SUBMERGED CULTIVATION OF MOREL MUSHROOM MYCELIUM IN WASTE SULFITE LIQUORS

by

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Kính dâng ba má

A mes parents

To my parents

ABSTRACT

Four species of morels (*Morchella crassipes, M. deliciosa, M. esculenta* and *Morchella* spp.) were cultivated in the waste sulfite liquors (NH_3-, Ca-, Mg-, Mixed- and Na-WSL)-ammonium phosphate dibasic-corn steep liquor media. Growth was found to be completely inhibited by calcium found in both Ca- and mixed-WSL and also by a large amount of dissolved SO_2 in Na-WSL. Growth generally occured in large pellets form in shake flasks but in dispersed form in the fermentors. The optimum initial pH for different species of morels in various WSL was found to be between 5.0 and 7.0. The concentrated WSL was found to completely inhibit growth due to its toxicity. The optimum dilution ratio for both growth and yield for the cultivation process was approximately 1:5 v/v. Very high yield (83.4% based on utilized carbohydrate) and dry weight (4.86 g/l) were obtained with *M. crassipes* in NH_3-WSL (1:5 v/v) medium. The highest yield (108.7%) and dry weight (15.0 g/l) were obtained with *M. crassipes* in SO_2-stripped Na-WSL (1:3 v/v) medium from shake flask experiments.

Freeze-dried morel mushroom mycelium (MMM) in WSL contained on a dry basis, 23.2-48.0% crude protein, 2.45-4.38% fat, 13.7-39.2% carbohydrate and 5.9-18.0% ash. The

spectrum of essential amino acids was comparable to the FAO standard, except for the levels of methionine and isoleucine. The gas-liquid chromatogram showed the presence of essential fatty acids such as linoleic and linolenic acids. All amino acids and fatty acids present in the fruiting bodies of the meadow mushroom (*Agaricus bisporus*) were found in the samples of MMM grown in WSL. A strong mushroom flavour (aroma and taste) was also conserved in the freeze-dried powdered samples. The flavour concentrate of *M. crassipes* grown in NH_3-WSL was recovered and compared with that of the meadow mushroom using gas-liquid chromatography, infrared spectrophotometry and ultraviolet absorption spectrophotometry. The flavour concentrates prepared from the freeze-dried samples lack low boiling compounds, while they were richer in high boiling components. Tentative interpretation of the infrared spectra revealed the presence of ketones, aldehydes, alcohols, phenols and esters in all flavour concentrates.

In shake flask experiments, the BOD_5 reductions were found between 43.8-62.7% for different MMM in various WSL. In the controlled batch fermentor, the reductions of BOD_5 and COD for the NH_3-WSL medium with *M. crassipes*, at dilution ratio 1:5 v/v, were 72.0% and 16.1% respectively after 14 days. Shake flask experiments on the Na-WSL adapted strain of *M. crassipes*, which was obtained by multiple

transfer technique. Maximum reduction of BOD_5 and COD of the Na-WSL was obtained by either stripping of SO_2 or γ-irradiating as pretreatment.

A continuous stirred tank fermentor (CSTF) used for cultivation of *M. crassipes* in NH_3-WSL was considered as a multivariable linear system around its operating point (quasioptimal conditions). Pulse testings on the inputs (x^3 = inlet jacket temperature, x^5 = inlet pH, x^6 = inlet substrate concentration) and their responses at the outputs (y^1 = biomass, y^2 = outlet temperature, y^3 = outlet jacket temperature, y^4 = outlet pH, y^5 = outlet substrate concentration) were used for numerical determination of the transfer function matrix of the CSTF system. From a chemical engineer's point of view, the resulting matrices were,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{0.228\, e^{-240s}}{(307s+1)} & \frac{1.637\, e^{-5s}}{(38s+1)^2(23s+1)} & \frac{0.256}{(75s+1)} \\ \frac{0.883}{(20s+1)} & 0 & 0 \\ \frac{0.965}{(18s+1)} & 0 & 0 \\ 0 & \frac{0.952}{(31s+1)} & \frac{0.064}{(32s+1)} \\ 0 & 0 & \frac{0.389}{(29s+1)^2} \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix}$$

and,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{(s-0.025)(s+0.907)e^{-240s}}{s(s+0.136)} & \frac{(s+14.731)(s^2-0.044s+0.019)e^{-5s}}{s(s-0.630)(s-0.388)} & \frac{(s-0.719)(s^2-0.056s+0.008)}{(s+0.019)(s-0.077)(s-0.899)} \\ \frac{(s+3.013)(s^2-0.335s+0.062)}{s(s+0.655)(s-0.988)} & 0 & 0 \\ \frac{(s+0.067)(s+108.826)}{(s+0.035)(s-894.000)} & 0 & 0 \\ 0 & \frac{(s+45.701)(s^2-0.093s-0.007)}{(s+0.070)(s-0.033)(s-4.588)} & \frac{(s+0.459)(s-163.188)}{(s+0.052)(s+1.023)} \\ 0 & 0 & \frac{(s+0.047)(s^2+0.177s+0.777)}{(s+0.064)(s+0.038)(s+0.804)} \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix}$$

for lag- and lead-lag transfer functions with dead time respectively. From a microbiologist's point of view, the resulting matrices were,

$$\begin{bmatrix} Y^1(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{0.259\,e^{-240s}}{(307s+1)} & \frac{1.720\,e^{-5s}}{(33s+1)^2} & \frac{0.655}{(94s+1)} \end{bmatrix} \begin{bmatrix} Y^2(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix}$$

and

$$\begin{bmatrix} Y^1(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{(s-0.083)e^{-240s}}{(s+0.181)(s-1.000)} & \frac{(s+0.0[illegible])(s^2+0.013s+0.028)e^{-5s}}{(s+0.043)(s+0.105)(s+0.016)} & \frac{(s+0.036)(s+0.319)}{(s+0.006)(s+0.587)} \end{bmatrix} \begin{bmatrix} Y^2(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix}$$

for lag- and lead-lag transfer functions with dead time respectively.

RESUME

Quatre espèces de morilles (*Morchella crassipes*, *M. deliciosa*, *M. esculenta* et *Morchella* spp.) ont été cultivées sur un milieu de culture composé de liqueur sulfitique usagée (LSU-NH_3, -Ca, -Mg, -Mixte et -Na), de phosphate dibasique d'ammonium, et d'une liqueur provenant du procédé industriel de maïs. On a trouvé que le calcium présent dans la LSU-Ca et la LSU-Mixte, ainsi qu'une grande quantité de SO_2 dissout dans la LSU-Na inhibaient totalement la croissance des dits champignons. En général, la croissance s'est faite sous forme de grosses boulettes en Erlen, sous agitation, mais sous forme dispersée dans les fermenteurs. On a trouvé qu'un pH initial comprise entre 5.0 et 7.0 était optimum pour les différentes espèces de morilles cultivées sur les types de LSU précités. Les LSU concentrées inhibent totalement, à cause de leur toxicité, la croissance des morilles. La dilution optimale basée sur la biomasse ainsi que le rendement du procédé de culture a été trouvée approximativement de 1:5 v/v. Un très grand rendement (83.4% basé sur l'hydrate de carbone utilisé) et un poids sec élevé (4.86 g/l) ont été obtenus avec *M. crassipes* cultivé sur de la LSU-NH_3 (1:5 v/v). Cependant, le meilleur rendement (108.7%) et le poids sec le plus élevé (15.0 g/l)

ont été obtenus en Erlen, sous agitation, pour la culture de *M. crassipes* sur de la LSU-Na (1:3 v/v) dont on avait éliminé le SO_2.

Le mycélium de la morille (MM) cultivée sur de la LSU, lyophylisé, contient à l'état sec, 23.2-48.0% de protéine brute, 2.45-4.38% de matière grasse, 13.7-39.2% d'hydrate de carbone et 5.9-18.0% de cendres. Le spectre des acides aminés essentiels est comparable au standard de la FAO, à l'exception des taux de méthionine et d'isoleucine. Une étude chromatographique en phase liquide-gazeuse a montré la présence des acides gras essentiels, comme les acides linoléique et linolénique. Tous les acides aminés et acides gras présents dans les champignons communs (*Agaricus bisporus*) ont également été trouvés dans le MM cultivé sur les LSU. Une forte saveur de champignon (arôme et goût) a persisté également dans les échantillons lyophylisés. Un extrait de la saveur de *M. crassipes* cultivé sur la LSU-NH_3 est préparé et comparé avec celui des champignons communs, en utilisant la chromatographie en phase liquide-gazeuse, la spectrophotométrie d'absorption dans l'infrarouge et dans l'ultraviolet. Les extraits préparés à partir des échantillons lyophylisés sont dépourvus de substances à basse température d'ébullition, tandis qu'ils sont riches en substances à haute température d'ébullition. L'interprétation des spectres d'absorption dans l'infrarouge a

révélé la présence des groupes cétone, aldéhyde, alcool, phénol et ester dans tous les échantillons d'extrait.

En Erlen, sous agitation, on a obtenu une réduction de la DBO_5 de 43.8-62.7% avec différents MM cultivés sur les LSU. Cependant, en fermenteur équipé d'un contrôle de pH, température, les réductions de la DBO_5 et de la DCO du milieu de culture pour la LSU-NH_3 (1:5 v/v) étaient de 72.0% et de 16.1% respectivement pour *M. crassipes* après 14 jours. En utilisant un souche de *M. crassipes* adaptée à la LSU-Na par la technique des transferts multiples, les réductions de 73.4% et de 33.0% respectivement pour la DBO_5 et la DCO du milieu de culture pour la LSU-Na (1:3 v/v) sont obtenues après 9 jours. La réduction maximum de la DBO_5 et de la DCO a été obtenue pour la LSU-Na dont on a éliminé le SO_2 ou que l'on a irradié par rayonnement gamma comme prétraîtement.

Un fermenteur type réacteur agité utilisé en continu pour la culture de *M. crassipes* sur un milieu comportant la LSU-NH_3, est considéré comme un système linéaire à plusieurs variables autour de son point d'opération (conditions quasioptimales). Les tests de pulsation sur les variables d'entrée (x^3 = la température du liquide de refroidissement à l'entrée, x^5 = le pH à l'entrée, x^6 = la concentration du substrat à l'entrée)

et leurs réponses à la sortie (y^1 = la biomasse, y^2 = la température du milieu de culture à la sortie, y^3 = la température du liquide de refroidissement à la sortie, y^4 = le pH à la sortie, y^5 = la concentration résiduelle du substrat) sont utilisées pour déterminer numériquement la matrice des fonctions de transfert du dit système. Du point de vue de l'ingénieur chimiste, les résultats sont,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \approx \begin{bmatrix} \frac{0.228\, e^{-240s}}{(307s+1)} & \frac{1.637\, e^{-5s}}{(38s+1)^2(23s+1)} & \frac{0.266}{(75s+1)} \\ \frac{0.883}{(20s+1)} & 0 & 0 \\ \frac{0.965}{(18s+1)} & 0 & 0 \\ 0 & \frac{0.952}{(31s+1)} & \frac{0.064}{(32s+1)} \\ 0 & 0 & \frac{0.389}{(29s+1)^2} \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix}$$

et,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \begin{bmatrix} \frac{(s-0.023)(s+0.007)e^{-240s}}{s(s+0.1[illegible]6)} & \frac{(s+14.737)(s^2-0.044s+0.019)e^{-5s}}{s(s-0.330)(s-0.098)} & \frac{(s-0.722)(s^2-0.056s+0.008)}{(s+0.013)(s-0.27[illegible])(s-0.333)} \\ [illegible] & 0 & 0 \\ [illegible] & 0 & 0 \\ 0 & \frac{(s+45.702)(s^2-0.033s-0.007)}{(s+0.070)(s-0.03[illegible])(s-4.338)} & \frac{(s+0.450)(s-103.259)}{(s+0.039)(s+1.62[illegible])} \\ 0 & 0 & [illegible] \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix}$$

pour les fonctions de transfert respectivement avec pôles seulement et avec pôles et zéros avec retard. Du point de vue du microbiologiste, les résultats sont,

$$\begin{bmatrix} Y^1(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{0.259\ e^{-240s}}{(307s+1)} & \frac{1.720\ e^{-5s}}{(33s+1)^2} & \frac{0.655}{(94s+1)} \end{bmatrix} \begin{bmatrix} Y^2(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix}$$

et,

$$\begin{bmatrix} Y^1(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{(s+0.083)e^{-240s}}{(s+0.182)(s+1.000)} & \frac{(s+0.051)(s^2+2.013s+0.029)e^{-5s}}{(s+0.065)(s+0.103)(s+0.018)} & \frac{(s+0.036)(s+0.319)}{(s+0.096)(s+0.539)} \end{bmatrix} \begin{bmatrix} Y^2(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix}$$

pour les fonctions de transfert respectivement avec pôles seulement et avec pôles et zéros avec retard.

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NOMENCLATURE

Abbreviations

APD = Ammonium Phosphate Dibasic.

BOD_5 = 5-Day Biochemical Oxygen Demand.

COD = Chemical Oxygen Demand.

CSL = Corn Steep Liquor.

CSTF = Continuous Stirred Tank Fermentor.

DNA = Deoxyribonucleic Acid.

DO = Dissolved Oxygen.

FAO = Food and Agriculture Organization.

FDA = Food and Drug Administration.

MMM = Morel Mushroom Mycelium (a).

PAG = Protein Advisory Group.

RNA = Ribonucleic Acid.

SCP = Single Cell Protein(s).

TCH = Total Carbohydrate(s).

TOC = Total Organic Carbon.

WSL = Waste Sulfite Liquor(s).

Symbols

a^i = constant in Eq. (5.1).

a_k = unknown parameter in Eq. (4.35).

AR = amplitude ratio (decibels).

b^j = constant in Eq. (5.1).

b_l = unknown parameter in Eq. (4.35).

B_k = unknown parameter in Eq. (4.25).

e_y = experimental errors involved in the Y_i.

F = operator for Fourier transformation.

$G(s), G(jw)$ = transfer function.

$|G(s)|, |G(jw)|$ = magnitude of the transfer function.

$\underset{\sim}{G}(s)$ = transfer function matrix.

K = lead-order of the system.

M = lag-order of the system.

N = number of data points.

P = pole of transfer function.

t = time (sec).

t_x = duration of the input pulse signal (sec).

t_y = duration of the output pulse signal (sec).

T, T_k = lag-order system time constant (sec^{-1}).

w, w_i = frequency (rad/sec).

$x^j(t)$ = input.

x^1 = aeration (vvm).

x^2 = agitation (RPM).

x^3 = inlet jacket temperature (°C).

x^4 = feed temperature (°C).

x^5 = inlet pH.

x^6 = inlet substrate concentration (g/l or ppm).

$\underset{\sim}{X}(s)$ = input vector.

$y^i(t)$ = output.

y^1 = biomass (g/l).

y^2 = outlet temperature (°C).

y^3 = outlet jacket temperature (°C).

y^4 = outlet pH.

y^5 = outlet substrate concentration (g/l or ppm).

$\underset{\sim}{Y}(s)$ = output vector.

Y_i = experimental value of the amplitude ratio in Eq. (4.24).

Z = zero of transfer function.

δ = dead time (sec).

Ω_b = maximum frequency (rad/sec) in Eq. (4.46).

Φ = phase shift (degree).

CHAPTER 1

INTRODUCTION

1.1 Foreword

The present exponential growth of the world population is the fundamental problem that man has to solve for his survival on this planet. As a consequence of this uncontrolled population growth, a tremendous pressure on natural resources and particularly on food supplies is being experienced. In addition, the environment is gradually being polluted by human and industrial wastes, disrupting considerably the world ecological balance. Man has to restore this balance if he is to survive. A schematic diagram as presented in Fig. 1.1 shows the survival cycle for man in his environment. Although, the mentioned problems are basically a consequence of the population growth, which is the major problem to be solved particularly in a long run, a short run solution might be to better utilize the available resources, and recycle as many raw materials as possible. As presented in the Fig. 1.1, wastes created by both human and industrial "populations" could be converted to raw material for industrial use or to new foods for humans. The role of engineers and scientists as well as governments is to develop and initiate processes that would be capable

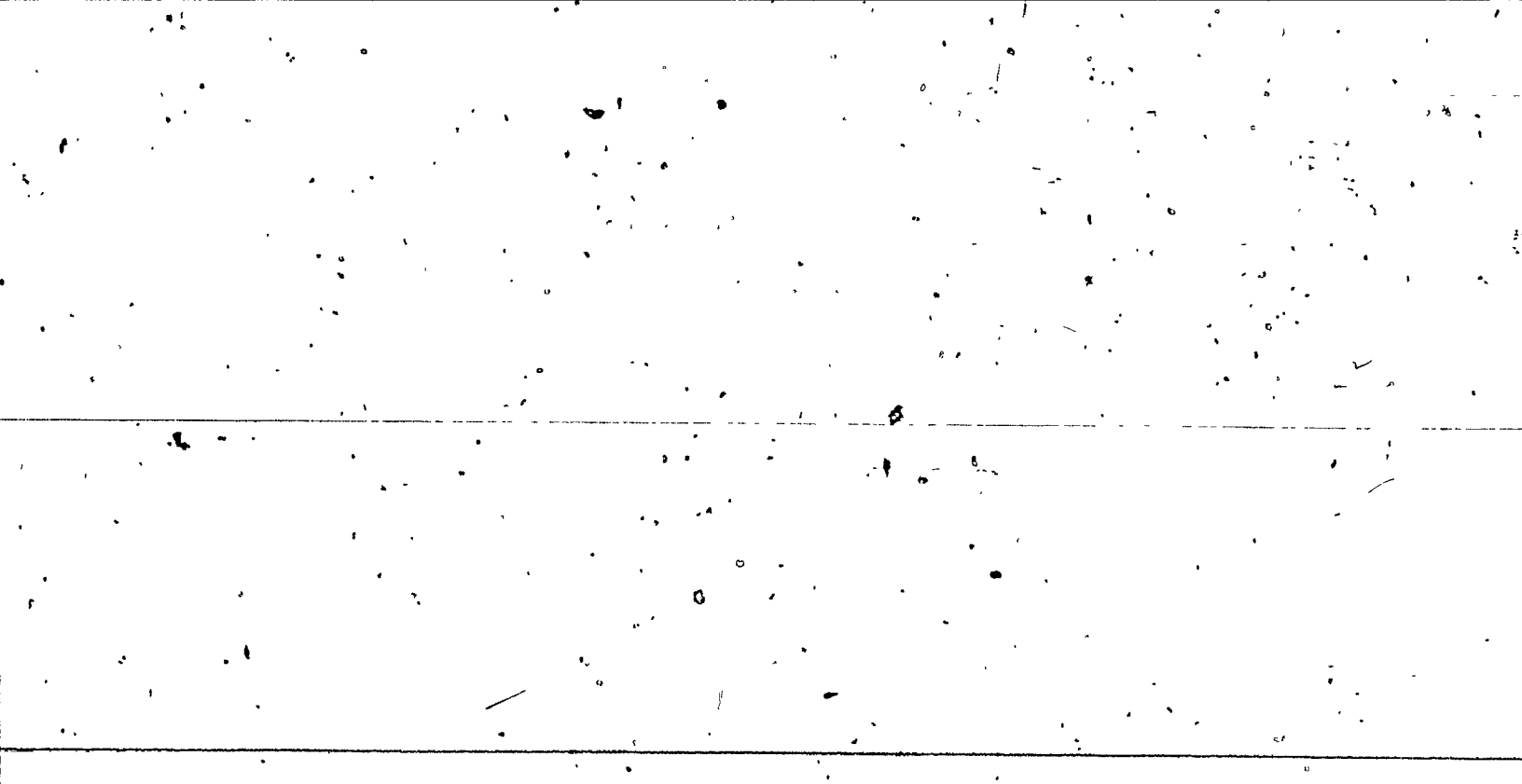
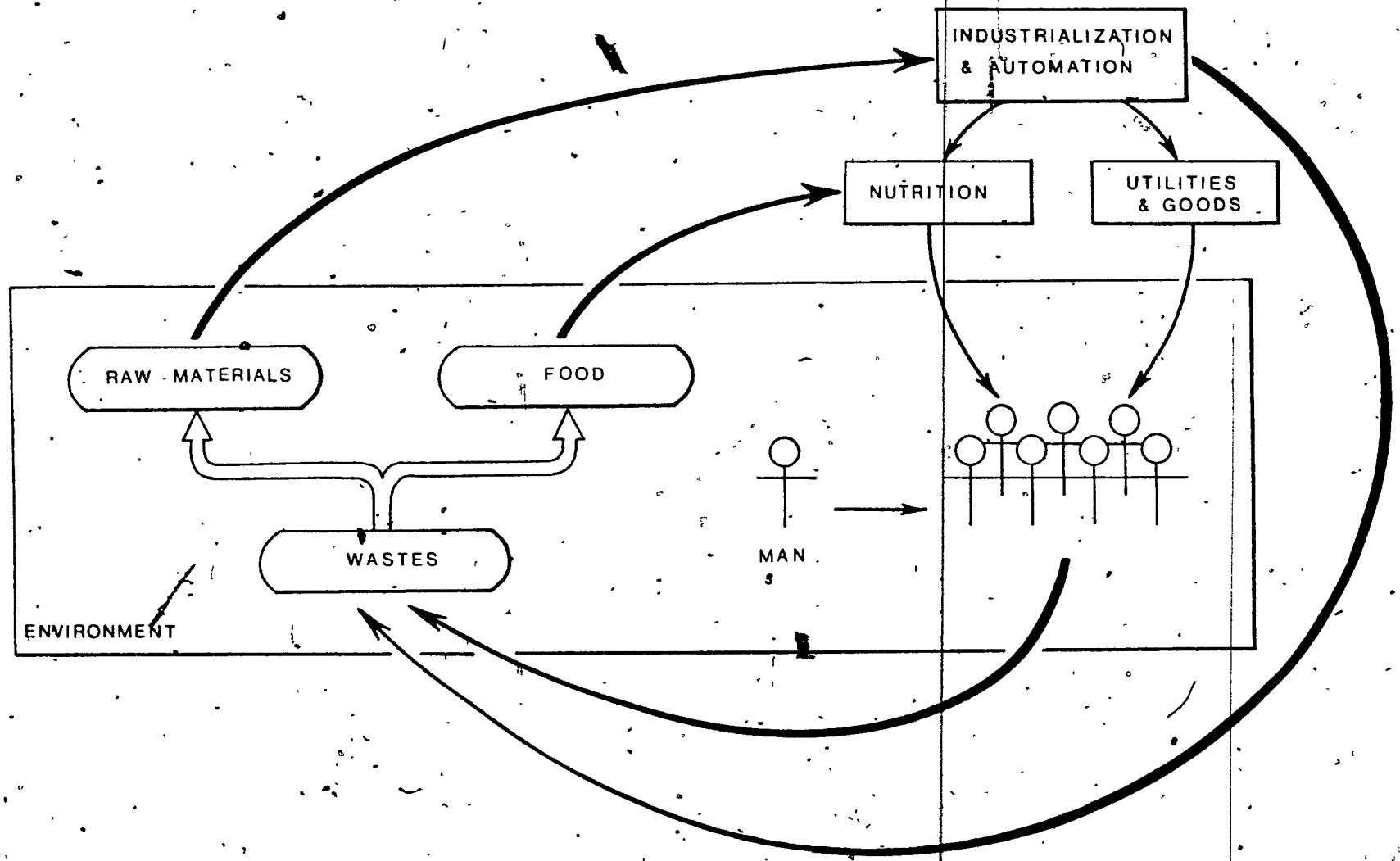


Figure 1.1 Survival Cycle for Man in his Environment.


INDUSTRIALIZATION
& AUTOMATION
NUTRITION
UTILITIES
& GOODS
RAW MATERIALS
FOOD
WASTES
MAN
ENVIRONMENT

of filling the "missing link". Using it as a theme, the objective of this work is to develop some information on the conversion of waste sulfite liquors (WSL) derived from pulp and paper industry into single cell proteins (SCP) of morel mushroom mycelium (MMM).

1.2 Morels

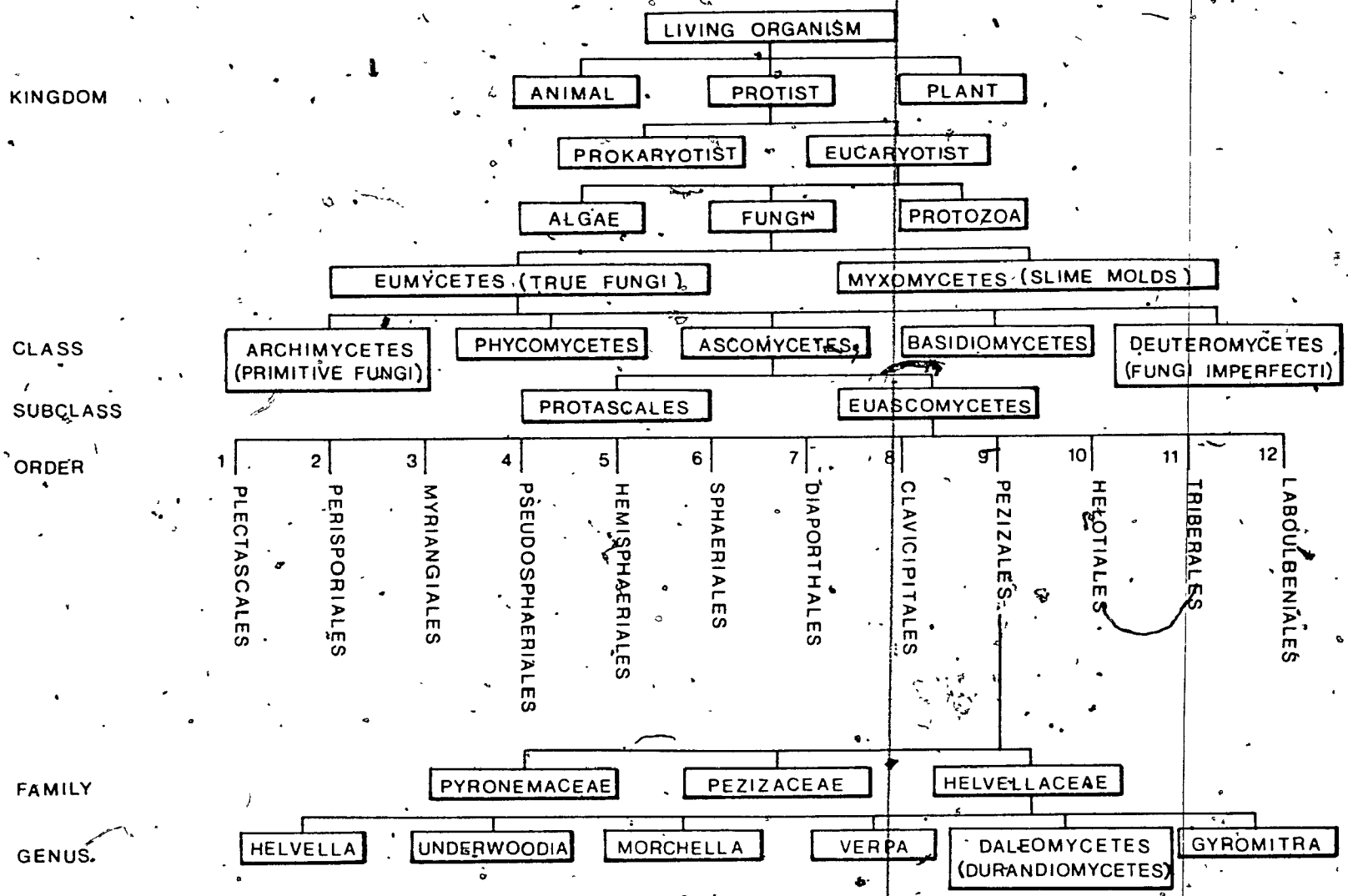
1.2.1 Early Work on the Cultivation of Morels

Morels are the fructifications of *Morchella* species (Fig. 1.2). The first cultivation of morels quoted was by baron d'Yvoire in 1889, [Heim (1936)]. He succeeded in producing in one year 300 morels on a 1.6m. x 6m surface of globe artichokes covered with apple marcs. The seeds used were fragments of 8 dry or fresh morels. Humidity and salpeter (KNO_3) were claimed as necessary factors for growth.

In 1896, Repin (1901) was able to isolate, conserve and transfer the mycelium of morels in long sterile culture tubes. He reported that the fructification of this mycelium was obtained within 5 to 8 years in the composts of apple marcs or decaying leaves which are rendered alkaline with sodium carbonate.

Later, Molliard (1905) performed experiments on a soil surface of 80cm x 80cm and he obtained 3 morels

Figure 1.2 Classification of Genus *Morchella* According to Gaumann (1952) and Bessey (1961).


LIVING ORGANISM
KINGDOM
ANIMAL
PROTIST
PLANT
PROKARYOTIST
EUCARYOTIST
ALGAE
FUNGI
PROTOZOA
EUMYCETES (TRUE FUNGI)
MYXOMYCETES (SLIME MOLDS)
CLASS
ARCHIMYCETES (PRIMITIVE FUNGI)
PHYCOMYCETES
ASCOMYCETES
BASIDIOMYCETES
DEUTEROMYCETES (FUNGI IMPERFECTI)
SUBCLASS
PROTASCALES
EUASCOMYCETES
ORDER
1 PLECTASCALES
2 PERISPORIALES
3 MYRIANGIALES
4 PSEUDOSPHAERIALES
5 HEMISPHAERIALES
6 SPHAERIALES
7 DIAPORTHALES
8 CLAVICIPITALES
9 PEZIZALES
10 HELOTIALES
11 TRIBERALES
12 LABOULBENIALES
FAMILY
PYRONEMACEAE
PEZIZACEAE
HELVELLACEAE
GENUS
HELVELLA
UNDERWOODIA
MORCHELLA
VERPA
DALEOMYCETES (DURANDIOMYCETES)
GYROMITRA

from 5kg of apple compost as substrate within 5 months. The mycelium of *M. esculenta* growing on carrots was used to seed the soil surface. The conidia of morels appeared 20 days after inoculation. This conidial form was identified earlier [Molliard (1904)] from *M. esculenta* var. *rotunda* Pers., and *M. conica* Pers., as *Costantinella cristata*. He claimed that the moisture and fermentable sugars were responsible for the growth of morels.

In 1905, Repin [Heim (1936)] explained that the required substrates for the growth of morels are not s[illegible]s but the cellulosic materials from trees and decaying woods. However, this point was clarified in the same year by Fron (1905). He established that the morels developed well in the presence of glucose, invert sugars and starch and moderately well in inulin (this explains the growth of morels on the globe artichokes performed by baron d'Yvoire). No growth took place on saccharose, levulose and mannitol. He also found that nitrogen, phosphate, calcium and/or magnesium salts are required for growth. Neither potassium nor iron salts are required for growth. The culture medium has to be neutral or slightly alkaline. This might be the first submerged cultivation of MMM (*M. conica*, *M. esculenta*, *M. vulgaris* var. *flava*) reported. The culture media were quoted as Knop liquid and Raulin liquid in which the concen-

tration of sugars was 5%. However, as techniques of aeration and agitation were not available at this time, the growth of mycelium took place on the surface of still liquid culture medium and on the vessel walls after 8 days of incubation.

Assays on the cultivation of morels (*M. esculenta* and *M. hortensis*) for commercial production were performed in the "meule" at Fontainebleau by Molliard [Costantin (1936)]. The record obtained was 23 morels of about 15g each (or 350g total) from each one square meter meule, and it was considered as feasible for industrial production.

Until 1928, Guitton's son [Heim (1936)] succeeded in recovering many pounds of morels from a compost containing a mixture of soil and apple marcs from a cider industry. This work might be considered as the first utilization of industrial waste materials for the cultivation of morels.

1.2.2 Taxonomy, Morphology, Cytology and Physiology

Morels are very familiar mushrooms to mushroom collectors. They grow on the ground, usually singly or in groups in open woods, orchards, and pastures during May and early June. In 34 years of collecting in Ohio, USA,

W.G. Stover found morels in the autumn only once [Gray (1970)]. Many mushroom eaters consider that morels have the finest flavour of all mushrooms [Groves (1958)]. The morels cannot be confused with other mushrooms due to their characteristic shape, size and color. The cap may have blonde, yellow, gray, black, brown, purple, green or white color [Heim (1936)], more or less elongated, usually rounded at the top, sometimes slightly conical, sometimes nearly globose, two to five inches long and three-quarters to one and one-half inches broad at the widest points, sometimes much larger. The surface is covered with rounded to irregular or somewhat elongated pits, sometimes arranged more or less in rows, more often irregularly arranged. The stem is two to three inches long and one-half to one inch thick, whitish, hollow, smooth or slightly scurfy, the surface even or wavy. The classification of species of morels is difficult as compared to other macromycetes mushrooms because of the variation of their form and color which depend upon environmental conditions. Details on the taxonomy of morels were reported and discussed by Boudier (1897), Lagarde (1923), Heim (1936) and Groves and Hoare (1953). New species of morels have also been identified [Heim (1966), Moravec (1970)]. Figure 1.3 shows the fruiting bodies of *M. crassipes* (the thick-footed morel) and *M. esculenta* (the sponge mushroom).

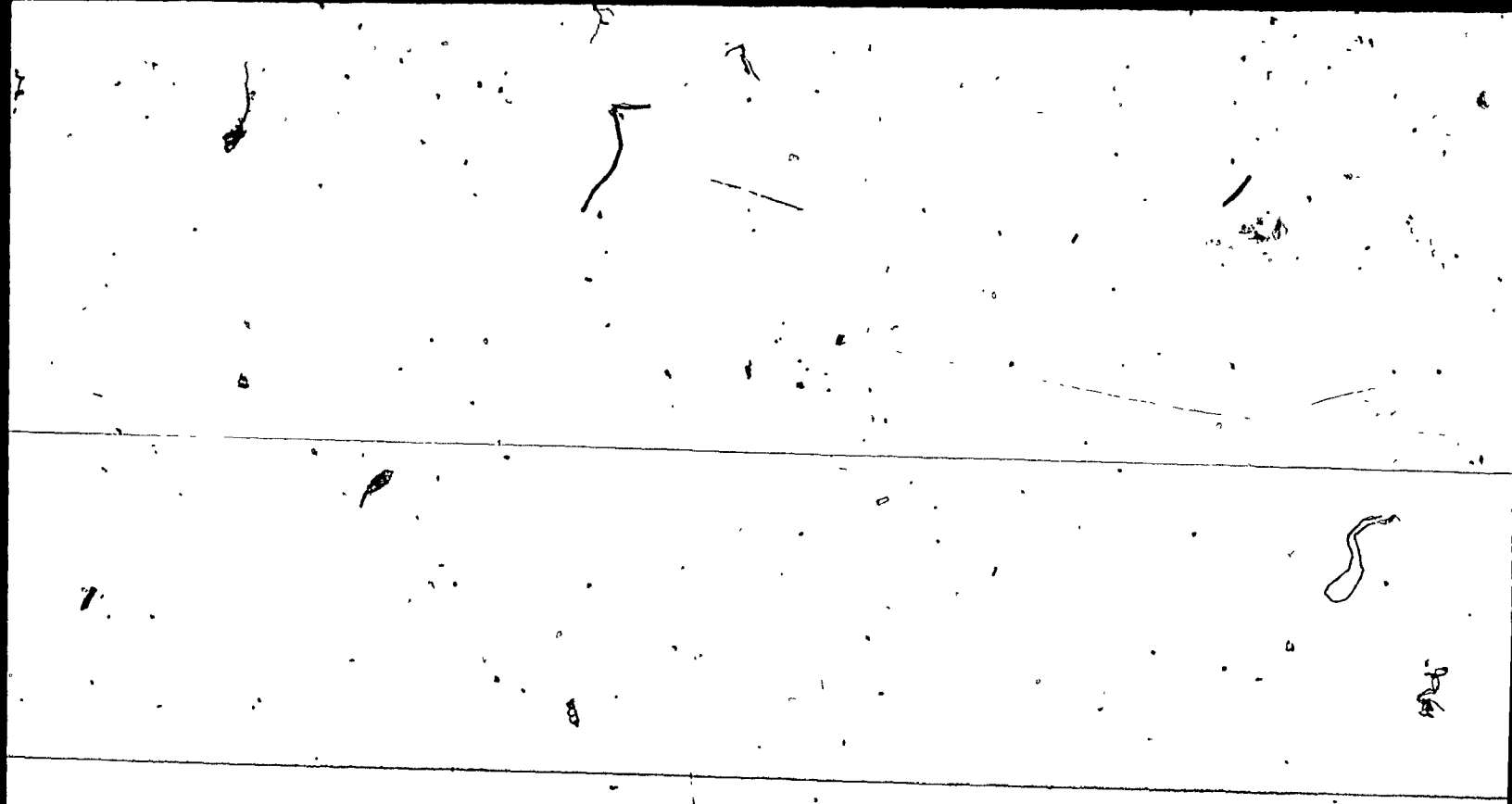
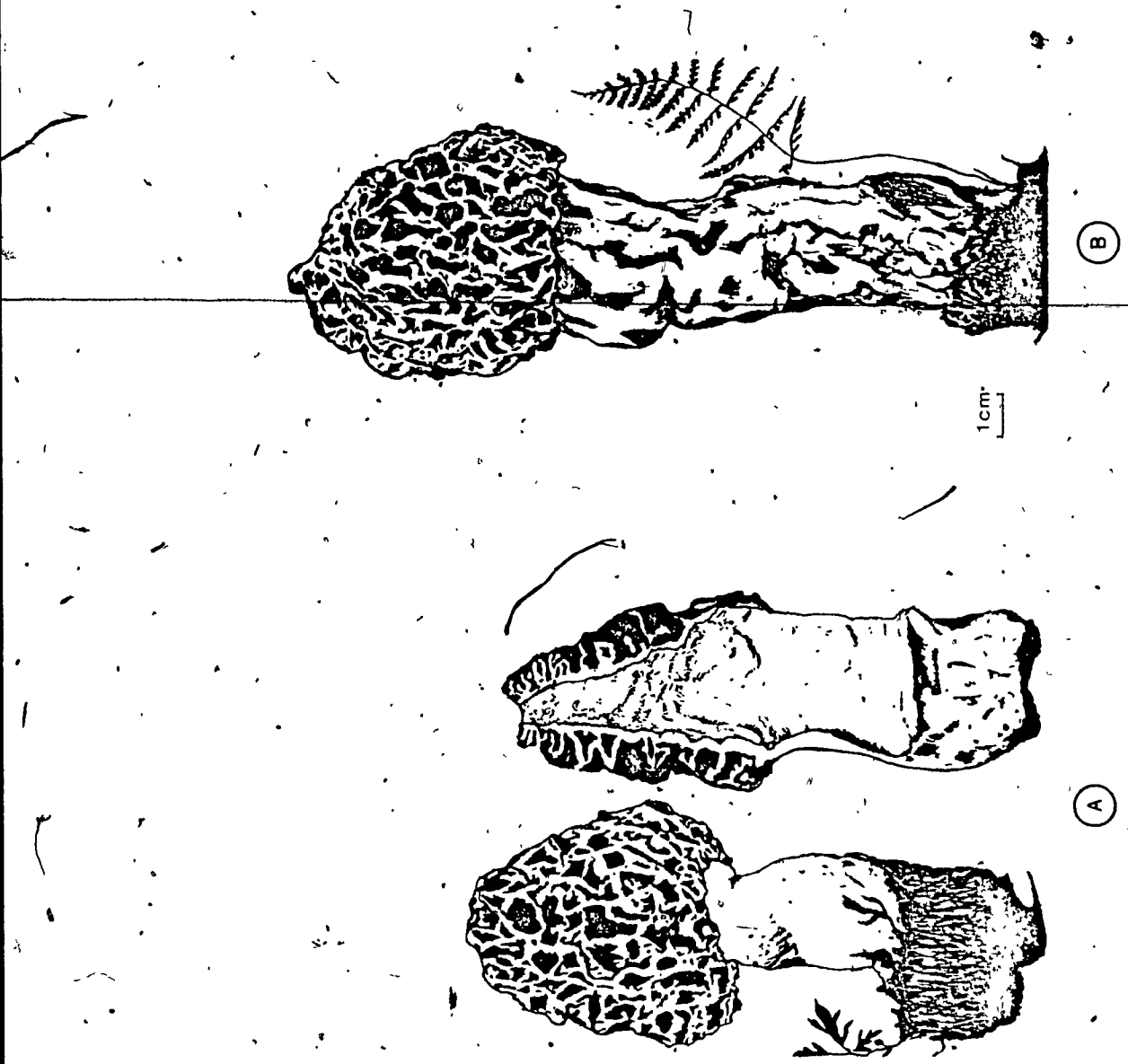


Figure 1.3 Fruiting Bodies of *M. esculenta* (A) and *M. crassipes* (B). From Atkinson (1911).


B
1cm.
A

The germination of the morel ascospores was observed by Molliard (1904) on *M. esculenta* var. *rotunda* Pers., *M. conica* Pers. and *M. deliciosa* Fr.. The germination took place at one extremity or more, usually two extremities of the spores. The filamentous mycelium ramifies rapidly, rests uncolored first and bears the roux color later. The cloison septa, external membrane and anastomosis were also recorded. Sclerotia are formed abundantly in pure culture, but rarely in soil cultivation.

The development of the fructification is initiated by purely vegetative processes and the sexual processes occur only among the vegetative hyphae in the young fruiting body. The sexual organs are lacking and copulation occurs somatogamously as in the case of *M. conica* Pers. and *M. esculenta* (L.) Pers. [Greis (1940)]. Copulation in *M. conica* Pers. normally occurs in the hypothecium and the plasmogamy results in subhymenium, and rarely in the stalk tissues just after its elongation. Although copulation occurs between adjacent hyphae composed of multinucleate cells, only one nucleus is required from each copulating cell in order to form the dikaryon. The dikaryon migrates into the ascogenous hyphae and undergoes conjugated divisions. The somatogamous copulation of *M. esculenta* (L.) can take place in the hymenium. The rest is similar to *M. conica*. Even somatogamous copulation is lacking in *M. elata* Fr.

[Greis (1940)] probably because of heterokaryosis. Any vegetative cell of the cap or of the subhymenium may form a dikaryon by the autogamous pairing of two of its own nuclei. The remaining nuclei degenerate. The ascogenous hyphae normally arise as lateral branches on the hypha germinating from the original dikaryotic cell, and they press through the hyphae of the young fruiting body until they become aligned to form the hymenium. Once the hymenium is formed, the asci are soon produced. The schematic diagram on the development of *Morchella* is shown in Fig. 1.4.

The life cycle of *Morchella* shows both sexual and asexual reproductions (Fig. 1.5). The asexual reproduction or conidial form of morels first discovered by Molliard (1904) was identified as an early named *Costantinella cristata* Matruchot [Costantin (1936)]. The sexual reproduction of *M. esculenta* and *M. deliciosa* was studied by Maire (1904, 1905) and Wakayama (1930) respectively. The basic steps are the same in both species except for some details as shown in Fig. 1.5.

Roze (1883) reported that *M. esculenta* Pers. was found parasitizing the rhizomes of the Jeruselum artichoke (Topinambour or Poire de Terre) *Helianthus tuberosus* L.. Jeruselum artichoke is known to have a high content of the polysaccharide inulin. However, Repin (1901)

Figure 1.4 Schematic Representation of the Development of *Morchella*. Two ascospores (SP) germinate to form a haploid mycelium (H). These haploid mycelia produce the fructification, and they copulate somatogamously in each successive fructification. The copulation results in the development of dikaryotic ascogenous hyphae (D) from the initial plasmogamy (P). From Gaumann (1952).

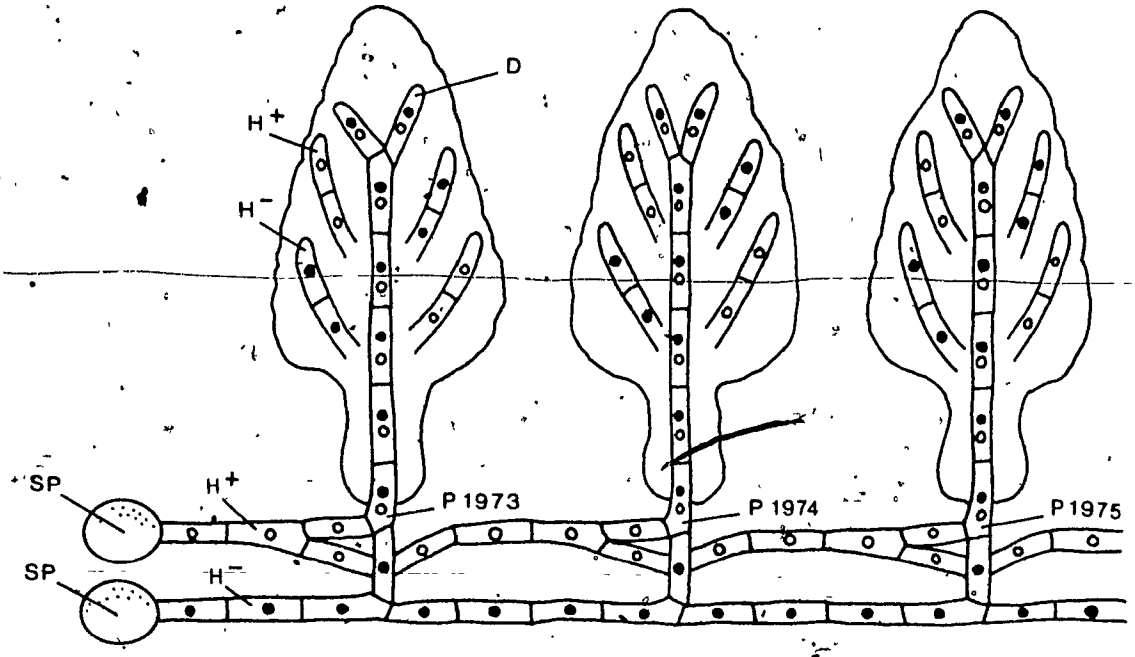
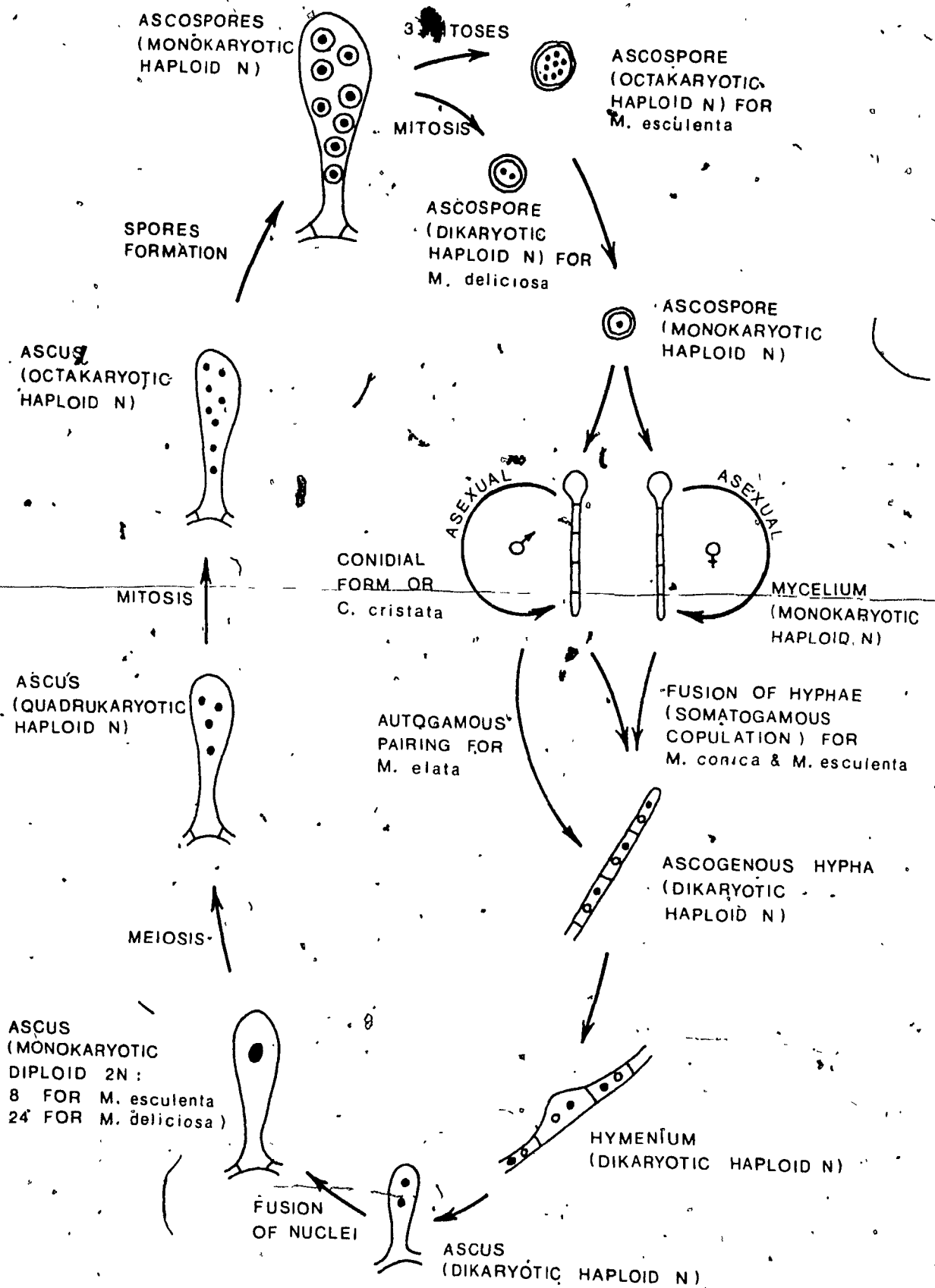

D
H+
H−
SP
H+
P 1973
P 1974
P1975
SP
H−

Figure 1.5 Schematic Representation of the Life Cycle of *Morchella*.


ASCOSPORES (MONOKARYOTIC HAPLOID N)
3 MITOSES
ASCOSPORE (OCTAKARYOTIC HAPLOID N) FOR M. esculenta
MITOSIS
ASCOSPORE (DIKARYOTIC HAPLOID N) FOR M. deliciosa
SPORES FORMATION
ASCOSPORE (MONOKARYOTIC HAPLOID N)
ASCUS (OCTAKARYOTIC HAPLOID N)
ASEXUAL
ASEXUAL
CONIDIAL FORM OR C. cristata
MYCELIUM (MONOKARYOTIC HAPLOID N)
MITOSIS
ASCUS (QUADRUKARYOTIC HAPLOID N)
AUTOGAMOUS PAIRING FOR M. elata
FUSION OF HYPHAE (SOMATOGAMOUS COPULATION) FOR M. conica & M. esculenta
ASCOGENOUS HYPHA (DIKARYOTIC HAPLOID N)
MEIOSIS
ASCUS (MONOKARYOTIC DIPLOID 2N : 8 FOR M. esculenta 24 FOR M. deliciosa)
HYMENIUM (DIKARYOTIC HAPLOID N)
FUSION OF NUCLEI
ASCUS (DIKARYOTIC HAPLOID N)

established in his work that the morels do not necessarily grow parasitically on roots of trees and can grow saprophytically. Also, fruiting bodies have been rarely reported to occur in culture, and never in pure culture.

Preliminary works on the physiology of *M. conica*, *M. esculenta*, and *M. vulgaris* var. *flava* were done by Fron (1905). Melin and Miden (1941) stated that thiamin has no effect on the growth of unisporic cultures of *M. conica*.

Brock (1951) investigated the physiology of *M. esculenta* mycelium grown in synthetic media composed of mineral salts and suitable carbon or nitrogen sources. A survey of many carbon and nitrogen sources was made. The fungus grew well on starch, maltose, fructose, turanose, glucose and sucrose, and moderately well on several others. Polyhydric alcohols and levorotatory methyl sugars supported little growth. Inulin was utilized in growth on agar but not in liquid culture. It is to be noted that some discrepancies were encountered between the results of Fron (1905) and Brock (1951) possibly due to strain differences and the basal media used. As nitrogen sources, cysteine-HCl, aspartic acid, asparagine, urea, sodium nitrite, various ammonium salts, alanine, glutamic acid-HCl, and sodium nitrate were favorable. Other amino acids and nitrogen compounds were intermediate or poor nitrogen sources. Ammonium citrate,

thiourea, hydroxylamine-HCl, and hydrazine-2HCl were toxic. The pH-growth curve for this organism was bimodal with maxima at pH 6.9 and 8.3 and a minimum at 7.5. The bimodal curve is attributed to the effect of pH on the fungus isoelectric point; the minimum point at pH 7.5 is interpreted as the isoelectric point for the cell colloid.

The favourable effect of a water extract of beechwood on the growth of *M. esculenta* Pers. ex St. Amans and *M. crassipes* Vent. ex Fries was reported by Robbins and Hervey (1959). The beneficial material in the wood extract was thermostable, non-volatile, not soluble in ethyl acetate or chloroform and present in a filtrate prepared by treating wood extract with activated charcoal (Norit A). The basal medium to which wood extract or filtrate was added contained casein hydrolysate, a variety of vitamins, purine and pyrimidine bases and mineral salts, including minor essential mineral elements. It was proposed, therefore, that the beneficial material in the filtrate from wood extract might be an unidentified growth substance [Robbins and Hervey (1963)]. Further investigation has demonstrated however, that the beneficial effect of the wood extract on *Morchella* is primarily due to its ash [Robbins and Hervey (1965)]. Manganese and calcium are primarily concerned. The ash of natural products is identified as the "filtrate factor" for this fungus.

Gilbert (1960) cultivated and reported some growth and flavour characteristics of nine species: *M. crassipes* (Vent.) Pers., *M. hortensis* (Pers.) Boud., *M. conica* Pers., *M angusticeps* Peck, *M. rimosipes* D.C., and *M. semilibera* D.C.. The mycelium grew slowly at 36° F but the optimal temperature range for growth was found between 55 to 70° F.

Factors affecting the growth of MMM (*M. hortensis*, *M. crassipes* and *M. esculenta*) in glucose, maltose and lactose media were studied by Litchfield *et al.* (1963a). The highest yield of *M. hortensis* from glucose was obtained with carbon to nitrogen ratios between 5:1 and 10:1 in the medium, pH 5.5 to 6.5, and an aeration rate of 0.08 mmole of oxygen per liter per minute.

1.2.3 Recent Work on Morels

The submerged cultivation of MMM was recently investigated by many authors. Sugihara and Humfeld (1954) reported the growth of *Morchella* spp., *M. crassipes* and other fungi in the laboratory. Various substrates have been used for the culture media. Szuecs (1956, 1958) developed the process for the cultivation of *M. esculenta*, *M. angusticeps* Pk, *M. bispora* Sor, *M. conica* Pers., *M. deliciosa* Fr., *Mr. hortensis* Boud., *M. crassipes* (Vent) Pers. and other fungi in molasses (beet molasses, black strap molasses, high test or Cuban inverted molasses, citrus

molasses and wood molasses), an extract of Jerusalem [*sic*] artichoke *H. tuberosus* and in a glucose medium. Reusser *et al.* (1958) grew *M. hybrida* in glucose, molasses and WSL media. It was found that high yields of dry matter and protein were obtained on molasses, while yields on WSL were slightly lower. In WSL, *M. hybrida* produced low yield of mycelium (16.3%) having a protein content of 37.5%. Cirillo *et al.* (1960) developed a process which utilizes WSL to grow various mushroom mycelia. However, it was claimed that *M. crassipes* failed to grow in WSL. The submerged culture growth of *M. crassipes*, *M. esculenta* and *M. hortensis* was also investigated using cheese whey, pumpkin canning waste and corn (Zea mays) canning waste as substrates [Litchfield and Overbeck (1963b)]. Highest yields were obtained with *M. hortensis* in all of the substrates studied. A carbon to nitrogen ratio of 8:1 in both cheese whey and pumpkin waste gave better yields with all three organisms than any other higher carbon to nitrogen ratio. Poor yields of all three organisms were obtained with corn canning waste as substrate. *M. hortensis* was selected as the culture of choice on the basis of its more efficient conversion of glucose and lactose components of the wastes to protein. The protein content of the mycelium of *M. hortensis* was approximately 35% when it was grown in cheese whey and pumpkin

wastes which had been supplemented with nitrogen and phosphorus. Falanghe *et al.* (1964) reported a low yield of *M. hybrida* submerged growth in soybean whey medium. Janardhanan *et al.* (1970) investigated the utilization of vegetable wastes for the cultivation of *M. crassipes*, *M. esculenta* and *Morchella* spp.. The cauliflower leaf extract was found to be the best medium. The mycelium had a characteristic morel flavour. Analysis of the mycelium indicated a rich source of protein, containing all the essential amino acids.

Methods for recovery of flavours from MMM have been investigated. Szuecs (1950) developed a method of separating the essence of mushroom from the mycelium (*M. esculenta*, *M. bispora* and other fungi) by steam distillation under reduced pressure. He claimed that the content of the mushroom essence in the mycelium is at least about 100 times that of the fruiting body or sporophore. Szuecs (1954) also developed a method of enhancing the mushroom flavour of these mushroom mycelia, by separating the freshly prepared mycelium from the nutrient medium, subjecting the mycelium to a chemical treatment, and then aging it until the characteristic mushroom flavour was substantially enhanced. Heinemann (1963) improved the culture medium and the

fermentation conditions for the submerged cultivation of MMM (*M. esculenta, M. crassipes, M. hortensis, M. angusticeps* and *M. rotunda*) in glucose medium. The dry weight of mycelium and the flavour score were claimed to be the highest with skim milk as compared with corn steep liquor (CSL) or milk protein hydrolysate. Kliss (1963) reported a commercial production of MMM (*M. esculenta*) by submerged cultivation in a glucose medium according to the process developed by Heinemann (1963). The production plant was located at Springfield, Missouri, USA (Special Products, Inc., Division of Producers Creamery Co.). The dried powder of this product was officially named "Powdered Morel Mushroom Flavouring" according to the Food and Drug Administration (FDA) regulation. The current market price was in the range of $3.60 per pound in 100lbs lots. This is far too costly for food or feed uses, but is competitive with the price of imported dried mushrooms for use as food flavouring in dehydrated foods. However, this production plant was discontinued (private communication with Dr. B. Heinemann of the Mid-America Dairymen, Inc., September 7, 1972). Bennett (1967) developed an improved process for the growth and production of mushroom tissue (*M. crassipes, M. hybrida* and other fungi) under sterile conditions in an aqueous nutrient fermentation medium. The mushroom tissue was treated with an aqueous solution of an oxidizing agent

consisting of sodium hypochlorite and hydrogen peroxide to sterilize the mushroom tissue against contaminating micro-organisms.

The chemical composition of MMM was also determined. The dried mycelia of *M. crassipes*, *M. esculenta* and *M. hortensis* grown in glucose medium, were subjected to proximate analyses and amino acid determination [Litchfield *et al.* (1963c)]. The dry samples contained 22.8 - 51.0% protein and 2.18 - 7.55% fat, depending upon the species. Amino acid contents were similar to those reported in literature for other fungi. Hatanaka and Terakawa (1968) reported the presence of β-alanine, γ-aminobutyric acid, and an unknown amino acid in the fruiting body of *M. esculenta*. Later, this unknown amino acid was isolated from *M. esculenta* and related species and identified as a new amino acid, *cis*-3-amino-L-proline [Hatanaka (1969)]. It was found in a free state in fruiting bodies of *M. esculenta*, *M. conica* and *M. crassipes*, as well as in their cultured mycelia. Its occurence seemed to be confined to the genus *Morchella*. The vitamin contents of the MMM was also reported [Szuecs (1954), Litchfield (1964)].

The consumption of the fresh natural morels is traditionally recognized as safe. The possible toxic effects of the "Powdered Morel Mushroom Flavouring" was also investigated. It was found that a person would have to eat 60 lbs per day to have any ill effects [Robinson and Davidson

(1959)]. However, one case has been reported on the illness caused by the consumption of alcohol at the same time that morels were eaten [Groves (1964)].

1.3 Waste Sulfite Liquors

1.3.1 Origin and Composition

Waste sulfite liquor (sulfite waste liquor, spent sulfite liquor, or red liquor) is the liquor which is separated from the sulfite pulping process after the cooking operation has been completed (Fig. 1.6).

The pulping process comprises an operation whereby wood, in the form of chips, is treated with a solution of a bisulfite, such as calcium, magnesium, ammonium or sodium, in an excess of sulfurous acid, under pressure (90-110 psig), at an elevated temperature (125-130°C), for an appropriate cooking time (6-12 hours) and with the proper acid concentration (5.6% free SO_2 and 1.15% combined SO_2). At the termination of the cooking operation, the individual fibers in each wood chip become softened, delighified and and easily separated from one another. The nature of the several chemical reactions involved in the solubilizing of lignin or delignification is still incompletely understood. It now appears that the two principal chemical reactions of sulfite pulping are those of sulfonation and hydrolysis,

PRODUCTION OF SO_2

- ▷ CRUDE SULFUR: $S + O_2 \longrightarrow SO_2$
- ▷ PYRITE:
  - $3FeS_2 + 8O_2 \longrightarrow 6SO_2 + Fe_3O_4$
  - $4FeS_2 + 11O_2 \longrightarrow 8SO_2 + 2Fe_2O_3$
- ▷ IRON PYRITE: $2Fe_2S_3 + 9O_2 \longrightarrow 6SO_2 + 2Fe_2O_3$
- ▷ PYRRHOTITE: Fe_7S_8

BASE

WATER

SO_2

PRODUCTION OF COOKING ACID

$SO_2 + H_2O \longrightarrow H_2SO_3$

LIME
- $CaO + H_2O \longrightarrow Ca(OH)_2$
- $Ca(OH)_2 + H_2SO_3 \longrightarrow CaSO_3 + 2H_2O$
- $CaSO_3 + H_2SO_3 \longrightarrow Ca(HSO_3)_2$

SLAKED LIME OR MILK OF LIME
- $Ca(OH)_2 + H_2SO_3 \longrightarrow CaSO_3 + 2H_2O$
- $CaSO_3 + H_2SO_3 \longrightarrow Ca(HSO_3)_2$

LIMESTONE OR CALCITE
- $CaCO_3 + H_2SO_3 \longrightarrow CaSO_3 + CO_2 + H_2O$
- $CaSO_3 + H_2SO_3 \longrightarrow Ca(HSO_3)_2$

MAGNESITE
- $MgCO_3 + H_2SO_3 \longrightarrow MgSO_3 + CO_2 + H_2O$
- $MgSO_3 + H_2SO_3 \longrightarrow Mg(HSO_3)_2$

MAGNESIA
- $MgO + H_2O \longrightarrow Mg(OH)_2$
- $Mg(OH)_2 + H_2SO_3 \longrightarrow MgSO_3 + 2H_2O$
- $MgSO_3 + H_2SO_3 \longrightarrow Mg(HSO_3)_2$

MAGNESIUM HYDROXIDE
- $Mg(OH)_2 + H_2SO_3 \longrightarrow MgSO_3 + 2H_2O$
- $MgSO_3 + H_2SO_3 \longrightarrow Mg(HSO_3)_2$

AMMONIA
- $NH_3 + H_2O \longrightarrow NH_4OH$
- $NH_4OH + H_2SO_3 \longrightarrow NH_4(HSO_3) + H_2O$

SODA - ASH
- $Na_2CO_3 + H_2SO_3 \longrightarrow Na_2SO_3 + CO_2 + H_2O$
- $Na_2SO_3 + H_2SO_3 \longrightarrow 2Na(HSO_3)$

DOLOMITE ($2CaCO_3 + 1MgCO_3$) ▶ DOLOMITE + $2H_2SO_3 \longrightarrow$ MIXED BISULFITE + $CO_2 + H_2O$

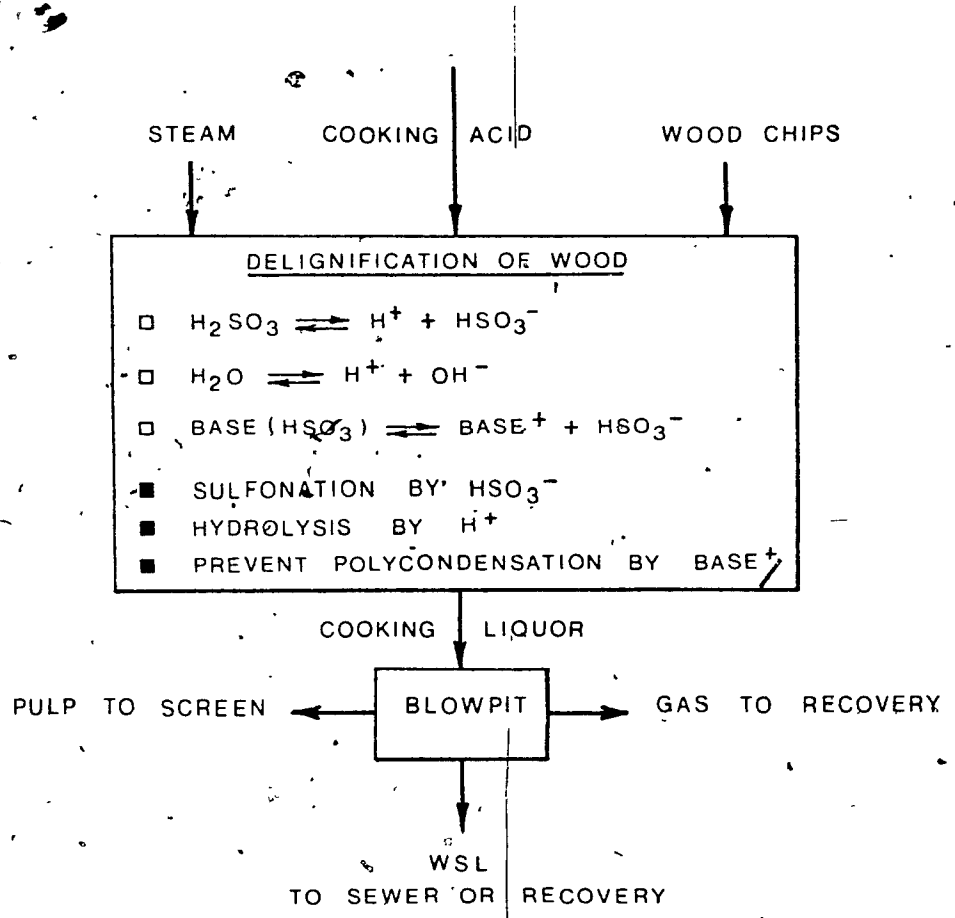


Figure 1.6 WSL From Sulfite Pulping Process.

although under certain conditions, the reaction of polycondensation may also be important.

In general, it seems that bisulfite ions in the reagent solution undergo reaction with the lignin structural units to form soluble sulfonate groups. It is not known how the sulfonate groups are attached to the lignin molecules. The hydrogen ions in the cooking liquor also play an important role in that they catalyze the hydrolysis or cleavage of lignin-lignin or lignin-carbohydrate linkages in the nearly infinite lignin network and thereby set free lignin sulfonate fragments which can now dissolve in the aqueous solution. In practice, the sulfonation and hydrolysis reactions proceed simultaneously. As sulfonate groups become attached to lignin, bisulfite ions or sulfurous acid molecules are consumed more or less stoichiometrically. Since the disappearing sulfurous acid is a relatively weak acid and limitedly dissociated, while the sulfonic acids being formed are strong acids and almost completely dissociated, the acidity of the cooking liquor tends to increase steadily as the sulfonation proceeds [Rydholm (1965)]. A certain degree of acidity is desirable to accelerate hydrolysis but it is necessary to control and limit the acidity to minimize progress of lignin polycondensation reactions which may give rise to difficulties in lignin removal and possibly, ultimately, to a burnt cook. To limit the acidity to desired

levels, a basic ion is used in the cooking liquor to serve as a buffer; and this ion, which may be calcium, magnesium, ammonium or sodium, should be present in such amounts as to be more than equivalent to the sulfonate and other strong acid groups which are formed.

Chemical changes also occur in the carbohydrate components of wood during the cooking operation with the hemicelluloses being hydrolyzed in preference to cellulose. The reason might be the existence in hemicelluloses of chemical linkages less resistant to hydrolysis or the existence of structures with a low degree or order of crystallinity. With gymnosperm woods, which are long-fibered and low in resin content, certain monomeric sugars are released, such as glucose, mannose, and possibly fructose, which are readily fermentable by yeasts. The yield of sugars obtained from the hemicelluloses in practice is only about one half to two thirds of the theoretical value. This loss appears to occur as a result of chemical reactions involving sugar aldonic and sulfonic acids. Complete discussion concerning the chemistry of the sulfite pulping process may be referred to Rydholm (1965). The chemical composition of the WSL from various sources is shown in Table 1.1. The concentration of the WSL in the digestor prior to pulp washing is around 12-16%, depending on the pulp grade and the liquor-to-wood ratio. The concentrated

Table 1.1 Major Components of WSL.

Components	Spruce (% of total solids) [Forss (1961)]	Spruce (% of total sugars) [Hägglund *et al.* (1928)]	Spruce (% of total sugars) [Sihtola *et al.* (1958)]	85% Western hemlock 15% White fir (% of total sugars) [Mulvany *et al.* (1951)]
Lignosulfonate	52			
Extractives	3			
Poly- and oligosaccharides	6			
Monosaccharides	23			
Galactose	3	4.2	9-12	10
Glucose	3	28.9	5-15	15
Mannose	11	42.7	50-60	48
Arabinose	1	17.0	3-5	6
Xylose	5		15-25	15
Fructose		4.0		< 2
Unidentified				4
Galacturonic acid		3.2		
Glucuronic acid	1			
Aldonic acid	4			
Sugarsulfonates	3			
Acetic acid	2			
Methanol	1			
Calcium	5			
Total	100	100.0		100

WSL then contains on a wet basis 3.5-4.5% sugars as monosaccharides and 7% of lignosulfonate. The rest are sugar derivatives, poly- and oligosaccharides and miscellaneous by-products.

1.3.2 Pollution Problem

A skunk and her kittens were travelling west
When dawn drew nigh and she stopped to rest
She found a place close by the town
Where she fixed a bed and they all lay down.

As the paper mill odor filled the air
Like rotten fish and burning hair
She awoke and crawled right out of bed
(You can't appreciate this unless you've
smelled the damned thing).

She turned to the kittens all seated there
Saying "Bow your heads, it's time for prayer
Lord, you gave us a weapon for self-defense
It was an awful odor, both potent and dense.

I'm a sow pole-cat of ripe old age
A cat that nobody keeps in a cage - -
But I can always recognize defeat
Compared with it, my smell is sweet.

The extent of the difference I realize
But I think it's partly a matter of size
So, Lord, I ask, if it be thy will
Make me as big as the kraft pulp mill".

Narcissus of Tuscaloosa.
[From Allan *et al.* (1972)]

Many would argue that the sentiments expressed in this poem are simply an emotional response on the part of one mill town resident, and not of much significance in approaching so important a question as pollution in the pulp and paper industry. The odor produced by a pulp mill is, in fact, only one aspect of the problem, and its importance in any terms but human comfort is debatable. Other sources of pollution stemming from the pulping process are no longer the subject of debate.

i) Water Pollution From Pulp and Paper Mills

Depending on the process, pulp mills use a maximum of 35,000 gallons of water per ton of kraft pulp and 60,000 gallons per ton of sulfite pulp (Table 1.2). Water is used in high pressure hydraulic jets for debarking, as cooling liquid for pumps and machines, in cooking, washing, and as a method of transporting fibers throughout the process. A great amount of variety of waste matter is discharged in these untreated waste water, i.e. suspended

Table 1.2 Effluent Volumes From Manufacture of Pulp and Paper Products. From Gehm (1965).

Process	US gals per ton
Pulp Manufacture:	
Kraft and soda pulps	15,000-35,000
Sulfite pulp	40,000-60,000
Semichemical pulp	30,000-40,000
Groundwood pulp	4,000-10,000
Deinked pulp	20,000-35,000
Pulp Bleaching:	
Kraft and soda pulps	15,000-60,000
Sulfite pulp	30,000-50,000
Neutral sulfite pulp	40,000-60,000
Paper Manufacture:	
Kraft papers	2,000-10,000
Paperboard	2,000-15,000
Tissues	8,000-35,000
White papers	20,000-40,000
Specialty papers	20,000-100,000

solids like fibers, bark, uncooked wood chips, and dirt; dissolved solids like carbohydrates and soluble wood matter; and cooking and bleaching chemicals. Although they are not toxic , these pulping pollutants have undesirable effects on waters.

Solid wastes sink to the bottom of slowly moving streams and lakes, where they form beds of sludge that are not only unpleasant but also ruin the quality of the water and aquatic life. They destroy the natural bottom habitat of fish, they blanket existing clam and oyster beds, and as they decay, they use up oxygen in the water and release toxic gases.

Carbohydrates dissolved from wood in the cooking process and drained from the pulp are even more polluting than solid wastes. These materials (sugars and sugar derivatives) are decomposed by the microorganisms in the water as long as there is oxygen present. However, if the concentrations of waste are too high, bacteria overfeed, overpopulate, and consume the available oxygen in the stream, often diminishing it to a level insufficient to support aquatic life. A strain of filamentous bacteria *Sphaerotilus natans* is usually found in streams receiving WSL and carbohydrate wastes [Amberg and Elder (1956)]. Extremely weak substrate concentration (0.5-1.0 ppm of sugars) is sufficient to

produce considerable sphaerotilus slime growth to reach levels that interfere with gill net operations of commercial fisheries. This biological decomposition process is further hampered by the chemicals dumped, leaked, or spilled from mills. Often waste chemicals react directly with oxygen in the water to form new chemical compounds. This reduces the amount of oxygen available for bacterial breakdown of organic mill wastes. The average waste load of a mill varies according to the process. From groundwood pulp, it is 30-60 pounds per ton of pulp; from unbleached kraft, 25-50 lb/ton; from unbleached sulfite, 550-750 lb/ton. Bleaching raises the BOD_5 (5 day biochemical oxygen demand) poundage by 25-250 lb/ton (Table 1.3). The BOD_5 indicates the level of water pollution. If it is too high, it adversely affects the complicated life chain in natural waters from bacteria to plankton to plants to fish life. The importance of this measurement can be seen by comparing human and industrial discharges. While an estimated 0.17 pound of oxygen is required by bacteria to decompose a daily human discharge of waste, nearly 1,000 pounds of oxygen are required for the decomposition of untreated wastes from one ton of bleached sulfite pulp. Thus an average sized sulfite mill that produces 200 tons of pulp each day, can create a BOD_5 load which is equivalent to that of raw wastes from one million people. Describing the

Table 1.3 Effluent Loads From Manufacture of Pulp and Paper. From Gehm (1965).

Process or Product	lb per ton of product	
	Suspended Solids	BOD_5
Kraft and soda pulp	20-30	25-50
Groundwood pulp	40-80	15-25
Sulfite pulp (no liquor recovery)	20-40	400-600
Neutral sulfite semichemical pulp	100-180	250-450
Textile fiber pulps	300-500	200-300
Straw pulp	400-500	400-500
Bleaching	6-35	12-200
Deinked pulp	500-800	100-130
Fine papers		
Tissue	50-100	15-30
Bond, mimeo, and printing	50-100	20-40
Glassine	10-15	15-25
Coarse papers		
Boxboard	50-70	20-40
Corrugating board	50-70	25-60
Kraft wrapping	15-25	5-15
Newsprint	20-60	10-20
Specialty papers		
Fiber	200-300	140-170
Asbestos	300-400	20-40
Roofing felt	50-100	40-60
Insulating board	50-100	150-250

BOD_5 of the waste output of specific mills in terms of population equivalents makes the measure more graphic. This equivalency however refers only to oxygen demand since industrial wastes do not usually contain pathogenic bacteria, nutrients, etc. present in domestic wastes.

Two other water pollutants, foam and coloring matter, are both annoying and damaging. The foam results from small amounts of resin, fatty acids, and chemicals in the effluent. It bubbles up from mill sewer outfalls, spreading brownish suds over the water. The dark color in mill effluent can be both distressing and harmful, for as it spreads over the surface of rivers and lakes, it can block out sunlight, thereby reducing the rate of photosynthesis and upsetting the ecological balance.

Until recently, mercury was another pollutant from pulp and paper manufacture. It was used as a fungicide to preserve pulp or chips in warm weather, as a slimicide, and as a catalyst in the manufacture of bleaching chemicals. However, since the mercury scare in mid 1970, the industry has almost totally discontinued using and discharging mercury.

ii) Economical Importance of Pulp and Paper Industry

An obvious reason for the environmental impact of the pulp and paper industry is its size. Pulp and

paper ranks as the third largest nondurable industry in the United States; in 1969 it had sales of $20.5 billion and produced 53.8 million tons of paper, or 576 pounds for every man, woman and child in this country, which population, that amounts to six per cent of the world's total, consumes almost 45 per cent of the world's paper [Allan *et al.* (1972)].

The Canadian pulp and paper industry is today, and has been for many years, Canada's leading manufacturing industry. It leads all others in the value of its production, as well as in employment, exports, and capital investment. It comprises some 60 companies operating some 130 mills in nine of the ten provinces, the exception being Prince Edward Island. Thus it is a national industry playing an important role in each of Canada's economic regions. The annual production of the industry is now valued at more than two billion dollars, or close to five per cent of Canada's gross national product. The province of Quebec is the leading producer, accounting for 37 per cent of the national output. Ontario is second with 24 per cent, followed by British Columbia with 23 per cent, New Brunswick, Nova Scotia, and Newfoundland with 14 per cent and Alberta, Saskatchewan and Manitoba with 2 per cent. The per capita consumption of paper and board in 1963 amount to 301 pounds in Canada, compared with 459 pounds in the United States and only 63 pounds on a world basis [Jones (1966)].

1.3.3 Pollution Control

i) State-of-the-Art

State-of-the-art pollution control is the highest level of control technologically and economically feasible. It is a flexible concept that changes as new and more efficient pollution control methods become available. In the pulp and paper industry today, with state-of-the-art effort and equipment, process water can be cleaned of over 90 per cent of its solid waste load (fibers, dirt, bark, and wood chips) and over 85 per cent of the oxygen-demanding dissolved solids and chemicals washed from the pulping and bleaching operations. Even the coloring matter in effluent can be greatly reduced by physical and chemical treatments. Before mill effluent is discharged into public waterways, it can be restored to a nontoxic and aerated condition.

Table 1.4 represents the major water pollution control methods and equipment in a state-of-the-art mill, their efficiencies and their approximate capital costs. This is not an exhaustive list of all possible equipment and mill modifications, but it includes the major categories of equipment. This state-of-the-art water pollution control system represents up to 10 per cent of the capital cost of a new mill. For older mills recovery and/or disposal systems may need extensive rebuilding in order to accommodate

Table 1.4 Water Pollution Control Methods. From Allan *et al.* (1972).

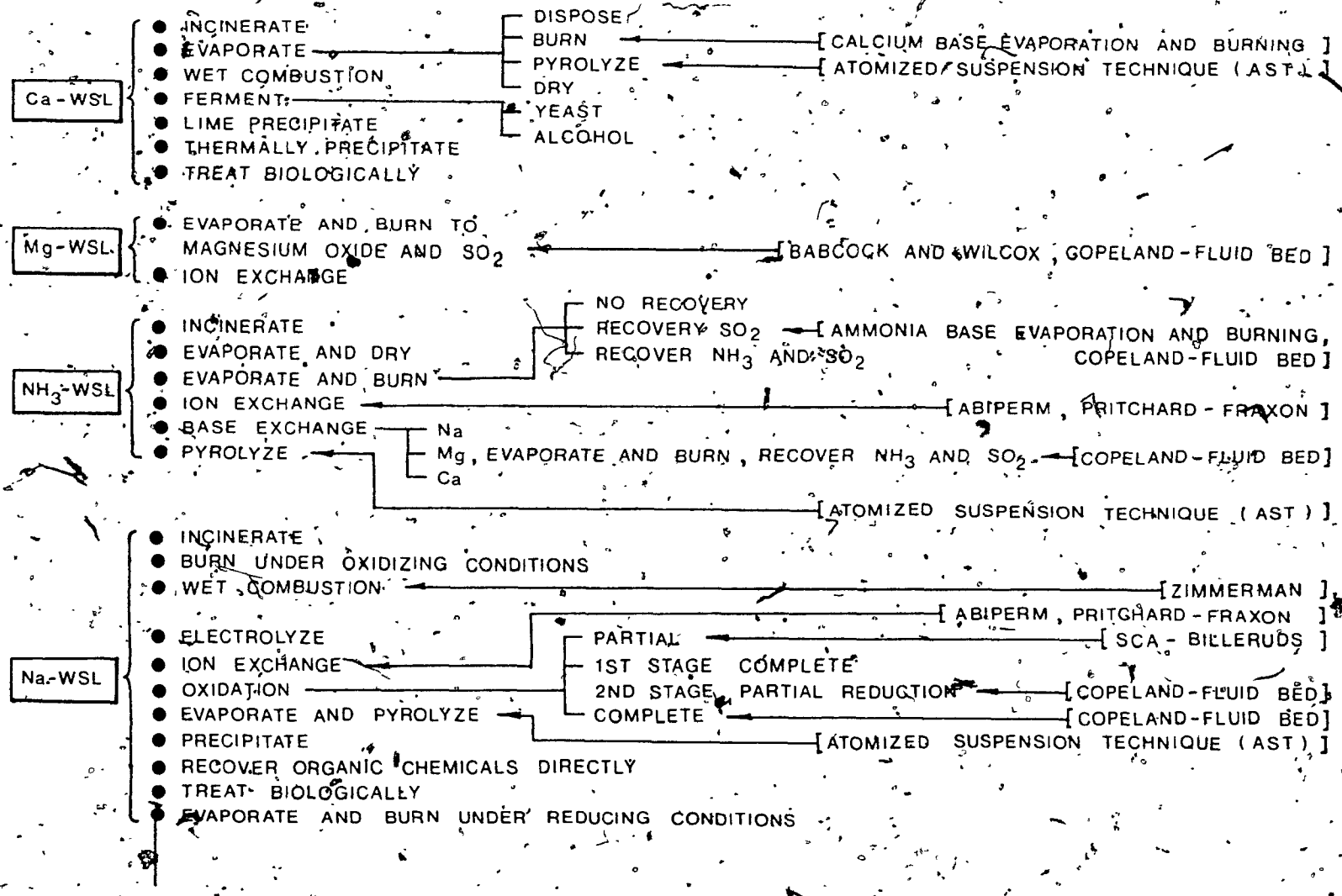
Method	Efficiency	Capital Cost (US $)
In-Plant Control:		
*Proper operation and maintenance of a mill.	Avoidance of spills and leakage and production at optimum capacity of the equipment.	
*Water recycling for reuse.	Saving fibers, chemical and heat losses.	
On-Site Effluent Treatment:		
*Primary (settling ponds, clarifiers).	75-95% solid removal and 25% BOD_5 reduction.	300,000-1,000,000
*Secondary (holding ponds or naturalization stabilization lagoons, aerated ponds or lagoons).	80-95% BOD_5 reduction.	700,000-2,000,000
Activated sludge.	80-95% BOD_5 reduction.	3,000,000-4,000,000
*Tertiary (color removal).	85-95% coloring matter removal.	System too new, accurate cost figures not available.
Out-of-Plant Water Treatment and Disposal:		
*Joint municipal-industrial treatment.	May include primary, secondary, and tertiary treatments.	
*Direct stream aeration.	Supplement to but not replacement for secondary treatment.	
*Spray irrigation.	Unacceptable because the chemicals and salts in the effluent are more likely to degrade soil quality.	
*Outfall pipes with diffusers (to ocean, river).	Unacceptable because oceans and large rivers do not have infinite assimilative capacity for wastes.	

the equipment. The costs of treatment vary widely depending not only on the age and size of mill but also on its location, type of operation, etc. Where a mill has been built with no thought of controlling waste disposal, it may take considerable effort even to find, much less plug up, all the discharge points. The industry's rule of thumb is that modifications to an older mill cost two to three times as much as installations in a new one.

ii) Available Recovery Processes

The classification of the recovery processes for various WSL is shown in Fig. 1.7. First, the choice of the recovery and treatment system depends on the base of the pulping liquor: calcium, magnesium, ammonium and sodium. At least one, and usually several recovery systems have been researched and developed for each of these base-liquor systems. Each base has different chemical and physical properties and therefore, a recovery system suitable for one base usually is not applicable to another.

The status of WSL recovery in Canada and United States in 1969 was the following [Perry and Woodland (1970)]: Of the 42 operating sulfite mills in Canada, only two had integrated chemical recovery systems for burning organic materials and recovery of heat and chemicals. Five other sulfite mills are recovering by-product chemicals from WSL


Ca-WSL
INCINERATE
EVAPORATE
WET COMBUSTION
FERMENT
LIME PRECIPITATE
THERMALLY PRECIPITATE
TREAT BIOLOGICALLY
DISPOSE
BURN
PYROLYZE
DRY
YEAST
ALCOHOL
[CALCIUM BASE EVAPORATION AND BURNING]
[ATOMIZED SUSPENSION TECHNIQUE (AST)]
Mg-WSL
EVAPORATE AND BURN TO MAGNESIUM OXIDE AND SO_2
ION EXCHANGE
[BABCOCK AND WILCOX, GOPELAND-FLUID BED]
NH_3-WSL
INCINERATE
EVAPORATE AND DRY
EVAPORATE AND BURN
ION EXCHANGE
BASE EXCHANGE
PYROLYZE
NO RECOVERY
RECOVERY SO_2
RECOVER NH_3 AND SO_2
[AMMONIA BASE EVAPORATION AND BURNING, COPELAND-FLUID BED]
[ABIPERM, PRITCHARD-FRAXON]
Na
Mg, EVAPORATE AND BURN, RECOVER NH_3 AND SO_2
Ca
[COPELAND-FLUID BED]
[ATOMIZED SUSPENSION TECHNIQUE (AST)]
Na-WSL
INCINERATE
BURN UNDER OXIDIZING CONDITIONS
WET COMBUSTION
ELECTROLYZE
ION EXCHANGE
OXIDATION
EVAPORATE AND PYROLYZE
PRECIPITATE
RECOVER ORGANIC CHEMICALS DIRECTLY
TREAT BIOLOGICALLY
EVAPORATE AND BURN UNDER REDUCING CONDITIONS
[ZIMMERMAN]
[ABIPERM, PRITCHARD-FRAXON]
PARTIAL
1ST STAGE COMPLETE
2ND STAGE PARTIAL REDUCTION
COMPLETE
[SCA-BILLERUDS]
[COPELAND-FLUID BED]
[COPELAND-FLUID BED]
[ATOMIZED SUSPENSION TECHNIQUE (AST)]

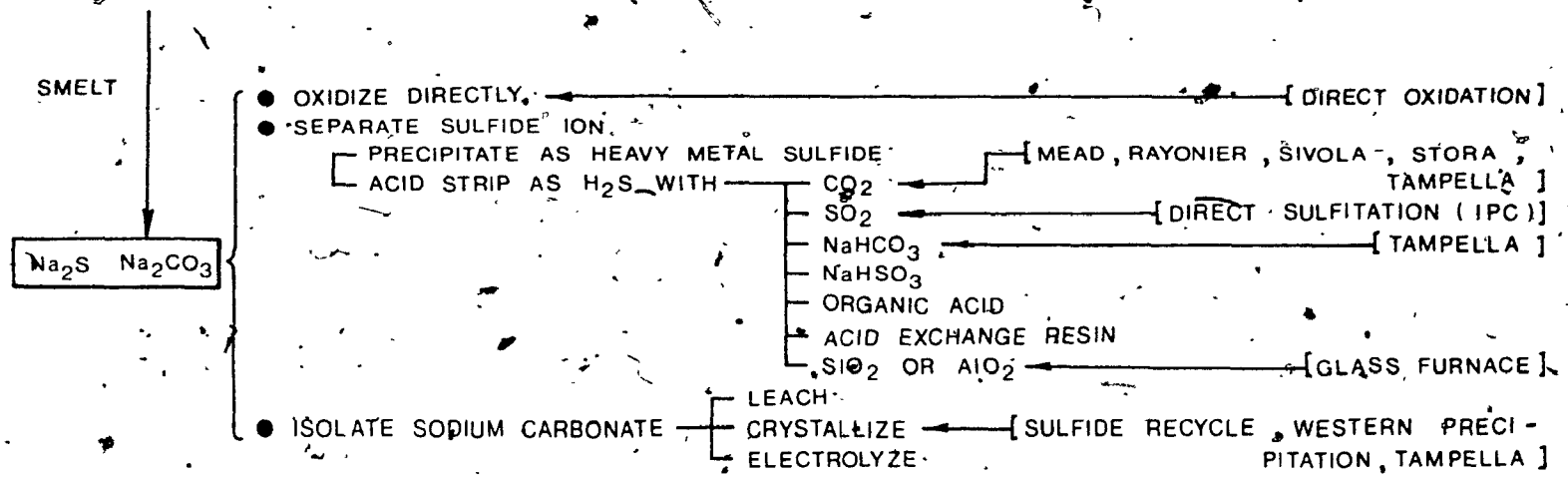


Figure 1.7 - Classification of Recovery Processes. From Perry and Woodland (1970).

for sale, which also results in a partial reduction in BOD_5 in the effluent. Of the remaining 35 mills, 10 are producing high-yield unbleached pulp for use in newsprint; these mills pose special problems with respect to potential recovery systems. The details are shown in Table 1.5. The comparable situation in United States sulfite mills is as follows: of 38 operating mills, 10 have integrated chemical recovery systems, 8 are recovering chemical by-products for sale (with partial BOD_5 reduction), and 20 are operating without recovery.

iii) Re-utilizations

Another approach to the pollution control is the development of methods to re-utilize the components, or their derivatives, in a form in which they can be sold at a profit. For example, WSL itself is being used to a limited but increasing extent as a road binder. In several plants in the United States and Canada, the fermentable sugars in WSL are being converted by microorganisms to produce industrial alcohol or fodder yeasts (food yeast, Torula yeast or Candida yeast). In other cases, WSL concentrates or solids are being sold for use as binders and adhesives, emulsifiers, soil modifiers, surface-active agents, and tanning materials, and for other purposes. By alkaline treatment, the lignin sulfonates in the liquors are being converted at several locations into the substance vanillin. This chemical is

Table 1.5 Status of Canadian WSL Recovery in 1969. From Perry and Woodland (1970).

Mill	Pulp Mill Capacity (tons per day)	Pulping Base	Products	By-products or Recovery System
Partial Chemical Recovery:				
*Domtar, Cornwall, Ont.	180	Ca-NH_3-	Fine paper, boxboard.	Vanillin.
*Ontario Paper, Thorold, Ont.	240	Na-	Newsprint.	Lignin, vanillin, alcohol.
*Anglo-Canadian, Quebec City, Que.	470	Ca-	Newsprint.	Lignosulfonates.
*CIP, Temiscaming, Que.	440	Ca-NH_3-	Dissolving & paper grade pulps.	Alcohol.
*CIP, Gatineau, Que.	450	Ca-NH_3-	Newsprint.	Alcohol.
Full Chemical Recovery:				
*Nova Scotia Pulp, Port Hawkesbury, N.S.	350(a)	Na-	Market pulp.	Stora Kopparberg.
*Spruce Falls Power & Paper, Kapuskasing, Ont.	200	Mg-	Newsprint.	B&W.

(a) Bleached production.

used in flavouring and cosmetics fields. Today, however, only a small fraction of the total available WSL solids is utilized. Table 1.6 summarizes the potential and available re-utilizations of WSL.

A brief review on the fermentative utilization of WSL by using various bacteria, yeasts and fungi for commercial production of chemicals, organic solvents and acids has been reported by Mueller (1970) and Mueller and Walden (1970). In recent years, research has also been conducted throughout the world on various species of yeasts such as *Candida (Torula* or *Torulopsis) utilis* [Ignat'eva and Sapotnitskii (1970), Rieche *et al.* (1970), Barta (1971a), Ivanyukovich and Kalyuzhnyi (1971), Kalyuzhnyi and Ivanyukovich (1971), Murko and Cezner (1971), Zol'nikova and Pebre (1971), Brabec and Radej (1972), Hojnos *et al.* (1972)], *Candida tropicalis* [Sapotnitskii *et al.* (1972)], *Cryptococcus diffluens* [Barta (1971b), Barta (1972), Hojnos *et al.* (1972)], and fungi such as *Aspergillus niger, A. oryzae, A. wentii, Rhizopus nigricans, R. stolonifer* [Negoita (1970)], and a species of lignivorous fungus *Mycelium sterilium* [Jilek *et al.* (1971)]. However, very few studies have been developed using fungi, particularly edible species. The submerged cultivation of MMM in WSL has been reported by Reusser *et al.* (1958) and Cirillo *et al.* (1960).

Table 1.6 Re-utilizations of WSL. From Calkin (1957).

Application	Comment
Briquet and ore binder.	At core baking temperature of 400-500° F the material becomes insoluble.
Adhesives.	Linoleum adhesive; compounded with a variety of materials to form adhesives.
Emulsifying agent.	Effective emulsifier of asphalt and oils, and additives to soaps; used as dispersant and adhesive for agricultural sprays.
Soil additive and modifier.	Aggregates soil into coarse groups, aiding penetration of water and air, and retention of water.
Extender for other organic chemicals.	Has found use as a resin (phenol-formaldehyde) extender.
Surface-active agent.	Disperses fine particles in water; useful in ceramic, cement, ore flotation, gypsum and oil-well drilling industries. Detergent in textile industry; dispersant in dyeing operations.
Sequestering agent.	Strongly sequesters ferric ion, cupric copper, and stannous tin.
Erosion preventative.	Acts as cement, binding soil particles to form crust, preventing wind damage to newly planted crops; used as a road binder.
Tanning agent.	Plays an important role in the tanning of leather; possibility of replacing much of vegetable tannins now in short supply.
Yeast and alcohol.	The fermentable carbohydrate constituents may be converted to ethyl and butyl alcohols, and acetic; propionic, butyric, and lactic acids by appropriate means. The yeasts grown in WSL are used as a supplementary diet for animals, especially poultry.
Chemical products.	Vanillin is prepared by the oxidation of softwood lignin, and a mixture of vanillin and syringaldehyde from hardwood lignin. Sulfite vanillin is used in flavouring vanillin to a large extent. Furfural is prepared by the conversion of pentose sugars at elevated temperatures in the presence of a mineral acid. It is now being used for nylon production, refining operations, butadiene extraction, and the manufacture of various chemicals.

1.4 Investigation Program

Because of the abundance and availability of WSL and because of the disposal problems associated with WSL, it is of particular interest to convert the organic material from these liquors to a valuable source of protein and flavouring.

Five different WSL, NH_3-, Ca-, Mg-, Na-, and mixed (Ca & Mg)- bases are used as substrates for the submerged cultivation of four species of morels, *Morchella* spp., *M. crassipes*, *M. deliciosa*, and *M. esculenta*. Due to the lack of available information for this particular system, preliminary studies are designed to develop data concerning the process and product (Chapters 2 and 3). The optimal growth conditions in terms of pH, dilution ratio (substrate concentration), composition of culture medium, pretreatment methods (SO_2-stripping, γ-irradiation of WSL), inoculum (original or adapted strain) on the yield of biomass and the reductions of BOD_5 and COD (chemical oxygen demand) of the WSL at the end of the cultivation processes are conducted in shake flask experiments and in batch fermentors. The protein and amino acids, fat and fatty acids, and carbohydrate contents in the biomass are determined in order to select the best culture system for an economical production. The flavour of the MMM in WSL is also extracted, analyzed and compared with that of fresh

meadow mushrooms. Chapters 4 and 5 deal with the dynamic behavior of one selected culture system in a continuous cultivation process. A continuous stirred tank fermentor (CSTF) is considered as a multivariable linear system around its operating point (optimal conditions gathered from preliminary studies). Pulse testings on the inputs (agitation, aeration, inlet jacket temperature, feed temperature, inlet pH, inlet substrate concentration) and their responses at the outputs (biomass, outlet medium temperature, outlet jacket temperature, outlet pH, outlet substrate concentration) are used for numerical determination of the system transfer function matrix. Finally, the conclusion and recommendations presented in Chapter 6 emphasize the advantages and disadvantages as well as the economic feasibility of this mycological waste treatment approach.

CHAPTER 2

MATERIALS AND METHODS FOR PRELIMINARY STUDIES

2.1 Materials

2.1.1 Waste Sulfite Liquors

The concentrated WSL samples were received from various pulp and paper mills. The insoluble solids were removed by filtration and the liquors were stored in closed plastic tanks for further use. Before utilization, the Na-WSL was stripped of SO_2 by boiling, and 60% (v/v) of liquor was evaporated. The concentrate was then diluted with distilled water to the original volume. The characteristics of the received WSL are presented in Table 2.1.

2.1.2 Stock Cultures

The original cultures of *Morchella crassipes* NRRL-2369, *M. deliciosa* NRRL-2601 and *M. esculenta* NRRL-2603 were received from the U.S. Department of Agriculture and that of *Morchella* sp. from the Department of Plant Science of the University of Western Ontario, London, Ontario, Canada. These pure cultures were maintained on

Table 2.1 Characteristics of WSL as Received.

WSL	Supplier	pH	TCH[d] (g/l)	BOD_5 (ppm)	COD (ppm)	TOC[e] (ppm)	SO_2 (%)	Color and odor
NH_3-	Canadian International Paper Co., Temiskaming, Que.	2.00[a] 2.30	44.0[a] 10.6	14,400[a] 15,800	116,300[a] 92,300	32,300[a]	0.047	Very dark brown.
Ca-	Domtar Fine Paper Ltd., Cornwall, Ont.	2.55[a]	50.0[a]	25,000[a]	129,600[a]			Brown.
Mixed-	Spruce Falls Powers & Paper Co., Ltd., Kapuskasing, Ont.	1.80[a]	70.0[a]	36,500[a]	160,600[a]			Light brown.
Mg-	The James Mclaren Co., Ltd., Buckingham, Que.[b]	3.85[a] 3.93	46.0[a]	18,900[a]	175,500[a] 132,700			Light brown.
Na-	Abitibi Paper Co., Iroquois Falls, Ont.	3.60 5.25[c]	15.4	28,500	100,200	36,800	0.282 0.022[c]	Light brown and acidic odor. Dark brown and no acidic odor.[c]

(a) Sample received in January-February 1971, otherwise received in January-February 1972.
(b) Mill shut down on May 31st, 1972.
(c) Values measured after stripping of SO_2 and diluting with distilled water.
(d) Total carbohydrates.
(e) Total organic carbon.

stock culture agar slants of the following composition: 2.5% glucose, 2.0% agar, 1.0% peptone, and 0.5% yeast extract. In the case of the adaptation experiments carried out on the Na-WSL, five per cent (v/v) of the concentrated liquor was added into the medium instead of glucose. Slants were inoculated with a portion of the mycelium, at least 0.5 cm^2, to minimize the probability of selecting variant types. They were incubated at room temperature for 6 to 8 days depending upon the fungal species and then were stored in the refrigerator (4° C). Fresh transfers of stock cultures were made every four months.

2.2 Cultivation Methods

2.2.1 Inoculum

The inoculum for shake flask experiments were prepared from the fresh growth on agar slants, using similar culture conditions as those described for stock cultures. The entire growth from 3 (6) slants was transfered aseptically to a sterile Waring-blender jar containing 150 ml of sterile distilled water and it was blended for 30 (45) seconds. This suspension was used to inoculate the culture media at a ratio of 5% (6.7%) v/v for studies on the effects of initial pH and dilution ratios (for further studies with Na-WSL).

For the adaptation tests with the Na-WSL, growth in one Erlenmeyer flask was blended for 30 seconds and used as inoculum for further flasks at a ratio of 6.7% v/v.

Growth in 4 Erlenmeyer flasks (800 ml of total culture medium) was used to seed large carboy-type bottles operated at a 16-liter volume. Relatively small pellets (1-3 mm in diameter) developed in the Erlenmeyer flasks so that blending was not necessary. Growth from Erlenmeyer flasks was also used to seed the fermentor at a ratio of 5% v/v.

2.2.2 Batch Cultivation

The shake flask experiments were performed in 500 ml or 1,000 ml Erlenmeyer flasks containing 150 ml, 200 ml or 300 ml of culture medium. The medium was prepared by diluting a stock solution of the following composition: 2% ammonium phosphate dibasic (APD) $(NH_4)_2HPO_4$ and 5% CSL per liter of concentrated WSL. In later experiments with Na-WSL, the CSL was entirely excluded. Various dilution ratios were prepared and the initial pH of the medium was adjusted to the desired value by addition of either HCl or NaOH solutions. The flasks were sterilized in the autoclave at 15 psig and 120° C for 20 minutes and then cooled to room temperature. In the case of the

Na-WSL medium, the resulting dark precipitate had to be removed from the clear filtrate after sterilization. The flasks were inoculated and were incubated at 200 RPM for 9 days at room temperature (23-28° C).

Larger laboratory-scale batch cultivations were also performed in a 5-gallon bottle fermentor containing 16 liters of culture medium. The culture medium for these experiments was prepared in the same way as for shake flask experiments, except the concentration of CSL was ten-fold lower. Cultivations were conducted at room temperature at an aeration rate of 4 liters of air per minute or 0.25 vvm (volume of air per broth volume per minute) for 9 days. No agitation was utilized other than that obtained from aeration.

The batch cultivation of *M. crassipes* in NH_3-WSL was performed in the 14-liter Chemap fermentor containing 10 liters of culture medium. The composition of the culture medium was the same as for the experiments in the 5-gallon bottle fermentor. The pH of the culture medium was adjusted to 6.4 using NaOH solution and it was then sterilized in place for 20 minutes at 120° C. An antifoam (AF-Dow Corning) solution was occasionally added to the fermentor when the foam was extensive. The cultivation was carried out at 0.25 vvm, 300 RPM and 23-24° C for aeration,

agitation and temperature respectively.

2.3 Analytical Methods

2.3.1 Determination of Biomass

For the determination of dry weight, the total biomass in the culture medium was filtered through dehydrated filter papers (by drying in the oven at 105° C for 2 hours). The filter paper with mycelia was dried in an oven at 105° C to constant weight. The initial inoculum and precipitate was subtracted from the total weight to obtain the dry weight of mycelia produced. For large recovery of product, fungal growth was filtered by gravity through cotton gauze, then washed twice with either 1% NaCl in distilled water or with distilled water, and was then dried in a New Brunswick Freeze-Dryer. The freeze-dried samples were ground into fine powders and stored in closed bottles at room temperature for further analyses.

2.3.2 Determination of Total Carbohydrates

The total carbohydrates (TCH) in the WSL samples or in the culture medium were determined colorimetrically by the anthrone reagent according to methods of Morris (1948) and Neish (1952). The filtrates were diluted to

the appropriate range (*ca* 20-200 mg/l of equivalent glucose) and samples were read after 15 minutes against distilled water at 540 nm. Results are expressed as equivalent glucose concentrations and always averaged from five separate sambles. The calibration curves for various WSL with their correction factors are shown in the Appendix A. The TCH in the dry MMM samples were determined based on the same reagent and procedure. Fine powder was weighed into a test tube, diluted with distilled water and allowed to react with anthrone-sulfuric acid reagent. A complete mixing of the sample and the reagent is essential.

2.3.3 Determination of Crude Protein

The organic nitrogen in the dry mycelium was determined by the Pregl-Parnas-Wagner micro-Kjeldahl method [Furman (1962)]. The dried powder of MMM (10.0 mg) was digested with concentrated sulfuric acid, potassium sulfate (as catalyst) and copper sulfate (for accelerating the digestion) for 30 minutes in order to convert the organic nitrogen into ammonium nitrogen. The ammonia was subsequently liberated by the addition of sodium hydroxide and distilled into a known amount of standard acid (0.01 N HCl) and the excess acid was determined by titration with standard alkali (0.01 N NaOH). Mycelial protein was assumed to contain 16% nitrogen and a factor of 6.25 was used to

convert the organic nitrogen to crude protein. No correction for other nitrogen-containing compounds in the cell was made. The results represent an average of three separate samples.

2.3.4 Determination of Total Lipids

Total lipids were extracted by shaking the dry mycelium powder (3.40-4.84 g) overnight with glass beads, glass powder and a solvent mixture of chloroform-methanol (2:1) in a tightly stoppered round bottom flask [Kosaric (1969)]. The extracts were separated from the solid portion by filtration and evaporated *in vacuo* to remove the solvent. The residue was redissolved in chloroform and the non-soluble portion was separated by filtration. The filtrate was finally evaporated to dryness *in vacuo* and weighed for total fats.

2.3.5 Determination of Moisture and Ash

The moisture and ash contents in dried mycelium powder were determined by the method described by Jacobs (1965). For the determination of moisture, the powder of MMM samples (402.5-992.5 mg) was dried in a dessicator in the presence of calcium chloride at 75° C to a constant

weight. The weight loss represents the moisture content. Ash was determined by ignition using a Bunsen burner.

2.3.6 Determination of Amino Acids

For amino acid determination, the dry mycelium (305.0-315.8 µg) was hydrolyzed by refluxing with 500 ml of 5.7 N HCl for 24 hours. The hydrolysate was dried in a rotary evaporator and then redissolved in an appropriate volume of buffer. Norleucine was added as internal standard and aliquot was applied to sample cartridge for analysis using Technicon TSM-1 Amino Acid Analyzer.

2.3.7 Determination of Fatty Acids

The fatty acids of MMM samples were analyzed by gas-liquid chromatography of their methyl esters. The transmethylation of individual MMM fatty acids was performed according to Carroll *et al.* (1968). The chloroform soluble lipid extracts of MMM (50-100 mg dry weight) were mixed with 10 ml of 10% acetyl chloride solution in methanol and then refluxed for about two hours at 70° C. Methyl esters were extracted three times with petroleum ether. Combined petroleum ether extracts were then washed with deionized water until neutral. They were further evaporated

to dryness *in vacuo* and redissolved in chloroform prior to injecting into the gas-liquid chromatograph. The gas-liquid chromatography was performed on an F&M Model 5750 Research Gas Chromatograph using flame ionization detector. An 8-foot long and 1/4-inch ID stainless steel column was packed with 10% EGSS-X on Chromosorb R. The carrier gas used was helium at a rate of 60 cc/min. The fatty acid methyl ester standards (National Heart Institute, Bethesda, Md., U.S.A.) were used for identification of MMM methyl ester fatty acids and also for testing the linearity of response in the gas-liquid chromatograph. Quantitization of the fatty acids was based on products of retention time and peak height [Carroll (1961)].

