

# Optimization of production of *Brucella abortus* S19 culture in bioreactor using soyabean casein digest medium

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## SUMMARY

A method of cultivating *Brucella abortus* S19 culture in bioreactor was attempted using three different media. Culture conditions in bioreactor were optimized by varying agitation and aeration parameters. Varying the aeration ranging from 0.5 vvm to 0.8 vvm and agitation rate ranging from 250 rpm to 400 rpm during bacterial growth was found to yield highest viable count within 48 hours of culture period. A count of  $>1 \times 10^{11}$  CFU per ml within 48 to 60 hours post seeding was obtained consistently in all five consecutive batches ( $P>0.05$ ) with  $6 \times 10^{11}$  CFU per ml being the maximum yield when the organism is grown in soyabean casein digest medium. *B. abortus* S19 maintained its smooth characteristics throughout its growth in bioreactor. The vaccine prepared with soyabean casein digest medium was found to be potent and safe with a protective index of 3.33 in mice. The vaccine was tested in 10 cattle calves of 3 to 13 months age and all the vaccinated animals were seropositive on 28, 60, 90, 120 and 150 days post-vaccination when analyzed by fluorescence polarization assay (FPA).

**KEY WORDS:** *Brucella abortus* S19, Bioreactor, Soyabean casein digest medium, Potency, Fluorescence polarization assay

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## INTRODUCTION

Brucellosis is a zoonotic disease that causes heavy economic losses and human suffering and is endemic in India (ICAR/PD-ADMS Annual report, 1999-2000). It causes abortion and infertility in bovines. The two live vaccines available for the control of brucellosis in cattle are *Brucella abortus* S19 and *Brucella abortus* RB51.

The viable count of *Brucella* is important during the cultivation and the batch reproducibility is crucial. Most of the currently used media for production of *B. abortus* are complex in nature. Therefore the yields in term of viable count vary

from batch to batch. A prerequisite to achieve high product yields in a fermentation process is to design an optimal production media and standardize a set of process operating conditions. Plommet (1991) studied minimal nutritional requirements and temperature limits for growth of *Brucella* species. In a saline basic medium containing thiosulphate, ammonium sulphate and glucose with addition of 2 or 4 vitamins (nicotinic acid, thiamin, pantothenic acid and biotin), 24/25 *B. suis*, 4/6 *B. melitensis* and 1/6 *B. abortus* strains were able to grow. Some strains, however, needed to be initially induced to grow in the presence of other ingredients, vitamins, amino acids, CO<sub>2</sub> or by prolonged incubation. *Brucella* is not a simple strict aerobic bacterium, it can adapt to various growth conditions like micro aerophilic, anaerobic (+ nitrate) and minimal media compositions. So there is necessity to find out a suitable medium for *B. abortus* S19 culture growth. *B. abortus* S19 was grown usually on a solid medi-

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um. Rousseau *et al.* (1987) reported that this method had several drawbacks like the cost of manpower, glassware and the possibility of dissociation because of the long growth period on a static moist surface. Culturing *Brucella* cells in a bioreactor has advantages over propagation on a solid media in Roux flasks. These include reduced contamination during inoculation and harvesting of culture. In addition, Corbel *et al.* (1979) reported that the production of antigen in fermenters eliminates the laborious process of handling large numbers of individual flasks and reduction in the risk of contamination. Van Drimmelen (1956; 1958) first used flat bottles on a reciprocating shaker and later a 250 liter vessel with surface aeration. Hauschild *et al.*, 1961; Angus, 1984; Zavenella *et al.*, 1984 reported batch production as well as continuous production in fermenters. Most commercial processes operate as a batch or a fed- batch system for large scale production. While it is still necessary to satisfy the nutritional requirements of the organism, it is essential at this scale to design a medium which is consistent, inexpensive and readily available. The present study deals with optimization of the production process of *B.abortus* S19 in a bioreactor using soyabean casein digest medium to obtain higher yields.

## MATERIALS AND METHODS

### Materials

Antifoam 289 was obtained from Sigma, USA; All fine chemicals were obtained from Merck, India; Potato infusion agar, Soyabean casein digest medium were obtained from Himedia, India; Tryptose agar and Dextrose Andrade's broth were obtained from Difco USA.

### Bacterial strains

The *B.abortus* S19 (vaccine strain) and *B.abortus* 544 biovar 1 (pathogenic strain) used in this study were obtained from the Animal Disease Research Laboratory (ADRL), National Dairy Development Board (NDDB), Anand, India. These strains were maintained in ADRL according to standard procedures as described by Alton *et al.*, (1988). *B.abortus* S19 vaccine (Bruvax) produced by Indian Immunological Limited was used as an internal reference vaccine for potency and immuno-

genecity testing in mice and cattle respectively. It was grown on potato infusion agar media (Himedia, India) using a surface culture method.

