J. Inst. Brew., September-October, 1989, Vol. 95, *pp.* 317-319

RAPID METHODS FOR THE DETECTION OF YEAST AND LACTOBACILLUS BY ATP BIOLUMINESCENCE

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Received 18 November 1988

Present plating methods for the detection of potential spoilage organisms in packaged beers require 2-3 days for yeast, and 5-7 days for lactic acid bacteria. Simple and inexpensive sterility tests based on bioluminescence have been developed for the detection in broth media of small numbers of yeasts in 1 day, and lactic acid bacteria, in both normal and heat-stressed conditions, in 3 days.

Key Words: *Bioluminescence, detection, lactic acid bacteria, yeast.*

INTRODUCTION

The brewing industry has for many years sought means of accelerating the traditional culture methods of microbiology. The approaches which have attracted most attention recently have been based on microscopy, and the detection of products of microbial metabolism.

This study utilized the type of bioluminescence whereby the light produced when ATP reacts with the firefly luciferin/luciferase enzyme system is measured. It is a well understood Methodology, and depends on the reactions:

$$\text{Luciferin} + \text{Luciferase} + \text{ATP} \rightarrow \text{Luciferin-Luciferase-AMP} + PP_i$$

$$\text{Luciferin-Luciferase-AMP} \rightarrow \text{Oxiluciferin} + \text{Luciferase} + \text{AMP} + CO_2 + \text{photon}$$

The light emitted is measured on a photometer at 562nm. It is proportional to the amount of ATP in the sample when all other reactants are in excess. The quantity of ATP in microbial cells has now been well established[2], being of the order of 1 fg(10^{-15}g)/bacterial cell, and 100 fg/yeast cell.

Bioluminescence has appeared on a number of occasions in the brewing literature. The ATP contents of brewing yeasts and bacteria[2], and factors affecting those contents[3] have been investigated. It fluctuates only within relatively narrow limits depending on the cell's physiological condition[3], so that the quantity of ATP in the sample correlates with the number of cells present. Various applications of bioluminescence to brewing microbiology have been reported[4,5,6,10].

Previous experience in this laboratory had shown us that very low levels of contamination by lactic acid bacteria (*Lactobacillus* and *Pediococcus*) could lead to spoilage of final packaged products. Our aim was therefore to develop methods which reduced the incubation times for sterility testing as much as possible, whilst ensuring that 1 colony-forming unit per volume of product sampled could be confidently detected, even if heat-stressed.

Detection of spoilage by forcing the product itself, and testing for growth after an adequate interval was shown to be of limited value. It enables detection of yeast spoilage of alcohol-free, relatively nutrient-rich lager in 24 h (unpublished results). However, for alcoholic beers and infections by lactobacilli, this approach offered only a small time saving over the standard forcing test, *i.e.* often longer than the current culture methods. A membrane filtration stage was therefore incorporated, followed by incubation in a broth growth medium.

EXPERIMENTAL

Organisms. Lactobacillus cultures were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, or isolated from brewery samples. Yeasts were obtained from the National Collection of Yeast Cultures, Norwich, or from brewery samples. Seeded samples were prepared by inoculating yeasts and lactic acid bacteria into WL broth (Difco) or Raka-Ray broth[9], and incubating at 27°C for 24 or 72 h respectively. They were then diluted in 0.25% NaCl to the required concentrations, and added to pasteurized beers. If the organisms were to be heat-stressed, approx 10^4 cells were heated in beer to 53-54°C for 1-4 min depending on thermal tolerance. The medium used for detection of lactobacilli was Raka-Ray broth supplemented with 10 ppm cycloheximide, 0.15% 2-phenylethanol, and 50 ppm vancomycin[7]. WL broth was used for yeast detection as directed.

Reagents. Despite its high-energy properties, the ATP molecule is extremely stable, and particularly heat-resistant; it is practically unaffected by boiling, and surface sterilization with methylated spirits. Autoclaved or sterile disposable containers and utensils are not necessarily ATP-free, and batches were checked before use by testing 100 μl residual or rinse water with 50 μl luciferin/luciferase: Relative Lights Units (RLU)<1000.

All water used was distilled, autoclaved, and sterile-filtered.

Tris-acetate buffer was prepared by autoclaving a 0.1M aqueous solution, adding EDTA (Sigma) to 2mM, and adjusting pH to 7.75 with 10M NaOH. Stock solutions of ATP (Sonco) were prepared by diluting 10μg ATP with 10 ml water, and storing in 2 ml aliquots at −18°C. Working solutions were prepared from stock solutions by a 500 fold dilution, and stored at 4°C prior to use. ATP solutions were stable for several months at 4°C, and even longer at −18°C.

Apyrase (Sigma) was diluted as directed with water. 5 ml aliquots were prepared which could be stored for 3 months at −18°C with minimal loss of activity. It was then further diluted 100 fold with water, and stored up to 1 week in 4°C.

Luciferin/luciferase (Sigma) was prepared as directed, and held at room temperature for approx 1 h until a satisfactory low blank reading was obtained. 5 ml aliquots were prepared, which were stable for 1 month at −18°C, or 3 days at 4°C.

Chlorohexidene gluconate (hibitane, ICI) was used as extractant at 0.3% in tris acetate buffer. Solutions were stable for 2-3 days, and performance was found to compare favourably with the previously-used extactant, NRB (Sonco). Cell breakdown occurs immediately, and the light output then remains constant for up to 1 h. Hibitane also inhibits apyrase activity[1], so that traces of residual apyrase after washing do not break down ATP in the sample.(However, attempts to simplify the method below by adding the extractant directly to samples with apyrase still present were unsuccessful, possibly be due to a difference in pH optima.) Hibitane is much less expensive than NRB, showed slightly better responses on membrane filters, but slightly poorer in other samples. Responses were slightly enhanced by rapidly heating the sample to 100°C, but this stage was not included in the method.

