Optimum parameters for overexpression of recombinant protein from *tac* promotors on autoinducible medium

Received for publication, October 15, 2007
Accepted, November 10, 2007

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Abstract

There ORF's from the megaplasmid pAO1 (Arthrobacter nicotinovorans) were overexpressed in Escherichia coli on two different mediums using tac promoter in order to establish the best conditions for biomass and protein production. The auto-inducible medium proved to be more efficient than standard Lauria-Bertani medium, allowing the cultures to reach higher optical density while the induction level was comparable with the one obtained using IPTG. The auto-induction moment can also be modified by changing the glucose content in the medium, showing that this complex medium is a god alternative for standard mediums.

Keywords: Auto-induction, Overexpression, Recombinant protein, Tac promoter, Escherichia coli, Glucose

Abbreviation:
IPTG: isopropyl-β-D-1-thiogalactopyranoside
ORF: open reading frame
LB medium: Medium Lauria-Bertani medium
DTT: Dithiothreitol
SDS: Sodium dodecyl sulfate
OD: optical density
SDS-PAGE: SDS -poliacrylamide gel electrophoresis
IMAC: immobilized metal affinity chromatography

Introduction

The fast development of DNA-sequencing methods has led to the accumulation of a huge number of gene sequences, but only the sequence of a given gene is not enough to describe its function. In order to understand the role of a gene in the genome of an organism some more information are therefore required. This general situation can be applied also for the pAO1 megaplasmid from *Arthrobacter nicotinovorans,* which has a length of 165 KB and it was completely sequenced by IGLOI et. al. [1] (the sequence can be found in the databases with GI 25169022). The same author describes two possible metabolic pathways encoded by genes situated on this plasmid. The group led by Brandsch has managed to fully describe the pathway for nicotine degradation and to characterize the enzymes involved (BRANDSCH [2]). This pathway consists of 12 % from the total number of described ORF's from pAO1.

The function of the second metabolic pathway is just a theoretical one, deduced from the homology of the genes with the sequences of some gene, which are known to be involved in the sugars degradation. There are no other experimental evidences, which argue for this function. Nevertheless, this pathway is interesting both from a theoretical and practical point of view. First of all, the GC content of the megaplasmid shows that it has a modular structure. It seems like it has appeared from an ancestral plasmid on which the nicotine-degrading pathway was attached (IGLOI et. al. [1]). A plasmid survives only if it encodes a selective advantage for the cell, so it is interesting to find what metabolic capacities the ancestral plasmid encodes and its origin.

Secondly, in this genus were described several enzymes involved in the sugar metabolism which also have interesting industrial application, as those studied by TRIMBUR et. al. [3], ATSUSHI et. al. [4], KAZUHISA et al. [5]. This shows that indeed this genus a good source for enzymes.

Most of the genes encode proteins and therefore the characterization of these molecules is a research area that has known a fast development in the last decades. A way to investigate the role and properties of these molecules which has become more and more used is cloning and overexpression of the gene in a different host followed by purification of the recombinant protein. This is the approach that we also choose in order to identify and characterize 4 ORF's from pAO1 megaplasmid. The 4 genes are ORF 24 (25% identity with a celulase precursor from *C. thermocellum*), ORF 38 (32% identity with 2-keto-gluconate-dehidrogenase), ORF 39 (56% identity with succinic semialdehyde dehidrogenase) and ORF 40 (63% identity with a COG-oxidase) [1]. This approach is not always straightforward; problems can appear mainly because the gene is expressed in a different organism, most of the time *E. coli* modified strains. Using synthetic un-metabolizable compounds as inducer gives a high and constant level of expression. This high un-phisiological intensity of expression destabilizes the cell metabolism and the newly synthesized polypeptide chain can not be folded correctly which lead to insoluble proteins known as inclusion bodies (APPLEBAUM et al. [6]). Also, *tac* based expression systems have the disadvantage of a base expression level disregarding the presence of the inducer. If the expressed protein is toxic for the cell, even a small level of expression can be fatal, can lead to plasmid lost or to mutations accumulation. These effects were observed by Grossman et. al. (1998) cited by STUDIER [7] when using T7 system.

