Scientific paper

Influence of the Media Composition on Behavior of pET Expression Systems

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† Dedicated to the memory of dr. Viktor Menart, Assistant Professor

Abstract

pET expression systems (Novagen) are the strongest tool available for production of recombinant proteins in bacteria *E. coli* and have been widely used for many years. It is relatively difficult to control them due to their efficiency enabling formation of large amounts of recombinant proteins.

Our work was focused on the influence of the media composition on the behavior of the pET3a expression system and we tried to select appropriate medium for inoculum preparation as well as appropriate production medium. We found out that media without glucose trigger unexpectedly high activity of *lac*UV5 promoter. Accumulation level of 15% of human granulocyte colony-stimulating factor (hG-CSF) in total cellular proteins was obtained in LBP medium (modified Luria-Bertani) without addition of IPTG inducer. Glucose addition into medium for inoculum preparation successfully represses expression of recombinant protein during inoculum preparation phase. Optimal optical density for high quality of inoculum is around OD_{600nm} = 4,0, when culture is in the middle of exponential growth phase. Presence of glucose is required also in production medium. GYSP medium containing glucose, enables by 25% higher recombinant protein accumulation level than LBP medium without glucose. In contrast to LBP medium it enables comparably high recombinant protein accumulation level also without addition of antibiotic into the production medium.

Keywords: *E. coli*, T7 promoter, media composition, catabolic repression, hG-CSF, IPTG

1. Introduction

pET expression systems, where expression of heterologous gene is controlled by *lac*UV5 repressor, are widely used for recombinant protein production. This is the strongest expression system available for expression in *E. coli* based on strong T7 promoter, which was developed by Studier and colleagues.[1–3] Induction of heterologous gene is achieved by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) into production media.[4,5] Target protein can exceed 50% of total cellular proteins. Such a strong promoter can negatively influence the host cell.[5] Leaky repression of T7 RNA polymerase can cause plasmid instability and consequently unstable expression of recombinant protein.

Even pET plasmid without insertion of recombinant protein can be toxic to the *E. coli* cell in the presence of IPTG.[5,6] Several strategies were developed to avoid above mentioned disadvantages. One approach is co-expression of T7 lysozyme, which inhibits T7 RNA polymerase by binding it.[7] Described approach usually reduces specific growth rate and requires addition of two different antibiotics into the medium to additionally ensure presence of plasmid bearing T7 lysozyme. Repression can also be improved by introduction of *lac*I operator sequence after T7 promoter.

Although it had been assumed for quite a long time that *lac*UV5 promoter is not cAMP mediated, it is clear now that *lac*UV5 promoter is activated in the late stationary growth phase.[5] This can be avoided by use of mutant *E. coli* BL21(DE3) strains with deactivated gene for adenylyl cyclase (*cya*), where improved expression and stability were achieved.[8] Another approach is catabolic repression by addition of glucose into growth media.[9,10]

While all these approaches have not been widely investigated for large-scale production, there are several problems connected to plasmid instability, especially when inoculum should be prepared in several steps. In the past we had noticed plasmid instability and loss of expression even in the case of pET19b plasmid, which contains *lac*I operator sequence downstream of T7 promoter, therefore we started to study parameters important for inoculum preparation more carefully. We focused on the investigation of the media composition and its influence on the expression level of recombinant protein. We tried to find a suitable medium for inoculum preparation where the basal expression level from pET expression plasmids would be lower than in widely used LB medium (Luria-Bertani). Furthermore, our goal was also to find production medium where stable accumulation level of recombinant protein could be obtained even without addition of antibiotic into production medium.

