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OPTIMAL CULTURE CONDITIONS FOR THE PROPAGATION OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* (APICOMPLEXA) IN PROTEIN DEFICIENT MEDIUM

LA PEYRE J.F.* & FAISAL M.*

Summary :

The protozoan, *Perkinsus marinus*, acclimated and proliferated in the culture medium JL-ODRP-1 without bovine serum albumin. The principal culture conditions for the optimal propagation of *P. marinus* in this protein deficient culture medium, were determined. The greatest growth rates of the parasite occurred at 28 °C, 661 mOsm/kg and pH 7.5. Decreasing seeding densities from 16 x 10^5 to 1 x 10^5 also increased growth rate. After several passages in the absence of 5 % CO_2 tension, the growth rate of *P. marinus* was similar to its original value in the presence of 5 % CO_2 tension. This protein deficient culture medium is ideally suited to study parasite-derived proteins since *P. marinus* extracellular proteins in culture supernatants were produced and were easily detected by gel electrophoresis and silver staining.

KEY WORDS : *Perkinsus marinus*, protozoan, culture, *in vitro*.

MOTS CLÉS : Perkinsus marinus, *protozoaire, culture*, in vitro.

Résumé : CONDITIONS OPTIMALES DE CULTURE D'UN PARASITE D'HUITRE, *PERKINSUS MARINUS* (APICOMPLEXA), DANS UN MILIEU PAUVRE EN PROTÉINE

Le protozoaire, Perkinsus marinus, *s'est acclimaté et a proliféré dans le milieu de culture JL-ODRP-1 sans albumine de sérum bovin. Les conditions principales de culture pour la prolifération optimale de* P. marinus *dans ce milieu pauvre en protéine ont été déterminées. Le taux de croissance du parasite était maximum à une température de 28 °C, une osmolarité de 661 mOsm/kg et un pH de 7,5; il a été augmenté en diminuant l'inoculum de 16 x 10^5 à 1 x 10^5 cellules/ml. Après plusieurs passages sans pression de 5 % CO_2, le taux de croissance des cellules était similaire à sa valeur originelle avec pression de 5 % CO_2. Ce milieu de culture, pauvre en protéine, est idéal pour étudier les protéines dérivées du parasite puisque des protéines extracellulaires ont été produites et ont pu être facilement détectées après séparation électrophorétique et coloration argentique.*

INTRODUCTION

Infection with the apicomplexan *Perkinsus marinus* has been associated with heavy mortalities in the eastern oyster, *Crassostrea virginica*. The breakthrough in propagating this deadly protozoan *in vitro* has enabled studies to identify its potential virulence factors. For example, we have recently demonstrated that *P. marinus* produces potent proteolytic factors *in vitro* (La Peyre and Faisal, 1995*a*; La Peyre *et al.*, 1995). The nature of the parasite–derived factors and their roles in pathogenesis of the disease remain to be determined. These investigations will require intensive biochemical analysis.

In our laboratory, *P. marinus* continuous cultures are maintained *in vitro* in the medium JL-ODRP-1 originally made up to resemble the known composition of oyster plasma (La Peyre *et al.*, 1993; La Peyre and Faisal, 1995*b*). This partly defined medium consists of more than 60 defined constituents and is supplemented with bovine serum albumin (BSA), cod liver oil, and yeastolate ultrafiltrate.

The culture medium JL-ODRP-1 presented two major advantages over commercial media used to propagate *P. marinus* in that: 1) the absence of animal sera eliminated problems associated with the use of complex, variable mixtures of proteins in controlled experiments (Barnes and Sato, 1980; Maurer, 1992), 2) each constituent of the medium could be easily adjusted to any desired value, a property essential to some studies such as those addressing the osmolality tolerance of *P. marinus* (O'Farrel *et al.*, 1995; O'Farrel, 1995).

Unfortunately, the presence of BSA in the culture media complicated the identification of *P. marinus* extracellular proteins (ECP) bands in samples analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, La Peyre and Faisal, 1995*a*). In addition, our observations and those of others (Dungan and Hamilton, 1995) suggest that batches of BSA show variable degrees of toxicities to cultured *P. marinus* cells. This study was, therefore, designed to determine the optimal culture conditions under which *P. marinus* would propagate in BSA-free JL-ODRP-1 medium and secrete ECP.

