

# High Density Yeast Fermentations with Flocculent Yeasts

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M. Clark Dale\* and Chongde Zhao<sup>1</sup>

\*Bio-Process Innovation, Inc.

<sup>1</sup>Ag & Bio Engineering Dept, Purdue Univ.  
W. Lafayette, IN 47906

## Abstract

Four strains of highly flocculent yeast for ethanol production from glucose/sucrose were obtained based on literature references. Each of these strains was evaluated for flocculent behavior and fermentation characteristics. Repeated batch fermentations were performed with settled cells reused ten times. Cell density built up to 22-25 g/l for each of the strains, allowing a 24 hour fermentation of a 20% glucose solution. Actual flocculation and fermentation performances were found to be fairly similar among all four strains, but one strain, *S. cerevisiae* BP-11 was chosen for further investigations as it produced slightly more ethanol than the other strains. Flocculation performance of strain BPY-11 as a function of Reynolds number in a 2 Liter stirred fermenter was studied. A Reynolds number of 50,000 was required to keep the cells in suspension in small flakes. At this Reynolds number there were few (0.2 g/l) single or un-flocculent yeasts, with the yeast observed to settle in a period less than a minute when the agitator was stopped. At a Reynolds number of 180,000, the turbulence was found to cause the de-flocculation of the yeast. After 16 hours, no large yeast flakes remained, with no settling of cells noted within 5 minutes of stopping the impellers. An interesting phenomena was noted in that the yeasts did not re-flocculate after the Reynolds was dropped to 50,000 over a period of 3 days.

## Introduction

The rates of ethanol production during a fermentation can be increased more or less linearly by increasing the density of active live yeast or bacteria in a reactor. During a normal batch ethanol fermentation with *S. cerevisiae* a final cell concentration of between 1.5 and 15 g/l cells is achieved. It is often noted that cell growth completely stops after a certain cell density is reached (Holzberg et al, 1967). The oxygen tension in the fermentation is important in these batch fermentations, as the cells will move towards biomass production as the amount of oxygen available to the cells increases. Trace oxygen can serve as a nutrient during the anaerobic fermentation of sugars, allowing the fermentation rate to increase with more cells produced. Cysewski and Wilke (1978) show an optimal oxygen tension of about 0.1 mm O<sub>2</sub>. To maintain a cell density higher than the natural maximum attained in the fermenter, methods for keeping the cells in the fermenter must be utilized. A high cell density can be maintained either by recycling cells (through membrane or centrifugal techniques) or by retaining or

immobilizing the cells within the reactor. Immobilization would seem to be advantageous as the capital expense of a cell recovery and recycle system can be eliminated. There has been a good deal of work over the last 10-15 years on immobilizing organisms to maintain a high cell density in the bioreactor. Immobilization can take one of several approaches, 1) entrapment within a gel bead or plate, 2) adsorption onto a solid matrix, or 3) self-agglomeration or flocculation into flakes or pellets.

1) gel entrapment- A large body of literature is available on lab and pilot scale attempts to immobilize cells within gel beads or sheets. These have had varying degrees of success, but longevity of the gel bead or sheet is a question which has not satisfactorily been addressed in the scale-up of these sorts of systems. Gas evolution by the fermenting cells has a disruptive effect on the cell matrix. Diffusion of sugar into the bead/sheet and ethanol from the bead are mass transfer inhibitions to achievable fermentation rates. Some studies have shown that cell growth occurs very near the surface of the bead.

2) adsorption onto a solid matrix- Our lab's previous work has focussed on adsorption of cells onto a fibrous matrix (Dale et al, 1987, 1991), run in a gas continuous mode. However, this sort of system is not tolerant to insoluble solids. Cells can also be adsorbed into porous glass or ceramic beads/matrices.

