Journal of Chemical and Natural Resources Engineering, 1:22-39

REVIEW : OVEREXPRESSION OF PROTEIN UNDER TRANSCRIPTIONAL REGULATION OF LAMBDA p^L PROMOTER SYSTEM IN *Escherichia coli*: CONSEQUENCES AND BIOPROCESS IMPROVEMENT APPROACHES

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Abstract: The attraction of employing thermal induction system in the production of heterologous protein is mainly due to 1) a higher expressed protein titer in comparison to constitutive system, and 2) a cleaner process since no chemical inducing agent is added. However, problems pertaining to high temperature and protein overexpression such as inclusion bodies formations, metabolic overload on the host, stress-related proteolysis on the expressed protein, and enhanced acetic acid secretion are commonly encountered. Several bioprocess approaches that include temperature upshift schemes, feeding strategies, timing of induction, two-stage culture mode, and stabilization of plasmid had been implemented to minimize some of these problems. The approaches are reviewed in this article.

Key Words: Plasmid instability, thermal induction, proteolysis, inclusion bodies, metabolic overload, *Escherichia coli*

1.0 INTRODUCTION TO INDUCIBLE and PROTEIN UNDER TRANSCRIPTIONAL REGULATION OF λ p^L PROMOTER SYSTEM

The expression of heterologous protein from a cloning vector may be regulated by a promoter. In the regulatable promoter, the binding of repressor protein on the operator (DNA sequence adjacent to promoter) inhibits the interaction between the RNA polymerase with the promoter [1]. As a result, the level of transcription is decreased. Georgiou [2] described that regulatable promoters can be either repressible or inducible. The affinity of this repressor protein towards the operator can be modulated by several factors including adjusting the concentration of small molecules such as nucleotides, amino acids, or sugars, or changes of culture physical environment such as temperature or osmotic pressure. In the inducible promoter system, these molecules (known as inducers) will bind to the repressor protein, and promote its detachment from the operator. On the other hand, transcription under the regulation of repressible promoter occurs when the level of these small molecules (known as co-repressors) is decreased.

Probably among the earliest work in the construction of plasmid cloning vehicles that promote gene expression from bacteriophage lambda p^L/cI promoter was accomplished by Bernard *et al.* [3] and Bernard and Helsinki [4]. In the p^L/cI promoter regulation system, the products of the *cI* and *cro* genes had been identified to being able

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to alternatively repress the initiation of transcription of this promoter [5] , [6], [7]. Thus, the control on p^L promoter by the temperature shift can be implemented if a cIts *cro*$^-$ background is employed in a cell. The *cI* gene may be located either on the host chromosome (e.g. in [8]) or on the second plasmid (e.g. in [9]), or on the vehicle itself [3]. In order to suppress the protein expression, the culture was usually grown at a temperature range between 28^{o}C and 32^{o}C [10], [11], [12]. The expression of protein was attained by increasing the temperature to between 38^{o}C and 42^{o}C [13], [14]. Figure 1 shows the example of lambda p^L promoter construct on a plasmid pBRG 401.

One of the attractions of using the lambda p^L promoter is the ability to express heterologous proteins up to 30% of total biomass [15]. In a study by Seeger *et al.* [16], a performance between the lambda p^L and the *lac* promoters on human basic fibroblast growth factor production in fed-batch culture has been compared. The result revealed that under comparable culture conditions, a 10-fold higher specific product concentration was obtained when product was expressed in the lambda p^L promoter in comparison to what was achieved under the *lac* promoter. Although 30% of the product was expressed as inclusion bodies under lambda p^L promoter, the remaining soluble portion of the protein level far exceeded to what has been expressed under the *lac* promoter.

