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Large-scale cultivation of *Leishmania infantum* promastigotes in stirred bioreactor

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ABSTRACT

Background & objectives: Bioreactors are practical tools that are used for economical, time-conserving and large-scale production of biomass from cell cultivation. They provide optimal environmental conditions such as pH and temperature required for obtaining maximum amounts of biomass. However, there is no evidence in the literature on the large-scale cultivation of *Leishmania infantum* parasites in the bioreactor. Therefore, the present study was undertaken to develop a new approach for obtaining *L. infantum* biomass by using pH and temperature controllable stirred bioreactor and to compare parasitic growth kinetics with classical method within erlenmeyers.

Methods: In order to obtain parasite biomass, a newly developed pH and temperature controlled stirred bioreactor was used and its efficacy was compared with a graduated classical scale-up method. Growth kinetics of parasites within erlenmeyers and bioreactors were determined by evaluating promastigote numbers using haemocytometer. The graduated scale enlargement of culture was followed by T25 flask, T75 flask, and 1 L erlenmeyer, respectively.

Results: Obtained results showed a 10-fold increase in the number of promastigotes within the conventional culture performed in 700 ml medium, while parasite numbers increased approximately 15 times due to initial inoculation amounts in the bioreactor culture performed in the 3.5 l medium. Thus, there was 7.5 times more biomass collection in bioreactor compared to classical method.

Interpretation & conclusion: It is postulated that constant culture pH and temperature in the bioreactor extends cultivation time. This could lead to significant increase in parasite numbers. Hence, pH and temperature controllable bioreactors provided acquisition of sufficient amounts of biomass in contrast to classical methods. Therefore, this type of bioreactors may substitute classical culture methods in the production of antigenic molecules for vaccine development.

Key words Culture; *Leishmania infantum;* process development; stirred tank bioreactor; visceral leishmaniasis

INTRODUCTION

Leishmaniasis, caused by compulsive intracellular *Leishmania* parasites, is one of the most important public health problems in the World[1]. According to the World Health Organization, it is endemic in about 98 countries. Globally every year, 1.5 million people are infected with cutaneous leishmaniasis, while about 500,000 people are suffering from visceral leishmaniasis, the fatal form of the disease. It is estimated that 60,000 people lose their lives every year due to leishmanisis-related complications[2–3]. It has not been possible to develop an effective and reliable vaccine that can provide protection from leishmaniasis despite ongoing work for long years[4].

Large-scale cultivation of *Leishmania* parasites is necessary to obtain sufficient amounts of antigenic molecules from parasites to use in molecular, genomic and immunological studies. In conventional large-scale cultivation technique, biomass is acquired in erlenmeyers by transferring parasite culture from flasks. However, production of parasite biomass in erlenmeyers possess some disadvantages such as rapid decrease in culture pH, uncontrollable temperature, high cost and excess labour. Especially instant pH variations result in inadequate production of parasite biomass[5–6].

Bioreactors have been recently used to obtain maximum biomass from culture. They can provide optimal environmental conditions such as pH and temperature for cell growth. Use of bioreactors is accepted as more economical, time- and labour-saving method in contrast to classical cultivation. Different models of bioreactors such as stirred, airlift, wave and membrane are preferentially used for suspended cultures[7]. Stirred bioreactors that can regulate pH and temperature are generally preferred for

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protozoan culture since they provide the most suitable conditions for parasite growth[8–12]. But there is no evidence in the literature on the large-scale cultivation of *L. infantum* in the bioreactors for production of antigens that will be utilized in the development of vaccines or diagnostic kits. Therefore, the aim of the study was to develop a new approach for obtaining *L. infantum* biomass by using pH and temperature controllable stirred bioreactor and to compare parasitic growth kinetics in the bioreactor with classical method.

MATERIAL & METHODS

Classical cultivation of L. infantum promastigotes

Leishmania infantum (EP 126) parasites preserved within cryobank were thawed by shaking in a water bath at 27 °C. All thawed cells were seeded into a T25 flask containing 5 ml RPMI 1640 medium with 10% FBS. Parasites were incubated in 27 °C for 6–7 days. Promastigotes were passaged for once in every week.