3) self-agglomeration/flocculation- The use of flocculent yeast flakes or pellets for yeast fermentation has been suggested by several researchers. We felt that this system is the simplest method for maintaining a high cell density in the reactor. Some yeast have the property of joining together in clumps or flocs, with these multi-cell clumps having a much more rapid settling velocity than single cells. Flocculation is an important factor in the brewing process. After the beer or wine has completed its fermentation, it is desirable to have the yeast settle out. Standard *S. cerevisiae* used for beer and wine fermentations are selected to have this post-fermentation flocculation characteristic. Standard wine or champagne yeast settle over a period of 150 to 300 minutes if there is no fermentation activity to suspend the cells (Arikan and Ozilgen, 1992), while flocculent cells tend to settle so quickly it is difficult to get an OD on the cells as the cells settle in one minute in a cuvette (Castellon and Menawat, 1990). There is a body of literature on flocculation available with a review of the literature available (Calleja, 1989) and a number of papers discussing the effects of sugars (Kihn et al, 1988), ions (specifically sodium as a deflocculant (Castellon and Menawat, 1990) and calcium as a pro-flocculant (Kihn et al, 1988a; 1988b; and Masy et al, 1990). A microscopic study of flocculating fission yeast was reported by Sowden and Walker (1987, 1989) where a "hairy" or "mucilaginous" coating of the yeast is described. Soares et al, 1992 show that cell-cell interaction is important with a cell suspension of  $2 \times 10^7$  cell/ml reaching a free cell density of  $0.5 \times 10^6$  cell/ml in 2 minutes while a suspension starting at  $1 \times 10^7$  cell/ml also settled to this same free cell density in 2 minutes.

The use of highly flocculent yeast for continuous reactors has been demonstrated by APV in a tower fermenter. Cell densities of 70-80 g/l were reported (Greenshield and Smith, 1970). Chen and Gong (1986) did further work on a flocculent tower type reactor, while Cysewski and Wilke (1977) used a simple settler with cell recycle to attain high cell densities in a stirred reactor.

To begin our experiments, literature descriptions of highly flocculent yeasts were used to select four strains which were kindly provided by C.L. Kurtzman of the NCAUR in Peoria, IL. These strains were 1) *S. cerevisiae* BPY- 11. 2) *S. bayanus* BPY 12 3) *S. cerevisiae* BPY-13. 4) *Schizos. japonicus* BPY- 14

### Methods and Materials

The yeast were grown micro-aerobically on a 50 g/l glucose medium supplemented with 3 g/l each of yeast extract, malt extract and peptone (YMP). Each 250 ml flask was filled with 150 ml of fermentation media. In subsequent repeated batch fermentations, the cells were allowed to settle for 15 minutes, after which the supernate broth was poured off, and a 22% glucose YMP media returned to the cells to bring the fermenter volume back to 150 ml. The flasks were held in an incubator at 30 C, and stirred with a magnetic stirrer at 180 RPM. Following the 10th consecutive fermentation, the settling/flocculation performance of the strains was compared.

Flocculation was measured by the settling speed as determined using a method developed in our lab. This method consists of filling a 50 ml graduated test tube with cell broth and observing the settling behavior over time, and taking samples of the the two layers. As time passes, the suspension divides into two layers, a supernate or top layer, and a cell concentrate (sedimenting) or bottom layer. The speed at which these layers form is a measure of the settling speed of the yeast. Kihm et al (1988) suggested measuring the OD of the supernate left in a tube after 5 minutes as being a measure of the flocculating nature of the yeast with the lower the OD the greater the degree of flocculation, but we feel our method gives a more accurate description of the settling behavior of the yeast floccs.

### Results

#### *Flocculation Characteristics*

After repeated batch fermentations using the various flocculating strains, the flocculation characteristics of each of the strains was noted. A clear 50 ml tube was filled with fermentation media, and observed as it separated into a supernate and sediment layer. The concentration of yeast in the sediment layer is shown in Figure 1. It can be seen that the sediment layer begins at a concentration of about 25 g/l and slowly compresses to a concentration of 70 to 85 g/l. Strain BPY-12 seems to compress more rapidly than the other 3 strains, but otherwise, similar performance was shown by each of the strains. The concentration of the supernate over time for each of the strains is shown in

Figure 2. Suspended cell concentration in the supernate drops from 7.5 to 3.8 over the first 8 minutes for strain BPY-13. Strain BPY-14 drops more slowly from 5 g/l to 3 g/l over 15 minutes. Strains BPY-11 and BPY-12 seem to be more completely in flocculant form, with the suspended cell concentration of BPY-11 dropping to under 2 g/l in 8 minutes, while the suspended cell concentration of strain BPY-12 is almost immediately under 2 g/l. Figure 3 shows the volume of the sediment layer in the tube versus time. Strain BPY-12 dropped within 2 minutes to 8 ml, while the other three strains settle gradually to a volume of about 18 ml. These settling velocity experiments indicated that strain BPY-12 seemed to settle more quickly, although all of the strains showed strong flocculent behavior. It was noted previously however, that strain BPY-12 did not flocculate at all, until stirred. Strain BPY-11 seemed to show perhaps the second best flocculation performance.

