STUDIES ON SPORULATION AND SPORE GERMINATION OF SELECTED BACTERIA AND AFLATOXIN PRODUCTION BY ASPERGILLUS FLAVUS IN METABISUIPHITE-TREATED MEDIA.

BY

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D E D I C A T I O N

Dedicated to the memory of my

late daddy

Pa Bukoye Oloyede

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A B S T R A C T

The effect of metabisulphite on sporulation and spore germination of Bacillus subtilis E 52, B. cereus W18 and on aflatoxin production by Aspergillus flavus IAR strain in selected media has been studied.

The incorporation of 600 ppm metabisulphite to nutrient broth was observed to have adequately prevented the growth and germination of B. subtilis and B. cereus spores in nutrient broth over a period of 72 hours. In unsweetened condensed (peak) milk, addition of 800 ppm of metabisulphite adequately prevented the growth of the two bacteria over a period of 5 days whereas in powdered milk (Nido, Nestle make), the addition of 500 ppm prevented the growth of the bacteria over a period of 7 days.

Matabisulphite concentrations of 500 and 600 ppm were found to prevent sporulation of B. subtilis and B. cereus respectively in a sporulating medium over a period of 72 hours. The same concentrations were found to prevent the synthesis of alkaline phosphatase and glucose dehydrogenase as well as reduced the protein and dipicolinic acid contents to undetectable levels in the two bacteria. The effect of the metabisulphite on the enzymes investigated, suggested that it caused leakages.

Incorporation of 600 ppm metabisulphite to buffered yeast extract medium prevented aflatoxin production by A. flavus over a period of 7 days, while 2,000 ppm of metabisulphite in powdered milk (Nido) prevented its growth for a period of 7 days.

INTRODUCTION

Worldwide population growth and improved standards of living call for increased food supply. To improve the standard of living, not only must the food production be increased but also attention would need to be directed towards better protection and preservation of foods from food spoilage microorganisms.

Food spoilage resulting from various agents cuts down tremendously the amount of food available to the world populations. Moreover, the consumption of microbial contaminated food items may cause serious health problems. Contamination by microorganisms and their by-products alone has been found to be responsible for about 90% of food borne diseases. The sporeforming bacteria like Clostridium perfringens, C. botulinum, Bacillus cereus and B. subtilis are a few examples of microorganisms responsible for food problems. Fungal contamination in foods and feeds is also well known. This implies that a constant microbiological examination of foods is essential as this may provide information concerning the quality of the food, the sanitary conditions under which the foods have been processed as well as to the effectiveness of the method of preservation.

Sporeforming bacteria are of special interest to the food industry because some of them are resistant to heat, radiation, chemical agents and other destructive agents. As a result, some may remain viable for many years in the absence of any detectable metabolism (85). Bacillus subtilis and B. cereus, for instance, can survive pasteurization

temperature and cause spoilage in refrigerated pasteurized products (46). Bacillus subtilis spores have also been found to be capable of withstanding the baking temperature of bread (26). This means they can germinate and grow in the loaves of bread if conditions are favourable. Moreover, the resistance exhibited by B. subtilis has endeared it to industries where it is frequently employed to test the quality control or to assess the preservative efficacy of processes (18).

Foods contaminated by spores, if allowed to germinate, can produce marked changes in appearance, flavour, odour and other qualities of the food. Bacillus subtilis and B.cereus are capable of causing proteolytic degradation in milk. Such a degradative change could lead to an undesirable change in the milk. If this kind of milk is fed to children it could pose a serious problem of infection since they have low resistance to infection. An undesirable change due to microbial synthesis could also occur in foods. Ropiness resulting from the production of a levan (a polymer of fructose) in bread is due to contamination by B. subtilis (26). This has a slimy consistency. It looks reasonable therefore, that any step which can arrest or reduce these problems and similar ones is a right step towards solving some aspects of food problems.

Fungal contamination can also pose a serious problem to the life of man if the situation is not arrested. Fungi are capable of producing secondary metabolites, which are known to be toxic to man and animals (51). Such substances are referred to collectively as mycotoxins. Mycotoxins show acute toxicity in large doses, cause liver cancer and

other diseases in sublethal doses in a wide range of animal species (96, 97). One of the mycotoxins of public health significance is aflatoxin and it is produced by Aspergillus flavus (11) and other species of Aspergillus. The ingestion of aflatoxin causes a disease known as aflatoxicosis, which has been reported in animals and, possibly, man (10). It is therefore, apparent that contamination of food and feeds by A. flavus could be a potential public health hazard.

Since the importance of food to man cannot be over emphasized, it is necessary that efforts be made to either avoid the contamination completely or create an environment which will make it unsuitable for the growth of the contaminating organisms in foods. It is not possible to completely prevent contamination but it is possible to prevent microbial growth. It is this latter approach that allows food preservative methods ranging from heat treatment and radiation to chemicals to be in common use today (27). Most microbial spores are highly heat resistant, hence use of heat alone has not achieved enough success in food preservation. Radiation has been applied in low doses and this is not enough to kill all the spores which are known to be radiation resistant (35). The use of chemicals might effectively supplement these other methods in solving the problems. Chemicals have been used with great success in preventing or delaying the spoilage of foods by microorganisms (46).

Metabisulphite (a source of sulphur dioxide) is one of such chemicals which has been used in foods and food products to serve as an antimicrobial preservative and as an antioxidant to prevent both enzymatic and non-

enzymatic discolouration (47). It has been applied in practice to a number of food items including fruit pulps and juices, dehydrated fruits and vegetables, fresh fruits such as grapes, wine, beer and preserves (47).

The concentrations of metabisulphite required to cause lethal and sublethal effects to some organisms under varying conditions have been studied and reported (2, 3, 87).

Even though metabisulphite has been employed in practice to a number of food items and the levels required to cause lethal effect to some pathogenic organisms have been reported its effects on sporeformers and on aflatoxin production have not been well documented. The present work, therefore, attempts to look into these areas using B. subtilis and B. cereus (sporeformers) as the test organisms and A. flavus as an aflatoxin - producing organism. In doing this, the effect of metabisulphite on sporulation has been investigated by relating its influence on certain biochemical events to sporulation. The biochemical events studied under the influence of metabisulphite include alkaline phosphatase synthesis, glucose dehydrogenase synthesis, dipicolinic acid content and protein content (55, 84, 92). Because of the problems associated with aflatoxins, the present research has been extended to include the effect of metabisulphite on aflatoxin production by A. flavus. The findings, we hope, may open up a new approach towards solving some aspects of food problems.

LITERATURE REVIEW

A major problem facing mankind today is inability to provide enough food to match the rapidly increasing world population. Most of the perishable foods begin to deteriorate shortly after harvest and hence become unsuitable for human consumption. Some deteriorations are accompanied by the production of poisonous agents while others inflict losses on the nutritive value of foods. Microbial contamination accounts for a large proportion of the food poisons known. Prominent among the microorganisms that can cause food poisoning are the sporeformers and aflatoxin producing organisms such as Aspergillus flavus (20) and A. parasiticus (94).

The most important sporeforming bacteria belong to the genera Bacillus and Clostridium (39, 71). Bacillus cereus, a sporeforming bacterium, has in recent years been incriminated as a food poisoning organism often associated with food items such as meat, vegetable soups, fried rice, ice cream and in milk (31). In relation to other food-borne pathogens, B. cereus ranked as the third most common cause of food poisoning in Hungary during the period 1960 - 1966 (33). This organism was responsible for 3,560 cases in 88 outbreaks representing 4.4% of total outbreaks recorded during this period. Ormay and Novotny (62) reported that between 1960 and 1968, B. cereus was responsible for 15.2% of total food poisoning cases of known aetiology in Hungary and 8.2% of total outbreaks. Huage (43) reported 4 outbreaks of B. cereus gastroentiritis in Norway which involved 600 persons. Vanilla sauce which was prepared and stored at room temperature for one day before being served was the food implicated.

Similar report was made in Netherlands by Goepfert, Spira and Kim (33) and the outbreak was attributed to consumption of contaminated mashed potatoes, vegetables, minced meat, liver sausage, puddings and soups. The ingestion of a large number of viable cells of B. cereus in food results in B. cereus gastroentiritis (28). This may be regarded as food infection. Other data seem to suggest intoxication instead of infection (28, 43, 53).

The problems posed by sporeforming bacteria are further made exacerbated by their resistance to various adverse conditions. It has been reported by Bond and Favero (12) that a temperature as high as 150°C for a period of 2.5 hours was required to kill about 90% of 5 x 10^2 spores of Bacillus species ATCC 27380. Gould (35) also observed that spores of some species may be up to 10^5 - fold more resistant to heat than are vegetative cells of the same species. It has also been possible to isolate a strain of B. cereus from cream that had been heated at 140°C for 2 seconds and when this strain was cultured and retested it was observed that one spore in 10^5 - 10^6 survived 135°C for 4 hours (25). The higher resistance of some spores to ultraviolet and ionising radiation (70) and also to high hydrostatic pressures (76) than their vegetative counterparts are well known. Spores have been reported killed by hydrostatic pressures as high as 176,000 p.s.i. (pounds per square inch) for 14 hours whereas the vegetative cells were killed by 88,000 p.s.i. for the same period (70). The higher resistance of spores than vegetative cells to a variety of chemical agents have also been reported. Such chemicals include methanol,

ethanol, acetone, ethylene oxide and chlorine water (70).

Spore formation is initiated following the exhaustion of a nutrient (35) and is influenced by several factors such as temperature, pH, oxygen requirements, manganese and the composition of the sporulation media. It has been observed that, although the sporulation pH and temperature ranges are narrower than those for normal growth, the optima pH and temperature for sporulation are identical to those required for normal growth (73). The same worker, reported that during sporulation the demand for oxygen consumption is increased and the presence of a high concentration of manganese stimulates sporulation. The latter has been reported in B. cereus (73) and in B. stearothermophilus (85). The composition of the sporulation media has a significant influence on sporulation by organisms. Russell (73) found that cooked meat medium based on Hartley's digest broth supported Clostridia sporulation more than any other media examined.

Sporulation is a multiphase process in which the changes occur in a fairly sequential manner (23). In this process, it is thought that a refracticle cell is first produced, followed by synthesis of dipicolinic acid (DPA; pyridine - 2, 6 - dicarboxylic acid) (65) and finally the development of heat resistant cells (38). However, the precise nature and sequence of the biochemical events that accompany sporulation are still poorly understood (57). Enzymes or simple substances strictly associated with sporulation and enzymes associated with both sporulation and vegetative growth are the two major classes of such biochemical events (91).

(a) Enzymes or simple substances strictly associated with sporulation include:-

(i) glucose dehydrogenase (75), adenosine deaminase and ribosidase (64).

(ii) Production of antibiotics (92) and appearance of new antigens (55).

(iii) production of exoprotease and a wall lytic enzyme (78).

(iv) Dipicolinic acid (DPA). This is present as a Ca - chelate in high concentration (5 - 15%) in all bacterial endospores (57). The roles DPA may play in spores have been grouped as non-specific (overall heat resistance) or specific [related to a given reaction or process] (57).

Various authors have implicated the DPA content of spores in heat resistance of spores (70, 73). It has been observed that when spores are heated there is a progressive loss of DPA from such spores (73). The rate of release of DPA is temperature dependent, varying from species to species with the most resistant species releasing the least DPA during such heat treatment. Gould and Dring (36) associated the heat resistance of spores with the Ca:DPA molar ratio. It has been found that as Ca: DPA molar ratio decreases, the heat resistance likewise decreases. DPA or Calcium-dipicolinate has also been implicated in the resistance of spores to chemical agents (70). It has been suggested that sensitive

sites or chemical groups in the spores are likely to be masked by calcium or DPA and thus responsible for the resistance of spores to alkylating agents (70).

It has been speculated that DPA is specifically involved in enzyme inhibition (37) and initiation of germination (69).

(b) Enzymes associated with both sporulation and vegetative growth. This class includes purine nucleoside phosphorylase in B. cereus (29), the DNA polymerase (24), alkaline phosphatase (32, 55, 84, 91, 92) in B. subtilis and the inorganic pyrophosphatase in B. megaterium (88).

Spore germination designates all the changes that take place during the conversion of a spore to a new vegetative cell. It involves the breakdown of the complexing structure which protects the vital functions, and the activation of enzymes that remain dormant in the intact spore (38). Two stages have been distinguished in the germination of spores. First, an initiation stage, in which the spores loose their heat resistance and become readily stainable second, an outgrowth stage in which the spore swells, ruptures, elongates and finally divides releasing the vegetative cells (38).

As mentioned earlier, A. flavus is one of the most important microorganisms that can cause food problems. The ability of A. flavus to produce aflatoxin has been well documented in literature (6, 42, 77).