Other previously reported extractants, acetone[5], dimethylsulphoxide[2], alcohol, tri-chloroacetic acid[8], and benzalkonium chloride[8] have to be removed before addition of lucifern/luciferase, because of inappropriate pH, or quenching of the light signal.

Method. Beer, 250 ml, was filtered through a 47 mm diam, 0.45 μm membrane. The membrane was transferred face-down to a 55 mm plastic Petri dish containing 2 ml of WL broth (for yeasts) or Raka-Ray broth (for lactobacilli). Cultures in WL broths were incubated aerobically for 24 h, and Raka-Ray anaerobically for 72 h, both at 27°C. Then 1 ml of apyrase was added, and after 1 h at room temperature, the membrane was re-inverted into a filter holder, excess reagent pipetted in, filtered, and washed with 100 ml water. The membrane was transferred to a sterile Petri dish, and 500 μl of hibitane added. 200 μl was withdrawn into a cuvette, mixed with 100 μl luciferin/luciferase, and the emitted light was read in the luminometer counting chamber.

Controls were included in each batch as follows.

Luciferin/luciferase reagent effectiveness-50 μl of luciferin/luciferase was tested with 200 μl of working ATP solution, containing 200 pg (RLU=100,000-300,000).

Hibitane contamination-200 μl was tested with 50 μl luciferin/luciferase. (RLU<1,000).

Extractant effectiveness-100 μl of hibitane was added to 100 μl of 0.25% NaCl containing 10^3 yeast cells, and 50 μl luciferin/luciferase. (RLU=50,000-200,000).

Sterile beer samples-2 samples were tested by whichever of the above methods was in use. (RLU<15,000).

Original concentrations of organisms were established by incubating duplicate membrane filters on the corresponding agars.

Instrument. A Berthold Biolumat LB 9500 was used, and emitted light was measured by integration mode over 10 s after a 2 s delay. Manual injection was used, to maximize utilization of luciferin/luciferase without its contamination.

TABLE I. *Bioluminescent Detection of Lactobacilli, No Heat Treatment*

Organism	Number detected by membrane filter
L.buchneri NCIB 8516	12
L.brevis NCIB 8561	3
Brewery Isolate 1	3
" " 2	1
" " 3	2
" " 4	1
" " 5	3
" " 6	3
" " 7	7
" " 8	11
" " 9	49
" " 10	19
" " 11	37
" " 12	5
" " 13	62
" " 15	49

TABLE II. *Bioluminescent Detection of Lactobacilli, After Heat Treatment*

Organism	Number detected by membrane filter
Brewery Isolate 2	13
" " 5	4
" " 6	48
" " 7	58
" " 10	36
" " 12	13
" " 13	62
" " 14	78
" " 16	49

TABLE III. *Bioluminescent Detection of Yeast*

Organism		Number detected by membrane filter
Saccharomyces cerevisiae (brewery strain)		2
Kleckera apiculata	NCYC 328	1
Saccharomyces cerevisiae	NCYC 396	2
Saccharomyces diastaticus	NCYC 447	9
Saccharomyces cerevisiae	NCYC 1175	1
Saccharomyces cerevisiae	NCYC 1177	3

Results And Discussion

In interpreting luminometry results as showing growth or no growth, a cut-off point had to be chosen. On the basis of our experience, samples showing more than three times the average of two control samples were taken as positive, i.e. growth had occurred. Results presented in Tables 1 and 2 are for *lactobacilli* in normal and heat-stressed conditions respectively; these were performed with different samples of the cultures. Results for yeasts are presented in Table 3. All are means of duplicates for both methods; all organisms tested were detectable by bioluminescence.

The methods described enable detection of low number of yeast in 1 day, and lactobacilli in 3 days. This compares favourably with the present standard methods, especially the 7-day *lactobacillus* incubation. Bioluminescence does not provide information on the type of organism detected. However, the medium employed contains the antibiotics 2-phenylethanol, cycloheximide, and vancomycin, thus inhibiting Gram-negative bacteria, many yeasts, and most Gram-positive bacteria except the heterofermentative *lactobacilli* which we wish to detect. An antibiotic-containing medium specific for yeast could similarly be developed. Where a positive result was obtained, saline rinsing of the first Petri dish used for incubating the sample into a growth medium often provided cultures for further testing, helping to counter the criticism that luminometry is a destructive methodology. The technique takes little longer to perform than existing methods, does not cause strain on the operator, and the cost of the above reagents is acceptable.

There is considerable variation in free ATP contents of different beers[3]. For the products used in these trials, sufficient ATP was present to require enzymic hydrolysis of free ATP in the method, in order to achieve maximum sensitivity. Raka-Ray medium caused considerable quenching of the luminescence signal. Most complex media, including beer itself, have this effect; it is minimized by the water wash recommended.

The development of bioluminescence methodology has occurred at an increasing pace over the last ten years in particular. Its introduction into routine usage had previously been hampered by the lack of reagents' availability and their high cost, problems which have now been largely overcome. The instrumentation is relatively inexpensive, and its potential range of applications within the brewing industry is wide.

Acknowledgement: The authors would like to thank the Directors of Bass PLC for permission to publish this paper.

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