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## Optimization of *Brucella abortus* Fermenter Cultural Conditions and LPS Extraction Method for Antigen Production

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**Abstract:** We modified *Brucella* fermentation medium from FAO/WHO for enhancing of *Brucella abortus* S99 biomass. The modified media composed of 15 g L<sup>-1</sup> peptic digest animal tissue, 15 g L<sup>-1</sup> pancreatic digest of casein, 10 g L<sup>-1</sup> yeast extract and 0.10 g L<sup>-1</sup> sodium bisulphate. Glucose was added during the incubation by fed-batch method (1-30 g L<sup>-1</sup>). Agitation speed and air flow rates were controlled at 300-500 rpm and 4-8 L min<sup>-1</sup>, respectively. Cell density was 9-10% and viable count was 3-3.3×10<sup>11</sup> mL<sup>-1</sup>. The modified conditions enhance the biomass production to more 2 times than the FAO/WHO method. Three methods were accomplished for LPS production: Extraction by butanol with enzymatic digestion, hot phenol extraction with enzymatic digestion, modified hot phenol with trichloroacetic acid (TCA) procedure. Yield of LPS extraction was 0.2, 0.8 and 1.3%, respectively. Method III results in a greater yield of LPS which is 6 and 1.5 times the yields of methods I and II, respectively. Protein contamination of LPS was <2, <2 and <2.9% and nucleic acid contamination of LPS was <1, <1 and <1.4%, respectively. The ketodeoxyoctonate content of LPS (in each of the three methods) was in agreement with ketodeoxyoctonate values obtained previously for highly purified LPS of *B. abortus*. According to present study, hot phenol with trichloroacetic acid (TCA) procedure is the most suitable procedure for large-scale LPS production from *Brucellae*, which can be employed for the production of *Brucella* biomass for vaccine and antigen preparations.

**Key words:** *Brucella abortus*, fermenter, LPS production, biomass

## INTRODUCTION

Brucellosis is one of the five common bacterial zoonosis in the world, especially in the developing countries, which is caused by organisms belonging to the genus *Brucella*, gram-negative, non-spore-forming, intracellular bacteria (Godfroid and Kasbohrer, 2002; Moreno, 2002; Refai, 2002).

*Brucella* is a facultative intracellular pathogen that causes abortion in cattle, goats and sheep and a febrile illness (undulant fever) in humans. Animal brucellosis is a serious problem worldwide and is endemic globally. In areas where it is endemic, human brucellosis is quite common but often not diagnosed.

There are two main methods for antigen production; cultivation on a solid media in Roux flasks and culture in a fermenter. Culture of *Brucella* cells in a fermenter has some advantages over

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propagation on a solid media including: decreasing the risk of contamination of culture during inoculation and harvesting; decreasing the infection risk to staff by contact with *Brucella* cells; having large volume of biomass and decreasing the labor and cost (Hendry *et al.*, 1985). Most commercial processes operate as a batch or a fed-batch system for large-scale productions. While it is still necessary to satisfy the nutritional requirements of the organism, it is essential on this scale to design a medium with constituents which are cheap and readily available. It is also desirable that they be consistent, stable and easy to store and handle (Dodge and Gerstner, 2002). Working with FAO/WHO protocol (Corbel and Hendry, 1983; Hendry *et al.*, 1985) for large volume production of biomass of *Brucella* is not satisfactory and gives low results. Therefore there is need for improvement and modification of the media and conditions for overproduction of biomass. A prerequisite for achieve high product yields in a fermentation process, is to design an optimal production media and a set of optimal process operating conditions. A strategy of fed-batch operation was adopted in order to improve production of biomass (Celik and Calik, 2004; Cheema *et al.*, 2002; Dodge and Gerstner, 2002). Also for large-scale preparation of LPS to utilize in production of diagnostic kits, vaccines and others, use of economic and high yield method is more preferable.

Lipopolysaccharide is a major constituent of the outer membrane of gram-negative bacteria such as *Brucella* and is known to activate phagocytic cells such as neutrophils and macrophages, to unregulated expression of adhesion molecules and produce a number of pro and anti-inflammatory cytokines (Campos *et al.*, 2004; Rittig *et al.*, 2003).

The most conspicuous structural defect that renders *Brucella* organisms avirulent, is the absence of the LPS (Freer *et al.*, 1996; Gorvel and Moreno, 2002).

Results clearly indicate that the LPS of *Brucella*, is required for virulence (Fernandez-Prada *et al.*, 2003; Pulendran *et al.*, 2001) and this molecule is one of the candidate antigens for diagnostic tests and *Brucella* subunit vaccines and as a carrier in vaccines (Bhattacharjee *et al.*, 2002; Goldstein *et al.*, 1992; Huang *et al.*, 1999; Porte *et al.*, 2003; Schurig *et al.*, 2002; Weynants *et al.*, 1997).

