Study of intracellular proteinases of some bacteria
by Dharam Vir Vadehra

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
MASTER OF SCIENCE in Dairy Manufacturing
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Abstract:
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Optimum pH for the various enzymes was found to be within the range of 6.0 to 7.0, although S. lactis showed a second peak at pH 5.5. ' The age of the cells was found to be a factor and proteinases production increased as the age of the cell increased up to 96 hours, Cells 144 hours old showed a decrease in proteinase production.

The addition of gelatin and casein to the medium used to grow the organisms, increased the proteolytic activity of the intracellular enzyme system, while the addition of Casitone (Difco) had no effect. The absence of carbohydrates from the growth medium also increased the proteolytic activity of these enzymes.

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ABSTRACT

Seven organisms (*Streptococcus lactis*, *Leuconostoc dextranicum*, *Lactobacillus casei*, *Proteus vulgaris*, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Staphylococcus aureus*) were selected and grown on liquid media. From these organisms the intracellular enzymes were separated and studied for their proteolytic activity on casein and/or whey protein substrates. The proteolysis was followed by the Folin-Ciocalteu method, with the results being expressed in micrograms of tyrosine and tryptophane.

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INTRODUCTION

Fermentations have been studied for a long time. Some fermentations, for example, the production of alcohol by yeast are well understood. Others such as cheese ripening are still somewhat of a mystery, although extensive work has been done in the area.

Quite a body of literature is available on the role played by such things as rennet and bacteria in cheese ripening. The rennet studies have been confined largely to the factors affecting the coagulation of milk, along with the advancement of some theories as to its role in the ripening process. The role played by bacteria in the fermentation of cheese has been studied primarily from the standpoint of bacterial populations at various stages of ripening and the resulting pH changes. These factors have been evaluated on the basis of organoleptic determinations of flavor and body and texture. Body changes have also been measured by such chemical methods as determinations of water soluble nitrogen, protease and peptide contents.

Bacterial enzymes have not been studied extensively but have been shown to be important. Both extracellular enzymes secreted into the surrounding medium and intracellular enzymes retained in the cell apparently play some part. Extracellular enzymes have been shown to be connected primarily with protein breakdown.

Only a few intracellular enzyme studies have been found in the literature reviewed. These studies have been devoted chiefly to the characterization of protein splitting enzymes and to some of the factors

such as pH, temperature and metal ions affecting their reaction.

In as much as in any fermentation there is a constant turnover of microorganisms i.e. some are dead while others are active vegetative cells, the role of enzymes remaining in the bacterial cells after they have become inactive is of interest and probably important in most fermentation, especially cheese ripening.

The purpose of this study was to investigate the intracellular proteinases of some bacteria commonly found in milk or starter as well as some common milk contaminants. The organisms selected which are commonly found in milk or starter were (1) Streptococcus lactis, (2) Leuconostoc dextranicum or (3) Lactobacillus casei. The common milk contaminants selected were (1) Proteus vulgaris, (2) Bacillus subtilis, (3) Pseudomonas fluorescens and (4) Staphylococcus aureus.

The effect of pH of the substrate; age of the bacterial cell; and composition of the medium used to propagate the cells on proteinase production and/or activity were studied.

REVIEW OF LITERATURE

Enzymes

Enzymes are proteins whose biological function is the catalysis of chemical reactions in living systems (34). Early investigations of enzymes go back to the work of Buchner as cited by Neilands and Stumpf (75) who in 1897 obtained a cell free preparation of yeast capable of fermenting sugars. The work of Harden and Young who demonstrated the role of a heat stable factor or coenzyme in certain reactions was another important contribution (41). The monograph by Harden is a good historical document on the early studies of enzymes (40).

Enzymes were first crystallized in 1926 (90) and by 1956 some 75 enzymes had been so prepared and their properties studied (30). All enzymes isolated to date have been identified as proteins. The monograph of Northop et al. (77) may be of interest.

Hoffmann (49) and Hoffmann and Thomas (50) have reviewed the literature on nomenclature and classification of enzymes. Lamanna and Mallette (61) suggested among other things a classification according to (a) the site of activity, and (b) the conditions governing the occurrence of enzymes, both of which are of interest in the present study.

