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Compound serum and hemin free medium for cultivation of *Leishmania tarentolae:* A recombinant protein expression system

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Serum free cultivation of *Leishmania* is cost-effective and improves large scale production of well-defined parasite material. Moreover, the production of recombinant pharmaceutical proteins requires cultivation of the host in a culture medium free of animal materials, so several culture media for *Leishmania tarentolae* expression system have been introduced. Some investigations have established the development of a serum-free, but hemin containing medium, based on yeast extract and buffer salts. Hemin is a substance of animal origin also interferes with nickel ions on Ni-NTA resin. In this study, *L. tarentolae* from Iranian lizard, cultivated in a compound serum and hemin free medium (LBR medium) and the growth parameters were determined. Here we report that LBR medium could obtain high maximal cell density of 1.8 × 108 cells ml-1 equivalent to that of hemin containing medium in our conditions. This compound medium was confirmed by successful expression of a 28 kDa his-tagged protein. With knowledge of the results, the easy-preparing culture medium could be used as a new culture medium for the production of recombinant proteins in *L. tarentolae*.

Key words: *Leishmania tarentolae*, serum free cultivation of *Leishmania*, protein expression, *Leishmania* culture media.

INTRODUCTION

The protozoan parasites of the genus *Leishmania* have a two-stage life cycle (Peters and Killick-Kendrick, 1987). Actively motile flagellated cells form, known as promastigotes is found in the sand flye's alimentary tract. The intracellular aflagellate amastigote form lives in mammalian host within a vacuole with lysosomal features known as parasitophorous vacuole (Galvao-Quintao et al., 1990; Sacks, 1989). *Leishmania tarentolae* is a parasite of the gecko *Tarentolae annularis* (Wallbanks et al., 1985) and has been introduced as a novel eukaryotic expression system for the production of recombinant proteins with mammalian-like post translational modification pattern (Fritsche et al., 2007).

The protozoan is non-pathogenic to humans (Breitling

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Table 1. Ingredients and preparation of nutrient media used for cultivation of *Leishmania tarentolae.*

Medium	Ingredients	Preparation
LBR	LB (40%), BHI (40%), $RPMI_{1640}$ (20%)	LB (25 g/l) and BHI (37 g/l) powders dissolved and autoclaved $RPMI_{1640}$ (100 g/l) dissolved and filtered Hemin was added to BHI (5 mg/l)
BHI-hemin	Brain Heart infusion + hemin	FBS was added to LBR (10%)
LBR-FBS	LB (40%), BHI (40%), $RPMI_{1640}$ (20%) + 10% FBS	FBS was added to BHI (10%)
BHI-FBS	Brain Heart infusion + FBS	

et al., 2002). The advantages of this system are easy handling, the higher growth rate and cultivation in lower cost medium compared to mammalian cells (Phan et al., 2009). Promastigotes are mainly cultivated in liquid media to which animal serum, blood or hemin is added (Limoncu et al., 2004). A commonly used medium is Brain Heart Infusion (BHI), partially supplemented with serum or hemin (Cox and Hardegree, 1976; Fritsche et al., 2007). The media supplemented with animal substances have a risk of contamination with viruses or prion proteins responsible for bovine spongiform encephalopathy (Grillberger et al., 2009).

In the current study, we tried to develop a nutrient medium free of animal substances for cultivation of *L. tarentolae* as a host for the production of recombinant proteins as therapeutic agents. The growth parameters of the wild type organism and an expressing type, such as the rate of cell division (r), time of doubling (t), and maximal cell density (Nmax) were determined.

MATERIALS AND METHODS

The experiment was performed on a wild and an expressing type of *L. tarentolae* from Iranian lizard. For expressing type, a ~ 28 kDa secretory protein was designed. The gene of interest was cloned into pLEXSY-neo2 (Jenabioscience, Germany) between *SalI* and *NheI* restriction sites. The signal peptide sequence of the *lmsap1* gene for secreted acid phosphatase (*lmsap1*) of *Leishmania mexicana* was used. After electroporation of the host (1300v/5ms) the transfected cells were selected by neomycin.

