FEASIBILITY OF USING CATALASE ACTIVITY AS AN INDEX OF MICROBIAL LOADS ON FOOD SURFACES

by

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B.S., Fu-Jen Catholic University, R.O.C. 1980

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Food Science

Department of Animal Sciences and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1985

Approved by :

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INTRODUCTION

Microorganisms in food and the environment have been a concern to human welfare ever since the beginning of time because they spoil food and cause food borne diseases. Today, controlling and/or detecting microbial activities in raw materials and food is still the main goal for the food microbiologist. There are four general methods to enumerate and quantitate microorganisms.

Food microbiologists are constantly searching for faster and better methods to determine microorganism in food for the purpose of quality control, food preservation, food production and prevention of food borne diseases.

Direct Count Methods

Direct count methods involve determination directly under a compound microscope of the number of cells in a sample drawn from the system being examined. Using a counting chamber (e.g. Petroff-Hausser Counting Chamber) with appropriate dilution factors, one can estimate total direct count of a sample. Alternately by knowing the microscope factor, one can also estimate total count by counts in part or all of the microscope field. Samples to be observed must contain at least 10^6 cells per ml for the organisms to appear in each microscopic field under oil immersion microscopy.

Viable Cell Count

Viability tests depend on the ability of microorganisms to grow and multiply in the environment provided by the liquid or solid media into which they had been inoculated. The environment is characterized by its chemical composition (inorganic salts, organic compounds, pH, oxygen concentration, etc.) and its physical characteristics (solid/liquid, temperature, agitation, atmospheric pressure, aerobic,

etc.). The most commonly used method for enumerating viable microbes as colonies is the standard plate count (SPC, American Public Health Association, 1981). This procedure involves a series of dilution of a sample in a buffer solution. Portions of each dilution was transferred to a petri dish containing medium. The inoculated dishes were incubated for a specified time interval and the number of colonies produced in the agar counted. By applying the appropriate dilution factor to the colony count, one can obtain the viable count in colony forming units (CFU) per ml, gm, or cm^2 of the sample. Automated spiral plate count method is an improvement of the traditional dilution procedure and was adopted by the Association Official Analytical Chemists (AOAC) in 1977 as an official first action method for foods and cosmetics. In this method a mechanical plater inoculates a rotating agar plate using a cam-activated syringe which dispenses a continuously decreasing volume of sample, resulting in a concentration range of up to 10,000 to 1 on a single agar plate.

Many microorganisms, accustomed to living in an aqueous environment, do not readily form colonies on solid media (ZoBell, 1946). Moreover, microbes which were injured but still metabolically active may not produce colonies (Postgate, 1969). Aggregates of microbes which had not been broken apart would produce a single colony although the aggregate may have hundreds of microorganisms. These problems were reduced by inoculating liquid media instead of solid media. Most probable number (MPN) technique requires that five replicates be made for 3 serial decimal dilutions. The observer records the number of turbid tubes at each dilution and then use a MPN table to estimate the number of viable microbes in the sample. The precision of the estimate increases with the number of replicates but the tedious procedure soon outweighted the benefit. Fung and Kraft (1969) had proposed a miniaturized system for the MPN technique.

Metabolic Activity Tests.

A feature common to all metabolic activity testing was the measurement of the loss of some chemical constituent, or the appearance of some metabolite, or the physical and chemical changes of the environment. Johnston Laboratories, Inc. (Cockeysville, MD.) designed automated and semiautomated bacterial detection systems (Bactec). This instrument was first adapted to detect organisms in blood and its usage was widened to analyze frozen orange juice concentrate (Hatcher et al., 1977) and raw hamburger samples (Previte et al., 1977).

Besides the metabolic compounds, small changes in heat production resulting from bacterial growth in liquids and food systems can be measured in a microcalorimeter (Sacks and Menefee, 1972). Mou and Cooney (1976) used calorimetry to measure fermentation processes. Lampi et al. (1974) used both radiometry and microcalorimetry to detect the microbial load in food. An instrument called Bactometer Microbial Monitoring system was developed by Bactomatic, Princeton, N.J. to measure the resistance to flow of an alternating electrical current through a food system. This impedance technique relied on the fact that metabolizing microorganisms alter the chemical composition of the growth medium and that these chemical changes caused a change in the impedance of the medium. Rowley et al. (1979) used impedance measurement to study cooked meat. Hardy et al. (1977) used this technique as an screening test for frozen vegetables. It has been used for estimation of coliforms in meat and dairy products (Firstenberg-Eden, 1983; Firstenberg-Eden et al., 1984).

Measuring Cell Constituents

A fourth method to estimate cell number is by monitoring cell constituents. The most commonly tested cell component was adenosine triphosphate (ATP). ATP is a chemical essential for energy metabolism in all organisms; this compound is quickly degraded after cell death . When firefly luciferin and luciferase come in contact with ATP, the ATP-luciferin-luciferase complex emits a light pulse which could be measured fluorometrically . The pulse intensity was proportional to the ATP concentration, which, in turn, was proportional to number of organisms that were present. Sharpe et al. (1970) had studied the ATP levels in foods and the relation to bacterial contents. Goldschmidt and Fung (1978) noted some difficulties in applying microbial ATP measurements to foods including interference from ATP of nonmicrobial origin and variation in ATP content with species, age and previous exposure to deleterious agents. Graumlich (1985) used bioluminescence to estimate the microbial populations in orange juice, but improved the methodology by applying reagents and ATPase to release and digest the ATP from somatic cells before releasing and measuring the microbial ATP.

The Limulus Amoebocyte Lysate (LAL) test used lysate prepared from amoebocytes of the horseshoe crab (Limulus polyphemus) which reacts with the endotoxin, present in the cell walls of Gram-negative bacteria, to form a gel (Levin et al., 1970). This reaction can be used to detect Gram-negative bacteria in various environments. The LAL test has applications in medical microbiology. Jay used it for meats (Jay, 1977; Jay, 1981). Dodds et al. (1983) used the LAL test and Catalasemeter method to study the microbial quality of vacuum-packed cooked turkey.

The purpose of this study was to utilize the enzyme catalase to rapidly estimate microbial counts in certain foods. Bacteria in the environment can be divided into catalase-positive and catalase-negative groups. Each group contains important bacteria. Since catalase is a constitutive enzyme of bacteria, the concentration increases as the number of bacteria increases. It is possible to use the catalase activity to estimate bacterial concentration under certain conditions. Two quantitative catalase tests, Catalasemeter method and Gas Column method were designed and developed by Biotechnology Group Ltd. and the food microbiology research group at Kansas State Univeristy, respectively, to estimate the bacterial load on certain foods.

Literature Review

Early History of Catalase Investigations

The enzyme, Catalase (H_2O_2 : H_2O_2 oxidoreductase; EC 1.11.1.6), has been the subject of investigation for more than a century and interests in the enzyme continue to the present time. The primary function of catalase is to destroy hydrogen peroxide according to the equation.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_{2(gas)}$$

Catalase action was first noted in animal tissues in 1818 by Thenard, the discoverer of hydrogen peroxide, who also found that finely divided metals had the same effect. Loew (1901) first established that the action was due to the effect of an individual, separable enzyme, which he named 'catalase'. The identity of catalase was unclear at that time, but Stern (1936) showed that protoheme, in fact, was involved in the reactions of liver catalase. Sumner and Dounce (1937) purified beef liver catalase, crystallized it and correlated its heme content with its catalatic activity. They were easily crystallizable heme enzymes with four iron atoms per molecule attached to protein and chelated to protoporphyrin IX, which may be isolated from bacterial catalase. Warburg (1949) proposed that iron took part in the catalase system. He suggested that catalase was a component of his 'oxygen-transporting iron', which contained protein. He therefore did not distinguish special catalatic or peroxidatic roles for the heavy metals.

Catalase is always present in an organism which employs the respiratory cytochrome system. This system derives energy from the interaction of oxygen with products in the Krebs Cycle. The oxygen is reduced incidentally to hydrogen peroxide which must be removed due to its toxic effects. Animals and plants all possess catalase activity. Among the organs of animals, kidney and liver had the highest catalase activity. Erythrocyte catalase had also been purified and studied

by Agner (1943). Molland (1947), cited an earlier study by Gottstein in 1893 who suggested use of the hydrogen peroxide decomposition reaction as a method of detecting bacteria. Most aerobic or facultatively anaerobic bacteria produce catalase. Some facultative anaerobes and all obligate anaerobes are catalase negative. Many of these cells had a small, but critical part of the enzyme missing. Some catalase negative bacteria can be made catalase positive by providing them with the missing iron molecule (heme-iron) as a nutrient (Whittenbury, 1964). A bacterial culture was considered catalase positive when gas bubbles were generated after a drop of 3% H_2O_2 was placed on the colony. A catalase-negative culture will not generate gas bubbles. Fung and Petrishko (1973) described a semi-quantitative catalase scale using capillary tubes and noted that bacterial species had different catalase activities.

Physiological Role of Catalase

Against the Lethality of Oxygen

Hydrogen peroxide was formed by cells in a two electron reduction of oxygen (flavoproteins act as catalyst). The absence of catalase formation of peroxides will poison the cell. Most of the O_2 (ground state) present in a biological system was reduced to water.

$$O_2 + 4\ e^- \longrightarrow 2\ H_2O$$

Some of the remaining O_2 was converted by a variety of mechanisms to hydrogen peroxide and superoxide.

$$O_2 + 2\ e^- \longrightarrow H_2O_2$$

$$O_2 + 1\ e^- \longrightarrow O_2^-$$

Hydrogen peroxide was a good oxidant, but it alone was not necessarily lethal. Superoxide, on the other hand, was a fairly poor oxidant and weak free radical. The problem that superoxide presented to the cell was that it can form

hydrogen peroxide and OH• by the function of Liganded divalent Transitional Metal (such as Ferric ion).

$$O_2^- + H_2O_2 + \text{L-dTM} \longrightarrow 2\ OH^\bullet$$

OH• was a strong free radical which had been shown to be toxic to bacteria. It was capable of starting free radical chain reactions with cellular components.

$$OH^\bullet + X_{\text{cell component}} \longrightarrow X^\bullet$$

The general defense mechanism of the cell against the production of OH• and hydrogen peroxide involved superoxide dismutase (SOD) and catalase. Recent research by Frederick and Fridovich (1981) discovered the third way to protect from oxygen lethality by means of a trace metal (manganese) in Lactobacillus plantarum.

The proposed functions of catalase include protective catalatic (decompose hydrogen peroxide only to water and oxygen), peroxidatic (catalysis of the oxidation of alcohols and other donors by hydrogen peroxide), and a mixture of the two. Heppel and Porterfield (1948) correlated the oxidation of nitrite by liver slices with the action of catalase in promoting peroxidatic "coupled oxidation". They considered this as an evidence for the availability of the flavoprotein-H_2O_2-catalase system for peroxidatic reactions in the mammalian cell. This evidence only showed that catalase had the ability to function peroxidatically in mammal. Another possible role of catalase derived directly from its chemical behavior. When catalase reacted catalatically with hydrogen peroxide and released water and oxygen, it involved "two-electron" transfer. The most effective donor for this reaction was a second molecule of hydrogen peroxide. The function of catalase was therefore likely to be as a catalyst of this oxidation, just as the function of peroxidase was probably as the catalyst of one-electron oxidation.

The theory that catalase only had a protective effect was criticized by Keilin and Hartree (1945). The enzyme failed to protect hemoglobin from attack by slowly generated peroxide; likewise, bacteria containing catalase can be killed by the action of the notatin (glucose oxidase) system (glucose oxidase oxidizes glucose and produces hydrogen peroxide). An example of the role of catalase in animals was the hereditary conditions known as 'acatalasemia'. Nishimura et al. (1959) reported that patients deficient in erythrocyte catalase were prone to oral gangrene. Recent observation of acatalasemia indicated the formation of a catalase variant, which possesses the normal specific activity, but with a tendency to dissociate into subunits (Aebi et al., 1974). A similar condition occurs for Rhodopseudomonas, which can be induced to form catalase by growing under aerobic conditions. Enzyme induction shows that catalase function in some microorganisms is necessary to survive in aerobic environment.

Differences Between Catalase and Peroxidase

Catalases are closely related to the peroxidases both structurally and functionally. They are all easily crystallizable heme enzymes with four iron atoms per molecule attached to protein and chelated to protoporphyrin IX. It was originally thought that the sole function of catalase was to protect the cell from high concentrations of peroxide produced by the respiratory chain and other oxidases. Keilin and Hartree (1945) discovered that very low concentrations of peroxide generated by the oxidase system in vitro could be readily used by catalase to oxidize alcohols and other donors, but a more complex role was suspected. It was subsequently found that free added peroxide could also be used by the enzyme to oxidize certain donors such as glycol, ethanol, isobutanol, nitrite, formate, acetate, etc.

Theorell (1947) had even suggested that the only difference in role between

catalase and peroxidase was the extra ability of catalase to decompose excess peroxides. This, however, ignored the possibility of differing donor specificity in the natural state. Generally, catalase was defined as possessing two functions; it reacts 'peroxidatic' when the concentration of peroxide was low, and reacts 'catalatic' when peroxide level was high.

Physical and Chemical Properties of Catalase

All catalases isolated to date contain four hematin groups on a protein of ca.240,000 dalton molecular weight. Sumner et al. (1940) showed that the most active preparations contained the least degraded hematin, and the activity of liver catalases in terms of hematin concentration seems to be the same as that of the corresponding blood catalases. Liver and blood catalase from the same species were serologically identical. Catalase enzymes in different animals differ in their protein component, as was shown by the development of 'anticatalase' antibodies in rabbits after injection of catalase derived from other animals (Tria, 1939). Bacterial catalase from Micrococcus lysodeikticus was claimed by Chance and Herbert (1950) to have higher reactivity than mammal catalase. Partial amino acid composition of horse catalase was reported by Bonnichsen (1947). He found the histidine, arginine, lysine, tyrosine, cystine, glutamic acid and aspartic acid contents of horse blood and liver catalase to be approximately the same.

Synthesis and Structure of Catalase

By use of iron isotopes injected into guinea-pigs. Theorell et al. (1951) traced the synthesis of liver catalase in liver and erythrocyte catalase in bone marrow, respectively. Regardless of the origin of catalase, the general properties were identical. de Duve (1974) reported that rat liver catalase synthesis consisted of a single autosomal determinant and proceeded in three distinct stages: 1)

synthesis of approximately 60,000 dalton apocatalase subunits, 2) intercalation of heme, and 3) formation of tetramers.

Data obtained to date indicated that catalase are oligomers (isoelectric point = 5.5) of a four tetrahedral arrangement with 60,000 dalton subunits. Each subunit consisted of a single polypeptide chain that associates with a single prosthetic group, ferric protoporphyrin IX. The subunits apparently functioned independently of one another; the resulting oligomer was relatively stable. Schroeder et al. (1969) reported the sequence of 505 amino acid residues of the subunit of bovine liver catalase (Fig. 1).

Recent studies by de Duve and Baudhuin (1966), Jones and Suggett (1968) and Barlow and Margoliash (1969) suggested that the prosthetic group was deeply intercalated but not necessarily strongly bound within the protein matrix; the functional groups at the active site were not readily accessible to modification and sulfhydryl groups were not essential to enzymatic activity. Different conformations of catalase in the ferric state played a key role in the expression of enzymatic activity specifically in (1) the decomposition of hydrogen peroxide into oxygen and water and (2) the peroxide-dependent oxidation of various substrates.

Figure 1. Amino Acid Sequence of the Sub-unit of Bovine Liver Catalase.

From Schroeder et al. (1969).

(Ala,Asx)-Asx-Arg-Asx-Pro-Ala-Ser-Asp-Gln-Met-Lys-His-Trp-Lys-Glu-Gln-Arg-Ala-Ala-Gln-Lys-Pro-Asp-Val-Leu-Thr-Thr-Gly-Gly-Gly-Asn-Pro-Val-Gly-Asp-Lys-Leu-Asn-Ser-Leu-Thr-Val-Gly-Pro-Arg-Gly-Pro-Leu-Leu-Val-Gln-Asp-Val-Val-Phe-Thr-Asp-Glu-Met-Ala-His-Phe-Asp-Arg-Glu-Arg-Ile-Pro-Glu-Arg-Val-Val-His-Ala-Lys-Gly-Ala-Gly-Ala-Phe-Gly-Tyr-Phe-Glu-Val-Thr-His-Asp-Ile-Thr-Arg-Tyr-Ser-Lys-Ala-Lys-Val-Phe-Glu-His-Ile-Gly-Lys-Arg-[Thr-Pro-Ile-Ala-Val-Arg-Phe-Ser-Thr-Val-Ala-Gly-Glu-Ser-Gly-Ser-Ala-Asp-Thr-Val-Arg-Asp-Pro-Arg-Gly-Phe-Ala-Val-Lys-Phe-Tyr-(Asx,Asx,Asx,Asx,Thr,Thr,Glx,Pro,Gly,Gly,Val,Ile,Leu,Phe,Phe,Trp)-Ile-Arg-Asp-Ala-Leu-Leu-Phe-Pro-Ser-Phe-Ile-His-Ser-Gln-Lys](Arg)-Asn-Pro-Gln-Thr-His-Leu-Lys-(Asx,Asx,Pro)-Met-Val-Trp-Asp-Phe-Trp-Ser-Leu-Arg-Pro-Glu-Ser-Leu-His-Gln-Val-Ser-Phe-Leu-Phe-Ser-Asp-Arg-Gly-Ile-Pro-Asp-Gly-His-Arg-His-Met-(His,Asx,Thr,Ser,Gly,Gly,Tyr,Phe)-Lys-Leu-Val-Asn-Ala-Asp-Gly-Glu-Ala-Val-Tyr-Cys-Lys-Phe-His-Tyr-Lys-Thr-Asp-Gln-Gly-Ile-Lys-Asn-Leu-Ser-Val-Glu-Asp-Ala-Ala-Arg-Leu-ala-His-Glu-Asp-Pro-Asp-Tyr-Gly-Leu-Arg-Asp-Leu-Phe-[(Asx,Thr,Ala,Ala,Ile)](Gly-Asn-Tyr-Pro-Ser-Trp)(Thr-Leu-Tyr)-Ile-Gln-Val-Met-Thr-Phe-Ser-Glu-Ala-Glu-Ile-Phe-Pro-Phe-Asn-Pro-Phe-Asp-Leu-Thr-Lys-Val-Trp-Pro-His-Gly-Asp-Tyr-Pro-Leu-Ile-Pro-Val-Gly-Lys-Leu-Val-Leu-Asn-Arg-Asn-Pro-Val-Asn-Tyr-Phe-Ala-Glu-Val-Glu-Gln-Leu-Ala-Phe-Asp-Pro-Ser-Asn-Met-Pro-Pro-Gly-Ile-Glu-Pro-Ser-Pro-Asp-Lys-Met-Leu-Gln-Gly-Arg-Leu-Phe-Ala-Tyr-Pro-Asp-Thr-His-Arg-His-Arg-Leu-Gly-Pro-Asn-Tyr-Leu-Gln-Ile-Pro-Val-Asn-Cys-Pro-Tyr-Arg-Ala-Arg-Val-Ala-Asn-Tyr-Gln-Arg-Asp-Gly-Pro-Met-Cys-(Asx,Asx,Asx,Asx,Glx,Pro,Pro,Gly,Gly,Ala,Met,Met,Tyr,Tyr)-Ser-Phe-Ser-Ala-Pro-Glu-His-Gln-Pro-Ser-Ala-Leu-Glu-His-Arg-Thr-His-Phe-Ser-Gly-Asp-Val-Gln-Arg-Phe-Asn-Ser-Ala-Asn-Asp-Asp-Asn-Val-Thr-Gln-Val-Arg-Thr-Phe-Tyr-Leu-Lys-Val-Leu-Asn-Glu-Glu-Gln-Arg-Lys-Arg-Leu-Cys-Glu-Asn-Ile-Ala-Gly-His-Leu-Lys-Asp-Ala-Gln-Leu-Phe-(Glx,Ile,)-Lys-Lys-Ala-Val-Lys-Asn-Phe-Ser-Asp-Val-His-Pro-Glu-Tyr-Gly-Ser-Arg-Ile-Gln-Ala-Leu-Leu-Asp-Lys-Tyr-Asn-Glu-Glu-Lys-Pro-Lys-Asn

Methods in Determining Catalase Activity

Catalase activity was usually determined by titrimetric, spectrophotometric or manometric methods. The most widely accepted standard to describe catalase activity is the Katalase-fahigkeit or Kat.f.(von Euler and Josephson, 1926).

Titrimetric Method

The titrimetric method developed by von Euler and Josephson (1926) was probably the best method to quantitate pure or partially purified catalase. A known quantity of hydrogen peroxide was mixed in phosphate buffer and cooled to 0°C. After adding catalase solution in the reaction vessel with vigorous stirring, 5 ml of reacting solution were withdrawn at specific time intervals (3, 6, 9 and 12 min). The reaction was stopped by the addition 5 ml of 2 N sulphuric acid. The mixture was then titrated with 0.005 N permanganate to the first persistent pink color.

Kat.F. = K / W W: W gram, dry weight of catalase

$K = (2.303/t) \text{Log}(X_o / X_t)$ min^{-1} at O°C

t: time

X_o: titration value in O time

X_t: titration value in t time

Spectrophotometric Method

In 1950 Chance and Herbert devised a method for determining the activity of catalase by direct measurements of the decrease of light absorption in the region 230 to 250 nm caused by the decomposition of hydrogen peroxide by catalase. This method was limited to the assay of catalase solutions that were pure enough to give negligible absorption at 230 to 250 nm in the concentrations required for the assay. This procedure involved the measurement of the difference of absorbance during 0, 10, 20, 30, 50, and 70 second by using 3 ml of 1:500 H_2O_2

and 1 µl of catalase solution. The catalase concentration was adjusted so that about half the peroxide is decomposed in about 30 seconds.

$K = (2.3/t_2 - t_1))$ Log (X_1/X_2)

t_1 and t_2 were the time corresponding to a pair of readings of the optical densities X_1 and X_2

Kat.F. = 60 K / 2.3 W

W: W gram of catalase in a final reaction mixture of 50 ml solution

Manometric Methods

This methods involved measurement of the amount of oxygen evolved by the catalase reacting with hydrogen peroxide solution. The reading was recorded as µl of oxygen evolved per minute, mainly used for the determination the reaction velocity constants. George (1948) used Barcroft manometers and pressure-gauge of the 'boat technique' to study the effect of the peroxide concentration and pH effect on the decomposition of hydrogen peroxide by catalase.