Large-scale parasite cultivation in erlenmeyer flasks

Scaling-up procedure for parasite culture was started with the addition of 1 million/ml *L.infantum* promastigotes into T25 flasks containing 5 ml RPMI 1640 medium with 10% FBS + 1 ml/l gentamicin[13]. After 3–4 days incubation, the number of promastigotes in cultures reached up to 8–10 million/ml parasites. Cell countings were performed by using a hemocytometer at inverted microscope (Olympus CKX41). Approximately, 4.5 ml of culture was added into T75 flasks from T25 flasks and 35 ml RPMI 1640 medium enriched with 10% FBS was added to initiate culture in larger flasks, starting with the number of 1 million/ml parasites. Following to 3–4 days incubation, the maximum number of parasites reached up to 8–10 million/ml in T75 flasks.

Then parasite cultures in the T75 flasks were transferred into a 1 L erlenmeyer flask for re-scale enlargement. The culture was initiated with inoculation of 1 million parasites into erlenmeyers flask including 330 ml brain-heart infusion medium with FBS (30 ml/l), hemin (5 mg/l), adenosine (26 mg/l) and gentamicin (1 ml/l). Following four-days of incubation, culture volume was in creased to 700 ml by including approximately 300 ml fresh media. Erlenmeyer cultures were shaken at room temperature.

Large-scale parasite cultivation in the bioreactor

A newly developed bioreactor (TIBO, Istanbul, Turkey) was used in the present study. This system consists of three components. The first component is the tip with pH and temperature measuring probes, the heater and the inlet and outlet holes and the second part contains a glass vessel and magnetic fish while the third and the main part has pH and temperature measurement systems, peristaltic pump, magnetic stirrer and main control panel for temperature, mixing speed, and pH adjustment. pH is kept under control by a peristaltic pump attached to the source of NaOH.

For cultivation in the bioreactor, a total of 3 l of the medium was prepared by adding 4% FBS, Hemin (0.00625 g/l), adenosine (0.0325 g/l) and gentamicin (1 ml /l) into brain-heart infusion broth (46.25 g/l). All the parts of the bioreactor were individually packaged and autoclaved. *Leishmania infantum* culture (400 ml) with the initial cell number of 1 million/ml was added to the glassware of bioreactor containing brain heart infusion medium. Electrical connections of the pH probe, the thermometer, and the heater were linked between bioreactor head and main control part. Autoclaved 0.5 M NaOH solution was bound to the peristaltic pump of the bioreactor for pH regulation. The pH was set at 7.25 from the control panel. The pH of the culture was not allowed to fall below 7.25 until the bioreactor was terminated. The culture temperature was set at 26 °C and kept constant until the end of the culture. The culture in the bioreactor was stirred at 70–90 rpm by using a magnetic stirrer throughout the process. The culture was terminated at the end of 9 days incubation. The number of parasites within erlenmeyer and bioreactor were counted by hemocytometer on an inverted microscope. Moreover, pH changes in both cultures were determined and compared at different time intervals

Ethical statement: Not applicable.

RESULTS

The steps of classical scaled-up procedure are demonstrated in figure 1. As mentioned, graduated scale enlargement of culture was followed from T25 flask to T75 flask, and 1-liter erlenmeyer, respectively. All the components of newly developed pH and temperature controllable fed batch stirred bioreactor are presented in Fig. 2. In each system, parasite cultivation was initiated with the parasite numbers of 1 million/ml. Subsequently, pH change and growth kintetics in erlenmeyer and bioreactor were compared. The pH dynamics of the cultures in the stirred bioreactor and in the erlenmeyer are presented in (Fig. 3). The culture pH was kept constant at 7.25 in the bioreactor with automatically addition of 0.5 M NaOH solution. On the other hand, culture pH in erlenmeyer decreased constantly to 6.1 with the effect of

