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ORIGINAL PAPER

Evaluation and monitoring of quorum sensing soluble mediators implicated in the regulation of bacterial growth in *Vibrio* strains

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Abstract

Quorum sensing (QS) is an ubiquitary regulation mechanism in the bacterial world implicated in intra and inter–bacterial communication and dependent upon the cellular density. In the present study, the authors have tried to elucidate the influence of soluble mediators accumulated in stationary phase cultures on the multiplication rate and growth curve of the homologous strains belonging to Vibrionaceae family. In this purpose, in the first step of the experiment, the growth curves of two bacterial strains, one non halophilic and one halophilic were comparatively established for the simple, control culture, culture treated by the homologous filtrate (containing soluble mediators) and culture treated by autoclaved filtrate (at 115^0 C and 130^0 C, respectively), incubated in small/big volumes at 4^0 C, 28^0 C and 37^0 C. Taking into account that the soluble mediators remain active in filtrate as well as in cultures treated at 115^0 C, for further experiments, there were used only simple, control cultures compared with cultures treated by simple filtrate, all of them being cultivated in small volumes and incubated at 37^0 C. The present study demonstrated that in stationary phase, bacterial cultures are accumulating soluble factors influencing the duration and aspect of the bacterial growth curve. In the most cases of the tested strains this influence consisted in the reduction of the multiplication rate and subsequently, of the culture density, the shortening of the lag phase and of the total duration of the growth curve. The synthesis of autoinducers proved to be dependent upon the bacterial strain, source of isolation (clinical case or aquatic environment), incubation temperature, volume of the culture medium, influencing the oxygenation surface.

Keywords: Quorum Sensing / non-halophilic / halophilic vibrios / autoinducers / lag phase / exponential phase.

Introduction

Quorum sensing (QS) is an ubiquitary regulation mechanism in the bacterial world implicated in intra and inter–bacterial communication and dependent upon the cellular density. The positive/negative regulation of bacterial virulence factors under the control of QS phenomenon is of crucial importance for the host-infectious agent inter-relation as well as for the understanding of the mechanisms of bacterial pathogenesis (1). The first and the best studied QS system was that mediated by pheromones of homo-serin-lactone (HL) structure, described in the Gram-negative bacteria (2, 14) and namely in bioluminiscent *V. fischeri* and *V. harveyi* (*Photobacterium*) with natural aquatic marine habitat as well as other *Vibrionaceae (V. cholerae, V. parahaemolyticus, V. alginolyticus, V. anguillarum*, *Aeromonas hydrophila),*

Enterobacteriaceae (Enterobacter sp., Serratia liquefaciens, Erwinia stuartii, E.carotovora, Yersinia enterocolitica), Pseudomonadaceae (Ps. aeruginosa) and *Agrobacterium tumefaciens* strains. It was demonstrated that in epidemic *V. cholerae* O1 and O139 (clinical and environmental strains), as well as in *V. cholerae* non O1 strains, the biofilm formation as well as the expression of certain soluble virulence factors (e.g. proteases) are under the control of (at least three) QS systems, exhibiting an unique hypostasis, their expression being present at low bacterial density and repressed at high cellular density, by comparison with other bacterial species (4, 8, 10, 11, 13).

The ability of the bacterial cells to modulate their multiplication rate correlated to the environmental changes is the key of the adaptation mechanism of *V. cholerae* strains to the aquatic / human host dual style of life (5, 7, 8). Considering these aspects, in case of *V. cholerae* strains it can be concluded that the QS mechanism can modulate the proper answer to stress conditions, playing an important role in bacterial surviving in the external medium (by biofilms formation when these microorganisms are exposed to marine water) (3, 4, 12, 15). The variation of QS systems is due to the selective pressure of the external medium and can be of benefit for the adaptation of *V. cholerae* strains to the variable conditions offered by the natural reservoirs.

In the present study, the authors have tried to elucidate the influence of soluble mediators accumulated in stationary phase cultures on the multiplication rate and growth curve of the homologous strains belonging to *Vibrionaceae* family.

Materials and Methods

i) Microbial strains

In this study there were isolated and analyzed thirteen *Vibrio* strains, out of which four were nonhalophilic strains (one *V. cholerae* serogroup O1 no. 200 / Tulcea clinical case, one *V. cholerae eltor* Inaba 35A, two *V. metschnikovii* no. 3303, 3561 from water sources) and nine halophilic from water sources (two *V. alginolyticus* no. 229 and 1560, three *V. fischeri* no.10, 754 and 898, two *V. anguillarum* no. 798 and 1545 and two *V. parahaemolyticus* no.1442 and 1671). One reference strain, i.e. *Escherichia coli* ATCC 29935, was used as negative control for oxidase reaction. All strains were preserved in the Microbial Collection of the National Reference Center for *Vibrio* of the National Institute for Research and Development in Microbiology and Immunology Cantacuzino.

ii) Methods

The isolated microbial strains were grown on BSA medium (Bile Salts Agar) and thereafter their biochemical features were identified by using an enlarged panel of 35 conventional biochemical tests: TSI (triple sugar iron agar- 5 tests), oxidase reaction, motility, growth at 37 ^{0}C, D-glucose (with /or without gas production assessed by Durham tube), ornithine-decarboxylase, lysine-decarboxylase, arginine-dihydrolase, lactose, sucrose, mannitol, mannose, maltose, arabinose, dulcitol, adonitol, inositol, sorbitol, trehalose, xylose and raffinose fermentation, Na citrate Simmons, nitrate reduction, indole production, methyl red and Voges Proskauer (on Clark medium) reactions, phenylalanine-desaminase, growth at different NaCl concentrations (i.e. 6%, 7,5%, 10 %), sensitivity to O/129 vibriostatic agent

(2,4-diamino-6, 7-diisopropylpteridine) 400 µg/disk (solution prepared in house), ONPG (orto-nitro-paraphenyl-galactopiranosidase) (16).

A positive ONPG reaction (in 30 min to 24 hrs) was considered as marker for the selection of nonhalophilic and halophilic *Vibrio* strains with functional QS systems as indicated in the literature (16).

For the selection of the optimal cultivation parameters, the growth curves of the bacterial cultures were established using different culture media such as: simple broth, TCBS (thioglycolate, citrate, bile salts, sucrose) and TSB (tryptone soy broth), the TSB medium being finally selected for further experiments.

The tested strains were cultivated on nutrient agar 2% for 24 hrs and from the fresh cultures there were prepared suspensions of 0.5 McFarland density in PBS (phosphate buffered saline). These suspensions were inoculated in TSB, incubated for 18 hrs at 37^0C. Subsequently the logarithmic phase cultures were centrifuged and sterilized by 0.22 µm membrane filtration in order to obtain the cell-free cultures, prospected to contain the highest amounts of QS soluble mediators (autoinducers).

For each strain, there were used simultaneously two 96 plastic well microplates ; each well of the first plate was inoculated with 90 µl TSB and 10 µl microbial suspension in order to obtain the growth curve in normal conditions whereas the second plate was used to put in light the possible role of autoinducers (accumulated in filtrate) to modulate the multiplication rate and growth curve of the homologous strains. In this purpose, each well from the second plate was inoculated with 90 µl filtrate obtained from 18 hrs bacterial cultures to which 10 µl of the homologous bacterial suspension were added.

The microbial cultures were harvested at different times of incubation (2 hrs, 6 hrs, 24 hrs, 48 hrs, 72 hrs) and spotted (10 µl of the adequate dilution) on solid media in order to establish the microbial growth curve and the time of logarithmic growth phase by performing viable cell counts.