In this work we want to adapt and optimize an expression system for the 4 genes to the local conditions from our lab. We focused mainly on there things: elimination of IPTG as inducer, maximizing the biomass and recombinant protein production and establishing the cultivation parameters so that the culture will be ready for harvest in the morning (eliminating the need to freeze the cells over night).

Material and Methods

Strains and plasmids. *Escherichia coli* XL1Blue (Stratagene) was used both as a host for plasmids and as an expression strain. This strain is resistant to tetracycline and overexpresses the lac repressor.

All the 4 ORF's were cloned in the expression vector pH6EX3 (BERTHOLD et al. [8]), behind a polylinker sequence encoding 6 histidine residues. The resulting plasmids were pH6EX3orf24*,* pH6EX3*orf38,* pH6EX3*orf39,* and pH6EX3orf40. The ORF's were placed under the control of tac operator and the resulting polypeptide had the his tag at the N-terminal region.

Growth conditions:

The strains were grown mainly on LB medium according to SAMBROOK et al. [9] and DUNCA et. al. [10], supplemented with ampicilin 50 microg/ml.

For protein expression two different mediums were used: LB medium as described and the auto-inducible medium described by STUDIER [7] with some modifications. The auto-inducible medium was prepared before used using the following solution sterilized solutions: 9m ml 1% peptone 0.5 % yeast extract, 5 ml 1 M Na_2HPO_4, 1 M NaH_2PO_4, 0,5 M $(NH_4)_2SO_4$, 4 ml 12,5 % glycerol, 1,25 % glucose, 5% lactose and 2 ml 1M MgSO4. In this paper we will refer to this medium as ZYP medium, as the original author does (STUDIER, [7]).

A pre-culture was prepared by inoculating 40 ml of LB medium and growth for 18 hours under continuos shaking at 37^0C. 5 ml from this saturated subculture were used to inoculate 100 ml of LB or ZYP medium, and then the culture was grown under continuous shaking for approximately 24 hour. When using LB medium the induction of protein expression was done after 2 hors of growth by adding IPTG in the medium to a final concentration of 1mM. Also, after 2 hour of growth, the temperature was reduced to 30^0C for both the LB and ZYP medium.

Growth curve

At regular time points 1 ml samples were taken from the culture in triplicates. The cells were harvested by centrifugation 5 min at 13000 rpm and re-suspended in 1 ml distillated water. The optical density was determined at 600 nm using distillated water as blank on a Pharmacia LKB Ultrospec plus spectrophotometer. For each sample the mean and standard error was calculated using the Analysis Toolpack module from Excel.

Quantitative analysis of the over expression level was done using the method described by SAMBROOK, [11] and also used by HASSAN, [12], STUDIER, [7].

1 ml samples were taken at specific timepoints from the culture and the OD600 was determined as described earlier. Samples for protein analysis were centrifuged for 5 min at 13000 rpm and the cells were re-suspended in Laemli buffer (SAMBROOK et al. [9]): 50 mM DTT, 2% SDS, 0,1% bromphenol blue, 10 % glycerol. The volume of buffer was chosen in such way that 5-20 microliter buffer to contain 0,08 OD600 units. Most of the

time 30 microliters were used at the beginning of the culture up to 200 microliters at the end (after 24 hours). The samples were heated at 95^0C for 10 minutes and then kept at -20^0C until further processing.

The protein content analysis was done using SDS-PAGE on 12% gels. The gels were cast according to (SAMBROOK et al. [9]) using a miniVertigel2 (Apelex, France) module, and were 10 ml in height, 8 cm in length and 1 mm thick. In this conditions, falowing the indications of SAMBROOK et al. [9] resulted in overloaded gels.

Generally a volume of 5 up to 20 microliters (equivalent of 0,08 OD600 units) of solution was loaded on the gel using a Hamilton syringe. The gel was run at 25mA/gel until the blue dye run out and afterwards was stained using Coomasie Brillant Blue R 250 0,25% in 45% methanol, 10% acetic acid for about 30 minutes. The destining was done in 30% methanol, 10% acetic acid and the gels were kept in 10% acetic acid until photographed.

