

## INFLUENCE OF CULTURE MEDIUM ON THE PRODUCTION OF EIF ANTIGEN FROM *LEISHMANIA CHAGASI* IN RECOMBINANT *ESCHERICHIA COLI*

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### ABSTRACT

With the advent of recombinant DNA technology, recombinant protein expression has become an important tool in the study of the structure, function and identification of new proteins, especially those with therapeutic functions. *Escherichia coli* has been the predominant prokaryote used in genetic engineering studies due to the abundance of information about its metabolism. Despite significant advances in molecular biology and immunology of infections, there are as yet no prophylactic drugs capable of preventing visceral leishmaniasis. It is therefore important to identify specific antigens in order to develop vaccines and diagnostic kits against this disease. The objective of this study was to evaluate the influence of culture medium on the production of eIF antigen from *Leishmania chagasi* in recombinant *Escherichia coli*. An induction procedure using IPTG was carried out in a series of trials, to observe the influence of culture medium (2xTY, TB) under expression of the recombinant eIF protein. Results showed that recombinant protein expression was associated to growth and that the highest eIF antigen expression was obtained in the 2xTY medium.

**Key words:** Cultivation, Recombinant protein; Visceral leishmaniasis, *Escherichia coli*

### INTRODUCTION

*Escherichia coli* is the most commonly used host strain for the expression of heterologous proteins and biocatalysts (12, 18). These recombinant proteins are usually synthesized intracellularly in either the cytoplasm or the periplasmic space (2), and overall productivity is a function of both cell density

and specific yield. Proteins such as interferons, interleukin and growth hormones are some of the recombinant proteins successfully expressed in *E. coli* (4). However, *E. coli* has a number of limitations in its expression system, including inability to carry out post-translational changes, common in eukaryotic cells, lack of secretion system for efficient release of the recombinant protein into the culture medium (1). Protein

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expression in cytoplasm often leads to inadequate protein structure and agglutination in insoluble inclusion bodies (16).

With the advent of recombinant DNA technology, recombinant protein expression has become an important tool in the study of the structure, function and identification of new proteins, especially those with therapeutic functions, allowing for the manufacture of drugs capable of controlling particular diseases (3, 14).

Leishmaniasis are illnesses caused by protozoa of the genus *Leishmania*, which, depending on the species, may produce cutaneous, mucocutaneous, diffuse or visceral manifestations (13). American visceral leishmaniasis or kala-azar is the most severe form of this disease. Untreated visceral leishmaniasis results in death within two years of initial infection (5). Despite significant advances in molecular biology and infection immunology, there are as yet no prophylactic drugs capable of preventing this disease, justifying the need to identify specific antigens, in order to develop vaccines and diagnostic kits against visceral leishmaniasis (11).

Thus, the objective of this study was to evaluate the influence of culture medium on the production of eIF antigen from *Leishmania chagasi* in recombinant *Escherichia coli* during batch fermentation using two different media (2xTY and TB).

## MATERIAL AND METHODS

### *Escherichia coli* strain

The *Escherichia coli* M15 strain with the eIF antigen used in this study was kindly donated by Dr. Mary Wilson (University of Iowa, U.S.A). The gene encoding the eIF antigen was expressed as a fusion protein containing histidine tag at the N-terminal end of the peptide using pQE-40 cloned in *E. coli* M15 cells (Qiagen, U.S.A). The strain was maintained on Luria-Bertani (LB) medium in the presence of ampicillin and kanamycin, as described elsewhere (16).

### Culture medium

Culture media were prepared with distilled water and

sterilized at 120°C for 20 minutes, while antibiotics and IPTG solutions were sterilized by filtration through a 0.22 µm membrane in aseptic conditions. After sterilization, solutions were stored at -20°C. 2xTY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) and Terrific broth (TB) complex cultivation medium (12 g/L tryptone, 24 g/L yeast extract, 0.004 mL/L glycerol, 12.54 g/L KH<sub>2</sub>PO<sub>4</sub>, 15 g/L K<sub>2</sub>HPO<sub>4</sub>, pH 7.0) were prepared according to Jordan *et al.* (7). For all experiments, media were used for batch cultivations in shake flasks supplemented with 0.1 g/L ampicillin (Invitrogen, Brazil) and 0.025 g/L kanamycin (Invitrogen, Brazil).

### Inoculum preparation

The stock of *E. coli* strain containing the eIF antigen was stored at -80°C in 50% glycerol. Two hundred microliters of stock was transferred to 50 mL of previously sterilized 2xTY and TB medium supplemented with 0.1 g/L ampicillin and 0.025 g/L kanamycin, using 250 mL Erlenmeyer flasks. Samples were kept in the shaker (37°C, 200 rpm) overnight. This suspension consisted of the initial inoculum batch cultivation carried out in a shaker flask.