2. Experimental

2. 1. Strain and Plasmid

Escherichia coli BL21(DE3) (Novagen), genotype: F^- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3) bearing plasmid pET3a/P-Fopt5 was used to overproduce hG-CSF. The optimized human G-CSF gene with codons optimized for expression in *E. coli* was prepared in house.[11] The optimized gene, designated as Fopt5, was properly inserted into the expression plasmid pET3a (Novagen) by directionally subcloning between restriction sites *Nde*I and *BamH*I resulting in plasmid pET3a/P-Fopt5. All DNA manipulations were performed according to Current Protocols in Molecular Biology.[12]

2. 2. Media

LBP medium (modified Luria-Bertani, where tryptone was replaced by phytone): 10 g/L Bacto phytone (Becton Dickinson), 5 g/L yeast extract (Becton Dickinson), 10 g/L NaCl (Sigma); LBP/amp100 medium: LBP medium supplemented by 100 mg/L ampicillin (Sigma);

LBPG: 10 g/L Bacto phytone (Becton Dickinson), 5 g/L yeast extract (Becton Dickinson), 10 g/L NaCl (Sigma), 2.5 g/L glucose (Sigma); LBPG/amp100 medium: LBPG medium supplemented by 100 mg/L ampicillin (Sigma); GYSP medium: 20 g/L Bacto phytone (Becton Dickinson), 5 g/L yeast extract (Becton Dickinson), 10 g/L NaCl (Sigma), 10 g/L glucose (Sigma), trace elements [($FeSO_4 \cdot 7H_2O$ (40 mg/L), $CaCl_2 \cdot 2H_2O$ (40 mg/L), $MnSO_4 \cdot nH_2O$ (10 mg/L), $AlCl_3 \cdot 6H_2O$ (10 mg/L), $CoCl_2 \cdot 6H_2O$ (4 mg/L), $ZnSO_4 \cdot 7H_2O$ (2 mg/L), $NaMoO_4 \cdot 2H_2O$ (2 mg/L), $CuSO_4 \cdot 5H_2O$ (1 mg/L), H_3BO_3 (5 mg/L)]; GYSP/amp100 medium: GYSP medium supplemented by 100 mg/L ampicillin (Sigma).

Replacement of tryptone by plant derived phytone did not result in any significant change in behaviour of production strain, such as expression level and growth rate.

2. 3. Shake Flask Culture

LBP/amp100 and LBPG/amp100 media were used to prepare inoculum for shake flask culture. GYSP, GYSP/amp100, LBP and LBP/amp100 media were used to grow bacteria and produce hG-CSF.

Bacterial culture from the frozen stock (–70 °C) was inoculated into appropriately sized Erlenmeyer flask containing 10–100 ml LBPG/amp100 or LBP/amp100 media in the ratio 1 : 500. The bacterial culture was grown overnight at 25 °C, 160 rpm in the linear incubator shaker. Inoculum was transferred into appropriately sized Erlenmeyer flask containing 10–200 ml GYSP, GYSP/amp100, LBP or LBP/amp100 medium in the ratio 1:20 and incubated 18–24 hours at 25 °C, 160 rpm in the linear incubator shaker.

Induction: Induction was performed by addition of IPTG (Sigma or GoldBioTechnology) to the final concentration of 0.4 mM IPTG. Inducer was added together with the inoculum or in the exponential phase, at OD_{600nm} ≈2.0–4.0 in LBP medium and at OD_{600nm} ≈3.0–6.0 in GYSP medium.

2. 4. Optical Density Determination

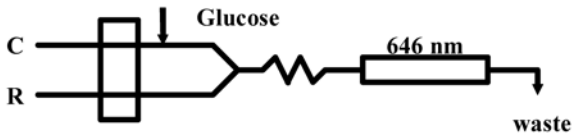
Growth of bacterial culture was monitored by measurement of optical density at 600nm using HP 8452A Diode Array Spectrophotometer (Hewlett Packard).

2. 5. Glucose Concentration Determination

Glucose consumption in growth media was monitored by Flow Injection Analysis,[13] based on the reaction of the glucose with glucose oxidase according to the following reaction:

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconic acid} + H_2O_2$$

The resulting peroxide reacts with another enzyme (peroxidase) and chromogen ABTS (2,2-azino-di-[3-ethylbenzthiazoline]-6-sulfonate) to produce a coloured product detectable spectrophotometrically at 646 nm. Reactions are fast and analytical data are obtained in several minutes.[14]



peristaltic pump reactor UV detector

C: 2500 U/l glucose oxidase (Sigma) in 0,05 M imidazole, pH 6,8 (Sigma); **R**: 2900 U/l peroxidase (Sigma) and 0.75 mM ABTS (Sigma) in 0,05 M imidazole, pH 6,8 (Sigma).