Gel densitometry and molecular weight determination

The stained gels were photographed and quantified using ImageQuant *TL* from *GE Healthcare*. We focused on expressing the amount of recombinant protein as percent from total protein. The molecular weight determination was done by running in parallel with the target protein a molecular weight protein marker from Sigma. The marker was used to construct a curve from which the molecular weight of the target protein was determined.

Results and Discussions

ZYP medium yields a better biomass production

The classical method for recombinant protein over expression in E.coli uses LB medium and growth of the induced cells for 3 to 6 hours at 30^0C (AUSUBEL et. al, [13]). This means that the cells must be harvested and then frozen till further processing, which can degrade specially multimer proteins. A simple way to eliminate the freezing step is to modify the overexpression protocol by reducing the incubation temperature and shaking speed (SAMBROOK et al. [9], (MILLARD et al., [14]), but this also means that the biomass and recombinant protein production will be smaller. STUDIER [7] propose an complex medium which maintains a culture in the growth faze for a longer period of time, increasing this way the usability interval and allowing growth and induction overnight.

The LB medium has the advantage of its simplicity, being cheap and easy to prepare. For this reasons it is now a classical medium for growing *E.coli*. But its simplicity also gives a big disadvantage: the maximum density of cells/ml it is pretty low. During the growth of the microorganisms, the nutrients from the medium are becoming scarcer and the cells are consuming the recombinant protein accumulated in excess. In the same time, in the medium toxic compounds tend to accumulate which leads to the death phase. Unlike the LB medium, the ZYP medium has a more complex composition, the glucose and glycerol being a more reliable carbon source and allowing a more massive accumulation of biomass. Table 2 shows that, disregarding the strain used, the biomass production after 24 hours was superior in ZYP medium compared to LB medium.

Table 1. Biomass production on LB and ZYP medium

Strain	LB medium		ZYP medium	
	Wet cells weight (mean, g/L)	Standard error	Wet cells weight (mean, g/L)	Standard error
pH6EX3	9,763	0,096	28,836	1,097
pH6EX3*orf38*	14,623	0,434	20,440	5,078
pH6EX3*orf39*	9,333	0,333	20,333	1,527
pH6EX3*orf40*	7,810	0,738	27,286	0,579

The numbers in table1 agree with the growth curves from figure 1. As it can be seen, using different amounts of pre-culture gave the same results: on ZYP medium the culture grows faster and reaches a greater optical density. After 24 hours of growth the optical density of the culture in LB medium was below 2, while on ZYP medium it reaches 2,5. Still, we did reached the saturation levels described by GRABSKI et al. [16] but not those described by

STUDIER, [7] on ZYP or by HASSAN et. al, [12] on LB medium. This is due to the fact that the shaker did not manage to assure the high aeration levels required by a fully saturated culture. Anyway, in the employed conditions growing the cells in ZYP medium allowed us to obtain a more rapid growth of the microorganism, a more saturated culture and more biomass. This proves that that this medium is mare economical efficient than standard LB medium.

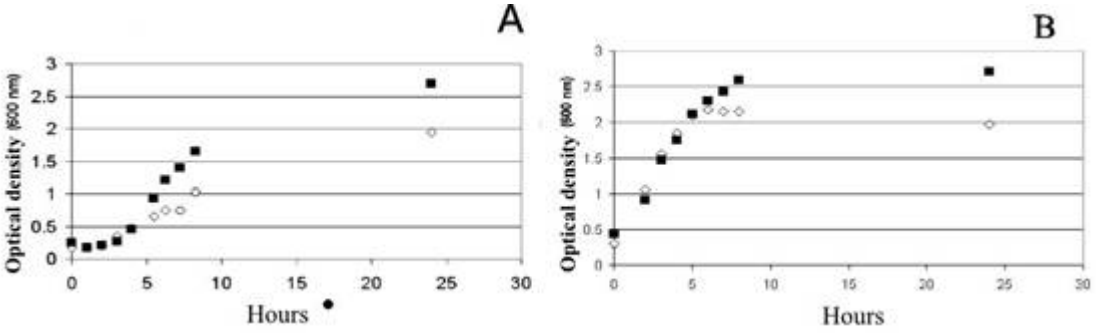


Figure 1. Growth curve of E. coli on ZYP medium. The culture was initiated using: A. 10-fold dilution of the pre-culture. B. 5-fold dilution of the pre-culture.

