

**Large scale growth of *E. coli* carrying the  
pMK 108 plasmid**

John Creasy

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Tennessee Scholars

Polymers are substances which consist of long chains of repeating units. They are useful in many capacities ranging from surgical implants to potato chip bags. Most usable polymers are inorganic. However, a number of biological molecules have very favorable physical properties when they are compared against standard metals and polymers - for example the tensile strength of bone or spider web is higher than that of steel on a pound for pound basis. Other biological molecules have molecular structures that suggest favorable bulk properties, but molecules are not available in bulk quantities for affordable prices. DNA is one such molecule. A large quantity of DNA is very difficult and expensive to produce. A good source of DNA is plasmid DNA, or small, circular strands of genetic code. Plasmid DNA can be purified in large quantities from the bacteria *E. coli*. This paper will address the problem of growing large amounts of *E. coli* while retaining the plasmid which is to be extracted and purified later.

## **Materials and Methods**

### *Organism*

The organism used was *Escherichia coli* MK 108, containing a genetically engineered plasmid pMK 108. pMK 108 carries the ampicillin resistant portion of the pBR 322 plasmid and the transposable element from the Mu phage including the ends necessary for transposition. This strain was genetically engineered by Craigie and Mizuuchi. The MK 108 cells were maintained at 4<sup>0</sup> temporarily on YT agar plates containing 50  $\mu\text{g ml}^{-1}$  ampicillin in order to retain the plasmid. For longer periods of time, the cells were stored at -70<sup>0</sup>.

### *Chemicals and enzymes*

Yeast extract, bacto-tryptone, and bacto-agar were obtained from Difco (Detroit, MI); chloramphenicol and chicken egg-white lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO); sodium ampicillin was bought from International Biotechnologies, Inc. (New Haven, CT); and sodium dodecyl sulfate was purchased from Pierre Chemical Company (Rockford, IL).

### *Media*

YT broth was used throughout the growth experiments. YT agar with and without  $50 \mu\text{g ml}^{-1}$  ampicillin was used for growth plates. All inoculums were grown in ampicillin-free YT broth. All but two cultures were grown in YT broth only; the two cultures grown on March 2nd and March 9th were grown in YT broth containing 50 and  $100 \mu\text{g ml}^{-1}$  ampicillin, respectively.

### *Growth conditions*

For each growth an inoculum was grown over night in YT broth. The inoculum was transferred to a 3 liter fermentor from Lab-line Bioengineering (Melrose Park, IL) containing 2 liters of media. Each growth was a batch fermentation. Growth time averaged between 8 and 12 hours. The agitation rate was 750 rpm. The initial pH of each growth was 7.0 and was not regulated except in the growth on March 2nd in which enough glacial acetic acid was added to bring the pH down to 6.5 after 6 hours of growth. Air was fed in at a constant rate beginning at 95% saturation with oxygen. All but two growths were grown in ampicillin-free YT broth, as stated above.

### *Analytical procedures*

Aproximately every two hours, samples were taken from each batch growth. The pH of the sample was determined as was the absorbance at 540 nm on a CARY 2220 spectrophotometer from Varian (Springvale, Australia). Various dilutions of each culture sample were plated on YT and YT-amp. plates. The plates were allowed to grow for about 6-8 hours. The cell number on each plate was recorded and used to produce the growth curves.

### *Harvesting of bacteria*

The bacterial cells from a 500-ml culture were harvested by centrifugation at 5000 rpm for 10 minutes at 4° in a Beckman (Fullerton, CA) JA-14 rotor placed in a Beckman J2-21 centrifuge. The supernatant was discarded and the pellet was homogenized in 50 ml STE (0.1 M NaCl, 10 mM Tris-Cl (pH 7.8), and 1 mM EDTA). The homologous solution was centrifuged on a Beckman JA-20 rotor under the same conditions as above. The homogenization and cetrifugation steps were repeated twice more to insure purity of cells. The pellet could then be stored at -70° in a cryofreezer for an indefinite period of time.

### *Lysis of cells and isolation of plasmid DNA*

The pellet harvested from a 500-ml culture was resuspended in 10 ml of solution I containing 5 mg ml<sup>-1</sup> lysozyme. [Solution I was made from 50 mM glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA (ethylenediamine tetraacetic acid).] The solution was transfered into a 50 ml polyallomer tube and allowed to stand at room temperature (25°) for 5 minutes. 20 ml of freshly made solution II was added. [Solution II consisted of 0.2 N NaOH and 1% SDS (sodium dodecyl sulfata).] The contents were mixed via several gentle inversions and then allowed to stand on ice for 10 minutes. 15 ml of an ice-cold solution of potassium acetate (3 M with respect to

potassium and 5 M with respect to acetate) was then added. The contents of the tube were mixed via several sharp inversions and then allowed to stand on ice for another 10 minutes. The solution was centrifuged on a Beckman JA-20 rotor at 10,000 rpm for 80 minutes at 4°. The cellular DNA and bacterial debris from lysis formed a tight pellet on the bottom of the polyallomer tube. The supernatant, which contained the plasmid DNA and some RNA was transferred in equal quantities into two new polyallomer tubes. 0.6 volumes of isopropanol was added to each tube. The solution was mixed well and allowed to stand at room temperature for 15 minutes. The plasmid DNA was recovered by centrifugation at 10,000 rpm for 30 minutes at room temperature. The supernatant was discarded, and the pellet was washed with room temperature 70% ethanol. The pellet was dried briefly in a vacuum desiccator and then dissolved in a total volume of TE (pH 8.0). This form of lysis is listed in Maniatis (1982) and is described by Birnboim and Doly (1979). The TE solution can be stored at either 4° or -70°. The absorbance of this solution can be taken at 260 nm and 280 nm. The ratio of  $Abs_{260}/Abs_{280}$  is indicative of whether the solution tested is mostly RNA or DNA. A ratio of 1.8 indicates that mostly DNA is present, while a ratio of 2.0 indicates that the solution is contaminated totally with RNA. Performed carefully, alkali lysis can produce an absorbance ratio of 1.8 and rather pure plasmid DNA.

## **Results and Discussion**

### *Repeats of previous results*

The first couple of months of the Fall semester of 1988 were spent learning laboratory procedures. Preparing plates; making broth and other solutions; plating cells; operating the autoclave, spectrophotometer, fermentor, etc.; lysing cells; and doing many other menial laboratory functions were all skills which took over half of the semester to acquire.

Most preliminary results were confirmed by textbook cases, and those that weren't were redone. Only after all these talents were learned could the first experimental growths be performed.

### *New results*

The conditions in the first four growths were all the same. The first two growths (November 30th and December 5th) were dry runs and used mainly for educational purposes. The next two runs (January 24th and February 7th) were more reliable and could be compared against one another. Exponential growth was reached after 6-8 hours. During this phase, in which the cell growth was the most rapid, percent free oxygen decreased constantly from 95 to 79. After log growth, during stationary phase, the percent oxygen gradually increased. The low amount of free oxygen during the heaviest period of growth is due to the increase in oxygen required by the aerobic *E. coli* for protein synthesis and replication. pH increased consistently throughout log phase and leveled off to about 9 when stationary phase was reached. The rise in pH could be attributed to excretion of alkali material by the bacteria into the media, the chewing up of the media, or both. Absorption measurements were not very helpful because after 4 hours of growth the ratio between cell bodies to the absorbance was no longer linear. Within the first 4 hours the number of cell bodies was not statistically different from the number of viable cells. By comparing the number of cells which grew on YT plates to the number of cells which grew on YT-amp. plates, the number of cells which did not retain pMK 108 could be determined. However, there was no recognizable difference between the two number of cells.

A couple of growth conditions were varied in the last two growths (March 2nd and March 9th). Firstly, ampicillin was added to the media to be used in the batch growths. Stationary phase was not reached until after 10-12 hours of growth, probably because many cells were destroyed

by the ampicillin, therefore causing the time to reach saturated growth to increase. In the March 2nd growth, in which  $50 \mu\text{g ml}^{-1}$  ampicillin was added to the media, glacial acetic acid was used to bring the pH from 8.5 down to 6.5. As a result the pH never got above 8.65. The cell density was also the highest ever reached,  $1 \times 10^{10}$  cells  $\text{ml}^{-1}$ . This seems to indicate the importance of pH control in order to maximize cell density. There was also a decrease in the number of plasmid carrying cells after 10 hours of growth. After the ampicillin was inactivated, the cells with the plasmid were disadvantaged in comparison to the plasmid-free cells (Dale and Smith, 1979). In the March 9th growth, in which  $100 \mu\text{g ml}^{-1}$  ampicillin was added, analysis yielded results similar to those obtained from growths without ampicillin, except that a longer log phase was seen on March 9th. Perhaps the ampicillin concentration could be increased even further, thereby increasing the plasmid copy number and amount of plasmid.

### Conclusion

There are ideal conditions which would maximize the cell density. Nevertheless, maximizing the cell density is useless unless the plasmid is retained. The physiological conditions also have a effect on the plasmid and the plasmid copy number (Cheah, *et al.*, 1987). My paper only covers the beginning of the study of growth conditions to maximize plasmid production. The next step could be to add chloramphenicol after log phase. This drug shuts down protein synthesis and allows plasmid replication to continue, thus increasing the plasmid copy number even more. The growths could also be scaled up to a larger batch (300 liters). Continuous-flow fermentation is another possibility (Wouters, *et al.*, 1980). Then isolation of DNA and cell lysis experiments need to be performed. The use of a modified form of alkali lysis is a likely area of

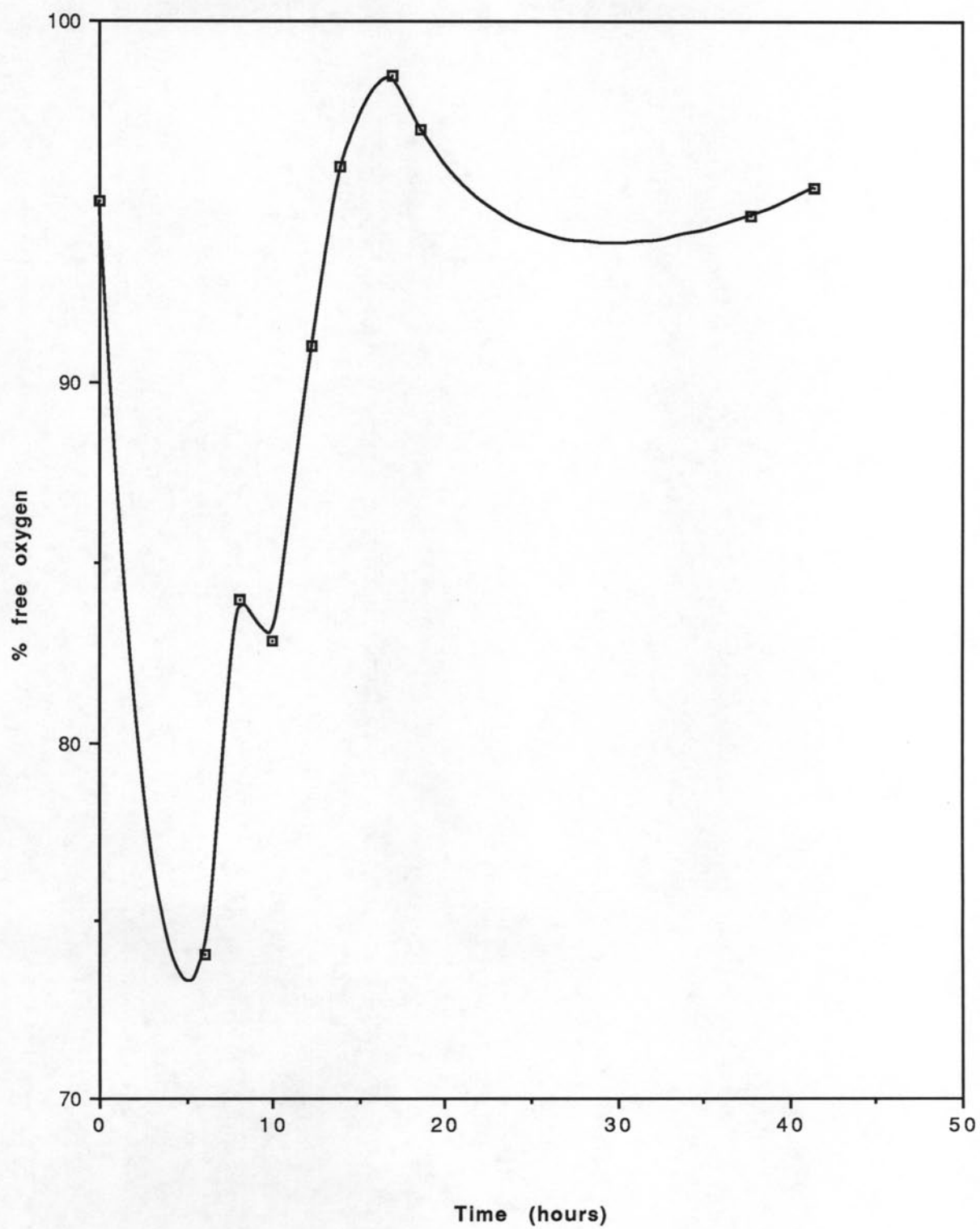
study (Lima, *et al.*, 1988). As parts are added to this project by other workers, another step in allowing the properties of biological molecules to be used as parts of manmade materials will be accomplished.

