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Assessment of Free-Living Nitrogen Fixing Microorganisms for Commercial Nitrogen Fixation

Prepared for
National Science Foundation
Research Applied to National Needs Program

by
Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California

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CONTENTS

I. ABSTRACT 1

II. INTRODUCTION 2

III. OBJECTIVES 4

IV. EXPERIMENTAL 5

A. Materials and Methods 5

1. Microorganisms 5
2. Culture Conditions 5
3. Oxygen Measurement 6
4. Growth Measurement 6
5. Glucose Analysis 7
6. Ammonia Analysis 7
7. Hydrogen Analysis 7
8. Reversion Analysis 7

B. Results & Discussion 7

1. Klebsiella pneumoniae 7

a. Glutamine Usage 7
b. Flask Cultures 9
c. Five Liter Experiments 12
d. Hydrogen Production 17
e. Theoretical Limits 18
f. Tolerance to Ammonia 19
g. Settling of Cells 19

2. Azotobacter vinelandii UW590 19

a. Effect of pH on Growth 19
b. Effect of Aeration on Growth of A. vinelandii UW590 . . . 22
c. Efficiency of Nitrogen Fixation 26

V. ECONOMIC EVALUATION 30

A. Economic Model 30

1. Conventional Fermentation 30
2. Lagoons 30
3. Ammonia Recovery 30

CONTENTS (Contd)

B. Results and Discussion 41

1. *Klebsiella pneumoniae* 41

a. *Klebsiella pneumoniae*, SK-25, in Conventional Fermentation 41
b. *Klebsiella pneumoniae*, SK-25, in Lagoons 50
c. Hydrogen and Other Product Formation by *Klebsiella pneumoniae* 53

2. *Azotobacter vinelandii* 56

a. *Azotobacter vinelandii*, UW590 56
b. *Azotobacter vinelandii*, UW590, in Lagoons 58

3. Photosynthetic Bacteria 64
4. Blue-Green Algae 65
5. Economic Comparison between Nitrogen Fixation and Anaerobic Digestion 67
6. *In Situ* Fertilization by Free-Living Microorganisms 71

a. Current Contributions 71
b. Derepressed Mutants 72
c. Economic Considerations 72

VI. CONCLUSIONS 74

VII. FUTURE RESEARCH NEEDS 77

VIII. UTILIZATION OF RESEARCH RESULTS 78

IX. PRODUCTS OF RESEARCH 79

X. PERSONNEL 80

XI. ACKNOWLEDGEMENT 81

XII. REFERENCES 82

CONTENTS (Contd)

		PAGE
Appendix I.	Data for K. pneumoniae in 250 ml Flask Cultures.	86
Appendix II.	Data for Low Density Fermentation, K. pneumoniae SK-25 (10 µg/ml initial gluNH$_2$).	88
Appendix III.	Data for High Density Fermentation, K. pneumoniae SK-25 (300 µg/ml initial gluNH$_2$)	90
Appendix IV.	Data for High Density Fermentation, K. pneumoniae SK-25 (500 µg/ml initial gluNH$_2$)	92
Appendix V.	Hydrogen Production by K. pneumoniae SK-25 (see Appendix II)	94
Appendix VI.	Analysis of Standard Hydrogen Mixtures	96
Appendix VII.	Data for A. vinelandii UW590 Fermentations	98
Appendix VIII.	Dry Weight Determinations of A. vinelandii UW590	100
Appendix IX.	Cost Estimate for K. pneumoniae in Conventional Fermentation at Low Ammonia Concentration.	104
Appendix X.	Cost Estimate for K. pneumoniae in Conventional Fermentation at High Ammonia Concentration	114
Appendix XI.	Cost Estimate for K. pneumoniae in Lagoon Fermentation at Low Ammonia Concentration	124
Appendix XII.	Cost Estimate for A. vinelandii in Conventional Fermentation	130
Appendix XIII.	Cost Estimate for A. vinelandii in Lagoon Fermentation for Theoretical Limits of Nitrogen Fixation	140

FIGURES

Figure		Page
1	Final Culture Density Versus Initial $GluNH_2$ Concentration . . .	8
2	Ammonia Production by 250 ml FlaskCultures of K. pneumoniae SK-25	10
3	Ammonia and Hydrogen Production by K. pneumoniae SK-25	14
4	pH Dependence of Growth of Azotobacter Vinelandii UW590	23
5	pH Dependence of Growth Efficiency of A. Vinelandii UW590. . .	24
6	Effect of Aeration on Growth of A. Vinelandii UW590.	25
7	Time Course of A. Vinelandii UW590 Growth at High Aeration . .	27
8	Growth Efficiency of A. Vinelandii UW590 versus Culture Density.	28
9	Diagram of Ammonia Still	39
10	Ammonia Recovery System from the Coking Industry	40
11	Continuous Fermentation by K. pneumoniae for Ammonia Production and Recovery.	42
12	Prediction of Continuous Operating Conditions From Batch Data for K. pneumoniae	43
13	Cost of Ammonia Production by K. pneumoniae as a Function of Ammonia Concentration in Conventional Fermentation	47
14	Cost of Ammonia Production by K. pneumoniae as a Function of Energy Consumption in Conventional Fermentation	48
15	Cost of Ammonia Production by K. pneumoniae as a Function of Ammonia Concentration in a Lagoon System.	51
16	Cost of Ammonia Production by K. pneumoniae as a Function of Energy Consumption for a Lagoon System	52
17	Prediction of Continuous Operating Conditions from Batch Data for A. vinelandii	57
18	Cost of Fixed Nitrogen as a Function of Efficiency for A. vinelandii in Conventional Fermentation	59
19	Cost of Fixed Nitrogen as a Function of Efficiency for A. vinelandii in a Lagoon System	61

TABLES

		PAGE
1.	Summarized Data for Flask Experiments using *Klebsiella pneumoniae*	11
2.	Data Summary For 5ℓ Fermentations.	16
3.	Ammonia Tolerance of *Klebsiella*	20
4.	Settling of *Klebsiella pneumoniae* SK-25	21
5.	Estimation of Fixed Capital Investment Cost.	32
6.	Estimation of Total Product Cost	33
7.	Format for Estimation of Capital Investment and Annual Cost for Lagoon Fermentations	34
8.	Design Criteria Selective ION Exchange Process for Ammonium Removal at the Upper Occoquan Plant.	36
9.	Regeneration and Regenerant Recovery System Design Criteria at the Upper Occoquan Plant.	37
10.	Estimated Costs of Selective ION Exchange at Upper Occoquan Plant.	38
11.	Capital Investment and Manufacturing Costs for *K. pneumoniae* in Conventional Fermentation	49
12.	Capital Investment and Manufacturing Costs for *K. pneumoniae* in a Lagoon System	54
13.	Capital Investment and Manufacturing Costs for *Azotobacter vinelandii* in Standard Fermentation Equipment	60
14.	Capital Investment and Manufacturing Costs for *A. vinelandii* in a Lagoon System	63
15.	Comparison of Product Value for Nitrogen Fixation Processes and Anaerobic Digestion	70

I. ABSTRACT

The importance of ammonia to the U. S. economy and the large fossil energy requirements of commercial ammonia production have focused research attention on biological systems for the production of ammonia and possibly hydrogen. Recent advances in microbial genetics suggest the possibility of adapting freeliving nitrogen-fixing microorganisms to the commercial production of fixed nitrogen.

This economic assessment indicates that ammonia production by Klebsiella pneumoniae is not economical with present strains and improving nitrogen fixation to its theoretical limits in this organism is not sufficient to achieve economic viability. Contamination and reversion of the mutant are major technical problems. This leads to sterilization requirements which are economically prohibitive. Ammonia is a low value product and has been obtained only in dilute solutions with biological systems. Since the value of both the hydrogen produced by this organism and the methane value of the carbon source required greatly exceed the value of the ammonia formed, ammonia (fixed nitrogen) should be considered the by-product and attention should be focused on other products. The production of hydrogen by Klebsiella or other anaerobic nitrogen fixers should receive additional study, since the value of hydrogen produced by Klebsiella greatly exceeds the value of the nitrogen fixed and since the activity of nitrogenase offers a significant improvement in hydrogen production.

At observed efficiencies, the production of fixed nitrogen in the form of cell mass by Azotobacter is also uneconomical and the methane value of the carbon substrate exceeds the value of the nitrogen fixed. Parametric studies indicate that as efficiencies approach the theoretical limits the economics may become competitive under the assumptions of the economic model employed. The use of nif-derepressed microorganisms, particularly blue-green algae, may have significant potential for in situ fertilization in the environment. Additional work is required to determine: 1) the extent of in situ nitrogen fixation when nif-derepressed strains are added to the environment and, 2) how effective these strains are in increasing crop yields through the production of substances other than fixed nitrogen which may enhance plant growth.

II. INTRODUCTION

Ammonia is a key chemical in the United States economy and is used in the manufacture of plastics, explosives, synthetic organic chemicals and fertilizers. Fertilizer constitutes the major use at 74 percent of the total 16.5 x 10^6 tons produced in the United States in 1975[1]. The application of nitrogen fertilizer is a critical factor in maintaining U.S. agricultural productivity. Hardy concludes that nitrogen fertilization is the single most important non-biological factor in increasing cereal grain yields.[2,3]

The majority of the commercially fixed ammonia is prepared by the Haber-Bosch process[4] which is well engineered and quite efficient. While current production is largely based on natural gas,[5] the process can be easily adapted to other fossil fuels at higher cost[6,7] so catastrophic dislocations in production are unlikely. Hardy[2] estimates that 500-600 ammonia plants could supply projected world wide needs through the year 2000. While the need for alternative technologies for nitrogen fixation is not acute, conventional ammonia plants are expensive, costing as much as 10^8 dollars per 1,000 ton per day capacity.[2] This fact together with recent interest in developing non-fossil fuel based technologies has focused attention on biological systems as an alternative means of fixing nitrogen. Global biological nitrogen fixation currently accounts for 175 x 10^6 metric tons of nitrogen fixed per year which exceeds the amount fixed by non-biological processes.[8] In addition 45 x 10^6 metric tons are produced biologically in agriculturally important settings.[8] Obviously efforts to enhance biological nitrogen fixation have considerable potential for reducing our fossil fuel based fertilizer usage.

One approach to enhancing nitrogen fixation in free-living microorganisms is the development of *nif*-derepressed strains. Free-living microbes produce ammonia only when fixed nitrogen is unavailable in the environment.[9-14] *Nif*-derepressed strains are insensitive to repression by ammonia and are, therefore, genetically enhanced for nitrogen fixation. Two general types of *nif*-derepressed microbes have been obtained during the last few years, namely control mutants and auxotrophs (glutamate or glutamine). Control mutants were obtained by selecting for *nif*-derepressed strains among revertents from mutant

strains of Azotobacter vinelandii which could not fix nitrogen.[15] Nitrogen fixation in these strains is not repressed by ammonia and the organisms grow normally on N_2 gas as the sole nitrogen source.[15] Nif-derepressed strains of Klebsiella pneumoniae[16-18] and Spirillum lipoferum[19] have been obtained by genetically blocking NH_3 assimilation. Such strains are auxotrophic (require glutamine or glutamate for growth) and excrete the ammonia produced by fixation of N_2 into the culture medium. The successful development of nif-derepressed strains raises the possibility of developing biological processes for commercial nitrogen fixation. This work examines the potential of nif-derepressed strains of Azotobacter vinelandii and Klebsiella pneumoniae for commercial nitrogen fixation.

III. OBJECTIVES

The overall objective of the study was to assess the potential of nif-derepressed, free-living microorganisms for nitrogen fixation. The study concentrated on available nif-derepressed mutants and relied heavily on the work of the laboratories of Dr. Raymond D. Valentine and Dr. Winston Brill for choosing culture conditions. The study was also intended to look beyond present nif-derepressed strains to assess general economic limitations and requirements of the concept of using free-living microorganisms for nitrogen fixation. The specific objectives of the are:

A. Determine optimum conditions in small-scale experiments for ammonia production in Azotobacter vinelandii UW590 and for ammonia and hydrogen production in Klebsiella pneumoniae SK-25

B. Scale ammonia and hydrogen production by Klebsiella pneumoniae SK-25 and ammonia production by Azotobacter vinelandii UW590 to a 5 liter system.

C. Determine cell growth rate, ammonia and hydrogen production rates, product yield and genetic stability of the systems mentioned above in the 5 liter system.

D. Model the systems for economic analysis on the basis of the data obtained in the 5 liter system. The maximum cost allowed for a carbon source at a given ammonia or hydrogen price will be the criterion for evaluation.

E. Adapt the economic model to include photosynthetic systems (bacteria and algae) for preliminary economic analysis assuming that derepressed mutants could be obtained at the nitrogenase activity of the parent organism.

F. Compare the economic models of the four classes of free living nitrogen fixing microbes (anaerobic-dark, aerobic-dark, anaerobic-light, aerobic-light) to determine the advantages and disadvantages of each type of system.

IV. EXPERIMENTAL

A. Materials and Methods

1. Microorganisms

Klebsiella pneumoniae SK-25 and SK-29 were obtained from Dr. Raymond C. Valentine. These strains were chosen due to their high ammonia production characteristics compared to other nif-derepressed strains of *Klebsiella*[17]. These strains are also among the most stable strains obtained by Valentine's group with a reversion frequency of less than one per 10^{10} cells.[20] *Azotobacter vinelandii* UW590 was obtained from Dr. Winston J. Brill. This organism was chosen from among the *Azotobacter* mutants[15] since it exhibits nitrogenase levels in the presence of ammonia which are characteristic of the parent strain growing on N_2 (fully derepressed).[2]

2. Culture Conditions

Klebsiella strains were maintained on Luria agar[22] to avoid selection for revertant strains. All *Klebsiella* experiments employed the minimal media of Yoch and Pengra[23] as modified by Streicher et. al.[24] except that the phosphate concentration was doubled to increase buffering capacity. Unless otherwise specified the temperature was controlled at $25^{o} \pm 1^{o}C$ and pH was maintained at 7.2 ± 0.2 in order to employ the conditions of maximum efficiency for ammonia production as determined by Andersen and Shanmugam.[18] The dialysis system employed by Andersen and Shanmugam was omitted since it is not amenable to scale up. Other deviations from their conditions are discussed in the text. Inocula were prepared by transferring a colony from the Luria slant with a flamed wire loop to 10 mls of Luria broth and allowing the culture to grow overnight at $37^{o}C$ in screw cap test tubes. These Luria broth cultures were used to inoculate volumes (1 percent inoculum) of minimal media supplemented with glutamine or glutamate (usually 100 mg/ml) and allowing the culture to grow to early stationary phase (18-24 hours). Preparation of this minimal media inoculum served to greatly reduce the carryover of fixed nitrogen from the Luria broth into the experiments.

Luria broth contains 10 g/l tryptone and 5 g/l yeast extract. The nitrogen (N) content of tryptone, yeast extract, glutamine and glutamic acid are 13.14 percent,[25] 9.18 percent,[25] 19.16 percent and 9.58 percent, respectively. A 1 percent inoculum of Luria broth culture would therefore contain a maximum of 0.1 g/l tryptone and 0.5 g/l yeast extract. On the basis of N content this carryover is equivalent to 93 µg/ml glutamate or 186 µg/ml glutamic acid. Using the minimal inoculum as a 1 percent transfer the carryover is reduced to 0.93 µg/ml glutamine or 1.86 µg/ml glutamate equivalent.

Azotobacter experiments employed a modified Burke's media[26] except that glucose (autoclaved separately) was substituted for sucrose. Unless otherwise stated pH was maintained at 7.2 ±0.1 and temperatures were controlled at 30°C. Azotobacter cultures were maintained on Burke's agar. Experiments were inoculated with log phase solution cultures (usually at densities around (A_{420} = 0.7). All 5 liter experiments employed a 7.5 liter New Brunswick bench top fermentor (Model 19) equipped with a pH controller and an O_2 monitor.

3. Oxygen Measurement

Oxygen concentration was measured by a recording New Brunswick dissolved oxygen analyzer. The instrument was zeroed prior to inoculation by sparging the culture with N_2 (Matheson ultrapure). The media was then saturated with air and the instrument spanned to 100 percent.

4. Growth Measurement

Where necessary, culture samples were diluted with distilled water to give absorbance values less than 0.6. The absorbance was measured on a Hitachi-Perkin Elmer-Coleman 139 spectrophotometer at 420 nm. Tube cultures were monitored with a spectronic 20 spectrophotometer. Dry weight measurements were obtained by filtering 10 to 30 mls of culture through a predried and weighed 0.45 µm metricel millipore filter (see Appendix VIII). The filter was then dried at 105°C and weighed.

5. Glucose Analysis

Cells were removed from culture samples by centrifigation at 12,000 g for 15 minutes, and the supernatants were stored frozen until analyzed by the Sigma 510 glucose assay (glucose oxidase and peroxidase enzyme assay).

6. Ammonia Analysis

The ammonia from one ml of culture supernatant was collected by micro-diffusion and analyzed by the Nesslers procedure as described by Burris.[27] In some cases ammonia was determined with an Orion Model 95-10 electrode.

7. Hydrogen Analysis

A 125 ml culture flask (actual volume = 135 mls) was stoppered with a butyl rubber stopper and evacuated. A hypodermic needle was inserted into the fermentor exhaust tubing and connected to a second needle with a latex tube. Sampling was accomplished by inserting the second needle into the stopper of the evacuated flask and the flask was stored until analysis (3 days maximum). Samples were then withdrawn from the flask and analyzed on a Hewlett-Packard 5830A gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column. The integral of the H_2 peak was then compared to standard mixtures of H_2 prepared by injecting pure hydrogen into stoppered culture flasks filled with air. When tightly stoppered, these flasks appeared to hold the H_2 in the standard mixtures for at least 10 days (see Appendix VI).

8. Reversion Analysis

Reversion was monitored by spreading 0.5 mls of culture on agar plates made with the minimal media described above supplemented with 1 g/l NH_3.

B. Results and Discussion

1. Klebsiella pneumoniae

a. Glutamine Usage. The effect of the initial glutamine concentration on the final culture density is shown in Figure 1. Cultures (10 mls) were inoculated with minimal media inoculum and grown in stationary screw cap tubes at room temperature on minimal media with varying

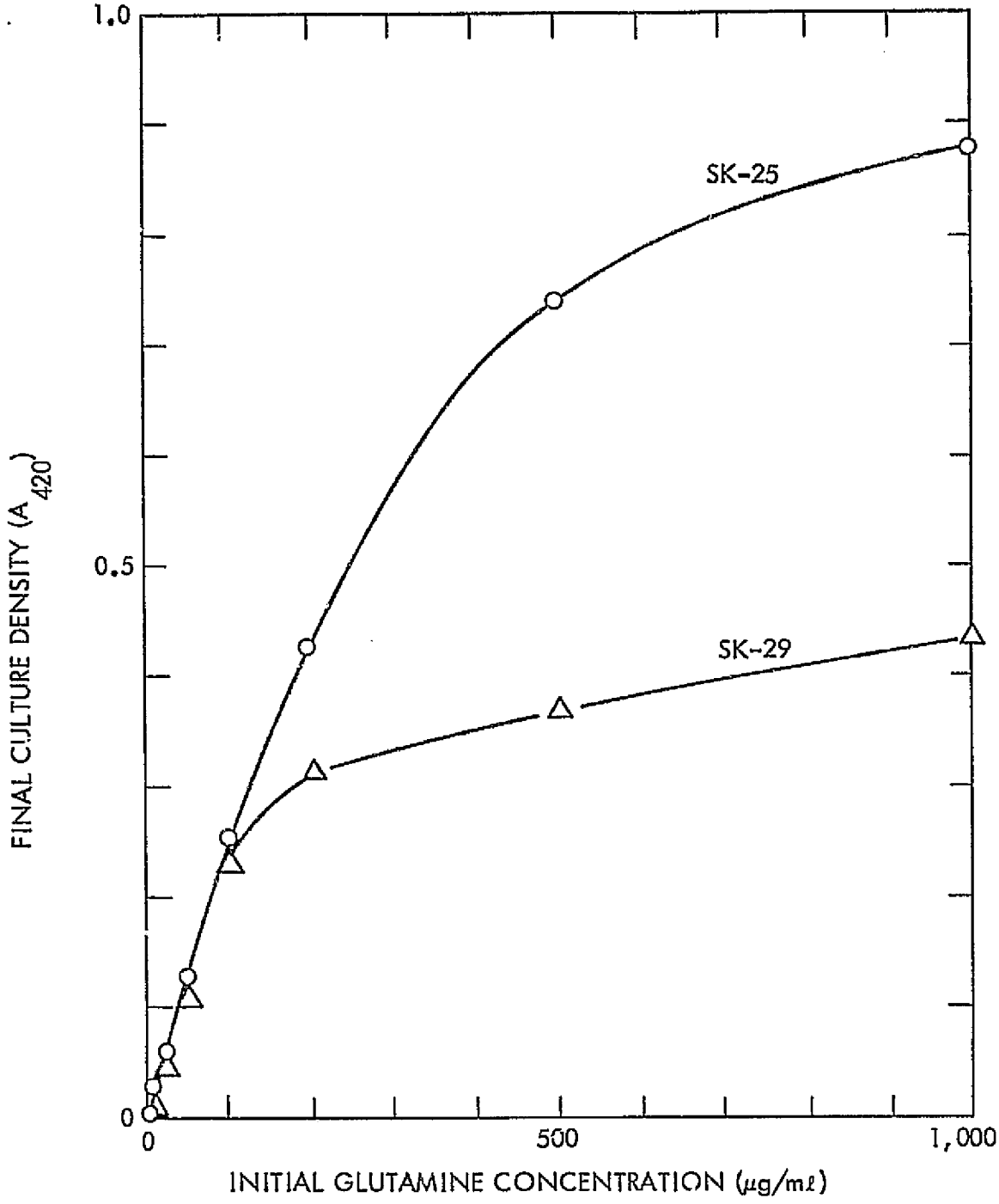

Figure 1. Final Culture Density Versus Initial $GluNH_2$ Concentration

amounts of glutamine. Growth was followed with a spectronic 20 spectrophotometer into stationary phase and at 35.5 hours the cultures were transferred to a 1 cm quartz cuvette and the culture density (A_{420}) was measured on a Hitachi (Model 139) spectrophotometer. These cultures, particularly the SK-29, tended to clump at high cell densities. This clumping may account for at least part of the difference in growth between the two strains and the decreased growth efficiency at high cell density. The important feature of the graph is the linear response of cell growth to glutamine concentration up to around 100 µg/ml glutamine. A growth efficiency of 0.255 A_{420}/100 µg glutamine/ml can be calculated from the slope of Figure 1.

b. Flask Cultures. Prior to scaling the Klebsiella fermentation to 5 liter capacity, flask cultures were run to compare glutamate and glutamine as nitrogen sources for cell growth. Experiments employed 250 mls of minimal media with 100 µg of amino acid per ml. Cultures were sparged with N_2 and inoculated with 3 mls (1.2 percent) of Luria broth inoculum. The pH of the cultures was controlled by manual addition of base and the pH fluctuated between 6.6 and 7.6. Optical density (420 nm) was measured with a spectronic 20 spectrophotometer and ammonia was determined with an Orion ammonia electrode (Model 95-10). Figure 2 presents the results for strain SK-25 growing on glutamine and Table 1 summarizes the results from the three cultures monitored in the experiment (see Appendix I for data). The total nitrogen added to the culture is calculated as glutamine or glutamate equivalent and includes the initial amino acid added, the periodic addition and the carryover of fixed nitrogen (110 µg glutamine or 220 µg glutamate equivalent/ml) from the Luria broth inoculum. Glutamine(glutamate) was added during growth, as indicated in Figure 2, in an attempt to periodically cycle the culture back into logarithmic growth phase to sustain nitrogenase activity. The addition of 30 µg and 33 µg/ml of glutamine at times 12 and 15 hours, respectively, did not appear to sustain logarithmic growth. The 50 µg/ml addition at 35.5 hours did produce significant additional growth but did not give a significant

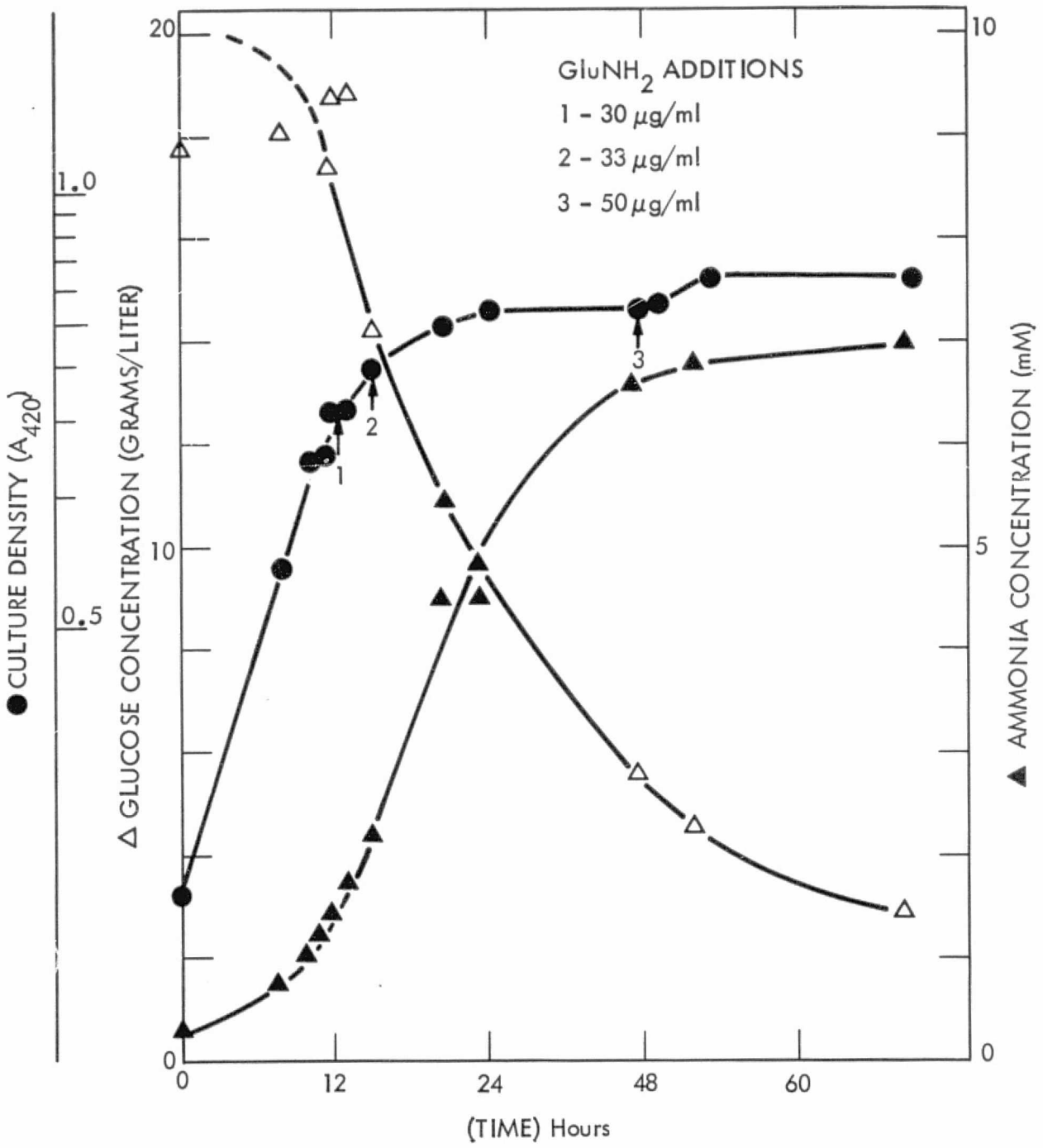

Figure 2. Ammonia Production by 250ml Flask Cultures of k. pneumoniae SK-25

Table 1. Summarized Data for Flask Experiments Using *Klebsiella pneumoniae*

	SK 25	SK 29	SK 29
Amino Acid Initial µg/ml	100 $gluNH_2$	100 $gluNH_2$	100 glu
Total N addition* µg/ml	320	320	425
Maximum growth A_{420}	0.66	0.66	0.46
NH_3 produced (mM)	6.7	7.4	4.4
Glucose consumed mg/ml	17	15	-
Moles glucose consumed per Mole of Ammonia formed	14.0	11.3	-
µMole NH_3 produced per µg glutamine (glutamate) added	0.021	0.023	0.010
A_{420} produced/100 µg amino acid addition.	0.206	0.206	0.11

*Includes Luria broth carryover from inoculation calculated as glutamine or glutamate equivalent on the basis of N content and all additions made up to 56 hours.

increase in NH_3 production. Table 1 shows that the overall efficiency of glucose utilization is 11.3 to 14 moles of glucose utilized per mole of NH_3 produced.

