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ПРИМЕНЕНИЕ БИОЛЮМИНЕСЦЕНТНОГО МЕТОДА ДЛЯ КОНТРОЛЯ КАЧЕСТВА ПИТАТЕЛЬНЫХ СРЕД И СТАНДАРТНЫХ БАКТЕРИАЛЬНЫХ ШТАММОВ (СТАНДАРТ NMX-AA-042-1987)

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Разработан биолюминесцентный метод для быстрого контроля качества бактериальных питательных сред и стандартных штаммов бактерий по определению АТФ, определено соотношение между уровнем АТФ и количеством бактерий, измеренным методом разведений и методом счета колониеобразующих единиц. Исследована кинетика роста *E. coli* в средах, содержащих бриллиантовый зеленый и лактозу, указанных в официальных стандартах Мексики (Mexican Official Norms (NOM)). Исследовано влияние pH, ионной силы и температуры на рост *E. coli* и смешанной культуры *E. coli* и *Pseudomonas spp*. АТФ определяли биолюминесцентным методом после обработки клеток диметилсульфоксидом; одновременно с измерением АТФ количество бактерий в образцах определяли методом счета колоний на чашках и методом разведений. Все измеренные параметры для сравнения роста бактерий в средах, содержащих бриллиантовый зеленый и лактозу, были статистически значимы. Сильное ингибирование роста бактерий в обеих средах наблюдалось в присутствии 5% NaCl. Отмечено увеличение удельного роста *E. coli* в присутствии *Pseudomonas spp.* Показано, что наблюдается хорошая корреляция между концентрацией АТФ и количеством бактерий в образце, измеренным методом разведений и методом счета колониеобразующих единиц.

INTRODUCTION

Organizations in charge of accrediting water quality monitoring labs nowadays encourage them to set strict controls to ensure efficient testing processes. Bacteriological testing includes enumeration of coliform bacteria that indicate fecal contamination of water. Microbiological analysis of water may be performed using different testing methods to examine samples for a variety of test parameters [1]. Regardless of the size, location or complexity of testing laboratory, the employed methods must be based upon recognized standard methods in order to provide quality results to the submitting agencies [2].

The parameters established in Mexican Official Norms (NMX-AA-042-1987) [3] for quantification of fecal water pollution are the MPN (Most Probably Number) and CFU (colony formed units on micropore filters). Both parameters are defined by microbiological analysis based on specific broths and solid media. The broths traditionally used in MNP quantification are Brilliant green bile (2%) and Lactose Broth. The incubation period was 24–48 h, but the results varied considerably. Although the tests required for evaluating these techniques can be done with standard bacterium, the viability and quality of such tests are uncertain at the start of the processes [3]. However, the use of modified medium as well as standard bacterial inoculum affects the precision of the assays. Hence, new methods of rapid microbial detection are emerging in modern microbiology. Traditional culture-based techniques demand lengthy protocols that cause notable delays in analysis, but as they are actually officially recognized, it is important to standardize these methods for rapid detection.

Adenosine triphosphate (ATP) or Relative luminescent units (RLU) technology is a new, rapid microbial detection method based on bioluminescence, which can meet the demands for sensitivity and at the same time could save time and money [4]. It is considered that this can be adapted for quality control of medium and bacterial strains applied in standard MPN and CFU assays. The bioluminescent ATP detection is based on reaction catalyzed by firefly luciferase (EC 1.13.12.7). It is the more sensitive method in comparison with other techniques used for biomass quantification, for example, absorbance measurements.

The bacterial growth kinetics data can be used for the development of the bioluminescence assay and this hypothesis formed the basis of this study.

Microbial growth kinetics, *i.e.*, the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (S), is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology or biotechnology, and therefore is an important part of the basic aspects of microbiology. Recently more ecologically oriented studies in the area of microbial growth and biodegradation kinetics demonstrated that many fundamental questions in this field still remains to be discovered [5]. Knowledge concerning the influence of environmental factors such as temperature, pH, salinity etc., on microbial growth is of crucial practical importance in the control of bioprocesses, for the safe handling of food, in waste water treatment and in bioremediation [6].

The aim of present study was to show that bioluminescent ATP monitoring of the microbial growth kinetics in the traditionally employed medium for MPN technique could be used to define the parameters for rapid quality control of the culture medium, as well as to correlate the ATP measurements to the viability of bacteria estimated by MPN and CFU methods.

MATERIALS AND METHODS

Luminometer TD-20/20 (USA) was used for bioluminescent measurement. Centrifuge (Thermo IEC Multi RF, USA) was used for pelletization of bacterial cells and an Incubator (Rios Rocha, Mexico) was employed for the growth studies at 37°C. The absorbance measurements were carried out using Hach (HACH, USA) spectrophotometer.

