PROTEOLYTIC ENZYMES FROM FERMENTATION OF FISH PLANT WASTES

by

HOWARD CHRISTOPHER WAH-ON

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Department of CHEMICAL Engineering

The University of British Columbia
Vancouver 8, Canada

Date Jan. 16, 1975

ABSTRACT

A clarified, nonheat coagulable fish medium derived from condensed fish solubles (B.C. Packers, Richmond, B.C.) was explored for its potential use as the major component of a fermentation medium for the production of proteolytic enzymes. The organism used was a pure strain of Sorangium. Factors affecting the bacterial metabolic activity and consequently the rate and extent of protease formation were studied.

Statistically designed experiments showed that both calcium and glucose had a significant effect on growth and protease formation, the latter supplementary nutrient being the more influential. Of the six carbohydrate sources (glucose, mannose, galactose, xylose, arabinose, and soluble starch) tested, only glucose and mannose were utilized for further growth. Neither initial pH (in the range 4 to 8) nor inoculum age (16 to 24 hrs) significantly affect the maximum yield of protease.

Kinetic studies with the optimum medium in 250 ml Erlenneyer flasks and in a 7-litre fermentor revealed that growth was biphasic with an initial fast growth followed by a slower secondary growth. The staged growth was attributed to the initial, fast utilization of easily available nutrients (such as amino acids) originally present in the medium, followed by a second, slower reaction in which the larger polypeptide units in the medium were digested. Since the inflection in the growth curve was entirely eliminated by increasing the agitation and/or aeration rate(s), it was thought that dissolved oxygen concentration and rate of oxygen

transfer might be the rate limiting factors.

Kinetic analysis of the data showed that product formation was not associated by any direct mechanism with carbohydrate utilization. Protease formation was highest with an agitation rate of 500 rpm and an aeration rate of 2 litre of air per minute, which corresponded to an oxygen transfer coefficient of 0.805 millimoles O_2/atm. min. ℓ. Recommendations for further work are suggested.

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NOMENCLATURE

Abbreviations

BSA	Bovine Serum Albumin
CFS	Condensed Fish Solubles
DNS	Dinitrosalicylic Acid
FPC	Fish Protein Concentrate
NB	Nutrient Broth
NBS	New Brunswick Scientific
OD	Optical Density
SCE	Salmon-canning Effluent
SCP	Single-cell Protein

Symbols

df	Statistical number of degrees of freedom
E	Enzyme
e	Enzyme concentration
ES	Enzyme - Substrate complex
F	F-test statistic
k_1	Forward reaction rate constant
k_2	Reverse reaction rate constant
k_3	Reaction rate constant
k_m	Michaelis-Menten constant
K_v	Volumetric transfer coefficient

Symbols (contd)

M	Molarity
MS	Statistical mean square
N	Normality
P	Product
S	Substrate
s	Concentration of total substrate
SS	Statistical sum of squares
SSE	Sum of squares of the residual error after some mathematical model has been applied to the experimental data
t	Time, usually in hours
v	Rate of product formation
x	Cell mass

Chapter 1

INTRODUCTION

1.1 Nature of the problem

The salmon-canning and the herring processing operations (either for reduction to meal and oil, or for food purposes) on the West Coast cause major problems of liquid waste disposal. Average effluent flow from these processing operations are of the order of 200 to 1000 U.S.G.P.M. for most plants [1]. These large wastewater flows result from the butchering, washing and processing of the product. The major pollutants in these effluents are flesh particles, scales, blood, slime and soluble proteins. When oily fish, such as menhaden, sardines, mackerel, and herring are processed, free oil in the form of an emulsion is also present as a contaminant in these wastewaters. Table 1.1, which is taken from Claggett [1] shows typical parameters of these wastewaters.

Previously, these effluents were discharged to waterways, or to sewage treatment plants; the proteinaceous matter decomposed rapidly, evolving CO_2, H_2S and other gases, thus giving rise to serious pollution problems. Owing to increasing pollution control legislation, other methods of disposing of these wastes have been studied. The more useful methods described so far have concentrated on recovering as much of the proteinaceous and oily material as possible. Claggett [1, 2, 3, 4, 5] apparently the most versatile investigator in this area, recently described a method in which

Table 1.1

PARAMETERS OF VARIOUS FISH PROCESSING PLANT EFFLUENTS

SPECIES PROCESSED	BOD		SUSPENDED SOLIDS		TOTAL SOLIDS	
	mg/l	#/1000# fish	mg/l	#/1000# fish	mg/l	#/1000# fish
Halibut	200	4	350	7.2	500	10.3
Grey Cod	435	2.2	300	1.5	600	3.0
Lingcod	460	4.1	235	2.2	550	5.2
Sole	200	1.4	125	0.8	300	1.9
Ocean Perch	75	0.7	50	1.3	85	2.2
Salmon Canning	3500	28	1500	12	3000	24
Food Herring	3850	22	3000	21	6000	42

the BOD load of salmon canning wastewaters could be reduced by over 90%. This method involves chemical treatment and air flotation. The sludge from this process is used for the production of a meal which could replace the conventional fish-meal in animal feed. One of the by-products from the fish meal production process is fish stickwater. At present this stickwater is used to make fish solubles which are used as vitamin supplements in animal feed.

In view of the rich protein content of the fish waste, another possible outlet is to use it as an inducer for the production of microbial proteases. Such a process would be quite contemporary and it has the potential of killing two birds with one stone; it can serve as a pollution abatement system as well as an enzyme production process. The scarcity of microbial enzymes together with their rising importance in industry provide the source of optimism for the development of an economically feasible process.

1.2 Fish Stickwater and Fish Solubles

An excellent account of the origin and properties of fish solubles is given by Soderquist [6]. A summary of the salient points is presented here.

Fish solubles are made from fish stickwater which is a by-product from fish-rendering processes. The fish and/or fish waste are steam-cooked to denature the fish protein. This material is then separated under pressure

into an aqueous extract and fish pulp. The fish pulp is dehydrated to a meal, containing about 8% moisture, which can be sold as a fertilizer or as an animal feed. The press water is screened to remove any solids and then passed through a gravity separator or a centrifuge. The sludge from this process is returned to the press cake and the clarified press liquor is passed to an oil centrifuge. The oil centrifuge yields two products, namely, the fish oil and the stickwater. The stickwater is treated with acid in holding tanks and then transfer to an evaporator. The evaporator reduces the water content from 95% to 50%. "Solubles" are produced in the form of a brown, somewhat viscous liquid with a mild, fishy odour. Figure 1.1 (taken from Soderquist) shows the process as described.

The approximate composition of stickwater and condensed fish solubles are given in Tables 1.2 and 1.3 respectively [7].

1.3 Fermentation Culture Medium

The nutritional requirements of micro-organisms are diverse because they differ inherently in ability to synthesize essential growth factors from simple nutrients. However, all micro-organisms demand water, carbon, nitrogen and mineral elements, as well as access to hydrogen and oxygen [8].

The development and formulation of culture medium is one of the most diverse steps of a fermentation programme; it requires a lot of frigging around. The type of medium used depends upon several interrelated

Figure 1.1

FISH RENDERING PROCESS

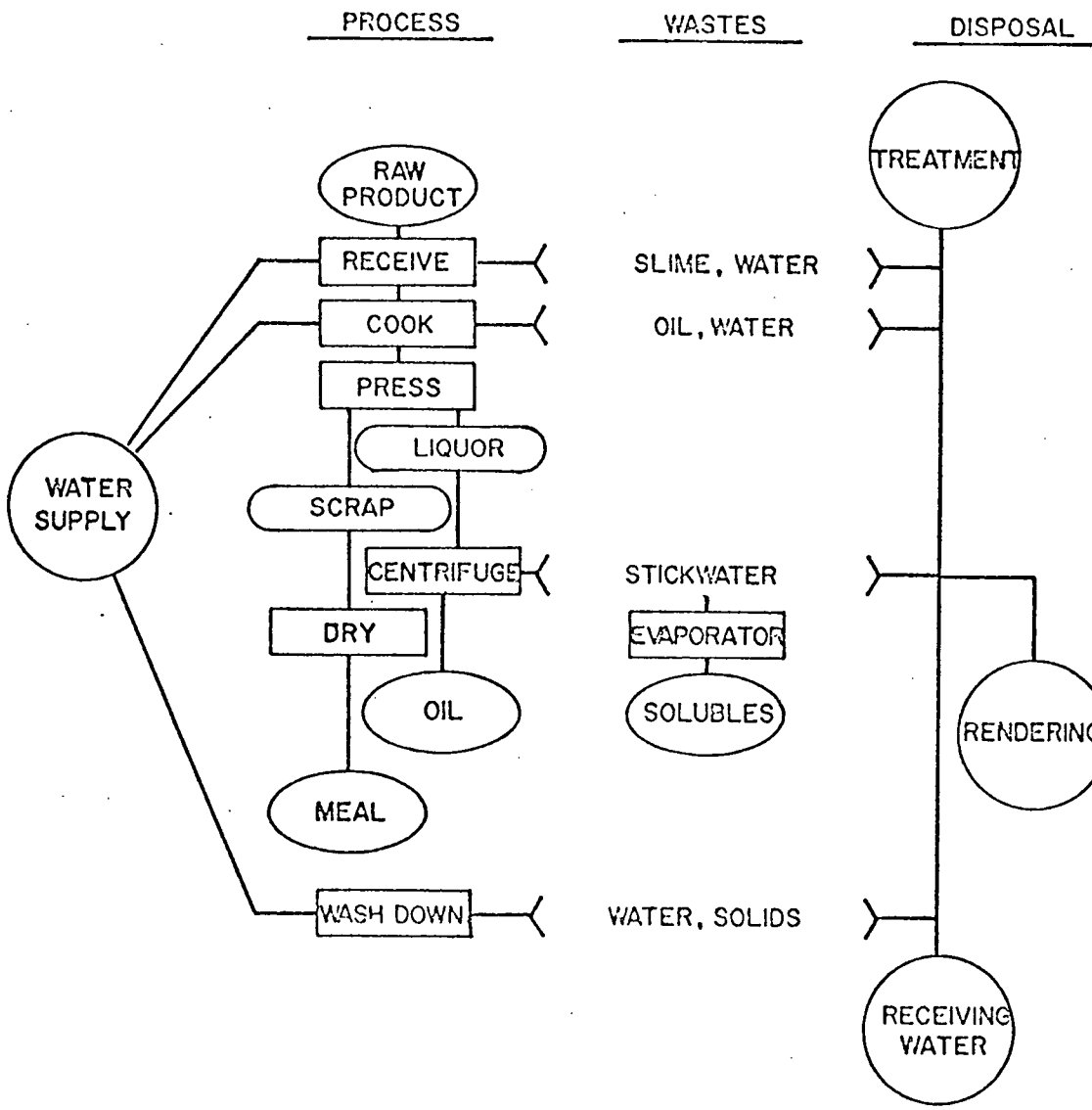


Table 1.2

TYPICAL ANALYSIS OF FISH STICKWATER

PARAMETER	VALUE
Total solids	5.6%
Ash	0.95%
Fatty substances	0.60%
Crude protein (N × 6.25)	3.5%

Table 1.3

TYPICAL ANALYSIS OF CONDENSED FISH SOLUBLES

PARAMETER	VALUE
Total solids	50.43%
Ash	8.86%
Fat	4.8%
Crude protein (N × 6.25)	33.85%
Sp. gr. at 20°C	1.20
pH	4.5

variables. Cost is almost always a factor in determining the suitability of any industrial fermentation medium. When large quantities of culture medium are required, the excellent and covenient types of standardized laboratory media as supplied by Difco, Oxoid, BBL, etc. are very uneconomical, it is therefore necessary to find alternative sources of nutrients. Obviously such materials, because they must be cheaper, cannot be as pure or as standardized as their laboratory counterparts. Industrial fermentation media are therefore usually complex [9]. Their compositions are usually formulated to promote the desired metabolic patterns of the micro-organism under examination; however, since knowledge of the latter is usually limited, development is done on an adhoc basis, although underlying principles can sometimes be incorporated.

A protease is an enzyme that hydrolyses proteins and therefore the fermentation medium must supply the nutritional stimulus to cause it to do so. The following sections discuss the suitability of salmon-canning wastewaters and fisheries by-products as the major component of a fermentation medium for protease production.

1.3.1 Salmon-Canning Wastewater as a Microbial Growth Medium

Strasdine and Melville [10] showed that salmon-canning effluent (SCE) can support the growth of six species of bacteria when used both as a component of a complex medium and as the sole source of available nitrogen. Tables 1.4 and 1.5, are adapted from Strasdine and Melville [10]. Table 1.4 indicates the total cell yield for each of the six species in media

containing the various nitrogen sources together with K_2HPO_4 (0.2%), $MgSO_4.7H_2O$ (0.02%), $FeSO_4.4H_2O$ (0.002%), $MnSO_4.4H_2O$ (0.002%) and dextrose (0.5%). Table 1.5 indicates the ability of SCE to serve in the capacity of a complete medium in which no other additions were necessary.

Based on these results, the authors suggest two possible uses for salmon-canning wastewaters, namely : (1) as an inexpensive source of available nitrogen for the microbial degradation and/or utilization of nitrogen-deficient wastes, and (2) as a microbiological medium per se for the production of crude enzyme preparations, and of single-cell proteins. The first suggestion has been worked on to a certain extent by Truong [11], whose main interest was in the production of Vitamin B_{12} and volatile acids (Propionic and acetic in his case) via fermentation of sulfite spent liquor. He was unsuccessful in growing Lactobacillus plantarum, Propionibacteria Freudenreichii and Propionibacteria Shermanii in a medium in which yeast extract was replaced by fish stickwater as the sole source of nitrogen. No work has been reported yet on the crude enzyme production potential of salmon-canning wastewaters.

Why not? The major difficulties which will obviously arise from the use of fish processing wastewaters as a fermentation substrate are (1) large volume of effluent to be processed, (2) rapid biodegradability of the wastes, (3) large fluctuations in composition, and (4) seasonal availability. The first two characteristics give rise to practical difficulties of storage while the third give rise to difficulties in media reproducibility. These problems are less apparent when the more concentrated

Table 1.4

CELL YIELD AS A FUNCTION OF NITROGEN SOURCE FOR

VARIOUS SPECIES OF BACTERIA

N-SOURCE / SPECIE	CELLS/ML OF MEDIA (log_{10})						
	SCE	TRYPTICASE	ACIDICASE	POLYPEPTONE	PEPTONE	PHYTONE	MEAT EXTRACT
Sorangium sp.	10.08*	6.99	9.51	9.37	9.23	8.29	8.68
P. putrefaciens	9.32	6.38	7.00	8.15	5.30	8.36	8.28
L. plantarum	8.18	7.88	5.30	8.11	9.18	7.26	6.46
A. aerogenes	8.67	7.96	7.79	9.63	9.00	9.04	9.57
Bacillus sp.	7.00	8.65	8.15	8.08	7.63	7.83	7.18
St. faecalis	7.79	7.04	7.30	8.69	7.72	8.23	7.63

* Underlined values indicate maximum cell yield.

Table 1.5

CELL YIELD OF SALMON-CANNING WASTE WATER AS A FUNCTION OF CELL NUMBER PER MILLILITER

	CELLS/ML ($\log_{10}$)	% OF MAXIMUM FROM TABLE 1.4	MEDIA GIVING MAXIMUM GROWTH
Sorangium sp.	9.34	19.1	SCE - S
A. aerogenes	8.60	9.3	polypeptone - S
Bacillus sp.	7.20	3.6	trypticase - S
P. putrefaciens	6.83	0.3	SCE - S
St. faecalis	6.76	1.2	polypeptone - S
L. plantarum	6.74	0.3	peptone - S

S : salts

fish stickwater and solubles are used.

1.3.2 Fish Stickwater as a Microbial Medium

Fish stickwater, as stated earlier, is an aqueous extract resulting from the pressing of cooked fish during fish meal production. Stickwater or presswater, as it sometimes called, is also the raw material for the manufacture of condensed fish solubles. Stickwater exhibits properties which are characteristic of both fish muscle extracts and the gelatin and glue-containing extracts of bones, cartilage, skin, and other connective tissues [12]. A typical analysis of some of the main constituents of stickwater is shown in Table 1.2.

The nitrogen-containing substances in stickwater consist largely of noncoagulable, water-soluble proteoses, peptones, highly dispersed protein particles, and nitrogenous extractives [6, 7]. These extractives include such compounds as ammonia, mono-, di, and trimethylamine, urea, amino acids, guanidine derivatives (creatine and arginine) and imidozole derivatives (histidine and carnosine). If the proportion of these extractives, which are not considered to be food protein sources, is unduly high, the conversion factor of 6.25, which assumes that the average protein contains 16.0% of nitrogen, gives a falsely high value for the protein content. The fatty substances are highly dispersed and are intimately tied to the protein particles to give the stickwater an opaque or milky appearance. These suspended particles can be precipitated by adding a flocculant like aluminium sulfate or by a change in the pH of the stickwater.

Although the stickwater contains many desirable biological growth factors, its highly perishable nature, even at very low temperatures, makes it very unpleasant to work with, and very difficult to store. It is a well recognised, but rarely-mentioned, fact that for a substrate to be industrially acceptable, the substrate must not only be appealing to the microbes but it must also not create an adverse employee reaction.

1.3.3 Fish Solubles as a Microbial Medium

Fish solubles, as mentioned earlier, is acidified and concentrated stickwater. Digestion with acid degrades the proteins present in stickwater to smaller molecules, thus making it more desirable as a bacteriological culture media. Acidification also reduces the problem of spoilage; concentration reduces storage space and enables a wider range of concentration to be examined.

Tables 1.6, 1.7, 1.8, adapted from Lassen [7], reflect the average content of some of the more important amino acids, vitamins and ash constituents respectively. These, together with some other unidentified growth factors [13] reveal the reason why condensed fish solubles have attained such prominence in the field of animal nutrition. However not much revenue is obtained fron this use.

Table 1.9 compares the prices of commercially available protein sources which can be used as the major component of a fermentation medium for protease production. Most obvious is the relatively low cost of

Table 1.6

AMINO ACID ANALYSIS OF CONDENSED FISH SOLUBLES

AMINO ACID	% CRUDE PROTEIN (N × 6.25)	ASSAY METHOD
Arginine	4.84	Mb.[a]
Histidine	5.79	Chem.[b]
Lysine	4.87	Mb.
Leucine	4.67	Mb.
Isoleucine	2.73	Mb.
Phenylalanine	2.33	Chem.
Tryptophan	0.35	Mb.
Methionine	1.51	Mb.
Threonine	2.55	Chem.
Cystine	0.58	Chem.
Glutamic acid	8.44	Chem.
Proline	6.70	Chem.

[a] Mb. = microbiological.

[b] Chem. = chemical.

Table 1.7

TYPICAL VITAMIN CONTENT OF WEST COAST FISH SOLUBLES

VITAMIN	µg/g	VITAMIN	µg/g
Riboflavin	22	Pyridoxin	12.5
Pantothenic Acid	84	Choline	1100
Thiamine	7.5	Folic Acid	0.23
Niacin	390	Vitamin B_{12}	0.47

Table 1.8

TYPICAL ANALYSIS OF FISH SOLUBLES ASH CONSTITUENTS

CONSTITUENT	%	CONSTITUENT	%
Potassium (K)	1.93	Iron (Fe)	0.0249
Sodium (Na)	1.87	Magnesium (Mg)	0.016
Phosphorous (P)	0.85	Copper (Cu)	0.007
Calcium (Ca)	0.0869	Iodine (I)	0.007
Manganese (Mn)	0.0869	Aluminum (Al)	0.005
TOTAL ASH		8.86%	

Table 1.9

ALTERNATE PROTEIN SUPPLY SOURCES [14, 15]

MATERIAL	PRICE/POUND* (cents)	PRICE/POUND PROTEIN (cents)
Soy Meal and Flour	3.5-6.5	8-14.8
Soy Protein Conc.	21.5	26.5-35
Fish Solubles	2.50	7.8
Fish Meal (feed grade)	6.3-8.5	10.5-14.2
Fish Protein Conc.	10-16	13-20
Cottonseed Flour	11	20
Wheat Flour	6.6	60
Dry Skim Milk	14.4-21.0	40-60
Yeast - Torula (sulfite waste)	15-16	27-29

* Based on 1969 market.

condensed fish solubles.

For the reasons cited above, this work, which is to investigate the protease production potential of condensed fish solubles, emerges.

1.4 Microbial Proteases

All proteolytic enzymes, or proteases, whether they are of animal, plant, or microbial origin are characterized by their ability to catalyze hydrolytic cleavage of peptide linkages between amino acids [16, 17, 18, 19]. These enzymes are generally classified into two distinct groups [20, 21], namely, the proteinases (or endopeptidases) and the peptidases (or exopeptidases). The proteinases are enzymes which attack internal peptide bonds in the high molecular weight polypeptide protein molecules; peptides such as proteoses and peptones are usually formed from this reaction. The peptidases are enzymes which attack terminal peptide bonds adjacent to free polar groups thus resulting in the liberation of free amino acids [17].

All micro-organisms which utilize proteins as nutrient materials produce proteolytic enzymes for the hydrolysis of the proteins to the amino acids necessary in their metabolism for the production of their own cellular and enzyme proteins. The majority of microbial enzymes studied to date have been extracellular, isolated in active form from the culture filtrates of the appropriate organism. These extracellular enzymes are predominantly endopeptidases and can be divided into three main groups, namely, acid,

neutral, or alkaline, depending upon the pH range in which their activity is greatest [22, 23, 24].

The few microbial proteases presently available commercially are from bacterial and fungal sources; they are usually sold in the crude from, which may contain a variety of other enzymes in varying amounts. Hence their enzymatic action is extremely complicated. Notwithstanding the impurities present, these microbial enzymes are enjoying wide applications in industry. Some of these applications [19, 20, 23], most of which were developed from empirical, trial and error methods, are in bread making, meat tenderizing, spot removal in cleaning, dehairing of hides, chill proofing of beer, plaque removal from teeth, and protein hydrolysate manufacturing. In 1969, over 4 million dollars' worth of proteolytic enzymes were marketed [25].

1.5 The Micro-organism Used - Sorangium 495

The organism chosen for this study was a soil bacteria belonging to the genus Sorangium. This organism, designated isolate No. 495, was isolated by Gillespie and Cook [26] in 1964. It was obtained for this study from G.A. Strasdine, Vancouver laboratory, Fisheries Research Board of Canada, who, as mentioned earlier, showed that this specie gave the highest cell yield in media containing SCE as the sole source of available nitrogen.

A perusal of "Bergey's Manual of Determinative Bacteriology" [27] shows that the genus Sorangium is a member of the family Sorangiaceae which belongs to the order Myxobacterales. Members of this Order can be distinguished from a true bacterium by two principal features : (1) its characteristic gliding, non-flagellar movement, which occurs only in contact with solid surfaces, and (2) its flexibility, due to the delicate nature of the cell wall. The vegetative cells of the Myxobacteria are gram-negative rods which during vegetative growth remain perfectly independent of one another, although they tend to be held in association by a thin layer of slime which they synthesize during movement and which keeps them together in a loose colony. Such a myxobacterial colony can give rise to structures known as fruiting bodies, which are formed by the co-operative action of many thousands of vegetative cells [28, 29]. Direct photometric methods of measuring growth are therefore impossible.

The vegetative cells of the family Sorangiaceae are short, rigid cells with blunt ends (1.2 × 2.5), and thus differ from the members of the other families (Myxococcaceae, Archangiaceae, and Polyangiaceae) which possess vegetative cells of the long, flexuous, tapered type (0.5 × 5 - 10) [30].

The Sorangium species are predominantly soil forms. Many isolates are strongly cellulolytic, although some do not possess this property. The work of Gillespie and Cook [26] and later, Whitaker, Cook and Gillespie [31] showed that their isolate, Sorangium 495, was able to to produce at least two extracellular enzymes which are separable by

chromatography on hydroxyapatite: (1) a lytic enzyme which effects complete lysis of various species of Staphylococcus, Bacillus, Sarcina, and Arthrobacter, and partial lysis of bacteria from several other genera; (2) a protease with hydrolytic activity towards casein and denatured hemoglobin but without lytic activity towards the above organisms. Gillespie and Cook [26] studied the change in proteolytic activity with changing pH for both casein and haemoglobin. Their results showed that the pH optimum for both substrates was at pH 8.5.

Katznelson et al [32] have also observed that certain soil nematodes were lysed by this strain of Sorangium. Subsequent work by Whitaker [33], Jurášek and Whitaker [34], Whitaker et al [35], Tsai et al [36], showed that there were two enzymes which were responsible for the high lytic activity. They called these two enzymes α- and β-lytic proteases.

Recently, owing to population increase and land shortage, much concern has been expressed about a world protein shortage in the future. Microbiologically-synthesized protein, usually termed single cell protein (SCP) due to the unicellular nature of its parent micro-organism, is one way of supplementing the conventional proteins from agriculture and the fishing industry [37, 38]. Micro-organisms have many attractions as protein supplements: they multiply rapidly, are easily handled, and can be grown econimically on inexpensive organic materials such as sulfite liquors, fish wastes, hydrocarbons, molasses, whey and other agricultural wastes [38]. However, before these SCP can be used as food for human consumption, the cell wall, which is resistant to digestion, must be

disrupted. Lytic enzymes from selected micro-organisms such as Sorangium 495 may prove valuable for this purpose. Another possible use for lytic enzymes is in the study of cell wall structure [39].

The enzyme used by Gillespie and Cook [26] in their study was obtained from shake cultures in flasks containing 50 - 500 ml. of medium, while that used by Whitaker and co-workers was produced in a fermentor containing 100 - 130 litres of medium. The composition of the culture medium (in gms/l) in most cases was as follows : Difco Casamino Acids, 10; Glucose, 1; K_2HPO_4, 1; KNO_3, 0.5; $MgSO_4 . 7H_2O$, 0.2; NaCl, 0.1; $FeCl_3 . 6H_2O$, 0.01; and NaOH to adjust the pH to 7.0 - 7.1 if necessary. The glucose was autoclaved separately, and the temperature of cultivation was 25°C. No attempt was made by these authors to examine conditions for maximal production of enzymes. The protein source, Casamino Acids, was rather expensive.

1.6 Nutrition of Myxobacteria

The myxobacteria are not very fastidious microorganisms [30, 40, 41]. They are ubiquitous inhabitants of normal soil, bark, and decaying plant materials. They are cosmopolitan, having been reported in the soils of Poland, Great Britain, Austria, Sweden, India, Russia and Japan [42]. The myxobacteria are capable of hydrolyzing particulate insoluble substrates, such as starch, glycogen, protein, cellulose, and bacterial cell walls. Recently, Martin and So [43] have shown that certain strains of myxobacter have the ability to utilize autoclaved keratinaceous substrates, in the

form of feathers and wool, as sole sources of carbon and nitrogen. It has been suggested, in the review by Dworkin [42], that the gliding motility and the nutritional dependence of the organism on its ability to solubilize insoluble macromolecules are related to each other. He said :

> An organism whose ecological niche is an aquatic one may depend on its flagella motility and random collisions to bring it into contact with soluble substrate molecules. A terrestrial organism dependent on insoluble macromolecules for its sustenance is obliged to creep or crawl to its food.

For building proteins, the fruiting myxobacteria require complex nitrogenous materials [41] or mixtures of amino acids [40]. Baur [44] reported that these organisms could be cultivated on media containing peptone. Also Loebeck [45], and Dworkin [40] have shown that although the essential nutritional requirements could be supplied by free amino acids, peptides and hydrolyzed proteins were better substrates for growth.

According to Dworkin [40], a small number of sugars including fructose, glucose and arabinose have negligible effect on the growth rate of some strains of myxobacteria; however, McDonald and Peterson [41] reported that glucose was distinctly the carbon source of choice for the two species they examined, and that the deletion of it from the medium in favor of any other carbon source resulted in marked inhibition of growth. Norén [46] has shown that his species could attack a large number of carbohydrates and that growth was stimulated by the presence of maltose and arabinose. Chase [47], on the other hand, was unable to demonstrate any effect of carbohydrates on the growth of his species in a defined

medium. It appears therefore that there are significant differences in responses to carbohydrate sources among strains of myxobacteria. While such may be the case for carbohydrates, there seems to be unanimous agreement [40, 41, 47] that, in general, the myxobacteria do not possess requirements for a particular vitamin or amino acid, nor do they appear to have any particularly exotic requirements for growth.

1.7 Objectives of this work

The objectives of the present work are :

1. To determine the suitability of condensed fish solubles as the major component of a fermentation medium for the growth of a pure strain of Sorangium 495.

2. To determine the pre-treatment required before the Sorangium specie will grow well on the fish waste medium.

3. To determine the amounts of supplementary nutrients (glucose, calcium, phosphate) required for optimum growth and protease production.

4. To determine which of the carbohydrate sources (hexoses, pentoses, and starch) can be utilized by the micro-organism for growth and protease production.

5. To determine the effects of agitation and aeration on the rates of growth and of production.

6. To determine from an analysis of the batch kinetic data the relationships between rates of growth, substrate utilization and protease production of the Sorangium specie.

Chapter 2

EXPERIMENTAL TECHNIQUES

2.1 General

Biological experiments are known for their large number of variables. Due to the tedious and time consuming nature of these experiments, it is impractical, although possible, to examine the effects of all the variables one can conceive, even with the help of factorial experimentation. The procedure adopted here was as follows : after using factorial design [48, 49, 50] to determine the most influential supplementary nutrient among those known to affect protease production, this important nutrient was then further studied at additional levels. In subsequent experiments, other process variables were studied one at a time. Such experiments assume that there is no interaction between the variables [49].

2.2 Preparation of Culture Medium

All work has been carried out with a single batch of condensed fish solubles. This material was supplied by B.C. Packers, Richmond, B.C. When not in use, the solubles were refrigerated at 6°C. Under these conditions spoilage was largely prevented. Table 2.10 shows the dry weight and pH of the solubles measured over a period of seven months. The negligible change in pH value supported the view that there had been negligible decomposition of the solubles.

Table 2.10

TIME VARIATION IN pH AND DRY WEIGHT OF REFRIGERATED CFS

DATE	pH	wt/vol,%
23/9/73	3.65	52.76
12/10/73	3.66	52.21
28/10/73	3.78	52.63
28/10/73	3.78	51.61
8/11/73	3.80	52.47
21/11/73	3.75	52.10
4/12/73	3.92	52.45
7/1/74	3.75	51.74
31/1/74	3.65	51.80
12/2/74	3.75	51.87
26/2/74	3.80	52.81
Average	3.75	52.20
σ	$\pm$ 0.0836	$\pm$ 0.43

The condensed fish solubles used in the fermentations were prepared by dilution with tap water. Almost all the runs were made with diluted fish solubles that had been filtered through Reeve-Angel glass fibre filter papers to give a clear media containing little or no coagulable proteinaceous materials. The average soluble protein content (measured as Bovine Serum Albumin equivalent) at various dilutions is shown in Table 2.11. The table also shows the average glucose content at these dilutions.

It was determined early in this study that the diluted solubles (filtered or unfiltered) could be used as a complete medium without any other additions. However, the addition of glucose as a carbon source increases the protease yield (Section 3.2).

The pH of the medium was adjusted to 7.0 with 2.5N or 6N Sodium Hydroxide before sterilization. Initially, sterilization was accomplished by autoclaving the fermentation mixture in direct contact with steam at 15psig. for 15 minutes when small volumes (100mls or less) were used, and for 30 minutes when large volumes (4 litres) were used. Later on, when the "Energy Conservation Act" came into effect (around December 1973), the maximum steam pressure attainable in the sterilizer was 10 psig; under such a crisis the sterilization time was increased by 15 minutes in both cases. There was a slight drop in the pH of the medium during sterilization. This is probably due to the loss of volatile bases. No attempt was made to re-adjust the pH before inoculation.

Table 2.11

ANALYSIS OF FILTERED CFS AT VARIOUS DILUTIONS

DILUTION (v/v)	PROTEIN mg BSA/ml	GLUCOSE mg/ml	DRY WEIGHT mg/ml
10/1000	1.87	-	-
20/1000	3.85	0.062	7.9
30/1000	5.22	-	10.8
40/1000	6.75	0.087	14.0
50/1000	8.66	-	18.06
60/1000	10.20	0.105	21.44

2.3 Inoculum Preparation

Cultures of the Sorangium species were maintained on a Brain Heart Infusion agar slant prepared by dissolving 37 gm of Brain Heart Infusion agar (Difco) and 15 gm Agar in 1,000 ml distilled water. After 2 - 3 days growth at 30°C, the cultures were stored at refrigeration temperature (6°C).

