rapid methods in food microbiology

EDITED BY
M.R. ADAMS AND C.F.A. HOPE

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M.R. ADAMS AND C.F.A. HOPE

Department of Microbiology, University of Surrey
Guildford, Surrey GU2 5XH (Great Britain)

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PREFACE

The increasing need for microbiological testing of foods, both in a regulatory context and in routine quality control, has only served to expose further the limitations of traditional techniques. Over recent years, this has prompted the development of methods identified as being "rapid"; an attribute which is conventionally taken to mean two different things. In some, it implies the result is obtained in a shorter time than with conventional plate-counting techniques, while in others a result is obtained, not necessarily sooner, but after less time is spent in the physical processing of the sample, the preparation of materials and the reading of results. Several rapid techniques possess both of these virtues to varying degrees.

This volume of Progress in Industrial Microbiology has its origins in two workshops held at the University of Surrey in 1986 and 1988. These provided participants with an opportunity to gain practical experience of rapid microbiological methods appropriate to food, in conjunction with a lecture programme outlining the background theory. In the time available, it was felt that many of the lectures could not do full justice to their subjects and that while authoritative reviews on individual techniques are published from time to time, a collection of up-to-date reviews in a single volume was needed.

In deciding which techniques to cover, we have restricted our attention to those for which there is a reasonable corpus of published information testifying to their utility, rather than include one-off speculations that may well prove ephemeral. Individual authors have been encouraged to give their thoughts on future developments in their own areas but, to allow some less restrained speculation about the future, we have devoted one chapter to an over-the-horizon view.

Considerable detail of actual or potential applications is given under the individual methods but, to provide an alternative viewpoint, we have also included chapters addressing the role of rapid methods in general. The first is concerned with a specific analytical problem - the quantitative estimation of filamentous fungi in food materials, while the second discusses the requirements of one particular branch of the food industry, brewing. We hope that this combination of approaches will give the reader not only a ready source of reference on the different techniques, but a wider view as to how they might be applied in different situations.

M R Adams and C F A Hope
University of Surrey, 1989

CONTENTS

ATP ESTIMATION

C J STANNARD
Leatherhead Food RA, Randalls Road, Leatherhead, Surrey KT22 7RY, UK

1. INTRODUCTION

Conventional microbiological techniques, and, indeed, some of the more rapid alternatives, rely on the amplification of microbial numbers by growth and division to provide a result. For example, a single cell must divide to form 10^{12} cells to produce a visible colony, 10^{6} cells to produce turbidity in a broth culture, or 10^{6}-10^{7} cells to produce a change in electrical conductivity. This dependence on growth ensures sensitivity, since a few microbial cells can be easily distinguished from food particles by their ability to divide; however, the time taken to obtain a result is limited by the generation time of the organism.

Faster detection of micro-organisms is possible if the cells are considered as a collection of compounds that can be assayed in minutes using a biochemical test. Several cell components have been used as the basis for such assays, for example, Gram-negative lipopolysaccharide (ref.1), haematin (ref.2) and adenosine triphosphate (ATP). The lack of amplification by growth in this type of test means that they are more prone to interference, eg, from non-microbial ATP in foods. In order for these assays to be useful, interfering compounds must be removed.

The aim of this chapter is to review the mechanisms of ATP assay and the

state of current instrumentation. The problems that occur when the assay is applied to foods, and the ways in which they can be overcome, are considered. The future needs to allow the more widespread acceptance of ATP assay are also discussed.

2. BIOCHEMISTRY

ATP is a molecule found in all forms of life, from bacteria to primates, and is extremely important for energy-transfer reactions in the cell. One reaction that utilises ATP is the production of light by fireflies (ref.3). The enzyme that catalyses the reaction, luciferase, and its substrate, luciferin, are present in the tail of the firefly. In the presence of ATP and magnesium ions, a luciferase-luciferin-AMP (adenosine monophosphate) complex is formed, together with pyrophosphate (PP), as shown in equation (1):

$$\text{luciferase} + \text{luciferin} + \text{ATP} \overset{Mg^{++}}{\rightleftharpoons} \text{luciferase-luciferin-AMP} + \text{PP} \qquad (1)$$