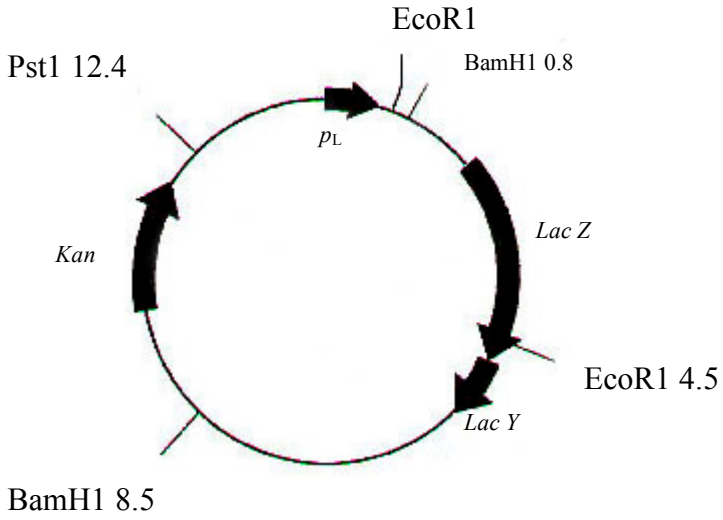

Figure 1 Schematic diagram of plasmid pBRG 401

In comparison to other induction methods such as pH stress [17], phosphate addition [18], or nitrogen limitation [19], side effects of elevated temperature such as proteolysis [20] and inclusion body formation [21] disfavor the employment of the p^L/cI promoter regulation system in heterologous protein production. This has motivated the effort to develop a p^L/cI$_{trp}$ expression system [22]. In this system, the supplement of tryptophan-riched medium blocks the production of lambda cI repressor molecules, and consequently activates the protein synthesis. This system is advantageous for large scale since trytophan-riched medium such as molasses is readily available at lower cost.

2.0 IMPLICATIONS OF PROTEIN OVEREXPRESSION IN *Escherichia coli* CULTURE

2.1 Inclusion Body Formation

Protein precipitate within bacterial cytoplasm was firstly reported by Williams *et al.* [23] during the overproduction of human insulin in *Escherichia coli* culture. The formation of these bodies was a result of unfolded polypeptide chains. These proteins form a crystalline array within the cell, which is also known as inclusion or refractile bodies. Under electron microscope, inclusion body can be easily recognized as darkly stained regions scattered within the cytoplasmic area [24]. The dense nature of this body may provide stearic hindrance toward protease [25], and facilitate the separation process [26]. However, it has to undergo time-consuming processes such as solubilization and refolding in order to restore the bioactivity of the protein. Although inclusion bodies usually occur with heterologous protein [27], it appears that this can also happen to any protein that is overexpressed within the cells [28]. A study by Corchero *et al*. [29] revealed that the solubility of overexpressed β-galactosidase fusion protein in *Escherichia coli* was greatly influenced by the position of the heterologous domain. In the lambda p^L promoter system, overexpression of heterologous products that lead to the formation of inclusion bodies has been reported by several authors [16], [21], [24].

2.2 Metabolic Overload

The overexpression of heterologous protein has been shown to significantly exerting a metabolic burden on the host. Metabolic burden (also referred as metabolic load or metabolic drain) may be defined as 'the portion of a host cell's resources, either in a form of energy such as ATP or GTP, or raw materials such as amino acids that is required to maintain and express foreign DNA, as either RNA or protein, in the cell' [30]. Cultures under extra metabolic burden are usually attributed by reduced growth rate or sometimes death. A study by Andersson *et al*. [31] reported that the induction of 0.1 mM IPTG on foreign gene-encoded plasmid marked a significant overall biomass reduction. However, in the uninduced culture, no significant effects of plasmid presence on the biomass yield. Their results also revealed that the number of dividing cells (colony forming ability) was influenced by plasmid presence and to a larger extent by induction. In a plasmid-host interaction study on *Escherichia coli* HB101 that harbored plasmid RSF1050 performed by Seo and Bailey [32], suggested that the maximum growth rates in LB and minimal media were lowered with increasing plasmid content per cell. Their report also indicated that the increased plasmid content per cell also significantly reduced the overall efficiency of plasmid gene expression, which was measured as the ratio of enzyme specific activity to plasmid content.