## Resources

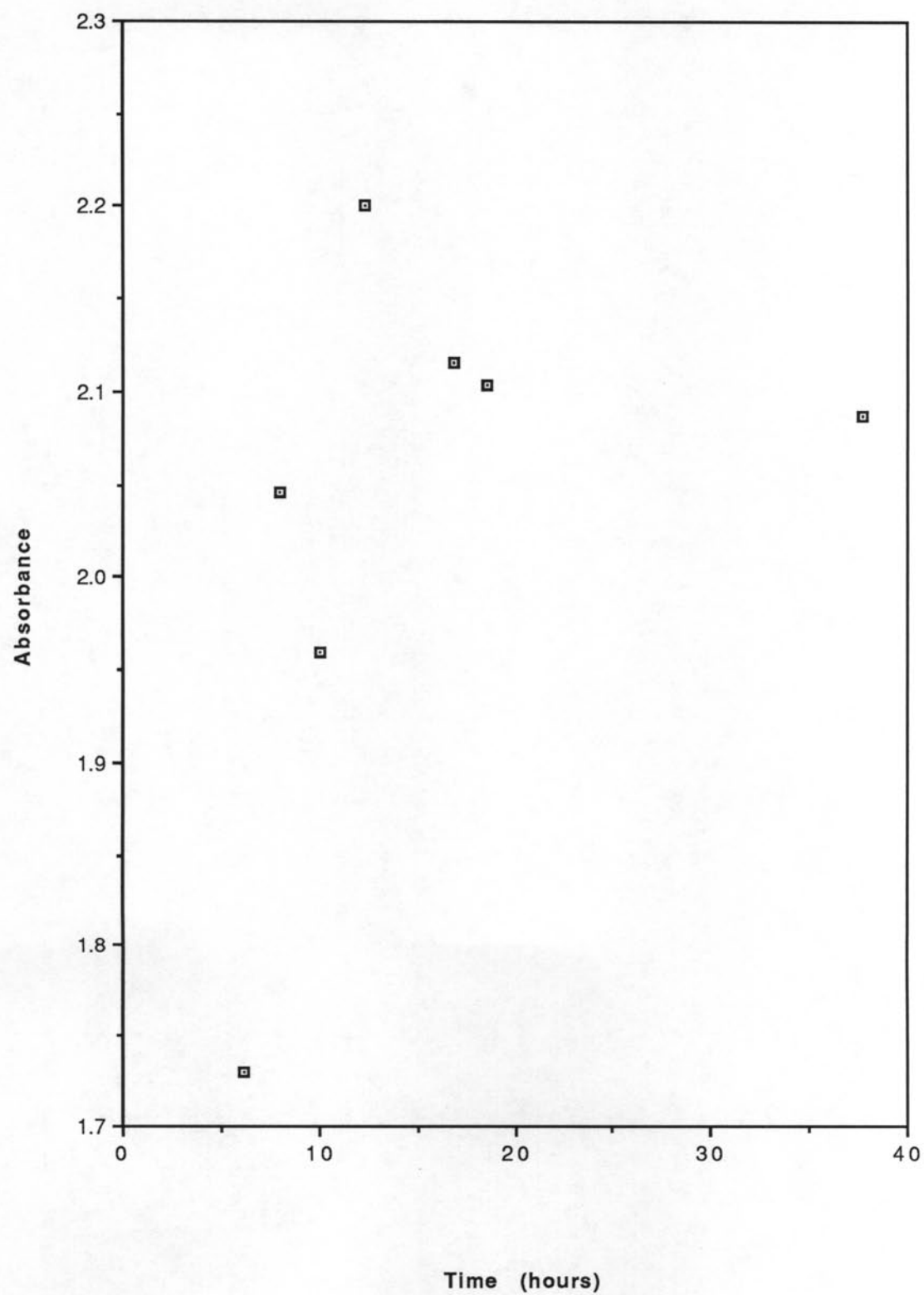
- BIRNBOIM, H. C. and DOLY, J. 1979. Purification of DNA using Alkali Lysis. *Nucleic Acids Res.* **7**, 1513.
- CHEAH, U. EONG, WEIGAND, WILLIAM A., and STARK, BENJAMIN C. 1987. Effects of recombinant plasmid size on cellular processes in *Escherichia coli*. *Plasmid* **18**, 127-134.
- CRAIGIE, R. and MIZUUCHI, K. 1985. The production of a vector containing phage *Mu* ends and ampicillin resistant region of pBR 322. *Cell* **41**, 867-876.
- DALE, J. W. and SMITH, J. T. 1979. The effect of a plasmid on growth and survival of *E. Coli*. *Antonie van Leeuwenhoek* **45**, 103-111.
- LIMA, W. F., ROSZAK, D. A., DRIVER, D. A., SELIGSON, D. B., and SHRAWDER, E. J. 1988. An effective preparative scale plasmid purification method. *Anal. Biochem.* **11**, 20-22.
- MANIATIS, T., FRITSCH, E. F., and SAMBROOK, J. 1982. "Molecular Cloning: A Laboratory Manual." p. 90, 91. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- WOUTERS, J. T. M., DRIEHUIS, F. L., POLACZEK, P. J., VAN ANDEL, J. G. 1980. Persistence of the pBR 322 plasmid in *Escherichia coli* grown in chemostat cultures. *Antonie van Leeuwenhoek* **46**, 353-362.

|    | Time (hours) | % Oxygen | Absorbance | YT       | YT-amp   |
|----|--------------|----------|------------|----------|----------|
| 1  | 0.000        | 95.000   |            |          |          |
| 2  | 6.117        | 74.100   | 1.730      |          |          |
| 3  | 8.033        | 84.000   | 2.046      |          |          |
| 4  | 10.033       | 82.900   | 1.960      |          |          |
| 5  | 12.270       | 91.000   | 2.200      | 1.29e+10 | 1.46e+10 |
| 6  | 13.917       | 96.000   |            |          | 9.98e+9  |
| 7  | 16.915       | 98.500   | 2.116      |          |          |
| 8  | 18.600       | 97.000   | 2.104      |          |          |
| 9  | 37.750       | 94.600   | 2.087      |          |          |
| 10 | 41.367       | 95.400   |            |          |          |