After reading the values, the results were transferred on a logarithmic scale on the computer in the purpose to establish the bacterial growth curve.

For the respective two strains the growth curves were performed comparatively in small volumes, using the 96 wells microplate method as well as in big volumes of 120 ml bacterial culture.

Results and Discussion

Microbial strains identification

It must be noticed that taking into account, as cited in the literature, that the classical phenotypic schemes of identification proved to be not enough discriminative for identifying certain *Vibrio* phenotypes, the molecular methods (e.g. sequencing of luxA gene) being highly recommended (17), in the present study the authors selected and used a broader panel of biochemical tests (i.e. 35) for a more appropriate phenotypic identification of the *Vibrio* strains that had to be further investigated. Using this enlarged panel, the analyzed strains could be identified as: *V. cholerae, V. metschinikovii, V. fischeri, V. parahaemolyticus, V. alginolyticus* and *V. anguillarum.*

Optimisation studies for establishing the experimental conditions to evidence the presence of soluble mediators and their influence on the microbial growth

In the purpose to evidence the presence of autoinducers responsible for the QS activity, the selection of *Vibrio* strains was based upon the ONPG reaction, out of 26 *Vibrio* strains, being selected 13 ONPG-positive strains.

In order to establish the influence of these autoinducers (accumulated in stationary phase bacterial cultures) upon the regulation of bacterial growth, the growth curves of the selected strains were comparatively determined by cultivation in simple broth and respectively, in the presence of culture filtrates.

In case of on one non-halophilic (*V. cholerae* O1 no. 200/Tulcea) and one halophilic (*V. fischeri* no. 10) strains, the influence of heat inactivated (at 115^0 C and 130^0 C) filtrates, as well as of the incubation temperature (4^0 C, 28^0 C and 37^0 C) on the bacterial multiplication rate and growth curves was also investigated. For the respective two strains the growth curves were performed comparatively in small volumes, using the 96 wells microplate method as well as in big volumes of 120 ml bacterial culture. Therefore, the growth curves of the three respective bacterial cultures when treated with heat inactivated filtrates at 130^0 C superposed perfectly upon the simple bacterial cultures, demonstrating that at 130^0 C, the autoinducer substance was inactivated. By comparison, the growth curve of the culture treated by heat inactivated filtrate at 115^0 C superposed perfectly over that obtained for the culture treated by filtrate, suggesting that the respective autoinducer was resistant at to 115^0 C, but not at 130^0 C. When studying by comparison the aspect of growth curve of simple cultures and treated by filtrate/autoclaved filtrate at 115^0 C, as function of the incubation temperature, no bacterial growth was registered at 4^0 C, in case of *V. cholerae* O1 no. 200 Tulcea strain, while at 28^0 C and 37^0 C the cultures treated by simple/ heat inactivated filtrate at 115^0 C cultivated in small volumes, developed similar growth curves (Fig. 1, 2). Concerning the control cultures, at 37^0 C the cellular densities were lower and constant, comparing to 28^0 C, with an expected earlier decline phase at this last temperature (Fig. 1-2).

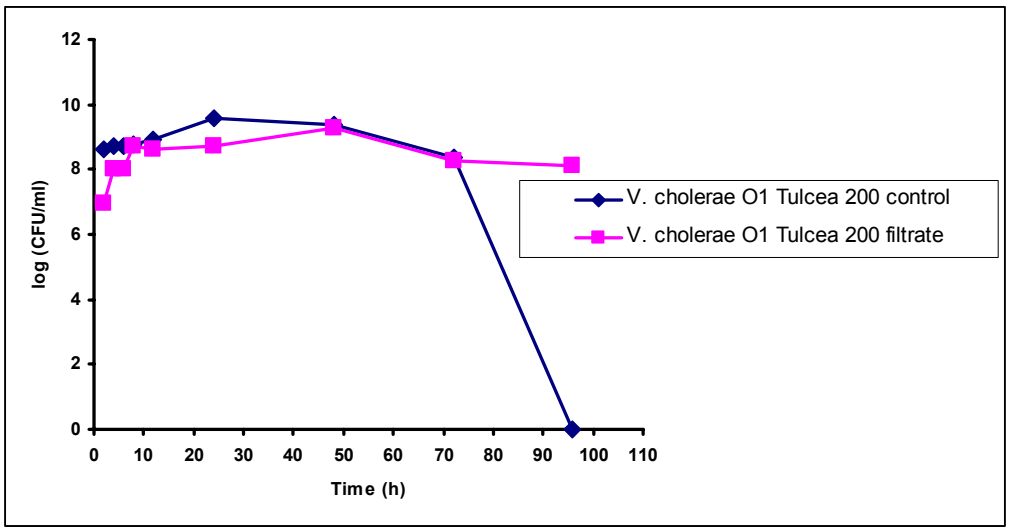

Figure 1. The comparative aspect of bacterial growth curves obtained after cultivation of the V. cholerae O1 no. Tulcea 200 strain in TSB and respectively in homologous filtrate and incubated at 28^0 C in small volumes

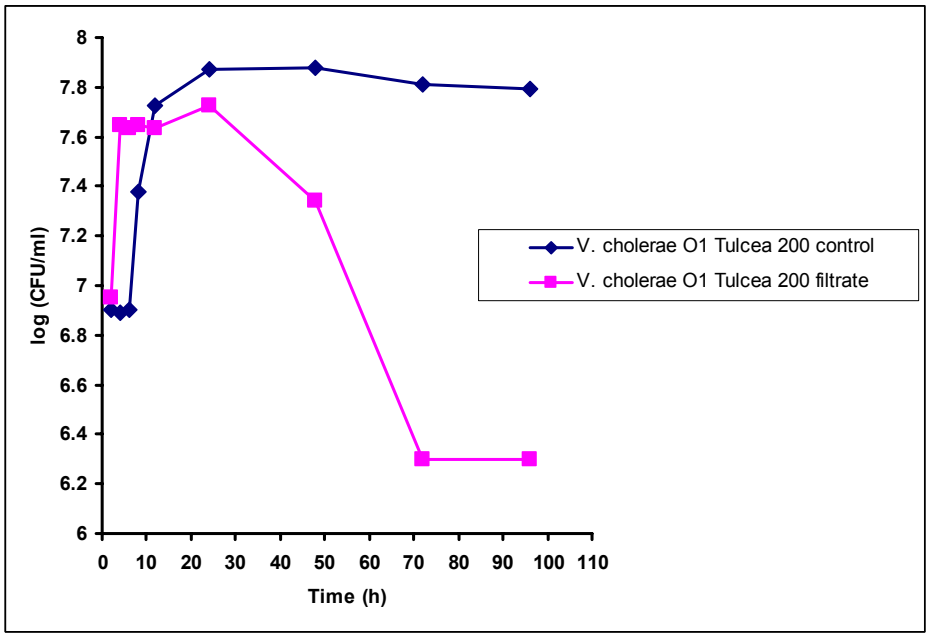

Figure 2. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. cholerae* O1 no. Tulcea 200 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C in small volumes.

In order to investigate the correlation between the level of autoinducer synthesis and the volume of the bacterial culture, there were used by comparison small (100 µl) and big (120 ml) culture volumes for the strains *V. cholerae* O1 no. 200/Tulcea and *V. fischeri* no.10.

When *V. cholerae* O1 no. 200/Tulcea strain was cultivated in small volumes and incubated at 37^0 C, a perfect superposing of the lag phase of the simple / filtrate treated cultures was obtained, whereas the exponential phase exhibited a lower level in case of the culture treated by filtrate than in the simple culture (Fig. 2). At 28^0 C, the two curves superposed perfectly (Fig. 1).