The effect of recombinant gene expression and plasmid amplification on growth kinetics of recombinant *E.coli* was also investigated by Betenbaugh *et al*. [33]. In their experiment, both gene expression and plasmid replication were regulated by the addition of IPTG and temperature upshift (38^oC), respectively. For comparison, the experiment was conducted in three different modes, 1) addition of IPTG, without temperature upshift, 2) temperature upshift, without addition of IPTG, and 3) simultaneous addition of IPTG and temperature upshift. In the first experiment, recombinant gene expression resulted in a significant loss of cell viability. On the other hand, cell viability in the temperature upshift experiment was not affected, although the product yield was much lower in comparison to the IPTG induction experiment. The expression in the temperature upshift experiment was due to the inability of the *lac* repressor to titrate out all repressor binding sites on the plasmids. Simultaneous induction by temperature and IPTG yielded 4400 times higher

than induction by IPTG alone, despite a 70% reduction in growth rate. Another study reported by Bentley *et al*. [34] indicated that the growth rate of *E.coli* RR1 expressing chloramphenicol-acetyl-transferase (CAT) from plasmid pBR322 (amp^r, cam^r, and tet^r) was decreased linearly with increasing foreign protein content, and was media independent. They also pointed out that the change in growth rate due to foreign protein expression also depended on the growth rate of the culture. In a kinetic study for the optimization of recombinant protein production by Kramer *et al*. [35], they established a simple kinetic relationship in describing the maximum specific content of recombinant protein, noted as '% F_p'. The value of $\%F_p$ might be determined by the ratio of recombinant protein production rate (q_p) to growth rate (μ). They concluded that it is impossible to gain a high ratio value (q_p/μ), at a rapid growth rate. According to them, both high expression rate and maintenance of cell metabolic activity could only be achieved at low growth rate.

The reduced volumetric production due to a lowered specific growth rate following the protein induction in the p^L/cI_{857} system has motivated Uhlin *et al*. [36] to develop a new cloning vector that exerts a smaller affect on host growth rate. The parent plasmid was from a runaway-replication mutant of plasmid R1, which had a temperature-dependent copy number per cell of five at 30°C, and increased to an uncontrolled plasmid number per cell at a temperature of 35°C. They reported that protein synthesis rate under this system was unchanged for several hours after the temperature upshift, while growth was maintained at an exponential rate. This phenomenon is most unlikely to happen in a culture using the p^L/cI_{857} promoter system. The simultaneous replication of plasmid and expression led to the synthesis of protein in proportion to gene dosage. In addition, since the runaway replication of the plasmid in rich media was lethal to the host, this property also contributed to the safety of the plasmids as a cloning vector.

2.3 Stress-related Intracellular Proteolysis

Proteolysis is essential for natural processes such as the degradation of non-functional proteins in order to provide building blocks for the synthesis of new protein [37]. Expression of heterologous protein and heat shock response among others has been identified to cause a notable increase in proteolytic activity. Proteins that appear to be 'foreign' or aberrant in *E.coli* have been shown to be more prone to proteolysis [38].

Heat shock response is a condition where organisms produce a number of new proteins (referred to as heat shock proteins) as a response to stress, while production of other proteins is suppressed [39], [40]. Since this observation was first noted from exposure of cells to super-optimal temperature, the term 'heat shock' has been adopted in many different cases. Environmental stresses such as nutrient starvation [41], toxic substances [42], and foreign protein expression [43] also has been identified to trigger heat shock responses. The major role of this response is to enable the cell to withstand this metabolic disruption [38]. Activities such as DNA replication, RNA synthesis, production, degradation and repair has been observed during this response [44], [45], [46]. Three types of heat shock proteins have been identified: protein regulators, proteases, and chaperones [47]. In inducible systems, heat shock-related proteolysis has also been proven to enhance intracellular product degradation [12], [20]. It has been shown that protease La, the product of the *lon* gene is the most important protease related to the heat shock response in *E.coli* [12]. The mechanism of the heat stress response and strategies to control the proteolysis in *E.coli* has been reviewed by Enfors [38]. In *E.coli*, the activity of protease La was found to be ATP-dependent [48]. A study by Yang *et al*. [49] on recombinant Staphylococcal Protein A revealed that intracellular proteolysis increased with increasing concentration of glucose up to 200 mg/l. This observation contradicts with the work that

reported starvation leads to high proteolytic activity [50]. Yang and Enfors [51] explained that in the presence of glucose, both product translation and proteolysis happened at the same time. However, the faster rate of translation over degradation masked the role of proteolysis, hence product accumulation was observed. When the glucose was exhausted, the translation would stop while proteolysis continued resulting in product loss. This explanation is further supported by the observation that only a minimal amount of glucose was needed to initiate energy-dependent proteolysis.