The complex is then oxidised:

$$\text{luciferase-luciferin-AMP} + O_2 \longrightarrow (\text{luciferase-luciferin-AMP})^{*}_{\overset{\|}{O}} + H_2O \qquad (2)$$

The oxidised complex is in an electronically excited state, and returns to its ground state with the emission of a photon of light:

$$(\text{luciferase-luciferin-AMP})^{*}_{\overset{\|}{O}} \longrightarrow \text{luciferase-luciferin-AMP}_{\overset{\|}{O}} + \text{light} \qquad (3)$$

The reaction sequence has been reviewed in more detail by various authors (refs 4, 5, 6).

The firefly light-producing reaction is extremely efficient, with approximately one quantum of light emitted for every luciferin molecule oxidised (ref.7). The reaction is also highly specific for ATP. Cross-reactions with other nucleoside triphosphates have been reported, but further investigation has shown this to be due to the presence of contaminating ATP in the preparation (ref.8), or phosphate-converting enzymes (ref.4).

Strehler and Totter (ref.9) were the first to realise the potential of the firefly luciferin-luciferase system for the accurate biochemical assay of ATP. Using a fluorimeter or a photomultiplier tube, they obtained a linear relationship between ATP concentration and light output. The assay can also be used to perform a variety of other biochemical tests if suitable conversions of the compound to ATP are done. Such compounds include ADP, AMP, c-AMP, phosphocreatine and glucose. Enzymes that use ATP can also be assayed, eg, creatine phosphokinase, myokinase, hexokinase, apyrase, creatine adenylic transphos-

phorylase (refs 9, 10, 11).

3. INSTRUMENTATION AND REAGENTS

Early work on the luciferin-luciferase assay for ATP depended on light detection using scintillation counters operating in the out-of-coincidence mode. Nowadays, several specialised instruments are available from a variety of manufacturers for photon counting. These instruments are generically termed photometers or luminometers.

The central part of photometer is the light detector. Small, portable instruments for field work are usually fitted with solid-state light detectors, which have moderate sensitivity (10^{-10}g, or 10^{5}fg). The more sensitive instruments contain photomultiplier tubes (PMTs) and are capable of detecting 10^{2}-10^{3} fg ATP, equivalent to approximately 10^{2}-10^{3} bacterial cells.

It is essential that the light detector be encased in a light-tight chamber. The light-emitting reaction occurs in a cuvette directly in front of the detector. The inner walls of the chamber are reflective to optimise the collection of photons at the photocathode of the PMT. Careful design of the chamber is essential to ensure that the cuvette containing the sample can be inserted and removed without the PMT being exposed to outside light. In many luminometers, it is possible to inject reagents whilst the cuvette is in the measuring position. To ensure reproducibility of mixing, pumps are used to inject reagents in many instruments. A schematic representation of a photometer is shown in Fig. 1.