Oxygen vs. Time  
11/30/88

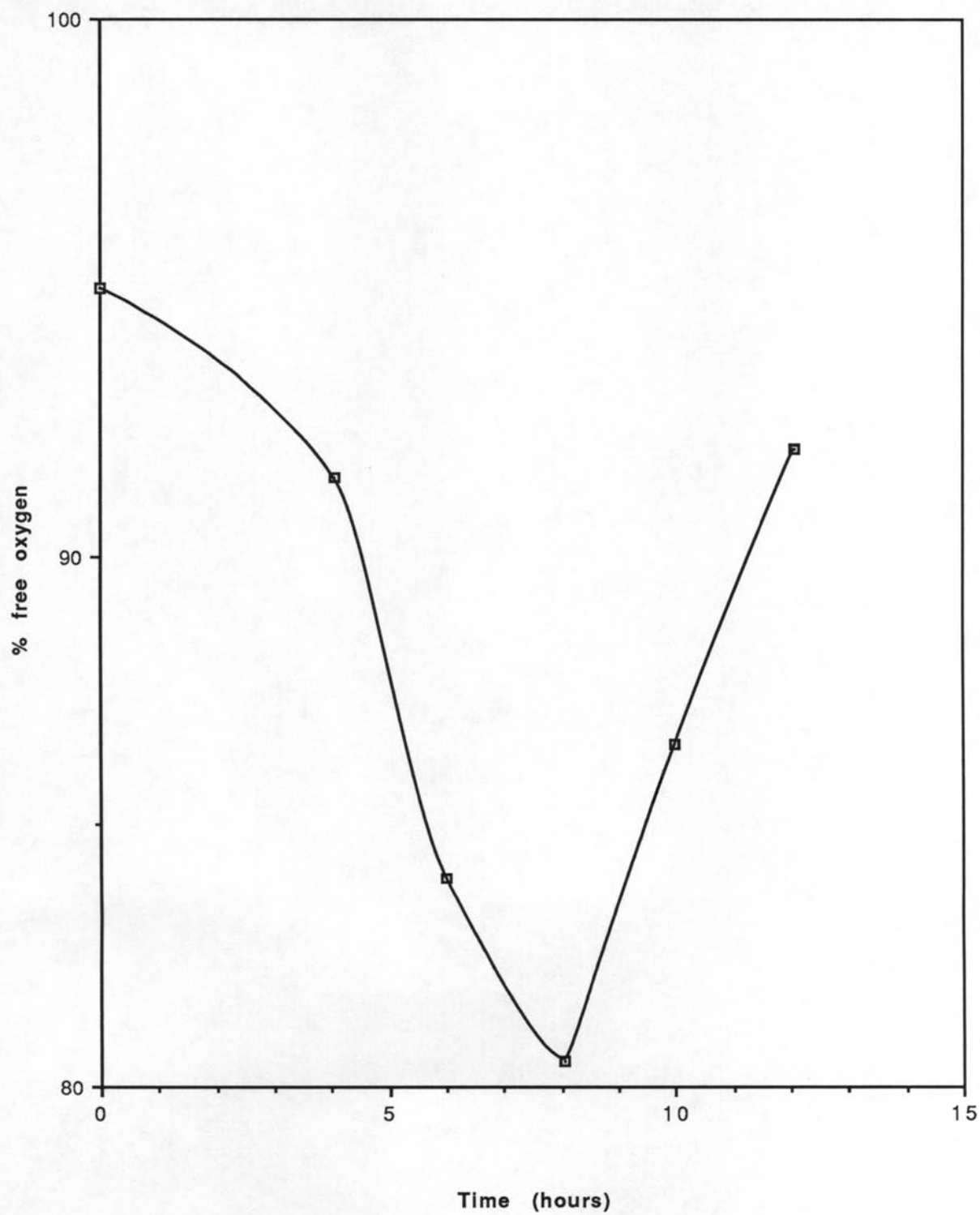


**Absorbance vs. Time**  
**11-30-88**

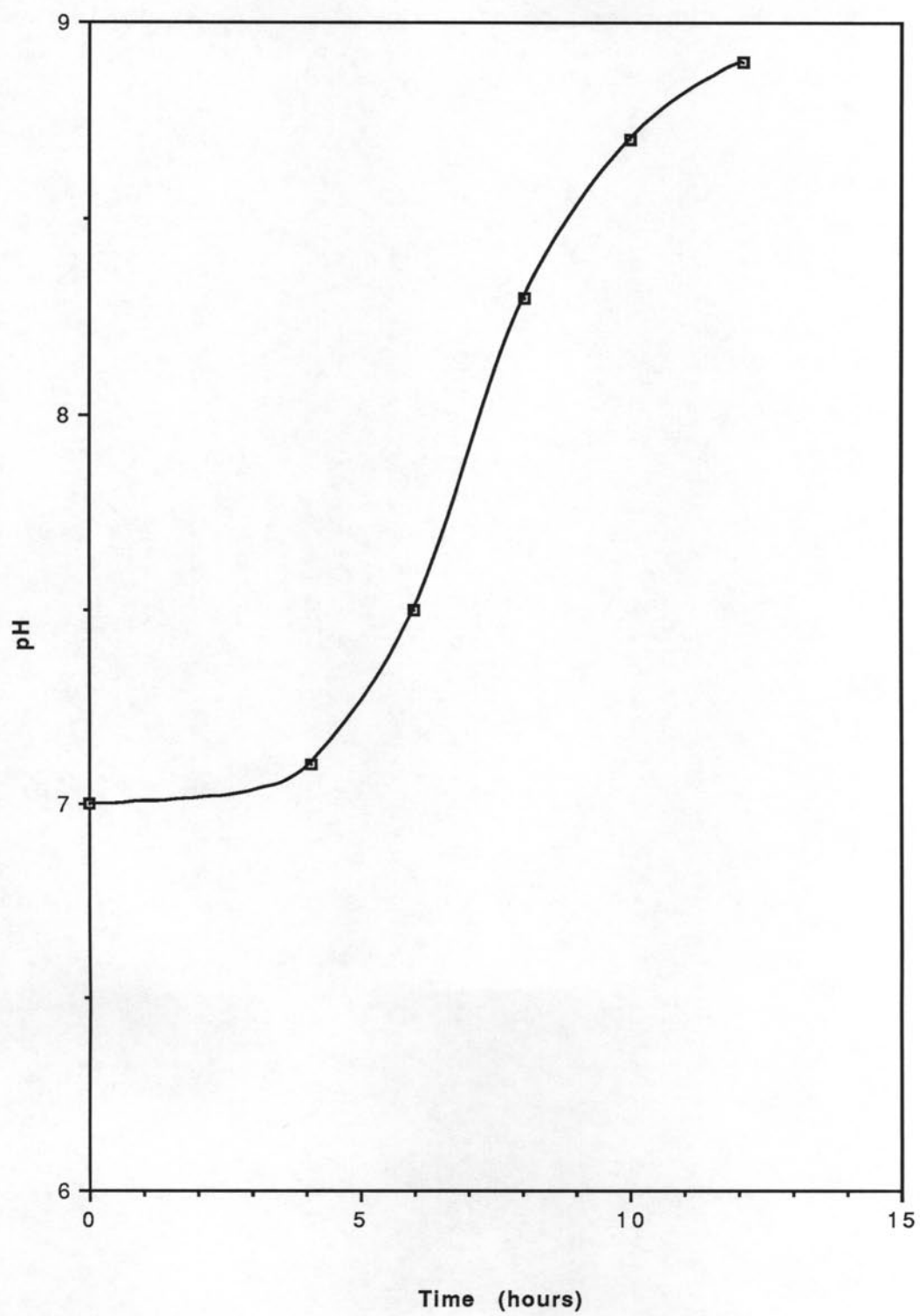


|   | Time (hours) | % Oxygen | Absorbance | YT      | YT-amp  |
|---|--------------|----------|------------|---------|---------|
| 1 | 0.000        | 95.000   |            |         |         |
| 2 | 1.750        | 95.500   | 0.020      | 7.20e+5 | 7.40e+5 |
| 3 | 3.817        | 97.500   | 0.260      | 1.60e+7 | 3.40e+6 |
| 4 | 5.833        | 50.700   | 1.270      |         |         |

