2011 International Conference on Environment and BioScience
IPCBEE vol.21 (2011) © (2011) IACSIT Press, Singapore

dps′: An H_2O_2 Inducible Promoter for High Level Protein Production in *Escherichia coli*

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Abstract. *Escherichia coli* is widely exploited as an expression system for the commercial production of several recombinant proteins. In its natural environment, *E.coli* has evolved a multifaceted response to combat oxidative stress: this involves strong induction of several promoters responsible for the synthesis of stress responsive proteins. In this work we have investigated the use of the *dps* promoter (*dps′*) towards achieving high level protein production. Expression of target proteins through *dps*' can be induced ≥10 fold by an inexpensive agent like hydrogen peroxide (H_2O_2) particularly in the early exponential phase of growth. At later stages of growth, expression through the *dps′* becomes constitutive suggesting that the production of endogenous H_2O_2 may circumvent the need for addition of an external inducer. This illustrates a cost effective and growth phase dependent promoter capable of directing high level protein production in *E.coli*.

Keywords: Protein expression, *dps*, hydrogen peroxide.

1. Introduction

Escherichia coli, a gram negative bacterium, is the most preferred industrial prokaryotic host for heterologous protein production. Starting with the synthesis of recombinant human insulin (Humulin) in 1978, *E.coli* dominates the world of protein synthesis even today. Although the expression of foreign proteins in *E.coli* is faster, cheaper and easier than in other systems, there is a need for development of better plasmids, hosts and candidate promoters, towards creating robust expression systems [1-3].

A low cost expression system that can synthesize large amount of foreign proteins has the potential to revolutionize large scale industrial protein production. Enzymes and hormones constitute the major targets. However, the metabolic load for targeted high level protein production affects native cellular activities [4, 5]. In addition, the characteristics of individual proteins often influence the extent of expression. Some proteins are toxic to *E.coli*; consequently they cannot be produced in large quantities [6]. Other induced proteins are either insoluble or are incorrectly folded [7, 8]. These issues have necessitated the development of more desirable expression systems using *E.coli* as a prospective host.

Gao et al have used a strategy wherein a gene encoding superoxide dismutase is induced in response to superoxide anion / paraquat [9]. On the other hand, Giacalone et al have developed a tightly controllable rhamnose based switch for the expression of toxic proteins [10]. Recently, Choi et al have demonstrated a robust cumate based inducible expression system that claims to be superior than the existing IPTG based expression system [11]. Despite the extensive use of *E.coli* as a host for protein production, the quest is on to develop a better expression system in *E.coli*.

Of the various promoters upregulated in response to oxidative stress, expression through *dps′* is induced to the highest level [12]. The level of *dps* transcripts increases about 180 fold upon induction by H_2O_2 making it an attractive candidate to be targeted for the purpose of high level protein production in *E.coli*. In this study, we have explored the ability of the *dps′* to bring about expression of foreign proteins in *E.coli*. In

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its natural context, the *dps* gene product (Dps) serves three basic functions; it acts as an iron storage protein, as a centre for ferroxidase activity, and protects the cells from harmful oxidative radicals by non-specifically binding the DNA [13, 14]. Dps is only the second known unstable *E.coli* protein (the first being σ^s) to be stabilized in accordance with starvation and oxidative stress signals encountered during physiological cell growth. Expression through *dps′* varies in accordance with internal metabolic activities and the surrounding *milieu* [15, 16]. This results in *dps′* exhibiting a differential expression profile as the culture progresses through various phases of growth. Also, expression of *dps* increases considerably when the cells are exposed to H_2O_2 [12]. This increase in expression is attributed to oxidation of the regulatory protein OxyR by H_2O_2, which subsequently acts as a transcriptional activator for the expression of *dps gene*.

We have used *E.coli* β-galactosidase protein as a reporter to monitor the expression through *dps′* - under influence of H_2O_2 - at the early and mid exponential phases of growth. β-galactosidase activity profile was correlated with the relative presence of β-galactosidase protein in soluble/insoluble fractions of cell lysate as observed using SDS-PAGE. The results obtained were further confirmed by using *Zymomonas mobilis* pyruvate decarboxylase (*Zm*PDC) as a heterologous target protein which was expressed under the control of *dps′*.