The two types, were cultivated in 25 and 75 cm^2 plastic cell culture flasks containing 10 and 50 ml nutrient broth for static and agitated cultures respectively. The agitated groups were cultivated in 75 cm^2 shaker flasks containing 50 ml nutrient broth at 26°C and 70 rpm on a shaker incubator. Primarily frozen cells were cultivated in each of the examined media and sub-passages were grown in new media. All of the groups were grown in the dark. Inoculations from the logarithmic phase growing agitated pre-culture were done. The inoculums were centrifuged (at 1000 × *g*, 20°C, and 5 min) and the pellets adjusted to 1×10^8 parasites/ml. The same ratio of the volume and count of parasites were inoculated into flasks containing each of examined media, so all of the studied groups were initiated at a cell density of 1×10^7 ml^{-1} and counted over 18, 42, 63, 89 h (Graph 1). All of the cultures were adjusted to pH of 7.5.

Medium preparation

A compound of three culture media was examined. LB medium (Mricomedia, EU) and BHI (Sisco, India) were dissolved and autoclaved, and $RPMI_{1640}$ (Gibco, Germany) dissolved and filtered. The LBR medium was prepared from each of the media with a concentration of 40, 40, and 20% sequentially (Table 1). Hemin (Jenabioscience, Germany) was added to BHI with a final concentration of 5µg l^{-1} from a sterile stock solution. Culture medium was supplemented with 10% of FBS (Gibco, Germany). To avoid bacterial contamination, 50 IU ml^{-1} Penicillin (Gibco, Germany) and 50µg ml^{-1} Streptomycin (Gibco, Germany) was supplemented to all of the culture media. For the selection of electroporated cells, 50 µg/ml Neomycin (Sigma, Germany) was added.

Leishmania growth assessment

Parasite growth was monitored daily and every 2-3 days the medium was changed. Continuous subcultures were maintained up to 10 days of cultivation.

Qualification and quantification of promastigotes were assessed by microscopic observation of the appearance and mobility of the parasite. Counting of fixed organisms was done using a haemocytometer. pH was measured by the pH meter (Milwaukee, MW 101, Romania).

Statistical analysis

SPSS. 17 were used to calculate the data. The evaluating of differences between groups was done using student's t-test. $P< 0.05$ was accepted as statistically significant. Mean specific growth rate (r) was calculated from the Equations below:

dN/dt = rN
Nt = No e^rt
tdouble = ln (2)/ r

RESULTS

In agitated cultures, the growth rate of all the groups was significantly higher than the statics, ($p<0.05$) due to better distribution of oxygen in agitated cultures. The maximum growth rate of agitated cultures was during 55-65 h (Figure 1) and of the statics was during 72-96 h. In the

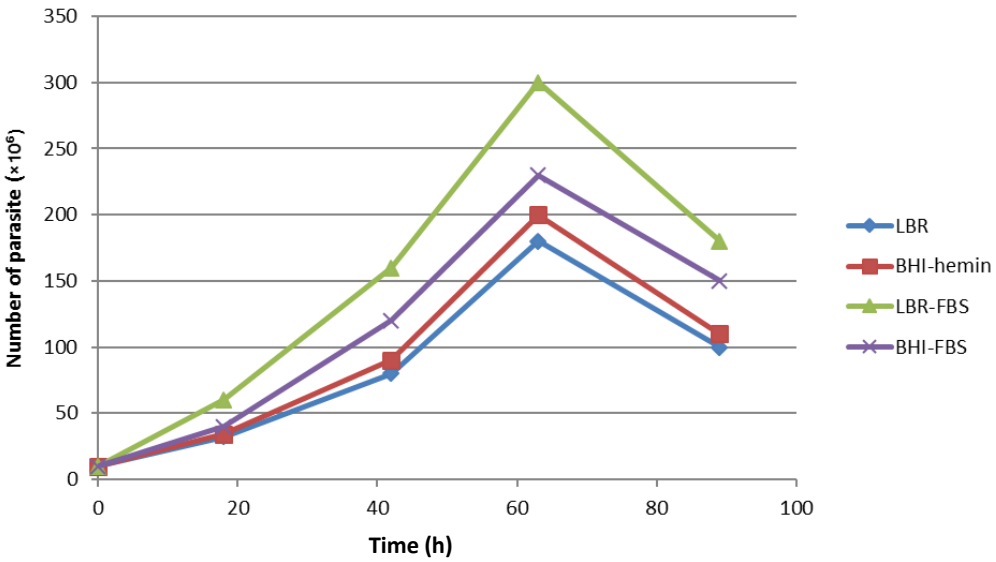

Figure 1. Reproduction of wild and expressing type of *Leishmania tarentolae* in various culture media from agitated cultures.

