding site recognizes the α-4-glycosidic bond linking the glucose moieties of maltose. MBP is essential for the energy dependent translocation of maltose and maltodextrins through the cytoplasmic membrane (Duplay *et al.*, 1984).

MBP is exported across the cytoplasmic membrane and localized in the periplasm. It has been proposed that the signal sequence is responsible for initiating export through the membrane. Mutations in the signal sequence prevent MBP export and provoke its accumulation in the cytoplasm.

The use of MBP as a fusion partner in the expression and purification of foreign proteins in *Escherichia coli* has many advantages. MBP and its precursor can be purified by affinity chromatography on crosslinked amylose and then the proteins can be eluted by competition with maltose. Hence, fusion proteins can be purified from cell extracts in one step with a high yield and high degree of purity. The materials used are easy to prepare and inexpensive and crosslinked amylose is reusable, making large-scale purification simple and feasible. Moreover, the purification takes place under very mild, physiological conditions, enabling the protein to maintain its full activity (Maina *et al.*, 1988). In contrast, purification by fusion to protein A necessitates the pH to be decreased to 3.0, with the risk of inactivation (Bedoulle and Duplay, 1988). Finally, MBP does not contain any cysteine residues that could interfere with the disulfide formation within the target peptide (Maina *et al.*, 1988).

MBP was found far superior to either thioredoxin (TrxA) or glutathione-S-transferase (GST) in increasing the solubility of passenger proteins that would otherwise accumulate within the inclusion bodies in the cell cytoplasm (Baneyx, 1999, Kapust and Waugh, 1999).

The fusion of target proteins to MBP has been improved in a system that allows the separation of the target domain by a site-specific proteolytic cleavage after purification. The fusion protein in this system contains the recognition sequence for the blood coagulation factor Xa. Factor Xa recognizes and cleaves the protein at the tetra-peptide Ile-Glu-Gly-Arg. The presence of the recognition site before the target domain in a fusion would allow the cleavage at that site, releasing the target protein without any N-terminal residues. This system has been employed for the expression of the prokaryotic β-galactosidase and the eukaryotic paramyosin. The lacI^q coded repressor strongly represses the *lac* promoter on plasmids they have used and allows cloning of genes that may be lethal (Maina *et al.*, 1988).

2.4. *Taq* I Restriction - Modification System

Restriction endonucleases cleave the DNA at specific sequences, which makes them the first line of defense of a bacterium against invading phages and valuable tools in various fields of molecular biology such as DNA mapping, sequencing or recombinant *in vitro* cloning. Therefore, it is desirable to obtain them in large amounts.

The isolation of a new thermophilic bacterium, *Thermus aquaticus* was described in by Brock in 1967). The optimum growth temperature of this microorganism is 70°C with generation time of 50 minutes. *Taq* I is a restriction endonuclease that has been purified from *Thermus aquaticus* YT-1 strain and is found to be very specific to the target sequence. *Taq* I restriction endonuclease recognizes 5' TCGA 3' sequence, binds specifically and cleaves between the thymine (T) and cytosine (C) residues leaving a 5' staggered end upon digestion. Its specificity is several million fold relative to sites that differ by only one base pair, a property common to the type II restriction endonucleases (Mayer and Barany, 1995). The enzyme cleaves bacteriophage lambda DNA at 121 sites, pBR322 at seven sites, SV40 at one site and bacteriophage ϕXl74 RF DNA at ten sites (Sato, *et al.*, 1977). It has an optimum temperature of 65° C and a molecular weight of

31612 Daltons. Kinetic analysis has shown that both high temperature and magnesium are required for specific binding and catalysis (Zebala *et al.*, 1992).

Taq I restriction endonuclease is a thermostable enzyme, maintaining its activity up to temperatures as high as 70°C. Moreover, *Taq* I restriction endonuclease is stable under conditions that would denature most of the proteins, it can cleave its canonical sequence in 50 per cent formamide or 7M urea at 65°C (Barany, 1988b). The advantage is the inactivation of nonspecific endo- and exonucleases at this temperature.

There are seven cognate sequences in the *Taq* I restriction endonuclease gene for the enzyme, while there are none in the methylase gene. This is thought to be important in the sequential expression of the two genes. The restriction endonuclease gene cannot be expressed until the DNA has become fully modified. When the restriction endonuclease is expressed, it cleaves its own DNA, interrupting its further transcription and hence preventing synthesis (Slatko *et al.*, 1987)

The *Taq* I restriction endonuclease and methylase genes were cloned and sequenced in *Escherichia coli.* The genes were cloned by selecting *in vitro* for self-modified plasmids. It was found that the restriction endonuclease gene is 702 bp in length coding for the enzyme of a molecular weight of 27523 Daltons. *Taq* I methylase was found to methylate adenine residues in the cognate sequence and hence sequences were no longer susceptible to cleavage by the restriction endonuclease. The strain dependent viability of the recombinant cells was studied and it was observed that the restriction endonuclease is not lethal in the absence of modification methylase. This could be due to the fact that the cells are grown at 37 °C and hence the restriction endonuclease can only nick the DNA at its recognition sequence, which is repairable by *Escherichia coli* ligases (Slatko *et al.*, 1987).

The *Taq* I restriction endonuclease gene was cloned under the control of the alkaline phosphatase (phoA) promoter to isolate and characterize *Taq* I restriction endonuclease mutants and to study specificity of protein DNA interactions. The aim of this study was to separate the methylase and the endonuclease, place the restriction endonuclease behind a strong promoter and to remove extraneous restriction sites thus allowing for subsequent two-codon insertion mutagenesis. When *Taq* I gene is introduced into a small pBR322 derivative, the recovery was 5000 U from five ml of culture. The *Taq* I restriction endonuclease gene was then cloned under the phoA promoter yielding 1000 U from five ml of culture. When the nucleotide sequences between phoA and *Taq* I gene were deleted the yield increased to 10000 U from five ml of culture. The endonuclease and phoA was then cloned under T7 promoter yielding 30000 Units from five ml culture. When the endonuclease gene was finally reunited with the methylase gene, the yield was 45000 units from five ml culture (Barany, 1987).

This study also revealed that insertion of two amino acids by two codon mutagenesis at position nine or ten had no effect on activity while in frame deletions between amino acid ten and 22 almost completely abolished activity, indicating the importance of the N terminus.

The *Taq* I endonuclease gene was fused to the first four codons of the alkaline phosphatase signal sequence and overproduced under the phoA promoter with a yield of five per cent of the total cellular proteins. The sequence of the *Taq* I restriction endonuclease gene sequence revealed a 14 bas pair hairpin structure. Replacement of the hairpin region by degenerate codons increased the yield. When the methylase gene was added again, the increase in yield was remarkable, overproducing the restriction endonuclease to 30 per cent of the cellular protein. The strains RRI, HB101 and MM294 were used in this study. It was observed that RRl strain tolerates *Taq* I methylase containing plasmids because it lacks an endonuclease that cleaves DNA containing N^6-methyladenine (Barany, 1988b).

The sequences of both *Taq* I methylase and restriction endonuclease were corrected and the C terminus of the methylase gene was shown to overlap with the N terminus of the restriction endonuclease gene, giving a restriction endonuclease of 842 base pairs and a methylase gene of 1363 base pairs (Figure 2.1). It was suggested that the overlap region allows for formation of a hairpin structure, which allows termination of transcription. Failure to translate a full-length methylase might result in premature transcription termination at the hairpin loop, preventing the synthesis of restriction endonuclease, which is lethal, since the methylase is not expressed. However, proper translation of the methylase would occlude formation of the hairpin, allowing transcription to continue.

Thus, the overlap with a hairpin region serves as a protector against degradation of DNA and hence loss of viability (Barany *et al.*, 1992).

1	ATGGCTTCCA	CACAAGCCCA	GAAAGCGCTC	GAAACTTTTG	AGCGTTTTCT
51	*CGCAAGCTTG*	*GACCTCGAGT*	*CCTACCAGCA*	*AAAGTACCGC*	*CCTATCAAAA*
101	CGGTTGAACA	AGACCTGCCT	AGGGAGCTGA	ACCCGCTTCC	GGACCTGTAC
151	GAGCATTATT	GGAAAGCGCT	TGAGGATAAC	CCTTCCTTCC	TGGGCTTCGA
201	AGAGTTCTTT	GACCACTGGT	GGGAAAAGCG	CCTACGGCCC	TTGGACGAGT
251	*TCATACGCAA*	*ATACTTTTGG*	*GGATGCTCCT*	*ACGCGTTTGT*	*TCGCTTGGGC*
301	CTCGAGGCTA	GGCTGTACCG	AACAGCCGTT	TCCATCTGGA	CTCAGTTTCA
351	CTTCTGCTAC	CGCTGGAACG	CCTCCTGCGA	GCTTCCTCTA	GAAGCTGCCC
401	CAGAACTCGA	CGCCCAAGGG	ATAGACGCGC	TGATTCATAC	AAGCGGGTCC
451	*TCAACAGGAA*	*TCCAGATCAA*	*AAAGGAAACT*	*TACCGTTCCG*	*AGGCCAAGAG*
501	CGAGAACCGC	TTTTTAAGGA	AGCAAAGAGG	CACCGCCCTC	ATCGAGATTC
551	CCTACACCCT	GCAGACACCA	GAGGAGCTCG	AAGAAAAAGC	CAAACGGGCA
601	AGAGTGAACG	GAGAAACCTA	CCGTCTATGG	GCCAAGGTTG	CACACCATTT
651	*GGACCGTCTA*	*GAAAACGGAT*	*TCGTCATTTT*	*TCGGGAAAGT*	*TATGTGAAAA*
701	GCATTGAGCT	TTTTCTCAG	AAAAACGCTC	CTACCCTATC	TGGGCTCATC
751	CGCTGGGACA	GGGTGGCCCA	GGAAGCCCTC	ACCGCCCCGT	GAGGTAGACA
801	CGAAGCACAA	GCCCCACAGC	AAAGAGCAGG	CCCACGGCAC	GG

Figure 2.1. The Nucleotide Sequence of the *Taq* I Restriction Endonuclease Gene (Barany *et al.*, 1992)

2.4.1. Purification of *Taq* I Endonuclease

The classical purification procedures in general consist of cell disruption, dialysis, adsorption, and several column chromatographic steps. The purification of *Taq* I endonuclease was partially achieved using a classical five-step protocol, which included adsorption onto phosphocellulose, fractionation with ammonium sulfate, dialysis, phosphocellulose column chromatography and gel filtration chromatography. They recovered 4000 U *Taq* I endonuclease from 200 g *Thermus aquaticus* cells (Sato, *et al.* 1977). A shorter two-step purification protocol was then reported using phosphocellulose column chromatography followed by hydroxyapatite chromatography with a yield of 8000 U *Taq* I endonuclease from 50 g *Thermus aquaticus* cells (Greene, *et al.*, 1978). Two-column purification using cation and anion high-performance liquid chromatography columns sequentially yielded 190000 U enzyme from 200 g *Thermus aquaticus* cells (Altıntaş, *et al.*, 1998).

MBP-*Taq* I endonuclease fusion protein was expressed in recombinant *Escherichia coli* cells. The fusion protein was purified by single-step amylose affinity chromatography. The eluates containing the MBP-*Taq* I fusion protein were collected and digested with Factor Xa to obtain the isolated *Taq* I endonuclease (Özkırımlı, 1998).

2.5. Expression Vectors Used in *E. coli*

2.5.1. The pET Vector System

The pET vector system of Novagen has been one of the most powerful expression systems available for producing recombinant proteins in *E. coli* over the past few years. The pET system is based on the T7 promoter-driven system developed by Studier, *et al.*, 1990.

The newer pET derivatives developed at Novagen were designed with enhanced features to permit easier subcloning, detection, and purification of target proteins. Two general categories of vectors are available: transcription and translation vectors. Transcription vectors are designed for the expression of target genes that already carry their own prokaryotic ribosome binding site and ATG start codon. Translation vectors contain the highly efficient ribosome binding site from the phage T7 major capsid protein. The translation vector names are distinguished from the transcription vector names by the addition of a letter suffix following the name, e.g. pET-28a(+), which denotes the reading frame relative to the BamH1 cloning site recognition sequence. Therefore, all vectors with the suffices a, b, c express from the GGA, GAT, ATC triplets, respectively. Vectors with a "d" suffix also express from the "c" frame, but contain an upstream NcoI cloning site in place of the NdeI site in that series for insertion of target genes directly into the ATG start codon.

In general, translation vectors are used for the expression of target genes derived from eukaryotic sources, and transcription vectors are used for target genes derived from prokaryotic genes, which usually carry compatible ribosome binding sites. Many strategies can be used for the subcloning a protein-encoding region of DNA into a pET vector for expression. The most convenient strategy is to use the unique restriction sites found in the multiple cloning region of the pET vectors for cloning inserts in a defined orientation using two different sites.

2.5.2. pMal-c2 Fusion And Purification System

The pMAL vectors contain the malE gene that encodes for MBP under the control of the strong inducible *tac* promoter and the translation initiation signals of MBP. The pMAL-c2 vector is 6646 base pairs long (Figure 2.2). It has an ampicillin resistance gene. There are approximately 20 copies of the vector per cell. The lacI^q gene on the vector encodes the *lac* repressor, which keeps expression low until the *tac* promoter is induced with IPTG. Unique restriction endonuclease sites between malE and the lacZα genes allow the insertion of coding sequences of interest, to generate fusions with foreign polypeptide. Periplasmic signal sequence of malE gene is deleted in pMAL-c2 resulting in the cytoplasmic expression of the fusion protein. β-galactosidase α-fragment activity of the malE-IacZa gene fusion results in blue colony formation when α-complementing hosts such as TB 1 are transformed with the pMAL-c2 vector. Insertion of a DNA fragment into the multiple cloning site interrupts the malE-lacZα gene fusion, destroying α–fragment activity. Hence recombinant colonies do not turn blue, allowing blue-to-white screening for inserts.

There is a spacer sequence coding for ten asparagine residues between the malE sequence and the polylinker. This spacer insulates MBP from the target gene increasing affinity binding to amylose resin. The vectors also have a sequence coding for the recognition site of the protease factor Xa. Factor Xa cleaves after the amino acids Ile-Glu-Gly-Arg allowing the protein of interest to be cleaved after purification. The polylinker includes an XmnI site superimposed on the sequence coding for the Factor Xa site. Digestion of the vector at the XmnI site yields a blunt end, and when this is used in cloning of a foreign gene, no vector-derived residues remain on the target protein upon cleavage with Factor Xa.

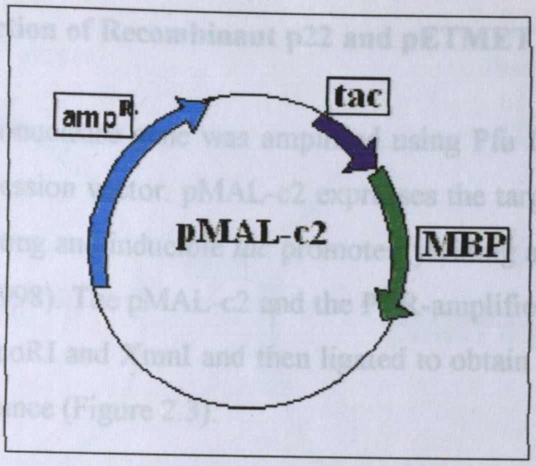
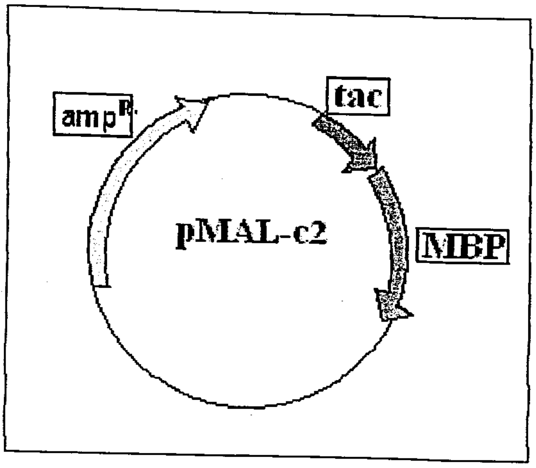


Figure 2.2. pMAL-c2 vector (6646 base pairs)

The M13 origin of DNA replication in the vector allows the production of single stranded DNA for sequencing and oligonucleotide directed mutagenesis.

The advantages of using pMAL-c2 vectors include the advantages of MBP fusion proteins as well as the advantages of C-terminal fusions. In this system, the MBP is fused to the N-terminal of the target gene, hence the transcriptional and translational signals that control expression are brought with the vector and are always identical, allowing predictability of expression. Moreover, transcription is under the control of the strong *tac* promoter which can be strongly repressed until induction with IPTG.



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2.6. Construction of Recombinant p22 and pETMET Plasmids

Taq I restriction endonuclease gene was amplified using Pfu DNA polymerase and cloned into pMAL-c2 expression vector. pMAL-c2 expresses the target protein with MBP under the control of the strong and inducible *tac* promoter, yielding a high recovery of the target protein (Özkırımlı, 1998). The pMAL-c2 and the PCR-amplified *Taq* I endonuclease gene were digested with EcoRI and XmnI and then ligated to obtain the new p22 plasmid conferring ampicillin resistance (Figure 2.3).

