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EFFECT OF GAS CONCENTRATION ON THE GROWTH OF AEROBIC AND ANAEROBIC MICROORGANISMS IN A FOOD-PACKAGE MODEL SYSTEM

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Luis Martin Rayas

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Bruce R. Harte

Major professor

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BY

Luis Martin Rayas

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ABSTRACT

EFFECT OF GAS CONCENTRATION ON THE GROWTH OF AEROBIC AND ANAEROBIC MICROORGANISMS IN A FOOD-PACKAGE MODEL SYSTEM.

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Growth of both Bacillus subtilis (aerobic) and Clostridium sporogenes (anaerobic) was determined individually and simultaneously using the same model system. Oxygen concentration in the head space was also monitored over the storage time. The parameters studied were: a) Initial oxygen content in the head space (1%, 5%, 21% oxygen and 21% oxygen with oxygen absorber <FreshPax™>); b) Bacterial growth in the model system (aerobic vs. anaerobic and mixture of both); c) Water activity <Aw> (0.96 and >0.99); and d) storage temperature (10 and 25°C).

Storage time, initial oxygen content in the head space, and abusive temperature (25°C) had a significant effect on bacterial growth ($P<0.01$). An oxygen absorber was effective in reducing oxygen content and preventing growth of aerobic microorganisms (Bacillus subtilis) at 25°C. Anaerobic growth was detected at 25°C with oxygen concentrations of 1% and <0.5% in the head space. Anaerobic growth was prevented at 10°C in jars at all oxygen head space concentrations including 1% oxygen and <0.5% oxygen. Aerobic growth was observed at 10°C in jars with initial oxygen concentrations of 1%, 5%, 21% with oxygen absorber, and 21% in the mixed culture. No growth of aerobic organisms was observed at 10°C for the samples containing pure culture and 21% initial oxygen in the head space. Oxygen level was reduced from 21% to about 0.1% in about 5 hours at 25°C and in approximately 12 hours at 10°C with an oxygen absorber, regardless of the water activity in the media.

To:

my wife, Ana Teresa; for her infinite love, help, and understanding;

my son, Luis Alberto; and

my parents, Luis and Irma; for their unconditional support and love

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INTRODUCTION

To control oxygen in a packaged product, vacuum, steam condensation, oxygen absorbers and gas flushing can be used. Oxygen leads to the growth of aerobic spoilage organisms and also is necessary for various oxidative reactions. Use of oxygen absorbers in concert with barrier packaging can be used to obtain oxygen levels of 0.01 % in a sealed package. However, growth of anaerobes must also be considered relative to food safety.

The market demand for prepared foods in optimal conditions has lead packaging and food scientists to work to increase the shelf life of perishable food products. The role of oxygen in degradation of perishable foods is well documented (Labuza, 1987; Brody, 1987). Although the kind of spoilage to be expected can often be predicted from the composition of the food (Ayres et al., 1980), microorganisms from many sources such as the product itself, environment (soil and water), man, and processing equipment may grow and cause quality changes, and create an unsafe product (Manomaiwiboon, 1990). Oxygen leads to the growth of aerobic spoilage organisms such as Bacillus bacteria and Penicillium and Aspergillus molds. By controlling the gases in the package the growth of surface molds is slowed when the oxygen content is below 1% (Brody, 1987). The use of carbon dioxide at high concentrations (>25 %) provides good control over the growth of aerobic organisms but may impart a flavor to the product (Brody, 1987) or when used with ground-roasted coffee, increases the chance that the package will burst (Labuza, 1987). Although carbon dioxide is bacterostatic and mycostatic, chalk-like yeast growth can occur on bread in storage (Brody, 1987).

Oxygen is also necessary for various oxidative reactions such as lipid oxidation and color change (Fennema, 1985), which can occur in potato chips, dried fish, beef jerky, semi-moist cookies, and other products. These reactions are important in food degradation and have economic importance. Thus far, the most common ways of controlling oxidative deterioration of lipids in foods are through either removal of the oxygen and gas-tight packaging (i.e., vacuum packing or replacing the air in the package with an inert gas, such as nitrogen); or the incorporation of a suitable antioxidant into the food (Stewart et al., 1982).

The current utilization of oxygen absorbers in the food industry is limited. "With respect to US. regulations, the Food and Drug Administration requires that the film used for oxygen absorber sachets be made of materials approved for use under 21CFR 175-178. In addition, any printing inks have to be from approved substances or shown not to be able to migrate into the food" (Labuza, 1987). Oxygen can be reduced to approximately 0.5 % by vacuum packaging or gas flushing. However, oxygen absorbers are capable of reducing residual oxygen in the package head-space to less than 0.01 % (Labuza, 1987). At 0.5% there is short term control of growth of aerobic organisms, but less control of oxidative chemical reactions. And in bread, because of the increase in packaging material barrier requirements, the cost of packaging for controlled atmosphere is approximately triple that of the chemicals and double that of pasteurization in Germany (Brody, 1987). There are data available to show that with use of oxygen scavenger sachets, a significant shelf-life improvement is attained (Labuza, 1987). The technology seems to work well for bakery products. At 0.01 % oxygen there is complete control of aerobic growth and effective control of oxidative reactions. This enhanced control is, in many cases, economically significant. However, whether such very low residual oxygen levels (0.01%) enhance the risk of the

outgrowth and toxin production of anaerobic organisms of food safety concern, such as Clostridium botulinum, has not been studied in the US. Koski (1988) suggested that there may be a question as to whether controlled atmosphere packaging and modified atmosphere packaging will gain acceptance in the US. not because there is a question whether the technology will be established, but, rather to what extent it can safely capture substantial segments of the US. food industry. Oxygen-free atmosphere at a product water activity greater than 0.92 can be conducive to the growth of many microbial pathogens, including Clostridium botulinum (Labuza, 1987), thus, use of the sachet under certain conditions can be dangerous. Furthermore, there are some Clostridia serotypes (B, E, and F) that can grow and produce toxin at temperatures as low as 3.3°C (Genigeorgis, 1987). In general, growth and toxin production will occur if products are held at temperature higher than 10°C (Goldoni et al., 1980). To date, most companies have relied on pH, water activity, temperature, and additives such as nitrite to ensure that there is no food safety concern when using oxygen absorbers. A variety of well known chemical additives ranging from sorbic acid to the benzoates can exert beneficial microbiostatic effects (Brody, 1987). However in Japan, where oxygen absorbers have had longer use, suppliers report that there is no greater danger at the lower residual oxygen levels (0.01%) generated by an oxygen absorber than at the higher levels (0.5%) generated by gas flushing. Reportedly, this opinion is based on the concept of oxidative reduction potential and findings that this factor is not substantially changed whether at 0.5% or 0.01% residual oxygen. However, this has not yet been substantiated in the US. under inoculum levels acceptable and customary to the US. food industry and academia. Also, there may be consumer resistance to the use of sachets in a food package. A concern may be preoccupation of consumers due to accidental ingestion and

potential toxicity of too much iron. This is minimized by use of the sachet concept as well as printing the words "Do not eat" on the sachet. The largest sachet contains only 7g of iron, so this would amount to an accidental consumption of only 0.1 g/kg for the standard 70-kg person, about 200 times less than the LD_{50} value of 16 g/kg body weight for rats (Labuza, 1987). When these negatives are overcome, use of this technology, (perhaps in other forms as well), will have significant impact for the US. food industry.

The hypothesis in which this study was based consisted on testing the microbial food safety concern existed in using oxygen absorbers in packages containing food products with high water activity and abused temperatures. To acomplish this, the present study had three specific objectives; First to evaluate growth of C. sporogenes and B. subtilis in a food-package model system inoculated with C. sporogenes (ATCC 11437) and/or B. subtilis (ATCC 9372) both individual and in a mixed system. These organisms were used to determine relative growth of aerobes/anaerobes in a perishable food product packaged with oxygen absorbers and modified atmospheres. The microbial load of each system was determined as influenced by: a) Storage temperature. b) Presence of oxygen absorber. c) Initial head space atmosphere. and d) Water activity. The second objective was to determine the effect of oxygen concentration and temperature on the growth of these organisms individually and in a mixed culture. A final objective was to determine the efficiency of the oxygen absorber utilized in this study as provided by Multiform Desiccants, Inc. This oxygen absorber used was "FreshPax™" type B-300, and tested under the influence of temperature and water activity.

LITERATURE REVIEW

There are at least five important factors which have an effect on the shelf life of a packaged food-product. These are: package barrier, head space gas content, thermal processing conditions, food composition and water activity (Aw), and storage temperature.

Effect of package barrier on the shelf life of a food-package system.

When specifying packaging materials, the following should be considered in terms of shelf life: Gas and water permeability; heat seal ability and adhesion; UV transmission properties; and chemical interactions (food / packaging interactions). Lewis (1989) defines packaging as the process of forming the packaging material into the desired shape, adding the product, establishing the gas atmosphere and sealing the package. Saunders (1988) reported that the use of suitable equipment, protective film, and suitable gas mixture, integrated with clean and appropriate handling, have allowed the food industry to achieve longer shelf life of food products. MAP packaging machines have been designed (Anonymous, 1978; Rice, 1991) to obtain optimum packaged food products. Examples of such machines include "Neutrafill" gas flushing system for packing potato products; 452 Flowpak system for packing bakery, tobacco, dairy/cheese, meat products, coffee/beverages, biscuit, snack food and vending packs; and SLS-VGF series fill/vacuumize/gas flush/seal machines for deli salads and vegetarian cuisine. Pinto (1979) reported that gas flush and vacuum lines are strikingly similar and the same equipment is often used for both systems.

According to Lundquist (1969) "a good vacuum package should have a minimum of 24-26 in Hg of vacuum in the packages and be formed from a film having a maximum oxygen permeability of 0.8 cc oxygen/100 sq. in/24 hr/73^{o}F - 50 % relative humidity". He indicated that a decrease in package vacuum below 24 in Hg will permit spoilage organisms to grow and as the oxygen permeability increases above 0.8 cc, the opportunity for yeast and mold to develop increases. Sacharow (1968) indicated that package permeability to gas and moisture has a major effect on shelf life of candies.

Morrison et al. (1977) reported that Owen-Illinois Co. developed a system for replacing vacuum-packing in metal cans with gas-flushing in composite cans with application to noodles, peanuts, soybeans, nuts, chips, and similar products. This system required low-initial investment, nitrogen usage was moderate, and the packed products had shelf lives equivalent to vacuum-packed products. More recently, use of wide mouth PET (polyester), polypropylene and polycarbonate jars has increased dramatically and will become more significant in the 1990's (Pack alimentaire '90, 1990).

Cabral et al. (1979) observed that metallized polyethylene terephthalate used as a protective barrier for potato chips yielded 33 days of shelf life under different conditions and had less oxidation, less moisture pick-up and better sensory grades than other potato chips. Labuza (1982) reported that potato chips having a moisture content of 3.57% were rejected by a taste panel because of their texture deterioration. These changes can be prevented by using of various moisture barriers.

Barnett et al. (1987) studied the effect on extended refrigerated storage on the shelf life of treated and non treated (2.3% potassium sorbate dip) boned trout (Salmo gairdneri) packaged in laminated high/low density "semi-

permeable" polyethylene bags in the presence of a CO_2-enriched modified atmosphere (MA). They found that the combination of packaging, refrigeration, and MA effectively doubled the shelf life of the fresh trout product. Potassium sorbate limited bacterial growth but did not extend shelf life beyond what was obtained with MA alone. The carbon dioxide content in the trout flesh appeared to be a function of CO_2 in the head space of the bags which was reduced by about 50 % during the first 20 days of storage.

Kautter et al. (1978) evaluated the botulism hazard in mushrooms packaged in commercial polyvinylchloride (PVC) film. They found of 1,078 packages examined that all were free of botulinum toxin.

Effect of head space gas content in a food-package system.

In all industrialized countries, today the demand for food products is generally oriented to the ready-to-eat or the ready-to-cook packaged foods, fresh or with fresh appearance, low processed and additives-free (Castelvetri, 1991). Modified or controlled atmospheres have been in existence since the 1920s (Smock, 1979). Modified atmosphere packaging (MAP) "is the enclosure of food products in gas-barrier materials, in which the gaseous environment has been changed to slow respiration rates, reduce microbiological growth, and retard enzymatic spoilage -with the final effect of lengthening shelf life" (Koski, 1988). Many studies (Hotchkiss, 1988; Silliker et al., 1980; Ginigeorgis, 1985; Hintlian et al., 1986; and Hobbs, 1979) confirm that the behavior of microorganisms in the presence of a MA is variable, in that: (1) not all microbial species are inhibited by a definitive atmosphere and, if inhibited, not with the same rate; (2) the effect of a definitive atmosphere depends on the nature of the food being packaged; and (3) particularly with non-heat processed foods, the possibility that MA supports

pathogens while delaying spoilage is still a matter of concern. Rothwell (1988) and Louis (1990) reported that these systems have been in use for a longer time and developed faster in Western Europe (Primarily The United Kingdom, Germany, Denmark, Sweden, Italy, Switzerland, Norway, Finland, and France), Japan, and Australia than the US. Lioutas (1988) explained that MAP systems have been in European markets for longer times due to (1) Geographically smaller countries/distribution systems; (2) European buyers shop more frequently; (3) Greater refrigerated food awareness/acceptance by the European consumer; (4) Total system/quantity approach for the MA technology; (5) Higher prices of food in European marketplace as compared to US. made MAP economically feasible; (6) Different driving forces for MA and refrigerated foods in Europe and US. (European market is retail driven while the US. market is packer/consumer driven); and (7) the European presence of large food chains dedicated to high-quality refrigerated foods.

More than half of all food products are perishable (Woods, 1987). There is evidence that MAP can extend the shelf life of many perishable products including meat, fish, poultry, and some forms of produce by 50-400 % (Hotchkiss, 1988). Zagory et al. (1988), Labell (1985), Anonymous (1990), Feldheim et al. (1979), Ohkata (1990), Schulz (1979), Anonymous (1979), Transfresh (1979), Rovema (1979), Anonymous (1977), observed extension in the shelf life of various food products such as snacks, cut chilled fruit, bread, fresh produce, shredded produce, and other foodstuffs by the use of MAP. Wolfe (1980), determined that in the case of fresh meat, fish, and poultry, the major components of concern resulting from dynamic changes are enzymatic aging processes, microbial spoilage, fat oxidation, and the various oxidation states of the myoglobin pigment. These changes can be prevented by using modified atmospheres (MA). Another

broad area of application is in packaging oxygen-sensitive powdered foods in gas-flushed composite cans since this contributes to increased shelf stability comparable to products in metal cans but at a more economical cost (Anonymous, 1980). Also for packaging raw and processed peanuts, Container Corp. of America's "Containervac" system (bag-in-box container and a machine that pulls a vacuum and then heat seals the bag) was tested and it was found that cold storage was unnecessary since it provides excellent sanitation and protection (Food and Drug Packaging, 1979). This system is in use for frozen fruits, ice cream flavorings and hops and has potential use for rice, beans, pecans, corn, and other foodstuffs.

Rice (1990), reported that modified atmosphere packaging has had success in marketing prepared, refrigerated potato products. The potato products were included in two varieties, hash browns and mashed potatoes and were first introduced in foodservice sizes, 20-lb. MAP gas-flushed barrier bags packed in corrugated cartons. Northern Star test marketed 20-oz, consumer-size versions of the hash browns in MAP bags and found that consumers responded well. Both products now are moving into national distribution. Rice (1990) also reported that Chinese entrees in CPET barrier trays (6" x 5" x 1 5/8" deep) and barrier film (anti-fog multi-layer structure with a nylon base) are being packaged in a carbon dioxide/nitrogen gas blend. This is accomplished manually by depositing the batch-cooked and cooled product into the trays, evacuating air and backflushing with the gas blend and the lidding film heat-sealed to the trays. The oxygen permeability exhibited was 0.04 cc/tray/day at 72^{o}F and 21% (ambient level) oxygen. The shelf life of the refrigerated trays was 21-day without use of MSG or freezing at refrigeration temperature. Foley (1984) demonstrated that for an oxygen-permeable air-evacuated packaging system of fresh high quality fish fillets

stored at 32°F, shelf life was 16-17 days. No significant odor buildup occurred. Flavor, texture and appearance were retained which translated to increased retailer sales and profits. The "Veryfrais" process was developed in France and combines MA and a new regenerating system for packing of fish and meat products and is available to the United States under licensing agreement (Louis, 1987).

Rothwell (1988) reported that the principal packaging systems for MAP are: horizontal form-fill-seal, rigid and semi-rigid "Atmospack"; horizontal & vertical form-fill-seal, flexible "Pillow-Pack," FFS pouches; pre-formed plastic tray or bag; form-fill-seal composite board/plastic tray or pre-formed board/plastic tray; pre-formed coated pulp tray; bag in box; darfresh vacuum skin packaging; Cryovac shrink vacuum bags; bivac vacuum shrink system; isopak vacuum skin system; and trayvac systems.

MAP is specially important in extending the shelf life of perishables at the retail level (Fig. 1). Sakamaki et al. (1988) studied the stability of oat cereal as influenced by selected barriers packaged with and without oxygen absorbers. They placed 50 g of oat cereal in plastic pouches made from either polyethylene (PE) with thickness of 84 μm or polyvinylidene chloride (PVDC)-coated polypropylene/polyethylene (PP/PE) with thickness of 86 μm. An oxygen absorber (OA) was introduced (consisting of iron powder, water and catalysts) into the package. The initial oxygen concentrations were 1, 5, or 21% for the second film, and lipid oxidation was monitored using a modified TBA test. They observed that since the oxygen absorbing ability of the scavenger (160 ml of oxygen/day at 21°C and 40% R.H.) was low compared to the oxygen permeability of the PE pouch (220m l/pouch/day), the OA was quickly overwhelmed by oxygen permeating through the poor barrier material. They concluded that in general, in the good

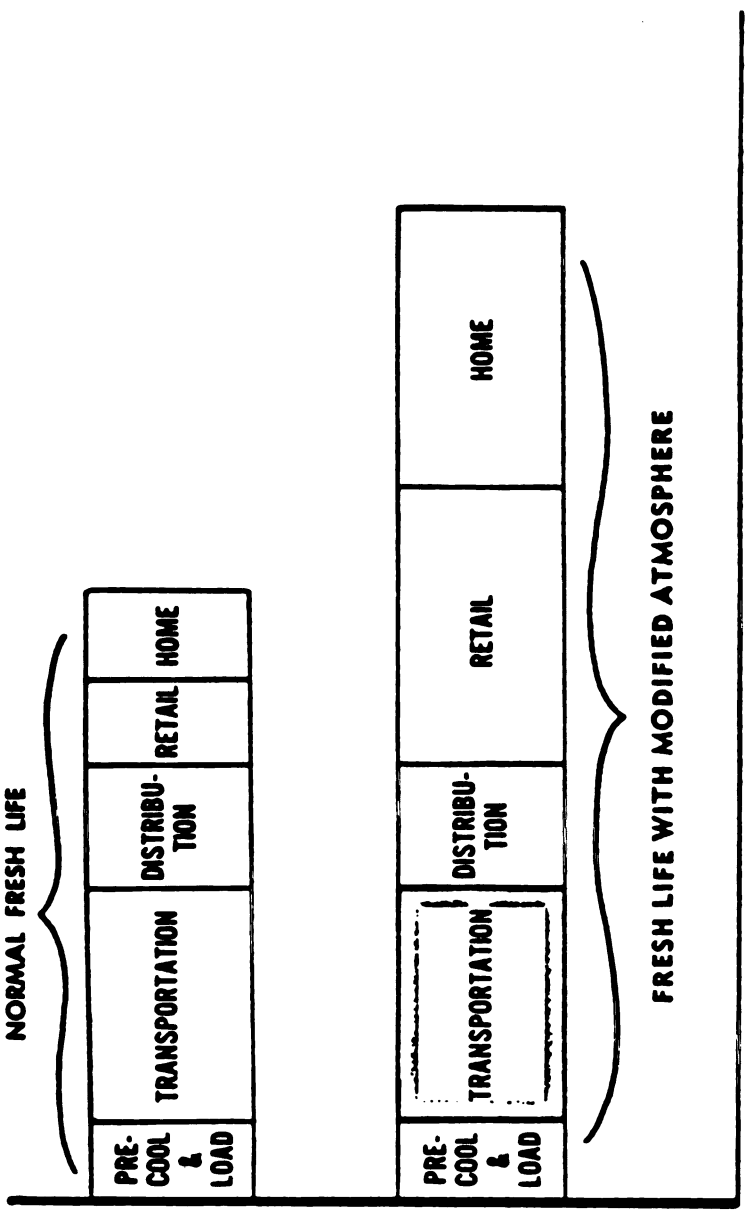

Figure 1: Comparison of normal shelf life and by using MAP of a perishable product

From: Report # 101 Trans Fresh Co. (undated)

barrier material (PVDC-coated PP/PE), oxygen absorber retarded or delayed lipid oxidation in the oat product during storage. A nitrogen gas flushed sample (1% initial head space oxygen concentration) showed deterioration after one month compared to samples with OA that prevented oxidative changes for up to three months in the PVDC-coated PP/PE barrier. Finally, they reported that there was a five-fold increase in the TBA index for product in the PE pouch without absorber compared to product in the PVDC-coated PP/PE package with absorber at 41^{o}C.

Effect of thermal processing and the shelf life of food-package products.

Concern has been expressed by regulatory authorities (Gill, 1988), food industry groups (NFPA, 1988) and others (Eklund, 1982) that MAP may create an undue safety hazard. This increased concern for safety is due to the increased knowledge concerning the survival and growth of pathogenic bacteria at refrigeration temperatures (Palumbo, 1987).

In research studies the use of C. botulinum as a test organism is often avoided, because of hazard potential if in-plant contamination by spores of C. botulinum were to occur (Manomaiwiboon, 1990). An alternative microorganism is C. sporogenes, which is a non toxic strain that has many physical and biochemical characteristics similar to C. botulinum. Table 1 shows a comparison of physiological requirements for these two clostridium species.

Elkund et al. (1988) demonstrated the feasibility of a heat-pasteurization process for certain vacuum-packaged hot-smoked fishery products to inactivate the spores of the non-proteolytic Group II C. botulinum types B, E, and F. They injected 10^{6} spores to already smoked salmon and then heat-pasteurized in a water bath held at a constant temperature. A total of 85, 65, and 55 min in the 185°F (85°C), 192°F (88.9°C), and 198°F (92.2°C) baths,

Table 1: Some physical characteristics of C. sporogenes and C. botulinum

Characteristics	*C. sporogenes*	*C. botulinum* (Types A, B, & F -proteolytic-)
H_2 produced	4	4
Indole produced	-	-
Lecithinase produced	-	-
Lipase produced	+	+
Nitrate reduced	-	-
Milk reaction	d	d
Meat digested	+	+
Acid produced from		
Amygdalin	-	-
Arabinose	-	-
Cellobiose	-	-
Fructose	-w	-w
Galactose	-	-
Glycogen	-	-
Inositol	-	-
Inulin	-	-
Lactose	-	-
Maltose	-w	-w
Mannitol	-	-
Mannose	-	-
Melezitose	-	-
Melibiose	-	-
Raffinose	-	-
Rhamnose	-	-
Ribose	-	-
Salicin	-	-
Sorbitol	-w	-
Starch	-	-
Sucrose	-	-
Trehalose	-	-
Xylose	-	-

Symbols: +, reaction positive for 90-100% of strains; -, reaction negative for 90-100% of strains; 4, represents abundant ("-" to "4+" scale); -w, weak reaction (pH of sugars 5.5-5.9)

From: Buchanan (1974)

respectively, prevented toxin production by type E during 21 day of incubation at 25°C. Longer times, 175, 85, and 65 min. respectively, were required to prevent toxin production by non-proteolytic type B. Toxin production by type E during 120 day of storage at 10°C was prevented by a 45 minute treatment in the 198°C (92.2°C) bath. They also found that outgrowth and toxin production by type E was prevented by a 55 minute process at 198°F (92.2°C) and type B was prevented by a 65 minute process. This process does not, however, inactivate the more heat-resistant proteolytic strains of C. botulinum Group I or necessarily other spore-former.

