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N. Economopoulos[a]; N. Athanassopoulos[a]; N. A. Katsanos[b]; G. Karaiskakis[b]; P. Agathonos[b]; Ch. Vassilakos[b]
[a] B. G. SPILIOPOULOS DISTILLERI S.A., PATRAS, GREECE [b] PHYSICAL CHEMISTRY LABORATORY UNIVERSITY OF PATRAS, PATRAS, GREECE

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A Plant Kinetic Study of Alcoholic Fermentation Using Reversed-Flow Gas Chromatography

N. ECONOMOPOULOS and N. ATHANASSOPOULOS

B. G. SPILIOPOULOS DISTILLERI S.A.
87 AKTI DIMEON, 263 33 PATRAS, GREECE

N. A. KATSANOS,* G. KARAISKAKIS, P. AGATHONOS, and CH. VASSILAKOS

PHYSICAL CHEMISTRY LABORATORY
UNIVERSITY OF PATRAS
P.O. BOX 1045, 261 10 PATRAS, GREECE

Abstract

The reversed-flow gas chromatographic sampling technique is used to study the kinetics of alcoholic fermentation in a factory in conjunction with measurements of suspended particles in the fermenting medium. It was found that the overall process consists of four phases which have different first-order rate constants during ethanol formation. The second phase is the slowest, with its rate constant being 4.3 and 13 times smaller than that of the first and third phases, respectively. There is also a decrease of suspended particles during the second phase. These results show that there is the possibility of increasing the rate constant during this phase, thereby increasing the overall production rate of ethanol significantly and thus lowering its cost of production.

INTRODUCTION

A detailed kinetic study of alcoholic fermentation in a factory, following the various steps which lead to the final product, may give valuable information about the mechanism of the whole process. This could help to reduce the total time of the process, thus lowering the cost of the ethanol produced. A mechanistic study of this kind cannot be based on conventional methods of chemical analysis, like distillation or traditional gas chromatography, because these methods may alter the composition of samples taken at intermediate times, and this will lead to erroneous conclusions

*To whom correspondence should be addressed.

regarding the mechanism. Two possible sources of error are the relatively high temperature used in the analysis and the effects of the filling materials used to pack the chromatographic columns on the analysis mixture. The reversed-flow gas chromatography (RF-GC) technique, recently developed, does not suffer from these two sources of error and can be used with advantage for the present purposes. It is a flow perturbation method which has been reviewed three times (*1–3*) and described in more detail in a book (*4*). A short description, together with a summary of recent applications of the technique, is given in the introduction of another paper (*5*).

The RF-GC method is based on narrow, extra chromatographic *sample peaks* superimposed on normal elution curves. The peaks are created by reversing the direction of carrier gas flow for short time intervals at various times during the experiment. Most physicochemical measurements are made by following the time dependence of the sample peak's height h, while the analytical applications make use of the infinity value h_∞, i.e., the limiting value with time of the peak height. The relation between concentration and h_∞ is given by the equation (*6*, *7*)

$$c(\mathrm{g}) = \frac{c(\mathrm{l})}{K} = \frac{v h_\infty}{2} (L_1/D + 1/k_c) \qquad (1)$$

where $c(\mathrm{g})$ is the gaseous concentration of the solute vapor in equilibrium with the bulk liquid phase, having concentration $c(\mathrm{l})$, K is the partition coefficient, v is the linear velocity of the carrier gas, L_1 is the length of the diffusion column (cf. Fig. 1), D is the diffusion coefficient of the solute vapor into the carrier gas, and k_c is the mass transfer coefficient for the evaporation of the solute from the liquid.

Equation (1) was employed for the purposes of the present paper. The alcohol concentration in the fermentation mixture is also expressed by the magnitude of $c(\mathrm{l})$, and this was measured in conjunction with measurements of the suspended particles, the number of which is proportional to the number of yeast cells in the fermentation medium.

EXPERIMENTAL

Reagents

Methanol, ethanol, 1-propanol, and ethyl acetate, used for identification purposes, were Merck products, while 2-methyl-1-propanol was obtained from B.D.H. All were of 99% purity. The carrier gas was helium of 99.99% purity from Linde (Athens, Greece).

Apparatus

The laboratory experimental setup for applying the RF-GC method is schematically outlined in Fig. 1. A Shimadzu 8AI gas chromatograph with a flame ionization detector was used. Inside its oven were placed all items of Fig. 1 except the four-port valve and the reference injector. The sections l, l', and L_1 of the sampling cell were stainless steel tubes of 4 mm i.d. and lengths $l = l' = 100$ cm and $L_1 = 51$ cm. All connections of columns to the valve were made with 1/16-in. stainless steel tubes. The fermentation mixture (0.5 cm^3) was placed in a glass tube of 4 mm i.d. which was connected to the stainless steel column L_1 with a 1/4-in. Swagelok union. The additional separation column, when used, was a 2 m × 4 mm i.d. stainless steel gas chromatographic column packed with 20 g of 5% carbowax 20M on chromosorb P 80–100 mesh. The carrier gas flow rate was measured with a bubble flowmeter. The pressure drops along the sampling column and the diffusion column were negligible. The signal from the detector was recorded by a strip-chart recorder Linseis (model L 6522).