The pETMET vector expressing *Taq* I methylase was constructed by E. Toksoy and K. Çavuşoğlu (1999) as shown in the Figure 2.4. The pMETaq vector was previously constructed by E. Toksoy. The *Taq* I methylase gene was PCR amplified and cloned into the BamHI site of the pBR322 vector to give pMETaq where *Taq* I methylase expression was under the control of the tetracycline gene promoter. The pET-28a(+) and pMETaq were digested with HindIII and SphI and then ligated to obtain the pETMET plasmid, which expresses *Taq* I methylase and confers kanamycin resistance.

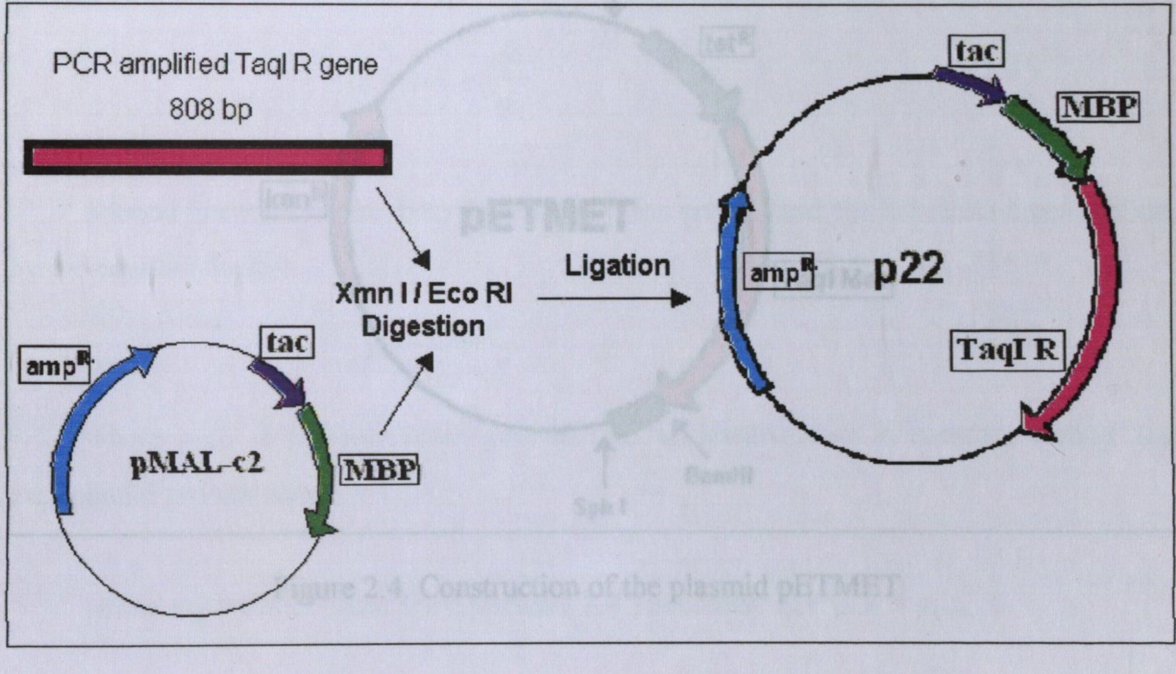

Figure 2.3. Construction of the plasmid p22 (Özkırımlı, 1998)

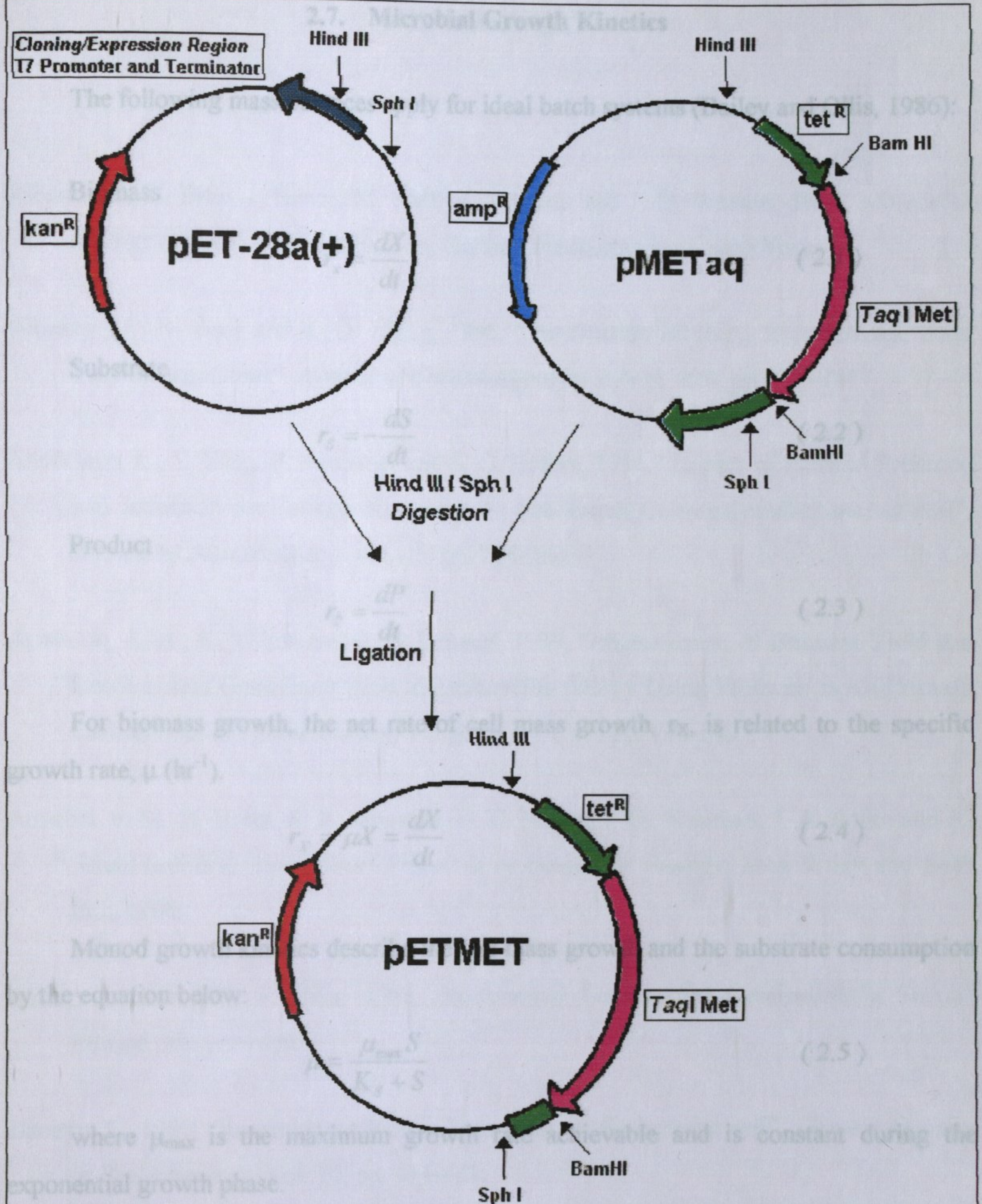

Figure 2.4. Construction of the plasmid pETMET

2.7. Microbial Growth Kinetics

The following mass balances apply for ideal batch systems (Bailey and Ollis, 1986):

Biomass

$$r_x = \frac{dX}{dt} \tag{2.1}$$

Substrate

$$r_S = -\frac{dS}{dt} \tag{2.2}$$

Product

$$r_P = \frac{dP}{dt} \tag{2.3}$$

For biomass growth, the net rate of cell mass growth, r_X, is related to the specific growth rate, μ (hr^{-1}).

$$r_X = \mu X = \frac{dX}{dt} \tag{2.4}$$

Monod growth kinetics describe the cell mass growth and the substrate consumption by the equation below:

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{2.5}$$

where μ_{max} is the maximum growth rate achievable and is constant during the exponential growth phase.

In the exponential phase the equation (2.4) becomes: $r_X = \mu X = \mu_{max} X$

The slope of ln X versus time during the exponential growth phase gives μ_{max}, which depends on the organism and the fermentation conditions.

3. MATERIALS

3.1 Bacterial Strains and Plasmids

The recombinant *Escherichia coli* strains *E. coli* TB1 [F$^-$, ara, Δ(lac-proAB), rpsL (Str ^{r}), ϕ80dlacZΔM15, hsdR (r_k^-, m_k^+)], *E. coli* ER2508 [lon:: Δ16Δ17, Δ(malB)zkb::tn5, Δ(lacZYA-argF)U169, Δ(mcrC-mrr)20, ara-14, galK2, rpsL 20, xyl-5, mtl1, supE44, leuB6, fhuA2], and *E. coli* XL-1 Blue [F′ : tn10 (tet^r), Δ(lac-proAB), lacI^q, recA1, endA1, gyrA96 (Nal ^{r}), thi-1, hsdR17(r_k^-, m_k^+), supE44, relA1, Δ(lac), λ^-] were used in this study. These strains were from our laboratory stock.

Recombinant plasmid p22 (Figure 3.1), where the expression of MBP-*Taq* I endonuclease fusion protein is under the control of the *tac* promoter, was constructed using pMAL-c2 (New England Biolabs) as the cloning vector, as described elsewhere (Özkırımlı, 1998), and kindly provided for this study.

Recombinant plasmid pETMET (Figure 3.2) expressing *Taq* I Methylase and conferring resistance to Kanamycin was constructed by E. Toksoy and K. Çavuşoğlu (1999) in our laboratory and kindly provided for this study.

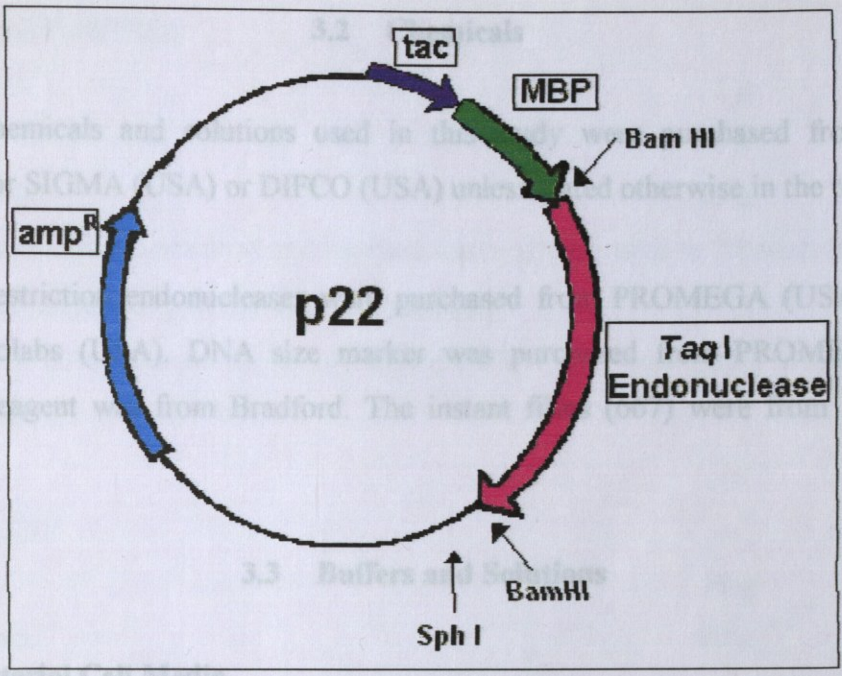

Figure 3.1. Plasmid p22 harboring *Taq* I endonuclease gene

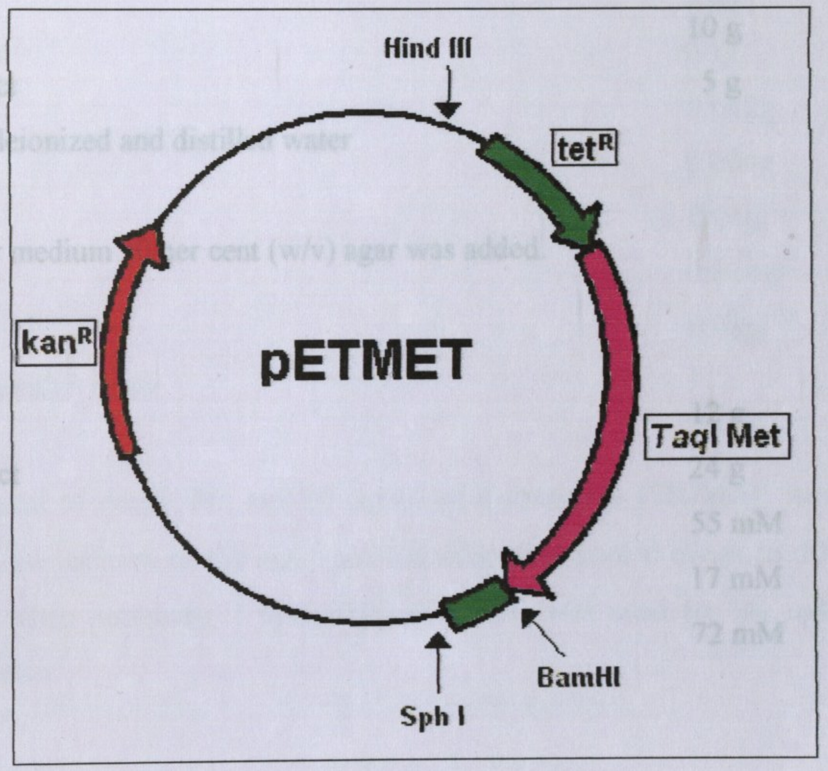

Figure 3.2. Plasmid pETMET harboring *Taq* I Methylase gene

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3.2 Chemicals

All chemicals and solutions used in this study were purchased from MERCK (Germany) or SIGMA (USA) or DIFCO (USA) unless stated otherwise in the text.

The restriction endonucleases were purchased from PROMEGA (USA) and New England Biolabs (USA). DNA size marker was purchased from PROMEGA (USA). Bradford Reagent was from Bradford. The instant films (667) were from POLAROID (USA).

3.3 Buffers and Solutions

3.3.1 Bacterial Cell Media

LB Medium (Luria-Bertani Broth)

NaCl	5 g
Tryptone	10 g
Yeast Extract	5 g

per liter of deionized and distilled water

For LB agar medium 20 per cent (w/v) agar was added.

TB Medium

Tryptone	12 g
Yeast Extract	24 g
Glycerol	55 mM
KH_2PO_4	17 mM
K_2HPO_4	72 mM

SB (Super Broth) Medium

NaCl	5 g
Tryptone	32 g
Yeast Extract	20 g

dissolved in 700ml of deionized and distilled water, pH adjusted to 7.0 with 50mM MOPS, after autoclaving at 121°C for 15 minutes

100 ml sterile 20% Glucose and 200 ml sterile 5X M9 Salts were added to one liter of final volume

2X YT Medium

Tryptone	16 g
Yeast Extract	10 g
NaCl	85 mM

SOC Medium

Tryptone	0.4g
Yeast Extract	0.1g
NaCl	0.012g
KCl	0.004g
$MgCl_2$	0.04g
$MgSO_4$	0.024g
Glucose	0.08g

in 20 ml of distilled water

100 µg/ml of Ampicillin and 60 µg/ml of Kanamycin (SIGMA), were added into either the liquid medium or the agar medium after they cooled down to 50°C following sterilization, when necessary. 1 mM IPTG (SIGMA) was used for the induction of the protein synthesis.

5X M9 Salts

$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g

per liter of deionized and distilled water

20 per cent (w/v) Glucose Solution

D-Glucose	20 g

per liter of deionized and distilled water

Autoclaved at 121°C and 1.02 atm for 3 min

3.3.2 Agarose Gel Electrophoresis Buffers

10X Loading Buffer

Bromophenol Blue	2.5 mg/ml

1per cent SDS in 1 ml Glycerol

5X TBE (Tris-Borate) Buffer

Trizma Base	445 mM
EDTA	10 mM
Boric Acid (pH 8.3)	445 mM

Ethidium Bromide	10 mg/ml

Agarose Gel (1.2%)

Agarose	1.2 g
0.5X TBE Buffer	100 ml

Stir and heat to boil until the solution is clear and transparent,

Cool down to 50°C, Add 5 μl Ethidium Bromide Solution.

