

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
Montana State University
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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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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	viii
ABSTRACT	ix
INTRODUCTION	1
REVIEW OF LITERATURE	3
Enzymes	3
Proteolytic enzymes and fermentation of cheese	4
Role of bacteria in ripening of cheese	6
Attempts to accelerate the cheese ripening	9
Contribution of bacteria to the development of flavor	10
Problem and purpose	12
EXPERIMENTAL PROCEDURES AND RESULTS	14
DISCUSSION AND CONCLUSIONS	40
SUMMARY	45
LITERATURE CITED	47
APPENDIX	55

LIST OF TABLES

Table		Page
I.	Average decrease in optical density X10 of milk (1:50 dilution) due to intracellular enzyme activity. Cells grown on medium I. Enzymes extracted by Toluene.	20
II.	Average decrease in optical density X10 of milk (1:50 dilution) due to intracellular enzyme activity. Organisms grown on medium II. Enzymes extracted by toluene.	21
III.	Decrease in optical density X10 of milk (1:20 dilution) as a result of intracellular enzyme activity. Organisms grown on medium I. Enzymes extracted by toluene.	22
IV.	Decrease in optical density X10 of milk (1:20 dilution) due to enzyme activity. Organisms grown on medium II. Enzymes extracted by toluene.	23
V.	Decrease in optical density X10 of milk (1:20 dilution) when the intracellular enzymes were extracted by acetone dry powder method. Organisms grown on medium I.	24
VI.	Summary of Tables I, II, III, IV and V.	25
VII.	Micrograms of tyrosine and tryptophane liberated as the result of intracellular enzyme activity on casein solution substrate.	28
VIII.	Micrograms of tyrosine and tryptophane liberated as a result of intracellular enzyme activity on whey protein solution.	29
IX.	Optimum pH, and micrograms of tyrosine and tryptophane liberated at various pH.	30
X.	Differences in amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 24 and 48 hours.	31
XI.	Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 48 and 96 hours.	32

Table		Page
XII.	Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 96 and 144 hours.	23
XIII.	Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 24 and 144 hours.	34
XIV.	Increase in amounts of tyrosine and tryptophane liberated by intracellular enzymes due to the presence of 0.5% gelatin in the growth medium.	35
XV.	Increase in the amounts of tyrosine and tryptophane liberated by intracellular enzymes due to the presence of 0.5% casein in the growth medium.	36
XVI.	Increase in the amounts of tyrosine and tryptophane liberated by intracellular enzymes due to the absence of carbohydrates in the growth medium.	37
XVII.	Increase in the amounts of tyrosine and tryptophane liberated by intracellular enzymes due to the presence of Casitone(Difco) in the growth medium.	38
XVIII.	Cultural characteristics of the organisms used.	56
XIX.	Media I and II used for growth of cells.	59
XX.	Composition of solid substrates.	60
XXI.	The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 24 and 48 hours and the differences.	61
XXII.	The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 48 and 96 hours and the differences.	62
XXIII.	The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 96 and 144 hours and the differences.	63
XXIV.	Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and gelatin rich medium.	64
XXV.	Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and casein rich medium.	65

Table		Page
XXVI.	Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and carbohydrate free medium.	66
XXVII.	Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and 0.5% Casitone rich medium.	67

LIST OF FIGURES

Figure		Page
1.	Peptide chain showing N terminal linkage and C terminal linkage and the points of attack by proteolytic enzymes.	5
2.	Standard curve of light absorbance vs. micrograms of tyrosine and tryptophane using Beckman B spectrophotometer.	27

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.

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.

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.

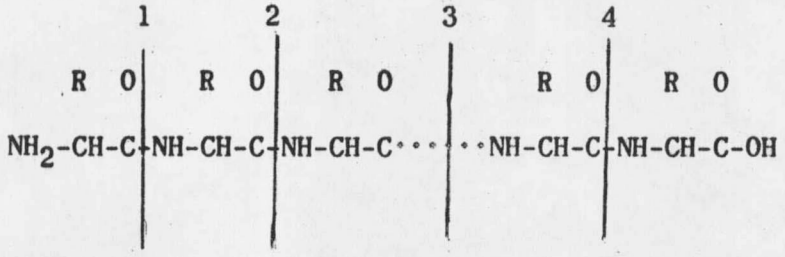


Figure 1. Peptide chain showing N terminal linkage and C terminal linkage and the points of attack by proteolytic enzymes.

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.

activity of the intracellular enzymes.

Effect of medium on proteolytic activity

Table I shows the decrease in optical density X10 (increase in protein breakdown) when the enzymes from cells grown on medium I were extracted from the bacterial cells by the toluene method and reacted with skim milk (1:50 dilution).

Table I

Average decrease in optical density X10 of milk (1:50 dilution) due to intracellular enzyme activity. Cells grown on medium I. Enzymes extracted by toluene.

Organism	Enzyme substrate reaction time						Difference between 0 & 192 hr.
	0 hr.	24 hr.	48 hr.	96 hr.	144 hr.	192 hr.	
Blank (no enzyme added)	4.7	4.5	4.4	4.2	4.1	4.2	0.50
1. *S. lactis*	5.2	4.8	4.7	4.6	4.5	4.5	0.70
2. *L. dextranicum*	6.4	6.0	5.9	5.7	5.7	5.7	0.70
3. *P. vulgaris*	6.0	5.8	5.6	5.4	5.2	5.2	0.80
4. *B. subtilis*	5.6	5.1	4.7	4.0	3.7	3.6	2.00
5. *P. fluorescens*	5.8	5.0	4.8	4.2	3.9	3.9	1.90
6. *S. aureus*	5.0	4.4	4.0	3.6	3.4	3.3	1.70

It will be noticed that the initial optical density is not the same for the various organisms. This is possibly due to slight differences in the particle sizes of various enzyme preparations. It will also be seen that the blank showed a slight decrease in optical density. This can possibly be attributed to minor denaturation changes which might be taking place during incubation.

These results show the change in the blank between 0 and 192 hours of reaction time to be 0.5 and the changes due to enzymes from organisms 1,2 and 3 to be only slightly greater than the blank. The enzymes from organisms 4,5 and 6 show a considerably greater breakdown with differences of 2.00, 1.90 and 1.70 respectively.

Table II shows the average decreases in optical density which were obtained when the enzymes mixtures were made from cells grown on medium II and extracted by toluene. Here again the proteolytic activity of enzymes from organisms 4,5 and 6 was considerably greater than organisms 1,2 and 3.

Table II

Average decrease in optical density X10 of milk (1:50 dilution) due to intracellular enzyme activity. Organisms grown on medium II. Enzymes extracted by toluene method.

Organism	Enzyme substrate reaction time						Difference between 0 & 192 hr.
	0 hr.	24 hr.	48 hr.	96 hr.	144 hr.	192 hr.	
Blank (no enzyme added)	4.10	3.90	3.80	3.70	3.60	3.60	0.50
1. *S. lactis*	4.80	4.50	4.50	4.30	4.20	4.20	0.60
2. *L. dextranicum*	4.60	4.50	4.30	4.10	4.00	3.90	0.70
3. *P. vulgaris*	4.50	4.30	4.00	3.80	3.60	3.60	0.90
4. *B. subtilis*	4.20	3.50	3.10	2.60	2.30	2.30	1.90
5. *P. fluoresens*	4.80	3.50	3.60	3.40	3.10	3.10	1.70
6. *S. aureus*	4.40	3.80	3.40	3.10	2.90	2.80	1.60

On comparing the values in table I and II it can be tentatively concluded that the enzymes from organisms grown on medium I had slightly

higher proteolytic activity than those grown on medium II as for example in B. subtilis the difference between 0 and 192 hour was 1.90 when grown on medium II and 2.00 when grown on medium I.

