RAPID ATP METHOD FOR THE SCREENING AND IDENTIFICATION OF BACTERIA IN FOOD AND WATER SAMPLES

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Previously, measurement of microbial Adenosine Triphosphate (ATP) has been applied to enumerating bacterial populations in water, wastewater, marine environments, urine, milk, and various foods, just to name a few. However, two universal difficulties were encountered in the application of this method: (1) Interference by non-microbial sources of ATP, and (2) Chemicals or metals in the sample. These two factors led to the limitation of sensitivity as well as false positives and negatives. Recent advances in instrumentation, reagents, and the inclusion of membrane filtration methods have resolved many of these problems.

Studies performed in various countries including the US, Canada, and Poland have shown the rapid 5 min ATP test to have a correlation of from 85%–95% to the standard 48 h APC. The sample volume can also be increased by use of a unique cell concentrator, allowing for detection of as few as 200 cfu/ml. Additionally, by varying the size of the filtration membrane the system has been shown to separate and quantify the amount of yeast and bacteria in beverage or other samples within 5 min. This technology may also be utilized for the specific identification of select organisms by use of select lysing reagents (*Listeria spp.*) in lieu of a total extracting reagent, as well as specific antibody coated beads or chemiluminescence. The specific assays can be accomplished withing 20 min with a sensitivity of less than 10^3 cfu/ml.

Introduction

Real time or near real time methods for detecting microorganisms are essential for implementation of a Hazard Analysis Critical Control Point (HACCP) program in any food and beverage plant (Cutter et al., 1996; Northcutt and Russell, 1996). ATP bioluminescence is widely used in rapid methods for assessment of cleanliness in food processing plants (Russell, 1995). Currently available methods, however, neither differentiate between bacterial and non-bacterial ATP, nor correlate with standard culture methods. Also, it has been suggested that residual sanitizers on surfaces may cause a significant reduction in the bioluminescence signal (Velazquez et al., 1996) by degrading Luciferin–Luciferase. Thus the presence of sanitizer residues on sampling sites could present a potential problem in underestimating the actual ATP signal and consequently effecting the cleanliness and actual hygiene status.

New Horizons Diagnostics (NHD, Columbia, Maryland) has developed a filtration-based bioluminescence technique which is able to separate bacteria from non-bacterial sources, thus able to detect bacterial ATP only (Siragusa et al., 1995, 1997). A hand-held Microluminometer (Model 3550i) is used for this purpose. The bacterial ATP is expressed as Relative Light Units (RLU) as it appears on the luminometer's digital readout. A good correlation exists between the counts (RLU) and corresponding bacterial Colony Forming Units (CFU). Evaluation studies were performed on over 1000 samples of beef, pork, and chicken carcasses by the researchers of the United States Department of Agriculture and the results were compared with conventional culture methods. A good correlation ($r = 0.92$) between the counts of bacteria on plates and the ATP from the luminometer for the same carcass sample was obtained (Siragusa et al., 1995; 1997; Cutter et al., 1996).

NHD has further advanced its ATP bioluminescence-based technology to detect ATP exclusively from yeast cells as well as to measure a mixed sample of yeast and bacterial. Yeast, the unicellular budding cell, is ubiquitous in the environment, being found on fruits, vegetables, and plant materials. The presence of yeast as a contaminant in the beverage industry has been a major concern. Rapid detection of yeast ATP employing NHD's luminometer would allow instituting proactive measures for quality assurance, i. e., implementing HACCP programs. This study describes the evaluation of a novel method to monitor yeast ATP as well as bacterial ATP, without the adverse affect of various chemicals, such as sanitizers.

Materials and Methods

Bacterial and Yeast strains: *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes*, and *Sacharomyces cereviciae.*

Sanitizers: Trichloroacetic acid and Sodium Hypochlorite (bleach) at concentrations of 5%, 2.5%, 1.25%, 0.625%, 0.312%, and 0.15%.

Rapid Microbial ATP Assay (PROFILE®): Microluminometer NHD Model 3550i (Fig. 1), Filtravette™ (.45u and 5.0u), Somatic Cell Releasing Agent (SRA),

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Fig. 1

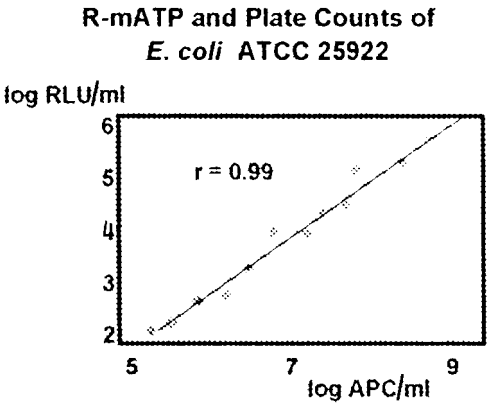

Fig. 2

Bacteria Cell Releasing Agent (BRA), Luciferin-Luciferase (LL) and cell concentrator with syringe.