### Culture medium

Two reference media (Medium A and Medium B) were used for comparison with the new Medium C. *Brucella* fermentation Medium 'A' was prepared following a method described earlier by Alton *et al.* (1988). It contains dextrose 3% (w/v), sodium dihydrogen phosphate 0.9% (w/v), disodium hydrogen phosphate 0.33% (w/v) and peptone 3% (w/v). *Brucella* fermentation Medium 'B' was prepared essentially following a method described earlier by Shapouri *et al.* (2007) and contained peptone-polypeptone 3% (w/v), pancreatic digest of casein 1.5% (w/v), peptic digest of animal tissue 1.5% (w/v), glucose 0.25% (w/v), yeast extract 1.0% (w/v) and sodium bi sulphate 0.01% (w/v). *Brucella* fermentation Medium 'C', Soyabean casein digest medium (Tryptone soya broth) was prepared as per the manufacture's (Himedia, India) instructions. It contains pancreatic digest of casein 1.7% (w/v), papaic digest of soyabean meal 0.3% (w/v), sodium chloride 0.5% (w/v), dipotassium hydrogen phosphate 0.25% (w/v) and dextrose (Glucose) 0.25% (w/v) per litre. Additionally, 20% (w/v) dextrose (Merck, India) stock solution was added to the medium to give a final concentration of 5% (w/v). All the media were sterilized at 1.2kg pressure, 121°C for 15 minutes and stored at  $\pm 4^{\circ}\text{C}$  till further use.

### Freeze drying stabilizer medium

Freeze drying stabilizer medium (FZ medium) was prepared using enzymatic digest of casein (Difco, USA) 2.5% (w/v), sucrose (Merck, India) 5.0% (w/v) and sodium glutamate (Merck) 1.0% (w/v). The medium was sterilized by passing through membrane filter (0.22  $\mu\text{m}$ ) and stored at 4 °C until further use.

### Preparation of master seed and working seed

Freeze-dried master culture of *B. abortus* S 19 was rehydrated in sterile phosphate buffered saline (PBS) containing 1% (w/v) sodium chloride, 0.025% (w/v) potassium chloride, 0.025% (w/v), anhydrous potassium dihydrogen phosphate and 0.1438% (w/v) anhydrous disodium hydrogen phosphate, pH 6.4 and inoculated on potato in-

fusion agar slants. The slants were incubated at 37°C for 72 hours and the culture was washed with 5 ml of PBS by gentle rotation. The harvest was transferred into the stabilizer medium aseptically and freeze dried. Freeze dried vials were stored at 4°C. Characterization of seed was performed according to methods described earlier by Alton *et al.* (1988). In brief, Gram staining, H<sub>2</sub>S production, oxidase test, urease test, acriflavine test and staining of colonies with crystal violet were carried out. Tests that discriminate *B. abortus* S19 from the reference *B. abortus* 544 biovar 1 strain were performed on tryptose agar (TA) plates supplemented either with Penicillin G (2.5 IU/ml), erythritol (1mg/ml) or thionin blue (2 µg/ml). The plates were incubated under 0% CO<sub>2</sub> and 5% CO<sub>2</sub> for *B. abortus* S19 and Strain 544, respectively.

### Preparation of seed culture

The freeze dried master culture was rehydrated in PBS and grown in 75 cm<sup>2</sup> tissue culture flasks containing Potato Infusion Agar for 72 h at 37°C. Then the culture was harvested by scrapping with 25 – 30 ml PBS and inoculated into two 1 liter conical flasks containing 250 ml of Soyabean casein digest medium. The flasks were incubated at 37°C for 20-24 hours at 220 rpm. The mid log phase culture was used to seed the bioreactor.

### *B. abortus* S19 production in bioreactor

Bioreactor (Bioflo - 3000, New Brunswick scientific, USA) with a working volume of 10 liters was used for the bulk production of *B. abortus* S19. Filter sterilized media was transferred aseptically to the previously sterilized bioreactor using sterile siphon head. The bioreactor was seeded with 500 ml suspension of *B. abortus* S19 with a viable count of 5x10<sup>8</sup> colony forming unit (CFU) per ml. Temperature was maintained at 36°C ±0.5°C. The aeration was maintained by sparging sterile compressed air. Antifoam 289 (Sigma) at 0.1% v/v was used. Optimization of the fermentation process was done in three stages.

In the first optimization stage, three different *brucella* fermentation media (Media A, Media B, Media C) were used with constant agitation (250 rpm) and constant aeration (0.5 vvm) throughout the culture period for 60 hours. The experiments were conducted in triplicate and viable counts of *Brucellae* were recorded. In the second stage, process optimization studies were con-

ducted with different aeration and agitation rates to maintain dissolved oxygen (DO) between 25% to 30% using the media yielding maximum viable counts in stage 1. Experiments were conducted in triplicates to check for consistency. The following parameters were studied:

*Method 1:* Constant agitation at 250 rpm and constant aeration at 0.5 vvm

*Method 2:* Constant agitation (250 rpm) and variation in aeration rates ranging from 0.5 vvm to 0.8 vvm.

*Method 3:* Variation in agitation rates ranging from 250 rpm to 400 and constant aeration (0.5vvm)

*Method 4:* Variation in agitation rates ranging from 250 rpm to 400 rpm and variation in aeration rates ranging from 0.5 vvm to 0.8 vvm.