Effect of substrate concentration on proteolytic activity

Table III shows the decrease in optical density on 1:20 dilution of milk. It will be noticed by comparing these data with Table I that the difference between 0 hour and 192 hour are much greater when the dilution was 1:20 as compared to 1:50 dilution. The increases range from 0.30 in S. lactis to 1:40 in S. aureus.

Table III

Decrease in optical density of milk (1:20 dilution) as a result of intracellular enzyme activity. Organisms grown on medium I. Enzyme extracted by toluene.

Organisms	Enzyme substrate reaction time						Difference between 0 & 192 hr.
	0 hr.	24 hr.	48 hr.	96 hr.	144 hr.	192 hr.	
Blank	8.5	8.2	8.1	8.0	8.0	8.0	0.50
1. S. lactis	9.0	8.4	8.2	8.2	8.1	8.0	1.00
2. L. dextranicum	8.8	8.2	8.0	8.0	7.8	7.7	1.10
3. P. vulgaris	9.0	8.5	8.4	8.1	7.8	7.7	1.30
4. B. subtilis	9.0	6.8	6.4	6.0	5.8	5.7	3.30
5. P. fluorescens	8.5	7.8	7.0	6.8	6.3	6.1	2.40
6. S. aureus	8.7	7.2	6.4	6.0	5.8	5.6	3.10

Table IV gives the decrease in optical density when the enzymes were reacted with the substrate (1:20 dilution of skim milk) but the cells in this experiment were grown on medium II instead of medium I. The results when compared to Table III show the values to be slightly lower in

all but one organism. The value for S. lactis is slightly but probably not significantly higher.

From the previous experiments it can be concluded that growth of the cultures on medium I and the use of 1:20 dilution of skim milk as an enzyme substrate gave the best results. It appears that substrate concentration was a factor in the lower values found when 1:50 dilution of skim milk was used.

Table IV

Decrease in optical density X10 of milk (1:20 dilution) due to intracellular enzyme activity. Organisms grown on medium II. Enzymes extracted by toluene method.

Organism	Enzyme substrate reaction time						Difference between 0 & 192 hr.
	0 hr.	24 hr.	48 hr.	96 hr.	144 hr.	192 hr.	
Blank	8.0	8.0	7.8	7.6	7.6	7.6	0.4
1. S. lactis	8.0	7.4	7.2	6.9	6.9	6.9	1.10
2. L. dextranicum	8.5	8.3	8.0	7.6	7.6	7.4	1.10
3. P. vulgaris	8.4	8.0	7.6	7.4	7.2	7.1	1.30
4. B. subtilis	8.0	7.2	7.4	6.0	5.2	5.0	3.00
5. P. fluorescens	8.0	7.4	6.0	6.5	6.0	5.7	2.30
6. S. aureus	8.5	7.5	6.5	6.1	5.8	5.5	3.00

Table V shows the decrease in optical density when the enzyme mixtures were prepared by using acetone dry powder method instead of toluene method. The results show an increase in the difference in optical density between 0 and 192 hours as compared to Table III which can only be attributed to this method of preparation of enzymes. The increases

varied from (.10 to .30) except for S. lactis which gave the same results.

Table V

Decrease in optical density of milk (1:20 dilution when the enzymes were extracted by acetone dry powder method. Organisms grown on medium I.

Organism	Enzyme substrate Reaction Time						Difference between 0 & 192 hr.
	0 hr.	24 hr.	48 hr.	96 hr.	144 hr.	192 hr.	
Blank	8.0	7.8	7.7	7.5	7.5	7.5	0.50
1. S. lactis	8.5	8.0	7.6	7.4	7.4	7.5	1.00
2. L. dextranicum	8.8	8.3	8.0	7.8	7.7	7.6	1.20
3. P. vulgaris	8.5	7.8	7.5	7.4	7.3	7.1	1.40
4. B. subtilis	9.0	7.1	6.5	6.0	5.7	5.5	3.50
5. P. fluorescens	8.7	7.8	7.2	6.5	6.2	6.1	2.60
6. S. aureus	8.5	7.7	6.5	6.0	5.4	5.1	3.40

Table VI is a summary of the previous four tables and shows that 1:20 dilution of skim milk as a substrate gave greater differences (column 1 and 3) than the 1:50 dilution skim milk substrate (column 2 and 4). On comparing the values in column 1 with those in column 3 it will be seen that medium I gave slightly greater differences as compared to medium II (at least for organisms 4,5 and 6). The differences in column 5 are comparatively higher than those in column 1 which is attributed to the acetone dry powder method of extraction of enzymes.

As medium I and the acetone dry powder method of extraction of enzymes appeared to give the higher values indicating greater proteolysis, they were used in the remaining part of the study.

Table VI

Summary of the results shown in Table I, II, III, IV, and V.

Differences in optical density between 0 and 192 hours

Organism	Method of extraction of enzymes: Toluene method, Medium I, Dil.1:20, Col.1	Toluene method, Medium I, Dil.1:50, Col.2	Toluene method, Medium II, Dil.1:20, Col.3	Toluene method, Medium II, Dil.1:50, Col.4	Acetone dry powder, Medium I, Dil.1:20, Col.5
1. S. lactis	1.00	.70	1.10	.60	1.00
2. L. dextranicum	1.10	.70	1.10	.70	1.20
3. P. vulgaris	1.30	.80	1.30	.90	1.40
4. B. subtilis	3.30	2.00	3.00	1.90	3.50
5. P. fluorescens	2.40	1.90	2.30	1.70	2.60
6. S. aureus	3.10	1.70	3.00	1.60	3.40

The Folin-Ciocalteu method

Since the optical density method gave only comparative results and no quantitative estimation of proteolysis could be obtained on this basis, the Folin-Ciocalteu method was used subsequently. This method as given by Lowry et al, (63) has been suggested by Cowgill and Pardee (20) and Davis and Smith (25) for the assay of proteolytic enzymes.

The following reagents were used. Reagent A: This was prepared by adding 1.0 ml of 2.7% sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) and 1 ml of 1% copper sulphate ($CuSO_4 \cdot 5H_2O$) to 100 ml of 2.0% sodium carbonate solution. Reagent B: This phosphomolybdotungstic acid reagent was made according to the directions of Folin and Ciocalteu (32).

Procedure: One milliliter of well mixed enzyme reacted substrate previously described was pipetted aseptically into a 10 ml centrifuge tube. Three milliliters of 5.0% trichloracetic were added to stop the enzyme activity and to act as a protein precipitant. These tubes were then centrifuged at 2,000 rpm for 10 minutes. Two-tenths milliliter of the supernatant were transferred to another tube and 0.8 ml 0.5 N NaOH added to make the volume to 1.0 ml. Five milliliters of Reagent A were then added to the above mixed, and allowed to stand for 15 minutes at room temperature. Five-tenths milliliter of Reagent B were added and the tubes shaken immediately. This mixture was allowed to stand undisturbed for one hour. At the end of this reaction time light absorption was determined by a Beckman B spectrophotometer at 750 Mu wave length. The ug of tyrosine and tryptophane were then determined by the aid of a standard curve.