The maximum efficiency of glucose utilization as determined between 15.5 and 23.5 hours in Figure 2 is about 11 moles glucose consumed per mole of NH_3 produced, which is substantially less efficient than the optimum of about 4 moles glucose per mole of NH_3 produced which Andersen and Shanmugam[18] report for several Klebsiella strains. The μ-moles of ammonia produced per μg of glutamine added is 10-20 fold less than that reported by Andersen and Shanmugam (0.28-0.41 μ moles NH_3/ μg glutamine used). The A_{420}/100 μg of glutamine added (0.206) in Table 1 indicates efficient conversion of glutamine to cell mass. The large differences in the efficiency of ammonia production between these cultures and the runs of Andersen and Shanmugam appear to involve nitrogen fixation rather than cell growth. The pH fluctuation of the experiment is an unlikely candidate for this inefficiency due to the broad pH optimum for nitrogen fixation with this organism.[18]

c. Five Liter Experiments. Andersen and Shanmugam have studied a number of derepressed mutants of Klebsiella pneumoniae.[18] In these studies the bacteria were maintained in a dialysis bag (25 mls) suspended in approximately ten volumes of media to provide for dissipation of waste products and better feeding of the cell suspension. The dialysis system gave significant increases in ammonia production efficiency over previous experiments.[16] The dialysis experiments yielded a value of about 4 moles of glucose utilized per mole of NH_3 produced during the peak efficiency period and 7-9 moles glucose per mole of ammonia for the overall fermentation. The moles of ammonia produced per mole of glutamine consumed calculated for K. pneumoniae SK-25 was approximately 40-60 (0.28 - 0.41 moles NH_3/gram glutamine).

While the dialysis apparatus of Andersen and Shanmugam was advantageous for their experimental goals, it is not amenable to scale-up for ammonia production. In order to approximate their conditions on a

larger scale, a conventional fermentation was run without the inclusion of the dialysis bag. In this experiment 20 µg/ml glutamine were added initially and an additional 1 µg/ml was added at 24 hour intervals whereas Andersen and Shanmugam employed 4-8 µg/ml initial and a continuous feed of 0.5 µg/ml per day. The culture was sparged with Matheson ultrapure N_2 at 150 - 180 mls per minute and stirred at 400 rpm. Analyses are described in methods. A one percent minimal medium inoculum was used as seed for the culture. The results are shown in Figure 3 (data in Appendix II).

Due to the low glutamine concentration of the medium the cells entered stationary phase at a low cell density (A_{420} = 0.057) and remained in stationary phase until revertants, capable of growth on ammonia overran the culture at around 120 hours.

The maximum rate of ammonia production occurs in early stationary phase. A maximum efficiency of 3.0 moles of glucose per mole of ammonia formed occurs between 80 and 90 hours. The overall efficiency of glucose utilization from 0-115 hours is 6.2 moles of glucose per mole of NH_3 produced. During this period the culture consumed 13 µg glutamine per ml to yield 13.2 moles of ammonia per mole of glutamine used. The glucose efficiency values from Figure 3, while somewhat less, show reasonable agreement with the data of Andersen and Shanmugan[18] and indicate that the dialysis apparatus is not required for attaining a relatively high efficiency system. Even though SK-25 is quite stable, less than one revertant per 10^{10} organisms,[20] five liter cultures routinely revert thus prohibiting large fermentations with this organism unless very inexpensive methods of growth control can be devised. While the slightly altered glutamine addition schedule (see methods) may have adversely affected the efficiency of glutamine usage, the low value compared to that of Andersen and Shanmugan is likely due to reversion of the culture.

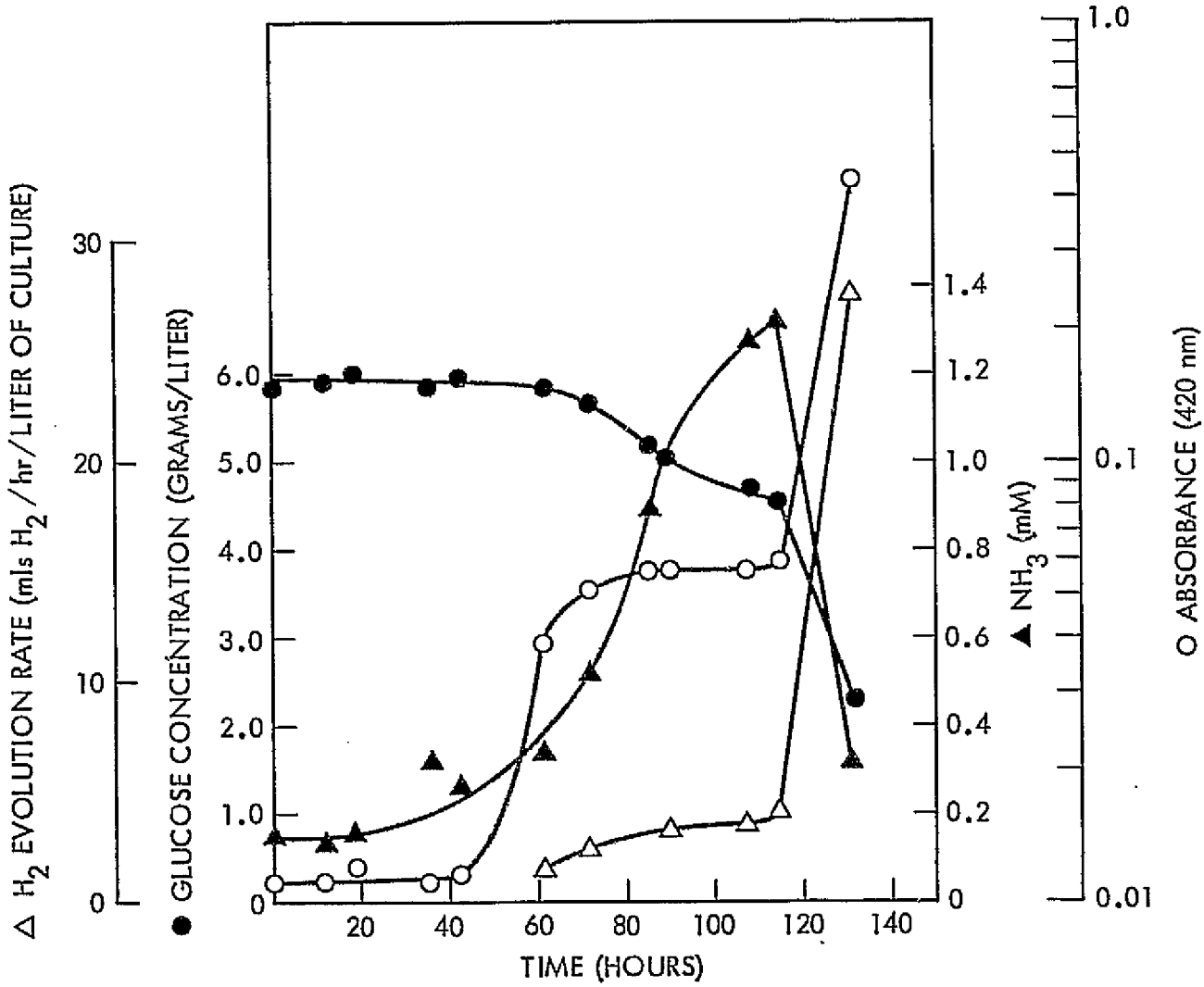

Figure 3. Ammonia and Hydrogen Production by K. pneumoniae SK-25

In an attemnt to increase the final ammonia levels in the media, fermentations with higher initial glutamine concentrations were run. Table 2 summarizes the data (see Annendices III & IV) for these experiments alonq with that of Figure 3. Table 2 indicates that utilization of glutamine for the production of cell mass is nearly as efficient at high culture densities as at low densities. While the final ammonia concentration is increased in high density cultures the efficiency drons such that more qlucose is consumed ner mole of ammonia formed. We expect that this decrease in efficiency at high culture density is due to fermentation rroducts which accumulate in the media.

Table 2. Data Summary for 5ℓ Fermentations

	A	B	C
Initial glutamine added (μg/ml)	10	300	500
Total glutamine added (μg/ml)	13	300	500
Maximum growth* (A_{420})	0.046	0.63	1.29
NH_3 produced≠ (mM)	1.17	1.0	5.4
Glucose Consumed≠ (g/l)	1.3	5.6	16.2
Moles glucose consumed per mole NH_3 formed	6.2	31	16.7
Moles NH_3 produced per μg glutamine added	0.090	0.0033	0.010
A_{420} produced/100 μg glutamine added	0.35	0.21	0.26

*Maximum stationary phase density.

≠Taken just prior to reversion, in A at 115 hrs., B at 48 hrs. and C at depletion of media glucose (41.5 hrs).

d. Hydrogen Production. The rate of hydrogen production per liter of culture shown in Figure 3 (data in Appendix V) parallels culture density as expected. The overall hydrogen production from 61.5-132 hours is 1.1 moles of H_2 per mole of glucose consumed. Since nitrogenase is only responsible for about 0.65 moles of H_2 per mole of NH_3 produced[18] and the production of one mole of NH_3 consumes 6.2 moles of glucose, the majority of the H_2 formed (91 percent) comes from the conversion of pyruvate to acetate + H_2 + CO_2.[26] One possible application of nif-derepressed heterotrophs is to enhance the production of H_2 from a carbon substrate. An estimate of the maximum obtainable increase in H_2 production due to nitrogenase can be calculated from the above data by assuming that a similar culture is grown in the absence of N_2 to divert all energy and reductant to the production of H_2 and that one mole of NH_3 is equivalent to 1.5 moles of H_2. The total H_2 produced would then be that observed plus the H_2 equivalent of the ammonia formed. Since 6.2 moles of glucose are required to produce one mole of NH_3, the total H_2 formed per glucose would be:

$$\frac{1.1 \text{ moles } H_2}{\text{mole glucose}} + \frac{1 \text{ mole } NH_3}{6.2 \text{ moles glucose}} \times \frac{1.5 \text{ moles } H_2}{\text{mole } NH_3} = 1.34 \frac{\text{moles } H_2}{\text{mole glucose}}$$

The hydrogen production attributable to nitrogenase under the above assumption is:

$$\frac{\frac{1.5 \text{ moles } H_2}{\text{mole } NH_3} + \frac{0.65 \text{ mole } H_2 \text{ (side rxn)}}{\text{mole } NH_3}}{\frac{6.2 \text{ moles glucose}}{\text{mole of } NH_3}} = 0.35 \frac{\text{moles } H_2}{\text{mole glucose}}$$

The expected percent increase in the production of H_2 by nitrogenase is:

$$\frac{\frac{0.35 \text{ moles } H_2}{\text{mole glucose}}}{1.34 \frac{\text{moles } H_2}{\text{mole glucose}} - 0.35 \frac{\text{moles } H_2}{\text{mole glucose}}} \times 100 = 35\%$$

The enhancement of H_2 production by nitrogenase is therefore substantial. The energy to power nitrogenase is presumably harvested at the expense of cell mass production and represents a valid increase in H_2 production efficiency.

e. Theoretical limits. A theoretical upper-limit for ammonia (or H_2) production can be made by assuming 2 ATP molecules used per $2e^-$ transferred and ignoring requirements for cell maintenance energy. Klebsiella pneumoniae SK-25 can obtain 2 moles of ATP and 2 moles of NADH by the fermentation of glucose to pyruvate and one additional ATP per mole of glucose from the clastic cleavage of pyruvate to H_2, CO_2 and acetate.[28] Assuming $3e^-$ transferred per molecule of NH_3 produced and 2 ATP molecules hydrolyzed per $2e^-$ transferred, one mole of glucose would yield sufficient ATP and more than enough reductant to produce one mole of NH_3 (or 3/2 moles of H_2). While this value cannot be achieved in practice it does represent an upper limit to the efficiency of the Klebsiella system.

f. Tolerance to Ammonia. Since the final ammonia concentration of the fermentation medium is an important economic parameter, and ammonia can be toxic to cellular processes, a preliminary experiment was conducted to determine the toxicity of ammonia to Klebsiella. The growth rate of 10 ml tube cultures of Klebsiella pneumoniae SK-25 was monitored on a spectronic 20. The doubling times at the various ammonia concentrations are shown in Table 3. These data indicate that much higher concentrations perhaps up to 0.1M could be tolerated by Klebsiella.

g. Settling of Cells. A preliminary settling experiment was run to determine if cell settling is possible as an inexpensive harvesting method for Klebsiella cells. The cells from the fermentation presented in Figure 3 were used to fill 300 ml beakers. The pH of the beakers was adjusted as shown in Table 4 and the cultures allowed to stand for 24 hours. After 24 hours an aliquot was removed from the center of the beaker and the A_{420} observed. A similar aliquot from which the cells were removed by centrifugation was used as a blank. Table 4 indicates that adjustment of cultures to pH 3.0 may provide conditions which are adequate for settling of cells.

2. Azotobacter vinelandii UW590

a. Effect of pH on Growth. While Azotobacter vinelandii UW590 is fully derepressed for nitrogenase synthesis,[21] all of the nitrogen fixed is used for cell growth. Measurements of free ammonia in growing and stationary cultures routinely yielded values below 0.1 mM even at high cell densities. The only product of nitrogen fixation considered for this organism therefore, is cell mass. Where cultures are grown with N_2 as the sole nitrogen source, cell growth is an indirect measure of nitrogen fixation. Figure 4 presents the effect of pH on the doubling time of Azotobacter vinelandii UW590 growing on N_2 and N_2 + 50 mM NH_3. Cultures (5 mls) were grown in 18 x 150mm culture tubes at an incline of approximately 20^0 from horizontal in a water bath shaker at 120 oscillations per minute and at 30^0C. Culture density was monitored in a spectronic 20 spectrophotometer and the

Table 3. Ammonia Tolerance of Klebsiella

NH_4CL mM	Doubling Time
0	5 hrs. 30 min.
1	5 hrs. 5 min.
10	5 hrs. 55 min.
50	5 hrs. 30 min.
100	6 hrs. 40 min.

Table 4. Settling of Klebsiella pneumoniae SK-25

pH	A_{420}*
1	0.16
3	0.08
5	0.46
7	0.54
9	0.42
11	0.40

*Absorbance of sample after 24 hours of settling. The initial absorbance was 0.49

doubling time was determined during the initial logarithmic growth phase. Figure 4 shows that cultures growing on ammonia are somewhat more resistant to lowered pH than those growing on N_2 indicating that the sensitivity of N_2 fixation is the factor which limits growth of Azotobacter on N_2 below pH 6.5. The marked increase in sensitivity of NH_4^+ grown cultures to high pH is somewhat surprising. This is possibly due to inhibition by free ammonia since the doubling time for these cultures increases near the expected titration curve of ammonia (pK_a = 9.25).

Figure 5 illustrates the effect of pH on efficiency of growth. Cultures were grown as described in the previous paragraph except the glucose concentration was lowered to 5g/l to facilitate the determination of glucose. At the end of growth (A_{420} < 0.7) the glucose remaining in the cultures was determined by the Sigma 510 method and subtracted from the initial glucose concentration. The relative efficiency is calculated as the optical density (A_{420}) produced per mg/ml of glucose utilized. These results indicate a pH optimum around 7.0 - 7.5 and a marked inhibition of growth efficiency (nitrogen fixation) below pH 6.5.

b. Effect of Aeration on Growth of A. Vinelandii UW590. Figure 6 shows the time course of cell mass production at various air flow rates indicating that the production of high cell densities requires high aeration (data in Appendix VII). The minimum doubling time for the organism on N_2 appears to be around 3 hours based on the optical density data.

Oxygen concentration is known to markedly affect the activity of nitrogenase in the Azotobacter genus. These organisms are obligate aerobes and fix nitrogen only in the presence of oxygen. High oxygen concentration on the other hand inhibits the nitrogenase enzyme, hence a distinct optimum O_2 concentration is observed. This optimum is variable and depends on the previous oxygen levels to which the culture was adapted.[20]

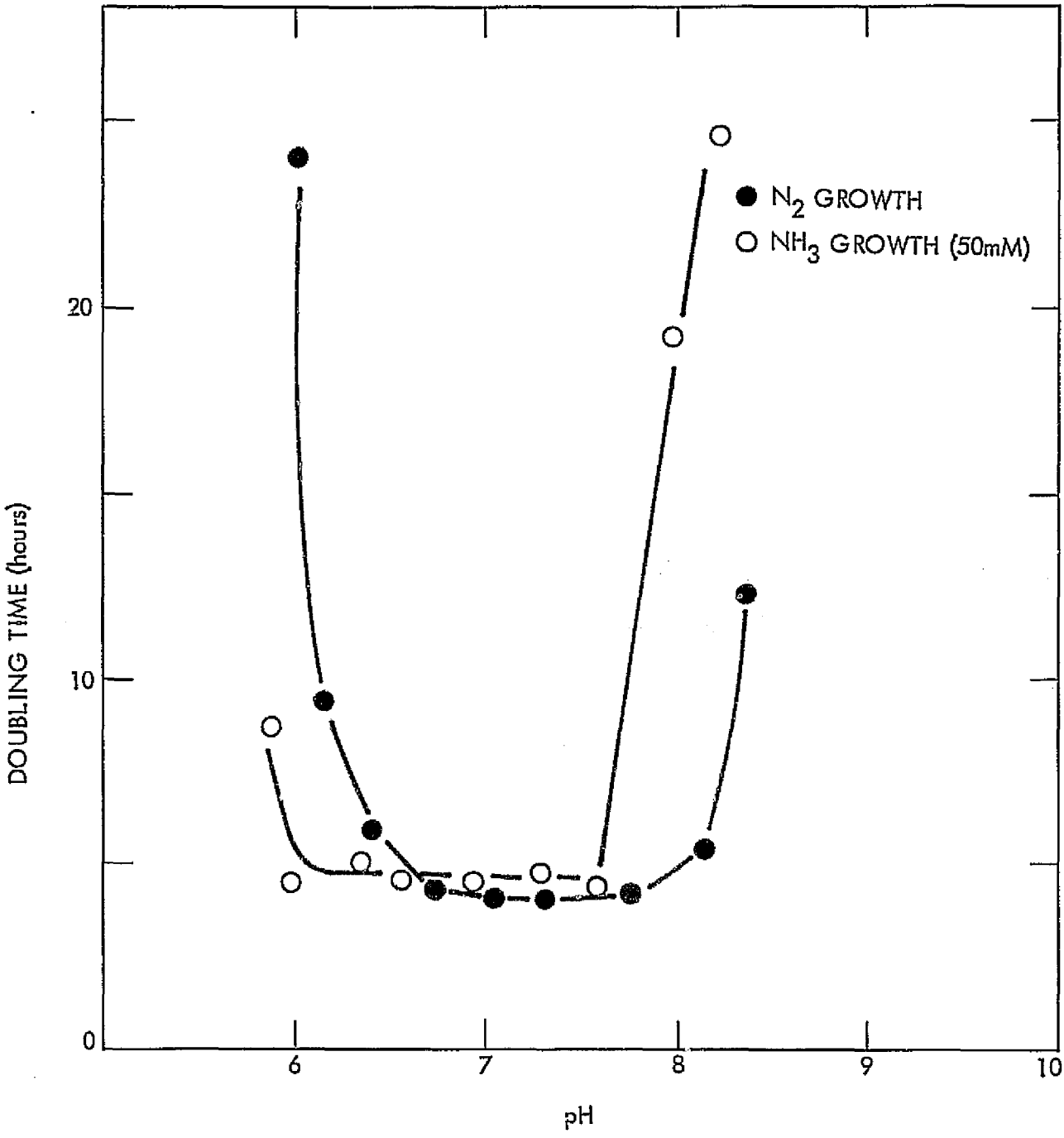

Figure 4. pH Dependence of Growth of Azotobacter vinelandii UW590

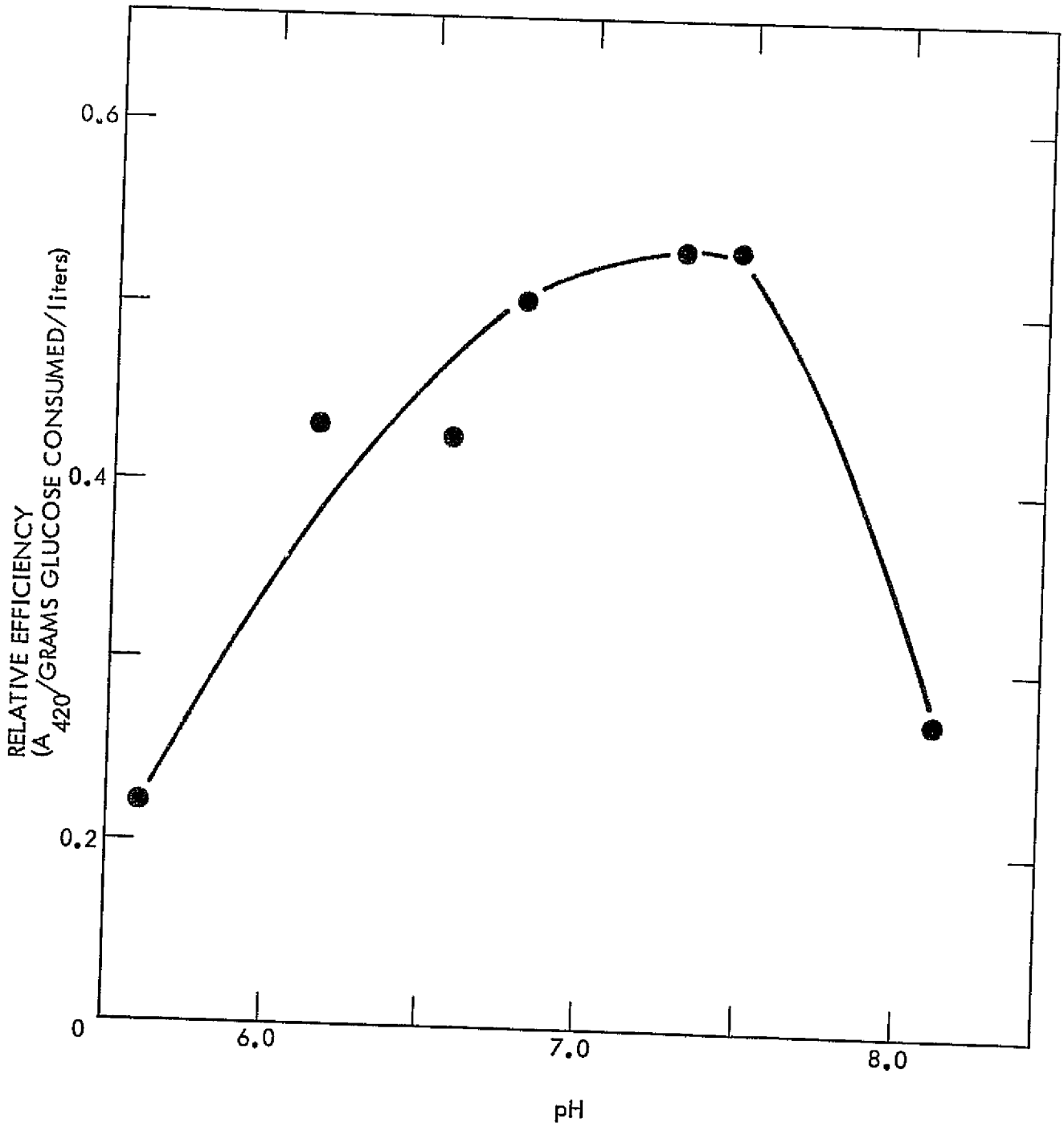

Figure 5. pH Dependence of Growth Efficiency of A. vinelandii UW590

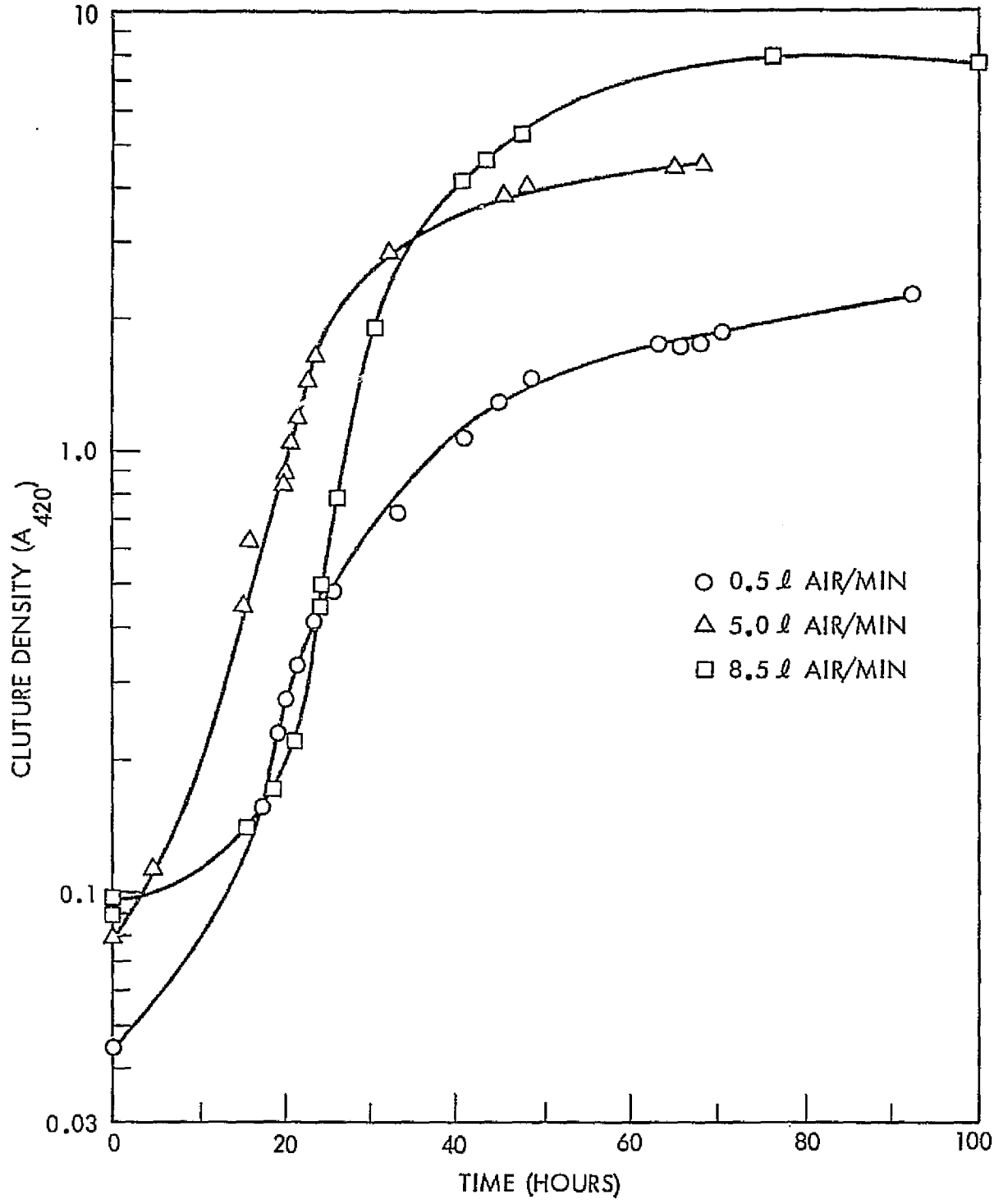

Figure 6. Effect of Aeration on Growth of A. vinelandii UW590

Figure 7 illustrates the variation of the efficiency of nitrogen fixation as a function of O_2 concentration.