### Fermentation Characteristics

The fermentation performance of the various strains was next compared by running repeated batch fermentations with the 4 strains. The cells were initially grown anaerobically in a 5% glucose YMP media. After 24 hours, the stirring was stopped, the cells allowed to settle, the clear supernate broth removed, and a 22% glucose YMP media added to the cell sediment in the bottom of the reactor. The flasks were incubated at 32 C. and stirred with a magnetic stirrer at 200 RPM. This was done repeatedly with each of the four strains. Glucose and ethanol concentrations as a function of time for the 8th consecutive repetition are shown in Figures 4 and 5. Again we see fairly similar behavior by the various strains, with strain BPY-12 lagging in fermentation speed slightly. Each of the strains except BPY-12 were able to completely utilize all the glucose within 24 hours. Strain BPY-11 reached the highest ethanol concentration of 93 g/l. The cell concentration in the media was about 25 g/l for each of the strains.

Based on these results, we felt that strain BPY-12 was the most flocculent, but BPY-11 was also good, as well as being perhaps a slightly better or more efficient ethanol producing organisms. We therefore selected strain BPY-11 for further investigation.

### Effect of reactor fluid turbulence on flocculation performance

A short experiment was performed to determine the effect of the reactor fluid dynamics on the flocculation/fermentation with strain BPY-11. A 2-liter Brunswick stirred fermenter was charged with growth media (30 g/l sucrose, YMP nutrients), and a cell crop grown for 17 hours. An air rate of 2 vvm (4 liters/min) was maintained during the aerobic experiments and a feed rate of 20 ml per hour (10 hour residence time). The reactor was run aerobically for 60 hours after which the medium was changed to 200 g/l glucose, and run anaerobically. The Reynolds number for a stirred reactor is defined as:

$$Re = D^2 N \rho / \mu$$

where D is the paddle diameter, N is the revolutions/sec, and  $\rho$  and  $\mu$  are the density and viscosity of the fluid in the vessel. A Reynolds number of over 10,000 generally is required to attain turbulence throughout the reactor (Geankoplis, 1980). Our 2 liter fermenter had a 6 bladed paddle with a diameter of 3.5 cm, and one or three paddles was used depending on the desired Reynolds number.

#### Aerobic operations

At a Reynolds number of 1000 (stirring speed of 26 RPM) all the cells formed a mass on the bottom of the reactor. These cells could be suspended by increasing the stirring speed. After such a resuspension, the cells settled within 30 seconds to the bottom of the fermenter. The cell density in the fermenter was about 1.6 g/l with about 160 ml of cells settled on the bottom of the fermenter. The brix of the feed dropped only about 8 g/l (26% sugar utilization).

After 17 hours, the speed of the paddles was increase to give a 10,000 Reynolds number. At a reactor Reynolds number of 10,000 most of the cells remained on the bottom of the reactor. The settled cell volume was estimated at 100 ml with a density of 78 g/l. The cell density of the reactor broth was 1.4 g/l. The largest suspended cell clump had a diameter of about 0.1 mm. After a brief (1 minute) high speed agitation to lift the cells from the bottom of the reactor, the cells settled within 47 seconds, with the cell density in the supernate broth measured at 0.5 g/l.

The Reynolds number was then increased to 50,000 (1,300 RPM). At this speed, all the cells/clumps were suspended in a fairly homogenous fashion, with only a few clumps drifting on the bottom of the reactor. A settling time of 30 seconds was measured after the stirrer was stopped. A sediment layer of about 80 ml. was measured, with a cell concentration in the supernate was measured at 0.2 g/l after 30 seconds, and 0.1 g/l after 2 minutes. This Reynolds number seemed to be the minimum required to maintain the cells in suspension.

The effects of higher levels of turbulence were next tested at a Reynolds number of 180,000, (RPM of 1,500 with three paddles) which is close to the maximum we are able to attain in our lab reactor. We noted that at this velocity, the cell clumps or flakes were actually being knocked apart, with settling velocity reduced and more cells remaining in the supernate layer. After 3 hours of operation, the settling time was noted to have increased to about 2 minutes (see Figure 6) while the concentration of the cells in the broth supernate had increased from the 0.1-0.2 concentration noted earlier, to 3-5 g/l as shown in Figure 6.