Also, from figure 1 panel B it can be seen that when the bacteria was grown on LB medium the death phase appears after approximately 6 hours after the culture was started with a 10 fold diluted pre-culture. On ZYP medium the growth phase continues also after 10 hours of incubation in the same conditions and after an other 14 hours the culture is in stationary phase, while the LB culture is in the death phase.

The typical phases for diauxic growth on mediums with lactose and glucose observed by ZARNEA [17] and KIMATA et. al, [18] could not be observed, probably due to the fact that the glycerol present in the medium could act as a carbon source.

The level of expression of recombinant proteins on ZYP medium is comparable with that on LB medium. The growing requirements for high amounts of purified proteins demands simplified methods for growing *E. coli* and ways to improve the recombinant protein expression levels as MILLARD et al., [14] shows. In the current paper we tried to adapt the method described by STUDIER, [7] for maximizing protein production for 3 ORF's cloned in pH6EX3 plasmid.

The most well known and used systems for protein expression are those derived from the *lac* promoter which have the advantage of being very easy to induce. Derived from the *lac* promoter is the *tac* promoter, a hybrid promoter combining the -35 region from the *trp* promoter and -10 region from the *lac* promoter. The resulting promoter is 5 times more powerful than the lac promoter (DE BOER et al., [14]) is, REECE, [19].

An third system is the so-called T7 expression system. It works by using the T7 bacteriophage RNA polymerize. A well presentation of the main promoter used to express proteins in different host as well as their advantages and disadvantages is done by MAKRIDES [18].

The *lac* derived expression system requires in the medium an inducer, mainly lactose or similar compounds. Traditionally, when grown on LB medium, protein expression is induced by using the un-metabolizable compound IPTG (Izopropy-β-D-1-thyogalactopyranosid). This method has several disadvantages, because IPTG is expensive and can not be used on industrial scale, it is highly toxic (VIITANEN et. al., [21]), and it gives a highly intense response.

To all this, there is another aspect worth mentioning. The promoter has a very low basal expression level in the absence of the inducer required in physiological conditions for constitutive expression of β-galactozid permeaze (ARTENIE, [22], and COJOCARU et al. [23]. This has unwanted effects as accumulation of mutation, plasmid lost or the impossibility of cloning the gene especially when ORF is toxic for the cell (Grossman et. all, cited by STUDIER, [7]).

The ZYP medium offers an alternative way to induce and auto-regulate the expression of *lac* derived promoters which does not have the drawbacks of IPTG. The system is based on a strict ratio of glucose/lactose ratio, which assures in the first stages of the culture development a powerful repression of the promoter and in the later stages a strong induction of expression. It has anyway a disadvantage due to the fact that the induction moment it is not strictly defined as it depends on the glucose utilization rate. The moment in which the glucose is eliminated from the medium depends on the speed that the culture grows. Also, the intensity of induction, although could be easily modified, some authors consider that it is hard to achieve on complex mediums (STUDIER, [7]). This is the reason why for every expressed gene it is required a process of trial and error does define the conditions for optimum growth, protein accumulation, and protein stability inside the cell.

Only 3 from all the 4 genes cloned could be identified as overexpressed on SDS-PAGE gels. As it can be seen in figure 2, the proteins are running in good accordance with the de theoretical molecular weight of 58.4 kDa for *orf38,* 51,2 kDa for *orf39* and 46 kDa for *orf40* (deduced from the aminoacid sequence with the application available on EXPASY servers GASTEIGER et. al. [24]).

