Review

A review of microbial injury and recovery methods in food

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ARTICLE INFO

Article history:
Received 12 February 2008
Accepted in revised form 23 April 2008
Accepted 25 April 2008
Available online 4 May 2008

Keywords:
Microbial injury
Recovery methods
Foodborne pathogens

ABSTRACT

The existence of injured microorganisms in food and their recovery during culturing procedures is critical. Microbial injury is characterized by the capability of a microorganism to return to normalcy during a resuscitation process in which the damaged essential components are repaired. Injury of microorganisms can be induced by sublethal heat, freezing, freeze-drying, drying, irradiation, high hydrostatic pressure, aerosolization, dyes, sodium azide, salts, heavy metals, antibiotics, essential oils, sanitizing compounds, and other chemicals or natural antimicrobial compounds. Injured microorganisms present a potential threat in food safety since they may repair themselves under suitable conditions. Detection of injured microorganisms can be important to practical interpretations of data in food microbiology. This review provides an overview of microbial injury in food and discusses the development of recovery methods for detecting injured foodborne microorganisms.

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Contents

1. Introduction .......................................................... 736
2. Injury of microorganisms .............................................. 736
   2.1. Overview ................................................................ 736
   2.2. Effects and changes of microbial cells ....................... 736
   2.3. Repair of injured cells ............................................. 737
3. Recovery and detection methods .................................... 737
   3.1. Overview ............................................................ 737
   3.2. Liquid-repair methods ............................................ 738
   3.2.1. Two-fold dilution (2FD) method ......................... 738
   3.3. Solid-repair methods .............................................. 739
   3.3.1. Pour-overlay plating method .............................. 739
   3.3.2. Surface-overlay plating method ......................... 739
   3.3.3. Thin agar layer (TAL) method ............................ 739
   3.3.4. Four-compartment thin agar layer (4-TAL) method ... 740
   3.3.5. Agar underlay method (Lutri plate recovery method) ... 740
   3.3.6. Membrane or solid-support-based method ............ 741
3.4. Modification of repair methods with addition of compounds ......................................................... 741
   3.4.1. Catalase .......................................................... 741
   3.4.2. Pyruvate .......................................................... 741
   3.4.3. 3,3’-Thiodipropionic acid (TDPA) ....................... 741
   3.4.4. Tween 80 with magnesium chloride .................... 741
   3.4.5. Oxyrase* ......................................................... 741
3.5. Membrane filtration and hydrophobic grid-membrane filtration methods ................................. 742
4. Conclusions .......................................................... 742
Acknowledgments ....................................................... 742
References .............................................................. 742

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1. Introduction

Microbial contamination of food is a major concern for the food industry, regulatory agencies and consumers. An estimated 76 million people contract foodborne illnesses each year in the United States; the estimated costs related to foodborne diseases in the United States is between $10 billion and $83 billion annually (USFDA, 2001).

Methods such as heating, freezing, drying, freeze-drying, irradiation, high hydrostatic pressure, fermentation, or the addition of antimicrobials and chemicals are commonly used to control bacterial contamination and pathogens. After these treatments, one population of microorganisms may be killed, another population may survive (non-injured), and a third population maybe sublethally injured (Wu et al., 2001a). The ability to detect foodborne pathogens is critical for final products that have undergone food processing. A good method should detect both normal and injured microorganisms (Foegeding and Ray, 1992). Injured organisms are potentially as important as their normal counterparts because they can resuscitate and become functionally normal in a favorable environment. Since injured cells may not grow well on selective detection media, a resuscitation step or repair of injured cells on non-selective media is necessarily incorporated with selective enumeration. Determining the presence of impaired microorganisms is important in many areas, such as the preservation and spoilage of foods, consumer protection, and the manufacturing of safe foods (Busta, 1976; Ray, 1989; Shintani, 2006). It should be noted that an injured cell is one which can repair the cellular damage (resuscitation) and regain its ability to form a colony in the presence of the selective agent; however, the dead cell cannot form a colony under any condition (Palumbo, 1989).

A population of surviving microorganisms, after a sublethal physical or chemical treatment, includes dead cells (lethally or irreversibly injured), uninjured cells (normal cells), and injured cells (stressed, sublethally or reversibly injured) (Fig. 1) (Ray, 1979, 1989; Russell, 1984; McFeters, 1989; Wu et al., 2001a, b; Bozoglu et al., 2004). Pathogens and spoilage organisms in foods can become injured within food products. Injury of microorganisms may result from food processing and handling procedures, such as thermal treatment, refrigeration, freezing, drying, and irradiation, from exposure to preservatives, acidity, and low water activity, or from being starved (Hurst, 1977; Foegeding and Ray, 1992; Jay et al., 2005). Therefore, determining the presence of impaired microorganisms is critical to the quality and safety of final food products. Microbiologists have been studying recovery of sublethally injured bacteria cells for more than 40 years (Jay et al., 2005).