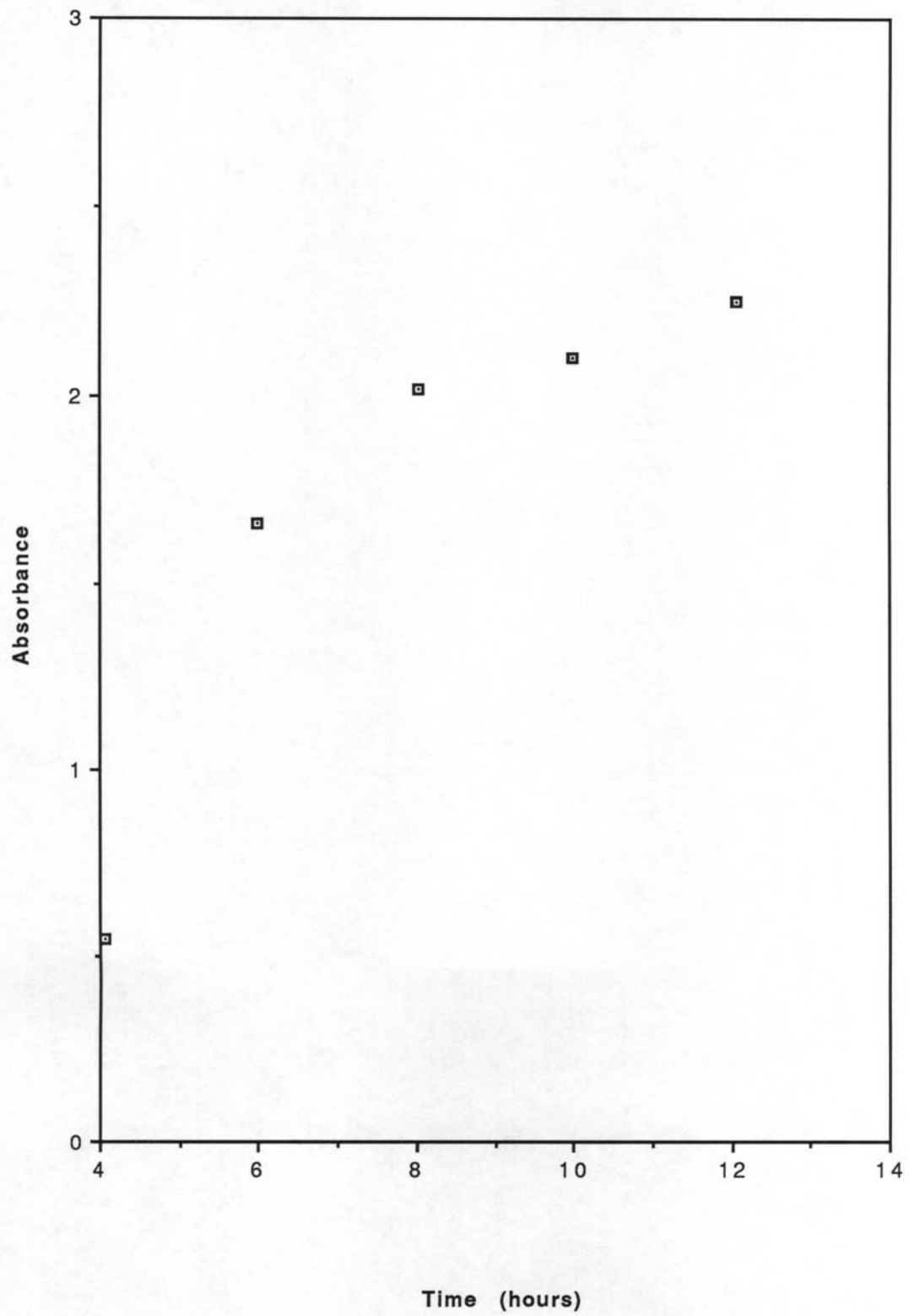
Oxygen vs. Time  
12/13/88



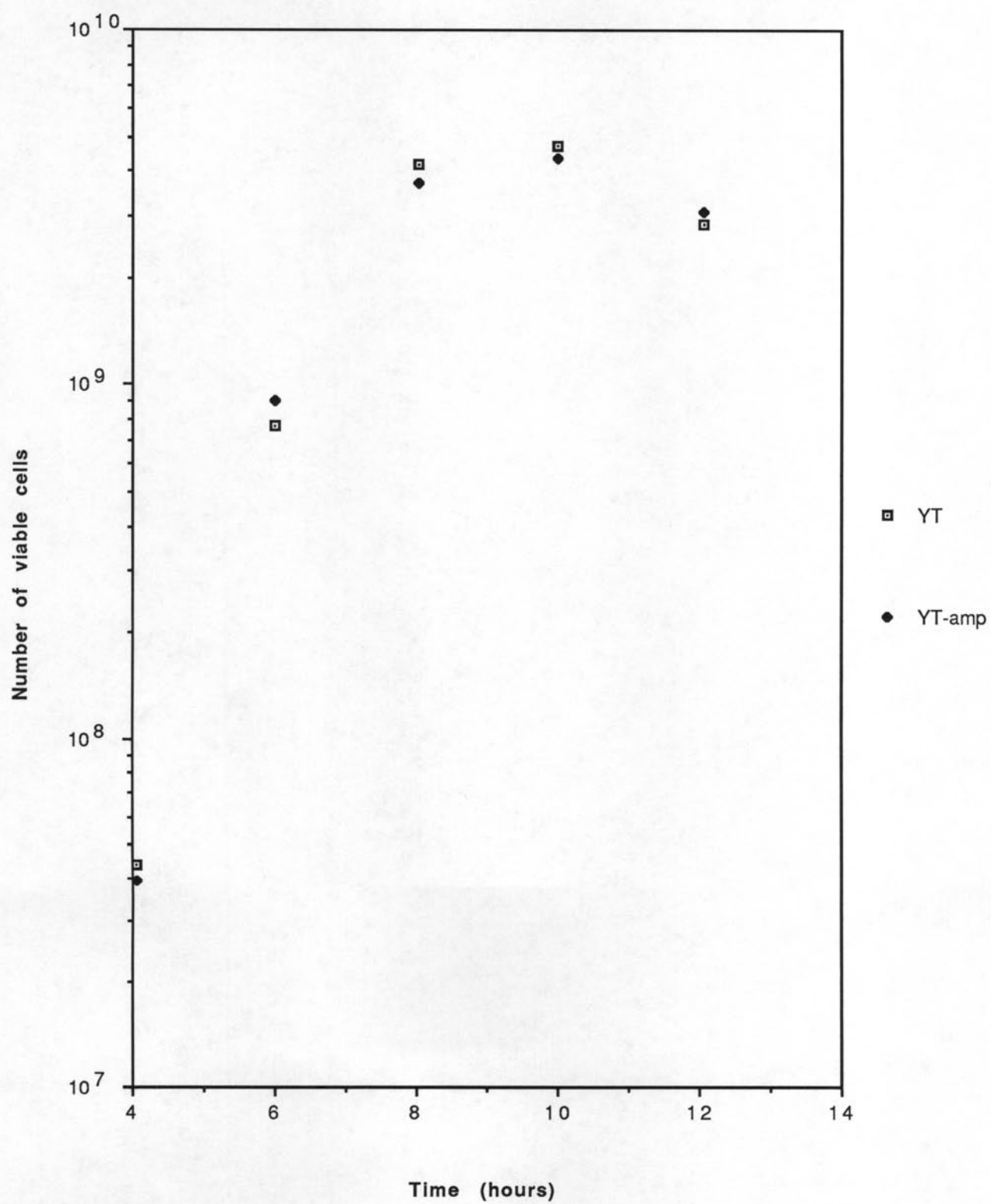
**pH vs. Time**  
**12/13/88**



**Absorbance vs. Time**  
**12/13/88**

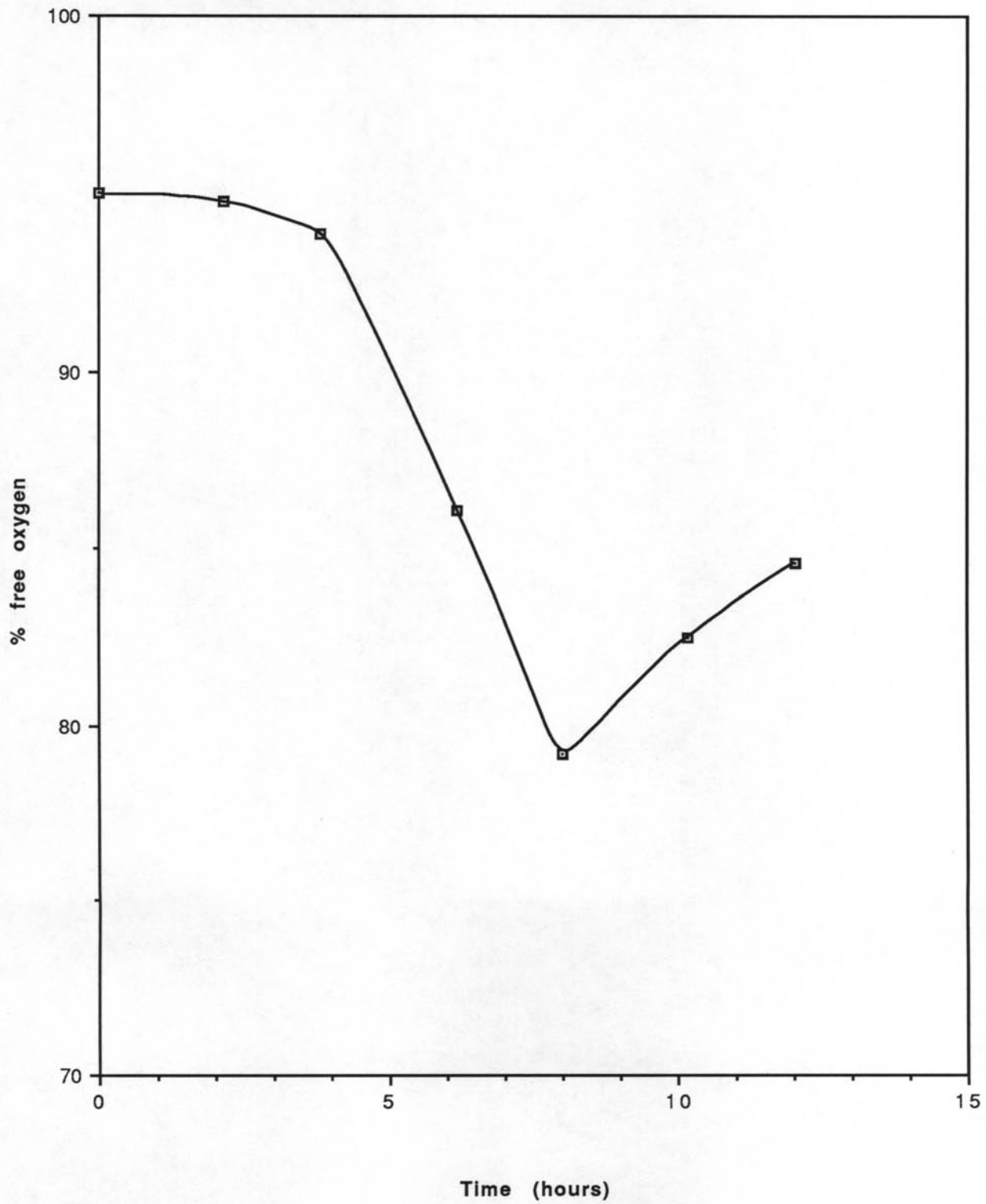


Growth Curve (from plates)  
12/13/88

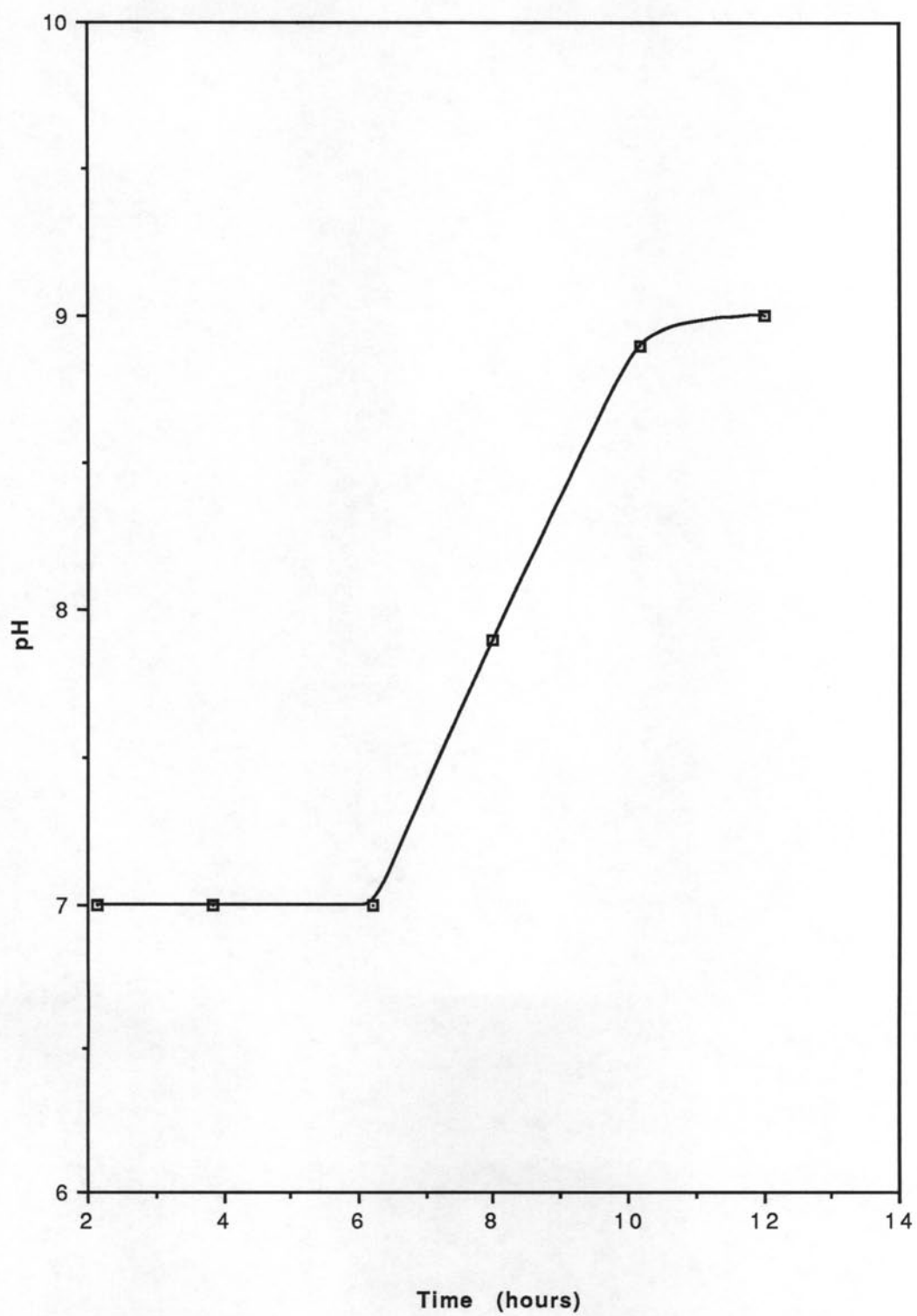


|   | Time (hours) | YT      | YT-amp  | % Oxygen | Absorbance | pH    |
|---|--------------|---------|---------|----------|------------|-------|
| 1 | 0.000        |         |         | 95.000   |            | 7.000 |
| 2 | 2.133        | 7.00e+5 | 2.80e+5 | 94.800   | 0.080      | 7.000 |
| 3 | 3.833        | 1.00e+7 | 7.00e+6 | 93.900   | 0.120      | 7.000 |
| 4 | 6.200        | 2.08e+8 | 2.64e+8 | 86.100   | 0.920      | 7.000 |
| 5 | 8.000        | 1.94e+9 | 1.58e+9 | 79.200   | 1.070      | 7.900 |
| 6 | 10.167       | 3.66e+9 | 4.20e+9 | 82.500   | 2.040      | 8.900 |
| 7 | 12.033       | 4.66e+9 | 4.90e+9 | 84.600   | 2.260      | 9.000 |

