PELLICLE FORMATION AND ESTER PRODUCTION BY *HANSENULA ANOMALA* - MILLS

PELLICLE FORMATION AND ESTER PRODUCTION BY HANSENULA ANOMALA

by

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I. INTRODUCTION

Hansenula anomala (Hansen) Sydow presents two interesting physiological phenomena: the formation of a pellicle on liquid media and the production of large amounts of ester.

Although the formation of the pellicle had been discussed in relation to colony form and cell hydrophobicity (Fabian & McCullough, 1934; Fabian & Wickerham, 1937; Wickerham, 1951; Tabachnick, 1953a; Windisch & Emeis, 1958), no quantitative determination of the pellicle for different colony forms of Hansenula anomala nor comparison of the physiology of the pellicle cells with the sedimented cells had been done. Various physiological roles had been attributed to the pellicle (Wickerham, 1951; Tabachnick, 1953a) but no experimental work had been done to verify these hypotheses. Factors influencing the formation of ester by Hansenula anomala had been studied at length by several authors; however, the results concerning the role of aeration in ester formation seemed questionable in view of the fact that suitable controls for the estimation of ester evaporation were not usually included.

These facts prompted us to carry out a study of pellicle formation by three different morphological strains of Hansenula anomala and to determine through the use of various inhibitors of pellicle formation the role played by the pellicle in ester production. Moreover, the effect of aeration on ester formation by these three strains of Hansenula anomala was investigated.

II. LITERATURE REVIEW

Considerable attention has been devoted, over a number of years, to the genus Hansenula. Designated as the genus Willia by Hansen (1904), it was characterized by hat-shaped or Saturn-shaped ascospores, gaseous fermentations and ester production. Renamed Hansenula by the Sydows in 1919, the genus was limited to species which assimilate nitrate, ferment sugars strongly and hydrolyze esculin by Stelling-Dekker (1931). The taxonomy of Hansenula, studied by Bedford (1942), was briefly reviewed by Gray (1949) and more recently by Wickerham (1951).

The phylogeny and biochemistry of the genus Hansenula was discussed at length by Wickerham and Burton (1962). These authors divide the genus Hansenula into five phylogenetic lines: 1) a homothallic and 2) a heterothallic chain of species, both of which evolved from habitats linked with coniferous through deciduous trees and, finally, into habitats where microorganisms are free living and highly competitive; 3) a homothallic and 4) a heterothallic chain, both evolved toward complete dependence upon coniferous trees and finally 5) a few species associated primarily with the animal body.

All species isolated from trees require vitamins for growth in chemically defined media. Species isolated from sources independent of trees are predominantly diploid, ferment three to five sugars, synthesize all the vitamins needed and often produce mat colonies. These yeasts represent an evolutionary line in which an increasing ratio of diploid to haploid cells, greater fermentative powers, an increasing ability to synthesize vitamins, and a change from mucoid to glistening butyrous and

finally to mat dry colonies indicate the sequence of evolutionary development within the genus.

Hansenula anomala is classified by Wickerham and Burton as a heterothallic yeast evolving towards a free-living state. It stands high in the second phylogenic line, requires no vitamins, rapidly ferments a number of sugars, and produces predominantly mat colonies.

In the present study, two aspects of the physiology of this yeast are reviewed in particular: the pellicle and colony characteristics of Hansenula yeast and the formation of ester by these organisms.

PELLICLE AND COLONY CHARACTERISTICS OF HANSENULA YEAST

Most Hansenula species produce a pellicle on liquid media. Fabien and McCullough (1934), using several genera of yeast including Willia (Hansenula) anomala concluded that the tendency to scum (pellicle) formation is due to the dissociation of the S form of the yeast to the R form and that scum formation took place in a shorter time at the higher temperatures than at the lower temperatures because the yeast dissociated more rapidly at the higher temperatures. The R form of the yeast produced scum at all temperatures. However, Scherr and Weaver (1953) pointed out in their review of the dimorphism phenomenon in yeast and yeast-like organisms that all other investigators of the phenomenon had found that lower temperatures rather than higher temperatures induced Y → M transformations.

The dissociation induced in Saccharomyces aceris-sacchari (Hansenula anomala) by lithium chloride was shown to be a gradual and reversible process by Wickerham and Fabian (1936). The rough forms obtained grew mainly at the surface of the liquid medium. Moreover, they possessed larger cells and produced bigger colonies on agar.

The amount of pellicle formed seems to correspond to the colony type of the strain employed. Fabian and Wickerham (1937) showed that the mat forms produce heavier pellicles on liquid media than the corresponding glistening forms. In Hansenula anomala, all degrees of transition are found between highly glistening and powdery mat extremes in colony appearance. Moreover, the authors concluded that homologous mat and glistening forms usually have approximately equivalent physiological activities from a qualitative though not from a quantitative standpoint. Glistening types were found to produce decidedly more alcohol than the corresponding mat types. Wickerham (1951) believes that the development of prominent pellicles by rough strains is a mechanism for the oxidation and utilization of the alcohol and organic acids produced.

Tabachnick (1953a), on the other hand, postulated that the cells in the surface film carried out the major portion of both ethanol and ethyl acetate production from glucose since in very low oxygen tensions very slow glucose utilization and little ester production resulted.

Hedrick (1960), in his study of the relative hydrophilic and hydrophobic characteristics of yeast cells through their sedimentation patterns in dilute aqueous saline solutions, postulated that the cellular surfaces of hydrophobic strains contain a preponderance of nonpolar groups such as $-CH_3$ and $-C_6H_5$. Cells with hydrophilic surfaces, on the other hand, have a preponderance of exposed polar groups, such as -OH, $-NH_2$, $-COO^-$, and $-OPO_3^-$. Two yeast strains employed in the present study were investigated by Hedrick; Y-1737 and Y-366. Hedrick observed that Y-1737 showed greated hydrophobicity than Y-366. Y-1737 formed growth clusters of many cells but the author concluded that aggregation of cells was not responsible for the shields (sedimented cell patterns)

in tubes with different amounts of saline.

Windisch and Emeis (1958) reported that Tween 80 by altering the surface tension prevented pellicle formation by a number of yeast genera including Hansenula.

ESTER PRODUCTION BY HANSENULA ANOMALA

In 1894, Hansen reported that Hansenula sp. produce a fruity odor during fermentation. The ester produced by Hansenula anomala was identified by Gray (1949) as ethyl acetate.

A detailed study of the chemistry and physiology of ester production by Hansenula anomala was presented by Tabachnick (1950) who concluded that ethyl acetate is not produced directly from glucose but is formed as the result of the aerobic utilization of ethanol accumulated in the fermentation of glucose. Tabachnick also noted that ester synthesis appeared to follow a separate pathway from hydrolysis. The esterase responsible for the hydrolysis is inhibited at low pH values permitting the accumulation of ester under these conditions.

Peel (1951) concluded from his experiments with washed suspensions of H. anomala cells that the amounts of ester formed were too large to be accounted for by the reversal of a simple esterase reaction and postulated a more exergonic mechanism of ester formation.

The acidic metabolic products of Hansenula anomala were shown to be, by Hughes (1952), acetic acid and a nonvolatile acid which was not identified.

Brock (1952) postulated from the respiration data obtained from glucose that one pathway of aerobic glucose oxidation in H. anomala follows neither the Embden-Meyerhof scheme nor the tricarboxylic acid

cycle but a scheme of the Warburg-Lipmann-Dickens type. This hypothesis was verified by Davis (1957) who determined that H. anomala oxidized intermediates of glycolysis, of the monophosphate pathway and of the tricarboxylic acid cycle. The author suggests that the monophosphate pathway is a major pathway in the initial dissimilation of glucose and that the pentose is split into 2-carbon and 3-carbon fragments with the 3-carbon fragments being converted to ethyl acetate via ethanol. Sova (1960) also concluded from his study of the mol ratios of CO_2 evolved/glucose utilized that both the glycolytic and the hexose monophosphate pathways are operative simultaneously in the hexose metabolism of Hansenula anomala. However, he proposed that ethyl acetate is produced through the glycolytic pathway since inhibition of the monophosphate pathway with atabrine resulted in enhanced ester production. Toy (1963) confirmed the presence of the glycolytic and hexose monophosphate pathways in Hansenula anomala and concluded that volatile acid accumulates when the monophosphate shunt is blocked and nonvolatile acid increased when glycolysis is inhibited.

Nordström (1961, 1962, 1963, 1964) in his intensive study of the mechanism of ester production in Saccharomyces cerevisiae also showed that ethyl acetate is not formed through the esterification of acetic acid by means of ethanol in the medium, but by an energy-demanding process requiring the participation of acetyl-CoA. The author demonstrated that the acetyl-CoA for ethyl acetate formation arises through the oxidative decarboxylation of pyruvic acid. Canterelli (1955) had already proposed that ester production in Hansenula is dependent on CoA; however, some of his results, notably the inhibitory effect of very low concentrations of 2,4-dinitrophenol and of malonate on ester production in

Hansenula anomala, may indicate that acetyl-CoA does not arise from the oxidative decarboxylation of pyruvic acid in this yeast.

Smith and Martin (1964) investigated the nature of the esterase present in ester-producing Hansenula and found that the partially-purified enzyme decomposed both aliphatic and aromatic esters. The esterase had a broad pH optimum, the maximum degradation of ethyl acetate occurring between pH 5 and 7. These authors failed, as had Tabachnick (1951), to produce an active cell-free preparation of the ester synthesizing enzyme.

Many investigators were especially interested in the effects of aeration on ester production. Kayser and Demalon (1909) stated that moderate excess of aeration favors ester production but that great excess results in decreased ester due to over-oxidation of ethanol. Fabian and Wickerham (1937) also concluded that when oxygen is low, ester production is low; when oxygen is abundant, no esters are formed. Sufficient oxygen to maintain normal growth produced the largest amounts of esters. In determining this, these authors used five liters of medium in a 6-liter flask with the only access to air being a 15 mm. hole tightly plugged with cotton, the same type of flask with a 50 mm. opening and thirdly, 5 liters of medium in a 16 inches square pan. In the first flask, at the end of 10 days, less than 0.02 gm. of ester per 100 ml. were formed. In the second flask, 0.58 gm. of ester per 100 ml. had been produced. In the pan no esters were present at the end of ten days. The authors, however, did not mention if ester determinations were made at earlier or later intervals nor did they discuss the possibility of ester evaporation.

Gray (1949) conducted slightly more elaborate aeration experiments. He added 100 ml. portions of medium to Erlenmeyer flasks of 125, 250, 500, 1000 and 2000 ml. capacity obtaining the results shown below after five days of incubation:

Flask Volume (ml.)	Total Ester (mg.)
125	109.5
250	176.0
500	94.3
1000	31.7
2000	5.2

From these results, the author concluded that increased aeration attained by increasing the surface/volume ratio resulted in a decided decrease in ester production. Again there is no mention of ester determinations made before or after the five-day period or of loss of the volatile ester. The time of maximum ester production was determined to be five days for cultures of 100 ml. in 250 ml. flasks and this was assumed to be the same for the other flasks. It was also shown that, in the 250 ml. flasks, after the ester had reached its maximum concentration, it disappeared from the fermentation medium at about the same rate as it had been produced.

The effect of excess aeration was also studied by Tabachnick and Joslyn (1953a) who noted that only 57% of the amount of ester formed in the standing cultures was found in the cultures incubated on the shaker. A control containing 1.60 mm. of ethyl acetate per 100 ml. showed only a 2% loss of ester by evaporation at the end of 12 hours. However, Tabachnick (1951) also pointed out that a much more rapid rate of ester evaporation occurs in cotton-stoppered 125 ml. Erlenmeyer flasks containing 20 ml. of medium and incubated on the shaker.

Sova (1960) found that over four times more ester was produced in 250 ml. flasks containing 100 ml. of medium when the cotton stoppers and the neck of the flasks were covered with aluminum foil. The author dismissed the loss of ethyl acetate by evaporation as a possible cause for this phenomenon relying solely on Tabachnick's (1953a) evidence with his 2500 ml. narrow-necked Fernbach flasks and neglecting Tabachnick's (1951) results with the 20 ml. of solution in 125 ml. flasks. Neglecting to verify this data under his own experimental conditions, he presented the results obtained with foil-covered flasks as further evidence in support in favor of the view that ethyl acetate is formed only under moderate conditions of aeration.

The results obtained by those investigating the effects of aeration on ester production seem to indicate, in general, that no ester is produced under anaerobic conditions (Peel, 1951; Tabachnick & Joslyn, 1953b) and that excess aeration leads to reduced ester production.

III. MATERIALS AND METHODS

CULTURES

The yeast cultures used were three strains of Hansenula anomala obtained from the Northern Regional Research Laboratory through the courtesy of Dr. L. J. Wickerham. These were given Macdonald College collection numbers: Y3221, Y3222 and Y3223.

An additional strain used in a few experiments was obtained through the courtesy of Dr. William D. Gray and was given number Y3224.

The yeast cultures were maintained on MY agar plates (this medium contains 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar) and were transferred every four to five days.

QUANTITATIVE DETERMINATION OF THE PELLICLE

The fermentation medium employed consisted of Difco Yeast Nitrogen Base, a chemically defined medium developed by Wickerham (1951), containing ammonium sulphate, minerals, trace elements and growth factors. This medium was dissolved in distilled water to give a ten-fold concentration stock solution and filter sterilized. A ten per cent glucose solution was sterilized by autoclaving for 15 minutes at 20 p.s.i. and 121°C. When the glucose solution had cooled, 40 ml. of the nitrogen base solution were dispensed into 360 ml. portions of the sugar solution and the resulting 400 ml. of complete medium were transferred to sterile 500 ml. pear-shaped separatory funnels.

Inoculum was prepared by transferring a loopful of yeast cells from a two to three day old MY plate culture to 10 ml. of the fermentation medium described above. After incubation on the shaker for 24

hours at 30°C., one ml. of the culture was used to inoculate 10 ml. of fresh medium which was incubated for 16 hours under the same conditions. The cells were then centrifuged and resuspended in enough water to give optical density measurements equivalent to 1.5-2.0 mg. dry weight of cells in the 2 ml. used as inoculum.