### Cultivation conditions

The cultivation inoculum (10% v/v) was transferred to 250 mL Erlenmeyer flasks and kept under agitation at 200 rpm for 8 hours at 37° C. To understand the IPTG induction effect on growth and eIF antigen expression, cultures were induced by the addition of a final concentration of 1mM IPTG when optical density (OD<sub>590nm</sub>) reached 0.5 (early log phase) (20). Samples were taken hourly from the shaker in which cultivated cells were harvested by centrifugation (Eppendorf centrifuge 5415D) at 16,100 G for 30 min. The precipitate was used to determine dry weight at 80°C until constant weight was achieved. The supernatant was used to determine protein concentration by the Lowry method (10). All assays were performed in duplicate.

### Recombinant protein purification

The encoding nucleotide sequence of the protein of

interest was inserted into the pQE-40 vector (Qiagen, U.S.A). The recombinant protein obtained is tagged to a histidine tail with high affinity for nickel ions. Therefore, batch mode purification by immobilized metal affinity chromatography (IMAC) used Nickel Sepharose 6 Fast Flow resin (Ge Healthcare, Brazil).

The purification process was as follows: 5 mL of the fermentation broth was centrifuged at 16,100 G for 30 minutes. The supernatant and cells were separated and stored at -20°C for further purification. For extracellular protein purification, samples were used directly, according to the following process. Intracellular proteins were first lysed with urea lysis buffer to release inclusion bodies (10 mM imidazole, 8 M urea, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, pH 8.0). During the lysis process, cells were put into an ice bath for 15 minutes, resuspended in lysis buffer and homogenized for 15-60 minutes. Cellular lysate was centrifuged at 16,100 G for 30 minutes at ambient temperature. The supernatant was used for purification processing. In this case, after washing, 1 mL of elution buffer (500 mM imidazole, 50 mM NaH<sub>2</sub>PO<sub>4</sub> 0.5 M NaCl, pH 7.4) was added and homogenized for 5 minutes. The resin was settled by means of centrifugation at 16,100 G for 5 minutes. The supernatant was removed and the process repeated four times to achieve complete desorption. The eluted product was used for quantitative (Lowry method) and qualitative (electrophoresis) analysis of the recombinant protein.

#### Protein determination and electrophoresis

Eluted fractions were collected and protein concentration was determined according to the Lowry method, using bovine serum albumin (BSA) as standard (8). Protein expression, assayed by electrophoresis in denaturing 15% polyacrylamide gel, was performed as described by Laemmli (9). A low molecular weight SDS calibration kit containing proteins (14.4 - 97.0 kDa) was used to provide standard molecular weight markers. Proteins were visualized by silver staining (17).

## RESULTS AND DISCUSSION

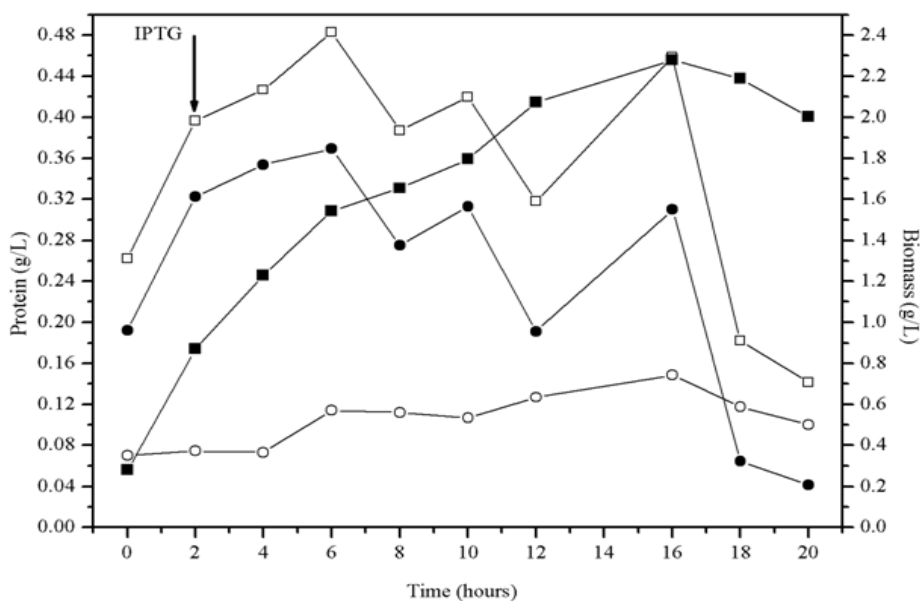
Experiments were carried out using IPTG as inducer in order to observe the influence of medium composition on the expression of the *Leishmania chagasi* eIF antigen. Figure 1 shows intracellular eIF protein, extracellular and total protein concentration (intra and extracellular) when 2xTY medium was used. It can be observed that eIF protein production was associated to growth behavior, with a maximum specific protein yield ( $Y_{p/x}$ ) of 0.37 g/g occurring after 6 h of cultivation. This corresponded to the maximum eIF protein fraction that cells could accumulate before initiating lysis. It was observed that the highest expression occurred after 6 hours of cultivation, with a protein content of 0.11 g/L, while maximum intracellular protein concentration was 0.37 g/L. In terms of location (intracellular or extracellular), it can be observed that cells retain most of their protein during the exponential growth phase. After this phase, a decrease in both intracellular and extracellular protein occurred. This may be due to cellular lysis resulting from protein saturation in the cell.