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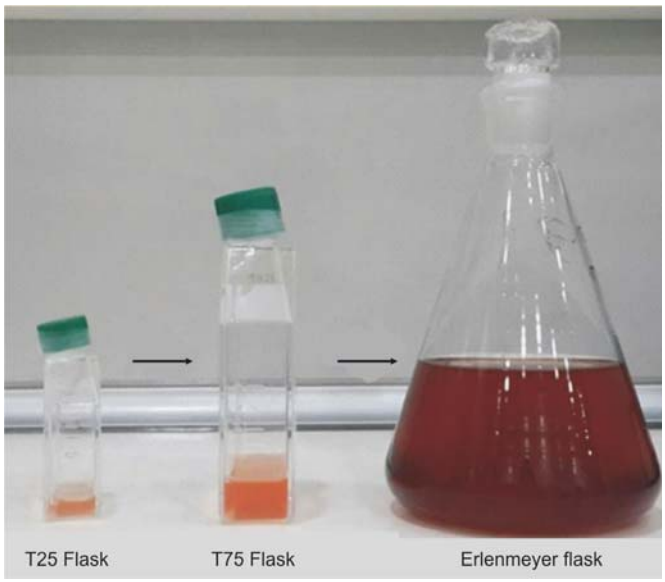


Fig. 1: Conventional scaled-up parasite culture beginning at T25 flasks and finished with erlenmeyer flasks.



Fig. 2: Components of the stirred bioreactor for the culture of *L. infantum* promastigotes; (a) NaOH solution; (b) A peristaltic pump; (c) Temperature set/display; (d) Inlet-outlet hole; (e) pH and temperature probe, and heater; (f) pH display/set; (g) Stirrer rpm; (h) On/Off and; (i) Stirrer.

diminished metabolite residues depending on vital activities of parasites (Fig. 3).

While performing classical large-scale cultivation in

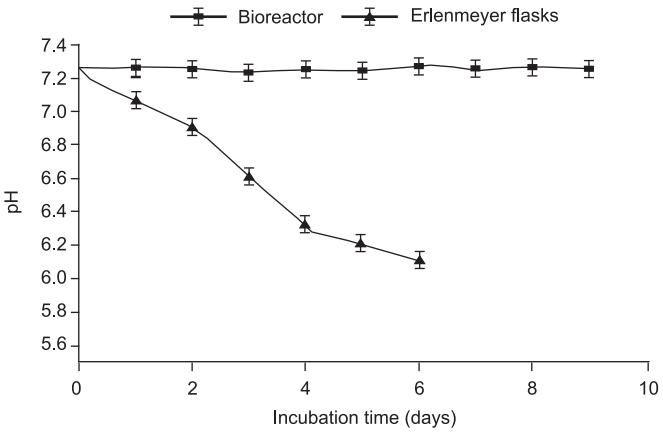


Fig. 3: The pH changes in the culture of *L. infantum* promastigotes performed in bioreactor and erlenmeyer flasks.

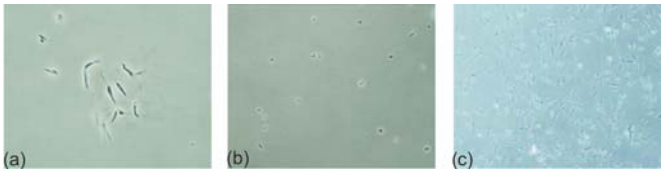


Fig. 4: The microscopic view (20×) of *L. infantum* parasites cultivated in erlenmeyers and bioreactors separately: (a) healthy promastigotes in erlenmeyer flasks at Day 3 of incubation; (b) parasites lost their morphologies at Day 6 of incubation and; (c) promastigotes that preserved compact and uniform shapes after cultivation in bioreactor for 9 days.

erlenmeyers, the number of parasites reached to 10 million/ml at the end of Day 3 of incubation. Subsequently, culture pH decreased to 6.6 resulting in a decline in the number of parasites. After six-days of incubation, it was observed that the morphological structures of the parasites deteriorated as a result of a decrease in the pH, while parasites were healthy at Day 3 of incubation (Figs. 4a and 4b). On the other hand, promastigotes preserved their compact and uniform shapes after cultivation within bioreactor at the end of Day 9 of incubation (Fig. 4c).