The cultures in the separatory funnels were incubated at 25 and 30°C. Every second or third day over a period of 11 to 14 days, three funnels were emptied for each strain of yeast used; 200 ml. were withdrawn through the stopcock so as not to disturb the pellicle and the remaining 200 ml. were washed out of the funnel with distilled water. The cells from the 200 ml. withdrawn through the stopcock are referred to as bottom cells and those in the remaining 200 ml. as top cells. The cells were centrifuged at 10,300 x g for fifteen minutes, washed twice and resuspended in 5 or 10 ml. of distilled water. Two ml. aliquots were dispensed into 10 ml. tared beakers and dried at 90°C. for 18-24 hours.

The sugar remaining was determined by taking the balling of the supernatant with a Brix saccharometer, after the cells were centrifuged. The pH was determined with a glass electrode pH meter.

DETERMINATION OF VIABILITY OF CELLS

Top and bottom cells were obtained as described above except that the fermentation medium contained 2% instead of 10% glucose. The centrifuged cells were stained with the methylene blue "dead cell" stain described by Townsend and Lindegren (1953). The stain is a single solution containing 0.2% methylene blue, 5% ethyl alcohol in 0.2M KH_2PO_4. Excess stain was mixed with the cells and the cells were allowed to stand for a

few minutes. The percentage of dead cells - cells which became blue - was calculated from a total count of approximately 1,000-4,000 cells obtained through the microscopic examination of two to three wet mounts.

RESPIRATORY STUDIES

Top and bottom cells, collected as indicated above, with 2% glucose in the fermentation medium, were resuspended to give 3.0-4.0 mg. of cells dry weight per ml. Standard manometric techniques (Umbreit et al., 1964) were used. One millilitre of HCl-KCl buffer at pH 2.5 (Colowick and Kaplan, 1955), 1.0 ml. of cell suspension, 1.0 or 0.9 ml. of water were dispensed into the main well of the Warburg flasks. In the center well, 0.2 ml. of 10% KOH was used and in the side arm, 0.1 ml. of a glucose solution containing 10 uM of glucose. The respiratory studies were conducted at 30°C. All values for gas exchange have been calculated in µl. of oxygen per hour per mg. dry weight of cells.

STUDY OF PELLICLE INHIBITORS

For these experiments, fermentations were carried out using 20 ml. of Yeast Nitrogen Base medium containing 2% glucose in 50 ml. Erlenmeyer flasks. The cells used for inoculum were taken from two to three day old MY agar plates, inoculated into 20 ml. of 5% glucose Yeast Nitrogen Base and incubated on the shaker at 30°C. for 16-18 hours. The cells were centrifuged, washed once and resuspended in distilled water to give an optical density reading equivalent to about 2 mg. of cells dry weight per ml. The flasks were inoculated with 0.2 ml. of this cell suspension.

In a series of fermentations, 0.05-0.2% Tween 80[1] (vol./vol.) was used. Supplement of 0.8% yeast extract was added to fermentations with and without Tween 80.

An ergosterol stock solution was prepared according to the method of Hesseltine et al. (1952) using 2.5 gm. of ergosterol with 250 ml. of Atlas Tween 80 and 500 ml. of 95% alcohol. Solution is effected by heating in a water bath at 60-70°C. and then the volume is made up to 1,000 ml. with 95% alcohol. Eight milliliters of this stock solution were used in 1,000 ml. of medium. An equivalent amount of alcohol was added to the cultures not receiving any ergosterol.

To other cultures, L (+) cysteine hydrochloride monohydrate was added to give a molar concentration of 10^{-2} to 10^{-5}. All these cultures were incubated at 25°C.: some were left still and others were placed on a New Brunswick rotary shaker, G 52, rotating in a two-inch circular orbit at 130 r.p.m.

ESTER DETERMINATION

The amount of ester produced in still and shaken cultures was determined using 20 ml. of the Yeast Nitrogen Base medium previously described with 2% glucose in 50 ml. Erlenmeyer flasks. Foam plugs (Identi-Plugs, Gaymar Co.) were used to stopper the flasks. The inoculum was prepared as described above. The cultures were incubated at 25°C.

The ester was determined by the colorimetric method outlined by Peel (1951) measuring the colour intensity with a Beckman model 9 colorimeter and a 590 mμ filter. Cultures were placed at 5°C. for

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[1]Supplied free of charge by the Atlas Powder Co., Brantford, Ontario.

20-30 minutes before removing the plugs to minimize loss of ester. The fermentation liquor was then clarified by filtering through a No. 3 Whatman filter paper. Ester determinations were always carried out immediately on the clarified solutions. For each ester determination, three separate flasks in the fermentation series were employed and duplicate determinations were done on each.

The effect of increased aeration on ester production was studied by increasing the surface-volume ratio using 20 ml. of medium in 25, 50, 125, 250, and 500 ml. Erlenmeyer flasks.

To minimize the loss of the volatile ester, fermentations were set up in which the air was distributed to the cultures through a manifold. The fermentation flasks were connected to the manifold and air reached the culture through a glass tube extending into the flask and ending one inch above the surface of the medium. Air left the flask through a glass tube at the top of the flask and was bubbled through 20 ml. of a solution of 1N hydroxylamine and 3N NaOH contained in a large test tube. The 250 ml. fermentation flasks contained 50 ml. of medium and were inoculated with 0.2 ml. of a cell suspension. The fermentation flasks in the 'train' were either left still allowing the pellicle to grow or else incubated on a New Brunswick rotary shaker, G 10, rotating in a one-inch circular orbit at 130 r.p.m. All 'train' fermentations were incubated at 30°C.

DETERMINATION OF GLUCOSE

The qualitative disappearance of glucose in the fermentation medium was followed by paper chromatography of 10 microliters of fermentation liquor using as solvent mixture n-butanol (50 parts),

pyridine (20 parts), and water (18 parts). The paper was sprayed with an aniline spray (1.3 ml. aniline and 0.6 ml. orthophosphoric acid in 100 ml. glacial acetic acid). The chromatogram was dried and heated at 100°C. for a few minutes. Ten microliters of 0.2% glucose were run as control.

DETERMINATION OF CARBON DIOXIDE PRODUCTION

Carbon dioxide was removed from the incoming air by bubbling through 400 ml. of 1N NaOH in a 500 ml. Erlenmeyer flask. The air leaving the fermentation flask was freed of moisture by passage through a U-tube containing anhydrous $Mg(ClO_4)_2$, then through a U-tube containing Indicarb, 6-10 mesh (Fisher), to remove the carbon dioxide, followed by another U-tube with the dessicant and finally a bubbler to regulate air flow. Fermentation flasks contained 50 ml. of Yeast Nitrogen Base containing 1.000 gm. of glucose. The Indicarb tubes were weighed daily and the fermentation stopped by pipetting in 20 ml. of 5N HCl when the Indicarb tubes showed no further increase in weight. The number of millimoles of CO_2 evolved was determined by the following calculation:

$$\frac{\text{Wt. increase of Indicarb tube in mg.}}{44} = \text{mM } CO_2$$

DETERMINATION OF ESTERASE ACTIVITY

Cells cultivated in the 'train' fermentations described above and having reached the beginning of ester peak production were centrifuged, washed once and resuspended in 50 ml. HCl-KCl buffered (pH 2.3) ester solution and reincubated under the same conditions as the train fermentations. Flasks containing only buffered ester solution were

used as controls. The ester remaining in the flasks after a given period of incubation was determined as described above.

DETERMINATION OF ESTER PRODUCTION IN THE PRESENCE OF ALKALINE NH_2OH

Fifteen milliliter beakers containing 10 ml. of 1N $NH_2OH.HCl$ and 1.25N NaOH were placed in 250 ml. Erlenmeyer flasks containing 50 ml. of Yeast Nitrogen Base with 2% glucose. Fluted filter paper was inserted in the beakers to insure greater surface area to the alkaline hydroxylamine solution. The flasks were stoppered with cotton plugs and aluminium foil. Water was dispensed into the beakers of the control cultures.

IV. EXPERIMENTAL RESULTS

CHARACTERISTICS OF THE COLONIES

Appendix Figures 1-4 show the colony appearance of the four strains of Hansenula anomala used.

No. Y3221 (NRRL Y-366) - produces smooth, even-edged colonies which may have a glistening or mat appearance depending on the age of the culture, the kind of medium and the amount of moisture present, Appendix Figure 1. The colonies are almost pure white. This strain will be referred to as the intermediate strain.

No. Y3222 (NRRL Y-1737) - produces rugose, coarsely convoluted colonies with a powdery appearance, Appendix Figure 2. This strain will be referred to as the rough strain.

No. Y3223 (NRRL Y-407) - produces smooth, glistening colonies which possess a creamy tinge, Appendix Figure 3. This strain will be referred to as the glistening strain.

No. Y3224 (No. 20 of the Ohio State Stock Culture Collection) - produces white, powdery colonies which are less rugose than those of Y3222, the rough strain, but are characterized by the formation of myceliar threads extending from the colonies into the agar, Appendix Figure 4.

CHARACTERISTICS OF THE PELLICLE

Quantitative Determination of the Pellicle

The total dry weight and the respective weights of the top and the bottom cells were determined. Accompanying changes in balling and

pH were noted as the fermentation progressed. Appendix Table I, II, and III list these changes for Y3221, Y3222, and Y3223 both at 25° and 30°C. and Figures 1-5 compare each of these aspects as they occur in the three yeast strains.

The weight of the bottom cells (Fig. 1) increases gradually at the beginning of the fermentation and exhibits a sudden increase towards the end of the fermentation, between the 9th and 14th day. The time of this remarkable change in the amount of bottom cells varies with cultures inoculated and incubated at the same time and under the same conditions. The increase in the amount of bottom cells is observable visually in the funnels and occurs during a 2-3 day interval in funnels belonging to the same fermentation batch.

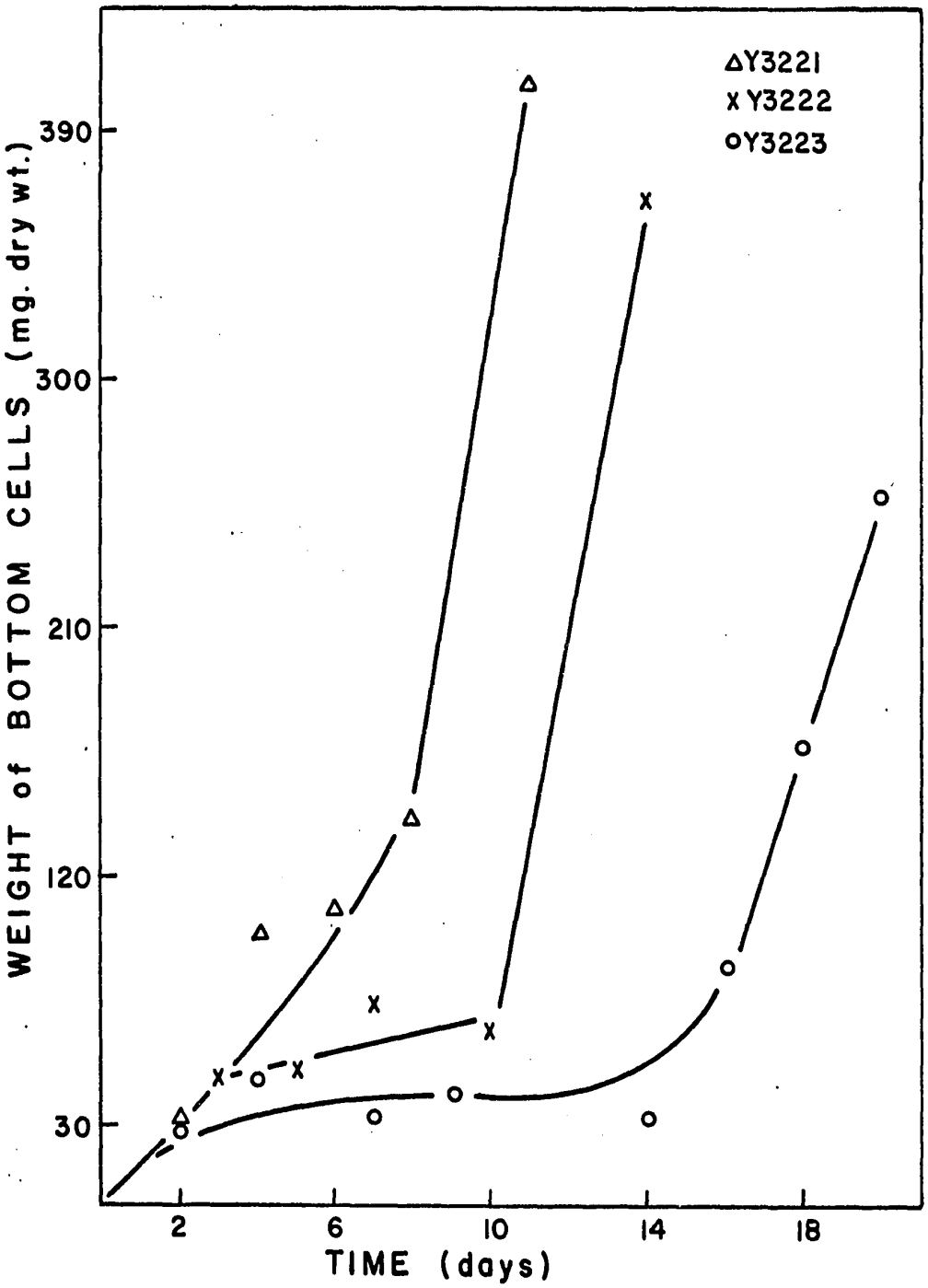
The appearance of the pellicles formed by the three strains is quite different and vary also depending on the media and the sugar concentration. The intermediate strain, Y3221, forms a white, delicate, lacelike film covering all the surface and climbing 2-3 inches on the walls of the flask, Appendix Figure 5. The pellicle is somewhat powdery and when disturbed breaks up into delicate, floating, hydrophobic flakes. The glistening strain, Y3223, forms a similar pellicle but after a longer period of time and this pellicle may remain incomplete and scanty when the glucose concentration is low. The pellicle formed by Y3222, the rough strain, is more membranous, thick and had a yellow tinge, Appendix Figure 6. Gas accumulates under both types of pellicles producing a large central bubble or several smaller ones if the glucose concentration is high enough. The intermediate strain forms its pellicle the fastest, within 24 hours; the rough strain may take 48 hours to produce one at 30°C. in 10% glucose medium

Figure 1

Figure 1

YIELD OF BOTTOM CELLS OF *HANSENUIA ANOMAIA*

Total dry weight of cells obtained from the bottom 200 ml. of fermentation broth from cultures consisting of 400 ml. of Yeast Nitrogen Base with 10% glucose in 500 ml. pear-shaped separatory funnels incubated at 30°C.