2.3.8 Analysis of Mushroom Flavour

The flavour concentrates were prepared from the following : (i) 10 g freeze-dried powder, and (ii) 100 g fresh pellets of MMM or fresh ground fruiting bodies. They were continuously extracted with 250 ml diethyl ether for 24 hours using Soxhlet apparatus. The extracts (solvent phase) were filtered and reduced to approximately 5 ml *in vacuo* and stored in the refrigerator (0° C) for further use.

The gas-liquid chromatography of flavour concentrates was performed on an F&M Model 5750 Research Gas Chromatograph provided with an 8-foot by 1/8-inch OD column packed with 20% Carbowax 20M on 60-80 mesh Chromosorb WAW-DMCS. The operating conditions were: temperature programming from 65 to 250° C at 10° C per minute, then isothermal for 40 minutes. Both injection port and detector temperatures were at 210° C.

The ultraviolet absorption spectrum of the flavour concentrates was determined by the Perkin-Elmer Model 124 Double Beam Grating Spectrophotometer.

The infrared spectrum of the flavour concentrates was determined by the Beckman Model IR-20 Double Beam Infrared Spectrophotometer. The diethyl ether (solvent) from the flavour concentrates was removed *in vacuo* and at 30° C. The residue was dissolved in an excess amount of spectral grade chloroform (treated with anhydrous magnesium sulfate). For IR spectrophotometry, the solution was treated again with anhydrous magnesium sulfate, then filtered and concentrated *in vacuo*. The thickness of the potassium bromide cells was 0.1 mm.

2.3.9 Determination of BOD_5

The BOD_5 determination in the WSL samples and

in the culture medium is based on the consumption of dissolved oxygen (DO) within 5 days in 300 ml standard BOD bottles at 20° C in the dark [Standard Methods (1967)]. The filtrates were diluted to the appropriate range (*ca* 10-50 ppm for BOD_5) and were neutralized to about pH 7.0. The dilution ratio was varied for each sample to obtain an approximate 50% depletion of oxygen after 5 days of incubation. The oxygen saturated distilled water containing 0.1% (v/v) of each of the phosphate buffer, magnesium sulfate, calcium chloride, ferric chloride solutions and 1% (v/v) of seed material was used as dilution water for determination. The seed material was the fresh supernatant taken from the aerator in the Greenway Pollution Control Center, London, Ontario, Canada. The DO was measured either by the polarographic method using the W.P.R.L. Type A 1672 Dissolved Oxygen Meter or by the YSI Model 54 Oxygen Meter. The results are averages of at least two separate samples.

2.3.10 Determination of COD

The COD of the WSL samples and the cell-free medium was determined by oxidizing the organic matter with potassium dichromate in a sulfuric acid-silver sulfate solution for 2 hours, and then titrating with ferrous

ammonium sulfate using ferroin indicator. Sulfamic acid is added in order to eliminate the interference of nitrites in the sample. Mercuric sulfate is also used to oxidize straight chain compounds and to eliminate chloride reactions [Standard Methods (1967)]. The samples were diluted to the appropriate range (*ca* 200-500 ppm) and analyses were always duplicated.

2.3.11 Determination of Total Organic Carbon

For the determination of total organic carbon (TOC), samples of WSL (0.1 ml) or cell-free media (0.4 ml) were evaporated to dryness at room temperature and were analyzed by the Coleman Model 33 Carbon-Hydrogen Analyzer. All combustible carbon was converted into carbon dioxide which was determined gravimetrically.

2.3.12 Determination of Sulfur Dioxide

The amount of SO_2 in the WSL was determined by the standard iodometric method. The sample was treated with an excess of I_2/KI solution, then the remaining iodine was titrated with a standard $Na_2S_2O_3$ solution.

CHAPTER 3

PRELIMINARY RESULTS AND DISCUSSION

3.1 Characteristics of Cultivation Process

3.1.1 Effect of Initial pH

Studies on the optimal initial pH were performed with four species of morels, *M. crassipes*, *M. deliciosa*, *M. esculenta*, and *Morchella* spp. on various WSL (NH_3-, Ca-, Mg-, mixed-, and Na-WSL) in 200 ml/500ml Erlenmeyer flasks within the pH range of 4.0 to 8.0. Early experiments in shake flasks using concentrated WSL as a culture medium were unsuccessful. Therefore the WSL was diluted in order to reduce the apparent toxicity level of the liquor. The stock culture medium was diluted to 1:10 (v/v) and used as the culture medium for the studies on the effects of initial pH. The results for NH_3-, Mg-, and Na-WSL are shown in Figs. 3.1 to 3.10 and Tables B.1 to B.10.

The dilution ratio, yield and efficiency are defined as,

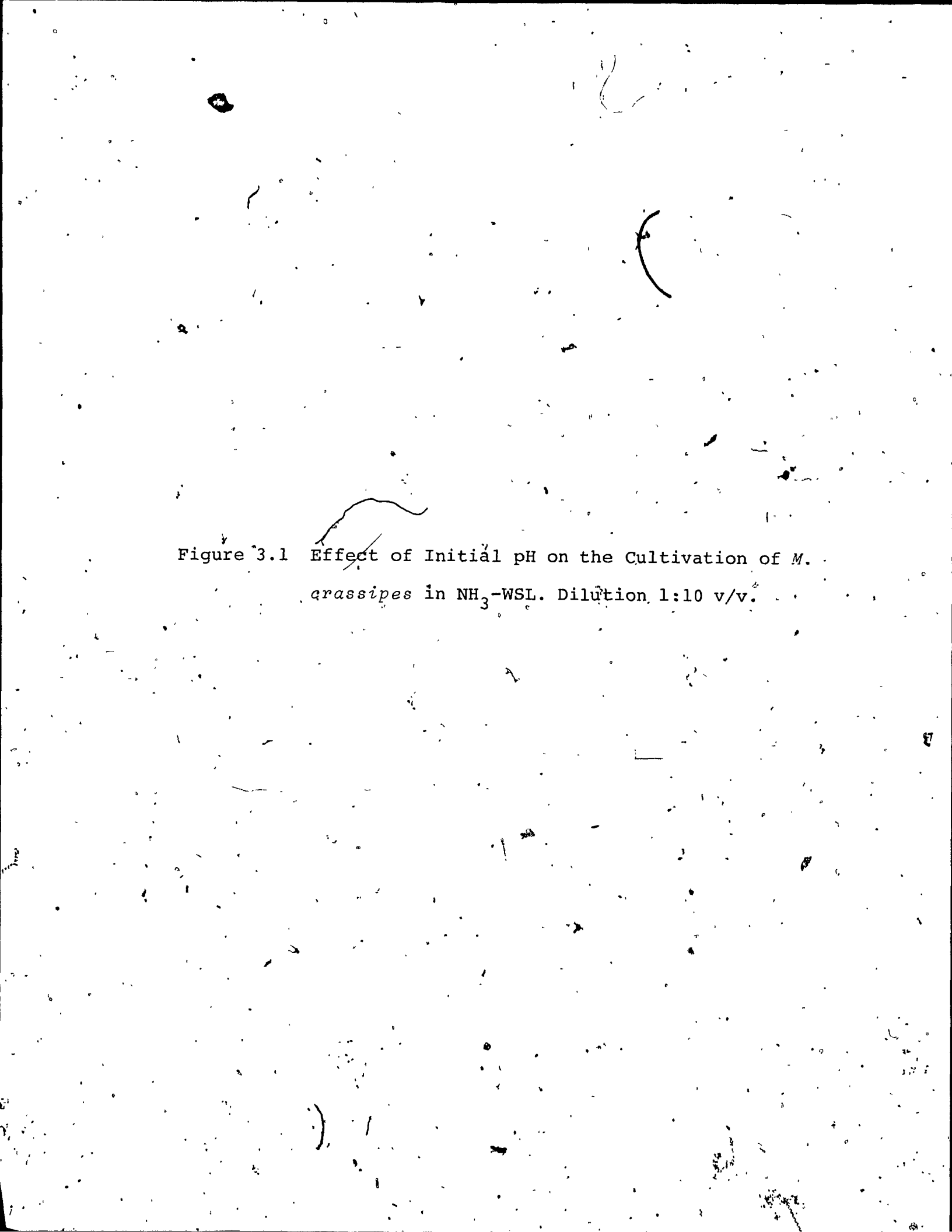


Figure 3.1 Effect of Initial pH on the Cultivation of *M. grassipes* in NH_3-WSL. Dilution 1:10 v/v.

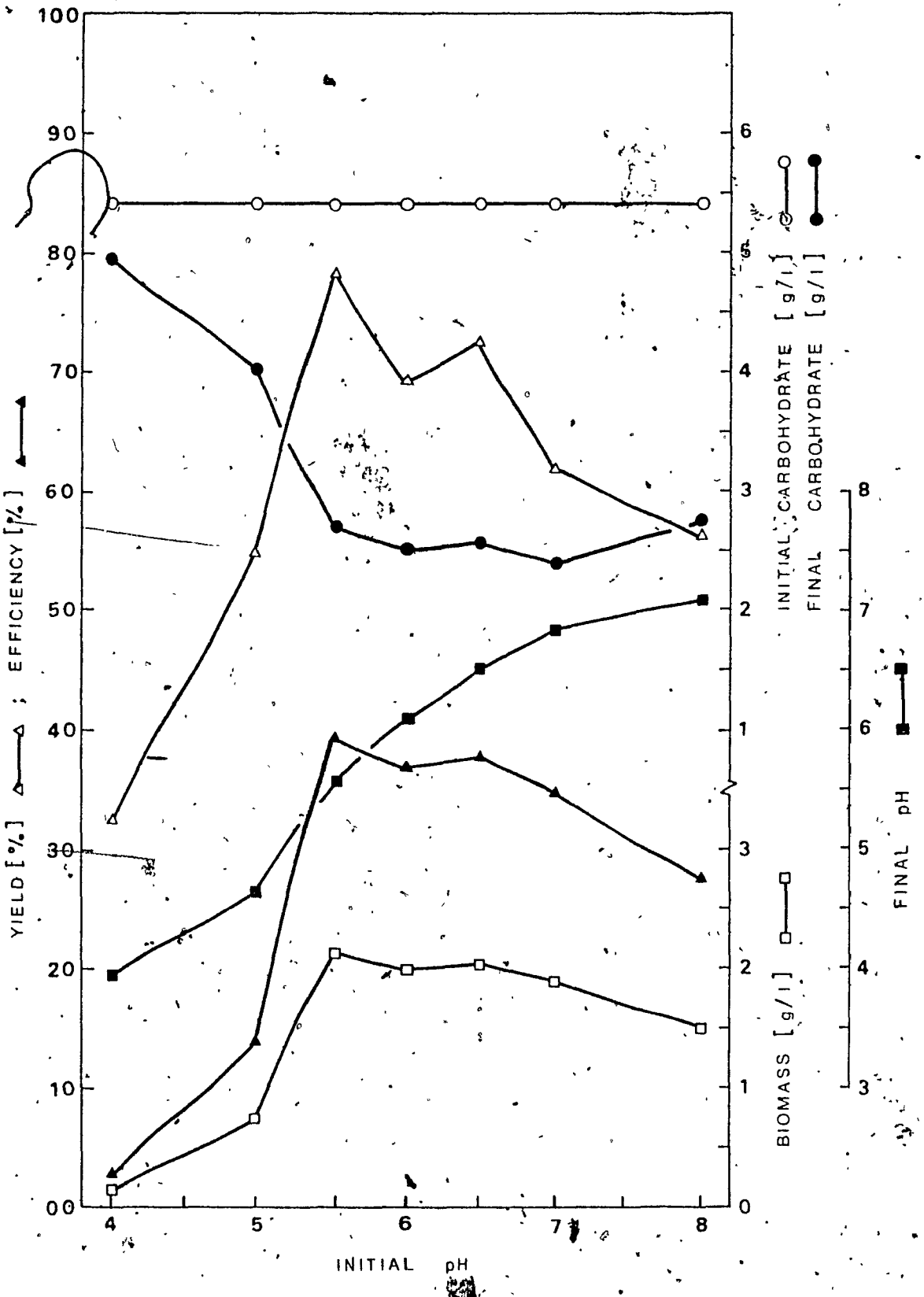

YIELD [%] ; EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

Figure 3.2 Effect of Initial pH on the Cultivation of *M. deliciosa* in NH_3-WSL. Dilution 1:10 v/v.

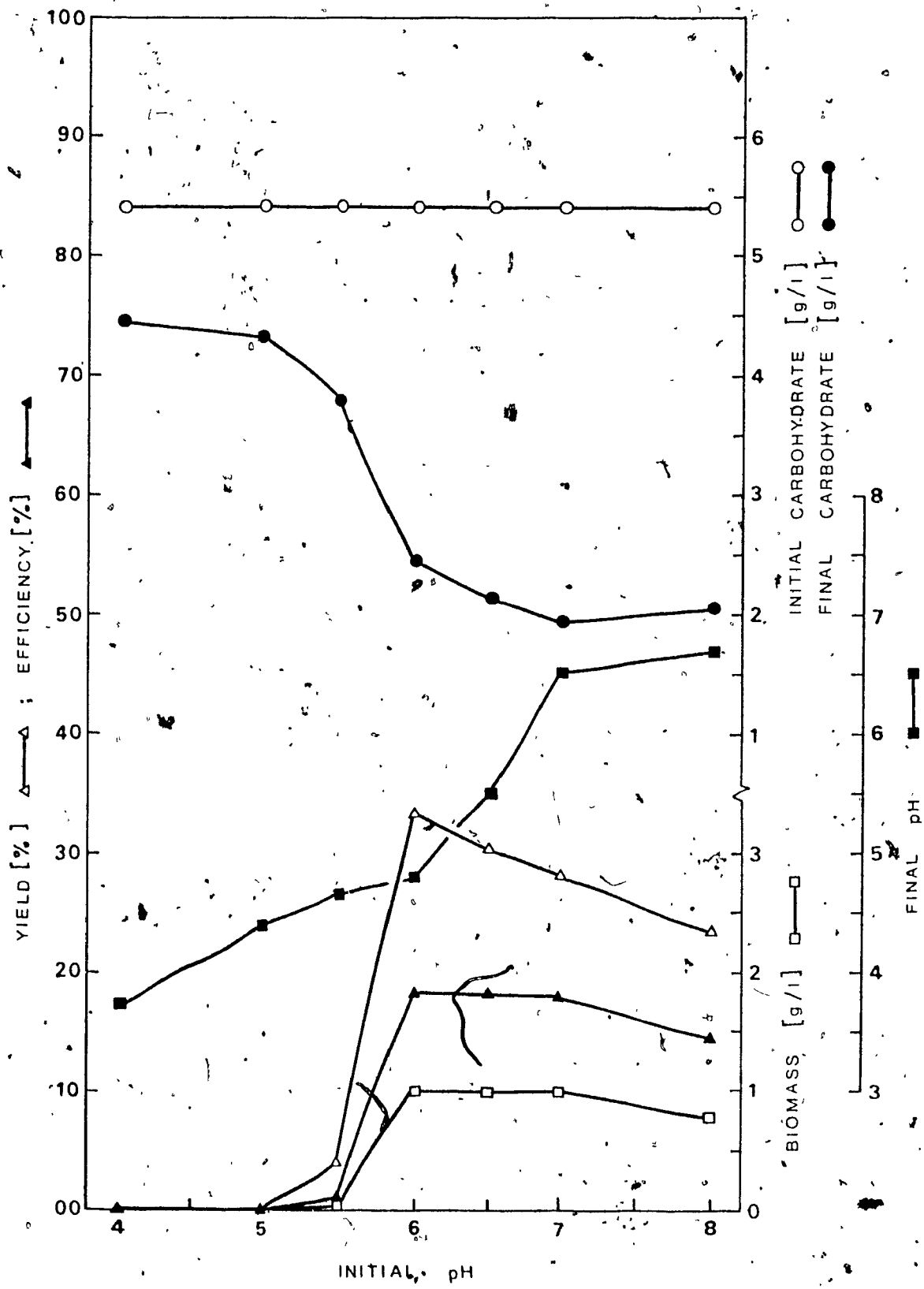
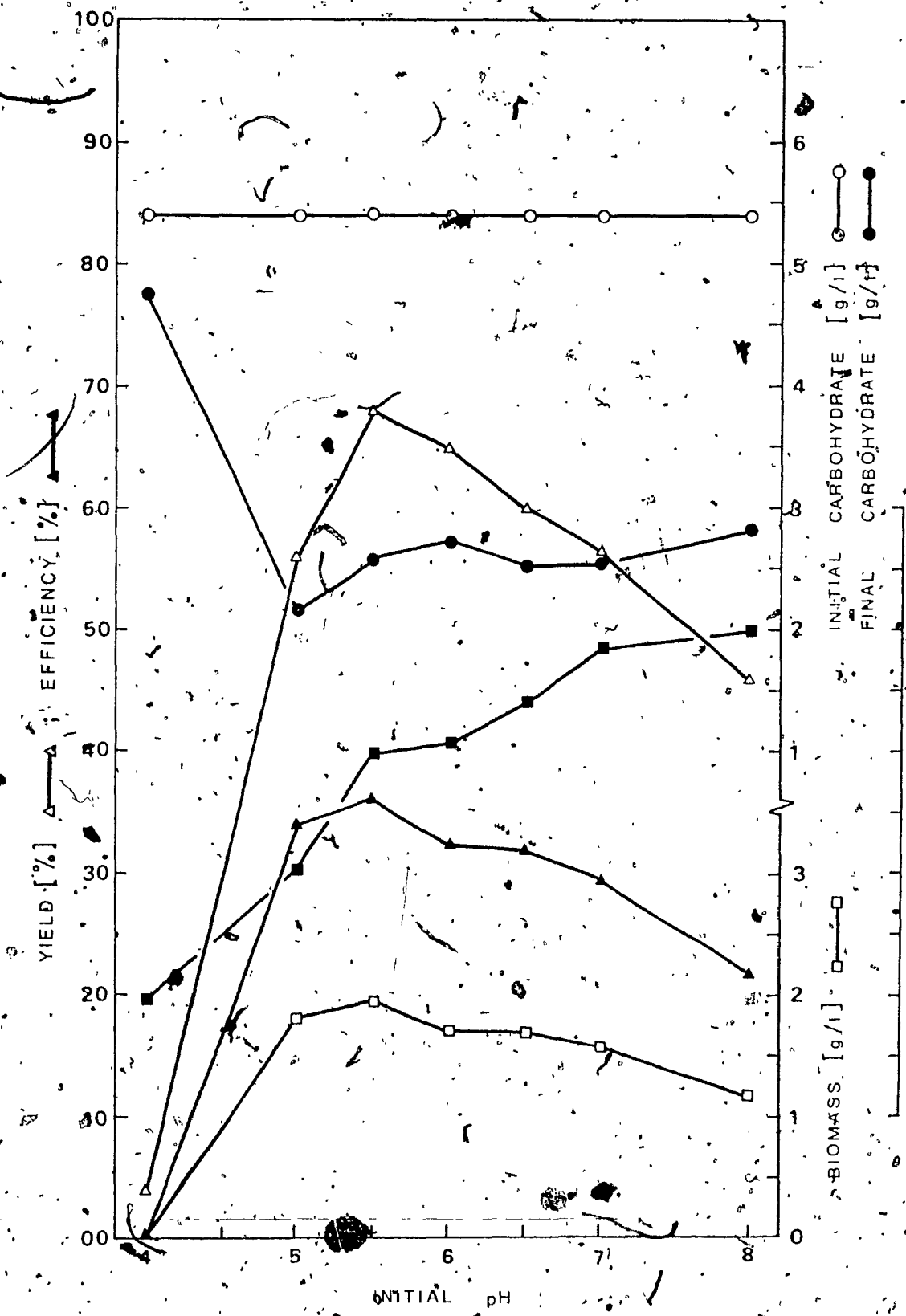

YIELD [%] ; EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

Figure 3.3 Effect of Initial pH on the Cultivation of *M. esculenta* in NH_3-WSL. Dilution 1:10 v/v.


YIELD [%]
EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

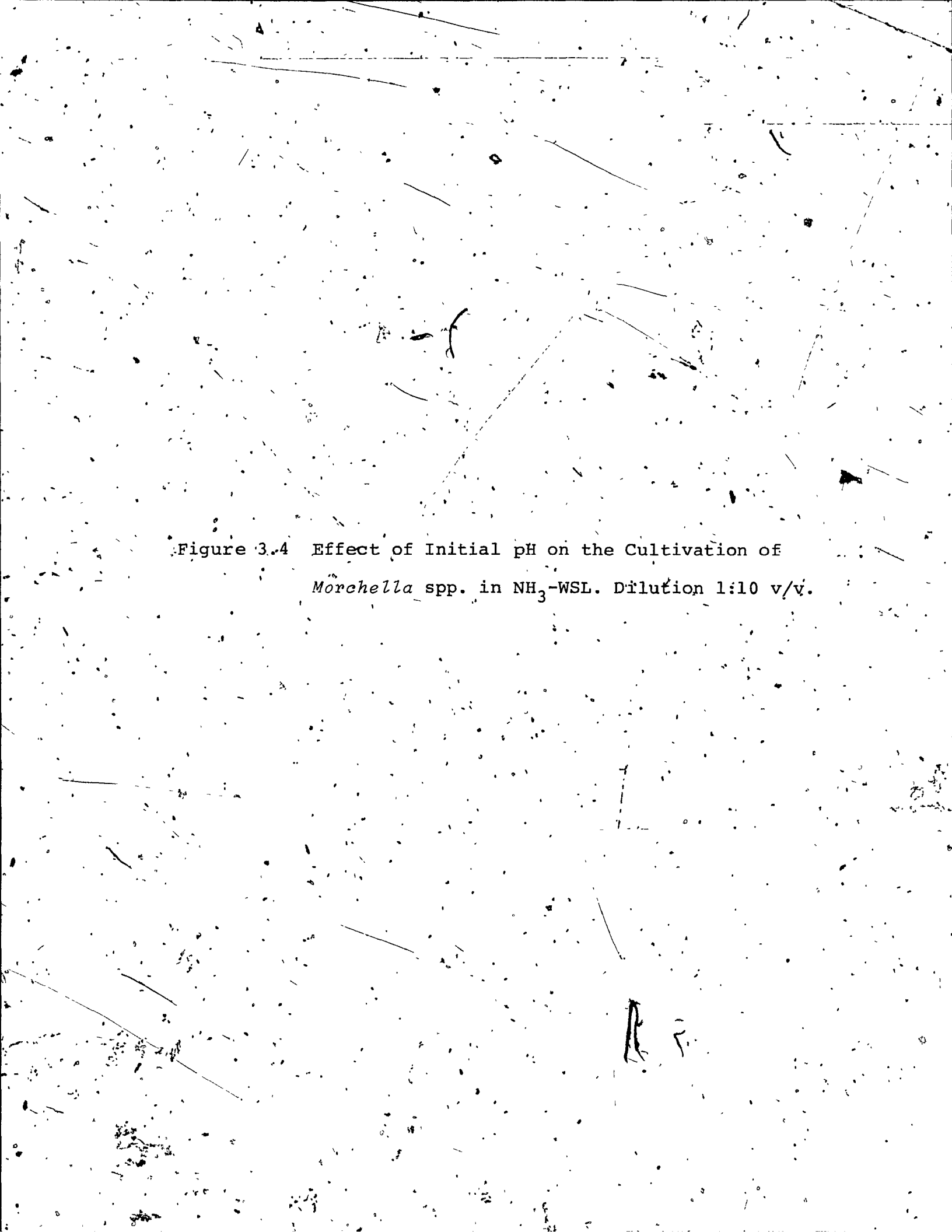
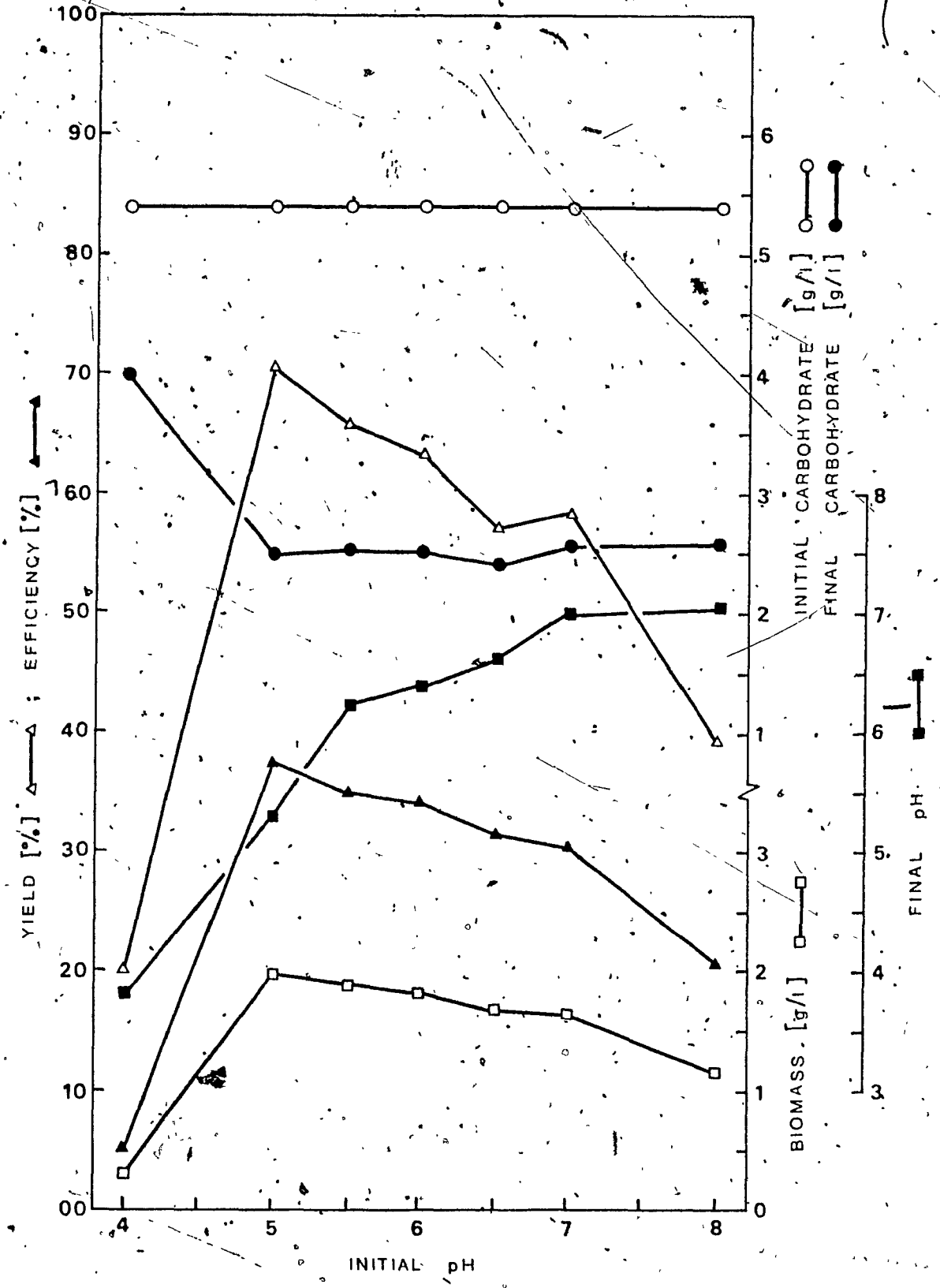


Figure 3.4 Effect of Initial pH on the Cultivation of *Morchella* spp. in NH_3-WSL. Dilution 1:10 v/v.


YIELD [%] △ ; EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

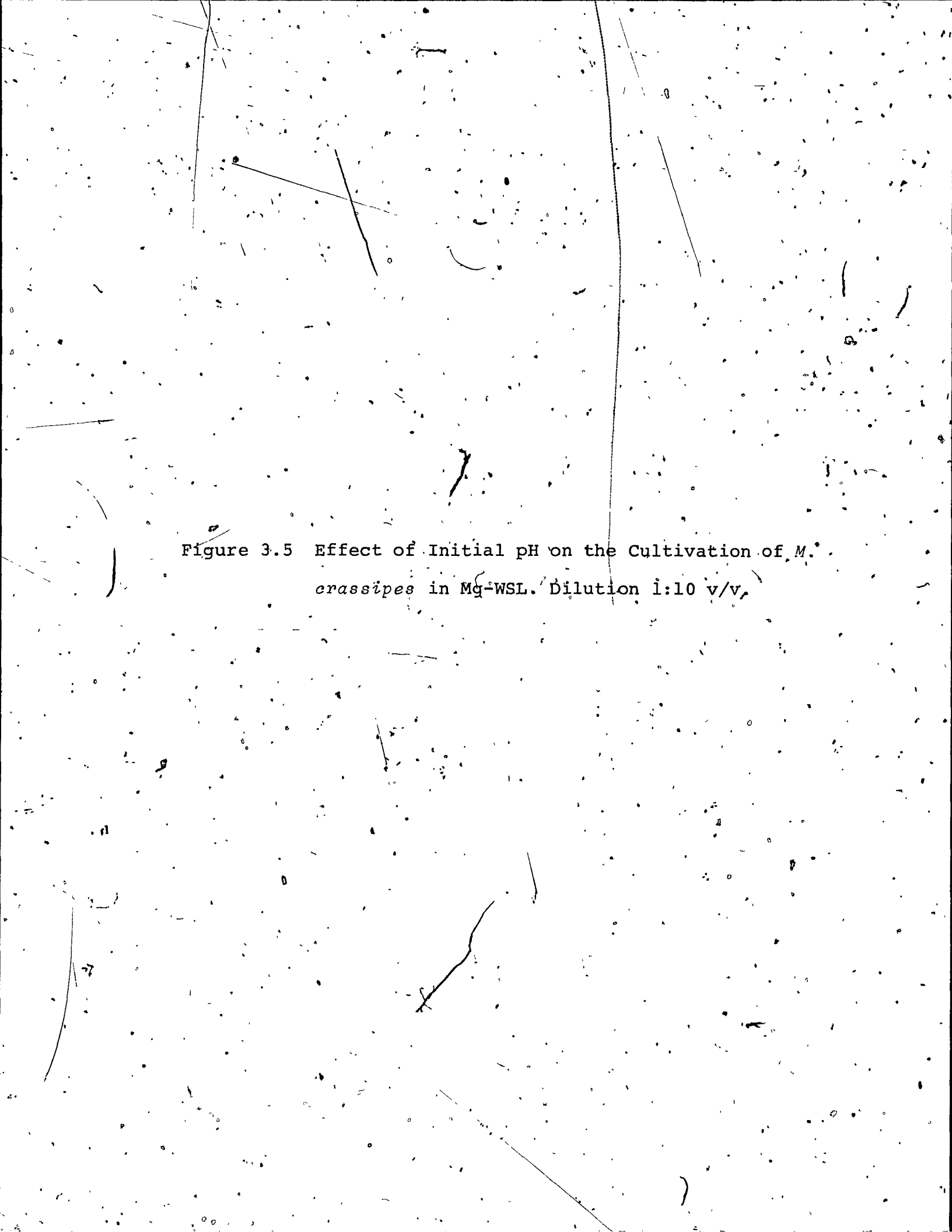
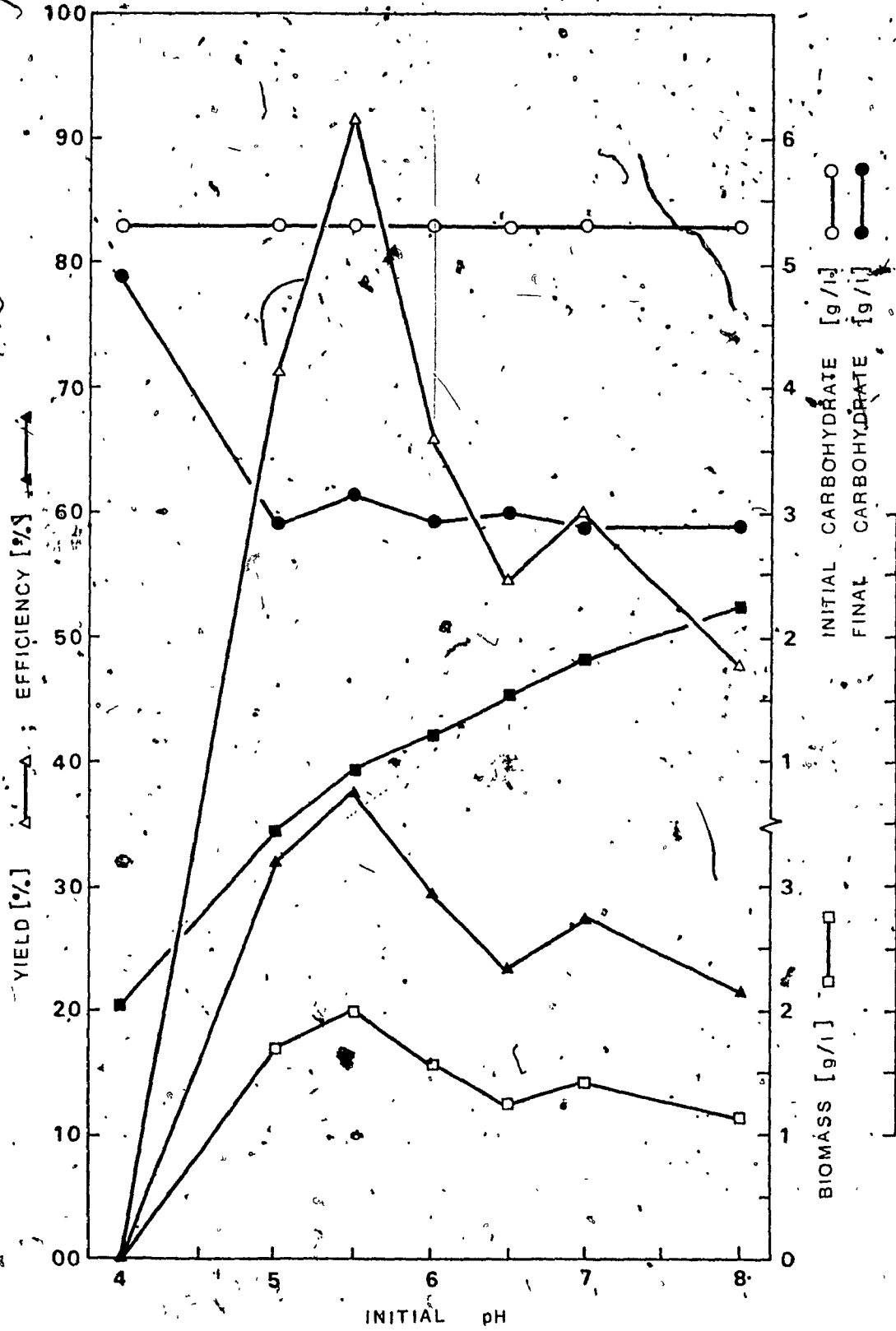


Figure 3.5 Effect of Initial pH on the Cultivation of *M. crassipes* in Mg-WSL. Dilution 1:10 v/v.


YIELD [%] ; EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

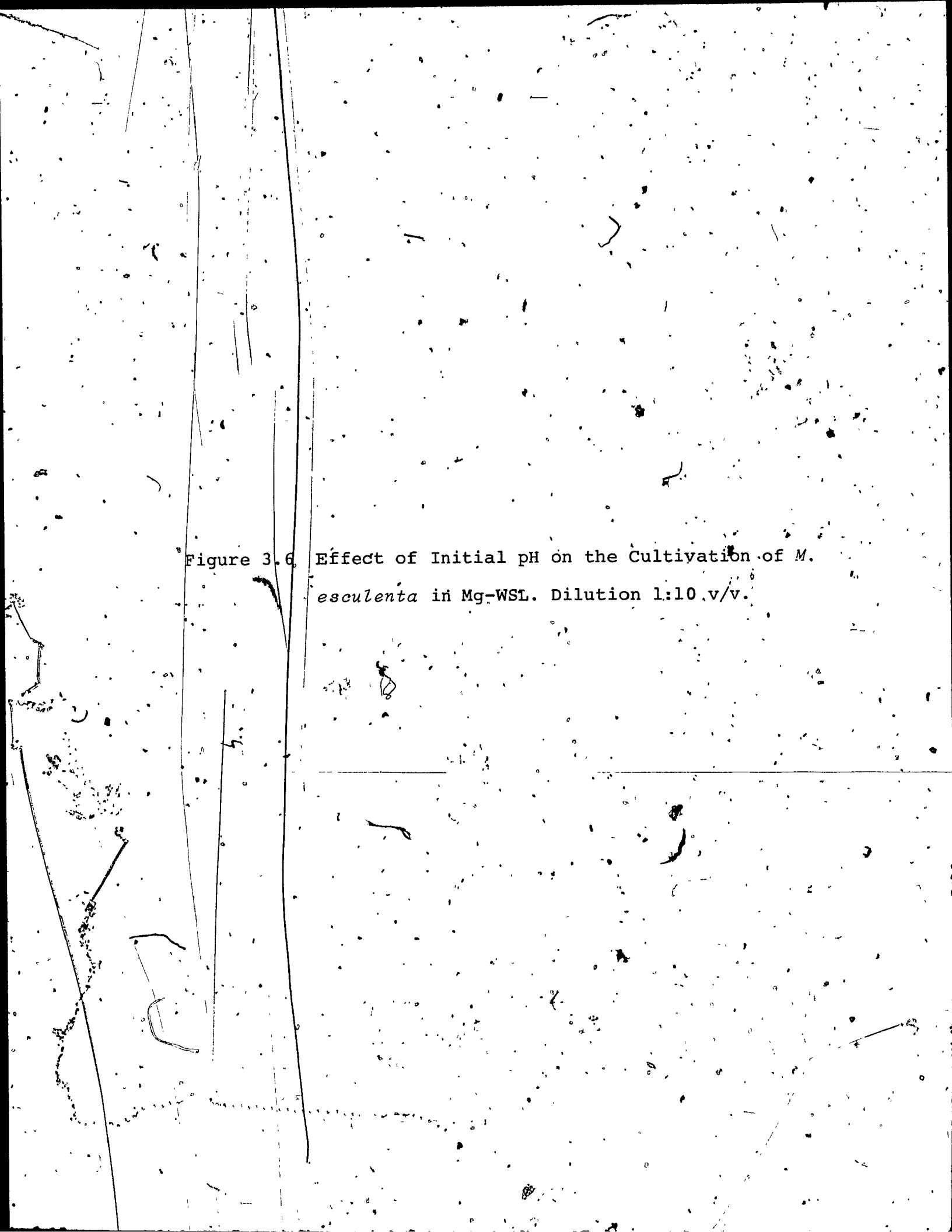


Figure 3.6 Effect of Initial pH on the Cultivation of *M. esculenta* in Mg-WSL. Dilution 1:10 v/v.

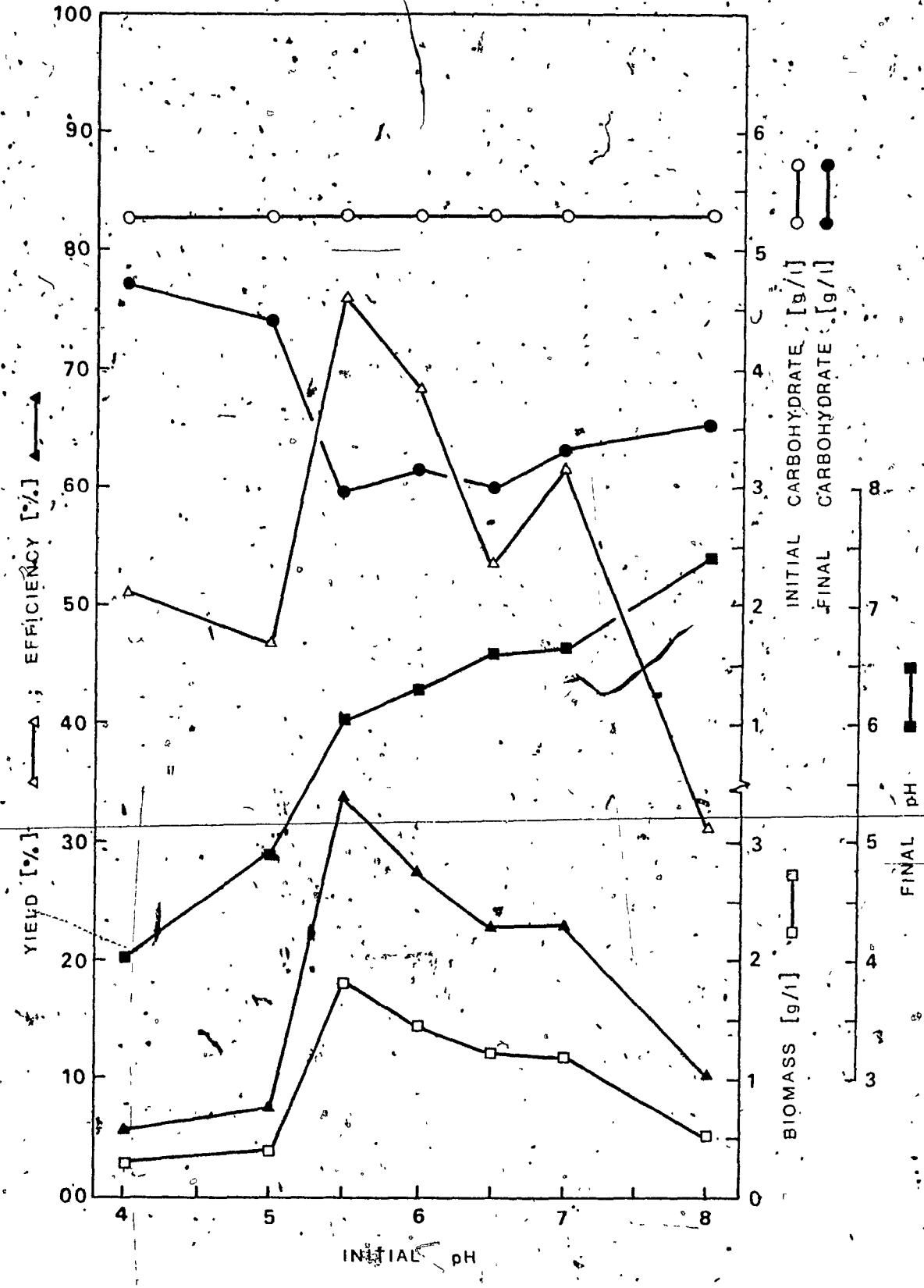
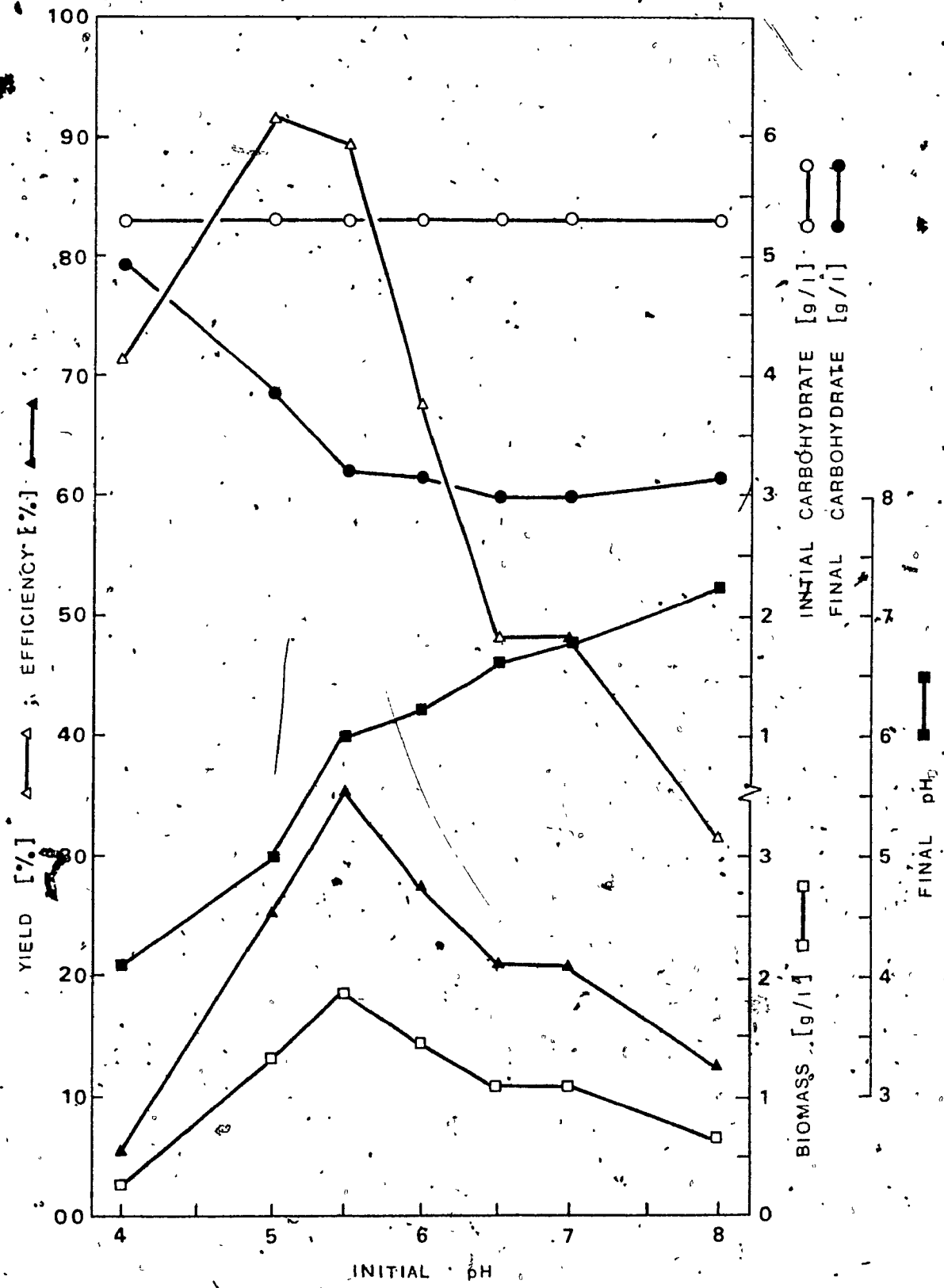

EFFICIENCY [%]
YIELD [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

Figure 3.7 Effect of Initial pH on the Cultivation of *Morchella* spp. in Mg-WSL, Dilution 1:10 v/v.


YIELD [%] △ EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l] □
FINAL pH ■
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

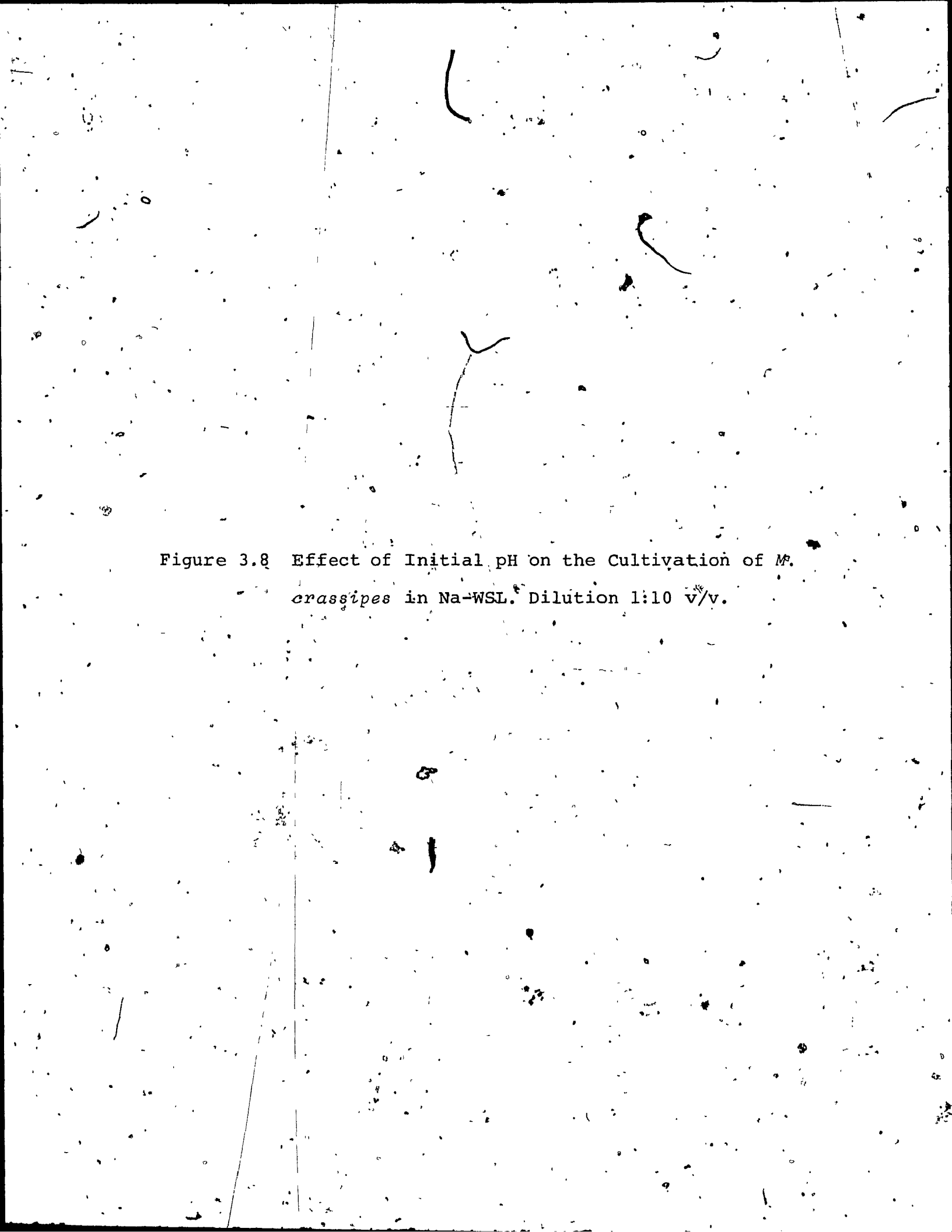


Figure 3.8 Effect of Initial pH on the Cultivation of *M. crassipes* in Na-WSL. Dilution 1:10 v/v.

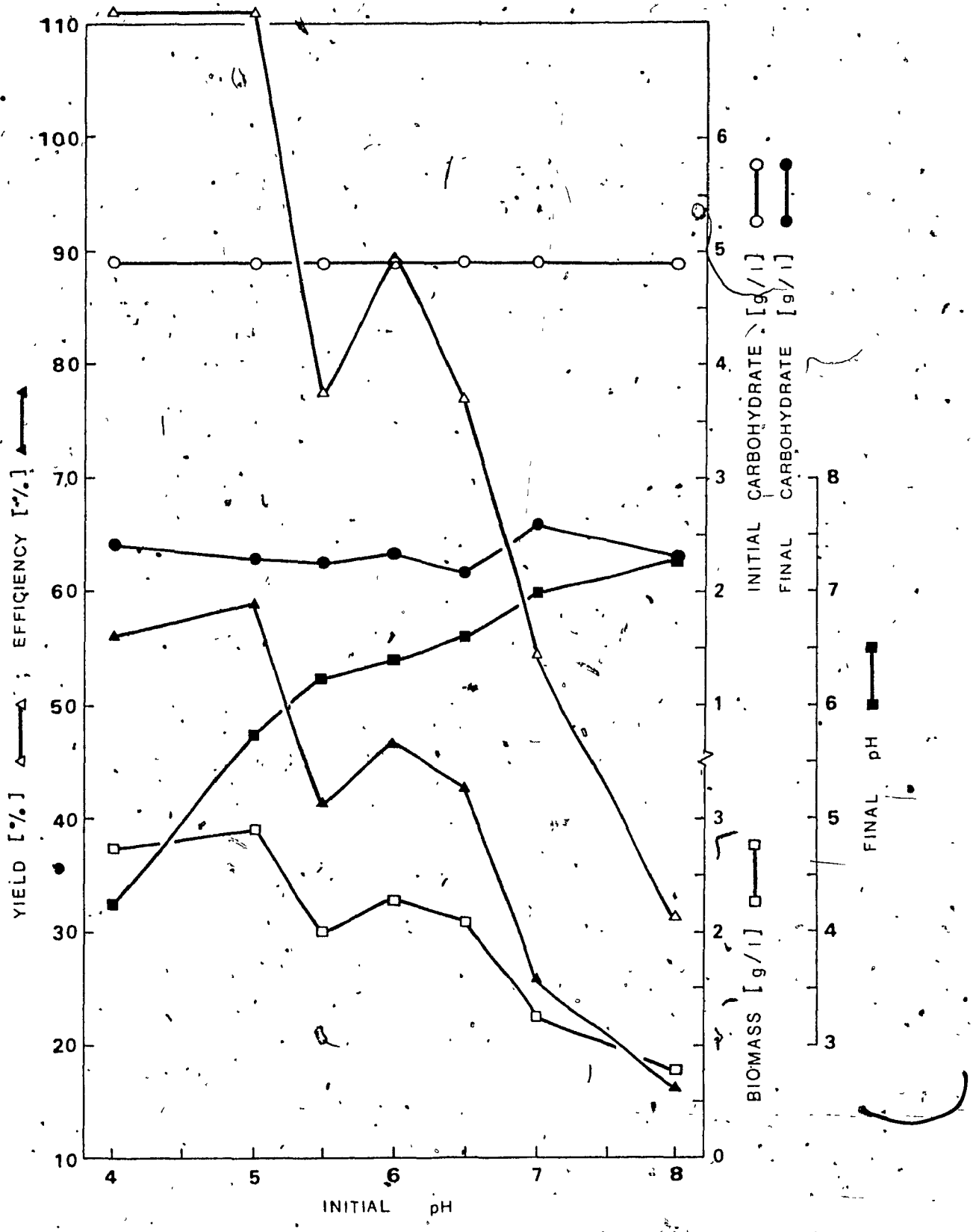
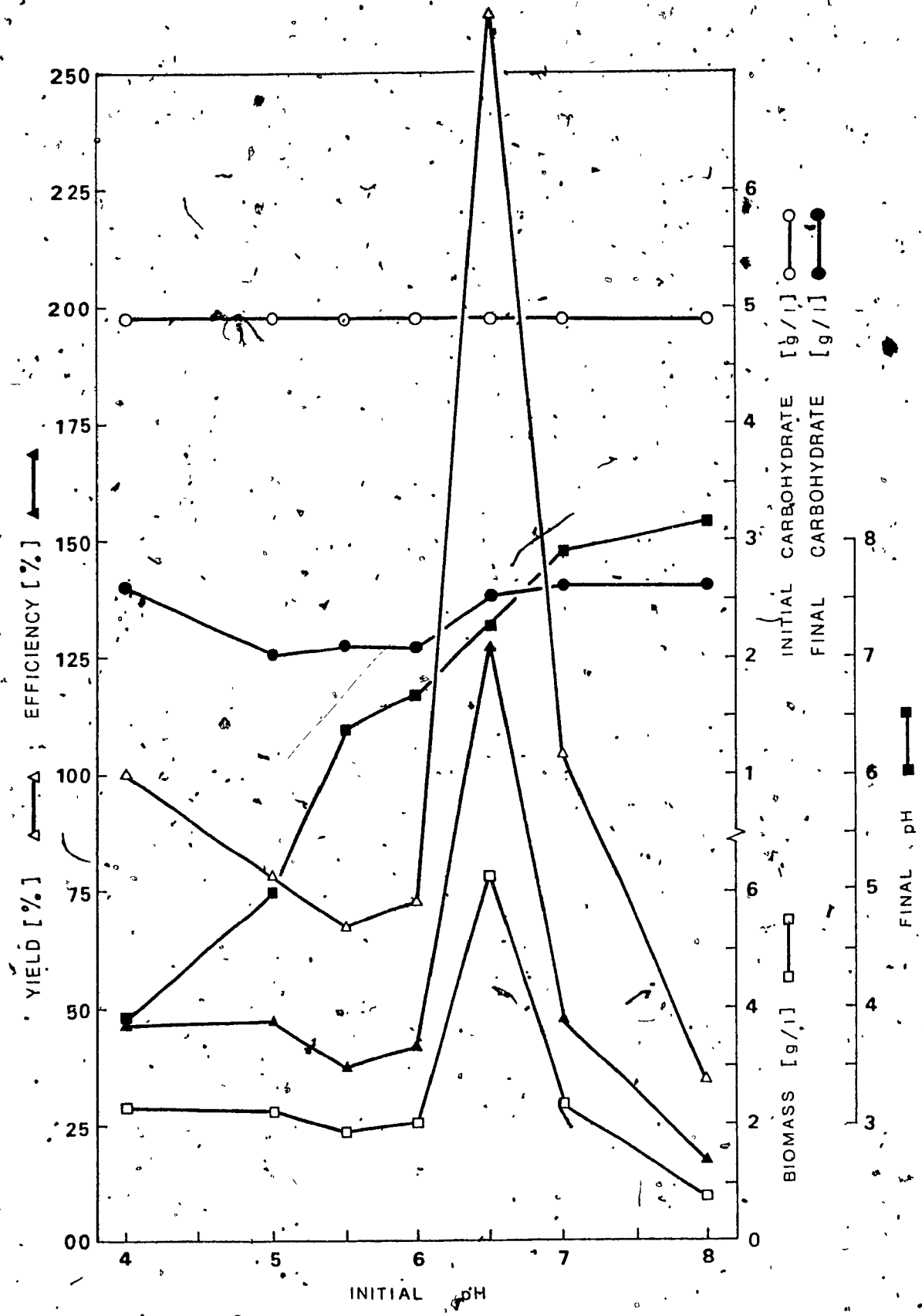

YIELD [%] ; EFFICIENCY [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
110
100
90
80
70
60
50
40
30
20
10
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

Figure 3.9 Effect of Initial pH on the Cultivation of *M. esculenta* in Na-WSL. Dilution 1:10 v/v.


250
225
200
175
150
125
100
75
50
25
00
YIELD [%]
EFFICIENCY [%]
6
5
4
3
2
1
0
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
8
7
6
5
4
3
FINAL pH
4
5
6
7
8
INITIAL pH

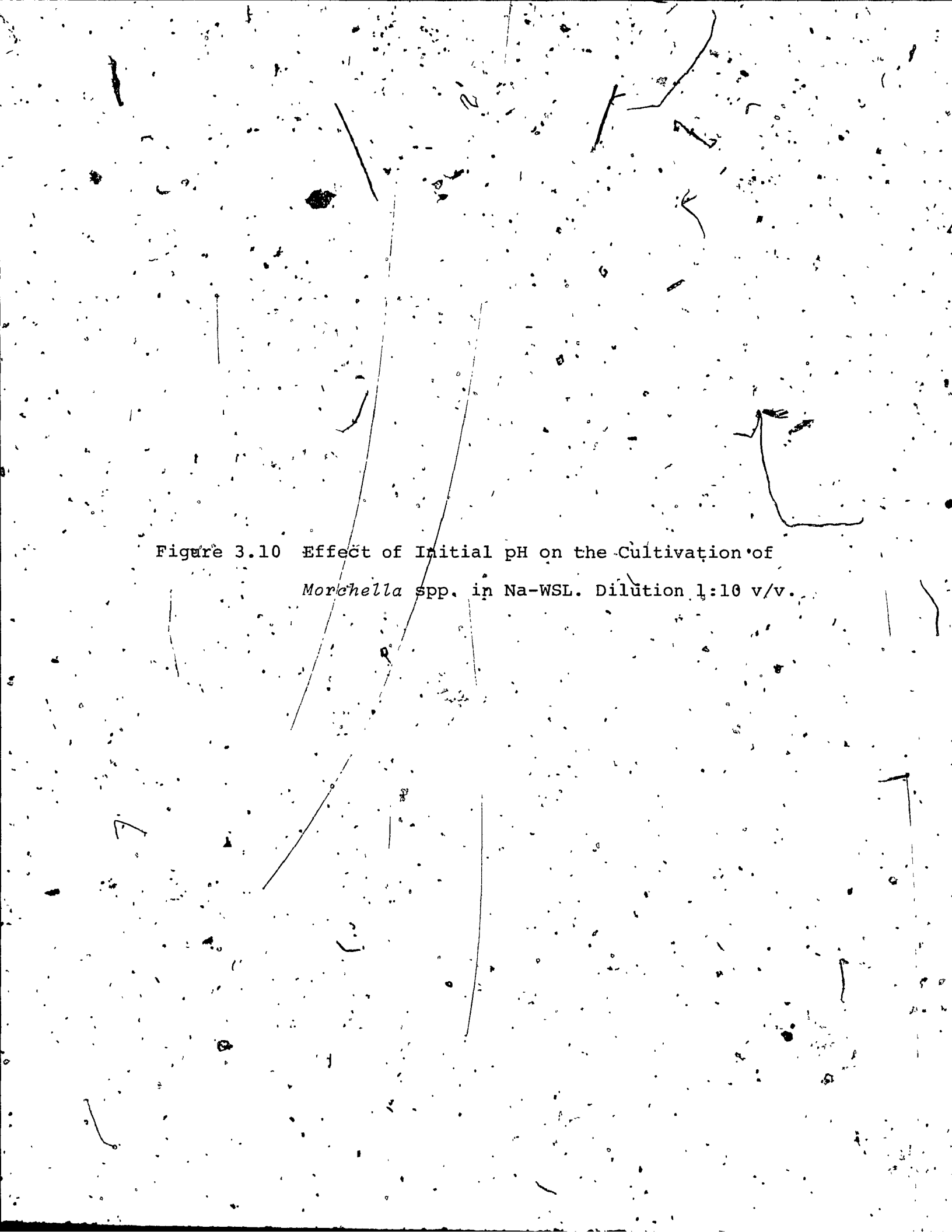
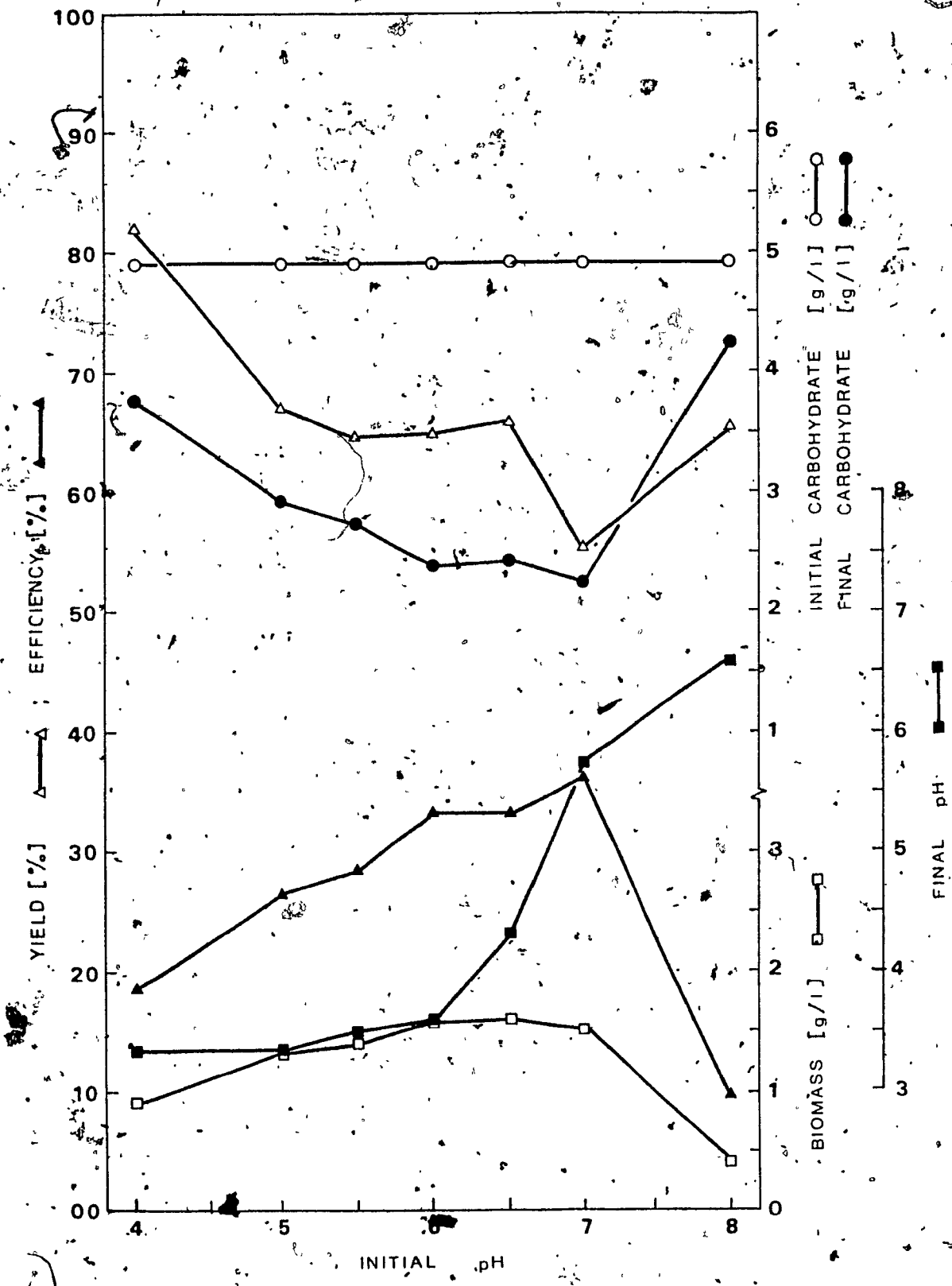


Figure 3.10 Effect of Initial pH on the Cultivation of *Morchella* spp. in Na-WSL. Dilution 1:10 v/v.


EFFICIENCY [%]
YIELD [%]
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
BIOMASS [g/l]
FINAL pH
INITIAL pH
100
90
80
70
60
50
40
30
20
10
00
6
5
4
3
2
1
0
8
7
6
5
4
3
4
5
6
7
8

Dilution ratio 1:x (v/v) = 1 volume of concentrated WSL

+

(x-1) volume(s) of distilled water (3.1)

$$\text{Yield [\%]} = \frac{\text{g of biomass produced}}{\text{100g of TCH utilized}} \quad (3.2)$$

$$\text{Efficiency [\%]} = \frac{\text{g of biomass produced}}{\text{100g of TCH supplied}} \quad (3.3)$$

It should not be confused the dilution ratio defined in Eq. (3.1) with the dilution rate which is associated with the continuous cultivation experiment (Chapters 4 and 5). The dilution rate is defined as,

$$\text{Dilution rate [time}^{-1}\text{]} = \frac{\text{Feed rate [volume/time]}}{\text{Reactive volume of fermentor [volume]}} \quad (3.4)$$

It was found that none of the four species of morels grew in Ca-WSL nor in the mixed-WSL even though high dilution ratios were applied. This fact may be due to the inhibition of growth by calcium compounds which are present in both of these WSL. *M. crassipes*, *M. esculenta*, and *Morchella* spp. grew well on NH_3-, Mg-, and Na-WSL,

while *M. deliciosa* grew very slowly in NH_3-WSL and yielded only a trace of mycelia in the Mg-WSL. Therefore *M. deliciosa* is excluded in all following experiments due to its slow growth rate.

The choice of optimal pH for these cultivations was based on the criterion of compromise between the yield (%) and the dry weight (g/l) of MMM, because their optimal values are not always at the same initial pH. The optimal initial pH's distribute regularly from 5.0 to 7.0, depending upon the species of morels and the WSL used. It is unusual that the initial pH below 5.0 seems to be the best for both yield (111.53%) and dry weight (2.900 g/l) of *M. crassipes* in Na-WSL. The highest yield (263.40%) and dry weight of biomass (6.269 g/l) was obtained with *M. esculenta* in Na-WSL at initial pH 6.5. A yield of this magnitude is thermodynamically impossible. Thus the data show that making such a calculation based on utilization of TCH does not adequately show the energy values utilized in the waste. There must other energy values being converted into biomass *eg.* resin and fatty acids, eugenols and *iso*-eugenols, etc. Very little or no effect of compositional differences between the NH_3-WSL and the Mg-WSL on the optimal initial pH were observed.

However, reports from previous workers confirmed that the optimal initial pH is specific for each species and each culture medium under particular cultivation con-

ditions. Fron (1905) reported that the required pH for growth of *M. conica*, *M. vulgaris* var. *flava*, and *M. esculenta* in Knop and Raulin medium containing 5% sugar should be neutral or slightly alkaline. The optimal pH range for *M. hortensis* growing in a glucose medium was found to be 5.5 to 6.5 by Litchfield *et al.* (1963a). However, Brock (1951) obtained the highest yield of *M. esculenta* in a glucose-ammonium chloride medium at pH 8.4 while Szuecs (1956) reported the best yield of the same species in a glucose synthetic medium at pH 6.5.

It is to be noted that from Figs. 3.1 to 3.10, two maximum values for dry weight were frequently encountered in the pH range from 4.0 to 8.0. This bimodal phenomenon confirms the results obtained by Brock (1951) with *M. esculenta* when cultured on glucose-sodium nitrate medium at different initial pH values. Brock attributed the bimodal curve to the effect of pH on the fungus isoelectric point and the minimum point between two maxima was interpreted as the isoelectric point for the cell colloids.

3.1.2 Effect of Dilution Ratio

An experiment on the effects of dilution ratio on the growth of *M. crassipes* and on the reduction of TCH in NH_3-WSL at optimal initial pH of 6.0 was conducted in

300 ml/1,000 ml Erlenmeyer flasks. Different dilutions of the stock culture medium and the results are shown in Fig. 3.11 and Table B.11.

The dilution ratio 1:5 (v/v) for NH_3-WSL was found to be the best for the production of biomass (4.856 g/l), reduction of TCH (53.8% based on the initial concentration of TCH of 10.82 g/l), yield (83.44%) and efficiency (44.88%). The utilization of TCH was higher at higher dilution ratios, however, in the corresponding biomass produced, yield and efficiency were lower at these values.

The biomass produced, yield and efficiency decreased for dilution ratios being higher or lower than 1:5 (v/v). The decrease in yield at a lower concentration of WSL suggested that the residual TCH primarily represents non-assimilable sugars. No growth took place in the concentrated WSL which is an indication of growth inhibition due to the toxicity of this liquor. The growth inhibition might be due to the presence of sulfur dioxide, sulfonic acid radicals, lignosulfonates and polyphenolic components in the medium which are known to be toxic for many fungi. Cirillo *et al.* (1960) claimed an unsuccessful growth of *M. crassipes* in concentrated WSL. The low yield (52.7% based on reducing sugars) of *M. hybrida* grown in diluted WSL was also reported by Reusser *et al.* (1958).

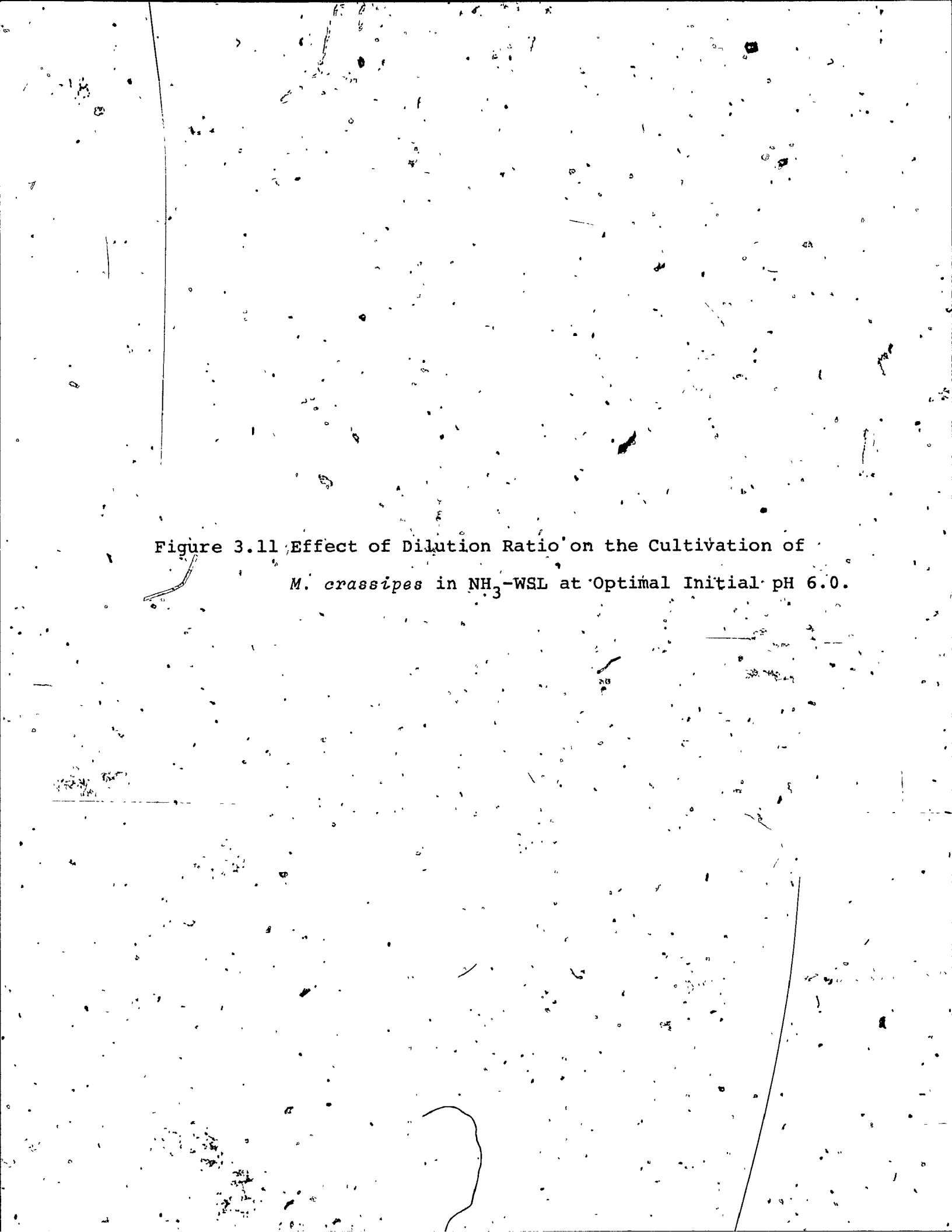
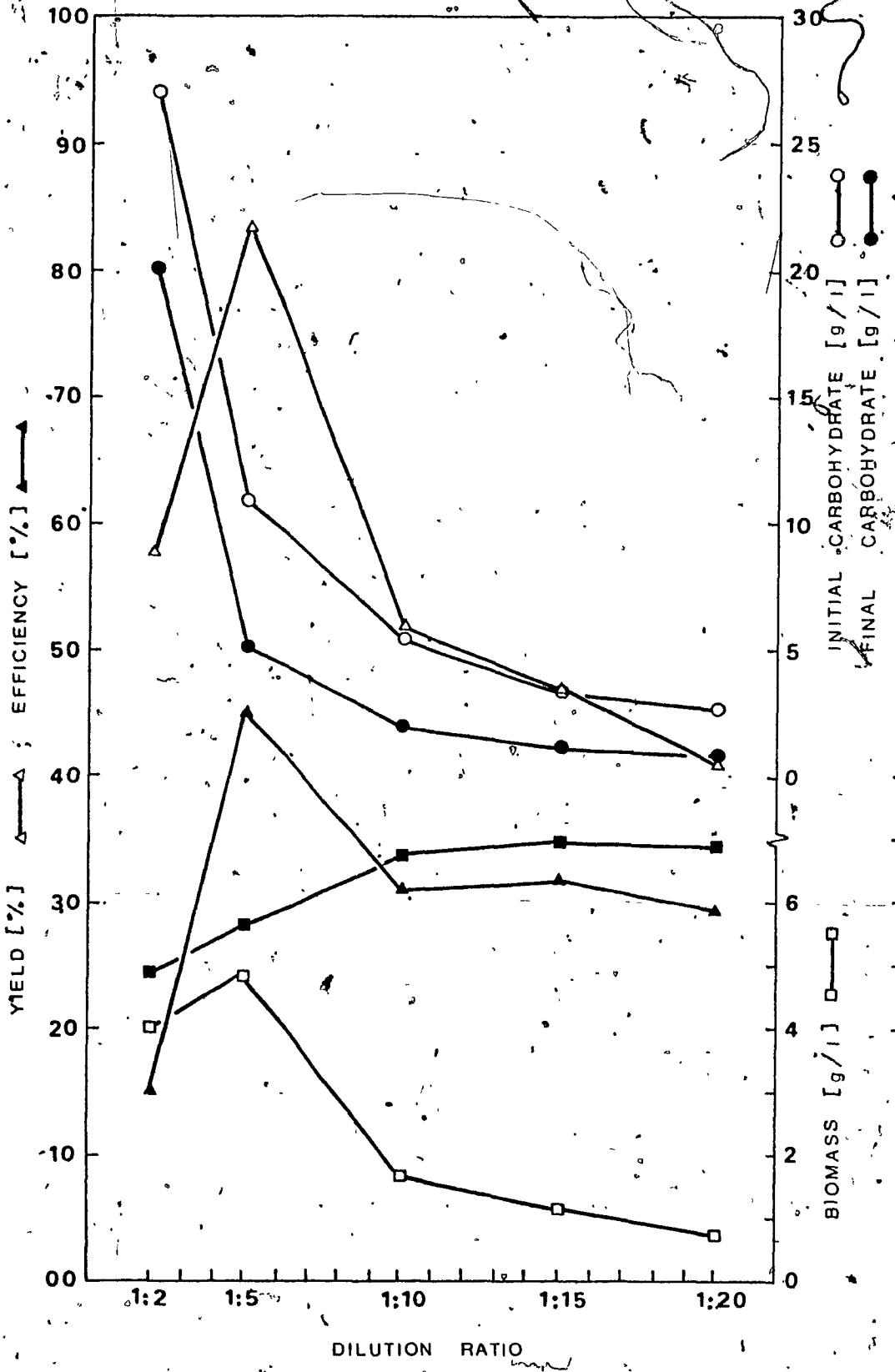


Figure 3.11 Effect of Dilution Ratio on the Cultivation of *M. crassipes* in NH_3-WSL at Optimal Initial pH 6.0.


100
90
80
70
60
50
40
30
20
10
00
YIELD [%] ; EFFICIENCY [%]
30
25
20
15
10
5
0
INITIAL CARBOHYDRATE [g/l]
FINAL CARBOHYDRATE [g/l]
7
6
5
FINAL pH
6
4
2
0
BIOMASS [g/l]
1:2
1:5
1:10
1:15
1:20
DILUTION RATIO

It should be noted that at the optimal conditions for initial pH and dilution ratio, the utilization of 53.8% of initial TCH (which is composed of assimilable and non-assimilable sugars) implies the reduction of BOD_5 of the NH_3-WSL by a value higher than 53.8%.

3.1.3 Effect of SO_2

The stripping of SO_2 in NH_3- and Mg-WSL was not necessary and all experiments were performed using these liquors as received. However, later experiments conducted in non-stripped Na-WSL were unsuccessful even at very high dilution ratios. The SO_2 content in these liquors was found to be 0.047% for the NH_3-WSL and 0.282% for the Na-WSL. It was necessary, therefore, to reduce the SO_2 content in the Na-WSL which was done by steam stripping. In this way the SO_2 content was reduced to 0.022% before cultivation. The SO_2 stripping was also used by Cirillo *et al.* (1960) for the cultivation of mushrooms in WSL.

3.1.4 Growth Forms

In shake flask experiments and in the 5-gallon bottle fermentor, growth of MMM took place in form of

pellets (Fig. 3.12). The growth of pellets is shown in Fig. 3.13. If the aeration is vigorous or in the agitated fermentor, the pellet size decreases and growth may take place in a more dispersed form (Fig. 3.14). Litchfield *et al.* (1963a) claimed that the yield of mycelia is lower when the growth is dispersed. Large pellets are preferable because their recovery and purification are simplified. One culture medium containing small pellets of about 1 mm diameter had been left to sediment by gravitation and it was found that all of the pellets settled down within less than 2 hours resulting in a clear supernatant (Fig. 3.15). The settling velocity and the 50% settling time for this test were 0.25 volume of packed biomass/volume of culture medium-min and 15 min respectively. Usually in large fermentors such as the 5-gallon bottles, the pellets are of large size (0.5-1.0 cm diameter) and are expected to be even larger in industrial-scale fermentors. It is, therefore, assumed that even a better settling rate may be obtained in the latter case. This is a great advantage for recovery processes as compared with yeasts or bacteria grown in WSL.

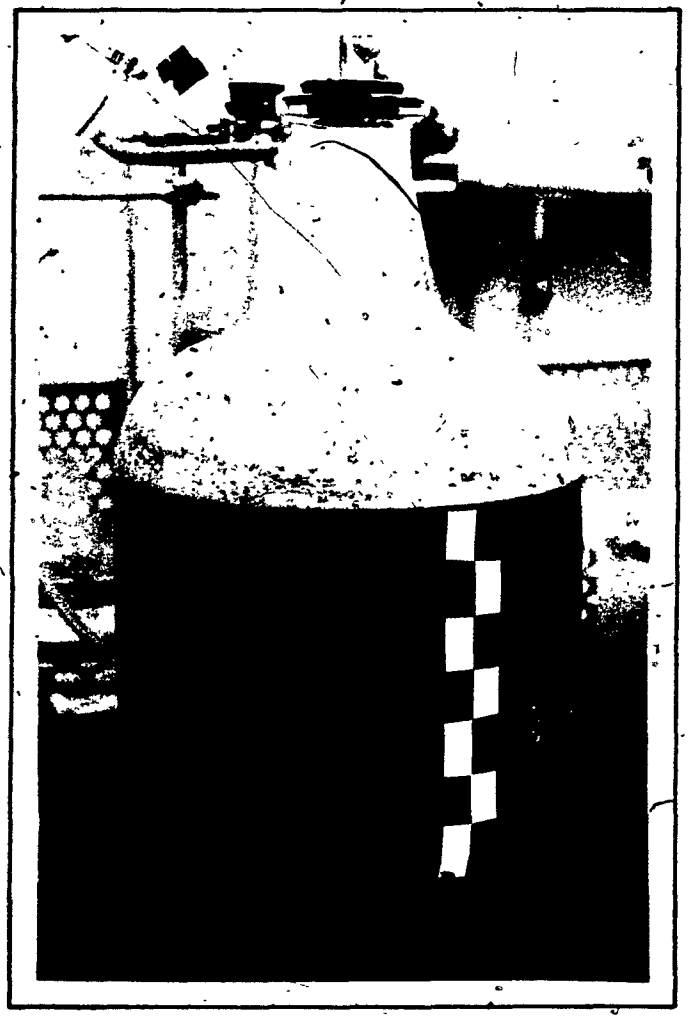
3.2 Characteristics of Product

3.2.1 Proximate Analysis

The chemical composition of the biomass was

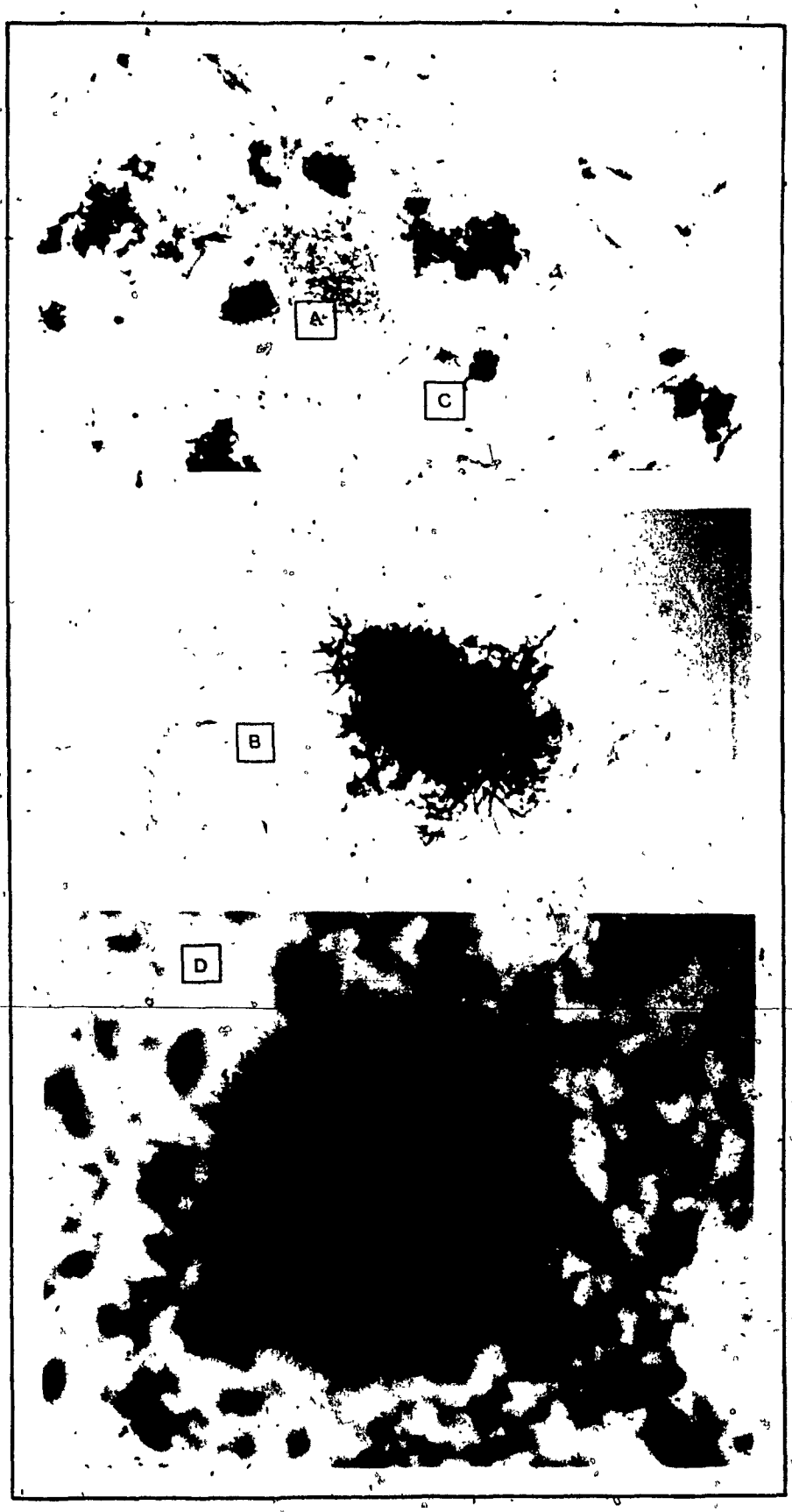
Figure 3.12 Pellets Growth of MMM in WSL in Shake Flask and in 5-Gallon Bottle Fermentor: (A) *M. crassipes* in NH_3-WSL after 17 days of incubation at 200 RPM and room temperature, (B) *M. crassipes* in Mg-WSL after 6 days of incubation at 0.25 vvm of air and room temperature.

A



B

Figure 3.13 Formation of *M. crassipes* Pellets in NH_3-WSL: Small portions of fungal hyphae grew and aggregated into groups of mycelium (A, x1) and then the initial type pellets formed (B, x2.5). Pellets may also be formed from small portions of agar bearing fungal hyphae (C, x1). Young pellet (D, x2.5) of 1 mm in diameter shown many growing tips of hyphae around its peripherical surface.


A
C
B
D

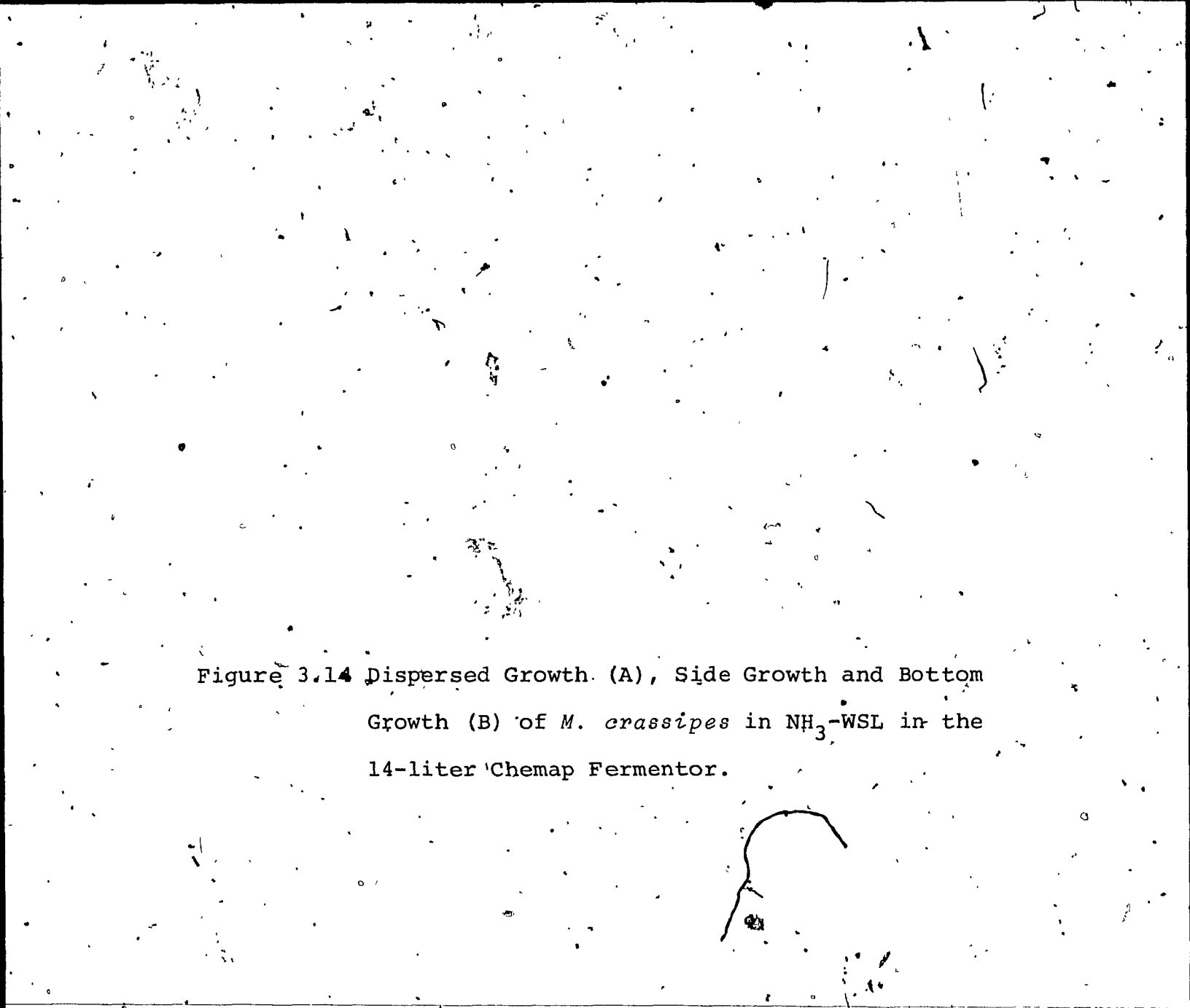
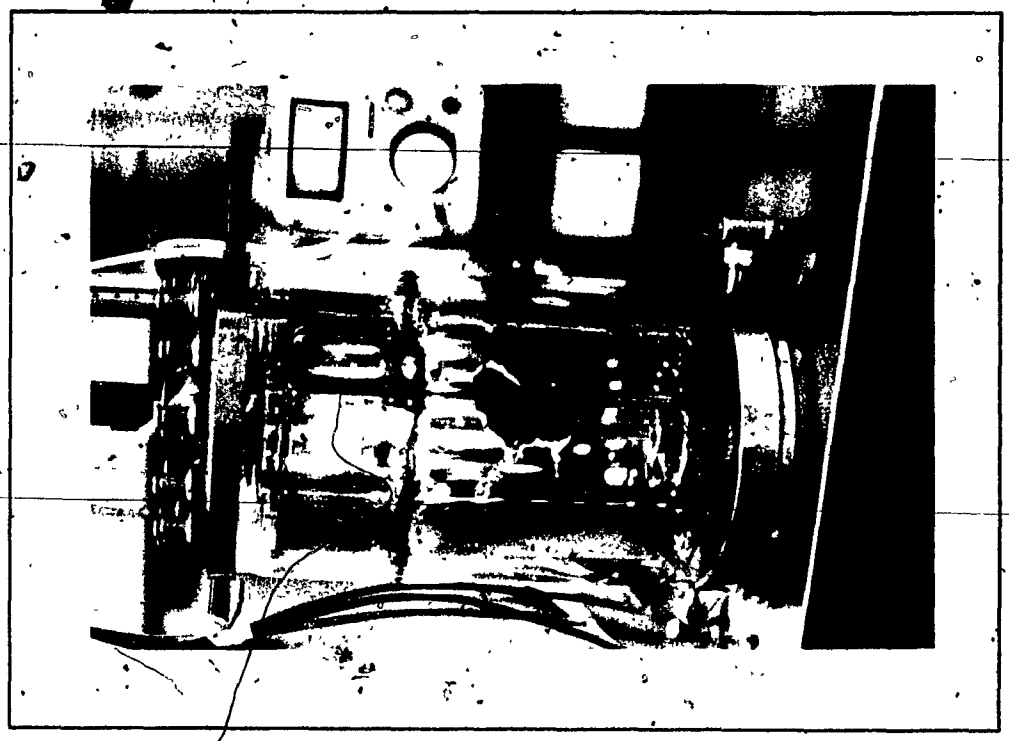
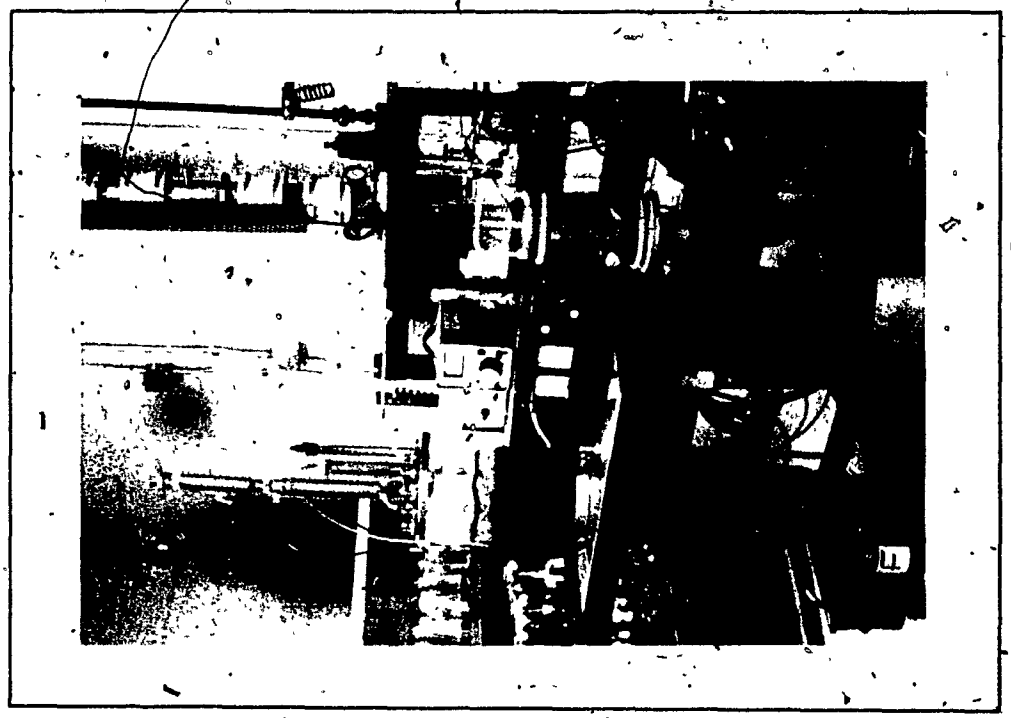


Figure 3.14 Dispersed Growth (A), Side Growth and Bottom Growth (B) of *M. crassipes* in NH_3-WSL in the 14-liter Chemap Fermentor.



B



A

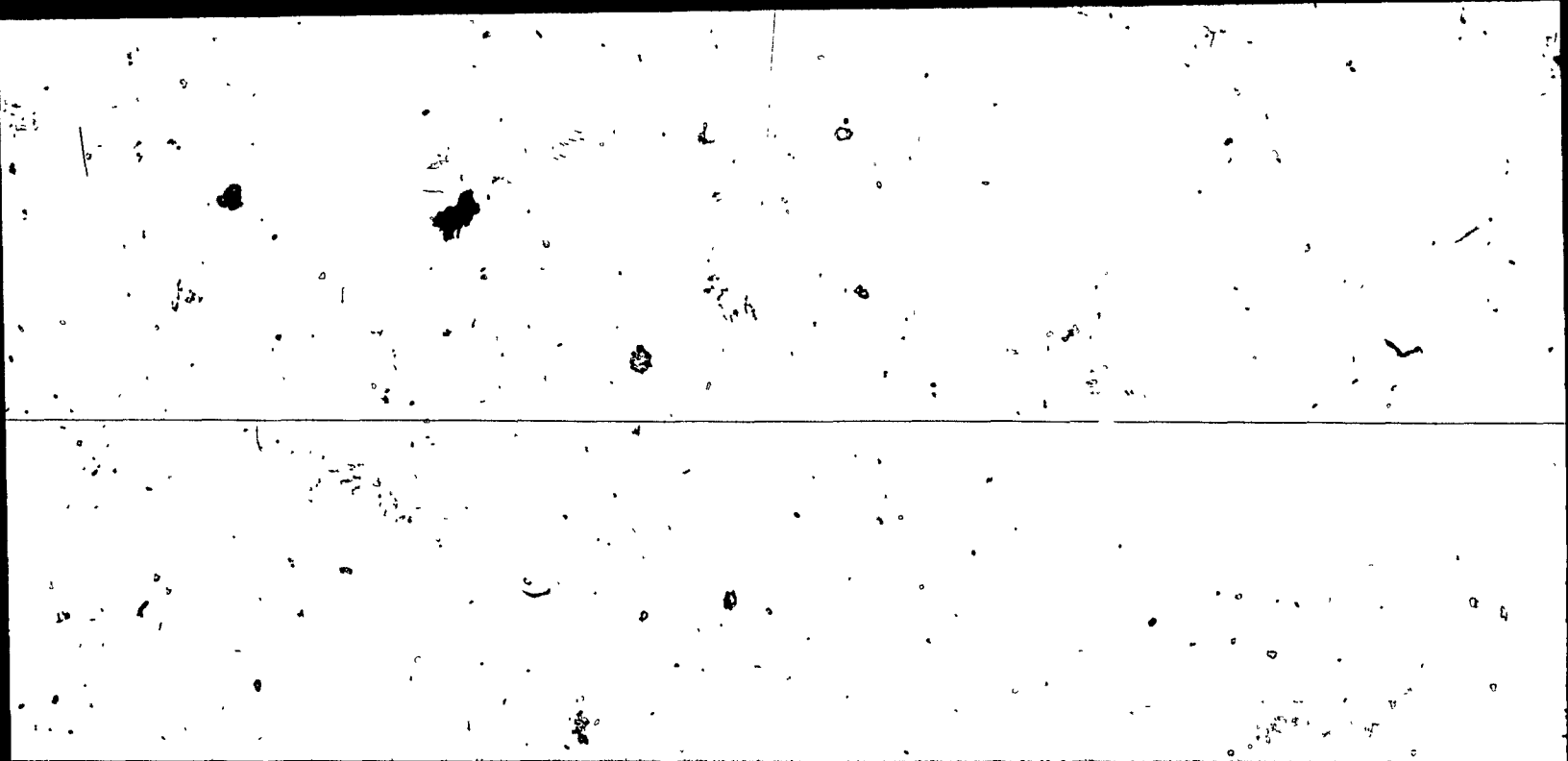
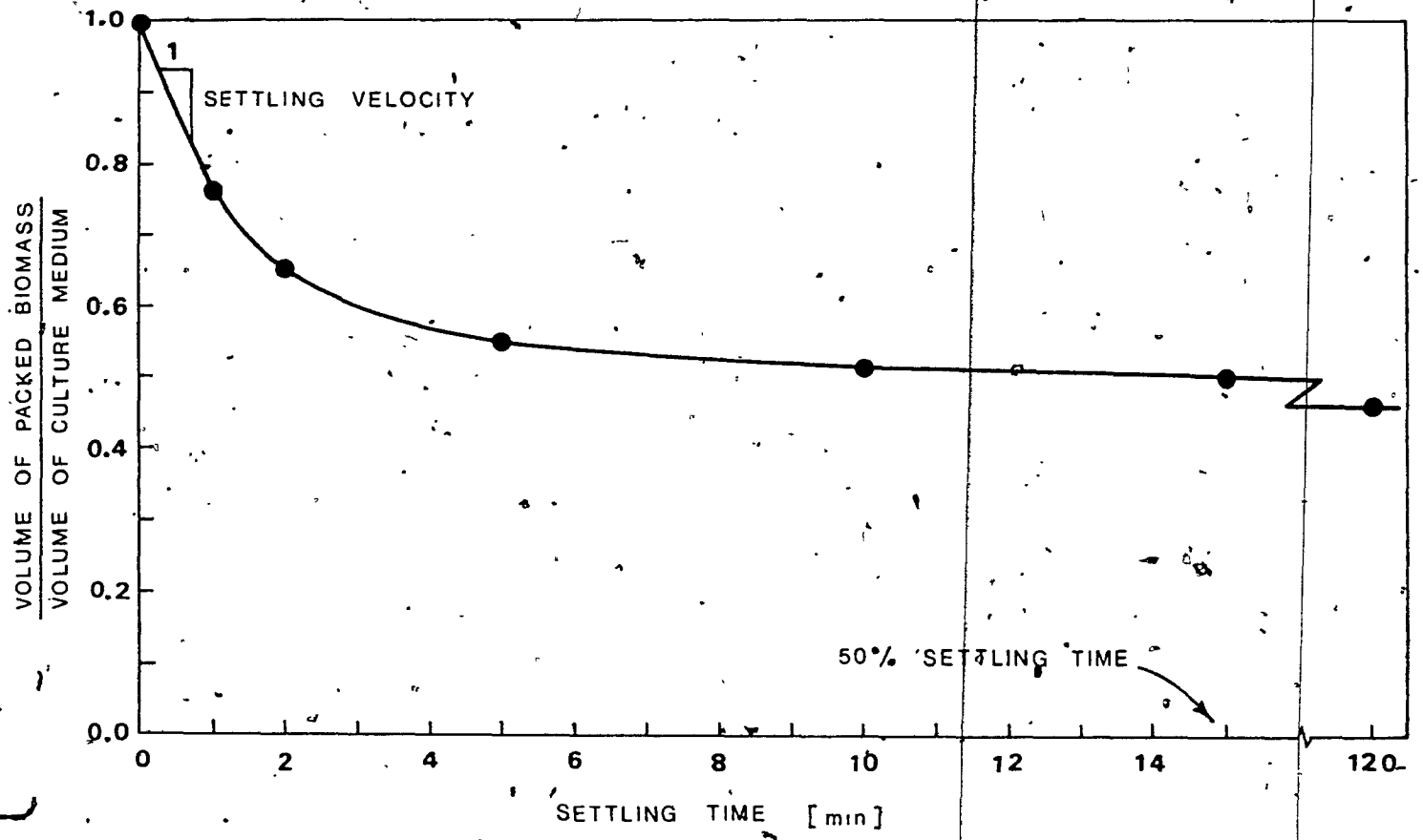


Figure 3.15 Settling of *M. crassipes* Pellets Grown in Mg-WSL. The test has been performed directly in the 5-gallon bottle fermentor as shown in Fig. 3.12.


1.0
0.8
0.6
0.4
0.2
0.0
1
SETTLING VELOCITY
VOLUME OF PACKED BIOMASS / VOLUME OF CULTURE MEDIUM
50% SETTLING TIME
0
2
4
6
8
10
12
14
120
SETTLING TIME [min]

analyzed in terms of protein, total fats, carbohydrates and ash. The commercial meadow mushrooms (fruiting bodies of *Agaricus bisporus*) and the sample of *Morchella* spp. grown in glucose were also analyzed for comparison. The results are shown in Table 3.1 together with values obtained from other sources.

It was found that freeze-dried MMM grown in WSL contain on dry basis 25.7-48.0% crude protein. *Morchella* spp. grown in Mg-WSL contained 48.0% of protein in comparison with 19.7% protein when grown in a glucose synthetic medium. This phenomenon is commonly found in other fungi and other species of morels. Reusser *et al.* (1958) reported that *M. hybrida* contained only 10.5% of protein when grown in a glucose medium, but 34.8% and even 37.5% in molasses and WSL media respectively. The protein content of *Morchella* spp. grown in Mg-WSL (48.0%) is slightly less than that of "Powdered Morel Mushroom Flavouring" (51.0%) [Litchfield *et al.* (1963a)] and *M. hybrida* grown in soybean wheys (48.3-54.7%) [Falanghe *et al.* (1964)], but it competes with fresh meadow mushrooms (46.3%).

Fat content was found to be 2.45% and 4.38% for *Morchella* spp. and *M. crassipes* respectively, grown in Mg- and NH_3-WSL. Values reported in literature were

Table 3.1 Composition of MMM Grown in Various Substrates (Dry Basis).

Species	Protein (%)	Fat (%)	Carbohydrate (%)	Ash (%)	Substrates	References
M. crassipes	30.6	3.07			Glucose	Litchfield *et al.* (1963a)
	30.1	3.35			Maltose	Litchfield *et al.* (1963a)
	29.8	3.72			Lactose	Litchfield *et al.* (1963a)
	22.8	7.55	51.45[a]	18.2	Glucose	Litchfield *et al.* (1963c)
	31.1		22.1	14.4	Mg-WSL	This work
	25.7	4.38	39.2	5.9	NH_3-WSL	This work
M. esculenta	31.1	1.88			Glucose	Litchfield *et al.* (1963a)
	29.6	1.32			Maltose	Litchfield *et al.* (1963a)
	30.0	1.93			Lactose	Litchfield *et al.* (1963a)
	25.0	3.31	54.39[a]	17.3	Glucose	Litchfield *et al.* (1963c)
	36.3		13.7	18.0	Mg-WSL	This work
M. hortensis	34.8	1.38			Glucose	Litchfield *et al.* (1963a)
	32.7	1.29			Maltose	Litchfield *et al.* (1963a)
	32.2	1.82			Lactose	Litchfield *et al.* (1963a)
	26.9	3.13	52.27[a]	17.7	Glucose	Litchfield *et al.* (1963c)
M. hybrida	10.5	6.0			Glucose	Reusser *et al.* (1958)
	34.8	1.3			Molasses	Reusser *et al.* (1958)
	37.5	0.8			WSL	Reusser *et al.* (1958)
	48.3-54.7				Soybean whey	Falanghe *et al.* (1964)
Morchella spp.	19.7		30.2		Glucose	This work
	48.0	2.45	22.9	8.0	Mg-WSL	This work
Powdered Morel Mushroom Flavouring	51.0	2.18	40.38[a]	6.44	Glucose	Litchfield *et al.* (1963c)
Meadow mushroom	46.3	2.84	19.2	7.8		This work

(*a*) Value calculated by difference, *i.e.* carbohydrate and fibrous materials.