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MATERIALS AND METHODS

Effects of BSA on the propagation of *Perkinsus marinus*

The cloned isolate of *P. marinus, Perkinsus*-1 (La Peyre *et al.*, 1993), was used throughout this study. Stock cultures of *Perkinsus*-1 cells were maintained in 75 cm^2 culture flasks (Corning Glass Works, Corning, NY) suspended in 50 ml of JL-ODRP-1 culture medium (4 mg/ml BSA, La Peyre *et al.*, 1993). The cultures were incubated at 28 °C in a humidified chamber in the presence of 5 % CO_2 tension. Cells were subcultured every four weeks at a seeding density of 10^6 cells/ml.

To compare the growth rate of *Perkinsus*-1 in BSA-containing and BSA-free JL-ODRP-1 media, cells harvested from the stock cultures were first rinsed and resuspended in either medium, and then propagated in 50 ml of the appropriate medium in triplicate flasks (75 cm^2). Samples of cultured cells (1.5 ml) were collected on days one, three, five, seven, nine, twelve and fifteen post-seeding. The morphology of cultured cells and type of division was observed with a Olympus (CK-2) inverted light microscope with phase contrast optics at a magnification of 400 X. The cell density in each sample was measured with a Bright-Line hemacytometer (Reichert, Buffalo, NY) after three passages through a 25 gauge needle. Viability was evaluated by staining with 0.005 % (w/v) of neutral red. The growth rate of *Perkinsus*-1 was further measured following the second, third and tenth subcultures in BSA-free medium to determine the ability of the protozoal cells to acclimate to the new medium.

Suitability of BSA-free JL-ODRP1 medium for ECP's identification

The protein profiles of cell-free culture supernatants were compared in JL-ODRP-1 in the presence and absence of BSA. Contents of four week old cultures containing *Perkinsus*-1 suspended in either medium were centrifuged at 800 g for 15 min and the supernatants collected, passed through a 0.2 µm filter. The supernatants from cultures with no BSA were further concentrated about 30 folds with a Macrosep™ 10K concentrator (Filtron, Northborough, MA). Supernatants were then stored at – 20 °C. The proteins in cell-free culture supernatants were separated by reducing SDS-PAGE on a protean II vertical slab gel unit (Bio-Rad, Richmond, CA) with an 8 % running gel and a 3 % stacking gel (Laemmli, 1970). Preparation and electrophoresis of the samples were done as described in La Peyre *et al.* (1995) except that mercaptoethanol was added to the sample buffer prior to boiling at 95 °C for 5 min.

Optimization of *P. marinus* growth in BSA-free medium

Stock cultures of *P. marinus*

All optimization experiments were conducted with cells subcultured at least ten times in BSA-free JL-ODRP-1. Stock cultures of *Perkinsus*-1 were maintained in BSA-free JL-ODRP-1 medium as described above.

Osmolality

Perkinsus-1 was propagated in media prepared with 10, 20, 30 or 40 g/L of seawater synthetic basal salt mixture (Sigma Chemical co.). Sodium bicarbonate (2 g/L, 42 mM) and Hepes (25 mM) were used as buffers in the media. The concentration of potassium chloride was adjusted to maintain a Na/K ratio of 23. The media final osmolalities were 372 ± 1, 661 ± 2, 963 ± 3 and 1273 ± 1 mOsm/kg. The pH of all culture media was adjusted to 7.5. The salt composition and osmolality of each medium are depicted in Table I. Cells cultured in the standard medium (*i.e.*, 22 g/L synthetic salts, 724 mOms/kg) were harvested, washed twice, and then resuspended in media with predesignated osmolalities. Flasks were then seeded and incubated in 5 % CO_2 atmosphere at 28 °C. Doubling time was calculated based on cellular growth between the first and seventh days of culture.

pH

Three pH values were compared initially for their ability to influence *Perkinsus*-1 propagation. The pH of the culture medium was adjusted to 7.0, 7.5 and 8.0 with either 1 N NaOH or HCl. Cells cultured in a medium of pH 7.5 and osmolality of 725 mOsm/kg were harvested, washed twice, and then resuspended in media with predesignated pH values. Flasks were seeded and incubated in 5 % CO_2 atmosphere at 28 °C. The propagation of cultured cells was assessed on days one, three, five, seven, nine, twelve and fifteen as described above.

Osmolality (mOsm/kg)	372 ± 1	661 ± 2	963 ± 3	1273 ± 1
SW synthetic salts[a] (g/l)	10.0	20.0	30.0	40.0
Sodium bicarbonate (g/l)	2.0	2.0	2.0	2.0
Potassium chloride (g/l)	0.103	0.161	0.218	0.273
Salinity[b] (~ppt)	14	24	34	44

[a] The seawater (SW) synthetic basal salt mixture was obtained from Sigma Chemical co. and did not contain sodium bicarbonate.
[b] Salinity of sea salt solution that has an osmolality equivalent to the osmolality of the tested medium.