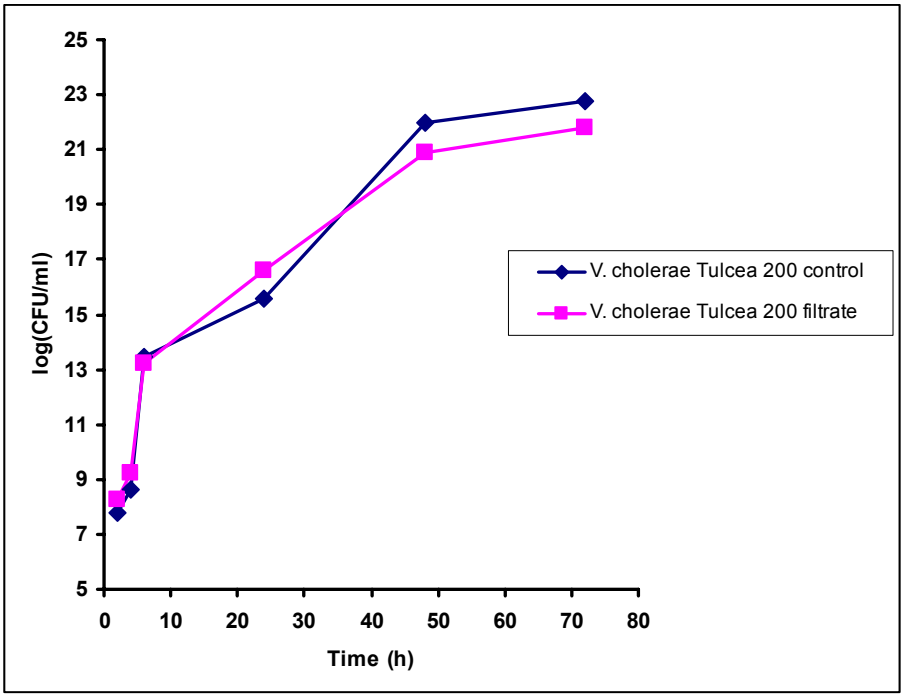

Figure 3. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. cholerae* O1 no. Tulcea 200 strain in TSB and respectively in homologous filtrate and incubated at 28^0 C cultivated in big volumes.

When cultivated in big volumes, the aspect of the growth curves was very different comparing to the results obtained in small volumes system cultivation, probably due to the different oxygenation condition offered by the big volume of culture medium. For the control culture, the growth curves obtained after incubation at 28^0 C and 37^0 C respectively, were relatively similar in terms of cell density and duration of growth phases, the only notable difference consisting in a longer exponential phase and a late stationary phase at 37^0 C (Fig. 3, 4). In exchange, the filtrate treated culture exhibited different features. At 28^0 C the growth curve was similar to that of the control culture, while at 37^0 C, the level of culture density was much lower when compared to the control culture, the lag phase was absent, the stationary phase very short and the decline was very early (after six hrs of incubation (Fig. 3, 4).

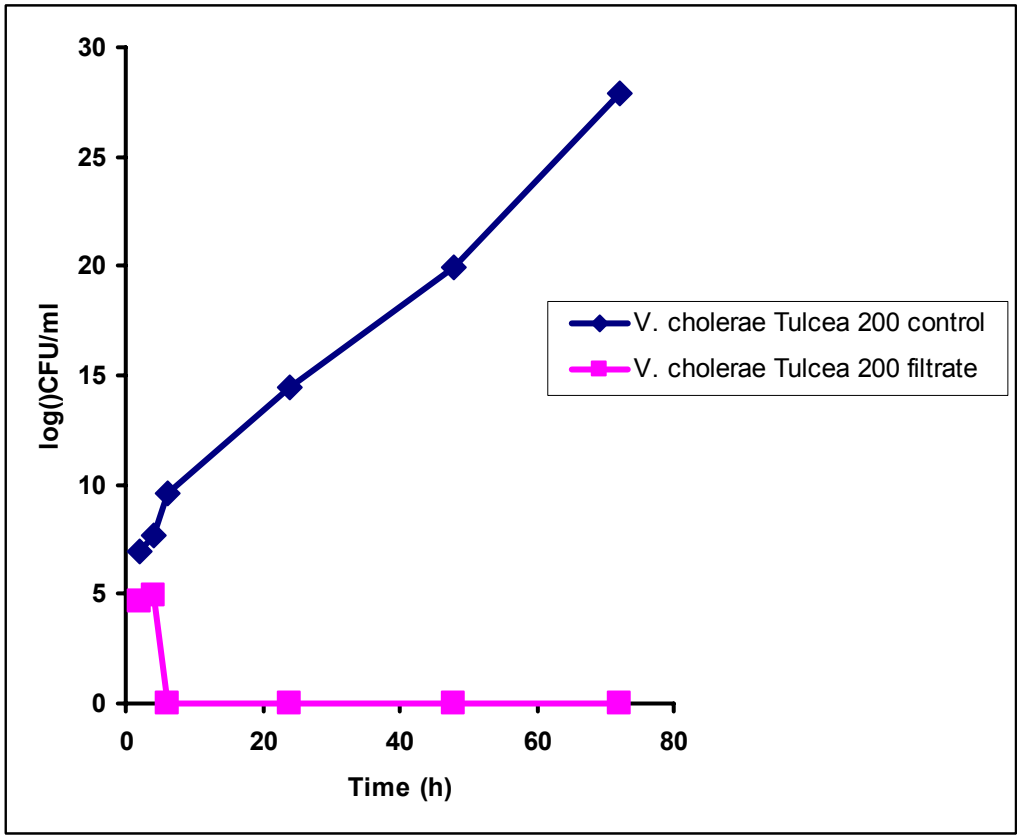

Figure 4. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. cholerae* O1 no. Tulcea 200 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C in big volumes

When *V. fischeri* strain no. 10 strain was cultivated in small volumes and incubated at 28^0 C, the growth curves of control/ filtrate treated cultures were almost superposing, while at 37^0 C, the filtrate treated culture exhibited an early but short exponential phase, followed by a longer stationary phase and an earlier decline phase (Fig. 5-7).

When cultivated in big volumes at 28^0 C, as well as at 37^0 C, the growth curves of filtrate treated cultures and control cultures were almost similar, the filtrate treated cultures developing the growth at lower densities (Fig. 7-8).

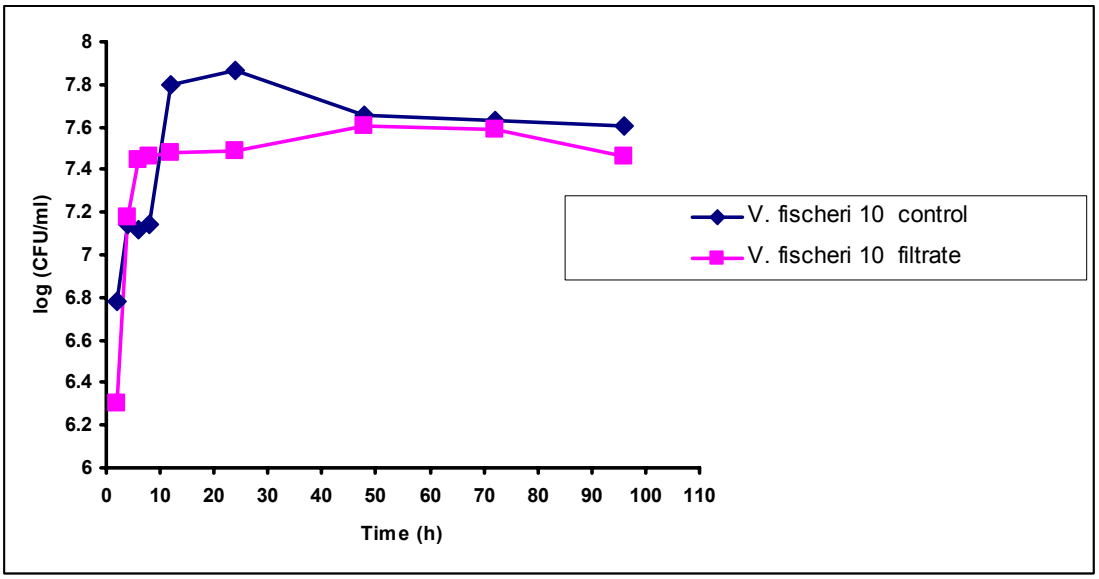

Figure 5. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 10 strain in TSB and respectively in homologous filtrate and incubated at 28^0 C cultivated in small volumes.