In this research, we improved fermentation media composition and optimized influencing factors for cells production from *B. abortus* S99 in batch fermenter and compared the various extraction methods of LPS from *B. abortus* S99 and improved the extraction method of LPS from *Brucella* for large-scale antigen production.

## MATERIALS AND METHODS

### Bacterial Strain and Culture Conditions

*B. abortus* S99 is obtained from the department of bacterial vaccines and antigens productions of Pasteur Institute of Iran. It is completely smooth and CO<sub>2</sub>-independent strain of *B. abortus* biovar 1. It was cultured in slant Brucella agar medium at 37°C for 72 h. The seed culture was grown in a 5 L flask containing 2 L of Brucella broth under aeration by a sparger at 37°C for 72 h.

### Fermentation

We modified FAO/WHO protocol (Corbel and Hendry, 1983; Hendry *et al.*, 1985) for enhancing the biomass production from *Brucella* (in department of bacterial vaccines and antigens productions, Pasteur Institute of Iran) and compared with primary protocol of FAO/WHO.

The modified fermentation medium was composed of 15 g L<sup>-1</sup> peptic digest of animal tissue (BBL), 15 g L<sup>-1</sup> pancreatic digest of casein (Gibco), 10 g L<sup>-1</sup> yeast extract (Gibco) and 0.10 g L<sup>-1</sup> sodium bisulphate. The pH of the fermentation medium was adjusted to 6.4. The cells from the seed flask culture were inoculated to be 5% (v/v) into a 60 L fermentor (Novo-Paljas, Contact-flow B.V., Netherlands) with a working volume of 40 L. Temperature was fixed at 36±1°C. Agitation speed was controlled at 300-500 rpm and air flow rate was controlled at 4-8 L min<sup>-1</sup>. Two hours after inoculation

of the seed culture, glucose was fed in the rate of  $1 \text{ g L}^{-1}$  and then increased up to  $30 \text{ g L}^{-1}$ . In this method antifoam was not used. In the process, cells are checked for purity and smoothness. After 60 h, the culture was harvested by centrifugation and cell density of suspension was determined by centrifuging 1 mL volumes in Hopkins vaccine tubes at 3000 g for 75 min (Alton *et al.*, 1988; Corbel *et al.*, 1979; Corbel and Hendry, 1983).

### **Extraction of LPS**

#### **Method I**

LPS was extracted by butanol (Morrison and Leive, 1978; Phillips *et al.*, 1989). Fifty grams of wet weight cells suspended in 400 mL of water-saturated n-butanol (99%) at 4°C. The aqueous phase obtained for LPS precipitation. Proteinase K (50 µg per 10 mg of protein) and both, DNase and RNase (50 µg per 1 mg of nucleic acid) was added to reduction of protein and nucleic acids contaminations.

#### **Method II**

LPS was extracted by the hot phenol (Corbel and Hendry, 1983; Hendry *et al.*, 1985). Fifty gram wet weight of cells suspended in 170 mL distilled water to 66°C followed by the addition of 190 mL of 90% (v/v) phenol to 66°C. After 30 min the mixture is centrifuged and the phenol layer is removed. The LPS is precipitated by cold methanol and is dissolved in 0.1 M Tris buffer for enzymatic digestion (with proteinase K, DNase and RNase), then it is freeze dried.

#### **Method III**

LPS was extracted by the hot phenol procedure with modification. Similar to method II, cells are suspended in water and added hot phenol. The phenolic phase was removed and 1/2 volumes of cold methanol was added to precipitate the proteins and nucleic acids. After centrifugation, 0.5 g per 10 mL of solution of trichloroacetic acid (TCA) was added and stirred for 30 min at 4°C and then dialyzed against distilled water; LPS precipitated afterwards.

### **Chemical Analysis**

The protein content of LPS was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The ketodeoxyoctonate content was determined by the Weissbach (1959) method. Nucleic acid was estimated by measuring the  $A_{260}$ . SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 14% PAGE (containing urea 4M) and stained with silver stain procedure (Tsal and Frasch, 1982).

### **Statistical Methods**

Significance of the differences between FAO/WHO media and our modified medium in biomass production was determined by one-way analysis of variance (ANOVA) ( $p < 0.01$ ).

## **RESULTS**

### **Biomass Production**

Comparing the modified fermentation media with the fermentation media of FAO/WHO, it is found that the former lacks sodium dihydrogen phosphate, disodium hydrogen phosphate, antifoam and glucose. In this modified media and fermenter conditions, glucose was added during the incubation time by fed-batch method. Composition of primary (FAO/WHO) and modified media are shown in Table 1.