The organism was grown at 30°C in two stages in 250 ml Erlenmeyer flasks, containing 100 ml of a Nutrient Broth (Peptone, 0.5%; Beef Extract, 0.3%) medium, to provide the inoculum for preliminary studies in shake flasks and for kinetic studies in 7-litre fermentors. The inoculum for the first stage was prepared by a transfer from the refrigerated culture. In this stage, the organism was induced to grow in the Nutrient Broth medium. When heavy growth was obtained (after about 1 - 2 days), 1 ml of this actively growing culture was used as the inoculum for the second stage which employed the same medium as the first stage. The inoculum for the fish medium was taken from the culture resulting from the second transfer. The transfer was made after 15 - 24 hrs growth, when the organism was in the exponential growth phase. The amount of inoculum used was, in ratio of inoculum to fresh medium, 1 : 100 for Erlenmeyer flasks and 1 : 40 for 7-litre fermentors.

Inoculum prepared in this way reduced the chance of contamination. This is especially important for myxobacteria because, even though several of the myxobacteria (including the specie used in this work) produce

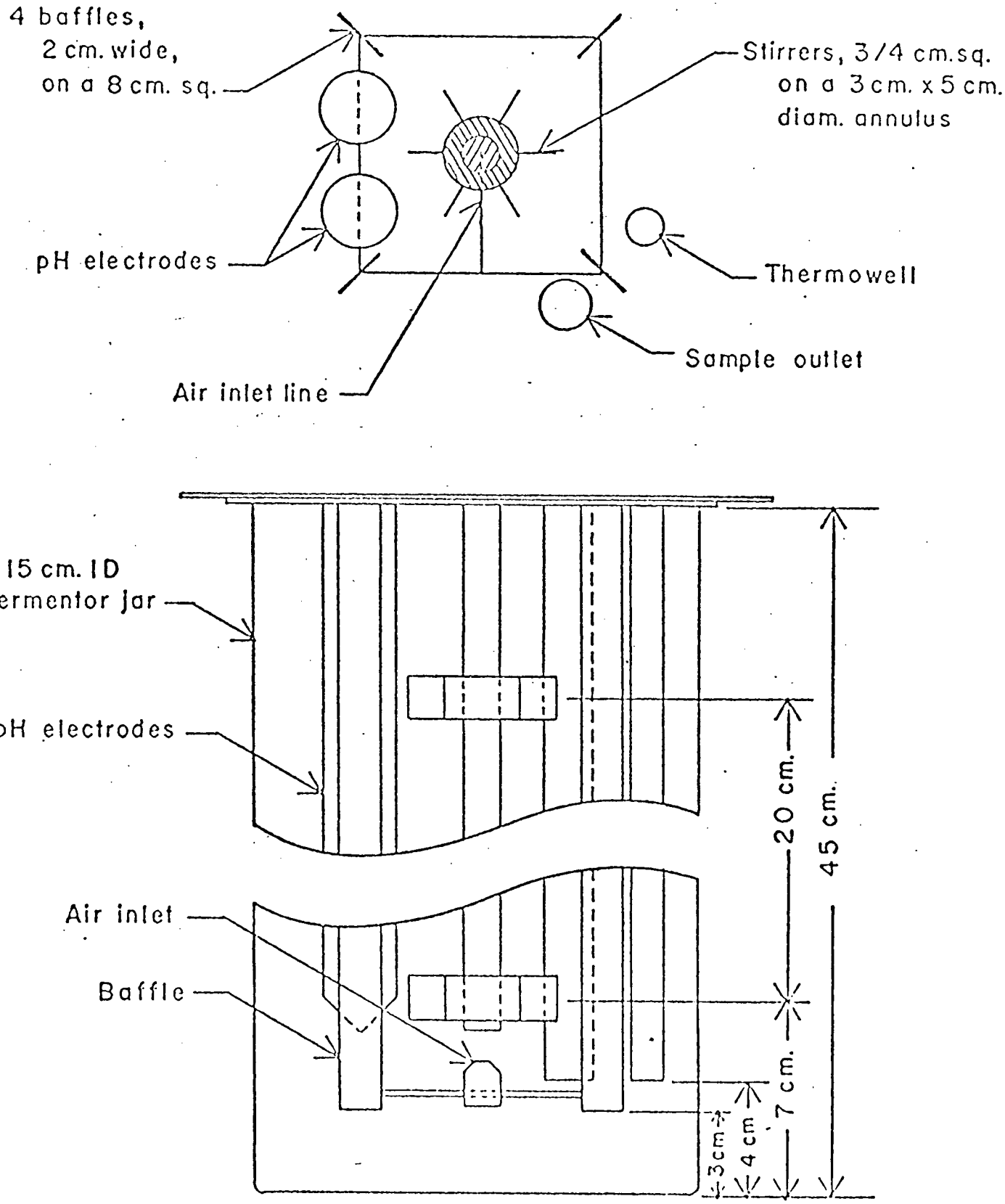
antibacterial and antifungal substances [42], they are poor competitors and can therefore be overgrown by the more rapidly growing eubacteria when both are placed on a rich medium [30].

2.4 Apparatus

Most of the preliminary experiments to test the influence of medium composition on yields of cells and enzymes were carried out in standard 250 ml Erlenmeyer flasks stoppered with non-absorbent cotton. The micro-organisms were cultivated on a New Brunswick Scientific (NBS) incubator-shaker working at 140 rpm with a gyrotory radius of 1/2 inch, at a constant temperature of 30°C.

For the study of agitation and aeration effects, a NBS model MF-207 bench top fermentor was used. This model consists of two 7-litre, cylindrical glass growth vessels, each 15 cm in diameter and 45 cm high. Four stainless-steel baffle-plates, each 42 × 2 cm, were fixed to the head plate. They were joined together by stainless-steel tubing at the bottom. The baffle plates were placed at right angles with their surfaces perpendicular to the fermentor walls and their lower ends 3 cm above the bottom of the vessel. The baffle assembly was utilized for the introduction of air. An agitator shaft with two adjustable impellers was centrally mounted through the head plate. The impellers were made of six 3/4 × 3/4 cm stainless-steel blades, set 60° apart with their flat surfaces perpendicular to the bottom of the vessel. Figure 2.2, adapted from Ferguson [51], is a schematic drawing of the fermentor

Figure 2.2 SCHEMATIC DRAWING OF 7-LITRE FERMENTOR



described above. With this unit, it was possible to ferment up to 4 litres of broth; control the temperature to within 1°C of a set point within a range of 10°C to 50°C; control the agitation rate from 10 to 900 rpm; and to control the air flow rate from 1 to 16 litre/min. [51, 52]. In addition, the fermentor could be sterilized with the medium in it; samples coulb be removed and additions could be made, under aseptic conditions, at any time during the fermentation [51].

2.5 Sampling Technique

1. Shake Flasks

Each treatment in an experiment was carried out in duplicate, samples of the two replicates were mixed at harvest time, the mixture was centrifuged to remove the organisms. The filtrate was saved for later assays.

2. 7-l Fermentor

A NBS model S20 sampling-inoculator was used to withdraw samples at various time intervals. This device consists of a removable glass collecting tube and a built-in air filter chamber. It can be sterilized with the Fermentor or separately.

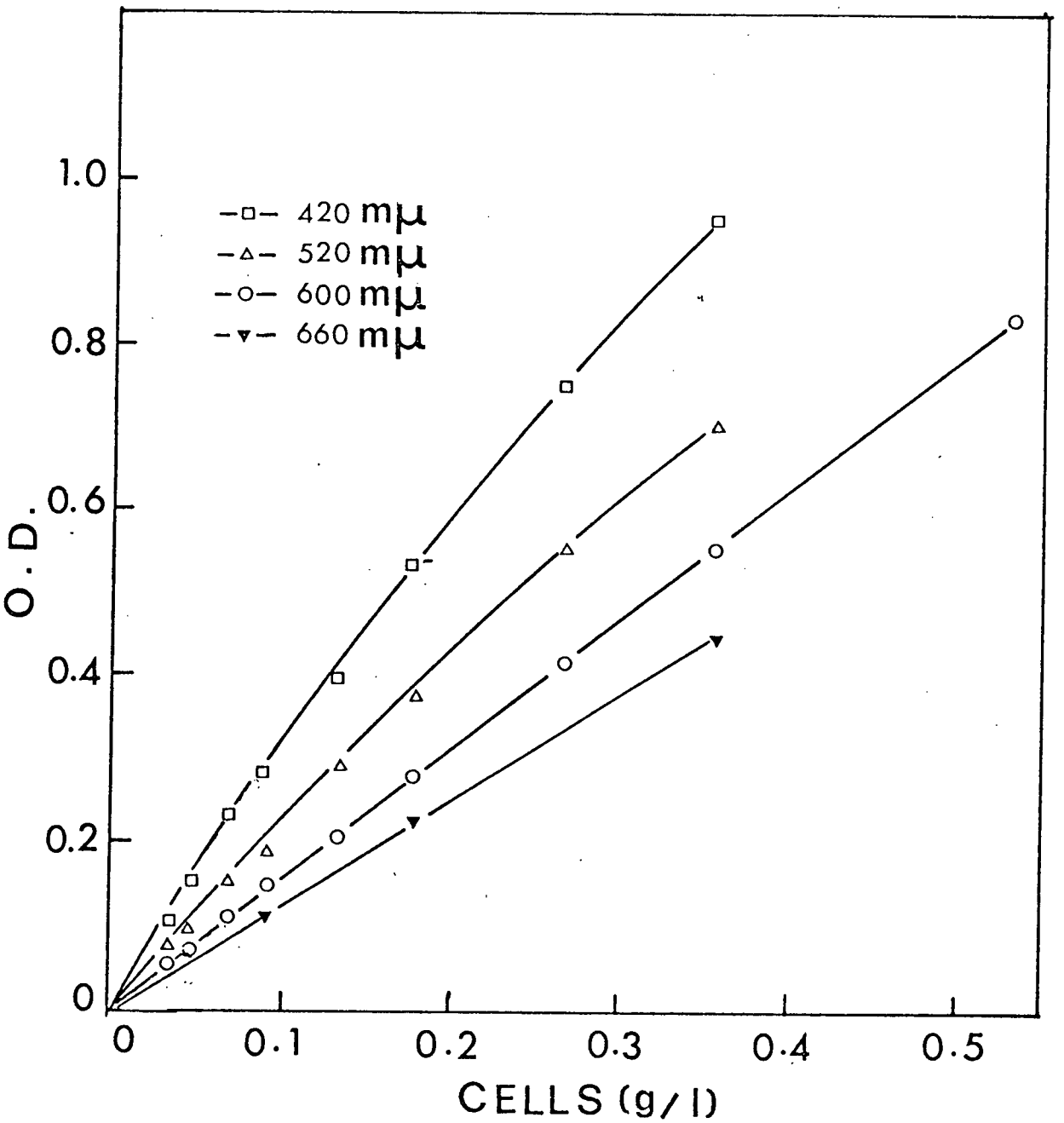
2.6 Measurement of Bacterial Growth

Several methods for the quantitative estimation of bacterial growth are available. Among them are : turbidity [53], dry cell weight [54], dilution or plate count [30], and ATP content. Viable counts are measures of cell concentrations (that is, number of individual cells per unit vol. of culture), and are essential only in problems where cell division is of interest. Turbidity and dry cell weight are measures of bacterial density (that is, dry weight of cells per unit volume of culture), and, according to Monod [55] are not equivalent to the cell concentration; this is because the average sizes of the cells vary considerably from one phase to another of a growth cycle. Since the fish medium was clarified prior to culture, bacterial density is sufficient as a measurement of growth for the purposes of this study. Due to the formation of slimy matter, and also since there was considerable color variation in the medium during growth, direct turbidimetric methods could not be used to follow growth.

Although a linear relationship was shown to exist between the turbidity of a washed cell suspension and the dry cell weight at various wavelengths (Figure 2.3), all the measurements reported here were made by direct weighing of the dry cells. The dry bacteria cells were obtained as follows. A sample of fermentation broth was centrifuged in an IEC international Centrifuge - Universal Model UV, fitted with an International Multispeed Attachment, for 10 minutes at 22,000 × G. The supernatant from this centrifugation was decanted and saved for later sugar analysis

Figure 2.3

TURBIDITY VS DRY CELL WEIGHT AT VARIOUS WAVELENGTHS



Note :- No attempt was made to test the reliability of this relationship. The samples used for these measurements were taken at one particular stage of growth, and the experiment was not repeated.

and proteolytic activity assay. The cells were re-suspended in distilled water using a vortex mixer and recentrifuged for 10 minutes at 22,000 × G to remove the wash water. This washing and centrifuging procedure was done twice. After the final washing, the cells were dispersed in distilled water and were then transferred to pre-weighted alumimum dishes. These were then dried at 95°C in a Fisher Isotemp oven for at least 12 hours.

2.7 Measurement of Sugar Concentration

Many colorimetric tests for reducing sugars and polysaccharides are available [56]. The two methods tested and used in this work were :

I. Phenol - Sulfuric Acid Reagent Method

This method, developed by Dubois et al [56], is based on the fact that simple sugars and their derivatives give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The authors claimed that :

> The method is simple, rapid, and sensitive, and gives reproducible results. The reagent is inexpensive and stable, and a given solution requires only one standard curve for each sugar. The color produced is permanent and it is unnecessary to pay special attention to the control of the conditions.

The above claims have been confirmed during the course of this work. However, in order to get reporducible results, it was necessary to

make the following cautionary notes :

(1) Owing to the high viscosity of the concentrated sulfuric acid, fast delivery of the acid resulted in a significant amount of the acid being left behind on the walls of the pipet (or burette). Care must be taken to ensure that the exact amount of acid is introduced.

(2) In as much as the reaction is brought about by the heat developed when the concentrated acid and water solutions are mixed, the shape and size of the reaction tube are important. Tubes with an internal diameter of 16 mm. were used. This diameter will allow good mixing without dissipating the heat too rapidly [55]. Good mixing is obtained by directing the stream of acid against the liquid surface rather than against the side of the test tube.

(3) The solution must be poured slowly into the Spectronic 20 tubes just before the readings are made. Rapid transfer resulted in entrained gas bubbles with low rise velocities, these bubbles, if not removed, will interfere with the spectrophotometer readings.

II. Dinitrosalicylic (DNS) Method

This method was developed by Sumner [57] in 1925 to detect reducing sugars in diabetic urine. In this method, the sugar solution was allowed to react with dinitrosalicylic acid reagent (Appendix II.1). This reagent contains sodium hydroxide, dinitrosalicylic acid, Rochelle salt, phenol and sodium bisulfite. According to Sumner [57], the Rochelle

salt prevented the dissolution of oxygen; this was desirable because the dissolved O_2 can destroy part of the sugar. Excess phenol was added so as to enhance the color produced by the sugar. Addition of sodium bisulfite helped to stabilize the color produced when the DNS - phenol solution was heated with glucose.

Figure 2.4 is a comparison of the phenol - sulfuric acid and the DNS tests for sugars. Although the agreement between the two methods was quite good, most of the sugar test results reported in this work were done by the DNS method for the following reasons :

(1) The phenol - sulfuric acid method requires the handling of a highly corrosive reagent. This necessitates the observation of certain obvious precautions which tends to slow down the process.

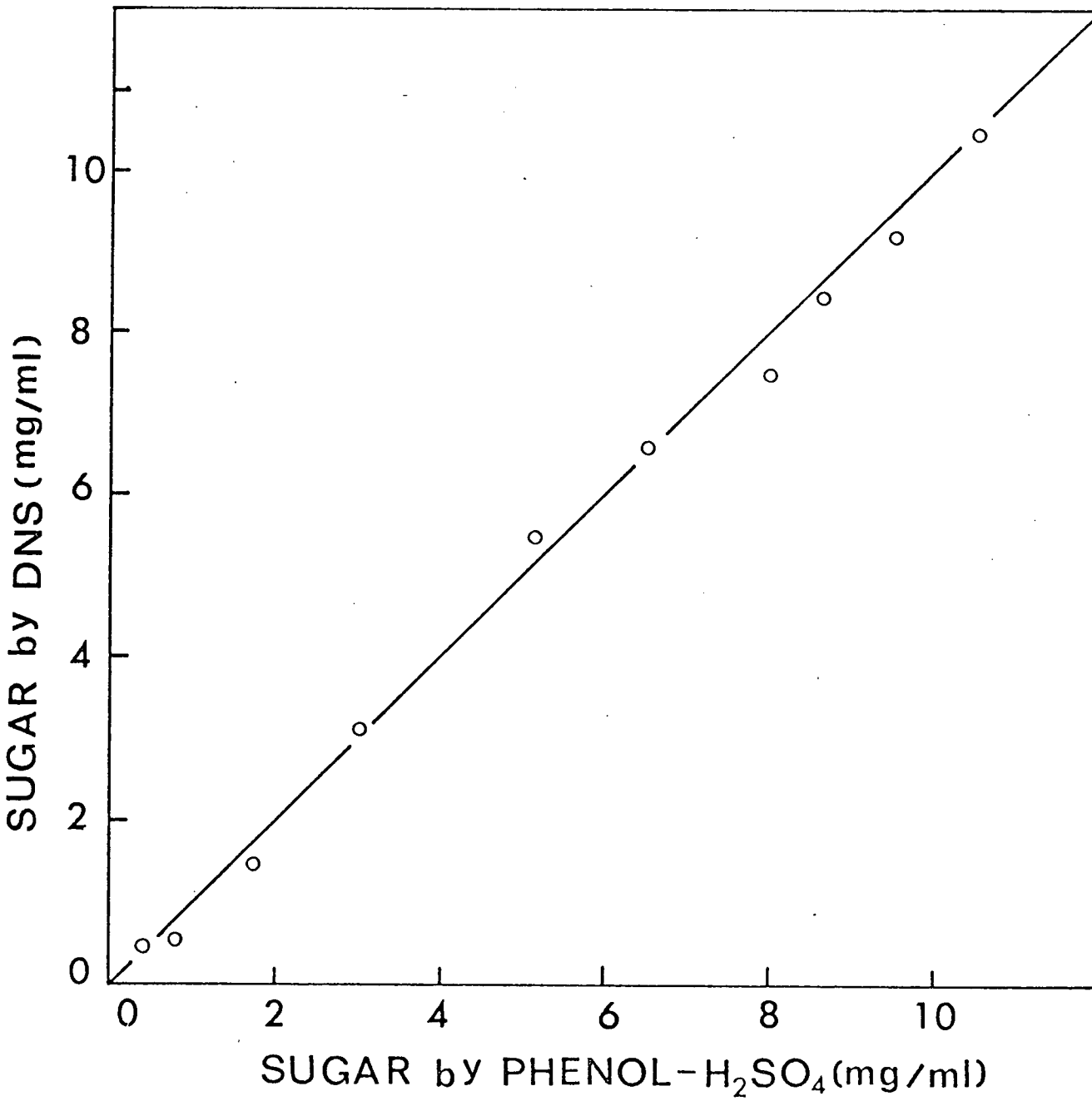
(2) The phenol - sulfuric acid method is four times more sensitive than the DNS method. Since the fermentation concentrations of sugar were usually in the milligram/ml range, more dilutions were required with the phenol - sulfuric acid method (range in μg/ml), resulting in a greater risk of error.

(3) When many samples were simultaneously measured, it was found that the DNS method was faster and easier to handle.

The details of the two test methods and the standard curves are given in Appendix II.1.

Figure 2.4

COMPARISON OF DNS AND PHENOL-H_2SO_4 ASSAYS



2.8 Measurement of Proteolytic Activity

An absolute quantitative determination of crude enzyme preparations by chemical or physical anslysis is not feasible because : (1) the structure of enzymes is generally not well known, (2) most enzymes occur only in trace amounts, (3) most enzymes simply give tests for proteins. Such tests do not distinguish them from other proteins. As a result enzyme quantity cannot be expressed in milligrams or moles of the substance as is valid for other different proteins. The amount of enzyme produced is invariably measured by the results it produces, that is, by its activity.

Enzyme activity is expressed in arbitrary units where the unit has been determined under specified conditions of substrate concentration, temperature, and pH, that is, under conditions where all factors which are known to influence enzyme activity are maintained constant if possible [58]. There often exist several kinds of units for one and the same enzyme depending upon whose method is employed; consequently, in many cases, it is not possible to compare measurements of one and the same enzyme because the different conditions of assay do not allow the use of equivalence calculation factors.

With a view to standardizing the enzyme unit, the Enzyme Commission of the International Union of Biochemistry [59] proposed the following definition of an enzyme unit :

> One unit of any enzyme is defined as that amount which catalyzes the transformation of one micromole of substrate per minute, or where more than one bond of a more complex substrate (eg. protein, poly-saccharide, etc.) is attacked one micro-equivalent of the group concerned per minute under well defined conditions. The temperature should be stated, and it is suggested that where practicable it should be 25°C. The other conditions, including pH and substrate concentration, should, where practicable, be optimal.

It was also recommended that enzyme assays be based, where practicable, upon measurements of initial rate of reaction, in order to avoid complications due, for instance, to reversibility of reactions or to formation of inhibitory products. The substrate concentration should be, whenever possible, sufficient for saturation of the enzyme, so that the kinetics in the assay approach zero order. Concentration of an enzyme in a solution should be expressed as units per millilitre.

Most of the methods which are used to determine the activity of proteolytic enzymes, and are suitable for routine analysis are based on a quantitative determination of the low - molecular weight products (amino acids and peptides) formed by the enzymatic hydrolysis of the substrate protein. Various modifications of Anson's colorimetric method [60] are currently enjoying wide application in research. In this method, the enzyme activity is assessed from the amount of tyrosine contained in the proteolysis products which are not precipitated by trichloroacetic acid. Tysosine is determined from the depth of color which it produces with

Folin's phenol reagent [61].

In view of the large number of samples to be analyzed, a simple and rapid method is desirable. After considering various alternatives, the modification of the Anson method used by Petrova and Vintsyunaite [62] was decided upon. The details of this method are given in Appendix II.2. Conditions in the reacting mixture were maintained as constant as possible during the assays. The reaction was carried out in a constant temperature water bath capable of automatically controlling temperatures within very close tolerances; all reagents were brought to the desired temperature before the reaction was started by mixing them. Adequate buffering was provided by a Tris - HCl buffer prepared as outlined in Appendix II.2. The reaction temperature was 30°C. The pH was buffered at 8.5 - the optimum found by Gillespie and Cook [26] for the proteolytic enzymes produced by the Sorangium specie used in this work.

... For a fixed initial substrate concentration and varying initial enzyme concentrations, the amount of substrate transformed in a given period of time may not be proportional to the amount of enzyme. This is a consequence of the elementary Michaelis-Menten scheme which, in general, may be expressed as [8] :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

The rate of product formation, v, in the enzyme reaction depicted above is given by :

$$v = \frac{k_3 es}{k_m + s} = \frac{k_3 e}{1 + k_m/s} \quad .$$

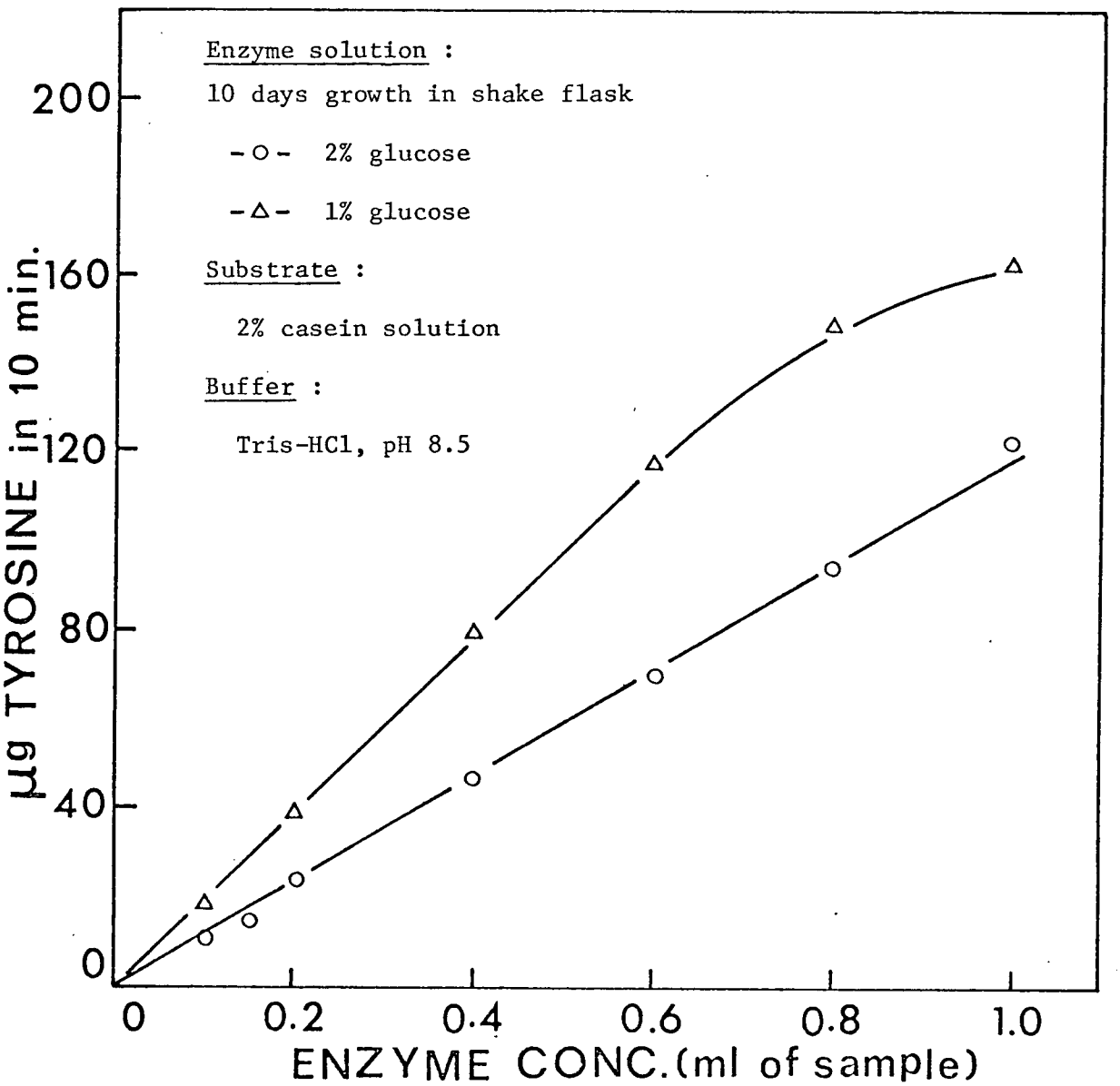
When conditions are such that $s >> k_m$, then $v = k_3 e$ and the reaction rate is directly proportional to the enzyme concentration. In such cases the reaction rate is of the first order as related to the enzyme concentration and of zero order as to the substrate concentration. It is therefore essential to establish the limit of enzyme concentration beyond which, maximum rate of breakdown of the enzyme-substrate complex is unattainable. Figure 2.5 is a plot of amount of tyrosine formed in 10 minutes versus crude enzyme concentration. It is apparent from this figure that under the conditions of this assay, beyond an enzyme concentration equivalent to 120 µg tyrosine, the substrate concentration used was not sufficient to maintain a constant maximum reaction rate during the whole incubation time. Fortunately, an absorbance reading of 0.9, the upper limit on the reliable portion of the spectrophotometer, corresponds to a tyrosine value of 70 µg; thus by diluting higher enzyme concentrations to give color values within the reliable portion of the Spectronic 20 will also ensure saturation of the enzyme.

2.9 Measurement of Protein Content

Many methods for the quantitative estimation of protein in solution are available. Among them are :

Figure 2.5

PROTEOLYTIC ACTIVITY VS ENZYME CONCENTRATION



1. UV Absorption

Most proteins enhibit a distinct ultraviolet light absorption maximum at 280 mμ, due primarily to the presence of tyrosine and tryptophan residues [16, 63]. Since the abundance of tyrosine and tryptophan varies from protein to protein, this method is in general not reliable. However reasonably reliable results can be obtained with very heterogeneous mixtures of proteins, because, in this case, it is unlikely that the mixture would be strikingly rich or poor in any particular amino acids. Nevertheless, direct photometry is not as specific as colorimetric methods, and is more liable to inaccuracy due to turbidity.

2. Colorimetric Methods

There are basically two colorimetric methods which are widely used for protein estimation. The chemical bases of these assays are :

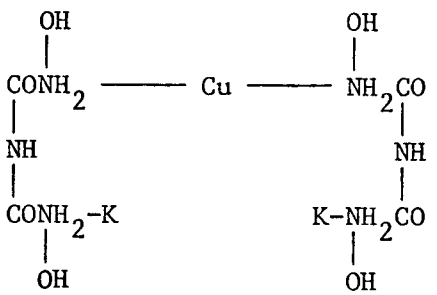
A. Folin

Phenols and phenolic amino acids such as tyrosine react with Folin's phenol reagent [61] in alkaline solution to form a reduced phosphomolybdic - phosphotungstic acid complex which is blue in color. In the presence of copper ions, additional color is produced through a biuret complex formation and through other mechanisms not presently understood [34]. This method suffers form the same disadvantages as the UV absorption method in that any variation in tyrosine content of the

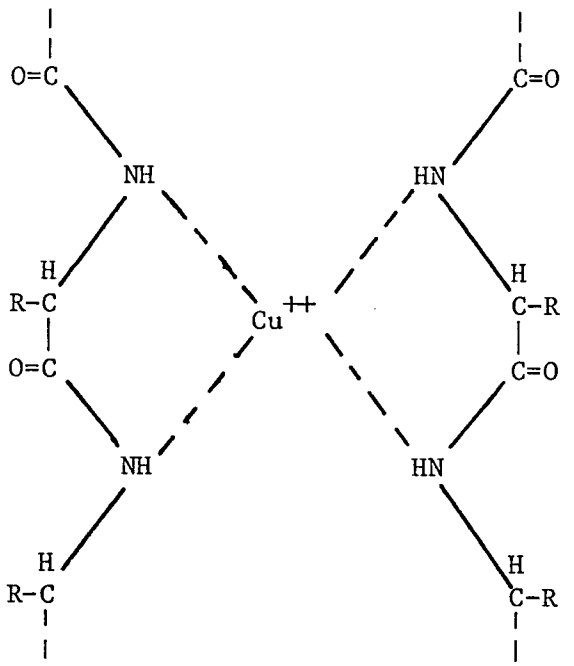
samples will invalidate the method.

B. Biuret

When biuret, whose structural formula is $H_2NCONHCONH_2$, is treated with an alkaline potassium copper tartrate solution, two biuret molecules are joined to form the following complex violet colored compound [63] :



Any compound that has in its molecular structure pairs of carbamyl groups ($-CONH_2$) linked through nitrogen or carbon (or peptide linkages) will show the above reaction [65]. Since protein contain these linkages, they react to give the characteristic biuret color. It is believed [16] that the chromophore, or light absorbing centre, is a complex between the peptide backbone and cupric ions :



Since peptide bonds occur with approximately the same frequency per gram of material for most proteins, deviations are encountered less frequently than with the Folin - phenol reagent. However, since the Folin reagent is about 100 times more sensitive than the biuret reaction [66], more material is required for assay by the latter method.

C. Chemical Method

One of the most widely used methods of protein determination is the Kjeldahl method, which is based on the quantitative estimation of total nitrogen. The method, as described in the Official Methods of Analysis [67], involves digestion of the protein with concentrated sulfuric acid in the presence of copper sulfate, mercuric sulfate, or some other suitable catalyst. The ammonia formed may be quantitatively determined

by removal from the digestion mixture through steam distillation followed by titration or addition of Nessler's reagent (mercuric potassium iodide in aqueous sodium hydroxide). The latter treatment permits ammonia to be determined colorimetrically.

For the photometric and colorimetric methods, the protein content is obtained by comparison against a standard solution of serum protein, usually crystalline Bovine Serum Albumin; in the Kjeldahl method, the average protein is calculated by assuming a nitrogen content of 16%. The details of the Biuret method, which was used throughout this work, are given in Appendix II.3.

Chapter 3

RESULTS AND DISCUSSION

3.1 Effect of Medium Constituents

Preliminary studies revealed that a medium consisting of diluted, condensed fish solubles alone could suffice as a substrate for good growth and protease production. However, it is a well known fact that the inclusion of certain ingredients in a fermentation medium may have an enhancing effect on proteolytic enzyme production. Merrill and Clark [68] and Haines [69] found that calcium was necessary for good protease yields, while Fukumoto and Negoro [70] showed that phosphate(s) could stimulate protease production also. Fukumoto et al [71] have shown that the effect of phosphates on enzyme production was very pronounced when carbohydrates were used as carbon sources.

In the present investigation, the factorial approach to experimentation was used to determine which of the above three ingredients were significant for further testing. Such an approach enables (1) variation of several factors at once in accordance with a pre-arranged pattern, thereby obtaining more information with possibly fewer runs, and (2) evaluation of interactions between variables, if any exist.