ΔY3221
X Y3222
O Y3223
WEIGHT of BOTTOM CELLS (mg. dry wt.)
390
300
210
120
30
2
6
10
14
18
TIME (days)

and the glistening strain, 96-120 hours.

The weight of the top cells (Fig. 2) rises quite rapidly and falls quickly once the maximum is reached. The glistening strain shows a less rapid increase in top cell weight. The sudden decrease in top cells coincides with the sudden increase in bottom cells and is related, obviously, to the sinking of the pellicle cells to the bottom at some time after the 9th day of fermentation.

The total weight of cells (Fig. 3) increases rapidly at first especially with Y3221 and Y3222; the glistening strain, Y3223, requires the longest amount of time to reach the maximum and Y3221, the intermediate strain, the least. Ultimately, all strains produce approximately equivalent biomasses.

The initial pH of 6.2 drops rapidly to very low values (Fig. 4). The rough strain, Y3222, gives the most rapid drop in pH and the lowest final pH.

Sugar disappears the most rapidly with Y3221, the intermediate strain, and the least rapidly with Y3223, the glistening strain (Fig. 5). The balling also reaches a lower value (1.9-0.0) with the intermediate strain before the pellicle falls to the bottom than with the rough strain where this occurs at a balling value between 2.3-3.0.

Cultures were incubated at 25°C. and 30°C. Equivalent total cell weights are obtained at both temperatures at the end of the fermentation but the total cell weight is less in cultures at 25°C. in the earlier stages of the fermentation. With Y3222, the total cell weights reach equal values by the 7th day; with Y3223, by the 18th day; with Y3221, by the 8th day. Strain Y3221 shows the greatest differences between the total weights of 2-6 day cultures at 30 and 25°C. The differences between total cell weights at 25 and 30°C. are due to a

Figure 2

Figure 2

YIELD OF TOP CELLS OF HANSENUIA ANOMALA

Total dry weight of cells from the top 200 ml. of fermentation broth from cultures consisting of 400 ml. of Yeast Nitrogen Base with 10% glucose in 500 ml. pear-shaped separatory funnels incubated at 30°C.

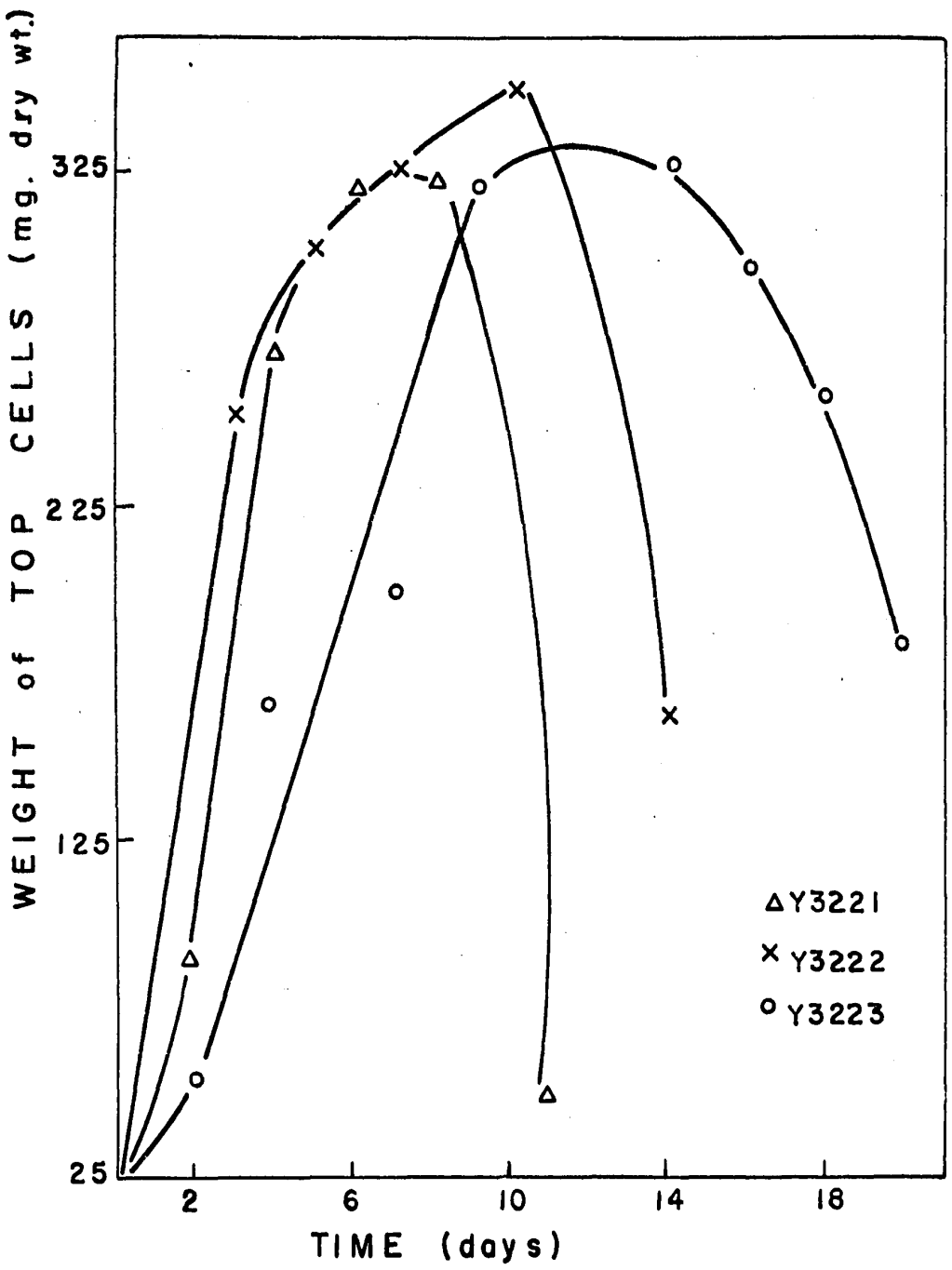

WEIGHT of TOP CELLS (mg. dry wt.)
325
225
125
25
2
6
10
14
18
TIME (days)
ΔY3221
× Y3222
o Y3223

Figure 3

Figure 3

TOTAL YIELD OF CELLS OF HANSENULA ANOMALA

Total dry weight of cells obtained from 400 ml. of Yeast Nitrogen Base with 10% glucose in 500 ml. separatory funnels at 30°C.

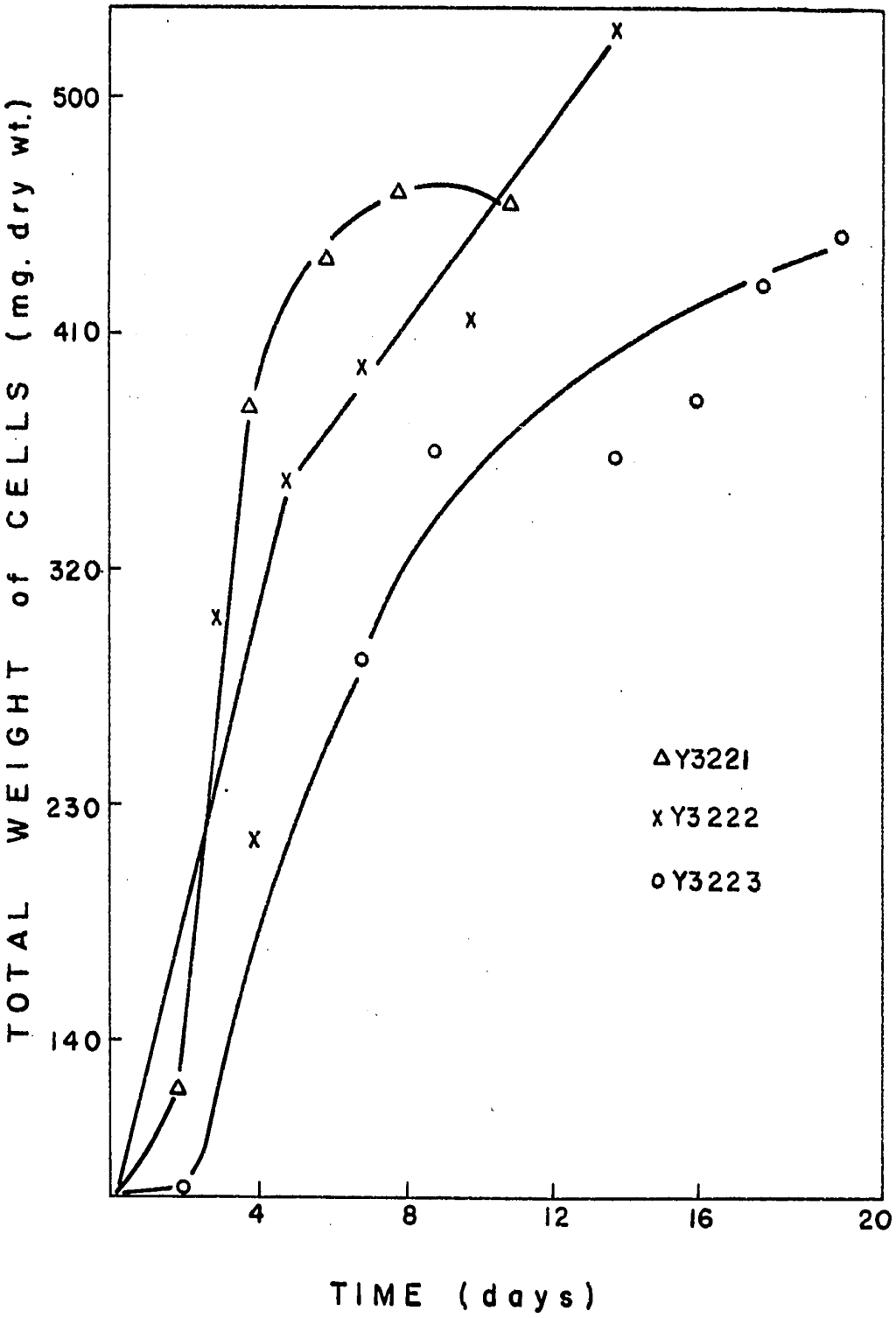

TOTAL WEIGHT of CELLS (mg. dry wt.)
500
410
320
230
140
4
8
12
16
20
TIME (days)
ΔY3221
xY3222
oY3223

Figure 4

Figure 4

CHANGES IN pH IN FERMENTATIONS OF HANSENULA ANOMALA

Fermentations were carried out in 500 ml. pear-shaped separatory funnels containing 400 ml. of Yeast Nitrogen Base with 10% glucose incubated at 30°C.

