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ВЛИЯНИЕ КОЛИЦИНА E_1 НА ВЫСВОБОЖДЕНИЕ АТФ ИЗ ШТАММОВ *E. coli* ПРИ ИХ СЕЛЕКТИВНОМ ОПРЕДЕЛЕНИИ БИОЛЮМИНЕСЦЕНТНЫМ МЕТОДОМ

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Показана возможность оптимизации биолюминесцентного анализа для селективного определения *E. coli* при использовании колицина E_1 для высвобождения АТФ. Показано, что колицин E_1 не ингибирует люциферазу, ванадат-ионы, используемые как ингибитор АТФ, при концентрации 0,05 мМ и менее не влияют на активность люциферазы. Колицин E_1 вызывает только ингибирование роста *E. coli* и не влияет на *Pseudomona aeruginosa, Enterococcus faecalis* и другие бактериальные штаммы. АТФ нельзя определять в присутствии колицина без ванадата. Определение АТФ становится возможным только в присутствии и колицина, и ванадата через 30 мин инкубации. Наблюдалась хорошая корреляция соотношений между концентрацией АТФ и количеством колониеобразующих единиц, полученных в присутствии колицина и ванадата, и с использованием диметилсульфоксида (ДМСО) для высвобождения АТФ.

INTRODUCTION

Among the various rapid methods proposed for evaluation of bacteriological quality of water and food, ATP bioluminescence was proved to be one of the most promising techniques. Several companies (Biotrace, Lumac, Promega, Sigma, NHD) produce ATP-bioluminescent reagent kits and equipment for analyzing samples for total bacterial count, but the bioluminescent method is not yet widely used for the selective detection of bacterial groups or microorganisms. The problem which limits the application of the bioluminescence assay for such detection is the absence of selectivity in the ATP release process, which is commonly carried out using DMSO or quaternary amine salts in the case of microorganisms [1] as well as non-ionic surfactants in the case of somatic cells [2]. New Horizons Diagnostics (NHD, Columbia, Maryland) had advanced its ATP bioluminescent technique to detect ATP exclusively from yeast cells, as well as to measure yeast ATP from a mixed sample of yeast and bacteria [3] using membrane filtration for separation of microorganisms based on the difference in cells size [3], but not based on selective ATP release from yeast cells. For specific identification of several bacteria and not for specific quantification, the use of selective medium followed by antibody coated beads is commonly applied [4].

In the present study, the application of bacetriocin as specific lysing reagent for *E.coli* is proposed. The bacteriocins are produced by various species of bacteria that exert a bactericidal activity on the same or closely related species [5]. Colicins are plasmid-encoded bacteriocins, which are produced by *E. coli* and other enteric bacteria. They exert a lethal effect on other bacteria including *E. coli* strains that lack the *Col* plasmid. These proteins bind to a cell surface receptor. Then the colicin E_1 inserts into the cytoplasmic membrane where it forms voltage-sensitive (trans-negative) channels that depolarize and deenergize the target cell [6].

The purpose of this study was to modify and optimize the bioluminescent assay for the selective detection of *E. coli* by employing colicin E_1 to release ATP.

MATERIALS AND METHODS

Reagents

Adenosine 5′-triphosphate (ATP), luciferin-luciferase kit for ATP detection, colicin E_1 and lactose broth were purchased from Sigma-Aldrich Chemical Co., USA. Brilliant green bile-, nutrient- broth, brilliant green- and EMB-agar were supplied by Bioxon, Mexico. Ammonium vanadate and sodium chloride were provided by ALQUIME, Mexico. All chemicals were of analytical grade.

Bacterial Strains and Inoculum Growth Conditions

The bacterial strains used in this study were: *Escherichia coli* ATCC 25952, *Enterococcus faecalis* ATCC 29212, *Pseudomona aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Klebsiella oxytoca* ATCC 49131and *Proteus vulgaris* ATCC 49132. The commercially provided strains were treated according to given instructions. Moreover, *E. coli* strain was isolated from fecal human samples using brilliant green and EMB agars. All *E. coli* strains were grown on 2% brilliant green bile broth. The other strains were grown on nutrient broth. All bacterial strains were incubated at 37°C for 6 to 8 h to obtain the inoculum for this study.