The report of the joint Food and Agricultural Organisation (FAO), World Health Organisation (WHO) and United Nations Environment Programme

(UNEP) conference on mycotoxins 1977 showed that Aspergilli are universally distributed in the environment and aflatoxin contamination in foods and feeds is very common (48). Wildman, stoloff and Jacobs (95) reported the presence of aflatoxin in substrates ranging from solid foods to commercially prepared fruit juices, bread, orange juice, tomato juice and pineapple juice. The presence of aflatoxins in Nigerian fruits such as pawpaw, citrus fruits, banana and tomato has been reported (9). Sorghum, sorghum flour, millet, wheat, corn, oats, soyabeans, cassava and other items have been reported to contain aflatoxin in varying concentrations (5, 81, 83). Food items such as milk, milk powder, groundnut products have also been reported to contain aflatoxins (8, 66).

The types of aflatoxins produced by A. flavus include aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 (80). Aflatoxin B_1 is the most potent variety and is then followed by G_1 which is about half as toxic as B_1. Aflatoxins B_2 and G_2 follow in that order of potency respectively (96). Aflatoxin B_1 has been found to be the most carcinogenic of the known hepatocarcinogens, producing cancer in rats when a diet containing as little as 15 ppb of aflatoxin B_1 was fed continuously for 68 weeks in 12 male rats (97). Newberne, Carlton and Abdelkaden (58) reported that metabolic products of A. flavus are toxic to animal species with induction of histopathological lesion such as hepatic parenchyma necrosis and proliferation of bile ductile cells. It has also been observed that aflatoxin has the highest toxicity for mammals, poultry and fish (16). In all these cases the target organ has been revealed to be the liver (56, 58). Aflatoxicosis

have not been well reported in man. However, Belchetz and Cohen (10) recently related pulmonary adenomatosis to aflatoxin inhalation from a case study. Hepatic dysfunction and also death have been reported as a result of aflatoxin consumption in an Indian village (50). Post-morten examination of tissues from patients suspected to have died of cancer or liver has linked aflatoxin to liver cancer while data on children who died in Thailand from an acute disease of unknown aetiology revealed aflatoxin B_1 as a causative agent of the mortality (82).

In view of the problems associated with sporeforming bacteria and aflatoxins, many investigations have been undertaken with a view to contributing towards solving some of these problems.

Vas (90) stated that the application of the canning method in the food industries is an attempt to bring about complete destruction of pathogenic organisms including spores. Cork and Gilbert (17) observed that increasing concentrations of sodium chloride (2, 4 and 8% w/v) in heating medium progressively reduced the heat resistance of spores of B. stearothermophilus. With increasing concentration of sodium chloride in tomato juice, it has been observed that there is a progressive increase in the death rate of B. coagulans var. thermoacidurans (70). Different chemicals are known to have prevented spore germination by acting at various stages in the germination process (63). Some of them such as chlorocresol and mixed esters of p-hydroxybenzoic acid, inhibit germination while cetrimide, quinacrine and phenyl mercuric nitrate inhibit postgerminative process. Phenols have also been found to possess sporicidal effect against spores

of B. subtilis (73). Borick and Fogarty (13) reported that using 0.1 ml of spore suspension equivalent to 10^8 spores per ml of B. subtilis E 152, B. globigii E 154 and B. cereus E 190 required 0.146 Mrad, 0.165 Mrad and 0.107 Mrad doses of gamma irradiation respectively to kill 90% of the spores.

In order to prevent production of aflatoxins, methods were employed which aimed at inhibiting the growth of A. flavus. Applegate and Chipley (7) observed that the growth and sporulation of two strains of A. flavus were greatly reduced in both wheat and synthetic media by exposing the organisms to ^{60}Co - gamma radiation dose of 300 krad, with complete inhibition at 400 - 600 krad. The application of a 50 krad dose of gamma irradiation to flour and subsequently to bread has been observed to cause a significant reduction in the growth of moulds that developed on bread during storage for a period of 20 weeks (14). The possibility of using chemical agents as a means of controlling aflatoxin production has also been explored; Rao and Harein (67) reported the prevention of aflatoxin production in stored grains using 20 ppm dichlorovos (an organophosphate insecticide). Chemicals such as ammonia, methylamine, sodium hydroxide, hydrogen peroxide and ozone have all been used with success to reduce a substantial amount of aflatoxins in contaminated oil seed meals but there appeared to be some reduction in the protein quality during most of the treatments (34). Sodium chloride (12% or greater), sodium acetate (4%) malonic acid (50 mM) and benzoic acid (0.4%) were are all found to effectively inhibit growth of and aflatoxin production

by A. flavus (89). Investigation carried out by Ogbadu (60) revealed that 0.3% of sodium benzoate, 0.17% of O-hydroxybenzoate and 0.1% of p-aminoethylbenzoate were inhibitory to both growth of and aflatoxin production by A. flavus in mashed fruits of Carica papaya (pawpaw).

Sulphur dioxide has long been accepted as a preservative and has been employed either as an antioxidant or as an antimicrobial agent (4, 15, 68). The various forms in which it can be introduced into foods include sodium, potassium, and calciumsalts of sulphite, bisulphite and metabisulphite (47).

Metabisulphite has been employed in practice as a food preservative to a number of food items including fruit pulps and fruit juices (1,000 - 2,000 ppm SO_2), dried fruits and vegetables (500 - 1,000 ppm SO_2) and concentrated fruit juices, wine, beer (100 - 500 ppm SO_2) (47). Okafor (61) observed that palm wine could be preserved for 6 months using 0.05% of sodium metabisulphite. The use of sodium metabisulphite has been suggested to offer a new option in the control of botulinal outgrowth in cured or non cured meat and poultry products, the addition of levels of 100 µg of sulphur dioxide per g of meat or greater levels are necessary to achieve significant inhibition when a target level of 100 botulinal spores per g was subjected to sulphur dioxide (87). Using pretreated bone meal samples, Abalaka and Deibel (3) observed that metabisulphite concentrations of 7,000 - 8,000 ppm rendered the product Salmonella - free for 21 days and 9,000 ppm achieved the same effect for 28 days.

The physico-chemical property of metabisulphite (source of SO_2) places it at advantage over other food preservatives because the presence of sulphur dioxide in food beyond a certain level causes an offensive odour to emanate from the food. The safety of sulphited foods has been evaluated (30). Its acute toxicity and chronic toxicity as well as its metabolism by mammals have been considered by some investigators (4). The estimate of acceptable daily intake of sulphur dioxide for man is 0.7 mg per kg bw (body weight) as SO_2 (47). There are available data to show that bisulphite is easily converted to sulphate in the liver of mammals (54). Claims for the mutagenic role of metabisulphite by some investigators (40, 41) are currently questionable. More investigations are needed to establish the possible mutagenic role of sulphur dioxide.

Although several contributions have been made by various investigators in trying to solve the problems posed by sporeforming bacteria and aflatoxin, there have been no report on the effect of metabisulphite on sporulation in B. subtilis and B. cereus (sporeformers) and on aflatoxin production by A. flavus. This, therefore, forms the basis for the present investigation.

MATERIALS AND METHODS

Microorganisms. Bacillus subtilis E 52, B. cereus W18 and Aspergillus flavus (IAR strain) were used for the investigation. Lyophilized cultures of B. subtilis and B. cereus from slant were obtained from the stock cultures in the laboratory of Dr. J.A. Abalaka, Department of Biochemistry, Ahmadu Bello University, Zaria, while A. flavus was procured from Institute of Agricultural Research, Ahmadu Bello University, Zaria.

Antimicrobial agent. Anhydrous sodium metabisulphite reagent grade (BDH chemicals Ltd. Poole England) was used as the lethal agent throughout the course of this investigation.

Food samples. Peak brand unsweetened full cream condensed milk (Coop condensfabrick Ficsland Leeuwarden, Holland) and Nido (powdered milk of Nestle make) purchased from one of the local supermarkets in Samaru, Zaria were used in carrying out further studies on the organisms.

Cultivation of Microorganisms. A small quantity of the lyophilized cultures of B. subtilis and B. cereus obtained from slants were suspended separately in 100 ml sterilised nutrient broth while A. flavus was suspended in sterile buffered yeast extract medium (BYEM, Table 1). Each cultured medium was incubated at 37°C for 24 hours in a Gallenkamp orbital incubator maintained at 100 rpm.

The microorganisms were re-isolated on solid media, re-cultured and confirmed for purity by microscopic examination (morphology and Gram stain reactions). Biochemical tests, involving catalase and oxidase reactions, were used to confirm the microorganisms.

Catalase reaction. A sample of each bacterial culture was dispensed into a test tube and to this was added 1 ml of 3% (v/v) hydrogen peroxide in

Table 1. Composition of BYEM

Ingredient	Percentage (w/v)
Yeast extract	0.5
Dextrose (D-glucese)	2.0
Ammonium sulphate	0.072
Ammonium dihydrogenorthophosphate (anhydrous)	0.026

accordance with the procedure of Deibel and Evans (19). A positive catalase reaction was indicated by the effervescence of gas bubbles ($H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2} O_2$).

Oxidase reaction. A sample of each bacterial culture was subjected to oxidase reaction in accordance with Kovac's procedure (49). Both organisms were found to be oxidase positive hence aerobic. Following the confirmation of the microorganisms, pure cultures were prepared by picking colonies of the organisms on nutrient agar and subculturing them in nutrient broth and working stock cultures were prepared by transfering cultures into fresh liquid media after every 72 hours.

Effect of metabisulphite on the growth of bacteria in nutrient broth and A. flavus in buffered yeast extract medium (BYEM). Sterile nutrient broth (100 ml) was dispensed into each of six - 250 ml Erlenmeyer flasks. Different amounts of metabisulphite were added to each flask to obtain final metabisulphite concentrations ranging from 100 - 500 ppm. One of the flasks without metabisulphite served as a control. The flasks were labelled appropriately.

From exponentially growing cultures, $2x10^7$ cells per ml of B. subtilis or B. cereus was inoculated into each of the six flasks and incubated at 37°C in a Gallenkamp orbital incubator maintained at 100 rpm. Five ml of each sample was taken at 4, 8, 12, 16, 24, 48 and 72 hours of incubation to estimate growth. This was done by recording the optical densities at 650 nm on an SP6 - 400 uv spectrophotometer (Pye Unican). The same procedure was adopted for estimating growth of A. flavus except that buffered yeast extract medium served as the growth medium. In the case of the bacteria, growth was recorded by using a calibration curve relating A_{650} to bacterial growth.

Effect of metabisulphite on the growth of Bacillus subtilis and B. cereus in peak brand unsweetened full cream condensed milk at different temperatures under aerobic and anaerobic conditions.

Pretreatment of the unsweetened full cream condensed milk (peak brand). Fifty-four cans of unsweetened full cream condensed milk were used for each microorganism. The cans of milk were divided into 9 groups such that each group contained six cans. All the cans were punctured in the centre of one of the sealed ends using sterile nail. The procedure was carried out in such a manner as to avoid spillage of the milk. The cans were resealed immediately with sterile cloth tapes.

Based on the net weight of the unsweetened full cream condensed milk (170g/can), a stock solution (17g/100ml) of sodium metabisulphite was prepared using distilled water. The appropriate volume of metabisulphite solution was removed from the stock solution and then added to the cans to obtain various metabisulphite concentrations ranging from 100 - 800 ppm, for the various groups. One group of cans to which no metabisulphite was added served as control. Sterile serological pipettes with pipette filler were used in carefully dispensing the required volume of metabisulphite into each can of unsweetened full cream condensed milk through the opening made at the centre of one of the sealed ends. To do this, the cloth tape covering the puncture was slightly opened to avoid contamination and resealed immediately after the addition. Each can of unsweetened full cream condensed milk was left for 24 hours and was carefully swirled round occasionally to allow for complete homogeneity of the metabisulphite.

Simulated contamination under aerobic and anaerobic conditions. Each can of the unsweetened full cream condensed milk was inoculated with $2x10^7$ cells/ml of an exponentially growing culture of B. subtilis or B. cereus. Each can was quickly resealed and carefully swirled round.

To create aerobic conditions, following inoculation with microorganisms, three cans of the unsweetened full cream condensed milk in each group were treated in such a way that the cloth tape covering the hole made at the centre of one of the sealed ends was removed and replaced immediately with sterile cotton wool which was held in position by using adhesive cloth tapes at the sides. The sterile cotton wool was not too thin to permit contamination nor was it too thick to exclude air passage into the unsweetened full cream condensed milk.

All the other cans remained tightly covered with the cloth tapes and the cans were further deaerated to create anaerobiosis. Cans were then incubated at desired temperatures (30°, 37° and $40^\circ C$) for 5 days following which viability was carried out on each can.

Effect of metabisulphite on the growth of Bacillus subtilis B. cereus and Aspergillus flavus in powdered milk (Nido; Nestle make).

Preparation of lyophilized cultures. The bacteria were grown in nutrient broth at 37°C while A. flavus was grown in buffered yeast extract medium at 30°C. Lyophilized cultures for all the microorganisms were prepared in accordance with the procedure of Weiss (93) as modified by Abalaka (1).