Working with FAO/WHO protocol for biomass production from *Brucella*, usually gives unsatisfactory results and very low cell quantity. In present research, fed-batch addition of glucose

Table 1: Comparison of the primary and modified *Brucella* fermentation medium ingredients

Primary medium (FAO/WHO)	Concentration	Modified medium	Concentration (g L <sup>-1</sup> )
Peptone	30 (g L <sup>-1</sup> )	Peptone	30
Yeast extract	10 (g L <sup>-1</sup> )	Pancreatic digest of casein	15
		Peptic digest of animal tissue	15
Glucose	30 (g L <sup>-1</sup> )	Yeast extract	10
Sodium dihydrogen phosphate	9 (g L <sup>-1</sup> )	Glucose*	1-30
Disodium hydrogen phosphate	3.3 (g L <sup>-1</sup> )		
Antifoam	0.20 (mL L <sup>-1</sup> )	Sodium bisulphate	0.10

\*: Glucose was added by fed-batch method

Table 2: Comparison of the cell density and viable cells count between the primary and modified methods for *Brucella* cells fermentation culture

Factor	Primary method	Modified method
Cell density (%)	4-5%	9-10%*
Viable count (mL)	1.3-1.6×10 <sup>11</sup>	3-3.3×10 <sup>11</sup> *

\*: Indicates a significant difference comparing to the primary method (p<0.01)

Table 3: Characteristics of *B. abortus* LPS that extracted with three methods

Methods	Yield of LPS (%)	Ketodeoxyoctonate rate (%)	Protein content (%)		Nucleic acid content (%)	
			Before treatment	After treatment	Before treatment	After treatment
I	0.2	1	<5	<2	<3	<1
II	0.8	1	<6	<2	<3	<1
III	1.3	0.9	<2.9*		<1.4*	

\*: In method III enzymatic treatment is not used

and increasing or decreasing of air flow and agitation rate, on the biomass production of *Brucella* were investigated and optimized. In the modified method, fermentation was completed after 60 h and cell density (packed cell volume) was 9-10% and viable count was 3-3.3×10<sup>11</sup> mL<sup>-1</sup> and also cells exist in correct colonial phase.

Results of cell density and viable cell count and comparison of these for primary and modified method were shown in Table 2. The modified conditions enhance the biomass production to more than 2 times the FAO/WHO method. In Fig. 1, comparison of the variation in cell concentration of the medium with cultivation time for both FAO/WHO (primary method) and modified methods are shown. This comparison confirms the enhancement of biomass in the modified method.

### LPS Extraction

The LPS of *B. abortus* S99 is extracted by three methods. In method III (modified hot phenol with trichloroacetic acid) we have the greatest yield of LPS (1.3%) which it is more than 6 and 1.5 times the yields of methods I and II, respectively. Ketodeoxyoctonate, which is a unique component of bacterial LPS, was found to be 0.9-1% by weight. Protein and nucleic acid contaminations of LPS extracted by three methods are given in Table 3. The protein and nucleic acid content of LPS extracted by methods I and II after enzymatic treatment were equal and low but the contaminations of LPS in method III without enzymatic digestion were slightly greater but consistently acceptable to use.

*B. abortus* LPS were analyzed with 14% SDS-PAGE gels with 4 M urea (Fig. 2) and stained by silver stain procedure, resulting in patterns similar to the patterns observed previously for the LPS of *Brucella* (Corbel *et al.*, 1979; Hendry *et al.*, 1985). Lanes 1 and 3 are pattern of 5 µg of *B. abortus* LPS extracted with methods I and II. Lane 4 is 10 µg of *B. abortus* LPS extracted with method III. Lane 2 is 5 µg of *E. coli* LPS.

In contrast to the LPS of *B. abortus*, the mature form of *E. coli* LPS migrates as a banded pattern rather than a smear, because the sugar units of *E. coli* LPS consist of four rather than one sugar molecule (Goldstein *et al.*, 1992).

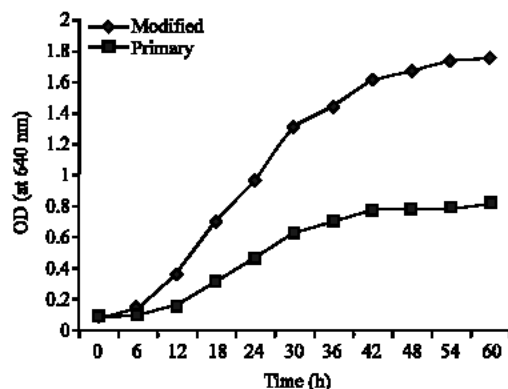


Fig. 1: Comparison of the variation in cell concentration of the medium with cultivation time for both FAO/WHO method (primary method) and our modified method



Fig. 2: SDS-PAGE of *B. abortus* LPS. Lane 1 and 3: Samples of 5 µg LPS extracted with methods I and II. Lane 4: 10 µg of LPS extracted with method III. Lane 2: 5 µg of *E. coli* LPS

## DISCUSSION

Control and eradication campaigns against brucellosis have led to demands for large quantities of *Brucella* cells, both for vaccine and antigens production.