Standard PROFILE® procedure: A sample suspension is transferred to the Filtravette™ (.45u for bacteria). Three (3) drops of Somatic Cell Releasing Agent (SRA) are added. The mixture is pushed through the Filtravette™ by a positive pressure device. Three (3) more drops of SRA are added and pressure-filtered to ensure the removal of interfering substances, free ATP, and somatic cell ATP. The Filtravette™ is then placed into the drawer slide of the Microluminometer (PROFILE®). Two (2) drops of Bacterial Releasing Agent (BRA) are added into the Filtravette™ to extract the microbial ATP. Immediately after the addition of the BRA, 50 μl of Luciferin-Luciferase (LL) is added and mixed by aspirating the fluid up and down three (3) times. The drawer slide is closed immediately. Light emission is measured with integration over ten (10) seconds. ATP is reported as Relative Light Units (RLUs), taken directly from the luminometer's digital readout (Fig. 1).

Assay with Sanitizers: A fifty (50) μl suspension of *S. aureus* and *E. coli* was transferred to the Filtravette™. Fifty (50) μl of sanitizer was then added to the bacterial (.45u) Filtravette™ and the standard PROFILE® procedure was performed. The experiment was repeated with various concentrations and types of sanitizers. The expelled liquid filtrate was then assayed for bacteria utilizing the standard PROFILE® procedure.

Assay with Yeast: Collect a two (2) ml sample of beer in a sterile syringe. Place a five (5) micron yeast Filtravette™ into the cell concentrator. Affix the cell concentrator to the syringe and expel the contents through the cell concentrator. Any yeast present will be collected in the Filtravette™. Then perform the standard PROFILE® procedure beginning with the SRA wash.

Table 1

Measurement of ATP vs. CFU (*S. aureus*)

Average Initial RLU (50 μl) Sample	Average RLU Sequence 2 Minutes Period 15 Seconds Intervals	Average Total Plate Count / ml
0	0	1.0×10^4
17	15, 18, 12, 6	5.5×10^4
545	532 468, 443 423 533 438, 417, 388	5.0×10^5
2725	2706, 2654, 2629, 2568, 2513 2482, 2430, 2378	7.5×10^6
16 500	16 456 16 337, 16 363, 15 962, 14 138 12 882	5.0×10^7

Table 2

Separation and Measurement Yeast and Bacteria in Beer

Sample	Average Yeast RLU's 2 ml Beer	Average Yeast CFU's 2 ml Beer	Average Bacteria RLU's 2 ml Beer	Average Bacteria CFU's 2 ml Beer
# A	15	2.0×10^2	250	7.5×10^4
# B	70	1.0×10^3	25	2.0×10^4
# C	950	8.5×10^3	95	4.5×10^4
# D	2500	1.8×10^4	2100	7.0×10^5
# E	9500	3.0×10^4	450	1.0×10^5

Results and Discussion

The rapid PROFILE® Luminescence System was challenged to perform with various samples under pristine, as well as stressed environments. The assay performed well; correlating to culture for both bacteria and yeast directly from a beverage sample. The separation/filtration method employed also demonstrated satisfactory performance when confronted with high salts and metals of the type normally found in the work environment. To date, this is the only 5 min bioluminescence system which correlates to standard culture methods as verified by various governmental laboratories.

Utilizing sodium hypochlorite (bleach) and trichloroacetic acid, a reduction of 94 to 96% in the bioluminescence signal was observed when concentrations

higher than 1% were used in conjunction with the standard non-filtered ATP method. It was further demonstrated that when the SRA wash step was employed, this quenching effect was significantly reduced or removed expanding the utility of this method.

Testing with a pure culture of *S. aureus* the PROFILE® ATP method could readily detect 10^5 CFU/ml with a 50 μl sample size. When the bacterial sample size increased to 2 ml, there was a 1 log increase in sensitivity, even in a beer sample that included yeast as well as bacteria. Results from a beer sample demonstrated the PROFILE® ATP system could readily detect 10^3 yeast per ml with a 2 ml sample. Subsequent testing has indicated sensitivity can be further improved by increasing the sample size as well as adjusting the voltage setting of the instrument.

The method described detects and enumerates generic bacteria or yeast. Several modifications can be employed that will allow specific identification. These may include antibody coated beads, chemiluminescence, or the use of specific lysing reagents. Recent preliminary data has demonstrated that a specific *Listeria spp.* enzyme may be employed in lieu of a generic bacterial releasing agent (BRA) to selectively lyse only Listeria. This method may be useful as a rapid environmental monitoring tool to determine the effectiveness of cleaning or identification of potential problem areas.

Conclusions

1. Real time or near real time methods for monitoring microorganisms are essential for implementation of a Hazard Analysis of Critical Control Point (HCCAP) program, determine contamination, or the quality of a food or beverage item.

2. Presence of salts, metals or other chemicals could present a potential problem in underestimating the actual ATP signal when select bioluminescence techniques are used; thereby mis- representing the actual hygiene status or presence of bacteria.

3. A rapid microbial ATP bioluminescence assay employing a hand held microluminometer has been developed by New Horizons Diagnostics Corp. (NHD) that can be used for near real time microbial load monitoring in food and beverage processing plants.

4. Employing NHD's bioluminescence technique, interfering residues could be removed/reduced, through filtration, to a level that does not significantly inhibit the bioluminescence signals. Consequently, the actual bacterial status of a surface is obtained.

5. NHD's filtration-based system detects and separates Yeast and Bacteria in less than two (2) minutes while providing good correlation ($> 90\%$) with conventional culture methods which require as long as 4–7 days.

6. Further advances in this system allow for increased detection limits as well as specific identification.

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