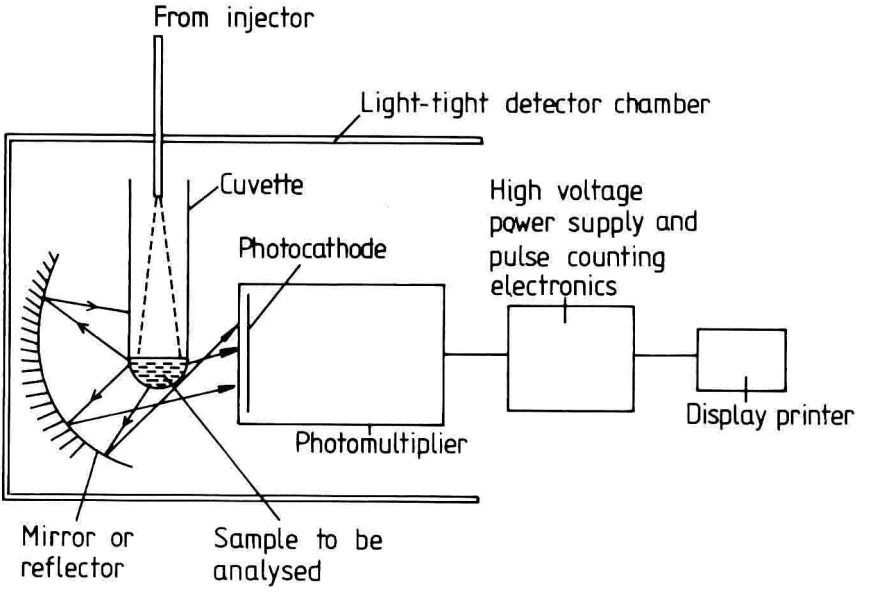

Fig. 1. Schematic representation of a photometer (after Stanley, 1983; ref.13).

Early photometers were single-sample instruments, requiring much manipulation by the operator. Most instruments can be linked to a computer to permit automated data collection and processing. The trend now is towards large instruments, where fifty or so samples can be analysed sequentially.

Many instrument manufacturers also supply reagents for ATP assay. These include firefly luciferin-luciferase, standard ATP and extractants (total or somatic). Most luciferin-luciferase preparations produce a near-constant light output a few seconds after mixing with sample (Fig.2). The height of the plateau or an integration of readings over a time interval (eg, 10s) can be used as a measure of ATP concentration.

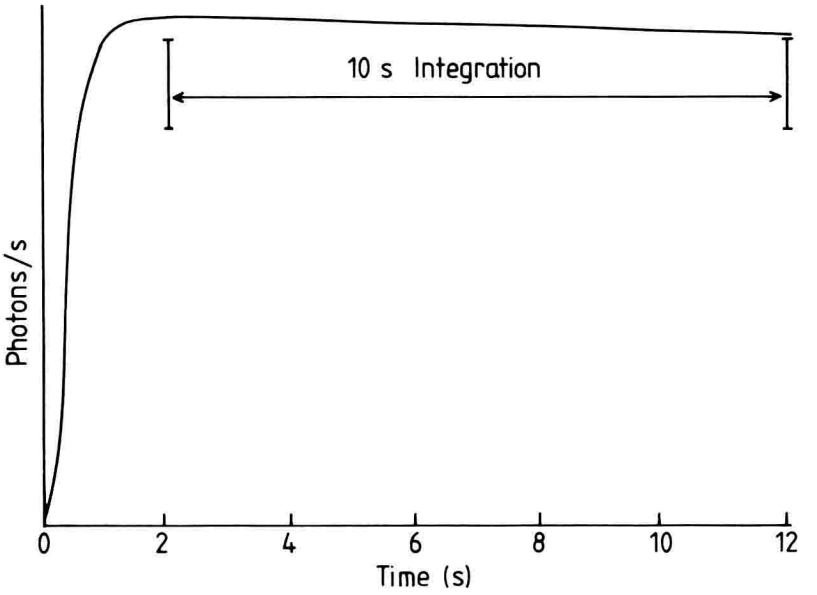

Fig. 2. Kinetic profile for light produced in the reaction of ATP with luciferin-luciferase reagent. Reagents were mixed at 0s. (after Stanley, 1983; ref.13).

Interference with the assay is common, and may be caused by several factors, eg, absorption of the light by coloured components, scattering of the light by particulate matter, or the presence of inhibitors in the sample. Under ideal conditions, a linear relationship between ATP concentration and light output can be obtained. Figure 3 shows such a relationship using a small, single-sample instrument (LKB 1250) and demonstrates its linearity over several $\log_{10}$ cycles of ATP concentration.

For a more thorough review of ATP instrumentation, see refs 12 and 13. A description of currently available instruments is given in ref. 14.