A basic principle to preserve perishable foods (Labuza, 1982) is to control or destroy microorganisms by: (1) lowering temperature to slow growth; (2) raise temperature to kill microorganisms; (3) remove or bind water to slow or prevent their growth; (4) lower pH to slow or stop their growth by adding acid or through fermentation; (5) Control O_2 or CO_2 level to control population; or (6) manipulate food composition to remove nutrients needed by the microbes. The use of heat to preserve foods is based on: (1) destruction of pathogens; (2) destruction of spoilage microbes; (3) denaturation of enzymes; and (4) softening of tissues to make them more digestible.

Different thermal processes used include: a) blanching: application of heat under mild conditions, or at high temperature for a short time, to achieve enzyme degradation and soften food tissues; b) pasteurization: mild heat treatment used to reduce the number of live microorganisms in the food so as to extend its shelf-life and destroy important pathogens (the product is not sterile); c) commercial sterilization: condition achieved by application of heat which renders food free of viable forms of microorganisms having public health significance, as well as any microorganisms of non-health significance capable of reproducing in

the food under normal non-refrigerated conditions of storage and distribution (Lopez, 1987); d) hot filling; and e) water bath cook.

Effect of food composition and water activity in the shelf life of a food-package system.

Most chemical reactions decrease in rate as the water content decreases (Labuza, 1982). Although various chemical treatments increase the shelf life of food products, there are some cases in which chemicals have deleterious effects on sensory or physical qualities of foods (Golden et al., 1987).

Both moisture and enzymes cause lipolysis of the triglyceride molecule into glycerol and free fatty acids (Labuza, 1982).

The microbiological load or state of the prepackaged food is a critical variable compounded in the term quality (Lioutas, 1988). Glass et al. (1991) examined the relationship between water activity (Aw) of fresh pasta and toxin production by proteolytic C. botulinum. They inoculated 4 types of fresh pasta (4 different Aw) with C. botulinum spores, packaged under modified atmosphere, and stored at either 4 or 30°C for 8 to 10 weeks. They found that no toxin was produced in any fresh pasta held at 4°C for up to 8 weeks. However, toxin was detected in meat tortellini with Aw of 0.99 and 0.95 at 2 and 6 weeks, respectively, when held at 30°C. Toxin was not detected in tortellini with an Aw of 0.94 or below when held at 30°C for 10 weeks. Also toxin was produced at 2 weeks in linguine at Aw 0.96 at 30°C. No toxin was found in linguine or fettucine (Aw 0.93 or 0.95) and held at 30°C during 10 or 8 weeks, respectively. Toxin was not detected in either filled or flat noodle pasta at any water activity, including the highest Aw (0.99 for filled pasta and 0.96 for flat noodles) held at 4°C for up to 10 weeks. They concluded that Aw of fresh pasta is a principal factor in preventing formation of

botulinan toxin for type A and B and minimum Aw for growth and toxin production of C. botulinum type A and type B is 0.95 and 0.94, respectively.

Shirazi et al. (1989) studied the feasibility of controlling in-package relative humidity (IPRH) using compounds possessing Type III sorption isotherm behavior on green tomatos. They placed sorbitol, xylitol, NaCl, KCl and $CaCl_2$ with one mature green tomato at 20°C in sealed packages for 48 days. By using different quantities of each compound they observed that IPRH was a function of the ratio of chemical to fruit mass. They found that relative humidities within control packages were in the range of 96 to 100% throughout the experiment, thus the humidity control system provided a method for stabilizing package humidities which could improve the efficacy of modified atmosphere packaging.

Sugar is used in the preservation of several foods, including sweetened condensed milk, jams, jellies, and other foodstuffs. Banwart (1989) reported that low levels of sucrose are utilized by many microorganisms as a source of energy, but high level of this chemical can have inhibitory properties. Thus, the preservative effect of sucrose is due to the development of osmotic forces and a lowered water activity. The control of microbial growth in acid medium products is currently helped by filling these products while still hot, by heating the surface by steam injection, spraying the surface with a chemical fungicide, or using a cover paper impregnated with a fungicide (Banwart, 1989).

Esteban et al. (1990), studied the water activities (Aw) of culture media used in food microbiology and found that fluid thioglycollate medium (from Difco) had a final Aw of 0.995 (pH=7.2), thus they considered it as a very high Aw medium.

Kuo et al. (1985) manufactured a chinese dried pork product with combinations of 15, 22.5, and 30% sugar and 0.5, 1.5, and 2.5% salt (9

treatments), and evaluated for microbiological, chemical and sensory properties at 0, 7, 14, and 21 days of storage at 4°C. They found that aerobic and anaerobic plate counts increased during storage, with the highest sugar and salt combination resulting in the least microbial growth. They obtained values for total anaerobes of 2.59 and 4.88 log CFU/g at 0 and 21 days respectively for pork product with Aw=0.81 (highest sugar and salt combinations), and values of 2.39 and 7.18 log CFU/g at 0 and 21 days respectively for product with Aw=0.88 (lowest sugar and salt combinations). For aerobes the values obtained were 3.55 and 6.11 log CFU/g at 0 and 21 days for the lowest Aw product (0.81) and 3.84 and 8.95 CFU/g at 0 and 21 days for product with the highest Aw (0.88).

Effect of storage temperature and time on the shelf life of a food-package system.

Labuza (1982) concluded that one of the major environmental factors which results in increased loss of quality and nutrition for most foods is exposure to increased temperature. Therefore, in order to predict shelf life, and to be able to put a shelf life date on a product, knowledge of the rate of deterioration as a function of environmental conditions (i.e. temperature) is necessary. Several authors (Mohr et al., 1980; Reichardt et al. 1982; Ratkowski et al.,1983) have studied microbe activity as a function of temperature, but there is little agreement of what is the best model to apply. Besides temperature, other environmental parameters affect the lag phase and the rate of growth of microorganisms, and include oxidation reduction potential of the food and the composition of the atmosphere in which the food is kept.

Labuza (1982) suggested that microorganisms constitute a major mechanism by which many foods, especially fresh ones, lose quality. This is because microbes are ubiquitous in the environment, and grow rapidly at ambient

temperature. In predictive modeling applications, models based upon lag time are most appropriate for pathogens with zero growth tolerance like C. botulinum, Salmonella, Listeria, etc. (Baker et al., 1990). Growth rate models are best for pathogens which must have significant increase in numbers to reach an infective/toxic dose or cause food spoilage (Baird-Parker et al., 1987).

Burdett et al. (1986) studied the growth kinetics of B. subtilis and found a doubling time of 120 min. at 35°C. They used a spectrophotometer at 600 nm to control growth of subcultures, so that cultures were inoculated from a single colony and periodically subcultured over 3 days to maintain an absorbancy of <0.3. They also found an exponential pattern of increase.

Various authors have shown (Clark and Lentz, 1969, 1973; Enfors and Molin, 1980; King and Nage, 1967, 1975; Paradis and Stiles, 1978; Eklund and Jarmund, 1983; Baker et al., 1985) that a high head space concentration of carbon dioxide in packaged foods has an inhibitory effect on the growth of aerobic spoilage microflora. However, elevated CO_2 levels can establish conditions where pathogenic organisms such as C. botulinum may survive (Daniels et al., 1985).

Molins et al. (1987) studied ham-type product made from soy-extended cured beef. The product was inoculated with C. sporogenes PA3679 and stored under wholesale (2-4°C) and retail (5°C) refrigerated storage conditions, during abuse-temperature holding for 24 and 48 hr at 24-25°C. They used Trypticase soy agar and BBL® Anaerobic GasPak Systems to enumerate C. sporogenes (30°C, 48 hr) and total viable spores (heat-shocked at 80°C for 20 min. followed by 30°C, 48 hr anaerobic incubation). Vegetative cells and viable spore counts were determined by counting typical rhizoid colonies characteristic of the culture. Samples held at 2-4°C did not exceed 10^4 CFU/g after 4 wk for mesophilic, psychrotrophic, anaerobic and lactic bacterial counts. Inoculated

PA3679 did not grow in the product during 24 or 48 hr of simulated mishandling (24-25°C). Molins et al. (1985) reported that the culture characteristic rhizoid colony formation of C. sporogenes had been selected for and was determined to be stable.

Silliker and Wolfe (1980) inoculated ground beef with enterococci, Salmonella, and S. aureus and then stored the meat under different gas atmospheres at either 10 or 20°C. After 10 days at 10°C, the number of Salmonella in samples stored in air increased by more than 3 log cycles, whereas in MA stored samples (60% CO_2, 25% O_2, 15% N_2) the number increased by approximately 0.5 log cycles. At 10°C there was no evidence that elevated levels of CO_2 increased the hazard from S. aureus, or enterococci. At 20°C, however, Salmonella grew almost equally as well in air or in MA stored samples. They also studied pork cubes into which C. botulinum spores had been inoculated and reported that elevated CO_2 level did not increase hazard from botulism. They reported that chicken cubes inoculated with C. botulinum spores grew in CO_2 and concluded that the CO_2 atmosphere did not enhance the potential hazard from botulism, but did not reduce it either. A recent study (Nelson et al., 1989) indicated that spores of B. subtilis (ATCC 9372) and C. sporogenes (ATCC 7955) were inactivated by exposure to oxygen gas plasma. Spores of C. sporogenes were grown under anaerobic conditions at 32-37°C in beef heart infusion media, whereas spores of B. subtilis were cultured aerobically on nutrient agar containing $CaCl_2$ and $MnSO_4$ at 32-37°C. Exposures of cultures at 50 and 200 watts plasma power settings, for 3 exposure periods, 5, 30, and 60 min. were studied. The results showed that greater than 3.4 spore logarithmic reductions (SLR) for a 30 min. exposure and greater than 3.5 SLR for a 60 min exposure were achieved in a 50 watt plasma for both microorganisms. Greater than 3.4 SLR was achieved for

both microorganisms at 200 watts for all exposure periods. They concluded that oxygen gas plasma may be feasible for inactivation of microorganisms that are resistant to other sterilization methods. Table 2 shows differential characteristics of both endospore-forming bacteria Bacillus and Clostridium.

Stier et al. (1981) inoculated salmon fillets with C. botulinum A, B, and E spores. They compared those fillets stored in air with those stored in MA for outgrowth and found that toxin production occurred in products stored in air and modified atmosphere at 27.2°C within 3 days. Outgrowth and toxin production did not occur at 4.4°C. They found that organoleptic spoilage generally preceded toxin formation. The time interval between toxigenesis and spoilage apparently was shortened with increased storage temperature. Luiten et al. (1982) observed that S. typhimurium was unable to grow on beef steaks stored at 10°C in an atmosphere containing 60% CO_2 and 40% O_2, although there was no die-off of the organism during the 9 day storage period. This is in contrast to air stored beef samples in which an approximately 3-log increase in the numbers of Salmonella was observed.

Dixon et al. (1987) found that there was no influence of carbon dioxide (pCO_2) partial pressure on the growth of C. sporogenes (NCIB 8053) when grown in a culture containing pancreatic digest of casein medium. Bacto fluid thioglycollate medium contains also pancreatic digest of casein (Difco, 1984). In media where optimal pCO_2 was observed for growth of the bacteria it was approximately 0.45 atm.

Gray et al. (1984) studied the combined effect of potassium sorbate and CO_2 on growth of S. aureus and Salmonella on chicken thighs. They noted that at 10°C, 100% carbon dioxide flushing resulted in S. aureus counts of 2 logs lower than on thighs packaged in air. Growth of Salmonella on chicken thighs

Table 2: Some differential characteristics of Bacillus and Clostridium genera

Characteristics	*Bacillus*	*Clostridium*
Rod-shaped	+	+
Diameter over 2.5 µm	-	-
Endospore produced	+	+
Motile	+	+
Stain Gram-positive at least in young cultures	+	+[h]
Strict aerobes	D	-
Strict anaerobes	-	+
Catalase	+	-

Symbols: +, 90 % or more of strains positive; -, 10% or less of strains positive; D, substantial proportion of species differ; [h], rarely Gram negative.

From: Buchanan (1974)

stored in high-barrier bags at 10°C was inhibited by 100% carbon dioxide but was not affected by vacuum packaging.

Baker et al. (1986) inoculated minced chicken meat with P. Fragi, S. typhimurium, S. aureus, or C. sporogenes and stored samples at 2, 7, and 13°C. Throughout an 18 day storage period, numbers of S. aureus were lower in 80% CO_2 MA than in air controls. Counts of C. sporogenes, which is culturally similar to proteolytic strains of C. botulinum, decreased under all conditions, but number in air-packaged samples declined more than in CO_2 indicating that MA had a protective effect on the organism. Hintliand and Hotchkiss (1987) noted that while modified atmospheres containing 5 or 10% oxygen inhibited the outgrowth of C. perfringes in sliced and cooked roast beef co-inoculated with P. fragi, S. typhimurium, S. aureus, and C. perfringes, the numbers were the greatest in air controls. It is probable that C. perfringes was able to grow following consumption of oxygen by P. fragi which grew to high numbers. The growth of C. perfringes was inhibited 1 log by a MA blend containing 75% CO_2 and 25% O_2 and by several log cycles by MA containing 2, 5, or 10% O_2, compared to growth in air.

The physiology of C. sporogenes (NCIB 8053) was studied by Lovitt et al. (1987a). They found that when batch cultures of the microorganism were incubated at 37°C with stirring at 250 rev/min, H_2 production was detectable in the cultures only under conditions of nutrient excess. They determined the microbial concentration by measuring absorbance at 520 nm and relating this to a standard curve. They also studied the growth and nutritional needs of C. sporogenes NCIB 8053 (Lovitt et al., 1987b). They incubated at 37°C with no shaking in Buchner flasks sealed with a rubber bung and fitted with a sample port, bladder and gas filters to allow flushing and evacuation of the flask head space. They used thioglycollate as a reducing agent in the media, and found that this media

supported the growth of this and several other strains of this species. The flasks were autoclaved at 121°C for 15 min, and after cooling, sterile supplements were added and the head space was filled with N_2/CO_2 (95:5 v/v). Growth in flasks was estimated by depositing samples in cuvettes and measuring optical density at 680 nm. They confirmed that the microorganism required 10 amino acids and one vitamin to grow, and was capable of growing in media containing no carbohydrates (although these stimulate growth). They also measured change in gas concentration in the head space using gas chromatography to estimate fermentation products.

Oxygen absorbers and oxidation in food systems.

Labuza (1982) reported that all fats are subject to deterioration by oxidative and hydrolytic rancidity leading to the formation of objectionable odors and flavors. Hydrolytic rancidity is also responsible for "soapy" flavors and facilitates deterioration by direct oxidation. Oxidative rancidity results in food spoilage associated with fat deterioration, i.e. presence of pungent or acrid odors. He also reported that in the case of potato chips, which are dried to a finished moisture level of 3% or less (which relates to an Aw of about 0.2), moisture gain leads to texture changes (sogginess) increased rate of oxidation, and loss of consumer acceptance. Moisture gain is a function of the package type and its permeability characteristics (Paine, 1991).

Labuza (1987) mentioned that only type E "Ageless™" sachets (oxygen scavenger) were in use in the United States (at that time). The patent that supports this product is US. patent 4,421,235 held by Mitsubishi (Moriya, 1983). Watanabe (1988) reported that sales of this oxygen scavenger have increased (up to 20% in 1987 over 1986), and reached 15 billion yen ($120 million) for some 350

million pieces. Several more patents have been issued in the US. that are related directly to oxygen scavengers (Takahashi et al., 1985; Farrel et al., 1985; and Otsuka et al., 1986). These more recently issued patents cover the oxygen absorbent, oxygen scavengers that can be included in a multilayer construction in which the scavenger remains passive until such time needed, and the oxygen absorbent packet.

In addition to Ageless™ (ferrous oxide that is oxidized to ferric oxide, -used in packaging of bakery items, precooked pasta, potato chips, nuts and coffee, etc.-), there are currently other oxygen absorber products in the market including "Oxysorb™" (absorbs oxygen by converting iron into iron oxide and hydroxide <0.01% O_2, -used with beef jerky, salami, peanut butter), "SmartCap Absorber™" (artificial gill for extracting oxygen from liquid, -for use in beverage packaging-), "Toppan®" (non-ferrous metal particles that replaces O_2 absorbed with CO_2); "Mega Mold" (oxygen scavenger plus mold/microbe prevention -e.g. yeast fungus and Bacillus subtilis-); and "Keplon®" (oxygen absorber -for use in beef jerky-). In Japan, the companies that produce oxygen absorbers (January 1986) were: Mitsubishi Gas Chemical Co., Inc (75.6% of market); Toa Synthetic Chemical Co., Ltd (5.9%); Toppan printing Co., Ltd (5.7 %); Nippon Sohda Co., Ltd (4.3%); Kepron (3.1%); Ohji Kakoh (2.6%); and Hakuyoh (1.25%). The price of the oxygen absorber depends on the amount of oxygen it absorbs.

Multiform Desiccants, Inc. (Buffalo, N.Y.) is assignee of a recent US. patent (Cullen et al. 1991). This patent allows the company to produce and sell "FreshPax™" (oxygen scavenger sachets), and is recommended for use in: breads, cookies, cakes, pizza, pastries, croissants, buns, powdered milk, sponge cakes, pastas (fresh and pre-cooked), cheeses, meats, quiche, Chinese fried noodles, dry seaweed products, wines, snacks, coffees, dry tea,

candies/confectioneries, beans, dried eggs, spices, dried fruits, dry baby foods, potato chips, nuts, fruits and vegetables (tomatoes, avocados, lettuce), chocolates, flour, dehydrated foods, and fish. The "FreshPax™" components are on the FDA's Generally Recognized As Safe (GRAS) list. Criteria to be considered when using this products include: (1) plastic film must be checked for its oxygen permeability (<20 cc/M^2 atmosphere per day or less is recommended), and (2) hermetic seals are essential. These sachets can reduce oxygen content to less than 0.1%. The oxidizing system made up of ferrous salts and an oxidation modifier conveniently delays primary oxidation for several hours until the absorbing packet is introduced into the food package (Anonymous, 1989). Therefore, the user does not have to be concerned with premature oxidation during handling or packaging of the absorbing system. In a recent publication (Anonymous, 1991) Mr. Ronald Idol, director of technology for Multiform Desiccants, Inc. described the newest innovation: "FreshMax™", "as one which will duplicate the oxygen scavenging performance of sachets currently placed in food packages". He referred to "FreshMax™" as a multi-functional gas scavenging technology that can be adhesive/backed with various adhesives, for numerous applications. This makes it far less likely to be ingested compared to sachets currently in use. The FreshMax™ (like other oxygen absorbing technologies), protects food from mold growth and rancidity that can result from oxidation, by absorbing available oxygen within a 24 hour period. It can also eliminate the need for costly food additives and/or preservatives. FreshMax™ can cost less than gas flushing or vacuum packaging which usually require a substantial capital investment (Anonymous, 1989).

Based on this review and by following the objectives stated in the introduction, the oxygen absorbers, temperature and high water activity influences

on the microbial growth were tested over time. Time intervals were established for sampling of head space percent oxygen and microbial load, so that a relation of all factors was obtained. The hypotesis was fulfilled in the parameters studied when final results were obtained and relations among the factors were analyzed.

MATERIALS AND METHODS

Package system preparation

One pint, wide mouth mason jars were obtained from the General Stores at M.S.U. The jars were prepared as follows (see figure 2): Three holes were made in each metal lid using a drill with a 13/64" diameter bit. One hole was positioned in the center of the lid and two at 3/4" from the center hole and opposite from each other. The holes were slightly enlarged using a metal shaft so that the edges left by the drill were dulled. Three rubber stopper septums, size 7 mm (Sigma Chemical Co., St. Louis, MO) were placed in the holes. Two borosilicate disposable pipettes (VWR Scientific Inc., San Francisco, CA) of 1 mm^3 were obtained and the cotton in the pipettes drawn down into the narrow part (using a wire). The pipettes were then cut from the narrow part so that about 2¼ inch segments were obtained. All cuts were made using a triangular file and dulled using sandpaper. The pipette segments were then placed into the 2 outside septums which left the center septum free.

The lids were placed on a table and the inside (facing the jar when assembled) sprayed with an enamel to prevent corrosion by high relative humidities. Special care was taken to cover the pipette hole segments with tape to prevent enamel clogging. Two hours between each enamel application were allowed. After the third application, the enamel was allowed to dry for 72 hours at room temperature.

After drying, the assembled lids were placed on the jars. The package model system was stored until used.

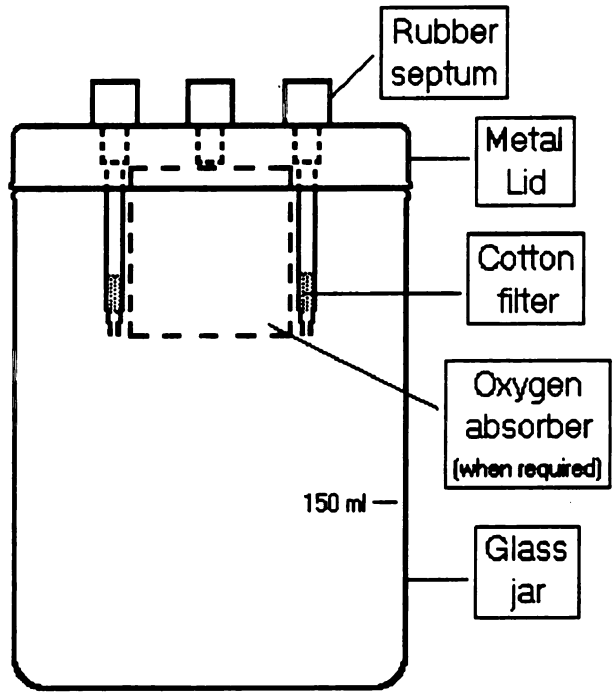

Figure 2: Package model system design.

Model preparation.

The model system utilized for this study was Bacto Fluid Thioglycollate Media (from Difco Laboratories, Detroit, MI). This media was chosen because it allows the growth of both bacteria used in this experiment (table 3). The media is formulated with Bacto Yeast Extract, Bacto Casitone and resazurin. Resazurin is used to indicate the status of oxidation or aerobiosis (OR indicator) in the media. Dextrose (glucose) is included in the Bacto Fluid Thioglycollate media because it has been shown (Difco manual, 1984; Lovit et al. 1987b) that most organisms grow earlier and have more vigorous growth in the presence of a carbohydrate.

The preparation of the media was performed according to the method suggested in the Difco manual (1984), and had a pH of 7.2.

In one series of samples the Aw was adjusted to 0.96 by addition of sucrose -measure of Aw was obtained using an electric hygrometer indicator (Hygrodynamics, Inc., Silver Spring, MD)- to the media before preparation (as powder). This yielded a final concentration of 40% (wt/vol) sugar with respect to the water. Once the media and sugar were mixed, the mixture was added to the water with constant agitation to prevent clumping. In the other series of samples the water activity was not adjusted and was >0.99.

C. sporogenes inoculum preparation

Clostridium sporogenes (ATCC 11437) was obtained as a freeze-dried culture from American Type Culture Collection (Rockville, MD). This microorganism was chosen because it closely resembles phenotypically to C. botulinum types A, B, and F, which are toxin producer proteolytic strains. The culture was activated using Bacto Fluid Thioglycollate (Difco) and the instructions

Table 3: Typical Cultural Response After* 18-48 Hours at 35°C

Organism	**Bacto Fluid Thioglycollate Medium**
Bacillus subtilis (ATCC 6633)	good to excellent
Clostridium sporogenes (ATCC 11437)	good to excellent

* these cultures may be incubated at 30-35°C for 2-7 days, if preferred, per USP.

From: DIFCO (1984)

for rehydration were followed according to the recommendation that accompanied the bacteria (ATCC, 1989). Once the culture was activated, an agar plate was prepared and inoculated with the microorganism and placed in anaerobic conditions at 37°C (±1°C) to allow grow of the organisms. From this plate, one single colony was taken using a platinum wire and deposited into fresh media to assure production of cells genetically similar for use during the study. Once the new batch was obtained, a series of stock cultures were prepared by aseptically (in front of a bunsen burner and using disposable sterile 1-ml pipettes -Corning Laboratory Science Co., Corning, NY-) mixing one part culture with 50% glycerol. The mixture was distributed into cryo-vials and closed so that 0.5 ml. per vial was obtained. All vials were stored at -80°F in a bio-freezer (Forma Scientific, Marietta, OH.) until needed in the experiment.