3.3.3 Sonication Buffer

Tris-HCl (pH 8.0)	20 mM
EDTA	10 mM
2–mercaptoethanol	1 mM
PMSF	0.15 mM

3.3.4 *Taq* I Digestion Mixtures

Digestion Reaction Mixture for Commercial *Taq* I

pUC18 (2 µg/µl)	1 µl
Buffer E (Promega)	2 µl
Acetylated BSA, 10 (µg/µl)	0.2 µl
sterile dH_2O	16.3 µl
Taq I	0.5 µl
Final Volume	20 µl

Digestion Reaction Mixture I for the Sample

pUC18 (2 µg/µl)	1 µl
sterile dH_2O	17 µl
Sample	2 µl
Final Volume	20 µl

Digestion Reaction Mixture II for the Sample

pUC18 (2 µg/µl)	1 µl
Multicore 10X Buffer	2 µl
sterile dH_2O	15 µl
Sample	2 µl
Final Volume	20 µl

3.4 Laboratory Equipment

Autoclaves	Eyela, Model MAC-601, Japan
Balances	Precisa 80A-200M, Switzerland Gec Avery
Camera	Polaroid DS-34 Direct Screen Instant Camera (Polaroid, USA)
Centrifuges	Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont, USA Centrifuge 5415 C Eppendorf, Germany Centrifuge NF 615, Nüve, Turkey
Cold Room	4°C VWR Scientific, VCR 422DBA, USA
Deepfreezers	-80°C, Hetofrig CL89, HETO, Denmark -20°C, Bosch, Germany -20°C, Arçelik, Turkey
Electroporation Equipment	Gene Pulser II, Pulse Controller II, Capaticance Extender II, (BIO-RAD, USA)
Electrophoresis Equipment	Horizon 58, Model 200, Horizontal Gel Electrophoresis Apparatus, BRL, USA
Fermenter	Bioflo III Batch/Continuous Fermenter, New Brunswick, England Bioflo 3000 Batch/Continuous Fermenter, New Brunswick, England

Filtration Unit	Millipore Vacuum Filter, USA
Ice Machine	Scotsman, AF-30, UK
Incubators	EN500, Nüve, Turkey FN500, Nüve, Turkey Lab-Line AMBI-HI-LOW Chamber, 3554-18, USA
Laminar Flow	HBB 2460 LaminAir, Holten, Denmark
Orbital Shakers	GFL 3032, Germany INNOVA 4340, New Brunswick Co., England
pH meter	HANNA Instruments, HI 8521, Singapore
Refrigerators	+4°C, ARÇELİK, Turkey +4°C, Ultra 1500, Simtel, Turkey
Sonifier	Labsonic 1510, B.Brown, Germany Model 250/450 Sonifier Branson Ultrasonic Co., USA
Spectrophotometer	Lambda 3 UV/VIS, Perkin-Elmer Cetus, USA DU 640 Beckman, USA
Thermo-cyclers	Thermal Reactor TR1, HYBAID, UK GeneAmp PCR System 9600, Perkin-Elmer Cetus, USA
Transilluminators	Reprostar II, CAMAG, Switzerland Foto/uv 15, Fotodyne, USA
Vortex	Elektromag, Turkey
Water Baths	HETO, CB 8-30e AT_{110}, Denmark

	HETO, CB 8-30e DT_1, Denmark
	HETO DT Hetotherm, Denmark
	Nüve, BM102, Turkey
Water Distillation System	MILLIPORE, Milli Ro Plus, USA
	MILLIPORE, Milli-Q UF Plus, USA
	GFL 2004 Germany

4. METHODS

The strains of *E. coli* were kept in glycerol solution at 70°C. Frozen glycerol cultures were used by streaking on LB agar plates for the preparation of master plates. Master plates were kept at 4°C after being sealed with parafilm. During the experiments, subplates produced from the master plates were used in order to remove one isolated colony when it was required. Preparation of plates, inoculation and all other experimental work that require sterile environment were carried out under sterile laminar flow.

4.1. Sterilization

Sterilization is a treatment that frees the treated object of almost all living organisms. During the experiments sterilized equipment was used. Pipetman tips, eppendorf tubes, centrifuge tubes, solutions and culture media were all steam sterilized at 1.02 atm and 121°C for 20 minutes in an autoclave. Glassware was dry air sterilized at 180°C for 2 hours in an oven.

4.1.1. Sterilization of Media

Different media were used for culture growth. The media were prepared in Erlenmayer flasks such that the total volume of the medium was always one fifth of the flask. The flask was sealed with a cotton stopper and wrapped with aluminum foil. The media were sterilized by autoclaving at 1.02 atm and 121°C for 20 minutes and thereafter left to cool down before using. All the solutions of the media were allowed to cool down after autoclaving, then they were combined aseptically under laminar flow.

4.1.2. Sterilization of the Fermentor

Motor drive was removed from the top of the vessel and placed on the motor mount at the top of the cabinet. Air lines and all probe cables were removed. Jacket and exhaust condenser water lines were disconnected. 100 ml of sterilized water was put into the fermentor to allow the probe tips to be moisturized during sterilization. The sterilization was performed at a temperature of 121°C and pressure of 15 psig (1.02 atm) for 25 minutes. The pH probe was calibrated prior to autoclaving, while the DO probe was calibrated after autoclaving.

4.2. Preparation of Preculture

Ten ml of sterilized liquid nutrient medium was inoculated with a single bacterial colony. The inoculating loop was sterilized by heating in a flame and cooled on the agar plate to avoid death of the living cells to be transferred. The resulting preculture was incubated in an orbital shaker at 37°C and 180 rpm until late-exponential phase for about 14 hours. This culture was used as an inoculum on a 1 per cent (v/v) basis.

4.3. Growth Conditions

In shake flask experiments the total volume of the culture was kept at 100-200 ml. The rate of agitation was 180 rpm and the temperature was 37°C in the orbital shakers used for these experiments. The pH was allowed to follow its natural course.

In fermentor experiments the volume of the culture medium was kept at 1.5L. A 3L New Brunswick Scientific, Bioflo 3000 Model Fermentor was used for the experiments. The bioreactor is equipped with built-in automatic feedback PID controllers for temperature, pH, foam, dissolved oxygen, and agitation. The agitation speed was 600 rpm, the temperature was at 37°C, and the pH was kept constant at 7.0 by buffering the system either with 5M NaOH or with 5M H_2SO_4. Foam control was achieved by using the 10per cent (v/v) silicone antifoaming agent. In the experiments with aeration, filtered air was supplied to the system at a flow rate of 0.5 L/min. The airflow rate is controlled by a valve and measured by a rotameter.

4.4. Determination of DNA Quantity and Quality

Spectrophotometric measurement and agarose gel electrophoresis methods were used to determine the quantity and quality DNA.

4.4.1. Spectrophotometric Measurement

DNA was diluted to a ratio of 1:500 with distilled water and the UV absorption of DNA was read at 260 nm using quartz cuvettes. Since 50 µg of double stranded DNA has an absorbance of 1.0 at 260 nm, the concentration of the DNA in the samples were calculated using the formula:

$$50\ \mu g/ml \times OD_{260} \times \text{dilution factor} = \text{Concentration in } \mu g/ml$$

The OD_{260} /OD_{280} ratio was also used to determine the purity of the DNA samples. A ratio of 1.8 was considered to be pure whereas higher and lower values suggested RNA and protein contamination respectively.

4.4.2. Agarose Gel Electrophoresis

Gels were prepared by boiling the appropriate mass of agarose in 0.5XTBE Buffer, cooling to 50°C, adding ethidium bromide to a concentration of 5 µg/ml and setting this in the gel former. Different dilutions of DNA samples were mixed with 0.5 volume of DNA loading buffer and loaded on the appropriate concentration of agarose gel and run at 150 V with the known amounts of DNA. Ethidium bromide intercalates DNA, and in this state fluoresces when illuminated by UV light. DNA was visualized by illuminating the gel with short wave UV light on a transilluminator. The unknown amount was estimated by comparing the intensity of the fluorescence in each sample.

4.5. Transformation of *Escherichia coli* Cells

4.5.1. Preparation of Competent Cells

100 ml LB was inoculated with one ml of overnight *E. coli* cell culture and was kept with vigorous shaking at 37°C until OD_{600} reached 0.5. The cells were then chilled on ice for thirty minutes before being centrifuged at 40°C at 6000 rpm, using SS34 type of rotor, for six minutes. The LB medium was discarded and the pellet was washed twice with 100 ml of sterile distilled water, kept on ice for ten minutes before centrifugation at 6000 rpm

for six minutes at 4°C using SS34 type of rotor. The cells were then washed with two ml of ten per cent glycerol centrifuged and resuspended in 200 µl of ten per cent glycerol. The competent cells were then aliquoted in 40 µl volume of samples and kept at -80°C for storage.

All centrifugations were carried out in SORVALL RC-5B refrigerated superspeed centrifuge (Dupont, USA).

4.5.2. Electroporation

Electroporation was employed to transform the competent cells with the recombinant plasmid (Maniatis *et al.*, 1989). The Biorad Electroporation Equipment was set at a voltage of 2.34 kV, resistance of 200 Ω and a capacitance of 25 µF. An aliquot of competent cells with one µl of the plasmid solution was transferred into a pre-chilled sterile 0.1 cm gap electroporation cuvette avoiding bubbling in the cuvette. Meanwhile, an aliquot of competent cells was transferred into another ice-chilled electroporation cuvette to serve as the negative control. The two samples were electroporated under the conditions specified above. Immediately after electroporation, 2 ml of ice-cold SOC medium was added onto them and they were incubated at 37°C water bath for two hours with gentle shaking time to time.

After the incubation, the LB plates containing 100 µg/ml ampicillin and 60 µg/ml kanamycin were spread with different volumes of the transformation mixture with the negative control. The plates were left at room temperature to diffuse for 10 minutes and were then incubated at 37°C overnight.

4.6. Detection of the Expression of *Taq* I Endonuclease

4.6.1. Preparation of Crude Cellular Extract

100 ml LB medium with ampicillin and kanamycin was inoculated with 1 ml overnight preculture of recombinant *E. coli* cells. When the OD_{600} reached between 0.8 and

0.9 the cells were induced by 1mM IPTG. After five hours of induction 10 ml culture was taken into a sterile glass centrifuge tube, centrifuged at 5000 rpm for 10 minutes, and the supernatant was removed. The wet cell pellet was resuspended in 1 ml sonication buffer and lysed by sonication of 30 sec with cooling on ice. The insoluble proteins and membranes were removed from lysate by centrifugation at 9000 rpm by using an Eppendorf microcentrifuge for two minutes. The supernatant, containing the crude extract was assayed immediately for the *Taq* I restriction endonuclease activity.

4.6.2. Measurement of *Taq* I Endonuclease Activity

Taq I restriction endonuclease activity was measured by incubating a series of dilutions of one µl of the cell extracts with one µg pUC18 at 65°C for one hour. *Taq* I restriction endonuclease digests pUC18 into four fragments, three of which can be identified by electrophoresis on an agarose gel. These three fragments are 1444, 736, and 476 base pairs long. Parallel digestions were carried out with the commercial *Taq* I endonuclease to compare the activities at different dilutions. The total volume of the reaction mixture was 20µl. Digestion mixtures prepared using commercial *Taq* I endonuclease were used as positive controls whereas undigested pUC18 plasmid was used as a negative control when checking the results of the digestions on 1.2 per cent agarose gel. One unit of *Taq* I endonuclease activity is defined as that required to digest one µg of lambda DNA to completion in one hour at 65°C.

4.7. Determination of the Protein Concentration

In order to determine the total protein content of cell extracts, the Bradford Dye-Binding Method was applied (Bradford, 1976). Biorad Bradford reagent was diluted at a ratio of 1:10. A series of dilutions of Bovine Serum Albumin (BSA) were prepared to a final volume of one ml. The dilutions of BSA were added to one ml of diluted Bradford reagent. After five minutes of incubation at room temperature, the absorbances of each sample were read at 595 nm against a blank containing only the diluted Bradford reagent. A standard curve showing protein amount in µg versus absorbance at 595 nm was obtained. The protein concentration in each sample was then calculated using this standard curve.

4.8. Determination of Growth Characteristics

Ten ml sterile LB medium containing 100 μg/ml ampicillin and 60 μg/ml kanamycin was inoculated with a single colony of recombinant *E. coli* cells, and it was left for overnight growth in an orbital shaker incubator at 37°C, 180 rpm. LB medium of 500 ml was inoculated with 5 ml of this preculture and incubated at 37°C, 180 rpm in an orbital shaker. One ml samples were taken from this culture every one hour and their absorbance values were read in Beckman spectrophotometer at 600 nm wavelength against a blank of sterile LB medium. When its OD_{600} value was between 0.8 and 0.9, the culture was induced with IPTG to a final concentration of 1 mM.

The growth of cells was followed either by measuring the optical densities at a wavelength of 600nm in DU640 Beckman Spectrophotometer. A calibration chart was prepared to correlate the dry cell weights to optical densities. One ml of cell suspension was centrifuged in a 1.5ml pre-weighed eppendorf tube at 14000 rpm and 10 minutes using an Eppendorf 5415C bench-top centrifuge. The supernatant was discarded and the eppendorf containing the cells was then dried at 70°C for minimum six hours and re-weighed. The difference in the weights gave the mass of cells in the sample of culture. For example, in LB medium, the correlation of dry weight of *E. coli* to optical density was:

$$\text{OD} = 0.482 \times \text{Dry Cell Weight} \qquad \text{for } E.\ coli \text{ TB1 Strain}$$

$$\text{OD} = 0.4009 \times \text{Dry Cell Weight} \qquad \text{for } E.\ coli \text{ XL1 Blue Strain}$$

4.9. Determination of Plasmid Stability

The plasmid stability of the cell culture was determined by the method of replica plating. The culture samples were appropriately diluted giving rise to growth as single colonies. These were spread on the LB plates to allow the growth of both plasmid containing and plasmid free cells. LB plates containing ampicillin were used to allow the growth of cells harboring the ampicillin – resistant plasmid 22. LB plates containing kanamycin were used to allow the growth of cells harboring the kanamycin – resistant plasmid pETMET. The cells harboring both of the plasmids were grown using LB agar plates containing both of the antibiotics. The cell colonies were counted on each plate. The ratio of the plasmid containing cells divided by the total cell number gave the percent of plasmid bearing cells.

4.10. Experimental Set-Up

4.10.1. Experiments in Shake Flasks

Different sets of experiments were performed in shake flasks. These experiments were conducted at 180 rpm and 37°C in orbital shakers either in GFL-3032 or in Innova 4340. The pH was allowed to follow its natural course.

The culture volumes were changed depending on the aim of the experiment, but the flasks were filled with nutrient medium to one – fifth of their total volume to allow good aeration, and the samples were withdrawn from the flasks under the Laminar Flow Cabinet to avoid contamination. The total volume of all the samples withdrawn was limited to 10 per cent of the culture medium to eliminate the change in total volume with time.

A series of experiments were conducted for the determination of the growth characteristics of the recombinants. In these experiments, the time course of cell growth, total protein concentration, specific and total activities of *Taq* I endonuclease were determined in LB medium.

Furthermore, in another set of experiments, fermentation characteristics of the recombinants were investigated in these shake flasks. In these experiments, the time course of cell growth, plasmid stability, total protein concentration, specific and total activities of *Taq* I endonuclease were determined in SB medium, in which fermentor experiments were conducted.

The total operation time was around 15 hours for the shake flask experiments whereas it was around 24 hours for fermentor experiments.

4.10.2. Fermentor Experiments

An inoculum of recombinant *Escherichia coli* TB1 cells (15ml) grown in a shake flask at 180 rpm and 37°C in SB medium until its late exponential phase was added into a sterile fermentor containing 1500 ml SB medium. The total volume of all the samples withdrawn was limited to 10 per cent of the culture medium to eliminate the change in total volume with time.

In fermentor experiments, the time course of cell growth, plasmid stability, total protein concentration, specific and total activities of *Taq* I endonuclease were followed by the analytical techniques described above.

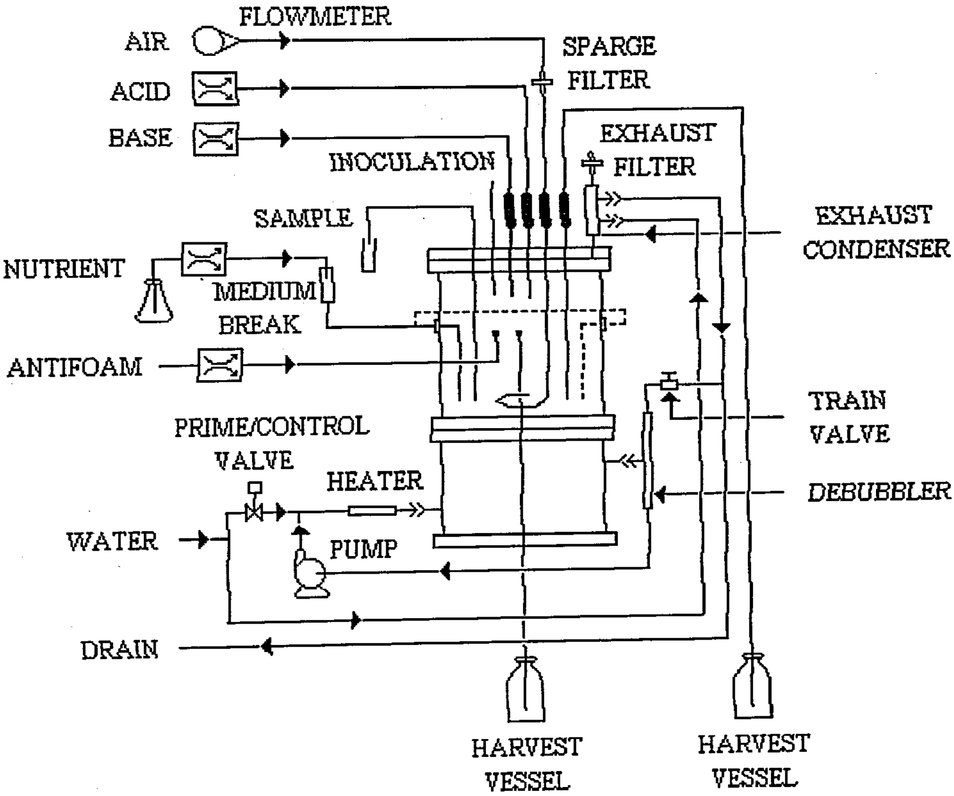


Figure 4.1. Fermentor Setup Diagram

4.10.3. Instrumentation and Control of the Fermentor

Bioflo 3000 (Figure 4.1) is a PC-ready multi-purpose fermentor that provides microprocessor control of major process parameters in one compact package. Designed for a wide range of microbial fermentations, Bioflo 3000 can be employed for both batch and continuous culture. The vessel part consists of a stainless steel head plate, a flanged glass tube vessel (2.5L) body which is detachable from the bottom – dished head. The dished – head is jacketed for circulation of temperature controlled water. Ports are provided in the head plate for inoculation, base and acid addition, antifoam addition, a thermowell for resistance temperature detector, a temperature sensor, a sparger, a sampling port, an exhaust condenser, dissolved oxygen and pH electrodes.

