# CONTROL OF *BACILLUS CEREUS* IN ENGLISH-STYLE CRUMPETS

By:

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***Dedicated to:***

***My mother (Haïfa), my father (Toufic), my sister and brother (Marie and Joseph)***

# Abstract

## CONTROL OF *BACILLUS CEREUS* IN ENGLISH-STYLE CRUMPETS

English-style crumpets (pH 6-8, $a_w$ 0.97-0.99) are a popular baked product enjoyed by consumers worldwide. However, over the past few years, outbreaks of food poisoning have been caused by the growth of *Bacillus cereus* in crumpets. This spore forming microorganism, which originates in flour, can easily survive the baking process and grow to $>10^6$ CFU/g within 3-5 days at ambient storage temperature. Therefore, control of this pathogen is essential to ensure the safety and marketability of English-style crumpets.

Initial studies were done to determine the effect of water activity ($a_w$), pH, modified atmosphere packaging (MAP), UV-light, bacteriocins, organic acids and esters, alone and in conjunction with each other, on the growth of *B. cereus* in model broth/agar systems. Control of *B. cereus* could be achieved through reduction of $a_w$ to <0.92 and pH to <5. However, both approaches would not be commercially viable due to changes in the sensory/textural qualities of the reformulated products. Preservatives, such as calcium propionate and potassium sorbate, also failed to inhibit the growth of *B. cereus* at pH levels >6; however, mono-lauryl maleate (MLM) inhibited the growth of *B. cereus* at alkaline pH (8-9). MAP, using 100% $CO_2$ and Ageless oxygen absorbents, alone and in combination with each other, also failed to inhibit the growth of *B. cereus* in agar media. However, a combination of an ethanol vapor generator/oxygen absorbent (Negamold®) and 100% $CO_2$ inhibited the growth of this pathogen for >35 days at ambient storage temperature. UV-light also proved effective in delaying the growth of *B. cereus* in agar plates.

Based on these initial studies, subsequent experiments focused on the control of *B. cereus* in English-style crumpets. While both UV-light and MLM (1000-3000 ppm) controlled the growth of this pathogen in model agar studies, they failed to inhibit its growth in English-style crumpets. Negamold®, in conjunction with 100% $CO_2$, inhibited the growth of *B. cereus* in crumpets stored at 30°C for >35 days. However, crumpets were organoleptically unacceptable since the ethanol content of crumpets was ~3.5% (w/w) i.e., higher than the approved maximum limit of 2% (w/w) for food. Pasteurization of the crumpet batter, prior to baking on a hot plate, also failed to control the growth of this pathogen; furthermore, textural changes also occurred in the final product.

In conclusion, *B. cereus* is a difficult microorganism to control in food using conventional preservation methods. Further studies are now under way to investigate novel methods to control the growth of this pathogen, particularly in high pH crumpets.

# RÉSUMÉ

# LE CONTRÔLE DU *BACILLUS CEREUS* DANS LES CRUMPETS ANGLAIS

Les crumpets anglais (pH 6-8, $a_w$ 0.97-0.99) sont des produits populaires, cuits au four et appréciés par des consommateurs dans le monde entier. Cependant, au cours de ces dernières années, des manifestations d'intoxication alimentaire ont été provoquées par la croissance du *Bacillus cereus* dans les crumpets. Cette spore formant le micro-organisme, et provenant de la farine, peut facilement survivre la cuisson et se diviser jusqu'à $>10^6$ CFU/g en une période de 3-5 jours, à température ambiante. Par conséquent, le contrôle de ce pathogène est essentiel afin d'assurer l'hygiène et la commercialité des crumpets anglais.

Des études préliminaires furent conduites afin de déterminer l'effet de l'activité de l'eau ($a_w$), du pH, du conditionnement sous atmosphère modifiée, de la lumière UV, des bactériocines, des acides organiques et des esters, séparément et en combinaison, sur la croissance du *B. cereus* dans des systèmes de bouillon/agar. Le contrôle du *B. cereus* fut réalisé sous une activité de l'eau $a_w < 0.92$ et un pH < 5. Cependant, ces deux approches ne seraient pas commercialement viables à cause des changements apportés à la qualité sensorielle et à la texture des produits reformulés. Les préservatifs tels que le propionate de calcium et le sorbate de potassium ne réussirent pas à empêcher la croissance du *B. cereus* à des pH supérieurs à 6; par contre, le maleate mono-laurique (MLM) a empêché la croissance du *B. cereus* sous un pH alkalin (8-9). L'utilisation du conditionnement sous atmosphère modifiée, avec du $CO_2$ 100% et des absorbants d'oxygène Ageless, seule ou en combinaison, n'a pas réussi non plus à empêcher la croissance du *B. cereus* dans de l'agar. Toutefois, l'utilisation en combiné d'un générateur de vapeur d'éthanol/absorbant d'oxygène

(Négamold®) et de $CO_2$ 100% empêcha la croissance du pathogène pendant plus de 35 jours, à température ambiante. La lumière UV a également été efficace dans le le retardement du *B. cereus* dans les contenants d'agar.

Suite à ces résultats préliminaires, des recherches ultérieures portèrent sur le contrôle du *B. cereus* dans les crumpets anglais. Alors que la lumière UV et le MLM (1000-3000 ppm) contrôlèrent la croissance du pathogène dans les études utilisant l'agar, ils ne furent pas efficaces lorsque appliqués sur les crumpets anglais. Le Negamold®, en combinaison avec du $CO_2$ 100%, a empêché la croissance du *B. cereus* dans les crumpets, sous une température de 30°C pendant plus de 35 jours. Cependant, les crumpets étaient organoleptiquement inacceptables puisque leur teneur en éthanol était ~3.5% (w/w) soit plus haut que la limite maximale acceptable de 2% (w/w) pour la nourriture. La pasteurisation de la pâte de crumpet, avant la cuisson sur plat chaud, n'a pas non plus réussi à contrôler la croissance de ce pathogène; en outre, des changements de texture se manifestèrent dans le produit final.

En conclusion, le *B. cereus* représente un micro-organisme difficile à contrôler dans la nourriture à l'aide de méthodes conventionnelles de conservation. Des études supplémentaires sont déjà entreprises afin de trouver de nouveaux moyens pour contrôler ce pathogène, en particulier dans des crumpets à pH élevé.

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### *1.1. Introduction*

Bakery products are a very important part of a balanced diet and account for ~20% of the daily dietary protein intake of the average Canadian consumer (Smith and Simpson, 1996). The importance of bakery products has increased over the past decade due to consumer demands for whole, natural grain and preservative-free products of nutritious value. Global consumption of bakery products, along with cereals, has increased at an average rate of 25% per annum since 1970. The average human consumption of cereals per annum is about 50% of the total production. In 1984-1986, out of 1677 Mt (metric ton) of the world's total domestic supply of cereals, 49% was used for human food, 37% for animal feed, 10% for processing and other uses, and 4% for seed. According to Hunt and Robbins (1989) spending on bakery products accounted for approximately 9% of total food expenditures in Canada. In 1997, the Canadian bread and bakery sub-sector shipped $2.30 billion of products, an increase of 36.5% from 1988 levels, and accounted for 4.2% of total food and beverage processing sector shipments (Canadian Bread and Bakery Industry, 2000). Meanwhile, the bakery industry in the United States marketed about $22 billion of products (Smith and Simpson, 1996). The various categories of bakery products, with specific examples of types within each category, are summarized in Table 1. The majority of bakery products are marketed fresh, both at the wholesale and the retail levels; however, others are stored frozen since they include more perishable ingredients, e.g., meat or cream. Bakery products, like most processed foods, are subject to physical, chemical, and microbiological spoilage over time. Each type of spoilage is briefly reviewed.

### *1.2. Spoilage of bakery products*

#### *1.2.1. Physical spoilage*

Physical spoilage is characterized by moisture loss or gain resulting in a loss of texture. This problem can be overcome by the use of appropriate packaging film i.e., low-density polyethylene (LDPE), which is a high barrier to moisture (Smith and Simpson, 1996). However, accumulation of moisture on a product's surface, if packaged too soon after baking, could result in conditions conducive to mold growth. Moisture loss and staling are of economic concern to the bakery industry. It has been estimated that ~$ 1 billion of products are lost annually due to staling (Kulp, 1979). Staling is characterized by a change in starch, from an amorphous to a crystalline form, at temperatures below 55°C. Starch, in its crystalline form, binds less water, which results in a toughening of the crust and hardening of the crumb, a loss of flavor and an opaqueness in the crumb (Kent and Evers, 1994). Staling is dependent upon the storage temperature with maximum staling occurring at 4°C. Thus, temperatures of –20°C and above 55°C are required to prevent staling (Kent and Evers, 1994). Emulsifiers, such as monoglycerides, are often added as anti-staling agents. Other surfactants, referred to as dough conditioners, such as calcium and sodium stearoyl-2-lactylate, are added to strengthen the dough properties during production.

#### *1.2.2. Chemical spoilage*

Chemical spoilage e.g., rancidity, occurs in products which have a high fat content. Rancidity can result in off-odors making them unpalatable and hence decreasing the shelf-life of products. Two types of rancidity can be distinguished namely, oxidative and hydrolytic.

Oxidative rancidity involves a breakdown of unsaturated fatty acids by oxygen through an autolytic free-radical mechanism. This in turn will lead to the formation of odorous aldehydes, ketones and short chain fatty acids (Smith and Simpson, 1996).

Hydrolytic rancidity, unlike oxidative rancidity, occurs in the absence of oxygen. It is enhanced by the presence of endogenous enzymes i.e., lipases and lipoxygenases and moisture. It results in the hydrolysis of triglycerides, release of glycerol, and malodorous short-chain fatty acids (Smith and Simpson, 1996).

**Table 1. Categories of bakery products found on supermarket shelves**

| Categories of bakery products | Types within each category |
|---|---|
| Unsweetened goods | Bread: sliced, crusty, part-baked, ethnic<br>Rolls: soft, crusty<br>Crumpets<br>English muffins<br>Croissants<br>Pizza base<br>Raw pastry |
| Sweet goods | Large cakes: plain, fruited<br>Pancakes<br>Doughnuts<br>Waffles<br>Cookies<br>Biscuits<br>American muffins<br>Buns<br>Wafers |
| Filled goods | Tarts: fruit, jam<br>Pies: meat, fruit<br>Sausage rolls<br>Pasties<br>Cakes: cream, custard<br>Pizza<br>Quiche |

Adapted from Blakistone (1998)

### *1.2.3. Microbiological spoilage*

Microbiological spoilage is often the major factor limiting the shelf-life of bakery products. Spoilage due to microbial growth causes economic losses for both manufacturers and consumers (Hickey, 1980). It is estimated that, in the U.S. alone, losses due to microbial spoilage are 1 to 3 % or over 90 million kg of products each year (Smith and Simpson, 1996). Among the intrinsic food-related factors, water activity ($a_w$) is the most important factor affecting microbial spoilage. The minimum $a_w$ value for the growth of spoilage microorganisms and the $a_w$ of selected baked goods is shown in Figure 1.

#### *1.2.3.1. Food spoilage concerns*

While mold growth is the most common form of spoilage in bakery products, yeast, and bacterial spoilage is also of concern. Mold growth usually occurs in products of high $a_w$. The majority of the molds found in white bread belong to the genus *Aspergillus* and *Penicillium* (Hartung *et al.*, 1973). Flour contains mold spores and therefore mold spoilage results from post-processing contamination. This occurs after baking of the product, during cooling and packaging, from contamination by airborne spores or contact with contaminated surfaces (Black *et al.*, 1993).

Yeast spoilage is due to wild species of yeast including *Pichia, Zygosaccharomyces,* and *Saccharomyces* strains (Graves *et al.*, 1967). Chalk bread occurs due to *Saccharomyces* spp., which produces white spots in the breadcrumb.

The major bacterial problem in high moisture bread is "rope" caused by *Bacillus subtilis* (also known as *Bacillus mesentericus*). Spores of *B. subtilis* can survive the baking process and then germinate and multiply when conditions are favorable for their growth (Volavsek *et al.*, 1992). For instance, stacking of bread or underbaking can both provide moisture favorable for germination and subsequent growth of vegetative cells to levels that cause rope. Rope development is fairly rapid, occurring within 12 hours of baking. Ropy bread is characterized by discoloration in the center of the loaf, softening of the crumb, and a sweet fruity odor which is similar to overripe pineapple (Kirschner and von Holy, 1989).

#### *1.2.3.2. Foodborne illness*

Although spoilage is of major concern to the food industry, bakery products have also been implicated in foodborne disease outbreaks. Egg-associated salmonellosis is a substantial public health problem. Unlike most *Salmonella* species, which contaminate only the eggshell, *Salmonella enteritidis* can be found inside eggs. In 1995 alone, 45,970 cases of salmonellosis were reported to the Centers for Disease Control and Prevention (Center for Disease Control and Prevention, 1996). The major source of *S. enteritidis* is grade-A table eggs (Shah *et al.,* 1991). Cheesecake prepared from raw eggs has been reported in numerous outbreaks, the source being the raw eggs contaminated with *S. enteritidis*. In Canada, the major incidents of foodborne diseases associated with bakery products arose from pizza and pasta products, and to a lesser extent, cake and breads. Examples of the foodborne illnesses associated with bakery products are summarized in Table 2.

**Figure 1. Minimum $a_w$ values for the growth of spoilage microorganisms in selected bakery products (Blakistone, 1998)**

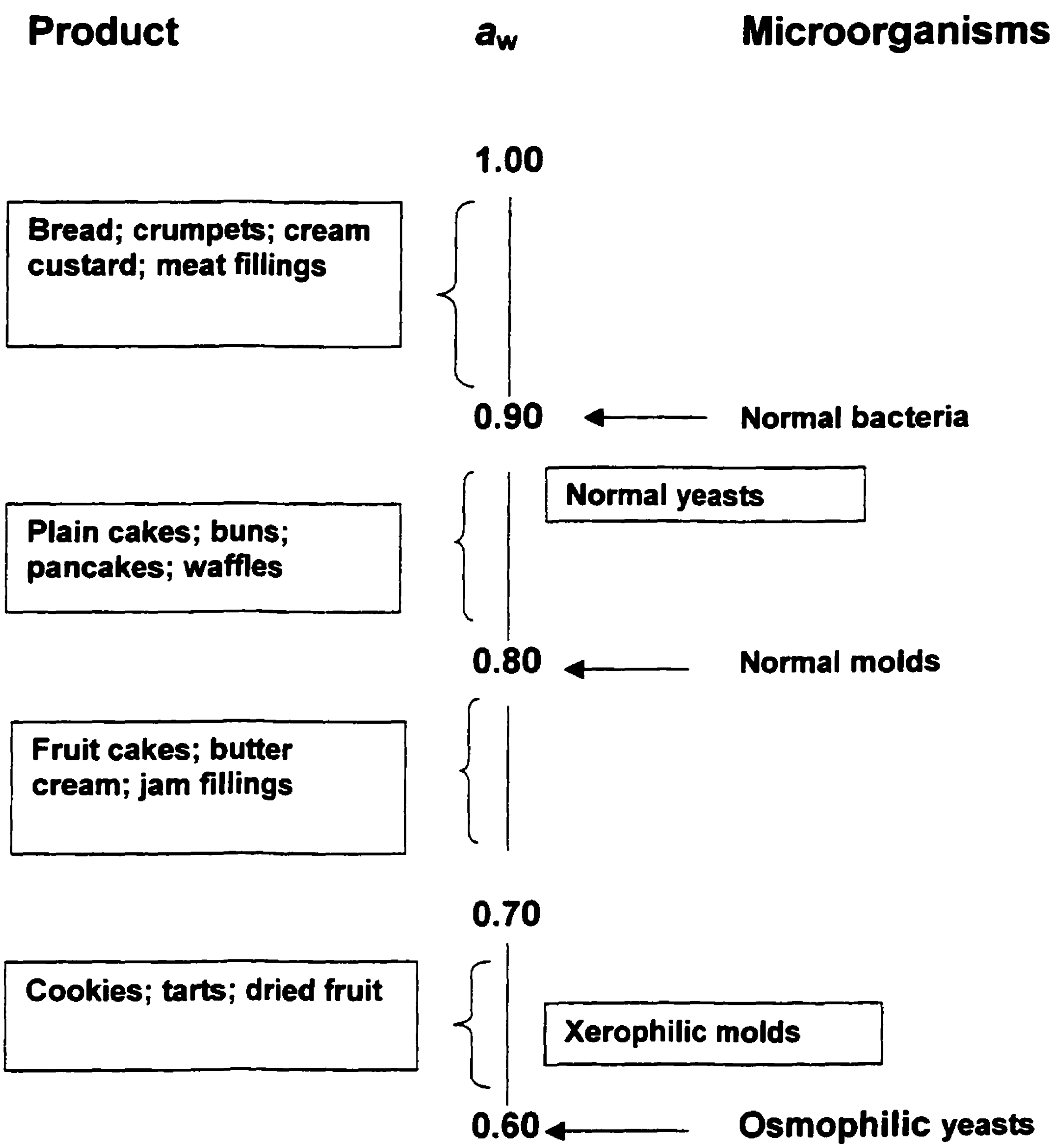


**Table 2. Bakery associated with foodborne incidents**

| Product | 1991 (%) | 1992 (%) | 1993 (%) |
|---|---|---|---|
| Pizza | 52 | 51.6 | 60.0 |
| Cakes: | | | |
| Cheese cake | 2.0 | 3.2 | 1.3 |
| Other cakes | 6.1 | 5.3 | 9.3 |
| Pasta products: | | | |
| Canned ravioli | 2.0 | 2.1 | 1.3 |
| Lasagna | 3.1 | 8.4 | 6.7 |
| Spaghetti | 2.0 | 6.3 | 4.0 |
| Other pasta | 6.1 | 4.2 | 4.0 |
| Breads | 4.1 | 1.1 | N/A[a] |
| Cookies | 4.1 | N/A | 1.3 |
| Cereal | 1.0 | N/A | 1.3 |
| Muffins | 1.0 | N/A | 1.3 |
| Pastries | 3.1 | 4.2 | N/A |
| Pies and tarts | 6.1 | 2.1 | N/A |

Adapted from Foodborne Disease-Reporting Center in Canada (1992-93)

[a] Not available

The incidence of foodborne illnesses in developed countries is less than in developing countries (Gavarni, 1987; Bean and Griffin, 1990). The major reasons for these lower incidences are better manufacturing practices, stricter regulations for the production and handling of foods, good sanitary practices, and regulatory and inspection agencies.

In 1992, two deaths occurred in Canada. A fifteen-year old girl died of cardiac arrest following a hemolytic uremic syndrome arising from an infection of *Escherichia coli* O157: H7 after eating a hamburger at a family barbecue. In the following year, three deaths occurred. One of them occurred following an outbreak of *Bacillus cereus*. Between the years 1991 to 1993 in Canada, incidents associated with meat represented the largest hazard category, followed by poultry, marine food, bakery products, and to a lesser extent, Chinese foods, salads, vegetables, sandwiches, dairy foods, and beverages. The most frequent food categories implicated in foodborne disease outbreaks by country are shown in Table 3. The number of outbreaks, along with the number of cases in each outbreak between 1991 and 1993, are summarized in Table 4. Certain food types are implicated with foodborne diseases more frequently than others, especially when food is prepared under specific conditions and the environment is conducive to the growth of specific bacteria. The presence of a pathogen in the raw material results in a greater chance of contamination of the finished product. The incidence of *Clostridium botulinum* (non-proteolytic type E) outbreaks is high in fish products, due to the presence of the bacteria in the marine environment. Similarly, fruits and vegetables are contaminated by *C. botulinum* (type A and B) present in soils. Staphylococcal food poisoning is considered to be one of the world's most frequently occurring foodborne diseases, caused by toxins of *Staphylococcus aureus. St. aureus* are Gram-positive cocci, facultative anaerobes which cause food intoxication. They are mesophiles with a growth temperature range of 7-48°C. They are able to grow at low $a_w$ (0.86), low pH (4.8), high salt concentrations up to 15 % and in the presence of $NO_2$. Some of the most commonly implicated foods involved in *St. aureus* outbreaks include ham, corned beef, salami, salads, cream fillings, and cheeses i.e., foods high in salt or sugar.