Figure 2 shows that maximum eIF concentration in TB medium was 0.35 g/L of total protein. The eIF protein expression demonstrated associated growth behavior, with maximum specific protein yield ( $Y_{p/x}$ ) of 0.095 g/g after 16 hours of cultivation. It can be observed that the highest intracellular expression occurred at 8 hours of cultivation. Additionally, cells retain most of their protein with an intracellular concentration of 0.15 g/L.

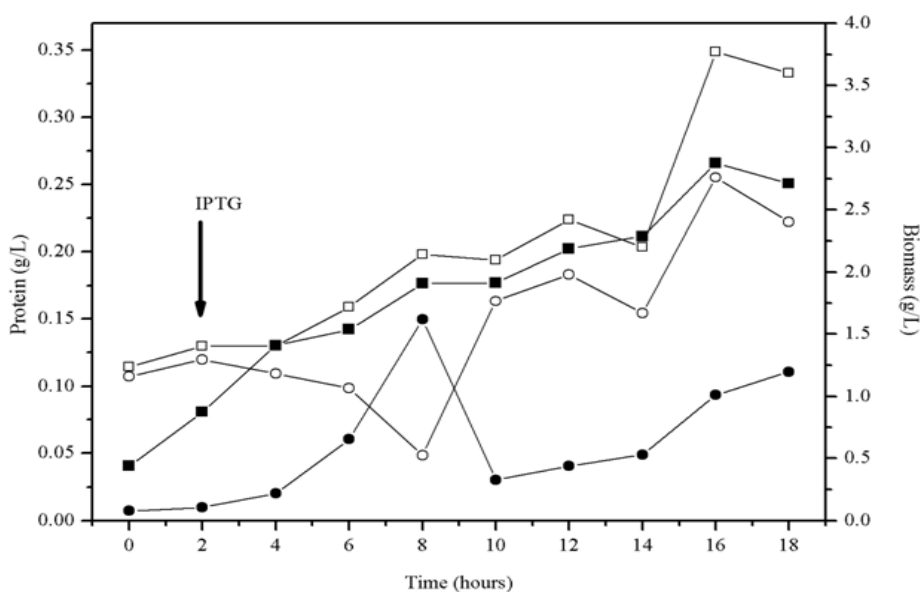
The influence of specific growth rate on eIF antigen expression was investigated. Figures 3 and 4 show the specific growth rate and protein concentration obtained during production of the eIF antigen in 2xTY and TB medium, respectively. It can be observed that eIF recombinant protein production is associated with reduction in specific growth rate of the culture during the post- induction period. The specific

growth rate results in both media are quite similar to those obtained by Panda *et al.* (15). Addition of amino acids in the presence of high tryptone concentration, as occurred in 2xTY medium, may reduce product degradation by proteases induced during amino acid limitation. Likewise, addition of casamino