For measuring the number of yeast cells and for determining the suspended solids in the fermentation mixture, a Steindorff optical microscope with counting cells and a spectrophotometer (Hach, model Drel/5) were used, respectively.

The plant equipment included tanks holding 5 and 25 m^3 mash for the yeast propagation steps, and tanks holding 200–250 m^3 mash for the final commerical fermentation. The temperature of each tank was controlled by

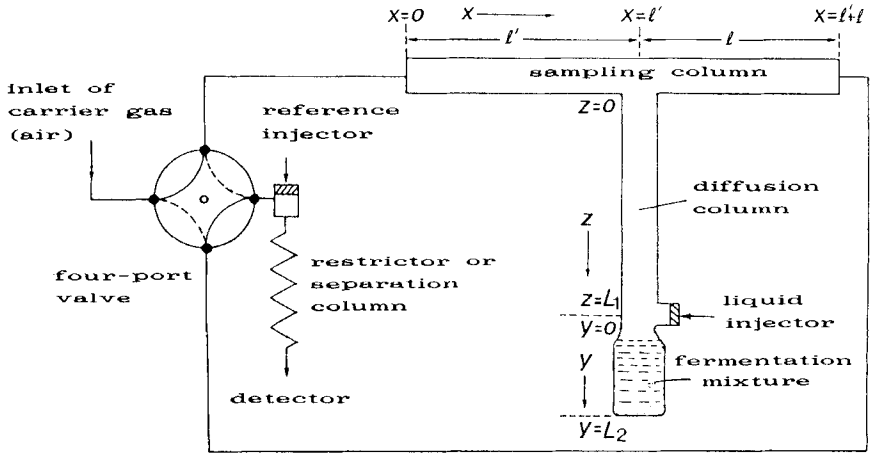

FIG. 1. Schematic representation of the cell and gas connections to measure the alcohol concentration in fermentation samples by the RF-GC method.

spraying cold water on its roof surface. All tanks were made of stainless steel.

Procedure

The stages of the plant work studied are shown in Fig. 2 and were the following. Sugar beet molasses was diluted to 20°Bé, acidified with sulfuric acid and heated to 80–90°C for 2 h. A quantity of this solution was further diluted to 9–10°Bé for the yeast propagation steps and supplemented with ammonium sulfate and ammonium mono-*H*-orthophosphate (0.2% each) to supply the nitrogen and phosphorus nutrients, respectively. Of this mash, 1.0–1.5 m^3 was added in a tank of 5 m^3 volume and was inoculated with ~10 kg of active dry bakers' yeast. The mash was fermented for 10–12 h at 30°C to a density of 5–6°Bé and then was filled with unfermented mash. When the density was decreased to 5–6°Bé, the whole content was inoculated in an ~15-m^3 volume of unfermented mash in a tank of 25 m^3 volume. After 10–12 h fermentation, the yeast propagation steps were completed and the whole mash of 20 m^3 volume was inoculated in a 20-m^3 volume of mash of 13–14°Bé in one of the final commercial fermentation tanks of ~250–300 m^3 volume. This mash was supplemented with decreased amounts of nutrients (0.2% ammonium sulfate and 0.1% ammonium mono-*H*-orthophosphate). Fermentation proceeded at 30°C. After 6–10 h fermentation periods, the addition of new unfermented mash was repeated in quantities of 10–25 m^3 until the entire amount of mash in the tank became ~200–250 m^3. Fermentation proceeded for a further 48–72 h at 30°C until the density of the fermented mash became constant. The ethanol concentration in the final product ("beer") was 7.8–8.2%. The "beer" then entered the distillation columns to give a final concentration of up to 95% ethanol.

Analysis of each sample by the RF-GC method was made by placing the liquid at the bottom of the diffusion column and waiting (15 to 25 min) until a monotonously rising concentration–time curve appeared in the recorder. Then, the chromatographic sampling procedure was started by reversing the direction of the carrier gas flow for 5 s, which is a shorter time period than the gas hold-up time in column sections l and l'. Examples of sample peaks created by the flow reversals are given in Fig. 3. The carrier gas flow-rate (corrected for column temperature and for any pressure drop in the separation column) was around 100 $cm^3\ min^{-1}$.

The height of the sample peaks finally reaches a maximum h_∞ and then remains constant as long as any liquid remains in the vessel. By drawing an appropriate calibration curve showing the relation between $c(l)$ and h_∞ (cf. Eq. 1), one can easily calculate the concentration of alcohol or any other product in the fermentation mixture.