**Table 3. Most frequent food categories implicated in foodborne disease outbreaks by country**

| Food | Countries |
|---|---|
| Meat | Canada (19%) |
| | United States (16%) |
| | Finland (34%) |
| | England/Wales (36%) |
| | Belgium (46%) |
| Poultry | Scotland (43%) |
| Bakery products | Poland (35%) |
| | Portugal (37%) |
| | Bulgaria (46%) |
| | Switzerland (47%) |
| Eggs/egg products | France (45%) |
| | Spain (62%) |
| Poisonous mushrooms | Hungary (47%) |
| Chinese foods | Netherlands (30%) |

Adapted from World Health Organization (1992) and Todd (1994).

**Table 4. Number of foodborne outbreaks/cases in Canada (1991-1993)**

| Microorganism | Number of outbreaks | | | Number of cases in outbreaks | | |
|---|---|---|---|---|---|---|
| | 1991 | 1992 | 1993 | 1991 | 1992 | 1993 |
| *Bacillus cereus* | 8 | 12 | 8 | 49 | 39 | 34 |
| *Campylobacter* spp. | 5 | 14 | 7 | 21 | 72 | 37 |
| *Clostridium botulinum* | 1 | 2 | - | 3 | 9 | - |
| *Escherichia coli* | - | 2 | - | - | 35 | - |
| *Listeria monocytogenes* | - | - | 2 | - | - | 4 |
| *Salmonella* spp. | 25 | 23 | 39 | 470 | 126 | 407 |
| *Staphylococcus aureus* | 18 | 22 | 6 | 162 | 281 | 37 |
| *Mold* | 4 | - | 1 | 83 | - | 4 |
| *Suspect yeasts* | 3 | - | - | 8 | - | - |

Adapted from Foodborne Disease-Reporting Center in Canada (1992-93)

### *1.3. Bacillus cereus*

#### *1.3.1. History*

*B. cereus* was first isolated and described by Frankland and Frankland in 1887 (Baird-Parker *et al.*, 1996). It was not until the 1950s that the role of *B. cereus* was firmly established as an agent of foodborne disease. Hauge (1950; 1955) was able to prove that this organism could actually cause foodborne illness by describing four outbreaks, all involving vanilla sauce. These outbreaks were characterized by watery diarrhea occurring 8-16 h after ingestion of the food. In 1971, an outbreak, characterized by nausea and vomiting, 1-5 h after ingestion of cooked rice, was reported. In all cases, *B. cereus* was implicated.

#### *1.3.2. Taxonomy*

The genus *Bacillus* is widely accepted as being a heterogeneous collection of Gram-positive, catalase positive spore-forming rods, which are facultative anaerobes but grow faster with the presence of oxygen (Claus and Berkeley, 1986). Group I *Bacilli* includes *B. cereus* and are defined as having a sporangium which is not swollen by the spore. Within this group are subdivisions of the species based on cell diameter. The large celled *B. cereus* has a cell diameter > 0.9 μm.

#### *1.3.3. Ecology*

*B. cereus* is widely distributed in nature. It is found in soil, dust, cereal crops, vegetation, animal hair, fresh water, and sediments (Kramer and Gilbert, 1989). Although its presence in fish products is not well established, it is found in other raw agricultural commodities, especially foods of plant origin which are the major source of *B. cereus*. A survey by Nygren (1962) revealed that 52% of 1546 food ingredients, 44% of 1911 cream and dessert dishes, and 52% of 431 meat and vegetable products were contaminated with *B. cereus*. This organism is also frequently found in milk and dairy products, particularly if cows are fed with silage or housed in barns. Approximately 9 to 48% of such products, including UHT-treated milk (48%), were found to contain *B. cereus*. The incidence of *B. cereus* in raw food is summarized in Table 5.

In processed foods, the presence of *B. cereus* is often associated with the ability of spores to survive the heat treatment process. The incidence of *B. cereus* in processed foods is shown in Table 6.

### *1.3.4. Growth and survival*

Strains of *B. cereus* vary widely in their growth and survival characteristics. Some strains are psychrotrophic (being able to grow at temperatures of 4-5°C), whereas other strains are mesophilic (can grow between 15°C and 55°C). The optimal temperature for growth ranges between 30-40°C. Similarly, the minimal pH for growth varies among strains and also depends on the acidulant. In general, growth does not occur at pH 4.8 in media acidified with hydrochloric acid (HCl), or at pH 5.6 in media acidified with lactic acid.

### *1.3.5. Food poisoning symptoms*

*B. cereus* causes two different types of poisoning: the diarrheal type, first recognized after a hospital outbreak caused by contaminated vanilla sauce in Oslo, Norway, (Hauge, 1950;1955), and the emetic type described twenty years later after several outbreaks associated with rice, in London, England (Mortimer and McCann, 1974).

The diarrheal illness has a longer incubation period, commonly 10-13 h, although incubation periods of 8-16 h have been reported. The gastroenteritis is usually mild with abdominal cramps, profuse watery diarrhea, rectal spasm and moderate nausea, usually without vomiting.

The emetic illness generally has an onset of ~1-5 h after consumption of the implicated food. Acute nausea and vomiting are the major symptoms of the emetic type illness with diarrhea being uncommon.

**Table 5. Incidence of *B. cereus* in raw foods**

| Food | Number positive/ tested | Spores/ gram | Reference |
|---|---|---|---|
| Raw milk | N/A | $0\text{-}10^{1}$ | Stadhouders & Driessen (1992) |
| Rice | 6/13 | $10^{2}\text{-}10^{3}$ | Lee & Chang (1980) |
| Flour | 25/25 | $0\text{-}10^{1}$ | Eyles *et al.* (1989) |
| Fish | 4/10 | $10^{2}\text{-}10^{4}$ | Kamat *et al.* (1989) |
| Meat chicken | 8/10 | $10^{3}-10^{4}$ | Kamat *et al.* (1989) |
| Beans | 5/9 | $<10^{2}\text{-}10^{3}$ | Blackey and Priest (1980) |
| Cocoa | 2/6 | $10^{3}-10^{5}$ | te Giffel *et al.* (1996) |
| Dried potatoes | 8/20 | $10^{2}-10^{3}$ | Kusunoki *et al.* (1977) |
| Powdered spices | 5/7 | $10^{3}-10^{4}$ | Kamat *et al.* (1989) |

**Table 6. Incidence of *B. cereus* in processed foods**

| Food | Incidence | Spores/gram | Reference |
|---|---|---|---|
| Bakery products | 9/10 | $10^3 - 10^4$ | te Giffel *et al.* (1996) |
| Boiled rice | 25/252 | $10^2$-$10^5$ | Gilbert and Parry (1977) |
| Chinese meals | 15/18 | $10^3 - 10^5$ | te Giffel *et al.* (1996) |
| Meat products | 9/10 | $10^2$-$10^4$ | te Giffel *et al.* (1996) |
| Milk | 56/157 | $10^1$-$10^4$ | te Giffel *et al.* (1996) |
| Pasta products | 4/8 | $10^4$ | te Giffel *et al.* (1996) |
| Pastries | 23/212 | $10^1$-$10^4$ | Kusunoki *et al.* (1977) |
| Ice cream | 48/100 | $10^1$-$10^3$ | Ahmed *et al.* (1983) |
| Vanilla slices | 55/133 | $10^2$-$10^3$ | Pinegar and Buxton (1977) |
| Egg yolk powder | 10/34 | $<10^2$-$10^3$ | van Netten and Kramer (1992) |

### *1.3.6. B. cereus toxins*

Recent studies have shown that at least two different *B. cereus* toxins exist. The diarrheal enterotoxin is heat labile (inactivated by 56°C for 5 min, but not 45°C for 30 min), unstable at pH values beyond the range of 4-11, and sensitive to proteolytic enzymes. Toxin activity is reduced with an increase in temperature. Little is known about the emetic toxin due to poor purification techniques. However, it has been shown to be stable at high temperatures (121°C for 90 min) and pH values ranging from 2-11 (Shinagawa, 1993). This toxin is most commonly found in rice and other starchy food products.

### *1.3.7. Outbreaks*

In the United States, federal regulatory agencies define foodborne disease as an outbreak "when two or more people become sick with a similar illness (symptoms) from the consumption of the same food(s) from the same source, and the epidemiological investigations implicate, either directly or indirectly, the same food(s) from the same source as the cause of the illness". However, in the case of botulism, for instance, due to a high fatality rate, even when only one person has the illness, it is considered an outbreak. For chemical poisoning, a single case is also considered an outbreak (Bibek, 1996).

The incidence of foodborne gastroenteritis from *B. cereus* is relatively high in some European countries (Bean *et al.,* 1990). In contrast, the incidence is relatively low in the United States. In the United States, it was first recognized as a causative gastroenteritis agent in 1969. However, between 1973 and 1987, 58 outbreaks, involving 1123 cases, were reported. Both the incidence of outbreaks and the number of cases per outbreak were low and no fatalities were reported. However, as the symptoms are not severe and last for only 12 h, many cases may not be reported. Moreover, foodborne illness transmitted through milk may affect only a few persons in many homes and, thus, would not be considered as an outbreak. Another factor may be that milk drinkers can acquire some immunity through continuous ingestion of small amounts of *B. cereus*.

The dominant type of illness from *B. cereus* differs from country to country, depending on the eating habits of its population. In Japan, for instance, the emetic illness is reported about ten times more frequently than the diarrheal illness (Shinagawa, 1993). In Europe and North America, the diarrheal type is most frequently reported (Kramer and Gilbert, 1989). Several reports indicate that some patients have suffered from both types of *B. cereus* illnesses simultaneously. Countries also have different reporting procedures for foodborne illnesses, making comparison for foodborne outbreaks among them a tedious task. The percentage of reported outbreaks and cases attributed to *B. cereus* in Japan, North America, and Europe vary from about 1 to 22 % of outbreaks and from about 0.7 to 33% of cases (reports from different periods between 1960 and 1992) (Aas *et al.*, 1992).

The Netherlands and Norway were among the first countries to report outbreaks due to *B. cereus*. Recent outbreaks in Norway were associated with stew with the infective dose being estimated at $10^4$ to $10^5$ CFU/g. Of the 17 people affected, 3 were hospitalized for 1-3 weeks (Granum, 1994). The second outbreak in 1995 involved 152 cases among competitors at the Norwegian junior ski championship (Granum *et al.*, 1993). The young athletes (16 to 19 years old) had the most severe symptoms, while their coaches and officials were not affected. The onset of symptoms for many patients was more than 24 h, and the duration of illness varied from two to several days.

### *1.4. Importance of B. cereus in cereal products*

#### *1.4.1. Pasta*

Studies in Australia have shown that the incidence of *B. cereus* in pasta products is ~50%. However, counts of this bacteria in pasta products were <$10^4$CFU/g i.e., below the levels which would be of public health significance

($10^5$CFU/g), (te Giffel *et al.*, 1996). The most conducive factor for growth of *B. cereus* is cooking temperature which can induce spore germination. Furthermore, the slow cooling of the product and storage of large amounts of food at temperatures between 10°C and 50°C will enhance the growth of vegetative cells. If food needs to be kept warm, it should be maintained at a temperature of 60°C to be safe. Conventionally, the drying temperature for pasta is about 55°C.

In some countries the drying temperature of pasta has been increased to 75°C and in other countries to 100°C. This has resulted in a better quality and safer product as well as a reduced drying time. Precautions which need to be adhered to in order to prevent the growth of either *B. cereus* or other microorganisms in pasta include:

(i) Frequent and thorough cleaning of machinery;
(ii) Pasta should be dried or refrigerated promptly after manufacture;
(iii) Liquid eggs, if used, should be pasteurized;
(iv) Pasta dough, or dried pasta dough, should always be boiled prior to consumption.

### *1.4.2. Rice*

Boiled rice, and more frequently fried rice, usually contains high levels of *B. cereus* ($10^3$-$10^4$CFU/g). According to the Food Disease-Reporting Center in Canada, fried rice was involved in a number of incidents from 1991 to 1993. However, the percentage of these incidents decreased from 8.2% in 1992 to 3.5 % in 1993. Factors conducive to the growth of *B. cereus* include, low cooking temperatures (<100°C), and the time elapsed between cooking and consumption. When rice is allowed to cool slowly, spores germinate and rapid cell multiplication occurs when rice is stored between 10-50°C before being served i.e., conditions which are favorable to the growth of *B. cereus*. Raw rice has a certain level of *B. cereus* naturally present upon harvesting. This initial level of bacteria is insufficient to cause illness; however, it can reach infectious levels when rice has been inadequately cooked or stored improperly. This problem could be easily overcome by cooling the food rapidly after cooking and storing it at <10°C. If it is to be consumed hot, rice should be maintained at 60°C or higher. In addition to strict temperature control, attention should be paid to good hygiene practices during preparation and storage of rice.

### *1.4.3. Bakery products*

#### *1.4.3.1. Bread*

Despite the frequent contamination of flour, bread is rarely a source of *B. cereus*. One survey found *B. cereus* in 1 of 46 bread samples tested (Rosenkvist and Hansen, 1995). This could be due to the fact that bread has a lower $a_w$ ($a_w$ <0.95). In another survey (te Giffel *et al.*, 1996), *B. cereus* was found at levels of $10^3$ and $10^4$ CFU/g in 9 out of 10 bakery products tested. However, it is possible that they were present in cream or custard filled products, which have sometimes been associated with food poisoning outbreaks (Fenton *et al.,* 1984). The presence of *B. cereus* in food at levels <$10^3$ CFU/g is considered innocuous since the minimum level required to cause an illness has been estimated to be >$10^5$ CFU/g (Hobbs and Gilbert, 1974). A study of bread production showed that growth of *B. cereus* was unlikely unless the dough contained a high level of spores. A lag time of at least 24 h occurred before growth commenced, and, preservatives, such as calcium propionate, were effective in delaying growth of *Bacillus* (Kaur, 1986). *B. cereus* can also be found in liquid and powdered egg yolk (van Netten *et al.*, 1990) and can seriously affect the shelf-life of products. According to the Foodborne Disease-Reporting Center in Canada, foodborne incidents related to bread have decreased from 4.1 % to 1.1 % between 1991 and 1992. Moreover, *B. cereus* was not related to any of the previously mentioned incidents including the year 1993.

### *1.4.3.2. Crumpets*

Crumpets are high moisture flour based products containing yeast, an aerating agent, or both, to give them a raised profile and open texture after baking. They are produced from flour, instant yeast, milk powder, baking soda, salt, and water. Greased cylindrical molds are filled with the crumpet batter and then baked on a hot plate, only cooking the bottom of the product. They have been implicated in many outbreaks of food poisoning. This is mainly due to the growth and toxin formation by naturally occurring *B. cereus*. The presence of *B. cereus* was noticeable after 5 days at ambient temperature (Jenson *et al.*, 1994). Spores in the batter which come in direct contact with the hot plate, do not survive the cooking temperature. However, surface spores can easily survive baking and, upon germination, produce toxin. Crumpets have a short shelf-life, i.e., < 6 days, due to the fact that crumpets have a non-acidic pH (6 to 8), a high moisture content (48 to 54%), and also a high water activity ($a_w$) (0.95 to 0.97). All of these factors make crumpets an ideal substrate for microbial growth. A recent study conducted by Jenson *et al.* (1994), proved the efficacy of using nisin, a bacteriocin produced by *Lactococcus lactis*, as an antimicrobial agent to control the growth of *B. cereus* in crumpets. It was the first time that nisin had been used in a flour-based product. As a result, regulations in Australia now allow the use of nisin in crumpets and similar high moisture flour-based products to control the growth of this important pathogen.

## *1.5. Control of B. cereus*

### *1.5.1. pH*

When microorganisms were implicated in food spoilage and foodborne diseases, methods to control their growth were investigated. One such method is pH.

In the unprocessed state, most foods, such as meat, fish, and vegetables are slightly acidic, most fruits are moderately acidic, while a few foods, such as egg white, are alkaline. The acidity of a food can be used to enhance its microbiological stability and to ensure its safety. For example, acidity is the primary factor in preserving acidic foods, such as yogurt, sauerkraut, and pickles. Examples of acid/alkaline foods which are contaminated by different microorganisms are shown in Table 7. Microorganisms vary in their ability to grow at low pH levels. The minimum pH for growth of different types of bacteria, molds and yeasts is summarized in Table 8.

In general, Gram-negative bacteria are more sensitive to low pH than Gram-positive bacteria, while yeast and molds are the least sensitive to acidic conditions. Fermentative bacteria are more resistant to lower pH than respiring bacteria, probably because they are able to resist changes in the external pH, as well as withstand slightly lower internal pH. The ability of yeast and molds to withstand low pH is also due to these factors (Baird-Parker, 1980). Some microorganisms in food, such as *Salmonella* strains, may be controlled by pH lower than 4.0, but even lower pH values or a combination of low pH and other factors, such as low temperature, are needed to control coliforms. In many meat products the growth of salmonellae is restricted by the growth of lactic acid producing organisms (i.e., *Lactobacillus* spp.). Since these foods have a high buffering capacity, large amounts of acid are produced without a corresponding decrease in pH. In poorly buffered foods, such as pickles, much less activity by similar acid producers will produce inhibitory concentrations of weak acids.

Most meat products do not have sufficiently low pH values and high salt concentrations to completely inhibit *C. botulinum* (Riemann *et al.*, 1972). However, *C. botulinum* does not grow in pickles containing acetic acid (0.9-1.8%, v/v) and outgrowth of spores is inhibited at pH 4.8, but not at pH 5.0.

When *B. cereus* was inoculated into canned foods, including fruit, vegetables, soups, and meat (pH values ranging from 3.64 to 6.3), no growth was observed below pH 5.0. At pH values above 5.15, the extent of growth was proportional to pH (Pivovarov and Kukhto, 1970). In laboratory media, *B. cereus* may grow over a pH range of 4.9-9.3 (Goepfert *et al.,* 1972). However, meat and rice products are less inhibitory than laboratory media, supporting growth at pH 4.35 (Raevuori and Genigeorgis, 1975). The effect of pH and NaCl on the growth of both *B. cereus* and *St. aureus* varies with the strain and growth medium used. At decreasing pH values, a larger inoculum is needed to initiate growth, while both high NaCl concentrations and extreme pH values are needed to inhibit the growth of *B. cereus* (Raevuori and Genigeorgis, 1975).

**Table 7. Some types of spoilage typically associated with foods of certain pH values**

| Type of food | Examples | pH range | Spoilage microorganism | Reference |
|---|---|---|---|---|
| Alkaline | Egg albumen | ~9.6 | *Pseudomonas* spp., *Aeromonas* spp. | Board (1969) |
| Neutral | Milk | 7.6-6.8 | *Bacillus* spp., *Micrococcus* spp. | Ingram (1962) |
| Low acid | Raw beef | 5.8-5.4 | *Staphylococcus, Micrococcus* spp. | Labadie *et al.* (1975) |
| Medium acid | Cheese | 4.5 | *Bacillus nigificans, Aerobacter* spp. | Jay (1970) |
| Acid | Mayonnaise | 4.1-3.0 | *Saccharomyces* | Jay (1970) |
| | Tomato, fruits | 4.0 | *Bacillus coagulans* | |
| High acid | Pickles, juice, salad dressing | 3.9-3.5 | Molds, yeast, some lactic acid bacteria | Jay (1970) |
| | Citrus fruits | 3.5-3.0 | *Penicillium italicum* | Eskin *et al.* (1971) |

**Table 8. Minimum pH for microbial growth**

| Microorganism | Minimum pH |
|---|---|
| **Gram-negative bacteria** | |
| *Escherichia coli* | 4.4 |
| *Pseudomonas spp.* | 5.6 |
| *Salmonella spp.* | 4.5 |
| *Vibrio spp.* | 4.8 |
| **Gram-positive bacteria** | |
| ***Bacillus cereus*** | **4.9** |
| *Bacillus stearothermophilus* | 5.2 |
| *Clostridium botulinum* | 4.6 |
| *Clostridium perfringens* | 5.0 |
| *Enterococcus faecalis* | 4.4 |
| *Lactobacillus spp.* | 3.8 |
| *Staphylococcus aureus* | 4.0 |
| *Listeria monocytogenes* | 4.6 |
| **Yeasts** | |
| *Candida spp.* | 1.5-2.3 |
| *Saccharomyces spp.* | 2.1-2.4 |
| **Molds** | |
| *Aspergillus spp.* | 1.6 |
| *Penicillium spp.* | 1.6-1.9 |

Adapted from Bibek (1996)

### *1.5.2. Water activity ($a_w$)*

The growth and metabolism of microorganisms requires the presence of water in an available form. The most useful measurement of the availability of water is water activity ($a_w$). The main objective of reducing $a_w$ in food is to prevent or reduce the growth of vegetative cells and germination and outgrowth of spores of microorganisms (Christian, 1980).