0.8% for *M. hybrida* grown in WSL [Reusser *et al.* (1958)] and 7.55% for *M. crassipes* grown in glucose [Litchfield *et al.* (1963c)]. It seemed that *M. crassipes* has the highest fat content (7.55%) of the five morel species studied (Table 3.1) and the amount of total lipid in each species depends highly upon the substrate used as a culture medium.

Analyses for mycelial carbohydrate by anthrone method gave values of 13.7% for *M. esculenta* grown in Mg-WSL and 39.2% for *M. crassipes* in NH_3-WSL, in comparison with 19.2% for meadow mushrooms.

Ash values were found to be as high as 18.0% for *M. esculenta* grown in Mg-WSL, or very low as 5.9% for *M. crassipes* in NH_3-WSL. Similar figures were found in literature, *i.e.* 18.2% for *M. crassipes* cultivated in glucose and 6.44% for *Morchella* spp., which was also cultivated in glucose [Litchfield *et al.* (1963c)]. This variation could be probably attributed to non-uniform and non-efficient growth of the mycelia that contain a hollow core which contains liquid of varying composition.

3.2.2 Amino Acids

Table 3.2 and Fig. 3.16 show the amino acid

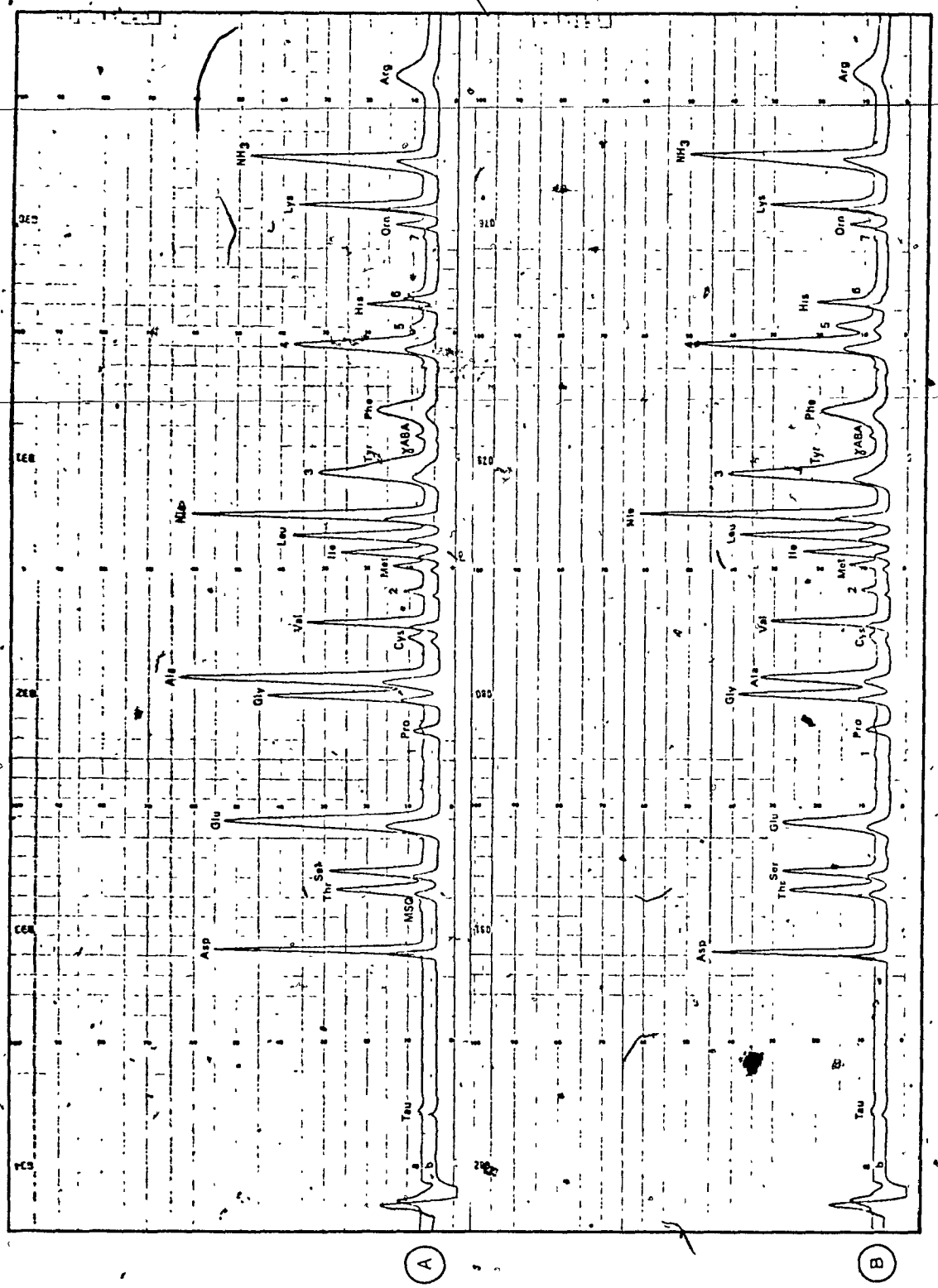
Table 3.2 Amino Acids of MMM and Meadow Mushroom (g/16g Nitrogen).

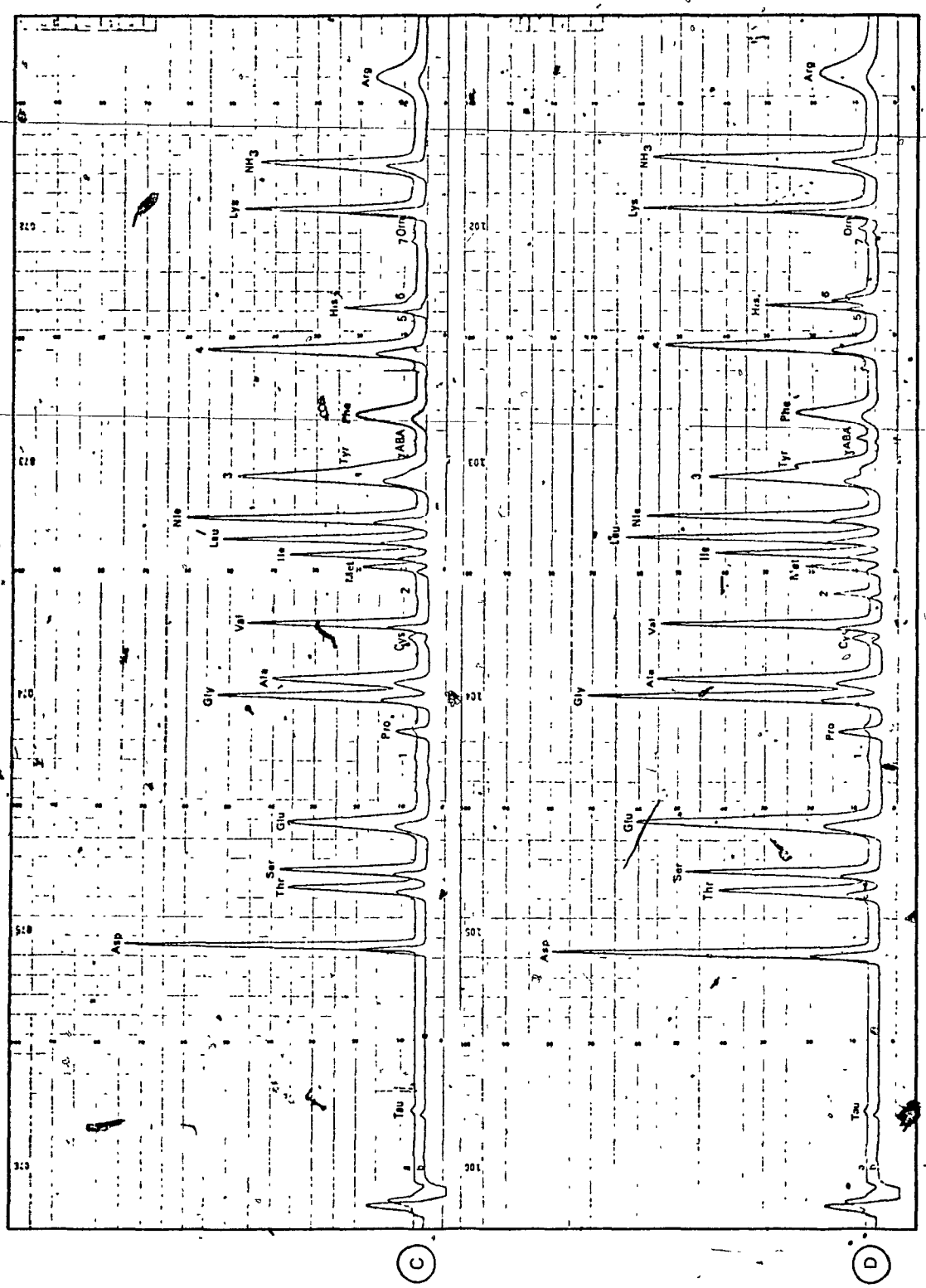
Aminb acids	*M. crassipes* in Mg-WSL	*M. crassipes* in NH_3-WSL	*M. esculenta* in Mg-WSL	*Morchella* spp. in Mg-WSL	*Morchella* spp. in Glucose
Tau.	0.47	0.59	0.47	0.34	0.80
Asp	6.01	5.87	8.11	8.23	5.19
MSO	0.18	-	-	-	0.29
Thr	3.55	4.14	4.65	4.32	2.85
Ser	3.30	3.96	4.35	4.51	2.74
Glu	15.72	8.33	9.04	13.29	19.57
Pro	3.04	3.96	3.85	4.15	2.62
Gly	3.51	3.82	4.15	4.32	2.97
Ala+	9.23	5.28	5.08	5.45	7.24
Cys	0.80	0.64	0.40	0.63	0.63
Val	3.73	4.14	5.08	4.61	2.91
Met	1.05	1.18	1.73	1.64	0.51
Ile	2.90	2.91	4.15	3.76	2.40
Leu	4.78	6.05	6.68	6.18	3.59
Tyr	*	*	*	*	*
γABA	0.51	0.59	0.30	0.51	0.51
Phe	3.30	5.82	3.92	3.57	2.05
His	2.06	1.96	2.26	2.44	1.54
Trp	*	*	*	*	*
Orn	0.58	0.77	0.10	0.14	0.51
Lys	5.07	5.55	6.94	6.78	4.79
NH_3	2.35	3.37	1.96	2.29	3.14
Arg	3.55	3.87	4.78	5.52	2.62

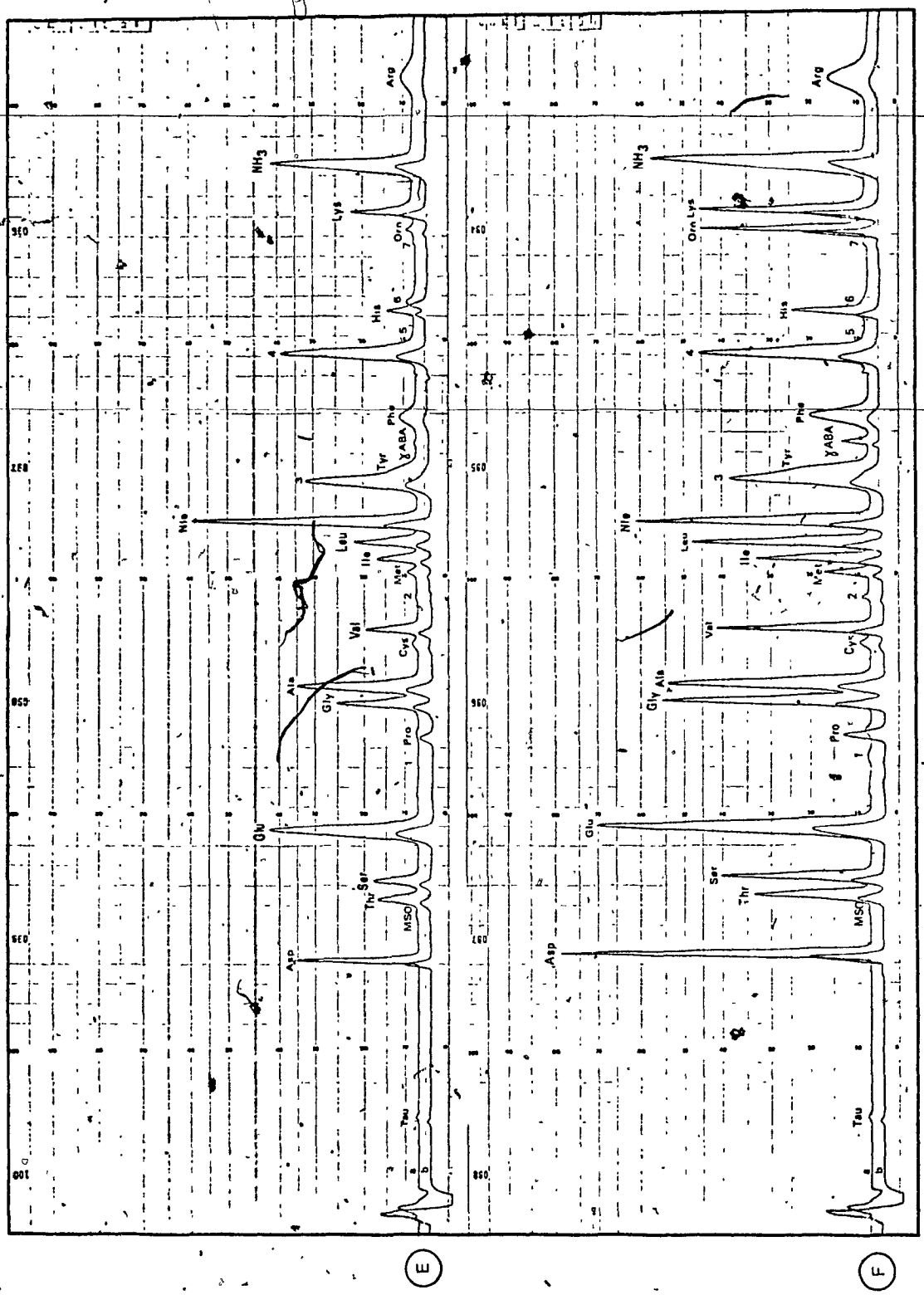
(+) Contains also an unknown partially resolved component.
(*) Present but not calculated.
(-) Not detected.
(*t*) Trace amount.
(*a*) From Litchfield *et al.* (1963c).

M. crassipes in Glucose[a]	*M. esculenta* in Glucose[a]	*M. hortensis* in Glucose[a]	Powdered Morel Mushroom Flavouring[a]	Meadow Mushroom	FAO Standard
				0.32	
4.82	4.97	4.63	6.02	7.04	
–	*t*	*t*	*t*	0.12	
2.99	2.98	2.68	3.37	3.08	2.9
3.08	3.05	2.82	3.90	3.37	
14.10	14.81	15.43	13.45	14.95	
5.11	4.15	4.52	4.78	3.62	
3.12	2.94	3.01	2.75	3.18	
6.16	4.81	4.45	11.12	5.25	
0.42	0.27	0.86	0.80	0.44	
3.04	3.36	2.94	3.86	3.18	4.3
1.01	0.90	0.69	1.41	1.00	2.3
2.87	2.70	2.40	3.45	2.61	4.3
5.57	5.12	5.03	6.06	4.27	4.9
1.70	1.72	1.85	2.49	*	
				1.10	
1.90	2.51	2.28	2.82	2.56	2.9
1.98	2.12	1.88	1.94	1.54	
1.48	0.86	0.98	1.43	*	1.4
				2.66	
3.46	3.84	3.02	5.38	4.79	4.3
				2.17	
3.00	7.95	3.96	4.32	3.42	

Figure 3.16 Spectra of Amino Acids of MMM and Meadow Mushroom: (A) *M. crassipes* in Mg-WSL, (B) *M. crassipes* in NH_3-WSL, (C) *M. esculenta* in Mg-WSL, (D) *Morchella* spp. in Mg-WSL, (E) *Morchella* spp. in glucose medium, and (F) Meadow mushroom. The chromatograms were measured at 570 nm (a) and 440 nm (b).


NH3
Lys
Orn
His
Arg
Tyr
Phe
γABA
Nle
Leu
Ile
Met
Val
Cys
Ala
Gly
Pro
Glu
Thr
Ser
MSO
Asp
Tau
A
B


Asp
Thr
Ser
Glu
Pro
Gly
Ala
Val
Cys
Ile
Leu
Nle
Tyr
Phe
γABA
His
Orn
Lys
NH3
Arg
Tau
C
D


NH3
Arg
Lys
Orn
His
Tyr
γABA
Phe
Nle
Leu
Ile
Met
Val
Cys
Ala
Gly
Pro
Glu
Ser
Thr
MSO
Asp
Tau
E
F

composition determined in this study and compared to literature data for other species.

The MMM grown in WSL contain all of the essential amino acids. The levels of threonine, phenylalanine and lysine are superior to the Food and Agricultural Organization (FAO) standard for all samples tested. Valine and leucine are superior to the FAO values for the samples of *Morchella* spp. and *M. esculenta* grown in Mg-WSL. The mushrooms which might be of nutritional interest on the basis of the essential amino acids content are *Morchella* spp. grown in Mg-WSL, *M. esculenta* in Mg-WSL and *M. crassipes* in NH_3-WSL even though the valine level of the latter is slightly below the FAO requirement. The levels of methionine and isoleucine are very low in all samples - these are common characteristics of all fungi. It is to be noted that isoleucine in *M. esculenta* grown in Mg-WSL is the highest among the other samples and it is very close to the FAO standard.

The non-essential protein amino acids were found in all samples of MMM grown in WSL. In an acid solution, cystine is present in the form of cysteine, asparagine and glutamine in the form of aspartic acid, glutamic acid and ammonia. The levels of non-essential protein amino acids are nearly the same for MMM in WSL, MMM in glucose medium, "Powdered Morel Mushroom Flavouring" and

the fresh meadow mushrooms, except for glutamic acid which is relatively low in the samples of *M. crassipes* grown in NH_3-WSL and *M. esculenta* grown in Mg-WSL.

Other amino acids and amines that are probably not associated with proteins [Hatanaka and Terakawa (1968)] are found in small or trace amounts. γ-aminobutyric acid and ornithine were found in trace amount in all MMM grown in WSL and in *Morchella* spp. grown in a glucose medium, while substantial amounts were found in the fresh meadow mushrooms. Methionine sulfoxide is present in trace amounts only in the samples of *Morchella* spp. grown in glucose, *M. crassipes* grown in Mg-WSL and in the meadow mushrooms. Taurine is present in trace amounts in all of the samples tested, including the meadow mushrooms. The retention time in all chromatograms was the same for α-amino-*n*-butyric acid and cysteine, and these two acids were not resolved on the column.

Hatanaka and Terakawa (1968) reported the presence of β-alanine and γ-aminobutyric acid and 4 unidentified ninhydrin positive substances in the fruiting body of *M. esculenta*. Hatanaka (1969) identified one of these compounds as a new amino acid having the structure *cis*-3-amino-*L*-proline. This amino acid was also found in the fruiting body of *M. conica* and *M. crassipes*, and in the

mycelium of *M. esculenta* and *M. conica*. Litchfield *et al.* (1963c) reported that the trace amounts of cysteic acid, ethanolamine, methionine sulfoxide, methionine sulfoxamine, homocitrulline and α-amino-*n*-butyric acid were detected in *M. esculenta*, *M. hortensis* and in the "Powdered Morel Mushroom Flavouring", but not in *M. crassipes*. The mycelia of *M. crassipes*, *M. esculenta* and *M. hortensis* contained 1.15, 2.00 and 2.00% respectively (dry weight basis) of α-aminoisobutyric acid, whereas the "Powdered Morel Mushroom Flavouring" did not contain this compound in a detectable concentration. A trace of 2,4-diaminobutyric acid was found in all four samples tested by these authors.

Seven unidentified ninhydrin positive substances were also found in various samples of MMM (Table 3.3). Compounds No. 3 and No. 4 are present in large amounts in all samples tested, including the fresh meadow mushrooms. They might be responsible for the typical mushroom flavour (taste and/or aroma). The compounds No. 6 and No. 7 were also common in all samples tested, but usually in trace amounts. Compound No. 1 is present in trace amounts in all samples except in *Morchella* spp. grown in a glucose medium. Compound No. 2 was found in all samples except in *M. esculenta* in Mg-WSL. Compound No. 5 was not detected in all

Table 3.3 Unidentified Ninhydrin Positive Compounds of MMM and Meadow Mushroom.

Sample	Unidentified Ninhydrin Positive Compound						
	1	2	3	4	5	6	7
M. crassipes in Mg-WSL	*t*	*	*	*	*	*	*t*
M. crassipes in NH_3-WSL	*t*	*	*	*	*	*t*	*t*
M. esculenta in Mg-WSL	*t*	*0*	*	*	*0*	*t*	*t*
Morchella spp. in Mg-WSL	*t*	*	*	*	*0*	*	*t*
Morchella spp. in glucose	*0*	*t*	*	*	*0*	*	*t*
Meadow mushroom	*t*	*t*	*	*	*0*	*t*	*t*

(*0*) Not detected.
(*t*) Trace amount.
(*) Present in substantial amount.

samples including the fresh meadow mushrooms, but it was found in substantial amounts in the species of *M. crassipes*. No attempt was made to further identify these substances.

3.2.3 Fatty Acids.

Table 3.4 and Fig. 3.17 show the fatty acid analyses as compared to values obtained from other sources. The chromatograms of fatty acids of MMM show 18 to 20 detectable substances as compared with 16 for the meadow mushrooms. Five saturated (myristic, pentadecanoic, palmitic, heptadecanoic and stearic) and four unsaturated (palmitoleic, oleic, linoleic and linolenic) fatty acids were identified in all samples tested. The rest of the peaks might represent various isomers, branched-chain or the higher molecular weight fatty acids.

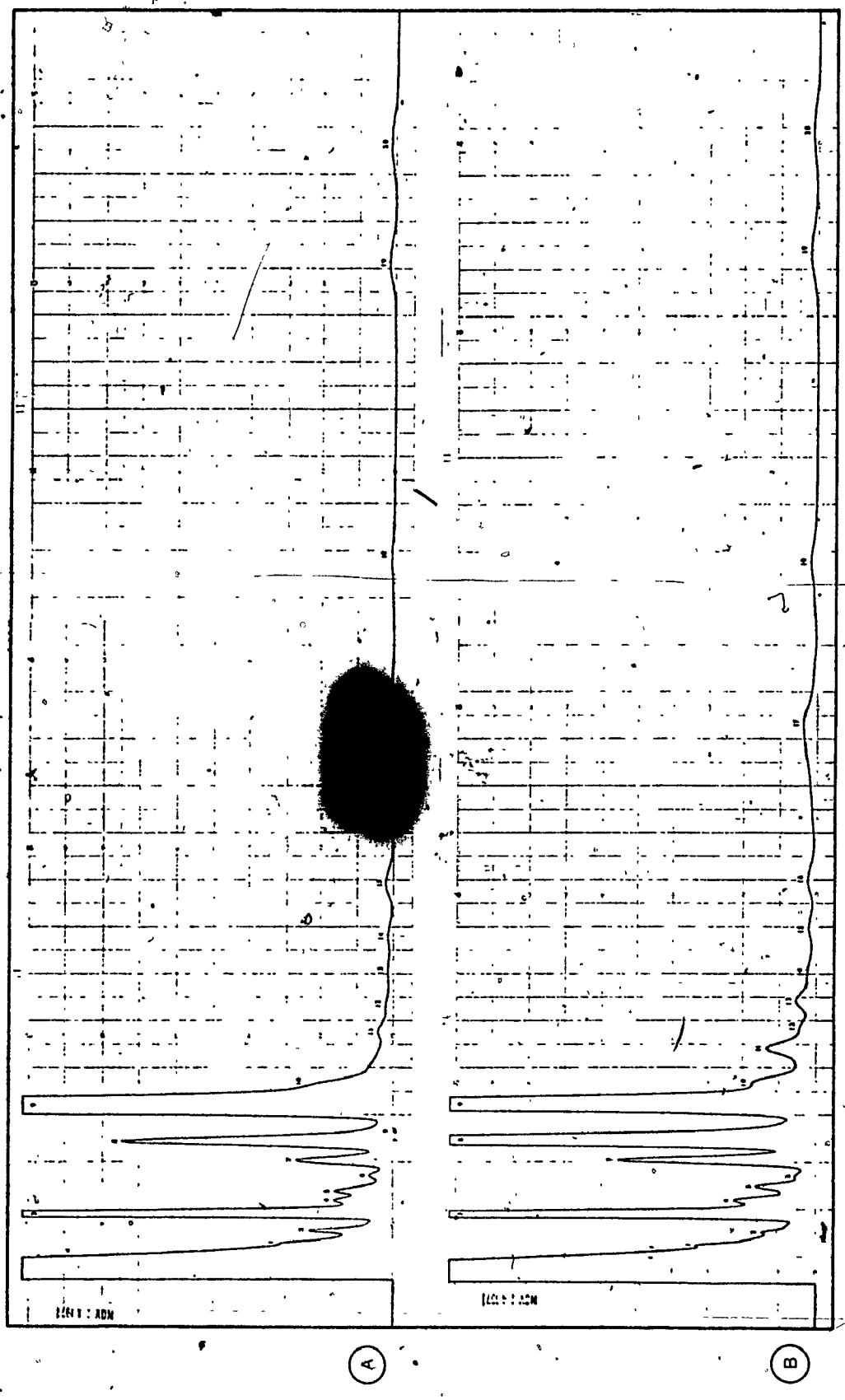
The essential fatty acids (linoleic and linolenic) are present in all samples of MMM grown in WSL. The concentration of linolenic acid is higher in the samples of *M. crassipes* grown in Mg-WSL and NH_3-WSL as compared to meadow mushrooms. As compared to the results of Ivanov and Blisnakova (1967) the linoleic acid content is particularly high in *Morchella* spp. grown in Mg-WSL and in the fruiting bodies of *A. bisporus*. The *M. crassipes* grown in Mg-WSL and NH_3-WSL contained a lower amount of

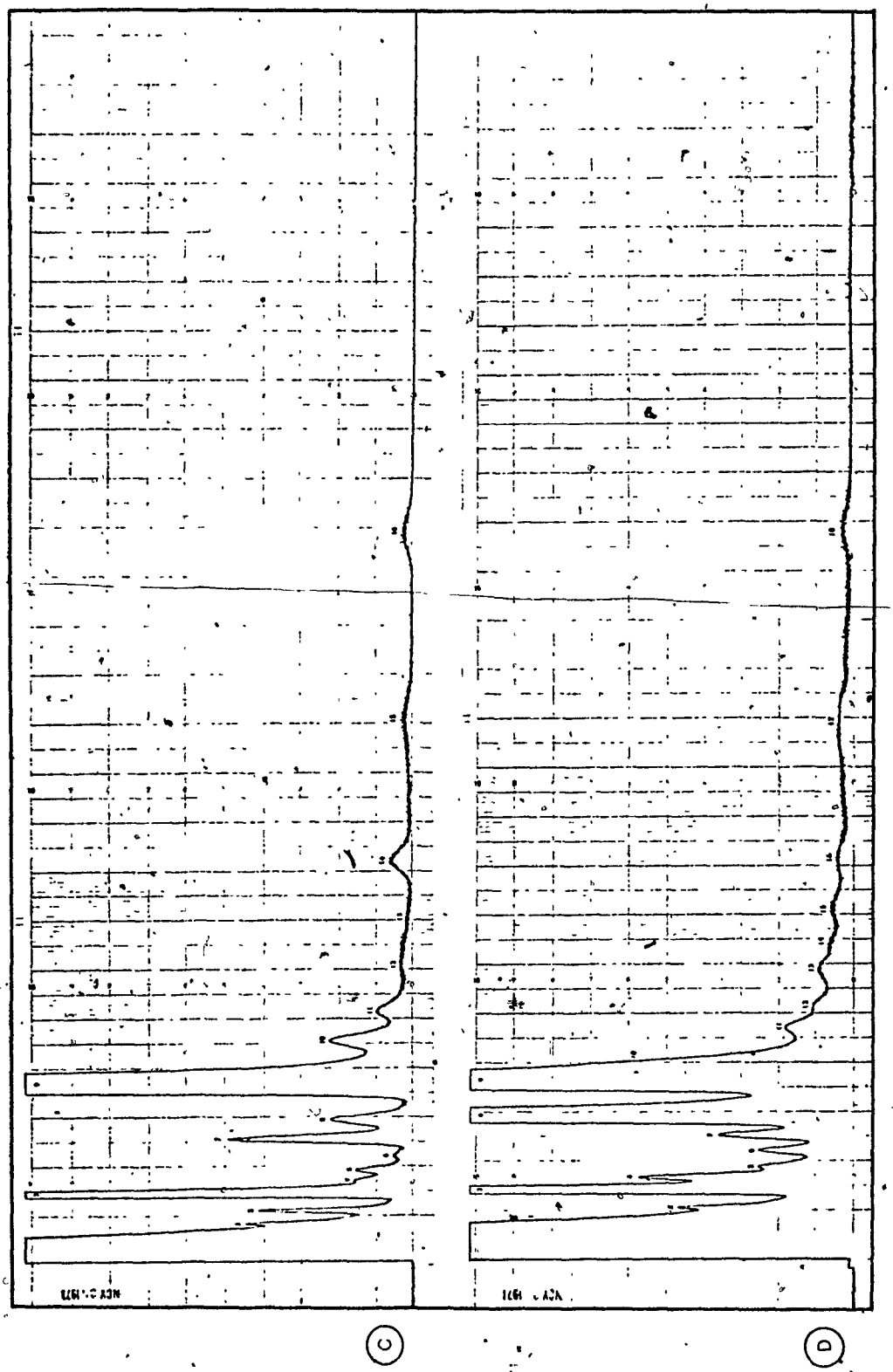
Table 3.4 Fatty Acids of MMM and Meadow Mushroom (% of total methyl esters).

Fatty Acids	Peak No.	*M. crassipes* in Mg-WSL	*M. crassipes* in NH_3-WSL	*Morchella* spp. in Mg-WSL	*M. esculenta*[a]	Meadow Mushroom
Myristic (14:0)	1	0.22	0.27	0.16	-	0.24
Pentadecanoic (15:0)	2	0.46	0.21	0.39	-	0.76
Palmitic (16:0)	3	11.79	12.92	9.69	14.1	8.69
Palmitoleic (16:1)	4	1.12	1.15	0.43	-	0.33
Heptadecanoic (17:0)	5	0.40	0.99	0.51	0.4	0.40
Stearic (18:0)	7	1.58	6.01	1.47	5.4	2.25
Oleic (18:1)	8	28.30	17.14	5.04	23.6	1.18
Linoleic (18:2)	9	47.40[+]	34.75[+]	72.02[+]	52.8	80.04
Linolenic (18:3)	11	4.25	8.50	1.20	-	2.88

(-) Not reported.
(+) Contains also an unknown partially resolved component.
(a) From Ivanov and Blisnakova (1967).

Figure 3.17 Spectra of Fatty Acids of MMM and Meadow Mushroom: (A) *Morchella* spp. in Mg-WSL, (B) *M. crassipes* in NH_3-WSL, (C) Meadow mushroom, and (D) *M. crassipes* in Mg-WSL.


A
B



linoleic acid, about one-half of the above two samples. The distribution of essential fatty acids is somehow more uniform in samples of *M. crassipes* in Mg-WSL and NH_3-WSL and *M. esculenta* [Ivanov and Blisnakova (1967)] while the heavy weight was confined to the linoleic acid alone as found in the samples of *Morchella* spp. in Mg-WSL and the meadow mushrooms. The levels of palmitic and oleic acids are superior in the MMM as compared to the meadow mushrooms.

The abundance of unsaturated and essential fatty acids adds to the nutritional quality of this biomass. In this respect MMM seems to be better than the commercial meadow mushrooms. The characteristic and pleasant flavour of MMM grown in WSL might also be partly attributed to this specific fatty acid spectrum.

3.2.4 Flavour

i) Characteristics of MMM Flavour

Three main categories of MMM flavour have been observed during experiments in our laboratory: (a) Highly volatile components with pleasant flower-like odor. These were detected around the fermentor near the exhaust air stream and also in the filtered MMM and even in the frozen sample. This flavour was only partially preserved if the

sample was stored in a frozen state. (b). Less volatile components which smell like ordinary mushrooms. This flavour characteristic is not lost even when the sample is stored in a closed flask at room temperature. (c) Non-volatile components which seem to be associated with the characteristic mushroom taste that is preserved in the air-dried samples. However, samples dried at 75° C had a strong peanut or similar taste.

Strong mushroom flavour is conserved in the freeze-dried powder samples. It was found that *M. crassipes* grown in Mg-WSL has a stronger flavour than that of *Morchella* spp. in Mg-WSL, and *M. esculenta* in Mg-WSL and that the flavour is more pleasant as compared to *Morchella* spp. grown in a glucose medium.

Gilbert (1960) characterized the odor of the effluent air from submerged cultures of MMM as follows: *M. hortensis* and *M. rimosipes*, aromatic or estery, *M. esculenta*, earthy, *M. angusticeps*, slippery elm, and other species, farinaceous. He further stated that *Morchella* spp. develop their characteristic flavour in the mycelium as well as in the sporocarp. He suggested that the flavour appears to be a genetically controlled characteristic in *Morchella* and the characteristic flavour of each species has been obtained in cultures of that species regardless

of the medium.

The flavour of the fresh MMM has also been reported by Gilbert (1960). According to taste panel evaluations, the flavour of the mycelium of *M. crassipes* was deemed the most delicious and was preferred to all others. The flavour of *M. esculenta* came second, and that of *M. hortensis* and the Szuecs strain, third and fourth. The taste of *M. angusticeps*, *M. conica*, *M. vulgaris* and *M. rimosipes* was acceptable but not as well liked as that of the first four. The two strains of *M. semilibera* tasted quite bland. Intensity of flavour was determined by defrosting the frozen mycelium, squeezing it and noting the persistence of the odor. In regard to intensity of flavour, the Szuecs strain was not surpassed. The flavour of this extract, as well as that in the freeze-dried samples of MMM was also conserved for a long period of time.

Litchfield *et al.* (1963a) reported that the dry, powdered mycelia of *M. esculenta*, *M. crassipes* and *M. hortensis* grown in glucose, maltose and lactose media have been subjected alone and in taste panel evaluation. It was found that the flavour of *M. esculenta* was the mildest and *M. crassipes* the strongest.

A comparison has also been made between the cooked flavour of fresh sporocarp and freshly cultured

M. esculenta mycelium [Gilbert (1960)]. The products were fried in butter in the same manner and submitted to a taste panel. The panel found the flavours similar but not identical. There was no preference for the sporocarp over the mycelial product.

ii) Chemical Composition and MMM Flavour

The relationship between protein as well as non-protein nitrogenous compounds of both mushroom mycelia and sporophores and flavour of various fungi has been reviewed by Litchfield (1967). However, there is a lack of comparative data on the amino acid contents of sporophores and mycelium and their extracts of the *Morchella* species.

The taste of individual amino acids was characterized [Kirimura *et al.* (1969), Solms (1969)] as being sweet, salty, sour, bitter, monosodium glutamate-like, sulfuric, or tasteless. The correlation between amino acids and taste is a very difficult task. It was found that some amino acids contribute to the inherent taste of foodstuffs; some specific patterns of amino acid mixtures intensify the taste of foodstuffs and increase the mouthful without losing their inherent taste. The buffer action of amino acids can also contribute to the

taste of foodstuffs.

Solms (1969) proposed an integrated flavour profile which is composed of four groups of substances, with close interrelationships, each of which should occur in a specific balance to give an overall flavour sensation. Amino acids, peptides and proteins are located at the base of this scheme, providing taste and tactile effects; certain compounds act as flavour potentiators and synergists, and they also act as a bridge between the non-volatile and volatile fractions.

Of the three main classes of foodstuffs, proteins, carbohydrates and lipids, lipids may be most important as sources of flavouring compounds but the least important for their own taste and aroma. Lipids of low volatility are tasteless, partly because they are insoluble in water. Fatty acids of low volatility (above C_{10}) do not taste acid or sour, nor do they have much aroma. Their flavour has been described as candlelike. Glycerol results from hydrolysis of lipids and has a sweet taste. The role of lipids as flavour precursors for their aldehyde, ketone, lactone and alcohol derivatives has been reviewed by Forss (1969).

The lipid fractions of mushrooms are also of interest in connection with taste and aroma development.

The typical flavour of mushrooms may well depend upon some products of auto-oxidation and/or esterification of unsaturated fatty acids such as palmitoleic, oleic, linoleic and linolenic acids. The essential oils and related constituents of various mushroom sporophores have been isolated [reviewed by Litchfield (1967)].

The carbohydrate constituents of the fruiting bodies of various mushrooms have been reported and reviewed [Litchfield (1967)]. Various reducing sugars are found as well as amino sugars, sugar alcohols, sugar acids, methylated sugars, methylated sugar acids, glycogen and hemicelluloses. These compounds most likely contribute to a sweet flavour and probably are not the major constituents of a typical fresh mushroom flavour.

The B-vitamin content of *Morchella* spp. has been determined [Litchfield (1964), Szuecs (1956)] and shown in Table 3.5. However, there is no evidence to indicate that these constituents have any major role as mushroom flavour constituents.

iii) Analysis of Flavour Extracts

The diethyl ether extracts of both fresh and freeze-dried samples of *M. crassipes* grown in NH_3-WSL

Table 3.5 B-Vitamin Composition of MMM (mg/100g dry weight).

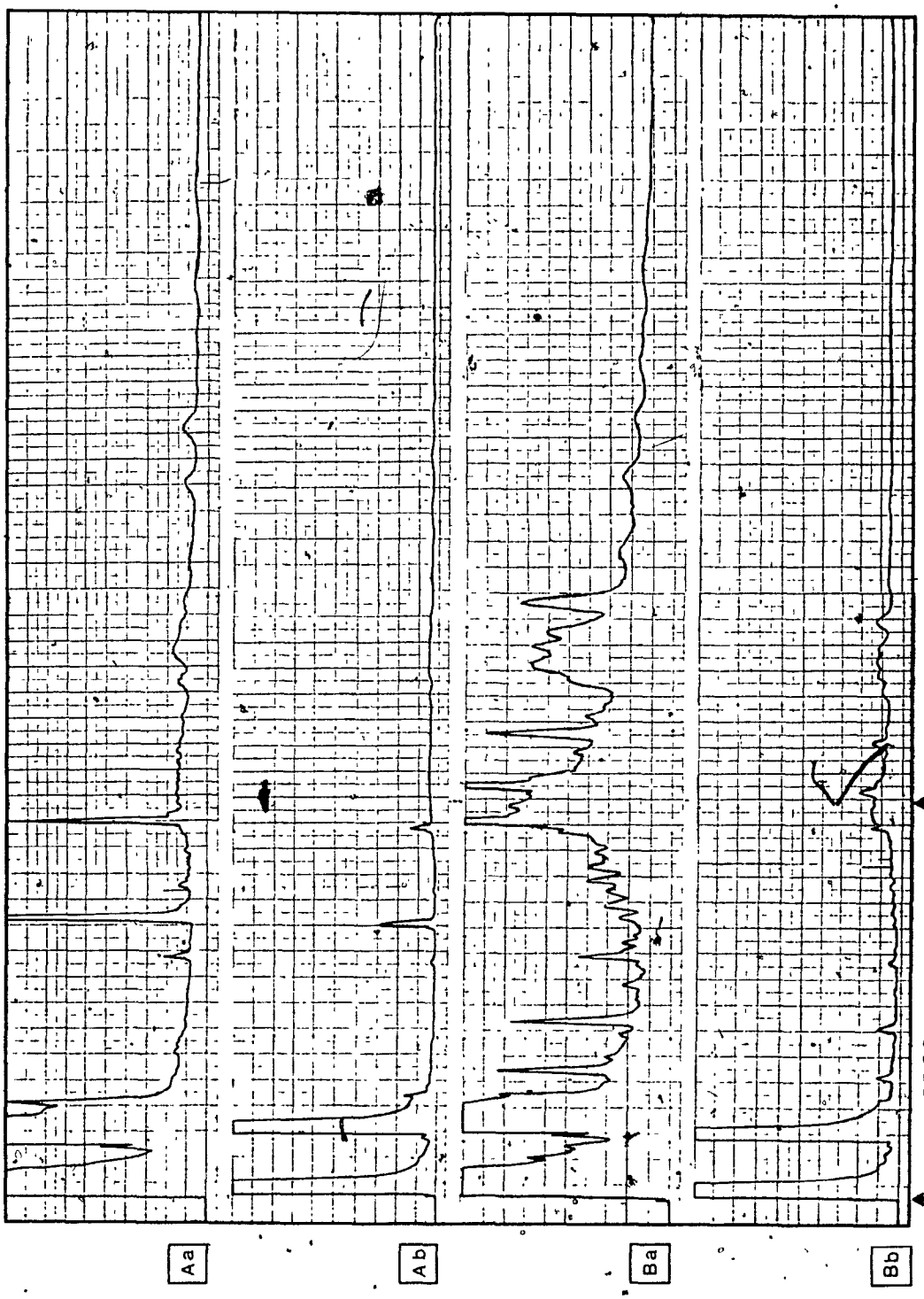
Vitamin	*M. esculenta* [Szuecs (1956)]	*M. hortensis* [Litchfield (1964)]
Thiamin	0.392	0.518
Riboflavin	2.46	1.31
Niacin	8.20	12.4
Vitamin B6 (as pyridoxine hydrochloride)	0.58	2.62
Pantothenic acid (as *p*-calcium pantothenate)	0.87	12.6
Choline		461.0
Folic acid	0.348	1.09
Inositol		178.0
Biotin	0.075	0.015

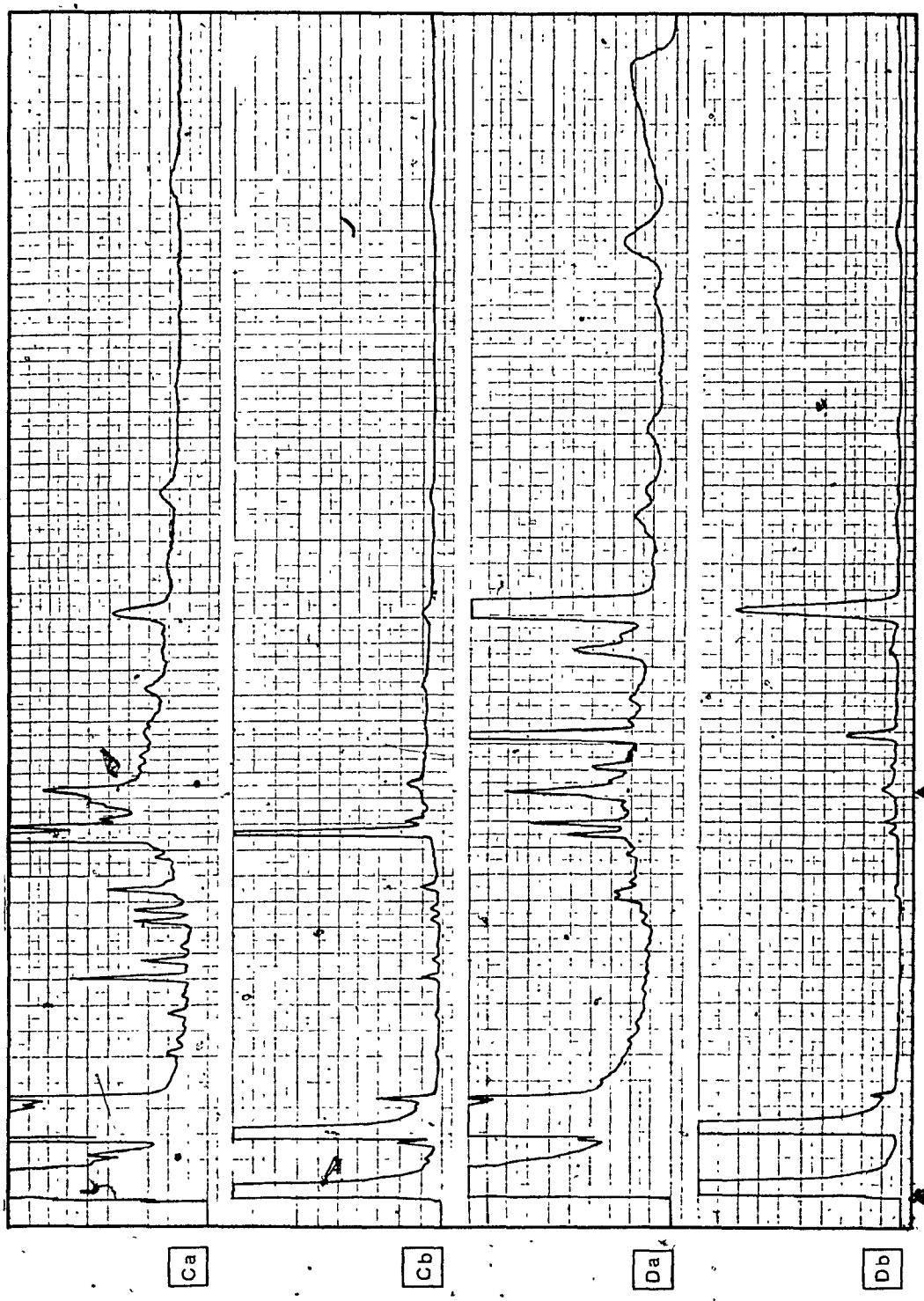
and meadow mushrooms were analyzed by gas-liquid chromatography, infrared spectrophotometry and ultraviolet absorption spectrophotometry.

The gas-liquid chromatograms (Fig. 3.18) showed more compounds in the freeze-dried sample of *M. crassipes* grown in NH_3-WSL as compared with fresh pellets. The freeze-dried samples of meadow mushrooms contained less components in the lower temperature region (below 250° C) but more compounds in the higher temperature region (250° C isothermal). It is suggested that the freeze-dried samples contain most of the low boiling compounds. The low boiling compounds which are not present in the freeze-dried samples are probably lost during the freeze-drying process.

The fact that more compounds were found in the freeze-dried samples of both mushrooms, particularly in the high temperature region of the gas-liquid chromatograms might be explained as follows: three categories of less-volatile and non-volatile flavour compounds were present in the mushrooms: (α) soluble in water only, (β) soluble in diethyl ether only, and (γ) soluble in both water and diethyl ether. The compounds in category (α) are not shown in all of the chromatograms; those in category (β) are shown in the chromatograms of fresh samples; and those in category (γ) are shown in the chromatograms

Figure 3.18 Gas-Liquid Chromatogram of the Ether Extract of MMM and Meadow Mushroom: (A) Fresh pellets of *M. crassipes* in NH_3-WSL, (B) Freeze-dried powder of *M. crassipes* in NH_3-WSL, (C) Fresh meadow mushroom, and (D) Freeze-dried powder of meadow mushroom; (a) Low attenuation, and (b) High attenuation.


Aa
Ab
Ba
Bb
65°C
PROGRAMMED
250°C
ISOTHERMAL


Ca
Cb
Da
Db
65°C
PROGRAMMED
250°C
ISOTHERMAL

of the freeze-dried samples. At the end of the Soxhlet extraction, two phases (solvent and water) were obtained from the fresh samples, while only one phase (solvent) was obtained from the freeze-dried samples. In the latter case, all compounds in the categories (β) and (γ) are expected to be present in the same chromatogram.

The gas-liquid chromatograms of MMM and meadow mushrooms showed many similar compounds. The distintive flavours of both fungi might be due to non-similar compounds and/or relative proportions of the similar compounds. It is to be noted that during the ether extraction, the volatile compounds of the flavour are lost, the remaining being mainly non-volatile compounds which are responsible for the taste rather than the aroma of mushrooms. The taste of mushrooms might play a superior role to the aroma in meal preparations.

The infrared spectra (Fig. 3.19) of the ether extracts of MMM and meadow mushrooms were similar in the functional group region (above 1,300 cm^{-1}). However, the finger print regions (909-1,300 cm^{-1}) were different for two fungi and also depend upon the preparation of the fresh or freeze-dried sample before extraction. The difference was also found in the ultraviolet absoption spectra (Fig. 3.20) of four samples tested.

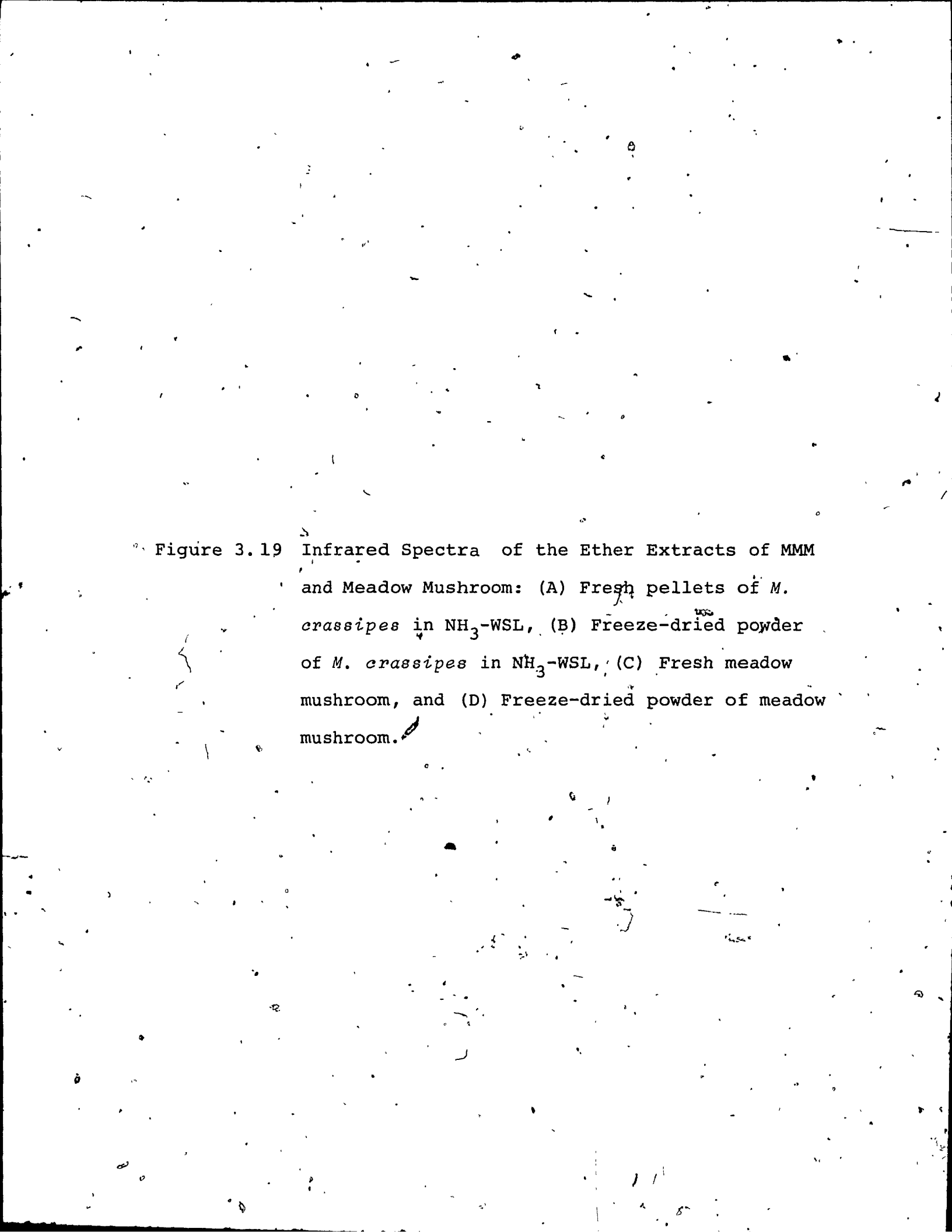
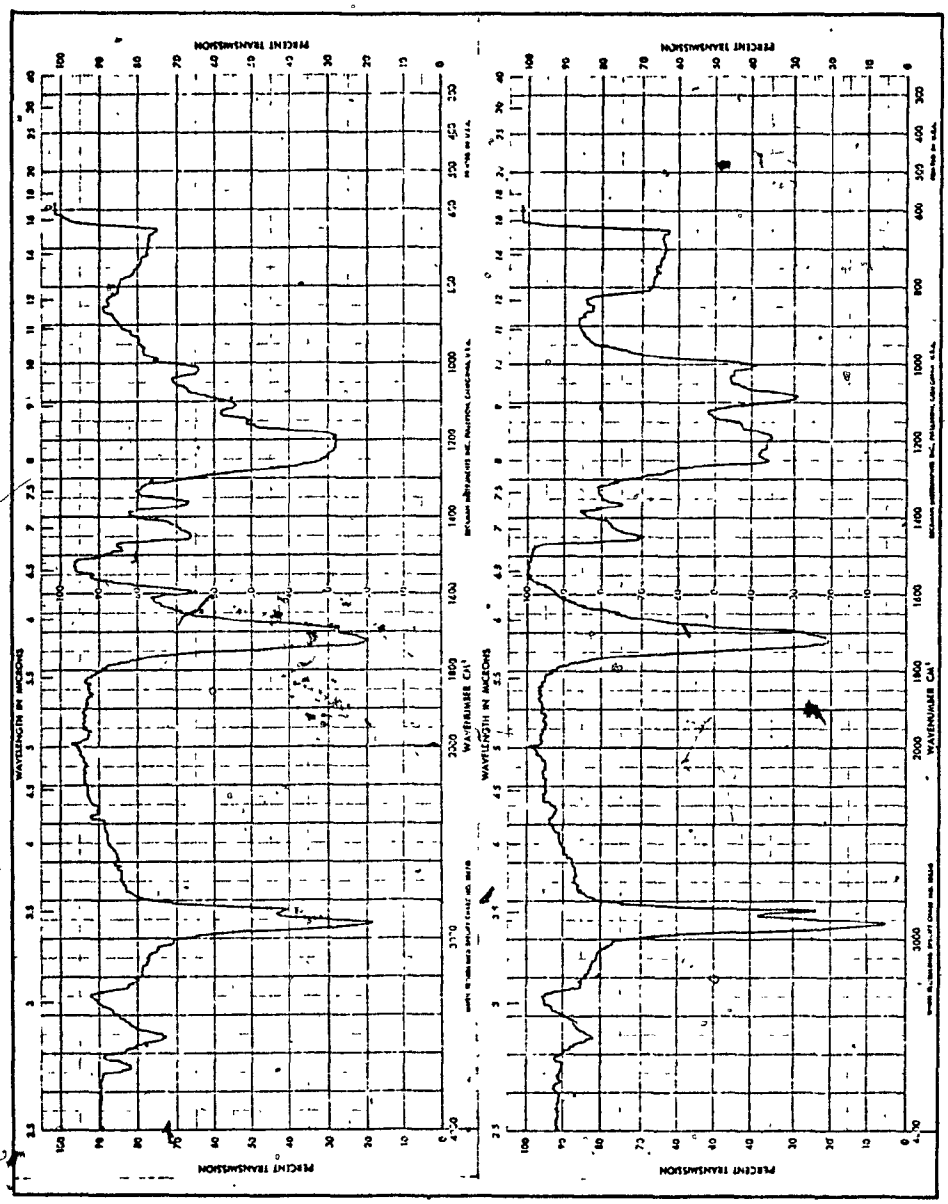
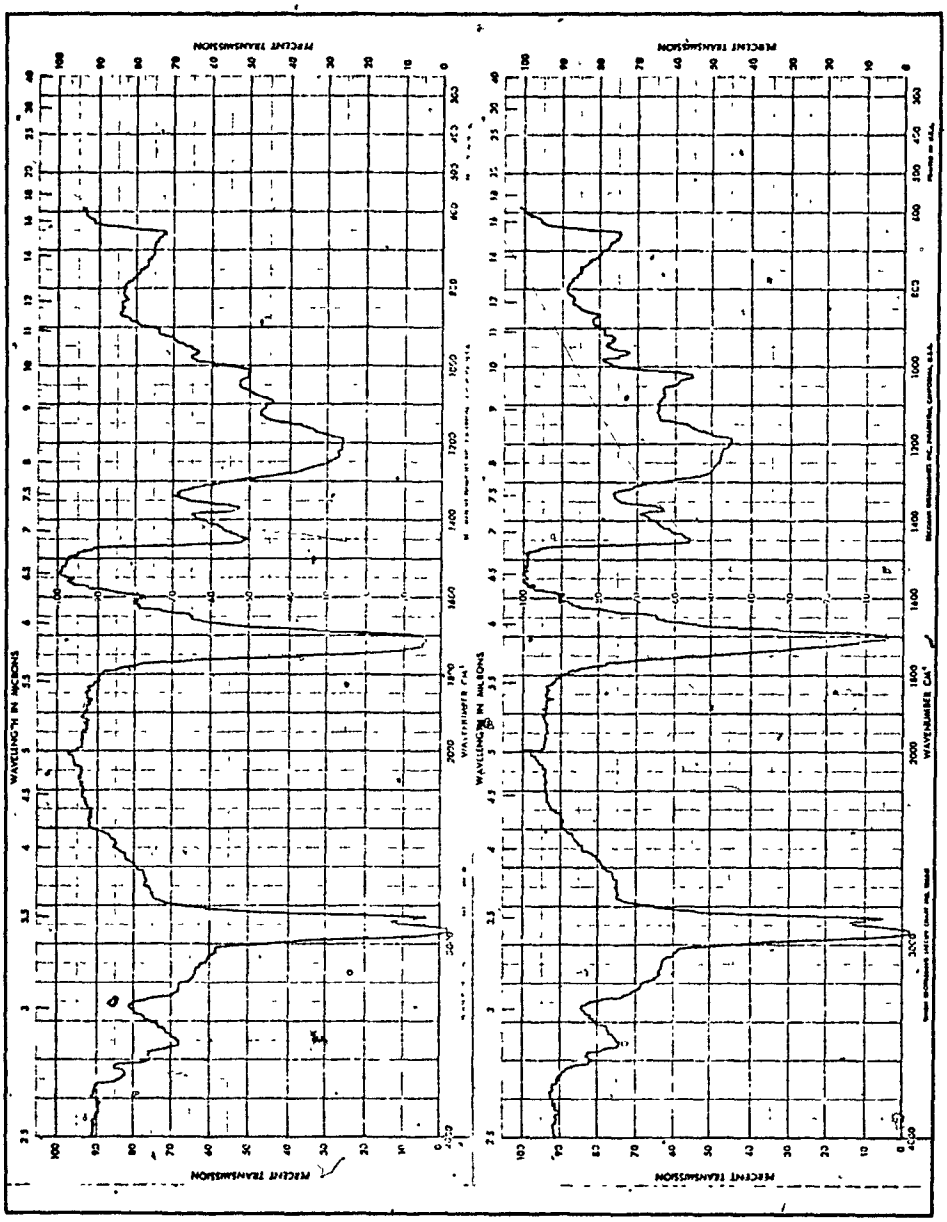


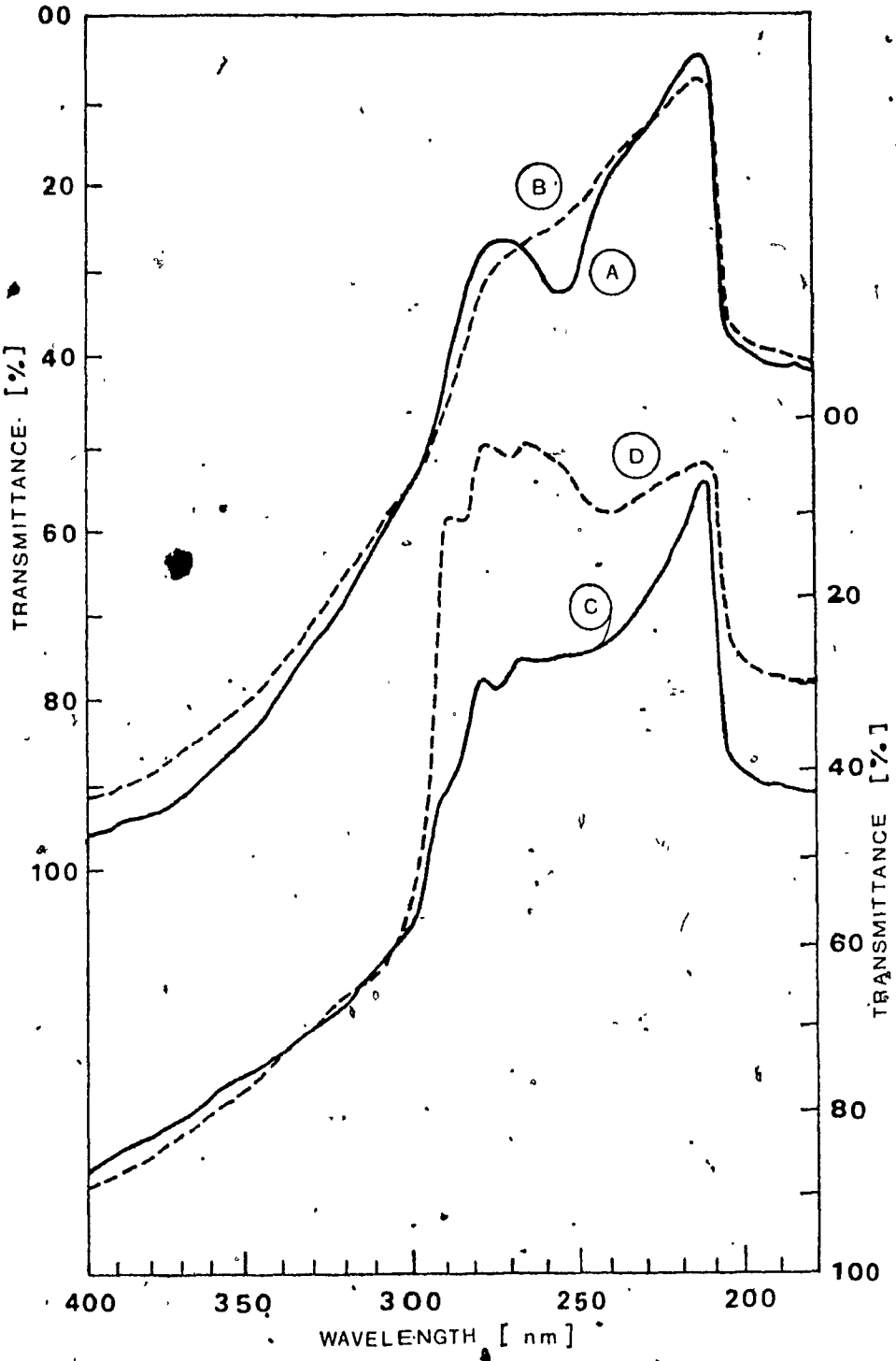
Figure 3.19 Infrared Spectra of the Ether Extracts of MMM and Meadow Mushroom: (A) Fresh pellets of *M. crassipes* in NH_3-WSL, (B) Freeze-dried powder of *M. crassipes* in NH_3-WSL, (C) Fresh meadow mushroom, and (D) Freeze-dried powder of meadow mushroom.


PERCENT TRANSMISSION
WAVELENGTH IN MICRONS
WAVENUMBER CM⁻¹
A
B


PERCENT TRANSMISSION
WAVELENGTH IN MICRONS
WAVENUMBER Cm⁻¹

(C) (D)

Figure 3. 20 Ultraviolet Absorption Spectra of the Ether Extracts of MMM and Meadow Mushroom: (A) Fresh pellets of *M. crassipes* in NH_3-WSL, (B) Freeze-dried powder of *M. crassipes* in NH_3-WSL, (C) Fresh meadow mushroom, and (D) Freeze-dried powder of meadow mushroom.


00
20
40
60
80
100
TRANSMITTANCE [%]
B
A
D
C
00
20
40
60
80
100
TRANSMITTANCE [%]
400
350
300
250
200
WAVELENGTH [nm]

Tentative interpretation of the infrared spectrograms of the ether extracts of mushroom flavours reveals the presence of the following functional groups: ketone, aldehyde, alcohol, phenol and ester. The strong absorption band at 1,725 cm^{-1} (A,B), 1,720 cm^{-1} (C) and 1,700 cm^{-1} (D) may be attributed to the C=O stretching vibration for ketones, aldehydes and esters. The C=O stretching vibration for aldehyde may be the absorption band in the 1,685-1,740 cm^{-1} region; the same for alcohols, phenols and esters in the 1,000 - 1,260 cm^{-1} region. The absorption band at 2,850 cm^{-1} might confine to the C-H stretching vibration of aldehydes. The O-H bending vibration of alcohol and phenol might be identified by the sharp absorption band at 1,370 cm^{-1} and a broad absorption band at the 650-769 cm^{-1} region. The multiple bands of moderate absorption in the 1,100-1,300 cm^{-1} might result from the C-C-C stretching and bending vibrations in the C-C(=O)-C group of ketones.

3.3 Characteristics of Used WSL

3.3.1 BOD_5 and COD Reductions of NH_3- and Na-WSL

The cultivation of *M. crassipes* in NH_3-WSL was conducted in a controlled batch fermentor under optimal initial pH and dilution ratio, for 15 days. The resulting

pH, BOD_5 and COD values of the cell-free medium are shown in Fig. 3.21 and Table B.12. At the 14th day, a 72.0% reduction in BOD_5 and a 16.1% reduction in COD were obtained. Previous experiments in shake flasks indicated a BOD_5 reduction of 60-65%, thus higher BOD_5 reduction was obtained in the batch fermentor. However, a maximum growth of mycelium was not achieved at the end of the cultivation due to side growth and bottom growth within the fermentor (Fig. 3.14). Higher reduction of BOD_5 and COD may be expected if the side and bottom growths were eliminated by using higher agitation rate and by reducing the foam level. It is also observed that the reduction of BOD_5 occurred in two steps and the higher rate of reduction was obtained between the 8th and 14th days (average of 210 ppm of BOD_5 per day) when the pH of the culture medium was around 5.0. In order to avoid the long lag phase, a continuous cultivation should be used and a higher rate of BOD_5 reduction might be expected.

Studies on the effects of *M. crassipes* on the characteristics of Na-WSL were conducted in shake flasks. The stock solution was diluted to 1:3 (v/v) and the initial pH for the culture medium was adjusted to 6.5. The changes in BOD_5, COD, TCH and TOC using different culture media and various inocula are shown in Table 3.6. The utilization of CSL for various WSL as previously reported

Figure 3.21 Kinetics of BOD_5 and COD Reductions of NH_3-WSL in the Batch Fermentor by *M. crassipes* at Optimal Initial pH 6.0 and Optimal Dilution Ratio 1:5 v/v.

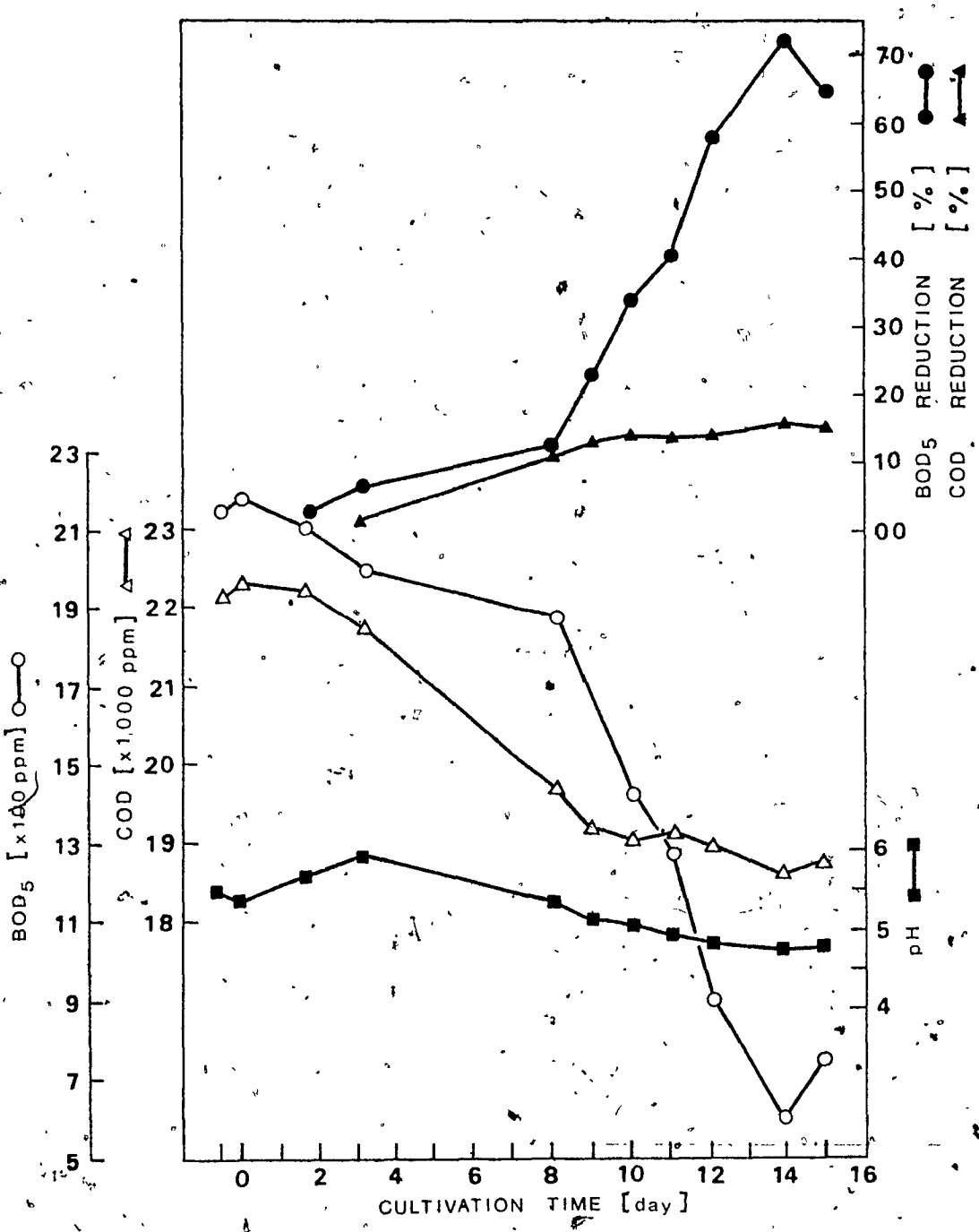

BOD5 [x100 ppm]
COD [x1,000 ppm]
BOD5 REDUCTION [%]
COD REDUCTION [%]
pH
CULTIVATION TIME [day]
0
2
4
6
8
10
12
14
16
23
21
19
17
15
13
11
9
7
5
70
60
50
40
30
20
10
00
6
5
4

Table 3.6 Effects of Various Culture Media and Various Strains of *M. crassipes* on the Characteristics[a] of Na-WSL in Shake Flask Experiments.

Culture Medium	Cultivation Time (day)	BOD_5		COD		TCH		TOC		Biomass (g/l)
		Average (ppm)	% Decrease	Average (ppm)	% Decrease	Average (ppm)	% Decrease	Average (ppm)	% Decrease	
A	0	31,580		110,000		19.0		38,390		
	9	17,750	43.8	90,640	17.6	17.4	8.4	32,240	16.0	6.4
B	0	30,620		100,000		18.4		36,000		
	9	7,550	73.4	67,000	33.0	4.6	75.0	22,350	37.9	15.0
C	0	32,360		102,000		20.1		36,570		
	9	17,890	44.7	87,000	14.7	11.8	41.3	31,610	13.6	7.8
D	0	31,250		104,620				38,050		
	9	8,270	73.5	72,430	30.8			24,820	34.8	12.6

(a) All values calculated for concentrated Na-WSL.
(A) WSL-CSL-APD medium with original culture.
(B) WSL-APD medium with third transfered culture.
(C) WSL medium with third transfered culture.
(D) WSL-APD medium with fourth transfered culture.

was found not to be necessary in the case of Na-WSL. Nonetheless, a culture medium should be enriched with inorganic salts of nitrogen and phosphorus for the mycelial growth. The comparison between two media B and C showed that the biomass produced in the culture medium with APD was nearly twice the biomass produced in the culture medium without APD. The reductions of BOD_5 and COD were also much higher when APD was used (73.4% of BOD_5 reduction and 33.0% of COD reduction for medium B as compared to 44.7% of BOD_5 reduction and 14.7% of COD reduction for medium C). A possible mutation and adaptation of *M. crassipes* grown in Na-WSL might be indicated by analysing the data from culture media A, B and D. The biomass produced was 6.4 g/l and the reduction of BOD_5 and COD were 43.8% and 17.6% respectively in medium A where the inoculum was prepared from agar slants. Results were considerably improved after using mycelium which was grown in shake flasks as the inoculum for other flasks. At the third transfer (medium C), the dry weight of mycelium obtained was 15.0 g/l and the reductions of BOD_5 and COD rose to 73.4% and 33.0% respectively. The data from media B and D showed that these results could not be improved by further transfers.

3.3.2 Effect of γ-Irradiation

500 ml of concentrated Na-WSL in a polyethylene

container were irradiated before cultivation for 22 hours at 20 Mrads in the Gamma Cell 220 (Atomic Energy of Canada Ltd.). The composition of the culture medium and the inoculum used were the same as for the medium B in Table 3.6. The BOD_5, COD, TCH and TOC of the culture media after γ-irradiation only, and both γ-irradiation and SO_2-stripping are shown in Table 3.7. The controls (media B and D) were SO_2-stripped Na-WSL media.

In A and B culture media, γ-irradiated Na-WSL medium gave slightly higher BOD_5 reduction, but the reductions of COD, TCH and TOC were lower. It might be suggested that when exposed to γ-irradiation, some fractions of lignosulfonates such as straight chain aliphatic compounds and aromatic hydrocarbons, which could not be completely degraded by the BOD_5 and COD methods, became assimilable by *M. crassipes*. The double-pretreated Na-WSL by γ-irradiation and SO_2-stripping did not improve the reductions of BOD_5, COD, TCH and TOC as shown by the culture media C and D. It is also shown that maximum reductions of BOD_5, COD, TCH and TOC could be obtained when either γ-irradiation or SO_2-stripping was used as the sole pretreatment of Na-WSL. The selection of either method will depend on the economic feasibility of each process.

Table 3.7 Effects of γ-Irradiation and SO_2-Stripping on the Characteristics[a] of Na-WSL in Shake Flask Experiments Using *M. crassipes*.

Culture Medium	Cultivation Time (day)	BOD_5		COD		TCH		TOC		Biomass (g/l)
		Average (ppm)	% Decrease	Average (ppm)	% Decrease	Average (ppm)	% Decrease	Average (ppm)	% Decrease	
A	0	19,530		76,850		14.6		27,690		
	9	4,520	76.8	56,890	26.0	5.6	61.6	19,570	29.3	7.7
B	0	21,520		83,830		17.0		30,940		
	9	5,880	72.7	59,880	28.6	4.2	75.3	20,170	34.8	10.5
C	0	17,690		76,380		12.0		26,210		
	9	5,280	70.1	57,770	24.3	5.2	56.7	19,250	26.5	4.7
D	0	18,860		76,380		14.0		27,380		
	9	4,900	74.0	52,790	30.9	3.2	77.2	17,560	35.9	6.7

(a) All values calculated for concentrated Na-WSL.
(A) γ-Irradiated medium.
(B) SO_2-Stripped medium.
(C) γ-Irradiated and SO_2-stripped medium.
(D) SO_2-Stripped medium.

It should be noted that the culture media B in Table 3.6 and B and D in Table 3.7 are similar. However, the initial values of BOD_5, COD, TCH and TOC for the media B and D in Table 3.7 are lower because the samples used contained less suspended solids due to the natural sedimentation occurring in the storage tank after one year period.

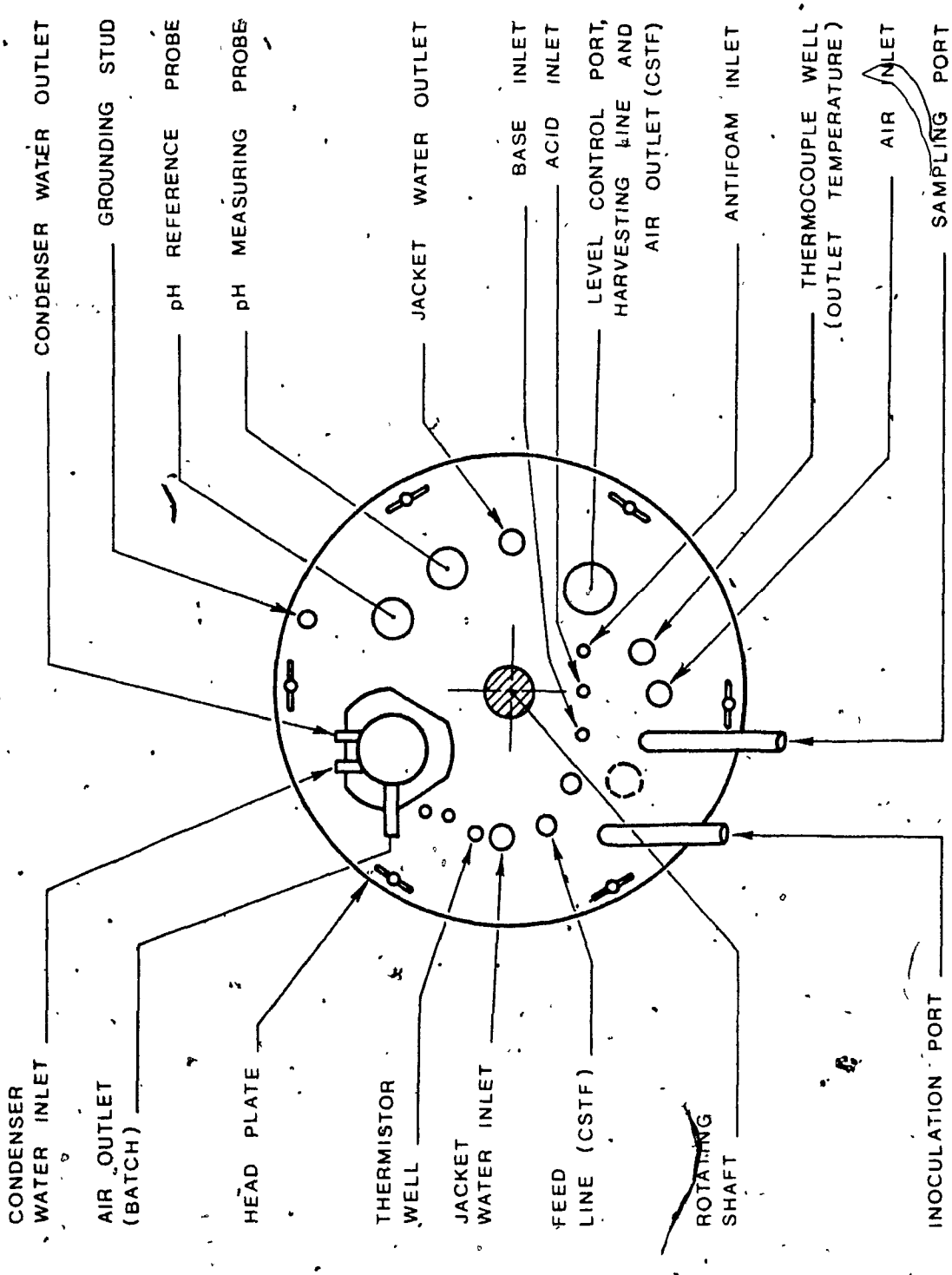
CHAPTER 4

INSTRUMENTATION AND METHODS FOR IDENTIFICATION OF THE CONTINUOUS CULTIVATION SYSTEM

4.1 Instrumentation

The studies on the continuous stirred tank fermentor (CSTF) system were conducted on the Modular Microferm Bench-Top Fermentor Series MF-114 containing 7.5 liters of the culture medium. The system of microorganism/substrate selected for this study was *M. crassipes* in NH_3-WSL, operated under quasioptimal conditions gained from preliminary studies. This system was operating under sterile conditions. The head plate penetration in Fig. 4.1 shows various injection ports to the inside of the fermentor jar. The volume of the culture medium was kept constant by using the harvesting line as the outlet air line during the continuous process. The foam was controlled partly by using a four blade turbine impeller as a mechanical foam breaker and partly by manual addition of an antifoam solution (Dow Corning Antifoam AF Emulsion) to the culture medium. Later, the antifoam (Dow Corning Antifoam C Emulsion) was directly added to the feeding medium.

Figure 4.1 Microferm MF-114 Head Plate Penetration.


CONDENSER WATER OUTLET
GROUNDING STUD
pH REFERENCE PROBE
pH MEASURING PROBE
JACKET WATER OUTLET
BASE INLET
ACID INLET
LEVEL CONTROL PORT, HARVESTING LINE AND AIR OUTLET (CSTF)
ANTIFOAM INLET
THERMOCOUPLE WELL (OUTLET TEMPERATURE)
AIR INLET
SAMPLING PORT
CONDENSER WATER INLET
AIR OUTLET (BATCH)
HEAD PLATE
THERMISTOR WELL
JACKET WATER INLET
FEED LINE (CSTF)
ROTATING SHAFT
INOCULATION PORT

4.1.1 Aeration

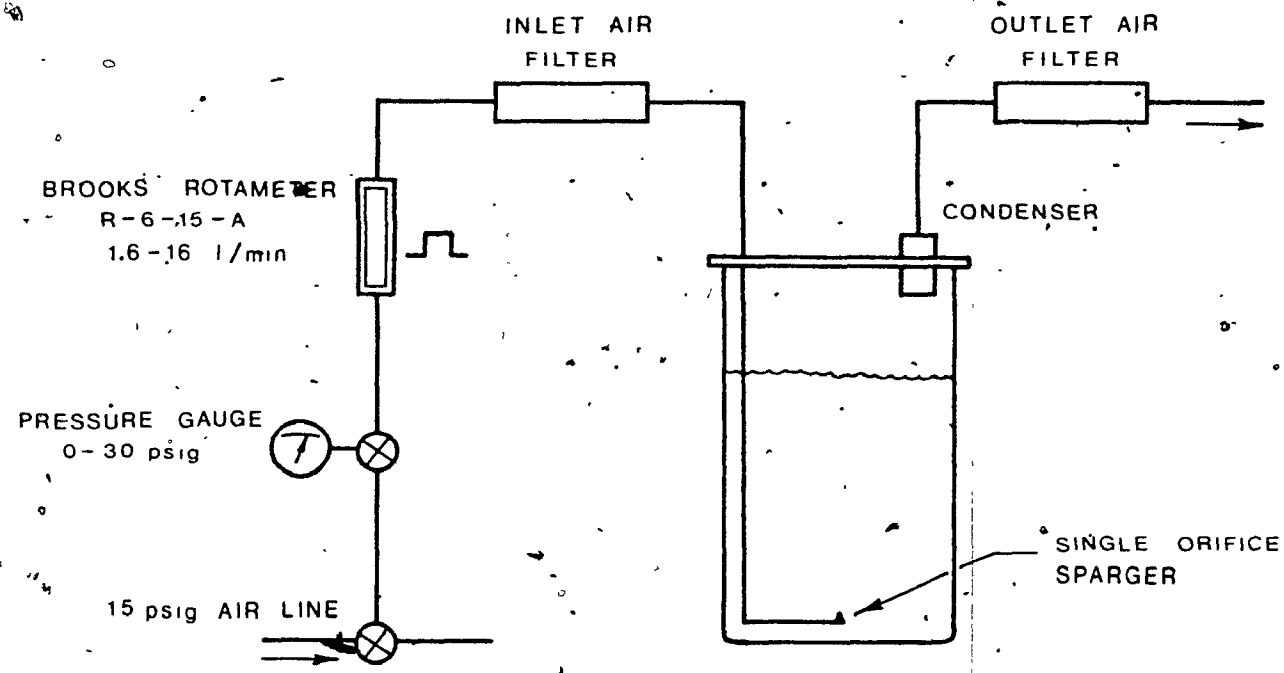
A schematic diagram of the aeration device for the CSTF is shown in Fig. 4.2. The inlet air was introduced through a pressure gauge of 0-30 psig, the Brooks rotameter No. R-6-15-A of 1.6-16.0 l/min, an inlet air filter, and it was then bubbled into the culture medium through a single-orifice sparger. The outlet air was passed through a condenser and then through an outlet air filter during the batch process or through a harvesting line during the continuous process.

The aeration rate was set at the desired value by the pressure gauge and the rotameter valves. The relationship between the aeration rate and the air pressure at the maximum opening of the air line and the rotameter valves is shown in Fig. C.1. The square pulses on the aeration rate could be performed by manipulating the pressure gauge valve.

4.1.2 Agitation

Agitation for the CSTF was performed with a 1/4 HP ball bearing drive motor coupled to a rotating shaft of the fermentor jar. The agitation rate was set at the desired value by the speed control knob. The rotation speed

Figure 4.2 Aeration Device and Measurement.


INLET AIR
FILTER
OUTLET AIR
FILTER
BROOKS ROTAMETER
R-6-15-A
1.6-16 l/min
CONDENSER
PRESSURE GAUGE
0-30 psig
SINGLE ORIFICE
SPARGER
15 psig AIR LINE

was controlled by a feedback control loop through a DC motor controller and was indicated by a tachometer. The schematic diagram for the control and measurement of the agitation device is shown in Fig. 4.3. The dimension and arrangement of the turbine impellers and the hollow baffles heat exchanger in the fermentor jar is shown in Fig. 4.4.

The square pulses on the agitation speed could be performed by manipulating the speed control set point.

4.1.3 Temperatures

The feed temperature, the outlet temperature, and the inlet and outlet jacket temperatures of the CSTF system were measured by TE miniature copper-constantan thermocouples of 1/8 inch in diameter and 8 or 12 inches long. The time constants for these thermocouples were found to be approximately 0.5 sec. for positive step responses from 0° C to 100° C and 1.0 sec. for negative step responses from 100° C to 0° C. The reference temperature source was held at 46.7 $\pm$ 0.1° C by the P.M. Tamson thermostatic water bath. The TE copper-constantan thermocouple wire was used and the reference junctions were made by soldering together the tips of these wires and coating with a thin layer of epoxy resin. These four temperatures were recorded on the Varian Aerograph Model 20 ten-inch, two-pen

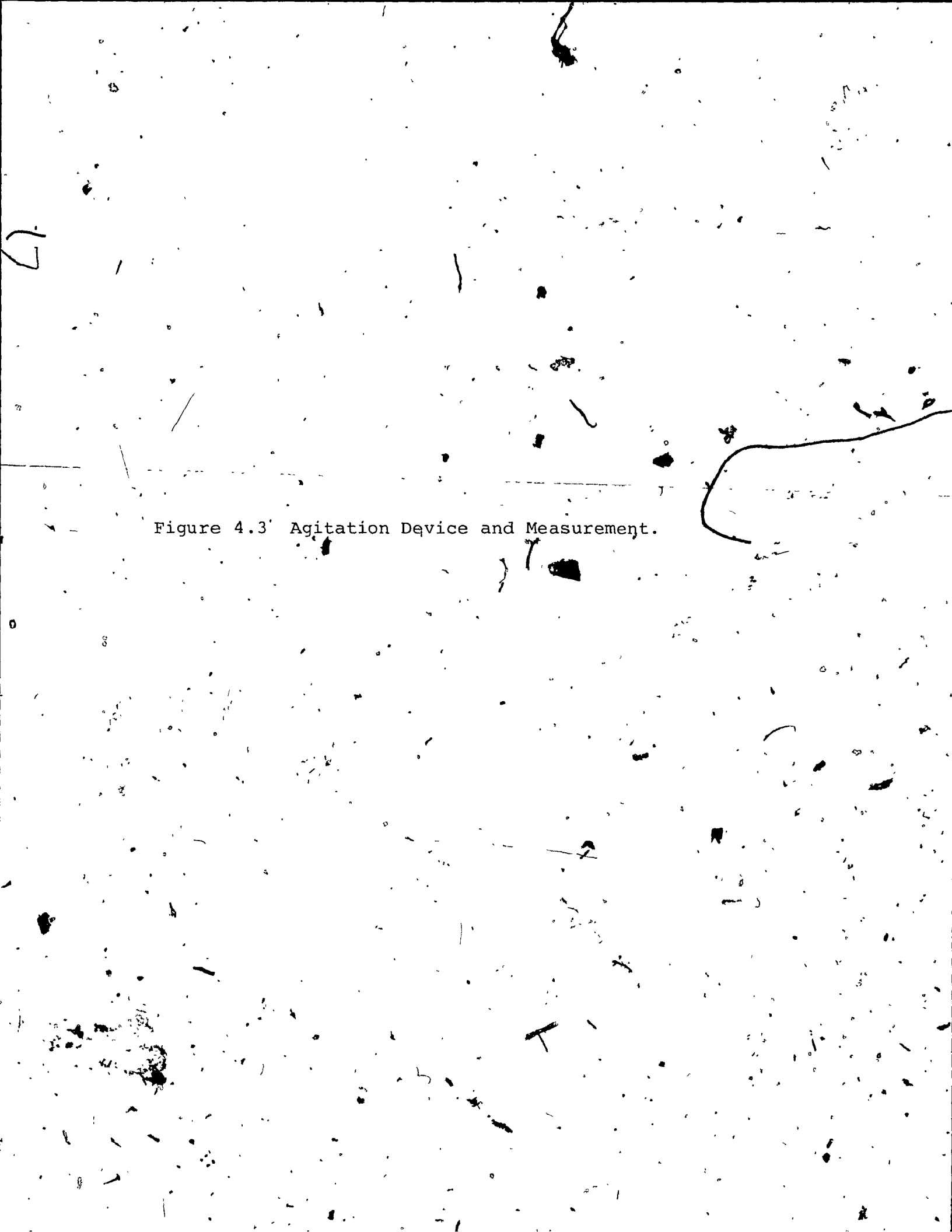


Figure 4.3 Agitation Device and Measurement.

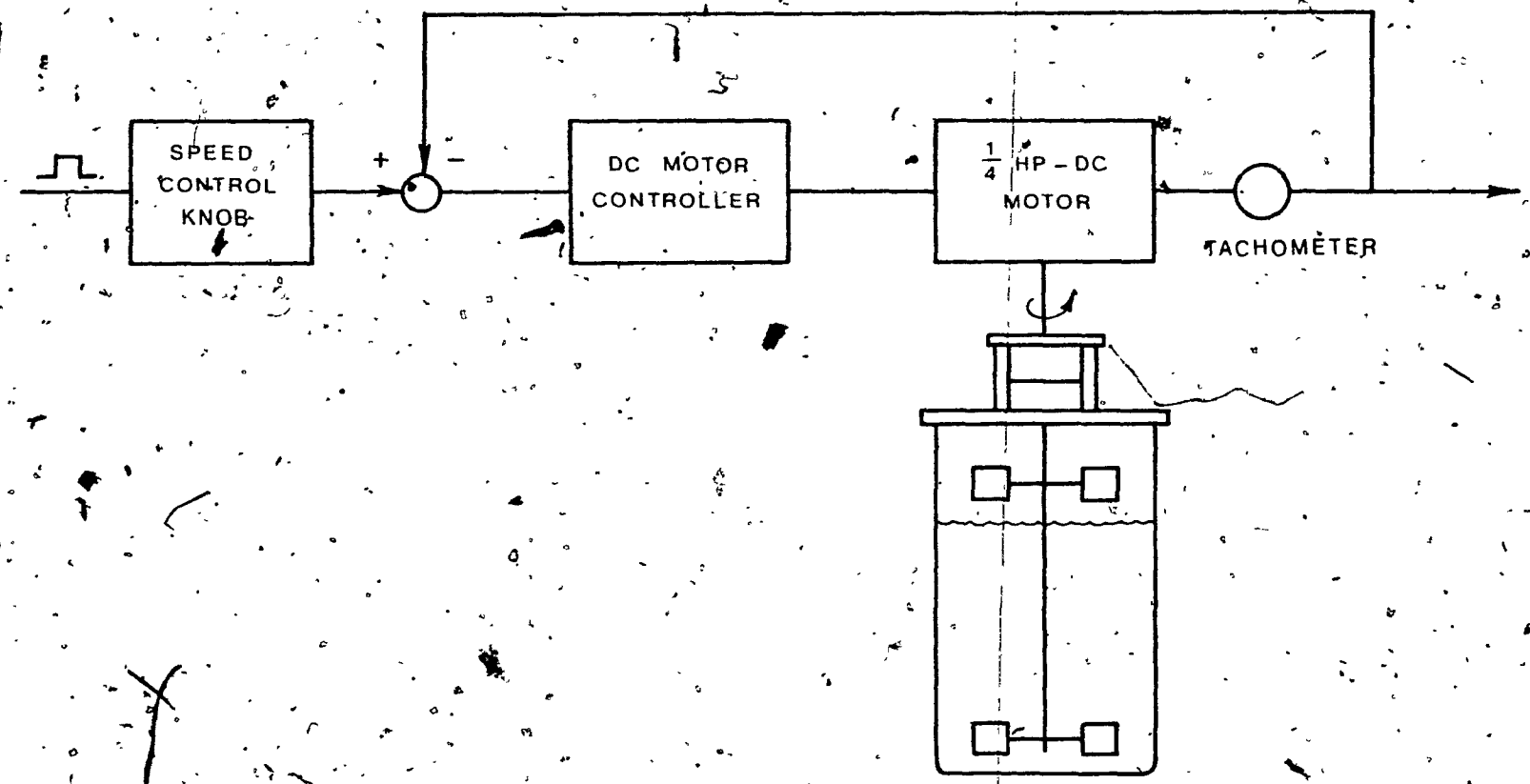
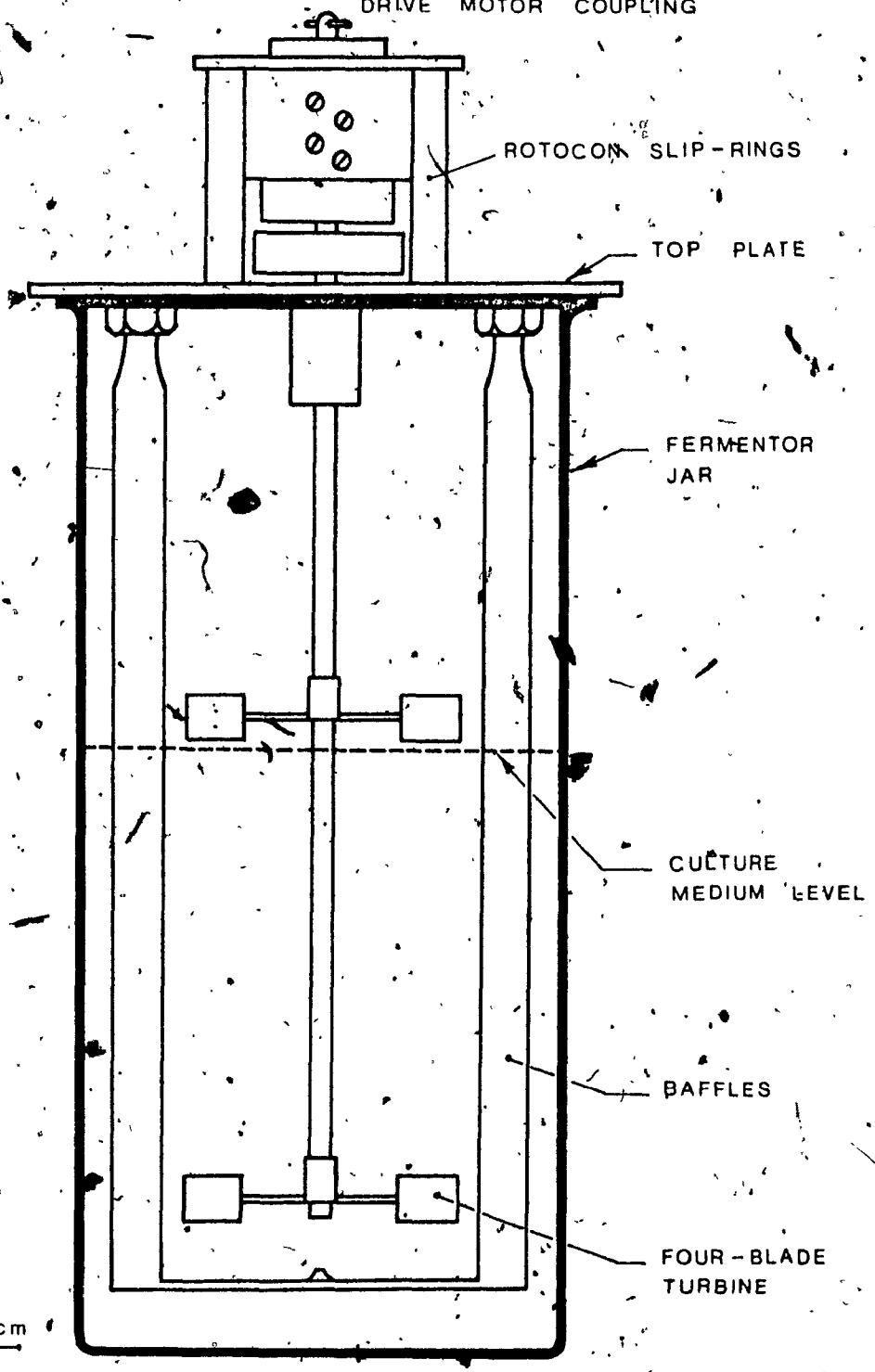

SPEED
CONTROL
KNOB
+
−
DC MOTOR
CONTROLLER
1/4 HP – DC
MOTOR
TACHOMETER

Figure 4.4 Agitation Device Arrangement.


DRIVE MOTOR COUPLING
ROTOCON SLIP-RINGS
TOP PLATE
FERMENTOR JAR
CULTURE MEDIUM LEVEL
BAFFLES
FOUR-BLADE TURBINE
1cm

strip chart recorder and the Honeywell Model Electronik 19 six-inch, two-pen strip chart recorder. Their calibration curves are shown in Figs. C.2 to C.5. The ambient temperature was followed by a wall thermometer installed at the fermentor console. A schematic diagram for the temperature measurements on the CSTF system is shown in Fig. 4.5.

The feed temperature was kept at the desired value by the Fisher temperature controlled water bath. The pulse on the feed temperature was performed by manipulating the temperature setting knob on this water bath (Fig. 4.5). The inlet jacket temperature was controlled through a feedback loop, using a solenoid valve to operate the heating coil around the inlet jacket water conduit. The temperature of the culture medium was detected by a thermistor and compared with the set point temperature at the temperature control knob. The difference between these two signals was used to operate the solenoid valve. The cold water was used partly for the inlet jacket temperature control and partly for the condenser at the outlet air stream from the fermentor jar. The flowrate of the jacket water was measured by the Brooks water flowmeter installed at the inlet port of the fermentor water jacket. The calibration curve for this water flowmeter is shown in Fig. C.6. The amplitude of the

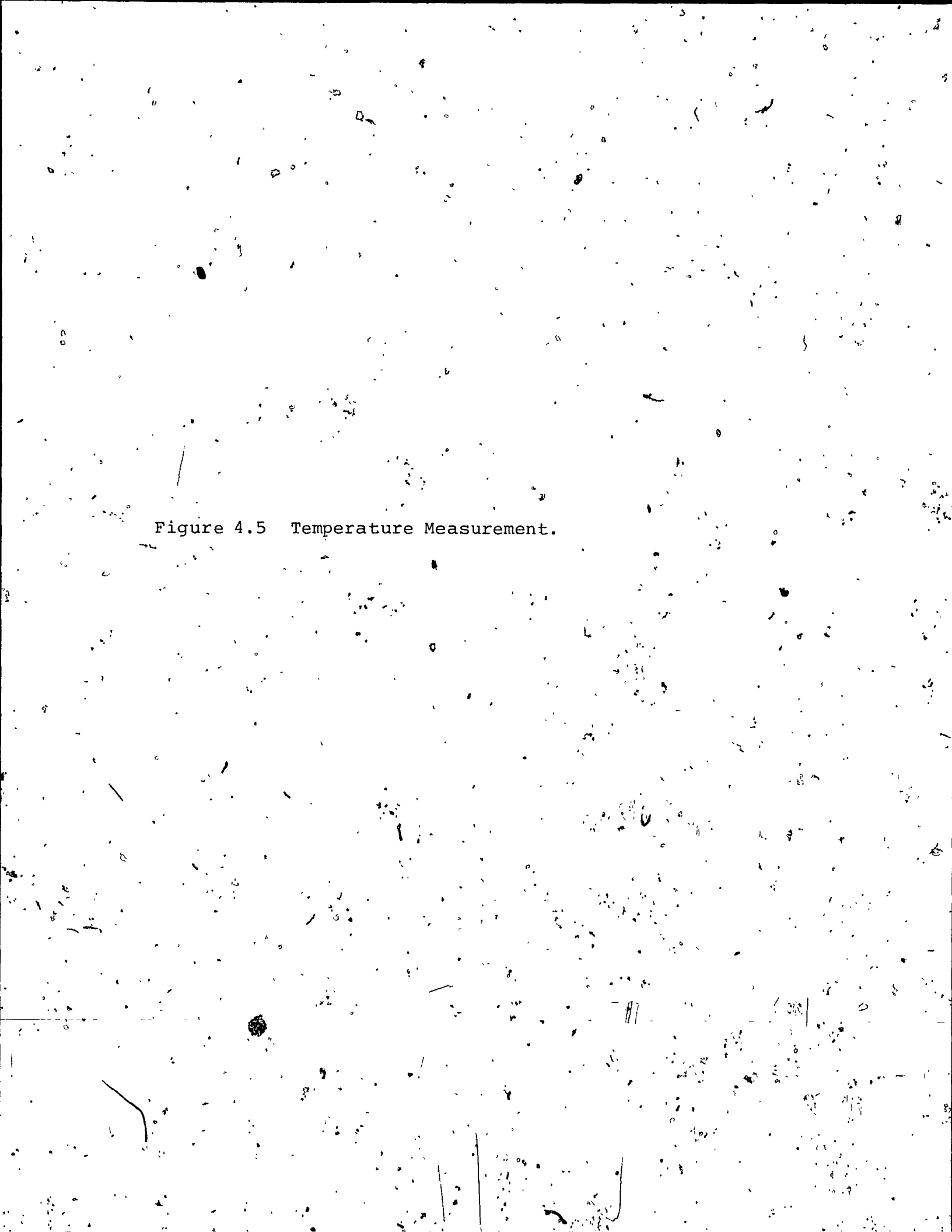
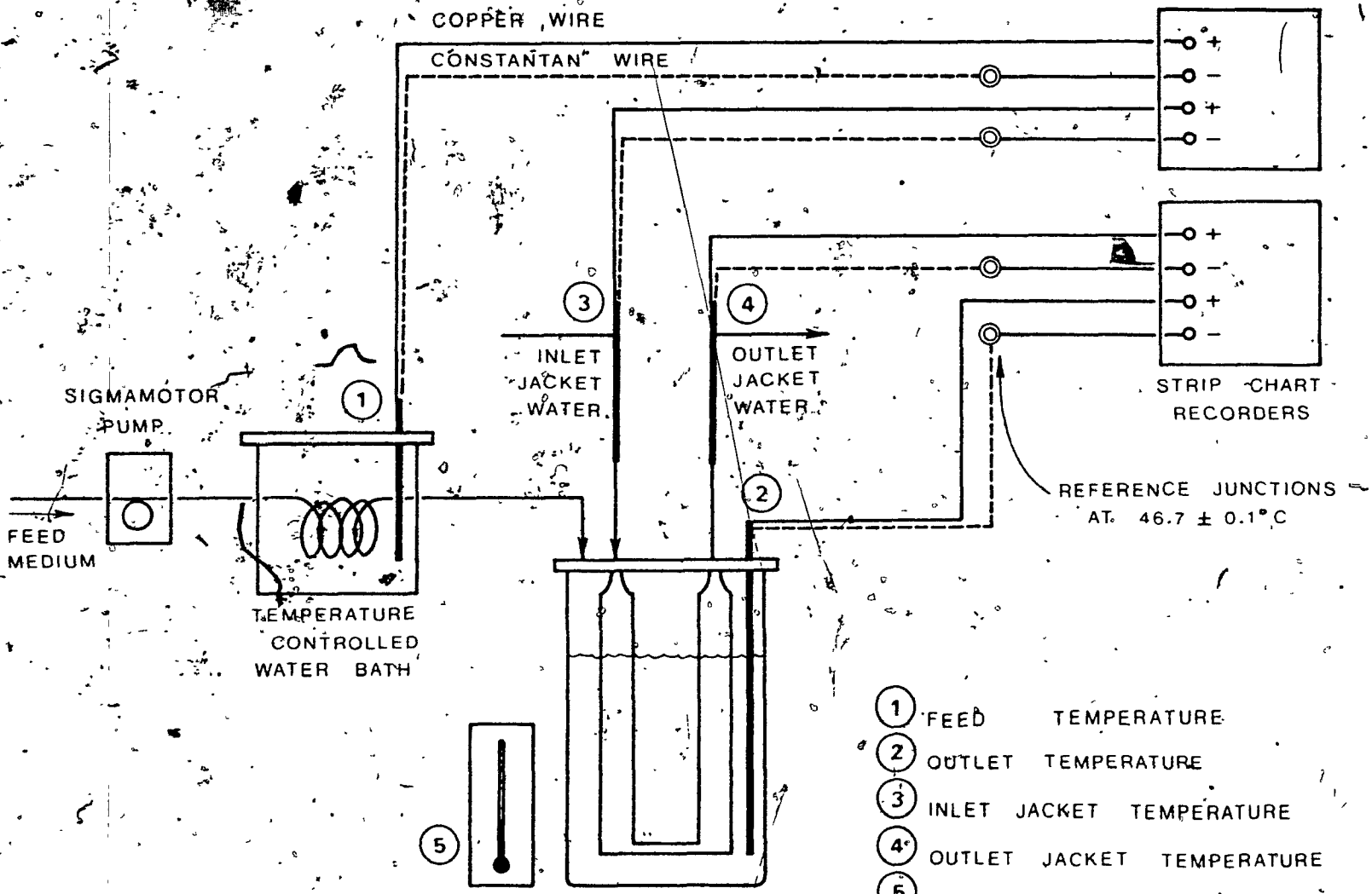


Figure 4.5 Temperature Measurement.