2.4 Increase of Acetic Acid Secretion

To date, four hypotheses have been proposed regarding acetic acid formation in *E.coli* culture. They are 1) limited capacity of oxygen uptake [52], 2) accumulation of $NADH_2$ switches carbon flow towards acetic acid [53], 3) combination of limited tricarboxylic acid cycle activity and limited capacity of the electron transport [54], and 4) limited overall capacity of oxidative metabolism [55]. The formation of acetic acid at high growth rates is unavoidable even though the oxygen supply is not limited [56], [57]. A significant increase of acetic acid secretion following protein overexpression under a thermal induction system in *E. coli* culture supplemented with glucose observed in several works [58], [59], was probably due to the inhibition of the tricarboxylic acid cycle.

A study by Kornberg [60] revealed that *E.coli* could also use acetate as a carbon source through a glyoxylate shunt. El-Mansi and Holms [61] suggested that the conversion of acetate to acetyl-CoA was catalyzed by reversible activity of two enzymes, phosphotransacetylase and acetate kinase. Other workers [62], [63] found that the conversion of acetate to acetyl-CoA could also be possibly mediated by acetyl-CoA synthetase. A report published by Aristos and co-workers[64] indicated that the accumulation of acetic acid ranging between 1 and 30 g/l in *E.coli* culture depends on the strain, growth medium composition and propagation (culture modes) techniques. In a study by Shiloach *et al*. [65], two strains of *E.coli*, namely *E.coli* BL21(λDE3) and *E.coli* JM109 (derived from K12) were compared for their pre-induction growth and acetate accumulation patterns. The result exhibited that *E.coli* JM109 released 10 g/l and 5.0 g/l acetic acid in batch and fed-batch culture respectively in comparison to only 2 g/l and less than 1 g/l secreted by *E.coli* BL21(λDE3). They concluded that *E.coli* BL21(λDE3) possessed a better acetate self-control mechanism. This attribute allowed the culture to grow at a desired pre-induction density in high glucose medium by using a simple batch technique.

Several works reported that the accumulation of acetic acid exerts an inhibitory effect on specific growth [66], [67] and production yield [68], [69]. The inhibitory effect of accumulated acetic acid on *E.coli* in the culture is comparable to the effect of ethanol on yeast [70], or propionic acid on *Bacillus subtilis* [71]. Acetic acid has been shown to inhibit cell growth completely in the range between 10 g/l to 20 g/l [57], [72], [73], [74]. A study by Koh *et al*. [75] revealed that acetate inhibition was more pronounced on a recombinant strain, and in a defined medium, than in a complex medium. Inhibition mechanism suggested that lipophilic protonated acetic acid (CH_3COOH) especially at pH lower than pK_a, could cross the cell membrane and acts as an uncoupling agent to the proton motive force between membrane [39]. Doelle *et al*. [39] pointed out that protonated acetic acid, which can pass through the cell membrane, and dissociates to an acetate ion and proton due to a higher internal pH. This lowers the internal pH and causes a net electroneutral proton influx. Since the medium is usually buffered, the decrease of intracellular pH is the possible reason for the uncoupling effect.

It has been well recognized that the formation of acetic acid is associated with high growth rate [57]. Dependence of the specific growth rate of *E.coli* on the

concentration of glucose has been described by using the Monod equation [76]. They pointed out that the saturation constant, K_s, divided specific growth rate into two parts. At glucose concentrations lower than K_s, the specific growth rate is linearly dependent on glucose concentration, while at concentrations above K_s, the specific growth rate becomes independent of glucose concentration. K_s value for *E.coli* growing on glucose is 2.2×10^{-5} M or 0.004 g/l, and at a concentration above about 0.045 g/l, specific growth reaches a maximum at slightly above 1.2 per hour. This extremely low K_s value has made the fermentative condition almost unavoidable. However, in a fed-batch practice, controlling the glucose level at 1.0 g/l or less in the culture broth could minimize acetic acid formation [57], [77].