Sodium chloride (ALQUIME, Mexico), sodium hydroxide (Jalmek, Mexico), hydrochloric acid (Jalmek, Mexico), dimethyl sulfoxide (Fisher Biotech, F-Scientific USA), adenosine triphosphate ATP, luciferin-luciferase kit for ATP detection (Sigma, USA), lactose and 2% brilliant green bile broths (Sigma, Fluka, Bioxon), nutrient and brilliant green agars (Bioxon, Mexico) were used in the present study and all reagents were of analytical grade.

Microorganisms, their preservation and inoculum preparation

In this study *Escherichia coli* (BD Bactrol plus ATCC 25922 Bioscience, USA) and *Pseudomonas aeruginosa* (BD Bactrol plus ATCC 27853 Bioscience, USA) strains were used. The commercially provided strains were treated according to given instructions. The *Escherichia coli* strain was maintained on 2% brilliant green brilliant bile agar and *Pseudomonas aeruginosa* on nutrient agar. The inoculum was prepared by incubation of *E. coli* in 2% brilliant green brilliant bile broth and *P. aeruginosa* in nutrient broth at 37°C for 6 to 8 h.

Medium employed in growth kinetics studies

E. coli was grown on 2% green brilliant bile and lactose broth, which are commonly employed in the Most Probable Number (MPN) technique recommended by Mexican Official Norm (NMX-AA-042-1987) and was used as control. *Pseudomona aeruginosa* was also grown on the same media to define their selectivity and to study their probable interference on the growth of *E. coli*. To demonstrate the influence of the quality of media on kinetics of growth, they were modified. The pH of 2% brilliant green bile broth was modified by changing the pH to 5.4 and 8.4 from 7.2+/–0.2 and the lactose broth was modified from 6.9 +/–0.2 to 5.4 and 8.4 using the drops of 5% HCl and 5% NaOH respectively. The ionic force of both media was increased by addition of 5% NaCl to the culture media. Moreover, both media were treated at high temperature on a microwave oven for 1 min.

Growth kinetics of E. coli

E. coli strain was grown on 7 ml of culture media listed above in tubes (10 ml volume) and with 0.1 ml of inoculum. All inoculated tubes were incubated at 37°C for 8–12 h. The growth of *E. coli* was determined every hour by means of A_{620} in all tubes using HACH spectrophotometer (Loveland, USA). All the assays were performed in triplicate for all the media under all conditions.

Bioluminescent assay for monitoring the growth kinetics of E. coli

E. coli was grown and multiplied under conditions described above. Considering that the presence of media influenced the bioluminescent detection, the biomass was separated from culture broth by means of centrifugation at 4000 g for 10 min at 4°C. Cells were broken with DMSO (10 ml of sample with 90 ml of DMSO) and the bacterial ATP released was detected by bioluminescent method. The bioluminescence assays were performed according to the techniques outlined in the Sigma luciferin-luciferase kit. The light emission was measured on a TD 20/20 luminometer (USA) with integration over 10 seconds and ATP was measured initially as Relative Light Units (RLUs). The activity of

the luciferin-luciferase was tested by using an ATP standard to obtain the calibration plot. The RLUs were proportional to the amount of ATP and the amount of ATP was proportional to the number of viable bacteria.

The obtained ATP kinetics was compared with the growth curves of bacteria obtained by spectrophotometric method. The assay was performed in triplicate for all the media employed in this study.

Assay for establishment of relationship between ATP, CFU and MPN

The relation between ATP detected, and CFU as well as MPN was defined by applying the bioluminescent assay, plate count and MPN technique respectively. Colony Forming Units (CFU) and MPN were evaluated according standard techniques by following the Official Mexican Norms [1, 6].

Different cell suspensions were used. The final concentrations of *E. coli* varied in range of $10^7 - 10^{15}$ CFU/ml. The ATP/CFU and ATP/MPN values were calculated from the slopes obtained in ATP (mol/ml) *vs* CFU/ml and ATP (mol/100 ml) *vs* MPN/100 ml coordinates respectively. All assays of this study were carried out in triplicate. Standard deviation and mean values were calculated for both plate count and bioluminescent ATP determination.

Statistical and mathematical analyses

Based on Monod's equation [7], the obtained kinetic curves were plotted on semi-logarithmic coordinates (ln-measured parameter (t)) vs time) to determine the specific growth rate values for each condition of *E. coli* growth. The specific rate was calculated as the slope of the line corresponded to the exponential phase of *E. coli* growth. The growth kinetics data were analyzed for multifactor variance analysis and the statistical significance was determined at 95% confidence limits.

RESULTS AND DISCUSSION

In the present study it was supposed that the kinetic data of bacterial growth can provide the necessary information for the development of a rapid assay that may be applied to quality control of medium and microbial strains.

Two media from Official Mexican Norm NMX-AA-042-1987 were used [1]; lactose broth was commonly employed for presumptive coliform quantification and 2% brilliant green bile broth was used for fecal coliform quantification. The kinetics of *E. coli* growth was studied by two different methods, *viz*., spectrophotometric and bioluminescent assays. In both cases, 4 artificially altered media for lactose broth as well as brilliant green bile broth were employed to compare the growth of *E. coli* with control (Figs 1–4). Both the media used are considered as the standard ones for the estimation of the microbial growth.