2. Injury of microorganisms

2.1. Overview

An injured cell can be defined as a cell that survives a stress but loses some of its distinctive qualities (Busta, 1976). Hartsell (1951) was among the first researchers to define injured cells as those capable of forming colonies on non-selective media, but not on selective media. Straka and Stokes (1959) also showed that supplementing the restricted medium with specific nutrients allowed the injured cells to regain the ability to multiply. Additionally, injured cells may show an extended lag phase, compared with non-injured cells, to repair damage and synthesize the proteins and nucleic acids needed for growth (Busta, 1976; Ray, 1989; Shintani, 2006). It should be noted that an injured cell is one which can repair the cellular damage (resuscitation) and regain its ability to form a colony in the presence of the selective agent; however, the dead cell cannot form a colony under any condition (Palumbo, 1989).

Fig. 1. Effects of sublethal treatments on microbial cells (Ray, 1979, 1989; Russell, 1984; McFeters, 1989; Bozoglu et al., 2004).

2.2. Effects and changes of microbial cells

When microorganisms undergo sublethal injury, some cellular changes may occur. Many structural and functional components of organisms are affected, such as cell wall, cytoplasmic membrane or inner membrane, ribosomes, DNA, RNA, tricarboxylic-acid-cycle enzymes as well as many other enzymes (Ray,
The cell membrane appears to be the component most commonly affected (Hurst, 1977; Jay et al., 2005). Most injured cells have damaged permeability barriers (surface structures and the cytoplasmic membrane) that render them susceptible to many selective agents or antimicrobials. For example, microbial inactivation by pulsed electric fields (PEF) is believed to be caused by the effects of PEF on the cell membranes. Sublethally injured cells would become leaky during PEF but reseal to some extent after treatment (Weaver and Chizmadzhev, 1996; García et al., 2003). Injured cells often lose some cellular material such as Mg²⁺, K⁺, amino acids, 260 nm absorbing material (nucleic acids), and 280 nm absorbing material (protein) through leakage into their surroundings (Hurst, 1977; Palumbo, 1989). For instance, frozen cells of Escherichia coli release amino acids, small molecular weight ribonucleic acids, and peptides. Heat-injured Staphylococcus aureus cells release potassium, amino acids, and proteins. Loss of intracellular compounds indicates damage to the cell membrane, which impairs growth and replication of a cell (Busta, 1976). Additionally, some injured cells encounter changed macromolecules within cells, and damage to the functional components that are related to their metabolic activities, thus causing metabolic injury (Ray, 1979; Jay et al., 2005). Landolo and Ordal (1966) and Allwood and Russell (1968) reported that ribosomal ribonucleic acid was degraded in heated cells of S. aureus and Salmonella Typhimurium. Heat-injured S. aureus have decreased catabolic capabilities and reduced activities of selected enzymes of glucose metabolism (Bluem and Ordal, 1969). Lipopolysaccharide molecules on the outer membrane of gram-negative bacteria are damaged by freezing due to destabilization of ionic bonds (Ray, 1986). Gomez and Sinskey (1973) reported that DNA breaks were observed in the heat injury of salmonella. Fung and Vanden Bosch (1975) also showed that injury due to freeze-drying of S. aureus S-6 cells caused breakdown of RNA replication. Acid injury has been observed to be different from heat or freeze injuries (Przybylski and Witter, 1979); leakage of cellular constituents following injury was not seen after acidification. There were no detectable amounts of 260- or 280-nm-absorbing materials leaked during the course of acid injury, but damage of ribonucleic acid was observed (Przybylski and Witter, 1979). Zayaitz and Ledford (1985) reported that coagulase and thermostable nuclease activities were reduced in injured S. aureus. Although acid injury did not affect cell membranes, RNA synthesis was effected (Jay et al., 2005). The similarities in bacterial cell injury by different treatments are indicated in Table 1.

It is important to note that not all cells in a population will endure the same amount of injury and not all forms of stress produce identifiable injuries (Jay et al., 2005). Damaged cells vary with the types of stress, the microbial species, the composition and consistency of the food, and storage conditions (Ray, 1979). Factors that influence injury to bacteria include elevated temperature, freezing, chilling, dehydration, freeze-drying, irradiation, acidity (pH), exposure to preservatives, contact with chemicals, Δw (water activity), and culture age (Busta, 1976; Martin and Myers, 1994). Therefore, to develop efficient recovery methods for injured microorganisms, those factors and variances should be considered.

### 2.3. Repair of injured cells

Sublethally injured cells have the capability to repair themselves and return to a normal physiological state with initiation of growth and cell division under favorable conditions. The restoration of lost capabilities in injured cells has been termed “resuscitation” because the cells are revived from apparent death (Hurst, 1984). Resuscitation was originally applied to repair in liquid media, and then was used with solid media. It has come to mean a brief period of incubation under optimal conditions which permits repair (Hurst, 1984; Jay et al., 2005). During repair, restoration of growth capabilities will occur before normal growth occurs. Many cellular modifications are reversed and losses of cell constituents are restored to the normal state during incubation (Busta, 1976). Ribosomes degraded during a heat treatment are regenerated (Tomlins and Ordal, 1971). Phospholipids are synthesized during recovery. Cell wall and protein synthesis appear necessary in the repair of damaged cells (Busta, 1976). The repair of cell ribosomes and membrane appears to be essential for recovery, at least from sublethal heat, freezing, drying, and irradiation injuries (Jay et al., 2005). Generally, most injured cells repair within 2–4 h at a suitable incubation temperature in a nutritionally rich non-selective medium. Moreover, the resynthesis of RNA lost during injury is critical in the first stage of repair (Ray, 1986; Fung and Vanden Bosch, 1975). Injury to pathogens eliminates their ability to cause disease, however, once the cells are repaired, pathogenicity is totally restored (Meyer and Donnelly, 1992). The repair of injured cells caused by different treatments is shown in Table 1.