Table I. — Salt composition and osmolality of media used in optimization experiment.

Effect of CO_2 tension

Propagation of *Perkinsus*-1 was compared in the presence or absence of 5 % CO_2 tension. Buffering of the medium in atmospheric CO_2 was provided by 4 mM of sodium bicarbonate and 10 mM of Hepes as recommended by Freshney (1987). In addition the Na/K ratio was maintained at 23. The salt and buffer concentrations in media prepared to propagate cells in 5 % CO_2 supplemented atmosphere and atmospheric CO_2 are displayed in Table II. Cells cultured in a medium of pH 7.5, osmolality of 725 mOsm/kg, and 5 % CO_2 were harvested, and then resuspended in the designated media. The flasks were seeded and then incubated at 28 °C in the presence or absence of 5 % CO_2 atmosphere. The propagation of cultured cells was assessed on days one, three, five, seven, nine, twelve and fifteen as described above. Several subcultures of *Perkinsus*-1 in atmospheric CO_2 were done to find out if the protozoal cells could acclimate to this new culture condition.

Temperature

Propagation of *Perkinsus*-1 cells, acclimated to the absence of 5 % CO_2 tension, was compared at 17, 23, and 28 °C. In addition, the ability of the cultured cells to survive at 4 and 36 °C was evaluated. Cells cultured in a medium of pH 7.5, and osmolality of 725 mOsm/kg were harvested and then resuspended in fresh medium. The flasks were seeded as described above and incubated at designated temperatures (also in the absence of 5 % CO_2). Doubling time was calculated based on cellular growth between the first and seventh days of culture.

Seeding density

The effect of seeding density on the growth rate of *Perkinsus*-1 was evaluated. Cells cultured in a medium of pH 7.5, osmolality of 725 mOsm/kg, in the presence of 5 % CO_2 were harvested, and then resuspended in fresh medium. The flasks were seeded at 1×10^5, 2×10^5, 4×10^5, 8×10^5 and 16×10^5 cells/ml then incubated at 28 °C in the presence of 5 % CO_2 tension. The propagation of the cultured cells was assessed on days one, three, five, seven, nine, twelve and fifteen as described above.

	5 % CO_2	Atmospheric CO_2
Synthetic salts	22.0 g	22.0 g
Sodium bicarbonate	2.0 g	0.336 g
Potassium chloride	0.1772 g	0.0296 g
Hepes (1 M solution)	25.0 ml	10.0 ml

Table II. — Salt and buffer composition per liter of media prepared for incubation at 5 % CO_2 or atmospheric CO_2 condition.

STATISTICAL ANALYSIS

Growth rate was expressed as doubling time (*i.e.*, the time for a population to double in number) during log phase. In this study, log phase was considered between day one and seven during the period of exponential growth. Doubling time data was $\log_{10}$ transformed and compared by one factor analysis of variance followed by SNK's multiple comparisons of means when significant differences ($p < 0.05$) were found.

RESULTS

PROPAGATION OF *P. MARINUS* IN BSA-FREE MEDIUM

Perkinsus-1 cells can proliferate without BSA. Their rate of growth, however, was significantly slower ($P = 0.0005$) compared to cells grown in medium supplemented with 4 mg/ml of BSA (Fig. 1). The doubling times (t_d) of cells grown in medium with and without BSA were 28.6 ± 0.7 h and 33.6 ± 1.7 h, respectively. *Perkinsus*-1 cells, however, could acclimate to BSA deprivation. Their growth rates increased in subsequent subcultures to attain a doubling time, after ten subcultures, of 29.9 ± 0.5 h which was similar to the growth rate of cells cultured in BSA-containing JL-ODRP-1 (Fig. 2). No apparent changes in the shape, size, or division pattern of cultured cells were observed due to the absence of BSA.