Moreover, to estimate the quality of standard *E. coli* strain, the assay was carried out on both broths in the presence of *Pseudomonas aeruginosa* (Fig. 5).

The results obtained with 2% brilliant green bile broth (Figs 1 and 2) demonstrated that both measurement methods detected the changes in growth kinetics of *E. coli*. The growth on modified broths was slower than the control media for most of the applied conditions. However, in the case of the heat treated medium the ATP detection demonstrated an increase in growth from 4 hours (Fig. 1, *b*). Similar observation was also made with absorbance method (Fig. 1, *a*), which did not present an increase on the measured parameter, but led to higher values of specific growth rate (Table 1). It was observed that the ATP detection was more sensitive to the changes in growth conditions and their effect on bacterial multiplication. Increase (Fig. 2, *b*) or decrease (Fig. 2, *d*) of pH, as well as an increase in the ionic force (Fig. 1, *d*) provoked several changes on ATP detection. The kinetic curves obtained from these three experiments did not exhibit a typical sigmoid behavior. An irregular pattern was detected; initially the ATP levels were higher (Fig. 2) or similar (Fig. 1, *d*) to control measurements. The increase might be related to some metabolic processes that led to the increase of ATP [8]. However, this was not reflected in a similar manner in absorbance measurements and also they indicated only the biomass quantity, but not its viability. This conclusion could be also confirmed by the decrease in ATP levels observed after 6 h of incubation, which was not recorded for the absorbance data (Figs 1 and 2).

The specific growth rates of the studied microorganism (Table 1) were calculated from the sigmoid kinetic curves obtained by both applied methods. The results correlated with the data on growth kinetics obtained in the previous experiment (Figs 1 and 2). The specific growth rate calculated from the growth kinetics obtained for control and modified 2% brilliant green bile broth was statistically different for all treatments at 95% confidence level. There was a complete inhibition of bacterial growth in the media modified with NaCl and a partial inhibition in the case of pH modified me-