Solutes are often used to reduce $a_w$ and they differ in their ability to do so. The amounts of NaCl, sucrose, glucose, and inverted sugar (%w/w) necessary to reduce the $a_w$ of pure water at 25°C to 0.99 are 1.74, 15.45, 8.9, and 4.11 g respectively. To reduce $a_w$ to 0.92, 11.9, 54.34, 43.72, and 32.87g of these solutes are required respectively. These solutes do not freely enter the microbial cell and thus have a greater inhibitory effect on microbial cells compared to solutes that enter freely into cells, (e.g., glycerol), which are required in higher amounts for similar inhibition.

Studies on minimal $a_w$ values to support the growth of specific microorganisms have generated conflicting data. This could be due to the inherent problems with different techniques to measure $a_w$. However, with modern electronic hygrometers, this problem is minimized (Sperber, 1983). Minimal $a_w$ values for growth, as well as the influence of $a_w$ on viability loss of microorganisms vary with the characteristics of the food and the food environment. In a homogeneous food, $a_w$ will remain unchanged provided other factors remain constant. However, a heterogeneous food, ingredients or items of different $a_w$ (e.g., sandwich or a meal with different items in the same package) will generate an $a_w$ gradient. This can lead to microbial growth in products preserved by $a_w$ alone and stored with products of high $a_w$ containing a preservative. In addition, condensation of water during storage with temperature fluctuation, followed by dripping of moisture onto food, can alter a safe $a_w$ level to an unsafe level. Microorganisms differ greatly in their minimal $a_w$ requirements for growth, sporulation, and germination (Table 9). In general, molds and yeasts can grow better at lower $a_w$ values than bacteria. Among pathogenic and spoilage bacteria, Gram-negative bacteria require a slightly higher $a_w$ than Gram-positive bacteria for growth. Sporulation by spore forming bacteria occurs at $a_w$ values in which the species-strain will grow. Toxin production may occur at the $a_w$ of growth or at a slightly higher $a_w$ than the minimum required for growth. Meanwhile, the minimum $a_w$ for microbial growth can vary with the type of solutes in a food. *C. botulinum* type E failed to grow below $a_w$ 0.97 when NaCl was used as a solute, but grew up to $a_w$ 0.94 when glycerol was the solute (Sperber, 1983). This is because glycerol enters freely inside the cell and thus does not cause as much osmotic stress as non-permeable NaCl, sucrose, and similar solutes.

Among microbial groups, halophiles, osmophiles, and xerophiles grow better at lower $a_w$ values. Halophiles, such as some *Vibrio* spp., need NaCl in varying amounts for growth. Osmophilic bacteria and xerophilic bacteria, yeasts, and molds grow at $a_w$<0.85, as they have no competition from bacteria. Optimum growth of most microorganisms in food occurs at $a_w$ greater or equal to 0.98. At $a_w$>0.98, Gram-negative bacteria, due to their faster growth rate, predominate if other needs for optimum growth are met. As the $a_w$ decreases to 0.97, Gram-positive bacteria, such as bacilli, lactobacilli, micrococci, and clostridia, will be predominant. Below $a_w$ 0.93, Gram-positive bacteria such as micrococci, staphylococci, enterococci, and pediococci, as well as yeasts and molds are able to grow. As the $a_w$ decreases below 0.86, osmophilic yeasts and xerophilic molds dominate. They will be able to grow in foods with $a_w$ up to 0.6; at an $a_w$ below 0.6, microbial growth stops (Sperber, 1983).

**Table 9. Minimal $a_w$ for microbial growth at optimum growth temperature**

| Microorganisms | | | |
|---|---|---|---|
| | $A_w$ | | $A_w$ |
| **Bacteria** | | **Yeasts** | |
| *Bacillus cereus* | 0.95 | *Saccharomyces cerevisiae* | 0.90 |
| *Bacillus stearothermophilus* | 0.93 | *Saccharomyces rouxii* | 0.62 |
| *Clostridium botulinum type A* | 0.95 | *Debaromyces hansenii* | 0.83 |
| *Clostridium botulinum type B* | 0.94 | | |
| *Clostridium botulinum type E* | 0.97 | **Molds** | |
| *Clostridium perfringens* | 0.95 | *Rhizopus nigricans* | 0.93 |
| *Escherichia coli* | 0.95 | *Penicillium chrysogenum* | 0.79 |
| *Salmonella spp.* | 0.95 | *Penicillium patulin* | 0.81 |
| *Vibrio parahaemolyticus* | 0.94 | *Aspergillus flavus* | 0.78 |
| *Staphylococcus aureus* | 0.86 | *Aspergillus niger* | 0.77 |
| *Penicillium fluorescens* | 0.97 | *Alternaria citri* | 0.84 |
| *Lactococcus viridescens* | 0.94 | | |

Adapted from Bibek (1996)

### *1.5.3. Preservatives*

Preservatives are used in food in relatively small amounts, either to kill undesirable microorganisms, to prevent or retard their growth. They vary greatly in their ability to act against different microorganisms. Preservatives that inhibit or retard microbial growth are called fungistatic or bacteriostatic. However, the allowable limits of preservatives used in foods, cannot completely kill all the microorganisms or prevent their growth for a long time during storage. There are two types of preservatives: chemical and natural.

The Code of Federal Regulations (CFR) defines a chemical preservative as "any chemical that tends to prevent or retard deterioration when added to food" (CFR, 1992). The concentrations of undissociated acid required to inhibit growth of most strains in microbiological media is illustrated in Table 10.

Natural preservatives are ingredients, such as cultured products, vinegar, or raisins. They are typically used in baked products marketed as "preservative-free" products. A good chemical or natural preservative must have several features, which ensures its acceptance and justifies its use as described by King (1981) in Table 11.

There is a wide variety of chemical preservatives being used in today's processed foods; however, not all preservatives are used in bakery products for suitability, and specificity purposes. The commonly used chemical preservatives in bakery products are calcium propionate, sodium propionate, sorbic acid, potassium sorbate, sodium diacetate, methylparaben, propylparaben, sodium benzoate, ethyl alcohol, and acetic acid.

**Table 10. Antimicrobial spectra of preservatives used in foods**

| Organic acids | Yeasts | Molds | *Enterobacteriacea* | *Micrococcaceae* | *Bacillaceae* |
|---|---|---|---|---|---|
| | **Minimum levels required for inhibition (% w/w)** | | | | |
| Acetic acid | 0.5 | 0.1 | 0.05 | 0.05 | 0.1 |
| Benzoic acid | 0.05 | 0.1 | 0.01 | 0.01 | 0.02 |
| Citric acid | >0.005 | >0.005 | >0.005 | 0.001 | >0.005 |
| Lactic acid | >0.01 | >0.02 | >0.01 | >0.01 | >0.03 |
| **Parabens:** | | | | | |
| Methyl | 0.1 | 0.1 | 0.2 | 0.4 | 0.2 |
| Ethyl | 0.1 | 0.05 | 0.1 | 0.1 | 0.1 |
| Propyl | 0.01 | 0.02 | 0.1 | 0.05 | 0.05 |
| Propionic acid | 0.2 | 0.05 | 0.05 | 0.1 | 0.1 |
| Sorbic acid | 0.02 | 0.04 | 0.01 | 0.02 | 0.02 |

Adapted from Chichester and Tanner (1972)

**Table 11. Characteristics of a good preservative**

| |
|---|
| Broad antimicrobial spectrum |
| Non-toxic to humans |
| Effective at low concentration |
| Little effect on pH |
| Free of odor, color, and flavor at levels used |
| Available in dry form |
| Water soluble |
| Non-corrosive |
| Stable during storage |
| No adverse effects on fermentation or loaf characteristics |
| Cost effective to use |

Adapted from King (1981)

#### *1.5.3.1. Chemical preservatives*

##### *1.5.3.1.1. Propionic acid and propionates*

Propionic acid and its salts, called propionates, were patented in 1939 as mold inhibitors. They have also been shown to be effective in controlling the rope-causing bacterium, *B. subtilis* (O'Leary and Kralovec, 1941). Although propionic acid has a higher antimicrobial activity, propionates are more commonly used in bakery products due to the fact that, unlike propionic acid, propionates are non-corrosive and possess only a slight odor which is not noticeable in baked goods. There are two types of propionates, sodium and calcium propionate. These are white, free flowing powders, and recently offered in low dust form. They are effective against molds, but have little activity against bacteria (with the exception of *B. subtilis*) and no activity against yeasts. The antimicrobial activity of calcium propionate is optimized at pH values between 4.5-6.0. They blend well with other baking ingredients and do not alter color, taste, volume, or baking time at normal use levels (Chichester and Tanner, 1972). Either calcium or sodium propionate can be used in yeast-leavened products; however, calcium propionate is preferred because of its contribution to nutritional enrichment, ease of handling, and cost effectiveness. The calcium ion can affect the leavening action of baking powder and other chemical-leavening agents. When sodium propionate is used, it replaces an equal weight of salt (Na chloride) in the formula (Hebeda and Zobel, 1996). Suggested levels of use of propionates in bakery products are shown in Table 12.

##### *1.5.3.1.2. Sorbic acid and sorbates*

Sorbic acid and its salts, called sorbates, are effective preservatives in a wide variety of foods. In baked goods, sorbic acid and potassium sorbate are utilized as antimicrobial agents. While potassium sorbate is substantially more water-soluble than sorbic acid, its potency is only 74% of the potency of sorbic acid on an equivalent weight basis. This means that 4 parts of potassium sorbate are needed for 3 parts of sorbic acid (Hebeda and Zobel, 1996). Sorbates are relatively tasteless and odorless and are not noticeable in baked goods at normal use levels.

Sorbic acid and sorbates have a broad-spectrum activity against yeasts and molds, but have little activity against bacteria, with the exception of *B. subtilis*. Sorbates are functional preservatives in foods of pH values ranging from 4-7 and with an optimum pH of 5.5-6, hence the antimicrobial activity of sorbates increases with an increase in acidity. Sorbates are twice as effective than propionates in inhibiting mold growth in bakery products, but have an adverse effect on yeast, reducing loaf volume and making dough sticky and difficult to process (Legan, 1993). This problem can be overcome by either spraying sorbate onto the product's surface after baking or mixing anhydrates of sorbic acid with fatty acids, such as palmitic acid. Moreover, sorboyl palmitate has also been successful in controlling mold growth without interfering in the fermentation process. The heat of the baking process hydrolyses sorboyl palmitate and releases sorbic acid which inhibits molds during storage (Sofos, 1989). The suggested levels of sorbates used in baked goods are shown in Table 13.

Sorbates can be applied to bakery products in different ways, including direct addition into the formulation, spraying onto the product as an aerosol, or incorporation into the packaging material (Sofos, 1989). The benefits of surface application of sorbate involve lowering yeast cost up to 20% in some systems, decreasing mixing time by 25%, reduced customer complaints due to spoilage in the home, reduced returns of unsold products, inhibition of mold growth, no adverse sensory qualities in the product (Monsanto Company, 1977).

**Table 12. Suggested use levels of propionates in baked goods**

| Product type | Propionate level oz/100 lb. flour |
|---|---|
| **Breads** | **Use calcium/sodium propionate** |
| White breads, buns, rolls, specialties | 2.5-5 |
| Dark bread, whole or cracked wheat rye breads, buns, rolls, etc. | 3-6 |
| English muffins | 6.5-12 |
| Pita bread | 4-5 |
| Tortillas/ soft tacos | 4-5 |

Adapted from Hebeda and Zobel (1996)

**Table 13. Suggested use levels of sorbates in baked goods**

| Product | Use level oz/100 lb. flour | Method of application |
|---|---|---|
| **Breads** | | |
| Breads, buns, rolls, etc. | 1-6 | Surface spraying |
| Tortillas | 1.25-3 | Dry blend |
| | **% of batter weight** | **Dry blend with:** |
| **Cakes** | | |
| Cheese cake | 0.1-0.3 | Sugar/milk powder |
| Chocolate cake | 0.1-0.3 | Flour |
| Fruit cake | 0.1-0.4 | Flour |
| **Pies** | | |
| Pie crusts | 0.05-0.1 | Flour/dough |
| Pie fillings | 0.05-0.1 | Flour/add and agitate after heating |

Adapted from Hebeda and Zobel (1996)

#### *1.5.3.1.3. Acetic acid and acetates*

Acetic acid is soluble in water and has a $pK_a$ value of 4.76. The main reasons for using it as a food preservative are its low cost, its availability, and its low toxicity. Salts of acetic acid, known as acetates, not only have antimicrobial activity, but they can be also used as acidulants, flavoring agents, and sequesterants. Purified acetic acid is corrosive, solid at about 60°F, and dangerous since its vapors are flammable and harmful if inhaled. For these reasons, it is always better to work with acetates in food products. It has been found that acetic acid has a greater activity against yeasts and bacteria than molds. Moreover, acetic acid and acetates have a greater antimicrobial activity as the pH is lowered, due to increasing quantities of the undissociated acid. In baked goods, sodium diacetate is preferred over acetate, because of its effectiveness against rope formation. However, in order to prevent interference with the leavening of yeasts, 0.4 % is usually used.

#### *1.5.3.1.4. Sodium benzoate*

Sodium benzoate has been used for over 80 years now and has been affirmed as Generally Recognized as Safe (GRAS). Sodium benzoate is more effective against bacteria and yeasts than molds, and has an antimicrobial activity that is optimized at a pH range of 2.5-4.0. This characteristic limits its use in bakery products to fruit fillings, jams, and jellies. In cases where lower sodium levels are required in a food product, potassium benzoate is used instead. Potassium benzoate can replace sodium benzoate in almost every application. However, to obtain the same efficacy as sodium benzoate, approximately 10% more potassium benzoate should be used (Hebeda and Zobel, 1996).

#### *1.5.3.1.5. Parabens*

These are alkyl esters of *p*-hydroxybenzoic acid and have been widely used in pharmaceutical products and cosmetics for many years. It was found that they could be used in some food products as antimicrobial agents. This is mainly due to the similarity of parabens to benzoic acid and their effectiveness over a broad pH range. They are more effective against molds and yeasts, but less effective against bacteria. However, molds and yeasts are more responsible for spoilage in an acidic environment, where they could be controlled by other less expensive antimicrobial agents.

The most common forms of parabens which are widely used in food products are methyl and propyl-parabens. They are both regarded as GRAS, and have a white color and present in the form of a free-flowing powder. Methyl-paraben has a faint color and an objectionable medicinal taste, while propyl-paraben is odorless. The use of parabens in baked goods is functionally limited, due to their activity against yeast that act as leavening agents for the dough. Basically, parabens are used as antimicrobial agents in cakes (particularly fruitcakes) pie crusts, non-yeast pastries, icings, fruit jellies and toppings at levels ranging from 0.01 to 0.1%.

### *1.5.3.2. Natural preservatives*

#### *1.5.3.2.1. Cultured products*

Cultured products are produced by fermenting either wheat or whey with food-grade bacterial cultures. Microorganisms produce different organic acids, such as propionic acid, acetic, and lactic acid as by-products of fermentation. The fermentation solution is neutralized to prevent the evaporation of the volatile organic acids, converted to their salt forms and then dried. The higher cost incurred by using natural cultured products, instead of chemical agents, has made their use uneconomical. Moreover, higher amounts must be applied when using natural antimicrobial agents to obtain a similar efficacy. For example, 15 g of a 20% natural calcium propionate is equivalent to 3 g of calcium propionate.

Nisin, a bacteriocin produced by *Lactococcus lactis,* has been widely used as a food preservative for many years now. It is accepted as safe for food in many countries and regarded as GRAS in the United States. Nisin has proved to be effective against Gram-positive bacteria, especially the spore formers such as *Bacillus and Clostridium* spp. In vegetative cells, nisin acts as a surface-active cationic detergent, destabilizes the cytoplasmic membrane. In spores, it allows them to germinate but inhibits outgrowth of the pre-emergent spore. In general, spore coats of *Bacillus* species, which are opened by mechanical pressure, are more sensitive to nisin than species whose spore coats are opened by lysis. At a molecular level, nisin modifies the sulfhydryl groups in the envelopes of germinated spores (Doyle *et al.*, 1997). Nisin is more soluble and stable at pH levels < 4, and usually loses its antimicrobial activity at pH levels >8. Nisin's efficacy is also dependent on the ease of its molecular dispersion in food systems, which is greatly affected by the lipid content and nature of lipid distribution in a food.

Food in which nisin is used include pasteurized processed cheese products, dairy desserts, cured meat and high moisture baked products, such as crumpets and pikelets, and canned foods where there is a need to control the outgrowth of bacterial spores (Jenson *et al.*, 1994). When added to crumpet batter at levels of 3.75 μg/g, nisin restricted the growth of naturally occurring *B. cereus* during ambient storage to levels below those capable of causing food poisoning (Jenson *et al.,* 1994). It was also found that the growth of 12 psychrotrophic strains of *B. cereus* in skim milk was inhibited by 6.25 μg at 7°C while 2 strains could grow in the presence of 1.25 μg (Dufrene *et al.*, 1995).

### *1.5.4. Irradiation*

It should be noted that, microorganisms vary greatly in their sensitivity to irradiation. For instance, molds are more sensitive than yeasts, which are more sensitive than bacterial cells; bacterial cells in turn are more sensitive than viruses. Among bacteria, Gram-negative are more sensitive than Gram-positive, and rods are more sensitive than cocci. Spores are quite resistant to irradiation, and this is mainly due to their low water content. Among the spore formers, spores of *Clostridium botulinum* type A and *Bacillus pumilus* are probably the most resistant to irradiation. Generally, *Bacillus* spores (aerobes) are more sensitive than *Clostridium* spores (anaerobes). Irradiation is most commonly used in foods like roast beef, pork, fresh fish, and gravy meals, to improve their microbiological and sensory quality. However, it is not a commercially viable method of control for bakery products.

### *1.5.5. Modified atmosphere packaging (MAP)*

Modified atmosphere packaging or MAP has become increasingly popular as a preservation method to extend the mold-free shelf-life of bakery products. MAP has been defined as "the enclosure of a food product in a high gas barrier film in which the gaseous environment has been changed or modified to slow respiration rate, reduce microbial growth, and retard enzymatic spoilage with the intent of extending shelf-life" (Young *et al.*, 1988). MAP technology has been more successful in the European countries than in North America. Currently, the United Kingdom is the leader in MAP technology, accounting for about half of the European market, followed by France which has approximately 25% of the MA packaged food market. As for North America, MAP is still in its infancy. This is mainly due to customer/regulatory concerns about the safety of this technology. Several factors have played a major role in the growth of MAP in Europe and these are:

#### *1. Developments in new polymeric barrier packaging materials*

New developments in polymer chemistry have produced packaging films with different barrier properties to air, water vapor, and gases. Also, the production of laminates has allowed variable heat sealability and strength as well as permeability characteristics. This has allowed food to be packaged/maintained under MA conditions (Smith and Simpson, 1996).