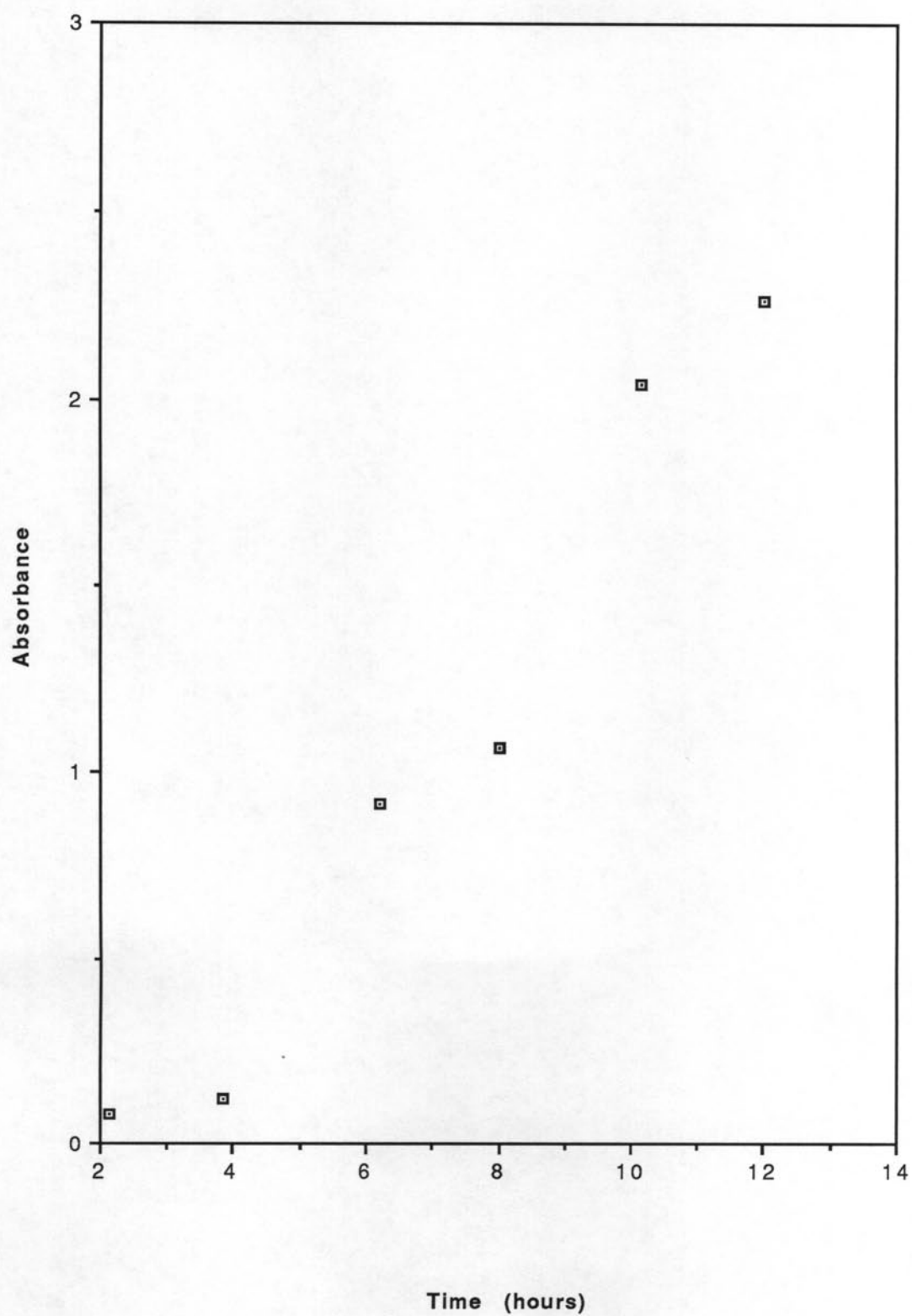
Oxygen vs. Time  
1-24-89



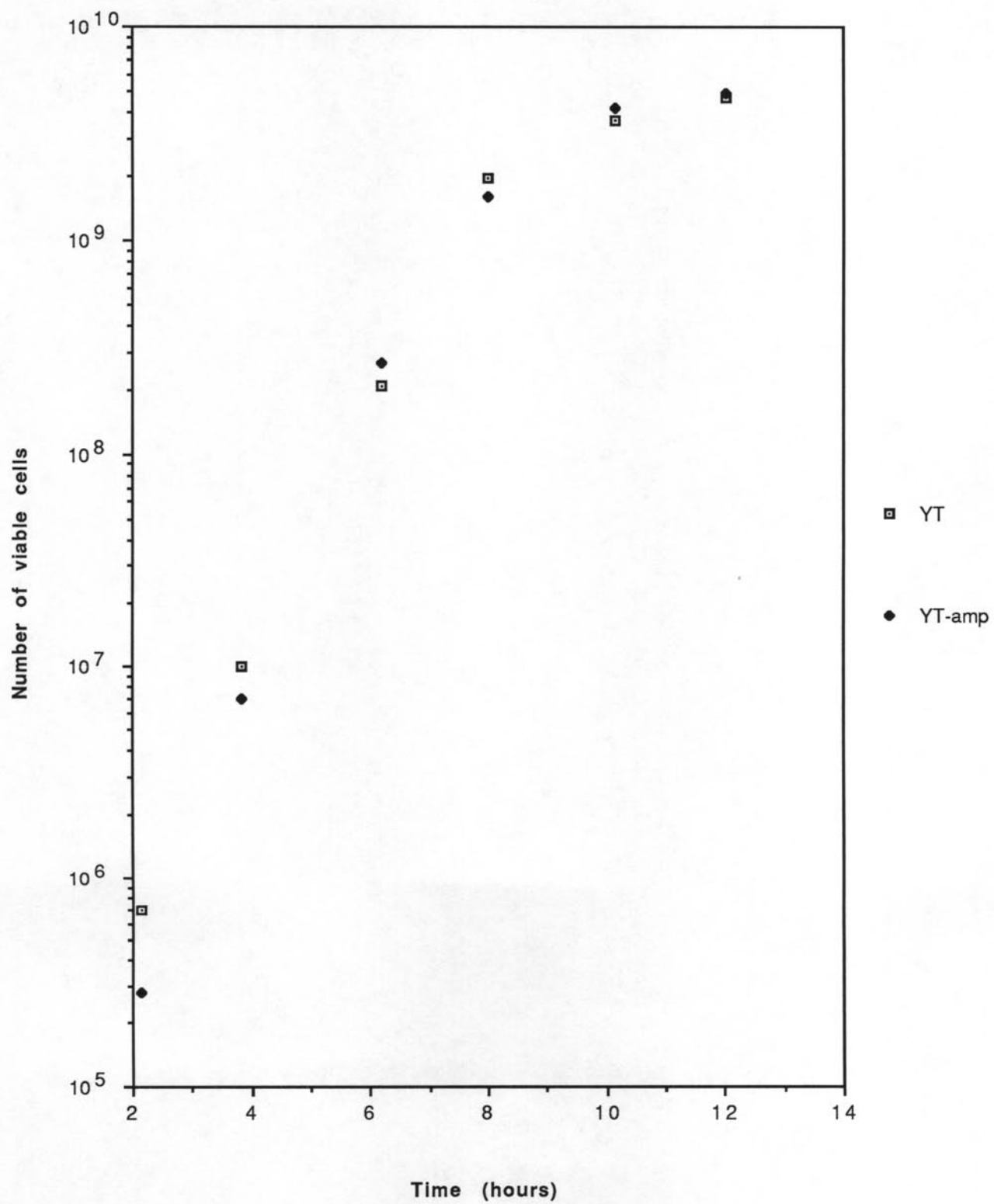
pH vs. Time  
1-24-89



**Absorbance vs. Time**  
**1-24-89**

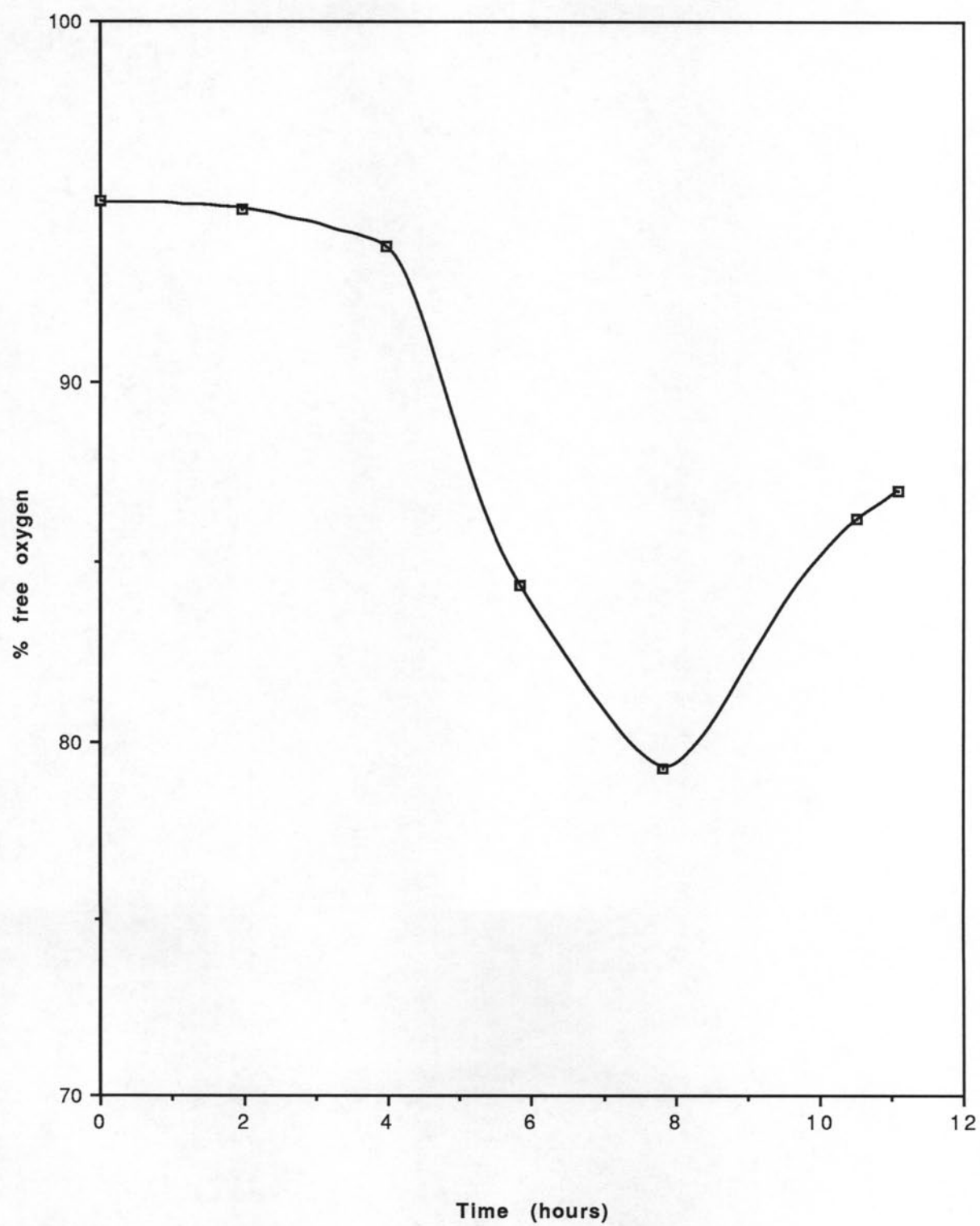


Growth Curve (from plates)  
1-24-89

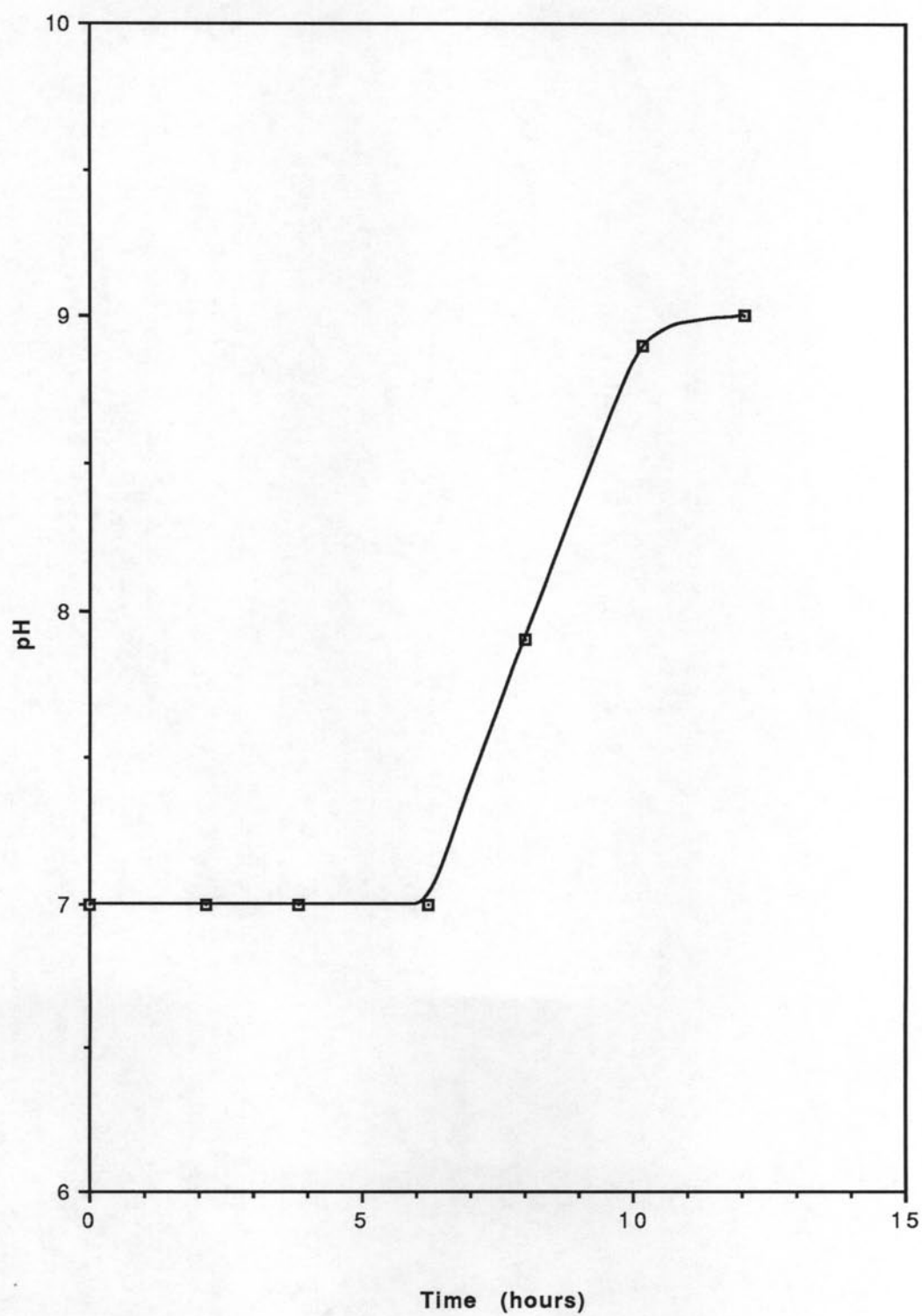


|   | Time (hours) | YT      | YT-amp  | % Oxygen | Absorbance | pH    |
|---|--------------|---------|---------|----------|------------|-------|
| 1 | 0.000        |         |         | 95.000   |            | 7.000 |
| 2 | 1.967        | 2.34e+6 | 2.38e+6 | 94.800   | 0.060      | 7.000 |
| 3 | 3.967        | 3.62e+7 | 3.74e+7 | 93.800   | 0.350      | 7.000 |
| 4 | 5.833        | 1.30e+9 | 1.40e+9 | 84.400   | 1.510      | 7.400 |
| 5 | 7.833        | 2.36e+9 | 2.48e+9 | 79.300   | 1.960      | 8.400 |
| 6 | 10.533       | 2.00e+9 | 1.94e+9 | 86.200   | 2.030      | 8.800 |
| 7 | 11.100       |         |         | 87.000   |            |       |

