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# Dehydrogenase Activity Measurement in Yeast Fermentation

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## ABSTRACT

The dehydrogenase activity was used as a measure of active biomass in preference to other biochemical parameters because of the simple, but accurate nature of the dehydrogenase test. After a considerable amount of experimental work on the dehydrogenase activity measurement technique and the consideration of utilization of the technique as a measure of the active biomass in yeast fermentation systems, an analytical procedure was developed. The developed procedure was first tested on two yeast cultures (*Kluyveromyces fragilis* and *Candida pseudotropicalis*) and then used for the determination of active yeast cells in single-cell protein reactors operating on cheese whey. The dehydrogenase enzymes appeared to be sensitive to the pH, the optimum being $7 \pm 0.2$. Because the optimum pH for the propagation of these yeasts is about $4.5 \pm 0.2$ and a pH variation of 3 U impairs enzyme activity, the pH of the medium must be adjusted. The incubation time is also a critical factor in determining the accuracy of the TTC test, especially at low-biomass concentrations. An incubation period of 80 min was found to be reasonable.

**Index Entries:** Cheese whey; batch fermentation; dehydrogenase activity; yeast; growth; lactose; pH; incubation time.

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## INTRODUCTION

The assessment of microbial activity is made possible to some extent by measuring the microbial mass. In practice, this may be carried out gravimetrically, optically, by plate count, or by direct microscopic count. However, even with pure cultures, the gravimetric and optical methods can lead to errors, since they include both living and dead cells as well as particulate organic matter (*1*). The direct microscopic count does not distinguish between living and dead cells, and is tedious to perform. Furthermore, standardization is difficult to achieve. The plate count is also very tedious to perform and requires at least 72 h to complete. Tests devised to distinguish between living and dead cells using fluorescent dyes may provide questionable results because of the presence of dye-adsorbing materials (*2*).

The activity of the coenzymes Nicotinamide Adenine Dinucleotide (NAD) and Flavin Adenine Dinucleotide (FAD), which act as intermediate electron acceptors, can be measured by the visible color changes of dyes. In the presence of a suitable electrode acceptor dye, the activity of NAD and FAD could, therefore, be measured by the visible color changes of the dye. One such a dye is the 2-,3-,5-Triphenyl Tetrazolium Chloride (TTC), which has been used in dehydrogenase activity studies by several authors (*2–5*).

The compound TTC has become especially important within the last two decades in biochemical, biological, and medical studies because its indicator property, with respect to the dehydrogenase activity, is considered to be a specific indicator reaction to all living cells. In the presence of dehydrogenase enzymes, TTC is reduced to triphenyl formazan (TF), a red dye that can be extracted by ethyl alcohol and determined colorimetrically. The reduction of the colorless TTC to red formazan is a clear indication of the existence of living organisms. The concentration and degree of the activity of these living organisms are reflected by the intensity of the red color (*2–5*).

Although, our earlier work (*5*) indicated that TTC test is a direct measurement of microbial activity, when the test was used to determine the activity of yeast cells, such as *Kluyveromyces fragilis* and *Candida pseudotropicalis*, in single-cell protein production reactors, no color was developed after the addition of the dye to samples containing living cells of the yeasts. After a considerable amount of experimental work on the technique, it was observed that the enzymatic activities of the yeasts were very sensitive to both the pH of the medium and the incubation time.

This article describes an analytical technique for the measurement of dehydrogenase activities for yeasts grown in cheese whey under aerobic conditions for the production of single-cell protein. The developed procedure was successfully tested with two yeasts and two bacteria.

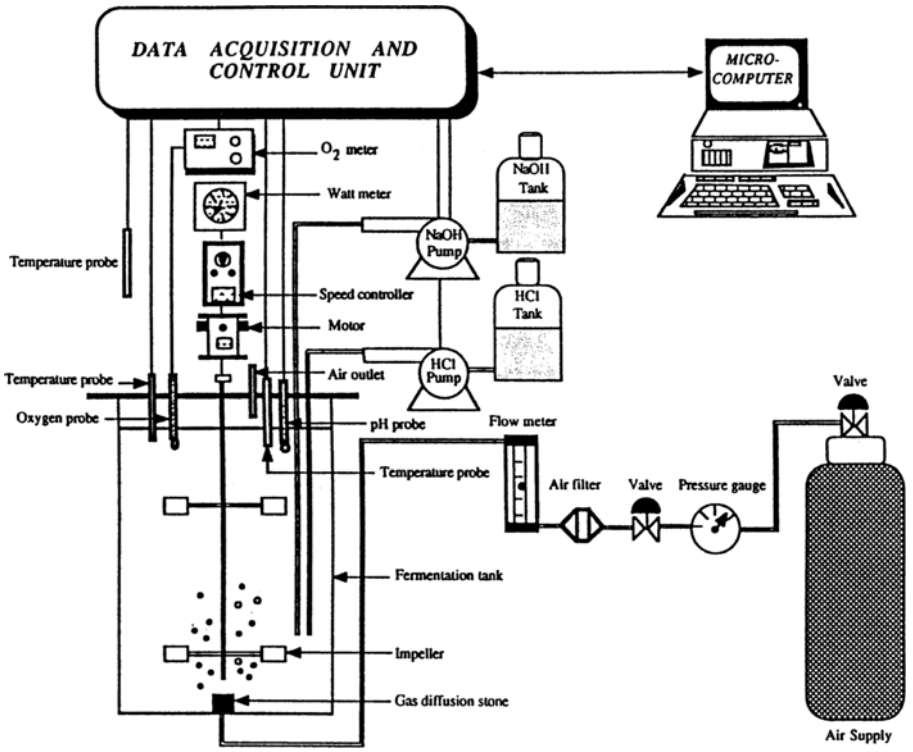


Fig. 1. Experimental setup.