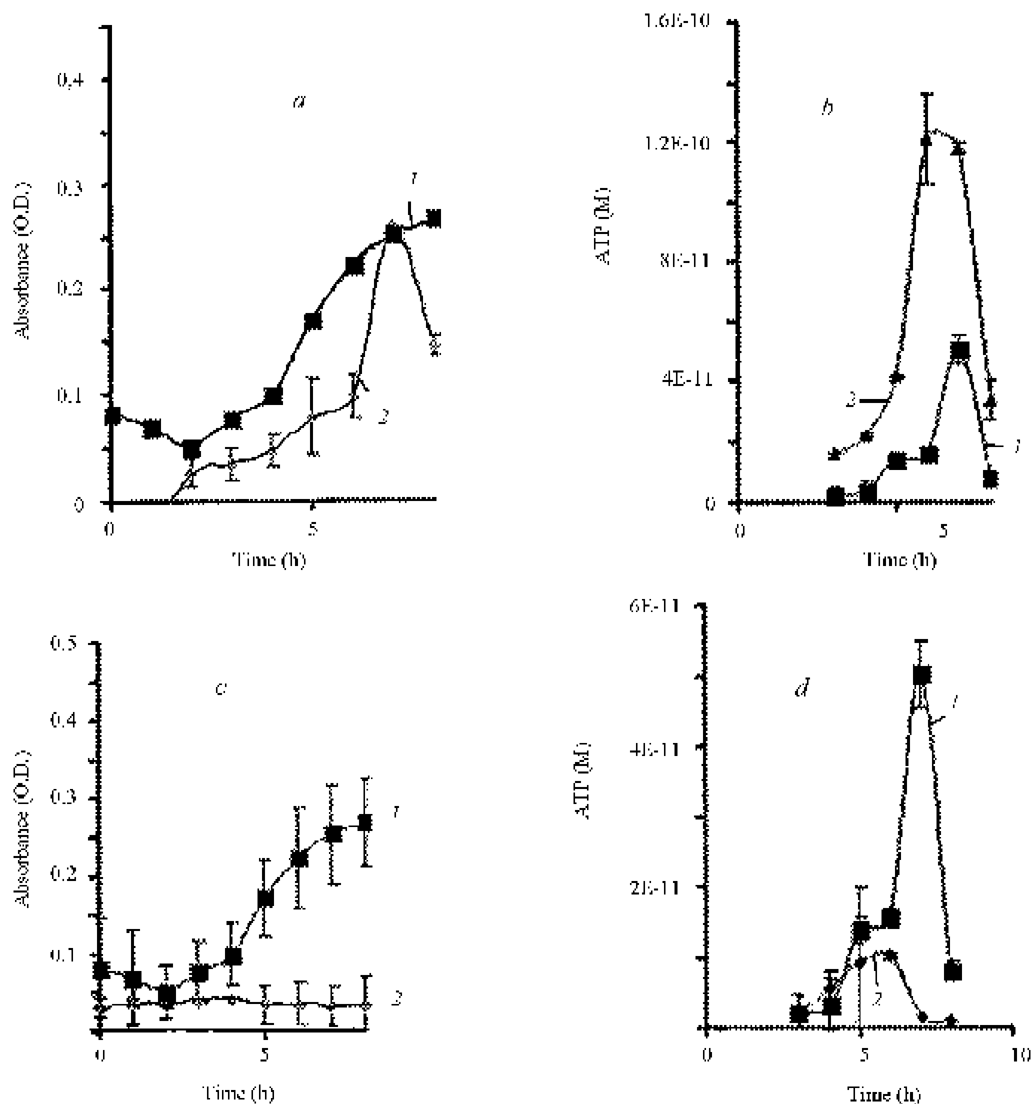

Fig. 1. Growth kinetics of *E. coli* ATCC 25952 on control (*1* – control) and modified (*2*) 2% brilliant green bile broth determined by spectrophotometric (left – *a, c*) and bioluminescent (right – *b, d*) assays: top, – comparison of control broth with heat treated medium (*a, b*); bottom, – with altered ionic force medium (*c, d*)

dia. Thus, the bioluminescent assay may be applied for the determination of the quality of brilliant green bile broth employed for the *E. coli* quantification as per Official Mexican Norm. The results showed that this assay was more sensitive and with high precision than the spectrophotometric assay. as ATP assay detected the viability of cells in stressful conditions and not the total biomass which also included dead cells. It can also be concluded that the parameters of growth kinetics, for example specific growth rate or the generation time, might be useful for the determination of the quality of culture medium as these values depended on the growth conditions. In other words, a change in value is always associated with the changes in the growth environment.

The obtained results are in accordance with earlier report, which also showed that the multiplication of *E. coli* in the media with NaCl was characterized by slower specific growth rate and lower growth yield [9].

Similar results were obtained for the *E. coli* growth on lactose medium with NaCl (Fig. 3, *c* and 3, *d*). Both the bioluminescent detection and turbidity measurement revealed a significant decrease in the growth of *E. coli*. However the difference in the growth kinetics in modified lactose broth was less than that observed for brilliant green bile broth. Figs 3, 4