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The reactor was left at this Reynolds number overnight, and the following morning (after 18 hours) it was noted that no flocs remained, with virtually no

cells settling after the stirrer was shut off. The Reynolds was then reduced to 50,000, and the reactor allows to run at a residence time of 8-10 hours. Over a period of the next 72 hours, the cells never re-flocculated. There was virtually no cells or clumps settling when the stirrer was stopped.

The reactor was then re-seeded and run anaerobically to determine if the aerobic results were representative of anaerobic fermentation. (Aerobic operation moves the fermentation toward yeast production, while anaerobic operation causes the sugars to be converted largely to ethanol). At a feed rate of 200 ml/hr (10 hour residence time) and a Reynolds number of 7,700, the cells were largely suspended. The brix in the reactor was reduced to 16 from the 20% feed. The cells maintained a small flake size of about 0.5 mm. After 16 hours, the stirring speed was increased to 300 RPM to generate a Reynolds number of 11,500. This more completely suspended all the floccs, and the brix was found to drop slightly to 15 over the next 24 hours.

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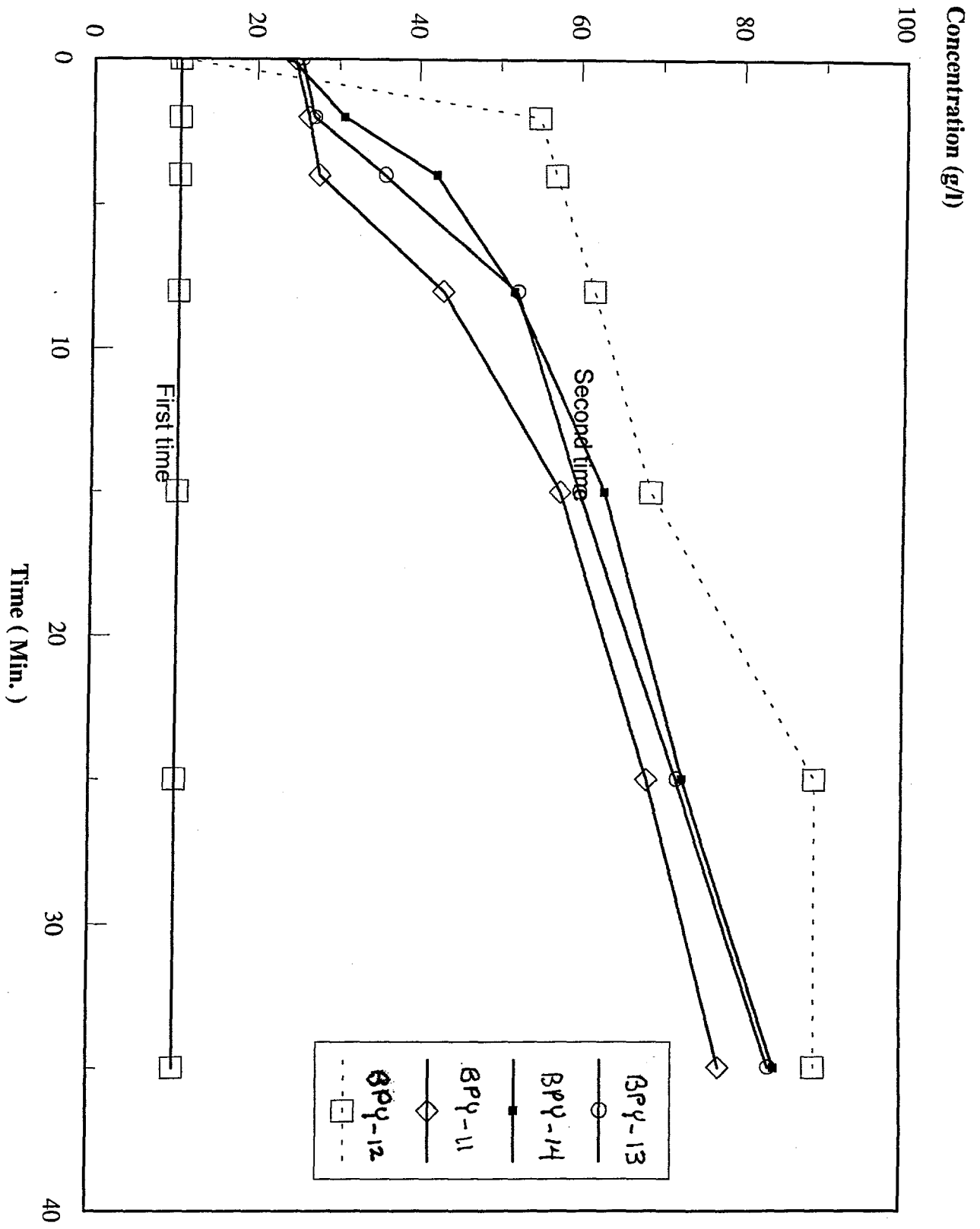
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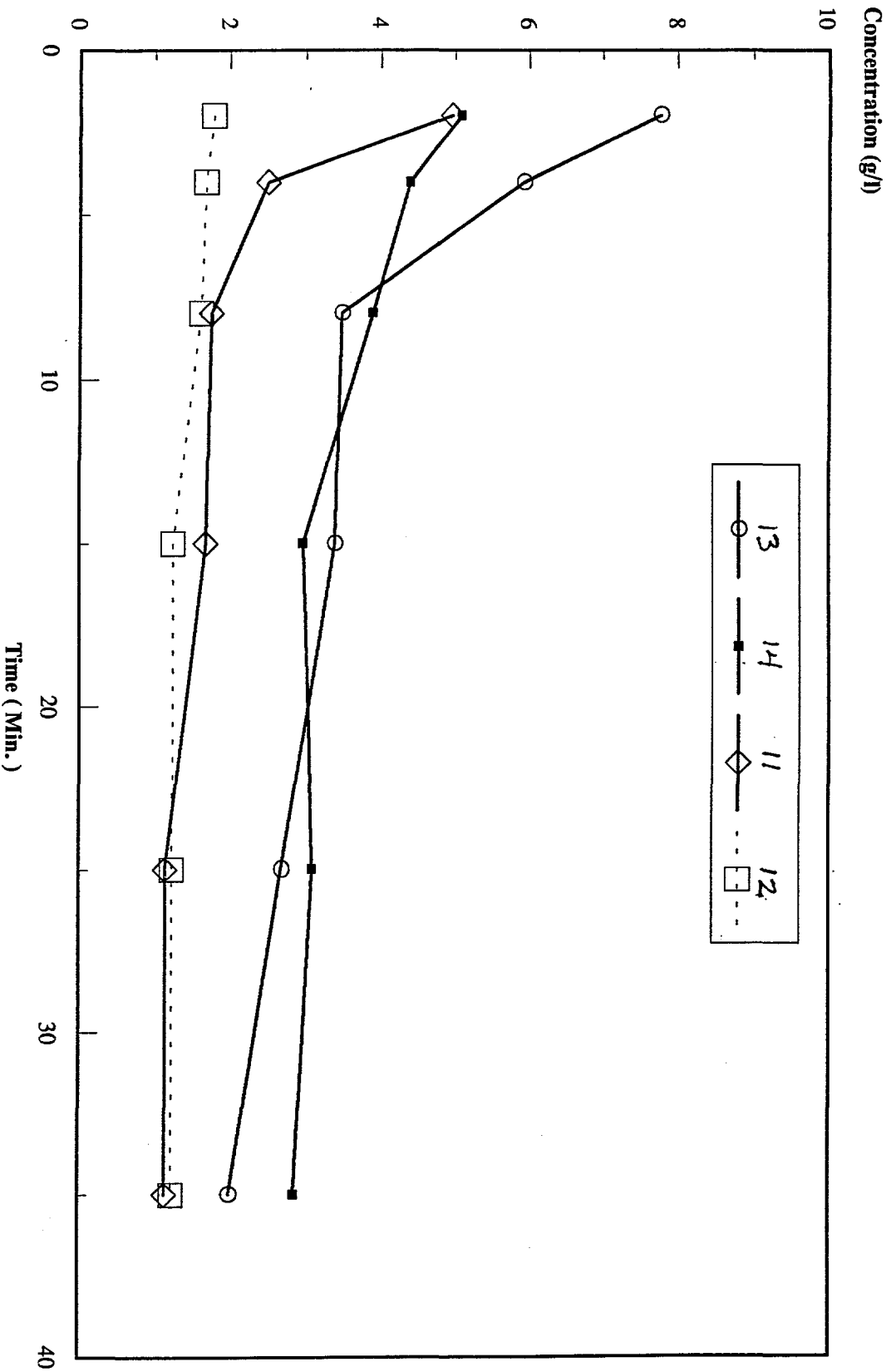
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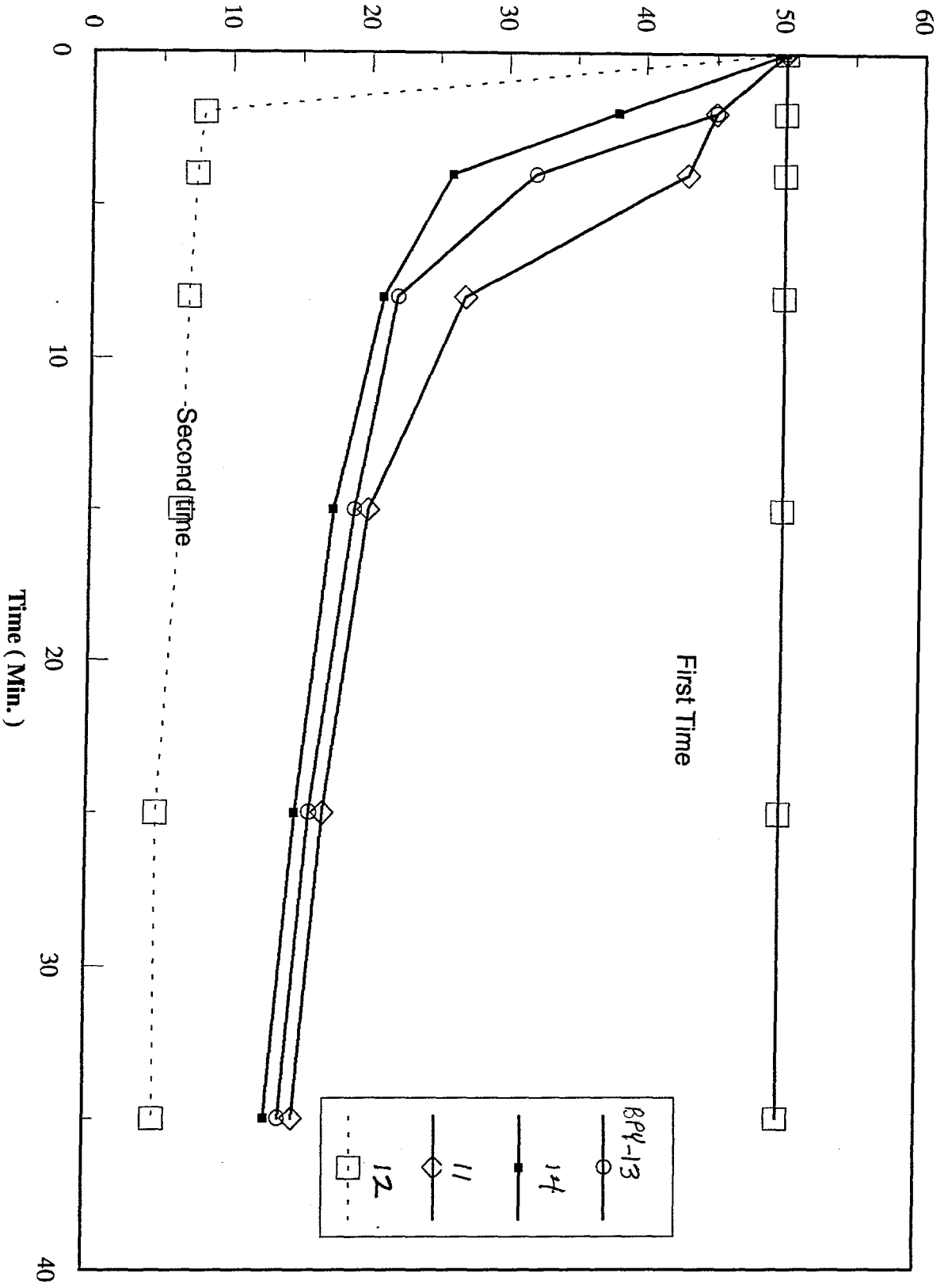


