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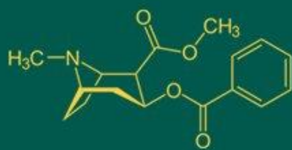

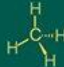
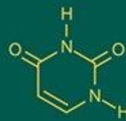
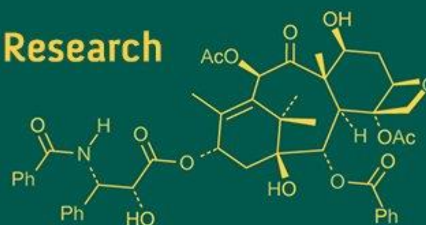

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Optimization of growth conditions for *Dichelobacter nodosus* in a modified reducing broth without gas phase

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Abstract
Virulent footrot represents as a significant contagious ailment affecting hooves of sheep, with *Dichelobacter nodosus* being the primary agent responsible for transmitting the disease. Vaccinations and various management practices are utilized to control the disease. *D. nodosus* is a highly fastidious anaerobic bacterium and it is very difficult to grow under laboratory conditions. For the mass production of bacteria as needed for vaccine manufacturing, it must be cultivated in a broth culture. In this study, the broth culture of *D. nodosus* was optimized and simplified under reducing conditions with three different strains belonging to serogroup B and E. The bacteria were grown in TAS broth supplemented with sodium carbonate (2 mg/ml) and differing levels of sodium thioglycolate (3 to 10 mM), with no gas supplementation, at 37 °C in an incubator. The most favorable growth conditions for the *D. nodosus* strain JKS-07B (serogroup B) were observed in a broth containing 6mM sodium thioglycolate with a pH of 4.8, before autoclaving and the subsequent addition of sodium carbonate. The bacterial cell density in the broths ranged from 1.9×10^8 to 2.5×10^8 CFU/ml, which seemed to be optimum for vaccine formulation.

Keywords: Broth culture, *D. nodosus*, Footrot, pH, Sodium thioglycolate

Introduction
Ovine virulent footrot is an important infectious bacterial disease in sheep that leads to lameness. The disease is marked by an initial stage of painful interdigital dermatitis, which can advance to the separation of hoof horn from the underlying tissue. This progression results in severe lameness, accompanied by a distinctive foul smell at all stages of the disease (Egerton *et al*., 1989; Kennan *et al*., 2011) [9, 14]. Footrot significantly diminishes the productivity, breeding efficiency, and overall welfare of the affected animals (Egerton *et al*., 2004, Nieuwhof and Bishop, 2005; Wassink *et al*., 2010) [7, 16, 29]. It is caused by the synergistic action of certain bacterial species, with *Dichelobacter nodosus* (*D. nodosus*) acting as the main transmitting agent (Thomas, 1964) [26]. The disease is a result of the interaction between the bacterium and the hoof epithelium, facilitated by warm and humid environmental conditions (Beveridge, 1941; Egerton *et al*., 1969) [1, 8]. The clinical manifestation varies based on the severity of lesions, which is influenced by the strains of *D. nodosus*. Virulent isolates of *D. nodosus* are distinguished by their capacity to produce heat-stable proteases (Stauble *et al*., 2014) [23]. Other organisms, particularly *Fusobacterium necrophorum*, may have a significant role in either aiding the development of footrot (Egerton *et al*., 1989) [9] or intensifying its severity (Witcomb *et al*., 2014) [30]. There are multiple serogroups of *D. nodosus* (A-I and M) distinguished by their type IV fimbrial antigen and immunity to the bacterium is always serogroup specific (Claxton *et al*., 1983; Claxton 1989; Ghimire *et al*., 1998) [4, 3, 12]. The expression of pili or fimbriae in bacteria is influenced by environmental factors such as temperature (Salzer *et al*., 2014) [20] and the consistency of media (Hendrickx *et al*., 2008) [13].
Footrot has been found to be endemic in Kashmir for last two decades, resulting in substantial economic losses for sheep farmers (Wani *et al*., 2004; Farooq *et al*., 2010; Wani