B. subtilis inoculum preparation

Bacillus subtilis (ATCC 9372) was obtained as a freeze-dried culture from American Type Culture Collection. The culture was taken and started using Bacto Fluid Thioglycollate (Difco Laboratoires, Detroit, MI) media by following the instructions for rehydration accompaning the bacteria (ATCC, 1989). Once the culture was obtained, an agar plate was prepared and inoculated with the microorganism and placed in aerobic conditions at 37°C (±1°C) to allow growth of the organism. From this plate one single colony was taken and deposited into fresh media to assure production of cells genetically similar for use in the study. Once the new batch was obtained, a series of stock cultures were prepared by aseptically (in front of a bunsen burner and by using disposable sterile 1-ml pipettes -Corning Laboratory Science Co., Corning, NY) mixing one part culture with 50% glycerol. The mixture was distributed into cryo-vials and closed so that

0.5 ml. per vial was obtained. All vials were stored at -80°F in a bio-freezer (Forma Scientific, Marietta, OH.) until needed in the experiment.

Experimental design (figure 3)

The package-model systems were labelled with the bacteria designation, water activity, initial oxygen in head space and storage temperature.

Approximately 150 ml of prepared media was placed into each jar. This was accomplished by transfering media from the erlenmeyer where it was prepared using a 150-ml beaker and gloves. Half of the jars were filled with media having a Aw of 0.96 and the other half a Aw of >0.99 for samples stored at 25°C. All jars stored at 10°C were filled with 150 ml of media with a Aw of >0.99.

A fresh package containing the oxygen absorbers (Multiform Desiccants, Inc. Buffalo, NY.) was opened (stored under vacuum and high barrier film) and one sachet per container was placed in a total of one quarter of the lot (32 and 16 jars for storage temperatures of 25° and 10°C, respectively). The sachet was attached using sterilizing condition-resistant tape between the two glass tubes so that the oxygen absorber was in the center of the lid (see figure 2). The food-package model systems were sterilized at 250°F for 15 minutes (commercial sterilization). After sterilization, the jars were allowed to cool at room temperature for 12 hours and then placed in the designated storage temperature rooms. Once the jars were sterilized they remained closed during the rest of the experiment.

Once the jars reached the storage temperature specified (by allowing sample jars a 12 hour period in the storage temperature rooms), the initial oxygen head space contents were obtained by mixing nitrogen and air from standardized tanks and flushing the mixed gas into the jars containing the media for the time

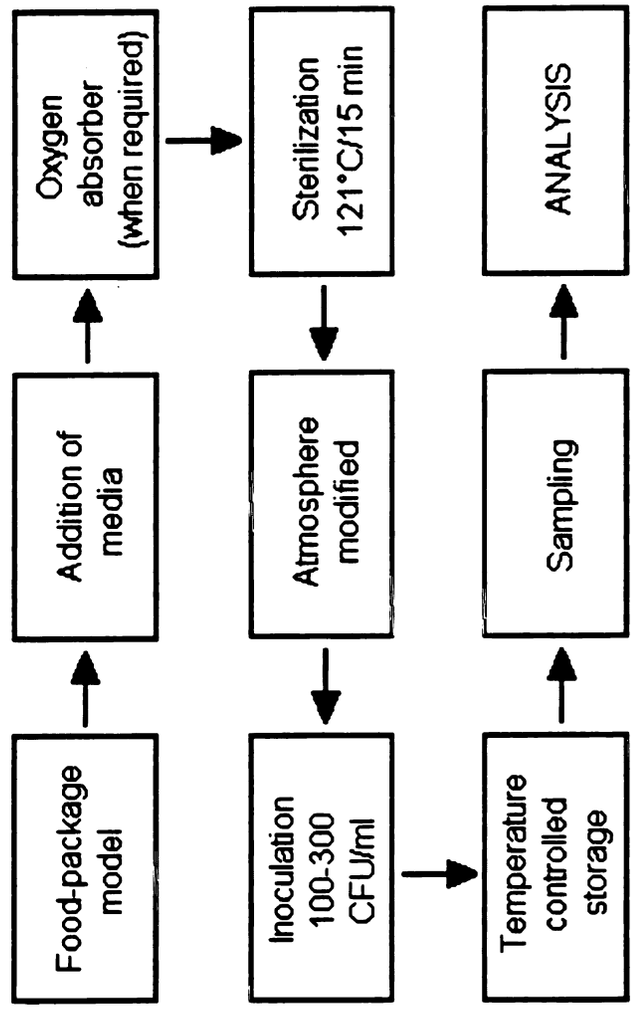

Figure 3: Experimental design used for the food-package model system setup

required to remove atmospheric gases to obtain the mixture specified. Four different oxygen systems were used: 1% O_2, 5% O_2, 21% O_2, and 21% O_2 with oxygen absorber.

The microorganisms were inoculated into the jars as follows: The jars were divided into four lots. The middle rubber septum was rubbed using a cotton swab with 70% ethanol, and then used to introduce the bacteria into the jars using 1cc tuberculin disposable syringes (Becton Dickinson & Co., Rutherford, NJ) and 20G1 Precision Glide™ needles (Becton Dickinson and Co., Rutherford, NJ) during the inoculation process. Inoculation of the microorganisms into the media and gas exchange was accomplished hermetically through the rubber septums. The first lot (32 jars) were used as controls, no microorganisms were added, a second lot was inoculated with Bacillus subtilis (ATCC 9372) at a concentration of approximately 10^2 CFU/ml. The third lot was inoculated with Clostridium sporogenes (ATCC 11437) in the same concentration of about 10^2 CFU/ml. The final lot was inoculated with a mixed culture containing approximately equal amounts (10^2 CFU/ml each) of both bacteria, B. subtilis and C. sporogenes. This was accomplished by adding first the aerobic and then the anaerobic microorganisms.

Oxygen absorbers used for study

Multiform Desiccants, Inc. supplied the appropriate size and formula of oxygen absorber for the study. The oxygen absorbers used were "FreshPax™" type B-300 and have the capability of absorbing 200 cc. of oxygen. The jars used after adding 150 ml of media had an approximate head space of 325 cc. (about 68 cc oxygen).

The oxygen absorber contains ferrous powder (with a 70-80% efficiency), salt (an electrolite), and water. Theoretically, the amount of ferrous powder contained in the B-300 type oxygen absorber (FreshPax™) is capable of absorbing 400 cc. of oxygen. The efficiency and reactions before and during packaging of the ferrous powder with the atmosphere makes necessary to lower the specification to assure 200 cc. of oxygen absorbed.

The basic reaction that takes place in the oxidation process is:

$$4\,Fe + 3\,O_2 + 6\,H_2O \longrightarrow 2\,Fe_2O_3 \cdot 3H_2O$$

Incubation (storage) conditions

In storage, all jars were agitated by constant rotation at 100 RPM by placing them in an incubator shaker (Lab-Line Instruments, Inc. Melrose Park, IL.) at room temperature (25 ±1°C) and in an open shaker (Eberbach, Co., Ann Arbor, MI.) at a temperature of 10 ±1°C. For the 25°C jars, samples were taken at 0, 24, 48, and 72 hr. for product at Aw>0.99; and at 0, 120, 240, 288, 336, 384 and 432 hr. for jars stored at Aw=0.96. For jars stored at 10°C samples were taken at 0, 1, 2, 3, and 4 weeks

Sampling

The jars containing the different media, microorganisms, and gas concentration were sampled in the same order every sampling period and the oxygen level in the head space measured at the specific time intervals cited above. This was performed utilizing a 0.5 cc series A Gas Syringe "Pressure-lok®" (Fisher Scientific, Pittsburgh, PA) and injecting the 0.5 cc head space gas sample into a Servomex series 1100 gas chromatograph (London, England). After the oxygen concentration had been determined, anaerobic and aerobic CFU's per

milliliter were determined under aerobic and anaerobic conditions, respectively, as follows: the jars were taken to the food microbiology laboratory and at least 2-4 samples of one ml each were taken from the media using a 20x4" long non-coring stainless steel needle (Popper & Sons, Inc., New Hyde Park, NY); the needles were pre-conditioned according to the following procedure: twelve needles were contained in a beaker with ethanol at 70% (v/v) near the flame of a bunsen burner. One needle was attached to a disposable 1 cc sterile tuberculin syringe (Becton Dickinson & Co., Rutherford, NJ.). The needle was then placed into 9 ml sterile peptone water (0.1% v/v) contained in 16x125 culture tubes (Corning Laboratory Science Co., Corning, NY) that were previously flamed using a bunsen burner to assure sterile conditions and then flushed 5 times to remove any 70% ethanol in the needle, the peptone water was then disposed of. One of the samples was placed in a disposable cuvette in an Spectronic 601 spectrophotometer (Milton Roy, Rochester, NY) and absorbance was measured at 620 nm, zeroing with one ml of pure media (no microorganims) stored under the same conditions as used in the study. This value was then compared to the values obtained for the standard curves (see appendix II) under optimal growth (37°C for B. subtilis and C. sporogenes, aerobically and anaerobically, respectively; absorbance value and CFU/ml were determined at specific time intervals) to determine the approximate number of total CFU/ml expected for the bacteria tested. A second 1 ml sample was taken and diluted according to the CFU/ml desired for the plate counts. One ml of the dilution media was taken and used to inoculate the agar used in the plate count. For samples in which the dilution was not needed additional 2 ml samples (1 ml each) were taken from the jars and used for inoculating the agar. Two replications were performed for each dilution. Dilutions were performed as necessary to obtain between 25 and 250 CFU's per milliliter (see appendix I). To

accomplish anaerobic conditions, BBL® microbiology generator (envelope with reagents and palladium catalyst for producing H_2 and CO_2) and BBL® GasPak disposable anaerobic indicator (Becton Dickinson and Co., Cockeysville, MD) systems were used. Media and incubating conditions for each type of microbial group were as follows:

- Aerobic Plate Count: Trypticase Soy Agar (DIFCO Laboratories, Detroit, MI), with aerobic incubation at 37°C for 48-72 hours.
- Anaerobic Plate Count: Trypticase Soy Agar with incubation at 37°C for 48-72 hours in anaerobic condition using petri desiccators and high vacuum silicone stopcock grease (American Scientific Products, McGraw Park, IL) with BBL® Microbiology System anaerobic condition generator (Becton Dickinson and Co., Cockeysville, MD) and BBL® GasPak disposable anaerobic indicator (Becton Dickinson and Co., Cockeysville, MD). Anaerobic condition was reached when the color of anaerobic indicator changed from blue to colorless.

Colonies from each plate were determined as described in appendix I, and calculated as the average colony forming units (CFU) per milliliter of culture medium. This number was calculated and reported as $\log_{10}$CFU for each sample.

Strains of B. subtilis and C. sporogenes were strictly aerobic and anaerobic, respectively. Because of this, growth of B. subtilis was used as a microbial indicator of presence of oxygen at any time in the closed system, and inversely, C. sporogenes was used to detect the absence of oxygen in the food-package system at the time of sampling.

At the end of the experiment, the final pH was measured using a pH meter model 240 (Corning Co., Corning, NY). pH in the media for jars containing B. subtilis as a pure culture had a final pH of 5.11 while for media in jars containing C. sporogenes as a pure culture had a final pH of 5.79.

Statistical analysis of data

To determine significant differences three factors were taken into account: a) Bacterial inoculation with 2 levels for determining growth of microbes (either pure B. subtilis and mixed culture with assay for B. subtilis or pure C. sporogenes and mixed culture with assay for C. sporogenes); and 4 bacterial inoculation conditions determining change in oxygen concentration in the head space (Control -no bacteria inoculated-, pure B. subtilis culture, pure C. sporogenes culture, and mixed culture), b) Initial Atmosphere with 4 levels (1%, 5%, and 21% O_2 in head space and 21% oxygen plus oxygen absorber), and c) Time (depending of number of time intervals sampled for each analysis). Two replications for each possible combination were performed so that the statistical test performed was a Completely Randomized Design with 3 factor factorial treatments for microbial growth analysis. Completely Randomized Design with 4 factor factorial was performed for analysis of change in oxygen level in the head space. The factors analyzed for the change in oxygen level in the head space were: a) Initial atmosphere concentration (1%, 5%, 21% and 21% + oxygen absorber); b) Bacterial inoculation (pure cultures of B. subtilis and C. sporogenes and mixed culture of these two bacteria); c) Temperature (10° and 25°C); and d) Time (0 and 24 hours). MSTAT-C statistical program, version 1.4 (1990) from the Department of Crop and Soil Sciences and Department of Agricultural Economics, Michigan State University was used as an aid for all statistical calculations in this study.

Effect of temperature and water activity on activity of oxygen absorbers

This study consisted of placing samples with different designations in constant temperature chambers for 24 hours. Sampling of the head space was

performed every hour for 12 hours and at 16 and 24 hours. The characteristics studied were: (1) Water activity <Aw> with values of 0.88 and >0.99, and (2) Temperature with two values: 10°C and 25°C. All jars contained media prepared as suggested by DIFCO (1984). The water activity was adjusted to 0.88 by adding sugar. The low water activity (0.88) was chosen because the water activity of 0.96 is not very different from the medium not adjusted (Aw >0.99) and still can support growth of many bacteria (only the more tolerant microorganisms would grow at 0.88); also, the difference of water activity was larger and therefore the evaluation of the water activity influence was more significant. At 10°C, a final concentration of 69.02% sugar (w/v) to water was needed and at 25°C, a final concentration of 65.51% sugar (w/v) to water was needed to reach this Aw.

Two replications were used for each designation. Half of the total jars included an oxygen absorber (FreshPax™) and the other half were used as the controls. This study was performed using constant temperature rooms located in the post harvest laboratory, Department of Horticulture at Michigan State University. Agitation was not provided to samples. Evaluation of % oxygen in the head space was performed as described for the microbial study.

The statistical analysis of this study was done using a two-way analysis of variance for % oxygen in the headspace over time and for each factor studied, such as: a) Presence or absence of FreshPax™; b) Water activity of the media (0.88 and >0.99); and c) Temperature (10°C and 25°C). MSTAT-C statistical program, version 1.4 (1990) from the Department of Crop and Soil Sciences and Department of Agricultural Economics, Michigan State University was used as an aid for the statistical calculations in this study.

RESULTS AND DISCUSSIONS

From preliminary studies (see appendix II), a spectrophotometric method was developed and used as a complementary aid to approximate the number of bacteria present at the moment of sampling. A growth curve of number of microorganisms (log CFU/ml) was compared to the absorbance level of samples over time using a spectrophotometer at 620 nm (this value was observed to give highest absorbance value for both bacteria in a range of 400-650 nm). Optimal conditions of growth for Bacillus subtilis and Clostridium sporogenes (37°C and aerobic and anaerobic conditions, respectively) were used to obtain a standard curve for each microorganism. Using the equation corresponding to each bacteria:

- Log B. subtilis CFU/ml = 1.775 (Absorbance) + 6.243138 and
- Log C. sporogenes CFU/ml = 1.65392 (Absorbance) + 6.583369

relating the absorbance value with the CFU/ml value from the standard curve an approximate CFU/ml number was obtained. Dilution corresponding to the CFU/ml value obtained from the equation was performed and dilutions at a level lower and higher of this value were also analyzed. Duplicate plate samples were prepared for the 3 dilutions selected. If absorbance value for the CFU/ml was low (<0.25), the calibration curve was not used and dilutions of 10^0, 10^1, 10^2, 10^3 and 10^4 were prepared, since it was observed that initial growth of bacteria was not detected by the spectrophotometer (very low absorbance value).

Growth of *Bacillus subtilis* in samples stored at abusive temperature of 25°C and water activity >0.99

Sample jars stored at 25 ±1°C were agitated at 100 RPM at all times. Samples of 1 ml were taken and aerobic plate count (APC) method was applied (see appendix I). The statistical results for samples cultured in media with a water

activity of >0.99 are shown in table 4. The calculated F-value was compared to standard F-test distributions to obtain significance. Significance was reported as a probability value for each factor. In this study, 99% probability ($P<0.01$) was used as the minimum significant level. The model used to analyze the data was a Completely Randomized Design using a three factor factorial. Storage time (0, 24, 48 and 72 hours); inoculum (whether B. subtilis was inoculated as a pure culture or combined with C. sporogenes as a mixed culture), and initial oxygen level (1%, 5%, 21%, and 21% + FreshPax™) in the head space were the factors statistically evaluated.

In this part of the study, the smallest dilution used was 10^0, and the largest dilution used was 10^{-8}. If no growth was detected in the smallest dilution, the $\log_{10}$ CFU number was reported to be less than 2.0 (<2.0). This value was substituted as ($\log_{10}$ CFU) 2.0 in the mean and statistical analysis corresponding to the approximate minimum number of microorganisms inoculated in the sample.

All factors and their interactions significantly influenced growth of Bacillus subtilis (ATCC 9372) at 25°C ($P<.01$). Time, oxygen, and their interaction were the primary factors that affected the growth of this aerobic bacteria. The maximum values for the number of microorganisms obtained for this and all other observations may not represent the real maximum value (the absolute maximum value could be in a specific instant between the sampling times stablished in this study). Figures 4 and 5 show the curves obtained for growth of B. subtilis at 25°C in media of Aw >0.99. The $\log_{10}$ number corresponding to the CFU/ml of B. subtilis at the time of sampling was used to construct growth curves . Numbers of B. subtilis were about the same in the jars containing 5 and 21% oxygen (initially) in the head space. The media used as the food model was capable of supporting the growth of both bacteria (table 3). In the presence of C. sporogenes in the

Table 4: Analysis of Variance for B. subtilis (log CFU/ml) at 25°C and Aw >0.99

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	3	142.380	47.460**
Inoculum	1	4.265	4.265**
Time x inoculum	3	6.696	2.232**
Oxygen	3	78.667	26.222**
Time x oxygen	9	90.549	10.061**
Inoculum x oxygen	3	5.198	1.733**
Time x inoc. x oxygen	9	19.737	2.193**
Error	32	1.144	0.036
Total	63	348.634	

Coefficient of Variation = 5.41 %

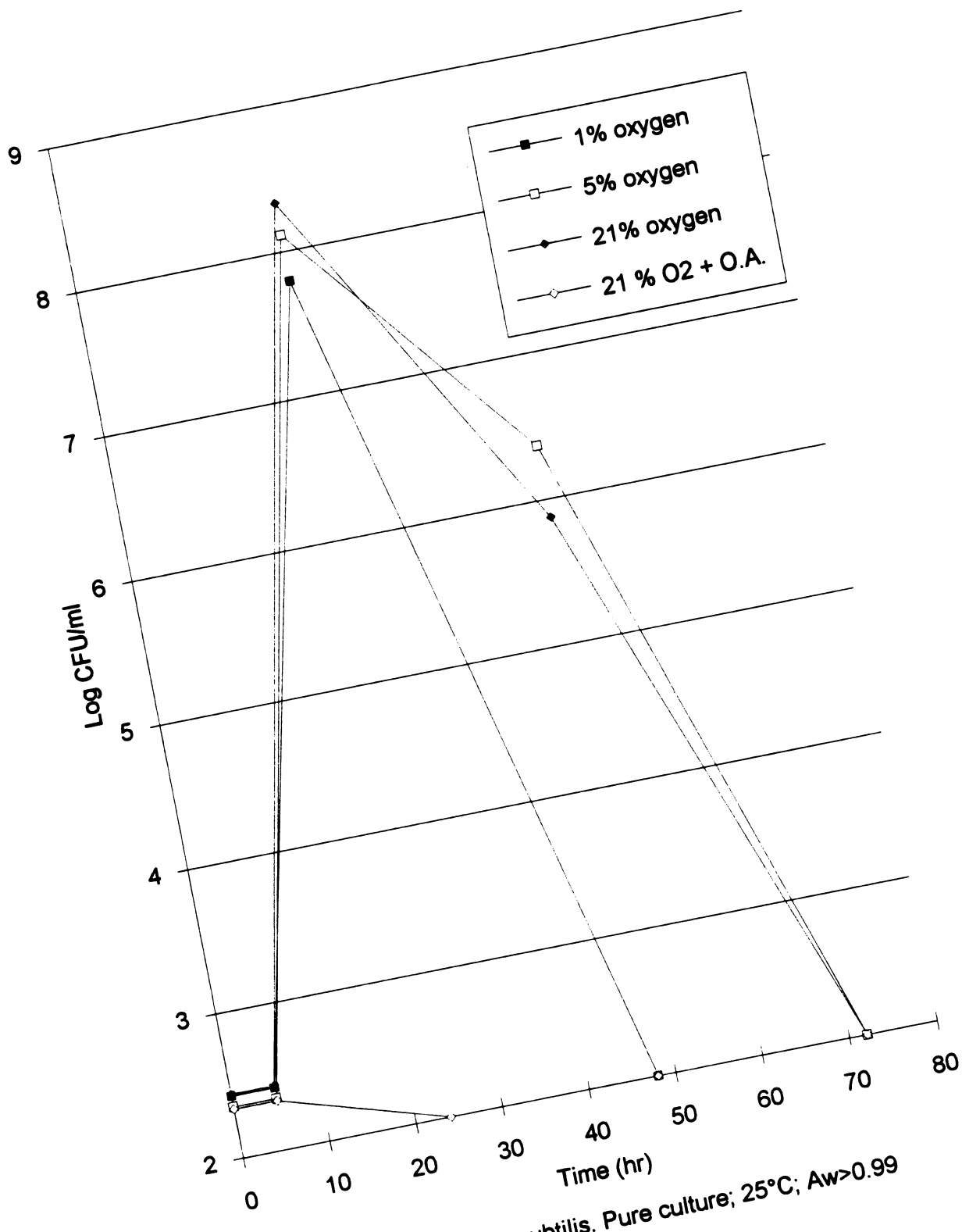

Figure 4: Bacillus subtilis. Pure culture; 25°C; Aw>0.99

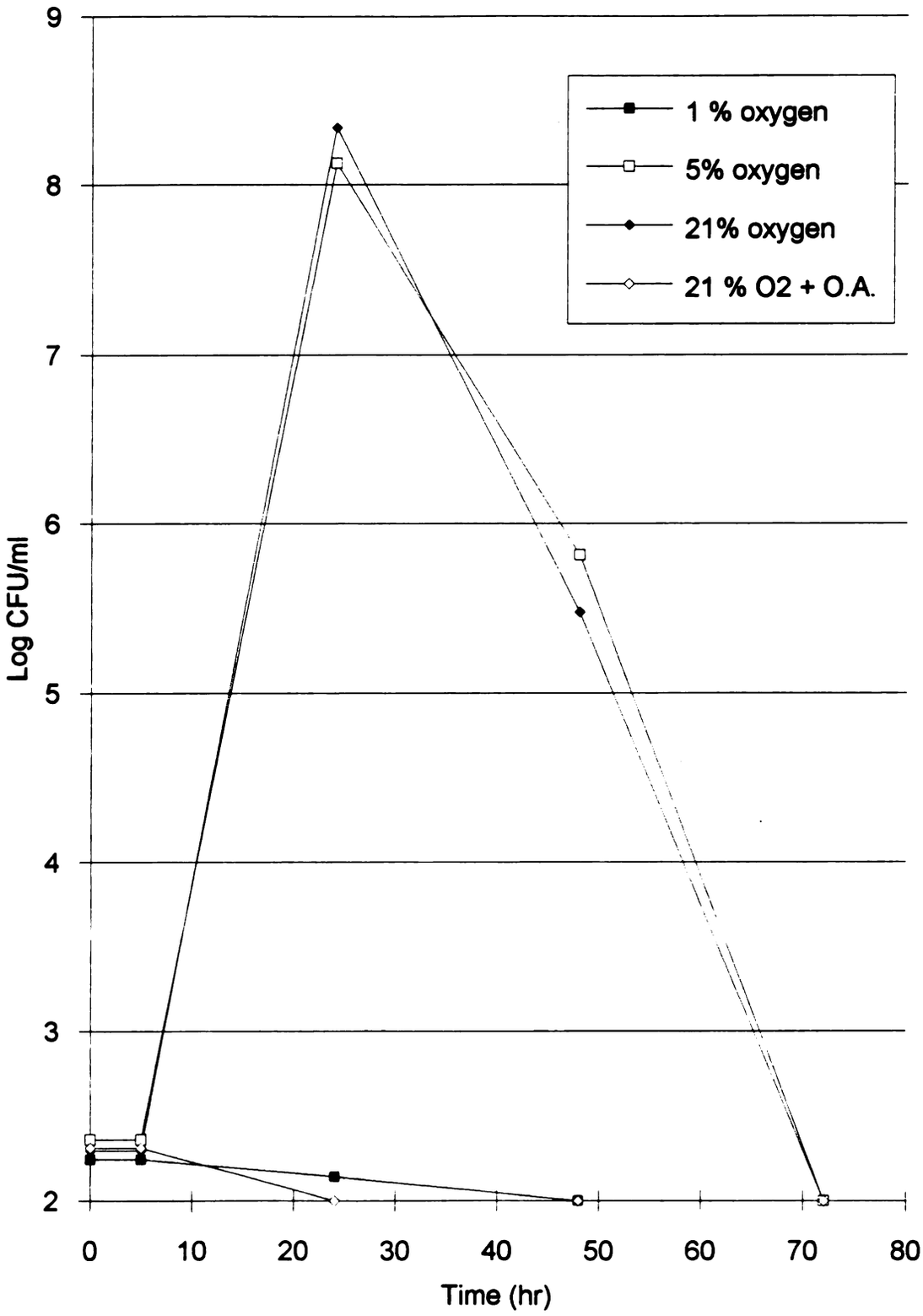

Figure 5: Bacillus subtilis. Mixed culture; 25°C; Aw>0.99

mixed culture, B. subtilis did not grow at 1% oxygen but grew when inoculated as a pure culture (figure 4). This may have occurred because C. sporogenes consumed the nutrients necessary for growth or build up of waste products occurred. Oxygen was shown to be a very important factor influencing the growth of both B. subtilis ($p<0.01$) and C. sporogenes (tables 4 and 5, respectively). No growth was observed in samples inoculated in the 21% initial oxygen in the head space + FreshPax™. This is attributed to the anaerobic atmosphere generated in the head space within 5 hours by the oxygen absorber that did not allow the growth of the aerobic bacteria.