Relative to the site of activity some enzymes may be limited to the confines of the cell and are called intracellular. Others are found on the surface of the cell and are called ectocellular, while those secreted in the medium supporting growth are known as extracellular. Most organisms exhibit all of these types.

Intracellular enzymes and the influence of medium on enzyme produc-

are of particular interest here.

Lamanna and Mallete (61) point out that the specific catalytic functions of intracellular enzymes are extensive and that these enzymes are responsible for the complete breakdown of the material into forms that can be utilized by the cells. These substances are generally first broken down by the extracellular enzymes in order that they may be transported through the cell wall by permeases.

Relative to conditions governing the occurrence of the enzymes, the role of a substrate of structurally analogous compounds as inducers or stimulators for enzymes is recognized (78). The synthesis of all enzymes is genetically determined but a given enzyme can be constitutive in one strain of organisms and induced in another. The chemical environment of the population determines which enzymatic reactions will occur, but only within the limits set by the genetic competence of the population. Stanier (89), Gale (36) and Spiegelman (88) have reviewed the literature on adaption by bacteria. Their work and the symposium on adaptation in microorganisms (26) may be consulted for details.

Proteolytic enzymes and the fermentation of cheese

The enzymes which hydrolyze proteins were among the first biological catalysts discovered (70). They have also continued to be prominent in the study of enzyme structure, kinetics, activity and mechanism of enzyme action (70). The term protease is usually employed as a general designation of enzymes capable of hydrolyzing peptide linkages. Johnson and Berger (53) point out that two kinds of proteases exist:

(1) Peptides which are capable of splitting off a single amino acid from one end or the other of the peptide chain, and, (2) proteinases which may split a linkage at any position in the chain yielding polypeptides. This phenomenon may influence the measurement of proteolytic activity by some methods. For example, the Folin-Ciocalteu method measures only the two amino acids tyrosine and tryptophane. It does not measure other products such as polypeptides or other amino acids. Figure 1 illustrates this phenomenon.

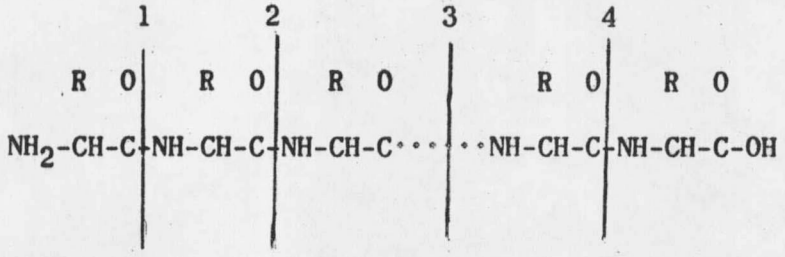

Figure 1. Peptide chain showing N terminal linkage and C terminal linkage and the points of attack by proteolytic enzymes.

Figure 1 shows a peptide chain with N terminal linkage (1) and C terminal linkage (4) which are hydrolyzed by appropriate peptidases and additional peptide bonds (2 and 3) which are hydrolyzed only by proteinases. Bergman (16) and Bergman and Fruton (17) have published detailed accounts of the classification and specificity of proteolytic enzymes.

Cheese ripening is a complex process which has never been fully explained, although, it has been quite extensively studied by chemists and bacteriologists.

During cheese ripening a number of changes take place and all major constituents of milk i.e. protein, fat and lactose undergo some changes (9). All these changes are responsible in some degree at least for the

characteristic body and flavor of cheese.

The chemical changes responsible for transforming the fresh curd into the final cheese are catalyzed by enzymes from three main sources (33): (a) rennet or other enzyme preparations, (b) microorganisms that grow in or on the surface of cheese, and (c) the milk itself.

Rennet is being considered in this review because it plays a part in the protein breakdown and flavor formation of cheese. Also, some of its properties are common to bacterial enzymes and the action of rennet and bacteria are related in that the conditions created by bacterial growth, particularly pH, influence the action of rennet (5,10). Also it is possible, although it has not been demonstrated, that rennet may serve somewhat the same function as extracellular enzymes of bacteria. It has been shown conclusively that rennin is a protease (18,80) and various authors (1,10,95) have noted that increasing amounts of rennet cause an increase in soluble nitrogenous products produced.