Effect of Colicin E_1 on the Growth of Different Bacterial Strains

Both E.coli strains (ATCC and local isolated) were grown on lactose broth and 2% brilliant green bile broth using 5 ml of media in tubes (of 10 ml) and 0.1 mL of inoculum. The used concentrations of colicin E_1 were 0, 4 and 10 U/mL from a stock solution (2000 U/ml). All inoculated tubes were incubated at 37°C for 8 h. The growth of *E.coli* was determined every hour by means of A_{620} in all tubes using HACH spectrophotometer (Loveland, USA).

The other bacterial strains listed above were grown in nutrient broth under the same conditions and the growth was measured in similar manner.

Effect of Colicin E_1 on ATP Release and the Vialbility of E.coli by Bioluminescence assay

The maximum bioluminescence intensity (I_{max}, mV) was detected using an EMILITE EL 1003 luminometer (Russia) and an internal ATP standard was used. In this assay local isolated *E. coli* strain was applied. It was multiplied for 12 h in 20 mL of 2% brilliant green bile broth. The cells were pelletized by means centrifugation and the pellet was suspended in 0.4 mL of physiological solution (0.9% of NaCl). This suspension was added to 0.32 ml of 2000 U/mL colicin E_1 solution and incubated at 37°C. From the resultant mixture, an aliquot of 0.01 mL was put on a luminometer cuvette containing 0.44 mL of sterile water and 0.05 ml of luciferin-luciferase reagent. ATP was measured immediately and after 15 min and at 1 h of incubation. After reading the bioluminescence intensity, 0.01 ml of 1 mM standard ATP solution was added to the same cuvette as internal standard and the bioluminescence signal was again detected. In addition, the total bacterial ATP was determined at the same manner using 0.01 ml of cell suspension and 0.09 ml of DMSO. The ATP concentration in the samples was calculated according to Ilyina et al. [2].

To estimate *E. coli* viability in the presence of colicin E_1, the plate counts (CFU/ml) were performed with all samples taken for bioluminescence detection, according to the standard microbiological method using the nutrient agar [7].

Sensitivity of Luciferase to the Presence of Colicin E_1 and Ammonium Vanadate

The bioluminescence assays were performed according to the technique outlined in the Sigma luciferin-luciferase kit [8]. The measurements were carried out using 0.01 ml ATP standard at 1 mM in the presence or absence of different concentrations of colicin E_1 (0, 10 and 100 U/ml) or ammonium vanadate (0, 0.03, 0.05, 0.1 and 1.0 mM). The maximum intensity measured in assays without colicin or vanadate was interpreted as control (100%) and compared with the values obtained in the presence of each compound.

Effect of Ammonium Vanadate on *E. coli* Growth

E. coli strain isolated previously in this study was grown on 5 ml of 2% brillant green bile broth HACH tubes with 0.1 ml of inoculum. The concentration of ammonium vanadate was varied in each three tubes from 0 mM to 0.05 mM. All inoculated tubes were incubated at 37°C for 12 h. The growth was measured every 1 hour as absorbance at 620 nm using HACH spectrophotometer (Loveland, USA).

Bioluminescent Assay to study the ATP Release from *E.coli* in the presence of Vanadate and Sodium Chloride

The bioluminescent assay was performed on both *E. coli* strains (ATCC and isolated) in order to compare the light emission in luciferin – luciferase reaction related to the release of ATP during the incubation of strains in the presence of vanadate and NaCl. The light emission was measured on a TD 20/20 luminometer (USA) with integration over 10 seconds, which provided more sensitivity in comparison with intensity measurement done previously on EMILITE luminometer. ATP is reported as Relative Light Units (RLU), taken directly from the luminometer's digital readout.

One mL of cell culture was centrifuged at 3000 rpm for 10 min at 4°C to separate the medium and to concentrate the cells. The pellet was suspended in 0.01 mL of isotonic saline solution (0.9 % of NaCl). The aliquot of 0.01 ml of cell suspension was added to 0.02 ml of ammonium vanadate at 0.1 mM and

0.07 ml of colicin E_1 (143 U/ml) with and without 0.01 ml of NaCl at 2 M. The ATP measurement was performed at 5, 10, 15, 30 and 60 min of incubation using an aliquot of 0.01 mL with 0.44 ml of ATP measurement buffer (pH 7.4) and 0.05 mL of luciferin-luciferase reagent. A control measurement was done by using DMSO as described above.