The system is equipped with built-in controls of pH, D.O., foam/level, agitation and temperature and includes pumps for acid, base, antifoam and nutrient addition. The reactor is easily removed for autoclaving. The agitation system is designed for high oxygen transfer rate (OTR) values. A powerful drive motor provides agitation in a range from 50-1200 rpm. In addition, an integral serial port permits remote communications or supervisory computer control, but this facility was not present in our experimental setup.

PID (Proportional-Integral-Derivative) controller regulates agitation speed. The motor automatically adjusts torque to maintain desired rpm over the full speed range, even with increasing culture viscosity. An optical encoder is coupled to the drive shaft for precise speed control. The high torque drive motor, large diameter turbine impellers and four flat baffles combine to achieve a high oxygen transfer rate of 350 mM O_2/L-hr. The PID control ranges are 50-1200 rpm for the agitation speed, 5 to 80°C for the temperature, 5-95 per cent for dissolved oxygen (DO), and 2.00-12.00 for the pH.

Sterile air is introduced into the medium through the ring sparger, and is controlled by the needle valve of the flowmeter. It is able to provide 1.5 working volume of sterile air through 0.2 μm replaceable cartridge filter, which is sterilizable with the vessel.

Electrodes that can be repeatedly steam-sterilized are available for pH, Redox potential, and dissolved oxygen and CO_2 partial pressures. Bioflo 3000 has a polarographic dissolved oxygen (DO) probe which is shown in the Figure 4.2.

The DO probe electrode measures the partial pressure or activity of the dissolved oxygen and not the dissolved oxygen concentration. An oxygen-permeable membrane separates the electrode internal devices from the medium.

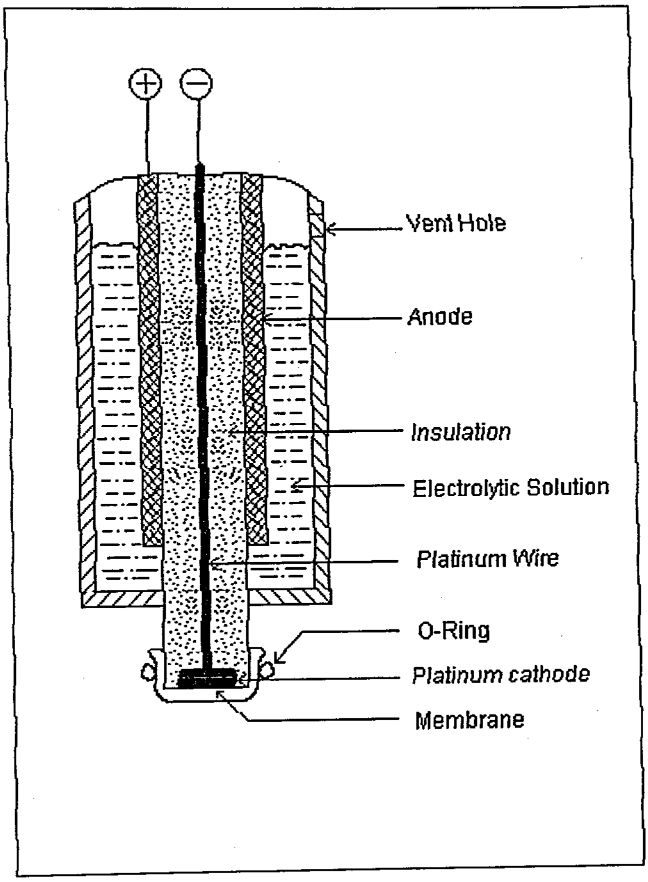


Figure 4.2: Dissolved Oxygen Probe

Both galvanic and polarographic types of electrodes feature the reduction of oxygen at the cathode surface (Bailey and Ollis 1986). Oxygen diffuses across the electrode membrane and is reduced to hydroxyl ions at the platinum cathode according to the following cathode reaction:

Cathode

$$\frac{1}{2}O_2 + H_2O + 2e^- \xrightarrow{Pt} 2OH^- \qquad (4.1)$$

The electrons necessary for this process are produced by a reaction at the silver anode:

Anode (polarographic)

$$Ag + Cl^- \longrightarrow AgCl + e^- \qquad (4.2)$$

At any given temperature, the current flow between cathode and anode is directly proportional to the level of oxygen outside the membrane.

Foam is controlled during batch fermentation by the antifoam probe, which is located in the headplate. The controller operates the peristaltic antifoam addition pump that adds chemical defoamer (silicone).

The exhaust gases pass into the exhaust condenser where moisture is removed and returned to the vessel. The remaining air passes through the 0.2 μm exhaust filter.

The hooded sampler is attached to a sampling tube that extends to the bottom of the vessel. The sampler has a rubber suction bulb to facilitate collection of representative samples without contamination.

4.10.4. Calibration of the pH Probe

After the electrode was connected to the probe cable, the power was switched to ON position when the agitation switch was OFF. The selector switch was set to pH and the mode switch to ZERO. pH probe was immersed into an external pH 7.00 buffer solution. After waiting for a certain time interval, the display was set to read the pH value of the buffer solution by INC / DEC switch. Then the probe was immersed into a second external buffer solution which is several pH units above or below the pH selected in the previous step (pH 4.00 was used in the experiments). The MODE switch was turned to SPAN.

Again by using INC / DEC switch, the display was set to the value of the second buffer solution.

After autoclaving, a sample from the medium was measured with an external pH-meter to check the calibration. If necessary, the zero (pH.7.00) calibration procedure is repeated.

4.10.5. Calibration of the Dissolved Oxygen Probe

The power was switched to ON position when the agitation switch was OFF. Minimum six hours of polarization of the DO probe is required before the calibration. Then the sterile medium inside the fermentor is aerated for minimum 30 minutes for 100% calibration. The selector switch was set to DO and the mode switch to ZERO. DO probe cable was disconnected form the electrode. After waiting for a certain time interval, the display was set to read DO value of 0 by INC / DEC switch. Then the electrode was connected to probe cable and MODE switch was turned to SPAN. By using INC / DEC switch, the display was set to 100.

5. RESULTS AND DISCUSSION

In the framework of this thesis, the production of MBP-*Taq* I restriction endonuclease fusion protein by genetically engineered cells that also express *Taq* I methylase gene was investigated both in shake flasks and fermentor under fully controlled operating conditions.

The *Taq* I restriction endonuclease was cloned in our laboratory into pMAL-c2 vector, which expresses the target protein as a fusion protein with Maltose Binding Protein (MBP), as a fusion partner (Özkırımlı, 1998). The MBP-*Taq* I fusion protein was targeted to the cytoplasm. Affinity of MBP to crosslinked amylose was employed to purify the protein using amylose column chromatography upon expression, as described in the theoretical background section. The cloned plasmid, named as plasmid p22, was kindly provided by E. Özkırımlı for this study. The co – expression of *Taq* I methylase gene was recommended to increase the *Taq* I endonuclease yield, by protecting the *Taq* I restriction endonuclease gene via methylation.

The *Taq* I methylase gene was PCR amplified and cloned into the pET-28a (+) vector conferring kanamycin resistance. This recombinant plasmid (pETMET) expressing the *Taq* I methylase enzyme was constructed by E. Toksoy and K. Çavuşoğlu (1999) in the Biochemical Engineering Laboratory, as described in the theoretical background section and was kindly provided for this study.

In order to investigate the effect of the co-expression of *Taq* I methylase on the production of *Taq* I restriction endonuclease, different strains of *E. coli* were transformed with both plasmids, namely p22 and pETMET. The productivity of the engineered cells *E. coli* (p22, pETMET) expressing *Taq* I endonuclease under the protective control of *Taq* I methylase was compared to those of the recombinant cells containing only single plasmid *E. coli* (p22).

5.1. Strain Selection

5.1.1. *Taq* I Endonuclease Activity of *E. coli* Strains Harboring p22

In this study, *Escherichia coli* host cells were transformed with the plasmid p22 to express MBP-*Taq* I endonuclease fusion protein. The host strain was selected by analyzing the *Taq* I restriction endonuclease activity of different strains bearing p22.

The plasmid p22 was obtained by cloning the *Taq* I restriction endonuclease gene into pMAL-c2 plasmid conferring ampicillin resistance, which expresses the MBP-*Taq* I fusion protein. Different recombinant strains of *E. coli* were obtained by introducing the plasmid p22 into host cells by electroporation technique. The transformed cells were spread on LB-agar plates supplemented with 0.1 mg/ml ampicillin at 37°C. Ten ml of LB (Amp) medium was then inoculated with a single recombinant colony bearing p22 overnight to serve as preculture. The cell cultures were grown in LB medium inoculated with the preculture in Erlenmeyer flasks in an orbital shaker (Innova 4340, USA) operated at 180 rpm. The IPTG induction was performed during the exponential phase of the growth when the optical densities of the culture broth at a wavelength of 600 nm were 0.9. The culture was induced by 1 mM IPTG. The cells were then incubated for five hours after the induction, as described by Özkırımlı, 1998. Cells were harvested by centrifugation and sonicated to obtain the crude cell extracts.

Different dilutions of the crude cell extracts were incubated with pUC18 at 65°C for one hour in sterile distilled water as described in the section 4.6.2. The digested samples were run on one per cent agarose gels prepared as described in the section 4.4.2. *Taq* I restriction endonuclease activity of the crude cell extracts was determined by comparing the results obtained from the digestion of pUC18 with different dilutions of commercial *Taq* I endonuclease (10 U/μl). The experiments were duplicated in all cases.

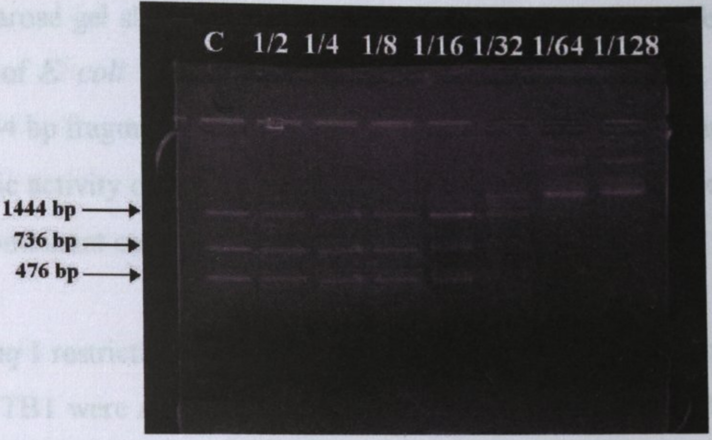


Figure 5.1. Digestion of pUC18 with different dilutions of commercial *Taq* I Restriction Endonucleases

Figure 5.1 shows the digestion of pUC18 with different dilutions of commercial *Taq* I Restriction Endonuclease (Promega) at 65°C for one hour in Buffer E provided by the manufacturer and BSA (Bovine Serum Albumin). The presence of the three bands of approximately 1444, 736 and 476 bp long digested with the dilutions up to 1/16 indicated the complete digestion of the plasmid pUC18 in one hour at 65°C.

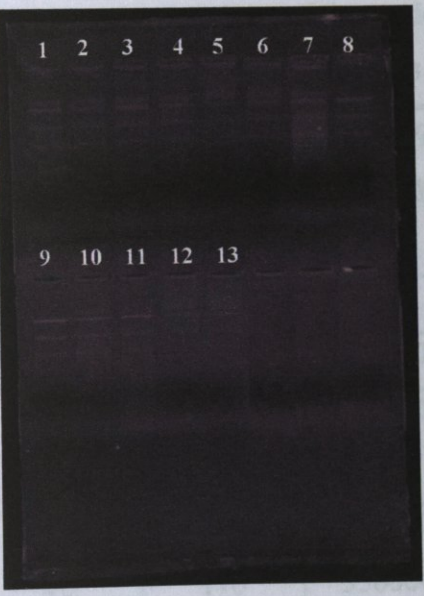


Figure 5.2. Digestion of pUC18 with different dilutions of cell extracts of *E. coli* TB1(p22)

Lanes 1-7:	Dilutions of 1/5, 1/6, 1/8, 1/10, 1/15, 1/16, 1/20, respectively.
Lanes 8-9:	Commercial *Taq* I digestion as positive control.
Lanes 10-13:	1/2, 1/4, 1/32, 1/64 dilutions

The agarose gel showing the digestion of pUC18 with the different dilutions of the cell extracts of *E. coli* TB1 (p22) is presented in the Figure 5.2. A band larger than the expected 1444 bp fragment was observed in samples diluted more than 32 fold. Therefore, the volumetric activity of the sample was found to be 3×10^5 U/L, produced by 4.1 g or 6.5 g of wet recombinant cells in two different experiments (Table 5.1).

The *Taq* I restriction endonuclease activities of the different strains of *E. coli* other than *E. coli* TB1 were also measured. All experiments were duplicated for accuracy. The resulting *Taq* I restriction endonuclease activities of the *E. coli* strains harboring p22, which express *Taq* I restriction endonuclease only are presented in Table 5.1. When *E. coli* XL1 Blue (p22) cells were used as host cells, 5.6×10^5 and 8×10^5 U/L of *Taq* I endonuclease was produced by 5.0 and 5.6 g wet cells, respectively in two separate experiments. *E. coli* ER2508 (p22) was found to produce 1.5×10^5 and 1.66×10^5 U/L *Taq* I endonuclease activity by 10.6 and 7.2 g wet cells respectively. The maximum specific *Taq* I endonuclease activities were 4773, 1148 and 505 U/mg by *E. coli* XL1 Blue (p22), ER 2508 (p22) and TB1 (p22), respectively.

Table 5.1: *Taq* I restriction endonuclease activity of the crude extracts of *E. coli* strains induced at OD_{600}=0.9 for five hrs

Wet Cell Weight (g/L)	Crude Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg/L)	Total *Taq* I Activity (U/L)	Specific Activity (U/mg)	U/g wet cell weight
E. coli TB1 (p22)						
6.5	50	7.6	382	300000	785	45802
4.1	30	8.7	261	300000	1148	72727
E. coli XL1 Blue (p22)						
5.0	20	12.4	247	800000	3234	267000
5.6	28	4.2	116	556000	4773	100000
E. coli ER 2508 (p22)						
10.6	50	5.9	297	150000	505	14085
7.2	50	6.9	345	166000	480	23024

Therefore, XL1 Blue and TB1 strains were selected as the host *E. coli* strains to investigate the effect of the co-expression of the methylase enzyme on the production of MBP-*Taq* I fusion protein displaying *Taq* I restriction endonuclease activity. *E. coli* TB1 and XL1 Blue strains were transformed with both p22 and pETMET and the production of the *Taq* I endonuclease activity by these recombinant cells were compared.

5.1.2. Transformation of *E. coli* Cells with p22 and pETMET

Competent cells of *E. coli* TB1 and *E. coli* XL1 Blue cells were prepared as described in the section 4.5.1 using LB medium. *E. coli* strains were transformed by two plasmids p22, and pETMET, harboring *Taq* I-R gene and *Taq* I-M gene respectively (Figure 5.3). The transformation was accomplished by electroporation technique as described in the 4.5.2. Since the plasmids p22 and pETMET confer resistance to ampicillin and kanamycin, respectively, the transformed cells were selected on LB-agar plates supplemented with ampicillin and kanamycin.

Two different approaches were employed for the transformation of *E. coli* cells with two plasmids. The first method was the simultaneous introduction of the two plasmids into the host cells by electroporation, and the second method was the sequential introduction of the two plasmids. When the two methods were compared with respect to *Taq* I endonuclease activity using *E. coli* TB1 as host, the simultaneous introduction method yielded colonies producing higher *Taq* I endonuclease activity, although less number of colonies formed after electroporation. No colonies were formed on LB (Amp, Kan) agar plates after electroporation by simultaneous introduction of the two plasmids into *E. coli* XL1 cells.

Since the concentrations of the isolated plasmids were different, different combinations of the amounts of plasmids between 0.5 µl and 2 µl were tested to increase the frequency of transformation. The *E. coli* XL1 Blue strain was transformed in two consecutive steps by the sequential introduction of plasmids; first electroporation was conducted using the plasmid p22, and then competent cells of recombinant *E. coli* XL1 (p22) were prepared. The second electroporation was then performed by transforming the recombinant *E. coli* XL1 (p22) cells by the second plasmid pETMET. This way, the cell death caused by the electroporation of the cells to be transformed with two different plasmids simultaneously was diminished by the introduction of this approach and enhanced the frequency of transformation.