2. Materials and Methods

All studies were performed in *E.coli* MG1655 Δ*lac*, obtained as a gift from Dr. D. J. Jin, NIH. The translational fusion vector pRB381, housing a promoter-less *lacZ* gene, was used to study the expression pattern through the *dps′* [17]. The upstream promoter region of *dps* (-197 to +5 from the translational start site of *dps*) was PCR amplified (*Techne TC-312 Thermocycler*) using the primers AACTGCAGCGAAAATTCCTGGCGAGCAGATAAAT and GCTCTAGACTCATAATTTCATATCCTCTT -GATG and was cloned in-frame with *lacZ* (encoding β-galactosidase) in pRB381 to give rise to pRB381-*dps′-lacZ*. Subsequently, the gene encoding *Zm*PDC (*pdc*) was PCR amplified from the vector construct pET43.1b-*Zm*PDC (Lab Stock) using the two primers ACTAGTCTAGACAGTTATACTGTCGGTACCTATT -TAG and AACCGGAATTCCTAGAGGAGCTTGTTAACAGGC and was cloned in-frame with *dps′* thus replacing the *lacZ* gene from pRB381-*dps′-lacZ* to give rise to pRB381-*dps′-pdc* [18]. These constructs were then transformed into the host and were used to monitor the effects of hydrogen peroxide induction upon the expression through *dps′* at different time points.

E.coli cells were cultivated in Luria Bertani (LB) broth containing 100μg/mL ampicillin at 37^0C under shaking conditions (250 rpm) and were challenged with 1mM H_2O_2 (*S. D. Fine-Chem Ltd.*) at either the early exponential phase (3 hrs.) or the mid exponential phase (6 hrs.) of growth. Samples were taken 20 minutes after the addition of H_2O_2. The cells were washed and resuspended in 50mM Tris buffer, subjected to sonication (*Equitron*® 100W) for 5 cycles of 10 seconds each and centrifuged at 12,000 rpm / 4^0C / 5 minutes. The supernatent (soluble fraction) was decanted into another tube and the pellet (insoluble fraction) was then washed once again with 50mM Tris buffer. Both supernatant and pellet were subjected to SDS-PAGE [19] and the gels were subsequently stained with Coomassie Brilliant Blue R-250. Densitometry studies were performed using ImageJ (version 1.42i, NIH, USA).

The β-galactosidase assay was performed as per the standard protocol [20]. β-galactosidase activity is measured in terms of Miller Units (MU). Readings plotted on graphs represent an average of three independent experiments and error bars represent the respective standard deviations.

3. Results and Discussion

As a first step towards attaining heterologous protein production, *E.coli* MG1655 Δ*lac* cells harboring the reporter fusion construct pRB381-*dps′-lacZ* were grown and challenged with H_2O_2 at the early (3 hrs.) and mid (6 hrs.) exponential phase to check for H_2O_2 mediated induction of protein expression. Cell lysates obtained from these induced cultures were subjected to SDS-PAGE analysis to study the behavior of host cells in response to H_2O_2 stress. The 3 hrs. old culture challenged with 1mM H_2O_2 showed ≥10 fold increase in the amount of target protein - β-galactosidase - being synthesized (Fig. 1, lanes 1 and 2). A large portion of β-galactosidase synthesized by the 3 hrs. old H_2O_2 induced culture, and also by the 6 hrs. old induced/uninduced culture, was found to be present in the insoluble fraction. Soluble fraction of cell lysate

obtained from the 3 hrs. old culture comprised only a small amount of protein (data not shown). Once the culture reaches mid exponential phase of growth, gene expression through *dps′* becomes constitutive thus resulting in similar levels of expression for both the untreated and the H_2O_2 treated cells. This suggests that heterologous protein synthesis proceeds without the need for exogenous H_2O_2 to be added to the growth medium.