To 20 ml of warm distilled water was added 4g of powdered milk of Nestle make (Nido). This was stirred thoroughly to ensure complete dissolution. Four ml of the powdered milk solution was pipetted into each of three - 100 ml round bottom flasks, one for each organism to be studied. Each was subjected to sterilization at 15lb per sq in for 2 minutes. This time was found to be sufficient enough to destroy any microorganism present as judged by the viable plate count method but was short enough to prevent coagulation of the milk. To each 4 ml of the sterile milk, o.1 ml of a 24-hour culture of the appropriate bacterial culture and A. flavus were added. The contents of each flask were then subjected to lyophilization. The number of microorganisms per g of lyophile was determined by resuspending a given quantity of the lyophilized culture in sterile 1% (w/v) peptone water (Difco), making serial decimal dilutions and plating out the dilutions. Bacterial counts and colony forming units (CFU)/g of the A. flavus were recorded following incubation at 37°C and 30°C respectively. Because of possible deterioration in viability of the lyophilized culture, the viability assay was repeated occasionally so as to ensure that the exact number of

microorganisms desired was often inoculated into samples.

Addition of metabisulphite and simulated contamination of the powdered milk (Nido). For each microorganism under investigation, several clean capped plastic bottles were taken and disinfected using chloroform. These were placed in an oven to dry. Into each of the sterile capped plastic bottles, was placed 10 g of the powdered milk (Nido) and the appropriate amount of metabisulphite added to obtain the desired concentration. Control sample (metabisulphite-free) was also set up. Each plastic bottle was quickly capped after addition of material to avoid possible contamination. The contents were thoroughly mixed, using sterile spatula and the plastic bottles were covered immediately after mixing. To each sample, the lyophilized bacterial or mould culture was added so as to obtain 10^3 cells/g for B. subtilis or B. cereus and 10^3 CFU/g for A. flavus. Each sample was again mixed thoroughly using sterile spatula and incubated at 30°C for 7 days. Viability assay was then carried out on each sample.

Selection of sporulation medium. Five different sporulation media were tried. These included those formulated by Donnellan, Nags and Levinson (21), Sterlini and Mandelstan (84), Schlessinger (79), Sadoff (74) and Abalaka (personal communication). The composition of each sporulation medium is as presented in Table 2. Each medium was tried for the spore formation by B. subtilis and and B. cereus. It was carried out by inoculating each 100 ml of the sterile sporulating medium with 1.0 ml of an exponentially growing culture of B. subtilis or B. cereus. The medium was then incubated at 37°C for 24 hours in a Gallenkamp orbital incubator maintained at 100 rpm. The refractility of the cells in each sporulating medium was determined by the Zeiss standard 14 Compound microscope in a wet mount using 0.2 ml of culture liquid. The medium that permitted the highest refractile bodies within the shortest time was selected as the best sporulation medium. After several trials, the one formulated by Sadoff (74) was selected and used throughout the entire investigation involving spore formation.

Table 2. Composition of the trial sporulation media

(a) Donnellan, Nags and Levinson (21)

Component	Concentration (mM)
Magnesium chloride	0.041
Manganese chloride	0.100
Ferric chloride/Ferrous chloride	0.00036
Sodium sulphate	0.750
Potassium dihydrogenorthophosphate	0.50
Calcium chloride	1.00
Ammonium nitrate	1.20
D-glucose	10.00
L-glutamic acid	10.00

(b) Sterlini and Mandelstam (84)

Component	Concentration (mg/litre)
Ferrous chloride	0.046
Magnesium sulphate	4.800
Manganese chloride	12.600
Ammonium chloride	535.000
Sodium sulphate	106.000
Potassium dihydrogenorthophosphate	68.000
Ammonium nitrate	96.000
Calcium chloride	219.000
L-glutamic acid	2.000
L-tryptophan	20.000

(c) Schlessinger (79)

Component	Concentration
Magnesium chloride	1.0 mM
Potassium dihydrogenorthophosphate	10.0 mM
Calcium chloride	0.7 mM
Manganese chloride	50.0 µM
Ferric chloride	1.0 µM
L-tryptophan	25.0 µg/ml
L-methionine	10.0 µg/ml

(d) Sadoff (74)

Component	Concentration (g/litre)
D-glucose	4.0
Yeast extract	2.0
Ammonium sulphate	4.0
Dipotassium hydrogenorthophosphate	1.0
Magnesium sulphate	0.8
Manganese sulphate	0.1
Zinc sulphate	0.01
Copper sulphate	0.01
Calcium chloride	0.10
Ferrous sulphate	0.001

(e) Abalaka (personal communication).

Component	Percentage (w/v)
Nutrient broth	0.80
Ammonium nitrate	0.08
Ammonium chloride	0.04
Calcium chloride	0.20
Ferrous sulphate	0.0005
Manganese sulphate	0.001
Magnesium sulphate	0.001
D-glucose	0.20

Effect of metabisulphite on the germination of Bacillus subtilis and B. cereus spores in nutrient broth.

Spores of Bacillus subtilis and B. cereus were produced in 100 ml Sadoff's (74) sporulation medium. For each bacterial organism, the sporulating culture was centrifuged in an ultracentrifuge (Beckman, JP 21) at 10,000g for 10 minutes. The supernatant obtained in each case was carefully decanted and the pellet retained. The pellet was resuspended in 5 ml of 1% (w/v) peptone water (Difco lab.). Each was heat shocked for 10 minutes at 65°C in a water bath. The heat-shock treated sample was allowed to cool before further treatment. A fraction of the heat-shocked resuspended pellet equivalent to 10^3 spores/ml was used in inoculating 100 ml of sterile nutrient broth containing metabisulphite concentrations ranging from 100 - 600 ppm. Control samples were also set up. All samples were incubated at 37°C. Five ml fractions were taken from each sample at 4, 8, 12, 16, 24, 48 and 72 hours of incubation. The optical densities were recorded each time at 650nm on SP6 - 400 uv spectrophotometer (Pye Unican) and transformed into bacterial population in counts per ml as previously described.

Effect of metabisulphite on protein content of Bacillus subtilis and B. cereus in nutrient broth (vegetative culture) and sporulation medium.

The protein content in the vegetative and sporulation cultures was determined using the Lowry, Rosebrough, Farr and Randall (52) method with slight modification.

Principle. There are two distinct steps which lead finally to the colour observed with proteins;

(a) reaction with copper in alkaline solution.

(b) reduction of phosphomolybdic-phosphotungstic acid reagent by the copper treated proteins.

The final colour intensity depends on the amount of tyrosine and tryptophan present in the protein.

Establishment of a standard curve. A portion (0.4 ml) of bovine serum albumin (BSA, sigma product) (0.1 mg per ml) was taken and diluted to obtain a stock solution of 4 µg per ml. Triplicates of 0.10, 0.20, 0.30, 0.40 and 0.50 ml of the stock solution were added to test tubes and the appropriate volumes of distilled water were added to obtain a final volume of 1.20 ml for each sample. To each tube, 2.5 ml of freshly prepared alkaline copper-tartarate solution (4 ml of 2% (w/v) $CuSO4.5H_2O$ and 4 ml of 4% (w/v) potassium-sodium tartarate solution added to 92.0 ml of 3% (w/v) sodium carbonate in 0.1M NaOH) were added, mixed and allowed to stand for 10 minutes at room temperature. Folin reagent diluted to 3 volumes (1 part + 2 parts of distilled water (0.3 ml) was added and mixed immediately. Time (30 minutes) was allowed for colour to develop at

room temperature. The absorbances were read on SP6 - 400 uv spectrophotometer (Pye Unican) at 660 nm. The average absorbance of each BSA standard was plotted against the corresponding concentration in µg per ml (Fig 1).

Determination of the protein content in bacterial samples. The total protein content of the vegetative and sporulating cultures in metabisulphite-treated and metabisulphite-free conditions were estimated for both bacteria. The vegetative culture was treated with metabisulphite concentrations ranging from 100 - 500 ppm while sporulating cultures were treated with metabisulphite concentrations ranging from 100 - 600 ppm. Five - ml portions were taken from each sample at 4, 8, 12, 16, 24, 48 and 72 hours of incubation. To determine the protein content in these samples, 0.2 ml of cultures was used in the assays. This replaced the standard BSA while all other components were added as in the standards. Each determination was done in triplicate and absorbance values were taken at 660 nm on SP6 - 400 uv spectrophotometer (Pye Unican). The absorbance values were averaged and were extrapolated on the standard curve to obtain the corresponding protein concentrations.

Effect of metabisulphite on Bacillus subtilis and B. cereus alkaline phosphatase activity in nutrient broth (vegetative culture) and in sporulation medium under aerobic and anaerobic conditions.

Alkaline phosphatase (a marker for sporulation) activity of both bacteria was determined for vegetative cells in nutrient broth and for spores in sporulation medium in accordance with the procedure of Warren (92) as further modified by Sterlini and Mandelstam (84).

Principle. Alkaline phosphatase formation in bacterial cultures is one of the biochenical events associated with growth and sporulation (Glenn and Mandelstam, 32). The enzyme is capable of acting on p-nitrophenyl phosphate to liberate p-nitrophenol in accordance with the equation;



The p-nitrophenol absorbs at 410 nm and this is used to monitor the activity of the enzyme.

Sample preparation. Six - 250 ml Erlenmeyer flasks were labelled appropriately depending on the metabisulphite concentration expected in each flask. Nutrient broth of 150 ml was dispensed into each flask to serve as the vegetative culture medium and sterilised in an autoclave 225 E (Laboratory Thermal Equipment, Greenfield, Oldhan, Lancs, England) at 15lbs per sq in. for 10 minutes. The appropriate amount of metabisulphite was added to the flasks to obtain final metabisulphite concentrations

ranging from 100 - 500 ppm. One flask without metabisulphite served as a control. An exponentially growing culture containing 2 x 10^7 cells/ml of B. subtilis or B. cereus was inoculated into each of the flasks using sterile serological pipettes. The flasks and their contents were incubated at 37°C in a Gallenkamp orbital incubator maintained at 100 rpm. For the anaerobic condition, flasks were flushed with carbon dioxide, properly sealed under nitrogen gas and were incubated at 37°C without shaking. Twenty ml fractions were removed from each flask at 4, 8, 12, 16, 24, 48 and 72 hours of incubation. These were dispensed into sterile bottles and covered before further treatments. Both B. subtilis and B. cereus grown under aerobic and anaerobic conditions were subjected to the same procedure. An identical procedure to that outlined above was used for spores in the sporulation medium in which B. subtilis and B. cereus were grown under aerobic and anaerobic conditions. The metabisulphite concentrations of 100 - 600 ppm were incorporated into the sporulation medium.

Treatment of samples for alkaline phosphatase assay.

Each sample obtained from the above procedure was subjected to centrifugation at 10,000 g for 15 minutes in an ultracentrifuge (Beckman, JP21). The supernatant obtained for each sample was retained and designated as fraction I. The pellet in each case was resuspended in 5 ml of 1.0M Tris-Hcl buffer pH 7.1. This was then subjected to disintegration in a sonicator (Model W185; Heat System ultrasonic, Inc.) for 90 seconds in three periods of 30 seconds separated by 15 seconds of cooling under ice immersion. The disintegrated pellet was designated as fraction II. Thus fractions I and II were obtained in both the vegetative and sporulation cultures under aerobic and anaerobic conditions for the two bacterial species.

Assay of alkaline phosphatase activity from the fractions.

Using the procedure of Warren (92) as further modified by Sterlini and Mandelstam (84), the alkaline phosphatase activity was assayed in the fractions at 4, 8, 12, 16, 24, 48 and 72 hours of incubation. For each determination, 1.0 ml of fraction I or II. was added to a sterile tube and two drops of toluene were added. The tube was stoppered and shaken thoroughly for 30 seconds. The mixture was kept in water bath set at 37°C for 30 minutes. After temperature equilibration, 1 ml of 1.0M Tris-Hcl buffer pH 7.1 and 1 ml of 0.1% (w/v) paranitrophenyl phosphate which served as the enzyme substrate were added. The mixture obtained was incubated for 15 minutes in a water bath maintained at 37°C and the reaction was stopped by the addition of 1.5 ml of 2M NaOH. The absorbance of each was read immediately on SP6 - 400 uv spectrophotometer (Pye Unican) at 410 nm. The activity in each case was calculated as µmoles/ml/minute. No attempt was made to purify the enzyme.

Effect of metabisulphite on Bacillus subtilis and B. cereus glucose dehydrogenase activity in vegetative culture and sporulation medium under aerobic and anaerobic conditions.

Glucose dehydrogenase is one of the many spore-specific proteins and its activity was determined in accordance with the modified method of Sadoff (74).

Principle. The spectrophotometric assay is based on the measurement of the rate of NAD^+ reduction at 340 nm in the presence of the dehydrogenase and saturating levels of glucose. D-glucose + NAD^+ + H_2O ⟶ gluconate + NADH + H^+.