Further, initiating with the Day 6 of cultivation, parasites tend to multiply within bioreactor and the numbers of parasites reached to 15 million/ml at the end of Day 9 of incubation due to constant pH. After Day 9, the number of parasites decreased. Nevertheless, it was observed that parasites in erlenmeyer showed very rapid growth at first three days of incubation in contrast to the bioreactor. It was determined that parasites reached to 10 million/ml on Day 3 in erlenmeyer, while they reached to the same number on Day 5 in the bioreactor. However, the number of parasites in erlenmeyer decreased gradually after third day of incubation. Figure 5 shows that the maximum number of parasites in erlenmeyer reached to 10 million at most, while the maximum number of parasites in the

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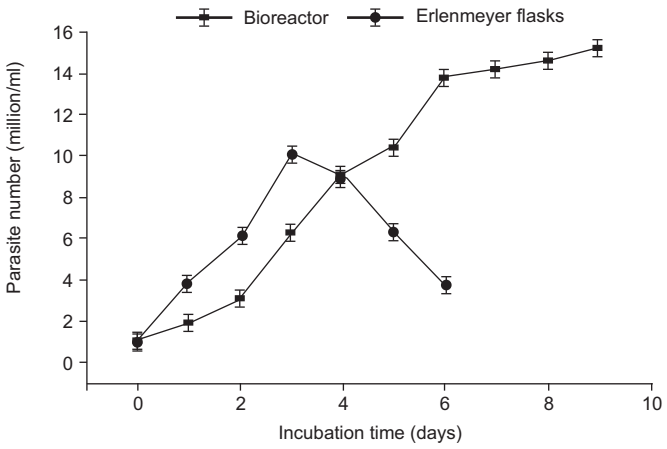


Fig. 5: Growth kinetics of *L. infantum* promastigotes cultivated in bioreactor and erlenmeyers at different time intervals.

bioreactor was 15 million. Obtained results showed that a 10-fold increase in the number of promastigotes was observed within the conventional culture performed in 700 ml medium, while parasite numbers increased approximately 15 times due to initial inoculation amounts in the bioreactor culture performed in the 3.5 l medium. Thus, it was observed that 7.5 times more biomass was collected in bioreactor compared to classical method.

DISCUSSION

Bioreactors have begun to be used in parasite culture in recent years in order to obtain high amounts of parasite pellets. In the literature, quite a few studies have been carried out on large-scale cultures of *Leishmania* parasites using bioreactors and there was no information about bioreactor based large-scale production of *L.infantum* promastigotes. Only in some studies, successful large-scale cultivation of *L. tarentolae* promastigotes has been achieved on bioreactors. *Leishmania tarentolae* is parasitic but non-pathogenic protozoan, and has shown promising results in developing a vaccination against leishmaniasis[1, 14]. Gazdag *et al*[12] have produced human Cu/Zn superoxide dismutase recombinant protein by using *L. tarentolae* parasites. Accordingly, the culture of parasites was carried out at 24 °C while keeping the pH constant in a bioreactor mixed with air. In another study, Fritsche *et al*[9] used large-scale cultivation of *L. tarentolae* parasites in a bioreactor using yeast extract as a medium. The pH was not controlled while culturing at 26 °C in a bioreactor mixed with a stirrer and air flow. By using this system, it was observed that the number of parasites increased by 20 times compared to the initial inoculated numbers. In our study, we used a batch stirred bioreactor in which the pH and temperature of the suspension culture of *L. infantum* promastigotes can be controlled for high yield and large-scale production. It was shown that cultivation in a stirred batch bioreactor, where the temperature and pH are constant, is more efficient than the static culture in regards to the production of *L. infantum* parasite pellet with higher yields. It was observed that the multiplication of the parasites in the bioreactor increased 15 times compared to the initial amounts. The difference in the number of parasites between the study by Fritsche *et al*[9] and our study may arise from diversities in parasite species, type of medium or number of parasites at the beginning.

