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Original Article

The Influence of Different Culture Media on the Growth and Recombinant Protein Production of Iranian Lizard Leishmania Promastigote

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Abstract

Background: *Leishmania* is a eukaryotic protozoan parasite belonging to the Trypanosomatidae family. The Iranian Lizard *Leishmania* (I.L.L.), which is non-pathogenic to mammals, shows great promise to be used as an expression system for recombinant protein production. Unlike other *Leishmania* strains, the ideal culture medium for I.L.L. has not been established, although it is commonly cultured in the $RPMI_{1640}$ medium.

Methods: We investigated the growth rate of the wild and recombinant I.L.L. in BHI, $RPMI_{1640}$, LB, and M199 media with and without FBS, hemin, or lyophilized rabbit serum. Subsequently, the expression rate of the recombinant protein in these media was compared.

Results: The growth rate of I.L.L. in $RPMI_{1640}$ medium and LB broth was similar and supplementation with 10% FBS did not affect the growth rate. The amount of protein expression in the LB medium was higher than in the other three media.

Conclusion: The LB broth is an appropriate medium for I.L.L. culture and recombinant protein production.

Introduction

Leishmania is a eukaryotic genus in the Trypanosomatidae family. Most species of *Leishmania* are not pathogenic to humans. However, a few species of this protozoan parasite are responsible for leishmaniasis. *L. tarentolae* is a nonpathogenic species and is used to produce recombinant proteins (1). Its post-translational modifications (PTM), and glycosylation patterns are similar to those of mammalian cells (2). Unlike mammalian cells, *Leishmania* has a higher growth rate and lower risk of contamination (3). Among the various *Leishmania* strains, *L. tarentolae* is widely used for the production of recombinant proteins. However, the yield is only up to 30 mg/L, which is the major limitation in its use as a factory for the production of recombinant proteins (3). *Iranian Lizard Leishmania* (I.L.L.) is a newly isolated strain of *Leishmania* that is non-pathogenic. I.L.L. has the potential to produce recombinant proteins as well (4). Our team has already produced several recombinant proteins using this strain (5-7).

Leishmania has a complex life cycle. It grows as a promastigote form (flagellar which replicates extracellularly) in the sandfly and as an amastigote form in the mammalian host (8). Unlike the amastigote form, the promastigote form is easy to handle and can be cultured in a simple medium. Therefore, the promastigote form is used for the production of recombinant proteins (9). Different media such as Brain Heart Infusion (BHI), Roswell Park Memorial Institute ($RPMI_{1640}$), Luria–Bertani (LB) broth, and Medium 199 (M199) are used for culturing *Leishmania* (10-12). To obtain better results, these media are sometimes supplemented with fetal bovine serum (FBS), lyophilized rabbit serum, or hemin (2). Although *Leishmania* grows well in these media, media supplemented with animal substances are at risk of contamination with viruses, prion proteins, or other proteins present in these substances which increase the cost of purification or cause immune responses (13, 14).

The cost of culturing *Leishmania* is supposedly lower than other eukaryotic hosts (15), but still, the same medium ($RPMI_{1640}$) is typically used in both cultures, which increases the final cost of recombinant protein production. Each *Leishmania* species has different suitable liquid media. For example, *L. tarentolae* is successfully cultured in hemin-enriched BHI, which avoids serum contamination and reduces the cost of recombinant protein production (16). Most often, *Leishmania* is cultured in $RPMI_{1640}$ enriched with FBS. FBS is the most expensive part of this medium and increases the cost of production. Recently, Nasiri et al. (17) introduced the use of lyophilized rabbit serum in culture media instead of FBS. Lyophilized rabbit serum is less expensive than FBS and Nasiri et al. have successfully used it for the cultivation of L. *major* (17).

Several culture media, such as the LB broth, are less expensive than $RPMI_{1640}$ and the use of these culture media can reduce the cost of *Leishmania* cultivation. This means that *Leishmania* can be cultured at almost the cost of a bacterial culture medium and recombinant proteins can be produced at a low cost. To date, there is no report of culturing I.L.L. in a culture medium other than $RPMI_{1640}$. The production rate of recombinant proteins in the LB medium is also important and needs to be investigated.