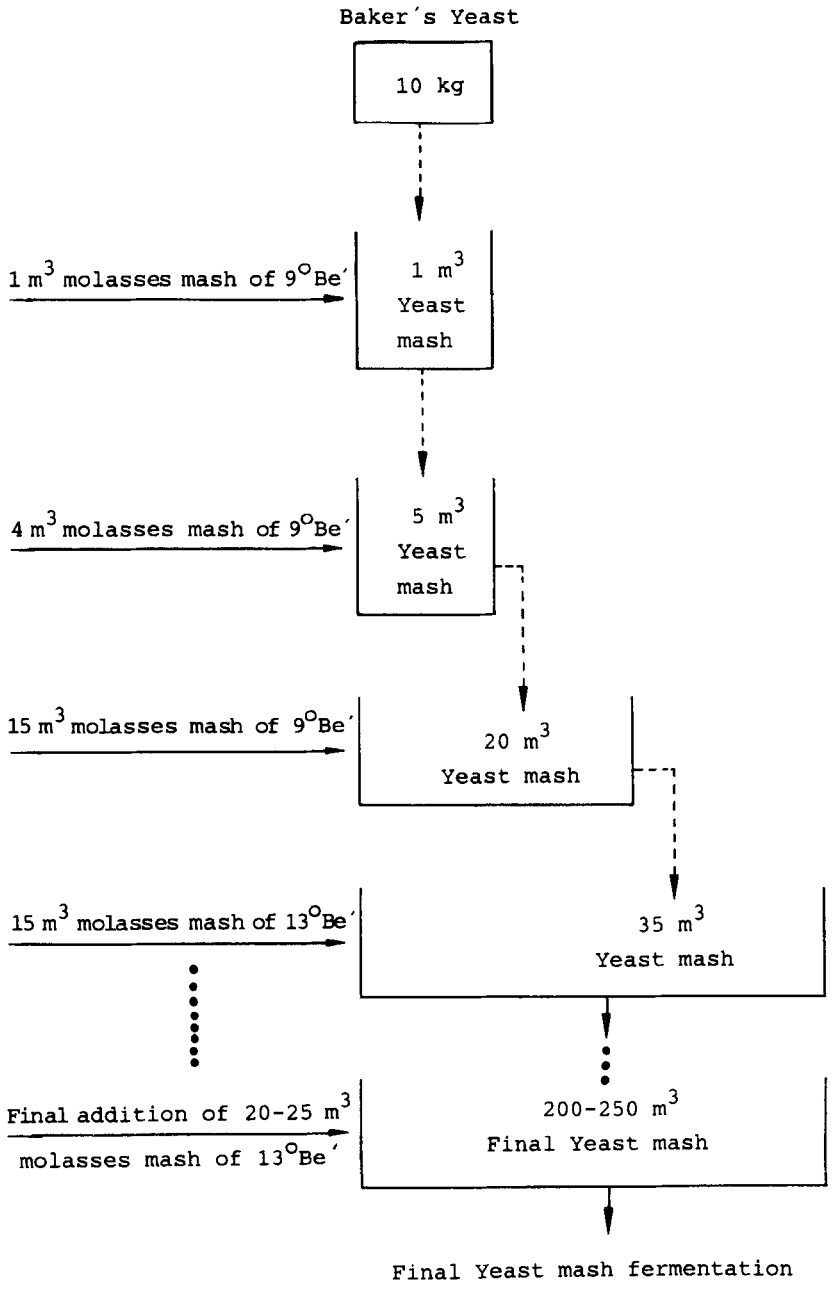

FIG. 2. Schematic diagram of yeast and molasses mash handling stages during distillery fermentations.