Sample preparation. The procedure employed in obtaining samples for alkaline phosphatase assay in vegetative culture and sporulation medium was followed. Twenty ml fractions were removed at 4, 8, 12, 16, 24, 48 and 72 hours of incubation from the metabisulphite-treated vegetative and sporulating cultures ranging from 100 - 600 ppm concentrations. The same was done for the metabisulphite-free vegetative and sporulating cultures. Fractions were taken from both B. subtilis and B. cereus vegetative culture and sporulation medium under aerobic and anaerobic conditions.

Treatment of samples for glucose dehydrogenase assay.

Samples (20ml) taken at time intervals above, were centrifuged at 10,000g for 15 minutes. The supernatant obtained was carefully decanted and designated as fraction I. The pellet left was resuspended in 5 ml of acetate buffer (0.05M, pH 5.0) and subjected to disintegration under a sonicator for 90 seconds in three periods of 30 seconds

separated by 15 seconds of cooling in ice solution. The disintegrated pellet obtained was designated as fraction II. Thus fractions I and II were obtained for each metabisulphite treated and control samples of vegetative and sporulating cultures. These fractions were used in the assay below.

Assay of glucose dehydrogenase activity. To assay for the crude enzyme activity, the following were added to a test tube in the order: 2.4 ml of 1M tris buffer pH 8, 0.4 ml of 1M glucose, 0.4 ml of NAD^+ (0.02M) and 0.4 ml of 0.0001M Manganese sulphate. The reaction system was mixed by inversion. Fractions I or II (0.4 ml) were added to the mixture and the reaction was allowed to proceed for 2 minutes before it was stopped using 1 ml of chlorobenzene. The absorbance values were recorded on SP6 - 400 uv spectrophotometer (Pye Unican) at 340 nm and the activity calculated as µmoles of NADH produced/ml of fractions I or II/minute. Like in alkaline phosphatase, no attempt was made to purify the enzyne. No activity was obtained for the assay carried out in vegetative culture and hence no values recorded.

Colorimetric estimation of the dipicolinic acid (DPA) content of Bacillus subtilis and B. cereus in sporulating medium under the influence of metabisulphite. The estimation of the DPA content of B. subtilis and B. cereus from metabisulphite-treated sporulating cultures were carried out according to the method of Janssen, Lund and Anderson (44).

Principle. The colorimetric method utilizes the colour complex formed by interaction of ferrous ion with dipicolinic acid (DPA). The complex of ferrous ion and DPA formed is unstable but with the addition of a reducing agent such as ascorbic acid, another complex results and this is stable for at least 2 hours and can therefore be measured colorimetrically.

Sample preparation. The sporulating medium formulated by Sadoff (74) was used. To this medium, various metabisulphite amounts were added to obtain concentrations ranging from 100 - 600 ppm for both organisms. Twenty ml portions of the spore suspension formed under aerobic and anaerobic conditions were taken at 4, 8, 12,16, 24, 48 and 72 hours of incubation at 37°C. These were later used in the estimation of the DPA content.

Establishment of a standard curve. In order to establish a standard curve (Fig.2) for the DPA estimation, volumes of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1 and 2.4 ml of the standard DPA stock solution (0.1g per litre) were dispensed into test tubes. Appropriate volumes of distilled water were added to each tube to obtain a final volume of 4.0 ml. To develop colour in each tube, freshly prepared 1.0 ml of

ferrous ammonium sulphate (1% w/v) and ascorbic acid (1% w/v) in 0.5M acetate buffer pH 5.5 was added. A yellowish brown colouration developed and the absorbance readings were taken at 440 nm on SP6 - 400 uv Spectrophotometer (Pye Unican) against a reagent blank. The blank contained every other component except DPA.

Estimation of the DPA content in bacterial samples. Spore suspension (20 ml) taken at different time intervals either from the ones subjected to aerobic or anaerobic conditions were autoclaved at 15lb per sq in for 15 minutes using autoclave 225E. All cultures (metabisulphite-treated and metabisulphite-free) were autoclaved alike. The suspension was allowed to cool and acidified with acetic acid (0.4 ml, 1.0N). The mixture was left at room temperature for 1 hour during which time aggregation of insoluble materials occured. A clear supernatant of the mixture was obtained after centrifuging at 15,000g for 10 minutes. Four ml of the supernatant obtained was pipetted using pipette filler. To this mixture, 1.0 ml of the colour reagent was added and the absorbance readings were recorded at 440 nm on SP6 - 400 uv Spectrophotometer. The absorbance readings were extrapolated to the standard curve to obtain the corresponding concentrations of the DPA.

Effect of metabisulphite on spore formation of Bacillus subtilis and B. cereus under aerobic and anaerobic conditions.

For each bacterium, seven - 250 Erlenmeyer flasks were taken and 100 ml of sporulating medium was dispensed into each. Six of the flasks were treated with different amounts of metabisulphite to obtain concentrations ranging from 100 - 600 ppm. The remaining flask served as a control. Each flask was inoculated with exponentially growing culture of 2 x 10^7 cells/ml of B. subtilis or B. cereus. These were incubated at 37°C in a Gallenkamp orbital incubator maintained at 100 rpm to achieve the aerobic condition. Five ml of the sporulating culture was taken from each flask at 4, 8, 12, 16, 24, 48 and 72 hours of incubation. Each was subjected to serial decimal dilutions and 0.2 ml of the appropriate dilution taken and the number of spores formed estimated using the Zeiss standard 14 compound microscope.

The same procedure as above was followed for the anaerobic condition except that the flasks used were flushed with carbon-dioxide, properly sealed under nitrogen gas and were incubated at 37°C without shaking.

Effect of metabisulphite on aflatoxin production by Aspergillus flavus.

In determining the effect of metabisulphite on aflatoxin production by A. flavus a number of steps were followed.

Growth of Aspergillus flavus and production of aflatoxin. Eight - 250 ml Erlenmeyer flasks were filled each with 100 ml of buffered yeast extract medium. These were sterilised in an autoclave and allowed to cool. Different amounts of metabisulphite were added to each of seven flasks to obtain metabisulphite concentrations ranging from 100 - 700 ppm. The eighth flask contained no metabisulphite and hence served as a control. Each flask was inoculated with 0.2 mls of A. flavus (obtained from stock culture) and incubated at 30°C on a rotary shaker at 100 rpm for 7 days. Twenty - mls of the suspension formed in each flask was removed on the 7th day of incubation and used in carrying out the study.

Extraction of aflatoxin from samples. In the extraction procedure, the method adopted by Elegbede (22) was followed. To each of the 20 ml sample obtained above, 100 ml of chloroform was added and shaken at 200 rpm on a rotary shaker for 24 hours. The clarified extract was then shaken with sodium bicarbonate (0.1M, 50 ml) in a separatory funnel. The lower chloroform layer obtained was drained into another separatory funnel and reextracted with 50 ml sodium bicarbonate. The lower chloroform layer was drained into a well labelled flask and retained.

The chloroform extract was evaporated under reduced pressure at 40°C using rotary evaporator fitted with a vacuum pump. The residue obtained was dissolved in aqueous methanol (85% (v/v), 100 ml). The

solution was transfered to a separatory funnel and extracted with 40 ml petroleum-ether 3 x (b.pt 60 - 80°C). The combined petroleum-ether extracts were further extracted with aqueous methanol (100 ml). The solution obtained was again extracted with chloroform (3 x 50 ml).

The chloroform extract was filtered through a bed of anhydrous sodium sulphate (BDH) (15g) and washed again with fresh chloroform (20ml). The filterate obtained was evaporated to dryness under reduced pressure at 40°C. The residue obtained was redissolved in benzene acetonitrile (98: 2v/v, 1.0 ml) until required for further use. The whole procedure as explained above was carried out in treating 20 ml samples of A. flavus cultures derived from the various metabisulphite concentration.

THIN LAYER CHROMATOGRAPHY OF SAMPLES.

(i) Preparation of chromatoplates. Kieselgel 60 PF (50g) was shaken with distilled water (100 ml) for 2 minutes in a glass stoppered flask. The resultant slurry was used to prepare thin layers on five glass plates (20 x 20 cm) using a spreader (Shandon Scientific Co. Ltd; London NW10, England) and plate leveller (Quickfit Model CR 7/1, Gallenkamp and Co. Ltd; London EC 2P 2ER, England). The plates were dried at room temperature for 30 minutes taking care to reduce the amount of dust that could contaminate the plates. The plates were then activated for two hours at 120°C, allowed to cool for 30 minutes and then stored in a plate cabinet.

Qualitative analysis of aflatoxins. To be able to identify the various types of aflatoxins produced by A. flavus, the extracted samples(30 µl

aliquot were spotted together with aflatoxin standards B_1 (0.02 µg), B_2 (0.02 µg), G_1 (0.02 µg) and G_2 (0.02 µg) on the prepared chromatoplates. The spots were applied 2 cm from the bottom of the thin layer chromatographic plate using a spotting template as a guide. Plates were developed in a tank containing toluene-ethyl acetate-formic acid (5:4:1v/v/v). After the solvent had advanced 15 cm from the origin the plates were removed from the tank.

The solvent was allowed to evaporate and the plates were viewed under long-wave (375 nm) and shortwave (254 nm) u.v. light. Spots in the samples corresponding in colour and R_f to the standards (see Table 3:1) were marked and the zone containing a particular aflatoxin was scrapped off from the plates and eluted with chloroform (5 ml).

For each aflatoxin type obtained, the name was written together with the metabisulphite concentration applied and kept in dark bottles in the refrigerator until the concentrations of aflatoxins were determined.

Determination of the aflatoxin concentration. The Velasco fluorotoxinmeter (VFM) (Neotec, Inc. U.S.A.) was used in determining the concentrations of the various aflatoxins produced by *A. flavus* in the metabisulphite-treated buffered yeast extract medium. It is a rapid and accurate method of determining aflatoxin concentrations in ppb as opposed to the cumbersome chromatographic methods that depend on comparative visual colour areas. It gives instant recordable numerical values.

Principle of VFM. The system makes use of a glass microcolumn which is packed with florisil, sand, silica gel and alumina. The detection of

aflatoxin is made by trapping it on the special florisil layer. The optical system comprises of an ultraviolet light source, various filters and two photodetectors. The filters allow only energy between 310 and 410 nm to pass through and the energy from the uv lamp is concentrated primarily at 365 and 405 nm. The energy at 365 nm (λ_1) which is the desirable energy is unaffected by the filter while that at 405 nm is severely attenuated. It is the unaffected energy (365 nm), that excites the florisil layer in the microcolumn. Once excited, at 365 nm, the florisil fluoresces at a second, longer wavelength (λ_2). The energy output at λ_2 is proportional to both the original excitation energy at λ_1 and the concentration in ppb of aflatoxin trapped by the florisil layer. Thus, by adjusting the excitation energy so as to maintain constant value, the energy output at λ_2 becomes a direct measure of the aflatoxin concentration in ppb. (Inst. Manual, VFM, 1974).

Calibration of VFM. The instrument was calibrated following the method adopted by Ogbadu, (60). Blank was prepared by wetting prepared micro-column with chloroform: methanol mixture (96:4 v/v) from the base. Aflatoxin standards B_1 (10 µg/ml), B_2 (10 µg/ml, G_1 (10 µg/ml) and G_2 (10 µg/ml) used were obtained from Elegbede, J.A. Biochemistry Dept., A.B.U. Zaria. Appropriate dilutions using chloroform as the solvent were made to obtain concentrations of 20 ng/ml for each aflatoxin standard. The absorbance value of each standard was read on CE 505 Cosil spectrophotometer at maximum absorption wavelength (365 nm) from which the corresponding concentration was calculated using the formula:

$$\text{Concentration of aflatoxin} = \frac{A \times M.W \times 1000 \times C.F.}{E}$$

where A = absorbance

M.W = Molecular weight of aflatoxin type

E = Molar absorptivity of the aflatoxin type

C.F.= Correction factor for the instrument

0.90 (Rodricks and Stoloff, 72).

To take the reading on the VFM, one ml of a particular aflatoxin standard was injected into the top of the chloroform wetted prepared microcolumn. This was allowed to drain and one ml of chloroform: methanol mixture (24:1 v/v) was injected and allowed to drain. The reading for each standard was taken while still wet and the expected 20 ppb was obtained.

Determination of the aflatoxin concentration produced by A. flavus. To determine the concentration of the aflatoxin extracted, one ml of each sample was injected unto the top of a chloroform wetted prepared micro-column. On draining, 1 ml of choroform: methanol mixture (24:1 (v/v) was injected and allowed to drain. Before taking the reading on the VFM, the reading for the particular aflatoxin standard was first taken and this was followed by that of the sample. For each determination, the microcolumn was turned to 180° after taking the first reading to obtain yet another reading. The average was taken as the concentration of the aflatoxin. Based on the concentration of each standard as calculated earlier on, the reading on the VFM was therefore corrected for using direct proportionality relationship.

RESULTS

Table 3. Growth of vegetative culture of Bacillus subtilis in metabisulphite-treated nutrient broth.