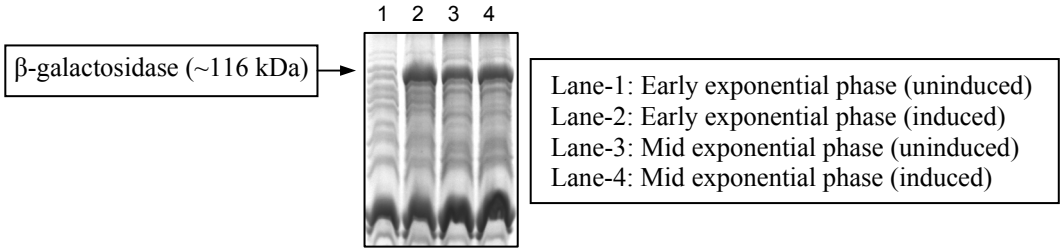

Fig. 1: SDS-PAGE profile of insoluble fraction of cell lysates obtained from H_2O_2 treated/untreated early and mid exponential phase cultures expressing β-galactosidase.

E.coli MG1655 Δ*lac*/pRB381-*dps′-lacZ* was further checked for induction of promoter expression upon exposure to H_2O_2 by measuring the β-galactosidase activity. The growth curve and the corresponding β-galactosidase activity profile are shown as a function of time in Fig. 2. The level of β-galactosidase activity varies as the culture goes through different phases of growth and it reaches a maximum at ~8 hrs. This variation in β-galactosidase activity levels could be attributed to the internal cellular mechanisms involved in regulation of expression of the *dps′* [14, 15]. Upon induction by H_2O_2 at the early (data not shown) or mid exponential phase (Fig. 2B), no significant increase in the level of β-galactosidase activity could be observed.

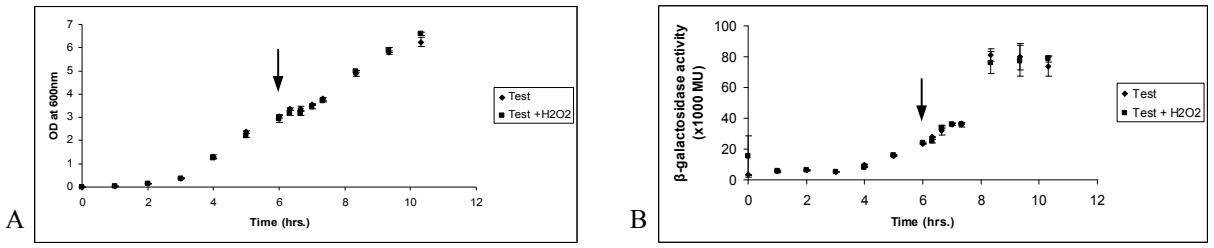

Fig. 2: Growth curve (A) and corresponding β-galactosidase activity (B) of *E.coli* MG1655 Δ*lac*/pRB381-*dps′-lacZ* treated (■) or untreated (♦) with H_2O_2. An arrow pointing downward indicates the time at which H_2O_2 pulse was added.

These results are not in contraction with those in Fig. 1. As observed from Fig. 1, most of the protein (β-galactosidase) synthesized is directed into inclusion bodies which constitutes insoluble fraction of the cell lysate. This suggests that the level of expression of target protein was high enough to probably direct β-galactosidase synthesized to the inclusion bodies due to which the distinct induction pattern observed at protein level (Fig. 1) could not be observed at the activity level (Fig. 2B). This is a common problem encountered while expressing proteins in large quantities which results in improper folding and aggregation of proteins thereby affecting the overall protein activity [21]. In fact the actual β-galactosidase activity levels, accounting additionally for the majority of protein activity which is lost as inclusion bodies in the insoluble fraction of cell lysate, could be much higher than the observed levels. Under the control of *dps′*, β-galactosidase activity reaches a maximum of ~80,000 MU as the cells pass through late exponential phase and approach the stationary phase of growth.