## MATERIALS AND METHODS

### Apparatus

The experimental apparatus used for single-cell protein production is shown in Fig. 1. The fermenter was designed to be completely mixed. The mixing system consisted of a mixing shaft, two flat-bladed turbine impellers, and a variable speed electric motor (Dayton Electric MFG Co. Model 4Z142). Four baffles were used in the fermenter to reduce vortexing and to improve the top-to-bottom turnover. The fermenter provided a liquid capacity of 4.8 L when the mixing device was submerged. Compressed air (Medigas Atlantic Limited Cat. No. T100172, composed of 78.084% $N_2$, 20.996% $O_2$, 0.033% $CO_2$, and 0.937% other gases) was supplied to the fermenter through a gas diffusion stone (Fisher Scientific Cat. No. 11-139B) of 26 mm height and 22 mm diameter, which was fixed at the bottom of the fermenter. The moisture and impurities in the air were $< 10$ ppm and 2 ppm, respectively. More information on the design and layout of the experimental apparatus and the pH control system can be found in Ben-Hassan et al. (*6,7*).

## Reagents for Dehydrogenase Activity Test

1. Tris buffer solution: A solution of 0.05*M* Tris (hydroxymethyl) aminomethane hydrochloride ($NH_2C[CH_2OH]_3 \cdot HCl$) is prepared by dissolving 6.057 g Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) in 20 mL 1.0*N* HCl and making up to 1.0 L with distilled water.
2. 2:3:5 Triphenyl tetrazolium chloride reagent (TTC-glucose): This solution contains 0.5% w/v triphenyl tetrazolium chloride ($C_{19}H_{15}N_4Cl$) and 1.0% w/v glucose ($CH_2OH[CHOH]_4CHO$) in aqueous solution. Because of light sensitivity, this solution should be fresh and kept in the dark at near 0°C. Solutions over 1 wk old should be discarded.
3. Standard solution of 0.0002*M* triphenyl formazan: This solution is made by dissolving 0.03 g triphenyl formazan ($C_6H_5N$: $NC[C_6H_5]:NNHC_6H_5$) in 500 mL ethyl alcohol.

## Triphenyl Formazan Standard Curve

1. A set (8–10) of solutions, containing 0.2–4.0 μmol triphenyl formazan/50 mL, is made by diluting with ethyl alcohol.
2. These solutions are used to prepare a curve of optical density (OD) against triphenyl formazan (TF) concentration.
3. The OD is read on a spectrophotometer at a wavelength of 484 μm.

## Stock Cultures

The freeze-dried pellets of *Kluyveromyces fragilis* (NRS 5790) culture was obtained from the Division of Biological Sciences, the National Research Council, Ottawa, Ontario, Canada, whereas the freeze-dried pellets of *Candida pseudotropicalis* (ATCC 8619) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. A pellet of *K. fragilis* was rehydrated in 5 mL sterilized YPD growth medium containing 1% yeast extract, 2% peptone, and 2% dextrose. A loop of this solution was streaked on an agar medium, containing 1% yeast extract, 2% dextrose, 2% peptone, and 2% agar, in a Petri dish. A pellet of *Candida pseudotropicalis* was rehydrated in 5 mL sterilized YM growth medium containing 0.3% bacto yeast extract, 0.3% malt extract, 0.5% bacto peptone, and 1.0% bacto dextrose. A loop of this solution was streaked on an agar, containing 0.3% bacto yeast extract, 0.3% malt extract, 0.5% bacto peptone, 1.0% bacto dextrose, and 2.0% bacto agar in a Petri dish. The Petri dishes were placed in a controlled environment incubator at 35°C and left until visual growth appeared (after about 48 h).

## Procedure for Dehydrogenase Activity Test

1. Prepare three, 100-mL glass-stoppered bottles, and mark them sample 1, sample 2, and control.
2. Pipet 10 mL of the sample into each of the three bottles.
3. Adjust the pH of the sample to 7.0 by the addition of 1*N* sodium hydroxide.
4. Add 5 mL of Tris buffer and 1 mL of TTC/glucose reagent to the bottles marked samples 1 and 2 and only 5 mL of Tris buffer to the control bottle.
5. Shake the three bottles and incubate in the dark at 20°C for 80 min (pink color will be present at the end of the incubation period for bottles marked samples 1 and 2). **Note: The incubation periods are very critical with this technique.**
6. Immediately add 10 mL of ethyl alcohol to stop the reaction.
7. Centrifuge the suspension at 5000 rpm for 20 min.
8. Transfer the suspension to a 50-mL volumetric flask.
9. Repeat two separate extractions of the retained solids each with 10 mL ethyl alcohol, resuspend the sediment, centrifuge, and transfer the pink supernatant to the 50-mL volumetric flask.
10. Make up the combined suspension to 50 mL with addition of ethyl alcohol.
11. Measure the optical density (OD) of the pink color of the ethyl alcohol solution of the samples 1 and 2 at 484 $\mu$m. Compare with a control in which the TTC/glucose reagent was omitted and is used as a blank to zero the spectrophotometer.
12. Obtain the formazan content from the standard OD/TF curve.