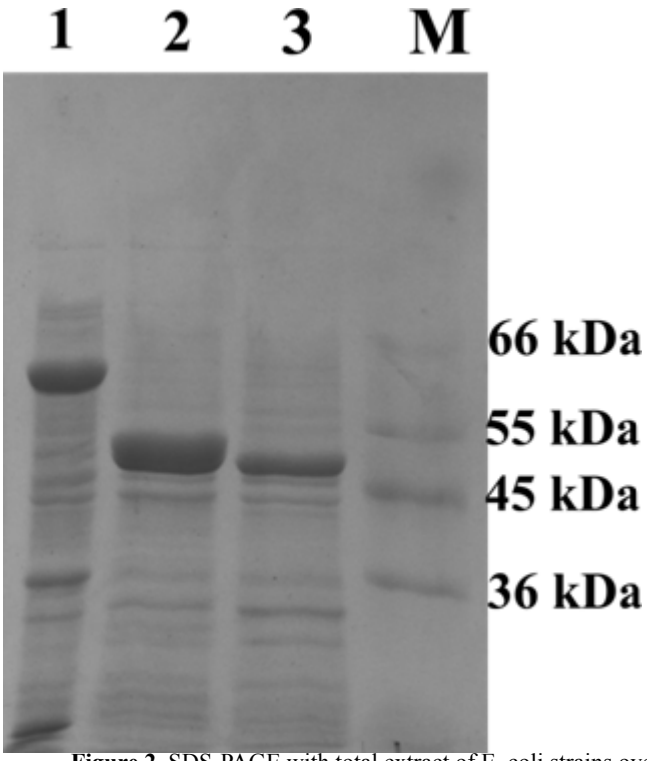
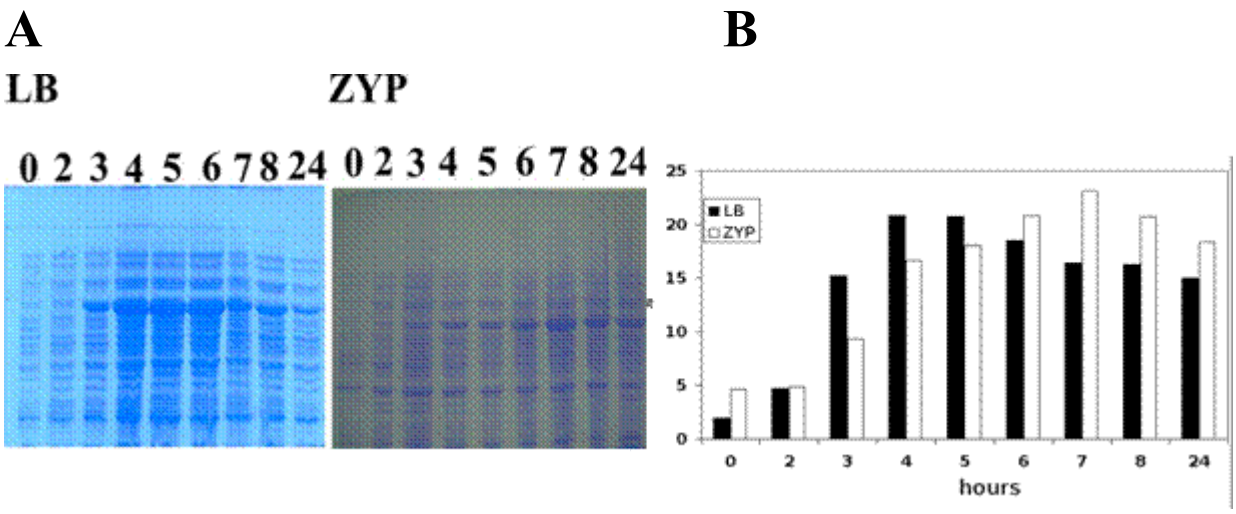


Figure 2. SDS-PAGE with total extract of E. coli strains over-expressing
1. pH6EX3*orf38*
2. pH6EX3*orf39*
3. pH6EX3*orf40*
Each sample consists of equivalent of 0,08 OD600 units. M-molecular weight markers from Sigma