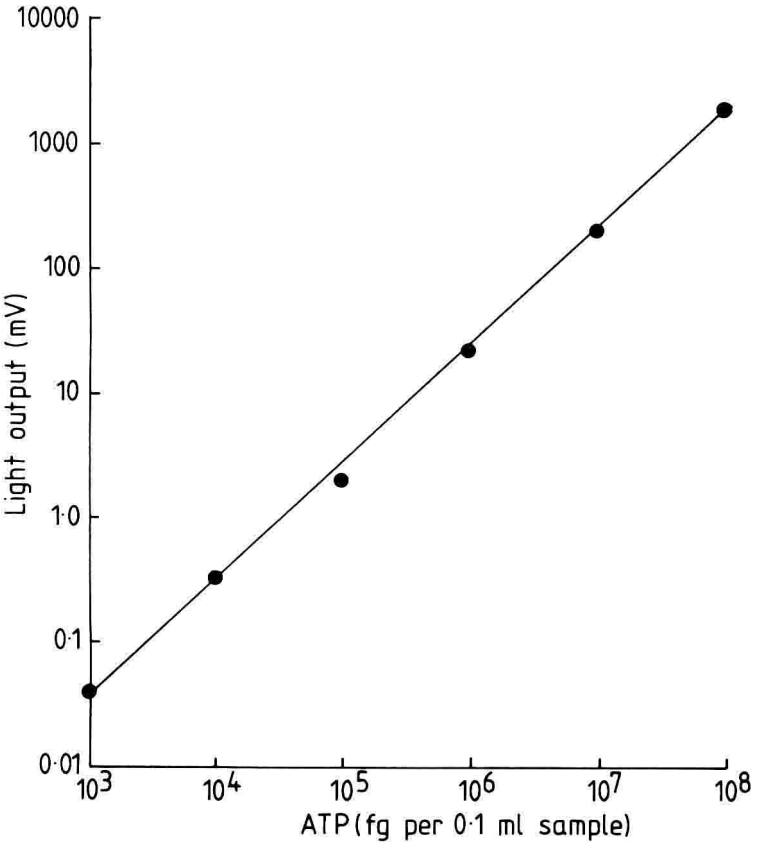

Fig. 3. Standard curve for ATP assay (LKB 1250 and LKB luciferin-luciferase).

4. APPLICATIONS

Since all living organisms contain ATP, the assay had potential for the detection or enumeration of micro-organisms. This proved simple for pure cultures, and many authors have recorded values of ATP per colony-forming unit (ATP/cfu) for a variety of organisms. For example, the mean ATP/cfu value for several species of bacteria was shown to be 0.47 fg, with a range of 0.22 to 1.03 fg (refs 15 and 16). More recent data (refs 17 and 18) show similar values. Yeast cells contain approximately 100 times as much ATP as bacteria (refs 17 and 18). Early reports (ref. 19) showed consistent ATP/cfu levels throughout the growth phase, although it is now recognised that ATP levels are highly dependent on growth conditions. Intracellular ATP falls in response to stress, eg, nutrient limitation, pH changes, presence of inhibitors, chilling, anaerobiosis (refs 20, 21, 22, 23, 24). Many of these factors are important in

the extension of shelf-life or preservation of foods, so it is necessary to remember that if ATP assay is to be used to determine microbial content, a resuscitation step may be necessary to optimise ATP levels. For example, micro-organisms separated from raw, chill-stored meat showed up to a ten-fold increase in ATP content after resuscitation in a glucose-containing broth (ref. 25).

Four different types of sample have been identified that might require microbiological testing using an ATP assay technique (ref. 26). These are:

i) Those containing microbial cells only, eg, starter cultures, activated sludge, process water and fermentation liquor.

ii) Those containing both microbial and somatic cells, eg, many foods, often raw.

iii) Those containing low levels of bacteria, eg, pasteurised or sterilised products, body fluids.

iv) Those containing a mixture of micro-organisms, where the detection of only one type is required, eg, the detection of pathogens or spoilage organisms.

All these categories of sample are routinely analysed in food microbiology laboratories, and research into ATP assay for all of them has been reported. The problems in dealing with each type will be considered in more detail.