## Plate Count Procedure

A loopful of each of the microorganisms was placed in a separate growth tube containing 50 mL sterilized cheese whey. The tubes were then placed in a controlled environment incubator at 35°C and left until visual growth appeared (after 48 h). Serial dilutions of $10^{-6}$, $10^{-7}$, and $10^{-8}$ were prepared from the yeast suspensions. A milliliter of each of the dilutions was placed in a sterile Petri plate (in duplicate). The agar medium was added to each plate and evenly mixed with the sample by gently swirling the plate six times in each direction (six times clockwise and six times counterclockwise). The agar medium used for *K. fragilis* contained 1% yeast extract, 2% dextrose, 2% peptone, and 2% agar, and that used for the *Candida pseudotropicalis* contained 0.3% bacto yeast extract, 0.3% malt extract, 0.5% bacto peptone, 1.0% bacto dextrose, and 2.0% bacto agar. The plates were allowed to solidify. They were then incubated in the inverted position at 35°C for 72 h. After incubation, the visible growth was counted as

colony forming units per milliliter (CFU/mL), according to the procedure described in the *Standard Methods for the Examination of Dairy Products* (*8*).

## Cheese Whey Collection and Preparation

The whey was obtained from the Farmer's Cooperative Dairy Plant in Truro, Nova Scotia. The whey was pumped from the plant cheese whey storage tank into 60-L plastic containers. The containers were subsequently sealed and transported to the Cold Storage Facility of the Biotechnology Laboratory at the Technical University of Nova Scotia, where they were stored at −25°C until used. The whey used in this study had 6.4% total solids, 5% lactose, 1.7% total nitrogen, 0.9% minerals, and a pH of 4.9. Raw whey was first pasteurized in several 250-mL Erlenmeyer flasks. The pasteurization process involved heating the whey to 70°C for 45 min and then cooling it to 1°C for 30 min. The processes of heating and cooling were repeated three times.

## Inoculum Preparation for Single-Cell Protein Experiment

Two Petri dishes each of the pure culture of *K. fragilis* and *C. pseudotropicalis* were transferred into a 250-mL sterilized Erlenmeyer flasks containing 100 mL pasteurized cheese whey. Ten flasks were used for each yeast in order to start with a large seed size and a high cell count. The Erlenmeyer flasks were capped with nonabsorbent cotton plugs and mounted on a controlled environment reciprocating shaker. The shaker was operated at a speed of 250 rpm for 48 h. A volume of 950 mL (approx 5% of the reactor volume) of the inoculum was transferred to the fermenter before the start of the experiment.

## Fermenter Operation

The fermenter was chemically sterilized using a 2% potassium *meta*-bisulfite solution and washed with hot distilled-deionized water several times before starting the experiment in order to remove any chemical traces. The fermenter was filled with pasteurized cheese whey and immediately inoculated using a 950-mL inoculum. The air flow and turbine drives were adjusted to 2VVM and 350 rpm, respectively. The dissolved oxygen, pH, and temperature were monitored continuously. Samples were drawn from the fermenter at zero time and every 2 h thereafter.

## Analyses

For each sample, the formazan yield, plate count, and lactose concentration tests were conducted. The formazan yield was carried out according to the analytical procedure developed in this study (previously described).

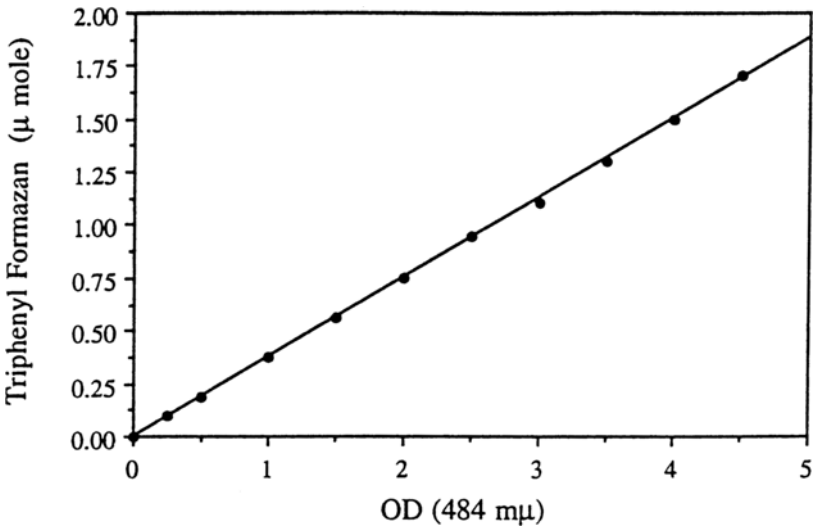


Fig. 2. Triphenyl formazan standard curve.

The plate count was carried out according to the procedures described in the *Standard Methods for the Examination of Dairy Products* (*8*). The lactose analysis was performed using a sugar Analyzer (YSI Model 27, Fisher Scientific Cat. No. 14-660). For each microorganism, the growth medium suggested in the *Compendium of Methods for the Microbiological Examination of Foods* (*9*) for the plate test was used as previously described.