Comparison of Bioluminescent Assay for the E. coli ATP detection with colicin, DMSO to the standard plate count technique

The relation between ATP detected using DMSO and colicin E_1 (after 30 min of incubation) and CFU was defined applying the bioluminescent assay and the plate count technique as described above. Different cell suspensions were used. The final concentrations for *E. coli* varied in range 10^7–10^{15} CFU/ ml. The ATP/CFU values were calculated from the slope obtained in ATP (mol/ml) vs CFU/ml coordinate.

All assays of this study were carried out in triplicate. The plate count and bioluminescent measurements were performed in duplicate. Standard deviation and mean values were calculated for both plate count and bioluminescent ATP determination.

RESULTS AND DISCUSSION

Initially, to demonstrate the sensitivity of isolated and ATCC *E. coli* strains to colicin E_1, the kinetics of *E. coli* growth in presence of bateriocin was determined. It was observed that all tested colicin concentrations inhibited the growth of both strains (Fig. 1). In the case of colicin added treatment, the optical density of the test cultures remained constant, while cell growth was recorded in the absence of colicin. Similar results were obtained on both lactose and 2% brillant green bile broths. The relatively low concentrations of colicin 4 and 10 U/ml completely inhibited the *E. coli* growth. The term "units" (U), as used herein, means the anti-microbial activity of the colicin defined as the reciprocal of the highest dilution causing complete inhibition of growth. The literatures [9] reported that the effect of colicin on bacterial strains was influenced by the relation between the bacteriocin and cell concentration, and for that reason it was decided to use 10 U/ml or greater concentration of colicin in the subsequent assays of this study to avoid its insufficient level.

Moreover, the obtained results demonstrated that the isolated *E. coli* as well as the ATCC strains were sensitive to colicin presence. As mentioned earlier, the colicin exerts a lethal effect on the bacteria that lack the *col* plasmid and can not produce this bacteriocin. The literatures [10] reported that in a population of colicinogenic bacteria most cells do not produce colicin. At any given time around 1/1000 of the cells produces colicin. This is lethal for the producing cells. Colicin production is thus a sort of molecular kamikaze attack against sensitive bacteria. But note that it is not the colicin itself which kills the producer cell. Colicin negative mutants also die when induced. Some other property which resides on the Col factor is lethal [10]. This may indicated that only

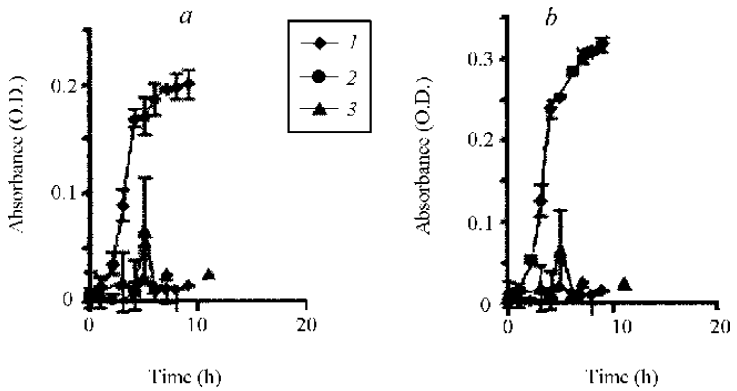

Fig. 1. Kinetics of the growth of *E. coli* (left – *Escherichia coli* ATCC 25952; right – isolated strain) on lactose broth in the presence and absence of colicin E_1: (*1*) control without colicin, and (*2, 3*) with 4 and 10 U/ml of colicin, respectively