Figure 1: Schematic presentation of the FIA apparatus for glucose determination.

2. 6. SDS-PAGE Analysis

Bacterial pellets from 4–8 ml of bacterial culture were resuspended in 1 ml 10 mM TrisHCl/pH = 8.0 buffer. Samples were mixed with 4X SDS-Sample buffer containing DTT, pH = 8.7 in ratio 1:4 and incubated 10 minutes at 95 °C. After 5 min centrifuging at 10000 rpm 5–10 µl samples were loaded onto SDS-PAGE and separated by electrophoresis. After Coomassie staining the content of recombinant hG-CSF was determined by densitometric analysis using a Bio-Rad Model GS-670 imaging densitometer.

3. Results and Discussion

3. 1. Growth Properties of the E. coli BL21(DE3) [pET3a/P-Fopt5] in Different Media at 25 °C

We investigated growth properties of the production strain *E. coli* BL21(DE3) [pET3a/P-Fopt5] in two different media. LBP is a variation of the well known Luria-Bertani medium, where tryptone is replaced by phytone and does not contain glucose, while GYSP is a richer production medium, where glucose supplement of 10 g/L is used. Bacteria were grown in media with or without antibiotic addition, as well as in media with or without addition of IPTG inducer. IPTG was added in different growth phases such as initial addition into production media or in exponential phase (OD_{600nm} 2.0–4.0 in LBP medium and 3.0–6.0 in GYSP medium). Specific growth rates were determined from growth curves for various growth conditions. Use of the GYSP medium enables at least 2-fold higher biomass in shake flask culture, which was at the beginning the main reason for choosing it as a production medium. Stationary growth phase was reached after approximately 12 hours of cultivation in LBP medium and after approximately 24 hours of cultivation in GYSP medium reaching optical density of 6.0–7.0 and 12.0–14.0

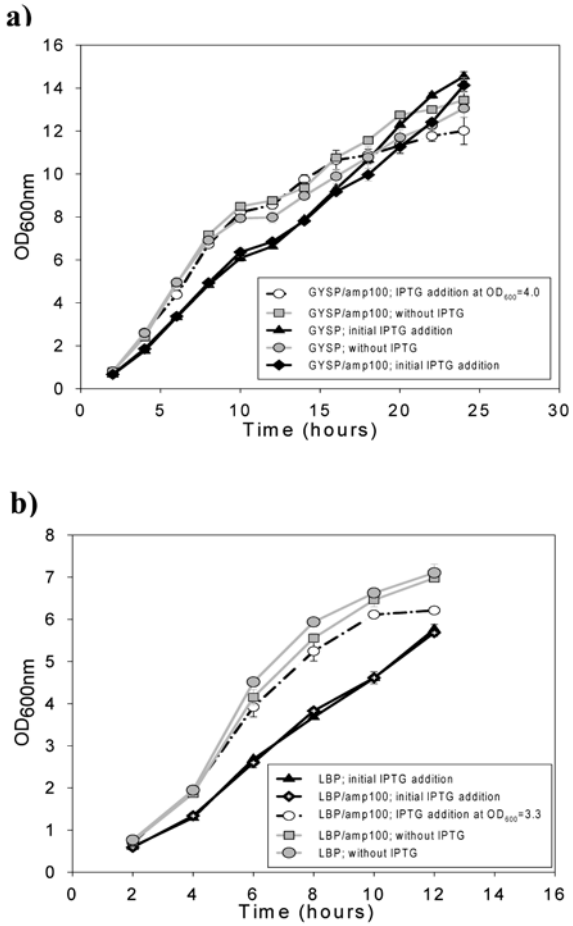


Figure 2: Growth curves of *E. coli* BL21(DE3) [pET3a/P-Fopt5] in different media at 25C; GYSP (a), LBP (b).

respectively. Comparison of specific growth rates showed that initial IPTG addition significantly reduced specific growth rates in both basic growth media (Figure 2). Interestingly, later IPTG addition did not influence the specific growth rates that were practically the same as without IPTG addition.