COPPER WIRE
CONSTANTAN WIRE
+
−
+
−
+
−
+
−
3
4
INLET JACKET WATER
OUTLET JACKET WATER
STRIP CHART RECORDERS
SIGMAMOTOR PUMP
1
2
REFERENCE JUNCTIONS AT 46.7 ± 0.1°C
FEED MEDIUM
TEMPERATURE CONTROLLED WATER BATH
5
1 FEED TEMPERATURE
2 OUTLET TEMPERATURE
3 INLET JACKET TEMPERATURE
4 OUTLET JACKET TEMPERATURE
5 AMBIENT TEMPERATURE

saw-tooth wave at the steady state value of the inlet jacket temperature was minimized by adjusting the main valve and the jacket water valve to their minimum opening. The pulse on the inlet jacket temperature was performed by manipulating the temperature control knob (Fig. 4.6).

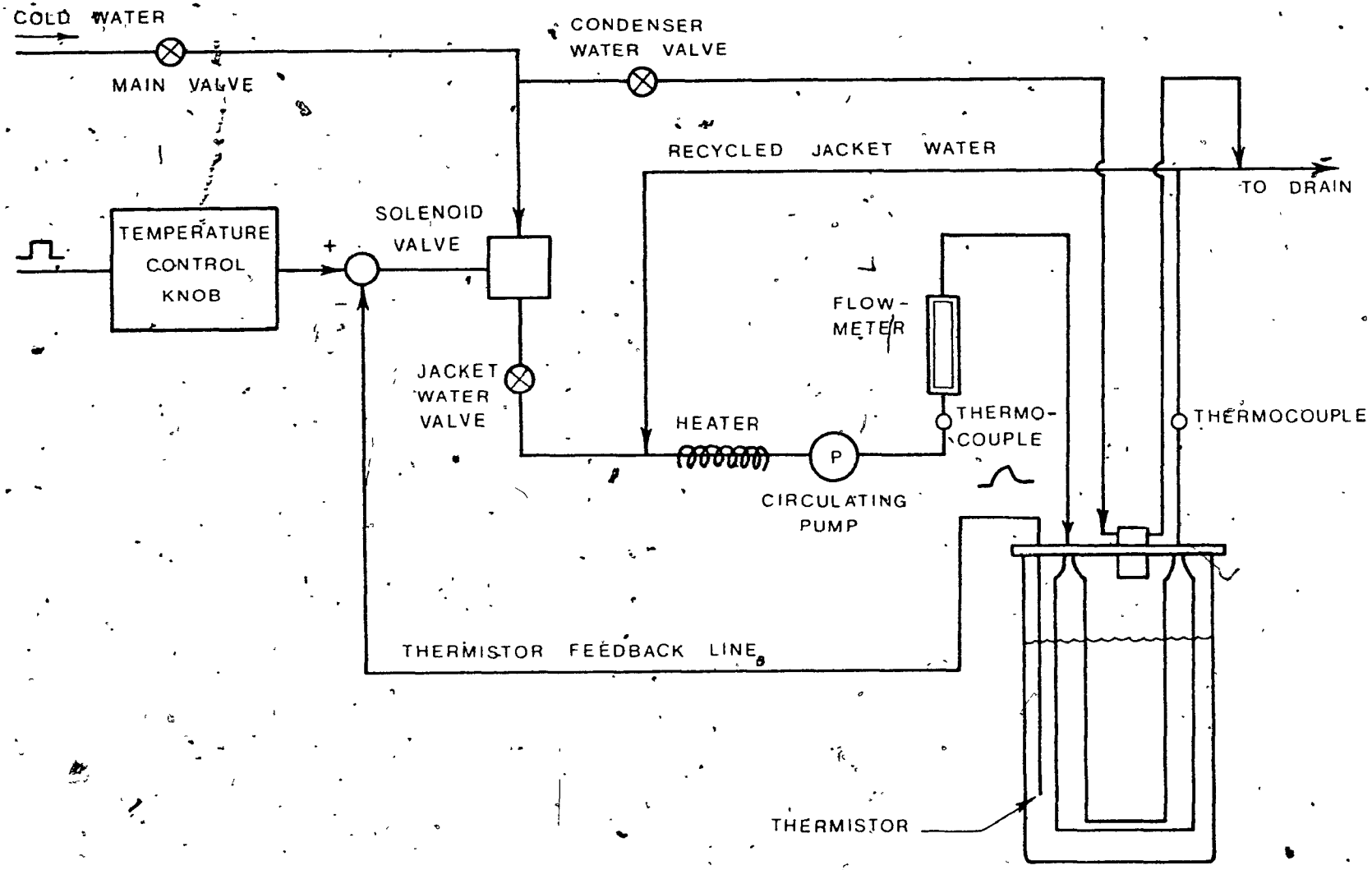
The flowrate of the feed medium was set at the desired values by the sigma-motor pump. The calibration curve for the feed pump is shown in Fig. C.7.

4.1.4 pH

The pH of the feed media and that of the samples taken from the harvesting line were measured by the Fisher Acumet Model 220 pH Meter at room temperature. The outlet pH, *i.e.* pH of the culture medium in the fermentor jar in the case of ideal mixing, was measured by the Leads & Northrup No. 117123 silver chloride glass electrode together with No. 117143 silver chloride reference electrode submerged in the culture medium. The value of pH was shown on the pH-indicator and also recorded on the pH-strip chart recorder. The calibration curve of the pH measurement system is shown in Fig. C.8.

The inlet pH of the system was adjusted to the desired value at the main feed tank. The square pulse

Figure 4.6 Jacket Water Flow and Temperature Control.


COLD WATER
MAIN VALVE
CONDENSER WATER VALVE
RECYCLED JACKET WATER
TO DRAIN
TEMPERATURE CONTROL KNOB
SOLENOID VALVE
+
−
JACKET WATER VALVE
FLOW-METER
HEATER
P
CIRCULATING PUMP
THERMO-COUPLE
THERMOCOUPLE
THERMISTOR FEEDBACK LINE
THERMISTOR

on the inlet pH was performed by replacing the main feed line by the supplement feed line, and then switching back to the original feed line after a certain period of time. The temperature, chemical composition and flow-rate of these two feed media should be identical except for their pH value. When the outlet pH is desired to be controlled, the Microferm Model pH-132 controller can be used. The pH of the culture medium was compared with the set point pH through the feedback loop and the difference between these values activated either one or another peristaltic pump for addition of acid or alkali solution to the fermentor jar.

A schematic diagram of the pH measurement and control for the CSTF system is shown by Fig. 4.7.

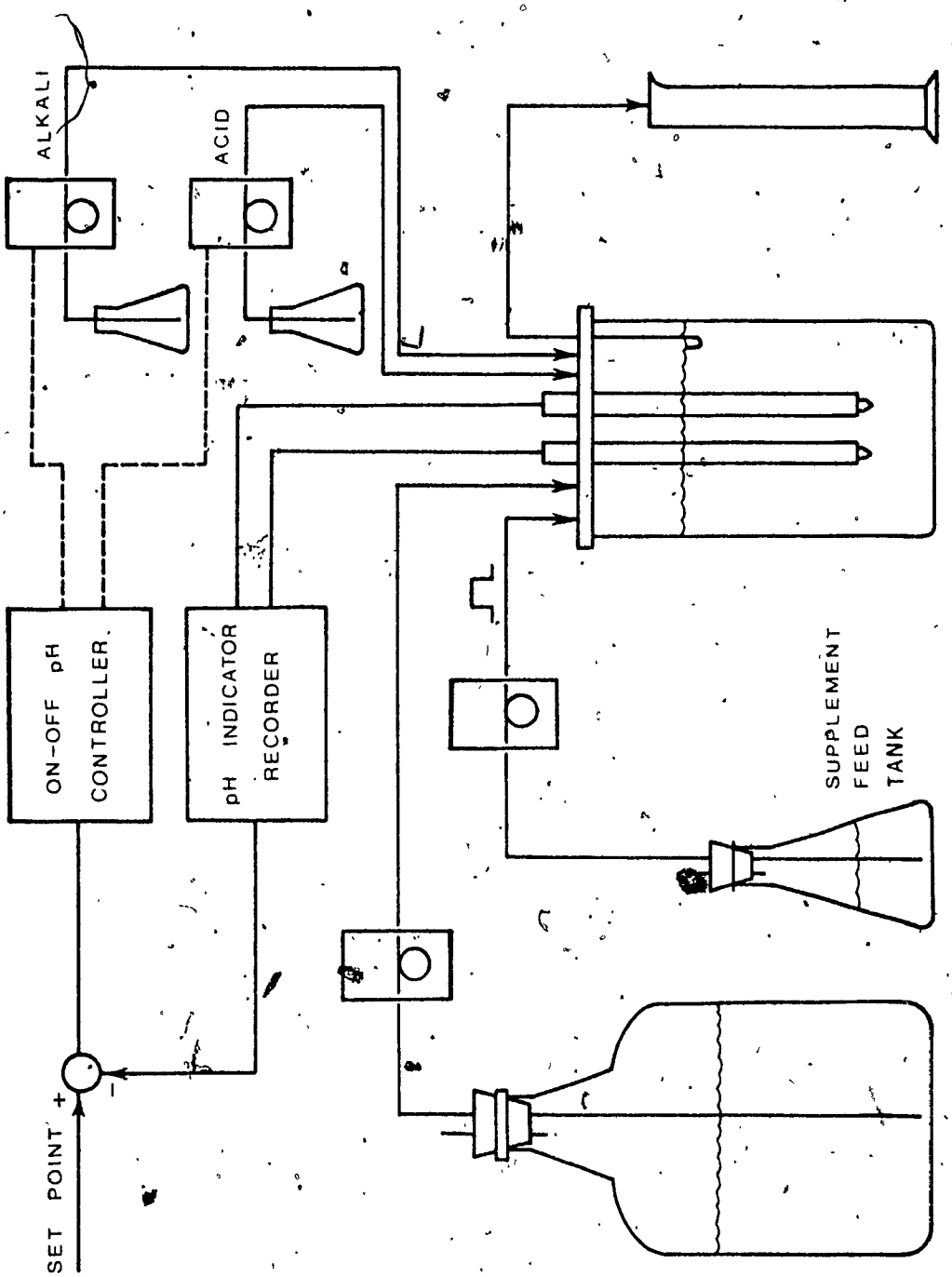
4.2 Cultivation and Analytical Methods

4.2.1 Cultivation Method

The inoculum for the CSTF system was prepared from a shake flask growth. Eight-day old growth in 3 Erlenmeyer flasks (600 ml of total culture medium) was blended for 30 seconds in 200 ml of their mother liquor and used to seed the fermentor.

The WSL-APD-CSL culture medium for the CSTF

Figure 4.7 pH Measurement and Control.


ALKALI
ACID
HARVESTING
ON-OFF pH CONTROLLER
pH INDICATOR RECORDER
SUPPLEMENT FEED TANK
SET POINT
+
-
MAIN FEED TANK

system was prepared in the same way as for the experiments in the previous batch fermentations. The pH of the culture medium was adjusted to 6.50 using a NaOH solution. The media in the fermentor as well as in the feed tank were sterilized in the autoclave at 120° C and 15 psig for 45 minutes.

The cultivation was carried out under quasi-optimal conditions for *M. crassipes* in NH_3-WSL as will be specified in the next Chapter. At the end of the exponential growth phase from the batch process, the fresh medium started to be continuously pumped from the feed tank into the fermentor jar. When the steady state for the continuous process was reached, the pulse tests on the desired inputs (independent variables) were performed.

4.2.2 Analytical Methods

The progress of fermentation was followed by analyzing and recording all inputs and outputs (dependent variables) at various time intervals. Aeration, agitation, temperature, and pH were recorded as described in paragraph 4.1. The biomass and TCH of the effluent from the harvesting line were determined by the procedures described in paragraph 2.3. The Gelman Type A glass fiber filter paper of pore size ~0.3μ was used for filtration

of cell mass instead of the regular filter paper to assure an accurate measurement of the biomass developed in a dispersed form in the CSTF system.

4.3 Numerical Methods

4.3.1 Frequency Response From Transient Response Using Pulse Technique

The general procedure for extracting the frequency response from the pulse testing data was described in detail by Clements and Schnelle (1963). The definition of the system transfer function using the Fourier transform is written,

$$G(j\omega) = \frac{F\{y(t)\}}{F\{x(t)\}} = \frac{\int_o^\infty y(t)e^{-j\omega t}dt}{\int_o^\infty x(t)e^{-j\omega t}dt} \qquad (4.1)$$

where,

$G(j\omega)$ = transfer function of the system

ω = frequency (rad/sec)

F = operator for Fourier transformation

$x(t)$ = input pulse signal to the system

$y(t)$ = output response signal from the system

t = time (sec)

Using Euler's relationship,

$$e^{-jwt} = \cos wt - j \sin wt \qquad (4.2)$$

the Eq. (4.1) becomes,

$$G(jw) = \frac{A-jB}{C-jD} = Re + j\,Im \qquad (4.3)$$

provided,

$$A = \int_o^{t_y} y(t) \cos wt\, dt \qquad (4.4)$$

$$B = \int_o^{t_y} y(t) \sin wt\, dt \qquad (4.5)$$

$$C = \int_o^{t_x} x(t) \cos wt\, dt \qquad (4.6)$$

$$D = \int_o^{t_x} x(t) \sin wt\, dt \qquad (4.7)$$

$$Re = \frac{AC+BD}{C^2+D^2} \qquad (4.8)$$

$$Im = \frac{AD-BC}{C^2+D^2} \qquad (4.9)$$

where,

t_x = duration of the input pulse signal (sec)

t_y = duration of the output response signal (sec)

The Bode diagram may be constructed from,

$$AR = -20 \log_{10} |G(jw)| = -20 \log_{10} \sqrt{Re^2+Im^2} \qquad (4.10)$$

$$\phi = \frac{\pi}{180} \tan^{-1}\left(\frac{Im}{Re}\right) \tag{4.11}$$

where,

AR = amplitude ratio (decibels)

ϕ = phase shift (degree)

Four integrals (4.4) to (4.7) were numerically evaluated throughout the wide frequency spectrum for w using Filon's method [Filon (1928)]. The Filon's quadrature formulas for $\int_a^b \Psi(t) \sin wt\, dt$ and $\int_a^b \Psi(t) \cos wt\, dt$ give the parabolic approximation to the pulse curves $\Psi(t)$ with an accuracy that is independent of the value of w and reduce to Simpson's rule as $w \to 0$. The curve $\Psi(t)$ is divided into an odd number, $2n + 1$, of points of intervals Δt. Denote these points by I_0, I_1, I_2, ..., I_{2n}, then

$$\int_a^b \Psi(t) \sin wt\, dt \cong \Delta t \{\alpha[\Psi(a) \cos wa - \Psi(b) \cos wb] + \beta S_{2n} + \gamma S_{2n-1}\} \tag{4.12}$$

$$\int_a^b \Psi(t) \cos wt\, dt \cong \Delta t \{\alpha[\Psi(a) \cos (wa + \frac{\pi}{2}) - \Psi(b) \cos (wb + \frac{\pi}{2})] + \beta S_{2n} + \gamma S_{2n-1}\} \tag{4.13}$$

provided,

for (4.12): $I_i = \Psi(t_i) \cdot \sin wt_i \quad i = 1, 2n$ (4.14)

for (4.13): $I_i = \Psi(t_i) \cos \omega t_i \quad i = 1, 2n$ (4.15)

and,

$$S_{2n} = \frac{1}{2} I_o + I_2 + \ldots + I_{2n-2} + \frac{1}{2} I_{2n} \tag{4.16}$$

$$S_{2n-1} = I_1 + I_3 + \ldots + I_{2n-1} \tag{4.17}$$

$$\theta = \omega \Delta t \tag{4.18}$$

$$\alpha = \frac{1}{\theta} + \frac{\sin 2\theta}{2\theta^2} - \frac{2 \sin^2 \theta}{\theta^3} \tag{4.19}$$

$$\beta = 2 \left[\frac{\cos^2 \theta + 1}{\theta^2} - \frac{\sin 2\theta}{\theta^3}\right] \tag{4.20}$$

$$\gamma = 4 \left[\frac{\sin \theta}{\theta^3} - \frac{\cos \theta}{\theta^2}\right] \tag{4.21}$$

The listing of a FORTRAN program for complete conversion of the transient response to the frequency response named program PULSE, is shown in Table D.1.

4.3.2 Transfer Function From Frequency Response Using Linear and Nonlinear Regression Techniques

Due to simpler expressions for the amplitude ratio and to the difficulty in determining the quadrant of angles on a digital computer, only the amplitude ratio versus frequency from the Bode diagram was curve fitted to the functional forms by the nonlinear regression technique.

i) Method of Law and Bailey (1963)

The transfer function was assumed as,

$$G(s) = \frac{1}{(T_1 s+1)\ (T_2 s+1)\ \ldots\ (T_k s+1)\ \ldots\ (T_M s+1)} \tag{4.22}$$

with the magnitude,

$$|G(s)| = |G(jw)| = \left[\prod_{k=1}^{M} (T_k^2\ w^2 + 1)\right]^{-1/2} \tag{4.23}$$

where,

T_k = system time constants (sec^{-1})

M = order of the system

$|G(s)| = |G(jw)|$ = magnitude of the transfer function

w = frequency (rad/sec)

s = variable in the domain of Laplace transform.

It is desired to fit an equation of the form,

$$Y_i = \phi\ (w_i,\ B_1,\ B_2,\ \ldots,\ B_k,\ \ldots,\ B_M)\ \pm e_y$$

$$i = 1,\ 2,\ \ldots,\ N \tag{4.24}$$

provided,

$$B_k = T_k^2 \tag{4.25}$$

where,

Y_i = dependent variable, experimental value of the amplitude ratio of $G(jw)$

w_i = independent variable, frequency (rad/sec)

N = number of data points

B_k = unknown parameters

e_y = experimental errors involved in the Y_i. These are assumed independent and normally distributed with mean zero and variance σ^2.

Assume that there is no error in the independent variables in Eq. (4.24); the method of least squares states that in order to obtain the best set of parameters, B_k, it is proper to seek an unrestricted minimum of the following,

$$S_a^2 = \sum_{i=1}^{N} (Y_i - \phi)^2 = \sum_{i=1}^{N} S_i^2 \tag{4.26}$$

If ϕ is expanded into a Taylor series about the parameters, B_k, the following is obtained by truncating all but the linear terms:

$$\phi^{(j+1)} \cong \phi^{(j)} + \left[\frac{\partial \phi}{\partial B_1}\right]^{(j)} \Delta B_1^{(j)} + \left[\frac{\partial \phi}{\partial B_2}\right]^{(j)} \Delta B_2^{(j)} + \ldots$$

$$+ \left[\frac{\partial \phi}{\partial B_k}\right]^{(j)} \Delta B_k^{(j)} + \ldots + \left[\frac{\partial \phi}{\partial B_M}\right]^{(j)} \Delta B_M^{(j)} \tag{4.27}$$

where superscript (j) refers to iteration (j). That is,

$\phi^{(j)}$ is ϕ evaluated using the parameters during iteration j. And,

$$\Delta B_k^{(j)} = B_k^{(j+1)} - B_k^{(j)} \tag{4.28}$$

The function ϕ in Eq. (4.24) may be replaced by the approximation in Eq. (4.27). If the resulting expression is substituted into Eq. (4.26) and if it is desired to seek an unrestricted minimum of S_a^2, that is,

$$\frac{\partial \sum_{i=1}^{N} S_i^2}{\partial B_k} \equiv 0 \tag{4.29}$$

M simultaneous linear algebraic equations are obtained which can be represented in the following matrix form,

$$\begin{bmatrix} \sum_{i=1}^{N} Z_1 Z_1 & \sum_{i=1}^{N} Z_1 Z_2 & \cdots & \sum_{i=1}^{N} Z_1 Z_M \\ \sum_{i=1}^{N} Z_2 Z_1 & \sum_{i=1}^{N} Z_2 Z_2 & \cdots & \sum_{i=1}^{N} Z_2 Z_M \\ \vdots & \vdots & & \vdots \\ \sum_{i=1}^{N} Z_M Z_1 & \sum_{i=1}^{N} Z_M Z_2 & \cdots & \sum_{i=1}^{N} Z_M Z_M \end{bmatrix} \begin{bmatrix} \Delta B_1^{(j)} \\ \Delta B_2^{(j)} \\ \vdots \\ \Delta B_M^{(j)} \end{bmatrix} = \begin{bmatrix} C_1 \\ C_2 \\ \vdots \\ C_M \end{bmatrix} \tag{4.30}$$

where,

$$Z_k = \left[\frac{\partial \phi}{\partial B_k}\right]^{(j)} \tag{4.31}$$

$$C_k = \sum_{i=1}^{N} \left[S_i (Z_k)_i\right]^{(j)} \tag{4.32}$$

The Eq. (4.30) may be written in the shorthand matrix notation,

$$\underset{\sim}{Z} \cdot \underset{\sim}{B} = \underset{\sim}{C} \tag{4.33}$$

and hence,

$$\underset{\sim}{B} = \underset{\sim}{Z}^{-1} \cdot \underset{\sim}{C} \tag{4.34}$$

If the scheme is converging to the values of B_k which minimize $S_a{}^2$, the vector $\underset{\sim}{B}$ will represent the differences given in Eq. (4.28) which can be solved for $B_k^{(j+1)}$. These new parameters will be better values of the B_k, and they can be used in the next iteration and the procedure repeated until the corrections, ΔB_k, become negligibly small.

Law and Bailey (1963) also developed the criteria to ensure the convergence and avoid a slow convergence during the iteration process, if there is a unique solution for B_k. The F-test on $S_a{}^2$ was used to select the simplest adequate representation among the first-, second- and third-order transfer functions for the system studied.

The listing of the FORTRAN program, entitled

program NLREG1, for this nonlinear regression technique is shown in Table D.2. This technique gives rapid results and provides a relatively simple method for comparing the transfer functions. However, if the parameters B_k are negative, it is impossible to determine T_k as shown by the Eq. (4.25).

ii) Method of Levy (1959)

The transfer function was assumed under the form,

$$G(s) = \frac{b_o+b_1 s+b_2 s^2+ \ldots + b_l s^l + \ldots + b_K s^K}{a_o+a_1 s+a_2 s^2+ \ldots + a_k s^k + \ldots + a_M s^M} \qquad (4.35)$$

where,

a_k, b_l = unknown parameters

M = lag-order of the system

K = lead-order of the system.

The Eq. (4.35) may be written,

$$G(j\omega) = \frac{\alpha+j\omega\beta}{\sigma+j\omega\tau} = \frac{N(\omega)}{D(\omega)} \qquad (4.36)$$

provided,

$$\alpha = b_o - b_2 \omega^2 + b_4 \omega^4 - \ldots \qquad (4.37)$$

$$\beta = b_1 - b_3 \omega^2 + b_5 \omega^4 - \ldots \qquad (4.38)$$

$$\sigma = a_o - a_2 w^2 + a_4 w^4 - \ldots \quad (4.39)$$

$$\tau = a_1 - a_3 w^2 + a_5 w^4 - \ldots \quad (4.40)$$

Let $F(jw)$ represent the experimental function of the frequency response curve,

$$F(jw) = R(w) + j I(w) \quad (4.41)$$

where,

$R(w)$ = real part of $F(jw)$

$I(w)$ = imaginary part of $F(jw)$

At any specific value of frequency, w_K, the error in fitting is then,

$$\varepsilon(w_K) = F(jw_K) - G(jw_K)$$

$$= F(jw_K) - \frac{N(w_K)}{D(w_K)} \quad (4.42)$$

Thus the problem is to minimize this error at each data point on the curve. To avoid difficulties, Levy (1959) modified the least squares philosophy and minimized the weighed error function, which is defined as,

$$E(w_K) = D(w_K) \cdot \varepsilon(w_K)$$

$$= D(w_K) \cdot F(jw_K) - N(w_K)$$

$$= A(w_K) + j B(w_K) \quad (4.43)$$

where,

$A(\omega_K)$ = the real part of $E(\omega_K)$,

$B(\omega_K)$ = the imaginary part of $E(\omega_K)$.

Using the Eqs. (4.36) and (4.41), the values of $A(\omega_K)$ and $B(\omega_K)$ become,

$$A(\omega_K) = \sigma_K R(\omega_K) - \omega_K \tau_K I(\omega_K) - \alpha_K \tag{4.44}$$

$$B(\omega_K) = \omega_K \tau_K R(\omega_K) + \sigma_K I(\omega_K) - \omega_K \beta_K \tag{4.45}$$

It is desired to minimize the sum of squares of the magnitude of $E(\omega_K)$ over the sampling frequencies,

$$\begin{aligned} E &= \sum_{K=0}^{\Omega} |E(\omega_K)|^2 \\ &= \sum_{K=0}^{\Omega} [A^2(\omega_K) + B^2(\omega_K)] \\ &= \sum_{K=0}^{\Omega} \{[\sigma_K R(\omega_K) - \omega_K \tau_K I(\omega_K) - \alpha_K]^2 \\ &\quad + [\omega_K \tau_K R(\omega_K) + \sigma_K I(\omega_K) - \omega_K \beta_K]^2\} \end{aligned} \tag{4.46}$$

where,

Ω = number of data points - 1

or,

$$\frac{\partial E_a}{\partial a_k} \equiv 0; \quad k = 1,2,\ldots, M \tag{4.47}$$

$$\frac{\partial E_a}{\partial b_l} \equiv 0; \quad l = 1,2,\ldots, K \tag{4.48}$$

Using expressions for α, β, σ, τ as defined in the Eqs. (4.37 to 40), and after simplification, $K + M$ simultaneous linear algebraic equations are obtained which may be represented in the following matrix form,

$$\begin{bmatrix}
\lambda_0 & 0 & -\lambda_2 & 0 & \lambda_4 & \ldots & T_1 & S_2 & -T_3 & -S_4 & T_5 & \ldots \\
0 & \lambda_2 & 0 & -\lambda_4 & 0 & \ldots & -S_2 & T_3 & S_4 & -T_5 & -S_6 & \ldots \\
\lambda_2 & 0 & -\lambda_4 & 0 & \lambda_6 & \ldots & T_3 & S_4 & -T_5 & -S_6 & T_7 & \ldots \\
0 & \lambda_4 & 0 & -\lambda_6 & 0 & \ldots & -S_4 & T_5 & S_6 & -T_7 & -S_8 & \ldots \\
\vdots & \vdots & \vdots & \vdots & \vdots & & \vdots & \vdots & \vdots & \vdots & \vdots & \\
T_1 & -S_2 & -T_3 & S_4 & T_5 & \ldots & U_2 & 0 & -U_4 & 0 & U_6 & \ldots \\
S_2 & T_3 & -S_4 & -T_5 & S_6 & \ldots & 0 & U_4 & 0 & -U_6 & 0 & \ldots \\
T_3 & -S_4 & -T_5 & S_6 & T_7 & \ldots & U_4 & 0 & -U_6 & 0 & U_8 & \ldots \\
\vdots & \vdots & \vdots & \vdots & \vdots & & \vdots & \vdots & \vdots & \vdots & \vdots &
\end{bmatrix}
\begin{bmatrix} b_0 \\ b_1 \\ b_2 \\ b_3 \\ \vdots \\ a_1 \\ a_2 \\ a_3 \\ \vdots \end{bmatrix}
=
\begin{bmatrix} S_0 \\ T_1 \\ S_2 \\ T_3 \\ \vdots \\ 0 \\ U_2 \\ 0 \\ \vdots \end{bmatrix} \tag{4.49}$$

provided,

$$\lambda_h = \sum_{K=0}^{\Omega} w_K^{\,h} \tag{4.50}$$

$$S_h = \sum_{K=0}^{\Omega} \omega_K^h \, R(\omega_K) \tag{4.51}$$

$$T_h = \sum_{K=0}^{\Omega} \omega_K^h \, I(\omega_K) \tag{4.52}$$

$$U_h = \sum_{K=0}^{\Omega} \omega_K^h \, [R^2(\omega_K) + I^2(\omega_K)] \tag{4.53}$$

$$h = 1, 2, \ldots, K \text{ (or } M)$$

The Eq. (4.49) may be written in the shorthand matrix notation,

$$\underset{\sim}{M} \cdot \underset{\sim}{N} = \underset{\sim}{D} \tag{4.54}$$

and hence,

$$\underset{\sim}{N} = \underset{\sim}{M}^{-1} \cdot \underset{\sim}{D} \tag{4.55}$$

The numerical value of the unknown parameters a_k and b_l may now be determined once the coefficients of the matrix $\underset{\sim}{M}$ and $\underset{\sim}{D}$ have been evaluated.

The listing of the FORTRAN program, entitled program LNREG2, prepared for the third order lead-lag system transfer function is shown in the Table D.3. Unfortunately, however, three serious deficiencies exist for this technique. Firstly, the order M of the transfer function must be accurately known beforehand. Secondly, if $\omega_K (0 < K < \Omega)$ extends over several decades, the elements

of M involving the lower frequencies are not very significant, so that a good fit cannot be obtained at the lower frequencies. Thirdly, if $G(s)$ has poles such that $|D(jw)|^2$ could vary widely throughout the frequency range, large errors can be introduced.

CHAPTER 5

TRANSFER FUNCTION MATRIX OF CONTINUOUS CULTIVATION SYSTEM OF *M. CRASSIPES* IN NH_3-WSL UNDER QUASIOPTIMAL CONDITIONS

5.1 Principle

Dynamic behaviour of the deterministic systems may be theoretically or experimentally determined [Himmelblau and Bischoff (1968)]. The procedures for development of mathematical models in order to characterize the process dynamics are usually based on the concept that any system could be described by one or a set of integrodifferential equations. For practical purposes, these integrodifferential equations are transformed and expressed in the frequency domain, under the name transfer function of the system. Among various techniques used in system modeling, the experimental methods, identification, have shown a great advantage for the complex systems. According to the basic principle of these identification methods, the transfer function of the system is deducted from the analysis of the informations obtained from the excitations and their response in the time domain.

The continuous cultivation (or fermentation) process is a complex system, in which multiple physico-chemical transport phenomena (heat, mass, momentum and electron transfers) and biochemical reactions occured. The CSTF system may be considered as a multivariable system with p inputs (independent variables) and q outputs (dependent variables). If the system is linear or linearized around its operating point (optimal conditions), the relationship between the ith output and the jth input may be described by the following nth order differential equation with constant coefficients,

$$a_o^i \frac{d^n y^i(t)}{dt^n} + a_1^i \frac{d^{n-1} y^i(t)}{dt^{n-1}} + \ldots + a_{n-1}^i \frac{dy^i(t)}{dt} + a_n^i y^i(t) =$$

$$b_o^j \frac{d^m x^j(t)}{dt^m} + b_1^j \frac{d^{m-1} x^j(t)}{dt^{m-1}} + \ldots + b_{m-1}^j \frac{dx^j(t)}{dt} + b_m^j x^j(t)$$

$$m < n; \; i = 1, 2, \ldots, q; \; j = 1, 2, \ldots, p \qquad (5.1)$$

where $y^i(t)$ = output,

$x^j(t)$ = input,

a^i, b^j = constants.

The differential equation in Eq. (5.1) provides a complete description of the system behaviour between the jth input and the ith output. Once the input and the initial conditions of the system are specified, the output response is

obtained by solving Eq. (5.1). However, it is apparent that the differential equation method of describing a system is rather cumbersome and of little practical use. Take the Laplace transform of both sides of Eq. (5.1) and assuming zero initial conditions,

$$[a_o^i s^n + a_1^i s^{n-1} + \ldots + a_{n-1}^i s + a_n^i] Y^i(s) =$$

$$[b_o^j s^m + b_1^j s^{m-1} + \ldots + b_{m-1}^j s + b_m^j] X^j(s) \qquad (5.2)$$

By definition, the transfer function of the system behaviour between the jth input and the ith output is the ratio of $Y^i(s)$ to $X^j(s)$, therefore,

$$G_{ij}(s) = \frac{Y^i(s)}{X^j(s)}$$

$$= \frac{b_o^j s^m + b_1^j s^{m-1} + \ldots + b_{m-1}^j s + b_m^j}{a_o^i s^n + a_1^i s^{n-1} + \ldots + a_{n-1}^i s + a_n^i}$$

$$= \frac{(\tau_{ij_1} s+1)(\tau_{ij_2} s+1) \ldots (\tau_{ijm} s+1)}{(T_{ij_1} s+1)(T_{ij_2} s+1) \ldots (T_{ijn} s+1)} \qquad (5.3)$$

where,

τ = lead-order time constant (sec^{-1})

T = lag-order time constant (sec^{-1}).

The characteristics of a linear system depend solely on the properties of system elements; therefore, the transfer function $G_{ij}(s)$ is a property of the system elements only, and is independent of excitation and initial conditions.

It should be noted that the Eq. (5.3) is defined with only the jth input in effect, while the other inputs are assumed to be zero. The ith output transform of the system is related to all the input transforms by,

$$Y^i(s) = G_{i1}(s)\,X^1(s) + G_{i2}(s)\,X^2(s) + \ldots + G_{ip}(s)\,X^p(s)$$

$$= \sum_{k=1}^{p} G_{ik}(s)\,X^k(s) \qquad i = 1,2,\ldots,q \qquad (5.4)$$

where $G_{ij}(s)$ is defined in Eq. (5.3). It is convenient to represent Eq. (5.4) by a matrix equation,

$$\underset{\sim}{Y}(s) = \underset{\sim}{G}(s) \cdot \underset{\sim}{X}(s) \qquad (5.5)$$

where,

$$\underset{\sim}{Y}(s) = \begin{bmatrix} Y^1(s) \\ Y^2(s) \\ \vdots \\ Y^q(s) \end{bmatrix} \qquad (5.6)$$

is a qx1 matrix and is called the transformed output vector,

$$\underset{\sim}{X}(s) = \begin{pmatrix} X^1 & (s) \\ X^2 & (s) \\ \vdots & \\ X^p & (s) \end{pmatrix} \tag{5.7}$$

is a px1 matrix and is called the transformed input vector,

$$\underset{\sim}{G}(s) = \begin{pmatrix} G_{11}(s) & G_{12}(s) & \cdots & G_{1p}(s) \\ G_{21}(s) & G_{22}(s) & \cdots & G_{2p}(s) \\ \vdots & & & \\ G_{q1}(s) & G_{q2}(s) & \cdots & G_{qp}(s) \end{pmatrix} \tag{5.8}$$

is a qxp matrix and is called the transfer function matrix.

Table 5.1 shows a list of common variables which may frequently be used as inputs [Eq. (5.7)] and outputs [Eq. (5.6)] for a CSTF system. It appears that the complete study of all system variables is extremely difficult if not impossible for this complex biophysico-chemical system. In general, a certain number of important variables are selected for study depending upon the specific objective of the individual CSTF process. The inputs and outputs for the *M. crassipes* in NH_3-WSL CSTF systems are

Table 5.1 Inputs-Outputs for CSTF System.

Inputs	Outputs
Fermentation conditions:	Products:
Temperatures	Biomass
Aeration	Cytology
Agitation	Morphology
Contamination	Intra and/or extracellular products
Foam	Final BOD, COD, TOC,...
Viscosity	Viscosity
Geometrical configuration	By-products:
Nutrients: (Quality and/or quantity)	Final pH
pH	Residual carbon
Carbon sources	Residual nitrogen
Nitrogen sources	Residual growth factors
Growth factors	Residual trace nutrients
Trace nutrients	Intra and/or extracellular by-products
BOD, COD, TOC,...	
Inoculum:	
Age	
Size	
Preparation method	

shown in Fig. 5.1. The objective is to determine the transfer function matrix $\underset{\sim}{G}(s)$ for this system.

Each element $G_{ij}(s)$ of the transfer function matrix in Eq. (5.8) was determined by the procedure shown in Fig. 5.2. The program PULSE converted the input $x^j(t)$ and output $y^i(t)$ from the time domain into the frequency domain in the form of a Bode diagram. Then the programs NLREG1 and LNREG2 were used to fit the Bode diagram to the form of either first-, second- or third-order lag with dead time,

$$G_{ij}(s) \underset{\sim}{=} \frac{K_{ij}\, e^{-\delta_{ij} s}}{\prod_{l=1}^{3} (T_{ijl}\, s + 1)} \qquad (5.9)$$

and also third-order lead-lag with dead time,

$$G_{ij}(s) \underset{\sim}{=} \frac{e^{-\delta_{ij} s} \prod_{k=1}^{3} (s - Z_{ijk})}{\prod_{l=1}^{3} (s - P_{ijl})} \qquad (5.10)$$

where P_{ijl} and Z_{ijk} are poles and zeros of the transfer function respectively.

5.2 Independence of Inputs

A priori, the variables selected as inputs for

Figure 5.1 Inputs-Outputs for the Continuous Cultivation of *M. crassipes* in NH_3-WSL System.

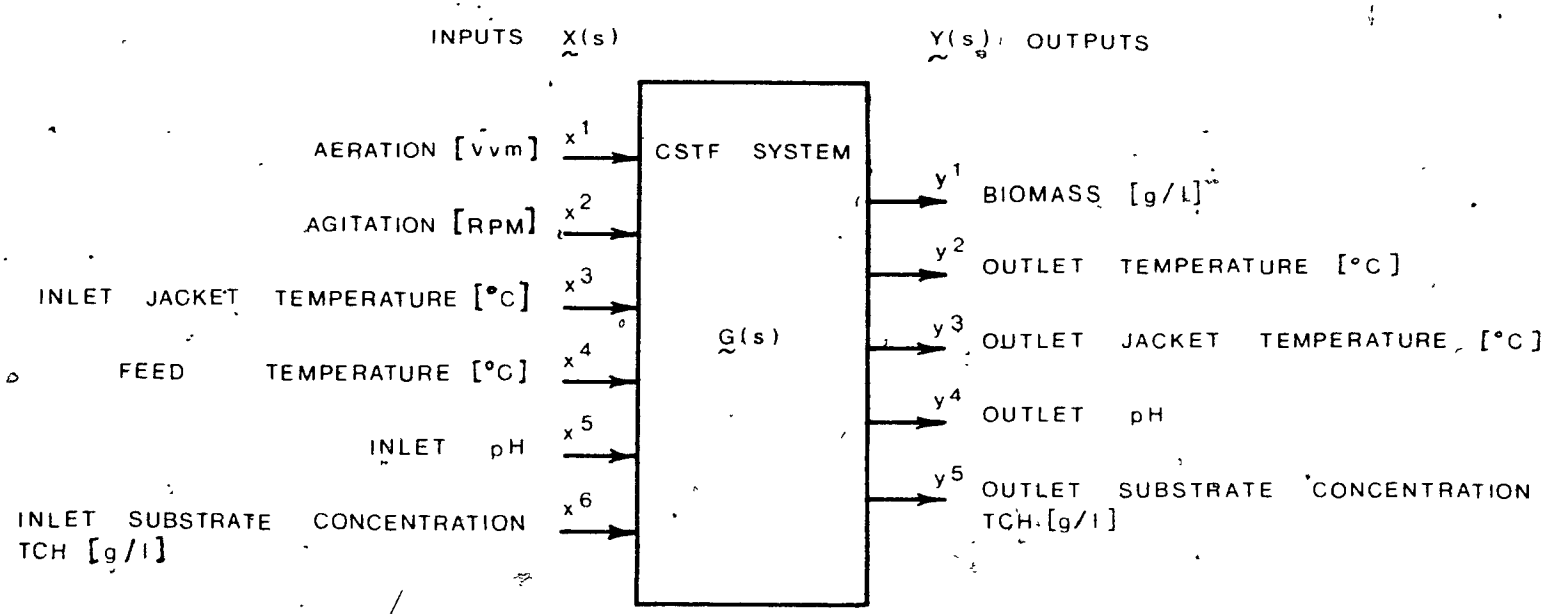
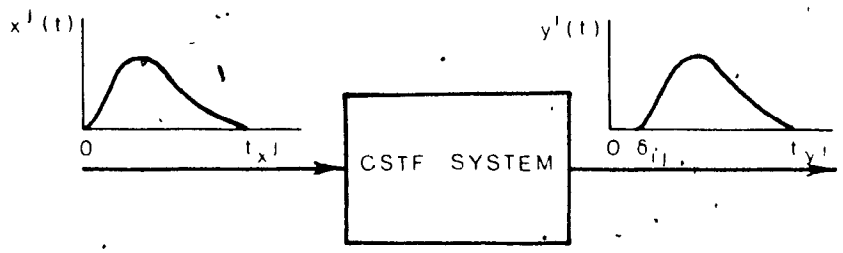
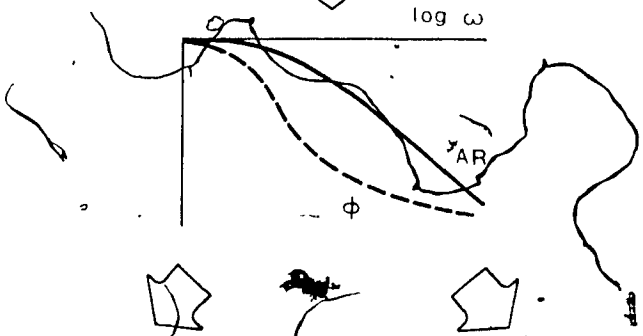

INPUTS X(s)
Y(s) OUTPUTS
AERATION [vvm]
AGITATION [RPM]
INLET JACKET TEMPERATURE [°C]
FEED TEMPERATURE [°C]
INLET pH
INLET SUBSTRATE CONCENTRATION TCH [g/l]
x1
x2
x3
x4
x5
x6
CSTF SYSTEM
G(s)
y1 BIOMASS [g/l]
y2 OUTLET TEMPERATURE [°C]
y3 OUTLET JACKET TEMPERATURE [°C]
y4 OUTLET pH
y5 OUTLET SUBSTRATE CONCENTRATION TCH [g/l]

Figure 5.2 Flowchart for Identification of CSTF System.



PROGRAM PULSE



PROGRAM NLREG1

PROGRAM LNREG2

$$G_{ij}(s) \cong \frac{K_{ij}\, e^{-\delta_{ij} s}}{\prod_{l=1}^{3} (T_{ijl}\, s + 1)}$$

$$G_{ij}(s) \cong \frac{e^{-\delta_{ij} s} \prod_{k=1}^{3} (s - Z_{ijk})}{\prod_{l=1}^{3} (s - P_{ijl})}$$

the CSTF system should satisfy the independence criterion, which could be expressed as,

$$x^j(t) \neq f[x^1(t), x^2(t), \ldots, x^{j-1}(t), x^{j+1}(t), \ldots, x^p(t)] \quad (5.11)$$

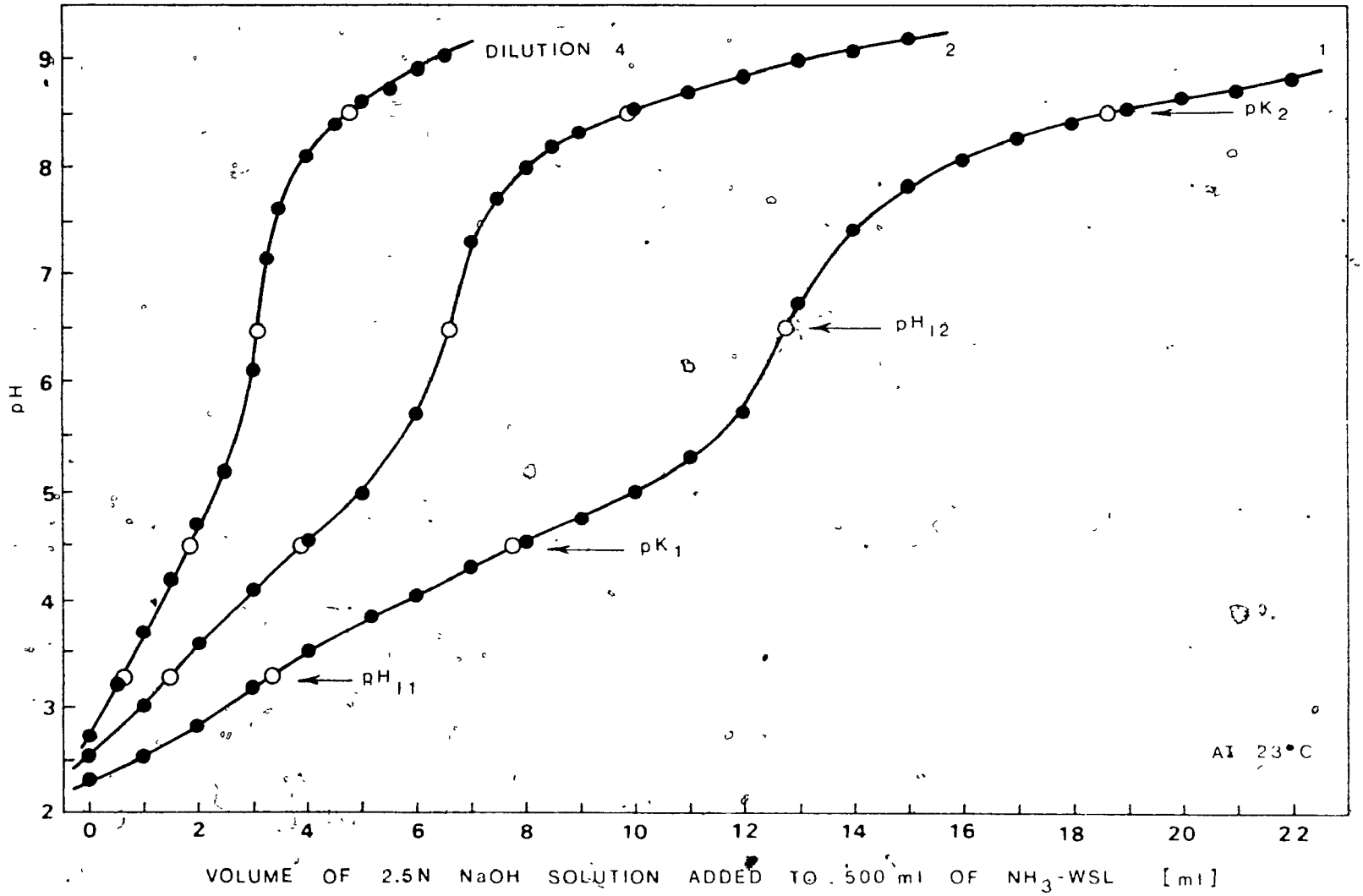
It is obvious that the aeration, agitation, inlet jacket temperature, feed temperature and inlet substrate concentration are independent of the rest of the other inputs cited in Fig. 5.1. The inlet pH is also independent of the rest of the other inputs except for the feed temperature. The tests should be performed in order to justify the independence hypothesis between pH and temperature of the feed medium.

The titration characteristics of NH_3-WSL at dilution 1, 2 and 4 are shown in Fig. 5.3. The dilution is defined as,

$$\text{Dilution}(1 + D) = 1 \text{ Volume of concentrated } NH_3\text{-WSL} + D \text{ Volume(s) of Distilled Water} \quad (5.12)$$

The titration curves of NH_3-WSL solutions show the characteristics of a polyprotonic solution, where in the pH range 2.0-9.0 two isoelectric points (equilibrium between positive and negative charges of dipolar or zwitterion forms of molecules) $pH_{I1} = 3.30$ and $pH_{I2} = 6.50$, two

Figure 5.3 Titration Characteristics of NH_3-WSL at Various Dilutions. Sample received in January-February 1972.


DILUTION 4
2
1
pK2
pH12
pK1
pH11
AI 23°C
pH
VOLUME OF 2.5N NaOH SOLUTION ADDED TO 500 ml OF NH3-WSL [ml]

isoionic points (equilibrium of proton donors and proton acceptors) pK_1 = 4.45 and pK_2 = 8.50 were observed.

The titration curves of NH_3-WSL solutions show a linear, high sensitive zone for pH between 5.5-7.5. This should be an ideal region for control purpose due to its high sensitivity and linearity. Furthermore, this range is coincided with the optimal initial pH range for *M. crassipes* in NH_3-WSL (paragraph 3.1.1) which is 5.5-6.5 for both yield and efficiency in shake flask experiments. Therefore, the value of initial pH 6.5 is selected as the optimal initial pH and also as a set point of pH controller for the continuous cultivation experiments.

The Figs. 5.4-5.6 show the results on the pH-temperature relationship for NH_3-WSL at dilutions 1, 2 and 4 and at original pH and at pH's 4.0, 5.5, 6.5, 7.5 and 9.0 of the solutions at 23°C. It was found that the inlet pH is a linear function of feed temperature and it could be expressed as,

$$pH\ (T) = \alpha T + \beta \qquad (5.13)$$

where the coefficients α and β depend upon specific pH value of the solutions at a specific dilution. The coefficient α was almost zero at the acidic pH of the NH_3-WSL

Figure 5.4 pH-Temperature Relationship of NH_3-WSL at Dilution 1. Samples were at its original pH (A) or adjusted to pH's 4.0 (B), 5.5 (C), 6.5 (D), 7.5 (E) and 9.0 (F) at 23 °C. Sample received in January-February 1972.

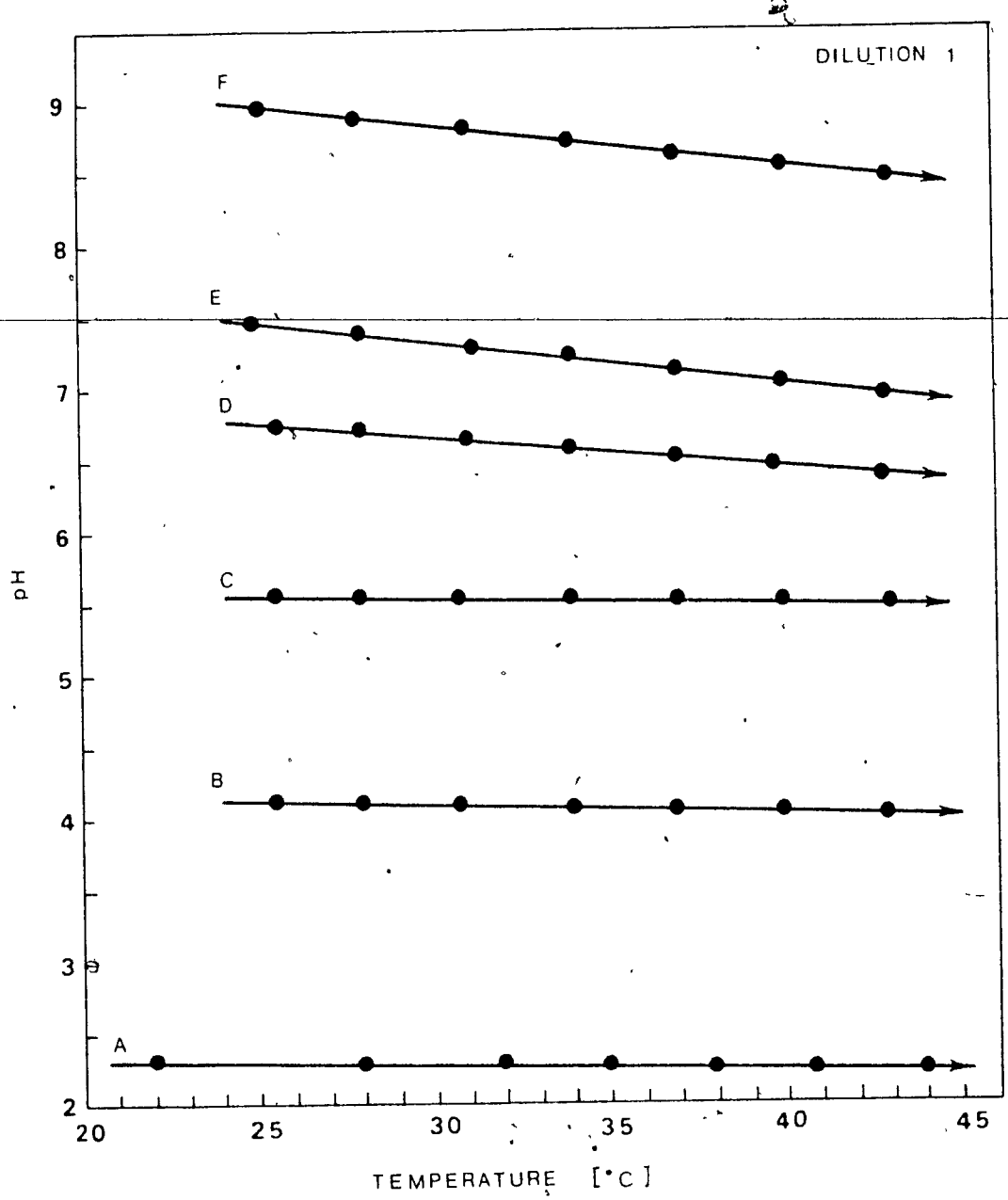

DILUTION 1
F
E
D
C
B
A
9
8
7
6
5
4
3
2
pH
20
25
30
35
40
45
TEMPERATURE [°C]

Figure 5.5 pH-Temperature Relationship of NH_3-WSL at Dilution 2. Samples were at its original pH (A) or adjusted to pH's 4.0 (B), 5.5 (C), 6.5 (D), 7.5 (E) and 9.0 (F) at 23 °C. Sample received in January-February 1972.

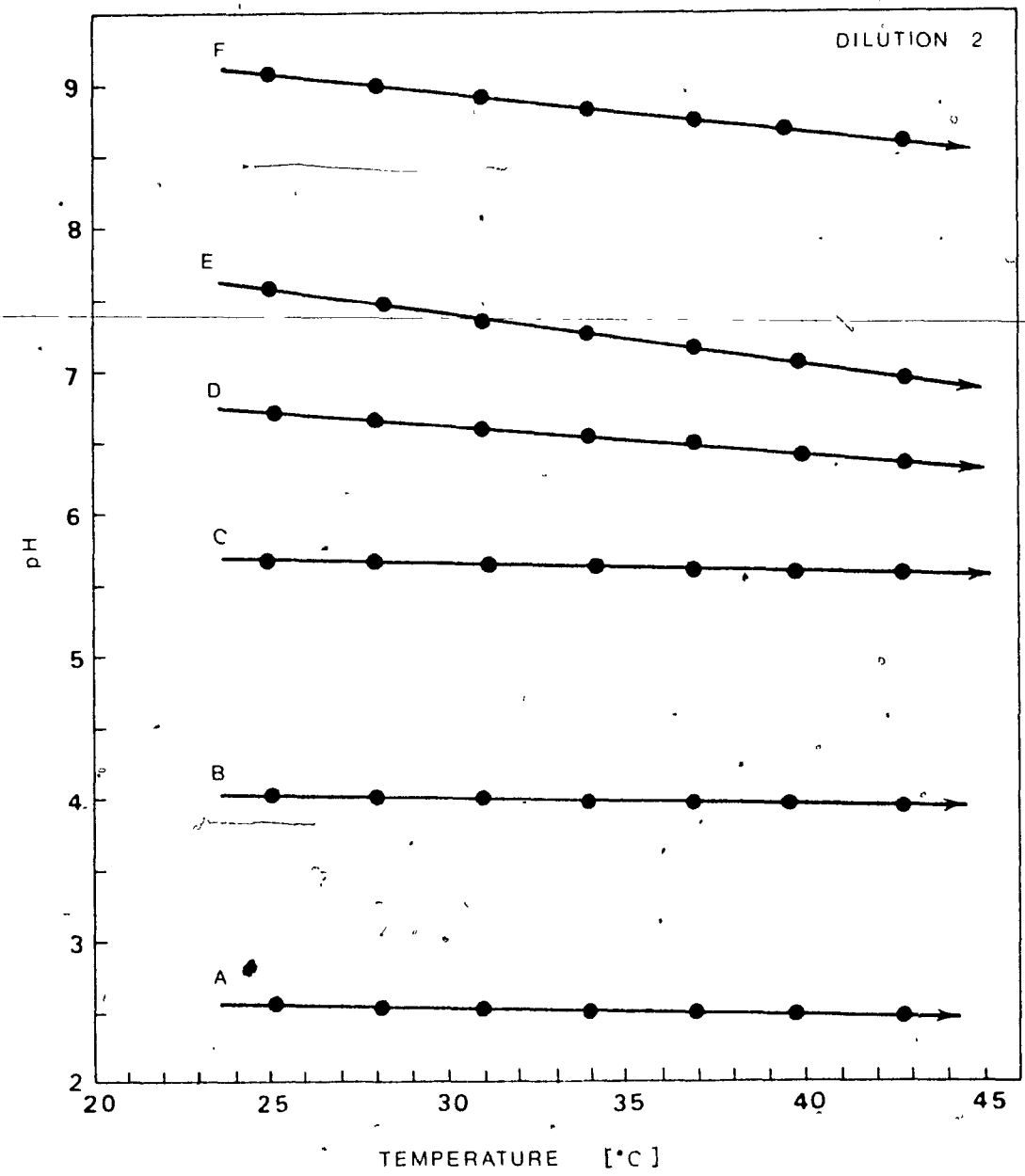
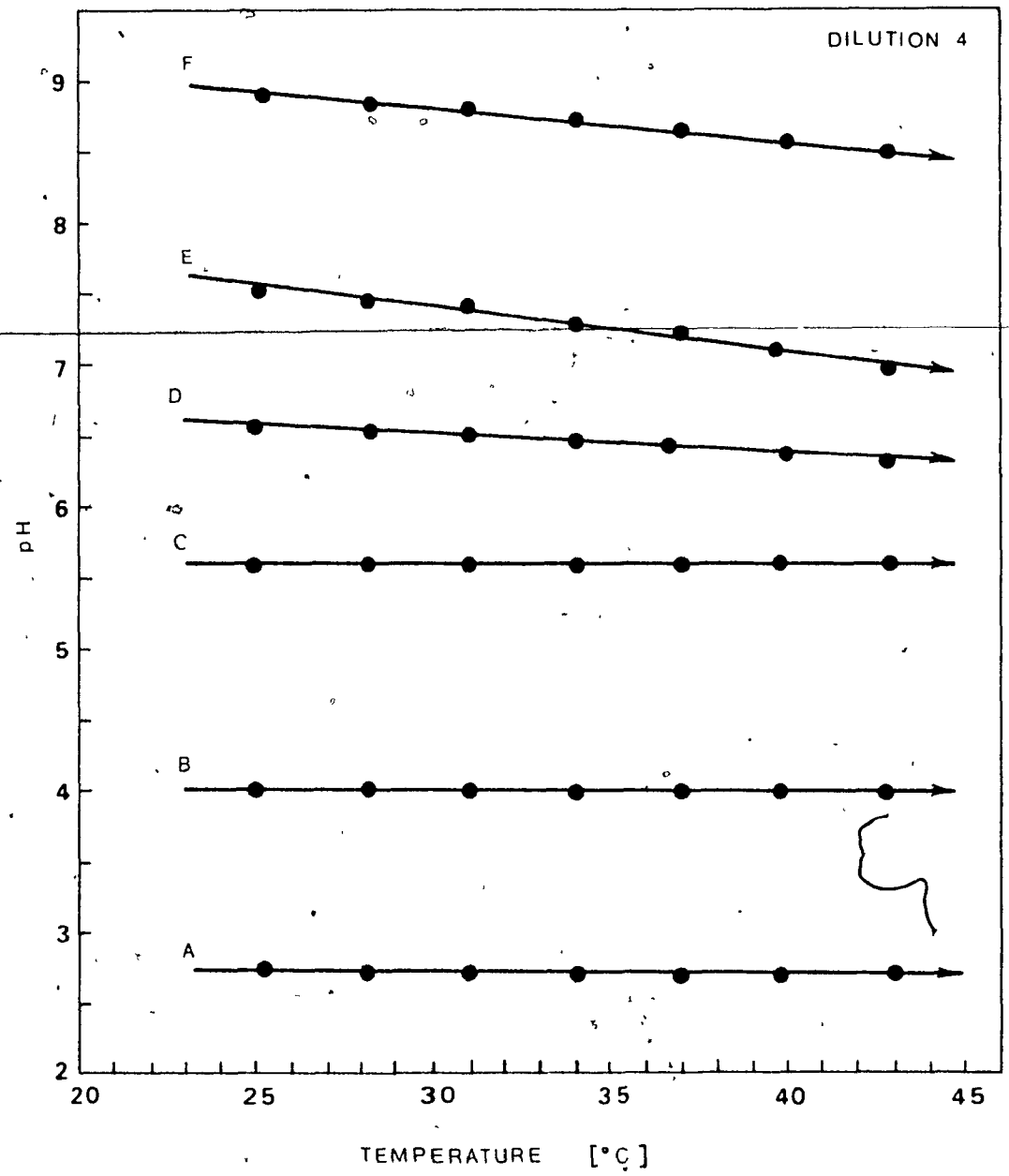

DILUTION 2
F
E
D
C
B
A
9
8
7
6
5
4
3
2
pH
20
25
30
35
40
45
TEMPERATURE [°C]

Figure 5.6 pH-Temperature Relationship of NH_3-WSL at Dilution 4. Samples were at its original pH (A) or adjusted to pH's 4.0 (B), 5.5 (C), 6.5 (D), 7.5 (E) and 9.0 (F) at 23 °C. Sample received in January-February 1972.


DILUTION 4
F
E
D
C
B
A
pH
9
8
7
6
5
4
3
2
20
25
30
35
40
45
TEMPERATURE [°C]

solutions, and increased with increasing pH. In general, at the optimal initial pH 6.50, the maximum value of α did not surpass 0.2/10 °C. Compared to the accuracy of the pH-Controller-Indicator-Recorder ($\pm$ 0.1 pH), the inlet pH could be taken to be more or less independent of the feeding temperature.

It should be noted that the results shown in Figs. 5.4-5.6 were obtained by raising the temperature to a desired value and then measuring the corresponding pH value. By lowering the temperature, the hysteresis phenomenon was observed (but not shown) for all pH-temperature curves. During the cooling process, the pH of the NH_3-WSL solutions had a tendency to stay constant at the final pH of the heating process.

5.3 Transfer Function Matrix

5.3.1 Static Data in the Vicinity of Quasioptimal Point Without Presence of Microorganisms

Studies on the static characteristics of the system in the vicinity of quasioptimal point are usually used for determination of the static gain K_{ij} of the transfer function $G_{ij}(s)$.

$$G_{ij}(s)\Big|_{ss} \equiv K_{ij} \tag{5.14}$$

The results from these static studies could also be useful to confirm or justify the validity of the hypothesis of linearity around the operating point. If the following functions,

$$y^i(t)\Big|_{ss} = y^i[\, x^1(t)\Big|_{ss}, x^2(t)\Big|_{ss}, \ldots, x^j(t)\Big|_{ss}, \ldots, x^p(t)\Big|_{ss}\,]$$

$$i = 1, 2, \ldots, q \tag{5.15}$$

are linear or linearized with respect to x^j, they could be approximated by,

$$y^i(t)\Big|_{ss} \cong A^j\, x^j(t)\Big|_{ss} + B^j \tag{5.16}$$

$$j = 1, 2, \ldots, p$$

where A^j and B^j are constants. The interactions between outputs at steady-state could then be deducted from these results. It should be noted that static studies are also helpful for selection of the most significant inputs and/or outputs for the dynamic studies. The insignificant or less significant inputs then could be disregarded after this screening procedure.

The static studies are also useful for searching the operating point, that is the optimal condition for

inputs. However, the quasioptimal conditions for inputs could be derived from the results in shake flask experiments for the system *M. crassipes* in NH_3-WSL. They were 2.0 l/min or 0.266 vvm for aeration (x^1), 300 RPM for agitation (x^2), 25.0°C for inlet jacket temperature (x^3) at constant flowrate of jacket water of 60 l/hr, 30.0°C for feeding temperature (x^4) at flowrate of feeding medium of 100 ml/hr, 6.50 for inlet pH before sterilization (x^5), and WSL at dilution 4 for the inlet substrate concentration (x^6). Therefore the static studies could be made on the CSTF system without presence of microorganisms in order to verify the linear characteristics described by Eq. 5.16 and to find out the significant inputs for the dynamic tests. It also could yield the information on the net effect of the microorganisms on the CSTF system when compared with the results from dynamic studies.

Figure 5.7 shows the static effects of the aeration rate (x^1) which was varied from 0 to 12 l/min on the outputs of the CSTF system without presence of microorganisms. Other inputs were kept constant at their optimal values such as x^2 = 300 RPM, x^3 = 25.0°C, x^4 = 30.0°C, x^5 = 6.50 and x^6 = WSL-APD-CSL medium. Because the medium had not been inoculated, the biomass (y^1) was always nil. The outlet temperature (y^2) stayed almost

Figure 5.7 Static Data at the Vicinity of Quasioptimal Point When Varying x^1.

x^1 = aeration rate,

x^2 = agitation speed,

x^3 = inlet jacket temperature,

x^4 = feed temperature,

x^5 = inlet pH,

x^6 = inlet TCH concentration,

y^1 = biomass concentration,

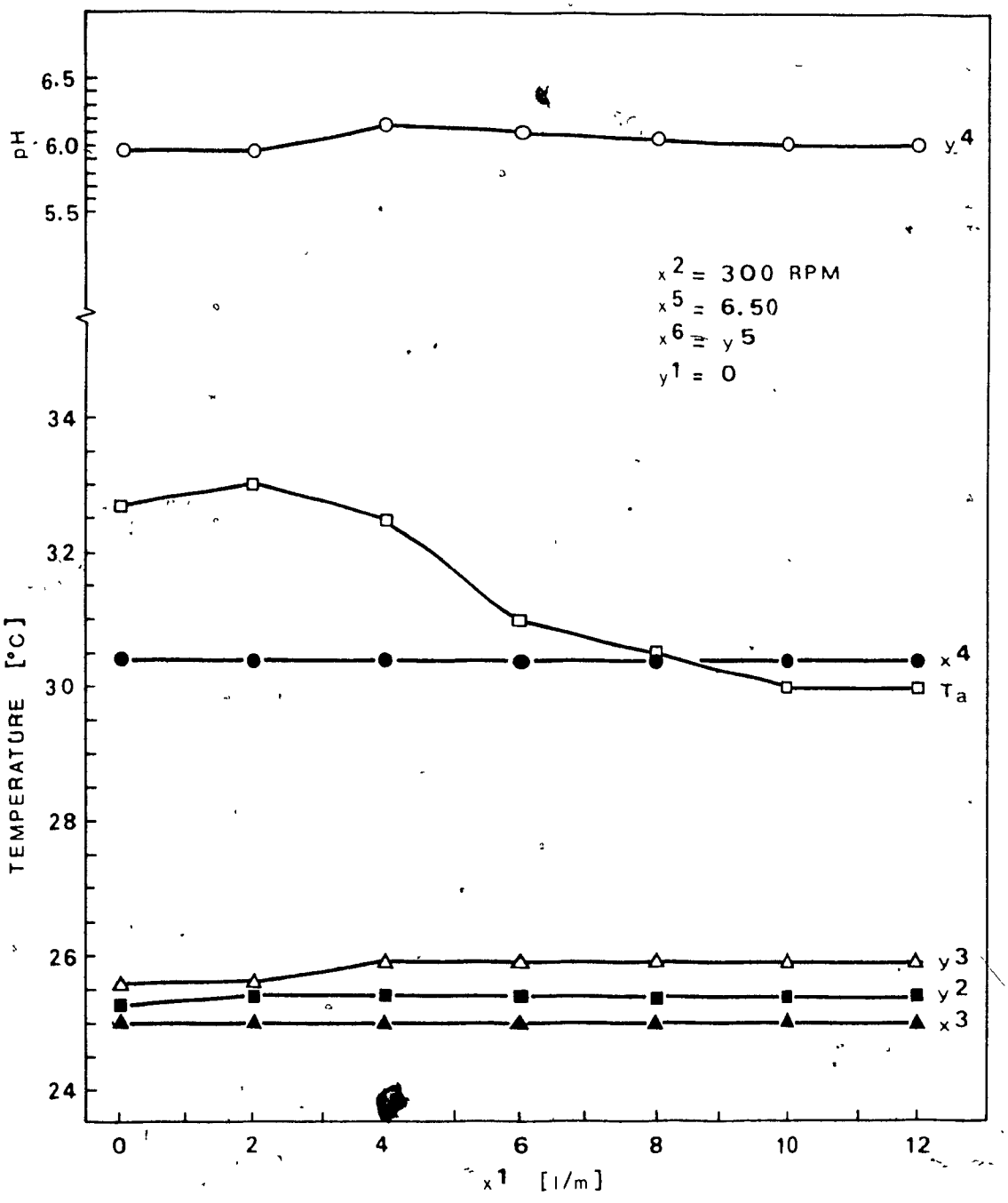
y^2 = outlet temperature,

y^3 = outlet jacket temperature,

y^4 = outlet pH,

y^5 = outlet TCH concentration,

T_a = ambient temperature.


6.5
pH 6.0
5.5
y_4
x_2 = 300 RPM
x_5 = 6.50
$x_6 = y_5$
y_1 = 0
34
32
30
28
26
24
TEMPERATURE [°C]
x_4
T_a
y_3
y_2
x_3
0
2
4
6
8
10
12
x_1 [l/m]

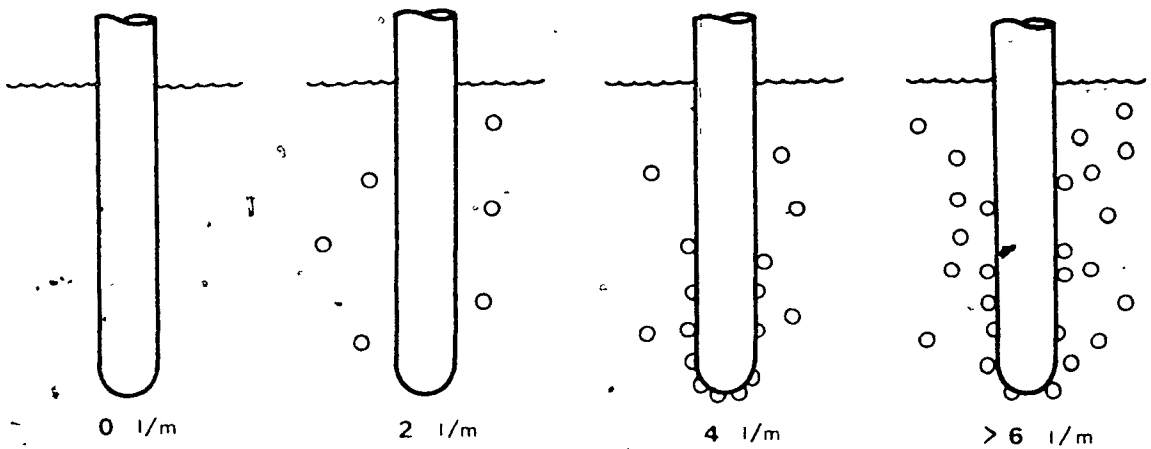
constant at 25.4°C within the range of aeration rates tested. The outlet jacket temperature (y^3) was found to be constant at 25.6°C for low aeration (0-2 l/min) and at 25.9°C for higher aeration. At constant inlet jacket temperature and feed temperature, the outlet temperature and the outlet jacket temperature have a tendency to increase at higher aeration rate ($\Delta y^2_{max} \cong 0.1$°C, $\Delta y^3_{max} \cong 0.3$°C). This fact could be explained as resulting from heat transfer from the aerated air and from the ambient environment to the culture medium. The ambient temperature (Ta) supplied heat to the culture medium and it obviously is a function of the aeration rate. The heat generated by agitation could affect the outlet temperature and then the outlet jacket temperature; however its constant amount due to a constant agitation (x^2 = 300 RPM) during this test did not have any effect on the variations of y^2 and y^3. The convection heat transfers on the wall of the fermentor jar depended upon Ta, x^2 and also y^2. During this test, although the high variation of ambient temperature from 30 to 33°C (ΔTa = 3°C), the effects on y^2 and y^3 were not significantly observed. The outlet pH (y^4) was at 5.97 for lower aeration (0-2 l/min), then it raised to a maximum value of 6.12 at 4 l/min and had a tendency toward 5.93 at higher aeration. The variation of pH could be due to the

variation of temperature in the culture medium. From the pH-temperature relationship presented in paragraph 5.2, the maximum variation of pH with temperature y^2 under this condition was insignificant (0.1°C x 0.02 pH/°C = 0.002 pH unit). Thus the major effect on the variation of pH was probably due to air bubbles in the culture medium which were sitting on and/or touching the tip of the pH electrodes. Due to the presence of cavities around the tip of the pH electrodes at high aeration rates, the pH electrodes may detect a less amount of protons $[H^+]$ in the solution, therefore the pH recorded was higher than its true value according to the definition,

$$pH = - \log_{10} [H^+] \qquad (5.17)$$

The details of this mechanism are shown in Fig. 5.8. Finally, the aeration obviously had no effect on the outlet substrate concentration ($y^5 = 4.75$ g/l) due to the absence of microorganisms in the culture medium. In summary, without presence of microorganisms in the culture medium, the aeration rates could be justified as having no effect on the outputs of the CSTF system. In other words, around the operating point ($x^1 = 2.0$ l/min), and applying the Eq. (5.16), it could be shown that,

Figure 5.8 Effect of Aeration Rate on pH of the Culture Medium (according to the observations shown in Fig. 5.7).



pH = TRUE pH

pH = TRUE pH

pH = pH_{max} > TRUE pH

pH < pH_{max} > TRUE pH

SITTING BUBBLES MORE THAN TOUCHING BUBBLES

SITTING BUBBLES LESS THAN TOUCHING BUBBLES

COMPLETE DETECTION OF $[H^+]$ BY pH ELECTRODES

INCOMPLETE DETECTION OF $[H^+]$ BY pH ELECTRODES

$$y^1(t)\Big|_{ss} \overset{\sim}{=} 0\ x^1(t)\Big|_{ss} + 0 \qquad (5.18a)$$

$$y^2(t)\Big|_{ss} \overset{\sim}{=} 0\ x^1(t)\Big|_{ss} + 25.4 \qquad (5.18b)$$

$$y^3(t)\Big|_{ss} \overset{\sim}{=} 0\ x^1(t)\Big|_{ss} + 25.6 \qquad (5.18c)$$

$$y^4(t)\Big|_{ss} \overset{\sim}{=} 0\ x^1(t)\Big|_{ss} + 6.00 \qquad (5.18d)$$

$$y^5(t)\Big|_{ss} \overset{\sim}{=} 0\ x^1(t)\Big|_{ss} + 4.75 \qquad (5.18e)$$

Figure 5.9 shows the static effects of agitation speed (x^2) which was varied from 0 to 800 RPM on the outputs at quasioptimal conditions for inputs as previously specified except for x^1 = 2.0 l/min for aeration rate and x^3 = 30.4-30.5°C for inlet jacket temperature. Because of the absence of microorganisms, the biomass was always nil. The outlet temperature was almost constant at 25.3-25.6°C at low agitation (<600 RPM) then increased to 27.0°C at higher agitation (800 RPM). The outlet jacket temperature was also constant at 25.3-25.6°C at low agitation (<400 RPM) and raised to 26.7-26.8°C at higher agitation (600-800 RPM). At constant inlet temperature (25.0-25.1°C) and inlet jacket temperature (30.4-30.5°C), the outlet temperature and the outlet jacket temperature had a tendency to increase at higher agitation. This probably came from the amount of heat generated by agitation,

Figure 5.9 Static Data at the Vicinity of Quasioptimal Point When Varying x^2.

x^1 = aeration rate,

x^2 = agitation speed,

x^3 = inlet jacket temperature,

x^4 = feed temperature,

x^5 = inlet pH,

x^6 = inlet TCH concentration,

y^1 = biomass concentration,

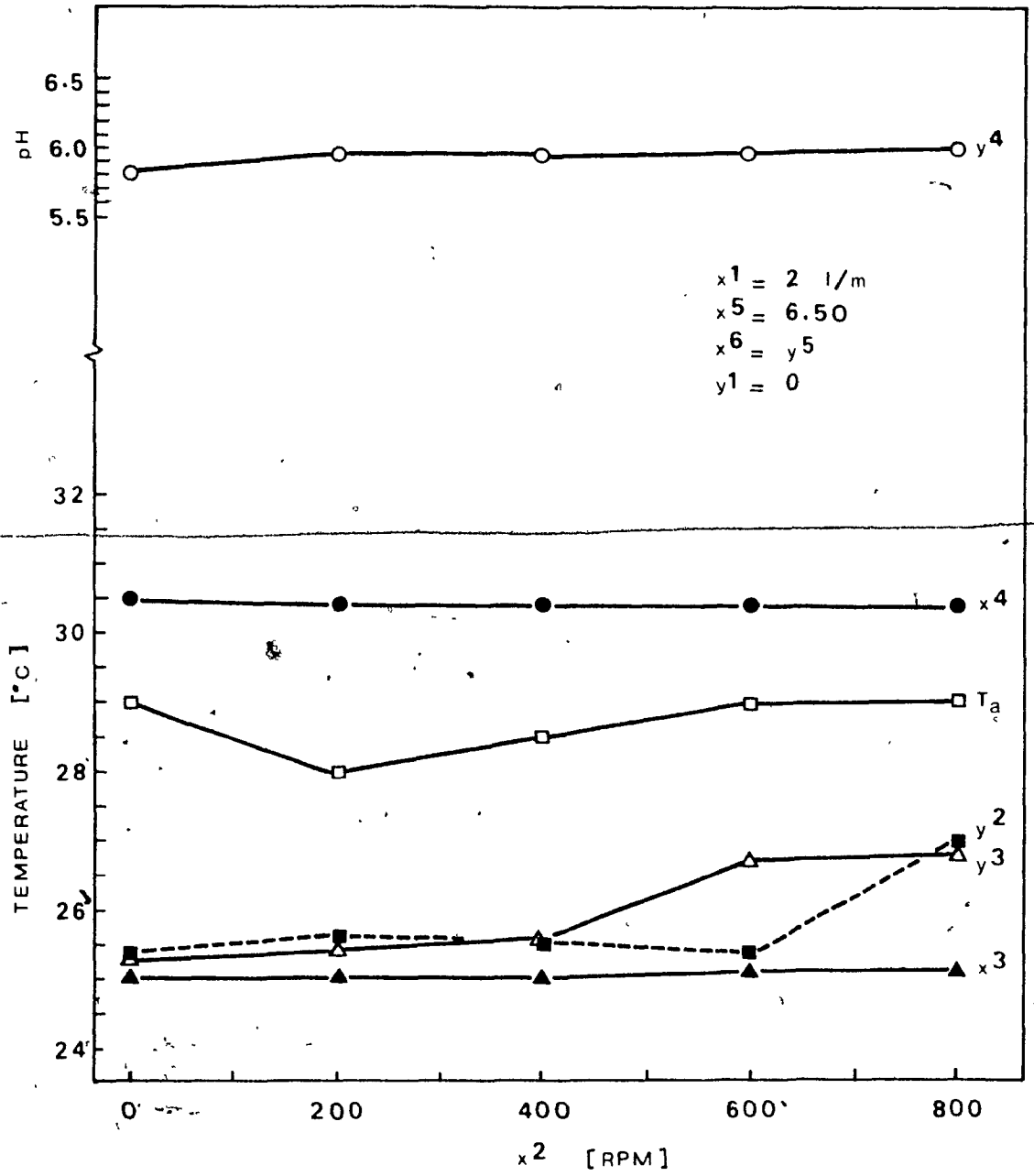
y^2 = outlet temperature,

y^3 = outlet jacket temperature,

y^4 = outlet pH,

y^5 = outlet TCH concentration,

T_a = ambient temperature.


pH
6.5
6.0
5.5
y4
x1 = 2 l/m
x5 = 6.50
x6 = y5
y1 = 0
32
30
28
26
24
TEMPERATURE [°C]
x4
Ta
y2
y3
x3
0
200
400
600
800
x2 [RPM]

which was the friction between the four-blade turbine impeller and the culture medium. From the previous discussion on the static effects of the aeration rate, the heat transfer from the aeration (depended on T_a), the heat transfer from the feed medium (depended on x^4), and the convection heat transfer at the wall of the fermentor jar (depended on T_a, x^2 and y^2) could be assumed to have an insignificant effect on the variation of y^2 and y^3. The outlet pH was 5.82 for still culture medium and had a value of 5.91-5.98 with agitation. This fact could be explained as mainly due to the air bubbles which were touching and/or sitting on pH electrodes as in the case of aeration shown in Fig. 5.8. The variation of the agitation speed could not have any effect on the substrate concentration of the culture medium due to the absence of the microorganisms. In summary, in the vicinity of quasioptimal point, the agitation speed could be considered as having no effect on the outputs of the CSTF system without the presence of microorganisms. In other words, around the operating point (x^2 = 300 RPM), and applying the Eq. (5.16), it could be shown that,

$$y^1(t)\Big|_{ss} \cong 0\ x^2(t)\Big|_{ss} + 0 \qquad (5.19a)$$

$$y^2(t)\Big|_{ss} \cong 0\ x^2(t)\Big|_{ss} + 25.6 \qquad (5.19b)$$

$$y^3(t)\Big|_{ss} \cong 0\ x^2(t)\Big|_{ss} + 25.5 \qquad (5.19c)$$

$$y^4(t)\Big|_{ss} \cong 0\ x^2(t)\Big|_{ss} + 5.95 \qquad (5.19d)$$

$$y^5(t)\Big|_{ss} \cong 0\ x^2(t)\Big|_{ss} + 4.75 \qquad (5.19e)$$

Figure 5.10 illustrates the static effects of varying the inlet jacket temperature from 23 to 40 °C when the CSTF system is operated at quasioptimal conditions. Because the culture medium had not been inoculated, the biomass was always nil. The outlet jacket temperature had the same value as the inlet jacket temperature. The outlet temperature was approximately the same as the outlet jacket temperature within the range of 23-29°C, but higher than that of outlet jacket temperature [$(y^2-y^3)_{max}$ = 1.5°C] at higher inlet jacket temperature (>32°C). The heat transfers come from the feeding medium (x^4 = 30.2-30.4°C), the aeration (depended on T_a = 29.5-30.5°C), the convection heat transfer at the wall of the fermentor jar (depended on T_a, x^2 and y^2), the heat generated by agitation (x^2 = 300 RPM) and the convection heat transfer from the jacket water (depended on x^2, x^3, and y^2). The fact that y^2 is slightly higher than y^3 at x^3 > 30.°C could be mainly due to the amount of heat generated by agitation.

Figure 5.10 Static Data at the Vicinity of Quasioptimal Point When Varying x^3.

x^1 = aeration rate,

x^2 = agitation speed,

x^3 = inlet jacket temperature,

x^4 = feed temperature,

x^5 = inlet pH,

x^6 = inlet TCH concentration,

y^1 = biomass concentration,

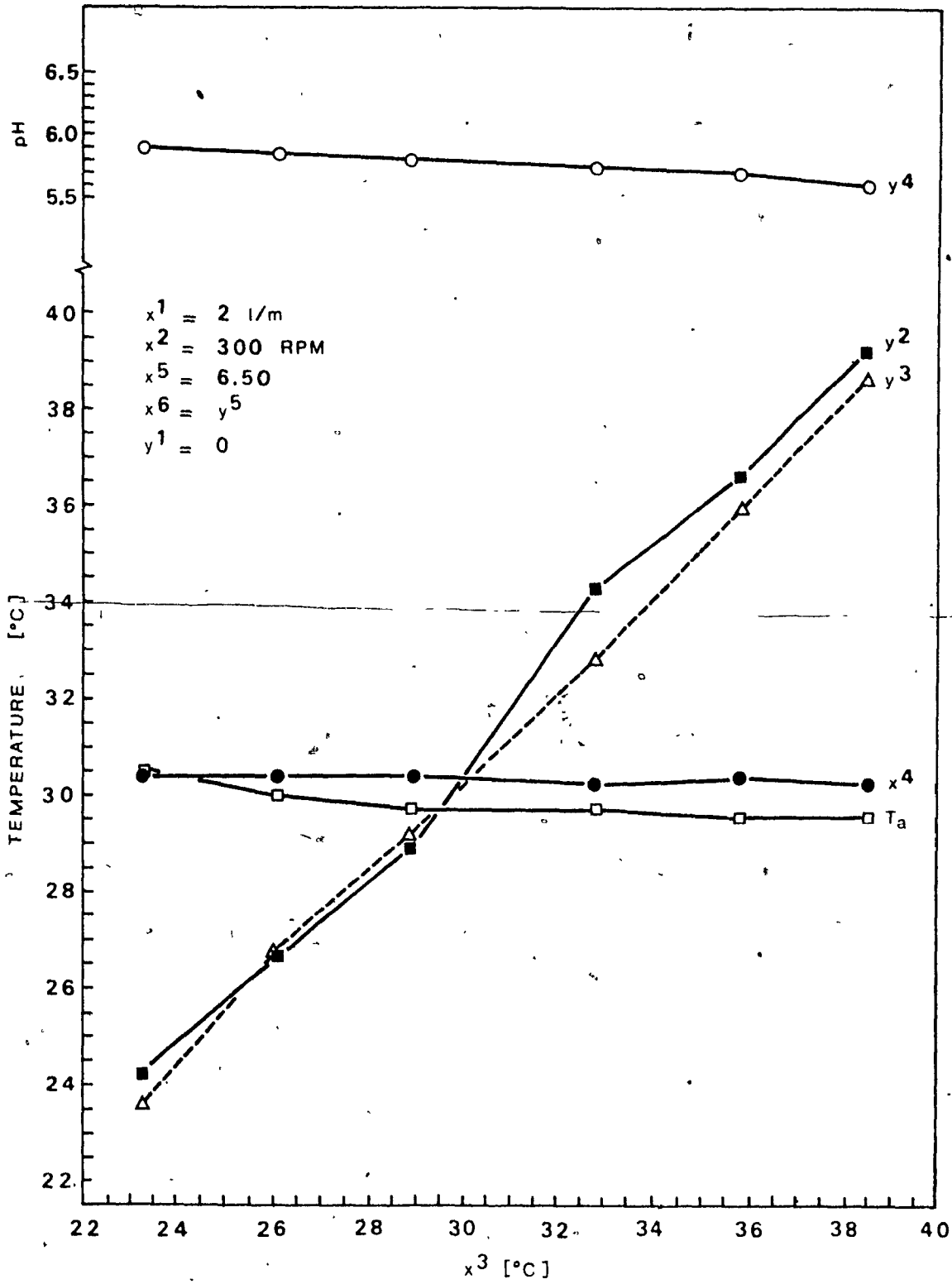
y^2 = outlet temperature,

y^3 = outlet jacket temperature,

y^4 = outlet pH,

y^5 = outlet TCH concentration,

T_a = ambient temperature.


pH
6.5
6.0
5.5
y4
x1 = 2 l/m
x2 = 300 RPM
x5 = 6.50
x6 = y5
y1 = 0
y2
y3
x4
Ta
TEMPERATURE [°C]
40
38
36
34
32
30
28
26
24
22
22
24
26
28
30
32
34
36
38
40
x3 [°C]

The outlet pH had a tendency to decrease from 5.90 to 5.60 with an increase in x^3 from 23°C to 38.5°C. This variation in pH was due to the pH-temperature dependence which was discussed in the paragraph 5.2. The variation of $\Delta y^4 \cong 0.30$ was comparable to that of the curve D in Fig. 5.6 within the same range of temperature difference of ~0.25. The outlet substrate concentration could not be expected to have any change because of the absence of microorganisms in the fermentor. In summary, it could be justified that the inlet jacket temperature did not have any static effect on the outputs y^1 and y^5 of the CSTF system, but linearly affected y^2, y^3, and y^4 at the vicinity of the operating point (x^3 = 25.0°C) when the microorganisms were not present. In other words, around the operating point, and applying the Eq. (5.16), it could be shown that,

$$y^1(t)\Big|_{ss} \cong 0\ x^3(t)\Big|_{ss} + 0 \qquad (5.20a)$$

$$y^2(t)\Big|_{ss} \cong 1.0\ x^3(t)\Big|_{ss} + 0.7 \qquad (5.20b)$$

$$y^3(t)\Big|_{ss} \cong 0.97\ x^3(t)\Big|_{ss} + 1.2 \qquad (5.20c)$$

$$y^4(t)\Big|_{ss} \cong 0.02\ x^3(t)\Big|_{ss} -5.86 \qquad (5.20d)$$

$$y^5(t)\Big|_{ss} \cong 0\ x^3(t)\Big|_{ss} + 4.75 \qquad (5.20e)$$

Figure 5.11 shows the static effects of the feed temperature which was varied from 22.5 °C to 70.0 °C on the outputs of the CSTF system operated under quasi-optimal conditions without the presence of microorganisms. The more or less constant value of the outlet temperature (y^2 = 25.2-25.7°C) and the outlet jacket temperature (y^3 = 25.3-25.9°C) resulted from the insignificant amount of heat transfered from the aeration (depended on T_a), the heat generated from agitation (x^2 = 300 RPM), the heat from the jacket water (x^3 = 24.7-25.1°C), the convection heat transfer at the wall of the fermentor jar (depended on x^2, y^2, and T_a), and the heat from the feed medium (depended on x^4). The fact that the high temperature of the feed medium (70°C) did not have any significant static effect on the outlet temperature could be due to the slow feeding rate of the fresh medium into the fermentor (feeding flowrate of 100 ml/hr or dilution rate of 0.0133 hr^{-1}). The outlet pH stayed almost constant at 5.80-5.83 during this test because of the insignificant variation of the outlet temperature. The outlet substrate concentration could not change because the microorganisms were not present in the culture medium. In summary, the feed temperature could be considered as having no static effect on the outputs of

Figure 5.11 Static Data at the Vicinity of Quasioptimal Point When Varying x^4.

x^1 = aeration rate,

x^2 = agitation speed,

x^3 = inlet jacket temperature,

x^4 = feed temperature,

x^5 = inlet pH,

x^6 = inlet TCH concentration,

y^1 = biomass concentration,

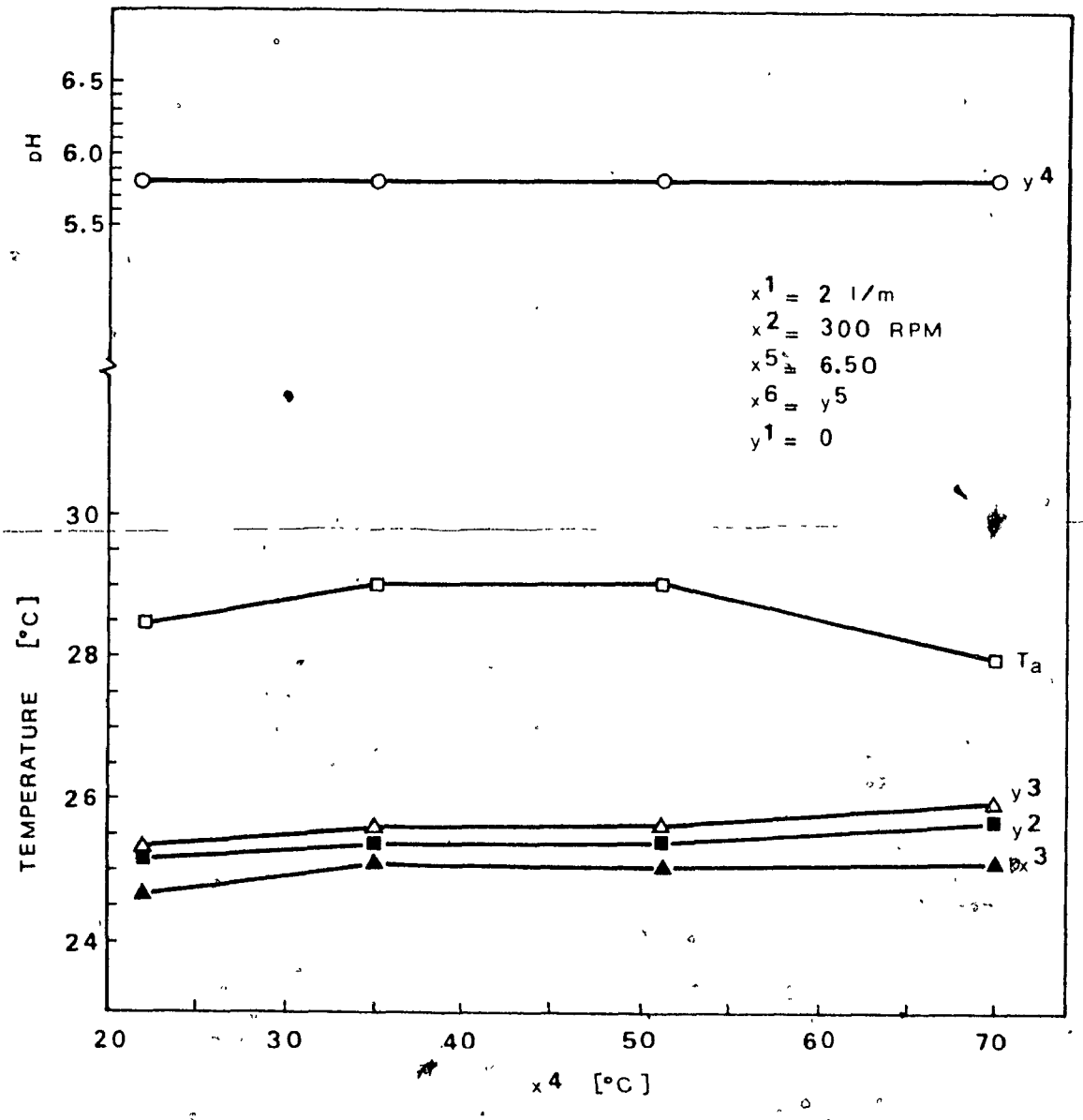
y^2 = outlet temperature,

y^3 = outlet jacket temperature,

y^4 = outlet pH,

y^5 = outlet TCH concentration,

T_a = ambient temperature.


pH
6.5
6.0
5.5
y 4
x 1 = 2 l/m
x 2 = 300 RPM
x 5 = 6.50
x 6 = y 5
y 1 = 0
30
28
26
24
TEMPERATURE [°C]
Ta
y 3
y 2
x 3
20
30
40
50
60
70
x 4 [°C]

the CSTF system without the presence of microorganisms. In other words, around the operating point (x^4 = 30.0°C), and applying the Eq. (5.16), it could be shown that,

$$y^1(t)\Big|_{ss} \cong 0\ x^4(t)\Big|_{ss} + 0 \qquad (5.21a)$$

$$y^2(t)\Big|_{ss} \cong 0\ x^4(t)\Big|_{ss} + 25.4 \qquad (5.21b)$$

$$y^3(t)\Big|_{ss} \cong 0\ x^4(t)\Big|_{ss} + 25.5 \qquad (5.21c)$$

$$y^4(t)\Big|_{ss} \cong 0\ x^4(t)\Big|_{ss} + 5.80 \qquad (5.21d)$$

$$y^5(t)\Big|_{ss} \cong 0\ x^4(t)\Big|_{ss} + 4.75 \qquad (5.21e)$$

Under quasioptimal conditions for the CSTF system and without the presence of microorganisms, the inlet pH obviously could not have any static effect on the outputs except the outlet pH should have the same value as the inlet pH at steady state. In other words, around the operating point (x^5 = 6.50 pH units), and applying the Eq. (5.16), it could be shown that,

$$y^1(t)\Big|_{ss} \cong 0\ x^5(t)\Big|_{ss} + 0 \qquad (5.22a)$$

$$y^2(t)\Big|_{ss} \cong 0\ x^5(t)\Big|_{ss} + 25.4\sim25.6 \qquad (5.22b)$$

$$y^3(t)\Big|_{ss} \cong 0\ x^5(t)\Big|_{ss} + 25.5\sim25.6 \qquad (5.22c)$$

$$y^4(t)\Big|_{ss} \cong 1.0\ x^5(t)\Big|_{ss} + 0 \qquad (5.22d)$$

$$y^4(t)\Big|_{ss} \cong 0\ x^5(t)\Big|_{ss} + 4.75 \qquad (5.22e)$$

A similar statement could be made for the static effects of the inlet substrate concentration on the outputs of the CSTF system. The only effect expected is that the outlet substrate concentration should have the same value as the inlet substrate concentration at steady state without the presence of microorganisms. In other words, around the operating point (x^6 = 4.75 g/l of TCH), and applying the Eq. (5.16), it could be shown that,

$$y^1(t)\Big|_{ss} \cong 0\ \ x^6(t)\Big|_{ss} + 0 \qquad (5.23a)$$

$$y^2(t)\Big|_{ss} \cong 0\ \ x^6(t)\Big|_{ss} + 25.4\sim25.6 \qquad (5.23b)$$

$$y^3(t)\Big|_{ss} \cong 0\ \ x^6(t)\Big|_{ss} + 25.5\sim25.6 \qquad (5.23c)$$

$$y^4(t)\Big|_{ss} \cong 0\ \ x^6(t)\Big|_{ss} + 5.80\sim5.95 \qquad (5.23d)$$

$$y^5(t)\Big|_{ss} \cong 1.0\ x^6(t)\Big|_{ss} + 0 \qquad (5.23e)$$

The static data in the vicinity of quasioptimal conditions without the presence of microorganisms led to the following matrix equation by combining the Eqs. (5.18)

to (5.23) together,

$$\begin{bmatrix} y^1(t) - 0 \\ y^2(t) - (25.5 \pm 0.1) \\ y^3(t) - (25.5 \pm 0.1) \\ y^4(t) - (5.90 \pm 0.10) \\ y^5(t) - 4.75 \end{bmatrix}_{ss} \simeq \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1.0 & 0 & 0 & 0 \\ 0 & 0 & 0.97 & 0 & 0 & 0 \\ 0 & 0 & 0.02 & 0 & 1.0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1.0 \end{bmatrix} \times \begin{bmatrix} x^1(t) - 2.0 \\ x^2(t) - 300 \\ x^3(t) - 25.0 \\ x^4(t) - 30.0 \\ x^5(t) - 6.50 \\ x^6(t) - 4.75 \end{bmatrix}_{ss} \quad (5.24)$$

It should be noted that the feed temperature did not have any effect on the outputs of the CSTF system, including the outlet temperature. Therefore, with the presence of microorganisms, this input should also have no effect on the outputs of the system, and it could be disregarded for the dynamic tests. This also implies that the temperature controlled water bath for the feed medium shown in Fig. 4.5 is not necessary for the experiments on this particular system.

It was also observed that the outlet pH had the value of less than 6.50 (pH before sterilization) in all cases during the static studies without the presence of microorganisms. Several tests have been performed to study the effects of sterilization on the pH of the culture medium and its stability with time. The results

are shown in Fig. 5.12. The pH after sterilization depended both on the holding time and on the volume of culture medium used. For the holding period of 20 minutes, the pH after sterilization of 100 ml of culture medium contained in a 500 ml Erlenmeyer flask dropped to 4.95 while that of 200 ml of culture medium in the same flask dropped to 6.07. The pH after sterilization was lower if the holding time is longer as seen by the values of 6.07 and 5.88 for 200 ml of culture medium in a 500 ml Erlenmeyer flask sterilized for 20 minutes and 45 minutes respectively. The culture medium in the feed tank (16 liters of culture medium in a 5 gallon carboy) yielded a pH of 5.85 after 45 minutes of sterilization. In all cases, the pH after sterilization was found to be stable within 9 days under aseptic conditions (variations of $\pm$0.1 pH unit).

5.3.2 Dynamic Data at the Quasioptimal Point

The continuous cultivation of *M. crassipes* in NH_3-WSL-APD-CSL medium was conducted for 975 hours ($\sim$ 41 days) in the CSTF system (Fig. 5.13) as described in Chapter 4. The progress of the cultivation was followed by sampling, measuring, analyzing and recording all variables during the experiment. The sampling frequency

Figure 5.12 Effects of Sterilization on the pH of the NH_3-WSL-APD-CSL Medium and Its Stability With Time.

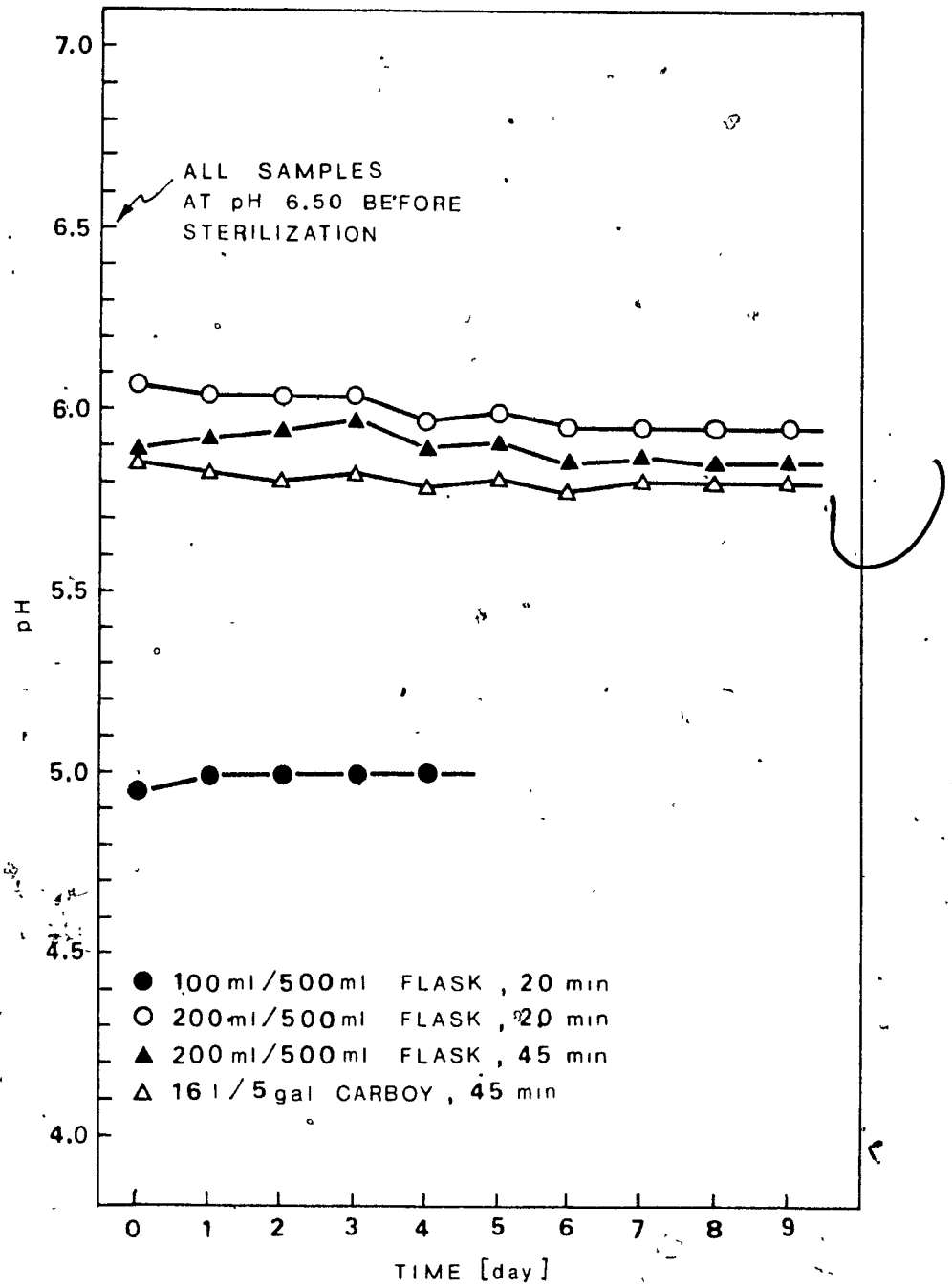
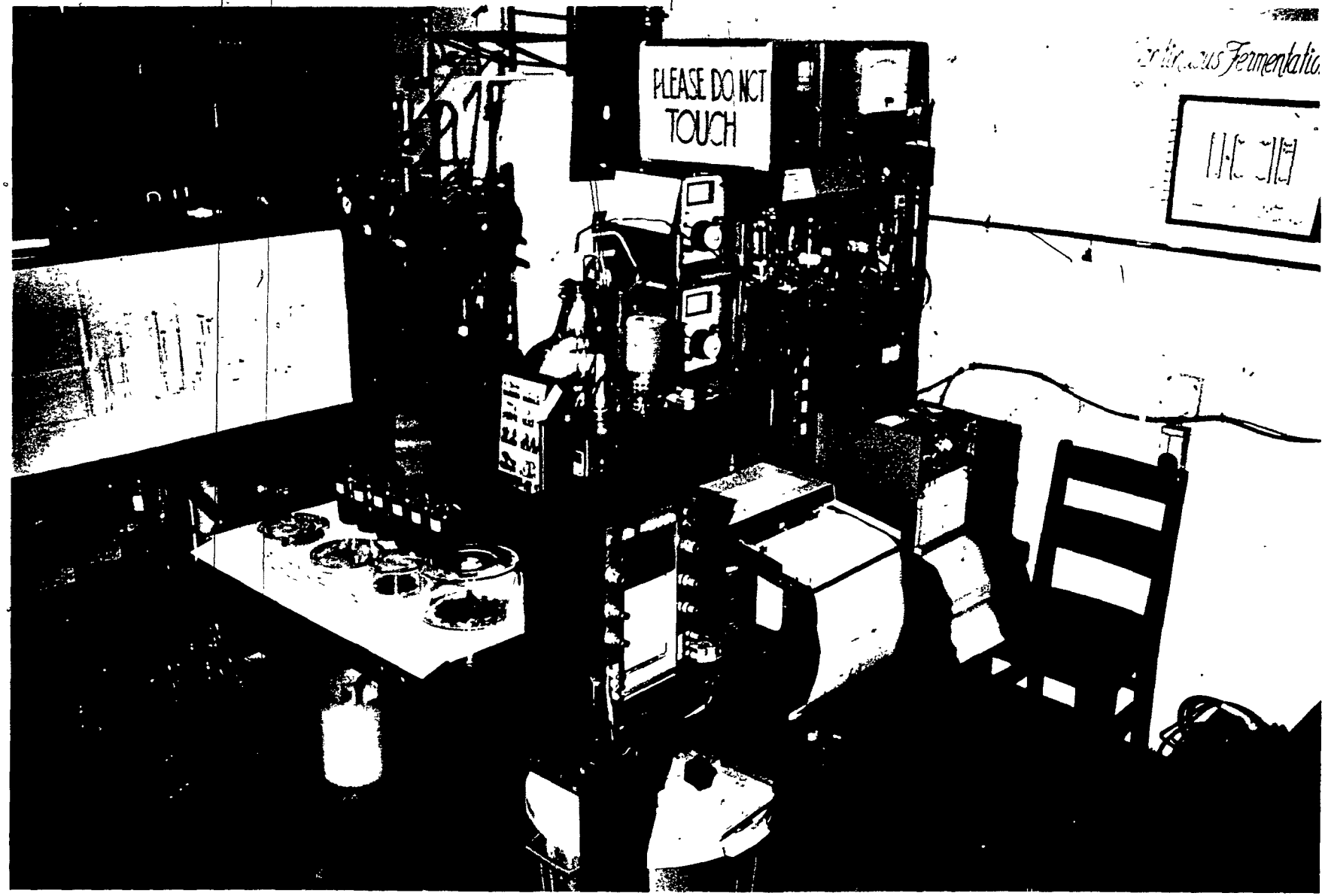

7.0
6.5
6.0
5.5
5.0
4.5
4.0
pH
ALL SAMPLES
AT pH 6.50 BEFORE
STERILIZATION
● 100 ml/500 ml FLASK, 20 min
○ 200 ml/500 ml FLASK, 20 min
▲ 200 ml/500 ml FLASK, 45 min
△ 16 l/5 gal CARBOY, 45 min
0 1 2 3 4 5 6 7 8 9
TIME [day]

Figure 5.13 The CSTF System Used for Continuous Cultivation of *M. crassipes* in NH_3-WSL.