Clostridium sporogenes evaluation of samples stored at 25°C and water activity >0.99

For C. sporogenes, statistical analysis of factors effecting growth is presented in table 5 for temperature of 25°C and water activity >0.99. The value of the mean square presented showed that as in the case of B. subtilis, all factors tested: time (0, 24, 48, and 72 hr); inoculum (either pure or mixed culture); oxygen level in the head space (1, 5, 21% oxygen or 21% O_2 plus oxygen absorber) were highly significant, and greatly influenced the growth of C. sporogenes. Figures 6 and 7 show the growth pattern of C. sporogenes as influenced by the various factors. In the pure culture the bacteria grew in sample jars containing atmospheres with an initial oxygen content of 1 and 5% O_2, and 21% O_2 plus "FreshPax™". Growth in jars containing 5% initial oxygen had total lower numbers compared to jars with 1% initial oxygen and 21% initial oxygen + oxygen absorber. It is sugested that the redox potential (Eh) in the media was the factor that influenced the growth of the bacteria. Banwart (1989) sugested that many anaerobic clostridia would grow at Eh levels from +85 to +160 even with presence

Table 5: Analysis of Variance for C. sporogenes (log CFU/ml) at 25°C, Aw >0.99

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	3	129.478	43.159**
Inoculum	1	4.378	4.378**
Time x inoculum	3	4.823	1.608**
Oxygen	3	51.953	17.318**
Time x oxygen	9	93.350	10.372**
Inoculum x oxygen	3	16.549	5.516**
Time x inoc. x oxygen	9	19.411	2.157**
Error	32	0.782	0.024
Total	63	320.723	

Coefficient of Variation = 4.65 %

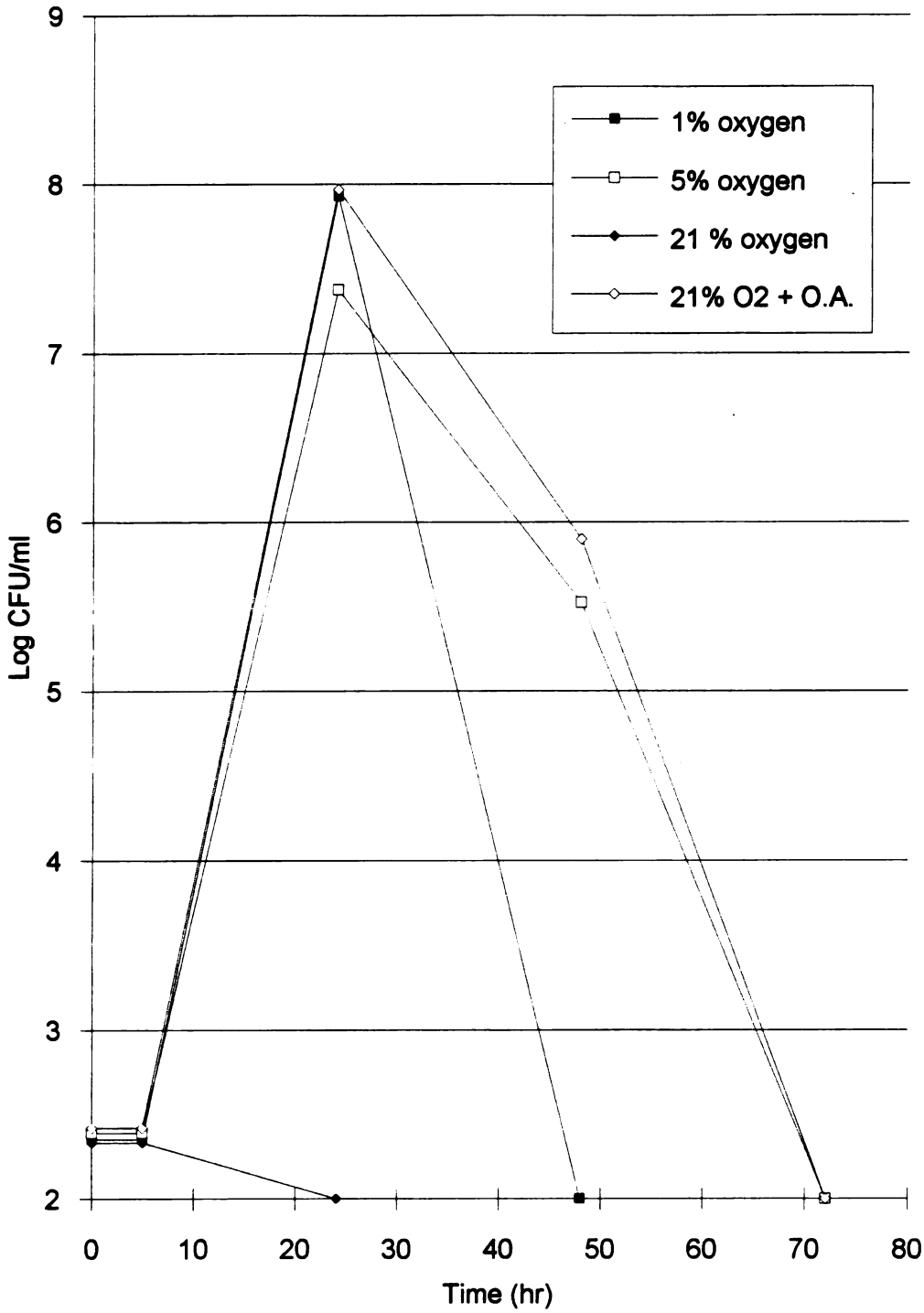

Figure 6: Clostridium sporogenes. Pure culture; 25°C; Aw>0.99

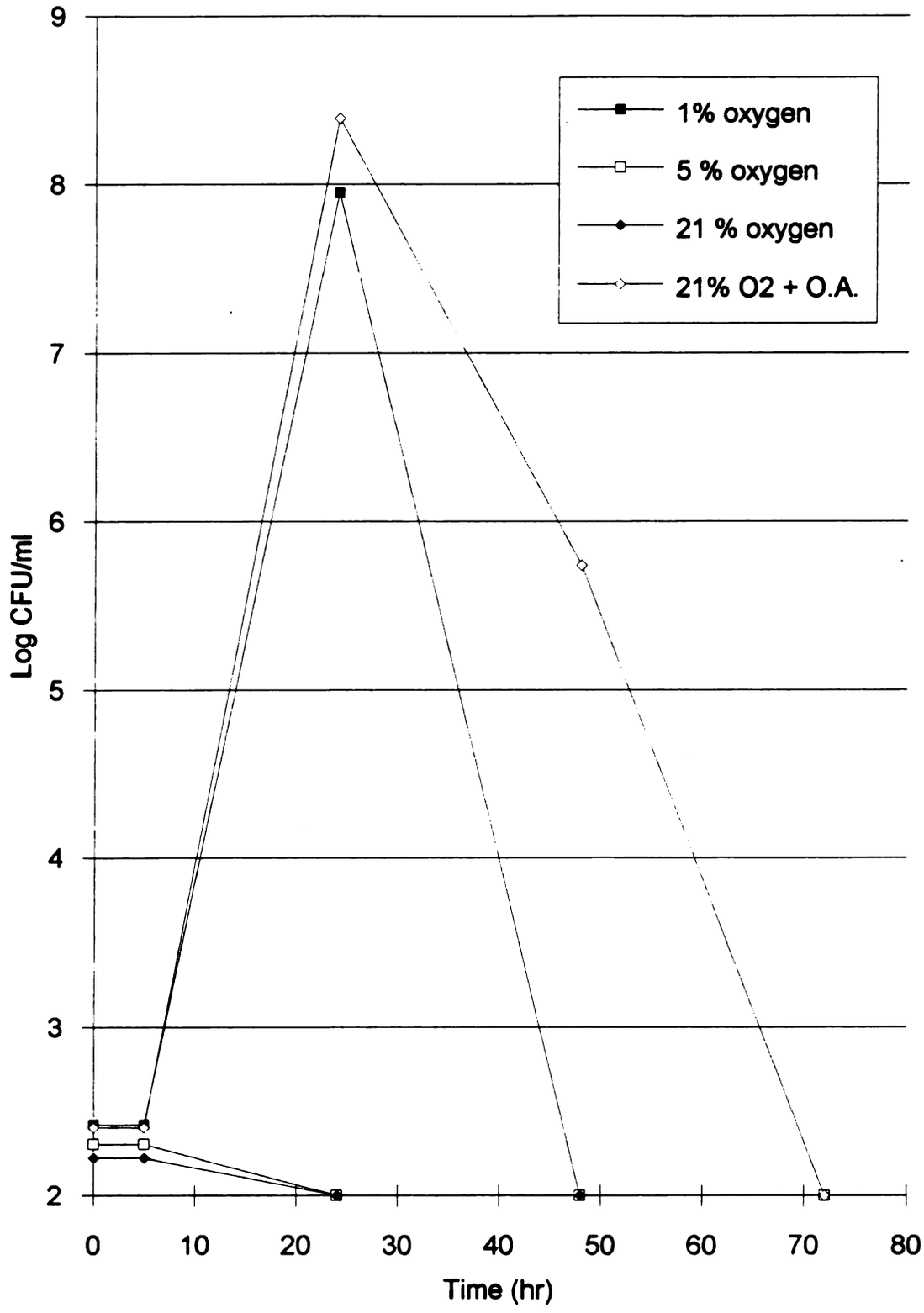

Figure 7: Clostridium sporogenes. Mixed culture; 25°C; Aw>0.99

of oxygen. The Eh value for the media at the end of the study for samples containing C. sporogenes as a pure culture was +86. Bacterial growth in jars with 1% oxygen in the head space reached an upper value of $\log_{10}$ CFU/ml of almost 8, which was similar to that found for the 21% oxygen + FreshPax™ samples. At 48 hours the value was less than 2. Values obtained were similar to those obtained by Molins et al. (1987) in a study with C. sporogenes PA 3679. One percent oxygen in the head space was found to be favorable for growth of C. sporogenes (figure 6). The level of bacteria after 24 hours corresponded to a spoilage level of 10^6 also used by Elkund et al. (1988) for C. botulinum. Since C. sporogenes resembles C. botulinum phenotipically (Buchanan, 1974), it can be differentiated only by toxin neutralization in mice, by polyacrylamide gel electrophoretic examination of soluble cellular proteins, or by gas chromatography of trimethylsilyl derivatives of whole cell hydrolysates. The toxin produced by C. botulinum is a protein with a characteristic neurotoxicity (Kautter et al., 1984). Kautter et al., (1984) reported that in the US, 766 botulism outbreaks were recorded from 1899 through 1977. These involved 1961 cases and caused 999 deaths. Of outbreaks in which the toxin type was determined, 199 were due to type A, 60 to type B, 32 to E, and 1 to type F.

A lower $\log_{10}$ CFU/ml value was obtained for C. sporogenes in jars with 5% oxygen but high numbers were held for one more day than at 1% initial oxygen (value of less than 2 reached at 72 hr). This is opposite to that observed for B. subtilis under the same incubation conditions. C. sporogenes did not grow at 5 % oxygen (figures 7 and 5, respectively) though Bacillus subtilis did (figure 5) in the mixed culture. This may have been because B. subtilis grew faster initially and consumed the nutrients required for growth of C. sporogenes since the system was closed and fresh media was not provided. Another possibility was the build up of

waste products by the bacteria that grew first, so that unfavorable conditions were achieved in the media and the other bacteria could not start their growth.

Growth of *Bacillus subtilis* in samples stored at abusive temperature of 25°C and water activity 0.96

For media with water activity of 0.96 (sugar level = 40%) and temperature of 25°C, only Bacillus subtilis grew. Table 6 shows the statistical results of this evaluation, for B. subtilis only. Factors considered in the calculations included: time (0, 0.21, 5, 10, 12, 14, 16, and 18 days); inoculum (B. subtilis in pure versus mixed culture); and oxygen level initially in the head space where growth was observed (5 and 21%). All factors excluding initial oxygen level and all interactions such as time-inoculum, time-oxygen, inoculum-oxygen, and time-inoculum-oxygen were highly significant and influenced the growth of the bacteria. Growth of B. subtilis was not significantly effected at 5 or 21% oxygen initial atmosphere at the statistical level of $P<0.01$. Time and whether or not the bacteria was inoculated as a pure culture or as a mixed culture with C. sporogenes were the most influential factors. The difference in time required to produce the same growth response was approximately 5 days: 5 and 10 days for the mixed and the pure culture, respectively (figures 8 and 9). A prolonged growth curve was observed with the reduced Aw (0.96). However, water activity was probably not the factor that influenced extension of the growth curve since Busta et al. (1984) reported that the approximate minimum Aw permitting growth (not optimum) of B. subtilis was 0.90. This number is far lower than the Aw in the media (0.96). B. subtilis did grow well in the media without sugar (figures 4 and 5). Therefore, extension in the growth response was probably because the metabolic rate (B.

Table 6: Analysis of Variance for B. subtilis (log CFU/ml) at 25°C and Aw = 0.96

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	8	273.464	34.183**
Inoculum	1	19.301	19.301**
Time x inoculum	8	70.497	8.812**
Oxygen	1	0.249	0.249*
Time x oxygen	8	21.694	2.712**
Inoculum x oxygen	1	4.813	4.813**
Time x inoc. x oxygen	8	16.887	2.111**
Error	36	1.369	0.038
Total	71	408.274	

Coefficient of Variation = 4.78 %

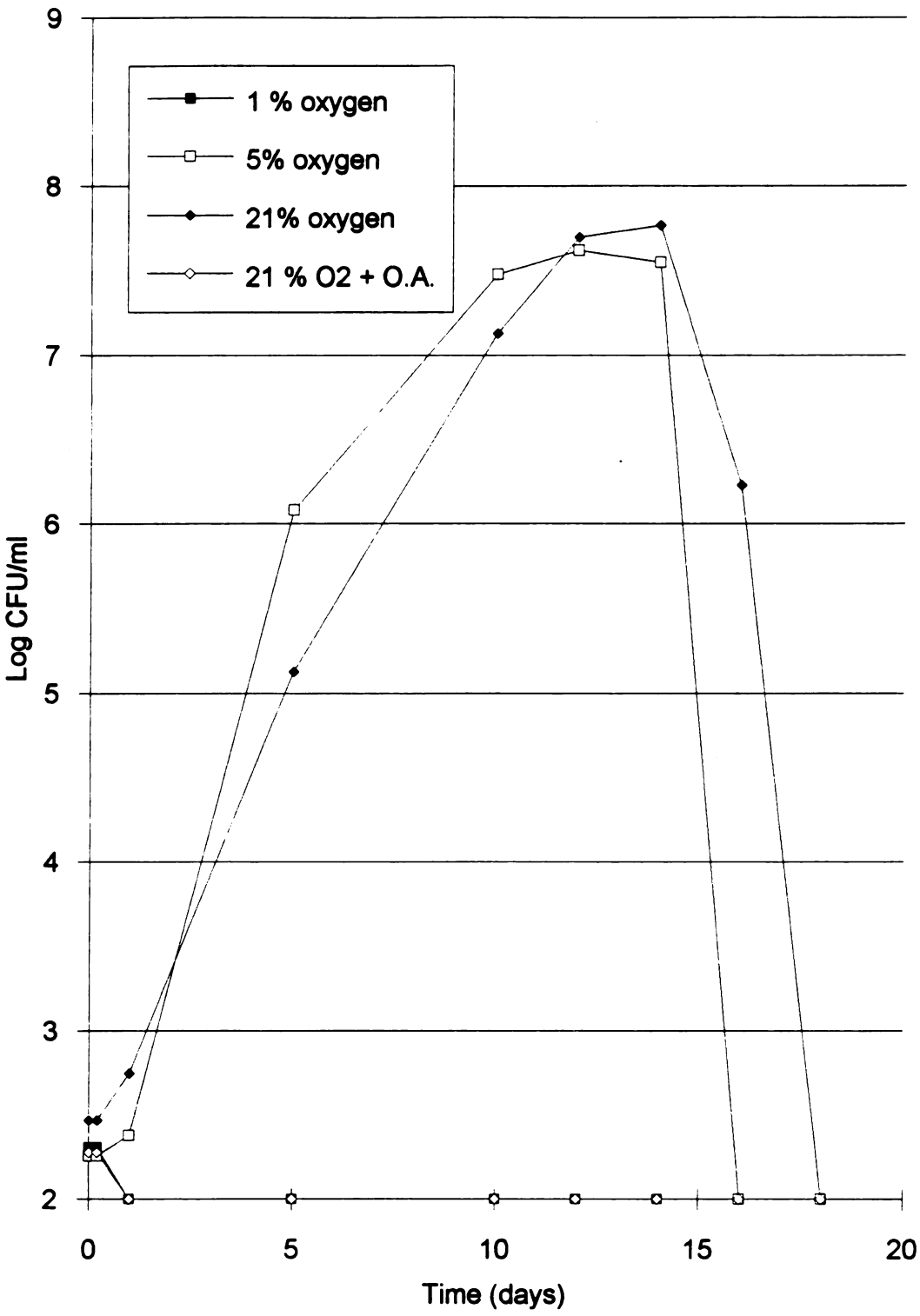

Figure 8: Bacillus subtilis. Pure culture; 25°C; Aw=0.96

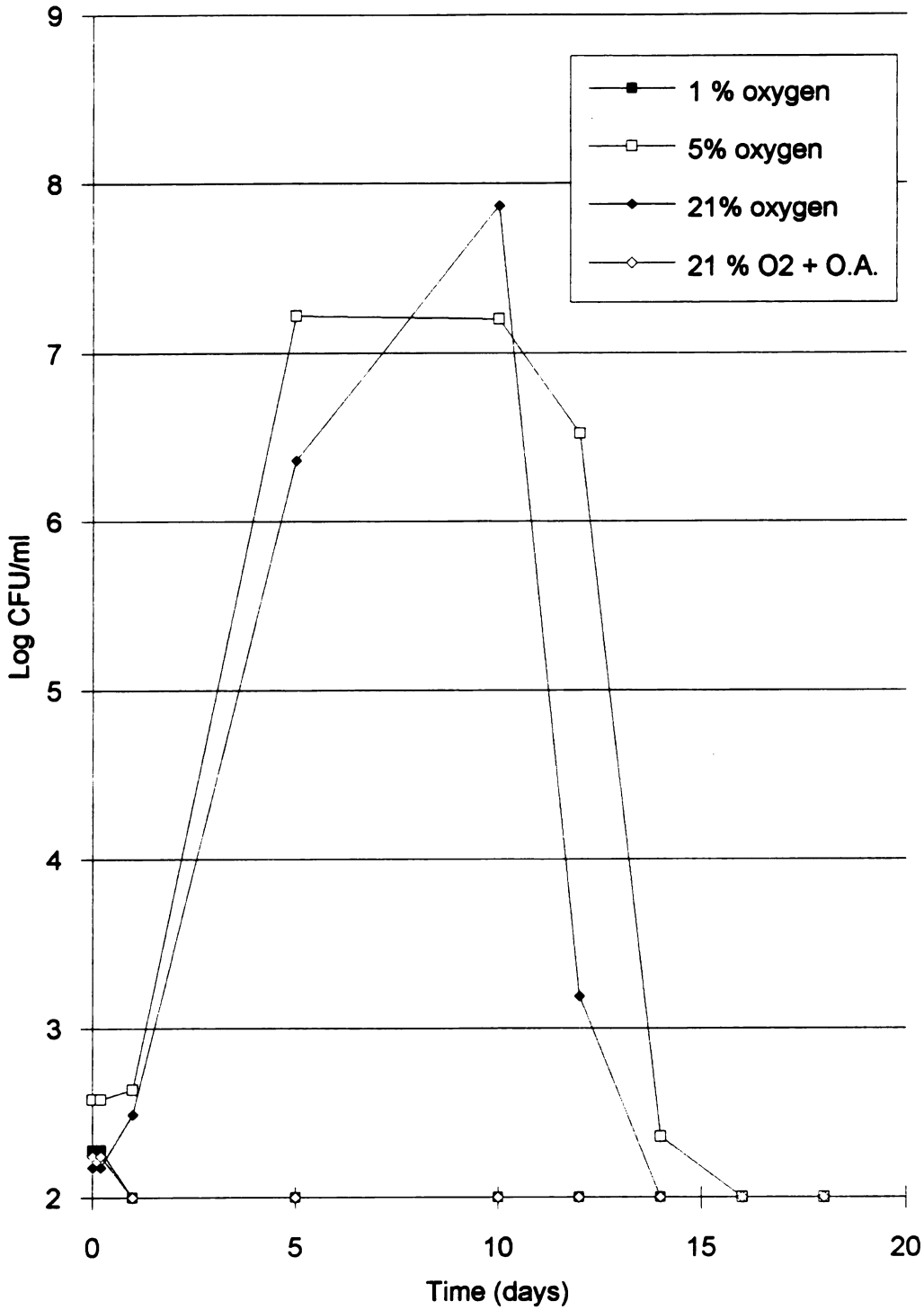

Figure 9: Bacillus subtilis. Mixed culture; 25°C; Aw=0.96

subtilis) was lowered due to the osmotic pressure created by the amount of sugar, as suggested by Pelczar et al. (1983) and Banwart (1989).

Change in head space oxygen content in media with Aw >0.99

Statistical evaluation of the change in oxygen level was based on analysis taken at 0 and 24 hours, in order to include more factors in the statistical analysis. Four factors were considered: Head space oxygen content (1%, 5%, 21%, and 21% plus oxygen absorber); inoculum (pure and mixed culture); temperature (10 and 25°C); and time (0 and 24 hour). Table 7 shows the analysis of variance for the factors considered and their interactions. From the statistical results, once comparison of the F values obtained versus the percent points in the F distribution table, it was found that all factors and interactions had a highy significant effect on the oxygen content within a 24 hour period.