In the present investigation, the following levels of the three factors were used :

Factor	Level 1	Level 2	Level 3
A. Glucose concentration (gm/100ml)	0	5	10
B. Phosphate concentration (ml 1M soln. K_2HPO_4)	0	0.3	0.7
C. Calcium concentration (ml 1M soln. $CaCl_2$ added)	0	0.5	1.0

One run was made at each combination of factors, thus giving rise to 27 experimental conditions, each of which consisted of 100 mls of a filtered, 1 : 50 v/v diluted, condensed fish solubles medium made up according to specifications. A white precipitate was formed when calcium chloride and potassium hydrogen phospate at the higher levels were added to the medium. No experiments were designed to examine what effect this might have on enzyme yield; in other words, the difference in enzyme production were attributed solely to the addition or deletion of the particular ingredient.

The order for running the 27 experiments was completely randomized by withdrawing numbers from a beaker. After 3 days growth at 30°C and 140 rpm in the incubator-shaker, the proteolytic activity of the culture filtrates was measured. The results are given in Table 3.12.

The model assumed for the analysis of this experiment is given by [72, 73] :

$$y_{ijk\ell} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijk\ell} ,$$

Table 3.12

RESULTS OF FACTORIAL EXPERIMENTS

PHOSPHATE LEVEL (B)	SUGAR LEVEL (A) 1 — CALCIUM LEVEL (C) 1	2	3	SUGAR LEVEL (A) 2 — CALCIUM LEVEL (C) 1	2	3	SUGAR LEVEL (A) 3 — CALCIUM LEVEL (C) 1	2	3
1	3.66*	1.39	1.41	2.04	0.95	0.57	0.0	0.13	0.0
2	2.92	1.47	1.79	1.64	1.06	0.73	0.0	0.0	0.0
3	2.19	1.06	0.89	2.38	0.98	0.63	0.0	0.08	0.0

* Values are in enzyme units / 10 cc.

where $y_{ijk\ell}$ represents the ℓ^{th} observation taken at the i^{th} level of factor A, j^{th} level of factor B, and the k^{th} level of factor C; μ is the overall mean, α_i the effect of the i^{th} level of factor A, β_j the effect of the j^{th} level of factor B, and γ_k the effect of the k^{th} level of factor C. ε_{ijk} measures the derivations of the observed $y_{ijk\ell}$ values in the $(ijk)^{th}$ cell* from the population mean μ. The other terms stand for interactions between the main factors α, β, and γ. It is usually assumed that the sums of the main effects as well as the sums of the two - way interaction effects summed on either subscript equal zero for any value of the other subscript (i.e. $\sum_{i=1}^{3} (\alpha\beta)_{ij} = 0$, etc.) and that the sum of the three - way interaction effects summed on any one of the subscripts is zero for any values of the other two subscripts (i.e. $\sum_{i=1}^{3} (\alpha\beta\gamma)_{ijk} = 0$, etc.). These restrictions will assure unique estimates for the parameters of the model.

In order that valid significance tests can be made, it is also assumed that the errors are values of independent and normally distributed random variables, each with zero mean and common variance σ^2.

The complete analysis of variance for this three-factor experiment is given in Table 3.13. The computational procedures for obtaining the sums of squares are spelled out in Appendix III.

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* Each treatment combination defines a cell. If there are n replications, each cell contains n observations.

Table 3.13

ANALYSIS OF VARIANCE

SOURCES OF VARIATION	SS	df	MS=SS/df	COMPUTED F
Main Effects				
A	15.72	2	7.86	131.0*
B	0.23	2	0.12	2.0
C	5.13	2	2.57	42.83*
Two Factor Interactions				
AB	0.90	4	0.23	3.83
AC	2.99	4	0.75	12.50*
BC	0.25	4	0.06	1.00
Three Factor Interactions				
ABC	0.51	8	0.06	

Note SS(ABC) was taken as SSE

$F_{2,8,0.05} = 4.46$ $F_{2,8,0.01} = 8.65$

$F_{4,8,0.05} = 3.84$ $F_{4,8,0.01} = 7.01$

* These factors are significant at the 0.01 level.

Note that since only one observation was made at each set of conditions, a within-cell mean square cannot be used as an estimate of the true variance of the system. Instead, the ABC interaction was assumed to be negligible and that SS(ABC) represents variation due solely to experimental error and thereby provides an estimate of the error variance. The F statistic in the Analysis of Variance table was calculated using MS_{ABC}.

The following conclusions can be made from the F ratio in the Analysis of Variance table :

(1) Sugar and calcium effects are significant at the 0.01 level.

(2) Interaction exists between the different sugar concentrations and different calcium concentrations.

(3) The F-ratio for glucose - phosphate interaction is barely below the 0.05 critical value; there is not enough evidence to conclude that interaction between these two factors does not exist.

(4) None of the other F-ratios are significant at either the 0.05 or 0.01 level.

3.2. Effect of Glucose Concentration

The analysis of variance table indicated that the amount of glucose present in the medium is the most important factor affecting enzyme

yield. The effect of this ingredient was further studied in an attempt to develop a medium yielding maximal proteinase production.

The media used for this study were both filtered and unfiltered, 1 : 50 v/v diluted, condensed fish solubles without added calcium salts or phosphates. Figures 3.6, 3.7 and 3.8 show the changes occuring during the course of fermentation when the glucose concentration was varied between 0 and 40 gm/1 in both filtered and unfiltered media. Since there was no easy method to separate the insoluble proteins from the bugs, the growth in the unfiltered media was not measured. The data for these figures are tabulated in Tables AI.1, AI.2 of Appendix I. Each reported value is the average of duplicate runs (Section 2-5).

The results indicate that, for both filtered and unfiltered media, the yield of protease was maximum in medium containing 1% glucose, while best growth was obtained with medium containing 2% glucose. It is clearly demonstrated that the effect of the added carbohydrate was one of increased growth. It is also apparent that protease production lags growth. The changes in the pH of the medium during incubation suggest that protease production is higher in media developing an alkaline reaction, while growth is generally higher in media developing an acid reaction. The drastic drop in enzyme yield on the eight day with media containing 3% or 4% glucose may be due to destruction of the enxyme when exposed to such low pH values (3.4 to 4.1). Comparison of Figure 3.6 and 3.8 indicates that the filtered fish solubles is a better medium for protease production.

Figure 3.6

EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY - UNFILTERED CFS

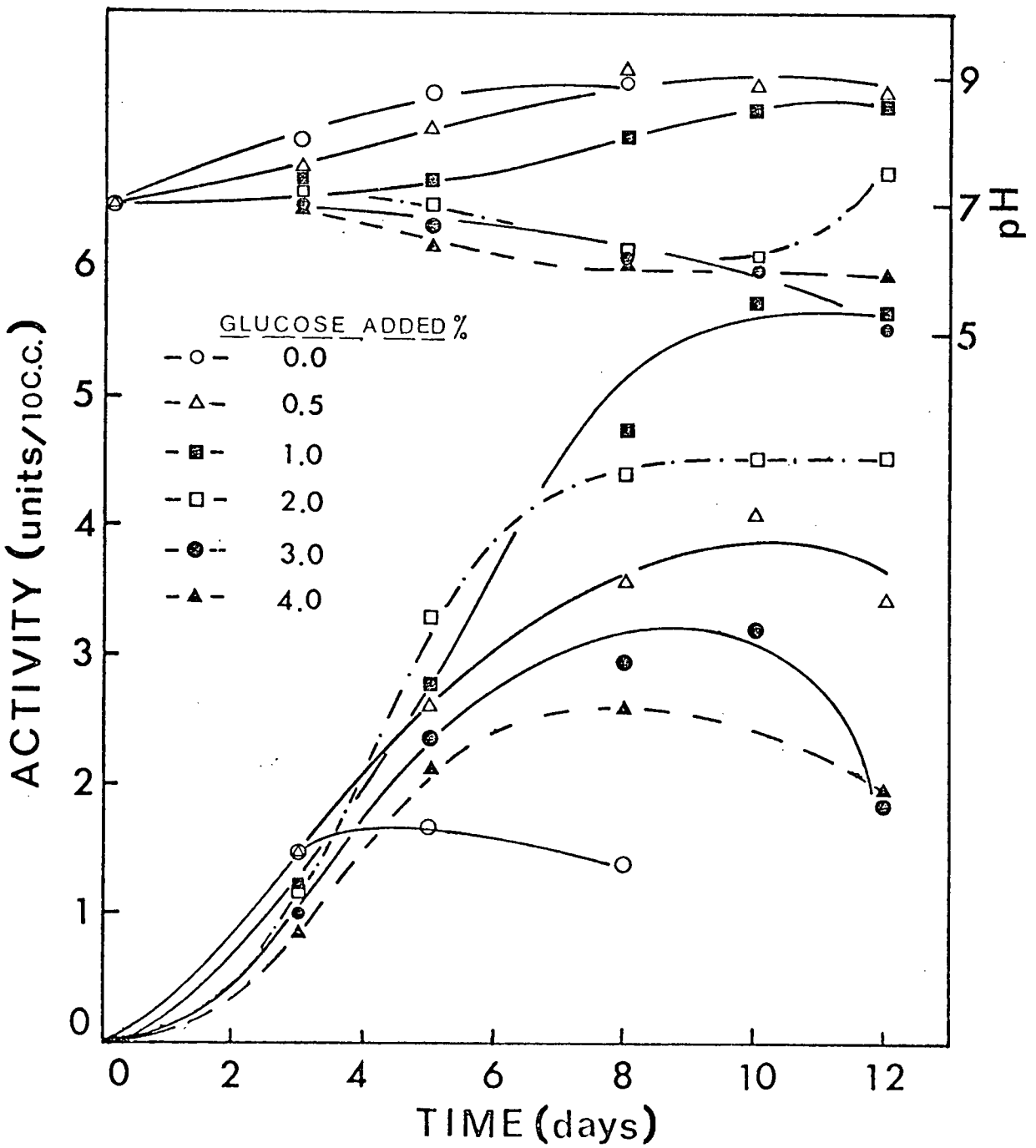


Figure 3.7

EFFECT OF GLUCOSE CONCENTRATION ON GROWTH - FILTERED CFS

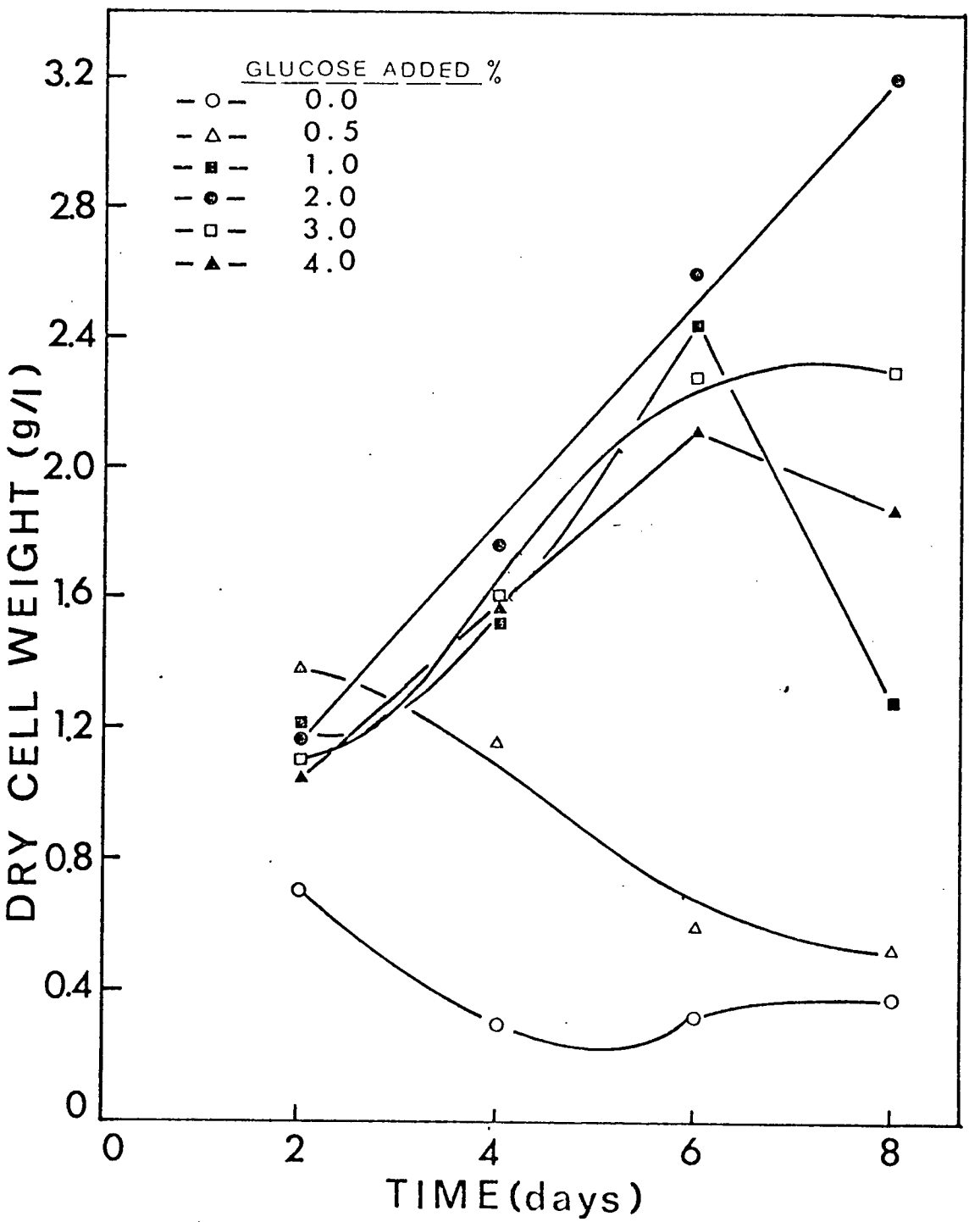
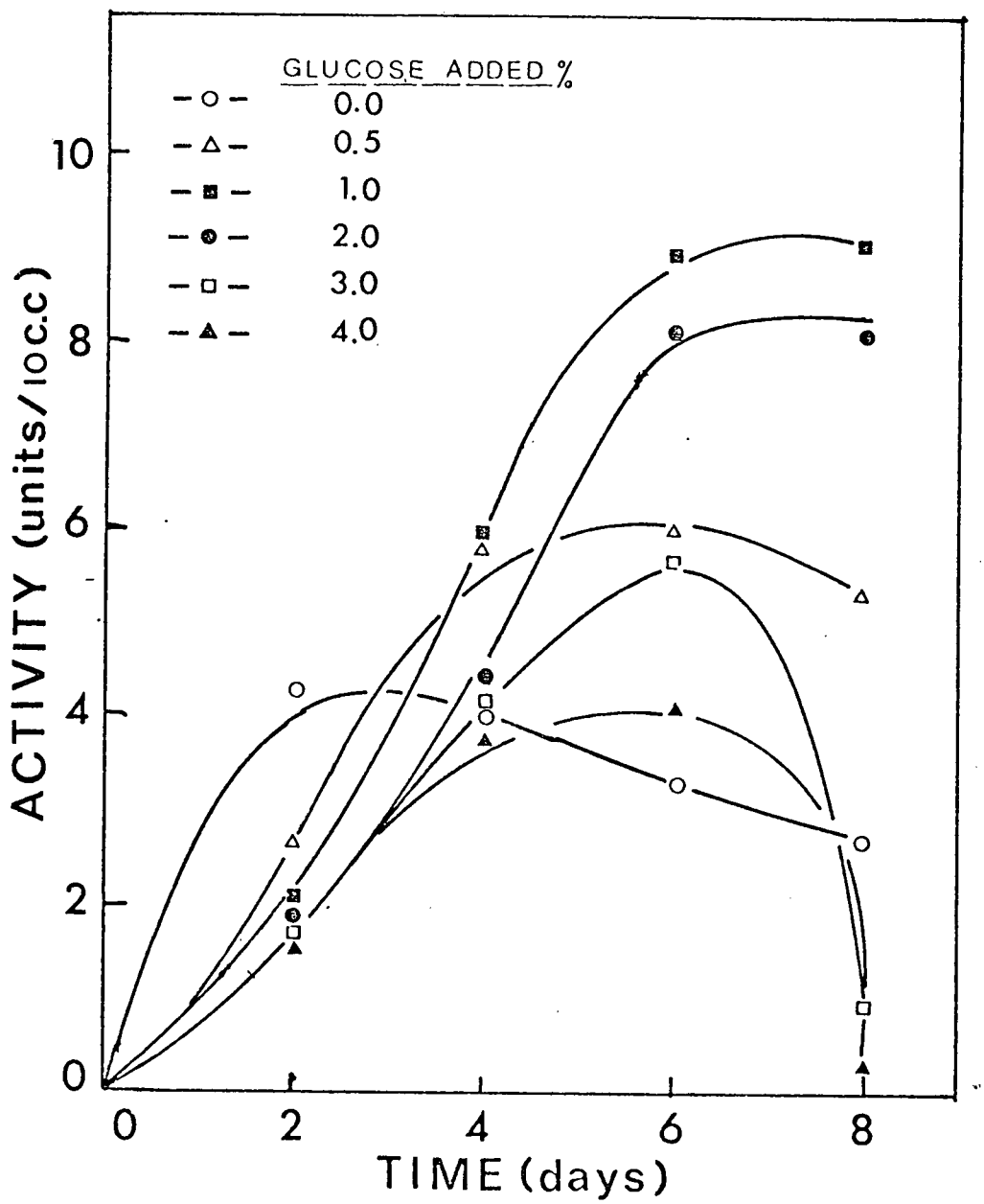


Figure 3.8

EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY - FILTERED CFS



It should be pointed out here that autoclaved glucose can inhibit the growth of certain species of bacteria [74]. All the experiments in this work were carried out with autoclaved glucose. The possible inhibitory effects of autoclaved sugars on the growth of Sorangium 495 were not taken into consideration.

3.3 Effect of Initial pH

The effects of initial pH (varying from 5 to 8) on growth and protease production were studied on filtered 1 : 50 v/v diluted, condensed fish solubles and containing 1% glucose. The initial pH of the medium was adjusted to various values by the addition of 2N sodium hydroxide. During the course of incubation, the pH was not adjusted but left to seek its own level, the final pH value (at the end of 8 days) representing chemical stabilization of the system. The dry cell weight and the proteolytic activity of the broth were measured at the fourth and eighth day of growth. The results are shown in Table 3.14. The results indicate that the initially established pH tends to rise (probably due to the formation of ammonia from the decomposition of protein) during the course of growth; the final pH being slightly above 8.0. The data show that an initial pH of 7.0 is best for enzyme production, while an initial pH of 8.0 is best for growth.

3.4 Effect of Condensed Fish Solubles Concentration

It has been reported [75, 76, 77] that many organisms produce

Table 3.14

EFFECT OF INITIAL pH

INITIAL pH	AFTER 4 DAYS			AFTER 8 DAYS		
	pH	DRY CELL WEIGHT gm/ℓ	PROTEOLYTIC ACTIVITY units/10cc	pH	DRY CELL WEIGHT gm/ℓ	PROTEOLYTIC ACTIVITY units/10cc
5	7.5	1.59	3.40	8.1	1.50	7.51
6	7.58	1.46	3.76	8.2	1.51	7.96
7	7.62	1.43	4.13	8.35	1.29	8.84
8	7.50	1.83	3.98	8.30	1.58	7.12

enzymes in response to the presance of a particular substrate in the culture medium - "adaptive production of enzymes." This evidence led to the belief that a medium rich in protein is required for the production of high yields of proteolytic enzymes. Figures 3.9 and 3.10, which show the effect of increasing the amount of condensed fish solubles on the time course of growth and protease production respectively, demonstrate that concentrations of filtered solubles having an average soluble protein content higher than 3.85 mg BSA/ml were markedly inhibitory for enzyme production. The data for these figures are tabulated in Table AI.3 of Appendix I.

The maximum protease production rates (slope of exponential phase of the production curves in Figure 3.10) in units/10 cc.hr. are plotted against protein concentration in Figure 3.11. Also plotted in the same figure is the maximum activity attained in each medium. It is apparent from this figure that at low protein levels, protease production is limited by availability of protein nitrogen (the final pH value of 5.9 also support this view); at high protein levels, rate of production and yield of enzyme are limited by substrate inhibition. That the detrimental effect of the higher solubles concentrations on protease formation could be attributed to inhibitory concentrations of protein or perhaps, of the organic nitrogen compounds, is indicated in Figure 3.9. This figure shows that growth at the higher protein concentrations was as good as, or better than, that obtained at the optimum concentration for protease formation.

Figure 3.9

EFFECT OF CONDENSED FISH SOLUBLES CONCENTRATION ON GROWTH

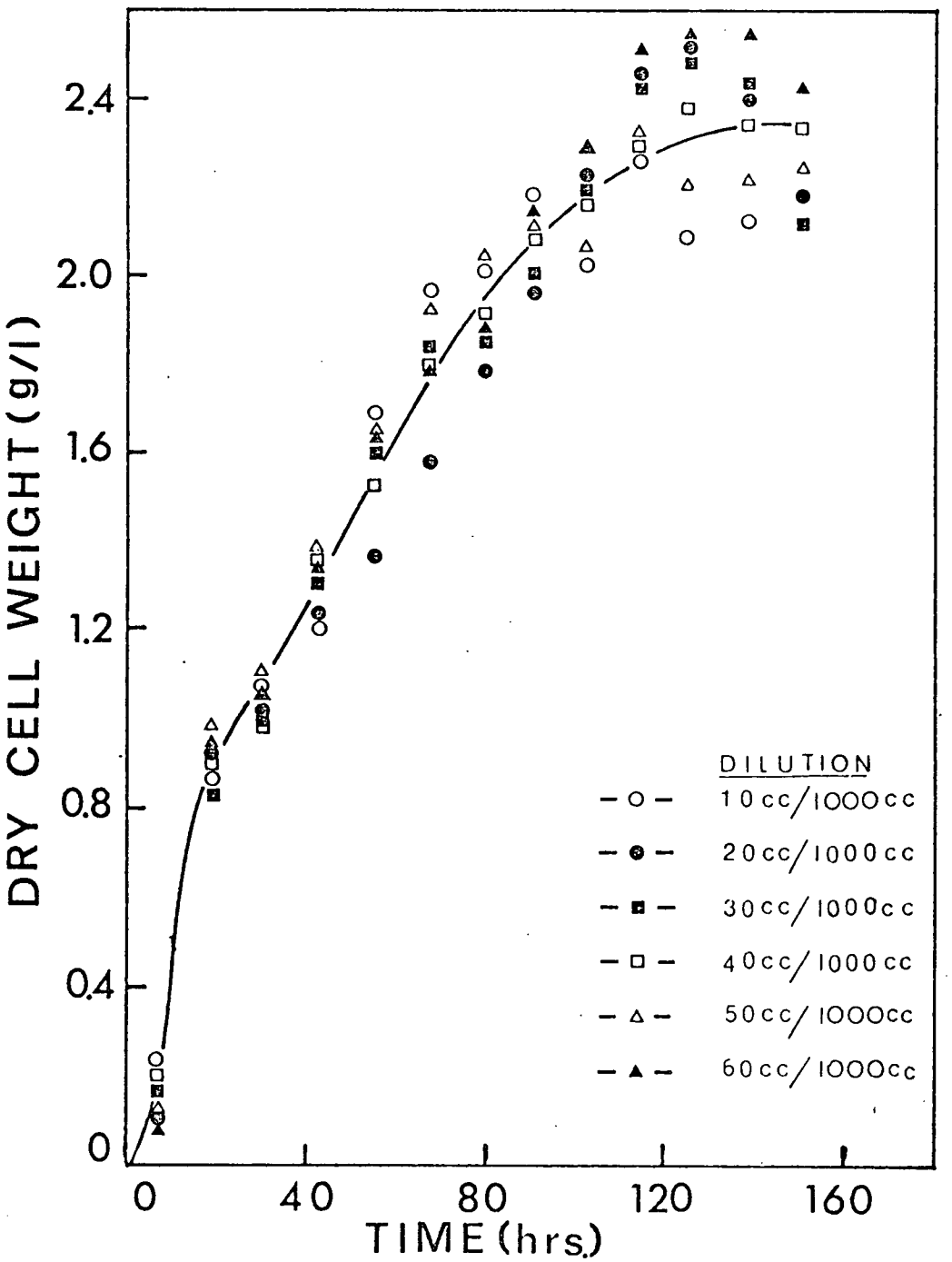


Figure 3.10

EFFECT OF CONDENSED FISH SOLUBLES CONCENTRATION ON PROTEASE FORMATION

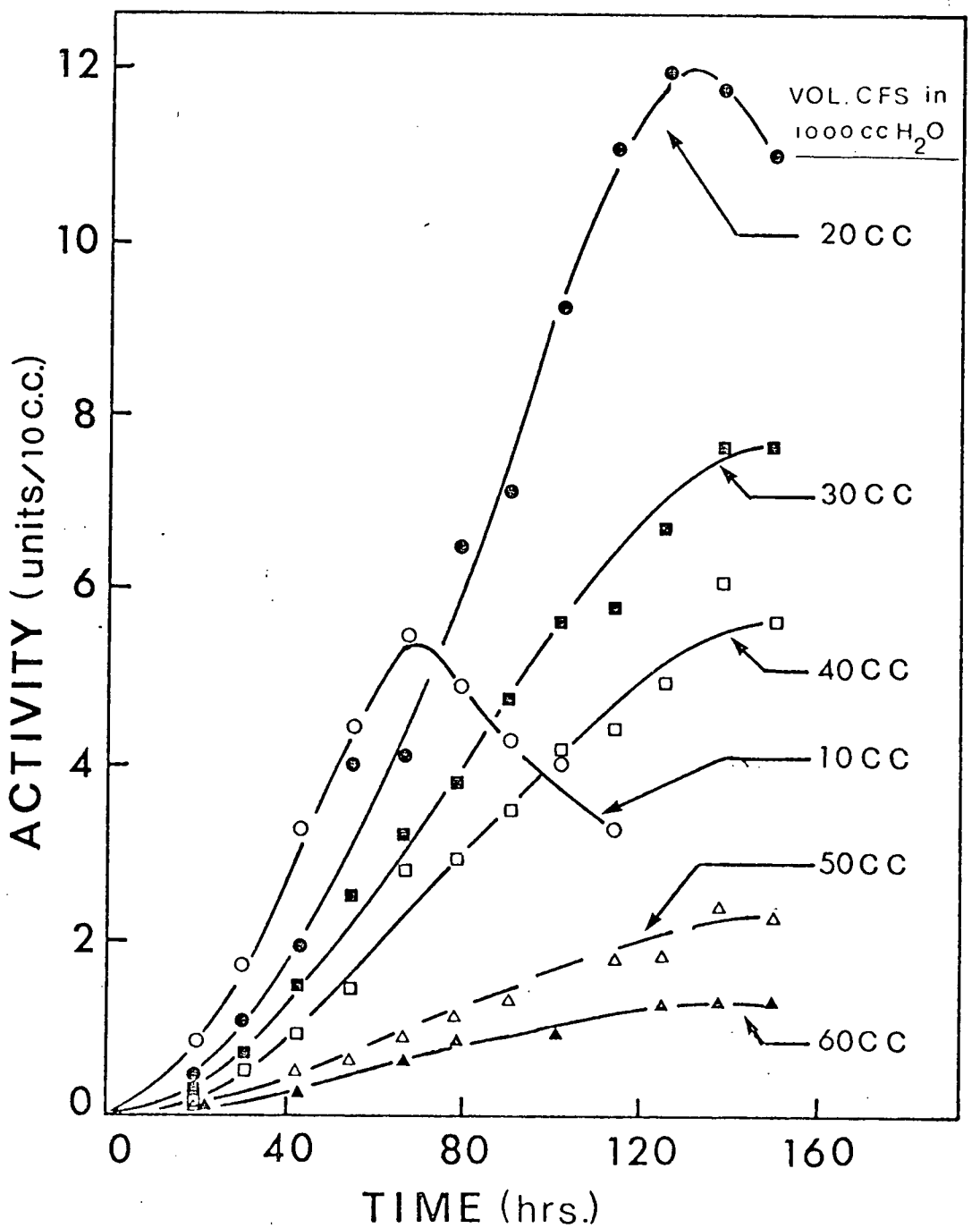
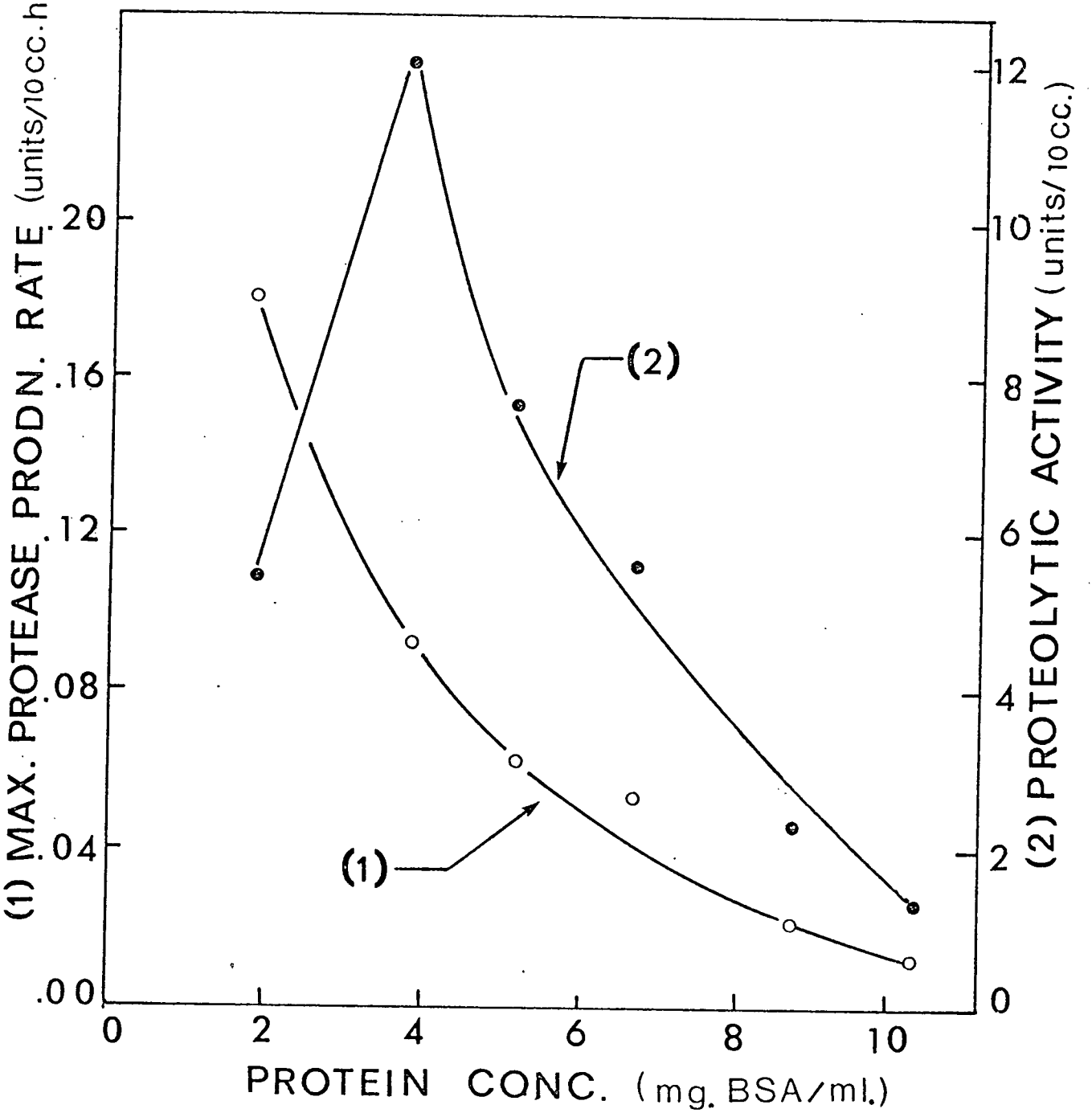


Figure 3.11

EFFECT OF PROTEIN CONCENTRATION ON ULTIMATE PROTEASE YIELD AND ON MAXIMUM RATE OF PROTEASE FORMATION



The results form these studies indicate that enzyme yield is affected by the ratio of carbon to nitrogen in the medium. Best results with Sorangium 495 were obtained in a fish solubles medium containing nitrogen equivalent to 3.85 mg BSA/ml, and 10 gm/l of glucose.

3.5 Effect of Carbohydrate Source

Glucose is commonly used as the energy and carbon source in microbiological media for laboratory growth experiments. However, industrial wastes high in carbohydrates are more suitable for commercial purposes. These wastes usually contain a mixture of monosaccharides; for example, in spent sulfite liquor, there are the hexoses - glucose, mannose and galactose, and the pentoses - xylose and arabinose. It was therefore thought to be useful to investigate the performance of cultures incorporating these different sugars as the carbon and energy sources. Since the McCurdy medium [78], a medium used for cultivating a wide spectrum of myxobacteria species, contains a high amount of soluble starch, the suitability of this polysaccharide as an energy supplement for the fish media was also evaluated.