Research has indicated that regardless of the nature of the stress imposed on a microbe, for injured vegetative cells: (a) the injuries are repaired when incubated in an appropriate environment, (b) the optimum temperature and time differ with the nature of the stressor, (c) the completely repaired cells regain normal resistance to the selective agents in the media, and (d) the repair process precedes cell multiplication. Therefore, it is desirable to allow injured cells to repair any damage before isolation or enumeration by customary procedures (Ray and Adams, 1984).

### 3. Recovery and detection methods

#### 3.1. Overview

For many years, recovery of injured microorganisms has been a major concern of microbiologists working with various applications from processed food to environmental samples. An ideal...
method to recover microorganisms in a food or environment should include both normal and injured organisms (Ray and Adams, 1984; Wu and Fung, 2001, 2003, 2006; Wu et al., 2001a, b). Non-selective agars allow the growth of both non-injured and sublethally injured cells, but cannot differentiate target pathogens from a mixed population. Many of the accepted methods used for isolation and enumeration of microorganisms in foods (selective media) do not allow for the repair of injured microorganisms and thus fail to detect them. Selective compounds such as surface-active agents, salts, antibiotics, sulfonamides, acids and dyes are added to solid and liquid media for the selective and differential detection of pathogenic, spoilage or other microorganisms from food. Those agents may inhibit the resuscitation of injured microorganisms. Thus, when such media are used, the injured microorganisms in the samples must be permitted to resuscitate in a suitable environment before exposure to selective agents (Ray, 1979; Read, 1979; Wu and Fung, 2001, 2003, 2006; Wu et al., 2001a, b). The comparison among different medium systems for recovery of non-injured and injured cells is indicated in Fig. 2.

Many methods have been developed to allow the repair of injured microorganisms before exposure to a selective medium (McDonald et al., 1983). The principles which should be considered in developing methods to detect injured microorganisms include: (a) the injured cells become temporarily susceptible to many selective compounds in the media, (b) this sensitivity may be due to the damage of the cytoplasmic membranes of the cells, (c) the injury is reversible and can be repaired in a nutritionally rich non-selective medium and repaired cells regain their resistance to the selective compounds and also their ability to multiply, (d) injured cells do not repair or multiply in the presence of the selective compounds, (e) injured cells could be enumerated or isolated in the selective media, if they are allowed to repair in a suitable environment before exposure to the selective environment, and (f) the surviving population constitutes both uninjured and injured cells (Busta, 1976; Ray, 1979; Foegeding and Ray, 1992).

In general, the repair methods can be grouped as either liquid or solid media repair methods (Ray, 1979; Ray and Adams, 1984). The liquid-repair method is effective for enumeration by the most probable number (MPN) technique and isolation of pathogens and indicator bacteria from different types of semi-preserved foods. The solid-repair method can be used for direct enumeration of organisms that are usually enumerated by the selective plating procedure. The principles and comparison between liquid-repair method and solid-repair method are indicated in Table 2.

### 3.2. Liquid-repair methods

In this method, a blended food sample is normally incubated in non-selective broth to facilitate repair. The time and temperature of incubation during the repair phase vary with the method of detection subsequently used. Generally, incubation at 25–37 °C is more effective for most mesophilic organisms, and 1–5 h incubation time (longer time is necessary for heat-stressed cells) have been used for optimal repair. This repair could be used efficiently as the initial step in selective isolation, followed by enumeration by direct plating or by the MPN technique for pathogenic and indicator bacteria from foods. However, the counts on subsequent selective plating media could be due to not only repair of the injured cells but also multiplication of uninjured cells, especially when the time of incubation is prolonged. Since uninjured and non-target cells can multiply before the population of interest recovers, this procedure may not be effective for regulatory purposes, especially when the enumeration is done by plating. Besides, the MPN method gives considerable variation in results, is time consuming (requires about 48–96 h), and uneconomical (in supplies and labor) (Ray, 1979; Ray and Adams, 1984).