It had been claimed that the manometric method gave values for the absolute rate of reaction that were too low. Nicholls (1959) reviewed former workers' data and concluded the difference between the three methods were very small if the same enzyme preparation and conditions were employed.

Nature of Catalase-Hydrogen Peroxide Compounds I, II and III

Discovery of Catalase-Hydrogen Peroxide Compounds I, II, and III.

Early observation by George (1947) indicated that when catalase was added to hydrogen peroxide there was an initial rapid evolution of oxygen in the first two minutes. After this, oxygen was given off at a steady rate which slowly decreased in the course of about an hour. He suspected that partial enzyme destruction might be responsible for the transition from the initial rapid rate

(alpha-activity) to the slower steady rate (beta-activity). Bonnichsen et al. (1947) compared a rapid titrimetric method by sampling the reacting solution in 15 second versust the traditional 3, 6, 9 and 12 minute of reaction time and found 50 fold larger Kat.F. value was obtained. They reported as excessive catalase dilution, large substrate concentration and low pH contributed to the inactivation of catalase during the decomposition of hydrogen peroxide and might influence the conversion of catalase to a less active form. Chance (1947) reported the catalase-hydrogen compound I obtained by the modified rapid method, which contributed to the rapid evolution of the oxygen while reacted with hydrogen peroxide. The reactions of catalase (Cat) with hydrogen peroxide were presented by Chance (1948).

$$Cat(OH)_4 + H_2O_2 \longrightarrow Cat(OH)_3OOH \text{ (Compound I)} + H_2O$$

$$Cat(OH)_3OOH + H_2O_2 \longrightarrow Cat(OH)_4 + H_2O + O_2$$

$Cat(OH)_4$:catalase, $Cat(OH)_3OOH$:catalase-hydrogen peroxide compound I

George (1948) used purified horse catalase to study the effect of peroxide concentration and other factors on the inactivation of catalase. He declared the effect he observed was not due to a decrease in the peroxide concentration, since it was quite marked in the experiments where there was a large excess of peroxide remaining. He also reported that by adding of dilute buffer solution or peroxide during the reaction, enzyme destruction played no part in determining these kinetic relationships. This argument continued until Chance in 1950 used the notatin system to study the catalase derivatives and activity with hydrogen peroxide. Finally he concluded that when catalase reacted with hydrogen peroxide it first formed the primary catalase-hydrogen peroxide compound I which had the necessary high dissociation constant to release the oxygen and to free the catalase. The formation of catalase-hydrogen peroxide compound II was slow. He presumed that in order to form significant amount of the compound II, the steady

state concentration of the primary complex must be maintained for some time. He suggested that as soon as a particular hematin-peroxide bond changed from compound I to compound II, one of the remaining free catalase hematins would combine with peroxide to form compound I. The process goes on until equilibrium. The compound II was catalatic inactive and contributed to the exponential decrease of reaction velocity constant during the course of the activity as determined by the Kat.F. value.

The tertiary compounds (catalase-hydrogen peroxide compound III) were obtained by Keilin and Hartree (1951) by the peroxide produced in the autoxidation of ascorbate. Compound III was produced under conditions of more rapid ascorbate oxidation. Chance (1952a) made compound III by adding a small amount of hydrogen peroxide to compound II formed in the reaction of catalase and methyl hydroperoxide. Compound III was produced only in the presence of excess hydrogen peroxide. No other reagents, not even alkyl peroxides, were effective. Compound III was rather inactive toward most hydrogen donors compared with compound II and hence acted as an inhibited form of the enzyme.

Nature of Compound I, II, and III.

Catalase hydrogen peroxide compound I had been regarded as either a complex between iron and peroxide (Chance and Fergusson, 1954) or as a higher oxidation state (Fe^{5+} or FeO^{3+}) of the iron (George, 1953). Both views were based on the 'models' thought to be appropriate. In the "peroxide complex" theory, the reaction of catalase was with acidic ligands.

$$\text{Catalase-Fe}^{3+}\text{H}_2\text{O} + \text{HA} \longrightarrow \text{Catalase-Fe}^{3+}\text{HA} + \text{H}_2\text{O}$$

This was regarded as appropriate because of its formal equivalence to the formation of a reversible Michaelis complex, in which the substrate become activated and to the inhibition of such complex formation by competitive inhibitors

reacting with the active site.

In the 'higher oxidation state' theory, hemoglobin (Hb) was oxidized to methaemoglobin and then to methaemoglobin peroxide.

$$Hb\text{-}Fe^{2+} \rightleftharpoons Hb\text{-}Fe^{3+} \xrightarrow{\text{oxidizing agent}} Hb\text{-}FeO^{2+}$$

This point of view was also considered appropriate because higher oxidation states of transition metals showed powerful oxidizing capabilities similar to those former observations. However, neither model was capable of explaining all the features of the catalatic reaction.

Further investigation was done by Nicholls (1961) who used purified horse liver and blood catalase to study the action of anions on the formation of catalase peroxide compounds I and II. He showed that anionic ligands can form complexes with the iron of compound I and that the iron retained all its usual patterns of reactivity and formed the usual derivatives of the enzyme. While studying reactions of azide with catalase, Nicholls (1964) further confirmed that during the peroxidatic oxidation of catalase, the iron was reduced by a product of donor oxidation, and in some cases, an oxidation product remained attached to it in the form of a complex. He suggested that the peroxide might be attached to the porphyrin ring. A ROOH moiety might be added across either of two different kinds of double bond by a methene bridge or a C-N bond in a pyrrole ring. In each of the positions, a tetrahedral carbon atom occurred with two possible positions for the (OOR) moiety which may be stabilized by the presence of either HOH or HXOH as a ligand on the iron. He concluded the peroxide moiety in compound I was primarily associated with the porphyrin ring. While the iron was retained, the ability to form complexes with donors and anionic ligands and the acceleration of compound II formation by anions may be regarded as a mobilization of the endogenous donor when HA replaced H_2O on the iron atom. The effect of bound hydrogen peroxide in compound I can also serve as an endogenous donor to form

the compound II complex at high concentrations of hydrogen peroxide which limit the breakdown of hydrogen peroxide from compound I (Fig. 2).

Jones et al. (1968) used ultracentrifugation and the sedimentation velocity patterns to study the molecular weight of catalase hydrogen peroxide II of Micrococcus lysodeikticus and reported that the formation of compound II involved a major disruption of the catalase molecule, and suggested that compound II may be a sub-unit species. Jones and Suggett (1968) proposed that compound I was an essential feature of both complex-formation steps which occurred substantially irreversibly. They proposed the reversible formation of a primary complex that undergoes an essentially irreversible transformation into a secondary complex before taking part in the redox reaction (Fig. 3). In this model, 'compound I' was regarded as a steady-state mixture of two species and assigned catalatic behavior to the tetrameric domain of catalase behavior. They further suspected the protonation-induced dissociation of catalase into four sub-units with subsequent irreversible deactivation of the subunits and represented the formation of compound II as a transition from the tetramers to the sub-unit domain and as a process in which a molecular hydrogen peroxide reacted with the tetrameric secondary complex. In this pathway, they reported at low substrate concentrations (as when hydrogen peroxide was generated by glucose oxidase system), compound II was largely formed from monomeric subunits (E/4) in a reaction with peroxide at a rate that was controlled by the protonation-induced dissociation of catalase. At higher substrate concentrations the enzyme dissociation was displaced towards the tetrameric domain. In the latter one, the rate of compound II formation was controlled by the rate of reaction of the secondary complex with peroxide. They suggested that compound III might result from the binding of peroxide by heme groups liberated in compound II formation. An alternative possibility was that its formation resulted from a reversible displacement by peroxide of the protein in a

Figure 2. The 'Bridge' Hypothesis.

From Nicholls (1964).

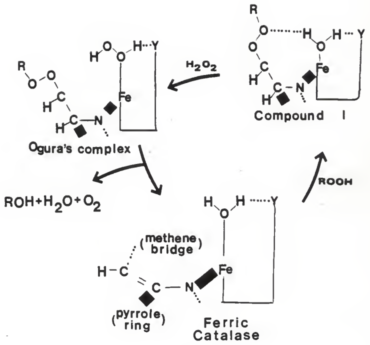
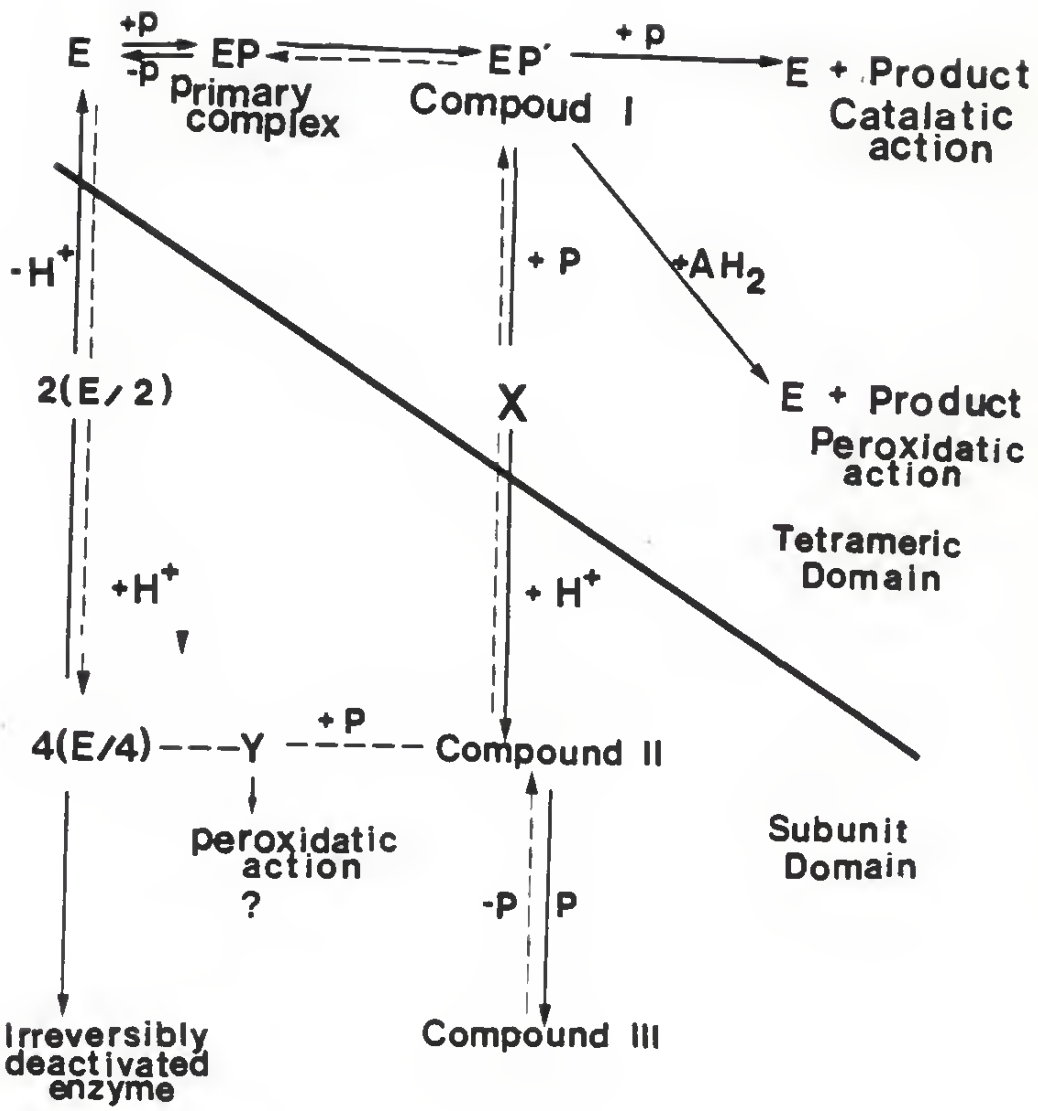

R
O
H
H
Y
O
O
C
H
Fe
C
N
H
Compound I
H_2O_2
ROOH
R
O
O
H
O
H
Y
H
C
Fe
H
C
N
Ogura's complex
ROH+H2O+O2
H
O
H
Y
(methene bridge)
H−C
Fe
C
N
(pyrrole ring)
Ferric Catalase

Figure 3. Revised Fromal Mechanism for Catalase Action.

From Jones and Suggett (1968).


E
+p
-p
EP
primary complex
EP'
Compoud I
+ p
E + Product
Catalatic action
-H+
+ P
+AH2
2(E/2)
X
E + Product
Peroxidatic action
Tetrameric Domain
+H+
+ H+
4(E/4)
Y
+P
Compound II
peroxidatic action
?
Subunit Domain
-P
P
Irreversibly deactivated enzyme
Compound III

monoheme subunit species.

Active Site of Oxygen Evolution.

Although detail of catalase action is not definitely established, a large amount of physicochemical evidence of the reaction is available. The mechanism of catalase action was elucidated with the discovery of that catalase as an enzyme involving iron atoms of the porphyrins. Theorell (1947) studied the valence state on the iron, the magnetic properties of the enzyme-substrate compounds, and the ionic or covalent nature of the intermediates with no emphasis on the protein part of catalase. Chance (1949) showed that the catalatic decomposition of hydrogen peroxide was not a chain reaction process. The decomposition of hydroperoxide to oxygen occurred on collision of catalase compound I with a second molecule of hydrogen peroxide or alkyl hydroperoxide. Compound I contained two oxidizing equivalents and probably reacted with the colliding peroxide via a concerted shift of four electrons; this resulted in a two electron donation by the free peroxide to the bound peroxide leaving water and oxygen (Fig. 4).

Jones and Suggett (1968) proposed that stereospecifically located acid-base functions in the active site might be the formation of a reactive intermediate. This hypothetical mechanism of catalatic reaction was shown on Figure 5.

Recent research on the identification of ligands at the fifth and sixth coordination positions of the prosthetic group had revealed more detailed and controversial arguments on the scission of the O-O bond. The three most popular hypothetical theories concerning the transfer of oxygen from compound I (EO) to the reductant (HXOH) were listed as follows and shown on Figure 6: (1) by the rearrangement of the "hydroxylated" intermediate (Hager et al., 1972), (2) by

Figure 4. Catalatic Action of Catalase to Release Oxygen.

From Chance (1949).

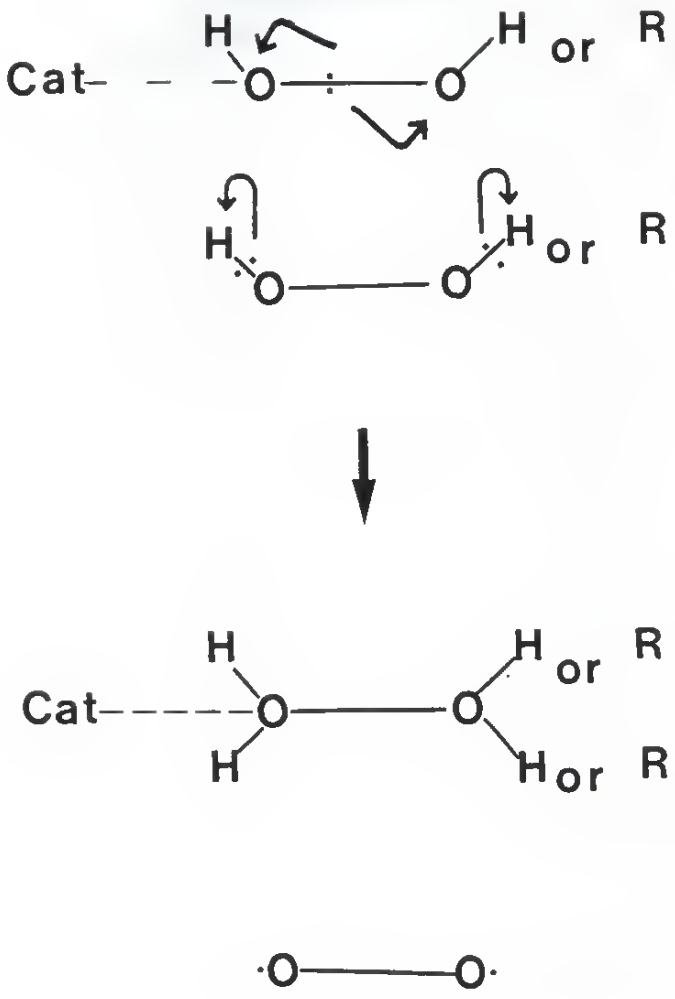
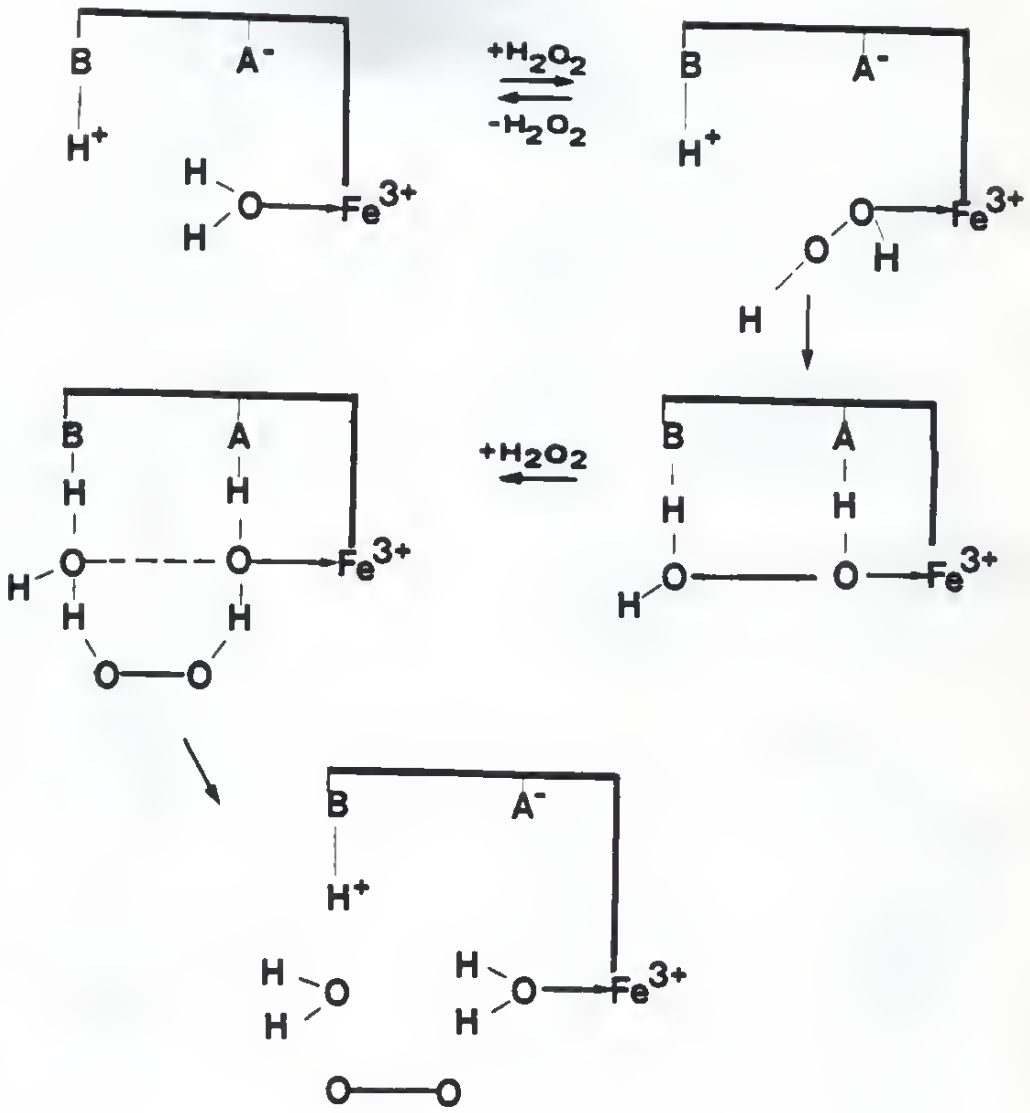

Cat
H
O
O
H
or
R
H
O
O
H
or
R
Cat
H
O
O
H
or
R
H
O
H
or
R
O
O

Figure 5. Hypothetical Scheme for the Role of Catalase Protein in Facilitating Catalase Action.

From Jones and Suggett (1968).


B
A⁻
H⁺
H
O
H
Fe3+
+H2O2
-H2O2
B
A⁻
H⁺
O
H
H
Fe3+
B
A
H
H
O
H
O
H
Fe3+
+H2O2
B
A
H
H
O
H
O
Fe3+
B
A⁻
H⁺
H
O
H
H
O
H
Fe3+
O—O

hydride transfer (Lowe and Ingraham , 1962., Nicholls, 1964), and (3) by inner sphere electron transfer (Hamilton, 1969).

These reactions were widely recognized in the "model" redox system, but still proof of the exact reaction mechanisms in releasing of the oxygen from catalase-hydrogen peroxide compound I is lacking.

Factors Affecting Catalatic Reactions

Effect of pH on Catalatic Reaction

Bonnichsen et al. (1947) studying horse blood catalase reaction rates at different pH conditions found that formation and decompositon of compound I was constant from pH 3 to 9. Although no apparent immediate inhibition of catalase in acidic conditions was observed, there was progressive decrease of the enzyme activity during the course of the reaction probably due to the formation of catalase-hydrogen compound II. A much more extensive loss of activity occurred when the substrate was presented, although the reason remained unclear. Chance (1952b) showed that the over-all catalatic reaction diminishes above pH 9, which suggested that pH affected the formation and dissociation of oxygen from compound I. If the inactivation of catalase protein due to partially reversible dissociation into subfragments was taken into account, the calculated reaction velocity of compound I was constant up to pH 12.

Effect of Temperature on Catalatic Reaction

The effect of temperature on the catalatic activity was probably due to the formation and decomposition of compound I. According to Bonnichsen et al. (1947) the Q_{10} value was 1.1 and the activation energy of intact catalase was 1,700 $\pm$ 100 cal corresponding to 6 % effective collisions of enzyme and substrate. Strother and Ackerman (1961) considered this explanation to be oversimplified because they observed that at very low temperatures, with glycerol used as an antifreezing

Figure 6. Hypothetical Theories of Transfer of Oxygen from Compound I (EO) to the Reductant (HXOH).