The efficiency of growth in Figure 7 is calculated as the grams of cells produced per gram of glucose used. This curve was generated using data obtained by extrapolating the dry weight curve on the basis of the optical density. (A_{420} = 1.0 corresponds to a culture dry weight of 0.38 g/l.) (See Appendix VIII.) Above optical densities of 1.0,cultures show a linear relationship between optical density and dry weight. Large deviations from linearity were observed, however, with some cultures below optical density 1.0. This phenomenon may be associated with rapid growth rates and /or growth at high O_2 concentrations.

During the initial phase of the culture, while the oxygen concentration is high, the growth efficiency remains low. Once the oxygen concentration has dropped to near zero the efficiency increases to a maximum and substantially declines as the culture density increases. This decline is presumably due to energy starvation of the culture by oxygen limitation. Figure 8 shows similar data for all three experiments shown in Figure 6. In each case the growth efficiency decreases with increasing culture density. No maximum is observed in the two low aeration runs because O_2 concentration had gone to zero and measurable decreases in the media glucose occurred at or past the period of peak efficiency. Figure 8 indicates that the operation of high density cultures at reasonable efficiencies requires considerable aeration of the medium. The shift of the efficiency curve to higher culture density with increasing aeration also indicates that the efficiency decrease with culture density is due to O_2 limitation.

c. Efficiency of Nitrogen Fixation. If one assumes that the cell mass produced in these experiments contains 10 percent nitrogen content, the maximum efficiency of fixation is around 50 mg N fixed per gram of glucose consumed which is at the high end of the reported values

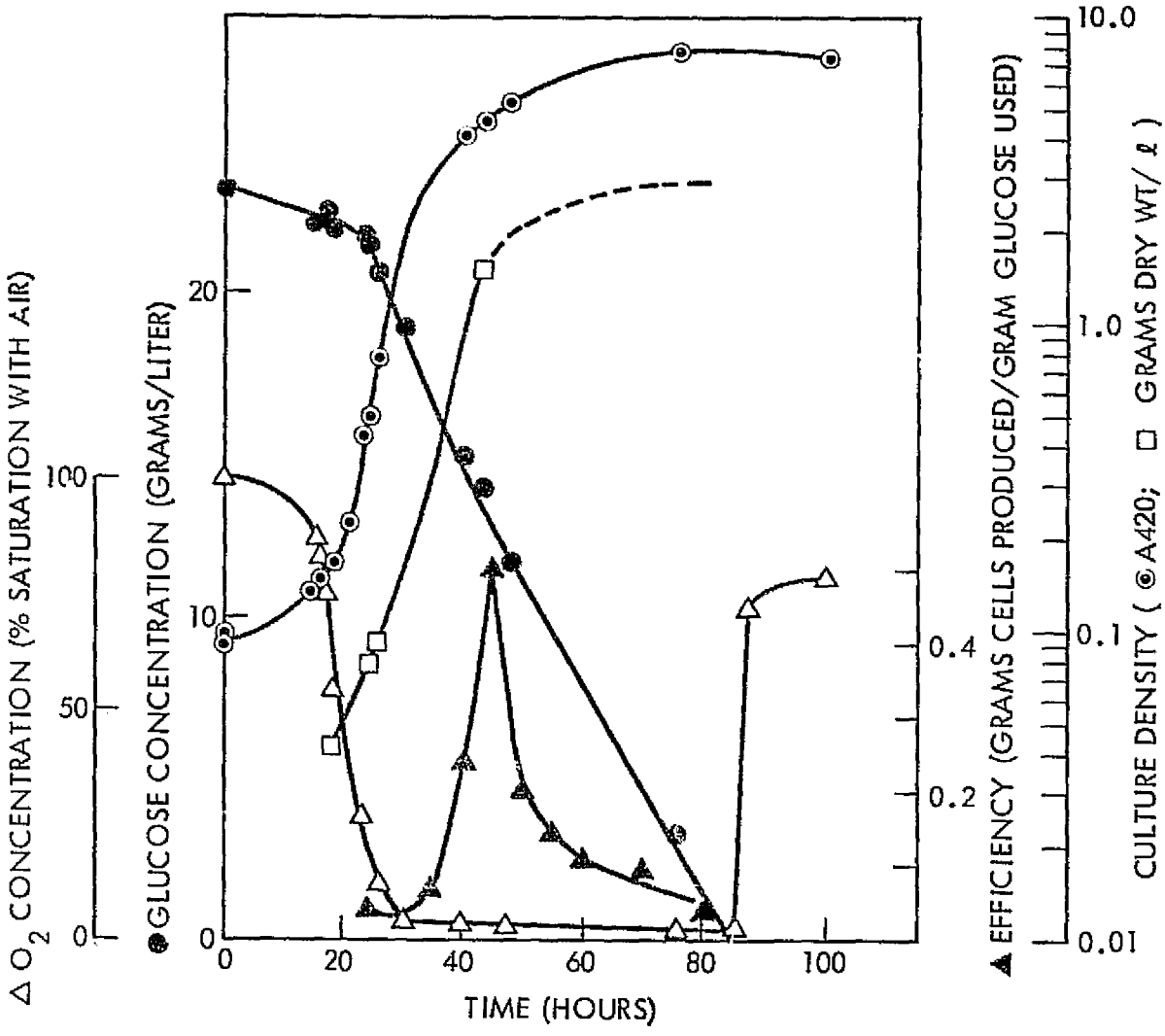

Figure 7. Time Course of A. vinelandii UW590 Growth at High Aeration

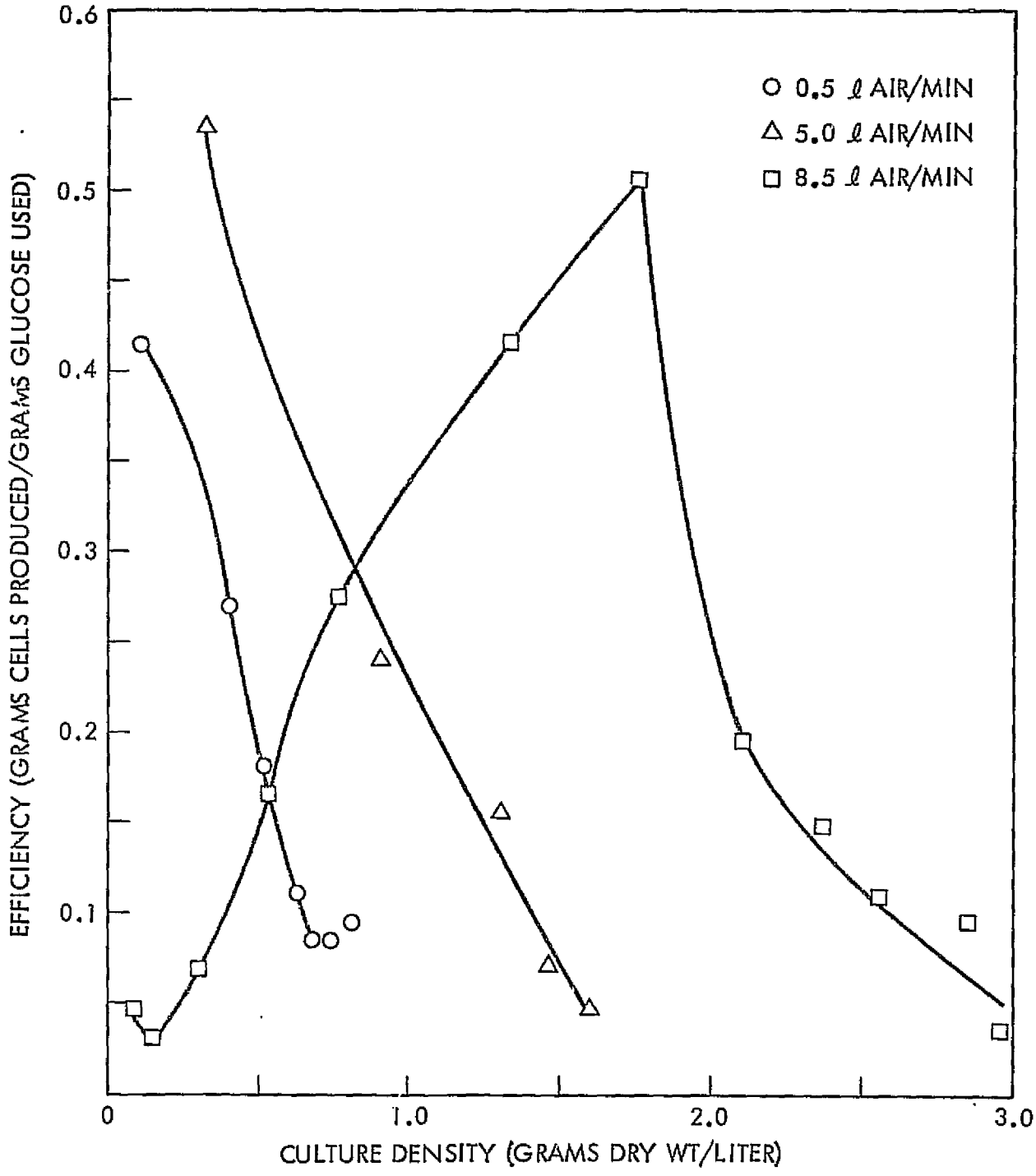

Figure 8. Growth Efficiency of A. vinelandii UW590 Versus Culture Density

for Azotobacter,[30,31] Leahy recently observed efficiency values with Azotobacter vinelandii UW590 approach 100 mg N per gram of glucose consumed which is roughly twice as efficient as the best reported values to date.[32] Since Leahy's values were based on cultures with very small consumption of carbohydrate, additional work appears appropriate to substantiate this unusually efficient value. If this work is confirmed, Leahy's conditions are the best reported values for Azotobacter to date.

Mulder[33] has proposed a theoretical efficiency limit of 280 mg N fixed/gram of glucose consumed (0.28 moles of glucose/mole of ammonia formed) for aerobic systems by assuming that two molecules of ATP are hydrolyzed per $2e^-$ transferred to nitrogenase, that $6e^-$ are required for the reduction of one N_2 molecule and that all of the energy from the oxidation of glucose (38 ATP equivalent) is available for nitrogen fixation.

V. ECONOMIC EVALUATION

For the engineering and economic assessment, calculations are based on experimental data obtained from 5-liter batch fermentations. Optimum conditions of temperature and pH were obtained from shake flask and test tube data and from literature results. Normal scale-up techniques of similar power input per unit volume of liquid or similar volumetric oxygen transfer coefficients were not used in going from shake flask or test tube to the 5-liter scale because of the small volumes involved. Normally these techniques are most suitable in scaling from laboratory and pilot plant equipment to industrial production. For the purpose of preliminary feasibility studies, the 5-liter scale is adequate; however, more important than scale would be to obtain consistently reliable data in continuous culture. Studies in continuous cultivation for an extended time period would yield valuable information on product yield, sterilization requirements and reversion difficulties.

The economic evaluation of the nif-derepressed mutants is based on a daily production of 9080 kgNH_4^+ (10 tons NH_4^+) for K. pneumoniae and 9080 kg Fixed N for A. vinelandii. This is a small scale for commercial nitrogen fixation where plants are normally in the 1000 ton/day capacity. Because of the vast quantities of liquid involved, which result from the dilute concentration of fixed nitrogen in the medium, capital investment is immense with conventional fermentation. In addition, annual costs are determined to a large degree by nutrient requirements; therefore, no advantage is apparent from larger scale is production. For lagoon systems some advantages for larger scale are likely in the areas of capital investment and operating labor.

A. Economic Model

1. Conventional Fermentation

Estimates of fixed-capital investment and total product costs for conventional fermentation are based on procedures discussed by Peters and Timmerhaus.[34] These approximate cost figures are basically predesign cost estimates suitable for determining feasibility of proposed investment. Estimated cost of processing equipment is based on published cost data corrected to the current cost index. Equipment installation, instrumentation and control, piping, buildings, service facilities, engineering and supervision, construction expenses and contingencies are estimated by a suitable percentage of purchased equipment, direct costs, etc. Raw material costs, in most cases, are obtained from Chemical Marketing Reporter. Utilities, maintenance, depreciation and other manufacturing costs are determined by suitable percentage of such items as fixed-capital investment. It is emphasized that these cost figures are only estimates and are dependent upon factors such as plant location and type of process. The predesign estimates are summarized in Tables 5 and 6.

2. Lagoons

Estimated capital and operational costs for lagoon fermentation are patterned after calculations of Benemann, et. al.,[35] for large algae ponds. In some cases manufacturer's bulletins and personal communication with suppliers are utilized. Table 7 outlines the basic format employed for estimation of capital investment and annual operating cost for lagoon fermentations.

3. Ammonia Recovery

For ammonia recovery, such methods as electrodialysis and reverse osmosis were not considered as a result of uneconomical cost figures reported by Dryden[36] for mineral removal from waters.

Table 5. Estimation of Fixed-Capital Investment Cost (from Peters and Timmerhaus[34])

I.	Direct Costs
	A. Equipment
	1. Purchased equipment (estimated from published cost data)
	2. Installation, including insulation and painting (25 percent of purchased equipment)
	3. Instrumentation and Controls, installed (10 percent of purchased equipment)
	4. Piping, installed (10 percent of purchased equipment)
	B. Buildings, process and auxiliary (20 percent of purchased equipment)
	C. Service facilities and yard improvement (40 percent of purchased equipment)
	D. Land (5 percent of purchased equipment)
II.	Indirect Costs
	A. Engineering and Supervision
	B. Construction Expenses
	C. Contingency
	Total Indirect Costs taken at 25 percent of total Direct Cost
III.	Fixed Capital Investment = direct costs plus indirect costs.

Table 6. Estimation of Total Product Cost (from Peters and Timmerhaus[34])

I. Manufacturing cost = direct production cost plus fixed charges plus plant overhead costs

   A. Direct Production Cost

      1. Raw materials (estimated from current market prices)
      2. Operating labor (estimated from manpower requirements)
      3. Direct Supervisory and Clerical Labor (10 percent of labor)
      4. Utilities (5 percent of total product cost)
      5. Maintenance and repairs (5 percent of fixed capital investment)
      6. Operating supplies (0.5 percent of fixed capital investment)

   B. Fixed Charges

      1. Depreciation (10 percent of equipment plus 2 percent of buildings)
      2. Local Taxes (2 percent of fixed capital investment)
      3. Insurance (one percent of fixed capital investment)

   C. Plant Overhead Costs (50 percent of labor, supervision and maintenance

II. General Expenses

   A. Administrative (15 percent of labor, supervision and maintenance)

   B. Distribution and Selling Cost (not considered)

   C. Research and Development (not considered)

   D. Financing (7 percent of capital investment)

III. Total Product Cost = manufacturing cost plus general expenses

Table 7. Format for Estimation of Capital Investment and Annual Cost for Lagoon Fermentations

I. Total Capital Investment

1. Growth Basin

a. Excavation ($1/yd^3)

b. Liner and Cover ($1/sq. ft.)

c. Panels (not used)

d. Pumps and Piping

e. Concrete ($1/sq. ft.)

2. Seed Tank

3. Settling Ponds

4. Ammonia Recovery System for K. pneumoniae

5. Contingencies

II. Annual Operating Costs

1. Capital (at 8 percent for 20 years)

2. Raw Materials

3. Power Requirements

4. Operating Labor

5. Maintenance (0.5 percent of capital investment)

6. Land (at $2000/acre)

Other methods of ammonia removal such as air stripping were judged impractical. For air stripping, air requirements are about 2.5 m^3/liter at a cost of $.00 5/1000 liter. Lime requirements for pH adjustment amount to an additional $.00 8/1000 liter. These costs are only for ammonia removal with no regard for recovery which would be excessive considering the vast quantity of air involved.

Selective ion exchange was selected as the method of choice for ammonia concentrations of 500 mg/l or less. Economics for ammonia recovery at low concentrations employing selective ion exchange are based on estimated costs at the Upper-Occoquan sewage treatment plant in Virginia.[37] Tables 8, 9, and 10 summarize basic design criteria, regeneration and regenerant recovery system, and estimated capital cost and operating expenses for 15 mgd and 22.5 mgd capacities.

For ammonia recovery at a concentration of 1000 mg/l, cost figures are based on equipment requirements from the coking of coal industry. In addition, economic data from the comparison of ammonia separation processes by Bonham and Atkins[38] were used. In the coking industry, ammonia is recovered as ammonium sulfate from a 1 percent ammonia solution. Steam stripping is employed in the lime still as depicted in Figure 9. From the still the gas is contacted with a 5-10 percent sulfuric acid solution, where ammonia is absorbed with formation of solid and crystalline ammonium sulfate. Lead-lined vessels are used for the saturator. Figure 10 depicts the saturator and final recovery system.

Table 8. Design Criteria Selective Ion Exchange Process for Ammonium Removal at the Upper Occoquan Plant (Virginia)[37]

Flow Rate	15 mgd (0.66 m^3/sec)
Beds in service	4
Beds in regeneration	2
Beds - backup capacity	2
Flow per bed	3.75 mgd (0.16 m^3/sec)
Bed loading rate	10.82 bed volumes/hr
	5.25 gpm/sf (3.6 l/m^2/sec)
Backwash rate	8 gpm/sf (5.41 m^2/sec)
Bed volumes to exhaustion	145
Average ammonia removal efficiency	95%
Average influent ammonia nitrogen concentration	20 mg/l
Average effluent ammonia nitrogen concentration	1 mg/l
Normal concentration of ammonia nitrogen at initiation of regeneration	2.5 mg/l
Clinoptilolite size	20 x 50 mesh
Clinoptilolite depth	4 ft (1.22 m)

Table 9. Regeneration and Regenerant Recovery System Design Criteria at the Upper Occoquan Plant (Virginia)[37]

Regeneration system[a]	
Number of regenerant tanks	4
Size of each tank	375,000 gals (1420 m^3)
Number of beds regenerated at once	2
Number of regeneration cycles per day	3.58
Regeneration bed volumes	39-44
Regenerant recovery system[a]	
Recovery system flow rate	1.080 gpm (68 l/sec)
Operation time per day	16 hr
Number of Units	2
Diameter	35 ft (10.66 m)
Overflow rate	800 gpd/sf (32.6 m^3/m^2/day)
Ammonia removal and recovery process[a]	
Number of ARRP modules	18
Liquid loading rate	760 gpd/sf (31.1 m^3/m^2/day)
Air to liquid loading rate	566 cf/gal (4234 m^3/m^3)
Media height	7.5 ft (2.29 m)
Removal efficiency at 10°C	90%
at 20°C	95%

[a]At 15 mgd flow rate

Table 10. Estimated Costs of Selective Ion Exchange at the Upper Occoquan Plant (Virginia)[37]

Item	Estimated costs, $/mil gal at 15 mgd (0.66 m^3/sec)	Estimated costs, $/mil gal at 22.5 mgd (0.99 m^3/sec)
Operating and maintenance[a]		
Chemicals		
NaOH	$ 26.80	$ 26.80
NaCl	7.10	7.10
H_2SO_4	9.80	9.80
	$ 43.70	$ 43.70
Income from sale of $(NH_4)_2$ SO_4 at $43/ton	$(12.60)	$(12.60)
Net chemical cost	31.10	31.10
Power, 18 HP/mil gal at $0.0192/kwh	6.90	6.90
Labor	17.70	17.70
Total, O & M	$ 55.70	$ 55.70
Capital[a]		
$4,470,000, 20 years at 7 percent	$ 77.22	$ 51.59
Total annual cost[a]	$132.92	$107.29

[a]August, 1974 costs

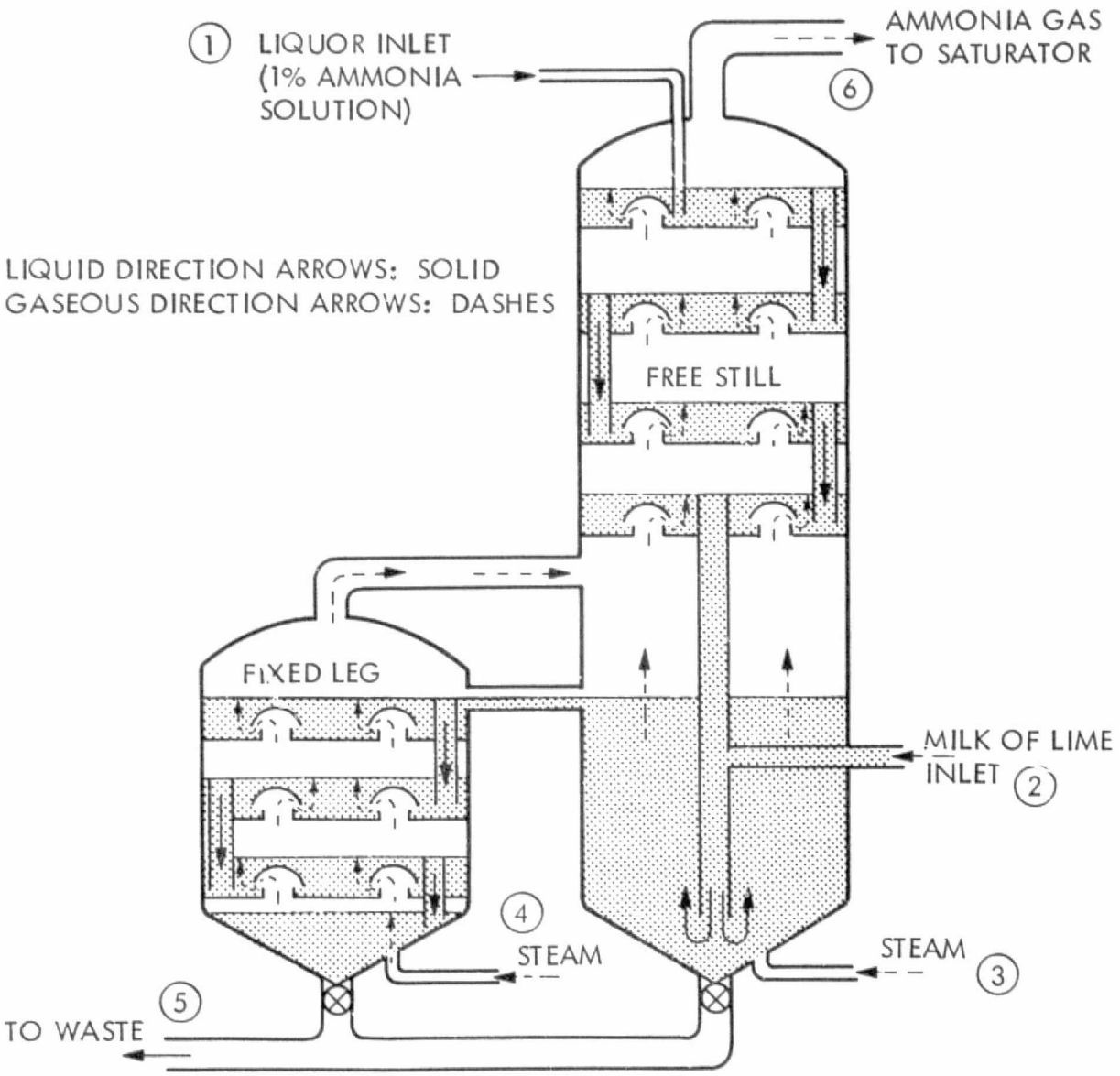

Figure 9. Diagram of Ammonia Still

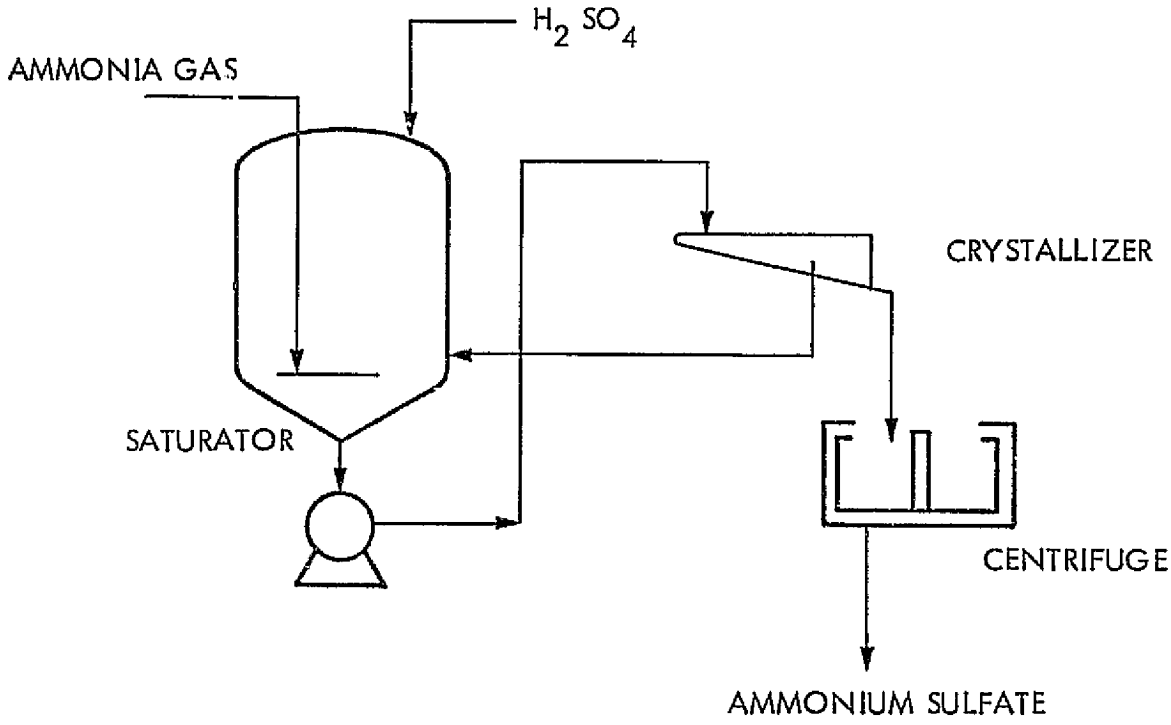

Figure 10. Ammonia Recovery System from the Coking Industry [39]

B. Results and Discussion

1. Klebsiella pneumoniae

a. Klebsiella pneumoniae, SK-25, in Conventional Fermentation. The continuous fermentation scheme for ammonia production is depicted in Figure 11. Settling basins are employed for cell recovery and both ion exchange and steam stripping are considered for ammonia recovery. In either case, ammonium sulfate is the final product.