#### Table 2: Comparison between liquid-repair methods and solid-repair methods (Speck et al., 1975; Ray, 1979; Foegeding and Ray, 1992; Wu and Fung, 2001; Wu et al., 2001a, b, 2008b)

<table>
<thead>
<tr>
<th>Repair methods</th>
<th>1. Samples are blended in a nonselective broth.</th>
<th>2. Incubated in the broth at optimum repair conditions.</th>
<th>3. Then transferred to selective environment for their selective growth.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4. Advantage:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. For isolation</td>
<td>b. For MPN-enumeration</td>
<td>c. For plating</td>
</tr>
<tr>
<td></td>
<td>5. Disadvantages:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Uninjured and non-target cells can multiply before the population of the interest recovers.</td>
<td>b. May not be effective for regulatory purposes, especially when enumeration is done by plating.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solid-repair methods</th>
<th>1. Blend the sample if necessary.</th>
<th>2. Traditional overlay method (OV):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a. Transfer aliquot to plates (0.1–3.3 ml/plate) or inoculate sample on solidified nonselective media after step b.</td>
<td>b. Pour nonselective media about 12 ml/plate</td>
</tr>
<tr>
<td></td>
<td>c. Incubate 1–3 h at room temperature.</td>
<td>d. Overlay with selective media about 10–12 ml/plate.</td>
</tr>
<tr>
<td></td>
<td>a. Overlay 14 ml TSA onto solidified selective media</td>
<td>5. Advantages:</td>
</tr>
<tr>
<td></td>
<td>b. Inoculate sample directly on prepared TAL plates.</td>
<td>a. Direct (so less variability when &gt;10/g or ml).</td>
</tr>
<tr>
<td></td>
<td>a. Variability at very low counts (&lt;30/g or ml)</td>
<td>c. Economical (less supplies and labor).</td>
</tr>
<tr>
<td></td>
<td>b. Colonies on OV plates may be small and inconvenient for isolating suspicious colonies from the plates for further confirmation.</td>
<td>d. Temperature of the melted selective overlay agar used on OV can further affect injured targets being resuscitated on the nonselective agar.</td>
</tr>
</tbody>
</table>

#### 3.2.1. Two-fold dilution (2FD) method

Kang and Siragusa (2001) reported a 2FD method for enumeration of total culturable bacterial cells and coliform counts including sublethally injured cells. The procedures include a two-fold serial dilution of samples in a 96-well microtiter plate using...
buffered peptone water. The 2FD set is then incubated at 37 °C for 3 h for resuscitation of injured cells, after which an equal volume of double strength selective broth is added to each well. The set is incubated for a further 13 h at 37 °C in the dark. The 2FD method saves time and space when compared with agar plate methods; however, it is unable to isolate microorganisms for further study without re-culturing of well contents (Kang and Siragusa, 2001).

3.3. Solid-repair methods

In traditional solid-repair methods, the blended sample is either pour-plated or surface-plated with a non-selective medium, and incubated for a suitable time (1–4 h) and temperature (25–37 °C) to facilitate repair. After repair, the plates are overlaid with 7–12 ml of a selective agar medium, which is specific for the type of microorganism, allowed to solidify, and then incubated. During incubation, the ingredients including the selective compounds from the selective medium will diffuse through the non-selective medium and create a selective environment throughout. Because the cells have already been repaired, they are not inhibited by the selective agents, and will multiply and form colonies. Only the target organisms can resist the selective environment and be enumerated (Ray, 1979; Ray and Adams, 1984). The solid-repair method can be used instead of a conventional selective enumeration procedure; it is more direct and economical compared to liquid-repair methods. However, the results are variable at very low counts (< 10 CFU/g) (Ray, 1979).

Some bacteria may form small colonies and picking isolated colonies that grow under the selective medium overlay is difficult to be picked for further characterization. TAL method, which involves pouring 5 ml of a non-selective medium (ca 12 ml) into a Petri dish Injured cells recover in 2-4 h before colonies are counted

Fig. 3. Traditional overlay method for recovery and detection of injured microorganisms.

Inoculate injured microorganisms

Pour 45–48 °C melted selective medium (7ml)

Nonselective medium (ca 12 ml)

Petri dish

Injured cells recover in 2-4 h on the nonselective medium incubated at a suitable temperature

1. Plate is first incubated at a suitable temperature (for example, 37 °C) for 2-4 h
2. Incubated plate is removed and a selective medium is poured on the top
3. The plate is re-incubated at a suitable temperature (for example, 37 °C) for 21 h before colonies are counted

Inoculated with 7–12 ml of a selective agar medium, when used in combination with the TAL, provides complete repair of the injured population without imposing problems caused by cell multiplication (Hartman et al., 1975; Speck et al., 1975; Kang and Fung, 1999, 2000). Previous reports showed that surface-overlay plating methods enumerated more coliforms than the pour-overlay plating procedure (Speck et al., 1975). This method is effective when a small volume of sample is used (0.1–0.5 ml per plate), therefore unsuitable for samples with low numbers of the specific organisms (Ray, and Adams, 1984). In addition, recovered colonies between two agar layers may be difficult to be picked for further characterization.

3.3.1. Pour-overlay plating method

The pour-overlay plating method was developed by Ray and Speck (1973) and Ray (1979). In this method, the blended sample (with phosphate diluent) is poured into 5 ml of trypticase soy agar (TSA) or plate count agar (PCA). The cells are immobilized upon solidification of the medium, and then incubated to facilitate repair (1 h at 25 °C for coliforms). After repair, the plates are overlaid with 10–12 ml of the selective medium (violet red bile (VRB) agar for coliforms), and after 15 min are incubated for the desired time and temperature (35 °C and up to 24 h for coliforms).