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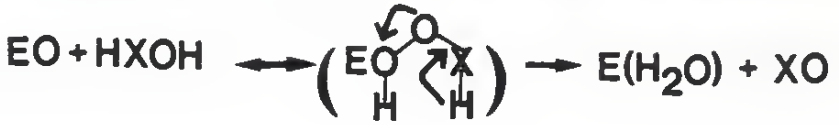
[1] From Hager et al. (1972).

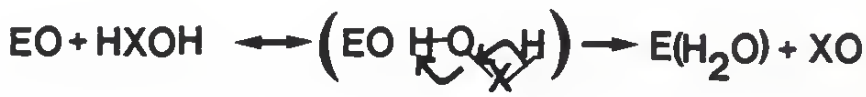
[2] From Lowe and Ingraham (1962), and Nicholls (1964).

[3] From Hamilton (1969).

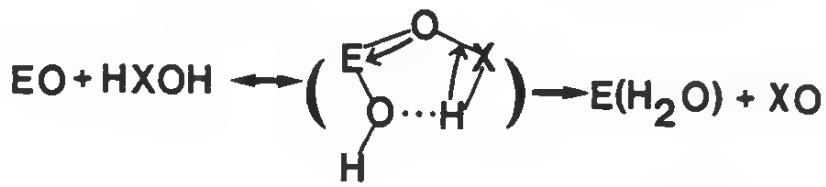
1. Rearrangement of Hydroxylated Intermediate



2. Hydride Transfer



3. Outer & Inner Sphere Electron Transfer



agent, the over all velocity to form compound I did not have a uniform Q_{10} relationship. The steady state of compound I was maximal and approximately constant between -4° and +26°C but decreased sharply beyond these limits.

Effect of Hydrogen Peroxide Concentration

George (1948) reported that the rate of decomposition of hydrogen peroxide by catalase was directly proportional to the enzyme concentration but the relation was more complex; below 0.06 M of H_2O_2, the rate was directly proportional to the concentration. Between 0.06 and 0.08 M the rate was maximum, and above 0.08 M the rate decreased. Ogura (1955) using purified horse liver catalase against varying concentrations of hydrogen peroxide found that the rate of the catalase reaction followed the first order reaction in respect to substrate concentrations.

Purified and Crystallized Catalase

Catalase was first crystallized from beef liver by Sumner and Dounce (1937). Other sources of catalase include liver of lamb, horse, human, guinea pig and pig as well as kidney of horse and beef erythrocytes. The method used to purify and crystallize catalase generally involved blending the source, precipitating or filtering the crude extracts, followed by adding the alcohol and chloroform to denature the hemoglobin, and then centrifuging the denatured hemoglobin from catalase solution. Ammonium sulfate was then added to precipitate catalase from solution. The enzyme was first dialyzed against phosphate buffer and then against several changes of cold distilled water. Crystalline catalase is obtained after three to five days of treatment.

Herbert and Pinsent (1948) crystallized microbial catalase from Micrococcus lysodeikticus by applying purified lysozyme to lyse the bacterial

suspension. The lysate was treated with alcohol in acetate buffer to separate catalase from other proteins. After centrifugation or filtration, chloroform was added to remove the remaining non-catalase protein. Catalase was isolated in the small top layer of the solution after addition of ammonium sulfate and sodium acetate. The top layer with catalase was removed and solid ammonium sulfate was added which resulted in another top layer of dark brown solution. Crystalline catalase would form after this dark brown solution wa0 dialyzed against acetate buffer at pH 5.6 and then dialyzed against distilled water at 0° C for 5 days. Although bacterial catalase has a different crystalline form with higher Kat.F value than animal catalase, the prosthetic groups, molecular weight and the number of hematin groups in the molecules are about the same.

History of Bacterial Catalase

According to Molland (1947) the historical account of bacterial catalase include 1) in 1893 Gottstein published the effect of hydrogen peroxide decomposition caused by microorganisms; 2) in 1906 the first systematic investigation into the catalase activity of different bacteria was made by Orla-Jensen when he traced back the origin of the catalase found in milk; 3) in 1908 Jorns studied bacterial catalase by titration with permanganate, and showed that a culture of Serratia marcescens which had been heated to 60-70° C for 5 minutes did not grow in subcultures, but still had strong catalase activity. Jorns tried to distinguish between endocatalase and ectocatalase by determining the activity of a broth culture and an equal quantity of filtrate from the culture. The difference was supposed to represent the endocatalase. He reported that in young cultures of Serratia the catalase was detected only in the culture, not in filtrate, as the culture grew older, an increasing amount of catalase was found in the filtrate, but always less than in the culture. To continue; 4) In 1921 D. Rywosz

compared the resistance to hydrogen peroxide in different strains of E. coli and explained the different resistant ability as due to the aerobic strains which contained catalase, while the anaerobic strains did not; and 5) In 1927 Kirchner studied the catalase production of Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli cultivated in different media and concluded from his experiments that the catalase content was strongly dependent on the composition of the medium, the chief factor being the hydrogen ion concentration. He also reported the presence of dextrose in the medium decreased the catalase production in the culture, but not because of the reduction of the cell number. Molland (1947) studied the producibility of catalase, hydrogen peroxide tolerance and the production of hydrogen peroxide for 37 species of bacteria by using the polarographic method. He concluded that the strains which produced most catalase were as a rule most resistance to hydrogen peroxide, but there were several exceptions such as Streptococcus salivarius which could tolerate 4 % hydrogen peroxide. One special observation he made was that applying sodium sulfide completely inhibited the catalase activity in the cell but could not inhibit the growth of bacteria. He suscepted catalase did not seem to be necessary for the maintenance of bacterial viability.

Synthesis of Catalase in Bacterial Cells

Factors affecting catalase activity in microbial cells have been studied by numerous workers with varied and contradictory results.

Addition of exogenous hydrogen peroxide had been reported to increase catalase activity in Rhodopseudomonas spheroides (Clayton, 1960), Escherichia coli and Salmonella typhimurium (Finn and Condon, 1975). Clayton (1960) studied the catalase synthesis in R. spheroides and reported that when the H_2O_2 was added to a moderately dense suspension (0.5-2.0 mg dry cell mass/ml) of R. spheroides,

catalase synthesis was directly or indirectly induced in 15-40 min. In the study of regulation of catalase synthesis in S. typhimurium, Enterobacter aerogenes and E. coli, Finn and Condon (1975) found the specific activity of catalase decreased during the logarithmic phase of growth and increased at the onset and during the stationary phase. The increase in catalase synthesis at the end of the exponential phase in S. typhimurium cells coincided with the lowest pH value reached by the culture. However, experiments in which pH was maintained at a constant neutral value (pH 7.0) did not alter the typical pattern of synthesis. They also reported that addition of H_2O_2 within the range of 1μM to 2mM in the exponential growth phase to cultures of S. typhimurium stimulated catalase synthesis with maximun production of catalase at 80μM. They proposed a hypothesis in explaning the stimulation effect of hydrogen peroxide as that H_2O_2 was not itself the direct inducer of catalase synthesis, but the direct inducer was synthesized after stimulation by H_2O_2 and by other circumstances. They considered the effect that a lag of 10 to 15 minutes after addition of H_2O_2 was observed in S. typhimurium before catalase synthesis was detected; the time lag between addition of inducer and detection of new enzyme was longer than generally accepted for enteric bacteria. For instance, induction of ß-galactosidase takes only 80 to 90 seconds in E. coli. Although the H_2O_2 would most likely be broken down by residual catalase and peroxidase in the first 2 min after addition, in this case, unless the messenger ribonucleic acid for catalase was unusually stable, they suspected that some compound synthesized on addition of H_2O_2 to exponentially growing cells was the actual inducer of catalase synthesis. However, Marie and Parak(1980) did not observe an increase of catalase activity of Micrococcus luteus by addition of hydrogen peroxide. They reported that addition of the heme precusors increased the catalase activity but the addition of glucose resulted in a repression effect. A similar observation was reported by Hassan and Fridovich (1978). They also

reported that growing E. coli cells in the presence of 8 mM adenosine 3',5'-cyclic monophosphoric acid (cAMP) reduced the effect of glucose and suggested the control mechanism of catalase biosynthesis in E. coli cell was an example of catabolite repression.

Aerobic Bacteria Flora Associated with Fresh Meat

Meat is one of the most perishable foods because it contains an abundance of nutrients required for the growth of bacteria. Since the healthy inner muscle of meats was regarded as sterile; bacterial spoilage of meat was generally limited to the surface through contamination during slaughtering, handling and processing (Ayres, 1955).

Because fresh meats is perishable, preservation of meat has been one of the most important concerns in human food history. Nowadays, in industrialized countries more meat is preserved by low temperatures than by any other method, and much more by chilling than by freezing. Thus, contemporary food microbiologists strive to understand and to control the microbial flora which are active in cold stored meats. Commercially, meats are stored between "-1.4 and 2.2°C", with preference in the lower temperatures range. Prompt and rapid cooling of meat renders the products less susceptible to spoilage by mesophilic microorganisms. Cold stored meats are generally spoiled by a group of microorganisms called psychrotrophs of which Pseudomonas is the most important. Other psychrotrophs included Alcaligenes, Micrococcus, lactic acid bacteria, Proteus, yeast and molds.

Early research work by Moran (1935) indicated that the bacteria commonly found at about 5°C or below belong to two groups, Achromobacter and Pseudomonas. However, Empey and Scott (1939) reported microbial floras of cold

stored beef indicated that Achromobacter accounted for 90% of the isolates from Australian beef stored at -1°C. Jensen (1944) reported that Pseudomonas rather than Achromobacter was responsible for the spoilage of refrigerated meat. Kirsch et al. (1952) found that the motile, non-pigmented members of Pseudomonas were the main organisms developed during storage of ground beef at 0°C to 2°C. Ayres (1955) resolved the confusion by pointing out that Achromobacter was reclassified in the 6th edition of Bergey's Manual of Determinative Bacteriology as non-pigmented Pseudomonas in 1948. Brown and Weidemann (1958) also reported that 170 out of 189 organisms isolated from chilled beef were species of Pseudomonas which were formerly recognized as Achromobacter. Jay (1967) also stated that large populations of fluorescent Pseudomonas were isolated from fresh and spoiled meat.

By investigating the relationships of temperature and microbial flora developing on refrigerated beef, Ayres (1960) claimed that at 10°C or lower, the bacteria responsible for production of slime were almost without exception pseudomonads; at 15°C or above, there was approximately an equal incidence of organisms of the genus Micrococcus and Pseudomonas. Marriott et al. (1967) reported that the genus Pseudomonas consisted of 95.8% of total isolates from prepackaged beef steaks; the rest were Achromobacter. At the species level P. fragi constituted 62% of total population, followed by 17% of P. geniculata.

Aerobic Bacterial Flora Associated with Fresh Poultry Meat

Similar to red meat, the inner muscle of poultry meat was also regarded as sterile. The bacterial flora of fresh poultry meats varied depending on the types of contamination involved. However, when these meats undergo low temperature spoilage, Pseudomonas was the primary spoilage organism. Recently, Lahellec et al. (1975) in studying 5920 strains of psychrotrophic bacteria isolated from chicken

reported that Pseudomonas constituted 30.5% of total isolates; Acinetobacter, 22.7%; Flavobacterium, 13.9%; Corynebacterium, 12.7%; Enterobacteriaceae, 5.8%; Micrococcaceae, 1.2%; yeasts, 8.9%; lactic acid bacteria, 2.2%. Although a great variety of organisms could be found on freshly slaughtered birds, a much smaller variety was responsible for the spoilage of refrigerated poultry meat (Ayres, 1955). Immediately after killing and processing, 75 to 80 % of the colonies recovered from chicken parts consist of chromogenic bacteria, mold, yeasts and sporeforming microorganisms. The proportion of chromogens and miscellaneous organisms decreases during storage, and within a few days after processing a rather uniform psychrotrophic flora caused off-odor and slime. Both indications of deterioration were closely associated with the growth and coalescence of colonies of several species of Pseudomonas.

Influence of Vacuum-Packaging on Bacterial Flora on Fresh Meat

The purpose of vacuum packaging is to prevent oxidation and to reduce spoilage by aerobic bacteria. Vacuum packaging is done by placing meat into gas-impermeable bags from which air is drawn by a vacuum pump. The residual trace oxygen in the bag is consumed by respiration of the tissue and replaced by carbon dioxide released from the muscle. However, Pierson et al. (1970) compared the bacterial count and flora of aerobically and anaerobically packaged beef steaks during 15 days storage at 3.3 °C, and found that 90 to 95 % of total count in anaerobic packaging were Lactobacillus while the fluorescent pseudomonads remained unchanged in numbers. Microbacterium thermosphactum and Gram-negative bacteria were found to decrease in number. Roth and Clark (1972) also reported that the flora of vacuum packaged samples consisted largely of lactobacilli (50 to 70 %), while for non-vacuum packaged meats they consisted mostly of pigmented pseudomonads and Microbacterium thermosphactum. They

compared the growth rates in vacuum and non-vacuum packages and reported that vacuum packaging of fresh beef reduced the growth rate of combined aerobes and anaerobes on meat to about 1/8 of that of meat packaged in gas-permeable film.

By studying the development of microbial flora on meat stored in vacuum bags at 0-2°C, Sutherland et al. (1975) reported that the microbial flora of lactic acid bacteria increased relative to the aerobic spoilage organisms, but the numbers of the aerobic spoilage bacteria continued to increase throughout the 9 weeks of vacuum storage. Hanna et al. (1977) also showed that, besides Lactobacillus, the microbial flora of refrigerated vacuum packaged lamb after 21 days storage were primarily Pseudomonas and Moraxella-Acinetobacter species. A recent study by Newton and Rigg (1979) showed that the storage life of the vacuum-packaged meat was inversely related to film permeability. The growth rates and final counts of Pseudomonas species increased with increasing film permeability. This study provided satisfactory evidence that aerobic bacteria survived from vacuum-packaged meat.

MATERIALS AND METHODS

Development of Gas Column Method

Preparation of Capillary Tubes

A 9 inch pasteur pipette was sealed at the narrow end by heat. After that the pipette was put into 100 °C oven for 60 min to inactivate all possible existing catalase. These tubes were then stored at 20 °C until needed.

Addition of Liquid Sample

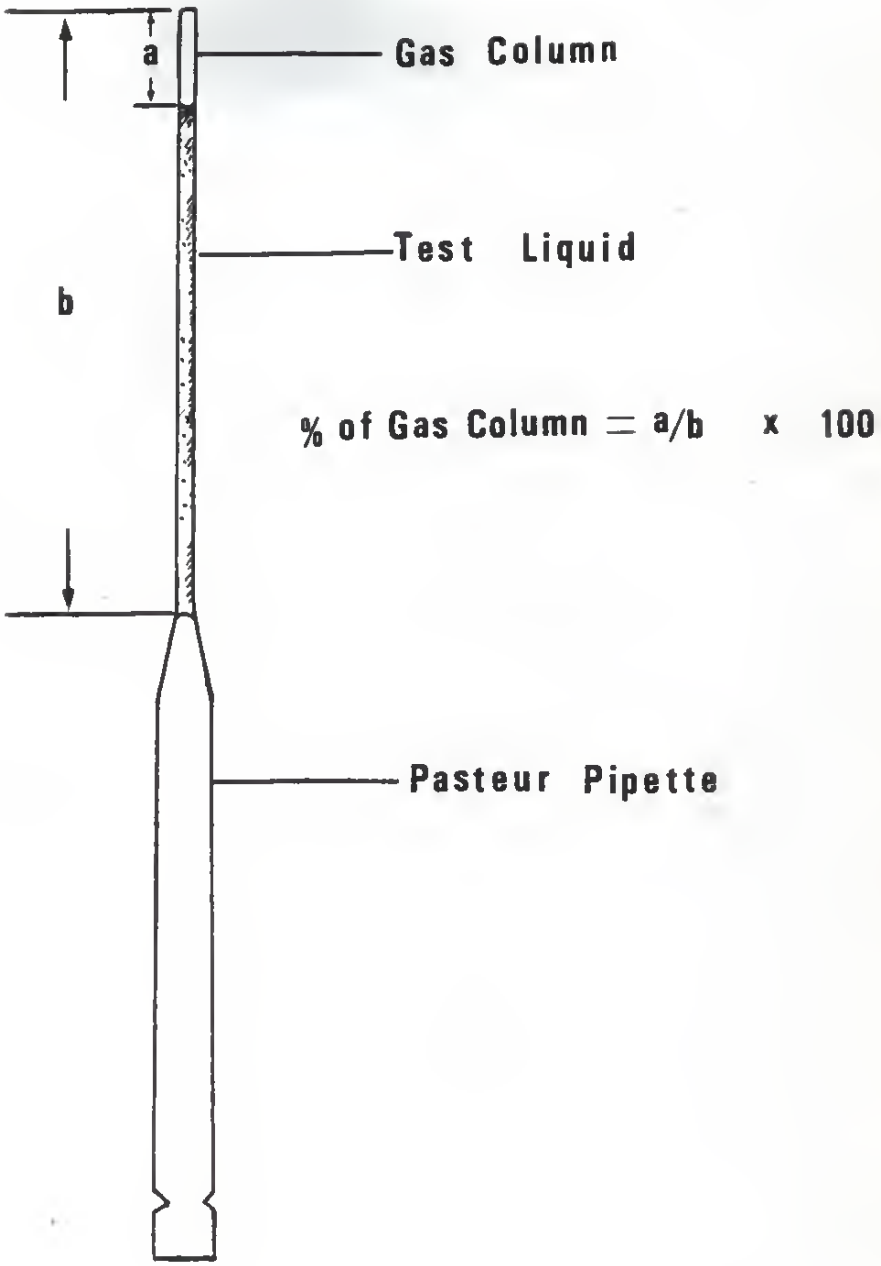
To the tubes already sealed, 10 µl of 30 % hydrogen peroxide (H_2O_2) were introduced from the wider end after which 90 µl of a liquid to be tested (bacterial culture or purified catalase) were introduced. The entire liquid column was then "shaken" into the narrow portion of the pipette. The pasteur pipette was then inverted so that gas can be trapped at the tip of the narrow tube. After fifteen minutes the length of the gas column and the total liquid were measured by use of an ocular micrometer or a ruler. The diagram of this system was shown on Figure 7. The gas column was expressed as percentage of total column.

Gas Column/Total Column x 100 = Percentage Gas Column (PGC).

Effect of Hydrogen Peroxide Concentration Upon the Gas Column Method

The influence of the concentration of hydrogen peroxide as well as different amounts of test catalase solution were studied. Commercial purified lyophilized Aspergillus niger catalase (Calbio Chem-Behring Co., San Diego, CA) was dissolved in 0.05 M phosphate buffer at pH 7.0. The range of concentration tested was from 200 catalase units/ml to 1 catalase unit/ml. Each concentration was tested 5 times. A comparative study using 50 µl of standard catalase solution and 50 µl of 3 % H_2O_2 was also conducted. The criteria for selecting the best ratio of this system involved the correlation between the log of the catalase

Figure 7. Diagram of Gas Column Method.


a
Gas Column
Test Liquid
b
% of Gas Column = a/b x 100
Pasteur Pipette

concentration and the log of the percent gas column. In judging the sensitivity of the system, the basic assumption was that the lower the detectable limit the more sensitive would be the system.

Catalasemeter with Paper Disc Method

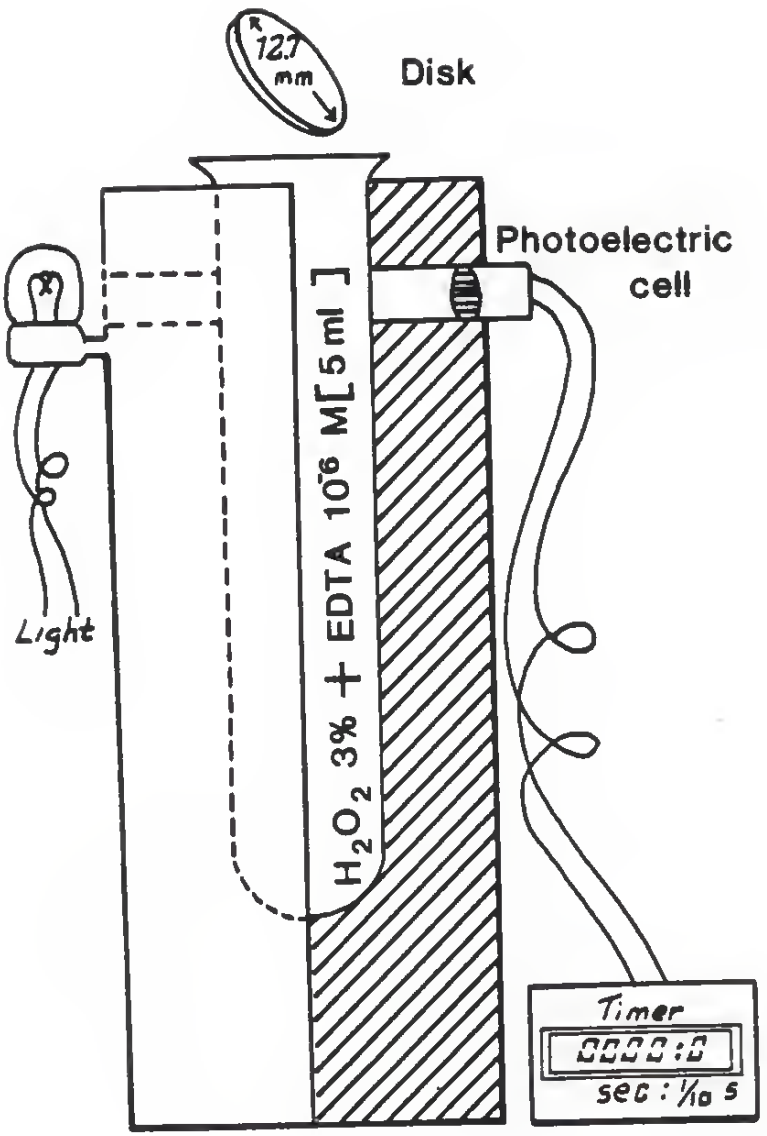
Catalasemeter

An instrument called the Catalasemeter was recently introduced commercially by Bio-Engineering Group Ltd. (Westport, Conn). The principle is the disc-flotation method proposed by Gagnon et al. (1959). It is a simple instrument to operate. First, an optically clean glass tube containing 5 ml of 3 % H_2O_2 (with 10^{-6} M EDTA) is introduced into a cavity in the instrument. A narrow beam of light passes through the liquid and light intensity is detected by a photoelectric cell attached to a digital timer. To perform the catalase test a paper assay test disc is saturated with the test liquid (0.12 ml) and dropped into the H_2O_2 solution. As the disc travels down the liquid by gravity the light path is disturbed and this activates the digital counter in the instrument. In the event the test liquid contains catalase, gas bubbles will be generated and trapped underneath the paper disc and cause the disc to float. When the disc floats past a light beam in the instrument the counter is stopped. The time lapse in seconds between activation and deactivation of the counter is the flotation time (FT) of the disc. The diagram of this system is showing in Figure 8.