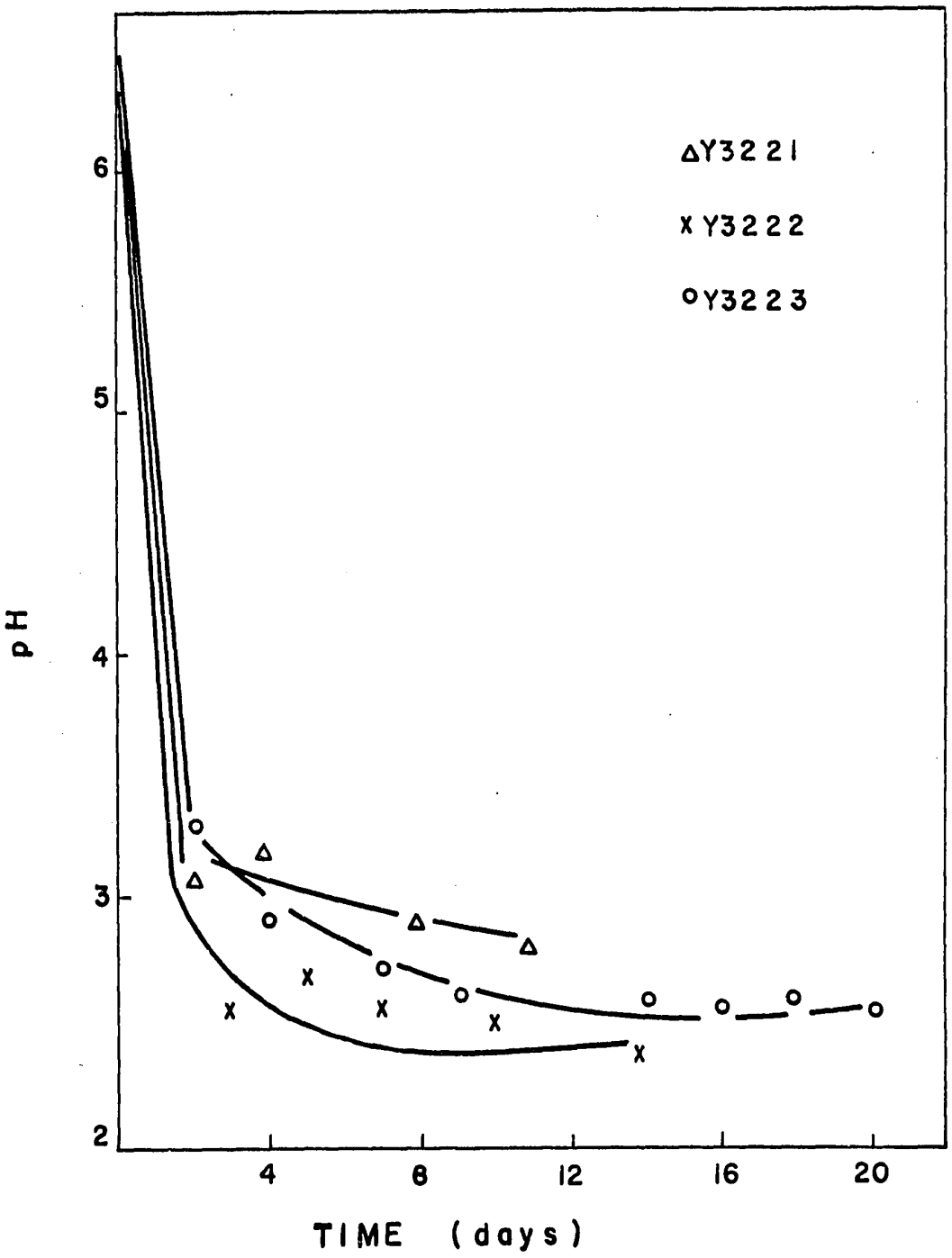
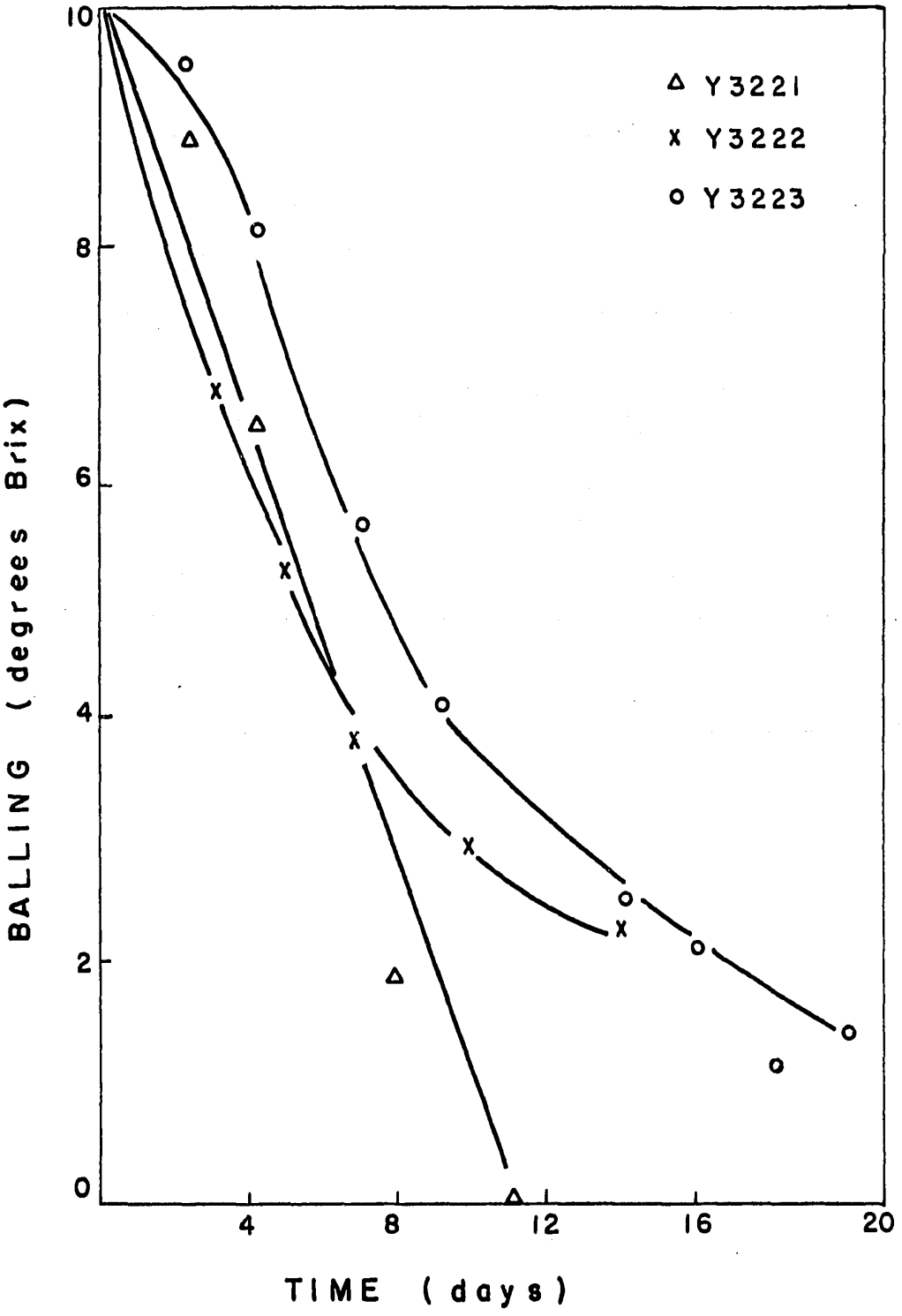

ΔY3221
X Y3222
O Y3223
pH
6
5
4
3
2
4
8
12
16
20
TIME (days)

Figure 5

Figure 5

CHANGES IN BALLING IN FERMENTATIONS OF HANSENULA ANOMALA

Fermentations were carried out in 500 ml. pear-shaped separatory funnels containing 400 ml. of Yeast Nitrogen Base with 10% glucose incubated at 30°C.


Δ Y3221
x Y3222
o Y3223
BALLING (degrees Brix)
TIME (days)
0
2
4
6
8
10
4
8
12
16
20

greater amount of top cells at the higher temperature rather than to a difference in bottom cell weights.

The only remarkable difference in pH values recorded for the cultures at 25 and 30°C. occurs with Y3221 when at 48 hours a pH of 5.0 is recorded at 25°C. and of 3.1 at 30°C. With all strains the balling falls more slowly at 25°C.

Reducing the air exchange in the funnel cultures by covering over the cotton plugs and the necks of the vessels with aluminium foil delayed the fall of the cells to the bottom of the funnels. In 4-day old cultures containing 2% glucose, 51% of the cells of the intermediate strain in the foil-covered funnels were found in the top 200 ml. of the medium as compared with 19% in the control cultures and 33% of the cells of the rough strain as compared with 25% in the controls. The total yield of cells produced in the foil-covered funnels was smaller than that in the controls (81% for the intermediate strain and 95% for the rough strain).

Viability of Top and Bottom Cells

Table 1 indicated the numbers of cells enumerated and the percentages of dead and living cells determined by the methylene blue stain and using Y3222, the rough strain. This yeast tends to form clusters (Appendix Fig. 7) which make it difficult to obtain quantitative counts of viability. These clusters are not too numerous up to the fourth day but predominate afterwards. Then, some small clusters containing 25-50 cells occur as well as many larger clusters whose diameter is 1/3-1/4 of the microscopic field using the oil-immersion lens (x1100). Some extremely large groups occupy the entire microscopic field. The results obtained with Y3222, the rough strain, indicate that at the beginning of the fermentation, bottom cells demonstrate

Table I

Viability of Top and Bottom Cells of Hansenula anomala, Y3222, incubated at 25°C.

Incubation time Days	Type of cells	Single cells dead/total	Single cells % dead	Groups of 2-10 dead/total	Groups of 2-10 % dead	Groups of more than 10 dead/total	Groups of more than 10 % dead
2	Top	68/1574	4.3	11/146	7.5	1/4	25.0
2	Bottom	101/1227	8.2	23/133	17.3	4/10	40.0
4	Top	148/791	18.7	22/131	16.8	17/54	31.5
4	Bottom	80/623	12.7	21/264	8.0	13/47	27.7

		Live cluster+ total	Live cluster+ per cent	½ dead cluster total	½ dead cluster per cent	Dead cluster total	Dead cluster per cent
6	Top	59	23.4	98	38.8	95	37.7
6	Bottom	50	38.8	41	31.8	38	29.5

+Clusters ranging in diameter from 1/4-1/3 of the microscopic field at 1100x

a greater proportion of dead cells. By the 4th day, the top cells account a larger proportion of dead cells. By the 6th day, cell clusters constituted most of the cell mass, so only the large clusters were taken into account. These clusters were not constituted entirely of cells of one category - all dead cells or all live cells. Therefore, in counting the clusters, those in which the majority of the cells were not stained were listed as live, those in which the majority of the cells were stained were listed as dead and those in which as many cells were stained as unstained were listed as $\frac{1}{2}$ dead. The results obtained in this way also indicate a greater percentage of dead cells among the top cells.

With strains Y3221 and Y3223, the problem of growth clusters was not encountered. Some attached cells in groups of 2-6 were found but no groups larger than this. Buds were counted as cells if they had attained the same size as the smallest single cells. Tables II and III indicate the viability of cells found among the bottom and the top cells of these two strains. The intermediate strain, Y3221, manifests an increasing percentage of dead cells among the top cells from the 4th day on. The glistening strain, Y3223, also shows a larger proportion of dead cells among the top cells after the third day but the difference is not as impressive. It must be noted, however, that these cultures of Y3223 possessed only slight islets in lieu of a pellicle so that the top cells examined would be in greater part cells suspended in the top 200 ml. of medium rather than pellicle cells in direct contact with the air.

Respiratory Activity of Top and Bottom Cells

Table IV indicates the oxygen uptake of cells of Y3221, Y3222

Table II

Viability of Top and Bottom Cells of Hansenula anomala, Y3221, at 25°C.

Incubation time Days	Type of cells	Dead cells/Total cells	Percentage of dead cells
2	Top	580/1631	35.6
2	Bottom	418/1344	31.6
3	Top	454/2055	22.1
3	Bottom	479/1937	24.7
4	Top	740/3496	21.2
4	Bottom	386/3020	12.4
6	Top	1318/3688	35.7
6	Bottom	534/3548	15.1

Table III

Viability of Top and Bottom Cells of Hansenula anomala, Y3223, at 25°C.

Incubation time Days	Type of cells	Dead cells/Total cells	Percentage of dead cells
3	Top	389/2734	14.2
3	Bottom	338/3099	10.9
6	Top	955/3063	31.2
6	Bottom	245/971	25.2

Table IV

Oxygen Uptake by Hansenula anomala, strains Y3221, Y3222 and Y3223+

Incubation time Days	Yeast strain	Top Cells		Bottom Cells	
		Endogenous	Substrate^	Endogenous	Substrate^
2	3221	13.9	54.1	10.7	47.6
3	3221	11.0	35.3	11.5	41.7
4	3221	15.7	38.4	18.6	40.8
6	3221	8.4	23.4	10.4	37.1
1	3222	15.6	43.6	14.0	43.0
2	3222	14.7	53.7	13.3	43.3
4	3222	13.1	37.1	15.9	33.2
6	3222	7.7	25.1	7.3	34.6
3	3223	13.1	28.2	11.1	30.0
4	3223	16.1	42.7	21.8	30.5
6	3223	12.4	41.4	16.3	34.1
7	3223	8.8	30.3	9.5	37.9

+Oxygen uptake is expressed as µl. of oxygen/mg. of cells dry weight/hr. at 30 C. in HCl-KCl buffer at pH 2.5. The cells were collected from pear-shaped separatory funnels containing Yeast Nitrogen Base medium with 2% glucose.

^Uptake with substrate is expressed as net uptake, i.e., as total uptake minus endogenous uptake.

and Y3223. Experiments carried out first at pH 6.0 using McIlwaine's buffer (Colowick and Kaplan, 1955) gave approximately the same results. In the experiments reported here, HCl-KCl buffer at pH 2.5 was used to simulate the conditions under which the cells normally respire. The endogenous uptake of Y3222, the rough strain, is slightly higher with the top cells on the first and second day and the top cells show greater activity on the substrate than the bottom cells on the second day. The endogenous uptake by bottom cells exceeds that of top cells on the fourth and on the sixth day both types of cells manifest an endogenous uptake that is lower than that found on previous days. On the sixth day, the bottom cells display a higher oxygen uptake than the top cells in the presence of the substrate.

With Y3221, the intermediate strain, the two-day old top cells also possess a higher endogenous respiration and a higher oxygen uptake with glucose as substrate than the bottom cells. The top cells of Y3221 seem to lose their enhanced respiratory powers sooner than those of Y3222. The bottom cells of Y3221 possess higher endogenous and higher substrate uptake than the top cells from the third day on.

The glistening strain, Y3223, shows higher endogenous uptake of the bottom cells from the fourth day on. The top cells, however, seem to retain an enhanced respiration in the presence of glucose up to the sixth day inclusively which represents a longer interval than that found with Y3221 and Y3222.

Using the data of Tables I, II and III and calculating oxygen uptake in relation with the percentage of live cells gives figures which are not qualitatively different from those of Table IV. These results seem to indicate that top cells initially oxidize glucose at

a more rapid rate than bottom cells but, as growth progresses, the exogenous uptake of the top cells slows down at a faster rate than that of bottom cells so that the bottom cells eventually oxidize glucose more quickly than the top cells.

These results can also be correlated with the speed of fermentation. The most rapid fermenter, Y3221, is the first to manifest higher oxygen uptake by the bottom cells. This occurs in the three-day old cultures. With Y3222, however, the bottom cells surpass the top cells only on the sixth day, and with Y3223, the slowest fermenter, on the seventh day.

Inhibitors of Pellicle Formation

Standing cultures of the three strains of Hansenula anomala produced no pellicles in cultures containing from 0.05-0.2% Tween 80. Considerably fewer cells were produced in still cultures containing Tween 80. Table V gives the total mg. of cells, dry weight, produced in cultures of the rough strain containing 0.2% Tween and the control cultures without Tween.

Table V

Total Yield of Cells of Hansenula anomala, Y3222, in the Presence of Tween 80

Days	Cultures with 0.2% Tween+	Control Cultures+
1	15.3	20.8
2	21.4	34.4
4	19.6	40.5

+Mg. dry wt. of cells in 20 ml. of Yeast Nitrogen Base medium containing 2% glucose and incubated at 25°C.

The addition of 0.5% Difco Yeast Extract to the fermentation medium did not overcome the inhibition on cell growth exercised by Tween 80 nor did it permit pellicle formation. Table VI indicates the results obtained with these cultures.

Table VI

Total Yield of Cells of Hansenula anomala, Y3222, in the Presence of Tween 80 and Yeast Extract

Days	Cultures with 0.2% Tween & 0.5% Y.E.+	Control Cultures & 0.5% Y.E.+
1	21.8	23.0
2	28.8	84.4
4	27.5	91.3

+Mg. dry wt. of cells in 20 ml. of Yeast Nitrogen Base medium containing 2% glucose and incubated at 25°C.

The addition of ergosterol to the fermentation medium with Tween 80 did not result in pellicle formation either. Andreasen & Stier (1953) found that both ergosterol and oleic acid (in the form of Tween 80) are required for the anaerobic growth of Saccharomyces cerevisiae in defined medium. However, the addition of ergosterol to the cultures of Hansenula anomala containing Tween 80 did not remove the inhibitory effects brought about by the surfacant.