### 2. *Extended market areas*

Recent marketing strategies emphasize the importance and necessity of globalization. The aim of the bakery industry is to satisfy the needs and demands of consumers on a regional basis. However, with new trends in marketing, it is essential that the bakery industry targets consumers on a national and even international basis. To achieve these goals, food packaging must be done in a way to help extend a product's shelf-life. MAP food can achieve such goals. A good example is Forecrest Foods of Calgary, Alberta, which produced crumpets. Prior to gas packaging, this product had a mold-free shelf-life of ~4 days. However, the mold-free shelf-life could be extended to 28-35 days through reformulation and gas packaging in 60% $CO_2$. This allowed the distribution of these crumpets all around Canada and into U.S. markets. Consequently, sales of crumpets quadrupled (Smith and Simpson, 1996).

### 3. *Consumer concerns about preservatives*

Many consumers nowadays are concerned about the use of preservatives in food and are demanding preservative-free food. The use of MAP is an alternative to preservatives and the gases used in MAP ($CO_2$, $N_2$) are all natural (Smith and Simpson, 1996).

### 4. *Increasing energy costs*

The conventional methods of preservation, such as freezing, can control microbial growth, enzymatic spoilage as well as mold growth. However, it is energy consuming. It has been estimated that MAP can reduce energy consumption by 18-20 % (Aboagye *et al.*, 1986).

### *5. Consumer perception of MAP*

A recent consumer survey conducted by Agriculture-Food Canada, on consumers' perception of MAP, indicated that Canadian consumers favored MAP technology over other methods of preservation and were willing to pay a premium for MAP food products (Smith and Simpson, 1996).

### *1.5.5.1. Methods for atmosphere modification*

Atmosphere modification process can be sub-divided into two categories: (1) Passive modification, and (2) Active modification.

### *1. Passive modification*

In commodity-generated or passive modification, the food product alters the original composition of the gases in the enclosed atmosphere. Usually, passive modification is suitable for fruits and vegetables since they respire and continue to do so even after harvesting. However, a critical factor in passive modification is the choice of packaging material.

Each film has certain characteristics which are suited for a specific kind of food and packaging requirements. In order to achieve such task, the film must allow $O_2$ to enter the package at a rate similar to its consumption by the product. Similarly, $CO_2$ must be allowed to escape of the product to offset the production of $CO_2$ by the product. It is important to maintain such balance to prevent rapid spoilage of the product. The most commonly used films for packaging of fresh produce include, low density polyethylene (LDPE) or polypropylene (PP). LDPE has higher gas permeability values while PP has greater clarity. However, both films are relatively inexpensive.

***2. Active modification***

To actively modify the atmosphere of a package, several methods can be employed. These include vacuum packaging, gas packaging (injection of gas mixtures), $O_2$ absorbents, $CO_2$ generators, and ethanol vapor generators.

### *1.5.5.1.1. Vacuum packaging*

Vacuum packaging is used in muscle foods to prevent the growth of aerobic spoilage microorganisms, including *Pseudomonas* and *Alteromonas* species. The product is packaged in a film of low $O_2$ permeability and then air is removed under vacuum. Under good vacuum conditions, oxygen in the package headspace is reduced to <1%, and carbon dioxide increases to ~10-20%. Vacuum packaging is not a suitable technology for most soft bakery products due to its crushability effect. However, it has been used in shortbread, a product with a hard texture.

### *1.5.5.1.2. Gas packaging*

Altering the gas composition of the atmosphere surrounding a food can result in an extension of its shelf-life. Gases used in packaging mainly include nitrogen and carbon dioxide.

Nitrogen is an inert gas which has no effect on microbial activity. It is used to prevent package collapse and oxidation of fats i.e., in potato chips, or oxidative rancidity in low $a_w$ bakery products and snacks.

Many gases can exert an antimicrobial effect namely, carbon dioxide, carbon monoxide, ozone, ethylene oxide, propylene oxide, nitrous oxide, and sulfur dioxide. Of these gases, only carbon dioxide is safe enough to be used in food for reasons of stability, toxicity, organoleptic quality, and economic feasibility.

Carbon dioxide is the most important gas used in gas packaging of bakery products. It is considered both bacteriostatic and fungistatic since it controls both mold and bacterial growth. Moreover, it can prevent the growth of insects in packaged and stored food products. It is highly soluble in water and fats, where it forms carbonic acid, which may result in a reduction of a product's pH (Smith and Simpson, 1996). Furthermore, $CO_2$ could be absorbed by the food product and cause package collapse. Mixtures of nitrogen and carbon dioxide are used to overcome this problem. The type of film used to package the food is also important in helping prevent the partial vacuum which is formed when the film is more permeable to $CO_2$ than to air e.g., laminated films based on nylon.

Carbon dioxide can inhibit the growth of many microorganisms and its inhibitory effect increases with increasing concentration. It interferes with various enzymatic and biochemical pathways necessary for metabolism; hence it affects growth of microorganisms.

The antimicrobial effect of $CO_2$ is dependent on the number and the type of microorganism present in food. It is therefore most effective against aerobic microorganisms. Anaerobic bacteria, such as *C. botulinum,* are not inhibited by high concentrations of $CO_2$.

Molds have different sensitivity to $CO_2$. Molds which contaminate products of low $a_w$ i.e., fruitcakes are more susceptible to $CO_2$ than *Penicillium* species, which predominate in products of higher $a_w$. Indeed, some species of *Penicillium*, e.g., *P. roquefortii*, are very resistant to the effects of carbon dioxide.

With respect to bacteria, $CO_2$ prevents the growth of aerobic spore forming bacteria in bread i.e., the rope causing *B. subtilis.* The inhibition is more related to the acidic environment created by $CO_2$ rather than its antimicrobial activity. It is possible for a facultative anaerobe which is acid tolerant i.e., *B. licheniformis,* to grow and cause rope spoilage even in the presence of carbon dioxide. Secondary problems can also occur in MAP products due to lactic acid bacteria. Ooraikul (1982) reported problems of swelling of the package caused by the growth of lactic acid bacteria in crumpets. To overcome this problem, products were refrigerated to prevent the growth of lactic acid bacteria prior to MAP. The advantages and disadvantages of gas packaging of food are shown in Table 14.

### *1.5.5.1.3. Oxygen scavengers*

Gas packaging can protect a food from deterioration by replacing oxygen inside the package. However, no matter how impermeable to oxygen the package is, oxygen can still be found in the package headspace due to (a) permeability of the packaging material; (b) ability of the food to trap air; (c) leakage through poor sealing; (d) inadequate gas flushing. For these reasons, oxygen absorbents or oxygen scavengers are used inside the package to maintain an oxygen-free environment.

Oxygen absorbents can be defined as "a range of chemical compounds introduced into the MAP package (not the product) to alter the atmosphere within the package" (Agriculture and Agri-Food Canada, 1990). They were developed in Japan by Mitsubishi Gas Chemical Co., and marketed under the name of Ageless (Smith *et al.*, 1990). Fine iron powder is placed inside a sachet (like a desiccant). The sachet material is highly permeable to oxygen and hence allows the iron inside the sachet to become oxidized and form non-toxic iron oxide i.e., rust. A range of oxygen absorbents can be employed according to the shelf-life desired. Some act rapidly, others slowly; some are used with moist food, others with dry; and some have a dual effect, such as oxygen absorption and $CO_2$ generation (Harima, 1990). The types and properties of Ageless oxygen absorbents are shown in Table 16. Several studies have shown that oxygen absorbents can extend the shelf-life of food. For instance, the mold-free shelf-life of white bread, packaged in polypropylene film, could be extended from 5 days to 45 days at room temperature by incorporating an oxygen absorbent sachet into the package. Pizza crust, which has a mold-free shelf-life of 2 days at 30°C, can be kept mold-free for more than 14 days at this temperature using appropriate oxygen absorbents (Smith *et al.*, 1990).

Some of the advantages and disadvantages of oxygen absorbents are listed in Table 15.

**Table 14. Advantages and disadvantages of gas packaging of food**

| Advantages | Disadvantages |
|---|---|
| ❑ Increased shelf-life | ❑ Initial high cost of films, equipment |
| ❑ Increased market area | ❑ Discoloration of meat pigments |
| ❑ Reduction in production/storage costs | ❑ Leakage |
| ❑ Improved presentation | ❑ Fermentation and swelling |
| ❑ Fresh appearance | ❑ Potential growth of organisms of public health significance |
| ❑ Clear view of the product | |
| ❑ Easy separation of slices | |

Adapted from Smith *et al.* (1990)

**Table 15. Advantages and disadvantages of oxygen absorbents**

| Advantages | Disadvantages |
|---|---|
| ❑ Inexpensive and simple to use | ❑ Free air-flow around sachet is needed |
| ❑ Non-toxic and safe to use | ❑ Cause package collapse |
| ❑ Prevent aerobic microbial spoilage | ❑ May promote growth of *C. botulinum* |
| ❑ Extend shelf-life of product | |
| ❑ Prevent rancidity/off-flavors in fats and oils | ❑ May cause flavor changes in:<br>▪ High moisture food<br>▪ High fat foods |
| ❑ Maintain flavor by preventing oxidation of flavor compounds | ❑ Consumer resistance to sachet use in packages. Possible misuse of the sachet |
| ❑ Maintain quality without additives | |
| ❑ Increase product shelf-life | |
| ❑ Increase distribution radius | |
| ❑ Reduce distribution losses | |
| ❑ Replace chemical pesticides to prevent insect damage of foods | |

Adapted from Smith *et al.* (1990)

**Table 16. Types and properties of Ageless oxygen absorbents**

| Type | Function | Moisture status | Water activity | Absorption speed (Day) |
|---|---|---|---|---|
| Z | Decreases $O_2$ | Self reacting | < 0.65 | 0.5-2 |
| S | Decreases $O_2$ | Self reacting | > 0.65 | 0.5-2 |
| SS | Decreases $O_2$ | Self reacting | > 0.85 | 2-3 (0-4°C)<br>10 (-25°C) |
| FX | Decreases $O_2$ | Moisture dependent | > 0.85 | 0.5-1 |
| FM | Decreases $O_2$ | Moisture dependent | > 0.85 | 0.5-1 |
| E | Decreases $O_2$<br>Increases $CO_2$ | Self reacting | <0.3 | 3-8 |
| G | Decreases $O_2$<br>Increases $CO_2$ | Self reacting | 0.3-0.5 | 1-4 |
| SE | Decreases $O_2$ /increases ethanol | Moisture dependent | >0.85 | 1-2 |

Adapted from Smith and Simpson (1996)

#### *1.5.5.1.4. Ethanol vapor generators*

A novel method of atmosphere modification is using ethanol vapor generators. Ethanol vapor generators release ethanol vapor into the headspace of the package to act as antimicrobial compound. The sachets contain absorbed or encapsulated ethanol in a carrier material and placed in a packaging film of selective permeability, thus allowing slow or rapid release of ethanol. They are sold under the trade name of Ethicap or Antimold 102 and are manufactured by Freund Co. Ltd., in Japan (Freund Technical Information, 1985). Ethicap consists of food grade alcohol (55%) by weight adsorbed on to silicon powder (35%) and contained in a sachet made of a copolymer of paper/ethyl vinyl acetate copolymer. Some sachets also have flavor compounds such as vanilla, in order to mask the odor of alcohol. A newly marketed type by the same company is called Negamold (dual function). Negamold absorbs oxygen from the headspace and releases ethanol. The size of the sachet varies from 0.6 to 6 g and is determined depending on many factors such as, (a) weight of food, (b) $a_w$ of food, and (c) desired shelf-life of the product. For instance, if the $a_w$ of a product is 0.95 and a shelf-life of (1-2 weeks) is desired, then a 2G size of Ethicap should be used for 100 g of product. For a longer shelf-life, a larger size Ethicap is used (4G) (Freund Technical Information, 1985). The types of alcohol generators manufactured are illustrated in Table 17.

When food is packaged along with an Ethicap sachet, moisture is absorbed from the food, ethanol vapor is generated from encapsulation and permeates into the package headspace. The amount of ethanol in the package headspace is a function of both the sachet size, and water activity of the product (Smith *et al.,* 1987). Ethicap is extensively used to extend the shelf-life of cakes and high moisture bakery products. For instance, a 5-20 times extension in mold-free shelf-life was observed for high- $a_w$ cakes depending on the size of Ethicap used (Freund Technical Information, 1985). Furthermore, results have shown that Ethicap exerts an anti-staling effect as well as an antimycotic effect (Freund Technical Information, 1985). Most of the studies conducted on Ethicap have concentrated on its use as an antimycotic agent. However, few studies have dealt with its potential to control food spoilage and food poisoning bacteria. Seiler and Russell (1993) reported that 1% ethanol by product weight delayed the onset of 'rope' caused by the growth of *B. subtilis*. They also reported that low concentrations of ethanol inhibited bacterial growth in both whipping cream and custard i.e., known vectors of food poisoning bacteria in filled bakery products. The advantages and disadvantages of ethanol vapor generators are outlined in Table 18.

Ethanol vapor generator technology is one of the most innovative "interactive packaging" technologies which shows promise to extend the shelf-life of food products and control microorganisms of public health significance. Ethanol vapor generators have also been shown to control at least 10 species of mold, including *Aspergillus* and *Penicillium;* 15 species of bacteria including *Staphylococcus, Salmonella,* and *E coli,* as well as three species of spoilage yeasts (Freund Technical Information, 1985).

**Table 17. Types of ethanol vapor generators**

| Type | Function | Application | $A_w$ of product |
|---|---|---|---|
| Ethicap | Generates ethanol vapor | Moisture dependent cakes/bread | > 0.85 |
| Negamold | Absorbs $O_2$/generates ethanol vapor | Moisture dependent cakes/bread | > 0.85 |

Adapted from Freund Technical Information (1985)

**Table 18. Advantages and disadvantages of ethanol vapor generators**

| Advantages | Disadvantages |
|---|---|
| ❑ Ethanol vapor can be generated without having to spray ethanol directly onto product surface prior to packaging | ❑ Absorption of ethanol from the package headspace into the product |
| ❑ Sachets can be conveniently removed from the package and discarded at the end of the storage period | |
| ❑ Eliminates the need for preservatives such as benzoates and sorbates to control yeast spoilage | |
| ❑ Controls mold spoilage and delay staling in bakery products | |

Adapted from Smith *et al.* (1987)

### *1.6. Objectives of research*

Bakery products, especially high moisture products such as crumpets, are very susceptible to mold spoilage within a few days at room temperature. The bakery industry is constantly exploring new techniques to extend the mold-free shelf-life and safety of baked products, while preserving organoleptic qualities. To date, food preservatives (potassium sorbate and calcium propionate) are added to achieve this objective. Most of these preservatives are effective at relatively low pH values i.e., below 6 and are of little value in controlling microbial spoilage in high pH products i.e., > 7. However, few studies have been done to control the growth of *B. cereus,* a pathogen implicated in several food poisoning outbreaks from the consumption of crumpets, a high moisture bakery product. Thus, the objectives of this research were:

1. To determine the effect and optimal levels of $a_w$, pH, bacteriocins, and preservatives alone, and in combination with each other, on the growth of *Bacillus cereus* in a model broth/agar system.

2. To determine the effect of modified atmosphere packaging (MAP) involving gas flushing, oxygen absorbents, and ethanol vapor generators on the growth of *B. cereus* in model agar systems.

3. To extrapolate the data obtained from 1 and 2 to extend the shelf-life and safety of English-style crumpets in challenge studies with *B. cereus.*

# Chapter 2

## Studies to Control the Growth of *B. cereus In Vitro*

### *2.1. Introduction*

Extensive studies have not been carried out on the control of *B. cereus*, despite the fact it is a microorganism of public health concern and it has been implicated in numerous foodborne illnesses associated with the consumption of bakery products (Jenson *et al.*, 1994). Several factors influence the growth of *B. cereus* including pH, water activity ($a_w$), antimicrobial agents, and gaseous atmosphere (Baird-Parker *et al.*, 1996).

Antimicrobial agents are chemical compounds which are either naturally present e.g., organic acids in citrus fruits, or are formed during processing e.g., lactic acid, or synthetically produced and added as generally regarded as safe (GRAS) ingredients to bakery products. The most commonly used chemical preservatives in bakery products are sorbates and propionates. Although sorbates are more effective against molds than against bacteria, they can inhibit the growth of *B. cereus* at acidic pH (5-6). Calcium propionate is widely used in bakery products, e.g., bread and rolls, since it does not affect the leavening process and the calcium contributes to the enrichment of the product (Hebeda and Zobel, 1996). Calcium propionate has been found to delay the germination of *B. cereus* spores and their subsequent growth and multiplication (Kaur, 1986).

$A_w$ and pH are also two important parameters influencing the growth and survival of bacteria. The majority of bacteria contaminating foods grow better at higher $a_w$ and basic pH; therefore, maintaining these two parameters below optimal levels could be used to inhibit the growth of *B. cereus* in bakery products.

Numerous studies have been carried out to examine the effect of combining preservatives and physical measures on the growth of microorganisms. This is commonly referred to as the "hurdle technology", a concept developed by Leistner (Bibek, 1996). The concept states that "several hurdles (inhibitory factors), even if individually unable to inhibit the growth of microorganisms, will nevertheless together prevent microorganisms' growth if incorporated into a substrate in sufficient number and height".

### *2.1.1. Objectives*

The objectives of this initial study were to determine the effect of $a_w$, pH, and chemical inhibitors, alone and in combination with each other, on the growth of *B. cereus* in broth.

## *2.2. Materials and methods*

### *2.2.1. Microorganism and inoculum preparation*

A strain of *B. cereus*, previously isolated from fish nugget ingredients (breadcrumbs and spices), was obtained from Health Canada, Ottawa, ON. The culture was grown overnight in Tryptic Soy Broth (TSB) (Difco, Michigan, USA) and then streaked onto polymyxin egg yolk mannitol bromothymol blue agar (PEMBA) plates (Oxoid LTD., Hampshire, England) and incubated at 30°C for 24 h to check for purity. Cultures grown on PEMBA were transferred every three weeks to ensure viability.

### *2.2.2. Growth curve*

The inoculum was prepared for the growth curve by transferring a loopful of pure culture from the PEMBA plates into 5 ml of TSB + 0.6% yeast-extract (TSBYE) (Difco, Michigan, USA) followed by incubation at 30°C for 24 h.

Appropriate dilutions were then made from the stock solution, using 9 ml of sterile peptone water (0.1% w/v) to give a working suspension of $10^3$ CFU/ml. One ml of this suspension was transferred to tubes containing 9 ml of TSBYE, which were incubated at 30°C. Tubes of TSBYE were monitored for growth every hour for ~16 hours by measuring the optical density (OD) using a spectrophotometer (Ultrospec 1000, Pharmacia Biotech. Cambridge, England) set at a wavelength 625 nm. Counts at each OD were made by preparing serial dilutions, again using 0.1% sterile peptone water, followed by surface plating onto Tryptic Soy Agar plates (TSA) (Difco, Michigan, USA). Plates were incubated at 30°C for approximately 24 h and colonies were enumerated using a Darkfield Quebec Colony Counter (AO Scientific Instruments, Quebec, Canada). Counts were expressed as Colony Forming Units per ml (CFU/ml).

### 2.2.3. Factors influencing growth of *B. cereus* ($a_w$, pH, and chemical inhibitors)

#### 2.2.3.1. Effect of water activity ($a_w$)

A preliminary study was done in a model broth system to determine the effect of $a_w$ on the growth of *B. cereus*. TSBYE was again used as the growth medium and appropriate amounts of glycerol and NaCl salt were added, on a weight per weight basis (% w/w), to reduce $a_w$ from 0.99 to 0.90 (Tables 19-20). The $a_w$ of the prepared media was checked using a Decagon $a_w$ meter (Decagon Device Inc., Pollman, Washington, USA) previously calibrated with a saturated solution of sodium chloride ($a_w$ 0.75). All measurements were carried out at ambient temperature (25°C) and had an accuracy of ± 0.1%. The $a_w$ adjusted media were then autoclaved at 121°C for 15 min and allowed to cool at room temperature. Tubes were inoculated, in duplicate, with 1 ml of the *B. cereus* stock suspension ($10^3$ CFU/ml) using an Eppendorf automatic pipette (Brinkmann Instruments Inc., Ontario, Canada). All tubes were then incubated at 30°C and checked for turbidity and OD at 625 nm daily for up to 35 d. Enumeration of *B. cereus* at each OD reading was monitored on PEMBA plates, as described previously. All plates were incubated aerobically at 30°C for 24 h and counts expressed as CFU/ml.