Following optimization of various parameters i.e. medium, seed volume, temperature, agitation and aeration rates, in the final stage five consecutive batches were prepared to complete the process optimization. Samples were collected at 12 hr intervals up to 60 hours to determine the viable count, pH, dissolved oxygen (DO), optical density and purity by colony characteristics and morphology.

### Downstream processing of the brucellae culture and preparation of freeze dried vaccine

The *Brucellae* in the culture broth were concentrated by micro filtration using 0.1 µm cassette (Sartorius, Germany). The purity was checked by inoculation in potato infusion agar slant, dextrose Andrade's broth and Gram's staining. The culture was concentrated and adjusted to 3x10<sup>11</sup> CFU/ml with FZ medium, filled in 2 ml tubular vials and freeze-dried. The freeze-dried vials were stored at 2°C to 8°C until further use.

### Quality control tests on final vaccine

#### *Identity and dissociation test*

The number of viable *brucellae* per ml of the final bulk concentrate as well as final freeze-dried vaccine was determined using a method described earlier by Alton *et al.* (1988). Test for dissociation (acriflavine test), identity and sterility were carried out as described earlier by Alton *et al.* (1988).

### Animal studies

#### *Safety in Guinea pigs*

The safety of *B. abortus* S19 vaccine was checked following the procedure described in OIE

Manual (2008). Briefly, groups of ten guinea-pigs each were given intramuscular injections (IM) of 1 dose of vaccine ( $5 \times 10^9$  CFU) diluted in PBS. The vaccine passes the safety test if none of the guinea pigs show any adverse effects and remain healthy.

### **Immunogenicity and potency studies**

#### *Animals*

BALB/C Mice (five to seven weeks old) and Guinea Pigs (275-350 grams) were obtained from National Institute of Nutrition, Hyderabad; Cross-bred female cattle calves: 3 to 12 months seronegative for *Brucella* were obtained from holding farm, Indian Immunologicals Limited, Hyderabad.

#### **Potency testing of *B. abortus* S19 vaccine in mice**

The potency of *B. abortus* S19 vaccine was tested in mice following the method described in OIE Manual (2008). Five to seven week old Balb/c mice were used for testing the vaccines. The animals were handled and cared for as per the Institutional Animal Ethics Committee recommendations.

The test was declared satisfactory if the immunogenicity value obtained in mice is significantly lower than that obtained in the unvaccinated controls and does not differ significantly from that obtained in mice vaccinated with the reference vaccine.

#### **Study of immune response in cattle for *Brucella* S19 vaccine**

Cross-bred female cattle calves of aged between 3 and 12 months were used in the vaccination experiment. Ten calves were administered 2 ml of *Brucella abortus* S19 vaccine containing a total of  $5 \times 10^{10}$  CFU subcutaneously (SC). Blood was collected on days 0, 28, 60, 90, 120, 150 and 180 days post vaccination (dpv). The sera samples were analyzed by FPA to determine the sero-conversion. Five calves of similar age group were kept as unvaccinated controls.

#### **Fluorescence polarization assay (FPA)**

The FPA was performed essentially following the method described earlier by Nielsen *et al.*, (2001) using a fluorescence polarimeter (Diachemix LLC, USA). Briefly, 1 ml of diluted reaction buffer was pipetted into glass test tubes to which, 10 $\mu$ l

of negative control reagent was added. The solution was mixed well and the mixture incubated at room temperature (RT) for 5 minutes. This mixture was further used as a blank for the equipment. 10 $\mu$ l of *Brucella* fluorescence polarization (FP) conjugate was added to the earlier mixture and further mixed. The mixture was then incubated at RT for 2 minutes. The reading obtained was expressed as millipolarization (mP) units. The above steps were repeated with negative control. The mean mP was calculated for the three replicates of the negative control, positive control and samples respectively. Positive and negative controls used for the test were supplied by the Diachemix LLC, USA. Serum samples collected on 0, 28, 60, 90, 120, 150 and 180 dpv were tested by FPA. The mean positive control must read between 120 and 250 mP. The mean negative control should read between 70 and 95 mP. The animals were declared sero-converted if the mean mP values above the mean negative control was more than 20 mP.

## **RESULTS**

The purity of the culture was confirmed by Gram's staining which revealed the presence of gram negative coccobacilli arranged either singly or in pairs. The culture did not require CO<sub>2</sub> for growth and was inhibited by penicillin G, thionin blue and *i*-erythritol.

### **Fermentation optimization studies**

#### *A) Use of different *Brucella* fermentation media (Media A, Media B and Media C)*

The optimization of fermentation media was done in order to obtain maximum viable count of *B. abortus* S19. The details of mean viable counts expressed as CFU per ml obtained are shown in Table 1. Medium C yielded  $1.50 \times 10^{10}$  CFU per ml in 60 h of fermentation as compared to reference Media A and B which yielded  $1.50 \times 10^9$  and  $8 \times 10^9$  respectively.

#### *B) Use of different process parameters*

Following the first optimization stage, *B. abortus* S19 was propagated in medium C coupled with different aeration and agitation rates. The mean viable counts observed at various time points were expressed as CFU per ml (Table 2).