The addition of 10^{-2}M cysteine resulted in complete inhibition of pellicle formation with Y3222, the rough strain. This concentration of cysteine resulted in a smaller biomass of yeast being

produced: after 53 hours, 20.3 mg. compared to 35.0 mg. of cells dry weight in 20 ml. of medium. With 10^{-3}M cysteine, a very slight film is formed and at 10^{-4}M, the pellicle appears within 24 hours and is normal. Similar results were obtained with Y3224 in spite of the differences in cell morphology between the two yeast strains.

The effect of these pellicle inhibitors on the formation of ester by the yeast will be presented in the following section.

ESTER PRODUCTION

Ester Production in Flask Cultures

Table VII gives the amounts of ester produced using 50 ml. Erlenmeyer flasks containing 20 ml. of medium and closed with foam stoppers in still culture.

Cultures incubated on the shaker produced more ester than still cultures. This ester was formed more rapidly and also disappeared sooner than the ester produced in still cultures. In Table VIII the amounts of ester produced in shaken cultures of Y3222 and Y3223, under the same conditions as the still cultures discussed above, are listed.

A large difference occurred in the total amounts of cells produced on the shaker and in still cultures. Table IX notes the total mgs. of cells obtained at different intervals in still and shaken cultures of Y3222 and Y3223.

Sugar also disappeared faster from the shaken cultures than from the still cultures. Disappearance of sugar in shaken cultures of Y3222 was noted within 22 hours whereas a small amount of sugar still remained after 48 hours in still cultures.

Table VII

Ester Produced in Still Culture+

Incubation time Hours	Y3221	Y3222	Y3223
16	0	1.7	0
20	1.4	3.7	0
24	2.5	6.5	0
30	4.5	7.4	0
42	6.0	12.7	0
48	6.1	15.0	0
52	-	13.6	0
66	5.0	-	0
72	5.3	13.4	0
90	5.2	7.3	1.4
96	-	7.5	2.9
120	9.1	1.8	-
142	7.8	1.3	6.0
166	4.0	0	9.8
186	0	0	5.4
213	0	0	7.1
237	0	0	2.2

+Ester is determined as µM/ml. Values of ester listed as "0" represent amounts of ester ranging from 0.0-1.0 µM/ml. Flasks were incubated at 25°C.

Table VIII

Ester Produced in Shaken Cultures+

Incubation time Hours	Y3222	Y3223
12	0	-
17	1.6	-
21	17.6	15.2
23	16.5	-
24	20.0	-
26	21.7	-
28	21.0	16.4
30	27.9	-
34	24.8	19.3
43	9.8	-
45	8.1	9.2
47	11.2	-
54	3.6	6.7

+Ester is determined as μM/ml. Values of ester listed as "0" represent amounts of ester ranging from 0.0-1.0 μM/ml. Flasks were incubated at 25°C.

Table IX

Total Amounts of Cells Obtained in Still and Shaken Cultures+

Incubation time Hours	Shaken Cultures Y3222	Shaken Cultures Y3223	Incubation time Hours	Still Cultures Y3222	Still Cultures Y3223
32	75.4	81.1	50	35.0	28.0
47	93.6	-	72	-	33.0
			165	36.5	35.0

+Mgs. dry weight of cells obtained from 20 ml. of medium in 50 ml. Erlenmeyer flasks incubated at 25°C.

Controls were used with the fermentations described above to determine the amount of ester evaporated off in a given interval of time from 20 ml. of solution containing 30-35 µM of ester/ml. in 50 ml. Erlenmeyer flasks, plugged in the same fashion as the flasks used in the actual fermentations and incubated under the same conditions. Flasks with plugs covered with aluminium foil were also included. Table X indicates the percentage of ester evaporated from the control flasks after 21 and 48 hours.

Table X

Per Cent Evaporation of Ester at 25°C.

Incubation time Hours	Shaker	Still	Foil-covered
21	64.5	61.6	-
48	91.1	81.9	2.9
72	-	-	6.4

As Sova (1960) had already remarked, foil-covered cultures gave remarkably more ester than those not so protected. Table XI lists the amounts of ester obtained in foil-covered cultures and Figures 6 and 7 compare the amounts of ester produced in the foil-covered cultures with the corresponding amounts found in the control flasks.

The effect of increased aeration obtained by increasing the surface-volume ratio was studied with Y3222, the rough strain. The results are listed in Table XII.

Figure 6

Figure 6

ESTER PRODUCED IN FOIL-COVERED AND CONTROL FERMENTATIONS OF HANSENULA ANOMALA

Twenty milliliters of Yeast Nitrogen Base containing 2% glucose in 50 ml. Erlenmeyer flasks at 25°C. were used. Control flasks were stoppered with foam plugs and 'foil-covered' flasks had in addition aluminium foil over the stoppers and around the necks of the flasks. The rough strain, Y3222, was used.

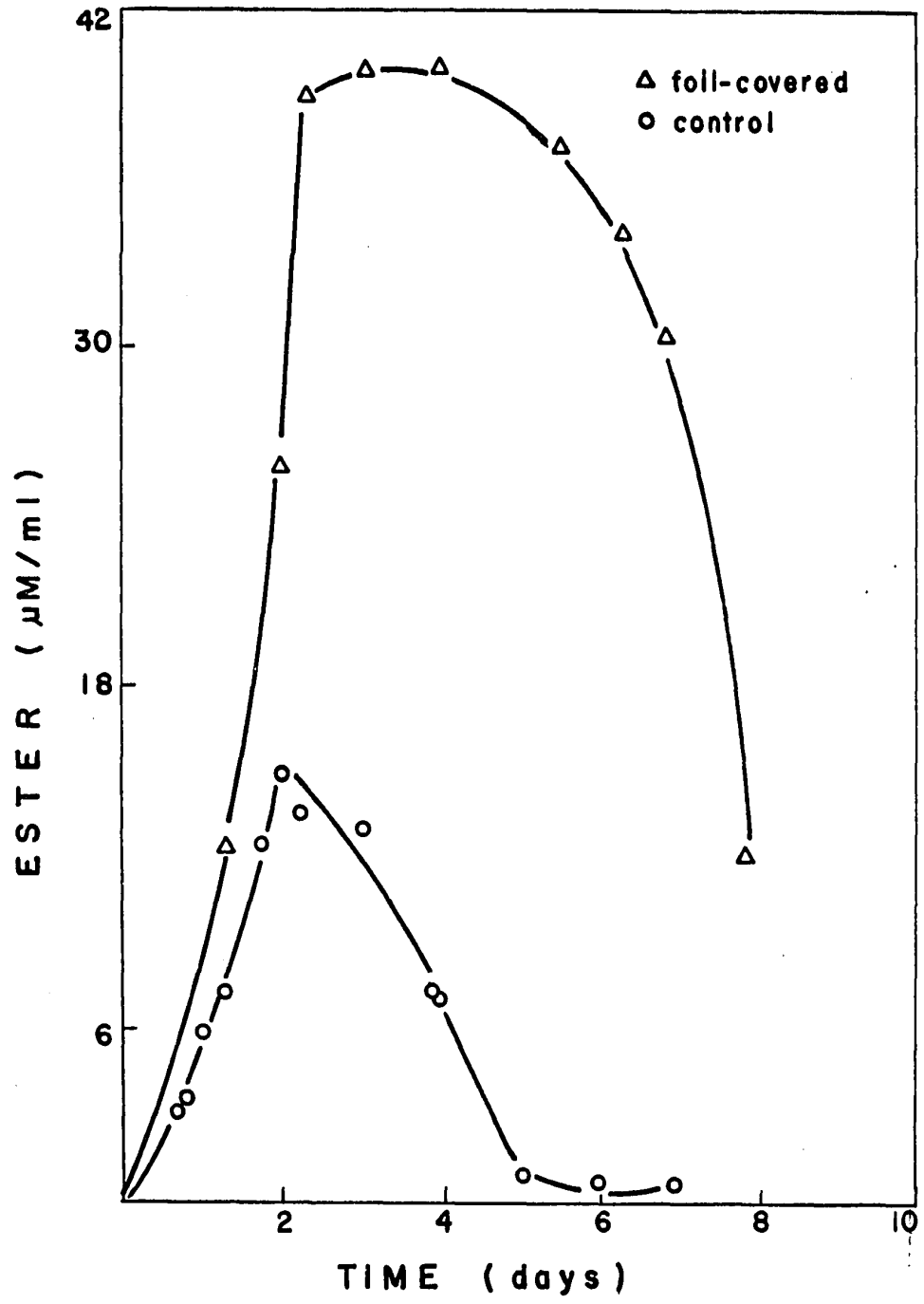

42
30
18
6
ESTER (μM/ml)
foil-covered
control
2
4
6
8
10
TIME (days)

Figure 7

Figure 7

ESTER PRODUCED IN FOIL-COVERED AND CONTROL FERMENTATIONS OF HANSENULA ANOMALA

Twenty milliliters of Yeast Nitrogen Base containing 2% glucose in 50 ml. Erlenmeyer flasks at 25°C. were used. Control flasks were stoppered with foam plugs and 'foil-covered' flasks had in addition aluminium foil over the stoppers and around the necks of the flasks. The glistening strain, Y3223, was used.

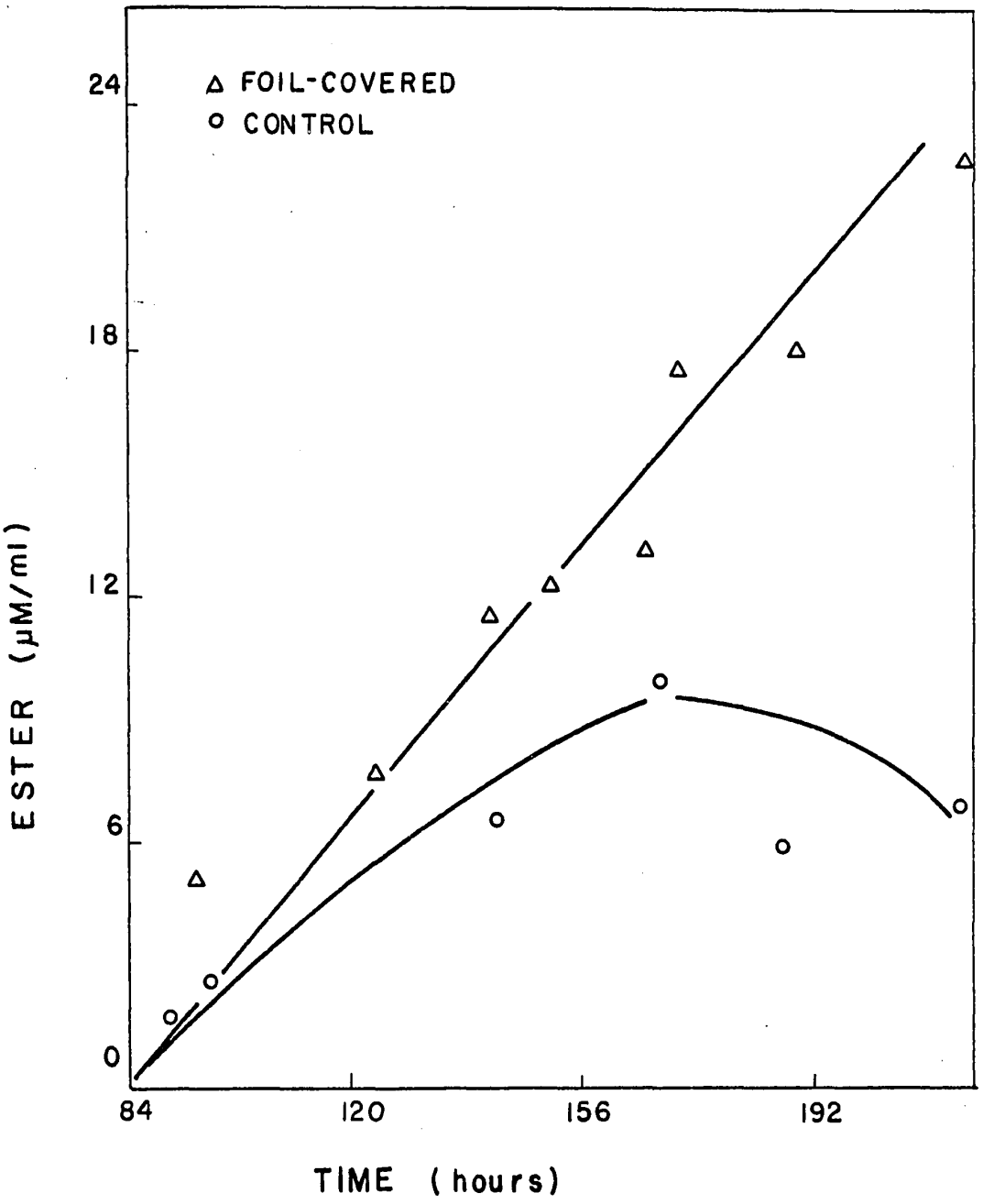

Δ FOIL-COVERED
o CONTROL
24
18
12
6
0
ESTER (μM/ml)
84
120
156
192
TIME (hours)

Table XI

Ester Produced in Foil-covered, Still Cultures+

Incubation time Hours	Y3222	Y3223
30	12.6	0
47	25.8	0
54	38.8	0
72	39.8	0
94	39.8	4.8
122	36.7	7.6
142	-	11.4
150	34.2	11.9
164	30.6	13.4
171	-	17.7
189	10.7	17.3
213	-	22.8

+Ester is expressed as μM/ml. Flasks were incubated at 25°C.

As can be noted from the above figures, the maximum ester production occurs progressively later in the course of the fermentation as the surface-volume decreases. Similar results were obtained with Y3221 and are listed in Table XIII.

Paper chromatographic examination for glucose remaining indicated that glucose had disappeared after 31 hours' incubation in the 500 ml. flask (see Fig. 8), after 44 hours in the 250 and 125 ml. flasks and after 68 and 124 hours in the 50 and 25 ml. flasks respectively

Table XII

Ester Obtained by Varying Surface-volume Ratio with Y3222, the Rough Strain+

Incubation time Hours	250 ml. flask	125 ml. flask	50 ml. flask	25 ml. flask
22	4.9	5.5	2.5	1.0
29	11.5	11.9	5.8	1.6
46	9.5	13.8	14.6	3.4
51	10.7	15.4	14.9	3.3
70	1.0	3.2	16.0	5.2
93	0	0	8.9	7.5
117	0	0	2.7	9.3

+Ester is expressed as μM/ml. Flasks were incubated at 25°C.

when Y3221, the intermediate strain was employed.