### *2.2.3.2. Effect of pH*

Tubes containing TSBYE were adjusted to pH 5, 6, 7, 8, and 9, using either 85% (v/v) lactic acid or 1N NaOH (v/v) as shown in Table 21. Following pH adjustment, the broths were autoclaved (15 min at 121°C), cooled to room temperature, and random samples checked for pH using a previously calibrated pH meter (Model 220, Corning Glassworks, New York, USA). All pH adjusted broths were then inoculated aseptically in a laminar flow cabinet (Labconco Corporation, Missouri, USA) with a fresh culture of *B. cereus* ($10^3$ CFU/ml), incubated aerobically at 30°C and checked daily for turbidity and OD as described previously.

### *2.2.3.3. Effect of potassium sorbate (KS) and calcium propionate (CP) at various pH levels*

The pH of broths was adjusted to pH 5, 6, 7, 8, and 9 as described previously. Appropriate amounts of KS (K&K Laboratories, Division of ICN Biomedicals Inc., Ohio, USA) and CP (ICN Biomedicals Inc., Ohio, USA) were added to give final concentrations ranging from 0 to 1000 ppm at increments of 250 ppm (Table 22). All media were then sterilized, cooled to room temperature and aseptically inoculated with $10^3$ CFU/ml of *B. cereus* as described previously. Inoculated broths were then incubated at 30°C for 24 h and checked for turbidity/OD at 625 nm daily for up to 35 d.

**Table 19. $A_w$ adjustment of TSBYE using glycerol**

| Weight of glycerol (g) | Weight of broth (g) | $A_w$ reading | Temperature (°C) |
|---|---|---|---|
| 32 | 68 | 0.90 | 25.1 |
| 31 | 69 | 0.91 | 21.3 |
| 29 | 71 | 0.92 | 22.0 |
| 26 | 74 | 0.93 | 21.8 |
| 23 | 77 | 0.94 | 20.7 |
| 20 | 80 | 0.95 | 25.5 |
| 17 | 83 | 0.96 | 25.3 |
| 11 | 89 | 0.97 | 24.9 |
| 5 | 95 | 0.98 | 24.0 |
| 3 | 97 | 0.99 | 25.1 |

**Table 20. $A_w$ adjustment of TSBYE using NaCl**

| Weight of NaCl (g) | Weight of broth (g) | $A_w$ reading | Temperature (°C) |
|---|---|---|---|
| 13.5 | 86.5 | 0.90 | 21.5 |
| 12.0 | 88.0 | 0.91 | 21.8 |
| 10.5 | 89.5 | 0.92 | 22.2 |
| 10.0 | 90.0 | 0.93 | 22.3 |
| 8.3 | 91.7 | 0.94 | 22.2 |
| 6.5 | 93.5 | 0.95 | 21.4 |
| 5.0 | 95.0 | 0.96 | 21.7 |
| 4.5 | 95.5 | 0.97 | 21.8 |
| 2.5 | 97.5 | 0.98 | 21.7 |
| 1.0 | 99.0 | 0.99 | 22.0 |

**Table 21. Levels of lactic acid or NaOH used to adjust pH of TSBYE**

| pH | 85% Lactic acid (ml) | 1N NaOH (ml) |
|---|---|---|
| 5 | 0.32 | N/A[a] |
| 6 | 0.21 | N/A |
| 7 | 0 | 0 |
| 8 | N/A | 1.01 |
| 9 | N/A | 3.01 |

[a] Not applicable

**Table 22. Levels of KS and CP added to 100 ml TSBYE to obtain final concentrations of KS and CP ranging from 0-1000 ppm**

| Weight of KS (g) | Weight of CP (g) | Final concentration (ppm) |
|---|---|---|
| 0 | 0 | 0 |
| 0.025 | 0.025 | 250 |
| 0.05 | 0.05 | 500 |
| 0.075 | 0.075 | 750 |
| 0.1 | 0.1 | 1000 |

### *2.2.3.4. Combined effect of $a_w$, pH, and CP, on the growth of B. cereus in TSBYE at 30°C*

***Experimental Design***

To determine the combined effect of $a_w$, pH, and CP on the growth of *B. cereus* in broth, a $2^K+2k+n$ Central Composite Rotatable design (CCRD) was used (Box *et al.*, 1978). The design consisted of 3 factors, each at 5 levels. The levels of each factor used in this study included $a_w$ (0.95-0.99), pH (5-9), and CP concentrations (0-1000 ppm). CP was chosen mainly since it is widely used in bakery products, it does not affect the leavening process, and previous studies by Kaur (1986) showed that CP delayed the germination and growth of *B. cereus* spores. The levels of these three factors were chosen based on the results of previous studies. The CCRD design and coded levels of each factor used in this design are shown in Table 23. Variable levels were coded –2, -1, 0, +1, +2, to facilitate statistical analysis. The coded and actual values of levels used in CCRD are shown in Table 24.

**Table 23. Coded level combinations for a 3 variable Central Composite Rotatable Design (CCRD) to control the growth of *B. cereus***

| Run # | $A_w$ | pH | Inhibitor Level |
|---|---|---|---|
| 1 | -1 | -1 | -1 |
| 2 | +1 | -1 | -1 |
| 3 | -1 | +1 | -1 |
| 4 | +1 | +1 | -1 |
| 5 | -1 | -1 | 1 |
| 6 | +1 | -1 | 1 |
| 7 | -1 | +1 | 1 |
| 8 | +1 | +1 | 1 |
| 9 | $-\alpha(-2)$ | 0 | 0 |
| 10 | $+\alpha(+2)$ | 0 | 0 |
| 11 | 0 | $-\alpha(-2)$ | 0 |
| 12 | 0 | $+\alpha(+2)$ | 0 |
| 13 | 0 | 0 | $-\alpha(-2)$ |
| 14 | 0 | 0 | $+\alpha(+2)$ |
| 15 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 |

**Table 24. Values of coded levels used in the CCRD**

| Variables | Levels | | | | |
|---|---|---|---|---|---|
| | -2 | -1 | 0 | +1 | +2 |
| ***$A_w$*** | 0.95 | 0.96 | 0.97 | 0.98 | 0.99 |
| pH | 5 | 6 | 7 | 8 | 9 |
| CP[a] (ppm) | 0 | 250 | 500 | 750 | 1000 |

[a] Calcium propionate

### *2.3. Results and discussion*

#### *2.3.1. Growth curve of B. cereus*

The growth curve of *B. cereus* is shown in Figure 2. Maximum growth (~$10^8$ CFU/ml) was reached after 14h at 30°C. These results are in agreement with Al-Zenki (1996) who observed that the maximum growth of *S. enteritidis* was reached after ~12h at 30°C. The relationship between OD and bacterial counts at each OD was determined using linear regression analysis and is shown graphically in Figure 3. There was a high correlation between OD and growth ($R^2$=0.9105) indicating that OD could be used as a reliable indicator to predict the maximum growth of *B. cereus*. Hence, OD was subsequently used throughout this study to determine the time taken to reach a maximum OD of 1.4 at 625 nm and a count of ~$10^8$ CFU/ml.

#### *2.3.2. Inhibitory effect of water activity*

The effect of $a_w$ on the growth of *B. cereus* at pH 7±0.2 and 30°C is shown in Tables 25-26. This study was conducted to test the inhibitory effect of $a_w$ alone without altering the pH of the media. Glycerol was chosen to reduce the $a_w$ since it has been shown to have no antimicrobial effect per se (Smith *et al.*, 1987). Inhibition of growth was proportional to the amount of glycerol added i.e., as $a_w$ was reduced, *B. cereus* growth was delayed. In broth of $a_w$ 0.99 (pH 7), a maximum OD of 1.4 at 625 nm was observed after 1 d at 30°C. However, when $a_w$ was reduced from 0.99 to 0.93, the growth of *B. cereus* was delayed and a maximum OD of 1.4 at 625 nm was observed after 3 d at 30°C. When the $a_w$ was further reduced ($a_w$<0.92), no growth was observed for >35 d (Table 25).

Almost identical results were observed in broth adjusted with NaCl. Growth occurred at $a_w$ 0.99 after 1 d at 30°C. However, when the $a_w$ was further reduced ($a_w$ 0.93), no growth was observed (Table 26).

The results of this preliminary study confirm the work of Marshall *et al.* (1971). They reported that *B. cereus* did not grow at $a_w$ 0.93 when NaCl was used to reduce the $a_w$ of the broth. However, when glycerol was used to reduce $a_w$, growth of *B. cereus* occurred at $a_w$ 0.93 but not at $a_w$ 0.92. However, studies conducted in food systems showed different results. Bryan *et al.* (1981), observed that rice samples from Cantonese-style restaurants, with an $a_w$ as low as $a_w$ 0.912, supported the growth of *B. cereus*. However, in food, moisture can migrate resulting in pockets of $a_w$ which may be sufficiently high to encourage growth.

**Figure 2. Growth curve of *B. cereus***

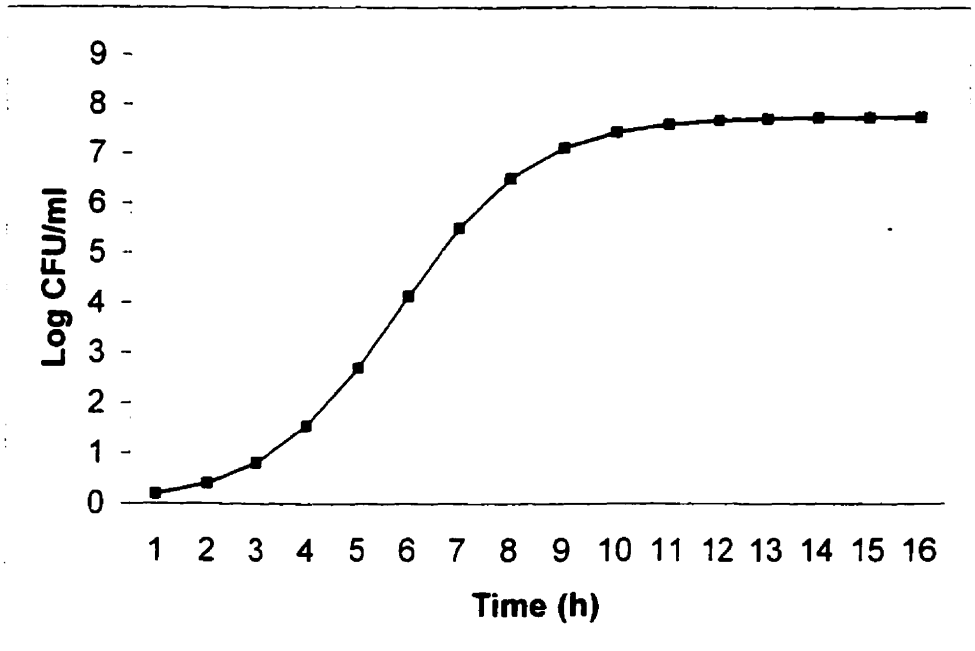



**Figure 3. Growth of *B. cereus* versus optical density @ 625 nm**

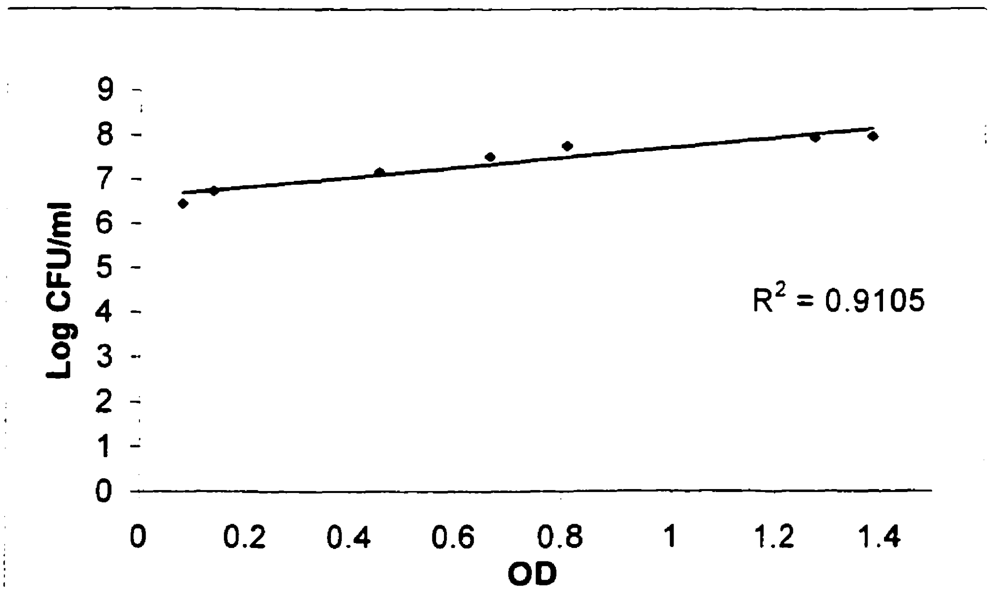


**Table 25. Effect of $a_w$, adjusted by glycerol, on the growth of *B. cereus* in TSBYE**

| $A_w$ | Maximum OD @ 625 nm | Time (day) to reach maximum OD |
|---|---|---|
| 0.99 | 1.4[a] | 1 |
| 0.98 | 1.4 | 1 |
| 0.97 | 1.4 | 2 |
| 0.96 | 1.4 | 2 |
| 0.95 | 1.4 | 2 |
| 0.94 | 1.4 | 2 |
| 0.93 | 1.4 | 3 |
| 0.92 | 0[b] | >35 |
| 0.91 | 0 | >35 |
| 0.90 | 0 | >35 |

[a] Maximum OD corresponding to a ~$10^8$ CFU/ml

[b] Minimum OD - no growth

**Table 26. Effect of $a_w$, adjusted by NaCl, on the growth of *B. cereus* in TSBYE**

| $A_w$ | Maximum OD @ 625 nm | Time (day) to maximum OD |
|---|---|---|
| 0.99 | 1.4 | 1 |
| 0.98 | 1.4 | 1 |
| 0.97 | 1.4 | 1 |
| 0.96 | 1.4 | 1 |
| 0.95 | 1.4 | 1 |
| 0.94 | 1.4 | 2 |
| 0.93 | 0 | >35 |
| 0.92 | 0 | >35 |
| 0.91 | 0 | >35 |
| 0.90 | 0 | >35 |

### *2.3.3. Inhibitory effect of pH*

The effect of pH on the growth of *B. cereus* in TSBYE is shown in Table 27. The optimum pH for growth of *B. cereus* in broth is pH 7, as the maximum OD was reached in 1 d at 30°C. However, growth was only slightly inhibited at pH levels between 6 to 9 as shown by the time to reach the maximum OD (1.5-3 d, Table 27). In broth adjusted to pH 5, growth of *B. cereus* was inhibited for >35 d at 30°C. However, it was not obvious if pH caused a lethal or a sublethal injury to the vegetative cells, i.e., if effect of pH was bacteriostatic or bactericidal. These results are in agreement with those previously reported by Chorin *et al.* (1997) who examined the growth of *B. cereus* in a model broth system using various strains of *B. cereus*. They reported that no growth was observed at pH 5, even at various water activities. However, at pH >5, growth was observed at all water activities investigated.

**Table 27. Effect of pH on the growth of *B. cereus* in TSBYE ($a_w$ 0.99) incubated at 30°C**

| pH | Time (day) to reach max OD @625 nm |
|---|---|
| 5 | >35 |
| 6 | 3 |
| 7 | 1 |
| 8 | 1-2 |
| 9 | 3 |

### *2.3.4. Antimicrobial effect of CP at various pH levels*

The antimicrobial effect of CP at pH 5 to 9 is shown in Table 28. The growth of *B. cereus* at pH 7 to 9 was noticeable after 1 d at 30°C, regardless of the levels of CP used. This lack of inhibition can be attributed to the total dissociation of CP at higher pH which prevents the acid from entering the cell membrane freely and interfering with cell metabolism. The antimicrobial activity of CP at pH 5 and 6 is also shown in Table 28. At pH 6, growth of *B. cereus* was observed after 1 d in control tubes and after only 2 d for all other levels of CP. This again confirms that the inhibitory effect of CP was pH dependent. Complete inhibition of *B. cereus* was observed at pH 5 - even with low levels of CP (250 ppm). However, it is not clear whether CP played a role in this inhibition since the growth of *B. cereus* was delayed for 35 d at this pH without CP (Table 28).

### *2.3.5. Antimicrobial effect of KS at various pH levels*

The effects of various concentrations of KS (0-1000 ppm) on the growth of *B. cereus* at pH 5 to 9 at 30°C are shown in Table 29. The results were similar to those observed for CP in the previous study (see section ***2.3.4***). Growth of *B. cereus* at pH 6 to 9 was observed after 1 d at 30°C. Again, this is due to the high pH and the dissociation of KS which is more effective at pH levels <6.5 (Sofos, 1989). Furthermore, KS is mostly effective against mold and yeast growth and has little or no effect on bacterial cells (with the exception of *B. subtilis*). As pH decreased to pH 6, the antimicrobial activity of KS was enhanced and the growth of *B. cereus* was delayed for ~2 d, regardless of KS concentration. At pH 5, growth of *B. cereus* was completely inhibited. Again, this could not be attributed to the effect of KS since this pH alone inhibited the growth of *B. cereus*.

It is evident that the antimicrobial activity of both CP and KS is pH dependent and decreases as the pH of the medium increases i.e., as it further moves away from its dissociation constant ($pK_a$ 4.76). This confirms the work conducted by Lahellec *et al.* (1981) on the growth effect of sorbate and selected antioxidants on toxigenic strains of *St. aureus*. Growth of *St. aureus* was inhibited at pH 5 in 50 ml Brain Heart Infusion (BHI) (Difco, Michigan, USA) by 1% (w/v) potassium sorbate. However, at pH 7, growth of *St. aureus* occurred, even in the presence of 5% (w/v) KS.

### 2.3.6. Combined effect of $a_w$, pH, and CP on the growth of *B. cereus* in TSBYE incubated at 30°C

In the previous studies the effect of each of $a_w$, pH, and level of inhibitor on the growth of *B. cereus* were examined one at a time. Although, hurdles used to inhibit bacterial growth are usually more effective when used in combination with each other. The combined effects of $a_w$, pH, and CP on the growth of *B. cereus* in TSBYE are shown in Table 30. The growth of *B. cereus* was visible in most treatment combinations after 1 d at 30°C. However, inhibition of growth was observed in only one treatment i.e., Run #11 which had an $a_w$ 0.97, a pH of 5, and 500 ppm CP (Table 30). In general, two or more environmental conditions will have a more inhibitory effect when combined than each parameter considered separately (hurdle technology). In this study, it is evident that no inhibition was observed even when several hurdles were used to control the growth of *B. cereus*. The inhibition observed in only one of the 16 treatments was mainly attributed to the inhibitory effect of low pH (pH 5) alone, and not to the other two factors namely, $a_w$, and CP concentration, since at higher pH levels (with similar levels for $a_w$, and CP), no inhibition was observed.

In general, mathematical models (first and second order polynomials) are generated to determine the optimal levels of the most significant factors to give a desired response (Box *et al.*, 1978). Since there was insufficient variation in the results, statistical analysis could not be done to quantify the combined effect of $a_w$, pH, and CP concentration on the growth of *B. cereus*.