For the design of a large-scale continuous fermentation based on batch data, the procedure as outlined by Aiba, et al[40] is used. It is emphasized that this design procedure is suitable only if the microbial characteristics in batch runs are representative of those in continuous operation. Aiba, et al[40] state: "For the rational design of a large-scale plant, however, there is no substitute for data obtained from continuous cultivation experiments in pilot-plant equipment."

Figure 12 represents typical batch data plotted in the form of dN/dt as a function of t where N is optical density and t is fermentation time. The operating line or material balance line on this graph establishes fermentation volume and residence time for a given volumetric flow rate and optical density in the continuous reactor system.

For cell population N = 1.2

$$NH_4^+ = 5.4 \text{ mMolar}$$
$$= 0.1 \frac{g}{\ell} NH_4^+$$

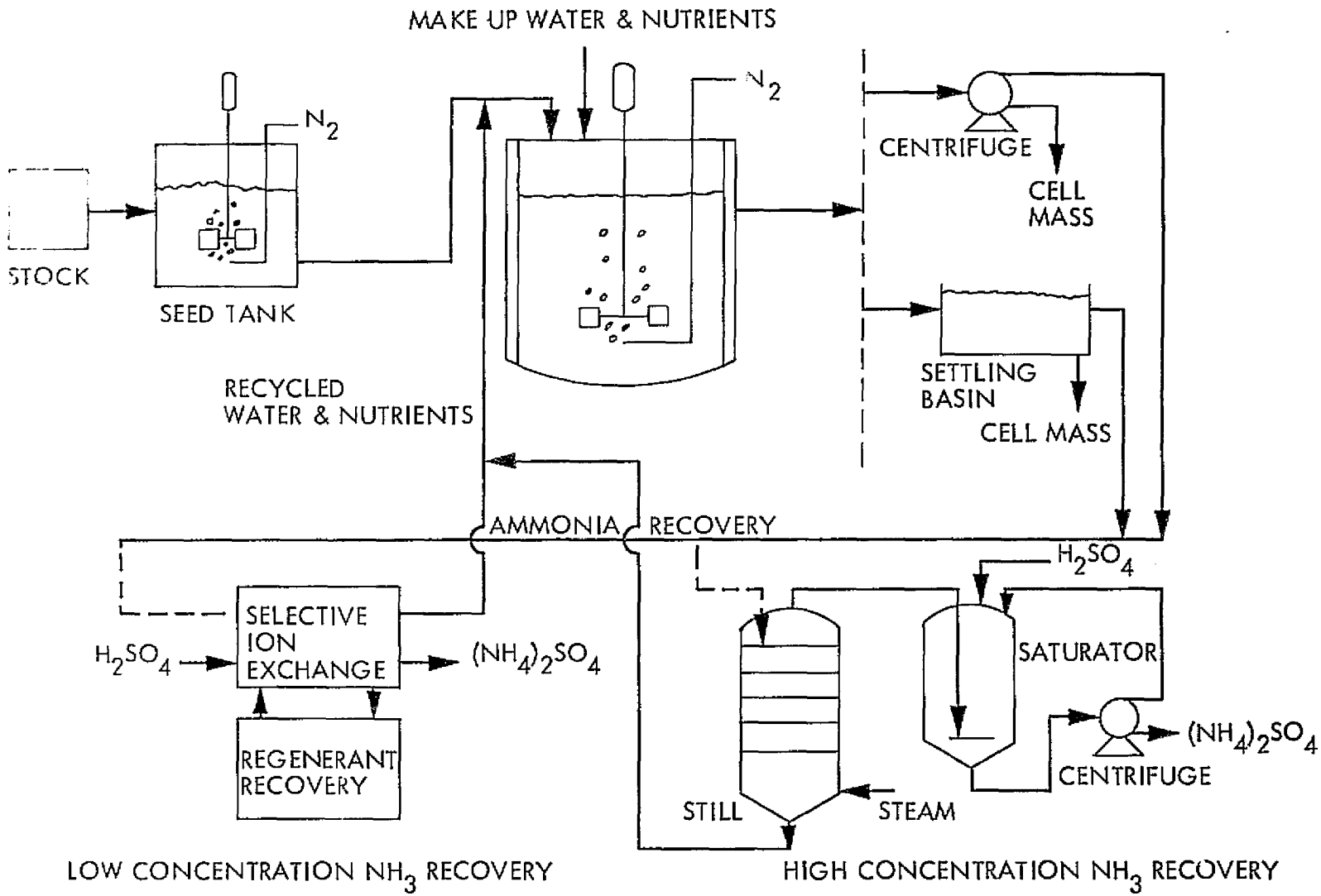

Figure 11. Continuous Fermentation by K. pneumoniae for Ammonia Production and Recovery

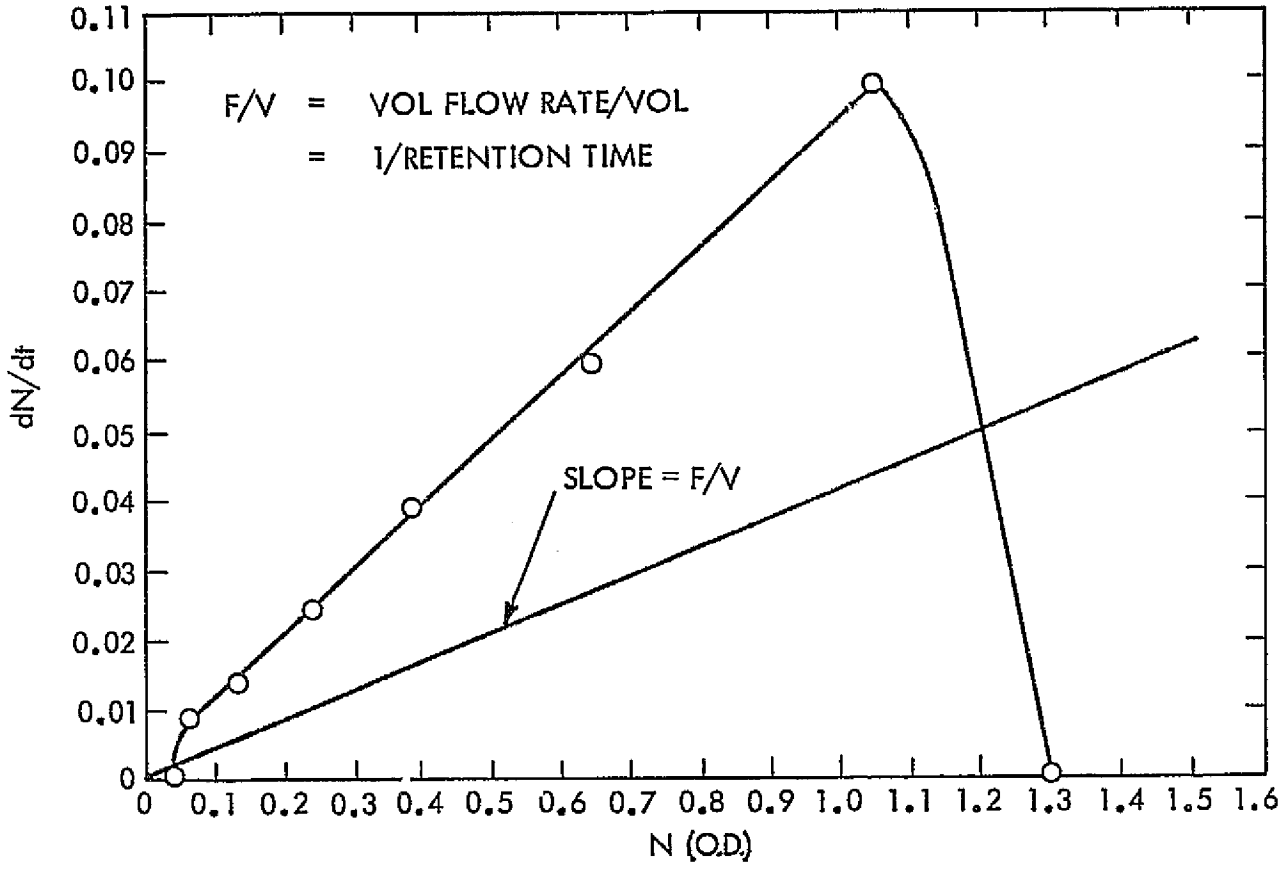

Figure 12. Prediction of Continuous Operating Conditions from Batch Data for K. pneumoniae

For 9080 kg/day NH_4^+ = 10 ton/day NH_4^+

$$10 \frac{\text{ton}}{\text{day}} \times 2000 \frac{\text{lb.}}{\text{ton}} \times 454 \frac{\text{g}}{\text{lb.}} \times \frac{\ell}{0.1\ \text{g}} = 9.1 \times 10^7 \frac{\ell}{\text{day}}$$

$$= 2.4 \times 10^7 \frac{\text{gal}}{\text{day}}$$

$$= 1 \times 10^6 \frac{\text{gal}}{\text{hr.}}$$

$$= 3.8 \times 10^6 \frac{\text{liter}}{\text{hr.}}$$

From dN/dt versus N plot

$$\frac{F}{V} = \frac{0.25}{1.2} = 0.042\ \text{hr}^{-1}$$

$$F = 3.8 \times 10^6 \text{ liter/hr}$$

$$V = 3.8 \times 10^6 \text{ liter/hr} \times \frac{\text{hr}}{0.042} = 90.5 \times 10^6 \text{ liters}$$

For cell population N = 1.1

$$NH_4^+ = 5.4 \text{ mMolar}$$

$$F = 3.8 \times 10^6 \text{ liter/hr.}$$

$$\frac{F}{V} = \frac{0.095}{1.1} = 0.086\ \text{hr.}^{-1}$$

$$V = \frac{3.8 \times 10^6}{0.086} = 43.6 \times 10^6 \text{ liter}$$

For the purpose of economic assessment several assumptions were desirable for ammonia production by conventional fermentation:

1) Glutamine costs are not included even though SK-25 requires glutamine or mixed amino acids for growth. Glutamine or glutamate requirements of available Klebsiella mutants exceed the value of the fixed nitrogen products; therefore a cheap amino acid source is needed. This requirement might be met economically with hydrolyzed fish scrap ($179/1000 kg at 60 percent protein) or possibly by hydrolyzed cell mass from the fermentation but these costs are not estimated since the use of these materials is speculative.

2) Sterilization costs are not included as steam sterilization of millions of gallons of media is prohibitively expensive for a cheap product such as ammonia.

3) Only carbohydrate, water, nitrogen gas, and recovery chemicals are included in raw material costs.

4) Settling ponds are suitable for cell separation and recovery. Definitive data are not available to indicate the feasibility of this; however, preliminary settling studies indicate that settling is possible with proper pH adjustment. Centrifugation for cell recovery is prohibitively expensive.[41]

5) Ammonia recovery is by selective ion exchange for ammonia concentrations of 500 mg/l and less, and steam stripping with sulfate precipitation is the method of choice for a concentration of 1800 mg/l.

6) Eighty percent recycle of water and unconsumed nutrients is assumed.

Sample calculations of cost estimates of Klebsiella pneumoniae in conventional fermentation are included for low and high ammonia concentrations in Appendices IX and X, respectively.

Figure 13 represents the effect of ammonia concentration on the product cost for glucose, molasses, and a free carbon source for a capacity of 9080 kg NH_4^+/day (10 tons NH_4^+/day). The experimental data from this study at high cell density corresponds to an NH_4^+ concentration of about 97 mg/l. For this NH_4^+ concentration $(NH_4)_2\ SO_4$ cost is in excess of $3,300/1000 kg if the carbohydrate is free. The current market price of $(NH_4^+)_2\ SO_4$ is $71/1000 kg. The lowest product cost in Figure 13 is represented by free carbohydrate and NH_4^+ concentration of 1800 mg/l corresponding to the maximum-level before growth inhibition was observed in test tube studies. Even assuming that this concentration could be attained in a fermentation system, carbohydrate is free, and that the assumptions listed above are reasonable, the economics are still grossly unfavorable.

Figure 14 depicts the effect of energy consumption on product cost for glucose and free carbohydrate. The energy consumption of 13.9 moles glucose/mole NH_4^+ corresponds to experimental data of this study at high cell density while 3.8 moles glucose/mole NH_4^+ corresponds to data from the literature [18] at low cell density. Calculations for the highest energy consumption noted in Figure 14 are based on a hypothetical NH_4^+ concentration of 1800 mg/l with fermentation time and glucose consumption assumed to be the same as observed in this study; this corresponds to an efficiency of 1.37 moles NH_4^+/mole glucose which is in excess of the theoretical limit of 1.0 for the anaerobic systems.

From Table 11, representing fixed capital investment (F.C.I.) and manufacturing cost for various NH_4^+ concentrations, it is apparent that F.C.I. is excessive for

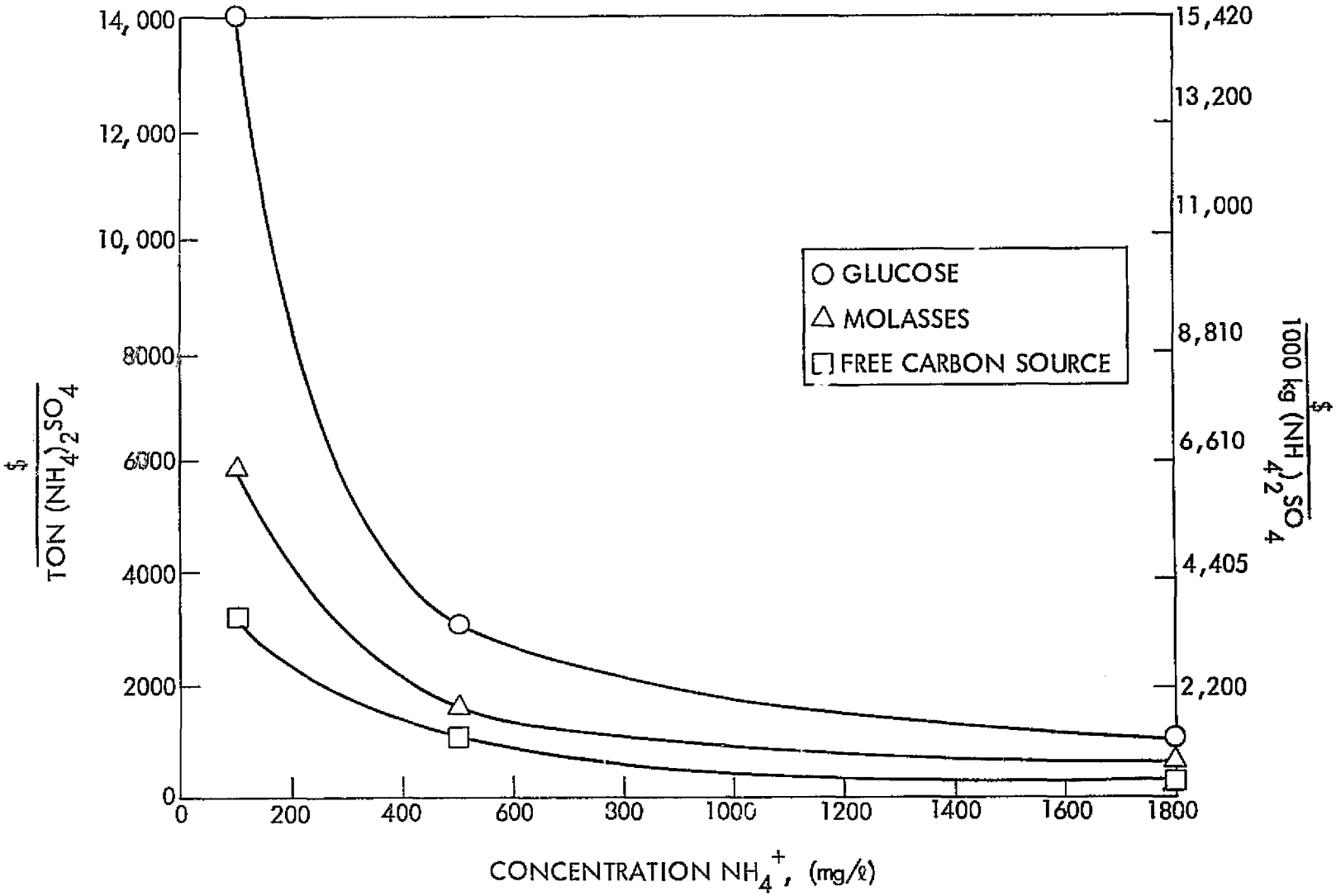

Figure 13. Cost of Ammonia Production by K. pneumoniae as a Function of Ammonia Concentration in Conventional Fermentation

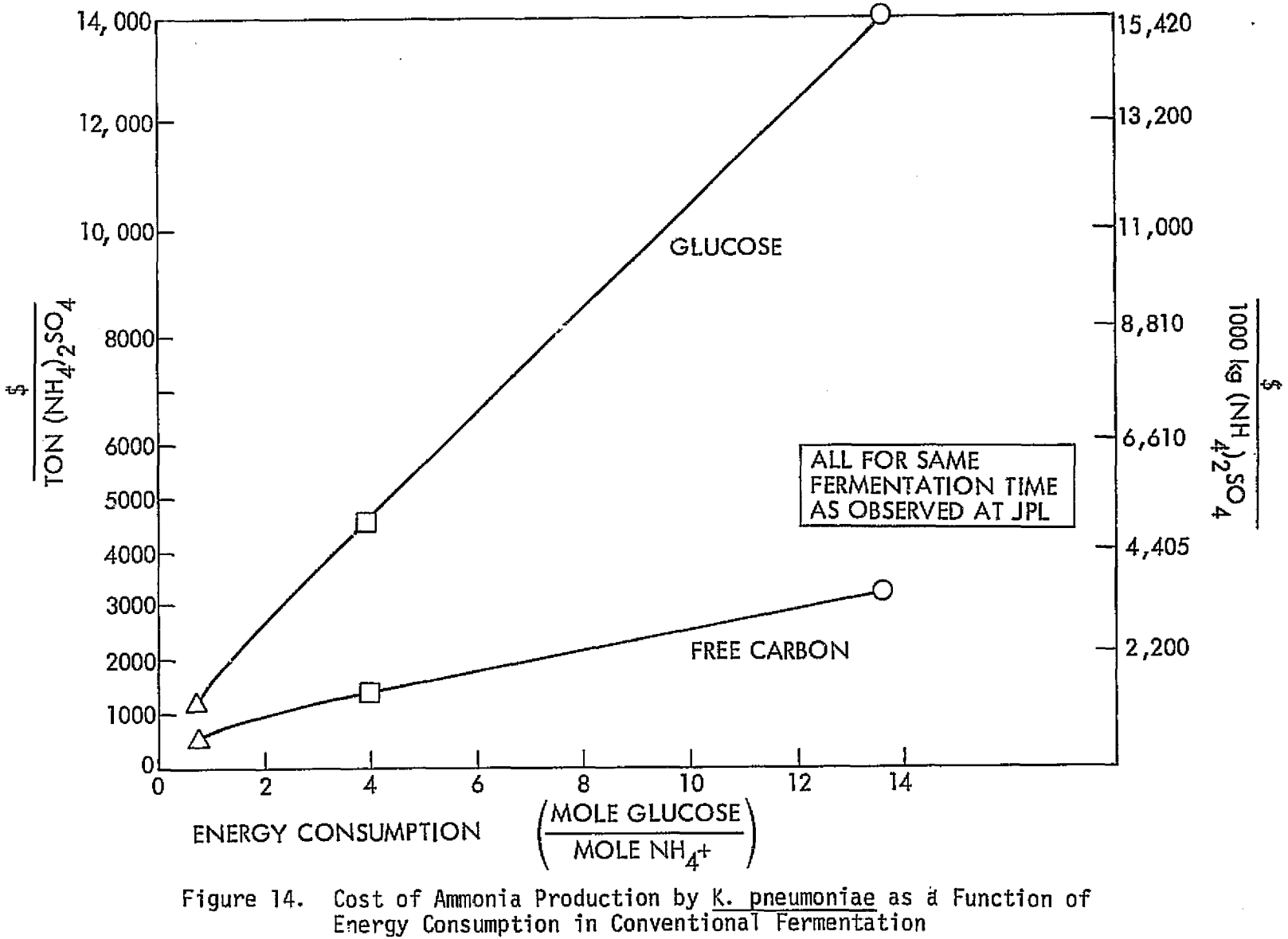

Figure 14. Cost of Ammonia Production by K. pneumoniae as a Function of Energy Consumption in Conventional Fermentation

TABLE 11. CAPITAL INVESTMENT AND MANUFACTURING COSTS FOR *K. PNEUMONIAE* IN CONVENTIONAL FERMENTATION (9080 kg NH_4^+/DAY = 10 TON NH_4^+/DAY)

NH_4^+ (mg/ℓ)	F.C.I. $ MILLION	MANUFACTURING COST $ MILLION		
		GLUCOSE	MOLASSES	FREE CARBON
97	144.2	199.8	81.4	44.9
500	34.4	43.9	21.2	14.4
1800	15.7	15.0	8.6	6.7

a conventional fermentation scheme. Large fermentation equipment is needed when producing a relatively cheap material such as ammonia in dilute solution. Manufacturing costs are extremely dependent on raw material requirements.

b. Klebsiella pneumoniae, SK-25, in Lagoons. The ammonia production scheme for K. pneumoniae growing in a lagoon is similar to that depicted in Figure 11 for conventional fermentation except for replacement of the stirred fermenter with a shallow plastic-lined lagoon. Some concrete support for the lagoon was considered in the economic assessment as well as a plastic cover for the anaerobic system. Settling ponds for cell separation as well as ammonia recovery techniques are the same as for the conventional fermentation. Sample calculations of cost estimates for K. pneumoniae in lagoons is presented in Appendix XI.

The effect of NH_4^+ concentration in the lagoon on product cost is presented in Figure 15. Product costs based on data of this study at high cell density are represented at an NH_4^+ concentration of 97 mg/l. An NH_4^+ concentration of 1800 mg/l corresponds to an upper limit before growth inhibition (Table 3). At the upper limit of NH_4^+ concentration, with free carbon, and with the same basic assumptions stated above, the product cost of $(NH_4)_2\ SO_4$ is \$100/1000 kg. For lower concentrations in the lagoon, production costs increase rapidly.

The effect of energy consumption on product cost is shown in Figure 16. Once again, the data of this study at high cell density correspond to an energy consumption of 13.9 moles glucose/mole NH_4^+. The lowest energy consumption considered, i.e., 0.73 moles glucose/mole NH_4^+, corresponds to 1800 mg NH_4^+/l with the assumption that the same amount of carbon would be consumed, 13.2 g/l, as was observed in the fermentation of high cell density of this study.