Two methods currently used are culture on a solid medium in Roux flasks and culture in a liquid medium (Hendry *et al.*, 1985). Handling of large volumes of culture presents the following hazards: (a) the risks of contaminating the cells during inoculation, harvesting and bottling and (b) the potential risk to staff who may become infected or sensitized by contact with *Brucella* cells.

The choice of method for producing cells will depend on the quantity of cells required and on the facilities available; whichever method is used, great care should be exercised (a) in maintaining cultures for seed material, in the correct colonial phase (smoothness) and (b) in the choice of medium used.

When large volumes of cells are required for vaccine or antigen production, the use of liquid medium has some advantage over propagation in Roux flasks because the laborious and costly process of handling large numbers of individual flasks is eliminated and the attendant risk of contamination is reduced (Corbel *et al.*, 1979; Hendry *et al.*, 1985).

In this study, optimum media composition and conditions were developed for the overproduction of brucellae cells for antigen production. Present results indicated that modified media and conditions

for batch fermenter increased cell quantity without incorrect colonial phase; also we found that incubation more than 60 h and agitation rate more than 500 rpm, decreased growth rate of the organisms.

In FAO/WHO protocol, the initial pH of medium is 6.6 but this tends to rise during the growth cycle and therefore it must be adjusted to pH 7.2-7.4 by the addition of sterile 0.1 M HCl. In modified media and conditions, the initial pH is 6.4 and rise to 7.42 at the end of fermentation, but this range of pH media is acceptable and no need to add HCl for pH adjustment.

A well known property of protein solutions is their propensity to foam when agitated. If the medium is allowed to foam it will occupy a larger volume, therefore the effective working volume of the fermenter is reduced. It may be necessary to reduce aeration and/or agitation rate, thus reducing the oxygen transfer rate with possible limitation to growth. If excessive foam formation occurs, it may enter the fermenter vent system thus posing a possible contamination risk. Addition of antifoam is necessary but antifoam compounds may inhibit growth and will de-gas the liquid and reduce oxygen availability (Celik and Calik, 2004).

In the FAO/WHO media antifoam is required but in our modified medium does not require antifoam, thus oxygen availability and growth rate of cells has not reduced.

Fed-batch addition of glucose after 2 h of inoculation of seed culture in the rate of 1 g L<sup>-1</sup> and increasing to 30 g L<sup>-1</sup>, increased the cell concentration. In fed-batch addition of glucose, buffer system ingredients of the primary medium, sodium dihydrogen phosphate and disodium hydrogen phosphate were deleted. It is frequently observed that at high concentration of substrate, the substrate also expresses growth inhibiting properties. However, prior to reaching the maximum population phase there is a stage during which substrate depletion causes a restriction in the growth rate (Celik and Calik, 2004; Cheema *et al.*, 2002; Dodge and Gerstner, 2002). In batch culture, as well as FAO/WHO protocol for *Brucella* fermenter culture, the microbial population are present in an environment in which initially all the substrates are present at excess concentrations, but in fed-batch method, the substrate (glucose) was added at low concentration and increased gradually, thus in this modified method we have not observed these undesirable effects. Compared to the primary method, these modified conditions, especially fed-batch addition of glucose increased the cell density to 9-10 from 4-5%.

The ketodeoxyoctonate content of LPS in this study is in agreement with ketodeoxyoctonate values obtained previously for highly purified LPS of *B. abortus* (Phillips *et al.*, 1989). The yield of LPS extraction in phenol-water partition methods were more than butanol-water partition method. Also modified hot phenol procedure have excess product than original hot phenol procedure. The protein and nucleic acid content of LPS that extracted with three methods were acceptable to use. For large-scale production of LPS the use of enzymatic treatment and butanol-water partition method has disadvantages like laborious, costly process and low yield. In the present study, we modified the extraction method for large scale production of *B. abortus* LPS: In the method III we use 1/2 volume of cold methanol and TCA for precipitation of proteins and nucleic acids instead of enzymatic treatment. Present data (Table 3) clearly showed that this method is suitable for large scale LPS production from brucellae.

In conclusion the presented research can be applied easily for production of *Brucella* biomass for vaccine and antigen preparations.

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