Standard Curve for Catalasemeter with Paper Disc Method

Standard catalase solution was prepared in the same manner as previously described. Concentrations tested ranged from 10^4 catalase units/ml to 10^{-4} catalase units/ml.

Figure 8. Diagram of Catalasemeter with Paper Disc System.


12.7 mm
Disk
Photoelectric cell
Light
H_2O_2 3% + EDTA 10^{-6} M[5 ml]
Timer
00000:0
sec : 1/10 s

Study of Gas Column and Catalasemeter with Paper Disc Method on Bacterial Cultures

Eight different species of bacteria (six catalase positive cultures and two catalase negative cultures as control) were studied. The following bacteria were tested both in the preliminary study of the Gas Column Method and Catalasemeter Method.

Organisms Tested			Optimal Growth Temp °C
1. Bacillus cereus	ATCC 14579	Catalase (+)	32
2. Escherichia coli	ATCC 25922	Catalase (+)	37
3. Lactobacillus brevis	ATCC 14869	Catalase (-)	37
4. Micrococcus luteus	ATCC 4689	Catalase (+)	26
5. Pseudomonas fluorescens	ATCC 13525	Catalase (+)	26
6. Salmonella typhimurium	ATCC 23566	Catalase (+)	37
7. Staphylococcus aureus	KSU 100	Catalase (+)	37
8. Streptococcus faecalis	ATCC 19433	Catalase (+)	37

All catalase positive cultures were kept in nutrient agar slants and two catalase negative cultures were kept in litmus milk at 4°C and transferred every three months. Working cultures were prepared by aseptically transferring one loopful of culture from stock culture into 20 ml of Brain Heart Infusion Broth (BHI, Difco) and incubated 24 hr at each organism's optimal growth temperature. At the end of this incubation, catalase activity was measured by Gas Column Method and Catalasemeter Method. Test cultures were prepared by aseptically transferring 1 ml of broth culture into 9 ml of dilution buffer solution. Each broth culture was shaken 40 times before liquid was drawn from the tube. Serial dilutions were made by pipetting 8, 6, 4 and 2 ml of diluted culture solution into another 2, 4, 6 and 8 ml of sterile dilution buffer solution, respectively. From the second diluted culture

solution another 1 ml was pipetted into another 9 ml of sterile buffer solution. The dilution process was continued until no further catalase activity could be detected. A comparative study of viable cell count using the Standard Method (Clark, et al., 1978) was done. Each broth culture was shaken 40 times before being pipetted into a dilution bottle containing 99 ml of phosphate buffer solution. Appropriate volumes of diluents (0.1 and 1 ml) were transferred into petri dishes; duplicates were made of each dilution. After that, 15 ml of 45 °C of melt Standard Plate Count Agar (SPC, Difco) were poured into each petri dish. The agar was allowed to solidify and incubated invertedly at 32°C for 24 hr. Plates with 30 to 300 colonies were counted after the incubation period.

Study of the Gas Column Method on Bacterial Cultures

Ten µl of 30 % hydrogen peroxide were added into each heat sealed capillary pasteur pipette. After all the hydrogen peroxide solution settled down into the capillary part of the pipette, 90 µl of test bacterial culture solution was added. After all the test liquid was shaken into the capillary part and homogenized with hydrogen peroxide solution, the tube was inverted. A 15 min reaction interval was allowed before the length of gas column and total liquid was recorded. The log viable cell count/ml and log % gas column has a positive correlation.

Study of the Catalasemeter with Paper Disc Method on Bacterial Cultures

Test culture solutions were prepared in the same manner as those used for Gas Column Method. A known amount of test liquid (0.12 ml) was transferred to a paper disc. The flotation time was recorded in seconds. Viable cell counts were made immediately after the test. The relation between flotation time and the number of cells was recorded. The log viable cell count/ml and the log flotation time in seconds has a negative correlation.

Preliminary Study of Catalase Activity on Cold Stored Meat

Chicken wings were purchased at local supermarket. Swab technique was applied in this sampling procedure. This technique involved wetting the sterile cotton swab (American Scientific Procedure. McGaw Park, IL) in a tube containing 5 ml of sterile pH 7.0 phosphate buffer. After swabbing the meat surface in a 2 X 4 cm^2 area in three direction, the cotton swab was broken into the original tube aseptically and shaken vigorously for 40 times. Comparative study of the modified psychrotroph count was conducted according to standard plate count procedure. The catalase activity was measured by both the Gas Column Method and Catalasemeter with paper disc method.

Effect of pH on Bovine's Blood Catalase and Bacterial Catalase

By Catalasemeter with Paper Disc Method

Phosphate buffer (0.05 M) solutions, ranging from pH 3.0 to pH 10.0 were prepared and measured by Model 43 pH meter (Beckman Instruments, Inc., Irvine, CA). Sterile bovine whole blood was obtained from Ruminant Microbiology Laboratory, Kansas State University. Two concentrations of catalase activities of blood solution were prepared; high with approximately 10^3 catalase units/ml and medium with approximately 10^0 catalase units/ml. Micrococcus luteus and Pseudomonas fluorescens were selected for comparison. Bacterial cultures were transferred from stock culture into 10 ml of Brain Heart infusion, and incubated 24-48 hr to allow the population to reach 10^8 cells/ml in order to have the maximum catalase activity. The test solution was prepared by adding 1 ml of bacterial or blood solution into 4 ml of phosphate buffer, and vigorously shaking 40 times. After waiting for 5 min to bring the tube into equilibrium, the catalase activity was measured by catalasemeter method. The flotation time at each pH was converted into catalase units by the formula of regression line obtained from a

standard curve. Percentage of remaining catalase activity was calculated as catalase units at a certain pH divided by catalase units at pH 7.0.

By Catalasemeter with Membrane Filter Method

Membrane filters (13 mm diameter) with pore size of 0.45 μm were obtained from Gelman Sciences Inc. (Ann Arbor, Michigan). This membrane filter was inserted in a filter adaptor and adjoined to a 3 ml disposable syringe; the test solution was then pipetted into the syringe. After filtering the test liquid, the membrane filter was assayed for its catalase activity by Catalasemeter (membrane filter method). Test solutions (blood and bacteria) were the same as used in the first part of this study and 0.05M phosphate buffer solutions of various pH (varied from 4.0 to 2.6) were used. The diagram of this system is showing on Figure 9.

Effect of Acidic Environment on Psychrotrophs

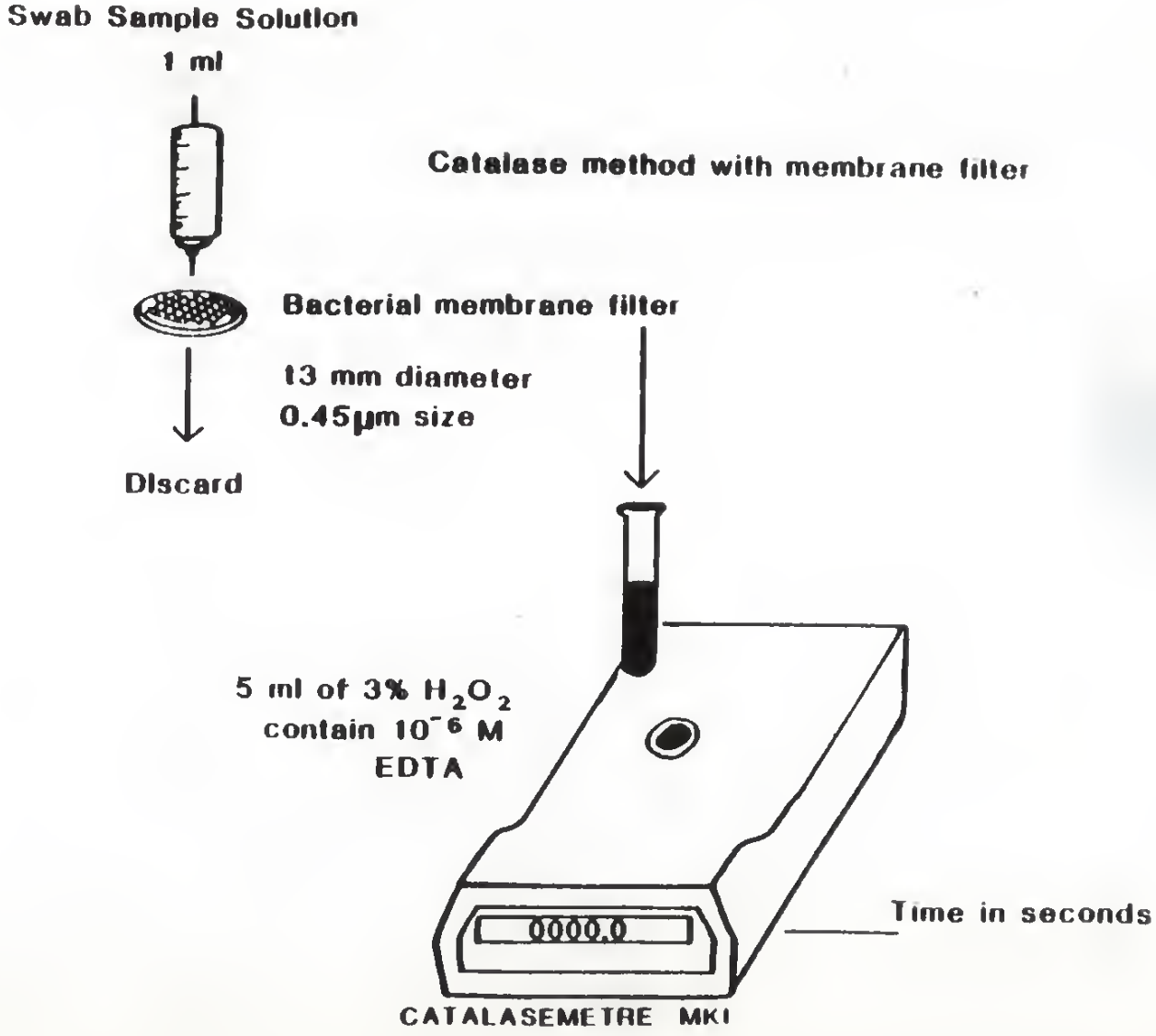
Effect of Acidic Environment on the Viability of Psychrotrophs

Influence of acidic environment on the viability of Pseudomonas fluorescens was tested. One ml of bacterial culture solution was added into 4 ml of 0.05 M pH 3.95 and 3.25 phosphate buffer (in order to obtain final pH of 4.00 and 3.30, respectively) for 0, 5, and 10 minutes. Viable cell counts were made immediately after 0, 5, and 10 minutes. Another viable cell count was made directly from the original cultural solution as control.

Effect of Acidic Environment on Catalase Activity of Selected Psychrotrophs

Since bacterial catalase activity was found to be much more resistant in acidic environment than blood catalase in preliminary studies, this phenomenon was applied as the key point to differentiate the blood catalase from bacterial

Figure 9. Diagram of Catalasemeter with Membrane Filter System.


Swab Sample Solution
1 ml
Catalase method with membrane filter
Bacterial membrane filter
13 mm diameter
0.45µm size
Discard
5 ml of 3% H_2O_2
contain 10^{-6} M
EDTA
0000.0
Time in seconds
CATALASEMETRE MKI

catalase. In order for practical usuage of catalase activities to predict the bacterial load in cold stored meat surface, standard curves of catalase activity in acidified samples versus bacterial numbers were made. Catalase activity of five psychrotrophic organisms were tested to build the base line in the Catalasemeter Method. Psychrotroph samples were transferred from stock culture into 10 ml of Brain Heart Infusion, aseptically. A 24-48 hr incubation period was done. A series of dilutions were made starting with pipetting 2 ml of culture into 18 ml of 0.05M pH 3.30 phosphate buffer. After shaking for 40 times and waiting for 5 minutes, different amounts of diluent were introduced into 3 ml disposable syringes. After the diluent was filtered through the 13 mm 0.45 μm membrane filter, catalase activity on the filters was assayed. Viable cell count using corresponding non-acidified culture liquid was made accordingly.

Organisms tested.

1. Micrococcus luteus ATCC 4698
2. Pseudomonas aeruginosa CS
3. Pseudomonas fluorescens ATCC 13525
4. Pseudomonas fragi 2F-36 KSU
5. Pseudomonas maltophilia CS

Study of Catalase Activity on Cold Stored Chicken Parts

Chicken wings, drumsticks and fryer parts were purchased from local grocery stores and stored at 7°C. Sampling was made every other day for 10 days after purchase. The swab method procedure was used for surface sampling. The sterile cotton swab was suspended in 2.5 ml of sterile phosphate buffer. Guided by a sterile template cut out of aluminum foil, the cotton swab was rubbed over a meat surface area of 2 X 4 cm^2 in three different directions. The cotton swab was

then aseptically broken into the tube and shaken 40 times in buffer solution. One ml of the swab sample solution (non acidified) was pipetted immediately into 9 ml of dilution buffer for a comparative study of viable cell count (incubated at 32°C for 24 hr) and modified psychrotroph count (incubated at 21°C for 23-25 hr). Another 1 ml of swab sample solution was then pipetted into 4 ml of pH 3.25 0.05M phosphate buffer (final pH 3.30) for acidification. After shaking 40 times and waiting for 5 min to bring the system to equilibrium, the solution was used for assaying catalase activity by the Catalasemeter with membrane filter method.

Statistical Methods for Analysis

A non-linear model (SAS program) was used to construct the standard curve and formula between logarithm of flotation time (sec) and logarithm of colony forming units/ml of data generated from five selected psychrotrophs, as well as viable cell count and modified psychrotroph count from cold stored chicken in pH 3.30 acidified treatment. Transformation of non-linear formula to linear formula was also accomplished.

RESULTS AND DISCUSSIONS

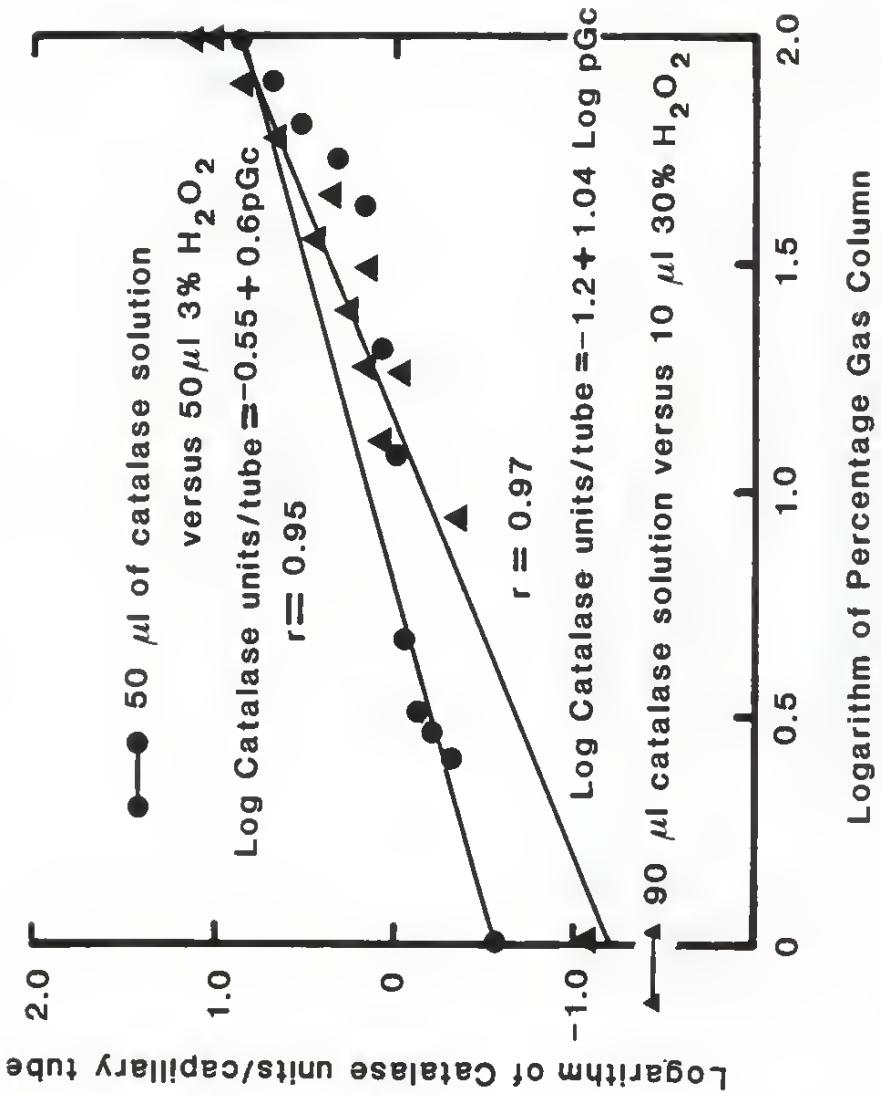
Study of Gas Column Method and Catalasemeter with Paper Disc Method on Purified Catalase

Development of Gas Column Method

Figure 10 shows the standard curve developed for catalase quantification by the capillary tube system identified as Gas Column Method. The curve was developed by serially diluting dissolved lyophilized catalase from 200 to 1 units/ml. Treatment 1 involved mixing 50 µl of diluted test solution with 50 µl of 3% hydrogen peroxide for reaction. In treatment 2, 90 µl of the diluted test solution was mixed with 10 µl of 30 % hydrogen peroxide. In both treatments, a direct linear relationship was observed between the logarithm of the percentage of gas column (PGC) and the logarithm of the catalase concentration. A tiny bubble was formed after testing time in the lower range whereas the entire liquid column was displaced by gas in the upper range of catalase concentration. The lowest detectable catalase concentration in treatment 1 was 0.28 catalase units/capillary tube and 0.06 catalase units/capillary tube for treatment 2. The correlation coefficient for the linear relationship of treatment 1 is $r = 0.95$ ($p<0.01$); and the equation for the regression line for this treatment is $\text{Log } Y = -0.55 + 0.6 \text{ Log PGC}$, where Y was the catalase units/capillary tube. The correlation coefficient for the linear relationship of treatment 2 is $r = 0.97$ ($p<0.01$); and the equation for the regression line for this treatment is $\text{Log } Y = -1.2 + 1.04 \text{ Log PGC}$. Every point in the graph represents the mean of five tests.

Correlation coefficients of both treatments gave similar results. According to the equation obtained, treatment 2 had a lower detectable limit than treatment 1; hence it was chosen for further experimentation.

Figure 10. Standard Curve of Two Treatments of Gas Column Method on Purified Aspergillus niger Catalase.


Logarithm of Catalase units/capillary tube
2.0
1.0
0
−1.0
0
0.5
1.0
1.5
2.0
Logarithm of Percentage Gas Column
50 μl of catalase solution versus 50μl 3% H_2O_2
Log Catalase units/tube = −0.55 + 0.6pGc
r = 0.95
r = 0.97
Log Catalase units/tube = −1.2 + 1.04 Log pGc
90 μl catalase solution versus 10 μl 30% H_2O_2

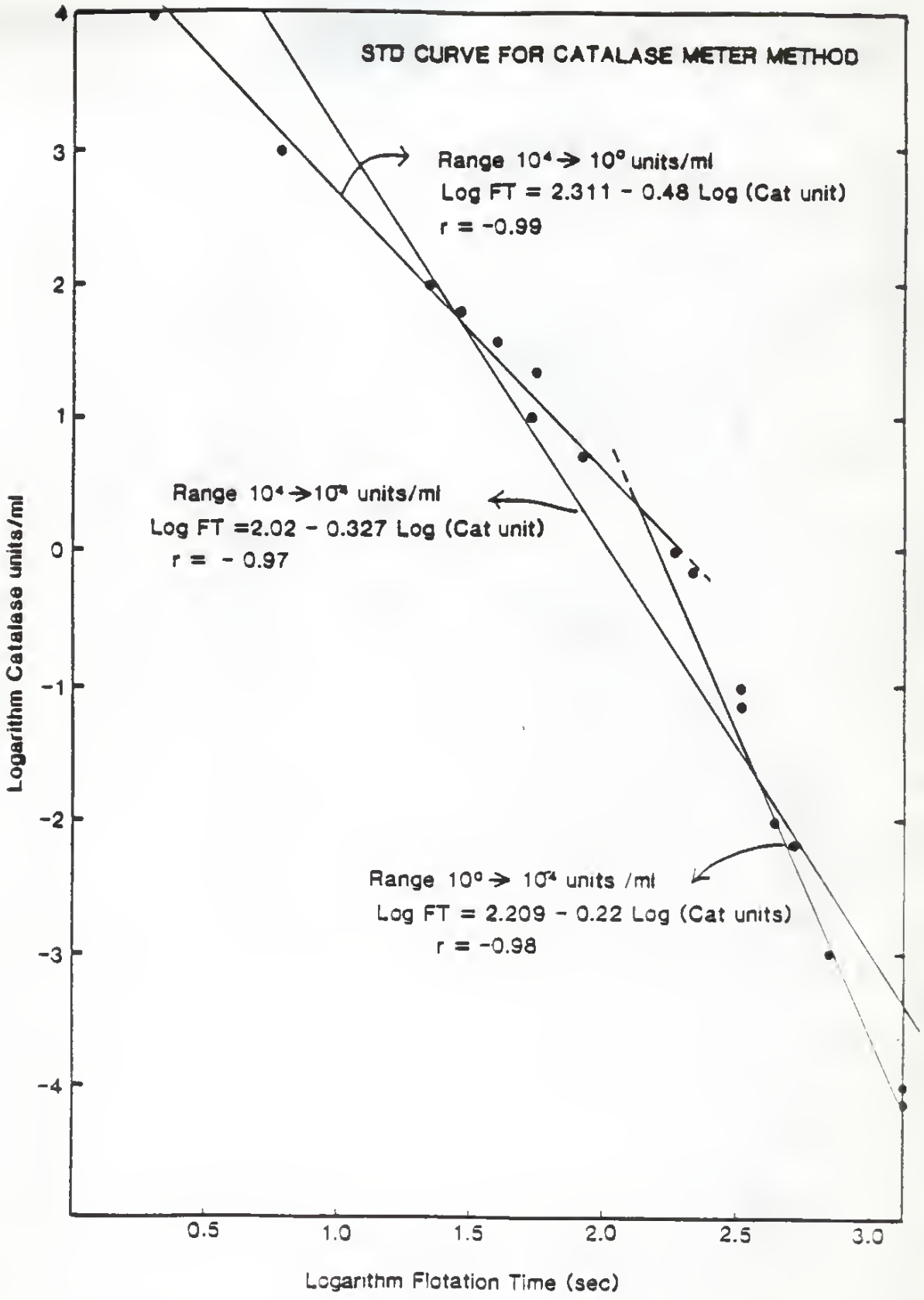
Study of Catalasemeter with Paper Disc Method

Figure 11 shows the standard curve developed for catalase quantification by the Catalasemeter Method with paper disc. The detectable range of catalase concentration in the Catalasemeter with paper disc method was between 10^4 to 10^{-4} catalase units/ml. At the lower limit the flotation time (FT) was 1385.2 sec. whereas at the higher limit the FT was 2.0 sec. Two linear slopes were observed between the logarithm of the FT and the logarithm of the catalase concentration tested. The correlation coefficient of the first slope (10^4 to 10^0 catalase units/ml) was $r = -0.99$ ($p<0.01$) and the formula for the regression line was log FT (sec) = 2.311 - 0.48 log catalase units/ml. The correlation coefficient of the second slope (10^0 to 10^{-4} catalase units/ml) was $r = -0.98$ ($p<0.01$) and the formula for the regression line was log FT = 2.209 - 0.22 log catalase units/ml. The overall correlation coefficient for the linear relationship between catalase units ranging from 10^4 to 10^{-4} per ml was $r = -0.97$ ($p<0.01$) and the formula for the regression line was log FT = 2.02 - 0.327 log catalase units/ml. Every point in the graph represents the mean of twenty tests.