Two factors and an interaction influenced predominantly in the change of oxygen concentration: Initial head space oxygen, time, and their interaction. The initial head space oxygen was important because of consumption of oxygen by B. subtilis. Figure 10 shows the change in the oxygen present in the head space at 0 and 24 hours for samples with 1% initial oxygen. The change in oxygen level was about the same magnitude for these samples and the final oxygen concentrations were below 0.2%, exept for the samples at 25°C containing C. sporogenes (this samples also had the highest final % oxygen of all, of about 1%). This reduction in oxygen may be due to oxygenation of the liquid media by a diffusion process, as suggested by Karel (1975). B. subtilis might have used some of the oxygen since it grew at the 1% level in the pure culture. The oxygen change in the 5% O_2 jars is shown in figure 11. Initially the media was in an anaerobic status as indicated by the resazurin indicator (which remained colorless) and with

Table 7: Analysis of Variance for % O_2 change in heaspace. Aw >0.99

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Headspace (A)	3	5250.062	1750.021**
Inoculum (B)	3	57.152	19.051**
headspace x inoculum	9	106.424	11.825**
Temperature (C)	1	36.643	36.643**
A x C interaction	3	107.852	35.951**
B x C interaction	3	55.552	18.517**
A x B x C interaction	9	109.733	12.193**
Time (D)	1	1876.478	1876.478**
A x D interaction	3	1886.054	628.685**
B x D interaction	3	55.681	18.560**
A x B x D interaction	9	109.988	12.221**
C x D interaction	1	61.138	61.138**
A x C x D interaction	3	97.419	32.473**
B x C x D interaction	3	49.728	16.576**
A x B x C x D interaction	9	110.230	12.248**
Error	64	1.033	0.016
Total	127	9971.167	

Coefficient of Variation = 1.59 %

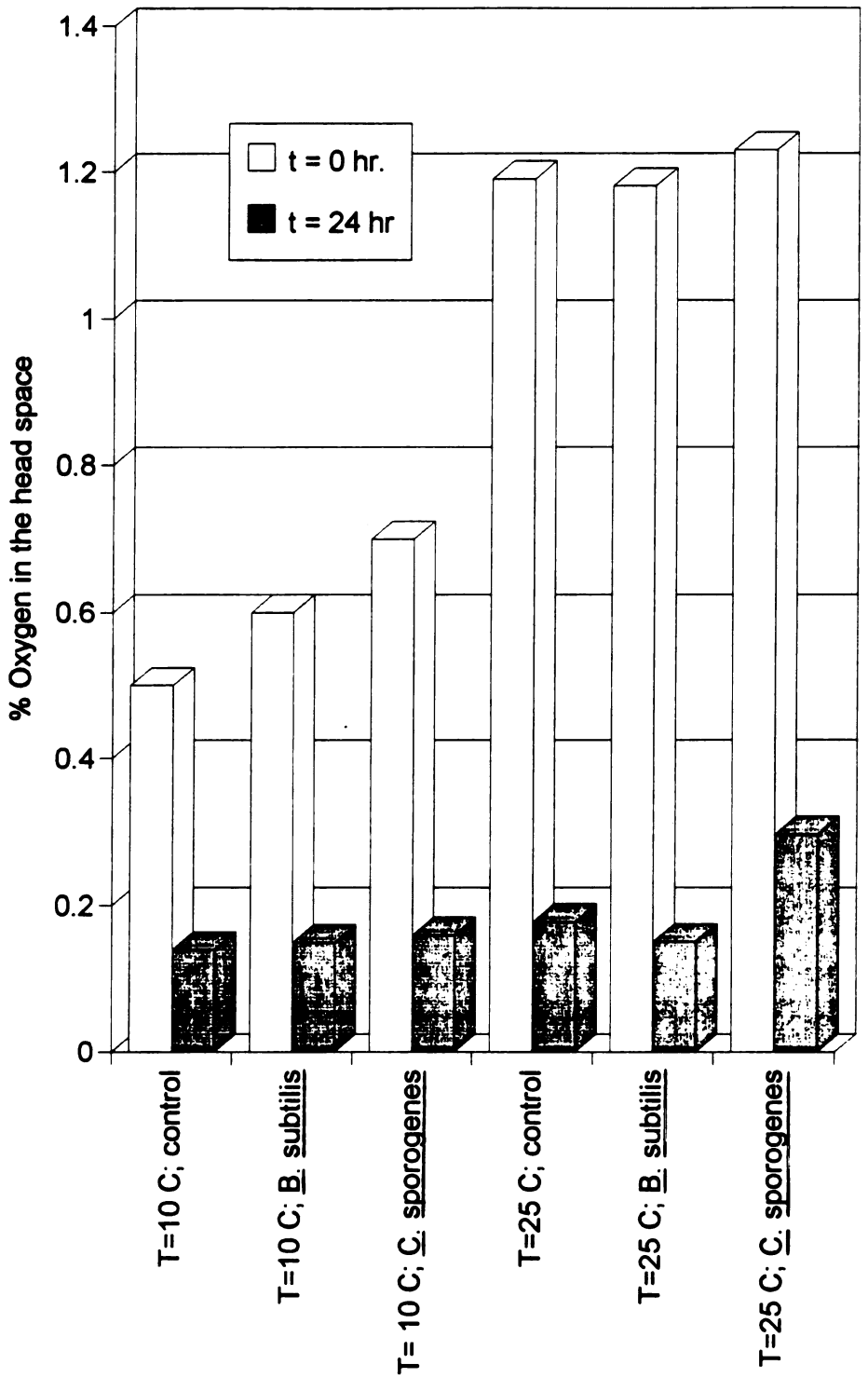

Figure 10: Oxygen change (%); 1% initial oxygen in the head space

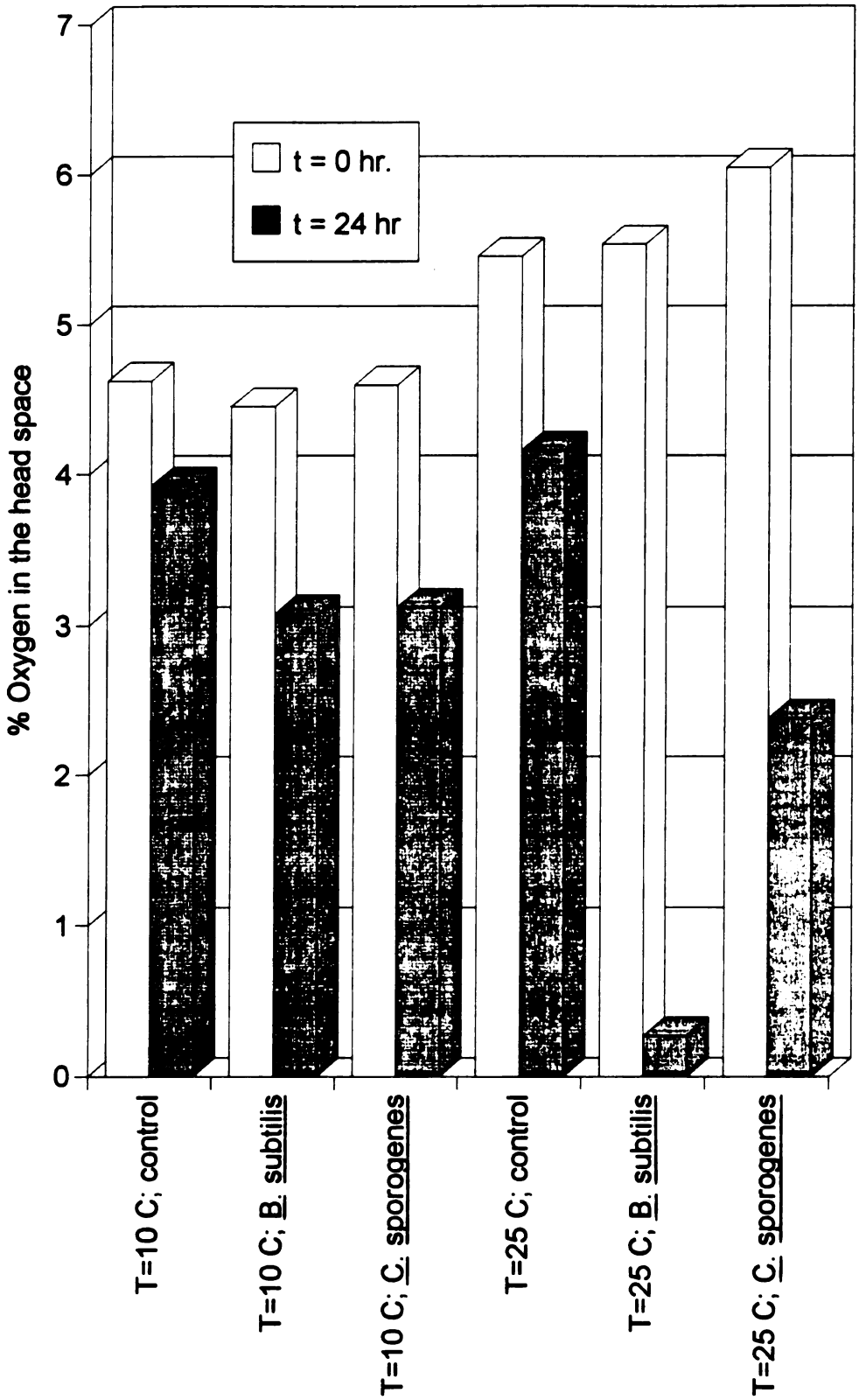

Figure 11: Oxygen change (%); 5% initial oxygen in the head space

introduction of the modified atmosphere and agitation, oxygen may have become dissolved into the media (indicator turned red, which indicates aerobiosis). Within 24 hours for all samples at 10°C and at 25°C for the control or in C. sporogenes samples, there was no significant difference in the percent oxygen change. There was a large change in the samples inoculated with B. subtilis at 25°C. This was probably because the oxygen was consumed by B. subtilis that grew to a value of >8 log CFU/ml (figure 4). The 21% initial oxygen present in the head space was lowered significatively in the jars containing B. subtilis at 25°C (figure 12). This was also found in the jars with 5% initial oxygen (figure 11). The oxygen in the 21% jars was reduced to less than 0.5%. At 25°C, in jars with an initial oxygen concentration of 21% the concentration was lowered to about 0.1% for some samples (figure 12). B. subtilis reduced the oxygen from 21% to levels similar to those obtained by vacuum or gas flushing systems (approximately 0.5%). This suggests that even if there was no initial growth of C. sporogenes in these jars, C. sporogenes could start growing. B. subtilis produces a toxin called "subtilin", which is a polypeptide with marked action against a wide range of Gram positive bacteria, and depending on concentration, it can be bacteriostatic or bactericidal. This antibiotic is known to prevent outgrowth of bacteria after germination. However, Ayres et al. (1980) reported that this toxin does not halt growth of C. sporogenes PA 3679. Thus, it is unlikely that this inhibited C. sporogenes in the jars containing both bacteria and where only B. subtilis was observed to grow. It is probable that in jars with 5% oxygen initially in the head space B. subtilis stopped growing because the oxygen was reduced and C. sporogenes did not grow because there was a lack of nutrients in the media.

In figure 13 is shown the change observed in the oxygen level for sample jars initially containing 21% oxygen + FreshPax™. In all samples, oxygen

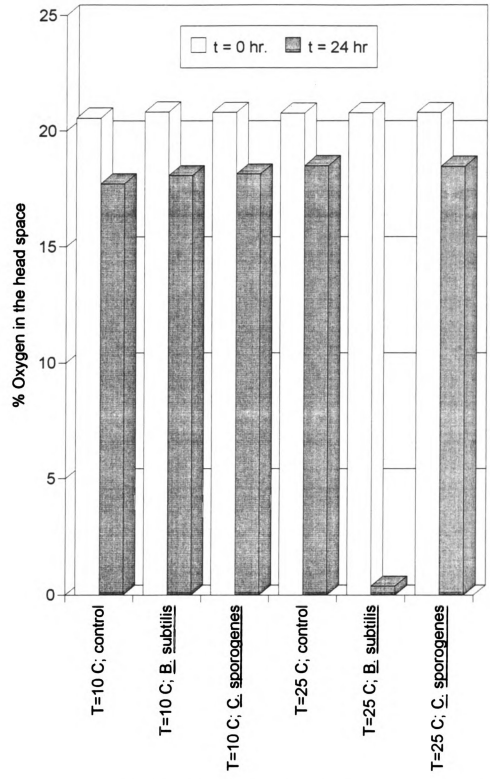

Figure 12: Oxygen change (%); 21% initial oxygen in the head space

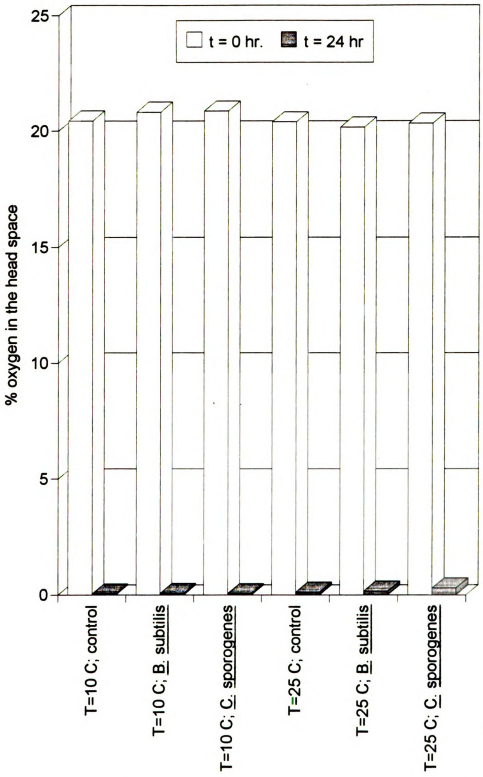

Figure 13: Oxygen change (%); 21% initial oxygen + FreshPax™

level fell to below 0.5%. Variations of 0.4 to 0.5 % oxygen were found in samples where C. sporogenes grew (figs. 6 and 7). All other jars had oxygen values less than 0.2% in the head space after only 5 hours at 25°C, and 12 hours at 10°C. No growth was observed for B. subtilis at any temperature when the oxygen absorber was used. C. sporogenes was found to grow rapidly at the 25°C storage temperature in the presence of the oxygen absorber (figures 6 and 7). Figure 14 shows the oxygen change in jars containing a mixed culture and either 1 or 5% initial oxygen in the head space. After 24 hours, initial oxygen in the head space was reduced to about the same level in samples with 1% initial oxygen, with growth observed for C. sporogenes (figures 6 and 7). B. subtilis grew in samples at 25°C and 5% oxygen (figures 4 and 5). In figure 15 is shown oxygen content in jars inoculated with the mixed culture and either 21% initial oxygen or 21% oxygen + FreshPax™. The oxygen (%) changed significantly in all jars at 25°C, and in jars stored at 10°C with oxygen scavenger. The only jars that maintained about the same oxygen levels as the controls were samples with bacteria and no FreshPax™ stored at 10°C. The decrease in oxygen from 21% to <1% in jars stored at 25°C without FreshPax™ is attributed to consumption of oxygen by B. subtilis.

Bacillus subtilis evaluation of samples stored at 10°C and water activity >0.99

All samples stored at 10°C were studied at a water activity of >0.99. In table 8 is shown the analysis of variance for the results collected. Time (0 to 4 weeks) and the oxygen level (1%, 5%, 21% and 21% plus oxygen absorber) affected significantly ($P<0.01$) the growth of B. subtilis at 10°C. The other factor, inoculum method and interactions of all possible combinations were non-

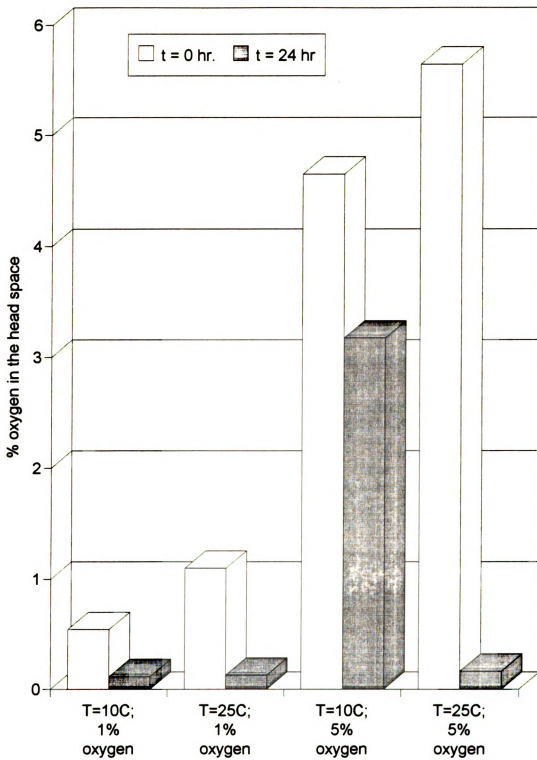

Figure 14: Oxygen change (%); mixed culture

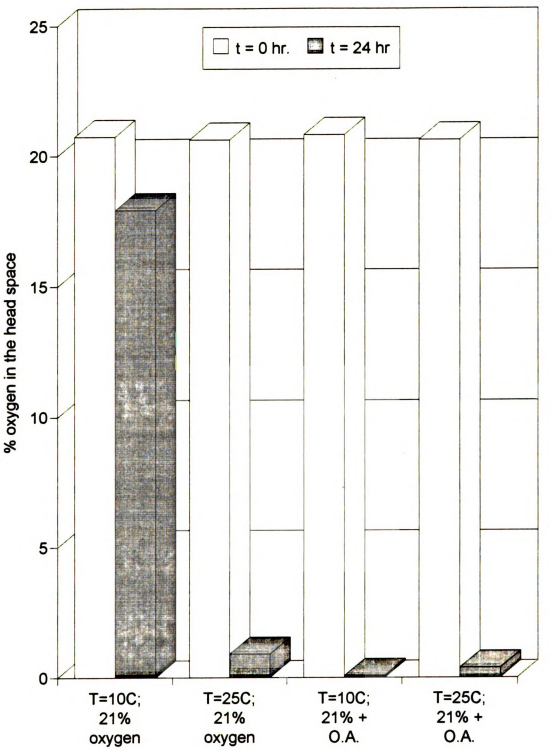

Figure 15: Oxygen change (%); mixed culture

Table 8: Analysis of Variance for B. subtilis (log CFU/ml) at 10°C and Aw >0.99

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	4	83.230	20.808**
Inoculum	1	0.018	0.018
Time x inoculum	4	0.200	0.050
Oxygen	3	42.835	14.278**
Time x oxygen	12	20.661	1.722
Inoculum x oxygen	3	12.660	4.220*
Time x inoc. x oxygen	12	13.937	1.161
Error	40	29.866	0.747
Total	79	203.407	

Coefficient of Variation = 24.63 %

significant, thus, they did not influence significantly ($P<0.01$) the growth of the bacteria at 10°C.

Figure 16 shows a continuous pattern for growth of B. subtilis (pure culture) at levels of about 10^5 CFU/ml through the fourth week in jar samples containing the oxygen absorber. Growth under anaerobic conditions was observed to yield large numbers; this was not expected since the bacteria was aerobic. This may be due to the presence of glucose in the media, and some authors, as Ayres et al. (1980) reported that under anaerobic conditions, species in the Bacillus group fermented sugars; and Buchanan (1974) reported that glucose permit a much restricted anaerobic growth of B. subtilis in complex media. Bacterial growth in the mixed culture was not detected until the third week, with levels attained of about 10^4 CFU/ml and at the fourth week about 3×10^4 CFU/ml. In these samples the oxygen was depleted to less than 0.1% in about 12 hours. By comparing figures 4 and 16, in which B. subtilis was inoculated as a pure culture, no growth was observed at 25°C and growth was observed with values of almost 10^5 for samples incubated at 10°C. This was attributed to various possible reasons: 1) pH change from 7.2 to 5.11. Banwart (1989) reported that the optimum pH for B. subtilis was markedly changed by altering the incubation temperature, so that the optimum pH was 7.4 at 37°C and 5.4 at 10°C; 2) Incubation time was longer at 10°C compared to 25°C. This probably allowed the bacteria to grow slowly initially in samples incubated at 10°C and lower the pH so that it generated a more favorable condition for growing and generating large number of cells.

Figure 17 shows growth of B. subtilis after week 2 for samples containing a mixed culture, with values of about 4×10^5 CFU/ml at the fourth week; these sample jars had 21% initial oxygen in the head space. No growth was observed for samples containing B. subtilis at any time when it was inoculated as a

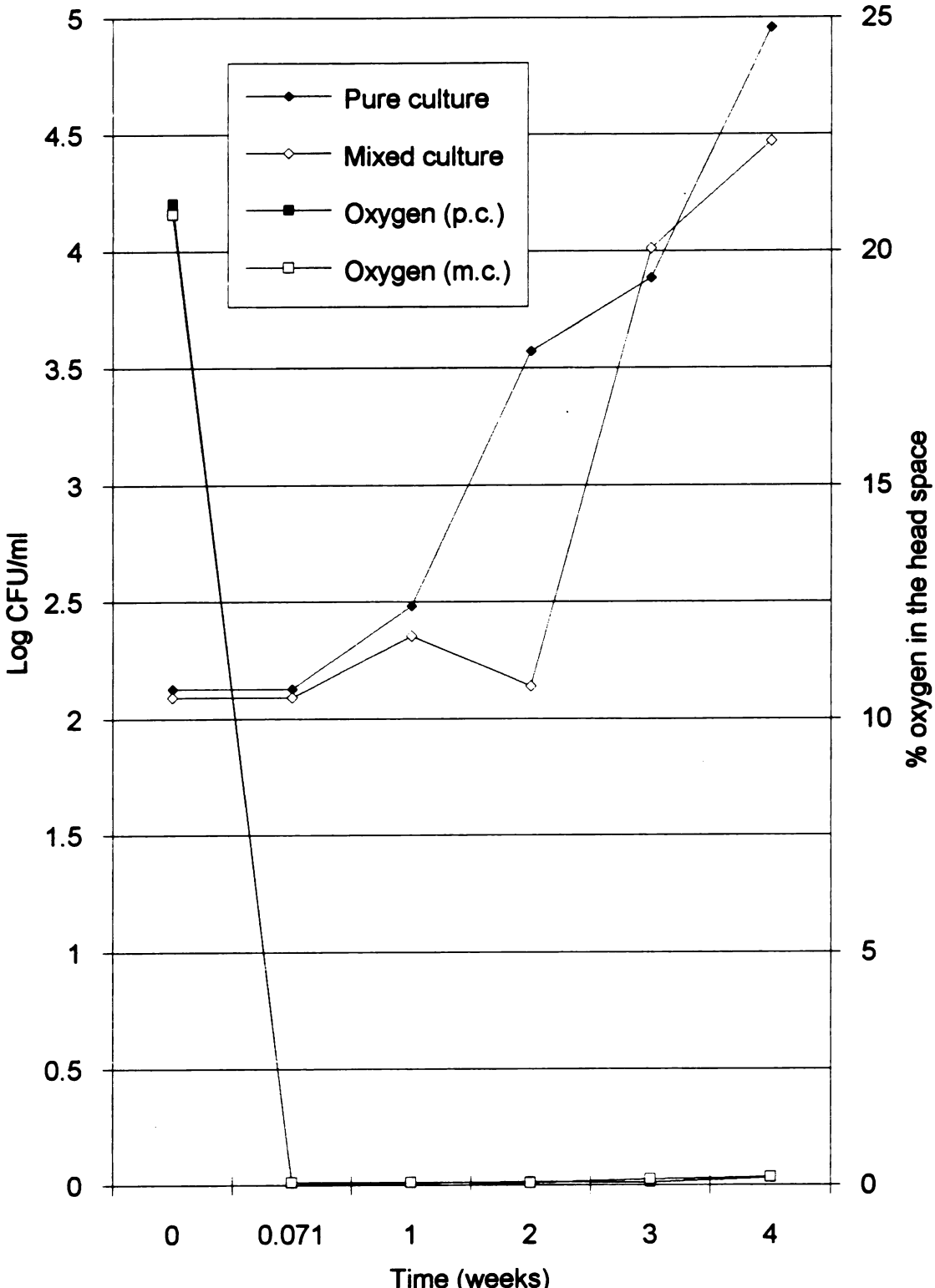

Figure 16: Bacillus subtilis CFU/ml and oxygen change (%). 10°C; 21% oxygen in the head space + FreshPax™