The survival of the protease of rennet during heating in milk has been studied by Pelotola and Autila (80). Amunstad (5) concluded that the proteases of rennet are able to act at a lower pH than those of bacteria especially in the presence of sodium chloride and therefore they continue proteolysis after the bacterial enzymes have ceased functioning. The reviews by Berridge (18), Aschaffenburg and Ling (7) and Aschaffenburg and Rowland (8) should be consulted for detailed treatment of the subject of the relationship of rennet to cheese making.

Role of bacteria in the ripening of cheese

Streptococcus lactis is the first species to bring about an important

change during the making and ripening of cheese (55,96). Lactic acid thus produced is important both in the manufacture and the ripening of cheese. It makes conditions favorable for the curdling of milk with rennet; the explusion of whey from the curd and the fusion of the curd particles (37). Hasting et al. (48) have shown that the organisms are concentrated in the curd after the coagulation of the milk. Milroy (71) pointed out that acid production also favors the proteolytic action of rennet extract.

The results of earlier workers (48,95) show that from the standpoint of numbers S. lactis is important in the early ripening period and lactobacilli are dominant later. However, recent studies by English workers using special media have shown that lactobacilli grow slowly but steadily from the beginning of ripening period.(65,73,85). Allen and Knowles (4) and Lane and Hammer (62) found that Lactobacillus casei added to pasturized milk made into cheese produced more rapid and extensive decomposition of protein than in cheese which was, they claim, devoid of lactobacilli.

The lactobacillus flora of Cheddar cheese is not usually added to the milk but apparently is derived from milk or may arise as a contaminant from air and other sources (74,81). Little is known of the part played by S. lactis in making conditions favorable for the growth of lactobacilli, however, associative growth of these bacteria has been demonstrated (31). Marshall (69) showed the importance of bacterial association in the souring of raw milk as early as 1903 and found that many bacteria commonly occurring in milk stimulated the growth and

fermenting activity of lactic acid bacteria. Nurmikko (76) showed that different strains of lactic acid bacteria could grow in symbiosis in a synthetic medium incapable of supporting the growth of either strain in pure culture. Dahiya and Speck (23) have more recently studied the symbiosis among lactic streptococci. They found that a combination of isolates from mixed strains of lactic streptococci showed different interaction when grown on milk. The interaction between the strains could not be predicted from the growth rate of the individuals isolated. Hansen (38) showed that the stimulation of L. casei by S. lactis or S. cremoris may play a role in the ripening of cheese in which the two latter species develop, die, disintegrate and are followed by L. casei.

The knowledge regarding the intracellular enzymes of the bacteria concerned with cheese ripening is not extensive, though it has been pointed out that they are important in cheese ripening and flavor development (9,33,37).

Van Der Zant and Nelson (93) reported that a cell free culture medium of S. lactis did not show proteolytic activity, however the intracellular enzymes of S. lactis showed a rapid increase in both soluble nitrogen and tyrosine and tryptophane when acted on casein and lactalbumin. In another study the same authors (94) have shown the presence of a heat labile proteolytic enzyme in the cell free extracts of S. lactis. They also reported that optimum activity against milk, casein and lactalbumin is near neutrality.

Rabin (84) has studied some of the factors influencing the formation of proteinases in S. liquefaciens, such as pH, essential amino acids and

the need for a water soluble synthetic medium.

Baribo and Foster (14) have published some of the characteristics of the intracellular proteinases of one strain each of L. casei, S. lactis, and Micrococcus freudenreichii. The microbial enzymes were compared with those of proteolytic enzymes extracted from one year old cheese. They concluded that the organisms used in their study possessed enzymes which could account for only a part of proteinases found in cheese and that enzymes from other sources are necessary to account for the remainder.

Kristofferson and Cole (59) reported that pasturization inactivates the milk enzymes and delays cheese ripening and flavor development.

Attempts to accelerate cheese ripening

Various attempts have been made to reduce the ripening period for cheese.

Hansen (39) noted that the addition of 0.05% of a milk culture of S. liquefaciens or 0.5% of a milk culture of an unidentified Micrococcus to pasteurized milk improved the flavor of cheese made from it, but it did not materially influence the nitrogenous decomposition. Harris and Hammer (46) found that out of 34 Micrococcus cultures, seven had an undesirable effect, 14 no effect and 13 had a desirable effect when inoculated into pasteurized milk for cheese making.