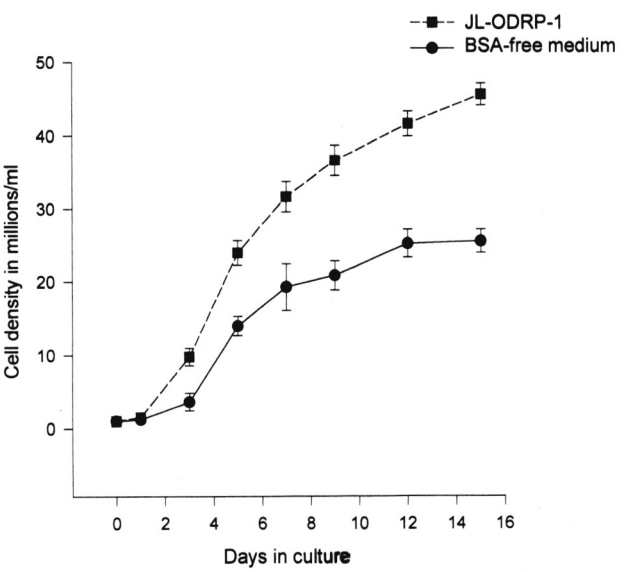

Fig. 1. – Propagation of *P. marinus* in JL-ODRP-1 medium with and without BSA. Cells were harvested after four weeks of culture in BSA-containing JL-ODRP-1 medium, washed twice, and then resuspended in either BSA-free or BSA-containing JL-ODRP-1. Triplicate flasks were seeded with 10^6 cells/ml and incubated in 5 % CO_2 atmosphere at 28 °C. The propagation of cultured cells was assessed on days 1, 3, 5, 7, 9, 12, and 15.

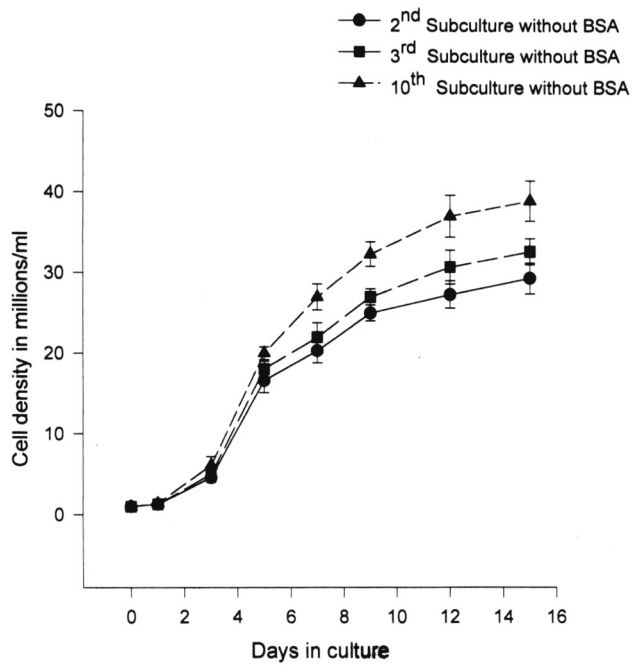

Fig. 2. – Acclimation of *P. marinus* to BSA-free JL-ODRP-1 culture medium. Cells were subcultured every two weeks at a seeding density of 10^6 cells/ml. Incubation temperature was 28 °C in the presence of 5 % CO_2 tension. The density of viable cells was measured on days 1, 3, 5, 7, 9, 12, and 15 after the second, third and tenth subculture.

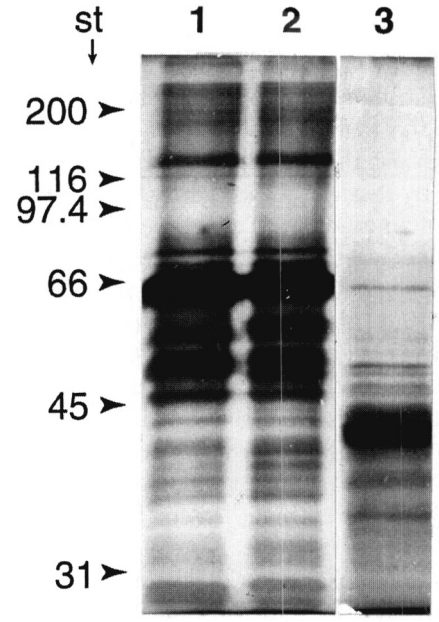

Fig. 3. – SDS-PAGE and silver staining of proteins in culture supernatants from flasks seeded with 1 × 10^6 cells/ml in JL-ODRP-1 medium with and without BSA. Flasks were maintained four weeks at 28 °C in 5 % CO_2. Lanes 1,2 and 3 contain 1 µg/lane proteins: 1) fresh BSA-containg JL-ODRP-1 medium, 2) inoculated JL-ODRP-1 medium, and 3) inoculated BSA-free JL-ODRP-1 medium.

Protein profiles of culture supernatants following SDS-PAGE

In the absence of BSA, *P. marinus* extracellular proteins (ECP) in culture supernatants were produced and were easily detected by SDS-PAGE electrophoresis (Fig. 3). In contrast, the presence of BSA and its breakdown products in the culture medium could evidently interfere with the analysis of *P. marinus* ECP bands. There were no proteins in the un-inoculated BSA-free culture medium.