A study by Neubauer *et al.* [78] demonstrated that the inhomogeneous environment in a large bioreactor influenced the formation of acetic acid in *E.coli* culture. They simulated the inhomogeneous condition by using a two-compartment reactor system consisting of a stirred-tank reactor (STR) and aerated plug flow reactor (PFR). Glucose was fed to the PFR or to the STR alternatively to simulate short-term glucose excess or starvation. It was found that there was no acetate accumulation in the culture because it was consumed in the STR part, where glucose was growth limiting. A significant acetate accumulation was noted when the oxygen in PFR was insufficient. They suggested that the short-term heterogeneities influenced both physiology and growth of cells in the culture.

Besides controlled-feeding of glucose, other efforts to minimize acetic acid formation have also been reported. Feeding of complex carbon-nitrogen substrates such as yeast extract during induction proved to suppress the formation of acetic acid [55] and enhanced productivity [79]. According to Han *et al.* [55], the addition of yeast extract reduced glucose uptake and hence resulted in reduced acetic acid formation. On the other hand, in a study of medium effects performed by Meyer *et al*. [80], *E.coli* K12D1 grown in a complex medium at a lower growth rate produced acetic acid, whereas no acetic acid was detected in cultures grown in defined medium. A comparable observation was also reported by Suarez *et al*. [81] indicating that the feeding of glucose with yeast extract produced a 10-times higher concentration of acetic acid concentration in comparison to the feeding of glucose alone. They concluded that the cell fermentative capacity was enhanced by yeast extract. Glycerol has also been used as an alternative to glucose in order to minimize acetic acid formation. Kwon *et al.* [82] found that glycerol-grown culture produced one-third of acetate than glucose-grown culture. They also found that intermittent feeding of glycerol was more effective in achieving high cell density than feeding an equal amount of glycerol continuously. In another study, Lee *et al.* [83] discovered that switching glucose to glycerol doubled the specific activity of chimeric fusion protein TP-40.

3.0 BIOPROCESS APPROACHES IN IMPROVING PROTEIN EXPRESSION IN λp^L PROMOTER SYSTEM

3.1 Temperature Upshift Schemes

Majority of the inductions using the λp^L promoter system in *Escherichia coli* cultures were performed at 42°C. However, the reason behind this was not quite clear. A study by Hecht *et al*. [84] found that the 'melting' temperature for protein repressor cI_{857} was 42°C. According to them, at a lower temperature, the amino terminal domain (which binds to DNA) was completely folded. When the temperature was increased up to 42°C, unfolding of this terminal domain increased sigmoidally. At 42°C, 50% of the molecule was unfolded. Further increase to 50°C showed more than 65% of this molecule unfolded.

They postulated that the binding characteristics of cI_{857} to the lambda p^L promoter also changed with these temperature induced conformational changes.

Studies have indicated that the *in vivo* protein synthetic capacity [85], the levels of major *E.coli* protein [86], the growth kinetics [87], and the regulation of ribosome synthesis [88] profoundly vary with temperature. They found that induction at temperatures lower than 42°C was more effective. For example, in a pilot-scale experiment of fusion protein SpA-β-galactosidase production performed by Strandberg *et al*. [89], a temperature of 40°C was used to induce the culture. Okita *et al*. [90] found that the optimal induction temperature was product dependent. In their investigation on the expression of two types of malaria antigens, namely RN1 and RN2, they observed that the optimal induction temperatures for RN1 and RN2 were at 39.5°C and 40°C, respectively.