The medium used in this experiment contained filtered, 2% v/v condensed fish solubles, and 1 gm/l of carbohydrate. The results are graphed in Figures 3.12, 3.13 and 3.14, and tabulated in Tables AI.4 and AI.5 of Appendix I. These results indicate that the organism grew more readily on mannose and glucose than on galactose, starch or pentoses. Figure 3.14 indicates that bacteriolysis is more pronounced in media containing starch or pentoses. The data demonstrate quite clearly that

Figure 3.12

EFFECT OF HEXOSES ON PROTEASE FORMATION

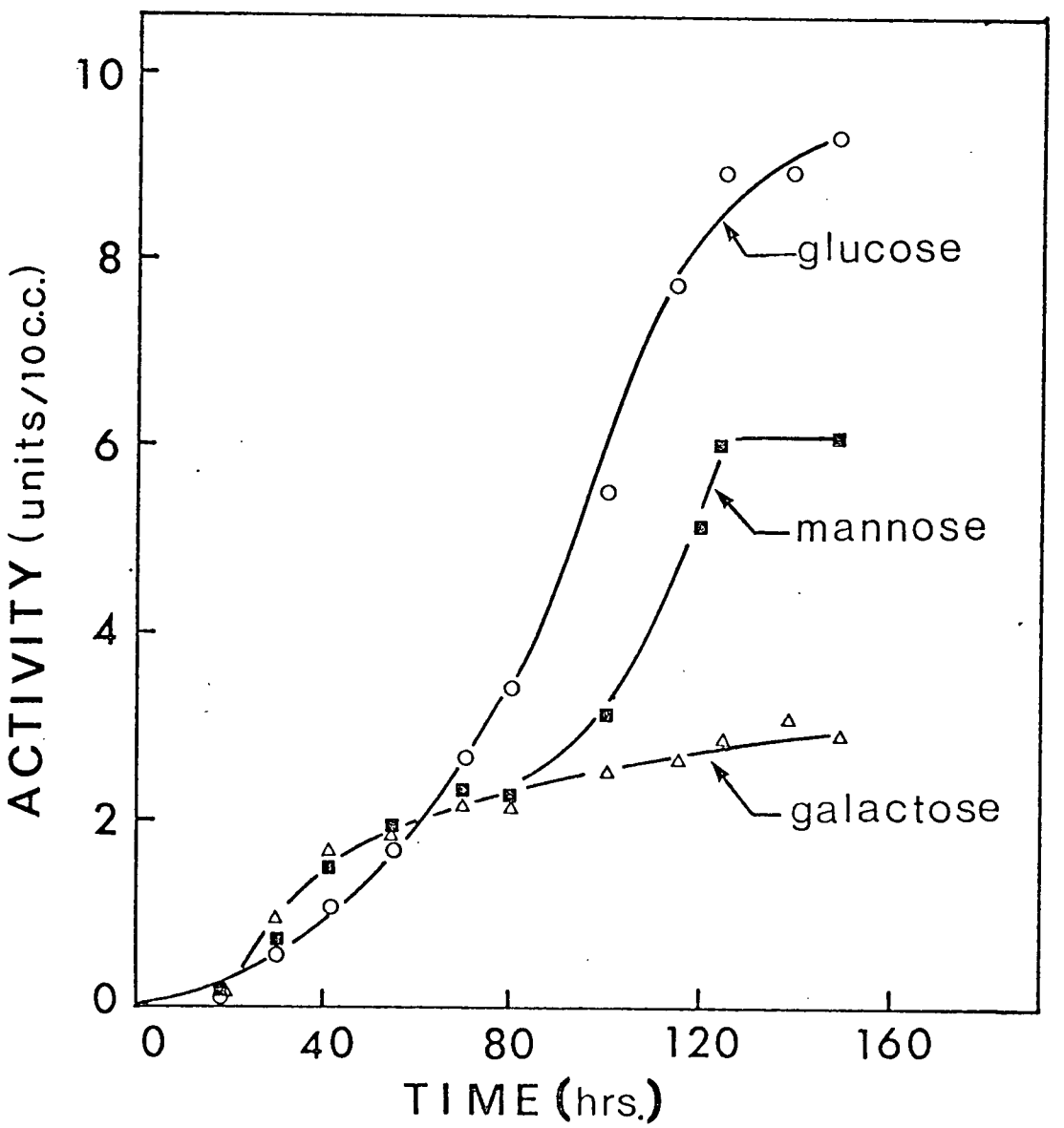


Figure 3.13

EFFECT OF SOLUBLE STARCH AND PENTOSES

ON PROTEASE FORMATION

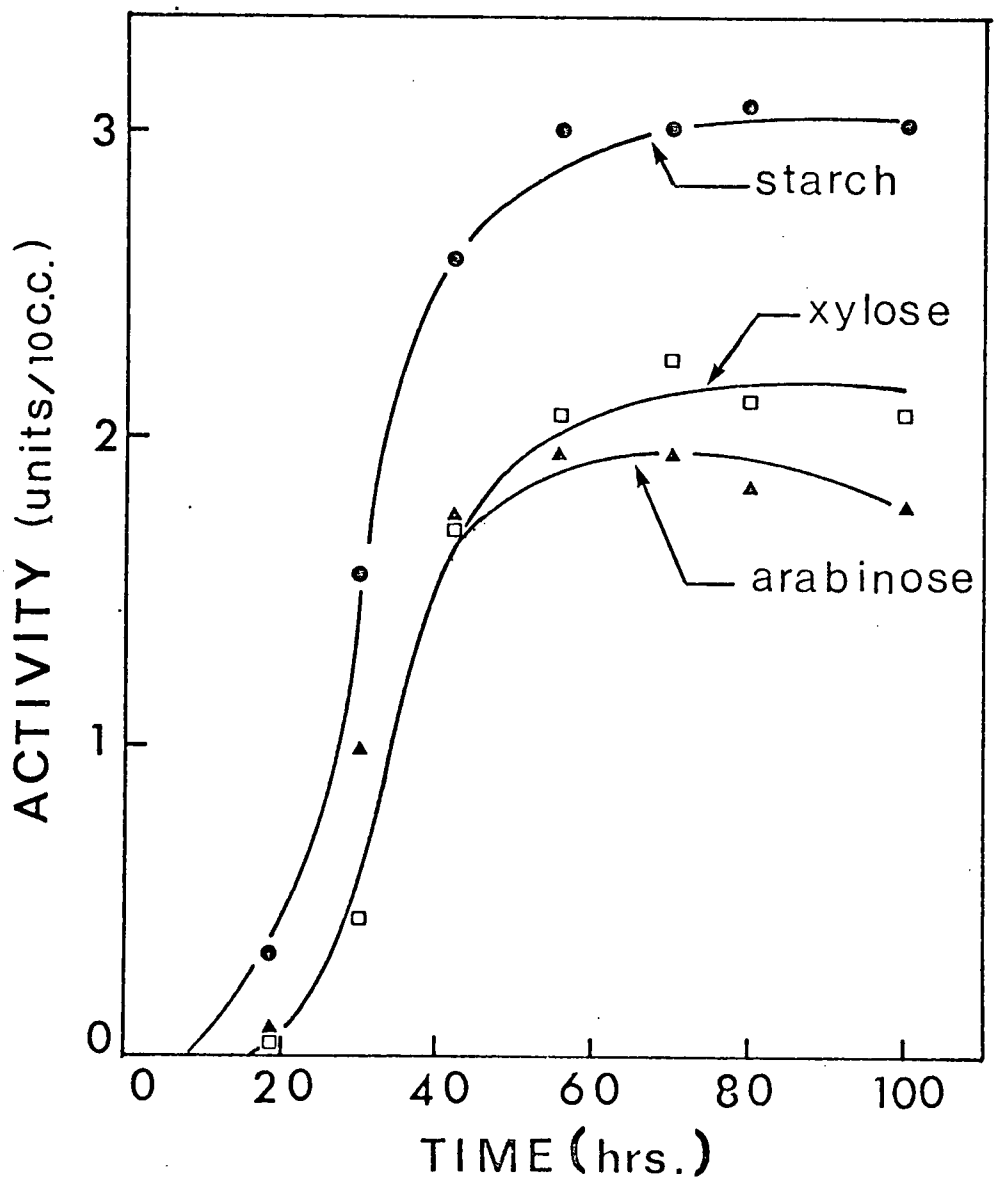
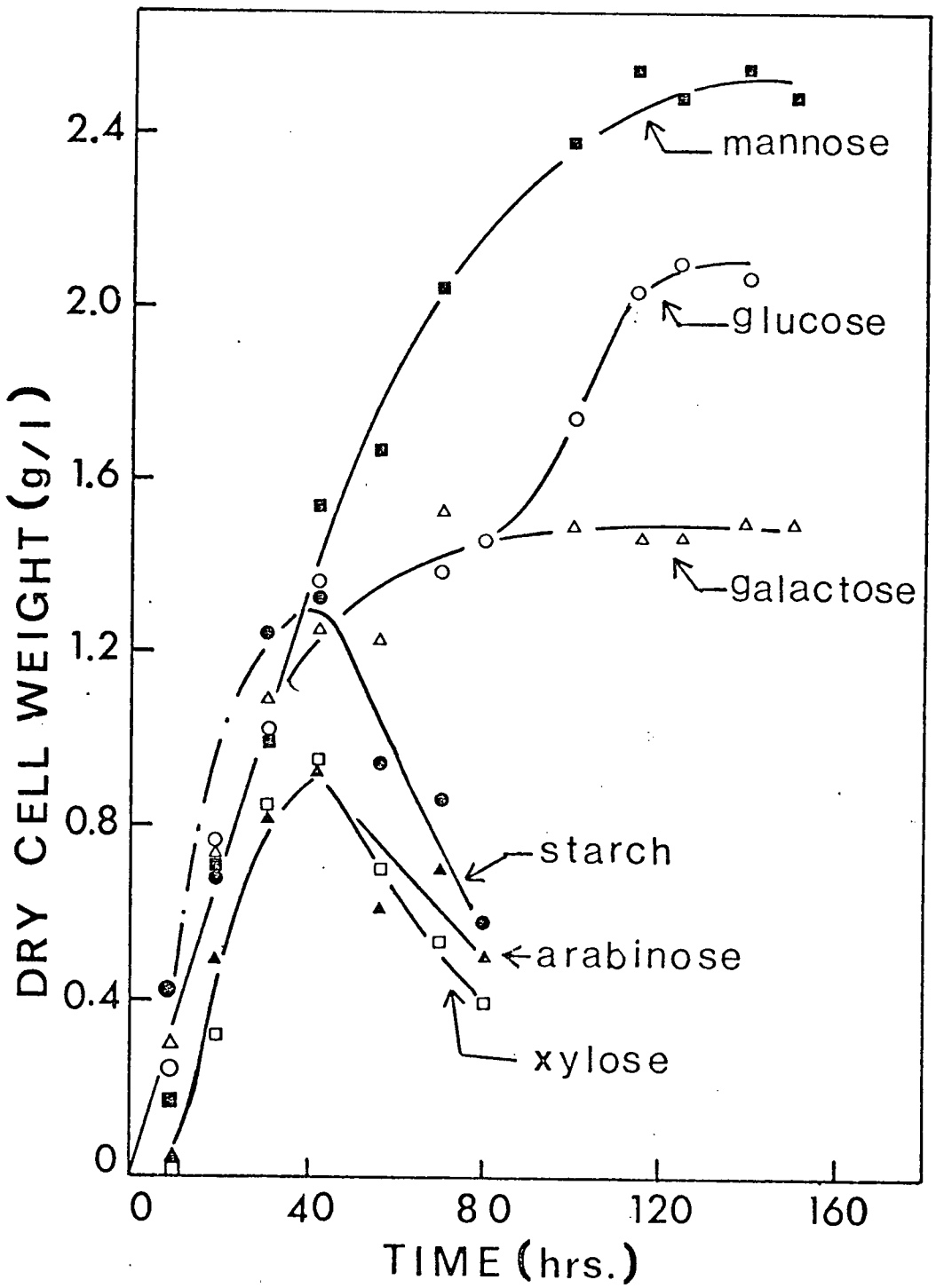


Figure 3.14

EFFECT OF CARBOHYDRATE SOURCE ON GROWTH



good growth is necessary for high yields of enzyme. It is also evident that glucose is better for protease formation, and mannose is better for growth. The biphasic nature of the protease production curve in medium containing mannose was not further investigated. The biphasic growth in glucose will be discussed in later sections.

Again, it should be noted that the effect of sterilization upon the different sugars was not taken into account in the above discussion. According to Davis and Rogers [79], autoclaving sugar solutions can cause changes in (1) optical activity, (2) pH, (3) caramelization and (4) reducing power. The degree of change differs from one sugar to another, the most sensitive being, amongst others, dextrose and arabinose.

3.6 Effect of Inoculum Age

Inoculum age between 16 and 24 hrs., which included ages from early log phase to deceleration phase of growth, was tested for effect on yield and rate of production. The changes occuring during the course of incubation in "optimal" medium (filtered, 2% v/v diluted condensed fish solubles with 1% glucose) are shown in Figures 3.15, 3.16 and 3.17 and are tabulated in Table AI.6 of Appendix I. These results indicate no significant effect on growth, protease production, and glucose utilization.

Figure 3.15

EFFECT OF INOCULUM AGE ON GROWTH

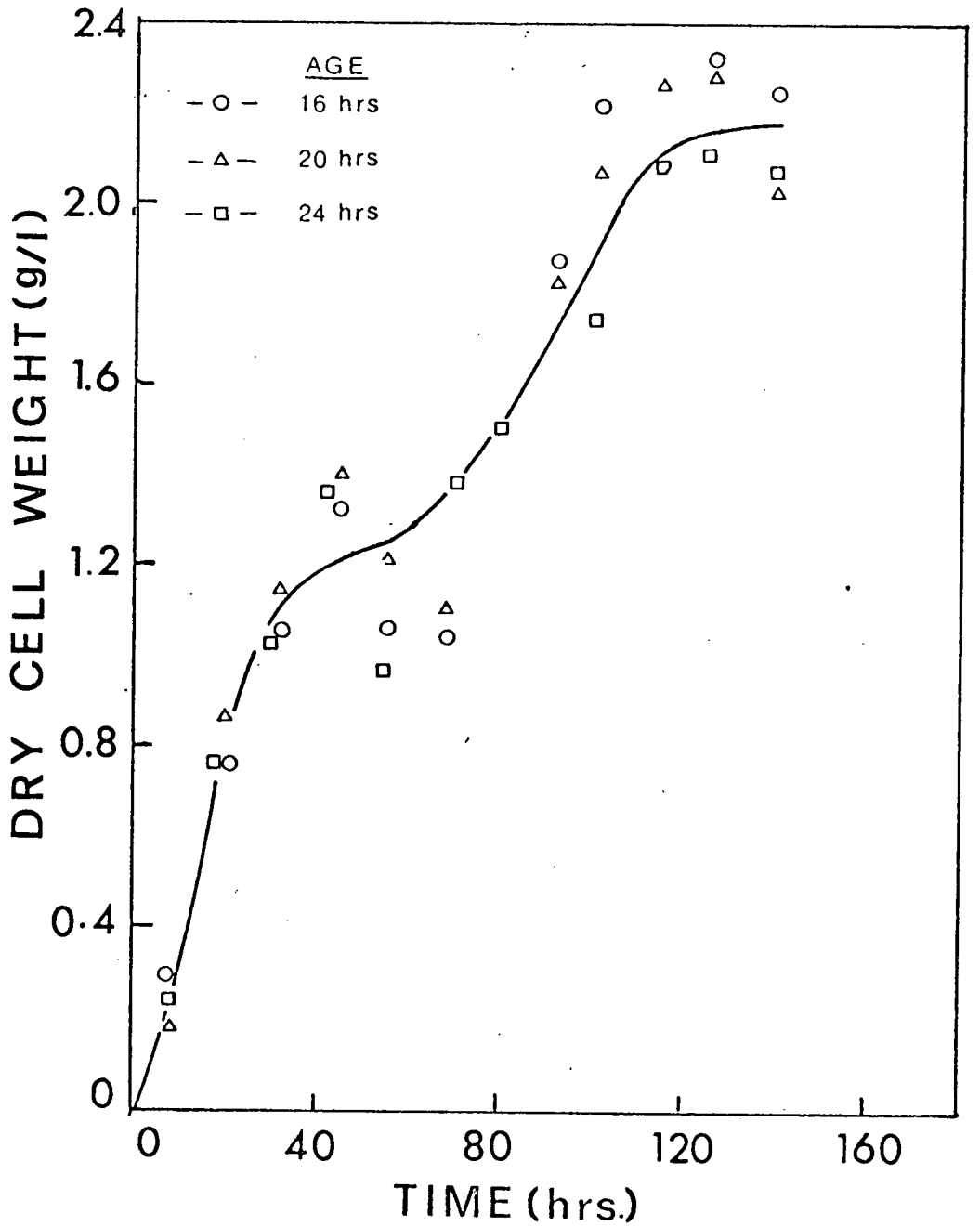


Figure 3.16

EFFECT OF INOCULUM AGE ON GLUCOSE UTILIZATION

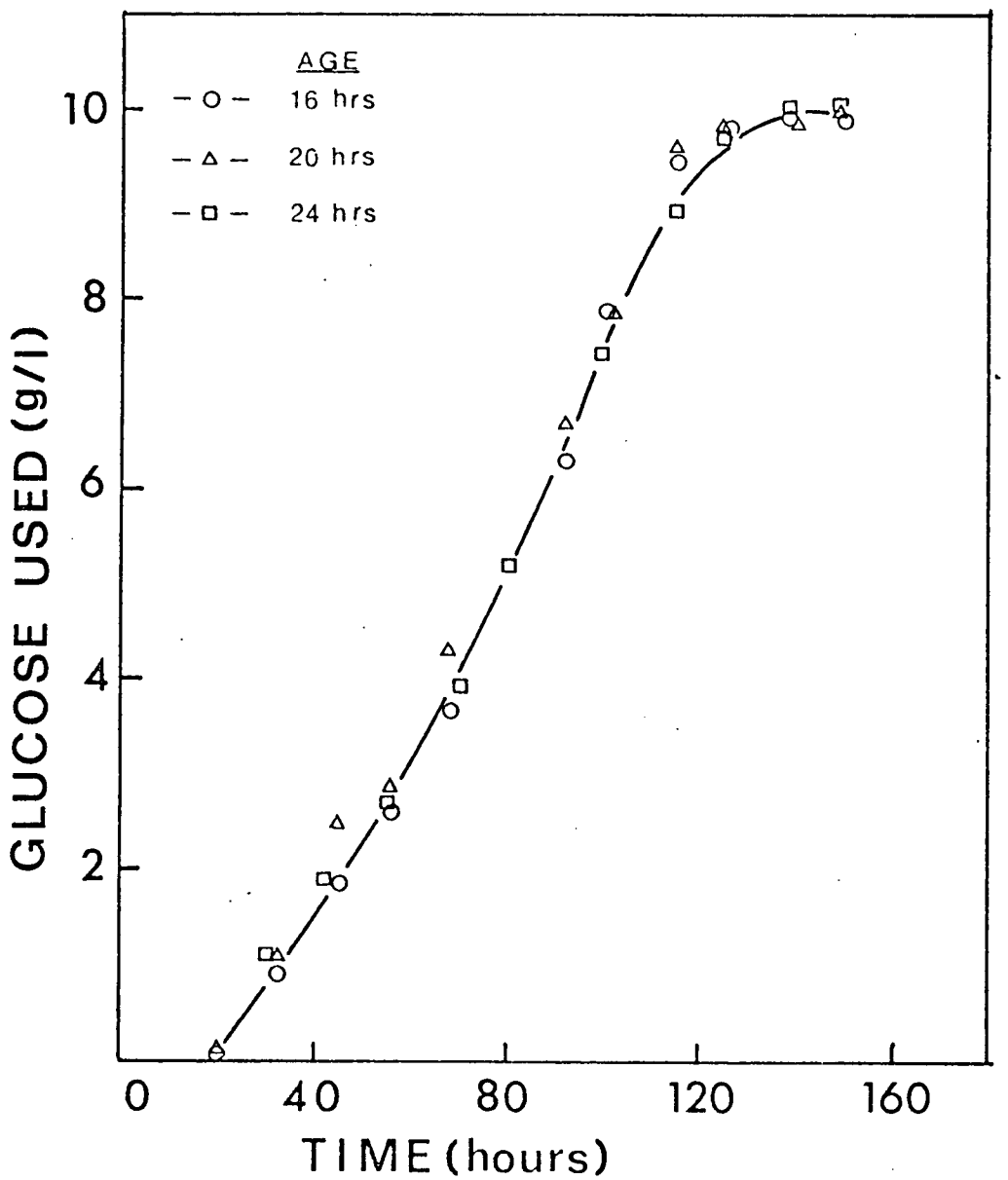
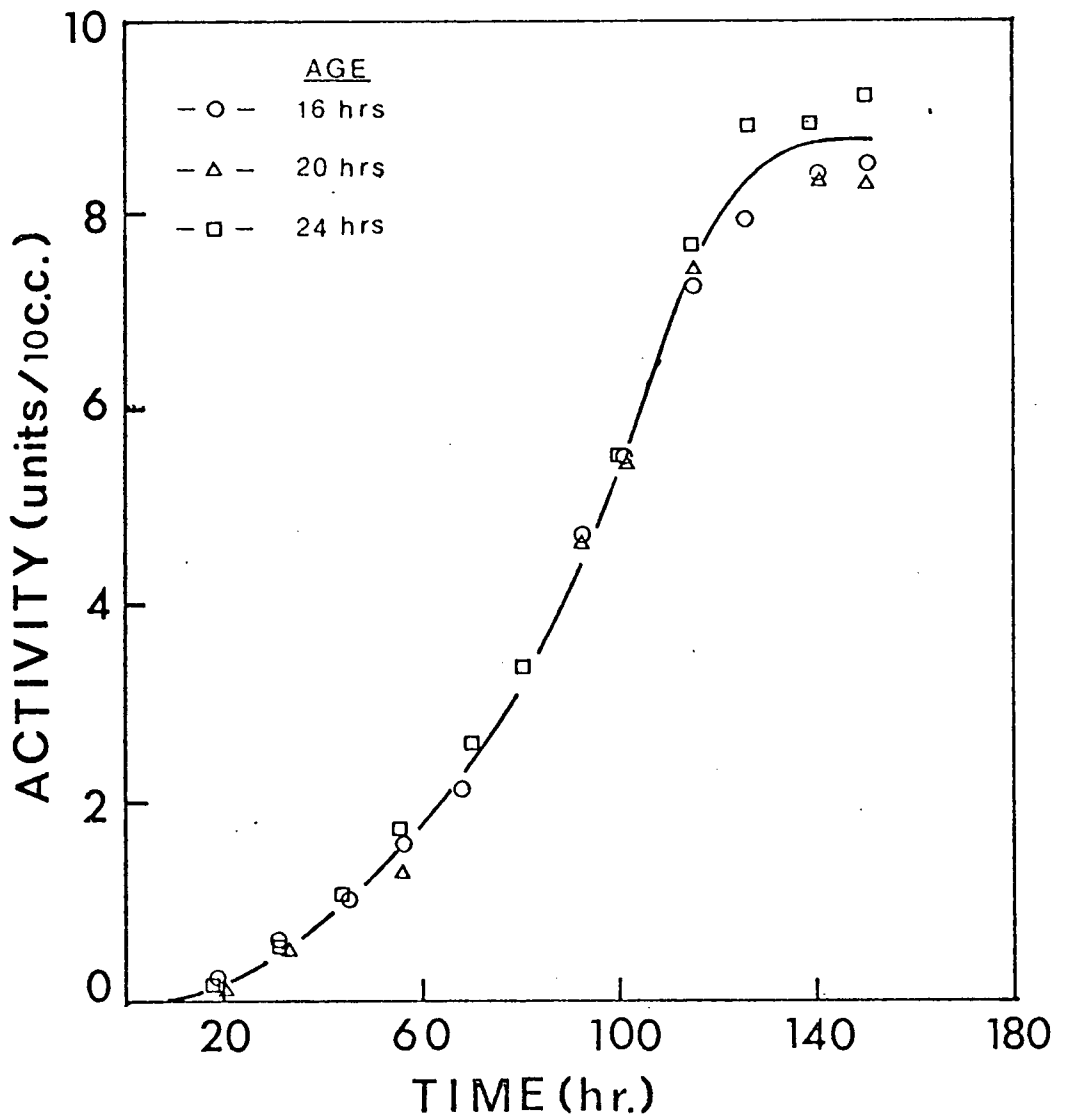


Figure 3.17

EFFECT OF INOCULUM AGE ON PROTEASE FORMATION



3.7 7-litre Fermentation Studies

All the above experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of fish medium. On the basis of these results, a set of "optimal" cultural conditions was chosen for maximum protease production. These "optima" are, at best, approximations, since it was not possible to study fully the interactions of the various factors that affect protease yields; also it was not possible to study the effects of agitation and aeration. In this section, experiments to investigate the influence of these two process variables on enzyme yields are described. The 7-litre fermentor described in Section 2.4 was used. A medium consisting of 4 litres of filtered, 2% v/v diluted condensed fish solubles with 1% glucose, and initial pH adjusted to 7.0, was used.

Eight 7-litre fermentations were run. One of these used a Nutrient Broth - glucose medium, the composition of which was, in gm/ℓ, peptone, 5; Difco Beef Extract, 3; and glucose, 10. The results of this run are presented in Figure 3.18 and Table AI.7 of Appendix I. The remainder of the runs were made with the fish solubles medium; agitation rate was varied from 300 rpm to 750 rpm. Five of these runs were made at an aeration rate of 1 ℓ/min while the other two were performed at an aeration rate of 2 ℓ/min. In no case was an entire run repeated.

Although an NBS automatic pH controller was available at the time these experiments were performed, no attempt was made to regulate the pH by the automatic addition of acid or base during the course of the

fermentation. It was thought that the high protein content of the medium provided the necessary buffer; the ratio of carbohydrate to protein was such as to keep the pH of the medium within ± 1 of neutrality. Due to the high content of surface active agents in the medium, there was considerable foaming. Since an automatic antifoam addition system was not available at the time, small amounts of defoaming agent in the form of a 2% solution of Antiform B was added, whenever necessary, through the inoculum port of the fermentor head with a sterilized syringe and hypodermic needle.

3.7.1 Effect of Agitation and Aeration on the Course of Fermentation

The time variations of pH, sugar utilization, proteolytic activity and dry cell weight for the 7-litre fermentations are presented in Figures 3.19 to 3.25 and are tabulated in Tables AI.8 to AI.14 of Appendix I. These results indicate that the relative amounts and times to maximum as well as the shape of the growth curve are affected by agitator speed and volumetric air flow rate. Sugar disappeareance, pH, and protease formation appear to be interrelated. An induction period was noted during which sugar utilization is small, pH rises, and protease formation has not yet begun. After this initial period, sugar concentration* decreases at a nonuniform rate toward a zero value. In the same interval, the pH is

---

* The figures are actually plotted in terms of sugar used rather than as sugar concentration.

Figure 3.18

7-LITRE FERMENTOR NB - GLUCOSE RUN

(Agitator speed : 400 rpm; Air flow rate : 1 ℓ/min)

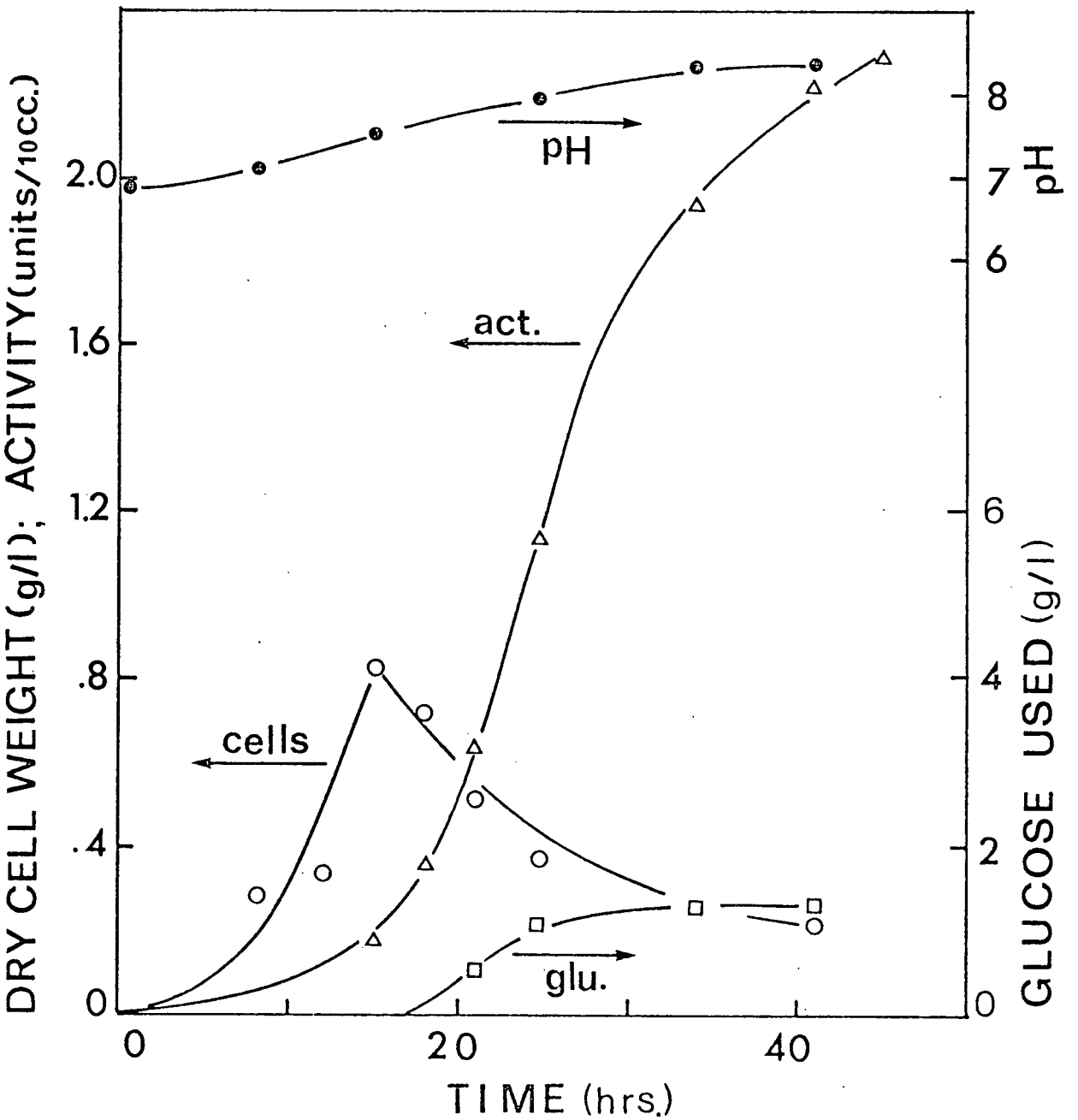


Table 3.19

7-LITRE FERMENTOR CFS RUN NO. 1

(Agitator speed : 300 rpm; Air flow rate : 1 ℓ/min)