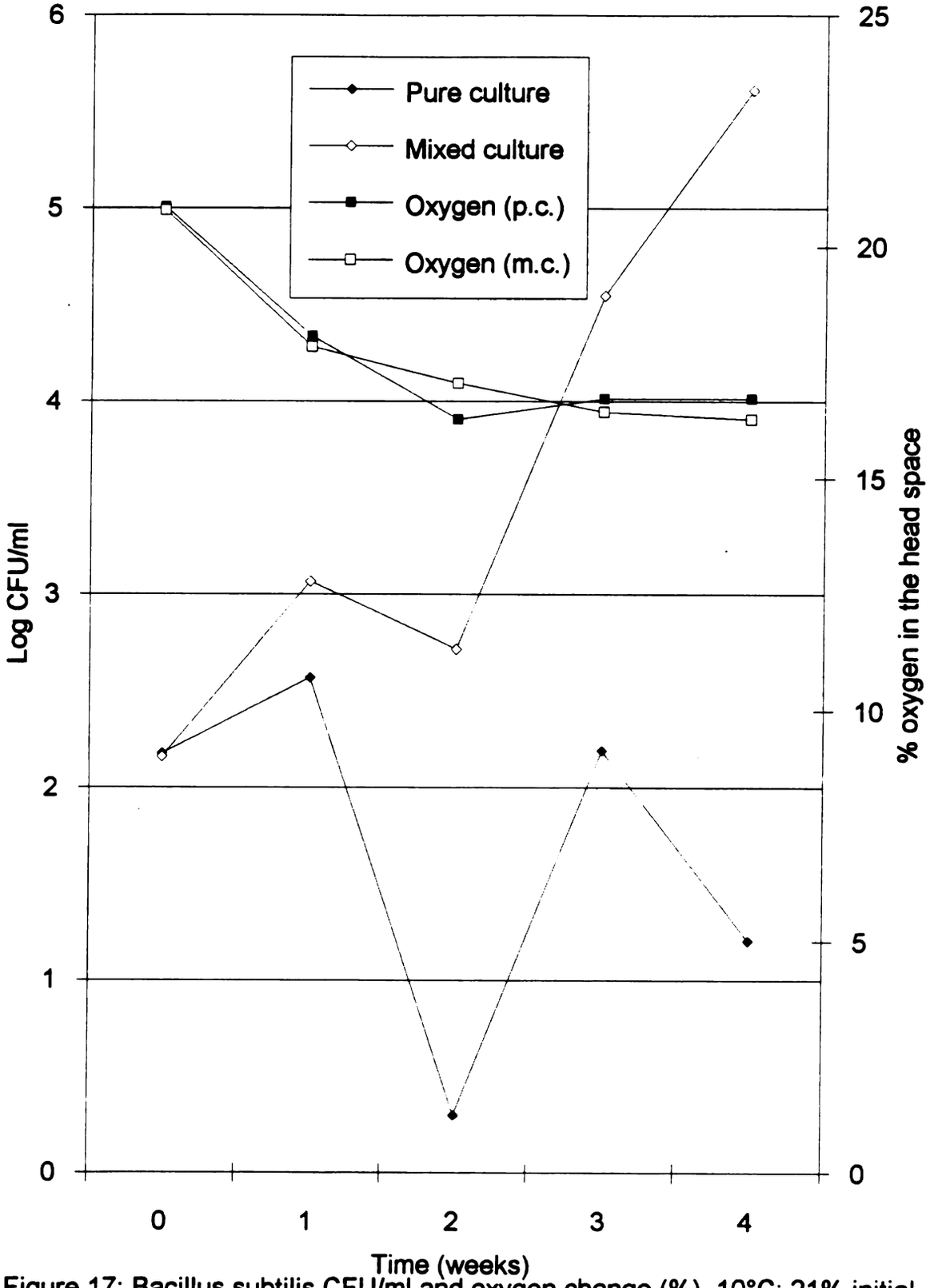

Figure 17: Bacillus subtilis CFU/ml and oxygen change (%). 10°C; 21% initial oxygen in the head space

pure culture over the same time period. This is sugested to dissolved oxygen had an inhibitory effect on growth of B. subtilis. Banwart (1989) reported that dissolved oxygen had an inhibitory effect on Bacillus fragilis, and showed that was independent of the oxidation-reduction potential. The oxido-reduction potential in the media with B. subtilis as a pure culture at the end of the study was of +99 mv, which is in the range of growth of +85 to +160 mv as reported by Banwart (1989). Oxygen level was about the same for samples containing pure culture and mixed culture during this period. Oxygen in the head space was reduced from 21% to about 16%. This is attributed to oxygen dilution in the liquid media which was also reported by Piper et al. (1989), who found that the amount of gas which partitions between water and the atmosphere is in equilibrium and inversely proportional to the temperature. Temperature was the most important factor affecting the oxygen concentration in water. The oxygen level in the head space of samples at 10°C at equilibrium was 16% while in samples stored at 25°C it was 18%. This indicates that more oxygen dissolved in the media at 10°C since all samples had about 21% initial oxygen in the jar head space.

Figure 18 shows the relationship between the log CFU/ml and time, and also the relationship between % oxygen and time for B. subtilis in samples with 5% initial oxygen in the head space. The most bacterial growth was obtained in sample jars with 5% initial oxygen in the head space as compared to the other initial oxygen concentrations at 10°C. Logaritmic growth was detected during the first 3 week period, with about 5.0×10^7 CFU/ml for samples containing pure culture and about 4.8×10^6 CFU/ml in the samples containing a mixed culture. By the fourth week this had decreased to 1×10^7 CFU/ml and 5.8×10^5 CFU/ml, respectively. Oxygen in the head space decreased from about 4.5% in both pure and mixed culture to about 2%. For the bacteria cultured at 10°C a decrease in

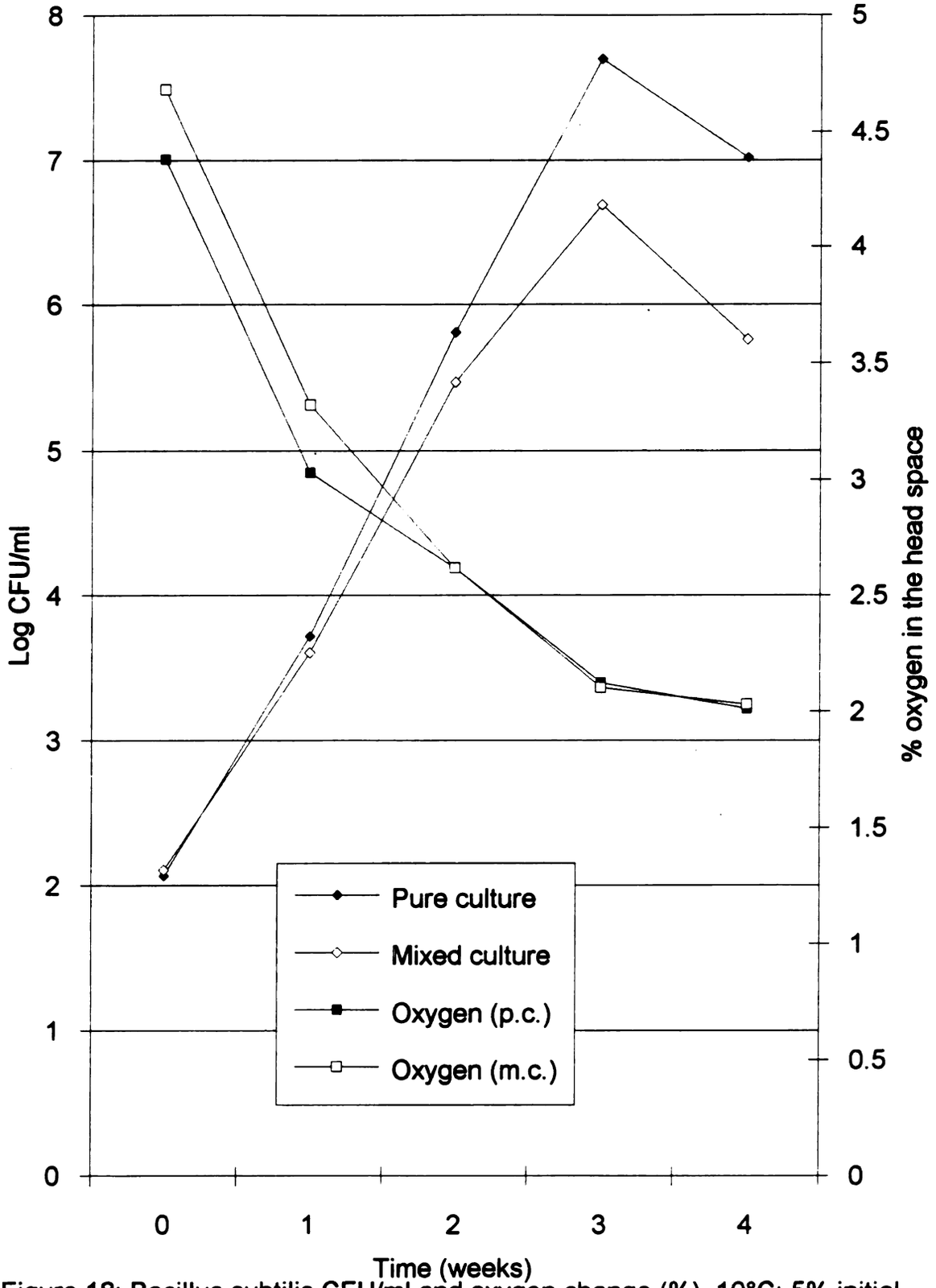

Figure 18: Bacillus subtilis CFU/ml and oxygen change (%). 10°C; 5% initial oxygen in the head space

oxygen was observed but not to the low levels (0.1%) found for the samples stored at 25°C (more bacterial growth) (see figures 4, 5, 11, and 12).

In figure 19 is shown the growth of B. subtilis at a concentration of 1% oxygen in the jar head space and at 10°C. Growth was observed to be logaritmic for bacteria inoculated as a pure culture while a slower rate was found for bacteria inoculated as a mixed culture. The levels attained at the fourth week were 3.2×10^6 and 2.3×10^4 CFU/ml for pure and mixed culture, respectively. Oxygen in the head space was lowered from about 0.55% to about 0.1% for both the pure and mixed culture.

Growth of Clostridium sporogenes in samples stored at 10°C and water activity >0.99

Analysis of variance for factors effecting growth of C. sporogenes at 10°C is presented in table 9. There was no single factor or interaction between factors that significantly influenced growth of this bacteria. Figures 20, 21, 22, and 23 present the growth curves for C. sporogenes (Log CFU/ml vs. time), and % oxygen change in the head space vs. time for samples stored at 10°C. No growth was observed in samples with initial oxygen in the head space of 21% and 5% (figures 21 and 22). Decreased log CFU/ml values from about 200 to 10 CFU/ml during the 4 week period was observed for samples containing either FreshPax™ or 1% initial oxygen.

The amount of oxygen in samples containing C. sporogenes was very similar to that in the controls (compare oxygen levels of figures 20-23 with figure 24). The oxygen levels in samples containing 21% initial oxygen was reduced to about 16%; for samples of 5% initial oxygen, oxygen levels were about

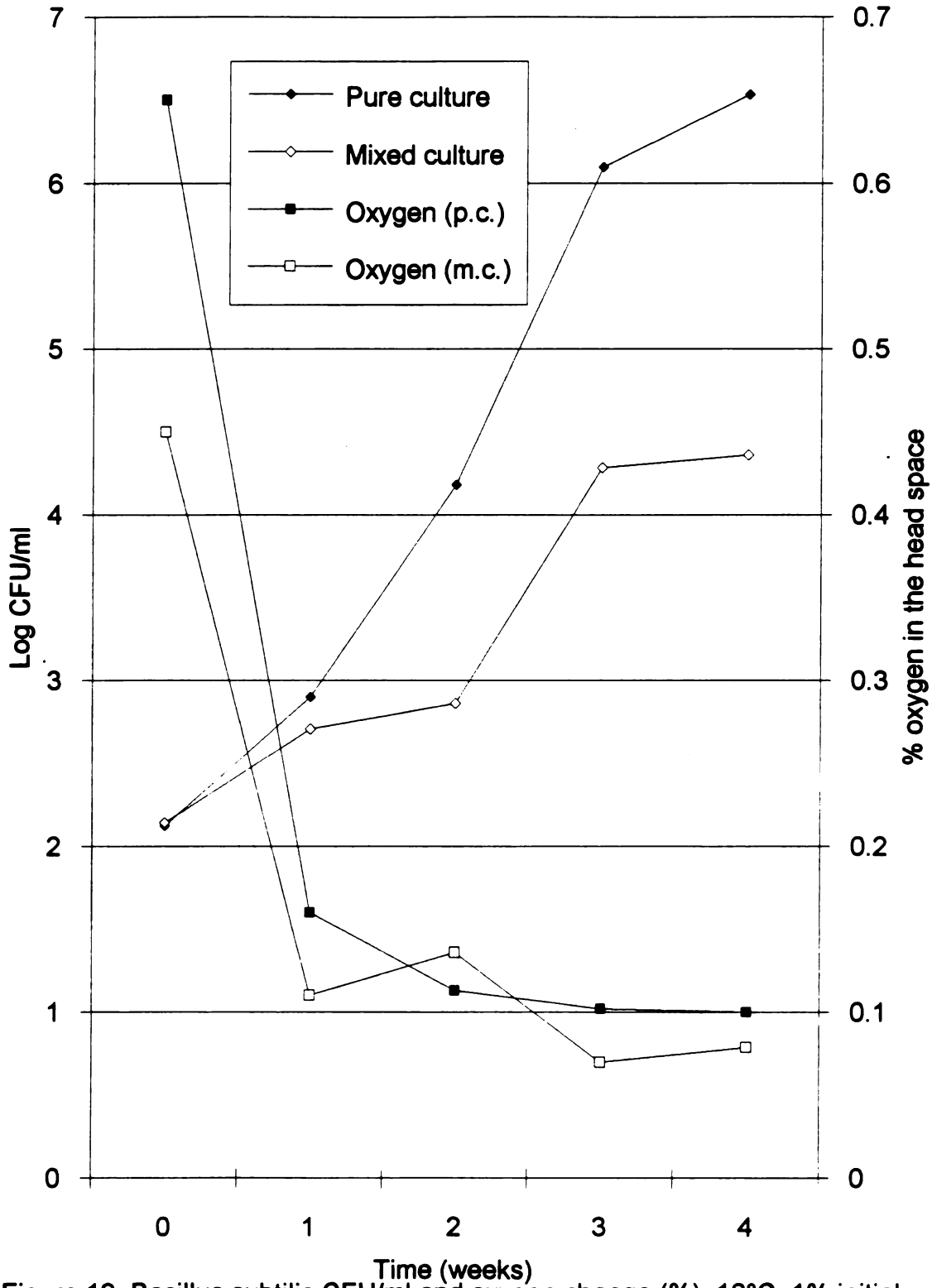

Figure 19: Bacillus subtilis CFU/ml and oxygen change (%). 10°C; 1% initial oxygen in the head space

Table 9: Analysis of Variance for C.sporogenes (log CFU/ml) at 10°C and Aw >0.99

FACTOR	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	4	0.002	0.001
Inoculum	1	0.000	0.000
Time x inoculum	4	0.001	0.000
Oxygen	3	0.001	0.000
Time x oxygen	12	0.005	0.000
Inoculum x oxygen	3	0.001	0.000
Time x inoc. x oxygen	12	0.006	0.001
Error	40	0.018	0.000
Total	79	0.034	

Coefficient of Variation = 1.05 %

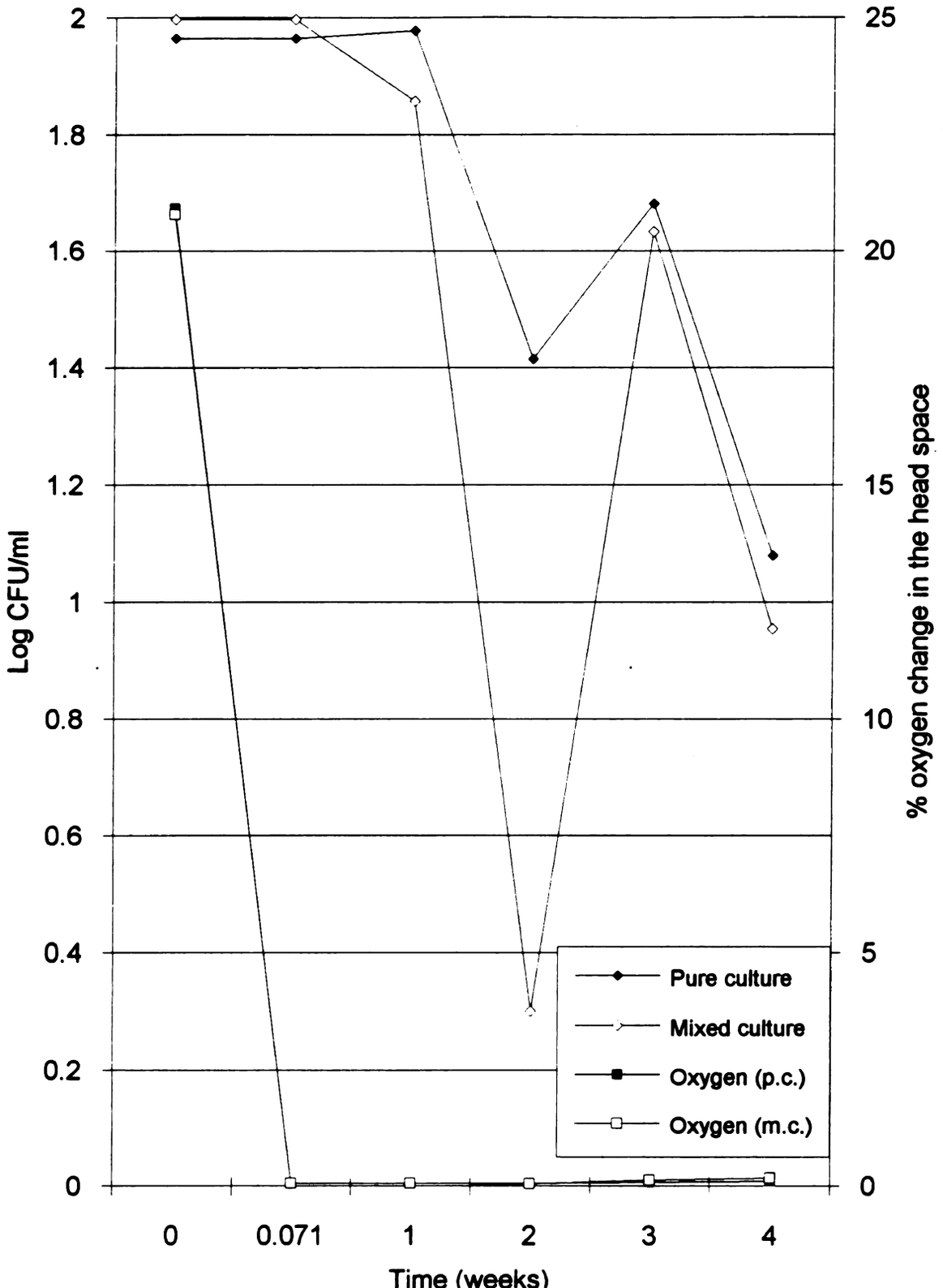

Figure 20: Clostridium sporogenes CFU/ml and oxygen change (%). 10°C; 21% initial oxygen in the head space + FreshPax™

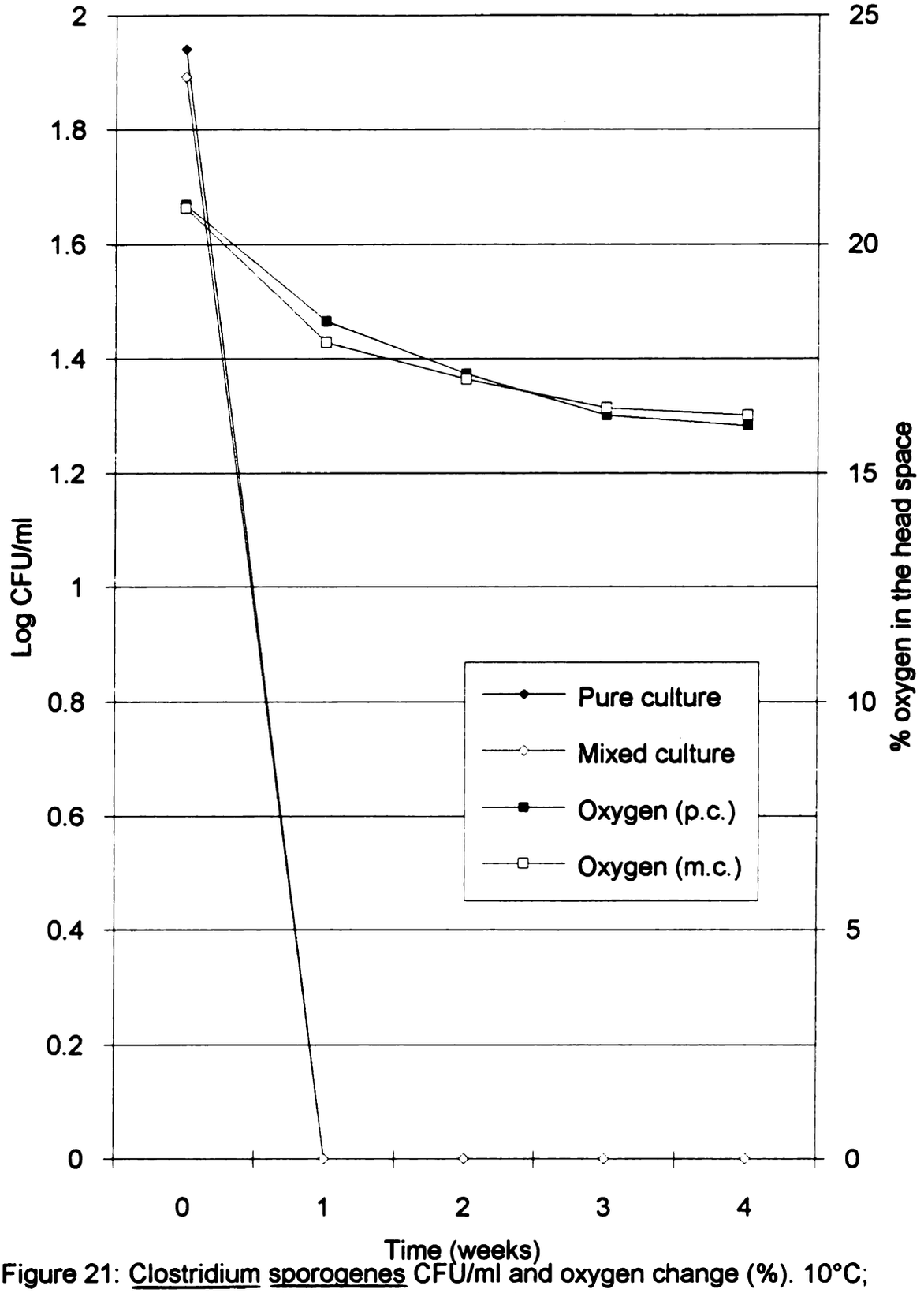

Figure 21: Clostridium sporogenes CFU/ml and oxygen change (%). 10°C; 21% initial oxygen in the head space