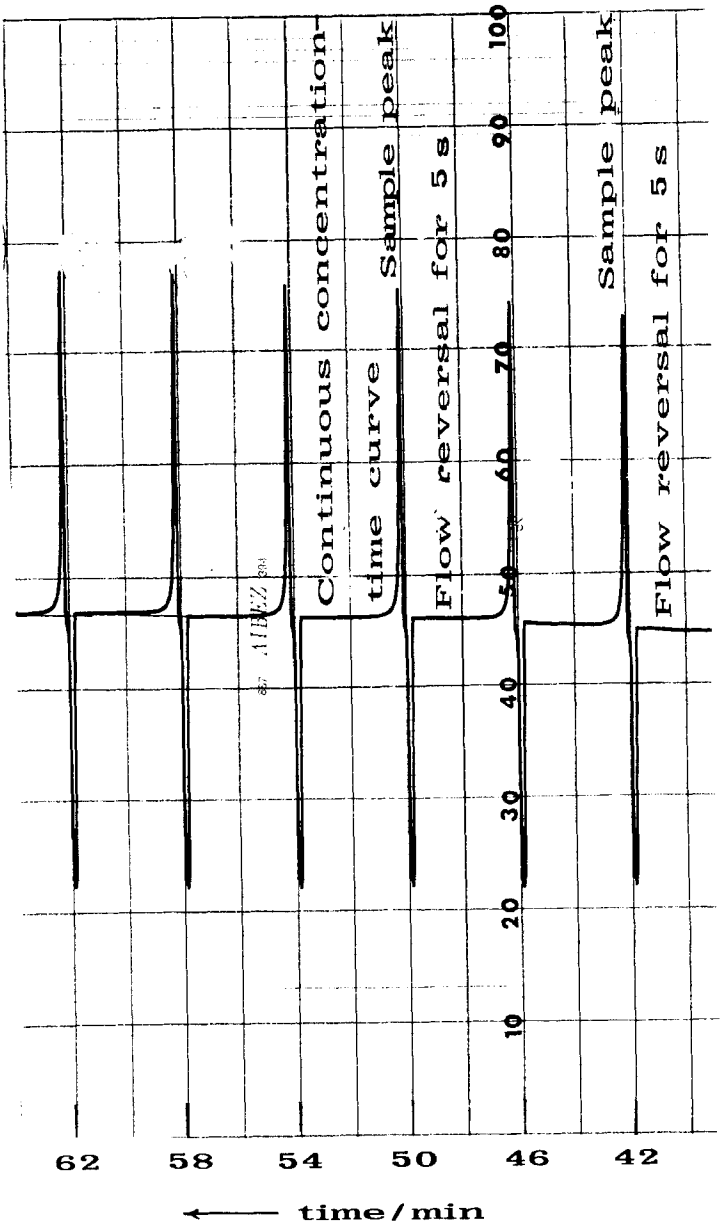

FIG. 3. A typical reversed-flow chromatogram with two sample peaks obtained during the analysis of Stage 10, after 27 h of total mash fermentation. The volume flow rate was 100 $cm^3 \cdot min^{-1}$ and the temperature 29.8°C.

Yeast population was determined by diluting the fermented mash and using the counting cells in the optical microscope. It was found that there is a linear correlation between the yeast population and the suspended solids in the mash.

The suspended solids were determined photometrically at 810 nm wavelength using a calibrated meter scale. Calibration was carried out with known yeast concentrations in unfermented mash.

RESULTS AND DISCUSSION

Calibration Curve

The calibration curve was constructed by carrying out RF-GC experiments at 56°C and a volumetric carrier gas flow rate of 100 $cm^3 \cdot min^{-1}$, using an ethanol–water mixtures of known alochol concentration (0.1 to 13% v/v). By plotting h_x vs ethanol concentration in the liquid, the line shown in Fig. 4 was obtained. This is a straight line with a correlation coefficient of $r = 0.998$, passing through the origin and having a slope of $b = 7994.59$. Therefore, $h_x = bc(l)$ and, according to Eq. (1), the value of b equals $2/Kv(L_1/D + 1/k_c)$. Thus, by increasing the carrier gas linear velocity v (i.e., the flow rate) and/or the length L_1 of the diffusion column (cf. Fig. 1), one can make the proportionality constant b as small as is consistent with the desired accuracy for measuring the ethanol concentration. Analogous calibration curves were obtained at lower temperatures, e.g., 30°C.

Initial Fermentation Stage

A preliminary study was made in the initial fermentation stage, not immediately after inoculation, but in the second vessel of 25 m^3, where the fermenting mash of volume ~5 m^3 is transferred 10 h after the reaction initiation and is mixed with ~15 m^3 of unfermented mash (cf. Fig. 2). The mixture remains there around 2 days, until enough ethanol is produced to weaken, but not completely weaken, the yeast cells. The concentration of ethanol in the liquid increases during the first 40 h and then tends to a limiting value of ~5.5% after 50 h.

First Fermentation Stage

The first fermentation stage was studied analytically from the moment when more mash was added to the inoculated liquid of 1.5 m^3, so that the total volume became 5 m^3, until the fermented mash (which by now had reached 20 m^3) was transferred to the large vessels with a volume of 250 m^3. The pH of the inoculation mashes was around 4, the temperature 30°C,