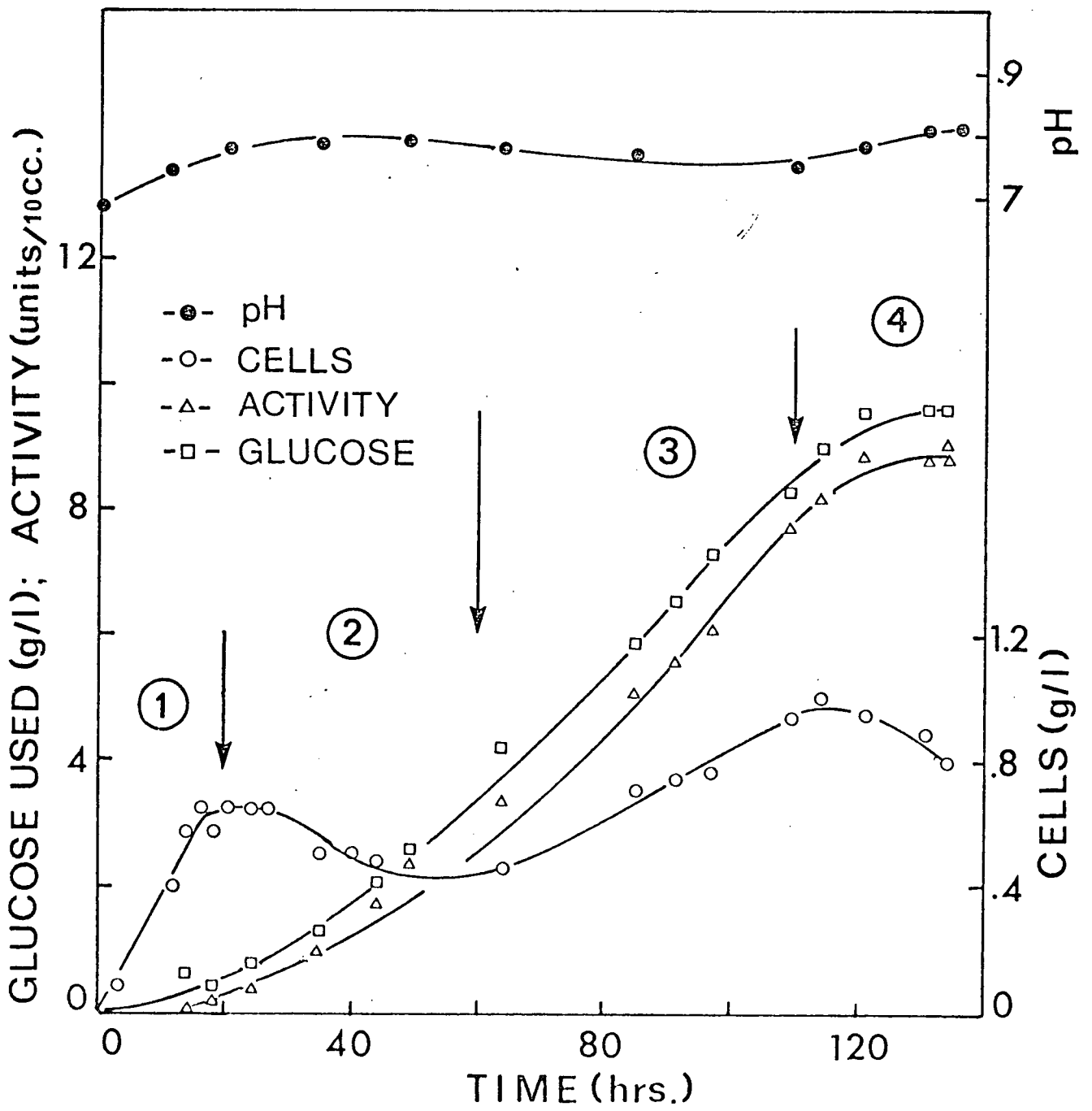


Figure 3.20

7-LITRE FERMENTOR CFS RUN NO. 2

(Agitator speed : 400 rpm; Air flow rate : 1 ℓ/min)

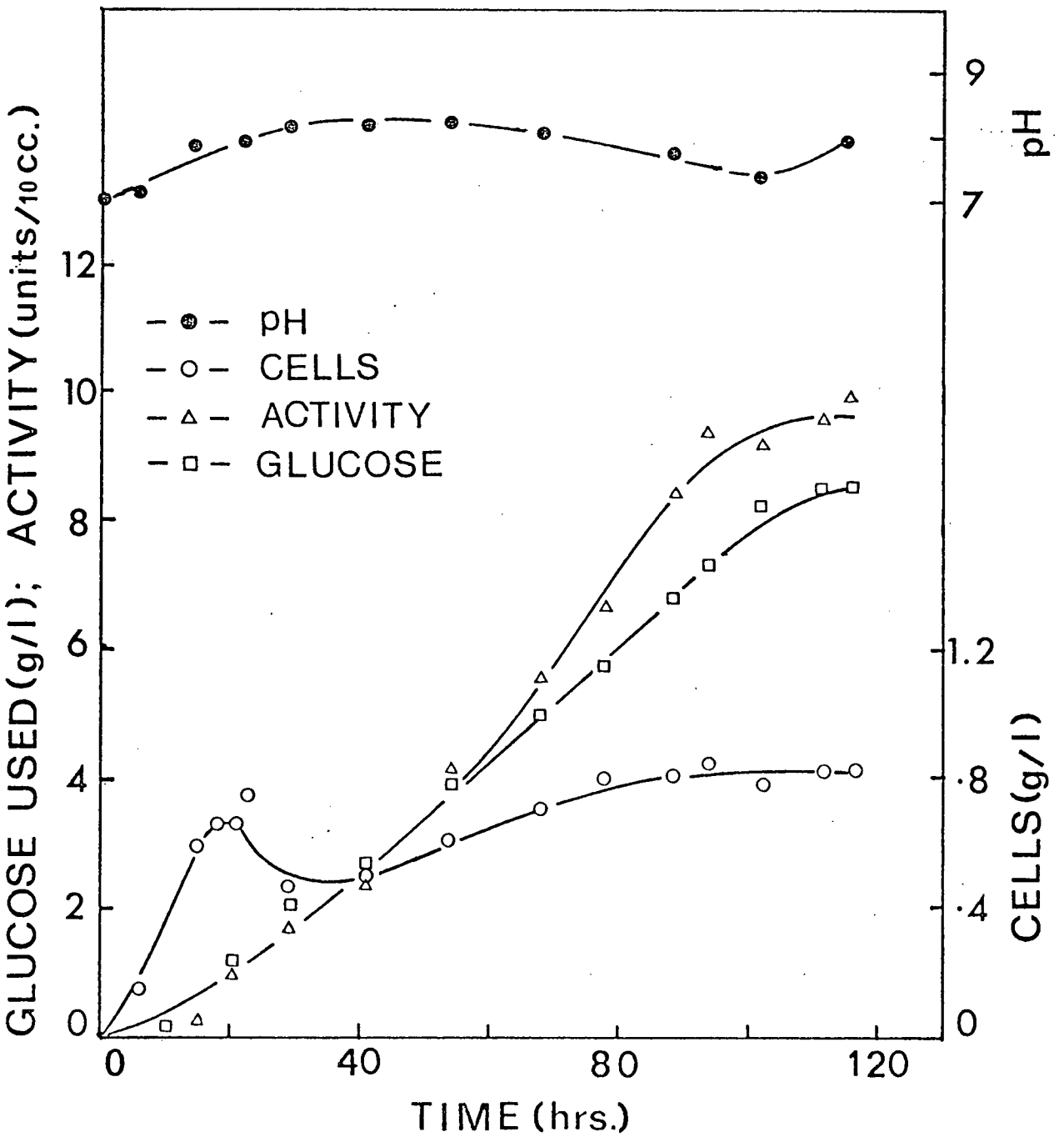


Figure 3.21

7-LITRE FERMENTOR CFS RUN NO. 3

(Agitator speed : 500 rpm; Air flow rate : 1 ℓ/min)

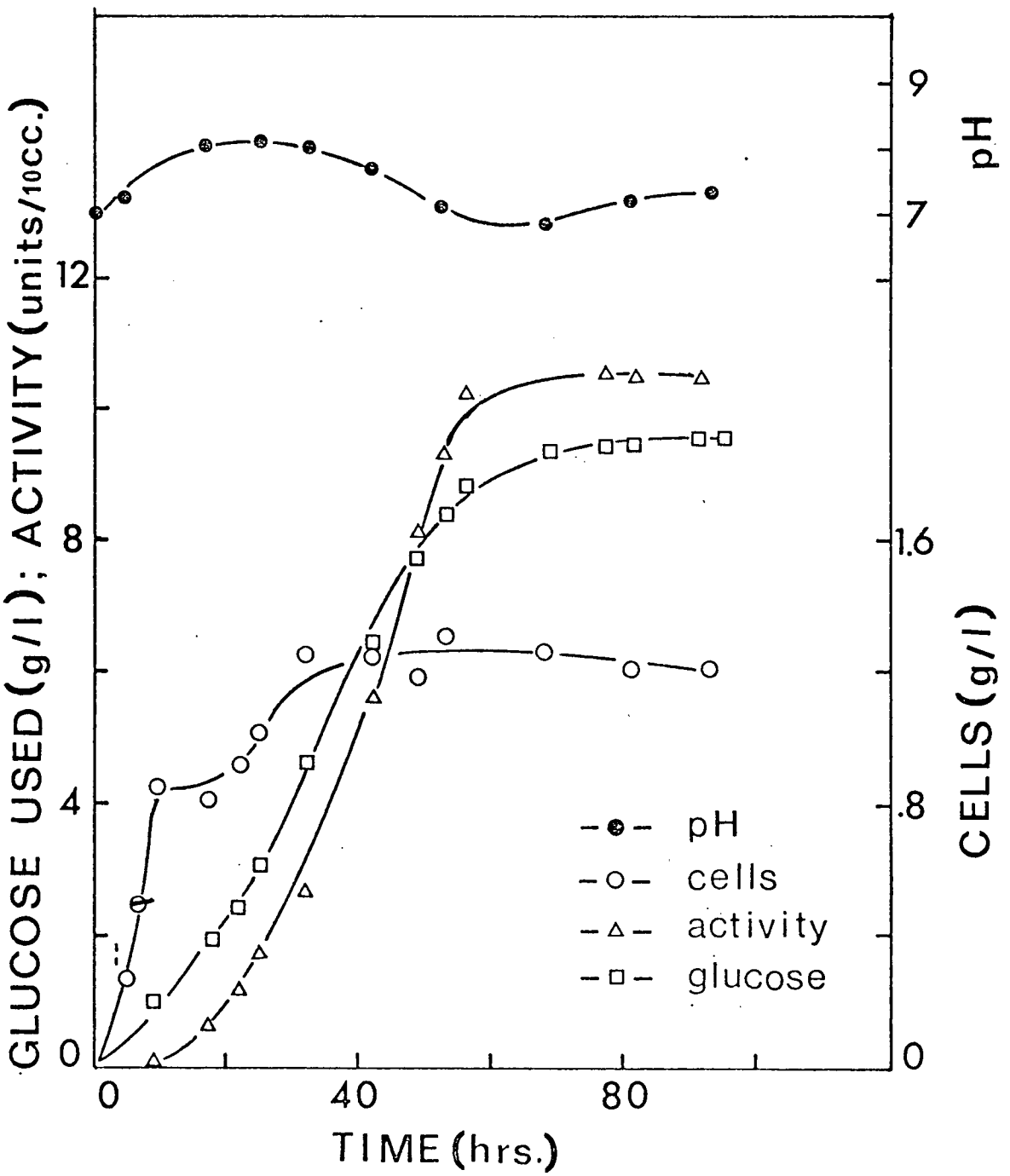


Figure 3.22

7-LITRE FERMENTOR CFS RUN NO. 4

(Agitator speed : 600 rpm; Air flow rate : 1 ℓ/min)

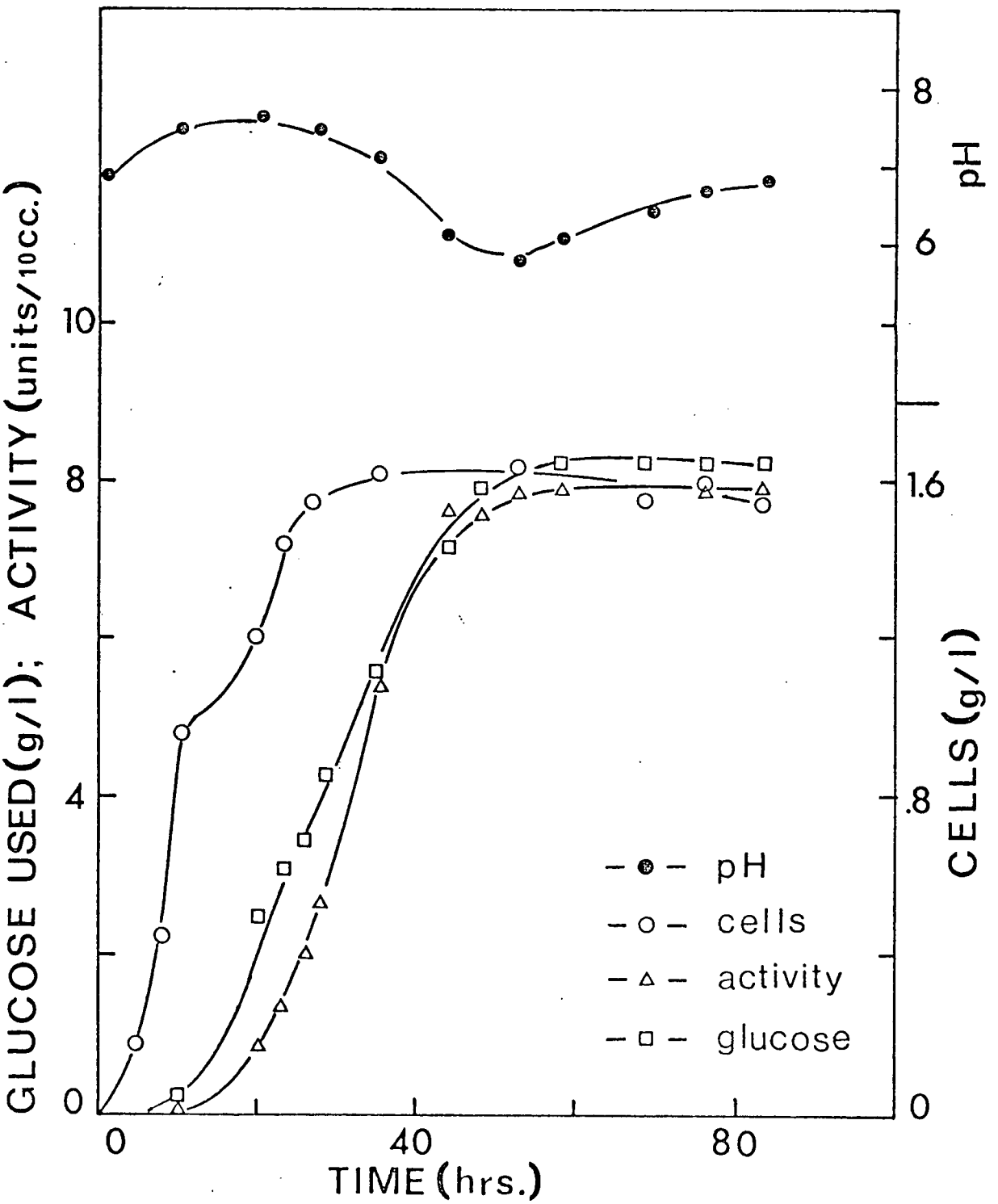


Figure 3.23

7-LITRE FERMENTOR CFS RUN NO. 5

(Agitator speed : 750 rpm; Air flow rate : 1 ℓ/min)

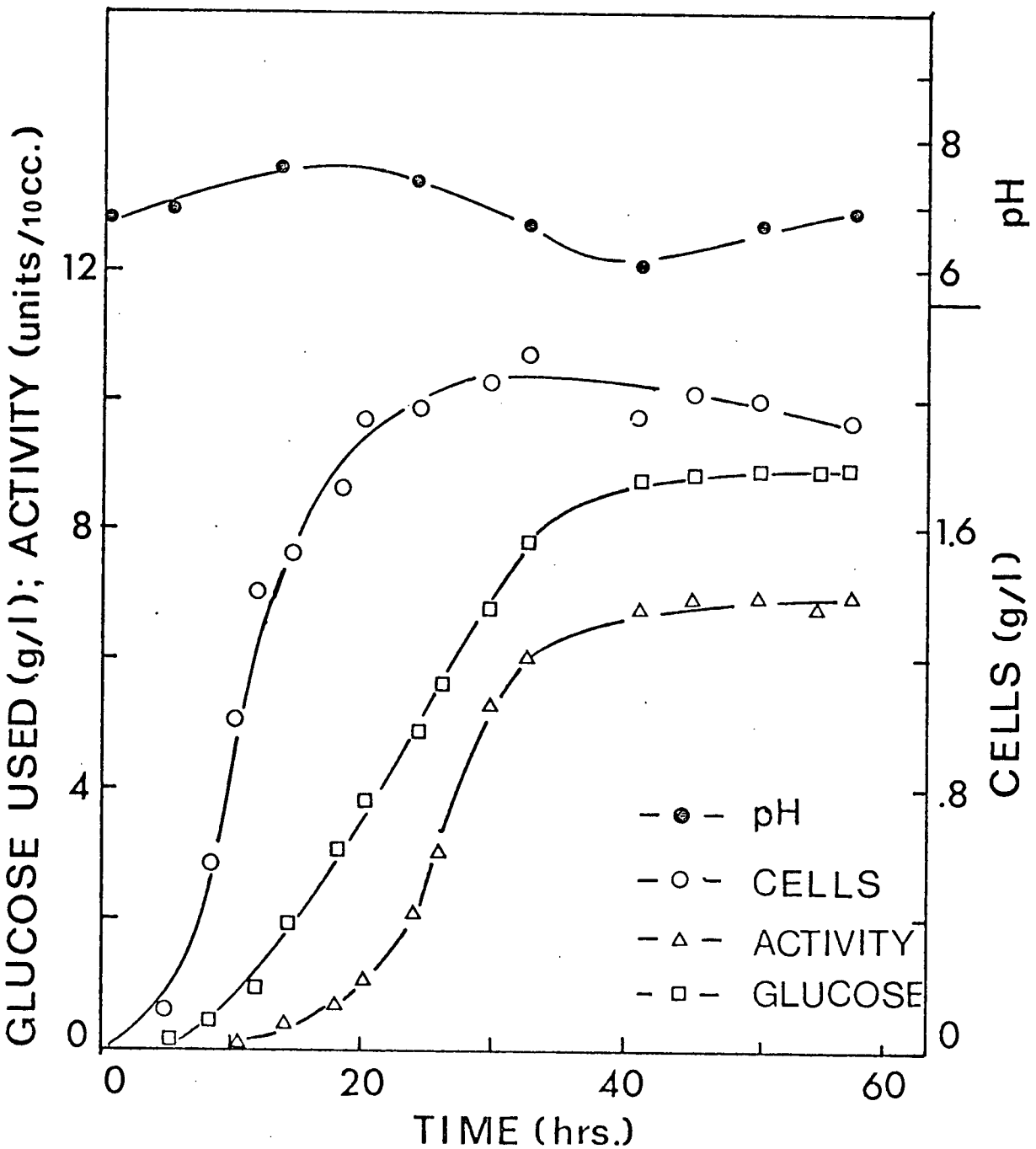


Figure 3.24

7-LITRE FERMENTOR CFS RUN NO. 6

(Agitator speed : 400 rpm; Air flow rate : 2 ℓ/min)

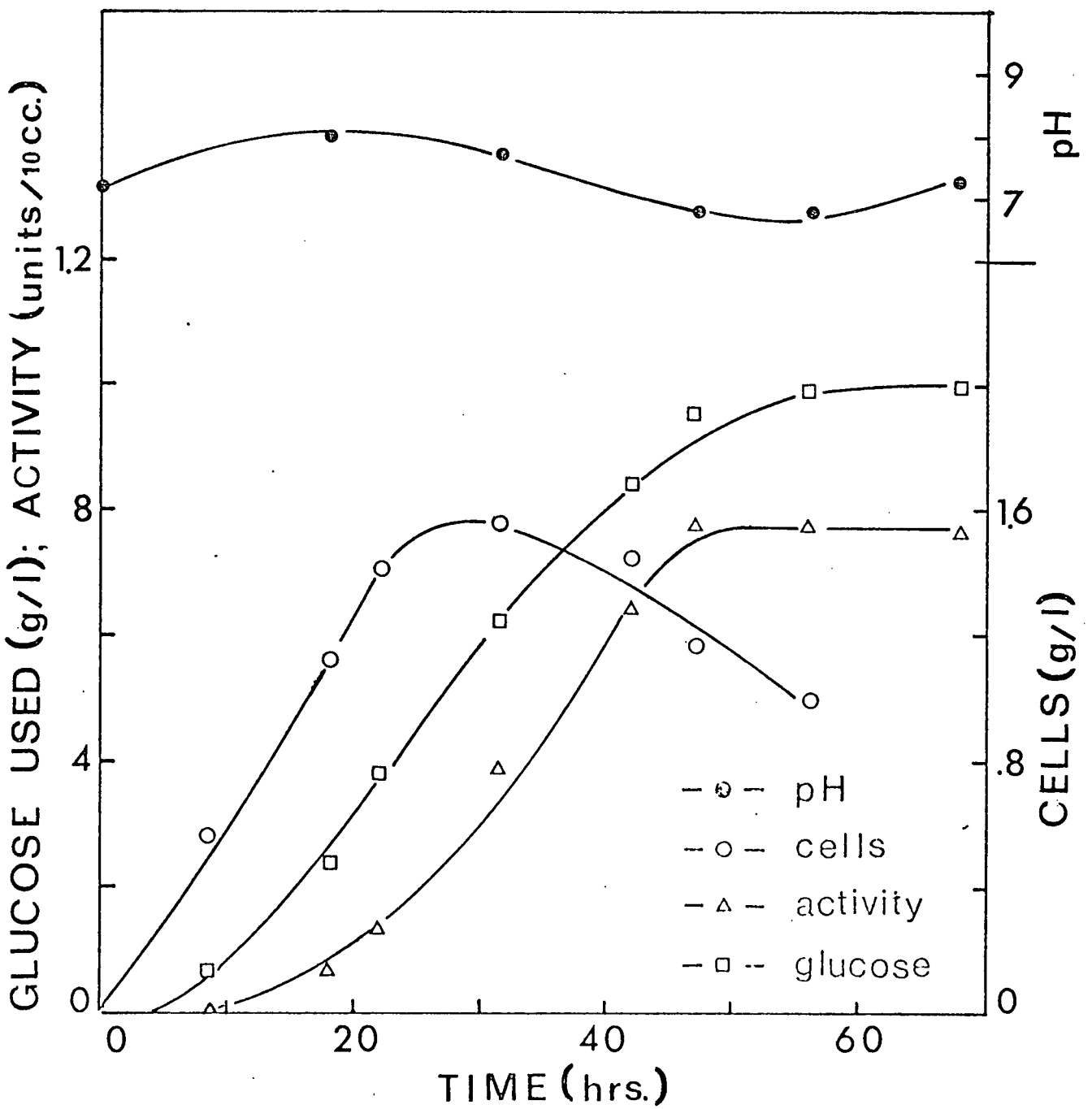
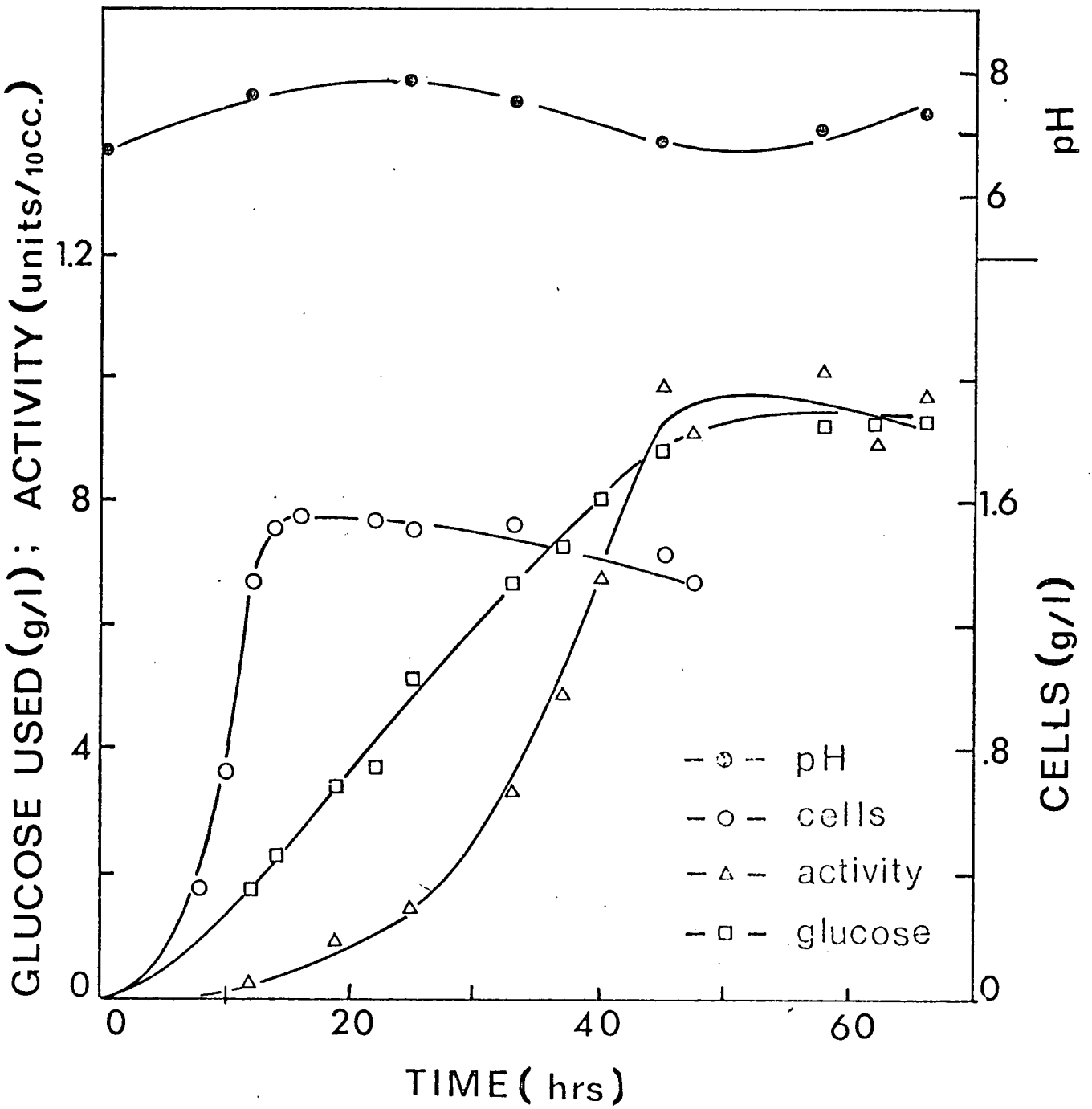


Figure 3.25

7-LITRE FERMENTOR CFS RUN NO. 7

(Agitator speed : 500 rpm; Air flow rate : 2 ℓ/min)



noted to reach a maximum and then decrease to a minimum. The concentration of enzyme rises in a sigmoidal fashion until a peak is reached. In all cases, the protease production curve lags behind the growth in a fashion similar to shake flask culturation.

The growth curve of Figure 3.19 can be divided up into 4 phases, namely: (1) phase of initial growth, (2) first stationary or death phase, (3) a phase of secondary growth and (4) a final deceleration and stationary phase. The duration of the second phase was greatly decreased, from 40 hours to 20 hours, as the agitator speed increased from 300 rpm to 400 rpm (Figures 3.19 and 3.20). At still higher agitation rates, 500 rpm and 600 rpm, the death phase of the second portion was completely eliminated; at the highest agitation rate, 750 rpm, the first and third phase meshed to give a sigmoidal growth curve. Increasing the aeration rate from 1 ℓ/min to 2 ℓ/min also completely eliminated the second phase (Figures 3.24, 3.25). The effect was therefore thought to be related to mass transfer limitation. However Figure 3.18 (Table AI.7 of Appendix I) indicated that the nature of the nitrogen source may have contributed to the overall effect.

As mentioned earlier, Figure 3.18 represents the results of a run made with peptone and meat extract as the nitrogen source; the agitation rate was 400 rpm and the aeration rate was 1 ℓ/min. This figure indicates that multiplication proceeded up to the 15^{th} hour, after which extensive lysis occurred, and very little glucose was utilized. The rise in the pH of the culture broth was obviously due to the products of nitrogen

metabolism. The large amount of unused glucose may have contributed to the extensive lysis observed after growth ceased [80, 81]. Nitrogen appears to be the limiting factor for growth and protease production in this case.

The first and second portions of the growth curve of Figures 3.19 and 3.20 correspond approximately, to the growth in the Nutrient Broth - glucose medium. These two portions of the growth curve may be interpreted as representing the depletion of free amino acids and nitrogenous bases initially present in the condensed fish solubles medium. After the depletion of the initial simple nitrogenous materials, bacterial metabolism appears to be limited by the rate at which the proteolytic enzyme can convert the larger polypeptides to simpler forms. Apparently, the rate of protein hydrolysis depends on the rate of enzyme production, which is in turn determined by the level of agitation. Although these reactions occur at different rates, they all proceed simultaneously. The difference in rates coupled with the initial presence of an easily available nitrogen source caused the staged nature of the growth kinetics. These results are consistent with Sperry and Rettger's [82] conclusions, which are quoted below :

> The inability of bacteria to decompose native proteins is not limited to aerobes and facultative anareobes, but even well-known and extremely active putrefactive anaerobes are devoid of this property. Solutions of native proteins may undergo complete hydrolysis, however, if they contain peptone or some other nitrogenous food material which

> readily furnishes the necessary nitrogen for bacterial development. In such instances the proteolysis of the native protein is the immediate result of the action of an enzyme which has been elaborated by the bacteria during the process of rapid multiplication. This multiplication is made possible by the nitrogen - containing material which is present along with the native protein.
>
> The resistance of native proteins to direct decomposition by bacteria is not due to any antiseptic properties of the proteins, but to a construction of the molecule which renders it relatively stable, the component parts being so firmly bound together that strong cleavage - producing agents, such as extreme heat, strong acids and/or alkalies, and enzymes, are required to change them so that bacteria may utilize their products for cell nutrition.

Rettger, Berman and Sturges [83] carried the matter a stage further when they showed that heat - coagulated albumin, and sometimes peptones are not available for direct utilization until further decomposed by enzyme action.

Of interest also are the findings of Archer et al [84]. While studying the solubilization of fish protein concentrate (FPC) by Bacillus Subtilis protease (Monzyme), they found that the overall kinetics could be described by a sequence of two first-order processes - an initial, fast reaction in which loosely bound polypeptide chains were cleaved, and a second, slower reaction in which more compacted protein was digested. Similar biphasic kinetics were obtained by Mihalyi and Harrington [85].

Figure 3.26 shows that when the Sorangium specie was grown in a fish solubles medium without added glucose, staged kinetics were not detected. A comparison of this figure with Figure 3.19 indicates that the maximum enzyme concentration attained in the glucose - deficient media corresponds to the value at the end of the second phase of growth shown in Figure 3.19. The results therefore suggest that the organism was unable, in the absence of a source of carbohydrate, to bring about breakdown of the larger polypeptide units present in the medium. The data points of this figure are tabulated in Table AI.15. Shake flask cultivation was used to obtain these results.