Preparation of the standard curve for the Folin-Ciocalteu method:

Sixty-three milligrams of tyrosine and 17 mg of tryptophane were dissolved in 100 ml of alkaline distilled water. This proportion of the two amino acids is the same as found in whole casein. One milliliter of the above solution was further diluted to 100 ml with distilled water. This solution gave a concentration of 8.0 ug per ml. From this 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 ml portions were then transferred into numbered tubes. Five-tenths normal NaOH were added into each to make up the volume to 1.0 ml. This gave concentrations of 0, 0.8, 1.6, 3.2, 4.8, 6.4 and 8.0 micrograms of tyrosine and tryptophane. The Folin-

Ciocalteu reaction was then run as described previously and micrograms of these amino acids were then plotted against light absorbance.

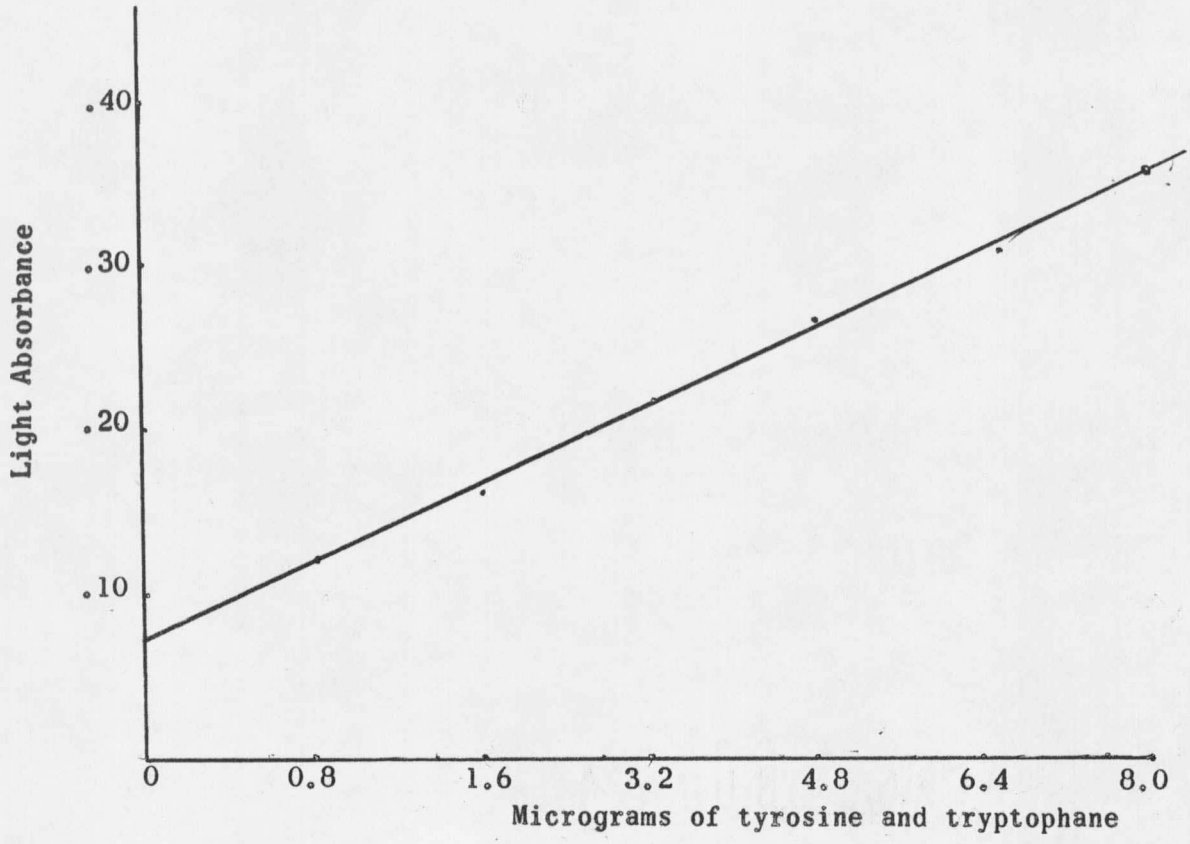


Fig. 2. Standard curve of light absorbance vs. micrograms of tyrosine and tryptophane using Beckman B spectrophometer.

Proteolytic activity using Folin-Ciocalteu method

All of the seven organisms were grown on medium I for 24 hours at 37 C. The cells were harvested, washed and the enzyme mixtures prepared by the acetone dry powder method, previously described. Ten milliliters of casein or whey protein solution (already described) were used as substrates. Two-tenths milliliter of Agromycin solution and 0.1 ml of

enzyme mixture were added to the substrates and the mixtures incubated at 37 C for 144 hours. One milliliter samples were drawn at 24, 48, 96 and 144 hours and assayed by Folin-Ciocalteu method. The results obtained are summarized in Table VII and VIII. Table VII shows that as the reaction time increased the amount of tyrosine and tryptophane liberated also increased. The blank showed an increase of 0.20 ug while the various enzymes mixture liberated from 1.20 ug to 2.40 ug of tyrosine and tryptophane. One finds from these results that the pattern of proteolytic activity is much the same as determined by the optical density method. Organism 2 gave the lowest value with the

Table VII

Micrograms of tyrosine and tryptophane liberated as the result of intracellular enzyme activity on casein solution substrate.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Micrograms of tyrosine and tryptophane			
1. S. lactis	1.35	1.70	1.80	1.90
2. L. dextranicum	1.25	1.35	1.40	1.40
3. L. casei	1.45	1.80	2.00	2.10
4. P. vulgaris	1.30	1.70	1.90	2.00
5. B. subtilis	1.50	2.10	2.75	3.00
6. P. fluorescens	1.50	2.20	2.80	3.10
7. S. aureus	1.40	2.15	2.70	2.95
Blank	0.75	0.90	0.90	0.90

liberation of 1.40 micrograms while 1, 3 and 4 followed next with values of 1.90, 2.10 and 2.0 micrograms. Organisms 5,6 and 7 had comparatively higher values of 3.0, 3.1 and 2.9 micrograms respectively. This table

represents the basic values and shall be used for comparison in the remaining part of this study.

Table VIII shows the micrograms of tyrosine and tryptophane liberated from whey protein solution, indicating that the enzymes can act upon this type of protein also. It will be seen that the pattern amongst different organisms is a little different as compared to Table VII, i.e. organism 2 and 4 show the lowest values of 1.20 ug, followed by organism 1 and 7 with 1.25 and 1.30 ug. Organism 3,5 and 6 are the higher group with 1.40, 1.50 and 1.60 ug of tyrosine and tryptophane. While on the casein substrate organism 2 had the lowest value of 1.4 ug, organisms 5,6 and 7 were the highest with 3.0, 3.1 and 2.90 ug of tyrosine and tryptophane.