Optimization of *P. marinus* in BSA-free JL-ODRP-1 medium

Osmolality

Perkinsus-1 cells propagated under all four media osmolality conditions. The rate of growth was significantly faster (P = 0.0069) in medium with an osmolality of 661 mOsm/kg as compared to growth rates in the media with the other three osmolality values tested (Table III). The doubling time was 30.1 ± 0.5 h for cells grown in the 661 mOsm/kg medium based on calculation of growth between day one and seven. In contrast, cells propagated in media with osmolalities of 372 and 1273 mOsm/kg during the first week of culture showed the lowest growth rates with a doubling time of 34.4 ± 1.8 h and 33.1 ± 1.2 h, respectively. The cell density of cultured cells after fifteen days was significantly greater (P = 0.018) in the medium of 661 mOsm/kg (33.4 ± 2.3 × 10^6 cells/ml than in the media of 372 mOsm/kg (23.9 × 4.1 × 10^6 cells/ml) and 1,273 mOsm/kg (23.3 ± 3.6 × 10^6 cells/ml).

Tested culture parameter (fixed conditions)	Doubling time[a] (h)
Osmolality (28 °C, pH 7.5, 5 % CO_2)	
372 mOsm/kg	34.4 ± 1.8
661 mOsm/kg	30.1 ± 0.5
963 mOsm/kg	31.0 ± 0.3
1,273 mOsm/kg	33.0 ± 1.2
pH (28 °C, 725 mOsm/kg, 5 % CO_2)	
7.0	32.4 ± 0.8
7.5	30.3 ± 0.5
8.0	34.5 ± 0.6
Temperature[b] (28 °C, pH 7.5, 725 mOsm/kg)	
4 °C	–
17 °C	97.6 ± 11.3
23 °C	33.6 ± 1.5
28 °C	29.2 ± 0.4
36 °C	–

[a] The doubling time was calculated between day one and seven.
[b] No 5 % CO_2 tension.

Table III. — Doubling times of *Perkinsus*-1 cells under varying culture conditions. Cells were seeded into 50 cm^2 flash at a density of 1 × 10^6 cells/ml.

pH

The growth rate of *P. marinus* was significantly greater ($P = 0.0006$) at pH 7.5 (t_d = 30.5 ± 0.5 h) than at either pH 7.0 (t_d = 32.4 ± 0.8 h) or 8.0 (t_d = 34.4 ± 0.6 h) (Table III).

Presence of 5 % CO_2

Upon transfer to atmospheric CO_2 conditions, the cultured cells showed a significant decrease ($p < 0.0001$) in their growth rate (t_d = 59.6 ± 3.2) as compared to cells cultured in the presence of 5 % CO_2 tension (t_d = 30.1 ± 0.5) (Fig. 4). In addition, the viability of cultured cells dropped by 25 % one week after their transfer from 5 % CO_2 tension. However, the growth rate and viability of the cells improved dramatically following subculturing. There was no apparent difference in the growth rate of *Perkinsus*-1 between cells grown with and without 5 % CO_2 tension after the third subculture (t_d = 29.9 ± 0.5) (Fig. 5). This finding suggests that *P. marinus* has the capacity to acclimate rapidly to changes in environmental conditions, such as changing CO_2.

Temperature

Propagation of *P. marinus* cells increased with increasing temperature between 17 and 28 °C (Table III). The growth rate of *P. marinus* between day one and day seven, was greater at 28 °C (t_d = 29.2 ± 0.4 h) than at 23 °C (t_d = 33.6 ± 1.5 h) or 17 °C (t_d = 97.6 ± 11.3 h). The cell density of cultured cells after fifteen days was highest at 28 °C (42 ± 3.1 × 10^6 cell/ml), intermediate at 23 °C (30.4 ± 3.0 × 10^6 cells/ml) and lowest at 17 °C (13.3 ± 1.2 × 10^6 cells/ml). Viability at 17, 23 and 28 °C was over 99 %. No growth was observed in cells maintained at 4 °C. The cultured cells maintained at 36 °C enlarged greatly (> 35 µm) during the first week of culture but did not divide. After two weeks at 36 °C, over 90 % of the cultured cells were dead.

Seeding density

The growth rate of cultured cells increased significantly ($p < 0.0001$) with decreasing seeding densities. During the first week of culture (day 1-7), cells seeded at 10^5/ml showed the greatest growth rate with a doubling time of 23.2 ± 0.7 h while cultured cells seeded at 16 × 10^5 cells/ml showed the lowest growth rate with a doubling time of 32.3 ± 0.5 h (Fig. 6). The growth rate of cells seeded at 10^5/ml was significantly greater than the growth rate of cells seeded at either 4, 8 or 16 × 10^5 cells/ml. A significant positive correlation ($r^2 = 0.910$; $p < 0.0001$) was found between seeding density and growth rate.