In comparison, the Catalasemeter Method gave a better correlation coefficient over all tested catalase concentration ranging from 10^4 to 10^{-4} catalase units/ml, while the Gas Column Method could only detect the catalase concentration ranging from about 200 to 1 catalase units/ml.

Gagnon et al. (1959) first utilized the flotation of a disc as a measurement of catalase activity. They used lyophilized bovine liver catalase to study this effect and reported only one slope in the graph. Since they used high concentrations of catalase, their observations corresponded to the first slope obtained in this study.

Figure 11. Standard Curve for Catalasemeter with Paper Disc Method on Purified Aspergillus niger Catalase.


STD CURVE FOR CATALASE METER METHOD
Range $10^4 \rightarrow 10^0$ units/ml
Log FT = 2.311 - 0.48 Log (Cat unit)
r = -0.99
Range $10^4 \rightarrow 10^3$ units/ml
Log FT =2.02 - 0.327 Log (Cat unit)
r = - 0.97
Range $10^0 \rightarrow 10^4$ units /ml
Log FT = 2.209 - 0.22 Log (Cat units)
r = -0.98
Logarithm Catalase units/ml
Logarithm Flotation Time (sec)
4
3
2
1
0
-1
-2
-3
-4
0.5
1.0
1.5
2.0
2.5
3.0

Study of Gas Column Method and Catalasemeter with Paper Disc Method on Bacteria Culture

Six catalase-positive bacteria and two catalase-negative bacteria were tested using the two catalase methods as well as the conventional viable cell count method.

Figures 12-17 show the logarithm of colony forming unit and the logarithm catalase activity studied by both Gas Column Method and Catalasemeter Method with paper disc on these six catalase-positive bacteria. As expected, catalase-negative bacteria (Lactobacillus brevis and Streptococcus faecalis) showed no responses in both catalase methods. Under the experimental conditions, the production of catalase varied with species tested. Micrococcus luteus was the highest catalase producing bacteria in this study. Pseudomonas fluorescens and Staphylococcus aureus were second in terms of catalase production. Escherichia coli and Bacillus cereus produced about the same amount of catalase activity. Salmonella typhimurium produced the least catalase among these six catalase-positive bacteria. Charbonneau et al. (1975) used the Catalasemeter to detect and measure bacterial catalase activity of Bacillus spp. and Enterobacteriaceae. Similar to this study they reported that the production of catalase varied with the species tested. They also reported that in the same species the ability to produce catalase also varied from strain to strain. In this study, catalase production increased with the increasing cell number, except in the case of Salmonella typhimurium which gave very weak catalase response until the cell number reached 10^7 cell/ml. This observation agreed with Finn and Condon (1975), that Salmonella typhimurium produced catalase on the late log phase and stationary phase.

The lower limit of detectable catalase activity on both catalase methods also varied among bacteria species. The apparent limit of sensitivity of the

Figure 12. Measurement of Catalase Activity of Bacillus cereus by Catalasemeter with Paper Disc Method and Gas Column Method.

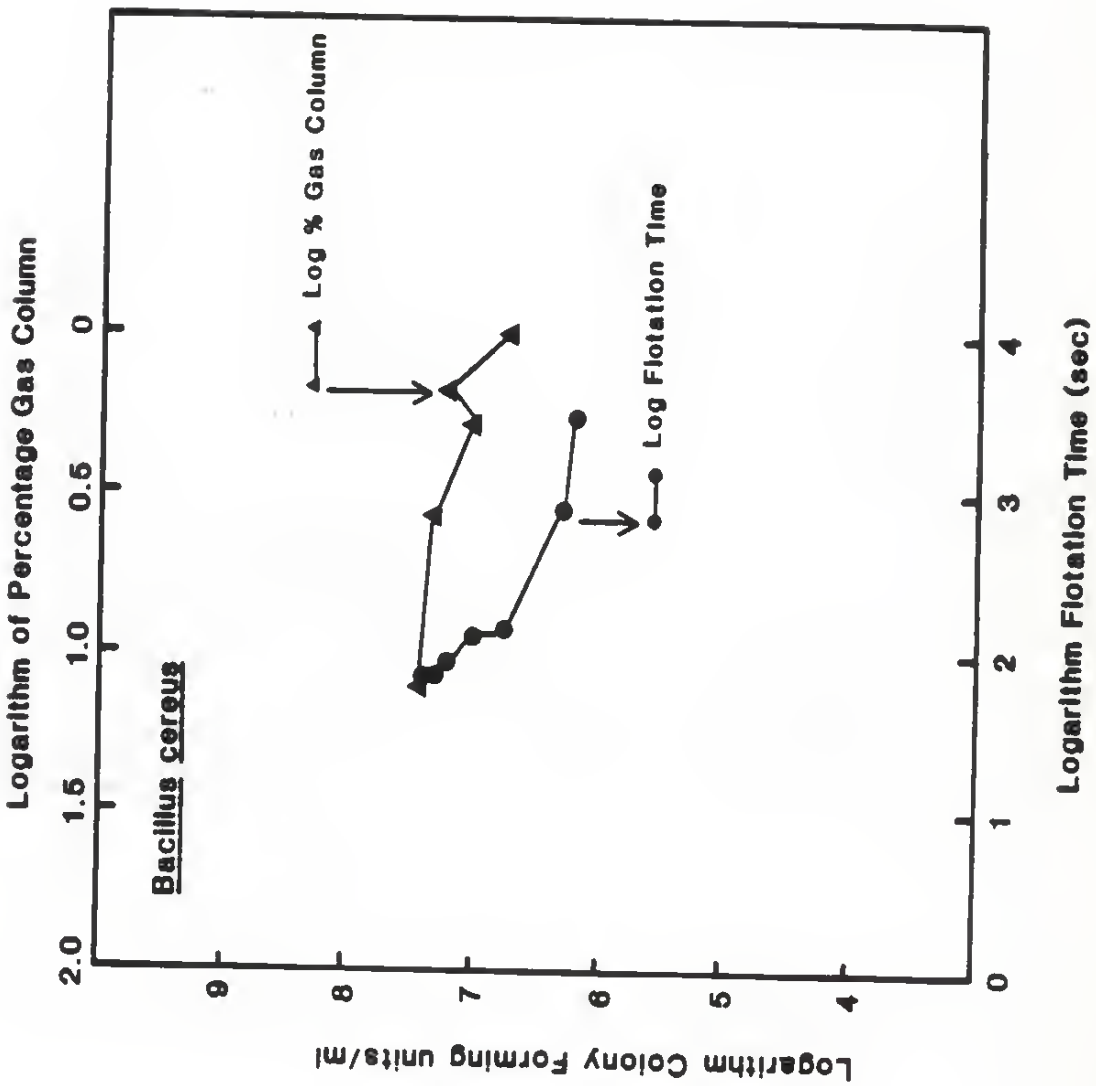

Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Log % Gas Column
Log Flotation Time
Bacillus cereus
9
8
7
6
5
4
Logarithm Colony Forming units/ml
0
1
2
3
4
Logarithm Flotation Time (sec)

Figure 13. Measurement of Catalase Activity of Escherichia coli by Catalasemeter with Paper Disc Method and Gas Column Method.

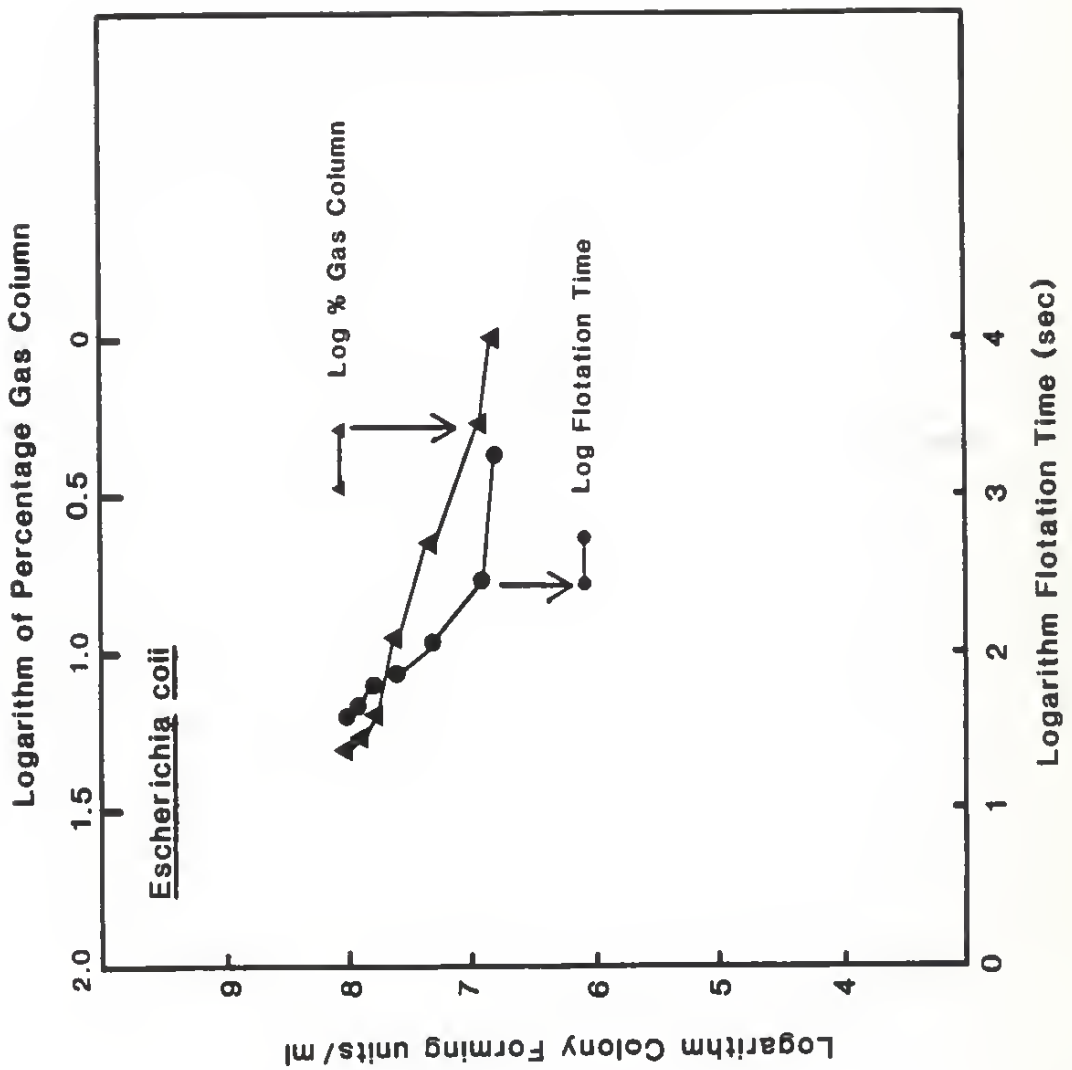

Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Escherichia coli
Log % Gas Column
Log Flotation Time
Logarithm Colony Forming units/ ml
9
8
7
6
5
4
0
1
2
3
4
Logarithm Flotation Time (sec)

Figure 14. Measurement of Catalase Activity of Micrococcus luteus by Catalasemeter with Paper Disc Method and Gas Column Method.

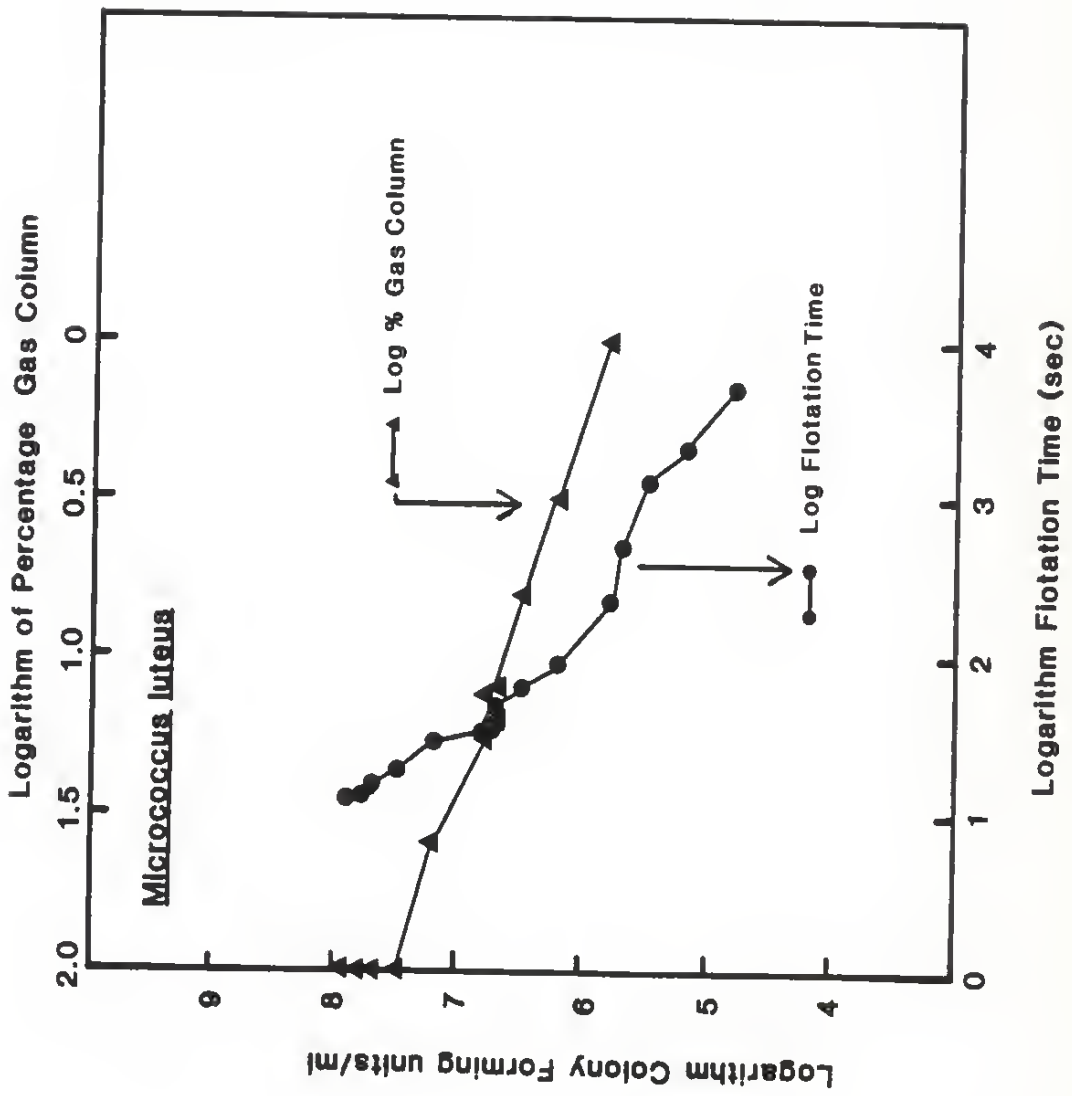

Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Micrococcus luteus
Log % Gas Column
Log Flotation Time
Logarithm Colony Forming units/ml
9
8
7
6
5
4
Logarithm Flotation Time (sec)
0
1
2
3
4

Figure 15. Measurement of Catalase Activity of Pseudomonas fluorescens by Catalasemeter with Paper Disc Method and Gas Column Method.

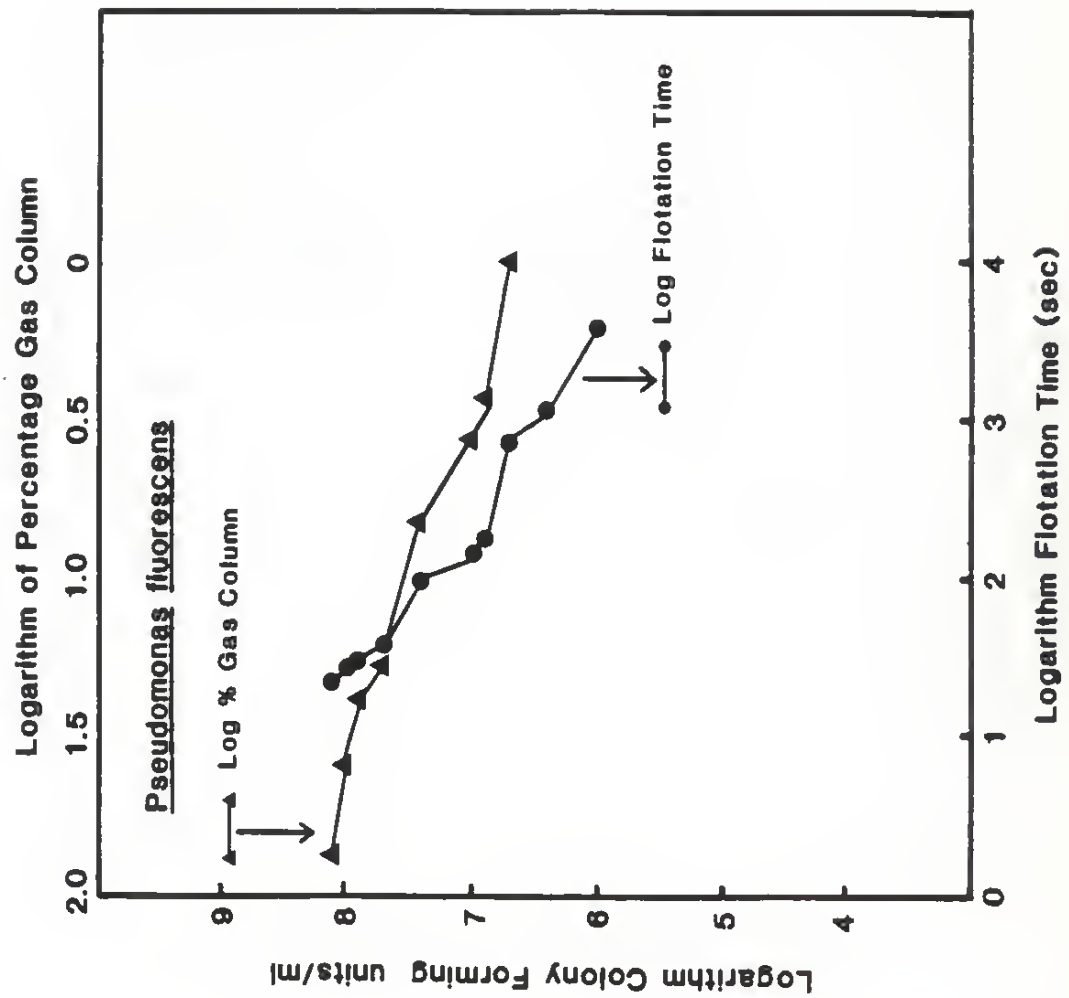

Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Pseudomonas fluoreascens
Log % Gas Column
Log Flotation Time
Logarithm Colony Forming units/ml
9
8
7
6
5
4
0
1
2
3
4
Logarithm Flotation Time (sec)

Figure 16. Measurement of Catalase Activity of Salmonella typhimurium by Catalasemeter with Paper Disc Method and Gas Column Method.