The overexpression of the fourth gene, ORF24 could not be detected on both the medium, indicating that the gene could be cloned in a wrong open reading frame or an very low expression level.
The expression levels achieved in the case of ORF 38 were quite high for the both mediums. When the cells were grown on LB medium, the overexpressed protein was visible after one hour from the induction reaching 15% of the total proteins. For two hours the protein accumulate in 20 %, but then it starts to degrade, falling back to 15% after 25 hours of growth. This fast induction produces a destabilization of the cell metabolism as the protein synthesis apparatus of the microorganism is not able to process the newly synthesized polypeptide chain. The result is formation of inclusion bodies APPLEBAUM et al. [6], highly compact insoluble protein formations. This is the case for this protein, which is totally insoluble when expressed on LB medium.
On ZYP medium, the induction is less intense. The protein appears on the gel after 3 hours, reaching only 7% of total proteins and after 8 hours of induction it reaches a maximum of 23%. After this peak, the protein start to degrade and as a result after 24 hours of culture it falls to 18%. An interesting question, which we plan to address in future studies, is the solubility of the protein when the microorganism is grown in this medium. The optimum harvesting time is at least after 8 hours from the culture initialization.



C

OD600/hours	0	2	3	4	5	6	7	8	24
LB	0.298	1.056	1.551	1.842	2.092	2.178	2.148	2.149	1.969
ZYP	0.437	0.916	1.466	1.746	2.107	2.300	2.430	2.583	2.712

Figure 3. A. Electrophoretic spectrum of total protein extracts during growth of E. coli pH6EX3orf38 on LB and ZYP medium. On each lane equivalent units of OD600 of culture at indicated time points were loaded on the gel. **B**. Recombinant protein accumulation during growth. **C**. Optical density of the culture at the indicated time points (which corresponds with sampling moments).

The production and accumulation of the transnational product of ORF 39 is shown in fig. 4. It can be observed that in this case, in 2 hours after induction (4 hours of growth) the protein accumulates in 14,5 of total proteins and only one hour later the amount doubles. The induction is very strong and after 8 hours of growth the protein attends its maximum, representing 59% from total proteins, this being the best moment for harvest.

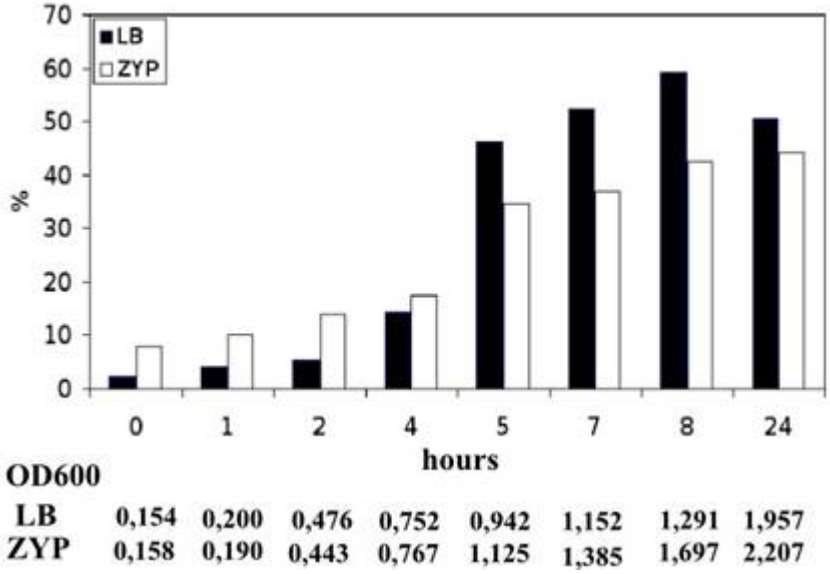
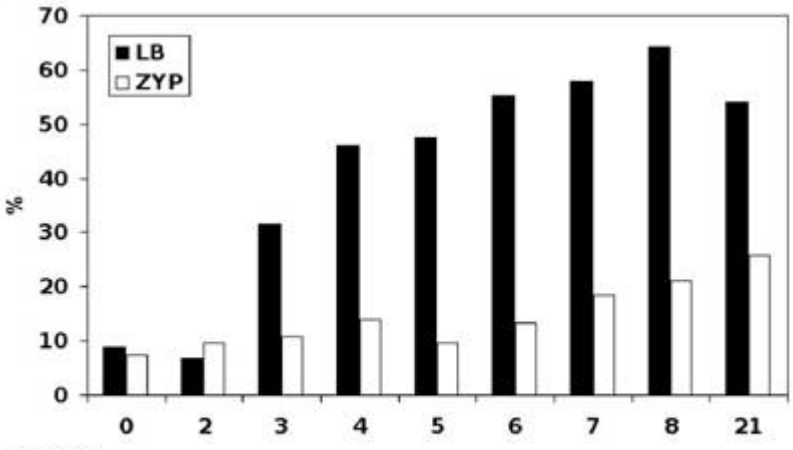