Table VIII

Micrograms of tyrosine and tryptophane liberated as a result of intracellular enzyme activity on whey protein substrate.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
1. S. lactis	1.10	1.15	1.20	1.25
2. L. dextranicum	1.00	1.10	1.20	1.20
3. L. casei	1.20	1.30	1.35	1.40
4. P. vulgaris	1.15	1.20	1.20	1.20
5. B. subtilis	1.30	1.40	1.40	1.50
6. P. fluorescens	1.35	1.40	1.40	1.40
7. S. aureus	1.20	1.20	1.30	1.30
Blank	0.50	0.60	0.60	0.60

Effect of pH on proteolytic activity

Whey solution was prepared, sterilized and cooled as previously described. Measured quantities of sterile tartaric acid were added aseptically to different portions to bring the pH to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 (the quantities of acid to be added were determined in a separate experiment. The pH were rechecked). One tenth milliliter of enzyme mixtures were added to each 10 ml of substrate and tubes incubated at 37 C for 15 days. One milliliter portion of the hydrolyzate was analyzed by Folin-Ciocalteu method.

Table IX

Optimum pH and the micrograms of tyrosine and tryptophane liberated at various pH.

Organisms	Optimum pH	*Micrograms of tryosine and tryptophane liberated						
		pH						
		7.0	6.5	6.0	5.5	5.0	4.5	4.0
1. S. lactis	**7.0 and 5.5	1.30	1.20	1.15	1.30	1.10	1.00	1.00
2. L. dextranicum	6.0	1.10	1.10	1.20	0.90	0.90	0.90	0.80
3. L. casei	6.5	1.40	1.50	1.30	1.20	1.20	1.00	0.90
4. P. vulgaris	**7.0	1.30	1.10	1.00	0.80	0.80	0.70	0.70
5. B. subtilis	6.5	1.55	1.60	1.40	1.20	1.00	0.90	0.80
6. P. fluorescens	6.5	1.45	1.50	1.40	1.30	1.00	0.90	0.80
7. S. aureus	**7.0	1.45	1.30	1.20	0.90	0.90	0.80	0.80
Blank		0.65	0.60	0.65	0.60	0.60	0.60	0.60

* The values given are after 15 days of reaction time.
** These values may not be optimum as no tubes were prepared at pH value above 7.0 because the pH during cheese ripening is never alkaline.

Table IX gives the results and shows that the optimum pH for various enzymes was in the range of pH 6.0 to 7.0. It will be noticed that there

was still some enzyme activity at lower pH ranges. The difference between the amounts liberated at optimum pH and pH 4.0 were .30, .40, .60, .60, .60, .90, and .65 micrograms for organisms 1, 2, 3, 4, 5, 6, and 7, respectively.

Effect of age of the culture on protein breakdown

The selected organism were grown on medium I at 37 C for 24, 48, 96, and 144 hours. The cells were harvested, washed and the enzyme mixture prepared by the acetone dry powder method as previously described. The enzyme mixture was then reacted on a sterile casein solution (already described) at 37 C. One milliliter portions were taken at regular intervals

Table X

Difference* in the amounts of tryosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 24 and 48 hours.

Organism	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Micrograms of tyrosine and tryptophane			
1. S. lactis	+.05	+.30	+.40	+.50
2. L. dextranicum	+.05	+.35	+.45	+.60
3. L. casei	+.15	+.40	+.40	+.40
4. P. vulgaris	+.00	+.20	+.30	+.30
5. B. subtilis	+.30	+.40	+.35	+.30
6. P. fluorescens	+.20	+.10	+.20	+.20
7. S. aureus	+.20	+.25	+.20	+.25

*Differences have been calculated on the basis of the same reaction time. and Folin-Ciocalteu reaction run. Tables X, XI, and XII shows the results obtained from these experiments.

Table X shows that all 48 hours old cultures showed an increase in the amounts of tyrosine and tryptophane liberated as compared to 24 hour

old cultures. However, the increase varied from organism to organism. Details of the actual amounts of tyrosine and tryptophane liberated are given in Table XXI in the Appendix.

Table XI gives the differences in the amounts of tyrosine and trypophane liberated between 48 and 96 hours old cultures. All the values are positive, indicating an increase in the amounts of free tyrosine and tryptophane. On comparing these values with Table X it will be seen that enzymes from organisms 1, 2, 3, and 4 showed lesser increase while organisms 5 and 6 showed a greater increase. The values for organism 7 were similar. These results indicate that enzymes from organisms 1, 2, 3, and 4 gain the maximum rate of activity between 24 and 48 hours while enzymes

Table XI

Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 48 and 96 hours.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Micrograms of tyrosine and tryptophane			
1. *S. lactis*	+.20	+.15	+.20	+.20
2. *L. dextranicum*	+.20	+.20	+.25	+.10
3. *L. casei*	+.10	+.20	+.20	+.20
4. *P. vulgaris*	+.05	+.10	+.10	+.00
5. *B. subtilis*	+.20	+.30	+.35	+.50
6. *P. fluorescens*	+.40	+.30	+.25	+.70
7. *S. aureus*	+.20	+.30	+.05	+.25

from organisms 5 and 6 obtained the maximum rate of proteolytic activity between 48 and 96 hours of age. The actual amounts of tyrosine and tryptophane liberated are given in Table XXII in the Appendix.

Table XII represents the differences in the amounts of tyrosine and tryptophane liberated from 96 and 144 hours culture. All the values are negative indicating that enzymes obtained from cells grown at 144 hours have lesser proteolytic activity as compared to cells grown for 96 hours. For details of the actual amounts of tyrosine and tryptophane liberated see Table XXIII in the Appendix.

Table XII

Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 96 and 144 hours.

Organism	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Micrograms of tyrosine and tryptophane liberated			
1. *S. lactis*	-.30	-.25	-.30	-.40
2. *L. dextranicum*	-.30	-.10	-.20	-.20
3. *L. casei*	-.30	-.65	-.65	-.70
4. *P. vulgaris*	-.05	-.10	-.20	-.30
5. *B. subtilis*	-.30	-.40	-.65	-.80
6. *P. fluorescens*	-.60	-.40	-.50	-.95
7. *S. aureus*	-.40	-.60	-.35	-.60

Table XIII shows the difference in the amounts of tyrosine and tryptophane liberated by 24 and 144 hours old cultures. The positive figures indicate that 144 hour old cultures had higher values while negative figures indicate that 24 hour culture had higher values by the amounts indicated. Others represent no change.

From Tables X, XI, XII and XIII it can be concluded that the proteolytic activity in all the organisms increased up to 96 hours of age, though the increases varied from one organism to another. The proteolytic

activity declined when the cells were 144 hours old. Only three organisms (1, 2 and 3) had higher values at 144 hours as compared to 24 hours. In organisms 6 and 7 the values were lower at 144 hours as compared to 24 hours, while organisms 4 and 5 showed no change.

Table XIII

Differences in the amounts of tyrosine and tryptophane liberated by intracellular enzymes extracted from cells grown for 24 and 144 hours.