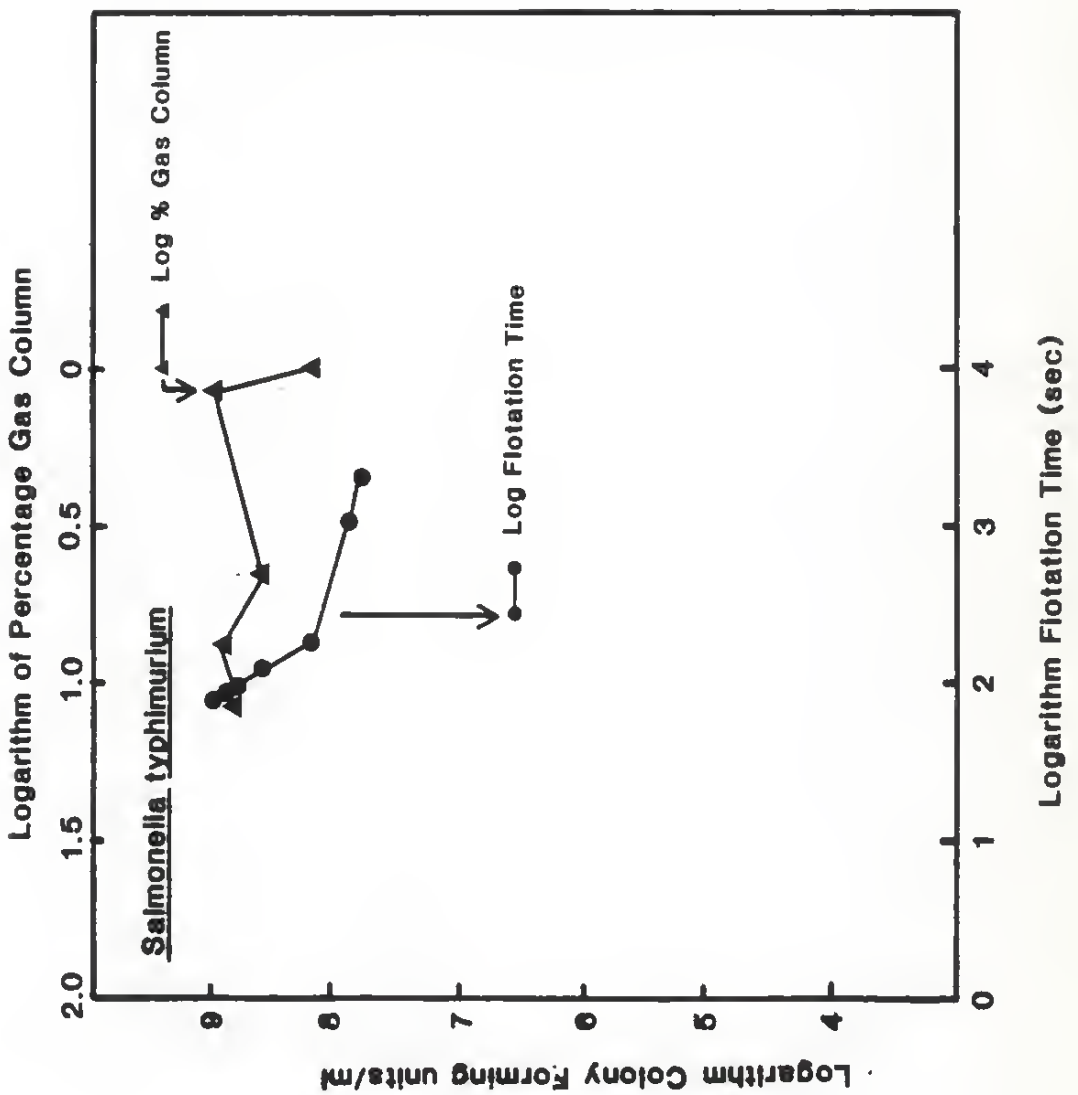
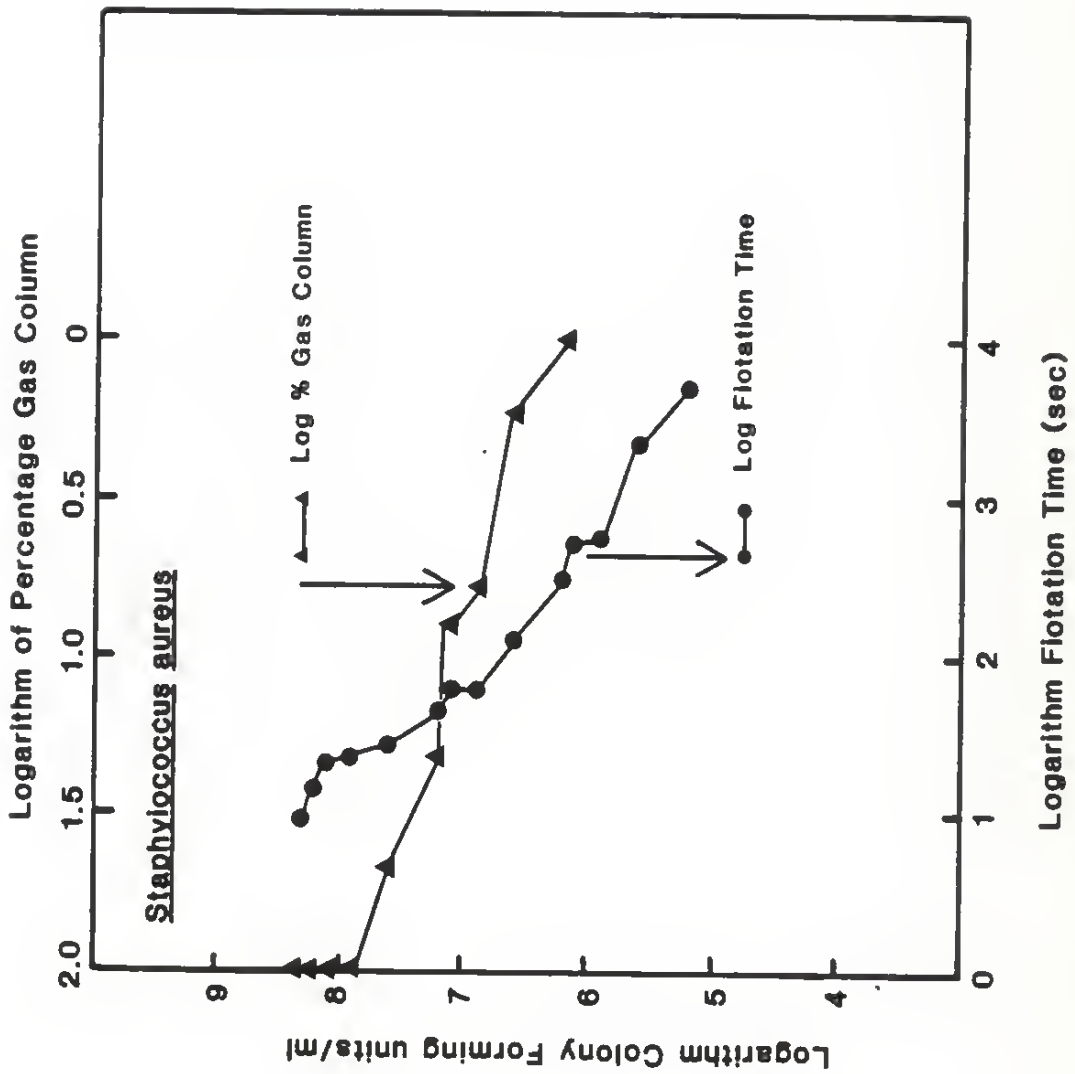

Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Log % Gas Column
Salmonella typhimurium
Log Flotation Time
Logarithm Colony Forming units/ml
9
8
7
6
5
4
0
1
2
3
4
Logarithm Flotation Time (sec)

Figure 17. Measurement of Catalase Activity of Staphylococcus aureus by Catalasemeter with Paper Disc Method and Gas Column Method.


Logarithm of Percentage Gas Column
2.0
1.5
1.0
0.5
0
Staphylococcus aureus
Log % Gas Column
Log Flotation Time
Logarithm Colony Forming units/ml
9
8
7
6
5
4
0
1
2
3
4
Logarithm Flotation Time (sec)

Catalasemeter and Gas Column Method for the catalase-positive bacteria was as follows: Micrococcus luteus (Log 4.8/ml for Catalasemeter, Log 5.8/ml for Gas Colum Method); Pseudomonas fluorescens (Log 6.0/ml for Catalasemeter, Log 6.8/ml for Gas Column Method); Staphylococcus aureus (Log 5.2/ml for Catalasemeter, Log 6.2/ml for Gas Column Method); Bacillus cereus (Log 6.2/ml for Catalasemeter, Log 6.7/ml for Gas Column Method); Escherichia coli (Log 6.8/ml for Catalasemeter, Log 6.9/ml for Gas Column Method); and Salmonella typhimurium (Log 7.9/ml for Catalasemeter, Log 8.2/ml for Gas Column Method).

In this investigation, both catalase methods had the potential to be used to estimate bacterial concentration under certain conditions. Some foods characteristically carry large populations of either catalase-positive or catalase-negative bacteria. By knowing the nature of the food or the test sample one can make use of catalase activities as a means of estimating bacterial populations, although the sensitivity of both methods needs to be improved before they can be actually applied on food samples.

Interference of Blood Catalase on Both Catalase Methods

For practical study of catalase activities in foods we monitored the psychrotrophic bacterial load in 44 cold stored (7° C) chicken wings. Figure 18 shows the logarithm of colony forming unit against logarithm of flotation time (second) in Catalasemeter with paper disc method. Figure 19 shows the logarithm of colony forming unit against logarithm of percentage of gas column in Gas Column Method. The Catalasemeter data showed a good negative correlation ($r = -0.64$, $p<0.01$) whereas the Gas Column method provided a reasonable positive correlation ($r = 0.57$, $p<0.01$). In both figures, some samples were examined which had bacterial numbers lower than $10^4/cm^2$ but possessed a certain amount of catalase activity (Fig. 18, and 19). The observation of low bacterial count with

Figure 18. Catalase Activity of Bacterial Samples from Cold Stored (7°C) Chicken by Catalasemeter with Paper Disc Method.

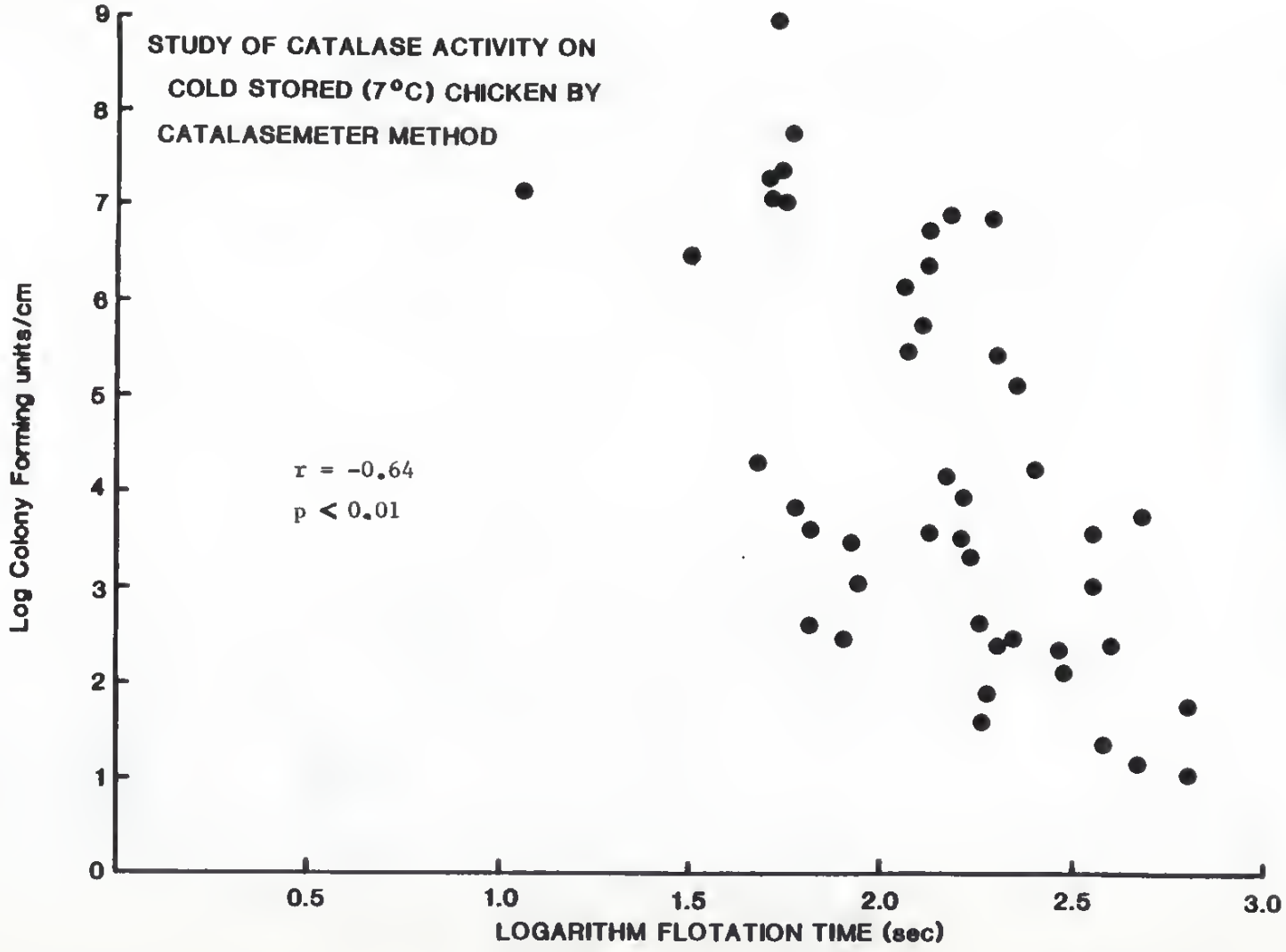
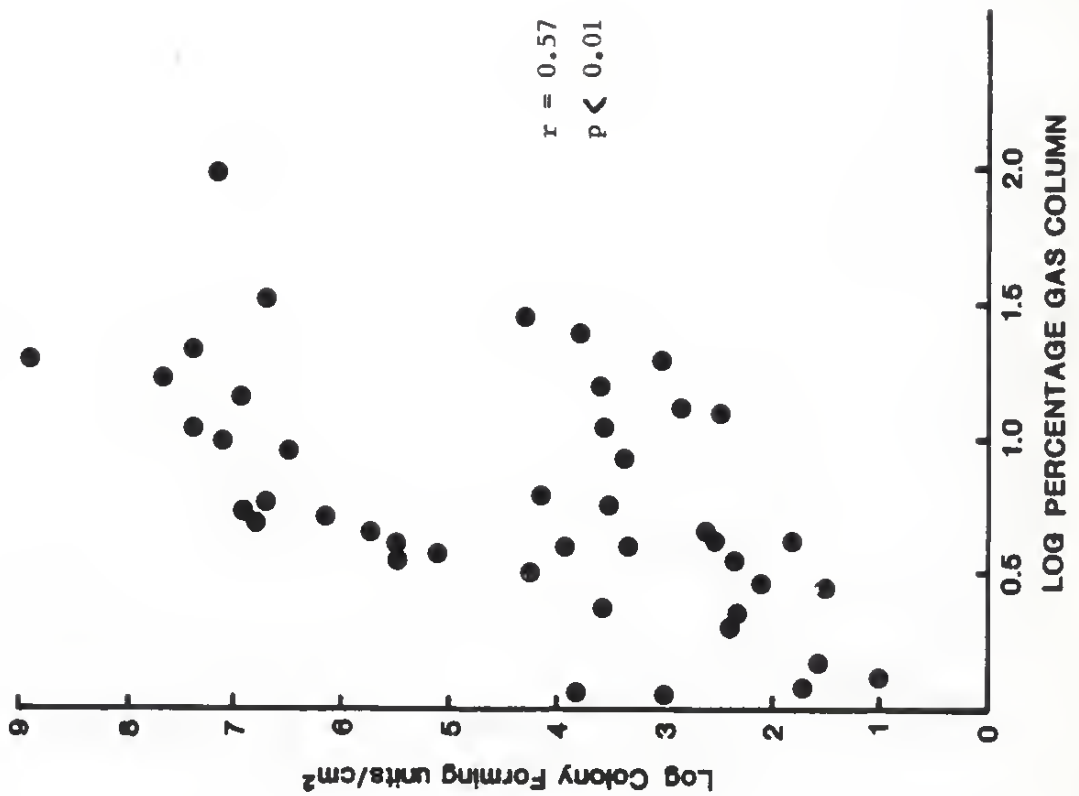

STUDY OF CATALASE ACTIVITY ON
COLD STORED (7°C) CHICKEN BY
CATALASEMETER METHOD
r = -0.64
p < 0.01
Log Colony Forming units/cm
LOGARITHM FLOTATION TIME (sec)
0
1
2
3
4
5
6
7
8
9
0.5
1.0
1.5
2.0
2.5
3.0

Figure 19. Catalase Activity of Bacterial Samples from Cold Stored (7°C) Chicken by Gas Column Method.


r = 0.57
p < 0.01
Log Colony Forming units/cm²
0
1
2
3
4
5
6
7
8
9
0.5
1.0
1.5
2.0
LOG PERCENTAGE GAS COLUMN

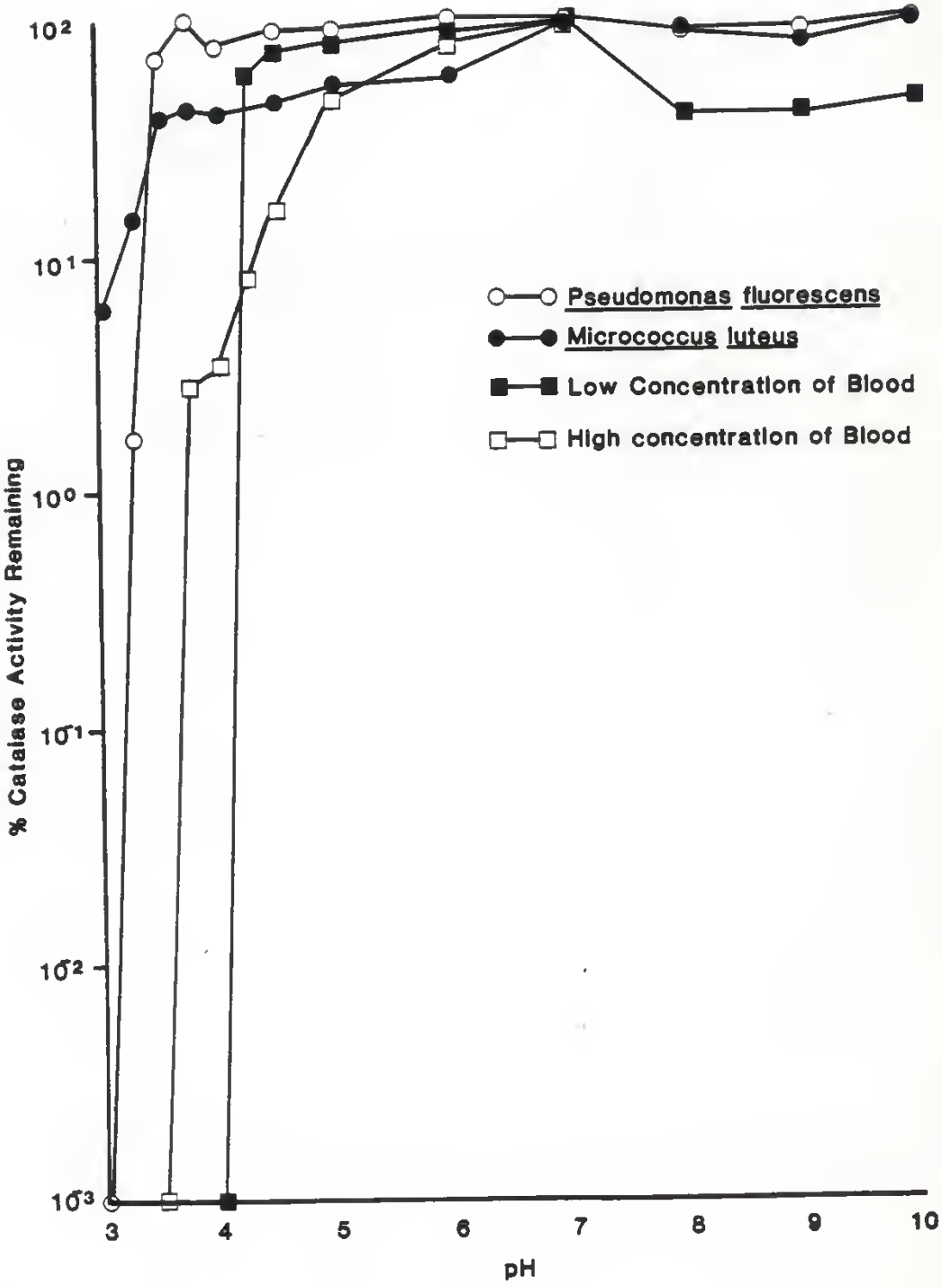
high catalase activity was in disagreement with the experimental results obtained from studying the catalase activity in a pure culture. The Micrococcus spp. was one of the strongest catalase producing bacteria and could only be detected when cell number was higher than 10^4 cell/ml. The interference from blood catalase was suspected, since chicken is subjected to contamination with blood on the surface during the slaughtering process. Two different patterns of catalase activity were observed by the Gas Column Method, which also indicated interference from blood catalase. Heterogeneity of natural flora in foods will also contribute to the difficulty in applying both catalase methods into practical usage. In order for the catalase method to be practical, one must be able to differentiate bacterial catalase against non-bacterial catalase and the nature of dorminant flora in food samples should also be understood.

Effect of pH on Bovine Blood Catalase and Bacterial Catalase Activity

By Catalasemeter with Paper Disc Method

Phosphate buffers (0.05M) with pH ranging from 3 to 10 were used to test two different concentrations of bovine's blood catalase and bacterial catalase. Micrococcus luteus and Pseudomonas fluorescens were compared because they are psychrotrophs and strong catalase producers. Figure 20 shows that the medium concentration of blood catalase (approximately 10^0 catalase units/ml) is inhibited at pH 4.0 and high concentration of blood catalase (approximately 10^3 catalase units/ml) is suppressed at pH 3.5. Catalase activity of Pseudomonas fluorescens is slowly decreasing along with descending pH, but still can be detected at pH 3.25, whereas Micrococcus luteus retains most of its activity even at pH 3.0. High alkaline environment showed no significant effect on both types of catalase activities. This observation shows that bacterial catalase is more resistant than bovine blood catalase to acidic environment. This characteristic can be used to

Figure 20. Inhibition Effect of Blood and Bacterial Catalase Activity in Different pH Environments by Catalasemeter with Paper Disc Method.