et al., 2019) [28, 10, 27]. Serogroups A, B, C, E, F and I of *D. nodosus* have been reported in India (Farooq *et al*., 2010, Sreenivasulu *et al*., 2013) [10, 22]. Among these serogroups, B was found to be predominant (>90%) in Kashmir (Wani *et al*., 2019) [27]. Based on this information, a serogroup B specific vaccine was formulated in our laboratory (unpublished data) using *D. nodosus* grown on plate culture. The challenge of maintaining anaerobic conditions complicates the growth of this bacterium on solid media in laboratory. Moreover, when grown in broth culture, the bacterium requires anaerobic and fastidious growth conditions. In this study, we optimized and simplified the growth conditions of *D. nodosus* in broth culture under reducing conditions.

Materials and Methods

Bacterial strains and media

The virulent strain JKS-07B of *D. nodosus* belonging to serogroup B (GenBank accession no NZ_SRJB00000000) (Qureshi *et al*., 2021) [18] and serotype B5 (Bhat *et al*., 2012) [2] was used as a reference strain in the present study for optimization of broth culture. In our laboratory, this strain was employed for vaccine formulation after being cultivated as a plate culture. Further, virulent strains JKS-11B (serogroup B) and JKS-25E (serogroup E) maintained in our laboratory were also included for optimization of broth culture. The strains were revived on TASH (tripticase arginine serine hoof) agar plates and their purity was checked. Subsequently, they were confirmed by serogroup specific PCR as described by Dhungyel *el al.* (2002) [5]. TAS broth was prepared by adding tryptose 15gm, proteose peptone 5gm, HM peptone B 5gm, yeast extract 5gm, L-arginine 5gm, DL-Serine 1.5gm and Magnesium sulphate heptahydrate ($MgSO_4.7\ H_2O$) 2 gm in 1 litre of distilled water. The media components were procured from HiMedia Laboratories Pvt Ltd, India. Appropriate concentration of sodium thioglycolate was added and pH was adjusted. The medium was taken in 500 ml screw capped bottles leaving little space for proper autoclaving. Additionally, a separate solution of 20% sodium carbonate (Na_2CO_3) was prepared and autoclaved. It was then aseptically added to the autoclaved broth at a rate of one ml per 100 ml, resulting in a final concentration of 2 mg per ml. (Skerman, 1975) [21]. The space left in the bottle prior to autoclaving was carefully filled, ensuring the absence of bubbles, up to the brim with broth autoclaved separately. The bottle was subsequently placed in the incubator under airtight conditions overnight to check its sterility. The pH of the broth and the reducing agent (sodium thioglycolate) were optimized as follows.

Optimization of pH

Initially, four TAS broths were prepared as described above with 0.1% sodium thioglycolate and pH was adjusted at 4.7, 4.8, 4.9 and 5.0, respectively before autoclaving and addition of Na_2CO_3.

Pieces of agar, each containing an equivalent number of colonies of *D. nodosus* (strain JKS-07B) from the TASH (trypticase arginine serine and hoof) agar plates, were inoculated into the broths, which were then incubated at 37˚C in a conventional laboratory incubator for 40 hrs. After incubation, the turbidity of each broth culture was measured at OD600 by a spectrophotometer (Biophotometer, Eppendorf, Germany). The experiment was repeated three times for each strain, with *D. nodosus* subcultured from a fully grown culture bottle at a ratio of 1 ml per 100 ml. The OD600 values of the inoculums for all the strains were standardized to ensure an equal number of bacteria. The broth with a specific pH, having a higher average OD 600 value compared to the others, was considered as optimal.

Optimization of reducing agent

TAS broths with optimized pH were prepared as above in triplicate, with varying concentration of sodium thioglycolate ranging from 3 to 10 mM. The broths were inoculated with freshly cultured *D. nodosus* strains @ 5 ml/ bottle and incubated as before at 37˚C for 40 hours. The turbidity of the broths was measured at OD600 by the spectrophotometer as above. The variations of OD600 values of broths were analyzed statistically by one way analysis of variance using SPSS software. The broth with a particular concentration of sodium thioglycolate, leading to a significantly higher OD600 value, was regarded as the optimum.