Organism	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Micrograms of tyrosine and tryptophane			
1. S. lactis	+.05	+.20	+.30	+.30
2. L. dextranicum	+.05	+.45	+.45	+.50
3. L. casei	+.05	+.05	+.05	+.10
4. P. vulgaris	.00	.00	.00	.00
5. B. subtilis	+.20	+.30	.00	.00
6. P. fluorescens	.00	.00	-.05	-.15
7. S. aureus	.00	-.05	-.10	-.10

Effect of gelatin in the growth medium on proteolytic activity

Medium I with .50% gelatin added was used for the cultivation of the organisms and the enzyme mixtures were prepared in the usual manner. The enzyme mixture from each organism was reacted with the casein substrate previously described and the Folin-Ciocalteu reactions was performed at intervals on 1.0 ml samples.

The results obtained have been summarized in Table XIV. The entries under each column of this table represent micrograms of tyrosine and tryptophane liberated in excess as compared to medium I (Table VII). The increase is attributed to the presence of gelatin in the growth medium. It

will be noticed that except for organisms 4 and 7 the increases were not very great, ranging from .10 to .30 micrograms. The increases for organisms 4 and 7 were .90 and .85 respectively.

For detail values for each medium at every reaction time see Table XXIV in the Appendix.

Table XIV

Increase in the amount of tyrosine and tryptophane liberated by intracellular enzymes due to the presence of 0.5% gelatin in the growth medium.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Increase in micrograms of tyrosine and tryptophane			
1. S. lactis	.05	.20	.20	.10
2. L. dextranicum	.05	.15	.15	.20
3. L. casei	.05	.10	.10	.10
4. P. vulgaris	.60	.70	.70	.90
5. B. subtilis	.45	.25	.20	.30
6. P. fluorescens	.25	.55	.05	.10
7. S. aureus	.70	.55	.80	.85

Effect of casein in the culture media on proteolytic activity

Five grams of casein (Merck) were dissolved in alkaline distilled water, and made up to 200 ml. Final pH of the medium was adjusted to pH 7.0 and the solution autoclaved. Eight hundred milliliters of medium I were mixed aseptically with the above. The final pH of the combined medium was 7.1.

Cultures of Proteus vulgaris, Pseudomonas fluorescens, Staphylococcus aureus and Bacillus subtilis were grown on the above medium. Cultures of Streptococcus lactis, Leuconostoc dextranicum and Lactobacillus

casei were grown on the above medium plus 0.2% calcium carbonate. Calcium carbonate served as a buffer for the acid produced by the above cultures. All cultures were grown for 24 hours at 37 C.

Cells were harvested at the end of incubation period and NaOH solution was used to dissolve the casein in instances where it precipitated. Excess calcium carbonate was removed by the use of an acid. The enzyme mixtures were prepared as usual and reacted with the casein substrate. Folin-Ciocalteu reaction was performed to follow the proteolytic changes. The results have been summarized in Table XV.

In Table XV the figures represent the micrograms of excess tyrosine and tryptophane liberated by cells when grown with added casein as compared to the medium I (Table VII). On comparing these values with those

Table XV

Increase in tyrosine and tryptophane liberated by intracellular enzymes due to the presence of .05% casein in the growth medium.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Increase in micrograms of tyrosine and tryptophane			
1. S. lactis	0.45	0.90	1.30	1.50
2. L. dextranicum	0.60	1.05	1.60	1.60
3. L. casei	1.15	0.40	1.60	1.80
4. P. vulgaris	0.40	0.90	0.95	1.10
5. B. subtilis	2.50	2.40	1.95	2.30
6. P. fluorescens	2.70	2.60	2.10	2.10
7. S. aureus	2.40	2.05	2.10	2.15

in Table XIV it will be seen that differences obtained by the addition of casein to the growth medium were much higher than those with gelatin. In

case of gelatin the increases ranged from .10 to .90 ug while in case of casein the increase ranged from 1.10 to 2.30 ug.

For details of the values obtained for each medium see Table XXV in the Appendix.

Effect of carbohydrate free medium on proteolytic activity

To study the effect of carbohydrates on the production of enzymes the following medium was used for growth of bacteria:

Trypticase	17.0 g
Polypeptone	5.0 g
Yeast extract	2.0 g
NaCl	5.0 g
Potassium hydrogen phosphate (dibasic)	2.5 g
Distilled water	1000 ml

The enzymes were prepared and reacted with the casein substrate and assayed by the Folin-Ciocalteu reaction. The results are shown in Table XVI.

Table XVI

Increase in the tyrosine and tryptophane liberated by intracellular enzymes due to the absence of carbohydrate in the growth medium.

Organisms	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Increase in micrograms of tyrosine and tryptophane			
1. *S. lactis*	.35	.70	.80	.85
2. *L. dextranicum*	.30	.40	.60	.70
3. *L. casei*	.35	.60	.65	.70
4. *P. vulgaris*	.20	.25	.30	.40
5. *B. subtilis*	.40	.50	.85	1.25
6. *P. fluorescens*	.90	1.00	1.10	1.40
7. *S. aureus*	.70	.65	.70	.60

In Table XVI the figures represent the micrograms of excess tyrosine and tryptophane liberated by enzymes from cells when grown on a carbohydrate free medium as compared to the basic medium (Table VII). On comparing the above results to Table XIV, XV, it will be noticed that these increases are higher than those shown in Table XIV (effect of gelatin) except in S. aureus; however these increases are comparitively much smaller than those in Table XV (effect of casein).

For details of values obtained from each medium see Table XXVI in the Appendix.

Effect of Casitone (Difco)* in the growth medium on proteolytic activity

To study the effect of Casitone in the medium on proteolytic activity the cultures were grown for 24 hours at 37 C on medium I plus 0.5% of Casitone (Difco, vitamin free). The enzyme mixtures were prepared

Table XVII

Increase in the tyrosine and tryptophane liberated by intracellular enzmes due to the presence of 0.5% Casitone (Difco) in the growth medium.

Organism	Enzyme substrate reaction time			
	24 hr.	48 hr.	96 hr.	144 hr.
	Increase in micrograms of tyrosine and tryptophane			
1. S. lactis	.00	.05	.05	.10
2. L. dextranicum	.00	.05	.00	.00
3. L. casei	.05	.05	.00	.00
4. P. vulgaris	.00	.05	.00	.00
5. B. subtilis	.05	.05	.10	.10
6. P. fluorescens	.00	.00	.00	.05
7. S. aureus	.00	.15	.15	.15

* Pancreatic digest of casein.

In Table XVII the figures represent the excess micrograms of tyrosine and tryptophane liberated by cells when grown on added Casitone as compared to the basic medium (Table VII). It can be concluded that Casitone did not affect the proteolytic activity in any appreciable amounts. Details of the values obtained are shown in Table XXVII of the Appendix.

On comparing Tables XIV, XV, XVI, and XVII it can be concluded that 0.5% casein in the growth medium showed maximum increase over a basic medium in the proteolytic activity of the enzymes and the carbohydrate free medium followed next. The presence of 0.5% gelatin in the growth medium increased the amounts of tyrosine and tryptophane liberated but except for P. vulgaris and S. aureus the quantities were not very signigicant. In case of 0.5% Casitone in the growth medium, the increases do not appear to be significant.