PLEASE DO NOT
TOUCH

depended upon critical periods during the continuous cultivation experiment. It was usually sampled at approximately every two hours during the pulse tests on the system. Figure 5.14 shows the dynamic data for six inputs $x^1, x^2, \ldots, x^6$ and five outputs $y^1, y^2, \ldots, y^5$. For the clarity of the graph, the curves were obtained by joining together the experimental data obtained. However, the numerical data for all inputs and outputs are shown in Table B.13 together with other variables which were measured and recorded during the continuous cultivation experiment, such as the ambient temperature (°C), air pressure for aeration (psig), jacket water flowrate (l/hr) and the dilution rate (hr^{-1}).

The experiment was started as a batch process. At the end of the exponential growth phase (at the 28th hour, maximum growth rate $\simeq$ 0.08 g/l-hr), the fresh medium from the feed tank No. 1 was fed into the fermentor at a flowrate of 100 ml/hr. The feed medium (16 liters) was prepared in a 5-gallon carboy as described in paragraph 4.2.1. However, the maximum fungal growth at this stage produced foam continuously and rapidly in the fermentor jar. A solution (10 g/l) of Dow-Corning Antifoam AF Emulsion had to be pumped into the fermentor jar at a flowrate of 100 ml/hr in order to completely

Figure 5.14 Progress of Continuous Cultivation of *M. crassipes* in NH_3-WSL : (A) Inputs, (B) Outputs.

x^1 = aeration rate,

x^2 = agitation speed,

x^3 = inlet jacket temperature,

x^5 = inlet pH,

x^6 = inlet TCH concentration,

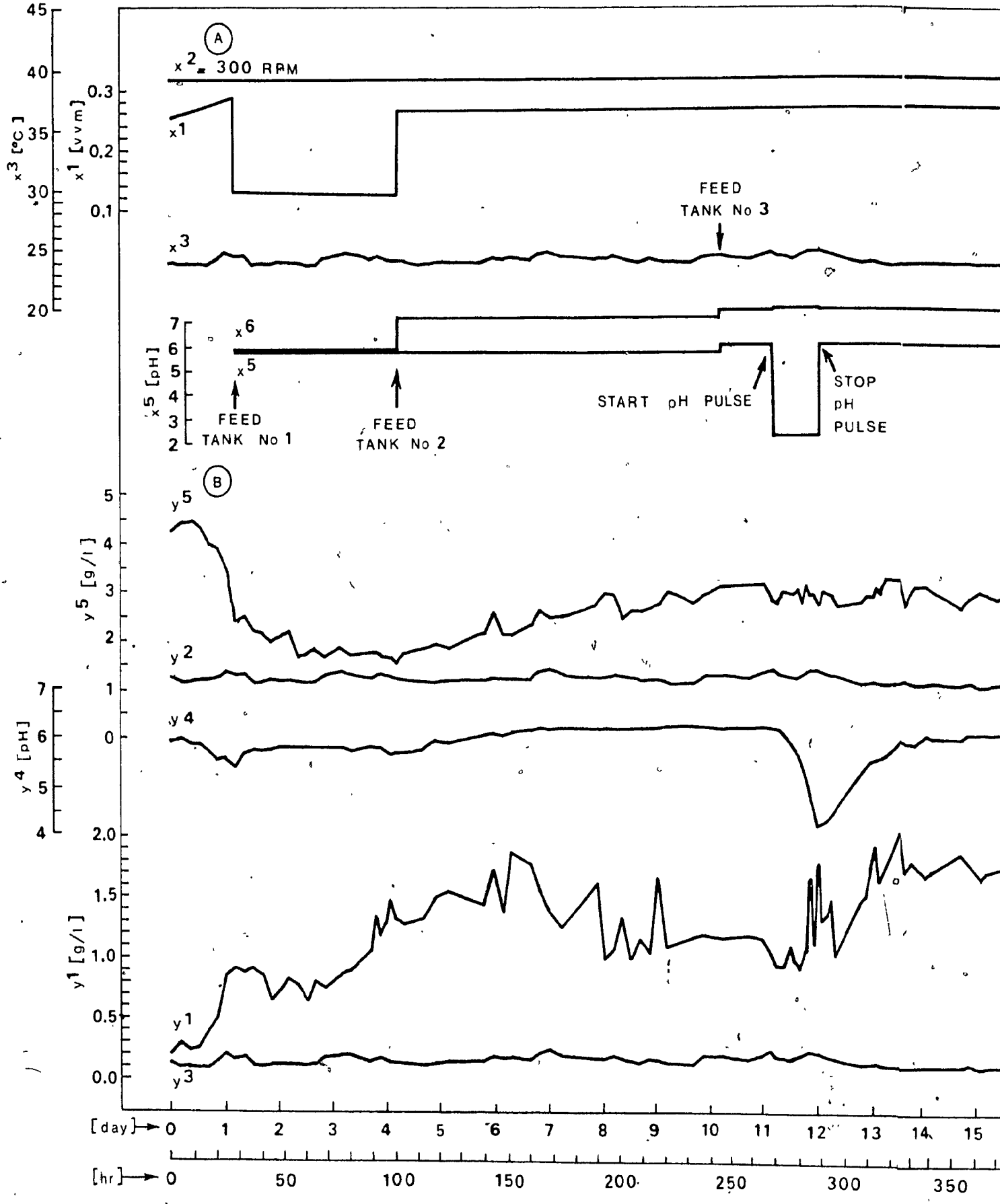
y^1 = biomass concentration,

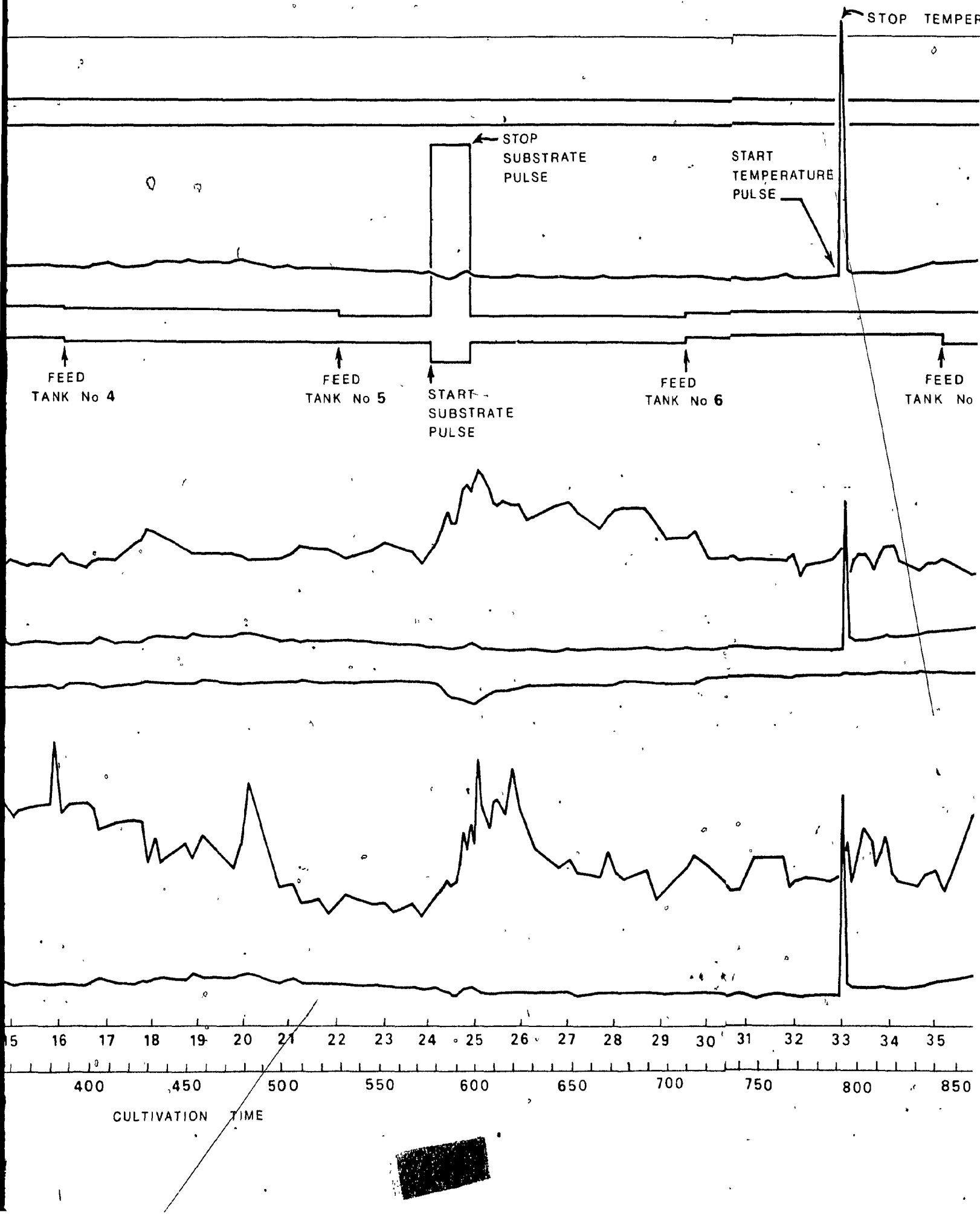
y^2 = outlet temperature,

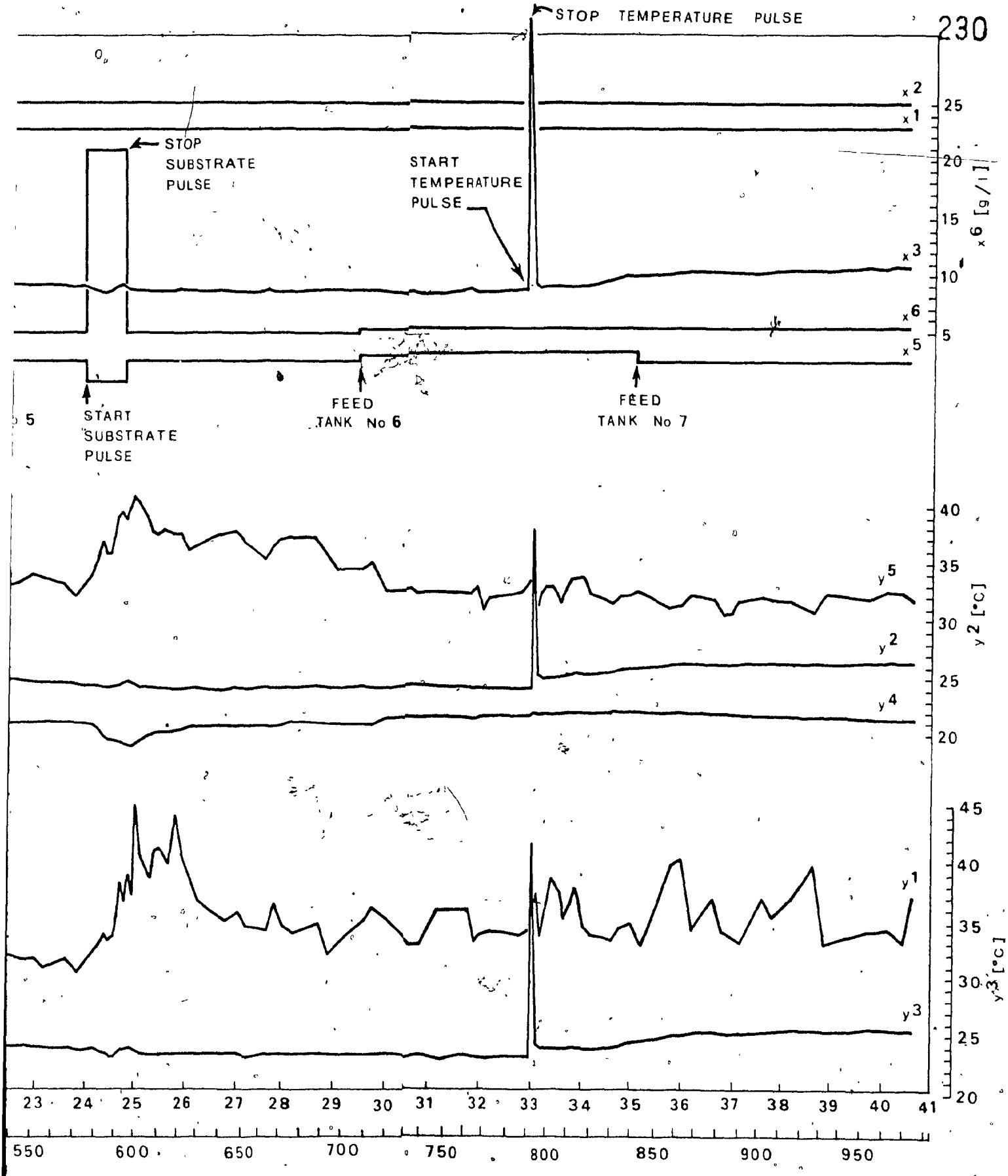
y^3 = outlet jacket temperature,

y^4 = outlet pH,

y^5 = outlet TCH concentration.


A
x2 = 300 RPM
x1
x3
x3 [°C]
x1 [vvm]
FEED
TANK No 3
x6
x5
x5 [pH]
FEED
TANK No 1
FEED
TANK No 2
START pH PULSE
STOP
pH
PULSE
B
y5
y2
y5 [g/l]
y4
y4 [pH]
y1
y3
y1 [g/l]
[day]
[hr]


STOP TEMPER
STOP
SUBSTRATE
PULSE
START
TEMPERATURE
PULSE
FEED
TANK No 4
FEED
TANK No 5
START
SUBSTRATE
PULSE
FEED
TANK No 6
FEED
TANK No
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
400 450 500 550 600 650 700 750 800 850
CULTIVATION TIME


STOP TEMPERATURE PULSE
STOP SUBSTRATE PULSE
START TEMPERATURE PULSE
START SUBSTRATE PULSE
FEED TANK No 6
FEED TANK No 7
x1
x2
x3
x5
x6
y1
y2
y3
y4
y5
x6 [g/l]
y2 [°C]
y3 [°C]
230
25
20
15
10
5
40
35
30
25
20
45
40
35
30
25
20
23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
550 600 650 700 750 800 850 900 950

control the foam. This resulted in a very high concentration of antifoam in the culture medium (5,000 ppm) and also reduced the level of substrate concentration of the feed line and the dilution rate of the CSTF system by one-half of the original. At the 100th hour of cultivation, the feed tank No. 2 was introduced. The Dow Corning Antifoam C Emulsion was used for the rest of the continuous cultivation experiment by direct addition to the feed tank at a concentration of 2 g/l of feed medium. This lower concentration of antifoam in the culture medium (2,000 ppm) was found to be effective in depleting the foam formation during the experiment and did not completely inhibit growth. The direct addition of antifoam into the feed tank also did not affect the level of substrate concentration of the feed medium and the dilution rate of the CSTF system. This procedure was used to prepare all feed tanks (No. 2 to No. 7, pH pulse tank and substrate pulse tank) for the whole experiment.

Pulse tests were performed when the steady state was reached for the cell mass concentration. The selection of the magnitude and the duration for the input pulse signals is very important. The magnitude of the pulse should not be too large in order to ensure the response of the system still within the linear zone

around its operating point. It should however not be too small either, so that a clear response signal from the system could be obtained. The duration of the pulse should not be too long because of possible physiological and morphological changes in the microorganisms, but it should not be too short either so that a distinguished response signal from the system could be assured. The selection of the pulse sign (positive or negative) has also to be justified for a specific variable.

The pH pulse (x^5) was applied to the CSTF system at the 268th hour. The negative pulse at pH 2.30 was selected because the original pH of the concentrated NH_3-WSL was 2.30 (Table 2.1). For a process operated in conjunction with a pulp and paper mill, the feed medium has to be prepared by diluting the concentrated NH_3-WSL and then adjusting the pH to 6.50 (before sterilization). Should a failure occur in the dilution and neutralization steps, a concentrated WSL might be introduced into the fermentor. Hence, the inlet pH drops to 2.30. Therefore, a pulse of a low pH value was selected in this study. The duration of the inlet pH pulse signal was decided based upon the observation of the outlet pH of the culture medium. At the 20th hour of the pH pulse, the outlet pH had the value of 4.18, which is the undesirable pH value for the growth of

M. crassipes in NH_3-WSL as previously found (Fig. 3.1). Therefore, the duration of the pH pulse was selected to last for 20 hours.

The positive inlet substrate pulse (x^6) at TCH concentration of 22.12 g/l was applied to the CSTF system at the 580th hour. The selection of the magnitude for this pulse signal was based on the same reasoning as for the pH pulse. The duration of the substrate pulse was kept the same as that of the pH pulse (20 hours).

The positive temperature pulse (x^3) was applied to the CSTF system at the 791st hour. The inlet jacket temperature was continuously increased to 46.5°C (the corresponding outlet temperature was 39.7°C) and then it was continuously lowered to its original operating value (23.0°C). The total duration of this pulse was 3 hours. The selection of the positive temperature pulse was as follows: It was found that *Morchella* did not grow at high temperatures (35-40°C) whereas it grew slowly at low temperatures ($\sim$ 0°C). Another possibility in actual operation could be a failure in the cooling system which would cause the temperature of a culture medium to rise up to $\sim$ 40°C. Therefore, a positive temperature pulse was selected in this study. Among the three pulse tests, the temperature pulse was the most critical for the CSTF

system. The undesirable consequence of this pulse was observed as an oscillatory response in the biomass concentration (three cycles are presented in Fig. 5.14 between 795-975th hours). In addition, the apparent biomass response at the 793rd hour was not a true response of the CSTF system. When the temperature of the culture medium reached the maximum value (39.7°C), some mycelia grew around the inner wall of the fermentor jar, collapsed and fell down into the culture medium and thus came out through the harvesting line.

The pulse test on the aeration(x^1) was not performed because higher aeration (for positive aeration pulse) creates excess foam in the fermentor which significantly changes the level of the culture medium and also could introduce contamination into the system. Lower aeration (for negative aeration pulse) could introduce contamination to the system as well due to back pressure to the fermentor, and also allows the growth of fungi in a pellet form, which makes uniform sampling through the harvesting tube impossible. The pulse test on the agitation (x^2) was not performed because higher agitation (for positive agitation pulse) continuously flushes the culture medium onto the inner wall of the fermentor, thus creating a large amount of wall growth, which would affect determination of the true biomass

concentration in the system. Lower aeration (for negative agitation pulse) also allows growth of fungi in a pellet form, which is undesirable as already mentioned. The pulse test on the feed temperature was not performed because it did not have any effect on the outputs of the CSTF system (Fig. 5.11).

The continuous cultivation experiment was stopped at the 975th hour. An amount of culture medium in the fermentor was transferred aseptically into a sterile flask. Streak plate tests and microscopic examination of this sample showed that no contamination occurred during the whole course of the continuous cultivation. It should be noted that the high values for the biomass between the 80-200th hours were probably due to the high concentration of antifoam which was adsorbed on the mycelia. Also, the positive biomass pulse responses from the pH pulse and the temperature pulse suggested that the optimal conditions found from shake flask experiments are not necessarily the optimal conditions for the continuous experiments. This could be understandable, because the cells grown in a batch process (shake flask) do not have the same physiological state as those grown in a continuous process (always in exponential phase).

5.3.3 Transfer Function Matrix

The smoothed data from the pulse tests on the inputs and their corresponding responses at the outputs were supplied to the program PULSE, then the resulting frequency data were supplied to both programs NLREG1 and LNREG2. Figures 5.15 to 5.25 show the experimental and smoothed data for each pair of input-output variables, and their resulting transfer functions from both regression techniques. The time increment (Δt) used to integrate each variable and the frequency range for the Bode diagrams are also shown in these Figures. It was found that the frequency range for the Bode diagrams are very low (maximum frequency of all transfer functions varied between 0.028-6.000 rad/hr). The low frequency range is a common characteristic for other physico-chemical systems and processes.

From a chemical engineer's point of view, the transfer function matrices for the CSTF system for *M. crassipes* in NH_3-WSL medium operated at the quasioptimal conditions are related to its inputs and outputs as,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \simeq \begin{bmatrix} \frac{0.293\, e^{-240s}}{(307s+1)} & \frac{1.037\, e^{-5s}}{(38s+1)^2(23s+1)} & \frac{0.256}{(75s+1)} \\ \frac{0.883}{(25s+1)} & 0 & 0 \\ \frac{0.968}{(18s+1)} & 0 & 0 \\ 0 & \frac{0.952}{(31s+1)} & \frac{0.064}{(32s+1)} \\ 0 & 0 & \frac{0.393}{(29s+1)^2} \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix} \qquad (5.25)$$

Figure 5.15 Transfer Functions Between y^1 and x^3.

y^1 = biomass concentration,

x^3 = inlet jacket temperature.

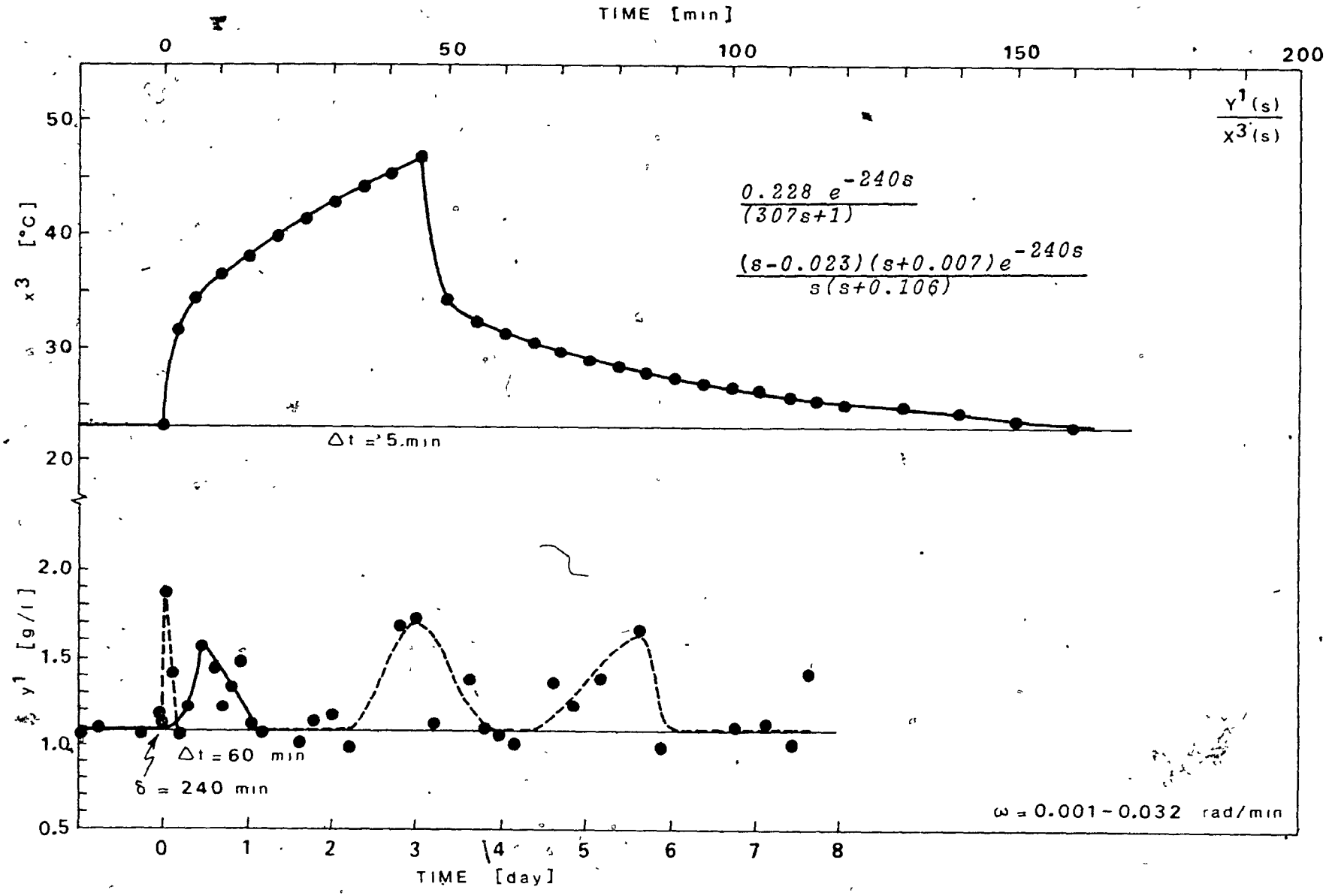

TIME [min]
0
50
100
150
200
$\frac{Y^1(s)}{X^3(s)}$
$\frac{0.228\ e^{-240s}}{(307s+1)}$
$\frac{(s-0.023)(s+0.007)e^{-240s}}{s(s+0.106)}$
x3 [°C]
50
40
30
20
Δt = 5 min
y1 [g/l]
2.0
1.5
1.0
0.5
Δt = 60 min
δ = 240 min
ω = 0.001 - 0.032 rad/min
0
1
2
3
4
5
6
7
8
TIME [day]

Figure 5.16 Transfer Functions Between y^1 and x^5.

y^1 = biomass concentration,

x^5 = inlet pH.

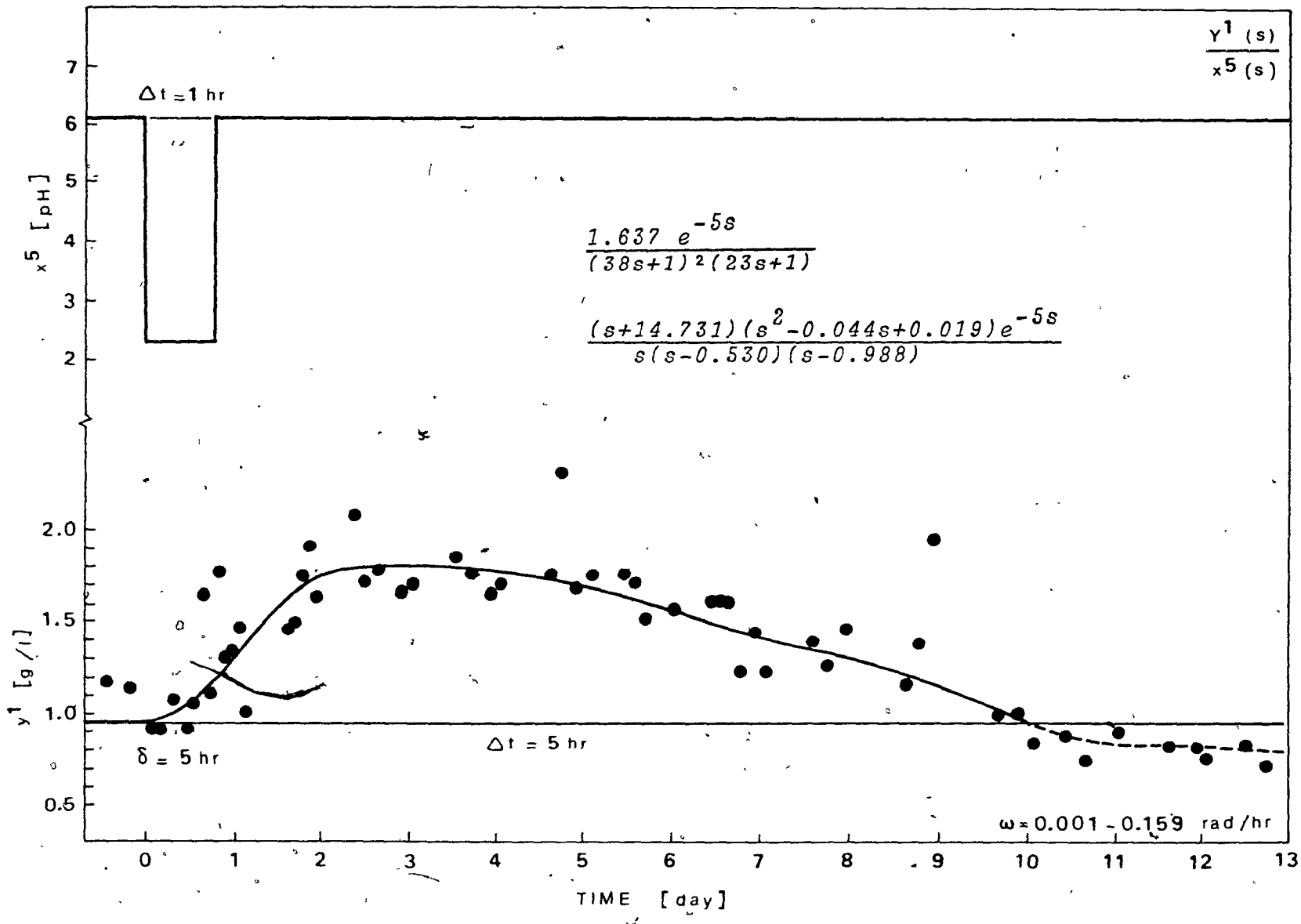

$\frac{Y^1(s)}{x^5(s)}$
$\frac{1.637\ e^{-5s}}{(38s+1)^2(23s+1)}$
$\frac{(s+14.731)(s^2-0.044s+0.019)e^{-5s}}{s(s-0.530)(s-0.988)}$
Δt = 1 hr
x^5 [pH]
y^1 [g/l]
δ = 5 hr
Δt = 5 hr
ω = 0.001 - 0.159 rad/hr
TIME [day]

Figure 5.17 Transfer Functions Between y^1 and x^6.

y^1 = biomass concentration,

x^6 = inlet TCH concentration.

Figure 5.18 Transfer Functions Between y^2 and x^3.

y^2 = outlet temperature,

x^3 = inlet jacket temperature.

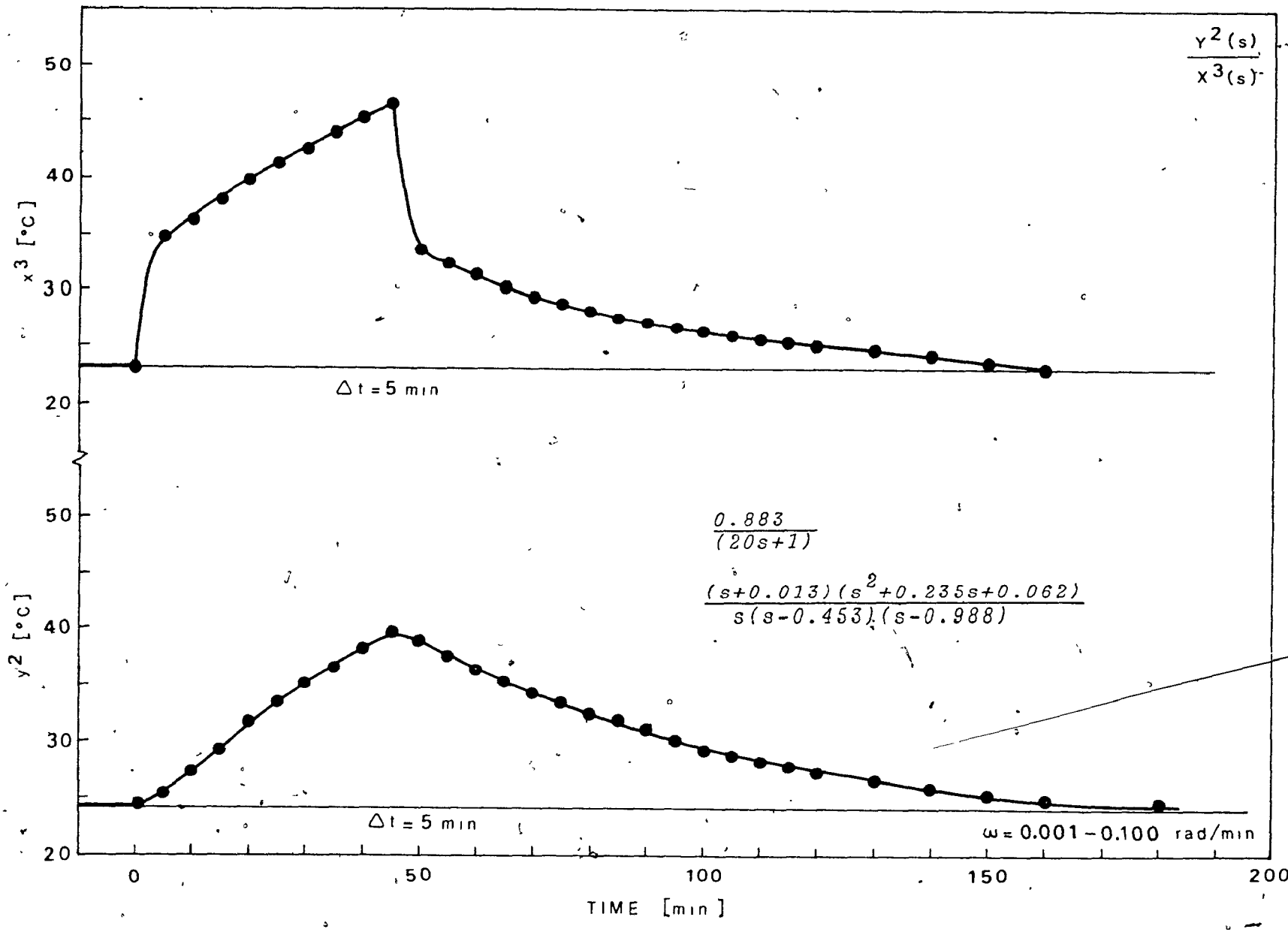

$\frac{Y^2(s)}{X^3(s)}$
x^3 [°C]
50
40
30
20
Δt = 5 min
$\frac{0.883}{(20s+1)}$
$\frac{(s+0.013)(s^2+0.235s+0.062)}{s(s-0.453)(s-0.988)}$
y^2 [°C]
50
40
30
20
Δt = 5 min
ω = 0.001 – 0.100 rad/min
0
50
100
150
200
TIME [min]

Figure 5.19 Transfer Functions Between y^3 and x^3.

y^3 = outlet jacket temperature,

x^3 = inlet jacket temperature.

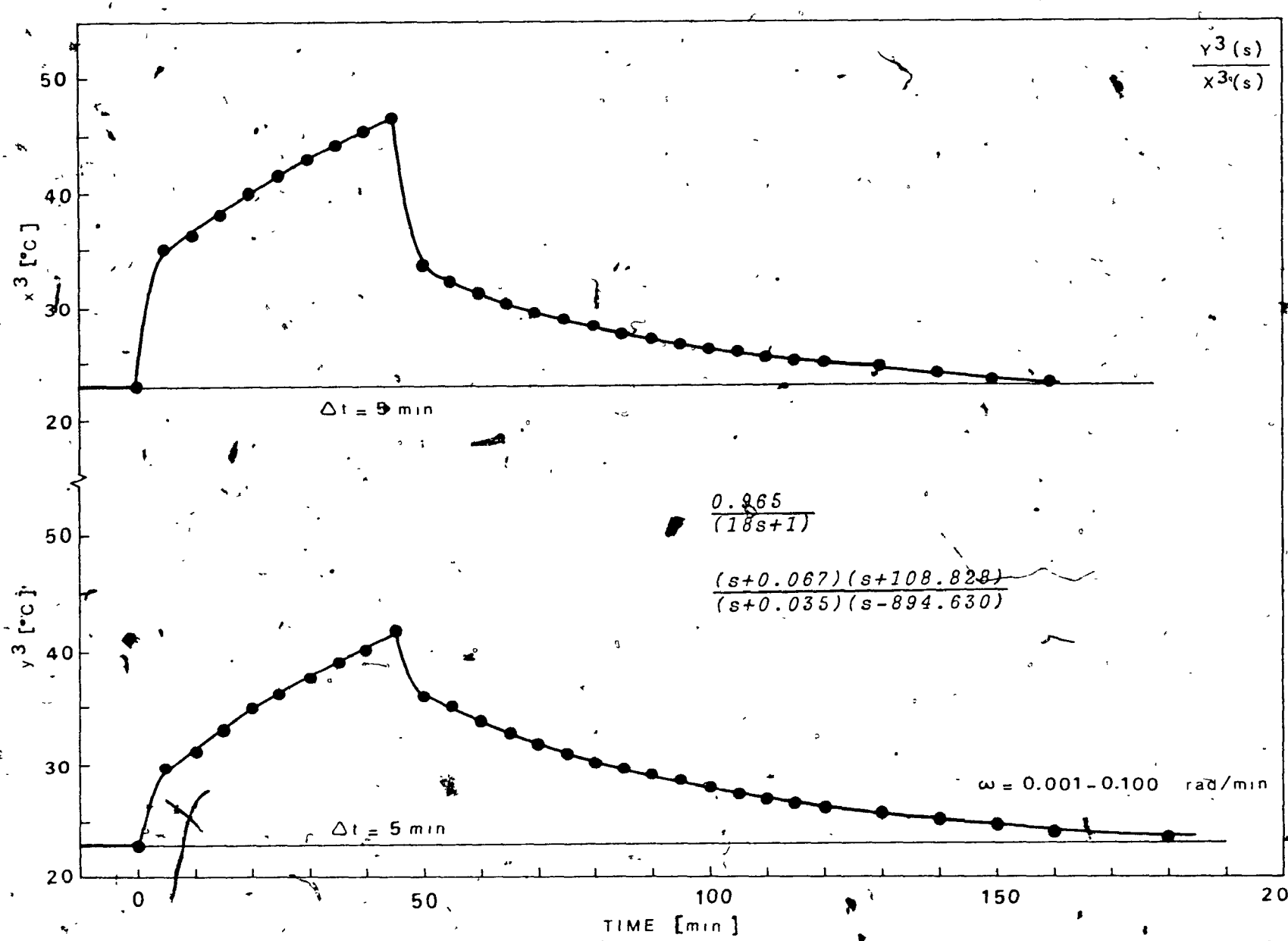

$\frac{Y^3(s)}{X^3(s)}$
x^3 [°C]
50
40
30
20
Δt = 5 min
$\frac{0.965}{(18s+1)}$
$\frac{(s+0.067)(s+108.828)}{(s+0.035)(s-894.630)}$
y^3 [°C]
50
40
30
20
ω = 0.001 - 0.100 rad/min
Δt = 5 min
0
50
100
150
200
TIME [min]

Figure 5.20 Transfer Functions Between y^4 and x^5.

y^4 = outlet pH,

x^5 = inlet pH.

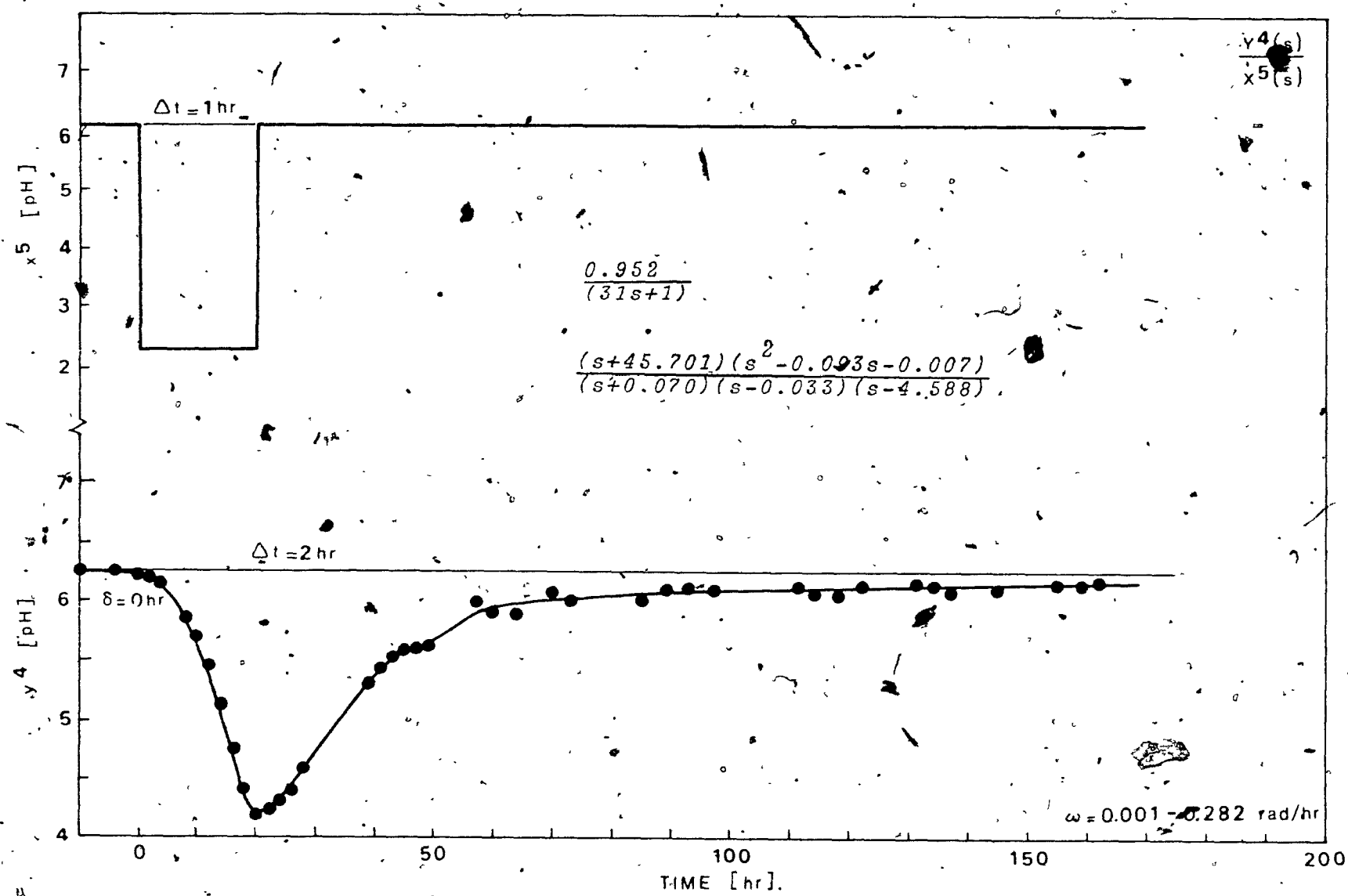

$\frac{Y^4(s)}{X^5(s)}$
$\Delta t = 1$ hr
x^5 [pH]
7
6
5
4
3
2
$\frac{0.952}{(31s+1)}$
$\frac{(s+45.701)(s^2-0.093s-0.007)}{(s+0.070)(s-0.033)(s-4.588)}$
$\Delta t = 2$ hr
$\delta = 0$ hr
y^4 [pH]
6
5
4
$\omega = 0.001 - 0.282$ rad/hr
0
50
100
150
200
TIME [hr].

Figure 5.21 Transfer Functions Between y^4 and x^6.

y^4 = outlet pH,

x^6 = inlet TCH concentration.

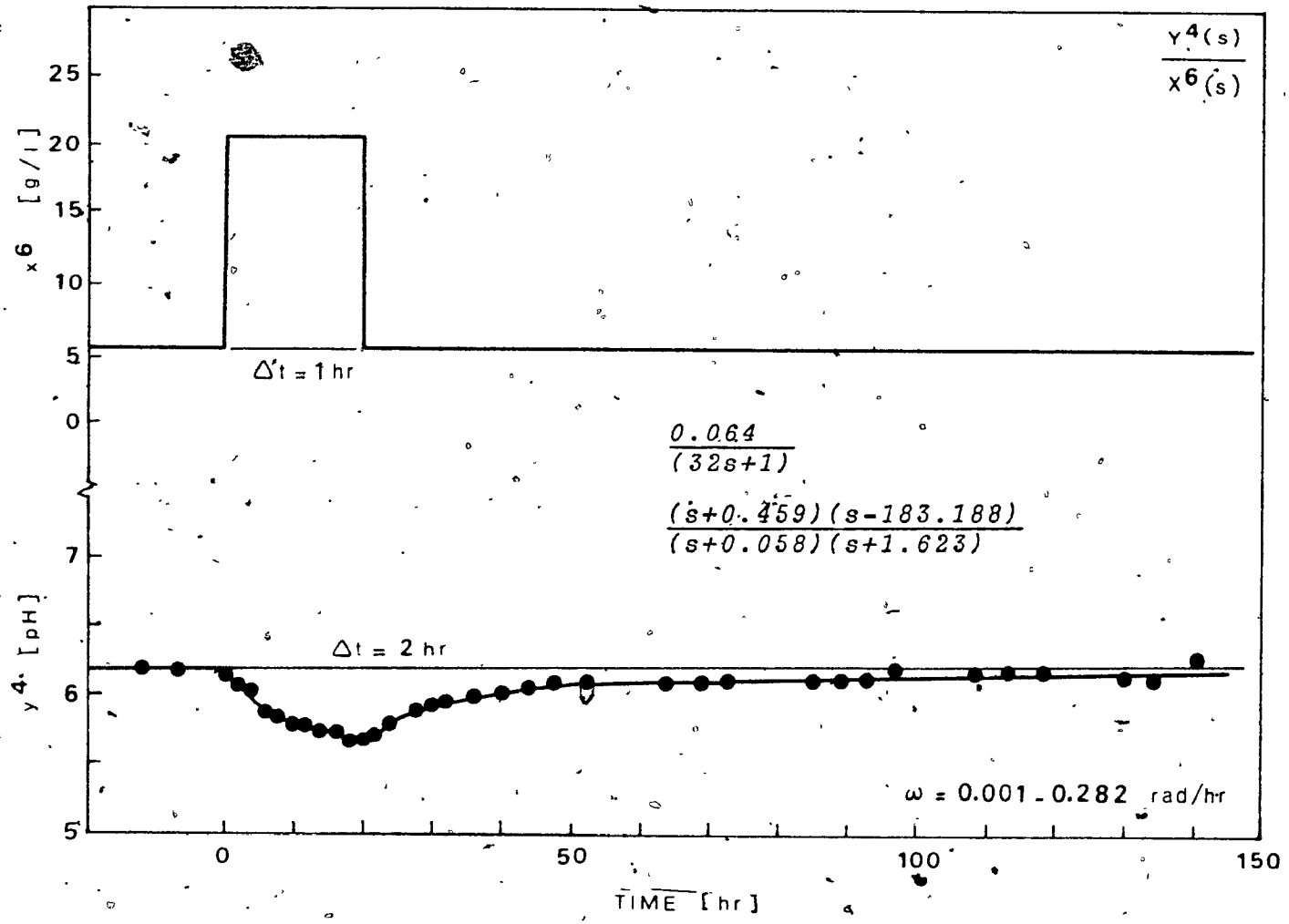

$\frac{Y^4(s)}{X^6(s)}$
$\frac{0.064}{(32s+1)}$
$\frac{(s+0.459)(s-183.188)}{(s+0.058)(s+1.623)}$
x^6 [g/l]
25
20
15
10
5
Δt = 1 hr
0
7
y^4 [pH]
6
5
Δt = 2 hr
ω = 0.001 - 0.282 rad/hr
0
50
100
150
TIME [hr]

Figure 5.22 Transfer Functions Between y^5 and x^6.

y^5 = outlet TCH concentration,

x^6 = inlet TCH concentration.

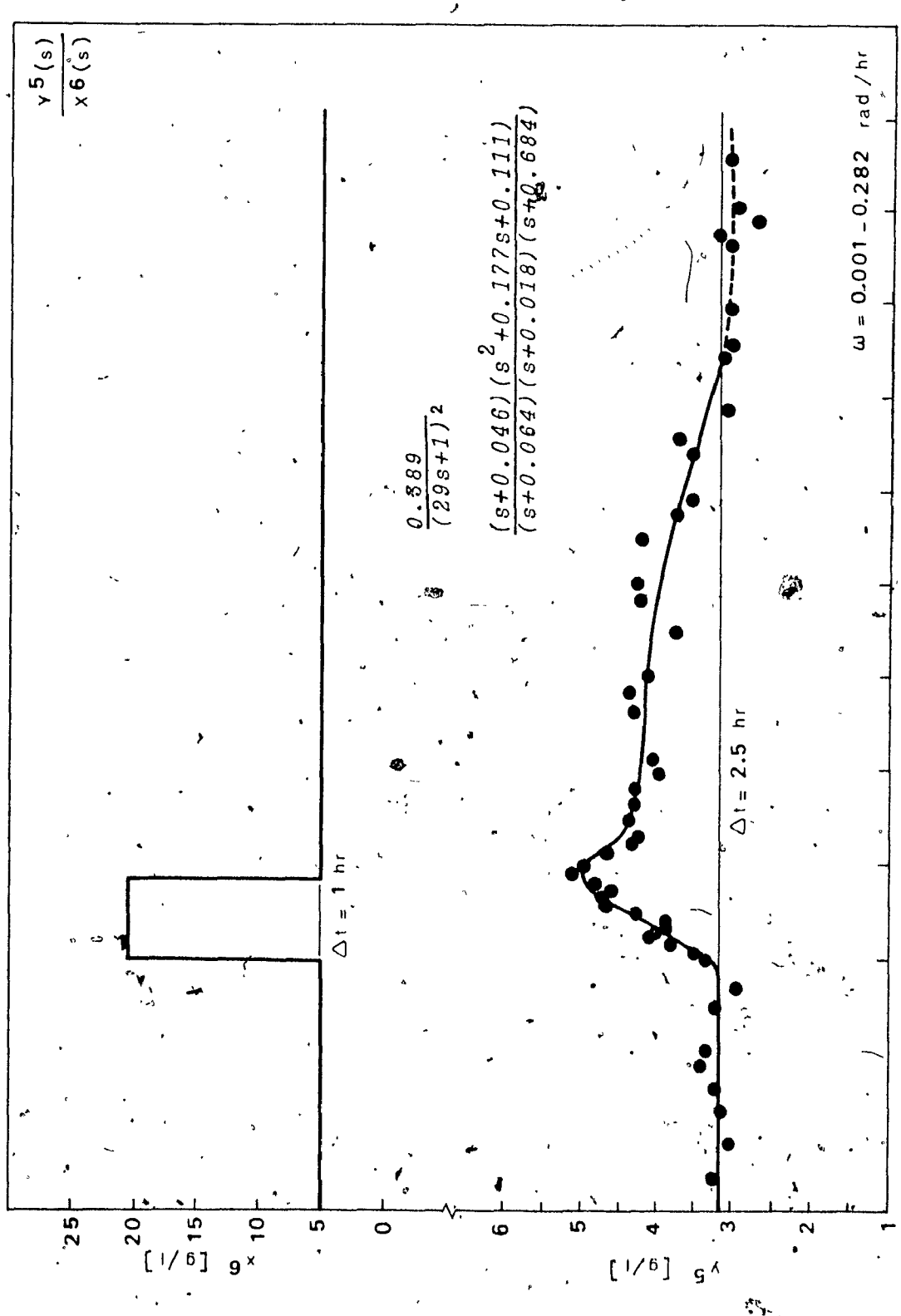

$\frac{Y^5(s)}{X^6(s)}$
$\frac{0.889}{(29s+1)^2}$
$\frac{(s+0.046)(s^2+0.177s+0.111)}{(s+0.064)(s+0.018)(s+0.684)}$
Δt = 1 hr
Δt = 2.5 hr
ω = 0.001_0.282 rad/hr
TIME [day]
y^5 [g/l]
x^6 [g/l]

Figure 5.23 Transfer Functions Between y^1 and y^2.

y^1 = biomass concentration,

y^2 = outlet temperature.

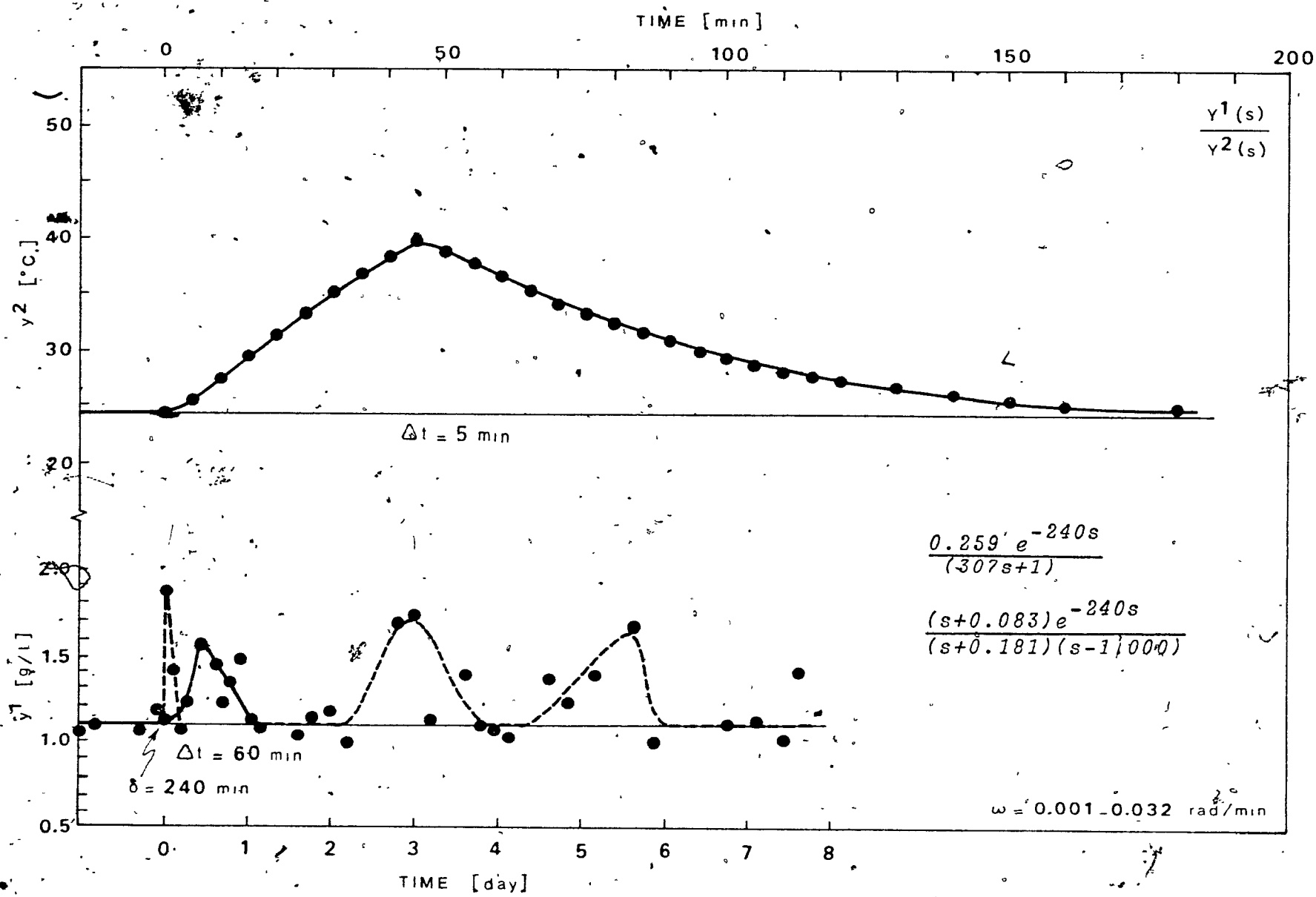

TIME [min]
0
50
100
150
200
y2 [°C]
50
40
30
20
Δt = 5 min
y1 [g/l]
2.0
1.5
1.0
0.5
Δt = 60 min
δ = 240 min
0
1
2
3
4
5
6
7
8
TIME [day]
Y1(s) / Y2(s)
0.259 e^-240s / (307s+1)
(s+0.083) e^-240s / (s+0.181)(s-1/000)
ω = 0.001-0.032 rad/min

Figure 5.24 Transfer Function Between y^1 and y^4.

y^1 = biomass concentration,

y^4 = outlet pH.

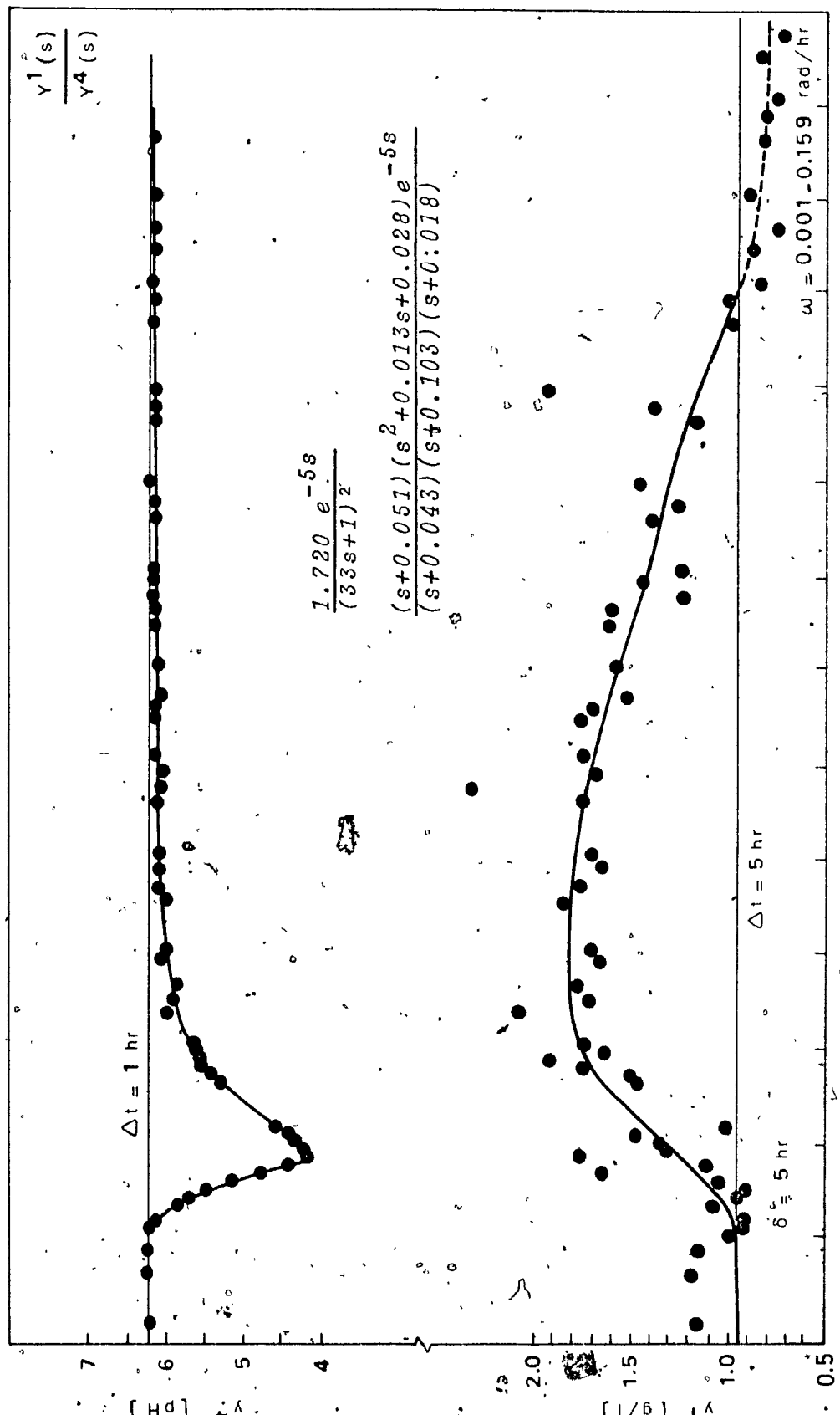
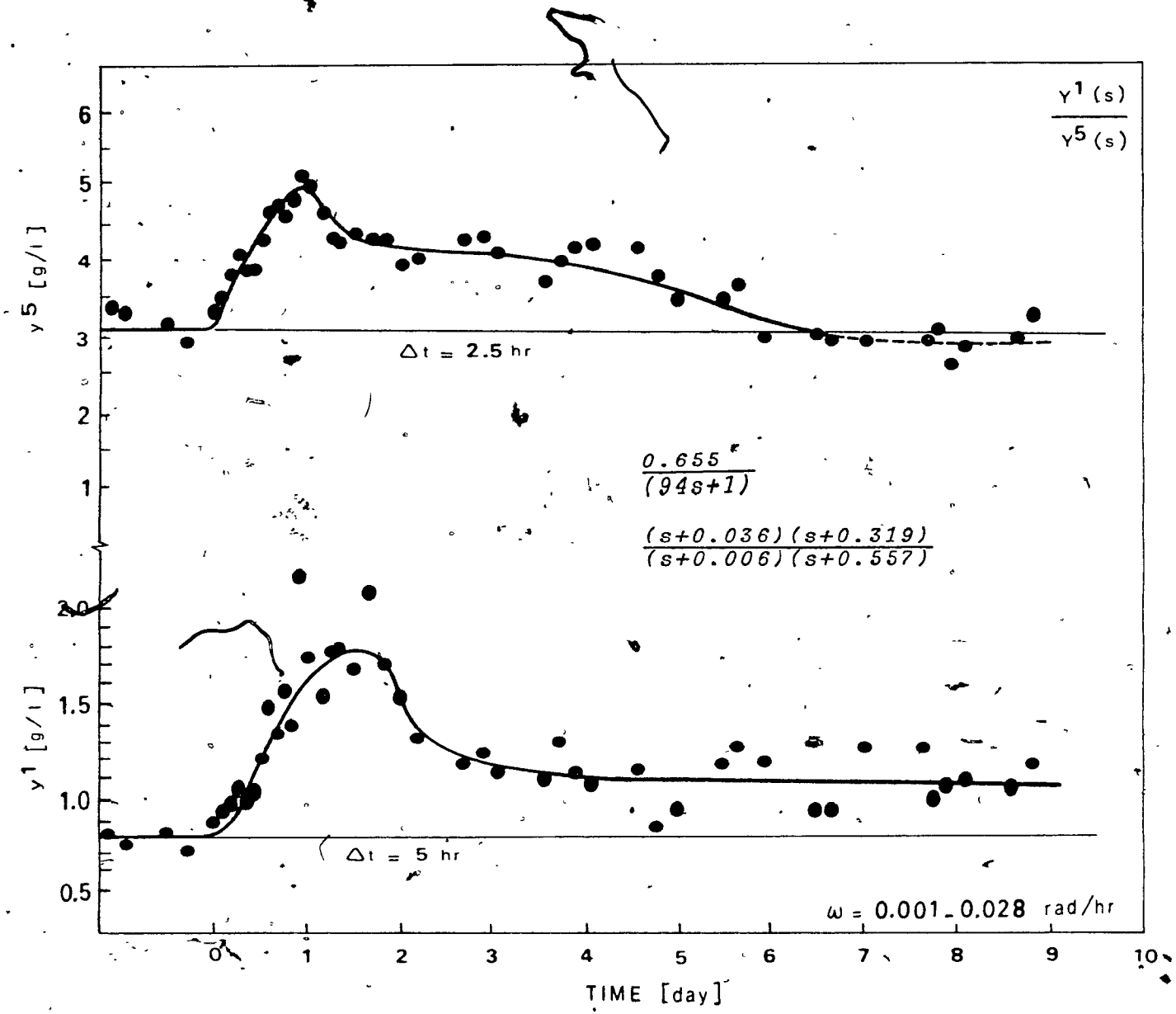

$\frac{Y^1(s)}{Y^4(s)}$
$\frac{1.720\ e^{-5s}}{(33s+1)^2}$
$\frac{(s+0.051)(s^2+0.013s+0.028)e^{-5s}}{(s+0.043)(s+0.103)(s+0.018)}$
Δt = 1 hr
Δt = 5 hr
δ = 5 hr
ω = 0.001-0.159 rad/hr
TIME [day]
y4 [pH]
y1 [g/l]
7
6
5
4
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 5.25 Transfer Functions Between y^1 and y^5.

y^1 = biomass concentration,

y^5 = outlet TCH concentration.


$\frac{Y^1(s)}{Y^5(s)}$
y^5 [g/l]
Δt = 2.5 hr
$\frac{0.655}{(94s+1)}$
$\frac{(s+0.036)(s+0.319)}{(s+0.006)(s+0.557)}$
y^1 [g/l]
Δt = 5 hr
ω = 0.001_0.028 rad/hr
TIME [day]

and,

$$\begin{bmatrix} Y^1(s) \\ Y^2(s) \\ Y^3(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \approx \begin{bmatrix} & & \\ & 0 & 0 \\ & 0 & 0 \\ 0 & & \\ 0 & & \end{bmatrix} \begin{bmatrix} X^3(s) \\ X^5(s) \\ X^6(s) \end{bmatrix} \tag{5.26}$$

However, from a microbiologist's point of view, the inputs and outputs of the CSTF system are different. The output is the cells or biomass (y^1) and the inputs are the variables which directly affect the cells. They are the temperature of the culture medium or the outlet temperature (y^2), the pH of the culture medium or the outlet pH (y^4) and the substrate concentration in the culture medium or the outlet substrate concentration (y^5). In this respect, the transfer function matrices are related to its inputs and outputs as,

$$\begin{bmatrix} Y^1(s) \\ \vdots \end{bmatrix} \approx \begin{bmatrix} \frac{0.259\, e^{-240s}}{(307s+1)} & \frac{1.790\, e^{-60s}}{(333s+1)^2} & \frac{0.68E}{(310s+1)} \end{bmatrix} \begin{bmatrix} Y^2(s) \\ Y^4(s) \\ Y^5(s) \end{bmatrix} \tag{5.27}$$

and,

$$\begin{bmatrix} y^1(s) \\ \vdots \end{bmatrix} \simeq \begin{bmatrix} \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots \end{bmatrix} \begin{bmatrix} y^2(s) \\ y^4(s) \\ y^5(s) \end{bmatrix} \tag{5.28}$$

It should be noted that the transfer function matrices from Eqs. (5.25) and (5.27) are more useful for practical purposes (design, operation and control) because the lead-order systems as shown in Eqs. (5.26) and (5.28) could only be artificially realized and only used for system compensations. The lead systems never exist for natural physicochemical or biological systems.

Most of the transfer functions in Eqs. (5.25) and (5.27) are best approximated by first-order system (time constants between 5.1-94 hours) with a dead time (between 0-5 hours). The second-order transfer functions were obtained between y^5 and x^6, and y^1 and y^4, and one third-order transfer function was obtained between y^1 and x^5. In practice, higher order transfer functions could always be approximated by a first-order transfer function with dead time. Seven transfer functions in Eq. (5.25) are nil because no response was observed at the outputs when its corresponding inputs were disturbed.

In recent years, a number of dynamic tests

on the inputs of several CSTF systems have been performed and their responses were observed and reported. Fuld *et al.* (1961) performed a periodic disturbance on the inlet pH and recorded the responses of the bacterial density and the substrate concentration of the system of *Lactobacillus delbrueckii* grown in a glucose synthetic medium. Gilley and Bungay (1968) also reported the response of the yeast count from a periodic disturbance on the dilution ratio of the system of *Saccharomyces cerevisiae* grown in a glucose synthetic medium. An impulse disturbance on the inhibitor concentration (ethanol) has been performed on the system of *Aerobacter aerogenes* grown in a glucose synthetic medium and their responses have been recorded for the biomass concentration, morphology (cell size) and the specific growth rate [Zines and Rogers (1970)]. The same type of impulse disturbance on the inhibitor concentration (potassium sorbate) and the responses on the outlet inhibitor concentration, outlet substrate concentration, biomass concentration and the yield coefficient have also been reported for the system of *Saccharomyces cerevisiae* grown in a molasses complex medium [Borzani and Vairo (1973)]. The pulse excitations on the dilution rate and on the inlet substrate concentration have been performed on the system of *Saccharomyces cerevisiae* grown in a glucose synthetic medium, and the

response of yeast count was recorded [Young *et al.* (1970)]. Most aperiodic disturbances have been performed using step signals. Zines and Rogers (1970) and Borzani and Vairo (1973) also performed step disturbances on the inputs of their previously described systems. Step disturbances on the inlet substrate concentration and on the dilution rate of the system of *Saccharomyces cerevisiae* grown in a glucose synthetic medium have been performed by Gilley and Bungay (1967), Regan *et al.* (1971) and Young and Bungay (1973). Gilley and Bungay (1967) recorded the response of a yeast count as a single output. Regan *et al.* (1971) reported the transient responses of the biomass concentration, the outlet substrate concentration and the product concentration in terms of ethanol, CO_2 and O_2 as outputs. Young and Bungay (1973) also recorded the responses of several outputs including the DO, outlet substrate concentration, biomass concentration, yeast count, specific growth rate and the cell composition in terms of protein and ribonucleic acid (RNA). George and Gaudy (1973) reported the responses of several outputs including the outlet pH, biomass concentration, COD and the cell composition in terms of protein, TCH, deoxyribonucleic acid (DNA), RNA, carbon, hydrogen and nitrogen from the step disturbance on the inlet pH of the system of mixed culture grown in a glucose synthetic medium. Several authors have also theoretically studied the step responses of the CSTF

systems. However, these authors had to assume a Monod or a variation of Monod growth equation for the system studied. Koga and Humphrey (1967) reported the response of the biomass concentration and the outlet substrate concentration when the system was disturbed by a step on the inlet substrate concentration and on the dilution rate. Yano and Koga (1969) reported the response of the biomass concentration, outlet substrate concentration and the product concentration on the step disturbance on the inlet substrate concentration where the phenomenon of substrate inhibition was taken into account. The responses of the biomass concentration and the outlet substrate concentration on the step disturbance on the temperature have also been studied by Topiwala and Sinclair (1971).

Although many experimental data on the dynamic testing of the CSTF system have been reported in literature, very few authors have attempted to identify their systems using these transient data. Zines and Rogers (1970) and Young and Bungay (1973) used a slightly different approach for their system identifications. The dynamic behaviour of their systems was *a priori* proposed by a mathematical model either under the form of a differential equation [Zines and Rogers (1970)] or in the form of a

transfer function [Young and Bungay (1973)]. The analog and/or digital simulations were used to estimate the parameters of the model by fitting the simulated data with the experimental data in the time domain. Another approach in system identification which is similar to that used in this thesis, had been used by Fuld *et al.* (1961), Gilley and Bungay (1968) and Young *et al.* (1970). This method consisted of converting the experimental data in the time domain into the frequency domain under the form of Bode diagrams, then the transfer function of the CSTF system could be determined from these Bode diagrams. Using this approach, Fuld *et al.* (1961) obtained a first-order lag transfer function between the bacterial density and the inlet pH of the culture medium with a time constant of 6.5 minutes while Gilley and Bungay (1968) found a third-order lag transfer function between the yeast count and the dilution rate, with the quadratic time constants of about 40 minutes and a first-order time constant of about 8 minutes. Young *et al.* (1970) revealed a second to third-order lag transfer function between the yeast count and the dilution rate, with a time constant in the range of 40 minutes, and a third-order lag transfer function between the yeast count and the inlet substrate concentration, with a time constant in the range of 40 minutes to one hour. It is interesting to note that the transfer functions

between the yeast count and the dilution rate of the CSTF system of *Saccharomyces cerevisiae* in a glucose synthetic medium obtained by the frequency response method [Gilley and Bungay (1968)] and by the pulse testing method [Young *et al.* (1970)] were very close.

The technical difficulties have also been confirmed by several pioneer authors in the field of CSTF system identification. The first type of difficulty is the stability of the system steady state. This stability was never achieved in the past for the CSTF system with fungi. Fuld *et al.* (1961) first attempted to use a fungus *Aspergillus ochraceus* for the CSTF system, however these authors had difficulty in attaining the steady state of the system and they decided to work with a bacterium (*Lactobacillus delbrueckii*). This difficulty was assumed to be due to a slow growth rate of the fungus as compared to that of bacteria and yeasts. The instability of the steady state in the continuous cultivation of fungi might also be due to their differentiation and branching during growth [Megee *et al.* (1970), Fencl (1970)] while the binary fission as in the case of bacteria is simpler. Regan *et al.* (1971) observed an oscillation in the transient response of the cell mass concentration of yeasts and suggested that the oscillation was associated with the budding cycle of the yeasts. Hence, all other studies

on the CSTF system identification have been conducted with unicellular microorganisms. It is also difficult to obtain accurate experimental data. Fuld *et al.* (1961) stated that the measurements of glucose concentration in their work were not sensitive enough to reveal the small changes that were occuring, therefore they were not able to determine the transfer function between the outlet substrate concentration and the inlet pH of the culture medium. Young *et al.* (1970) stated that the transfer function between the specific growth rate and the substrate concentration could not be determined from their experimental data because the cell number is not an accurate parameter for the cell mass during transient periods. In fact, at present there are no experimental procedures available that would enable accurate determination of this transfer function. This inaccuracy of the experimental data usually yielded an oscillated result. Sinclair *et al.* (1971) postulated that most of the short period oscillatory behaviour in a continuous culture could be explained as being the result of poor sampling techniques or bad practice in the application of pH, foam, temperature or DO control.

The experimental results from this work showed that the technical difficulties on the stability of the

steady state and on the accuracy of the experimental data also occurred for some variables. High fluctuations in the cell mass concentrations are mainly due to the non-homogeneous nature of the mycelial growth in the fermentor (Appendix E). In addition, the cell mass concentrations did not come back to its original steady state values after the pulse was applied (pH pulse and substrate pulse) and also gave rise to an oscillatory response (after the temperature pulse). These facts could be due to the physiological changes of the microorganisms because of natural mutation and/or different operating conditions (during the pulse tests). This shows that the step response method is not suitable for identification of the CSTF system, because in this method, the microorganisms are subjected to different operating conditions for a long period of time (infinity). They certainly do change their physiological and morphological properties, as reported by Zines and Rogers (1970). The frequency response method was also not successful for identification of the CSTF system for fungi [Fuld *et al.* (1961)] because of the slow growth rate of the mycelium. The complete response from a single pH pulse took place for more than 10 days (Fig. 5.16), therefore a periodic input would give a highly fluctuated response resulting from superimposure of a series of output pulses.

However, the most difficult technical part in this work was due to the fact that *Morchella* is extremely susceptible to contamination, and the NH_3-WSL is very attractive for other fungi. The contaminants, that frequently occurred in the system were bacteria and other fungi (*Aspergillus*).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

6.1.1 Significance

The results of this work demonstrate an alternate possibility for utilizing edible fungi in a biological treatment of WSL from the pulp and paper industry. Besides all the advantages which have been mentioned in Chapters 3 and 5, there are two significant achievements which need to be stressed.

First, it has been reported that *M. crassipes* does not grow in WSL (the base has not been specified) medium which is neutralized to pH 5.0 with calcium carbonate and supplied with 0.1% of monobasic ammonium phosphate as source of nitrogen and phosphate [Cirillo *et al.* (1960)]. It was shown in this work that *M. crassipes* can be cultivated in NH_3-, Mg- and Na-WSL culture media and that a high yield can be obtained as compared to other species of morels. The wild fruiting *M. crassipes* found in nature have the largest bodies when compared to other morels (Fig. 1.3).

The second significant point is that since the first attempt to identify the CSTF system using frequency response method was unsuccessful [Fuld *et al.* (1961)], no further work was carried out to identify a continuous cultivation of fungi. The results from Chapter 5 demonstrate that due to the slow growth rate of fungi, the frequency response method is not suitable for identification of the CSTF system for fungi. Using the pulse testing method, the transfer function matrix of the CSTF system for fungi can be obtained. This provides a breakthrough in technology, because the knowledge of the transfer function matrix (or a dynamic behaviour) of a system is necessary and essential for design, operating (prediction) and control of the industrial continuous cultivation of fungi.

6.1.2 MMM/WSL Process

The submerged cultivation of MMM in WSL, which is named the MMM/WSL process, consists of a combination of the pulp and paper waste treatment process and single cell protein (SCP) production process. It is probable that most present processes being developed for SCP production are similar in broad outline but differ in detail such as in choice of microorganisms, substrate, fermentation

conditions, harvesting and purification procedures etc. Therefore, the description of the MMM/WSL process (Fig. 6.1) could be based on the process flow diagram for SCP production from hydrocarbons previously reported [Wang (1968), Bennett *et al.* (1969), Humphrey (1970)].

i) Description

The MMM/WSL process is operated under completely aseptic conditions so that all microorganisms are excluded except the specific one inoculated into the system (*Morchella*). The process is run continuously with partial recycle. A certain portion of the mother liquor from the fermentor effluent is recycled in order to supply the residual nutrient to the fermentor and in the same time to dilute the concentrated WSL coming from the digester. This recycled mother liquor could also be used to prepare the mineral salts solution for the fermentor. The sequence of processing steps is the following: (a) liquid and solid raw materials reception and storage, (b) medium preparation, (c) air and liquid medium sterilization, (d) fermentation, (e) cell harvesting and washing and (f) water removal from biomass. The material is produced in a single fermentor and the product separation and recovery are achieved in a single stream. Aseptic

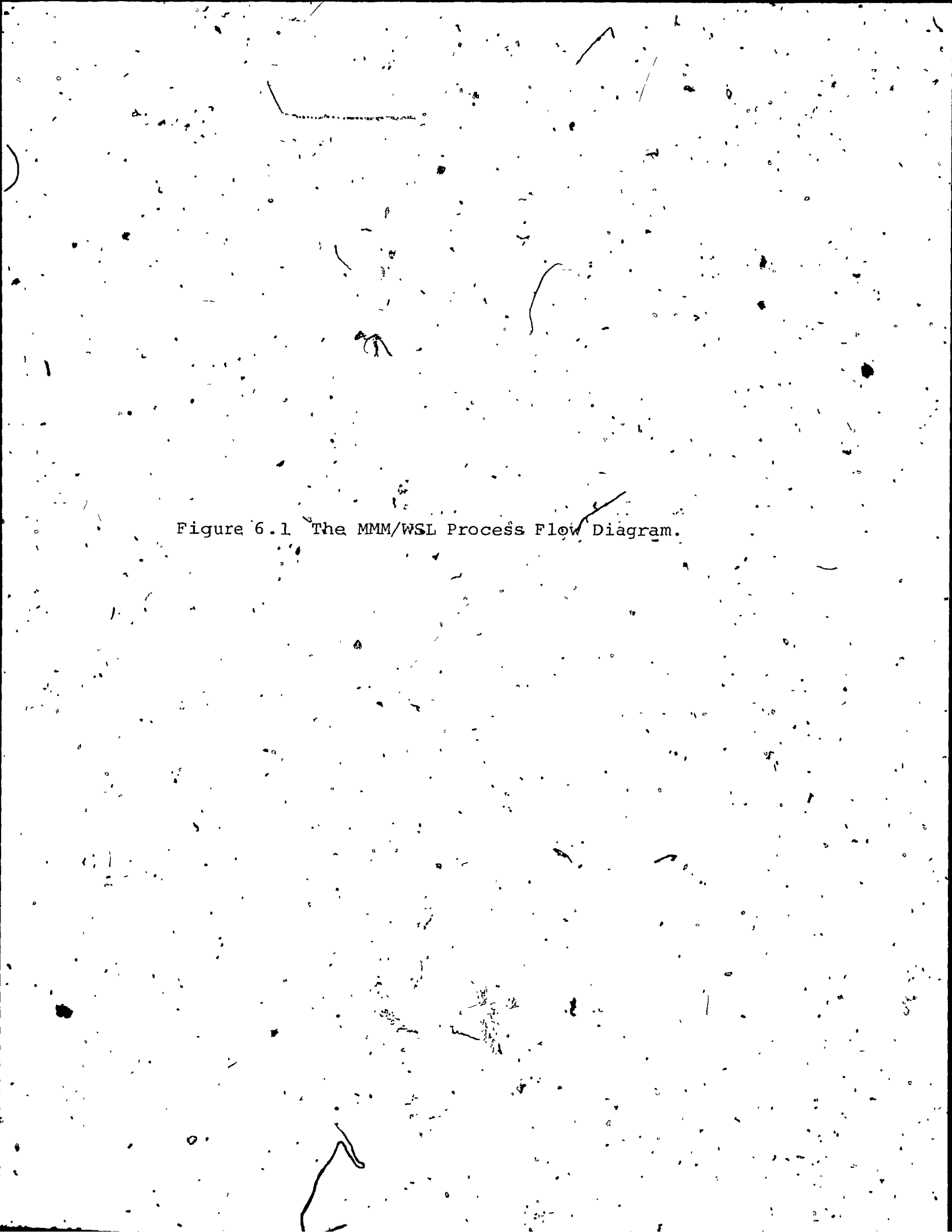
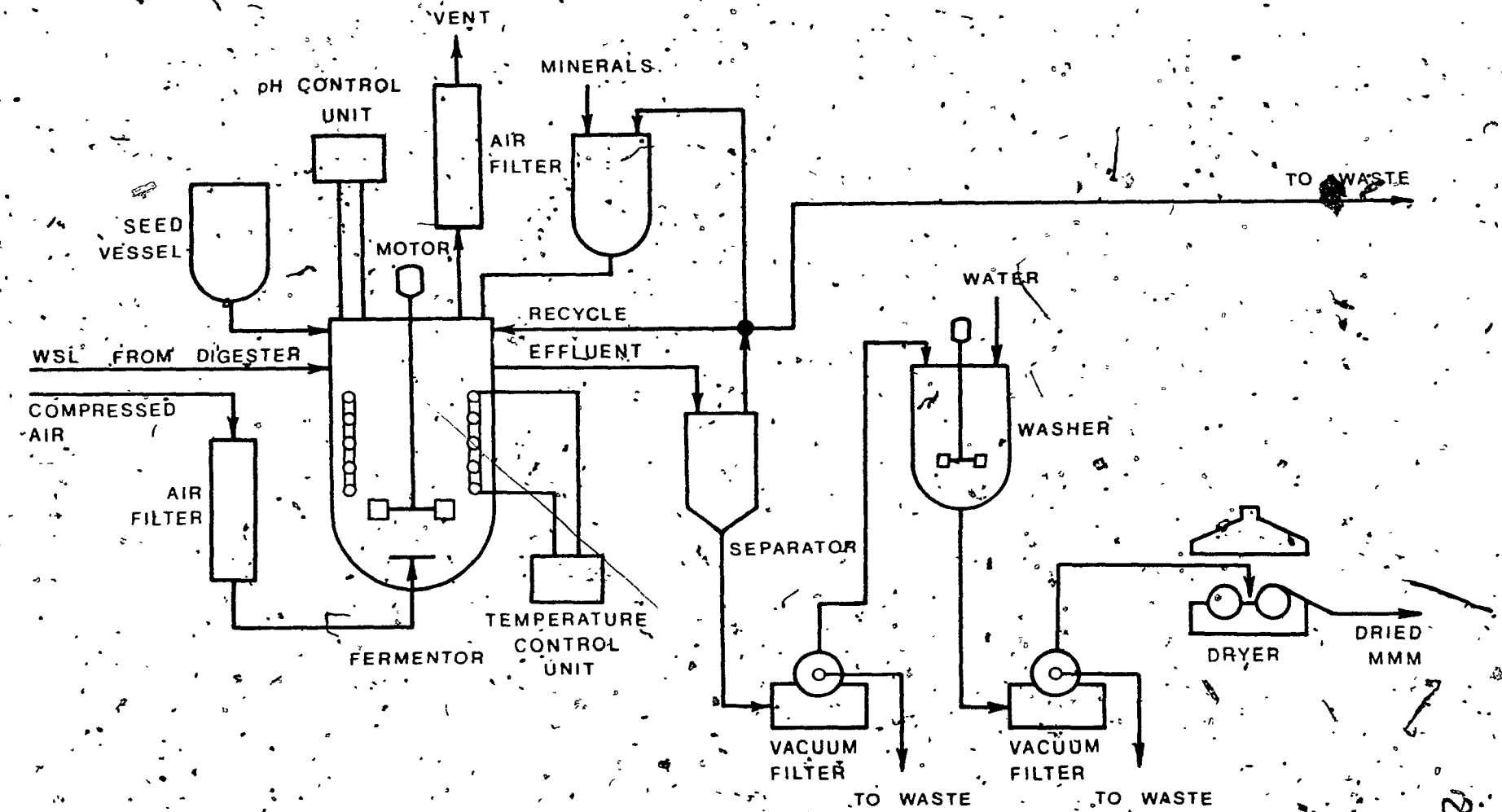


Figure 6.1 The MMM/WSL Process Flow Diagram.


VENT
MINERALS
pH CONTROL UNIT
AIR FILTER
SEED VESSEL
MOTOR
TO WASTE
WATER
RECYCLE
EFFLUENT
WSL FROM DIGESTER
COMPRESSED AIR
WASHER
AIR FILTER
SEPARATOR
TEMPERATURE CONTROL UNIT
FERMENTOR
DRYER
DRIED MMM
VACUUM FILTER
VACUUM FILTER
TO WASTE
TO WASTE

conditions are maintained only on the fermentor and accessories. Thereafter, normal precautions, as experienced by the general food industry practices, are observed by maintaining a high standard of cleanliness. Major plant items are made of stainless steel to facilitate this clean operation and thus ensure an acceptable end product.

ii) Fermentation

Mineral nutrients and growth factors (APD and CSL) essential for cell propagation are steam sterilized and fed continuously at a controlled rate to the fermentor as an aqueous solution. A metered stream of WSL passes directly to the fermentor at a controlled rate. Sterilization of WSL is not necessary as it is received as sterile from the digester.

All the oxygen necessary for cell growth is provided from a continuous stream of air blown into the fermentor. This is sterilized by filtration. Excess air and the carbon dioxide formed in the fermentor pass through a sterile filter before release to the atmosphere. This after-filter is provided to prevent any possible back contamination which may occur during low or temporary loss of air flow.

The pH of the culture medium is controlled by a pH control unit. The transfer of nutrients and oxygen to the cells is achieved in the fermentor by intimate mixing using mechanical agitators. Economic operation has been achieved for this step of the process by experimenting with agitator type and position and with air sparging rate.

The heat released during the fermentation together with the relatively low fermentation temperature of about 25°C necessitate external cooling during summer. In the winter period, however, a heating system is required to maintain this operating temperature.

iii) Harvesting

The product stream of fermentation broth is processed in a settling-type separator unit to yield a fungal stream, of about 15 per cent weight solids and a mother liquor. The mother liquor is pumped to the mineral tank, to the fermentor as nutrients recycle and to the waste stream for further treatment such as the tertiary treatment plant. The fungal stream is further concentrated in a vacuum filter, washed with water in a washing vessel and reconcentrated in a vacuum filter. The fungal filter cake is then passed to the dryer. The

dried powder, containing 5-6 weight per cent of moisture is discharged from the dryer, cooled and conveyed to bulk storage.

6.1.3 Feasibility of MMM/WSL Process

i) Technical Feasibility

Several important factors have to be considered for selection of microorganisms to be used as food. The technical requirements consist of (a) rapid growth, (b) simple media, (c) suspended culture, (d) simple separation, (e) freedom from infection and stable fermentation, (f) effecient utilization of energy source and (g) disposable effluent. The MMM/WSL system meets all of the above requirements. The growth rate of MMM is certainly slower than that of bacteria and yeasts, however it is rapid if compared with the mass doubling time of plant or animal feedstocks (Table 6.1). Ideally, commercial SCP production should operate at aseptic conditions. However, in the MMM/WSL process, the steam sterilization of the fermentor and its accessories is required. This procedure is widely used in the food and pharmaceutical industries. Therefore, the sterilization of equipment and the precautions for plant sanitation

Table 6.1 Mass Doubling Times. From Humphrey (1969).

Organisms	Time For Doubling in Mass
Bacteria & yeasts	20-120 minutes
Fungi & algae	2-6 hours
Grass & some plants	1-2 weeks
Chickens	2-4 weeks
Hogs	4-6 weeks
Cattle	1-2 months
People	3-6 months

could be considered as normal operations in any food industries. The contamination during the fermentation progress could be eliminated by treating the fungal mycelium with an aqueous solution of an oxidizing agent selected from the group consisting of sodium hypochlorite and hydrogen peroxide [Bennett (1967)].

The physiological and organoleptic requirements are the following: (a) capable of genetic modification, (b) non-toxic, (c) good flavour and taste, (d) highly digestible, (e) high nutrient content and (e) protein, fat and carbohydrate content of high quality. The MMM/WSL also meets all of the above requirements. However, complete information on the toxicity and nutritional quality for the conditions (b) and (d) are not available at present.

Concerning the process itself, there are several advantages as compared to other similar processes. The most pronounced advantages are the following: First, it would certainly be economically desirable to eliminate the medium sterilization step because this would reduce the capital investment cost of the sterilization equipment as well as utility and operating costs. The MMM/WSL process possesses this advantage due to the sterile nature of the WSL coming from the digestors.

Secondly, the operating cost is associated with the heat evolution during the fermentation [Wang (1968)]. The metabolic conversion of carbohydrates in the MMM/WSL process proceeds *via* oxidation to yield, as end product, fungal proteins, carbohydrates, lipids and B-vitamins, while carbon dioxide and water are liberated. The overall chemical reaction may be written as follows:

$$1.8n\ CH_2O + 0.8n\ O_2 + 0.19n\ NH_4^+ + \text{other essential elements (P, K, S, etc.)} \rightarrow n(CH_{1.7}O_{0.5}N_{0.19}Ash) + 0.8n\ CO_2 + 1.3n\ H_2O + 80{,}000n\ Kcal$$

This may be compared with the reaction for SCP process using hydrocarbons as substrate:

$$2n\ CH_2 + 2n\ O_2 + 0.19n\ NH_4^+ + \text{other essential elements (P, K, S, etc.)} \rightarrow n(CH_{1.7}O_{0.5}N_{0.19}Ash) + n\ CO_2 + 1.5n\ H_2O + 200{,}000\ Kcal$$

The molar heat release corresponds to about 3,000 Kcal and 7,600 Kcal respectively per Kg dry weight of biomass. The essential differences to note are that the use of hydrocarbon substrates instead of carbohydrates, requires a supply of about two and a half times as much atmospheric oxygen and releases over twice as much heat in the reaction.

Also, because the two liquid reaction phases for the former process are virtually immiscible, sufficient agitation must be provided to disperse the smaller volume of the hydrocarbon phase thoroughly into the larger volume of the aqueous phase. These are advantages of the MMM/WSL process as compared to the hydrocarbon fermentation process relative to operating costs [Bennett *et al.* (1969)].

As far as plant location is concerned, the usual site-finding considerations still apply. The plant should be near the pulp and paper mills, provided that transportation cost of the final product to the market is not out-of-line, and that process utilities are available at the site.

ii) Economic Feasibility

The most valuable economic analysis of the MMM/WSL process should be based on the data obtained from a pilot-plant test unit. Unfortunately, these data are not available at present. However, the production cost of SCP from the MMM/WSL process could be estimated based on a similar process. Wang (1968) proposed a list of production costs for SCP from purified *n*-paraffins. However, due to the present inflationary pressures (TIME, April 8, 1974) and the energy (oil) crisis (TIME,

November 9, 1973 and TIME, January 21, 1974), these item costs are not true anymore. The labor cost index and the plant cost index [Anonymous (1974)] show that the operating cost and the capital investment costs increased at least 60% and 40% respectively as compared to 1968. The price of hydrocarbon feedstock has also increased by 547% since 1968 (TIME, October 14, 1974). Thus, a revised estimation of the production cost for SCP from *n*-paraffins based on the most recent available cost indices is shown in Table 6.2.

The total production cost was approximated at 19-34 cents per pound (390-680 US dollars per ton) of biomass or 39-68 cents per pound (770-1,360 US dollars per ton) of crude microbial protein. As compared to the price of available food grade protein-rich products shown in Table 6.3, it is evident that crude SCP containing 50% protein must sell for no more than 50 cents per pound (1,000 US dollars per ton). Additional processing, such as cell purification through selective solvent extraction, or manipulations of cell properties for food formulations, or reduction of cell nucleic acids, would undoubtedly add to the production cost. While there is presently no way of knowing exactly how much this additional processing would cost, all these uncertainties will be resolved at some near-future date.

Table 6.2 Estimates of Production Costs for SCP.
Basis: 100,000 tons/year.

Item	Cost per pound (¢/lb)		
	SCP from *n*-Paraffins (50% Protein)	MMM/WSL (30-40% Protein)	
		With Capital Investment	Without Capital Investment
Raw Materials:			
Substrate	11.7 - 22.8	0.0 - 0.0	0.0 - 0.0
Others	1.0 - 1.0	1.0 - 1.0	1.0 - 1.0
Subtotal	12.7 - 23.8	1.0 - 1.0	1.0 - 1.0
Operating and Utilities:			
Aeration and agitation	2.0 - 3.2	6.0 - 9.6	6.0 - 9.6
Cooling	1.6 - 1.6	2.40 - 2.88	2.40 - 2.88
Recovery	0.4 - 0.8	1.17 - 1.56	1.17 - 1.56
Drying	0.64- 0.96	1.92 - 2.88	1.92 - 2.88
Others	0.32- 0.64	0.96 - 1.92	0.96 - 1.92
Subtotal	4.96- 7.20	12.45 - 18.84	12.45 - 18.84
Capital Investment:			
Fermentation	0.49- 0.98	1.47 - 2.94	0.0 - 0.0
Recovery	0.51- 0.70	1.53 - 2.10	0.0 - 0.0
Drying	0.07- 0.07	0.21 - 0.21	0.0 - 0.0
Cooling	0.31- 0.62	0.93 - 1.86	0.0 - 0.0
Others	0.28- 0.56	0.84 - 1.68	0.0 - 0.0
Subtotal	1.66- 2.93	4.98 - 8.79	0.0 - 0.0
Total Cost:	19.32- 33.93	18.43 - 28.63	13.4 - 19.84

Table 6.3 Price of Available Food Grade Protein-Rich Products.

Product	Price[a] (June 1973-1974) (¢/lb)	Protein Content (%)	Price of Pure Protein ($/lb)
Cottonseed meal	6.00 - 7.75	41.0[a]	0.15 - 0.19
Soybean (whole)	7.45 -18.30	40.0[b]	0.19 - 0.46
Corn (kernel)	3.45 - 5.60	8.06 - 10.88[c]	0.32 - 0.70
Wheat	4.00 - 9.15	7.0 - 18.0[d]	0.23 - 1.38
Milk (cow's fluid)	7.50	3.50 - 3.58[e]	2.01 - 2.14
Rice (rough)	9.0 -30.0	7.6 - 14.6[f]	0.62 - 3.95
Eggs (whole)	25.85 -57.50	12.8 - 13.4[g]	1.93 - 4.49
Meat (whole cuts of fresh muscles, beef, pork, lamb, veal)	74 - 134	12.8 - 19.9[h]	3.72 -10.47
Potatoes	4.3 -19.2	0.7 - 4.6[i]	0.94 -27.43
SCP from *n*-paraffins[j]	19.32 -33.93	50	0.39 - 0.68
MMM/WSL[j] (with capital investment)	18.43 -28.63	30 - 40	0.47 - 0.96
MMM/WSL[j] (without capital investment)	13.45 -19.84	30 - 40	0.34 - 0.67

(a) From Commodity Year Book (1974).
(b) From Wolf and Cowan (1971).
(c) From Matz (1969).
(d) From Shellenberger (1969).
(e) From Henderson (1971).
(f) From Beachell and Matz (1969).
(g) From Powrie (1973).
(h) From Lushbough and Schweigert (1960).
(i) From Smith (1968).
(j) From Table 6.2.

The estimate for production cost of SCP from the MMM/WSL process is shown in Table 6.2. It is obvious that the cost of WSL for substrate is nil. The corrected operating and utilities costs for cooling and recovery are estimated from the available data for SCP grown in carbohydrate substrates [Wang (1968)]. The rest of the cost items is assumed to be the same as for the SCP from *n*-paraffins. It should be noted that the real costs for some of these items are expected to be less than the assumed values, as carbohydrate fermentations are generally easier to operate than the hydrocarbon fermentations. Also product recovery is simpler. Due to the slow growth rate of fungi as compared to that of yeast (Table 6.1) used for hydrocarbon fermentations, another correction factor has to be made. The operating and utilities costs as well as the capital investment were assumed to be threefold higher than those estimated for the production of SCP from *n*-paraffins. The resultant estimate for total production cost of SCP from MMM/WSL process on the basis of 100,000 tons/year plant is 19-29 cents per pound (370-580 US dollars per ton) of biomass containing 30-40% protein or 46-96 cents per pound (930-1,910 US dollars per ton) of crude fungal protein.

The greatest economic advantage of the MMM/WSL process is due to the fact that it is a combination of a pulp and paper wastes treatment plant and a SCP production plant. If the treatment of pulp and paper wastes is obligated, then the net capital investment for the MMM/WSL process is that for the pulp and paper wastes treatment plant. The capital investment for WSL treatment plant is not available but it could be estimated based on a similar activated sludge waste water treatment process [Eckenfelder and Barnard (1971), Evans and Wilson (1972)]. The operating and utilities costs for the MMM/WSL process are also much reduced because they are shared with those for the pulp and paper wastes treatment plant. In this respect, the total production cost for SCP from MMM/WSL process will certainly be less than 14-20 cents per pound (270-400 US dollars per ton) of biomass or less than 34-66 cents per pound (160-300 US dollars per ton) of crude fungal proteins. Compared to the price of available food grade protein-rich products in Table 6.3, the SCP from the MMM/WSL process would be economically feasible.

Concerning the ideal plant size for SCP production, at the present time, a 10,000 tons/year size for animal feeds is not economical. Under favorable economic conditions, a 100,000 tons/year size is much more attractive. There is roughly a 40% price difference

in product from the two sized plants for a 10% return on investment. The expected return on investment, balance of payments for a country, prime interest rate on capital necessary for securing process equipment, etc., can greatly influence the economic feasibility. The capital costs for a 100,000 tons/year plant are reported to be of the order of 20 million US dollars. If the capital can be obtained within a given country and if the SCP production can be used to enhance a balance of payments, then the attractiveness of the SCP process will be increased [Anonymous (1972a)].

This MMM/WSL process thus appears attractive because of its capability to produce high quality protein product at lowest cost from the readily available raw materials. In the same time, it reduces the pollution load from the pulp and paper industry. If, with the passage of time, developments in this process bring the same degree of improvement as has been possible in many other new processes, then the highest hopes for this will have been fulfilled.

6.2 Recommendations

As mentioned earlier, a pilot-plant test unit is necessary to provide data for technical and economical

scale-up of the production plant. The pilot-plant would also provide materials for the toxicity and digestibility tests, and for the product utility studies.

There is adequate evidence to indicate that some species of yeasts, algae, fungi and bacteria can be safe and useful sources of proteins, vitamins and minerals for animal and human consumption. However, the safety of such materials depends on the microorganisms selected, the quality of the substrates utilized and the conditions of growth. This point has recently been stressed by the Protein Advisory Group (PAG) of the United Nations System [Anonymous (1973a)]. Whether or not a given product is safe can be determined by following the procedures specified by the PAG Guideline No. 6 [Anonymous (1972 a,b)]. In general, it consists of acute toxicity tests, subchronic toxicity tests, chronic toxicity tests, carcinogencity tests and multiple generation tests on animals such as rats and/or mice. The value of SCP for human consumption should be confirmed by the testing procedure outlined in the PAG Guideline No. 7 [Anonymous (1973b)]. It should be noted that the above guidelines are directed to the evaluation of protein foods for human nutrition rather than for animal feeding, and they are general recommendations rather than a series of mandatory procedures. Previous history of use of the source material

may be taken into account, but in the light of new safety criteria and potentially different conditions of use, apparently safe experience in prior cases is not necessarily sufficient to eliminate the need for preclinical testing.

To be truly significant the studies should be conducted on the SCP product as made on a production scale rather than on laboratory batches. In any case, the physical and chemical identity of the industrial product should be established to be essentially the same as that of the material tested experimentally.

The utility of specific SCP products depends primarily on composition, price and the intended applications. The potential applications of SCP, the major factors that affect its nutritional value and some factors influencing its acceptability have been proposed [Lipinsky and Litchfield (1970)]. They could be used as the scheme for future investigations on the applications of the SCP from the MMM/WSL process. The last big hurdle probably will be that of acceptance, which depends on such factors as personal and cultural idiosyncrasies of consumers, and flavour, color and appearance of products.

APPENDIX A

CORRECTION FACTOR FOR ANTHRONE CARBOHYDRATE IN WSL

A.1 Introduction

When carbohydrates are treated with anthrone (9, 10-Dihydro-9-oxoanthracene) in a strong solution of sulfuric acid, a blue color is obtained. The mechanism of this reaction is not completely known. It is believed that under the action of heat and acid, furfural or hydroxymethyl-furfural is formed from the carbohydrates; these aldehydes then react with the anthrone to give the characteristic blue color [Neish (1952)]. Determination of carbohydrates using the anthrone reagent is preferred because the method is simple and rapid, anthrone is highly specific for carbohydrates and there is no interference by acetoin which is the case when copper reduction method is used [Neish (1952)].

Morris (1948) and Neish (1952) reported a positive reaction with all pure mono-, di- and polysaccharides tested, as well as with all samples of dextrins, dextrans, starches, cellulose, plant polysaccharides and gums. Positive reactions were also obtained with Pneumococcus

polysaccharides of types II and III (but not type I), with all glucosides tested, and with the acetates of mono-, di-, and polysaccharides. No noncarbohydrates tested gave the characteristic blue color; a red color was produced by ordinary aliphatic aldehydes, by polyvinyl alcohol and by proteins. The common solvents gave no color, though solutions containing dioxane became fluorescent. The aldonic acids and sugar alcohols likewise produced no color.

The waste sulfite liquors used for the submerged cultivation of MMM contain complex colouring materials ranging from creamy to black. Therefore, a correction factor when dealing with these liquors is required. The factor was determined and the analysis is shown in this Appendix.

A.2 Materials and Methods

A.2.1 Waste Sulfite Liquors

The color characteristics of the received WSL are shown in Table A.1. Before utilization, they were filtered through 0.8μ millipore filter paper in order to remove the suspended particulates which could interfere with the spectrophotometric reading.

A.2.2 Spectrophotometry

The spectrophotometer used for the preparation

Table A.1 Color Characteristics of WSL as Received.

WSL	Supplier	Color	Maximum Transmitted Wavelength (nm)	Per Cent Transmittance Against Distilled Water at Maximum Transmitted Wavelength (%)
NH_3-	Canadian International Paper Co., Temiskaming, Que.	Very dark brown	- 685	0.0[a] 4.0
Ca-	Domtar Fine Paper Ltd., Cornwall, Ont.	Brown	658[a]	15.2[a]
Mixed-	Spruce Falls Powers & Paper Co., Ltd., Kapuskasing, Ont.	Light brown	646[a]	25.2[a]
Mg-	The James Mclaren Co., Ltd., Buckingham, Que.[b]	Light brown	605[a] 595	20.0[a] 44.0
Na-	Abitibi Paper Co., Iroquois Falls, Ont.	Light brown	590	18.1

(a) Samples received in January-February 1971, otherwise received in January-February 1972.
(b) Mill shut down on May 31st, 1972.

of the calibration curves of glucose was the Bausch & Lomb Spectronic 20 Colorimeter/Spectrophotometer equiped with the light source GE 1630, the phototube CE A9'5 and the filter combination. The visible spectra of WSL at different dilutions were obtained from the Perkin-Elmer Model Coleman 124 Double-Beam Grating Spectrophotometer operated as a single beam instrument to measure the spectral intensity of radiant energy emitted by the light source (tungsten lamp). These spectra were recorded by the Perkin-Elmer Model Coleman 165 Strip Chart Recorder. The cell thickness used was 10 mm.

A.2.3 Analytical Method

The reagent and procedure for quantitative determination of total carbohydrate is based on a method described by Morris (1948) and Neish (1952) using a slight modification. The anthrone reagent was prepared by dissolving 2.000 g of anthrone crystals in one liter of 95% sulfuric acid. This reagent could be kept at room temperature for 5 days for routine use.

Two ml of a dilute aqueous solution, containing 0-200 mg/l of glucose or its equivalent, were pipetted into a round bottom tube (size 13x100 mm) with a cap having a teflon-faced rubber liner, assuring a highly inert sealing surface, then 4 ml of the anthrone reagent were syringed

in using a "zippette" device. The tube was then capped and tipped over for three times in order to mix the solution and then it was kept for 15 minutes at room temperature. The reacted mixture was poured into the Spectronic 20 Colorimeter test tube (light path 11.67 mm, length 10 cm) and read against a blank (prepared with 2 ml of distilled water) at 540 nm. Standard calibration curves were prepared by plotting log per cent transmittance against glucose concentration.

Due to the high viscosity of 95% sulfuric acid for the anthrone reagent, the utilization of pipet caused a great error during transfering of the reagent to the sample tube. Moreover, the reacted mixture frequently resulted in several nonuniform layers in the sample tube due to imperfect mixing. The utilization of a hypodermic syringe for transfering the reagent into the sample tube by submerging the hypodermic needle into the sample solution could provide an accurate volume of reagent and a perfect mixing, but it creates relatively stable air bubbles in the reacted mixture and a large number of needles are required for multiple measurements.

The technique used in this work is more convenient, it required a minimum amount of sample and reagent, provided a perfect mixing and could be easily and accurately performed. It was found that the 15 minute period for the

reaction is not critical, but the initial temperatures of the reagent and the sample could cause drastic errors. The initial temperatures for both reagent and sample at 22-25° C (room temperature) are recommended and were used for this work.