Since the cells are immobilized, any cell multiplication during the repair phase will not influence the final count (Ray, 1979). The pour-overlay plating can be used for samples with low or high counts of the specific type of microorganisms. However, it is not suitable for organisms which are enumerated by surface-plating method (Ray and Adams, 1984).

3.3.2. Surface-overlay plating method

The surface-overlay method was developed by Speck et al. (1975) and Hartman et al. (1975) to allow for recovery from injury before subsequent enumeration (Fig. 3). Injured cells are first plated on 12 ml of non-selective medium such as TSA and incubated 2–4 h at a suitable temperature (2 h at 35 °C for coliforms) to allow the repair and resuscitation of injured microorganisms. A layer of selective agar (7–12 ml) is then overlaid on top of the resuscitated cells. After solidification of the overlaid medium, the plates are incubated further at a suitable temperature (35 °C for coliforms) for 21–24 h. This is termed the overlay (OV) resuscitation method. Repair on non-selective medium followed by the use of the selective medium allow more complete repair of the injured population without imposing problems caused by cell multiplication (Hartman et al., 1975; Speck et al., 1975; Kang and Fung, 1999, 2000). The non-selective medium inhibited by the selective agents (Kang and Fung, 1999, 2000; Wu and Fung, 2006). The traditional solid-repair methods include pour-overlay plating methods and surface-overlay plating methods. Recent developments include thin agar layer (TAL) method (Kang and Fung, 1999, 2000; Wu and Fung, 2001; Wu et al., 2001a, b), agar underlay method (Kang and Siragusa, 1999), four-compartment TAL method (Wu and Fung, 2003, 2006), and membrane or solid-support-based method (Blackburn and McCarthy, 2000; Kang, 2002).

3.3.3. Thin agar layer (TAL) method

Due to the cumbersome procedures of traditional recovery methods, Kang and Fung (1999) developed a novel one-step procedure, TAL method, which involves pouring 5 ml of a non-selective medium onto a prepoured and solidified pathogen-specific selective medium. This method was further modified by overlaying 14 ml of non-selective medium (TSA) onto a prepoured selective medium to recover injured foodborne pathogens (Fig. 4) (Kang and Fung, 2000; Wu and Fung, 2001; Wu et al., 2001a, b). The top layer (TSA) provides a favorable environment for injured cells to resuscitate and become functionally normal in the first few hours of incubation. Then, the resuscitated target microorganisms can interact with the selective agents in the bottom layer to develop typical reactions while other microorganisms are inhibited by the selective agents (Kang and Fung, 1999, 2000; Wu and Fung, 2001; Wu et al., 2001a, b). The non-selective medium (TSA) does not hinder typical color of colonies produced by target microorganisms. This is an improvement over the cumbersome two-step overlay (OV) method’s complicated manipulations and allows isolation of single colonies for further characterization. TAL also eliminates any chance of further sublethal injury from heat contributed by molten agar. Our previous studies have shown significant value of TAL method in recovering heat-, acid-, and cold-injured E. coli O157:H7, Listeria monocytogenes, S. Typhimurium, S. aureus, and Yersinia enterocolitica (Wu and Fung, 2001; Wu et al., 2001a,b). Examination of selectivity has shown that selective agar, when used in combination with the TAL, provides
TAL has broader applications for controlling various foodborne pathogens (Wu, 2007; Wu et al., 2008a, b). Recently, we have used TAL as a simpler procedure than MPN. Duan et al. (2006) reported a TAL-based double-layer agar plate (overlaying an equal volume non-selective medium (10 ml) onto a selective medium) was as effective as the MPN method for recovering heat- and cold-injured bacteria. The phenomenon was observed for pure culture as well as for mixed culture studies (Wu and Fung, 2001, 2003; Wu et al., 2001a, b). Chang et al. (2003) utilized the TAL to effectively recover L. monocytogenes, S. Typhimurium, and Campylobacter coli associated with pork surface. Hajmeer et al. (2001) showed that TAL was more effective than MacConkey sorbitol agar in recovering NaCl-injured E. coli O157:H7. Yuste and Fung (2003) demonstrated that TAL and TSA had similar effectiveness in recovering S. Typhimurium, S. aureus and Y. enterocolitica treated with apple juice with cinnamon (mainly acid injury). In addition, TAL can differentiate or select target bacteria while TSA cannot. Duan et al. (2006) reported a TAL-based double layer agar plate (overlaying an equal volume non-selective medium (10 ml) onto a selective medium) was as effective as the MPN method for recovering heat- and cold-injured Vibrio parahaemolyticus and was a simpler procedure than MPN. Recently, we have used TAL as an efficient recovery method when studying antimicrobial properties of natural ingredients for controlling various foodborne pathogens (Qiu and Wu, 2007; Wu et al., 2008a, b). TAL has broader applications than other selective-agar-based microbial culture techniques, which rely on growth media with selective agents that might be toxic to injured target cells. In addition, TAL can differentiate or select target bacteria while TSA cannot. Kang and Siragusa (1999) utilized an agar underlay procedure for culture sublethally injured bacteria, including E. coli O157:H7, Salmonella, fecal enterococci, and coliforms using a specially designed Lutri plate (LP; Starkville, MS) reported by Colwell and Speidel (1985). The procedure uses a non-selective agar underlay with a selective medium. In a two-chambered Petri dish, the LP, a non-selective agar is inoculated with a population of sublethally heat-injured bacteria. After a 2-h repair incubation period, selective agar is added to the bottom chamber of the LP and incubated. By diffusing through the non-selective top agar, selective agents from the underlay medium impart selectivity to the system. Unlike traditional overlay plating methods for injury repair, the agar underlay procedure allows the typical selective-medium colony morphology to develop and allows colonies to be more easily picked for further characterization. The agar underlay method exhibits slower diffusion of selective agents from the selective agar underlay into the non-selective recovery agar than the TAL technique or the traditional overlay (OV) method, but the procedures are more complicated than the TAL method. Chang et al. (2003) compared the effectiveness of three repair methods, OV, TAL and LP for recovery of freeze-injured S. Typhumurium, C. coli and L. monocytogenes in suspension or associated with fresh pork surfaces and found that TAL was the best recovery method that allowed the repair, selective isolation, and enumeration of freeze-injured bacteria studied. TAL also offers several advantages, such as convenience of use and preparation, and is more economical than OV and LP (Chang et al., 2003).