We aimed to analyze different culture media for the cultivation of I.L.L. as a host for recombinant protein production. We also tried to determine the growth rate and recombinant protein production rate of I.L.L. in these culture media.

Materials and Methods

Leishmania species

The I.L.L. is of the non-pathogenic species belonging to the genus *Leishmania* and subgenus L. (Sauroleishmania), used in this experiment was isolated from *Agama agilis*, an Iranian lizard in Varamin Province, by Kazemi et al. (18) and transfected with a plasmid encoding a ~150 kDa secretory protein by Jalali et al. (19). The study was conducted in the Cellular & Molecular Biology Research Center of Shahid Beheshti University of Medical Sciences in 2020, Tehran, Iran

Culture media

The following seven conventional culture media were used in this study: Medium 199 (M199) (Gibco BRL, France), $RPMI_{1640}$ medium (RPMI) (Gibco BRL, France), Luria-Bertani broth (LB) (Miller Luria Bertani broth, HiMEDIA, INDIA) (hereinafter referred to as the LB broth), BHI (Sigma Aldrich, USA), Luria-Bertani medium prepared according to the Sambrook & Russell protocol (1 g peptone, 0.5 g yeast extract and 1 g NaCl were suspended in 100 ml distilled water), Novy-MacNeal-Nicolle medium (NNN) (prepared from 1.4% agar (Merck) and 0.6% NaCl (Merck) suspended in 100 ml distilled water and after autoclaved, 31% defibrinated rabbit blood was added to the prepared solution (20), and BRL which was previously introduced by Jalali et al. (LB (40%), BHI (40%), $RPMI_{1640}$ (20%)) (21). All these media were used without other supporting substrates or supplemented with different concentrations of FBS (Gibco BRL, France), and lyophilized rabbit serum (Razi Vaccine and Serum Research Institute, Islamic Republic of Iran), or defined concentrations of hemin (5 mg/l). To avoid bacterial contamination, penicillin 50 IU/ml and streptomycin 50 µg/ml were added to each medium.

Growth curves

The 20 µl samples from each culture flask were collected and mixed with the same volume of phosphate-buffered saline (PBS) containing 0.2% glutaraldehyde. The I.L.L. densities were determined at daily intervals by counting the fixed I.L.L. in a Neubauer chamber under a light microscope for ten consecutive days unless the number of viable cells reached almost zero. in addition, to confirm the counts in the Neubauer chamber, optical density was measured at the wavelength of 600 nm (OD600) once daily for 10 days. Cell viability was determined daily using erythrosine B staining (Merck, Germany) to distinguish between live cells, which exclude the dye, and dead cells, which absorb the dye (incubated for 15 min on ice with precooled 0.4% erythrosine B staining solution at a 1:1 ratio).

Continuous cultivation of promastigotes

Promastigotes of I.L.L. in the mid-log phase, previously cultured in NNN medium, were concentrated by centrifugation at 2,500 g for 10 min and washed twice with sterile PBS. I.L.L. was counted using a Neubauer chamber (hemocytometer) and diluted in another medium to a final concentration of 6.8×10^6 parasites per milliliter. Each medium was prepared in triplicate and incubated at 24-26°C. The growth curve of I.L.L. in four main media was generated using GraphPad Prism 9.0.0 (GraphPad software).

Protein expression analysis

Transfected and non-transfected *Leishmania* were cultured in serum-free media for 48 h. A protein secreted by *Leishmania* was precipitated with ice-cold trichloroacetic acid following the protocol mentioned in our previous work (21) and resuspended in an SDS-PAGE loading buffer. The sample was run on a 10% SDS PAGE gel. Densitometric measurement of protein yield was performed using Im-

ageJ.1.8.0_172 software, and yield was calculated using a standard curve.

Statistical analysis

GraphPad Prism 9.0.0 (GraphPad Software) was used to calculate the data. The student's *t*-test was used to evaluate the differences between groups. $P< 0.05$ was accepted as statistically significant.

Results

I.L.L. grew at similar rates in LB broth and $RPMI_{1640}$ medium, and the difference between growth rates was not significant ($P = 0.75$). However, I.L.L. grew slightly faster in the M199 medium than in other media, although the difference was not significant ($P = 0.13$). I.L.L. in BHI had a lower maximum cell density than the other three media, but again the difference was not significant ($P = 0.39$) (Fig.1).