**Table 28. Antimicrobial effect of CP on the growth of *B. cereus* in TSBYE at pH 5-9 and incubated at 30°C**

| Level of CP (ppm) | Time (day) to reach max OD @ 625 nm | | | | |
|---|---|---|---|---|---|
| | pH | | | | |
| | 5 | 6 | 7 | 8 | 9 |
| 0 | >35 | 1 | 1 | 1 | 1 |
| 250 | >35 | 2 | 1 | 1 | 1 |
| 500 | >35 | 2 | 1 | 1 | 1 |
| 750 | >35 | 2 | 1 | 1 | 1 |
| 1000 | >35 | 2 | 1 | 1 | 1 |

**Table 29. Antimicrobial effect of KS on the growth of *B. cereus* in TSBYE at pH 5-9 and incubated at 30°C**

| Level of KS (ppm) | Time (day) to reach max OD @ 625 nm | | | | |
|---|---|---|---|---|---|
| | pH | | | | |
| | 5 | 6 | 7 | 8 | 9 |
| 0 | >35 | 1 | 1 | 1 | 1 |
| 250 | >35 | 1 | 1 | 1 | 1 |
| 500 | >35 | 2 | 1 | 1 | 1 |
| 750 | >35 | 2 | 1 | 1 | 1 |
| 1000 | >35 | 2 | 1 | 1 | 1 |

**Table 30. Combined effect of $a_w$, pH, and CP on the growth of *B. cereus* in TSBYE and incubated at 30°C**

| Run # | Factors | | | Time (day) to reach max OD @ 625 nm |
|---|---|---|---|---|
| | $A_w$ | pH | CP (ppm) | |
| 1 | 0.96 | 6 | 250 | 1 |
| 2 | 0.98 | 6 | 250 | 1 |
| 3 | 0.96 | 8 | 250 | 1 |
| 4 | 0.98 | 8 | 250 | 1 |
| 5 | 0.96 | 6 | 750 | 1 |
| 6 | 0.98 | 6 | 750 | 1 |
| 7 | 0.96 | 8 | 750 | 1 |
| 8 | 0.98 | 8 | 750 | 1 |
| 9 | 0.95 | 7 | 500 | 1 |
| 10 | 0.99 | 7 | 500 | 1 |
| **11** | **0.97** | **5** | **500** | **NG[a]** |
| 12 | 0.97 | 9 | 500 | 1 |
| 13 | 0.97 | 7 | 0 | 1 |
| 14 | 0.97 | 7 | 1000 | 1 |
| 15 | 0.97 | 7 | 500 | 1 |
| 16 | 0.97 | 7 | 500 | 1 |

[a] No visible growth after 35 d at 30°C

### *2.4. Conclusion*

In conclusion, the use of $a_w$ as a means of food preservation is not new. Glycerol and NaCl were both tested for their inhibitory effect on the growth of *B. cereus* with NaCl being slightly more inhibitory than glycerol. However, this study has shown that $a_w$ is not a viable method to control the growth of *B. cereus* since large amounts of salt or glycerol would be required for complete inhibition of this pathogen and this may severely affect the organoleptic and textural qualities of the food.

Most processed food products on the market have a low pH (more acidic) to increase their shelf-life and to ensure the safety of the food product. This study has shown that the growth of *B. cereus* was inhibited by low pH (pH 5) - with or without preservatives. However, many bakery products e.g., English-style crumpets, are formulated to pH levels >8 to enhance color. Hence, other methods need to be considered to control the growth of *B. cereus* in such high pH products.

# Chapter 3

# Further Studies to Control the Growth of *B. cereus In Vitro*

### *3.1. Introduction*

The introduction of new high-barrier plastic packaging materials has resulted in the development of new preservation methods of food products, such as modified atmosphere packaging (MAP). The desire of consumers for fresh, ready-to-eat, and preservative-free foods, has drastically increased the demand for MAP products which has become one of the most prominent methods of food preservation.

The objective of MAP is to control or reduce the growth of undesirable microorganisms in food. In addition, MAP retards enzymatic and respiratory activity of fresh foods, hence, extending the shelf-life and reducing spoilage losses. Most of the studies related to MAP have focused on determining the optimum gas mixture ($CO_2$; $O_2$; $N_2$) for specific products (Devlieghere *et al.*, 1998). The most important component of the gas mixture is $CO_2$ due to its antimicrobial activity (Devlieghere *et al.*, 1998).

MAP technology has been applied to different food products including meat, poultry, bakery products and vegetables. However, few studies have examined the effect of MAP on the growth of pathogenic bacteria i.e., *B. cereus* in bakery products. Daifas *et al.* (1998) reported that MAP packaged bakery products supported the growth of proteolytic strains of *C. botulinum*, regardless of the atmosphere in the packaged product.

Ethanol, or ethyl alcohol ($C_2H_5OH$), has been used as an antimicrobial agent since alcoholic fermentation was employed to preserve fruits (Shelef and

Seiter, 1993). Ethanol is bactericidal at high concentrations (60-75%) and has an inhibitory activity at lower concentrations (5-20%). However, it has been reported that ethanol is not sporicidal (Banwart, 1989).

Lactic acid bacteria are well known for their production of antimicrobial proteins and peptides called bacteriocins. Nisin, an antibacterial polypeptide, is produced by some strains of *Lactococcus lactis* subsp. *lactis*. Nisin has been widely used in many countries and is generally regarded as safe (GRAS) in the U.S. (Jenson *et al.*, 1994). It is effective in controlling the growth of Gram-positive bacteria; however, it is not inhibitory against yeasts, fungi, or Gram-negative bacteria (Hurst and Hoover, 1993). Foods in which nisin is used as a preservative include pasteurized processed cheese products and canned foods (Jenson *et al.*, 1994).

Ultra-violet light is a physical method used to control the growth of undesirable microorganisms. UV-light has been shown to be lethal in the near 260 nm region by forming thymine dimers on bacterial DNA, thereby blocking further DNA replication. It has been demonstrated that UV-light is effective in killing microorganisms contaminating the surfaces of a variety of materials (Kuo *et al.*, 1997). In addition, UV-light has been applied to various food products e.g., bakery products, to prevent mold growth (Smith, 1993). Stermer *et al.* (1987) reported that UV-light was effective in reducing the bacterial load on the surface of fresh meat. Bank *et al.* (1991) also showed that *Salmonella* growing on agar plates were susceptible to UV-light.

Numerous studies have been carried out on the antifungal and bactericidal properties of fatty acids and their esters. It has been found that the activity of various esters can be altered by the environment in which their potency is measured. For instance, the antimicrobial activity of esters was markedly reduced in the presence of proteins which was attributed to the formation of the detergent-protein complex (Kabara, 1978).

### *3.1.1. Objectives*

The objectives of this study were to determine the effect of modified atmosphere packaging (MAP), ethanol vapor generators (alone and in combination with gas flushing), UV-light, bacteriocins, and mono-lauryl maleate on the growth of *B. cereus* in model broth and agar systems.

## *3.2. Materials and methods*

### *3.2.1. Inoculum and media preparation*

A stock suspension of *B. cereus* was prepared as described previously (see section ***2.2.2***). Appropriate dilutions of this stock suspension were made using 0.1% sterile peptone water to obtain a working suspension of $10^3$ CFU/ml. *B. cereus* media or PEMBA plates were prepared, sterilized at 121°C for 15 min, supplemented with *B. cereus* selective supplement (SR009E Oxoid Ltd., Hampshire, England), and stored in the dark according to the manufacturer's specifications. The pH of the media was maintained at pH 7.2±0.2.

### *3.2.2. Effect of MAP on the growth of B. cereus in PEMBA*

To determine the effect of MAP on the growth of *B. cereus*, PEMBA plates were inoculated with $10^3$ CFU/ml of *B. cereus* using a spread plate technique. All plates were packaged in duplicate, in 210 x 210 mm high gas barrier Cryovac bags ($O_2$ transmission rate: 3-6 cc/m$^2$, 24 h, 1 atm at 4.4°C, 0% RH) obtained from Cryovac Sealed Air Corporation Mississauga, Ontario. The packaged plates were divided into two sets. The first set was packaged using the following gaseous atmospheres: Air, 25%$CO_2$, 50%$CO_2$, 75%$CO_2$, 100%$CO_2$ (balance $N_2$). Gas Packaging was done using a Multivac chamber type, heat seal packaging machine (Model KM100-3M, Wolfertschwenden, Germany). The desired gas mixture was obtained using a Smith's proportional gas mixer (Model 299-028, Tescom Corp., Minneapolis, USA). The second set of plates was packaged under similar gaseous conditions. However, prior to gas flushing, an Ageless Type $FX_{100}$ oxygen absorbent (Mitsubishi Gas Co., Tokyo, Japan) was taped to the inside of each package, and packages were gas flushed as described previously. All packages were incubated at 30°C and checked daily for visible signs of *B. cereus* growth for up to 35 d.

### *3.2.3. Effect of ethanol vapor generators alone, and in combination with 100% $CO_2$ on the growth of B. cereus*

In this study, inoculated PEMBA plates were packaged in duplicate, in Cryovac bags as described previously (see section ***3.2.1***). The packages were then divided into two sets. In the first set, plates were packaged with either 2, 4, or 6G Ethicap®, a commercially available ethanol vapor generator (Freund Industrial Co. Ltd., Tokyo, Japan), or 200, 400, or 600S sachets of Negamold® (Freund Industrial Co. Ltd., Tokyo, Japan). In the second set, PEMBA plates were packaged with either 3, or 6G Ethicap® and 100% $CO_2$ and 300 or 600S Negamold® and 100% $CO_2$. All sachets were taped to the inside of each package prior to gas flushing and sealing with an impulse heat sealer (E82163 Tish-300. Abtec Inc. Ohio, USA). Packages were stored at 30°C, and checked daily for visible signs of growth of *B. cereus* for up to 35 d.

### *3.2.4. Headspace gas analysis*

Headspace gas ($CO_2$, $O_2$, and $N_2$) analysis was done on all gas packaged and oxygen absorbent packaged PEMBA plates. Gas samples were withdrawn using a 0.5 ml gas-tight Pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, Louisiana, USA) through a septum previously attached to the outside of each package. Headspace gas was analyzed with a Varian gas chromatograph (Model 3400, Varian Inc., Ontario, Canada), fitted with a thermal conductivity detector (TCD), and using Porapak Q (80-100 mesh) and Molecular sieve 5 A

(80-100 mesh) columns in series (Supelco, Canada Ltd.). Helium was used as carrier gas at a flow rate of 30 ml/min. The column oven was set at 60°C, the injector at 100°C, and the detector filament at 170°C. Resultant peaks of $CO_2$, $O_2$, and $N_2$ were recorded using a Hewlett Packard integrator (Model 3390A, Hewlett Packard Co., Avondale, USA).

### *3.2.5. Headspace ethanol*

Headspace ethanol was done using a Varian gas chromatograph fitted with a flame ionization detector (FID), and a Nukol column (30M x 0.53mm) (Supelco, Canada Ltd.). Helium was again used as the carrier gas at a flow rate of 30 ml/min. The column temperature was set at 60°C and the injector port temperature at 100°C. Resultant peaks of ethanol were recorded on a Hewlett Packard integrator, and the concentrations of ethanol were extrapolated from an existing standard curve.

A standard ethanol curve was made from 80% (v/v) stock solution of ethanol. Standard ethanol solutions of: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, and 10.0% (v/v) were prepared from this stock solution. These standard solutions were placed in 250 ml volumetric flasks and wrapped with parafilm to prevent the escape of ethanol vapor. Prior to measuring the headspace, a septum was placed on the parafilm and 0.5 ml of each standard solution was injected into the Varian chromatograph as described previously. The results of the average of 3 injections, from each standard solution, were used to generate a standard curve of area versus ethanol concentration shown in Figure 4.

### *3.2.6. Effect of UV-light on the growth of B. cereus in PEMBA*

To determine the effect of UV-light on the growth of *B. cereus*, PEMBA plates were swabbed with a suspension of *B. cereus* equivalent to 0.5 McFarland standard suspension (Remel, Lenexa, Kansas, USA). All plates were then exposed, uncovered, to UV-light (intensity of UV-light ~620$\mu W/cm^2$) in the laminar flow cabinet, for 1, 2, 3, 5-10, 20, and 30 min (Philips UV, TUV 30w/G30 $T_8$, Holland) at a distance of 50 cm above the inoculated plates. In the second study, the distance between the plates and the UV lamp was only 2 cm and the selected time intervals were 10, 20, 30 sec, and 1, 2, 3, 4, 6, 8, and 10 min. All UV treated plates were incubated at 30°C and checked daily for visible signs of *B. cereus* growth for up to 35 d.

### 3.2.7. Effect of nisin, Alta, and Per/Lac on the growth of B. cereus in TSBYE

Alta 2341 is a pediocin which is produced by *Pediococcus acidlactici.* The commercial product is produced by culturing savory flavor precursors such as, yeast extract, corn syrup, and vegetable protein. This is achieved using a food grade fermentation process (Quest Int. Technical Information). Perlac 1911 is produced by *Lactococcus lactis* and is derived from the fermentation of glucose solids. It is a multifunctional food ingredient which improves flavor, water retention, and shelf-life of various foods (Quest Int. Technical Information). To determine the effect of nisin, Alta, and Per/Lac on the growth of *B. cereus*, the pH of tubes containing TSBYE was adjusted to pH 5.5, 6, 7, 8, and 9 as described previously (see section ***2.2.3.2***). Appropriate amounts of nisin (Nisaplin, Applin & Barett LTD., Technical Dept., Dorset, England) with an activity of $10^6$ International unit per gram (IU/g), were added aseptically on a weight per volume basis (%w/v) to the sterilized broths to give final concentrations of 0-500 ppm at increments of 100 ppm (Tables 31). Similarly, Alta and Per/Lac (Quest International Ltd., Quebec, Canada) were added at concentrations of 0-500, and 0-10000 ppm (as per manufacturer's recommendations) respectively (Table 32). All broths were inoculated with 0.1 ml of a working suspension of *B. cereus* to give a final inoculum of $10^2$ CFU/ml. Broths were then incubated at 30°C and checked daily for turbidity/OD for up to 35 d. Growth of *B. cereus* was confirmed on PEMBA plates as described previously.

### *3.2.8. Effect of mono-lauryl maleate (MLM) on the growth of B. cereus in TSBYE*

MLM is a solid, long chain ester (16 C) with an active *cis* form (McGill University, Food Science and Agricultural Chemistry, Quebec, Canada). To determine the effect of MLM on the growth of *B. cereus*, the pH of TSBYE was adjusted to pH 8 and pH 9 using 1N NaOH as described previously. Concentrations of 0.1%, 0.2%, and 0.3% of MLM (%w/v) were added to the broths to obtain final concentrations of 1000, 2000 ppm, and 3000 ppm respectively (Table 33). All broths were autoclaved (121°C for 15 min), and inoculated with 0.1 ml of a working suspension of *B. cereus* to give a final inoculum of $10^2$ CFU/ml. Broths were then incubated at 30°C, and checked for turbidity/OD daily for up to 35 d. Growth of *B. cereus* was again confirmed on PEMBA plates as described previously.

**Figure 4. Standard curve of headspace ethanol vapor**

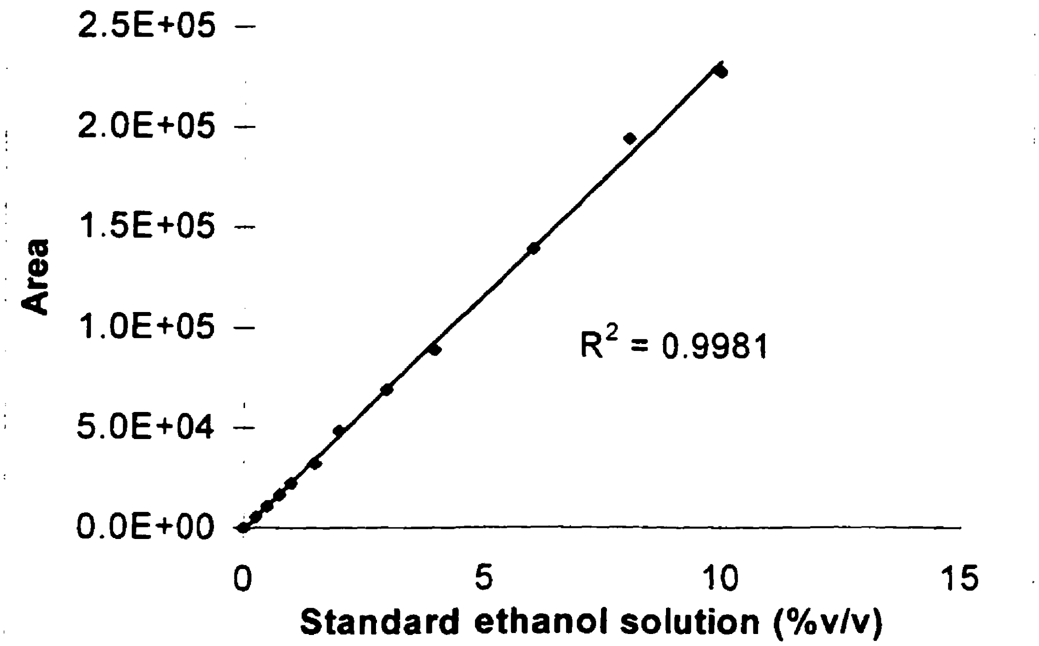


**Table 31. Levels of nisin added to 100 ml TSBYE**

| Levels of nisin (g) | Final concentration (ppm) |
|---|---|
| 0 | 0 |
| 0.01 | 100 |
| 0.02 | 200 |
| 0.03 | 300 |
| 0.04 | 400 |
| 0.05 | 500 |

**Table 32. Levels of Alta and Per/Lac added to 100 ml TSBYE**

| Levels of Alta and Per/Lac (g) | Final concentration (ppm) |
|---|---|
| 0 | 0 |
| 0.01 | 100 |
| 0.02 | 200 |
| 0.03 | 300 |
| 0.04 | 400 |
| 0.05 | 500 |
| 0.30 | 3000 |
| 0.50 | 5000 |
| 1 | 10000 |

**Table 33. Levels of MLM added to 100 ml TSBYE**

| Levels of MLM (g) | Final concentration (ppm) |
|---|---|
| 0 | 0 |
| 0.1 | 1000 |
| 0.2 | 2000 |
| 0.3 | 3000 |

### *3.3. Results and discussion*

#### *3.3.1. Effect of MAP on the growth of B. cereus in PEMBA at 30°C*

The effect of MAP on the growth of *B. cereus* in PEMBA plates is shown in Table 34. In all air-packaged plates, growth of *B. cereus* was observed after 1 d at 30°C. Colonies on PEMBA plates were large and blue in color due to the presence of bromothymol blue pH indicator. Growth also occurred in gas packaged plates after 1-2 d at 30°C regardless of the $CO_2$ concentration (Table 34). Colonies were smaller in size and the media was yellow in color which was attributed to the lower pH caused by dissolution of $CO_2$ in the media. These results are in partial agreement with the study of Bennik *et al.* (1995) who reported no significant difference in lag times for *B. cereus* packaged under various gaseous atmospheres. They observed that growth of *B. cereus* was slightly reduced in 20% $CO_2$ compared to 0% $CO_2$.

Similar results were observed in plates packaged with Ageless FX oxygen absorbents i.e., growth occurred after 1 d at 30°C, under all gaseous atmospheres (Table 35). These results show that *B. cereus* is capable of growing under reduced oxygen levels even in the presence of high $CO_2$.

#### *3.3.2. Effect of ethanol vapor generators alone, and in combination with 100% $CO_2$, on the growth of B. cereus in PEMBA at 30°C*

The effect of ethanol vapor on the growth of *B. cereus* is shown in Table 36. Growth of *B. cereus* was observed in all treatments after 1 d at 30°C. However, the effect of Negamold was more inhibitory than Ethicap. Unlike Negamold, which has a dual action, Ethicap produces only ethanol vapor (Freund Technical Information, 1985); therefore, headspace $O_2$ in packages containing Ethicap was higher (Table 36). Furthermore, studies in our laboratory have shown that higher levels of ethanol vapor had a plasticizing effect on the film structure and could influence its oxygen transmission rate (OTR). It was found that as the size of the sachet increased (hence the level of ethanol vapor), the OTR of the film increased (El-Khoury, 1999). This explains the increase in headspace $O_2$ observed in packages with larger Negamold sachets (Table 36). These results are in agreement with those observed by Notermans *et al.* (1990) who studied the effect of EtOH on the growth of *B. cereus* in an eggnog-like product. Their results showed that growth of *B. cereus* occurred at 22°C, even in the presence of 7% EtOH.