3.3.5. Agar underlay method (Lutri plate recovery method)

Kang and Siragusa (1999) utilized an agar underlay procedure to culture sublethally injured bacteria, including E. coli O157:H7, Salmonella, fecal enterococci, and coliforms using a specially designed Lutri plate (LP; Starkville, MS) reported by Colwell and Speidel (1985). The procedure uses a non-selective agar underlay with a selective medium. In a two-chambered Petri dish, the LP, a non-selective agar is inoculated with a population of sublethally heat-injured bacteria. After a 2-h repair incubation period, selective agar is added to the bottom chamber of the LP and incubated. By diffusing through the non-selective top agar, selective agents from the underlay medium impart selectivity to the system. Unlike traditional overlay plating methods for injury repair, the agar underlay procedure allows the typical selective-medium colony morphology to develop and allows colonies to be more easily picked for further characterization. The agar underlay method exhibits slower diffusion of selective agents from the selective agar underlay into the non-selective recovery agar than the TAL technique or the traditional overlay (OV) method, but the procedures are more complicated than the TAL method. Chang et al. (2003) compared the effectiveness of three repair methods, OV, TAL and LP for recovery of freeze-injured S. Typhumurium, C. coli and L. monocytogenes in suspension or associated with fresh pork surfaces and found that TAL was the best recovery method that allowed the repair, selective isolation, and enumeration of freeze-injured bacteria studied. TAL also offers several advantages, such as convenience of use and preparation, and is more economical than OV and LP (Chang et al., 2003).
3.3.6. Membrane or solid-support-based method

Blackburn and McCarthy (2000) reported a membrane transfer of E. coli O157:H7 from the TSA onto sorbitol MacConkey (SMAC) agar and showed the recovery of sublethally injured E. coli O157:H7 cells was improved by a factor of 3 log. First, a portion of samples was spread onto a membrane, which had been placed on the surface of a TSA plate. Following resuscitation of injured cells at 37 °C for 4 h, the membrane was transferred to the surface of a SMAC plate. After incubation for a further 20 h, typical color reactions of colonies were visualized (Blackburn and McCarthy, 2000). Kang (2002) reported a membrane filter holder (MFH) method using a specific apparatus (membrane filtration holder obtained from QA Life Sciences, Inc., San Diego, CA) to recover heat-injured E. coli O157:H7 and S. Typhimurium. MFH was composed of compartments A and B divided by a water diffusible stainless steel net. Melted non-selective agar (5 ml) was first poured into compartment A. Following solidification, the agar surface was then inoculated with injured cells. The plate was incubated for 3 h for resuscitation of injured cells and then 20 ml of melted selective agar was poured into compartment B. The differential and selective agents from the compartment B underlay then diffused into the compartment A chamber. The MFH method is an improvement with a commercially available apparatus for the agar underlay method since the LP used for the agar underlay method is not commercially available. However, like traditional two-step overlay methods, the procedures may be cumbersome because the method requires separated incubation steps and plates may not be prepared ahead of time.

3.4. Modification of repair methods with addition of compounds

During the repair process of injured cells, some inhibitors with specific actions may affect the repair mechanism (Russell, 1984). Flowers and Ordal (1979) reported that hydrogen peroxide (H₂O₂) formed during respiration, or peroxides produced in culture media, may be highly toxic to injured cells. Injured cells may have reduced catalase and superoxide dismutase activity, which may explain the toxic effects of relatively low peroxide levels in media (Andrews and Martin, 1979). Addition of catalase, pyruvate or other chemicals that will react with and remove H₂O₂ in the selective media (H₂O₂ decomposers) has been found to increase the detection of stressed staphylococci in foods (Martin et al., 1976; Flowers and Ordal, 1979; Foegeding and Ray, 1992). Magnesium (Mg) is required for repair after sublethal injury (Hurst, 1977). The incorporation of Tween 80 and magnesium chloride in selective medium as well as iron supplementation have been shown to increase the recovery of injured microorganisms (Murthy and Gaur, 1987). Therefore, these compounds could be incorporated into the development of repair methods to increase the capability of recovery.