Number of cells in the flasks seeded with 1 × 10^5 cells/ml increased about 404 fold after fifteen days in culture. In contrast, the number of cells in the

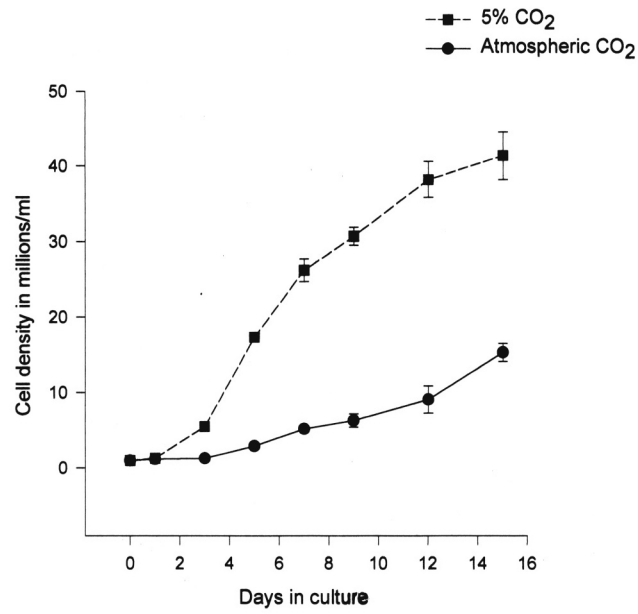

Fig. 4. – Propagation of *P. marinus* in BSA-free JL-ODRP-1 medium in the presence and absence of 5 % CO_2. Cells cultured in a medium of pH 7.5, osmolality of 725 mOsm/kg, and 5 % CO_2 were harvested, and then resuspended in the appropriate medium. The flasks were seeded with 10^6 cells/ml and then incubated at 28 °C in the presence or absence of 5 % CO_2. Cell densities were measured on days 1, 3, 5, 7, 9, 12, and 15.

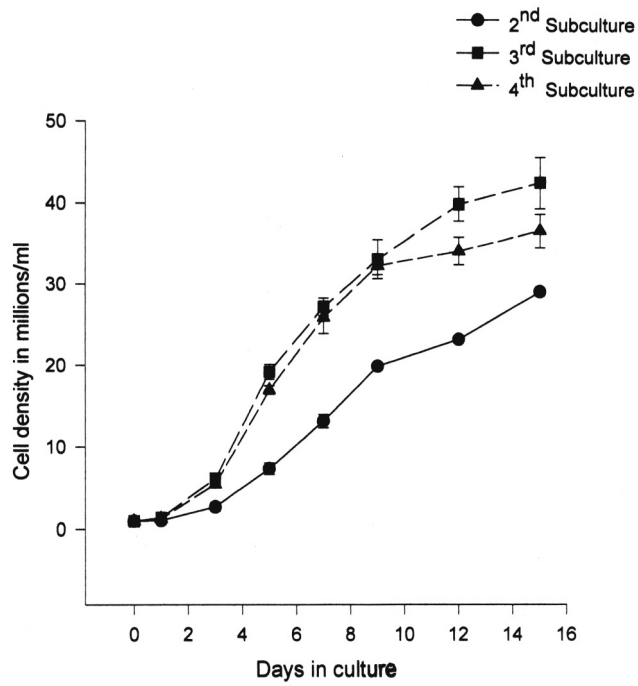

Fig. 5. – Acclimation and propagation of *P. marinus* in the absence of 5 % CO_2. Cells were subcultured every two weeks at a seeding density of 10^6 cells/ml in the absence of 5 % CO_2, at 28 °C. The number of viable cells was evaluated on days 1, 3, 5, 7, 9, 12, and 15 after the second, third and fourth subculture.