TABLE 1 - Mean viable counts (CFU/ml) of *B. abortus* S19 during standardization of production medium in Bioreactor.

Hours of culture	Medium A		Medium B		Medium C	
	Mean	SD	Mean	SD	Mean	SD
0	5.00X10 <sup>6</sup>	4.36X10 <sup>5</sup>	4.00X10 <sup>6</sup>	2.65X10 <sup>5</sup>	3.80X10 <sup>6</sup>	4.58X10 <sup>5</sup>
12	2.97X10 <sup>5</sup>	2.68X10 <sup>5</sup>	2.83X10 <sup>5</sup>	1.89X10 <sup>5</sup>	4.83X10 <sup>4</sup>	2.84X10 <sup>5</sup>
24	6.00X10 <sup>6</sup>	4.00X10 <sup>6</sup>	1.83X10 <sup>6</sup>	7.64X10 <sup>5</sup>	3.52X10 <sup>6</sup>	3.26X10 <sup>6</sup>
36	4.33X10 <sup>7</sup>	3.21X10 <sup>7</sup>	1.78X10 <sup>8</sup>	2.79X10 <sup>8</sup>	3.30X10 <sup>8</sup>	4.07X10 <sup>8</sup>
48	1.19X10 <sup>9</sup>	1.22X10 <sup>9</sup>	5.33X10 <sup>9</sup>	2.52X10 <sup>9</sup>	7.67X10 <sup>9</sup>	1.53X10 <sup>9</sup>
60	1.50X10 <sup>9</sup>	5.00X10 <sup>8</sup>	8.00X10 <sup>9</sup>	1.80X10 <sup>9</sup>	1.50X10 <sup>10</sup>	5.00X10 <sup>9</sup>

\*Significant differences compared to other media.

TABLE 2 - Mean viable counts (CFU/ml) of *B. abortus* S19 per ml obtained during optimization process using *Brucella* broth fermentation medium C.

Method 1: Constant agitation rate and constant aeration.

Method 2: Constant agitation and variation in aeration.

Method 3: Variation in agitation rate and constant aeration.

Method 4: Variation in agitation rate and variation in aeration.

Hours of culture	Method 1	Mean ±SD	Method 2	Mean ±SD	Method 3	Mean ±SD	Method 4	Mean ±SD
0	3.70x10 <sup>6</sup>	3.8x10 <sup>6</sup>	4.40x10 <sup>6</sup>	4.20x10 <sup>6</sup>	4.20x10 <sup>6</sup>	4.40x10 <sup>6</sup>	3.60x10 <sup>6</sup>	4.00x10 <sup>6</sup>
	3.90x10 <sup>6</sup>	±0.1x10 <sup>6</sup>	4.20x10 <sup>6</sup>	±0.20x10 <sup>6</sup>	4.20x10 <sup>6</sup>	±0.346x10 <sup>6</sup>	4.40x10 <sup>6</sup>	±0.40x10 <sup>6</sup>
	3.80x10 <sup>6</sup>		4.00x10 <sup>6</sup>		4.80x10 <sup>6</sup>		4.00x10 <sup>6</sup>	
12	1.80x10 <sup>8</sup>	2.00x10 <sup>8</sup>	5.40x10 <sup>7</sup>	5.00x10 <sup>7</sup>	3.20x10 <sup>7</sup>	3.50x10 <sup>7</sup>	1.80x10 <sup>8</sup>	2.00x10 <sup>8</sup>
	2.20x10 <sup>8</sup>	±0.20x10 <sup>8</sup>	5.20x10 <sup>7</sup>	±0.52x10 <sup>7</sup>	3.50x10 <sup>7</sup>	±0.30x10 <sup>7</sup>	2.30x10 <sup>8</sup>	±0.264x10 <sup>8</sup>
	2.00x10 <sup>8</sup>		4.40x10 <sup>7</sup>		3.80x10 <sup>7</sup>		1.90x10 <sup>8</sup>	
24	5.80x10 <sup>8</sup>	5.50x10 <sup>8</sup>	3.10x10 <sup>8</sup>	3.5x10 <sup>8</sup>	1.40x10 <sup>7</sup>	1.50x10 <sup>7</sup>	0.70x10 <sup>9</sup>	1.00x10 <sup>9</sup>
	5.30x10 <sup>8</sup>	±0.26x10 <sup>8</sup>	3.40x10 <sup>8</sup>	±0.458x10 <sup>8</sup>	1.80x10 <sup>7</sup>	±0.264x10 <sup>7</sup>	1.20x10 <sup>9</sup>	±0.264x10 <sup>9</sup>
	5.40x10 <sup>8</sup>		4.00x10 <sup>8</sup>		1.30x10 <sup>7</sup>		1.10x10 <sup>9</sup>	
36	1.90x10 <sup>9</sup>	2.00x10 <sup>9</sup>	0.50x10 <sup>9</sup>	1.00x10 <sup>9</sup>	9.30x10 <sup>8</sup>	9.00x10 <sup>8</sup>	3.10x10 <sup>10</sup>	3.50x10 <sup>10</sup>
	2.40x10 <sup>9</sup>	±0.360x10 <sup>9</sup>	0.60x10 <sup>9</sup>	±0.78x10 <sup>9</sup>	9.20x10 <sup>8</sup>	±0.435x10 <sup>8</sup>	3.60x10 <sup>10</sup>	±0.360x10 <sup>10</sup>
	1.70x10 <sup>9</sup>		1.90x10 <sup>9</sup>		8.50x10 <sup>8</sup>		3.80x10 <sup>10</sup>	
48	2.00x10 <sup>9</sup>	2.09x10 <sup>9</sup>	4.50x10 <sup>10</sup>	5.00x10 <sup>10</sup>	2.20x10 <sup>9</sup>	2.50x10 <sup>9</sup>	2.30x10 <sup>11</sup>	2.50x10 <sup>11</sup>
	2.30x10 <sup>9</sup>	±0.25x10 <sup>9</sup>	5.50x10 <sup>10</sup>	±0.50x10 <sup>10</sup>	2.40x10 <sup>9</sup>	±0.360x10 <sup>9</sup>	2.60x10 <sup>11</sup>	±0.173x10 <sup>11</sup>
	1.80x10 <sup>9</sup>		5.00x10 <sup>10</sup>		2.90x10 <sup>9</sup>		2.60x10 <sup>11</sup>	
60	2.20x10 <sup>10</sup>	2.00x10 <sup>10</sup>	4.30x10 <sup>10</sup>	4.00x10 <sup>10</sup>	0.70x10 <sup>10</sup>	1.00x10 <sup>10</sup>	3.60x10 <sup>11</sup>	4.00x10 <sup>11</sup>
	2.30x10 <sup>10</sup>	±0.43x10 <sup>10</sup>	3.90x10 <sup>10</sup>	±0.264x10 <sup>10</sup>	1.20x10 <sup>10</sup>	±0.264x10 <sup>10</sup>	4.10x10 <sup>11</sup>	±0.360x10 <sup>11</sup>
	1.50x10 <sup>10</sup>		3.80x10 <sup>10</sup>		1.10x10 <sup>10</sup>		4.30x10 <sup>11</sup>	