Table 2. Growth parameters of wild and expressing type of *Leishmania tarentolae* during exponential phase in various nutrient media.

Type of culture	medium	Nmax ×10^6 [cells/ml]	r [h^{-1}]	t double [h]	*P value*
Agitate	LBR	180	0.066	10.5	
	BHI-hemin	200	0.069	10	*P*=0.07
	LBR-FBS	300	0.099	7	
	BHI-FBS	230	0.077	9	*P*=0.001
Static	LBR	120	0.046	15	
	BHI-hemin	125	0.049	14	*P*=0.28
	LBR-FBS	200	0.063	11	
	BHI-FBS	150	0.053	13	*P*=0.003

agitated cultures there was no significant differences between Nmax of BHI-Hemin and LBR media (p=0. 07), as it reached 2×10^8 and 1.8×10^8 ml^{-1}, respectively, while in the groups of FBS supplemented media was significantly different (p= 0.001). The Nmax increased to 2.3×10^8 ml^{-1} for BHI-FBS, but 3×10^8 ml^{-1} for LBR-FBS medium. These results were observed in the static cultures with differences in maximal growth rate (Table 2).

The doubling time of agitated cultures in LBR and BHI-hemin was 10.5 and 10 h with mean specific growth rate of 0.066h^{-1} and 0.069 h^{-1}, respectively, while in LBR-FBS medium was 7 h with mean specific growth rate of 0.099 h^{-1}.

There were no growth differences between wild and expressing type of the parasite (Figure 1). The produced protein secreted into LBR medium was purified by Ni-NTA affinity chromatography (QIAGEN, Germany). We could not purify the protein secreted into BHI-hemin medium (Figure 3). The yield of the produced protein was 2 mg/l, related to the number of parasites/ml, so there were no differences between BHI-hemin and LBR medium according to the same yield of parasites/ml grown in both media.

In all of them the pH shifted down from 7.5 to 6. Although the cells could grow at pH 8 and more which declined to pH 6 during the metabolism, but the better growth rate was achieved at pH 7.5. The comparison between the prices of media related to the number of parasites/ml showed BHI-hemin costs 5 times and FBS-BHI more than 5 times of LBR medium.

Summarizing, the results showed that the growth rate

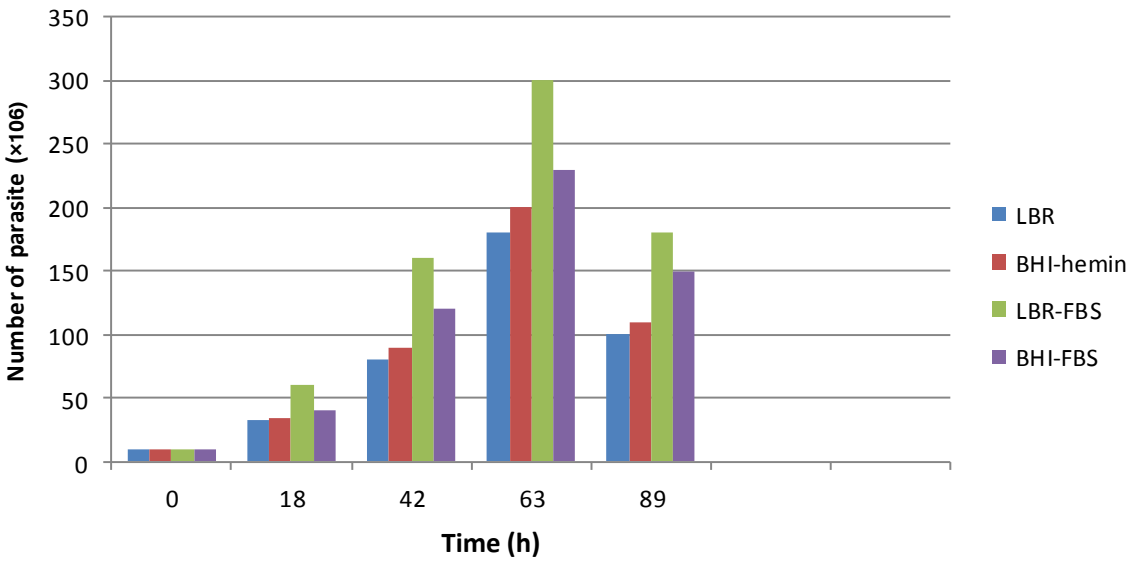

Figure 2. Growth behavior of wild and expressing type of *Leishmania tarentolae* in various nutrient media from agitated culture.