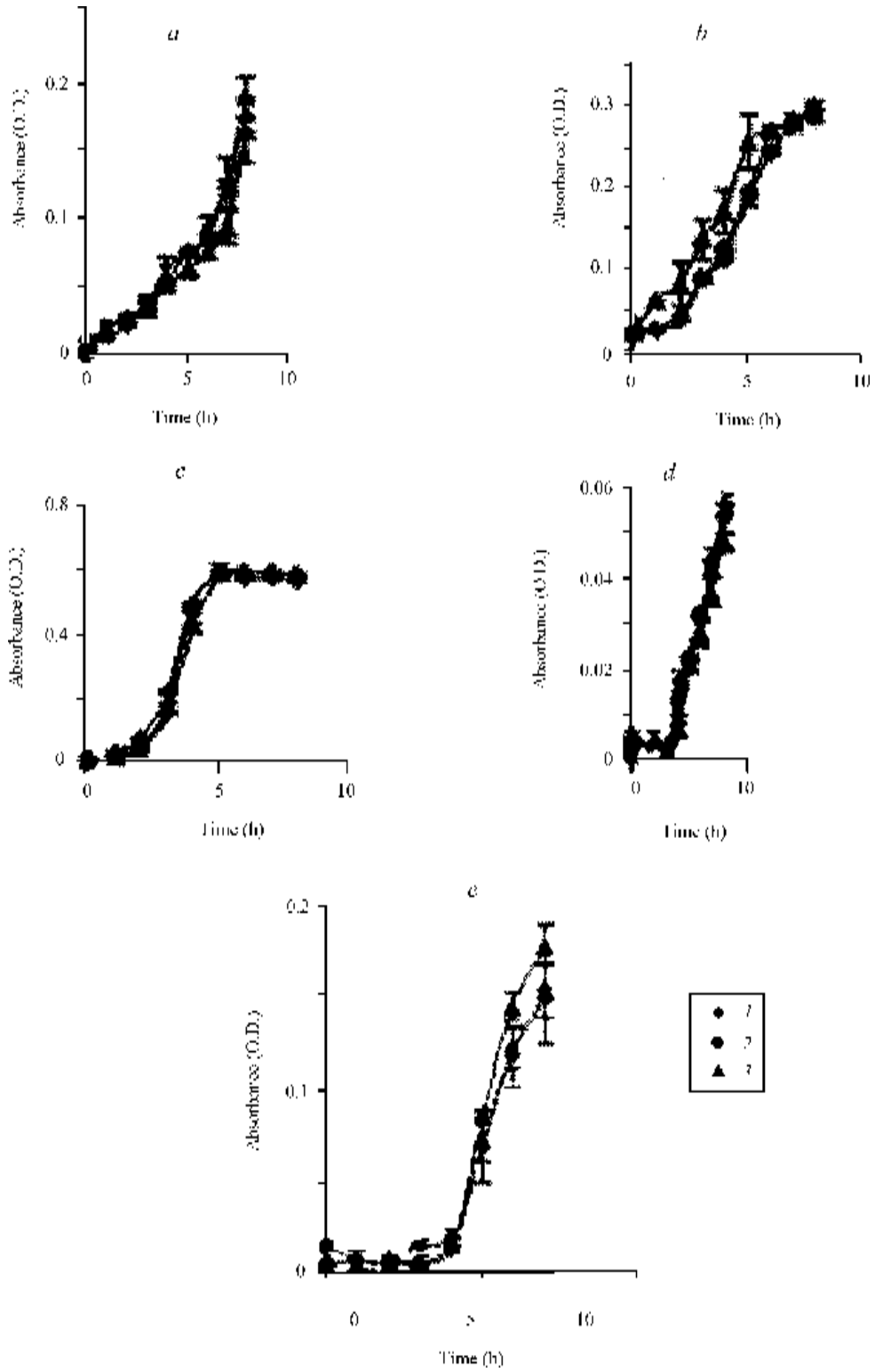

Fig. 2. Kinetics of the growth of *Proteus vulgaris* ATCC 49132(*a*), *Klebsiella oxytoca* ATCC 49131(*b*) *Enterococcus faecalis* ATCC 29212 (*c*) , *Pseudomona aeruginosa* ATCC 27853 (*d*), *Staphylococcus aureus* ATCC 29213 (*e*) on nutrient broth in the presence and absence of colicin E_1: (*1*) control without colicin, and (*2, 3*) with 4 and 10 U/ml of colicin, respectively

Table 1

Effect of Different Concentrations of Colicin E_1 on the Bioluminescent Intensity Measured with 1 mM ATP Standard Solution

Colicin E_1 concentration, U/mL	I_{max}, mV
0.0	390.4+/–10.2
10	379.3+/–13.2
100	386.2+/–9.4

a small percent of enteric bacteria might be resistant to colicin. It is significantly less than the statistical variability of common microbiological methods applied actually for fecal contamination monitoring.