3.7.2 Effect of Agitation and Aeration on Ultimate Yields

The effects of agitation and aeration on ultimate yields are summarized in Table 3.15. The highest protease production occurred at 500 rpm with 1 litre of air introduced per min. However, more significantly at the same agitator speed and 2 litres of air per minute, almost the same ultimate protease yield was obtained but at a much faster rate. Other agitation rates resulted in lower productivity. The percentage of initial sugar utilized at the end of fermentation was noted to be approximately the same at all levels of agitation and aeration tested; however the highest sugar consumption rate occurred at 750 rpm with 1 litre of air per minute. Aeration rates and agitation rates are interdependent in their effect on cell metabolism - for example, at 600 rpm and an air flow of 1 ℓ/min, total sugar consumption, protease production, and growth are almost

Figure 3.26

SHAKE FLASK FERMENTATION - GLUCOSE DEFICIENT MEDIUM

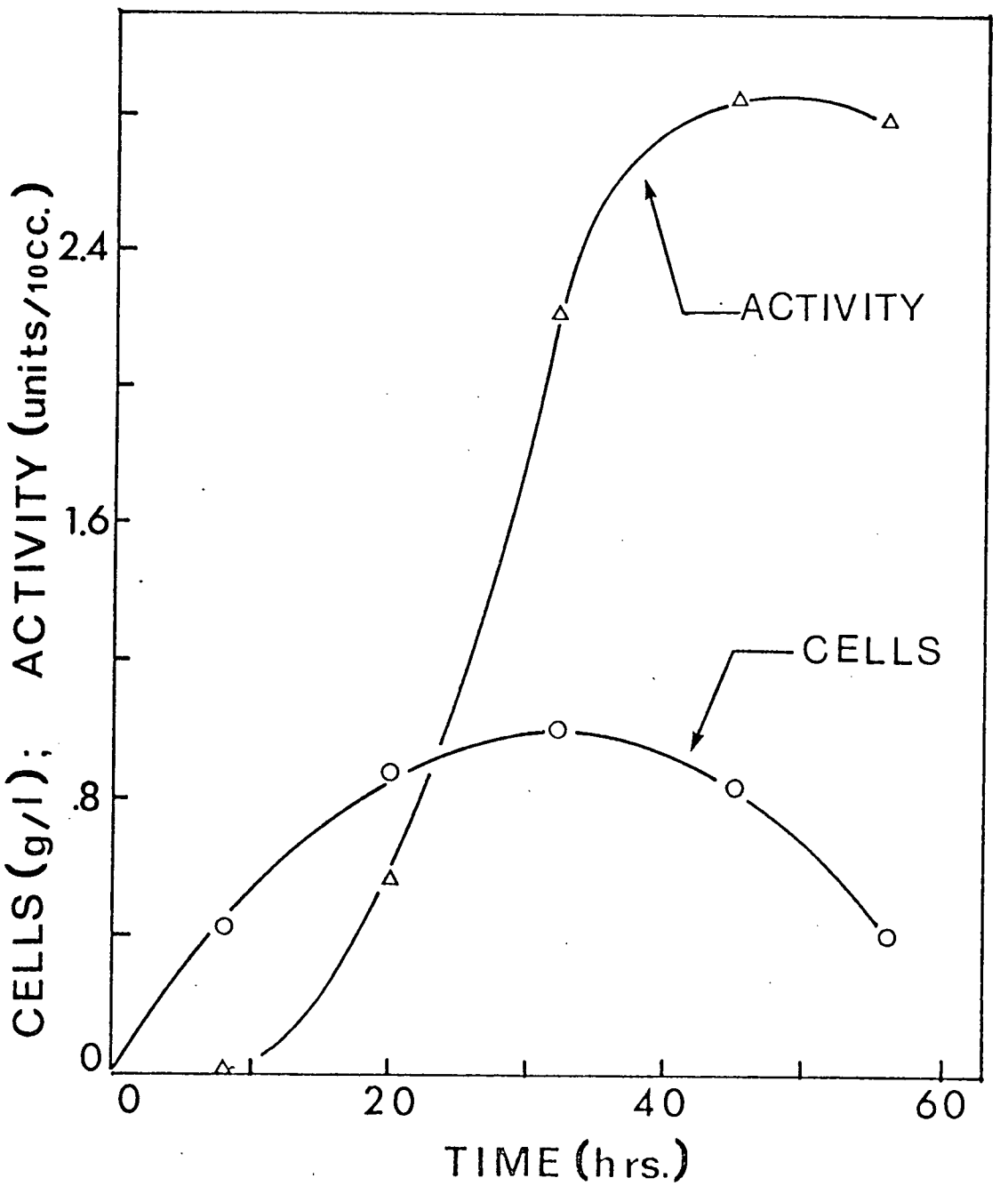


Table 3.15

EFFECT OF AGITATION AND AERATION ON ULTIMATE YIELDS

MEDIA	AIR FLOW RATE (ℓ/min)	AGITATOR SPEED rpm	DRY WEIGHT MAX (g/ℓ)	DRY WEIGHT TIME TO MAX (hrs)	PROTEOLYTIC ACTIVITY MAX (units/10cc)	PROTEOLYTIC ACTIVITY TIME TO MAX (hrs)	SUGAR UTILISATION MAX (%initial)	SUGAR UTILISATION TIME TO MAX (hrs)
FILTERED 2% v/v CONDENSED FISH SOLUBLES + 1% GLUCOSE	1	300	1.00*	$113\frac{3}{4}$	8.84	134	96.3	134
		400	0.85*	94	9.95	$116\frac{1}{2}$	95.0	$116\frac{1}{2}$
		500	1.29	$52\frac{3}{4}$	10.52	$68\frac{1}{2}$	95.4	77
		600	1.62	35	7.82	$52\frac{3}{4}$	94.4	$57\frac{3}{4}$
		750	2.05	$29\frac{1}{2}$	6.94	45	92.4	45
	2	400	1.55	$31\frac{1}{2}$	7.74	47	94.3	56
		500	1.55	16	9.86	45	94.1	$47\frac{1}{2}$
ABOVE MEDIUM minus 1% GLUCOSE	SHAKER INCUBATOR		1.00	32	2.85	45	-	-
NB plus 1% GLUCOSE	1	400	0.83	15	2.28	45	16.0	34

* Global maximum

identical to those at an air flow of 2ℓ/min and 400 rpm.

Figure 3.27 is a plot of cells produced, and proteolytic activity against glucose used. No relationship between the three variables is apparent.

3.7.3 Effect of Agitation and Aeration on Rate of Oxygen Transfer

The functions of agitation and aeration in aerobic fermentors are mainly to create a large air - liquid interfacial area and to reduce the resistance to oxygen diffusion by reducing the thickness of the liquid film surrounding each bubble [86].

The correlation of Yamada et al [87] was used to calculate the values of K_v, the volumetric oxygen transfer coefficient, shown in Table 3.16. An attempt to correlate the K_v values with the maximum yield and the maximum rate of protease formation is shown in Figure 3.28. This method of correlation was unsuccessful in bringing together data for the various systems studied. It appears therefore that mass transfer is not the only controlling factor in the production of proteolytic enzyme or that Yamada's correlation for K_v is not applicable to our fermentation system, and that a further investigation of this subject is necessary.

Figure 3.27

ACTIVITY AND CELL YIELD VS SUGAR USED

FOR VARIOUS 7-LITRE FERMENTOR RUNS

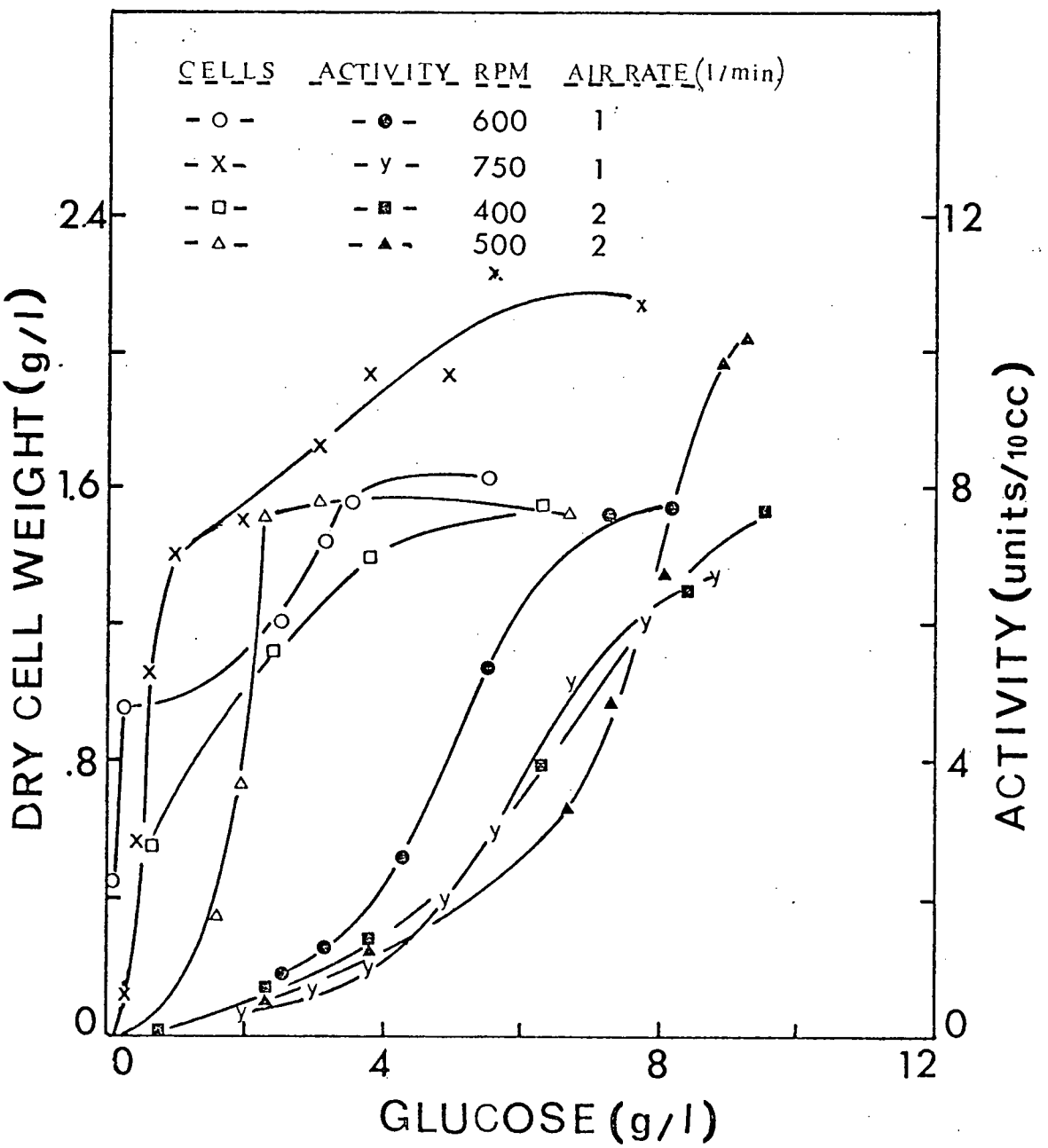


Table 3.16

K_v VALUES ATTAINED IN 7-L FERMENTATION RUNS

AIR FLOW RATE ℓ/min	AGITATOR SPEED rpm	K_v millimoles/atm. min. litre	(dp/dt)max units/100cc.hr
1	300	0.177	1.20
	400	0.369	1.40
	500	0.654	3.50
	600	1.043	3.70
	750	1.847	5.65
2	400	0.452	3.60
	500	0.805	6.90

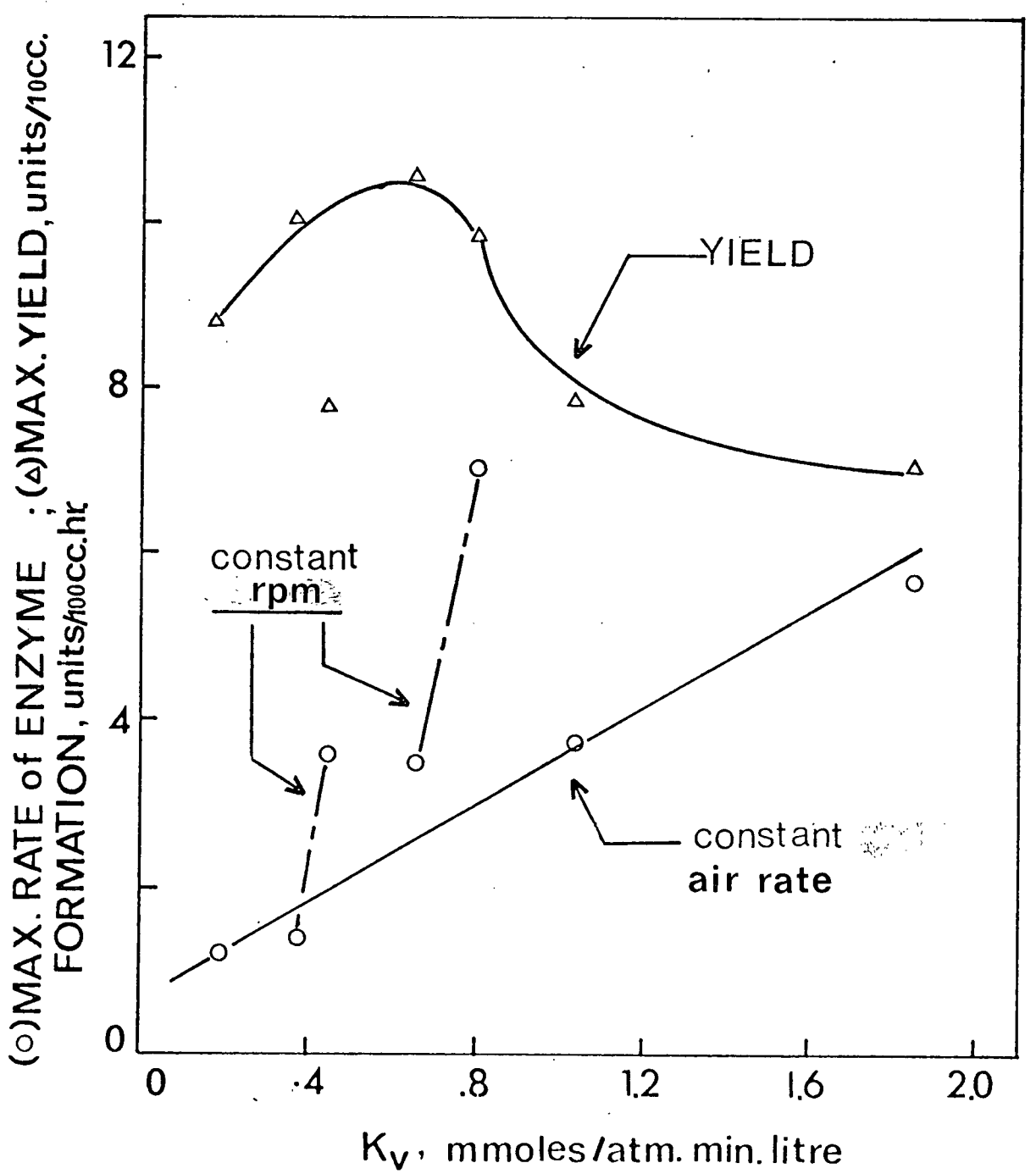
Correlation used was :

$$K_v = 8.06 \times 10^{-8} \times v^{0.3} \times N^{2.56}$$

This empirical relationship was obtained by Yamada et al (87) using a stainless steel,8-ℓ capacity fermentor fitted with a single hole sparger and a disk type agitator.

Figure 3.28

MAXIMUM YIELD AND MAXIMUM RATE OF ENZYME PRODUCTION AS A FUNCTION OF K_v



Chapter 4

FERMENTATION KINETICS

Batch kinetic data are needed not only to develop basic understanding of fermentation processes but also to permit rational design of continuous fermentation systems. The fermentation of fish solubles to produce enzymes has so far not received any quantitative treatment; consequently no kinetic analysis of this fermentation can be found in the literature. This chapter attempts to analyse further some of the results presented in the previous sections. In particular, it is the hope of this chapter to determine the relationships, if any, between the different rates and to see how these rates vary with respect to agitation and aeration.

Instantaneous (or volumetric) rates of growth, sugar utilisation, and enzyme production were obtained by the graphical differentiation [88] of curves of figures showing the time changes in these variables. The specific rates were determined by dividing the volumetric rates by the cell mass at that time. The specific rates are more useful for kinetic analysis since they put everything on a comparable basis, ie. per unit mass of cells.

Complete rate patterns, on both volumetric and specific rate bases, for fermentations at 500 rpm at 1 ℓ/min, 750 rpm at 1 ℓ/min and 500 rpm at 2 ℓ/min, are presented in Tables 4.17 to 4.22 and graphed in Figures 4.29 to 4.34. They were calculated from the data presented in Figures 3.21, 3.23 and 3.25.

Table 4.17

7-LITRE FERMENTATION - CFS RUN NO. 3*

VOLUMETRIC RATES OF

GROWTH		SUGAR UTILIZATION		PROTEASE FORMATION	
TIME (hrs)	dx/dt (g/ℓ.hr×10)	TIME (hrs)	ds/dt (g/ℓ.hr×10)	TIME (hrs)	dp/dt (units/cc.hr×100)
2.5	0.44	4.0	0.70	7.0	0.0
5.0	0.84	10.5	1.10	12.0	0.6
7.0	1.24	20.0	1.55	16.0	0.8
8.5	1.60	30.5	1.90	24.0	1.6
9.5	0.32	40.0	2.10	29.5	2.0
10.5	0.18	48.5	1.50	34.0	2.4
14.0	0.0	61.5	0.50	40.5	2.85
18.0	0.10	80.0	0.10	47.0	3.35
23.0	0.44	92.0	0.00	51.0	3.80
26.5	0.29			53.5	2.10
29.5	0.20			62.5	0.40
35.0	0.08			75.0	0.00
44.0	0.02				
55.0	0.00				

* Agitator speed : 500 rpm

Air flow rate : 1 ℓ/min.

Table 4.18

7-LITRE FERMENTATION - CFS RUN NO. 3*

SPECIFIC RATES OF

TIME (hrs)	GROWTH $\frac{1}{x}\frac{dx}{dt}$ (g/g.hr×10)	SUGAR UTILIZATION $\frac{1}{x}\frac{ds}{dt}$ (g/g.hr×10)	PROTEASE FORMATION $\frac{1}{x}\frac{dp}{dt}$ (units/mg.hr×100)
2.5	4.89	5.56	0.78
5.0	3.11	2.78	0.74
7.0	2.53	1.83	-
8.5	2.22	1.35	-
10.0	0.33	1.28	0.55
14.0	0.0	1.49	0.83
18.0	0.12	1.69	1.16
20.0	0.22	1.74	1.31
23.0	0.46	-	1.46
27.5	0.27	1.62	1.67
30.0	0.19	1.65	1.78
32.5	-	-	1.91
35.0	0.07	1.65	2.07
40.0	0.03	1.65	2.38
45.0	-	1.46	2.64
50.0	-	1.11	2.72
55.0	-	0.79	1.61
60.0	-	0.56	0.75
65.0	-	0.36	0.32
75.0		0.12	0.0

*Agitator speed : 500 rpm

Air flow rate : 1 ℓ/min.

Table 4.19

7-LITRE FERMENTATION - CFS RUN NO. 5*

VOLUMETRIC RATES OF

GROWTH		SUGAR UTILIZATION		PROTEASE FORMATION	
TIME (hrs)	dx/dt (g/ℓ.hr×10)	TIME (hrs)	ds/dt (g/ℓ.hr×10)	TIME (hrs)	dp/dt (units/cc.hr×100)
2.3	0.24	7.4	1.0	12.8	0.6
6.5	0.81	12.0	2.2	21.3	2.05
9.0	1.80	18.5	3.05	25.3	5.65
10.7	2.34	24.4	3.70	28.0	4.80
13.5	1.12	30.0	3.00	30.5	2.80
16.7	0.64	36.5	0.95	36.0	0.80
20.7	0.39	45.0	0.25	42.5	0.35
26.8	0.10	55.0	0.0	47.5	0.20
32.5	0.0			52.5	0.0

* Agitator speed : 750 rpm

Air flow rate : 1 ℓ/min.

Table 4.20

7-LITRE FERMENTATION - CFS RUN NO. 5*

SPECIFIC RATES OF

TIME (hrs)	GROWTH $\frac{1}{x}\frac{dx}{dt}$ (g/g.hr×10)	SUGAR UTILIZATION $\frac{1}{x}\frac{ds}{dt}$ (g/g.hr×10)	PROTEASE FORMATION $\frac{1}{x}\frac{dp}{dt}$ (units/mg.hr×100)
2.5	5.75	5.75	0.0
5.0	4.43	4.43	0.0
7.5	3.57	3.14	0.0
10.0	2.59	2.13	0.19
12.5	1.14	1.70	0.34
15.0	0.57	1.69	0.47
17.5	0.34	1.75	0.68
20.0	0.21	1.78	0.96
22.5	0.13	1.80	1.37
25.0	0.07	1.79	2.46
25.5	-	-	2.77
27.5	0.04	1.65	2.42
30.0	0.02	1.45	1.74
32.5	0.0	0.89	0.89
35.0	0.0	0.61	0.61
40.0	0.0	0.28	0.28
45.0	0.0	0.10	0.10

* Agitator speed : 750 rpm

Air flow rate : 1 ℓ/min.

Table 4.21

7-LITRE FERMENTATION - CFS RUN NO. 7*

VOLUMETRIC RATES OF

GROWTH		SUGAR UTILIZATION		PROTEASE FORMATION	
TIME (hrs)	dx/dt (g/ℓ.hr×10)	TIME (hrs)	ds/dt (g/ℓ.hr×10)	TIME (hrs)	dp/dt (units/cc.hr×100)
2.7	0.26	2.5	0.85	12.6	0.15
6.8	0.86	6.5	1.55	21.5	1.10
9.5	1.71	11.0	2.10	29.0	2.57
11.5	3.10	17.6	2.50	34.5	3.85
12.5	1.38	26.0	2.30	38.8	5.10
12.8	0.97	35.0	2.10	42.0	6.90
15.0	0.25	42.0	1.65	43.7	3.80
18.0	0.0	47.5	0.80	44.7	2.30
		52.2	0.20	48.5	0.60
		55.0	0.0	52.5	0.0

* Agitator speed : 500 rpm

Air flow rate : 2 ℓ/min.

Table 4.22

7-LITRE FERMENTATION - CFS RUN NO. 7*

SPECIFIC RATES OF

TIME (hrs)	GROWTH $\frac{1}{x}\frac{dx}{dt}$ (g/g.hr×10)	SUGAR UTILIZATION $\frac{1}{x}\frac{ds}{dt}$ (g/g.hr×10)	PROTEASE FORMATION $\frac{1}{x}\frac{dp}{dt}$ (units/mg.hr×100)
1.5	7.0	20.0	-
2.5	6.0	16.8	-
5.0	4.46	9.6	-
7.5	3.05	5.0	-
10.0	2.41	2.88	-
11.5	2.87	2.08	-
12.5	1.00	1.70	0.07
15.0	0.19	1.61	0.16
17.5	0.0	1.62	0.32
20.0	-	1.62	0.52
25.0	-	1.56	1.10
30.0	-	1.49	1.82
35.0	-	1.39	2.69
40.0	-	1.20	3.90
45.0	-	0.84	1.75
50.0	-	0.31	0.31

* Agitator speed : 500 rpm

Air flow rate : 2 ℓ/min.