The amount of cells produced increases as the surface-volume ratio increases. Figure 8 depicts the decrease in percentage transmission with time in the different size flasks.

The amount of evaporation of the ester formed also increases as the surface-volume ratio increases. Figure 9 illustrates this fact.

Ester Production in 'Train' Cultures

The significant loss of ester incurred during the course of fermentations necessitated the investigation of ways of minimizing loss of volatile products while maintaining high oxygen levels. The 'train' fermentation using alkaline hydroxylamine to catch the volatile ester gave very good recovery with control solutions of

Figure 8

Figure 8

CHANGES IN CELL DENSITY WITH INCREASING AERATION

The intermediate strain, Y3221, was used in 20 ml. of Yeast Nitrogen Base, containing 2% glucose, in Erlenmeyer flasks of different sizes. The flasks were stoppered with cotton and incubated at 25°C.

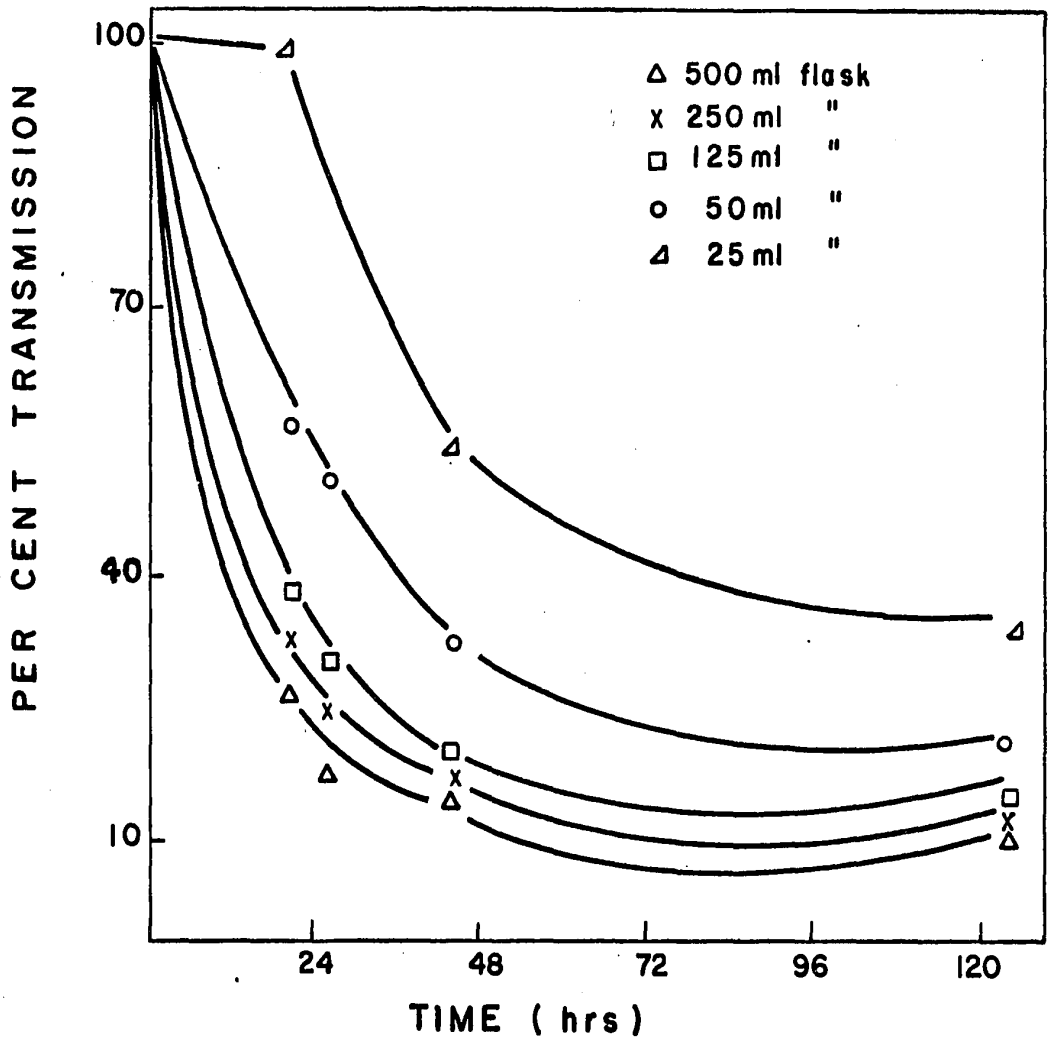

100
70
40
10
PER CENT TRANSMISSION
24
48
72
96
120
TIME (hrs)
Δ 500 ml flask
x 250 ml "
□ 125 ml "
o 50 ml "
Δ 25 ml "

Figure 9

Figure 9

EVAPORATION OF ESTER AS AFFECTED BY THE SURFACE-VOLUME RATIO

Each Erlenmeyer flask contained 20 ml. of ester solution having 34.4 μM of ester/ml. The flasks were plugged with cotton and incubated at 25°C.

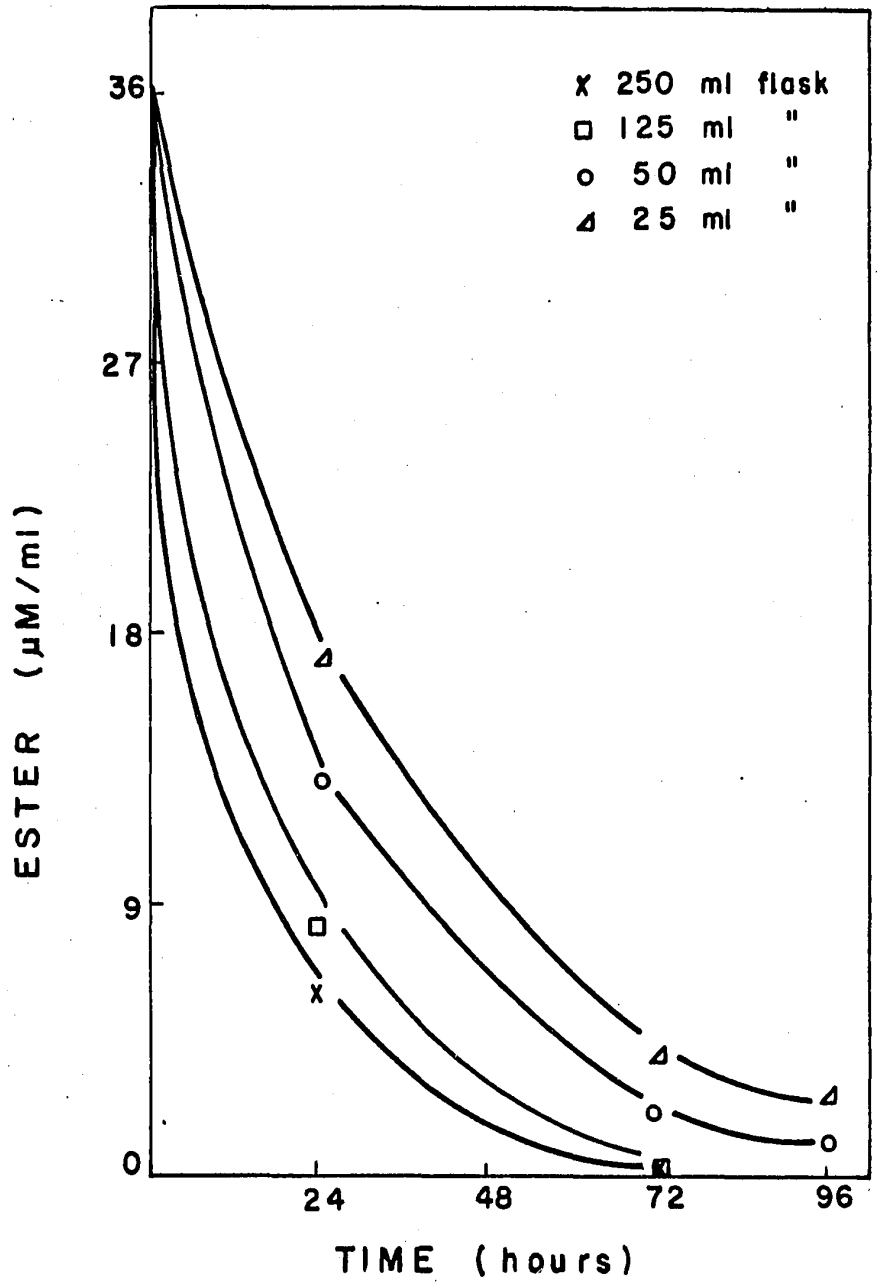

X 250 ml flask
□ 125 ml "
o 50 ml "
Δ 25 ml "
36
27
18
9
0
ESTER (μM/ml)
24
48
72
96
TIME (hours)

Table XIII

Ester Obtained by Varying Surface-volume Ratio with Y3221, the Intermediate Strain+

Incubation time Hours	250 ml. flask	125 ml. flask	50 ml. flask	25 ml. flask
20	2.3	2.7	1.4	0
25	4.2	4.1	1.9	0
31	5.6	4.1	2.0	0
44	7.8	6.0	4.4	0
51	7.9	5.6	4.1	0
68	6.4	5.7	2.8	1.6
124	0	6.8	9.1	1.4
140	0	0	7.8	2.1
164	0	0	4.0	2.8
196	0	0	0	3.1
239	0	0	0	3.4

+Ester is expressed as μM/ml. Flasks were incubated at 25°C.

ester. The percentage of ester recovered with 50 ml. ester solution in 250 ml. flasks using hydroxylamine in the 'train' fermentation and with the same volume of solution in 250 ml. foil-covered flasks is noted in Table XIV.

Table XV compares the amounts of ester produced in the 'train' fermentations and the still foil-covered flask fermentations using 50 ml. of medium in 250 ml. Erlenmeyer flasks and yeast Y3221, the intermediate strain, and Y3222, the rough strain. Each flask received about 60 ml. of air/minute.

Table XIV

Percentage of Ester Recovered in Control Flasks at 30°C.

Incubation time Hours	'Train' Fermentation (NH_2OH)		Foil-covered Flasks
	Still	Shaken	Still
24	105.5	100.4	87.5
48	99.6	94.6	-
72	98.3	92.3	85.3

Foil-covered cultures not only received less oxygen than the cultures stoppered only with cotton plugs but also accumulated more carbon dioxide within the flasks. This might account in part for the high ester production in the foil-covered flasks and to verify this, beakers containing alkaline hydroxylamine were placed in foil-covered flasks as described above. The amounts of ester produced in these and the control cultures of Y3221, the intermediate strain, are shown in Figure 10. As can be noted, less ester was produced in the cultures with the hydroxylamine wells. Similar results were obtained with Y3222, the rough strain. The numbers of cells produced in flasks with hydroxylamine wells and in the control flasks as determined by turbidity readings are approximately the same.

Determination of esterase activity at a low pH value (2.3) similar to that found in the actual fermentations revealed that ester was hydrolyzed to a much greater extent in the shaken 'train' fermentation than in the still 'train' fermentations or in the foil-covered flasks. The foil-covered flasks exhibited less hydrolysis than the still 'train' fermentations. The rough strain, Y3222,

Figure 10

Figure 10

THE EFFECT OF ABSORPTION OF CO_2 ON ESTER PRODUCTION

The intermediate strain, Y3221, was grown in 250 ml. Erlenmeyer flasks containing 50 ml. of Yeast Nitrogen Base with 2% glucose and having a well containing 10 ml. of alkaline hydroxylamine (1.0N $NH_2OH.HCl$ and 1.25N NaOH). Control flasks had water in the wells. All flasks were covered with foil.

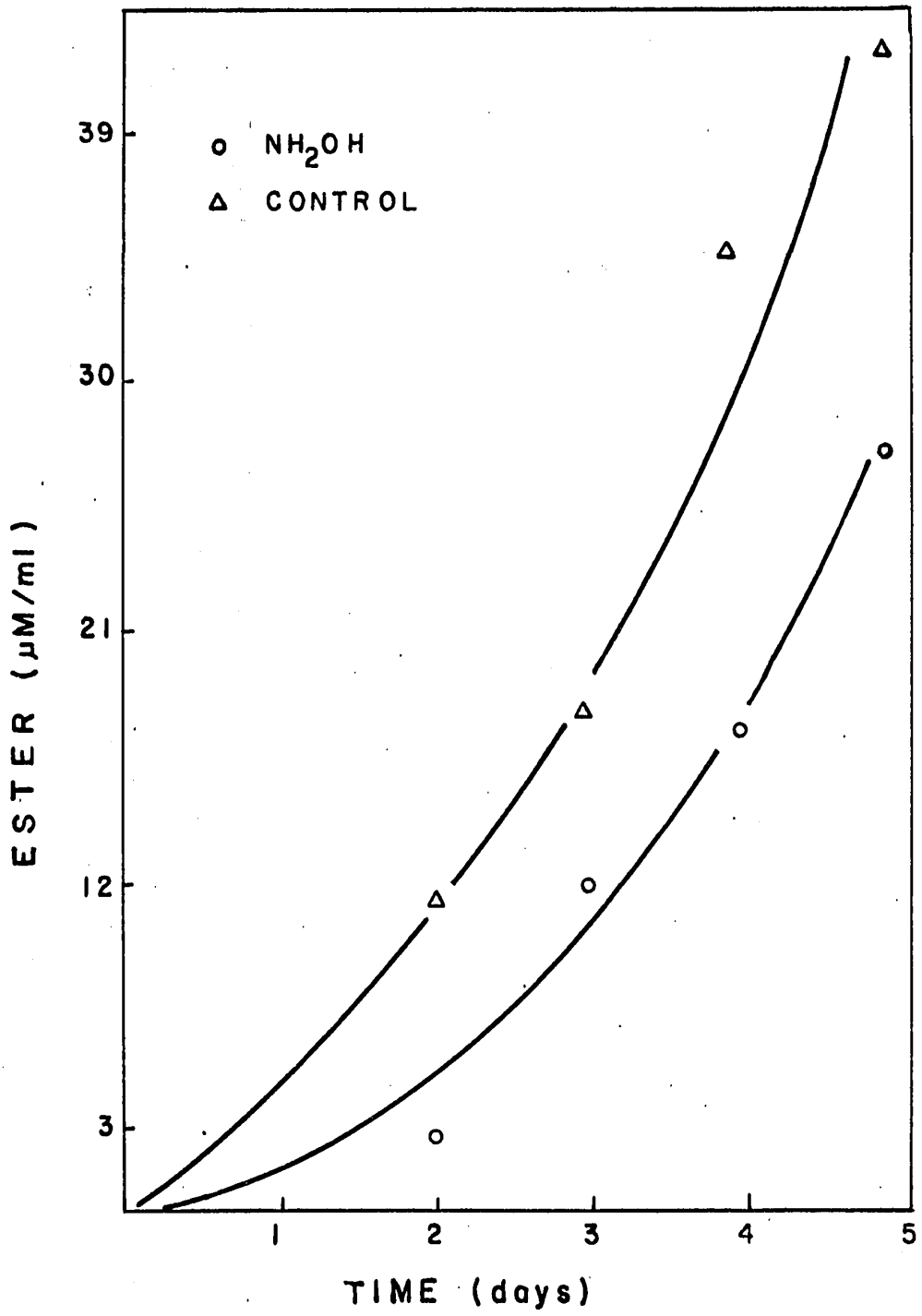

o NH2OH
Δ CONTROL
39
30
21
12
3
ESTER (μM/ml)
1
2
3
4
5
TIME (days)