## RESULTS

### Dehydrogenase Activity

The triphenyl formazan standard curve is shown in Fig. 2. Three replicates were carried out and the coefficient of variability (CV) was very small (0.1–0.5%). Within the range used in this study, a linear relationship between the triphenyl formazan concentration and the optical density was observed. The dehydrogenase activity was used as a measure of the active biomass in preference to other biochemical parameters because of the simple but accurate nature of the dehydrogenase test. The procedure was tested with two yeasts (*K. fragilis* and *C. pseudotropicalis*).

The pH of the medium and the incubation period were the critical factors in determining the accuracy of the TTC test. The maximum yield of the triphenyl formazan was obtained after 80 min for both *K. fragilis* and *C. pseudotropicalis* (Fig. 3). The optimum pH for the growth of the yeasts *K. fragilis* and *C. pseudotropicalis* is $4.5 \pm 0.2$. When using the TTC test to determine the activity of the yeast cells in the samples obtained from the

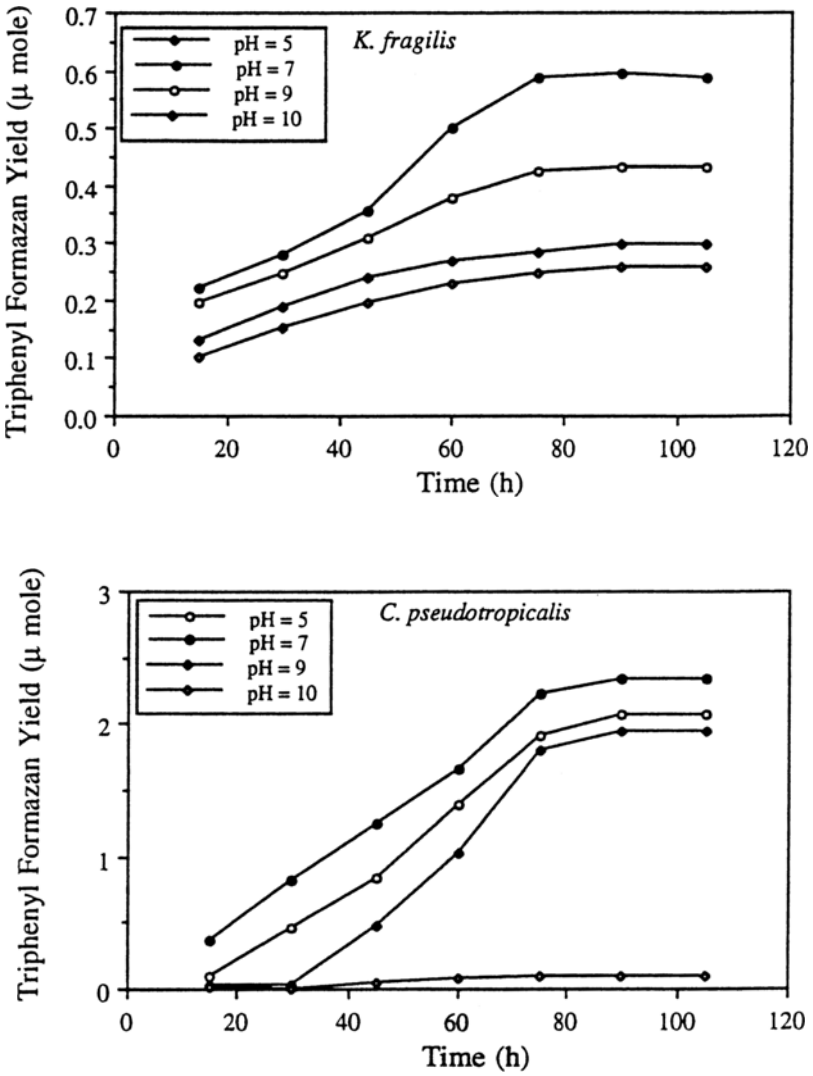


Fig. 3. Effect of incubation time on formazan yield at various pH.

single-cell protein reactors (pH=4.5), no color was developed after the addition of the dye to the samples containing living yeast cells (as was determined by the plate count test). It was concluded that the enzymatic activity was sensitive to the pH of the medium, and 1*N* sodium hydroxide was used to adjust the pH. The results obtained in Fig. 4 indicated that the optimum pH range for dehydrogenase activity for *K. fragilis* and *C. pseudotropicalis* is $7.0 \pm 0.2$. A pH variation of 3 U impairs enzyme activity. Similar observations were reported by Patel et al. (*10*) for a purified secondary alcohol dehydrogenase enzyme obtained from cultures of *Pichia sp.*

After a considerable amount of experimental work on the dehydrogenase activity measurement technique, and consideration of the utiliza-