Figure 4.29

VOLUMETRIC RATES VS TIME FOR FERMENTOR RUN NO. 3

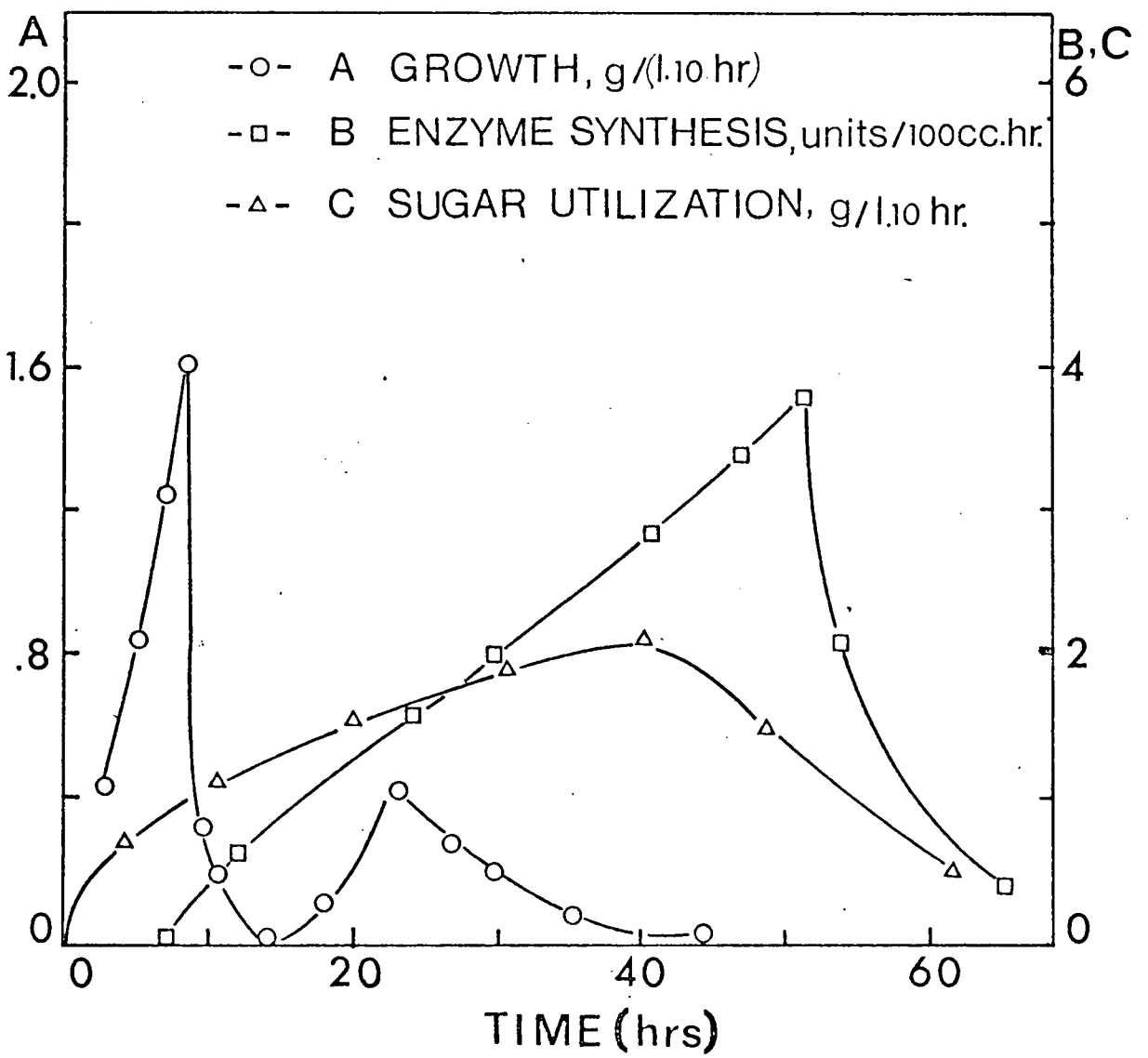


Figure 4.30

SPECIFIC RATES VS TIME FOR FERMENTOR RUN NO. 3

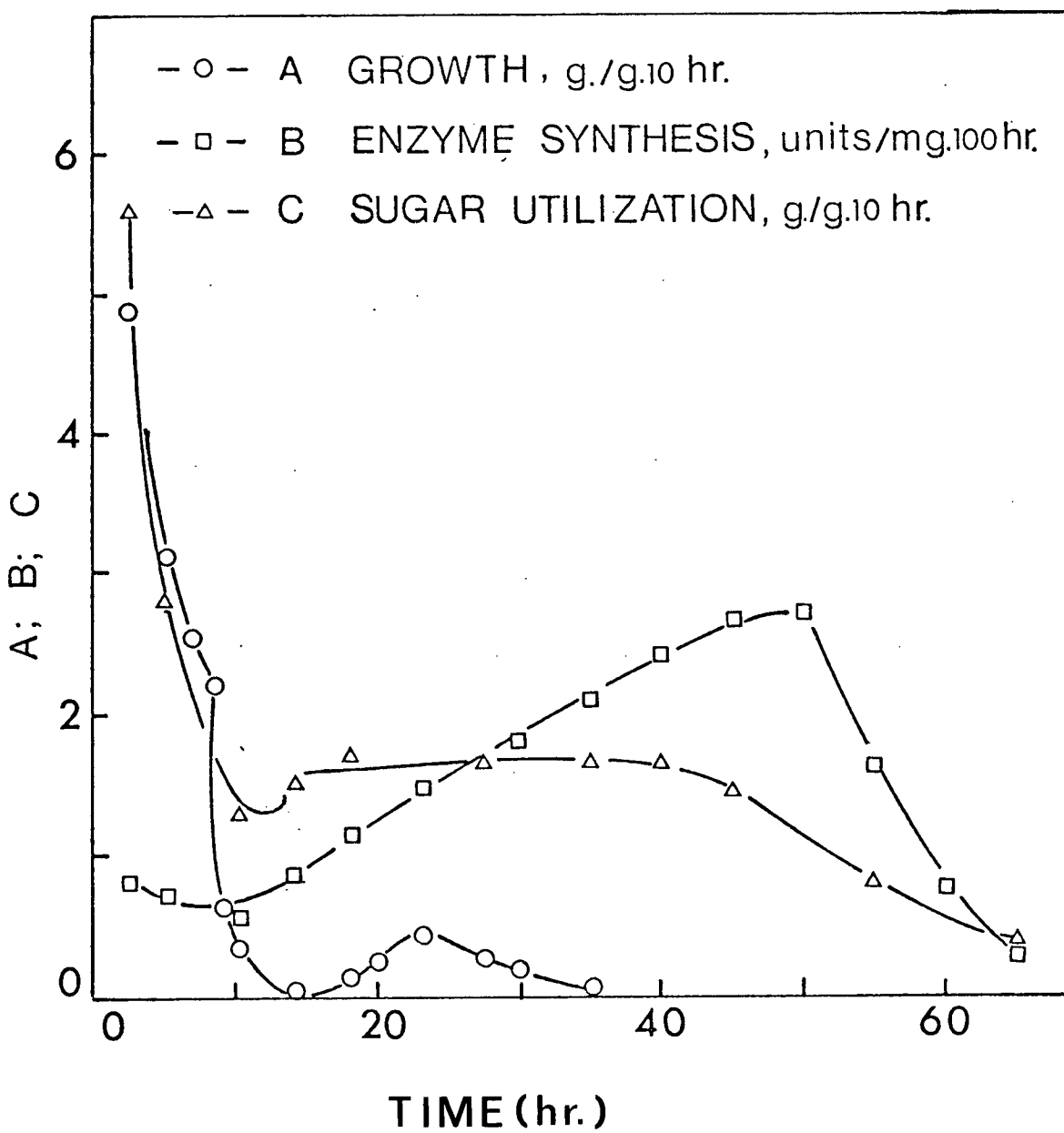


Figure 4.31

VOLUMETRIC RATES VS TIME FOR FERMENTOR RUN NO. 5

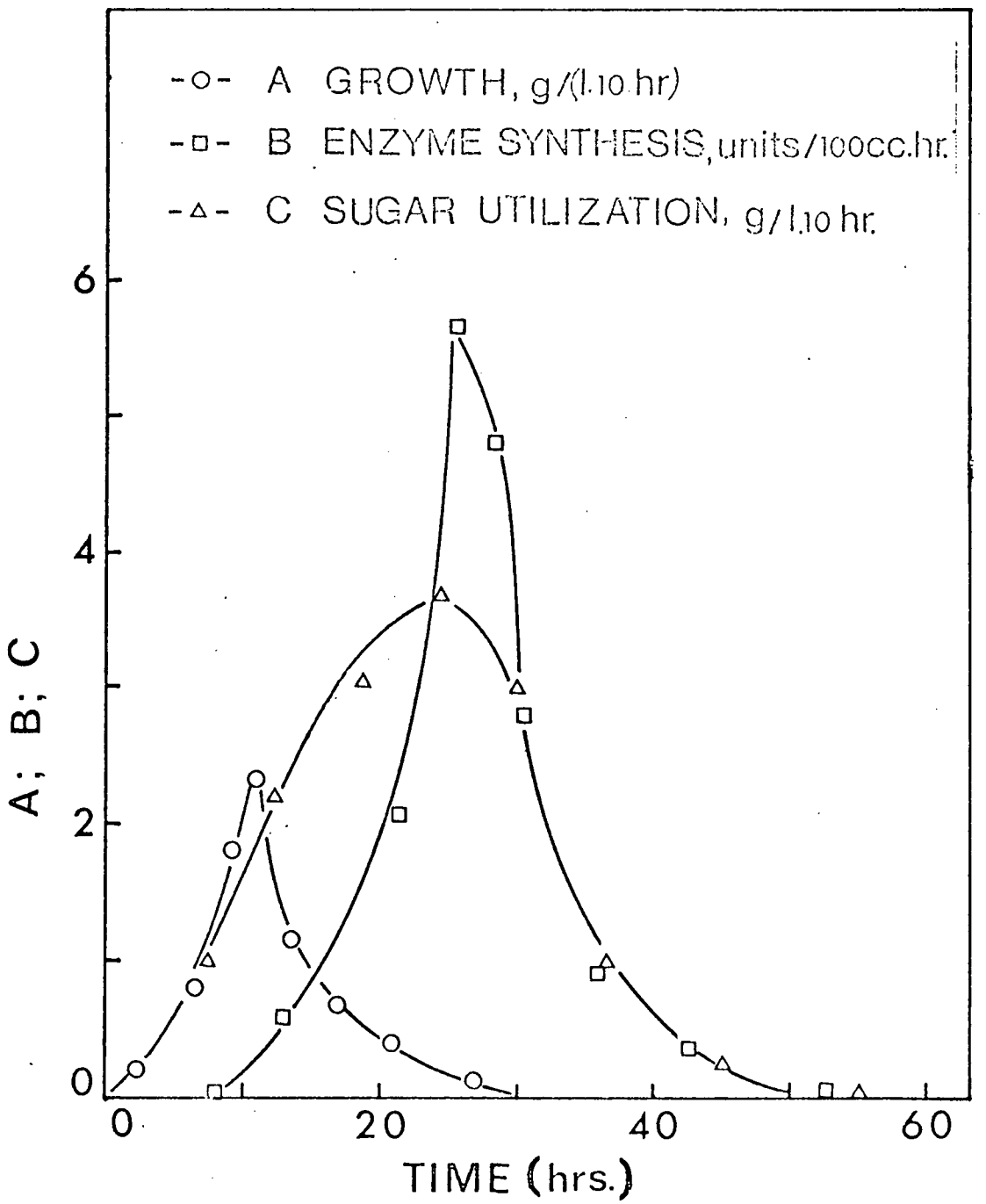


Figure 4.32

SPECIFIC RATES VS TIME FOR FERMENTOR RUN NO. 5

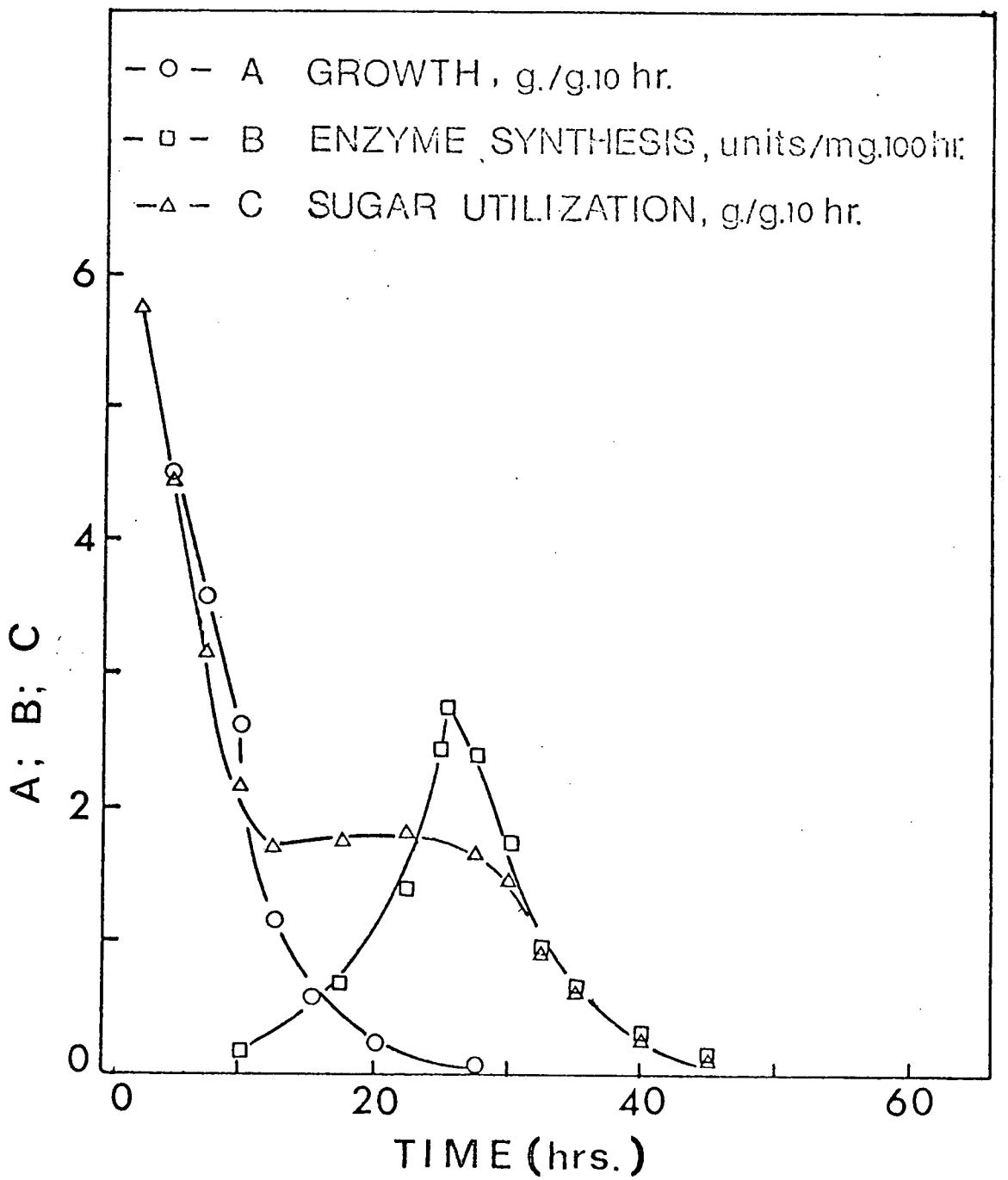


Figure 4.33

VOLUMETRIC RATES VS TIME FOR FERMENTOR RUN NO. 7

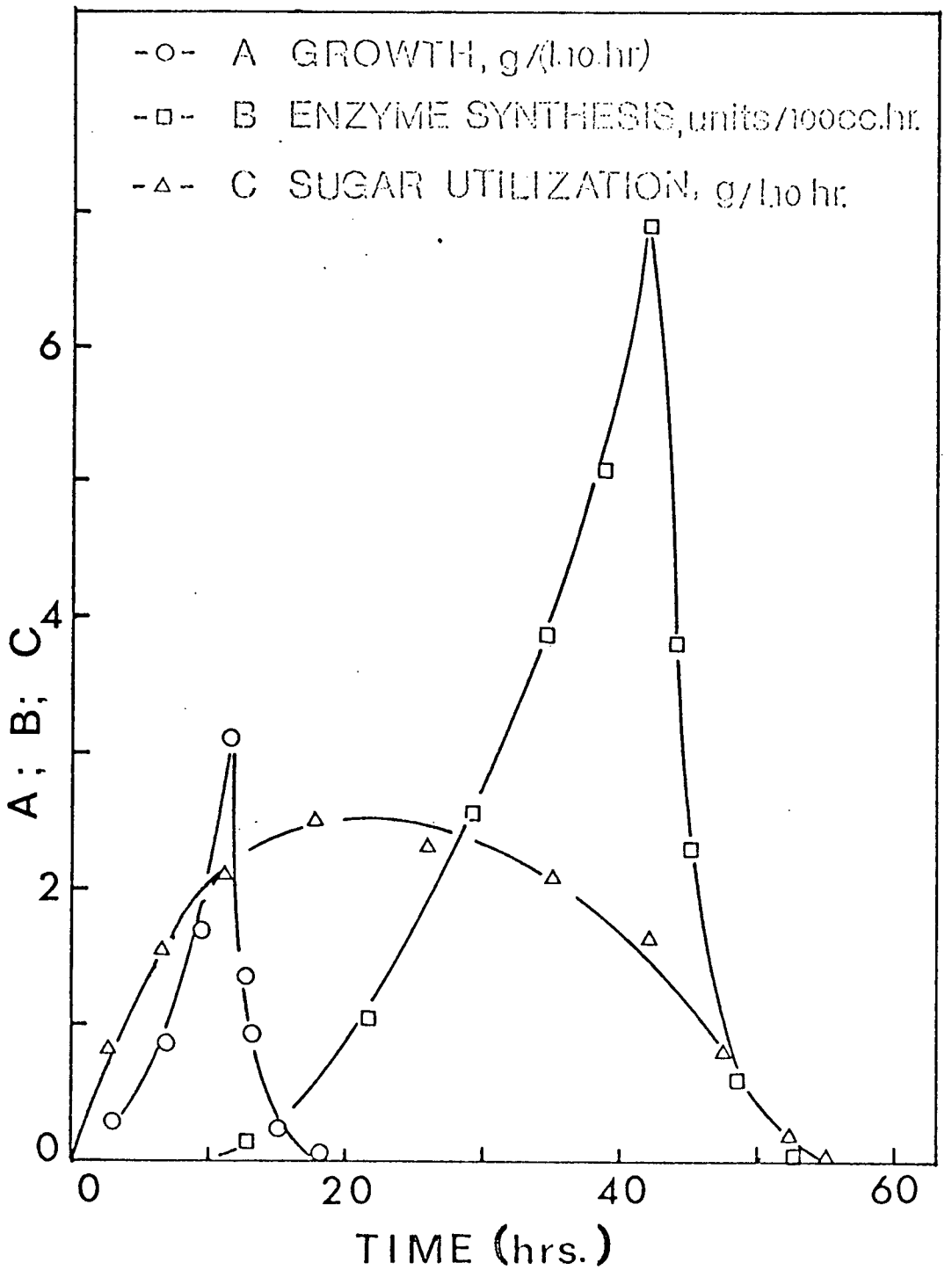
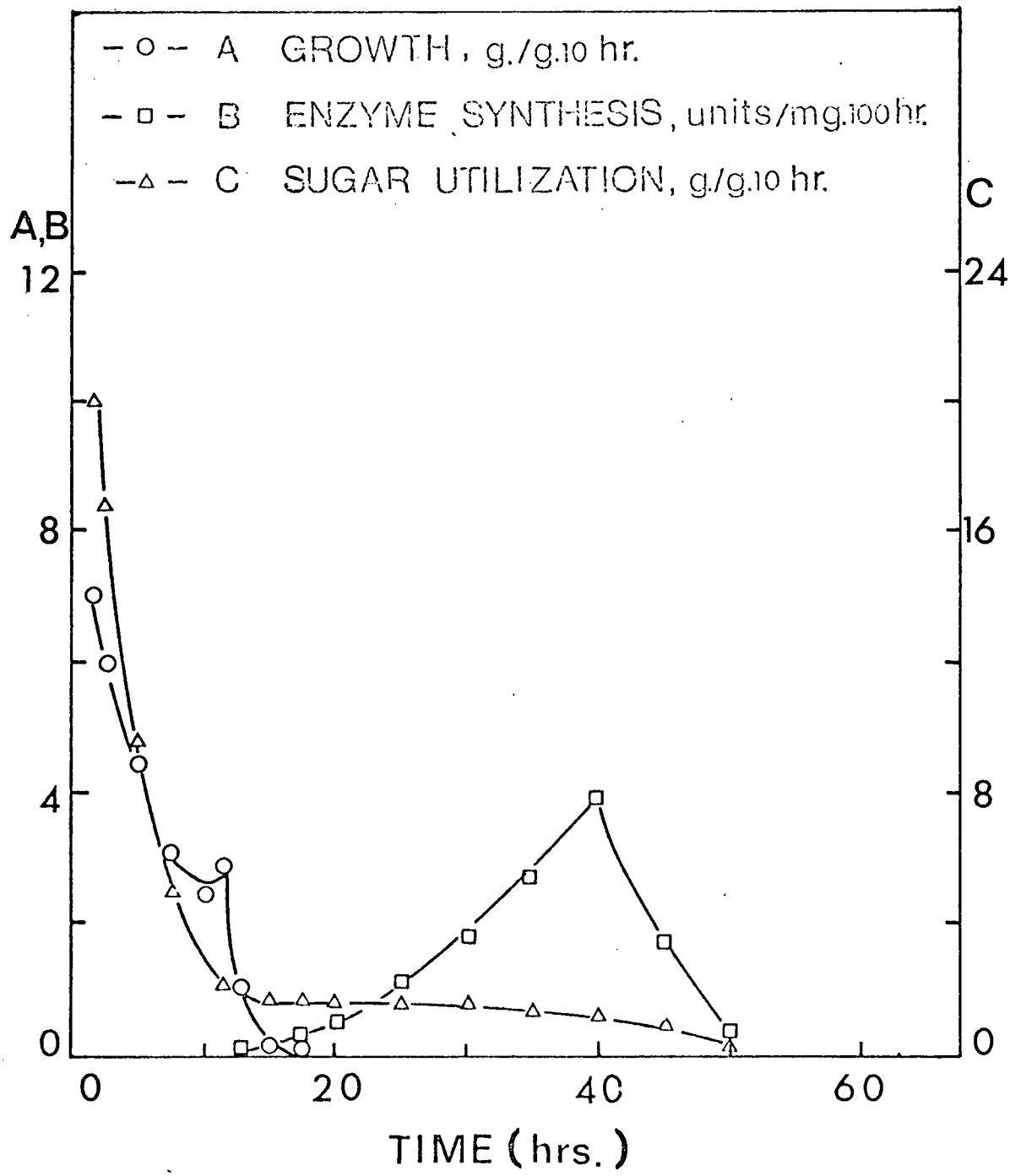


Figure 4.34

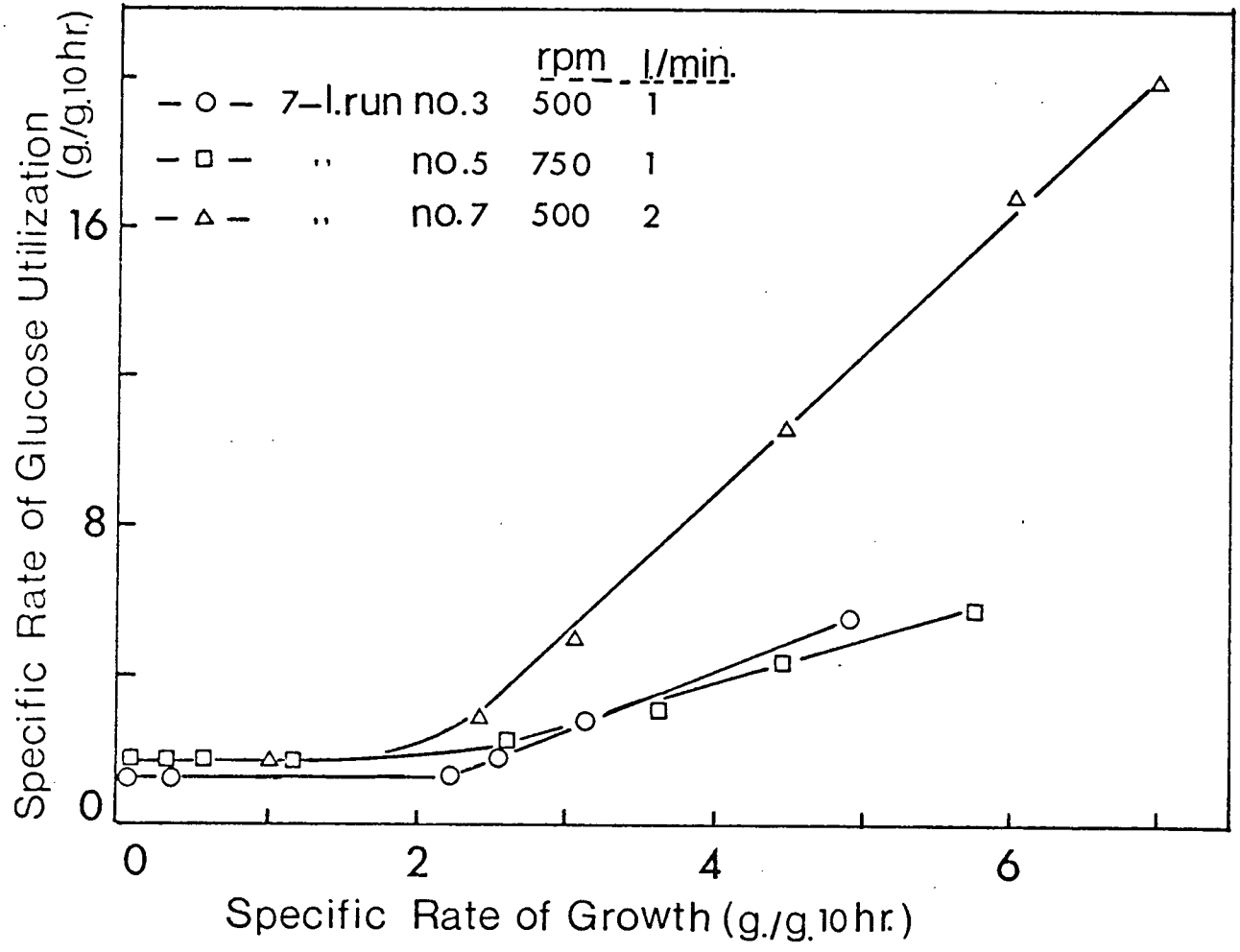
SPECIFIC RATES VS TIME FOR FERMENTOR RUN NO. 7



The rate curves of these figures show two distinct phases in the fermentation system under consideration. In the initial phase, sugar is used primarily for cell multiplication with practically no production of enzyme; in the second phase, when the growth rate has decreased to a zero value, enzyme accumulation is maximum. This is most evident in the specific rate plots. Another point, also demonstrated quite clearly in the specific rate patterns, is the close coincidence of growth and sugar utilization rates in the initial stages (10-15 hours). During the period of enzyme production, both the dry cell weight and the specific rate of carbohydrate utilization remained constant. The relationship between the specific rate of glucose utilization $\left(\frac{1}{x}\frac{ds}{dt}\right)$ and the specific rate of growth $\left(\frac{1}{x}\frac{dx}{dt}\right)$ is furthered demonstrated in Figure 4.35. This plot suggests that during the early stages of the fermentation (up to the 10^{th} hour) the rate of carbohydrate consumption per cell is directly proportional to the specific rate of growth. The constant of proportionality depends more on the air flow rate than on the agitator speed. From the 10^{th} hour to the early stages of the final stationary growth phase, the rate of glucose consumption is constant at 1.682 g/g. hr × 10. This value, obtained by averaging all values of $\frac{1}{x}\frac{ds}{dt}$ between 1.6 and 1.8, was the same for all three cases examined. During the period of constant specific glucose utilization, glucose consumption and protease production had just entered their logarithmic phases (Figures 3.21, 3.23 and 3.25). No direct relationship between carbohydrate oxidation rate and protease production rate is apparent. One can therefore speculate that one of the purposes served by the glucose

Figure 4.35

SPECIFIC RATE OF GLUCOSE UTILIZATION VS SPECIFIC RATE OF GROWTH FOR VARIOUS FERMENTOR RUNS



was to supply the energy required for the endergonic reaction of synthesis. A comparison of the specific rates of enzyme production for the three cases examined indicates that (1) agitator speed has a greater influence on this rate than does air flow rate. For example, at a stirring rate of 500 rpm and 1 ℓ/min, the rate of enzyme production per gm dry cell weight increased slowly and steadily to its maximum value after 50 hours, and at 750 rpm and the same aeration rate, the maximum was attained after 25 hours, while at 500 rpm and 2 ℓ/min, time to maximum was 40 hours; and (2) The metabolic activity of the organism during the early part of the fermentations is highest at an aeration rate of 2 litre air per minute.

The kinetic patterns obtained in this study are quite complex. Additional studies on the effects of dissolved oxygen concentration and rate of oxygen transfer to the medium are needed to elucidate the various limiting steps.

Chapter 5

CONCLUSIONS

This study has shown that the batch production of extracellular protease by Soranguim 495 can be achieved in a medium containing condensed fish solubles as its major component. The condensed fish solubles, obtained from B.C. Packers, Richmond, B.C., was diluted and any solid material filtered off. The filtered media was found to give better protease yields than the unfiltered media. A dilution ratio of 20 cc condensed fish solubles to 1000 cc of water, containing soluble protein equivalent to 3.85 mg BSA/ml, was found to be the optimum for enzyme yield.

Prolific growth of the organism was obtained in both filtered and unfiltered diluted condensed fish solubles. Statistically designed experiments showed that while additional phosphate has no significant effect on protease production, this was not so for glucose and calcium. The amount of supplementary glucose was found to have a large influence on both growth and protease production. The one tailed F-test also indicated that there was significant interaction between the different sugar concentrations and the different calcium concentrations. The complete analysis of variance is given in Table 3.13.

Further experimental runs with medium containing several levels of glucose (0% to 4%) indicated that a two fold increase in coseinolytic activity was obtained by increasing the initial sugar content from 0.062

mg per ml, originally present in the medium, to 10 mg per ml. That the additional glucose exerted its influence by increasing the growth of the organism was readily apparent from visual observation and from the two phase nature of the growth curve. The data also illustrated that addition of glucose retarded protease formation. Later results disclosed that adequate agitation and/or aeration not only eliminated the staged growth but also increased both the yield and rate of protease formation.

Glucose and mannose supported the growth of the organism. Best growth was achieved with mannose. Best protease yield occurred with glucose. The medium containing mannose gave a biphasic enzyme production curve while the medium containing glucose gave a biphasic growth curve. Soluble starch, galactose, xylose and arabinose were not utilized. Initial pH (in the range 4 to 8) did not significantly affect the maximum yield of proteases nor did inoculum age (16 to 24 hours). Reproducible results have been obtained with shake flask cultivation.

7-litre fermentation studies indicated that the inflection in the growth curve observed at the lower agitation and aeration rates and in shake flask cultivation may be a complex result of initial amino acid (and possibly small polypeptides) utilization on one hand and enzymatic digestion of larger polypeptide units plus enzyme secretion and oxygen limitation on the other. Highest productivity was obtained with an agitation rate of 500 rpm and an aeration rate of 2 litre of air per minute; this corresponded to an oxygen absorption coefficient of 0.805 millimoles O_2/atm.min.ℓ.

This value was obtained using the correlation of Yamada et al [87].

When the kinetic data were presented on a per unit of cell mass basis (that is specific rate basis), it transpired that the two processes, growth and protease synthesis, were independent. During the phase of enzyme production, the specific rate of glucose utilization remained at a constant value of 1.682 g/g. hr × 10. It was therefore apparent that product formation was not associated by any direct mechanism with carbohydrate utilization. Enzyme production was affected more by rate of agitation than aeration; the reverse holds for sugar consumption.

The kinetic patterns are quite complex, and further work is necessary to elucidate the various mechanisms involved.

Chapter 6

RECOMMENDATIONS

(1) Agricultural wastes high in carbohydrate, such as fruit wastes, should be investigated with the view to replacing glucose as a supplementary nutrient. The acid hydrolyzed condensed fish solubles contains a large amount of protein hydrolysates and other easily available nitrogen compounds which are not present in the stickwater that Truong [11] used, unsuccessfully, as a replacement for yeast extract. It is therefore recommended that condensed fish solubles and not stickwater should be used as a replacement for yeast extract.

(2) Additional studies on the effects of dissolved oxygen concentration and rate of oxygen transfer to the medium should be carried out. Hopefully data from these results will clarify the major features of the mechanism of the system so that a working theoretical model can be formulated. It is recommended that these studies be carried out with better pH and foam control.

(3) The effect of filter - sterilized and autoclaved sugars on growth and enzyme producing ability of the Sorangium specie should be investigated.

(4) The effect of calcium ions on protease yields should be further investigated.

(5) In order to develop useful applications of the Sorangium protease, its specificity and optimum conditions of action should be studied. In this connection, it is suggested that the enzyme be tested for its ability to hydrolyze fish to produce FPC for human consumption and to produce fish hydrolysates for use in bacteriological culture media. A more meaningful cost estimate for an enzyme production process can be made when such information is obtained.

(6) Experimentation with other microorganisms, such as those associated with industrial fermentation processes (for example, Bacillus subtilis, and Aspergillus oryzae), is recommended.

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Appendix I

EXPERIMENTAL DATA

Table AI.1

EFFECT OF GLUCOSE CONCENTRATION (FILTERED CFS)

GLUCOSE CONCEN-TRATION	TIME (days)	pH	DRY CELL WEIGHT (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
0%	2	7.9	0.70	96.0	4.24
	4	8.9	0.30	90.5	4.00
	6	8.9	0.32	74.0	3.27
	8	8.9	0.38	60.3	2.67
0.5%	2	7.5	1.38	60.2	2.66
	4	8.3	1.16	131.0	5.79
	6	8.4	0.59	135.0	5.97
	8	8.9	0.53	119.5	5.28
1.0%	2	7.42	1.20	46.8	2.07
	4	7.66	1.52	135.0	5.97
	6	7.80	2.44	202.5	8.96
	8	8.25	1.28	204.5	9.04
	10	8.20	0.80	164.5	7.18
2%	2	7.32	1.18	42.1	1.86
	4	7.31	1.76	100.0	4.42
	6	6.70	2.60	183.0	7.98
	8	5.80	3.20	180.0	7.85
	10	7.25	3.17	118.5	5.08
	12	7.60	-	128.0	5.66
3%	2	7.25	1.10	40.0	1.77
	4	6.71	1.60	92.5	4.09
	6	5.95	2.28	127.0	5.60
	8	4.10	2.30	20.3	0.90
4%	2	7.20	1.06	35.8	1.58
	4	6.21	1.60	84.0	3.71
	6	5.25	2.11	92.0	4.07
	8	3.40	1.87	6.0	0.26

Inoculum age : 24 hours
Dry cell weight at t = 0 : 0.0085 gm/ℓ

Table AI.2

EFFECT OF GLUCOSE CONCENTRATION (UNFILTERED CFS)

GLUCOSE CONCENTRATION	TIME (days)	pH	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
	3	8.0	33.0	1.46
0%	5	8.75	37.8	1.67
	8	8.90	31.5	1.39
	3	7.55	33.0	1.46
	5	8.20	59.2	2.62
0.5%	8	9.10	80.5	3.56
	10	8.90	92.5	4.09
	12	8.75	77.0	3.40
	3	7.45	26.2	1.158
	5	7.40	63.5	2.807
1.0%	8	8.12	107.5	4.752
	10	8.50	129.5	5.724
	12	8.61	128.0	5.658
	3	7.25	26.2	1.158
	5	7.0	74.2	3.280
2.0%	8	6.2	100.0	4.420
	10	6.2	102.5	4.531
	12	7.61	103.0	4.56
	3	7.15	21.5	0.950
	5	6.60	54.0	2.387
3.0%	8	6.10	66.9	2.957
	10	6.00	73.0	3.227
	12	5.10	42.8	1.892
	3	7.09	20	0.884
	5	6.40	48.1	2.126
4.0%	8	6.15	59.6	2.634
	10	-	-	-
	12	5.95	44.0	1.945

Table AI.3

EFFECT OF CFS CONCENTRATION

TIME (hrs)	DILUTION : 1:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	DILUTION : 2:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/100cc)
7	0.12	1.2	0.053	0.16	1.2	0.053
19	0.86	18.9	0.835	0.92	9.4	0.415
30	1.06	39.0	1.724	1.00	24.8	1.096
43	1.20	73.3	3.240	1.22	45.0	1.989
55	1.68	100.0	4.420	1.36	91.7	4.053
67	1.96	122.5	5.414	1.58	94.0	4.155
$79\frac{1}{4}$	2.02	110.5	4.884	1.78	146.0	6.453
$90\frac{1}{4}$	2.18	96.0	4.243	1.96	162.5	7.183
$102\frac{1}{4}$	2.02	91.3	4.035	2.18	209.5	9.260
$114\frac{3}{4}$	2.26	73.0	3.227	2.46	252.5	11.161
$125\frac{1}{2}$	2.08	58.6	2.590	2.52	271.5	12.000
138	2.12	60.0	2.652	2.44	265.5	11.735
150	1.74	56.8	2.511	2.18	249.0	11.006
	Initial pH : 7.0 Final pH : 5.9			Initial pH : 7.0 Final pH : 8.0		

Table AI.3 (Contd)

EFFECT OF CFS CONCENTRATION

TIME (hrs)	DILUTION : 3:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	DILUTION : 4:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
7	0.16	0.5	0.022	0.20	1.6	0.071
19	0.84	7.1	0.314	0.90	4.5	0.199
30	0.98	15.6	0.690	0.98	12.0	0.530
43	1.30	32.0	1.414	1.34	21.8	0.964
55	1.64	56.0	2.475	1.52	31.8	1.406
67	1.84	72.5	3.205	1.80	63.3	2.798
$79\frac{1}{4}$	1.86	86.5	3.823	1.90	66.7	2.948
$90\frac{1}{4}$	2.00	107.0	4.729	2.08	78.3	3.461
$102\frac{1}{4}$	2.16	127.0	5.613	2.16	94.0	4.155
$114\frac{3}{4}$	2.44	131.5	5.812	2.28	99.7	4.407
$125\frac{1}{2}$	2.50	150.0	6.630	2.38	112.3	4.964
138	2.44	172.5	7.625	2.34	138.7	6.130
150	2.12	172.5	7.625	2.34	127.0	5.613
	Initial pH : 7.0 Final pH : 8.4			Initial pH : 7.0 Final pH : 8.32		

Table AI.3 (Contd)

EFFECT OF CFS CONCENTRATION

TIME (hrs)	DILUTION : 5:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	DILUTION : 6:100 v/v DRY WEIGHT (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
7	0.14	0.07	0.003	0.06	-	-
19	0.98	3.5	0.155	0.94	3.3	0.146
30	1.10	4.2	0.186	1.06	4.5	0.199
43	1.38	10.3	0.455	1.34	7.8	0.345
55	1.64	13.6	0.601	1.64	7.8	0.345
67	1.92	21.4	0.946	1.80	15.0	0.663
$79\frac{1}{4}$	2.04	26.9	1.189	1.88	19.8	0.875
$90\frac{1}{4}$	2.10	29.5	1.304	2.18	19.5	0.862
$102\frac{1}{4}$	2.06	-	-	2.28	20.9	0.924
$114\frac{3}{4}$	2.30	41.5	1.834	2.52	11.8	-
$125\frac{1}{2}$	2.22	40.5	1.790	2.54	29.1	1.286
138	2.20	53.4	2.360	2.54	28.4	1.255
150	2.24	51.2	2.263	2.42	29.3	1.295
	Initial pH : 7.0 Final pH : 8.40			Initial pH : 7.0 Final pH : 8.40		

Table AI.4

EFFECT OF HEXOSE SUGARS (GLUCOSE)

TIME (hrs)	DRY WEIGHT (gm/ℓ)	GLUCOSE CONCENTRATION (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
0	0.0085	10.4	-	-
8	0.24	10.4	-	-
$18\frac{1}{2}$	0.76	-	3.5	0.16
30	1.02	9.2	13.1	0.58
42	1.36	8.45	23.8	1.05
$55\frac{1}{2}$	-	7.50	37.2	1.64
70	1.38	6.55	59.8	2.64
80	1.50	5.40	76.8	3.40
$100\frac{1}{2}$	1.74	3.10	124.5	5.50
115	2.03	1.40	175.0	7.74
125	2.10	0.60	202.5	8.95
139	2.06	0.50	202.5	8.95
149	-	0.50	209.0	9.24

Initial pH : 7.0
Final pH : 8.12

Inoculum age
= 24 hours

Dry cell weight
at t = 0 : 0.0085 gm/ℓ

Table AI.4 (Contd)

EFFECT OF HEXOSE SUGARS

TIME (hrs)	GALACTOSE DRY WEIGHT (gm/ℓ)	GALACTOSE TYROSINE (µg/ml)	GALACTOSE PROTEOLYTIC ACTIVITY (units/10cc)	MANNOSE DRY WEIGHT (gm/ℓ)	MANNOSE TYROSINE (µg/ml)	MANNOSE PROTEOLYTIC ACTIVITY (units/10cc)
8	0.30	-	-	0.18	-	-
$18\frac{1}{2}$	0.74	3.6	0.159	0.70	2.1	0.093
30	1.09	20.8	0.919	1.00	16.3	0.720
42	1.25	36.8	1.626	1.54	34.6	1.529
$55\frac{1}{2}$	1.22	41.6	1.839	1.67	42.8	1.892
70	1.52	48.2	2.130	2.04	50.8	2.245
80	-	47.8	2.113	-	49.6	2.192
$100\frac{1}{2}$	1.48	56.5	2.497	2.38	71.3	3.151
115	1.46	58.8	2.599	2.54	115.5	5.105
125	1.46	63.8	2.820	2.48	135.7	5.998
139	1.50	69.0	3.00	2.55	111.5	-
149	1.40	63.8	2.820	2.48	137.5	6.078
	Initial pH : 7.00 Final pH : 7.30			Initial pH : 7.00 Final pH : 8.12		

Table AI.5

EFFECT OF PENTOSES AND STARCH

TIME (hrs)	XYLOSE DRY WEIGHT (gm/ℓ)	XYLOSE TYROSINE (µg/ml)	XYLOSE PROTEOLYTIC ACTIVITY (units/10cc)	ARABINOSE DRY WEIGHT (gm/ℓ)	ARABINOSE TYROSINE (µg/ml)	ARABINOSE PROTEOLYTIC ACTIVITY (units/10cc)
8	0	-	-	0.02	-	-
$18\frac{1}{2}$	0.32	1.3	0.057	0.50	1.9	0.084
30	0.85	10.0	0.442	0.82	22.4	0.990
42	0.94	38.3	1.693	0.92	39.4	1.741
$55\frac{1}{2}$	0.70	47.0	2.077	0.60	44.0	1.945
70	0.54	50.8	2.245	0.70	43.8	1.936
80	0.40	48.0	2.122	0.50	41.6	1.839
$100\frac{1}{2}$	-	47.0	2.077	-	40.0	1.768
	Initial pH : 7.00 Final pH : 8.90			Initial pH : 7.00 Final pH : 8.90		

STARCH

8	0.42	-	-
$18\frac{1}{2}$	0.68	7.3	0.323
30	1.25	34.6	1.529
42	1.31	58.1	2.568
$55\frac{1}{2}$	0.95	68.0	3.006
70	0.85	68.0	3.006
80	0.57	70.6	3.121
$100\frac{1}{2}$	-	68.8	3.041
	Initial pH : 7.00 Final pH : 9.10		

Table AI.6

EFFECT OF INOCULUM AGE

(1) Inoculum Age : 16 hours

TIME (hrs)	DRY WEIGHT (gm/ℓ)	GLUCOSE CONCENTRATION (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
0	0.007	10.3	-	-
8	0.30	10.3	-	-
20	0.76	10.3	1.7	0.075
32	1.04	9.4	13.3	0.588
45	1.32	8.45	22.6	0.999
56	1.06	7.70	35.2	1.556
68	1.04	6.65	48.8	2.157
92	1.88	3.97	106.7	4.716
$101\frac{1}{2}$	2.21	2.40	123.5	5.459
$115\frac{1}{2}$	2.44	0.78	165.5	7.315
126	2.32	0.50	180.0	7.956
140	2.25	0.45	190.0	8.398
150	2.00	0.42	192.5	8.508