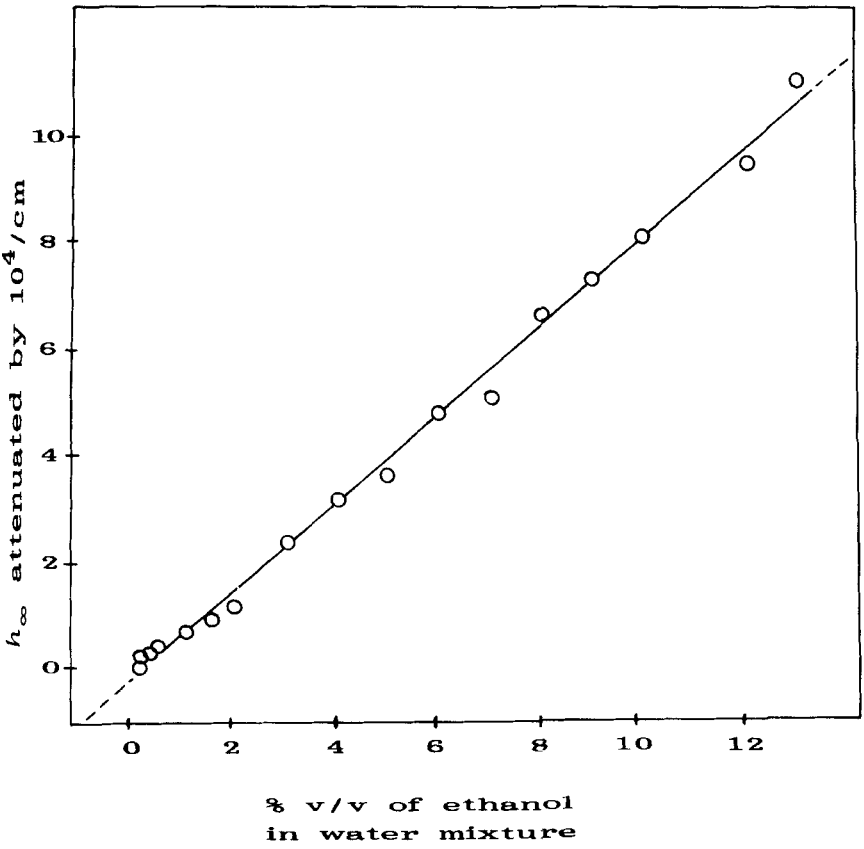

FIG. 4. Calibration curve for measuring the concentration of ethanol in the fermentation mixtures from the infinity sample peak height h_∞ according to the RF-GC method at 56°C.

and the Bé 9–10°. After having recorded all conditions and the concentration of the liquid in ethanol at various times, some of the above parameters were changed.

(1) Increased amounts of nutrients salts (0.4% ammonium sulfate and ammonium mono-*H*-orthophosphate instead of the usual 0.2%) were added to the mash, and this caused an increase in both the amount of ethanol produced and the rate of fermentation of this stage.
(2) The temperature was lowered to 27–27.5°C, and this caused a decrease in the fermentation rate. The final solution of this stage was ready to be transferred to the next vessel 22–23 h after inoculation, whereas

16 h were enough at 30°C. The final concentration of ethanol was also smaller (2.3% instead of the usual 3–3.2%) in spite of the fact that the number of yeast cells remained practically constant. A temperature rise to 32–33°C increased the fermentation rate significantly, but this was accompanied by the appearance of other kinds of microorganisms which produce methanol and higher alcohols. It seems, therefore, that 30°C is the optimum temperature.

(3) The behavior of the fermentation was studied at lower pH values (3.8 instead of 4), the other factors (salts and temperature) remaining at the initial levels. Although an increase in rate was not observed, the final concentration of ethanol was higher (3.8% instead of the usual 3–3.2%). Naturally, the lower pH value requires greater amounts of sulfuric acid to be added to the mash, after inoculation and the activation of yeast cells. If the pH is increased (~4.5–5.0), the fermentation is somehow accelerated, but other kinds of microorganisms are produced, which are undesirable, because they cause the formation of other alcohols.

Overall Fermentation Process

After the initial and the first fermentation stages, the overall process was studied for the 161 h needed to go through the stages listed in Table 1. Samples were taken from the mixture three times a day and subjected to measurements of their temperature and pH, their °Bé value, the amount of suspended particles, and their % v/v content in ethanol, as described in the Experimental Section. The temperature varied between 27 and 28°C and the pH between 3.8 and 4.1. The variation of °Bé, the ethanol content, and the suspended particles with time is shown in Table 2. The various stages correspond to those of Table 1. In the 1st phase (Stages 1 to 6), the limiting height h_∞, and therefore the ethanol content, increases significantly with time, while the quantity of suspended particles shows up and down variations, reaching finally a maximum value of 2.9 $g \cdot dm^{-3}$. During the 2nd phase (Stages 7 to 10), the quantity of ethanol shows only a small increase with time, thus forming a transition state in the overall process. In this phase the suspended particles decrease steadily with time. These two observations show that a possible acceleration of ethanol production can be brought about during the 2nd phase by adding increased amounts of the nutrients salts (ammonium sulfate and ammonium mono-*H*-orthophosphate), thus increasing the rate of formation of ethanol without the danger of significantly increasing the number of yeast cells. Moreover, the temperature can be raised to 32–33°C during the second phase, thereby considerably increasing the fermentation rate but again without increasing the number of yeast cells to an undesirable level.