The effect of ethanol vapor generators, combined with 100% $CO_2$, on the growth of *B. cereus* is shown in Table 37. This study was conducted to determine the required sachet size (in conjunction with 100% $CO_2$) which could inhibit the growth of *B. cereus* in PEMBA at 30°C. As shown in Table 37, the most effective treatment was Negamold (NM) 600S and 100% $CO_2$ followed by NM 300S. The growth of *B. cereus* was delayed for over 35 and 5 d at 30°C, respectively. However, Ethicap did not have similar inhibitory activity since the growth of *B. cereus* was observed after only 2 d at 30°C, regardless of the sachet size. These results are in agreement with those reported by Smith *et al.* (1987) who showed that the growth of *Saccharomyces cerevisiae* was inhibited in potato dextrose agar (PDA) plates when a combination of $a_w$ 0.90 and 1.52% (v/v) ethanol vapor was used. They concluded that ethanol vapor does exert an antimicrobial effect on yeast growth and activity and that the concentration of headspace ethanol required for inhibition was dependent on the water activity of the system.

**Table 34. Effect of gas packaging on the growth of *B. cereus* in PEMBA plates stored at 30°C**

| Packaging conditions | Days to growth | Headspace @ time of growth | | |
|---|---|---|---|---|
| | | $CO_2$ % | $O_2$ % | $N_2$ % |
| Air | 1 | 5.1 | 14.85 | N/A[a] |
| 25% $CO_2$[b] | 1 | 20.9 | 3.75 | N/A |
| 50%$CO_2$ | 1 | 31.6 | 7.35 | N/A |
| 75%$CO_2$ | 1 | 71.4 | 0.2 | N/A |
| 100%$CO_2$ | 1-2 | 97.1 | 0.5 | N/A |

[a] Not available

[b] Balance $N_2$

**Table 35. Combined effect of gas packaging and an oxygen absorbent Ageless (FX) on the growth of *B. cereus* in PEMBA plates stored at 30°C**

| Packaging conditions | Days to growth | Headspace @ time of growth | | |
|---|---|---|---|---|
| | | $CO_2$ % | $O_2$ % | $N_2$ % |
| Air + Ageless FX | 1 | 0.8 | 0 | N/A |
| 25% $CO_2$+ Ageless FX | 1 | 9.8 | 0 | N/A |
| 50% $CO_2$+ Ageless FX | 1 | 33.1 | 0 | N/A |
| 75% $CO_2$+ Ageless FX | 1 | 55.1 | 0 | N/A |
| 100%$CO_2$ + Ageless FX | 1-2 | 97.0 | 0.4 | N/A |

**Table 36. Effect of Ethicap® and Negamold® on the growth of *B. cereus* in PEMBA at 30°C**

| Packaging conditions | Days to growth | Headspace @ time of growth | | | |
|---|---|---|---|---|---|
| | | Ethanol (% v/v) | $CO_2$ % | $O_2$ % | $N_2$ % |
| $EC_2$ | 1 | 6.0 | 5.3 | 14.1 | N/A |
| $EC_4$ | 1 | 6.3 | 6.1 | 12.8 | N/A |
| $EC_6$ | 1 | 7.9 | 5.7 | 13.3 | N/A |
| $NM_2$ | 1 | 1.3 | 1.1 | 0.8 | N/A |
| $NM_4$ | 1 | 8.1 | 1.4 | 0.6 | N/A |
| $NM_6$ | 1 | 11.2 | 0.6 | 3.9 | N/A |

**Table 37. Effect of Ethicap® and Negamold®, in combination with 100% $CO_2$, on the growth of *B. cereus* in PEMBA at 30°C**

| Packaging conditions | Days to growth | Headspace @ time of growth | | | |
|---|---|---|---|---|---|
| | | Ethanol (% v/v) | $CO_2$ % | $O_2$ % | $N_2$ % |
| $EC_3$ +100%$CO_2$ | 2 | 0.8 | 94 | 0.9 | N/A |
| $EC_6$ +100%$CO_2$ | 2 | 1.4 | 95 | 1.0 | N/A |
| $NM_3$ +100%$CO_2$ | 5 | 4.7 | 92 | 0.9 | N/A |
| $NM_6$ +100%$CO_2$ | >35 | 6.9 | 93 | 1.0 | N/A |

### *3.3.3. Effect of UV-light on the growth of B. cereus in PEMBA plates stored at 30°C*

The effect of UV-light on the growth of *B. cereus* in PEMBA plates is shown in Tables (38-39). Growth of *B. cereus* was observed in all plates exposed to UV-light for 10 min or less at a distance of 50 cm from the UV source. However, for exposure times of >10 min, complete inhibition of *B. cereus* was observed.

In the second study, placing plates at a distance of 2 cm from UV source, growth of *B. cereus* was detected in plates exposed to UV-light for <2 min. However, for exposure times of >2 min, no growth was observed. Therefore, distance from the UV source appeared to influence the exposure time necessary to inhibit the growth of *B. cereus*. However, when the agar medium was cut and added to BHI broth, growth occurred within 1 week, indicating that the antimicrobial effect of UV-light was bacteriostatic rather than bactericidal. Similar results were reported by Kuo *et al.* (1997) who found that counts of *S. typhimurium*, in brilliant green agar (BGA) plates decreased by 6 log units after exposure to UV-light for 1 min or longer. They also reported that there was no growth in plates exposed to UV-light for >10 min.

**Table 38. Effect of UV-light (50 cm from UV source) on the growth of *B. cereus* in PEMBA at 30°C**

| UV treatment (min) | Growth detection |
|---|---|
| 0 | +[a] |
| 1 | + |
| 2 | + |
| 3 | + |
| 5 | + |
| 6 | + |
| 7 | + |
| 8 | + |
| 9 | + |
| 10 | -[b] |
| 20 | - |
| 30 | - |

[a] *B. cereus* growth occurred after 24h at 30°C

[b] No growth observed at 30°C for 35 d

**Table 39. Effect of UV-light (2 cm from UV source) on the growth of *B. cereus* in PEMBA at 30°C**

| UV treatment (min) | Growth detection |
|---|---|
| 0.17 | + |
| 0.34 | + |
| 0.50 | + |
| 1 | + |
| 2 | - |
| 3 | - |
| 4 | - |
| 6 | - |
| 8 | - |
| 10 | - |

### *3.3.4. Effect of nisin on the growth of B. cereus in TSBYE at 30°C and various pH levels*

The effect of nisin on the growth of *B. cereus* in TSBYE at 30°C is shown in Table 40. The growth of *B. cereus* at pH 7-9 was observed after 1 d at 30°C, regardless of the concentration of nisin. This could be attributed to pH since at neutral or alkaline pH, nisin becomes practically insoluble and inactivated, even at room temperature. Rayman *et al.* (1981) reported that the effectiveness of nisin decreased with increasing pH. They observed that as the pH increased to pH>7, less nisin remained in solution. Hall (1966) reported that the solubility of nisin was ~ 4% at pH 5. However, at pH 7 and 8 nisin became practically insoluble.

At pH 6, the antimicrobial activity of nisin was noticeably enhanced and the growth of *B. cereus* was inhibited at all concentrations.

At pH 5.5, complete inhibition was observed for over 35 d at 30°C. At this pH, the solubility and antimicrobial activity of nisin was also enhanced. Furthermore, this effect was due to the antimicrobial effect of nisin alone since growth of *B. cereus* was detected at pH 5.5 in all control tubes. Therefore, nisin would appear to act in a similar manner to organic acids i.e., it is effective at acidic but ineffective at neutral or alkaline conditions.

**Table 40. Effect of nisin on the growth of *B. cereus* in TSBYE at 30°C and various pH levels**

| Nisin levels (ppm) | Time (day) to reach max OD @ 625 nm | | | | |
|---|---|---|---|---|---|
| | pH | | | | |
| | 5.5 | 6 | 7 | 8 | 9 |
| 0 | 1 | 1 | 1 | 1 | 1 |
| 100 | >35 | >35 | 1 | 1 | 1 |
| 200 | >35 | >35 | 1 | 1 | 1 |
| 300 | >35 | >35 | 1 | 1 | 1 |
| 400 | >35 | >35 | 1 | 1 | 1 |
| 500 | >35 | >35 | 1 | 1 | 1 |

### *3.3.5. Effect of Alta and Per/Lac on the growth of B. cereus in TSBYE at 30°C and various pH levels*

The effect of Alta and Per/Lac on the growth of *B. cereus* in TSBYE is shown in Table 41. At pH 5.5-9, the growth of *B. cereus* was observed after 1 d at 30°C, regardless of the concentration of either Alta or Per/Lac. Despite being active only against Gram-positive bacteria, pediocins are usually active against cells closely related to the producer strain (Green *et al.*, 1997). Furthermore, at pH levels greater than pH 5.5, the inhibitory activity of these pediocins decreased dramatically, even at high concentrations (3000-10000 ppm). Lower pH levels (5.5-6) did not enhance the antimicrobial activity of either Alta or Per/Lac, since the cell counts were still >$10^9$CFU/ml.

### *3.3.6. Effect of MLM on the growth of B. cereus in TSBYE at pH 8-9*

The effect of MLM on the growth of *B. cereus* is shown in Table 42. Most chemical preservatives used in the previous studies i.e., sorbates and propionates, were ineffective in controlling the growth of *B. cereus* at alkaline pH (pH>7). Thus, in order to overcome this problem, it was necessary to choose a chemical preservative, which is known for its antimicrobial properties at alkaline conditions i.e., phenolic esters or monoesters. Some of these esters have been shown to exert an antimicrobial activity at a wider pH range (3-8) i.e., parabens, than most of the other organic acid preservatives, which are usually effective at pH<6.5 (Russell and Gould, 1991). Hence, MLM was chosen and tested for its antimicrobial activity against the growth of *B. cereus* in TSBYE at 30°C and pH 8-9. MLM inhibited the growth of *B. cereus* in broth at alkaline conditions for > 35 d at 30°C. MLM is insoluble in water at room temperature, partially soluble in tris-HCl buffer (pH 9), and completely soluble in TSBYE after autoclaving for 15 min at 121°C. It should be noted that the antimicrobial activity of MLM was not altered by heat (121°C, 15 min). Therefore, based on this initial study, MLM would appear to be an effective antimicrobial agent to control the growth of *B. cereus*, particularly at high pH levels (>7).

Table 41. Effect of Alta & Per/Lac on the growth of *B. cereus* in TSBYE at 30°C

| | Time (day) to reach max OD @ 625 nm | | | | |
|---|---|---|---|---|---|
| | pH | | | | |
| | 5.5 | 6 | 7 | 8 | 9 |
| **Alta levels (ppm)** | | | | | |
| 0 | 1 | 1 | 1 | 1 | 1 |
| 100 | 1 | 1 | 1 | 1 | 1 |
| 200 | 1 | 1 | 1 | 1 | 1 |
| 300 | 1 | 1 | 1 | 1 | 1 |
| 400 | 1 | 1 | 1 | 1 | 1 |
| 500 | 1 | 1 | 1 | 1 | 1 |
| | 1 | 1 | 1 | 1 | 1 |
| **Per/Lac levels (ppm)** | | | | | |
| 0-500 | 1 | 1 | 1 | 1 | 1 |
| 3000 | 1 | 1 | 1 | 1 | 1 |
| 5000 | 1 | 1 | 1 | 1 | 1 |
| 10000 | 1 | 1 | 1 | 1 | 1 |

**Table 42. Effect of MLM on the growth of *B. cereus* in TSBYE at 30°C and pH 8-9**

| MLM levels (ppm) | Time (day) to reach max OD @ 625 nm | |
|---|---|---|
| | pH | |
| | 8 | 9 |
| 0 | 1 | 1 |
| 1000 | >35 | >35 |
| 2000 | >35 | >35 |
| 3000 | >35 | >35 |

### *3.4. Conclusion*

In conclusion, this study has clearly shown that modified atmosphere packaging is not a viable method to control the growth of *B. cereus* at neutral pH (~7) and elevated storage temperatures (30°C). Even at high $CO_2$ concentrations (>50%), *B. cereus* growth was observed after only 1 d at 30°C. Despite being effective in controlling the growth of molds i.e., *Penicillium notatum* and *Aspergillus niger* (Hasan, 1997), oxygen absorbents, alone and in combination with $CO_2$, also failed to inhibit the growth of *B. cereus* in agar plates.

Similar trends were observed with ethanol vapor generators. Growth of *B. cereus* was observed after 1 d at 30°C in plates packaged with Ethicap and Negamold. However, Negamold, with its dual action, could be more effective in inhibiting the growth of *B. cereus* since it maintained low oxygen levels inside the package. The effect of Negamold may become more pronounced if used in combination with other inhibitors i.e., gas packaging (hurdle technology).

The growth of *B. cereus* was inhibited by UV-light in PEMBA plates. Exposure to UV-light for only 2 min was sufficient to inhibit the growth of *B. cereus*; however, the distance of the plates from the UV source was critical in determining exposure times of plates to UV-light. Furthermore, it has been found that the antimicrobial effect of UV-light was bacteriostatic and not bactericidal since growth occurred within 1 week, when cuts from the inoculated and irradiated media were added to BHI broth.

Alta and Per/Lac, commercially produced pediocins, did not exert any antimicrobial activity against the growth of *B. cereus*, even at acidic pH (pH 5.5). However, nisin inhibited the growth of *B. cereus* at acidic pH (5.5-6) for over 35 d at 30°C. Growth of *B. cereus* however, was observed in TSBYE at alkaline conditions (pH >7) after only 1 d at 30°C.

Mono-lauryl maleate (MLM) inhibited the growth of *B. cereus* at high pH (pH 8-9) in TSBYE for over 35 d at 30°C. However, MLM was insoluble in TSBYE at room temperature, but completely soluble after autoclaving for 15 min at 121°C.

# Chapter 4

# Effect of Packaging and Processing on the Growth of *B. cereus* in English-style Crumpets

## *4.1. Introduction*

English-style crumpets are snack-type, high moisture bakery product which have been implicated in food poisoning outbreaks due to the growth of, and toxin production by, *B. cereus* (Jenson *et al.*, 1994). Due to their high water activity ($a_w$ 0.95~0.97), high moisture content (48~54%), and neutral to alkaline pH (pH 6.5~8), crumpets have a short shelf-life of under 6 d (Jenson *et al.*, 1994). Crumpets are cooked on the bottom only on a hot griddle and are consumed within few days, prior to mold or bacterial spoilage. When a longer shelf-life is desired they are frozen (Ooraikul and Stiles, 1991). Various methods have been examined in previous chapters to control the growth of *B. cereus* in media. The more successful methods in broth/agar plate studies were now extrapolated to control the growth of this pathogen in fresh crumpets.

### *4.1.1. Objectives*

The objectives of this study were to determine the effect of ethanol vapor generators, in combination with gas packaging ($CO_2$), UV-light, and mono-lauryl maleate, on the growth of *B. cereus* in English-style crumpets.

### *4.2. Materials and methods*

#### *4.2.1. Preparation of spore inoculum/sample inoculation*

A strain of *B. cereus*, used previously in section ***2.2.1***, was used throughout this study. A spore suspension of *B. cereus* was prepared using a modified method of Thayer and Boyd (1994). *B. cereus* was sub-cultured from PEMBA plates (agar base, Oxoid LTD., Hampshire, England) onto sporulation media (Thayer and Boyd, 1994). The media consisted of 3.5 g of peptone, 4.0 g of nutrient broth, 1.0 of dextrose, and 15 g of agar/L of distilled water. The pH was adjusted to 6.55 with either 1N NaOH or 1 N HCl prior to sterilization. The *B. cereus* strain was spread on the agar and incubated for 1 week at 30°C. The cells and spores were washed from the agar plates with ~50 ml of sterile 0.1% peptone. The suspension was heated at 65°C for 30 min and washed 3 times with sterile 0.1% peptone and centrifuged (12,100 x *g* for 20 min) between washings using a IEC HN-S centrifuge (Damon/IEC Division, Massachusetts, USA). The suspension was heated a second time at 70°C for 30 min to eliminate vegetative cells and stored at 4°C until use. Spore suspensions were adjusted to approximately 7-log spores/ml, and appropriate dilutions of this stock suspension were then made using 0.1% peptone to obtain a working suspension of ~$10^3$ spores/ml.

#### *4.2.2. Crumpet formulation and inoculation*

High moisture, English-style crumpets, used in this study ($a_w$ 0.990, pH 8.55), were prepared from all-purpose flour, sugar, instant yeast, reconstituted dry milk, salt, and baking soda, according to the method of Witty and Schneider Colchie (Table 43). All ingredients were weighed on a Mettler Toledo Scale (PB-3001, Switzerland) in round stainless steel bowls, placed in a Hobart Mixer (A-200, Hobart Canada Inc., Don Mills Ontario), and mixed for 5 min until a homogeneous batter was formed. The batter was then inoculated with ~$10^3$

spores/g prior to baking. Crumpets were then baked by pouring 65 g of batter into greased cylindrical molds on an oiled griddle (Moffat appliances, Montreal, Quebec, Canada) pre-heated to 204°C. Crumpets were baked for approximately 9 min until the surface was no longer wet and holes had formed on the top of the crumpets. Following baking, crumpets were cooled to room temperature, and then packaged in Cryovac bags.

**Table 43. Ingredients used in crumpet formulation**

| Ingredient | Amount (g) | Brand name |
|---|---|---|
| Flour | 1200 | Robin Hood Inc. |
| Milk powder | 110 | Carnation, Nestlé |
| Salt | 12 | Sifto |
| Sugar | 3.2 | Redpath |
| Water | 1300 | - |
| Yeast | 18 | Fleischmanns (Quick-rise) |
| Baking soda | 10 | - |

#### *4.2.3. Effect of 600S Negamold® in combination with 100%$CO_2$, on the growth of B. cereus in crumpets*

All inoculated and control crumpets were packaged by placing 2 crumpets (~50 g each) into 210 x 210 mm high gas barrier Cryovac bags ($O_2$ transmission rate: 3-6 cc/$m^2$, 24 h, 1 atm at 4.4°C, 0% RH; ethanol transmission rate; 0.21 g/ $m^2$/24h, 1 atm at 25°C). Crumpets were packaged with 600S sachets of Negamold® (Freund Industrial Co. Ltd., Tokyo, Japan) and 100%$CO_2$. Gas flushing was done as described previously (see section ***3.2.2***). The ethanol sachets were added to the bags immediately prior to gas flushing and sealing. All packaged samples were stored at 30°C. Duplicate samples of inoculated and control crumpets were analyzed after 0, 3, 5, 7, 14, 21, 28, and 35 d.

#### *4.2.4. Headspace gas analysis*

Changes in headspace $O_2$ and $CO_2$ were monitored using a previously calibrated Oxygen/Carbon Dioxide Analyzer (Servomex, Food Package Analyzer Series 1400, Minneapolis, USA). Samples of headspace gas were withdrawn using a 0.5 ml gastight pressure-Lok® syringe through a septum affixed to the outside of each package.

#### *4.2.5. Headspace ethanol*

Changes in headspace ethanol were monitored using a Varian gas chromatograph (see section ***3.2.5***). The concentration of headspace ethanol (% v/v) was determined from a standard curve ($R^2$= 0.9981) generated by analyzing, in triplicate, standard solutions of food grade ethanol ranging in concentration from 0.25 to 10% (v/v) (section ***3.2.5***).