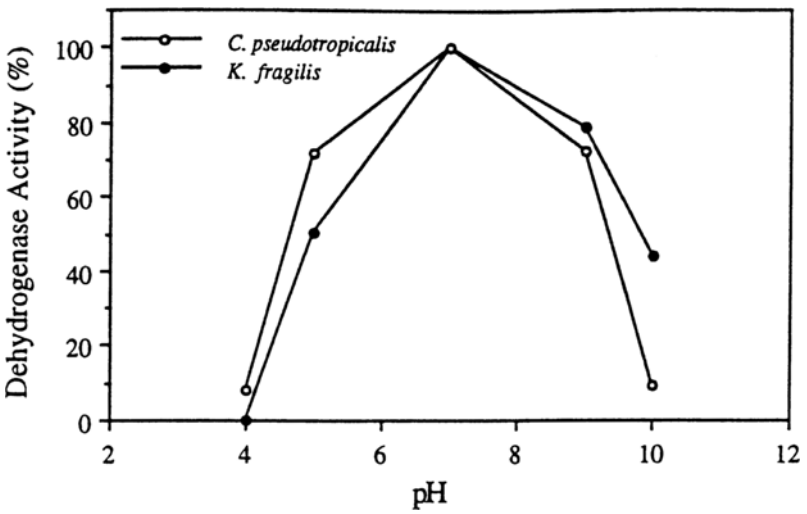


Fig. 4. Effect of pH on the dehydrogenase activity level at an 80-min incubation period.

tion of the technique for the determination of biomass activity in yeast fermentation systems, the analytical procedure previously described was developed. Three replicates were always carried out, and the repeatability of the test was very good since the coefficient of variability was very small (CV = 0.55–2.81%). The relationship between the dehydrogenase activity (as measured by the formazan yield at a pH of 7.0 and an incubation period of 80 min) and the concentration of active cells of these microorganisms (as measured by the plate count) appeared to be linear for all microorganisms as shown in Fig. 5. It can be described in the following formula:

$$Y = FX \tag{1}$$

where $Y$ is the formazan yield ($\mu$mol), $F$ is the yield coefficient, determined from the slope of the line ($\mu$mol cell$^{-1}$), and $X$ is the microbial population (cell). The formazan yield coefficient ($F$) was found to be $0.354 \times 10^{-9}$ and $0.394 \times 10^{-9}$ $\mu$mol cell$^{-1}$, for *K. fragilis* and *C. pseudotropicalis*, respectively.

## Yeast Growth

Microbial growth is the orderly increase in the quantity of all components of the microbial cell. After a microorganism (microbial cell) has almost doubled in size and in the amount of each of its components, it divides into two daughter cells. The time required for one cell to grow and divide into two cells is referred to as the doubling time or the generation time. However, the mode of doubling time in yeast culture is by budding whereby a mother cell produces a bud. When the bud is nearly as large as the mother cell, it separates from the mother cell. Consequently, the growth of microorganisms is measured in terms of an increase in the number of cells, rather than the increase in the size of a single organism.

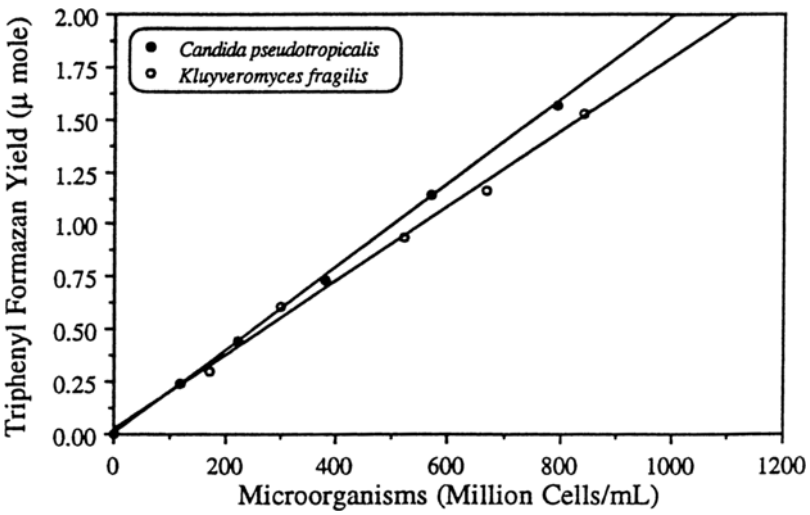


Fig. 5. Correlation between the dehydrogenase activity as measured by formazan yield and the concentration of the active yeast cells as measured by plate count.

The growth curves of the yeasts *K. fragilis* and *C. pseudotropicalis* were determined from the plate count test (Figs. 6a and 7a) and the formazan yield test (Figs. 6b and 7b). The important feature of this curve is that the four principal phases encountered in the history of a microbial culture grown in a batch operation can be clearly recognized. These were:

1. The lag phase, which represents the time for the yeast cells to acclimatize themselves to the new environment;
2. The exponential growth phase, during which the growth rate had a constant maximum value;
3. The stationary phase, during which the growth rate was zero; and
4. The death phase, during which the yeast cells died faster than new cells were produced.

For both yeasts, the formazan yield curve was almost identical to the growth curve obtained from the plate count test. The relationship between the formazan yield and the number of cells was linear as shown in Fig. 8. The coefficient of correlation ($R^2$) was 0.99 and 0.97 for *K. fragilis* and *C. pseudotropicalis*, respectively. The formazan yield coefficient for *K. fragilis* and *C. pseudotropicalis* was $0.350 \times 10^{-9}$ and $0.400 \times 10^{-9}$ $\mu$mol cell$^{-1}$, respectively. These results are in good agreement with those obtained from Fig. 5. They also confirm the observation made by Ghosh et al. (4) and Ghaly et al. (5) that the dehydrogenase activity per unit biomass synthesized is constant for a given substrate-organism system at all observed specific growth rates (i.e., in the lag, exponential, stationary, and death phases).