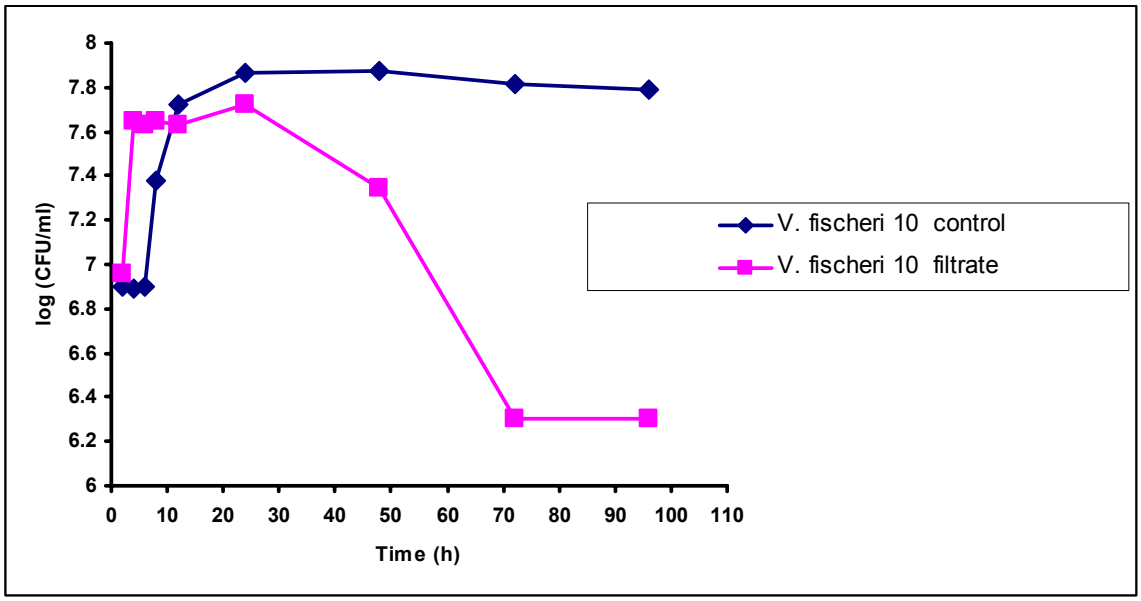

Figure 6. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 10 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C cultivated in small volumes.

When *V. fischeri* no. 10 strain was cultivated in big volumes at 28^0 C, as well as at 37^0 C, the growth curves of control/filtrate treated cultures superposed (Fig. 7, 8).

The pH measurements of the bacterial cultures during the tests, revealed that the autoinducer expected activity was present between the limits of 5.7 –7.5 in *V. fischeri* and 6.4 – 8.4 in *V. cholerae* serogroup O1 no. 200 Tulcea.

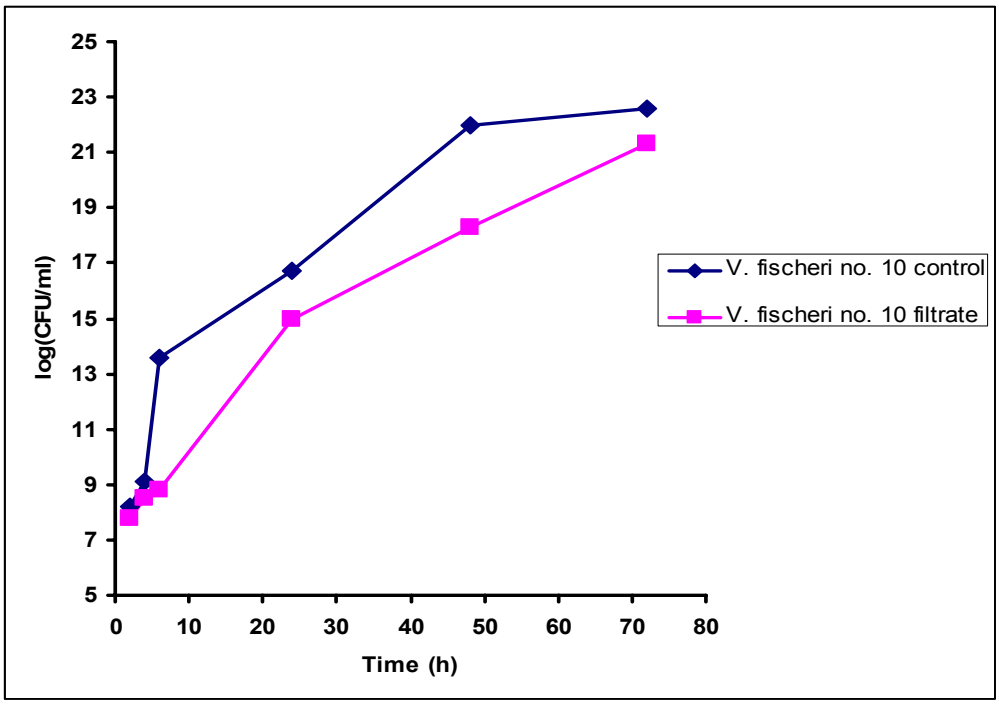

Figure 7. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 10 strain in TSB and respectively in homologous filtrate and incubated at 28^0 C cultivated in big volumes.

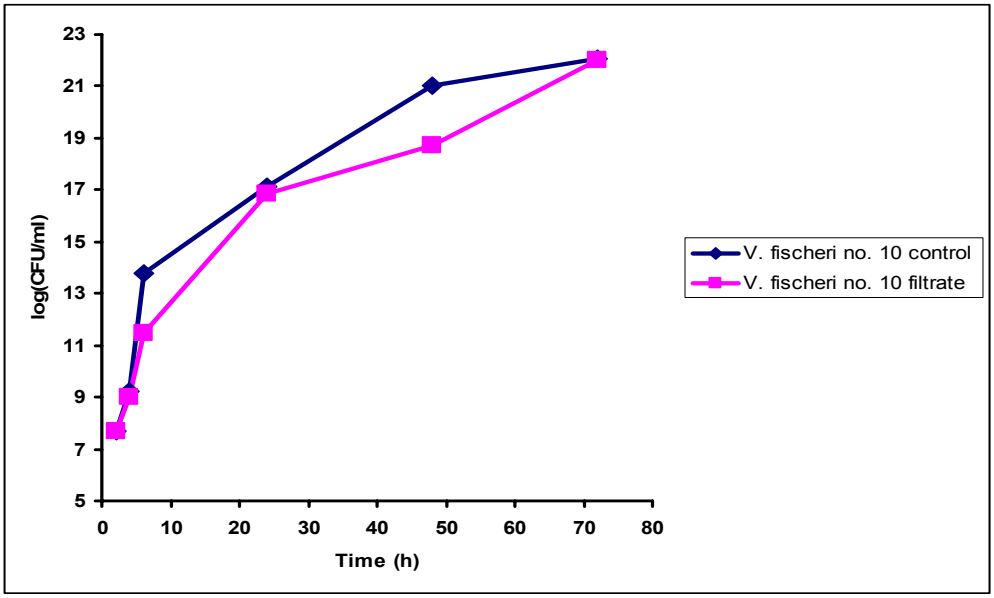

Figure 8. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 10 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C cultivated in big volumes.