<sup>a</sup>Yields in Method 4 are significant as compared to the other methods.

TABLE 3 - Viable counts (CFU/ml) of *B. abortus* S 19 per ml obtained during production of five consecutive batches in stirred tank bioreactor using the optimized conditions.

Hours of culture	Batch 1*	Batch 2*	Batch 3*	Batch 4*	Batch 5*	Mean cfu/ml $\pm$ SD
0	$3.50 \times 10^6$	$5.00 \times 10^6$	$4.50 \times 10^6$	$5.20 \times 10^6$	$3.80 \times 10^6$	$4.40 \times 10^6 \pm 7.38 \times 10^5$
12	$1.50 \times 10^9$	$8.00 \times 10^8$	$3.00 \times 10^9$	$2.00 \times 10^9$	$5.60 \times 10^8$	$1.57 \times 10^9 \pm 9.81 \times 10^8$
24	$3.37 \times 10^9$	$5.00 \times 10^9$	$5.20 \times 10^9$	$8.00 \times 10^8$	$2.50 \times 10^9$	$3.37 \times 10^9 \pm 1.83 \times 10^9$
36	$4.43 \times 10^{10}$	$7.00 \times 10^{10}$	$2.00 \times 10^{10}$	$1.70 \times 10^{10}$	$6.00 \times 10^{10}$	$4.23 \times 10^{10} \pm 2.36 \times 10^{10}$
48	$5.45 \times 10^{11}$	$1.00 \times 10^{11}$	$3.70 \times 10^{11}$	$2.00 \times 10^{11}$	$4.50 \times 10^{11}$	$3.33 \times 10^{11} \pm 1.82 \times 10^{11}$
60	$6.00 \times 10^{11}$	$2.00 \times 10^{11}$	$1.50 \times 10^{11}$	$3.50 \times 10^{11}$	$3.00 \times 10^{11}$	$3.20 \times 10^{11} \pm 1.75 \times 10^{11}$

\*No significant difference was noticed between the batches.

Varying the aeration ranging from 0.5 vvm to 0.8 vvm and agitation rate ranging from 250 to 400 rpm during bacterial growth was found to yield the highest viable counts of  $4.0 \times 10^{11}$  CFU per ml while other combination of process parameters like constant aeration and agitation, constant agitation and variation in aeration, variation in agitation rate and constant aeration gave  $2.0 \times 10^{10}$ ,  $5.0 \times 10^{10}$ , and  $1 \times 10^{10}$  CFU per ml respectively.

### C) Batch consistency

The viable counts of *B. abortus* S19 for five consecutive batches at varying time points such as

12, 24, 36, 48 and 60 hours post inoculation in stirred tank bioreactor were presented in Table 3. The yield obtained was  $>1.0 \times 10^{11}$  CFU per ml within 48 to 60 hours post seeding in all the five batches with  $6.0 \times 10^{11}$  CFU per ml being the maximum yield.

The mean yields obtained after 48 and 60 hours were  $3.33 \times 10^{11}$  and  $3.20 \times 10^{11}$  CFU per ml, respectively.