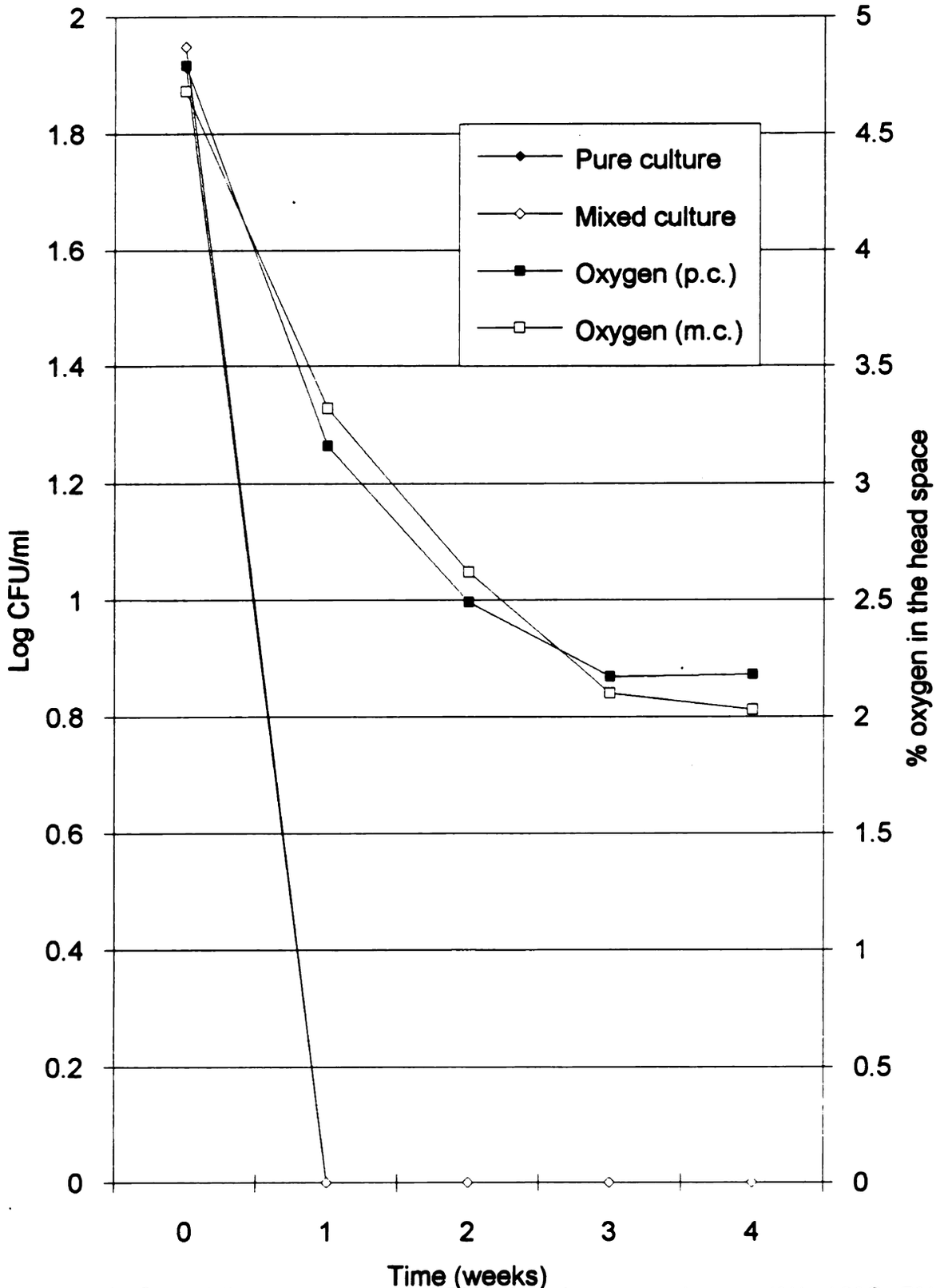

Figure 22: Clostridium sporogenes CFU/ml and oxygen change (%). 10°C; 5% initial oxygen in the head space

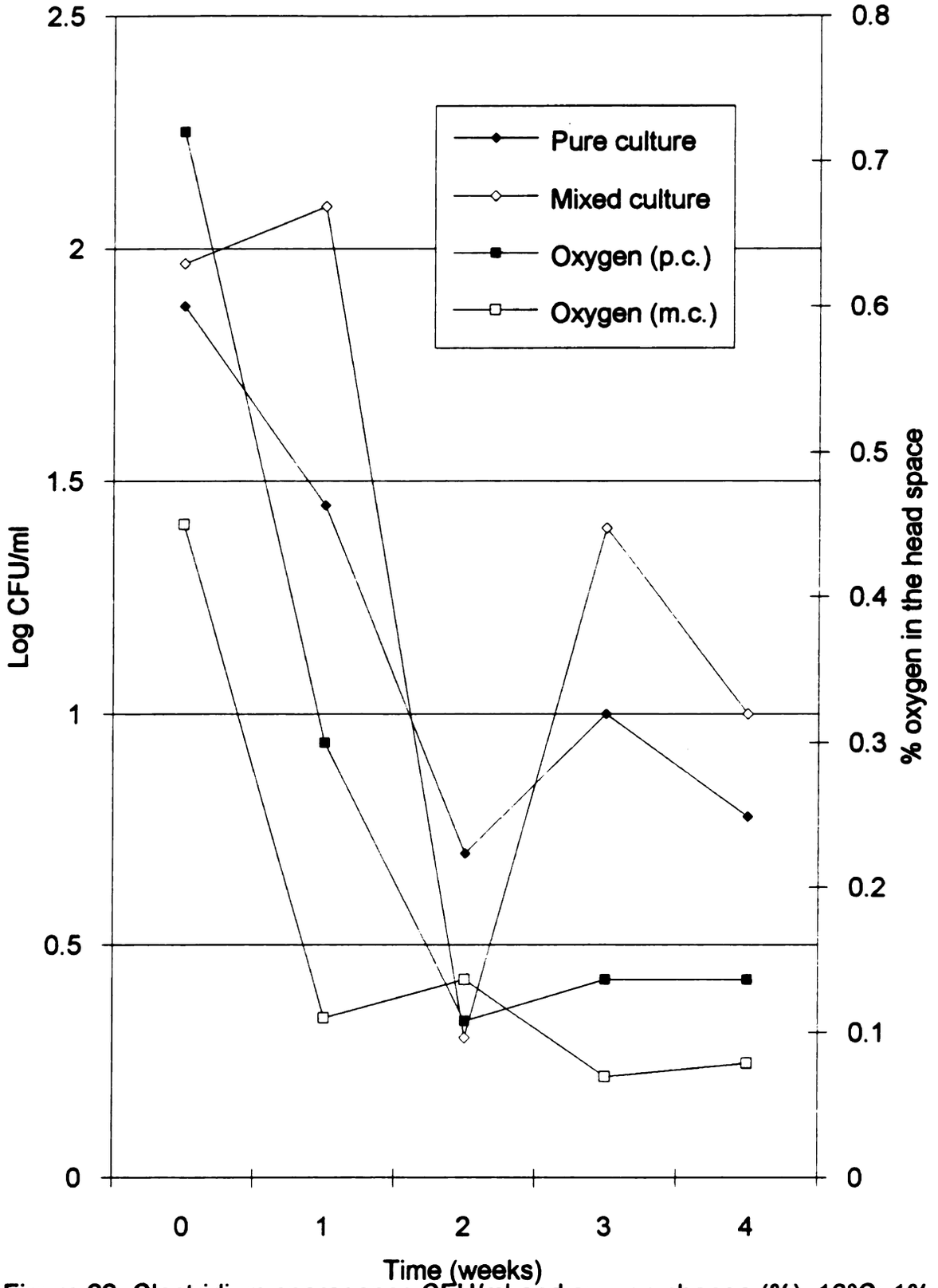

Figure 23: Clostridium sporogenes CFU/ml and oxygen change (%). 10°C; 1% initial oxygen in the head space

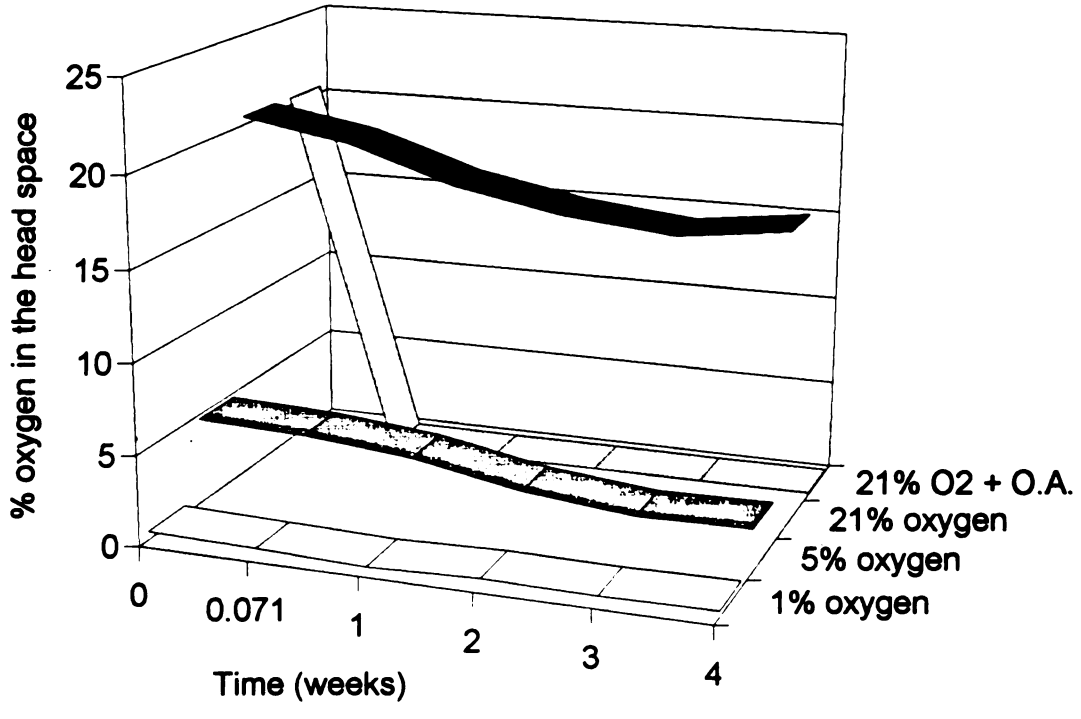

Figure 24: Oxygen change (%) in the head space for control samples. 10°C

2%; for samples with 1% initial oxygen, oxygen levels were about 0.3%; and for all samples containing FreshPax™, the oxygen level was reduced to less than 0.1%.

Altough no growth of C. sporogenes was observed, a comparison with C. botulinum strains in terms of growth and toxin production can be performed as follows: Kautter et al. (1984) reported that the optimum temperature for C. botulinum growth and toxin production of the proteolytics (all A, and some B and F strains) is close to 35°C, while that of the nonproteolytics (all E, and remaining of B and F strains) is approximately 26°C. They reported that nonproteolytic types B, E, and F are able to produce toxin at refrigeration temperature (3°C to 4°C), but toxins of the nonproteolytic do not manifest maximum potential toxicity until they are activated with trypsin; and toxins of the proteolytics generally occur in fully, or close to fully, activated form.

Kautter et al. (1984) concluded that a food can contain viable C. botulinum and still not be capable of causing botulism, and as long as the organisms do not grow, toxin is not produced. The usual vehicles of botulism are those foods which are processed to prevent sopilage, and which are not usually refrigerated. Also, unless the temperature is very precisely controlled and kept below 3°C, refrigeration will not prevent growth and toxin formation by nonproteolytic strains.

Temperature effect on oxygen absorber

Table 10 shows the two-way analysis of variance for % oxygen in sample head space as a function of time and in the presence of FreshPax™. The statistical analyses presented in tables 10, 11, and 12 were calculated for the first 12 hours (0, 1, 2, ..., 12 hours). The oxygen absorber decreased the % oxygen

Table 10: Two-way Analysis of Variance for % oxygen in the head space over time and in presence of FreshPax™

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	12	235.96	19.664
Oxygen absorber	1	1624.91	1624.908**
Error	12	217.77	18.147
Total	25	2078.64	

Coefficient of Variation = 34.40 %

Table 11: Two-way Analysis of Variance for % oxygen in the head space over time and as affected by water activity (Aw)

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	12	235.96	19.664**
Water activity	1	0.32	0.318*
Error	12	0.68	0.057
Total	25	236.96	

Coefficient of Variation = 1.92 %

Table 12: Two-way Analysis of Variance for % oxygen in the head space over time and as affected by Temperature

SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Time	12	235.96	19.664**
Temperature	1	18.23	18.226**
Error	12	12.17	1.014
Total	25	266.36	

Coefficient of Variation = 8.13 %

change in the jar head space with a significance at the 1% level compared to sample jars without FreshPax™. Further orthogonal contrast for presence vs. no presence of FreshPax™ showed an F value of 89.539 with effect of -7.905 and a probability of 0.0%, which confirmed the highly significance effect of the FreshPax™ on the % oxygen in the head space. Figure 25 shows the % oxygen for samples stored at 10°C and 25°C containing media with a Aw of >0.99. Controls % oxygen did not change over time. Jars containing FreshPax™ had oxygen levels in the head space of less than 0.1% in about 7 hours for samples stored at 25°C and in about 12 hours for samples stored at 10°C.

In table 11 is shown the statistical evaluation for the influence of analysis of variance for % oxygen head space as affected by time and water activity (Aw). Water activity did not influence significantly ($P<0.01$) the % oxygen in the head space of the samples. Orthogonal contrast was performed on the water activity factor and an effect of 0.111 with an F value of 5.616 and probability of 3.5% was found. This indicates that at a 1% level of significance, water activity did not influence the % oxygen in the head space of the samples studied. Results for samples with water activity of 0.88 are shown in figure 26. By comparing figures 25 and 26 it is shown that values were very similar under the same conditions. Controls had about the same level of oxygen over the time period studied. For samples containing oxygen absorber and stored at 25°C % oxygen in the head space decreased to less than 0.1% in about 5 hours while for samples stored at 10°C the % oxygen in the head space decreased to the same amount in about 12 hours.

Table 12 shows the two-way analysis of variance for % oxygen in the head space as a function of time and temperature. Temperature was observed to significantly ($P<0.01$) affect the activity of the oxygen absorber. Orthogonal

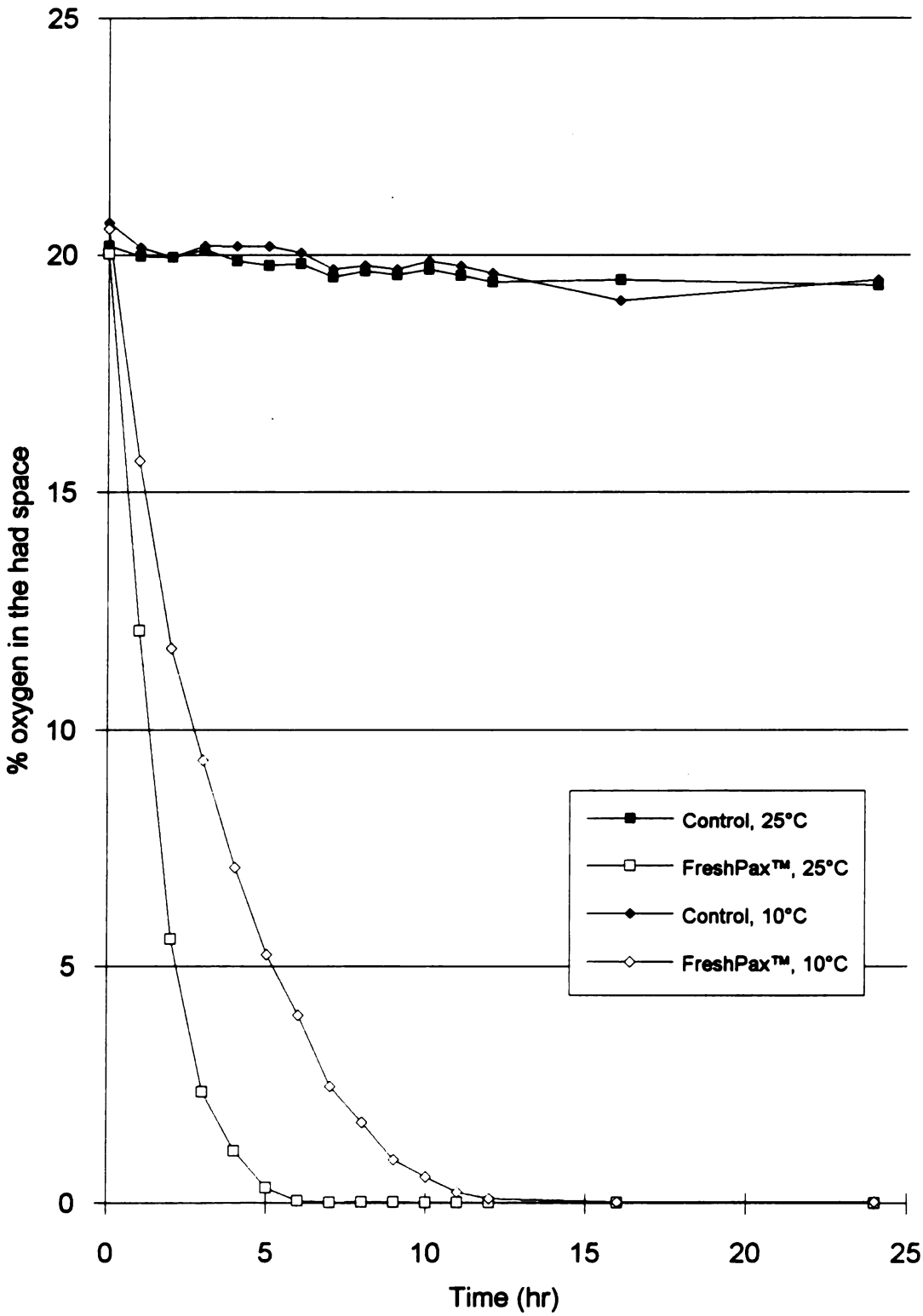

Figure 25: Temperature effect on oxygen absorber. Aw>0.99

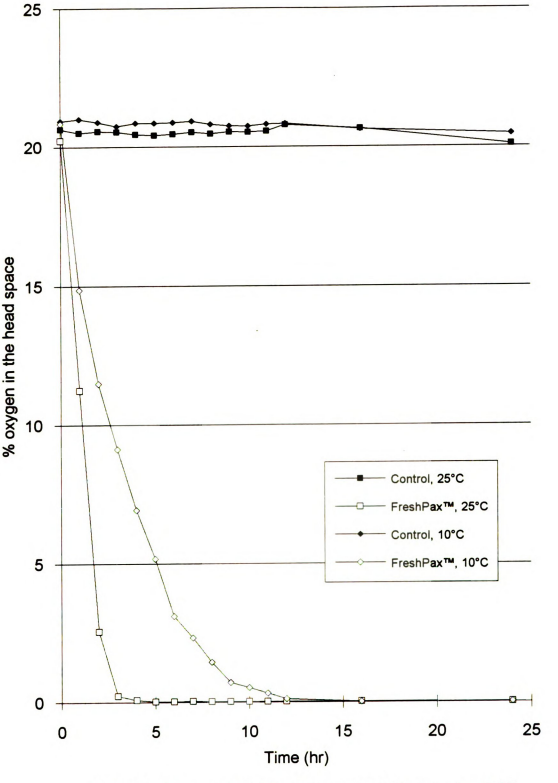

Figure 26: Temperature effect on oxygen absorber. Aw=0.88

contrast for the temperature effect yielded a statistical effect of 0.837 with and F value of 17.971 and a probability of 0.1%, which confirmed that it had a significant effect at the 1% level of significance. By comparing figures 25 and 26 a difference was observed at 10 and 25°C for samples containing FreshPax™.

CONCLUSIONS

A food-package model system was inoculated with B. subtilis ATCC 9372, C. sporogenes ATCC 11437, and a mixture of both. Growth of the microorganisms as influenced by several factors (time, inoculum, and oxygen level) was determined. Change in percent oxygen in the head space of the samples was also determined. It can be concluded that:

1) The effect of different inoculum (pure vs. mixed cultures) was significant at the level of $P<0.01$ for both B. subtilis and C. sporogenes in the temperature abusive condition (25°C). Growth of aerobes rapidly increased with oxygen head space levels of 1, 5, and 21% for the pure culture and at 5 and 21% for the mixed culture. Anaerobes increased rapidly at 1 and 5% and 21% + oxygen scavenger for pure culture samples. Anaerobes also increased rapidly at oxygen levels of 1% and 21% + oxygen absorber for the mixed culture.

2) The effect of two Aw (40% sugar -Aw=0.96- and Aw>0.99) influenced the growth of B. subtilis. At the low Aw, growth was extended to about 16 to 18 days. This Aw also prevented the growth of C. sporogenes.

3) Time was found to have a highly significant effect ($P<0.01$) for all inoculums for samples stored at 25°C. Bacterial numbers increased with time and values of about 10^7 CFU/ml were obtained as a maximum.

4) Oxygen level (1, 5, 21, and 21% + FreshPax™) was found to have a highly significant effect on bacterial growth at the level of $P<0.01$. B. subtilis did not grow at 25°C even at high initial values of oxygen (21%) when the oxygen absorber was present because anaerobic conditions were obtained very rapidly (approximately 5

hours) which prevented aerobic growth. Anaerobic bacteria were able to grow in media containing high initial oxygen content (21%) after the oxygen was scavenged. Interactions of time-inoculum and time-oxygen level were also found to be highly significant.

5) The effect of FreshPax™ in jars containing 21% initial oxygen was found to be highly significant ($P<0.01$). Oxygen was lowered to less than 0.5% in the headspace after 5 hours at 25°C and after 12 hours at 10°C.

6) At 25°C and media with Aw of 0.96 (40% sugar) the oxygen level was not significant on growth of B. subtilis in sample jars with 5% and 21% initial oxygen in the head space.

7) At 25°C and media of Aw 0.96, growth of B. subtilis was delayed and CFU/ml values of 1×10^7 for samples containing 21% and 5% initial oxygen in the head space were not obtained until 12 days had passed.

8) Samples stored at 10°C (media Aw >0.99) did not support the growth of C. sporogenes even under anaerobic conditions. Numbers of C. sporogenes cells decreased for sample jars with initial oxygen concentrations of 1% and 21% with the oxygen absorber. For samples where the initial oxygen concentrations were 5% and 21%, no growth was observed at any time during weeks one to four.

9) At 10°C B. subtilis growth was detected through the 4 week incubation for samples containing 1% and 5% initial oxygen and reached $>10^6$ CFU/ml for the pure culture and for the mixed culture with 5% initial oxygen. Samples inoculated with mixed culture and 1% initial oxygen had values of about 2.3×10^4 CFU/ml for B. subtilis at the fourth week. In samples containing 21% initial oxygen growth of B. subtilis was detected for the mixed culture after the second week. No growth was observed in samples containing pure culture and 21% initial oxygen. Growth

was observed (3.1 x 10^4 CFU/ml at the fourth week) for samples containing FreshPax™.

10) No water activity -Aw- effect was observed to influence the FreshPax™ effectiveness in the values studied (0.88 and >0.99).

11) Temperature was observed to effect the effectiveness of the oxygen absorber but only in the short time (the first 12 hours), since after 12 hours the oxygen levels are similar.

12) FreshPax™ (oxygen absorbers) could be used in foods to remove the oxygen in the head space only if refrigeration below 3°C is always maintained once the oxygen absorber is introduced and the package is closed. Also in products held above 3°C if the food, naturally or by design, is acid, has low water activity, a high sodium chloride concentration, an inhibitory sodium nitrite concentration, or two or more of these in combination.

APPENDIX

APPENDIX I

Standard Colony Count Methods

Based on Busta et al (1984), each sample in the study was taken and diluted according to the dilution recommended by the use of spectrophotometer technique in a range of 10^{-1} to 10^{1} (3 dilutions total). The dilution was related to the absorbance value for each specimen analyzed. Duplicate plates from the sample were individually counted and calculated as units of CFU per milliliter sample following these steps. Reported number of CFU per milliliter sample was averaged from the CFU value calculated from each plate of the same.

1) If there was no colony growth on all plates at the same dilution -the CFU number was reported as less than 100 CFU per milliliter.

2) If colony growth in each plate was less than 25 colonies in all dilutions - the actual number of colonies appearing on each plate was counted and reported as estimated CFU per milliliter sample.

3) If colony growth in each plate ranged between 25-250 colonies - the number of colonies on plate was counted and reported as CFU per milliliter sample.

4) If the growth in all plates was more than 250 colonies - then it was reported as more than 2.5×10^{6} CFU per milliliter. An estimated number of colonies in plate was obtained from the average count of 4 (four) 1x1 cm squares selected randomly in the most diluted solution, then multiplying this number by the plate surface area (in cm^2).

APPENDIX II

Spectrophotometric Method

A spectrophotometric method was developed and used as a complementary aid to approximate the number of bacteria present at the moment of sampling. This was performed to avoid utilization of extra of petri dishes and to save time at the moment of sampling. Growth of B. subtilis and C. sporogenes was monitored by using an absorbance method (figure 27) and a plate count method (figure 28). The relationship between log CFU/ml and absorbance for B. subtilis and C. sporogenes is shown in figures 29 and 30 as obtained in thioglycollate medium at 37°C. Aerobic and anaerobic conditions were set for B. subtilis and C. sporogenes, respectively (optimal growth conditions). A growth curve of number of microorganisms as $\log_{10}$ CFU/ml was compared to the absorbance level of samples over time using a spectrophotometer at 620 nm (this value was observed to give highest absorbance value for both bacteria in a range of 400-650 nm). Optimal conditions of growth for Bacillus subtilis and Clostridium sporogenes (37°C, aerobic and anaerobic conditions, respectively) were used to obtain a standard curve for each microorganism (see figures 31 and 32).