4.1 Samples containing microbial cells only

This type of sample is the most simple to analyse using ATP assay as there is no ATP present from other sources. Several examples have been quoted in the literature for ATP analysis of this type of sample in the food industry. The assay has proved useful in fermentation control, where progress of fermentation in terms of biomass production can easily be determined by measurement of ATP. Viable yeasts for wort pitching during the brewing process can be quantified by ATP assay (ref. 27), permitting more consistent yeast pitching. In this case, ATP assay was apparently more reliable than conventional percentage solids determination, since it was unaffected by extraneous solids and dead yeast cells. ATP values per yeast cell do not differ markedly during the fermentation of brewery wort by ale and lager yeasts (ref. 28), but a percentage solids value also has a contribution from dead cells and debris.

ATP assay has also been used as a biomass measurement for Trichoderma reesei during fermentation (refs 29 and 30), correlating well with cellulose consumption, absorbance and dry weight. Micro-organisms are also present in high numbers in activated sludge, and ATP results have been shown to agree well with suspended solids values for brewery wastewater (ref. 31).

4.2 Samples containing microbial and somatic cells

The successful enumeration of organisms in pure cultures using ATP assay led to attempts to utilise the assay for the same purpose in foods. However, early workers were hindered by interference from large quantities of non-microbial ATP in foods, which in many cases masked the ATP from bacteria (refs 17 and 32). Table 1 shows ratios of non-microbial to microbial ATP in a variety of foods, calculated on the presumption that each organism present (as detected by the colony count) contains 1 fg ATP. The ratio can be as high as infinity (eg, for concentrated orange juice), where ATP levels are high but no micro-organisms are detected by conventional methods. Similar results were also found during our own early studies; some of these data are also shown in Table 1. This problem delayed further efforts to utilise the ATP assay for enumeration of micro-organisms in foods by almost a decade.

TABLE 1

Ratios of non-microbial to microbial ATP in foods.

Food	Ratio[a] Food ATP : Bacterial ATP
[b]Orange juice (super-concentrated)	∞ : 1
[b]Ice cream	40,000 : 1
[b]Comminuted meat	12,000 : 1
[b]Peas (frozen)	1,200 : 1
[b]Milk	15 : 1
[b]Chicken noodle soup mix (dried)	3,500 : 1
[b]Bacon	300 : 1
[c]Steak	11,000 : 1
[c]Steak	1,500 : 1

[a]Calculated using a value of 1 fg ATP per colony-forming unit.
[b]Data from ref. 17.
[c]Leatherhead Food RA data.

Early researchers concluded that, in order to solve this problem for foodstuffs, a means of differentiating between microbial and non-microbial ATP was necessary. There are two possible approaches to this:

i) To extract and destroy non-microbial ATP selectively, before extraction and assay of microbial ATP.

ii) To separate micro-organisms from interfering food components (especially non-microbial ATP) before extraction and assay of their ATP.

Both these approaches have been studied for foods and will be described in more detail.

The first approach is dependent on the differential resistance of somatic (ie, food) cells and microbial cells to extractants. A mild extraction procedure is used first, usually treatment with a surfactant preparation (refs 26, 33-36), although ultrasonics have also been used (ref. 37). This releases ATP from the somatic cells. It can then be measured if required, for example to detect mastitis problems in raw milk (ref. 33) where non-microbial ATP was found to correlate well with somatic cell count as determined by the Coulter counter. If microbial ATP is to be assayed, somatic ATP is destroyed by an ATP-degrading enzyme (eg, apyrase). Microbial ATP is then extracted from cells using a stronger surfactant, and assayed.

Several examples of this type of method have been reported for foods, for example, raw milk (ref. 34), fruit juices (ref. 26) and raw meat (refs 35-38). In general, a result can be obtained in approximately 45 minutes, although for raw milk a more rapid test was designed to analyse milk in incoming bulk tankers, where a result could be obtained in 5 minutes by increasing the apyrase concentration (ref. 39). In the case of fruit juices, the pH must be raised from ca 3.5 to ca 6.5 by the somatic extractant to allow the apyrase to function (ref. 40). Reported results for meat appear variable, since some authors obtained poor correlations between ATP content and colony count except at high levels of contamination ($>10^7$ cfu/ml, where 1 ml represents 0.5 cm^2 of meat surface; ref. 38), in comparison with others who report good correlations for levels of contamination $>10^5$ cfu/g (ref. 35) for meats from a single processor ($r = 0.97-0.99$), although for retail samples correlations were rather lower ($r = 0.80-0.87$).