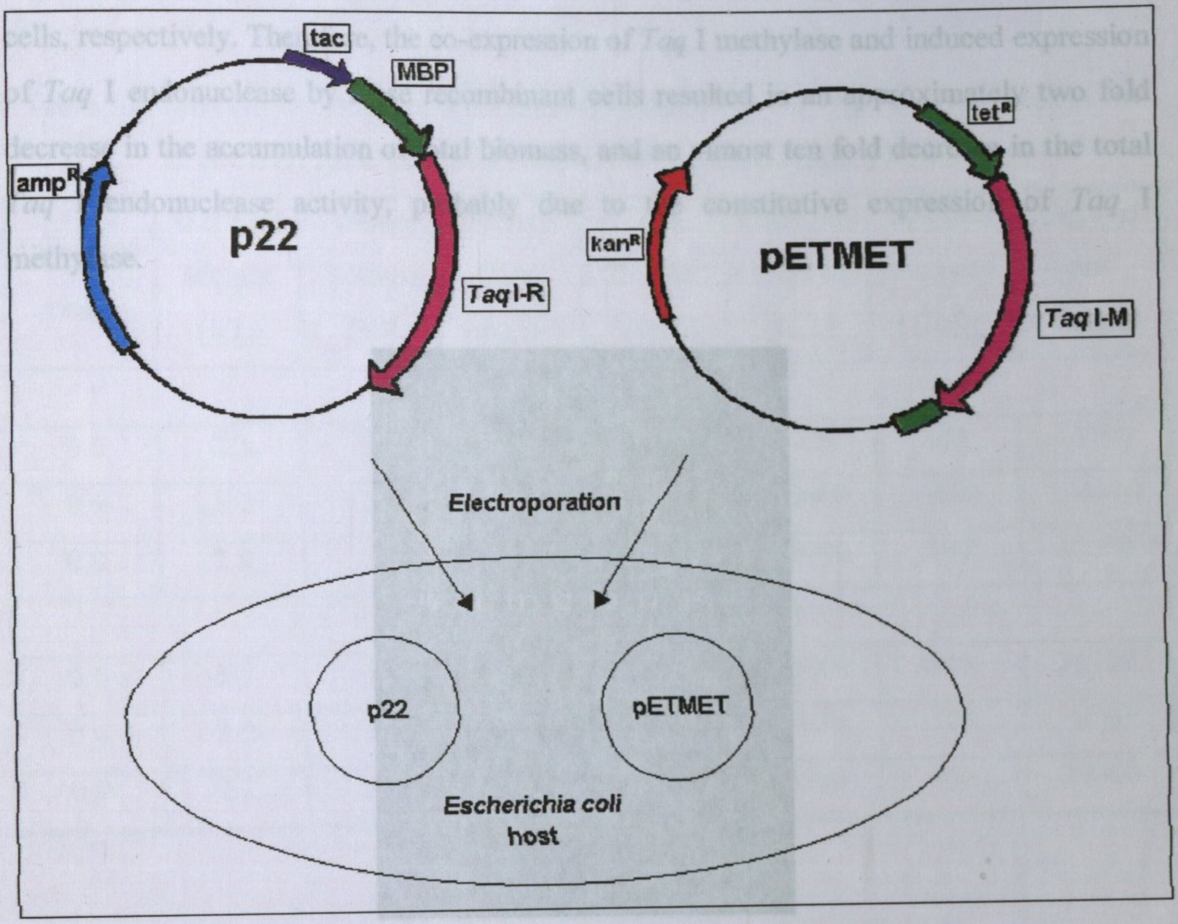


Figure 5.3. Transformation of *E. coli* cells with two plasmids

After electroporation, the competent cells were grown in SOC medium for two hours at 37°C in a water bath with gentle shaking. Then the cells were spread on LB agar plates supplemented with ampicillin and kanamycin and incubated at 37°C overnight. Ten ml of sterile LB (Amp, Kan) medium was inoculated with a single colony of transformed bacteria. The preculture was incubated overnight and 10 ml were used to inoculate 100 ml of LB (Amp, Kan) medium. This culture was grown until OD_{600} of 0.9 and induced with 1mM IPTG for five hours. The cells were then harvested by centrifugation and after sonication, the *Taq* I endonuclease activity was measured as described.

Figure 5.4 shows the digestion of pUC18 vector with different dilutions of the samples. The *Taq* I endonuclease activities obtained by the two recombinant strains, namely *E. coli* TB1 (p22, pETMET) and *E. coli* XL1 Blue (p22, pETMET), were calculated to be 1.25×10^5 U/L and 0.63×10^5 U/L, and produced by 2.8 g/L and 2.1 g/L wet cells, respectively. Therefore, the co-expression of *Taq* I methylase and induced expression of *Taq* I endonuclease by these recombinant cells resulted in an approximately two fold decrease in the accumulation of total biomass, and an almost ten fold decrease in the total *Taq* I endonuclease activity, probably due to the constitutive expression of *Taq* I methylase.

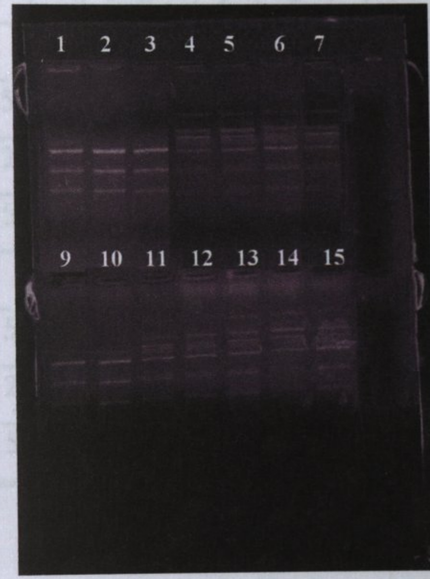


Figure 5.4. Digestion of pUC18 with different dilutions of cell extracts of *E. coli* TB1 (p22, pETMET) and XL1 (p22, pETMET)

Lanes 1-7:	TB1 strain dilutions: 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64
Lanes 9-15:	XL1 Blue strain dilutions: 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64

In order to investigate the effect of the induction time on the production of the *Taq* I endonuclease by the two recombinant strains *E. coli* TB1 (p22, pETMET) and *E. coli* XL1 Blue (p22, pETMET), IPTG induction was performed at OD_{600} values of 0.5 and 0.6, in addition to 0.9 as described. The results are presented in Table 5.2 and in Figure 5.5. The highest *Taq* I endonuclease activity was obtained from 3g/L wet cell weight of *E. coli* TB1 with a specific *Taq* I endonuclease of activity 4680 U/mg and a total *Taq* I endonuclease activity of approximately 4.3×10^5 U/L when they were induced at their early exponential phase of their growth at OD_{600} 0.6. Early induction has also resulted in the production of higher total *Taq* I endonuclease activities by *E. coli* XL1 (p22, pETMET) cells. These cells were found to produce 1.25×10^5 U/L of endonuclease activity when they are induced at OD_{600} 0.5 for five hours.

Table 5.2. Effect of the induction time on the production of the *Taq* I endonuclease of the *E. coli* TB1 (p22, pETMET) and *E. coli* XL1 Blue (p22, pETMET)

Induction OD600	Wet Cell Weight (g/L)	Crude Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg/L)	Total *Taq* I Activity (U/L)	Specific Activity (U/mg)	U/g wet cell weight
E. coli TB1 (p22, pETMET)							
0.5	2.3	50	1.4	72	31250	434	13500
0.6	3.0	21	4.3	91	428600	4680	144000
0.9	2.8	50	4.2	211	125000	592	45000
E. coli XL1 Blue (p22, pETMET)							
0.5	3.5	50	4.8	241	125000	518	36100
0.6	2.9	22	4.2	91	13636	150	4690
0.9	2.1	50	2.9	143	62500	436	29300

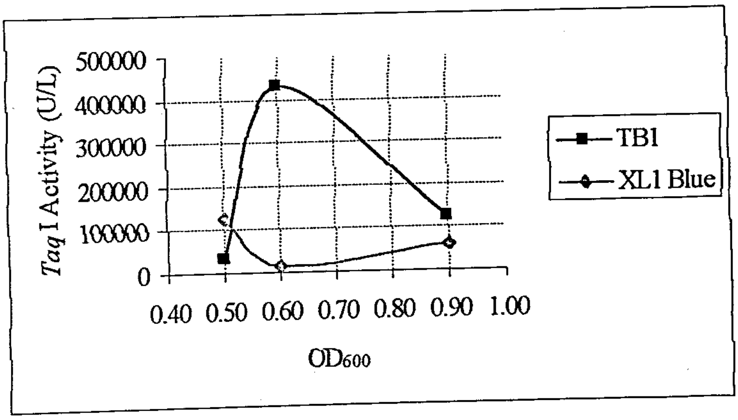


Figure 5.5. Effect of Induction Time on the Total *Taq* I Endonuclease Activity (U/L) in crude extracts of *E. coli* strains TB1 and XL1 Blue harboring two plasmids

5.1.3. Growth Characteristics of Recombinant *E. coli* TB1 and XL1 Blue Harboring p22 and pETMET

The growth characteristics of the recombinant *E. coli* strains TB1 (p22, pETMET) and XL1 Blue (p22, pETMET) harboring two plasmids were investigated by conducting shake flask experiments in LB medium. The growth pattern was followed spectrophotometrically.

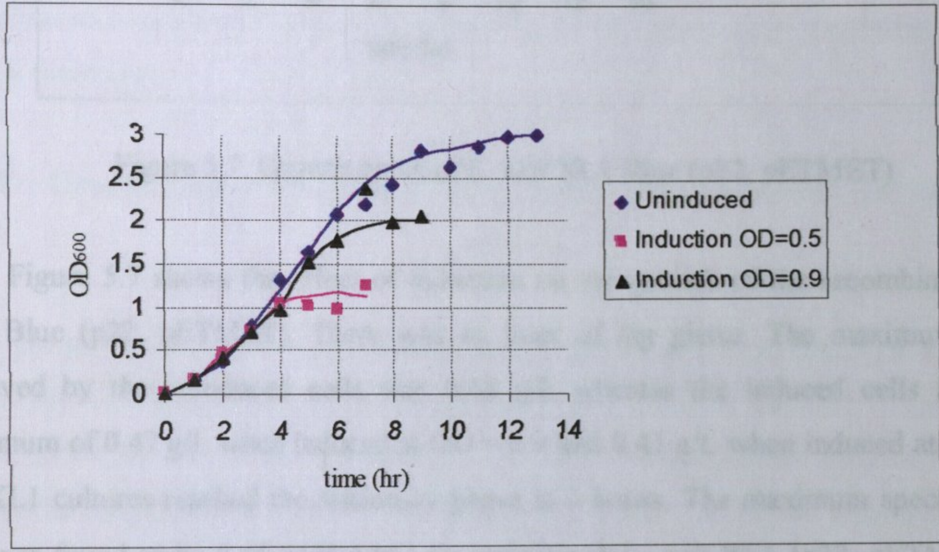


Figure 5.6. Growth curve of *E. coli* TB1 (p22, pETMET)

Figure 5.6 shows the effect of induction on the growth of the recombinant *E. coli* TB1 (p22, pETMET). One hour of lag phase was observed. The maximum biomass (dry cell weight) achieved by the uninduced cells was 1.45 g/L whereas the induced cells achieved a maximum of 1.0 g/L when induced at OD 0.9, and 0.60 g/L when induced at 0.5. The uninduced culture reached stationary phase in 9 hours, whereas the induced cultures reached the stationary phase in 4 hours when induced at OD =0.5, and 6 hours when induced at OD=0.9. The maximum specific growth rate, μ_{max}, calculated from the slope of the natural logarithm of dry cell weight (biomass) versus time graph of the exponential phase was found to be 0.70 (±5%) hr^{-1} for uninduced *E. coli* TB1 (p22, pETMET) cells. Induction of these cells at OD_{600} 0.5 and 0.9 resulted in a decrease in the maximum specific growth rate of these cells and μ_{max} values of 0.40 (±5%) hr^{-1} and 0.54 (±5%) hr^{-1} were calculated respectively.

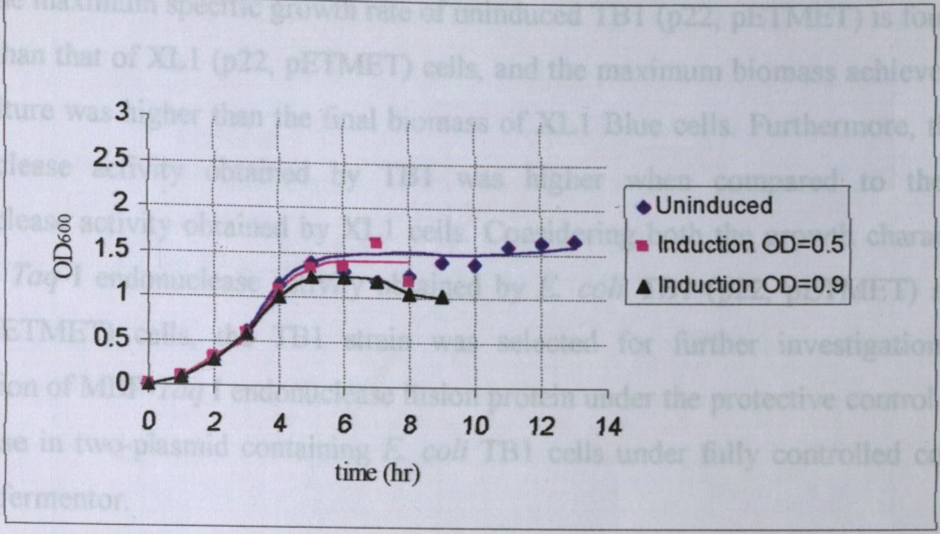


Figure 5.7. Growth curve of *E. coli* XL1 Blue (p22, pETMET)

Figure 5.7 shows the effect of induction on the growth of the recombinant *E. coli* XL1 Blue (p22, pETMET). There was an hour of lag phase. The maximum biomass achieved by the uninduced cells was 0.66 g/L whereas the induced cells achieved a maximum of 0.47 g/L when induced at OD = 0.9 and 0.41 g/L when induced at 0.5. All of the XL1 cultures reached the stationary phase in 5 hours. The maximum specific growth rate was found to be 0.68 (±5%) hr^{-1} for uninduced *E. coli* XL1 (p22, pETMET) cells. Induction of these cells at OD_{600} 0.5 and 0.9 resulted in a decrease in the maximum specific growth rate of these cells and μ_{max} values of 0.62 (±5%) hr^{-1} and 0.67 (±5%) hr^{-1} were calculated respectively.

Therefore, it has been concluded that the expression of the MBP-*Taq* I endonuclease and *Taq* I methylase retarded the cell growth. The maximum biomass achieved is higher in the case of the recombinant TB1 (p22, pETMET) cells than that of the XL1 Blue cells when induced in the late exponential phase at OD_{600}=0.9. The recombinant XL1 (p22, pETMET) cells was found to achieve a higher biomass than the biomass achieved by the TB1 cells when the cells were induced at the early exponential phase of their growth at OD_{600}=0.5. In both cases, the uninduced cells yielded the highest biomass concentration than the induced cells. The growth patterns of the uninduced cells and the cells induced at OD 0.9 were almost identical because the cells reached the stationary phase at OD 1.0.

The maximum specific growth rate of uninduced TB1 (p22, pETMET) is found to be higher than that of XL1 (p22, pETMET) cells, and the maximum biomass achieved by the TB1 culture was higher than the final biomass of XL1 Blue cells. Furthermore, the *Taq* I endonuclease activity obtained by TB1 was higher when compared to the *Taq* I endonuclease activity obtained by XL1 cells. Considering both the growth characteristics and the *Taq* I endonuclease activity obtained by *E. coli* TB1 (p22, pETMET) and XL1 (p22, pETMET) cells, the TB1 strain was selected for further investigation of the production of MBP-*Taq* I endonuclease fusion protein under the protective control of *Taq* I methylase in two-plasmid containing *E. coli* TB1 cells under fully controlled conditions using a fermentor.

5.2. Growth Characteristics of the Host and the Recombinant *E. coli* TB1 (p22, pETMET)

The growth behavior of the recombinant *E. coli* TB1 (p22, pETMET) is compared to that of the host organism *E. coli* TB1 in two different media, LB and TB without any induction. In the case of the recombinant TB1 (p22, pETMET), the cells were grown in both media supplemented with 0.10 mg/ml ampicillin and 0.06 mg/ml kanamycin. 500 ml of LB or TB medium was inoculated with 5 ml of overnight preculture and cells were grown at 37°C and agitated with 180 rpm in an orbital shaker. Optical density at 600 nm wavelength was followed to investigate the growth (Figure 5.8).