Taking into account the above mentioned results, for further experiments, there were used only simple, control cultures compared with cultures treated by simple filtrate, all of them being cultivated in small volumes and incubated at 37^0 C.

Concerning the two *V. metschnikovii* strains, different aspects were observed and namely:

i)in case of the strain no. 3651, when the culture was treated by filtrate (obtained in the stationary phase), the lag period completely disappeared, the slope of the exponential growth

(log) being more abrupt by comparison to the control culture, the stationary phase developed at lower densities, the decline period also starting at a lower bacterial density, suggesting the influence of soluble mediators, which accumulated in high densities inducing the decrease of the multiplication rate (Fig. 9).

ii) in case of the strain no. 3303, the lag and exponential periods superposed in both experimental conditions (i.e. simple bacterial culture and culture treated by filtrate respectively), the stationary phases being 24 hrs shorter and the decline phase 24 hrs earlier in the second case (Fig. 10).

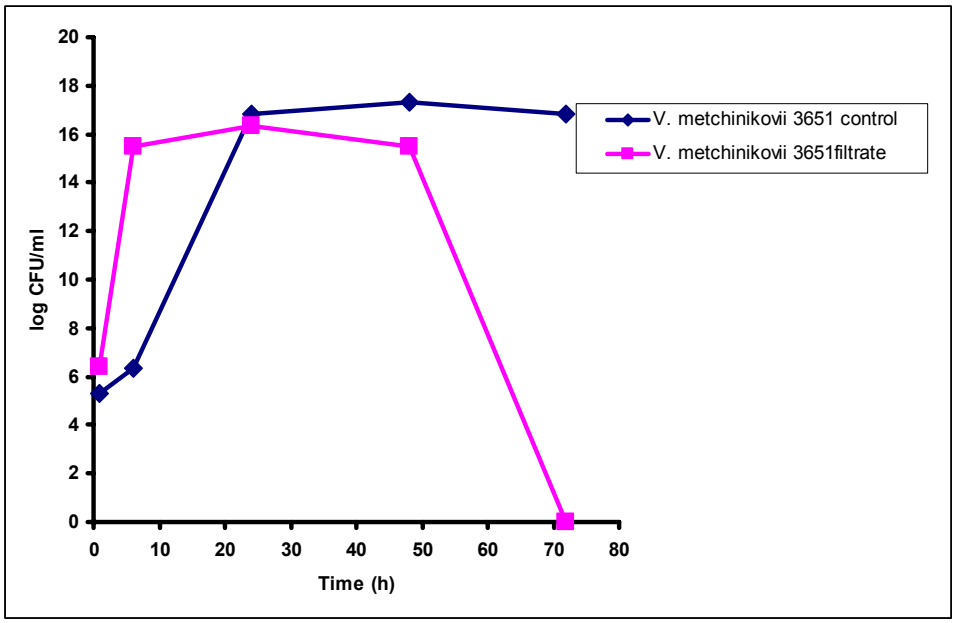

Figure 9. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. metschinikovii* no. 3651 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

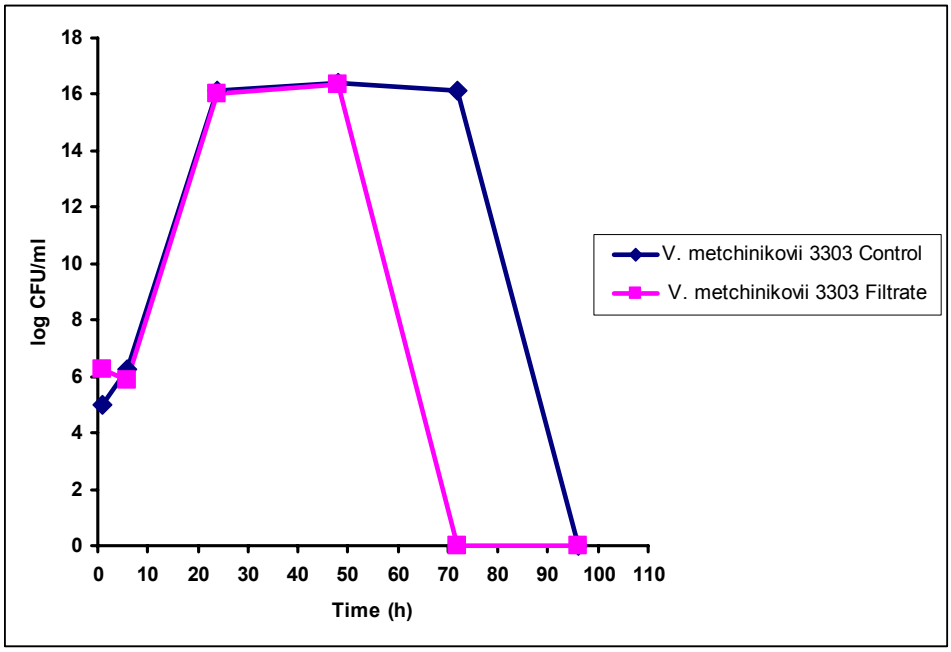

Figure 10. The comparative aspect of bacterial growth curves obtained after cultivation of the V. metschinikovii no. 3303 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

Concerning the two *V. parahaemolyticus* strains, there were observed also different aspects:

i) in the case of the strain no.1442, the culture treated by the respective filtrate displayed almost similar features with the simple bacterial culture, but curiously, the density levels of filtrate treated cultures were higher than of those obtained in the control conditions (Fig. 11).

i) in the case of the strain no.1671, the culture growth curve treated by filtrate exhibited similar lag and exponential phases with the control culture, the stationary phase was missing and the decline phase being 24 hrs in advance comparing to the control culture (Fig. 12).

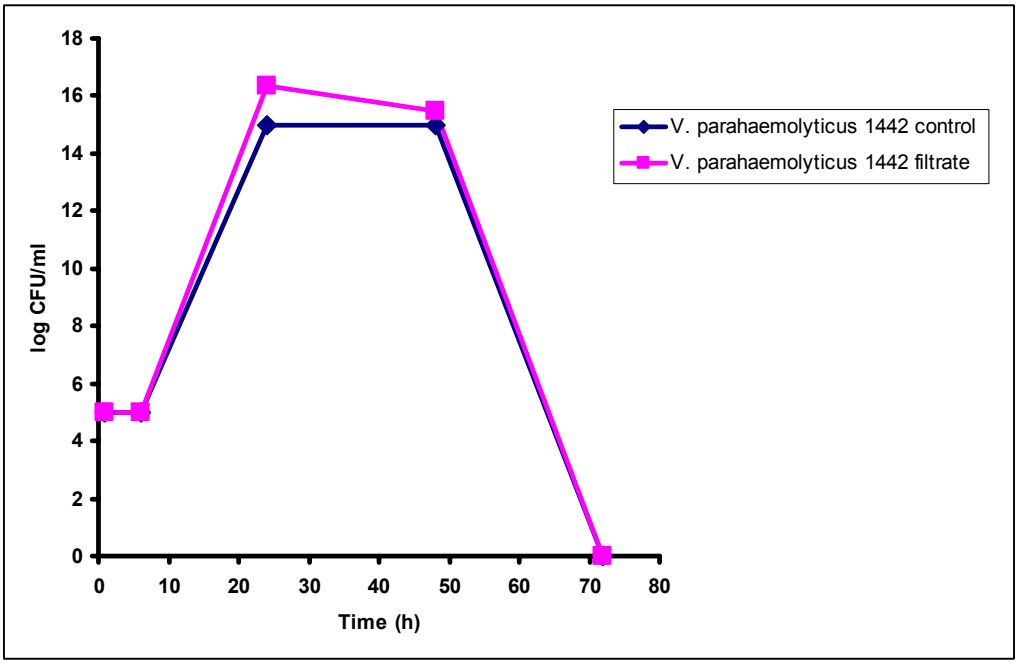

Figure 11. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. parahaemolyticus* no. 1442 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