3. 2. Production of hG-CSF in Different Media

Several experiments were performed in order to check influence of media composition on the accumulation level of recombinant protein. Production was studied in two different basic growth media, LBP (containing no glucose) and GYSP (containing glucose). Furthermore, influence of IPTG addition in different growth phases on the efficiency of induction production was investigated. In addition, influence of presence or absence of selection pressure on hG-CSF accumulation was studied.

Cells were harvested after 12 hours of cultivation in LBP media and after 24 hours of cultivation in GYSP media, when culture reached stationary growth phase.

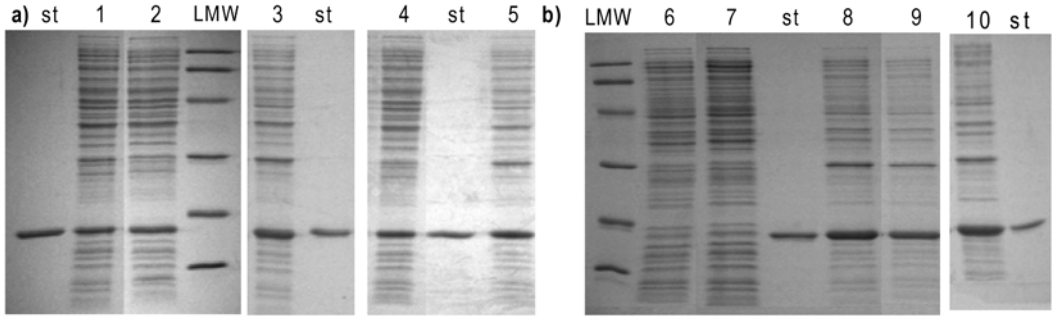


Figure 3: Production of hG-CSF by *E. coli* BL21(DE3) [pET3a/P-Fopt5] in different variations of LBP media (a), and in GYSP media (b).

(st: standard hG-CSF 0.6 µg; LMW: LMW protein standard (BioRad); Lane 1: LBP/amp100, IPTG addition at OD_{600nm} = 3.3; Lane 2: LBP/amp100, without IPTG addition; Lane 3: LBP/amp100, initial IPTG addition; Lane 4: LBP, without IPTG addition; Lane 5: LBP, initial IPTG addition; Lane 6: GYSP/amp100, without IPTG addition; Lane 7: GYSP, without IPTG addition; Lane 8: GYSP, initial IPTG addition; Lane 9: GYSP/amp100, initial IPTG addition; Lane 10: GYSP/amp100, IPTG addition at OD_{600nm} = 4.0).

As shown in Figure 3a (Lane 2, 4), a surprisingly high expression level of hG-CSF was noticed in LBP medium without IPTG addition, which reached almost 15% of hG-CSF of total cellular proteins. Some basal expression was expected due to the leaky promoter, but the observed expression level was much higher than it was expected. Since basic LBP medium did not contain glucose, we concluded, that strong cAMP mediated activation of lac*UV5* promoter occurred resulting in efficient induction of heterologous gene expression even without IPTG addition.

Unexpectedly low expression level was obtained using LBP/amp100 medium with later addition of inducer IPTG, which reached 14% of hG-CSF in total cellular proteins (Figure 3a, Lane 1). Using the same medium with initial IPTG addition resulted in high expression level, namely 30% of hG-CSF in total cellular proteins and was higher than in the case where antibiotic was omitted reaching hG-CSF accumulation level of 25% in total cellular proteins.

Expectedly no accumulation of hG-CSF in basic GYSP medium without addition of inducer IPTG was noticed (Figure 3b, Lanes 6, 7). It seems that glucose supplement successfully represses expression of heterologous gene. Addition of IPTG into GYSP medium resulted in high accumulation levels of hG-CSF, namely between 35% and 40% and was not dependent on the growth phase in which IPTG was added.

Addition of antibiotic into GYSP medium did not increase hG-CSF accumulation level, which demonstrates stability of the production strain under described conditions. Our results show that GYSP medium containing glucose is an excellent choice for production medium. Its use enables comparably high accumulation level of recombinant protein even without addition of antibiotic into the production medium.