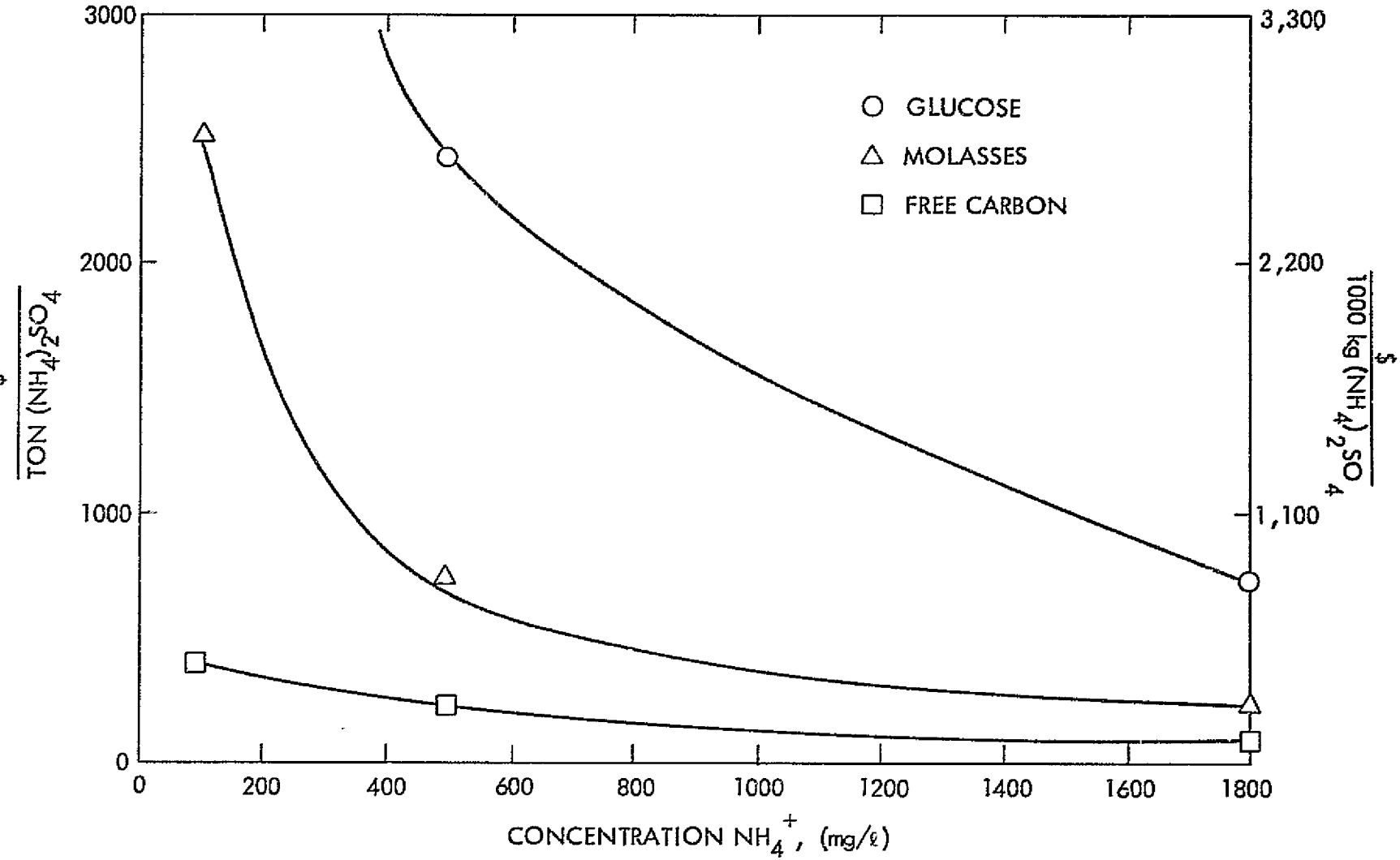

Figure 15. Cost of Ammonia Production by K. pneumoniae as a Function of Ammonia Concentration in a Lagoon System

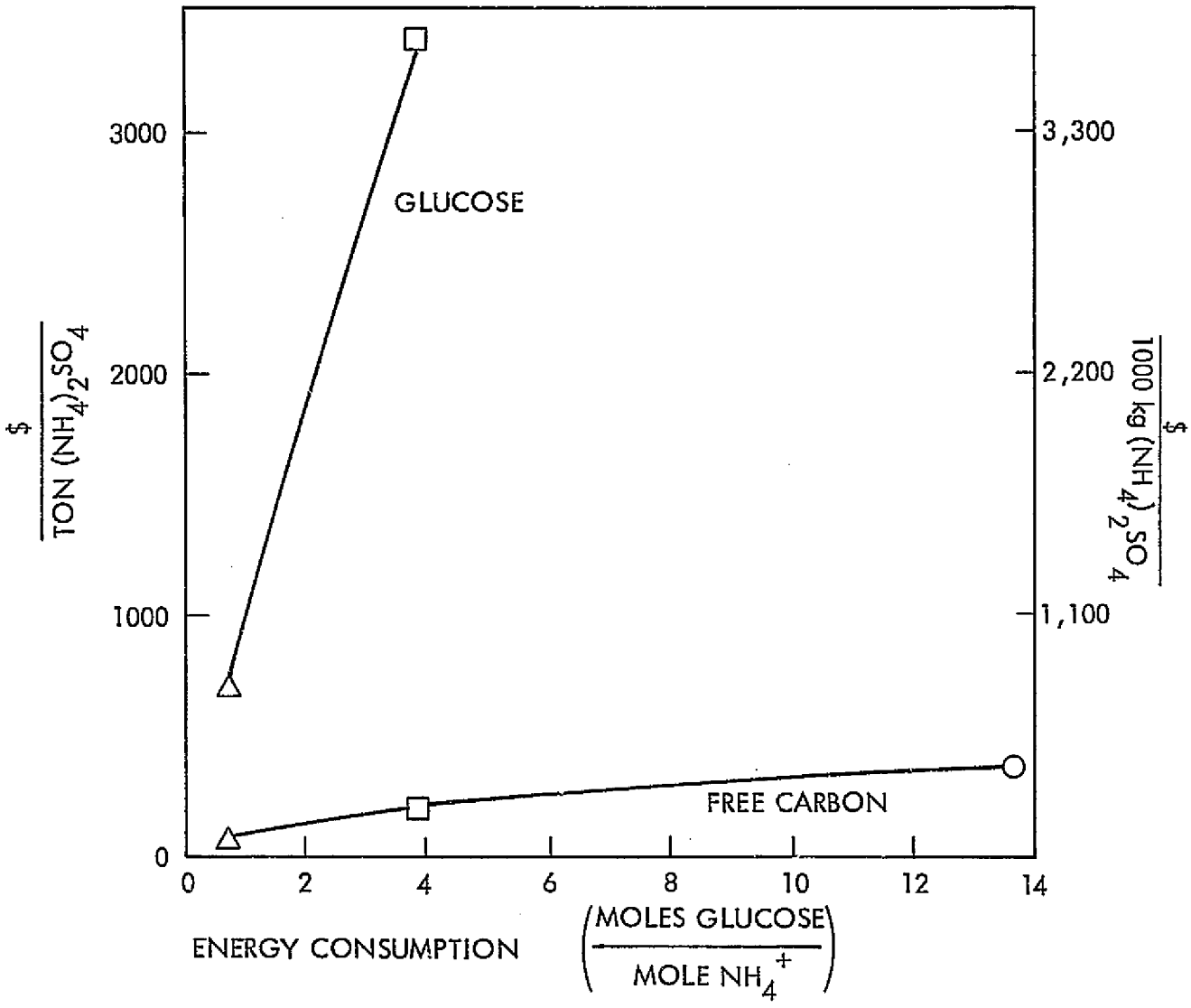

Figure 16. Cost of Ammonia Production by K. pneumoniae as a Function of Energy Consumption for a Lagoon System

Since capital costs are much less for a lagoon than for agitated steel vessels, the economics for the lagoon system become much more dependent on raw material costs. As a result of this, the difference in product cost for glucose and a free carbon source is even more significant than for a conventional fermentation system. Table 12 presents the results of NH_4^+ concentration on fixed capital investment and manufacturing costs for the lagoon system.

c. Hydrogen and Other Product Formation by Klebsiella pneumoniae. The most promising application of nif-derepressed mutants (of Klebsiella) appears to be the enhancement of hydrogen production since the value of hydrogen produced in the fermentation (1.1 moles of H_2/mole glucose consumed) at $4.40/kg is roughly 20 fold greater than that of the ammonia produced, and the H_2 equivalent of ammonia (assuming 1.5 moles H_2 per mole of ammonia) is 3.5 times that of ammonia.

From Figure 3, (much higher levels of H_2 production would be reached at higher concentrations of cell mass).

NH_3 = 1.3 mMole/l

$$H_2 = 1.1 \frac{\text{mole } H_2}{\text{mole glucose}}$$

Cell mass (O.D = 0.06) = 0.02 g/ℓ

glucose consumed = 1.5 g/ℓ

Accetate production is about 1 mole/mole glucose from ref.[28].

Product value/10^6 liter

$$H_2 \qquad 1.1 \frac{\text{mole } H_2}{\text{mole glucose}} \times \frac{\text{mole}}{180\text{g}} \times \frac{1.5\text{g}}{\ell} \times \frac{1\text{b}}{454\text{g}} \times \frac{2\text{ g}}{\text{mole}} \times$$

$$\frac{\$2}{1\text{b}} \times 10^6 \text{ liter} = \frac{\$80.70}{10^6 \text{ liter}}$$

TABLE 12. CAPITAL INVESTMENT AND MANUFACTURING COSTS FOR K. PNEUMONIAE IN A LAGOON SYSTEM (9080 kg NH_4^+/DAY = 10 TON NH_4^+/DAY)

NH_4^+ (mg/ℓ)	F.C.I. $ MILLION	MANUFACTURING COST $ MILLION		
		GLUCOSE	MOLASSES	FREE CARBON
97	8.7	152	38.6	5.2
500	2.85	32.6	9.9	3.07
1800	3.05	9.41	3.08	1.22

$$\underline{NH_3} \quad 1.3 \frac{mMole}{\ell} \times 10^6 \text{ liter} \times \frac{mole}{10^3 \text{ mMole}} \times \frac{17g}{mole}$$

$$\times \frac{lb}{454g} \times \frac{\$0.075}{lb} = \frac{\$3.65}{10^6 \text{ liter}}$$

This corresponds to a hydrogen value twenty-two times greater than that of ammonia. For high cell density, with greater carbohydrate utilization and, consequently, greater hydrogen yield, the ratio of hydrogen to ammonia value is even greater. The consideration of hydrogen as a valuable product of the K. pneumoniae fermentations could significantly affect the economic feasibility of large scale nitrogen fixation by the free-living microbe. In order to adequately measure the significance of hydrogen production, consideration would have to be given to the technical and economic problems associated with hydrogen recovery, purification, compression, storage, etc. If only product value is considered with no regard for attendant hydrogen recovery and purification costs, then it is obvious that annual manufacturing costs are less than product value as the cost of carbohydrate becomes free. In fact, it would appear at first blush that a carbohydrate cost of about $ .04/kg would yield hydrogen of comparable value. The activity of nitrogenase in present strains provides a significant increase over wild type H_2 production. Hydrogen production would also be simplified since contaminating microbes would be unable to use the product in the anaerobic environment. Using a non-auxotroph, culture growth could be limited by addition of ammonia, and problems of reversion, amino acid supplementation, and sterilization could be largely eliminated. While wild type cultures could presumably be employed, nif-derepressed control mutants similar to A. vinelandii UW590 would be preferred since nitrogenase would not be repressed by fixed nitrogen contamination of the carbon material utilized. A by-product of this process would be acetate which could be used as a fermentation substrate or presumably harvested as methane. Hydrogen production in conjunction with anaerobic digestion should be explored, possibly in a two-stage fermentation system, as a potential application for suitable Klebsiella mutants.

2. Azotobacter vinelandii

a. Azotobacter vinelandii, UW590, in Conventional Fermentation. The economic assessment of A. vinelandii in both the lagoon and conventional fermentation employed several assumptions:

1) Sterilization costs are not included. This may not be a severe restriction or limitation on the actual economic feasibility since nitrogen is not included in the media and, therefore, only nitrogen fixing organisms could contaminate.

2) Settling ponds are suitable for cell separation and recovery.

3) Eighty percent of the water and unconsumed nutrients are recycled.

4) Surface aeration is adequate for cell growth in the lagoon. Experimental data show that final cell density increases with aeration; therefore, surface aeration in a lagoon may be totally inadequate for high yield of cellular mass.

5) Growth rates in the lagoon are assumed to be one-half the rate observed in a stirred fermenter.

Figure 17 depicts dx/dt as a function of x where x is cell density and t is time for an A. vinelandii fermentation at high aeration. As explained for the Klebsiella fermentations, this type of graph is used to predict continuous fermenter conditions on the basis of batch data.

Sample calculations of cost estimates for A. vinelandii in conventional fermentation appear in Appendix XII.

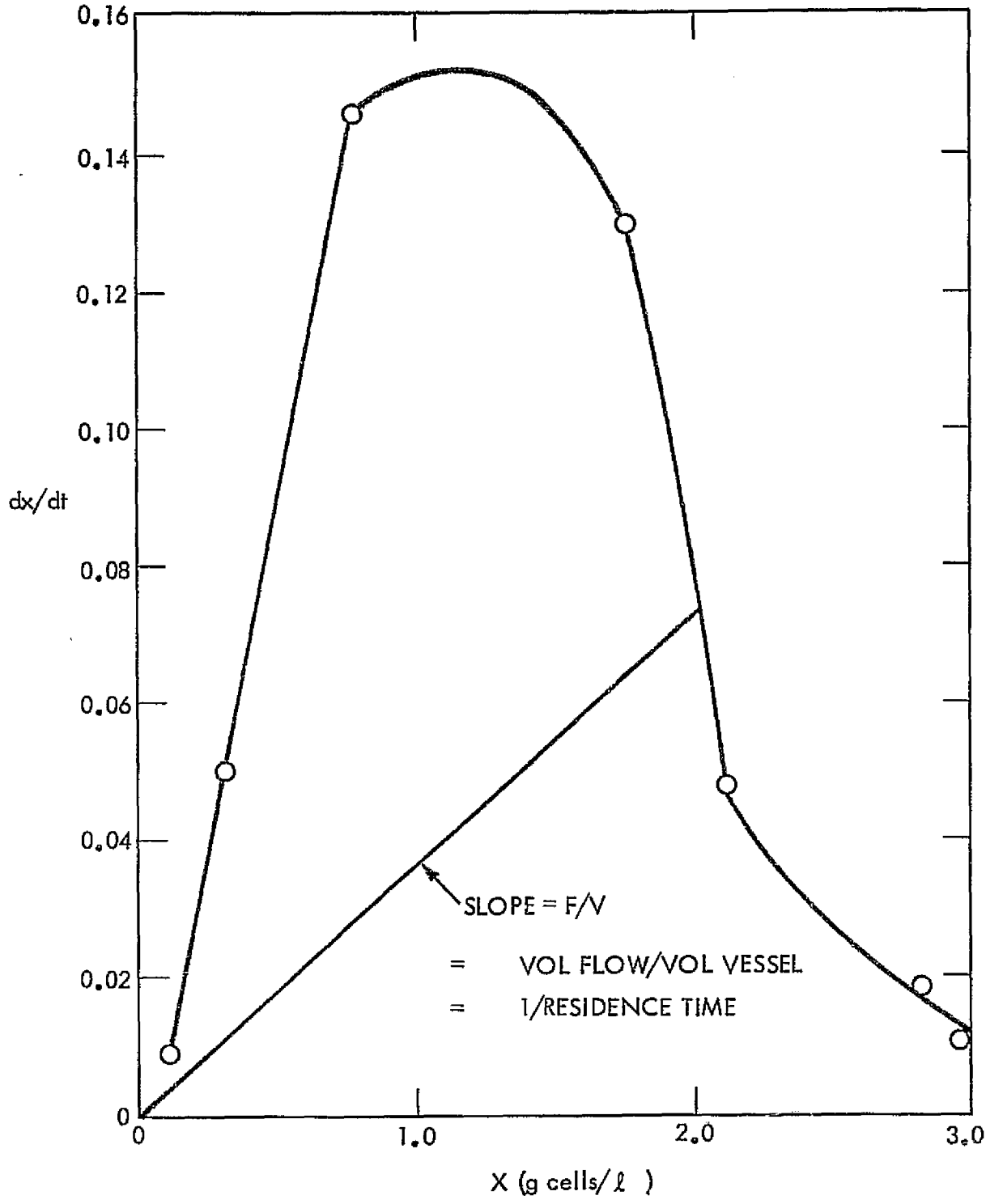

Figure 17. Prediction of Continuous Operating Conditions from Batch Data for A. vinelandii

For conventional fermentation, Figure 18 demonstrates the effect of nitrogen fixation efficiency on the cost of producing fixed nitrogen. The data of this study correspond to an overall fermentation efficiency of 18.7 mg fixed N/g carbohydrate. Higher efficiencies were noted at various times during the course of the batch fermentations. For a free carbohydrate, the cost estimates indicate approximately $33/kg fixed N for the efficiency of 18.7. Fixed N for fertilizer purposes is priced at about $.20/kg and is considerably higher if intended for animal feed.

According to Mulder,[33] the theoretical maximum efficiency for free-living nitrogen fixation in an aerobic system is 280 mg N/g carbohydrate. At this efficiency, and for a free carbohydrate, the cost of fixed N by conventional fermentation is $2.50/kg fixed N. This assumes similar fermentation times as observed at an efficiency of 18.7.

Capital investment and manufacturing costs for A. vinelandii in conventional fermentation equipment are summarized in Table 13. Capital investment is observed to be a function of ammonia concentration decreasing rapidly from $166.4 million for an efficiency of 18.7 mg N fixed/g carbohydrate to $11.3 million at a theoretical efficiency of 280 mg N fixed/g carbohydrate. Manufacturing costs are dependent on ammonia concentration and carbohydrate cost.

b. Azotobacter vinelandii, UW590, in Lagoons. From the graph of fixed N cost as a function of nitrogen fixation efficiency, Figure 19, it is obvious that a free or extremely cheap carbohydrate is mandatory for any chance of favorable economics. From the data of this study which correspond to an efficiency of 18.7 g fixed N/g carbohydrate, the cost of fixed N is in excess of $2.20/kg even for free carbohydrate could be theoretical efficiency of 280 mg fixed N/g carbohydrate could be attained and a free carbohydrate used, then the cost of fixed N becomes about $.20/kg or comparable in price to present fertilizer

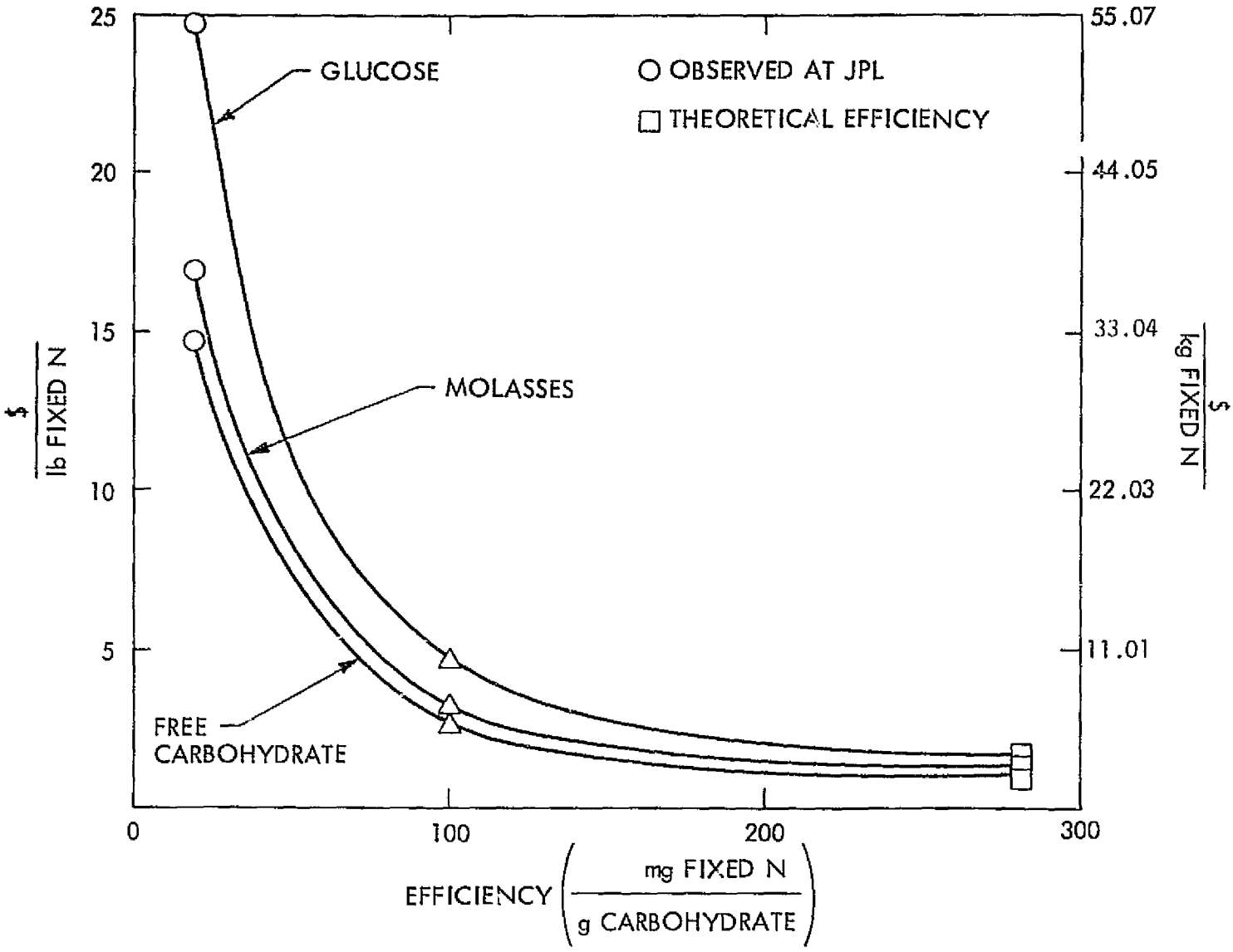

Figure 18. Cost of Fixed Nitrogen as a Function of Efficiency for A. vinelandii in Conventional Fermentation

Table 13.

Capital Investment and Manufacturing Costs
for Azotobacter vinelandii in Standard Fermentation Equipment

EFFICIENCY	F.C.I.	MANUFACTURING COSTS		
$\left(\frac{\text{mg N FIXED}}{\text{g CARBOHYDRATE}}\right)$	$ MILLION	$ MILLION		
		GLUCOSE	MOLASSES	FREE CARBON
18.66	166.4	181	124	108
100	31.5	33.5	22.5	19.3
280	11.3	13.4	9.5	8.3

CAPACITY: 9080 kg NH_4^+/DAY = 10 TONS NH_4^+/DAY

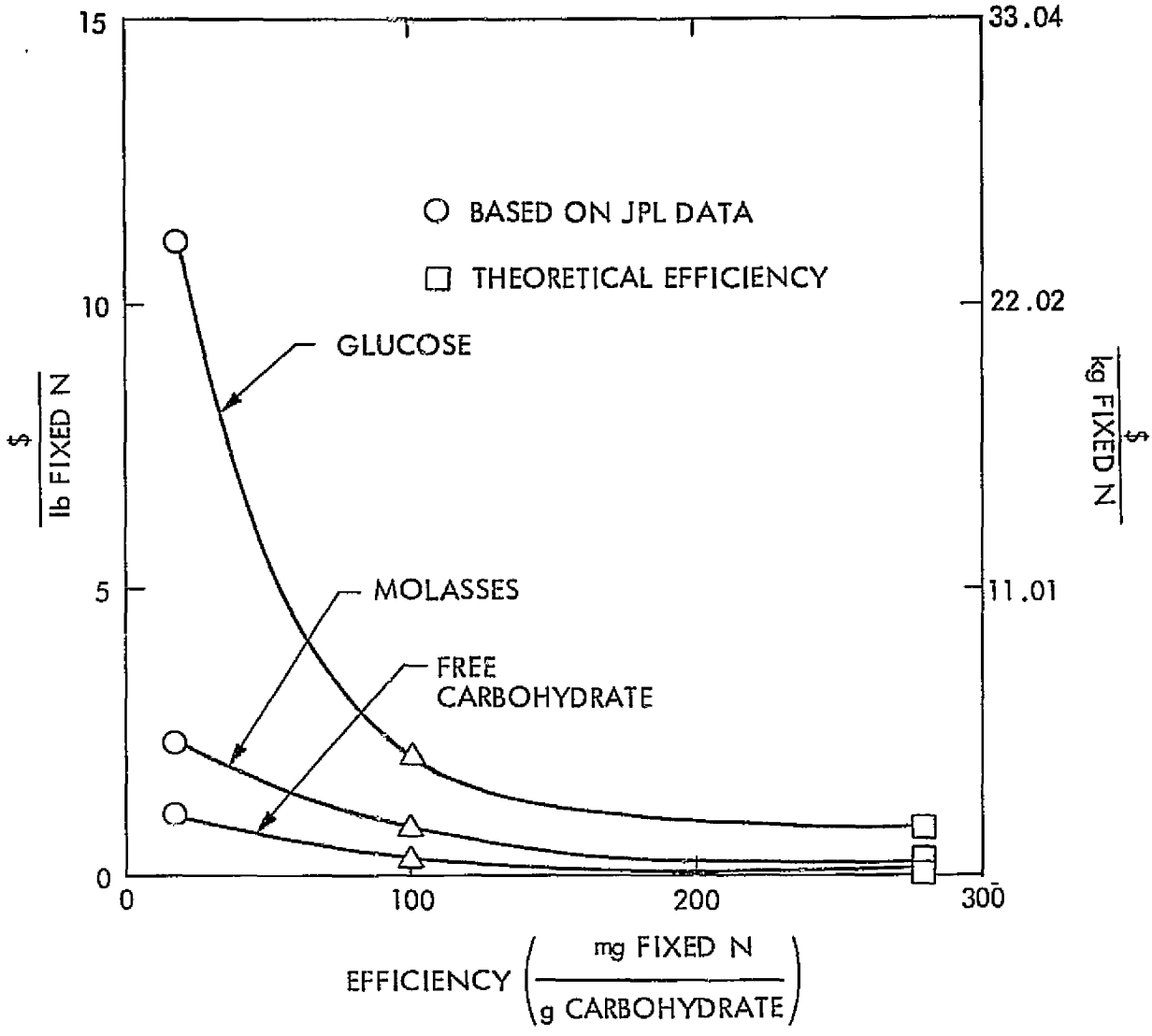

Figure 19. Cost of Fixed Nitrogen as a Function of Efficiency for A. vinelandii in a Lagoon System

nitrogen. However, the severe assumptions imposed on the economic assessment must be realized; they may correspond to an unrealistic and impractical situation as data are not available on A. vinelandii adaptation in lagoons, settling properties of the organism, growth rates and nitrogen fixation efficiencies in lagoon conditions, etc.

Capital investment and manufacturing costs for A. vinelandii growth in lagoons are tabulated in Table 14 for a capacity of 9080 kg fixed N/day=10 ton fixed N/day.

Sample calculations of cost estimates for capital investment, manufacturing cost, and total product cost for A. vinelandii in lagoons are included in Appendix XII.

TABLE 14. CAPITAL INVESTMENT AND MANUFACTURING COSTS FOR A. VINELANDII IN A LAGOON SYSTEM (9080 kg FIXED N/DAY = 10 TON FIXED N/DAY)

EFFICIENCY	F.C.I.	MANUFACTURING COST		
(mg N) / (g CARBOHYDRATE)	$ MILLION	$ MILLION		
		GLUCOSE	MOLASSES	FREE CARBON
18.66	1.67×10^6	81.1	17.1	8.1
100	410,000	15.3	4.8	1.7
280	168,000	5.6	1.85	0.75

3. Photosynthetic Bacteria

Since these microorganisms can obtain ATP by cyclic photophosphorylation, they are expected to be somewhat more efficient in nitrogen fixation than heterotrophs. These bacteria are not capable of splitting water and therefore require fixed carbon or a suitable inorganic source of electrons for growth and nitrogen fixation.[42]

The study of nitrogen fixation in photosynthetic bacteria appears to be somewhat neglected compared to other types of free-living bacteria. Weare and Shanmugam[43] have recently measured nitrogen fixation by Rhodospirillum rubrum in the presence and absence of methionine sulfoximine (MSX). MSX is a glutamate analogue that inhibits ammonia assimilation thus derepressing nitrogenase synthesis and causing ammonia excretion by the organisms. They observed a maximum activity of 15 nMole C_2H_2 reduced per minute per mg of cell protein in the absence of MSX. By adding glutamate or NH_3 in the presence of the analogue, complete derepression of nitrogen as was observed and an excretion of 5.5 µMoles NH_3/ml was measured during an 80-hour period. No data on the amount of substrate (electron source) were presented.