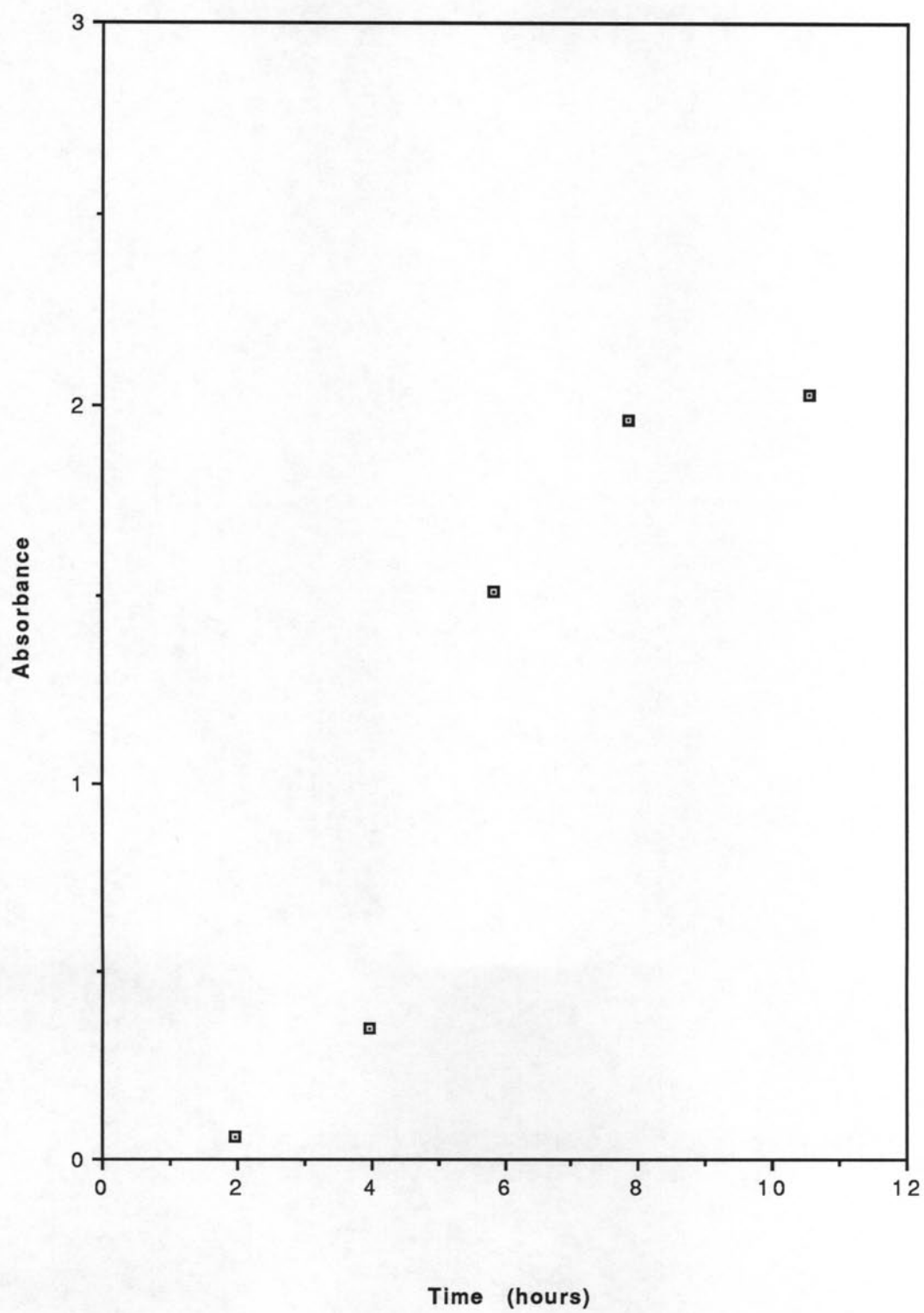
Oxygen vs. Time  
2-7-89



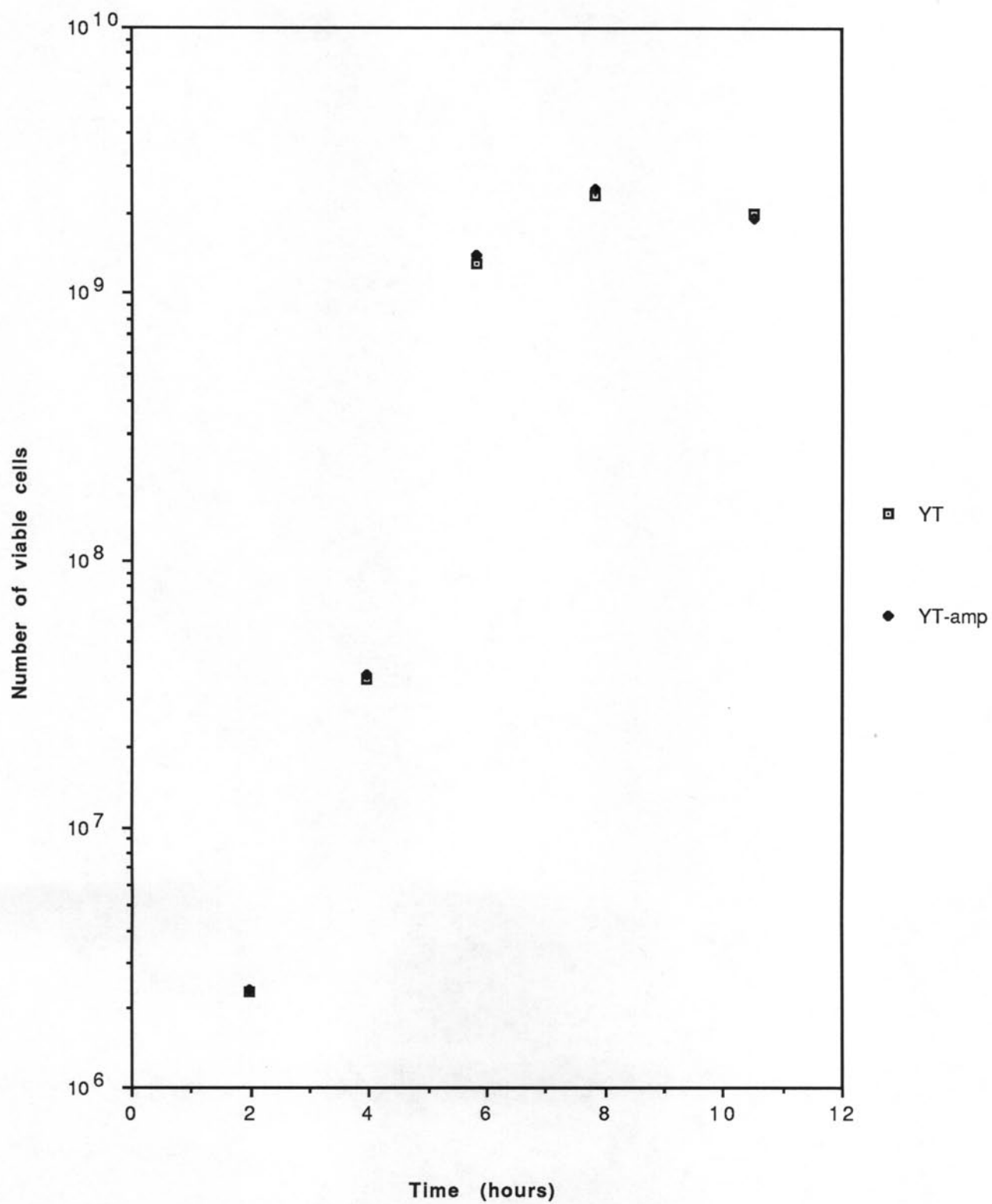
pH vs. Time  
2-7-89



**Absorbance vs. Time**  
**2-7-89**

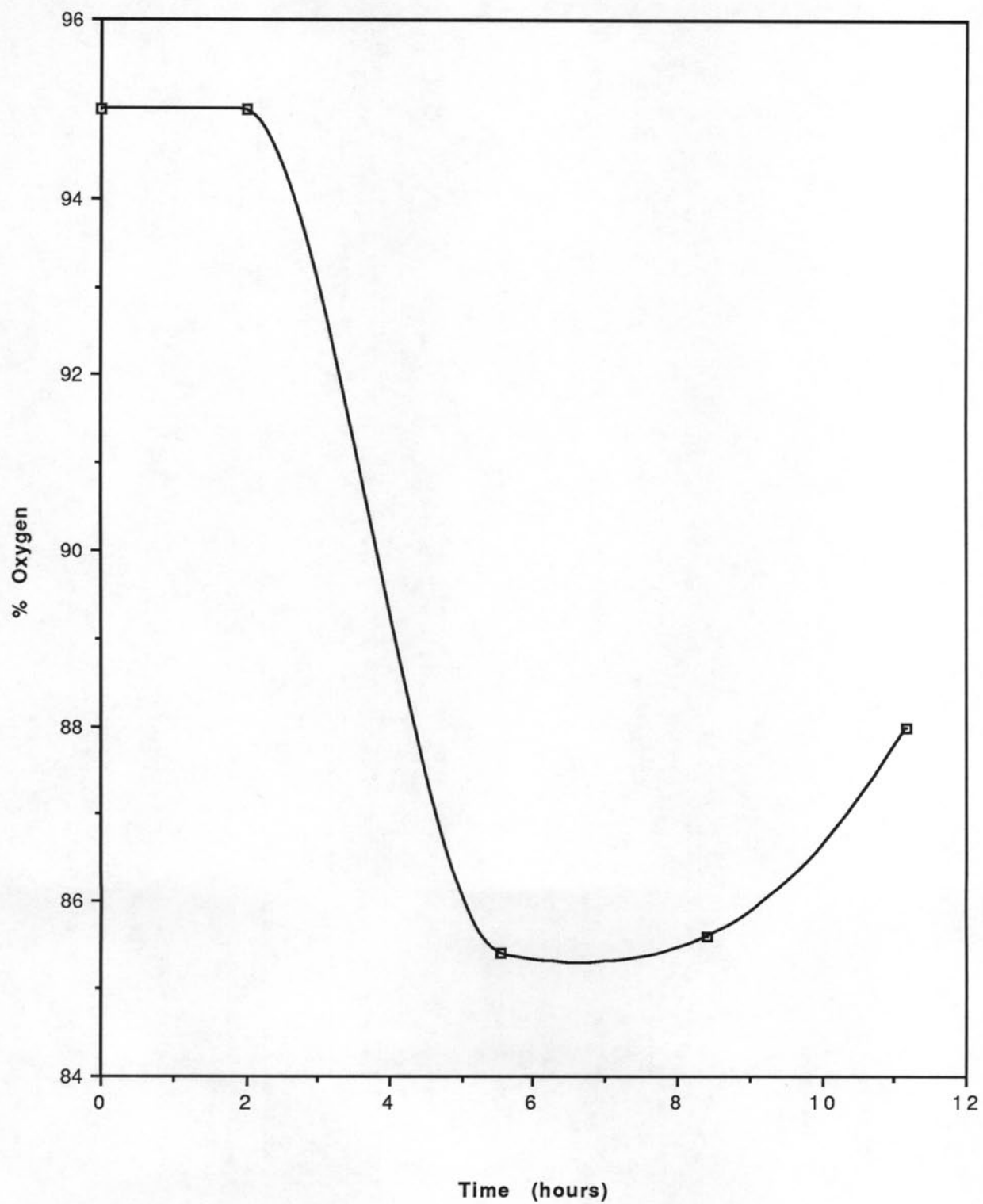


Growth Curve (from plates)  
2-7-89

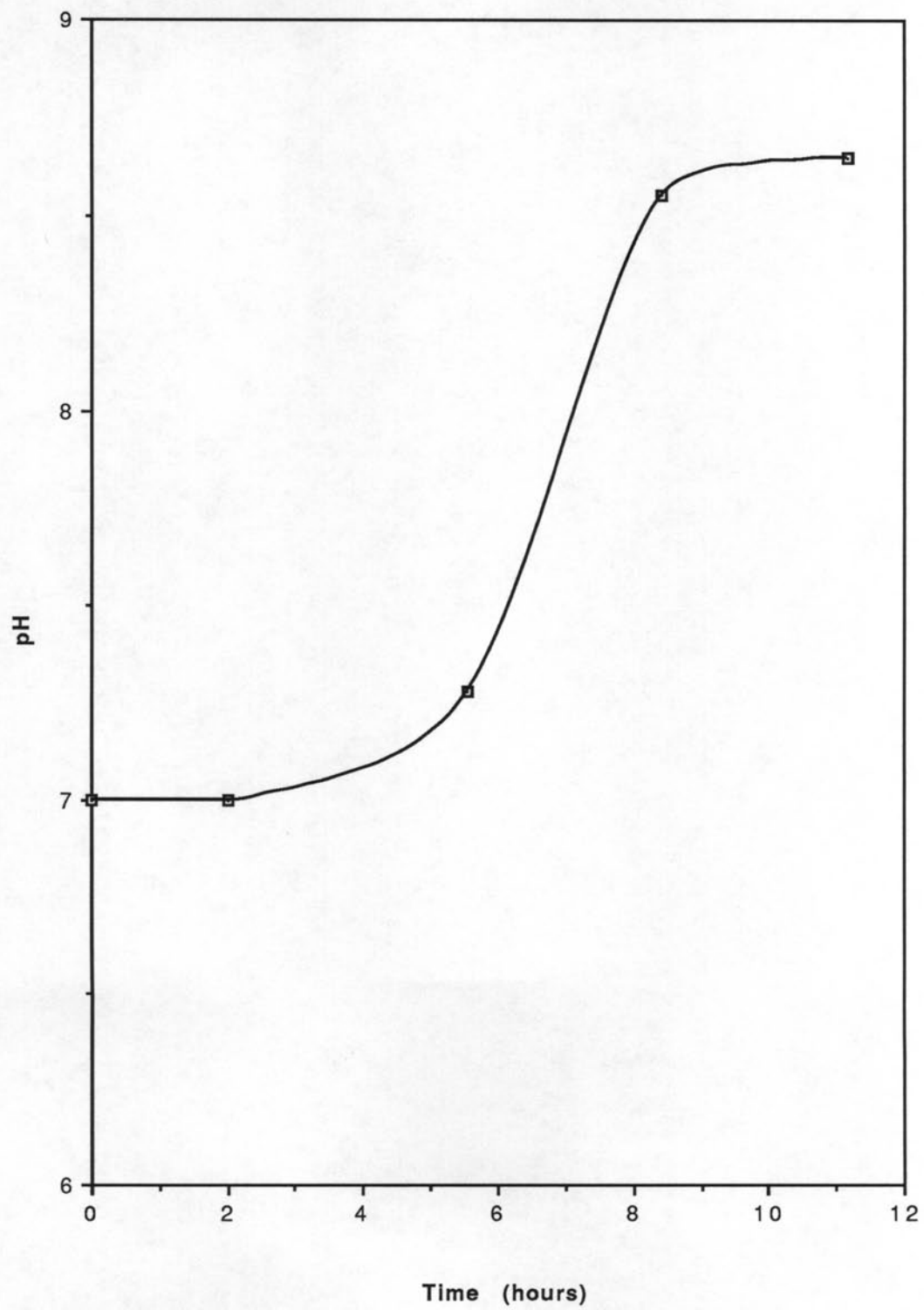


|   | Time (hours) | YT       | YT-amp   | % Oxygen | Absorbance | pH    |
|---|--------------|----------|----------|----------|------------|-------|
| 1 | 0.000        |          |          | 95.000   |            | 7.000 |
| 2 | 2.000        | 5.72e+6  | 7.28e+6  | 95.000   | 0.012      | 7.000 |
| 3 | 5.550        | 2.26e+9  |          | 85.400   | 1.970      | 7.280 |
| 4 | 8.417        | 4.80e+9  | 4.40e+9  | 85.600   | 3.770      | 8.550 |
| 5 | 11.167       | 1.18e+10 | 1.00e+10 | 88.000   | 8.200      | 8.650 |