The results obtained with different Gram positive and negative strains mentioned above also demonstrated that colicin E_1 did not affect the growth of other tested strains. The kinetics of growth was same both in the presence and absence of colicin (Fig. 2). It clearly demonstrated the selective action of the colicin E_1 on *E.coli* and demonstrated the possibility of the use of colicin for selective quantification of *E. coli.*

In assay performed with ATP standard, it was observed that the presence of colicin at the test concentration (10 U/ml) as well as at 10-fold greater level (100 U/ml) did not influence the bioluminescence intensity measured on EMILITE luminometer (Table 1). This showed that the compound applied at mentioned concentration did not affect the luciferase reaction. At the same time, it was observed that the luciferase activity was strongly inhibited in the lactose and 2% brillant green bile broths due to their composition. Due to this fact, the cells were separated by means centrifugation for all subsequent bioluminescent assays.

In the assay performed with isolated *E. coli* strain to study the ATP release in the presence of colicin it was demonstrated the absence of bioluminescence intensity in the samples incubated for 60 min (Table 2). The control measurements done with DMSO and plate count technique verified the presence of viable bacteria and showed ATP detection after DMSO lysis. Both parameters (CFU/ml and ATP) decreased approximately at 10-fold level after 15 min of incubation. It means that only 10% of cells were viable after 15 min of incubation with colicin.

The sensitivity of ATP detection on EMILITE luminometer was insufficient to perform the measurement of ATP present after 60 min of incubation. The CFU counts were decreased at 99.6% in comparison with initial data that indicated the lethal effect of bacteriocin on *E. coli* strain. The colicin provoked the death of cells, with rapid hydrolysis of ATP, which impeded the measurement of ATP liberation in the incubation medium. It is known that the ATP hydrolysis is performed by cellular ATP-ases, which are inhibited by DMSO but not by colicin.

Based on the obtained results, the vanadate, an ATP-ase inhibitor was used to study the sensitivity of luciferase activity. Vanadate ions are known inhibitor of a wide spectrum of ATP-ases [11]. Results presented in Table 3 showed that luciferase activity was affected by the presence of vanadate at 1 mM. In the presence of concentrations less than 0.1 mM of vanadate, the bioluminescence signal was comparable to the one detected in the absence of vanadate (Table 3). *E. coli* growth kinetics also demonstrated that 0.05 mM vanadate did not affect the cell viability and growth (Fig. 3). Based on these results, 0.05 mM vanadate concentration was se-

Table 2

ATP detected in the presence of colicin and DMSO for the isolated *E. coli* strain and the quantification by plate count technique (CFU/ml)

Incubation time, min	ATP, mM in colicin treatment	ATP, μM in sample treated with colicin after DMSO lysis	CFU/ml
0	0	32.7+/-7.9	4.14E+10
15	0	3.23+/-0.001	3.29E+9
60	0	0	1.63E+8

Table 3

Effect of Different Concentrations of Ammonium Vanadate on the Bioluminescent Intensity Detected with 1 mM ATP standard solution

Vanadate concentration, mM	I_{max}, mV
0	188.6 +/- 10.2
0.03	177.0+/- 4.2
0.05	186.8+/- 10.2
0.1	171.5+/- 6.4
1	171.5+/- 6.4

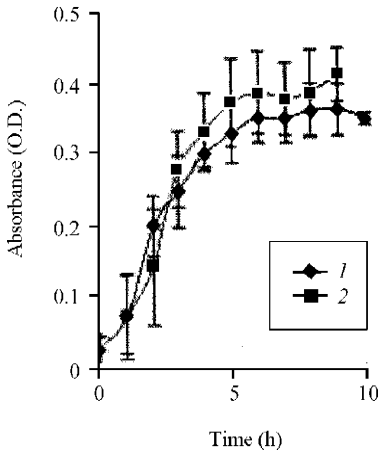

Fig. 3. Kinetics of the growth of *E.coli* in the presence and absence of ammonium vanadate (*1*) control without vanadate, and (*2*) with 0.05 mM vanadate

lected for further studies as it did not influence the luciferase activity.