% Catalase Activity Remaining
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
3
4
5
6
7
8
9
10
pH
Pseudomonas fluorescens
Micrococcus luteus
Low Concentration of Blood
High concentration of Blood

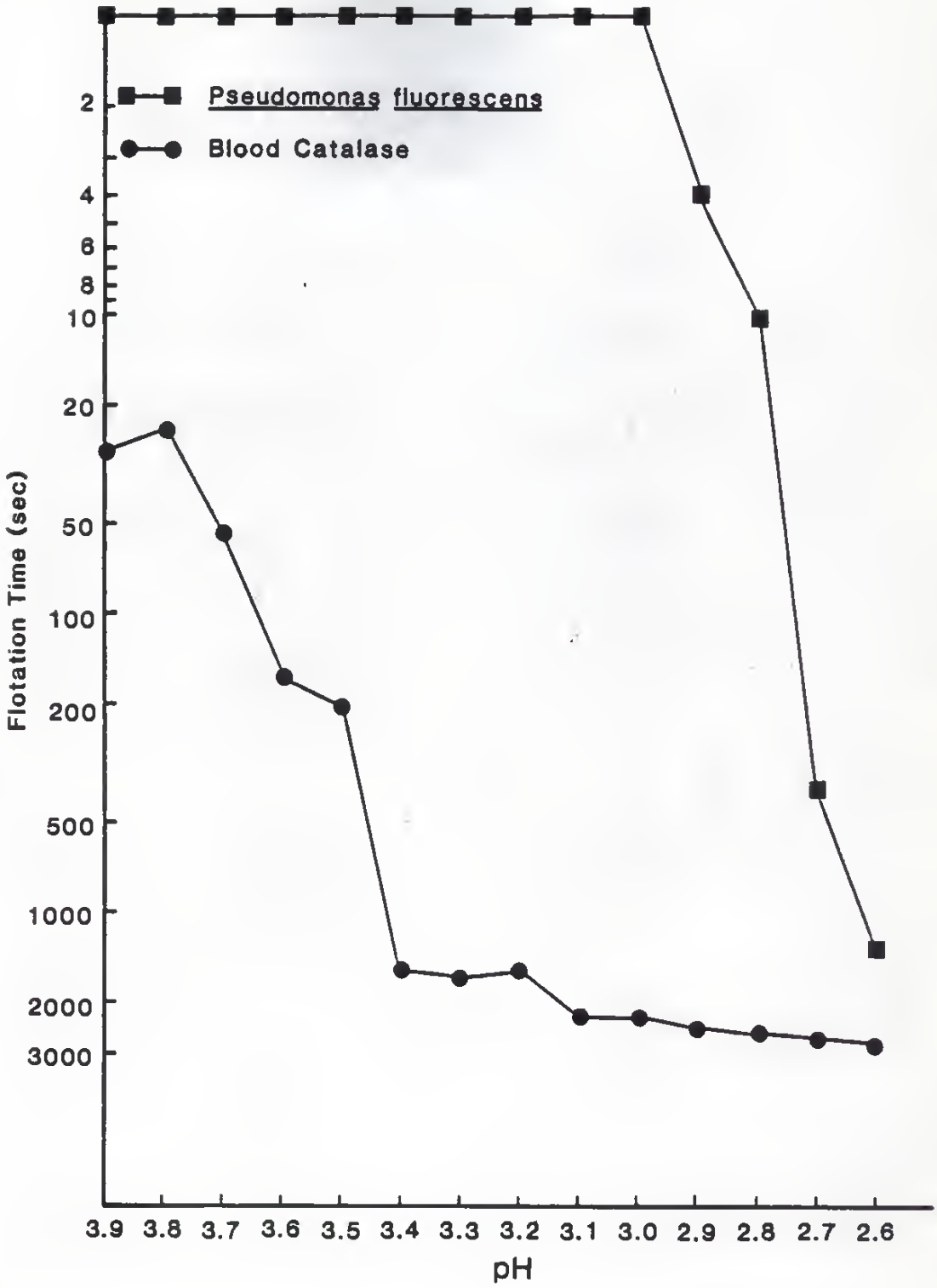
differentiate bacterial catalase from blood catalase in monitoring bacterial loads on meat. Since the acidified treatment not only can suppress the blood catalase activity but also the bacterial catalase activity, sensitivity of both Gas Column Method and Catalasemeter with paper disc method were affected. Improvement of the sensitivity of Catalasemeter Method was done by using a 13 mm 0.45 μm pore size membrane filter instead of the paper disc.

By Catalasemeter with Membrane Filter Method

A membrane filter is uniform in pore size, thin and able to trap particles which are larger than its pore size. These characteristics overcome some of the disadvantages of paper disc such as lack of uniformity, limited sample size and requiring more gas bubbles to float. Figure 21 shows that by introducing this membrane filter into Catalasemeter system, not only the bacterial (Pseudomonas fluorescens) catalase activity but also the blood catalase activity can be detected in more acidic environment. This data also pointed out that the blood catalase activity cannot be totally inhibited but only repressed to some extent; when pH is less than 3.30 with about the same activity as the test buffer solution was lowered to pH 2.6. The pH 3.30 is the critical point to suppress the blood catalase least and affect bacterial catalase, hence it was chosen for further experimentation.

Previous reports on the inhibition of catalase activity in an acidic environment involved purified or chemically defined catalase. No report has been made to study how to differentiate the crude blood catalase and bacterial catalase in acidic environment. Possible explanation of this observation is that the bacterial cell wall is able to resist environmental changes to some extent to protect the catalase which exists in the plasma; while the erythrocyte had only the fragile, semipermeable cell membrane which fails to effectively protect the catalase in the acidic environment.

Figure 21. Blood and Bacterial Catalase Activity in Acidic Environments by Catalasemeter with Membrane Filter Method.


Pseudomonas fluorescens
Blood Catalase
Flotation Time (sec)
2
4
6
8
10
20
50
100
200
500
1000
2000
3000
3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6
pH

The possible mechanism of acidified treatment that decreased both types of catalase activity was the dissociation of intact catalase (tetramer) into subunits of catalase (dimer or monomer) first proposed by Jones and Suggett (1968). This dissociation reaction was increased along with increasing acidified treatment. Both the dimer and monomer subunits of catalase can also decompose hydrogen peroxide into water and release oxygen but with a relatively slow reaction rate, which was reported in the discovery of catalase-hydrogen peroxide compound II and III. This phenomenon was observed in the study of blood catalase activity in the acidified sample by membrane filter. The activity of blood catalase was suppressed to some extent but could not be totally inhibited in test conditions because these subunits (dimer or monomer) of catalase still can react with hydrogen peroxide, but with much slower rates.

Viability of Psychrotrophs in Acidified Samples

Table 1 shows the viable cell count of Pseudomonas fluorescens put in pH 4.00 and 3.30 in 0.05 M phosphate buffer for 0, 5 and 10 minutes. The pH value of 3.30 was attained by adding 1 ml of swab buffer solution (approximate pH 6.75) into 4 ml of 0.05 M pH 3.25 phosphate buffer, giving a final pH for the mixture of about 3.30. The number of Pseudomonas fluorescens did not change at 0 and 5 min and was slightly decreased at 10 min in pH 4.00 phosphate buffer but was strongly decreased in pH 3.30 phosphate buffer even at 0 time for which the viable cell count was made immediately after mixing. This result suggested that pH 3.30 which was required to suppress the blood catalase activity could not be used directly as swab solution for viable cell count. This could be resolved by first taking an aliquot of the swab for viable cell count then acidifying the remaining sample for catalase activity measurement.

Table 1. Viability of Pseudomonas fluorescens in 0.05M pH 4.00 and pH 3.30 Phosphate Buffer

pH	Time (min)	Viable Cell Counted/ml
Control		4.2×10^8
4.00	0	4.4×10^8
	5	5.0×10^8
	10	3.5×10^8
3.30	0	2.2×10^6
	5	1.7×10^6
	10	1.3×10^6

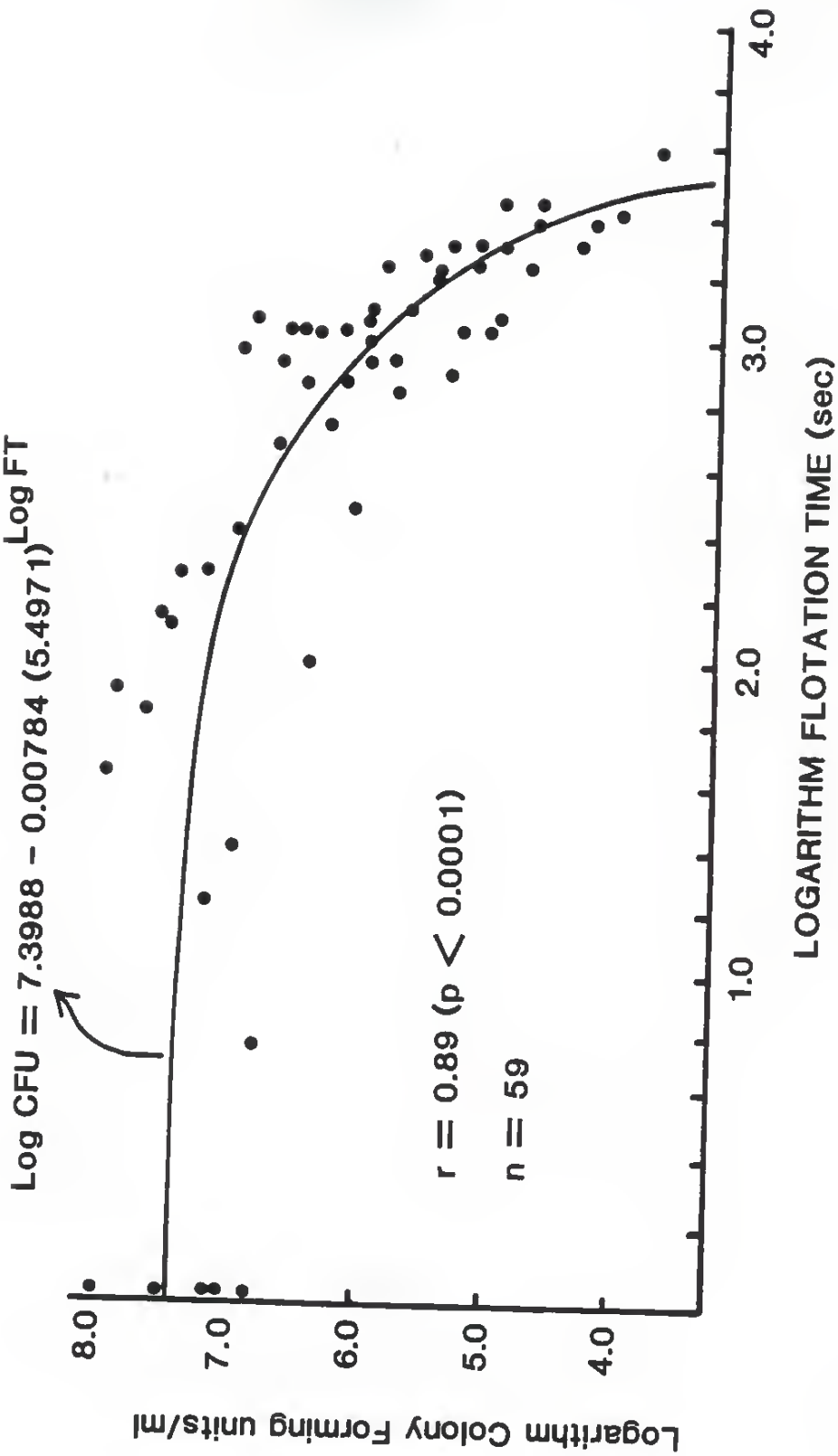
Catalase Activity of Selected Psychrotrophs and Cold Stored Chicken in Acidified Samples by Catalasemeter with Membrane Filter Method

Catalase Activity of Selected Psychrotrophs

Figure 22 shows the relationship between logarithm of flotation time and the logarithm of colony forming units/ml of five selected psychrotrophs. Among these bacteria, Micrococcus luteus had the strongest catalase activity, while four different strains of Pseudomonas had similar but not identical catalase activity. By using the membrane filter instead of paper disc, the sensitivity of Catalasemeter was highly increased and overcame the disadvantages of acidified treatment which was required to decrease the bovine blood catalase activity but also affected bacterial catalase activity to some extent. The detectable limit of this system for each selected psychrotroph was as follows : Micrococcus luteus (Log 3.8/ml), Pseudonomas aeruginosa (Log 4.7/ml), P. fluorescens (Log 4.7/ml), P. fragi 2F-36 (Log 5.0/ml) and P. maltophilia (Log 3.4/ml). Statistical analysis of the bacterial catalase activity to build the standard curve was done by non-linear model. The correlation coefficient for the prediction value (obtained from the standard formula) against observed value is $r = 0.89$ ($p<0.0001$).

In comparision, the membrane filter gave more sensitive results than the paper disc method. Paper disc can only absorb about 0.1 ml of test solution hence can only reveal about 1/10 of the catalase activity of test solution. Membrane filter had no limit on the ability to concentrate the test solution, hence increased times of activity of the test solution depended on how the test solution been filtered.

Figure 22. Catalase Activity of Five Selected Psychrotrophs in pH 3.30 Phosphate Buffer Using Catalasemeter with Membrane Filter Method.


Log CFU = 7.3988 – 0.00784 (5.4971)^Log FT
r = 0.89 (p < 0.0001)
n = 59
Logarithm Colony Forming units/ml
8.0
7.0
6.0
5.0
4.0
1.0
2.0
3.0
4.0
LOGARITHM FLOTATION TIME (sec)

Catalase Activity of Cold Stored Chicken

Figures 23 and 24 show the relationship between logarithm of flotation time and the logarithm of colony forming units/cm^2 of viable cell count (32°C, 24 hr) and modified psychrotroph count (21°C, 23-25 hr) from 59 cold (7°C) stored chicken samples, respectively. A typical sigmoidal curve pattern is observed on both figures. The detectable limit in this methodology is about 1.6 x $10^3/cm^2$ for viable cell count and 4.0 x $10^3/cm^2$ for modified psychrotroph count. Statistical analysis of both sets of data to build the standard curve was made. The correlation coefficient between the prediction value against observed value for both the population of viable cell and modified psychrotroph was $r = 0.93$ ($p<0.0001$).

In comparison, the catalasemeter with paper disc method without acidified pre-treatment (Fig. 18) and the catalasemeter with membrane filter method with acidified pre-treatment (Fig. 23, 24); acidified treatment gave a satisfactory effect in suppressing the blood catalase activity.

In some instances high catalase activity was observed when bacterial concentration was low in cold stored chicken samples (Fig. 23 and 24) but not on the five selected psychrotrophs (Fig. 22). Possible explanations for the low bacterial concentration with a somewhat higher catalase activity from chicken samples compared with the laboratory cultures are 1). the remaining blood catalase tends to elevate the catalase activity when the chicken is still fresh; and 2). bacteria in the presence of heme precusor or heme products may increase the catalase production (Marie and Parak, 1980). The scattering effect observed at high bacterial concentrations on cold stored chicken sample is probably due to food particles that were decomposed by the bacteria and then existed in swab solution. These deteriorated food particles could block part of the membrane filter and make the filtering process much difficult to achieve. When these particles were trapped on membrane filter, the weight of membrane filter would increase in an

Figure 23. Catalase Activity of Viable Cell Population from Cold Stored (7°C) Chicken Samples in pH 3.30 Phosphate Buffer Using Catalasemeter with Membrane Filter Method.

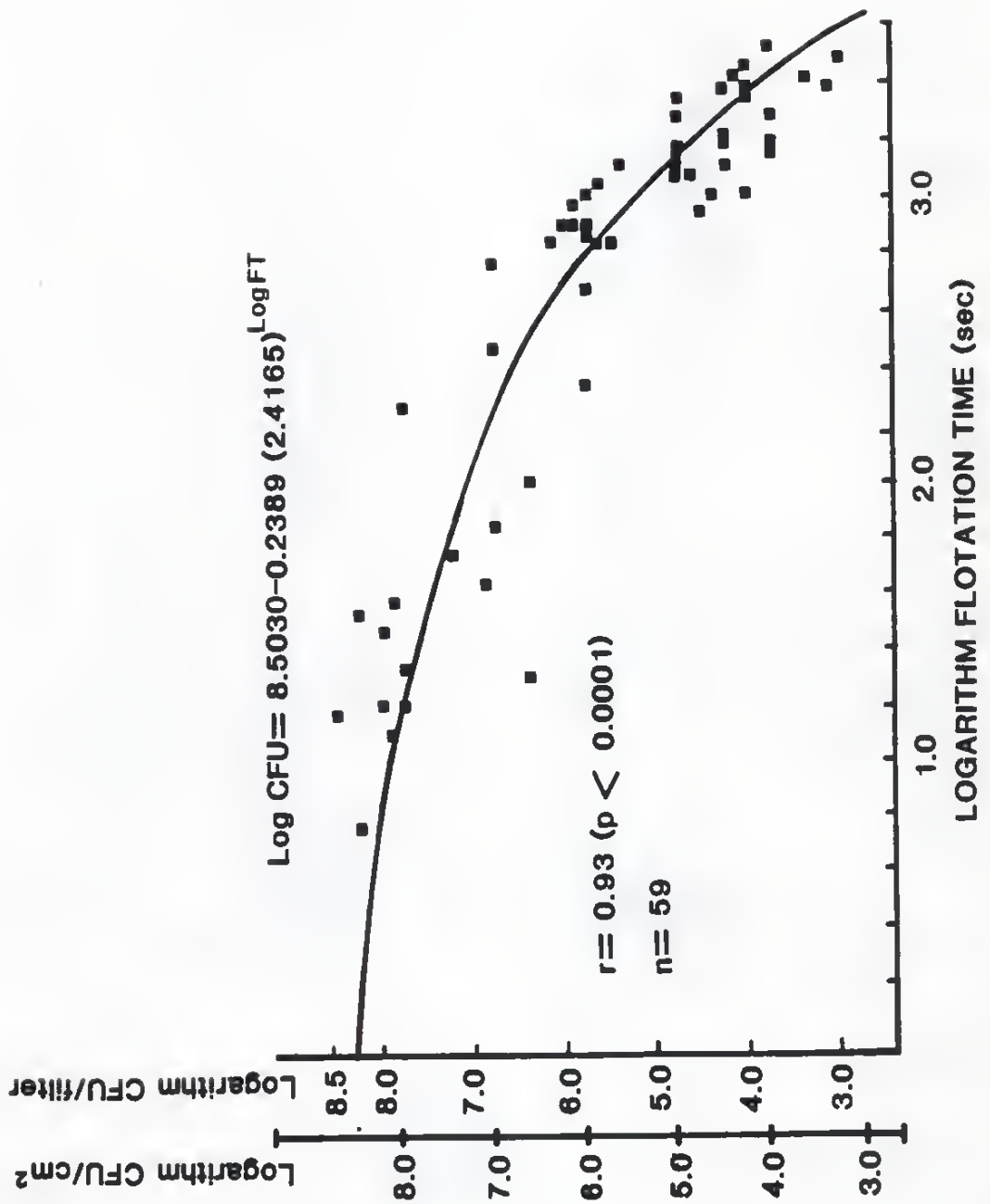
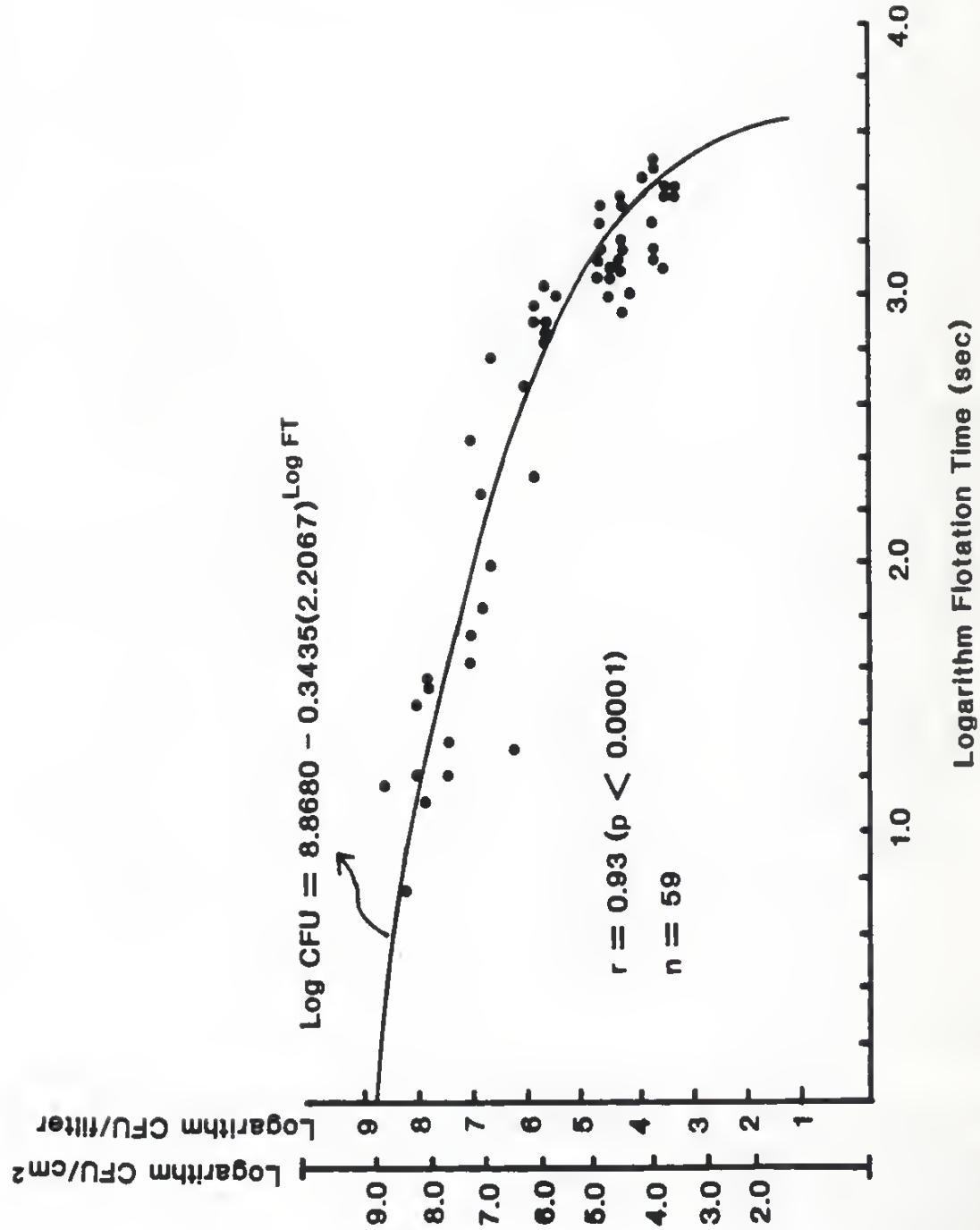

Log CFU= 8.5030–0.2389 (2.4165)^LogFT
r= 0.93 (p < 0.0001)
n= 59
LOGARITHM FLOTATION TIME (sec)
1.0
2.0
3.0
Logarithm CFU/cm²
Logarithm CFU/filter
8.5
8.0
7.0
6.0
5.0
4.0
3.0

Figure 24. Catalase Activity of Psychrotroph Population from Cold Stored (7°C) Chicken Samples in pH 3.30 Phosphate Buffer Using Catalasemeter with Membrane Filter Method.