Dahlberg and Kosikowski (24) isolated a strain of S. faecalis that fermented lactose rapidly and they advocated its use as a starter organism. Another investigation (27) in which S. liquefaciens was used resulted in pronounced bitterness in cheese.

The use of special proteolytic enzymes (pepsin, trypsin) in cheese

manufacture and their effect from the standpoint of accelerated ripening has been studied by Freeman and Dahle (35). Babel et al., (12) used an enzyme preparation from chicken proventriculi to coagulate milk and used a modified Edam process in the making of a new variety of cheese (Savoureux).

Kristoffersen has pointed out in a recent symposium on flavor chemistry (58) that the work done so far would indicate that we can only shorten the ripening period at the cost of flavor.

Contribution of bacteria to the development of flavor

Mabbitt (64) points out that the known metabolic products of different species of homofermentive lactic acid bacteria which occur in cheese such as streptococci, lactobacilli and pediococci are so qualitatively similar that it is difficult on this basis to suppose that one species or strain would be more important than the other. Small differences in minute amounts of odoriferous or flavorful substances such as diacetyl which are produced can be important.

Most of the work on streptococci has been done on the production of acid. The chief interests have been, the selection of suitable strains for acid production (11), control of phage infection (42,101), and other causes of slowness (11). Less work has been done on their relation to flavor. Perhaps the main contribution of starter organism to flavor production is indirect and attention has been focused on the proteolytic enzymes of cells (6,103). Importance of lactobacilli to flavor has been reported by Naylor and Sharpe (73), Mabbitt and Zielinska (67) and Johns and Cole (52). Dacre (21,22) has reported on the presence of pediococci in New Zealand cheese and has shown its possible relation to flavor (21,22).

Alford and Frazier (2,3) have shown the relationship of micrococci to flavor. Occurrence of Staphylococcus aureus in raw milk cheese has also been reported, but its relation to flavor is not evident (91,92).

Though most of the observations lead one to believe that bacteria are important in flavor, reports to the contrary have been published (64,66).

Individual studies have been conducted to determine the relationship between proteolysis and flavor (13,24,43,44,47,56,87), lipolysis and flavor (15,82,51) and carbonyl compounds and flavor (28,29,45,60, 97,98).

Mulder, a Dutch investigator (72) has proposed a component balance theory of cheese flavor, which in short states that characteristic cheese flavor is not related to a single compound but mixtures of compounds coming from the degradation of fat, protein and lactose. Silverman and Kosikowski (86), have confirmed the above view.

Keeny and Day (54) have evolved another hypothesis of cheese flavor formation. They suggest that a slow chemical interaction over a long period of ripening between amino acids and dicarboxy compounds could lead to the production of flavorful aldehydes. Their trials of various compounds have indicated that methional has a cheese like flavor. This compound can possibly be derived as follows:

$$\underset{\text{Methionine}}{CH_3SCH_2CH_2\underset{\underset{NH_2}{|}}{C}HCOOH} + \underset{\text{Pyruvic acid}}{CH_3COCOOH} \longrightarrow \underset{\text{Methional}}{CH_3SCH_2CH_2CHO} + \underset{\text{Alanine}}{CH_3\underset{\underset{NH_2}{|}}{C}HCOOH} + CO_2$$

Witting and Batzar (102) however, have disputed the cheesiness of methional while Oro et al. (79) have reasserted the importance of this compound. Jackson and Morgan (51) have found 3 methyl butunal in cheddar cheese and thought that it contributed to flavor. It is not certain by which mechanism these aldehydes are formed though McCleod and Morgan (68) report their production from amino acids by certain strains of S. lactis.

Kosikowski and Mocquot (57) state that it should be noted that all the evidence now suggests that typical flavor of cheese is due to complex mixtures of components and success of tasting trials will depend on the care given to obtain the correct component balance.

Problem and Purpose

In recent years there has been a renewed interest in rapid cheddar cheese processing, both during manufacturing and ripening (59,99). This has led to the use of higher temperatures for cooking the curd and has encouraged the use of heat tolerant enterococci, Streptococcus faecalis as starter organisms (24,27). Higher curing temperatures as a means of decreasing the ripening period have also been investigated (4,27). In most of the studies the natural flavor of the cheese could not be produced however, and thus there is still a great deal of interest in reducing the duration of the ripening period without affecting the flavor.