The success of stirred bioreactor systems on the acquisition of high yield biomass from culture has been demonstrated on various cell types. Darvishi *et al*[15] used *Yarrowia lipolytica* as a cultivator using a bioreactor for recombinant laccase production. It was found that protein production was fast and higher in the stirred bioreactor where pH, temperature, and oxygen can be controlled than conventional cultures in the flasks. In another study, Agabalian *et al*[16] performed the cultures of skin-derived precursors (SKPs) cells in a stirred bioreactor. The bioreactor culture at constant pH was found to be 5 times more proliferative in contrast to the static culture proliferation. When SKPs in both cultures were examined, there was no difference in their ability to regenerate dermal stem cell niches in deeply rooted hair follicles. Tang *et al*[17] examined the importance of pH and temperature for MethA tumor cell culture in a stirred bioreactor. It has been shown that this type of bioreactor lead to a significant reduction in the mean specific growth rate when culture pH and temperature are not controlled.

Like in batch bioreactors, controlling pH is an important factor that enhances the yield in fed-batch bioreactors. The pH is indirectly kept at the optimal values by addition of the culture medium in the bioreactor. In several studies, it was indicated that cell proliferation gradually increased in pH-controlled fed-batch bioreactors when compared to conventional cultures where pH was not controlled. Olmer *et al*[18] examined the importance of using stirred bioreactors against static cultures in the production of human pluripotent stem cells. In this study, a regular cell increase was observed in the bioreactor as a result of daily nutrient enrichment and pH of the medium was kept at optimum values. When the nutrient was not included in the static culture, there was a significant decrease in the number of cells due to reasons such as the decline in pH and oxygen levels and also negative effects of metabolic wastes[18]. In our developed bioreactor system, it is estimated that the number of parasites increased over time compared to the static culture, because the pH

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was kept constant and the amount of dissolved oxygen in the medium was higher than the normal culture. In a similar study, Rafiq *et al*[19] studied the culture of human mesenchymal stem cells (hMSCs) in a fed-batch bioreactor. In the bioreactor, the pH varied from 6.7 to 7.2, which is the vital value for cells due to the addition of daily nutrient. They showed that higher amounts of cell density were obtained in the bioreactor than in the flask culture. In another study, Surrao *et al*[20] comparatively investigated the growth of human skin-derived precursor cells in a stirred bioreactor and in static culture. It has been shown that cell density increased extensively in bioreactor than in static cultures, and culture pH, which is not controlled in bioreactor decreased more slowly than static culture, and low pH affects cell viability and density[20]. These results corroborate with the present study outcome i.e., sufficient amounts of biomass can be obtained by keeping the culture at optimum pH range.

Uncontrolled pH in cell culture tends to decrease throughout the culture process due to increased waste metabolites. The pH ranged between 7.2 and 7.4 is accepted as optimal for parasite culture and stimulates the multiplication of parasites. In our study, we observed that medium pH dropped to the critical levels such as 6.5 in classical cultivation at fourth day of incubation. The slowdown in parasite numbers following a rapid increase may be explained by the variations in medium pH. On the other hand, constant pH in bioreactor up to nine days facilitated an increase in parasite number. It is known that in conventional cultivation physiological conditions change during growth and multiplication of parasites since glucose is consumed and the pH decreases with lactose production. Furthermore, the cellular activities may stop due to the excessive pH drop[20–22]. Van Hellemond *et al*[23] have shown that trypanosomatids produce mainly partially oxidized products such as pyruvate, succinate, and acetate during their energy metabolism. Especially, the abundance of nutrients causes organic acid production, while cells consume more glucose than they can catabolize. For this reason, keeping the pH constant throughout the culture for longer period is important for obtaining the maximum number of cells. We were able to achieve a high-efficiency parasite pellet in the bioreactor by keeping the pH constant in this study. This result shows how controlled pH applications are important for the production of parasites in the bioreactor.

CONCLUSION

For the first time in this study, the large-scale culture of *L. infantum* promastigotes was performed in a stirred bioreactor in which pH and temperature can be controlled. The number of parasites obtained as a result of the bioreactor culture was compared with the number of parasites obtained with the standard culture. The result revealed that the *L. infantum* promastigotes can be cultivated with higher efficiency in the bioreactor. This indicates that the use of a bioreactor system in a large-scale culture of *L. infantum* parasites is appropriate. More intensive use of such systems in the future will allow the collection of high amounts of biomacromolecules-specific to *L. infantum* parasites. For this reason, it is thought that the bioreactors may play an important role in the production of molecules that can be used for the treatment or protection of the disease.

Conflict of interest: None

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