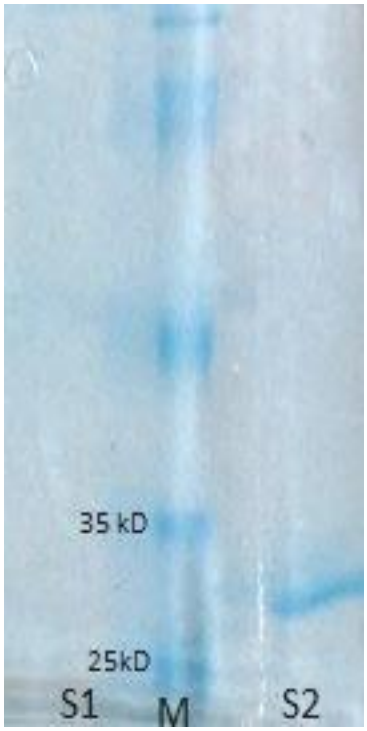

Figure 3. SDS-PAGE of The purified protein from BHI-hemin (S1) and LBR (S2).

and Nmax in the LBR and BHI-Hemin media were similar. Although the two FBS supplemented media have increased the growth rate and Nmax but FBS supplemented LBR achieved significantly better parameters (Figure 2).

DISCUSSION

Many liquid culture media have been established for the *in vitro* cultivation of *Leishmania* species. Fetal bovine serum (FBS) is the main part and the most expensive ingredient of *Leishmania* culture media. Several substances have been used to replace the FBS for successful cultivation of *Leishmania,* which originated from animals (Nasiri et al., 2012). Also hemin have been introduced as an alternative substance to produce a large amount of the parasite (Fritsche et al., 2007).

L. tarentolae has been developed as a novel eukaryotic protein expression system (Basile and Peticca, 2009). Using serum or other animal derived components (like hemin) in cultures is problematic, because of high cost, macromolecule contamination, risk of virus or prion contamination (Van der Valk et al., 2004) particularly when it is used to produce a therapeutic protein (Daniell et al., 2005).

So the cultivation of the host requires media free of animal substances for therapeutic purposes (Grillberger et al., 2009) as well as ability of increasing cell density resulted in higher protein production (Klatt et al., 2013).

Some studies have developed a serum free, but hemin supplemented media (Fritsche et al., 2007). Hemin is a substance of animal origin (Fritsche et al., 2007) also, as stated in the instructions of his-selection from Sigma-Aldrich; iron may displace nickel ions from the resin in the Ni-NTA affinity chromatography during the purification of secretory his-tagged proteins and therefor increases the number of non-specific proteins binding to the column.

We demonstrated that an easy-preparing compound medium (LBR) could obtain the same growth rate and cell density of hemin containing BHI medium without animal

materials. The maximal cell density of 1.8×10^8 $ml^{-1}$for Iranian lizard *Leishmania* cultivated in LBR was achieved. Besides, the LBR supplemented with FBS could significantly improve maximal cell density of the parasite than BHI-FBS. Investigation of Fritsche et al. (2007) on the *L. tarentolae* laboratory strain p10 (Jena Bioscience, Germany) reported a maximal cell density of 8.5×10^8 to 1×10^9 ml^{-1}, cultivated in LEXSY BHI and hemin (Fritsche et al., 2007). We could not achieve such cell density despite the use of BHI supplemented with LEXSY hemin. Meehan et al. (2000) displayed maximum cell density using BHI-hemin medium of 2×10^8 ml^{-1} (2000), which is consistent with our results. Moreover, we could successfully grow an expressing type of the parasite and purify a secretory his-tagged protein using Ni-NTA affinity chromatography, so using LBR medium for the production of recombinant proteins in *L. tarentolae* is more economical. The advantages of this system like easy handling, high similarities of posttranslational protein modifications to mammalian cells, and also ability to grow in animal-substance free media, according to our results, is to be considered (Klatt et al., 2013).

Conclusion

In conclusion the serum and hemin free LBR medium can be used for the cultivation of *L. tarentolae* as an alternative to BHI-hemin medium and can also apply to FBS supplemented media to obtain better growth parameters.

Conflict of interests

The author(s) did not declare any conflict of interest.

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