Functioning of this expression system was further confirmed using *Zm*PDC as a heterologous protein being synthesized under the influence of *dps′* (pRB381-*dps′-pdc*). The SDS-PAGE profile of *Zm*PDC observed in Fig. 3 (insoluble fraction of cell lysate) confirmed the H_2O_2 based induction of the early exponential phase culture of *E.coli*. The amount of protein synthesized using *dps′* was estimated to be induced by ≥10 fold upon addition of 1mM H_2O_2. Also, as observed in case of pRB381-*dps′-lacZ* (Fig. 1), there was no significant induction observed when the culture grown to mid exponential phase was treated with 1mM H_2O_2.

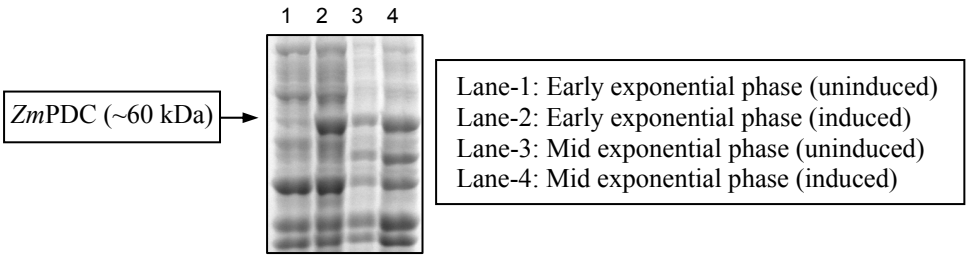

Fig. 3: SDS-PAGE profile of insoluble fraction of cell lysates obtained from H_2O_2 treated/untreated early and mid exponential phase cultures expressing *Zm*PDC.

Significant induction of gene expression during early exponential phase is of great advantage while dealing with synthesis of proteins that pose problems during purification and/or downstream processing. This is due to the presence of certain growth phase specific proteins that get expressed highly during the mid/late exponential or stationary phase of growth [22, 23]. Furthermore, it is highly advantageous for the large scale production of proteins that are potentially toxic to the cells and demand an alarming rate of synthesis so that host cells could accumulate maximum amount of protein before the toxic effect of target protein is triggered [6, 10]. A 10 fold increase in the production of such toxic proteins could be largely beneficial at the commercial scale.

Interestingly, as observed from Fig. 1 and Fig. 3, the amount of protein expressed in H_2O_2 treated 3 hrs. old culture was equivalent to that of the uninduced/induced 6 hrs. old culture. H_2O_2 constitutes a prominent factor responsible for oxidation of a transcriptional activator - OxyR - which in turn activates transcription through the *dps′*. In the case of cells growing in mid exponential phase, endogenously generated H_2O_2 would possibly have accumulated to a level equivalent to that in case of H_2O_2 treated early exponential phase cells [24]. Consequently, the magnitude of expression through *dps′* is perceived to be similar. However, even in the case of early exponential phase culture, a time lapse of ~20 minutes is required for the cells to sense oxidative stress signal (H_2O_2) from their surrounding *milieu* and ultimately transform it to achieve induction of protein synthesis through the *dps′*.

Unlike other commercially explored inducers viz. IPTG or Cumate, H_2O_2 is spontaneously degraded by host cells thus eliminating the additional burden to be dealt with during the process of downstream processing and/or subsequent purification of the target protein. It renders the amount of protein synthesized to be an exclusive function of biomass being accumulated during the bacterial growth. In other words, more the biomass higher will be the yield.

4. Conclusion

We have developed a simple and cheap H_2O_2 inducible plasmid based expression system that can be used for large scale production of heterologous proteins in *Escherichia coli*. Expression of foreign proteins can be induced in the early exponential phase by addition of low levels of H_2O_2 with ≥10 fold induction of protein synthesis occurring within 20 minutes of addition. It has a propensity towards becoming a constitutive expression system as the cells reach mid/late exponential phase of growth thus leading to expression of target proteins without the need for purposeful addition of exogenous H_2O_2. In other words, induction naturally triggers on once biomass had been accumulated.

5. Acknowledgements

We thank Prof. Ding Jun Jin, NCI Frederick, NIH for providing us the strain *E.coli* K-12 MG1655 *lacX74* (DJ480) referred here as *E.coli* MG1655 Δ*lac*. Also, P.P.S. thanks CSIR for a fellowship.

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