The optical density (OD), DO values and viable count of the broth observed at varying time points of bacterial culture for five consecutive batches are presented in Figure 1.

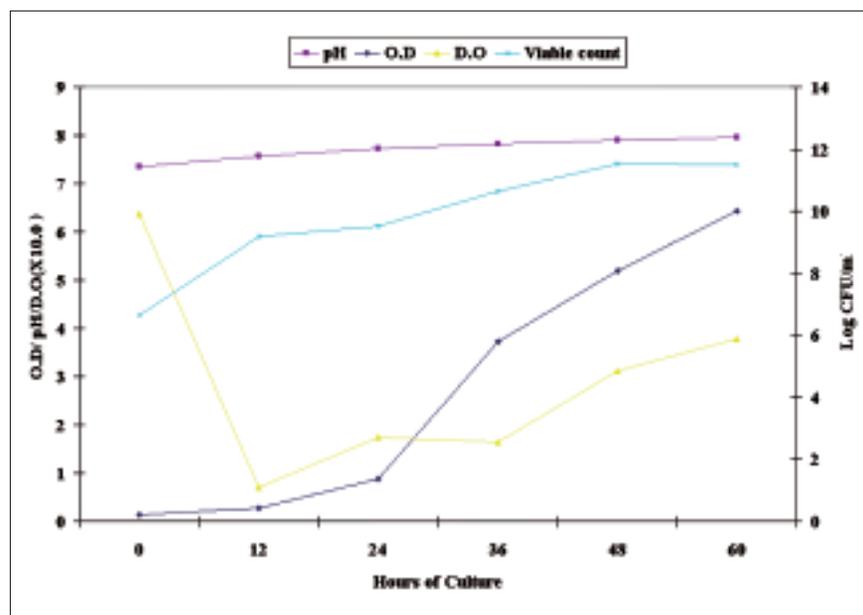


FIGURE 1 - Fermentation Profile of Five consecutive batches of *Brucella abortus* S19 in 10 L fermenter

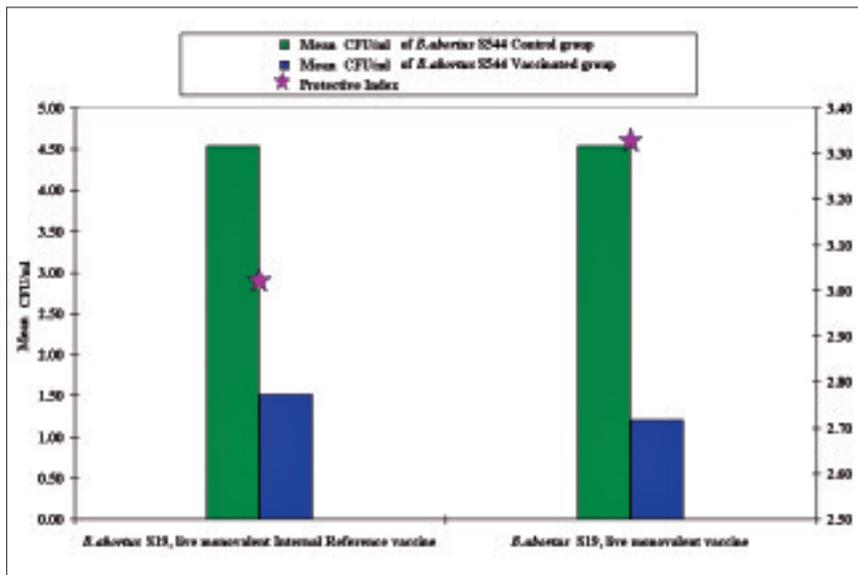


FIGURE 2 - Protective index of the of *B. abortus* S19 Internal Reference and Experimental vaccine in mice.

### Quality control tests on the bulk and freeze-dried vaccine

Bacterial culture was clarified and concentrated by micro filtration using 0.1 µm membrane cassettes and the volume was reduced to 1 liter (10X concentration). The bacterial concentrate was subjected to Gram’s staining which revealed the presence of typical gram-negative cocco-bacilli. Colony morphology characteristic of *brucellae* was observed when the concentrate was inoculated onto potato infusion agar slope and exam-

ined 5 days later. Acid and gas production was not observed when the concentrate was inoculated into Dextrose Andrade’s broth, indicating the purity of the culture (data not shown).