The same cannot be said for production in LBP medium, where lower accumulation level of hG-CSF was obtained when selection pressure was not applied.

3. 3. Leaky Expression During Inoculum Preparation – Comparison of Inoculum Quality in LBP/amp100 and LBPG/amp100 Media

We performed additional experiments in order to study influence of glucose addition into inoculum medium on the quality of inoculum. We noticed that inoculum growth time could be critical for the quality of the inoculum, which influenced the protein accumulation level in production phase.

Inoculum quality was compared with inoculum grown in the LBPG/amp100 medium containing glucose and in the LBP/amp100 medium without glucose supplement. We wanted to determine optimal inoculum growth time and decide about the most appropriate medium.

As shown in Figure 4, glucose was depleted from LBPG/amp100 medium after 17 hours of cultivation, when culture reached the stationary growth phase at OD_{600nm} between 7.0 and 8.0.

Growth curves were similar for both media and cultures reached stationary phase after 17 hours of cultivation in both cases.

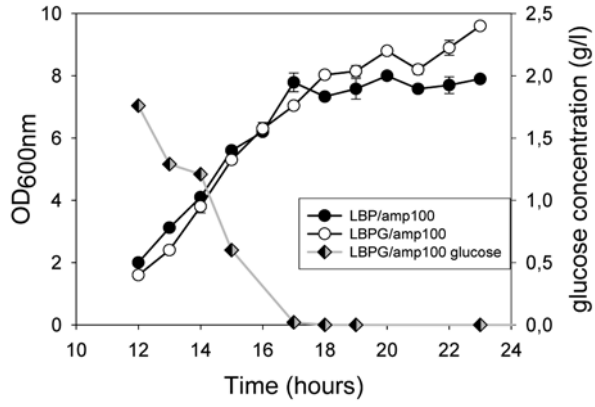


Figure 4: Growth curves and glucose consumption during inocula preparation in LBP/amp100 and LBPG/amp100 growth media.

Cultivation was carried out at 25 °C; samples were taken every 1 hour starting after 12 hours of cultivation at OD_{600nm} around 2.0.

Results show that strong cAMP-mediated activation of *lac*UV5 promoter occurred in the LBP/amp100 medium without glucose in exponential phase. As presented in Figure 5, high hG-CSF accumulation level was observed after 15 hours of cultivation at OD_{600nm} around 5.0 (Figure 5a, lane 4). In the LBPG/amp100 medium containing glucose, expression of hG-CSF was not noticed after 23 hours of cultivation, when culture was in the stationary phase, although glucose was depleted after 17 hours of cultivation.

Preparation of inoculum in a way that basal expression of recombinant protein is blocked is crucial for stable and high expression and accumulation level of recombinant protein in production phase. It is known that high basal expression usually leads to loss of plasmid and consequently to loss of production.[5,15] Our results show that glucose supplement of 2.5 g/L into modified Luria-Bertani medium LBP/amp100 successfully represses basal expression of recombinant protein and suggests necessity of glucose addition into media for inoculum preparation when production of recombinant protein is carried out by use of the expression plasmids pET, which are very widely used due to their efficiency.

It is also important that inoculum is transferred into fresh production media before stationary growth phase is reached. Suitable optical density for inoculum transfer into fresh media is around $OD_{600nm} \approx 4.0$, when inoculum is in the mid-exponential growth phase. At this point we have not observed significant cAMP-mediated activation of *lac*UV5 even in the LBP/amp100 medium without glucose supplement.

4. Conclusions

Although pET expression plasmids have been widely used for recombinant protein production for many years, little attention has been paid to selection of suitable media for inoculum preparation and recombinant protein production. Complex media such as LB (Luria-Bertani) or TB (Terrific broth) not containing glucose are usually the first choice for cultivation of recombinant *E. coli* with the aim of recombinant protein production. Our results show necessity of glucose addition into media for production of recombinant proteins by using pET expression plasmids in the inoculum preparation phase as well as in the production phase. Choice of proper conditions, including appropriate media composition, is crucial to control behaviour of very powerful pET expression plasmids.