An induction temperature profile was implemented in some studies suggesting that instantaneous exposure to the inducer might lead to intolerable stress on the host as well as plasmid instability. A report by diPasquantonio *et al.* [11] mentioned that there is an optimal length of exposure time at 42°C in order to get maximum production. They discovered that downshifting the temperature to 37°C after 30 to 45 minutes of exposure at 42°C yielded the highest and most stable expression. Horiuchi *et al.* [14] reported similar results. They found that by lowering the induction temperature from 41°C to 38°C in combination with adjusting the pH from 5.5 to 5.65 stabilized the production of β-galactosidase. A study by Lamotte *et al.* [58] suggested that profiled induction performed at the right phase of growth might result in high production. They reported a 27% increase in recombinant UDP-glucuronosyltransferase production if a lag phase at 35°C was followed by an exponential growth phase at 42°C and a stationary phase at 38°C at in comparison with the typical 28°C growth followed by 42°C induction in late batch culture. Brandis and co-workers [9] introduced a process control scheme using cell density and metabolic activity in order to determine the time onset of temperature induction and time of cell harvest in the production of transforming growth factor-alpha (TGF-α). They used culture florescence derived from the reduced form of intracellular NADPH to determine cell density and metabolic activity. Prior to the experiment, a normalized fluorescent unit (NFU) was calibrated to the corresponding cell density. The changes of temperature set points were determined by the response from the culture fluorescence that was measured online. In order to minimize heat shock effects, a three-stage temperature phase, i.e. 32°C-42°C, 42°C (20 minutes), and 42°C –37°C as implemented in their work. Their three-stage temperature scheme had been adopted by other researchers with some modifications. For example, Cockshott and Bogle [59], [91] ramped their culture temperature from 30°C to 38°C within one hour, and maintained at 38°C for the next hour, and instantaneously upshifted to 42°C for the rest of the induction period. In a study of human interleukin production-2 in *E.coli* culture by Chung *et al*. [8], a similar temperature scheme as Brandis *et al*. [9] was carried out, except the initial temperature of 30°C was used.

In other inducible systems such as the *lac* promoter system, where chemicals such as isopropyl-β-D-thiogalactoside (IPTG) or lactose is used as an induction agent, similar strategies of profiled induction was also implemented for the same reason as in the lambda p^L promoter system. In a fed-batch study, a gradual feeding of IPTG and phenylalanine resulted in a significant increase in chloramphenicol acetyltranferase production in comparison to pulsed feeding [92]. The effect of IPTG concentration, timing and duration of induction has been reviewed [93].

3.2 Feeding of Substrate

Numerous fermentation studies focussed on the employment of fed-batch culture mode for the production improvement in *E. coli* cultures. The application of fed-batch has been

reviewed and discussed in Yamane and Shimizu [94] and Minihane and Brown [95] respectively. One of the advantages of using fed-batch in the lambda p^L promoter system is to control the growth at a predetermined constant rate. This is usually achieved by feeding the limiting substrate at the exponential rate. Several studies showed that controlling the specific growth rate before and during induction tremendously improved the productivity. A study by Ryan *et al*. [96] demonstrated that a higher pre-induction growth rate favored better cell viability, greater cell mass yields, and increased cloned gene expression. However, a study by Zabriskie *et al.* [97] revealed that the production yield of recombinant malaria antigen was independent of pre-induction specific growth rate.

Investigations of the influence of pre-induction specific growth rate in continuous culture were also reported. In a study of recombinant α-consensus interferon production by Curless *et al.* [98], showed that the expression level increased 4-fold as the pre-induction dilution rate was increased from 0.025 per hour to 0.2 per hour. On another chemostat study on the production of granulocyte macrophage colony stimulating factor by Curless *et al.* [50], indicated that increased pre-induction specific growth rate resulted in high expression in the cytoplasm. They hypothesized that increasing pre-induction specific growth rate would enhance the ribosomal machinery for protein synthesis. This permits cells to synthesize proteins quicker for growth and maintenance. The cell would use the same machinery to express the heterologous protein and hence higher production was seen at a higher rate of growth. Their observation was supported by Herbert [98] as much as he found that the ribosome concentration within cells increases with increasing specific growth rate.

The importance of controlling the post-induction specific growth rate to improve production has been reported by Lim and Jung [10]. They found that either step-wise or constant medium feeding at late exponential phase of batch culture resulted in dramatic extension of the production period. This was due to maintaining a sustained specific growth rate to overcome the harsh conditions imposed during induction. An investigation conducted by Wong *et al.* [100] using the T7 promoter system (induction was achieved by introducing IPTG to the culture) found that linearly changing the post-induction feeding rate resulted in a better production of adhesive protein. They also reported that growth was independent of the nutrient feeding rate.