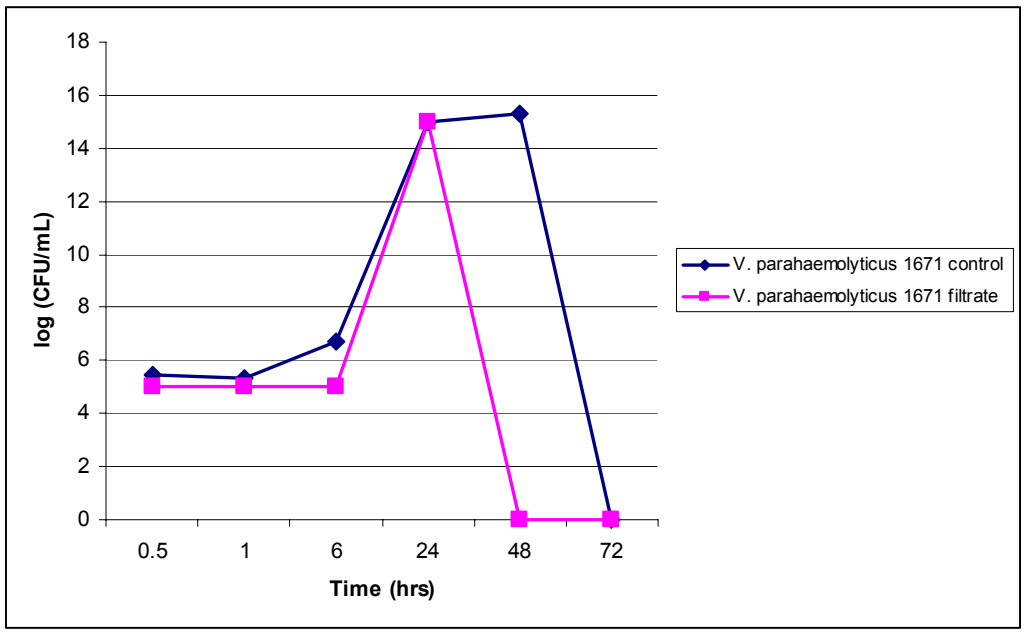

Figure 12. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. parahaemolyticus* no. 1671 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

Concerning the two *V. alginolyticus* strains no. 229 and 1560, in case of the both strains, the culture growth, when treated by filtrate was almost completely inhibited, as revealed by the very short exponential phase in case of *V. alginolyticus* no. 229 (Fig. 13) followed directly, in case of both strains by the decline phase (Fig. 13-14).

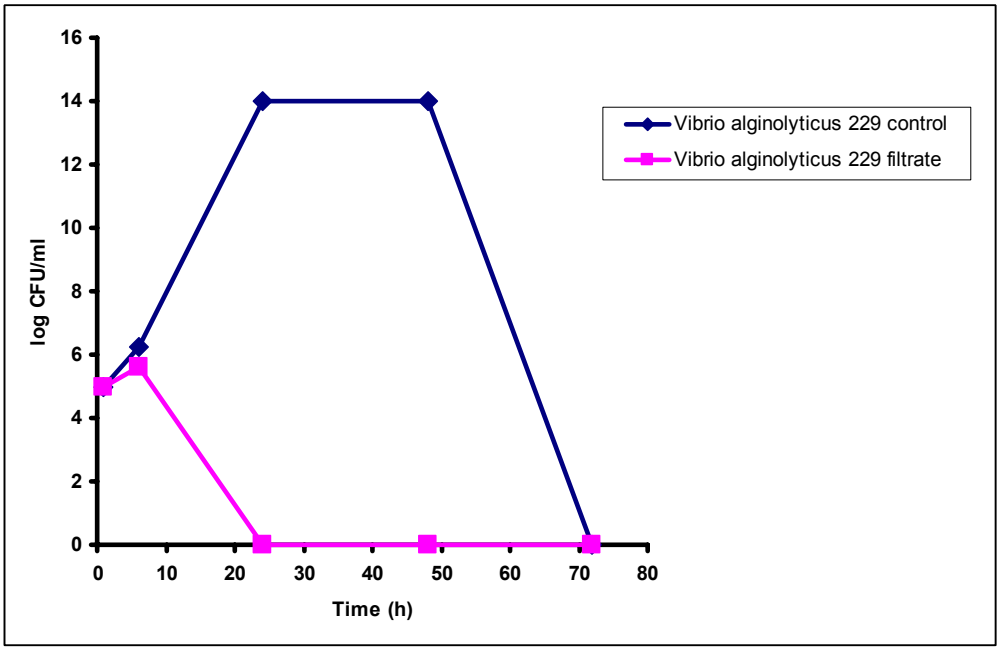

Figure 13. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. alginolyticus* no. 229 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

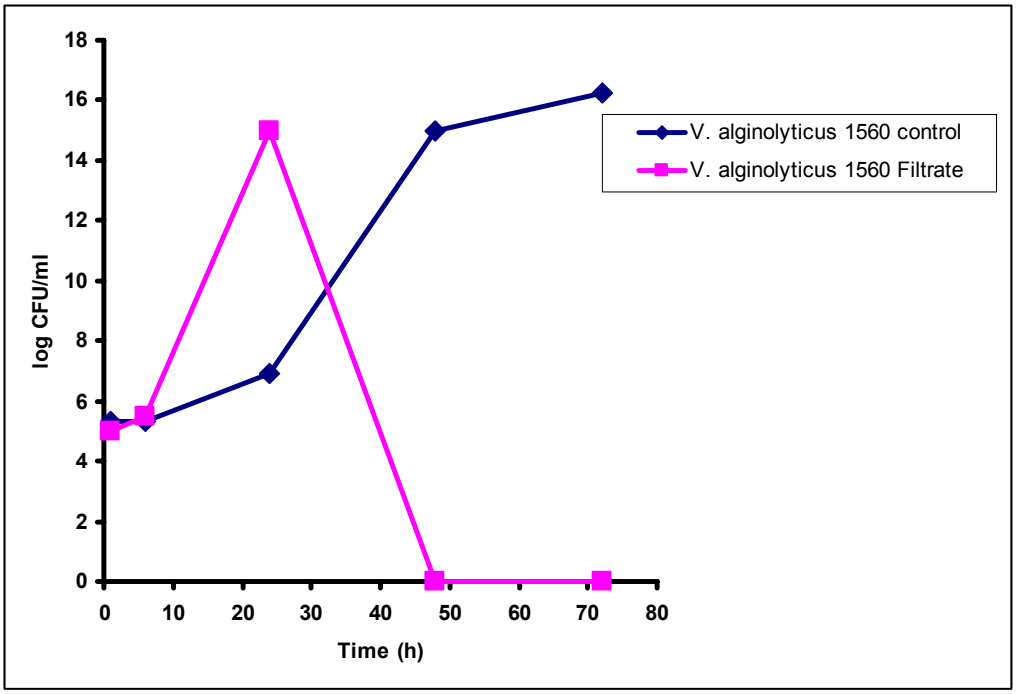

Figure 14. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. alginolyticus* no. 1560 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

When studying the two *V. fischeri* strains no. 754 and 898, similar features have been noticed, the lag and the exponential phases superposing almost perfectly in case of the

bacterial growth curve of the simple culture / culture treated by filtrate, whereas the decline phase was much in advance, in case of the culture treated by filtrate by comparison with the simple culture (Fig. 15-16).