Concentration of metabisulphite (ppm)	Bacterial population (counts/ml)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	6.7×10^4	4.0×10^5	1.8×10^6	3.6×10^6	1.3×10^7	4.4×10^7	$>10^9$
100	6.7×10^4	2.7×10^5	1.0×10^6	1.9×10^6	7.4×10^6	2.6×10^7	7.8×10^7
200	3.3×10^4	1.3×10^5	6.0×10^5	1.3×10^6	4.8×10^6	1.6×10^7	5.1×10^7
300	—[a]	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 4. Growth of vegetative culture of Bacillus cereus in metabisulphite-treated nutrient broth

Concentration of metabisulphite (ppm)	Bacterial population (counts/ml)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	1.0×10^5	6.0×10^5	2.3×10^6	6.4×10^6	1.5×10^7	6.5×10^7	$>10^9$
100	6.7×10^4	4.7×10^5	1.5×10^6	3.2×10^6	9.2×10^6	3.7×10^7	6.7×10^7
200	$3,3\times10^4$	2.7×10^5	1.1×10^6	2.1×10^6	6.6×10^6	2.4×10^7	5.0×10^7
300	3.3×10^4	2.0×10^5	9.0×10^5	1.3×10^6	3.8×10^6	1.3×10^7	2.8×10^7
400	—[a]	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a]No viable bacterial count was recorded.

Table 5. Viability of bacteria at different temperatures in metabisulphite-treated unsweetened full cream condensed milk (peak).

Conc. of metabisulphite (ppm)	*Bacillus subtilis*						*Bacillus cereus*					
	Aerobic			Anaerobic			Aerobic			Anaerobic		
	Temperature °C						Temperature °C					
	30	37	40	30	37	40	30	37	40	30	37	40
	Bacterial count/ml			Bacterial count/ml			Bacterial count/ml			Bacterial count/ml		
0	4.7×10^{7}	6.3×10^{7}	2.3×10^{7}	2.5×10^{6}	3.3×10^{6}	1.3×10^{6}	2.2×10^{8}	1.3×10^{8}	1.6×10^{7}	2.5×10^{6}	1.9×10^{6}	8.7×10^{5}
100	6.5×10^{6}	1.6×10^{7}	3.6×10^{6}	1.2×10^{5}	5.2×10^{5}	9.0×10^{4}	1.1×10^{8}	2.5×10^{7}	2.6×10^{6}	4.7×10^{5}	2.5×10^{5}	9.0×10^{4}
200	5.9×10^{6}	5.5×10^{6}	8.7×10^{5}	2.2×10^{4}	3.6×10^{4}	1.8×10^{3}	5.1×10^{7}	8.5×10^{6}	1.2×10^{6}	5.6×10^{4}	3.5×10^{4}	5.8×10^{3}
300	1.5×10^{6}	1.6×10^{6}	5.6×10^{5}	1.8×10^{4}	1.9×10^{4}	9.5×10^{2}	3.9×10^{6}	2.7×10^{6}	2.9×10^{5}	1.3×10^{4}	9.2×10^{3}	9.5×10^{2}
400	5.0×10^{5}	5.2×10^{5}	1.6×10^{4}	4.7×10^{3}	4.4×10^{3}	1.7×10^{2}	1.4×10^{6}	3.1×10^{5}	1.7×10^{5}	4.3×10^{3}	6.2×10^{2}	2.8×10^{2}
500	3.8×10^{3}	2.0×10^{4}	1.3×10^{3}	—[a]	8.0×10^{2}	—	7.0×10^{5}	7.1×10^{4}	2.9×10^{4}	2.2×10^{2}	1.0×10^{2}	—
600	—	1.1×10^{3}	—	—	—	—	5.5×10^{4}	4.3×10^{3}	1.2×10^{3}	1.0×10^{2}	—	—
700	—	—	—	—	—	—	2.0×10^{3}	—	—	—	—	—
800	—	—	—	—	—	—	—	—	—	—	—	—

[a]No viable bacterial count was recorded.

Table 6. Viability of bacteria and Aspergillus flavus in metabisulphite-treated powdered milk (Nido) at 30°C

Concentration of metabisulphite (ppm)	Bacterial count per g		Mould count[a] per g
	Bacillus subtilis	Bacillus cereus	Aspergillus flavus
0	4.5×10^5	7.2×10^5	2.6×10^8
100	3.8×10^4	6.1×10^4	4.5×10^7
200	3.2×10^4	5.5×10^3	3.5×10^6
300	4.2×10^2	2.8×10^3	2.7×10^6
400	—[b]	1.5×10^2	1.5×10^6
500	—	—	9.3×10^5
600	—	—	8.4×10^5
700	—	—	6.7×10^4
800	—	—	4.3×10^4
900	—	—	3.9×10^4
1,000	—	—	2.5×10^3
2,000	—	—	—

[a]Mould counts recorded as colony forming units (CFU)/g

[b]No bacterial count was recorded.

Table 7. Germination of Bacillus subtilis spores in metabisulphite-treated nutrient broth.

Concentration of metabisulphite (ppm)	Bacterial population (counts/ml)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	4.3×10^5	2.5×10^6	5.8×10^6	8.5×10^6	1.3×10^7	2.8×10^7	4.8×10^7
100	1.3×10^5	1.3×10^6	4.8×10^6	6.8×10^6	1.1×10^7	2.2×10^7	3.9×10^7
200	6.7×10^4	9.3×10^5	4.4×10^6	6.5×10^6	1.0×10^7	2.1×10^7	3.6×10^7
300	3.3×10^4	6.0×10^5	1.3×10^6	2.5×10^6	5.2×10^6	1.2×10^7	2.3×10^7
400	—[a]	—	—	5.3×10^5	1.4×10^6	3.6×10^6	6.0×10^6
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 8. Germination of Bacillus cereus spores in metabisulphite-treated nutrient broth.

Concentration of metabisulphite (ppm)	Bacterial population (Counts/ml)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	2.7×10^5	1.9×10^6	5.5×10^6	8.1×10^6	1.5×10^7	3.1×10^7	5.0×10^7
100	1.7×10^5	1.1×10^6	3.7×10^6	5.6×10^6	1.1×10^7	2.4×10^7	3.8×10^7
200	1.0×10^5	8.0×10^5	3.1×10^6	5.1×10^6	9.2×10^6	2.0×10^7	3.5×10^7
300	1.0×10^5	4.7×10^5	2.5×10^6	3.9×10^6	6.6×10^6	1.5×10^7	2.4×10^7
400	3.3×10^4	4.0×10^5	1.8×10^6	3.2×10^6	5.4×10^6	1.2×10^7	2.2×10^7
500	—[a]	—	4.0×10^5	1.2×10^6	2.2×10^6	5.6×10^6	9.6×10^6
600	—	—	—	—	—	—	—

[a]No viable bacterial count was recorded.

Table 9. Growth of *Aspergillus flavus* in metabisulphite-treated buffered yeast extract medium

Concentration of metabisulphite (ppm)	Absorbance at 650 nm						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	0.04	0.10	0.21	0.56	0.93	1.82	> 2.0
100	0.02	0.05	0.13	0.37	0.61	0.97	> 2.0
200	0.02	0.03	0.09	0.28	0.53	0.87	0.95
300	0.01	0.02	0.06	0.19	0.38	0.61	0.76
400	0.01	0.01	0.04	0.08	0.24	0.42	0.59
500	—[a]	—	0.03	0.05	0.13	0.20	0.37
600	—	—	—	—	—	—	—

[a]No viable organism was detected.

Action of metabisulphite on growth of and spore germination by microorganisms. Tables 3 - 9 show the growth of the tested microorganisms (Bacillus subtilis, B. cereus and A. flavus) in various metabisulphite-treated media.

Generally, it was observed that the metabisulphite concentration required to prevent the growth of B. subtilis (300 ppm) in nutrient broth was lower than that required for growth of B. cereus (400 ppm) (Tables 3 and 4 respectively). For these same microorganisms, it was found that the prevention of the germination of their spores required metabisulphite concentrations of 500 ppm for B. subtilis and 600 ppm for B. cereus (Tables 7 and 8 respectively).

The optimum temperature for the growth of B. subtilis in unsweetened full cream condensed milk under aerobic and anaerobic conditions was found to be 37°C. At this temperature, the metabisulphite concentrations required to stop growth under aerobic and anaerobic conditions were found to be 700 and 600 ppm respectively. In the case of B. cereus the optimum temperature for the growth was found to be 30°C under aerobic and anaerobic conditions but the metabisulphite concentrations required to prevent growth in the respective cases were found to be 800 and 700 ppm (Table 5).

In powdered milk (Nido; Nestle make), the mould (A. flavus) was found to be more resistant to metabisulphite than the two bacteria studied. Whereas metabisulphite concentration of 500 ppm stopped the growth of the two bacteria, a higher metabisulphite concentration

(2000 ppm) was required to stop the growth of A. flavus (Table 6).

The growth of A. flavus in buffered yeast extract medium was prevented by a metabisulphite concentration of 600 ppm for over a period of 72 hours (Table 9).

For all the organisms studied, concentrations of metabisulphite lower than those that caused lethal effect caused decrease in growth with increase in metabisulphite concentrations.

Table 10. Protein content of vegetative culture of *Bacillus subtilis* in metabisulphite-treated nutrient broth

Concentration of metabisulphite (ppm)	Protein content (µg/ml culture)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	0.26	0.65	1.05	1.85	1.94	2.25	2.30
100	0.05	0.08	0.36	0.68	0.80	0.97	1.05
200	—[a]	—	0.21	0.50	0.53	0.70	0.85
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 11. Protein content of vegetative culture of *Bacillus cereus* in metabisulphite-treated nutrient broth

Concentration of metabisulphite (ppm)	Protein content (µg/ml culture)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	0.43	0.75	1.28	2.19	2.36	2.58	2.64
100	0.05	0.14	0.42	0.72	0.94	1.05	1.24
200	0.05	0.06	0.26	0.31	0.48	0.56	0.64
300	0.05	0.05	0.18	0.24	0.28	0.34	0.43
400	—[a]	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a]No protein was detected.

Table 12 Protein content of Bacillus subtilis in metabisulphite-treated sporulating culture

Concentration of metabisulphite (ppm)	Protein content (µg/ml culture) Sampling time (hours)						
	4	8	12	16	24	48	72
0	0.31	0.71	0.91	1.45	1.71	1.91	2.05
100	0.06	0.13	0.22	0.48	0.70	0.87	0.92
200	0.05	0.08	0.11	0.35	0.41	0.58	0.66
300	—[a]	—	0.05	0.16	0.24	0.35	0.43
400	—	—	—	0.07	0.11	0.27	0.29
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 13. Protein content of Bacillus cereus in metabisulphite-treated sporulating culture

Concentration of metabisulphite (ppm)	Protein content (µg/ml culture) Sampling time (hours)						
	4	8	12	16	24	48	72
0	0.51	0.82	1.04	1.65	1.95	2.08	2.12
100	0.09	0.31	0.41	0.69	0.88	0.93	0.99
200	0.05	0.20	0.36	0.43	0.51	0.68	0.73
300	0.05	0.11	0.19	0.34	0.43	0.51	0.59
400	0.05	0.05	0.07	0.28	0.35	0.36	0.39
500	—[a]	—	0.05	0.09	0.19	0.21	0.25
600	—	—	—	—	—	—	—

[a]No protein was detected.

Table 14a Alkaline phosphatase activity of metabisulphite-treated vegetative cultum of Bacillus subtilis under aerobic condition in nutrient broth.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	0.12	0.12	0.25	0.37
100	—	—	0.12	0.62	1.12	1.24	1.24
200	—	—	0.25	1.49	1.86	2.48	2.61
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 14b Alkaline phosphatase activity of metabisulphite-treated vegetative culture of Bacillus subtilis under aerobic condition in nutrient broth.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.12	0.62	2.61	4.72	5.96	6.83	7.20
100	0.12	0.12	0.50	1.98	2.73	3.60	3.72
200	0.06	0.12	0.37	0.87	1.49	1.99	1.99
300	—[c]	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a] Activity assayed from fraction 1 (culture supernatant)

[b] Samples were taken from nutrient broth at time intervals indicated

[c] No activity was detected

[d] Activity assayed from fraction II (disintegrated pellet)

Table 15a Alkaline phosphatase activity of metabisulphite-treated vegetative culture of Bacillus cereus under aerobic condition in nutrient broth.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a](Umoles/ml/min) Sampling time (hours)[b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.12	0.12	0.12	0.25	0.25
100	—	—	0.62	0.99	1.24	1.86	2.11
200	—	0.12	0.87	2.36	2.73	3.60	3.60
300	—	0.12	0.99	2.48	2.85	3.97	4.10
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 15b Alkaline phosphatase activity of metabisulphite-treated vegetative culture of Bacillus cereus under aerobic condition in nutrient broth.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d](Umoles/ml/min) Sampling time (hours)[b]						
	4	8	12	16	24	48	72
0	0.25	0.99	2.98	5.09	6.45	7.32	7.82
100	0.12	0.37	1.61	2.98	4.84	5.09	5.21
200	0.12	0.12	0.62	0.99	2.05	2.36	2.73
300	0.12	0.12	0.37	0.75	1.61	1.73	2.23
400	—[c]	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a]Activity assayed from fraction I (culture supernatant)

[b]Samples were taken from nutrient broth at time intervals indicated.