The purpose of this study was to explore ways in which the intracellular proteinases of the starter organism could be increased. If successful, this should increase the rate of protein breakdown and decrease the curing time. With this end in view it was proposed to in-

vestigate the following factors which might effect the production of intracellular proteinases, (a) the age of the cell, (b) the presence of casein, gelatin and Casitone in the growth medium and (c) the absence of fermentable carbohydrate from the growth medium. It was also proposed to determine the optimum pH for the enzyme substrate reactions. Some common milk contaminants were included in the study in order to obtain some basic information about the intracellular enzymes of these bacteria.

EXPERIMENTAL PROCEDURE AND RESULTS

Seven cultures of different organisms were selected for this work. The selection was based on the physiological characteristics of some organisms commonly present in milk and of some common milk contiminants.

1). Organisms selected which commonly occur in milk or starters were:

   a. Streptococcus lactis (BB10)

   b. Leuconostoc dextrinicum (NDRL B-640)

   c. Lactobacillus casei (U. of Indiana No. 14696)

2). Organisms selected which commonly occur in milk or milk products as contaminants were:

   a. Proteus vulgaris (BB151)

   b. Bacillus subtilis (ATCC 6093)

   c. Pseudomonas fluorescens (U. of Indiana)

   d. Staphylococcus aureus (BB137)

The cultural characteristics of above organisms have been summarized in Table XVIII of the appendix.

Growth of cells: The cultures were taken from agar slants and were 12 hours old when inoculated into 1000 ml of liquid medium I or II where they were incubated at 37 C for 24 hours. Medium I differed from medium II in that the former had a papaic digest of soymeal while the latter contained beef extract. The complete compositions of media I and II are given in Table XIX in the Appendix. The Bacillus subtilis cultures were shaken continuously to provide better aerobic conditions for growth.

The cells were harvested at the end of the incubation period by centrifuging the culture at -2 C in a Serval refrigerated centrifuge, revolving at 4,000 rpm. The mass of cells so obtained was washed 4 or more times with sterile normal saline (0.9% Nacl) to remove the extracellular enzymes. The washed cells were then suspended in 3 ml of sterile normal saline solution and the suspension stored in the refrigerator for not more than 24 hours. Prior to use, the saline was decanted, leaving the packed cells in the bottom of the tube.

Extraction of intracellular and ectocellular enzymes

The intracellular and ectocellular enzymes were extracted by either the toluene method or the acetone dry powder method.

Toluene method: Each culture used here was grown on medium I or II as indicated above and the cells were harvested and washed in the manner previously described. To 1.0 g of packed cells 1 ml of toluene was added for the purpose of autolyzing the cells. It also served as a preservative. The mixture was then frozen for subsequent work.

Acetone dry powder method: The enzymes extracted by this method were made from cells grown on medium I only and were harvested as indicated above. One gram of packed bacterial cells was suspended in 3 ml of normal saline (sterile) and mixed until a homogenous mixture was obtained. This mixture was then added drop-wise to 100 ml of cold (5 to 0 C) acetone with vigorous stirring. After the entire mixture has been added, the stirring was stopped and the suspension allowed to settle for 15 minutes. The supernatant (acetone) was then decanted and

the remainder filtered through a Whatman #50 filter using a Buchner funnel under suction to enhance the process. The eluate was washed with 20 ml of cold acetone (5 to 0 C).

The cells as obtained above were then aseptically transferred to a sterile watch glass and dried in a dessicator under vacuum for 36 hours. The dried cells were ground in a sterile mortar and pestle and the powder obtained suspended in 3 ml sterile normal saline. The mixture was then frozen to preserve it for further experimental work.

Preparation and selection of the substrate

Both solid and liquid substrates were tried for the purpose of measuring proteolytic activity of the enzyme mixtures:

Solid substrate:

1. 2.5% casein agar.
2. Bacto staphylococcus medium No. 110.
3. Skim milk agar.
4. a. 5% skim milk agar.
   b. 2% skim milk agar.