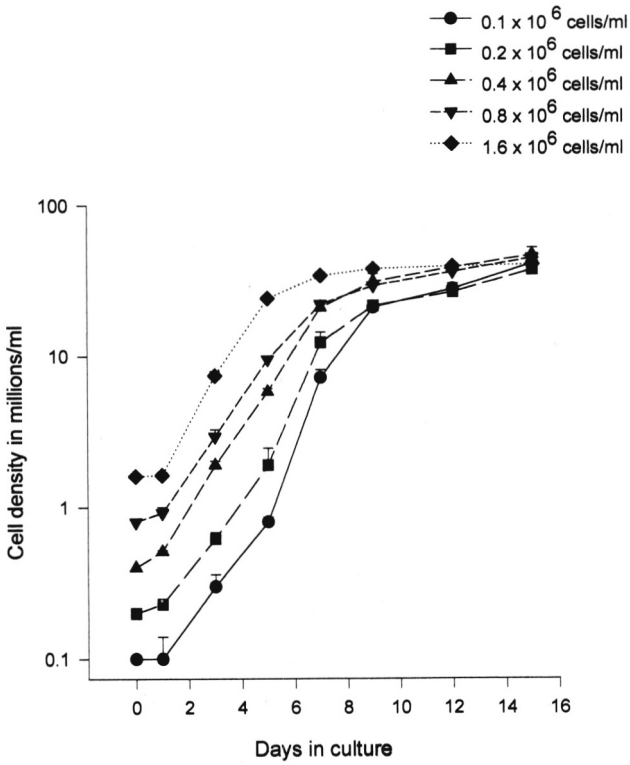

Fig. 6. – Propagation of *P. marinus* cells in BSA-free JL-ODRP-1 media in flasks seeded at 1, 2, 4, 8, 16 × 10^5 cells/ml. Cells cultured in a medium of pH 7.5 and osmolality of 725 mOsm/kg were harvested, washed twice, and added to flasks at the various seeding density. Flasks were incubated in 5 % CO_2 atmosphere at 28 °C. Cell densitites were measured on days 1, 3, 5, 7, 9, 12, and 15.

flasks seeded with 2 × 10^5, 4 × 10^5, 8 × 10^5 and 16 × 10^5 cells/ml increased about 187, 114, 55 and 25 fold respectively after fifteen days in culture. Interestingly, the lowest calculated doubling time for *Perkinsus-1* cells was 16.9 ± 0.1 h and occurred between day five and day seven in flasks seeded at the lowest cell density (1 × 10^5 cells/ml). Based on this finding, propagation of *Perkinsus*-1 is clearly accelerated if the number of seeded cells is lowered.

DISCUSSION

Our data showed that *P. marinus* can acclimate and propagate despite the absence of BSA from the culture medium. The doubling time for *Perkinsus*-1 was 16.9 ± 0.1 h between day five and seven, when seeded at a density of 10^5 cells/ml. This doubling time is comparable to the growth rates reported for *P. marinus* cultured in the commercial medium DME: Ham's F-12 nutrient mixture (1:1 v/v), a mixture of Dulbecco's modified eagle's medium and Ham's F-12 nutrient mixture, supplemented with 5-10 % fetal bovine serum (Gauthier and Vasta, 1993; Dungan and Hamilton, 1995). Moreover, *P. marinus* produced ECP in BSA-free medium that could be easily analyzed by SDS-PAGE.

The principal culture conditions for the optimal propagation of *P. marinus* in BSA-free JL-ODRP-1 medium were determined in this study. Previous studies of Dungan and Hamilton (1995) and Gauthier and Vasta (1995) have reported optimal conditions of *P. marinus* growth in protein-rich culture media (*i.e.*, FBS). It is difficult, however, to compare the findings obtained by these two studies and our results. Differences in the composition of culture media used, *P. marinus* isolates, seeding densities, assays used to obtain doubling times are among the factors that make a comparison between results of these three studies inaccurate. Therefore, only major differences between our findings and previously published reports will be discussed.

Temperature showed the greatest effect on the growth of *P. marinus in vitro*. In our study, the propagation of *P. marinus* increased as the temperature of incubation increased from 17 to 23 to 28 °C. At 36 °C however, the cultured cells enlarged, were unable to divide, and eventually died. Gauthier and Vasta (1995) reported that *P. marinus* optimal growth, as measured by optical density, was at 28-32 °C and decreased dramatically at 38 °C. In contrast to our findings, Gauthier and Vasta (1995) were unable to detect cellular growth at 20 °C. Based on the assessment of cellular growth during the first three days of culture, Dungan and Hamilton (1995) found near-maximal proliferation of cells between 15-35 °C. Caution must be exercised when interpreting Dungan and Hamilton (1995) results since *a*) their assay is based on the formation of formazan from tetrazolium salts by mitochondrial dehydrogenase, and *b*) data were collected after three days of culture.