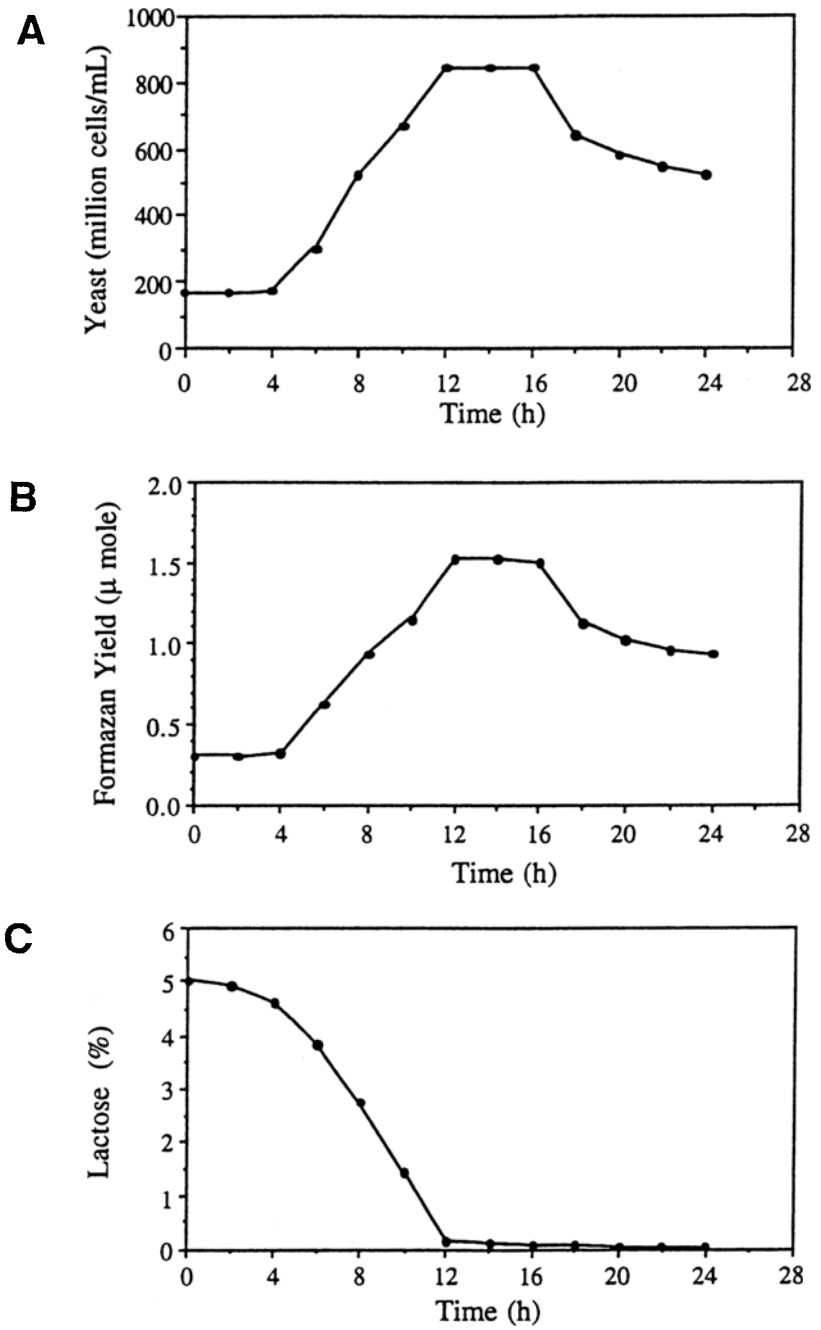


Fig. 6. Batch culture formazan yield, active *K. fragilis* cell number, and lactose concentration.