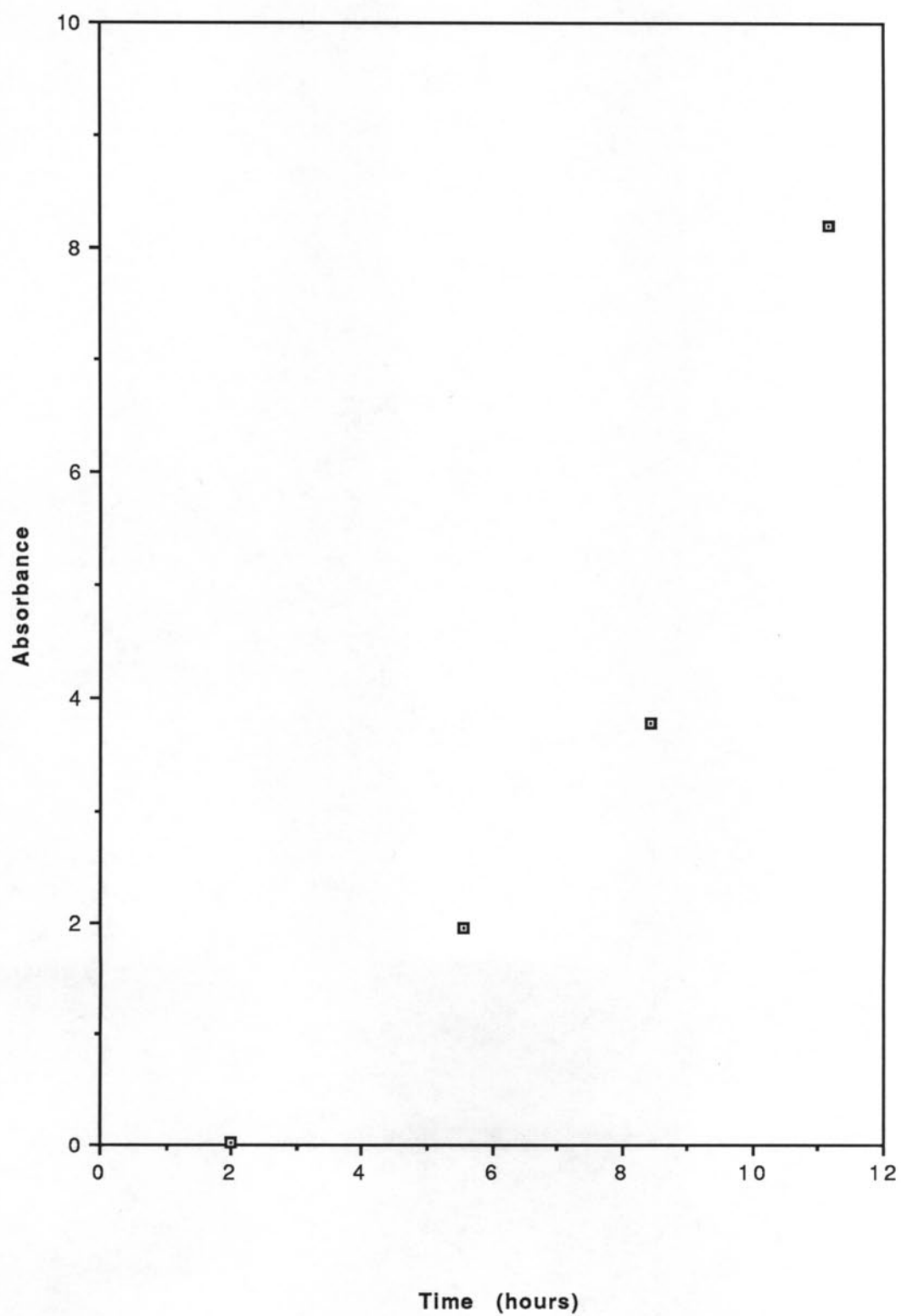
Oxygen vs. Time  
3/2/89



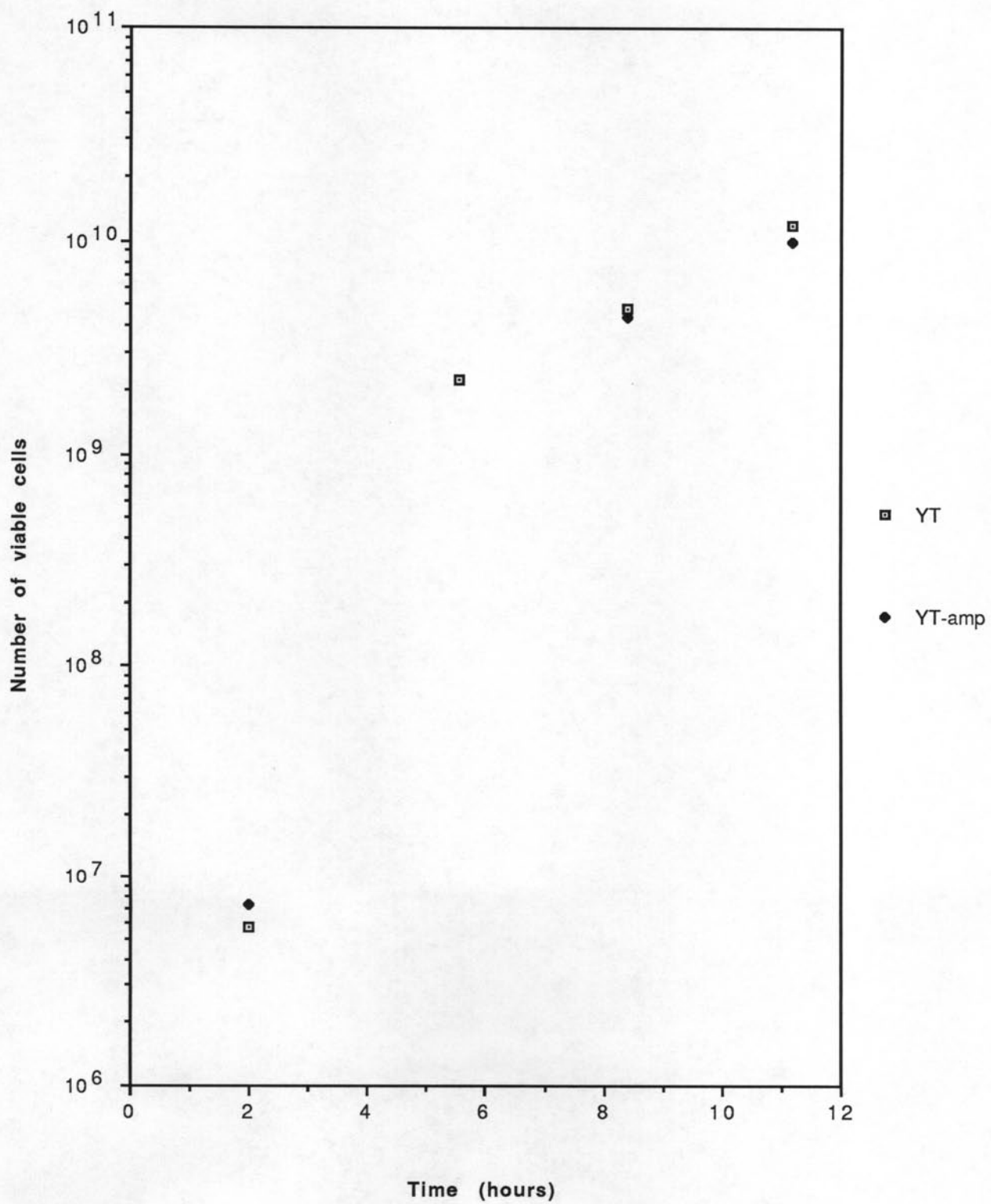
pH vs. Time  
3/2/89



**Absorbance vs. Time**  
**3/2/89**

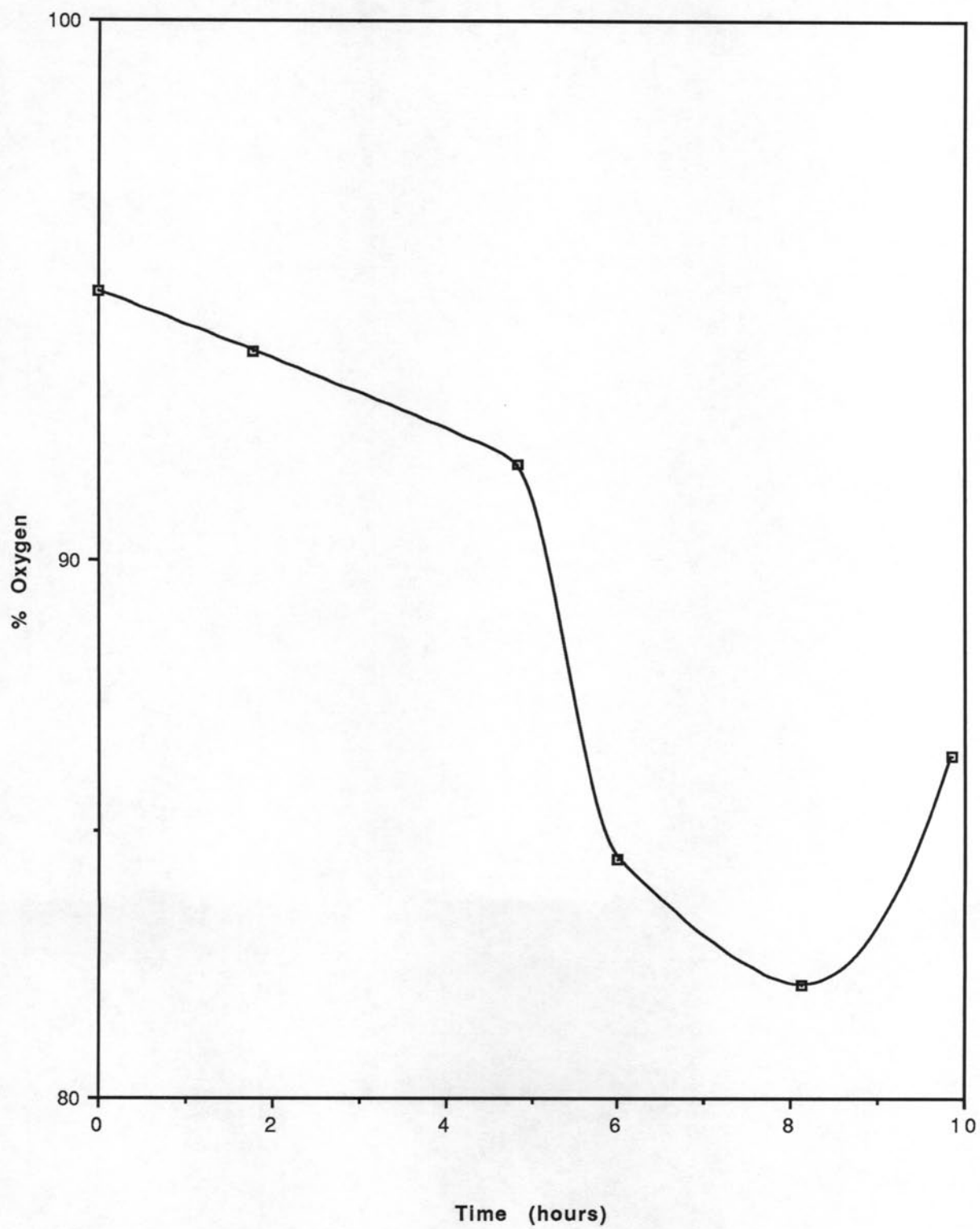


Growth Curve (from plates)  
3/2/89

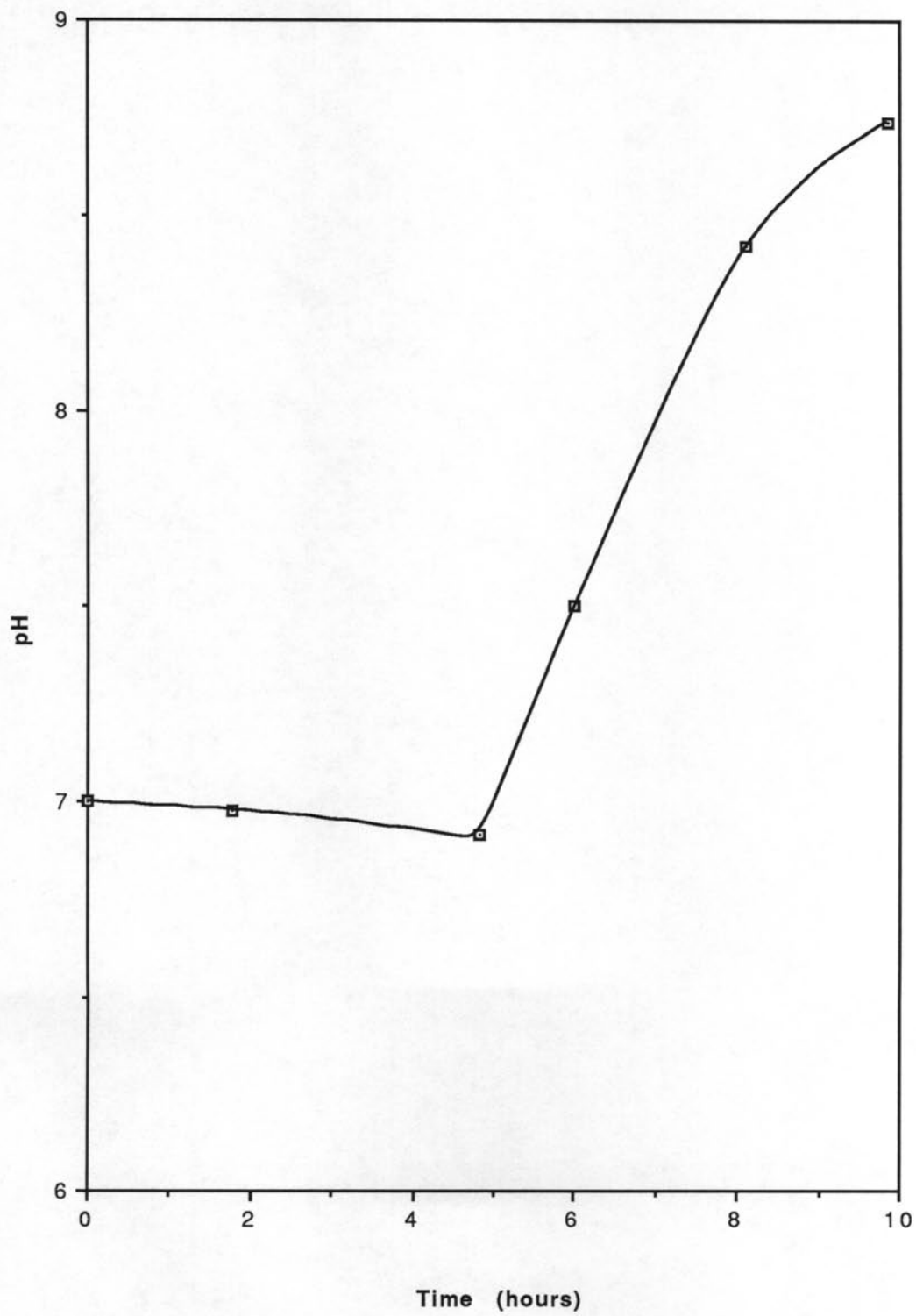


|   | Time (hours) | YT      | % Oxygen | Absorbance | pH    |
|---|--------------|---------|----------|------------|-------|
| 1 | 0.000        |         | 95.000   |            | 7.000 |