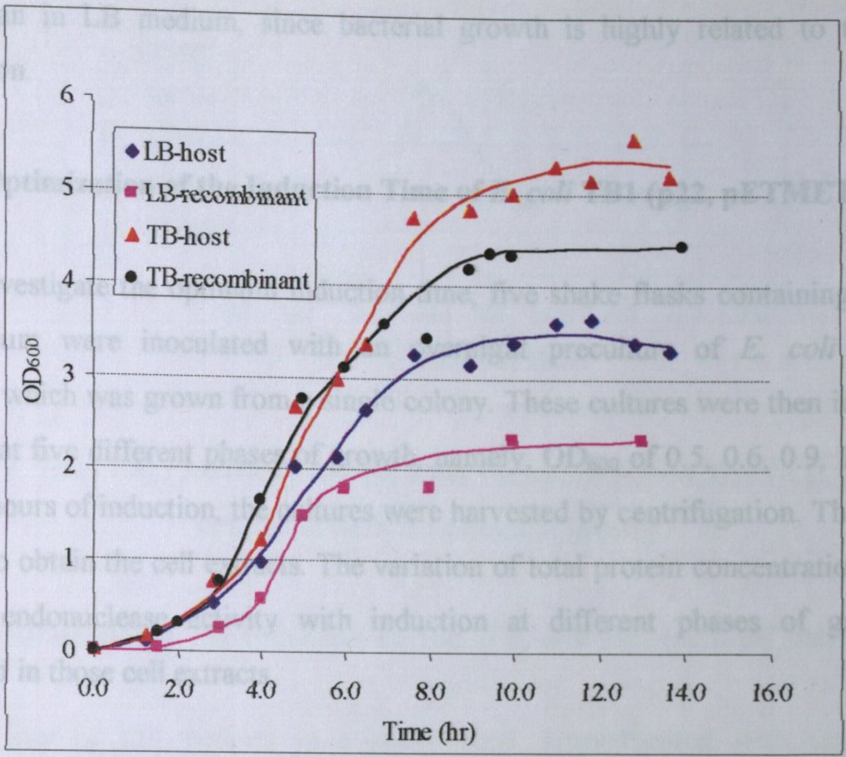


Figure 5.8. Uninduced growth of recombinant and host *E. coli* TB1 cells

In the LB medium, the host cells reached to a maximum OD$_{600}$ of 3.3 with one hour of lag phase, whereas the recombinant cells reached to an OD of 2.2 with two hours of lag phase. The host cells in LB medium reached the stationary phase in eight hours and the recombinant cells in six hours. In the TB medium, which is richer than LB, the final optical density of the host cells were as high as 5.1, whereas the OD of the recombinant cells were 4.5, both with one hour of lag phase. Both cultures in TB medium reached the stationary phase in eight hours.

The specific growth rate, μ_{max}, values were 0.78 (±5%) hr^{-1} for the host and 0.70 (±5%) hr^{-1} for the recombinant *E. coli* TB1 cells grown in the LB medium. In the TB medium, the specific growth rate of the host cells was 0.80 (±5%) hr^{-1}, and 0.73 (±5%) hr^{-1} for the recombinant cells.

When the final biomass concentrations and the specific growth rates were considered, the host cells grew faster than the recombinant cells in both LB and TB media. The biomass concentrations and the specific growth rates were higher in the richer TB medium than in LB medium, since bacterial growth is highly related to the nutrient concentration.

5.3. Optimization of the Induction Time of *E. coli* TB1 (p22, pETMET) in LB

To investigate the optimum induction time, five shake flasks containing LB (Amp, Kan) medium were inoculated with an overnight preculture of *E. coli* TB1 (p22, pETMET), which was grown from a single colony. These cultures were then induced by 1 mM IPTG at five different phases of growth, namely, OD_{600} of 0.5, 0.6, 0.9, 1.0, and 1.4. After five hours of induction, the cultures were harvested by centrifugation. The cells were sonicated to obtain the cell extracts. The variation of total protein concentration and *Taq* I restriction endonuclease activity with induction at different phases of growth were investigated in those cell extracts.

The total protein concentration of the cell extracts varied between 100 mg/L and 200 mg/L. The total protein concentration linearly increased as the induction OD_{600} increased from 0.6 until 1.0 and then remained constant.

The specific *Taq* I endonuclease activity for the cultures induced at OD_{600} 0.5, 0.6, 0.9, 1.0, and 1.4 were 3900, 9350, 2040, 1890, and 1655 U/mg, respectively. The total *Taq* I endonuclease activities of those cultures were 4×10^5, 8.6×10^5, 3.2×10^5, 3.75×10^5 and 3.2×10^5 U/L when the cells were induced at 0.5, 0.6, 0.9, 1.0, and 1.4, respectively (Figure 5.9).

The maximum *Taq* I endonuclease activity (8.6×10^5 U/L) was obtained when the culture was induced at OD 0.6 (Figure 5.9). The optimum induction time to maximize the *Taq* I endonuclease activity was found as the early exponential phase.

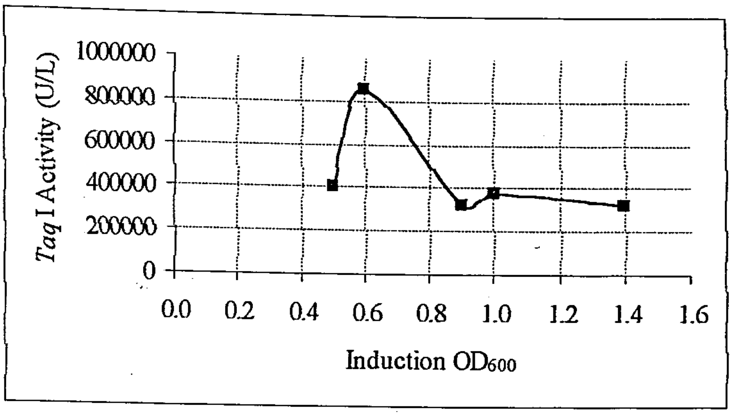
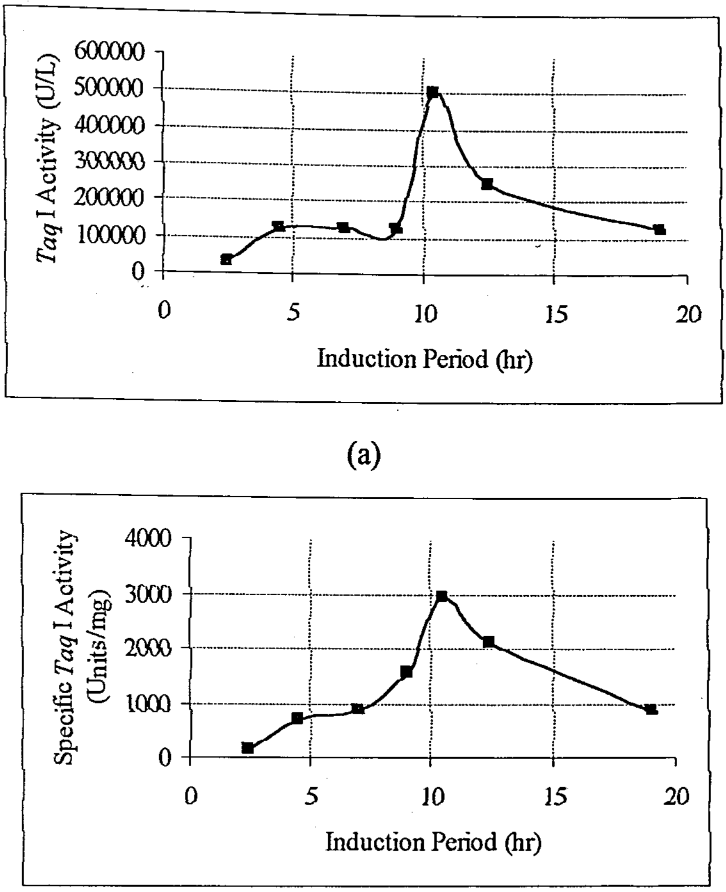


Figure 5.9. Total *Taq* I Activity Units per Liter Culture of *E. coli* TB1 (p22, pETMET) induced at different phases of growth

5.4. Optimization of the Induction Period of *E. coli* TB1 (p22, pETMET) in LB

One liter of LB medium in a shake flask supplemented with ampicillin and kanamycin was first inoculated with 10 ml of overnight preculture of *E. coli* TB1 (p22, pETMET) to investigate the effect of the induction period on the growth behavior, plasmid stability, total protein, and *Taq* I endonuclease activity. The cells were grown at 37°C and 180 rpm in shake flasks. When the OD reached 0.6 in the early exponential phase, induction was carried out by the addition of IPTG to a final concentration of 1mM. Then 10 ml of samples were harvested by centrifugation and the protein concentration and the *Taq* I endonuclease activity were measured in the cell extracts.

The *Taq* I endonuclease activity shows its peak value at the tenth hour after induction in terms of 3000 U/mg specific *Taq* I activity, 5×10^5 U/L total *Taq* I activity, and 3.5×10^5 U/g wet recombinant cells (Figure 5.10a,b). Therefore, the optimum induction period to produce the MBP-*Taq* I fusion protein from *E. coli* TB1 (p22, pETMET) harboring two plasmids was 10 hours.



(a)

(b)

Figure 5.10. The effect of Induction Period on the Production of *Taq* I Endonuclease by *E. coli* TB1 (p22, pETMET): (a) Variation of Total *Taq* I Endonuclease Activity, (b) Variation of Specific *Taq* I Endonuclease Activity with Induction Period

The total *Taq* I endonuclease activity obtained from the shake flask experiments using *E. coli* TB1 (p22) culture as 3×10^5 U/L (Table 5.1). Therefore, the co-expression of *Taq* I-Methylase was found to increase the total *Taq* I-Restriction Endonuclease activity in the shake flask experiments when the cells were induced at their early exponential phase for 10 hours. In addition, the specific *Taq* I activity was approximately three folds increased along with the employment of the *Taq* I restriction – modification system.

5.5. Plasmid Stability of *E. coli* TB1 (p22) and *E. coli* TB1 (p22, pETMET) in LB

The plasmid stability of the recombinant *E. coli* TB1 (p22) and TB1 (p22, pETMET) cultures were investigated as described in Methods Section 4.4. The plasmid stability was calculated as the per cent ratio of plasmid bearing cells grown on selective antibiotic media (LB-agar plates) to all of the cells grown on non-selective media. For the *E. coli* TB1 (p22) cells, the selective media was LB-agar supplemented with ampicillin. For the *E. coli* TB1 (p22, pETMET) cells, the stability of the plasmids p22 and pETMET were calculated using LB (Amp, Kan) medium. These results were very close to each other, the plasmid stability was around hundred per cent until induction, and made a sharp fall to zero after induction (Figure 5.11). Therefore the presence of pETMET does not seem to protect the stability of plasmids under these conditions. The cause of the plasmid loss may be due to the segregational or structural instability of the pETMET. The expression of Taq methylase needs also to be tested in further experiments.

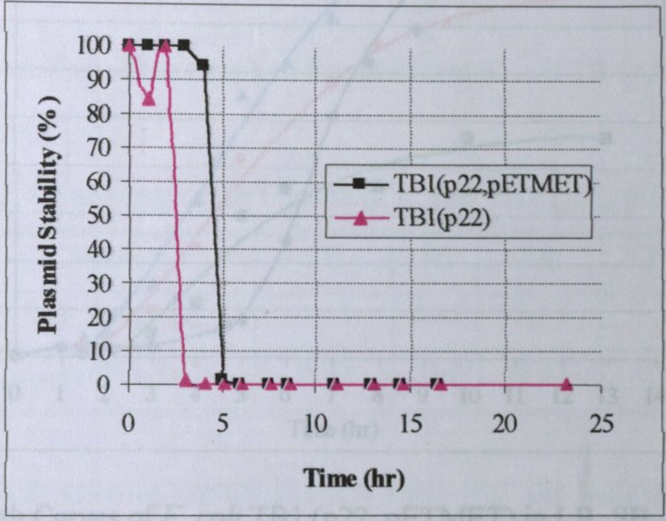


Figure 5.11. Plasmid Stability of the recombinant cells grown in the Shake Flasks

5.6. Media Selection

In order to select the best medium formulation for the fermentor experiments to reach high cell density cultures, the three rich media SB, TB, YT, and LB were compared via shake flask experiments. The formulations of these media are presented in the Materials section 3.3.1. 200 ml of cultures supplemented with Ampicillin and Kanamycin were inoculated with 2 ml of preculture of *E. coli* TB1 (p22, pETMET). The growth behavior of these uninduced cells were followed by measuring OD_{600} during 14 hours until the growth reached stationary phase.

The *E. coli* TB1 (p22, pETMET) cultures grown in SB, TB, YT, and LB media reached to a maximum OD of 4.8, 4.5, 4.0, and 2.4, respectively as seen in the Figure 5.12. The time required to reach the stationary phase of growth were 10, 9, 8, and 6 hours. The lag phases were four, two, two, and three hours for the SB, TB, YT, and LB media, respectively. The corresponding biomass concentrations and the maximum specific growth rates calculated from the slope of the natural logarithm of biomass concentration versus time (Figure 5.12) are presented in the Table 5.3.

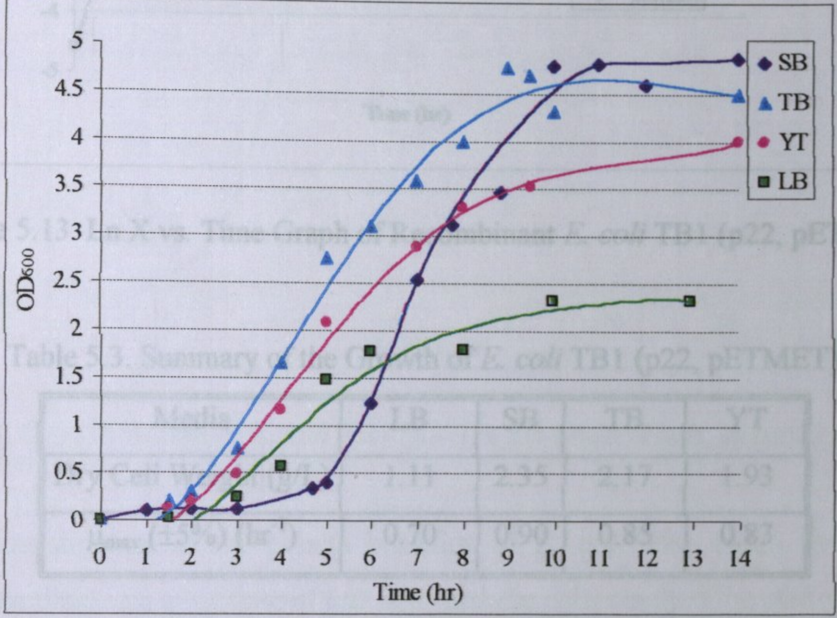


Figure 5.12. Growth Curves of *E. coli* TB1 (p22, pETMET) in LB, SB, TB, and YT Media

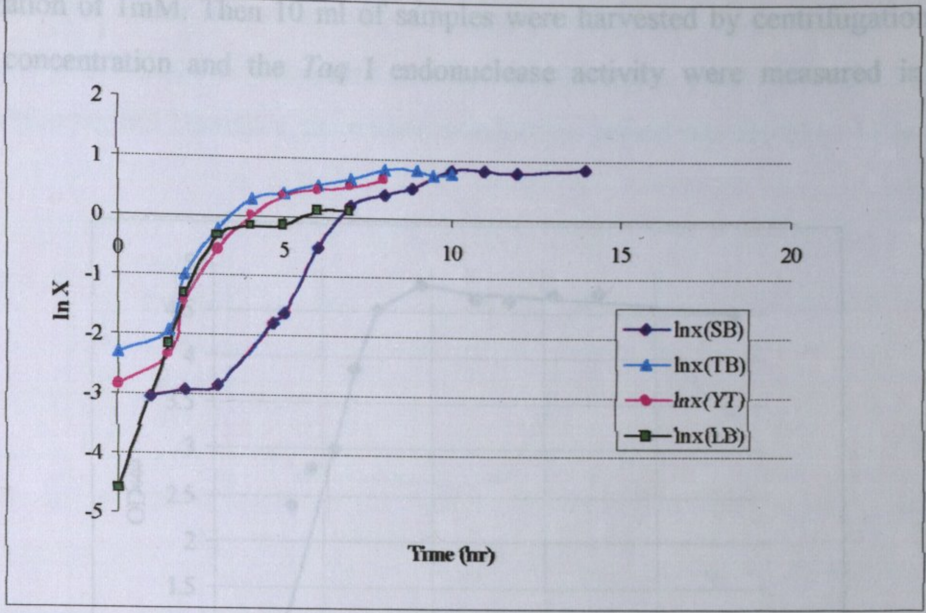


Figure 5.13. Ln X vs. Time Graph of Recombinant *E. coli* TB1 (p22, pETMET)

Table 5.3. Summary of the Growth of *E. coli* TB1 (p22, pETMET)

Media	LB	SB	TB	YT
Dry Cell Weight (g/L)	1.11	2.35	2.17	1.93
μ_{max} (±5%) (hr^{-1})	0.70	0.90	0.85	0.83

The SB medium gave the highest biomass value with the highest maximum specific growth rate, although the recombinant cells were in the lag phase for five hours. Therefore, for fully controlled fermentation experiments SB medium was selected to obtain high cell density cultures, although a lag time was observed in the above Figure 5.12. SB medium was also widely used for obtaining high cell density *Escherichia coli* cultures in literature (Aristidou, 1999).