The freeze-dried vaccine was subjected to quality control check to determine the viable *Brucellae* count which was found to be  $2.6 \times 10^{11}$  CFU per 5 doses. The *Brucellae* when subjected to acriflavine test showed that the colonies are intact with no evidence of dissociation. The Gram’s staining revealed the presence of gram negative

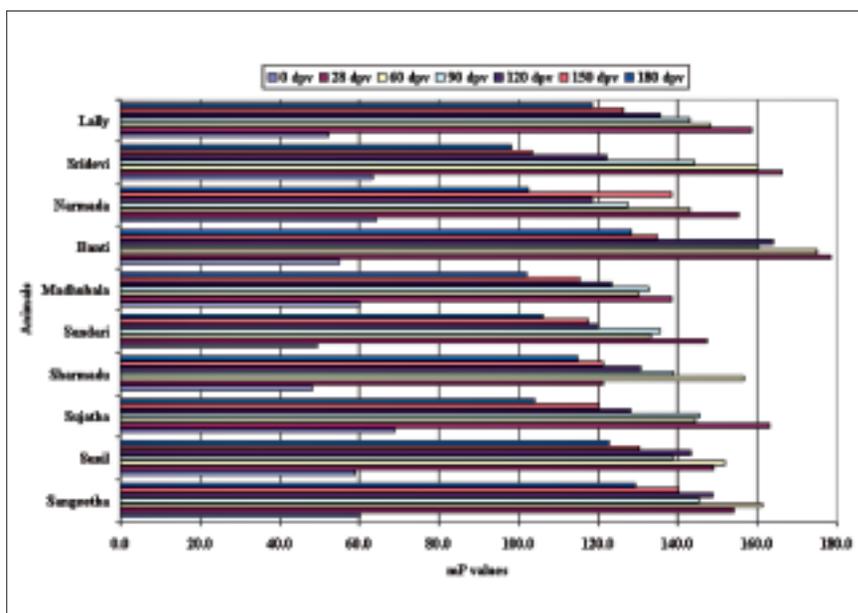


FIGURE 3 - Immunological response in cattle after vaccination with *B. abortus* S19 vaccine prepared using the cultures obtained from stirred-tank bioreactor.

coco-bacilli arranged either singly or in pairs. The bacterial morphology was homogenous throughout the smear and no pleomorphic forms were observed. No acid and gas production was observed on days 2, 4 and 7 post-inoculation when sterility test was carried out using Dextrose Andrade's broth. Further sub culturing on day three by spreading the inoculated broth on potato infusion agar slope and incubation for 5 days at 37°C revealed colonies characteristic of *brucella*.

### Animal studies

#### *Safety in guinea pigs*

The vaccine when subjected to guinea pig inoculation did not result in adverse effects or mortality thereby indicating that the vaccine was safe.

#### **Potency testing in mice**

*B.abortus* S19 vaccine gave more than 2 log<sub>10</sub> protection two weeks post challenge in mice (Figure 2). Splenomegaly was observed in unvaccinated control mice challenged with *B.abortus* S544 while splenomegaly was absent in vaccinated mice.

#### **Immunological response in cattle**

*B.abortus* S19 vaccine was tested in 10 cattle calves and the antibody response was analyzed by FPA on 28, 60, 90, 120, 150 and 180 dpv (Figure 3). All the vaccinated animals were seropositive on day 28 post primary vaccination with mean FPA Value 150 mP and remained seropositive on days 60, 90, 120 and 150 post-vaccination followed by decrease in mean FPA values to 110 mP on 180 dpv.

The vaccine was found to be safe. All the unvaccinated control calves were sero negative in FPA for *B.abortus* antibodies through out the duration of experiment.

## DISCUSSION

Control and eradication campaigns against brucellosis have led to demands for large quantities of *brucella* cells, both for vaccine and antigen production Shapouri *et al.*, (2007). Vaccination with *B.abortus* S19, live vaccine is a valuable aid in brucellosis control. The manufacture of *B.abortus* S19 vaccine is based on a seed lot system (OIE Manual, 2008).

The origin, source and maintenance conditions of *B.abortus* S19 seed culture influence the final quality of S19 commercial vaccines (Bosserey, 1985, Bosserey, 1991, Grillo *et al.*, 2000). The sensitivity to *i*-erythritol is considered as an important differential characteristic of S19, and is used for differentiating the *B.abortus* S19 vaccine strain from *B.abortus* biovar1 field strains. Grillo *et al.*, (2000) reported that inadequate subculture or maintenance of seed strains for both *B.melitensis* Rev1 and *B. abortus* S19 anti-*brucella* live vaccines can result in a loss of virulence and/or immunogenicity. Mukherjee *et al.* (2005) reported that the presence or absence of the 702 bp deletion in the *ery* locus of S19 strains had no correlation with either the rates of spontaneous mutation to *i*-erythritol resistance or with the residual virulence in Balb/c mice. So different strains with in *B.abortus* S19 have different characteristics. There is a need to optimize growth parameters for each strain.

The use of liquid medium for large scale antigen production has advantages as it avoids handling of several roux bottles when the propagation is done in solid medium and consequent risk of contamination. In this study, three different liquid media viz., reference Medium A, Medium B and the new Medium C were screened to achieve maximal yields before proceeding to optimization of process parameters. *Brucella* fermentation medium A and B yielded 1.5x10<sup>9</sup> and 2.4x10<sup>9</sup> CFU per ml, respectively whereas *brucella* fermentation medium C yielded 1.5x10<sup>10</sup> CFU per ml after 60 hours of culturing in bioreactor. Batches were taken with different aeration and agitation rates in triplicate using the medium C in order to optimize the process and improve the process consistency. However, varying agitation rate and constant aeration yielded 1x10<sup>10</sup> CFU per ml after 60 hours of culturing, while varying agitation and aeration rate had yielded a higher growth 4x10<sup>11</sup> CFU per ml after 60 hours of culturing.