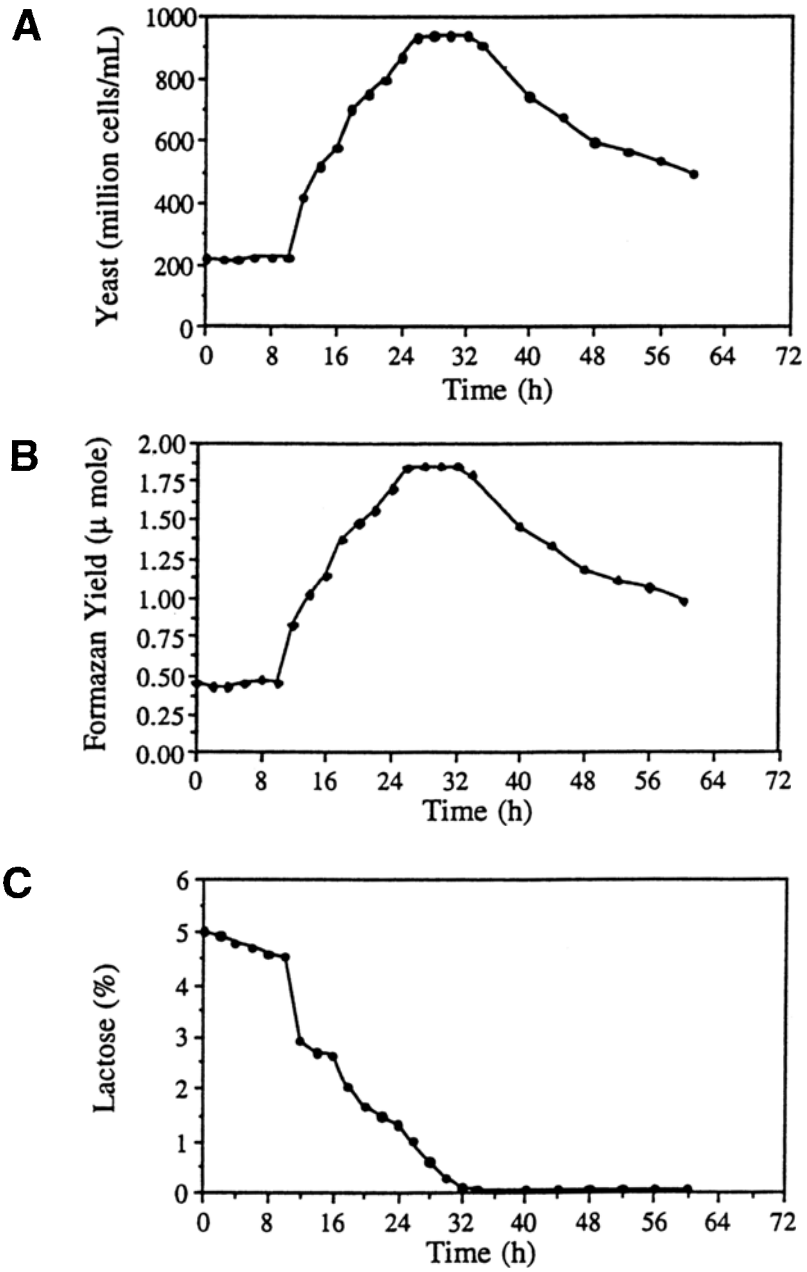


Fig. 7. Batch culture formazan yield, active C. *pseudotropicalis* cell number, and lactose concentration.

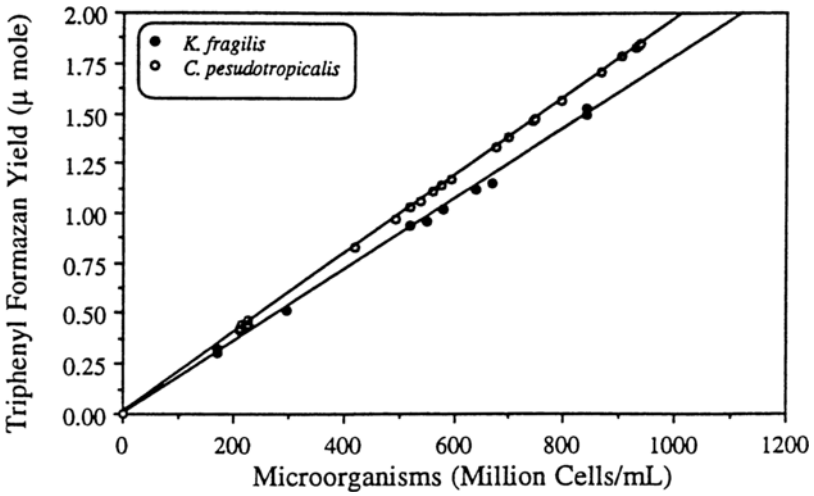


Fig. 8. Formazan yield vs cell number of *K. fragilis* and *C. pseudotropicalis* during batch fermentation of cheese whey.

The natural logarithm of the number of active cells in the exponential growth phase was plotted against the time for each yeast as shown in Fig. 9. This was done to linearize the exponential portion of the growth curve. The specific growth rate ($\mu$) was then calculated from the slope of the linear portion of the exponential growth phase using the Recognition Procedure of the Statistical Package MINITAB from the Statistical Library of the Technical University of Nova Scotia. The duration of the lag phase was also obtained from the intersect with X axis. The values of the specific growth phase and the lag phase were found to be 0.22 and 0.06 $h^{-1}$, and 3.50 and 9.00 h for *K. fragilis* and *C. pseudotropicalis*, respectively. The long lag period observed with *C. pseudotropicalis* was apparently caused by the long storage period of the inoculum.

## Lactose Utilization

For both yeasts, the reduction of lactose displayed three distinct stages that corresponded to the lag, exponential growth, and stationary phases of the yeast growth curve (Figs. 6C and 7C). During the first stage, there was a slow reduction in the lactose concentration, since the latter was utilized mainly for cell respiration and cell endogenous growth (individual cell growth). The second stage was a period of rapid lactose reduction in which lactose was utilized by the yeast cells for both endogenous cell growth (cellular cell growth) and cell mass growth (cell multiplication) as well as cell respiration. In the third stage, the concentration of lactose was very low and, thus, an insignificant reduction of lactose was achieved during this period. As a result of lack of substrate, the microbial growth