The ammonia concentration of 5.5 µmoles NH_3/ml for R. rubrum is very similar to levels observed for K. pneumoniae; however, the photosynthetic bacteria have a somewhat slower excretion rate. Therefore, economics for large scale production of ammonia by R. rubrum would be more unfavorable than those presented for K. pneumoniae if a cost for the carbon source is not considered. Assuming a suitable mutant of R. rubrum could be obtained, the requirement of an electron source other than water would make the process uneconomical.

4. Blue-Green Algae

Nitrogen fixation in blue-green algae is completely powered by solar energy. Electrons of the reduction are obtained by the splitting of water and ATP is produced by photophosphorylation. While these microorganisms do not require a source of fixed carbon for energy and reductant, the addition of fixed carbon appears to promote algal growth.

Benemann,et al[35] have studied the growth of algae on sewage for subsequent digestion and methane production. For growth rate of 20 tons algae/acre/year, = 1.816 x 10^5 kg/acre/year a 50-acre pond yields a methane product valued at about $20,000/year ($2.0/10^6 BTU). Annual operating cost with land costs included amounts to $37,000/year. No credit is taken for the fertilizer content of the digester residue or effluent from the anaerobic system. As the price of natural gas increases, algae growth for methane will continue to look more and more cost effective. Furthermore, the ecological benefit of wastewater clean-up adds additional economic credit to the algae system.

Large-scale algae growth in lagoons for fertilizer usage appears to be less economically attractive than growth for methane recovery. Oswald[44] reports a cost figure of 11¢/kg for algae of which 6.5¢/kg is for growth and 4.5¢/kg is for harvesting. If algae are assumed to be 10 percent nitrogen, the fertilizer value of algae would be about 2¢/kg. Application of the algae to the crop land, rice paddy, etc., where additional photosynthesis, nitrogen fixation and growth would occur, could greatly enhance the fertilizer value of the algae.

The growth of algae for protein value would show greater economic feasibility than either methane or fertilizer production as a result of the greatly enhanced value of algae as animal protein. Naturally, this assumes acceptability of the algae for animal feed and a market for the product.

Comparison of A. vinelandii growth in lagoons for fertilizer value with photosynthetic blue-green algae shows them comparable if a high

nitrogen fixation efficiency in excess of 100 mg fixed N/g carbohydrate can be obtained with a free carbohydrate source. This comparison is made with the same severe assumptions and restrictions imposed on the A. vinelandii fermentations as discussed previously.

Blue-green algae have several advantages over the derepressed mutants of this study: 1) algae are photosynthetic; therefore a free carbon source is readily available; 2) sterilization is not a requirement with the algae, although strain selection for growth in ponds appears to be of increasing significance; 3) harvesting of blue-green algae is expensive; however, considerable data are available and work is in progress; and 4) sewage is a well established growth medium for algae.

5. Economic Comparison Between Nitrogen Fixation and Anaerobic Digestion

It is of interest to compare the value of ammonia produced by K. pneumoniae, the value of A. vinelandii cell mass, and methane produced from the anaerobic digestion of carbohydrate. Necessary calculations for this comparison are as follows:

K. pneumoniae (high cell density - data from JPL)

$$\frac{\text{mole } NH_4^+}{13.9 \text{ mole glucose}} \times \frac{\text{mole glucose}}{180 \text{ g}} \times \frac{18 \text{ g } NH_4^+}{\text{mole } NH_4^+} \times \frac{132 \text{ g } (NH_4)_2 SO_4}{36 \text{ g } NH_4^+}$$

$$\$71.60/10^3 kg \times 100 \text{ kg} = \$.19/100 \text{ kg glucose}$$

K. pneumoniae (low cell density - data from JPL)

$$\frac{\text{mole } NH_4^+}{6.2 \text{ mole glucose}} \times \frac{\text{mole glucose}}{180 \text{ g}} \times \frac{18 \text{ g } NH_4^+}{\text{mole } NH_4^+} \times \frac{132 \text{ g } (NH_4)_2 SO_4}{36 \text{ g } NH_4^+}$$

$$\$71.60/10^3 kg \times 100 \text{ kg} = \$.42/100 \text{ kg glucose}$$

K. pneumoniae (theoretical limit)

$$\frac{1 \text{ mole } NH_4^+}{1 \text{ mole glucose}} \times \frac{\text{mole glucose}}{180} \times \frac{18 \text{ g } NH_4^+}{\text{mole } NH_4^+} \times \frac{132 \text{ g } (NH_4)_2 SO_4}{36 \text{ g } NH_4^+}$$

$$\$71.60/10^3 kg \times 100 \text{ kg} = \$2.62/100 \text{ kg glucose}$$

A. vinelandii (Data from JPL)

$$\frac{2 \text{ g cells}}{\ell} \times \frac{\ell}{10.72 \text{ g glucose}} \times \$.022/\text{kg cells} \times \frac{10^3 \text{g}}{\text{kg}} \times 100 \text{ kg glucose}$$

$$= \$0.41/100 \text{ kg glucose}$$

A. vinelandii (theoretical fixed N)

$$\frac{280 \text{ mg N}}{\text{g glucose}} \times \frac{\text{g}}{10^3 \text{ mg}} \times \frac{\text{g cells}}{0.1\text{g N}} \times \$0.22/\text{kg cells} \times 100 \text{ kg glucose}$$

$$= \$6.16/100 \text{ kg glucose}$$

For Anaerobic digestion

$$C_6H_{12}O_6 \rightarrow 3\ CO_2 + 3\ CH_4$$

$$1 \text{ g glucose} \times \frac{\text{mole glucose}}{180 \text{ g glucose}} \times \frac{3 \text{ mole } CH_4}{\text{mole glucose}} \times \frac{22.4 \text{ liter}}{\text{mole}} = \frac{0.37 \text{ liters } CH_4}{\text{g glucose}}$$

Therefore,

$$100 \text{ kg glucose} \times \frac{0.37 \text{ liters } CH_4}{\text{g glucose}} \times \frac{\$.07}{10^3 \text{ liter}} \times \frac{10^3 \text{g}}{\text{kg}} = \$2.59/100 \text{ kg glucose}$$

Table 15 summarizes the value of the product, either $(NH_4)_2\ SO_4$, cell mass, or methane, per 100 kg. of glucose consumed for K. pneumoniae and A. vinelandii fermentations and anaerobic digestion (theoretical value assuming digester gases are 50 percent CH_4 and 50 percent CO_2). The theoretical methane yield for glucose is about 0.37 liters of CH_4 per g of glucose. For waste materials such as poultry manure, 0.25 liters of CH_4 have been obtained per g of volatile solids.[45] It is apparent from Table 15 that anaerobic digestion of carbonaceous material yields methane of greater value than the nitrogenous products of nitrogen fixation by the presently available derepressed mutants. By increasing the nitrogen content of digester effluent and residue, it may be possible to enhance the value of these materials as fertilizers without inhibiting methanogensis by developing suitable nif-derepressed mutants. Ammonia concentrations for swine[46] and poultry manure[45] digestion reach levels of 1500-3000 mg/ℓ. However, digester operation has been observed to cease for swine manure digestion at an ammonia concentration of 2000 mg/ℓ.[46] Ammonia loss from the digester effluent when exposed to the air would also decrease its value as a fertilizer. From these facts it would appear that increasing nitrogen content in the digester would have no benefit and may even be detrimental for wastes of high nitrogen content. However, for wastes of low nitrogen concentration, enhancement of nitrogen fixation may prove beneficial.

TABLE 15. COMPARISON OF PRODUCT VALUE FOR NITROGEN FIXATION PROCESSES AND ANAEROBIC DIGESTION

	VALUE OF PRODUCT/100 kg GLUCOSE CONSUMED $(NH_4)_2SO_4$
KLEBSIELLA PNEUMONIAE	
DATA FROM JPL (HIGH CELL DENSITY)	$0.19
DATA FROM JPL (LOW CELL DENSITY)	$0.42
THEORETICAL	$2.62
AZOTOBACTER VINELANDII	(CELL MASS)
DATA FROM JPL	$0.41
THEORETICAL	$6.16
METHANE FROM ANAEROBIC DIGESTION	$2.59

6. In Situ Fertilization by Free-Living Microorganisms

a. Current Contributions

The contribution of fixed nitrogen to the ecosystem by free-living, nitrogen-fixing heterotrophs has been recently reviewed.[47] While heterotrophs appear to fix significant amounts of nitrogen in some cases, the large carbon requirement for fixation limits the economic importance of this class of microorganisms. Mulder and Brotonegoro[47] aptly point out that fixation of 100 KgN/ha would consume 10,000 kg of fixed carbon, apparently assuming 10 mg N fixed per gram of carbon consumed. Even assuming the best efficiency reported for Azotobacter[32] (100 mg N/gram carbon) the weight of carbon required is still an order of magnitude greater than the weight of nitrogen fixed. However, the fact that fixed carbon is available in many environments, both from decaying plant materials as well as exudates from live plants, suggests that efforts to enhance the fixation of nitrogen by heterotrophs is worthwhile and that improved strains could yield small but perhaps significant increases in in situ nitrogen fertilization.

Since blue-green algae do not require a fixed carbon source for growth and nitrogen fixation, they would seem to be the most important contributors of fixed nitrogen to the environment. Studies in the United Kingdom indicated that algal crusts were responsible for the majority of nitrogen fixed (10-45 kg/ha/year) in wheat soils.[48] Similar data have been published for Swedish soils.[49] Even values of fixation as high as 88 kg N/ha in fields of sugar cane and maize during a 75 day period have been reported.[50] Since algal crusts fix nitrogen only when moist,[48] the soil surface would appear to be less optimal than an aqueous environment for nitrogen fixation. This can be readily seen in the comparison of nitrogen fixation between flooded and upland rice.[51] In the flooded environment during the wet season 57 kg N/ha were observed whereas only 7 kg N/ha were fixed in upland soils. The values for fixation during the dry season were 63 and 5, respectively. As indicated above, the economic importance of blue green-algae in providing fixed nitrogen to rice culture is substantial. The literature on this subject was recently reviewed by Fogg,[52] and the effect of algalization on crop yield was treated by Vankataraman.[53] A number of studies showed substantial improvements in yield as a result of algalization (addition of

algal biomass to soils). In addition to fixing nitrogen, algae[54] and Azotobacter[55] produce growth substances and vitamins which may be largely responsible for the observed yield increases, particularly when high rates of chemical fertilization are involved.[53] The application of algae is reported to replace 40 percent of the nitrogen fertilizer required by rice crops, where one kg of algae/ha is equivalent to the application of 30 kg/ha of ammonium sulfate.[56] Obviously additional efforts to enhance in situ fertilization with blue green algae is warranted.

b. Derepressed Mutants

While current levels of nitrogen fixation in soils by free-living microorganisms can be significant, the addition of chemical fertilizers can substantially inhibit fixation.[57] Balandreau,et al, however, report that ammonium sulfate additions up to 40 ppm (120 kg/ha) did not inhibit nitrogen fixation but that additions higher than this level led to severe reductions of fixation even after the ammonia was utilized, suggesting modifications in the equilibrium of the microflora.[58] While the threashold for repression of nitrogen fixation is quite high, substantial advantages may be obtained by inoculating fields with nif-derepressed strains. The most promising approach would be to isolate the microorganism which normally predominates in the environment, obtain derepressed mutants and inoculate with the mutant strains as suggested by Brill.[55] Such an approach would require control mutants similar to A. vinelandii UW590 since amino acid auxotrophs would not survive in the environment. The principal questions to be answered in this kind of application are 1) the survival capability of the mutant compared to the wild type strain and 2) the benefit derived from the derepressed type compared to that of the wild type strain. Strains which produce and excrete excess ammonia would also be very advantageous in such in situ applications.

c. Economic Considerations

The growth of microorganisms solely for their nitrogen content, to be used as fertilizer, does not appear economical. The observation that microorganisms produce growth promoting substances and that substantial fixation may occur in the field, however, indicates that such an analysis may be simplistic and that the actual benefit received from the application

of cell mass to fields may be much more attractive than predicted on the basis of nitrogen content alone. If such is the case, the analysis of this report may be considered as the cost to grow an inoculum which then will be added to the field. This material will serve not only as a fertilizer but as a nitrogen fixing agent and the extent of growth and survival in the enviornment will determine the fertilizer value of the material as indicated above. This approach has been pursued with blue green algae. The potential enconomic benefit can be seen from a cursory analysis assuming that 1 kg of algal inoculum can replace 30 kg of ammonia sulfate.[56] If this cost of growing the algae is 5¢/lb.[44], than 5¢ of worth of algae replaces $0.97 worth of ammonium sulfate. While many other costs and complications are likely to arise in a rigorous evaluation, the concept appear to have considerable potential.

VI. CONCLUSIONS

K. pneumoniae

Ammonia production. Several factors render the present strains of K.pneumoniae unsuitable for application. Firstly, the amino acid requirement places a significant burden on the economics, assuming that a satisfactory, low cost amino acid substitute for glutamate/glutamine could be obtained. Otherwise, the burden is prohibitive. Secondly, the genetic stability of the present mutants is insufficient for even 5¢ scale ammonia production. Presumably this could be corrected by obtaining the appropriate deletion mutants but a third consideration, namely sterilization, would still render ammonia production uneconomical. Any contaminating microorganisms would quickly overrun the fermentation since the high efficiency production of ammonia relies on stationary cultures at low optical densities. If the rate of ammonia production were increased markedly and high density culutures could be used, batch processes relying on high initial inoculum and short duration runs may avoid sterilization requirements for the final batch media. However, the economic projections presented, which assumed such improvements, do not indicate that such development of the organism for ammonia production alone is worthwhile. Development of a very inexpensive method of inhibiting microbial growth, which would not affect nitrogenase activity, could be employed after the culture entered stationary phase, but again the economic projections presented do not justify such activity.

Other Products. The production of ammonia by Klebsiella does not use all of the energy available in the carbon substrate. Products other than ammonia include cell mass, H_2, acetate and other organic materials. The organic remainder could possibly find application as media for an aerobic fermentation or in the production of methane. Separation of these organics from the solution is likely uneconomical due to the low concentration involved.

As discussed previously the value of the H_2 produced in the medium greatly exceeds the value of the fixed nitrogen and even the H_2 equivalent of NH_3 is worth more than the ammonia. The methane value of the carbon energy source also exceeds the value of the fixed nitrogen produced. These considerations

suggest that ammonia should be considered the by-product and attention should focus on the production of H_2 and other materials.

A. vinelandii

Large scale production of fixed nitrogen by existing strains does not appear economical. Realistic efficiencies for A. vinelandii are in the range of 10-50 mg fixed N/gram of carbohydrate consumed. If efficiencies approaching theoretical limits could be achieved in inexpensive lagoons, with minimum aeration and a free carbon source, economics of the process may become competitive. At theoretical efficiency the value of the fixed nitrogen as fertilizer exceeds the value of the carbon substrate for methane production.

The use of a derepressed mutant in such a system is beneficial only if the waste material contains a significant amount of fixed nitrogen which would repress nitrogenase synthesis. Sterilization is not required since the only product is cell mass and contaminating strains are expected to have only a marginal negative impact when the material employed contains fixed nitrogen.

General

Capital investment is prohibitive for conventional fermentation employing stainless steel vessels. This is primarily due to the vast capacity required in dealing with a low value product in dilute solutions. Any application of free-living microorganisms for commercial nitrogen fixation will require considerable ingenuity in devising low cost "low technology" methods. Fortunately, microorganisms are amenable to such low technology methods and may find application in countries where large capital outlay is unfeasible.

While the economics of nitrogen fixation by Azotobacter might become economical by improving the efficiency of the organism and by price increases of commercial nitrogen, the occurrence of truly free carbon materials is unlikely in a commercial setting. Free carbon on the other hand may be available on a small-scale such as to the individual farmer who needs fixed nitrogen. This would be particularly true in the foreign setting and efforts to increase

the efficiency of *Azotobacter* or other aerobic nitrogen fixers are needed.

The use of *nif*-derepressed microbes in *in situ* production of ammonia is a potentially attractive application. Experiments to quantitate the survival of control type *nif*-derepressed mutants in the field are required in order to evaluate the benefit of soil inoculation with these strains. Due to the high carbohydrate requirements of heterotrophs, *nif*-derepressed strains of blue-green algae would appear to be the organisms of choice for *in situ* fertilization. Phototrophs, however, are limited to surface environments so *nif*-derepressed heterotrophs may still be useful in fixing nitrogen in dark environments.

Several future research needs are obvious as a result of this study:

1) The economic assessment of hydrogen production by K. pneumoniae should be carefully evaluated taking into account the various technical and economic problems associated with hydrogen recovery, purification, compression and storage.

2) The enhancement of the nitrogen content of digester residue and effluent and the production of hydrogen by K. pneumoniae in the anaerobic digester should be explored as means of augmenting product value from conversion of carbonaceous wastes.

3) The environmental adaptation and utilization of free-living nitrogen fixing microbes in situ should be examined. Competition of A. vinelandii UW590 with the parent strain (OP) should be explored as well as the adaptation and survival of this organism in soils.

4) Low technology applications and use in less developed countries should be evaluated. Large scale commerical production of fixed nitrogen was considered in this study: however, potential low technology uses in both domestic and foreign countries are obvious, although the economic feasibility of these uses remains unknown.

5) Photosynthetic blue-green algae have many advantages relative to the heterotrophic and phototrophic bacteria for large-scale production of fixed nitrogen and for low technology utilization. Nif-derepressed algae with similar genetic modifications as the A. vinelandii UW590 should be developed and examined for potential economic utilization.

VIII. Utilization of Research Results

Several university investigators have been made aware of the findings of this study throughout its duration.

This project was presented in its very early stages at a Grantees-Users Conference in May 1976, Charlottesville, Virginia. The completed project was presented at the Symposium on Biotechnology in Energy Production and Conservation, Gatlinburg, Tennessee, May 10, 1978.

Dissemination of the results of this study will be carried out by wide distribution of the final report and by discussion with appropriate industrial companies, university investigators and government agencies.

IX. Products of Research

(1) B. O. Stokes and C. J. Wallace, "Assessment of Free-Living Nitrogen-Fixing Microorganisms for Commercial Nitrogen Fixation" Final Report, National Science Foundation Research Applied to National Needs, Grant No. AER-7607093, May 1978.

(2) C. J. Wallace and B. O. Stokes, "Assessment of Nif-Derepressed Microorganisms for Commercial Nitrogen Fixation", presented at Symposium on Biotechnology in Energy Production and Conservation, Gatlinburg, Tennessee May 10-12, 1978. To appear in Biotechnology and Bioengineering.

(3) B. O. Stokes, C. J. Wallace and J. J. Kalvinskas, "Assessment of Nitrogen Fixing Microorganisms for Ammonia Synthesis", published in Proceedings of Grantees- Users Concerence, Charlottesville, Virginia, May 19-21, 1976.

X. Personnel

(1) Dr. J. J. Kalvinskas, Project Manager
(2) Dr. B. O. Stokes, Principal Investigator
(3) Dr. C. J. Wallace, Co-Investigator
(4) Mr. W. W. Schubert, Scientist
(5) Mr. A. Hatter, Technician

A total of five (5) scientists and engineers were involved in this project from its inception. In addition to the personnel with day-to-day contact and responsibility to the project, members of the Steering Committee, Drs. A.A. App, J. Newton, D. Isenberg, and R. Valentine, gave valuable guidance and direction. Additional advice and consultation were given by Drs. K. Shanmugan, K. Anderson and W. Brill.

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APPENDIX I

DATA FOR *K. pneumoniae* IN 250 ml FLASK EXPERIMENTS

APPENDIX I

DATA FOR K. pneumoniae IN 250 ml FLASK EXPERIMENTS

	SK-25				SK-29					SK-29		
Time Hours	gluNH$_2$ Added µg/ml	A_{420}	*Glucose g/ℓ	NH_3 mM	gluNH$_2$ Added µg/ml	A_{420}	*Glucose g/ℓ	NH_3 mM	Glutamate Added µg/ml	A_{420}	*Glucose g/ℓ	NH3 mM
0	100	0.025	17.7	0.3	100	0.030	27.8	0.15	100	0.025	21.6	0.15
8		0.14	18.1	0.7		0.034	14.3	0.28		0.20	-	0.15
10		0.24	26.1	1.1		0.42	17.4	0.84		0.26	19.1	0.31
11		0.25	17.4	1.2		0.42	15.3	0.86		0.28	-	0.44
12	30	0.31	18.8	1.4	30	0.43	15.3	1.1	30	0.31	18.8	0.60
13		0.32	18.8	1.8		0.45	14.3	1.2		0.32	16.0	0.76
15	33	0.40	14.3	2.2	33	0.46	18.8	1.8	33	0.36	-	1.0
20.5		0.50	10.9	4.4		0.50	13.6	3.7		0.38	-	2.1
23.5		0.55	9.7	4.5		0.54	12.2	4.7		0.40	-	2.6
35.5	50	0.54	5.6	6.6	50	0.56	5.9	5.4	42	0.40	7.6	3.7
37		0.55	-	-		0.58	-	-		0.42	-	-
40.5		0.66	4.5	6.9		0.66	5.6	6.6		0.46	-	4.2
56.5		0.64	2.9	7.0		0.64	5.2	7.6		0.46	-	4.6

Conditions: 1.2 percent Luria Broth Inoculum Room Temperature (see text).

*20 g glucose/ℓ added initially.

APPENDIX II

DATA FOR LOW DENSITY FERMENTATION
K. pneumoniae SK-25,
(100 µg/ml initial gluNH$_2$)

APPENDIX II

DATA[≠] FOR LOW DENSITY FERMENTATION, K. pneumoniae SK-25, (10 µg/ml initial gluNH$_2$[*])

Time (hours)	A_{420}	Glucose (g/ℓ)	NH_3 (mM)**	Viable Cells (10^6/ml)	Revertants (No./ml)
0	0.11	5.8	0.15	-	0
12	0.11	5.9	0.13	0.01	0
18	0.12	6.0	0.16	0.02	0
36	0.11	5.8	0.32	-	0
43	0.13	6.0	0.26	0.2	0
61.5	0.39	5.8	0.34	-	0
72	0.53	5.6	0.52	3.9	0
86	0.57	5.2	0.90	2.6	4
89	0.57	5.0	1.01	6.4	280
108	0.57	4.7	1.27	5.5	-
115	0.59	4.6	1.32	4.3	-
132	0.44	2.3	0.2	-	-

[≠]Data for H_2 production in Appendix V.

* 1 µg/ml gluNH$_2$ added at 18 hours, 72 and 90 hours.

Conditions:

1 percent inoculum from minimal media supplemented with 100 µg gluNH$_2$/ml, culture grown at 25°C with stirring rate of 400 rpm.

**Ammonia determined by Nesslers procedure.

APPENDIX III

DATA FOR HIGH DENSITY FERMENTATION, K. pneumoniae SK-25,
(300 µg/ml initial glutamine)

APPENDIX III

DATA FOR HIGH DENSITY FERMENTATION, K. pneumoniae SK-25,
(300 µg/ml initial glutamine)

Time Hours	A_{420}	NH_3 (mM) **	*Glucose (g/l)	Revertants (No./ml)	Viable Cells (10^6/ml)
0	0.06	0.40	25.6	0	3
1.5	0.04	0.39	25.4	0	12
4.0	0.04	0.41	21.6	0	10
6.0	0.03	0.43	26.4	0	4
8.0	0.04	0.40	24.4	0	7
11.0	0.02	-	-	0	-
17.5	0.05	-	-	0	-
19.3	0.07	-	-	0	-
22.5	0.08	-	-	0	-
23.2	0.09	1.1	25.8	0	7
26.4	0.13	1.3	18.6	0	-
34.0	0.23	1.9	22.2	2	57
38.0	0.37	1.4	15.4	6	206
40.5	0.51	-	-	-	-
41.0	0.63	1.4	23.0	-	-
41.5	-	-	-	15	275
43.5	0.69	1.4	20.4	22	492
45.7	0.69	1.4	20.6	-	-
46.5	0.69	1.3	18.6	53	626
48.0	0.68	1.4	19.4	165	585
56.5	0.65	1.2	17.4	4,000	415
67.5	0.61	1.2	15.6	-	514
76.0	0.58	0.97	10.4	-	-
91.5	0.93	-	7.6	-	-
112.5	1.3	0.74	2.2	-	-

* Conditions: 25°C, 350 rpm, 5 percent inoculum grown on minimal medium supplemented with 300 µg/ml glutamine, 25 g/l glucose initial.

**Nesslers procedure.

APPENDIX IV

DATA FOR HIGH DENSITY FERMENTATION, *K. pneumoniae* SK-25.
(500 µg/ml initial glutamine)

APPENDIX IV

DATA FOR HIGH DENSITY FERMENTATION, *K. pneumoniae* SK-25,
(500 µg/ml initial glutamine)

Time (hours)	A_{420}	NH_3 (mM)	Glucose (g/l)	Viable Cells (10^6/ml)	Revertants (No./ml)
0	0.071	0.158	16.4	-	-
1	0.072	0.36	14.4	-	-
3	0.090	0.43	14.5	-	-
12	0.160	13.0	13.0	5.3	-
19.5	0.35	2.4	11.7	29.0	-
28	0.73	5.2	7.5	182	4
33	1.24	5.4	1.6	390	25
41.5	1.36	5.6	0.25	860	85
57.5	1.22	5.6		880	2.1×10^3
67.8	1.22	5.6		830	1.5×10^5
93	1.20	7.4		340	2.1×10^7
139	0.88	7.4			
140	-	7.2			
142	0.85	-		7.4	1×10^8
145	0.85	6.9			
146	-	6.2			
164	0.78	7.0			

Note:

1. Inoculated with 300 mls (6%) of a minimal media inoculum containing 500 µg/ml. glutamine.
2. Stirring rate 500 rpm.
3. Slow sparging with N_2 (100-200 mls/min)
4. Viable cells counted by making serial dilutions into 0.25 mM Pi at pH 7.0 transferring 1 ml to petri dish and mixing with Luria agar (45°C).
5. NH_3 determined with NH_3 electrode.