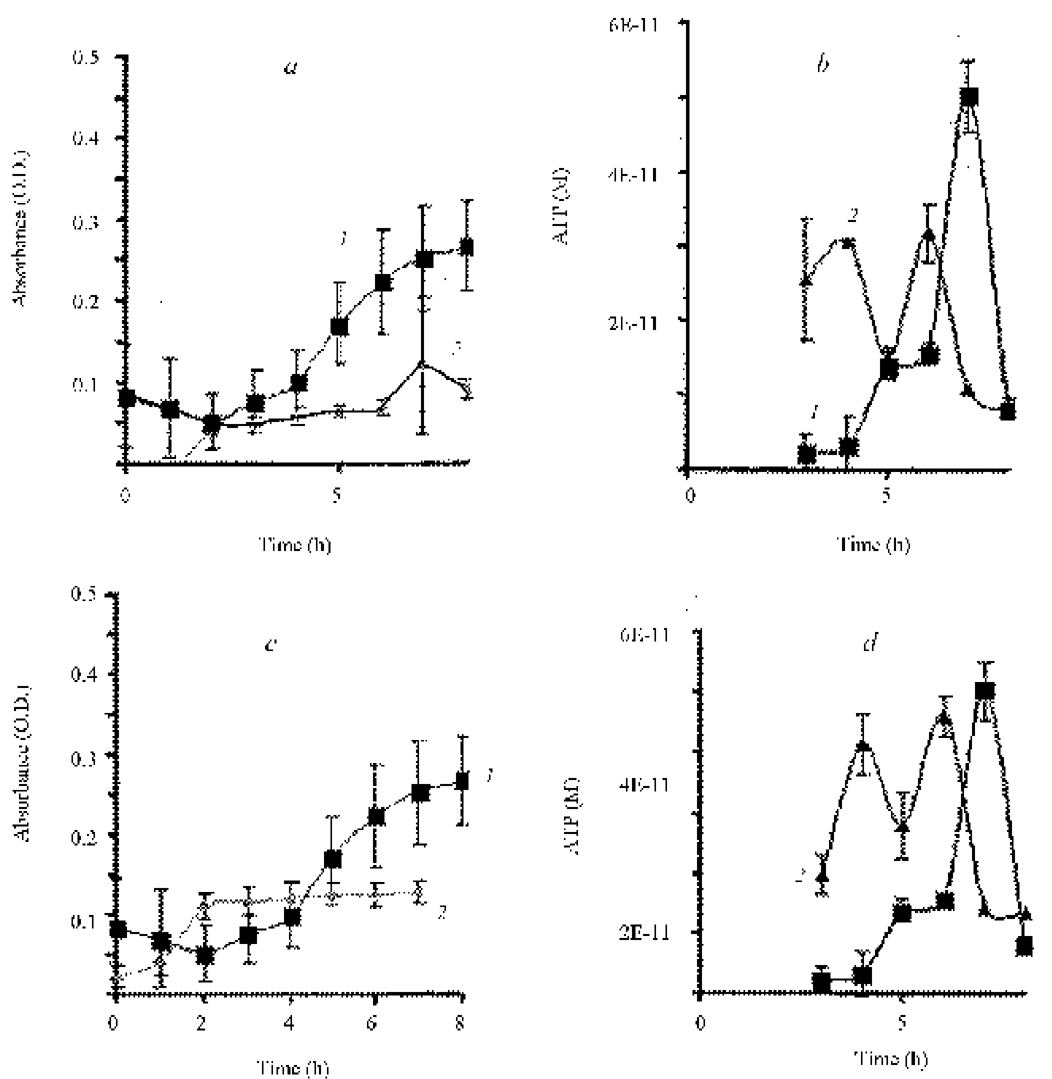

Fig. 2. Growth kinetics of *E. coli* ATCC 25952 on control (*1* – control) and modified (*2*) 2% brilliant green bile broth measured by spectrophotometric (left – *a, c*)and bioluminescent (right – *b, d*) assays: top – comparison of control with medium at pH 5.4 (*a, b*); bottom,– with medium at pH 8.4 (*c, d*)

showed a similar kinetic behavior for control and altered media except ionic force modified medium (Fig. 3, *d*). When compared to control, a statistically significant difference in specific growth rates was observed for most of the applied conditions, except for the lactose broth at pH 8.4. The difference in specific growth rate due to the changes in broth conditions are presented in Table 2.

The growth kinetics studies with rigorous statistical analysis permitted the prediction of the quality of culture medium. As mentioned before, the bioluminescent assay has various advantages in comparison with other standard assays, because it reflects the cell viability in response to the changes in environmental conditions [10].

Fig. 5 showed that the bioluminescent assay might be also useful in the prediction of the microbial contamination in the standard strain of *E. coli*. The kinetic curves (Fig. 5) and the Table 3 demonstrated that the *E. coli* multiplication in 2% brilliant green bile broth was decreased in the presence of *P. aeruginosa*. Brilliant green bile broth was more selective than lactose broth [3] and impeded the multiplication of *P. aeruginosa*. The contamination of *E. coli* strain was observed by the appearance of lag period in absorbance measurements as well as changes the ATP levels. The specific growth rate calculated from ATP kinetics was significantly less, when compared to that of control (Table 3). Thus, biolumi-

Table 1

Specific growth rate of Escherichia coli ATCC 25952 in 2% brilliant green bile broth* and in modified broths

Medium	Measurement method	Specific growth rate (μ), 1/h	Standard deviation (S)	Statistical significance
Control	O D ATP	0.358 0.728	0.0296 0.0452	
Heat treated medium	O D ATP	0.332 0.847	0.0176 0.0707	+ +
Medium at pH 5.4	O D ATP	0.096 Incalculable	0.0799 0.0954	+ +
Medium at pH 8.4	O D ATP	0.021 Incalculable	0.0083 0.0502	+ +
Medium with 5% NaCl	O D ATP	Incalculable Incalculable	0.0257 0.0933	+ +

*Medium recommended by Mexican Official Norm for MPN analysis of coliforms in water samples;
O – Optical ensity; ATP – Adenosinetriphosphatereleasebyusingdimethylsulfoxide(MSO);
+ enotesastatisticallydifference(statisticalsignificancecanbeindicatedinthevaluesitself); – enotesa nonstatisticallydifference.