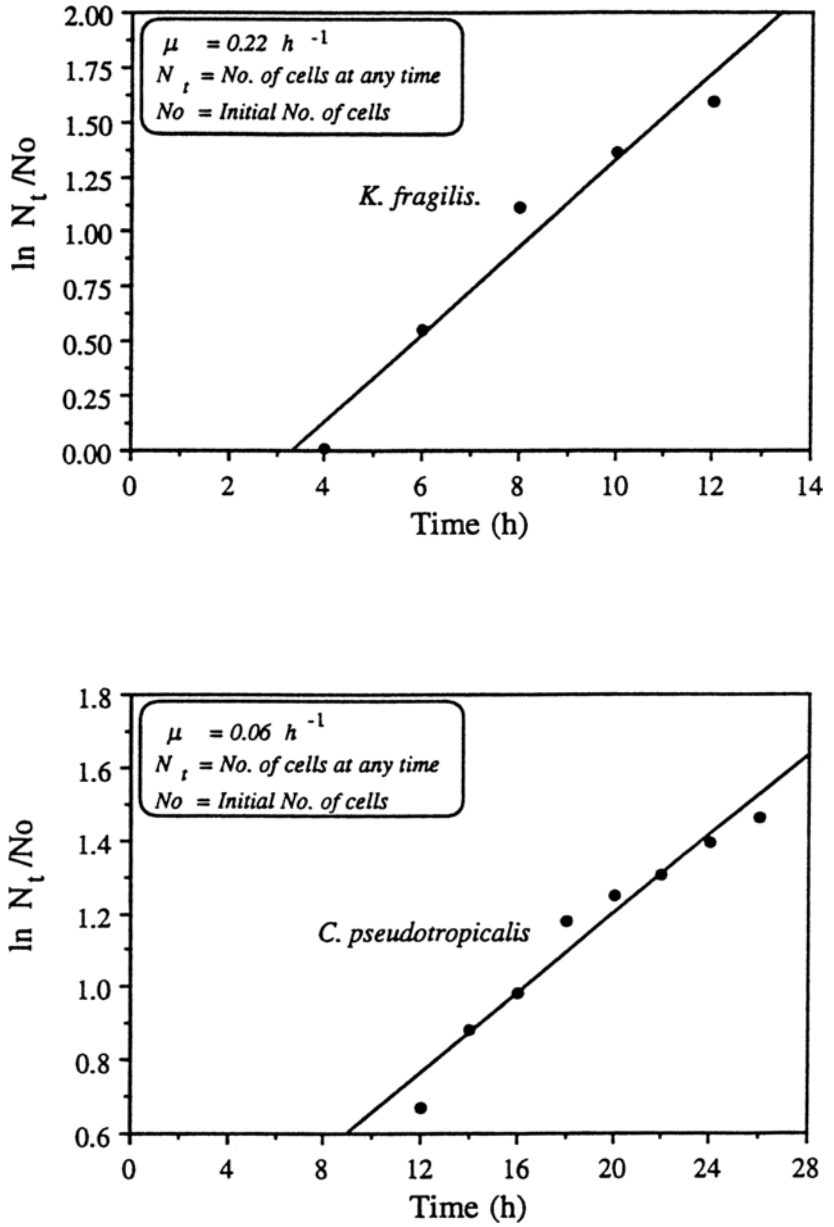


Fig. 9. Determination of specific growth rates of *K. fragilis* and *C. pseudotropicalis*.

remained stationary for a period that was then followed by the death and/or the sporulation of the vegetative cells of the yeast (*11*). Burgess (*12*) reported that the lactose consumption under batch condition using a laboratory-scale tower fermenter was fairly low in the first 2 h (while the yeast was adapting to the new substrate) and then increased rapidly. In this

study, the period of slow lactose consumption was 4 and 10 h for *K. fragilis* and *C. pseudotropicalis*, respectively.

The initial value of lactose was 5.0% (which is equal 50 g/L). Since the liquid volume was 4.8 L, the total amount of lactose in the fermenter at zero time was 240.0 g. The lactose was reduced from 5.0 to 0.05% after 16 h by *K. fragilis* and after 32 h by *C. pseudotropicalis*. This resulted in lactose reductions of about 99% (of the initial lactose in the fermenter). Mahmoud and Kosikowski (*13*) found that the rate of lactose utilization was faster under aerobic than under anaerobic condition for all yeasts. The highest percentage of lactose utilized by *K. fragilis* was 99.7% aerobically and 60.9% anaerobically.

## CONCLUSIONS

The following conclusions are made from this research:

1. A modified technique for the measurement of dehydrogenase activities of yeast, using Triphenyl Tetrazolium Chloride (TTC), has been developed. The technique is easy to perform and provides indirect measurements of the yeast population in relatively short time with high accuracy (2 h for the TTC test as compared to 72 h for the tedious and sometimes inaccurate plate count).
2. The formazan yield is influenced by both the pH of the medium and incubation time. A pH of $7.0 \pm 0.2$ and an incubation period of 60 min are recommended for dehydrogenase activity measurements in yeast fermentation.
3. The measurement of yeast dehydrogenase activity in cheese whey can be used as a measure of the yeast population. The formazan yield curve was almost identical to the growth curve obtained from the plate count test. The coefficient of correlation ($R_2$) was 0.99 and 0.97 for *K. fragilis* and *C. pseudotropicalis*, respectively.
4. The four principal phases (lag, exponential, stationary, and death) encountered in the history of a microbial culture grown in a batch operation were clearly recognized in the *K. fragilis* and *C. pseudotropicalis* growth curves.

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