1. Comparison of Cell Concentration in Down Layer

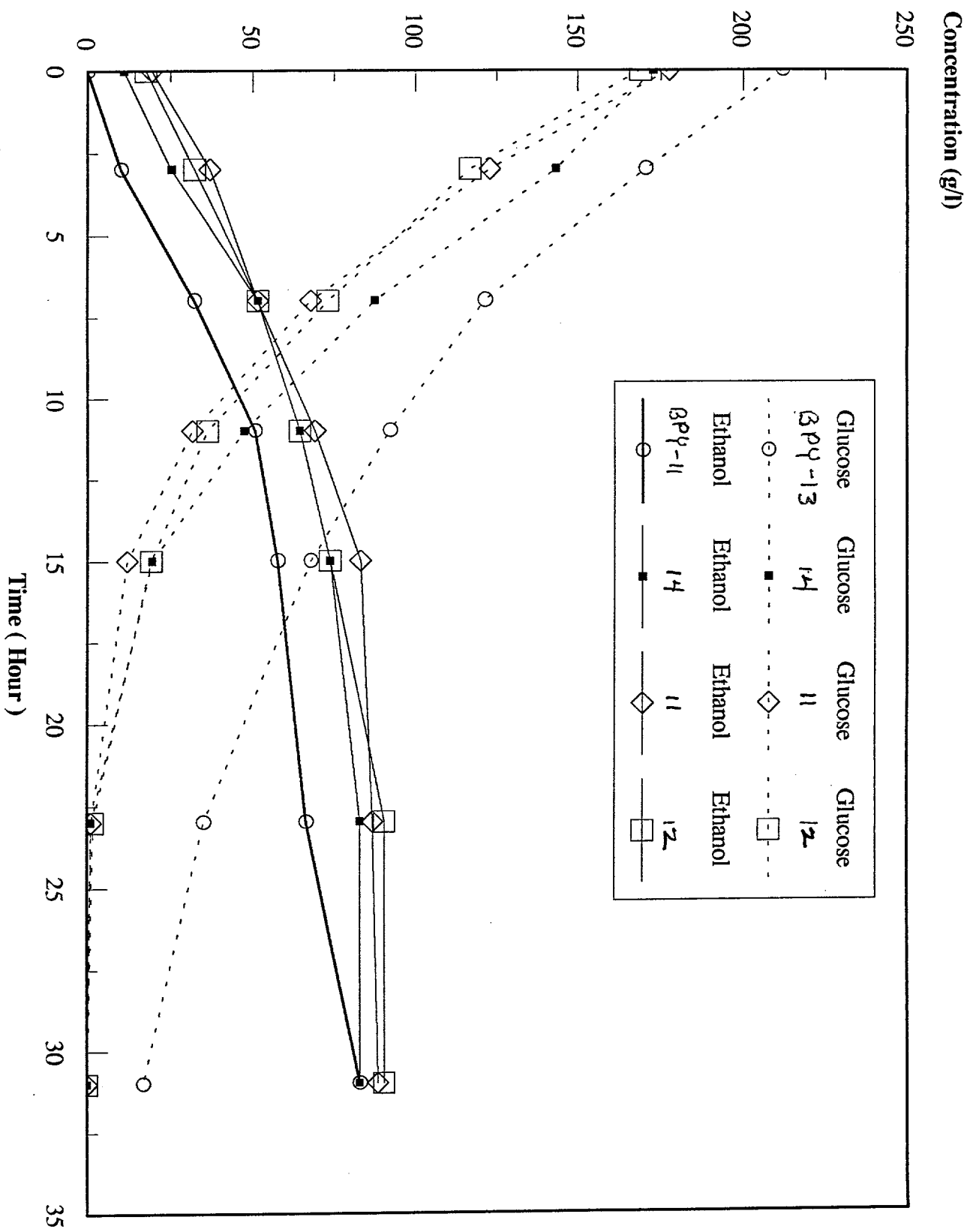


2. Comparison of Cell Concentration in up Layer

Sediment Layer of 50 ml Ferment Solution



3. Comparison of Volume of Down Layer



4. Comparison of Fermentation with Four Strains