Initial pH : 7.0
Final pH : 8.1

Table AI.6 (Contd)

EFFECT OF INOCULUM AGE

(2) Inoculum age : 20 hours

TIME (hrs)	DRY WEIGHT (gm/ℓ)	GLUCOSE CONCENTRATION (gm/ℓ)	TYROSINE (µg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)
0	0.0085	10.3	-	-
8	0.18	10.3	-	0
20	0.86	10.0	3.2	0.141
32	1.14	9.2	12.6	0.557
45	1.40	7.8	22.2	0.981
56	1.20	7.45	29.4	1.300
68	1.10	6.00	47.0	2.077
92	1.82	3.63	104.3	4.610
$101\frac{1}{2}$	2.06	2.46	124.5	5.503
$115\frac{1}{2}$	2.26	0.70	170.0	7.514
126	2.28	0.52	180.5	7.978
140	2.03	0.49	190.0	8.398
150	1.80	0.44	187.0	8.265

Initial pH : 7.0
Final pH : 8.1

Table AI.7

7-LITRE FERMENTATION - NB - GLUCOSE RUN

(Agitator speed : 400 rpm; Air flow rate : 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	7.00	0.023	-	-	8.1
8	7.10	0.28	-	-	8.1
12	7.40	0.34	-	-	-
15	7.50	0.83	4.0	0.177	7.8
18	7.62	0.71	8.2	0.362	8.1
21	7.66	0.50	14.3	0.632	7.6
$24\frac{3}{4}$	7.87	0.37	25.5	1.127	7.0
34	8.35	0.12	43.6	1.927	6.8
41	8.22	0.22	50.2	2.219	6.8
45	8.45	0.07	51.5	2.276	7.4

Table AI.8

7-LITRE FERMENTATION - CFS RUN NO. 1

(Agitator speed : 300 rpm; Air flow rate : 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	6.82	0.023	-	-	10.00
3	6.92	0.090	-	-	-
$11\frac{1}{2}$	7.38	0.40	-	-	-
14	7.53	0.57	1.5	0.066	9.40
16	7.62	0.66	-	-	-
18	7.70	0.58	5.4	0.239	9.60
$20\frac{1}{2}$	7.75	0.65	-	-	-
24	7.80	0.65	7.6	0.336	9.20
27	7.83	0.65	-	-	-
35	7.85	0.51	21.0	0.928	8.70
40	7.80	0.52	-	-	-
$43\frac{3}{4}$	7.85	0.48	39.0	1.724	7.90
49	7.92	0.48	54.5	2.409	7.40
$63\frac{1}{2}$	7.78	0.46	75.0	3.315	5.80
$85\frac{1}{4}$	7.70	0.72	114.0	5.039	4.10
91	7.70	0.74	126.0	5.569	3.50
97	7.62	0.77	137.5	6.078	2.70
$109\frac{1}{2}$	7.52	0.94	167.5	7.400	1.70
$113\frac{3}{4}$	7.48	1.00	184.2	8.132	0.99
$121\frac{1}{4}$	7.82	0.95	199.5	8.012	0.44
$131\frac{1}{4}$	8.10	0.88	197.0	8.702	0.39
134	8.10	0.80	200.0	8.819	0.37

Table AI.9

7-LITRE FERMENTATION - CFS RUN NO. 2

(Agitator speed : 400 rpm; Air flow rate L 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	7.0	0.023	-	-	9.00
6	7.1	0.15	-	-	9.00
15	7.85	0.59	4.8	0.212	8.95
$17\frac{1}{2}$	7.85	0.66	9.5	0.420	8.55
$20\frac{1}{2}$	7.95	0.66	21.5	0.950	7.85
23	7.92	0.75	30.2	1.335	7.85
29	8.20	0.46	37.0	1.635	6.90
41	8.22	0.49	54.0	2.387	6.25
54	8.25	0.61	93.5	4.133	5.00
68	8.12	0.71	126.5	5.591	4.00
$78\frac{1}{4}$	8.00	0.80	151.0	6.674	3.27
$88\frac{1}{2}$	7.75	0.81	190.0	8.398	2.20
94	7.61	0.85	211.5	9.348	1.64
102	7.36	0.78	207.5	9.172	0.77
$111\frac{1}{2}$	7.83	0.82	217.0	9.591	0.50
$116\frac{1}{2}$	7.98	0.83	225.0	9.945	0.45

Table AI.10

7-LITRE FERMENTATION - CFS RUN NO. 3

(Agitator speed : 500 rpm; Air flow rate : 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	6.88	0.023	-	-	9.9
5	7.20	0.270	-	-	9.2
7	7.42	0.49	-	-	9.9
9	7.65	0.86	1.0	0.044	8.9
$17\frac{1}{2}$	8.15	0.81	15.5	0.685	7.95
22	8.15	0.91	26.2	1.158	7.50
25	8.15	1.08	39.0	1.724	6.90
32	8.03	1.25	60.5	2.674	5.30
42	7.70	1.20	127.0	5.613	3.53
$49\frac{1}{4}$	7.40	1.18	183.5	8.111	2.20
$52\frac{3}{4}$	7.08	1.29	211.5	9.348	1.48
$56\frac{1}{4}$	6.80	1.10	231.0	10.210	1.07
$68\frac{1}{2}$	6.92	1.25	252.5	11.14	0.54
77	7.20	1.20	238.0	10.52	0.46
81	7.28	1.20	235.5	10.41	0.42
$91\frac{1}{4}$	7.33	1.20	235.5	10.41	0.37
94	7.35	1.21	231.0	10.21	0.37

Table AI.11

7-LITRE FERMENTATION - CFS RUN NO. 4

(Agitator speed : 600 rpm; Air flow rate : 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	6.9	0.023	-	-	8.7
$4\frac{1}{4}$	7.0	0.18	-	-	8.7
8	7.25	0.45	-	-	8.7
10	7.45	0.96	0.7	0.031	8.5
20	7.70	1.20	20.7	0.915	6.2
23	7.65	1.44	30.3	1.340	5.6
26	7.60	1.55	45.6	2.016	5.2
28	7.50	1.56	59.2	2.617	4.4
35	7.20	1.62	122.5	5.415	3.2
44	6.15	1.50	172.5	7.624	1.5
48	5.72	1.56	171.0	7.558	0.8
$52\frac{3}{4}$	5.85	1.65	177.0	7.823	0.55
$57\frac{3}{4}$	6.05	1.59	178.5	7.890	0.49
$68\frac{1}{2}$	6.40	1.55	184.0	8.133	0.47
76	6.70	1.58	177.5	7.846	0.45
$83\frac{1}{4}$	6.85	1.54	178.5	7.890	0.43

Table AI.12

7-LITRE FERMENTATION - CFS RUN NO. 5

(Agitator speed : 750 rpm; Air flow rate : 1 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	6.80	0.023	-	-	9.6
5	6.98	0.120	-	-	9.4
$8\frac{1}{4}$	7.30	0.57	0.7	0.031	9.2
10	7.40	1.06	1.0	0.044	9.0
12	7.44	1.40	8.0	0.354	8.65
14	7.65	1.51	8.2	0.362	7.65
16	7.70	1.50	14.8	0.654	7.05
18	7.62	1.72	15.8	0.698	6.55
20	7.50	1.93	23.0	1.017	5.80
24	7.40	1.97	46.2	2.042	4.70
26	7.22	2.23	68.0	3.006	4.00
$29\frac{1}{2}$	7.00	2.05	118.6	5.242	2.87
$32\frac{1}{2}$	6.60	2.14	135.5	5.989	1.84
41	6.08	1.93	152.0	6.718	0.82
45	6.25	2.01	157.0	6.939	0.73
50	6.75	1.99	157.0	6.939	0.72
$54\frac{1}{2}$	6.82	1.83	152.5	6.741	0.67
57	6.90	1.86	157.0	6.939	0.65

Table AI.13

7-LITRE FERMENTATION - CFS RUN NO. 6

(Agitator speed : 400 rpm; Air flow rate : 2 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	7.20	0.023	0	0	10.55
$8\frac{1}{2}$	-	0.55	0.7	0.031	9.90
18	8.02	1.12	15.0	0.663	8.20
22	8.02	1.40	29.5	1.304	6.80
$31\frac{1}{2}$	7.70	1.55	89.0	3.934	4.27
42	7.22	1.44	146.5	6.475	2.12
47	6.75	1.16	175.0	7.735	1.05
56	6.80	1.10	175.0	7.735	0.60
68	7.30	1.27	172.5	7.624	0.51

Table AI.14

7-LITRE FERMENTATION - CFS RUN NO. 7

(Agitator speed : 500 rpm; Air flow rate : 2 ℓ/min)

TIME (hrs)	pH	DRY WEIGHT (gm/ℓ)	TYROSINE (μg/ml)	PROTEOLYTIC ACTIVITY (units/10cc)	GLUCOSE CONCENTRATION (gm/ℓ)
0	6.70	0.023	-	-	9.75
8	7.30	0.35	-	-	8.15
10	7.50	0.73	-	-	7.80
12	7.70	1.34	5.2	0.230	8.00
14	7.70	1.51	11.8	0.522	7.50
16	7.80	1.55	18.5	0.818	6.70
19	7.90	-	22.2	0.981	6.35
22	7.88	1.53	28.5	1.260	6.00
25	7.90	1.50	32.0	1.414	4.63
33	7.70	1.52	74.8	3.300	3.10
37	7.50	-	110.0	4.862	2.50
40	7.38	-	152	6.718	1.70
45	6.90	1.42	223	9.857	0.88
$47\frac{1}{2}$	6.90	1.33	205	9.061	0.58
58	7.10	-	229	10.122	0.52
62	7.20	-	203	8.973	0.56
66	7.30	-	220	9.724	0.49

Table AI.15

SHAKE FLASK FERMENTATION - GLUCOSE DEFICIENT MEDIUM

TIME (hrs)	DRY CELL WEIGHT (gm/ℓ)	PROTEOLYTIC ACTIVITY units/10cc
0	0.008	0
8	0.41	.009
20	0.88	.559
32	1.00	2.20
45	0.83	2.85
56	0.40	2.78

Appendix II

Analysis Procedures :

II.1 Measurement of Glucose Concentration

II.2 Measurement of Proteolytic Activity

II.3 Protein Estimation with the Biuret Reagent

Appendix II

II.1 Measurement of Glucose Concentration

II.1.1 Phenol - Sulfuric Acid Reagent Method [56]

Reagents :

(1) Sulfuric Acid - Reagent grade 95.5%, conforming to ACS specifications, specific gravity 1.84.

(2) Phenol - 5% solution in water.

Apparatus :

(1) Fast delivery 5-ml pipet, to deliver 5 ml of concentrated sulfuric acid in 10 to 20 seconds. Can be prepared by cutting a portion off the tip of a standard 5 ml pipet.

(2) Set of tubes with internal diameter between 16 and 20 mm.

(3) Spectronic 20 tubes or a set of matched colorimeter tubes.

Procedure :

Standard Curve

(1) Prepare 1 ml glucose solutions containing between 10 and 70 µg of glucose.

(2) Add 1 ml of 5% phenol solution to each.

(3) Add 5 ml of concentrated H_2SO_4 rapidly; the stream of acid is directed against the liquid surface rather than the side of the

test tube in order to obtain good mixing.

(4) Allow to stand for 10 minutes at room temperature.

(5) Shake and place in water bath set at 30°C for 10 to 20 minutes.

(6) Transfer slowly to colorimeter tubes and measure the absorbance at 490 mμ in spectronic 20.

(7) Plot A_{490} versus μg glucose and make a least square fit to the data, as shown in Figure AII.36.

Unknown

Repeat steps 2 to 6 with unknown sample, and obtain the sugar concentration from Figure AII.36.

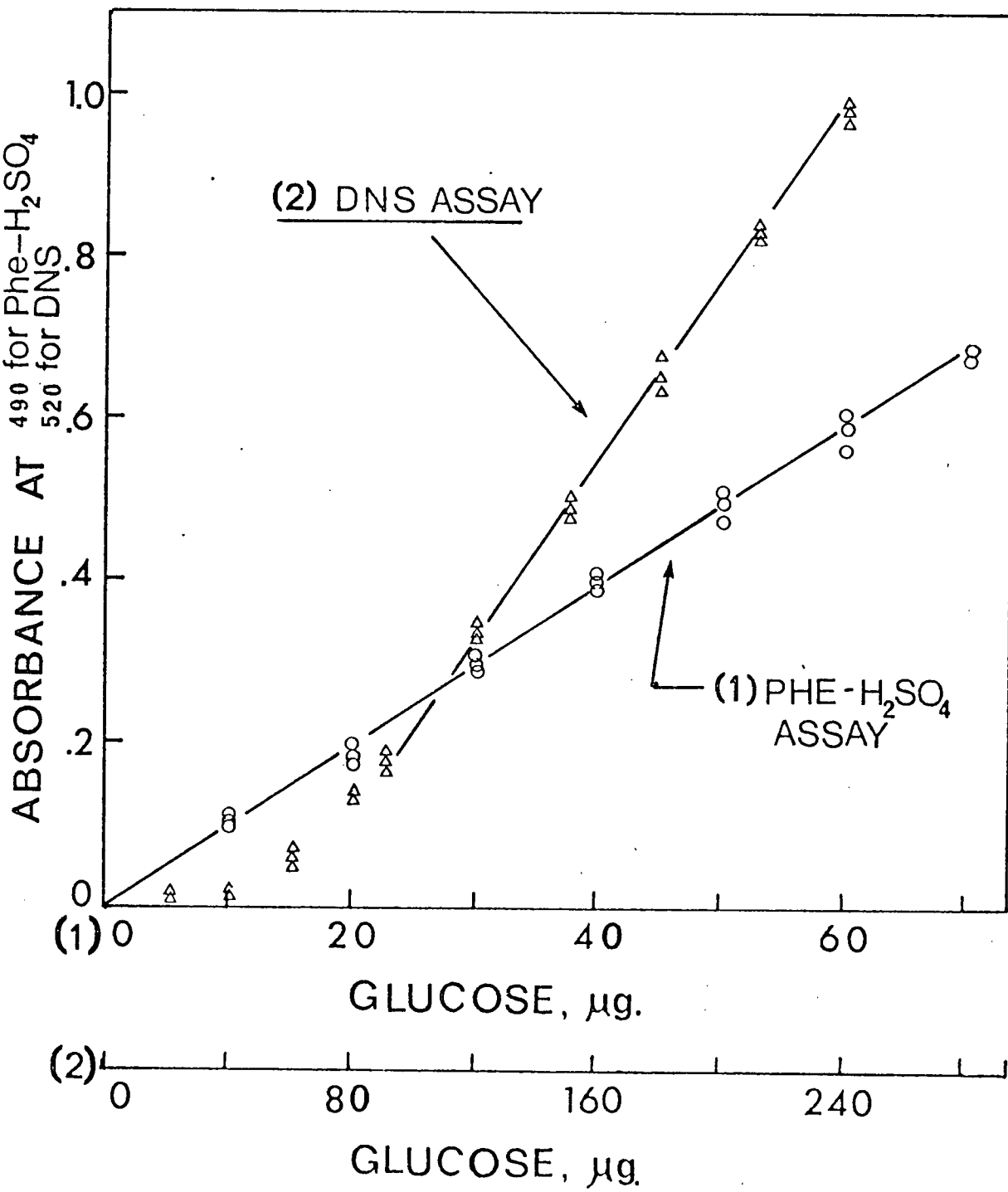
II.1.2 Dinitrosalicylic Method [57]

Reagents :

Dinitrosalicylic acid reagent prepared as follows : To 300 ml of 4.5 per cent sodium hydroxide, and 880 ml of 1 per cent dinitrosalicylic acid add 255 g of Rochelle salt. To 10 g of crystalline phenol, add 22 ml of 10 per cent sodium hydroxide. Add water to dissolve. Dilute to 100 ml and mix. To 69 ml of this solution add 6.9 g of sodium bisulfite and add to the dinitrosalicylic acid solution. Mix well until all of the Rochelle salt has dissolved. Keep tightly stoppered in well filled bottles. The reagent will last for at least one year. Three ml of the reagent should contain by titration, with

Figure AII.36

GLUCOSE TESTS STANDARD CURVES



phenolphthalein as indicator, the equivalent of 5 to 6 ml of tenth normal sodium hydroxide.

Apparatus :

(1) Set of test tubes with internal diameter between 16 and 20 mm.

(2) Boiling water bath.

(3) Spectronic 20 tubes or a set of matched colorimeter tubes.

Procedure :

Standard Curve

(1) Prepare 1 ml glucose solutions containing between 0.04 and 0.3 mg of glucose.

(2) Add 3 ml of the dinitrosalicylic acid reagent to each, mix well.

(3) Capped and heat for 5 minutes in the boiling water.

(4) Cool immediately in running water for 3 minutes.

(5) Transfer to colorimeter tubes and measure absorbance at 520 mμ.

(6) Plot A_{520} versus mg of glucose and make a least square fit to the data, as shown in Figure AII.36.

Unknown

Repeat steps 2 to 5 with the unknown sample, and obtain the sugar concentration from Figure AII.36.

II.2. Measurement of Proteolytic Activity [62]

Reagents :

(1) 5% solution of tricholoacetic acid (CCl_3COOH)

(2) 6% solution of sodium carbonate, Na_2CO_3

(3) Folin's reagent prepared as follows : 100 gm of sodium tungstate and 25 gm of sodium molybdenate are put into a 2-litre round - bottomed flask fitted with a reflux condenser with a ground - glass joint. 700 ml of water, 50 ml of 85% orthophosphoric acid (sp. gr. 1.689), and 100 ml of concentrated hydrochloric acid are added. The solution is boiled for 10 hr. After cooling, 150 gm of lithium sulfate, 50 ml of water, and several (5-6) drops of bromine water are added. The open flask is heated until the excess bromine evaporates. The solution should then be yellow. If the solution is green, the treatment with bromine water is repeated. The cooled solution is brought to 1 litre with water. The reagent should be 2N in acid. The acid concentration is checked by titration of tenfold-diluted Folin's reagent with 0.1N NaOH solution using phenolpthalein as an indicator. The prepared reagent is stored in a dark bottle. The working solution is prepared before the activity determinations by adding four volumes of water to one volume of Folin's

reagent. Note that the concentrated Folin's reagent may be obtained commercially from Fisher Scientific Co.

(4) 0.025M tris-HCl Buffer, pH 8.5 prepared as follows : Pipette 25 ml of 0.2M stock tris (24.24 gm of tris (hydroxymethyl) aminomethane in 1000 ml of H_2O) into 200 ml volumetric flask. Add 0.1N HCl to it until the pH falls to 8.5. Make up to 200 mls with water.

(5) 2% Casein solution prepared as follows : Suspend 2 gm of "Hammarsten" casein in about 45 mls of the 0.025M tris-HCl buffer. Stir vigorously with a magnetic stirrer. The pH of the suspension drops to about 7; add a small amount of 6% sodium carbonate solution to elevate the pH to about 8 so as to facilitate the dissolution of the casein. Heat the suspension in a water bath at 65°-70°C till the solid particles dissolve. After cooling, bring the pH of the solution to 8.5 and make up to 100 mls with the tris-HCl buffer.

(6) Tyrosine solution : 10 mg of tyrosine is dissolved in 5 ml of 0.1N alkali, transferred to a 25-ml measuring flask, and brought to the mark with water. This solution, containing 400 µg of tyrosine per ml, is used to prepare solutions of lower concentration containing 5 to 100 µg of tyrosine per ml.

Procedure

Construction of Calibration Curve

One ml batches of tyrosine solutions of different concentration are taken and to these are added 4 ml of 6% sodium carbonate solution and 1 ml of five-fold - diluted Folin's reagent. These solutions are immediately mixed and left for the color to develop - for 30 min at room temperature (20 to 25°C). A control is simultaneously set up for the reagents; in the control the tyrosine solution is replaced by water. The depth of color is determined on a photocolorimeter at wavelength 660 mμ in colorimeter tubes. Plot the data and draw a least square straight line through the points, as shown in Figure AII.37.

Determination of Activity

(1) Bring 2 mls of 2% casein solution and 1 ml of enzyme solution (culture filtrate), in separate test tubes, to 30°C.

(2) Add the casein solution to the enzyme solution, mixed, and immediately start stop watch. Incubate for 10 mins at 30°C.

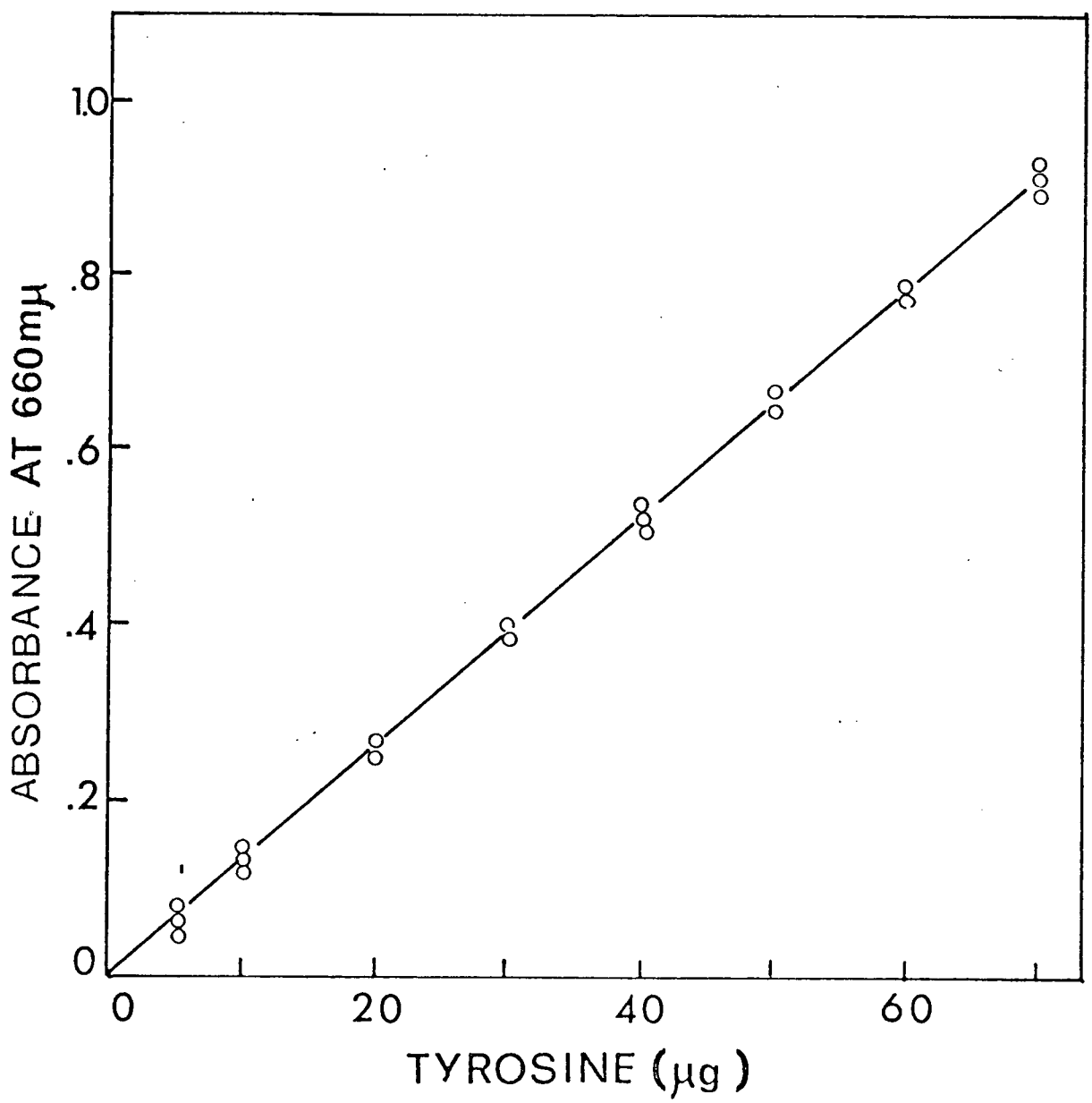
(3) Stop the reaction by adding 5 ml of 5% trichloroacetic acid solution as rapidly as possible; mixed and placed in 30°C bath for about 2 to 5 minutes.

(4) Filter the mixture through Whatman #3 filter paper, to remove the white precipitate.

(5) Determine the amount of tyrosine in the filtrate using the method outlined in "Construction of Calibration Curve" - 1 ml of filtrate is used instead of 1 ml of tyrosine solution.

Figure AII.37

TYROSINE TEST STANDARD CURVE



(6) A control is simultaneously set up by adding first 5 ml of trichloroacetic acid to the enzyme solution and then 2 ml of casein solution. The solution is mixed, filtered, and 1 ml of the filtrate instead of 1 ml of water is used as the blank in the tyrosine assay.

Calculation of activity of enzyme preparations

In this method the enzyme unit is defined (following the recommendations of the Enzyme Commission of the Inter. Union of Biochemistry) as the amount which in one minute forms proteolysis products which are not precipitated with trichloroacetic acid and contain one microequalent of tyrosine. Therefore the number of enzyme units per ml of the investigated solution will be :

$$E = (a \times 8)/(181 \times 10) = a \times 0.00442 .$$

Where E is the enzyme unit (μeq. tyrosine/ml. min); a is the number of μg of tyrosine found from the calibration curve; 8 is the dilution factor after precipitation with trichloroacetic acid; 181 is the microgram molecular weight of tyrosine and 10 is the proteolysis period in minutes.

II.3 Protein Estimation with the Biuret Reagent [66]

Reagents

(1) Standard solution of Bovine Serum Albumin (BSA). Dissolve 0.25 gm of crystalline BSA in 25 mls of H_2O. This solution, containing 10 mg of BSA per ml, is used to prepare solutions of lower concentration,

containing 1 to 10 mg/ml.

(2) Biuret Reagent (may be obtained commercially from BBL) : Dissolve 1.50 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) and 6.0 g of sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) in 500 ml of water. Add, with constant swirling, 300 ml of 10% sodium hydroxide. Dilute to 1 litre with water, and store in a paraffin-lined bottle. This reagent should keep indefinitely but must be discarded if, as a result of contamination or of faulty preparation, it shows signs of depositing any black or reddish precipitate.

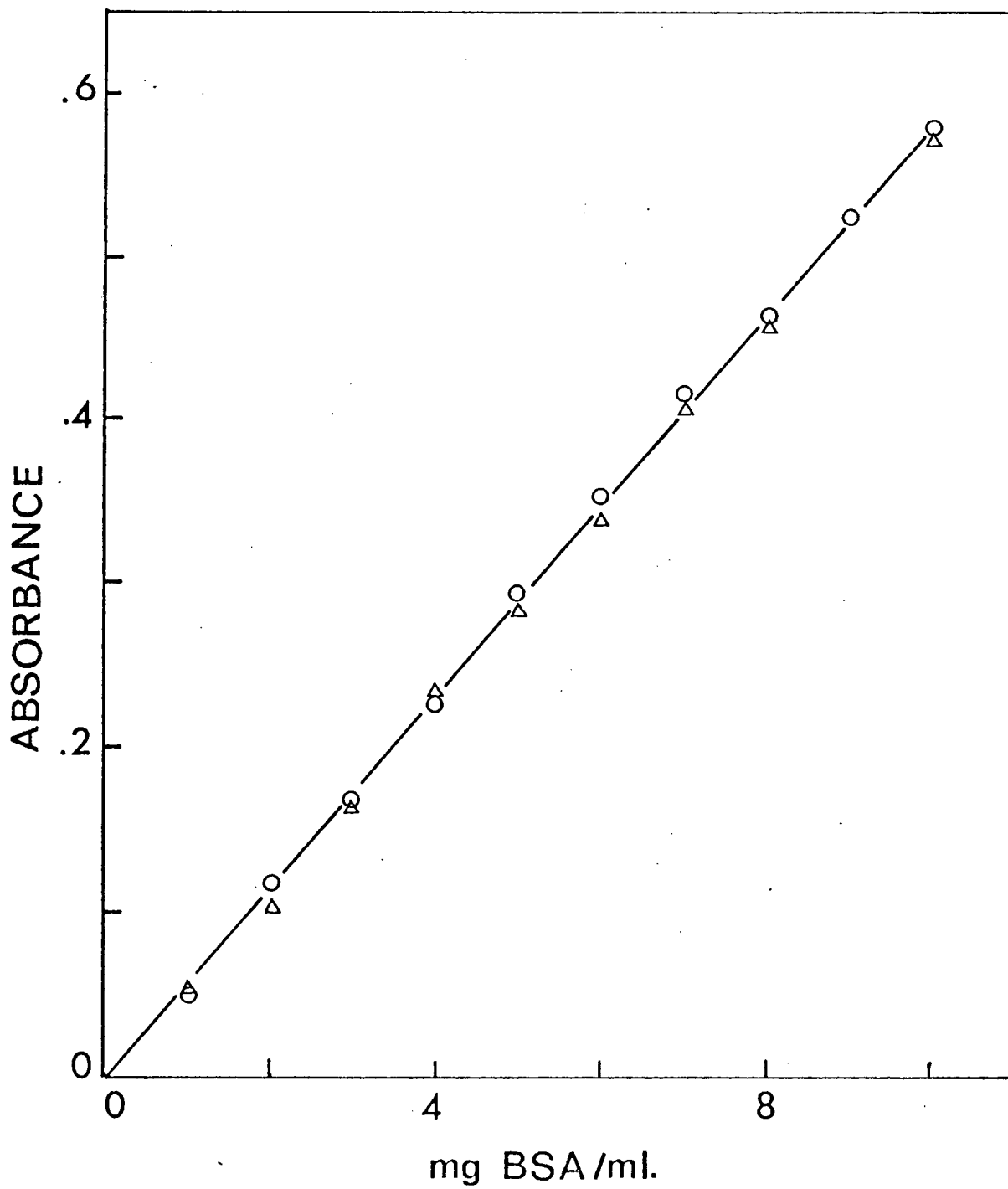
Procedure

To 1.0 ml of a solution containing 1 to 10 mg of protein per ml add 4.0 ml of biuret reagent, mix by swirling, and allow to stand for 30 minutes at room temperature (20 to 25°C). Measure the per cent transmission at 540 mμ, with the Spectronic 20, against a blank consisting of 4.0 ml of biuret reagent plus 1.0 ml of water.

The concentration of protein in the sample is obtained by reference to a calibration curve previously established with a clear solution of serum protein (Figure AII.38.)

Figure AII.38

PROTEIN TEST STANDARD CURVE



Appendix III

COMPUTATION OF SUM OF SQUARES

The treatment sum of squares* is subdivided into the three main effect sums of squares SSA, SSB, SSC, the three two-way interaction sums of squares SS(AB), SS(AC), and SS(BC), and the three-way interaction sum of squares SS(ABC). To facilitate the calculation of these sums of square the following two-way tables of totals and subtotals were constructed :

	B			
A	1	2	3	Total
1	6.46	6.18	4.14	16.78
2	3.56	3.43	3.99	10.98
3	0.13	0.0	0.08	0.21
Total	10.15	9.61	8.21	27.97

	C			
A	1	2	3	Total
1	8.77	3.92	4.09	16.78
2	6.06	2.99	1.93	10.98
3	0.0	0.21	0.0	0.21
Total	14.83	7.12	6.02	27.97

* When there is no replication, the total sum of squares is used.

B	C 1	2	3	Total
1	5.70	2.47	1.98	10.15
2	4.56	2.53	2.52	9.61
3	4.57	2.12	1.52	8.21
Total	14.83	7.12	6.02	27.97

Correction term, $CT = \frac{(27.97)^2}{27} = 28.97$

$SST = 3.66^2 + 1.39^2 + \ldots + 0.08^2 - CT = 25.73$

$SSA = \frac{1}{9}(16.78^2 + 10.98^2 + 0.21^2) - CT = 15.72$

$SSB = \frac{1}{9}(10.15^2 + 9.61^2 + 8.21^2) - CT = 0.23$

$SSC = \frac{1}{9}(14.83^2 + 7.12^2 + 6.02^2) - CT = 5.13$

$SS(AB) = \frac{1}{3}(6.46^2 + 3.56^2 + \ldots + 0.08^2) - CT - SSA - SSB = 0.90$

$SS(AC) = \frac{1}{3}(8.77^2 + 6.06^2 + \ldots + 0.0^2) - CT - SSA - SSC = 2.99$

$SS(BC) = \frac{1}{3}(5.70^2 + 4.56^2 + \ldots + 1.52^2) - CT - SSB - SSC = 0.25$

$SS(ABC) = SST - SSA - SSB - SSC - SS(AB) - SS(AC) - SS(BC) = 0.510$