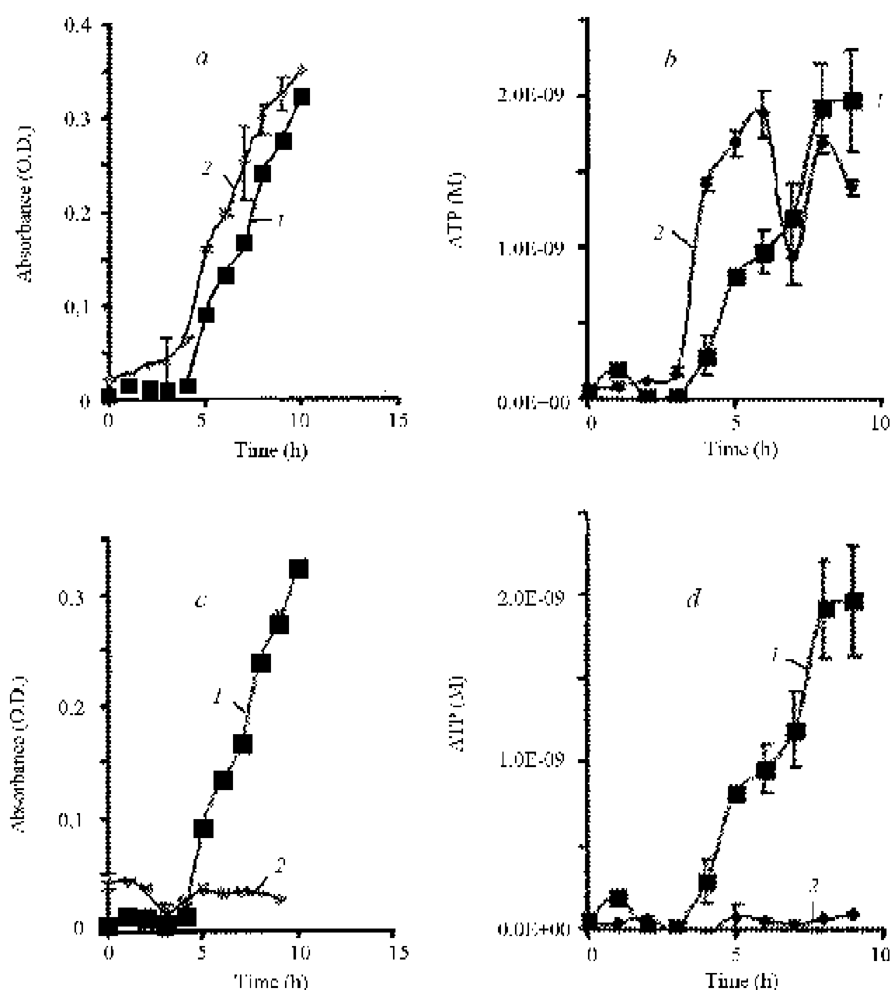

Fig. 3. Growth kinetics of *E. coli* ATCC 25952 on control (*1* – control) and modified (*2*) lactose broth detected by spectrophotometric (left – *a, c*) and bioluminescent (right – *b, d*) assays: top,– comparison of control with heat treated medium (*a, b*); bottom,– with altered ionic force medium (*c, d*)

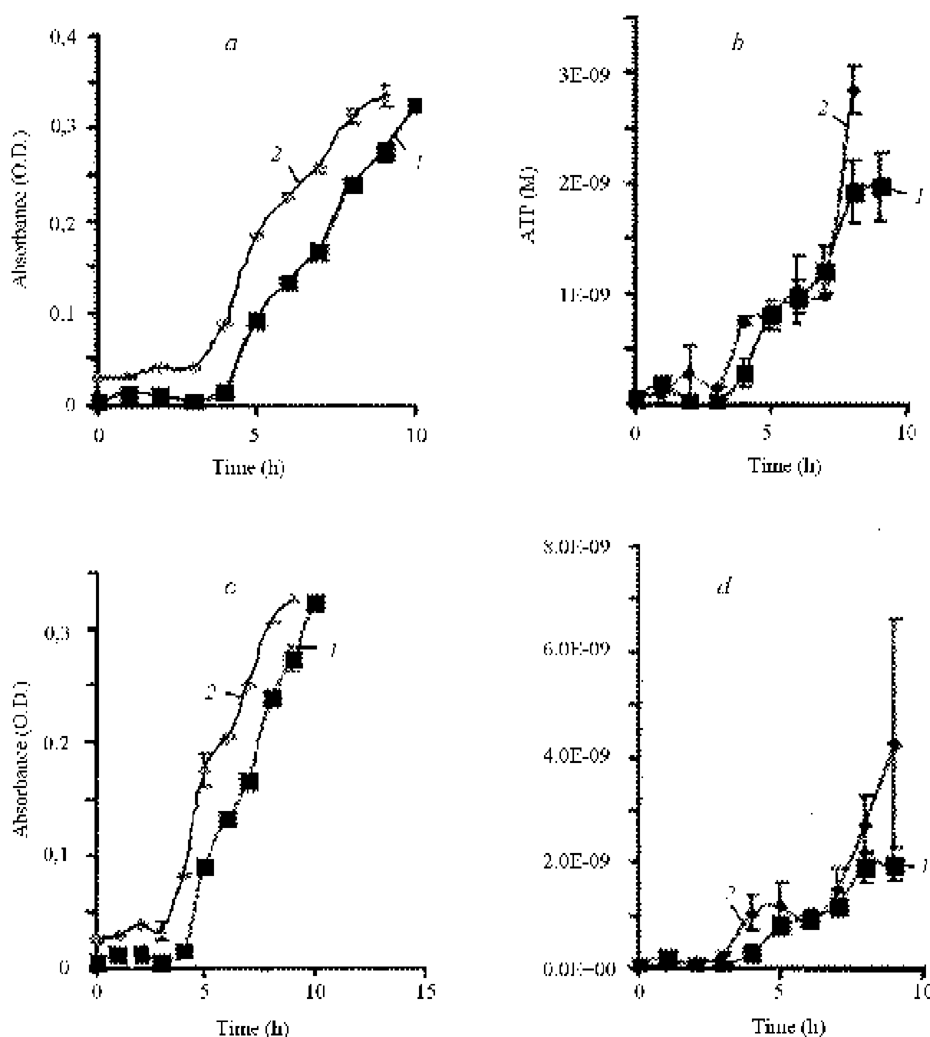

Fig. 4. Growth kinetics of *E. coli* ATCC 25952 on control (*1* – control) and modified (*2*) lactose broth detected by spectrophotometric (left – *a, c*) and bioluminescent (right – *b, d*) assays: top – comparison of control broth with medium at pH 5.4 (*a, b*); bottom – with medium at pH 8.4 (*c, d*)