Further improvements like increasing the seed rate from 3% to 5%, temperature at 36°C ±0.5°C, agitation ranging from 250 rpm to 400 rpm and aeration ranging from 0.5 vvm to 0.8 vvm were established. The minimum and maximum viable count obtained were 1x10<sup>11</sup> and 5.45x10<sup>11</sup> CFU per ml, 1.5x10<sup>11</sup> and 6x10<sup>11</sup> CFU per ml after 48 and 60 hours of culturing respectively during five

consecutive batches. The mean viable CFU per ml obtained was  $3.43 \times 10^{11}$  and  $3.20 \times 10^{11}$  after 48 and 60 hours of culturing, respectively. The ideal harvesting time lies between 48-60 hours. Celik *et al.* (2004) reported that addition of antifoam results in reduced  $O_2$  availability there by inhibiting the growth of S19. Our results indicate that addition of antifoam did not result in a significant reduction in viable count in agreement with the results of Alton *et al.* (1988).

*Brucella* broth fermentation Medium C was used successfully to optimize the yields without affecting the colonial phase. No correlation between OD and viable counts could be established. This could probably be attributed to the presence of non-viable cell mass. The results of colony counting clearly show that harvesting at 48 hours after inoculation was found more appropriate than after 60 hours.

In this study controlling agitation speed and air flow rates at 250-450 rpm and 0.5-0.8 vvm, yielded a mean viable count of  $3.43 \times 10^{11}$  to  $3.2 \times 10^{11}$  CFU per ml respectively. The results agree with the results of Alton *et al.* (1988) and Shapouri *et al.* (2007) who obtained viable counts of  $2.5-3.0 \times 10^{11}$  and  $3.0-3.3 \times 10^{11}$  CFU per ml after 48 hours and 60 hours of culturing, respectively using other media compositions. Van Drimmelen (1956) obtained viable brucella organisms of  $1.9-2.5 \times 10^{11}$  CFU per ml after growing the S19 culture for 70 hours in fermenter. Hauschild *et al.* (1961) reported that *B. abortus* S19 culture was grown in continuous culture for 3 weeks and obtained  $2.0 \times 10^{11}$  CFU per ml.

The highest count recorded for an autoclaved medium was  $1 \times 10^{11}$  per ml after 48 hours by Zavanella *et al.* (1984).

Lyophilized vaccine is considered superior to liquid vaccine because of its stability and longer shelf life. The optimal pre lyophilization cell suspension contained  $2-3.5 \times 10^9$  CFU per ml (Angus, 1984). Lyophilization was carried out with an initial bacterial count of  $3 \times 10^{11}$  CFU per 5 doses. The viable *brucellae* count was found to be  $2.6-2.8 \times 10^{11}$  CFU per 5 doses after freeze drying process. The loss due to freeze drying ranged from 6.7% to 8.6%. The freeze-dried vaccine complied with OIE standards for dissociation, identity, sterility and safety when it was subjected to various quality control tests (OIE Manual, 2008).

The potency of the freeze-dried vaccine was evaluated in mice model (Bosserey *et al.*, 1984). Because of the high costs and long time span of the experiments in natural hosts, mice have been used to test the potency of vaccines against brucellosis (Montaraz *et al.*, 1986). OIE approved protocol allows the prediction of the safety and immunogenicity of *B. abortus* S19 vaccine. In the present study mice models were used to study the protective index of *B. abortus* S19. The *B. abortus* S19 internal reference vaccine gave the protective index value 3.02. The *B. abortus* S19 vaccine prepared from soyabean casein digest medium gave more than  $2 \log_{10}$  protection i.e., 3.33 which indicated that vaccination with *B. abortus* S19 induced protective immunity that allowed the animal to clear 90% of the wild type challenge organism within the first two weeks. The results of the present study corroborate with the specification of immunogenicity test prescribed in the OIE Manual (2008).

Lyophilized vaccine was tested in cattle calves and sero-conversion was checked by FPA. Cattle calves tested by FPA prior to vaccination with *B. abortus* S19 vaccine were sero negative for *brucella* antibodies.

All the cattle calves vaccinated with S19 elicited positive response 28 days after vaccination and remained sero-positive during 60<sup>th</sup> to 150 dpv. On 180 dpv antibody levels in 40% of the animals decreased to lower level. These results indicate that cattle vaccinated with S19 vaccine produce antibodies that can be detected by FPA.

The findings of the present study corroborate with the results of Stevens *et al.* (1994) who reported that cattle generally had positive or suspect response during 2 to 10 weeks after vaccination with S19. The results of this study are in accordance with the findings of Aguirre *et al.* (2002) who reported that FPA reached a maximum of 68.5% positive animals at week 4 and then declined slowly thereafter.

The results of the present study demonstrated that *B. abortus* S19 can be cultivated using Soyabean casein digest medium in stirred tank bioreactor. The organism maintained its morphological characteristics throughout the fermentation process. Potency in mice and immunogenicity in cattle was similar to that of vaccine produced in potato infusion agar medium using surface culture method.

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