[c]No activity was detected.

[d]Activity assayed from fraction II (disintegrated pellet)

Table 16a Alkaline phosphatase activity of vegetative culture of Bacillus subtilis in metabisulphite-treated nutrient broth under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a](Umoles/ml/min) Sampling time (hours)[b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	—	0.06	0.06	0.06
100	—	—	0.12	0.99	1.61	1.99	2.23
200	—	—	—	—	—	—	—
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 16b Alkaline phosphatase activity of vegetative culture of Bacillus subtilis in metabisulphite-treated nutrient broth under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d](Umoles/ml/min) Sampling time (hours)[b]						
	4	8	12	16	24	48	72
0	—[c]	0.25	1.99	3.10	4.10	5.71	5.96
100	—	—	0.37	1.37	2.11	2.61	2.98
200	—	—	—	—	—	—	—
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a]Activity assayed from fraction I (culture supernatant)

[b]Samples were taken from nutrient broth at time intervals indicated.

[c]No activity was detected.

[d]Activity assayed from fraction II (disintegrated pellet).

Table 17a Alkaline phosphatase activity of vegetative culture of Bacillus cereus in metabisulphite-treated nutrient broth under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	—	—	0.06	0.12
100	—	—	—	0.74	0.99	1.61	1.74
200	—	—	0.62	1.49	2.36	3.10	3.35
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

Table 17b Alkaline phosphatase activity of vegetative culture of Bacillus cereus in metabisulphite-treated nutrient broth under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	0.62	1.99	3.85	5.34	6.21	6.58
100	—	—	1.37	2.11	2.98	3.48	4.10
200	—	—	0.25	0.74	0.87	1.24	1.74
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (culture supernatant)

[b] Samples were taken from nutrient broth at time intervals indicated.

[c] No activity was detected

[d] Activity assayed from fraction II (disintegrated pellet)

Table 18a Alkaline phosphatase activity of Bacillus subtilis in metabisulphite-treated sporulating culture under aerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (Umoles/ml/min)						
	Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.12	0.12	0.25	0.25	0.25
100	—	0.12	1.12	2.61	3.23	3.32	3.32
200	—	0.62	1.61	4.10	4.72	4.84	4.84
300	—	0.62	2.23	4.22	5.09	5.09	5.21
400	—	—	—	4.47	5.71	5.96	5.96
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 18b Alkaline phosphatase activity of Bacillus subtilis in metabisulphite-treated sporulating culture under aerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (Umoles/ml/min)						
	Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.25	2.11	4.84	8.01	9.31	9.56	9.68
100	0.12	1.49	2.36	3.60	5.09	5.83	6.21
200	0.12	0.37	1.49	1.74	2.61	2.85	2.98
300	0.12	0.25	0.62	0.99	1.49	1.99	1.99
400	—[c]	—	—	0.12	0.25	0.37	0.37
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet).

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Table 19a Alkaline phosphatase activity of Bacillus cereus in metabisulphite-treated sporulating culture under aerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	0.12	0.12	0.25	0.25	0.25	0.25
100	—	0.50	2.98	3.35	3.85	4.10	4.10
200	—	1.37	3.35	4.10	4.59	4.84	4.96
300	—	1.61	4.10	4.59	5.21	5.59	5.71
400	—	1.74	4.47	4.96	5.96	6.58	6.83
500	—	—	4.72	5.34	6.08	7.20	7.20
600	—	—	—	—	—	—	—

Table 19b Alkaline phosphatase activity of Bacillus cereus in metabisulphite-treated sporulating culture under aerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.37	2.98	7.20	9.18	10.50	10.90	11.20
100	0.25	2.11	3.35	5.09	5.96	6.21	6.45
200	0.12	0.75	2.23	3.10	3.60	4.22	4.34
300	—[c]	0.25	1.12	2.11	2.61	3.10	3.35
400	—	0.12	0.50	1.12	1.24	1.24	1.37
500	—	—	—	0.25	0.37	0.37	0.37
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating culture at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet)

Table 20a Alkaline phosphatase activity of Bacillus subtilis in metabisulphite-treated sporulating culture under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	—	0.06	0.06	0.06
100	—	—	0.37	1.61	2.36	2.73	2.98
200	—	—	0.99	2.73	3.48	4.10	4.10
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 20b Alkaline phosphatase activity of Bacillus subtilis in metabisulphite-treated sporulating culture under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	2.73	4.72	6.08	6.70	6.83
100	—	—	1.61	2.48	2.98	3.35	3.48
200	—	—	0.50	0.75	1.37	1.74	1.74
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet)

Table 21a Alkaline phosphatase activity of Bacillus cereus in metabisulphite-treated sporulating culture under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [a] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	—	—	0.06	0.06
100	—	—	0.62	1.49	2.36	2.85	3.10
200	—	—	1.37	2.48	3.35	3.85	3.85
300	—	—	2.48	3.72	4.84	4.96	4.96
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 21b Alkaline phosphatase activity of Bacillus cereus in metabisulphite-treated sporulating culture under anaerobic condition.

Concentration of metabisulphite (ppm)	Alkaline phosphatase activity [d] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	6.33	7.82	8.94	9.31	9.43
100	—	—	2.73	5.46	5.71	5.96	5.96
200	—	—	1.49	3.48	3.97	4.10	4.34
300	—	—	—	1.12	1.24	1.24	1.37
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet).

Table 22a Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus subtilis under aerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [a] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.01	0.01	0.01	0.01	0.02	0.02	0.02
100	0.01	0.04	0.09	0.15	0.19	0.20	0.20
200	0.02	0.08	0.14	0.20	0.28	0.35	0.36
300	0.02	0.10	0.16	0.24	0.33	0.40	0.41
400	0.02	0.10	0.21	0.27	0.39	0.42	0.43
500	—[c]	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 22b Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus subtilis under aerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [d] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.07	0.20	0.48	0.65	0.67	0.67	0.68
100	0.03	0.11	0.29	0.41	0.44	0.46	0.46
200	0.01	0.03	0.18	0.24	0.28	0.29	0.29
300	0.01	0.01	0.13	0.16	0.19	0.20	0.21
400	—[c]	—	0.05	0.08	0.11	0.13	0.13
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected

[d] Activity assayed from fraction II (disintegrated pellet).

Table 23a Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus cereus under aerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [a] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.01	0.01	0.01	0.02	0.02
100	0.01	0.06	0.17	0.23	0.25	0.26	0.28
200	0.01	0.11	0.19	0.27	0.28	0.31	0.33
300	0.01	0.12	0.20	0.31	0.36	0.38	0.39
400	0.01	0.12	0.22	0.34	0.40	0.41	0.43
500	0.01	0.13	0.24	0.37	0.42	0.42	0.45
600	—	—	—	—	—	—	—

Table 23b Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus cereus under aerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [d] (Umoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	0.08	0.22	0.53	0.71	0.78	0.81	0.82
100	0.04	0.10	0.26	0.39	0.44	0.48	0.49
200	0.02	0.03	0.19	0.30	0.39	0.41	0.41
300	0.01	0.01	0.15	0.20	0.25	0.27	0.29
400	0.01	0.01	0.11	0.14	0.19	0.21	0.21
500	—[c]	—	0.03	0.07	0.09	0.11	0.12
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant)

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet)

Table 24a Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus subtilis under anaerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [a] (μmoles/ml/min)						
	Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.005	0.005	0.005	0.005	0.01
100	—	—	0.06	0.10	0.17	0.19	0.20
200	—	—	0.11	0.19	0.25	0.26	0.31
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 24b Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus subtilis under anaerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [d] (μmoles/ml/min)						
	Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.42	0.53	0.59	0.61	0.63
100	—	—	0.22	0.30	0.32	0.36	0.38
200	—	—	0.05	0.09	0.13	0.15	0.16
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet).

Table 25a Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus cereus under anaerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [a] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	—	0.01	0.01	0.01	0.01
100	—	—	0.08	0.15	0.20	0.22	0.24
200	—	—	0.13	0.21	0.27	0.28	0.30
300	—	—	0.22	0.30	0.34	0.36	0.39
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 25b Glucose dehydrogenase activity of metabisulphite-treated sporulating culture of Bacillus cereus under anaerobic condition.

Concentration of metabisulphite (ppm)	Glucose dehydrogenase activity [d] (μmoles/ml/min) Sampling time (hours) [b]						
	4	8	12	16	24	48	72
0	—[c]	—	0.49	0.59	0.63	0.64	0.66
100	—	—	0.20	0.27	0.32	0.35	0.35
200	—	—	0.11	0.15	0.21	0.24	0.25
300	—	—	—	0.03	0.09	0.11	0.12
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a] Activity assayed from fraction I (sporulating culture supernatant).

[b] Samples were taken from sporulating medium at time intervals indicated.

[c] No activity was detected.

[d] Activity assayed from fraction II (disintegrated pellet)

Table 26a Dipicolinic acid (DPA) content of Bacillus subtilis in metabisulphite-treated sporulating medium under aerobic condition.

Concentration of metabisulphite (ppm)	DPA content (µg/ml culture supernatant)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	—[a]	40.5	115.5	151.5	166.5	168.0	168.0
100	—	—	60.0	94.5	112.5	115.5	121.5
200	—	—	21.0	42.0	52.5	52.5	55.5
300	—	—	—	19.5	21.0	21.0	21.5
400	—	—	—	12.0	17.5	19.5	19.5
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

Table 26b Dipicolinic acid (DPA) content of Bacillus subtilis in metabisulphite-treated sporulating medium under anaerobic condition.

Concentration of metabisulphite (ppm)	DPA content (µg/ml culture supernatant)						
	Sampling time (hours)						
	4	8	12	16	24	48	72
0	—[a]	27.5	86.5	105.0	122.5	132.5	140.5
100	—	—	39.5	48.5	69.5	86.5	89.5
200	—	—	—	8.5	16.5	16.5	16.5
300	—	—	—	—	—	—	—
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a]No DPA detected.

Table 27a Dipicolinic acid (DPA) content of Bacillus cereus in metabisulphite-treated sporulating medium under aerobic condition.

Concentration of metabisulphite (ppm)	DPA content (µg/ml culture supernatant) Sampling time (hours)						
	4	8	12	16	24	48	72
0	—[a]	62.5	124.5	175.5	187.5	190.5	192.5
100	—	—	75.0	100.0	142.5	145.5	145.5
200	—	—	25.5	47.5	57.0	67.5	69.0
300	—	—	—	16.5	33.0	40.5	40.5
400	—	—	—	12.0	24.0	33.0	33.0
500	—	—	—	—	7.5	7.5	9.0
600	—	—	—	—	—	—	—

Table 27b Dipicolinic acid (DPA) content of Bacillus cereus in metabisulphite-treated sporulating medium under anaerobic condition.

Concentration of metabisulphite (ppm)	DPA content (µg/ml culture supernatant) Sampling time (hours)						
	4	8	12	16	24	48	72
0	—[a]	39.5	99.5	126.5	149.5	158.5	158.5
100	—	—	58.0	72.5	89.5	125.0	125.5
200	—	—	17.5	31.5	45.5	58.5	59.5
300	—	—	—	7.5	18.5	18.5	19.5
400	—	—	—	—	—	—	—
500	—	—	—	—	—	—	—
600	—	—	—	—	—	—	—

[a]No DPA detected.

Table 28. Relative degree of sporulation in metabisulphite-treated cultures of Bacillus subtilis and Bacillus cereus under aerobic condition.

Concentration of metabisulphite (ppm)	Degree (%) of sporulation in organism													
	Bacillus subtilis							Bacillus cereus						
	Sampling time (hours)							Sampling time (hours)						
	4	8	12	16	24	48	72	4	8	12	16	24	48	72
0	12.9	32.3	45.2	64.5	77.4	90.3	100	14.7	38.7	50.2	67.5	80.5	93.3	100
100	5.6	19.4	35.5	58.1	72.6	82.3	95.2	5.8	19.9	38.2	60.4	76.4	86.5	96.7
200	3.2	12.9	24.2	32.3	48.4	59.7	64.5	3.1	12.4	20.8	31.2	62.4	74.2	80.4
300	2.4	8.9	14.5	17.7	25.8	30.6	38.7	2.2	7.5	16.3	20.1	40.2	50.8	60.6
400	0	0	0	3.2	9.7	13.7	19.4	0.6	3.5	8.2	12.4	27.7	33.5	40.8
500	0	0	0	0	0	0	0	0	0	3.2	6.9	11.1	19.8	24.4
600	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Percentage sporulation at 72 hour for control was fixed at 100 while others were calculated relative to this.