nescent assay is an useful tool for the rapid prediction of the contamination of standard *E. coli* strain, which normally require long period of time in traditional method. The lactose broth was not very selective for *E. coli* growth or for the multiplication of coliforms [3]. The increase in ATP level as well as in absorbance values are related to the growth and multiplication of *P.aeruginosa*. Therefore, an increase in specific growth rate in lactose broth could be used as the indication of the contamination of standard strain with *P. aeruginosa*.

The results from this study demonstrated that the specific growth rate calculated from the kinetics of ATP measurement can be applied for monitoring the quality of culture medium and standard strains. The values obtained in our control treatments were in accordance with the results reported previously for medium containing lactose [6, 11, 12], which was in the range from 0.38 to 1.20 h^{-1} for different formulations.

The ATP detection might also be useful for quantification of *E. coli* in the inoculums used for different microbiological assays, as well as a reference point for standardization of the conventional MPN and CFU methods.

In the present study we defined the relation between ATP and the conventional microbial quantification methods such as CFU and MPN methods and the results are presented in Table 4. This data was in accordance with results of an earlier report (*13*). In spite of the observed correlation between the mentioned parameters, the values were characterized by high standard deviation (Table 4). The accuracy and the repro-

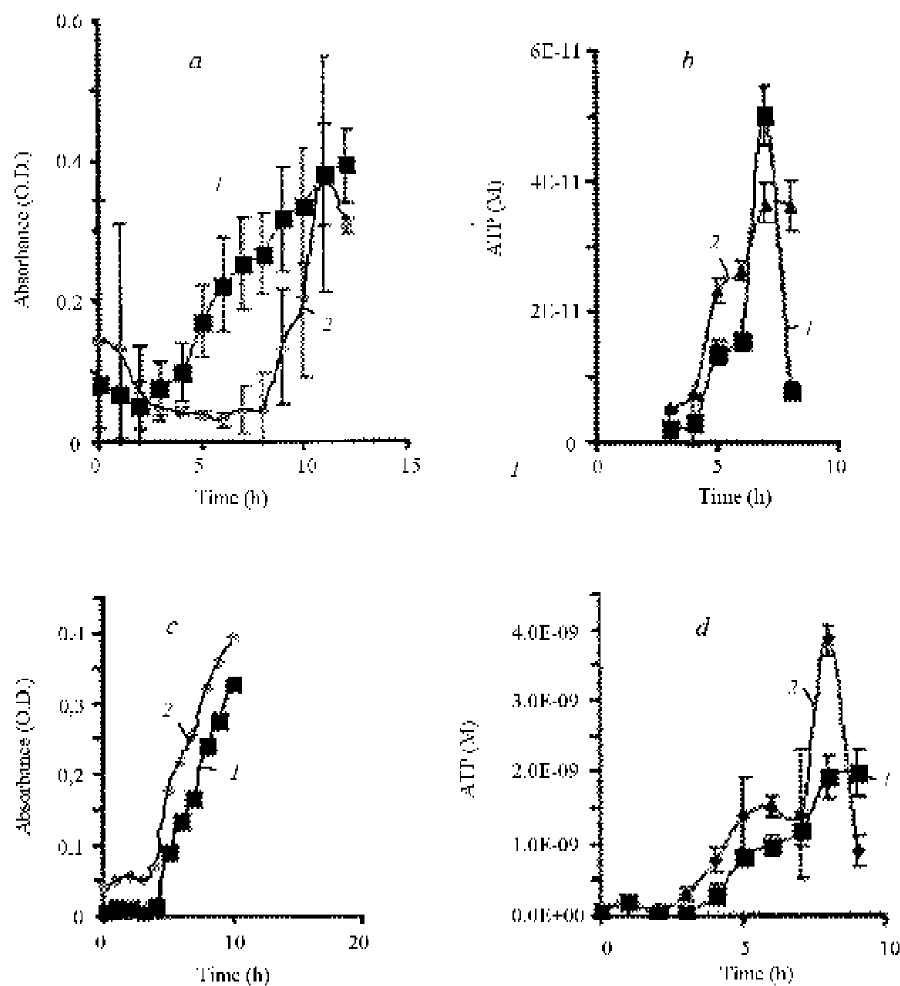

Fig. 5. Growth kinetics of *E. coli* ATCC 25952 growth (*1* – control) in the presence of *Pseudomona aeruginosa* ATCC 27853 (*2*) detected by spectrophotometric (left – *a, c*) and bioluminescent (right – *b, d*) assays on: top – 2% brilliant green bile broth (*a, b*); bottom – on lactose broth (*c, d*)

Table 2

Specific growth rate of *Escherichia coli* ATCC 25952 in lactose broth* and in modified broths