A.3 Results and Discussion

A.3.1 Effects of WSL on the Calibration Curves of Glucose

The effects of WSL on the calibration curves of glucose were recognized only when using distilled water with presence of WSL instead of distilled water alone. Various amounts of WSL in distilled water (1:1,000 to 4:1,000 v/v) were used for seven samples of five different WSL (NH_3-, Ca-, Mg-, Mixed- and Na-bases) and the results are shown in Figs. A.1 to A.7. It was found that the calibration curves for glucose in presence of WSL deviated from the calibration curves for glucose without the presence of WSL. This deviation was not detected at low concentration of WSL as in the case of NH_3-WSL (Fig. A.2 at 1:1;000 and 2:1,000 v/v) and became more and more pronounced at higher concentrations of WSL (Figs. A.1-A.7). There existed a maximum concentration of WSL, beyond this the deviation of the standard calibration curves did not increase furthermore as in the case of Na-WSL (Fig. A.7 at

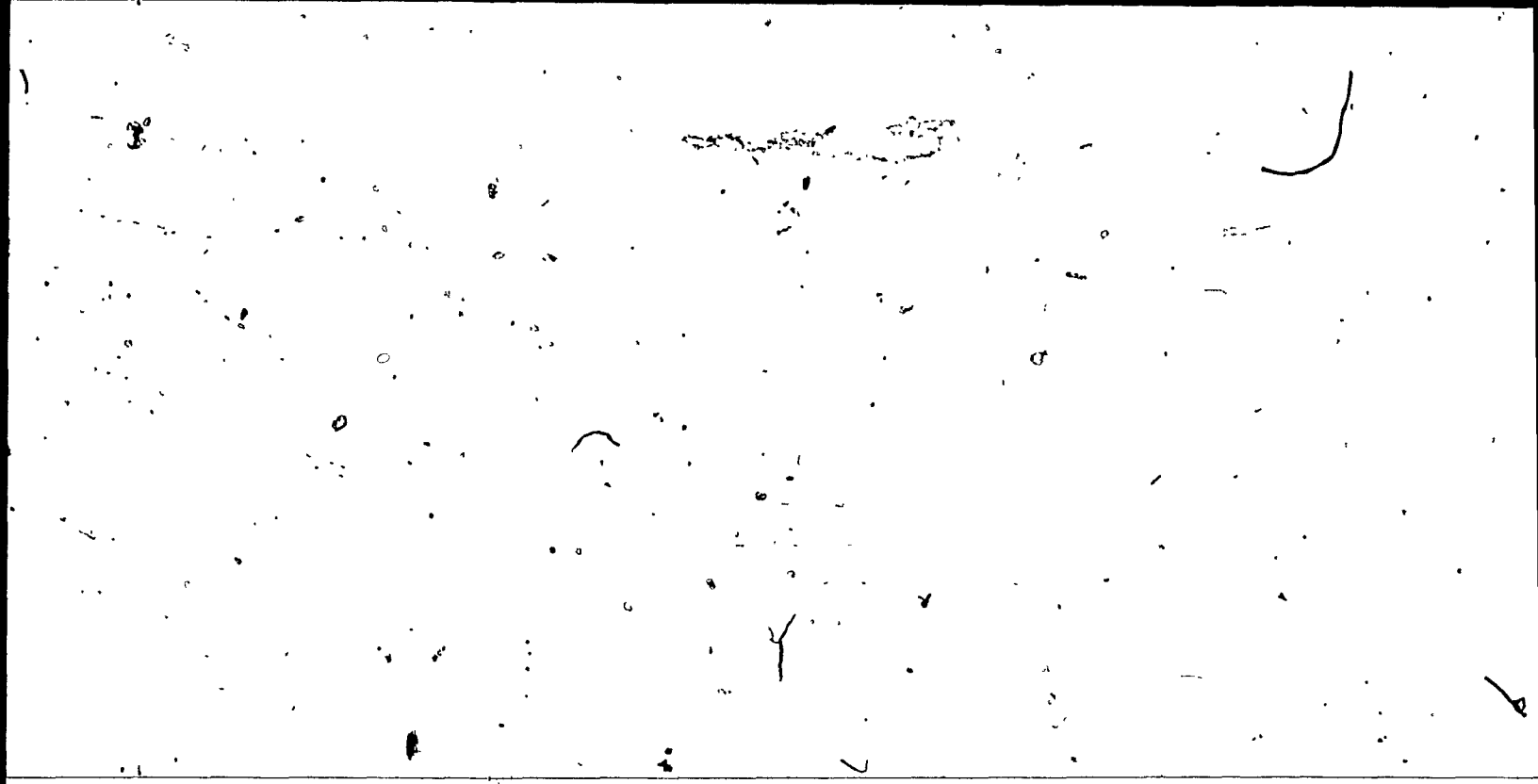


Figure A.1 Calibration Curves for Glucose in NH_3-WSL. Sample received in January-February 1971.

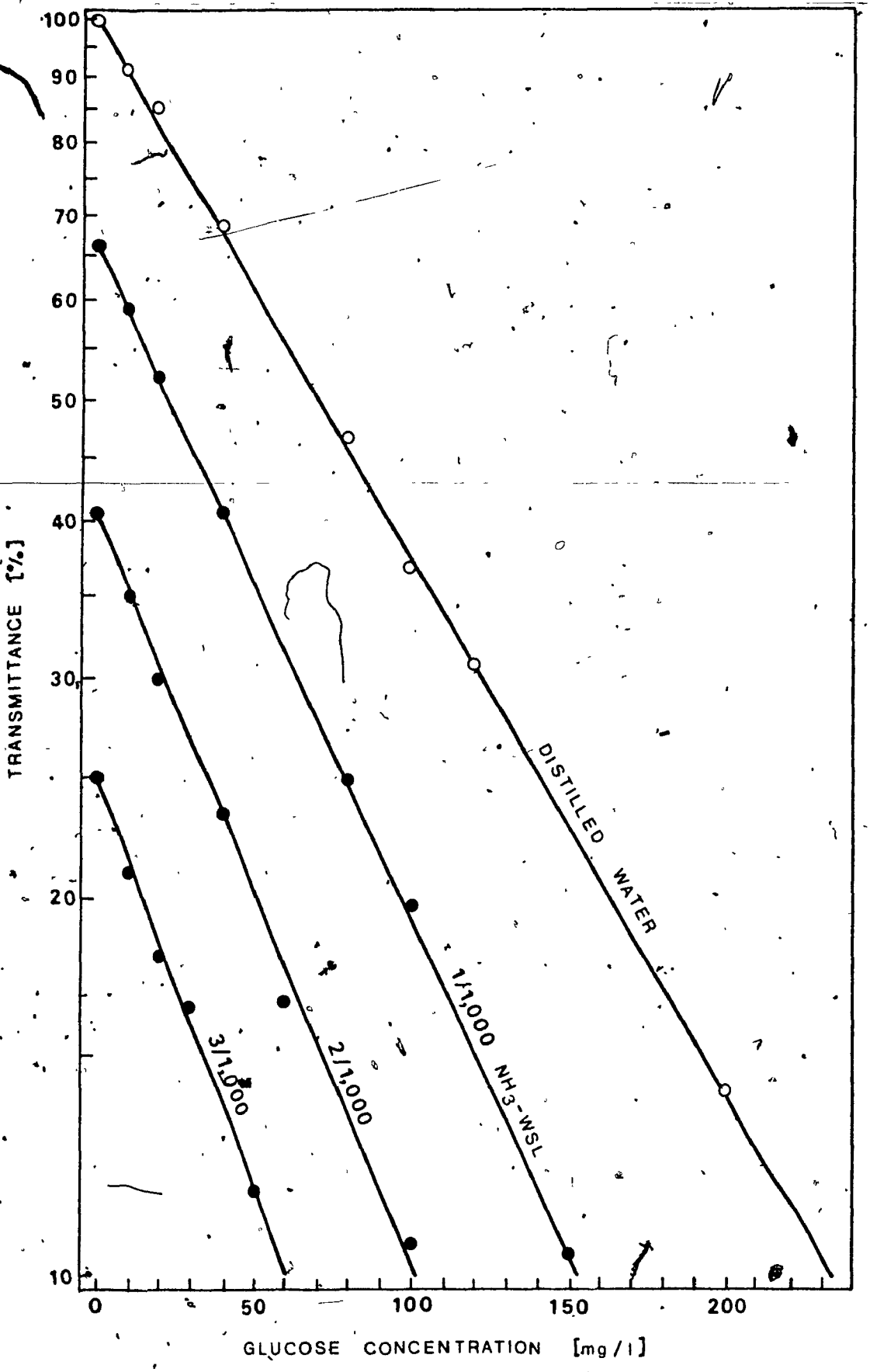

100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/l]
DISTILLED WATER
1/1,000 NH3-WSL
2/1,000
3/1,000

Figure A.2 Calibration Curves for Glucose in NH_3-WSL. Sample received in January-February 1972.

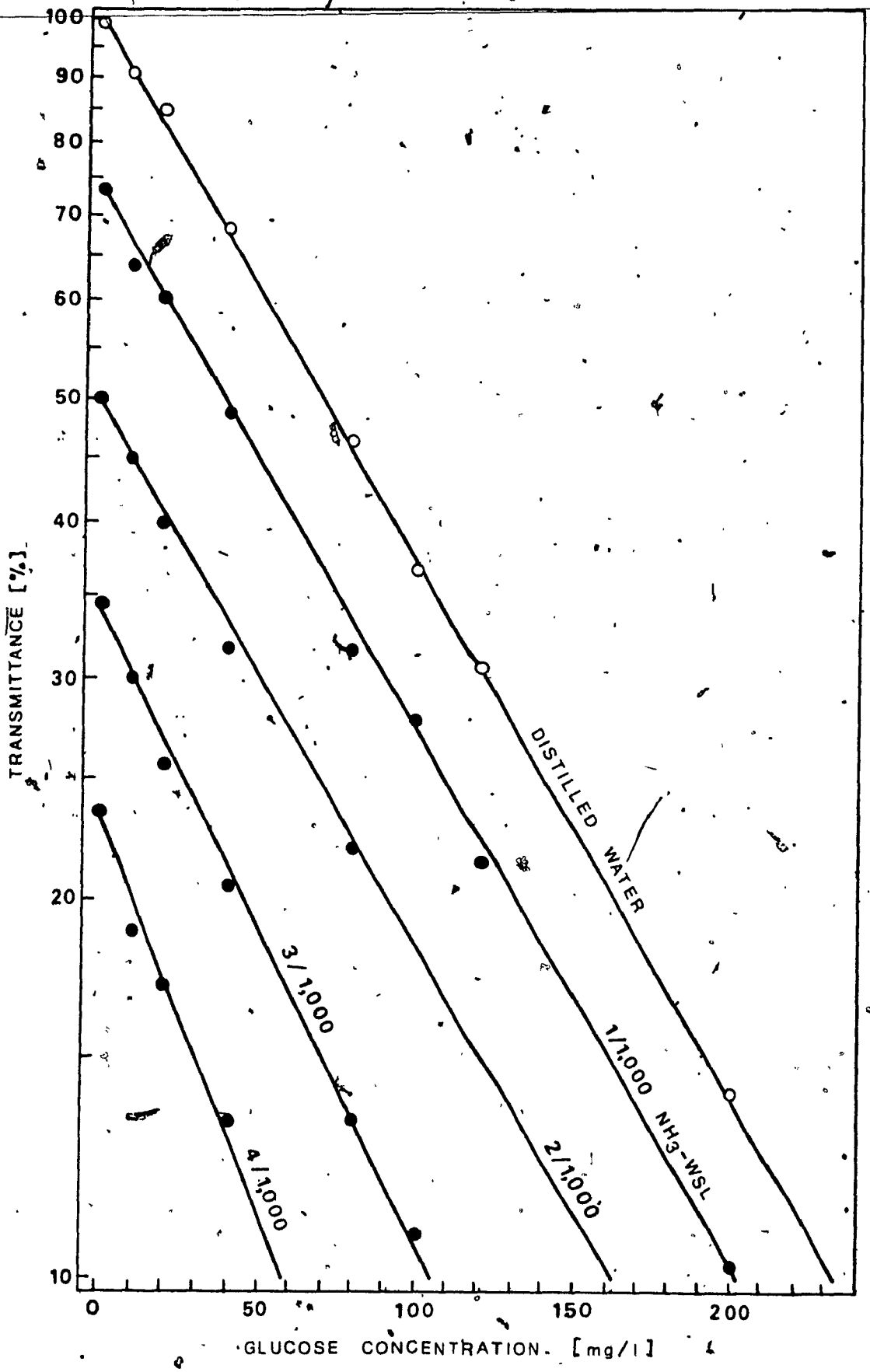

100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION. [mg/l]
DISTILLED WATER
1/1,000 NH3-WSL
2/1,000
3/1,000
4/1,000

Figure A.3 Calibration Curves for Glucose in Ca-WSL.

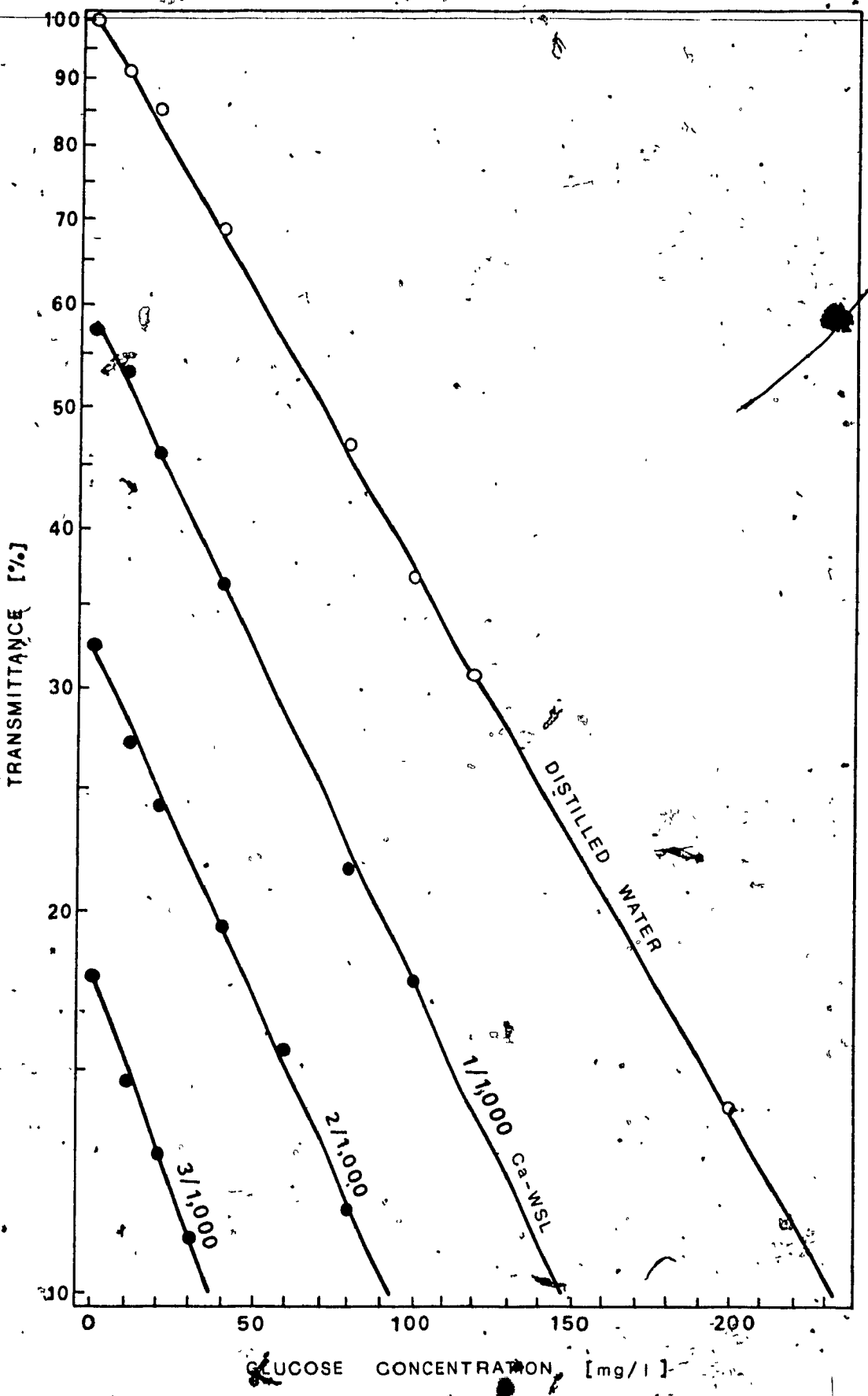

100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/l]
DISTILLED WATER
1/1,000 Ca-WSL
2/1,000
3/1,000

Figure A.4. Calibration Curves for Glucose in Mg-WSL.
Sample received in January-February 1971.

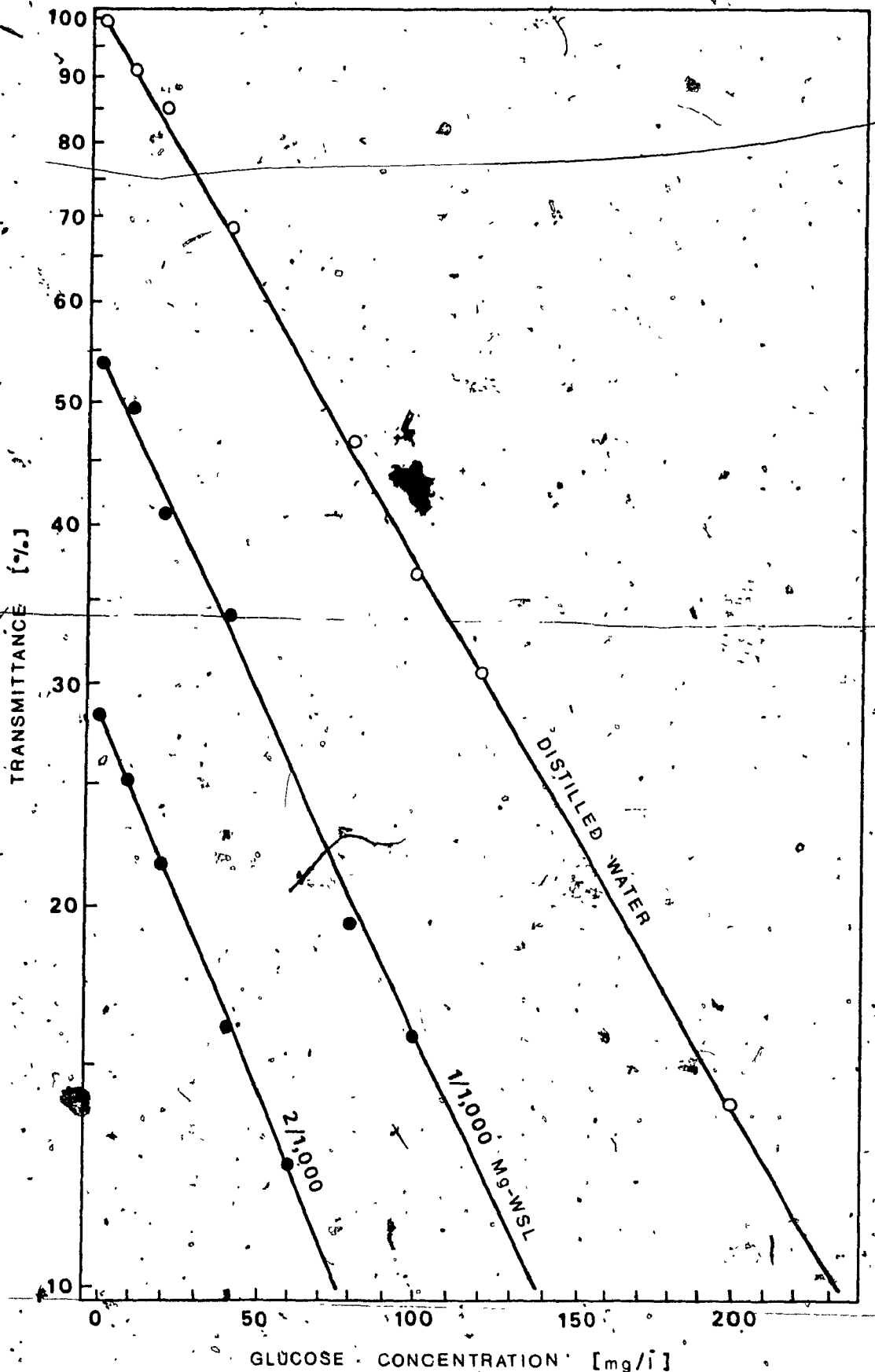
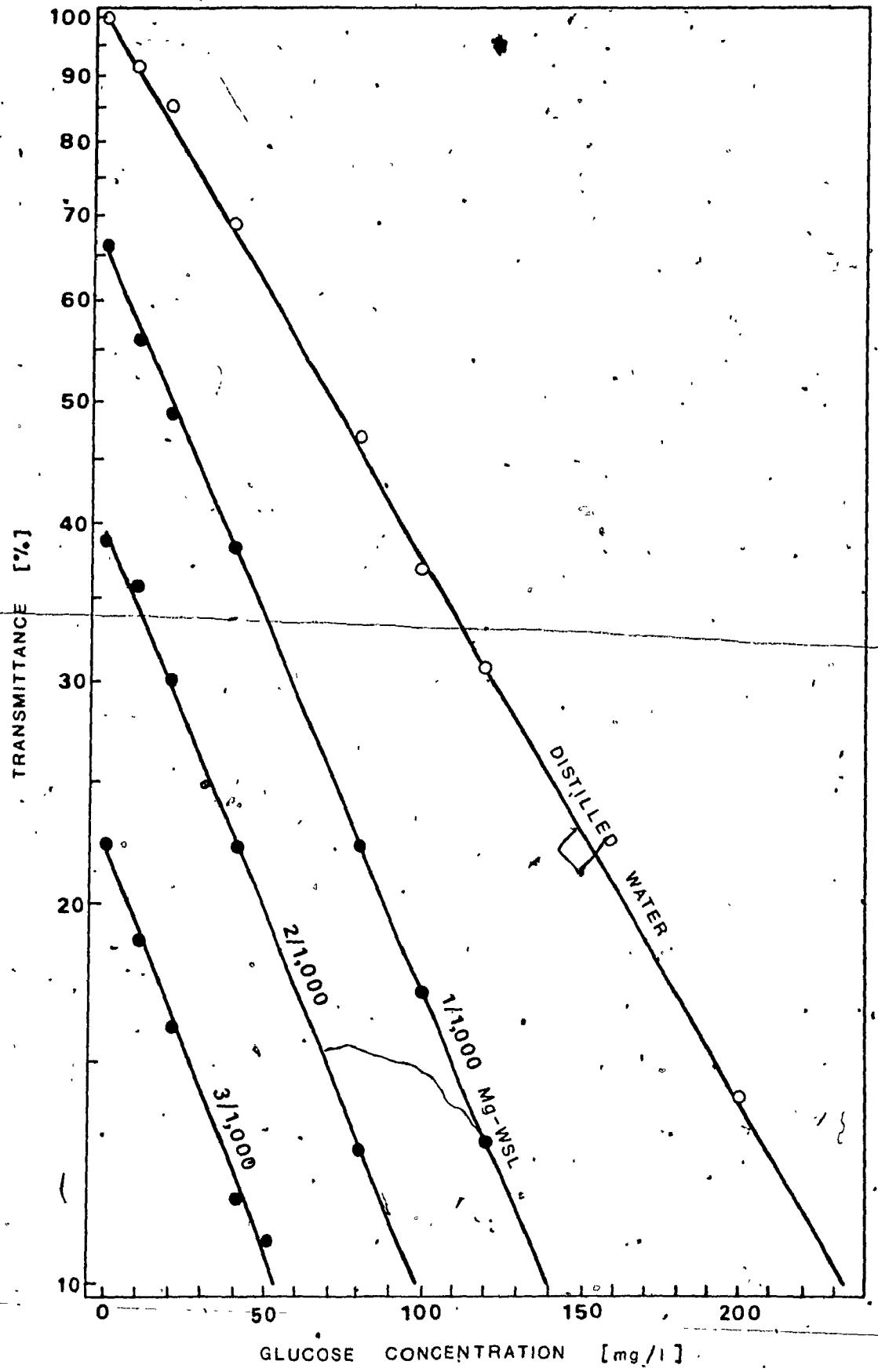

100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/l]
DISTILLED WATER
1/1,000 Mg-WSL
2/1,000

Figure A.5 Calibration Curves for Glucose in Mg-WSL.
Sample received in January-February 1972.


100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/l]
DISTILLED WATER
1/1,000 Mg-WSL
2/1,000
3/1,000

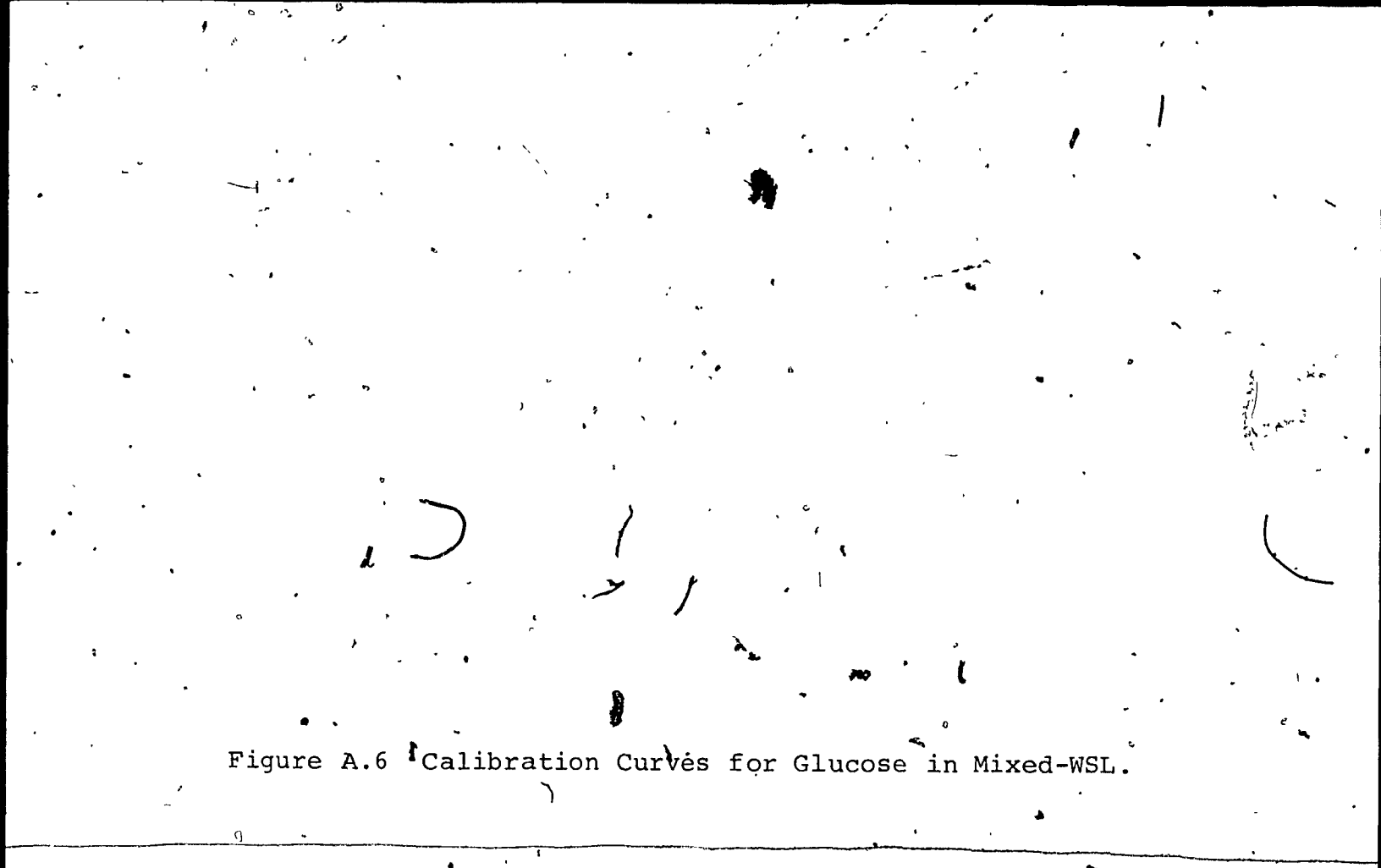


Figure A.6 Calibration Curves for Glucose in Mixed-WSL.

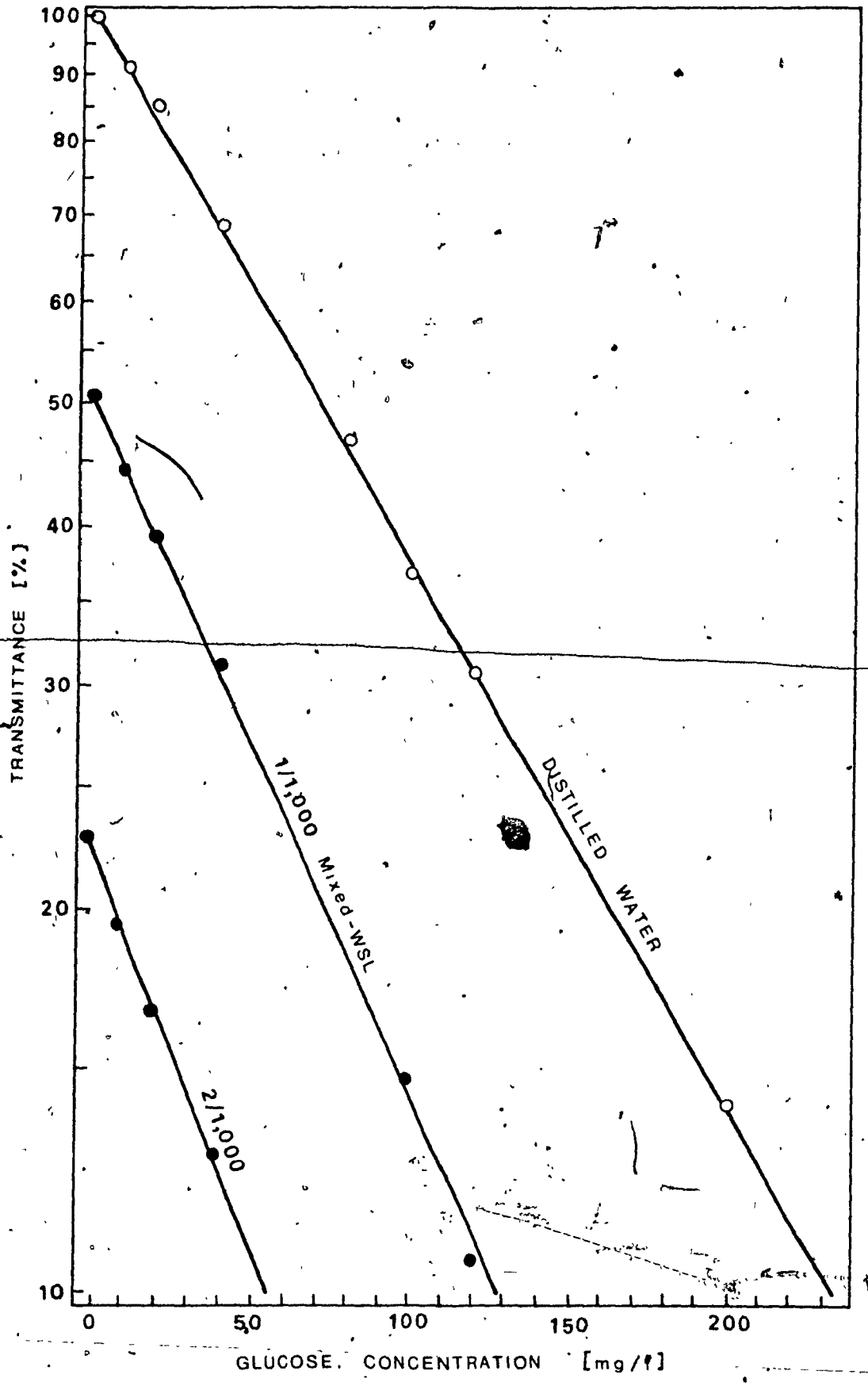
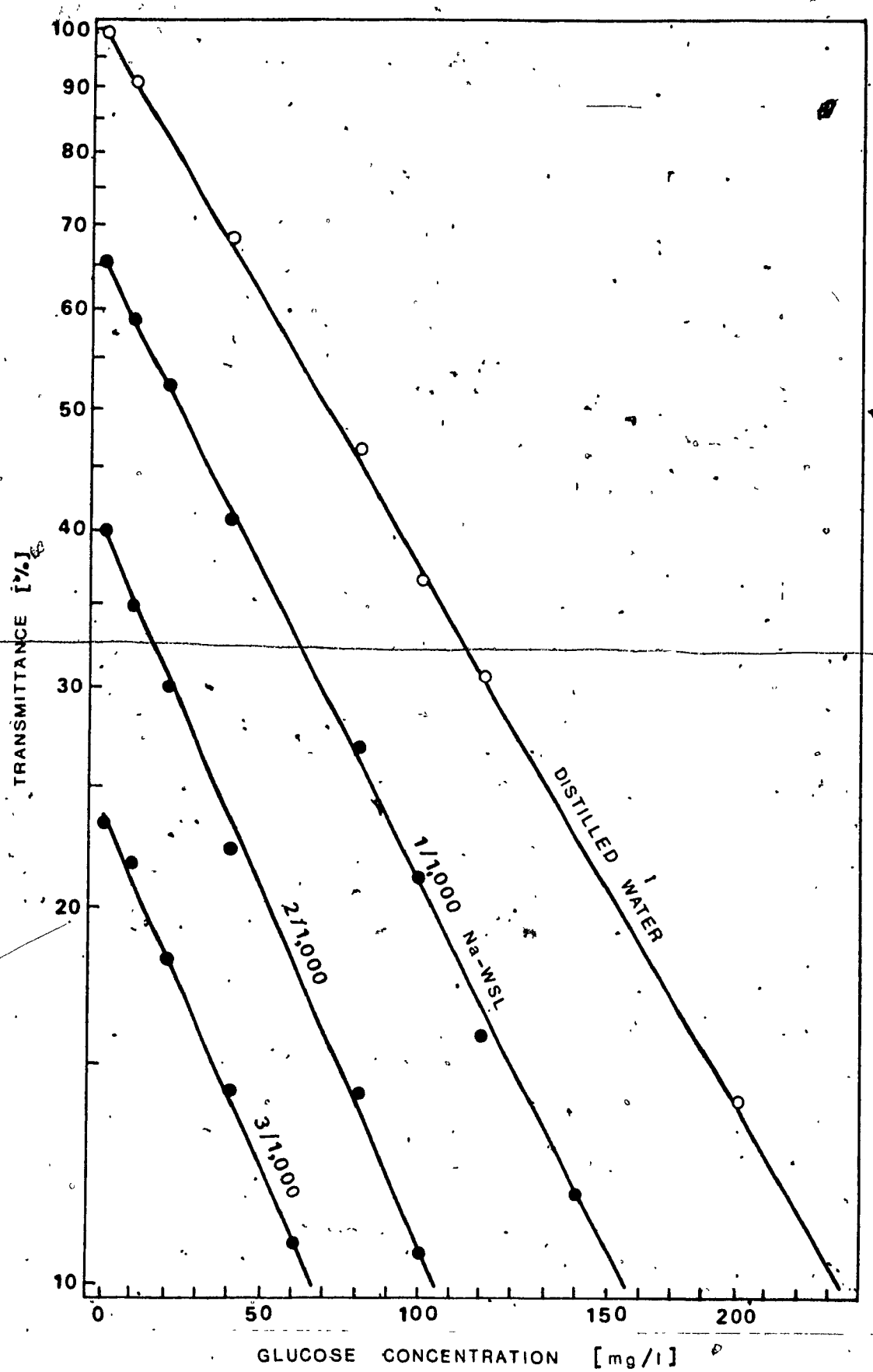

100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/dl]
DISTILLED WATER
1/1,000 Mixed-WSL
2/1,000

Figure A.7 Calibration Curves for Glucose in Na-WSL.


100
90
80
70
60
50
40
30
20
10
TRANSMITTANCE [%]
0
50
100
150
200
GLUCOSE CONCENTRATION [mg/l]
DISTILLED WATER
1/1,000 Na-WSL
2/1,000
3/1,000

2:1,000 and 3:1,000 v/v).

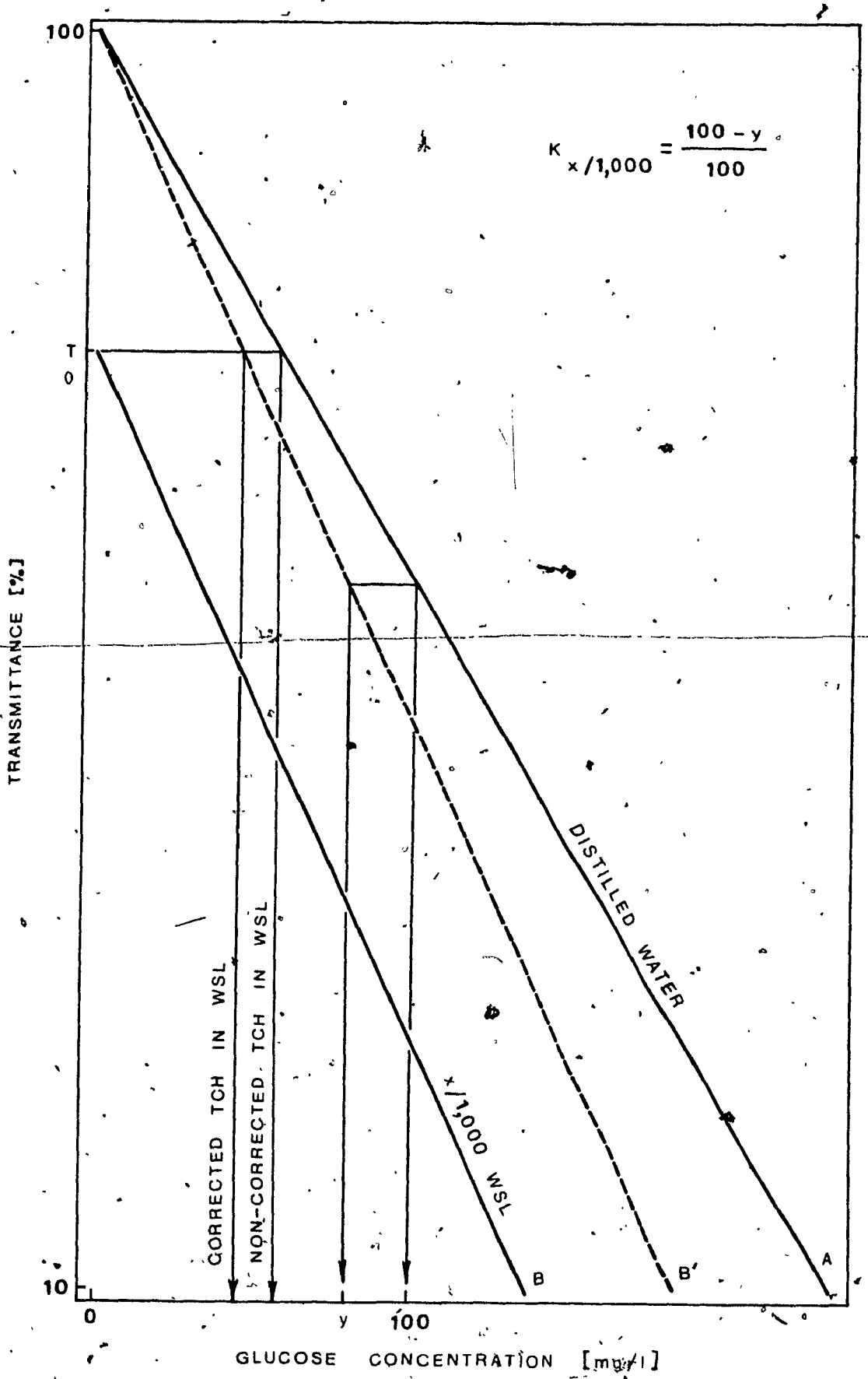
A.3.2 Correction Factor for the Calibration Curves

In Fig. A.8, the straight line A represents the calibration curve of glucose prepared with distilled water and the line B represents the calibration curve of glucose with the presence of x:1,000 v/v of WSL. The per cent transmittance T (straight line B) represents the amount of total carbohydrate expressed as glucose which existed in WSL. The straight line B' is parallel with B and passes through the point of 100 per cent transmittance. If the WSL were free of carbohydrate, the line B could be superimposed to B'. The difference in the slopes of A and B' was probably due to the interference of the color of WSL present in distilled water. The correction factor for the calibration curve with the presence of WSL is defined as the amount of glucose concentration which had to be subtracted from the value obtained using the standard calibration curve with distilled water alone. From Fig. A.8, the correction factor is defined as,

$$K_{x:1,000}[\%] = \frac{100 - y}{100} \qquad (A.1)$$

where,

Figure A.8 Calculation of the Correction Factor for Anthrone Carbohydrate in WSL.


100
T
0
10
0
y
100
$K_{x/1,000} = \frac{100 - y}{100}$
TRANSMITTANCE [%]
GLUCOSE CONCENTRATION [mg/l]
CORRECTED TCH IN WSL
NON-CORRECTED TCH IN WSL
x/1,000 WSL
DISTILLED WATER
A
B
B'

$K_{x:1,000}$ = correction factor of the calibration curve when x:1,000 v/v WSL present,

y = amount of glucose concentration obtained from standard calibration curve of glucose with the presence of x:1,000 v/v WSL corresponding to 100 mg/l of glucose concentration obtained from the standard calibration curve of glucose with distilled water alone.

The correction factor for all samples of WSL at various concentrations (1:1,000 to 4:1,000 v/v) has been calculated by Eq. (A.1) and the results are shown in Fig. A.9. It was found that the correction factors depended upon the different bases of WSL and also upon different batches of the same WSL. However, within the practical dilution range of WSL for the determination of total carbohydrate (1:1,000 - 4:1,000 v/v) the correction factors varied from 15 to 40%, which is very significant. In all WSL samples, the correction factor became more significant at higher concentrations of WSL in the solution.

A.3.3 Colors of WSL

Figures A.10 to A.16 show the results from the spectrophotometric studies of seven samples of WSL in the visible range at different dilutions. The dilution is

Figure A.9 Correction Factors for Anthrone Carbohydrate in WSL at Different Concentrations. (*a*) Samples received in January-February 1972, otherwise received in January-February 1971.

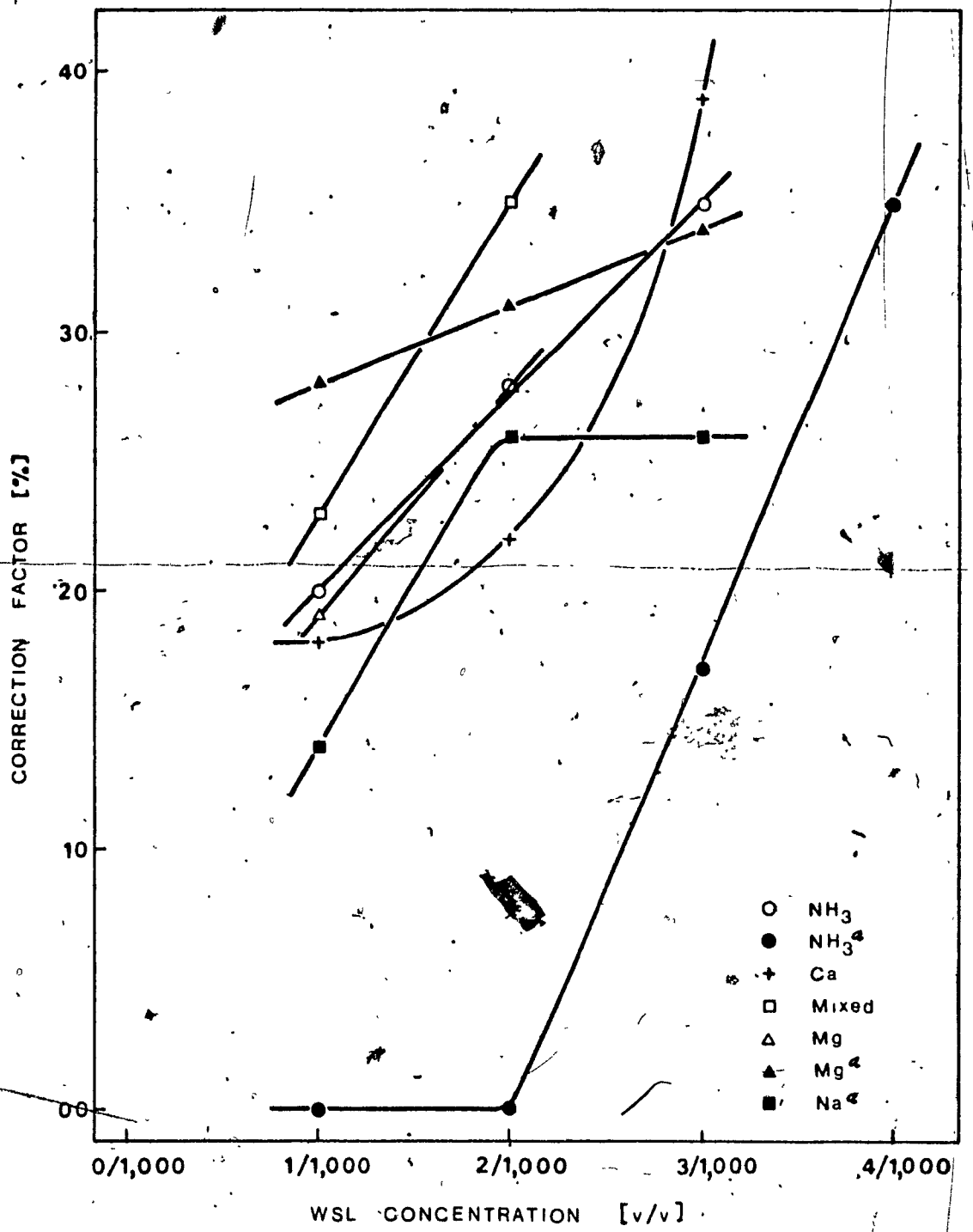

40
30
20
10
00
CORRECTION FACTOR [%]
0/1,000
1/1,000
2/1,000
3/1,000
4/1,000
WSL CONCENTRATION [v/v]
NH3
NH3d
Ca
Mixed
Mg
Mgd
Nad

Figure A.10 Isochrome Locus of NH_3-WSL From Their Visible Spectra at Different Dilutions. Sample received in January-February 1971.

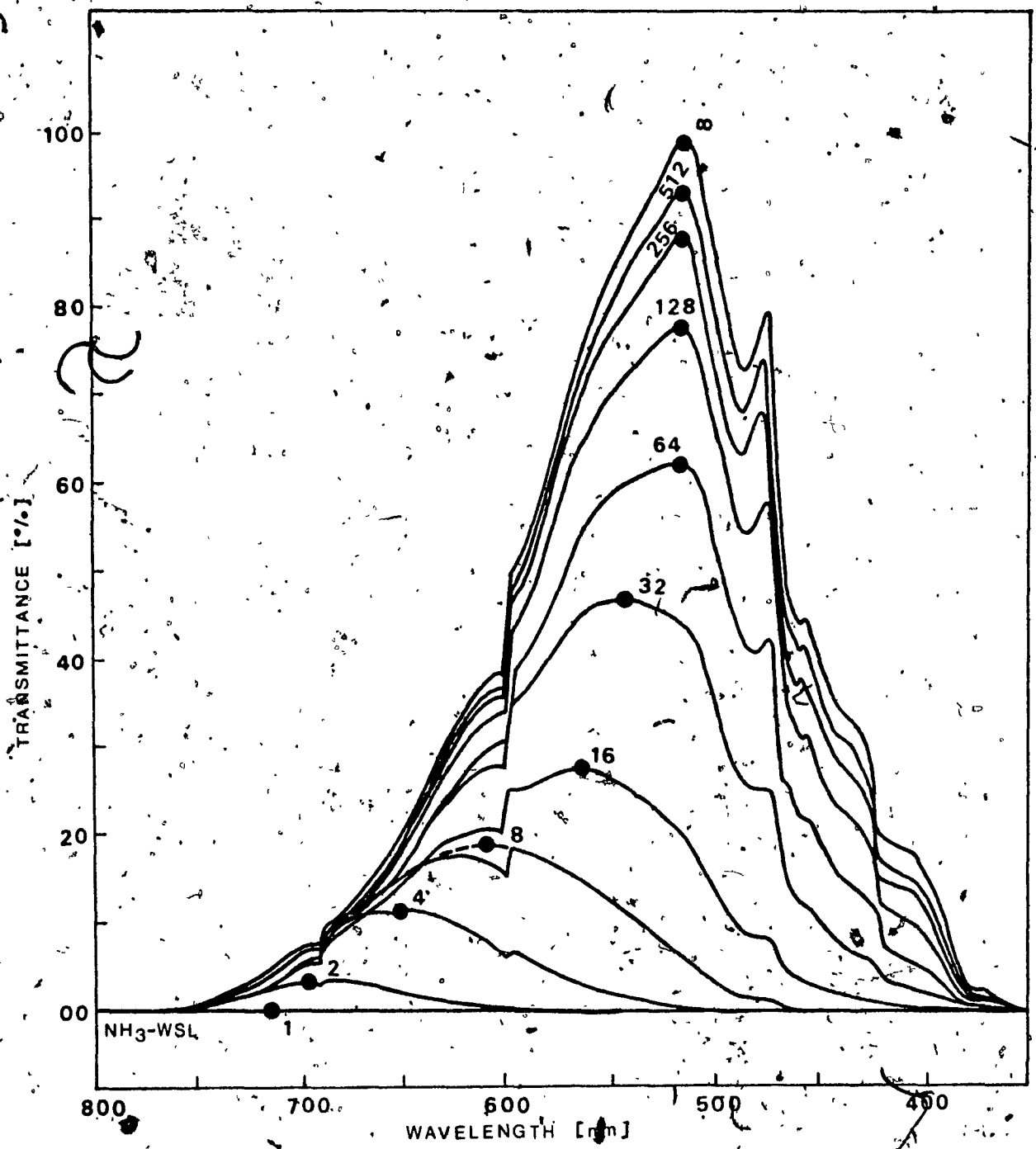

100
80
60
40
20
00
TRANSMITTANCE [%]
800
700
600
500
400
WAVELENGTH [nm]
NH3-WSL
1
2
4
8
16
32
64
128
256
512
∞

Figure A.11 Isochrome-Locus of NH_3-WSL From Their Visible Spectra at Different Dilutions. Sample received in January-February 1972.

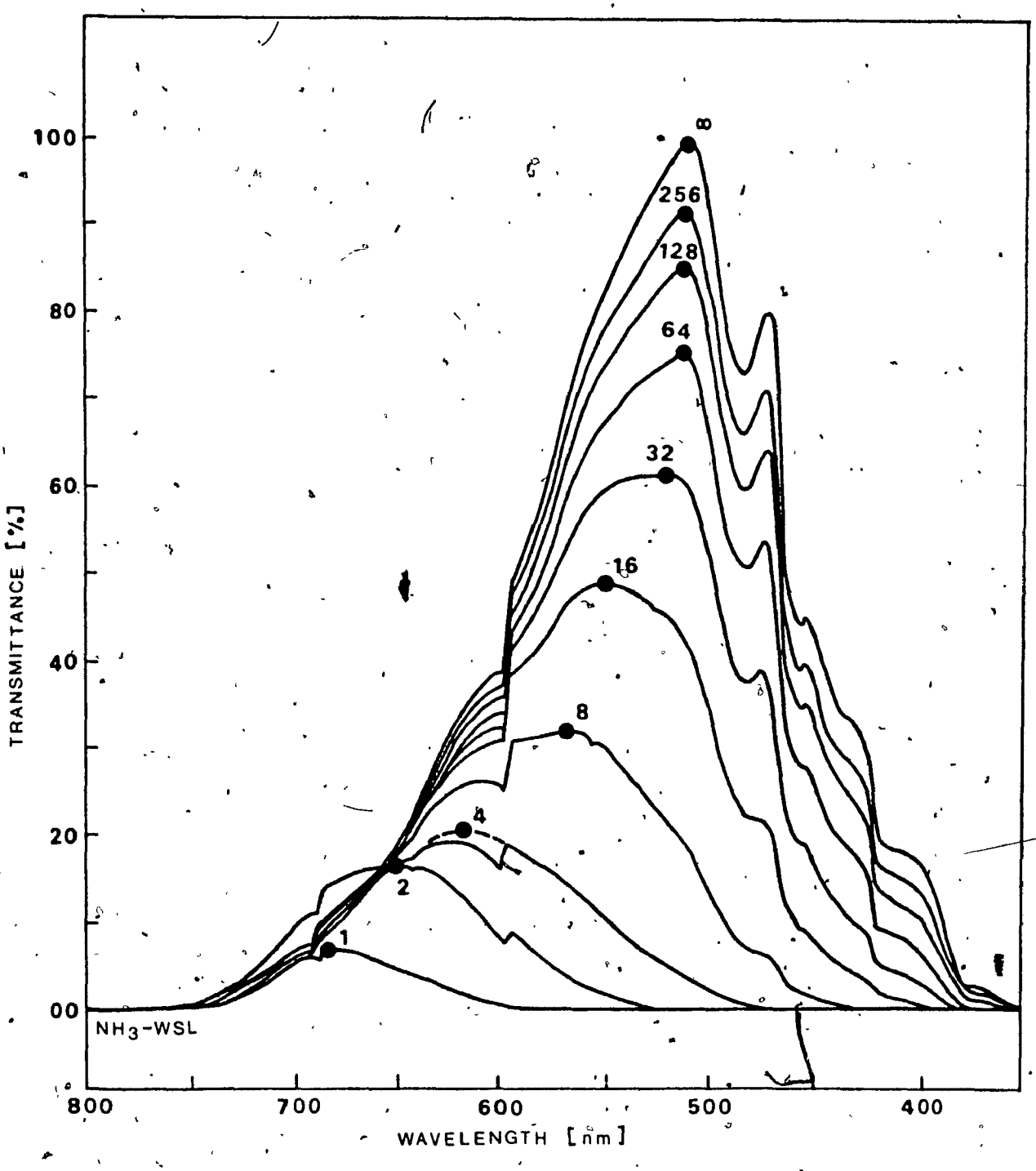

100
80
60
40
20
00
TRANSMITTANCE [%]
∞
256
128
64
32
16
8
4
2
1
NH3-WSL
800
700
600
500
400
WAVELENGTH [nm]

Figure A.12 Isochrome Locus of Ca-WSL From Their Visible Spectra at Different Dilutions.

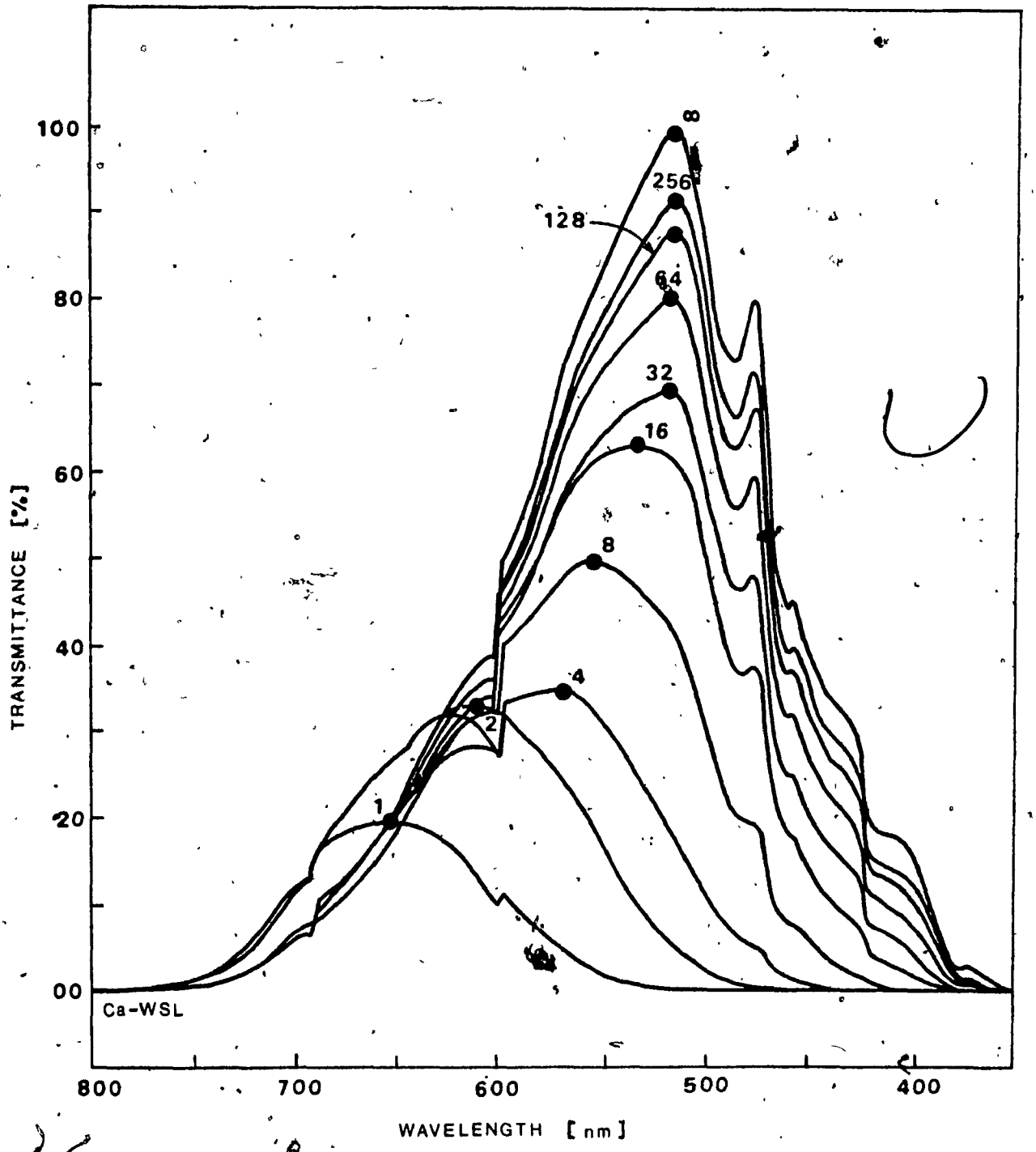

100
80
60
40
20
00
TRANSMITTANCE [%]
800
700
600
500
400
WAVELENGTH [nm]
Ca-WSL
∞
256
128
64
32
16
8
4
2
1

Figure A.13 Isochrome Locus of Mg-WSL From Their Visible Spectra at Different Dilutions. Sample received in January-February 1971.

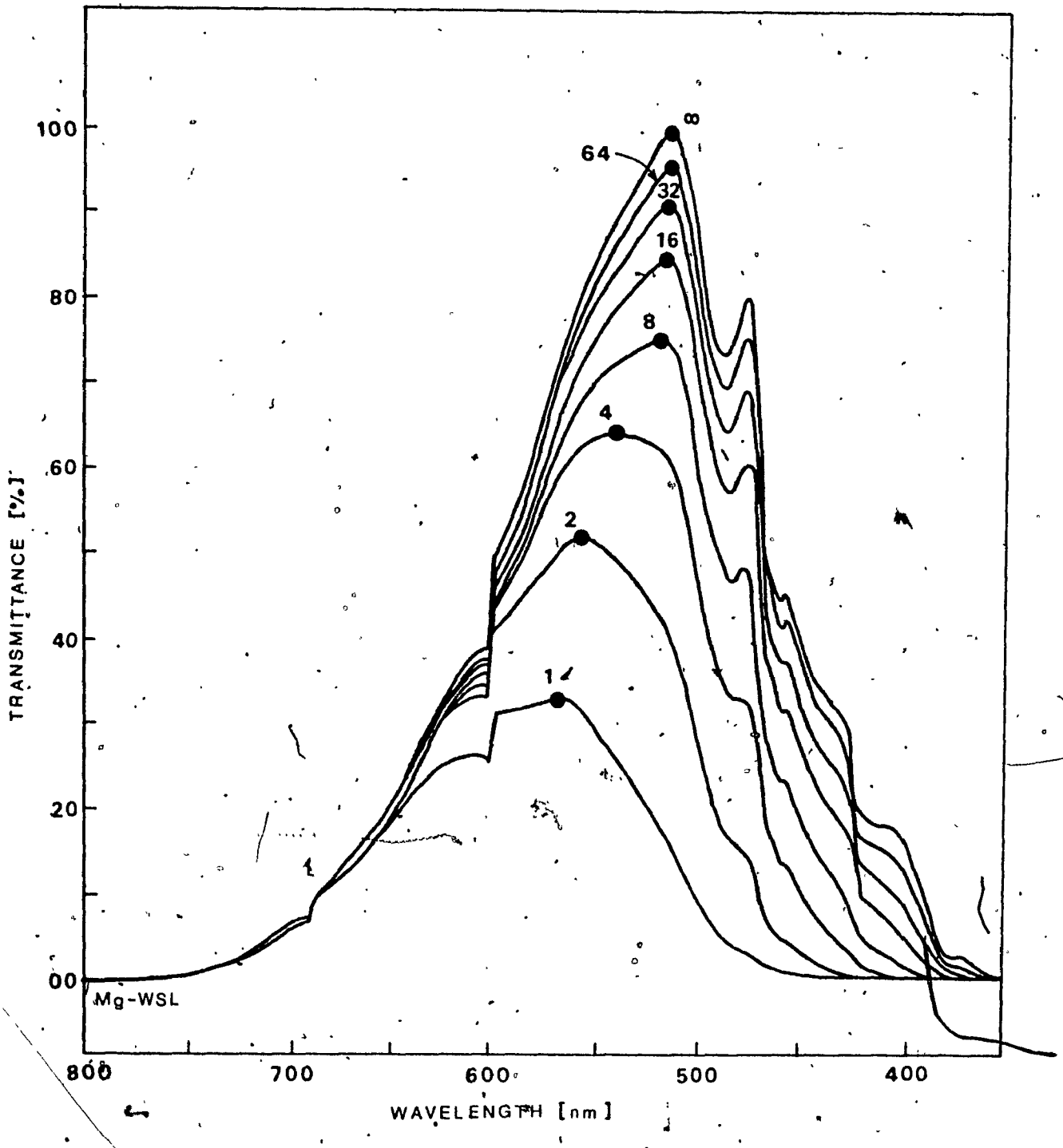

100
80
60
40
20
00
TRANSMITTANCE [%]
∞
64
32
16
8
4
2
1
Mg-WSL
800
700
600
500
400
WAVELENGTH [nm]

Figure A.14 Isochrome Locus of Mg-WSL From Their Visible Spectra at Different Dilutions. Sample received in January-February 1972.

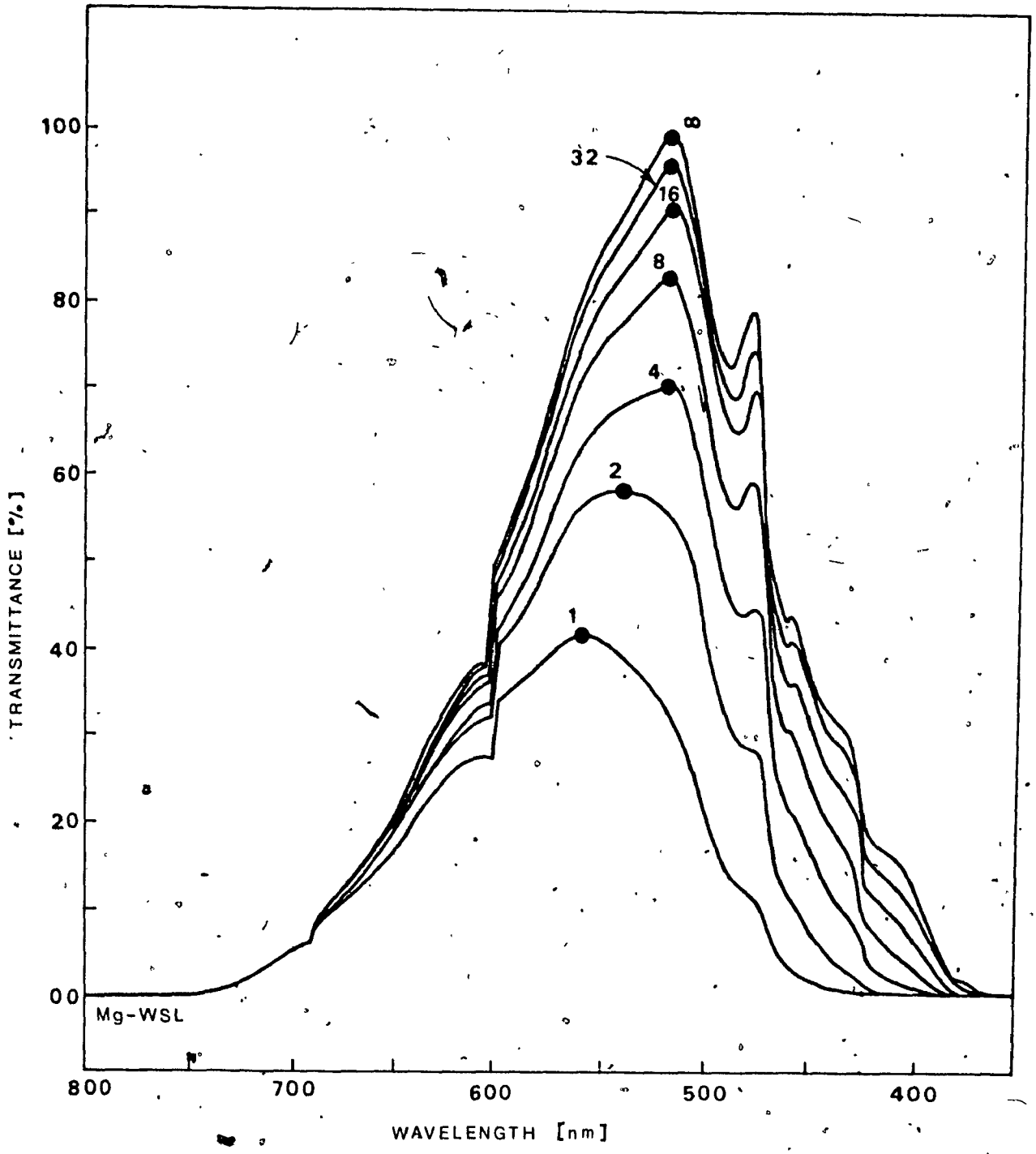

100
80
60
40
20
00
TRANSMITTANCE [%]
800
700
600
500
400
WAVELENGTH [nm]
Mg-WSL
∞
32
16
8
4
2
1

Figure A.15 Isochrome Locus of Mixed-WSL From Their Visible Spectra at Different Dilutions.

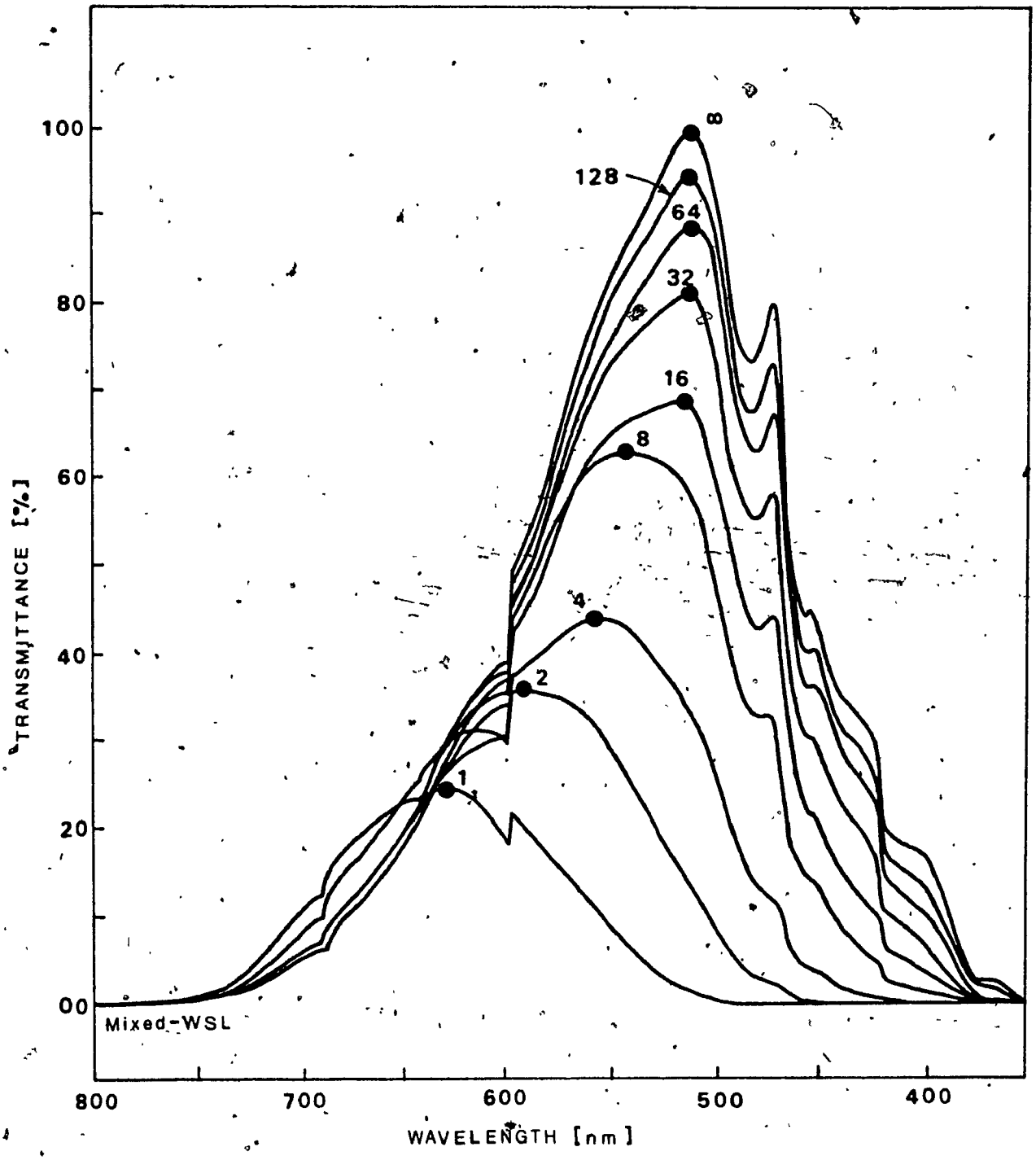
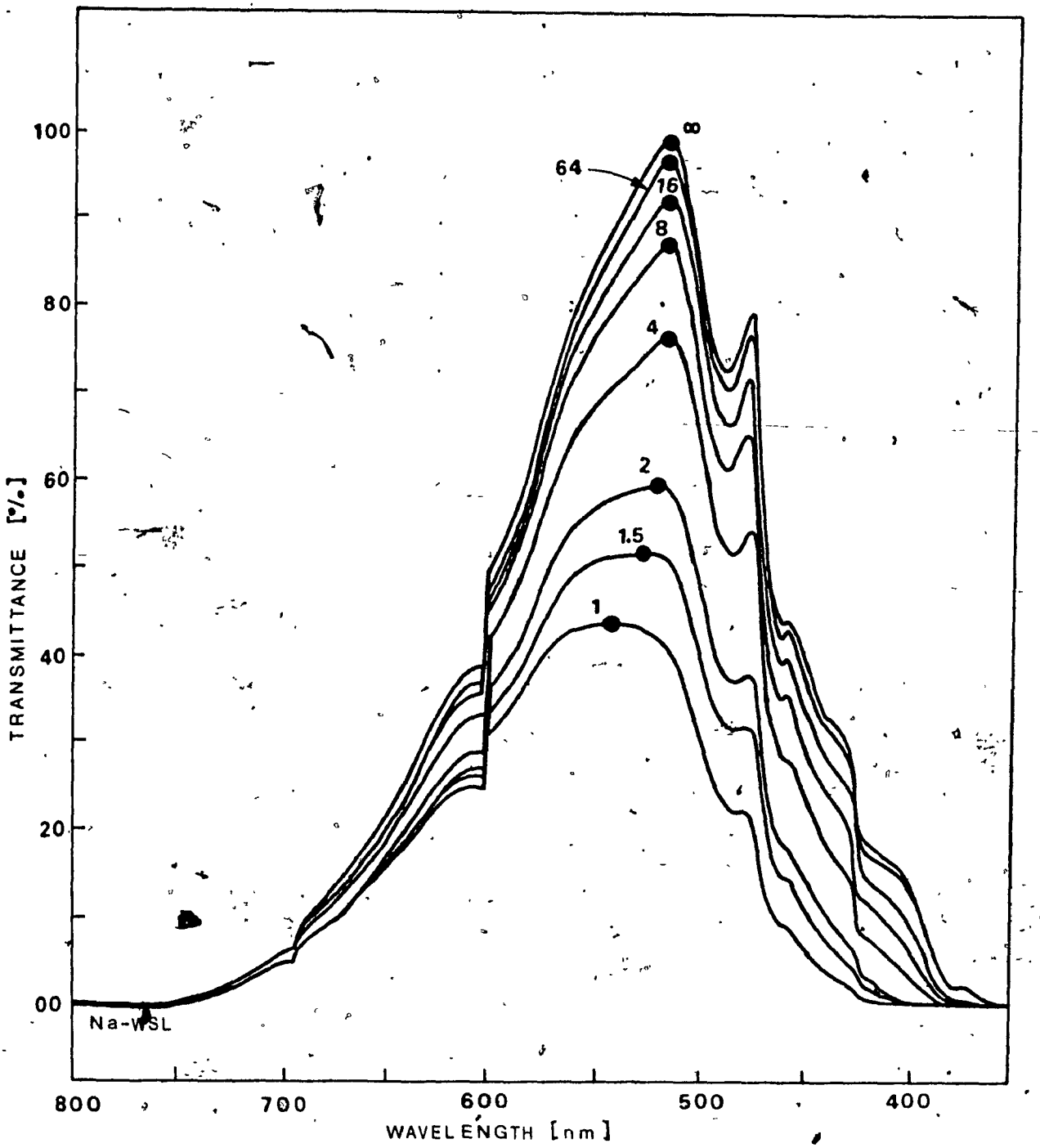

100
80
60
40
20
00
TRANSMITTANCE [%]
800
700
600
500
400
WAVELENGTH [nm]
Mixed-WSL
∞
128
64
32
16
8
4
2
1

Figure A.16 Isochrome Locus of Na-WSL From Their Visible Spectra at Different Dilutions.


100
80
60
40
20
00
TRANSMITTANCE [%]
800
700
600
500
400
WAVELENGTH [nm]
∞
64
16
8
4
2
1.5
1
Na-WSL

defined as,

Dilution D = 1 Volume of concentrated WSL
+ D Volume(s) of distilled water. (A.2)

From this definition, dilution 1 referred to concentrated WSL samples and dilution ∞ to distilled water. The maximum transmitted wavelength from the visible spectrogram may be taken to represent the color of WSL. It was found in all samples tested that the maximum transmitted wavelength always shifted from the values for concentrated samples (Table A.1) toward the maximum transmitted wavelength of distilled water (515 nm) as the dilution increased. The total amount of shifted band vary from 75 nm for Na-WSL (16.7% of visible band) to 200 nm for NH_3-WSL (44.5% of visible band), and the significance of this shifting phenomenon depends upon the intensity (darkness or lightness) of the colors observed with the naked eyes. The locus of the maximum transmitted wavelengths of WSL at different dilutions could be tentatively named the isochrome locus of WSL. It should be noted that the term isochrome means that they should have the same maximum transmitted wavelength at all dilutions, which is not true; if isochrome is not used, it means the color of WSL has changed with dilutions, which is probably not true either. A better term needs to be searched for future use in order to overcome this paradox.

The plot of the maximum transmitted wavelength versus dilution of seven samples of WSL in Fig. A.17 shows the same pattern for the color of WSL. The following empirical equation has been used to fit the experimental data,

$$\Lambda = \Lambda_s \left[1 + \left(\frac{D_c}{D}\right)^2\right]^{\frac{n}{2}} \qquad \text{(A.3)}$$

where,

Λ = maximum transmitted wavelength of WSL solution (nm)

Λ_s = maximum transmitted wavelength of distilled water (515 nm)

D = dilution as defined by Eq. (A.2)

D_c = critical dilution of WSL, which is the intersection between two asymtotes of the curve

n = chromatic index of WSL.

The best fit of the experimental data is shown by a family of curves in Fig. A.17 together with their values of D_c and n. The general shape of the curves could be interpreted as following: At lower dilutions (or higher concentrations) the color of WSL solution is dominated by the color of WSL itself, therefore the maximum transmitted wavelength of the WSL solution is shifted toward the visible range. As the dilution increases to its critical dilution D_c, the color of WSL solution is dominated by the green color of distilled

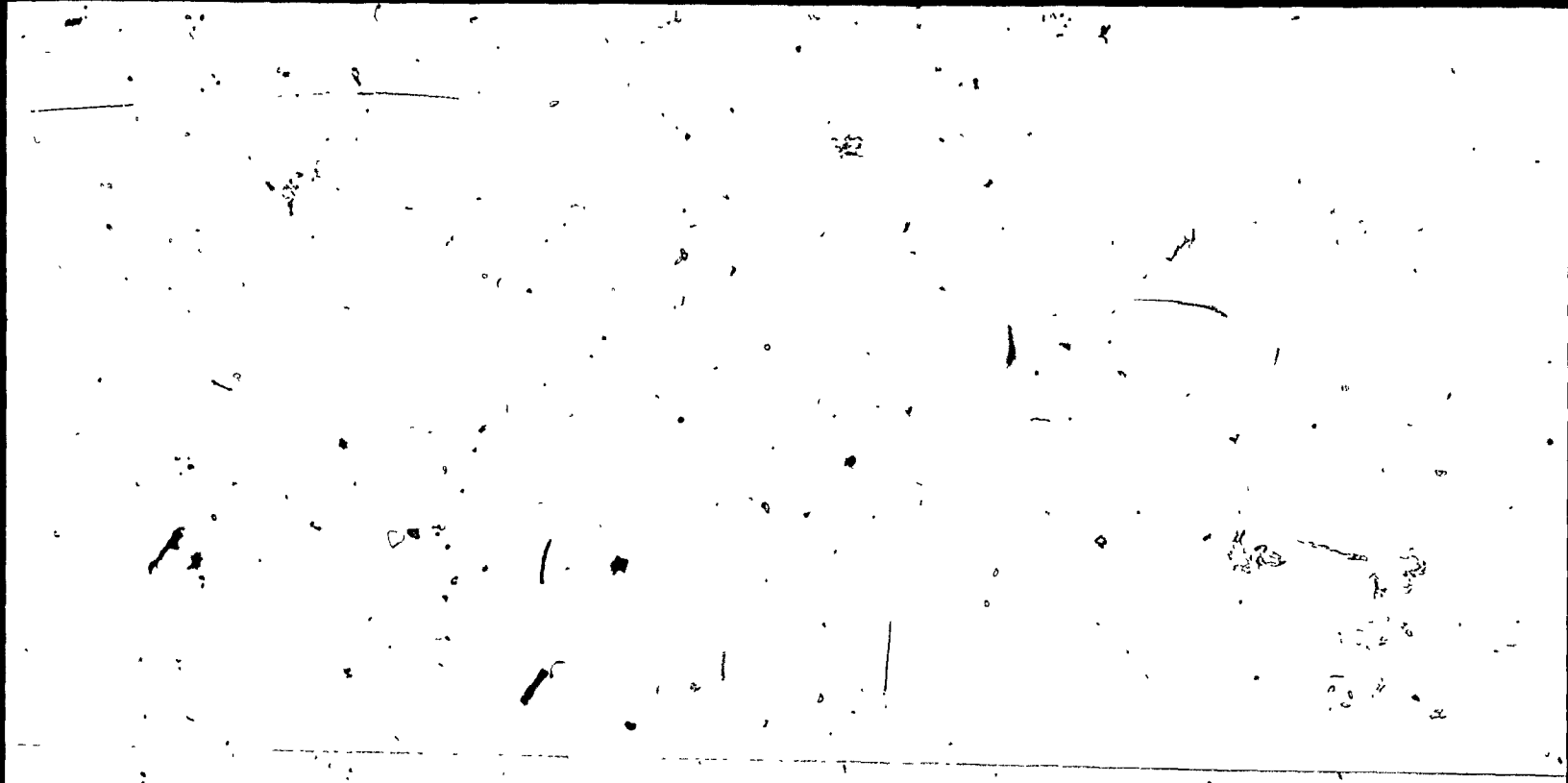
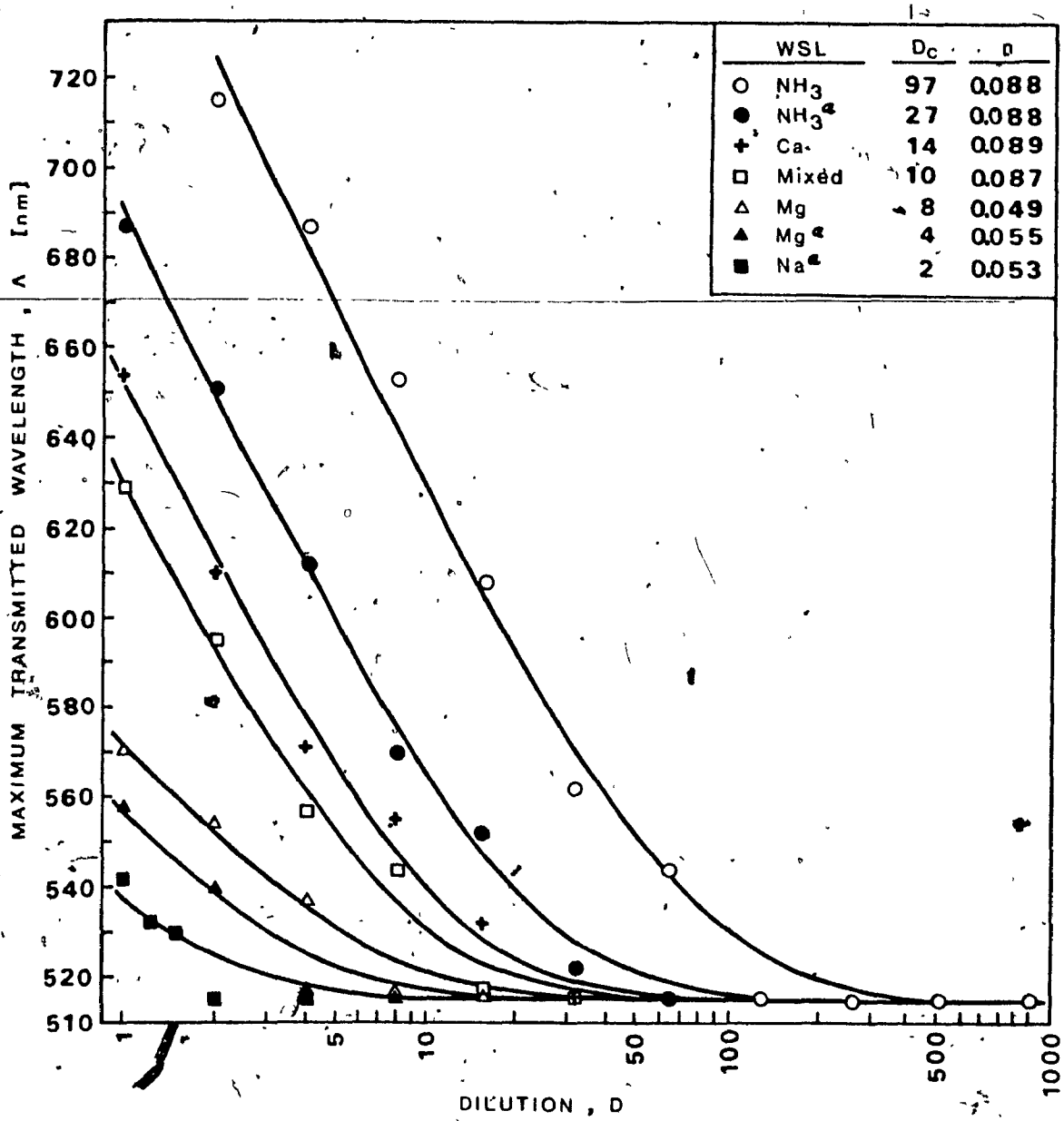


Figure A.17 Dilution Dependance of Colors of WSL. (a) Samples received in January-February 1972, otherwise received in January-February 1971.


WSL
Dc
n
NH3 97 0.088
NH3a 27 0.088
Ca 14 0.089
Mixed 10 0.087
Mg 8 0.049
Mga 4 0.055
Naa 2 0.053
MAXIMUM TRANSMITTED WAVELENGTH , λ [nm]
720
700
680
660
640
620
600
580
560
540
520
510
1
5
10
50
100
500
1000
DILUTION , D

water (green color of the sea or the ocean as the evidence), therefore the maximum transmitted wavelength of WSL solution at dilutions higher than critical dilutions was constant at 515 nm. The shifting characteristics of WSL are represented by their chromatic index n and they are expected to be the same for each base of WSL. In fact, it was found that n=0.088 for both samples of NH_3-WSL and n=0.049 and n=0.055 for two different samples of Mg-WSL.

APPENDIX B

TABULATED RESULTS

Table B.1 Effect of Initial pH on the Cultivation of *M. crassipes* in NH_3-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.05	3.94	5.41	4.95	0.46	0.150	32.61	2.77
5.01	4.67	5.41	4.03	1.38	0.759	55.00	14.03
5.48	5.57	5.41	2.68	2.73	2.144	78.53	39.63
6.02	6.09	5.41	2.53	2.88	2.002	69.51	37.01
6.50	6.52	5.41	2.59	2.82	2.047	72.59	37.84
7.04	6.83	5.41	2.38	3.03	1.879	62.01	34.73
8.00	7.11	5.41	2.74	2.67	1.503	56.29	27.78

Table B.2 Effect of Initial pH on the Cultivation of *M. deliciosa* in NH_3-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.07	3.72	5.41	4.47	0.94	0.000	0.00	0.00
5.01	4.38	5.41	4.32	1.09	0.000	0.00	0.00
5.49	4.66	5.41	3.80	1.61	0.060	3.73	1.11
6.05	4.80	5.41	2.45	2.96	0.991	33.48	18.32
6.51	5.49	5.41	2.15	3.26	0.997	30.58	18.43
7.10	6.51	5.41	1.94	3.47	0.983	28.33	18.17
8.00	6.69	5.41	2.08	3.33	0.781	23.45	14.44

Table B.3 Effect of Initial pH on the Cultivation of *M. esculenta* in NH_3-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.03	3.97	5.41	4.76	0.65	0.027	4.15	0.50
5.00	5.03	5.41	2.18	3.23	1.815	56.19	33.55
5.50	5.98	5.41	2.54	2.87	1.958	68.22	36.19
6.00	6.07	5.41	2.72	2.69	1.743	64.80	32.22
6.48	6.40	5.41	2.53	2.88	1.727	59.97	31.92
7.03	6.86	5.41	2.58	2.83	1.596	56.40	29.50
8.04	7.01	5.41	2.84	2.57	1.182	45.99	21.85

Table B.4 Effect of Initial pH on the Cultivation of *Morchella* spp. in NH_3-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.00	3.80	5.41	4.02	1.39	0.278	19.99	5.13
5.02	5.32	5.41	2.50	2.91	2.046	70.32	37.82
5.51	6.25	5.41	2.54	2.87	1.890	65.85	34.93
6.00	6.39	5.41	2.52	2.89	1.834	63.45	33.89
6.50	6.63	5.41	2.43	2.98	1.703	57.13	31.47
7.02	6.98	5.41	2.57	2.84	1.658	58.37	30.63
8.00	7.06	5.41	2.57	2.84	1.119	39.39	20.68

Table B.5 Effect of Initial pH on the Cultivation of *M. crassipes* in Mg-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.00	4.04	5.30	4.88	0.42	0.000	0.00	0.00
5.00	5.45	5.30	2.92	2.38	1.702	71.49	32.10
5.49	5.94	5.30	3.14	2.16	1.984	91.86	37.44
6.00	6.23	5.30	2.92	2.38	1.569	65.91	29.60
6.52	6.53	5.30	3.00	2.30	1.252	54.43	23.62
7.05	6.84	5.30	2.88	2.42	1.456	60.15	27.47
7.99	7.26	5.30	2.92	2.38	1.137	47.75	21.44

Table B.6 Effect of Initial pH on the Cultivation of *M. esculenta* in Mg-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.00	4.04	5.30	4.72	0.58	0.298	51.38	5.62
4.97	4.90	5.30	4.41	0.89	0.417	46.85	7.87
5.50	6.02	5.30	2.94	2.36	1.801	76.31	33.98
6.00	6.30	5.30	3.16	2.14	1.466	68.50	27.66
6.48	6.58	5.30	3.02	2.28	1.223	53.64	23.08
7.05	6.66	5.30	3.33	1.97	1.218	61.83	22.98
8.0	7.43	5.30	3.54	1.76	0.551	31.31	10.40

Table B.7 Effect of Initial pH on the Cultivation of *Morchella* spp. in Mg-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.05	4.07	5.30	4.92	0.38	0.272	71.58	5.13
5.00	4.97	5.30	3.85	1.45	1.328	91.59	25.06
5.51	5.97	5.30	3.21	2.09	1.864	89.19	35.17
6.00	6.22	5.30	3.17	2.13	1.441	67.65	27.19
6.51	6.63	5.30	3.00	2.30	1.106	48.09	20.87
7.00	6.79	5.30	3.00	2.30	1.109	48.22	20.92
8.00	7.21	5.30	3.17	2.13	0.669	31.41	12.62

Table B.8 Effect of Initial pH on the Cultivation of *M. crassipes* in Na-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.00	4.27	4.91	2.42	2.49	2.764	111.00	56.29
5.00	5.78	4.91	2.31	2.60	2.900	111.53	59.06
5.50	6.23	4.91	2.28	2.63	2.035	77.18	41.44
6.00	6.41	4.91	2.34	2.57	2.300	89.49	46.84
6.50	6.61	4.91	2.17	2.74	2.101	76.67	42.79
7.00	7.02	4.91	2.58	2.33	1.268	54.42	25.82
8.00	7.23	4.91	2.33	2.58	0.806	31.24	16.41

Table B.9 Effect of Initial pH on the Cultivation of *M. esculenta* in Na-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied).
4.00	3.94	4.91	2.62	2.29	2.302	100.52	46.88
5.00	5.02	4.91	2.05	2.86	2.262	79.09	46.06
5.50	6.40	4.91	2.13	2.78	1.866	67.12	38.00
6.00	6.68	4.91	2.09	2.82	2.072	73.47	42.19
6.50	7.29	4.91	2.53	2.38	6.269	263.40	127.67
7.00	7.92	4.91	2.63	2.28	2.379	104.34	48.45
8.00	8.17	4.91	2.63	2.28	0.796	34.91	16.21

Table B.10 Effect of Initial pH on the Cultivation of *Morchella* spp. in Na-WSL. Dilution 1:10 v/v.

pH		Carbohydrate			Dry Mycelium		
Initial	Final	Initial (g/l)	Final (g/l)	Used (g/l)	Concentration (g/l)	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
4.00	3.34	4.91	3.78	1.13	0.924	81.76	18.81
5.00	3.34	4.91	2.95	1.96	1.309	66.78	26.65
5.50	3.48	4.91	2.76	2.15	1.388	64.55	28.26
6.00	3.58	4.91	2.40	2.51	1.632	65.01	33.23
6.50	4.27	4.91	2.46	2.45	1.619	66.08	32.97
7.00	5.74	4.91	2.24	2.67	1.479	55.39	35.29
8.00	6.57	4.91	4.28	0.63	0.412	65.39	9.83

Table B.11 Effect of Dilution Ratio on the Cultivation of *M. crassipes* in NH_3-WSL Initial pH 6.00

Dilution Ratio (v/v)	Final pH	Carbohydrate			Dry Mycelium		
		Initial (g/l)	Final (g/l)	Used (g/l)	Concentration	Yield (g/100g TCH used)	Efficiency (g/100g TCH supplied)
1:2	5.44	27.05	20.00	7.05	4.043	57.34	14.94
1:5	5.85	10.82	5.00	5.82	4.856	83.44	44.88
1:10	6.37	5.41	2.19	3.22	1.684	52.31	31.13
1:15	6.48	3.61	1.17	2.44	1.153	47.25	31.94
1:20	6.44	2.71	0.77	1.94	0.801	41.28	29.55

Table B.12 Kinetics of BOD_5 and COD Reductions of NH_3-WSL in the Batch Fermentor by *M. crassipes* at Optimal Initial pH 6.00 and Optimal Dilution Ratio 1:5 v/v.

Cultivation Time (day)	pH	BOD_5		COD	
		(ppm)	% Reduction[a]	(ppm)	% Reduction[a]
BI[b]	5.43	2,145	0.0	22,133	0.0
0[c]	5.37	2,175		22,324	
1 2/3	5.64	2,100	2.1	22,239	
3 1/6	5.90	1,995	7.0	21,756	1.7
8 1/6	5.30	1,875	12.6	19,686	11.1
9 1/6	5.11	1,650	23.0	19,220	13.2
10 1/6	4.98	1,425	33.5	19,028	14.0
11 1/6	4.88	1,275	40.5	19,130	13.6
12 1/6	4.74	900	58.0	18,940	14.4
14	4.75	600	72.0	18,574	16.1
15	4.77	750	65.0	18,772	15.2

(*a*) Based on the values before inoculation.
(*b*) Before inoculation.
(*c*) After inoculation.

Table B.13 Data From the Continuous Cultivation of *M. crassipes* in NH_3-WSL Medium Under Quasioptimal Conditions.

(BI) Before inoculation.
(AI) After inoculation.
(*a*) Start feed tank No. 1.
(*b*) Start feed tank No. 2.
(*c*) Start feed tank No. 3.
(*d*) Start pH-pulse.
(*e*) Stop pH-pulse and re-start feed tank No. 3.
(*f*) Start feed tank No. 4.
(*g*) Start feed tank No. 5.
(*h*) Start substrate pulse.
(*i*) Stop substrate pulse and re-start feed tank No. 5.
(*j*) Start feed tank No. 6.
(*k*) Start temperature pulse.
(*l*) Stop temperature pulse and re-start feed tank No. 6.
(*m*) Start feed tank No. 7.