5.7. Growth of *E. coli* TB1 (p22) in SB Shake Flasks

One liter of SB medium in a shake flask with ampicillin was first inoculated with an overnight preculture of *E. coli* TB1 (p22) to investigate the growth pattern, plasmid stability, *Taq* I endonuclease activity, and total protein. When the OD reached 0.6 in the early exponential phase, induction carried out by the addition of IPTG to a final concentration of 1mM. Then 10 ml of samples were harvested by centrifugation and the protein concentration and the *Taq* I endonuclease activity were measured in the cell extracts.

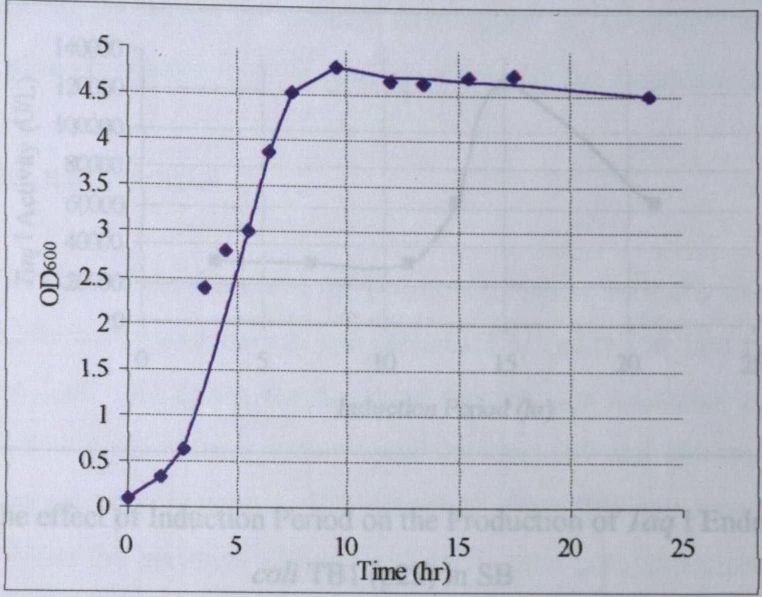


Figure 5.14. Growth Pattern of *E. coli* TB1 (p22) in SB Shake Flask, Induced at OD_{600}=0.6

The investigation of the growth behavior of *E. coli* TB1 (p22) in SB medium indicated that there was a lag time of one hour and the cells reached the stationary phase in 9 hours (Figure 5.14). The maximum specific growth rate was 0.94 (±5%) hr^{-1} with the final biomass concentration of 2.2 g/L. The maximum specific growth rate of the *E. coli* TB1 (p22, pETMET) cells in SB medium was previously calculated as 0.79 (±5%) hr^{-1} (Table 5.3). The decrease in the specific growth rate is expected because of the constitutive expression of *Taq* I methylase in the system with two plasmids.

5.8. Optimization of Induction Time of *E. coli* TB1 (p22) in SB Shake Flasks

The variation of total protein concentration and *Taq* I endonuclease activity with the induction period of *E. coli* TB1 (p22) cells grown in SB shake flasks was investigated prior to the fermentor experiments. The protein concentration reached to a maximum of 400 mg/L. The peak of the total *Taq* I endonuclease activity (1.25×10^5) was reached when the induction period was 15 hours producing 20833 U per gram wet recombinant cells and then the *Taq* I endonuclease activity decreased to 0.63×10^5 U/L (Figure 5.15). The maximum specific *Taq* I endonuclease activity was 374 mg/L, observed in the 15th hour of the induction period. Therefore, the optimum induction period was found as 15 hours.

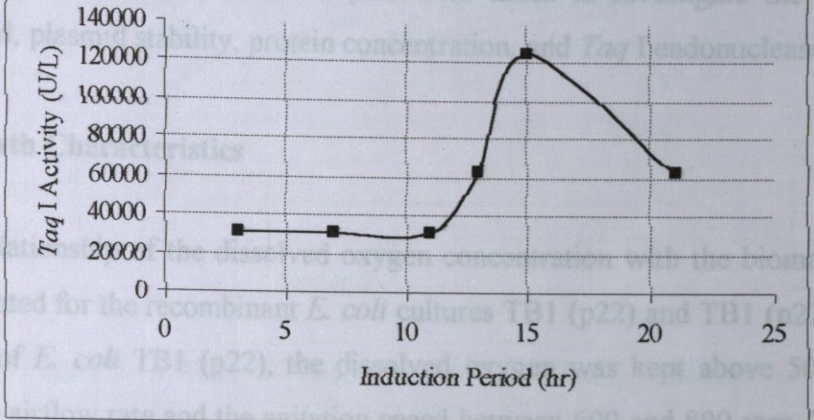


Figure 5.15. The effect of Induction Period on the Production of *Taq* I Endonuclease by *E. coli* TB1 (p22) in SB

5.9. Fermentation Characteristics of *E. coli* TB1 (p22) and *E. coli* TB1 (p22, pETMET) in SB

In the shake flask experiments, to maintain a constant operating environment for cell growth is not possible, regarding two important parameters; pH and dissolved oxygen. Acidification of the growth media, which is inevitable due to the acetate produced, creates an extra burden on cell growth. Therefore, a set of experiments was carried out in a well – controlled environment of pH and temperature under fully aerobic conditions. Two fermentor experiments were conducted to compare the *E. coli* TB1 culture bearing single and two plasmids, in which the effect of *Taq* I methylase co-expression on the production of MBP-*Taq* I endonuclease was investigated.

The volume of the BioFlo 3000 Bench – Top Fermentor with PID control was 2.5 L. 1.5 L of SB medium was inoculated with 15 ml of overnight recombinant *E. coli* culture. For *E. coli* TB1 (p22) cultures 0.1 mg/ml ampicillin was used to select the recombinant cells, whereas for *E. coli* TB1 (p22, pETMET) additional 0.05 mg/ml kanamycin was used to select the recombinant cells harboring both p22 and pETMET. The cells were grown at then the *Taq* I endonuclease activity decreased to 0.63×10^5 U/L (Figure 5.15). The maximum specific *Taq* I endonuclease activity was 374 mg/L, observed in the 15th hour of the induction period. Therefore, the optimum induction period was found as 15 hours.

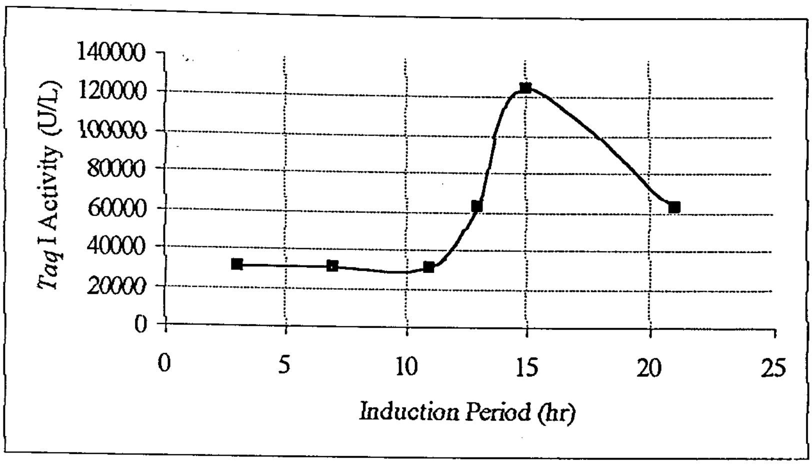


Figure 5.15. The effect of Induction Period on the Production of *Taq* I Endonuclease by *E. coli* TB1 (p22) in SB

5.9. Fermentation Characteristics of *E. coli* TB1 (p22) and *E. coli* TB1 (p22, pETMET) in SB

In the shake flask experiments, to maintain a constant operating environment for cell growth is not possible, regarding two important parameters; pH and dissolved oxygen. Acidification of the growth media, which is inevitable due to the acetate produced, creates an extra burden on cell growth. Therefore, a set of experiments was carried out in a well – controlled environment of pH and temperature under fully aerobic conditions. Two fermentor experiments were conducted to compare the *E. coli* TB1 culture bearing single and two plasmids, in which the effect of *Taq* I methylase co-expression on the production of MBP-*Taq* I endonuclease was investigated.

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37°C and induced at an OD of 0.6 by 1mM IPTG. The dissolved oxygen was kept above 50 per cent by changing the agitation speed between 600 – 800 rpm with a constant aeration rate of 0.5 vvm. The pH of the culture broth was kept at 7.0 by the addition of 5M NaOH and 5M H_2SO_4 when necessary. The foaming was prevented by using a 10 per cent silicone antifoam agent. 13 ml of samples were taken to investigate the growth rate, biomass yield, plasmid stability, protein concentration, and *Taq* I endonuclease activity.

5.9.1. Growth Characteristics

The relationship of the dissolved oxygen concentration with the biomass formation was investigated for the recombinant *E. coli* cultures TB1 (p22) and TB1 (p22, pETMET). In the case of *E. coli* TB1 (p22), the dissolved oxygen was kept above 50 per cent by changing the airflow rate and the agitation speed between 600 and 800 rpm. Therefore, as the biomass concentration increased, DO decreased from 100 per cent to 50 per cent (Figure 5.16). When the plasmids p22 and pETMET were both expressed, the dissolved oxygen control system of the fermentor had some problems. Therefore, the dissolved oxygen concentration could not be maintained above 50 per cent as in the case of single plasmid expression, which resulted in some fluctuations in the dissolved oxygen content of the fermentation culture (Figure 5.17). In both cultures, dissolved oxygen was consumed by the cells fastest in the exponential phase, and the biomass increased in the same ratio as the dissolved oxygen decreased. As seen in the Figure 5.17, during the lag phase for three hours, oxygen was not consumed since no biomass had formed.

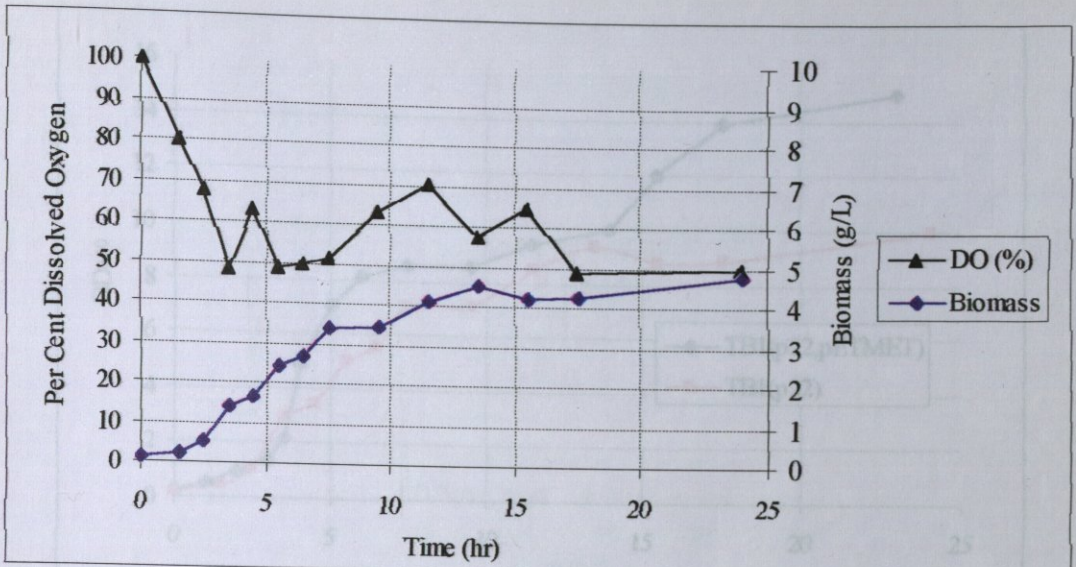


Figure 5.16. Growth Pattern of *E. coli* TB1 (p22) in Bioreactor

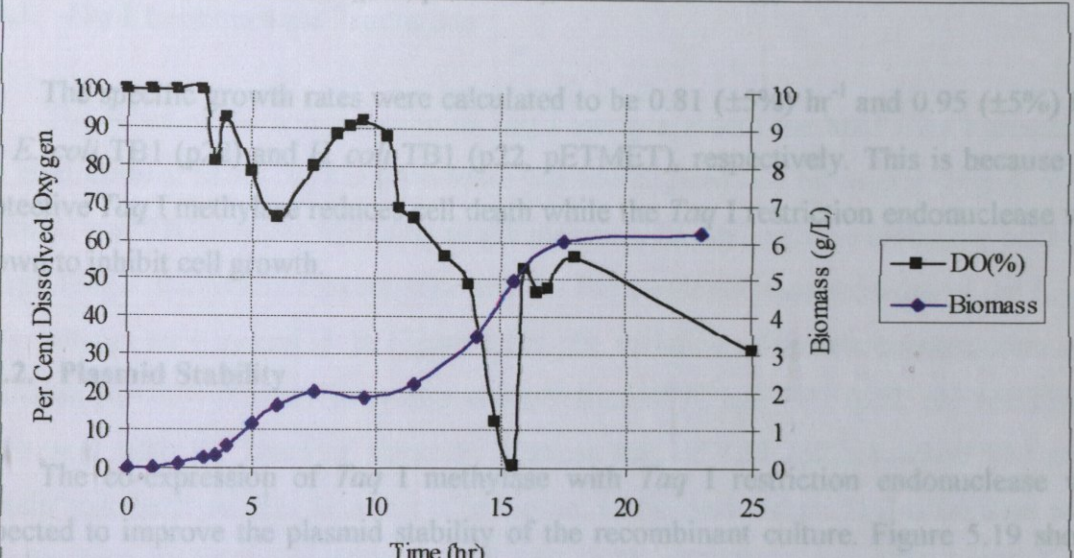


Figure 5.17. Growth Pattern of *E. coli* TB1 (p22, pETMET) in Bioreactor

The final biomass concentrations for the *E. coli* cultures TB1 (p22) and TB1 (p22, pETMET) were 4.8 g/L and 6.3 g/L, respectively. The biomass formed during the fermentation of the *E. coli* culture TB1 (p22) was lower than the TB1 (p22, pETMET) culture, although the dissolved oxygen in the fermentation of *E. coli* TB1 (p22, pETMET) had some values below 30 per cent, which was necessary for cell life (Figure 5.18).

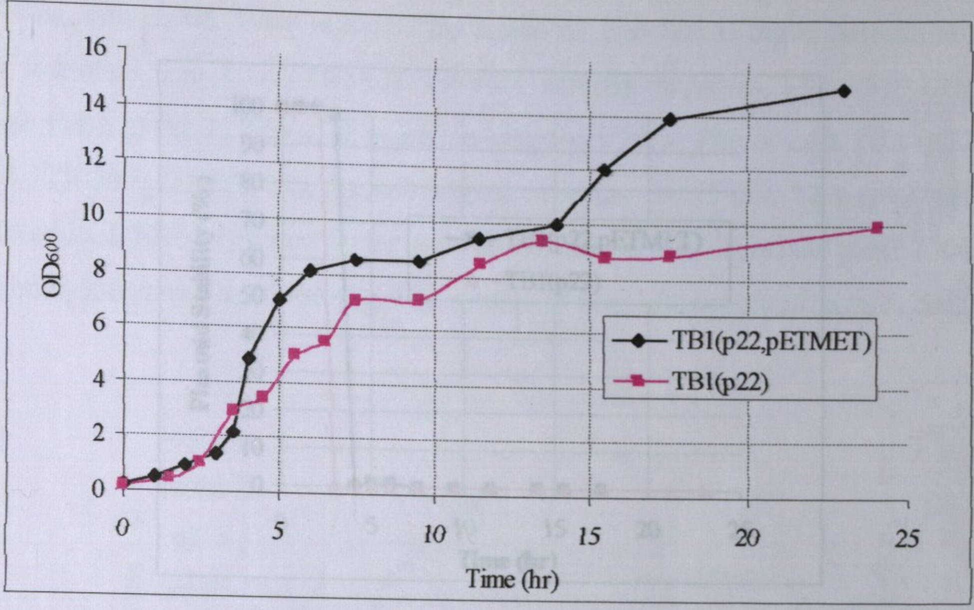


Figure 5.18. Comparison of Biomass Formation in the Fermentation of *E. coli* TB1 (p22) and TB1 (p22, pETMET), Induced at OD$_{600}$=0.6

The specific growth rates were calculated to be 0.81 (±5%) hr^{-1} and 0.95 (±5%) hr^{-1} for *E. coli* TB1 (p22) and *E. coli* TB1 (p22, pETMET), respectively. This is because the protective *Taq* I methylase reduces cell death while the *Taq* I restriction endonuclease was shown to inhibit cell growth.

5.9.2. Plasmid Stability

The co-expression of *Taq* I methylase with *Taq* I restriction endonuclease was expected to improve the plasmid stability of the recombinant culture. Figure 5.19 shows that the plasmid stability was affected directly by induction and made a sharp fall to zero after induction. In the Figure 5.11 and Figure 5.19, the slight difference seen between the TB1 (p22) and TB1 (p22, pETMET) cultures was caused by the difference in induction times; the culture bearing two plasmids had one to two hours of lag time in coming to the same optical density with the TB1 (p22) culture. In conclusion, both cultures had zero per cent ratio of plasmid bearing cells to plasmid – free cells after induction, regardless of the growth conditions of shake flask or fermentor. Furthermore, the protective effect of the presence of *Taq* I methylase on the apparent stability of p22 was not observable under these conditions.