TABLE 1
Stages and Phase of the Alcoholic Fermentation

Stage	Phase	Description
1	1st	After inoculation of 1 m^3 mash of 9°Bé with dry yeast in a tank of 5 m^3
2	1st	After addition of 4 m^3 mash of 9°Bé into 1 m^3 fermenting mash in a tank of 5 m^3
3	1st	After inoculation of 15 m^3 mash of 9°Bé with the 5-m^3 fermenting mash in a tank of 20 m^3
4	1st	After inoculation of 15 m^3 mash of 13°Bé with the 20-m^3 fermenting mash in a tank of 250 m^3
5	1st	After addition of 20 m^3 mash of 13°Bé to a fermenting volume of 55 m^3
6	1st	After addition of 25 m^3 mash of 13°Bé to a fermenting volume of 80 m^3
7	2nd	Same as above but to a fermenting volume of 105 m^3
8	2nd	Same as above but to a fermenting volume of 130 m^3
9	2nd	Same as above but to a fermenting volume of 155 m^3
10	2nd	Same as above but to a fermenting volume of 180 m^3
11	3rd	After final addition of 25 m^3 mash to a total fermenting mash volume of 205 m^3
12	3rd	After 15 hours of total mash fermentation
13	3rd	After 27 hours of total mash fermentation
14	4th	After 24.5 hours of total mash fermentation
15	4th	After 70 hours of total mash fermentation

TABLE 2
The Values of °Bé and h_∞, the % v/v Ethanol Content, and the Quantity of Suspended Particles in the Fermenting Must as a Function of Time during the Stages of Table 1

Stage	Time (h)	°Bé	h_∞ (cm)	% Ethanol	Suspended particles ($g \cdot dm^{-3}$)
1	12.5	—	1660	2.60	2.00
2	18.5	5.7	2300	3.64	2.70
3	25.5	6.5	2120	3.31	1.50
4	35.5	5.6	3080	4.49	2.95
5	43.5	6.3	3120	4.52	2.40
6	50.5	5.7	3600	4.91	2.90
7	59.5	6.2	3560	4.88	2.40
8	67.5	6.2	3760	5.04	2.30
9	72.5	6.2	—	—	2.25
10	83.5	6.3	3800	5.08	2.15
11	91.0	5.7	4160	5.48	2.05
12	96.0	5.3	4320	5.67	2.05
13	108.0	4.4	4960	6.43	2.20
14	115.5	4.0	5120	6.60	2.40
15	161.0	3.5	5440	6.94	2.55

During the 3rd phase (Stages 11–13 of Table 1), the liquid content in ethanol increases linearly with time and with a large slope, whereas the quantity of suspended particles shows only a small increase. Therefore, no action for increasing the production rate of ethanol is recommended in this phase. In the 4th phase (Stages 14 and 15 of Table 1), the fermentation rate is small, approaching a limiting value in the ethanol content of the mixture. So is the quantity of the suspended particles. Obviously, no increase of the rate is advisable in this phase for the same reason as before, i.e., because it will lead to an increase in the number of yeast cells, which will result in an increase in the content of by-products.

Chemical Kinetics

The RF-GC technique lends itself to an easy study of the chemical kinetics during the 1st, 2nd, and 3rd phases of the overall process. As mentioned before, the infinity value h_∞ of the sample peak's height is proportional to the ethanol concentration in the liquid at time t after reaction initiation. If by h_∞° we denote the value of h_∞ obtained after the end of the reaction, i.e., after 161 h (cf. Table 2), it can be easily shown that, assuming first-order kinetics, the integrated rate equation has the form

$$\ln (h_\infty^\circ - h_\infty) = \ln h_\infty^\circ - kt \qquad (2)$$

This equation can be used to plot the data of Table 2 and see whether the fermentation process is first order with respect to the precursor species forming the ethanol. In that case the reaction rate constant k can be calculated from the slope of the predicted linear plot. Figure 5 shows the plot of Eq. (2).

It is clearly seen from this figure that in all three phases the reaction obeys Eq. (2), but with a different slope, i.e., rate constant k. The 4th phase comprises only two experimental points (cf. Table 2), and one of them (Stage 15) has been taken as the h_∞° value for plotting Eq. (2); the second was incorporated into the 3rd phase. By using linear regression analysis, the rate constants in the three phases were computed and found to be as follows:

Phase:	1st	2nd	3rd
$10^3 k/\mathrm{h}^{-1}$:	17.7 ± 2.5	4.1 ± 1.7	53.4 ± 4.8

The $\pm$ values are standard errors.