Catabolic repression with glucose addition is an approach, which can be also applied in the industrial production of recombinant proteins such as biopharmaceuticals. It does not require addition of additional antibiotic into growth media and does not reduce specific growth rate, which are not desired in large-scale production.

5. Acknowledgements

This article is dedicated to the memory of our dear colleague and inspiring leader Dr. Viktor Menart who unexpectedly passed away on Feb. 1st, 2007 leaving behind a lot of unfinished work. Dr. Viktor Menart pioneered recombinant DNA technology in Lek Pharmaceuticals d.d. and played important role in the development of modern Biotechnology in Slovenia. We are truly grateful for the opportunity to work with him.

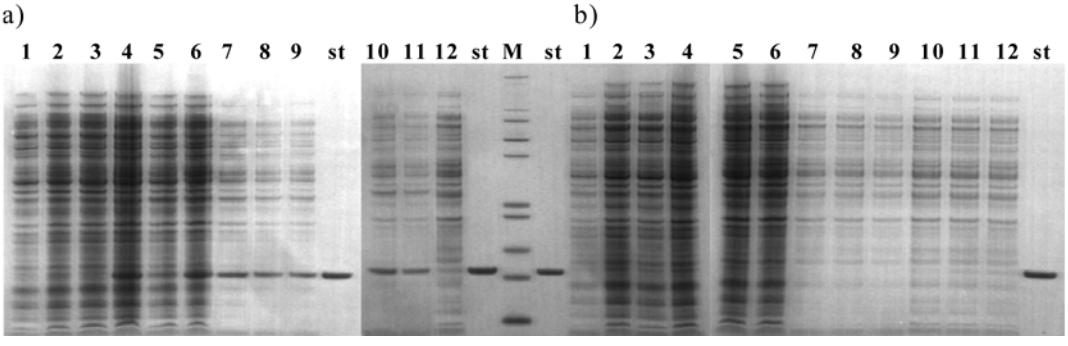


Figure 5: SDS-PAGE of inoculum cell lysates. Cells were grown in LBP/amp100 medium (a) and in LBPG/amp100 medium (b) and were harvested after 12–23 hours of cultivation.

(Lanes 1–12: inoculum after 12–23 hours of cultivation in **a)** LBP/amp100 medium, **b)** LBPG/amp100 medium; **st:** 1 μg hG-CSF standard; **M:** Mark 12 standard (Invitrogen))

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Povzetek

Ekspresijski sistemi pET proizvajalca Novagen predstavljajo najučinkovitejše orodje za pridobivanje rekombinantnih proteinov v bakteriji *E. coli* in so že dolga leta v široki uporabi. Zaradi njihove izjemne učinkovitosti, ki omogoča nastajanje velikih količin rekombinantnega proteina, jih je relativno težko kontrolirati.

V našem delu smo se osredotočili na vpliv sestave gojišča na obnašanje ekspresijskega sistema pET3a in poskušali izbrati primerno gojišče za pripravo inokuluma kot tudi primerno gojišče za proizvodnjo. Ugotovili smo, da gojišča brez glukoze sprožajo nepričakovano visoko aktivnost promotorja *lacUV5*. V gojišču LBP (modificirano gojišče Luria-Bertani), smo dobili že brez dodatka induktorja IPTG akumulacijo hG-CSF, ki ustreza približno 15 % celotnih proteinov. Dodatek glukoze v gojišče za pripravo inokuluma uspešno zavira izražanje rekombinantnega proteina med fazo priprave inokuluma. Optimalna gostota biomase za kakovosten inokulum je okrog OD_{600nm} = 4,0, ko je kultura v sredini eksponentne faze rasti. Prisotnost glukoze je potrebna tudi v produkcijskem gojišču. Gojišče GYSP, bogato z glukozo, omogoča za 25 % višjo akumulacijo rekombinantnega proteina kot LBP, ki ne vsebuje glukoze. Za razliko od LBP omogoča enako visoko akumulacijo hG-CSF tudi brez dodatka antibiotika v produkcijski fazi.