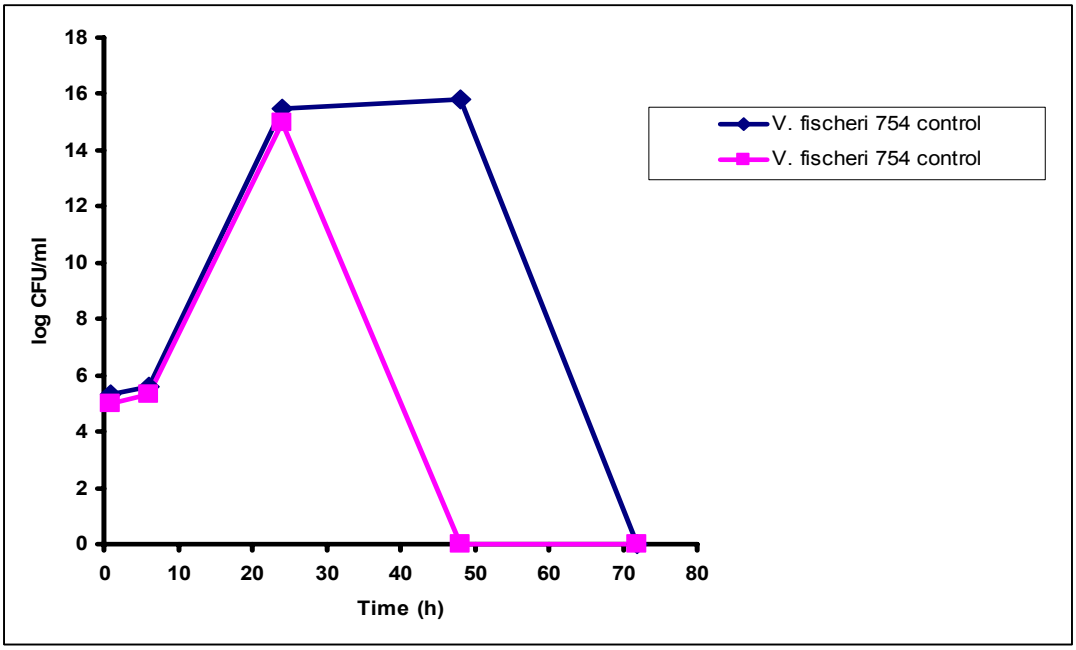

Figure 15. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 754 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

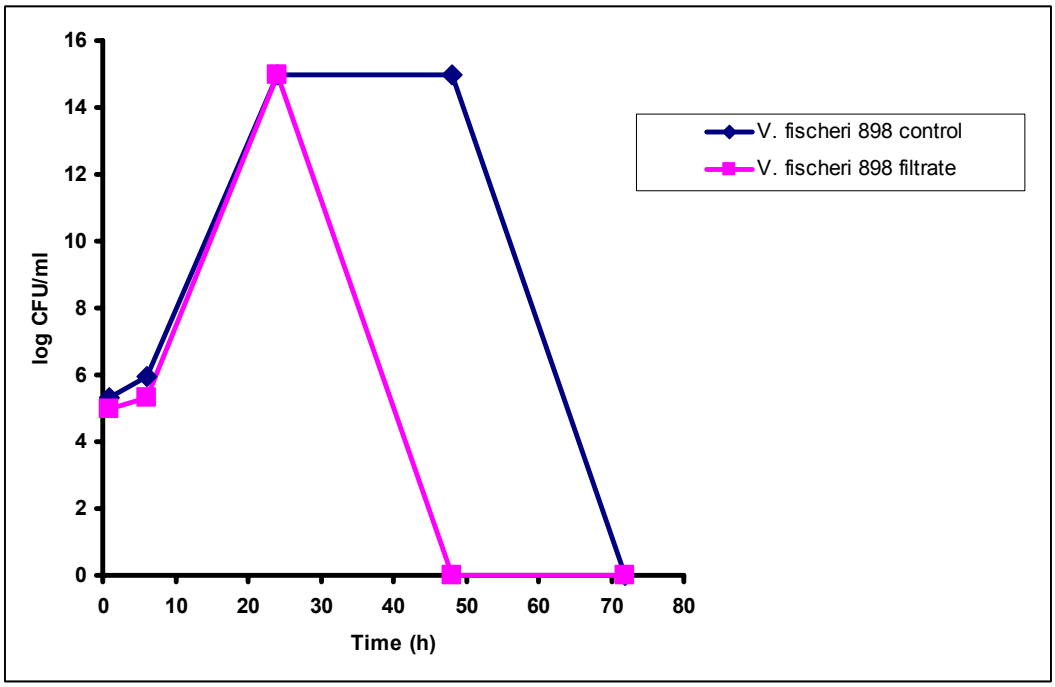

Figure 16. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. fischeri* no. 898 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

Concerning the two *V. anguillarum* strains no. 798 and 1545 the results were very different for the two strains. If in case of the strain no. 1545, the growth curves of the simple

and filtrate treated bacterial culture superposed almost perfectly (Fig. 17), in case of the strain no. 798, the result were very similar to those obtained for *V. fischeri*, where the lag and exponentially phases superposed perfectly in case of simple and filtrate treated cultures, but in case of the culture treated by filtrate, the stationary phase completely disappeared, the decline phase being in advance with 24 hrs (Fig. 18).

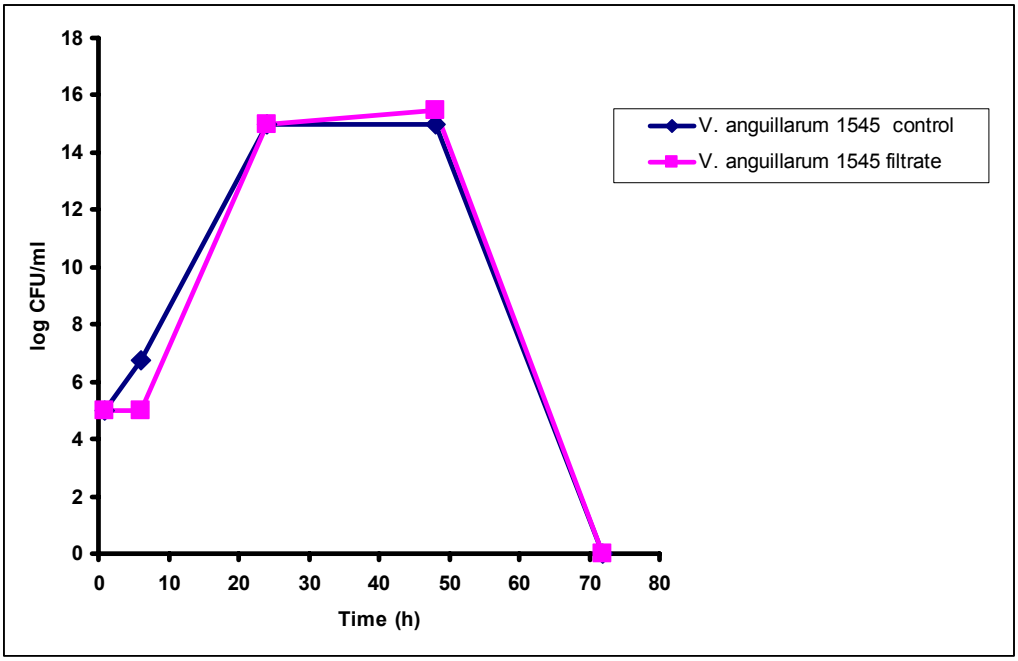

Figure 17. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. anguillarum* no. 1545 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