These rate constants confirm and place on a quantitative basis the arguments of the previous subsection "Overall Fermentation Process." The proper activation energy for the process cannot be calculated since the

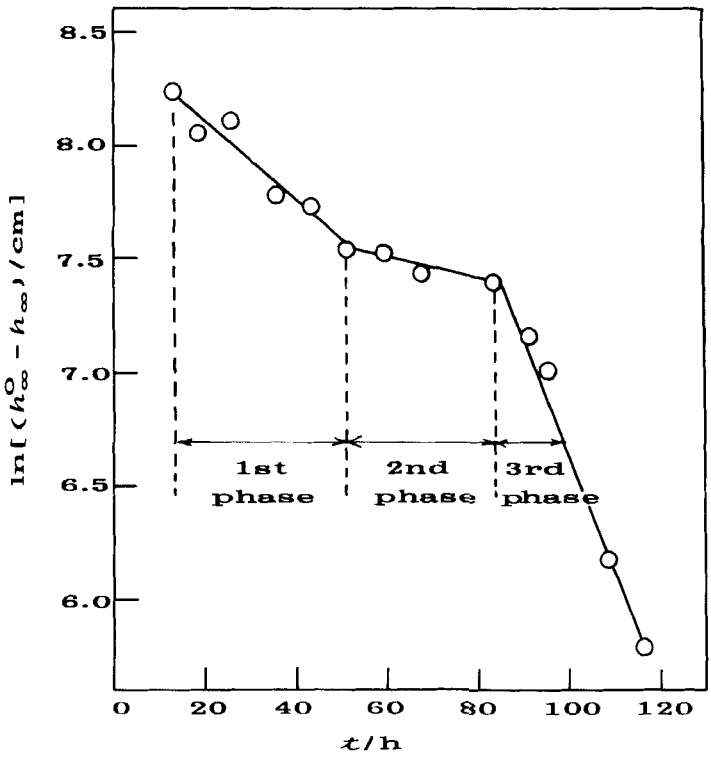

FIG. 5. First-order plot of the data of Table 2 according to Eq. (2).

temperature can be varied only within narrow limits, for reasons given in the previous subsection.

Studies with a Separation Column

In another study of the overall fermentation process, a separation column packed with carbowax 20M on chromosorb P (cf. the Experimental Section) was used in place of the restrictor (cf. Fig. 1) to separate the ethanol from other volatile fermentation products. An example of a reversed-flow chromatogram of this type is given in Fig. 6. It shows that each flow reversal is accompanied by two sample peaks, one of ethanol and one of ethyl acetate, the latter being one of the main by-products of the process. The identification was made by injecting mixtures of ethanol/methanol, ethanol/ethyl acetate, ethanol/1-propanol, and ethanol/2-methyl-1-propanol through the reference injector (cf. Fig. 1). A cross-check of the

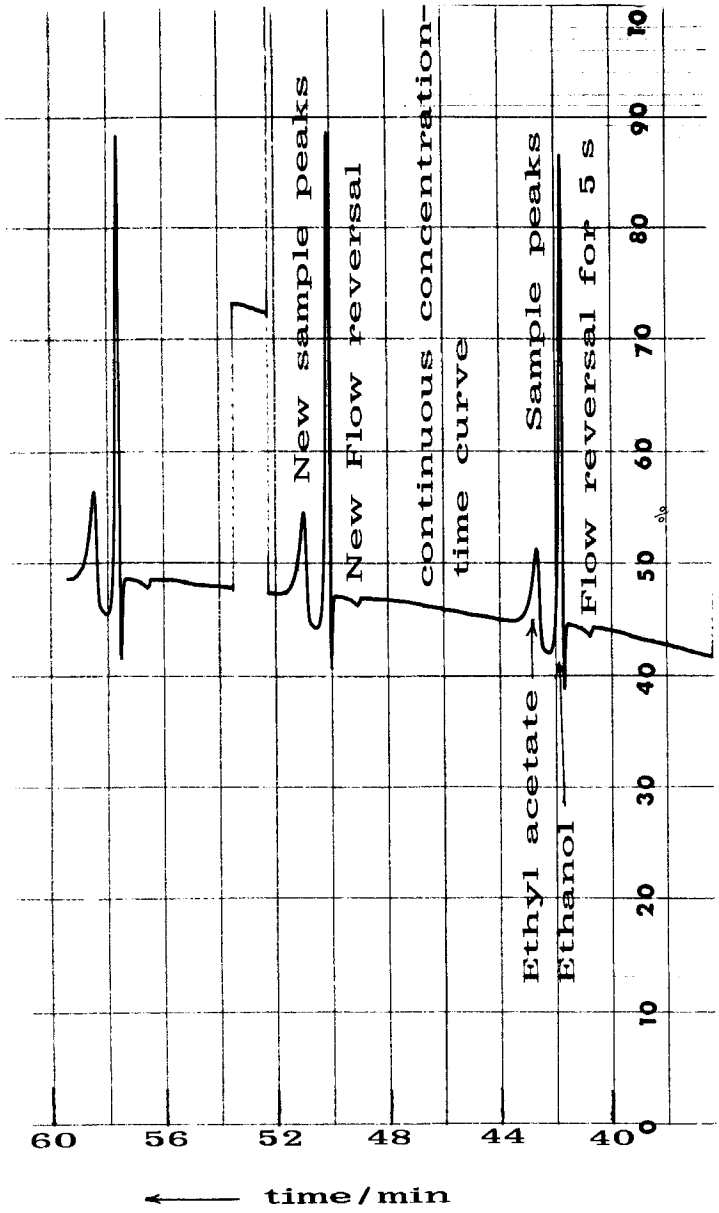

FIG. 6. A typical reversed-flow chromatogram obtained in the presence of a carbowax 20M/chromosorb P separation column placed before the detector. The volume flow rate was 100 $cm^3 \cdot min^{-1}$, and the temperature of the column was 105°C.