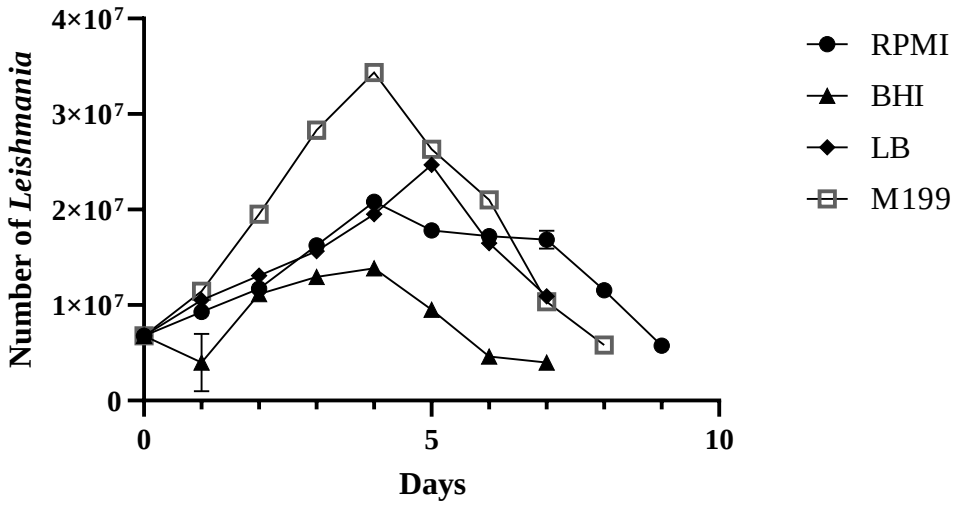

Fig. 1: The proliferation of I.L.L. in different culture media

I.L.L. in the BRL medium reached a stationary phase at a lower parasite density than I.L.L. in the LB broth; however, the difference was not significant ($P = 0.36$). I.L.L. grew at similar rates in the agitated and static LB broths ($P = 0.76$). In addition, we compared the growth rate of I.L.L. in the ready-to-use LB broth with LB, which was prepared according to the Sambrook protocol, and the difference was not significant ($P = 0.30$) (Fig. 2). Normally, 10% FBS improves the growth rate of *Leishmania*, but 10% FBS did not influence the growth rate of I.L.L. and more than 10% FBS was needed to improve the growth rate of I.L.L. in LB broth and $RPMI_{1640}$ medium. There was no significant difference between the growth rate of LB broth without supporting substrates and LB broth supplemented with 10% FBS (P-value= 0.89). Besides, the LB broth supplemented with both hemin and 10% FBS could influence the growth rate of I.L.L.

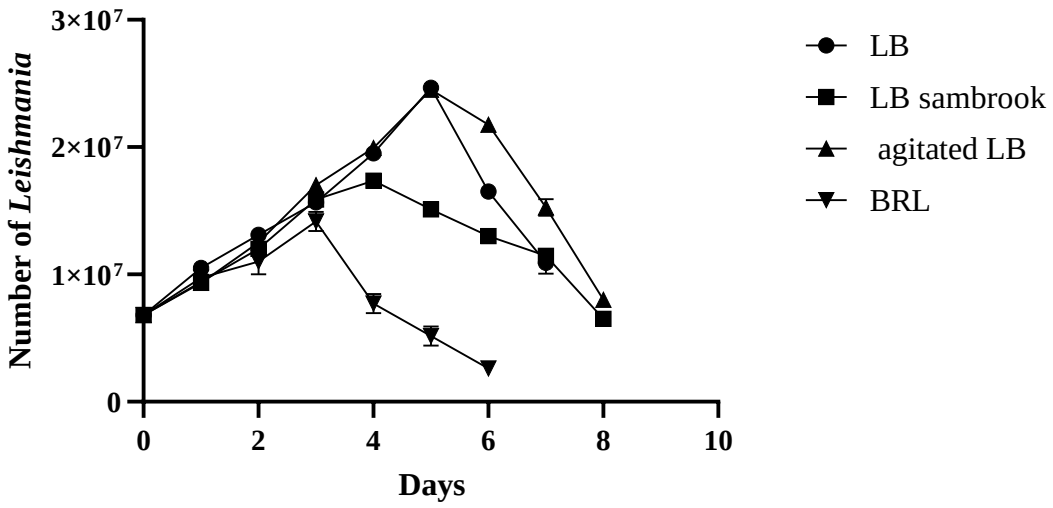

Fig. 2: Growth profile of cultured I.L.L. under different conditions for LB broth and for BRL medium