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
0(BI)	BI	0.250	300	-	-	-	0.054	-
0(AI)	AI	0.253	300	24.0	-	-	0.197	24.9
4	1	0.256	300	23.8	-	-	0.281	24.5
8	2	0.260	300	23.8	-	-	0.217	24.6
12	3	0.263	300	23.8	-	-	0.235	24.8
16	4	0.267	300	23.8	-	-	0.370	24.8
20	5	0.270	300	24.2	-	-	0.483	25.0
24	6	0.274	300	24.8	-	-	0.857	25.5
28	7	0.278	300	24.5	5.77	5.40	0.898	25.2
32	8	0.282	300	24.5	5.77	5.40	0.868	25.3
36	9	0.133	300	23.7	5.77	5.40	0.899	24.5
40	10	0.133	300	23.8	5.77	5.40	0.851	24.7
44	11	0.133	300	23.8	5.77	5.40	0.638	24.8
48	12	0.133	300	24.0	5.77	5.40	0.721	24.7
52	13	0.133	300	23.9	5.77	5.40	0.822	24.7
56	14	0.133	300	23.9	5.77	5.40	0.763	24.6
60	15	0.133	300	23.7	5.77	5.40	0.637	24.6
64	16	0.133	300	23.8	5.77	5.40	0.785	24.7

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
0(BI)	BI	-	5.90	4.75	-	-	-	-
0(AI)	AI	24.2	5.95	4.23	29.5	1.5	49	-
4	1	23.8	5.97	4.43	28.5	1.7	49	-
8	2	23.9	5.90	4.46	28.0	1.5	49	-
12	3	23.9	5.86	4.30	28.0	1.5	62	-
16	4	23.9	5.72	3.95	28.7	1.5	62	-
20	5	24.4	5.57	3.80	30.7	1.5	68	-
24	6	24.9	5.60	3.45	30.5	1.5	55	-
28[a]	7	24.6	5.40	2.63	29.7	1.5	62	-
32	8	24.7	5.70	2.52	29.0	1.5	62	0.0320
36	9	23.9	5.77	2.20	28.0	1.2	62	0.0286
40	10	23.9	5.74	2.15	28.3	1.2	62	0.0258
44	11	24.0	5.74	1.97	-	-	-	0.0258
48	12	24.1	5.80	2.07	28.7	1.3	62	0.0333
52	13	24.0	5.83	2.15	28.5	1.3	62	0.0276
56	14	24.0	5.82	1.65	28.7	1.3	62	0.0296
60	15	23.9	5.80	1.70	28.0	1.4	62	0.0296
64	16	24.0	5.80	1.85	29.5	1.4	62	0.0267

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
68	17	0.133	300	24.4	5.77	5.40	0.746	25.2
76	18	0.133	300	24.7	5.77	5.40	0.865	25.5
80	19	0.133	300	24.7	5.77	5.40	0.886	25.3
89	20	0.133	300	24.2	5.77	5.40	1.054	24.9
91	21	0.133	300	-	5.77	5.40	1.355	-
93	22	0.133	300	24.4	5.77	5.40	1.177	25.3
95	23	0.133	300	-	5.77	5.40	1.266	-
97	24	0.133	300	24.1	5.77	5.40	1.455	25.0
99[b]	25	0.266	300	-	5.80	5.34	1.316	-
103	26	0.266	300	24.0	5.80	5.34	1.277	24.8
112	27	0.266	300	23.8	5.80	5.34	1.314	24.6
118	28	0.266	300	24.0	5.80	5.34	1.500	24.6
124	29	0.266	300	24.0	5.80	5.34	1.598	24.8
139	30	0.266	300	24.0	5.80	5.34	1.432	24.8
143	31	0.266	300	24.4	5.80	5.34	1.724	25.0
147	32	0.266	300	24.3	5.80	5.34	1.364	25.0
151	33	0.266	300	24.4	5.80	5.34	1.857	25.0
160	34	0.266	300	24.2	5.80	5.34	1.757	25.0
164	35	0.266	300	24.8	5.80	5.34	1.546	25.6

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
68	17	24.6	5.80	1.67	31.0	1.5	62	-
76	18	24.8	5.80	1.85	30.0	1.7	62	0.0267
80	19	24.8	5.74	1.73	30.0	1.5	62	0.0267
89	20	24.3	5.80	1.77	29.5	1.5	62	0.0250
91	21	-	5.80	1.77	-	-	-	-
93	22	24.5	5.78	1.67	30.0	1.5	62	0.0267
95	23	-	5.70	1.65	-	-	-	0.0258
97	24	24.2	5.65	1.65	29.0	1.5	62	0.0267
99[b]	25	-	5.68	1.55	-	-	-	0.0267
103	26	24.1	5.70	1.75	28.5	2.0	62	0.0138
112	27	23.9	5.82	1.85	27.7	3.7	62	0.0174
118	28	24.0	5.95	1.95	29.0	3.7	62	0.0125
124	29	24.2	5.90	1.85	-	-	-	0.0136
139	30	24.2	6.05	2.17	28.5	3.0	62	0.0140
143	31	24.6	6.10	2.60	29.0	3.5	62	0.0136
147	32	24.5	6.05	2.15	28.8	3.3	62	0.0136
151	33	24.6	6.10	2.13	28.8	3.0	62	0.0131
160	34	24.4	6.15	2.35	29.0	2.3	62	0.0131
164	35	24.9	6.20	2.65	30.7	2.5	62	0.0127

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
168	36	0.266	300	25.0	5.80	5.34	1.369	25.7
173	37	0.266	300	24.5	5.80	5.34	1.239	25.2
189	38	0.266	300	24.3	5.80	5.34	1.590	25.0
193	39	0.266	300	24.3	5.80	5.34	0.973	25.0
197	40	0.266	300	24.5	5.80	5.34	1.063	25.2
201	41	0.266	300	24.3	5.80	5.34	1.334	25.1
205	42	0.266	300	24.1	5.80	5.34	0.975	25.0
209	43	0.266	300	24.0	5.80	5.34	1.133	24.8
213	44	0.266	300	24.3	5.80	5.34	1.032	25.0
217	45	0.266	300	24.2	5.80	5.34	1.661	24.9
221	46	0.266	300	24.0	5.80	5.34	1.077	24.5
232	47	0.266	300	24.0	5.80	5.34	1.153	24.7
237	48	0.266	300	24.5	5.80	5.34	1.176	25.3
245[c]	49	0.266	300	24.6	6.12	6.12	1.143	25.3
258	50	0.266	300	24.3	6.12	6.12	1.178	25.0
264	51	0.266	300	24.6	6.12	6.12	1.139	25.4
268[d]	52	0.266	300	24.8	2.30	6.15	0.983	25.7
270	53	0.266	300	24.5	2.30	6.15	0.915	25.3
272	54	0.266	300	–	2.30	6.15	0.905	–

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temperature (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
168	36	25.2	6.17	2.50	30.5	3.2	58	0.0131
173	37	24.7	6.20	2.54	29.5	3.5	62	0.0116
189	38	24.4	6.20	2.80	29.2	2.7	62	0.0133
193	39	24.4	6.20	3.02	29.5	2.5	58	0.0136
197	40	24.6	6.18	2.96	-	-	-	0.0136
201	41	24.4	6.20	2.50	29.2	2.5	62	0.0129
205	42	24.2	6.20	2.65	28.8	2.5	62	0.0125
209	43	24.1	6.22	2.64	29.3	3.4	62	0.0131
213	44	24.4	6.23	-	30.0	2.5	58	0.0145
217	45	24.3	6.23	2.80	29.0	2.7	62	0.0133
221	46	24.0	6.26	3.05	29.3	2.5	58	-
232	47	24.0	6.27	2.82	29.0	2.8	65	0.0160
237	48	24.6	6.25	2.97	3.05	3.3	62	0.0129
245[c]	49	24.7	6.24	3.15	30.0	3.8	71	0.0133
258	50	24.4	6.25	-	30.0	3.0	62	0.0143
264	51	24.7	6.26	3.20	30.3	2.7	62	0.0143
268[d]	52	25.1	6.22	2.86	30.0	2.7	62	0.0174
270	53	24.6	6.22	3.77	-	-	-	-
272	54	-	6.15	3.05	-	-	-	0.0123

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
276	55	0.266	300	24.3	2.30	6.15	1.069	25.0
278	56	0.266	300	-	2.30	6.15	0.954	-
280	57	0.266	300	24.6	2.30	6.15	0.904	25.3
282	58	0.266	300	-	2.30	6.15	1.048	-
284	59	0.266	300	24.9	2.30	6.15	1.652	25.7
286	60	0.266	300	-	2.30	6.15	1.106	-
288	61	0.266	300	24.8	6.12	6.12	1.766	25.6
290	62	0.266	300	-	6.12	6.12	1.304	-
292	63	0.266	300	24.6	6.12	6.12	1.335	25.3
294	64	0.266	300	-	6.12	6.12	1.467	-
296	65	0.266	300	24.4	6.12	6.12	1.003	25.2
307	66	0.266	300	24.0	6.12	6.12	1.463	24.6
309	67	0.266	300	-	6.12	6.12	1.496	-
311	68	0.266	300	-	6.12	6.12	1.745	-
313	69	0.266	300	24.0	6.12	6.12	1.920	24.8
315	70	0.266	300	-	6.12	6.12	1.634	-
317	71	0.266	300	23.8	6.12	6.12	1.747	24.6
325	72	0.266	300	23.8	6.12	6.12	2.079	24.6
328	73	0.266	300	23.8	6.12	6.12	1.711	24.8

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temperature (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
276	55	24.3	5.85	3.02	29.5	4.2	58	0.0131
278	56	-	5.70	3.10	-	-	-	0.0138
280	57	24.7	5.47	2.80	29.7	4.2	62	0.0138
282	58	-	5.14	3.18	-	-	-	0.0140
284	59	25.1	4.78	2.96	30.3	3.8	75	0.0140
286	60	-	4.42	2.95	-	-	-	0.0133
288	61	24.9	4.18	2.75	30.0	3.8	62	0.0145
290	62	-	4.23	3.05	-	-	-	0.0151
292	63	24.7	4.32	3.00	29.7	4.0	75	0.0131
294	64	-	4.40	2.97	-	-	-	0.0148
296	65	24.4	4.60	2.78	29.5	4.0	75	0.0143
307	66	24.0	5.30	2.84	28.5	3.4	43	0.0145
309	67	-	5.42	2.95	-	-	-	0.0140
[illegible]	68	-	5.53	2.93	-	-	-	0.0145
313	69	24.1	5.57	3.13	28.5	3.5	46	0.0154
315	70	-	5.60	3.04	-	-	-	0.0143
317	71	23.9	5.63	3.30	-	-	-	0.0143
325	72	23.8	6.00	3.28	27.5	5.0	62	0.0136
328	73	23.8	5.90	2.83	28.7	5.0	62	0.0143

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
332	74	0.226	300	23.8	6.12	6.12	1.776	24.6
338	75	0.226	300	23.8	6.12	6.12	1.652	24.6
341	76	0.226	300	23.8	6.12	6.12	1.705	24.7
353	77	0.266	300	23.8	6.12	6.12	1.852	24.5
357	78	0.266	300	23.8	6.12	6.12	1.756	24.7
361	79	0.266	300	23.7	6.12	6.12	1.647	24.3
365	80	0.266	300	23.8	6.12	6.12	1.706	24.4
379	81	0.266	300	23.7	6.12	6.12	1.738	24.4
382	82	0.266	300	23.7	6.12	6.12	2.329	24.3
386	83	0.266	300	23.7	6.12	6.12	1.666	24.3
390	84	0.266	300	23.6	5.92	5.77	1.741	24.3
399	85	0.266	300	23.6	5.92	5.77	1.753	24.4
402	86	0.266	300	24.0	5.92	5.77	1.700	24.4
405	87	0.266	300	24.2	5.92	5.77	1.515	24.9
413	88	0.266	300	23.7	5.92	5.77	1.572	24.4
423	89	0.266	300	23.7	5.92	5.77	1.610	24.5
427	90	0.266	300	23.8	5.92	5.77	1.597	24.5
430	91	0.266	300	24.0	5.92	5.77	1.214	24.8
434	92	0.266	300	24.3	5.92	5.77	1.439	25.0

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
332	74	23.9	5.87	3.17	29.0	4.2	62	0.0140
338	75	23.9	6.07	3.14	29.0	4.6	62	0.0154
341	76	23.9	6.00	3.07	28.7	4.0	62	0.0145
353	77	23.9	6.00	2.70	29.0	4.2	58	0.0143
357	78	24.0	6.10	2.95	29.0	4.2	62	0.0148
361	79	23.7	6.10	3.07	28.7	4.5	75	-
365	80	23.8	6.10	2.97	28.0	4.2	68	0.0140
379	81	23.7	6.12	2.85	28.5	4.7	62	0.0133
382	82	23.7	6.06	3.02	-	-	-	0.0143
386	83	23.8	6.05	3.15	28.7	4.7	58	0.0140
390	84	23.7	6.15	2.93	28.0	4.0	65	0.0116
399	85	23.7	6.15	2.80	27.8	4.5	65	0.0127
402	86	24.0	6.13	2.98	29.5	4.0	58	0.0121
405	87	24.3	6.07	3.04	30.0	4.5	62	0.0125
413	88	23.8	6.10	3.00	28.3	4.5	62	0.0127
423	89	23.7	6.14	3.38	28.0	4.2	75	0.0123
427	90	24.0	6.15	3.46	-	-	-	0.0123
430	91	24.0	6.18	3.71	29.5	4.0	62	0.0118
434	92	24.3	6.16	3.63	30.2	4.0	58	0.0123

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
437	93	0.266	300	24.2	5.92	5.77	1.223	25.0
450	94	0.266	300	24.2	5.92	5.77	1.392	24.8
454	95	0.266	300	24.5	5.92	5.77	1.251	25.2
459	96	0.266	300	24.3	5.92	5.77	1.455	25.0
475	97	0.266	300	24.2	5.92	5.77	1.152	25.0
479	98	0.266	300	24.6	5.92	5.77	1.375	25.3
483	99	0.266	300	24.5	5.92	5.77	1.942	25.2
500	100	0.266	300	23.8	5.92	5.77	0.982	24.5
506	101	0.266	300	24,0	5.92	5.77	1.012	24.7
510	102	0.266	300	23.7	5.92	5.77	0.829	24.5
519	103	0.266	300	23.8	5.92	5.77	0.878	24.7
524	104	0.266	300	23.8	5.92	5.77	0.740	24.6
533[g]	105	0.266	300	23.7	5.90	5.15	0.899	24.6
547	106	0.266	300	23.6	5.90	5.15	0.817	24.5
553	107	0.266	300	23.5	5.90	5.15	0.820	24.3
557	108	0.266	300	23.5	5.90	5.15	0.750	24.3
568	109	0.266	300	23.5	5.90	5.15	0.836	24.2
573	110	0.266	300	23.3	5.90	5.15	0.712	24.0
580[h]	111	0.266	300	23.4	5.00	22.12	0.901	24.0

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temperature (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
437	93	24.4	6.18	-	30.4	4.0	75	0.0127
450	94	24.2	6.14	-	30.0	4.0	62	0.0123
454	95	24.7	6.16	3.15	30.5	3.7	62	0.0133
459	96	24.3	6.20	3.14	29.6	4.2	62	0.0140
475	97	24.3	6.13	3.13	30.0	4.2	62	0.0128
479	98	24.6	6.15	3.07	30.0	4.5	75	0.0170
483	99	24.7	6.15	2.98	30.0	4.5	65	0.0133
500	100	23.8	6.18	3.04	29.7	5.2	62	0.0129
506	101	24.2	6.18	3.14	29.5	5.3	62	0.0133
410	102	23.8	6.23	3.33	28.5	5.2	71	0.0125
519	103	23.9	6.16	-	28.5	5.2	71	0.0129
524	104	23.8	6.17	3.22	29.7	4.0	75	0.0123
533[g]	105	23.7	6.18	3.02	29.5	5.0	75	0.0080
547	106	23.7	6.20	3.21	30.0	5.0	62	0.0114
553	107	23.6	6.20	3.38	29.5	5.0	62	0.0125
557	108	23.6	6.20	3.33	-	-	-	0.0118
568	109	23.6	6.18	3.21	29.7	4.6	62	0.0119
573	110	23.3	6.20	2.95	30.7	4.8	68	0.0123
580[h]	111	23.4	6.16	3.33	30.0	5.0	62	0.0100

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
582	112	0.266	300	23.3	5.00	22.12	0.945	24.0
584	113	0.266	300	-	5.00	22.12	0.980	-
586	114	0.266	300	23.0	5.00	22.12	1.044	23.9
588	115	0.266	300	22.8	5.00	22.12	0.997	23.8
590	116	0.266	300	22.8	5.00	22.12	1.026	23.8
592	117	0.266	300	-	5.00	22.12	1.204	-
594	118	0.266	300	23.2	5.00	22.12	1.483	24.0
596	119	0.266	300	-	5.00	22.12	1.339	-
598	120	0.266	300	23.4	5.00	22.12	1.558	24.3
600	121	0.266	300	23.2	5.90	5.15	1.388	24.2
602	122	0.266	300	-	5.90	5.15	2.153	-
604	123	0.266	300	23.0	5.90	5.15	1.735	23.8
608	124	0.266	300	23.0	5.90	5.15	1.523	23.9
610	125	0.266	300	-	5.90	5.15	1.771	-
612	126	0.266	300	-	5.90	5.15	1.794	-
616	127	0.266	300	-	5.90	5.15	1.662	-
620	128	0.266	300	22.8	5.90	5.15	2.078	23.8
624	129	0.266	300	23.0	5.90	5.15	1.700	23.8
628	130	0.266	300	23.0	5.90	5.15	1.527	23.8

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temperature (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
582	112	23.2	6.10	3.48	29.0	5.0	62	0.0119
584	113	–	6.03	3.78	–	–	–	0.0119
586	114	23.0	5.90	4.08	28.0	4.7	65	0.0119
588	115	22.8	5.85	3.85	28.0	4.5	65	0.0123
590	116	22.8	5.80	3.84	28.5	4.5	75	0.0121
592	117	–	5.78	4.27	–	–	–	0.0118
594	118	23.3	5.75	4.67	30.0	5.0	62	0.0118
596	119	–	5.73	4.73	–	–	–	0.0118
598	120	23.5	5.70	4.58	31.0	5.0	68	0.0114
600	121	23.2	5.68	4.78	30.0	4.8	62	0.0114
602	122	–	5.73	5.10	–	–	–	0.0133
604	123	23.0	5.82	4.96	29.7	4.5	62	0.0121
608	124	23.0	5.90	4.67	29.7	4.8	71	0.0129
610	125	–	5.93	4.34	–	–	–	0.0125
612	126	–	5.96	4.23	–	–	–	–
616	127	–	6.00	4.37	–	–	–	–
620	128	23.0	6.03	4.29	29.0	4.6	62	0.0123
624	219	23.0	6.07	4.28	28.5	5.2	71	0.0121
628	130	23.0	6.10	3.95	–	–	–	0.0133

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
632	131	0.266	300	23.0	5.90	5.15	1.319	23.9
644	132	0.266	300	23.0	5.90	5.15	1.164	23.7
649	133	0.266	300	22.8	5.90	5.15	1.239	23.9
653	134	0.266	300	22.8	5.90	5.15	1.120	23.8
665	135	0.266	300	22.8	5.90	5.15	1.080	24.0
669	136	0.266	300	23.0	5.90	5.15	1.302	23.8
673	137	0.266	300	22.8	5.90	5.15	1.114	23.9
677	138	0.266	300	22.3	5.90	5.15	1.057	23.8
680	139	0.266	300	23.0	5.90	5.15	1.142	24.0
694	140	0.266	300	23.0	5.90	5.15	0.865	23.8
699	141	0.266	300	23.0	5.90	5.15	0.959	23.8
711	142	0.266	300	23.0	6.20	5.66	1.169	24.0
715	143	0.266	300	23.0	6.20	5.66	1.276	23.8
722	144	0.266	300	23.0	6.20	5.66	1.182	23.8
735	145	0.266	300	22.8	6.20	5.66	0.950	24.0
739	146	0.266	300	22.8	6.20	5.66	0.953	24.0
748	147	0.266	300	22.6	6.20	5.66	1.275	23.9
763	148	0.266	300	23.0	6.20	5.66	1.270	24.0
766	149	0.266	300	23.0	6.20	5.66	1.002	24.0

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
632	131	23.0	6.10	4.03	–	–	–	0.0133
644	132	23.0	6.10	4.29	29.2	4.8	71	0.0129
649	133	23.0	6.10	4.35	29.2	4.5	62	0.0133
653	134	22.8	6.12	4.10	29.0	4.8	65	0.0129
665	135	23.0	6.12	3.70	29.2	4.3	62	0.0131
669	136	23.0	6.12	4.00	29.3	4.3	62	0.0114
673	137	23.0	6.12	4.18	28.7	5.2	71	0.0133
677	138	23.0	6.20	4.25	28.0	5.0	62	–
689	139	23.0	6.17	4.18	29.2	4.7	58	0.0129
694	140	23.0	6.18	3.79	–	–	–	0.0127
699	141	23.0	6.20	3.49	29.0	4.3	71	0.0119
711	142	23.0	6.16	3.52	–	–	–	–
715	143	23.0	6.16	3.67	–	–	–	0.0133
722	144	23.0	6.32	3.04	29.7	5.0	62	0.0133
735	145	22.8	6.30	3.07	29.0	5.0	65	–
739	146	23.0	6.30	2.98	–	–	–	0.0129
748	147	22.7	6.35	3.01	26.5	4.7	71	0.0138
763	148	23.0	6.35	3.04	29.0	3.0	62	0.0100
766	149	23.0	6.35	3.20	29.0	2.8	62	0.0129

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
769	150	0.266	300	22.7	6.20	5.66	1.058	24.0
773	151	0.266	300	22.7	6.20	5.66	1.092	24.0
786	152	0.266	300	23.0	6.20	5.66	1.064	24.0
791	153	0.266	300	23.0	6.20	5.66	1.177	24.0
792[k]	154	0.266	300	-	6.20	5.66	1.116	-
793[l]	155	0.266	300	-	6.20	5.66	1.869	-
795	156	0.266	300	23.5	6.20	5.66	1.414	25.1
797	157	0.266	300	23.2	6.20	5.66	1.052	24.2
799	158	0.266	300	23.3	6.20	5.66	1.218	24.8
803	159	0.266	300	-	6.20	5.66	1.659	-
807	160	0.266	300	23.3	6.20	5.66	1.439	25.0
809	161	0.266	300	-	6.20	5.66	1.205	-
811	162	0.266	300	23.3	6.20	5.66	1.323	25.1
814	163	0.266	300	23.3	6.20	5.66	1.481	25.3
817	164	0.266	300	23.3	6.20	5.66	1.118	25.2
820	165	0.266	300	-	6.20	5.66	1.055	-
831	166	0.266	300	23.8	6.20	5.66	1.009	25.6
835	167	0.266	300	24.0	6.20	5.66	1.131	25.7

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
769	150	22.8	6.38	2.68	27.0	2.8	62	0.0133
773[h]	151	22.9	6.40	2.86	27.0	2.8	65	0.0133
786	152	23.0	6.42	3.07	27.2	3.2	62	0.0160
791	153	23.0	6.43	3.38	26.8	4.0	62	0.0178
792[k]	154	-	6.46	3.33	-	-	-	0.0178
793[l]	155	-	6.47	2.96	-	-	-	0.0123
795	156	24.0	6.45	2.78	26.7	3.8	62	0.0133
797	157	23.7	6.45	3.07	26.4	3.7	62	0.0133
799	158	23.7	6.46	3.22	26.8	3.6	62	0.0138
803	159	-	6.47	3.19	-	-	-	0.0114
807	160	23.6	6.48	2.82	26.2	3.3	62	0.0133
809	161	-	6.52	3.22	-	-	-	0.0133
811	162	23.7	6.50	3.38	27.8	3.4	62	0.0129
814	163	23.8	6.50	3.38	27.6	3.2	68	0.0129
817	164	23.7	6.50	3.39	27.0	3.0	62	0.0133
820	165	-	6.50	3.04	-	-	-	-
831	166	23.8	6.55	2.82	27.2	4.0	62	0.0129
835	167	24.0	6.52	3.01	29.0	4.2	71	0.0133

Cultivation Time (hr)	Sample No	x^1 (vvm)	x^2 (RPM)	x^3 (°C)	x^5	x^6 (g/l)	y^1 (g/l)	y^2 (°C)
840	168	0.266	300	24.2	6.20	5.66	1.178	25.7
845m	169	0.266	300	24.2	5.82	5.73	0.979	25.8
859	170	0.266	300	24.4	5.82	5.73	1.681	26.1
864	171	0.266	300	24.4	5.82	5.73	1.729	26.2
869	172	0.266	300	24.6	5.82	5.73	1.109	26.2
879	173	0.266	300	24.5	5.82	5.73	1.384	26.2
883	174	0.266	300	24.5	5.82	5.73	1.091	26.2
887	175	0.266	300	24.5	5.82	5.73	1.052	26.2
891	176	0.266	300	24.5	5.82	5.73	0.994	26.2
903	177	0.266	300	24.5	5.82	5.73	1.364	26.2
908	178	0.266	300	24.6	5.82	5.73	1.217	26.2
916	179	0.266	300	24.7	5.82	5.73	1.381	26.2
927	180	0.266	300	24.7	5.82	5.73	1.663	26.3
933	181	0.266	300	24.6	5.82	5.73	0.981	26.2
954	182	0.266	300	25.0	5.82	5.73	1.105	26.2
963	183	0.266	300	24.8	5.82	5.73	1.117	26.3
970	184	0.266	300	24.9	5.82	5.73	1.009	26.3
975	185	0.266	300	24.8	5.82	5.73	1.419	26.3

Cultivation Time (hr)	Sample No	y^3 (°C)	y^4	y^5 (g/l)	Ambient Temptera-ture (°C)	Air Pressure (psig)	Jacket Water Flowrate (l/hr)	Dilution Rate (hr^{-1})
840	168	24.2	6.54	3.04	28.7	4.0	62	0.0136
845[m]	169	24.4	6.53	3.12	27.8	4.0	71	0.0104
859	170	24.9	6.53	2.77	29.3	4.0	71	0.0119
864	171	25.0	6.50	2.80	28.7	3.8	68	0.0114
869	172	25.1	6.50	3.02	29.0	3.6	71	0.0127
879	173	25.2	6.48	2.96	29.2	3.3	62	0.0119
883	174	25.0	6.48	2.63	29.5	4.0	65	0.0129
887	175	25.0	6.47	2.70	29.0	4.5	65	0.0133
891	176	25.1	6.47	2.88	28.8	4.7	62	0.0133
903	177	25.1	6.46	2.98	28.0	4.6	65	0.0118
908	178	25.2	6.45	2.94	29.3	4.2	62	0.0076
916	179	25.3	6.43	2.89	30.0	4.0	71	0.0129
927	180	25.3	6.40	2.72	-	-	-	0.0178
933	181	25.3	6.40	3.07	29.4	4.4	62	0.0123
954	182	25.4	6.37	2.96	28.7	4.3	75	0.0123
963	183	25.3	6.37	3.13	28.7	4.3	62	-
970	184	25.3	6.37	3.10	29.7	4.0	62	0.0133
975	185	25.3	6.37	2.92	29.0	4.2	71	0.0118

APPENDIX C

CALIBRATION OF INSTRUMENTS

C.1 Pressure-Flowrate Relationship of Aeration.

C.2 Calibration of Temperature.

C.3 Calibration of Temperature.

C.4 Calibration of Temperature.

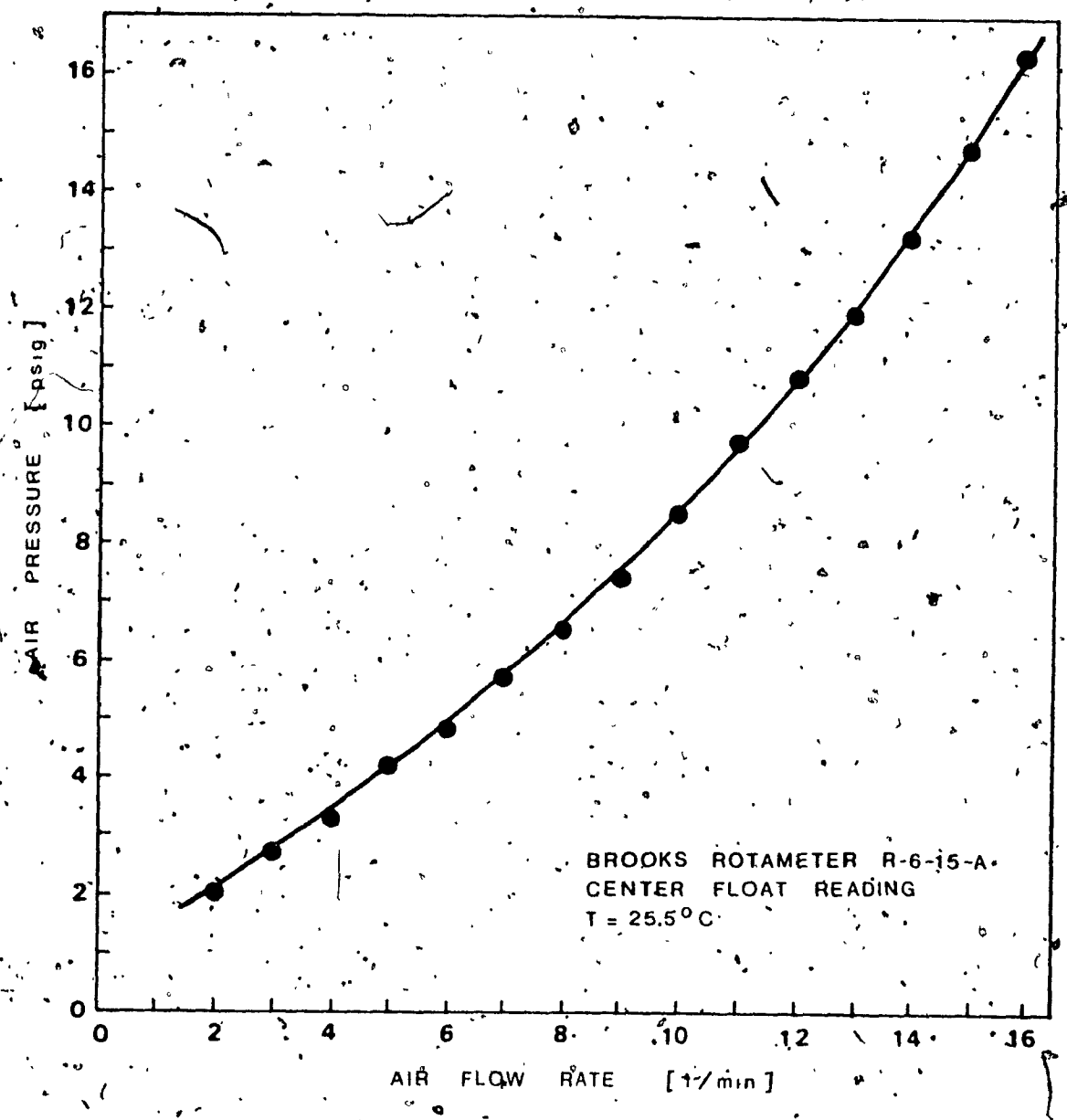
C.5 Calibration of Temperature.

C.6 Calibration of Jacket Water Flowmeter.

C.7 Calibration of Feed Pump.

C.8 Calibration of pH Measurement System.

Figure C.1 Pressure-Flowrate Relationship of Aeration.


AIR PRESSURE [psig]
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12
14
16
AIR FLOW RATE [l/min]
BROOKS ROTAMETER R-6-15-A
CENTER FLOAT READING
T = 25.5°C

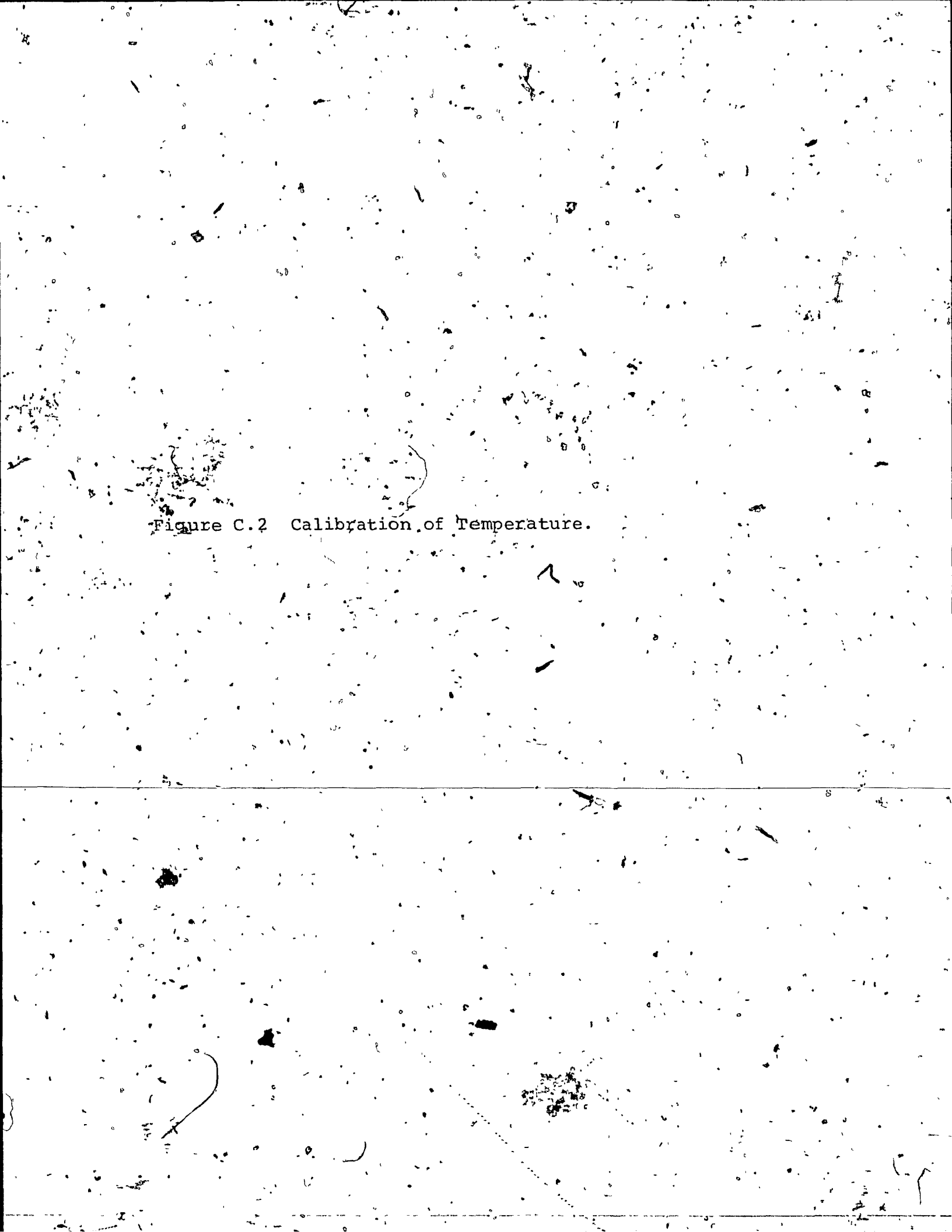


Figure C.2 Calibration of Temperature.

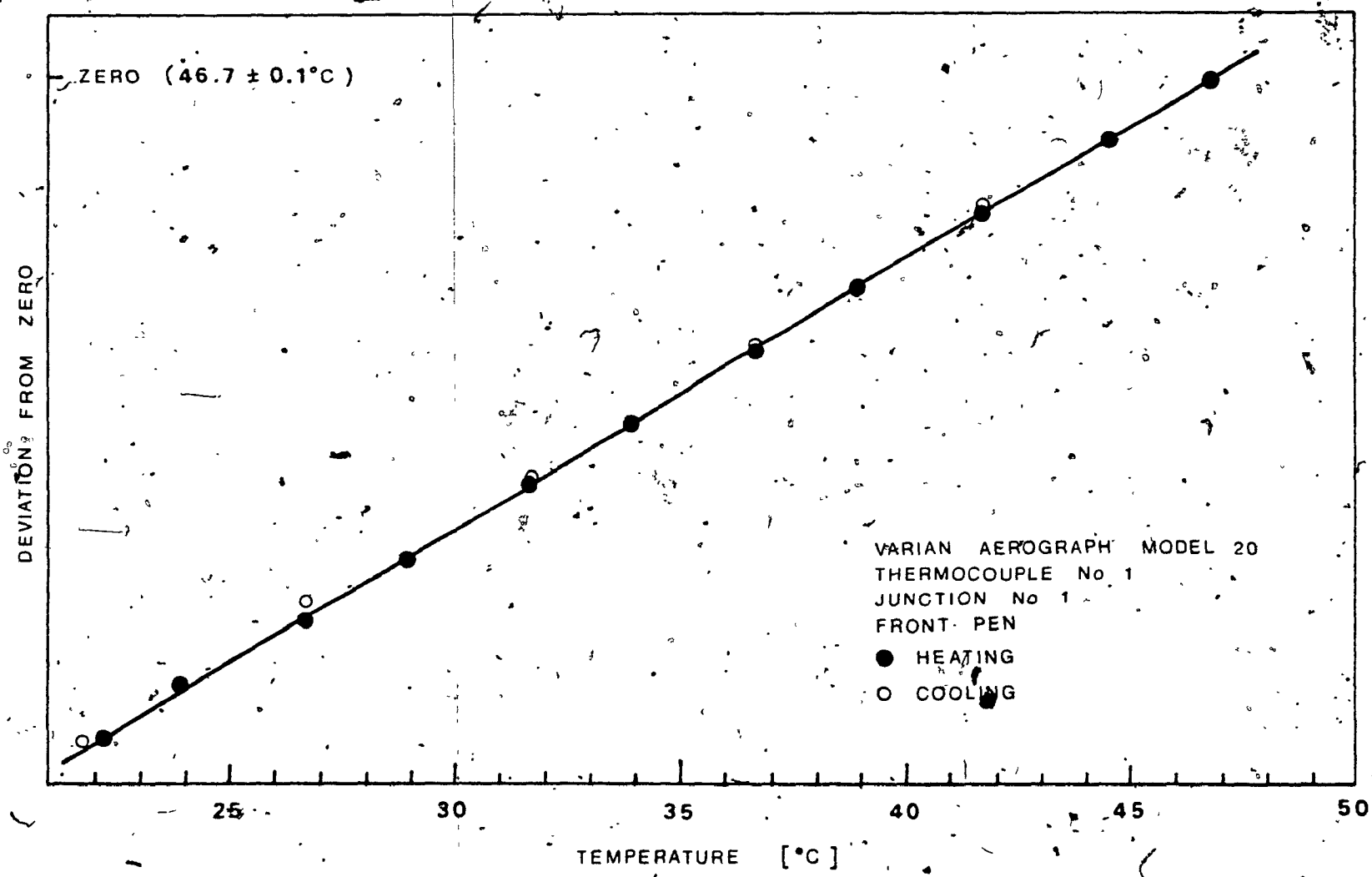

ZERO (46.7 ± 0.1°C)
DEVIATION FROM ZERO
VARIAN AEROGRAPH MODEL 20
THERMOCOUPLE No 1
JUNCTION No 1
FRONT PEN
HEATING
COOLING
25
30
35
40
45
50
TEMPERATURE [°C]

Figure C.3 Calibration of Temperature.

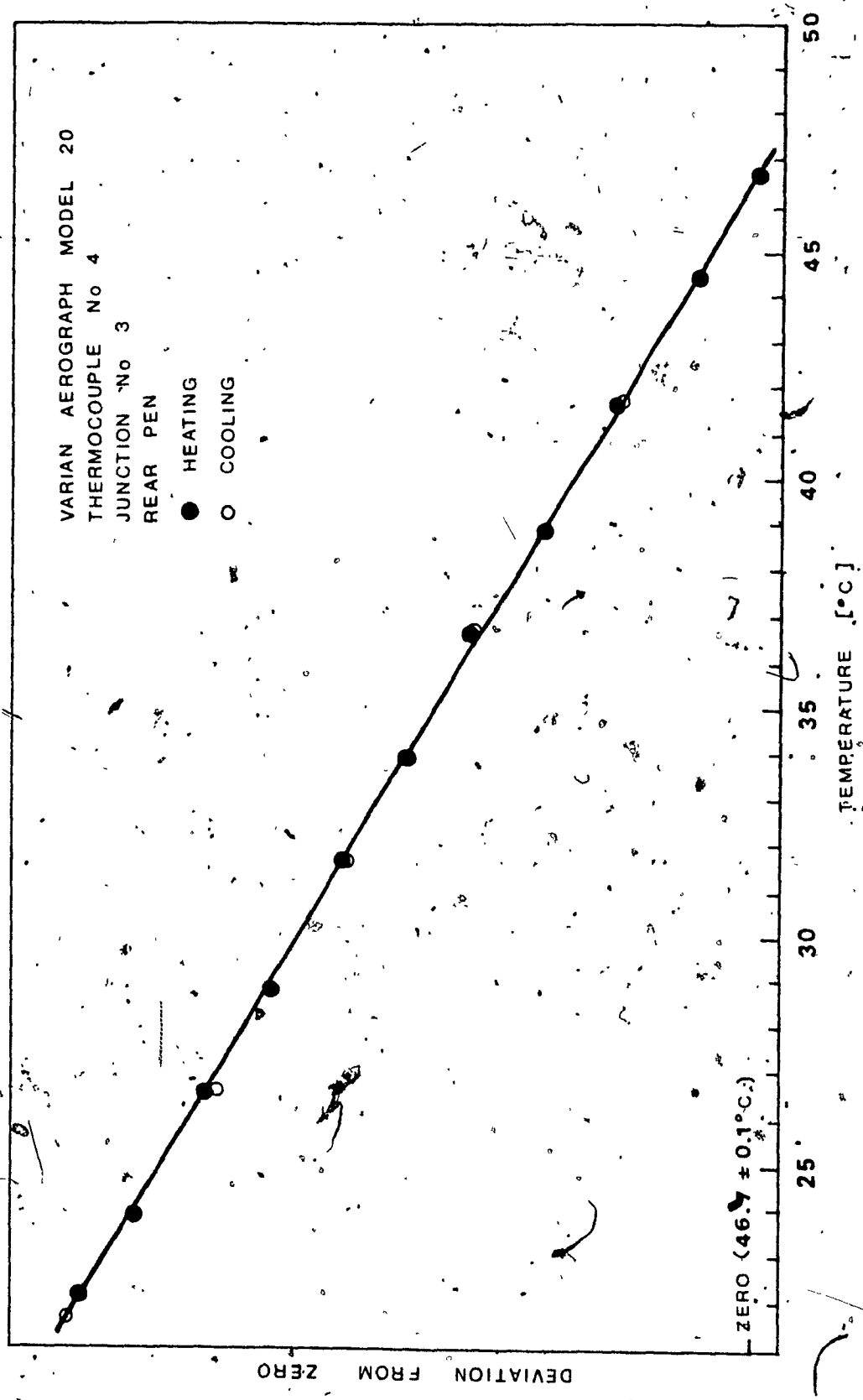

VARIAN AEROGRAPH MODEL 20
THERMOCOUPLE No 4
JUNCTION No 3
REAR PEN
● HEATING
○ COOLING
ZERO (46.7 ± 0.1°C.)
DEVIATION FROM ZERO
TEMPERATURE [°C]
25
30
35
40
45
50

Figure C.4 Calibration of Temperature.

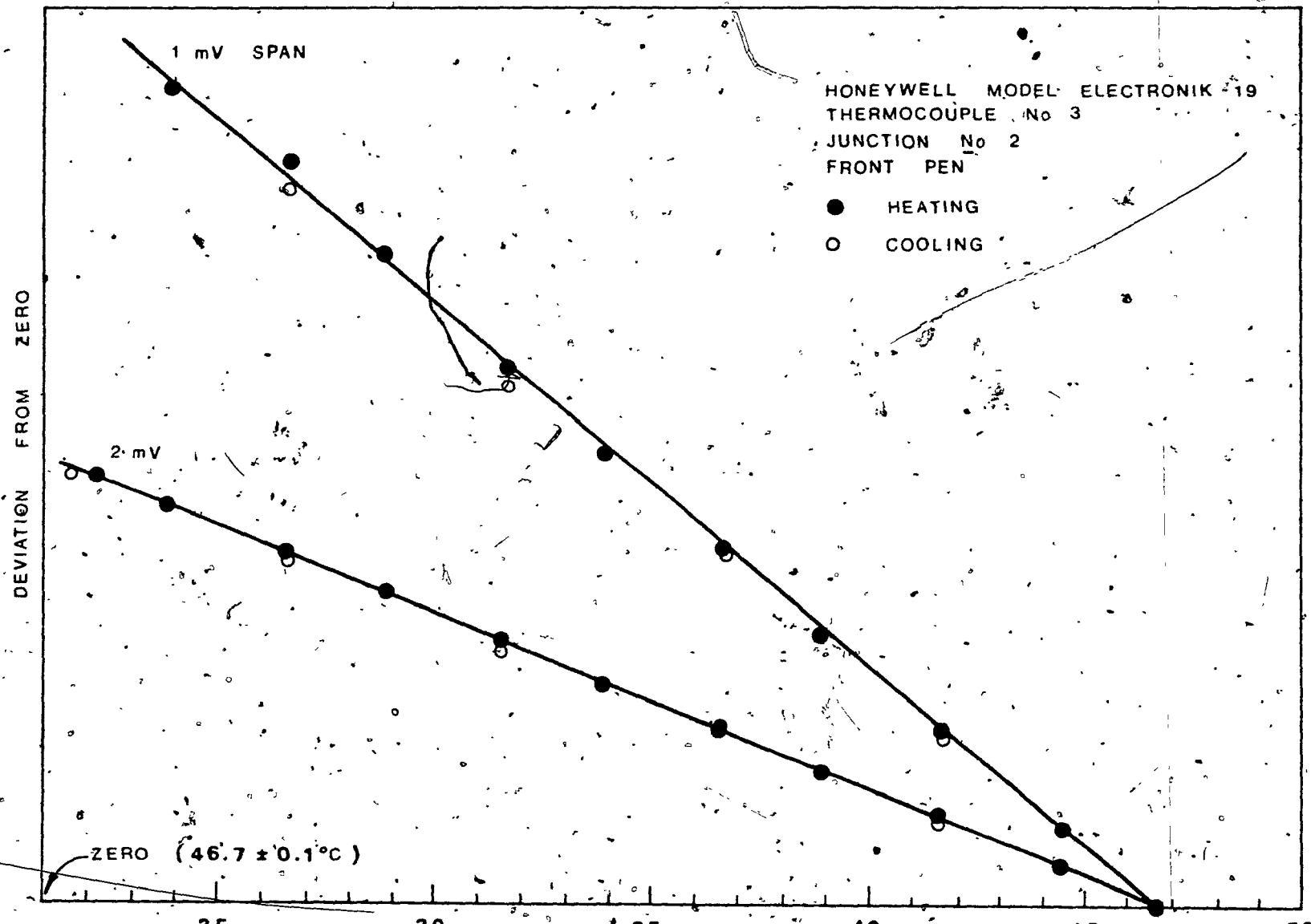

1 mV SPAN
2 mV
HONEYWELL MODEL ELECTRONIK 19
THERMOCOUPLE No 3
JUNCTION No 2
FRONT PEN
HEATING
COOLING
DEVIATION FROM ZERO
ZERO (46.7 ± 0.1°C)
25
30
35
40
45
50
TEMPERATURE [°C]

Figure C.5 Calibration of Temperature.

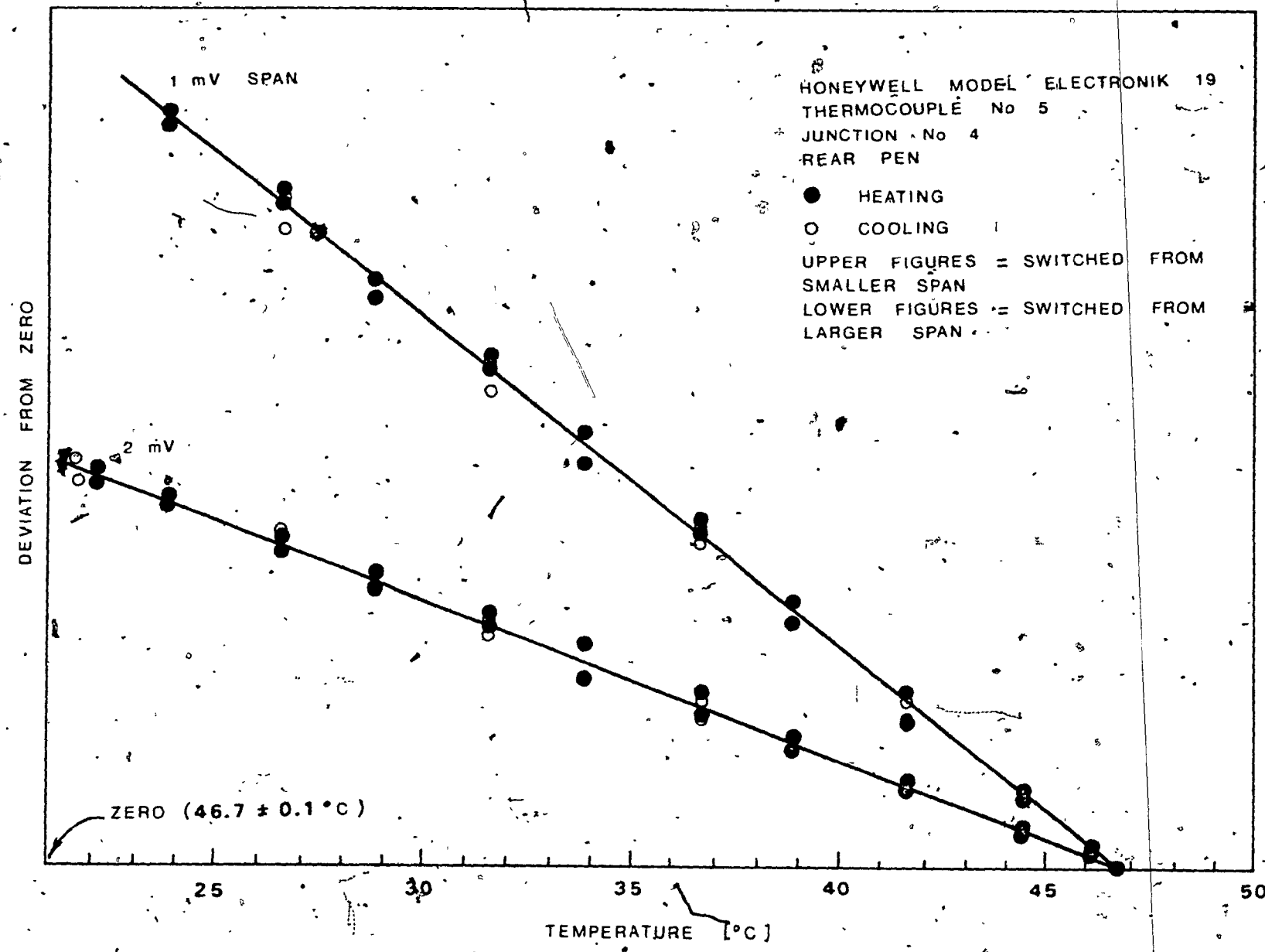

1 mV SPAN
2 mV
HONEYWELL MODEL ELECTRONIK 19
THERMOCOUPLE No 5
JUNCTION No 4
REAR PEN
HEATING
COOLING
UPPER FIGURES = SWITCHED FROM SMALLER SPAN
LOWER FIGURES = SWITCHED FROM LARGER SPAN
DEVIATION FROM ZERO
ZERO (46.7 ± 0.1 °C)
25
30
35
40
45
50
TEMPERATURE [°C]

Figure C.6 Calibration of Jacket Water Flowmeter.

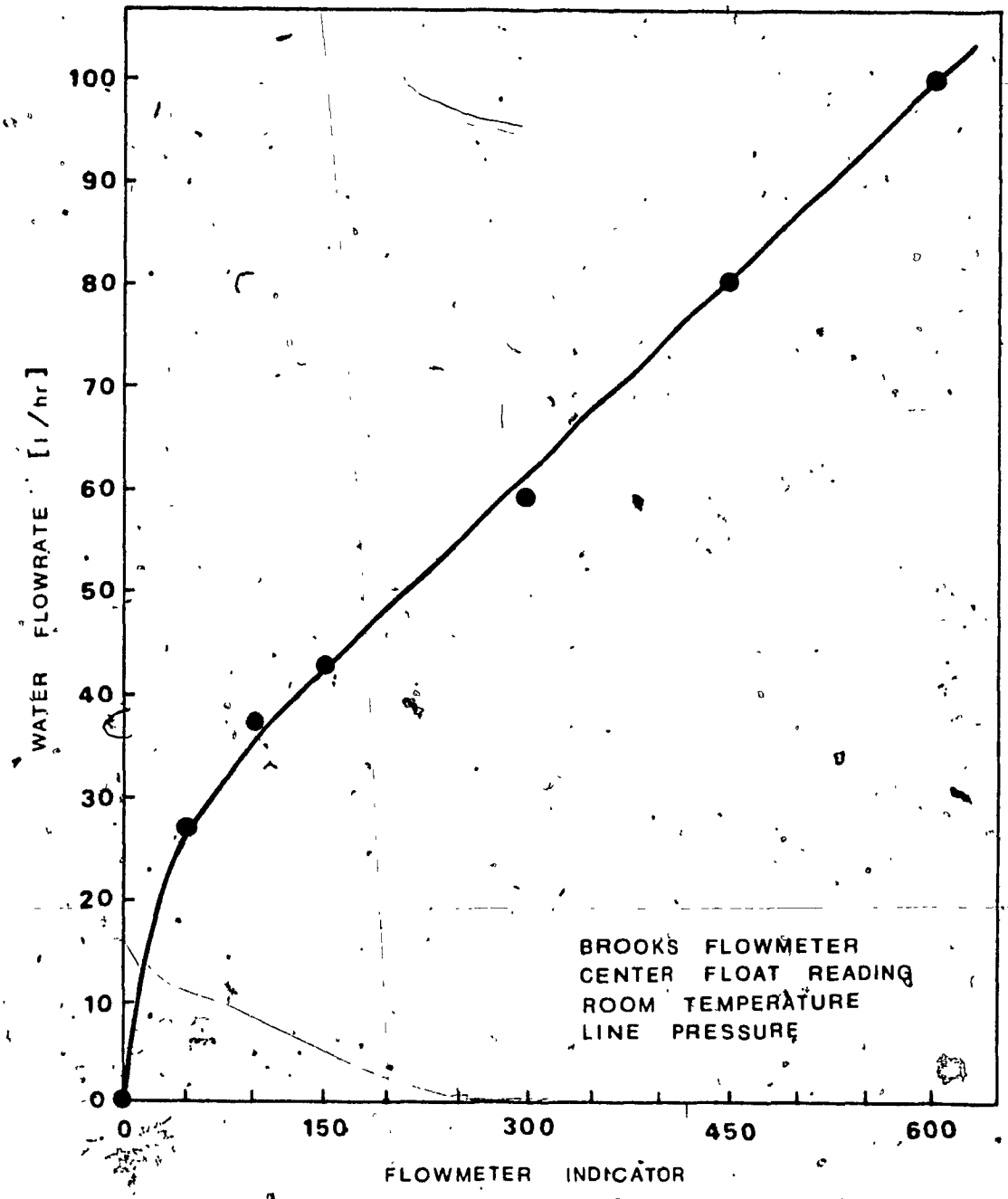

WATER FLOWRATE [l/hr]
100
90
80
70
60
50
40
30
20
10
0
0
150
300
450
600
FLOWMETER INDICATOR
BROOKS FLOWMETER
CENTER FLOAT READING
ROOM TEMPERATURE
LINE PRESSURE

Figure C.7 Calibration of Feed Pump.

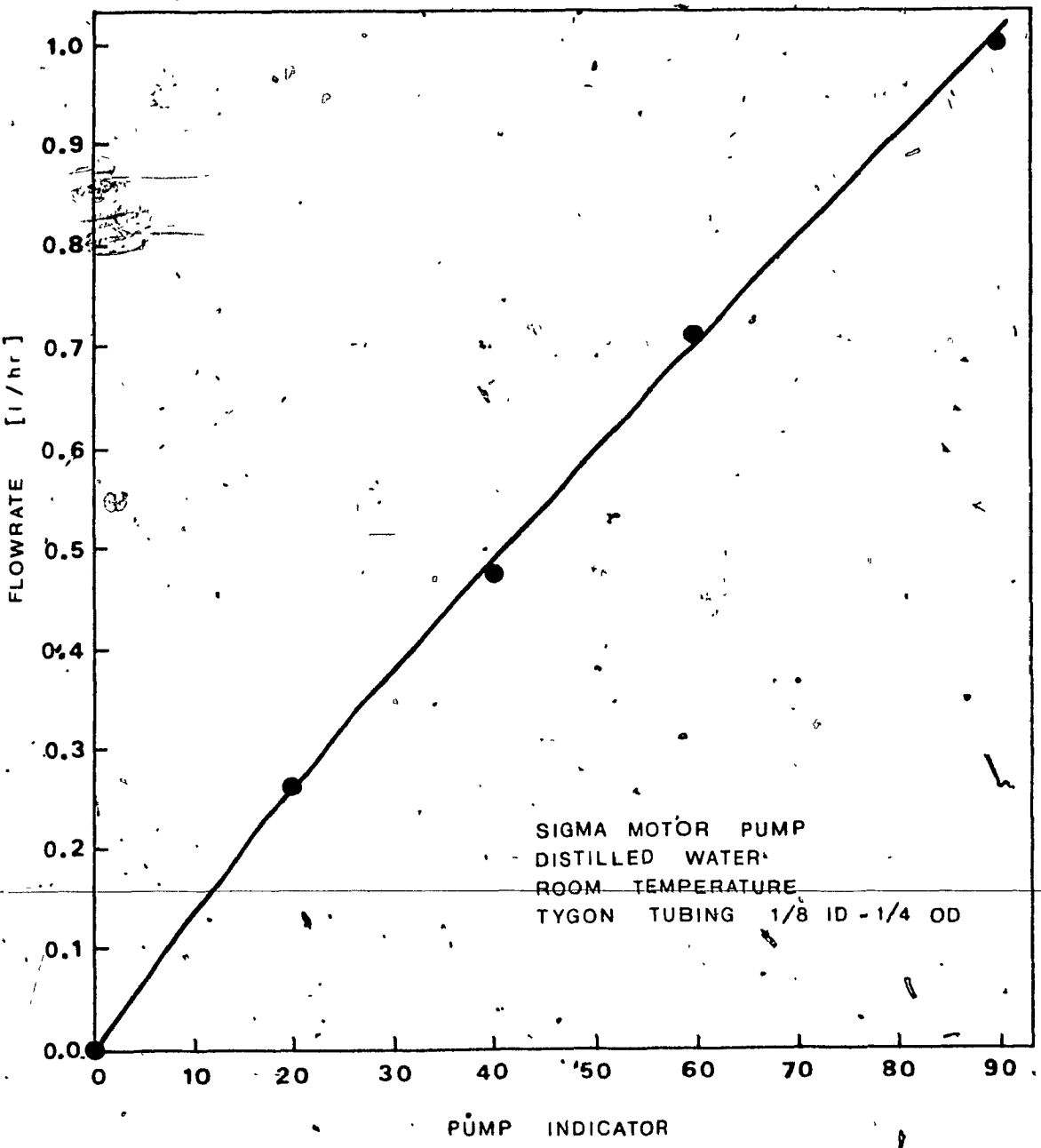
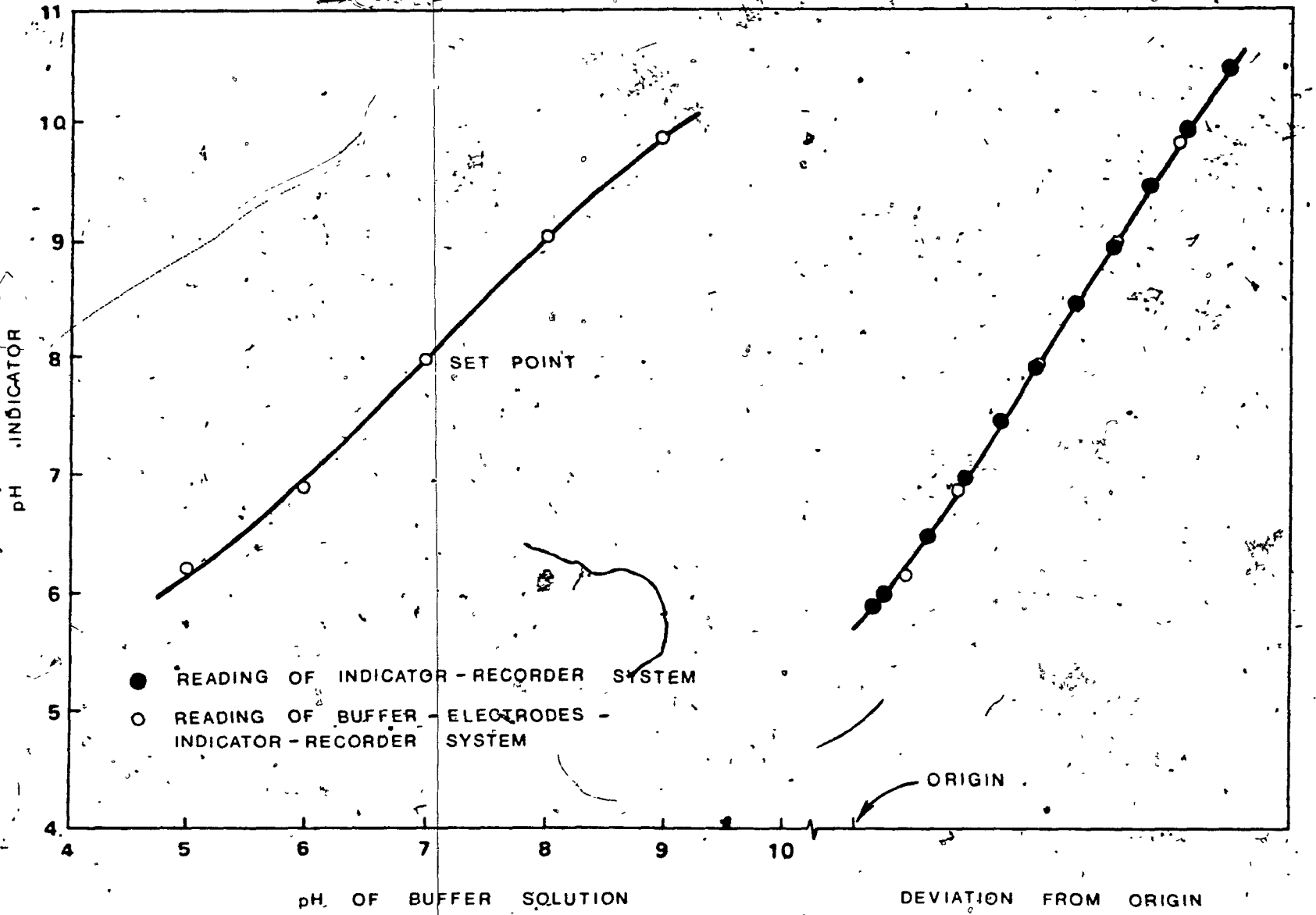

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FLOWRATE [l / hr]
0
10
20
30
40
50
60
70
80
90
PUMP INDICATOR
SIGMA MOTOR PUMP
DISTILLED WATER
ROOM TEMPERATURE
TYGON TUBING 1/8 ID - 1/4 OD

Figure C.8 Calibration of pH Measurement System.


11
10
9
8
7
6
5
4
pH INDICATOR
SET POINT
READING OF INDICATOR - RECORDER SYSTEM
READING OF BUFFER - ELECTRODES - INDICATOR - RECORDER SYSTEM
ORIGIN
4
5
6
7
8
9
10
pH OF BUFFER SOLUTION
DEVIATION FROM ORIGIN

APPENDIX D

LISTING OF COMPUTER PROGRAMS

D.1 Program PULSE.

D.2 Program NLREG1.

D.3 Program LNREG2.

D.4 Testing of Computer Programs.

D.1 Program PULSE

```
C     ********************
C     *                  *
C     *  PROGRAM PULSE   *
C     *                  *
C     ********************
C
C
C     FREQUENCY RESPONSE FROM TRANSIENT RESPONSE USING PULSE
C     TECHNIQUES.
C
C     REFERENCE W.C. CLEMENTS,JR., AND K.B. SCHNELLE,JR.
C     (1963)  I$ EC PROCESS DESIGN AND DEVELOPMENT, 2  94-102.
C
C     PROGRAM INPUTS  DATA CARDS ARE IN THE FOLLOWING ORDER,
C
C     1  IDENTIFICATION CARD,FORMAT 20A4.
C
C     1  CARD FOR WI,WF,DW,TR,TC,TD,FORMAT 6E10.3
C
C          WI = LOWER LIMIT OF FREQUENCY RANGE,
C          WF = HIGHER LIMIT OF FREQUENCY RANGE,
C          DW = LOGARITHMIC INCREAMENT OF FREQUENCY,
C          TR = DURATION OF INPUT SIGNAL,
C          TC = DURATION OF OUTPUT SIGNAL,
C          TD = PURE LAG BETWEEN INPUT AND OUTPUT SIGNALS.
C
C          SUGGESTED VALUES FOR FIRST TRIAL
C          WF=5.0/TR,
C          WI=0.001*WF,
C          DW=0.05.
C
C     1  CARD FOR XS,YS,FORMAT 2F20.5
C
C          XS = STEADY STATE VALUE OF INPUT SIGNAL,
C          YS = STEADY STATE VALUE OF OUTPUT SIGNAL.
C
C     1  CARD FOR NX,NY,FORMAT 2I5
C
C          NX = NUMBER OF DATA POINTS FOR INPUT SIGNAL,
C          NY = NUMBER OF DATA POINTS FOR OUTPUT SIGNAL.
C
C          NX AND NY SHOULD BE ODD NUMBERS.
C
C     1  CARD FOR N1R,N2R,N1C,N2C,RP,CP,FORMAT 4I5,2F10.3
C
C          N1R = HALF NUMBER OF DIVISIONS IN THE FIRST
C                SECTION FOR INPUT SIGNAL,
C          N2R = HALF NUMBER OF DIVISIONS IN THE SECOND
C                SECTION FOR INPUT SIGNAL,
C          N1C = HALF NUMBER OF DIVISIONS IN THE FIRST
C                SECTION FOR OUTPUT SIGNAL,
```

```
C           N2C = HALF NUMBER OF DIVISIONS IN THE SECOND
C                 SECTION FOR OUTPUT SIGNAL,
C           RP  = DURATION OF INPUT SIGNAL FIRST SECTION,
C           CP  = DURATION OF OUTPUT SIGNAL FIRST SECTION.
C
C           NUMBER OF DIVISIONS SHOULD BE EVEN.
C           IF THERE IS ONLY ONE SECTION, PUT N1R=0 AND/OR
C           N1C=0.
C
C     NX CARDS FOR X(I),FORMAT F10.4
C
C           X(I) = VECTOR OF INPUT SIGNAL DATA.
C
C     NY CARDS FOR Y(I),FORMAT F10.4
C
C           Y(I) = VECTOR OF OUTPUT SIGNAL DATA.
C
C     PROGRAM OUTPUTS- CARDS AND PRINT-OUTS ARE IN THE FOL-
C     LOWING ORDER,
C
C     DATA CARDS FOR THE PLOTTER
C
C     1 IDENTIFICATION CARD,FORMAT 20A4.
C     1 TITLE CARD.
C     1 CARD FOR LMAX,FORMAT I3
C
C           LMAX = NUMBER OF POINTS FOR BODE DIAGRAM.
C
C     LMAX CARDS FOR W,CBAR,DPHI,FORMAT 3E15.8
C
C           W    = FREQUENCY,RADIAN/TIME UNIT,
C           CBAR = AMPLITUDE RATIO,DECIBELS,
C           DPHI = PHASE LAG,DEGREES.
C
C     DATA CARDS FOR PROGRAMS NLREG1 AND LNREG2
C
C     1 IDENTIFICATION CARD,FORMAT 20A4.
C     1 CARD FOR LMAX,FORMAT I3.
C     LMAX CARDS FOR W,AR,DPHI,FORMAT 3E15.8
C
C           AR = MAGNITUDE OF TRANSFER FUNCTION
C
C     PRINT-OUT OF PROGRAM INPUTS.
C     PRINT-OUT OF VECTORS L,S,W,AR,DPHI,DBAR
C
C           L = LTH POINT OF BODE DIAGRAM,
C           S = NORMALIZED FREQUENCY CONTENT.
C
C     PRINT-OUT OF BODE DIAGRAM FOR AMPLITUDE RATIO.
C     PRINT-OUT OF BODE DIAGRAM FOR PHASE LAG.
C
C     PROGRAM SUBROUTINES
C
C     SUBROUTINE SOM.
C     SUBROUTINE RPLOT.
C
C
      PROGRAM PULSE(INPUT,OUTPUT,PUNCH,TAPE5=INPUT,TAPE6=OUT
     1PUT,TAPE7=PUNCH)
C
      DIMENSION X(100),Y(100),IFMT(20)
```

```
      DIMENSION DBTRA(100,2),PHITRA(100,2)
      DIMENSION AR(100),PH(100)
C
    1 FORMAT(6F10.4)
    2 FORMAT(2F20.5)
    3 FORMAT(2I5)
    4 FORMAT(4I5,2F10.3)
    5 FORMAT(F10.4)
    6 FORMAT(//,2X,1HL,7X,1HS,11X,1HW,10X,2HAR,10X,2HPH,9X,
     14HDBAR,8X,4HDPHI)
    7 FORMAT(I4,6F12.5)
    8 FORMAT(1H1)
    9 FORMAT(20A4)
   11 FORMAT(1H1,23X,26HAMPLITUDE RATIO ; DECIBELS)
   12 FORMAT(1H1,23X,19HPHASE LAG ; DEGREES)
   13 FORMAT(3E15.8)
   14 FORMAT(I3)
   15 FORMAT(20X,28HBODE DIAGRAM FROM PULSE TEST)
   16 FORMAT(/10X,24HYOU HAVE A NEGATIVE GAIN)
C
C     READ AND PRINT THE PROGRAM INPUTS
C
      READ(5,9) IFMT
      READ(5,1) WI,WF,DW,TR,TC,TD
      READ(5,2) XS,YS
      READ(5,3) NX,NY
      READ(5,4) N1R,N2R,N1C,N2C,RP,CP
      READ(5,5) (X(I),I=1,NX)
      READ(5,5) (Y(I),I=1,NY)
C
      WRITE(6,8)
      WRITE(6,9) IFMT
      WRITE(6,1) WI,WF,DW,TR,TC,TD
      WRITE(6,2) XS,YS
      WRITE(6,3) NX,NY
      WRITE(6,4) N1R,N2R,N1C,N2C,RP,CP
      WRITE(6,5) (X(I),I=1,NX)
      WRITE(6,5) (Y(I),I=1,NY)
C
C     CALCULATE THE FREQUENCY CONTENT OF THE INPUT SIGNAL AT
C     ZERO FREQUENCY
C
      W=0.0
C
      CALL SQM(UR,0.0,RP,TR,1.0,W,XS,X,N1R,N2R)
C
      SO=ABS(UR)
C
C     CALCULATE THE FREQUENCY CONTENT OF THE INPUT AND OUT-
C     PUT SIGNALS
C
      WRITE(6,8)
      WRITE(6,9) IFMT
      WRITE(6,8)
      W=WI
      PI=3.14159
      A1=0.0
      DO 60 L=1,75
      W=W*10.**DW
      IF(W.GT.WF) GO TO 70
C
```

```
      CALL SOM(UR,0.0,RP,TR,1.0,W,XS,X,N1R,N2R)
      CALL SOM(XR,0.0,RP,TR,0.0,W,XS,X,N1R,N2R)
      CALL SOM(UC,0.0,CP,TC,1.0,W,YS,Y,N1C,N2C)
      CALL SOM(XC,0.0,CP,TC,0.0,W,YS,Y,N1C,N2C)
C
      IGAIN=1
      IF(UC/UR.GT.0.) GO TO 25
      UC=-UC
      XC=-XC
      IGAIN=-1
   25 AR(L)=SQRT((UC**2+XC**2)/(UR**2+XR**2))
      PH(L)=ATAN((UC*XR-XC*UR)/(UC*UR+XC*XR))
C
C     CALCULATE THE PHASE LAG
C
      PRE=UC*UR+XC*XR
      PIM=UC*XR-XC*UR
      A2=ATAN2(PIM,PRE)
      IS=-1
   30 D=ABS(A2-A1)
      IF(D.LT.PI/2.) GO TO 40
      A2=A2+FLOAT(IS)*PI
      D1=ABS(A2-A1)
      IF(D1.GT.D) IS=-IS
      GO TO 30
   40 IF(TD.EQ.0.) GO TO 45
      TPHA=-W*TD*180./PI
      GO TO 46
   45 TPHA=0.0
   46 DPHI=A2*57.2958+TPHA
      A1=A2
C
C     CALCULATE THE AMPLITUDE RATIO
C
      S=SQRT(UR**2+XR**2)/S0
      DBAR=20.*ALOG10(AR(L)/AR(1))
      WRITE(6,7) L,S,W,AR(L),PH(L),DBAR,DPHI
C
C     LOAD THE PLOT MATRIX
C
      DBTRA(L,1)=W
      PHITRA(L,1)=W
      DBTRA(L,2)=DBAR
      PHITRA(L,2)=DPHI
      LMAX=L
      IF(S-0.2) 70,70,60
   60 CONTINUE
   70 CONTINUE
C
C     PERFORATE THE CARDS
C
      WRITE(7,9) IFMT
      WRITE(7,15)
      WRITE(7,14) LMAX
      DO 80 L=1,LMAX
      WRITE(7,13) DBTRA(L,1),DBTRA(L,2),PHITRA(L,2)
   80 CONTINUE
C
      WRITE(7,9) IFMT
      WRITE(7,14) LMAX
      DO 90 L=1,LMAX
```

```
      WRITE(7,13) DBTRA(L,1),AR(L),PHITRA(L,2)
   90 CONTINUE
C
C     PLOT THE BODE DIAGRAMS
C
      IF(IGAIN.EQ.(-1)) WRITE(6,16)
      WRITE(6,11)
      WRITE(6,15)
      WRITE(6,9) IFMT
C
      CALL RPLOT(2,LMAX,DBTRA)
C
      WRITE(6,12)
      WRITE(6,15)
      WRITE(6,9) IFMT
C
      CALL RPLOT(2,LMAX,PHITRA)
C
      STOP
      END
C
C
C
      SUBROUTINE SOM(Z,A,P,B,AI,W,SS,Y,N1S,N2S)
C     ******************************************
C
C     CALCULATE THE INTEGRAL OF F(T)*SIN(W*T)*DT OR
C     F(T)*COS(W*T)*DT BY FILON METHOD.
C
C     REFERENCE  L.N.G. FILON(1928)  PROC.ROY.SOC.EDENBURG,
C     49  38-47.
C
C     Z    = INTEGRAL OF F(T)*SIN(W*T)*DT OR F(T)*COS(W*T)*DT,
C     A    = LOWER LIMIT OF INDEPENDENT VARIABLE T,
C     P    = DURATION OF THE FIRST SECTION OF F(T),
C     B    = UPPER LIMIT OF INDEPENDENT VARIABLE T,
C     AI   = 0.0 FOR SIN, 1.0 FOR COS,
C     W    = FREQUENCY,RADIAN/TIME UNIT,
C     SS   = STEADY STATE VALUE OF F(T),
C     Y    = FUNCTION F(T),
C     N1S  = HALF NUMBER OF DIVISIONS IN THE FIRST SECTION
C            OF F(T),
C     N2S  = HALF NUMBER OF DIVISIONS IN THE SECOND SECTION
C            OF F(T).
C
C
      DIMENSION Y(120)
      RES=0.0
      IN=1
C
C     CALCULATE THE NUMBER OF DATA POINTS FOR EACH SECTION
C
      IF(N1S.LE.0) IN=2
      DO 190 J=IN,2
      IF(J.EQ.2) GO TO 110
      KL=2*N1S+1
      AN=N1S
      JH=1
      GO TO 120
  110 KL=2*(N2S+N1S)+1
      AN=N2S
```

```
      JH=2*N1S+1
  120 CONTINUE
C
C     CALCULATE THE INCREAMENT DT AND THE COEFFICIENTS
C     ALPHA,BETA,GAMMA
C
      DT=(FLOAT(2-J)*(P-A)+FLOAT(J-1)*(B-P))/(2.*AN)
      T=W*DT
      TSQ=T*T
      TCUBE=TSQ*T
      IF(T-0.75) 140,130,130
  130 CONTINUE
C
      SINUST=SIN(T)
      SIN2T=SIN(2.*T)
      COSINT=COS(T)
C
      ALPHA=1./T+SIN2T/(2.*TSQ)-2.*SINUST**2/TCUBE
      BETA=2.*((COSINT**2+1.)/TSQ-SIN2T/TCUBE)
      GAMMA=4.*(SINUST/T-COSINT)/TSQ
      GO TO 150
C
  140 ALPHA=TCUBE*(2./45.+TSQ*(-2./315.+TSQ*(2./4725.)))
      BETA=2./3.+TSQ*(2./15.+TSQ*(-4./105.+TSQ*(2./567.+TSQ*
     1(-4./22275.))))
      GAMMA=4./3.+TSQ*(-2./15.+TSQ*(1./270.+TSQ*(-1./11340.+
     1TSQ*(1./997920.))))
  150 CONTINUE
C
C     CALCULATE THE INTEGRAL
C
      S2N=0.0
      S2N1=0.0
      F1=FLOAT(2-J)*A+FLOAT(J-1)*P
      AIP=AI*1.570796
C
      DO 160 K=JH,KL,2
      FKJH=FLOAT(K-JH)
      AIK=(Y(K)-SS)*SIN(W*(F1+FKJH*DT)+AIP)
  160 S2N=S2N+AIK
C
      KM=KL-1
      JH=JH+1
C
      DO 170 K=JH,KM,2
      FKJH=FLOAT(K-JH)
      AIK=(Y(K)-SS)*SIN(W*(F1+FKJH*DT)+AIP)
  170 S2N1=S2N1+AIK
C
      FKM=FLOAT(KM)
      S2N=S2N-0.5*((Y(JH)-SS)*SIN(W*F1+AIP)+(Y(KL)-SS)*SIN(W
     1*(F1+FKM*DT)+AIP))
C
      FKM=FLOAT(KM)
  190 RES=RES+DT*(ALPHA*((Y(JH)-SS)*COS(W*F1+AIP)-(Y(KL)-SS)
     1*COS(W*(F1+FKM*DT)+AIP))+BETA*S2N+GAMMA*S2N1)
      Z=RES
      RETURN
      END
C
C
```

```
C
      SUBROUTINE RPLOT(M,L,A)
C     **********************
C
C     PLOT THE BODE DIAGRAM ON THE PRINTER.
C
C     M = TOTAL NUMBER OF INDEPENDENT AND DEPENDENT VARIABLES,
C     L = NUMBER OF DATA POINTS,
C     A = VECTOR(MATRIX) OF INDEPENDENT AND DEPENDENT VARIABLE(S).
C
C
      DIMENSION A(100,2),YPR(8)
      INTEGER OUT(71),ANG(11),BLANK
      DATA ANG/1H*,1H1,1H2,1H3,1H4,1H5,1H6,1H7,1H8,1H9,1H0/,
     1BLANK/1H /
C
    2 FORMAT(1H ,F8.4,1X,71A1)
    3 FORMAT(1H ,11X,8(10H.         ))
    4 FORMAT(1H0,1X,8F10.1)
C
C     SCALE THE DEPENDENT VARIABLE(S)
C
      YMIN=A(1,2)
      YMAX=YMIN
C
      DO 40 I=1,L
      DO 40 J=2,M
      IF(A(I,J)-YMIN) 20,10,10
   10 IF(A(I,J)-YMAX) 40,40,30
   20 YMIN=A(I,J)
      GO TO 40
   30 YMAX=A(I,J)
   40 CONTINUE
      YSCAL=(YMAX-YMIN)/70.
C
C     PRINT THE CURVE(S)
C
      DO 70 I=1,L
      DO 50 IX=1,71
   50 OUT(IX)=BLANK
C
      DO 60 J=2,M
      JP=INT((A(I,J)-YMIN)/YSCAL)+1
      OUT(JP)=ANG(J-1)
   60 CONTINUE
   70 WRITE(6,2) A(I,1),(OUT(IZ),IZ=1,71)
C
C     PRINT THE SCALE
C
      WRITE(6,3)
      YPR(1)=YMIN
      DO 80 KN=1,6
   80 YPR(KN+1)=YPR(KN)+YSCAL*10.
      YPR(8)=YMAX
C
      WRITE(6,4) (YPR(IP),IP=1,8)
      RETURN
      END
```

D.2 Program NLREG1

```
C     ********************
C     *                  *
C     *  PROGRAM NLREG1  *
C     *                  *
C     ********************
C
C
C     DIRECT CURVE FITTING FOR TRANSFER FUNCTION
C
C                          1
C          G(S) = ------------------------
C                 (T1*S+1)(T2*S+1)(T3*S+1)
C
C     FROM BODE DIAGRAM (MAGNITUDE VS FREQUENCY) USING NON-
C     LINEAR REGRESSION TECHNIQUE.
C
C     REFERENCE  V.J. LAW, AND R.V. BAILEY (1963)  CHEM.ENG.
C     SCI., 18  189-202.
C
C     PROGRAM INPUTS  DATA CARDS ARE IN THE FOLLOWING ORDER,
C
C     1 SERIE OF CARDS FROM PROGRAM PULSE.
C     1 CARD FOR T(1),TOL,TD,FORMAT 3F10.3
C
C            T(1) = STARTING VALUE FOR THE FIRST PARAMETER,
C            TOL  = TOLERANCE FOR PARAMETERS T(J),
C            TD   = PURE LAG BETWEEN INPUT AND OUTPUT SIGNALS.
C
C     PROGRAM OUTPUTS  CARDS AND PRINT-OUTS ARE IN THE FOL-
C     LOWING ORDER,
C
C     DATA CARDS FOR THE PLOTTER
C
C     1 IDENTIFICATION CARD,FORMAT 20A4.
C     1 TITLE CARD.
C     1 CARD FOR N,FORMAT I3
C
C            N = NUMBER OF POINTS FOR BODE DIAGRAM.
C
C     N CARDS FOR W,XMDB,ARDB,PH,DPHI,FORMAT 5E15.8
C
C            W    = FREQUENCY,RADIAN/TIME UNIT
C            XMDB = AMPLITUDE RATIO FROM PULSE,DECIBELS,
C            ARDB = AMPLITUDE RATIO FROM NLREG1,DECIBELS,
C            PH   = PHASE LAG FROM PULSE,DEGREES,
C            DPHI = PHASE LAG FROM NLREG1,DEGREES.
C
C     PRINT-OUT OF PROGRAM INPUTS.
C     PRINT-OUT OF VECTORS W,XMDB,ARDB,PH,DPHI.
C     PRINT-OUT OF SELECTED ORDER FOR TRANSFER FUNCTION.
C     PRINT-OUT OF BODE DIAGRAMS FOR AMPLITUDE RATIO.
```

```
C     PRINT-OUT OF BODE DIAGRAMS FOR PHASE LAG.
C
C     PROGRAM SUBROUTINES AND FUNCTION SUBPROGRAMS
C
C     SUBROUTINE GMPRD.
C     SUBROUTINE MINV.
C     SUBROUTINE RPLOT.
C     FUNCTION RPHI.
C     FUNCTION RZ.
C
C
      PROGRAM NLREG1(INPUT,OUTPUT,PUNCH,TAPE5=INPUT,TAPE6=OUT
     1PUT,TAPE7=PUNCH)
C
      DIMENSION XAR(100),AR(100),W(100),CAR(100),PH(100)
      DIMENSION SA(3),CSA(3),NSA(3),B(3),KC(3)
      DIMENSION DB(3),NB(3),Z(3),C(3),D(3),T(3),AD(3)
      DIMENSION R(3),L1(3),M1(3),CZ(9),BI(3)
      DIMENSION SPZZ(3,3),A(100,3),IFMT(20),TK(3,3)
      DIMENSION TT(3,3),DPHI(100),Q(100,3)
      REAL NSA,NB
    1 FORMAT(I3)
    2 FORMAT(5E15.8)
    3 FORMAT(5F14.5)
    4 FORMAT(//,5X,25HIF THE SYSTEM ORDER IS = ,I2,/)
    5 FORMAT(10X,11HPARAMETER  ,1HT,I1,2H =,F10.3,/)
    6 FORMAT(//5X,35HF-TEST ON THE SUM OF SQUARES OF THE,
     116H RESIDUAL ERRORS,//)
    7 FORMAT(10X,3HSA(,I1,8H)/SA(3)=,F10.5,5X,3HDF=,I3,1H,,
     1I3,/)
    8 FORMAT(10X,40HSUM OF SQUARES OF THE RESIDUAL ERRORS SA,
     12H =,E10.3)
    9 FORMAT(3F10.3)
   10 FORMAT(3F15,5)
   11 FORMAT(20A4)
   12 FORMAT(3E15.8)
   14 FORMAT(/,5X,24HRESULTS FROM F-TEST ARE ,5X,F4.2,5X,F4.2,
     1//,5X,29HTHE SELECTED SYSTEM ORDER IS ,I2)
   16 FORMAT(1H1)
   18 FORMAT(5X,26H**WARNING**THE PARAMETER T,I2,
     138H IS COMPLEX , THIS IS ITS SQUARE VALUE)
   19 FORMAT(23X,19HPHASE LAG , DEGREES)
   20 FORMAT(23X,26HAMPLITUDE RATIO , DECIBELS)
   21 FORMAT(9X,37HBODE DIAGRAMS FROM PROGRAMS PULSE AND,
     17H NLREG1)
   22 FORMAT(10X,22HNUMBER OF ITERATION = ,I3)
   23 FORMAT(9X,18HNO CHOICE WAS MADE)
   24 FORMAT(///,7X,1HW,13X,4HXMDB,10X,4HARDB,10X,2HPH,12X,
     14HDPHI,//)
C
C     READ AND PRINT PROGRAM INPUTS
C
      READ(5,11) IFMT
      READ(5,1) N
      READ(5,12) (W(K),XAR(K),PH(K),K=1,N)
      READ(5,9) T(1),TOL,TO
C
      WRITE(6,16)
      WRITE(6,11) IFMT
      WRITE(6,1) N
      WRITE(6,10) (W(K),XAR(K),PH(K),K=1,N)
```

```
      WRITE(6,9) T(1),TOL,TD
      WRITE(6,16)
C
C     CALCULATE THE NORMALIZED MAGNITUDES
C
      RAR=XAR(1)
      DO 30 K=1,N
   30 XAR(K)=XAR(K)/RAR
C
C     CALCULATE THE SQUARES OF MAGNITUDES
C
      DO 40 I=1,N
      AR(I)=XAR(I)**2
   40 CONTINUE
C
C     INITIALIZE THE B(K)
C
      B(1)=T(1)**2
      B(2)=0.
      B(3)=0.
      KC(1)=0
      KC(2)=0
      KC(3)=0
      DO 200 J=1,3
      IF(J.GT.1) B(J)=B(J-1)/2.
C
C     CALCULATE THE SUM OF SQUARES
C
      SA(J)=0.0
      DO 50 K=1,N
      PW2=W(K)**2
      CAR(K)=RPHI(J,B,PW2)
      SA(J)=SA(J)+(AR(K)-CAR(K))**2
   50 CONTINUE
      DO 170 I=1,10
      IMAX=I
      ALPHA=1.0
      BETA=0.25
C
C     CALCULATE THE MATRIX Z AND THE VECTOR C
C
      DO 52 M=1,J
      DO 51 L=1,J
   51 SPZZ(L,M)=0.0
   52 C(M)=0.0
      DO 65 K=1,N
      PW2=W(K)**2
      DO 55 KL=1,J
      Z(KL)=RZ(J,KL,B,PW2)
   55 CONTINUE
      DO 60 M=1,J
      DO 59 L=1,J
   59 SPZZ(L,M)=SPZZ(L,M)+Z(L)*Z(M)
   60 C(M)=C(M)+(AR(K)-CAR(K))*Z(M)
   65 CONTINUE
      IF(J.EQ.1) GO TO 90
      DO 70 M=1,J
      AC(M)=C(M)
   70 CONTINUE
      KL=1
      DO 75 M=1,J
```

```
      DO 75 L=1,J
      CZ(KL)=SPZZ(L,M)
      KL=KL+1
   75 CONTINUE
C
C     INVERT THE MATRIX Z AND THE VECTOR B
C
      CALL MINV(CZ,J,DD,L1,M1)
      CALL GMPRD(CZ,AC,R,J,J,1)
      DO 80 M=1,J
      DB(M)=R(M)
   80 CONTINUE
      GO TO 95
   90 DB(1)=C(1)/SPZZ(1,1)
   95 CONTINUE
C
C     CALCULATE D(K)
C
      DT=0.0
      DO 100 M=1,J
      D(M)=DB(M)*C(M)
      DT=DT+D(M)
  100 CONTINUE
C
C     CHECK IF DT IS POSITIVE
C
      IF(DT.GE.0.0) GO TO 120
      DT=-DT
      DO 110 M=1,J
      DB(M)=-DB(M)
  110 CONTINUE
  120 CONTINUE
  125 CONTINUE
C
C     CALCULATE NEW B(K)
C
      DO 130 M=1,J
      BI(M)=ALPHA*DB(M)
      B(M)=B(M)+BI(M)
  130 CONTINUE
C
C     TEST FOR THE FINAL RESULTS
C
      DO 132 K=1,J
      IF(ABS(BI(K)).GT.TOL) GO TO 135
  132 CONTINUE
      GO TO 180
  135 CONTINUE
C
C     EVALUATE NEW SUMS OF SQUARES
C
      NSA(J)=0.0
      DO 140 K=1,N
      PW2=W(K)**2
      CAR(K)=RPHI(J,NB,PW2)
      NSA(J)=NSA(J)+(AR(K)-CAR(K))**2
  140 CONTINUE
C
C     USE CRITERIA FOR RAPID CONVERGENCE
C
      DSA(J)=SA(J)-NSA(J)
```

```
      CRITER=CSA(J)-BETA*DT*ALPHA*(2.0-ALPHA)
      IF(CRITER.GE.0.0) GO TO 150
      ALPHA=ALPHA/2.
      GO TO 125
  150 SA(J)=NSA(J)
      DO 160 M=1,J
      B(M)=NB(M)
  160 CONTINUE
  170 CONTINUE
  180 CONTINUE
      WRITE(6,11) IFMT
      WRITE(6,4) J
      DO 181 K=1,3
      TT(J,K)=0.0
  181 CONTINUE
      DO 185 M=1,J
      IF(B(M).GT.0.) GO TO 182
      KC(J)=1
      T(M)=B(M)
      WRITE(6,18) M
      GO TO 185
  182 T(M)=B(M)**0.5
      TT(J,M)=T(M)
  185 CONTINUE
C
C     PRINT THE RESULTS
C
      DO 190 M=1,J
      WRITE(6,5) M,T(M)
  190 CONTINUE
      WRITE(6,8) SA(J)
      WRITE(6,22) IMAX
      DO 195 JOR=1,J
      TK(J,JOR)=T(JOR)
  195 CONTINUE
  200 CONTINUE
C
C     PERFORM THE STATISTIC TESTS
C
      WRITE(6,6)
      R2=SA(2)/SA(3)
      R3=SA(1)/SA(3)
      NN1=N-1
      NN2=N-2
      NN3=N-3
      LM1=2
      LM3=1
      WRITE(6,7) LM1,R2,NN2,NN3
      WRITE(6,7) LM3,R3,NN1,NN3
      ANN1=NN1
      ANN2=NN2
      ANN3=NN3
      F2=-0.05*ANN3-0.003*(ANN1-40.0)+3.68
      F1=-0.05*ANN3-0.003*(ANN1-40.0)+3.68
      IF(R2.LT.F1) GO TO 210
      K=3
      IF(KC(K).NE.1) GO TO 230
  210 IF(R3.LT.F2) GO TO 220
      K=2
      IF(KC(K).NE.1) GO TO 230
  220 K=1
```

```
      IF(KC(I).EQ.1) WRITE(6,23)
  230 CONTINUE
      WRITE(6,14) F1,F2,K
C
C     CALCULATE THE PHASE LAG
C
      J=K
      PHIO=0.0
      PI=3.14159
      DO 235 I=1,N
      PHI=-ATAN(W(I)*TT(J,1))-ATAN(W(I)*TT(J,2))-ATAN(W(I)*
     1TT(J,3))
      IS=-1
  231 D=ABS(PHI-PHIO)
      IF(D.LT.PI/2.) GO TO 232
      PHI=PHI+FLOAT(IS)*PI
      D1=ABS(PHI-PHIO)
      IF(D1.GT.D) IS=-IS
      GO TO 231
  232 IF(TD.EQ.0.) GO TO 233
      TPHA=-W(I)*TD*180.0/PI
      GO TO 234
  233 TPHA=0.0
  234 DPHI(I)=PHI*57.2958+TPHA
      PHIO=PHI
  235 CONTINUE
C
C     LOAD THE PLOT MATRIX
C
      WRITE(6,16)
      WRITE(6,11) IFMT
      WRITE(6,24)
C
      PW1=W(1)**2
      DO 238 J=1,N
      PW2=W(J)**2
      A(J,1)=W(J)
      DO 236 L=1,K
  236 B(L)=TK(K,L)**2
      RPHI1=RPHI(K,B,PW1)
      A(J,2)=20.*ALOG10(SQRT(RPHI(K,B,PW2)/RPHI1))
      A(J,3)=20.*ALOG10(XAR(J))
      C(J,1)=W(J)
      C(J,2)=PH(J)
      C(J,3)=DPHI(J)
      WRITE(6,3) A(J,1),A(J,3),A(J,2),PH(J),DPHI(J)
  238 CONTINUE
C
C     PERFORATE THE CARDS
C
      WRITE(7,11) IFMT
      WRITE(7,21)
      WRITE(7,1) N
      DO 240 J=1,N
      WRITE(7,2) A(J,1),A(J,3),A(J,2),PH(J),DPHI(J)
  240 CONTINUE
C
C     PLOT THE BODE DIAGRAMS
C
      WRITE (6,16)
      WRITE(6,20)
```

```
      WRITE (6,21)
      WRITE(6,11) IFMT
C
      CALL RPLOT(3,N,A)
C
      WRITE(6,16)
      WRITE(6,19)
      WRITE(6,21)
      WRITE(6,11) IFMT
C
      CALL RPLOT(3,N,Q)
C
      STOP
      END
C
C
C
      SUBROUTINE GMPRD(A,B,R,N,M,L)
C     *****************************
C
C     MULTIPLY TWO GENERAL MATRICES TO FORM A RESULTANT GE-
C     NERAL MATRIX.
C
C     A = NAME OF FIRST INPUT MATRIX,
C     B = NAME OF SECOND INPUT MATRIX,
C     R = NAME OF OUTPUT MATRIX,
C     N = NUMBER OF ROWS IN A,
C     M = NUMBER OF COLUMNS IN A OR ROWS IN B,
C     L = NUMBER OF COLUMNS IN B.
C
C     REMARK  ALL MATRICES MUST BE STORED AS GENERAL MATRI-
C     CES, MATRIX R CANNOT BE IN THE SAME LOCATION AS MATRIX
C     A OR MATRIX B.
C
C
      DIMENSION A(1),B(1),R(1)
C
      IR=0
      IK=-M
      DO 10 K=1,L
      IK=IK+M
      DO 10 J=1,N
      IR=IR+1
      JI=J-N
      IB=IK
      R(IR)=0
      DO 10 I=1,M
      JI=JI+N
      IB=IB+1
   10 R(IR)=R(IR)+A(JI)*B(IB)
      RETURN
      END
C
C
      SUBROUTINE MINV(A,N,D,L,M)
C     **************************
C
C     INVERT A GENERAL MATRIX.
C
C     REFERENCE  THE STANDARD GAUSS-JORDAN METHOD IS USED.
```

```
C     THE DETERMINANT IS ALSO CALCULATED. A DETERMINANT OF
C     ZERO INDICATES THAT THE MATRIX IS SINGULAR.
C
C     A = INPUT MATRIX, DESTROYED IN COMPUTATION AND REPLACED
C        BY RESULTANT INVERSE.
C     N = ORDER OF MATRIX A,
C     D = RESULTANT DETERMINANT,
C     L = WORK VECTOR OF LENGTH N,
C     M = WORK VECTOR OF LENGTH N.
C
      DIMENSION A(1),L(1),M(1)
C
C     SEARCH FOR LARGEST ELEMENT
C
      D=1.0
      NK=-N
      DO 80 K=1,N
      NK=NK+N
      L(K)=K
      M(K)=K
      KK=NK+K
      BIGA=A(KK)
      DO 20 J=K,N
      IZ=N*(J-1)
      DO 20 I=K,N
      IJ=IZ+I
   10 IF(ABS(BIGA)-ABS(A(IJ))) 15,20,20
   15 BIGA=A(IJ)
      L(K)=I
      M(K)=J
   20 CONTINUE
C
C     INTERCHANGE ROWS
C
      J=L(K)
      IF(J-K) 35,35,25
   25 KI=K-N
      DO 30 I=1,N
      KI=KI+N
      HOLD=-A(KI)
      JI=KI-K+J
      A(KI)=A(JI)
   30 A(JI)=HOLD
C
C     INTERCHANGE COLUMNS
C
   35 I=M(K)
      IF(I-K) 45,45,38
   38 JP=N*(I-1)
      DO 40 J=1,N
      JK=NK+J
      JI=JP+J
      HOLD=-A(JK)
      A(JK)=A(JI)
   40 A(JI)=HOLD
C
C     DIVIDE COLUMN BY MINUS PIVOT (VALUE OF PIVOT ELEMENT
C     IS CONTAINED IN BIGA)
C
   45 IF(BIGA) 48,46,48
   46 D=0.0
```

```
      RETURN
   48 DO 55 I=1,N
      IF(I-K) 50,55,50
   50 IK=NK+I
      A(IK)=A(IK)/(-BIGA)
   55 CONTINUE
C
C     REDUCE MATRIX
C
      DO 65 I=1,N
      IK=NK+I
      IJ=I-N
      DO 65 J=1,N
      IJ=IJ+N
      IF(I-K) 60,65,60
   60 IF(J-K) 62,65,62
   62 KJ=IJ-I+K
      A(IJ)=A(IK)*A(KJ)+A(IJ)
   65 CONTINUE
C
C     DIVIDE ROW BY PIVOT
C
      KJ=K-N
      DO 75 J=1,N
      KJ=KJ+N
      IF(J-K) 70,75,70
   70 A(KJ)=A(KJ)/BIGA
   75 CONTINUE
C
C     PRODUCT OF PIVOTS
C
      D=D*BIGA
C
C     REPLACE PIVOT BY RECIPROCAL
C
      A(KK)=1.0/BIGA
   80 CONTINUE
C
C     REINTERCHANGE ROW AND COLUMN
C
      K=N
  100 K=(K-1)
      IF(K) 150,150,105
  105 I=L(K)
      IF(I-K) 120,120,108
  108 JQ=N*(K-1)
      JR=N*(I-1)
      DO 110 J=1,N
      JK=JQ+J
      HOLD=A(JK)
      JI=JR+J
      A(JK)=-A(JI)
  110 A(JI)=HOLD
  120 J=M(K)
      IF(J-K) 100,100,125
  125 KI=K-N
      DO 130 I=1,N
      KI=KI+N
      HOLD=A(KI)
      JI=KI-K+J
      A(KI)=-A(JI)
```

```
  130 A(JI)=HOLD
      GO TO 100
  150 RETURN
      END
C
C
C
      SUBROUTINE RPLOT(M,L,A)
C     ***********************
C
C     PLOT THE BODE DIAGRAM ON THE PRINTER.
C
C     M = TOTAL NUMBER OF INDEPENDENT AND DEPENDENT VARIABLES,
C     L = NUMBER OF DATA POINTS,
C     A = VECTOR(MATRIX) OF INDEPENDENT AND DEPENDENT VARIABLE(S).
C
C
      DIMENSION A(100,3),YPR(8)
      INTEGER OUT(71),ANG(11),BLANK
      DATA ANG/1H*,1H1,1H2,1H3,1H4,1H5,1H6,1H7,1H8,1H9,1H0/,
     1 BLANK/1H /
C
    2 FORMAT(1H ,F8.4,1X,71A1)
    3 FORMAT(1H ,11X,8(10H.  ))
    4 FORMAT(1H0,1X,8F10.1)
C
C     SCALE THE DEPENDENT VARIABLE(S)
C
      YMIN=A(1,2)
      YMAX=YMIN
C
      DO 40 I=1,L
      DO 40 J=2,M
      IF(A(I,J)-YMIN) 20,10,10
   10 IF(A(I,J)-YMAX) 40,40,30
   20 YMIN=A(I,J)
      GO TO 40
   30 YMAX=A(I,J)
   40 CONTINUE
      YSCAL=(YMAX-YMIN)/70.
C
C     PRINT THE CURVE(S)
C
      DO 70 I=1,L
      DO 50 IX=1,71
   50 OUT(IX)=BLANK
C
      DO 60 J=2,M
      JP=INT((A(I,J)-YMIN)/YSCAL)+1
      OUT(JP)=ANG(J-1)
   60 CONTINUE
   70 WRITE(6,2) A(I,1),(OUT(IZ),IZ=1,71)
C
C     PRINT THE SCALE
C
      WRITE(6,3)
      YPR(1)=YMIN
      DO 80 KN=1,6
   80 YPR(KN+1)=YPR(KN)+YSCAL*10.
      YPR(8)=YMAX
C
```

```
      WRITE(6,4) (YPR(IP),IP=1,8)
      RETURN
      END
C
C
C
      FUNCTION RPHI(J,B,PW2)
C     **********************
C
C     CALCULATE THE SQUARE OF MAGNITUDE OF TRANSFER FUNCTION.
C
C     J   = ORDER OF TRANSFER FUNCTION,
C     B   = SQUARE OF PARAMETER T,
C     PW2 = SQUARE OF FREQUENCY.
C
      DIMENSION B(3)
C
      RPHI=1.
      DO 10 I=1,J
      RPHI=RPHI/(B(I)*PW2+1.)
   10 CONTINUE
      RETURN
      END
C
C
C
      FUNCTION RZ(J,KL,B,PW2)
C     ***********************
C
C     CALCULATE Z(J) FOR MATRIX Z.
C
C     J   = ORDER OF TRANSFER FUNCTION,
C     KL  = ORDER OF TRANSFER FUNCTION (KL=1,J),
C     B   = SQUARE OF PARAMETER T,
C     PW2 = SQUARE OF FREQUENCY.
C
      DIMENSION A(3),B(3)
C
      DO 10 I=1,J
      A(I)=1./(B(I)*PW2+1.)
   10 CONTINUE
      RZ=-PW2
      DO 20 I=1,J
      RZ=RZ*A(I)
   20 CONTINUE
      RZ=RZ*A(KL)
      RETURN
      END
```

D.3 Program LNREG2

```
C     **********
C     *        *
C     *  PROGRAM  LNREG2  *
C     *        *
C     **********
C
C
C     DIRECT CURVE FITTING FOR TRANSFER FUNCTION
C
C                                  2     3
C          (                BO+B1*S+B2*S +B3*S
C        G(S) = ------------------------------
C                                  2     3
C                       AO+A1*S+A2*S +A3*S
C
C     FROM BODE DIAGRAMS USING LINEAR REGRESSION TECHNIQUE.
C
C     REFERENCE  E.C. LEVY (1959)  IRE TRANSACTIONS ON AUTO-
C     MATIC CONTROL, AC-4  37-43.
C
C     PROGRAM INPUTS  DATA CARDS ARE IN THE FOLLOWING ORDER,
C
C     1 SERIE OF CARDS FROM P[redacted] PULSE.
C     1 CARD FOR TD,FORMAT F[redacted]
C
C           TD = PURE LAG B[redacted]NPUT AND OUTPUT SIGNALS.
C
C     PROGRAM OUTPUTS  CARDS [redacted]NT-OUTS ARE IN THE FOL-
C     LOWING ORDER,
C
C     DATA CARDS FOR THE PLOTTER
C
C     1 IDENTIFICATION CARD,FORMAT 20A4.
C     1 TITLE CARD.
C     1 CARD FOR N,FORMAT I3
C
C           N = NUMBER OF POINTS FOR BODE DIAGRAM.
C
C     N CARDS FOR W,XMDB,ARDB,PH,DPHI,FORMAT 5E15.8
C
C           W    = FREQUENCY,RADIAN/TIME UNIT,
C           XMDB = AMPLITUDE RATIO FROM PULSE,DECIBELS,
C           ARDB = AMPLITUDE RATIO FROM LNREG2,DECIBELS,
C           PH   = PHASE LAG FROM PULSE,DEGREES,
C           DPHI = PHASE LAG FROM LNREG2,DEGREES.
C
C     PRINT-OUT OF PROGRAM INPUTS.
C     PRINT-OUT OF VECTORS W,XMDB,ARDB,PH,DPHI.
C     PRINT-OUT OF TRANSFER FUNCTION COEFFICIENTS.
C     PRINT-OUT OF BODE DIAGRAMS FOR AMPLITUDE RATIO.
C     PRINT-OUT OF BODE DIAGRAMS FOR PHASE LAG.
```

```
C
C     PROGRAM SUBROUTINES
C
C     SUBROUTINE MINV.
C     SUBROUTINE POLRT.
C     SUBROUTINE RPLOT.
C
C
C
      PROGRAM LNREG2(INPUT,OUTPUT,PUNCH,TAPE5=INPUT,TAPE6=OUT
     1PUT,TAPE7=PUNCH)
C
      DIMENSION A(7,7),B(7),BB(7),IX(7),IY(7)
      DIMENSION W(100),XM(100),PH(100),IFMT(20)
      DIMENSION ARDB(100),DPHI(100),XMDB(100)
      DIMENSION ARPL(100,3),PHPL(100,3)
      DIMENSION COF(4),AAA(4),BBB(4),RRA(3),RIA(3),RRB(3),RIB(3)
      DIMENSION XMM(100),AR(100)
    1 FORMAT(I3)
    2 FORMAT(3F15.5)
    3 FORMAT(3E15.8)
    4 FORMAT(15X,1HB,I1,3H = ,F10.5,6X,1HA,I1,3H = ,F10.5)
    5 FORMAT(F10.4)
    6 FORMAT(9X,6HZ    = ,F10.5,4X,6HBB1  = ,F10.5,4X,6HBB2 = ,
     1F10.5)
    7 FORMAT(9X,6HZ1   = ,F10.5,4X,6HZ2   = ,F10.5,4X,6HZ3  = ,
     1F10.5)
    8 FORMAT(9X,6HP    = ,F10.5,4X,6HAA1  = ,F10.5,4X,6HAA2 = ,
     1F10.5)
    9 FORMAT(9X,6HP1   = ,F10.5,4X,6HP2   = ,F10.5,4X,6HP3  = ,
     1F10.5)
   11 FORMAT(20A4)
   12 FORMAT(1H1,23X,26HAMPLITUDE RATIO , DECIBELS)
   13 FORMAT(1H1,23X,19HPHASE LAG , DEGREES)
   15 FORMAT(9X,37HBODE DIAGRAMS FROM PROGRAMS PULSE AND,
     17H LNREG2)
   16 FORMAT(1H1)
   17 FORMAT(5E15.8)
   18 FORMAT(5F14.5)
   19 FORMAT(///,9X,36HRESULTANT TRANSFER FUNCTION UNDER PO,
     114HLYNOMIALS FORM,//,41X,1H2,5X,1H3,/,29X,9HB0+B1*S+B,
     29H2*S +B3*S,/,22X,26HG(S) = ----------,/,41X,
     31H2,5X,1H3,/,29X,18HA0+A1*S+A2*S +A3*S,///)
   20 FORMAT(///,9X,36HRESULTANT TRANSFER FUNCTION UNDER PO,
     114HLES-ZEROS FORM,//,29X,18H(S-Z1)(S-Z2)(S-Z3),/,22X,
     225HG(S) = ----------,/,29X,15H(S-P1)(S-P2)(S-,
     33HP3),//,36X,1H2,/,29X,19H(S-Z)(S +BB1*S+BB2),/,9X,
     42HOR,11X,26HG(S) = ----------,/,36X,1H2,/,
     529X,19H(S-P)(S +AA1*S+AA2),///)
   21 FORMAT(///,7X,1HW,13X,4HXMDB,10X,4HARDB,10X,2HPH,12X,
     14HDPHI,//)
C
C     READ AND PRINT PROGRAM INPUTS
C
      READ(5,11) IFMT
      READ(5,1) N
      READ(5,3) (W(I),XM(I),PH(I),I=1,N)
      READ(5,5) TD
C
      WRITE(6,16)
      WRITE(6,11) IFMT
```

```
      WRITE(6,1) N
      WRITE(6,2) (W(I),XM(I),PH(I),I=1,N)
      WRITE(6,5) TD
      WRITE(6,16)
C
C     CALCULATE THE POLYNOMIALS COEFFICIENTS
C
      PI=3.14159
      DO 24 I=1,7
      DO 24 J=1,7
   24 A(I,J)=0.
      B(1)=0.
      DO 25 K=1,N
      XX=PH(K)*PI/180.
      ZM=XM(K)*SIN(XX)
      RE=XM(K)*COS(XX)
      A(3,1)=A(3,1)+W(K)**2
      A(5,1)=A(5,1)+ZM*W(K)
      A(6,1)=A(6,1)+RE*W(K)**2
      A(6,2)=A(6,2)+ZM*W(K)**3
      A(6,3)=A(6,3)-RE*W(K)**4
      A(3,3)=A(3,3)-W(K)**4
      A(5,5)=A(5,5)+XM(K)*XM(K)*W(K)**2
      A(6,6)=A(6,6)+XM(K)*XM(K)*W(K)**4
      A(4,4)=A(4,4)-W(K)**6
      A(7,3)=A(7,3)-ZM*W(K)**5
      A(7,4)=A(7,4)-RE*W(K)**6
      A(7,7)=A(7,7)-XM(K)*XM(K)*W(K)**6
   25 B(1)=B(1)+RE
      A(1,3)=-A(3,1)
      A(2,2)=A(3,1)
      A(4,2)=-A(3,3)
      A(2,4)=A(3,3)
      A(5,3)=-A(6,2)
      A(7,1)=A(6,2)
      A(6,4)=A(7,3)
      A(5,2)=-A(6,1)
      A(7,2)=A(6,3)
      A(5,4)=-A(6,3)
      A(1,5)=A(5,1)
      A(3,5)=A(6,2)
      A(2,6)=A(6,2)
      A(1,7)=-A(6,2)
      A(4,6)=-A(7,3)
      A(3,7)=A(7,3)
      A(2,5)=-A(6,1)
      A(1,6)=A(6,1)
      A(4,5)=A(6,3)
      A(3,6)=-A(6,3)
      A(3,7)=-A(6,3)
      A(7,5)=A(6,6)
      A(5,7)=-A(6,6)
      A(1,1)=FLOAT(N)
      B(2)=A(4,1)
      B(3)=A(5,1)
      B(4)=A(6,2)
      B(5)=0.0
      B(6)=A(5,5)
      B(7)=0.0
C
      CALL MINV(A,7,D,IX,IY)
```

```
C
      DO 46 I=1,7
      BB(I)=0.
      DO 46 J=1,7
   46 BB(I)=BB(I)+A(I,J)*B(J)
      DO 60 I=1,4
      AAA(I)=BB(I)
   60 CONTINUE
      BBB(1)=1.0
      DO 61 I=2,4
      BBB(I)=BB(I+3)
   61 CONTINUE
C
      CALL POLRT(AAA,COF,3,RRA,RIA,IER)
      CALL POLRT(BBB,CCF,3,RRB,RIB,IER)
C
C     CALCULATE THE AMPLITUDE RATIO
C
      XM1=XM(1)
      PHIO=0.0
      DO 70 I=1,N
      WG=W(I)
      AG=BB(1)-BB(3)*WG**2
      BG=WG*(BB(2)-BB(4)*WG**2)
      CG=1.0-BB(6)*WG**2
      DG=WG*(BB(5)-BB(7)*WG**2)
      REG=(AG*CG+BG*DG)/(CG**2+DG**2)
      ZIMG=(BG*CG-AG*DG)/(CG**2+DG**2)
      AR(I)=SQRT(REG**2+ZIMG**2)
C
C     CALCULATE THE NORMALIZED MAGNITUDES
C
      XMM(I)=XM(I)/XM1
      XMDB(I)=20.0*ALOG10(XMM(I))
C
C     CALCULATE THE PHASE LAG
C
      PHI=ATAN(ZIMG/REG)
      IS=-1
   65 D=ABS(PHI-PHIO)
      IF(D.LT.PI/2.) GO TO 66
      PHI=PHI+FLOAT(IS)*PI
      D1=ABS(PHI-PHIO)
      IF(D1.GT.D) IS=-IS
      GO TO 65
   66 IF(TD.EQ.0.) GO TO 68
      TPHA=-WG*TD*180.0/PI
      GO TO 69
   68 TPHA=0.0
   69 DPHI(I)=PHI*57.2958+TPHA
      PHIO=PHI
   70 CONTINUE
C
C     LOAD THE PLOT MATRIX
C
      DO 75 I=1,N
      ARDB(I)=20.0*ALOG10(AR(I)/AR(1))
      ARPL(I,1)=W(I)
      ARPL(I,2)=XMDB(I)
      ARPL(I,3)=ARDB(I)
      PHPL(I,1)=W(I)
```

```
      PHPL(I,2)=PH(I)
      PHPL(I,3)=DPHI(I)
   75 CONTINUE
C
C     PERFORATE THE CARDS
C
      WRITE(7,11) IFMT
      WRITE(7,15)
      WRITE(7,1) N
      DO 80 I=1,N
      WRITE(7,17) W(I),XMDB(I),ARDB(I),PH(I),DPHI(I)
   80 CONTINUE
C
C     PRINT THE RESULTS
C
      WRITE(6,11) IFMT
      WRITE(6,19)
      DO 90 I=1,4
      II=I-1
   90 WRITE(6,4) II,AAA(I),II,BBB(I)
      WRITE(6,20)
      IF(RIA(1).EQ.0.0) GO TO 98
      BB1=-2.0*RRA(1)
      BB2=RRA(1)**2+RIA(1)**2
      IF(RIA(2).EQ.0.0) GO TO 93
      Z=RRA(3)
      GO TO 94
   93 Z=RRA(2)
      GO TO 94
   94 WRITE(6,6) Z,BB1,BB2
      GO TO 110
   98 IF(RIA(2).EQ.0.0) GO TO 106
      Z=RRA(1)
      BB1=-2.0*RRA(2)
      BB2=RRA(2)**2+RIA(2)**2
      WRITE(6,6) Z,BB1,BB2
      GO TO 110
  106 Z1=RRA(1)
      Z2=RRA(2)
      Z3=RRA(3)
      WRITE(6,7) Z1,Z2,Z3
      GO TO 110
  110 IF(RIB(1).EQ.0.0) GO TO 118
      AA1=-2.0**RRB(1)
      AA2=RRB(1)**2+RIB(1)**2
      IF(RIB(2).EQ.0.0) GO TO 113
      P=RRB(3)
      GO TO 114
  113 P=RRB(2)
      GO TO 114
  114 WRITE(6,8) P,AA1,AA2
      GO TO 130
  118 IF(RIB(2).EQ.0.0) GO TO 126
      P=RRB(1)
      AA1=-2.0*RRB(2)
      AA2=RRB(2)**2+RIB(2)**2
      WRITE(6,8) P,AA1,AA2
      GO TO 130
  126 P1=RRB(1)
      P2=RRB(2)
      P3=RRB(3)
```

```
      WRITE(6,9) P1,P2,P3
      GO TO 130
  130 WRITE(6,16)
      WRITE(6,11) IFMT
      WRITE(6,21)
      DO 135 I=1,N
      WRITE (6,18) W(I),XMDB(I),ARDB(I),PH(I),DPHI(I)
  135 CONTINUE
C
C     PLOT THE BODE DIAGRAMS
C
      WRITE(6,12)
      WRITE(6,15)
      WRITE(6,11) IFMT
C
      CALL RPLOT(3,N,ARPL)
C
      WRITE(6,13)
      WRITE(6,15)
      WRITE(6,11) IFMT
C
      CALL RPLOT(3,N,PHPL)
C
      STOP
      END
C
C
C
      SUBROUTINE MINV(A,N,D,L,M)
C     **************************
C
C     INVERT A GENERAL MATRIX.
C
C     REFERENCE  THE STANDARD GAUSS-JORDAN METHOD IS USED.
C     THE DETERMINANT IS ALSO CALCULATED. A DETERMINANT OF
C     ZERO INDICATES THAT THE MATRIX IS SINGULAR.
C
C     A = INPUT MATRIX, DESTROYED IN COMPUTATION AND REPLACED
C       BY RESULTANT INVERSE.
C     N = ORDER OF MATRIX A,
C     D = RESULTANT DETERMINANT,
C     L = WORK VECTOR OF LENGTH N,
C     M = WORK VECTOR OF LENGTH N.
C
      DIMENSION A(1),L(1),M(1)
C
C     SEARCH FOR LARGEST ELEMENT
C
      D=1.0
      NK=-N
      DO 80 K=1,N
      NK=NK+N
      L(K)=K
      M(K)=K
      KK=NK+K
      BIGA=A(KK)
      DO 20 J=K,N
      IZ=N*(J-1)
      DO 20 I=K,N
      IJ=IZ+I
   10 IF(ABS(BIGA)-ABS(A(IJ))) 15,20,20
```

```
   15 BIGA=A(IJ)
      L(K)=I
      M(K)=J
   20 CONTINUE
C
C     INTERCHANGE ROWS
C
      J=L(K)
      IF(J-K) 35,35,25
   25 KI=K-N
      DO 30 I=1,N
      KI=KI+N
      HOLD=-A(KI)
      JI=KI-K+J
      A(KI)=A(JI)
   30 A(JI)=HOLD
C
C     INTERCHANGE COLUMNS
C
   35 I=M(K)
      IF(I-K) 45,45,38
   38 JP=N*(I-1)
      DO 40 J=1,N
      JK=NK+J
      JI=JP+J
      HOLD=-A(JK)
      A(JK)=A(JI)
   40 A(JI)=HOLD
C
C     DIVIDE COLUMN BY MINUS PIVOT (VALUE OF PIVOT ELEMENT
C     IS CONTAINED IN BIGA)
C
   45 IF(BIGA) 48,46,48
   46 D=0.0
      RETURN
   48 DO 55 I=1,N
      IF(I-K) 50,55,50
   50 IK=NK+I
      A(IK)=A(IK)/(-BIGA)
   55 CONTINUE
C
C     REDUCE MATRIX
C
      DO 65 I=1,N
      IK=NK+I
      IJ=I-N
      DO 65 J=1,N
      IJ=IJ+N
      IF(I-K) 60,65,60
   60 IF(J-K) 62,65,62
   62 KJ=IJ-I+K
      A(IJ)=A(IK)*A(KJ)+A(IJ)
   65 CONTINUE
C
C     DIVIDE ROW BY PIVOT
C
      KJ=K-N
      DO 75 J=1,N
      KJ=KJ+N
      IF(J-K) 70,75,70
   70 A(KJ)=A(KJ)/BIGA
```

```
   75 CONTINUE
C
C     PRODUCT OF PIVOTS
C
      D=D*BIGA
C
C     REPLACE PIVOT BY RECIPROCAL
C
      A(KK)=1.0/BIGA
   80 CONTINUE
C
C     REINTERCHANGE ROW AND COLUMN
C
      K=N
  100 K=(K-1)
      IF(K) 150,150,105
  105 I=L(K)
      IF(I-K) 120,120,108
  108 JQ=N*(K-1)
      JR=N*(I-1)
      DO 110 J=1,N
      JK=JQ+J
      HOLD=A(JK)
      JI=JR+J
      A(JK)=-A(JI)
  110 A(JI)=HOLD
  120 J=M(K)
      IF(J-K) 100,100,125
  125 KI=K-N
      DO 130 I=1,N
      KI=KI+N
      HOLD=A(KI)
      JI=KI-K+J
      A(KI)=-A(JI)
  130 A(JI)=HOLD
      GO TO 100
  150 RETURN
      END
C
C
C
      SUBROUTINE POLRT(XCOF,COF,M,ROOTR,ROOTI,IER)
C     ********************************************
C
C     COMPUTE THE REAL AND COMPLEX ROOTS OF A REAL POLYNOMIAL.
C
C     XCOF   = VECTOR OF M+1 COEFFICIENTS OF THE POLYNOMIAL
C              ORDERED FROM SMALLEST TO LARGEST POWER,
C     COF    = WORKING VECTOR OF LENGTH M+1,
C     M      = ORDER OF POLYNOMIAL, MAXIMUM 36,
C     ROOTR  = RESULTANT VECTOR OF LENGTH M CONTAINING REAL
C              ROOTS OF THE POLYNOMIAL,
C     ROOTI  = RESULTANT VECTOR OF LENGTH M CONTAINING THE
C              CORRESPONDING IMAGINARY ROOTS OF THE POLYNOMIAL,
C     IER    = ERROR CODE WHERE
C                0 = NO ERROR,
C                1 = M LESS THAN 1;
C                2 = M GREATER THAN 36,
C                3 = UNABLE TO DETERMINE ROOT WITH 500 ITERATIONS
C                    ON 5 STARTING VALUES,
C                4 = HIGH ORDER COEFFICIENT IS ZERO.
```

```
C
C
      DIMENSION XCOF(1),COF(1),ROOTR(1),ROOTI(1)
      IFIT=0
      N=M
      IER=0
      IF(XCOF(N+1)) 10,25,10
   10 IF(N) 15,15,32
C
C     SET ERROR CODE TO 1
C
   15 IER=1
   20 RETURN
C
C     SET ERROR CODE TO 4
C
   25 IER=4
      GO TO 20
C
C     SET ERROR CODE TO 2
C
   30 IER=2
      GO TO 20
   32 IF(N-36) 35,35,30
   35 NX=N
      NXX=N+1
      N2=1
      KJ1=N+1
      DO 40 L=1,KJ1
      MT=KJ1-L+1
   40 COF(MT)=XCOF(L)
C
C     SET INITIAL VALUES
C
   45 XC=.005C0101
      YC=.010C0101
C
C     ZERO INITIAL VALUE COUNTER
C
      IN=0
   50 X=XO
C
C     INCREMENT INITIAL VALUES AND COUNTER
C
      XC=-10.0*YO
      YC=-10.0*X
C
C     SET X AND Y TO CURRENT VALUE
C
      X=XO
      Y=YO
      IN=IN+1
      GO TO 59
   55 IFIT=1
      XPR=X
      YPR=Y
C
C     EVALUATE POLYNOMIAL AND DERIVATIVES
C
   59 ICT=0
   60 UX=0.0
```

```
      UY=0.0
      V=0.0
      YT=0.0
      XT=1.0
      U=COF(N+1)
      IF(U) 65,130,65
   65 DO 70 I=1,N
      L=N-I+1
      XT2=X*XT-Y*YT
      YT2=X*YT+Y*XT
      U=U+COF(L)*XT2
      V=V+COF(L)*YT2
      FI=I
      UX=UX+FI*XT*COF(L)
      UY=UY-FI*YT*COF(L)
      XT=XT2
   70 YT=YT2
      SUMSQ=UX*UX+UY*UY
      IF(SUMSQ) 75,110,75
   75 DX=(V*UY-U*UX)/SUMSQ
      X=X+DX
      DY=-(U*UY+V*UX)/SUMSQ
      Y=Y+DY
   78 IF(ABS(DY)+ABS(DX)-1.0E-05) 100,80,80
C
C     STEP ITERATION COUNTER
C
   80 ICT=ICT+1
      IF(ICT-500) 60,85,85
   85 IF(IFIT) 100,90,100
   90 IF(IN-5) 50,95,95
C
C     SET ERROR CODE TO 3
C
   95 IER=3
      GO TO 20
  100 DO 105 L=1,NXX
      MT=KJ1-L+1
      TEMP=XCOF(MT)
      XCOF(MT)=COF(L)
  105 COF(L)=TEMP
      ITEMP=N
      N=NX
      NX=ITEMP
      IF(IFIT) 120,55,120
  110 IF(IFIT) 115,50,115
  115 X=XPR
      Y=YPR
  120 IFIT=0
      IF(X) 122,125,122
  122 IF(ABS(Y)-ABS(X)*1.0E-04) 135,125,125
  125 ALPHA=X+X
      SUMSQ=X*X+Y*Y
      N=N-2
      GO TO 140
  130 X=0.0
      NX=NX-1
      NXX=NXX-1
  135 Y=0.0
      SUMSQ=0.0
      ALPHA=X
```

```
      N=N-1
  140 L1=1
      L2=2
      CCF(L2)=COF(L2)+ALPHA*COF(L1)
  145 DO 150 L=2,N
  150 CCF(L+1)=COF(L+1)+ALPHA*COF(L)-SUMSQ*COF(L-1)
  155 RCOTI(N2)=Y
      RCOTR(N2)=X
      N2=[illegible]
      IF([illegible]) 160,165,160
  160 Y=-Y
      SUMSQ=0.0
      GO TO 155
  165 IF(N) 20,20,45
      END
C
C
C
      SUBROUTINE RPLOT(M,L,A)
C     ***********************
C
C     PLOT THE BODE DIAGRAM ON THE PRINTER.
C
C     M = TOTAL NUMBER OF INDEPENDENT AND DEPENDENT VARIABLES.
C     L = NUMBER OF DATA POINTS.
C     A = VECTOR(MATRIX) OF INDEPENDENT AND DEPENDENT VARIABLE(S).
C
C
      DIMENSION A(100,3),YPR(8)
      INTEGER OUT(71),ANG(11),BLANK
      DATA ANG/1H*,1H1,1H2,1H3,1H4,1H5,1H6,1H7,1H8,1H9,1HO/,
     1BLANK/1H /
C
    2 FORMAT(1H ,F8.4,1X,71A1)
    3 FORMAT(1H ,11X,8(10H         ))
    4 FORMAT(1HO,1X,8F10.1)
C
C     SCALE THE DEPENDENT VARIABLE(S)
C
      YMIN=A(1,2)
      YMAX=YMIN
C
      DO 40 I=1,L
      DO 40 J=2,M
      IF(A(I,J)-YMIN) 20,10,10
   10 IF(A(I,J)-YMAX) 40,40,30
   20 YMIN=A(I,J)
      GO TO 40
   30 YMAX=A(I,J)
   40 CONTINUE
      YSCAL=(YMAX-YMIN)/70.
C
C     PRINT THE CURVE(S)
C
      DO 70 I=1,L
      DO 50 IX=1,71
   50 OUT(IX)=BLANK
C
      DO 60 J=2,M
      JP=INT((A(I,J)-YMIN)/YSCAL)+1
      OUT(JP)=ANG(J-1)
```

```
   60 CCNTINUE
   70 WRITE(6,2) A(I,1),(OUT(IZ),IZ=1,71)
C
C     PRINT THE SCALE
C
      WRITE(6,3)
      YPR(1)=YMIN
      CC 80 KN=1,6
   80 YPR(KN+1)=YPR(KN)+YSCAL*10.
      YPR(8)=YMAX
C
      WRITE(6,4) (YPR(IP),IP=1,8)
      RETURN
      END
```

D.4 Testing of Computer Programs

In order to test the validity of the computer programs, a system of a known transfer function

$$G(s) = \frac{1}{(2s + 1)(5s + 1)} \qquad (D.1)$$

was simulated on the TR-10 Analog Computer (Fig. D.1). The unit half-sine input signal was also simulated on the same computer (Fig. D.1) and both input and output signals were recorded on strip chart recorders (Fig. D.2). These data were truncated as shown in Fig. D.2 and used to feed the computer programs. The result from the program PULSE is shown in Table D.1 and also compared with the theoretical Bode diagram (amplitude ratio) in Fig. D.3 for the transfer function in Eq. (D.1). The print-outs from the programs NLREG1 and LNREG2 are shown in Tables D.2 and D.3. The resulting transfer functions are,

$$G(s) = \frac{1}{(2.157s + 1)(4.736s + 1)} \qquad (D.2)$$

from the program NLREG1 and,

$$G(s) = \frac{(s-3.164)(s^2-1.137s + 0.703)}{(s+0.197)(s-0.238)(s+2.531)} \qquad (D.3)$$

from the program LNREG2 respectively.