Using the equation corresponding to each bacteria:

- Log B. subtilis CFU/ml = 1.775 (Absorbance) + 6.243138 and
- Log C. sporogenes CFU/ml = 1.65392 (Absorbance) + 6.583369

relating the absorbance with the CFU/ml, from the standard curve an approximate CFU/ml for the bacteria analyzed was obtained. This value was then used as a

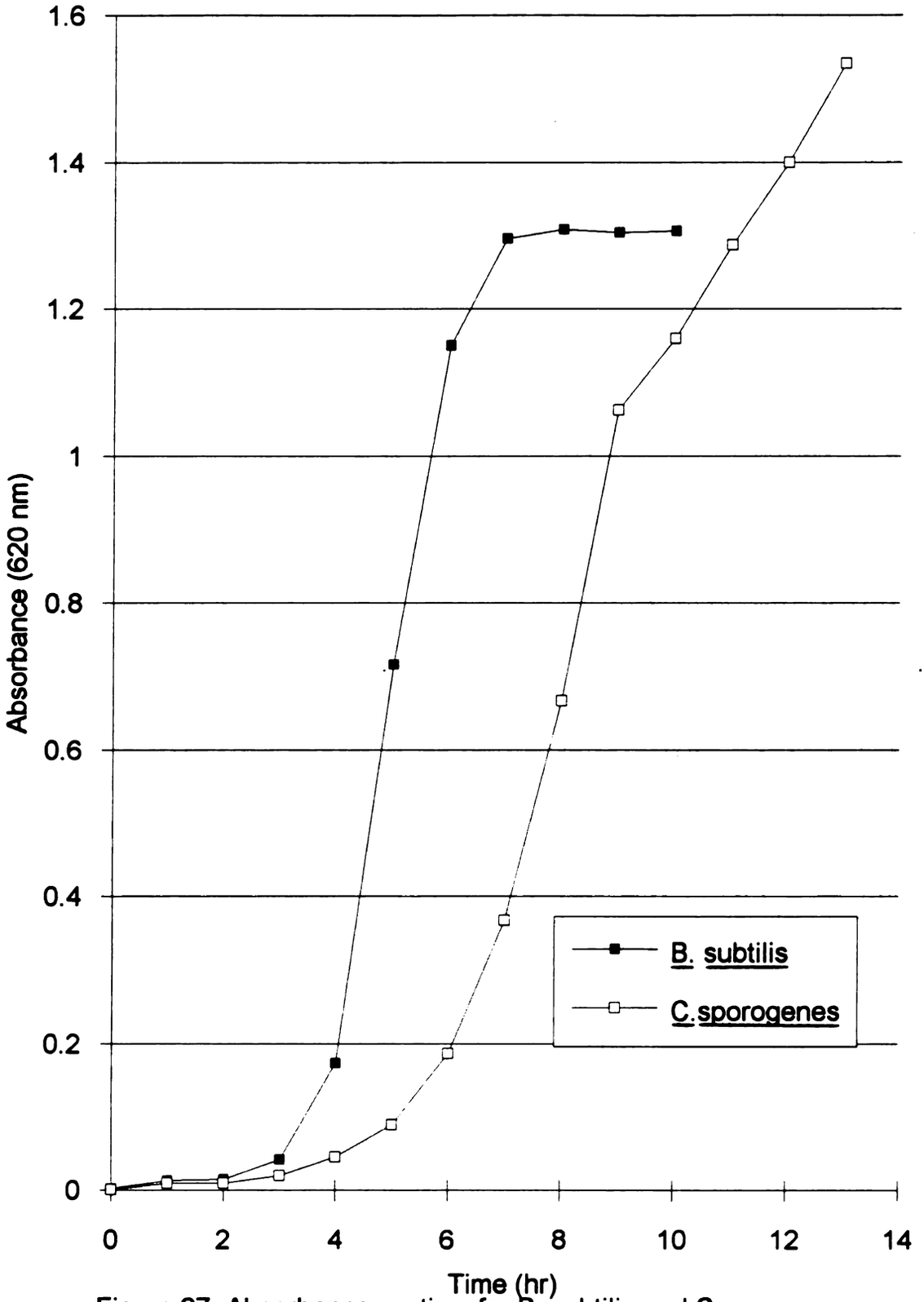

Figure 27: Absorbance vs. time for B. subtilis and C. sporogenes.
Thioglycollate medium; 37°C

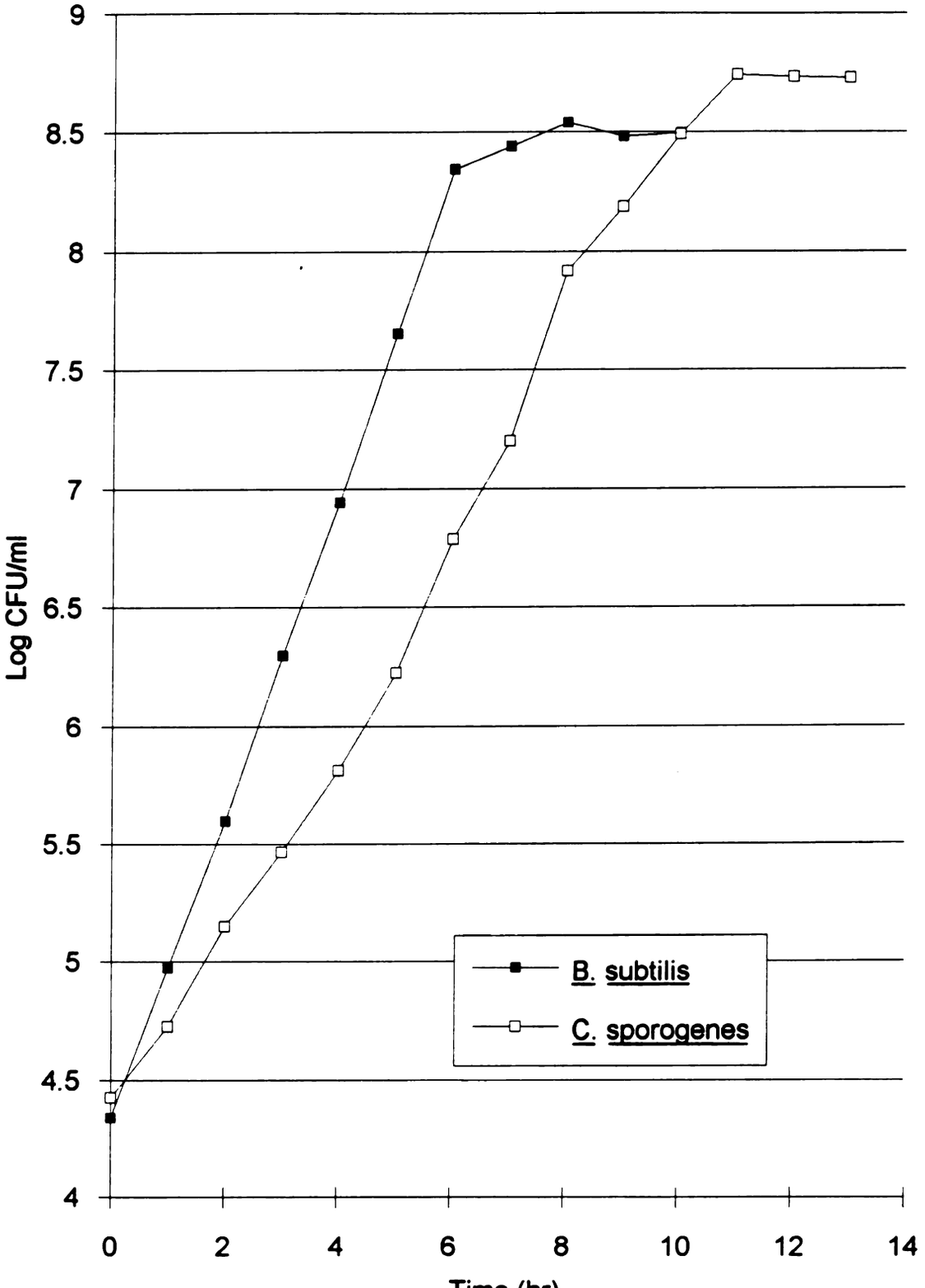

Figure 28: Log CFU/ml vs. time for B. subtilis and C. sporogenes.
Thioglycollate medium; 37°C

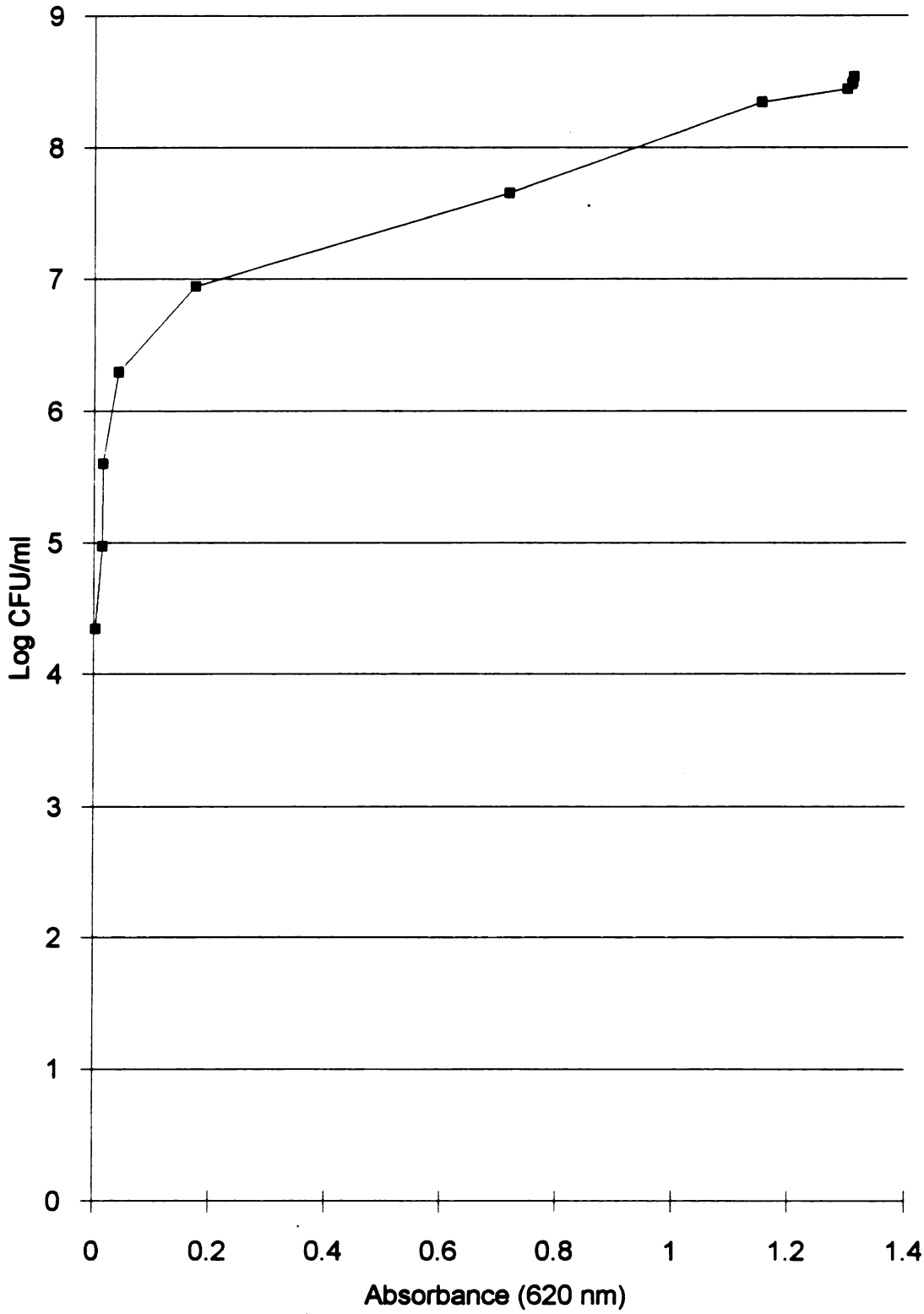

Figure 29: Log CFU/ml vs. absorbance for Bacillus subtilis. 37°C

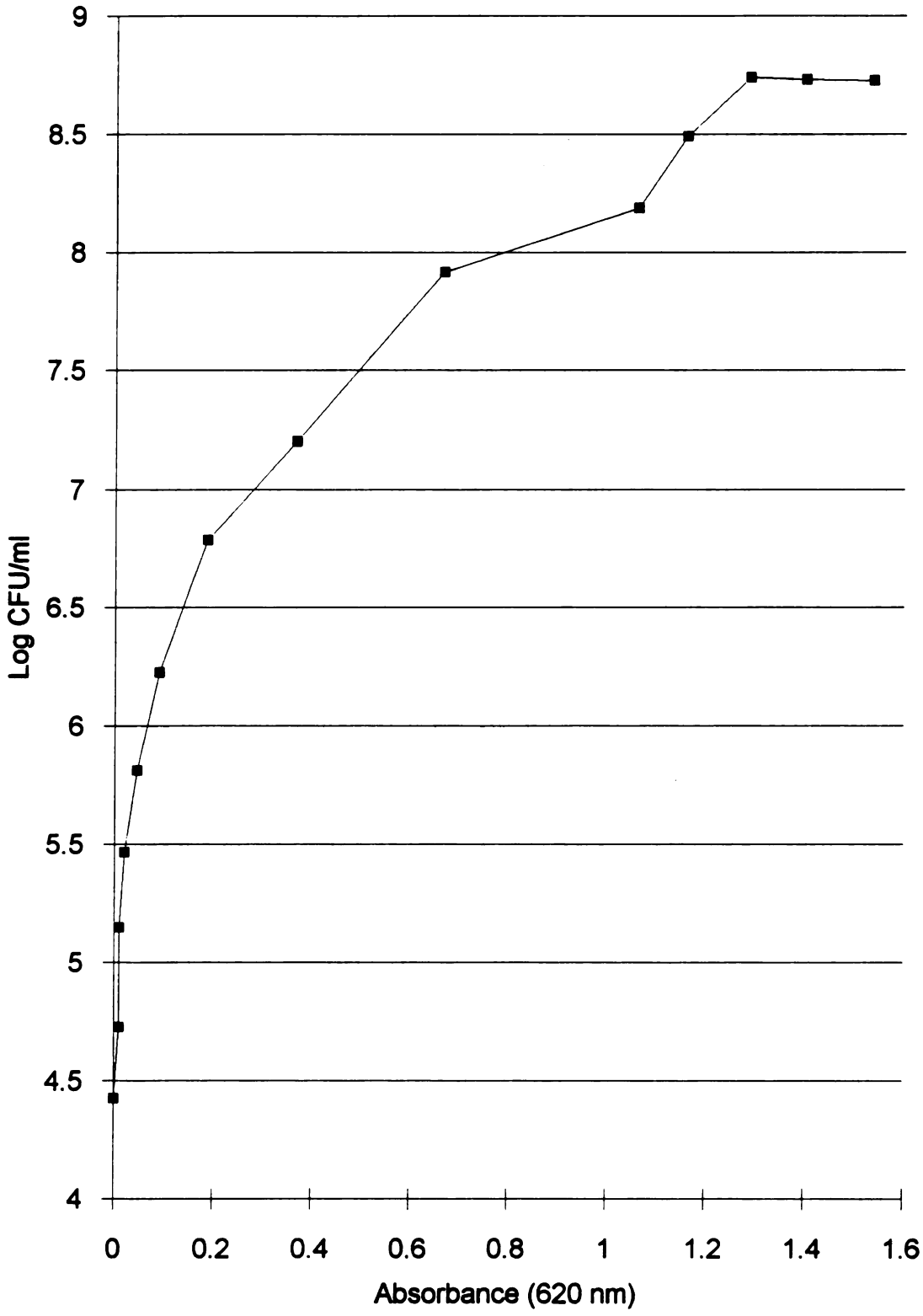

Figure 30: Log CFU/ml vs. absorbance for C. sporogenes. 37°C

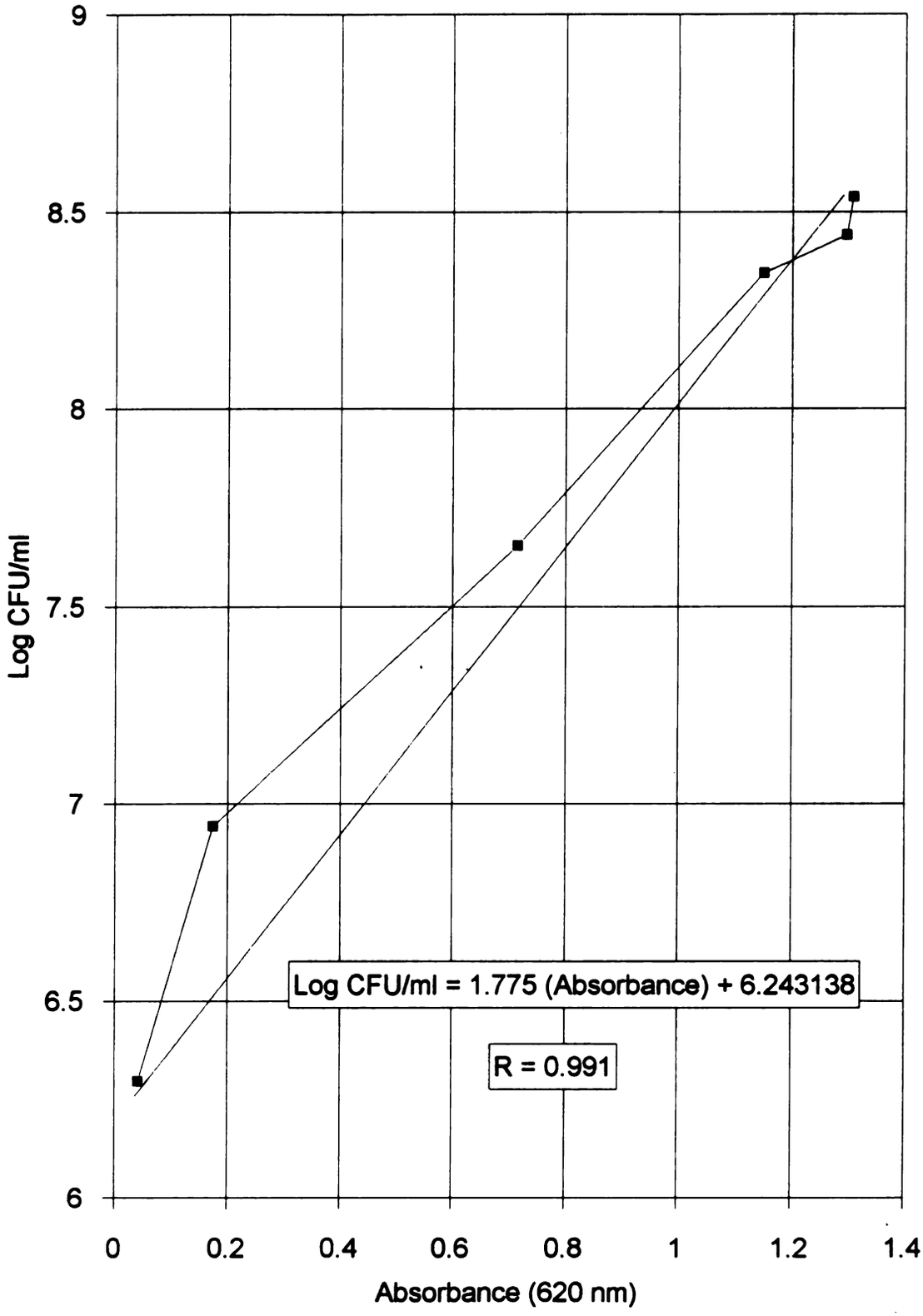

Figure 31: Standard curve for Bacillus subtilis

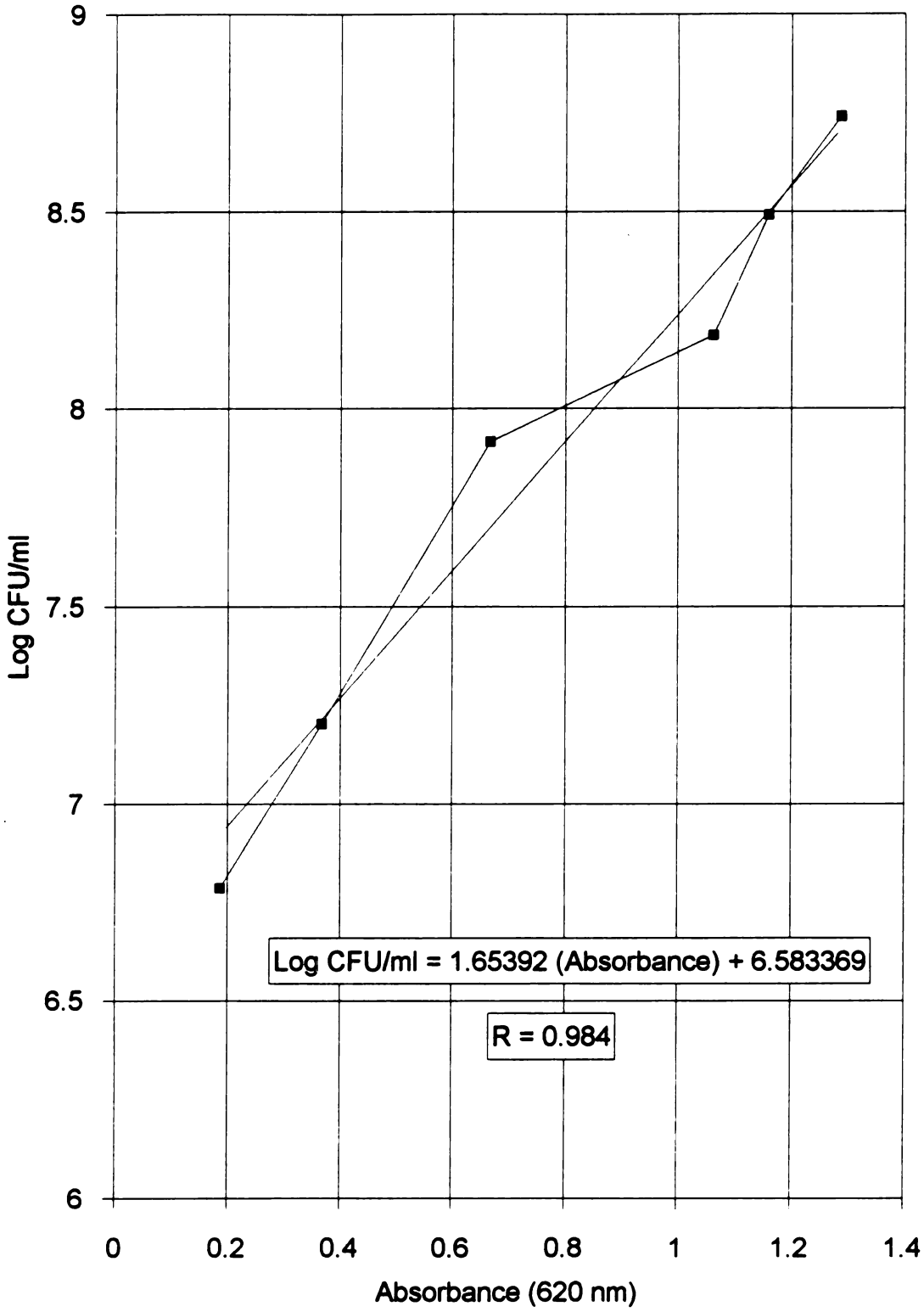

Figure 32: Standard curve for Clostridium sporogenes

base idea for making the respective dilutions as follows: 1) Absorbance value was put in the equation depending on the bacteria tested; 2) A log CFU/ml value was obtained; 3) Inverse of the log CFU/ml value was performed (10^X) so that a CFU/ml value was obtained for the bacteria; 4) From this number, depending on the exponent, a dilution corresponding to yield 100 CFU/ml was performed (log CFU/ml = 2), also a dilution of 10 CFU/ml and 1000 CFU/ml (log CFU/ml = 1 and 3, respectively), to assure plates with 25-250 colonies counts.

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