### *4.2.6. Effect of UV-light on the growth of B. cereus in crumpets*

To determine the effect of UV-light on the growth of *B. cereus* in crumpets, crumpets were inoculated, in duplicate (~50g each), and placed uncovered in sterile petri dishes. All crumpets, with the exception of control crumpets, were exposed to UV-light at a distance of 2 cm from the UV source for 0-12 min at 1-min intervals. All samples were then packaged in Cryovac bags, stored at 30°C and monitored for growth of *B. cereus* on PEMBA plates.

### *4.2.7. Effect of MLM on the growth of B. cereus in crumpets*

To examine the effect of MLM on the growth of *B. cereus*, crumpet batter was again prepared and inoculated with a working suspension of ~$10^3$ spores/g as described previously in section ***4.2.2***. Appropriate amounts of MLM were dissolved in 10 ml of sterile water at 70°C and added to the batter at concentrations of 0 to 3000 ppm at 1000 ppm increments (Table 44). Crumpets were then baked, allowed to cool and packaged in Cryovac bags (section ***4.2.2***). All samples were incubated at 30°C and checked for *B. cereus* growth using PEMBA.

### *4.2.8. Effect of batter pasteurization on the growth of B. cereus in crumpets*

To determine the effect of pasteurization on the growth of *B. cereus*, the batter was inoculated with ~$10^3$ spores/g as described previously. In the first batch, the batter was baked directly, without pasteurization, and was used as a control. In the second batch, the batter was pasteurized in a water bath at 75°C for 30 min. Crumpets were then prepared as described previously. All crumpets were then packaged in Cryovac bags and incubated at 30°C and checked for growth of *B. cereus* as outlined in section ***4.2.4***.

### *4.2.9. Sampling of crumpets*

On the appropriate sampling day, crumpets were aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of sterile 0.1% peptone water and stomaching for 1 min using a Stomacher Lab Blender 400 (Seward Medical Stomacher, London, UK). Decimal dilutions were subsequently prepared from this initial dilution. Growth of *B. cereus* was monitored by spread plating 0.1 ml of appropriate decimal dilutions of the 1:3 homogenized samples, in duplicate, on *B. cereus* agar (PEMBA). All plates were incubated at 30°C for 24 h and checked for visible growth of *B. cereus*.

### *4.2.10. Ethanol content of crumpets*

In order to monitor changes in the ethanol content of crumpets, the column, carrier gas, carrier gas flow rate and column temperature were set as described previously (see section ***3.2.5***) for headspace ethanol analysis. However, the injector port temperature was increased to 150°C. The ethanol content of crumpets was analyzed by injecting 1 μl of supernatant of the centrifuged homogenized sample (1:3 dilution) into the chromatograph. Peaks were recorded and analyzed with the Hewlett-Packard integrator. The ethanol content of crumpets (% w/w) was determined from a standard curve ($R^2$=0.994) generated by analyzing, in triplicate, standard solutions of 95% food grade ethanol ranging in concentration from 0.25-10% (w/w) (Figure 5).

**Figure 5. Standard curve for ethanol content in crumpets**

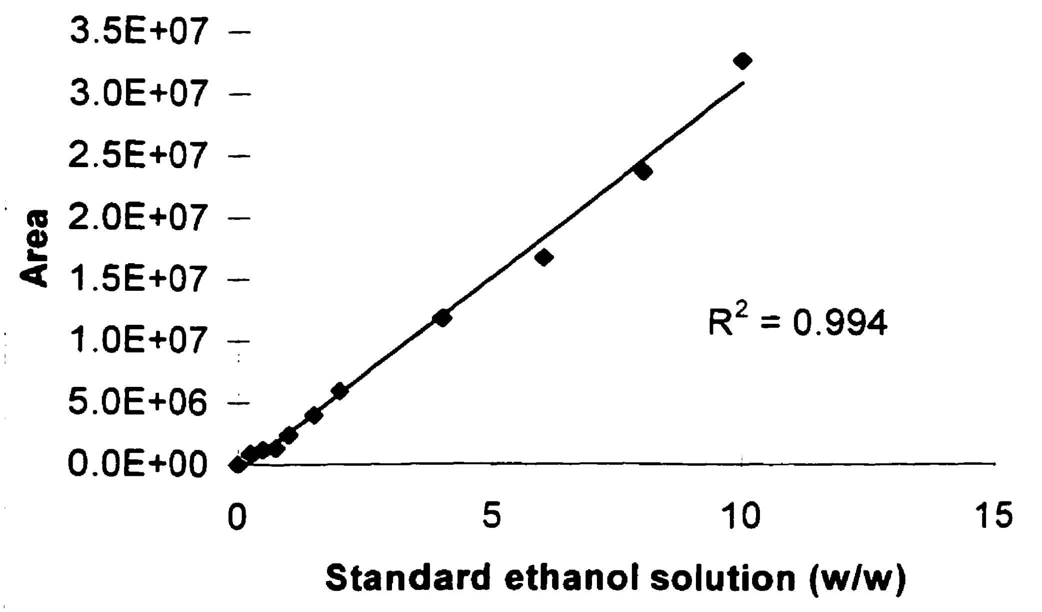


**Table 44. Levels of MLM added to 100g of crumpet batter to obtain final concentrations of 0-3000 ppm**

| MLM levels (g) | Final concentration (ppm) |
|---|---|
| 0 | 0 |
| 0.1 | 1000 |
| 0.2 | 2000 |
| 0.3 | 3000 |

### *4.3. Results and discussion*

#### *4.3.1. Effect of Negamold (600S) in combination with 100% $CO_2$ on the growth of B. cereus in crumpets*

Studies were conducted to determine the combined effect of Negamold and 100% $CO_2$ to control the growth of *B. cereus* in crumpets. It was previously observed that the growth of *B. cereus* was inhibited for 35 d when 600S Negamold sachets were used in combination with 100% $CO_2$ in PEMBA media. These packaging conditions were subsequently chosen to determine their effect on the growth of *B. cereus* in English-style crumpets.

It is evident that a combination of $CO_2$ and ethanol vapor was effective in controlling the growth of *B. cereus*, as growth was inhibited for >35 d of at 30°C. The average headspace ethanol in crumpets was ~6% (Table 45), while the average headspace $CO_2$ level was ~90% (Figure 6-7). These results are in agreement with those reported by Smith *et al.* (1987) who reported that ethanol vapor could be used to extend shelf-life and keeping quality of apple turnovers. They observed that the growth of *S. cerevisiae* was completely inhibited for 21 d at 25°C when apple turnovers were packaged with ethanol vapor in conjunction with $CO_2$. Furthermore, they found that the level of ethanol decreased from an initial level (d 1) of 1.8% to 0.5% after 21 d at 25°C in products packaged with a combination of ethanol vapor generators and 60% $CO_2$ (balance $N_2$).

**Table 45. Effect of Negamold ($NM_6$)/100% $CO_2$ on the growth of *B. cereus* in crumpets**

| Packaging conditions | Days to growth | Average headspace after 35 d storage | | |
|---|---|---|---|---|
| | | Ethanol (% v/v) | $CO_2$ % | $O_2$ % |
| $NM_6$+100% $CO_2$ | >35 | 5.9 | 90.2 | 0.3 |

**Figure 6. Changes in headspace ethanol over a 35 d storage period**

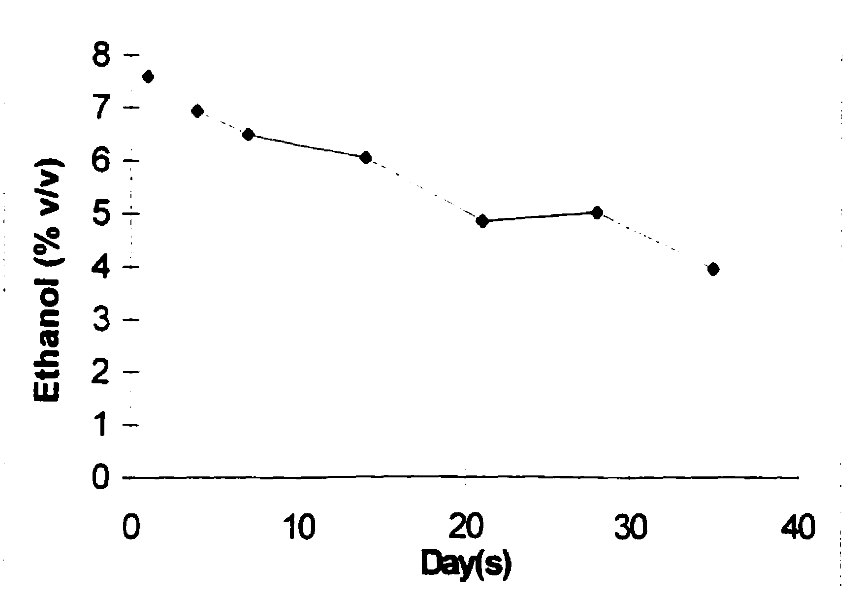
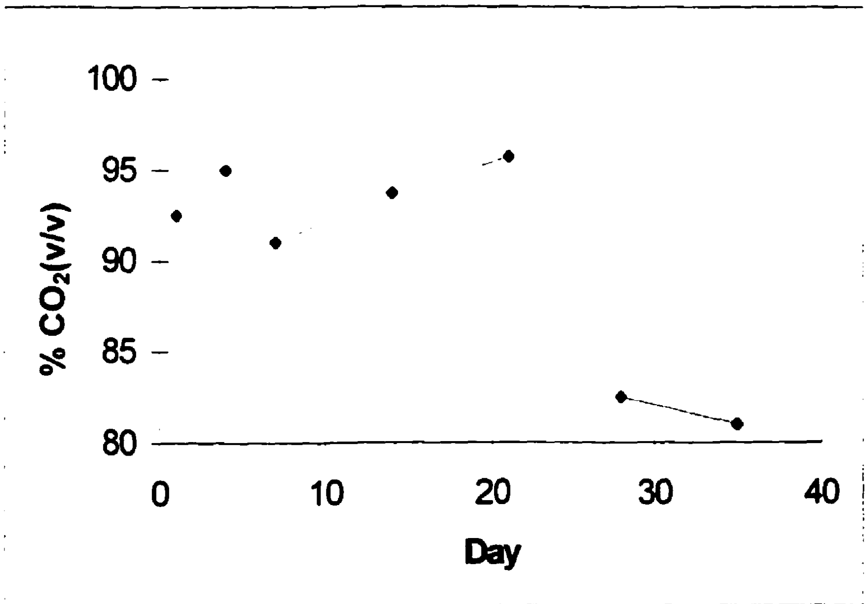



**Figure 7. Changes in headspace $CO_2$ over a 35 d storage period**



### *4.3.2. Ethanol content of crumpets*

The ethanol content of crumpets packaged with 600S Negamold+100% $CO_2$ was analyzed at each sampling day. It was observed that at the end of the storage period the ethanol content of crumpets reached ~3.4% (Day 28). In this study, the ethanol content of crumpets at the end of shelf-life exceeded the permitted level of 2% (w/w) in bakery products (Federal Register, 1974). Higher levels of ethanol found in crumpets could be due to their higher $a_w$ (0.990). Daifas *et al.* (2000) reported that the amount of ethanol generated and absorbed in a medium or product was greater at a higher $a_w$. Furthermore, they showed that the ethanol content of crumpets packaged without ethanol increased from 0.7% after baking to ~3% (w/w) at the end of storage and may be attributed to fermentation by yeast (Daifas *et al.*, 2000).

### *4.3.3. Effect of UV-light on the growth of B. cereus in crumpets at 30°C*

The effect of UV-light on the growth of *B. cereus* in crumpets is shown in Table 46. Growth of *B. cereus* was observed after only 1 d at 30°C at all exposure times. Previous studies on the effect of UV-light on the growth of *B. cereus* in PEMBA plates placed at either 2 or 50 cm away from the UV source showed that the distance from the UV source influenced the exposure time needed to cause inhibition of *B. cereus*. Consequently, crumpets in this study were placed at a distance of 2 cm. However, while UV-light was effective in controlling the growth of *B. cereus* in PEMBA plates, it was not effective in crumpets. Since the surface of PEMBA plates is smooth, UV-light was perhaps more effective in reducing the number of bacteria on plates. Conversely, the porous surface of a crumpet may prevent the UV rays from penetrating it, thus enabling bacterial cells to survive the UV treatment. These results are in agreement with those reported by Stermer *et al.* (1987) who compared the effectiveness of UV-light on beef round samples (rough surface) and inoculated agar plates (smooth surface). They found that UV-light was very effective in reducing bacteria on agar plates inoculated with various species of *Staphylococcus*, *Pseudomonas*, and *Micrococcus* (a 90-99.9% kill rate was obtained depending on UV dose). However, lower inactivation levels were obtained when beef round samples were treated with UV-light. They concluded that UV-light is more effective on agar plates than on meat (Stermer *et al.*,1987).

**Table 46. Effect of UV-light[a] and distance (2 cm from source) on the growth of *B. cereus* in crumpets stored at 30°C.**

| UV treatment (min) | Days to growth ($>10^6$ CFU/g) |
|---|---|
| 0 | 1 |
| 1 | 1 |
| 2 | 1 |
| 3 | 1 |
| 4 | 1 |
| 5 | 1 |
| 6 | 1 |
| 8 | 1 |
| 9 | 1 |
| 10 | 1 |
| 11 | 1 |
| 12 | 1 |

[a]Intensity of UV-light ~620μW/$cm^2$

### *4.3.4. Effect of MLM on the growth of B. cereus in crumpets at 30°C*

The effect of MLM on the growth of *B. cereus* in crumpets is shown in Table 47. MLM has been shown to inhibit the growth of *B. cereus* in PEMBA plates, even at a highly alkaline pH (~9). However, due to the complexity of the food system, different results were obtained in crumpets. The growth of *B. cereus* occurred in all samples after only 1 d at 30°C, regardless of the level of MLM. This may be due to poor solubility and dispersion of MLM in the batter. Kato and Shibasaki (1975) reported that the antimicrobial activity of several fatty acids, and their esters, was dependent on the nature of the food, especially if it is comprised of starch, gelatin, and albumin. Crumpets are also high in starch and this may have contributed to the reduced antimicrobial activity of MLM in crumpets compared to PEMBA plates. Furthermore, MLM, being more lipophilic, was perhaps unavailable to exert an antimicrobial activity in the aqueous phase of the food where most microbial growth is likely to occur. These results are in agreement with those reported by Robach *et al.* (1981). They reported that monolaurin exerted antimicrobial activity in laboratory media. However, monolaurin had no effect on the growth of yeasts, molds, psychrotrophic bacteria, and coliforms in cottage cheese.

### *4.3.5. Effect of pasteurization on the growth of B. cereus in crumpets at 30°C*

The effect of batter pasteurization (75°C for 30 min) on the growth of *B. cereus* in crumpets is shown in Table 48. The growth of *B. cereus* occurred after only 1 d at 30°C. The internal temperature of the batter, as measured using a portable thermometer, was only 50°C after 30 min. Therefore, this pasteurization treatment failed to heat-shock the spores, which would then become vegetative cells, hence making them more susceptible to the baking temperature. Spore cells survived the baking temperature and growth of *B. cereus* reached

$>10^6$ CFU/g after only 1 d at 30°C. Furthermore, the textural quality of the batter/crumpets was affected as a result of this pasteurization treatment.

**Table 47. Effect of MLM on the growth of *B. cereus* in crumpets at 30°C**

| MLM levels (ppm) | Days to visible growth |
|---|---|
| 0 | 1 |
| 1000 | 1 |
| 2000 | 1 |
| 3000 | 1 |

**Table 48. Effect of pasteurization on the growth of *B. cereus* in crumpets at 30°C**

| Conditions | Days to visible growth |
|---|---|
| Control | 1 |
| Batter pasteurized @ 75°C[a] for 30 min | 1 |

[a] Internal temperature: ~50°C

### *4.4. Conclusion*

In conclusion, ethanol vapor generators, combined with 100% $CO_2$, were effective in delaying the growth of *B. cereus* for 35 d or longer. However, the headspace ethanol of the packages was high (~6%), and the content of ethanol in crumpets was an average of ~3.5% after 28 d. Crumpets were also organoleptically unacceptable since the ethanol content had surpassed the maximum limit (~2% w/w) approved for food.

UV-light did not inhibit the growth of *B. cereus* in crumpets due to the crumpets' rough and porous surface.

MLM also failed to inhibit the growth of *B. cereus* in crumpets; however, further studies are required to improve the solubility/dispersion of MLM in crumpets.

Pasteurization of the crumpet batter proved to be ineffective as well in controlling the growth of *B. cereus* in crumpets. Furthermore, pasteurization had a deleterious effect on the texture and organoleptic quality of crumpets.

# Chapter 5

## General Conclusion

Crumpets are high moisture bakery products which have been implicated in several food poisoning outbreaks involving *Bacillus cereus*. This microorganism is a spore former which is commonly found in flour and milk, ingredients used in the making of crumpets. In the baking process the bottom of the crumpet is fully baked, while the remainder of the crumpet, especially the surface, receives a partial baking treatment. Consequently, *B. cereus* spores can easily survive the baking temperature and subsequently germinate and grow to levels of $10^5$ CFU/g within 3-5 days at ambient temperature. Therefore, methods to control this pathogen are essential to ensure the safety of such minimally processed food products.

Preliminary studies clearly indicated that $a_w$ was not a viable method to control the growth of *B. cereus*. Large amounts of salt or glycerol would be required for complete inhibition of this pathogen and such levels may severely affect the organoleptic and textural qualities of crumpets. This study has also shown that the growth of *B. cereus* was inhibited at pH 5. However, many bakery products e.g., English-style crumpets, are formulated to pH levels >8 to enhance color. Hence, other methods need to be considered to control the growth of *B. cereus* in such high pH products. However, the ability of *B. cereus* to grow over a wide pH range, 5 to 9, made it difficult to control using conventional preservation methods, such as organic acids and their salts. This study showed that neither calcium propionate nor potassium sorbate were effective in controlling the growth of this pathogen at pH> 5.

Further studies also showed that modified atmosphere packaging was not a viable method to control the growth of *B. cereus* at neutral pH (~7) and elevated storage temperatures (30°C). *B. cereus* is a facultative anaerobe, hence it could grow with or without the presence of oxygen. *B.* cereus was resistant to the antimicrobial activity of high $CO_2$ concentrations (100%), with growth being observed after only 1 d at 30°C. Similar results were observed when an oxygen absorbent sachet (FX) was used introduced to the package. Furthermore, when ethanol vapor generators were used, growth of *B. cereus* was observed after 1 d at 30°C in plates packaged with Ethicap and Negamold. However, the effect of Negamold was enhanced when used in combination with 100% $CO_2$ and inhibition occurred in media and crumpets for over 35 d at 30°C. However, the ethanol content of crumpets was greater than the maximum allowable limit for food, i.e., ~2% (w/w). Thus, crumpets were rejected based on their organoleptic acceptability.

Alternative methods to control the growth of *B. cereus* included UV-light, bacteriocins, organic esters and batter pasteurization. Many of these methods were highly effective in media but not in crumpets, which is mainly due to the complexity of the food system. For instance, while UV-light inhibited the growth of *B. cereus* in agar, it failed to inhibit its growth in crumpets due to their rough and porous surface. Esters, such as mono-lauryl maleate, proved effective in broth, but they did not inhibit the growth of *B. cereus* in the crumpet batter at levels of 3000 ppm probably due to solubility and dispersion problems. Finally, pasteurization of the crumpet batter was not a viable method since it had a deleterious effect on the texture and organoleptic quality of the crumpets. The internal temperature reached in the pasteurization treatment (~50°C) was insufficient to heat-shock the spore cells and hence make them more susceptible to the baking temperature.

In conclusion, *B. cereus* is a difficult microorganism to control, especially in high pH crumpets. Further studies are now underway to examine the effect of other barriers, such as sorbic hydroxamic acid, an antimicrobial agent which has a high $pK_a$ value (~12). Therefore, this preservative will remain in the undissociated and active form at high pH levels found in certain crumpet formulations (pH ~8). Moreover, the effect of L-alanine on spore germination and a novel antimicrobial gum will be examined, alone and in conjunction with each other, to control the growth of *B. cereus* in both agar plates and in high pH English-style crumpets.

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