Table 29. Relative degree of sporulation in metabisulphite-treated cultures of Bacillus subtilis and Bacillus cereus under anaerobic condition.

Concentration of metabisulphite (ppm)	Degree (%) of sporulation in organism.													
	Bacillus subtilis							Bacillus cereus						
	Sampling time (hours)							Sampling time (hours)						
	4	8	12	16	24	48	72	4	8	12	16	24	48	72
0	0	14.5	23.6	45.5	58.2	83.6	100	0	24.3	40.4	58.7	76.2	90.1	100
100	0	4.5	14.6	34.5	56.4	72.7	89.1	0	22.5	32.1	44.2	68.3	78.4	92.5
200	0	0	6.4	11.8	30.9	45.5	56.4	0	14.6	20.2	26.5	36.9	52.1	62.6
300	0	0	0	0	0	0	0	0	4.7	10.1	13.2	17.8	20.9	26.8
400	0	0	0	0	0	0	0	0	0	0	0	0	0	0
500	0	0	0	0	0	0	0	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Percentage sporulation at 72 hour for control was fixed at 100 while others were calculated relative to this.

Action of metabisulphite on some biochemical events associated with sporulation and/vegetative cells.

Tables 10 - 27 show the influence of metabisulphite on some biochemical events associated with spores and/vegetative cells. It was observed that while the protein content in the sporulating cultures of B. subtilis and B. cereus were reduced to an undetectable level by metabisulphite concentrations of 500 and 600 ppm respectively (Tables 12 and 13) the vegetative cultures required only metabisulphite concentrations of 300 and 400 ppm respectively to achieve similar effects (Tables 10 and 11).

Generally, it was observed in both vegetative and sporulating cultures that, as the alkaline phosphatase activity in the cell extract decreased, there was an increase in its activity in the supernatant. Also the level of alkaline phosphatase in sporulating cultures is higher when compared to its level in vegetative culture (Tables 14 - 21).
Below the metabisulphite concentrations that stopped alkaline phosphatase activity (300 ppm for B. subtilis and 400 ppm for B. cereus both under aerobic condition) it was observed that the alkaline phosphatase activity in the cell extract (pellet) decreased with increasing metabisulphite concentrations while the alkaline phosphatase activity in the supernatant increased with increasing metabisulphite concentrations (Tables 14 and 15). Similar trends were observed for the cultures under anaerobic condition, except that the concentrations that stopped the activity of the enzyme were found to be lower under anaerobic condition (Tables 16 and 17). The trend observed with the alkaline phosphatase

activity in the sporulating culture was not strikingly different from the vegetative culture except that much higher metabisulphite concentrations were required to stop the alkaline phosphatase activity under sporulating condition than in the vegetative culture (Tables 18 - 21).

As in the case of alkaline phosphatase activity, the activity of glucose dehydrogenase was also observed to increase in the supernatant and to decrease in cell extract as the metabisulphite concentration increases until the synthesis of the enzyme was finally stopped. This observation was common to both aerobic and anaerobic conditions (Tables 22 - 25).

In all cases of investigation, it was noticed that higher concentrations of metabisulphite were required to cause biochemical lesions in B. cereus than in B. subtilis. All other concentrations below that required to cause complete inhibition of enzyme synthesis were observed to cause an increase in the activity of the enzymes in the supernatant and a decrease in the activity in the cell extract with increasing metabisulphite concentrations.

Similarly, it was observed that the metabisulphite concentrations that reduced the dipicolinic acid content of the bacteria to undetectable level were generally higher for B. cereus than for B. subtilis under aerobic and anaerobic environments (Tables 26 and 27).

In drawing up the relative degree of sporulation (Tables 28 and 29) the degree of sporulation in control sample at 72 hour was taken to be 100% while sporulation under other conditions was estimated relative

to the control. It was observed that for B. subtilis and B. cereus there was a drop in sporulation for the metabisulphite-treated cultures under aerobic and anaerobic conditions. Also at metabisulphite concentrations of 500 ppm for B. subtilis and 600 ppm for B. cereus under aerobic; 300 and 400 ppm for B. subtilis and B. cereus respectively under anaerobic condition, no spores were observed. Also high incidence of sporulation at a particular time interval seems to be followed by high level of enzyme and DPA synthesis.

Table 30. Concentrations of aflatoxin standards as determined on CE 505 Cosil spectrophotometer and Velasco fluorotoxinmeter (VFM).

Aflatoxin Standards	solvent	λ max	Abs. on CE 505	Mol. weight	Calculated[a] Concentration nℊ/ml	E	Conc. on VFM (ppb)
Aflatoxin B_1	Chloroform	365	1.40	312	20.0	19800	20
Aflatoxin B_2	Chloroform	365	1.45	314	19.6	20900	20
Aflatoxin G_1	Chloroform	365	1,16	328	20.0	17100	20
Aflatoxin G_2	Chloroform	365	1.22	330	20.0	18200	20

[a] Calculated concentration = $\frac{A \times M.wt \times C.F. \times 1000}{E}$

where A = absorbance

M. wt = Molecular weight

C.F. = Correction factor

E = molar absorptivity of aflatoxin type

Table 31. The relative mobility and fluorescence characteristics of aflatoxins extracted from the buffered yeast extract medium

Aflatoxins	R_f values	Colour under u.v. light	
		Longwave (375nm)	Shortwave (245nm)
Aflatoxin B_1	0.62	faint blue	faint blue
Aflatoxin B_2	0.60	faint blue	faint blue
Aflatoxin G_1	0.41	blue-green	blue-green
Aflatoxin G_2	0.42	blue-green	blue-green

Table 32. Concentrations of aflatoxins produced by *Aspergillus flavus* in metabisulphite-treated buffered yeast extract medium.

Concentration of metabisulphite (ppm)	Concentration of aflatoxin (ng/ml)			
	B_1	B_2	G_1	G_2
0	31.8	18.0	24.6	15.9
100	22.3	13.7	17.4	11.2
200	18.6	10.4	15.9	7.8
300	11.2	6.7	9.6	4.9
400	8.9	3.5	6.3	2.8
500	3.8	1.9	2.4	0.8
600	—[a]	—	—	—
700	—	—	—	—

[a]No aflatoxin obtained.

Effect of metabisulphite on aflatoxin production by *A. flavus*.

Table 32 reveals that at metabisulphite concentration of 600 ppm, no aflatoxin was detected. It was also observed that below 600 ppm of metabisulphite, the level of aflatoxins detected decreased with increasing metabisulphite concentrations. Aflatoxin B_1 was most abundant while G_2 was found to be the least abundant in the extract.

D I S C U S S I O N

Action of metabisulphite on growth and spore germination of microorganisms. The data have revealed that lower metabisulphite concentrations were required to prevent the growth of B. subtilis and B. cereus in nutrient broth (Tables 3 and 4) than the prevention of the spore germination of the two bacteria in the same medium (Tables 7 and 8). This observation seems to agree with the fact that spores are generally known to be more resistant to most chemicals than their vegetative counterparts (70). The high resistance of spores to chemicals has been attributed to a number of factors which are still speculative.

The difference in the degree of susceptibility of B. subtilis and B. cereus to metabisulphite treatment is difficult to explain since it has not been possible to correlate differences in susceptibility of the organisms to either morphological or physiological characteristics. It is not out of place to state that resistance is an inherent genetic characteristic that varies from species to species.

The finding that the bacteria (B. subtilis and B. cereus) thrived better in unsweetened full cream condensed milk (peak) under aerobic conditions than anaerobic conditions is note worthy. A higher metabisulphite concentration is required to suppress the growth of the organisms under aerobic condition than under anaerobic condition. Bacillus subtilis and B. cereus respectively required 700 and 800 ppm metabisulphite concentrations under aerobic condition but required 600 and 700 ppm metabisulphite

concentrations under anaerobic condition (Table 5). It may be infered that by keeping acrobic organisms which cause food spoilage under anaerobic conditions, one may minimise the extent of the bacteria growths and may therefore be an added advantage to the preservation of food with metabisulphite. It therefore follows, that the metabisulphite concentration that is capable of eliminating the organisms under aerobic condition should be able to eliminate them under anaerobic condition provided the medium is the same. It is important to mention here, that in most homes in this country, only few can afford the usage of a whole tin of unsweetened condensed milk in a day and to avoid its spoilage by microorganisms, the hole made on the can of milk is usually covered with paper after a very small quantity has been taken. The paper cannot prevent the growth of organisms in the milk hence spoilage can easily occur. The addition of an antimicrobial agent such as metabisulphite by the food industries may reduce considerably post-processing contamination of this nature.

The mould (A. flavus) required higher metabisulphite concentration than the bacteria in order to stop their growth. However, the differences in media composition may be an important factor. In attempting to eliminate these three organisms studied from a particular food, it is only the metabisulphite concentration that is active against the mould that is likely to be the most important since such a concentration will be active against the two bacteria. The growth of the three organisms in powdered milk (Nido; Nestle make) still revealed that A. flavus was more resistant to metabisulphite treatment (2000ppm) than the two bacteria (500 ppm)

employed in this investigation (Table 6). The powdered milk (Nido) was selected as one of the media for this investigation because in most homes in this country where peak milk or other forms of milk is a luxury, the powdered milk (Nido) is the milk of choice. The Nido milk usually cannot be consumed all at once but in bits until it is used up. Exposure of the milk could lead to contamination by food spoilage microorganisms which then grow to cause food problems under suitable conditions. The incorporation of metabisulphite might help to minimise this problem of post processing contamination. It has been shown that 2000 ppm of metabisulphite (equivalent to 674 ppm SO_2) is considered unharmful to animal and human tissues (2) and therefore the incorporation of this level of metabisulphite into food for the purpose of eliminating moulds is desirable.

Action of metabisulphite on some biochemical events associated with sporulation and/vegetative cells. Metabisulphite was found to have an inhibitory effect on protein synthesis since the protein content continued to decrease with increasing concentrations of metabisulphite (Tables 10 - 13). Judging from the role of protein in general metabolism this inhibition of protein synthesis may contribute significantly to the antimicrobial action of metabisulphite. Since enzymes are proteins, the synthesis of some enzymes important in general metabolism might also be inhibited.

Generally, a very striking point observed with the effect of metabisulphite on alkaline phosphatase and glucose dehydrogenase activities is the fact that as the activities of the two enzymes assayed from the cell extract decreased there was an increase in their activities assayed from the

supernatant. The observation leads credence to the speculation that the metabisulphite might be acting on the bacterial cell membrance, thereby causing the leakage of these enzymes into the medium. Possibly this may be one of the mechanisms by which metabisulphite exerts its antimicrobial effect. Lucker, Mosser and Hanson (53) have earlier postulated leakages of ATP into the surrounding medium when bacterial cell membrane is subjected to sulphite. Since ATP may be in a common pool with other metabolites and enzymes, the effect of metabisulphite may be to cause leakage of vital metabolites.

The metabisulphite concentrations required to stop alkaline phosphatase activity under aerobic condition when assayed from sporulating or vegetative cultures were always found to be higher than the metabisulphite concentrations required to achieve the same effect under anaerobic conditions. Oxygen facilitates spore formation (73) and therefore under anaerobic condition there would be less spore formation and less alkaline phosphatase production. Implicit in this statement is the fact that there appears to be some correlation between sporulation and alkaline phosphatase level (55). This possibly explains why a lower metabisulphite concentration was required to stop alkaline phosphatase activity under anaerobic condition than the metabisulphite concentration required under aerobic condition. A similar explanation might account for the observation in which glucose dehydrogenase activity assayed from the cultures maintained under aerobic condition was shown to require more metabisulphite to stop its synthesis than in the cultures maintained under anaerobic condition (Tables 22 - 25).

The observation that the metabisulphite concentrations required to stop alkaline phosphatase activity in vegetative cells under aerobic condition were more than the concentrations required under anaerobic condition was not unexpected as the organisms are more or less aerobes.

The reaction of the bacterial spore alkaline phosphatase to metabisulphite treatment still reveals the higher resistance of spores to chemical treatment than their vegetative counterparts. While the B. subtilis spore and B. cereus spore alkaline phosphatase synthesis were stopped by metabisulphite concentrations of 500 and 600 ppm respectively under aerobic condition (Tables 18 and 19) their vegetative counterparts required 300 and 400 ppm respectively (Tables 14 and 15). Moreover, the higher level of the alkaline phosphatase in sporulating culture than the vegetative cells is suggestive of the enzyme being involved in sporulation. This was found to be the case with glucose dehydrogenase whose activity was not detected at all throughout the period of investigation in the vegetative culture.

Metabisulphite was observed to have an inhibitory effect on the dipicolinic acid (DPA) content of the bacterial spores since the DPA level continued to decrease with increasing concentrations of metabisulphite until its synthesis was finally stopped. Under aerobic condition, at metabisulphite concentrations of 500 ppm (B. subtilis) and 600 ppm (B. cereus) and under anaerobic condition, at metabisulphite concentrations of 300 ppm (B. subtilis) and 400 ppm (B. cereus), the DPA content was reduced to an undetectable level (Tables 26 and 27). Since metabisulphite

was found to reduce the level of DPA in the bacterial spore to an undetectable level, spore resistance as reported with heat and some chemicals (70) may not be significant. Metabisulphite, therefore, has a promising future in the control of microorganisms in food.