APPENDIX V

HYDROGEN PRODUCTION BY K. pneumoniae SK-25
(See Appendix II)

APPENDIX V

HYDROGEN PRODUCTION BY K. pneumoniae SK-25
(See Appendix II)

Time (hours)	% H_2* in. Exhaust Gas	N_2 Flow Rate	H_2 Production ml/hr.	Liters Culture Remaining	H_2 mls/hr./ℓ	mole H_2 / mole glucose **
61.5	0.068	158	6.45	4.4	1.46	
72	0.114	156	10.7	4.4	2.43	0.82
89	0.138	174	14.4	4.3	3.35	0.68
108	0.147	≈174	15.3	4.3	3.56	1.51
115	0.166	≈174	17.3	4.2	4.12	1.44
132	1.11	≈174	116	4.2	27.6	0.96

*Determined by comparing integration units from GC to standard mixtures (Appendix VI).

**Sample calculation

$$(72 - 61.5 \text{ hrs}) \times \frac{1.46 + 2.43}{2} \frac{\text{mls } H_2}{\text{hr-}\ell} \times \frac{180 \frac{\text{g glucose}}{\text{mole glucose}}}{5.85\text{-}5.65 \text{ g glucose used.}}$$

$$\times \frac{1}{22.4 \times 10^3 \frac{\text{mls } H_2}{\text{mole } H_2}} = 0.82$$

The average value from 61.5-132 hours may be obtained by omitting the glucose terms in the above calculations, calculating the moles of H_2 produced for each time period, summing the contributions from all of the time periods and dividing by the glucose used between 61.5 and 132 hours. Such a calculation yields 1.1 moles H_2/mole glucose consumed.

APPENDIX VI

ANALYSIS OF STANDARD H_2 MIXTURE

APPENDIX VI

ANALYSIS OF STANDARD H_2 MIXTURE

Sample	% H_2	Integration Units G.C. (thousands)		
		0 Days	5 Days	11 Days
1	1.0	12.6	13.4	12.9
2	0.1	1.3	0.94	0.2
3	0.01	0.16	0.40	0.14

Samples were prepared by injecting pure H_2 gas into 135 ml cultures flasks capped with butyl rubber stoppers with a syringe (1.35 mls, 0.135 mls and 0.014 ml for Samples 1, 2 and 3). These samples were analyzed (see methods) immediately and at 5 and 11 days. Sample 1 shows no loss of hydrogen over the 11 day period. Sample 2 lost about 85 percent of the hydrogen over the 11 day period. Visual inspection of the stopper revealed that the seal was not as tight as Sample 1 and may be responsible for the leakage. The low levels of H_2 in Sample 3 lead to inaccuracies in analysis which disallow conclusions on leakage. The preparation samples in Appendix V involved the evacuation of the flasks which insured adequate seating of the stoppers so significant leakage of samples which required storage is unlikely.

APPENDIX VII

DATA FOR A. vinelandii FERMENTATIONS

APPENDIX VII
DATA FOR A. vinelandii FERMENTATIONS

0.5 ℓ Air/min.					5.0 ℓ Air/min.					8.5 ℓ Air/min.				
ΔT hours	O_2 (%)	A_{420}	Dry Wt (g/ℓ)	Glucose (g/ℓ)	ΔT hours	O_2 (%)	Dry Wt (g/ℓ)		Glucose (g/ℓ)	ΔT Hours	O_2 (%)	A_{420}	Dry Wt. (g/ℓ)	Glucose (g/ℓ)
0	100	0.045		18.7	0		0.078		21.0	0	100	0.090		23.2
0	100	0.045		18.8	0		0.078		19.6	16.3	87	0.143		22.2
16.5	3	0.152		18.7	8.5		0.107		-	17.2	83	0.153		-
16.7	2	0.178	0.05	-	18.1	8	0.450		19.6	18.4	75	0.171	0.043	22.4
18.7	2	0.234	0.09	18.6	19.3	1.8	0.625		19.2	21.1	54	0.227		22.0
19.8	2	0.280		18.7	20.4	1	0.850		19.0	23.8	27	0.440	0.080	21.8
21.0	2	0.327	0.11	18.6	20.8	1	0.880	0.34	-	24.4	24	0.497	0.093	21.6
22.8	2	0.407	0.13	18.3	21.6	1	1.01		18.8	26.3	12	0.770		20.8
24.8	2	0.492		17.9	22.7	1	1.20	0.65	18.2	30.2	4	1.92		19.0
32.5	2	0.715	0.27	17.6	24.1	1	1.43		18.2	40.4	4	4.17		15.0
40.5	2	1.06	0.36	16.9	26.0	1	1.67		17.4	43.3	4	4.54	1.52	14.0
44.5	2	1.34		16.8	34.6	1	2.84		15.6	47.3	3	5.24		11.8
47.6	2	1.47		16.6	42.1	1	-		13.6	76.1	2	7.90		3.4
62.8	1	1.76		16.4	47.1	1	3.85		12.8	99.8	78	7.47		0.0
65.8	1	1.22		-	48.4	1	4.02	1.51	-	112.8	80	9.06		0.0
67.8	1	1.76		-	64.9	1	5.47		7.0					
69.8	1	1.85		14.9	66.3	1	-	2.01	-					
92.5	1	2.24		13.2	67.3	1	5.45		-					

Conditions: 30°C, pH 7.2 and 350 rpm.

APPENDIX VIII

DRY WEIGHT DETERMINATION OF *AZOTOBACTOR vinelandii* UW590

APPENDIX VIII

DRY WEIGHT DETERMINATION OF *Azotobacter vinelandii* UW590

Figures 1 and 2 show plots of the dry weight data from Appendix VII. With the exception of two points of the 8.5 % aeration run a good linear relationship is observed. Due to oversight the filters for this run were not preweighed. The final dry weight was corrected by subtracting the observed weight loss (0.4 mg) of other filters from the same lot. Such filters typically lose 0.4-0.6 mg each upon drying. The dry cell weight of the divergent points after subtraction of the 0.4 mg correction was 2.4 and 2.8 mg, so the potential error in weight correction does not appear adequate to account for the divergence of these points.

Azotobacter cells are known to change optical properties during growth which makes absorbance measurements of cell concentration unreliable (W.J. Brill, personal communication). Our data indicate that growth at high oxygen concentration may cause the phenomenon and that cultures with limited O_2 show a linear relationship between cell concentration and absorbance.

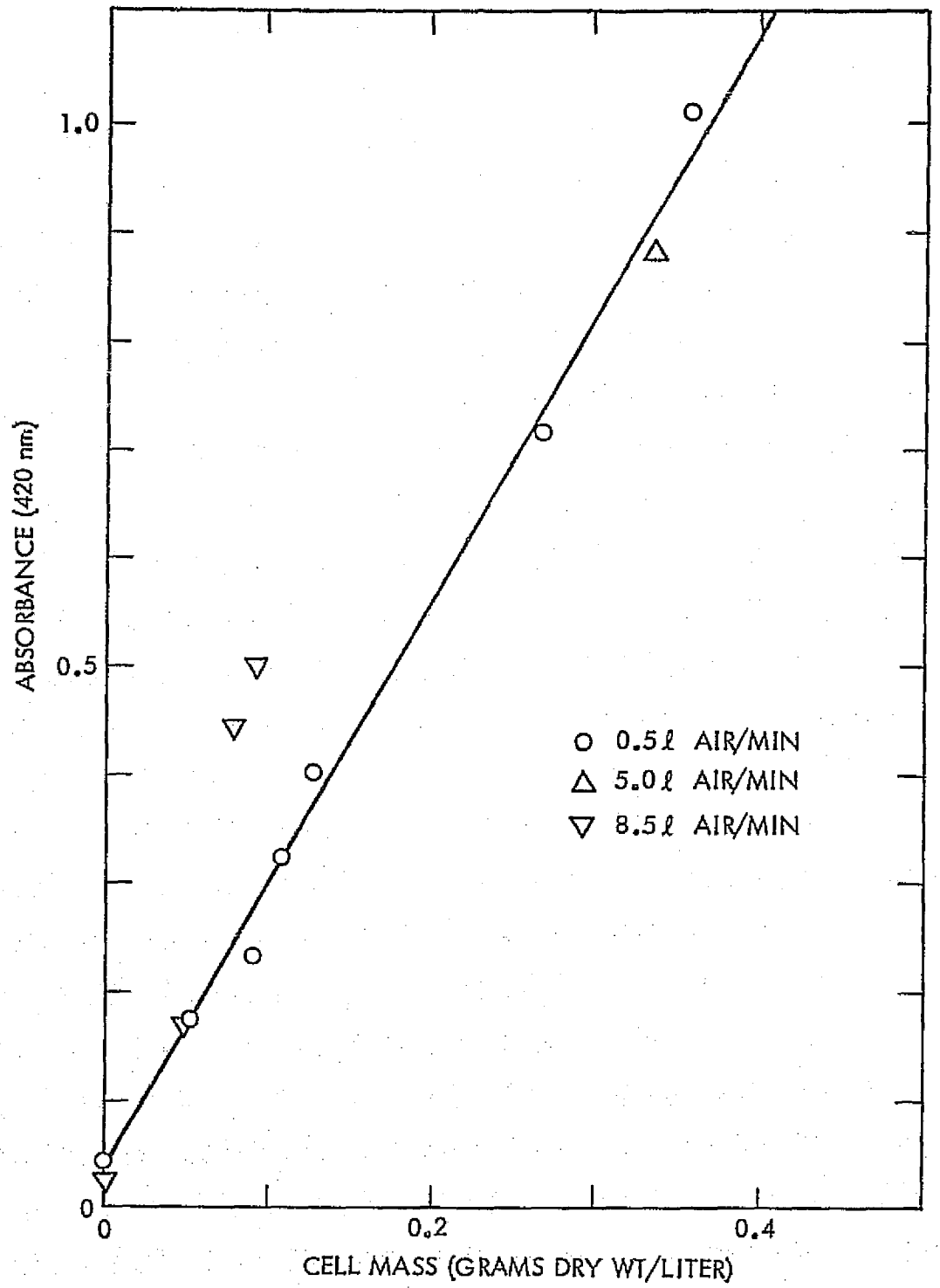

Figure 1. Absorbance (0-1.0) Versus Culture Dry Weight A. vinelandii UW590

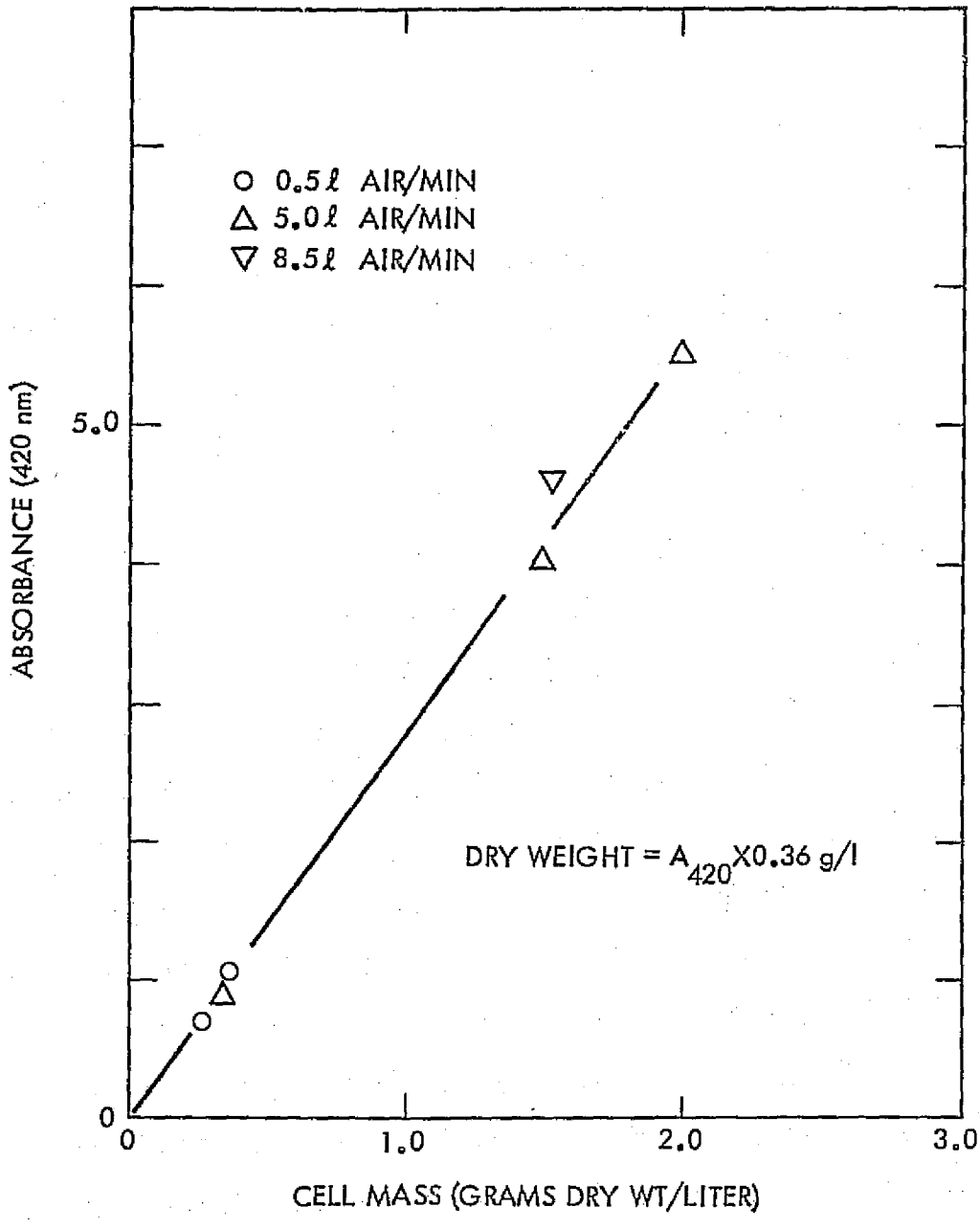

Figure 2. Absorbance (0.0-10.0) Versus Culture Dry Weight A. vinelandii UW590

APPENDIX IX

COST ESTIMATES FOR *K. pneumoniae* IN CONVENTIONAL FERMENTATION AT LOW AMMONIA CONCENTRATION

APPENDIX IX

COST ESTIMATES FOR K. pneumoniae IN CONVENTIONAL FERMENTATION AT LOW AMMONIA CONCENTRATION

Capacity: 10 tons NH_4^+/day

Concentration: 5.4 mMolar NH_4^+ = 97 mg NH_4^+/ℓ

Volumetric Flow: 24×10^6 gal/day

I. Direct Costs

1. Purchased Equipment

a. Fermenters

Total volume = 11.5×10^6 gal for optical density of 1.1

110 vessels at 100,000 gal/vessel

Agitated jacketed vessel (Stainless Steel)

100,000 gal. = $250,000 (1968) extrapolated from Fig. 13-90 (ref. 34)

Marshall and Swift index 1968 ≈ 300

1978 = 520

Therefore, 100,000 gal. = $\frac{520}{300}$ (250,000) = $433,000

Fermenter Cost = 110 (433,000) = 47.6×10^6

b. Seed Tank

Cell population in seed vessel = 10^9 cells/ml.

Cell population in freshly inoculated fermenters = 3×10^6 cells/ml.

$$10^9 \frac{\text{cells}}{\text{ml}} \times V_{seed} = 3 \times 10^6 \frac{\text{cells}}{\text{ml}} \times 11.5 \times 10^6 \text{ gal.}$$

$$V_{seed} = 35{,}000 \text{ gal.}$$

35,000 gal. = \$120,000 (1968) extrapolated from Fig. 13-90 (ref. 34)

M&S Index 1968 ≈ 300

1978 = 520

Therefore, 35,000 gal. = $\frac{520}{300}$ (120,000) = \$208,000.

c. Settling Pond

(Assumptions: tenfold increase in concentration in settling basin; residence time about one-half growth time)

$$24 \times 10^6 \frac{\text{gal}}{\text{day}} \times 0.5 \text{ day} = 12 \times 10^6 \text{ gal} = \text{volume lagoon}$$

$$= 1.6 \times 10^6 \text{ ft}^3$$

depth = 10 ft.

$$1.6 \times 10^6 \text{ ft}^3 \times \frac{1}{10 \text{ ft}} = 1.6 \times 10^5 \text{ ft}^2$$

$$= 3.57 \text{ acres}$$

From cost estimates of Benemann, et. al., cost ≈ \$10,000 (negligible compared to other equipment costs).

d. Ammonia Recovery System (selective ion exchange)

Capital cost = $4,470,000 (1974)
from ref. 37 for 22.5 mgd capacity.

Marshall and Swift index = 398.4 (1974)
= 520 (1978)

$$\text{Capital Cost} = \$4{,}470{,}000 \times \frac{520}{398.4}$$

$$= \$5{,}830{,}000$$

Summary of Equipment Costs:

Fermenters	47.6×10^6
Seed tank	0.2×10^6
Settling pond	Negligible
Ammonia recovery system	5.8×10^6
Total	52.6×10^6

A. Equipment

1.	Purchased Equipment	52.6×10^6
2.	Installation (25% of 1.)	13.1×10^6
3.	Instrumentation and control (10% of 1.)	5.2×10^6
4.	Piping (10% of 1.)	5.2×10^6
5.	Electrical (10% of 1.)	5.2×10^6
		81.3×10^6

	Subtotal from A. Equipment	$81.3 x 10^6
B.	Buildings (20% of 1.)	10.5 x 10^6
C.	Service facilities, etc. (40% of 1.)	21.0 x 10^6
D.	Land (5% of 1.)	2.6 x 10^6
	Total Direct Cost	$115.4 x 10^6

II. Indirect Costs (25 percent of T.D.C.)

A. Engineering and Supervision
B. Construction Expense and Contractor Fee
C. Contingency

$ 28.9 x 10^6

Fixed Capital Investment $144.2 x 10^6

III. Manufacturing Cost

A. Direct Production Costs

1. Raw Materials

Media	Concentration	Price
Glucose	15 g/ℓ	$15.3/100 lb.
NaCl	2.0 g/ℓ	$2.19/100 lb.
KH_2PO_4	0.75 g/ℓ	$297/300 lb.
Na_2HPO_4	6.25 g/ℓ	$166/183 lb.*
$FeSO_4$	10 mg/ℓ	$52/ton
Na_2MoO_4	10 mg/ℓ	$1.88/lb.*
$MgSO_4$	200 mg/ℓ	$8.50/100 lb.
Glutamine	500 µg/ℓ	$16.80/100g*
Water (20% loss)	-	$0.20/$10^3$ gal.

*specialty chemicals

Consider only carbohydrates, nitrogen gas, water, and recovery chemicals,

$$\text{water} = 24 \times 10^6 \frac{\text{gal}}{\text{day}} \times 0.20 \times \frac{\$0.20}{10^3 \text{ gal}} = \$1000/\text{day}$$

$$\text{glucose} = 13.2 \frac{\text{g}}{\ell} \times \frac{\text{lb.}}{454\text{g}} \times \frac{3.79\ell}{\text{gal}} \times \frac{24 \times 10^6 \text{ gal}}{\text{day}} \times \frac{\$15.3}{100 \text{ lb}} = \$405{,}000/\text{day}$$

N_2 gas (assumed that saturation of media is adequate) solubility (30^oC) = 3.1×10^{-4} mole N_2/mole solution

$$3.10 \times 10^{-4} \frac{\text{mole } N_2}{\text{mole water}} \times \frac{\text{mole water}}{18 \text{ lb water}} \times \frac{8.33 \text{ lb}}{\text{gal}} \times 24 \times 10^6 \frac{\text{gal}}{\text{day}}$$

$$\times 28 \frac{\text{lb}}{\text{mole}} \times \frac{\$0.0366}{\text{lb.}} = \$3{,}530/\text{day}$$

Ion exchange chemical:

$$H_2SO_4 = 2.72 \frac{\text{lb. } H_2SO_4}{\text{lb. } NH_4^+} \times 2000 \frac{\text{lb.}}{\text{ton}} \times 10 \frac{\text{ton } NH_4^+}{\text{day}} \times \frac{\text{ton}}{2000 \text{ lb.}}$$

$$\times \frac{\$50}{\text{ton}} = \$1{,}360/\text{day}$$

$$NaCl = \frac{7.10}{9.80} \times \$1{,}360 = \$985/\text{day}$$

Cost ratio from ion exchange recovery system of ref. 37.

$$NaOH = \frac{26.80}{9.80} \times \$1,360 = \$3,720/day$$

Cost ratio from ion exchange recovery system of ref. 37.

Raw materials = \$415,000/day

= \$151 x 10^6/year

2. Operating Labor

	Men/shift
Seed tank	1
Fermenters	40
Micro Lab.	4
Ion Exchange system	2
Utilities	2
Control Lab.	2
Chemical Storage	2
Misc.	2
	55

Tech.	55/shift x 3 shifts x \$15,000/yr.	=	\$2,500,000
Supervision	10/shift x 3 shifts x \$25,000/yr.	=	750,000
Janitorial, etc.	10/shift x 3 shifts x \$12,000/yr.	=	360,000
			\$3,600,000/yr.

3. Supervisory and clerical help (10 of labor) — \$ 400,000/yr.

4. Utilities (5 of product cost) — \$10 x 10^6/yr.

5. Maintenance and repair (5 of F.C.I.) — \$7.1 x 10^6/yr.

6. Operating Supplies (0.5 of F.C.I.) — \$0.7 x 10^6/yr.

\$172.8 x 10^6

B. Fixed Charges

1. Depreciation (10% of equipment +2% of buildings) = 5.4×10^6

2. Local Taxes (2% of F.C.I.) = 2.9×10^6

3. Insurance (1% of F.C.I.) = 1.4×10^6

C. Plant-overhead Costs (50% of labor + supervision + maintenance) = 5.6×10^6

$\$15.3 \times 10^6$/yr.

II. General Expenses

A. Administrative (15% of labor + supervision + maintenance) $\$ 1.7 \times 10^6$

B. Interest (7% of F.C.I.) 10.0×10^6

$\$11.7 \times 10^6$/yr.

III. Total Product Cost = $\$199.8 \times 10^6$/yr.

Glucose for Carbohydrate

$$\frac{\$199.8 \times 10^6}{\text{yr.}} \times \frac{\text{day}}{10 \text{ ton } NH_4^+} \times \frac{\text{yr.}}{365 \text{ days}} \times \frac{36 \text{ tons } NH_4^+}{132 \text{ ton } (NH_4)_2 SO_4}$$

$$= \frac{\$14,900}{\text{ton } (NH_4)_2 SO_4}$$

Molasses for Carbohydrates

$$13.2 \frac{g}{\ell} \times \frac{3.79}{\text{gal}} \times \frac{24 \times 10^6 \text{ gal}}{\text{day}} \times \frac{\text{lb.}}{454 g} \times \frac{\text{ton}}{2000 \text{ lb.}} \times \frac{2 \text{ ton molasses}}{\text{ton sugar}}$$

$$\times \frac{\$35}{\text{ton}} = \$92,500/\text{day}.$$

Raw Material:

Molasses	$ 92,500
Water	1,000
H_2SO_4	1,360
NaCl	985
NaOH	3,780
N_2	3,530
	$103,000/day = $37.6 x 10^6/yr.

Manufacturing cost:

Raw Materials	$37.6 x 10^6/yr.
Operating labor	3.6 x 10^6
Supervisory, etc.	0.4 x 10^6
Utilities	5.0 x 10^6
Maintenance	7.1 x 10^6
Operating Supplies	0.7 x 10^6
	$54.4 x 10^6/yr.
Fixed Changes	15.3 x 10^6
General Expenses	11.7 x 10^6
	$81.4 x 10^6/yr.

Total Product Cost

$$\frac{\$81.4 \times 10^6}{\text{yr.}} \times \frac{\text{day}}{10 \text{ ton } NH_4^+} \times \frac{\text{yr.}}{365 \text{ days}}$$

$$\times \frac{36 \text{ ton } NH_4^+}{132 \text{ ton } (NH_4)_2 SO_4} = \frac{\$6080}{\text{ton } (NH_4)_2 SO_4}$$

Free Carbohydrate

Raw Materials = $3,900,000/yr.

Manufacturing cost:

Raw materials	$ 3.9 x 10^6
Operating labor	3.6 x 10^6
Supervisory, etc.	0.4 x 10^6
Utilities	2.2 x 10^6
Maintenance	7.1 x 10^6
Operating Supplies	0.7 x 10^6
	$17.9 x 10^6/yr.
Fixed Charges	$15.3 x 10^6
General Expenses	11.7 x 10^6
	$44.9 x 10^6/yr.

Total Product Cost:

$$\frac{\$44.9 \times 10^6}{\text{yr.}} \times \frac{\text{day}}{10 \text{ ton } NH_4^+} \times \frac{\text{yr.}}{365 \text{ days}}$$

$$\times \frac{36 \text{ ton } NH_4^+}{132 \text{ ton } (NH_4)_2 SO_4} = \$3{,}350/\text{ton } (NH_4)_2 SO_4$$

APPENDIX X

COST ESTIMATES FOR *K. pneumoniae* in CONVENTIONAL FERMENTATION AT HIGH AMMONIA CONCENTRATION

APPENDIX X

COST ESTIMATES FOR K. pneumoniae IN CONVENTIONAL FERMENTATION AT HIGH AMMONIA CONCENTRATION

Capacity: 10 ton NH_4^+/day

Concentration: 0.1 molar = 1,800 mg NH_4^+/ℓ

Volumetric flow: 24×10^6 gal/day

$$10 \frac{\text{ton } NH_4^+}{\text{day}} \times \frac{2000 \text{ lb}}{\text{ton}} \times \frac{454 \text{g}}{\text{lb}} \times \frac{\ell}{1.8 \text{g}} \times \frac{\text{gal}}{3.79 \ell} = 1{,}331{,}000 \text{ gal/day}$$

$$= 55{,}500 \text{ gal/hr.}$$

For optical density = 1.1, $\frac{F}{V} = 0.086$ from Figure 12 .

$$F = 55{,}500 \text{ gal/hr.}$$

$$V = \frac{55{,}500 \text{ gal/hr.}}{0.086 \text{ hr}^{-1}} = 645{,}000 \text{ gal.}$$

I. Direct Costs

1. Purchased Equipment

a. Fermenters

Total volume = 645,000 gal.