Most exponential feeding algorithms were developed on a single limiting substrate [72], [101], [102], [103]. In practice, the desired growth rate can be achieved easily in cultures that use a defined medium where only single carbon source is fed. But when complex carbon-nitrogen substrates such as tryptone, yeast extract or peptone are used together with the principal carbon sources, algorithm based on a single limiting substrate is no longer valid. This is due to the fact that the predictability of the specific growth rate becomes poor as the fermentation time proceeds. Consequently, either over or under-feeding phenomenon takes effect. If this happens before the induction, either substrate starvation or a Crabtree-like effect may occur. To overcome these problems, the feeding rate is usually coupled with a feed back control to correct any possible deviation. Culture conditions such as pH [104], dissolved oxygen [[73], 105]; [106]; [107], acetate concentration [66], biomass concentration and culture volume [108], glucose concentration [71]) has been successfully used as a feedback input to control the growth rate. Details of feeding methods with and without feedback control has been reviewed by Lee [109].

3.3 Timing of Induction

A number of studies have shown that the timing of induction has a great influence on protein expression. Delaying the culture time, and thus induction time results in not only a high cell concentration but may also result in accumulating metabolic byproducts such as

acetate, fumarate, citrate, lactate, and succinate to an intolerable level [66]. The timing of induction also influences final volumetric production since induction imposes extra metabolic burden on the growing cell. For example, in a batch culture study of *E.coli* producing β-galactosidase by Kamasawa *et al.* [110], temperature upshift at hour 3 and hour 5.5 produced 0.75 and 1.2 Units/ml of the enzyme respectively. Their experiment also showed a significantly lower final cell concentration at hour 3 induction in comparison to that achieved at hour 5.5. In a study by Lee *et al.* [83] on chimeric fusion protein production in glycerol-grown *E.coli*, increasing yeast extract concentration from 1% to 4% has significantly delayed the induction time, so higher cell density could be achieved before induction. They suggested that by increasing the yeast extract concentration, initiation of a metabolic switch from yeast extract to glycerol could be shifted reasonably far ahead. As a result, the final titer of the chimeric protein was doubled from 400 mg/l to 850 mg/l. In another study by Seo *et al.* [107], a batch culture of *E.coli* producing human interleukin-2 was induced during three different growth phases. They found that the final interleukin-2 concentrations of 0.21, 0.39, and 0.24 g/l corresponded to an induction time at early exponential (hour 8), mid-exponential (hour 10), and late exponential phase (hour 14) growth respectively. Lim and Jung [10] have established a relationship between the initial specific expression rate and specific growth rate. Their results revealed that the initial specific production was exponentially increased as induction was engaged in the order of stationary late, middle, and early exponential phases of batch cultures.

3.4 Two-stage Culture

One of the key features of inducible systems is the potential separation of growth and induction phases. Traditionally, both phases are carried out in a single bioreactor. This is somewhat unfavorable since studies in the growth phase may potentially affect the production phase, and vice versa. A complete physical separation of these two phases can be achieved by two-stage fermentations. This approach offers high operational flexibility since different modes and feeding strategies can be implemented in both phases independently. This also permits the optimal conditions to be accommodated for both growth and production stages simultaneously. In a study of α-amylase production by Chang *et al.* [103], a two-fold increase in volumetric concentration was achieved in a two-stage, cyclic fed-batch fermentation in comparison to production in an ordinary fed-batch system. In their study, a portion of broth from the first stage (growth phase) was transferred to the next stage for induction. Both stages were then fed with fresh medium to maintain a high cell density and productivity at the same time. In a study of *trp A* protein production, Siegel and Ryu [13] have employed a two-stage continuous fermentation to examine the effects of variables such as temperatures and dilution rates on productivity. Among the results, they found that at the induction temperature of 41.7^{o}C, the productivity in two-stage fermentation was 60% of the maximal expression at 20 hour, while in a single-stage, the expression was essentially stopped at 10 hours. Increased productivity with increasing dilution rates was also reported by them. According to them, these productivity improvements and prolonged period of expression were due to minimizing of plasmid instability during high expression. They also claimed that the two-stage system allows the investigation of the effect of fermentation parameters of recombinant gene expression under a better controlled environment than in continuos single-stage or batch culture.