DISCUSSION AND CONCLUSIONS

The first part of this study was concerned with the determination of proteolytic activity of the intracellular enzymes of the organisms selected. It was found that the enzymes from all the organisms had some activity though it varied greatly from organism to organism e.g. Streptococcus lactis could liberate only 1.90 ug of tyrosine and tryptophane while Pseudomonas fluorescens liberated 3.10 ug.

As far as lactic acid organisms are concerned it reaffirms the belief that starter organisms may be responsible for at least part of the protein breakdown during cheese ripening. In cottage cheese lactic acid bacteria (S. lactis and Leuconostoc dextranicum) are always present and Pseudomonas, Proteus and Bacillus species may be encountered as contaminants. This latter group could bring about rather extensive protein breakdown after autolysis. This may be one of the causes of bitter and other off flavor in low count cottage cheese, since some peptones have been reported to have bitter taste. Chromatographic studies of the hydrolyzate should be done to identify the peptones in order to confirm the possibility of off flavors being due to these substances.

Raw milk Cheddar cheese is recognized as ripening faster than pasteurized milk cheese. Most of the selected organisms are present in raw milk in smaller or larger numbers. During pasteurization the organisms are destroyed and proteinases would probably be inactivated. On the other hand in raw milk cheese, the bacteria usually get a chance to grow before they are inactivated by an unfavorable pH (6.8 to 4.3) thus their number would be higher. This study has shown these bacterial cells on death and autolysis will liberate proteinases. The action of these enzymes can in part be the cause of faster curing, as the pH studies of bacterial pro-

teinases showed, some activity in the lower pH range.

Both whey protein solution and casein solution substrate were attacked by these enzymes. A comparison between the values obtained cannot be made since there was a difference in the concentration of the two.

The study of the effect of pH of the substrate on the enzyme activity revealed that most of the enzymes had their optimum pH in the vicinity of 6.0 and 7.0, though S. lactis had two peaks, one at pH 7.0 and another at pH 5.5. These values for S. lactis are in accordance with the study of Baribo and Foster (14). P. fluorescens, Lacobacillus casei and B. subtilis showed an optimum pH at 6.5 Staphylococcus aureus and P. vulgaris showed the maximum activity at pH 7.0 (this may not be optimum since no pH higher than 7.0 were studied). It was also observed that the organisms still had some activity at a lower pH (4.5 - 5.0).

The effect of the age of the cells on the intracellular proteinases was also investigated and it was found that as the cells grew older, up to 96 hours of age, the enzyme activity increased. In cells 144 hours old the proteolytic activity of the enzymes decreased in all cases. It was observed that in 144 hour old cells S. lactis, L. dextranicum and L. casei still had higher proteolytic activity than 24 hour old cells. P. fluorescens and S. aureus showed a decrease in values as compared to 24 hour old cells, while P. vulgaris and B. subtilis had similar values at 24 and 144 hours.

The effect of age could probably be explained from the fact that carbohydrases are the first enzymes to be formed and proteinases build up later. As to why the enzyme activity fell after a certain peak one

explanation that could be given is that old autolyzed cells might serve as a substrate for some other type of enzymes. It follows, based on the results of this study that old cultures might be of more value as an inoculum than the young cultures if fast protein breakdown is desired. However, since the cultures are generally added primarily for the purpose of acid production and it is desired to have maximum acid production in minimum time, old cultures would be disadvantageous under these circumstances.

Some of the environmental factors which could enhance the proteolytic activity of intracellular proteinases were also studied. Gelatin 0.5% was added in the growth medium and the enzymes prepared from such cells had a higher proteolytic activity than those grown in the absence of gelatin. The increases varied from .10 to .90 ug of tyrosine and tryptophane for the different organisms. This was a little surprising because none of the organisms selected showed any liquefaction of gelatin when grown on it. Perhaps gelatin contained some other protein in minute quantities which might have stimulated the production of proteinases.

Casein 0.5% was also added to the growth medium. The enzymes extraced from the cells grown on such a medium showed the largest increase in proteolytic activity of any substance studied. The increase varied from 1.10 ug to 2.30 ug of tyrosine and tryptophane. In S. lactis, L. dextranicum and L. casei two factors i.e. casein and calcium carbonate were involved so that the increases of 1.50, 1.60 and 1.80 ug were probably due to their combined affect. It appears that the presence of a substrate increases the production of proteinases.

The presence of 0.5% Casitone (Difco) gave no appreciable increase. The maximum increase was .15 ug of tyrosine and tryptophane in S. aureus

enzymes. This observation seems to indicate that undigested protein increases the formation of proteinases in the organisms studied.

Absence of fermentable carbohydrate in the growing medium also affects the proteolytic activity of the intracellular enzymes. The increases of tyrosine and tryptophane were .85 ug in S. lactis, .70 ug in L. dextranicum and L. casei, .40 ug in P. vulgaris, 1.25 ug in B. subtilis, 1.40 ug in P. fluorescens and .60 in S. aureus. No exact explanation can be given for the above results. In the lactic acid bacteria, however, the addition of carbohydrate in the growing medium might lead to the inhibition of deaminases production, pH might be a factor here. But this does not explain the results obtained with non-fermenting bacteria.

In conclusion it can be said that pH studies showed that optimum for enzyme activity for various organisms was in the vicinity pH 6.0 to 7.0. Study of the effect of age of cells revealed an increase in proteolytic activity till the cells were 96 hours old, growing them for another 48 hours however showed an appreciable decrease. The presence of gelatin and casein and the absence of carbohydrate in the growth medium increased the proteinases while Casitone did not have an appreciable effect.

Suggested further work

Combination of more than one factor should be tried to see the combined effect on the production of proteinases, e.g. absence of carbohydrate and addition of casein; age of the cells might be varied for each combination. Several such combinations can be tried.

An important thing to verify would be to see whether this increased amount of proteinase results in off flavors or speeds up development of desirable flavor. Additions of the acetone dry powders from various

organisms to the milk used for making experimental cheese would be one approach.

Comparisons between the proteinase production of the cells on these special media and the cells occurring in normal cheese would be interesting, and a study to determine the effect of temperature on the activity of these enzymes may be of value in explaining the influence of pasteurization on the rate of cheese ripening.

SUMMARY

Seven cultures selected on the basis of their common occurrence in milk or starters and contaminants of milk and milk products were used for this study. The organisms were Streptococcus lactis, Leuconostoc dextranicum, Lactobacillus casei, Proteus vulgaris, Bacillus subtilis, Pseudomonas fluorescens and Staphylococcus aureus. Each organism was grown on medium I and/or medium II for 24 hours at 37 C. At the end of the incubation period the cells were harvested, washed four times and the enzyme mixtures prepared by toluene and acetone dry powder method. The proteolytic activity of the enzymes was determined by the optical density method using a diluted skim milk substrate.

Cells grown on medium I and enzymes extracted by acetone dry powder method gave the best results. The Folin-Ciocalteu method was adapted for following the proteolysis in the latter part of the work since this method could give a quantitative determination of proteolytic activity expressed in terms of the amounts of tyrosine and tryptophane liberated.

Casein (1.5 mg/ml) solution and whey protein solution (.57 mg/ml) were used as substrates. Studies of pH were done on whey protein solution while the remaining part of the study was done using casein solution as a substrate. It was found that optimum pH for various enzymes fell in the vicinity 6.0 to 7.0 though S. lactis showed a second peak at pH 5.5.