Figure 4. Production and accumulation of ORF 39 encoded protein. With black is represented the amount (%) of protein accumulated when the cells were grown on LB medium, with white on ZYP. At the bottom is the optical density of the culture in the moment when the samples were taken

On ZYP medium the induction is again less strong. The recombinant protein is visible only after 5 hours of growth and the accumulation is quite strong. After 24 hours it reaches 44,3 %. Although the expression level is smaller then on the cells are grown on LB medium it is balanced by the high amount of biomass, and the ZYP medium is a god alternative to the traditional medium.

The same behavior could be observed also when the E. coli cells were expressing the ORF 39. Immediately after induction, the translation product starts to accumulate reaching 31 % of total protein after 1 hour and 64% often 8 hours of growth. After an other 13 hours (21 hours of culture) the amount of recombinant has a small fall, sign that the culture has exhausted the nutrients from the medium and started to use the excess protein.

On the ZYP medium the autoinduction takes place much late, the protein being visible on the gel only after 7 hours shaking and it reaches only 25% of total protein in the 21 hours old culture (figure 5).



LB 0,262; 0,257;0,454;0,465; 0,551;0,718;0,788;0,884;2,074;
ZYP 0,215; 0,257;0,416;0,528; 0,797;1,183;1,413;1,596;2,471;

Figure 5. Production and accumulation of ORF 39 encoded protein. With black is represented the amount (%) of protein accumulated when the cells were grown on LB medium, with white on ZYP. At the bottom is the optical density of the culture in the moment when the samples were taken.

Changing the auto-induction moment is feasible. This low level of production and accumulation has made us to undergo some experiments to modify the moment of expression. STUDIER, [7] assumes that this is possible, but he also supposes that a precise and fine-tuning will not be possible because of the start of induction depends on several factors (strain, aeration, and aminoacid content). As we described earlier, the autoinduction is the result of combining glucose and lactose. The last one is responsible for the intensity of induction and the glucose, in an indirect way, for the moment of induction. By catabolic repression, glucose represses the tac promoter and does not allow the protein to be expressed. Being preferred over lactose by the cell, it is slowly degraded and the inhibitory effect disappears. We realized 3 variants of YZP medium, in which the glucose concentration was 0,05%; 0,025 %; 0,0125 %. The variants were inoculated from the same pre-culture and used at the same time. Under the condition used, the protein became visible on SDS-PAGE gels after 6 hours of growth on the medium containing 0,0125% glucose and after 7 hours on the others two. The protein accumulation levels were also different in the 24 hours old cultures, being 54,9 % in the 0,125 % glucose medium, 44,16% in the 0,025 % glucose medium and 25,84% in the standard 0,05 % glucose medium. Probably the moment of autoinduction on the 0,025% glucose medium is somewhere between 6 and 7 hours. This shows that reducing the amount of glucose in the medium had the desired effect, allowing the autoinduction to happen earlier and the protein to accumulate to higher levels. Modifying the autoinduction moment by altering the glucose content in the medium is a feasible method.

Conclusions

The auto-inducible medium is used to express proteins using the T7 expression system but this is the first report of successful usage of this medium to express proteins placed under the control of *tac* promoter. This medium proved to have several advantages over the standard LB medium:

1. the biomass production was superior and the level of overexpression was similar to those obtained when using LB medium. This way less culture volume is needed for the same amount of protein expressed
2. the culture is ready to be harvest early in the morning, eliminating the need to freeze the cells.

The possibility to control the moment of induction was also addressed, and we showed that by changing the glucose content in the cell, the moment of induction can be modified according to ones needs.

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