Figure D.1 Computer Circuit for Analog Simulation of the Unit Half-Sine Function and the System $G(s) = 1/(2s + 1)(5s + 1)$.

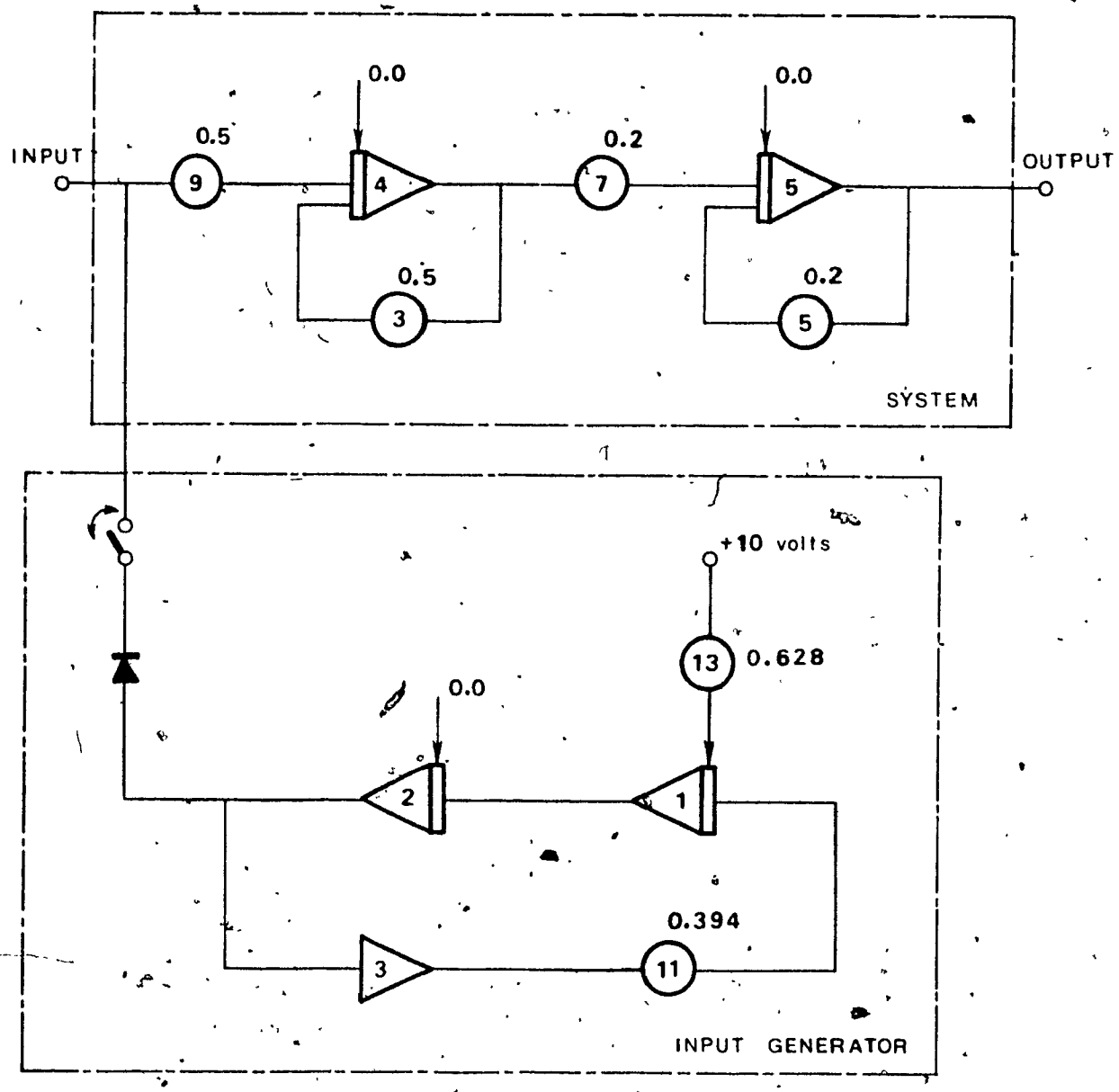

INPUT
0.5
9
0.0
4
0.5
3
0.2
7
0.0
5
0.2
5
OUTPUT
SYSTEM
+10 volts
13
0.628
0.0
2
1
0.394
3
11
INPUT GENERATOR

Figure D.2 Input and Output From the Simulated System $G(s) = 1/(2s + 1)(5s + 1)$: (A) Input and (B) Output.

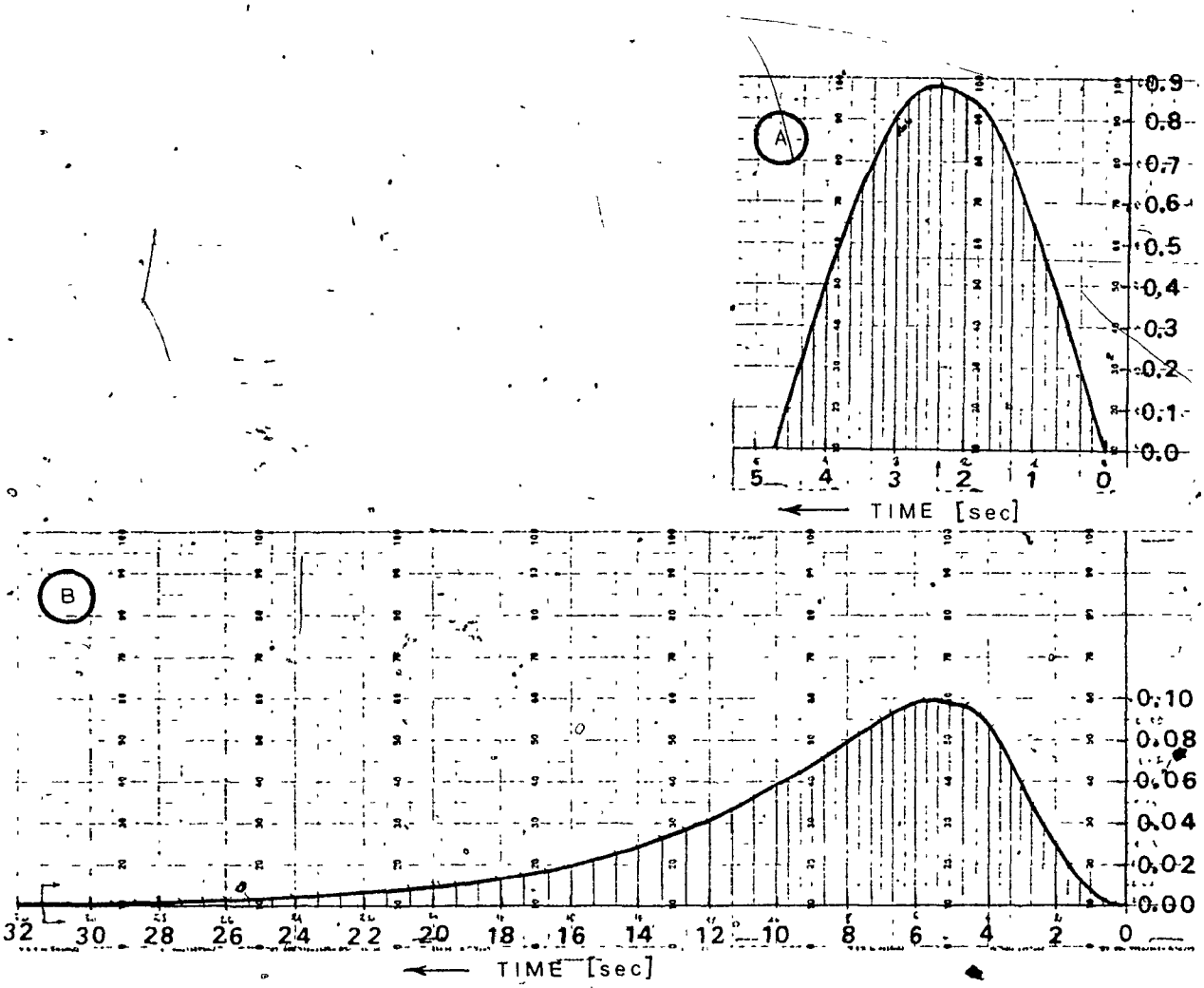

A
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5
4
3
2
1
0
TIME [sec]
B
0.10
0.08
0.06
0.04
0.02
0.00
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
TIME [sec]

Table D.1 Typical Print-Out From the Program PULSE

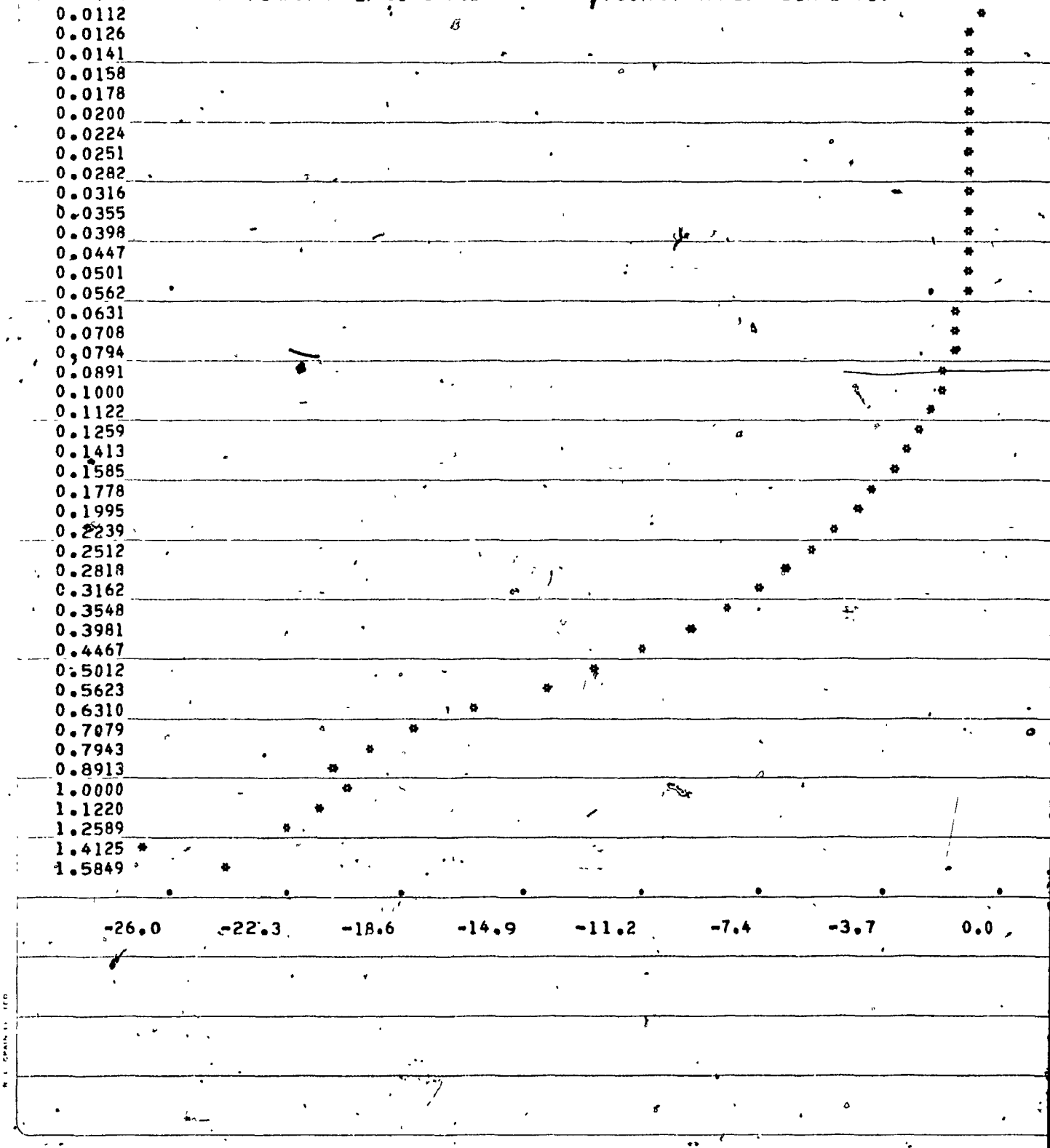
```
TEST THE PROGRAM  ,INPUT=HALF-SINE PULSE        ,OUTPUT=ANALOG SIMULATION
   0.0100   10.0000     0.0500     4.6667   31.3334     0.0000
            0.00000                0.00000
 29   63
  0   14   15   16      0.000      10.000
    0.0050
    0.1000
    0.1900
    0.2800
    0.3700
    0.4500
    0.5400
    0.6200
    0.6850
    0.7450
    0.7900
    0.8300
    0.8600
    0.8750
    0.8850
    0.8850
    0.8700
    0.8400
    0.8000
    0.7540
    0.6900
    0.6300
    0.5600
    0.4800
    0.4000
    0.3000
    0.2100
    0.1000
    0.0200
    0.0000
    0.0010
    0.0030
    0.0070
    0.0120
    0.0200
    0.0290
    0.0380
    0.0490
    0.0600
    0.0680
    0.0780
    0.0860
    0.0920
    0.0960
    0.0980
    0.0990
    0.0990
    0.0980
    0.0950
    0.0920
    0.0900
```

0.0860
0.0830
0.0790
0.0750
0.0710
0.0680
0.0650
0.0610
0.0580
0.0520
0.0460
0.0410
0.0360
0.0320
0.0280
0.0250
0.0220
0.0190
0.0170
0.0150
0.0130
0.0120
0.0110
0.0090
0.0080
0.0070
0.006
0.0060
0.0050
0.0040
0.0035
0.0032
0.0030
0.0026
0.0023
0.0020
0.0019
0.0018
0.0017
0.0016
0.0015

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION

L	S	W	AR	PH	DBAR	DPHI
1	0.99994	0.01122	0.36390	-0.07131	0.00000	-4.08567
2	0.99992	0.01259	0.36375	-0.08000	-0.00360	-4.58358
3	0.99990	0.01413	0.36356	-0.08974	-0.00812	-5.14199
4	0.99987	0.01585	0.36332	-0.10067	-0.01382	-5.76817
5	0.99984	0.01778	0.36302	-0.11293	-0.02099	-6.47024
6	0.99979	0.01995	0.36264	-0.12666	-0.03002	-7.25725
7	0.99974	0.02239	0.36217	-0.14206	-0.04138	-8.13926
8	0.99967	0.02512	0.36157	-0.15930	-0.05567	-9.12743
9	0.99959	0.02818	0.36082	-0.17862	-0.07366	-10.23411
10	0.99948	0.03162	0.35988	-0.20024	-0.09630	-11.47289
11	0.99935	0.03548	0.35871	-0.22443	-0.12477	-12.85863
12	0.99918	0.03981	0.35723	-0.25146	-0.16057	-14.40750
13	0.99897	0.04467	0.35539	-0.28164	-0.20557	-16.13688
14	0.99871	0.05012	0.35308	-0.31530	-0.26210	-18.06514
15	0.99837	0.05623	0.35021	-0.35275	-0.33306	-20.21136
16	0.99795	0.06310	0.34664	-0.39435	-0.42200	-22.59466
17	0.99742	0.07079	0.34223	-0.44040	-0.53330	-25.23327
18	0.99675	0.07943	0.33680	-0.49119	-0.67221	-28.14313
19	0.99591	0.08913	0.33017	-0.54691	-0.84493	-31.33585
20	0.99485	0.10000	0.32214	-0.60765	-1.05856	-34.81605
21	0.99352	0.11220	0.31256	-0.67332	-1.32085	-38.57830
22	0.99185	0.12589	0.30130	-0.74358	-1.63962	-42.60421
23	0.98975	0.14125	0.28833	-0.81788	-2.02189	-46.86113
24	0.98711	0.15849	0.27374	-0.89543	-2.47270	-51.30466
25	0.98379	0.17783	0.25779	-0.97541	-2.99408	-55.88689
26	0.97963	0.19953	0.24084	-1.05714	-3.58494	-60.56957
27	0.97440	0.22387	0.22329	-1.14036	-4.24208	-65.33748
28	0.96786	0.25119	0.20553	-1.22545	-4.96189	-70.21291
29	0.95966	0.28184	0.18782	-1.31393	-5.74482	-75.28270
30	0.94941	0.31623	0.16997	-1.40856	-6.61234	-80.70464
31	0.93662	0.35481	0.15119	-1.50940	-7.62922	-86.48211
32	0.92070	0.39811	0.13137	1.53545	-8.84964	-92.02516
33	0.90094	0.44668	0.11272	1.45321	-10.17934	-96.73735
34	0.87651	0.50119	0.09621	1.37034	-11.55527	-101.48521
35	0.84643	0.56234	0.07963	1.28461	-13.19776	-106.39705
36	0.80963	0.63096	0.06278	1.23155	-15.26367	-109.43739
37	0.76495	0.70795	0.05044	1.27511	-17.16406	-106.94149
38	0.71123	0.79433	0.04255	1.29226	-18.64242	-105.95901
39	0.64745	0.89125	0.03677	1.38984	-19.90950	-100.36786
40	0.57294	1.00000	0.03825	1.37382	-19.56776	-101.28583
41	0.48774	1.12202	0.03575	1.20975	-20.15451	-110.68633
42	0.39300	1.25893	0.03091	0.80287	-21.41716	-133.99902
43	0.29155	1.41254	0.01816	0.01958	-26.03569	-178.87822
44	0.18842	1.58489	0.02476	0.60177	-23.34377	-145.52081

AMPLITUDE RATIO , DECIBELS
BODE DIAGRAM FROM PULSE TEST
TEST THE PROGRAM , INPUT=HALF-SINE PULSE , OUTPUT=ANALOG SIMULATION



PHASE LAG , DEGREES

BODE DIAGRAM FROM PULSE TEST

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION

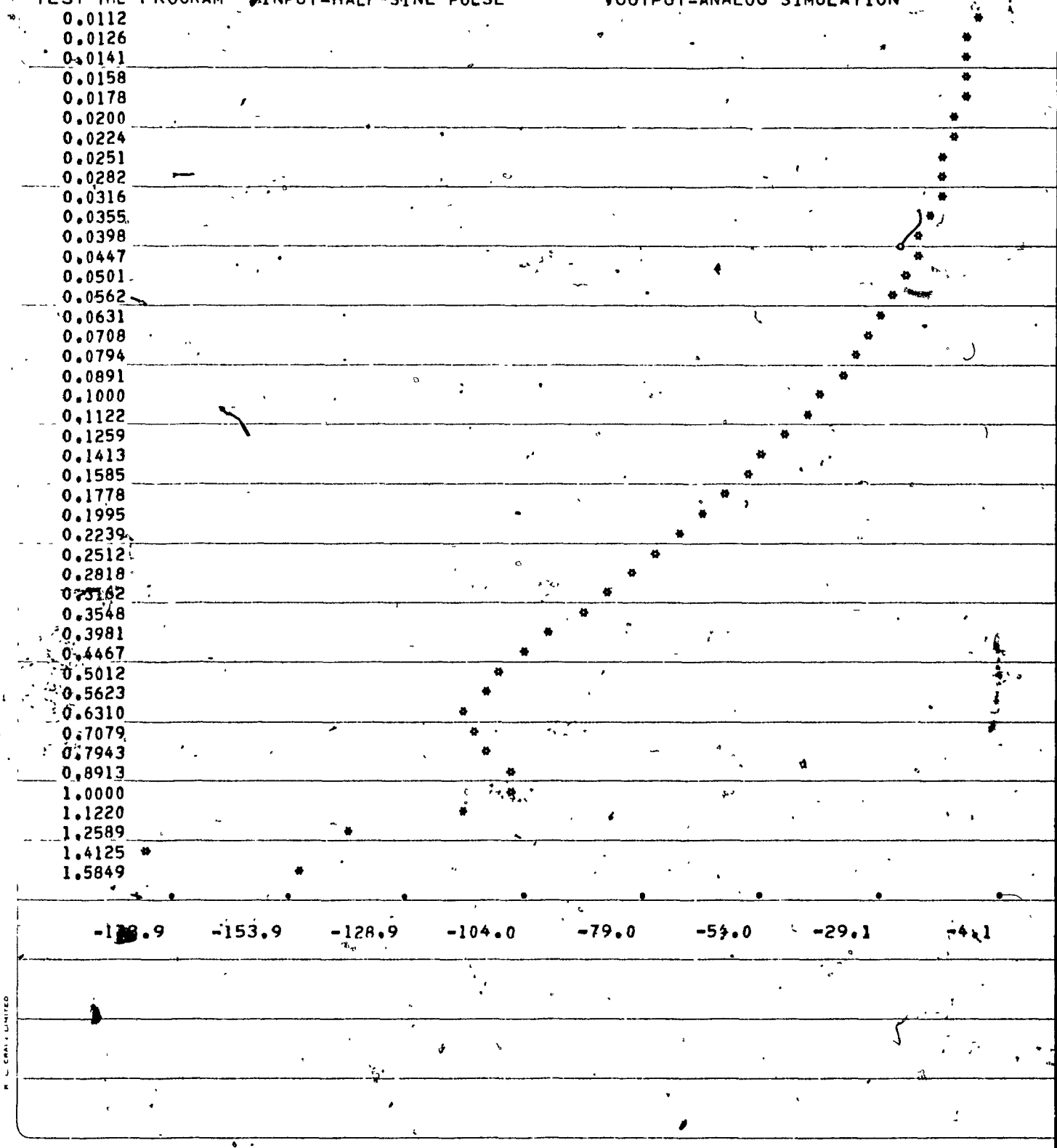


Figure D.3 Bode Diagrams (Amplitude Ratio) of Theoretical and Simulated System $G(s) = 1/(2s + 1)(5s + 1)$.

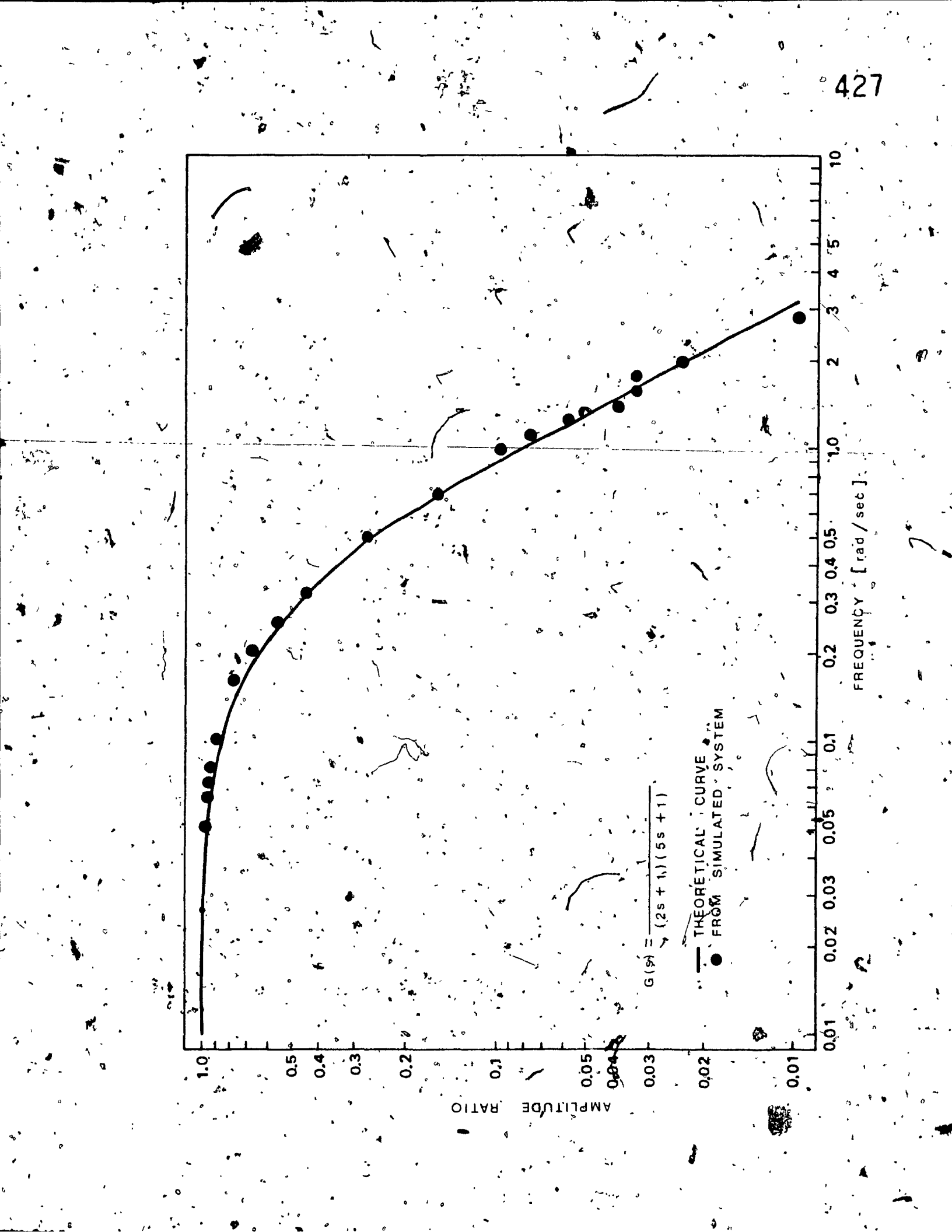

AMPLITUDE RATIO
1.0
0.5
0.4
0.3
0.2
0.1
0.05
0.04
0.03
0.02
0.01
FREQUENCY [rad/sec]
0.01
0.02
0.03
0.05
0.1
0.2
0.3
0.4
0.5
1.0
2
3
4
5
10
THEORETICAL CURVE
FROM SIMULATED SYSTEM
G(s) = 1 / (2s + 1)(5s + 1)

Table D.2 Typical Print-Out From the Program NLREG1

```
TEST THE PROGRAM  ,INPUT=HALF-SINE PULSE        ,OUTPUT=ANALOG SIMULATION
44
        0.01122         0.36390        -4.08567
        0.01259         0.36875        -4.58358
        0.01413         0.36356        -5.14199
        0.01585         0.36332        -5.76817
        0.01778         0.36302        -6.47024
        0.01995         0.36264        -7.25725
        0.02239         0.36217        -8.13926
        0.02512         0.36157        -9.12743
        0.02818         0.36082       -10.23411
        0.03162         0.35988       -11.47289
        0.03548         0.35871       -12.85863
        0.03981         0.35723       -14.40750
        0.04467         0.35539       -16.13688
        0.05012         0.35308       -18.06514
        0.05623         0.35021       -20.21136
        0.06310         0.34664       -22.59466
        0.07079         0.34223       -25.23327
        0.07943         0.33680       -28.14313
        0.08913         0.33017       -31.33585
        0.10000         0.32214       -34.81605
        0.11220         0.31256       -38.57830
        0.12589         0.30130       -42.60421
        0.14125         0.28833       -46.86113
        0.15849         0.27374       -51.30466
        0.17783         0.25779       -55.88689
        0.19953         0.24084       -60.56957
        0.22387         0.22329       -65.33748
        0.25119         0.20553       -70.21291
        0.28184         0.18782       -75.28270
        0.31623         0.16997       -80.70464
        0.35481         0.15119       -86.48211
        0.39811         0.13137       -92.02516
        0.44668         0.11272       -96.73735
        0.50119         0.09621      -101.48521
        0.56234         0.07963      -106.39705
        0.63096         0.06278      -109.43739
        0.70795         0.05044      -106.94149
        0.79433         0.04255      -105.95901
        0.89125         0.03677      -100.36786
        1.00000         0.03825      -101.28583
        1.12202         0.03575      -110.68633
        1.25893         0.03091      -133.99902
        1.41254         0.01816      -178.87822
        1.58489         0.02476      -145.52081
    5.000       0.001       0.000
```

```
TEST THE PROGRAM  ,INPUT=HALF-SINE PULSE        ,OUTPUT=ANALOG SIMULATION

    IF THE SYSTEM ORDER IS =  1

        PARAMETER  T1 =     5.661

        SUM OF SQUARES OF THE RESIDUAL ERRORS SA = 0.198E-01
        NUMBER OF ITERATION =   5
TEST THE PROGRAM  ,INPUT=HALF-SINE PULSE        ,OUTPUT=ANALOG SIMULATION

    IF THE SYSTEM ORDER IS =  2

        PARAMETER  T1 =     4.736

        PARAMETER  T2 =     2.157

        SUM OF SQUARES OF THE RESIDUAL ERRORS SA = 0.938E-03
        NUMBER OF ITERATION =   7
TEST THE PROGRAM  ,INPUT=HALF-SINE PULSE        ,OUTPUT=ANALOG SIMULATION

    IF THE SYSTEM ORDER IS =  3

    **WARNING**THE PARAMETER T 3 IS COMPLEX , THIS IS ITS SQUARE VALUE
        PARAMETER  T1 =     4.688

        PARAMETER  T2 =     2.278

        PARAMETER  T3 =    -0.191

        SUM OF SQUARES OF THE RESIDUAL ERRORS SA = 0.930E-03
        NUMBER OF ITERATION =  10

    F-TEST ON THE SUM OF SQUARES OF THE RESIDUAL ERRORS

        SA(2)/SA(3)=    1.00882      DF= 42, 41

        SA(1)/SA(3)=   21.29769      DF= 43, 41

    RESULTS FROM F-TEST ARE:      1.62      1.62

    THE SELECTED SYSTEM ORDER IS: 2
```

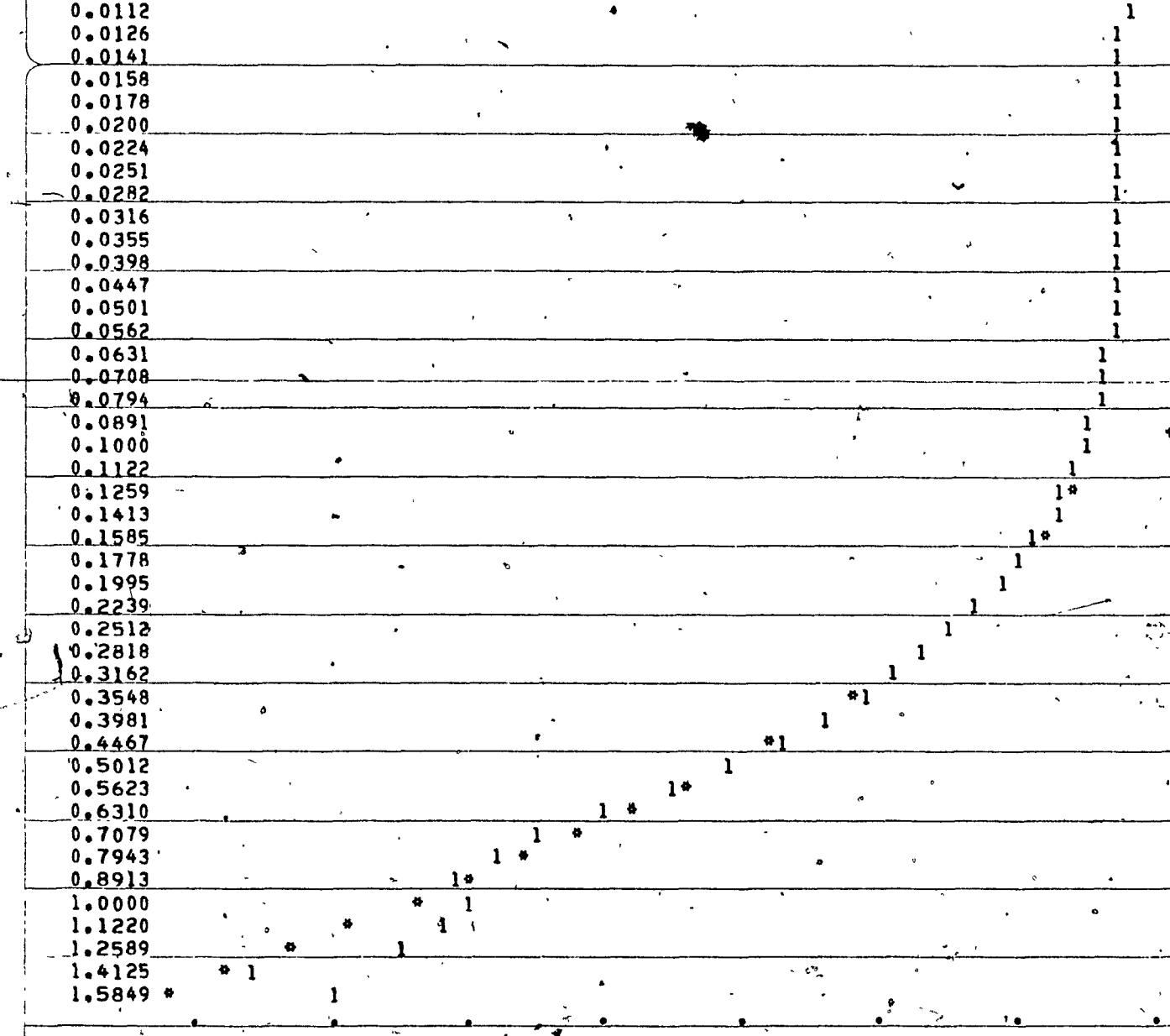
TEST THE PROGRAM *,INPUT=HALF-SINE PULSE *,OUTPUT=ANALOG SIMULATION

W	XMDB	ARDB	PH	DPHI
0.01122	0.00000	0.00000	-4.08567	-4.42807
0.01259	-0.00360	-0.00382	-4.58358	-4.96747
0.01413	-0.00812	-0.00863	-5.14199	-5.57231
0.01585	-0.01382	-0.01468	-5.76817	-6.25043
0.01778	-0.02099	-0.02229	-6.47024	-7.01055
0.01995	-0.03002	-0.03185	-7.25725	-7.86237
0.02239	-0.04138	-0.04387	-8.13926	-8.81666
0.02512	-0.05567	-0.05897	-9.12743	-9.88532
0.02818	-0.07366	-0.07792	-10.23411	-11.08146
0.03162	-0.09630	-0.10169	-11.47289	-12.41949
0.03548	-0.12477	-0.13149	-12.85863	-13.91506
0.03981	-0.16057	-0.16881	-14.40750	-15.58514
0.04467	-0.20557	-0.21546	-16.13688	-17.44787
0.05012	-0.26210	-0.27368	-18.06514	-19.52244
0.05623	-0.33306	-0.34621	-20.21136	-21.82875
0.06310	-0.42200	-0.43632	-22.59466	-24.38704
0.07079	-0.53330	-0.54793	-25.23327	-27.21720
0.07943	-0.67221	-0.68564	-28.14313	-30.33799
0.08913	-0.84493	-0.85480	-31.33585	-33.76591
0.10000	-1.05856	-1.06148	-34.81605	-37.51399
0.11220	-1.32085	-1.31245	-38.57830	-41.59030
0.12589	-1.63962	-1.61502	-42.60421	-45.99650
0.14125	-2.02189	-1.97687	-46.86113	-50.72652
0.15849	-2.47270	-2.40579	-51.30466	-55.76552
0.17783	-2.99408	-2.90930	-55.88689	-61.08942
0.19953	-3.58494	-3.49439	-60.56957	-66.66511
0.22387	-4.24208	-4.16713	-65.33748	-72.45128
0.25119	-4.96189	-4.93244	-70.21291	-78.39981
0.28184	-5.74482	-5.79389	-75.28270	-84.45750
0.31623	-6.61234	-6.75361	-80.70464	-90.56783
0.35481	-7.62922	-7.81222	-86.48211	-96.67275
0.39811	-8.84964	-8.96882	-92.02516	-102.71437
0.44668	-10.17934	-10.22101	-96.73735	-108.63665
0.50119	-11.55527	-11.56495	-101.48521	-114.38721
0.56234	-13.19776	-12.99550	-106.39705	-119.91905
0.63096	-15.26367	-14.50638	-109.43739	-125.19231
0.70795	-17.16406	-16.09049	-106.94149	-130.17549
0.79433	-18.64242	-17.74023	-105.95901	-134.84625
0.89125	-19.90950	-19.44782	-100.36786	-139.19143
1.00000	-19.56776	-21.20561	-101.28583	-143.20647
1.12202	-20.15451	-23.00639	-110.68633	-146.89435
1.25893	-21.41716	-24.84350	-133.99902	-150.26415
1.41254	-26.03569	-26.71102	-178.87822	-153.32965
1.58489	-23.34377	-28.60373	-145.52081	-156.10788

AMPLITUDE RATIO , DECIBELS

BODE DIAGRAMS FROM PROGRAMS PULSE AND NLREG1

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE , ,OUTPUT=ANALOG SIMULATION



PHASE LAG , DEGREES

BODE DIAGRAMS FROM PROGRAMS PULSE AND NLREG1

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION

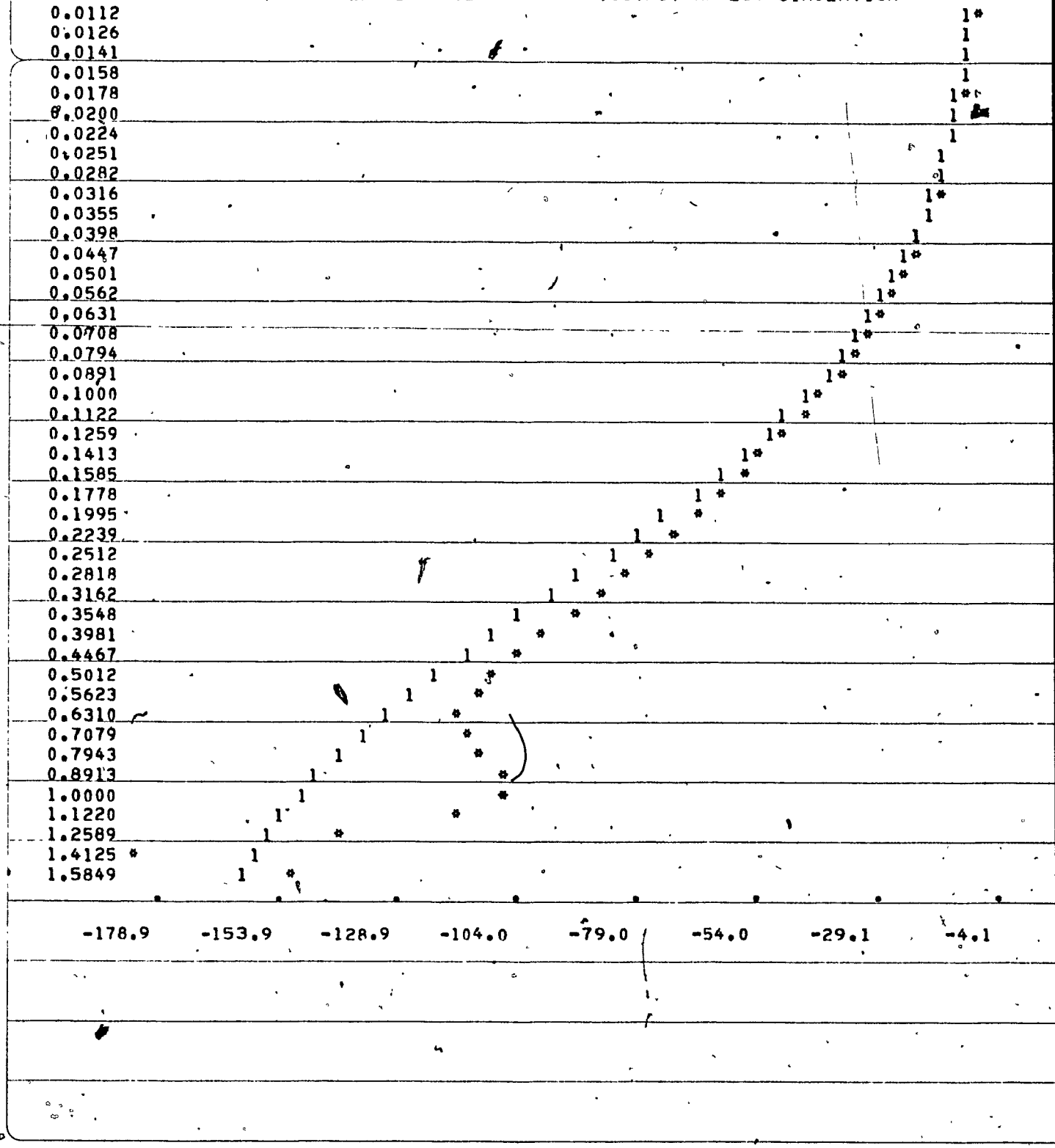


Table D.3 Typical Print-Out From the Program LNREG2

```
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44
        0.01122         0.36390        -4.08567
        0.01259         0.36375        -4.58358
        0.01413         0.36356        -5.14199
        0.01585         0.36332        -5.76817
        0.01778         0.36302        -6.47024
        0.01995         0.36264        -7.25725
        0.02239         0.36217        -8.13926
        0.02512         0.36157        -9.12743
        0.02818         0.36082       -10.23411
        0.03162         0.35988       -11.47289
        0.03548         0.35871       -12.85863
        0.03981         0.35723       -14.40750
        0.04467         0.35539       -16.13688
        0.05012         0.35308       -18.06514
        0.05623         0.35021       -20.21136
        0.06310         0.34664       -22.59466
        0.07079         0.34223       -25.23327
        0.07943         0.33680       -28.14313
        0.08913         0.33017       -31.33585
        0.10000         0.32214       -34.81605
        0.11220         0.31256       -38.57830
        0.12589         0.30130       -42.60421
        0.14125         0.28833       -46.86113
        0.15849         0.27374       -51.30466
        0.17783         0.25779       -55.88689
        0.19953         0.24084       -60.56957
        0.22387         0.22329       -65.33748
        0.25119         0.20553       -70.21291
        0.28184         0.18782       -75.28270
        0.31623         0.16997       -80.70464
        0.35481         0.15119       -86.48211
        0.39811         0.13137       -92.02516
        0.44668         0.11272       -96.73735
        0.50119         0.09621      -101.48521
        0.56234         0.07963      -106.39705
        0.63096         0.06278      -109.43739
        0.70795         0.05044      -106.94149
        0.79433         0.04255      -105.95901
        0.89125         0.03677      -100.36786
        1.00000         0.03825      -101.28583
        1.12202         0.03575      -110.68633
        1.25893         0.03091      -133.99902
        1.41254         0.01816      -178.87822
        1.58489         0.02476      -145.52081
  0.0000
```

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION

RESULTANT TRANSFER FUNCTION UNDER POLYNOMIALS FORM

$$G(S) = \frac{B0+B1*S+B2*S^2+B3*S^3}{A0+A1*S+A2*S^2+A3*S^3}$$

B0 =	0.45118	A0 =	1.00000
B1 =	-0.87265	A1 =	1.25075
B2 =	0.87281	A2 =	-20.97845
B3 =	-0.20292	A3 =	-8.42205

RESULTANT TRANSFER FUNCTION UNDER POLES-ZEROS FORM

$$G(S) = \frac{(S-Z1)(S-Z2)(S-Z3)}{(S-P1)(S-P2)(S-P3)}$$

OR

$$G(S) = \frac{(S-Z)(S^2+BB1*S+BB2)}{(S-P)(S^2+AA1*S+AA2)}$$

Z =	3.16431	BB1 =	-1.13701	BB2 =	0.70267
P1 =	-0.19745	P2 =	0.23759	P3 =	-2.53104

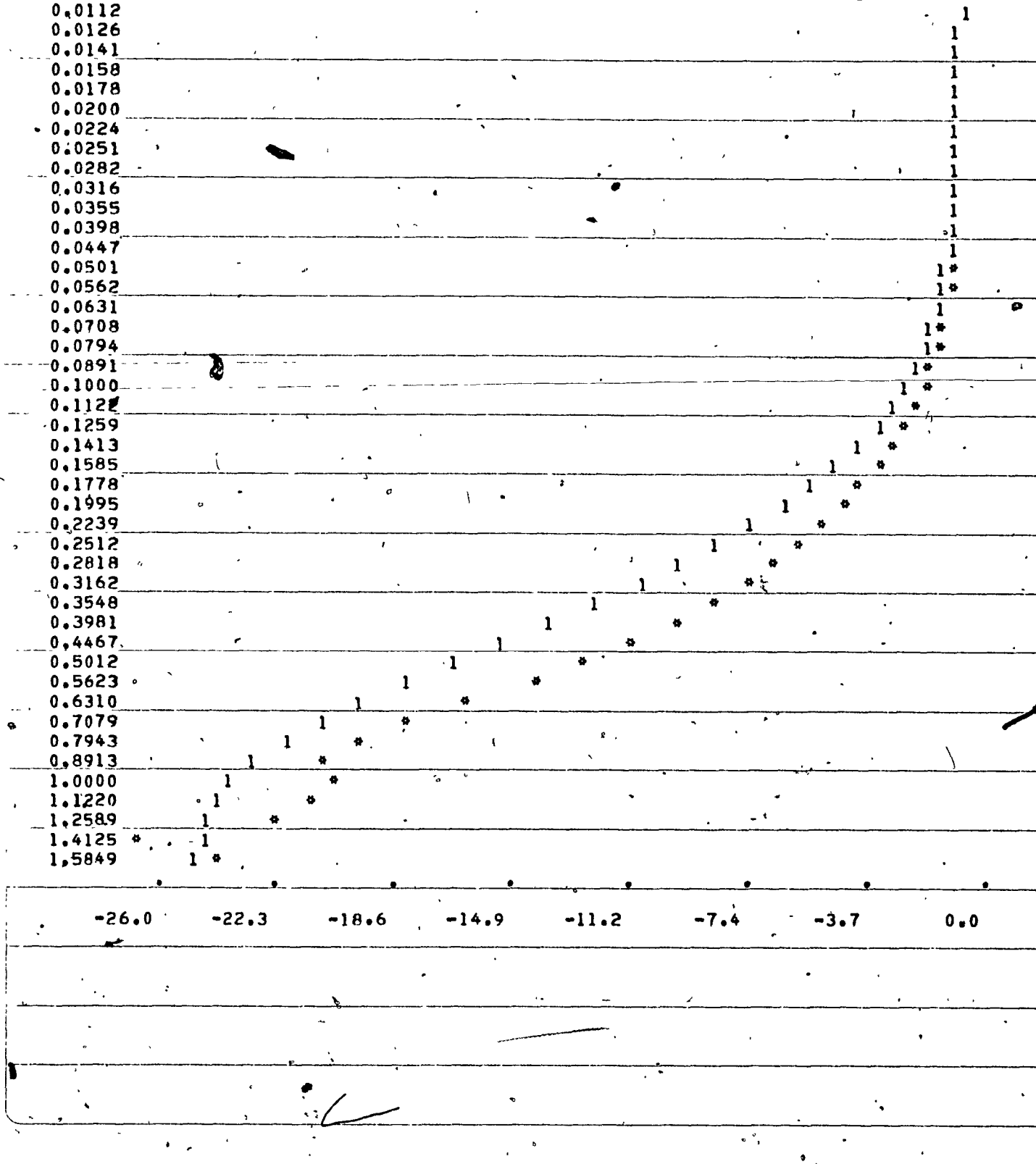
TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION

W	XMDB	ARDB	PH	DPHI
0.01122	0.00000	0.00000	-4.08567	-2.04605
0.01259	-0.00360	-0.00616	-4.58358	-2.29530
0.01413	-0.00812	-0.01391	-5.14199	-2.57479
0.01585	-0.01382	-0.02365	-5.76817	-2.88814
0.01778	-0.02099	-0.03590	-6.47024	-3.23940
0.01995	-0.03002	-0.05130	-7.25725	-3.63304
0.02239	-0.04138	-0.07065	-8.13926	-4.07406
0.02512	-0.05567	-0.09494	-9.12743	-4.56797
0.02818	-0.07366	-0.12543	-10.23411	-5.12086
0.03162	-0.09630	-0.16365	-11.47289	-5.73940
0.03548	-0.12477	-0.21152	-12.85863	-6.43092
0.03981	-0.16057	-0.27141	-14.40750	-7.20334
0.04467	-0.20557	-0.34622	-16.13688	-8.06524
0.05012	-0.26210	-0.43946	-18.06514	-9.02575
0.05623	-0.33306	-0.55542	-20.21136	-10.09456
0.06310	-0.42200	-0.69918	-22.59466	-11.28180
0.07079	-0.53330	-0.87679	-25.23327	-12.59796
0.07943	-0.67221	-1.09524	-28.14313	-14.05381
0.08913	-0.84493	-1.36254	-31.33585	-15.66035
0.10000	-1.05856	-1.68759	-34.81605	-17.42895
0.11220	-1.32085	-2.07998	-38.57830	-19.37163
0.12589	-1.63962	-2.54971	-42.60421	-21.50177
0.14125	-2.02189	-3.10668	-46.86113	-23.83513
0.15849	-2.47270	-3.76008	-51.30466	-26.39152
0.17783	-2.99408	-4.51772	-55.88689	-29.19680
0.19953	-3.58494	-5.38525	-60.56957	-32.28532
0.22387	-4.24208	-6.36554	-65.33748	-35.70236
0.25119	-4.96189	-7.45810	-70.21291	-39.50648
0.28184	-5.74482	-8.65872	-75.28270	-43.77139
0.31623	-6.61234	-9.95910	-80.70464	-48.58711
0.35481	-7.62922	-11.34650	-86.48211	-54.06018
0.39811	-8.84964	-12.80312	-92.02516	-60.31200
0.44668	-10.17934	-14.30507	-96.73735	-67.47342
0.50119	-11.55527	-15.82092	-101.48521	-75.67264
0.56234	-13.19776	-17.31043	-106.39705	-85.01168
0.63096	-15.26367	-18.72471	-109.43739	-95.52872
0.70795	-17.16406	-20.01019	-106.94149	-107.15244
0.79433	-18.64242	-21.11806	-105.95901	-119.66813
0.89125	-19.90950	-22.01722	-100.36786	-132.73204
1.00000	-19.56776	-22.70411	-101.28583	-145.94677
1.12202	-20.15451	-23.20217	-110.68633	-158.96508
1.25893	-21.41716	-23.55135	-133.99902	-171.56109
1.41254	-26.03569	-23.79500	-178.87822	-183.63900
1.58489	-23.34377	-23.97070	-145.52081	-195.19673

AMPLITUDE RATIO , DECIBELS

BODE DIAGRAMS FROM PROGRAMS PULSE AND LNREG2

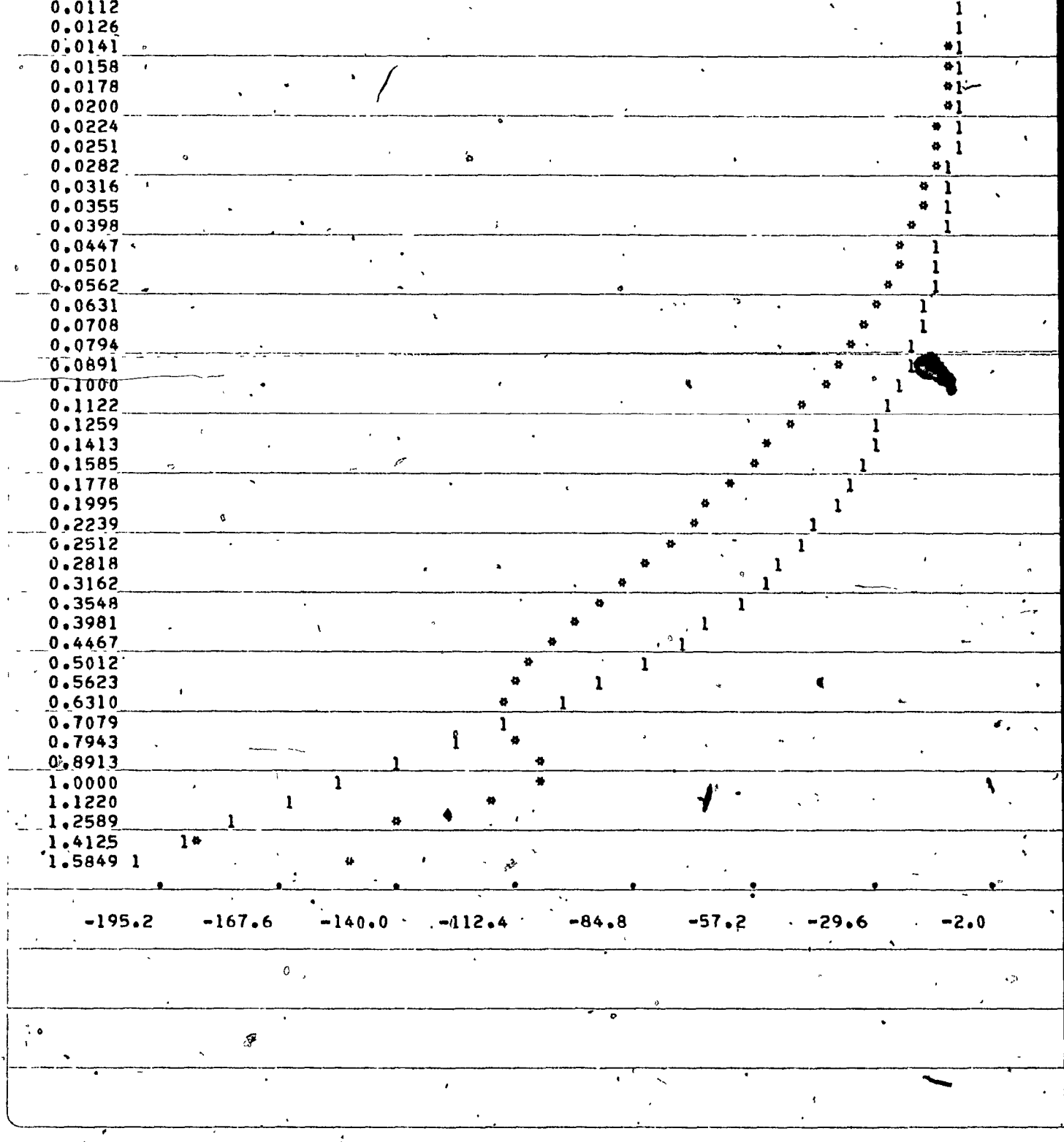
TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION



PHASE LAG , DEGREES

BODE DIAGRAMS FROM PROGRAMS PULSE AND LNREG2

TEST THE PROGRAM ,INPUT=HALF-SINE PULSE ,OUTPUT=ANALOG SIMULATION



APPENDIX E

TREATMENT OF DATA

Given a set of n measurements or observations, $x_1, x_2, \ldots, x_n$, there are several ways to describe their center (middle or centered location). Foremost among these is the arithmetic mean, although other kinds of "averages" are sometimes used for special purposes.

The arithmetic mean or, more succinctly, the mean $(\bar{x})$ is defined by,

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i \qquad \text{(E.1)}$$

It should be noted that the Eq. (E.1) would yield the mean of the distribution of a random variable which assumes the values of x_i with equal probabilities of $1/n$.

The variance (s^2) and the standard deviation (s) are defined as,

$$s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2 \qquad \text{(E.2)}$$

and,

$$s = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2} \qquad \text{(E.3)}$$

The standard deviation and the variance are measures of absolute variation, that is, they measure the actual amount of variation present in a set of data, and they are

dependent on the scale of measurement. To compare the variation in several sets of data, it is generally desirable to use measures of relative variation, the coefficient of variation (CV) being defined as,

$$CV = \frac{s}{\bar{x}} \times 100 \qquad \text{(E.4)}$$

It should be noted that this measure, which gives the standard deviation as a percentage of the mean, is independent of the scale of measurement.

The Eqs. (E.1) to (E.4) were applied to the experimental data from the continuous cultivation of *M. crassipes* in NH_3-WSL. The complete results are shown in Table E.1.

At the end of the continuous cultivation experiment (975 hours), thirty 100 ml samples of the culture medium have been collected through the sampling port which is used for the batch process. These samples were analyzed for pH, biomass and TCH concentrations as shown in Table E.2. The treatment of these data provided the standard deviations for the outlet pH, the biomass concentration and the outlet TCH, which are shown in Table E.1.

High fluctuations of the biomass during the continuous cultivation experiment could be due to either the nonhomogeneous nature of fungal mycelium in the fermentor or the sampling technique. A test has been performed

Table E.1 Treatment of Experimental Data From the Continuous Cultivation of *M. crassipes* in NH_3-WSL.

Variable (x_i)	Cultivation Period (hr)	Number of Data
Aeration rate (vvm)	103-975	estimated
Agitation speed (RPM)	0-975	estimated
Inlet jacket temperature (°C)	0-975	162
Inlet pH	28-975	8
Inlet TCH (g/l)	28-975	8
Biomass (g/l)	975	30
Outlet temperature (°C)	0-975	estimated
Outlet jacket temperature (°C)	0-975	estimated
Outlet pH	975	30
Outlet TCH (g/l)	975	30
Dilution rate (hr^{-1})	28-99	16
	99-975	150
Ambient temperature (°C)	0-975	147
Air pressure (psig)	0-28	7
	28-99	15
	99-975	125
Jacket water flowrate (l/hr)	0-975	146
Culture volume (l)	-	30

Mean ($\bar{x}$)	Variance (σ^2)	Standard Deviation (σ)	Coefficient of Variation (CV)
0.266	<0.0011	<0.033	<12.41
300	<100	<10	<3.33
23.9	0.39	0.6	2.61
5.82	0.1322	0.36	6.25
5.66	0.1283	0.36	6.33
1.077	0.0214	0.146	13.60
-	0.39	0.6	2.61
-	0.39	0.6	2.61
6.33	0.0001	0.01	0.18
3.39	0.0568	0.24	7.04
0.0277	0.0000	0.0023	8.44
0.0131	0.0000	0.0014	11.34
29.0	0.96	1.0	3.39
1.5	0.01	0.1	4.95
1.4	0.02	0.1	9.67
4.0	0.63	0.8	19.74
64	30	6	8.59
7.505	0.0005	0.024	0.32

Table E.2 Treatment of Data From Thirty Samples of Culture Medium at the End of the Continuous Cultivation of *M. crassipes* in NH_3-WSL.

Sample No.	Outlet pH	Biomass (g/l)	Outlet TCH (g/l)
1	6.35	1.339	3.02
2	6.34	1.442	3.25
3	6.34	1.262	3.25
4	6.34	1.286	3.52
5	6.33	1.276	2.75
6	6.33	1.140	3.62
7	6.33	1.243	3.38
8	6.33	1.023	3.71
9	6.33	1.192	3.43
10	6.33	1.053	3.25
11	6.33	1.053	3.38
12	6.33	1.033	3.38
13	6.33	1.041	3.52
14	6.33	1.030	3.46
15	6.33	0.925	3.20
16	6.33	0.933	3.32
17	6.33	1.182	3.62
18	6.32	1.165	3.82
19	6.33	0.977	3.75
20	6.33	1.034	3.52

Sample No.	Outlet pH	Biomass (g/l)	Outlet TCH (g/l)
21	6.33	0.946	3.15
22	6.33	1.030	3.62
23	6.33	1.028	3.62
24	6.32	0.942	3.46
25	6.33	0.981	3.32
26	6.32	1.069	3.32
27	6.33	1.052	3.35
28	6.32	0.900	3.39
29	6.33	0.829	3.35
30	6.33	0.900	2.90
$\bar{x}$	6.33	1.077	3.39
σ^2	0.0001	0.0214	0.0568
σ	0.01	0.146	0.24
CV	0.18	13.60	7.04

to clarify this point. A suspension of homogeneous particles which have more or less the same size as the fungal mycelium (Silica Gel H for Thin Layer Chromatography according to Stahl, size 10-40 μ) has been prepared in the fermentor at the concentration approximately equal to the concentration of the biomass at the end of the continuous cultivation experiment. Thirty samples of this suspension have been collected through the sampling port which is used for the batch process. These samples were analyzed similarly as for the biomass determination and the results of their dry weights are shown in Table E.3. It is interesting to see that at almost the same concentration of solids (1.077 g/l of biomass; 1.100 g/l of Silica Gel), the standard deviation of the biomass (0.146 g/l) was almost six fold higher than that of the Silica Gel (0.025 g/l). This is obviously due to the nature of two different solids. Hence, the high fluctuation in the biomass values are definitely due to the nonhomogeneous nature of the mycelial growth. If the unicellular microorganisms were used in the continuous cultivation experiment, much smoother results would be expected.

The outlet pH of the culture medium has been continuously recorded throughout the continuous cultivation experiment. However, due to the high fluctuation of these values, which are shown in Fig. E.1, they could not be useful. Therefore, all reported pH values were obtained from the harvested samples which are measured with the Fisher

Table E.3 Treatment of Data From Thirty Samples of Silica Gel Suspension.

Sample No.	Dry Weight (g/l)	Sample No.	Dry Weight (g/l)
1	1.075	16	1.072
2	1.087	17	1.107
3	1.100	18	1.131
4	1.093	19	1.054
5	1.119	20	1.090
6	1.164	21	1.089
7	1.065	22	1.093
8	1.155	23	1.121
9	1.106	24	1.127
10	1.074	25	1.077
11	1.090	26	1.110
12	1.095	27	1.110
13	1.110	28	1.105
14	1.070	29	1.098
15	1.110	30	1.096

$\bar{x} = 1.100$

$\sigma^2 = 0.0006$

$\sigma = 0.025$

$CV = 2.24$

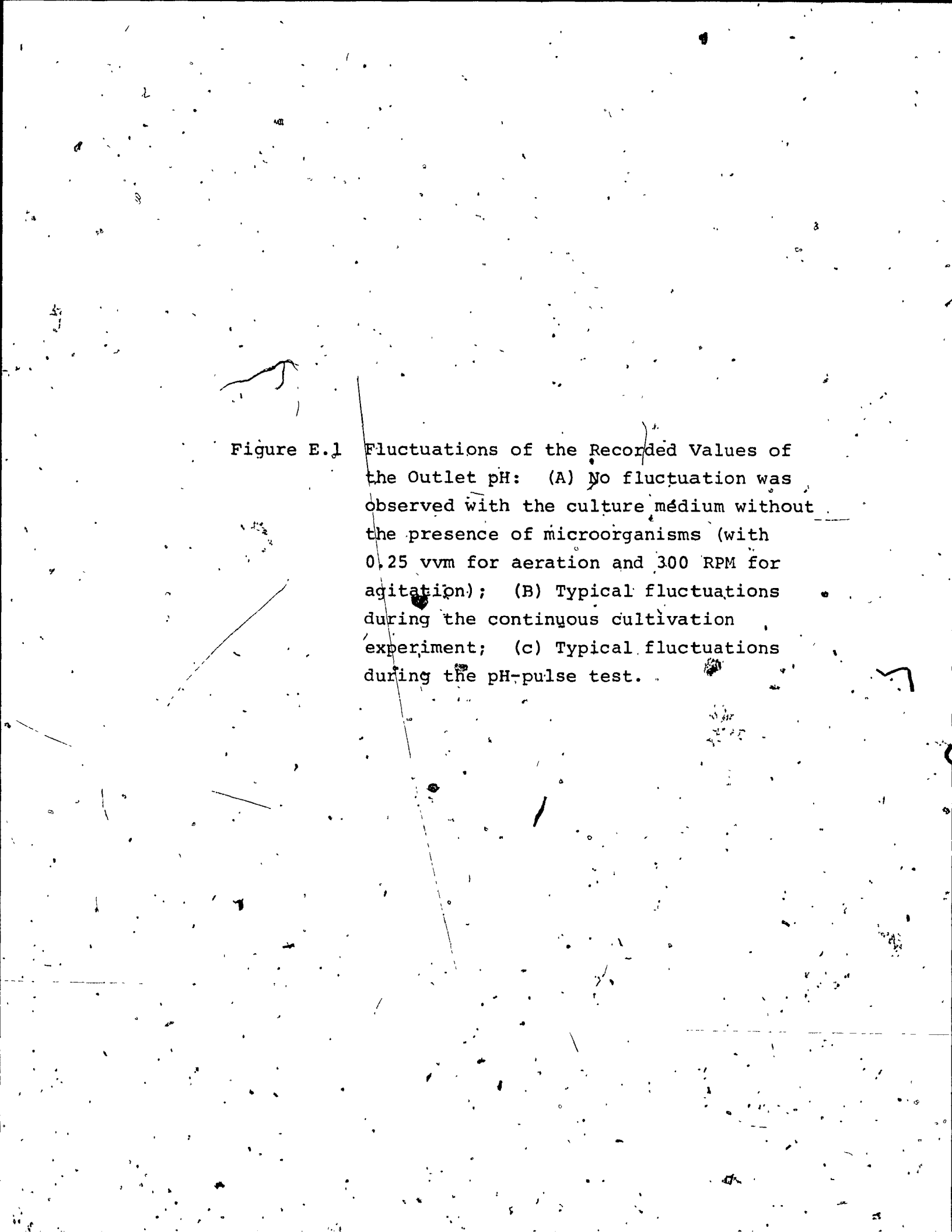
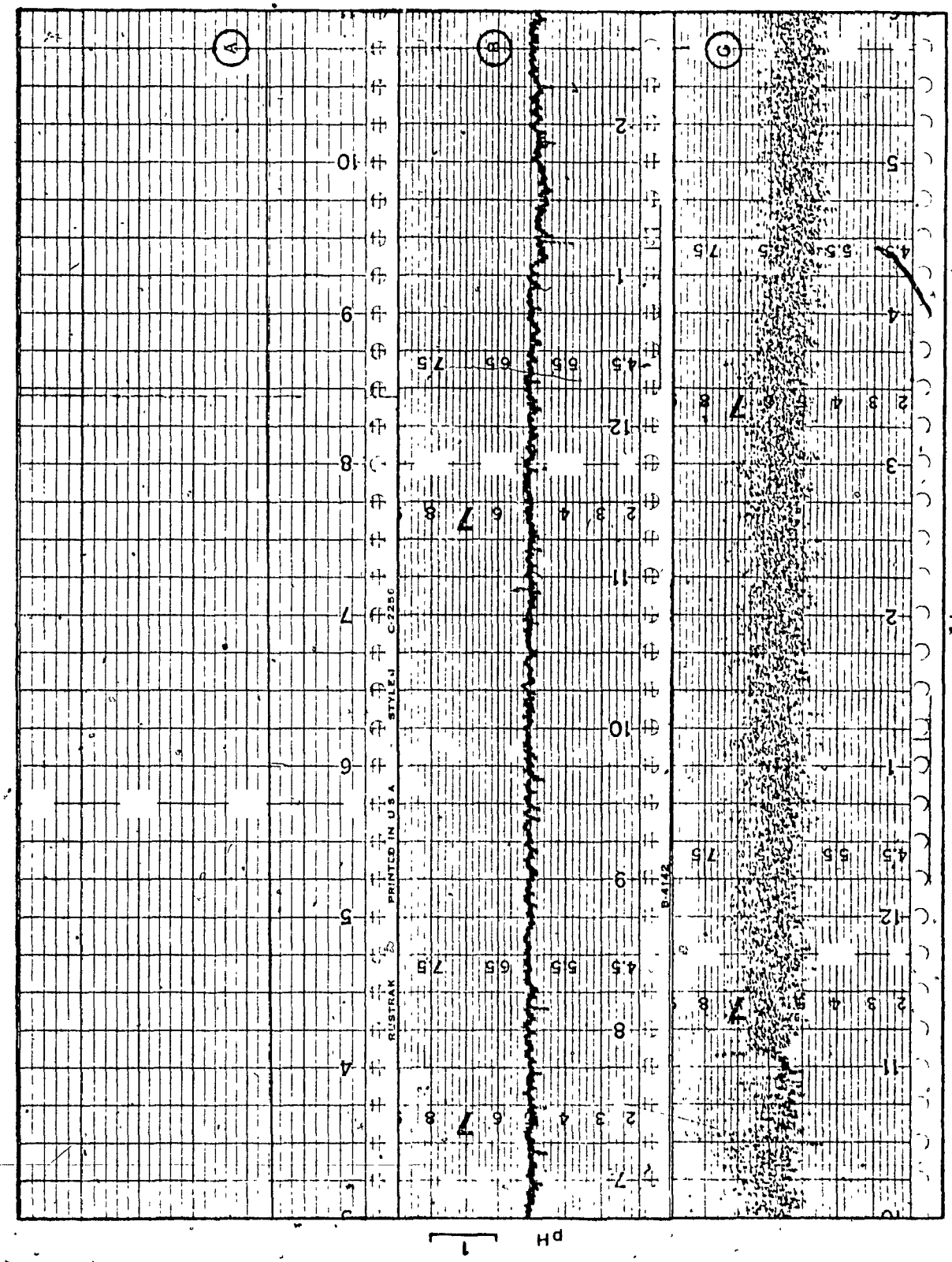


Figure E.1 Fluctuations of the Recorded Values of the Outlet pH: (A) No fluctuation was observed with the culture medium without the presence of microorganisms (with 0.25 vvm for aeration and 300 RPM for agitation); (B) Typical fluctuations during the continuous cultivation experiment; (c) Typical fluctuations during the pH-pulse test.


A
B
C
pH
1
TIME
1 hr
RUSTRAK
PRINTED IN U.S.A.
STYLE J
C-2256
P-4142

Acumet Model 200 pH Meter at room temperature. This provided an accurate value of the outlet pH with the standard deviation of approximately 0.01 at the mean value of 6.33 pH units (Tables E.1 and E.2).

The fluctuation of the culture medium volume in the fermentor jar was tested by operating the CSTF system with distilled water. At each interval of approximately one hour, the aeration and agitation were turned off and the level of the still water in the fermentor was recorded. Thirty values of the operated volume of the fermentor were shown in Table E.4 and the treatment of these data is shown in TablesE.1 and E.4.

The dilution rate during the continuous cultivation experiment has been followed by recording the time necessary for collecting each sample (100 ml). The calculated feeding rate at each sampling period is divided by the mean value of the culture medium volume (Table E.1) to yield the corresponding dilution rate. The data for the dilution rate are shown in Table B.13 and the results from the treatment of these data are shown in Table E.1.

The tolerances of the aeration rate and the agitation speed are not specified by the manufacturer. However, during the continuous cultivation experiment, the agitation speed set at 300 RPM rarely reached the value of 290 RPM or 310 RPM. The aeration rate set at 2.0 l/min

Table E.4 Treatment of Data for the Culture Medium Volume.

Reading No.	Volume (l)	Reading No.	Volume (l)
1	7.600	16	7.500
2	7.550	17	7.500
3	7.550	18	7.500
4	7.500	19	7.500
5	7.500	20	7.500
6	7.500	21	7.500
7	7.500	22	7.500
8	7.500	23	7.500
9	7.500	24	7.500
10	7.500	25	7.500
11	7.500	26	7.500
12	7.500	27	7.500
13	7.500	28	7.500
14	7.500	29	7.500
15	7.450	30	7.500

$\bar{x} = 7.505$

$\sigma^2 = 0.0005$

$\sigma = 0.024$

$CV = 0.32$

also rarely fluctuated to the level of 1.75 1/min or 2.25 1/min. Therefore, under the assumption that the values of the aeration rate and the agitation speed are normally distributed throughout the fermentation course, their standard deviations are certainly less than 0.033 vvm and 10 RPM respectively.

The inlet pH and inlet TCH during the continuous cultivation experiment were obtained from the samples taken from seven feed tanks. These data are shown in Table E.5 and the results from the treatment of these data are shown in Tables E.1 and E.5.

The accuracy for the temperature measurement systems used for the continuous cultivation was ± 0.1°C. However, the inlet jacket temperature depended on the temperature of tap water as shown in Fig. 4.6. Therefore, the accuracy of the inlet jacket temperature will be less than expected. The recorded data for the inlet jacket temperature and the ambient temperature are shown in Table B.13 and the results from the treatment of these data are shown in Table E.1. The standard deviations of the outlet temperature and the outlet jacket temperature could be assumed to be the same as that of the inlet jacket temperature.

The air pressure for aeration and the jacket

Table E.5 Treatment of Inlet pH and Inlet TCH Data From the Feed Tanks.

Feeding	Inlet pH	Inlet TCH (g/l)
1	5.77	5.40
2	5.80	5.34
3	6.12	6.12
4	5.92	5.77
5	5.90	5.15
6	6.20	5.66
7	5.82	5.73
pH-Pulse	2.30[a]	6.15
Substrate-Pulse	5.00	22.12[a]
$\bar{x}$	5.82	5.66
σ^2	0.1322	0.1283
σ	0.36	0.36
CV	6.25	6.33

(a) Excluded for the treatment of data.

water flowrate were also recorded throughout the continuous cultivation experiment (Table B.13). The treatment of these data is shown in Table E.1.

BIBLIOGRAPHY

Allan, L., Kaufman, E.K. and Underwood, J. (1972): Pollution in the Pulp and Paper Industry, The Council on Economic Priorities, New York.

Amberg, H.R. and Elder, R. (1956): Intermittent Discharge of Spent Sulfite Liquor, J. San. Eng. Division, Proc. Am. Soc. Civil Eng., paper No. 929.

Anonymous (1972a): PAG Statement No. 4 on Single Cell Protein, Protein Advisory Group of the United Nations System, United Nations.

Anonymous (1972b): Guideline for Preclinical Testing of Novel Sources of Protein, PAG Guideline No. 6, Protein Advisory Group of the United Nations System, United Nations.

Anonymous (1972c): Suggestions for Revision of PAG Guideline No. 6 "Preclinical Testing of Novel Sources of Protein", Report on the Second Meeting of the PAG *ad hoc* Working Group on Single Cell Protein, Moscow, U.S.S.R., 7-10 June 1971, Document 3.14/15: 18-20.

Anonymous (1973a): Single-Cell Protein: Which One Do You Mean?, PAG Bulletin, Protein Advisory Group of the United Nations System, United Nations, *3*(1): 23-25.

Anonymous (1973b): PAG Guideline No. 7 for Human Testing of Supplementary Food Mixtures, PAG Bulletin, Protein Advisory Group of the United Nations System, United Nations, *3*(3): 12-19.

Anonymous (1974): Plant Costs Zoom, Chem. Eng., *81*(13): 136-137.

Atkinson, G.F. (1911): Mushrooms, Henry Holt Co., New York.

Barta, J. (1971a): [Utilization of Yeast Proteins as Nutrients], Prum. Potravin., *22*: 321-325.

Barta, J. (1971b): [Higher Yields of Yeast Proteins Made From Sulfite Waste Liquor], Kvasny Prum., *17*: 145-150.

Barta, J. (1972): [*Cryptococcus diffluens*, a Yeast for Fermentation of Spent Sulfite Liquors I.], Pap. Celul., *27*(12): V70-V71.

Beachell, H.M. and Matz, S.A. (1969): Rice, in "Cereal Science", S.A. Matz Ed., The AVI Publishing Co., Inc., Westport, Connecticut: 172-223.

Bennett, B.A. (1967): Mushroom Production, U.S. Patent No. 3,328,911.

Bennett, I.C., Hondermarck, J.C. and Todd, J.R. (1969): How BP Makes Protein From Hydrocarbons, Hydrocarb. Proc., *48*: 104-108.

Bessey, E.A. (1961): Morphology and Taxonomy of Fungi, Hafner Publishing Co., New York.

Borzani, W. and Vairo, M.L.R. (1973): Observations of Continuous Culture Responses to Additions of Inhibitors, Biotechnol. Bioeng., *15*: 299-308.

Boudier, M. (1897): Révision Analytique des Morilles de France, Bull. Soc. Mycol. France, *13*: 129-153.

Brabec, J. and Radej, Z. (1972): [Nitrogen in Combined Calcium-Ammonium Sulfite Spent Liquors and Its Microbiological Utilization], Pap. Celul., *27*(10):V41-V43.

Brock, T.D. (1951): Studies on the Nutrition of *Morchella esculenta* Fries, Mycologia, *43*: 402-422.

Calkin, J.B. (1957): Modern Pulp and Paper Making, 3rd Ed., Reinhold Publishing Corp., New York.

Carroll, K.K. (1961): Quantitative Estimation of Peak Areas in Gas-Liquid Chromatography, Nature, *191*: 377-378.

Carroll, K.K., Cutts, J.H. and Murray, E.G.D. (1968): The Lipids of *Listeria monocytogenes*, Can. J. Biochem., *46*: 899-904.

Cirillo, V.P., Hardwick, W.A. and Seeley, R.D. (1960): Fermentation Process for Producing Edible Mushroom Mycelium, U.S. Patent No. 2,928,210.

Clements, W.C. and Schnelle, K.B. (1963): Pulse Testing for Dynamic Analysis, I&EC Process Design and Development, *2*: 94-102.

Commodity Year Book (1974): Commodity Research Bureau, Inc., New York.

Costantin, J. (1936): La Culture de la Morille et sa Forme Conidienne, Annales des Sciences Naturelles, Botanique et Biologie Végétales, *18*: 111-140.

Eckenfelder, W.W. and Barnard, J.L. (1971): Treatment-Cost Relationship for Industrial Wastes, Chem. Eng. Prog., *67*(9): 76-85.

Evans, D.R. and Wilson, J.C. (1972): Capital and Operating Costs-AWT, J. WPCF, *44*: 1-13.

Falanghe, H., Smith, A.K. and Rackis, J.J. (1964): Production of Fungal Mycelial Protein in Submerged Culture of Soybean Whey, Appl. Microbiol., *12*: 330-334.

Fencl, Z. (1970): Comments on Differentiation and Product Formation in Molds, Biotechnol. Bioeng., *12*: 845-847.

Filon, L.N.G. (1928): On a Quadrature Formula for Trigonometric Integrals, Proc. Roy. Soc. Edenburg, *49*: 38-47.

Forss, K. (1961): The Composition of a Spent Spruce Sulphite Liquor, Diss., Abo (Findland), from Rydholm (1965).

Forss, D.A. (1969): Role of Lipids in Flavors, J. Agr. Food Chem., *17*: 681-685.

Fron, G. (1905): Sur les Conditions de Développement du Mycélium de la Morille, Acad. des Sci. à Paris, Compte Rendus Hebd. des Séances, *140*: 1187-1189.

Fuld, G.J., Mateles, R.I. and Kusmierek, B.W. (1961): A Method for the Study of the Dynamics of Continuous Fermentation, S.C.I. Monograph No. 12, Soc. Chem. Industry, London: 54-77.

Furman, N.H. (1962): Standard Methods of Chemical Analysis, 6th Ed., D. Van Nostrand Co., Inc., New York, *1*.

Gaumann, E.A. (1952): The Fungi, A Description of Their Morphological Features and Evolutionary Development, Hafner Publishing Co., New York.

Gehm, H.W. (1965): Industrial Waste Water Control, Chapter 20, C.F. Gurnham Ed., Academic Press, New York.

George, T.K. and Gaudy, A.F. (1973): Response of Completely Mixed Systems to pH Shock, Biotechnol. Bioeng., *15*: 933-949.

Gilbert, F.A. (1960): The Submerged Culture of *Morchella*, Mycologia, *52*: 201-209.

Gilley, J.W. and Bungay, H.R. (1967): Oscillatory Growth Rate Responses of *S. cerevisiae* in Continuous Culture, Biotechnol. Bioeng., *9*: 617-622.

Gilley, J.W. and Bungay, H.R. (1968): Frequency Response Analysis of Dilution Rate Effects on Yeast Growth, Biotechnol. Bioeng., *10*: 99-103.

Gray, W.D. (1970): The Use of Fungi as Food and in Food Processing, CRC Critical Review in Food Technol., *1*: 225-329.

Greis, H. (1940): [Fruiting Bodies of *Morchella*]. Jahrbucher fur Wissenschaffliche Botanik, *89*: 245-253.

Groves, J.W. and Hoare, S.C. (1953): The Helvellaceae of the Ottawa District, Can. Field-Naturalist, *67*: 95-102.

Groves, J.W. (1958): Mushroom Collecting for Beginner, Canada Dept. of Agric., Pub. No. 861.

Groves, J.W. (1964): Poisoning by Morels When Taken With Alcohol, Mycologia, *56*: 779-780.

Hagglund, E., Klingstedt, F.W., Rosenqvist, T. and Urban, H. (1928): [Carbohydrate Constituents in the Easily Hydrolyzable Hemicelluloses From Spruce], Z. Physiol. Chem., *177*: 248-263.

Hatanaka, S.I. and Terakawa, H. (1968): Biochemical Studies on Nitrogen Compounds of Fungi I. Distribution of Some Nonprotein Amino Acids 1, Bot. Mag. Tokyo, *81*: 259-266.

Hatanaka, S.I. (1969): A New Amino Acid Isolated From *Morchella esculenta* and Related Species, Phytochemistry, *8*: 1305-1308.

Heim, R. (1936): La Culture des Morilles, Rev. Mycol. Paris, Suppl. 1: 10-11 and 19-25.

Heim, R. (1966): Quelques Ascomycetes Remarquables V. Morilles Tropicales (Pacifique Sud), Bull. Soc. Mycol. France, *48*: 442-449.

Heinemann, B. (1963): Process and Composition for Growing Mushroom Mycelium Submerged Fermentation, U.S. Patent No. 3,086,320.

Henderson, J.L. (1971): The Fluid-Milk Industry, 3rd ed., The AVI Publishing Co., Inc., Westport, Connecticut.

Himmelblau, D.M. and Bischoff, K.B. (1968): Process Analysis and Simulation, Deterministic Systems, John Wiley & Sons, Inc., New York.

Hojnos, J., Bogdanova, M. and Borisek, R. (1972): [Production of Nutrient Proteins From Beech Magnesium Bisulfite Spent Liquors I. Discontinuous Fermentation], Pap. Celul., *27*(7-8):V31-V35.

Humphrey, A.E. (1969): Engineering of Single Cell Protein: State of the Art, Chem. Eng. Prog. Symp. Ser., *65*(93): 60-65.

Ignat'eva, O.I. and Sapotnitskii, S.A. (1970): [Important Factor in Reducing Pollution of Natural Water], Bum. Prom., (5): 9-11.

Ivanov, St. A. and Blisnakova, L. (1967): [Fatty Acid Composition of a Lipid Concentrate From Commercially Grown *Morchella esculenta* Pers. and *Coprinus comatus* Fr. Mycelium], Natura Plovdiv., *1*: 39-40.

Ivanyukovich, V.A. and Kalyuzhnyi, M. Ya. (1971): [Utilization of Nitrogen of Sulfite Liquors During Growth of Feed Yeasts], Sb. Tr. Vses. Nauch.-Issled. Inst. Gidroliza Rast. Mater., (21): 115-118.

Jacobs, M.B. (1965): The Chemical Analysis of Food and Food Products, 3rd Ed., D. Van Nostrand Co., Inc., New York.

Janardhanan, K.K., Kaul, T.N. and Husain, A. (1970): Use of Vegetable Wastes for the Production of Fungal Protein From *Morchella* Species, J. Food Sci. Technol., *7*: 197-199.

Jilek, R., Kominek, J., Prochazka, H., Fuska, J., Sevcik, V., Macko, U. and Katzer, J. (1971): [Degradation of Sulfite Liquor With Ligniyorous Fungi], Czech. Patent No. 140,576.

Jones, D. (1966): The Canadian Pulp and Paper Industry, Canadian Council of Resource Ministers, Montreal, Background paper B18-1.

Kalyuzhnyi, M.Ya. and Ivanyukovich, V.A. (1971): [Digestion of Sulfite Liquors With Different Bases by Feed Yeasts], Sb. Tr. Vses. Nauch.-Issled. Inst. Gidroliza Rast. Mater., (19): 39-46.

Kirimura, J., Shimizu, A., Kimizuka, A., Ninomiya, T. and Katsuya, N. (1969): The Contribution of Peptides and Amino Acids to the Taste of Foodstuffs, J. Agr. Food Chem., *17*: 689-695.

Klis, J.B. (1963): Real Mushrooms in Powder Form, Food Proc., *24*: 99-101.

Koga, S. and Humphrey, A.E. (1967): Study of the Dynamic Behavior of the Chemostat System, Biotechnol. Bioeng., *9*: 375-386.

Kosaric, N. (1969): Studies on the Chemical Structure of Phospholipids Isolated From *Listeria monocytogenes*, Ph.D. Thesis, The University of Western Ontario, Canada.

Lagarde, J. (1923): Discomycètes de France I. Les Morilles, La Pensée Française, Paris.

Law, V.J. and Bailey, R.V. (1963): A Method for the Determination of Approximate System Transfer Functions, Chem. Eng. Sci., *18*: 189-202.

Levy, E.C. (1959): Complex-Curve Fitting, IRE Trans. Automatic Control, AC-4(1): 37-43.

Lipinsky, E.S. and Litchfield, J.H. (1970): Algae, Bacteria and Yeasts as Food or Feed, CRC Critical Reviews in Food Technol., *1*: 581-618.

Litchfield, J.H., Overbeck, R.C. and Davidson, R.S. (1963a): Factors Affecting the Growth of Morel Mushroom Mycelium in Submerged Culture, Agric. Food Chem., *11*: 158-162.

Litchfield, J.H. and Overbeck, R.C. (1963b): Submerged Culture Growth of *Morchella* Species in Food Processing Waste Substrates, Proc. 1st Intern. Cong. Food Sci. and Technol. (London), Gordon and Breech, New York, B-6, Part II: 511-520.

Litchfield, J.H., Vely, V.G. and Overbeck, R.C. (1963c): Nutrient Content of Morel Mushroom Mycelium: Amino Acid Composition of the Protein, J. Food Sci., *28*: 741-743.

Litchfield, J.H. (1964): Nutrient Content of Morel Mushroom Mycelium; B-Vitamin Composition, J. Food Sci., *29*: 690-691.

Litchfield, J.H. (1967): Morel Mushroom Mycelium as a Food-Flavoring Material, Biotechnol. Bioeng., *9*: 289-304.

Lushbough, C.H. and Schweigert, B.S. (1960): The Nutritive Content and the Nutritional Value of Meat and Meat Products, in "The Science of Meat and Meat Products", American Meat Institute, W.H. Freeman and Co., San Francisco: 185-211.

Maire, R. (1904): Sur les Divisions Nucléaires Dans l'Asque de la Morille et de Quelques Autres Ascomycètes, Compte Rendus Hebd. des Séances et Mémoires de la Soc. de Biol. Paris, *56*: 822-824.

Maire, R. (1905): Recherches Cytologiques sur Quelques Ascomycètes, Annales Mycologici Editi in Notitiam Scientiae Mycologicae Universalis (Berlin), *3*: 123-157.

Matz, S.A. (1969): Corn, in "Cereal Science", S.A. Matz Ed., The AVI Publishing Co., Inc., Westport, Connecticut: 39-77.

Megee, R.D., Kinoshita, S., Fredrickson, A.G. and Tsuchiya, H.M. (1970): Differentiation and Product Formation in Molds, Biotechnol. Bioeng., *12*: 771-801.

Melin, E. and Miden, G. (1941): [*Morchella conica* Pers., an Aneurin-Autotrophic Mushroom], Svensk Botanisk Tidskrift, *35*: 333-336.

Molliard, M. (1904): Mycélium et Forme Conidienne de la Morille, Acad. des Sci. à Paris; Compte Rendus Hebd. des Séances, *138*: 516-517.

Molliard, M. (1905): Production Expérimentale de l'Appareil Ascospore de la Morille, Acad. des Sci. à Paris, Compte Rendus Hebd. des Séances, *140*: 1146-1148.

Moravec, J. (1970): [*Morchella pragensis* Smotlacha 1952-Smrz Prazsky, Little Known Kind of *Morchella* Dill. ex St. Amans], Ceska Mykol., *24*: 32-39.

Morris, D.L. (1948): Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent, Science, *107*: 254-255.

Mueller, J.C. (1970): Fermentative Utilization of Spent Sulphite Liquor. A Review and Proposal, Pulp Pap. Mag. Can., *71*: 72-76.

Mueller, J.C. and Walden, C.C. (1970): Microbiological Utilisation of Sulphite Liquor, Proc. Biochem., *5*(6): 35-42.

Mulvany, P.K., Agar, H.D., Peniston, Q.P. and McCarthy, J.L. (1951): A Chromatographic Study of Sulfite Waste Liquor Sugars, J. Am. Chem. Soc., *73*: 1255-1257.

Murko, D. and Cezner, V. (1971): [Possibility of Producing Feed Proteins From Yugoslavian Spent Liquors], Kem. Ind., *20*: 163-166.

Negoita, D.R. (1970): [Manufacture of Food Proteins From Spent Bisulfite Liquor, Using *Aspergillus* and *Rhizopus*], Celul. Hirtie, *19*: 146-153.

Neish, A.C. (1952): Analytical Methods for Bacterial Fermentations, N.R.C. Can., Report No. 46-8-3 (2nd Revision).

Perry, F.G. and Woodland, L.R. (1970): An Approach to Selection of a Sulphite Chemical Recovery System, Pulp Pap. Mag. Can., *71*: 45-53.

Powrie, W.D. (1973): Chemistry of Eggs and Egg Products, in "Egg Science and Technology", W.J. Stadelman and O.J. Cotterill, Eds., The AVI Publishing Co., Inc., Westport, Connecticut: 61-90.

Regan, D.L., Roper, G.H. and Moss, F.J. (1971): Response of Continuous Cultures to Stimuli in Glucose Feed Rate and Dilution Rate, Biotechnol. Bioeng., *13*: 815-824.

Répin, C. (1901): La Culture de la Morille, Rev. Gen. Sci. Pures et Appl. & Bull. Assoc. Fr. pour Avanc. Sci. Paris, *12*: 595-596.

Reusser, F., Spencer, J.F.T. and Sallans, H.R. (1958): Protein and Fat Content of Some Mushrooms Grown in Submerged Culture, Appl. Microbiol., *6*: 1-4.

Rieche, A., Lorenz, M., Martini, A.E. and Hilgetag, G. (1970): [Work-up of Spent Sulfite Liquor With High Solid Content], Ger. Patent No. 1,931,233.

Robbins, W.J. and Hervey, A. (1959): Wood Extract and Growth of *Morchella*, Mycologia, *51*: 356-363.

Robbins, W.J. and Hervey, A. (1963): Unidentified Filtrate Growth Substances for Several Fungi, Mycologia, *55*: 59-64.

Robbins, W.J. and Hervey, A. (1965): Manganese, Calcium and Filtrate Factor for *Morchella crassipes*, Mycologia, *57*: 262-274.

Robinson, R.F. and Davidson, R.S. (1959): The Large-Scale Growth of Higher Fungi, Adv. Appl. Microbiol., *1*: 261-278.

Roze, E. (1883): Le Parasitisme du *Morchella esculenta* Pers. sur l'*Helianthus tuberosus* L., Bull. Soc. Bot. de France, *30*: 139-143.

Rydholm, S.A. (1965): Pulping Processes, Interscience Publishers, New York.

Sapotnitskii, S.A., Fomenko, T.K., Ignat'eva, O.I. and Khutorshchikov, I.S. (1972): [Cultivation of Yeasts on Two-Stage Cooking Liquor of Larch Wood], Gidroliz. Lesokhim. Prom., *25*(2): 5-6.

Shellenberger, J.A. (1969): Wheat, in "Cereal Science", S.A. Matz Ed., The AVI Publishing Co., Inc., Westport, Connecticut: 1-38.

Sihtola, H., Anthoni, B. and Rosqvist, E. (1958): Monosaccharides in Technical Sulphite Cooking Liquor, Pap. Puu, *40*: 493-495.

Smith, O. (1968): Chemical Composition of the Potato, in "Potatoes: Production, Storing, Processing", O. Smith Ed., The AVI Publishing Co., Inc., Westport, Connecticut: 59-109.

Solms, J. (1969): The Taste of Amino Acids, Peptides, and Proteins, J. Agr. Food Chem., *17*: 686-688.

Standard Methods for the Examination of Water and Wastewater Including Bottom Sediments and Sludges, 12nd Ed., Am. Public Health Assoc., Inc., New York.

Sugihara, T.F. and Humfeld, H. (1954): Submerged Culture of the Mycelium of Various Species of Mushroom, Appl. Microbiol., *2*: 170-172.

Szuecs, J. (1950): Essence of Mushroom and Its Preparation, U.S. Patent No. 2,505,811.

Szuecs, J. (1954): Method of Enhancing Mushroom Mycelium Flavor, U.S. Patent No. 2,693,664.

Szuecs, J. (1956): Mushroom Culture, U.S. Patent No. 2,761,246.

Szuecs, J. (1958): Method of Growing Mushroom Mycelium and the Resulting Products, U.S. Patent No. 2,850,841.

Topiwala, H. and Sinclair, C.G. (1971): Temperature Relationship in Continuous Culture, Biotechnol. Bioeng., *13*: 795-813.

Wakayama, K. (1930): Contributions to the Cytology of Fungi II. Cytological Studies in *Morchella deliciosa* Fr., Cytologia Tokyo, *2*: 27-36.

Wang, D.I.C. (1968): Protein From Petroleum, Chem. Eng., *75*(18): 99-108.

Wolf, W.J. and Cowan, J.C. (1971): Soybeans as a Food Source, CRC Critical Reviews in Food Technology, CRC Press, Cleveland, Ohio.

Yano, T. and Koga, S. (1969): Dynamic Behavior of the Chemostat Subject to Substrate Inhibition, Biotechnol. Bioeng., *11*: 139-153.

Young, T.B., Bruley, D.F. and Bungay, H.R. (1970): A Dynamic Mathematical Model of the Chemostat, Biotechnol. Bioeng., *12*: 747-769.

Young, T.B. and Bungay, H.R. (1973): Dynamic Analysis of a Microbial Process: A Systems Engineering Approach, Biotechnol. Bioeng., *15*: 377-393.

Zines, D.O. and Rogers, P.L. (1970): The Effect of Ethanol on Continuous Culture Stability, Biotechnol. Bioeng., *12*: 561-575.

Zol'nikova, G.G. and Pebre, S.K. (1971): [Improving the Operation of a Yeast Cultivation Section], Gidroliz: Lesokhim. Prom., *24*(5): 16-18.

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B) Refereed Papers:

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2) N. Kosaric, A. LeDuy and J.E. Zajic (1973): Submerged Culture Growth of Edible Mushrooms on Waste Sulphite Liquors, Can. J. Chem. Eng. 51: 186-190.
3) A. LeDuy and J.E. Zajic (1973): Sampling Procedures for Non-Newtonian Fermentation Broths, Biotechnol. Bioeng., 15: 579-581.
4) A. LeDuy and J.E. Zajic (1973): A Geometrical Approach for Differentiation of an Experimental Function at a Point: Applied to Growth and Product Formation, Biotechnol. Bioeng., 15: 805-810.
5) A. LeDuy, A.A. Marsan and B. Coupal (1974): A Study on the Rheological Properties of a Non-Newtonian Fermentation Broth, Biotechnol. Bioeng., 16: 61-76.
6) A. LeDuy, N. Kosaric and J.E. Zajic (1974): Morel Mushroom Mycelium Growth in Waste Sulfite Liquors as Source of Protein and Flavouring, Can. Inst. Food Sci. Technol. J., 7: 44-50.

7) A. LeDuy, N. Kosaric, K. Muzika and J.E. Zajic (1974): Treatment of Waste Sulfite Liquors by Morel Mushrooms, Water-1974, (in press).

8) N. Kosaric, J.E. Zajic, B. Volesky, A. LeDuy and T. Higgs (1974): Biochemical-Microbiological Synthesis of Proteins for Human and/or Animal Consumption, Proceeding of the 9th International Symposium of Zootechny, (in press).

C) Scientific Papers Delivered at Symposia and Conferences:

1) N. Kosaric and A. LeDuy: Submerged Culture Growth of Edible Mushrooms on Waste Sulphite Liquors, paper presented at the 21st Conference of the Canadian Society for Chemical Engineering (C.S.Ch.E.), Montreal, October 12-15, 1971.

2) A. LeDuy, N. Kosaric and J.E. Zajic: Morel Mushroom Mycelium Growth in Waste Sulfite Liquors as Source of Protein and Flavouring, paper presented at the 16th Conference of the Canadian Institute of Food Science and Technology (C.I.F.S.T.), Vancouver, May 30-June 1, 1973.

3) A. LeDuy, N. Kosaric, J.E. Zajic and W.Y. Svrcek: Identification of Continuous Cultivation Systems Using Pulse Techniques, paper presented at the 56th Conference of the Chemical Institute of Canada (C.I.C.), Montreal, June 4-6, 1973.

4) A. LeDuy, N. Kosaric, K. Muzika and J.E. Zajic: Treatment of Waste Sulfite Liquors by Morel Mushrooms, paper presented at the 23rd Conference of the Canadian Society for Chemical Engineering (C.S.Ch.E.), Vancouver, September 9-12, 1973.

5) N. Kosaric, J.E. Zajic, B. Volesky, A. LeDuy and T. Higgs: Biochemical-Microbiological Synthesis of Proteins for Human and/or Animal Consumption, paper presented at the 9th International Symposium of Zootechny, Milano, Italy, April 15-17, 1974.

6) A. LeDuy: A Proposed Method for the Specification of Rheological Properties of Power-Law-Obeying Non-Newtonian Food Fluids, paper presented at the 17th Conference of the Canadian Institute of Food Science and Technology (C.I.F.S.T.), Montreal, June 10-12, 1974:

7) V.T. Xuyen and A. LeDuy: Dynamic Batch Process Identification for Cell Growth, paper presented at the 24th Conference of the Canadian Society for Chemical Engineering (C.S.Ch.E.), Ottawa, October 20-23, 1974.

D) Miscellaneous Scientific Contributions:

1) A. LeDuy: Elaboration of Polysaccharide From Sucrose by Fermentation With *Pullularia pullulans*, M.Sc.A. Thesis, Universite de Sherbrooke, 1972.

2) J.E. Zajic, N. Kosaric, A. LeDuy and K. Muzika: Mycological Approach to Waste Treatment, Final Report Submitted to the Canadian Forestry Service, Department of the Environment (CPAR Project No. 54, Contract No. 25), March 1973.

3) J.E. Zajic and A. LeDuy: Pullulan, Encyclopedia of Polymer Science and Technology, Interscience Publishers, John Wiley & Sons, Inc. (in press).