In all, there was a parallelism between sporulation and the levels of the enzymes (alkaline phosphatase and glucose dehydrogenase) as well as the level of DPA. This was because as the degree of sporulation increased so also the levels of the enzymes and the level of DPA increased. Moreover, the same level of metabisulphite prevented the synthesis of the enzymes, reduced the DPA to undetectable level and stopped sporulation in the two bacteria. The biochemical events are therefore involved in sporulation and hence may serve as good indices in the food industries where the preservative efficacy of antimicrobial agents are tested.

It would seem that in all cases, the metabisulphite causes leakage of the bacterial components at sublethal dose, the degree of leakage increasing as the metabisulphite concentrations increase. At higher but still sublethal metabisulphite concentration, the synthesis of enzymes and proteins was inhibited even though viability was not completely affected and finally, as the lethal dose was approached the cells failed to show any sign of viability.

Effect of metabisulphite on aflatoxin production by Aspergillus flavus.

At metabisulphite concentration of 600 ppm, no aflatoxin was detected and at this same metabisulphite concentration, no growth of A. flavus was observed in the buffered yeast extract medium (Tables 9 and 32). It appeared that the metabisulphite inhibited the growth of the mould, resulting in less aflatoxin production. In more acidic medium such as coke (pH 4.5) compared to buffered yeast extract medium (pH 6.5) lower metabisulphite concentration would be required to stop aflatoxin production since metabisulphite is more active at low pH values than at high pH values (53). The metabisulphite concentration of 600 ppm still falls safely within the level tolerated by humans (47). It implies, therefore, that the possible use of metabisulphite in minimising aflatoxin production by A. flavus in drinks like orange juice and carbonated beverages might have a promising future.

CONCLUSIONS AND SUMMARY

Metabisulphite concentration of 600 ppm was found to be the maximum metabisulphite concentration required to adequately prevent the growth and the germination of spores of Bacillus subtilis and B. cereus over a period of 72 hours under aerobic condition in nutrient broth.

In unsweetened condensed (peak) milk, the metabisulphite concentration required to stop the growth of B. subtilis over a period of 5 days under aerobic condition was observed to be 700 ppm while B. cereus required a metabisulphite concentration of 800 ppm over the same period. However, under anaerobic condition, metabisulphite concentrations of 600 and 700 ppm were observed to stop growth of B. subtilis and B. cereus respectively over a period of 5 days in unsweetened condensed milk. It is felt that keeping foods under anaerobic condition especially in the presence of metabisulphite may therefore be an added advantage to the preservation of food.

The reaction of B. subtilis, B. cereus and A. flavus to metabisulphite treatment in powdered milk (Nido; nestle make) showed that the mould was most resistant, requiring metabisulphite concentration of 2,000 ppm to stop its growth over a period of 7 days. It may be suggested that when the mould (A. flavus) and the tested bacteria are to be eliminated with metabisulphite, it is the concentration required by the mould that is of importance since this concentration will eliminate the tested bacteria.

It was observed that metabisulphite concentrations of 500 and 600 ppm were required to stop spore formation of B. subtilis and B. cereus

respectively over a period of 72 hours under aerobic condition in sporulating medium. Lower metabisulphite concentrations achieved same effect under anaerobic condition. It was also obsdrved that the same metabisulphite concentrations of 500 and 600 ppm were required to stop alkaline phosphatase, glucose dehydrogenase activities as well as reducing the protein and DPA contents to undetectable levels in B. subtilis and B. cereus respectively under aerobic condition over a period of 72 hours. Lower metabisulphite concentrations were observed to achieve same effect under anaerobic condition for the two bacteria. A parallelism may therefore be drawn between the degree of sporulation and the levels of enzymes and DPA investigated since as there is increase in degree of sporulation so also there is an increase in the levels of the enzymes and the DPA level. It is felt that the biochemical events investigated may serve as good indices in food industries where the preservative efficacy of antimicrobial agents are tested. The action of metabisulphite on the enzymes suggests that metabisulphite acts by causing leakages of these enzymes.

Aflatoxin production by A. flavus in buffered yeast extract medium was prevented by metabisulphite concentration of 600 ppm when incubated for a period of 7 days. The same 600 ppm metabisulphite concentration stopped the growth of A. flavus for over a period of 72 hours in the same medium. The metabisulphite thus inhibits both growth and aflatoxin production.

SUGGESTIONS FOR FURTHER STUDIES

Although the results obtained so far have shown that metabisulphite is a promising antimicrobial agent, there is still the need to carry out further studies along the following suggestions:

1. The effect of composition of various media and metabisulphite on sporulation and spore germination.
2. The influence of metabisulphite on spore formation and germination over a longer duration than employed in this investigation.
3. The influence of natabisulphite on calcium ion uptake of bacterial spores and on the formation of DPA.
4. The effect of metabisulphite on different aflatoxigenic strains of *Aspergillus*.
5. The effect of metabisulphite on the organoleptic quality of a number of food products.

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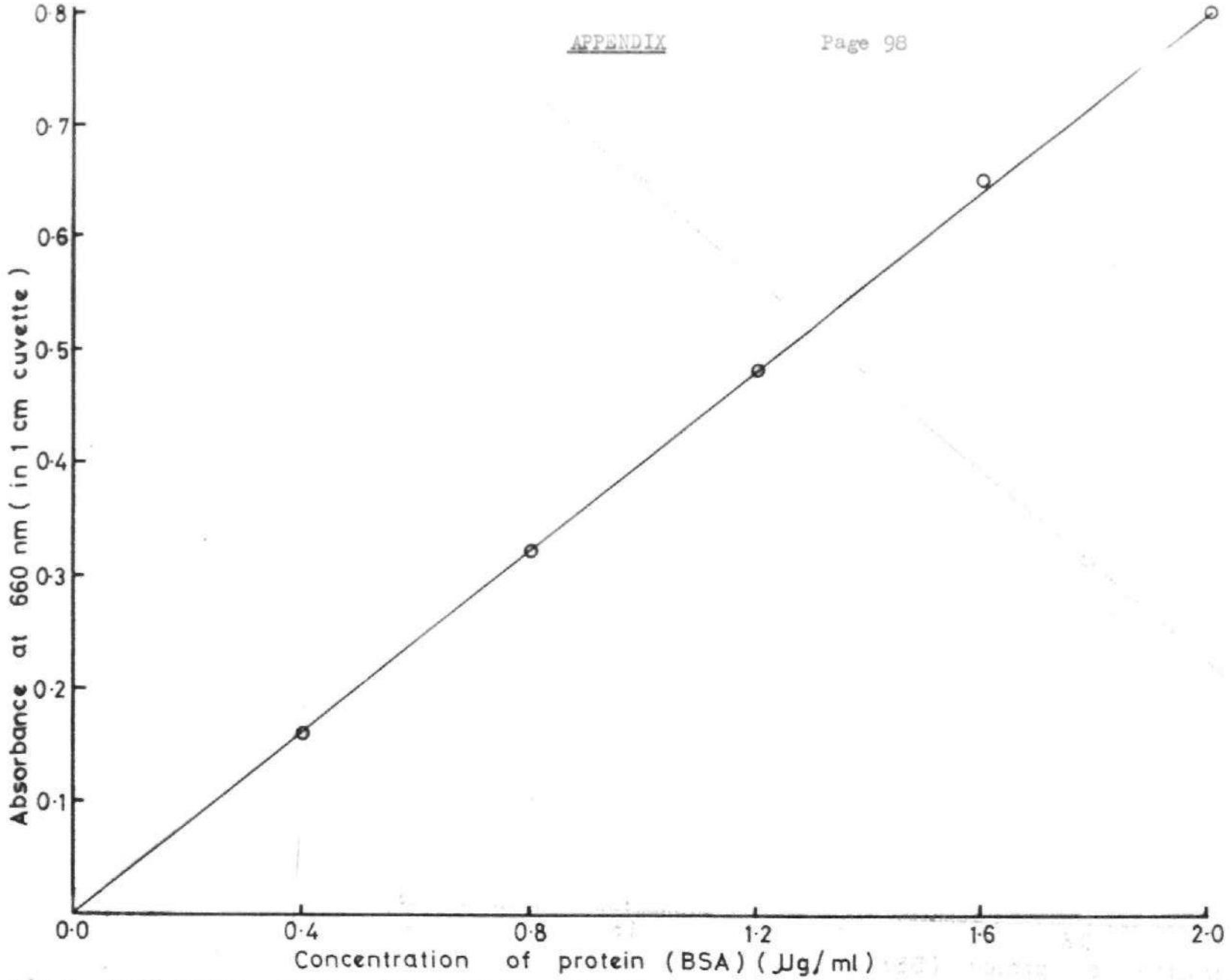


Fig. 1. Calibration curve for protein estimation

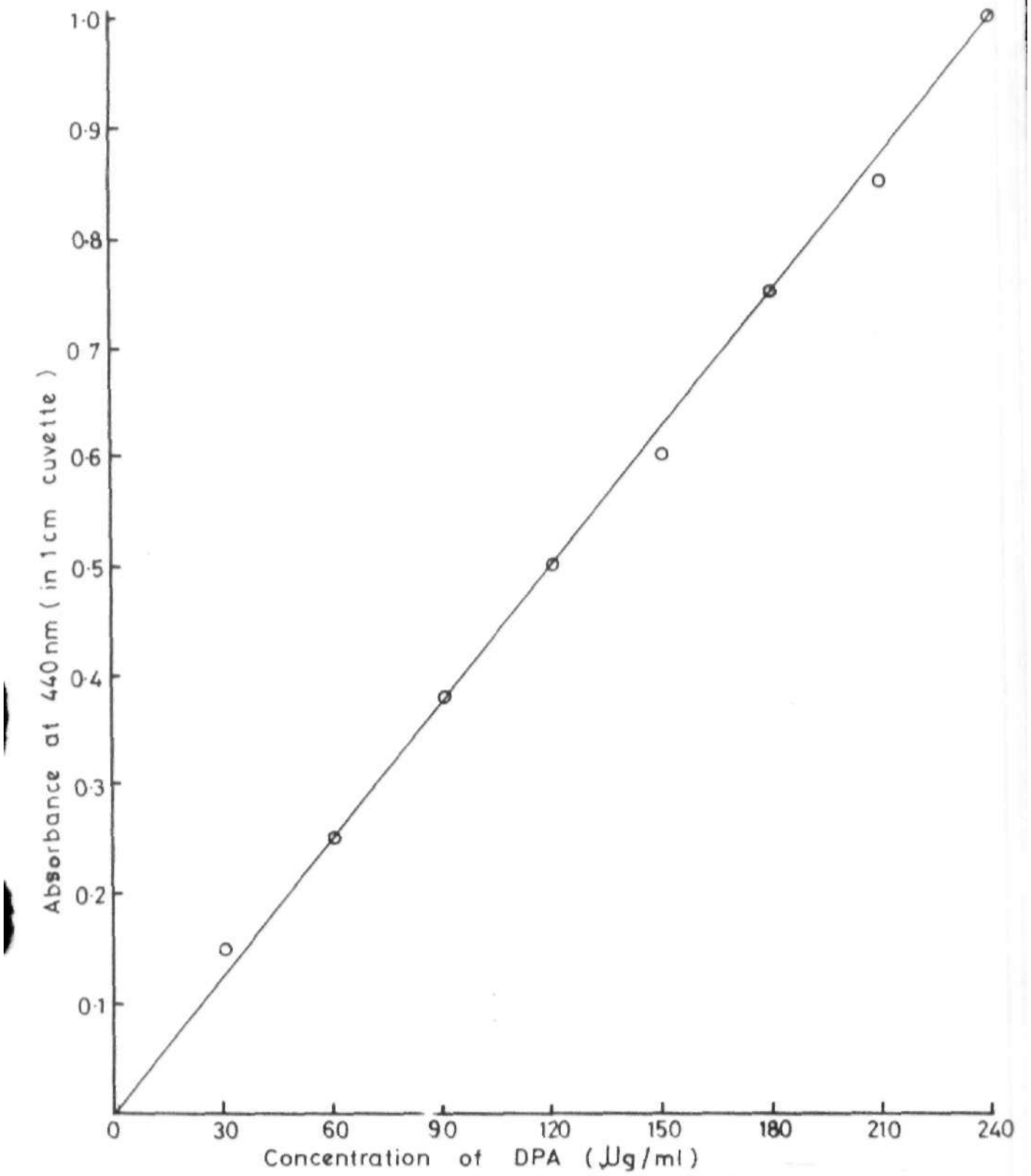


Fig. 2. Calibration curve for dipicolinic acid (DPA) estimation