In order to increase the sensitivity of detection, the next assay was carried out using TD 20/20 luminometer (USA) instead of EMILITE. It was demonstrated that the use of vanadate improved the ATP release from *E. coli*. However, ATP was detected only after 30 min of incubation, in the presence as well as the absence of NaCl (Fig. 4). RLU´s detected after 5 min of incubation were significantly less than the values obtained with DMSO as control (Fig. 4). Similar results were detected at 10 and 15 min of incubation. The RLU´s detected after 30 min and 60 min of incubation was similar to the control values measured with DMSO. The detected bioluminescence increased approximately by 1.5-fold after 60 min of incubation in the presence of colicin and vanadate,

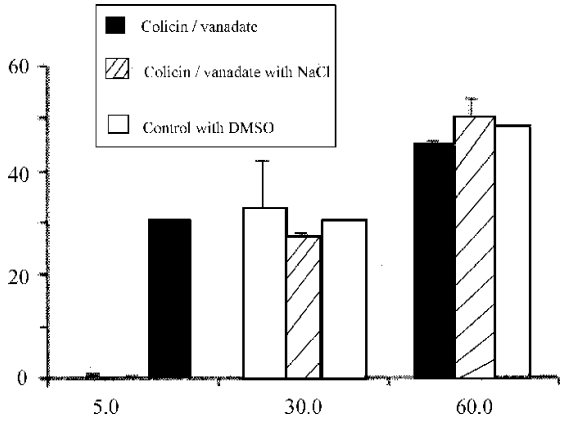

Fig. 4. ATP release during incubation of *E. coli* with colicin/vanadate in the presence and absence of NaCl. Control measurement of ATP with DMSO was carried out in the same cell suspension at the defined incubation time

as well as in control suspension without bacteriocin, which could be related to the cell adaptation process [12]. Although in this case the average value was greater in the presence of NaCl, there was no statistical variation. Similar results were obtained for both *E. coli* strains. It demonstrated that colicin/vanadate could be applied for the ATP release from *E. coli*, and thereby the selective bioluminescent detection of *E. coli*.

Colicin E_1 is a bactericidal protein that binds to specific receptors in the outer membrane of *E. coli* cells and affects membrane associated, energy-coupled activities of the bacterial cells [5, 6]. The results in Fig. 4 demonstrated the effect of vanadate in the inhibition of the ATPase which hydrolyzed intracellular ATP after the formation of ion channels (Table 2) and prevented its release [11].

The accuracy and the reproducibility of the bioluminescent determination of bacterial cells were high, whereas values of plate counts for the same samples varied in the range 11–51%. The significant variation in the plate count data is probably due to clumping of bacteria in the solid agar media. The specific content of ATP (mol/CFU) was calculated from the slope obtained in the ATP vs CFU coordinate, using the ATP values detected with colicin and DMSO. The obtained linear regressions were ATP (mole/mL) = 5.96E–19*x* (CFU/ml) with a standard deviation of 9% and ATP (mole/ml) = 6.50E–19*x* (CFU/mL) with a standard deviation of 5.48 %, for colicin and DMSO, respectively. The values of ATP content calculated in this study correlated with an earlier report [13]. The ATP/CFU demonstrated a good correlation between

the ATP concentrations detected with DMSO and with colicin E_1 in the presence of vanadate and this showed that assay with colicin E_1 could be used as an indicator of total ATP release in case of coliforms.

In conclusion, the results of this study demostrated the possibility of the use of colicin E_1 for the selective bioluminescent detection of *E. coli,* which are employed as bioindicators of fecal coliforms.

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EFFECT OF COLICIN E_1 ON ATP RELEASE FROM *E. coli* STRAINS FOR ITS SELECTIVE DETECTION BY BIOLUMINSECENCE ASSAY

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The purpose of this study was to demonstrate that the bioluminescent assay could be optimized for selective *E. coli* detection by employing colicin E_1 for ATP release. Colicin E_1 is a bacteriocin, which is an extracellular proteinaceous antimicrobial substance that exerts a bactericidal activity on the same or closely related species. Considering the sensitivity of luciferase to various inhibitors, it was proved that the colicin E_1 did not inhibit luciferase at different concentrations. Further, the luciferase activity was not affected by vanadate ions (using as ATP-ase inhibitor) at 0.05 mM concentration or less. By means of spectrophotometric assay, it was demonstrated that colicin E_1 caused only inhibition of *E.coli* proliferation without an effect on *Pseudomona aeruginosa, Enterococcus faecalis* and other bacterial strains. It was observed that in the presence of colicin without vanadate, ATP was not detected. ATP quantification was possible after 30 minutes of incubation and only in the presence of both vanadate and bacteriocin. The ATP/ CFU relations obtained with colicin/vanadate demonstrated a good correlation with the values detected using dimethyl sulfoxide (DMSO) for the release of ATP.