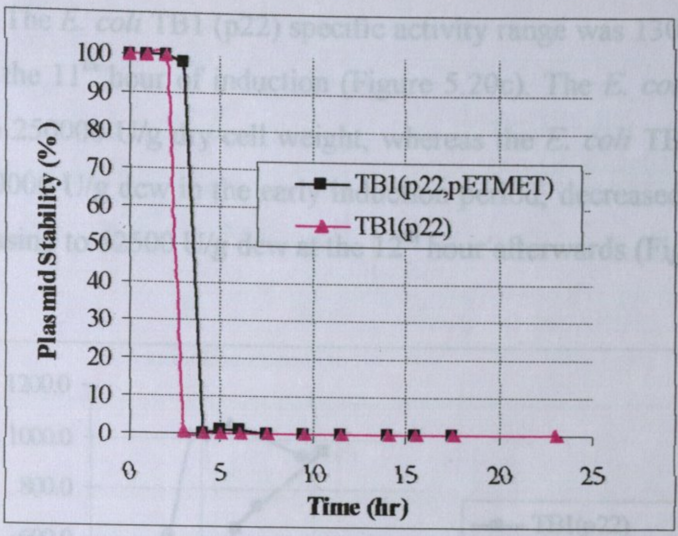


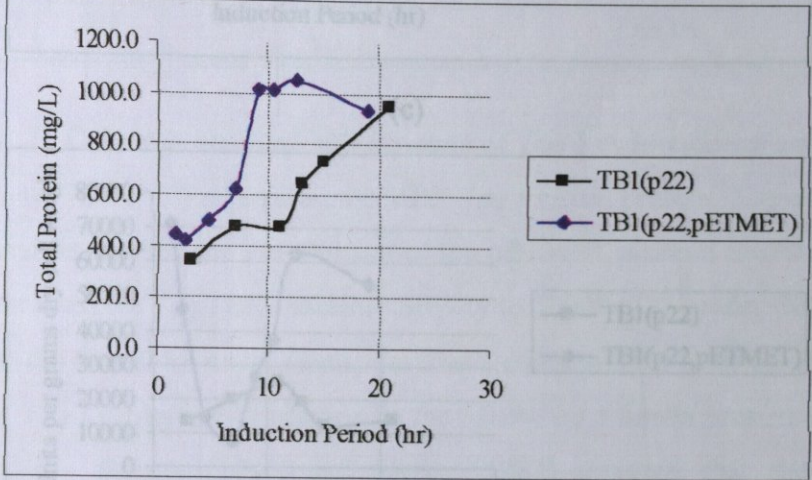
Figure 5.19. Plasmid Stability of the recombinant *E. coli* cells grown in the Fermentor

5.9.3. *Taq* I Endonuclease Production

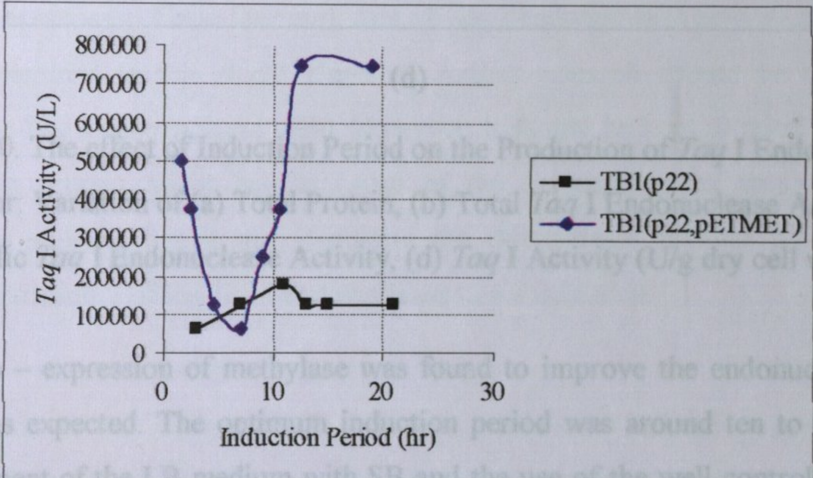
The effect of the co-expression of *Taq* I methylase with the MBP-*Taq* I protein on the production of MBP-*Taq* I endonuclease was investigated in a bioreactor (Figure 5.20). In the *E. coli* TB1 cultures harboring single plasmid p22, and cultures harboring both p22 and pETMET, the protein concentration and the *Taq* I endonuclease activities of the *E. coli* TB1 cultures were measured. In Figure 5.20a, the variation of protein concentration with induction period is shown. The protein concentration of *E. coli* TB1 (p22, pETMET) was 1050 mg/L after 11 hours of induction whereas that of TB1 (p22) reached 950 mg/L protein concentration after 21 hours of induction, as the peak value. This is because more proteins were to be expressed in *E. coli* TB1 (p22, pETMET) than TB1 (p22), i.e. in addition to *Taq* I restriction endonuclease, *Taq* I methylase was also expressed.

The effect of the induction period on the *Taq* I endonuclease activity was investigated. The *E. coli* TB1 (p22) culture maximum total *Taq* I endonuclease activity was 1.75×10^5 U/L, when the cells were induced for 10 hours. On the other hand, the *E. coli* TB1 (p22, pETMET) culture produced a maximum total *Taq* I endonuclease activity of 7.5×10^5 U/L (Figure 5.20b). The specific *Taq* I endonuclease activities of the *E. coli* TB1 (p22) and TB1 (p22, pETMET) were compared. The specific *Taq* I activity of the strain harboring two plasmids was at the maximum value at the second hour of induction with

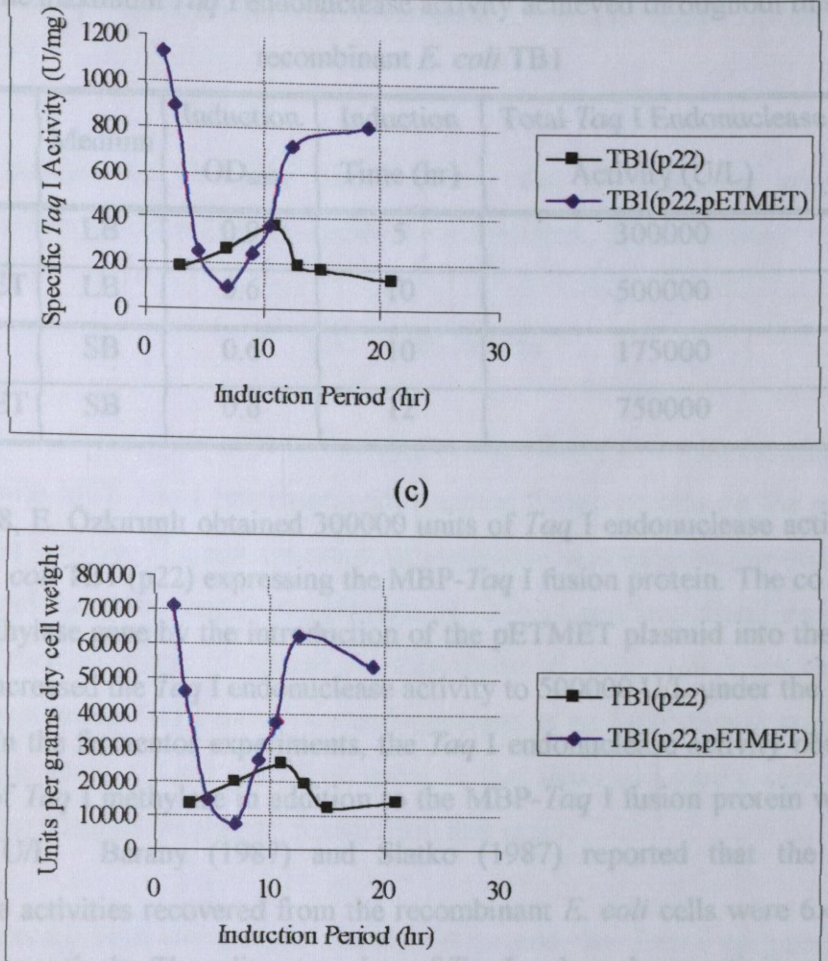
1130 U/mg, falls to 100 U/mg at the 7th hour, and rises to 805 U/mg maximum at the 19th hour of induction. The *E. coli* TB1 (p22) specific activity range was 130 – 370 U/mg, with the peak value at the 11th hour of induction (Figure 5.20c). The *E. coli* TB1 (p22) strain yielded 125000 to 250000 U/g dry cell weight, whereas the *E. coli* TB1 (p22, pETMET) strain produced 70000 U/g dcw in the early induction period, decreased until 7300 U/g at the 7th hour, increasing to 62500 U/g dcw at the 12th hour afterwards (Figure 5.20d).



(a)



(b)



(c)

(d)

Figure 5.20. The effect of Induction Period on the Production of *Taq* I Endonuclease in Bioreactor: Variation of (a) Total Protein, (b) Total *Taq* I Endonuclease Activity, (c) Specific *Taq* I Endonuclease Activity, (d) *Taq* I Activity (U/g dry cell weight)

The co – expression of methylase was found to improve the endonuclease activity production as expected. The optimum induction period was around ten to twelve hours. The replacement of the LB medium with SB and the use of the well-controlled fermentor systems have increased the productivity of the cells 1.5 fold (Table 5.4).

Table 5.4. The maximum *Taq* I endonuclease activity achieved throughout this study with recombinant *E. coli* TB1

Plasmid	Medium	Induction OD_{600}	Induction Time (hr)	Total *Taq* I Endonuclease Activity (U/L)	Growth
p22	LB	0.9	5	300000	shake flask
p22+pETMET	LB	0.6	10	500000	shake flask
p22	SB	0.6	10	175000	fermentor
p22+pETMET	SB	0.6	12	750000	fermentor

In 1998, E. Özkırımlı obtained 300000 units of *Taq* I endonuclease activity per liter culture by *E. coli* TB1 (p22) expressing the MBP-*Taq* I fusion protein. The co – expression of *Taq* I methylase gene by the introduction of the pETMET plasmid into the *E. coli* TB1 (p22) cells increased the *Taq* I endonuclease activity to 500000 U/L under the same growth conditions. In the fermentor experiments, the *Taq* I endonuclease activity obtained by the production of *Taq* I methylase in addition to the MBP-*Taq* I fusion protein was increased to 750000 U/L. Barany (1987) and Slatko (1987) reported that the total *Taq* I endonuclease activities recovered from the recombinant *E. coli* cells were 6×10^6 U/L and 1×10^6 U/L respectively. These literature data of *Taq* I endonuclease activity are higher than the results obtained in this study, therefore further research should be carried out to maximize the *Taq* I endonuclease yield. However, it should be kept in mind that MBP tail of the fusion protein might be the cause of the presentation of these lower *Taq* I endonuclease activity, but it may facilitate the downstream process by reducing the purification procedure of the *Taq* I endonuclease to a single step.

6. CONCLUSION AND RECOMMENDATIONS

The conclusions that are drawn from the experimental results and the recommendations for related future studies are summarized in this section.

6.1. Conclusions

The aim of this study was to investigate the effect of the co-expression of *Taq* I methylase with MBP-*Taq* I restriction endonuclease fusion protein on the resulting *Taq* I endonuclease activity. The co-expression of methylase was expected to protect the *Taq* I restriction endonuclease gene from cleavage by its own product, therefore increasing the *Taq* I endonuclease yield. Therefore, in this study, *E. coli* cells were transformed by pMAL-c2 expressing *Taq* I restriction endonuclease as an MBP-fusion protein (p22) and pETMET containing a *Taq* I methylase gene, so that these two genes were co-expressed. The following conclusions may be drawn from this study:

Considering both the growth characteristics and the *Taq* I endonuclease activity obtained with single and double plasmids, the TB1 strain was selected among *E. coli* XL1 Blue and ER2508 for further investigation of the production of MBP-*Taq* I endonuclease fusion protein under the protective control of *Taq* I methylase in two-plasmid containing *E. coli* TB1 host cells under fully controlled conditions using a fermentor. *E. coli* TB1 strain was transformed with both p22 and pETMET to investigate the *Taq* I endonuclease activity produced by these recombinant cells.

When the final biomass concentrations and the specific growth rates were considered, the host cells grew faster than the recombinant cells in different media.

The optimum induction time to maximize the *Taq* I endonuclease activity was found as the early exponential phase. The co-expression of *Taq* I methylase was found to increase the total *Taq* I restriction endonuclease activity in the shake flask experiments when the cells were induced at their early exponential phase for 10 hours. In addition, the specific

Taq I activity was approximately three folds increased along with the employment of the *Taq* I restriction – modification system.

Both cultures of *E. coli* TB1 (p22, pETMET) and *E. coli* TB1 (p22) had zero per cent ratio of plasmid bearing cells to plasmid – free cells after induction, regardless of the growth conditions of shake flask or fermentor. The presence of pETMET does not seem to protect the stability of plasmids under the fermentation conditions. The cause of the plasmid loss may be due to the segregational or structural instability of the pETMET. The expression of Taq methylase needs also to be tested in further experiments.

Among the LB, YB, SB, and YT media, the SB medium gave the highest biomass value with the highest maximum specific growth rate, although the recombinant cells were in the lag phase for five hours. Therefore, for fully controlled fermentation experiments SB medium was selected to obtain high cell density cultures.

The specific growth rate of *E. coli* TB1 (p22, pETMET) was higher than that of *E. coli* TB1 (p22) grown in SB in fermentor. This is because the protective *Taq* I methylase reduces cell death while the *Taq* I restriction endonuclease was shown to inhibit cell growth. Analysis of the growth characteristics of the host and recombinant *E. coli* TB1 (p22, pETMET) showed that the host *E. coli* TB1 cells grew faster than the recombinant cells. The results obtained in this study indicate that although the expression of the gene encoding *Taq* I restriction endonuclease retards cell growth, the co-expression of *Taq* I methylase with the MBP-*Taq* I endonuclease improved the growth of the recombinant *E. coli* TB1 (p22) strain in bioreactor by protecting the cell DNA from cleavage by the product, *Taq* I endonuclease.

The co – expression of methylase was found to improve the endonuclease activity production as expected. The optimum induction period was around ten to twelve hours. The replacement of the LB medium with SB and the use of the well-controlled fermentor systems have increased the productivity of the cells 1.5 fold.

The maximum total *Taq* I endonuclease activity (0.75×10^6 U/L) could be reached when the recombinant cells *E. coli* TB1 (p22, pETMET) were induced at the early exponential phase for 12 hours, when the cells were grown in SB medium at 37°C using a Bioflo 3000 fermentor with fully controlled operation conditions with the agitation 600-rpm, dissolved oxygen above 50 per cent and pH 7.0. Growth of *E. coli* TB1 (p22, pETMET) in LB-shake flask yielded 0.5×10^6 U/L total *Taq* I endonuclease activity when induced at the early exponential phase for 10 hours, while Özkırımlı obtained 0.3×10^6 U/L (1998). Barany (1987) and Slatko (1987) reported that total activities recovered from the recombinant *E. coli* cells were 6×10^6 U/L and 1×10^6 U/L respectively. Although the 0.75×10^6 U/L *Taq* I endonuclease activity is close to that of Slatko, further research should be carried out to maximize the *Taq* I endonuclease yield. However, the MBP tail of the fusion protein might be the cause of the presentation of these lower *Taq* I endonuclease activity, although the MBP fusion provides a simpler and easier purification procedure with single-step amylose affinity chromatography.

6.2. Recommendations

In this study, the maximum achieved *Taq* I endonuclease activity produced by the recombinant *E. coli* TB1 (p22, pETMET) was 0.75×10^6 U/L, which is slightly below the previously reported by Slatko (1987). Further research should be carried out to improve the *Taq* I endonuclease yield.

The fermentor experiments may be conducted with a variety of growth conditions such as modifications in the media formulations in LB and SB, changing the glucose, salts tryptone and yeast extract concentrations. Fructose instead of glucose can also be tried since Aristidou, *et al.* (1999), achieved 40 per cent increase in biomass and 65 per cent in recombinant protein yield in *E. coli* by reducing acetate formation.

The poor plasmid stability both in the fermentor and shake flask experiments should be improved. In large-scale cultivations, a small portion of the cells can inactivate the antibiotic in the medium and hence the plasmids free cells can propagate. Therefore, increasing the ampicillin and kanamycin concentration, or feeding the antibiotics regularly in certain time intervals can be utilized. On the other hand, the expression of *Taq* I methylase should be monitored throughout the fermentation period. It is possible that the segregational or structural instability of the pETMET plasmid may be the cause of the poor plasmid stability.

The *Taq* I endonuclease activity of the MBP-*Taq* I fusion protein was measured in the crude cell extracts. Amylose affinity column chromatography followed by the cleavage with Factor Xa should be used for the isolation of the native *Taq* I endonuclease.

In order to transform the *E. coli* cells with two different plasmids, the *Taq* I methylase and MBP-*Taq* I endonuclease genes may be cloned together in a single plasmid conferring ampicillin resistance. Hence, investigation of the *E. coli* cells having single and two plasmids can give a comparison of the two systems.

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