However, the difference was not significant compared to the LB broth without supporting substrates (P = 0.75). Supplementation with hemin and 10% FBS could not improve the parasite density of I.L.L. in the BHI medium, and their growth rate compared with the growth rate of I.L.L. in the BHI medium without supporting substrates was not significant (P = 0.77) (Fig.3).

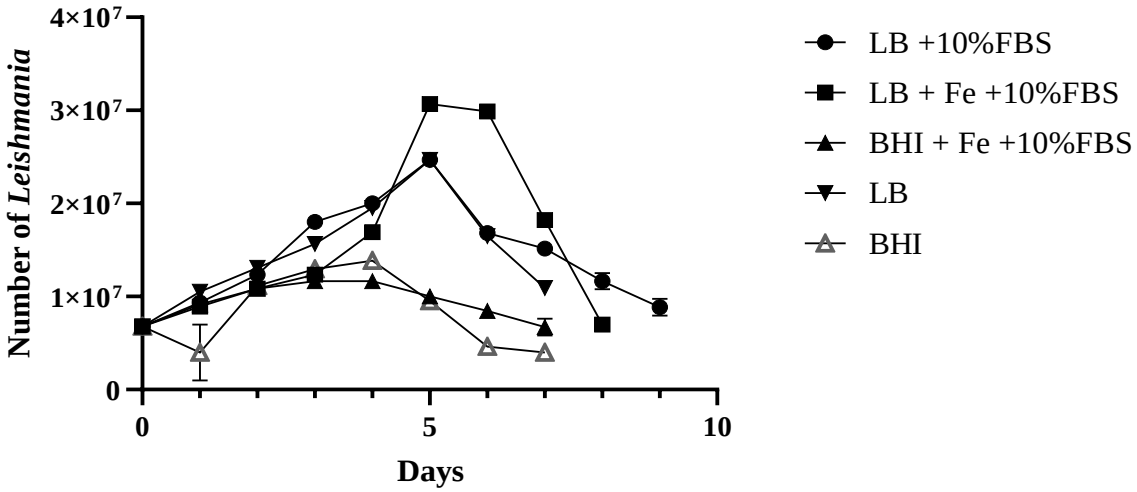

Fig. 3: I.L.L. growth curves in media supplemented with different substrates

We further investigated the proliferation capacity of I.L.L. in four main media supplemented with 10% FBS. The addition of 10% FBS improved parasite density in M199 and BHI media; however, the difference in growth rate from media without supporting substrates was not significant (P = 0.23 and 0.14 respectively). Addition of 10% FBS did not change the parasite density in LB and $RPMI_{1640}$ media (P = 0.89 and 0.93 respectively). LB broth, $RPMI_{1640,}$ and BHI supplemented with 10% FBS showed almost overlapping growth curves (Fig. 4).