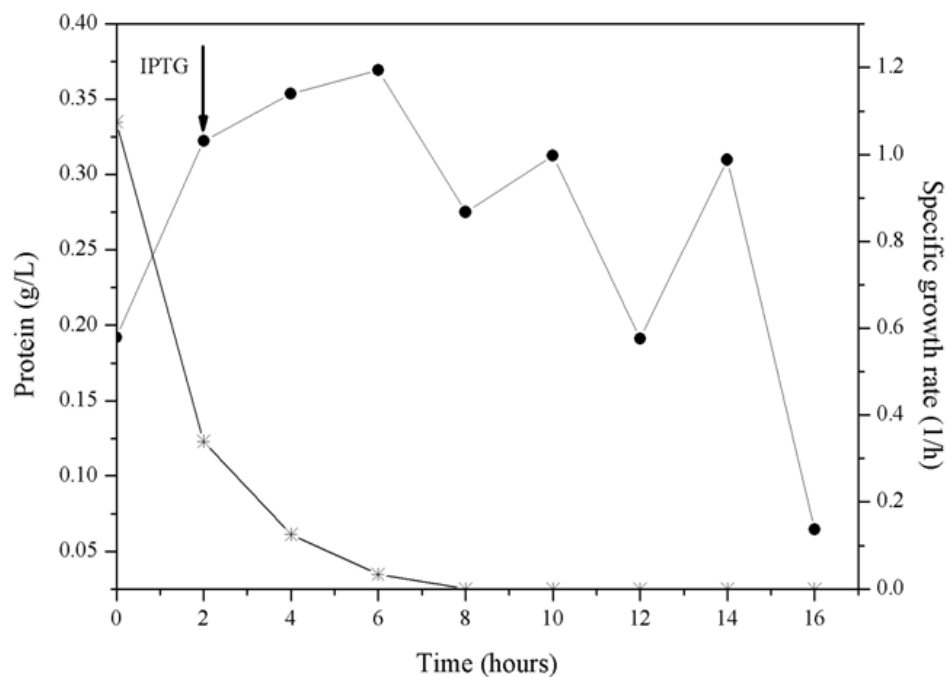
acids or peptone may enhance recombinant protein stability or synthesis, resulting in high protein levels compared to TB medium rich in yeast extract, which supplies bacteria with nitrogen and organic and inorganic nutrients, favoring only cell growth (6).



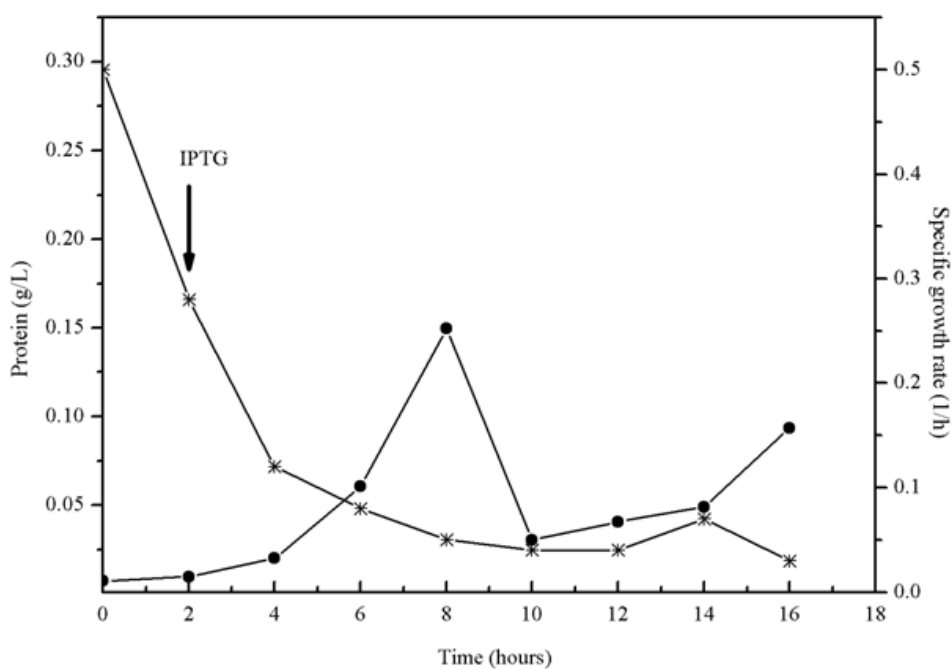
**Figure 1.** Curves of IPTG-induced eIF protein production in 2xTY: Intracellular (●), Extracellular (○), Total Protein (□) and Biomass (■).



**Figure 2.** Curves of IPTG-induced eIF protein production in TB medium: Intracellular (●), Extracellular (○), Total Protein (□) and Biomass (■).