Perkinsus marinus could be propagated in media with a wide range of osmolality. Our results indicated that the propagation of *Perkinsus*-1 was greater at 661 mOsm/kg (~24 ppt) than at either 372 mOsm/kg (~14 ppt) or 963 mOsm/kg (~34 ppt). In comparison, Dungan and Hamilton (1995) reported near optimal proliferation of *P. marinus* between 475 mOsm/kg (~17 ppt) to 959 mOsm/kg (~34 ppt) with a maximum at 794 mOsm/kg (~28 ppt). Moreover, Gauthier and Vasta (1995) found that growth of *P. marinus* was significantly lower at 18 ppt than at 24 or 30 ppt but not 36 ppt. From these studies it would appear that *P. marinus* growth is greatest in salinities in the range of 24-30 ppt. It is important to note, however, that the results obtained represent growth rates from cells cultured from stock solutions at 724 mOsm/kg (La Peyre and Faisal, this study), 650 mOsm/kg (Dungan and Hamilton, 1995) and 960 mOsm/kg (Gauthier and Vasta, 1995) and then transferred to the designated osmolalities without acclimation.

In our study, seeding density dramatically influenced the growth rate of *P. marinus*. The growth rate of *Perkinsus*-1 significantly increased as the seeding density decreased from 16×10^5 to 10^5 cells/ml. Presumably, the decreasing cell density increases the amount of nutrients available per cell. In contrast to this finding, Gauthier and Vasta (1995), using a protein-rich medium, reported decreasing growth rates with decreasing seeding densities.

The optimal pH for the propagation of *P. marinus in vitro* was 7.5 in our study which is within the range of oyster plasma pH. Our optimal pH agrees with that reported by Dungan and Hamilton (1995) but is higher than the optimal pH of 6.6 reported by Gauthier and Vasta (1995).

In conclusion, *P. marinus*, clearly, grew well in the protein deficient medium, BSA free JL- ODRP-1. It was also evident that *P. marinus* can tolerate and then acclimate to acute changes in culture conditions. The BSA-free culture medium is ideally suited to study parasite-derived proteins since their detection and purification is greatly simplified. Using the optimal conditions determined in this study, *P. marinus* was grown, with a short doubling time and without the need for exogenous proteins that can potentially interfere with biochemical analysis and interpretation of experimental data.

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REFERENCES

BARNES D. & SATO G. Methods of growth of cultured cells in serum-free medium. *Analytical Biochemistry*, 1981, *102*, 255-270.

DUNGAN C.F. & HAMILTON R.M. Use of tetrazolium-based cell proliferation assay to measure effects of *in vitro* conditions on *Perkinsus marinus* (Apicomplexa) proliferation. *Journal of Eukaryotic Microbiology*, 1995, *42*, 379-388.

FRESHNEY R.I. Culture of animals cells. A manual of basic techniques. Second edition, Wiley-Liss Inc., New York, 1987, 397 p.

GAUTHIER J.D. & VASTA G.R. Continuous *in vitro* culture of the eastern oyster parasite *Perkinsus marinus*. *Journal of Invertebrate Pathology*, 1993, *62*, 321-323.

GAUTHIER J.D. & VASTA G.R. *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *Journal of Invertebrate Pathology*, 1995, *66*, 156-168.

LA PEYRE J.F., FAISAL M. & BURRESON E.M. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *Journal of Eukaryotic Microbiology*, 1993, *40*, 304-310.

LA PEYRE J.F. & FAISAL M. *Perkinsus marinus* produces extracellular proteolytic factor(s) *in vitro*. *Bulletin of the European Association of Fish Pathologists*, 1995*a*, *15*, 28-31.

LA PEYRE J.F. & FAISAL M. Improved method for the initiation of continuous cultures of the oyster pathogen *Perkinsus marinus* (Apicomplexa). *Transactions of the American Fisheries Society*, 1995*b*, *124*, 144-146.

LA PEYRE J.F., SCHAFHAUSER D.Y., RIZKALLA E.H. & FAISAL M. Production of serine proteases by the oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *Journal of Eukaryotic Microbiology*, 1995, *42*, 451-458.

MAURER H.R. Towards serum-free chemically defined medium for mammalian cell culture, *in*: *Animal cell culture, a practical approach*. Freshney, R.I. (ed.), IRL Press at Oxford University Press, 1992, 15-46.

O'FARRELL C.L. Osmotic tolerance and volume regulation in cultured cells of the oyster pathogen *Perkinsus marinus*. Master thesis, College of William and Mary, Williamsburg, Virginia, 1995.

O'FARRELL C.L., LA PEYRE J.F. & BURRESON E.M. Acute osmotic tolerance of cultured cells of the oyster pathogen *Perkinsus marinus* acclimated to low salinity. *Journal Shellfish Research*, 1995, *14*, 274.

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