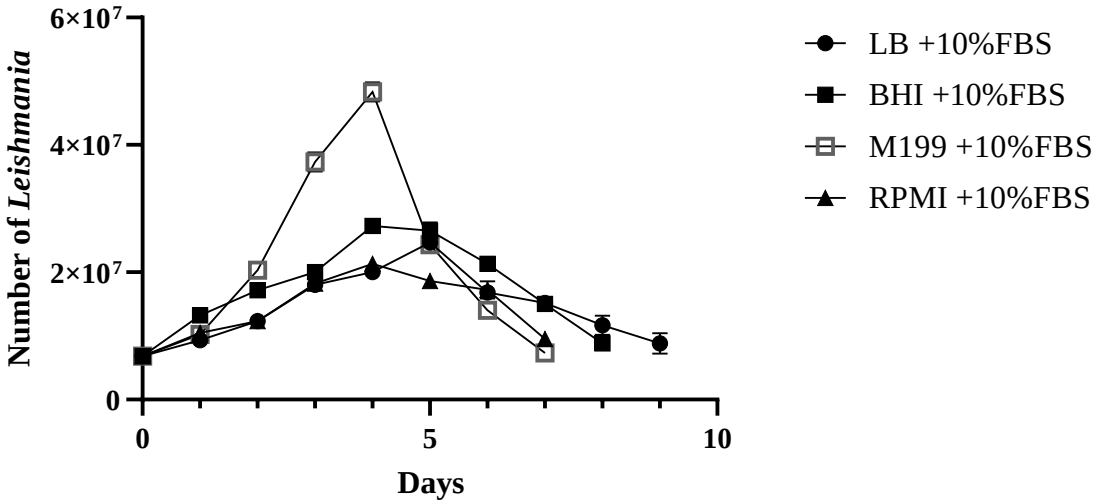

Fig. 4: The proliferation of I.L.L. in 4 media supplemented with FBS

Fig. 5 shows the growth curve of I.L.L. cultured in LB broth supplemented with 5-10% lyophilized rabbit serum alongside LB supplemented with 10% FBS. These cultures had a similar growth curve; however, increasing the lyophilized rabbit serum slightly improved the parasite density and growth rate of I.L.L., but these differences were not significant (P = 0.13 and 0.08 respectively).

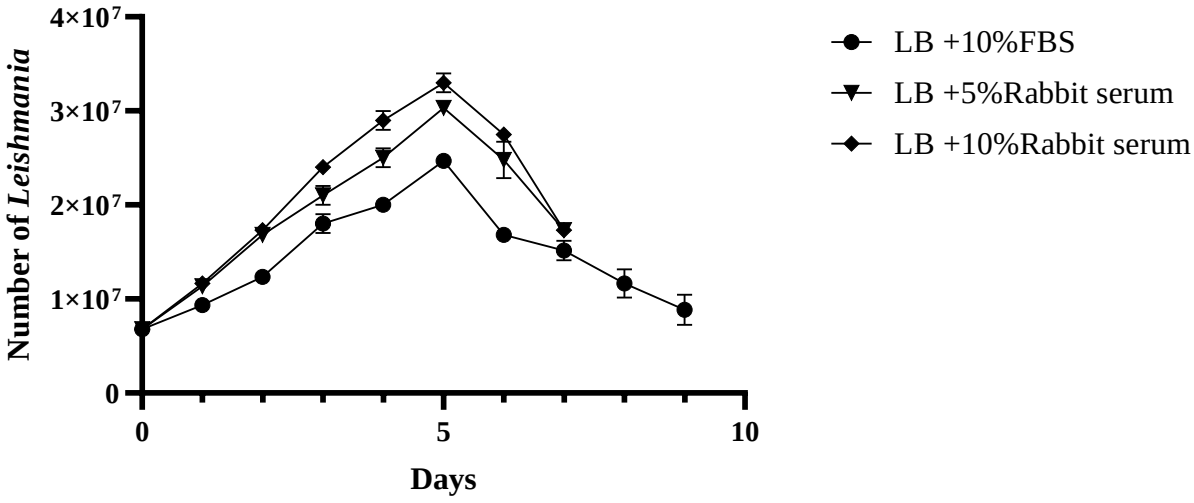

Fig. 5: Growth curves of I.L.L. in LB broth containing 10% FBS or 5-10% lyophilized rabbit serum

Finally, we tried to demonstrate the effect of more than 10% FBS on the growth rate of I.L.L., because 10% FBS did not change the growth rate of I.L.L. in LB broth and $RPMI_{1640}$ medium. We found that increasing FBS by 10% stimulated the growth rate of I.L.L. in the aforementioned media. LB supplemented with 30% FBS showed no significant difference in growth rate with LB broth without supporting substrates; however, LB

supplemented with 50% FBS had a significant influence on growth rate with LB broth without supporting substrates (P = 0.13 and 0.01 respectively) (Fig.6.a). Both the RPMI media supplemented with 30% and 50% FBS had a significant impact on the growth rate with RPMI media without supporting substrates (P = 0.03 and 0.01 respectively) (Fig.6.b).