Details of these media are given in Table XX in the Appendix.

The enzyme mixtures previously described were thawed and applied to the 5 ml of solid substrate in a 100 mm peteri dish in the form of a streak on its surface. The plates were incubated for 24, 48, and 96 hours at 37 C and then were examined for a clear zone around the streak as evidence of proteolysis.

None of the above four substrates showed any evidence of proteo-

lytic activity and thus the solid substrates were discarded and liquid substrates tried. No exact explanation as to why the above substrates did not work can be given but possibly insufficient direct contact between the substrate and the enzyme or the adsorption of enzymes to agar might be the cause.

Liquid substrates:

1. Diluted skim milk. Fresh pasteurized skim milk was diluted in the ratio of 1:20 and 1:50 with distilled water. These concentrations were arbitrarily picked but were within the range of light transmission used in analyzing the end product. Seven milliliters of this diluted milk medium were dispersed in screw cap tubes and sterilized in an autoclave for 20 minutes at 15 lbs pressure and 120 C.

2. Casein solution. This substrate was prepared by dissolving 1.5 g of casein (Merck) in distilled water. Heat and N/10 NaOH were used to aid in getting the casein into solution; distilled water was added to make the volume to 250 ml. The initial pH of the solution was 9.5. This was adjusted to pH 7.0 by adding 10% tartaric acid.

Twenty-five milliliters of the above solution were diluted to 100 ml with distilled water which gave a concentration of 1.5 mg of casein per ml. This solution was dispensed in 10 ml quantities in screw cap tubes and autoclaved for 20 minutes.

3. Whey protein solution. This solution was prepared by precipitating the casein at its isoelectric point from pasteurized

skim milk with lactic acid. The casein was spun down in a centrifuge and 10 ml of the supernatant liquid was taken up in 90 ml of distilled water. This gave a concentration of about 0.57 mg of whey protein in 1.0 ml of the solution. The final pH of this solution was adjusted to pH 7.0 by the addition of N/20 NaOH.

General procedure for handling substrates

To all the above substrates 0.2 ml of Agromycin* solution was added for every 10 ml portion. This antibiotic solution was made by suspending 0.33 g of Agromycin in 50 ml of sterile phosphate buffer (pH 7.0). The mixture was shaken vigorously and allowed to stand for 2 hours. The clear supernatant liquid was then used. The Agromycin was added to destroy any living cells present in the enzyme mixture.

Measurement of proteolytic activity

The following methods for the measurement of proteolytic activity were tried:

1. Viscosity measurement of gelatin.
2. Formal titration.
3. Light absorption.
4. Folin - Ciocalteu.

Methods (1) and (2) were dropped after very preliminary trials as neither methods gave reproducible results.

(3) Light absorption method: Seven milliliters of 1:20 and 1:50

*Agromycin 100 is the trade name of the Chas. Pfizer Co. product which has the following composition: Streptomycin 15%, Oxytetracycline 1.5%, Total inert ingredients 83.5%.

dilution of skim milk previously described were used. One-tenth milliliter of enzyme mixture was added to each 7 ml of these substrates and the tubes were incubated at 37 C for varying lengths of time. The optical density was determined by a Lumentron Photoelectric Colorimeter* at regular intervals to follow the protein breakdown. The tubes were inverted two times to mix and suspend the entire contents uniformly, before taking the readings. Inverting of the tubes was also done once every 12 hours to expose the maximum substrate surface.

Optical density roughly means the amount of light that will be absorbed by a solution. As the proteolytic action of the enzyme mixtures progressed and casein broke into simpler compounds, the light absorbed by the solution decreased. Thus a fall in optical density was a measure of proteolytic activity, though it did not give a quantitative measurement. The method was used however to evaluate the effect of media used for the growth of the organisms; the concentration of substrate and methods of preparing the enzyme mixtures.

(4) Folin-Ciocalteu method: This method measures the amount of free tyrosine and tryptophane in a solution. Quantitative estimations of these two amino acids can be made using a standard curve. Details of the procedure will be discussed later.

This method was used to determine the effect of pH of the substrate on the enzyme activity, also to determine the effect of the age of the bacterial cell and the effect of modified media on the production and/or

*The optical density scale in this instrument reads from 0 to 20.