Log CFU = 8.8680 – 0.3435(2.2067)^Log FT
r = 0.93 (p < 0.0001)
n = 59
Logarithm CFU/cm2
Logarithm CFU/filter
Logarithm Flotation Time (sec)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
9
8
7
6
5
4
3
2
1
1.0
2.0
3.0
4.0

irregular fashion thus requiring more gas bubbles to float it and causing the data scattering.

Statistical Analysis

A non-linear model was used to analyze the relationship between log colony forming units/filter (log CFU) and log flotation time (log FT) obtained from the empirical data of the 59 chicken samples for both population of viable cell and modified psychrotrophs and five selected psychrotrophs. Relationship between log CFU and log FT for three sets of data is shown below :

1. Selected Psychrotrophs

   $\text{Log CFU/ml} = 7.3988 - 0.0084\ (5.4971)^{\log FT}$

2. Viable Cell Count of 59 Chicken Sample

   $\text{Log CFU/filter} = 8.5031 - 0.2389\ (2.4165)^{\log FT}$

3. Modified Psychrotroph Count of 59 Chicken Sample

   $\text{Log CFU/filter} = 8.8680 - 0.3435\ (2.2067)^{\log FT}$

It was found that linear relationships can be obtained by transforming these non-linear equations in such a fashion as Log (A' - Log CFU) = Log B' + C' x (Log FT). By this transformation, these non-linear equations were translated into linear ones as shown below :

1. Selected Psychrotrophs

   $Y_0 = \log (9.0 - \log \text{CFU/ml}) = (0.2300) + 0.2981\ (\log FT)$

2. Viable Cell Count of 59 Chicken Samples

   $Y_1 = \log (9.0 - \log \text{CFU/filter}) = (-0.8112) + 0.7206\ (\log FT)$

3. Modified Psychrotroph Count of 59 Chicken Samples

   $Y_2 = \log (9.0 - \log \text{CFU/filter}) = (-1.1094) + 0.8129\ (\log FT)$

The purpose of this transformation is to ease the complicated formula of the non-linear model for practical in-plant usuage. After the transformation, the correlation coefficients are $r = 0.72$ ($p<0.0001$) for selected psychrotrophs (Fig. 25); $r = 0.91$ ($p<0.0001$) for viable cell count (Fig. 26) and $r = 0.92$ ($p<0.0001$) for modified psychrotroph count (Fig. 27) from 59 chicken samples. These equations obtained from both linear and non-linear models, can be used by the food microbiologist to estimate the microbial load on chicken sample in a comparatively short time period to enhance the quality control and product utilization procedures.

Figure 25. Relationship Between Observerd and Predicted Value of Selected Psychrotrophs after Linear Transformation.

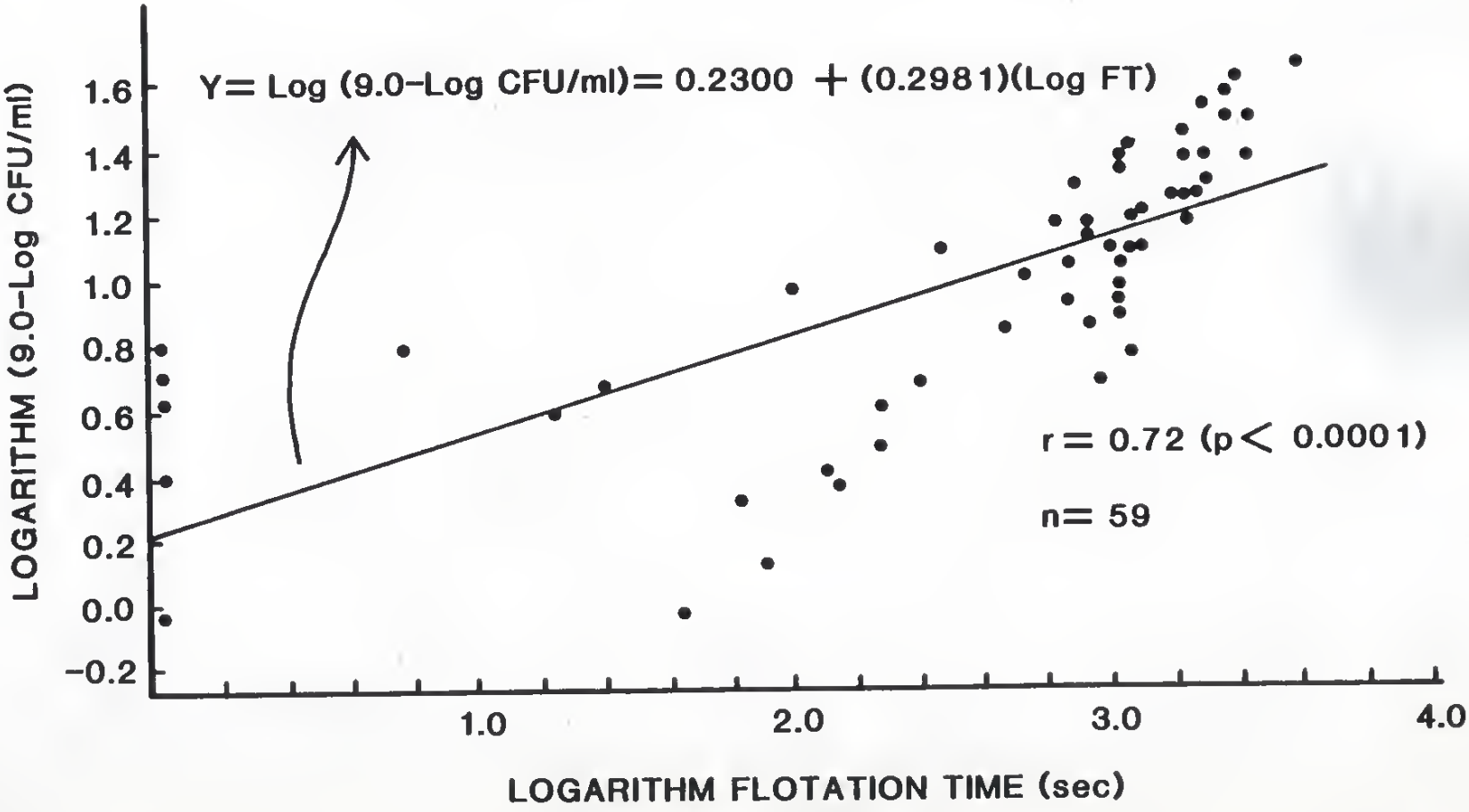

Y= Log (9.0-Log CFU/ml)= 0.2300 + (0.2981)(Log FT)
r= 0.72 (p< 0.0001)
n= 59
LOGARITHM (9.0-Log CFU/ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
1.0
2.0
3.0
4.0
LOGARITHM FLOTATION TIME (sec)

Figure 26. Relationship Between Ovserved and Predicted Value of Viable Cell Population from Cold Stored Chicken after Linear Trasnformation.

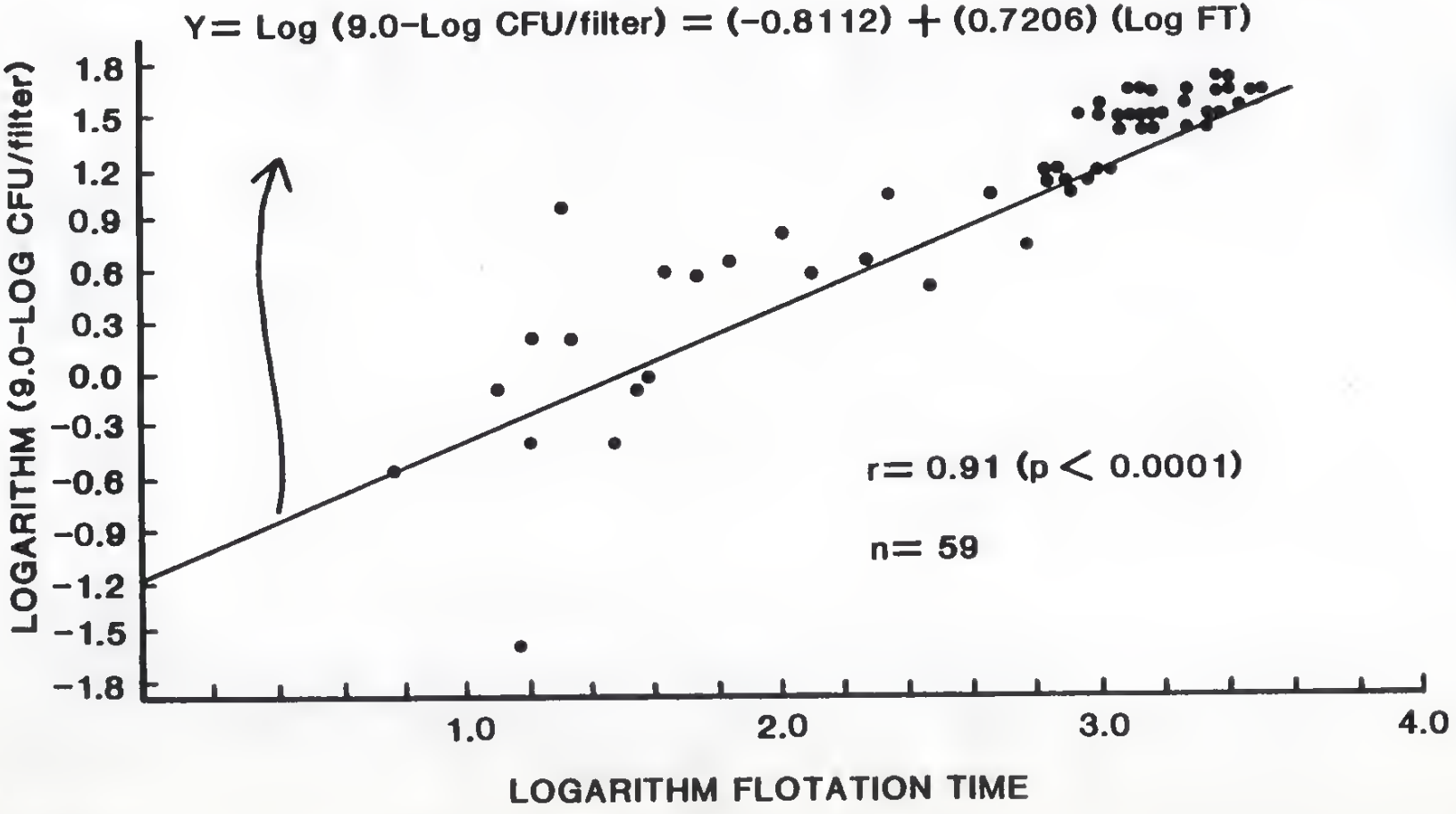
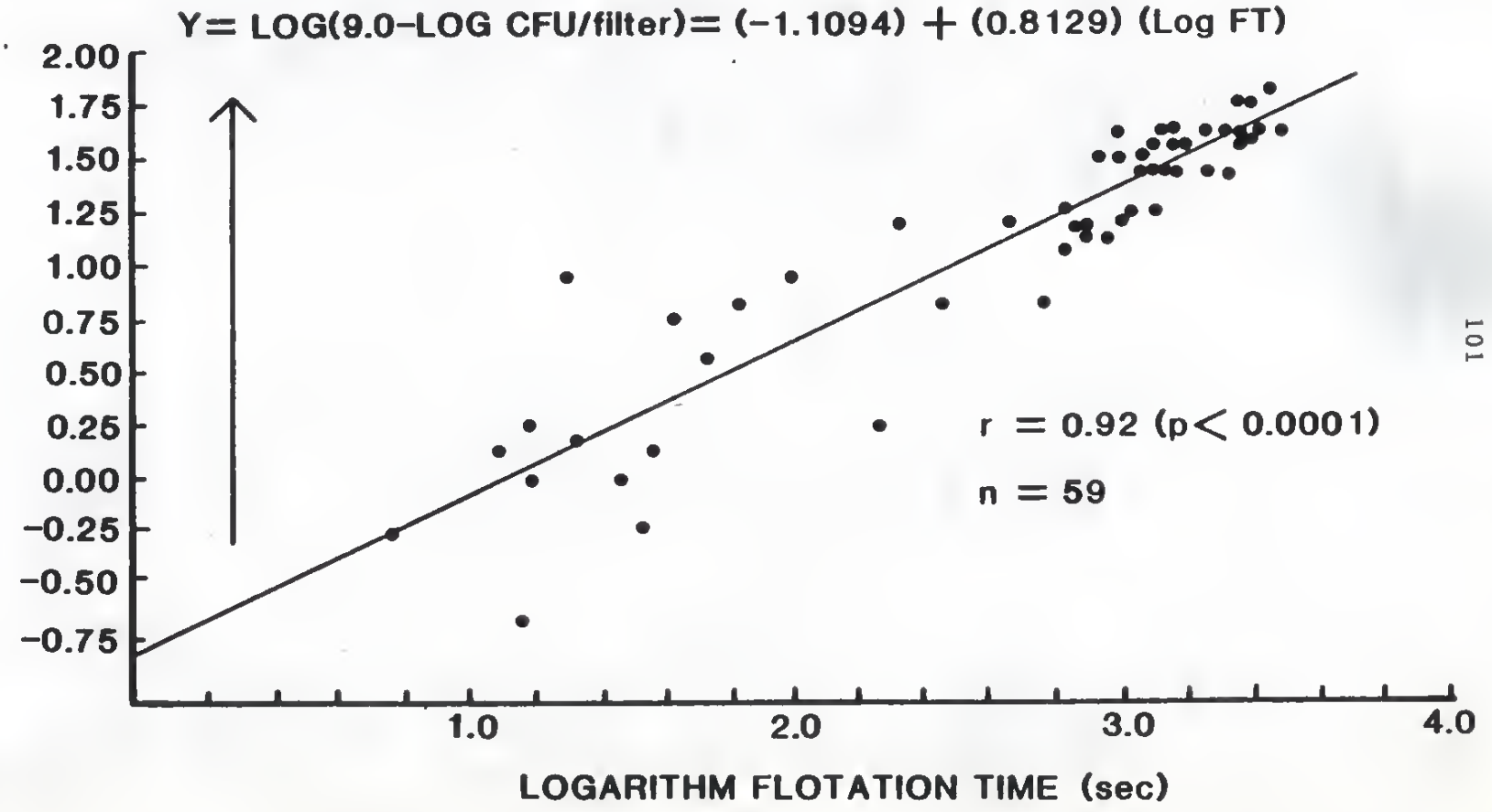

Y= Log (9.0-Log CFU/filter) = (-0.8112) + (0.7206) (Log FT)
LOGARITHM (9.0-LOG CFU/filter)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
-0.3
-0.6
-0.9
-1.2
-1.5
-1.8
r= 0.91 (p < 0.0001)
n= 59
1.0
2.0
3.0
4.0
LOGARITHM FLOTATION TIME

Figure 27. Relationship Between Observed and Predicted Value of Psychrotroph Population from Cold Stored Chicken after Linear Transformation.


Y= LOG(9.0-LOG CFU/filter)= (-1.1094) + (0.8129) (Log FT)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
r = 0.92 (p< 0.0001)
n = 59
1.0
2.0
3.0
4.0
LOGARITHM FLOTATION TIME (sec)

CONCLUSIONS

1. This research presents evidence that it is possible to estimate microbial loads of certain foods by measuring catalase activities.

2. The Gas Column method is a rapid and fairly accurate method to estimate the bacterial load on certain food sample. Although the range of detectable catalase concentration was limited from 0.06 to 0.9 catalase units/capillary tube, this method is easy to perform, flexible, rapid (gives the reading in 15 min), and saves both materials and space.

3. Catalasemeter with paper disc method posseses a wider detection range as compared with Gas Column method. In the study with purified catalase from 10^{4} and 10^{-4} catalase units/ml, there was concomitant decrease in flotation time as the concentration of catalase increased. Although the enzyme activity was more sigmoidal than straight line, the overall correlation coefficient for the linear relationship was $r = -0.97$ ($p<0.01$).

4. In the study of catalase producing ability of eight species of both catalase positive and catalase negative bacteria by both Gas Column method and Catalasemeter with paper disc method, different species of catalase positive bacteria had different catalase producing ability. Catalase negative bacteria gave no response in both Gas Column method and Catalasemeter with the paper disc method. Among the bacteria tested, Micrococcus luteus had the strongest catalase producing ability, Staphylococcus aureus and Pseudomonas fluorescens were the second, Escherichia coli and Bacillus cereus were next and Salmonella typhimurium was the least catalase producing organism.

5. Activity of bacterial catalase and bovine blood catalase was measured from pH 3 to 10 by Catalasemeter with paper disc method. Lowering the pH to less than 4.0 can differentiate blood from bacterial catalase. High alkaline environment showed little effect on both bacterial and bovine blood catalase activity. Using the membrane filter instead of paper disc in Catalasemeter method to further examine the effect of acidic environment (pH 4.0 to 2.6) on both catalase activities, showed that lowering pH to 3.30 can suppress the bovine blood catalase, while having little affect on bacterial catalase.

6. Study of five selected psychrotrophs by Catalasemeter with membrane filter method after acidified treatment showed a typical sigmodial pattern. Within these five tested bacteria, the catalase producing ability was about the same. In the upper part of the curve, larger variance was observed when bacterial population was higher than 10^6 per ml; in the lower part of the curve, the variance was relatively smaller. The non-linear correlation coefficient was $r = 0.89$ between the prediction value and observation value.

7. In the study of 59 cold (7°C) stored chicken samples, typical sigmoidal patterns were also observed for both viable cell count and modified psychrotroph count. The upper part of the curve had a larger variance than the lower part, especially for those organisms having counts larger than 10^7/filter in both population (viable cell count and modified psychrotroph count). The non-linear correlation coefficient for both curve was $r = 0.93$ between the prediction value and observation value.

8. Transformation of the non-linear equations of five selected psychrotrophs, viable cell count and modified psychrotroph count from 59 cold

stored chicken samples was made in order to ease and facilitate the application of in-plant usage of this method. Correlation coefficients of these non-linear equation between predicted and observed cell number/filter were $r = 0.72$ ($p<0.0001$) for five selected psychrotroph, $r = 0.91$ ($p<0.0001$) for viable cell count and $r = 0.92$ ($p<0.0001$) for modified psychrotroph count from cold stored chicken samples.

9. The surface microbiological quality of chicken can be estimated in mins by using the Catalasemeter method with membrane filter. Psychrotroph population from chicken samples were log 8-9 CFU/cm^2 (extremely high counts) with a flotation time of less than 27 sec; when the flotation time was less than 185 sec, bacteria count were log 7-8 CFU/cm^2 (very high counts); when the flotation time was less than 584 sec, the bacterial counts were log 6-7 CFU/cm^2 (high count); when the flotation time was less than 1326 sec, the bacterial counts were log 5-6 CFU/cm^2; when the flotation time is less than 2515 sec, the bacterial counts were log 4-5 CFU/cm^2; and when the flotation time is longer than 4246 sec, the bacterial counts were less than log 3 CFU/cm^2 (very low count).

10. Catalasemeter method with membrane filter can be used to estimate the microbial load on certain food with accurate and rapid results. This method also has some other advantages such as flexibility, ease of performance and savings of materials and space.

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ACKNOWLEDGEMENTS

The author expresses his thanks and appreciation to Dr. Daniel Y. C. Fung, the major professor, for his suggestion, encouragement, guidance and help throughout his study at this period. Without his untiring patience and advice, this work would not have been accomplished.

Sincere acknowledgement is also expressed to Dr. Ike J. Jeon and Dr. Donald H. Kropf for their serving on the supervisory committee. Without their instruction and precious opinions, this work would never be accomplished. The author is also indebted to Dr. Dallas E. Johnson Department of Statistics, Kansas State University for his suggestions and gracious help on the statistical analysis of the data.

Special thanks also extended to the Food Chemistry and Food Microbiology group in K.S.U. for their friendship and lively discussions.

Thanks are to his parents, Mr. and Mrs. Tien-Fu Wang and his mother-in-law, Mrs. A-Zo Chang for their love and support. Thanks also are to his cousin, Professor and Mrs. Mike and JoAnn Lin for their assistance and support.

Most of all the author wishes to express his gratitude to his truly beloved wife, Yueh-Jau Wang, for her love, understanding, assistance and encouragement during this project at Kansas State University.

FEASIBILITY OF USING CATALASE ACTIVITY AS AN INDEX OF MICROBIAL LOADS ON FOOD SURFACES

by

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B.S., Fu-Jen Catholic University, R.O.C. 1980

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Food Science

Department of Animal Sciences and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1985

ABSTRACT

Preliminary study of two catalase activity tests, Gas Column Method and Catalasemeter Method with paper disc, were made using purified catalase and eight strains of both catalase positive and catalase negative bacteria to test the sensitivity and accuracy of both methods. Both Gas Column method and Catalasemeter methods indicate reasonable responses and show potential to be used to estimate bacterial concentration under certain conditions. For example, cold stored meat characteristically carried large populations of catalase-positive bacteria.

In the study of catalase producing ability of eight species of both catalase positive and catalase negative bacteria by both Gas Column method and Catalasemeter method, different species of catalase positive bacteria had different catalase producing ability, and catalase negative bacteria gave no response in both method. Among the tested bacteria, Micrococcus luteus, Staphylococcus aureus and Pseudomonas fluorescens had high catalase producing ability; Escherichia coli, Bacillus cereus and Salmonella typhimurium had low catalase producing ability.

A strong interference of blood catalase activity was suspected in the preliminary study of 44 cold stored chicken wings. In order to differentiate blood catalase and bacterial catalase activity; both were studied at different pH environments. The environment of pH 3.30 was found to be an adequate pretreatment to suppress the blood catalase and yet had the least effect on bacterial catalase activity.

The sensitivity of the Catalasemeter with paper disc method was increased by using the membrane filter (13 mm diameter with 0.45μm pore size) instead of the filter paper disc. Cold stored (7°C) chicken parts and five selected psychrotrophs were used to study the catalase activity after acidified treatment, by the Catalasemeter with membrane filter method. A comparative study of viable

cell count (for both chicken and selected bacteria) and modified psychrotroph count was performed against catalase activities. All correlation coefficients relating logarithm of flotation time (sec) to logarithm of colony forming units/filter for both viable cell count and modified psychrotroph count ranged from $r = 0.89$ to $r = 0.93$. ($p<0.0001$)

The catalasemeter method provided a guideline for rapidly estimating microbial loads in cold stored chicken to indicate their suitability for sale or further processing. Using the Catalasemeter with membrane filter, less than 20 minutes are needed to estimate microbial loads, while 24-48 hr are required for results using the standard plate count procedure.