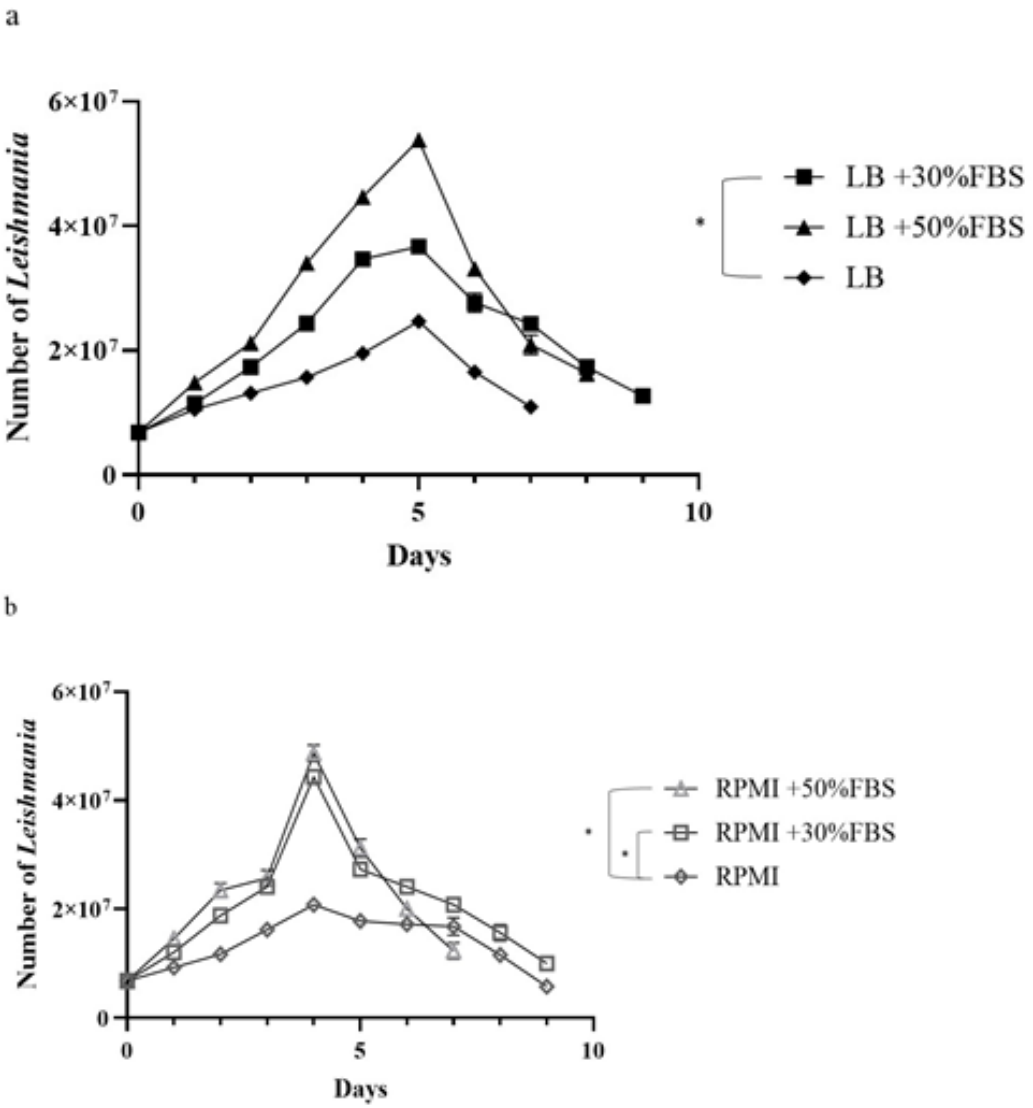

Fig. 6: Growth curve of I.L.L. in; a. LB broth and; b. $RPMI_{1640}$ medium enriched with 30-50% FBS (* for $P < 0.05$)

Subsequently, we tried to determine the expression level of ~150.0 kDa anti-IL-2Rα in the four aforementioned basic media in SDS-PAGE gel. We analyzed the amount of protein expressed in these media. To avoid the growth rate of each medium changing the amount of protein expressed, we cultured I.L.L. in one medium, scaled it up, centrifuged and precipitated it, and then cultured it in each medium. After 48 h, the protein was extracted and analyzed (Fig. 7).