The effect of age on intracellular proteinases was determined by growing cells on medium I for 24, 48, 96 and 144 hours. Enzymes were prepared at the end of each incubation period and reacted with the casein substrate. Amounts of tyrosine and tryptophane liberated were

compared and it was found that S. lactis, L. dextranicum and L. casei showed a maximum rate of increase during the 24 and 48 hour period though the increase continued up to 96 hours. B. subtilis and P. fluorescens showed a maximum rate of increase during the 48 and 96 hour period, while S. aureus gave a very similar increase during the 24 to 48 hour period and the 48 to 96 hour period. P. vulgaris did not show any increase beyond 48 hours period. However, all the organisms showed an appreciable decrease when the cells were 144 hours old.

The organisms were also grown on medium I with 0.5% additional amounts of gelatin, casein and Casitone (Difco). The cells were grown for 24 hours and enzymes prepared and assayed. It was found that gelatin and casein both increased the proteinases but the increase in case of casein was much greater. Casitone on the other hand did not bring about any appreciable change. Deletion of carbohydrates from medium I also increased the proteolytic activity of the enzymes.

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APPENDIX

TABLE XVIII

CULTURAL CHARACTERISTICS OF THE ORGANISMS USED

Streptococcus lactis BB10

Shape, size, arrangement, and relation to O_2	-	Ovoid, 0.70u in diameter (a/v); in pairs or short chains. Aerobic
Staining characteristics motility	-	Gram positive non-motile
Agar colonies	-	Small, less than 1 mm slightly mucoid, Sub-surface colonies lenticular
Carbohydrates fermentation	-	Acid from: glucose, maltose, lactose, sucrose, no acid from raffinose and sorbitol
Litmus milk	-	Acidified and curdled. Litmus reduced before curdling. No digestion.
Remarks	-	Growth was luxuriant.

Leuconostoc dextranicum NRRL B-640

Shape, size, arrangement, and relation to O_2	-	Spherical 0.86u in diameter in short chains mostly. Aerobic
Staining characteristics motility	-	Gram positive. Non-motile.
Agar colonies	-	Small, gray, circular, slightly raised, entire.
Carbohydrates fermentation	-	Acid from: glucose, fructose, galactose, maltose, sucrose.
Litmus milk	-	Slight acid. No coagulation. No reduction of litmus.
Remarks	-	Growth fair, in enriched medium.

Lactobacillus casei - U. of Indiana 14696

Shape, size, arrangement, and relation to O_2.	-	Long rods, occurring in chains some single cells seen. Micro-aerophillic
Staining characteristic Motility	- 	Gram positive. Non-motile.
Agar colonies	-	Poor growth on surface. Pin point colonies.
Carbohydrates fermentation	-	Acid from: glucose, fructose. mannose, galactose, sucrose.
Litmus milk	-	Acid and coagulation. Litmus reduced, partial digestion.
Remarks	-	Growth was abundant on enriched medium.

Proteus vulgaris BB 151

Shape, size, arrangement, and relation to O_2	-	Stright rods, .75 x 2.0 u occurring in chains. Aerobic
Staining characteristic Motility	- 	Gram negative. Motile.
Agar colonies	-	Ameboid, opaque, gray.
Carbohydrates fermentation	-	Acid and gas from glucose, fructose, galactose, maltose. No acid or gas from lactose.
Litmus milk	-	Slight acid, becoming markedly alkaline, quick peptonization.

Bacillus subtilis ATCC 6093

Shape, size, arrangement, and relation to O_2	-	Rods, .7 x 2.5 u generally singly, not encapsulated. Spores .70 u in diameter. Aerobic
Staining characteristic Motility	-	Gram positive. Uniformly stained. Motile.
Agar colonies	-	Rough, opaque, dull spreading, slightly gray.
Carbohydrates fermentation	-	Acid but no gas from glucose, sucrose and mannitol. No acid from lactose.
Litmus milk	-	Slow peptonization, becoming alkaline.
Remarks	-	On broth it gave wrinkled rough film.

Pseudomonas fluorescens U. of Indiana

Shape, size, arrangement, and relation to O_2	-	Rods, 0.4 x 1.5 u occurring singly and in pairs. Aerobic.
Staining characteristic Motility	-	Gram negative. Motile.
Agar colonies	-	Comparatively large, slimy, reddish layer becoming reddish gray.
Carbohydrates fermentation	-	Acid from glucose.
Litmus milk	-	No coagulation becoming alkaline.
Remarks	-	On broth turbid flocculent and greenish pellicle and gray sediment.

Staphylococcus aureus BB 137

Shape, size, arrangement, and relation to O_2	-	Spherical, 1.0 u in diameter, occurring in irregular clumps. Some short chains also seen. Aerobic.
Staining characteristic Motility	-	Gram positive. Non-motile.
Agar colonies	-	Circular, smooth, white entire.
Carbohydrates fermentation	-	Acid from: glucose, lactose, sucrose, mannitol. No acid from raffinose, salicin.
Litmus milk	-	Acid coagulation.
Remarks	-	Coagulase positive

Table XIX

Media I and II used for growth of cells

Medium I

Trypticase Soy Broth BBL	30.0 g
Dextrose	5.0 g
Yeast extract	2.0 g
Distilled water	1000.0 ml
pH	7.2

Medium II

Bacto Beef extract	3.0 g
Bacto Tryptone	5.0 g
Dextrose	7.5 g
Yeast Extract (Difco)	2.0 g
Potassium hydrogen phosphate (Dibasic)	2.5 g
Distilled water	1000.0 ml
pH	7.1

Table XX

Composition of solid substrates

1. Casein agar:

Casein	2.5 g
Agar (Difco)	1.5 g
Distilled water	100.0 ml
pH	7.0

2. Bacto Staphylococcus Medium 110 (dehydrated) Stone's reaction for the detection of liquefaction of gelatin was performed.

3. Milk agar:

Bacto Agar	1.5 g
Skim Milk	100.0 ml
pH	7.0

4. Diluted milk agar:

a)

Skim Milk	5 ml
Agar (Difco)	1.5 g
Distilled water	100.0 ml
pH	7.0

b)

Skim Milk	2 ml
Agar (Difco)	1.0 g
Distilled water	100.0 ml
pH	7.0

Table XXI

The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 24 and 48 hours and the differences.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.35	1.40	+.05	1.70	2.00	+.30	1.80	2.20	+.40	1.90	2.40	+.50
L. dextranicum	1.25	1.30	+.05	1.35	1.70	+.35	1.40	1.85	+.45	1.40	2.00	+.60
L. casei	1.45	1.60	+.15	1.80	2.20	+.40	2.0	2.40	+.40	2.10	2.50	+.40
P. vulgaris	1.30	1.30	+.00	1.70	1.90	+.20	1.90	2.20	+.30	2.00	2.30	+.30
B. subtilis	1.50	1.80	+.30	2.10	2.50	+.40	2.75	3.10	+.35	3.00	3.30	+.30
P. fluorescens	1.50	1.70	+.20	2.20	2.30	+.10	2.85	3.00	+.15	3.10	3.30	+.20
S. aureus	1.40	1.60	+.20	2.15	2.40	+.25	2.70	2.90	+.20	2.95	3.20	+.25

I - cells grown for 24 hours.
II - cells grown for 48 hours.