3.4.1. Catalase

The effect of catalase in recovery and selective media has yielded various results with different organisms. This may be attributed to the organisms used, the method of injury and the media used for recovery. Rayman et al. (1978) reported that the recovery of heat-injured Salmonella Senftenberg was improved by TSA supplemented with catalase. Mossel et al. (1980) indicated that only a very small effect of catalase on the recovery of freeze-injured Enterobacteriacea was observed. Andrews and Martin (1979) studied catalase activity on S. aureus during thermal injury and recovery and found that the decrease in catalase activity during recovery, rather than injury, may be responsible for the increased sensitivity of injured staphylococci to H₂O₂. Martin et al. (1976) reported that the presence of catalase on the surface of selective media overcame the effects of H₂O₂ to increase enumeration of injured and normal microorganisms.

3.4.2. Pyruvate

The presence of sodium pyruvate in the media has been shown to increase enumeration of injured microorganisms. Baird-Parker and Davenport (1965) reported that incorporation of pyruvate into selective media enhances the recovery of S. aureus. Rayman et al. (1978) reported that the addition of pyruvate to non-selective tryptic soy agar greatly increased recovery of heat-injured Salmonella Senftenberg. The proposed mode of action of the sodium pyruvate is via the degradation of the metabolic by-product H₂O₂, rather than through supplementation of a required nutrient (Martin et al., 1976; McDonald et al., 1983).

3.4.3. 3'-Thiodipropionic acid (TDPA)

McDonald et al. (1983) reported that TDPA could be used as a supplement for modified repair detection procedures due to the effects on improvement of the recovery of injured cells in both selective and non-selective media and its low cost. One percent of TDPA is the suggested addition to the medium before sterilization, and the pH should be adjusted to 7.3. TDPA, through neutralization of the toxic effect of H₂O₂, allows better enumeration of injured microorganisms.

3.4.4. Tween 80 with magnesium chloride

Lachia (1984) replaced the egg yolk in Baird-Parker medium with Tween 80 and magnesium chloride and showed recovery of both thermally stressed and acid stressed cells of S. aureus. Murthy and Gaur (1987) reported that VRB agar containing Tween 80 and magnesium chloride was significantly better than conventional VRB agar for recovering coliforms after freezing. During injury to the microorganisms, there is damage to the cell membrane and to the ribosomes due to loss of magnesium ions (Hurst, 1977). Tween 80, being a lipid and surfactant, would repair the damaged cell membrane and adding magnesium chloride to the medium compensates for magnesium loss (Murthy and Gaur, 1987).

3.4.5. Oxyrase®

The utility of a sterile suspension of bacterial membrane fragments and their associated enzymes as reagents for elimination of dissolved oxygen was first reported by Adler and Crow (1981). After the addition of membrane fractions, the redox potential in a medium is reduced to −200 to −300 mV becoming completely anaerobic within about a minute. The partially purified membrane fragments from E. coli are now commercially available as Oxyrase® from Oxyrase, Inc. (Mansfield, OH). A unit of Oxyrase® per milliliter reduces dissolved oxygen at the rate of 1%/s at 37 °C, pH 8.4 in 40 mM phosphate buffer and 50 mM sodium lactate in an air saturated solution. The activity of Oxyrase® increases with a temperature up to 55 °C and the effective range of pH is 6–9. Oxygen reducing membrane or Oxyrase® has been used to remove dissolved oxygen from media for culturing anaerobes and to stimulate growth of pathogenic facultative anaerobes. It also improves growth of heat-, cold-, chemical-, and radiation-injured microorganisms (Hoskins and Davidson, 1988; Ali and Fung, 1991; Yu and Fung, 1991; Tuitemwong, et al., 1994; Patel and Beuchat, 1995; Thippareddi, et al., 1995; Wonglumsom, et al., 2000a; Huang, et al. and Fung, 2001). Yu and Fung (1992) reported that heat-injured and healthy cells of L. monocytogenes were significantly recovered in Fraser Broth supplemented with 0.01 units/ml of Oxyrase® compared with control broth without the supplement; oxygen is potentially toxic to injured cells (Shoemaker and Worsley, 1984).
Dubos (1929) and Nelson (1944) indicated that the low redox potential was responsible for allowing growth of heat-injured facultative microorganisms. Knabel et al. (1990) reported that use of the strictly anaerobic Hungate technique significantly increased recovery of heat-injured L. monocytogenes compared with aerobically incubated controls. Hence, Oxyrase® could be considered as another supplement to the TAL method to increase the capability for recovery of injured pathogens. Wu et al. (2004) developed a thin agar layer Oxyrase method (TALO, overlaying 14 ml of TSA with 1:30 dilution of “Oxyrase® for Agar” onto a prepoured pathogen-specific, selective medium) for recovering and detecting Y. enterocolitica from inoculated non-frozen and frozen ground pork samples. The TALO method showed more sensitivity and a greater recovery capability than the selective medium cefsulodin irgasan novobiocin. Wu and Fung (2004) reported the TALO method showed a higher percentage increase of recovery than the TAL method for heat-injured E. coli O157:H7, L. monocytogenes, S. Typhimurium, and Y. enterocolitica, due to stimulation by Oxyrase® in growth of pathogens.