Medium	Measurement method	Growth specific rate (μ), 1/h	Standard deviation (S)	Statistical significance
Control	O D ATP	0.314 0.303	0.0007 0.0028	
Heat treated medium	O D ATP	0.215 0.1385	0.0077 0.02192	+ +
Medium at pH 5.4	O D ATP	0.177 0.2015	0.0084 0.0530	+ +
Medium at pH 8.4	O D ATP	0.189 0.350	0.0268 0.0021	+ --
Medium with 5% NaCl	O D ATP	Incalculable Incalculable	0.0063 0.113	+ +

*Medium recommended by Mexican Official Norm for MPN analysis of coliforms in water samples; + Denotes a statistical difference; – Denotes a non-statistical difference; OD – Optical Density; ATP: Adenosine triphosphate release by using dimethyl sulfoxide (DMSO).

Table 3

Specific growth rate of *Escherichia coli* ATCC 25952 contaminated with *P. aeruginosa* ATCC 27853 in 2% brilliant green bile* and lactose broth*

Srain	Applied medium modification	Measured sign	Growth specific rate (μ), 1/h	Standard deviation (S)	Statistical significance
Control *E. coli*	Lactose	O D ATP	0.314 0.303	0.0007 0.0028	
Strain growth *E.coli* with *Pseudomona aeruginosa*	Lactose	O D ATP	0.415 0.465	0.0042 0.0240	+ +
Control *E. coli*	2% Brilliant green bile	O D ATP	0.358 0.728	0.0296 0.0452	+ +
Strain growth *E. coli* with *Pseudomona aeruginosa*	2% Brilliant green bile	O D ATP	0.491 0.51	0.0518 0.0820	+ +

*Media recommended by Mexican Official Norm for MPN analysis of coliforms in water samples; + Denotes a statistically difference; – Denotes a Non statistically difference; OD – Optical Density; ATP: Adenosine triphosphate release by using dimethyl sulfoxide (DMSO).

ducibility of the bioluminescent determination of bacterial cells were high. But the results obtained by plate count technique varied in the range of 9–51% and this was due to the clamping of bacteria in solid medium. MPN technique is a statistical approach for the coliform quantification and the values in MPN table showed a wide range of possible coliform count [3].

The results showed that the ATP bioluminescence assay provided a rapid means of enumerating total numbers of viable bacterial cells. ATP is a rapid, sensitive and simple method in comparison with the conventional microbiological techniques and could be used as a complement or a substitute assay. As per the Official Mexican Norms, water samples can be stored only for six hours by the laboratories for microbiological analysis. The conventional coliform assays have duration of 24 and 48 h for presumptive and confirmative evaluation, respectively. For this reason, the quality control aspect on the culture medium and standard strain is avoided and the use of any modified methods might not lead to reliable results and proper conclusions.

From the results of this study it is suggested to use the specific growth rate as a parameter to check the quality of culture media with a short and reliable analysis of bioluminescence assay and by interpretation of the data by computational analysis.

The specific growth rate of *E. coli,* as standard strain in brilliant green bile broth and lactose broth aided to meet the Mexican Norms for coliforms.

Table 4

Correlation between ATP bioluminescence method, and conventional methods such as Colony forming units (CFU)* and Most Probable Number (MPN)* for detection of coliforms in drinking water

Culture Media	ATP/MPN +/- standard deviation(s)	ATP/CFU +/- standard deviation (s)
2% Brilliant Green bile broth with *E. coli* - control	5.54E-19 +/- 3.70E-19	7.20E-19 +/-2.07E-19
Lactose broth with *E. coli* - control	3.49E-19 +/- 1.1E-19	5.02632E-19 +/-6.81E-19

*From Mexican Official Norms.

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APPLICATION OF BIOLUMINESCENCE METHOD FOR QUALITY CONTROL OF CULTURE MEDIA AND BACTERIA APPLIED TO STANDARD NORM (NMX-AA-042-1987)

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The aim of the study was to develop a bioluminescence assay for the rapid monitoring of the quality of the bacterial culture medium and quality of standard bacterial strain by way of ATP detection, as well as to define the relationship between ATP and the bacterial population estimated by MPN and CFU methods. The growth kinetics of *E. coli* in the Brilliant green bile and Lactose broth, suggested by the NOM (Mexican Official Norms) were studied. The influence of pH, ionic force, and temperature was also evaluated for *E. coli* and *E. coli* co-inoculated with *Pseudomonas spp*. ATP was detected by the bioluminescent method after dimethyl sulfoxide treatment and simultaneously the bacteria present in the samples were enumerated by plating and MPN techniques. All the parameters tested to compare the growth of bacteria in brilliant green bile and lactose broth, were statistically significant. A strong inhibition of growth was observed in both media containing 5% NaCl. An increase in specific growth constant of *E. coli* was noted in presence of *Pseudomonas spp*. The relationship between ATP, CFU and MPN of the cultures was established and it was observed that the ATP assay has good correlation with CFU and MPN techniques.