Table XXII

The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 48 and 96 hours and the differences.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.40	1.60	+.20	2.00	2.15	+.15	2.20	2.40	+.20	2.40	2.60	+.20
L. dextranicum	1.30	1.50	+.20	1.70	1.90	+.20	1.85	2.10	+.25	2.00	2.10	+.10
L. casei	1.60	1.70	+.10	2.20	2.40	+.20	2.40	2.60	+.20	2.50	2.70	+.20
P. vulgaris	1.30	1.35	+.05	1.90	1.80	+.10	2.20	2.10	+.10	2.30	2.30	.00
B. subtilis	1.80	2.00	+.20	2.50	2.80	+.30	3.10	3.45	+.35	3.30	3.80	+.50
P. fluorescens	1.70	2.10	+.40	2.30	2.60	+.30	3.00	3.25	+.25	3.30	3.90	+.60
S. aureus	1.60	1.80	+.20	2.40	2.70	+.30	2.90	2.95	+.05	3.20	3.45	+.25

I - cells grown for 48 hours
II - cells grown for 96 hours

Table XXIII

The micrograms of tyrosine and tryptophane liberated by intracellular enzymes from cells grown for 96 and 144 hours and the differences.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.60	1.30	-.30	2.15	1.90	-.25	2.40	2.10	-.30	2.60	2.00	-.60
L. dextranicum	1.50	1.20	-.30	1.90	1.80	-.10	2.10	1.90	-.20	2.10	1.90	-.20
L. casei	1.70	1.40	-.30	2.40	1.75	-.65	2.60	1.95	-.65	2.70	2.00	-.70
P. vulgaris	1.35	1.30	-.05	1.80	1.70	-.10	2.10	1.90	-.20	2.30	2.00	-.30
B. subtilis	2.00	1.70	-.30	2.80	2.40	-.40	3.45	2.80	-.65	3.80	2.00	-.80
P. fluorescens	2.10	1.50	-.60	2.60	2.20	-.40	3.25	2.75	-.50	3.90	2.95	-.95
S. aureus	1.80	1.40	-.40	2.70	2.10	-.60	2.95	2.60	-.35	3.45	2.80	-.65

I - cells grown for 96 hours
II - cells grown for 144 hours

Table XXIV

Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and gelatin rich medium*.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.30	1.40	+.10	1.70	1.90	+.20	1.80	2.00	+.20	1.90	2.00	+.10
L. dextranicum	1.25	1.30	+.05	1.35	1.50	+.15	1.40	1.55	+.15	1.40	1.60	+.20
L. casei	1.45	1.40	-.05	1.80	1.70	-.10	2.00	2.00	.00	2.10	2.20	+.10
P. vulgaris	1.30	1.90	+.60	1.70	1.40	-.30	1.90	2.60	+.70	2.00	2.90	+.90
B. subtilis	1.50	1.95	+.45	2.10	2.60	+.50	2.75	2.95	+.20	3.00	3.30	+.30
P. fluorescens	1.50	1.75	+.25	2.20	2.40	+.20	2.80	2.85	+.05	3.10	3.20	+.10
S. aureus	1.40	2.10	+.70	2.15	2.70	+.55	2.70	3.50	+.80	2.95	3.90	+.95

I - cells grown on basic medium I for 24 hours
II* - cells grown on basic medium I - 0.5% gelatin for 24 hours

Table XXV

Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and casein rich medium*.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.30	1.80	+0.45	1.70	2.60	+0.90	1.80	3.10	+1.30	1.90	3.40	+1.50
L. dextranicum	1.25	1.85	+0.60	1.35	2.40	+1.05	1.40	3.00	+1.60	1.40	3.00	+1.60
L. casei	1.45	1.60	+0.15	1.80	3.20	+2.40	2.0	3.60	+1.60	2.10	3.90	+1.80
P. vulgaris	1.30	1.70	+0.40	1.70	2.6	+0.90	1.90	2.85	+0.95	2.00	3.10	+1.10
B. subtilis	1.50	4.0	+2.5	2.10	4.5	+2.40	2.75	4.70	+1.95	3.00	5.30	+2.30
P. fluorescens	1.50	4.2	+2.7	2.20	4.8	+2.60	2.80	4.90	+2.10	3.10	5.20	+2.10
S. aureus	1.40	3.8	+2.4	2.15	4.2	+2.05	2.70	4.80	+2.10	2.95	5.10	+2.15

I - The organisms were grown on medium I for 24 hours.
II* - The organisms were grown on medium I plus 0.5% casein for 24 hours.

Table XXVI

Comparative amounts (micrograms) tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and carbohydrate free medium.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.30	1.70	+0.35	1.70	2.40	+0.70	1.80	2.60	+0.80	1.90	2.75	+0.85
L. dextranicum	1.25	1.55	+0.30	1.35	1.75	+0.40	1.40	2.00	+0.60	1.40	2.10	+0.70
L. casei	1.45	1.80	+0.35	1.80	2.40	+0.60	2.00	2.65	+0.65	2.10	2.80	+0.70
P. vulgaris	1.30	1.50	+0.20	1.70	1.95	+0.25	1.90	2.20	+0.30	2.00	2.40	+0.40
B. subtilis	1.50	1.90	+0.40	2.10	2.60	+0.50	2.75	3.60	+0.85	3.00	4.25	+1.25
P. fluorescens	1.50	2.40	+0.90	2.20	3.20	+1.00	2.80	3.90	+1.10	3.10	4.50	+1.40
S. aureus	1.40	2.10	+0.70	2.15	2.80	+0.65	2.70	3.40	+0.70	2.95	3.55	+0.60

I - The organisms grown on medium I for 24 hours.
II - The organisms grown on carbohydrate free medium for 24 hours.

Table XXVII

Comparative amounts (micrograms) of tyrosine and tryptophane liberated by intracellular enzymes prepared from cells grown on medium I and casein rich medium*.

Organisms	Enzyme substrate reaction time											
	24 hours			48 hours			96 hours			144 hours		
	I	II	Diff.	I	II	Diff.	I	II	Diff.	I	II	Diff.
S. lactis	1.30	1.35	+.00	1.70	1.75	+.05	1.80	1.85	+.05	1.90	2.00	+.10
L. dextranicum	1.25	1.25	.00	1.35	1.30	-.05	1.40	1.40	.00	1.40	1.40	.00
L. casei	1.45	1.40	-.05	1.80	1.85	+.05	2.00	2.00	.00	2.10	2.10	.00
P. vulgaris	1.30	1.30	.00	1.70	1.60	-.10	1.90	1.90	.00	2.00	2.00	.00
B. subtilis	1.50	1.55	+.05	2.10	2.20	+.10	2.75	2.85	+.10	3.00	3.10	+.10
P. fluorescens	1.50	1.50	.00	2.20	2.20	.00	2.80	2.80	.00	3.10	3.15	+.05
S. aureus	1.40	1.40	.00	2.15	2.00	+.15	2.70	2.85	+.15	2.95	3.10	+.15

I - cells grown on medium I for 24 hours
II* - cells grown on medium I plus 0.5% Casitone for 24 hours

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