| 2 | 1.783        | 2.08e+6 | 93.900   | 0.066      | 6.980 |
| 3 | 4.833        | 9.00e+7 | 91.800   | 0.340      | 6.920 |
| 4 | 6.000        | 1.90e+9 | 84.500   | 2.280      | 7.500 |
| 5 | 8.117        | 6.60e+9 | 82.100   | 4.200      | 8.420 |
| 6 | 9.850        | 9.40e+9 | 86.400   | 4.200      | 8.740 |

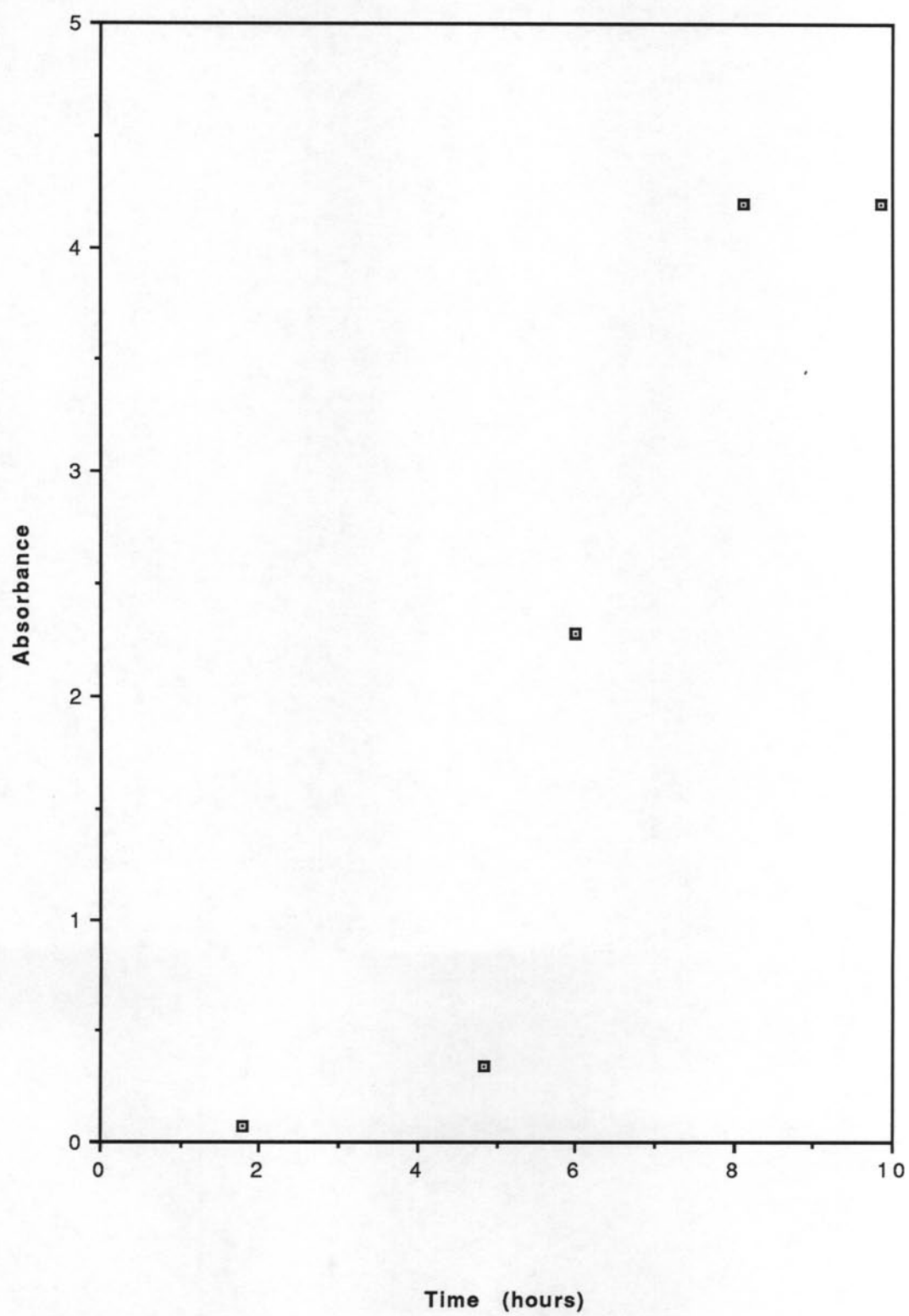
Oxygen vs. Time  
3/9/89



**pH vs. Time**  
**3/9/89**



**Absorbance vs. Time**  
**3/9/89**



Growth Curve (from plates)  
3/9/89