3.5. Membrane filtration and hydrophobic grid-membrane filtration methods

Membrane filtration (MF) technology was introduced into the United States in 1951 (Goetz and Tsuneshi, 1951). It has been utilized in a wide variety of applications over several decades. The membrane filter techniques described by Goff et al. (1972) have been used as repair methods by filtering the diluted sample through a sterile membrane filter (450 nm), incubating the filter first on a non-selective medium and then on a selective medium. The technique can be used for samples with low or high numbers of a specific type of microorganism. The deleterious effects of inhibitory and toxic materials during repair can be avoided. The major drawback of the method is that food materials may clog the pores during filtration, especially with lower dilutions. Anderson and Baird-Parker (1975) introduced a membrane filter-plating method. A 1 ml portion of the blended sample is spread over a 450 nm pore membrane filter, placed on a non-selective medium, and incubated at optimal temperature for 4 h to facilitate repair. The filter is then transferred to a selective agar medium and incubated for 18 h. The major advantage of this method is enumeration of organisms within a short period of time. However, inhibitors and toxic materials present in food would affect repair. The membrane filter may not adequately contact the selective agar and other organisms can interfere with the growth of the target organisms in areas lacking good contact with selective components.

Sharpe and Michaud (1974) introduced the concept of hydrophobic grid-membrane filter (HGMF) as a bacterial counting tool. HGMF is essentially a membrane filter imprinted with hydrophobic grids to form 1600 individual growth compartments. The hydrophobic grids (e.g. wax) prevent the lateral spread of colonies and incubated for 18 h. The major advantage of this method is enumeration of organisms within a short period of time. However, inhibitors and toxic materials present in food would affect repair. The membrane filter may not adequately contact the selective agar and other organisms can interfere with the growth of the target organisms in areas lacking good contact with selective components.

Sharpe and Michaud (1974) introduced the concept of hydrophobic grid-membrane filter (HGMF) as a bacterial counting tool. HGMF is essentially a membrane filter imprinted with hydrophobic grids to form 1600 individual growth compartments. The hydrophobic grids (e.g. wax) prevent the lateral spread of colonies so that even large numbers of colonies remain as discrete square colonies rather than producing a confluent lawn. The count on the HGMF is determined by an MPN calculation, since two or more microbes may land in one square during the filtration step (Sharpe and Michaud, 1975; Sharpe et al., 1978). The system was formerly made by QA Laboratories (San Diego, CA) as ISO-GRID® and currently is manufactured by Neogen Corp. (Lansing, MI). The application of the HGMF technique to enumerate microorganisms in food has been used widely.

In this system, the food suspension is passed first through a specific apparatus to remove debris and then filtered through the membrane. The inoculated membrane is then placed on the appropriate media for repair and recovery at suitable incubation temperatures (Entis et al., 1982; Peterkin and Sharpe, 1980; Sharpe et al., 1979a, b). The HGMF technique was also utilized to study the effect of stress and resuscitation recovery of indicator bacteria from foods by placing filters on a non-selective medium for a several-hour resuscitation period and then transferring to an appropriate selective medium (Brodsky et al., 1982). This is quite cumbersome and complicated with a long wait between each manipulation. Wu and Fung (2004) applied the ISO-GRID® HGMFs directly with TAL and TALO to reduce the cumbersome procedures and also allow the TAL method to detect low numbers of microbes in food due to the concentration effect of filtration and the capability of the HGMF to cover 3 log units of cells (1–1600 cells).

4. Conclusions

The significance of injured microorganisms in food should not be ignored. Injured cells may be present but escape detection because they do not develop in selective media. The potential for hazard is still a concern because injured foodborne microorganisms are capable of repair and toxin production. Many rapid methods for the detection of pathogens from food are now commercially available. The detection limits are such that they require an enrichment stage, prior to testing, to allow the target organism to multiply. The enrichment conditions are particularly important when testing food samples in which injured cells may be present. However, many rapid methods that recommend the use of direct selective enrichment with or without elevated incubation temperature may give false-negative results. The incorporation of a resuscitation stage using a non-selective pre-enrichment medium or an effective recovery method improves the detection rates of these rapid assays. Therefore, appropriate recovery procedures should be incorporated into current detection and enumeration methods and adopted for regulatory and quality control purposes to ensure that a true microbiological analysis is obtained.

Acknowledgments

This work was supported by the USDA FAS/ICD/RSED Scientific Cooperation Research Program with agreement number 58-31484-4106 and Maine Agricultural and Forest Experiment Station at the University of Maine with external publication number 3005. The author thanks Dr. Daniel Y.C. Fung for advice on the study and Elizabeth Dodge for assistance with editing.

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