3.5 Stabilization of Plasmid

Plasmid instability may be classified as either segregational or structural. Segregational instability is caused by biased distribution of the plasmid to the daughter cells from the dividing mother cell [111]. Consequently, the culture will eventually be overwhelmed by the plasmid-free segregants. Structural instability is signified by the disappearance of the plasmid expression activity while the plasmid still exists in the cell. In *E. coli*, the structural instability is typically attributed to the presence of movable genetic elements known as transposons. Transposons such as TnA and Tn10 that usually carry drug-resistance gene(s) have been recognized to promote deletion of DNA in their immediate vicinity [112]. The insertion of a transposon into a gene or a resultant partial deletion of a gene by transposon activity would certainly interrupt the functionality of the gene, and hence affect the expression. It is also important to note that structural instability may not be detected through a conventional plate method. This is due to the fact that the interruption on the gene of interest may not necessarily affect the marker genes. As far as structural instability is concerned, no known bioprocess strategy has been proposed to overcome this problem. The best solution may be an appropriate choice of a strain. Nevertheless, it has been reported that the addition of selective pressure may 'persuade' the cell to retain the plasmid [111].

Several factors that contribute to plasmid instability have been observed. Environmental conditions such as growth temperature [113], decreasing dilution rates [114], dissolved oxygen concentration [115], starvation for essential amino acids [116], sulfate limitation [117], and pH [118] have been recognized to affect plasmid stability. In a case of a system that employs a high copy number plasmid, segregational loss may occur due to plasmid multimerization. Ryan and Parulekar [119] have reported that this phenomenon was partially responsible for the plasmid segregational loss in a continuous culture of *E.coli* JM103 harboring plasmid pUC8.

Strategies in maintaining the stability of a recombinant plasmid have been well discussed. Imanaka and Aiba [120] have proposed that the stability of a recombinant plasmid can be ensured by the following factors: 1) employment of temperature-dependent plasmid and strain, 2) providing selective pressure in the culture, 3) no transposable element in plasmid, and finally 4) employment of a mutation deficient (*rec*$^-$) strain. Kumar *et al*. [121] suggested that strategies for improving plasmid stability in bioreactors can be divided into two approaches, namely cellular/molecular strategies and bioprocess strategies. The cellular/molecular strategies could be implemented in three ways. The first is by modulating the genes for stable maintenance during segregational steps. This is achieved by the insertion of the *par*A loci [122] or *cer* function [123] into the plasmid. The second approach is by employing post-segregational killing or inhibiting functions or compounds [124, 125], and finally the integration of the plasmid in the chromosome [126]. In bioprocess strategies, control of plasmid instability is achieved by one of these three following approaches, 1) inhibiting the growth advantage of plasmid-free segregants by employing two-stage fermentation [13] or recycling technique [127], 2) separation of plasmid-free segregants by selective flocculation [128] or aqueous two-phase system [129], and 3) whole-cell immobilization [130].

4.0 CONCLUSIONS

Despite the attraction of employing the lambda p^L promoter system in the overproduction of heterologous proteins in *E.coli*, deleterious effects pertaining to high temperature such as increased proteolytic activity, metabolic overload imposed on the host, formation of inclusion bodies, and excessive secretion of acetic acid have been proved to be among the key factors that lower the overall productivity. Various bioprocess strategies have been

introduced to address these problems that include systematic ramping and timing of induction temperature, controlling the growth rate by employing fed-batch mode, separation of the growth and the production phase, as well as stabilization of plasmid. It is worth mentioning that some of the techniques are only applicable to only a few cases, while others may work satisfactorily to certain scales of production. Nevertheless, as the production scale increases, the most cost effective and economic strategy(ies) will eventually dictates the choice of strategy.

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