Results

Effect of pH on the growth of *D. nodosus*

In this study, the pH of the TAS broth was initially adjusted to 4.7, 4.8, 4.9, and 5.0, before autoclaving and the subsequent addition of Na_2CO_3 (2 mg/ml) to optimize the pH using *D. nodosus* strain JKS-07B. Visible turbidity was evident after 24 hrs of incubation in all the bottles in which the pH was adjusted to 4.7, 4.8 and 4.9, before autoclaving and the subsequent addition of Na_2CO_3. Nevertheless, growth was only observed after 40 hours of incubation in the bottles where the pH was adjusted to 5.0. Details of the OD600 value of different broth cultures after 40 hrs of incubation are given in Table 1. The optimal growth was noted at a pH of 4.8. The final pH of TAS adjusted to pH 4.8 before autoclaving was found to be 6.8-6.9 after addition of Na_2CO_3. There was fluctuation of pH, if Na_2CO_3 was added to the TAS broth before autoclaving and consistent results were not obtained.

Effect of sodium thioglycolate concentration on the growth of *D. nodosus*

To assess the effect of sodium thioglycolate on the growth of different strains of *D. nodosus*, the pH of the broths was adjusted to 4.8 before autoclaving and the addition of Na_2CO_3, with varying concentrations of sodium thioglycolate. Growth of *D. nodosus* in the broths varied with variations in the concentration of sodium thioglycolate. Details of the OD600 values of different strains are given in Table 2. The differences in OD600 values among various broths with different concentrations of sodium thioglycolate were statistically significant ($p<0.05$). The most favorable growth was observed in the broth containing 6 mM sodium thioglycolate. Similar results were shown by all the three strains of *D. nodosus*. Growth of *D. nodosus* strain JKS-07B was found to be better in comparison to strain JKS-11B and JKS-25E. The above condition gave consistent results in our subsequent cultures of *D. nodosus*. Further, the broth could be subcultured up to 10 weeks from the culture bottles stored at $4^{O}C$. However, it took longer time to see visible turbidity at later stages of subcultures.

The concentration of *D. nodosus* in the broth culture ranged approximately from 1.9×10^8 to 2.5×10^8 CFU/ml of broth culture. The bacteria in the broths were generally shorter and wider than the agar cultures. There was little or no lysis of bacilli after about 64 hrs of incubation.

Discussion

Dichelobacter nodosus is a Gram negative, non-spore-forming rod and obligate anaerobic bacterium (Garrity *et al*., 2005; Raadsma and Egerton, 2013) [11, 19], sensitive to dryness and requires special medium for growth, which makes its culturing and isolation difficult. Hoof agar, TAS agar and Eugon agar are suggested for its isolation (Stewart and Claxton, 1982) [24]. Cultivating on solid medium requires a considerable amount of time and the strict maintenance of anaerobic conditions. Moreover, the reduced yield on solid media and the cumbersome harvesting procedure, in comparison to liquid media, contribute to increased costs in vaccine production. To overcome these challenges and facilitate the production of large biomass, liquid culture is a more advantageous option. Thomas (1963) [25] employed a liquid medium containing a reducing agent, acid-hydrolyzed horn or wool, and auto-digested pancreas or trypsin, which facilitated substantial growth of cultures within 3 to 5 days under nitrogen. This medium was further modified by enriching it with yeast and liver extracts, bovine blood, amino acids and various protein digests (Parsonson *et al*., 1967; Marsh and Claus, 1970; Egerton, 1974) [17, 15, 6]. Skerman (1975) [21] used TAS medium which also included lab-lemco, proteose peptone, yeast extract, $MgSO_4$, thioglycolic acid and Na_2CO_3 for the growth of *D. nodosus*.