Table XV

Ester Production in 'Train' Fermentations and in Foil-covered Flasks+

	Incubation time Hours	'Train' Fermentation		Foil-covered Flasks
		Still	Shaken	Still
Y3221 Intermediate Strain	19	-	12.3	-
	24	-	31.0	5.1
	31	-	38.7	-
	43	-	43.1	-
	48	20.6	-	-
	72	25.2	36.2	17.8
	96	35.6	-	30.8
	120	41.6	-	33.5
	144	36.2	-	42.7
	168	-	-	40.3
Y3222 Rough Strain	20	-	13.5	-
	22	-	17.9	-
	25	-	21.3	-
	30	-	31.6	-
	35	-	33.6	-
	45	23.2	32.7	-
	51	26.5	30.9	22.2
	72	54.1	-	48.5
	96	49.5	-	45.8
	120	48.9	-	32.3

+Fifty milliliters of Yeast Nitrogen Base with 2% glucose in 250 ml. Erlenmeyer flasks at 30°C. were used. Ester is expressed as μM/ml.

exhibited much greater esterase activity than Y3221, the intermediate strain. However, actual fermentation conditions could not be completely replicated in the method used to measure esterase activity since the cells resuspended in the buffered ester solution in the still cultures settled to the bottom of the flasks whereas in the actual fermentations, the pellicle was present and could have been more actively engaged in ester hydrolysis than the bottom cells. However, as the esterase is known to be active under anaerobic conditions in cell-free extracts (Smith & Martin, 1963), the enhanced esterase activity in shaken flasks could be due simply to better mixing of the medium and cells.

Determination of carbon dioxide production in still and shaken 'train' fermentations indicated that the still cultures produced 1.5 mM of CO_2 per mM of glucose utilized whereas the shaken cultures gave 2.5 mM of CO_2 per mM of glucose utilized.

Ester Production in the Presence of Pellicle Inhibitors

No ester nor pellicle were produced by any of the yeast strains in still cultures containing 0.5 to 2.0 ml. of Tween 80 per liter. The addition of yeast extract (0.5%) or ergosterol to the medium did not reverse this effect. Incubation of cultures containing Tween on the shaker resulted in the production of ester and cells in amounts proportional to that produced in the control cultures.

Cysteine in a 10^{-2}M concentration completely inhibits pellicle formation and also ester production. The effect of this concentration of cysteine is not completely reversed when the cultures are incubated on the shaker. Only one third of the number of cells

obtained in the control cultures appear in the cysteine-containing cultures. The disappearance of glucose from the medium is also greatly delayed by the presence of cysteine. Ester is produced in cysteine-containing cultures incubated on the shaker, but amounts to only one third of that found in the control flasks.

Concentrations of 10^{-4} and 10^{-5}M cysteine permit complete pellicle formation and the production of ester in amounts equivalent to those found in the controls. The presence of 10^{-3} cysteine results in lighter film production and the reduction of ester by half. Shake cultures containing $10^{-3.5}$M cysteine actually produce more ester than the controls (20.8 vs. 29.4 μM/ml.).

Whereas control cultures of Y3222, the rough strain, produced a pellicle and ester within 24 hours, those containing 0.5% yeast extract produced no pellicle and no ester before 48 hours even though more cells and more rapid glucose utilization were noted in the cultures supplemented with yeast extract.

V. DISCUSSION

From the study of the pellicle in relation to colony form, the following two observations were made. First, the S → R change occurring at higher temperatures and responsible for scum formation mentioned by Fabian and McCullough (1934) was never encountered in the present study. Pellicle cells formed at 30°C. and as old as six weeks were streaked repeatedly on agar plates and consistently produced colonies characteristic of the strain employed. No dissociation was ever observed so that this cannot be the sole factor governing pellicle formation. Secondly, visual observation of pellicles produced by mat and glistening strains seemed to indicate that the mat forms produced heavier pellicles than the corresponding glistening forms (Fabian & Wickerham, 1937) but the quantitative data obtained in the present study indicate that with the strains used approximately equal amounts of top cells were produced by each. The total yield of cells obtained with the rough strain, however, is slightly larger.

The enhanced respiratory activity of the top cells at the beginning of the fermentation seems to point out their active role in the metabolism of the yeast as suggested by Wickerham (1951) and Tabachnick (1953). It is interesting to note that the pellicle cells do not appear to possess this enhanced respiratory activity at the very onset of the fermentation as indicated by the data obtained on the first day with Y3222 and on the third day with Y3223. At this time there is as yet little or no ester formed.

The role of the pellicle in ester formation is also suggested by the results obtained with the inhibitors of pellicle formation.

Cultures containing Tween 80 produce no pellicle, fewer cells and no ester. Since these three effects are completely reversed by incubating the cultures on the shaker, they must be due to the partial anaerobic conditions existing in still cultures possessing no pellicle. The pellicle would then be the agent responsible for maintaining the aerobic conditions needed in still cultures for ester production.

The results obtained using cysteine as pellicle inhibitor are less straight-forward since the inhibition brought about by a cysteine concentration large enough to inhibit pellicle formation (10^{-2}M) is not completely reversed when the cultures are incubated on the shaker. There is, however, here also, a correlation between the amount of pellicle produced and the ester formed: no pellicle results in no ester formation; slight pellicle, in reduced ester production; complete pellicle, in normal ester production. There is also a partial or complete reversal of the inhibition of ester production with increased aeration by incubation on the shaker. The role of cysteine on the morphological interconversions between the yeast-state and the filamentous state (Y→M) was studied by Nickerson & Van Rij (1949), Nickerson & Mankowski (1953) and Nickerson (1963) using mainly Candida albicans. The authors obtained with a strain of H. anomala inhibition of the Y M phenomenon with 10^{-3}M cysteine. Similar changes were noted in the present work when we used 10^{-2}M cysteine with strain Y3224.

Besides its action on cell morphology, cysteine exerts a number of other effects. It is a strong reducing agent and also a chelating agent. It may remove from the medium Zn^{++} which is known

to be indispensible to H. anomala for ester production (Miwa, 1961).

Organic nutrients are also known to act as chelating agents (Jones, 1964) through the action of amino acids and other metal-binding metabolites. This may account for the delay in pellicle formation and ester production in cultures containing yeast extract. The yeast extract may also possess some surface-active material which would account for the same phenomena.

Thus, the results obtained with inhibitors of pellicle formation seem to indicate the role played by the pellicle in enhancing the aeration of still cultures thus permitting the formation of ester in these cultures.

The maximum value of ester produced in shaken 50 ml. flasks is 1.9 and 2.0 times as great as that formed in the still flasks for Y3222 and Y3223 respectively. Even though the amount of evaporation occurring in the shaken flasks is much greater than that found in the still flasks, the ester content of the shaken cultures drops much faster than that of still cultures. This might indicate that the esterase activity in the shaken flasks exceeds that of the still cultures.

The foil-covered 50 ml. flask cultures give a maximum ester value which is 1.4 times that obtained in the shaken cultures of Y3222. However, the amount of evaporation of ester occurring in the foil-covered cultures is much less than that found in the shaken flasks and the long period of time during which the ester present retains its high value seems to indicate only weak or no esterase activity. The results obtained using alkaline hydroxylamine to diminish the carbon dioxide tension in these cultures may indicate

that carbon dioxide plays a role in ester accumulation.

The results obtained with increasing aeration by varying the surface-volume ratio force us to reach conclusions which are quite different from those expressed by Gray (1949) and outlined in the Literature Review. The peak of ester production occurs progressively earlier as the surface-volume ratio increases. The maximum ester values attained in the various flasks, however, does not vary very much with the different degrees of aeration. The increased amount of evaporation occurring as the aeration augments accounts for some differences.

Controls used to determine the amount of ester evaporation occurring under various conditions indicated that loss of ester was considerable under many of the conditions employed by previous workers. This would account for the very low values for ester obtained with increased aeration by Fabian & Wickerham (1937) and Gray (1949). Better control of evaporation afforded by the foil, covering the stoppers of the fermentation flasks and, perhaps, the accumulation of CO_2 within the flasks account for Sova's (1960) enhanced ester production in these foil-covered flasks.

The shaken 'train' fermentations with Y3221, the intermediate strain, give the same amount of ester as the still 'train' fermentations but with Y3222, the rough strain, give 0.6 times less ester than the still train fermentations. It is interesting to note that the rough strain, Y3222, gives ester values for 'train' cultures incubated on the shaker whereas Y3221 , the intermediate strain, gives high values, a fact which can be correlated to the much stronger esterase activity present in the rough strain than found in

the intermediate strain in shaken flasks. The foil-covered cultures do not give a maximum ester value exceeding that obtained in the 'train' fermentations.

Previous workers (Wickerham & Fabian, 1937; Gray, 1949; Tabachnick & Joslyn, 1953a; Sova, 1960) concluded that maximum ester production occurs under reduced or moderate aeration. However, the results obtained in this study of the effect of aeration on ester production indicate that as much, if not more, ester is produced with increasing aeration but that, once ester evaporation is controlled, effects such as increased esterase activity with greater aeration and increased carbon dioxide tension with restricted aeration must be taken into account.

VI. SUMMARY

Three strains of Hansenula anomala, with different morphological and colony characteristics and designated as glistening, intermediate and rough strains, were studied with respect to pellicle formation and ester production. The following conclusions were evident.

1. The amount of top cells produced by each strain was approximately the same regardless of colony characteristics.

2. Top cells showed a greater respiratory activity than the bottom cells at the beginning of fermentation and a smaller respiratory activity at the end of the fermentation.

3. The use of inhibitors of pellicle formation showed that still cultures possessing no pellicle formed no ester but that ester was produced in shaken cultures in the presence of the inhibitor indicating the role played by the pellicle in enhancing the aeration of still cultures thus permitting the formation of ester in these fermentations.

4. With increased aeration obtained by varying the surface-volume ratio, the peak of ester production occurred progressively earlier and the maximum ester values attained in the various flasks did not vary much with the different degrees of aeration.

5. The amount of ester lost through evaporation in both still and shaken cultures was found to be considerable. 'Train' fermentations were designed to provide a controlled air supply and to give a complete recovery of ester.

6. 'Train' fermentations with the intermediate yeast strain incubated on the shaker gave as high ester production as still 'train' fermentations and still 'foil-covered' flasks. The shaken 'train'

fermentations with the rough strain produced less ester than the still fermentations; however, the strong esterase activity noted in the shaken flasks could account for the lower ester production in the shaken flasks.

7. Results obtained in this study of the effect of aeration on ester production indicate that as much, if not more, ester is produced with increasing aeration. Once ester evaporation is controlled, effects such as increased esterase activity with greater aeration and increased carbon dioxide tension with restricted aeration must be taken into account.

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VIII. APPENDIX

Appendix Table I

Quantitative pellicle determination and fermentation data for Hansenula anomala, Y3221[+]

Incubation time Days		pH	Balling	Yield of Cells, mg. dry weight		
				Top	Bottom	Total
A[++]	2	3.10	9.0	89.3	34.1	123.4
	4	3.20	6.5	272.3	110.9	383.2
	6	-	-	321.3	119.0	440.2
	8	2.90	1.9	324.1	140.3	464.4
	11	2.80	0.0	51.6	407.5	459.1
B[+++]	2	5.00	9.8	54.0	30.0	84.0
	4	3.20	9.0	102.8	68.9	171.7
	6	2.85	7.0	171.5	115.3	286.8
	8	2.75	4.3	254.4	166.3	420.7
	13	2.68	0.5	102.0	373.4	475.4

[+]Fermentations were carried out in 500 ml. pear-shaped separatory funnels containing 400 ml. of Yeast Nitrogen Base medium with 10% glucose. The yield of top and bottom cells represents the total dry weight of the cells found in the top and bottom 200 ml. of medium respectively.

[++]Incubated at 30°C.

[+++]Incubated at 25°C.

Appendix Table II

Quantitative pellicle determination and fermentation data for *Hansenula anomala*, Y3222+

	Incubation time Days	pH	Balling	Yield of Cells, mg. dry weight		
				Top	Bottom	Total
A+	3	2.55	6.8	254.6	47.3	301.9
	5	2.68	5.3	303.9	49.9	353.8
	7	2.57	3.9	326.4	73.4	399.8
	10	2.49	3.0	353.2	64.2	417.4
	14	2.37	2.3	164.6	365.1	529.7
B+	3	2.80	8.7	172.0	40.2	212.2
	5	2.66	7.5	263.1	46.5	309.6
	7	2.52	6.1	288.8	104.1	392.9
	9	2.56	2.5	314.5	143.0	457.5
	11	2.48	1.5	320.9	148.4	469.3
	15	2.53	0.8	223.8	280.6	503.7

+See legends of Appendix Table I.