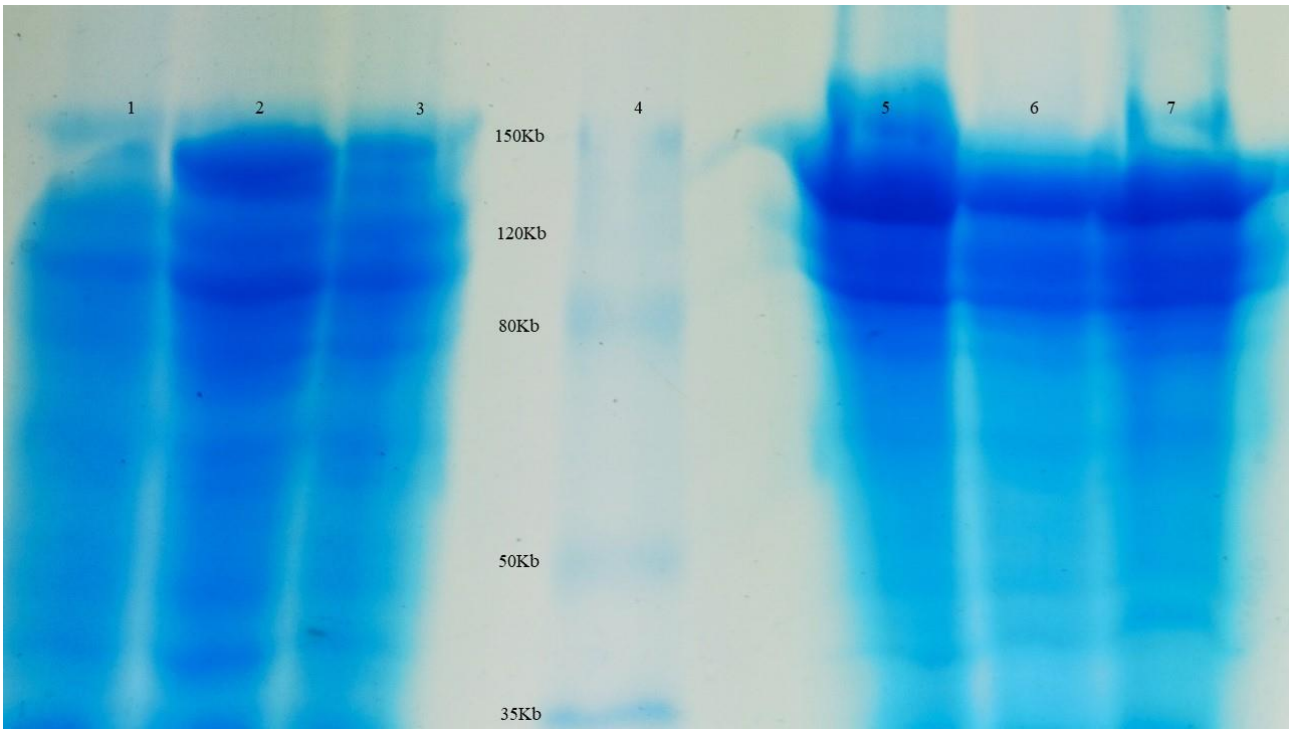

Fig. 7: The SDS PAGE indicated the presence of the recombinant ~150 kDa anti-IL-2Rα antibody in different media. Lane 1, wild type of I.L.L. in LB broth; lane 2, recombinant anti-IL-2Rα antibody in BHI broth; lane 3, wild type of I.L.L. in BHI broth; lane 4, protein size marker (kDa); lane 5, recombinant anti-IL-2Rα antibody in LB broth; lane 6, recombinant anti-IL-2Rα antibody in m199 medium; and lane 7, recombinant anti-IL-2Rα antibody in RPMI medium

The protein yield in BHI, LB, m199, and RPMI media was compared using ImageJ.1.8.0_172 software and the result showed that the I.L.L. cultured in LB broth expressed a slightly higher amount of the recombinant protein compared with the I.L.L. cultured in M199. We also demonstrated that the I.L.L. cultured in the M199 medium expressed more protein than the I.L.L. cultured in the RPMI medium. Meanwhile, the I.L.L. cultured in RPMI and BHI broth produced almost the same amount of protein.