However, some problems with this type of technique have been reported. For example, in the case of raw milk, the ATP assay was shown to be adequate in estimating bacterial concentrations $>10^5$ cfu/ml, but for lower levels the variation in micellar ATP not destroyed by the enzyme treatment confuses bacterial ATP detection (ref. 41). The situation could be improved partially but not totally by sonication of the milk, and a proteolytic enzyme treatment. An additional problem with milk is that large numbers ($>10^5$/ml) of somatic cells produce sufficient ATPase to hydrolyse ATP (ref. 42). Pseudomonas fluorescens, a common spoilage agent of milk, also has a high ATPase activity. Since the bacterial release agent only partially inactivated the apyrase, these workers identified two possible problem areas - the partial hydrolysis of bacterial ATP, and variability in internal standardisation. They concluded that ATP results from milks with a high somatic cell content should be interpreted with caution.

In fruit juices, the low pH (3-3.5) causes problems with apyrase activity. The juice must be heavily buffered to permit the apyrase to function

efficiently. Stannard (ref. 43) and Graumlich (ref. 44) both found that a centrifugation step improved the extraction of non-microbial ATP from fruit juices, with a limit of sensitivity of 10^3–10^4 cfu/ml, as shown in Fig. 4.

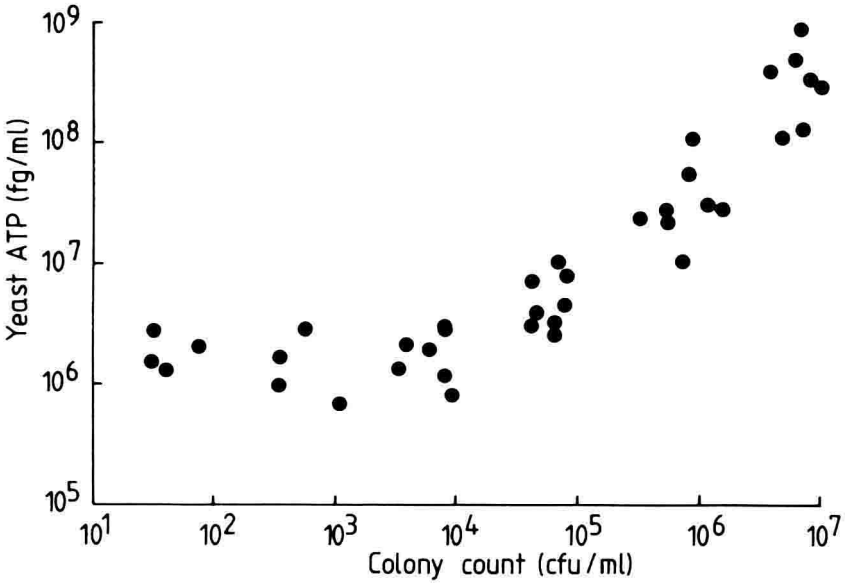

Fig. 4. Relationship between yeast ATP and colony count for artificially contaminated pasteurised orange juice (from ref. 43).

The second approach to the problem of interference from non-microbial ATP is to separate the organisms from the food before extraction and assay of their ATP. Several separation procedures have been attempted.

Clear, non-particulate liquids pose no problems in separation since they can be easily filtered; organisms are collected on the filter and interfering compounds (eg, free, non-microbial ATP) in the filtrate are removed. This approach has been used for carbonated beverages (refs 45, 46, 47), where filtration was used to isolate yeasts in the samples for ATP assay. Good correlations have been demonstrated between ATP levels and colony-forming units. Artificially contaminated beverages were used to generate standard curves of ATP versus colony count, since the ATP/cfu values were dependent on the type of beverage. The authors concluded that environmental factors (eg, pH, sugar content, carbonation levels, flavouring agents, preservatives) in the beverage were influencing cellular ATP levels. This method is envisaged as a presence/absence test for yeasts in carbonated beverages, ie, with no