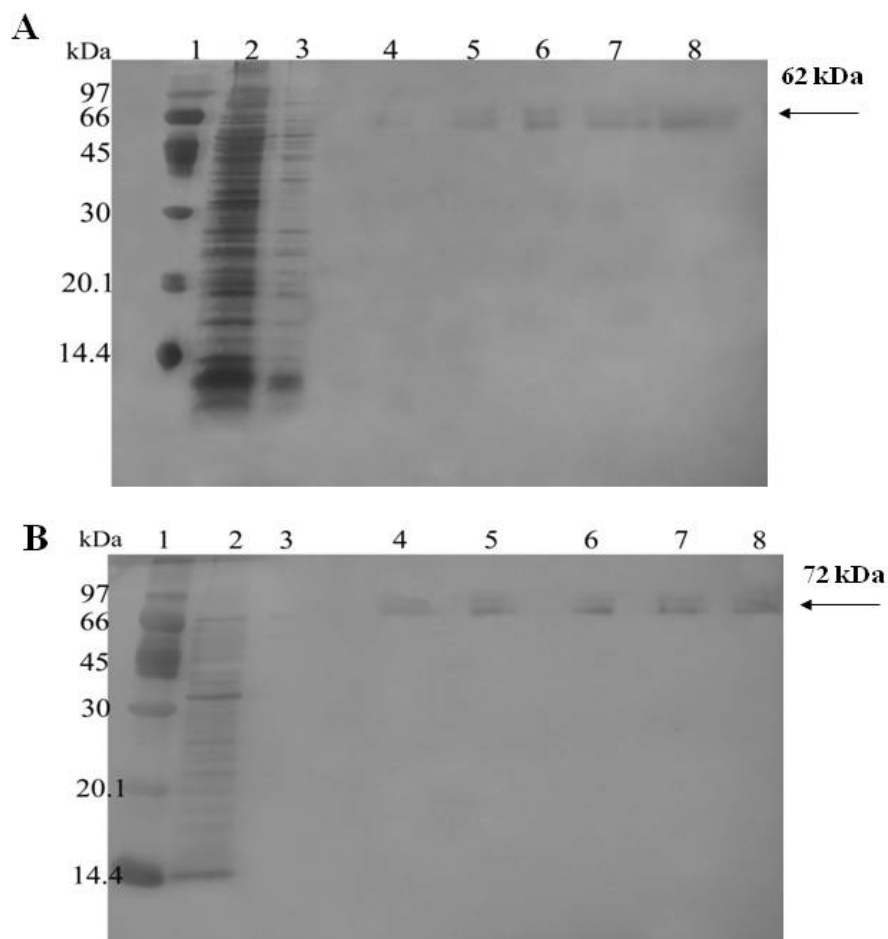
**Figure 3.** Curves of IPTG-induced intracellular production of the eIF recombinant protein (●) and specific growth rate (\*) in 2xTY medium.



**Figure 4.** Curves of IPTG-induced intracellular production of the eIF recombinant protein (●) and specific growth rate (\*) in TB medium.

Figure 5A shows the eIF antigen as a protein band during the second elution (line 4-8) with a molecular mass of approximately 62 kDa. In this case, extracellular protein concentration in 2xTY medium was 0.37 g/L. Figure 5B depicts the eIF antigen as a protein band with a molecular mass of approximately 72 kDa. In this case, extracellular protein concentration in TB medium was 0.15 g/L. The difference in molecular mass of the same protein in different cultivation media could be a result of different medium composition. The increase in eIF molecular weight in the TB medium may be due to the presence of amino acids in particular histidine (19). According to Kubar and Fragaki (8), the difference in molecular weight with different cultivation media is due to the association between the

protein of interest and a number of components present in the medium. Moreover, it can be observed that fusion of the eIF protein to the histidine tail does not lead to post-translational processing by *E. coli* (19). In conclusion, it was found that eIF antigen production is associated to growth, with maximum expression 4 hours after culture induction in 2xTY medium. High protein accumulation was also observed during the exponential growth phase. Recombinant protein production in *E. coli* in 2xTY medium was considerably higher than in TB medium due to the addition of amino acids and high tryptone concentration. This may reduce product degradation by proteases induced during amino acid limitation.



**Figure 5.** SDS-PAGE analysis of IPTG-induced eIF recombinant protein expression in 2xTY medium (A) and TB medium (B). Lane 1: protein molecular weight standards; Lane 2: total proteins; Lane 3: proteins not binding to the resin; Lane 4-8: eluted protein.

## NOMENCLATURE

|                     |   |
|---------------------|---|
| IPTG                | Isopropyl- $\beta$ -D thiogalactopyranoside                 |
| kDa                 | quilo daltons (1000 daltons)                                |
| $X_{\max}$          | Maximum cell concentration ( $\text{g}\cdot\text{L}^{-1}$ ) |
| $\mu_{\text{xmax}}$ | Maximum specific growth rate ( $\text{h}^{-1}$ )            |
| $Y_{\text{p/x}}$    | Specific cell protein yield ( $\text{g/g}$ )                |

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