Discussion

Large-scale and low-cost production methods are required for the production of recombinant proteins (22). The use of prokaryotic expression systems to produce recombinant proteins is very affordable, but the production of recombinant proteins with correct PTM and proper folding in a prokaryotic system remains a major challenge (23). Although mammalian expression systems such as the CHO cell lines can be used to overcome the aforementioned challenges, the expression of recombinant proteins in these systems increases the cost and risk of contamination with animal viruses and decreases the speed and yield of recombinant protein production (24). *Leishmania*, the newly introduced host, can be used as an expression system to produce recombinant proteins with correct PTM and folding. *Leishmania* has several advantages, for example, it grows faster than mammalian cells, no expensive equipment is needed, and the risk of contamination is lower (25). However, *Leishmania* is usually cultured in expensive media, which may affect the final cost of recombinant protein production.

Wild types and mutants (expressing proteins) of I.L.L. showed almost the same growth pattern in the different media studied. Analysis of OD of all culture media confirmed the result of Neubauer counting of cells. The shape and motility of I.L.L. were checked under an inverted microscope and except in a medium with more than 10% FBS, I.L.L. had normal shape and good motility. In media with 30-50% FBS, the I.L.L. had a circular shape. Based on our experiment, we found that the I.L.L. in the M199 medium had a better growth rate than in the other three main media. The growth rate of I.L.L. in LB broth and $RPMI_{1640}$ medium was the same. Moreover, agitated and static cultures had the same growth rate, which means that agitated culture does not improve the growth rate of I.L.L. In addition, the LB broth showed better results than the LB prepared according to the Sambrook protocol. One of the most surprising points of our experiments is that the LB broth supplemented with 10% FBS did not change the growth rate of I.L.L. compared to LB without other additional substrates. However, the addition of hemin to this medium improved the multiplication rate of I.L.L. In contrast, the addition of hemin to BHI did not improve the I.L.L. growth rate. Similarly, BHI supplemented with only 10% FBS showed the same growth rate as LB broth and $RPMI_{1640}$ medium supplemented with 10% FBS. Likewise, M199 supplemented with 10% FBS showed the best growth rate of the other supplemented media. The addition of 5–10 % lyophilized rabbit serum to LB broth slightly improved the growth rate of I.L.L. compared to LB supplemented with 10% FBS. This means that LB broth enriched with 5–10 % lyophilized rabbit serum is more effective than LB broth and $RPMI_{1640}$ with 10% FBS. In addition, we observed that increasing the FBS content in LB and $RPMI_{1640}$ had a positive effect on the growth rate of I.L.L. This suggests that more than 10% FBS is required to increase the growth rate of I.L.L. The protein yield in the four media was compared using ImageJ.1.8.0_172 software, and the result showed that the I.L.L. cultured in the LB broth expressed a much larger amount of the recombinant protein than the I.L.L. cultured in all three other media.

Based on the lower price of LB broth compared to $RPMI_{1640}$ medium, the same growth rate and maximum cell density of I.L.L. in both media, and also no positive effect of 10% FBS – the most expensive part of the culture media – on the growth rate of I.L.L. in these two media, it seems that using LB broth for culturing I.L.L. as a host cell can reduce the final cost of producing recombinant proteins. In addition, we found that more than 10% FBS is required to increase the growth rate of I.L.L. Alternatively, lyophilized rabbit serum can be used. To achieve a higher growth rate, the M199 medium can also be used. However, the difference between the amount of recombinant protein produced in the LB broth and the M199 medium needs to be accurately determined to compare the cost-effectiveness of producing proteins in each of the two media.

Leishmania needs aerobic conditions and because of that, the main limitation of this study was the cultivation of *Leishmania* in a large amount of media. Meanwhile, the doubling time of the *Leishmania* is 7 hours, which means more time is needed to reach a higher number of the *Leishmania* compared to *E.coli*. Hemin is light-sensitive, therefore, the *Leishmania* must be cultivated in the dark, in addition, its half-life is short and needs to be added to medium every 14 days.

Conclusion

The LB broth could be used as a cost-effective medium for culturing I.L.L. and producing recombinant proteins by this host.

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Conflict of interest

The authors declare that there is no conflict of interest.

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