products was made with a distilled sample on a conventional gas chromatograph using a 25 m × 0.53 mm vitreous silica capillary column containing bonded phase BP20.

The results of the fermentation studies are given in Table 3, and the first-order plot, using the mean value of the last two stages as h_∞°, is shown as Fig. 7. A noticeable dispersion of the experimental points around the linear regression line is seen from this figure. Its slope gives $k = (20.0 \pm 3.6) \times 10^{-3}\ h^{-1}$. This rate constant does not differ significantly from that of the 1st stage determined in the previous overall run (17.7 ± 2.5) since their difference is less than one standard error. The rate constants of the other two stages do not appear distinctly in this run, owing most probably to a perturbation of the samples by the analytical column.

CONCLUSION

The new technique of RF-GC can be used not only for determining the ethanol content in a fermenting mash but also for studying the detailed kinetics of the overall process which pertains to the fermentation mechanism in a factory.

The study can be divided into four phases, each with a different first-order rate constant.

The most interesting phase is the 2nd, which has the smallest rate constant value and therefore the smallest ethanol production rate. Simulta-

TABLE 3
The Infinity Value h_∞ for the Ethanol Content as a Function of Time in the Presence of an Additional Analytical Column

Stage	Time (h)	h_∞ (cm)	Description of stage
1	17.5	92	From a fermenting mash of 5 m^3
2	27.5	160	From a fermenting mash of 20 m^3
3	35	188	From a fermenting mash of 35 m^3
4	42	232	From a fermenting mash of 60 m^3
5	59	176	From a fermenting mash of 100 m^3
6	65	264	From a fermenting mash of 125 m^3
7	75	212	From a fermenting mash of 150 m^3
8	84.5	256	From a fermenting mash of 160 m^3
9	95	272	From a fermenting mash of 170 m^3
10	112	296	From the final total mash of 190 m^3
11	121	328	9 hours after the final addition of mash
12	163	308	51 hours after the final addition of mash

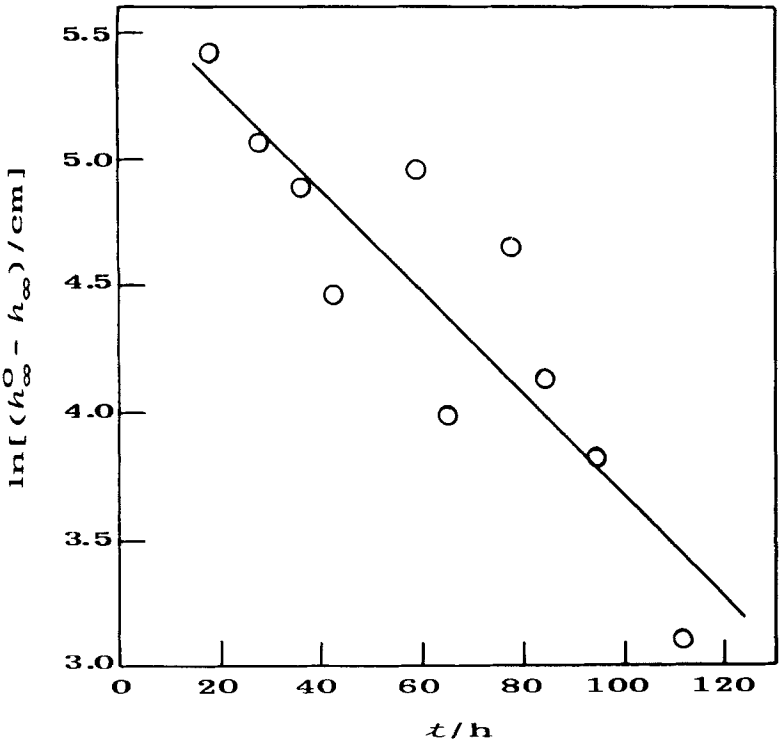

FIG. 7. First-order plot of the data of Table 3 according to Eq. (2).

neously, there is a significant decrease in the number of suspended particles during this phase. Therefore, the addition of increased amounts of nutrient salts, a small rise in temperature, and a small increase in pH during the 2nd phase of the overall process may cause a significant increase in the overall production rate of ethanol, thus lowering its cost.

Acknowledgments

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