The fastidious and anaerobic nature of the organism poses a challenge for mass production of vaccines containing well-piliated organisms of representative serogroups of *D. nodosus*. In this study, we have addressed this problem by growing the organism in TAS broth in presence of Na_2CO_3 and reducing agent sodium thioglycolate. Although *D. nodosus* was grown in TASH agar at pH 7.8-8.0 in our laboratory, it was unable to grow at this pH in TAS broth. Therefore, we utilized the TAS medium as described by Skerman (1975) [21], but without the gas phase. Various pHs, adjusted before autoclaving and addition of Na_2CO_3 into the broth, were experimented with to achieve the maximum growth and it was observed that pH of 4.8 (initial) exhibited optimal growth. After optimizing pH, different concentrations of sodium thioglycolate were tested to find the minimum concentration that would yield maximum growth and make the process cost effective. It was found that a concentration of 6mM of sodium thioglycolate yielded the maximum growth. Skerman used 10 mM of sodium thioglycolate in his experiment and found that results were better when sodium thioglycolate was added into the media before autoclaving (Skerman, 1975) [21]. However, he recommended thioglycolic acid solution as reducing agent as it gave more reproducible results. In our specific scenario, we noticed that the growth of *D. nodosus* was both sluggish and reduced in TAS broth supplemented with 10 mM sodium thioglycolate. The concentration of *D. nodosus* varied from 1.9×10^8 to 2.5×10^8 CFU/ ml in broth culture in the present study. Even though we excluded CO_2 from the broth, our results were comparable to those reported by Skerman (1975) [21], who observed a range of 1.75×10^8 to 8.0×10^8 CFU/ml of broth across the 20 strains of *D. nodosus* investigated. The increased bacterial concentration observed in Skerman's study could be attributed to the use of CO_2 gas or potential variations among the strains of *D. nodosus*. We also observed that the bacteria in the broths were shorter and wider than the agar cultures with occasional filamentous forms and there was no extensive lysis of the bacilli after prolonged incubation of about 64 hrs.

Table 1: Effect of pH on growth of *D. nodosus* in TAS broths containing 0.1% soduim thioglycolate before autoclaving and addition of Na_2CO_3.

Strain	pH	Broth 1	Broth 2	Broth 3	Average
JKS-07B	4.7	0.596	0.580	0.550	0.575
	4.8	0.772	0.712	0.798	0.761
	4.9	0.649	0.690	0.595	0.645
	5.0	0.378	0.401	0.435	0.405

Table 2: Effect of sodium thioglycolate concentration (mM) on the growth of different strains of *D. nodosus* in TAS broth.

Strain	Sodium thioglycolate Concentration (mM)	ODs			Average ODs
		Broth 1	Broth 2	Broth 3	
JKS-07B	3	0.612	0.67	0.58	0.620
	4	0.791	0.786	0.753	0.776
	5	0.772	0.812	0.75	0.778
	6	0.917	0.99	0.873	0.926
	7	0.805	0.776	0.83	0.804
	8	0.739	0.725	0.77	0.745
	9	0.714	0.78	0.693	0.729
	10	0.701	0.75	0.66	0.704
JKS-11B	3	0.63	0.58	0.56	0.590
	4	0.68	0.59	0.628	0.633
	5	0.65	0.694	0.678	0.674
	6	0.767	0.73	0.794	0.764
	7	0.752	0.663	0.715	0.710
	8	0.694	0.689	0.728	0.703
	9	0.659	0.629	0.679	0.656
	10	0.673	0.649	0.642	0.654
JKS-25E	3	0.513	0.572	0.43	0.505
	4	0.681	0.69	0.643	0.671
	5	0.71	0.697	0.682	0.696
	6	0.86	0.81	0.794	0.821
	7	0.765	0.782	0.743	0.763
	8	0.747	0.713	0.711	0.719
	9	0.693	0.637	0.667	0.684
	10	0.639	0.678	0.694	0.678

Conclusion

Dichelobacter nodosus exhibited optimal growth when cultured in TAS broth containing sodium thioglycolate, supplemented with Na_2CO_3, and in the absence of CO_2 gas phase. Optimal pH and a precise concentration of the reducing agent sodium thioglycolate were effective in promoting rapid and maximum growth, thereby simplifying the growth of the bacterium in broth media.

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Declaration of Conflict of Interest

The authors declare that they have no known conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

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