~7 vessels at 100,000 gal/vessel

Agitated Jacked Vessel (Stainless Steel)

= $433,000/vessel (same calculation as Appendix IX)

Fermenter cost = 7 x $433,000 = $3,030,000

b. Seed Tank

$$\frac{0.645 \times 10^6 \text{ gal/day}}{24 \times 10^6 \text{ gal/day}} \times 35{,}000 \text{ gal} = 940 \text{ gal} \approx 1000 \text{ gal} = \$20{,}000 \text{ (1967 - Ref. 34)}$$

Apply M&S Index

$$\text{Cost} = 20{,}000 \times \frac{520}{300} = \$35{,}000$$

c. Settling pond

(Assumptions: tenfold increase in concentration in settling basin; residence twice about one-half growth time.)

$$1.33 \times 10^6 \frac{\text{gal}}{\text{day}} \times 0.5 \text{ day} = 670{,}000 \text{ gal} = \text{volume lagoon}$$

$$= 90{,}000 \text{ ft.}^3$$

$$\text{depth} = 10 \text{ ft.}$$

$$90{,}000 \text{ ft.}^3 \times \frac{1}{10 \text{ ft.}} = 9{,}000 \text{ ft.}^2 = 0.2 \text{ acre}$$

$\sim$\$1,000 from Ref.35
(Considered negligible)

d. Ammonia Recovery System (steam stripping)

Total flowrate = 1,331,000 gal/day x 0.8 = 1,065,000 gal/day.

	Estimates (Ref. 34)
Stripper (bubble-cap stainless steel)	$250,000
Saturator (stainless or lead-lined)	200,000
Crystallizer	200,000
Centrifuge (basket)	20,000
	$670,000

From Ref. 38, utility requirements for steam stripping are $1.14 x 10^6/year for an ammonia removal system of 4,667,000 gal/day at 4,800 mg NH_3/l. For the USS Engineers process, utility requirements for the same flow and concentration are $1.45 x 10^6/year. The differential investment for the USS Engineers process is $2.7 x 10^6 over the cost for basic steam stripping.

Therefore, $\frac{1.14}{1.45}$ = 0.80 or 80% utilities required for basic steam stripping

Total utilities system USS Engineers

$$= \frac{\$2.7 \times 10^6}{0.2} = \$13.5 \times 10^6$$

Total ammonia processed by the biological scheme is 10 tons/day or about one-tenth that of the Ref. 38; therefore, utilities estimated as 1/7 of USS Engineers requirement

$$\frac{\$13.5 \times 10^6}{7} = \$1.93 \times 10^6$$

Basic equipment + utilities system = 670,000 + $1,930,000

$\sim \$2.6 \times 10^6$

Summary of equipment costs:

Fermenters	$3,030,000
Seed tank	35,000
Settling pond	Negligible
Ammonia Recovery System	2,600,000
Total	$5,700,000

A.	Equipment	
	1. Purchased Equipment	$5,700,000
	2. Installation (25% of 1.)	1,430,000
	3. Instrumentation and control (10% of 1.)	570,000
	4. Piping (10% of 1.)	570,000
	5. Electrical (10% of 2.)	570,000
		$\$8.84 \times 10^6$
B.	Buildings (20% of 1.)	$1,140,000
C.	Service facilities, etc. (40% of 1.)	2,280,000
D.	Land (5% of 1.)	285,000
	Total Direct Cost	$\$12,6 \times 10^6$
II.	Indirect Costs (25% of T.D.C.)=	$\$ 3.1 \times 10^6$
	Fixed Capital Investment =	$\$15.7 \times 10^6$

III. Manufacturing Cost

A. Direct Production Cost

1. Raw Materials

Consider only carbohydrate, nitrogen gas, water, and recovery chemicals.

$$\text{water} = 1{,}331{,}000 \frac{\text{gal}}{\text{day}} \times 0.2 \times \frac{\$0.20}{10^3 \text{ gal}} = \$53/\text{day}$$

$$\text{glucose} = 13.2 \frac{\text{g}}{\text{l}} \times \frac{\text{lb}}{454\text{g}} \times 3.79 \frac{\text{l}}{\text{gal}} \times 1{,}331{,}000 \frac{\text{gal}}{\text{day}}$$

$$\times \frac{\$15.3}{100 \text{ lb}} = \$22{,}440/\text{day}$$

$H_2 SO_4$ (same as from Appendix X) = \$1,360/day

$$N_2 = \$3{,}530 \times \frac{1.3 \times 10^6 \text{ gal/day}}{24 \times 10^6 \text{ gal/day}} = \$191/\text{day}$$

$$\text{Lime} = \frac{2 \text{ lb}}{25 \text{ lb } (NH_4)_2 SO_4} \text{ from Ref. 39}$$

$$\frac{2 \text{ lb. lime}}{25 \text{ lb}} \times \frac{10 \text{ tons } NH_4^+}{\text{day}} \times \frac{2000 \text{ lb}}{\text{ton}} \times \frac{132 \text{ ton } (NH_4)_2 SO_4}{36 \text{ ton } NH_4^+}$$

$$\times \frac{\text{ton}}{2000 \text{ lb}} \times \frac{\$25}{\text{ton}} = \$73/\text{day}$$

Raw Materials $\approx$\$25,000/day

= \$8.8 x 10^6/yr

2. Operating Labor

Seed Tank	1
Fermenter	4
Micro Lab.	2
Ammonia recovery	1
Utilities	2
Control lab.	2
Chemical storage	2
Miscellaneous	1
	15

Tech.	15 x 3 x \$15,000 =	\$ 675,000
Supervision	3 x 3 x \$25,000 =	225,000
Janitorial	3 x 3 x \$12,000 =	108,000
		\$1,008,000

3. Supervisory and clerical help (10% of labor) \$100,000

4. Utilities 600,000

5. Maintenance and repairs (5% of F.C.I.) 790,000

6. Operating supplies (0.5% of F.C.I.) 80,000

\$11.4 x 10^6

B. Fixed Charges

1. Depreciation (10% of equipment +2% of buildings)	$907,000
2. Local Taxes (2% of F.C.I.)	314,000
3. Insurance (1% of F.C.I.)	160,000

C. Plant-overhead Costs

(50% of labor + supervision + maintenance)	950,000
	$2.3 x 10^6

II. General Expenses

A. Administrative

(15% of labor + supervision + maintenance)	$ 285,000

B. Interest (7% of F.C.I.)	1,100,000
	$1.3 x 10^6

III. Total Product Cost = $15 x 10^6/yr.

Glucose for Carbohydrate

$$\frac{\$15 \times 10^6/yr}{13{,}383 \text{ ton/yr.}} = \$1{,}100/\text{ton } (NH_4)_2\ SO_4$$

Molasses for Carbohydrate

Molasses = $5,130/day

Raw Materials

Molasses	$5,130
Water	53
H_2SO_4	1,360
N_2	191
Lime	73
	$6,807/day = $2.5 x 10^6/yr.

Manufacturing Cost:

Raw Material	$2.5 x 10^6
Operating labor	1.0 x 10^6
Supervisory, etc.	0.1 x 10^6
Utilities	0.5 x 10^6
Maintenance	0.8 x 10^6
Operating supplies	0.08 x 10^6
	$5 x 10^6

Total Product Cost = $8.6 x 10^6/yr.

$$\frac{\$8.6 \times 10^6/yr.}{13,383 \text{ ton/yr.}} = \$640/\text{ton } (NH_4)_2\ SO_4$$

Free Carbohydrate

Raw Materials = 612,000/yr.

Manufacturing cost:

Raw Materials	0.6×10^6
Operating labor	1.0×10^6
Supervisory, etc.	0.1×10^6
Utilities	0.5×10^6
Maintenance	0.8×10^6
Operating supplies	0.08×10^6
	3.08×10^6

Total Product Cost = $\$6.7 \times 10^6$/yr.

$$\frac{\$6.7 \times 10^6/\text{yr.}}{13{,}383 \text{ ton/yr.}} = \$500/\text{ton } (NH_4)_2\ SO_4$$

APPENDIX XI

COST ESTIMATES FOR K. pneumoniae IN LAGOON FERMENTATION AT LOW AMMONIA CONCENTRATION

APPENDIX XI

COST ESTIMATES FOR K. pneumoniae IN LAGOON FERMENTATION AT LOW AMMONIA CONCENTRATION

Capacity: 10 tons NH_4^+/day

Concentration: 5.4 mMolar NH_4^+ = 97 mg NH_4^+/ℓ

Volumetric flow: 24×10^6 gal/day

Same growth rate as observed in fermenter

Depth of Lagoon = 4 ft.
Residence time = 23 hours

Volume growth basin:

$$24 \times 10^6 \frac{\text{gal}}{\text{day}} \times \frac{23}{24} \text{ day} = 23 \quad 10^6 \text{ gal} = 3.07 \times 10^6 \text{ ft.}^3$$

Area of growth basin:

$$3.07 \times 10^6 \text{ ft.}^3 \times \frac{1}{4 \text{ ft.}} = 0.77 \times 10^6 \text{ ft.}^2 = 17 \text{ acres}$$

Total Capital Investment:

1. Growth basin

   a. Excavation and levee (at 1.00 yd.^{3}) = 20,000

   b. Liner and cover (at \$1.0/ft.^{2})

$$2 \times 0.77 \times 10^6 \text{ ft.}^2 \times \frac{\$1}{\text{ft}^2} = \$1.5 \times 10^6$$

(A cost of \$0.40/ft.^{2} was quoted by a supplier; however, installation and reinforcement would increase this value.)

c.	Panels	not used
d.	Pumps and piping	\$25,000
e.	Concrete (at 1.00/sq. ft.)	\$10,000
		\$1.5 x 10^6

2. Seed Tank

35,000 gal. from Appendix IX = \$208,000

3. Settling Pond

From Appendix IX = \$10,000

4. Ammonia Recovery

From Appendix IX = \$5,830,000

Capital Cost:

Growth Pond	1.5 x 10^6
Seed Tank	0.2 x 10^6
Settling ponds	Negligible
Ammonia Recovery	5.8 x 10^6
	7.5 x 10^6
Contingencies (15%)	1.2 x 10^6
	\$8.7 x 10^6

Raw Materials (Consider only glucose, nitrogen gas, water, and recovery chemicals)

From Appendix IX = $415,000/day

Operating Labor:

Ion Exchange	2
Pond, Seed Tank, Micro Lab, etc.	1
	3

Tech.	3/shift x 3 shifts x $15,000	= $135,000
Supervision	1/shift x 3 shifts x $25,000	= 75,000
Janitorial	1/shift x $12,000	= 12,000
		$222,000/yr.

Power Requirement

Ion Exchange

18 hp/10^6 gal. at $0.0192/kwh (from Ref. 37) = $\frac{\$6.20}{10^6 \text{ gal/day}}$

= $60,000

Pumping (lagoon, etc) = $100/day = $36,000

10 hp/10^6 gal.

Total = $96,000/yr.

Maintenance

(0.5% of Capital Cost)

$0.005 \times 8.7 \times 10^6 = \$40{,}000$

Land at \$2000/acre = \$34,000

Annual Cost

1.	Capital (at 8% for 20 yr.)	= \$ 900,000
2.	Raw Materials	= 151,000,000
3.	Power	= 96,000
4.	Labor	= 222,000
5.	Maintenance	= 40,000
6.	Land	= 3,800
		\$152 x 10^6/yr.

Product Cost

Glucose

$$\frac{\$152 \times 10^6/\text{yr.}}{13{,}383 \text{ ton/yr.}} = \$11{,}385/\text{ton } (NH_4)_2\ SO_4$$

Molasses

From Appendix IX, Raw Materials = $\$37.6 \times 10^6$/yr.

Annual cost is $\$38.6 \times 10^6$

$$\frac{\$38.6 \times 10^6/\text{yr.}}{13{,}383 \text{ ton/yr.}} = \$2530/\text{ton } (NH_4)_2\ SO_4$$

Free Carbohydrate

From Appendix IX, Raw Materials = $3,900,000

Annual Cost = $5.2 x 10^6

$$\frac{\$5.2 \times 10^6/\text{yr.}}{13{,}383 \text{ ton/yr.}} = \$386/\text{ton } (NH_4)_2\ SO_4$$

APPENDIX XII

COST ESTIMATE FOR *A. vinelandii* in CONVENTIONAL FERMENTATION

APPENDIX XII

COST ESTIMATE FOR *A. vinelandii* in CONVENTIONAL FERMENTATION

Capacity: $10 \frac{\text{tons fixed N}}{\text{day}}$

Cell Density: 2 g/ℓ

Efficiency = $18.7 \frac{\text{mg fixed N}}{\text{g glucose}}$ (observed at JPL)

Volumetric Flow = $10 \frac{\text{tons N}}{\text{day}} \times \frac{2000 \text{ lb}}{\text{ton}} \times \frac{454 \text{g}}{\text{lb}} \times \frac{\ell}{2 \text{g cells}}$

$$\times \frac{\text{g cells}}{0.1 \text{g N}} \times \frac{\text{gal}}{3.79\ell} = 11.98 \times 10^6 \text{ gal/day}$$

$$= 500{,}000 \text{ gal/hr.}$$

From dN/dt versus N (Figure 17)

$\frac{F}{V} = 0.035 \text{ hr}^{-1}$

F = 500,000 gal/hr.

$V = 500{,}000 \times \frac{1}{0{,}036} = 13.9 \times 10^6$ gal.

I. Direct Costs

1. Purchased Equipment

13.9×10^6 gal = 139 vessels at 100,000 gal/vessel

Cost/vessel = $433,000 (1978) from Appendix IX.

Cost = 139 x $433,000 = $60.2 x 10^6

2. Seed Tank

Cell population = 10^9 cells/ml

Inoculated fermenter = 3×10^6 cells/ml

$$V_{seed} = \frac{3 \times 10^6 \times 13.9 \times 10^6}{10^9} = 41{,}700 \text{ gal.}$$

From Ref. 34

Cost = \$165,000 (1967)

M&S Index 1968 = 300

1978 = 520

$$\text{Cost} = \$165{,}000 \times \frac{520}{300} = \$290{,}000$$

3. Settling Basin

(Assumptions: tenfold increase in concentration in settling basin; residence time about one-half growth time)

$$11.98 \times 10^6 \frac{\text{gal}}{\text{day}} \times \frac{13.9}{24} \text{ day} = 6.94 \times 10^6 \text{ gal.}$$

= vol. lagoon

Depth = 10 ft.

$$6.94 \times 10^6 \text{ gal} \times \frac{\text{ft.}^3}{7.48 \text{ gal}} \times \frac{1}{10 \text{ ft.}} = 92{,}800 \text{ ft.}^3$$

= 2.06 acres

Cost (excavation, levee, etc.) = \$8,000

Second settler of about 1/5 capacity of first settler could be used.

Area = 0.5 acre

Cost = $2,000

Equipment Cost:

1.	Fermenters	60.2×10^6
2.	Seed Tank	0.3×10^6
3.	Settling ponds	Negligible
	Total	$\$60.5 \times 10^6$

A.	Equipment	
1.	Purchased equipment	$\$60.5 \times 10^6$
2.	Installation (25% of 1.)	15.1×10^6
3.	Instrumentation and control (10% of 1.)	6.0×10^6
4.	Piping (10% of 1.)	6.0×10^6
5.	Electrical (10% of 1.)	6.0×10^6
		$\$93.6 \times 10^6$
B.	Buildings (20% of 1.)	$\$12.1 \times 10^6$
C.	Service facilities, etc. (40% of 1.)	24.2×10^6
D.	Land (5% of 1.)	3×10^6
	Total Direct Cost	$\$132.9 \times 10^6$
II.	Indirect Costs (25% of T.D.C.)	$\$ 33.3 \times 10^6$
	Fixed Capital Investment =	$\$166.2 \times 10^6$

III. Manufacturing Cost

A. Direct Production Cost

1. Raw Materials

Media	Concentration	Price
KH_2PO_4	0.2 g/ℓ	\$297/300 lb.
K_2HPO_4	0.8 g/ℓ	\$1/lb.
Glucose	20 g/ℓ	\$0.152/lb.
$MgSO_4 \cdot 7\ H_2O$	0.2 g/ℓ	\$8.50/100 lb.
$CaCl_2 \cdot 2\ H_2O$	0.09 g/ℓ	\$67/ton
$FeCL_2 \cdot 6\ H_2O$	1 mg/ℓ	\$4.70/100 lb.
Na_2MoO_4	0.1 mg/ℓ	\$1.88/lb.

Water (20% loss) = 2.40×10^6 gal/day

$$2.40 \times 10^6 \frac{\text{gal}}{\text{day}} \times \frac{\$0.20}{10^3 \text{ gal}} = \$480/\text{day}$$

Glucose

$$13 \frac{\text{g}}{\ell} \times \frac{\text{lb}}{454\text{g}} \times \frac{3.79\ell}{\text{gal}} \times 11.98 \times 10^6 \frac{\text{gal}}{\text{day}} \times \frac{\$0.153}{\text{lb}} = \$200{,}000/\text{day}$$

KH_2PO_4

$$0.2 \frac{\text{g}}{\ell} \times 0.2 \times \frac{1}{454} \times 3.79 \times 11.98 \times 10^6 \times \frac{\$297}{300 \text{ lb}} = \$4{,}000/\text{day}$$

$MgSO_4 \cdot 7\ H_2O$ = $ 340/day

$CaCl_2 \cdot 2\ H_2O$ = $ 60/day

$FeCl_2 \cdot 6\ H_2O$ = $ 4.70/day

$Na_2M\ O_4$ = $ 19/day

K_2HPO_4 = $16,000/day

Air (8.5 ℓ/min in a 5 liter fermenter)

$$8.5\ \frac{\ell}{\text{min}} \times \frac{60\ \text{min}}{\text{hr}} \times \frac{24\ \text{hr}}{\text{day}} \times \frac{\text{gal}}{3.79\ell} \times \frac{\text{ft.}^3}{7.48\ \text{gal}} = 432\ \frac{\text{ft}^3}{\text{day}}\ \text{for 5 liters}$$

$$432\ \frac{\text{ft}^3}{5\ell} \times \frac{3.79\ell}{\text{gal}} \times \frac{13.9 \times 10^6\ \text{gal}}{\text{day}} = 4500 \times 10^6\ \frac{\text{ft}^3}{\text{day}}\ \text{at}\ \frac{\$0.03}{10^3\ \text{ft}^3}$$

= $130,000/day

Considerably lower aeration rates may be suitable. For example, in aeration tanks for waste treatment air requirements to insure adequate mixing and aeration vary from 20-30 scfm/1000 ft.^{3} of tank volume.[57]

Many of these raw materials could be purchased at lower price for the vast quantities required for the fermentations. Furthermore, cheaper chemicals could be used in place of certain nutrients.

Raw materials (all chemicals considered at the concentrations and prices stated) = $350,000/day

2. Operating labor:

	Men/Shift
Seed Tank	1
Fermenters	40
Micro. Lab.	4
Utilities	2
Control Lab.	2
Chemical Storage	2
Miscellaneous	2
	63

Tech.	63/shifts x 3 shifts x $15,000	=	$2,800,000
Supervision	10/shifts x 3 shifts x $25,000	=	750,000
Janitorial	10/shifts x 3 shifts x $12,000	=	360,000
			$3,900,000/yr.

3. Supervisory and clerical help (10% of labor) = $ 390,000

4. Utilities (5% of Product Cost) = 8,600,000

5. Maintenance and repair (5% of F.C.I.) = 8,300,000

6. Operating supplies (0.5% of F.C.I.) = 800,000

$150 x 10^6

B. Fixed Charges

1. Depreciation = 6.2×10^6

2. Local Taxes = 3.3×10^6

3. Insurance = 1.7×10^6

C. Plant-Overhead Costs

$$= \frac{6.3 \times 10^6}{\$17.5 \times 10^6}$$

II. General Expenses

A. Administrative = $\$ 1.8 \times 10^6$

B. Interest = $\frac{11.65 \times 10^6}{\$13.5 \times 10^6}$

Total Product Cost = $\$181 \times 10^6$/yr.

Glucose for Carbohydrates

$$10 \frac{\text{tons N}}{\text{day}} \times \frac{365 \text{ days}}{\text{yr.}} \times \frac{2000 \text{ lb}}{\text{ton}} = 7{,}300{,}000 \text{ lb/yr.}$$

$$\frac{\$181 \times 10^6/\text{yr.}}{7{,}300{,}000 \text{ lb/yr.}} = \$24.8/\text{lb fixed N}$$

Molasses as Carbohydrate

Manufacturing costs:

Raw Material	$71,200,000
Labor	3,900,000
Supervisory and Clerical	390,000
Utilities	5,000,000
Maintenance and repair	8,300,000
Operating supplies	800,000
	$89,500,000

Total Product Cost = $121 x 10^6

$$\frac{\$121 \times 10^6/\text{yr.}}{7{,}300{,}000 \text{ lb/yr.}} = \$16.6/\text{lb Fixed N}$$

Free Carbohydrate

Manufacturing costs:

Raw Materials	$55,080,000/yr.
Labor	3,900,000
Supervisory and clerical	390,000
Utilities	5,000,000
Maintenance and repair	8,300,000
Operating supplies	800,000
	$74 x 10^6

Total Product Cost = $105 x 10^6

$$\frac{\$105 \times 10^6/\text{yr.}}{7{,}300{,}000 \text{ lb/yr.}} = \$14.4/\text{lb Fixed N}$$

APPENDIX XIII

COST ESTIMATE FOR *A. vinelandii* IN LAGOON FERMENTATION FOR THEORETICAL LIMITS OF NITROGEN FIXATION

APPENDIX XIII

COST ESTIMATE FOR *A. vinelandii* IN LAGOON FERMENTATION FOR THEORETICAL LIMITS OF NITROGEN FIXATION

Capacity: $10 \frac{\text{ton Fixed N}}{\text{day}}$

Glucose consumption same as observed: 10.72 g/ℓ

Efficiency: 280 mg Fixed N/g glucose

Residence time in lagoon assumed to be twice that observed in fermenter.

Surface aeration only.

$$10.72 \frac{\text{g glucose}}{\ell} \times 280 \frac{\text{mg N}}{\text{g glucose}} \times \frac{\text{g}}{10^3 \text{ mg}} = 3 \frac{\text{gN}}{\ell}$$

$$= 30 \frac{\text{g cells}}{\ell}$$

$$10 \frac{\text{ton N}}{\text{day}} \times \frac{2000 \text{ lb}}{\text{ton}} \times \frac{454 \text{g}}{\text{lb}} \times \frac{\ell}{30 \text{g cells}}$$

$$\times \frac{\text{g cell}}{0.1 \text{g N}} \times \frac{\text{gal}}{3.79 \ell} = 798{,}000 \text{ gal/day}$$

$$= 33{,}250 \text{ gal/hr.}$$

$\frac{F}{V} = 0.018$ (growth rate = $\frac{1}{2}$ that in fermenter)

$$V = 33{,}250 \frac{\text{gal}}{\text{hr.}} \times \frac{1}{0.018} = 1{,}847{,}000 \text{ gal.}$$

$$= 247{,}000 \text{ ft.}^3$$

Area of growth basin:

$$= 247{,}000 \text{ ft.}^3 \times \frac{1}{4 \text{ ft.}} = 61{,}800 \text{ ft.}^2$$

$$= 1.37 \text{ acres}$$

Total Capital Investment

1. Growth Basin

a.	Excavation	$ 2,000
b.	Liner (at $1.0/ft.^{2})	61,800
c.	Pumps and piping	5,000
d.	Concrete	1,000
		$69,800

2. Seed Tank

$$V_{\text{seed Tank}} = \frac{1.847 \times 10^6}{10^9} \times 3 \times 10^6 = 5{,}500 \text{ gal.}$$

From Ref.[34] = $43,000 (1967)

Apply M&S Index

$$= 43{,}000 \times \frac{520}{300}$$

$$= \$75{,}000 \text{ (1968)}$$

3. Settling Pond

Residence time = $\frac{1}{2}$ that of growth basin

= 27.7 hrs.

$$798{,}000 \frac{\text{gal}}{\text{day}} \times \frac{27.7}{24} \text{ day} = 921{,}000 \text{ gal.}$$

depth = 10 ft.

$$921{,}000 \text{ gal} \times \frac{\text{ft.}^3}{7.48 \text{ gal}} \times \frac{1}{10 \text{ ft.}} = 12{,}300 \text{ ft.}^2$$

= 0.3 acre

Cost = $1,000

4. Contingencies (15%)		$22,000
	Total	$168,000

Operating Labor	Men/Shift
Seed Tank	1
Micro. Lab.	1
Ponds and Misc.	1
	3

Tech.	3 shifts x 3 shifts x $15,000	= $135,000
Supervision	1 shift x 3 shifts x $25,000	= 75,000
Janitorial, etc.	1 shift x 1 shift x $12,000	= 12,000
		$222,000

Power Requirements $3/day = $1,000 (est.)

Maintenance (0.5% of capital) = 840

Land at 2000/acre = 3,400

Raw Materials:

$$\text{Water} = 798{,}000 \frac{\text{gal}}{\text{day}} \times 0.2 \times \frac{\$0.20}{10^3 \text{ gal}} = \$32/\text{day}$$

$$\text{glucose} = 13 \frac{\text{g}}{\ell} \times \frac{\text{lb.}}{454\text{g}} \times \frac{3.79}{\text{gal}} \times 798{,}000 \frac{\text{gal}}{\text{day}} \times \frac{\$0.153}{\text{lb.}}$$

$$= \$13{,}250/\text{day}$$

KH_2PO_4 = $ 264/day

$MgSO_4$ = $ 23/day

$CaCl_2$ = $ 4/day

$FeCl_2$ = Negligible

$NaMoO_4$ = Negligible

K_2HPO_4 = $ 1,065/day

$14,700/day = $5.34 x 10^6/yr.

Annual Cost:

1.	Capital (at 8% 20 yr)	$17,100
2.	Raw Materials	5.34×10^6
3.	Power	1,000
4.	Maintenance	840
5.	Operating labor	220,000
5.	Land	350
		$\$5.58 \times 10^6$/yr.

Product Cost:

Glucose

$$\frac{\$5.58 \times 10^6/\text{yr.}}{7{,}300{,}000\ \text{lb/yr.}} = \$0.76/\text{lb.N}$$

Molasses

$$13\ \frac{g}{\ell} \times \frac{3.79\ell}{\text{gal}} \times \frac{798{,}000\ \text{gal}}{\text{day}} \times \frac{\text{lb.}}{454g} \times \frac{\text{ton}}{2000\ \text{lb.}} \times \frac{2\ \text{lb molasses}}{\text{lb sugar}}$$

$$\times \frac{\$35}{\text{ton}} = \$3{,}030/\text{day}$$

Raw Materials = $4,418/day

= 1.6×10^6/yr.

Annual Cost = 1.85×10^6/yr.

$$\frac{\$1.85 \times 10^6/\text{yr.}}{7{,}300{,}000\ \text{lb/yr.}} = \$0.26/\text{lb.N}$$

Free Carbohydrate

Raw Materials = $505,000/yr.

Annual Cost = $745,000

$$\frac{\$745,000/yr.}{7,300,000\ lb/yr.} = \$0.10/lb.$$