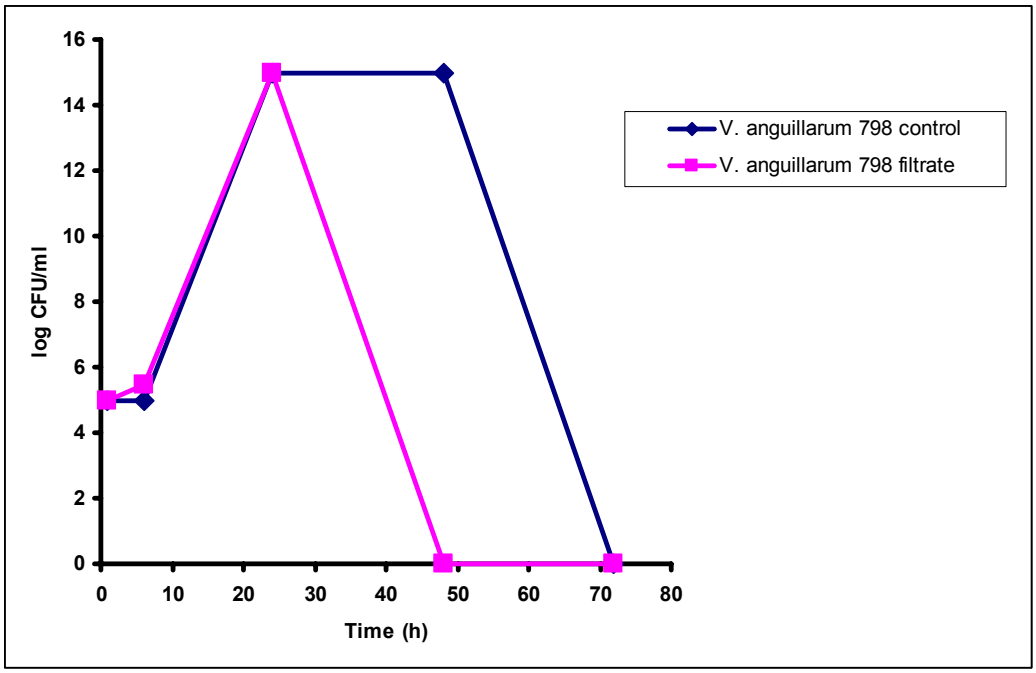

Figure 18. The comparative aspect of bacterial growth curves obtained after cultivation of the *V. anguillarum* no. 798 strain in TSB and respectively in homologous filtrate and incubated at 37^0 C.

Conclusion

The current study demonstrated that in stationary phase, bacterial cultures are accumulating soluble factors influencing the duration and aspect of the bacterial growth curve. In the most cases of the tested strains this influence consisted in : reduction of the multiplication rate and subsequently, reduction of the culture density, shortening of the lag phase and of the total duration of the growth curve, with an earlier occurrence of the decline phase. The synthesis of autoinducers proved to be dependent upon the bacterial strain, source of isolation (clinical case or aquatic environment), incubation temperature, volume of the culture medium, as well as the oxygenation surface.

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Selected References

1. BOARDMAN B.K., MEEHAN B.M., FULLNER SATCHELL K.J. Growth phase regulation of *Vibrio cholerae* RTX toxin export. J.Bacteriol. 2007, 189(5), 1827-35.
2. BRUHN J.B., DALSGAARD I., NIELSEN K.F., BUCHOLTZ C., LARSEN J.L., GRAM L. *Quorum sensing* signals molecules(acylated homoserine lactones) in gram-negative fish pathogenic bacteria. Dis. Aquat. Organ. 2005, 65(1), 43-52.
3. DAVIES D.G., PARSEK M.R., PEARSON J.P., IGLEWSKI B.H., COSTERTON J.W.,GREENBERG E.P. The Involvement of cell-to cell signals in the development of a bacterial biofilms. Science 1998, 280, 295-298.
4. HAMMER B.K., BASSLER BL. *Quorum sensing* controls biofilm formation *Vibrio cholera*. Mol. Microbiol. 2004, 51(5) 1521.
5. HENKE J.M., BASSLER B.L. Three parallel *quorum-sensing* systems regulating gene expression in *Vibrio harveyi*. J. Bacteriol. 2004, 186(20), 6902-14.
6. JOELSSON A., LIU Z., ZHU J. Genetic and phenotypic diversity of *quorum sensing* systems in clinical and environmental isolates of *Vibrio cholerae*. Infect. Immun. 2006, 74(2), 1141-7.
7. KRUKONIS E.S., DIRITA V.J. From motility to virulence: *Sensing* and responding to environmental signals in *Vibrio cholerae*. Curr.Opin. Microbiol. 2003,6(2), 186-90.
8. LIU Z., STIRLING F.R., ZHU J. Temporal *quorum-sensing* induction regulates *Vibrio cholerae* biofilm architecture. Infect. Immun. 2007, 75(1), 122-6.
9. LYNCH M.J., SWIFT S., KIRKE D.F., KEEVIL C.W., DODD O., WILLIMAS P. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila* Environ. Microbiol. 2002, 4(1), 18-28.
10. MILLER B. MELISSA, KAREN SKORUOSKI, LENZ H.D., TAYLOR, K.R., BASSLER B.I. Parallel *Quorum-sensing* System converge to regulate Virulence in *Vibrio cholerae*. Cell 2002, 110,305- 314.
11. MILLER M.B., SKORUPSKI K., LENZ D.H., TAYLOR R.K., BASSLER B.L. Parallel *quorum sensing* systems converge to regulate virulence in *Vibrio cholera*. Cell 2002, 110(3),3003-14.
12. MILTON D.L. *Quorum sensing* in vibrios: complexity for diversification. Int. J.Med. Microbiol. 2006, 296(2-3), 61-71.
13. SWIFT S., LYNCH M.J., FISH L., KIRKE D.F.,TOMAS J.M., STEWART G.S., WILLIAMS P. *Quorum sensing*- dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect. Immun. 1999, 67(10), 5192- 9.
14**.** WHITEHEAD N.A., BARNARD A.M.I., SLATER H., SIMPOSON N.J., SALMOND G.P. *Quorum-sensing* in gram-negative bacteria FEMS Microbiol. Rev. 2001, 25, 365- 404.
15. ZHU J., MEKALANOS J.J. *Quorum sensing*-dependent biofilms enhancing colonization in *Vibrio cholerae* Dev.Cell. 2003, 5(4), 647-56
16. *** Metode de laborator de uz curent vol. II Ministerul Sănătății. Academia de Ştiinţe Medicale Ed. Medicală Bucureşti 1977, 181-182.
17. URBANCZYK H., AST J.C. , HIGGINS M.J., CARSON J., DUNLAP **P.V.** Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov.Int. J. Syst. Evol. Microbiol., 2007, 57, 2823-2829