Appendix Table III

Quantitative pellicle determination and fermentation data for Hansenula anomala, Y3223+

	Incubation time Days	pH	Balling	Yield of Cells, mg. dry weight Top	Bottom	Total
A+	2	3.30	9.5	55.7	29.8	85.5
	4	2.90	8.1	166.7	48.4	215.1
	7	2.70	5.7	201.6	33.5	283.3
	9	2.62	4.2	321.9	43.2	365.1
	14	2.60	2.3	330.2	32.0	362.2
	16	2.56	2.2	298.5	88.0	386.5
	18	2.62	1.2	261.3	166.0	427.3
	20	2.52	1.6	187.2	258.0	445.2
B+	6	2.85	8.0	133.4	34.3	173.7
	8	2.74	6.7	173.9	34.2	208.1
	10	2.62	4.6	248.2	41.3	289.5
	15	2.71	1.8	249.6	64.3	313.9
	18	2.62	1.3	324.3	96.3	420.6
	21	2.56	0.5	276.8	185.3	462.1

+See legends of Appendix Table I.

Appendix Figure 1

Appendix Figure 2

Appendix Figure 1

COLONIES OF Y3221, THE INTERMEDIATE STRAIN

Five-day old colonies on MY agar incubated at room temperature.

These colonies are often mat and not glistening as they appear here.

Appendix Figure 2

COLONIES OF Y3222, THE ROUGH STRAIN

Five-day old colonies on MY agar incubated at room temperature

Appendix Figure 1

COLONIES OF Y3221, THE INTERMEDIATE STRAIN

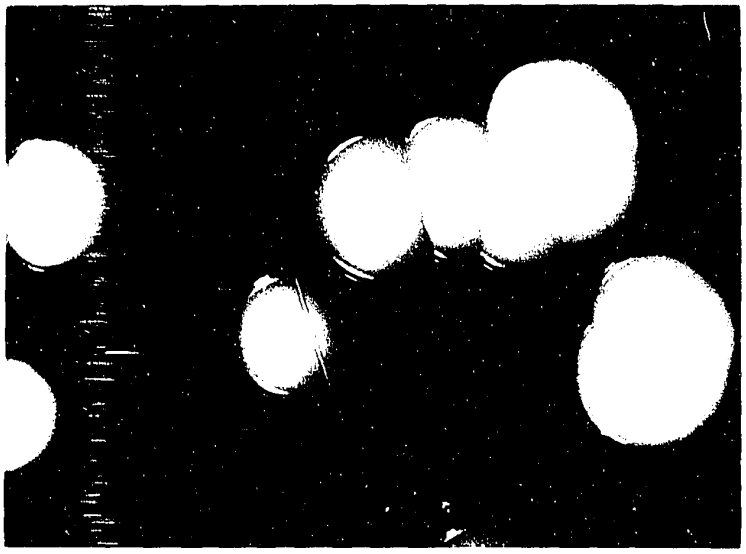
Five-day old colonies on MY agar incubated at room temperature.

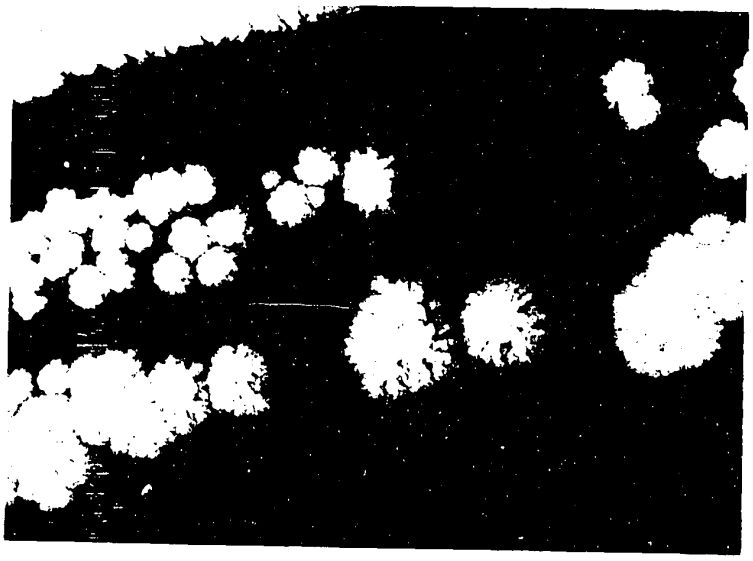
These colonies are often mat and not glistening as they appear here.

Appendix Figure 2

COLONIES OF Y3222, THE ROUGH STRAIN

Five-day old colonies on MY agar incubated at room temperature





Appendix Figure 3

Appendix Figure 4

Appendix Figure 3

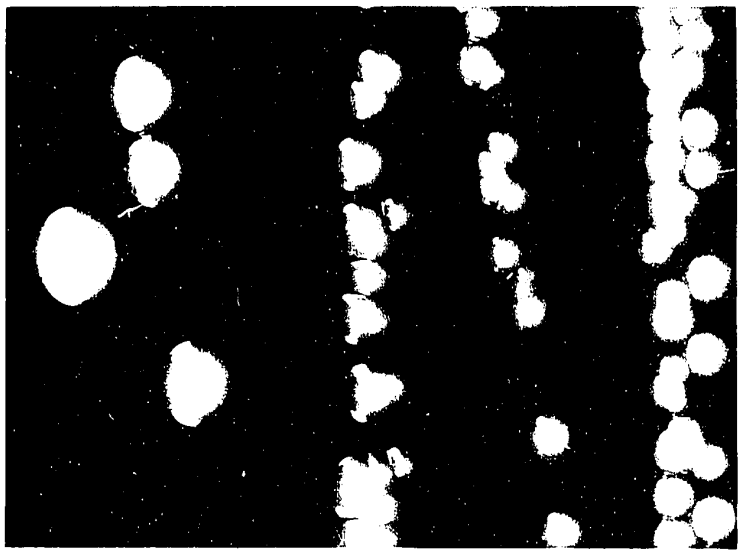
COLONIES OF Y3223, THE GLISTENING STRAIN

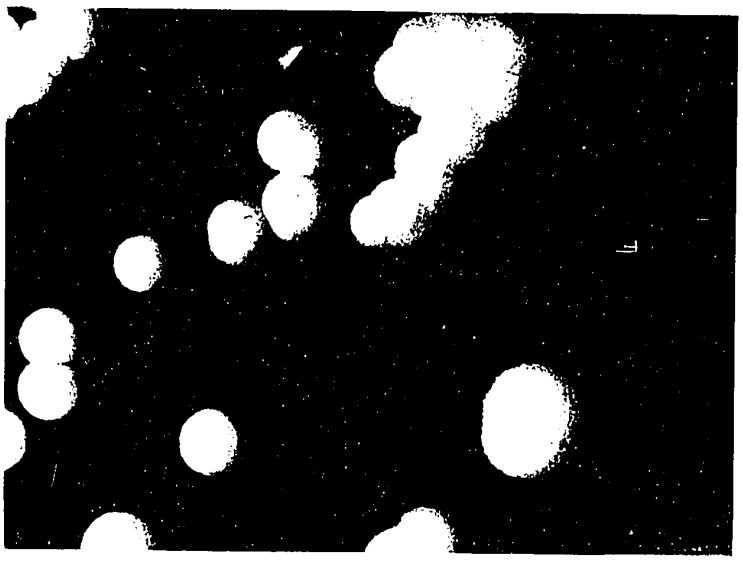
Five-day old colonies on MY agar incubated at room temperature.

Appendix Figure 4

COLONIES OF Y3224

Five-day old colonies on MY agar incubated at room temperature.





Appendix Figure 5

Appendix Figure 6

Appendix Figure 5

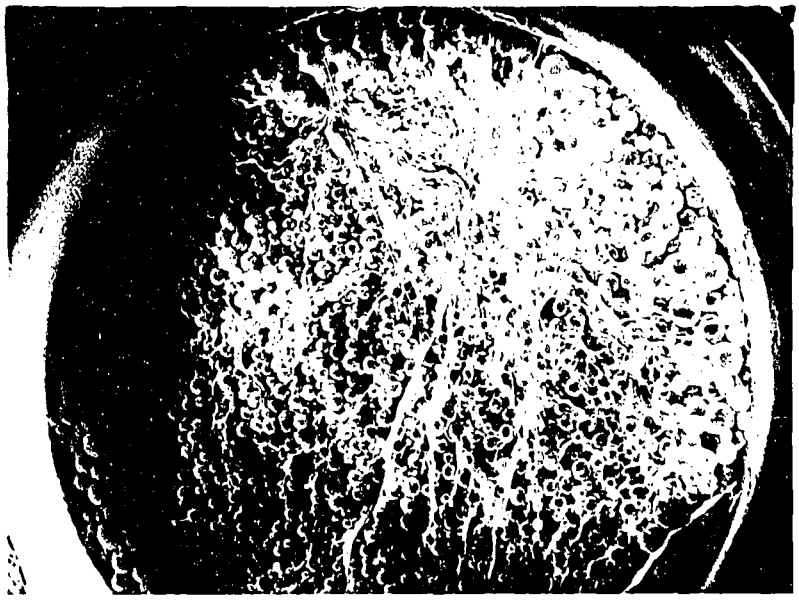
PELLICLE PRODUCED BY Y3221, THE INTERMEDIATE STRAIN

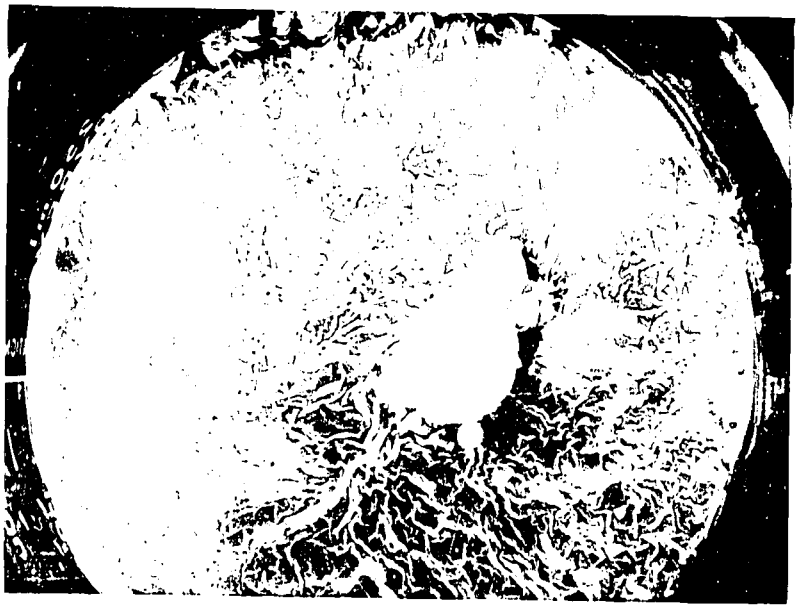
Four-day old fermentation of 400 ml. of Yeast Nitrogen Base containing 5% glucose in a 500 ml. beaker incubated at room temperature.

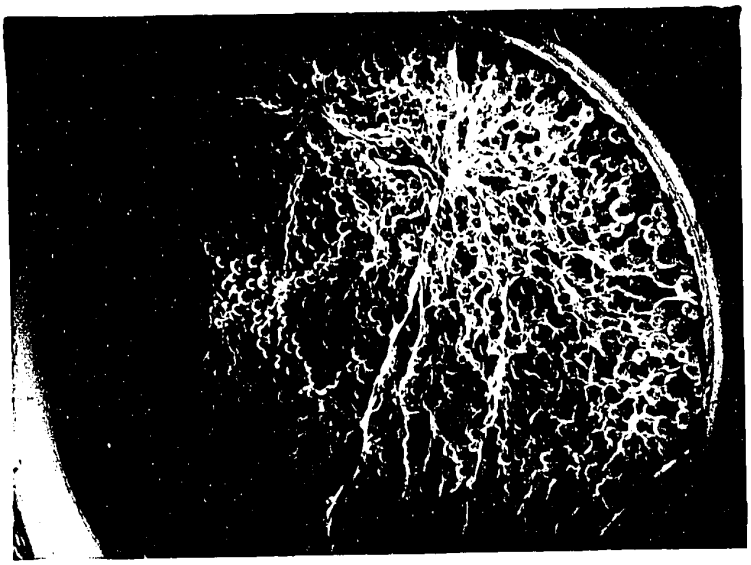
Appendix Figure 6

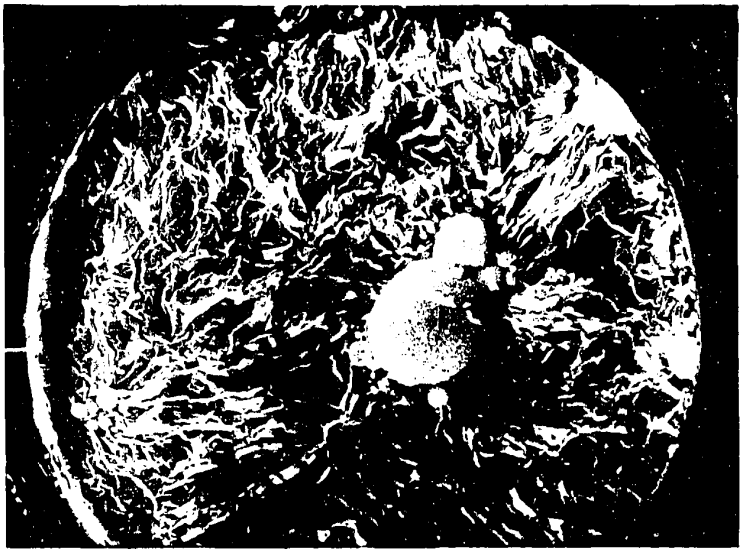
PELLICLE PRODUCED BY Y3222, THE ROUGH STRAIN

Four-day old fermentation of 400 ml. of Yeast Nitrogen Base containing 5% glucose in a 500 ml. beaker incubated at room temperature.









Appendix Figure 7

Appendix Figure 7

CELL CLUSTER OF Y3222, THE ROUGH STRAIN

Taken from the top 200 ml. of a funnel fermentation on the 4th day of incubation.